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SECTION I

TOPICS

- Biodiversity, genepool protection and conservation of medicinal and aromatic plants (MAP)
- Biotechnology, cultivation, industrial processing of MAP
- Ecobiology of MAP
- Quality control of MAP

THE SPECTRUM OF BIOFORMS AND THE AREAL-GEOGRAPHIC STRUCTURE OF THE MEDICINAL PLANTS FROM THE WESTERN PART OF THE BANATIAN MOUNTAINS

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Summary

The knowledge of the flora of pharmaceutic interest is a prerequisite for its rational valorization and protection. Our study presents the medicinal plats from the Western part of the Banatian Mountains, and to analyze their spectrum of bioforms and areal-geographic structure. The researches point out the presence of 241 species employed in modern and traditional phytotherapy. The analysis of the floristic elements shows the predomination of Eurasian species (43%), followed by European (9,5%) and Circumpolar (9,1%) elements.

Keywords: *medicinal plants, Banat region, bioforms, repartition*

Introduction

In today's Europe, where industrial development, pollution and urban agglomerations are continuously extending, the rich medicinal flora of Romania represents a priceless asset. In this concept, the knowledge of the flora of pharmaceutic interest becomes imperative, representing a prerequisite for its rational valorization and protection. The conservation of biodiversity, which includes the protection of medicinal plants, is presently taking place within the legal framework of the "Convention on International Trade in Endangered Species of Wild Fauna and Flora" (CITES), adopted in 1973 [1]. In order to safeguard the resources of medicinal plants for the future, assessing the vegetal genepool of therapeutic interest through the inventory of plants by regions is of major importance, reflecting the territorial distribution of the species.
The Banatian Mountains, situated in the West of Romania, offer favorable conditions for the development of medicinal plants; although having low altitudes, of 600-1400 m, their degree of anthropization is yet reduced. Approximately 60% of their surface is covered by forests, the rest being occupied by pastures, primary or as a result to the disafforesting. The relief is varied, with the configuration of the terrain being generally undulated. The prevailing orientation of the slopes is NNE-SSW [2]. The studied territory is situated in an area with humid, temperate climate, soft winters, exposed to obvious Mediterranean influences. Average annual temperatures vary between 8.8˚C in the area of hills with low altitude, and 5.4˚C at heights of about 1000m, in the mountain area [3]. The vegetation of the Banatian Mountains is represented by the beech level and by the common oak - bitter level [4].
The aim of the present study was to identify the medicinal plats (MP) from the Western part of the Banatian Mountains, known for their vegetal diversity, and to outline their spectrum of bioforms and areal-geographic structure. This approach brings a contribution to the identification of the critical and/or valuable components of the genepool of MP existing in the researched area.

Material and methods

The study was carried out during March 2002 - November 2005, evaluating the medicinal flora from a surface of approximatively 1500 km^2 (Dognecei Mountains, Ezeriş Depression, Aninei Mountains, Oraviței and Bozoviciului Hills).

The identification of the species was mainly performed based on Flora Republicii Populare Române (1952-1976) [5] and Flora ilustrată a României (2000) [6], other sources being also employed [7]. The classification of bioforms and the determination of the areal-geographic structure was carried out as indicated in [6].
The inclusion of medicinal species in the study was based upon the description of their utilization by works of modern and traditional phytotherapy [8-13]. Species employed in local traditional medicine were also taken into account.

Results and discussions

Following the study of the wild vascular flora from the Western part of the Banatian Mountains, 241 species utilized in the modern and traditional phytotherapy were identified. These plants are integrated into 70 botanical families. A large number of plants belong to the *Asteraceae* (24 species), *Rosaceae* (22), *Lamiaceae* (20), *Fabaceae* (12), *Scrophulariaceae* (10), *Ranunculaceae* (8) and *Apiaceae* (8) families, as presented below :

- *Alismataceae: Alisma plantago-aquatica* L.;
- *Alliaceae: Allium ursinum* L.
- *Apiaceae*: *Angelica archangelica* L., *Anthriscus cerefolium* (L.) Hoffm., *Anthriscus sylvestris* (L.) Hoffm., *Conium maculatum* L., *Daucus carota* L., *Eryngium campestre* L., *Pimpinella saxifraga* L., *Sanicula europaea* L.;
- *Araceae: Arum maculatum* L.;
- *Araliaceae: Hedera helix* L.
- *Aristolochiaceae: Aristolochia clematitis* L., *Asarum europaeum* L.;
- *Asclepiadaceae: Vincetoxicum hirundinaria* Medikus
- *Aspleniaceae: Asplenium adiantum-nigrum* L., *Asplenium scolopendrium* L., *Dryopteris filix-mas* (L.) Schott;
- *Asteraceae: Achillea millefolium* L.s.l., *Arctium lappa* L., *Artemisia absinthium* L., *Artemisia annua* L., *Artemisia vulgaris* L., *Bellis perennis* L., *Bidens tripartita* L., *Carlina acaulis* L., *Cichorium intybus* L., *Cirsium arvense* (L.) Scop., *Erigeron canadensis* (L.) Pers., *Eupatorium cannabinum* L., *Hieracium pilosella* L., *Matricaria discoidea* D.C., *Matricaria recutita* L., *Mycelis muralis* (L.) Dumort, *Onopordum acanthium* L., *Petasites hybridus* (L.) Gaertner*, Solidago virgaurea* L., *Tanacetum vulgare* L., *Taraxacum officinale* agg., *Telekia speciosa* (Schreber) Baumg, *Tussilago farfara* L., *Xanthium spinosum* L.;
- *Balsaminaceae : Impatiens noli-tangere* L.;
- *Betulaceae: Alnus glutinosa* (L.) Gaertner, *Betula pendula* Roth.;
- *Boraginaceae*: *Anchusa officinalis* L., *Cynoglossum officinale* L., *Echium vulgare* L., *Pulmonaria officinalis* agg., *Symphytum officinale* L.;
- *Brassicaceae : Alliaria petiolata* (Bieb.) Cavara et Grande, *Capsella bursa-pastoris* (L.) Medik., *Dentaria bulbifera* L., *Erysimum cheiranthoides* L., *Nasturtium officinale* R.Br.;
- *Cannabaceae: Humulus lupulus* L.;
- *Caprifoliaceae*: *Sambucus nigra* L., *Sambucus ebulus* L., *Viburnum lantana* L., *Viburnum opulus* L.;
- *Caryophyllaceae: Saponaria officinalis* L.;
- *Celastraceae: Evonymus europaeus* L.;
- *Colchicaceae: Colchicum autumnale* L.*;*
- *Convallariaceae: Polygonatum odoratum* (Miller) Druce;
- *Convolvulaceae*: *Calystegia sepium* (L.) R.Br., *Convolvulus arvensis* L.;
- *Cornaceae: Cornus mas* L., *Cornus sanguinea* L.;
- *Corylaceae: Corylus avellana* L.;
- *Crassulaceae: Jovibarba heufelii* (Schott) A. et D. Löve, *Sedum acre* L.;

- *Cupressaceae: Juniperus communis* L.;
- *Dennstaedtiaceae: Pteridium aquilinum* (L.) Kuhn;
- *Dioscoreaceae: Tamus communis* L.;
- *Dipsacaceae: Knautia arvensis* (L.) Coulter, *Scabiosa ochroleuca* L.*, Succisa pratensis* Moench;
- *Equisetaceae: Equisetum arvense* L.;
- *Ericaceae: Calluna vulgaris* (L.) Hull, *Vaccinium myrtillus* L.;
- *Euphorbiaceae: Euphorbia amygdaloides* L., *Euphorbia cyparissias* L., *Euphorbia helioscopia* L., *Mercurialis perennis* L.;
- *Fabaceae: Anthyllis vulneraria* L., *Coronilla varia* L., *Genista tinctoria* L.; *Genistella sagittalis* (L.) Gams, *Laburnum anagyroides* Medik., *Lotus corniculatus* L., *Medicago falcata* L., *Melilotus* officinalis (L.) Pallas*, Ononis spinosa* L., *Sarothamnus scoparius* (L.) Wimmer*, Trifolium arvense* L*, Trifolium pratense* L.;
- *Fagaceae: Quercus petraea* L., *Quercus robur* L.;
- *Fumariaceae : Corydalis cava* (L.) Schweigg et Koerte, *Corydalis solida* (L.) Clairv.;
- *Gentianaceae: Centaurium erythraea* L., *Gentiana cruciata* L.;
- *Geraniaceae: Erodium cicutarium* (L.) L'Hérit, *Geranium macrorrhizum* L., *Geranium robertianum* L.;
- *Hypericaceae: Hypericum perforatum* L.;
- *Juglandaceae: Juglans regia* L.;
- *Lamiaceae: Ajuga reptans* L., *Ballota nigra* L., *Galeopsis tetrahit* L., *Glechoma hederacea* L., *Lamium album* L., *Leonurus cardiaca* L., *Lycopus europaeus* L., *Melissa officinalis* L., *Mentha aquatica* L., *Mentha longifolia* L., *Mentha pulegium* L., *Origanum vulgare* L., *Prunella vulgaris* L., *Salvia glutinosa* L., *Teucrium chamaedrys* L., *Thymus balcanus* Borbas, *Thymus glabrescens* Willd., *Thymus jankae* Celak., *Thymus pannonicus* All., *Thymus pulegioides* L.;
- *Liliaceae: Convallaria majalis* L., Ruscus aculeatus; *Veratrum album* L.;
- *Loranthaceae: Viscum album* L.;
- *Lythraceae: Lythrum salicaria* L.;
- *Malvaceae: Althaea offcinalis* L., *Malva sylvestris* L.;
- *Oleaceae: Fraxinus excelsior* L., *Fraxinus ornus* L., *Ligustrum vulgare* L., *Syringa vulgaris* L.;
- *Onagraceae: Chamerion angustifolium* (L.) Holub., *Epilobium hirsutum* L., *Epilobium palustre* L., *Epilobium parviflorum* Schreber, *Oenothera biennis* L.;
- *Orchidaceae: Orchis morio L.;*
- *Oxalidaceae: Oxalis acetosella* L.;
- *Papaveraceae: Chelidonium majus* L., *Papaver rhoeas* L.;
- *Phytolaccaceae: Phytolacca americana* L.;
- *Pinaceae: Abies alba* Miller, *Picea abies* (L.) Karsten, *Pinus sylvestris* L.;
- *Plantaginaceae: Plantago lanceolata* L., *Plantago major* L., *Plantago media* L.;
- *Poaceae*: *Anthoxanthum odoratum* L., *Elymus repens* (L.) Gould;
- *Polygonaceae: Polygonum aviculare* L.s.l., *Polygonum hydropiper* L., *Rumex acetosa* L., *Rumex acetosella* L., *Rumex crispus* L.;
- *Polypodiaceae: Polypodium vulgare* L.;
- *Primulaceae: Anagallis arvensis* L., *Lysimachia nummularia* L., *Lysimachia vulgaris* L., *Primula elatior* (L.) Hill, *Primula veris* L.;
- *Ranunculaceae: Anemone nemorosa* L., *Anemone ranunculoides* L., *Clematis recta* L., *Clematis vitalba* L., *Helleborus odorus* Waldst. et Kit., *Helleborus purpurascens* Waldst. et Kit., *Hepatica nobilis* Schreber, *Ranunculus ficaria* L.;
- *Rhamnaceae: Frangula alnus* Mill., *Rhamnus cathartica* L.;

- *Rosaceae: Agrimonia eupatoria* L., *Alchemilla vulgaris* L. emend Fröhner, *Alchemilla xanthochlora* Rothm., *Cerasus avium* (L.) Moench*, Crataegus laevigata* agg*, Crataegus monogyna* agg., *Filipendula ulmaria* (L.) Maxim*, Fragaria vesca* L., *Geum urbanum* L., *Potentilla alba* L., *Potentilla anserina* L., *Potentilla argentea* L., *Potentilla erecta* (L.) Räusch., *Potentilla recta* L., *Potentilla reptans* L., *Prunus spinosa* L., *Pyrus pyraster* (L.) Burgsd., *Rosa canina* L., *Rubus fruticosus* L*, Rubus idaeus* L., *Sanguisorba officinalis* L., *Sorbus aucuparia* L.;
- *Rubiaceae: Cruciata laevipes* Opiz, *Galium aparine* L., *Galium mollugo* L., *Galium odoratum* (L.) Scop., *Galium verum* L;
- *Salicaceae : Populus nigra* L., *Populus tremula* L., *Salix alba* L., *Salix caprea* L., *Salix cinerea* L., *Salix fragilis* L., *Salix purpurea* L.;
- *Scrophulariaceae : Digitalis grandiflora* Miller, *Euphrasia officinalis* L., *Linaria vulgaris* L., *Scrophularia nodosa* L., *Verbascum densiflorum* Bertol., *Verbascum phlomoides* L., *Veronica beccabunga* L., *Veronica chamaedrys* L., *Veronica officinalis* L., *Veronica spicata* L.;
- *Simaroubaceae: Ailanthus altissima* (Miller) Swingle;
- *Solanaceae: Atropa belladonna* L., *Physalis alkekengi* L., *Scopolia carniolica* Jacq., *Solanum dulcamara* L.;
- *Taxaceae: Taxus baccata* L.*;*
- *Tiliaceae: Tilia cordata* Miller, *Tilia platyphyllos* Scop., *Tilia tomentosa* Moench;
- *Triliaceae: Paris quadrifolia* L.;
- *Ulmaceae: Ulmus minor* Mill.;
- *Urticaceae: Parietaria officinalis* L., *Urtica dioica* L., *Urtica urens* L.;
- *Valerianaceae: Valeriana officinalis* L.;
- *Verbenaceae: Verbena officinalis* L.;
- *Violaceae: Viola odorata* L., *Viola tricolor* L.

The analysis of the biologic type of the identified medicinal plants reveals that the hemichryptophyte species predominate (44%), being followed by the phanerophytes (22%), geophytes (11%), therophytes (7,4%) and chamaephytes (6,6%) (fig. 1). The high percentage of hemichryptophytes and hemitherophytes indicates the situation of the researched territory in an area of temperate climate, but is also connected to the herbaceous nature of most medicinal plants, which present in the pastures and in the herbal cover of the forests. The phanerophytes, ligneous plants, also make up a considerable proportion from the totality of medicinal species. Therophytes, plants which go through their life cycle in a single vegetation period, are indicative of a more or less arid climate ; their repartition is conditioned by zoo-anthropoid influences and the existence of territories where the vegetal cover is discontinuous and ready to be occupied by annual plants.
The areal-geographic structure of the medicinal flora from the investigated territory shows the participation of numerous categories of elements with different florogenetic elements, offering at the same time information about the complex genepool of the flora from this area. The analysis of the floristic elements outlines the predomination of Eurasian species, who make up 43% (103 species) of the total number of MP (fig.2). To this category pertain widely distributed species, available in large quantities for gathering: *Achillea millefolium, Arctium lappa, Betula pendula, Chelidonioum majus, Cichorium intybus, Filipendula ulmaria, Fragaria vesca, Galium verum, Lamium album, Leonurus cardiaca, Lycopus europaeus, Melilotus officinalis, Plantago lanceolata, Primula veris, Salix alba, Tussilago farfara, Viola tricolor*. Against their background intervened in different phytohistoric stages European (9,5%) and Circumpolar (9,1%) elements. Among the 40 European species that were identified, common MP can be cited, like: *Ajuga reptans, Alchemilla vulgaris and A. xanthochlora, Anthyllis vulneraria, Bellis*

perennis, Covallaria majalis, Corylus avellana, Fraxinus excelsior, Ononis spinosa, Quercus robur, Q. petraea, Rosa canina, Viscum album. Circumpolar elements (22 species) are less frequent in the flora of the Banatian Mountains than the previous, are represented by: *Alisma plantago-aquatica, anagallis arvensis, Artemisia vulgaris, Asplenium scolopendrium, Chamerion angustifolium, Epilobium palustre, Erysimum cheiranthoides, Hepatica nobilis, Juniperus communis, Sanguisorba officinalis, Viburnum opulus.*

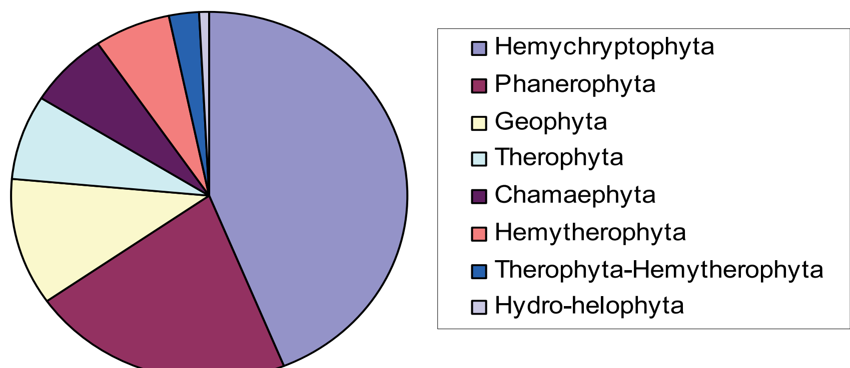

Fig. 1. The spectrum of bioforms for the medicinal flora of the Western part of the Banatian Mountains

The cosmopolite species (6,2 %) and the adventive ones (2,9%) also have a notable presence among the medicinal species. Although not represented through a large number of species, they occur in large populations. Among the 15 identified cosmopolite MP, *Capsella bursa-pastoris, Convolvulus arvensis, Equisetum arvense, Erodium cicutarium, geranium robertianum, Polygonum aviculare, Rumex acetosa, Urtica dioica* are very widespread. A particular problem is posed by *Pteridium aquilinum*, an extremely invasive fern, toxic to livestock, that continuously reduces the usable surface of pastures. The adventive species are represented by: *Ailanthus altissima, Erigeron canadensis, Matricaria discoidea, Oenothera biennis, Physalis alkekengi, Phytolacca americana* and *Xanthium spinosum*.

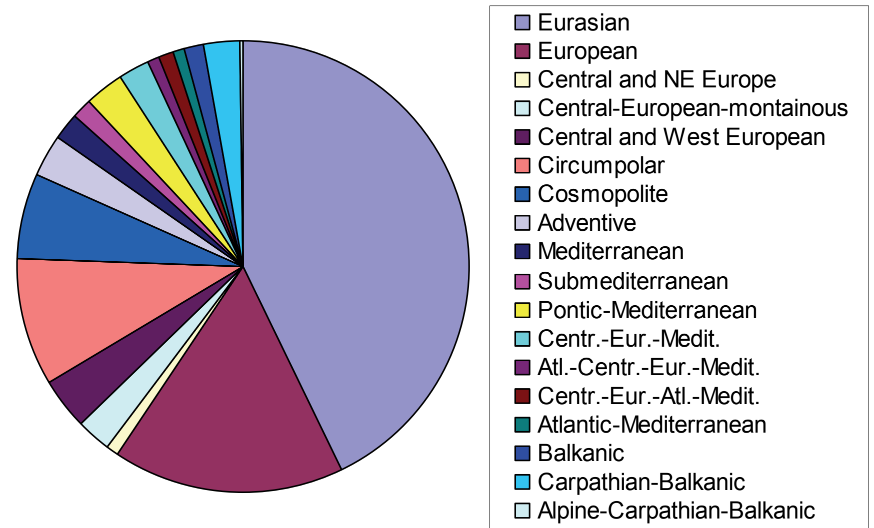

Fig. 2. The spectrum of floristic elements from the medicinal flora of the Western part of the Banatian Mountains

An important number of species, among which: *Ruscus aculeatus, Cotinus coggygria, Fraxinus ornus, Parietaria officinalis, Laburnum anagyroides, Sarothamnus scoparius, Tamus communis, Taxus baccata* testify to the Southern, Mediterranean, influence of the medicinal flora of the Banatian Mountains. There have been identified 3 taxa of Mediterranean, 3 of Submediteranean, 7 of Pontic-Mediteranean, 5 of Central European – Mediteranean, 5 of Central European – Atlantic – Mediteranean and 2 of Atlantic – Mediteranean origin. The Balkanic (*Helleborus odorus, Thymus jankae, Tilia tomentosa*) and Carpathian-Balkanic species (*Helleborus purpurascens, Jovibarba heufelii, Scopolia carniolica, Syringa vulgaris, Telekia speciosa, Thymus balcanus, Geranium macrorrhizum*) are represented in the researched area by only 10 taxons; however, they particularize the local medicinal flora when compared to other regions [14,15].

Conclusions

The rich medicinal flora (241 species) growing in the Western part of the Banatian Mountains shows the participation of numerous categories of elements with different florogenetic elements, offering at the same time information about the wealth of this area's genepool. The majority of medicinal plants pertain to the hemichryptophytes (44%), and are Eurasian, European and Circumpolar elements. An important number of species (25), among which: *Ruscus aculeatus, Cotinus coggygria, Fraxinus ornus, Parietaria officinalis, Laburnum anagyroides, Sarothamnus scoparius, Tamus communis, Taxus baccata* testify to the Southern influence of the medicinal flora of the Banatian Mountains. Along with the Balkanic and Carpathian-Balkanic species, they particularize the local medicinal flora.
The protection of the genepool of wild-growing MPs, according to international conventions like CITES ensures to some degree the sustainability of MP resources in the future. However, given the continuous augmentation of the demand for MPs and the incapacity of these plants to meet the ever increasing economic necessities, their growth in cultures will be representing a lasting solution for insuring the availability of sufficient plant material.

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RARE AND VULNERABLE SPECIES OF PHARMACEUTIC INTEREST FROM ANINEI MOUNTAINS (ROMANIA)

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Summary

The present study aimed at identifying the endangered, vulnerable, rare and endemic taxa from the flora of the Aninei Mountains, establishing in accordance to the criteria of the World Conservation Union, the Red List of Vascular Plants (RLVP) from this area. Ninety-five taxa were noted, among which five endemic and six sub endemic species were pointed out. The RLVP also contains several medicinal plants, wild-growing in the region; among these, Angelica archangelica is the most threatened one due to its gathering for pharmaceutical purposes.

Keywords: *rare and vulnerable taxa, endemic plants, medicinal species, Angelica archangelica*

Introduction

The protection of biodiversity represents a problem of present interest on a global level due to the continuous expansion of human impact on the environment.The knowledge of the rare/vulnerable species, as well of the endemic ones, is highly important from a scientific point of view, as critical and valuable genepool components are identified and their efficient protection is secured. The relevance of this matter goes beyond the purely theoretic, scientific aspect: The elucidation of the specific, particular biochimism of these plants, generally little researched, may reveal a series of new vegetal molecules with a large array of implications.
The plant kingdom is known to be a source of compounds with therapeutic activity [1]. Recent developments in the field of analytical chemistry and molecular biology make researches possible to be carried out on minute amounts of plant material [2] ; as such even rare plants may be investigated without putting them at risk. In case of positive findings, subsequent cultivation of the relevant plants could provide sufficient plant material for further large-scale research and economic utilization. A successful example in this regard is edelweiss (*Leontopodium alpinum* Cass.), a worldwide protected species. Investigated on the base of ethnobotanical sources [3], it was proved to contain an impressive array of compounds with therapeutic activity [4,5] ; researches are still ongoing. Cultures existing in countries like Switzerland provide today the necessary amounts of prime matter for the pharmaceutical and cosmetic industry.
Other applications emerging from the analysis of rare and endemic plants are in the area of chemotaxonomy, giving the possibility to define phylogenetic connections between species, as well as in to uncover specific chemovars and geographic races.
The premises for the potential valorization under any aspect remain however the knowledge of the present status of the rare/vulnerable plant patrimony. In this concept, we proceeded to the identification of these plants in the Aninei Mountains, an area situated at the interference of air masses with maritime and continental character, having a particular carstic relief, and hosting a series of rare Romanian and European species [6,7]. The species-specific mapping of the areas where vulnerable medicinal plants grow was also performed.

Material and Methods

The red list of medicinal vascular plants containing also other numerous plants has been elaborated according to our research and to bibliographical information vailable from: *The*

World Conservation Monitoring Centre (WCMC) [8], *The World Conservation Union* (IUCN) [9], the *Law nr. 13/1993* regarding Romania joining the *European Habitats* and *Wild Life Convention [10], The* red *list of vascular plants in Romania* - Oltean et al.(1994) [11]; *The* red *list of rare, vulnerable endangered, and disappeared vascular plants in Romanian flora* - N. Boşcaiu et al. (1994) [12]; *The red list of the Romanian plants occurring in meadow, including endemic and sub-endemic species* (*Tracheophyta*) - G. Negrean (2001) [13] and other sources [14-18]. The periclitation categories used in this work are :

Endangered = **E**: taxa in danger of extinction ; their survival is improbable if the causal factors continue to operate

Vulnerable = **V**: taxa considered to be ready to pass in the endangered category in the near future, if the causal factors continue to operate

Rare = **R**: taxa with small world-wide populations, which presently are not threatened, but under risk (due to their restrictive areal). In case of certain species, Oltean et al (1994) use the supplementary hybrid category **V/R**: ***vulnerable and rare***.

Indeterminate = **I**: taxa known to pertain to one of the previous categories, but for which there is not sufficient information to specify the category

Insufficiently known = **K**: taxa which are suspected but not definitely known to pertain to one of the above categories (due to lack of information)

Relative to the local peculiarities of the flora, the endemism code used in this work is the one applied by UNEP-WCMC:

A: areal restrained to only one area; only the taxa known within the limits of the national territory are included (i.e. the species endemic to Romania)

B: areal extended to more than one area in the same region; this category includes taxa whose areal does not exceed the limits of Europe

b: subendemic taxa ; their areal exceed the limits of the national territory, but is extending only to the zones situated nearby.

The nomenclature used for the listed taxa is the one established in Flora Europaea [19].
Mapping procedures were performed according to the TK25 system, used in the mapping of the Flora of Central Europe [20,21].

Results and Discussions

The investigation of the flora of the Aninei Mountains revealed the presence of 95 taxa that could be included in one of the periclitation categories according to criteria of the World Conservation Union. Among these, 1 taxon is endangered at European level; 5 taxa are vulnerable; 80 taxa are rare; 7 taxa are vulnerable and rare; 1 taxon has indeterminate status; and 1 taxon has an "insufficiently known" status (table 1). Regarding the distribution areal of the taxa from the RLVP, 5 are endemic to Romania, 6 are subendemic, whereas 6 have a repartition limited to the European territory. The endemic element is mostly represented by species and subspecies that vegetate on rocks of limestone: *Athamanta turbith* ssp. *hungarica*, *Linum uninerve* and *Sorbus borbasii*; *Dianthus giganteus* ssp. *banaticus* vegetates in the mesophyllous pastures from Cheile Nerei, Cheile Gârliştei, the Lisovacea reservation, whereas *Primula auricula* ssp. *serratifolia* is present on poor, scheletic soils, in the reservations Izvorul Bigăr, Ducin and Lisovacea.

The taxa from the RLVP belong to 28 botanical families; about one quarter of the rare or vulnerable species (25.3%) are *Orchidaceae*. Other families, like *Brassicaceae* (7.4%), *Liliaceae* (6.3%), *Apiaceae* and *Caryophyllaceae* (5.2%) account for a smaller number of species.

Comparing the number of taxa from the RLVP established in the this study with the data received from the Environment Protection Agency Resita (APMR) [22], it can be noted that the our list, elaborated according to IUCN and UNEP-WCMC criteria, includes a significantly larger number of species. In fact, the number of vegetal taxa protected on the territory of all

the eight mixed reservations from the Aninei Mountains, and accounted for by the APMR, is only 59, in comparison with 95. The most evident difference appears in case of the reservation Cheile Nerei-Beuşniţa, where only 16 taxa are noted on the APMR lists [22], whereas 82 taxa could be identified as being protected on a European level. In order to insure a more efficient protection of rare and vulnerable species within the legal framework, the data from the present study will be transmitted to the relevant agency.

Table 1. The Red List of Vascular Plants from the Aninei Mountains

SPECIES / FAMILY	LOCALIZATION	IUCN Categ.
Acanthus balcanicus Heywood et I.B.K. Richardson (*Acanthaceae*)	Cheile Nerei	V/R
Aegilops neglecta Req. ex Bertol. (*Poaceae*)	Beuşniţa Basin	R
Aethionema saxatile (L.) R.Br. var. *heterocarpus* Beck. (*Brassicaceae*)	Cheile Nerei, Pânza Albinii, Cârşia Beuşniţei	R
Allium carinatum L. (Liliaceae)	Cheile Nerei	K
Allium moschatum L. (*Liliaceae*)	Cheile Nerei, Beul V.	R
Alyssum montanum L. ssp. *gmelinii* (Jordan) Hegi et Schmidt (*Brassicaceae*)	Pk.Cetăţuia, Cheile Nerei	R
Alyssum wierzbickii Heuffel (*Brassicaceae*)	Pleşiva Pk. , Cornetul Înalt Pk.	B R
Anacamptis pyramidalis (L.) Rich. (*Orchidaceae*)	Beuşniţa Basin	V/R
Angelica archangelica L. (*Apiaceae*)	Bârzava and Miniş meadows	V
Anthericum liliago L. (*Liliaceae*)	Cheile Nerei	R
Aquilegia nigricans Baumg. (*Ranunculaceae*)	Valea Rea	V
Astragalus onobrychis L. *ssp. banaticus* (Roch.) Jav. (*Fabaceae*)	Cheile Nerei	R
Athamanta turbith (L.) Brot. ssp. *hungarica* (Borb.) Tutin (*Apiaceae*)	Cârşa Beuşnitei, Cârşa Cornetului Înalt Pk., Cheile Nerei (Cârşa Şoimului)	A R
Campanula grossekii Heuffel (*Campanulaceae*)	Cheile Nerei	R
Carex brevicollis D.C. (*Cyperaceae*)	Lespedea Plateau,Beul Sec V.	I
Centaurea atropurpurea Waldst. et Kit (*Asteraceae*)	Cheile Nerei	R
Centaurea calvescens Pancic (*Asteraceae*)	Cheile Nerei	B R
Cephalaria laevigata (Waldst. et Kit.) Schrader (*Dipsacaceae*)	Cheile Nerei, Beul Sec V.	R
Cerastium banaticum (Rochel) Heuffel (*Caryophyllaceae*)	Cheile Nerei, Beul Sec V.	R
Coronilla emerus L. ssp. *emeroides* (Boiss. et Spruner) Hay. (*Fabaceae*)	Cheile Nerei	R
Corylus colurna L. (*Corylaceae*)	Cheile Caraşului; Ch. Nerei; Pleşiva Pk.	V/R
Crocus banaticus Gay (*Iridaceae*)	Cheile Nerei	R
Crocus flavus Weston (*Iridaceae*)	Cheile Nerei	V
Dactylorhiza incarnata (L.) Soo (*Orchidaceae*)	Cheile Nerei	R
Dactylorhiza maculata (L.) Soo (*Orchidaceae*)	Cheile Nerei	R
Dactylorhiza sambucina (L.) Soo (*Orchidaceae*)	Cheile Nerei	R
Dianthus giganteus D'Urv. ssp. *banaticus* (Heuffel) Tutin (*Caryophyllaceae*)	Cheile Nerei; Ch. Gârliştei; Lisovacea	A R
Dianthus petraeus Waldst et Kit. ssp. *simonkaianus* (Petrfi) Tutin (*Caryophyllaceae*)	Cheile Nerei, Beu V.	B R
Dianthus trifasciculatus Kit. ssp. *deserti* (Prod.) Tutin (*Caryophyllaceae*)	Beul Sec V., Cheile Nerei, Cetăţuia Pk; Cheile Gârliştei	B R
Digitalis ferruginea L. (*Scrophulariaceae*)	Lespedea Plateau	V/R

SPECIES / FAMILY	LOCALIZATION	IUCN Categ.
Dorycnium pentaphyllum Scop. ssp. *germanicum* (Gremli) Gams (*Fabaceae*)	Cheile Nerei	B R
Echium russicum J.F. Gmelin (Boraginaceae)	Cheile Nerei	B E
Epipactis helleborine (L.) Crantz (Orchidaceae)	Bigăr Spring, Gura Golumbului	R
Epipactis microphylla (Ehrh.) Schwarz (*Orchidaceae*)	Cheile Nerei	R
Epipactis palustris (L.) Crantz	Cheile Nerei	R
Epipogium aphyllum Schwarz *(Orchidaceae*)	Cheile Nerei	R
Erysimum comatum Pancic. (*Brassicaceae*)	Cheile Nerei	R
Erysimum crepidifolium Reichenb. (*Brassicaceae*)	Cheile Nerei, Sasca Montană	R
Euphorbia myrsinites L. (*Euphorbiaceae*)	Cheile Nerei, on Pânza Albinii	V/R
Gymnadenia conopsea (L.) R.Br. (*Orchidaceae*)	Cheile Nerei	R
Himantoglossum hircinum (L.) Sprengel (*Orchidaceae*)	Beuşniţa Basin	R
Iris reichenbachii Heuffel (*Iridaceae*)	Cerbonia	b R
Jovibarba heufelii (Schott) A.et D. Löve (*Crassulaceae*)	Cheile Nerei, Cheile Caraşului, Ogaşul Ursului, on lime rocks	R
Leucojum vernum L. (*Amaryllidaceae*)	Platoul Pleşiva	V
Limodorum abortivum (L.) Schwarz (*Orchidaceae*)	Cheile Nerei	R
Linum uninerve (Rochel) Jav. (*Linaceae*)	Cheile Nerei	A R
Listera ovata (L.) R.Br. (*Orchidaceae*)	Cheile Nerei	R
Lunaria annua L. ssp. *pachyrhiza* (Borbas) Hayek (*Brassicaceae*)	Beuşniţa Basin, Cheile Nerei, Bel Sec V., Sasca Montană	R
Lychnis viscaria L. ssp. *atropurpurea* (Griseb.) Chater (*Caryophyllaceae*)	Cheile Nerei, near the Beul Bridge	R
Neottia nidus-avis (L.) L.C.M. Richard (*Orchidaceae*)	Cheile Nerei	R
Onobrychis alba (Waldst. et Kit.)Desv. (*Fabaceae*)	Beul Bridge; Beul V.	R
Onosma heterophylla Grieseb. (*Boraginaceae*)	Beul V., Cetăţuia Pk., Sasca Ro	b R
Ophrys fuciflora (F.W. Schmidt) Moench (*Orchidaceae*)	Cheile Nerei	R
Ophrys scolopax Cav. ssp. *cornuta* (Steven) Canus (*Orchidaceae*)	Cheile Nerei	R
Orchis coriophora L. (*Orchidaceae*)	Nera V.	R
Orchis laxiflora Lam. ssp. *elegans* (Heuffel) Soo (*Orchidaceae*)	Nera V.	R
Orchis morio L. (*Orchidaceae*)	Cheile Nerei, Cheile Caraşului	R
Orchis pallens L. (*Orchidaceae*)	Cheile Nerei	R
Orchis papilionacea L. (*Orchidaceae*)	Cheile Nerei	R
Orchis purpurea Hudson (*Orchidaceae*)	Cheile Nerei	R
Orchis tridentata Scop. (*Orchidaceae*)	Cheile Nerei	R
Ornithogalum sphaerocarpum A. Kerner *(Liliaceae)*	Lespedea Plateau, Beul Sec V.	R
Paeonia mascula (L.) Miller (*Paeoniaceae*)	Cheile Nerei, Ducin V.	V/R
Peucedanum longifolium Waldst. et Kit. (*Apiaceae*)	Cheile Nerei	b R
Piptatherum holciforme (Bieb.) Roemer et Schultes (*Poaceae*)	Beuşniţa	R
Platanthera bifolia (L.) L.C.M. Richard (*Orchidaceae*)	Cheile Nerei	R
Primula auricula L. ssp. *serratifolia* (Rochel) Jav. (*Primulaceae*)	Bigăr Spring; Ducin, Lisovacea	A R

SPECIES / FAMILY	LOCALIZATION	IUCN Categ.
Ranunculus flabellifolius Heuffel ex Reichenb. (*Ranunculaceae*)	Beuşniţa Basin	b R
Rorippa prolifera (Heuffel) Neilr. (*Brassicaceae*)	Nera V.	R
Rosa stylosa Desv. (*Rosaceae*)	Cheile Nerei, Beuşniţa Basin	R
Rubus bifrons Vest ex Tratt. ssp. *banaticus* (Nyar.) Ciocârlan	Sasca Română	R
Ruscus aculeatus L. (*Liliaceae*)	Cheile Caraşului, Ch. Nerei; Lisovacea, Bigăr Spring; Ducin	R
Ruscus hypoglossum L. (Liliaceae)	Ch. Nerei, Poiana Florii; Pleşiva Plateau; Păuleasca-Poiana Florii area; Bigăr Spring; Lisovacea	R
Satureja montana ssp. *kitaibelii* (Wierzb.) P.W.Ball (*Lamiaceae*)	Cheile Nerei, Cheile Caraşului	R
Saxifraga bulbifera L. (*Saxifragaceae*)	Pleşiva Pk.	R
Saxifraga marginata Sternb. (Saxifragaceae)	Pleşiva Pk.	R
Scrophularia heterophylla Willd. ssp. *laciniata* (Waldst. et Kit.) Maire et Petitmengin (*Scrophulariaceae*)	Cheile Nerei	R
Scutellaria velenovskyi Rech.fil. (*Lamiaceae*)	Beuşniţa Basin	R
Secale montanum Guss. (*Poaceae*)	Pleşiva Mică Pk.	R
Sedum cepaea L. (*Crassulaceae*)	Cheile Nerei; Cheile Caraşului	R
Sempervivum marmoreum Griseb. (*Crassulaceae*)	Cheile Nerei; Cheile Caraşului	R
Sempervivum montanum L. (*Crassulaceae*)	Pleşiva Mică Pk., Cornetul Înalt Pk., Pleşiva Pk.	R
Seseli gracile Waldst. et Kit. (*Apiaceae*)	Cheile Nerei	b R
Seseli rigidum Waldst. et Kit. (*Apiaceae*)	Cheile Nerei	b R
Sesleria filifolia Hoppe (*Poaceae*)	Cheile Nerei, Ch. Caraşului, Ch. Minişului	R
Silene flavescens Waldst. et Kit. (*Caryophyllaceae*)	Cheile Nerei	R
Silene saxifraga L. ssp. *petraea* (Waldst. et Kit.) Gusul. (*Caryophyllaceae*)	Cârşa Beuşniţei, Cornetul Înalt Pk.	R
Sorbus borbasii Jav. (*Rosaceae*)	Cârşa Beuşniţei, Cornetul Înalt Pk.	A R
Spiranthes spiralis (L.) Chevall. (*Orchidaceae*)	Cheile Nerei	R
Symphytum ottomanum Friv. (*Boraginaceae*)	Cheile Nerei, V. Rea	R
Taxus baccata L. (*Taxaceae*)	Cheile Nerei, under Cârşa Şoimului, Beuşniţa Basin; Bigăr Spring	V/R
Thymus dacicus Borb. (*Lamiaceae*)	Cheile Nerei	V
Valerianella coronata (L.) D.C. (*Valerianaceae*)	Cheile Nerei, betwen the Beul Bridge and Sasca Română	R
Verbascum vandasii (Rohlena) Rohlena (*Scrophulariaceae*)	Cheile Nerei	R
Veronica crassifolia Wierzb. ex Heuffel (*Scrophulariaceae*)	Cheile Nerei	R

A - endemic to Romania; b - subendemic; B – European areal; E - endangered; V - vulnerable; R - rare; V/R - rare and vulnerable; I - indeterminate; K – insufficintly known (definition of terms detailed under Material and Methods); Pk. – peak; V. – valley

The analysis of the bioforms reveals the presence of a large number of geophytes among the rare plants (36%); this fact is mostly due to the import number of *Orchidaceae* among the

species of the RLVP. The hemichryptophytes (34%) testify to the belonging of the studied mountains to an area with temperate climate; the therophytes (5%), indicative of a more or less arid climate and zoo-anthropic influences, have the lowest occurrence among the rare plants.

The flora of the studied region displays many thermophile elements, which either resisted in these refuges during the glaciations, or migrated later into the Balkans. Southern species, intricate with Balkanic, Eurasian, European, Central European, Continental, Pontic, Atlantic and others, constitute complex phytocenoses on different altitude floors. Among the rare Balkanic taxa identified in the Aninei Mountains are: *Acanthus balcanicus, Alyssum wierzbickii, Centaurea atropurpurea, Crocus flavus, Iris reichenbachii, Onosma heterophylla, Silene saxifraga* ssp. *petraea* etc. Together with the Daco-Balkanic taxa (*Saxifraga marginata, Scophularia heterophylla ssp. laciniata, Sempervivum marmoreum, Thymus dacicus*), the Balkanic elements represent more than one third of the taxa listed in the RLVP. The Dacic elements are especially present on the rocky slopes, as well as in the reliquary forest phytocenoses. Also on the limestone rocks, Southern, Mediterranean elements can be encountered (*Sedum cepaea*). The phytocenoses from the subxerophyllous turkey oak and italian oak forests, and the xerotermophyllous bushes, as well as the saxicol ones, are the richest in rare species; the reservations established on the territory of the Aninei Mountains mostly protect the floristic diversity of these formations.

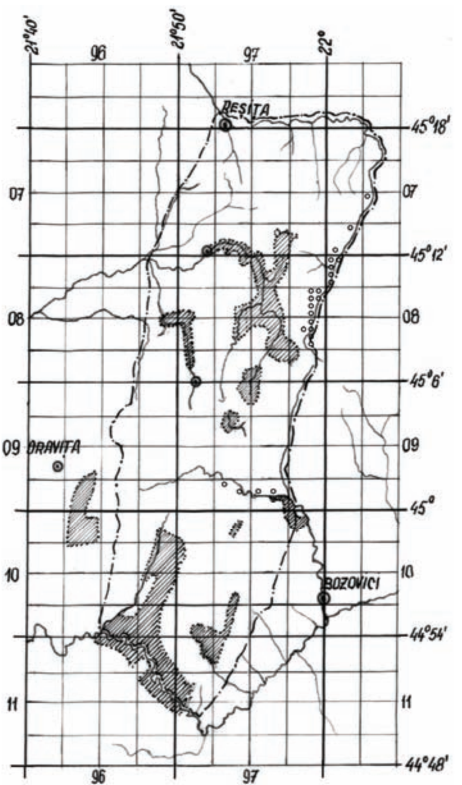

Fig. 1. The repartition of *Angelica archangelica* in the Aninei Mountains (lined surface: reservations)

Among the rare/vulnerable species that vegetate in the investigated mountains, some have an already firmly established place among the medicinal plants (*Angelica archangelica, Ruscus aculeatus, Orchis morio*), other are known in the folk medicine of the area (*Acanthus balcanicus* – respiratory diseases, *Jovibarba heufelii* – ear ailments). Most of the species listed on the RLVP have not been investigated from a phytochemical point of view, but researches performed on related taxa suggest a promising phytotherapeutic potential. Some examples in this regard are : *Sedum* sp. and *Sempervivum* sp. [23], *Ruscus hypoglossum* [24], *Silene* sp. [25], *Erysimum* sp. [26].

Well-known for the stimulating effect upon the digestion, the subterranean parts of *Angelica archangelica* have been gathered since hundreds of years. Even today, when this plant is under the protection of the law in Romania, roots and rhizomes of angelica are still taken from wild-growing plants, for pharmaceutical purposes. In the Aninei Mountains, this species occurs sporadically, in groups of 2-3 individuals, along the superior course of the Miniş river, but mostly on the inferior course of the Bârzava, starting from the Bârzăvița canton till after Văliug, in the area of the Breazova lake (fig. 1). No inhabitant or representative of the Sylvic Protection knows the fact that this plant is under protection. This situation should be changed, else the danger of the disappearance of the species becomes imminent.

Other medicinal species, like *Ruscus aculeatus, Orchis morio, Acanthus balcanicus* are only rarely utilized in local folk medicine, and their populations are stable. *Jovibarba heufelii* enjoys a good protection due to its vegetation on rather inaccessible, steep lime slopes; additionally its utilization by locals is very limited. However, the existence of *Taxus baccata*

is more and more endangered, as its populations decline both in areal, as well in the number of individuals.

Conclusions

In the flora of the Aninei Mountains, 95 taxa were included on the Red List of Vascular Plants, due to their rare/vulnerable/endangered status. The completion of the lists of the relevant Environment Protection agency will insure their better protection.
Supplementary measures must be taken in case of plants gathered in medicinal purposes, especially *Angelica archangelica*; information of the representatives of the Sylvic Protection upon the occurrence and status of these species will be performed. The introduction of angelica into culture in the Bârzava meadow could also be a good solution, as the vegetation conditions are favorable and the area has a good accessibility.

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MORPHOLOGICAL RESEARCHES REGARDING THE INFLUENCE OF TOPSIN M TREATMENTS ON *MENTHA LONGIFOLIA* (L.) HUDS. SPECIES. NOTE 2.

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Summary

Topsin M is a common systemic fungicide used as protective/curative substance for alimentary and medicinal plants. That's why it's relevant to evaluate the influence of Topsin M upon the morphological features of Mentha longifolia, a volatile oil producing medicinal plant and parental species for the hybrid Mentha × piperita. We noticed statistical significant variations (Oneway Anova) of the dimensional features of the leaves from Topsin M treated plants comparing to the control.

Keywords: *Mentha longifolia, Topsin M, morphological features*

Introduction

The abusive and disordered employment of pesticides have the potential to harm human health and the environment and pose ecological risk to ecosystems.
Because of possible health effects, widespread use and insufficient data, pesticide monitoring in plants is necessary. Some of the pesticide monitoring aspects refere to the investigation of the pestide influence on the morphological features of medicinal plants.
Data from literature report that pesticides may affect morphological features of plants, as following:

- smaller and fewer leaves, smaller axial organs (carbamate and amido-type erbicides (5);
- depigmentations along the nervures, necrosis at the edge of the leaves (urea and sulphonylurea derivates; alchyl N-phenylcarbamates and alchyl-N-phenylthiocarbamates) (1, 2, 3);
- variation of the lenght of the leave limb and the lenght of the whole plant (fungicides) (4).

Topsin M is a common systemic fungicide used as protective/curative substance for alimentary and medicinal plants. That's why it's relevant to evaluate the influence of Topsin M upon the morphological features of *Mentha longifolia*, a volatile oil producing medicinal plant and parental species for the hybrid *Mentha × piperita*.

Materials and Methods

Plant materials were brought from the experimental lots in "Anastasie Fatu" Botanical Garden, Iasi. In this experimental area, parallel cultures of *Mentha longifolia* (L.) Huds. have been made in 2001-2003 period.
Thus, every year there have been two experimental fields: a field which had no pesticide treatment (control field) and a field which had been treated with pesticide.
The antifungal treatment was achieved in vegetative phase by spraying a wettable powder of Topsin M 70 PU (TM) (Oltchim Rm. Valcea-Romania) as 0,1% and 0,4% aqueous solutions.
Investigated samples of *Mentha longifolia* are presented in table 1:

Table 1. Samples of *Mentha longifolia*

Nr.	Sample	Codification
1.	Control 2001	M.l. M 2001
2.	Treatment TM 0,1% 2001	M.l. TM 0,1% 2001
3.	Control 2002	M.l. M 2002
4.	Treatment TM 0,4% 2002	M.l. TM 0,4% 2002
5.	Control 2003	M.l. M 2003
6.	Treatment TM 0,1% 2003	M.l. TM 0,1% 2003
7.	Treatment TM 0,4% 2003	M.l. TM 0,4% 2003

The morphological study was achieved through the analysis of the parameters concerning aspect, colour and dimensions of the leaves (officinal product) from both treated and untreated plants, as following:

- the shape;
- the edges, base and top;
- covered hairs for the both faces of leaves;
- the length and breadth (cm), depending of the leaf insertion: top, middle or the base of stem. For each type of leave, for each variant of treatment and for each control, there have been made 10 measurements of the studied parameter.

The dimensional parameters data were statistically analysed through the *Oneway Anova* method using J.M.P. Programme 5.0.1.2. (SAS Institute, Cary N.C., S.U.A).

Results and discussion

Mentha longifolia leaves from both untreated (fig. 1) and TM 0,1% (fig. 2) and TM 0,4% (fig. 3) treated plants have the some morphological features of the leave.

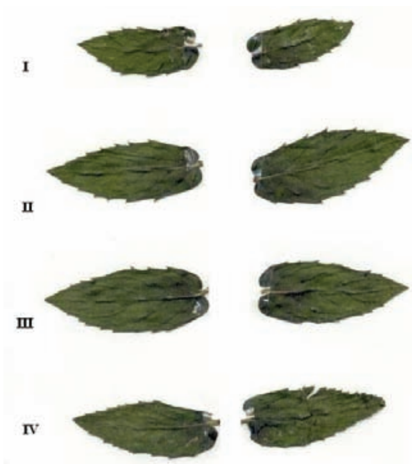

Fig. 1. Control plants

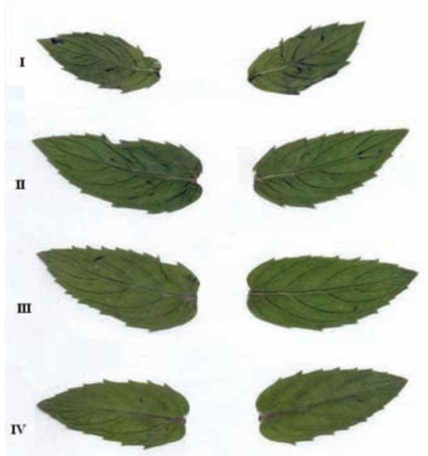

Fig. 2. TM 0,1% treated plants

Fig. 3. TM 0,4% treated plants

The leaves lanceolate or oblong were sessile and arranged opposite on stem.
The tip of the limb is sharp and the base is narrowed.The edges of the leaves are convex and almost parallel and have triangular teeth, 1-4 mm distanced forward orientated.
The nervation is pennate and proeminent on the lower surface. Leaves have covered hairs placed especially along the nervures on the lower surface.
The leaves are dark green on the upper surface and lighter on the lower surface.
The powdered leaves have an aromatic, characteristic smell.
The dimensional parameters of both untreated and treated vegetal material are shown in table II, as minimal and maximal values registered for each type of leave.

Table 2. The dimensional parameters (cm) of foliar limb of *Mentha longifolia*

Year	Sample	Top leaves		Middle leaves		Basal leaves	
		lenght	breadth	lenght	breadth	lenght	breadth
2001	M.l. M	2.0 – 2.5	0.9 – 1.7	3.0 – 4.2	1.2 – 1.6	2.8 – 4.1	1.0 –1.7
	M.l. TM 0,1%	2.4 – 2.9	1.4 - 1.9	3.2 – 4.2	1.3 – 1.8	3.0 – 4.4	1.2 – 1.9
2002	M.l. M	2.3 – 2.9	1.1 – 1.6	3.5 – 4.3	1.2 – 1.8	3.0 – 4.3	1.3 – 1.6
	M.l. TM 0,4%	1.9 – 2.9	0.8 – 1.4	3.6 – 4.2	1.1 – 1.6	3.0 – 4.1	1.2 – 1.6
2003	M.l. M	1.7 – **2.3**	0.7 – 1.2	3.5 – 4.3	1.4 – 1.6	3.0 – **5.0**	1.3 – 1.9
	M.l. TM 0,1%	2.3 – **3.3**	1.2 – 1.9	3.8 – 4.4	1.7 – 2.0	2.9 – **3.8**	1.3 – 1.8
	M.l. TM 0,4%	2.4 – **4**	1 - 1.9	3.9 – 4.8	1.2 – 2.1	2.7 – **3.6**	1.3 – 1.9

The dimensional parameters data were statistically analysed through the *Oneway Anova* method using J.M.P. Programme 5.0.1.2. (SAS Institute, Cary N.C., S.U.A) (6).
Statistical data (x - mean; SD - standard deviation; p*- indicator for evaluation of statistical significance) were presented in tables 3 and 4:

Table 3. Biometrical data of *Mentha longifolia* leaves – the length of foliar limb (cm)

Nr.	**Sample**	**Top leaves**			**Middle leaves**			**Basal leaves**		
		x	**SD**	**p***	**x**	**SD**	**p***	**x**	**SD**	**p***
1.	M.l. M 2001	2.23000	0.176698	**0.0006**	3.71000	0.369534	0.6643	3.22000	0.456557	0.6619

2.	M.l. TM 0,1% 2001	2.57000	0.188856		3.78000	0.339280		3.32000	0.545283	
3.	M.l. M 2002	2.61000	0.228279	0.3450	3.82000	0.342540	0.4504	3.39000	0.472464	0.8614
4.	M.l. TM 0,4% 2002	2.49000	0.317805		3.92000	0.225093		3.43000	0.535516	
5.	M.l. M 2003	2.10000	0.240370	**0.18409**	4.02000	0.274064	- 0.16502	4.39000	0.645411	**0.47821**
6.	M.l. TM 0,1% 2003	2.77000	0.394546		4.14000	0.206559		3.39000	0.369534	
7.	M.l. TM 0,4% 2003	2.94000	0.602218	**0.35409**	4.46000	0.283627	**0.15498**	3.17000	0.333500	**0.69821**

It is considered that the results are statistically significant if **p*** ***is lower than 0.05***, in case of 2001 and 2002 samples, or if **p* has a positive value** for 2003 samples

Table 4. Biometrical data of *Mentha longifolia* leaves – the breadth of foliar limb (cm)

Nr.	Sample	Top leaves			Middle leaves			Basal leaves		
		x	SD	p*	x	SD	p*	x	SD	p*
1.	M.l. M 2001	1.24000	0.250333	**0.0008**	1.41000	0.137032	**0.0400**	1.30000	0.262467	**0.0855**
2.	M.l. TM 0,1% 2001	1.63000	0.176698		1.57000	0.182878		1.52000	0.278089	
3.	M.l. M 2002	1.36000	0.171270	0.0150	1.48000	0.161933	0.3386	1.42000	0.113529	0.3618
4.	M.l. TM 0,4% 2002	1.15000	0.177951		1.40000	0.200000		1.47000	0.125167	
5.	M.l. M 2003	1.01000	0.172884	**0.18511**	1.52000	0.063246	**0.14982**	1.69000	0.196921	- 0.19197
6.	M.l. TM 0,1% 2003	1.44000	0.195505		1.84000	0.107497		1.66000	0.142984	
7.	M.l. TM 0,4% 2003	1.34000	0.279682	**0.08511**	1.72000	0.234758	**0.02982**	1.61000	0.246982	- 0.14197

The statistical data reveal that the following dimensional variations compared to the corresponding controls are significant:

- increase in lenght and breadth of the top leave limb at the 0,1% treated plants;
- increase in breadth of the middle leaves limb at 0,1% treated plants as well as an increase of lenght and breadth of the same leave at 0,4% treated plants;
- decrease in lenght of basal leaves limb at TM 0,1% and TM 0,4% treated plants;
- increase in breadth of basal leaves at TM 0,1% treated plants.

Conclusions

Antifungal treatment with Topsin M did not affect morphological features of *Mentha longifolia* leaves, excepting dimensional features.

We noticed statistical significant variations (*Oneway Anova*) of the dimensional features of the leaves from Topsin M treated plants comparing to the control, as following:

- lenght and breadth of top leaves limb increase at 0,1% TM treated plants;
- lenght and breadth of middle leaves limb increase at 0,4% TM treated plants;
- only the breadth of middle and basal leaves limb increase at TM 0,1% treated plants;
- length of basal leaves limb decrease at TM 0,1% treated plants.

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CONSERVATION OF MEDICINAL AND AROMATIC PLANTS IN EUROPE – A REVIEW OF CURRENT PROGRESS

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Summary

Conservation directives of ECP/GR MAP WG, which are congruent with Global Strategy for Plant Conservation (GSPC) and European Plant Conservation Strategy (EPCS), are presented. In situ conservation of MAP genetic resources in member countries will help to maintain the biodiversity of endangered, rare and vulnerable species. Domestication of these species is an important task of conservation programs and involves regeneration of the collected seed material of surveyed populations in order to assure the MAP material for their characterization, evaluation and cultivation in the future.

Keywords: medicinal and aromatic plants, conservation, directives, descriptors

Introduction

Studies on the uses of natural genetic resources in different cultural environments show a strong relation between dietary habits and health of local population. Many wild plants were used by indigenous peoples both as a food and medicine (Johns, 1999; Pieroni and Heinrich, 2003; Rivera *et al*., 2005). Besides characterization of environmental factors that affect existence and distribution of natural genetic resources, the knowledge on traditional habits, of ethnobotanical and general ethnographic characteristics of a particular area all serve as a professional ground for prospecting for those wild genetic resources that can be of use in a diet and/or medicine of local inhabitants (Heinrich, 2002; Heinrich, 2003; Bremner *et al*., 2004; Heinrich *et al*., 2005). The existence of traditional medicine basically depends on plant species diversity and the related knowledge of their use as herbal drugs. In addition, both plant species and traditional knowledge are important to the herbal medicine trade and the pharmaceutical industry, whereby plants provide raw materials and the traditional knowledge prerequisite information (Tabuti *et al*. 2003). Together with growth in global demand for medicinal plants and in local demand for plant based traditional medicines, the pressure on the existing populations of medicinal plants has increased tremendously during the last few decades (http://www.tifac.org.in/offer/tlbo/rep/S061.htm). Historically, most of these plants grew in wild as a natural component of vegetation of a particular region. The necessary plant material (roots, barks, leaves) have been collected and sold by the local people to the traders and the industry and exporters purchased them from traders. Since there was no scientific system of collecting or regenerating these plants in past, several plants have either been completely lost or have become endangered.

In order to stop further biodiversity loss of natural resources of medicinal and other socio-economically important plants, modern scientific methods must evaluate remaining stocks of these populations in their natural habitats and secure their sustainable and permanent use (Johns and Eyzaguirre, 2000; Johns and Eyzaguirre, 2002; Johns, 2002; Troppman *et al*., 2002) in order to conserve an essential part of our natural and cultural heritage.

The main general aim and long-term goal of conservation of target species in their natural habitats is to protect, manage and monitor the selected populations in the direction of maintenance of the natural evolutionary processes, thus allowing new variation to be generated in the gene pool that will allow the species to adapt to changing environmental

conditions such as global warming, changed rainfall patterns, acid rain or habitat loss (Heywood, 2004).
In many countries the excessive collection of wild plants was often combined with a loss of habitat that resulted in a severe decrease of certain plant species. For that reason domestication of wild medicinal and other socio-economically important plants is necessary to allow intensive production to meet high demand - especially in the period of global trade, massive and expanding markets for medicinal and herbal products. In the history of human civilisation domestication of wild edible plant species and their agricultural production was the key anthropogenic contribution to the production of agricultural surpluses, capable of supporting urban communities. The maintenance and sustainable management of natural habitats - ecosystems and biological resources is possible only after the basic decision change from wild-gathering to agricultural production of any raw materials that has been subjected to the growing commercial demand.

MAP biodiversity conservation in the light of global strategies and of EU legislation
When considering conservation and use of plant genetic resources of potential use in food and agriculture (that have been assigned also as neglected, underutilized species, minor crops or medicinal and aromatic plant species), community legislation that has an immediate legal effect on the member states shall take into account relevant international processes, developments and agreements, in particular as regards: the Convention on Biological Diversity, the International Treaty on Plant Genetic Resources for Food and Agriculture, the FAO's Global Plan of Action for the Conservation and Sustainable Utilisation of Plant Genetic Resources for Food and Agriculture, the European Plant Conservation Strategy and programmes implemented under international frameworks such as the European Cooperative Programme for Crop Genetic Resources Networks (ECP/GR), and the Consultative Group on International Agricultural Research (CGIAR).
The Convention on International Trade in Endangered Species (CITES), which layed down provisions for the protection of endangered species of flora and fauna, represents controls on international trade in specimens of these species and is the basis of a worldwide policy on protection of endangered species (Council Regulation (EC) No. 338/97, Commission Regulation (EC) No. 939/97, Commission Regulation (EC) No.1808/2001, Commission Regulation (EC) No 1497/2003**,** Commission Regulation (EC) No 834/2004).
Council Regulation (EEC) No 2092/91 of 24 June 1991 on organic production of agricultural products (Annex I, point 4) lays down provisions on sustainable collection of edible plants and parts thereof, growing naturally in natural areas, forests and agricultural areas, postualating that the collection should not affect the stability of the natural habitat or the maintenance of the species in the collection area. In addition, the German Federal Agency for Nature Conservation (BfN), WWF and TRAFFIC and the Medicinal Plant Specialist Group (MPSG) of the IUCN Species Survival Commission have developed a strong base of expertise and knowledge and prepared practical standards and performance criteria in the field of sustainable medicinal and aromatic plant wild-crafting (Klingestein *et al*., 2004). Their efforts have resulted in an International Standard for Sustainable Wild Collection of Medicinal and Aromatic Plants (ISSC-MAP) (Final draft of April 2006). However, when wild collecting is the source for covering the demand on the expanding market of raw materials, especially in the period of lacking the attention to illegal and unsustainable trade, one of the major concerns still remains the risk of the loss of the plant species' ability to regenerate, when quantities of harvested material exceed the population limit.
On 26 June 2003, EU farm ministers adopted a fundamental reform of the Common Agricultural Policy (CAP, Official journal L270 – 10/21/2003). On September 29, 2003, the Agriculture Council formally adopted the legal texts of the June 2003 CAP Reform

agreement. The CAP's objectives include helping agriculture to fulfil its multifunctional role in society: producing safe and healthy food, contributing to sustainable development of rural areas, and protecting and enhancing the status of the farmed environment and its biodiversity. The reform will completely change the way the EU supports its farm sector (Council regulation (EC) No. 1698/2005).

The International Treaty on plant genetic resources for food and agriculture came into force on 29 June 2004. Each country that ratifies the Treaty will develop the legislation and regulations, needs to implement the Treaty. Treaty's objectives are the conservation and sustainable use of plant genetic resources for food and agriculture and the fair and equitable sharing of benefits derived from their use for sustainable agriculture and food security, in harmony with the CBD and GSPC. The Treaty resumes the GSPC's definition of sustainable management and use of plant diversity that should integrate social and environmental considerations, such as fair and equitable sharing of benefits and the participation of indigenous and local communities. All actors involved benefit, in many ways:

- farmers and their communities, through Farmers' Rights;
- consumers, because of a greater variety of foods, and of agriculture products, as well as increased food security;
- the scientific community, through access to the plant genetic resources crucial for research and plant breeding;
- international Agricultural Research Centres, whose collections the Treaty puts on a safe and long-term legal footing;
- both the public and private sectors, which are assured access to a wide range of genetic diversity for agricultural development;
- the environment, and future generations, because the Treaty will help conserve the genetic diversity necessary to face unpredictable environmental changes, and future human needs.

Long-term goals in this field are managed by numerous global strategic documents, which were used in preparation of international conventions or national programs, i.e. World Conservation Strategy (1980, IUCN, UNEP, WWF), Caring for the Earth (1991, IUCN, UNEP, WWF), Global Biodiversity Strategy (1992 WRI, IUCN, UNEP) and especially directives for conservation of medicinal plants: Guidelines on The Conservation of Medicinal Plants (1993, WHO, IUCN, WWF), where the significance of ecology, identification and traditional use of plants, as well as cultivation and conservation of plants both *in situ* and *ex situ* are strongly emphasized (Skoberne, 2002). Together with WHO Guidelines on good agricultural and collection practices (GACP) for medicinal plants (WHO, 2003) these guidelines offer the background supporting documents for many national and international initiatives, programs and frameworks, aimed at improving the knowledge on distribution, abundance, sustainable management and use of medicinal plants worldwide.

In April 2002 the European Plant Conservation Strategy (EPCS) was recognised as a contribution to the Global Strategy for Plant Conservation (GSPC), adopted by the CBD (Decision VI/9). The European Plant Conservation Strategy (EPCS) is a joint initiative of the Council of Europe and Planta Europa Network, and will significantly help to raise the profile of the Planta Europa Network's efforts to protect plants in Europe. It contributes also to the Convention of Biological Diversity and the Pan European Biological and Landscape Diversity Strategy (PEBLDS). The Planta Europa Network, at its 4th Planta Europa conference (Valencia, September 2004), set itself a challenging agenda to be achieved by 2007. Among seven 'Critical Targets', which were identified to provide the framework for Planta Europa activities in next three years, the target 7 applies to the identification of best practice for conservation and sustainable use of medicinal and other socio-economically important plants and the promotion to relevant policy makers. This critical target corresponds to Target 3.1 of the EPCS.

The role of ECP/GR in conservation and sustainable management and use of MAPs

Conservation programs, aimed at conserving of natural heritage, at improvement of the knowledge on the MAPs genetic variability and improving biological knowledge, including MAPs-related user safety, as well as sustainable management and use of MAPs should be promoted in the EU countries.

The International Plant Genetic Resources Institute (IPGRI) is committed to promote the use of biodiversity for the greater well-being of people. Although medicinal plants are not a preponderant part of IPGRI's Agenda, the Steering Committee of the European Cooperative Programme for Plant Genetic Resources Networks (ECP/GR), which operates through nine broadly focused networks (http://www.ecpgr.cgiar.org/Index.htm), agreed on the establishment of the Medicinal and Aromatic Plants Working Group (MAP WG) (in October 2001) in order to facilitate European collaboration in the field of conservation of natural resources of MAPs and their sustainable use. Up to now the ECP/GR MAP WG met twice, the first meeting was held in Gozd Martuljek, Slovenia, in September 2002 and the second in Strumica, Macedonia, in December 2004. After the 1st meeting, the selection of 10 target species/genera that will be used as model species (*Achillea millefolium* agg., *Artemisia absinthium*, *Carum carvi*, *Gentiana lutea*, *Hypericum perforatum*, *Melissa officinalis*, *Mentha* spp., *Origanum vulgare*, *Salvia officinalis*, *Thymus* spp.) has been made, enabling development of specific MAP descriptors. MAP descriptor lists, harmonized with EURISCO descriptors, have been prepared and proposed to the members as a working scheme during the second meeting. The members, interested in the same species/genus, work on characterization of specimens *in situ* and on evaluation of accessions within their gene banks. The reports, obtained after evaluation of morphological, cytological and genetic characterization *in situ* and evaluation of accessions *ex situ* in each of the member countries will be presented during the next meeting of the WG, which is planned for 2007.

The Group is expected to contribute to the development of the conservation strategy of MAPs at the European level. Legislative limitations, implemented by EU trade regulations on endangered MAPs and low knowledge of biological conditions and biodiversity status urge for coordinated action and involvement of European experts and scientists. Efforts are to be carried out in partnership with a variety of actors (at local, national and international level) and deploying a variety of tools that contribute more effectively towards the common scope – conservation of MAPs and their habitats in the European region.

The inventory and monitoring of endangered MAP species and their *ex situ* maintenance, the study of intra-specific diversity, the assessment of threats, preparation of relevant descriptors and successive evaluation of ecotypes are just some of the research programmes that would contribute to the knowledge on current gene pools of MAPs and the degree of vulnerability in individual countries. The field work should be restricted to the areas, which have been assessed at the national level of the relative importance for each natural habitat type in member countries.

Best practice for the conservation and sustainable use of medicinal plants (and other sociologically important plants) that in majority occur outside protected areas (protected area *per se* already represents a protection measure) would be the study and identification of risks of biodiversity loss, promotion of appropriate conserving measures to relevant policy makers and policy-based conservation actions.

Monitoring the impacts with bad influence on the status of the endangered species could be considered as an important factor in notification of biodiversity drop. Plant species can be endangered due to the loss of habitats because of natural succesion (reforestation) or due to direct extermination (collecting, meliorations, agricultural activities, infrastucture, etc.). For the conservation of the natural species populations some extra measurement are needed. The

in-situ and *ex-situ* measures should be coordinated, especialy the propagation of the plants for the reintroduction and establishing of suitable environmental conditions for their further domestication. The study of the level of endangerness of species populations are the basis for their effective conservation. For threatened species (whether of known economic importance or not) ecogeographical surveying should be undertaken, the extent of the genetic representation in the natural habitat assessed, actions undertaken to control or remove the factors that cause the threats and the detailed management or recovery should be planned and implemented through natural resource managers, local communities and policy makers.
Seed material from rare and vulnerable species should be collected and tranferred to appropriate *ex situ* collections, where the plants should be propagated. Thereafter, morphological, citological, usage and chemical characteristics of plants in different developmental stages would be recorded. In case of not threatened species ecogeographical surveying should be undertaken to establish the amount and distribution of genetic variation, potential changes in population size in natural habitat (*in situ*) as well as to assess the conservation needs. Domestication of wild species that indicate a trend in economic importance should be promoted, independently on the species endangerness status.
In this context one of the most important scopes of the ECP/GR MAP WG are to develop professional standards/criteria, which could be of immediate use in member countries in order to follow up current status of MAP populations in their natural habitats and which would point out, which of the measures should be addressed to the management of target species in order to control/prevent its biodiversity loss.

ECP/GR MAP WG results (2002 – 2006)

The MAPs WG working plan covers activities related to documentation, characterization/ evaluation, *ex situ*, *in situ* and on farm conservation of MAPs. Harmonization of methodologies for evaluation of natural plant populations and their habitats is based on the principles of a descriptor system. Descriptors needed to be set up on the basis of observations and measurements of floristic and phytocoenological data, morphological, chemotaxonomic, cytological and genetic variables. These should meet the criteria of population characters significance and distinctiveness.
A descriptor-based approach consists of five categories of descriptors, with the following descriptor categories:

1. *Passport and Collecting descriptors* describe all parameters which have been obtained during inventarization and/or survey (mapping) of genetic resources and provide basic information for managing of accession, including registration, identification, ethno botanical and ethnographic data. Collecting descriptors comprise all data registered at field working, and which describe natural habitats together with natural genetic resources.
2. *Management descriptors* consist of prescriptions and technical instructions for preparation of genetic material and conditions for long-term maintenance of accessions. Protocols for preparation (pre-condition) of genetic material, its maintenance and multiplication (regeneration), based on international standards and regulations, are to be set up.
3. *Environment and Site descriptors* explain environmental (geographic, soil-pertaining, topographic, phytocoenological, micro-climatic) and habitat-specific parameters, which are important for distribution and assessment of abundance of populations, characterization, evaluation as well as for assessment of useful properties of a particular genetic source, which can be attributed to the interaction between ecotype and environment.
4. *Characterization descriptors* express morphological, taxonomic, cytological, chemical, production (biomass/m^2) characteristics of respective specimens (20 – 25 entities per population) of genetic source and are crop-specific. Characterization descriptors have

been prepared for 10 model species that MAP WG selected for the study during the first phase.

5. *Evaluation descriptors* will be used in *ex situ* evaluation of genetic resources of a specimen under study (20 – 25 entities per population) and consist of observations/measurements of plant biomass (fresh and dry in g/plant), of regeneration potential (weight of 1000 seeds, possibility for vegetative propagation), of earliness (measured by developmental stage), of determinations of the contents of the secondary metabolites and of evaluation of sensitivity of a genetic source for abiotic and biotic stress factors, with a characterization of a stress factor.

The WG members agreed that development of relational databases would advance the work on evaluation of MAP species. All data collected in the process of inventarization, mapping and evaluation of natural genetic resources and of genetic resources *ex situ* would be jointly interlinked by relational pointers into such an information database (national and/or international).

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MEDICINAL SPECIES OF MACROMYCETES RECORDED IN THE REPUBLIC OF MACEDONIA

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Summary

Fungi have a long history of usage in traditional medicine. The content of medicinal species (about 200 species) has not been researched enough in Macedonia. Therefore, in the present study an attempt has been made to present the medicinal species of macromycetes recorded in the Republic of Macedonia together with the literature data for their effects in human therapy. Their large number facilitates selection of those characterised by a significant medicinal quality.

Keywords: *medicinal, macromycetes, R. Macedonia*

Introduction

The territory of the Republic of Macedonia has been relatively poorly investigated from mycological aspect. Until now, few systematic analyses of fungi have been made what explains the fungia in some regions of the country (e.g. Pelister, Jakupica, Galicica, Kozuf, Sar Planina etc).

The first mycological paper in the Republic of Macedonia was published by Ranojevic [1] in 1909. Sydow determined fungi collected by Bornmüller in Macedonia in 1921 [2]. The published papers by Lindtner [3-5], are of great importance. He investigated micromycetes, but also he collected macromycetes and the results were partially published by Pilàt & Lindtner in 1938 [6] and 1939 [7]. Litschauer [8] in 1939 gave a great contribution to the investigation of corticoid fungi, especially from the genus *Tomentella* from Sar Planina and Korab. Minev [9] in 1953 explored mycosis on pine and fir trees in Pelister. About higher and lower fungi, parasites and saprophytes on forest trees, the following authors were writing: Tomasevic [10] (1955), Grujoska [11-13] (1970, 1973), Serafimovski & Grujoska [14] (1959), Koleva-Sekutkovska [15] (1959), Serafimovski and al. [16] (1968), Grujoska & Papazov [17, 18] (1974, 1975) and Papazov [19, 20] (1973, 1983). Higher fungi from the Macedonian pine forests on Pelister were investigated by Tortic in 1967 [21, 22] and 1987 [23], and also on Jakupica by Tortic and Cekova in 1975 [24] and by Sylejmani in 1980 [25]. Lignicolous fungi in the sub-Mediterranean region of Macedonia were explored by Tortic & Karadelev [26] in 1986, as well as on Kozuf, Nidze, Pelister and Galicica by Karadelev [27, 28]. Tortic included 585 species of macromycetes in the first Macedonian mycoflora [29]. Recently Karadelev has continued the investigations on fungi in various Macedonian areas [30-34].

Fungi represent a very heterogeneous group of organisms. However, studies to date have been concerned mainly with Ascomycota and Basidiomycota. The other phyla have been studied insufficiently. Thus far, in the Republic of Macedonia approximately 1,250 species of fungi have been recorded [35]. The majority of them belong to the phyla Ascomycota (130) and Basidiomycota (1,050).

This plentiful gene fond of fungi might be a potential source for isolation of active principles with antioxidative, antitumor, immunomodulative, antiinfective and other actions [36-57].

Material and methods

The Division of Mycology within the Faculty of Natural Sciences (Institute of Biology), possesses a large collection of macroscopic fungi called Fungi Macedonici. The collection contains approximately 10,000 specimens as well as a fungi database named MACFUNGI.
Literature data for the antioxidative, antitumor, immunomodulative, antiinfective and other actions of fungi found on the territory of the Republic of Macedonia are included in the following databases: PubMed, Chemical Abstracts and Hinari.

Results and discussion

By synthesis of the secondary metabolites the enormous practicability of fungi as biosystems that humans can manipulate for their own benefit, could obviously be emphasized.
Although the majority of these metabolites were isolated from representatives of the subdivisions Zygomycotina and Ascomycotina, recently investigations have been transferred to the subdivision Basidiomycotina. The advantage of the higher fungi is that in most of the cases cultivation in laboratory is not necessary. Their macroscopic fruiting bodies could easily be identified in nature and collected if necessary. Especially interesting are the species that extensively grow on various substrates or forest phytocenoses.
According to the action of their metabolites fungi could be divided into: bazidiomycetes with antibacterial, antifungal, antiviral and anticancer (cytostatic) action

1. Bazidiomycetes with antibacterial action
Berdy (1974) [36] quoted 3222 antibiotics, out of which 140 were isolated from representatives of the subdivision Basidiomycotina.
From the submerged culture of *Merulius tremelosus* the following antibiotics have been isolated: merulic acid A, B, C (also present in *Phlebia radiata*) and merulidial who acts bactericidally on Gram-positive (*Micrococcus roseus, Corynebacterium insidiosum, Bacillus brevis, B. subtilis, Streptomyces viridochromogenes, Sarcina lutea*), Gram-negative (*Proteus vulgaris*) and antifungal on micelial fungi and yeasts [37].
From *Stereum hirsutum* hirsutic acid was isolated with effects on *Micrococcus pyogenes*, *Corynebacterium diphteriae* and *Neisseria meningitis*, from *S. rameale* - ramealin, active against some Gram-positive bacteria, and from the related *Xylobolus frustulatus* antibiotic frustulosinol, active against *Staphylococcus aureus, Bacillus mycoides, B. subtilis, Vibrio cholerae*, and in higher concentration on some fungi [38].
From the poroid species of *Hirschioporus pargamenus* antibiotics biformin and biformic acid were reported with wide spectra of action on Gram-positive bacteria, *Mycobacterium phlei* and against fungi; on *Piptoporus betulinus* - poliporenic acid A and C, active against Gram-negative bacteria (*Escherichia coli*) and *Mycobacterium phlei*. Extract from this fungus is successfully used in therapy of brucellosis. From the species *Heterobasidion annosum* phytotoxins fomanosin and fomanocsin were isolated with antibacterial action, and from *Pycnoporus cinnabarinus* - cinabarin active against Gram-positive bacteria and *Mycrococcus pyogenes*. From *Gloeophyllum trabeum* termofilin was isolated, active against cocci, and from *G. sepiarium* antibiotic lenzitin was reported with vigorous bactericide and bacteriostatic action on many Gram-positive and Gram-negative bacteria. From the species *Laetiporus suphureus* and *Phellinus igniarius* sulfuridin was reported with bacteriostatic action on Gram-negative bacteria [39, 40, 41].
From Agaricales the following antibiotic compounds were isolated: *Agrocybe aegerita* - egeritin, with wide spectra of action; *Marasmius alliaceus* - aliakolid, with antibacterial and antifungal action and crystal aliacol A and B; *Hypholoma fasciculare* - fasciculol D with

action on *Staphyllococcus aureus* and *Klebsiella pneumoniae*. From the species *Agaricus bisporus* agaritin was isolated [42]; from *A. campester*, kampestrin, which was used against typhoid and some other bacteria; from *A. xanthodermus*, psaliotin with bactericidal action on Gram + and Gram - bacteria. From the culture of *Colibia peronata* antibiotic compound was isolated active against *Staphyloccocus aureus;* the species *Coprinus picaceus* is used as a producer of a picace acid, active against micrococcus and typhoid. From the *Lactarius deliciosus* antibiotic compound named as laktaroviolin was isolated, active against *Mycobacterium tuberculusis*, as well as laktarazulen and laktarofulven, till from the related species *Lactarius rufus* a compound active against micrococcus was isolated. From the species *Lepista nebularis* antibiotic nebularid was isolated with action on *Mycobacterium tuberculosis*, and from the related *Lepista nuda,* nudic acid A and B with action against Gram + and Gram - bacteria. From dried fruit bodies water extracts of *Lucopaxillus giganteus* and *Leucopaxillus candidus* antibiotics klitocibin A and B were isolated with bacteriostatic effect on penicillin-resistant bacteria. From the species *Marasmius scorodonius* skorodonin was isolated with inhibition action on yeast and bacteria [43].
From lignicolous Gasteromycetales, the folowing antibiotics were isolated: from *Cyathus striatus* - striatin A, B and C, active against Gram + aerobic and anaerobic bacteria, some Gram - bacteria and against Fungi imperfecti [44].
Musilek (1981)[45] investigated 338 culture races from 195 species of Bazidiomycetes on representatives of Gram + bacteria (*Bacillus subtilis*), Gram - bacteria (*Escherichia coli*) and fungi (*Candida pseudotropicalis*), and 101 of them or 51.7% showed antibiotic activity.

2. Antibiotics with antifungal action

Among the above mentioned: merulidial, frustulozinol, biformin, egeritin and striatin, which besides antibacterial poses and antifungal action, from some species of macromycetes are isolated compounds with purely antifungal action. From *Gloeophyllum sepiarium* oosplakton was isolated, from *Omphalotus olearius* iludin M and S and lampterol, from *Oudemansiella mucida* mucidermin, active against *Candida* spp. (*C. albicans, C. tropicalis, C. pseudotropicalis, C. guilermondii, C. Krusei* etc), dermatophytes (*Trichophyton*, *Epidermophyton*, *Microsporum* etc) and against some keratomycosis. This species poses affirmed antifungal action on 30 dermatophytes. From the same species preparation oudemansin was isolated, and from *Oudemansiella radicata* oudenon was isolated which beside antifungal posed antihypertensive action, too [46, 47].

3. Bazidiomycetes with antiviral action

From poroid species *Pyrofomes demidoffii* antiviral compound active against poliomyelitis entitled as fomecin A was isolated [48].

4. Bazidiomycetes with cytostatic action

Based on the investigation of 7000 collections of cultures of micromycetes, their cytostatic action was verified on sarcoma 180, adeno carcinoma 755 and leukaemia L-1210, anticancer compounds were evidenced in 50 species from 20 various genuses. In these investigations the following species were enclosed: *Tricholoma rickenii, Lepista luscina, Coprinus ephemerus, Lentinus lepideus, Schizophyllum commune, Clitopilus passeckerianus, Oudemansiella radicata, Gloeophyllum sepiariumn,* and the representatives from the genuses: *Irpex, Merulius, Polyporus, Boletus, Corticium, Stereum, Poria, Pholiota, Agaricus* and *Morchella* [49,50].
From the representatives of ordo Aphyllophoralles the following anticancer compounds were isolated: from *Merulius tremellosus* - merulidial, active against carcinoma Ehrlich; from the extracts of *Fomes fomentarius* and *Piptoporus betulinus*, preparations for treatment of carcinoma of mammary gland, and carcinoma Sticker are prepared and from *Inonotus*

obliquus - inotodiol with proved cytostatic action and oblikvol with cytostastic effect on sarcoma in mice. From dried and minced imperfect fruit bodies of *I. obliquus* preparation noted as "Befungin" is prepared in Russia. It is used for healing various types of carcinomas, and also has tonic action and normalises the disorganized enzymes functions [51, 52].
From the genus Agaricales the following anticancer compounds are detected: from *Omphalotus olearius* - iludin M and S; *Pleurotus ostreatus*; *Flammulina velutypes* - flamulin; *Marasmius alliaceus* - aliakol A and B; *Armillaria mellea* s.l.- peptidoglucan fraction B; *Agaricus campestris* - agaridoksin, *Amanita phalloides* - amanulin. From the water extract of dried fruit bodies of *Boletus edulis* compounds active against some types of sarcoma are isolated. From *Phalus impudicus* antitumour compound is described which has manifested good results in healing of skin cancer. Antibiotic nebularin isolated from *Lepista nebularis* has showed antitumour action. From the fruit body of *Tylopilus felleus* compound with antimicotic activity was tested on cancer cells [43, 53, 54, 55, 56].
From lignicolous Gasteromycetales anticancer compounds were verified in *Lycoperdon pyriforme* [57].
Almost all of the above mentioned species are registered in the Republic of Macedonia. They are with different distribution and frequency. In adequate conditions extensively grow of some species occur. In beech forests, generally in autumn, large quantities of the following species appear: *Marasmius alliaceus, Oudemansiella mucida, O. radicata.* They grow only on Fagus, but the parasite *Armillaria mellea* s.l. in addition to its beech extensive grow it is found on oak and Molika-pine forests (Pelister), too. It is of special interest the extensive presence of *Fomes fomentarius* which in some parts of Europe became rare, but in Macedonia grows as a parasite and saprophyte on 10 hosts, most often on *Fagus sylvatica*. *Fomes fomentarius* is particularly frequently found on the beech forests on mountain Kozuf. From the other species the following are frequently found on Fagus: *Hypholoma fasciculare, Merulius tremellosus, Pycnoporus cinnabarinus* and *Pleurotus ostreatus,* although they could be found on other substrates, too.
The following non - specific and wide-spread species could be distinguished: *Stereum hirsutum*, in Macedonia grows as a saprophyte on 28 various kinds of trees and bushes, *Trametes versicolor* on 21 species, *Schizophyllum commune* on 11, *Flammulina velutipes* on 11 (especially frequent on cultivated trees and bushes) and *Laetiporus sulphureus* (prefers Salix spp.) on 6 different substrates.
From the coniferous species, the following could be found *Heterobasidion annosum*, as a parasite on the roots of Abies, Picea and Pinus (rare Fagus) and *Gloeophyllum sepiarium*, as a saprophyte on Picea and *Pinus peuce*.
From the substrate specific species are: *Agrocybe aegerita* that's grows frequently as a saprophyte on the trunks of Populus spp., *Phellinus igniarius* as a parasite on *Salix* spp, *Pyrofomes demidoffii* on *Juniperus excelsa*, *Piptoporus betulinus* and *Inonotus obliquus* on Betula spp. *Inonotus obliquus* species is still not registered in Macedonia, probably because of poor investigation on its host.
Rare species in Macedonia or those insufficiently investigated are the following: *Cyathus striatus* (frequently on beams and prepared wood), *Gloeophyllum trabeum (Populus), Trichaptum biforme (Betula, Fagus), Lentinus lepideus (Pinus peuce), Lycoperdon pyriforme (Fagus), Omphalotus ollearius (Quercus)* and *Xylobolus frustulatus (Quercus).*

Conclusions

In this paper selection has been made of compounds with antibacterial, antifungal, antiviral and cytostatic effects, isolated from the medicinal species of macromycetes recorded in the Republic of Macedonia.

Some of the registered medicinal species: *Armillaria mellea* s.l., *Fomes fomentarius, Marasmius alliaceus, Stereum hirsutum, Trametes versicolor* etc, very frequently could be found on beech and oak forests in mass quantities on the territory of the Republic of Macedonia.
Their large number will facilitate selection of those characterised by a significant medicinal quality for our further investigations.

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AN OVERVIEW ON THE ALBANIAN MEDICINAL AND AROMATIC PLANTS INDUSTRY

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Summary

Albania offers a wide range of Aromatic and Medicinal plants which presently sell to the International markets mainly as bulk dried and essential oils. Over 95% of Albanian Medicinal and Aromatic plants are wild collections grown all over the country. The most important export items are Sage, Oregano, Juniper, Thyme, Savory and Laurel. Transitional developments in the country, over the last decade, contributed to a new design of this industry which in the meantime introduced several challenges. Considering the maxim "Don't put all your eggs in one basket!", it is important to search for new ventures although these are always risky. While Albania is on the way to become an EU member country, the Albanian herb and spice industry businesses should start preliminary preparations preceding the integration process, which means firstly an effective management system to ensure that MAP harvesting from the wild and protected areas are properly administered, and secondly more information on the international plant trade and regulation.

Keywords: *Albania, herbs, industry, spices, trade*

Introduction

Albania, part of the lush Mediterranean basin, has a very rich flora with about 3250 native vascular plant species, distributed in 165 families and about 910 genera (Paparisto et al., 1989). This number comprises about 30% of the ca. 11.600 European species. Albania offers a wide range of Aromatic and Medicinal Plants (MAPs) giving the country a competitive trade advantage. About 250 different plant species are wild harvested for medicinal and aromatic use in Albania (Vaso, 1997). The MAP biodiversity includes, to a considerable extent, indigenous species. An excellent example is *Gentiana lutea* which is a plant named after Illyrian King Gent; such a name was inherited over the centuries and is very popular even to date.

Local use of Medicinal and Aromatic Plants has deep roots and a long tradition in Albania in treating human diseases. It appears that at least a century ago man valued herbs as medicinal agents as noticed by the British traveller Edith Durham, who was astonished by the knowledge owned by some local people on the north Albania on the use of MAP in remedies for disease (Durham, 1910; 1923). MAPs have been an important Albanian export commodity for many years. Until the early 1990s, the purchase of cultivated or wild harvested MAPs and trade in these materials were exclusively state controlled. State organisations and authorities sold the purchased plant material to the central, state-owned "Agroexport", which exported either the dried MAP raw material or distillations thereof (Qendro et al., 2004). Albanian MAP industry experienced significant changes during the transition towards the free market economy; originally functioning under the centralized system as an auxiliary economic sector, this industry has now become one of the weightiest components of the agricultural overall exports. Many exports are shipped to Western European countries, and a large number of private companies have taken over the formerly state-controlled trade.

However, it is important that for the harvesting and trade of MAPs, the ecological, the social and the economic issues should be considered. We discuss the actual trends in MAPs harvesting and trade. Some recommendation that might have a positive impact on the

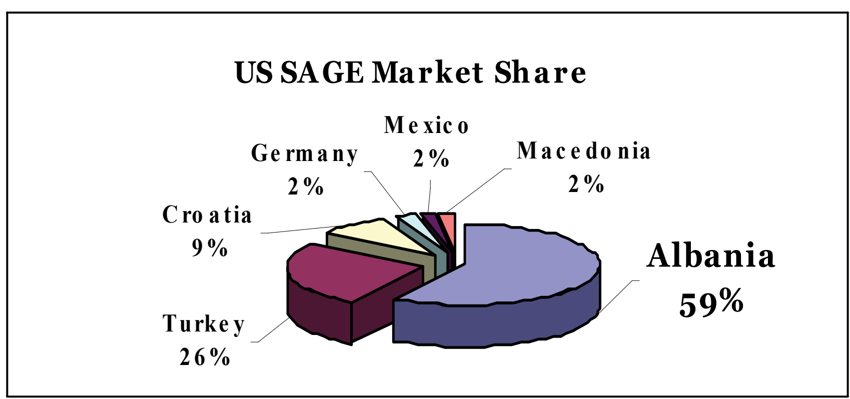
conservation of the MAPs species diversity and the welfare of the harvesters and the economy of the country are given.

Material and methods

This paper represent a review of the most relevant and recent scientific reports and studies about the Medicinal and Aromatic Plant in Albania as well as relevant legislation and seeks to gain understanding of MAP collection scale and intensity, trends, likely future trends etc. The review is based on the information collected from local-level stakeholders and from individuals at the various departments of the General Directorate of Forest and Pastures, Ministry of Agriculture and Food, Ministry of Environment, international and national non-governmental organizations with an interest in MAP exploitation and private enterprises involved in the business of MAP.

Results and discussions

1.1. Albanian MAP Industry Profile
Restructured and adjusted to synchronize with the international market demands, trends and developments, the industry diversified the products supplied thus continuing to maintain its position as a “demanded supplier”. Wild collections, which enjoy excellent biological characteristics, are processed and sold to the international markets as a) dried bulk, b) essential oils, and c) fresh herbs.
Albania’s major export MAPs are Sage, Thyme, Oregano, Savory, Hawthorns, Red clover, Lemon balm, Rose hips, Rosemary, etc. Sage has always been and still is the dominating export item. United States Department of Agriculture data indicate that Albania is the main international Sage supplier to the US providing 60% of total imports (Figure 1). It is assumed that another 20 % of Sage that enters the US market is of Albanian origin which transits European countries.



Source: USDA, Foreign Agricultural Services, 2005

Fig. 1. US Sage Imports by countries

Albanian MAP Industry businesses have intensified their business initiatives and efforts towards increasing industry’s competitiveness capacities worldwide by heavily focusing on continuous increase of quality of final products, value adding, technology upgrade, marketing and market learning and business development and expansion.

1.2. Albanian MAPs Industry Challenges

MAPs in Albania represent an export industry; as such it is difficult to handle even in developed countries. Albania is still e developing country and the transition derived changes contributed to a new design of the industry, and introduced in the meantime several challenges which while facing them served as a great learning experience for the industry businesses for their successful survival and performance.

Migration of harvesting power towards urban areas diminished well experienced labor thus affecting the quality of harvesting raw material. In addition, inapt physical infrastructure directly influenced the increase in the overall productions costs. Moreover, legal framework on the other hand hasn't been very stimulating particularly delayed VAT reimbursement.

It is worth noting though external factors that driven by low purchasing prices interfere with the quality of the products exported from Albania thus indirectly impairing the reputation of the country.

1.2.1. Market place Timing: The most important Key to profitability

Marketplace timing is a key factor that is directly linked to the business profitability as if high quality spices, herbs and essential oil products are delivered on time that ensures long standing partnerships and sound reputation of supplying country. As most of the Albanian herb and spice businesses were born during transition, lack of international business management and industry specifics experience and inadequate market information slowed down the integration of these businesses into the worlds market, yet excellent biological properties and wide range of the Albanian herb and spice flora helped international markets still place inquires for these items. With time (16 years after break down of system), Albanian herb and spice businesses leant to be responsive to immediate market demands and honor the contractual agreements even though numerous are the cases of missing trade business opportunities which if captured would have significantly contributed to countries' overall export values (USD).

1.3. Albanian MAPs Industry Highlights

Despite the challenging working environment, the MAP industry in Albania developed and progressed thus contributing to the prosperity of all the levels involved in the harvesting, processing and export of the MAPs in the country. The United States Department of Agriculture data depicts a significant annual increase in export of the Albanian Sage (Table 1). It is noted a slight drop down in quantity of sage exports in 2004 versus 2003 (severe droughts and dollar drop down), yet the dollar value is increased due to value adding / further processing of this product in the country. Whereas the highest increase ever in dollar value is recorded in 2005 (it obviously triples the export figures of the previous year). This shows serious engagements and investments of the industry businesses for higher quality and diversified products.

Value adding has become the driving force of the MAP industry in Albania; as a result at present the industry supplies to international markets, in some cases for the first time ever:

a) higher quality final products,

b) organically certified MAPs (EU and NOP certified),

c) further processed products – ASTA Quality and Rubbed Sage,

d) new packaged formulations (herbal curative teas and cocking spices).

Table 1. US Sage imports from Albania

No	Year	Quantity (MT)	Value (000 USD)	% Increase in Value
1	2003	1,610.6	2,578	
2	2004	1,431	2,852	**10**
3	2005	2,007	4,297	**34**

Source: USDA, Foreign Agricultural Services, 2005

Additionally, technology upgrade has been major component of companies' investments and focus consisting in processing and production capacities expansion and improved plants operations and management. This led to an increase in volumes and improved quality of exported products which in turn contributed to higher business profit margins.
Apart from sage, the most frequently collected MAP species and herbs include oregano (*Origanum vulgare*), thyme (*Thymus vulgaris* spp.), Bay tree leaves (*Laurus nobilis*), *Juniperus* spp., Urtica spp., *Hypericum perforatum*, *Viscum album*, *Lavandula officinalis* and rosemary (*Rosmarinus officinalis*). An estimation of the annual quantity exported as dried raw material and their value is given in the Table 2.

Table 2. Annual quantities of MAPs exported from Albania lbania

Year	Quantity [tones]	Value (000 USD)
1997	4 000	5 583
1998	8 000	9 063
1999	8 875	10 303
2000	7 411	9 950
2001	9 980	11 737
2002	9 500	14 760
2003	8 800	12 850

(Source: Qendro et al., 2004)

Immense are industry businesses efforts to perform direct export sales to final export markets in order for more dollar value to remain in the country; and those market segments previously overlooked because of their size are being considered and dealt with which in turns increases overall businesses profit margins.

1.4. Albanian MAP Industry Opportunities

MAPs natural resources in Albania are estimated to be three times higher than what is presently being harvested and exported (FAO, 1993, Kathe et al., 2003). This definitively represents great potential for future development in the export volumes which will certainly bring more dollar value in the country as well.
Along with increased export volumes, MAPs industry is in the position to offer much a diverse export products basket supplying competitive items like properly cleaned products, oleoresins, organic products, cultivated based dried and essential oils, etc.
Training and education of all levels of the industry will definitively contribute to quality improvement from the collection all the way to final processing and production. Training will help educate businesses cope up with the up to date international standards and requirements thus immediately react and better answer market needs; it will also help educate on the sustainable use of MAPs in order for next generations to still enjoy and benefit from these natural resources.

ASTA specifications, GMP and GHP systems implementation will definitively help MAP industry businesses in Albania increase quality control and assurance thus not only remaining competitive but also increasing their share in the global markets.

1.5. Cultivating Medicinal Herbs as a Small Enterprise

Albania's key competitive advantage in the world markets is the wild collections. With dwindling supplies from natural sources and increasing global demand, the MAPs will need to be cultivated to ensure their regular supply as well as conservation. Moreover, most wild spices, herbs and essential oils crops are labor intensive; therefore, less challenging alternatives which would significantly reduce labor, time and overall costs and would increase the quality of final products have been explored and experimented over the years in Albania. Prior to the political changes in 1991, rosemary (*Rosmarinus officinalis*) was cultivated on a fairly large scale by some co-operatives; once the land had reverted to private property again, the cultivation of rosemary almost ceased to exist Recently rosemary is once again the most commonly cultivated species. Other major export herb and spice plant species such as lavender (*Lavandula officinalis*), thyme (*Thymus vulgaris*), coriander (*Coriandrum sativum*) and *Satureja montana* are being cultivated in various eco-zones of the country as small scale commercial activities undertaken by leading export businesses and/or associations which basically intend to:

a) Supply valued added products to international markets;
b) Utilize non-productive lands;
c) Create job opportunities for non migrating rural community;
d) Reduce pressure on plants populations;
e) Increase control of companies over quality and supply
f) Increase profitability

However, practically speaking cultivation is not seen as a trendy development of the Albania's herb and spice industry; it is basically seen as a small enterprise activity and applies to fast-growing, not space-demanding, and high-yielding species are economically attractive to commercial growers.

Since many of the MAPs are grown under forest cover and are shade tolerant, agroforestry might offers a convenient strategy for promoting their cultivation and conservation. Several approaches are feasible: integrating shade tolerant MAPs as lower strata species in multistrata systems; cultivating short cycle MAPs as intercrops in existing stands of plantation tree-crops and new forest plantations; growing medicinal trees as shade providers, boundary markers, and on soil conservation structures; interplanting MAPs with food crops; involving them in social forestry programs; and so on (Rao et al., 2004).

1.6. Risks and Problems for the Grower

There are about 7-10 individual herb and spice businesses that are presently cultivating. While their interest on cultivation tends to increase, these growers are faced with challenges that affect initiative and performance of such activities. Critical demolishing factors that influence large scale/extensive cultivation in Albanian primarily are:

1. Land segmentation;
2. Lack of "Contractual farming" practices;
3. Differences in quality and yield introduced by imported herb and spice chemo-types;

Conclusions

Rapid changes in the global economy represent a big challenge for the Albanian herb and spice industry businesses. Within this challenging business environment, intensive and serious

efforts are being made to increase their competitiveness capacities in order to maintain the existing trade links and/or capture new market segments. As a result of over-harvesting, land conversion, erosion and other factors, the populations of some MAP species traditionally collected in the region have declined considerably; some species have become rare, threatened or vulnerable. It is important to search for new ventures although these are always risky. Albanian herb and spice industry businesses should start preliminary preparations preceding the integration process, which means firstly an effective management system to ensure that MAP harvesting from the wild and protected areas are properly administered, secondly shift to cultivation of MAPs and thirdly, more information on the international plant trade and regulation.

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MEDICINAL PLANT COLECTION FROM THE BOTANICAL GARDENS OF THE STATE UNIVERSITY OF MEDICINE AND PHARMACY

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Summary

More than 200 taxons are included in the collection of the medicinal plants which represent the authohthone and aloohthone flora and contain different Biological active substances: hetherosids, volatile oils, alkaloids, cumarins, etc.

Keywords: *State Medicine and Pharmacy University, Medicinal plants*

Introduction

The Medicinal plants and the Biological active substances from them, which are used in treatement of different diseases, don't have adversed effects practicaly. That's why the interest to know and research these plants in the main of creating medicial preparates is increasing. The SMPU have a more than 200 taxons collection of medicinal plants, reprezenting different floristic zones. This collection is situated at 20 km S-W from Chisinau in a silvic zone of moldavian Woods.
The main tasks of the collection are:

- Introducind and researcing the aloohthone and autohthone medicinal plants, included in the „Pharmaceutical Botany" and „Pharmacognosy" study program, and, also those plant which are disapearing from the spontaneons flora.
- Biological, Fithochemical, Pharmacological, investigations to find valne plants in elaborating of the medical preparates.
- Study practical training for students, couresworks, licence and doctorate thesis.

Material and methods

Species of Medicinal plants from *Lamiaceae, Apiaceae, Asteriaceae, Solanaceae, Rosaceae* the initial semen material obtained from spontaneus flora or other similar foreign collections. The law of omolog row in ereditar variability, Biological, Fenological, Biomorfological, Phythochemical and over methods were used to create and study the plants from the collection.

Results and discussions

The collection of Cultivating Medicinal Plants Center of SMPU "Nicolae Testemitanu" contain the Medicinal plant with different principles activities:
Hetherosids: *Adonis vernalis L., Bordago officinalis L,. Brassica juncea (L.) Czern., Convalaria majalis L., Digitalis lanata Ehrh., D.purpurea L., Galium verum L., Rhamnus cantharctica L., Rheum palmatum L., Rumex confertus Willd., Sambucus nigra L.*etc.
Saponins: *Aesculus hippocastanium L., Calendula officinalis L., Epilobium parviflorum Schreb., Equisetum arvense L., Glycyrrhiza glabra L., Hedera helix L., Ononis spinosa L., Primula veris L., Saponaria officinalis L., Verbascum thapsiforme Schrad* etc.

Flavonoids: *Aronia melanocarpa (Michx.) Elliot, Crataegus monogyna Jacq., Fagopyrum sagittatum Hilib., Polygonium aviculare L., P. hidropipir L., P.persicaria L., Ruta graveolens L., Scutellaria biacalensis Georgi, Viola tricolor L.* etc.
Anthocyanids: *Althea rosea L., Centaurea cyanus L., Rosa galica L., Paeonia officinalis L., Papaver rhoeas L* etc.
Cumarins: *Ammi majus L., Angelica arhangelica L., Anethum graveolens L., Levisticum officinale Koch., Melilotus officinalis (L.) Pall.* etc.
Tannins: *Agrimonia eupatoria L., Alnus glutinosa (L.) Gaertn., Bergenia crassifolia (L.) Fritsch., Cotinus coggygria Scop., Geum aleppicum Jacq., G.urbanum L., Potentilla alba L., P.erecta (L.) Hampe, Quercus robur L., Salix alba L., Sanguisorba officinalis L.* etc.
Lipids: *Althaea officinalis L., Amygdalus communis L., Arachis hypogaea L., Brassica nigra (L.) Koch, Carfamus trictorius L., Helianthus annuus L., Cyperus esculentus L., Linum usitatissimum L., Oenothera biennis L., Ricinus communis L.* etc.
Volatile oils: *Achillea millefolium L., Agastache foeniculum O. Kuntze, Anisum vulgare Gaertn., Artemisia balchanorum Krasch., Calamintha nepeta (L.) Savi, Carum carvi L., Cephalophora aromatica Schrad., Coriandrum sativum L., Dracocephalum moldavica L., Foeniculum vulgare Mill., Hyssopus officinalis L., Lavandula vera Dc., Inula helenicum L., Iris florentina L., Majorana hortensis Moench., Melissa officinalis L., Nepeta cataria Dum., Ocimum basilicum L., Origanum vulgare L., Rosa damascena Mill., Rosmarinum officinalis L., Salvia officinalis L., S.sclarea L., Satureja montana L., Thymus serpyllum L., T.vulgare L., Tagetes signata Bartl.,* etc.
Alkaloids: *Atropa belladonna L., Berberis vulgaris L., Chelidonium majus L., Conium maculatum L., Datura inoxia Mill., D. stramonium L., Ephedra distachya Bunge, Calega officinalis L., Glacium flavum Grantz., Macleya microcarpa (Maxim.) Fedde, Phytolaca americana L., Papaver somniferum L., Scopolia carniolica Jacq., Symphytum officinale L., Vinca minor L.* etc.
Bitter substances: *Artemisia absinthium L., Centaurum umbellatum Gilib., Cichorium inthybus L., Marrubium vulgare L.* etc.
Vitamins : *Chaenomeles japonica L., Hippophae rhamnoides L., Ribus nigrum L., Rosa canina L., Sorbus aucuparia L* etc.
The CMPC of SMPU „Nicolae Testemitanu" conteins also the Medicinal plants with pharmacological actions:
Sedativ: *Cannabis sativa L., Filipendula ulmaria (L.) Maxim., Leonurus cardiaca L., Passiflora coerulea L., Valeriana officinalis L.* etc.
Stimulating, tonic, hepatoprotective: *Aralia mandshurica Rupr. et Maxim., Cynara sclolymus L., Echinacea purpurea (L.) Moench, Eleuterococcus senticosus Maxim., Helichrysum arenarium (L.) Moench, H.italicum (Roth) Guss., Hypericum perforatum L., Rubia tinctorum L., Schizandra chinensis L., Silybum marianum (L.) Gartn., Withania somnifera (L.) Dun.* etc.
Insecticide and vermyfuge: *Artemisia taurica, Chenopodium ambrosioides L., Koellia virginiana (L.) Mac C., Pyrethrum cinerariaefolium Trev., Tanacetum vulgare* etc.

Conclusion

The Medicinal plants collection of SMPU is a reproduction of valuarele taxones base in the main of creating a pharmaceutical brand in industrial plantation; a model for introducing and maintaining of the authohthone and aloohthone genofound of the plants; a base for acquainting the students with the protection of the spontanens flora, in special the medicinal plants.

FUNCTIONAL CORRELATIONS OF THE BIOTIC AND ABIOTIC FACTORS IN THE EVOLUTION AND THE PRODUCTIVITY OF *VERBASCUM* POPULATIONS

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Summary

Investigations on the spreading of Verbascum population in the Moldavian sub-Carpathian area have revealed a vertical zone of the species of this genus, on three parallel altitude levels north-south wise. The number-related development and the bio productivity of Verbascum populations allow for significant correlations with the biotope ecological conditions. Biometric characteristics are significantly positively correlated with temperature and significantly negatively with altitude. Traditional phytotherapeutic uses refer to treatment of digestive, kidney, breathing and skin-related disorders in humans and animals. Transplant proved to be the optimal method to restore Verbascum lychnitis, V. thapsus and V. phlomoidesa populations.

Keywords: *Verbascum, correlations, biometry, bioproductivity, restoration*

Introduction

The study of the herb fund has been initiated as a result of some surveys of the use in folk medicine as well as a result of the fact that there have been very few studies on these genus s on a national and, sometimes, international level. Species of *Verbascum* are very important for the phytotherapeutic range of digestive, kidney, and skin-related disorders. Hence, the management of habitats, the inventory of populations, the study of their bio productive potential and their morphometric properties became mandatory. In compliance with CEE Directive 92-43, in order to protect, preserve, and restore the populations facing extinction, quantifying the role of each environmental factor in the development, spread and bio productivity of *Verbascum* populations within their natural habitats became a must. Furthermore, it has appeared as mandatory to design methodologies to obtain conventional cultures of such valuable species.

Material and methods

The material used was made up of samples of each *Verbascum* species collected from their natural habitats, spread along three north-south wise altitude levels within the Moldavian sub-Carpathians. The morphometric characteristics used in the study of biotope / bioproductivity functional correlations sum up biometric determinations on some examples from among 60 populations, with four predominant species, investigated in 18 localities. Data related to the biotope ecological conditions, i.e. temperature and vegetative season precipitation averages over 90 years were obtained from the Târgu Neamț and Piatra Neamț Hydrological Units, whereas altitude was determined at each collection point by means of an altimeter. The etno-phytotherapeutic surveys regarding the use of these plants in traditional medicine were conducted via 215 questionnaires and 44 frequency sheets over three years in 20 localities representative for the *Verbascum* species spreading area. In order to correlate ecological data with the biometrical ones the relationships existing between two sets of data were used – scaled so as to be independent from the unit measure. The correlated calculation of the population uses the covariance of two data series which divide by the product of their standard deviations (1 BERND MARKERT, -1988).

Results and discussions

1. *Verbascum* species habitats in Moldovian Sub-Carpathians (numbering as per CEE Directive 92-43)

3. fresh waters on banks and in the riverbed gravel:

32 river banks – natural or semi-natural dynamics river sections; *Verbascum lychnitis, Verbascum densiflorum, Verbascum phlomoides*

3260 river banks in mountain plains with *Ranunculion fluitantis* and *Callitricho-Batrachian* vegetation *Verbascum lychnitis, Verbascum densiflorum, Verbascum phlomoides*

4. heaths and temperate thickets

4030 European dry lands *Verbascum lychnitis, V. densiflorum, Verbascum phlomoides*

6. natural grassy formations and semi-natural grazing fields

62 semi-natural dry grass formations and bushy area covering facies *Verbascum lychnitis, Verbascum densiflorum, Verbascum phlomoides*

6210 semi-natural dry grassland and bushy area on lime layer covering facies; *Verbascum lychnitis, Verbascum densiflorum, Verbascum phlomoides*

6220 *pseudo-steppe with grass and annual Thero-Brachypodietea plants*: Verbascum nigrum*

6230 *grassland rich in Nardus species on mountain area silica sublayers, (and of sub-mountain continental Europe); *Verbascum nigrum, Verbascum lychnitis*

8. detritus and rocky regions

8110 rocky detritus at the lower mountain floor: *Verbascum lychnitis, ssp.lychnitis, ssp. kanitzianum, V. nigrum, ssp. nigrum, ssp. abietum, V. phlomoides, V. speciosum*, *V. thapsus*

9. forests: 91 temperate Europe forest rims: *Verbascum nigrum, V. lychnitis*

2. *Verbascum* species in Neamt County Sub-Carpathian area

In the Moldavian sub-Carpathians in the Neamt County area, species are spread in areas somewhat parallel with the evolution of relief in the landscape, namely north-south wise, as follows:

- on the strip from and in the mountains, in the Vânători, Mănăstirile Neamțului, Secu, and Sihăstria areas,
- on the upper valleys of the brooks Calu, Iapa, Nechit, and of the Tazlău river *Verbascum nigrum, ssp. nigrum, ssp. abietum* frequently grow, whereas *Verbascum speciosum*, *Verbascum thapsus, Verbascum densiflorum* seldom grow;
- in the central Moldavian sub-Carpathian area *Verbascum lychnitis* is the predominant species in compact populations in the lower valleys of the brooks Secu, Agapia, Văratic, Cracău, Almaş, Calu, Iapa, and of the Neamț, Bistrița, and Tazlău rivers, in mixtures or separated from individuals of *Verbascum phlomoides* and *Verbascum thapsus,* most notably at forest rims and on silica-based poor but well-drained soils;
- in arid spots in the eastern extremity of the sub-Carpathians – in localities such as Grumăzeşti, Roznov, Secuieni. Hociungi, Români, Siliştea, Costişa, Podoleni, Mărgineni, next to compact *Verbascum lychnitis* populations and *Verbascum thapsus* individuals where few *Verbascum phlomoides, Verbascum olimpycum* and *Verbascum pulverulentum* may occur;

3. Ecological conditions provided by the Moldavian sub-Carpathian biotopes for *Verbascum* species

Biotopes soils - the spreading areas of the *Verbascum* species genus have in all cases powerfully levigated, silica-based, lithic soils - with much skeleton on profile, *silt and erratic soils*, notably *underdeveloped soils*

the Moldavian sub-Carpathian topoclimates include two complex categories:

- the complex topoclimate of the Moldavian sub-Carpathian depression;
- the complex topoclimate of the Moldavian sub-Carpathian hills;

which according to the general atmosphere circulation, and against the general temperate continental climate context, the Moldavian sub-Carpathian fall into: - *arid-influenced climatic province sector* – which includes almost the entire area of the Moldavian sub-Carpathians with frequent polar-o or artic-originated cold air advections determining frosts, hoar frost and early or late snowfalls. In summer, the continental tropical air advections determine temperatures above 30 – 35^0 C and a poor precipitation regime; *climatic province sector with Baltic influence* in the northern part of the surveyed area, characterised by a higher nebulosity and more frequent richer precipitations, whereas in winter temperature drop below – 30^0 C and the earliest and latest frosts. *According to the active sub-adjacent area characteristics: with elementary topoclimate,* includes in the complex topoclimate, they can also be natural or antropic.

4. Functional correlations among biotic and abiotic factors within the *Verbascum* species habitats

The vertical zoning of *Verbascum* species is determined by the ecological conditions provided by the biotope, i.e. altitude, temperature, precipitations, and soil, which induce biometric characters reflecting functional correlations among these factors. (3. HELLER R., 1969)

Table 2. Ecological and biometric variation limits of *Verbascum* species in Moldova sub Carpathian habitats

		Altitude	Temp	Precipitations	Length (cm)				Weight (g)			
		m	°C	mm	root	stem	flower	total	root	stem	flower	total
Verbascum nigrum 470–650 m	Min	470	17.23	28,2	20	30	18	**68**	15	25	20	**60**
	Mean	531.8	18.44	67	26	37.7	25.5	**89.2**	19.2	29.3	28.8	**77.3**
	Max	650	19.14	105.2	35	62	50	**147**	23	52	60	**135**
Verbascum thapsus 500-650 m	Min	500	17.88	28.2	21	60	43	**124**	21	130	152	**303**
	Mean	525.6	18,63	67	27.8	62.4	47	**137.2**	24.3	144.5	156.4	**325.2**
	Max	650	18.93	105.2	32	68	52	**152**	30	153	160	**343**
Verbascum thapsus 245-350 m	Min	245	19.98	28.2	30	63	49	**142**	29	154	159	**342**
	Mean	277.6	20.38	67	36.	69.4	53.3	**159.4**	32.3	158.6	164.3	**355.2**
	Max	350	20.72	105.2	45	75	58	**178**	37	166	170	**373**
Verbascum thapsus 200-210 m	Min	200	20.96	27.3	49	76	59	**184**	38	163	159	**360**
	Mean	202	21.02	65.48	51	78.4	61.4	**190.8**	40.8	165	165	**370.8**
	Max	210	21.03	108.4	55	81	66	**202**	45	168	172	**385**
Verbascum phlomoides 480-500 m	Min	480	18.86	28.2	50	58	70	**178**	52	118	175	**345**
	Mean	490	18.88	67	52	60	71	**183**	54	125	177	**356**
	Max	500	18.93	105.2	54	62	72	**188**	56	133	179	**368**
Verbascum phlomoides 400-430 m	Min	400	18.93	28.2	46	51	66	**163**	51	125	179	**355**
	Mean	415	18.93	67	47	55	67	**169**	53	126	179	**358**
	Max	430	18.93	105.2	48	60	69	**177**	55	127	180	**362**
Verbascum phlomoides 320-350 m	Min	320	20.33	27.3	49	56	70	**175**	50	120	160	**330**
	Mean	335	20.36	65.48	50	57	71	**178**	50	125	169	**344**
	Max	350	20.40	108.4	51	59	72	**182**	50	130	181	**361**
Verbascum lychnitis 500-650 m	Min	500	17.88	28.2	21	65	68	**154**	29	156	170	**355**
	Mean	528,7	18.73	67	26.4	69.5	71.9	**167.8**	32.9	160.1	175.1	**368.1**
	Max	550	18.93	105.2	30	72	78	**180**	38	165	180	**383**
Verbascum lychnitis 245-350 m	Min	245	19.98	28.2	28	68	75	**171**	36	160	175	**371**
	Mean	292,6	20.48	67	35.3	75.1	79.5	**189.9**	42.8	167.3	182.3	**392.4**
	Max	350	20.72	105.2	40	78	83	**201**	50	174	188	**412**
Verbascum lychnitis 200-210m	Min	200	20.6	27.3	39	78	72	**189**	51	178	181	**410**
	Mean	202	21.02	65.5	41.4	79.4	75.4	**196.2**	52.2	178.6	185.2	**416**
	Max	210	21.03	108.4	44	82	80	**206**	54	180	190	**424**

Data collected for each habitat and each population are construed as averages of 20 measurements on the whole plant and on its organs. Due to space economy, the development of such data is given in Table 1 as selections of limits of the ecological and biometric parameter variation limits of *Verbascum* spontaneous populations.

Correlations for *Verbascum nigrum* were not calculated as its habitats enjoy relatively homogenous biotope conditions. From among the *Verbascum* species it has the smallest sizes and is spread at 450-550 m, on the forest rim.
In order to quantify the contribution of the environmental parameters represented by their numerical values to the spread and development of morphometric and bioproductive characteristics of *Verbascum* populations, correlation coefficients of the ecological data series were correlated with the biometrical data series.
Correlations of the ecological data series – the biometrical data series of the most frequent species in the survey area, i.e., *V. thapsus, V. phlomoides* and *V. lychnitis,* are given in the correlation coefficient matrices (I), whose values show the role of the environmental factor in the development and bioproductivity of these populations, within their natural habitats (1 - BERND MARKERT, 1988). Hence:

Table 3. Correlation matrix of biometric and ecological data in *V. thapsus* populations

Verbascum thapsus	altitude, m	T °C	pp mm	Length cm			Weight g		
				root	stem	flower	root	stem	flower
altitude, m	1								
T °C	-0.84955	1							
pp mm	0.22487	0.22418	1						
root, cm	-0.70648	0.91924	0.38891	1					
stem, cm	-0.62075	0.87496	0.47064	0.98755	1				
flower, cm	-0.64461	0.89117	0.48965	0.98805	0.98940	1			
root, g	-0.69968	0.92101	0.43701	0.98616	0.98248	0.99442	1		
stem, g	-0.66255	0.95355	0.46451	0.91644	0.89549	0.92043	0.93338	1	
flower g	-0.50561	0.79426	0.71653	0.81315	0.83050	0.85875	0.85777	0.86972	1

Plant sizes with *V. thapsus* species negatively correlate with altitude, and the correlation coefficient values vary between -0.62 and -0.71 for length/altitude, and between -0.5 and 0.70, for the series weight/altitude, respectively. This fact is determined by the drop in temperature as altitude grows, an environmental factor influencing plant growth and development. In this particular case, strong correlations are established between the biometrical data series and temperature with coefficients varying between + 0.79 and + 0.95 (Table 1).

Table 4. Correlation matrix of biometrical and ecological data in *V. phlomoides* population

Verbascum phlomoides	altitude, m	T °C	pp mm	Length cm			Altitude g		
				root	stem	flower	root	stem	flower
altitude, m	1								
temp. °C	-0.90904	1							
pp. mm	0.87518	-0.9972	1						
root, cm	-0.98691	0.82995	-0.78571	1					
stem, cm	-0.96608	0.77061	-0.72058	0.99508	1				
flower cm	-0.95544	0.74553	-0.69338	0.99053	0.99926	1			
root, g	-0.60385	0.21677	-0.14286	0.72449	0.78920	0.81224	1		
stem, g	-0.53882	0.13878	-0.06402	0.66762	0.73808	0.76348	0.99685	1	
flower g	-0.42677	0.01110	0.06401	0.56702	0.64582	0.67471	0.97856	0.99180	1

Functional relations of *V. phlomoides* between environmental and their biometrical results in population are materialised via strongly negative correlations in the case of length / altitude data series (I between -0.95 and – 0.98), in moderately negative correlations of the weight / altitude data series (I between –0.43 and -0.60), which means the growth of biomass quantity along with the decrease of the habitat altitude. This species, too, records significant positive

correlations between the length / temperature data series (I = +0.74 – +0.83), plant growth being stimulated by the rise in temperature.

Table 5. Biometrical and ecological correlation matrix in *V. lychnitis* populations

Verbascum lychnitis	altit, m	T °C	pp mm	Length cm			Weight g		
				root	stem	flower	root	stem	flower
altitude, m	1								
temp. °C	-0.99755	1							
pp. mm	0.71748	-0.6665	1						
root, cm	-0.99999	0.99641	-0.7272	1					
stem, cm	-0.99818	0.99142	-0.7582	0.99892	1				
flower, cm	-0.25885	0.32639	0.4871	0.24523	0.20009	1			
root, g	-0.98223	0.96652	-0.8354	0.98477	0.99176	0.07296	1		
stem, g	-0.93538	0.90808	-0.9174	0.94026	0.95501	-0.0994	0.98513	1	
flower, g	-0.90261	0.93075	-0.3477	0.89646	0.87499	0.64942	0.80578	0.69206	1

Plants in *V. lychnitis* populations are highly sensitive to drops in temperature along with altitude. This is the reason why the strongest morphometric functional interactions recorded with this species high morphometric / biotope functional interactions with negative values of altitude correlation coefficients ranging between -0.90 and -0.99, and positive values of temperature correlation coefficients, ranging between + 0.90 and + 0.99., respectively.

5. Therapeutic uses of *Verbascum* plants in the Neamt County

Etnophytotherapeutic investigations conducted as questionnaires and frequency sheets covered the western edge of the territory – such as in Nemţişor, Mănăstirea Neamţului, Agapia, Văratec – convent and village, Bălţăteşti, Dobreni, Almaş, Piatra Şoimului, Nechit, Tazlău, as well as in the southern and eastern areas covering such localities as Dumbrava Deal, Săvineşti, Slobozia, Roznov, Români, Siliştea. Such investigations resulted in the following:

In the Vânători area as well as the areas between the Neamt, Secu, and Sihăstria monasteries, *Verbascum* plants are occasionally used to treat inflammations of the lever under various forms such as ointments, tinctures and tea (of leaves and flowers), and more rarely of root as tincture or decoct.

In the areas of Agapia, Văratec Bălţăteşti, Bodeşti, Dobreni, Roznov – Borleşti and Tazlău, the root, thinly cut, is notably added to animal food to drain the gall when animals suffer from "jaundice".

In Tazlău, people use a mullein-based ointment to calm down sore throat, remission of inflamed ganglions, to treat haemorrhoids and stop hair loss. Mullein root decoct in broth is administered in depressions, in gall bladder colic, to fight hair loss, and as compress on the neck and chest in case of inflammation of said areas.

6. *Verbascum* population restoration methods: in conventional cultures

With a view to carrying out the culture on the experimental field, small-sized individuals were chosen form among the spontaneous populations the species: *lychnitis, phlomoides, thapsus* and *nigrum,* which were planted again on the location chosen so as to meet the natural habitat ecological requirements.

Ecological conditions in the location

The soil the experiment was conducted on is an antropic one, created on a clay erratic sublayer having a heavy structure due to the earth layers added from the A horizon of some brown-acid and brown – mesobasic soils, with a slightly acid pH – acid (pH ≤5.5), rich in humus (3.2 – 4.15%).

Ground ***temperature*** and the ***number of cloudy days*** are the other ecological requirements in the experimental area field and are detailed in Table 6.

Table 6. Multi-annual climatic data for the experimental field location

Parameter: average over 32 years 1963-1995	Month in the vegetative period					
	May	June	July	August	September	October
cloudy days	10.20	7.90	6.80	5.70	6.60	8.80
Ground temperature °C	17.0	21.1	22.9	22.0	16.6	9.6

Multiplication Method

In the case of this genus, we have applied the method of **multiplication by replanting** of some individuals of all species recorded in the surveyed area. (2. CAUX S., 1993-1994)
It was not necessary to experiment the multiplication methods by sowing and division in order to pass into conventional culture the species of *Verbascum* genus as nature offered us the results of a gigantic multiplication via vegetative multiplication and seeds.

Multiplication Results

The individuals collected from spontaneous flora having similar sized with those had when they were planted were installed in the experimental field and some 90% of them survived. During the first year, all *Verbascum nigrum, Verbascum lychnitis,* and *Verbacsum phlomoides* individuals accepted the new habitat and developed aerial parts displaying biometrical data higher than those in the field.
During the second and third years of the experiment, the *V. lychnitis* şi *V. phlomoides*, individuals germinated whereas the *V. nigrum* individuals did not adjust to the new location conditions and disappeared. The *V. nigrum* species developed populations in mountain areas on forest rims where hill meets the mountain.
Biometrical data of the individuals in the conventional cultures recorded increased values in their length of up to 133% and in weight of up to 170%, according to the biometrical data collected:

Table 7. Development of biometric characteristics in *Verbascum* conventional culture

thread planted	thread output	length planted	length output	weight planted	weight output	Increase		
						threads	length	weight
Number		cm		g		%		
Verbascum lychnitis								
Almaş population – planted on 23/062004								
3	3	146	170	190	233	-	116	122
Girov population – planted on 06/07/2004								
4	4	153	200	400	612	-	131	153
Verbascum thapsus								
Almaş population – planted on 06/07/2004								
1	1	138	165	375	480	-	120	128
Girov population– planted on 06/07/2004								
1	1	116	155	250	375	-	133	150
Verbascum phlomoides								
Girov population – planted on 06/07/2004								
3	3	131	168	170	288	-	128	169

Conclusions

Verbascum species are spread over the entire outer-Carpathian territory of the Neamt County, on three north-south-wise altitude levels, covers.
Biometrical data of *Verbascum* populations enter highly negative correlations with the habitat altitude and highly positive ones with temperature and the number of clear-sky days.

The investigations on the status of *Verbascum* populations conducted over three years resulted in confirming several situations which justify the passing of the relevant species to conventional cultures, notably:

- climatic conditions and altitude favours especially the development of some species in plant associations within spontaneous flora to the detriment of those species relevant to phytotherapy and can severely reduce the development of herb populations;
- the stationeries where they naturally develop undergo changes of their destination by means of antropic intervention;
- herb collection in wild life associations disturb the ecosystem and may trigger the extinction of herb species and associated ones.
- plants of *Verbascum* genus are used all over the Neamt County under different forms such as ointments, tinctures, and tea – leaves and flowers – to treat sore throat, swollen ganglions, swollen throat, haemorrhoids; moreover, they are used as infusions or decoct to treat inflammations of the lever and as fresh root to treat animal “jaundice.”

The optimal method to obtain *Verbascum* conventional cultures is to transplant some individuals from wild life to locations meeting ecological requirements similar to those offered by the natural habitat of the species.

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NUMERICAL AND MORPHOSTRUCTURAL CHARACTERISTICS OF *TRIGONELLA FOENUM-GRAECUM* (2N=16) MITOTIC CHROMOSOMES

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Summary

Trigonella foenum-graecum L. is an important medicinal species. The karyological study have showed that 2n=16. We identified following types of chromosomes: ***m, sm*** *and, sometimes,* ***st*** *(only in early metaphase). So, we can appreciate that the karyotype of this species is enough symmetric and less evolved.*

Keywords: *Trigonella foenum-graecum, karyotype, chromosomes*

Introduction

Trigonella foenum-graecum L. (*Fabaceae*) species is very interesting one, for its pharmaceutical, industrial and alimentary valences (Pârvu, 2005).
Foenum-graecum name means Greek Hay, the plant being used to scent inferior hay. The name of the genus, *Trigonella*, is derived from the old Greek name, denoting “three-angled” (the trait of its corolla). The seeds of fenugreek have been used all through the ages and were held in high repute among the Egyptians, Greeks and Romans for medicinal and culinary purposes. Compounds extracted from the plant have shown cardiotonic, hypoglycemic, diuretic, antiphlogistic and hypotensive activity. One of its alkaloids, called “trigonelline”, has shown potential for use in cancer therapy.
The cytogenetic analysis of this species is a part of complex investigations about the chromosomal features of medicinal and aromatic plants, made in our laboratory (Băra et al., 1986, 1987; Lu and Băra, 1993; Căpraru et al., 2004a, b; Cîmpeanu et al., 2004). The cytogenetic data, and, especially, those regarding the satellites chromosomes number, are different from one author to another. For this reason we have proposed to establish some morphostructural characteristics of the chromosomes at this species.

Material and methods

The study was carried out using seeds of *Trigonella foenum graecum* L., from 2005 harvest. For germination the seeds were placed in Petri dishes on filter paper moisted with distilled water, at thermostat (22^0C). The root tips were collected when had about 10-20 mm length, and pre-treatment was assured placing them in 0.2% colchicines, for 2 hours, in the dark, at room temperature. After that, the seeds were washed with distilled water and then fixed in fresh Carnoy solution, for 24 hours at room temperature. Until analyse, the roots were stored in 70% Ethanol, at 4^0C, in the refrigerator.
Before squashing, root tips were softened in HCl 50%, at room temperature. The most part of root tips have cut and squashed in 45% acetic acid. The staining was performed with Carr solution (Cîmpeanu et al., 2002). Images have taken with Nikon Eclipse 600 bright field microscope 100x objective in oil immersion, with a digital camera Cool Pix Nikon.

Results and discussions

The metaphases (with well-spread chromosomes - with less than two overlaping) study, revealed 2n=16, situation which is in conformity with literature data (Floria et al., 1997; Tiță and Pădureanu, 2003, Dundas et al., 2006).
Establishing of eight homologous pairs had made using the arms ratio (q/p), the centromeric index, the difference between long arm and short arm (q-p) and the relative length.
The arms length it was established by Adobe Photoshop 8.0 programme. Therefore, the total length of each chromosome is represented by the sum of two arms (including the centromere length.
Analyzing the results (table 1), in the metaphase with highly degree of condensation chromosomes (fig. 1), the total length of chromosomes is between 2.79 µm (first pair) and 2.18 µm (last pair), and the decreasing rate of the total length, from first to last pair, is varying from 0.14 µm (between first and second pair) to 0.01 µm (between sixth and seventh pairs). The relative length of the eight pairs of chromosomes, established according formula: (TL/HSL) x 100, it was between 14.18 and 11.09.
About the arms ratio, the minimum value it was recorded at first pair (1.02µm), and the maximum (2.56µm) at second pair, and the difference from the long arm to the short arm it was 0.03µm (first pair), and 1.16µm (second pair).
The centromeric index was between 49.46 (first pair) and 28.11 (second pair).
According to arms ratio, centromeric index, and relative length we established two morphological types of chromosomes, namely: **m** (I, VI and VIII pairs) with arms ratio between 1.02 and 1.59, and also the type **sm** (II, III, IV, V, VIII pairs), which has arms ratio varying from 1.78 to 2.56.

Table 1. The chromosome traits - *Trigonella foenum-graecum* late metaphase

Chromosomes		Total length		Long arms		Short arm		Arm ratio (q/p)	Arm difference (q-p)	Centromeric index	Relative length
Pair	Type	µm	Limits of variability	µm	Limits of variability	µm	Limits of variability				
I	m	2.79	2.71-2,87	1.41	1.38-1.44	1.38	1.33-1.44	1.02	0.03	49.46	14.18
II	sm	2.65	2.60-2.71	1.91	1.88-1.93	0.75	0.72-0.72	2.56	1.16	28.11	13.47
III	sm	2.56	2.54-2.57	1.77	1.77-1.77	0.79	0.77-0.80	2.25	0.99	30.72	12.98
IV	sm	2.54	2.54-2.54	1.66	1.66-1.66	0.88	0.88-0.88	1.89	0.78	34.65	12.91
V	sm	2.38	2.32-2.43	1.52	1.49-1.55	0.86	0.083-0.88	1.78	0.67	36.00	12.07
VI	m	2.30	2.21-2.38	1.41	1.38-1.44	0.89	0.83-0.94	1.59	0.53	38.56	11.66
VII	sm	2.29	2.27-2.32	1.49	1.49-1.49	0.80	0.77-0.83	1.86	0.69	34.93	11.64
VIII	m	2.18	2.15-2.21	1.19	1.16-1.22	0.99	0.99-0.99	1.20	0.20	45.41	11.08
HSL		19.68									

HSL – haploid set length

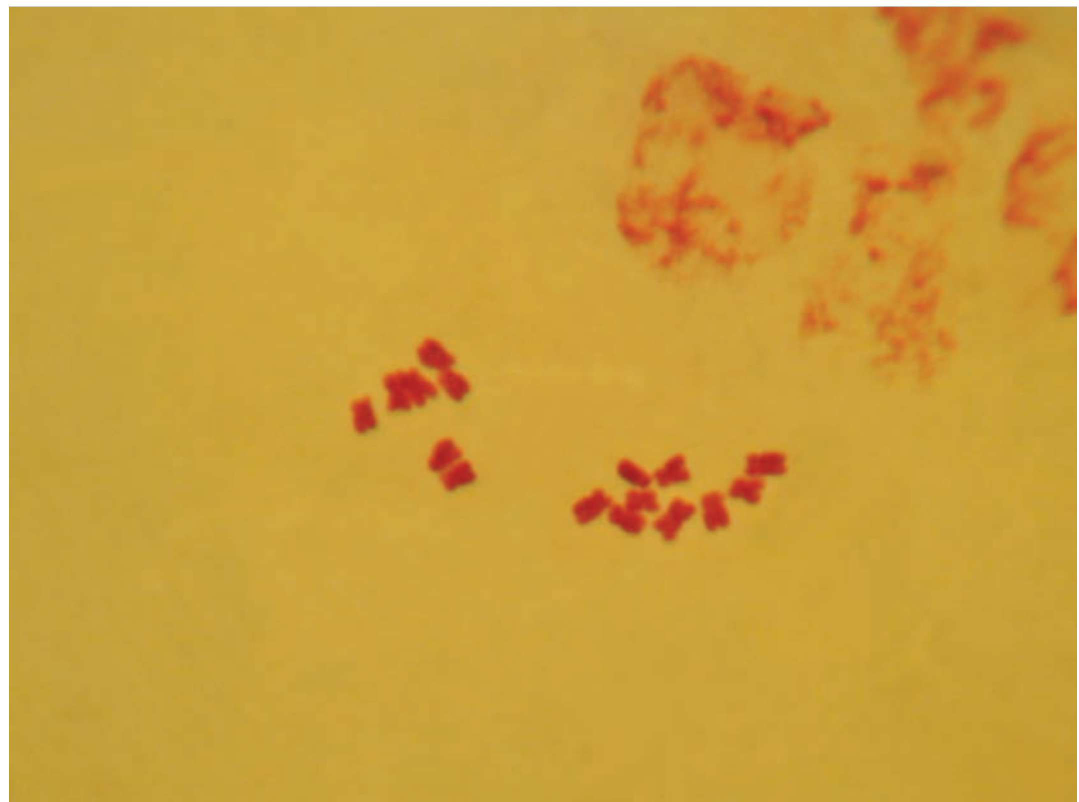

Fig. 1. *Trigonella foenum-graecum* late metaphase

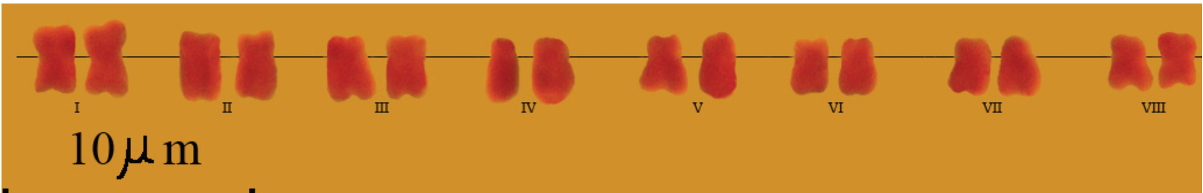

Fig. 2. The karyotype of late metaphase

In table 2 there are data for the homologous pairs from the metaphase in which the chromosomes had a small degree of condensation (fig. 3). The total length was between 4.31μm (first pair) and 2.85 μm (eighth pair) and the relative length was between 14.50μm and 9.78μm. The decreasing chromosomes length rate was little, because the 5th and 6th pairs had the same length, the decreasing rate being assured by the rest of chromosomes, the biggest one being between 7th and 8th (0.46μm).

Arms ratio was between 1.08 (2nd pair) and 3.50 (5th pair), and the arms difference, for the same chromosomes pairs, it was 0.17μm and 1.93μm. The centromeric index had values between 48.00 (2nd pair) and 22.22 (5th pair).

Unlike the anterior metaphase, in this case it was possible to identify three morphological types of chromosomes: three **m** (II, VI, VII) pairs with r=1.08-1.65, four pairs **sm** (I, III, V, VIII) with r=1.91-2.60 and one pair of type **st** (V) which had r=3.50.

In both analysed metaphases, shown as karyotypes (figures 2 and 4), we did not identified chromosomes with satellites, in the spite of the fact that Țiță and Padureanu (2003), have specified the presence of secondary constriction at the long arm of chromosomes of first and second pairs. Martin (www.bab.com.tr) emphasized both metaphases with chromosomes without satellites and metaphases in which chromosomes have secondary constriction (first pair), but at the short arm.

Table 2. The chromosome traits - *Trigonella foenum-graecum* early metaphase

Chromosomes		Total length		Long arms		Short arm		Arm ratio (q/p)	Arm difference (q-p)	Centro meric index	Relative length
Pair	Type	µm	Limits of variability	µm	Limits of variability	µm	Limits of variability				
I	sm	4.31	4.25-4.36	2.98	2.93-3.04	1.33	1.33-1.33	2.25	1.66	30.77	14.81
II	m	4.14	4.09-4.14	2.15	2.10-2.15	1.99	1.99-1.99	1.08	0.17	48.00	14.25
III	sm	3.98	3.92-4.06	2.87	2.90-2.93	1.10	1.02-1.13	2.60	1.77	27.78	13.68
IV	sm	3.54	3.37-3.65	2.32	2.21-2.38	1.22	1.16-1.27	1.91	1.10	34.38	12.16
V	st	3.48	3.43-3.54	2.71	2.65-2.76	0.77	0.77-0.77	3.50	1.93	22.22	11.97
VI	m	3.48	3.37-3.54	2.15	2.10-2.15	1.33	1.27-1.38	1.63	0.83	38.10	11.97
VII	m	3.31	3.15-3.43	1.99	1.93-2.04	1.33	1.22-1.38	1.50	0.66	40.00	11.40
VIII	sm	2.85	2.80-2.89	1.93	1.88-1.93	0.92	0.91-0.93	2.09	1.01	32.04	9.78
HSL		29.09									

HSL – haploid set length

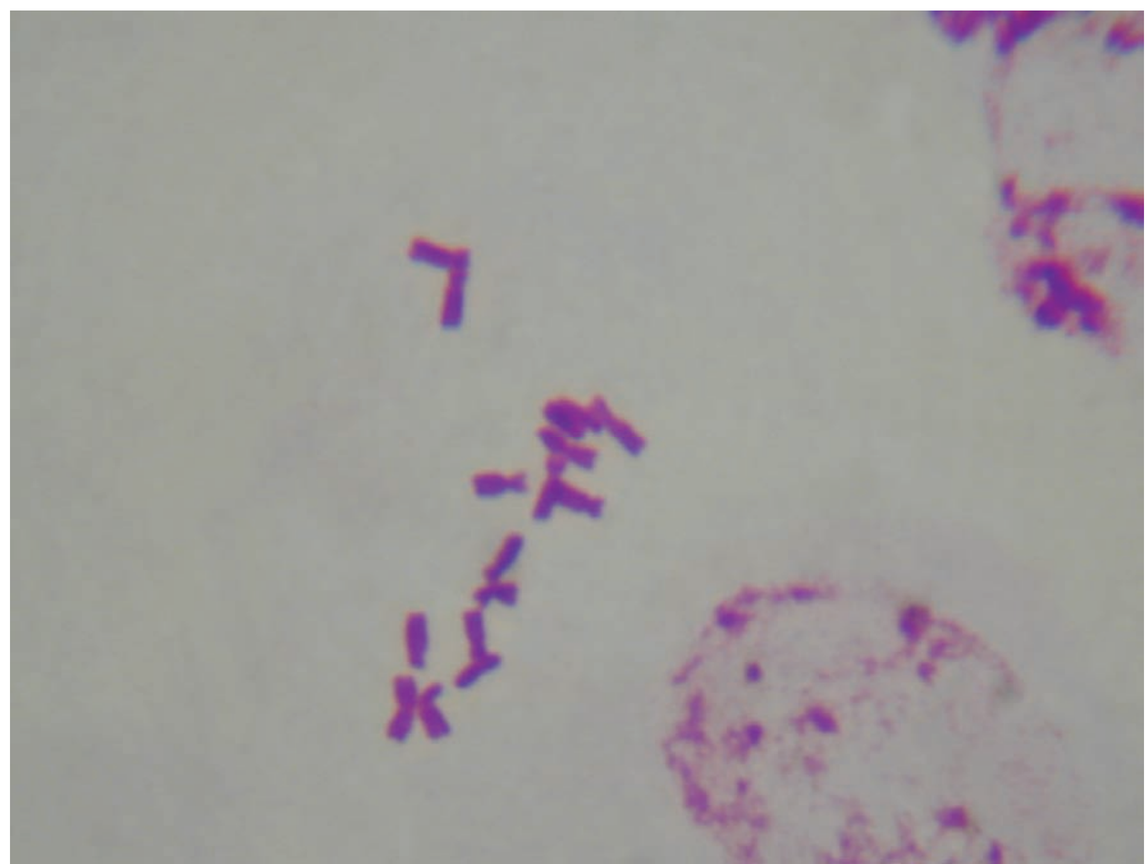

Fig. 3. *Trigonella foenum-graecum* early metaphase

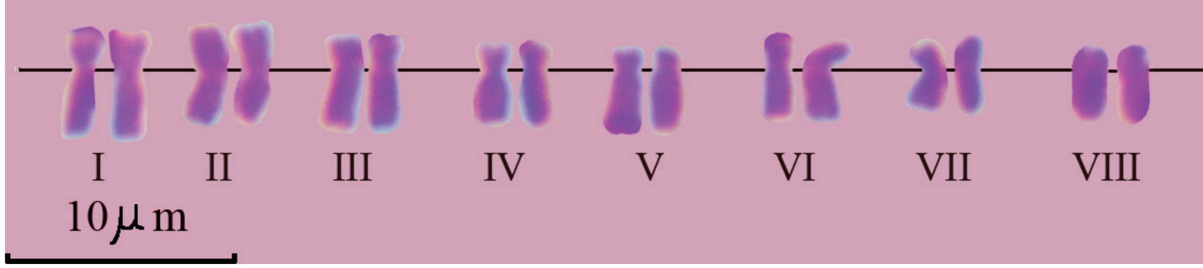

Fig. 4. The karyotype of early metaphase

Conclusions

The number of chromosomes from the root apex cells of *Trigonella foenum –graecum* L. is 2n=16.

In both analysed metaphases we have identified the following types of chromosomes: **m** (median), **sm** (submedian) and **st** (subtelocentric), this last type being signalised only in early metaphase.

The karyotype of this species is fairly symmetric being less evolved

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MORPHOLOGICAL AND MOLECULAR GENETIC APPROACH OF *ELYMUS REPENS* AND ITS RELATED *POACEAE* SPECIES

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Summary

Certain representatives of Poaceae (Gramineae) family, which includes a plant with known medicinal characteristics, Elymus (Agropyron) repens, have been chosen for a study case of molecular taxonomic approach in the biodiversity domain. The resulted molecular variance has proven a relative good informational potential of the simple RAPD method: only one of the suggested arrangement of the Elymus taxa representatives was in accordance of the known morphological based classification.

Keywords: *biodiversity, RAPD markers, Triticae tribe, red list*

Introduction

Taxonomic classification is a major task of the biodiversity domain, which includes with great responsibility economic important plants, as medicinal species are. Up to now, taxonomy was conducted by the analysis of morphological characters, which frequently showed a rather limited variation leading to controversial discussions concerning the classification of related taxa [1, 13]. Modern, molecular genetic approaches, such as random amplified polymorphic DNA (RADP) analysis [1, 5, 9, 11] have been recently introduced for completing the peculiar information regarding genetic similarities of plant genomes.

In this paper we present a synthesis of our preliminary molecular taxonomic study of certain representatives of *Poaceae* (*Gramineae*) family, which includes a plant with known medicinal characteristics. A study case of a very common plant in our country, used as medicinal, *Elymus (Agropyron) repens* respectively and its relatives [2, 4,6], is presented as a model for the molecular approach in the domain of biodiversity. Our choice is based on the frequent problems encountered with the taxa of *Triticae tribe* from *Poaceae* (*Gramineae*) family, which come obvious from their numerous synonyms. Another motivation of this choice is based on the complex chorological and sozological aspects regarding the endemic and endangered position of some of these taxa in the Red List [2, 10].

The morphological features of spike and spikelets represented the main criteria routinely used up to now for the taxonomic classification for *Triticae tribe* such as:

1.Elymus repens (L.) Gould. Synonyms – *Agropyron repens* (L.) Beauv; *Triticum repens* L., *Elytrigia repens* (L.) Nevski. It is one of the most frequent *Elymus* species, found from the steppe zone up to the mountain area in bushes and grasslands. Our accessions were from the Botanical Garden, Bucharest [4].

2. Elymus elongatus (Host) Runemark subsp. *elongatus.* Synonyms- *Agropyron elongatus* (Host.) Beauv.; *Triticum elongatum* Host. It is represented by numerous populations located in the South Romania and Dobrogea region, where is growing in grasslands and see shore sands.

3. Elymus farctus (Viv.) Runemark ex Melderis subsp. *bessarabicus* (Savul. &Rayss*)* Melderis. Synonyms- *Agropyron junceum (L.)* Beauv. Var. *bessarabicum* (Savul. &Rayss) Anghel & Morariu*, Agropyron bessarabicum* Savul. &Rayss, *Elytrygia juncea* subsp. *bessarabicum* (Savul.&Rayss) Tzvelev. It is represented practically only by few populations with a decreasing number of entities, which suggested that from the sozological point of view this species is critically endangered by the extensive touristic and building activities. Its accessions were made from Constanta (Eforie Sud/see shore sands).

4.*Elymus sabulosus* (Bieb.) Tzvelev. Synonyms- *Leymus racemosus* (Lam.) Tzvelev subsp. *sabulosus* (Bieb.) Tzvelev; *Elymus sabulosus* (Bieb.); *Elymus arenarius* L. subsp. *sabulosus.* It is a pontic species, which grows sporadicly in Romania in the Dobrogea see shores. It is considered an endangered species, considering the high antropic pressure in its habitats. Its accessions were made from Constanta (Eforie Sud).
5. *Agropyron brandzae* Pantu & Solac. Synonyms- *Agropyron cristatum (L.) Gaertner* subsp. *brandzae* (Pantu & Solac.) Melderis. It is related with *Agropyron ponticum* and is an endemic species for Dobrogea region, where it grows on sunny and stoney slopes. Its accessions were from Constanta-Cotul Vaii.
The molecular approach partially confirmed such morphological hypotesis and suggested certain different arrangements of the taxa based on the polymorphic loci revealed by different primers used in PCR random amplifications (RAPD).

Material and methods

Plant material. Herbarium specimens and fresh leaves from *Triticae* tribe accessions mentioned above (1 to 5) were used for DNA isolation.
DNA isolation. The Promega kit for DNA purification have been use for DNA preparation followed the protocol provided by company (Promega).
RAPD markers. RAPD profiles were obtained using 4 primers which amplify certain conserved minisatellite regions for core sequence minisatellite regions [14,15], in a standard PCR mixture reaction (50mM of each dNTP, 50 ng of genomic DNA, 1mM of each primer, Taq DNA polymerase and 10X standard PCR buffer). The PCR conditions reaction (Biometra thermocycler) was specifically set up for each primer and the PCR products were separated by electrophoresis through 1.5% agarose gels comparatively with a molecular marker (100/1000 bp ladder), stained with ethidium bromide and photographed under ultraviolet light with a Polaroid camera.
Data Analysis
Four RAPD profiles were obtained which showed different extent of polymorphism. The polymorphic bands were scored as a 0 (absence) and 1 (presence). The binomial matrix was used to calculate the level of polymorphism (percentage of polymorphic bands) for each sample and to compute similarities between individuals using the Jaccard's similarity coefficient, calculated as $J = a/(n-d)$, where a is the number of positive matches (i.e. the presence of a band in all samples), d is the number of negative matches (i.e. the absence of a band in all samples), and n is the total sample size including both the numbers of matches and „unmatches". The genetic distances were calculated as $GD = 1-J$ using the data from the Jaccard's similarity coefficient matrix (Table 1 to 4, represented the matrix for each sample and each primers) [16]. The minimum, maximum and mean genetic distance between the individuals for each population were compared in order to describe the molecular variance within the populations. A distance matrix was calculated using a simple Euclidian distance measure and all date were represented as a dendrogram (tree) that will give information's regarding the linkage between taxa.

Results and discussions

The study shows variable (3-11) extent of polymorphism depending on the type of the primers. The maximum number of polymorphic bands (11) corresponded to the primer 2. The genetic relatedness among species was obtained by dendrograms showing the cluster arrangements.
The four polymorphic patterns are represented in Fig.1 to 4. The informational potential of the resulted dendrograms are relatively good as they reproduce 2 of 4 the morphological based

classification of the *Elymus* species. The results are based on the measurements of the RAPD generated DNA fragment length and the corresponding likelihood Jaccard coefficients, represented in the tables 1-4.

A.The RAPD pattern and the corresponding dendrogram generated by the primer 1.

Table 1. Jaccard coefficients calculated with the RAPD pattern generated by the primer 1

P1	1	2	3	4	5
1	0	0,858	0,775	0,834	0,66
2		0	0,25	0,834	0,858
3			0	0,66	0,775
4				0	0,834
5					0

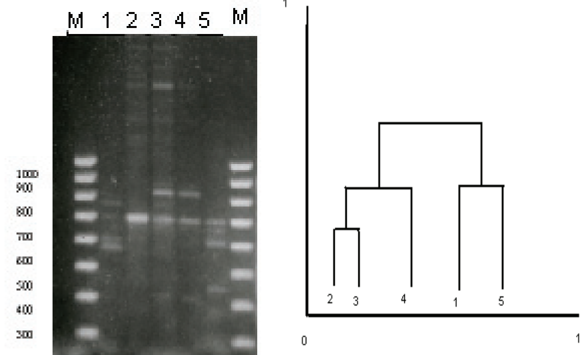
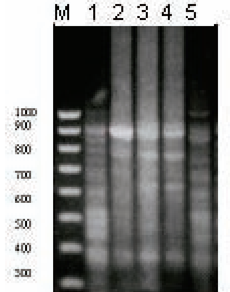


Fig.1. a) RAPD polymorphisms with primer 1. 1-5 samples*: 1.Agropyron repens,2Elymus elongatus.3Elymus bessarabicus,4 Elymus sabulosus, 5Agropyron brandzae, M-molecular marker. b)* dendrogram based on Jaccard similarity coefficient

B. The RAPD pattern and the corresponding dendrogram generated by the primer 2.

Table 2. Jaccard coefficients calculated with the RAPD pattern generated by the primer2

P2	1	2	3	4	5
1	0	0,637	0,88	0,5	0,77
2		0	0,363	0,446	0,63
3			0	0,77	0,88
4				0	0,72
5					0



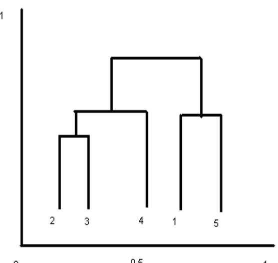


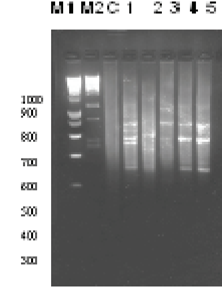
Fig.2.a) RAPD polymorphisms with primer 2. 1-5 samples*:1.Agropyron repens, 2Elymus elongatus.3Elymus bessarabicus,4 Elymus sabulosus, 5Agropyron brandzae, M-molecular marker. b)* dendrogram based on Jaccard similarity coefficient

By scoring the presence of bands 1:1 for the 5 *Elymus* representatives which are compared in pairs, the following arrangements have been suggested: (*2,Elymus elongatus:3. Elymus bessarabicus*) and (*2.Elymus elongatus:3Elymus bessarabicus:4 Elymus sabulosus,*) and (*1.Agropyron repens*: *5. Agropyron brandzae*).

C.The RAPD pattern and the corresponding dendrogram generated by the primer 3

Table 3. Jaccard coefficients calculated with the RAPD pattern generated by the primer3

P3	1	2	3	4	5
1	0	0,875	0,78	0,75	0,705
2		0	0,756	0,67	0,5
3			0	0,67	0,9
4				0	0,56
5					0



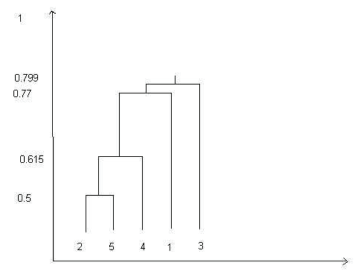


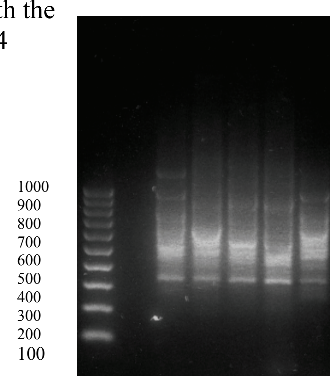
Fig.3.a) RAPD polymorphisms with primer 3. 1-5 samples*: 1. Agropyron repens, 2Elymus elongatus.3 Agropyron brandzae,4 Elymus sabulosus, 5 Elymus bessarabicus, M1.-molecular marker,100bp ladder, λPstI, M2.-Smart. b)* dendrogram based on Jaccard similarity coefficient

D.The RAPD pattern and the corresponding dendrogram generated by the primer 4

Table 4. Jaccard coefficients calculated with the RAPD pattern generated by the primer 4

P4	1	2	3	4	5
1	0	0,57	0,8	0,57	0,66
2		0	0,50	0,33	0,2
3			0	0,8	0,33
4				0	0,2
5					0

M C 1 2 3 4 5



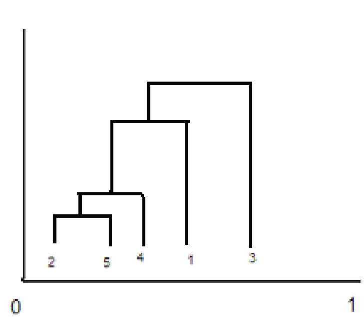


Fig.4.a) RAPD polymorphisms with primer 4. 1-5 samples:*1.Agropyron repens,2Elymus elongatus.3 Agropyron brandzae,4 Elymus sabulosus, 5 Elymus bessarabicus, M1.-molecular marker 100bp ladder. b)* dendrograme based on Jaccard similarity coefficient

By scoring the presence of bands 1:1 for the 5 *Elymus* representatives which are compared in pairs, the following arrangements have been suggested: (*2,Elymus elongatus: 5. Agropyron brandzae*), (*2,Elymus elongatus: 5. Agropyron brandzae:4 Elymus sabulosus)* and (*1.Agropyron repens: 4 Elymus sabulosus: 5. Agropyron brandzae:2.Elymus elongatus).* The accession *3, Elymus bessarabicus,*is suggested by the later two polymorphic pattern as a distinct entity.

Conclusion

Molecular methods have proven to be powerful tool in taxonomy to test morphological hypotheses at different taxonomic levels. In some cases, molecular data have confirmed previous morphological concepts or helped to decide in favor of one morphological hypothesis. In other cases, molecular studies have led to new arrangements of morphologically circumscribed entities, or were not at all supported by morphology.
The present study represents a first approach of taxonomy based on molecular methods. A simple RAPD marker showed rather relative informational potential as only two of four dendrograms reproduced the known morphological based classification.
Further investigations using new RAPD markers such codominant DNA markers, (microsatellites or AFLP's: amplified fragments length polymorphisms) would give elucidating information about the complete picture of genetic distances such as the levels of heterozygosity in the populations of the investigated plants.

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REVIEW OF SOME USEFUL METHODS IN TAXONOMICAL INTERPRETATION OF DIFFICULT TAXA OF MEDICINAL AND AROMATIC PLANTS. CASE: *THYMUS* L.

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Summary

To evaluate the significance of some well accepted methods in taxonomic evidence of botanically complicated taxa of medicinal plants, a number of populations of thyme species was studied in their morphological and anatomical traits, as well as in analyses in histo-chemistry, the main components of essential oils and molecular markers. Results confirmed a high variability in all tested characters, suggesting existence of morpho- and chemotypes.

Keywords: *Thymus, morphology, anatomy, phytochemistry, molekular markers*

Introduction

It is known that there is a problem with many taxa (genera, species, and especially intra/species categories) regarding both their botanical classification and determining in the nature. Usually, such genera include a great number of species, whereas in some cases they are not obligatory diversifed. The botanically complicated from the aspect of their classification and identification are genera such as *Hieracium* L., *Viola* L., *Verbascum* L., *Ranunculus* L., *Orchis* L., *Dactylorhiza* Necker ex Nevski, *Atriplex* L., *Campanula* L., *Orobanche* L., *Festuca* L., etc. Similar situation refers also to the medicinal and aromatic plants and may cause complications in wild collection and thus in further evaluation of their drug quality, as well as in collecting and descriptors development of accessions for gene bank needs. The following taxa of medicinal and aromatic plants may be stressed in regard of suspicious taxonomical interpretation of their species: *Mentha* L., *Achillea* L., *Hypericum* L., *Satureja* L., *Micromeria* Benth., *Allium* L., *Geranium* L., *Rosa* L., *Thymus* and some others. Difficulties in botanical determination of populations of these taxa are related to high morphological plasticity and variability in morphological features, occurrence of many similar (overlapping) characters, breeding between the genetically related species leading to numerous hybrids and frequent apomixis. On the other hand, the problems might be linked with insufficiently developed and/or often confused dichotomous keys , as well as too sophisticated tools and expensive methodologies in survey of biochemical, molecular and genetic traits. Although the structural (morphological) characters are usually only criterion for taxonomic evidence (Judd et al., 1999), there is a set of useful methods in specimen identification, including histological features, such as the structure of sieve-element plastids (Behnke 1994), general leaf anatomy (Carlquist, 1961, Barthlott, 1990, Rathnam, 1976), and secretory structures (Metcalfe, 1966), followed by embriology, chromosome number and structure (Nogueira et al., 1995), palynology, determining of secondary compounds (Adams, 1977, Dalgren, 1983, Stuessy, 1990), and recent approaches in molecular systematic (Sytsma and Hahn, 1997, Rice et al., 1997, etc.)

1.1. Why *Thymus* L. is complicated taxa for taxonomic evidence of its species?

Genus *Thymus* is one of most important genera as regards number of species within the Lamiaceae family. This family comprises more or less 220 genera. *Thymus* belongs to tribe

Mentheae, subfamily Nepetoideae. Although the number of species within this genus varies depending on taxonomical viewpoint, there is more than 200 species.
Thymus is distributed throughout the arid, temperate and cold regions of the Old World north of the Equator and on the coasts of Greenland (Morales, 1989). However the central area of this genus surrounds the Mediterranean Sea. According to Jalas (1971), *Thymus* is divided into eight sections: *Micantes*, *Mastichina*, *Piperella*, *Teucrioides*, *Pseudothymbra*, *Thymus*, *Hyphodromi* and *Serpyllum*. The first five are only found on the Iberian Peninsula, in the northwest of Africa and the Macronesian Region. Section *Hyphodromi* extends through Mediterranean area and comprises around 60 species. Three subsections can be distinguished in this section: *Subbracteati*, *Serpyllastrum* and *Thymbropsis*. Section *Serpyllum* appears to be the oldest in the genus. Around 150 species belongs to this section. They occur throughout the area of the genus. This group is taxonomicaly difficult, and Jalas (1971) divided it into 7 subsections (*Insulares, Pseudopiperellae Isolepides, Isolepides, Kotschyani, Pseudomarginati, Alternantes, Serpyllu*).
In Flora of Serbia the total of 31 species of genus *Thymus* is listed with more than 60 varieties, which mostly grow in various meadow and pasture communities and dry, sunny rocky habitats both on limestone and serpentine.
Genus *Thymus* is famous of high diversity of its species, including intra-species (population) variability and a number of described subspecies, varieties and forms. Difficulties in their taxonomical interpretation are related to high morphological variability of populations in many morphological, micromorphological traits and secondary compounds (chemical polymorphism), caused by influence of both environmental factors and genetic variation due to the frequent hybridization leading to variable chromosome number and expressed gynodioecy, a sexual polymorphism in which natural populations contain two type of plants – females and hermaphrodites (Thompson, 2002). It was shown that high female frequency causes increased heterozygosity (Gouyon and Couvet, 1987), thus increasing the population variability. Moreover, expressed variability in many morphological features even within a single plant does not allow simple and proper determination of a population. Additional problems are caused by often confuse botanical keys comprising a plenty of characters (leaf and calyx shape and size, indumentum, inflorescences, branching, etc.) and out of some are overlapping and /or insufficiently obvious both in native (fresh) and herbarium accessions. Taxonomic evidence for lower taxonomic categories (varieties, forms) is even more suspicious, insecure and difficult. It is clear that improper botanical determination of *Thymus* species might cause confusion in evaluation of a drug quality, especially because a majority of raw material at the markets of medicinal and aromatic plants originates from wild collecting.
The aim of this study was to highlight the significance of some frequently used methods in taxonomic interpretation of complicated taxa of medicinal and aromatic plants, focused on genus *Thymus* L.

Material and methods

Plant material of several populations and species of the genus *Thymus* L. was collected from their natural habitats from Serbia during the vegetation season in 2002-2003, and from the living collection of the Royal Botanical Gardens Kew for DNA sequencing.
In order to evaluate the various methodological approaches in taxonomic evidence of *Thymus* populations/species, accessions were studied through plant morphology, anatomy, histochemisry, essential oil composition, and DNA sequencing.
For morphological studies, herbarium specimens were used for measurements of leaf length and width (n=70). Within the survey of leaf and stem anatomical traits (n=50), the plant material was fixed in FAA, followed by the standard paraffin procedure, microtome sectioned

on 7-10 µm and stained with safranine and aniline blue, while the image analysis was done with software LEICA IM1000. Micromorphological research of leaf and calyx glands was conducted by SEM. Essential oils were obtained by hydro-distillation of dried aerial parts of plants collected in blossoming stage (June 2004), with yields covering a range between 0.1 and 0.8 ml/100 g of plant material. Chemical analysis of essential oil samples was performed by GC-FID (Gas Chromatography - Flame Ionization Detector) and GC-MSD (Gas Chromatography/Mass Selective Detector). Histochemical investigations were performed on fresh plant material by use of standard procedures for different chemical compounds, including sesquiterpene lactones (conc. sulphuric acid) (Geissmann et al. 1971), total lipids (Nile blue and Sudan) (Cain, 1947 and Jensen 1962), carbohydrates (PAS reaction) (O'Brian et al. 1981) and proteins (Ninhidrin-Schif reagens)(Ruzin 1999). In molecular analysis fresh plant material of each specimen was dried in 5g Silica gel. Total DNA was extracted and purified according to standard protocols. The internal transcribed spacer (ITS) regions of 18S-26S nuclear rDNA was amplified as described in Baldwin (1992). Amplified region was then sequenced and analyzed with PAUP software.
Obtained results were statistically processed by descriptive statistics, analysis of variance (ANOVA, LSD test) and cluster analyses based upon the Euclidean distances using program package Statistica 5.0.

Results and discussion

Degree of variability and corresponding clustering of surveyed accessions of the genus *Thymus* L. was separately analyzed and discussed for each applied method.

1. Morphology
Results of variability in leaf length, width and length/width ratio (ANOVA) have shown that all used characters were statistically significant ($p < 0.001$), i.e. might be important in discrimination of *Thymus* populations. According to the cluster analysis (Fig.1), the *Th. pannonicus*, was separated from the other analyzed populations, and in the less extent the endemic species *Th. dacicus*. This corresponds with the limited and geographically isolated habitats of these species in the studied region of the northern Serbia (Vojvodina).

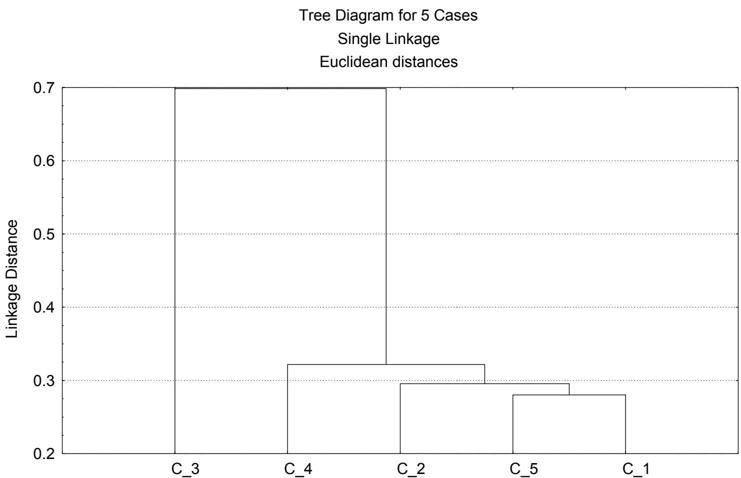

Fig. 1. Cluster for leaf size character of different *Thymus* populations
C1- *Thymus glabrescens* C2 – *Th. glabrescens* C3 – *Th. pannonicus* C4 – *Th. dacicus* C5 – *Th. marschallianus*

2. **Plant anatomy**

Regarding stem and leaf anatomy the following traits were analyzed: stem diameter, the width of stem ribs and diameter of the central cylinder, and the length of upper and lower epidermis, the width of palisade tissue and leaf thickness, respectively.

2.1 Stem anatomy

According to the features in stem anatomy significant differences were obtained in all studied characters, particularly in stem diameter (the highest in population of the *Th. pannonicus* - 1640.75µm, and the lowest in population of the *Th. dacicus* -933.35 µm). The highest diameter of the central cylinder was found in population of *Th. marschallianus* (954.85 µm). All studied characters of stem anatomy were statistically significant ($p < 0.001$), and thus might be relevant for species discriminating

1.1. Leaf anatomy

It was shown that investigated populations differ in all studied characters of leaf anatomy which all were statistically significant ($p < 0.001$). The greatest variability was found in leaf thickness ranging from 154.14 ± 15.75 to 204.83 ± 14.45 in *Th. marschallianus* and *Th. glabrescens*, respectively.

Considering relation between all surveyed anatomy traits (stem and leaf anatomy) and population discrimination (Fig. 2), population of *Th. pannonicus* exhibited much different anatomy traits, whereas populations of the same species, the *Th. glabrescens* were grouped together.

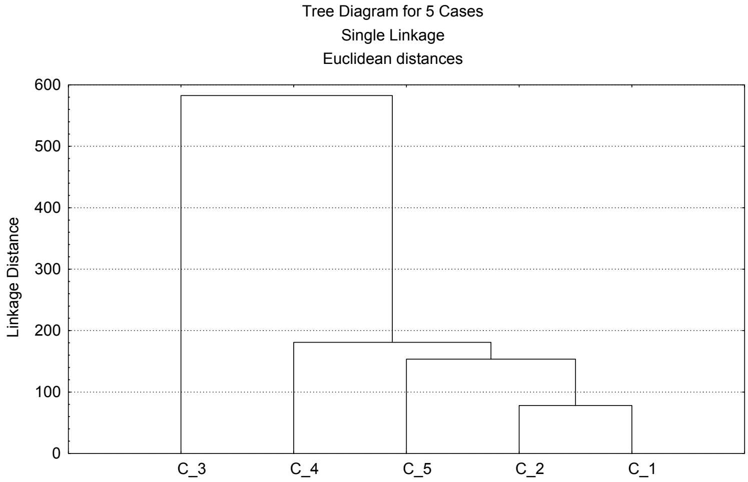

Fig. 2 Clustering of thyme populations according to stem and leaf anatomy features

C1- *Thymus glabrescens* C2 – *Th. glabrescens* C3 – *Th. pannonicus* C4 – *Th. dacicus* C5 – *Th. marschallianus*;

1.3 Micromorphology – studies on gland number and size

The presence of leaf glands and their size at lower and upper leaf epidermis and calyx varied among the studied *Thymus* populations, whereas the counting of the leaf glands of the upper and lower epidermis per mm^2 of three populations collected from three habitats of Stara mountain on the southeast of Serbia, of the endemic species *Thymus vandasii* pointed out the existence in intra-specific variability in this character, ranging from 1.15 ± 0.72 to 13.51 ± 0.86 and from 5.88 ± 1.35 to 5.88 ± 1.35 in upper and lower epidermis, respectively. According to the size of glands present at leaves and calyx studied in 10 different populations/species of the genus *Thymus* (Fig. 3.) grouping in two clades was obtained, where different populations of the same species such as *Th. marschallianus* and *Th. glabrescens* occurred in both groups, suggesting that gland size varies not only among different species, yet also among populations of the single species. Therefore the characters such as gland number and size could not be recommended in discrimination of *Thymus* species.

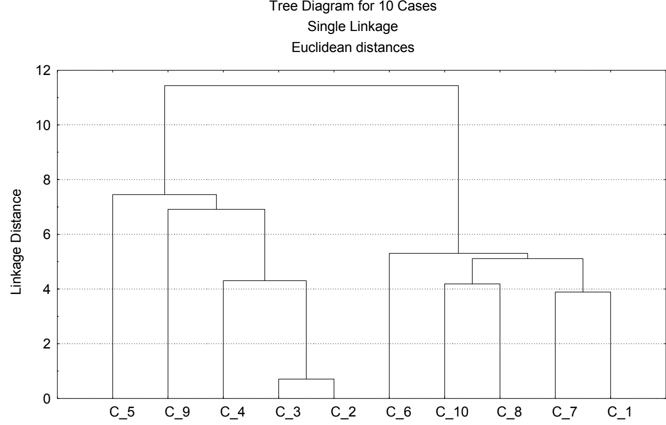

Fig. 3. Cluster according the size of leaf and calyx glands
C1- *Thymus pannonicus*; C2- *Th. glabrescens* C3- *Th. glabrescens* C4- *Th. glabrescens* C5- *Th. marschallianus* C6- *Th. pannonicus* C7- *Th. glabrescens* C8- *Th. marschallianus* C9- *Th. dacicus* C10- *Th. vandasii*

4. Histochemistry

Histochemical analysis of the target components has not revealed any differences among studied species (Tab. 1).

Table 1. Histochemical determining of some compunds in *Thymus* populations

Population	Lipids	Carbohydrates	Proteins	Sesquiterpene lactones
Th. glabrescens	+	+	+	+
Th. marschallianus	+	+	+	+
Th. pannonicus	+	+	+	+
Th. dacicus	+	+	+	+
Th. glabrescens	+	+	+	+

Studies on sesquiterpene lactones by use of conc. sulfuric acid have only confirmed that leaf glands are the structures responsible for secondary compounds (essential oil) accumulation It is possible that some more subtle methods in histochemistry should be performed, targeting the specific secondary compounds to evaluate eventual differences among different *Thymus* populations. This could be especially important for identifying of phenolic components knowing that *Thymus* taxa are characterized as either phenolic or non-phenolic (Stahl-Biskup, 2002).

5. Essential oils analysis

To evaluate the importance of the chemical composition of essential oil of *Thymus* populations the reliable group of seven components (thymol, ρ-cimene, linalool, γ-terpinene, geraniol, nerolidol and α-terpineol) was chosen in eight different species, including of some previously studied (Kulevanova, 1996). Clustering of surveyed populations (Fig. 4) showed grouping of species in two distinct clades, one comprising the *Th. tosevii*, *Th. moesiacus*, *Th. jankae* (all classified into the sub-section Pseudomarginatii), and *Th. pannonicus* of the section Isolepides. The second group included species of the section Isolepides, such as: *Th. glabrescens*, *Th. marschallianus* and *Th. dacicus*. It might be concluded that there is a relation between taxonomic position and the determinants of essential oils in the *Thymus* species. Nevertheless, many studies underlined the strong impact of environmental conditions on the essential oil composition, as the monoterpene variation may represent an adaptive strategy in relation to environmental variation, contributing to the spatial and evolutionary dynamics of the chemotypes (Thompson, 2002). It was postulated that that in some cases the participation

of particular essential oil component might be discriminator trait for *Thymus* species corresponding with their position within certain sections (Sáez and Stahl-Biskup, 2002), at least allowing the rough classification of *Thymus* taxa into the phenolic and non-phenolic group (Stahl-Biskup, 2002). This might explain the grouping of the *Th. pannonicus* within the "non-phenolic" group (absence of thymol) rather than group of genetically related species of the section Isolepides (Fig. 4). However, the critical approach of genetic determinacy in chemotype variation is needed, especially in the frame of adaptive responses of *Thymus* populations to specific habitat conditions, including soil and climate characteristics (Gouyon et al., 1986, Sáez, 1996), which was evident according to position of two different populations of the *Th. glabrescens* within the cluster (Fig. 4).

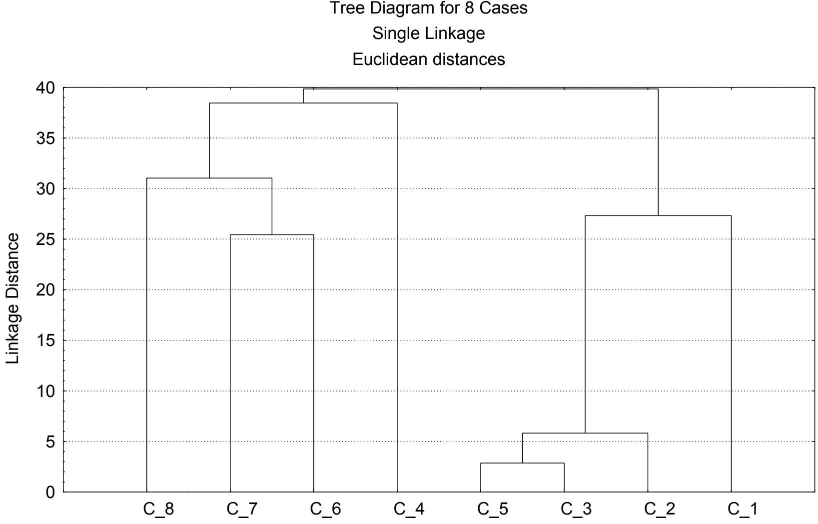

Fig. 4. Clustering of some Thyme populations and species according to main components of essential oils

C1- *Thymus glabrescens* C2 – *Th. glabrescens* C3 – *Th. marschallians* C4 – *Th. pannonicu* C5 – *Th. dacicus* C6 – *Th. tosevii* C7 – *Th. moesiacus* C8- *Th. jankae*

6. **The validity of chromosome number**

In the genus *Thymus* following chromosome numbers are known: *2n*= 24, 26, 28, 30, 32, 42, 48, 50, 52, 54, 56, 58, 60, 84 and 90, corresponding to the diploid, tetraploid and hexaploid levels. The secondary basic numbers x= 14 and x= 15 probably originated from a basic number x= 7. The most frequent numbers are *2n*= 28, 30, 56 and 60. Aneuploidy has been an important phenomenon during the evolution of this genus and is responsible for the other numbers. There are a lot of interesting cases of different levels within the same species. This is true for *Th. mastichina* with *2n*= 30, 60; *Th. vulgaris 2n*= 28, 58; *Th. zygis*, *Th. leptophyllus*, *Th. glabrescens*, *Th. longicaulis*, *Th. praecox 2n*= 28,, 56; *Th. algeriensis 2n*= 30, 56; *Th. comptus 2n*= 26, 52; *Th. zygoides 2n*= 60, 90; *Th. longidentatus2n*= 30, 90; *Th. striatus* and *Th. herba-barona 2n*= 28, 56, 84. The latter is most remarkable because the chromosome numbers studied in the Western Mediterranean populations resulted to be *2n*= 28 in Mjorca, *2n*= 56 in Corsica and *2n*= 84 in Sardinia. Although the chromosome number has not been determined within this study, it should be considered as an important character in Thymus populations discrimination, but is it still unclear whether in some species chemical polymorphism and high ploidy levels are related (Sáez and Stahl-Biskup, 2002).

7. **Molecular analysis – DNA sequencing**

The internal transcribed spacer (ITS) regions of 18S-26S nuclear rDNA are considered to be rapidly evolving, but also have highly conserved areas that can be aligned over a broad

taxonomic scale. Thus, it have become a major focus of comparative sequencing at the specific and generic levels in angiosperms. Having in mind that analysis of ITS gave good results in *Nepeta* (Lamiaceae) studies (Jamzad et al., 2003), we conducted screening survey and analysis of several *Thymus* species and populations from Serbia and RBG Kew living collection that are native to Iberian peninsula and British isles. Although thyme samples were from different geographical locations and members of different sections and subsections within the genus, the analysis showed that there is no significant difference in ITS region (Fig. 5), but it can be concluded, based on limited evidence, that the genus is monophyletic.

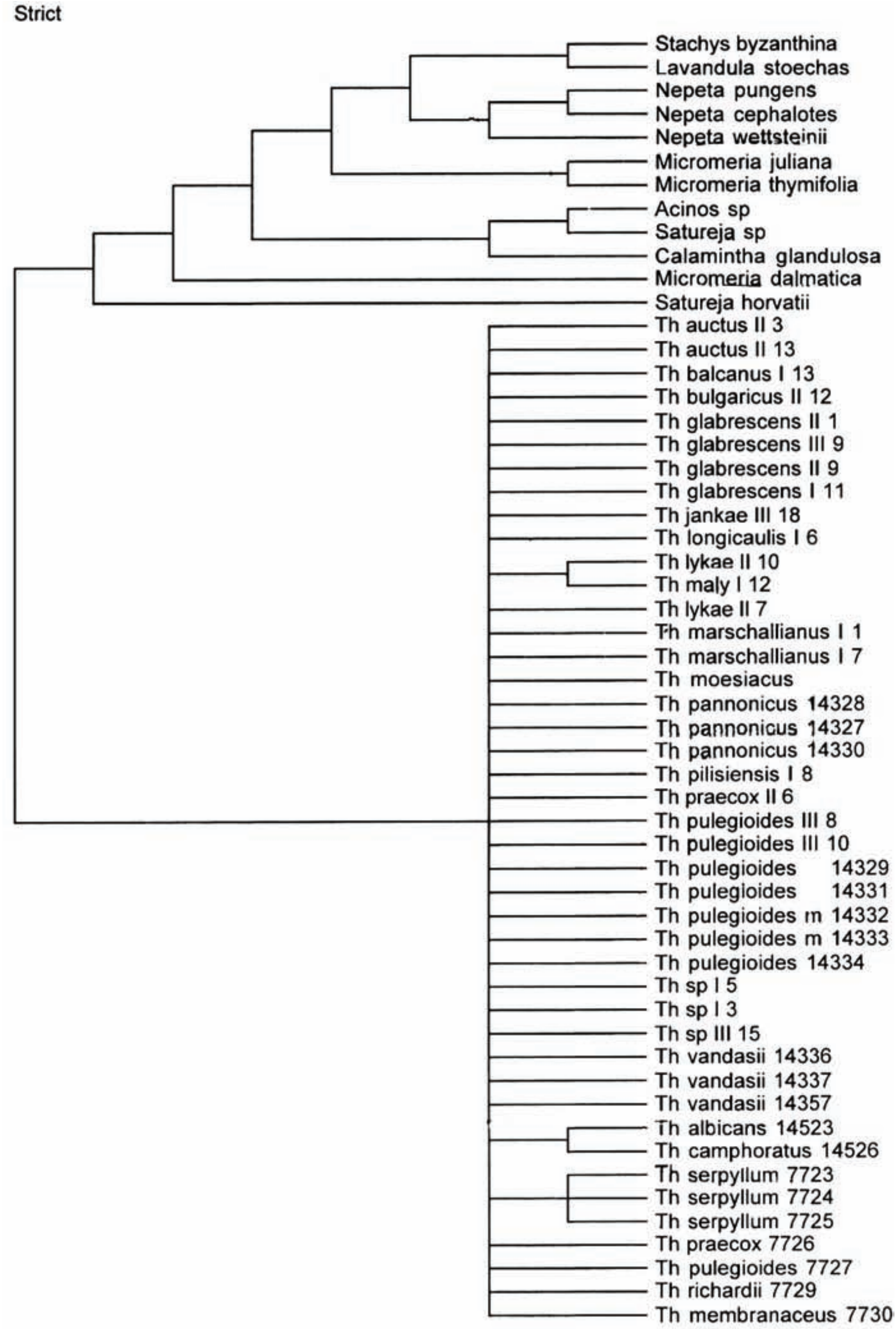

Fig. 5. Strict consensu tree for 41 *Thymus* sample and outgroups based on ITS nuclear region

For better delimitation on lower taxonomical level (species/population level) analysis of amplified fragment length polymorphism (AFLP) should be conducted. AFLP technique has a

potential for populational studies where fine scale monitoring of individual genotypes is important (Travis et al., 1996).

Conclusions

Results on infra-specific and population variability in *Thymus* L., obtained due to performing of different methods acceptable for taxonomic evidence, such as morphology, anatomy, histo-chemistry, essential oil analysis, and DNA sequencing, suggest that traditional methodological principles in taxa identification, in first morphological and anatomy traits should be combined and used as a set of characters. Chemical polymorphism based upon the content of particular components of essential oils should be estimated in relation to environment, and in further research should be correlated with molecular (genetic) studies. AFLP analysis should be conducted to establish more clear delimitation between different species, and to make clear correlation between different sections and subsections within the genus. At se same time, further anatomical and morphological investigation should back up molecular marker analysis, and result in congruent determination keys and descriptors for thyme species.

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ESTABLISHMENT AND MAINTENANCE OF CALLUS OF *STEVIA REBAUDIANA* UNDER ASEPTIC ENVIRONMENT

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Summary

The callus culture of leaves of Stevia rebaudiana was initiated and maintained on different strengths of Murashiage and Skoog (MS) medium supplemented with various phytohormones. Kinetin in combination with Naphthalene Acetic Acid (NAA) and 2,4-D showed better results in callus initiation whereas combined application of Benzyl adenine or 6-benzyl amino purine (BAP) and NAA showed most satisfactory results of callus maintenance in half strength of MS medium..

Keywords: *Stevia, stevioside, phyto-hormones, tissue culture*

Introduction

The worldwide demand for high potency sweeteners increased in present years by introducing Stevia as a modern crop from wild plant. *Stevia rebaudiana* is commonly known as candy leaves or natural non-caloric sweet plant as was officially discovered by Dr. M.S. Bertoni in early 20th century (1905) (Bertoni MS, 1905).
Stevioside and rebaudiosides are the active principal constituent of diterpene glycosides with differing sugar molecule attached as found in the leaves of Stevia plant and are responsible for high sweetness activity. Stevioside abundantly available in leaf of Stevia (13-20% in dry leaves) which shows 250-350 times sweeter and rebaudioside content in less quantities (1-3% in dry leaves) which shows 350-450 times sweeter while that of fresh healthy leaves only 30 times sweeter than table sugar. This sweetness property of Stevia focused multipurpose uses in human life. Due to sweetness and no side effect it can be used as an artificial sweeteners. Other uses viz. hypoglycemic, cardiovascular, anti-microbial, digestive tonic, dental and skin care (Mowrey D, 1992).
These beneficial uses focused Stevia's importance and its availability throughout the year, but the main problem lies with Stevia seeds which show wide variation in the stevioside content probably due to gene segregation. To avoid segregation and to improve the yield of stevioside it is necessary to propagate a genetically homogeneous population from a selected elite plant with desirable characters. Micro-propagation techniques can serve as an alternative method to conventional techniques that can give disease free plant and assist in the preservation of germplasm by storing the *in vitro* developed propagules under low temperature.
Literature survey reveals initiation and establishment of callus from explant of Stevia by using various combinations of phyto-hormones but there is no such comparative tissue culture study on Stevia for initiation and maintenance in different strength of MS medium with different concentration of phyto-hormones so far been reported (Handro *et al*., 1977 and Ferreira and Handro, 1988). Geuns JMC (2003) and Nepovim *et al* (1998), reported stevioside content depends on biomass yield and method of cultivation, same thought followed in this present study for improving more amount of callus in suitable media. Therefore it is worthwhile to develop the callus in different strength of MS medium and medium selection with better callus initiation and their maintenance. HPTLC analysis study reveals that confirmation.

Materials and methods

Selection of Plant material: Stevia plants, grown in the green house of the herbal garden, University of Agricultural Sciences, Gandhi Krishi Vigyana Kendra, Bangalore, were selected as the source of mother plants. Plants parts like apical buds, axillary buds, and stem segments were the explants sources initially for shoot production .Leaf discs were used for the callus induction.

Chemicals: All the chemicals i.e. growth regulators, agar used for the culture media preparation were of analytical grade, obtained from the Hi-Media company.

Glass wares: Various glasswares like the Erlenmeyer flasks, test tubes, Beakers, pipettes, funnels, measuring cylinders and glass rods etc. were used. Glass bottles and jars with poly propylene caps were also used.

Preparation of Glass wares : All the glass ware were soaked in 5% Chromic acid, overnight and were washed in running tap water followed by rinsing with double distilled water .The cleaned glasswares were then dried in forced draft hot air oven at 100^0 C temperature and were stored in dust proof cupboards until further use. Clean and dry glasswares were used for all kinds of tissue culture studies throughout the present research work..

Preparation of Stock solution: The stock solution of auxins- Indole acetic acid (IAA), Naphthalene Acetic Acid (NAA), Indole-3-butyric acid (IBA) and 2,4, Dichlorophenoxy acetic acid (2-4-D) were dissolved separately in minimal quantity of redistilled ethyl alcohol and volume was made up with double distilled water.

The stock solution of cytokinins-kinetin, Benzyl adenine or 6-benzyl amino purine (BAP) was dissolved in a few drops of 0.1 N HCl and volume was made up with double distilled water.

Stock solution of Poly vinyl pyrrolidone (PVP) was made (100 mg L^{-1}) by dissolving 0.1 N HCl or 0.1N NaOH .

All the above stock solutions were stored in a refrigerator at 5-6^0C then kept back to the ambient temperature before using them for the media preparation.

Preparatoin of Culture Medium: Various combinations of MS media were prepared with double distilled water with sugar concentration 3 g lt^{-1} along with different concentration of required growth hormones. 0.7% Agar was used as gelling agent. The media was then dispensed into culture tubes, 100ml EM flask, 200ml bottles with 15 ml ,40 ml and 60 ml media and PVP 5ml, 10ml and 15ml were added respectively to avoid browning of explants and were avoided using cotton plugs or Laxbro plastic caps and made air tight. They were then sterilized in an autoclave at a temperature of 121^0 C and a pressure of 103.4 kPa for 20 minutes. The containers with cultured media were stored in a dust proof sterile chamber.

Preparation of explants for Inoculation and Incubation: The selected explants were washed thoroughly under running tap water for 15 minutes. Explant was treated with Bavistin (0.1%) for 15 minutes (Patil *et al.*, 1996). The explants were washed with single distilled water and then it was taken to the laminar airflow chamber. The working bench of laminar airflow was sterilized using 70% alcohol. The treated plant materials were subjected to sterilization using 0.1% $HgCl_2$ for 5 minutes and repeatedly washed with sterile double distilled water. Then a known weight of tissue or a uniform plant material (leaf, axillary bud or meristem) were dissected and inoculated to the medium. The inoculated tubes, flasks and bottles were then incubated in the culture room at 25 ± 2^0C under fluorescent light with an intensity of 60 U E in m^{-2} Sec^{-1}. A photoperiod of 16 hours of light and 8 hours of darkness was maintained through an automatic timer, with a constant relative humidity of 65- 75% using an air-cooling system.

Growth measurement: In each replication of different treatments for an individual experiment, uniform explant tissues were inoculated and were incubated at 25 ± 2^0C. Each time a fixed number of replications were taken for the record. Weight of the callus and

maintenance of the same were also done 30 days after inoculation and after every 16 days of subculturing respectively.

a) Effect of different combinations of auxin and cytokinin (kinetin) on callus induction of *Stevia rebaudiana* in different strengths of MS medium.
Treatments: Different strengths of MS Basal medium along with auxins (IAA, IBA, NAA, 2,4 D) 1 mg/ L, cytokinin (Kinetin) at 0.1, 0.2 and 0.3 mg/ L were used in combination.
Methodology: Leaf discs of 3 mm size were sterilized and inoculated in culture tubes containing 15 ml media.
Observations: 30 days after inoculation, observations were recorded. Results were tabulated in Table-1 with figures (Fig- 1 and 2).

b) Effect of different combination of auxin and cytokinins for callus maintenance of *Stevia rebaudiana* in half and single strength of MS medium.
Treatments: MS Basal medium along with auxins IAA, NAA, 2,4,D at 0.1 mg L^{-1} and cytokinins, Kinetin and BAP at 1 and 2 mg lt^{-1} were used in combination.
Methodology: As explained above.
Observations: After 30 days callus growth for different treatments were recorded that were tabulated in Table-2 with figures (Fig-3 and 4).

Results and discussions

a) Effect of different combination of auxins and Kinetin as cytokinins on callus induction of *Stevia rebaudiana* in different strengths of MS medium:
The results (Table 1) show that the growth of callus was found independent due to an increased strength of MS media. Here, half strength of MS medium had given similar trend as like that of single strength of MS medium. Indole -3- butyric acid (IBA) and Kinetin combinations did not show any good response as like that of other combinations. 2,4-D 1.0 mg/L and Kinetin 0.2 mg /L combinations showed highest callus growth in both half (2.20 cm) and single strength (2.25 cm) MS medium. Whereas, IAA 1.0 mg/L + Kinetin 0.3 mg/L combination showed response to all strengths of MS media, but in terms of survival rate, 2,4-D and Kinetin showed good response among all. However, the overall growth response was better in single strength MS medium than others but maintenance of callus was better in half strength MS medium. Hence half strength MS medium was selected for callus growth.

Table 1. Effect of hormones on callus induction

Treatments	Callus response on MS media			Callus diameters (cm)			Maintenance of callus		
	½	1	2	½	1	2	½	1	2
NAA1.0+Kinetin0.1	No	No	No	--	--	--	--	--	--
NAA1.0+Kinetin0.2	Yes	Yes	No	1.00	1.08	--	++	+	--
NAA1.0+Kinetin0.3	Yes	Yes	No	1.12	1.13	--	+	+	--
IAA1.0+Kinetin 0.1	No	No	No	--	--	--	--	--	--
IAA1.0+Kinetin0.2	No	Yes	No	--	1.50	--	--	+	--
IAA1.0+Kinetin0.3	Yes	Yes	Yes	2.01	2.10	1.70	+	+	--
IBA1.0+Kinetin 0.1	No	No	No	--	--	--	--	--	--
IBA1.0+Kinetin0.2	No	No	No	--	--	--	--	--	--
IBA1.0+Kinetin0.3	No	Yes	Yes	--	1.70	1.60	--	+	--
2,4D1.0+Kinetin0.1	Yes	Yes	No	2.10	2.20	--	--	--	--

2,4D1.0+Kinetin0.2	Yes	Yes	No	2.20	2.25	--	++	++	--
2,4D1.0+Kinetin0.3	Yes	Yes	Yes	1.70	1.80	0.96	--	+	--

IBA= Indole butyric acid; IAA= Indole acetic acid; NAA=Naphthoxy acetic acid; 2,4 D = 2, 4 Dichloro acetic acid; (--) Dried; (+) Upto first subculture; (++) Upto second subculture;

b) Effect of different combination of auxin and cytokinins for callus maintenance of *Stevia rebaudiana* in half and single strength of MS medium.

The results (Table 2) show that 2,4-D 0.1 mg L^{-1} and Kinetin 2 mg L^{-1} gave higher callus growth of 5.10 cm and 4.90 cm respectively in half and single strength of MS medium respectively and least one was IAA 0.1 mg L^{-1} and BAP 1.0 mg L^{-1} combinations for half strength (1.00 cm) and NAA 0.1 mg L^{-1} and Kinetin 2.0 mg L^{-1} combination for single strength (1.03 cm). Callus culture in combinations of 2,4-D, IAA with Kinetin and BAP were dried after 2 weeks of inoculation, while combination of NAA with BAP and Kinetin were of viable in nature and green in color, but more stable was recorded with NAA and BAP combinations in both half and single strength MS media. Therefore, it may be concluded that half strength MS medium was proved superior for callus maintenance in hormone combinations of NAA and BAP.

Table 2. Effect of hormones on maintenance of callus

Treatments	Callus initiation		Callus growth (cm)		Maintenance of callus	
	½MS	1 MS	½MS	1 MS	½MS	1 MS
IAA0.1+Kinetin1.0	No	No	--	--	--	--
IAA0.1+Kinetin2.0	Yes	Yes	1.90	1.95	--	+
IAA0.1+BAP1.0	Yes	Yes	1.00	1.10	--	--
IAA0.1+BAP2.0	No	Yes	--	1.05	--	--
NAA0.1+Kinetin1.0	No	No	--	--	--	--
NAA0.1+Kinetin2.0	No	No	--	--	--	--
NAA0.1+BAP1.0	Yes	Yes	3.10	2.81	++	+
NAA0.1+BAP2.0	Yes	Yes	4.30	4.25	++	++
2,4D0.1+Kinetin1.0	Yes	Yes	1.80	1.80	+	--
2,4D0.1+Kinetin2.0	Yes	Yes	5.10	4.90	+	+
2,4D 0.1+BAP1.0	Yes	Yes	2.10	2.15	--	--
2,4D 0.1+BAP2.0	Yes	Yes	2.03	3.00	+	--

Table 3. HPTLC analysis data for micro propagated callus study

Treatments	Fresh weight(g)	Dry weight(g)	% stevioside
Half MS medium	2.72	0.512	5.60
Full MS medium	2.31	0.331	4.45

The production of Stevioside in callus cultures has been positively reported by Komatsu *et al* (1976) while Handro *et al* (1977) reported that their culture were not sweet but in this present study effect of MS medium at half and full strength was evaluated on sweet stevioside content in Stevia.

From the Table 3, it was found that half MS medium was better (5.60 %) than full strength (4.45 %) in respect of stevioside content. The results of the present investigation has confirmed the earlier results reported by Nepovim *et al*., (1998) and Geuns (2003).

Conclusions

The NAA 0.1 + BAP 2.0 treatment combination has been proved superior in callus initiation, growth and maintenance. The use of half MS medium was found better with respect to fresh weight, dry weight and percentage of stevioside content compared to full MS medium irrespective of different treatments.

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INTERACTION EFFECT BETWEEN PHOSPHORUS AND ZINC ON THEIR AVAILABILITY IN SOIL IN RELATION TO THEIR CONTENTS IN *STEVIA REBAUDIANA* GROWN IN INDIAN SUBTROPICS

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Summary

A greenhouse experiment was conducted at the Indian Institute of Horticultural Research (IIHR), Bangalore, India, to study the interaction effect between phosphorus and zinc on their availability in soil in relation to their contents in Stevia rebaudiana. The results show that the amount of available P and Zn content in soil has been found to increase initially and, thereafter, the amount of the same decreased with the progress of plant growth up to 60 days irrespective of treatments. The amount of P and Zn in soils showed an increase with their separate applications either as soil or foliar spray while that of the same value significantly decreased both in soils and plants due to their combined applications, suggesting a mutual antagonistic effect between Zn and P affecting each other's availability in soil and content in the stevia plant.

Keywords: *interaction, phosphorus, soil, stevia, zinc*

Introduction

Stevia (*Stevia rebaudiana* Bertoni) is a sweet herb (medicinal plant) native of Paraguay. It belongs to the family Compositae and is fast becoming a major source of high-potency bio-sweetener for the rapidly growing market of "natural" foods, replacing chemical sweeteners (Saccharine) and even table sugar (Mandal *et al* ., 2005). Roy *et al*.(2005) also reported that the leaves of stevia are free from carbohydrates and calories and, hence, it can be used safely by diabetic patients. It has now been recognized that stevia has many uses for human beings, especially when it acts as a sugar substitute for those persons with blood sugar problems.

In view of the above fact, cultivation of stevia is gradually gaining importance in India, having no sufficient agronomic management practices. Any practice of a nutrient management, which either decreases or increases the supply of another nutrient element or its absorption from the soil by plants or translocation and mobility within the plant, will influence its nutrition and, thereby, the nutrient use efficiency and crop yields (Takkar *et al*., 1985). The use of NPK fertilizers and well-rotten FYM are common practice for cultivation of any crop including medicinal plants like stevia. However, the efficiency of applied P rarely exceeds 30% and that of most of the micronutrient cations more than 10%. Therefore, their repeated applications over the years lead to their buildup and interactions in soils and plants, affecting agricultural production (Takkar *et al*., 1985). But the use of micronutrient fertilizers, especially Zn, for cultivation is still limited; rather, no information about this is available. The heavy use of P fertilizers may have some adverse or favourable effect on the availability of applied Zn in soils as well as its effect on plants (Takkar *et al*., 1985 and Das and Mandal., 1986). Hence, the interaction effect between Zn and P is still very much contradictory. Therefore, it is worthwhile to study the interaction effect between P and Zn on their availability in soil in relation to their contents in stevia.

Materials and methods

Cuttings of the stevia plant were collected from Gandhi Krishi Vigyan Kendra, Bangalore and were used as a test plant. Before planting, initial soil samples were analysed for pH (Soil:Water, 1:2.5), organic carbon, CEC, available P, and DTPA-Zn by following the methods described by Jackson (1973) and Lindsay and Norvell (1978). After extracting the soil samples, Zn and P were determined with the help of an atomic absorption spectrophotometer (Perkin Elmer model AAnalyst 100) and spectrophotometer, respectively. The relevant physicochemical properties of soils were: pH, 8.9; organic carbon, 3.8 g/kg; available P_2O_5, 38 kg/ha; CEC, 14.4 Cmol(p^+)/kg; DTPA-extractable Zn, 0.42 mg/kg.
Thirty-two earthen pots (15-kg capacity) were taken and 10-kg powdered soil collected from the Indian Institute of Horticultural Research Farm, Hessaraghata, Bangalore was filled in each pot and the following treatments were: T_1 – absolute control, no application of Zn and P; T_2 – application of P_2O_5, but no application of Zn; T_3 – soil application of Zn as $ZnSO_4$ @ 10 kg/ha, but no application of P_2O_5; T_4 – foliar application of Zn as $ZnSO_4$ @ 0.2% solution, but no application of P_2O_5; T_5 – both soil ($ZnSO_4$ @ 10 kg/ha) and foliar ($ZnSO_4$ @ 0.2%) of Zn, but no application of P; T_6 – soil application of both Zn as $ZnSO_4$ @ 10 kg/ha and P_2O_5 as single super phosphate (SSP) @ 30 kg/ha; T_7 – foliar application of Zn as $ZnSO_4$ @ 0.2% along with soil application of P_2O_5 at 30 kg/ha; T_8 – both soil (Zn as $ZnSO_4$ @ 10 kg/ha) and foliar (Zn as $ZnSO_4$ @ 0.2%) application of Zn along with basal application of P_2O_5 as SSP @ 30 kg/ha. Each treatment was replicated four times in a completely randomised design (CRD). There were 32 pots (8 × 4) altogether. The pots were placed in net house in order to monitor growth of the plant after cuttings of the stevia plant were put in each pot. Then the plants were allowed to grow for a period of 60 days. The periodic collection of soil and plant samples was made and analysed for pH, DTPA-extractable Zn, and available P by following the method as mentioned earlier.

Results and discussion

The results (Table 1) show that the amount of available P content in soil has been found to increase initially and, thereafter, the amount of the same gradually decreased up to 60 days of plant growth irrespective of treatments. The magnitude of such changes, however, varied with treatments; the greater amount in the treatment where only P was applied as basal. The results further indicated that the increased amount of available P was found to be significantly decreased with the application of Zn and P; the greater decrease (1.74 mg/kg) with both soil and foliar application of Zn along with basal application of P to the soil at 60 days of plant growth. Such decrease might be due to the antagonistic effect between Zn and P in soils forming insoluble compounds, $Zn_3(PO_4)_2$ resulting in the low amount of P in the available pool (Takkar *et al*., 1985, Mandal and Haldar., 1980 and Das, 2004).

Table 1. Interaction Effect Between P and Zn on the Changes in Available P Content (mg/kg) in Soil

Treatments	Days after Plant growth				
	15	30	45	60	Mean
–Zn, –P	1.64	2.14	1.86	1.74	1.85
–Zn, +P	3.26	3.68	2.48	1.97	2.85
Zn(S), –P	1.58	1.79	1.57	1.46	1.6
Zn(F), –P	1.49	1.55	1.50	1.44	1.50
Zn(S+F), –P	1.60	1.64	1.54	1.48	1.57
Zn(S), +P	2.78	2.96	2.32	1.84	2.48

Zn(F), +P	2.66	2.88	2.41	1.81	2.44
Zn(S+F), +P	2.69	2.77	2.11	1.74	2.33
Mean	2.21	2.43	1.97	1.69	
p (0.05)	0.14	0.21	0.17	0.11	

The results (Table 2) show that the amount of DTPA-extractable Zn content also showed almost a similar trend of changes to that of available P, but the absolute amount of Zn was recorded much lower than that of available P in soil. Considering the effect of different treatments, it was found that the amount of Zn content in soil was maintained highest (1.36 mg/kg) in the treatment where Zn was applied as both soil and foliar spray in the absence of P, which was closely followed by the treatment T_3 (1.22 mg/kg) where Zn was applied to the soil in the absence of P. The relatively lower amount of Zn was maintained in treatments where combined application of Zn, both as soil and foliar, and P was made. Such decrease in the amount of Zn due to combined application of P and Zn might be explained as the antagonistic effect between them. It has been reported that the interaction between Zn and P occurred in soil because added P decreased the available Zn content in plants. An increasing level of P in soils significantly decreased shoots and grain yields of maize (Takkar *et al*., 1985).

The results (Table 3) show that the amount of Zn in stevia plants gradually decreases after attaining a maximum value at 30 days of plant growth, which might be due to the dilution effect resulting from the greater biomass production at the later period of growth. The amount of Zn content was maintained highest (4.62 mg/kg) in the treatment T_5 where Zn was applied as both soil and foliar spray in the absence of P with the simultaneous highest total biomass production (23.34 g), which suggests that the contribution of Zn content in plants towards biomass production was far greater than that of P content. However, such decrease in Zn content in stevia plants due to P application might be explained by restricting the translocation of Zn from roots to other parts of the stevia plant resulting from the interference of applied P in the metabolic processes of plants (Takkar *et al*., 1985). An excess of P inhibits Zn uptake, first by curtailing its translocation through endodermis into root xylem, and finally and more importantly, by lowering its rate of absorption through the epidermal or surface cell layer of the root (Takkar *et al*., 1989).

Table 2. Interaction Effect Between P and Zn on the Changes in Zn Content (mg/kg) in Soil

Treatments	Days after Plant growth				
	15	30	45	60	Mean
–Zn, –P	0.44	0.52	0.43	0.46	0.46
–Zn, +P	0.46	0.50	0.44	0.48	0.47
Zn(S), –P	0.82	1.47	1.78	1.22	1.32
Zn(F), –P	0.48	0.55	0.68	0.62	0.58
Zn(S+F), –P	0.88	1.54	1.89	1.36	1.42
Zn(S), +P	0.70	0.82	0.86	0.88	0.81
Zn(F), +P	0.54	0.63	0.59	0.51	0.57
Zn(S+F), +P	0.79	0.82	0.89	0.83	0.83
Mean	0.63	0.86	0.95	0.80	
p (0.05)	0.16	0.12	0.28	0.23	

Table 3. Interaction Effect Between P and Zn on the Zn Content (mg/kg) in Plant

Treatments	Days after Plant growth				
	15	30	45	60	Mean
–Zn, –P	2.61	2.82	2.58	2.10	2.53
–Zn, +P	2.30	2.48	2.27	1.96	2.25
Zn(S), –P	4.54	4.86	4.42	4.28	4.5
Zn(F), –P	4.91	5.18	4.76	4.40	4.81
Zn(S+F), –P	4.98	5.89	4.78	4.62	5.07
Zn(S), +P	2.58	2.78	2.36	2.11	2.46
Zn(F), +P	4.23	4.58	3.82	3.77	4.1
Zn(S+F), +P	2.48	2.86	2.24	2.08	2.42
Mean	3.58	3.93	3.40	3.17	
p (0.05)	1.12	1.26	1.14	1.22	

The results (Table 4) show that the amount of P content in stevia plants has been found to increase initially and, thereafter, the amount of the same decreased up to 60 days of plant growth. Such variation in the P content, however, varied with treatments. The highest (1.98 mg/kg) and lowest (1.46 mg/kg) amount of P was maintained in T_7 and T_4 treatments where Zn was applied as foliar along with basal application of P and foliar spray of Zn in the absence of basal application P, respectively. Zinc fertilization depressed P concentration in plants, but the interactive effect of P on Zn was more pronounced than that of Zn on P (Takkar *et al.*, 1985). It was also reported that reduced synthesis of some organic acid complexes at high P levels partly might be the cause for P-induced Zn deficiency and also partly due to reduction of unit absorption rate of Zn by roots putting restraint on the functional requirement of Zn by plants (Takkar *et al.*, 1989).

Table 4. Interaction Effect Between P and Zn on the P Content (mg/kg) in Plant

Treatments	Days after Plant growth				
	15	30	45	60	Mean
–Zn, –P	1.74	1.92	1.80	1.58	1.76
–Zn, +P	2.40	2.68	2.42	1.94	2.36
Zn(S), –P	1.76	1.89	1.77	1.60	1.76
Zn(F), –P	1.78	1.86	1.80	1.46	1.73
Zn(S+F), –P	1.71	1.88	1.78	1.58	1.74
Zn(S), +P	1.96	2.05	1.84	1.71	1.89
Zn(F), +P	2.37	2.74	2.48	1.98	2.39
Zn(S+F), +P	1.97	2.10	1.87	1.73	1.92
Mean	1.96	2.14	1.97	1.70	
p (0.05)	0.38	0.27	0.18	0.21	

Conclusions

The interaction effect between P and Zn did not show any positive effect on their availability in soil as well as their contents in the *Stevia rebaudiana* plant. The results clearly suggested that the application of Zn only, as both soil and foliar spray, was found superior over that of P only as basal application in relation to their availability as well as contents in plants, while that of the same content in soil and plant was recorded significantly lowest when Zn was applied as both soil and foliar spray in the presence of P, suggesting an antagonistic relationship between Zn and P in relation to their availability in soil and contents in plants.

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GERMAN CHAMOMILE *CHAMOMILLA RECUTITA* (L.), A SUITABLE CROP FOR CONVERSION PERIOD

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Summary

In organic production conversion period is the most difficult time for producers. To overcome that period with minimum loss of income it is very important to choose appropriate crop. In this experiment 9 producers are chosen and they start with organic production on 11 ha. After two years of production it was obvious that chamomile is excellent crop for conversion period because does not need protection from pests and disease and does not need mineral fertilizers. Yield was between 1600 and 2500 kg/ha of pure chamomile flowers and between 1500 and 2200 kg/ha flowers with petals. After drying producers put on the market chamomile like conventional but anyway they earn more than with some cereal crops. This experiment shows that chamomile is for sure suitable crop for conversion period.

Keywords: *Chamomile, conversion period, organic agriculture*

Introduction

Chamomile, *Chamomilla recutita* (L.) Rauschert, family Asteraceae, is a native of southern and eastern Europe. Today, however, this species has spread throughout most of the European continent. The plant has considerable ecological amplitude and can adapt to less than optimum climatic and soil conditions (Salamon, 1992). Chamomile is one of the most important medicinal plants in the world, grown primarily for the flowers which are used as a drug (*Flos Chamomillae)*. This chamomile drug has anti-inflammatory, antiseptic, stimulative, carminative, spasmolytic and sedative activity (Salamon, 1994).
In Bosnia and Herzegovina medicinal plants are cultivated on surface which is between 200 and 300 ha. Most of these cultivation areas are chamomile fields (Companies' Catalogue, 2006).
In conversion period, which is obligatory in organic production, the most important question is how to overcome that phase with minimum loss of income and how to avoid use of mineral fertilizers and pesticides. Pests and diseases in medicinal plant production do not represent problems and those species do not have big demand for nutrients. Weeds are the biggest problem in this production (Znaor, 1996). In chamomile field it is possible to find more than 40 weed species, but just some of them are important (*Veronica persica Poir., Rumex crispus L., Sonchus asper (L.) Mill., Convolvulus arvensis L.)* (Monograph, 1997). *Capsella bursa-pastoris* is the most important weed because its cycle is compatible with chamomile cycle, but this problem can be solved with proper agro technique (Muminovic, 1998). The intention of this assessment was to find out could chamomile be a suitable crop to overcome the problems which appears during conversion period.

Material and methods

The cultivation and production in conversion period has two main complex questions: how to overcome that phase with minimum loss of income and how to avoid use of mineral fertilizers and pesticides. Proper choice of crop can solve those problems. This field trial was carried out on 9 farms, totally surface 11 ha, in the village Liskovac, North Bosnia and Herzegovina, near the city Nova Gradiska. This region is the biggest flat area in Bosnia and Herzegovina and it

is characterized with intensive and modern agriculture production. This locality has temperate continental type of climate with annual mean temperature 11˚C and total precipitation 820 mm. It is on 92 m a. s. l. altitude.
Prevailing soil type in that village is pseudogley with following characteristics: pH (KCl) from 4.1 to 7.0; content of humus from 1,5 % to 3,4 %; from 0,5 to 16,3 mg/100 g P_2O_5; from 17,4 to 41,5 mg/100 g K_2O (Table 1).

Table 1. Soil analysis from 9 farms

Farm	pH		Humus %	P_2O_5 mg/100 g	K_2O mg/100 g
	H_2O	KCl			
1.	5.7	4.6	1.8	4.1	17.4
2.	6.4	5.5	2.4	16.3	41.5
3.	5.2	4.5	2.8	9.2	39.2
4.	6.1	4.7	3.4	2.8	20.5
5.	5.4	4.6	1.9	4.8	25.8
6.	5.3	4.1	1.8	1.2	22.5
7.	7.1	7.0	3.0	2.1	23.7
8.	6.3	5.2	2.2	0.5	30.2
9.	6.2	5.2	1.5	2.0	26.3

As we can see from the table 1, except one farm, every field has acid reaction of the soil. This is common characteristic of that region. Also we can see that percentage of humus is not high which is due to general maintenance of the soil and agro technique which farmers apply as well as characteristics of pseudogley type of soil.
On most of the farms preceding crops were cereals, wheat or barley. Farmers in that region produce wheat, barley, maize or they have cattle and in that case sow fodder crops. Fields were mainly free from weeds and with proper crop rotation every year but farmers, also used herbicide to control weeds. On farm number 4 were a huge number of *Ambrosia artemisifolia* plants, which are completely covered the field. This farmer mowed *Ambrosia artemisifolia* and prepares soil on time for chamomile sowing. This was the first time that those farmers did chamomile production.
Chamomile was propagating by direct sowing in the field, over the soil surface, in the growing season 2003/2004 and again in the 2004/2005 in dry farming conditions. On the bigger fields farmers used sowing machines and on the smaller fields they sow by hands. We use 12 kg/ha of chamomile seed, variety Banatska, which is only present on the market in that period in Bosnia and Herzegovina. Farmers finished sowing of chamomile on September 25th in the year 2003 and on September 14th on the year 2004. Fertilizers were not used.
Harvesting was done with chamomile harvester when chamomile was in full flowering. This harvester is a propelled machine which is carried by tractor and is used for direct picking of chamomile flowers. Capacity for harvesting is 2,5 ha per day. After harvesting, biomass was transported on vibrating machine which separate flowers with small stalks from flowers which have long stalks. Finally, flowers with small stalks go on stalk cuter. All biomass was dried at 40˚C, to attain 14 % of moisture content. Pure flowers went on the market like pure chamomile flower tea and flowers with stalks were pulverized and used for filter bags. After first year, some farmers decide to produce cucumbers till next sowing of chamomile and some just left soil to have a rest.

Results and disscusion

The chamomile has germinated in autumn and developed leaf rosette, safely overwinters under snow cover and starts growing at the end of the winter, when air and soil temperature went up. The harvest of chamomile crop was at the end of May. Yield of fresh chamomile varies from farm to farm (Table 2).

Table 2. Yield of fresh chamomile on 9 farms in two years

Farm	Size of the field (ha)	Yield of pure flowers (kg)		Yield of flowers with stalks (kg)	
		2004	*2005*	*2004*	*2005*
1.	0.8	1494	1528	1416	1556
2.	1	1769	1684	1643	1590
3.	1.3	2714	2905	2580	2764
4.	2.4	3932	4070	3616	3832
5.	1.5	2563	2625	2541	2791
6.	0.5	847	930	768	831
7.	1	2549	2362	2140	2068
8.	1.5	2728	2886	2623	2745
9.	1	1683	1740	1558	1798
AVERAGE:		**1843**	**1884**	**1717**	**1816**

If we recalculate yield on kg/ha we can see that the yield of pure fresh flowers in the first year was between 1638 kg/ha (farm 4) and 2549 kg/ha (farm 7) and for the second year was between 1684 kg/ha (farm 2) and 2362 kg/ha (farm 7). Yield of fresh flowers with stalks was between 1506 kg/ha (farm 4) and 2140 kg/ha (farm 7) in the first year and between 1590 kg/ha (farm 2) and 2126 kg/ha (farm 3) in the second year.
Highest yield of chamomile was obtained on farm number 7. Better yield on that farm could be consequence of several factors, among which neutral pH value and higher fertility of the soil are the most important ones.
In that period price of fresh chamomile pure flowers was 0.25 €, and farmers got 0.10 € for flowers with stalks. If we take in consideration that in chamomile production everything is mechanized and that for preparation of the soil, sowing and harvesting farmers does not need more than 4 days/ha, than we can say that this is very secure and profitable production. In addition, in Bosnia and Herzegovina farmers get subvention for medicinal plant production, which is 750 €/ha.
There were no problems with pests and diseases on any farm. Also, there was not a problem to find buyers for chamomile flower on domestic market. After second year of chamomile production, these 9 producers made together 4 draying chambers which have enough capacity to dry all chamomile flowers from their fields.
Chamomile crop succeeds in overcoming weeds due to its earlier start and rapid growth. There were no significant problems with weeds, not even on field which was full of *Ambrosia artemisifolia*. Even on that field chamomile crop prevail over weeds.

Conclusion

The chamomile yield varied from 1638 kg/ha to 2549 kg/ha. The price of chamomile flower is good and farmers can earn more money than if they produce cereals. Beside that, after harvesting of chamomile there is more than tree months for second crop which also can bring some income. If we take into consideration that this production is completely mechanized,

and that there is no serious problems with pests, diseases and weeds and also that chamomile does not need mineral fertilizers we can recommend it as a suitable crop for conversion period.

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ANATOMICAL STUDIES ON *RESEDA LUTEA* L.

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Summary

Reseda lutea L. (Resedaceae), with a wide distribution area in Turkey, is used as a natural dye in dyeing industry. Although this medicinal plant is used in life stock feeding and honey production in many parts of the world, it was reported that it is seen as a weed in some parts of the world. The aim of this study is to determine anatomical characteristics of root, stem and leaf of R. lutea in cross sections. As a result of the study, it was identified that anatomical characteristics of the species resemble to other members of the family Resedaceae, to which this species belong. The most distinguishing characteristic of the species in anatomical structure is the presence of myrosin cells, which is also present in root, stem and leaf of other members of the family. The leaf is bifacial, stomata in the leaves show mesophytic, anomocytic and amphistomatic characteristics. It is interesting that leaves of the species do not have glandular cells, but they are seen in cross sections taken from the stem.

Keywords: *Anatomy, medicinal and useful plant, Reseda lutea.*

Introduction

Resedaceae family is represented by 6 genera in the world. Among them only genus *Reseda* L. is distributed naturally in Turkey. Genus *Reseda* contains approximately sixty species throughout the world. In Turkey the genus is represented by 15 species, including *Reseda lutea* L., 1 sub-species and 7 varieties (Davis 1965, Davis *et al.* 1988, Özhatay *et al.* 1994).
R. lutea is generally distributed in temperate zones in the world. General distribution areas of the species can be defined as follows: From South, West and Middle Europe to Finland, Norway and Sweden; Great Britain; Mediterranean Basin and Anatolia; Southwest Asia, Former Soviet Union countries and Afghanistan; Chile, USA; Australia and New Zealand; South and North African countries (Dogan 2001).
R. lutea is distributed naturally on open rocky slopes and wet areas, and on changing environments as a result of anthropogenic activities like roadsides, rail road sides, garbage dump areas, around agricultural areas, artificial ditches and grave yards, etc. Our study sample, *R. lutea* is distributed densely in Turkey as a ruderal plant.
For centuries the species has been widely used by local people as a source of natural dye in carpet and rug industry in this country (Anonymous 1991, Dogan 2001). Therefore, in some places in Turkey, the species has a special place in locals' economic lives. Although it is not common nowadays, according to Bonnier (1934), this species has been used by locals due to its diuretic, sedative and sudorific characteristics.
We can summarize the economical importance of *R. lutea* in the light of related literature:

- As a medicinal plant (Bonnier 1934),
- In daily life, young leaves of plant goods in salad and raw as an edible plant (Kirk 1975, Kunkel 1984),
- In carpet and rug industry, as a source of natural dye (Uğur 1988, Anonymous 1991, Öztürk & Özçelik 1991),
- In animal farming, as a grazing plant and stock food source (Moghaddam 1977, Heap *et al.* 1995),
- In honey production, due to its high nectar secretion (Jablonski *et al.* 1992),
- In combating with the erosion, as a primer succession plant (Bruns & Jochimsen 1989,

Jochimsen & Janzen 1991, Heap *et al*. 1995),
On the other hand, the species is seen as a weed in some parts of the world, especially in cultivated areas (Abdallah & Dewitt 1978, Forbes & Mathews 1985, Heap *et al.* 1995, Dogan *et al.* 2002).
In this study, due to its economical importance and wide distribution in Turkey, it was aimed to investigate the anatomical characteristics of *R. lutea* by using samples collected from Western part of Anatolia.

Material and methods

Plant samples of our study material *R .lutea* were collected from 5 different West Anatolian cities from 25 m to 1500 m altitude from the sea level. These localities are as follows:

Canakkale
1. A1 Ecebat, between Alcitepe-Eceabat, near the field, 50 m.

Balikesir
2. B1 Savastepe, Sogucak Village, near the field, 400 m.

Izmir
3. B1 Konak, in the park.

Denizli
4. C2 Honaz Mountain, Kocapinar Village, near the field, 1500 m.

Kutahya
5. B2 Gediz, Abideler Village, 625 m.

Collected plant samples were identified taxonomically according to "Flora of Turkey and the East Aegean Islands" (Davis 1965) and "The Biology of Australian Weeds" (Heap *et al.* 1995).
After identification, the plant materials were put into 70% alcohol solution to preserve their characteristics for later anatomical investigation. Anatomical sections of the material, then, were taken from root, stem and leaves. The sections were put into sartur reactive and milon reagent. Then, photos of the sections were taken under the light microscope by using microphotography apparatus (Figures 1-9).

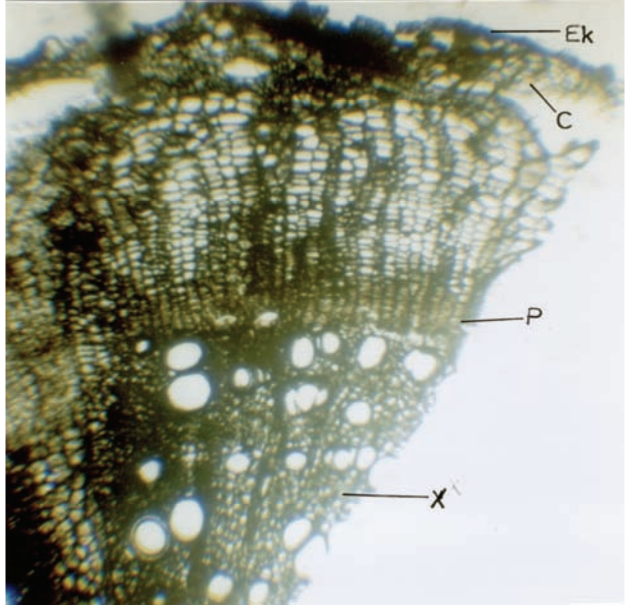

Fig. 1. A cross section of the root of *R. lutea* (3.2 x 6.3). Ek-Eksodermis, C-Cortex, P-Phloem, X-Xylem.

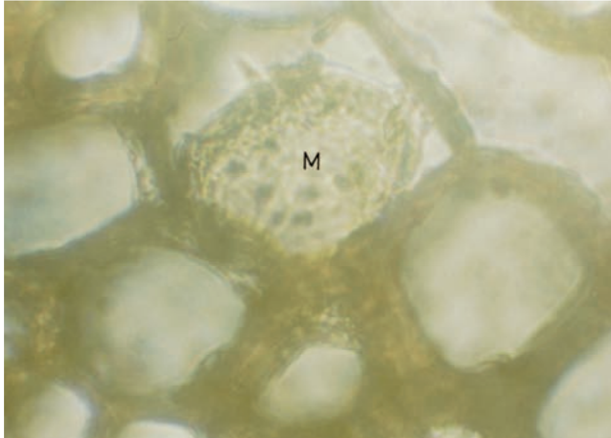

Fig. 2. A cross section of the central part of root of *R. lutea* (40 x 6.3). M-Myrosin cell

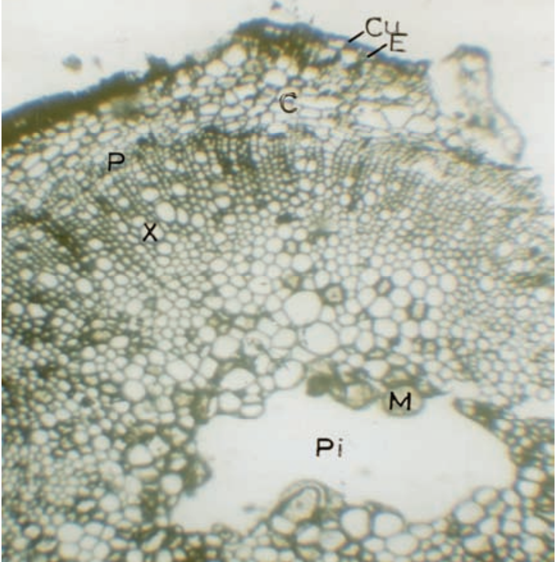

Fig. 3. General appearance from the cross section of the stem of *R. lutea* (3.2 x 6.3). Cu-Cuticle, E-Epidermis, C-Cortex, P-Phloem, X-Xylem, M-Myrosin cell, Pi-Pith space.

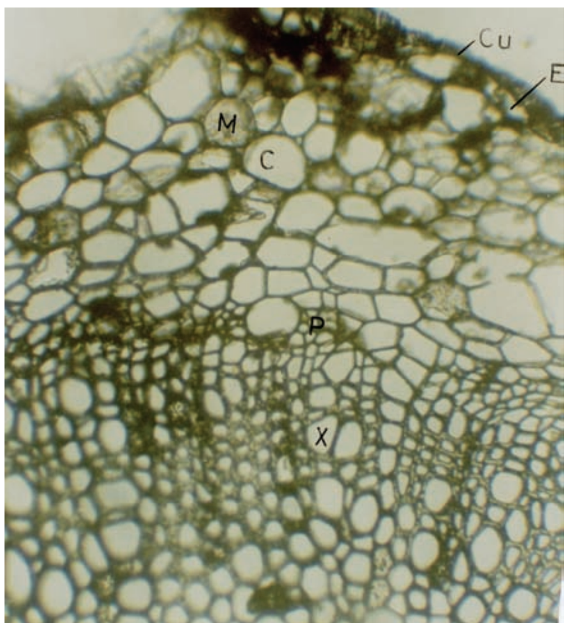

Fig. 4. Detail appearance from the stem of *R. lutea* (10 x 6.3). Cu-Cuticle, E-Epidermis, C-Cortex, P-Phloem, X-Xylem, M-Myrosin cell.

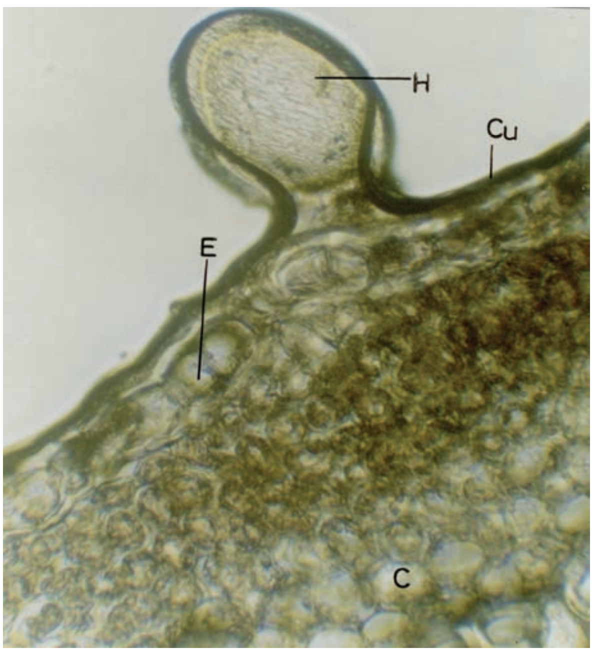

Fig. 5. A cross section of the stem (10 x 6.3). Cu-Cuticle, H-Hair, E-Epidermis, C-Cortex.

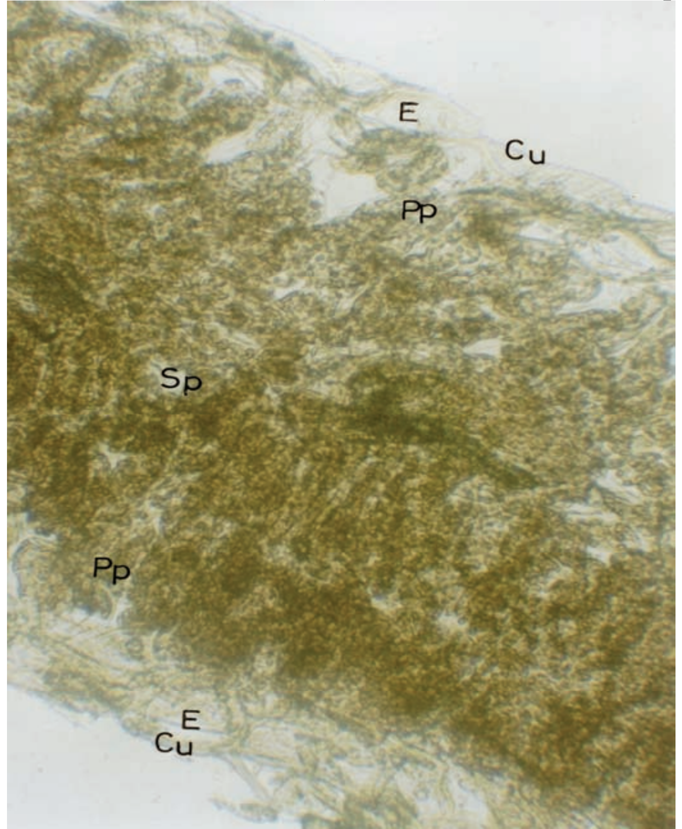

Fig. 6. A cross section of the leaf of *R. lutea* (10 x 6.3). Cu-Cuticle, E-Epidermis, Pp-Palisade parenchyma, Sp-Spongy parenchyma

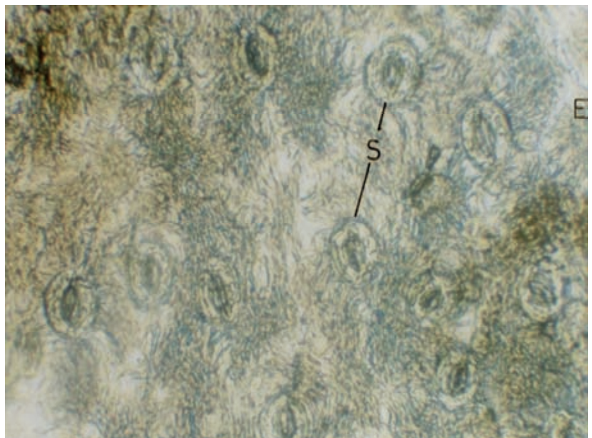

Fig. 7. Upper epidermis with stomata in the transverse section of the leaf of *R. lutea* (10 x 6.3). E-Epidermis, S-Stoma.

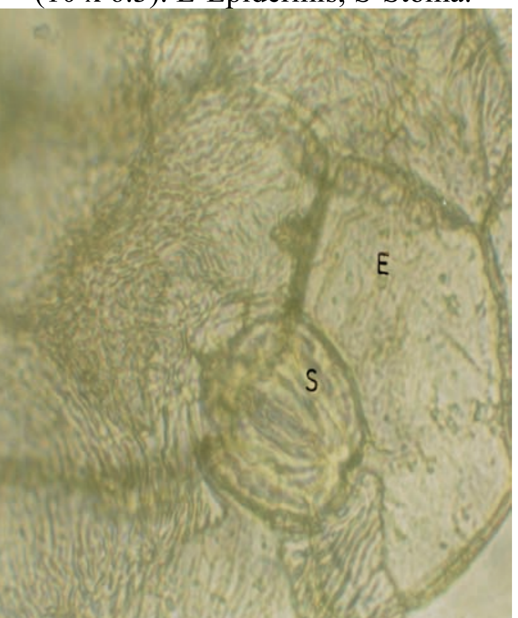

Fig. 8. Lower epidermis with stomata in the transverse section of the leaf of *R. lutea* (10 x 6.3). E-Epidermis, S-Stoma.

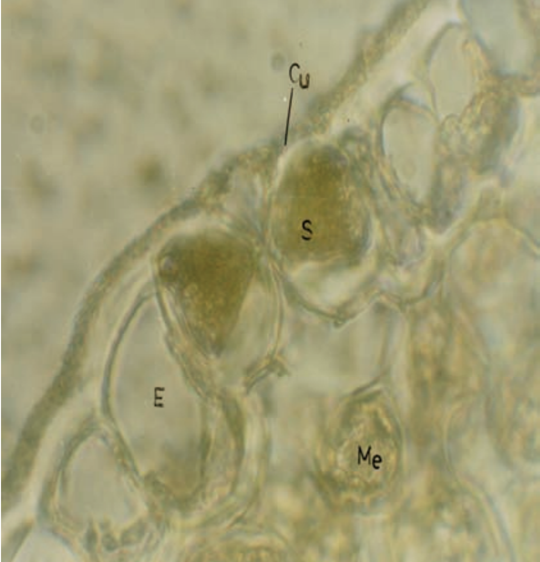

Fig. 9. A cross section of the leaf of *R. lutea* (40 x 6.3). Cu-Cuticle, E-Epidermis, S-Stoma, Me-Mesophyll.

Results and discussion

In the investigation of the anatomical structure of the cross section of the root of *R. lutea,* a lignified and broken shaped exodermis is seen on the outer surface. Epidermis cells cannot be seen clearly. Parenchymatous cortex shows a structure which is squeezed in a narrow place. Vascular region covers a large area. Among them phloem tissue covers a very narrow place and cannot be distinguished. Xylem tissue, on the other hand, covers a very large space and extends to the pith. Endodermis and pericycle are not clear, therefore, cannot be distinguished. In the cortex and pith regions of the root, secretion cells (myrosin cells) are seen.

In the cross section taken from the stem of *R. lutea*, a thin layer of cuticle and epidermis layer, which is formed by thickened membranous smaller cells, are seen on the outer surface of the stem. Glandular hairs are seen in some places in the epidermis layer. Just below the epidermis layer, cortex layer, which is formed by non-homogenous cells, takes place. Cortex tissue contains high amount of myrosin cells. The phloem tissue, squeezed between the cortex and xylem tissues, covers a very narrow place in the stem. Xylem tissue, on the other hand, covers a very large space in the form of a well-arranged circle. Like in the cortex region, among the cells close to the pith, ample amount of myrosin cells are identified.

In the cross section of the leaves of *R. lutea*, the outer surface of both upper and lower surface of the leaf is covered by the cuticle layer. Just below the cuticle layer, epidermis layer, which is formed by one-layer of bigger cells, is seen. Upper and lower membranes of the epidermis cells are thickened. The leaf shows an equifacial structure. That means that palisade parenchyma cells are present on both upper and lower part of the leaf and a layer of spongy parenchyma in between them. It is very difficult to distinguish the palisade and spongy parenchyma cells from each other in the mesophyll structure. While spongy parenchyma cells cover a little space, palisade parenchyma cells cover a wider space in the mesophyll**.** Superficial sections taken from the leaf to investigate the structure of the stomata show that stomata are of Ranunculaceous (anomocytic) type. Stomata are seen on both sides (amphistomatic type) of the leaf. Stomata show mesophytic character in the cross sections of the leaf. In other words, stomata cells and epidermis cells are on the same level. As in root and stem cross sections, myrosin cells are present in leaf mesophyll tissue. Crystal structure could not be seen in the leaf cross sections.

In our literature review, no detailed study about the anatomical structure of *R. lutea* could be found. Only one literature, Metcalfe & Chalk (1957), reported the general structure of the family Resedaceae and some of *Reseda* species other than *R. lutea*. Bonnier (1934), Metcalfe & Chalk (1957), Fahn (1967), Gibbs (1974) and Jorgensen (1995) stated that, like in the members of the families Caricaceae, Caparaceae and Brassicaceae, presence of myrosin cells in the members of the family Resedaceae is characteristic. Parallel to this statement, in this study it was identified that in the anatomical cross sections of *R. lutea*, in the root cortex and pith regions, in the stem cortex and pith regions, and in the leaf mesophyll tissues myrosin cells are present. The equifacial structure of the leaf, identified in this study, was not mentioned by Metcalfe & Chalk (1957). They only reported that palisade parenchyma cells and spongy parenchyma cells cannot be distinguished clearly from each other. Our other findings about the anatomical structure of the root, stem and leaf of *R. lutea* investigated in the cross sections in this study show a parallelism with the findings of Metcalfe & Chalk (1957) studied on family level.

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INVESTIGATIONS ON THE CULTIVATION TECHNOLOGY OF *WITHANIA SOMNIFERA DUN.* (ASHWAGANDHA)

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Keywords: *ashwagandha, cultivation technology*

Introduction

Withania somnifera Dun. is an exotic sub-shrub of the Solanaceae family, 30-150 cm high, the branches covered with white sparkled hairs, sempervirent in its natural habitat. Its habitat is extending from the dry regions of India to Asia Minor, the submediteranean regions and The Canary Islands. The specialists focused on this species due to its multiple use in the traditional Indian and African medicine- from bitter tonic to anti-tumoural. The drug mostly used is represented by the roots as well as the bark, leaves and seeds. The active principles present in the plant (especially in the roots) are used for the depurative and diuretic action, to fight juvenile and grown-up debility. It also has an anti-inflamatory, antihelmitic and aphrodisiac action and is further used to treat alcoholism and emphysematous dyspnoea, to meliorate nervous exhaustion and tiredness, etc. (1). The most interesting use in traditional medicine is that as a plant with anti-tumoural virtues, the leaves being reported as used in local applications to treat tumours. Recently, some authors have shown that *Withania somnifera* Dun. Has adaptogene, tonic, analgesic, antipyretic, anti-inflamatoty properties being used in the treatment of rheumatism, ulcer, rheumatoid arthritis, asthma (2).

Material and method

To establish the main elements of technology for this species, we achieved at S.C.D.A. Secuieni a series of experiments in which we observed the influence of the best epoch, of the nutrition space and of fertilization on the plant growth and development as well as of the *Withania somniferum* Dun. yield.
To establish the optimum cultivation epoch, we studied three variants at different times: V1-cultivated at April 20; V2- cultivated at April 30 and V3-May 10.

For the optimum fertilization level we placed five variants:
V1-fertilized with 20 t/ha manure;
V2-fertilized with 30 t/ha manure;
V3-fertilized with 40 t/ha manure;
V4-fertilized with fertilizer with **N**50 **P**50;
V5-non-fertilized control.

To determine the optimum nutrition space we cultivated six variants;
V1-50 cm between the rows and 25 cm between the plants in the row;
V2-50 cm between the rows and 40 cm between the plants in the row;
V3-50 cm between the rows and 50 cm between the plants in the row;
V4-70 cm between the rows and 25 cm between the plants in the row;
V5-70 cm between the rows and 40 cm between the plants in the row;
V6-70 cm between the rows and 50 cm between the plants in the row.

Results obtained

The experiments performed at Secuieni, between 2002 and 2003, showed that the cultivation epoch influenced both the growth and development of the plants and the biomass production per surface unit. The differences registered among the three epochs, taking into account the number of days necessary to the phenophase stages, maintain themselves till budding after which an evening in the plant development takes place. For the whole vegetation period, the plants of *Withania somnifera* Dun. need a number of 126-136 days (fig. 1). The greatest dry weight was obtained in the first epoch and the smallest in the last.(fig. 2).

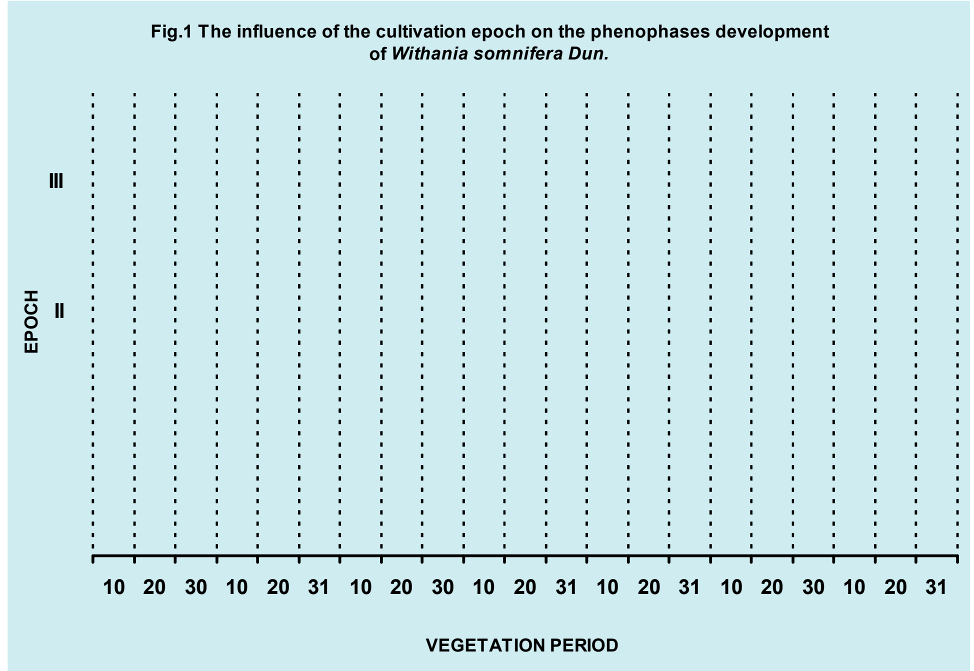


Fig.1 The influence of the cultivation epoch on the phenophases development of *Withania somnifera Dun.*

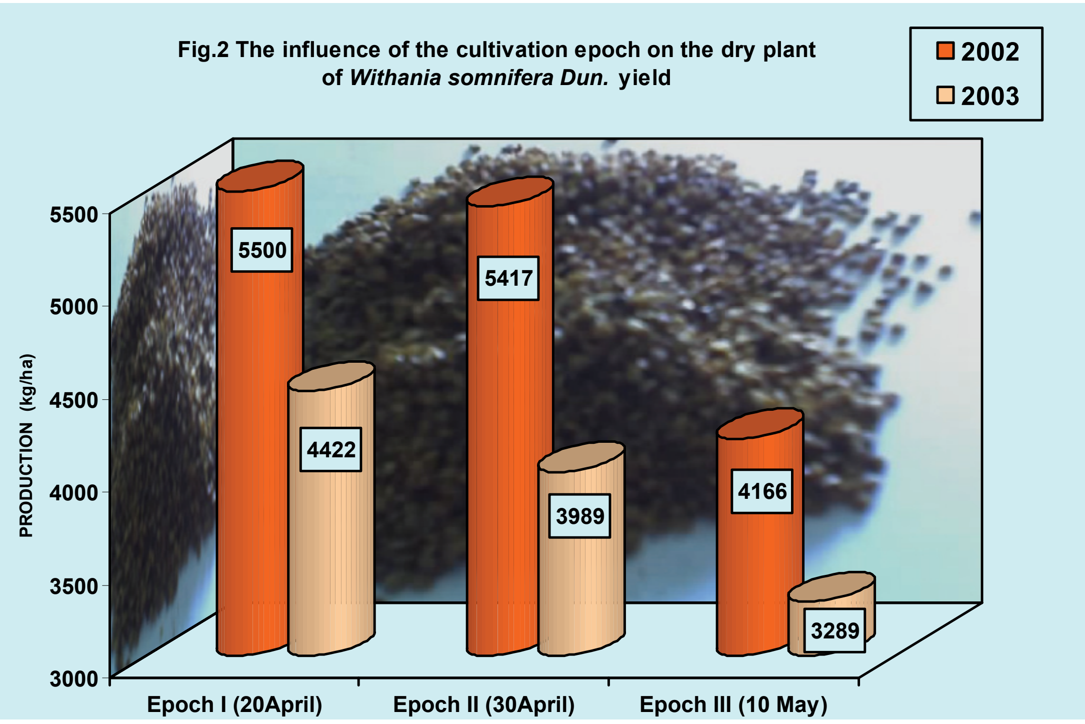
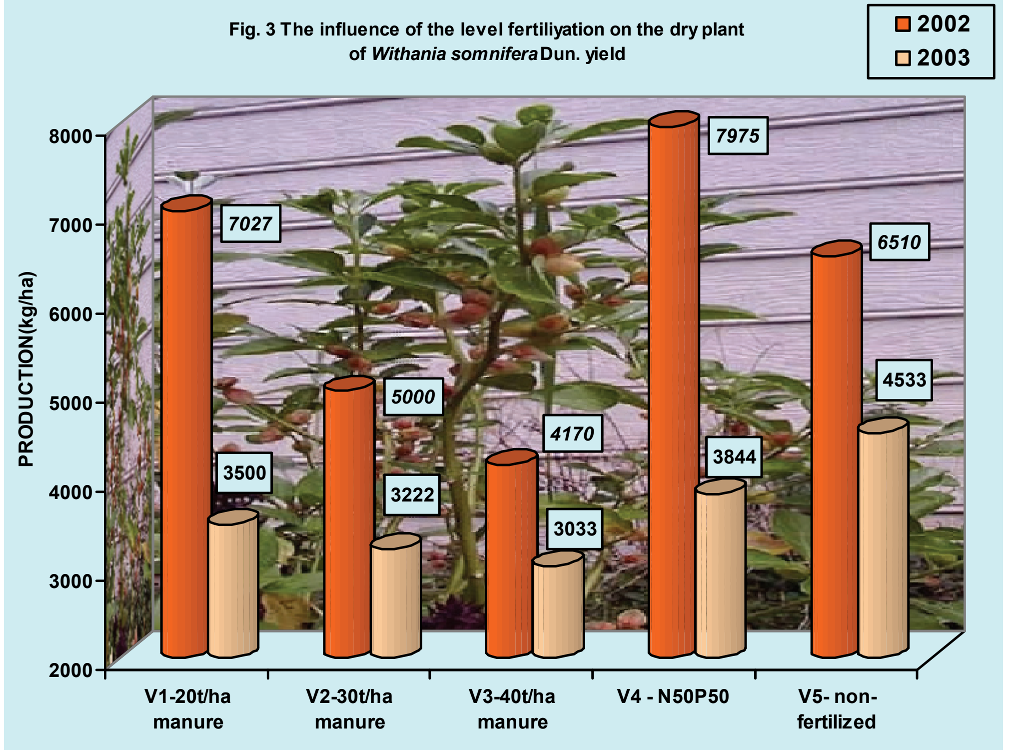


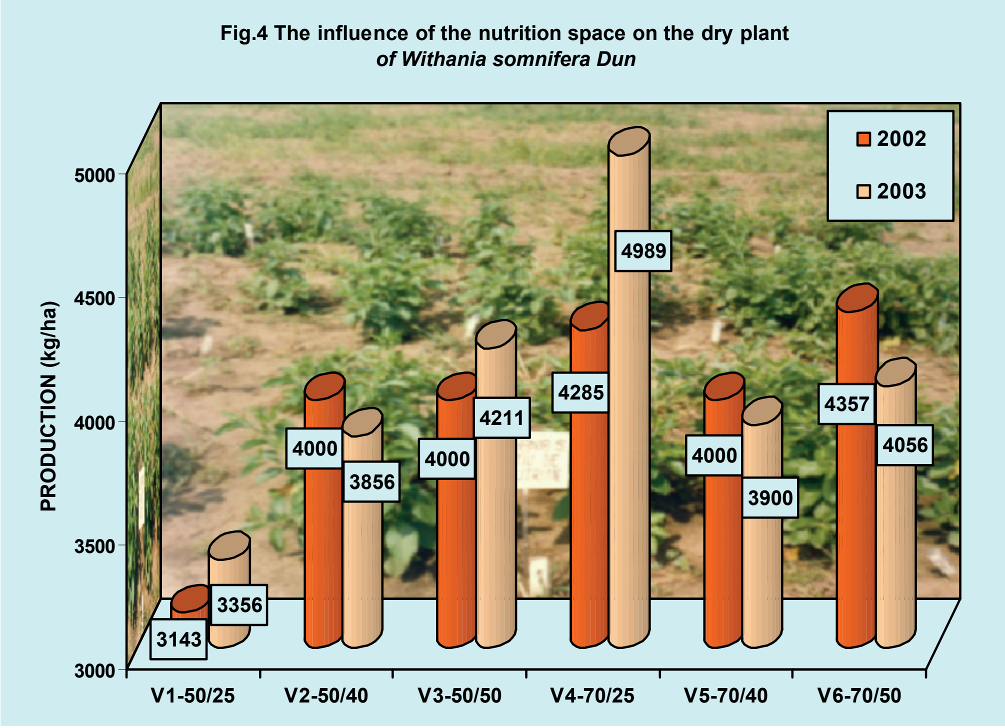
Fig.2 The influence of the cultivation epoch on the dry plant of *Withania somnifera Dun.* yield

The results obtained after fertilization with manure and fertilizer showed that the species is not productive with high fertilizing doses, the yield being inferior to that of the control in the case

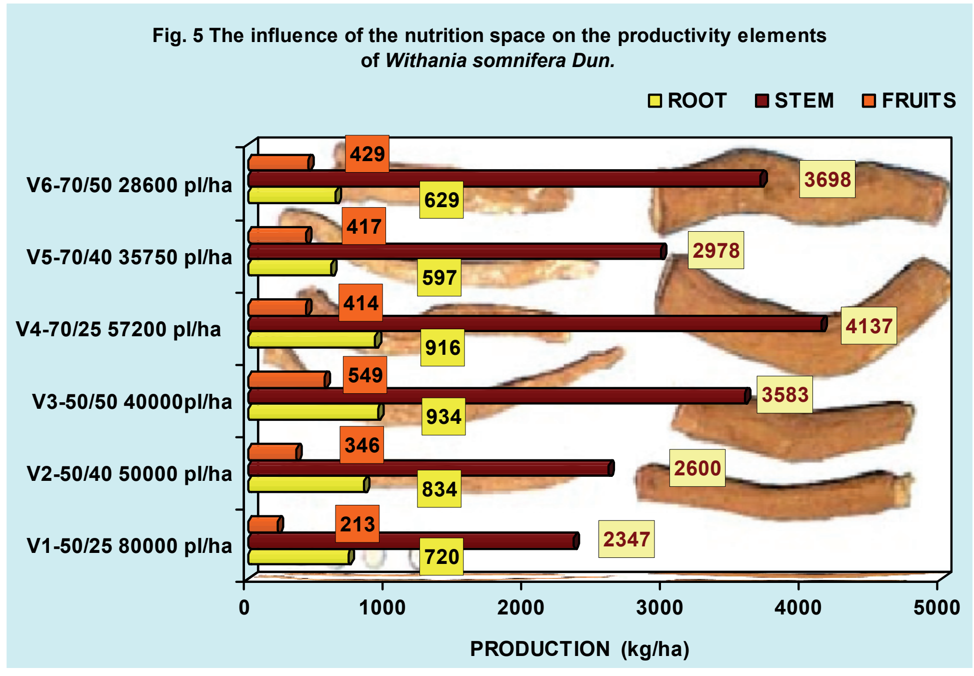
the plants were treated with 30 and 40 t/ha manure. The highest productions were obtained by the variants fertilized with N50P50 (fig. 3). We must also admit that the droughty conditions of 2003 lead to a bad capitalization of the manure and the fertilizer.



As to the nutrition space, the highest yield of dry weight was obtained by the variants cultivated at the distance of 50 cm between the rows and 50 cm between the plants in the row and by the variants cultivated at 70 cm between the rows and 25 cm between the plants in the row (fig. 4).



The biomass production was determined in the experiment regarding the nutrition space on different organs of the plant. Thus, the greatest yield of roots, stems and fruit per ha were obtained by the variants mentioned in the previous paragraph (fig. 5).



Conclusions

- *Withania saomnifera* is a plant that likes a warm climate but also needs raised humidity especially in the early vegetation periods;
- *W. somnifera* may be cultivated in the interval April, 20-May, 10 but we must mention that in the warm and droughty springs it may be cultivated in the first period of the interval so that the plants beneficiate of the humidity accumulated during winter and the early spring. This will assure the plant a rapid start, a good spring and good subsequent development. In cold and humid springs, cultivation will be performed in the last period of the interval to assure the necessary thermic conditions;
- The plant does not need a fertile soil; organic fertilizers determine the decrease of the dry weight yield, compared to the control;
- Being a rather high plant, it prefers a greater nutrition space (50/50cm or 70/25 cm) for a better branching and for the necessary luminosity.

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BIODIVERSITY AND PROTECTION OF THE MEDICINAL AND AROMATIC PLANTS IN BULGARIA

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Summary

Medicinal and aromatic plants present very important part of the Bulgaria's biodiversity. The scientific investigations and the activity of the Bulgarian government for the sustainable use and protection of their resources have been discussed in this paper.

Keywords: *Medicinal and aromatic plants, biodiversity, protection*

Current status of the Bulgaria's biodiversity

At the beginning of 21th century the Bulgarian botanists have faced at some challenges as:
- Globalization of the science and the contribution of the regional sciences
- Innovation of new methods and technologies
- Development of interdisciplinary and applied investigations
- Application and implementation of an ecological approach

Globalization of the science gives us a possibility to exchange scientific information and to apply the new criteria for an assessment of the research results. Bulgarian scientists would give a significant contribution to the European botanical science with further investigations of the Bulgarian flora (Palamarev, 2002).

The Bulgarian vascular flora includes 3 900 species, belonging to 916 genera and 159 families. The current data shows that 186 species are Bulgarian and 312 species- Balkan endemic plants (Petrova et al., 2005).

Medicinal and aromatic plants (MAP) present a significant part of the Bulgaria's biodiversity and they play very important role in the everyday life of men- phytomedicines, herbal teas and other natural products.

Bulgaria is a traditional producer and supplier of high quality medicinal herbs, essential oils, etc. According to the Law on Medicinal plants (2000) 739 vascular plants are used for medical purposes, as spices, for cosmetic products or as food. 29 rare and valuable species are included in this list also (2004). The major part of this biodiversity presents *Magnoliophyta*- 658 species. There were remarked some families with big species richness: *Asteraceae*- 76, *Lamiaceae*- 68, *Rosaceae*- 68, *Fabaceae*- 42, *Apiaceae*- 40, etc. *Polypodiophyta* includes 14, *Equisetophyta*-5 and *Lycopodiophyta*- 3 species. One species from *Algae* (*Cystoseira barbata* (Good et Vood) Ag) and one species from *Lychenes* (*Cetraria islandica* (L.)Ach.) have been included in this list also (Gussev, 2005).

The MAP's resources have been strongly influenced by the climatic changes, erosion, fires, agriculture and by the over-exploitation. The problem of these resources and their long-term development was one of the main aspect of the National Strategy for biodiversity protection (Hardalova et al., 1994).

Legislative activity of the Bulgarian government

The Law on Medicinal plants (2000) has determined the MAP they may be collected, the rules for sustainable use and protection, as well as the cultivation of some valuable plants. The amounts of medicinal herbs for personal and for economic usage and licence taxes are

regulated also. The Ministry of Environment and waters (MEW) is responsible for the law implementation and it's Regional Inspectorates of Environmental Protection control this process. The sustainable use of MAP's resources is regulated also by the Law on Protected Areas (1998). The system of protected areas in Bulgaria is comparatively well developed. At present, there are 3 National Parks, 8 Nature Parks, 16 Biosphere Reserves under law protection (Peev et al., 1998). The protected areas should be developed for the purposes of nature protection according to the National and European legislation (Kathe et al., 2003). However , it depends on their category. Few years ago Management Plans for two Bulgarian National Parks have been elaborated. The state of the populations of some rare and threatened plants, as well as the resources of some valuable MAP, have been carried out (Vitkova, Evstatieva, 2000, Evstatieva, Vitkova, 2000).

Bulgaria is the only country in Europe, which has a National System of quotas with aim of management the collection of wild MAP (Lange, 1998). The MEW annually issues quotas for some economically valuable plants as: *Paeonia peregrina* Mill., *Frangula alnus* Mill., *Primula veris* L., *Berberis vulgaris* L., *Galium odoratum* (L.) Scop., *Atropa bella donna* L., *Betonica officinalis* L., *Carlina acanthifolia* All., *Sedum acre* L., *Alchemilla vulgaris* complex. For 26 other species the collection of raw material from the natural habitats has been strongly forbidden.

The National System for Environmental Monitoring, which has developed since 1994, has a great significance. The data about MAP's collection includes the species-specific quantities collected in some regions with rich stock of valuable medicinal herbs.

In relation with the implementation of national and international legislative norms, concerning the biodiversity conservation, the MEW initiated an establishment of a National Biodiversity Monitoring System (Stanimirova, Ivanova, 2005). At present, this system is at state of development and the monitoring of the MAP resources should be considered successfully in future.

MAP with conservation significance are of a great interest for the Bulgarian botanists. According to the Law on Biodiversity (2002) 61 species are protected and their collection from the Nature is forbidden. 43 species are included in the list of CITES (Gussev, 2005). 76 rare and endangered MAP are presented in the Red Data Book of Bulgaria (1984). May be this number will be changed in future, because of the new edition of this book, supported by the MEW. The species have been evaluated according to IUCN Red List Categories and Criteria.Version 3.1 (2001).

The habitat conservation plays an important role for the protection of Map's resources. Bulgaria is an actively participant in the "Natura 2000" system of protected areas and it is in the process of adopting the EU "Habitats, Fauna and Flora" Directive. Bulgaria has ratified most of the important international conventions related to nature conservation and environmental protection- Ramsar Convention, CITES, Bern Convention, Convention on Biological Diversity, etc. Handbook for determination of habitats with European significance in Bulgaria was published (Kavrukova et al., 2005).

One of the most effective manner for protection and conservation of the MAP is the cultivation of some rare and valuable species. Bulgaria has an old tradition for cultivation of some economically valuable plants as: *Mentha piperita* L., *Melissa officinalis* L., *Valeriana officinalis* L., *Matricaria recutita* L., etc. In the last years the interest for cultivation of rare MAP is increasing rapidly. Some protected plants as: *Ruta graveolens* L., *Sideritis scardica* Griseb., *Rhodiola rosea* L., *Alchemilla mollis* (Buser) Rothm. have been cultivated successfully in small plantations (Evstatieva, Hardalova, 2004).

Scientific investigations

At present, the main centre for scientific investigations and protection of Map's resources in Bulgaria is the Institute of Botany, BAS, Department of Applied Botany. The research projects, supported by the MEW and the National Research Foundation for mapping the MAP's resources, have been carried out by the Bulgarian botanists. The management programs for the sustainable use and protection of MAP on the territory of the National Parks have been established also (Genova, 2004).
Two experimental fields for ex-situ conservation of rare and threatened MAP were created some years ago in the Institute of Botany, BAS (Evstatieva, Hardalova, 2004) One of them is close to Sofia (550 m a.s.l.) and the second is in the Rhodopes mountains (1 550 m a.s.l.). Other two collections for ex situ and in vitro conservation of rare and threatened plants were created in the Institute for Plant Genetic Resources in Sadovo (Koeva et al., 2005). These collections could be used as a source for seeds, vegetative materials for cultivation of plants in small plantations.
The research project "Alternative Approaches of bioproduction of alkaloids and active substances from Bulgarian rare and threatened medicinal plants", supported by NATO under the science for Peace Programme, was finished successfully in 2006. Five-years monitoring of natural populations of *Leucojum aestivum* L. was performed. In vivo and in vitro multiplied original plant material were adapted in ex-situ collection of the Institute of Botany, BAS. Bulgarian *Leucojum aestivum* Database containing data about diversity, population characteristics, soil parameters in some habitats and galanthamine content was elaborated.

Further goals

There are some very important goals in the field of protection and conservation of MAP in front of the Bulgarian botanists:

- To develop the management plans for the Map's resources in the protected areas
- To elaborate the certificated system for the MAP according to the requirements of the European market
- To develop the monitoring system and to organize the database for some rare, protected and threatened MAP.
- To organize courses, workshops for education and training of collectors, traders and all people who have interest in sustainable use and protection of Map's resources**.**

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SOME CONSIDERATIONS REGARDING THE *IN VITRO* BEHAVIOUR OF *LAVANDULA ANGUSTIFOLIA* L. SPECIES

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Summary

The main reaction of explants (shoot tips, nodes, leaves) of Lavandula angustifolia L. on varied hormonic formuli of MS medium was callogenesis. Nodes and shoot tips provided neoplantlets only on hormone-free MS and sporadically on MS with B_{02} (0.2 mg/l), A_2 (2 mg/l IAA), KN_1 (1 mg/l kinetine and 0.5 mg/l NAA) and N_2 (2 mg/ NAA). A friable cream callus, high proliferative in light and also in darkness was obtained on A_2. Its capacity of fresh biomass accumulation was tested on varied hormonic formuli.

Keywords: *lavander, callogenesis, caulogenesis, micropropagation*

Introduction

Lavander (*Lavandula angustifolia* L.) is a perennial semi-bushy herb from the Lamiaceae family, of mediterranian origin, grown in Romania for its active principles within its inflorescences, especially for its volatile oil (that comprises mostly linalool and linalil acetate). Lavander active principles have an antiseptic, carminative, sedative, antispastic, diuretic, colagogue etc (1, 2, 4-9). Though lavander cultures can be accomplished with plants provided generatively or vegetatively (by means of cuttings), *in vitro* micropropagation of this species is important in view of eventual valuable genotypes multiplication. Callus production by means of *in vitro* cultures and the selection of cell lines that produce certain substances (e.g. biotine) appear to be up-to-date topics regarding this species, (3, 10, 11). All this being considered, our aim was to investigate the morphogenetic reaction of some lavander explants within *in vitro* cultures, the possibility of inducing indirect organogenesis via callus and somatic embryogenesis, to evaluate the capacity of biomass accumulation in callus cultures, to evince the cytogenetic outcome of the *in vitro* regenerants on varied hormonic formuli, as well as their behaviour in field conditions. This paper presents the reaction of this species *in vitro* and also the estimate of biomass accumulation in lavander callus cultures.

Material and methods

The source of explants to initiate the *in vitro* cultures of *Lavandula angustifolia* was represented by plants from the Research Centre for Medicinal and Aromatic Plants from Fundulea subsequently cultivated in soil pots in laboratory conditions at the 'Stejarul' Research Centre of Piatra Neamt. The explants (shoot tips) were sterilized for 20-25 minutes in chloramine-T (solution, 5%), then rinsed twice with sterile distilled water and inoculated on Murashige-Skoog medium, hormone-free or supplemented with 0.2 mg/l BAP. The MS medium was solidified with agar (8.5 g/l) and the carbon source of the nourishing medium was saccharose (25 g/l). The neoplantlets obtained on this culture media represented the source of explants to diversify the experiences of *in vitro* testing on varied hormonic variants of MS. The cultures were initiated in Erlenmeyer vials of 100 ml (B type) and then incubated in a culture room with half-climatised conditions (temperature of 23 to 25º C, light intensity of about 2000 lux, permanent illumination). We also evaluated the capacity of biomass accumulation in callus cultures by means of a callus line provided by stems inoculated on a culture medium supplemented with 2 mg/l IAA. The callus was cultivated in enlightened

rooms and also in the absence of light and the data from table 1 represent the average estimate of 3 vials for each hormonic variant. Our tests' results are displayed in tables 1, 2 and figure 1 (a-f).

Results and discussions

In vitro culture initiation at lavander in the previously mentioned conditions did not raise any particular difficulties. We may state from the beginning that the most significant morphogenetic reaction of the tested explants (shoot tips, nodes, leaves, stem fragments) on the most varied hormonic variants of the MS medium (A, B, BA_1, BB_2, BD_1, BG_1, BN_1, D_2, KN_1, N_2) was callus generation (mostly friable, cream or green), (table 2, fig. 1-a-f). Sometimes even the shoots transfered on MS medium without growth regulators for enrooting provided roots at base and also a layer of friable callus, more or less developed. The most appropriate medium formuli for callus induction from stems were BG_1, BN_1, BD_1 and B_{02}. Leaf fragments provided callus intensively on D_2. Stem callus as well as leaf callus proliferated very intensively by transfering it on fresh media supplemented with BAP (0.5-2 mg/l), with BAP (1 mg/l)+IAA (0.5 mg/l), with BAP (1 mg/l)+GA (0.5 mg/l), BAP (1 mg/l)+IBA (0.5 mg/l), kinetine (1 mg/l)+NAA (0.5 mg/l). The callus provided by leaves had no organogenetic capacity. Friable cream or green stem callus grown in light conditions (especially on nourishing media supplemented with BAP) turned green gradually, became more consistent (breakable) and formed caulogenetic isles that generated multiple shoots frequently. The friable cream callus produced at the base of nodes cultivated on media with 2 mg/l IAA (that was considered a callus line with proliferation peculiarities). Shoots provided by indirect organogenesis (by means of callus) from stem (nodes) callus were resistant to enrooting, disregarding the root-inducing medium formula).
Shoot tips and nodes inoculated on varied hormonic formuli also generated roots, forming neoplantlets: sporadically on media with cytokinins and a high frequency of enrooting on media with auxins (IAA, NAA) or cytokinins and auxins (BAP+IAA, BAP+NAA, kinetine+NAA), (table 2). The highest percentage of root formation was registered on MS medium supplemented with 2 mg/l NAA. This medium formula also favoured the obtaining of the most vigorous neoplantlets that had an average of 5 to 7 basal shoots. A quite satisfactory frequency of enrooted shoots was observed on hormone-free MS medium (1d). We consider that in order to micropropagate this species the best solution is to initiate and maintain the culture only on hormone-free MS medium as lavander is very sensitive to hyperhydria. This phenomenon brings a great difficulty in shoots enrooting which is sometimes impossible. Supplying the culture medium with phytoregulators obviously increases the risk to induce hyperhydria. At the same time the use of hormone-free MS medium only inhibits callus formation at shoot base, as well as root generation. Researches to discover the most appropriate hormonic formula for enrooting the shoots obtained by indirect organogenesis via callus are in progress. Neoplantlets' accommodation to septic environment took a short period of time and the biological material losses did not exceed 10 %, (fig. 1f).
During our researches we offered a special attention to two stem callus lines that were very semblable as consistency and colour (friable, cream-greenish), though their proliferation capacity was very different. They were provided on MS medium supplemented with 2 mg/l IAA (A_2) and respectively on media supplemented with 1 mg/l kinetine and 0.5 mg/l NAA (KN_1). The friable cream callus obtained on A_2 transfered on BA_1 (1 mg/l BAP+0.5 mg/l IAA) enhanced its luxurious multiplication, it gained a foamy aspect and this character was maintained for about 2 years. Our purpose was to use this 2 callus lines to induce somatic embryogenesis. In this view they were cultivated on hormone free MS, in a culture room with no light (fig.1b).

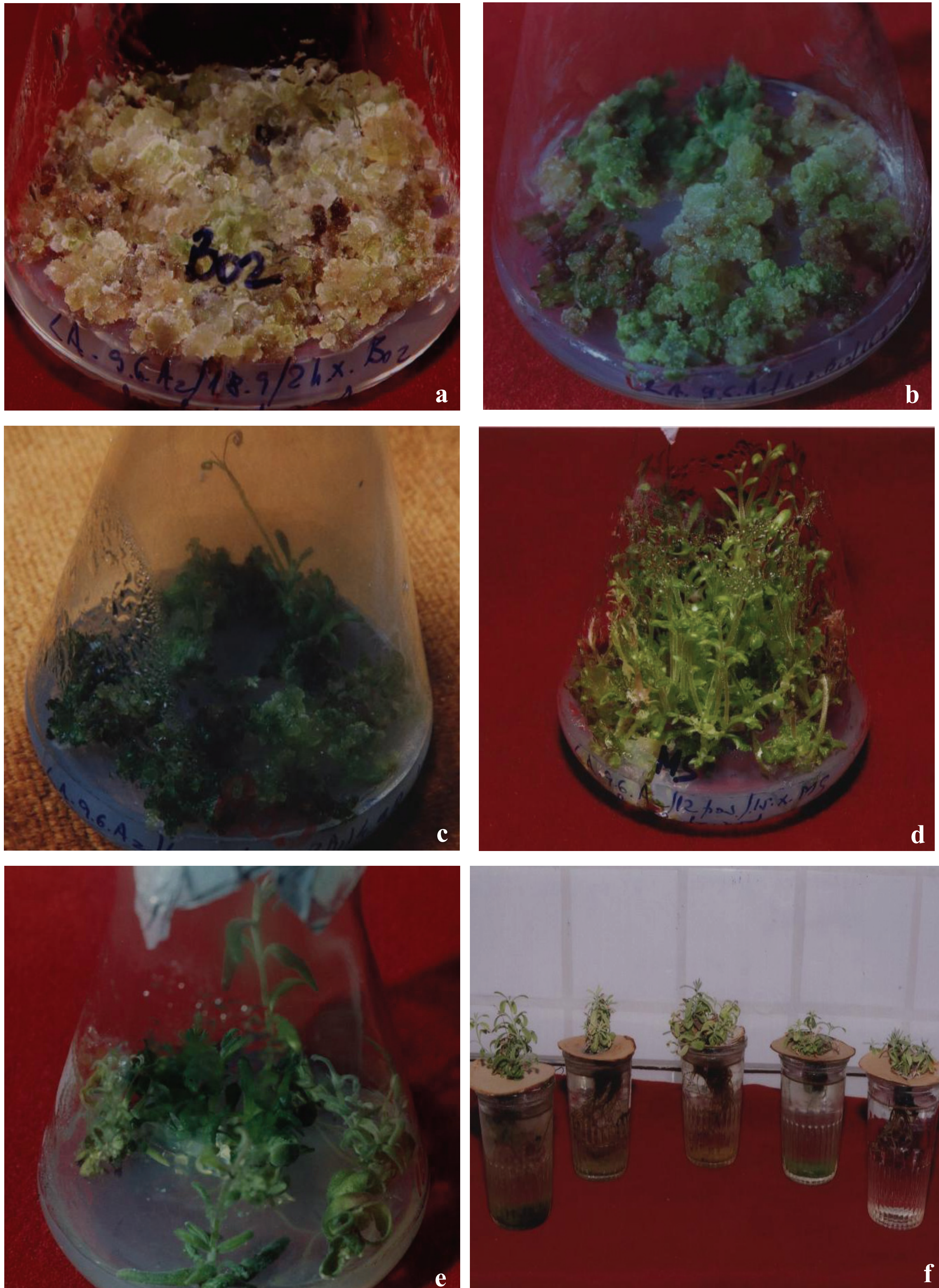

Fig. 1. a – Friable foamy callus provided by nodal explants on B_{02} culture medium; b – Caulogenetic callus generated by nodal explants on medium with IAA and transfered on media comprising Kinetine and IBA; c – Regeneration of shoots from nodal callus passed on media supplemented with BAP; d – Indirect multiple shooting (from friable nodal callus obtained on A_2 formula) on MS basal medium; e – Neoplantlets with callus formation provided on IBA medium; f – *In vitro* regenerants accommodated in a hydroponic system

The A_2 line (kept in darkness) maintained its features though it was repeatedly passed for 4 months: high proliferation speed (in a period of 14 days it filled about half of the culture vial and deprived the medium of its nutrients) and light-cream colour. The attempts to induce somatic embryogenesis were unsuccessful. Growing this type of callus in light and its repeated transfers on MS formula with BAP (0.2-0.5 mg/l) or with BAP (0.5 mg/l)+kinetine (0.5 mg/l) improved its consistency. It turned green and caulogenetic callus isles appeared on its surface, leading to shoot formation subsequently, (fig. 1c). Even after 2 years of *in vitro* subcultivation, A_2 callus line maintained its caulogenetic capacity.

Table 2. Biomass evaluation in *in vitro* lavander callus cultures

Hormonic variant	Growth regulators (mg/litre)				Biomass supply (g/vial)
	BAP	Kinetine	GA	NAA	
A. *Callus cultures exposed to permanent illumination*					
MS	-	-	-	-	9.4452
BK	0.5	0.5	-	-	13.5589
BG_1	1.0	-	0.5	-	14.3526
KN_1	-	1.0	-	0.5	11.9016
B. *Callus cultures maintained in darkness conditions*					
MS	-	-	-	-	12.7420
B	0.5	-	-	-	11.6152
BG_1	1.0	-	0.5	-	14.9952
KN_1	-	1.0	-	0.5	12.2952

The high proliferation speed of A_2 callus cells determined us to evaluate its capacity of accumulating fresh biomass within a fortnight, in light and also darkness conditions on MS (control) medium and on certain hormonic formuli in view of its potential utility – production of important active principles. It was very interesting issue is that callus biomass values achieved in light and also in darkness conditions are very similar on the same medium formuli. It was ascertained that on hormone free MS medium a greater callus biomass was obtained compared to the one produced in the absence of light. In the first case some of the nutrients are probably consumed during the cell differentiation processes and not only for their own multiplication. The medium formula that provided the highest biomass both in light conditions (14.35 g/vial) and darkness (14.99 g/vial) was the one comprising 1 mg/l BAP and 0.5 mg/l GA (BG_1), (table 1).

The KN_1 callus line cells were also unable to produce somatic embryos by their repeated cultivation on MS medium (in the absence of light). In this case callus displayed a lower cell proliferation speed; it turned friable and cream, compact and whitish and some callus regions generated shoots (etiolated) even in darkness conditions, shoots that got their natural colour. In a period of 3 months this callus line lost its cell multiplication capacity gradually.

Conclusions

Our investigations regarding the *in vitro* behaviour of *Lavandula angustifolia* L. evinced that:

- Nodes and shoot tips provided neoplantlets on hormonic formuli that comprised cytokinins (BAP and kinetine) and auxins (IAA, IBA and NAA) alone or combined; the top efficiency was accomplished on MS medium supplemented with 2 mg/l NAA;
- Callogenesis process is very frequent at this species and was induced from all the plants parts on every hormonic formula tested, the callus was friable, cream or green and caulogenetic;

- A stem callus line (with a high proliferation speed, biomass efficiency and caulogenesis) was isolated. This line maintained its features even after two years of *in vitro* subcultivation.
- We consider that the the results of our research depended very much on the genotype of the biological material used to initiate the *in vitro* cultures of *Lavandula angustifolia.*

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Table 1. Morphogenetic reaction of some lavander explants on varied hormonic formuli of Murashige – Skoog medium

Var	Type of explant	Hormonic formula	Growth regulators (mg/l)							Morphogenetic reaction and proliferation speed
			BAP	GA	IAA	IBA	NAA	KIN	2,4-D	
1.	Nodes and shoot tips	A			2.0					Vigorous neoplantlets (++) with multiple shoots (+++); friable cream callus (+++) at stem basis
2	"	B	0.2							Neoplantlets (+); compact green-brownish callus (+) at contact surface with nourishing medium; multiple shooting (+++)
3	"	BA	1.0		0.5					Multiple shooting (+); neoplantlets sporadically (+); friable green callus (++) that sporadically differentiated shoots (+)
4	"	BB	1.0			0.5				Friable cream callus (++) that degenerated in time; caulogenesis was inhibited on this medium formula
5	"	BD	1.0						0.5	Friable cream and green callus (++) that degenerated in time
6	"	BG	1.0	0.5						Multiple shooting (+++); friable cream callus (+++)
7	"	BN	1.0				0.5			Neoplantlets (+) with multiple shoots (++); cream and green callus (++)
8	"	IB				2.0				Neoplantlets (++), cream friable callus (+)
8	"	N					2.0			Vigorous neoplantlets (++) with multiple shoots (++)
9	"	KN					0.5	1.0		Neoplantlets (++), multiple shooting (++); friable but also hard consistency (granulated) cream and green callus (++)
10	"	MS								Neoplantlets (++) - multiple offshoots (+), cream greenish friable callus (+)
11	Internodes	BD	1.0						0.5	Friable cream and green callus (++) on the entire explants' surface
12	"	D							2.0	Quite friable callus (++), cream and green callus on the whole explants' surface
13	Leaves	BD	1.0						0.5	Compact cream and green callus (++) on the entire explants' deprived of organogenetic capacity
14	"	D							2.0	Friable cream and green callus (++), no organogenetic capacity
15	Node callus provided on A medium	B	0.2 -0.5							Friable green callus (++) that frequently provides multiple shoots (+++)
16	"	KN			2.0		0.5	1.0		Friable foamy cream callus (++++), high proliferative
17	"	N					2.0		2.0	Friable foamy cream callus (++++), high proliferative
18	"	MS								Friable cream-whitish callus in darkness, green in light (++++) that frequently provided shoots in light conditions (++) and seldom in dark conditions (+)
19	Node callus provided on KN	MS								Callus cultures maintained in darkness displayed a good proliferation speed (++), turned cream-whitish, semi-compact and generated frail shoots sporadically (+)
20	"	B	0.2							Friable callus of hard consistency (++) that frequently produced multiple shoots (++)
21	Leaf callus generated on D_2	B BG	0.5 1.0	 0.5						Friable green callus (++++) with cream-brownish or white callus isles, no organogenetic capacity

A=IAA; B=BAP; BA=BAP+IAA; BB=BAP+IBA;BD=BAP+2.4-D; BN=BAP+NAA; D=2.4-D; BK=BAP+kinetine; BG= BAP+giberellic acid; IB=IBA; KN=Kin+NAA; N=NAA;
(+) poor reaction; (++) good reaction; (+++) very good reaction

CARROT FRUIT ESSENTIAL OIL AND SUPERCRITICAL FLUID EXTRACT - THE CHEMICAL COMPOSITION AND ANTIMICROBIAL ACTIVITY

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Summary

The composition of Carrot fruit (Daucus carrota, cultivar Chanteney) essential oil obtained by hydrodistillation and its extract obtained by supercritical carbon dioxide extraction at 313 K and 10 MPa were examined. The qualitative and quantitative analyses of the essential oil and supercritical extract were performed using GC and GC/MS methods. Antimicrobial properties of the oil and the extract were investigated against nine strains of bacteria and one strain of yeast.

Keywords: *Carrot seed, Daucus Carota* L., *supercritical extract, essential oil, antimicrobial activity*

Introduction

Supercritical fluid extraction (SFE) of active compounds from plant material is a promising field for the industrial application of SFE [1] since it has certain advantages over steam distillation and solvent extraction. Steam distillation can lead to thermal degradation and partial hydrolysis of some essential oil compounds, while SFE can be performed at lower temperatures, thereby preserving original extract composition and properties.
Carrot fruit (seed) essential oil is widely used as a flavour ingredient in most major food categories. It is also used as a fragrance component in perfumes, cosmetics, and soaps. Conventional method for carrot essential oil isolation is steam distillation of dried fruits. The oil has anthelmintic, antiseptic, carminative, depurative, diuretic, emmenagogue, hepatic, stimulant, tonic, vasodilatory and smooth muscle relaxant actions [2]. Moreover, the essential oils of some varieties were proved to have antibacterial and fungicidal activities [3-12].
In this investigation antimicrobial activity of carrot fruit SFE extract was investigated using 10 species of microorganisms (9 strains of bacteria and 1 strain of yeast) and compared to the antimicrobial activity of the essential oil obtained by the hydrodistillation. The MICs were determined by agar dilution method and broth dilution method.

Material and methods

Fruits of *Daucus Carota* L. (Chanteney) produced in northern Serbia and examined by the Agricultural Station Novi Sad, Serbia (320-6-00015-89/2002-04) were used in experimental studies. The plant material moisture content was 9.1% wt. Carrot fruit essential oil was isolated by hydrodistillation in Clevenger-type apparatus for 4 hours, up to the point at which the oil contained in the herbaceous matrix was exhausted.
Extractions with SC CO_2 were carried out in the Autoclave Engineers Screening System shown in Fig. 1. The Supercritical Extraction Screening System is designed for small batch research runs using CO_2 as the supercritical medium. Liquid CO_2 is supplied from CO_2 cylinder (T) by a siphon tube. The CO_2 is pumped into the system by the liquid metering pump (LP) untill the required pressure is obtained. Back preassure regulators are used to set the system pressure (in extractor – E, and separator - S). The extractor vessel (150 ml) is filled with the plant material from which a substance is to be extracted. Heaters are supplied on the extractor vessel for temperature elevation. The SC CO_2 flows through the extractor and enters

the separator vessel. Samples of the extracted substance can be taken by opening the ball valve located at the bottom of the vessel. A flowmeter is provided to indicate the flow rate of CO_2 being passed through the system and the flow can be adjusted by micrometering valve. The CO_2 continues to flow out of the separator through the flowmeter/totalizer and out to atmosphere. Carrot fruit was fine milled and sieved to the particle diameter of 0.5 mm. Mass of the plant sample was 47g and the solvent flow rate was 0.3 kg/h in all experiments. Extractions were carried out at temperatures of 313 and 323 K and pressures of 9 and 10 MPa.

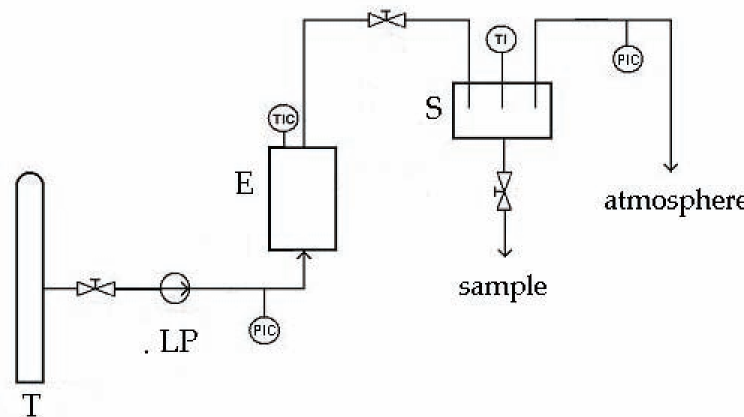

Fig. 1. Schematic presentation of The Autoclave Engineers Screening System: T – CO_2 storage tank; LP – high pressure liquid pump; E – extractor vessel; S – separator vessel.

GC-FID Analysis. Qualitative analyses of the samples were carried out using a Hewlett-Packard GC (FID) analytical systems. Model HP-5890 Series II, equipped with a split-splitless injector, HP-5 capillary column (25 m · 0.32 mm, film thickness 0.52 µm) and a flame ionization detector (FID), was employed. Hydrogen was used as carrier gas (1 ml/min, measured at 210 °C). Sample solutions in ethanol (1 µl) were injected in split mode (1:30). The injector was heated at 255°C, the detector at 300°C, while the column temperature was linearly programmed from 50-285°C (4.3°C/min). **GC-MS Analysis.** Quantitative analyses were carried out under the same analytical conditions. HP 5890 Series II gas chromatograph with HP G 1800C GCD Series II (GC-FID) detector, equipped with a split/splitless injector (250°C) and a HP-5MS column (30 m x 0.25 mm x 0.25 µm film thickness). Carrier gas (He) flow rate was 1 ml/min while column temperature was linearly programmed in a range of 40-240°C at a rate of 4°C/min. Transfer line was heated at 260°C. Electron impact mass spectra (70 eV) were acquired in m/z range 45-450. A library search and mass spectral deconvolution and extraction were performed using NIST AMDIS (Automated Mass Spectral Deconvolution and Identification System) software version 2.4.

Determination of antimicrobial activity. The investigation of the antimicrobial effects has been performed on referential strains of *Staphylococcus aureus* ATCC 6538 P, *Enterococcus faecalis* ATCC 29212, *Bacillus subtilis* ATCC 6633 BB, *Bacillus cereus* ATCC 11778, *Listeria monocytogenes* ATCC 19115, *Rhodococcus equi* CAPM 6312, *Escherichia coli* ATCC 25922, *Salmonella enteritidis* ATCC 13076, *Pseudomonas aeruginosa* ATCC 27853, *Candida albicans* ATCC 10231 (Becton Dickinson). Mueller Hinton agar (BioLife), Mueller Hinton broth (Becton Dickinson), Brain Heart Infusion agar (Merck), Sabouraud dextrose agar (BioLife) and Sabouraud dextrose broth (BioLife) were used for the investigation. Active substance of gentamicin sulfate (purity 685 µg/mg, Sigma) by Becton Dickinson was used for comparative investigations of referential strains sensitivity. 2,3,5-Triphenyltetrazoliumchliorid (Merck), final concentration at 50 mg/l, was added to Mueller Hinton broth to obtain bacterial growth visibility.

Antimicrobial effects of carrot fruit oils obtained by SFE and hydrodistillation were investigated by agar dilution method and broth dilution method. The preparation of the

investigated bacteria suspension was performed according to prescribed references by NCCLS for bacterial sensitivity to antibiotics investigation [13]. Each assay in this experiment was performed in triplicate.

Results and discussion

The content of essential oil in carrot fruit obtained by hydrodistillation was 0.44%. In the case of SFE the highest extraction yield was obtained at 313 K and 10 MPa, and the extract obtained at these conditions has been chosen for further antimicrobial studies. Experimental data of SFE from carrot fruit in two outermost cases (the highest yield at 313 K and 10 MPa and the smallest yield at 323 K and 9 MPa) are presented in Fig. 2.

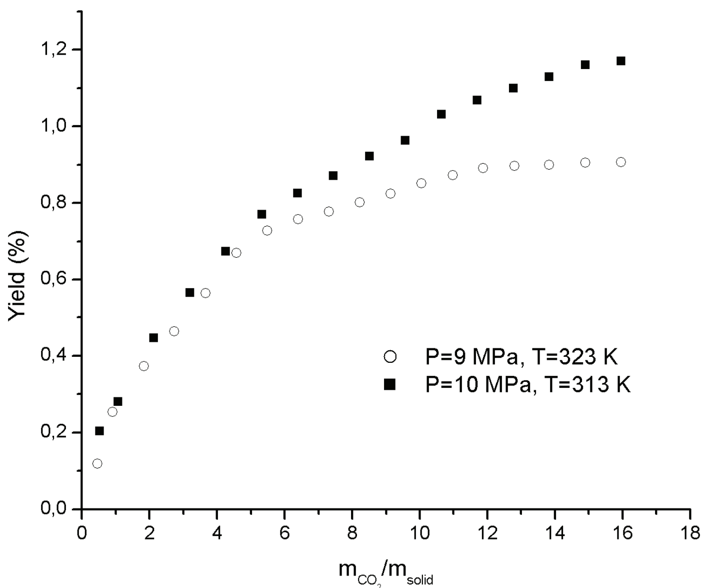

Fig. 2. Yield of total extract as a function of the specific amount of solvent m_{CO_2}/m_{solid} (kg CO_2/ kg herbaceous material) for SFE from carrot fruit.

The chemical composition of the carrot fruit essential oil and SFE extract is presented in Table 1. Carotol was the main component of SFE extract as well as the essential oil. SFE extract was characterized with higher contents of carotol and heavier compounds than the essential oil. As can be seen from Table 1 light components present in essential oil were not detected in SFE extract.

Table 1. Chemical composition of essential oil and supercritical extract of Carrot seed

Peak No.	Compound	RI	Essential oil, area (%)	Supercritical extract area (%)
1	α-Thujene	931	0.32	-
2	α-Pinene	939	7.95	-
3	Camphene	953	0.71	-
4	Thuja-2,4(10)diene	957	0.46	-
5	Sabinene	976	18.7	1.07
6	β-pinene	980	t	-
7	β-Myrcene	991	1.35	-
8	E-3-Caren-2-ol	995	0.46	
9	Car-2-ene	1001	0.55	-

10	o-Cymene	1021	1.38	-
11	Limonene	1027	1.68	-
12	γ-Terpinene	1062	0.58	-
13	Terpinolene??	1080	0.37	-
14	Linalool	1098	0.40	-
15	3(10)-Caren-2-ol	1111	0.31	0.67
16	Born-5-en-2-ol	1125	1.14	t
17	Pinocarveol	1139	1.37	t
18	*cis* -Verbenol	1144	2.76	1.13
19	Sabina keton	1158	1.30	0.96
20	Pinocarvone	1162	0.45	-
21	4-Terpineol	1176	0.47	-
23	Myrtenol	1193	1.15	0.51
24	Verbenone	1204	1.08	0.87
25	*trans*-Carveol	1217	0.33	t
28	Geraniol	1254	0.82	-
29	Bornyl acetate	1285	0.56	0.65
30	Cuminalcohol	1287	0.86	t
31	γ-Terpinene-7-ol	1325	0.35	1.27
32	α-Terpineol acetate	1350	0.36	0.89
33	Isoledene	1373	1.1	-
34	Geranyl acetate	1383	4.40	7.22
35	β-Caryophyllene	1418	5.04	6.47
36	(Z)-β-Farnesene	1443	0.63	0.96
37	α-Humulen	1448	0.43	0.54
38	(E)-β-Farnesene	1458	0.51	0.79
39	γ-Muurolen	1470	1.29	2.16
40	Curcumene	1483	0.79	0.78
41	β-Selinene	1485	4.18	6.66
42	α-Selinene	1494	1.08	1.93
43	β-Bisabolene	1508	1.11	2.85
44	β-Sesquiphellandrene	1524	0.86	0.65
45	Aromadendrene epoxide	1565	0.38	0.68
46	Caryophyllene oxide	1584	4.42	7.38
47	Carotol	1594	20.3	30.28
48	Humulene epoxide	1606	t	0.72
49	Daucol	1638	0.97	2.46
50	Longifollenaldehyde	1678	0.70	1.07
51	Juniper camphor	1691	0.72	-
52	Aristolone	1756	t	1.49
53	Nonadecene	1900	-	0.46
54	Methyl palmitate	1927	-	0.83
55	Palmitic acid	1968	-	2.77
56	Methyl oleate	2128	-	0.61
57	Oleic acid	2132	-	3.17
58	Stearic acid	2137	-	1.60
59	Tricosane	2300	-	0.49
60	Methyl arachate	2322	-	0.55

61	Bis (2-ethylhexyl) phtalate	2540	-	0.66
62	Heptacosane	2700	-	t
63	Nonacosane	2900	-	0.60
64	Stigmasterole	3332	-	t
65	Sitosterole	3408	-	t
TOTAL			97.13	94.86

trace, t<0.01

Results of the antimicrobial activity obtained by agar dilution method and broth dilution method were identical, and they are presented in Table 2. The investigation of carrot seed oil antimicrobial effects by agar dilution method and broth dilution method served to establish the antimicrobial effect of this oil on some Gram-positive bacteria in certain investigated concentrations, as well as on *C. albicans* ATCC 10231. The investigated essential oil and SFE extract in applied concentrations failed to show antimicrobial effects on Gram-negative bacterial strains included in this investigation, as well as on *Enterococcus faecalis* ATCC 29212 in MIC values less then 1280 μg/ml. These results are in accordance with results of Staniszewska et al. [3] which showed that essential oil had higher antimicrobial activity against Gram-positive bacteria and *Candida albicans* then against Gram-negative bacteria. As can be seen from Table 2, SFE extract was more effective than the essential oil in the case of *Bacillus cereus* ATCC 11778 and *Rhodococcus equi* CAPM 6312. The grow of *Bacillus subtilis* ATCC 6633 BB was more inhibited with carrot seed essential oil than it was inhibited with SFE extract of carrot seed.

Table 2. The values of minimal inhibitory concentrations of carrot seed oils obtained by agar dilution method and broth dilution method (average deviation ± 4 μg/ml for oil samples and ± 0.02 μg/ml for gentamicin)

Source	Strain	SFE extract MIC*	Essential oil MIC*	Gentamicin MIC*
Gram-positive bacteria		640	640	≤ 4
ATCC 6538 P	*Staphylococcus aureus*			
CAPM 6312	*Rhodococcus equi*	160	320	≤ 4
ATCC 11778	*Listeria monocytogenes*	640	640	≤ 4
ATCC 6633 BB	*Bacillus subtilis*	160	80	≤ 4
ATCC 11778	*Bacillus cereus*	80	640	≤ 4
ATCC 29212	*Enterococcus faecalis*	>1280	>1280	≤ 4
Gram-negative bacteria		>1280	>1280	≤ 4
ATCC 25922	*Escherichia coli*			
ATCC 13076	*Salmonella enteritidis*	>1280	>1280	≤ 4
ATCC 27853	*Pseudomonas aeruginosa*	>1280	>1280	≤ 4
Yeast		640	640	-
ATCC 10231	*Candida albicans*			

*MIC value presents minimally inhibitory concentration [μg/ml]

Conclusions

The highest extraction yield in the SFE of carrot fruits was obtained at 313 K and 10 MPa. Content of carotol, as well as the content of heavier weight compounds was higher in SFE extract than in the oil obtailed by hydrodistillation. The essential oil and SFE extract had

higher antimicrobial activity against Gram-positive bacteria and *Candida albicans* then against Gram-negative bacteria. SFE extract was much more effective than the essential oil in the case of *Bacillus cereus* ATCC 11778 and *Rhodococcus equi* CAPM 6312 but the grow of *Bacillus subtilis* ATCC 6633 BB was more inhibited with carrot seed essential oil than it was inhibited with SFE extract of carrot seed.

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GENETICS - AMELIORATION RESEARCHES FOR AROMATIC AND MEDICINAL PLANTS IN MOLDOVA REPUBLIC

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The studies carried out in the area of genetics and breeding of aromatic and medicinal plants in Moldova have resulted in the development and registration of new varieties. The genetic and breeding researches have been more profound and effective in Sage Clary (Salvia sclarea L.). The development, evaluation and use of initial valuable breeding material, that includes inbreeding and male sterile lines, the lines that consolidate male sterility, simple hybrids, three line hybrids, double line hybrids, back-crosses and step-wise hybrids have allowed the development of some high-effective hybrids with an enhanced producing capacity. Among them, there are late-ripening varieties named Victor and Nataly-Clary, a variety with a medium ripening named Dacia-99. These varieties reach the ripening state gradually and together with the early-ripening variety Dacia-50, that was registered earlier, form a conveyer during harvesting, which allows a gradual harvesting of each variety and ensures a substantial reduction of raw material and essential oil losses. The varieties Victor and Dacia represent simple hybrids, while Nataly-Clary is a very complex hybrid, developed through a step hybridization. All three varieties are suitable for both processing technology of raw material and production of essential oil through distillation and production technology of concrete through organic solvent extraction. A different vegetative period, gradual ripening allows the expansion of the harvesting period up to 27 days. In its turn, this contributes to the increase of the areas occupied with sage and processing of a higher quantity of raw material while expanding industrial processing capacities. The producing capacity of new Salvia sclarea L. varieties in two years of cultivation is 13.1-19.5 t/ha of inflorescence and 35/45 kg/ha of essential oil with a high concentration of litalite acetate (65-75%) and sclareoli (6-12%), as well as 85-127 kg/ha of concrete with a high content of sclareoli (60-70%). The efficiency of these varieties grows substantially when, following essential oil distillation, the wastes are extracted with organic solvents resulting in concrete with a sclareol concentration of more than 55-60%.

When the plantations are sown with new sage varieties, the seed rate is 5-6 kg/ha of the 1st class as opposed to other varieties which need a sowing rate of 10-12 kg/ha of seeds. The plantations of new varieties can be exploited 2-3 years. By the third year of vegetation, their yielding capacity makes 6-9 t/ha of raw material and 8.4-15.7 kg/ha of essential oil in relation to the cultivar. These two elements of growing technique reduce the expenditures for laying and exploiting the plantations and respectively, enchances the crop efficiency.

The studies carried out in the area of genetics and breeding in lavender (*Lavandula angustifolia Mill.*) have started with the development of new genotypes through polycross hybridization using some germ plasma resources from France, Crimea, and Moldova. Three new varieties of *L. angustifolia*, named Moldoveanca-4, Alba-7 and Vis magic-10 have been developed up to now, which are resistant to frost, wintering and drought. The production of inflorescences and the content of essential oil (4.231-5.229%) are higher in the new varieties that in the control cultivar Chisinau-90, while the production of essential oil makes 130-200 kg/ha.

The other essential oil species, as well as medicinal one cultivated in Moldova is dill (*Anethum graveolens L.*). During 1996-2000, when the markets of essential oils had been lost by this country and the essential oil crop plantation were annihilated , dill oil was exported every year/ An early dill variety , named Ambasador, has been developed to increase the efficiency of cultivation and processing of this species. It is characterised by

average yields of raw material making 10.5 t/ha, those of essential oil of 88.9 kg/ha with a carvone concentration of 39.8%. The variety was registered in 2004. The dill varieties that have been cultivated up to the present have a producing capacity of only 50-60 kg/ha of essential oil with a concentration of the principal component (carvone) making only 29.5%.

The works performed on *Salvia officinalis L.* have resulted in the development of an early ripening variety, named Miracol that is resistant to drought, frost and wintering. It was registered in 2005. It can be used to produce pharmaceutical raw material of *Folium Salviae* and *Herba Salviae* and essential oil – *Oleum Salviae*. The producing capacity of the variety Miracol is 8.4 C.M./ha of dry leaves (13% of humidity) or 18 kg/ha of essential oil. In case of two yields, the producing capacity of the variety is even higher.

Among the medicinal plant species under study, there is milk thistle (*Silybum marianum (L)Gaert).* Individual selections and a subsequent hybridization have afforded an early-ripening variety named Argintiu and registered in Moldova in 2004. The variety is resistant to drought, while simultaneous maturation of fruits, in the majority of inflorescences, contributes to mechanical harvesting. The average fruit producing capacity (*Fructus Cardui Marianus*) of the Argintiu variety is 890-1000 kg/ha under conditions of cultivation using no fertilizers.

Germ plasma resources of different genetic and geographic origin have been used to develop genotypes with new characteristics and properties in merry golds (*Calendula officinalius L.*) A complex hybridization has produced many varieties, two of them – Natali and Diana with large inflorescences, with a producing capacity of more than 10 M.C./ha. The varieties are distinguished by the tubular flower colour. Thus, the tubular as well as ligulate flowers are orange in the Natali variety, while the tubular flowers are brown and the ligulate flowers are orange in the Diana variety. The concentration of the active matter in the new *Calendula officinalis L*. cultivars is much higher than that in the local population cultivated in Moldova, which served as control. The distinction between them, as for this index, is as follows: the flavone concentration in the Natali variety is 0.873%, being higher in the Diana variety which contains 0.624%, while the content of polyphenols is relatively lower (0.988%) than in the Diana variety in which the polyphenol concentration is 1.038%.

Conclusions

- The studies carried out in the area of genetics and breeding of aromatic and medicinal plants in the Republic of Moldova have resulted in the development of new high-efficient varieties.
- The new varieties of *Salvia sclarea L.* that are early-, medium- and late-ripening allows the production of 13-19 t/ha of raw material and 32-41 kg/ha of essential oil or 85-126 kg/ha of concrete. The new elements of the growing technique of the new varieties reduce the cost of the production substantially.
- New varieties of *Lavandula angustifoliia Mill*, named Moldoveanca/-4, Alba-7 and Vis Magic-10 have been developed. Their producing capacity is 130-200 kg/ha ofessential oil in relation to the variety.
- The works carried out on breeding of *Anethum graveolens L.* have resulted in the development of the variety named Ambasador with a producing capacity of 10,5 t/ha of raw material and 88.9 kg/ha of essential oil with a carvone content of 39.8%.
- A new variety named Maricaol has been developed in *Salvia officinalis L.* which contributes to a producing capacity of 8.9 M.C/ha of essential oil at a single harvesting.
- In *Silybum marianum L*. the researches conducted have provided for a new variety named Argintiu, which is early ripening with a producing capacity of approximately 900 kg/ha of fruits.

- Individual hybridizations and selections have resulted in the development of two new varieties of *Calendula officinalis L*. – Natali and Diana. They ensure a production of more than 10 M.C./ha of dry inflorescences with flavone content of 0.624-0.873% and polyphenols of 0.988-1.038%. At plantation sowing, the seed rate is by 50% lower for the new varieties.

MICROMORPHOLOGICAL ANALYSES OF *LAMIUM ALBUM* L. TRIHOMES

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Summary

Micromorphology and distribution of trichomes on stem, leaves and calyx of Lamium album L. were investigated using scanning electron microscopy. On the stems, leaf and calyx two types of trichomes were found: nonglandular uniseriate elongated trichomes and glandular peltate trichomes with 4-celled heads.

Keywords: *Lamium album, Lamiaceae, glandular trichomes*

Introduction

The trichomes of the species which belong to the subfamily Lamioideae were rarely examined. One of the representatives from the subfamily is *Lamium.* According to available literature data, the trichomes of the *L. album* have not been yet examined.
As a part of the micromorphological, anatomical and chemical research of the *Lamium* species, in this paper the results of the structure of glandular and non glandular trichomes are reported.
Lamium album L. (Lamiaceae) is a perennial herb widely distributed in Europe (Ball, 1972). It is used in folk medicine as blood tonic, antispasmodic and antiinflamatory agent. This species contains iridoids, flavonoids, phenolic acids, tanins, saponins and triterepenes as secondary compounds. The tanins in *Lamium* are responsable for its tranquillising, mildly astringent and haemostatic action, while saponins are responsable for a mild expectorant action. It may be used for sore throats and inflamed gums, as a compress for wounds, haemorrhoids, eczema and burns. It is useful remedy in menorhhagia and intestinal bleeding. It is also used in the treatment of abnormal vaginal discharge (Bartram, 1995; Chevallier, 1996; Weiss, 1991).

Material and methods

Aerial parts of the analyzed plants were collected at the flowering stage in July 2004 in Botanical Garden "Jevremovac" in Belgrade. A voucher specimen has been deposited in the Herbarium of the Institute of Botany and Botanical Garden "Jevremovac", Faculty of Biology, University of Belgrade, Serbia and Montenegro.

Scanning electron microscopy (SEM)
Small segments of stem, leaves and calyx were coated with a thin layer of gold (ion sputtering coating) in BALTEC-SCD 005 Sputtering Device. Observations were carried out on a JEOL JSM 6460 LV scanning electron microscope at 20 kV.

Results and discussion

On the stems two types of trichomes were found: nonglandular uniseriate elongated trichomes and glandular peltate trichomes with 4-celled heads. Abaxial and adaxial leaf surfaces were sparsely covered with nonglandular uniseriate elongated trichomes. Glandular peltate

trichomes with 4-celled heads were found on adaxial leaf surface (Table 1). Calyx was densely covered with numerous simple nonglandular uniseriate elongated trichomes among which the peltate glandular trichomes were distributed (Table 2).

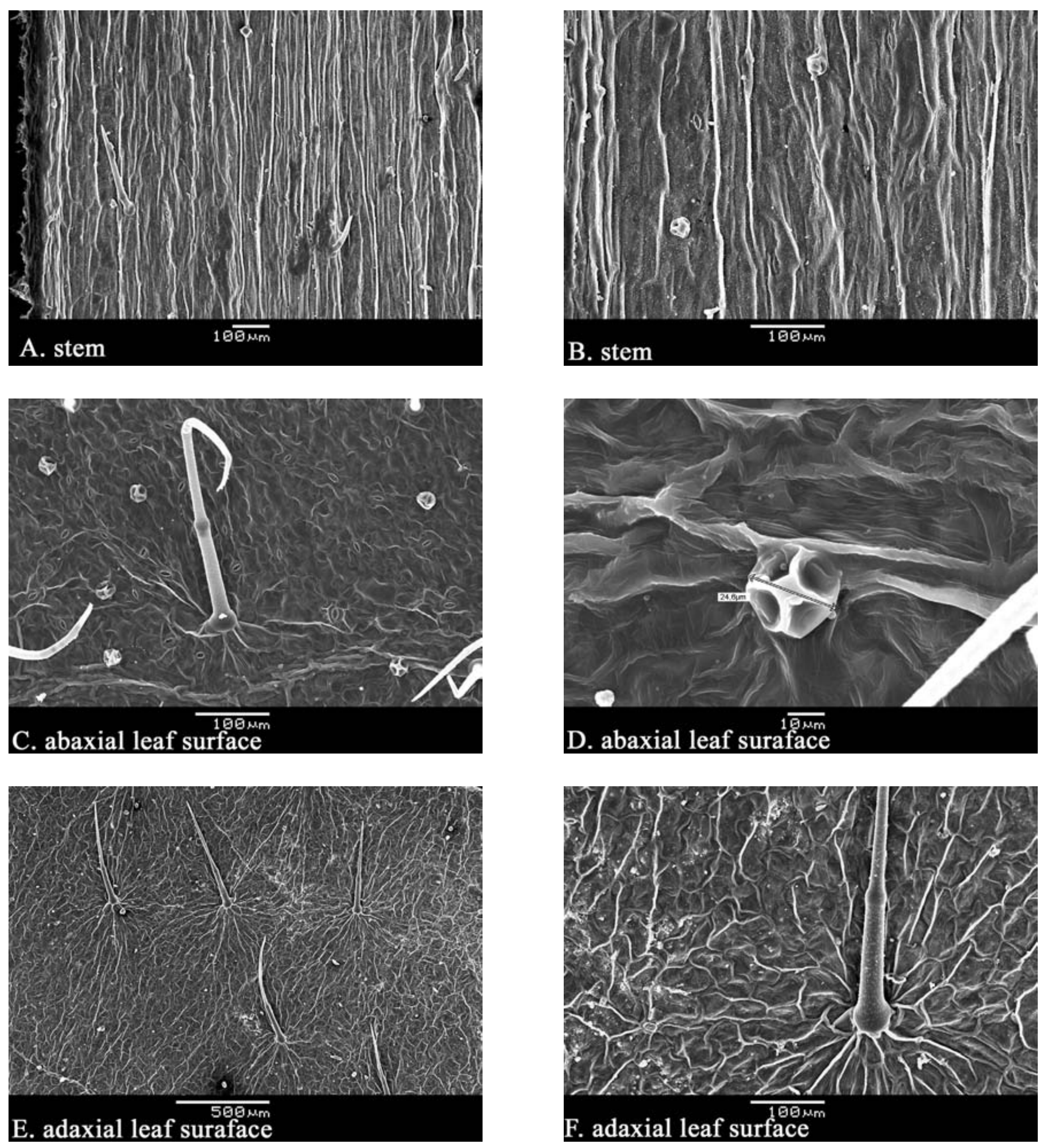

Table 1. SEM micrographs of stem and both leaf surfaces of *Lamium album.*

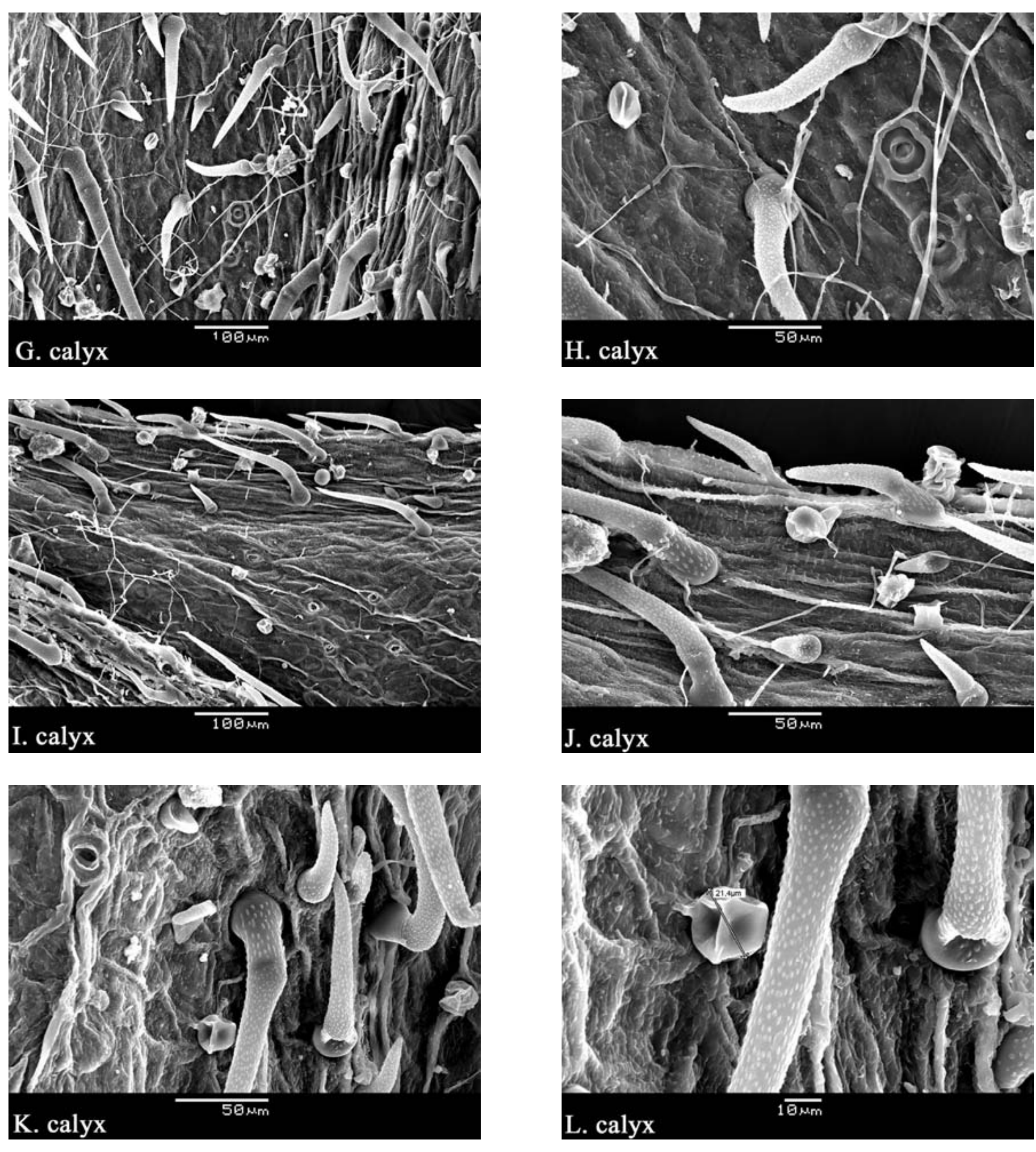

Table 2. SEM micrographs of calyx of *Lamium album.*

The trichomes are widely distributed over the aerial parts in many plant families (Fahn, 2000). Their structure can vary widely among species (Werker, 2000). In the Lamiaceae family there are two main types of glandular trichomes: peltate and capitate (Fahn, 2000). Numerous studies of the trichomes in the Lamiaceae family have been carried out, from morphological, ultrastructural, histochemical and chemical point of view (Werker, 2000 and references cited therein; Hallahan, 2000; Spring, 2000; Fahn, 2000). Many Lamiaceae species have been investigated because of their high content of essential oils, which are widely used in pharmaceutical preparation, perfumery and cosmetics. *L. album* is essential oil poor species. Glandular peltate trichomes covered all vegetative organs but calyx is densely covered with them. The small amount of essential oil produced by glandular trichomes on calyx and leaf surface may act as a protection of the inner flower parts and leaf of *L. album* against herbivores and pathogens.

Conclusion

Since the floral parts are used for medicine purposes as tinctures, the further analysis of calyx glandular trichomes chistochemistry, as well as essential oil compounds, could provide more data for medicinal usefulness.

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CULTIVATION OF MEDICINAL, AROMATIC AND SPICY PLANTS IN SLOVAKIA AFTER JOIN THE EUROPEAN UNION

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Summary

European Union represents the biggest unique market with medicinal, aromatic and spicy plants (MASP) in the world. It was about 120,000 tons (e.g. 200 million USD) of this plant material during the years 1991-2004. Paper is orientated on the current status and situation in the field of MASP grown and produced in Slovakia. Growing areas of these plants were the lowest in 1999 (467.44 ha) and the highest in 2003 (851.85 ha). Global production varied between 222.2 (2000) and 1380.2 tons (2004). Average yields ranged from 0.32 (2000) to 2.60 t.ha^{-1} (2004). Acute problem is to increase and stabilize the production and to obtain stronger position on global herbal market. The objectives of future medicinal plant strategy are: (1) To ensure the quality of MASP material used as the source for herbal medicine to improve the quality, safety and efficacy of finished herbal products; (2) To improve national and/or regional good agricultural practice, processing guidelines, publications about MASP and related standards for operating procedures; (3) To encourage and support the sustainable cultivation and collection of good quality MASP, in ways that respect and support the conservation of the environment. As an alternative for MASP producers can be the organic production of MASP. Demand for organic products is still increasing in EU as well as the consumption of natural substances.

Keywords: *cultivation, medicinal, aromatic, spicy plants, production*

Introduction

Cultivation of medicinal, aromatic and spicy plants (MASP) has recorded long tradition in the agri-ecological conditions of Europe. It was originated in Mediterranean, where many MASP species were produced in the past. From the point of view biodiversity, there are approximately 130 – 150 MASP species cultivated in Europe and 150 – 170 MASP species are collecting from their natural resources nowadays. As the most cultivated MASP in Europe are: *Carum carvi* L., *Coriandrum sativum* L., *Foeniculum vulgare* Mill., *Silybum marianum* (L.) Gaertn., *Pimpinella anisum* L., *Artemisia absinthium* L., *Matricaria recutita* L., *Hypericum perforatum* L., *Mentha piperita* L., *Melissa officinalis* L., and *Lavandula angustifolia* Mill.

Producers in most Central European countries are in close contact with processors, which determine requirements for type and amount of demanded MASP raw material. Different types of private or state producers cultivate the MASP species in Slovakia:

- Specialized farms for MASP cultivation;
- Farms produce MASP supplementary;
- Agricultural companies with their own processing of MASP products or semi-products
- Industrial processing organizations – pharmaceutical, food or cosmetics, including global companies. They secure the required amount of raw material by contracts with individual producers;
- Research, educational organizations or botanical gardens;
- Gardens on small areas (less than 400 m^2).

The importance of MASP is varied. It is orientated to different using of processed plants, their parts or utilizing of active ingredients in these areas:

- Human and veterinary medicine, pharmaceutical and cosmetics industry use the medicinal plants as a raw material;

- Food industry (e.g. brewery), tobacco industry, cosmetics industry use aromatic plants as a raw material;
- Food industry (producing of foods, canning industry, alcohol production) as well as homes are using of spicy plants.

Materials and methods

Documentation materials were used from (1) statistical data obtained from official statistic reports of Statistical Office of the Slovak Republic during the years 1997 – 2005 and (2) results of Farm Structure Census, which was done in 2000 – 2002. Source material was analyzed by methods of selection, comparison and synthesis. Evaluated results during the analysis of relevant data founded by a study of foreign and domestic literature resources were orientated to the target group: medicinal, aromatic and spicy plants (MASP) cultivated on agricultural or arable land in Slovak Republic. At the same time, method of comparison was used to compare status of MASP cultivation and production during the pre-accession period with a period after Slovak Republic joins the European Union in 2004.

The survey and research was done in the framework of projects, which have been funded by GA SPU 705/02180 and by VEGA 1/1343/04.

Results and discussion

Current status of MASP cultivation in Europe

European Union represents one of the biggest unique markets with medicinal, aromatic and spicy plants (MASP) in the world. It imports about 120,000 tons with the value of 200 millions US$ in average during 1991 – 2004 (UN Comtrade, 2004). This market exchanges increase every year between 5 – 10 % (Lange, 1998). The most important importer within European MASP market is Germany with more than 45,000 $t.year^{-1}$ (e.g. 38 % of global European import). It is followed by France with 17 % and Italy with 9 % of global import (Commonwealth Secretariat, 2001). Germany is also the biggest (re-) exporter of MASP within EU (Tables 1 and 2). It exports circa 15,000 $t.year^{-1}$ to the other EU countries and to the USA. The next big exporters are: France, Poland, Hungary, and Czech Republic etc. The most important non-European suppliers of MASP to the EU countries from the point of view value of global import of these commodities are: USA (15.80 %), India (8.00 %), China (7.45 %), Bulgaria (6.44 %) and Egypt (5.47 %).

Table 1. The cultivation acreage of medicinal, aromatic and spicy plants (MASP) in selected European countries in 2003 (UN COMTRADE, 2004)

Country	Acreage [ha]	Country	Acreage [ha]
Belgium	100	Poland	30,000
France	25,000	Austria	4,300
Netherlands	2,500	Slovakia	1,500
Ireland	50	Slovenia	100
Hungary	37,500	Switzerland	150
Germany	12,000	Great Britain	4,000

Current status of MASP cultivation in Slovak Republic

Cultivation of MASP as a part of special plant production is a main activity to obtain required amount and quality of domestic MASP species when the protection of natural resources is increasing. Because of multi-year results of research and their application in agricultural

practice, the technology of MASP cultivation of 30 species is described in details. About 50% of these species belongs to produce of high capacity drugs.

Table 2. Market with MASP in selected EU countries in 2003 (UN COMTRADE, 2004)

Country	Export [t]	Import [t]	Balance (I – E) [t]	Export value [$]	Import value [$]
Slovakia	603.2	347.8	-255.4	1,206,720	971,495
Germany	16,729.9	45,700.5	28,970.7	73,449,000	100,720,000
France	8,150.0	18,234.3	10,084.3	52,500,880	48,902,956
Belgium	1,935.4	4,795.5	2,860.1	19,888,968	23,879,468
Poland	14,469.9	4,755.2	-9,714.7	27,935,000	8,069,000
Czech Republic	767.2	2,835.7	2,068.5	2,487,306	7,202,909
Hungary	3,012.9	983.1	-2,029.7	6,845,000	3,060,000
Austria	1,625.7	2,160.9	535.2	4,975,798	7,590,191
Italy	2,216.8	11,509.1	9,292.2	10,530,507	38,672,596
Latvia	5.5	180.7	-255.4	41,207	714,030

Ministry of Agriculture of Slovak Republic in cooperation with Research Institute of Agri-ecology in Michalovce published "Development program of production and processing of medicinal, aromatic and spicy plants in Slovak Republic" (Šalamon, 2000). Prognosis of MASP acreage (Table 3), improvement of technology in production and processing of MASP as well as analysis of MASP industry in Slovakia are given in the document.

Table 3. Prognosis of MASP cultivation area as enlarging in Slovak Republic (Šalamon, 2000)

Years	Unit	1970-1980	1980-1990	1990-2000	2000-2010	2010-2020
Cultivation acreage	ha	150	350	370	1,500	2,500
Production	t	165	385	410	1,650	3,000

Table 4. Development of harvested acreage and production of cultivated medicinal plants in Slovak Republic (1997-2005)

Year	Harvested acreage [ha]	Total yield [t]	Yield [$t.ha^{-1}$]
1997	527.37	328.9	0.62
1998	540.55	439.9	0.81
1999	467.44	475.2	1.02
2000	696.56	222.2	0.32
2001	623.98	873.1	1.40
2002	601.65	989.9	1.65
2003	851.85	821.4	0.96
2004	531.07	1380.2	2.60
2005	*709.71		

* Harvested acreage in 20th May 2005.

Development of cultivation acreages of MASP has oscillated in the last decades. The MASP were cultivated in the 1989 at the area of 408.3 ha, in 2000 it was 783.6 ha and in 2004: 540.4 ha. Cultivation of MASP in Slovak Republic according to Statistical institute of Slovakia during 1997 – 2005 is presented in Table 4.

The harvested acreages of aromatic plants are presented in Table 5.

Table 5. Development of harvested acreage and production of aromatic plants in Slovak Republic (1997-2005)

Year	Harvested acreage [ha]	Total yield [t]	Yield [$t.ha^{-1}$]
Humulus lupulus L.			
1997	816.41	742.1	0.91
1998	151.29	261.4	1.73
1999	238.30	233.6	0.98
2000	273.39	95.7	0.35
2001	246.16	188.1	0.76
2002	317.60	297.6	0.94
2003	318.41	323.2	1.02
2004	307.82	363.8	1.18
2005	*310.66		
Nicotiana tabacum L.			
1997	649.16	994.1	1.53
1998	958.87	1487.4	1.55
1999	834.89	1288.6	1.54
2000	1133.57	1870.4	1.65
2001	1245.27	1986.9	1.60
2002	1099.83	2020.1	1.84
2003	1079.55	1932.1	1.79
2004	934.51	1298.2	1.39
2005	*957.33		

* Harvested acreage in 20th May 2005.

As the most cultivated spicy plant was red pepper (*Capsicum annuum* L.) despite the fact, that acreage decreased from 2,289 ha (1975) to 254 ha (2003). Global acreage of red pepper was the lowest in 2003: 254 ha with yield of pepper fruits about 228.1 t. The largest harvested area was 718 ha (1998) with yield of 1,024 t (Table 6).

Table 6. Development of red pepper (*Capsicum annuum* L.) acreage and its production in Slovak Republic (1997-2005).

Year	Harvested acreage [ha]	Total yield [t]	Yield [$t.ha^{-1}$]
1997	551.67	666.3	1.21
1998	718.12	1024.2	1.43
1999	560.34	827.0	1.48
2000	536.28	540.2	1.01
2001	333.06	482.1	1.45
2002	272.52	377.8	1.39
2003	254.00	228.1	0.90
2004	460.32	450.2	0.98
2005	*463.38		

* Harvested acreage in 20th May 2005.

Caraway (*Carum carvi* L.) is the second most produced spicy plant in Slovak Republic. Statistical data are documented from 1998 (Table 7).

Table 7. Development of harvested acreage of Caraway (*Carum carvi* L.) and its production in Slovak Republic (1997-2005)

Year	Harvested acreage [ha]	Total yield [t]	Yield [t.ha^{-1}]
1997	-	-	-
1998	90.26	49.1	0.54
1999	80.03	19.3	0.24
2000	51.00	14.5	0.28
2001	117.00	75.5	0.65
2002	258.28	216.3	0.84
2003	174.31	37.9	0.22
2004	249.78	54.6	0.22
2005	*166.82		

* Harvested acreage in 20th May 2005.

Main aspects that determined cultivation of MASP in Slovak republic are:

- Market demand – production depends on requirements of processors;
- Supplier – consumer contracts;
- Prices of production;
- Development of processing subjects;
- Competition;
- Availability of traditionally required or introduction of non-traditional plant species;
- Macro-economic processing conditions – support possibilities of business activities (EU funds, state subsidies, tax benefits etc.).

Balance of foreign trade with MASP and their products obtained always-negative values (Table 8) because of higher import of these commodities. Slovakia imported about 5,048.5 t of MASP raw material or products. In comparison to the year 1997 (1,308.4 t) import was increased almost four times. This fact confirms higher consuming of these commodities in the country.

Main MASP supplier countries to Slovak Republic are: Czech Republic, Bulgaria, Poland, Croatia, Romania, and Ukraine. Export in 2004 was about 211.9 t and in comparison to 2003 (603.2 t) significantly decreased. Export of domestic MASP products is orientated mainly to EU market: Czech Republic, Poland, Italy, Germany, Hungary, and Australia as well. Very important factor when to export these commodities is optimizing of delivery-supply relationships with the aim of purchase guarantee.

Conclusion

Cultivation of medicinal, aromatic and spicy plants (MASP) in Slovak Republic after the EU accession knots at the pre-accession period. Situation in agricultural subjects is dramatically developing. Reserves in supporting of MASP producers are necessary to solve systematically through Ministry of Agriculture. Stabile realization of production with optimum qualitative parameters and existence of products with competition ability, strengthens the position at the domestic market and creates the better position to success at the European trade. One of the alternatives for MASP producers could be production of these commodities in organic (ecological) farming systems. Realization of organically certified products as well as the

consuming of natural products has recorded increasing demand in the EU trade during the last years.

Table 8. Balance of foreign trade with MASP in Slovak Republic (UN Comtrade, 2004).

Year	Export [t]	Import [t]	Balance (I – E) [t]	Export value [$]	Import value [$]
1994	507.3	3,030.1	- 2,522.8	1,629,466	1,772,966
1995	560.3	2,859.1	- 2,298.8	1,624,248	2,104,958
1996	675.2	3,235.1	- 2,559.9	1,601,461	1,780,203
1997	429.8	1,308.4	- 878.6	1,015,778	890,356
1998	478.3	1,980.7	- 1,502.4	1,055,366	1,126,275
1999	536.0	5,092.6	- 4,556.6	1,052,977	2,071,783
2000	456.1	4,657.7	- 4,201.6	720,065	1,718,252
2001	538.7	4,334.1	- 3,795.4	835,739	1,636,996
2002	293.6	4,697.2	- 4,403.5	614,437	2,092,672
2003	603.2	5,389.5	- 2,522.8	1,206,720	2,994,152
2004	211.9	5,048.4	- 4,836.6	423,710	3,732,313

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CONSIDERATIONS ON THE HISTO-ANATOMICAL STUDY OF THE LEAVES OF *CYNARA SCOLYMUS* L. TREATED WITH METHYLTHIOPHANATE (TOPSIN M)

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Summary

The purpose of this study was to investigate the histo-anatomical modifications of the leaves of Cynara scolymus L treated with thiophanate-methyl, compared with the untreated sample. There were three applications and two variants of treatment: 0.1 %, Topsin M70, a concentration used in agriculture and 0.4 % Topsin M70, for observing whether the limits of concentration between the modifications induced by thiophanate-methyl remain acceptable for the plant. Cross sections through the petiole and limb (the officinal product Cynarae folium), made at different levels were used, together with superficial sections for the upper and lower epidermis. The modifications observed were rather quantitative than qualitative, the general picture showing that the development of leaves was obviously stimulated.

For the treated plants, the comparative study revealed the following aspects: the thickness of petiole increased, the hypodermic walls of collenchymas are more developed, more specialized conducting bundles appear, with a larger diameter of the xylem vessels, cambium layer's activity is more intensive; the form and dimensions of the median nervure are modified, the mesophyll tends to thicken, the pallisade parenchyma being much more developed; the glandular and nonglandular trichomes are more numerous per unit area of leaf surface, the epidemic cells are many but more smaller and stomata more numerous per unit area, with guarding cells of smaller dimensions.

Keywords: *Cynara scolymus L, thiophanat-methyl, histo-anatomical modifications, leaf structure.*

The therapeutically importance of the artichoke being unanimously recognized (Stănescu Ursula, 2004) its introduction in intensive cultures require, in some cases, the application of certain phito-sanitary treatments (Mititiuc, 2000; Verze Maria, 2003), once known that the newly-created microclimate favourizes mass development of pathogenic agents such as: *Septoria scolymii* Pass., *Ascochyta cynarae* Maffei., *Ramularia cynarae* Sacc.(Săvulescu Alice, 1967), *Bremia lactucae* Regel.(Corda, Franceschini, Fiori, 1983). Thiophanate-methyl has quite a large action spectrum (on the *Septoria, Ramularia, Bremia, Sclerotinia* species included), the literature of the field providing several data on the successful application of such a fungicide, as well as of its main metabolite-carbendazime (Marras et all, 1983)
As most of the researches devoted to methyl-thiophanate have been mainly directed towards metabolism, toxicity or estimation of the maximum residual potential in the vegetal material (Marras şi colab., 1983), and considering the fact that, generally, pesticides induce morpho-anatomical modifications in the treated plants (Georgescu, Sanda, 1964; Niță Mihaela,. 2003; Aprotosoaie Clara, 2002), the present paper analyzes the possible histo-anatomical modifications that the fungicide and/or its metabolite might induce at the level of the vegetative organs – at the level of the leaf, especially (the *Cynarae folium* pharmaceutical product) – versus the untreated control sample, the structure of which has been described in several papers of vegetal anatomy (Solereder, 1899; Rácz, Pèter, Sebe, 1967; Rácz, Pèter, 1968; Metcalfe, Chalk, 1972; Napp-Zinn, 1974 Rugină, Toma, 1989 ; Toma, Rugină 1998).

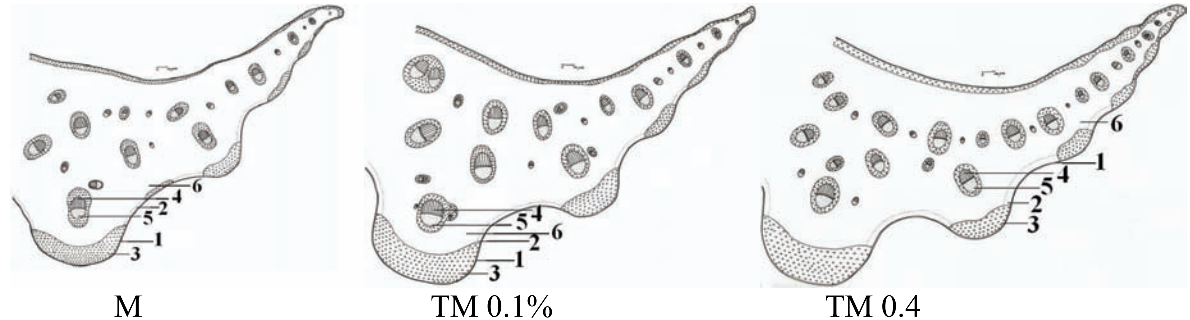
Materials and methods

The material taken into study has been cultivated in the „Anastasie Fătu" Botanical Gardens of Iaşi in parallels whit the treated plants (Topsin in 0.1% concentration – as used in agriculture, and 0.4 % concentrated Topsin, respectively, which is the value recommended for other fungicides, similar to methyl thiophanate), a batch of untreated plants – the control – has

been also involved. Fungicide's administration as a wettable powder, in doses of 1000 l/ha, was performed three times (at intervals of 7 and 10 days), in the moment in which the foliary system of the artichoke was already well developed, the basal rosette having 5-6 nomophyls. For the obtention of cross-sections, at different levels, through the leaf (petiole, limb), as well as of superficial sections, the vegetal material harvested twice, at 7 days after the second and, respectively, third treatment (July 23 and August 3) has been fixed and conserved in 70% ethanol, after which it has been processed according to the methods currently employed.

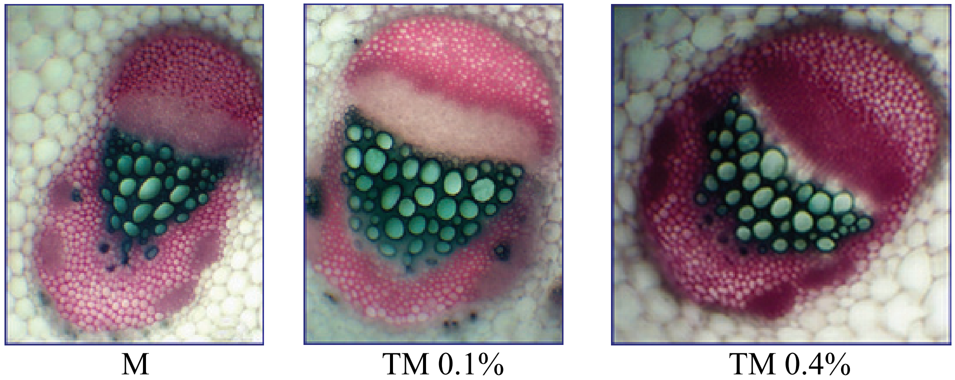
Results and discussion

The petiole (fig. 1-2). In the control sample (M), the contour of the cross-section (medium level) is V-shaped, with highly divergent and increasingly thinner arms. The abaxial side evidences 5-7 obtuse ribs, while the adaxial one – a large and deep ditch. The epidermis evidences iso-diametric cells, rare stomata, prominent on the inferior part, where the glandular and nonglandular trichomes (long, with a thin and flexuose terminal cell) are much more numerous; the short nonglandular trichomes, with an extremely wide basal cell, are very rare. In the abaxial ribs (8-9 layers) and at the end of the arms, a thick belt of angular cholenchyma may be noticed while, between the ribs, the hypodermis is of the chlorenchymatic type. In the fundamental, homogenous parenchyma, there appear numerous conducting bundles (22-24), the big alternating with the small ones, all of them arranged as a double, large-open arch.



M TM 0.1% TM 0.4

Fig. 1. Diagrams of cross-sections through the petiole, medium level, August 3; - 1 epidermis; 2- chlorenchyma; 3- cholenchyma; 4- xylem; 5- liber; 6- fundamental parenchyma.



M TM 0.1% TM 0.4%

Fig. 2. Cross-sections, petiole, medium level, conducting bundle (Oc. 10x Ob.10), August 3.

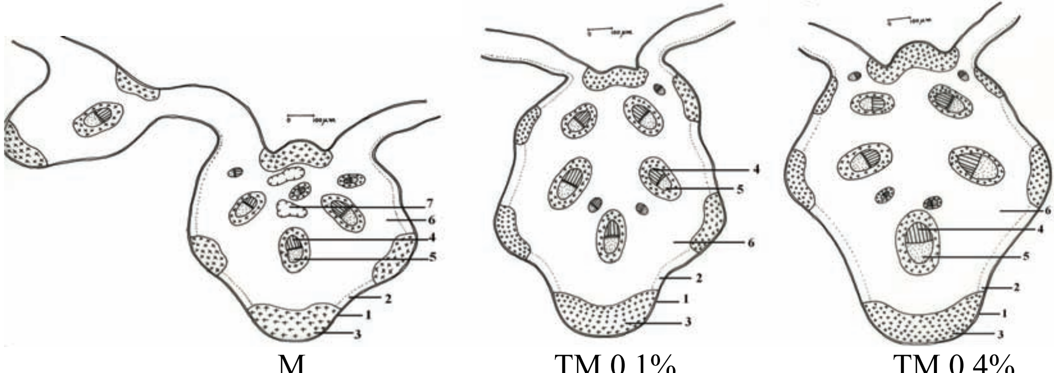
With the exception of the very small ones, each conducting bundle shows, at both poles, a thick girdle of mechanical elements, with moderately-thick, yet non-lignified walls, resembling a transitive cholenchyma. At the xylemic pole of the large bundles, in the mechanical tissue, two very small conducting bundles of closed collateral type may be noticed; in the other bundles, of open collateral type, the tracheogenesis process is not

completed yet. Each conducting bundle is surrounded by a parenchymatic pod at the level of which, in the xylemic part, 5 secreting channels are visible.
Following treatments with 0.1% and 0,4 % Topsin M70, the petiole·s thickness increases considerably, the cholenchyma girdles show several (12-13) layers, and the degree of the cellular walls' thickening increases, too. At the level of the epidermis, the stomata are highly prominent on the inferior side, while the glandular and nonglandular trichomes are more numerous on the surface unit.
The conducting fascicles, much larger in size, are more numerous (46-48); generally after treatments, the number of small conducting bundles increases; cholenchymatization is much more intensive at the bundles' poles, the mechanical tissue being here a typical angular cholenchyma; the xylem vessels are larger and more numerous, with thicker and more intensely lignified walls; the cambial activity is stimulated (3-4 layers), several xylem vessels being still immature. In the perifascicular pod, secretory ducts (1-2) do appear and, in front of the liber, their number (7 on the whole) is higher after the 0,1% TM treatment.

Table 1. Variation - under treatment with 0.1% and 0.4 % Topsin M - of some numerical indices in the petiole (medium level) of the *Cymara scolymus* leaves taken over on August, 3

Variant	Thickness (µm)	No. of cholenchyma layers	No. of conducting bundles	Diameter of the xylem vessels (µm)
Cy. sc. M	4100-4300 7300-7500 (x2)	8-9	34-37	(30) 50-60
Cy. sc. TM 0,1 %	4500-5300 7600-7800 (x2)	12-13	35-40 (42)	(50) 70-90
Cy. sc. TM 0,4 %	4700-5500 7800-8000 (x2)	10-11	46-48	(50)70- 90

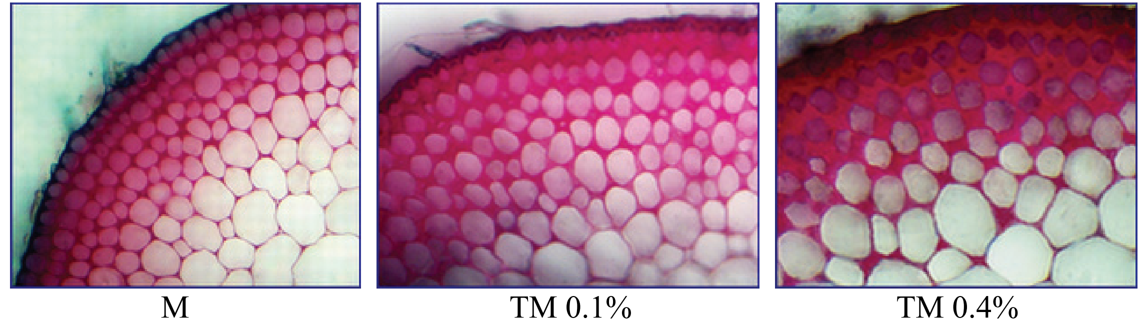
The limb (fig. 3-7). In the control sample (M), the median nervure is prominent on the inferior side, where the long and fine nonglandular trichomes, as well as the glandular ones, whit the cells of the secretory gland in tiers, submerged towards the basis of the hair, are more numerous on the unit of surface. In the abaxial ribs (3), girdles of angular cholenchyma with 3-4 layers may be observed while, at the adaxial side a cholenchyma ridge appears. The median nervure shows 1-2 aeriferous cavities and 5-6 conducting bundles, all of them evidencing a mechanical tissue at both poles. The limb as such shows an epidermis with small cells, slightly elongated tangentially on the inferior part and larger ones, visibly elongated tangentially, on the superior part. The hairs are much more numerous in the inferior epidermis (the secretory ones occurring in excavations through the epidermis); equally numerous are the stomata, although they are present on both sides of the limb (aphistomatically). The mesophyll (5-6 layers) is differentiated into untypical, bi-layered pallisade parenchyma on the superior part and pluri-stratified spongy parenchyma on the inferior one (heterofacial bifacial limb). The pallisade parenchyma with relatively law cells (only two times higher then large), with straight or slightly curled lateral walls, represents about 45-50% of the mesophyll's thickness (fig. 5).



M TM 0.1% TM 0.4%

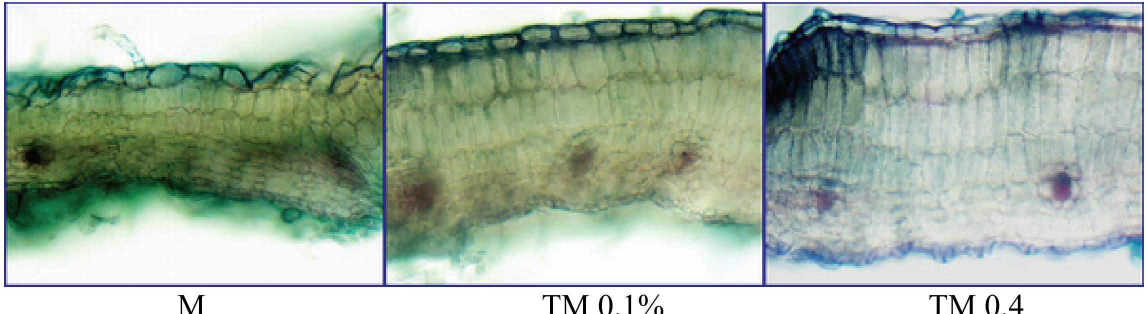
Fig. 3. Diagrams of cross-sections, limb, medium level, Aug. 3;-1 epidermis; 2chlorenchyma; 3- cholenchyma; 4- xylem; 5- liber; 6- fundamental parenchyma; 7- aeriferous gap.

As a result of treatments (0.1%, 0.4 % TM), the nervures become much thicker, especially in the anterior-posterior plane, the auriferous cavity is absent, while the more compact fundamental parenchyma includes several conducting bundles (7-8) 5 abaxial ribes are visible, each having 5-6 layers of cells with a higher cholenchymatization degree. The limb is thicker, which occurs especially at the expense of the pallisade parenchyma (cells' growing in length being stimulated), which is three-layered, representing 65-70% (in 0.1% TM treatments) and, respectively, 75-80% (in 0.4% TM treatments) of the mesophyll's thickness. At the level of the superior epidermis, the walls are slightly thicker, the glandular and nonglandular trichomes are more numerous per unit of surface, the bi-seriated secretory ones prevailing between the secondary nervures and towards the margin of the limb; also here, short nonglandular trichomes, with a much larger basis and thickened walls are visible, as well, in the upper epidermis.



M TM 0.1% TM 0.4%

Fig. 4. Cross-sections through the limb, medium level, cholenchyma, foto: (Oc. 10x Ob. 20), August, 3.

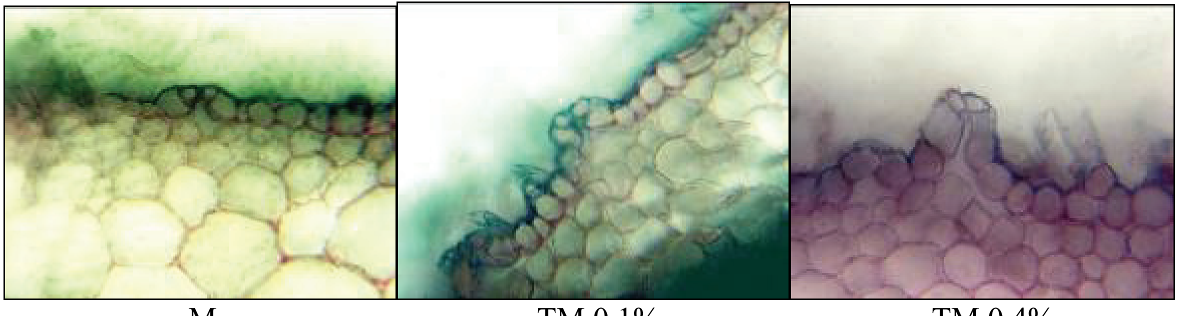


M TM 0.1% TM 0.4

Fig. 5. Cross-sections through the limb, medium level, mesophyll foto: (Oc.10x Ob. 20), August, 3.

Table 2. Variation under treatment with 0.1% and 0.4 % Topsin M of some numerical indices in the limb (median nervure, medium level) of the *Cymara scolymus* leaves taken over on August, 3

Variant	Diameter of the median nervure (µm)	No. of cholenchyma layers	No. of conducting bundles	No. of layers of the limb	Thickness of the limb (µm)	Thickness of the pallisade (µm)
Cy. sc. M	2100/2500	3-4	5-6 (7)	6-7	250-270	130-140
Cy. sc. TM 0,1 %	2400/3900	6-7	6-8	7-8	290-300	180-200
Cy. sc. TM 0,4 %	3200/5100	5-6	7-9	8-9	290-320	210-240



M TM 0.1% TM 0.4%

Fig. 6. Cross-sections through the limb, medium level, stomata, foto: (Oc.10x Ob. 40), Julay, 23.

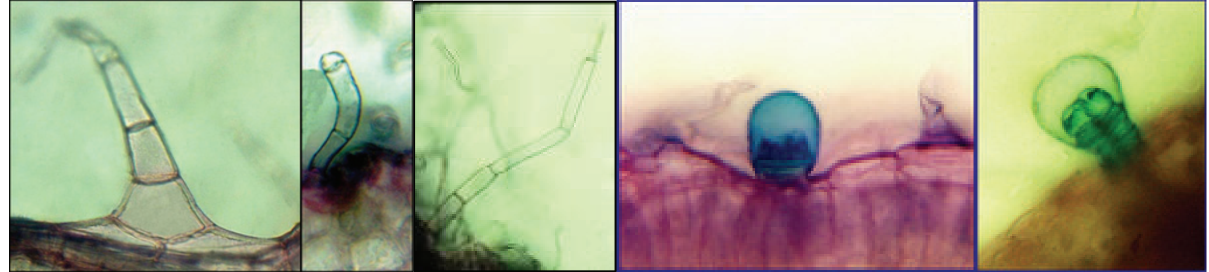


Fig. 7. *Cynara scolymus*: nonglandular trichomes, (**a, b, c**) and glandular trichomes: uniseriated- d, biseriated- e

Superficial section epidermis (fig. 8, 9). In the control sample, the superior epidermis evidences polygonal cells with straight lateral walls; amonocytic-type stomata; pluricellular nonglandular trichomes of various thickness, with a very long flexuose, terminal cell, some of them being shorter, pluricellular, uniseriated, with the basal cell evidencing thickened walls; the uni- or biseriated secretory trichomes are very rare. The inferior epidermis shows irregularly-shaped cells, with winding lateral walls; the stomata are more numerous on the surface unit; the nonglandular trichomes (of both categories) are numerous and only very rarely biseriated. The number of cells per unit of surface is more reduced in the superior epidermis than in the inferior one.

The treatments (0.1%, 0.4 % TM) result in more numerous epidermic cells on the unit of surface, being therefore smaller; the number of stomata increases significantly, in parallels with a reduction in the size of the annex cells. The glandular and nonglandular trichomes are more numerous on the surface unit.

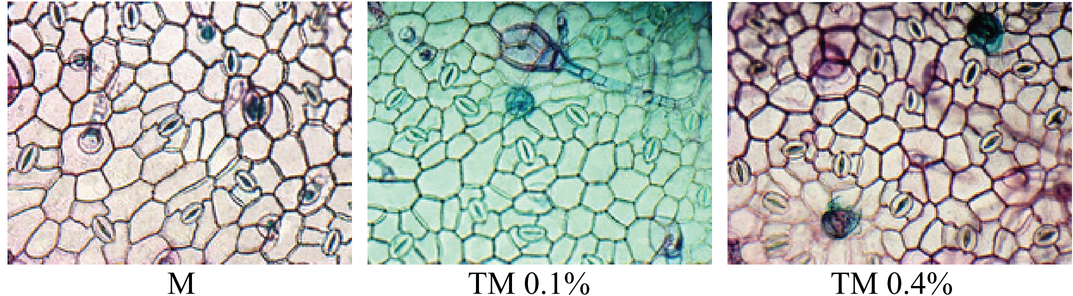


Fig.8. Superficial sections of the limb (superior epidermis) medium level, August, 3.

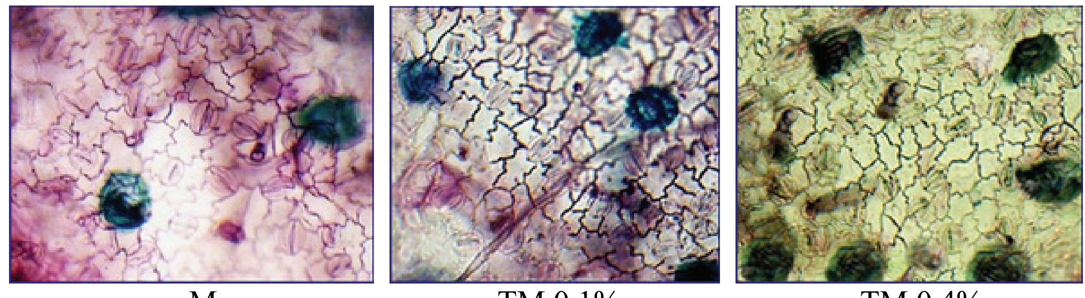


Fig.9. Superficial sections of the limb (inferior epidermis) medium level, August, 3.

Table 3. Numerical data on the epidermis of the *Cymara scolymus* taken over on August, 3(per unit of 10x40 microscopic field)

Sample	Superior epidermis		Inferior epidermis	
	No. of cells	No. of stomata	No. of cells	No. of stomata
Cy. sc. M	35	4	115	26
Cy. sc. TM 0,1 %	38	5	120	29
Cy. sc. TM 0,4 %	52	7	126	32

Conclusions

The histo-anatomical modifications induced by the treatment with Topsin M are most frequently of quantitative type, the plants free from the negative effects of the pathogenic agents having still to face another stressing factor, namely the anti-fungi treatment, especially in concentrations of 0.4%, applied for establishing the concentration limits between which the modifications induced by the fungicide remain acceptable for the plant.

The general picture of the response reactions should differentiate, in a clear-cat manner, between the objective of a higher production, as a result of more reduced losses, and the one related to the direct influence of the substance upon the plant, when some parts of the phyto-sanitary product may have a stimulating effect, as actually asserted by the literature of the field (Baicu 1972).

For the treated plants, the comparative study put into evidence the following aspects: petiole's thickness increases, cholenchymatization is more pronounced, more numerous conducting bundles, with larger xylem vessels, do appear, cambial activity is more intense; the median nervure modifies its shape and sizes, the mesophyll gets thicker, while the pallisade parenchyma is better developed; the glandular and nonglandular trichomes are more numerous on the unit of surface; the epidermic cells are numerous and smaller, while the stomata are more numerous on the unit of surface, the latter ones having annex cells of lower size.

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EPCA TOWARDS VALUE ADDING OF MAP's IN ALBANIA

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Keywords*: Albania, MAP's, EPCA, Essential oils, Cultivation.*

Aromatic and Medicinal Plants still continue to be a significant component of the Albania's overall agricultural exports. Blessed by mother nature, Albania represents a very diverse MAP's basin, most of which are basically harvested, processed and exported as bulk. There has also been some product's diversification activity going on in the country during the last 12- 13 years which primarily consisted in the essential oils extraction. The essential oils too were exported yet to a smaller amount as compared to the dried items.

Production of essential oils is estimated to be somewhat between 15-20 tons a year which represent a wide range of products such as Sage Oil, Thyme, Lavender, Juniper, Myrtle, Savory, Laurel, etc. It is worth noting that oils' characteristics largely depend on the growing and harvesting areas of the raw material. Still, the Albanian essential oils are highly preferred due to their properties. Steam distillation is the major production practice employed in oils extraction.

There have been efforts undertaken recently by EPCA members towards value adding of the essential oils through organic certification. For the first time Albania (EPCA members) successfully exported Organic Essential Oils; this not only increased the range of MAP products from Albania but also enabled to capture more dollar value as premium directly originating from the Organic Certification. In the general view Albania has previously exported organically certified herb and spice products which fall in the category of dried and fresh items.

Albanian conventional and recently organic essential oils are marketed mainly to the EU countries. Nevertheless, few major industry players are recently making efforts trying direct export of these items to other international markets outside EU. EPCA members have conducted a number of visits in the USA and England in order to get acquainted with markets specifications and standards and explore new partnership opportunities.

Albanian essential oils producers are also endeavoring to upgrade existing oils production technology and increase production capacities.

However, generally speaking, Albanian herb and spice businesses are faced with too many obstacles which originate from the local business environment/infrastructure as well as the outside buyers' pressure. The latter, keeping sufficient stocks in the warehouse do not sing

contractual agreements with Albanian producers until the beginning of the next production season, which on the other hand forces the Albanian producers to sell very cheap as they want to get rid off old production prior to the new production season. Another critical aspect that is becoming a serious risk to many significant and correct Albanian essential oils producers is that some whole sale buyers form Europe have started to buy from Albanian sporadic and amateur oils producers, who sell cheap/under cost hoping to capture any market segment; which has led to unfair competition within the country.

In these circumstances the Essence Producers and Cultivators Association (EPCA) was founded to better serves the needs of its membership thus assisting this industry move ahead and overcome the challenges. EPCA intends to promote new partnerships on fair trade basis. In addition, EPCA is looking at low cost production alternatives not through illegal production and export practices but through extraction technology upgrade, proper harvesting timing of high quality raw material, thus abandoning inappropriate practices of employing very law quality raw material.

EPCA has about 70 regular fee paid members from all over the country including 12 significant industry players.

Along with the progress of this industry, EPCA recognizes that conservation of natural resources is a must as that is the only way for the industry to grow and develop and continue to be a significant income source for thousands of rural families.

EPCA considers fundamental sustainable use of aromatic and medicinal plants in order for the industry to have a sound present and future and equally important to save Mother Nature from overexploitation of these resources. Therefore, EPCA has targeted this issue as one of its major goals which summarized are as follows:

a) preservation of biodiversity,
b) quality improvement, and
c) increased incomes.

EPCA is also working on the identification of value adding alternatives besides actual essential oils production which in other terms represents further processing of harvested wild MAP's. Driven by these incentives and also knowing the recent developments of this industry in the region and other countries, EPCA members are exploring recently opportunities for small scale cultivation of these plants. As a result, few EPCA members have initiated cultivation of several herb and spices in different location from North to South. The most important items that are experimented for cultivation are Sage, Thyme, Oregano, Lemon balm and Rosemary.

It is worth noting here that areas under cultivation areas are still small and wild production leads countries' overall exports and is likely to do so in the long run as wild crafts comprise a significant competitive advantage for Albania.

EPCA is however promoting cultivation only if such activities are commercially viable and make sense for the selected items. Equally important to value adding, EPCA looks at the

cultivation activities as an important means particularly for the preservation of endangered species.

Albania, just like many other countries, is faced with the erosion of the “natural gene banks” of some important plant species. And environmental damages repair takes time, efforts and investments. Therefore, EPCA is closely collaborating with various Albanian institutions working in the same field such as Research Institute of Forests and Pastures, Directorate General of Forests and Pastures, Ministry structure, etc,.

EPCA has organized special events bringing together representatives of these
institutions and its membership where MAP’s preservation is the most frequently discussed topic.

EPCA members have been assisted over the years from various USAID funded projects through training seminars (harvesting timing and techniques, quality control and assurance. marketing and value adding), new market connections and promotion of plants’ properties and their use in the country.

In the light of sustainable use of MAP’s in Albania, EPCA considers crucial continuous education and training of its members in order to ensure proper handling of these plants and also know-how transfer to the recently engaged power into harvesting and processing. Harvesting timing and techniques are key issues that the whole supply chain (collectors up to final exporters) needs on-going education and guidance. Through these educational programs major processors and exporters, who are also being exposed to the requirements and specifications of the international buyers, can apply more pressure to the collection levels towards proper MAP’s harvesting. In order to increase awareness of its members, EPCA is presently using media channels to address issues of concern, and is planning to produce promotion materials that would reach and help train the targeted audience (rural community) prior to and during the harvesting and production season.

Bottom line - EPCA sees value adding as process that requires money, time, skills and initiatives. In addition EPCA considers value adding every single effort made by its members and nonmembers and the cluster related players (research institutions, ministry structures, etc) to improve the business environment, quality of the harvested and processed products, upgrade the production technologies, which will definitively contribute to an increase of the country’s overall reputation, wellness and profile of final products.

Major Albanian production and export companies, EPCA members, are working on creating a “brand” name for their products in order to soon export “Albanian Products” under their brand name, as parallel with the biological cleanliness of these products it has to be ensured the cleanliness throughout their handling, processing and marketing.

EPCA is also exploring potential fractionation of the essential oils to further processed products such as oleoresins and is working on the identification of market needs and opportunities for these products.

THE PRODUCTION OF DRUG ACTIVE CONSTITUENTS THROUGH BIOTECHNOLOGICAL METHODS

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Summary

Increasing demand for plant based drugs and active constituents cause for decrease or even extinction of the source. This situation directed us to find biotechnological methods to obtain or produce natural drug active constituents. Our main goal is to produce valuable natural drug constituents via plant tissue culture methods. We have several projects. One of our projects focused on producing cucurbitacin B from Ecballium elaterium, the other one is the production of shikonin derivatives from Arnebia densiflora. Cucurbitacin B was amplified in suspension culture via elicitors. We also have A. densiflora callus cultures which produce red callus that is the sign of shikonin and its derivatives.

Keywords: *Cucurbitacin B, shikonin derivatives, Ecballium elaterium, Arnebia densiflora, drug active constituents*

Introduction

Plant originated folk medicines have been widely used to cure diseases and disease symptoms for ages. Rising desire for plant based drugs and drug active constituents one of the reason for the reduction of medicinal plants in their source. Particularly endemic plants, which can not be cultivated beyond their habitat because of their seed features, plant selectivity, and excessive collection, might become endangered. Natural active compounds are generally low in their sources. This situation cause excessive collection or even destruction of the plant source to have enough amounts of effective compounds. Accordingly, these circumstances led us to find different ways by using biotechnological techniques to protect the source and to obtain as well as amplify the compounds without destroying the natural source. Production of desired compounds via plant tissue culture is one of the biotechnological methods. Our main goal is to produce or increase pharmacologically effective drug active-constituents by using biotechnological methods. We have several projects, only two of them were elucidated in this paper.

Ecballium elaterium is known as squirting cucumber has variety of biological activity *in vitro* (Miro *et al*. 1995) such as analgesic, antipyretic (Agil *et al*. 1995), anti-hepatotoxic (Agil *et al.* 1999), anticancer, anti-inflammatory (Jayaprakasam *et al*. 2003). The plant extract and the fruit juice have been widely used to treat various diseases, such as hemorrhoids and specifically sinusitis, in the Mediterranean region (Baytop 1984). In clinical studies, application of crude-diluted-fruit juice on volunteers having sinusitis showed good recovery (Cingi *et al* 1983). Despite of the success in treatment of sinusitis, some toxicities and edemas in upper respiratory tracks were reported. This brought deeper research on active compounds of the crude extracts. It has been identified that triterpenic cucurbitacin B is the active phytochemical (Yesilada *et al*. 1997). Our studies focused on establishing callus and suspension cultures by using plant tissue culture methods to determine and increase the cucurbitacins. Previously, callus and suspension cultures were established in our laboratory (Toker *et al* 2003; Memisoglu *et al* 2004.). Cucurbitacin B was determined in our suspension cultures and some elicitors were applied to increase it. Successful preliminary results were observed. We will continue trying different elicitors to obtain the best results.

Arnebia densiflora is one of the endemic plants that can produce shikonin and its derivatives in Mediterranean region, specifically in Greece and in Turkey, (Davis 1978 and 1988). Shikonin is a phytochemical compound, which is valuable in pharmacy for its pharmacological effects, in food science and textile as a colorant because of its red color. In Far East, shikonin was produced from *Lithospermum erythrorhizone* in plant tissue culture (Tabata *et al*. 1974; Mizukami *et al*. 1978; Bozan *et al*. 1996).
Root extracts of some of the Boraginaceous plants, especially root extracts of *A. densiflora,* have been used as folk medicines to cure wounds, burns, hemoroids and diverse skin diseases (Bozan *et al*. 1996; Yesilada *et al.*1996). Oily root extracts have been used as ingredients of skin tonics for their protective roles against harmful sunlight. Extracts contain naphtoquinones as colored compounds, which were used in textile, cosmetology and food industry as a colorant (Futagoishi and Abe 1973). Shikonin and some shikonin derivatives have anti-tumor (Katti *et al.* 1979), anti-inflammatory (Chen *et al*. 2001; Singh *et al*. 2003), anti-microbial, anti-oxidant (Assimopolou *et al*. 2004), anti-HCV (Chou *et al*. 2003; Ho *et al*. 2003) (against hepatit-C), and anti-HIV-1 activities, which were shown scientifically (Chen *et al.* 2003). Specifically anti-HIV-1 activity brought the idea of its usage as a drug active substance to develop new drugs to cure this disease. Although some shikonin derivatives can be synthesized chemically, that is not feasible yet (Papageorgiou *et al 1999*). In Far East, particularly in Japan and in China, *Lithospermum erythrorhizon*, later on *Arnebia euchroma* have been used to produce shikonin and its derivatives via tissue culture methods (Tabata *et al*. 1974; Mizukami *et al*.1978; Hechun *et al* 1991; Shiyun *and* Songsheng 1994). According to a study to determine the alkannin and shikonin contents of the eighteen Boraginaceous plants, the highest naphtaquinone ratio was detected in the root barks of *A. densiflora (Yesilada et al.*1996*)* This result led us to focus on *A. densiflora*.

Material and methods

Ecballium elaterium (L.) A. Rich. plant samples were collected from Ankara, Turkey in May through October. A voucher specimen was placed in the Herbarium of Pharmacy Faculty at the Gazi University. In each cultivation period fresh explants (stem, root, leaf) were utilized, wich were available in wild from April to October. Stem and stem nodes were surface sterilized as described in Toker 2003. Cucurbitacins were analyzed in dried callus material.
Dried callus material (100–220 mg per sample) was extracted with chloroform at room temperature, and then evaporated to dryness in vacuo. Dried extract was dissolved in methanol and filtered through membrane filters (0.45 µm, Althec). Quantitative determination of cucurbitacin B was performed by HPLC, comparing with authentic sample.
Arnebia densiflora (Nordm.) Ledeb. plant samples were collected from Eskisehir-Sivrihisar Turkey in May when they were flowering. All the parts of the plant were used as an explant to produce callus. Media were prepared from stock solutions. All the hormones utilized in plant cell culture were purchased from Sigma unless othewise were expressed from different brand. All the solvents utilized were HPLC grade and they were either supplied by Sigma or Merck. Methyl jasmonate 95% was purchased from Aldrich, yeast extract was purchased from Fluka BioChemika.

Results and discussion

Ecballium elaterium

Callus Production: First of all, callus was obtained from stem nodes and leaf explants and fruit of *E. elaterium* using media supplemented with BA and NAA in different ratios (Toker *et al.* 2003). Root and fruit explants was not very succesful to produce callus. Highest calli

production level was obtained from stem nodes. Therefore stem nodes were choosen as an explant for further experiments. Plant parts were analyzed by HPLC for their cucurbitacin content. Stem node contained 0.01% cucurbitacin B, whereas our calli culture from stem nodes produced 1.26% cucurbitacin in supplemented with 1 mg/l BA and 0.1 mg/l NAA. We aimed to produce more cucurbitacin by applying elicitors. Therefore, callus were transfered to the liquid medium to have suspension culture which has advantages to apply elicitors to produce secondary metabolites (Gundlach *et al.* 1992, Verpoorte *et al*. 2002).

Suspension Culture, Production and Application of Elicitors: Suspension cultures were analyzed for their cucurbitacin B contents. Cucurbitacin B was not observed in the cells any of the suspension cultures. Therefore we decided to apply elicitors to stimulate or amplify cucurbitacin B formation in the suspension cultures. Yeast extract, methyl jasmonate and potassium dihydrogen phosphate were added to suspension cultures as elicitors. Methyl jasmonate affected the levels of cucurbitacin in cell suspension cultures. When it is compared to the plant stem nodes, addition of methyl jasmonate brought about 43-54 fold increase in cucurbitacin B level in the suspension culture Although methyl jasmonate was found to be an effective elicitor in this study, the highest cucurbitacin B yields was obtained when dihydrogen phosphate (340 mg/l) was added to the suspension culture as an elicitor on day 12. The amplification of the cucurbitacin B level was 230 fold greater than the plant stem nodes (Fig. 1).

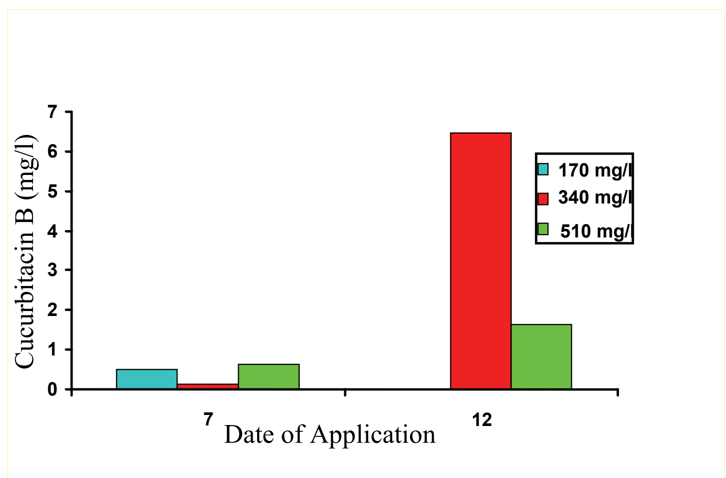

Fig. 1. Effects of potassium dihyrogen phosphate on the cucurbitacin B level of *E. elaterium* cell suspension culture

Arnebia densiflora

Calli Production: Various parts of the plant, root, leaf and flower axis were used as explants to produce calli (Kurnaz 2002). Sterilization of the explants was the major problem during our studies. Different concentrations of sodium hipochloride were applied to sterilize the explants but there was always a contamination problem. Different concentrations of plant hormones, IAA, Kinetin, 2,4D and NAA were used in different combinations in MS, LS and SH media. Our preliminary results showed that LS and MS were the most succesful media to produce calli from all the explants. As for explants, flower axis responded best in MS media supplemented with Kinetin $0.3x10^{-6}$M, NAA 10^{-5}M. According to our unpublished preliminary results, flower axis was the best choice as an explant to produce pink-red callus (Fig. 2). Since shikonin is a red pigment, pink-red colored callus may be the sign of the formation of shikonin and its derivatives.

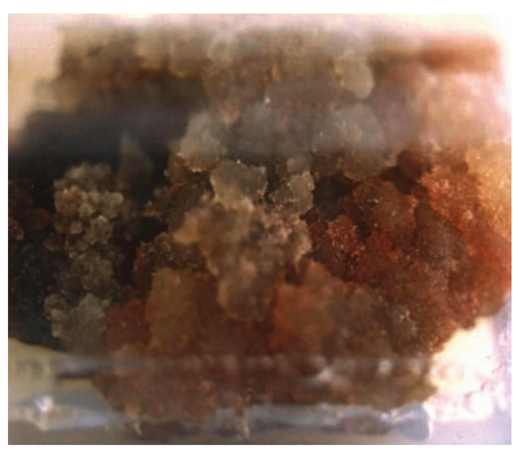

Fig. 2. Pink-red callus was produced from flower axis in MS media suplemented with Kinetin $0.3x10^{-6}$M, NAA 10^{-5}M.

Conclusions

In conclusion, stem explants of *E. elaterium* formed better callus than the other organs of the plant and the highest cucurbitacin B content was detected in first subculture in a medium containing 1 mg/l BA and 0.1 mg/l NAA. The cucurbitacin B ratio of the stem increased from 0.01% in the original plant to 1.126% in callus. Dihydrogen phosphate was the best elicitor to stimulate the cucurbitacin production in the *E. elaterium* suspension culture. Our prelminary resulst showed that cucurbitacin level increased upto 230 fold with the effect of potassium dihydrogen phosphate. Different elicitors will be tried to get the highest cucurbitacin B level in the suspension cultures in further. We will also anaylze cucurbitacins other than cucurbitacin B, which they have pharmacological effects.
As for *A. densiflora*, we had serious sterilization problems. Despite using more than 30% of sodium hypochloride, most of the explants were lost due to contamination. Although there was a persistent contamination problem owing to features of the plant, flower axis responded best to the studies for callus production. Even if the diffrent media were applied, MS medium supplemented with Kinetin $0.3x10^{-6}$M and NAA 10^{-5}M was the best choice to produce colored callus culture from the flower axis of *A. densiflora*. The calli are being analyzed for their phytochemical composition by using series of phytochemical methods, in particular for their shikonin and shikonin derivatives. These are only preliminary results, our ongoing study on the production of pink-red callus stably and produce shikonin and its derivatives in large scale.

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SLOVAK VARIETY OF MAJORAN „*MARCELKA*", ITS CULTIVATIONS AND ESENTIAL OIL CHARACTERISTICS

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Summary

Sweet marjoram plants of the Slovak variety „MARCELKA" were started to cultivate on the East-Slovakian Lowland. The content of essential oil into the dry herbs is 1.2 %. A wide range of secondary metabolites (terpinen-4ol: 6%, α-terpinene: 28%, γ-terpinene: 16%, sabinene: 2%, limonene: 6%, cineole: 7% a linalool: 2%) presents its composition. They are giving to plants very nice and strong fragrance.

Keywords: *Sweet Marjoram, essential oil and its composition*

Introduction

Sweet marjoram is cultivated for its strong aromatic drug (*Majorane herba*). It is mixture of dry leaves and stems with characteristic aroma. It comes from North-western Africa or Eastern Mediterranean and was cultivated by ancient Egyptians, Greeks and Romans.
Specific crops represent only a small share in overall agricultural output in Slovakia, but their lack causes difficulties. They never are the priority of agriculture, but only a supplement to vegetable production. (Kučerová, 1999).
The cultivation of medicinal, aromatic and spicy plants on arable land is from point of view of history the latest branch of agricultural products. Although there is elaborated technology for cultivating of 30 species, the fact is that only 15 of them are cultivated on a large scale. The places of production are located in mountain and marginal areas mostly, and only a small part of production places are in the maize and beet areas. So, we might say that these specific plants are grown in economically more demanding conditions. (Haban, 1996).
The aim of our research is the study of qualitative and quantitative composition of essential oil as well as the aspects of the Sweet marjoram cultivation (*Majorana hortensis* L.), its variety „*MARCELKA*" in selected area of East Slovakia lowland.

Materials and methods

Cultivation and seed production technology

There is only one variety which is allowed for cultivating – „*MARCELKA*". The locality of Streda nad Bodrogom (the Eastern Slovakia) was selected for its breeding. In elaborated technological process for seed production, a light-textured soil was chosen, rich in humus, with neutral pH. The soil of the mentioned area complies with the norms. It is light-textured, rich in humus and not crustaceous. pH is between 6.7 – 7.3. Content of tolerable nutriments in 1 kg of soil: P=50 $mg.kg^{-1}$, K=51 $mg.kg^{-1}$, Mg=674 $mg.kg^{-1}$. The content of humus is 3.27 %.
For growing from seedlings, the seed was seeded into cold frames. The germination of the seed is 85%. When three leaves appeared, the seedlings were planted. This started at the beginning of May. Distance between lines are 0.25 x 0.35m = 120 000 plants on 1 ha.
The plants were cultivated four times in the early state. Phosphorus, which is important for development of the seed, was used for fertilization. The soil was fertilized with 80kg´s of nitrogen, in two doses. The first dose was added before seeding, the second in the time of last hoeing. The growth was irrigated at need.

Seed harvesting was realized at the time a plant started to dry-up, and the lower third of a plant got brown. Above ground parts of a plant were picked. The general principles for aromatic-oil drugs were applied. Some 0.25 – 0.30 m of over-ground part of a plant are cut. Raw material is dried-up in thin layers in shade and draught. The harvest is 200-300 kg's of seed per hectare at the purity 98 %. The price of one kg of seed is 18 euros per 1 kg.

Specification of equipment, instruments and other tools needed for problem solution
The aromatic-oil from obtained samples of marjoram dry tops was isolated by means of hydro distillation. Apparatus by COOKING and MIDDLETONE modification from years 1932-1934 was used (by PALISH modification in 70´s). The distill apparatus belongs to the most frequently used equipment for isolation of essential oils in the European laboratories.
Consequently, the essential oil was dehydrated with anhydrous potassium Sulphate, and separated from n-hexane through the use of differential distillation. The mixture is heated in distillation bulb (weighed on analytic scales) to the boiling point of dissolvent and evaporates consequently. The vapor is led into the condenser. The condensate is continuously led-away. Since the boiling point of n-hexane is 69 ^{o}C, (it means temperature lower than boiling points of aromatic-oil components), the dissolvent evaporates at firstly. Thus the essential oil remains in the distillation bulb, and is dehydrated in desiccator's cabinet.
Main components of the oil were determined by means of gas chromatography, through the use of equipment by Carlo Erba Instruments, type GC 6000 Vega Series 2, with FID detector and capillary tube of length of 30 m, internal diameter 0, 53 mm, and with state steady thickness of 0, 1 mm. Experimental conditions of determination: the inner temperature of injector- 150° C, detector space temperature – 200° C, feed volume 0, 6 ml, and nitrogen as a carrier gas. The temperature course: 130°C – 0 min., 5 ^{o}C.min up to 200 ^{o}C, 10 min. 200°C, 10°C.min up to 220°C, 15 min. 220°C. Identification of marjoram essential oil main components was realized by the help of retentive times of authentic reference standards.

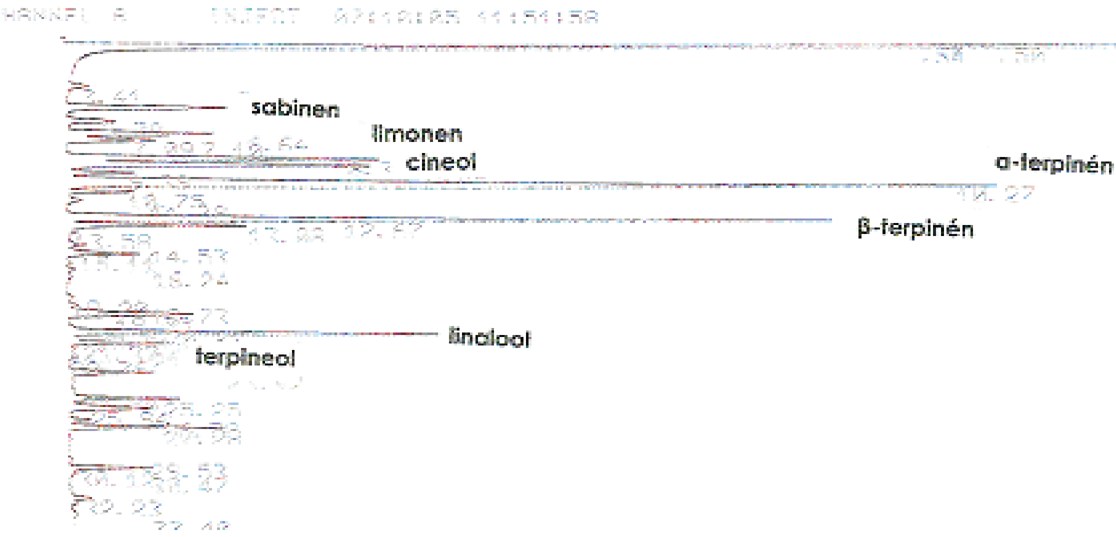
Results and discussion

The marjoram essential oil was analyzed through the use of gas chromatography (Picture 1.) this physical-chemical method showed very interesting results of composition of oil, not described in our literature up to now. For the first time, the wider secondary metabolites spectrum was described: the amount of terpineol – 6%, α-terpinene – 28%, β-terpinene – 16%, sabinene – 2%, limonene – 6%, cineole – 7%, linalool – 2%, (Picture 2.)
The oil was isolated from dry stem, with volume of 1.2 %. Similar rates was reached by cultivators in Poland (1.2 – 1.6 %), France (1.2 %), Lower rates was reached in Hungary (0.5 – 1.3 %), the higher rates in Russia (2.1 %). (Habán, 1996)
One of the most important properties of α-terpinene and β-terpinene is their antioxidant effect (Chen et al., 2004). They protects human organism from reactive forms of oxygen. Free oxygen radicals react with molecules of proteins, fats and nucleic acids, what leads to changes in their structure. This might cause a formation of many diseases among others the rheumatic disease, disease of nervous system (Alzheimer disease, Parkinson d., schizophrenia, Down's syndrome), creation of neoplasms and acceleration of ageing process.
The plant *Meleuca altermifiolia*, cultivated in Australia, examined for its antibacterial and antipyretic effects. WONG and his team (2001) determined that the α-terpinene has the mentioned antioxidant effect, and its volume in aromatic-oil is 8, 82%. The main component of marjoram aromatic-oil is α-terpinene with volume 28%.
Sweet marjoram of various origins was experimentally cultivated in Quedlinburg (Germany). Qualitative-quantitative analysis of aromatic-oils through the use of GC showed various chemo types characterized by following volumes of components: carvacrol (33 – 88 %), 1, 8-

cineole (58 – 64 %), thymol (60 – 64 %), γ- terpinene (17 - 48%), terpinene-4-ol (10 – 24 %), and *cis*-sabinene (17 – 51 %) (Baranska et al., 2005). Slovak variety of sweet marjoram – "*MARCELKA*" has different aromatic-oil composition; the main component is α-terpinene. This is the condition for identification of new marjoram chemo type.

Conclusion

The variety, which is cultivated in East Slovakia lowland locality (Streda nad Bodrogom), gives stabile values in comparison with other foreign varieties. The sum of terpenes is about 50%, what gives the plant a sharp and pleasant aroma. High content of α-terpinene (28%), is a precondition for identification of new marjoram chemo type and considerable properties of used raw material and essential oil content.



Picture 1. The GC-chromatograph of the marjoram essential oil composition.

Picture 2. Marjoram plants before flowering stage.

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THE *IN VITRO* MORPHOGENETIC REACTION OF *ECHINACEA PURPUREA* MOENCH.

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Summary

Plantlets and cotyledons from germinated seeds were cultivated in vitro on media of initiation (MS, B_{02}, B_2) providing neoplantlets which represented the source of sterile explants – mainly shoot tips and nodes, but also leaf, internode, petiole and callus fragments. The best reaction obtained from shoot tips and nodes was on BN medium – multiple shooting with the most vigorous shoots and roots. We also tested the organogenetic capacity of the friable foamy callus. This species registered a low growth speed and development compared to other species that we have tested.

Keywords: *Echinacea purpurea, explants, callus, neoplantlets*

Introduction

Echinacea purpurea is a herbaceous plant originating from the South regions of the United States of America. It is widely cultivated in Europe, Australia, New Zealand and North America. *Echinacea* pharmaceutical products are used to prevent and treat especially respiratory, urogenital and skin infections [5]. Its positive effects rezide in its active principles from roots and aerial parts that stimulate immunity. These properties drew the attention of numerous researchers (doctors, pharmacists, biologists). The *in vitro* micropropagation of this species is an opportunity to provide new valuable vitroclones with a superior content of active principles.

Material and methods

The first step in the *in vitro* culture initiation at *Echinacea purpurea* (L) Moench was represented by providing plantlets from germinated seeds in December 2004. The seeds were brought from the Agro-Zootechnical Research Centre Secuieni (county of Neamt), crop of 2003. Plantlets and cotyledons were inoculated on media of initiation MS, B_{02} (0.2 mg/l BAP) and B_2 (2 mg/l BAP), generating sterile neoplantlets that were the explant source to test the morphogenetic reaction on varied hormonic formuli.
We used Erlenmeyer vials (100 ml, type B) for the *in vitro* cultures. The carbon source was represented by saccharose (25 g per litre of culture medium) and the agar was used to solidify the media.
Culture incubation was accomplished in a half-climatised room (temperature 23º to 25º C, light intensity of about 2500 lux, permanent illumination – white artificial light).
Our experiments were carried out in the Genetic Laboratory of the University of Bacau. Our results are presented in table 1 and figures 1-8.

Results and discussions

We mention that this species registered a low growth speed compared to other species. The explants we mostly tested were shoot tips and nodes from the shoots regenerated on media of initiation. Explants were sterilised with mercury chloride for 5 minutes, subsequently emerged in chloramine-T (for 12 minutes), then rinsed twice with sterile distilled water. We

encountered no difficulties regarding explant sterilisation as the number of infections was very small.
We used the basal hormone-free Murashige-Skoog (1962) medium to initiate the *in vitro* cultures but also two variants of MS supplemented with small amounts of BAP: B_{02} (0.2 mg/l BAP) and B_{05} (0.5 mg/l BAP). These culture media stimulated the growth and development of shoots that were the explant source to test the morphogenetic reaction of *Echinacea purpurea* on varied hormonic formuli of nourishing media.
Shoot tips and nodes were the most utilized explants we used, but we also observed the leaf, internode and callus explants' reaction on media comprising varied combinations and concentrations of phytohormones added to the basal MS medium.
The reaction on MS medium (control medium) was a less representative one, the generated shoots did not present any secondary branches, they were frail, with thick short roots (no secondary roots). We also tested the explants' reaction on media supplemented either with cytokinins or with auxins or with combinations of the two phytohormones. On culture media comprising only BAP in small amounts (0.2 mg/l) we noticed a good caulogenetic reaction, still lower than on media containing BAP and kinetine. Augmenting BAP amount from the nourishing media (up to 2.0 mg/l) the intensity of caulogenesis is enhanced. B medium (with BAP) induces multiple shooting (3-4 basal branches/shoot). It seldom generates friable green granulated callus at shoot base. Root growth was inhibited on this medium of culture. We noticed (not only on this nutritive formula) that basal leaves are thick and augmented, turning into compact mossy structures. Media comprising IAA favoured the growth of strong roots within the medium of culture (fig. 4).
Associating BAP with an auxin (IAA) root genesis was stimulated, though the roots were frail and short. These roots were absent on the previous medium formula. Replacing IAA with 2.4-D combined with BAP we observed compact, cream-yellowish callus at the base of explants which did not provide shoots or roots (fig.7). A good callus genesis was registered on BN_1 medium (with BAP – 1 mg/l and NAA 0.5 mg/l) which favoured the formation of granulated green-yellowish callus, presenting a low proliferation capacity. Only the nodal explants provided few poorly developed shoots and seldom roots.
On BN medium comprising a much smaller quantity of NAA (0.01 mg/l) the reaction was more different than on the previous one, providing the best caulogenetic reaction (fig.2). We observed the multiple shooting phenomenon (strong green shoots with large leaves). The roots were thick, short, white and branchless. We seldom noticed that the offshoots presented callus at their basis. We may assess that this culture medium registered the best results regarding shoot formation compared to the other variants used to test this species. A favourable morphogenetic reaction was registered on BK medium formula, a combination of BAP (1 mg/litre) and kinetine (0.5 mg/litre), which induced multiple shooting (fig.1). The offshoots are vigorous, with a compact hard green callus provided at their basal part. Few red hue roots without secondary branches were provided. Shoot formation on BK is less represented than on B_2 and more intense than on B_{02} (B_2>BK>B_{02}).
The association of BAP and giberellic acid was not a successful one, the general reaction being poor with few frail neoplantlets, with a tendency to degenerate; root genesis was completely inhibited. A similar reaction was evinced on KN medium with kinetine (1 mg/l) and NAA (0.5 and 1 mg/l)(fig.3). Leaf fragments inoculated on a medium supplemented with moderate amounts of IAA provide long, thick, green-whitish roots. That was the best rhysogenetic reaction of foliar explants. They sometimes provide granulated dark green callus. Shoots induction was a difficult process and they were frail. Culture media with another auxin (2.4-D) favoured callus formation in case of using leaf explants, on their entire surface and sporadically they provided roots (fig.6).

Varied amounts of BAP added into the nourishing medium did not induce the differentiation of leaf explants, that grew and thickened into a slightly callussed structure. The best caulogenetic reaction of leaves was registered on BA medium (with BAP and IAA), the neoplantlets were generated by indirect shooting providing a granulated green callus. Shoot differentiation was a difficult process on BN_1 medium too. The slightly callussed and enlarged leaves obtained on BK medium were transfered on B_2 where they induced shoot formation, the higher amounts of cytokinine (2 mg/l BAP) stimulated stem genesis.
The leaf explants were also tested on KN formula, when a friable foamy white-greenish callus with shoot buds was obtained. Passed on B_2 medium this callus provided shoots. Testing the reaction of petiole explants on KN medium we noticed the presence of well developed short roots without callus formation.
Internode explants inoculated on BN medium provided a foamy green callus that proliferated but it didn't differentiate. We also tested the reaction of callus obtained on KN and passed on a medium formula with cytokinin and giberellin (BG) but we did not succeed to induce differentiation, eventually this callus degenerated.
The best caulogenetic reaction is by far the one from BN medium (1.0 mg/l BAP and 0.01 mg/l NAA, followed by the reaction on BK, B_2 and BA. The best nourishing formula to induce roots was the one with auxin IAA (in a higher amount: 2 mg/litre of medium).
We stress the fact that this species is quite refractory to *in vitro* cultivation, growth processes were slow. It was a challenge to obtain vigorous offshoots.
Neoplantlets accommodation to septic conditions was accomplished in a hydroponic system (fig.8), but most of them did not survive the shock of the *ex vitro* medium.
The data displayed wihin this paper are preliminary studies that we aim to continue with biochemical and cytogenetical tests on this valuable species.

Conclusions

1. Testing the response of shoot tips and nodes we observed that the best caulogenetic reaction and multiple shooting was provided on nourishing formuli containing BAP (1 mg/l) and NAA (0.01 mg/l), while the most vigorous roots grew on media with higher amounts of IAA (2 mg/l).
2. The combination between BAP (1 mg/l) and NAA (0.5 mg/l) was the most efficient to induce callus (low proliferative).
3. The best rhysogenesis of foliar explants was enhanced by auxin IAA (2 mg/l), the most vigorous stems from leaf explants were induced by BAP combined with IAA, while the formula KN provided foamy regenerative callus.
4. Internode fragments used as explants only generated callus that proliferated but did not differentiate.
5. This species is quite refractory to *in vitro* cultivation, the accommodation to septic environment being also a difficult process.

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Table1. The morphogenetic reaction of some *Echinacea purpurea* types of explants within *in vitro* cultures

Var	Explant	Hormonic formula	Growth regulators (mg/litre of medium)						The morphogenetic reaction and proliferation speed
			BAP	2.4-D	GA	IAA	Kin	NAA	
1	Shoot tips and nodes	MS	-	-	-	-	-	-	Caulogenesis, poor reaction (+); shoots' growth is slow, only in height; root formation (+), thick, short roots (1-2 cm), no secondary roots
2	—"—	A	-	-	-	0.5-2.0	-	-	The offshoots transfered to A_{05} had strong thick roots (++) in the absence of callus ; A_2 medium induced the formation of long (3-4 cm), thick roots (++) ; sporadically short secondary roots (featherlike arrangement)
3	—"—	B	0.2-2.0	-	-	-	-	-	Caulogenesis (++), strong shoots, sometimes granulated friable green callus (+) at their basis; roots are absent; basal leaves belonging to explants grow, thicken, turn hard and mossy; shoots are fewer than on BK medium formula, but their are more vigorous
4	—"—	BA	1.0	-	-	0.5	-	-	Caulogenesis (++); well developped leaves; frail short roots appear sporadically (+)
5	—"—	BK	1.0	-	-	-	0.5	-	Multiple shooting (++), vigorous shoots. Compact hard green callus is present at shoot base. Sometimes reddish roots without branches (+)
6	—"—	BD_1	1.0	0.5	-	-	-	-	Callus (+) compact, cream-yellowish in the basal area of explants; the reaction of explants is very poor.
7	—"—	BG_1	1.0	-	0.5	-	-	-	Caulogenesis (+); roots are absent; shoots display a tendency to degenerate on this medium formula
8	—"—	BN_1	1.0	-	-	-	-	0.5	Low proliferative, granulated green-yellowish (++); nodal explants provide frail shoots (+); indirect root formation sporadically (+)
9	—"—	BN	1.0	-	-	-	-	0.01	Multiple shooting (+++), vigorous dark-green offshoots, with large leaves; thick white roots deprived of secondary branches (++), of 1-2 cm in lenght; shoots are seldom slightly callussed at base (friable green callus (+))
10	—"—	KN_1	-	-	-	-	1.0	0.5-1.0	Caulogenesis (+) with feeble offshoots, very low growth speed; roots are absent
11	Leaves	A	-	-	-	0.5	-	-	Root genesis (++) with thick green-whitish roots of 1-2 cm in lenght; leaf explants sporadically provided granulated dark-green callus; poorly developed shoots (+)
12	—"—	B	0.2-2.0	-	-	-	-	-	Leaves grow, thicken, have a slightly callussed aspect, but do not differentiate
13	—"—	D	-	2.0	-	-	-	-	Leaf explants form callus on their entire surface; friable granulated green-yellowish callus (++); few roots not well developed, without branches; adventitious roots (+)
14	—"—	BA	1.0	-	-	0.5	-	-	Neoplantlets (++) obtained by indirect caulogenesis; friable granulated green callus that provides multiple shooting (++)
15	—"—	BN	1.0	-	-	-	-	0.01	Leaves grow but hardly diffrentiate shoots (+) by indirect caulogenesis
16	—"—	BK	1.0	-	-	-	0.5	-	The leaves are slightly augmented, starting to form small isles of friable green callus; this callus provides frail shoots on B_2 (+)
17	—"—	KN	1.0	-	-	-	-	1.0	Foamy white-greenish callus (++), with shoot buds, regenerative on B_2; petiole explants provide strong short whitish roots by indirect root genesis (++)
15	Internodes	BN	1.0	0.5	-	-	-	0.01	Foamy green callus (+) with embryoids; it proliferates but does not differentiate
16	Callus	BG	1.0	-	0.5	-	-	-	Foamy light-green callus (++) obtained on KN; subsequently transfered on BG it degenerated

A=IAA; B=BAP; BA=BAP+IAA; BD=BAP+2.4-D; BN=BAP+NAA; D=2.4-D; BK=BAP+kinetine; BG= BAP+giberellic acid; IB=IBA; KN=Kin+NAA; N=NAA
(+) poor reaction; (++) good reaction; (+++) very good reaction

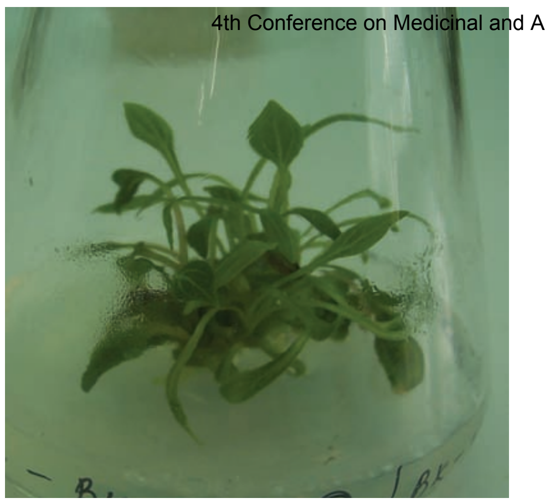
Fig.1 Vigorous dark-green shoots on BK medium formula

Fig. 2 Multiple shooting on BN medium formula

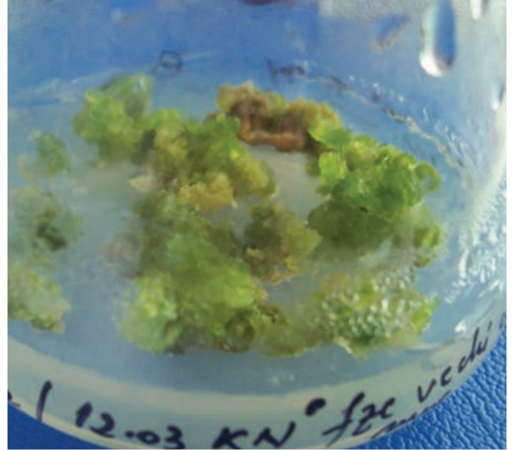
Fig.3 White greenish callus with shoot buds (KN)

Fig 4 – Vigorous roots provided on A medium formula

Fig.5 Shoot tips on MS medium

Fig. 6 Friable green callus provided by leaf explants on D medium

Fig. 7 Slightly callused nodal explants on BD medium

Fig. 8 Accomodation to septic environment (hidroponic system)

CONTENT AND QUALITY OF ESSENTIAL OIL IN DIFFERENT MINT CULTIVARS

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Summary

Mint is one of the most important medicinal plant species. Its roll and significance in pharmaceutical, cosmetic and food industry is of great importance. To determine possibility of increasing yield of essential oil per area of cultivated plants, three different mint cultivars were tested, Mitcham, and two Moldavian cultivars: N-310 and U-2M. The data, along with those dealing with chemical composition of isolated oils, showed that cultivar N-310 deserves special treatment in the cultivar selection process.

Keywords: *mint, essential oil, chemical composition, yield*

Introduction

Mint is one of the main medicinal plants. Mint, as a medicinal plant species, has not been accidentally classified among the oldest plant species in the cultivation system. Its essential oil was used for medical purposes by the ancient Egyptians in 410 BC. In England, this plant species was used as early as 1696 (Kišgeci *et al.*, 1998).

Today, mint has one of the leading positions, both by quantity and trade, and by its multipurpose application. Thanks to its medicinally recognised properties, it is very important in the pharmaceutical, cosmetic, food, and other industries. For this reason, it is very attractive on the market.

The greatest producers of this cosmopolitan plant species are USA, Russia and Brazil. In Europe, the highest production level is in England. In Serbia, it is cultivated on more than 1,000 hectares, with a trend of increased production.

Modern pharmacy uses peppermint leaf (*Mentha piperitae folium*), aboveground parts (*Mentha piperitae herba*) and the essential oil (*Aetheroleum menthae piperitae*) for the preparation of various medicinal formulations and teas.

In the aim of finding the most favourable genotypes for peppermint cultivation, multiannual all-inclusive research has been carried out, aiming at the increased yield, and especially the higher percentage of essential oil and its quality.

Material and methods

To achieve the aim of attaining the higher yield per unit area and of increasing the essential oil content compared to the oil content in mint cultivar YU-Mitcham, we tried the introduction of several peppermint cultivars from the neighbouring countries, mainly from the Republic Moldavia. During 2001, 2002 and 2003, experiments were carried out with the cultivars N-310, U-2M and the standard, in our country the most widespread cultivar, YU-Mitcham. The cultivar YU-Mitcham, the so-called English mint, was the most cultivated mint cultivar in the former Yugoslavia and in Serbia. It originates from England, but in our conditions, thanks to its multiannual cultivation, it has adopted some authentic characteristics, by which it probably differs from the variety of this cultivar cultivated in other countries (Maksimović *et al.,* 1998).

During the three-year field experiments at Obrenovac in 2001, Bajina Bašta in 2002 and Pančevo in 2003, the growth and development of the above cultivars were monitored, and the

ratio of fresh and dried matter, leaf output, yield, content of essential oil and its quality were determined.
Field experiments were performed by the usual methodological procedures. The basic soil tillage and preparation were carried out as usual. The applied cropping practices and protection were identical as the measures commonly carried out in plantation production. The yield and the essential oil determination were performed based on the first harvest.
Content of essential oil in the mint samples was determined in a Clevenger type apparatus, by hydrodistillation, whilst composition of isolated oils was determined by GC/FID and GC/MS. GC/FID analysis of the oils was carried out on a GC HP-5890 Series II apparatus, equipped with split-splitless inlet and automatic liquid sampler (ALS), attached to HP-5 column (25 m x 0.32 mm, 0.52 µm film thickness) and fitted to FID. Carrier gas flow rate (H_2) was 1 ml/min, split ratio 1:30, injector temperature was 250°C, detector temperature 300°C, while column temperature was linearly programmed from 40-240°C (at rate of 4°/min). The same analytical conditions were employed for GC/MS analysis, along with column HP-5MS (30 m x 0.25 mm, 0.25 µm film thickness), using HP G 1800C Series II GCD system. Instead of hydrogen, helium was used as carrier gas. Transfer line was heated at 260°C. Mass spectra were acquired in EI mode (70 eV), in m/z range 40-400. For quantification purposes, area percent reports obtained by FID were used as the base.

Results and discussion

The analysis of the yield results, presented in Table 1, shows clearly the relatively low variability of the average yield of the cultivars studied. The interval of variation of the average values ranges from 3,335 kg/ha for the cultivar YU-Mitcham, to 3,675 kg/ha for the cultivar U-2M. It is evident that all the three analysed cultivars attained the high yields of the dried mint leaves.
It is interesting that the domestic peppermint cultivar approached the Moldavian selections significantly, and the reason of such yield volume is probably the extremely high fertility of the cultivar YU-Mitcham in 2001 at Obrenovac, where the yield of 4,024 kg/ha of dried leaves is the usual value for this peppermint cultivar. According to the data by Maksimović (1991) on the experiments with mint cultivar YU-Mitcham in Pančevo in 1989, the yield of this cultivar varied within the interval 758-1379 kg/ha of dried leaves from the first harvest. Similar data were obtained also in the research by Stepanović *et al.* (1995), in the experiments at Pirot and Pančevo. Perhaps the elevated yields, compared to the common yields, were the consequence of the exceptional cultivar practices and intensive fertilisation, as well as irrigation, which was performed at two occasions in 2001 at Obrenovac.
The ratios of fresh and dry substance are rather non-uniform, both by years and between the analysed cultivars. It is especially interesting that a very low interval of variation occurred in the ratios of stem and leaf, both by years and between the cultivars, which can be explained also by the adequate soil management and crop cultivation.
The content of essential oil on the average varied within the limits from 2.05% in the cultivar YU-Mitcham, to 3.01% in the cultivar N-310. The lowest oil content of averagely 2.05% was determined in mint cultivar YU-Mitcham, and a significantly higher content (average 3.01%) was determined in mint cultivar N-310 (Table 1).
Recorded data are in harmony with the data obtained by Maksimović *et al.* (1998), Stepanović *et al.* (1995) and Tucakov (1997). It is important to emphasise that the cultivars, which attained the elevated yield (U-2M and N-310), had simultaneously also the higher percentage of essential oil, so these cultivars also achieved a significantly higher yield of oil per unit area compared to the cultivar YU-Mitcham.

Regarding the essential oil quality characteristics of the studied mint cultivars, the marked variability of individual components should be emphasised (Table 2). The most manifested variability was measured in the most important component – menthol. Menthol content in the oils varied widely from 26.61% in the cultivar YU-Mitcham 2003, to 71.02% in the cultivar N-310, produced at Obrenovac in 2001.
The comparison of the results on the percentage of menthol in three different mint cultivars shows the noticeably higher menthol content in the cultivar N-310, which is by more than 100% higher compared to the cultivar YU-Mitcham, in which the average menthol content was 36.80%. The highest percentage of menthone was determined in the domestic cultivar YU-Mitcham (14.16 - 34.15), and almost twice as low quantities during all the three studied years were attained by the cultivar N-310. Menthofuran, isomenthone and neomenthol (MF+IM+NM), as the important components of peppermint essential oil, are presented collectively, and their highest presence was determined in the Moldavian mint U-2M. The content of these three components is in harmony with the data by Tasić *et al*. (1998), but in his data the menthyl acetate content in industrial distillation was higher by 7.1% in the cultivar YU-Mitcham. As for the other components, the relatively high percentage of limonene 1.82 - 8.19% in the Moldavian mint U-2M should be emphasised, which is a positive component from the aspect of quality characteristics of mint essential oil.
In our analysis of the significance of individual components of peppermint essential oil, we decided on the selection of the 16 analysed components. Although many other components have been analysed, we have not taken them in consideration due to their relatively minor percentage.

Conclusions

The analysis of the yield results shows clearly the relatively low variability of the average yield of the studied cultivars. The interval of variation of the average values ranges from 3,335 kg/ha for the cultivar YU-Mitcham, to 3,675 kg/ha for the cultivar U-2M.
The content of essential oil on the average varied within the limits from 2.05% in the cultivar YU-Mitcham, to 3.01% in the cultivar N-310. The lowest oil content of averagely 2.05% was determined in mint cultivar YU-Mitcham, and a significantly higher content (average 3.01%) was determined in mint cultivar N-310.
Regarding the essential oil quality characteristics of the studied mint cultivars, the marked variability of individual components should be emphasised. The most manifested variability was measured in the most important component (menthol).
Variability of contents of other registered constituents was present, but recorded differences were quite usual.

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Table 1. Yield and content of essential oil in studied peppermint cultivars

Cultivar	Dried to fresh mass ratio			Leaf to stem ratio			Dried leaf yield (kg/ha)				Oil content (%)			
	2001	2002	2003	2001	2002	2003	2001	2002	2003	avg	2001	2002	2003	avg
N-310	3.84 : 1	3.79 : 1	3.69 : 1	50.48 : 49.2	56.8 : 43.2	52.4 : 43.6	3585	3168	4122	3625	3.35	2.17	3.52	3.01
U-2M	3.54 : 1	3.58 : 1	3.62 : 1	48.9 : 51.1	55.15 : 44.85	51.4 : 49.6	3724	3437	3863	3675	2.97	1.98	3.64	2.86
YU-Mitcham	3.76 : 1	3.81 : 1	3.83 : 1	52.63 : 47.37	56.73 : 43.27	50.52 : 49.48	4024	3186	2796	3335	1.83	2.07	2.26	2.05
avg							3778	3264	3594	3545	2.72	2.07	3.14	2.64

Table 2. Composition of essential oils of three studied peppermint cultivars

Constituent (%)	Cultivars											
	N-310			U-2M			YU-Mitcham					
	2001	2002	2003	2001	2002	2003	2001	2002	2003	minimum	average	maximum
α-thujene	-	-	-	-	-	0.04	-	0.10	0.07	0.04	0.07	0.10
α-pinene	0.26	0.86	0.55	0.50	0.61	0.68	0.07	0.84	0.72	0.07	0.47	0.86
β-pinene	0.40	0.93	0.62	0.60	0.81	1.01	0.18	1.33	1.13	0.18	0.76	1.33
limonene	1.15	2.39	1.58	1.82	4.12	8.19	0.56	1.64	1.02	0.56	4.38	8.19
menthone	8.51	8.29	10.39	13.32	9.25	22.13	14.16	19.16	34.15	8.29	21.22	34.15
MF+IM+NM*	6.65	5.37	5.64	22.10	15.90	6.16	5.68	7.29	7.79	5.37	13.74	22.10
menthol	71.02	66.31	68.78	51.79	50.81	37.57	48.90	34.89	26.61	26.61	48.82	71.02
terpinene-4-ol	0.41	0.46	0.55	0.70	0.71	1.16	0.55	2.16	1.78	0.41	1.29	2.16
isomenthol	0.22	0.27	0.21	0.68	1.83	0.41	0.19	1.05	0.44	0.19	0.62	1.05
α-terpineol	0.31	0.33	0.34	0.32	0.51	0.41	0.13	0.87	0.72	0.13	0.50	0.87
pulegone	1.27	1.19	1.17	0.56	0.66	1.54	2.77	0.63	0.38	0.38	1.58	2.77
menthyl acetate	4.73	6.30	6.12	1.15	4.17	4.66	6.72	5.16	3.79	1.15	3.94	6.72
piperitenone	0.02	-	-	0.07	0.05	-	0.11	-	0.03	0.02	0.07	0.11
β-caryophyllene	0.45	0.46	0.28	0.88	0.71	0.38	1.65	1.88	1.74	0.28	1.08	1.88
germacrene D	0.50	0.53	0.41	0.44	0.71	1.39	0.13	2.13	2.29	0.13	1.21	2.29
bicyclogermacrene	0.61	0.60	0.41	0.77	0.66	0.64	0.49	1.01	0.92	0.41	0.71	1.01

*MF+IM+NM = menthofuran+isomenthone+neomenthol

CONSERVATION OF EASTERN EUROPEAN MEDICINAL PLANTS: *ARNICA MONTANA* IN ROMANIA

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Summary

The purpose of the project is to develop a model for the sustainable production and trade of Arnica montana, in Gârda de Sus commune, Apuseni Mountains (Romania), resulting in benefits for biodiversity and livelihoods. The principles of which can be used to inform the development of conservation approaches and methodologies for other endangered medicinal and aromatic plants and their habitats. This model can be tested on other species and will have conservation benefits for habitats, extending beyond benefits for the targeted species. Ecological management and human management are equally challenging and essential for the success of the project. Based on experiences from a previous project and preliminary scientific data on arnica distribution, growth and reproduction in the area, key components for successful project implementation have been identified: research on arnica ecology, trade chain, socio-economic context and drying methods; training and capacity building; development of a local resource management and trade association; development and construction of arnica drying facilities.

Keywords: *Arnica montana, medicinal plants, conservation, land use management*

Project Background

Southeast Europe is one of Europe's most important and richest biodiversity hot-spots. Large and relatively unspoilt natural or semi-natural ecosystems contribute to this remarkable level of biodiversity as do traditionally managed cultural landscapes.
Following a long tradition, the local rural population in most Southeast European countries has collected and used a variety of wild plant and mushroom species for medicinal, cosmetic and nutritional purposes, be it on subsistence level or for trade. Today, Southeast Europe is by far the most important European source region of medicinal plants collected from the wild. Bulgaria, Albania, Croatia and Romania in particular provide the European market with considerable amounts of raw material: On average, over 36,000 tonnes of pharmaceutical plants are exported from East and Southeast Europe every year (Lange, 2003).
As elsewhere in Europe, however, the destruction or conversion of habitats and an increasing demand for raw material, have piled on the pressure on medicinal plant resources in the Balkans. WWF has expressed concern over this development, which is likely to be further aggravated in countries preparing for accession to the European Union. The World Wide Fund for Nature (WWF), the University of Agriculture and Veterinary Medicine (USAMV) and Babes-Bolyai University (UBB) in Cluj have initiated the project "Conservation of Eastern European Medicinal Plants: *Arnica montana* in Romania" to make this problem more widely known and develop a model for the sustainable use of medicinal plants from the wild. The project is scheduled for a three years' period (April 2004 – March 2007) and funded by the Darwin Initiative, UK. The project is being carried out at field level with the community of Gârda-de-Sus in the Apuseni Mountains (Transylvania).

Vision

Sustainable use of our natural resources is one of the big challenges of our time and will become increasingly important. This challenge is inextricably linked to local livelihoods and to economic viability, without which the use of natural resources and ultimately nature

conservation cannot be managed in a sustainable way. We hope that the project 'Conservation of Southeast European Medicinal Plants: *Arnica montana* in Romania' will be a model project for the entire region and beyond. Close co-operation of all people and groups directly and indirectly linked to the project will, however, be required to make full use of its great potential.

Goals of the Project

The main goal of the project is to develop a model for the sustainable production of and trade in *Arnica montana* in Gârda-de-Sus resulting in benefits to both biodiversity and livelihoods. The project will seek to extract principles and lessons from its work, that can be applied to the conservation of Arnica at other sites, as well as other species of medicinal plants and their habitats.
To achieve these goals, the project team will work with the community of Gârda-de-Sus in developing and establishing a community-based Arnica management including all local stakeholders. As one of the key elements of the project, a Resource Management and Trade Association (RMTA) will be established at Gârda-de-Sus. The mechanisms and procedures to set up the RMTA will be developed by the local stakeholders, in particular farmers, collectors and people with key functions in the village, in co-operation with the project team from Cluj and WWF-DCP, Romania Office. Before any research is carried out, the suggested topics of research will be discussed with the local stakeholders and modified, if need be. Ultimately, the project aims at developing an effective management system for Arnica, that promotes sustainable sourcing and trade and results in higher revenues for both farmers and collectors and at establishing a profitable community-based resource management, which continues working effectively after the end of the project. The Arnica management will have to be included in the management system of the Apuseni Mountains Nature Park administration. First contacts with the park authorities have already been established.

Project Components

The development of fair and considerate ecological and social management systems is equally challenging and essential for the success of the project. Based on experiences from a previous project and preliminary scientific data on Arnica distribution, growth and reproduction in the area, key components for successful project implementation have been identified:
- Training and capacity building
- Development of a local Resource Management and Trade Association (RMTA)
- Development and construction of Arnica drying facilities
- Research on Arnica ecology, trade chain, socio-economic context and drying methods

All project components will be implemented in parallel as they are interdependent. Regular evaluation of successes, threats, challenges and lessons learnt will enhance targeted and effective project development and implementation.

Training and capacity building

Training is a process of mutual learning and teaching. The members of the project team will closely work together with farmers (land-owners) and collectors (mostly children and elderly women) and train them in basic scientific and technical knowledge of Arnica ecology, post-harvest treatment of Arnica flowers and possibilities of sustainable resource management; the local farmers and collectors will teach the project team their traditional knowledge with regard to Arnica growth, flowering patterns and meadow management. Together, project team and farmers / collectors will then be able to work out monitoring methods and a local

management plan for the sustainable use of Arnica meadows. In a second step, the people who work in the RMTA will be trained in order to train others (capacity building). In addition, the capacity of young scientists from USAMV and UBB will be built in community-based and interdisciplinary approaches to conservation and in technical and scientific skills.

Development of a local Resource Management and Trade Association (RMTA)

Successfully establishing a local RMTA will be a key milestone of the project. Plans include the development of such an association together with interested local farmers / families, who express interest in the sustainable use of Arnica on the meadows of their village. Important tasks of the RMTA will be the development and implementation of a local Arnica management plan, the setting of annual harvest quotas and developing a detailed concept for value-added products (e.g. local drying facility/ies of Arnica flowers), negotiate with traders and, if need be, between farmers and collectors. The RMTA will also have to agree on mandatory rules with regard to membership. It is important that the RMTA be developed and set up through a fully participatory process and that the administration of the regional nature park takes part in this process. Eventually, company partnerships may be developed between the association and traders and / or herbal companies.

Development and construction of Arnica drying facilities

Local drying facilities for Arnica flowers are an important component of the concept, as quality dried Arnica flower is a processed and refined product that can be sold at considerably higher prices as compared to fresh Arnica. The project seeks to add value to Arnica by building adequate drying facilities close to collection sites. Methods and scale of drying are matters of discussion.
Experimental drying in a demonstration trying facility is planned for the first field season for to convince collectors / farmers of the usefulness of the method and ease setting up more solid drying facilities during the second year.

Research on Arnica ecology, trade chain, socio-economic context and drying method

Research is, together with traditional knowledge, an important cornerstone in the development of a resource sustainability concept. Several research items have been suggested:
- Ecological sustainability of Arnica and link to farm management and tenure rights
- Socio-economic context and community attitudes at Gârda-de-Sus
- Analysis of the trade-chain for Arnica flowers from Gârda-de-Sus
- Analysis and development of adequate drying and storage methods for Arnica flowers

These research items will be suggested by the project team to local stakeholders and interested people; these items may be modified and adjusted according to the current actual needs. It is planned that, within the research topics, two masters thesis be written by students from Cluj. The topics will be conceptually developed together with all project partners and the students' local academic supervisors.

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ELECTRON BEAMS TREATMENT OF *SPIRULINA PLATENSIS* TO ENSURE THE MICROBIAL SAFETY

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Summary

Spirulina platensis has high protein content and therefore, a high nutritional value. Taking into account that nowadays it is used as substitute and/or supplement in human diets, it should have a proper hygienic quality. The treatment with accelerated electrons is already applied on various foods for decontamination and extension of shelf life, being considered an ecological and non-expensive one. The aim of the paper is to discuss the electron beams application on Spirulina platensis and the evolution of modifications during its storage. The results of our study showed that the treatment with accelerated electron beams on Spirulina platensis is suitable to ensure the microbiological safety.

Keywords: *Spirulina platensis, electron beam treatment, microbial safety*

Introduction

Spirulina platensis is a green-blue microalga known by its high nutritional value and considered as one of the most complete natural sources of proteins, vitamins, minerals and other nutrients [1]. It has been recognized to be a more than potential alimentary source [2] to be used as substitute and/or supplement in conventional diets [3].
Due to its nutritional value, the recently increasing demand for *Spirulina* as a health-beneficial food has motivated interest in the improvement in its hygienic quality.
Electron beam treatment is already applied to various foodstuffs for decontamination and extension of shelf life [4, 5]. In addition, the major advantage of the linear accelerators as compared with gamma irradiators is that they can simply be turned off when is not in use [6]. Therefore, we have proposed to discuss in our paper the electron beams (e-beams) application on *Spirulina platensis* and the evolution of modifications during its storage.

Materials and methods

The samples of *Spirulina platensis* have been irradiated in plastic package at room temperature and atmospheric pressure in accelerated electron beams of 6 MeV. Doses up to 20 kGy, with a dose rate of approximately 2 kGy/minute, have been obtained.
Microbial contamination has been measured according to the method described by Romanian Pharmacopoeia [7].
Chlorophylls have been determined by a spectrophotometric method using acetone as a solvent. The filtered acetone extracts have been read as follows: $\lambda = 662$ nm for *a* chlorophyll and $\lambda = 644$ nm for *b* chlorophyll.
C Phycocyanin content have been established according to Boussiba's method [8], being analysed with a Carl Zeiss Jena spectrophotometer. The characteristic absorbance of C phycocyanin has been read at $\lambda = 620$ nm.
Fatty acids determination. The lipids contained in *Spirulina* biomass have been transformed into fatty acids by alkaline hydrolysis. Their salts have been refined by extraction with petroleum ether. After acidifying, the free acids have been extracted with petroleum ether and the residuum obtained after concentration has been subjected to esterification using methanol

and acidic catalysis. The obtained methyl esters have been extracted with heptane and injected into a gas chromatograph SHIMADZU GS-MS having a column with stationary phase MACROGOL 20000 Ø = 0.25 mm, l = 30 mm using He_2 as carrier gas $t_{injector} = 250^0C$, $t_{column} = 160\text{-}250^0C$. A QP 5000 mass spectrometer has been used as a detector.
Antioxidant activity has been identified by enzymatic measurement (superoxide dismutase – SOD) in guinea pig brain homogenate Activity of SOD (EC. 1.15.1.1) has been determined by standard method, following the catching action of free radicals in a generating system of them.
All measurements have been carried out immediately after irradiation and after 6 months storage at room temperature.

Results and discussion

The number of bacterial colonies decreased when e-beam dose increased immediately after treatment; no microorganisms survived after 20 kGy dose. The decrease had a linear evolution (Fig. 1), D_{10} (the decimal reduction dose) being at 9.64 kGy. The 5 kGy and 10 kGy treated samples showed a permissible level of microbial load according to Romanian Pharmacopoeia.

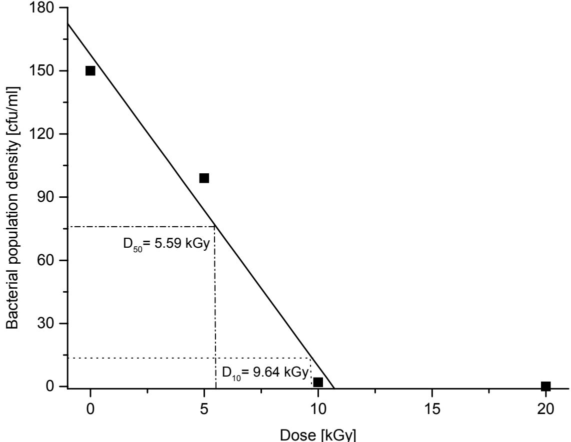

Fig. 1. Bacterial contamination of *Spirulina* before and immediately after treatment

The control sample had a very high fungal contamination level (Table 1), but after 5 kGy dose appeared a drastic reduction of it. In comparison to bacteria behaviour to e-beam treatment, these results suggested us that fungi are more sensitive in the 01.-5 kGy range.

Table 1. Fungal contamination [CFU/ml] of *Spirulina*

Doza [kGy]	0	5	10	20
Immediately after irradiation	TNTC*	11	2	1
After 6 months storage	24	12	4	1

*TNTC = too numerous to count

During storage period of e-beam treated *Spirulina* the microbiological situation remained unchanged, being in accepted limits. This could be expected because *Spirulina* is a relative dry product. All the data indicated that the treated samples were stable from microbiological point of view.
The content of *a* and *b* chlorophylls did not vary importantly with e-beam dose immediately after treatment (Table 2). During storage time, the chlorophyll content decreased, especially in the case of chlorophyll *b*.

Table 2. Content in *a* and *b* chlorophylls [mg/100g] of *Siprulina*

Dose	Chlorophyll *a*		Chlorophyll *b*	
months / [kGy]	0	6	0	6
0	3.3	2.9	30.0	18.6
5	3.2	2.4	29.5	12.4
10	3.2	2.3	29.3	19.6
20	3.1	2.0	30.5	21.3

No significant changes of C phycocyanin content have been observed immediately after e-beam treatment (Fig. 2), but for all treatment dose appeared a decrease in phycocian content during storage time.

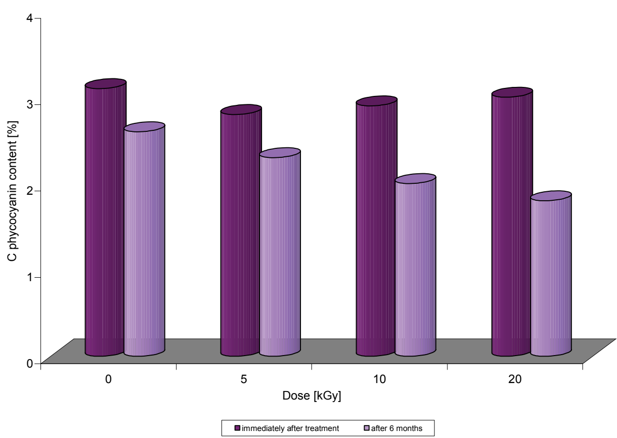

Fig. 2. Phycocyanin content of *Spirulina plantensis*

Tabel 3 shows the content in fatty acids of *Spirulina*. The data did not indicate very important changes of composition in fatty acids both immediately after treatment and after 6 months storage. However, after 6 months storage a slight tendency to decrease has been remarked for the following saturated fatty acids: palmitoleic, oleic, linoleic, linolenic, EPA (eicosapentaenoic acid) and DHA (docosahexaenoic acid).

Table 3. Fatty acids content [%] in *Spirulina platensis*

Dose	Palmitic		Palmitoleic		Stearic		Oleic		Linoleic		Linolenic		EPA		DHA	
months / kGy	0	6	0	6	0	6	0	6	0	6	0	6	0	6	0	6
0	44.3	44.0	2.4	1.8	2.0	2.0	14.0	13.7	16.0	10.2	17.0	13.2	8.1	6.3	3.8	1.9
5	44.0	44.3	2.3	1.6	1.7	1.9	13.4	12.8	16.1	10.0	17.1	15.3	8.3	6.7	3.4	2.0
10	42.5	44.5	2.1	1.7	2.1	2.1	14.1	13.7	15.9	10.0	16.8	14.9	8.2	6.0	3.2	2.3
20	42.9	44.2	2.2	1.5	1.8	1.9	13.9	12.9	15.8	9.8	16.8	14.8	8.1	6.0	3.4	2.2

The content of “like-SOD” compounds decreased significantly with the increase of dose for all chosen doses immediately after treatment (Fig. 3). For the same dose treatment the SOD activity did not change during storage period.

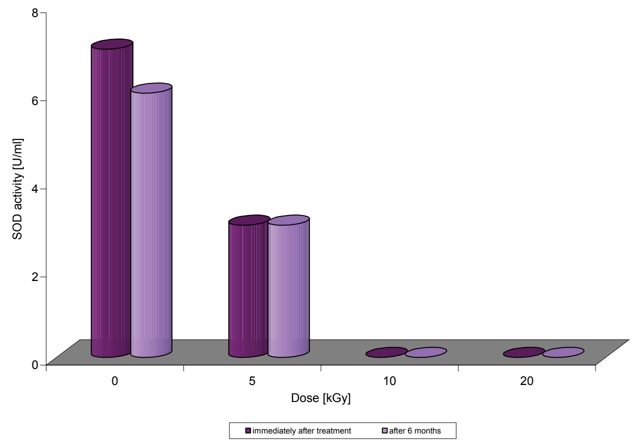

Fig. 3. Variation of “like-SOD” enzyme content of *Spirulina*

Conclusion

The results of our study showed that the treatment with accelerated electron beams on *Spirulina platensis* is suitable mean to ensure the microbiological safety. The dose range may be established taking into account for which application (e.g. food supplements, pharmaceutical products, etc) the microbial safety require to be ensured.

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THE INFLUENCE OF ELECTRON BEAMS ON THE SEA BUCKTHORN BERRIES

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Summary

Sea buckthorn is well known for its quality with almost unlimited potential for the cure of some diseases. Due to the fact that it is necessary to harmonise the products across the international regulations regarding microbial contamination and active biologic compounds, we initiated a study with respect to the action of electron beam treatment on sea buckthorn berries. Sea buckthorn berries were treated with electron beams up to 40 kGy and microbial contamination, flavonoid derivates, carotenoid and protein contents as well as antioxidant activity were evaluated. The study revealed that after 3 kGy irradiation, the sea buckthorn microbial load was reduced under permissible level according European Pharmacopoeia without important alteration in active principles.

Keywords: *sea buckthorn, electron beams, microbial contamination*

Introduction

Sea buckthorn is well known for its quality with almost unlimited potential for the cure of some diseases. Berries contain many bioactive substances and can be used in the treatment of several diseases such as cardiovascular disease, cancer, and acute mountain sickness [1, 2]. Generally, the microbiological background of medicinal plants depends on several environmental factors and exerts an important impact on the overall quality of the products [3]. Nowadays there are very clear international regulations concerning the problematic of microbial contamination of medicinal plants and for this reason it is necessary to harmonise the products across these in order to have good quality products. As radiation technology is increasingly recognized as an effective way in reducing microbiological contamination of medicinal plants [4], we initiated a study with respect to the action of electron beam (e-beam) treatment on sea buckthorn berries.

Materials and methods

Material
Sea buckthorn berries from Romanian spontaneous flora have been transformed in powder state by controlled drying at temperatures lower than 60^{0}C and then grounding.
Irradiation
Sea buckthorn powder has been packed up in polyethylene bags and treated in electron beams with doses up to 40 kGy, at room temperature and atmospheric pressure. It has been used a linear electron accelerator which has the mean energy of 6 MeV, the peak intensity of 75 mA at a repetition rate of 50-100 Hz.
Microbial load has been measured on liquid plant extracts according to the method described by Romanian Pharmacopoeia [5].
Quantitative determination of total *flavonoid derivatives* has been performed using colour reaction of them with $AlCl_3$ and reading the extinction at λ = 430 nm. The total flavonoid content has been expressed in rutin.
Determination of *carotenoids* has been carried out in benzene extracts of sea buckthorn by a spectrophotometrical method. It has been measured the absorbance of samples at λ = 460

using for standardization solution of β-carotene and then the carotenoid content has been calculated.
Soluble protein measurements have been performed suspending the sea buckthorn powder in water, filtration and precipitation of filtrate with ethanol.
Antioxidant activity has been identified by enzymatic measurement (superoxide dismutase – SOD) in guinea pig brain homogenate. Activity of SOD (EC. 1.15.1.1) has been determined by standard method, following the catching action of free radicals in a generating system of them.

Results and discussion

Table 1 shows the contamination with microorganisms before and after different dose treatment. It can be noted the samples presented decrease of total number of bacteria with the increase of irradiation dose.

Table 1. Microbial contamination level in sea buckthorn before and after treatment

Dose [kGy]	0	3	5	10	20	40
Bacterial count, [CFU/ml]	320	260	250	90	0	0
Fungal count, [CFU/ml]	10	10	0	0	0	0

The total number of filamentous fungi has been much more less in comparison to that of bacteria. Up to 3 kGy no modification could be observed, but after 5 kGy dose no fungi number was remarked.
Therefore, we observed that both the number of bacteria and fungi decreased with the increase of dose, being in the accepted limits of Romanian Pharmacopoeia after 3 kGy treatment.

Table 2. Total content of flavonoid derivatives

Dose [kGy]	Flavonoid derivatives [g%]
0	0.582
3	0.540
5	0.483
10	0.480
20	0.426
40	0.305

Total content of flavonoids suffered a decrease after sea buckthorn treatment, slight decrease up to 5 kGy and accentuated one at doses higher than 20 kGy.
Figure 1 shows the carotenoid levels in sea buckthorn before and after e-beam treatment. These results suggested that at relative low doses, up to 10 kGy, the carotenoid content is not affected significantly. But, after treatment with 20 kGy we observed that an important decrease appeared in the content of carotenoids.
The soluble protein content was not affected by e-beam treatment in the dose range chosen by us.
Figure 2 shows the variation of antioxidant potential of sea buckthorn as related to e-beam dose. It was noted a slight decrease of antioxidant potential with the increase of treatment dose. The SOD value of non-treated sea buckthorn was relative low and it is remarkable that even at low e-beam dose of 3 kGy this value decreased drastically.

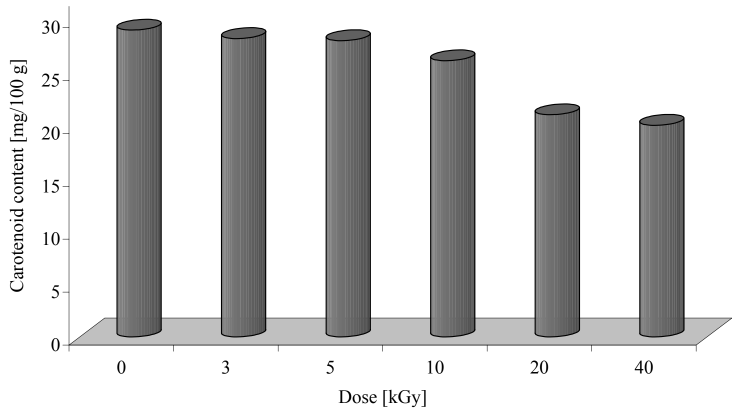

Fig. 1. Carotenoid content of sea buckthorn before and after treatment

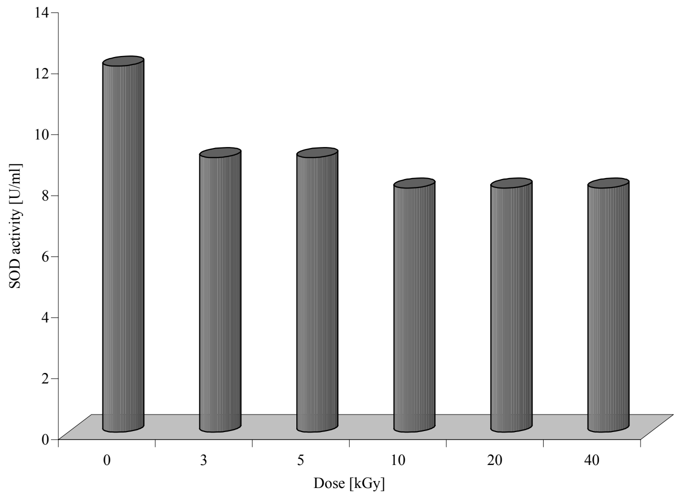

Fig. 2. Variation of SOD activity with e-beam dose

Conclusion

The study revealed that after treatment with 3 kGy, the sea buckthorn microbial load was reduced under permissible level according European Pharmacopoeia without important alteration in active principles up to 10 kGy.

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STUDY ON MEDICINAL AROMATIC PLANTS IN THE AREA OF NATIONAL PARK OF LLOGORA

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Summary

The area of National Park of Llogora is distinguished for the rich flora. About 1,400 species or 42,4 % of the Albanian flora inhabit the area. The presence of some endemic, subendemic, relic, rare and threatened species improves the biodiversity values in this area. There are about 115 medicinal and aromatic plant species grown in this area; 22 medicinal plants are actually more used in the region. Some of them are affected by the phenomena of genetic erosion and 20 species of this region are included in the National Red Data Book.

Keywords: *Flora, Medicinal plants*

Introduction

The National Park of Llogora lies in the Northwest of Çike-Lungare mountain range. Çika Mountain (2045 m) and the Peak of Qore (2018 m) are the highest peaks of the zone that gradually get down to the Qafë e Llogorasë (1027 m) and the valley of Dukat to Northwest. The rocks are mostly hard limestone of different age, but in the Eastern part of Sazani island, valley of Dukat and Jonufer there are small areas with more or less soft schist.
The region has a richer vegetation than any area of comparable size in Albania. A number of factors that have contributed to this high biodiversity are:

- The flora contains Tertiary species
- The isolation of land masses (island, mountain ranges, etc) as a result of changes in the sea level. This has resulted in habitat fragmentation, isolation and migration of species.
- The proximity of other floras, especially the Aegean flora from which migration has taken place.
- The influence of man, which goes back several thousand years and has resulted in complex mosaic of natural, semi-natural and man-made habitats promoting high diversity.

The National Park of Llogora (including Massif of Rrëzë e Kanalit, Karaburun Peninsula, Valley of Dukat, the Sazani island and Orikum Lagoon extends over an area of nearly 300 km²) is characterized by a high diversity of habitats and plant communities such as marine, lagoons, sand-dunes, coastal rocks, evergreen mediterranean forests or different evolutive stades of their (maquis, garrigue, phryganas and mediterranean pseudo-steppes) deciduous oak forests, coniferous forests dominated by *Pinus nigra*, *Abies borisii-regis*, *Pinus leucodermis* and alpine pastures. It represent one of the most interesting places of Albania. The presence of some endemic, subendemic, relic, rare and threatened species improves the biodiversity values in this area.
The studied region is distinguished for the rich flora incomparable to any other area in the country. About 1,400 species or 42,4 % of the Albanian flora inhabit the region.
The Mediterranean element is certainly the most widely spread flora in this region, with 439 species or 31,4 % of the total number of the regional flora, which reports on the strong influence of the Adriatic and Ionian seas on the flora of the region and mainly on the forests and Mediterranean scrubs (maquis, frigana, etc.).

Material and methods

The medicinal aromatic plants of the Llogora National Parc will analyse based on releves and on the data reported in the literature. This analysis will conduct with the support of Flora of Albania (Paparisto & al.,1984-2000) and Flora Europaea (Tutin & al., 1964-1980). The nomenclature follow is that by Paparisto in Flora of Albania.
The vegetation releves follow the phytosociological method of Zurich-Montpellier school (Braun- Blanquet, 1932).

Results and discussion

There are about 115 medicinal and aromatic plant species grown in this area. They have been used for therapeutic uses since ancient time. First they were used as fresh plants, and later as extracts and liquids. Except from the historical phases, the use of medicinal plants has already possed through, these plants are being used widely nowdays under the names: medicinal plants, medical teas, and medical drugs. The last category which is mainly made up of dried plants parts serves to prepare the medicaments.

List of medicinal plants of the Park

Acanthus spinosus L.
Ajuga reptans L.
Alkanna tinctoria Tausch.
Alisma plantago-aquatica L.
Alnus glutinosa (L.) Gaerth.
Anacamptis pyramidalis
Anagallis arvensis L.
Arbutus unedo L.
Asparagus officinalis L.
Asplenium trichomanes L.
Althaea officinalis L.
Ammi visnaga Lam.
Apium graveolens L.
Arctium lappa (l.) Willd.
Artemisia vulgaris L.
Bellis perennis L.
Borago officinalis L.
Bryonia dioica Jacquin
Buxus sempervirens L.
Capsella bursa pastoris (L.)M.
Centaurium pulchellum Sw
Centaurium umbellatum Cilib
Ceterach officinarum Lam.&D
Cichorium intybus L.
Colchicum automnale L.
Cotinus coggygria Scop.
Capparis spinosa L.
Ceratonia siliqua L.
Clematis vitalba L.
Colutea arborescens L.
Convollaria majalis L.
Convolvulus arvensis L.
Cornus mas L.
Corydothymus capitatus (L.)
Crithmum maritimum L.
Crataegus monogyna Jac.
Cupressus sempervirens L.
Cynodon dactylon Pers.
Datura stramonium L.
Daucus carota L.
Dictamnus albus L.
Digitalis lanata L.
Dryopteris filix mas (L.)Sch.
Ecballium elaterium (L.) A.R.
Ephedra distachya L.
Equisetum arvense L.
Echium vulgare L.
Eryngium campestre L.
Eryngium maritimum L.
Foeniculum vulgare Mill.
Fragaria vesca L.
Gentiana lutea L.
Glaucium flavium Crantz.
Glycyrhiza glabra L.
Galega officinalis L.
Hypericum perforatum L
Ilex aquifolium L.
Inula viscosa L..
Juniperus communis L.
Juniperus oxycedrus L.
Laurus nobilis L.
Leonurus cardiaca L.
Lycopus europaeus L.
Lotus corniculatus L.
Malva sylvestris L.
Matricaria chamomilla L.
Melilotus officinalis (L.) Pall.
Melisa officinalis L.
Myrtus communis L.
Mercurialis perennis L.
Nasturtium officinale R. Br.
Nerium oleander L.
Ononis spinosa L.
Origanum vulgare L.
Orchis sp.
Papaver rhoeas L.
Plantago lanceolata L.
Plantago major L.
Polygonum aviculare L.
Polypodium vulgare L.
Prunella vulgaris L.
Prunus spinosa L.
Punica granatum L.
Pistacia lentiscus L.
Pistacia terebinthus L.

Rhus coriaria L.
Rosa canina L.
Rubus fruticosus L.
Rubus idaeus L.
Ruta graveolens L.
Ruscus aquleatus L.
Salvia officinalis L.
Sambucus ebulus L.
Sambucus nigra L.
Saponaria officinalis L.
Satureja montana L.
Salicornia herbacea L.
Salix alba L.
Salix fragilis L.
Salix purpurea L.
Sideritis roeseri Boiss.
Smilax aspera L.
Spartium junceum L.
Taraxacum officinale Web.
Tussilago farfara L.
Taxus baccata L.
Teucrium chamaedrys L.
Teucrium pollium L.
Trifolium pratense L.
Urginea maritima (L.) Bak.
Urtica dioica L.
Viola odorata L.
Viola tricolor L.
Viscum album L.
Verbena officinalis L

Table 1. Medicinal plants which are actually more used in the region

Nr.	***Albanian name***	***Latin name***	***Population sizes*** *(approx.in ha)*	***Yield*** *(approx. in tons)*
1	Murrizi njëbërthamësh	*Crataegus monogyna*	595	11
2	Mëllagë e egër	*Malva sylvestris*	28	0.8
3	Shtogu i zi	*Sambucus nigra*	50	2.4
4	Thundërmushka	*Tussilago farfara*	17	1
5	Lavandula	*Lavandula vera*	28	6
6	Kin fushe	*Centaurium pulchellum*	33	2.5
7	Gjethedelli i madh	*Plantago major*	53	5
8	Sherbela	*Salvia officinalis*	953	198
9	Rozmarina	*Rosmarinus officinalis*	58	35
10	Dafina	*Laurus nobilis*	170	61
11	Melisa (Barblete)	*Melissa officinalis*	5	0.1
12	Bishtkali	*Equisetum arvense*	27	2.2
13	Çaji i malit	*Sideritis roeseri*	650	10.9
14	Lulebasani	*Hypericum perforatum*	38	4.8
15	Mendra gjethegjatë	*Mentha longifolia*	33	6.9
16	Njëmijëfletëshi	*Achillea millefolium*	26	4.5
17	Trumza	*Satureja montana*	2929	344
18	Zhumbricë	*Thymus serpyllum*	57	17
19	Shëngjini	*Salvia sclarea*	3	4.4
20	Borziloku	*Ocimum basilicum*	56	80.8
21	Dëllinjë e kuqe	*Juniperus oxycedrus*	300	106
22	Konopica	*Vitex agnus-castus*	12	3.8
23	Kullumbri	*Prunus spinosus*	15	2
24	Trëndafil i egër	*Rosa canina*	105	117
25	Salep	*Orchis sp.*	39	1.4
26	Luledele	*Bellis perennis*	3	0.1
27	Gjembgomari	*Silybium marianum*	18	0.3
28	Rigon i bardhë	*Origanum vulgare*	25	2.8

Threatened Medicinal plant species included in the National Red Data Book" (Vangjeli et al., 1995)

Threats facing medicinal plant species in this region are - very similar across the country.

- A largely unmonitored trade
- Destructive harvesting techniques
- Habitat loss and habitat changes
- Over – exploitation

If over – exploitation takes place these impacts may lead to decreasing population sizes, decrease in genetic diversity and finally to the extinction of the species. An additional impact in this region has been the deregulation of state-controlled commerce resulting in an increase of wild-collection.

A wrong practice, for "their forage improvement" in this region (Dukat, Karaburun) are the illegal burns, especially in so-called "Phrygana" or "Winter Pastures". This practice has been conducted for many years. The real damage is a reducing of medicinal plant species distribution, and vegetation covered these habitats. This practice has also a very bad effects on soil erosion and increasing of desertification. The uprooting the entire wild plant for harvesting is another wrong practice in this region. Threats are different, either from their limited area of distribution, or the threats of their habitats, or the explotation.

The following table (Tab.5) give the list of threatened medicinal plant species by "Red Book" of Albania (Vangjeli et al., 1995), Degree of threats based in categories of IUCN and distribution in region.

Table 2. List of threatened medicinal plant species by "Red Book" of Albania (Vangjeli et al., 1995)

Nr.	Plant name	Degree of threat by IUCN	Distribution in Region
1	*Agrimonia eupatoria*	E	National Park of Llogora
2	*Capparis spinosa*	E	Coastal Rocky Places, Vlora Bay
3	*Convallaria majalis*	E	National Park of Llogora
4	*Colchicum autumnale*	E	Everywhere, From Coniferuous Mediterranean forests Poro up to 1000 m above sea-level, but allways very rare
5	*Digitalis lanata*	E	National Park of Llogora
6	*Dryopteris filix-mas*	E	National Park of Llogora
7	*Ephedra distachya*	E	Coastal Rocky Places, Vlora Bay
8	*Gentiana lutea*	CR	National Park of Llogora
9	*Hyoscyamus niger*	E	National Park of Llogora
10	*Hypericum perforatum*	E	Everywhere,up to 1000 m above sea-level, but rare
11	*Laurus nobilis*	E	Shrubs of Uji i Ftohtë and Sazani island, often cultivated
12	*Orchis sp.*	E	Everywhere, From Coniferuous Mediterranean forests Poro up to 1000 -1500 m above sea-level, but allways very rare
13	*Origanum vulgare*	E	Everywhere, from 0 up to1500 m above sea-level,mostly in open calcareous places. Collection every year in a very considerable quantity is the reason of the high degree of threat
14	*Salvia officinalis*	E	Everywhere, from 0 up to1500 m above sea-level,mostly in open calcareous places. Collection every year in a very considerable quantity is the reason of the high degree of threat
15	*Sambucus nigra*	E	Along mountainous streams of Llogara National Park
16	*Satureja montana*	E	Everywhere, from 0 up to1500 m above sea-level,mostly in open calcareous places. Collection every year in a very considerable quantity is the reason of the high degree of threat
17	*Sideritis raeseri*	E	National Park of Llogora, Mountains of Qorre and Çika
18	*Valeriana officinalis*	E	National Park of Llogora, rocky places
19	*Viscum album*	E	National Park of Llogora, semiparasite, mostly in trees of the *Pinus nigra*
20	*Adianthum capillus-veneris*	V	National Park of Llogora, in wet and rocky places

Collection and trade of medicinal plant species

Prior to 1992, trade structure was hierarchically organised. Rural collectors gathered the bulk of produce, which was collected by local branches, or mobile units, of the district Produce Collector Enterprises. Another body, the district forestry enterprise, had responsibility for collecting medicinal and aromatic plant material from cultivated areas and forests. If not for use within the country, from district level, plant material generally came next under control of the Agroexport Enterprise.

Nowdays, the majority of medicinal and aromatic plants, being wild-collected in this region are still sold by rural collectors to the local dealer. From the local trader, plant material pass to a Vlora District trader, before release onto the domestic market, or to one of the main companies in Albania involved in international trade in medicinal and aromatic plant material.

Much of the wild collection of medicinal plants takes place on state lands in the hill and mountain areas of the region. collection is undertaken by all members of a family, but most commonly by women within forest villages, together with children (period of school hollydays), retired people and in some instances stock herders. Medicinal plants collection is regarded by the inhabitants as a valuable means of supplementing low family incomes.

Legislation

The collection and export of medicinal plants before 90'-ties was controlled by the governmental instruments. After 90'-ties a number of lows were ratified which made an important step forward in the field of legislation. The following laws influence directly or indirectly on the medicinal plants:

- For Protected areas, Law No.8906, dt.6.06.2002.
- For environmental protection, Law No. 8934, dt. 05.09.2002.
- For forestry and forest service policy, Law No.7623, 1992
- For protection of natural medicinal, aromatic and tanning plant species fund, Law No. 7722, 1993
- About pastures and meadows, Law No. 7917, 1995

All these laws are of considerable importance regarding the protection and sustainable use of the fond of the natural, medicinal and aromatic plant species. Law no.7722, of the year 1993 considers directly these group of plants of high economic importance, however it needs further improvements. The natural medicinal, aromatic and tanning plant species are declared National Property under the Article 1. Every October, the Minister of Agriculture with a declaration could stop or limited amount of medicinal plants which should be collected or prohibited during the consecutive year or further on. For that purpose the compilation of a species list is attached to the decision; Article 4.

Conclusions

Medicinal and aromatic plants play an important role in everyday life in this region; many people are consuming phytomedicines, herbal teas etc. 20 medicinal and aromatic plants are included in National Red Data Book.

Harvesting techniques may often exacerbate the threat to medicinal and aromatic plants, by causing unnecessary levels of damage, as in the cases of *Salvia officinalis* , where uprooting of the whole plant to use only aerial parts of the plant causes unnecessary depletion of population levels of the species, as well as damage to the top-soil.

Habitat changes across most parts of region have also eroded species'population levels. This implies an urgent need for designating more protected areas in this region, in particular in those areas with a high biodiversity of medicinal and aromatic plants and in those where threatened species occur.

The trade has altered from a strictly state-organised and controlled commerce, to one of privatised competition and associated uncontrolled collection. Harvest from the wild has in many cases increased as a result of a general decrease in cultivation of medicinal and aromatic plant cultivation.

Cultivation of medicinal and aromatic plants is a very effective means, if not the most promising one, to satisfy the market's expanding demand in future, while reducing or eliminating pressure on their wild populations. In the case of plant species that are critically endangered through over-exploitation (*Gentiana lutea)* this is certainly the only method to stop their decline and to secure their long term survival, if there is no substitute plant material.

The trade in medicinal and aromatic plants is huge, largely unmonitored, and that wild-harvesting predominates. Training of local people dealing with collection of medicinal plants regarding these issues is very important

Public awareness of the trade in medicinal and aromatic plants and the impact on wild-populations of plant species is slight. It is highly likely that many uses are unsustainable and hence threatening taxa. Lack of data for assessment of the conservation status of species is resulting in delayed protection measures: conservation action has usually only begun once it is known that a species is already threatened. Public awareness and educational material should be developed to inform government authorities, traders, phytopharmaceutical companies, scientists, collectors, cultivators and consumers on threats facing specific medicinal and aromatic plant species in trade and on the legal status of the plants in use.

Some others duties:

- Legislation enforcement, particularly for rare and threatened medicinal plant species
- There is a particular need to produce a simple brochure describing sustainable harvesting, the most effective drying conditions etc. for collectors and middlemen
- A periodical monitoring, based on indicators techniques and scientific criteries, aiming given the right recomendations to the governmental authorisation
- Further research should be carried out on medicinal and aromatic plants exploited in order to improve knowledge of trade volumes and structures, source countries, and the impact on their wild populations.

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MORPHO-PHYSIOLOGICAL, PRODUCTION AND QUALITY TRAITS OF THE NAPOCA CULTIVAR OF *ECHINACEA PALLIDA* NUT.

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Summary

The "Napoca" cultivar (Echinacea pallida Nutt.) has been created at U.S.A.M.V. Cluj-Napoca through repeated mass selection from echinacea that had been imported to our country and studied in yield trials (with the temporary name of CN 11/992).

Keywords: *Echinacea pallida Nutt., Napoca echinacea cultivar, mass selection*

Introduction

The biologic factor (cultivar, hybrid, population) and the cultural value of the breeding material, together with ecological and technological factors are conditions for good productivity and good quality of the raw material of the medicinal and aromatic plants. Many valuable medicinal and aromatic plant cultivars have been created in Romania, in others there are a lot of cultivars that are cultivated but there are also a lot of medicinal plants which are only represented by local populations or cultivars (1,2,3,7)
Therefore, especially in the case of the plants only represented by local populations, it is necessary that more research should be done in order to create new cultivars. The main objectives of the breeding programmes for these plants refer to the production level, the quality of the raw material (the content of active principles), the uniformity of the material and its technological maturity, the capacity to adapt and resist factors such as diseases, pests, falling, excessive cold or drought etc., the processing of the raw material etc. (2, 3, 7 etc.)
Starting with 1993, the Research Centre for Hop and Medicinal Plants- USAMV Cluj-Napoca has also initiated breeding programmes, along with the research on the biotechnologies and the crop technology used for medicinal and aromatic plants

Material and methods

This paper presents the first Romanian cultivar of Echinacea pallida Nutt – „**Napoca**", created by the Research Centre for Hop and Medicinal Plants- USAMV Cluj-Napoca. This species has been used for obtaining very important medical products which enhance the immunity system by mobilizing the leucocytes, by increasing the activity of the phagocytosis, by inhibiting virus multiplication. It is used as raw material for obtaining anti-virus, imunostimulents, antitumorale.products etc (3, 4, 8).

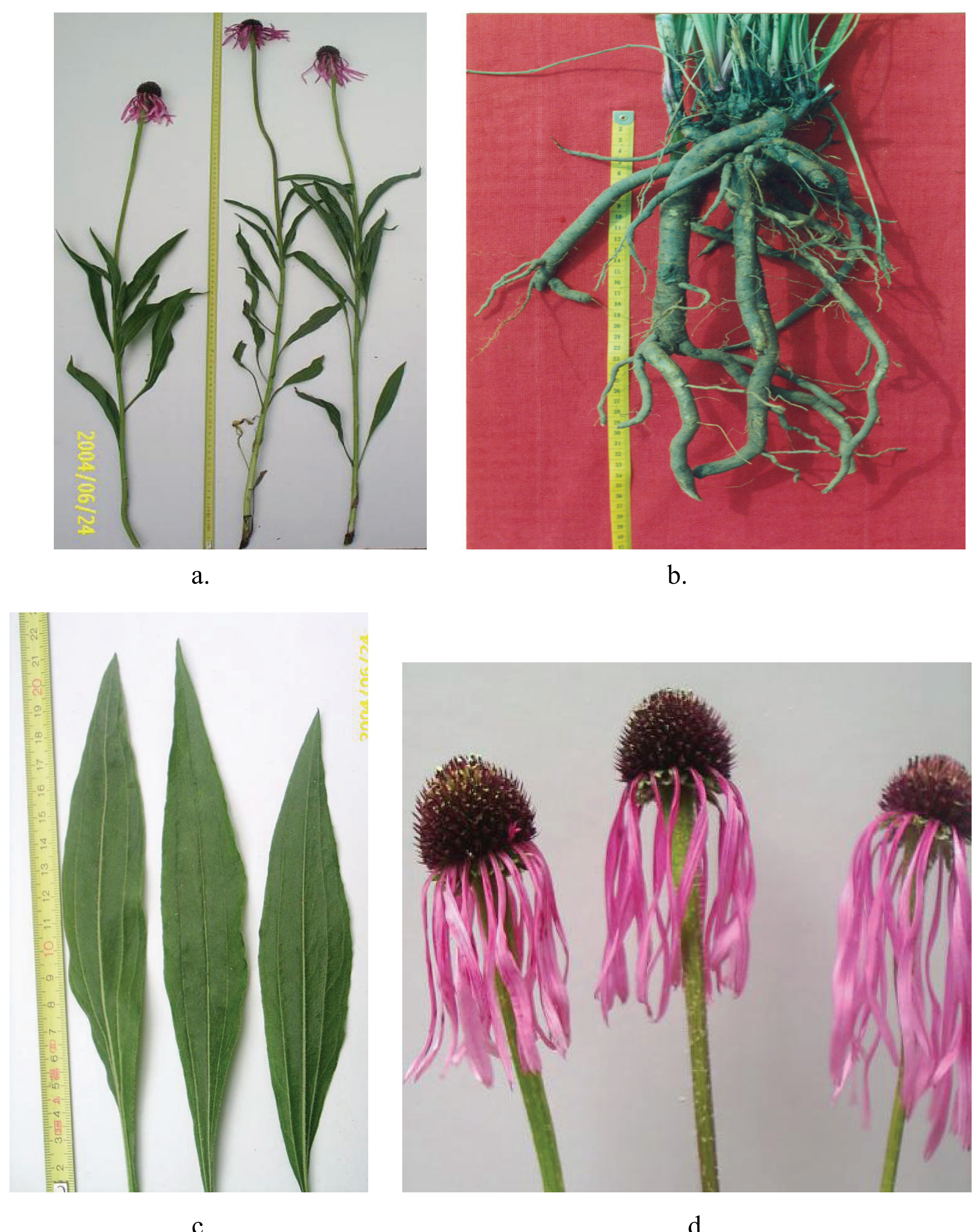
Results and discutions

The Napoca cultivar- the first Romanian *Echinacea pallida* Nutt.cultivar, tested in the network of the National Institute for Cultivar Testing and Certificating (at Cluj-Napoca and Dej), in 2004 and 2005.

Sistematycs - The 'Napoca' cultivar(*Echinacea pallida* Nutt) has been created at U.S.A.M.V. Cluj-Napoca through repeated mass selection from Echinacea that had been imported to our country and studied in yield trials (with the temporary name of CN 11/992) (4, 5, 6).
The biologic material used for obtaining the Napoca cultivar was presented from the morpho-physiological point of view, as well as from the point of view of the content of active principles in our former papers. (4, 5, 6).
Stages of the breeding programme. The breeding of this plant was initiated in 1992 by the Research Centre for Hop and Medicinal Plants- USAMV Cluj-Napoca and was then developed after 1995 (4). In 2004 and 2005, the Napoca cultivar was registered in the network of the National Institute for Cultivar Testing and Certificating (at Cluj-Napoca and Dej), in order to obtain the certificate for national recognition in the official list of cultivars.
The main morpho-physiological, productivity and quality features of the 'Napoca' *Echinacea pallida* Nutt. cultivar (5, 6) and fig. 1:

- The vegetation period. It is a semi late cultivar, with a vegetation period of 134-145 days in the second and third years; in the first year the root system is formed, and also a leaf rosette and flower stems with a reduced number of flowers, the complete flowering only beginning in the second year of vegetation;
- The root. It has got a tap root, with 1-3 vertical gray-brownish main roots (which are the actual raw material, harvested in the years 2-3);
- The part above the soil looks like a bush
- Stems are 80-120 cm long, sometimes with branches, 6-8 mm thick, with 8-12 internodes, the last one being 30-40 cm long, ended with a inflorescence;
- The leaves from the rosette are linear lanceolate with a petiole of 8-18 cm length, with a blade of 8 cm length and 3-6 cm width, with three nervures on the dorsal side;
- The inflorescence is ovoid, thorny when dry with involucral leaflet at the bottom (in two rows), conical receptacle (1,5-2 cm high, with a diameter of 2-3 cm); the ray flowers are pink-violet in colour, sterile (5-8 cm length and 2-5 mm in width); the disc flowers are tubular, orange and hermaphrodite (4-6 mm in length). The flowering takes place in July-August, pollination is made by insects;
- The fruit is a sided achene, 2-4 mm in length, whitish with rudimentary pappus;
- The whole plant is covered with rough hairs, 1.8-2 mm long (made of 3-5 cells), wider-bottomed and narrow-ended, that give rigidity to the plant.
- The raw root yield is of 4,5-5,2 t/ha (1,3-1,5 t/ha when dry);
- The content of active principles is high: imunostimulent polysaccharides (6,1-6,4 g %), phenil-propanic derivates (0,5 – 0,6 g %), volatile oil (0,9-1,2 ml/100g) etc., which enhance the immunity system (by mobilizing the leucocytes, by increasing the activity of the phagocytosis, by inhibiting virus multiplication), the raw material being used for obtaining anti-virus, imunostimulent, antitumorale products etc.

Area. The "Napoca"cultivar of *Echinacea pallida* Nutt. is recommended to be cultivated in Transylvania (all areas), using plantlets obtained from the seed with high biological and cultural value produced by the Research Centre for Hop and Medicinal Plants- USAMV Cluj-Napoca



a. b.

c. d.

Fig. 1. The “Napoca”cultivar of *Echinacea pallida* Nutt.:
a. plants during the flowering period; b. roots; c. stems; d. inflorescence

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ECOPHYSIOLOGICAL RESEARCH ON SOME MEDICINAL PLANTS FROM BĂLTENI FOREST (VASLUI COUNTY)

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Keywords: *ecophysiology, medicinal plants, pigments, mineral elements, active substances*

Introduction

Medicinal plants have been the subject of many phytochemical, agro-biological, and pharmaceutical studies, and many botanists from our country dealt with their study, distribution, and introduction in botanical gardens (Bârcă C., 1981; Dobrescu C., 1984; Gheorghiță G.,1979; Tóth – Tünde Ecaterina, 2003).
According to many biochemical and physiological investigations, numerous Romanian spontaneous species would successfully replace foreign vegetal products, due to their high contents in active substances (Stratu A., 2002; Robu T., 2002, 2004; Milică C., 2001; Toth E. 2003; Tóth – Tünde Ecaterina, 2003; Burzo I., 2005). During the last years, the interdisciplinary and complex investigation of the natural and disturbed forests from Moldavia have gained momentum in order reveal on ecophysiological bases the phytocoenological bioproductive potential and the biochemical and physiological changes (Murariu A 2002,2003).
This research aims to show the physiological behaviour of some medicinal plants from Bălteni natural reserve, through the determination of the contents in water, dry substance, assimilatory pigments, and total mineral elements.

Material and methods

The representative characteristics of Bălteni natural reserve is the presence of the shrub *Evonymus nana* – a glacial relict discovered at the beginning of the past century by I. C. Constantinescu – and other species such as *Leucoyum aestivum*, *Iris graminea*, *Tulipa biebersteiniana*, *Fritilaria Montana*, *Ulmus laevis* (Chifu 2002). Together with these rare species, unique for the vegetation of Moldavia, grow numerous medicinal plants, some of which make the object of our study: *Tilia tomentosa*, *Rosa canina*, *Sambucus nigra*, *Achillea millefolium*, *Hypericum perforatum*, *Urtica dioica*, *Lamium album*, *Geum urbanum*, *Galium verum*, *Tussilago farfara*, *Consolida regalis*, and *Matricaria chamomilla*.
The vegetal material, consisting of leaves, was sampled during June and July, 2004. The fresh leaves were weighed and dried (105 ^oC) for the assessment of the contents in free water and relative dry weight.
The assimilatory pigments were extracted from leaves with acetone 85 % in the presence of $CaCO_3$. Subsequently, the extract was cleared through filtration. The identification of the pigment components was carried out with a spectrophotometer (wavelengths: 663 nm for chlorophyll a, 645 nm for chlorophyll b, and 427 nm for carotenoids).
The total mineral elements were investigated through the ash analysis resulted from the incineration of the dry vegetal material at 450 ^oC

Results

The research regarding the contents in water, dry weight, pigments, and mineral elements of the leaves, revealed certain differences among the analysed species, which are both taxonomically and ecologically dissimilar (Table 1.).

Table 1. Ecological Characteristics towards edafic and climatic factors

Species	Family	Soil trophicity	pH	Soil humidity	Exigencies of light and temperature
Tilia tomentosa Moench	Tiliaceae	3	3-4	3(4)	he,st
Rosa canina L.	Rosaceae	3	3-4	(1) 2-3	he,hs
Sambucus nigra L.	Caprifoliaceae	3	2-4	3-4	he,hs
Achillea milllefolium L.	Compositae	2-3	2-3	2-4	he
Hypericum perforatum L.	Hypericaceae	1	2-4	2-3	he
Urtica dioica L.	Urticaceae	3 (N)	2-5	3-4	he,hs
Lamium album L.	Labiatae	3 (N)	2-4	3	he,hs
Geum urbanum L.	Rosaceae	3	2-4	2-4	he,hs
Galium verum L.	Rubiaceae	1	2-4	2	he
Tussilago farfara L.	Compositae	2-3	2-3	3-4	he
Matricaria chamomilla L.	Compositae	2	2-3	3	he, st
Consolida regalis L.	Ranunculaceae	3	4	2	he, tf

The data show that the analysed species are highly tolerant to edafic factors and relatively not very demanding with regard to the climatic factors. Most of the species are heliophilous (helio-sciaphilous) and sub-thermophilous, with an ecological optimum in biotopes sufficiently lit during the active growing period.

The forest vertical stratification is primarily influenced by light intensity which decreases from top to bottom. In these conditions, the heliophilous species from the top storey had the cytoplasm less hydrated (58 % in *Rosa canina*, *Tilia tomentosa*) because of the intense transpiration; whereas the helio-sciaphilous and sciaphilous species from the storeys underneath the canopy had the cytoplasm more hydrated (65 – 80 % in *Lamium album* and *Tussilago farfara*, Table 2.).

Table 2. Physiological particularities in some medicinal plants from Bălteni forest.

Species	Water %	Dry weight %	Assimilatory pigments (mg/g fresh matter)						Mineral elements %	Organic matter %	Active substances (after Burzo, 2005 and Murariu 1991)
			Chlorophyll a	Chlorophyll b	Carotenoids	Total	a/b	(a+b)/c			
Tilia tomentosa	58.23	41.77	2.07	0.89	0.64	3.60	2.32	4.62	7.77	34	Flowers (essential oils, phenolic acids, mucilage,pigments,flavonoids, saponins)
Rosa canina	58.94	41.06	2.51	1.57	0.88	4.96	1.59	4.63	5.68	35.38	Fruits (tannins, ascorbic acids, carotenoidicand flavonoidic pigments, organics acids)

Sambucus nigra	74.62	25.38	1.75	0.67	0.52	2.94	2.61	4.65	10.80	14.58	Leaves (phenolic acids,tannis, essential oils, alkaloids)
Achillea milllefolium	76.17	23.83	1.43	0.55	0.40	2.38	2.60	4.95	11.64	12.19	Inflorescențand leaves (inulin, alkaloids, vitamins,essenti al oils, tannins, resins, mineral elements)
Hypericum perforatum	58.58	41.42	1.66	0.64	0.64	2.94	2.59	3.59	13.96	27.46	Resins, tannins, ascorbic acids, cafeic acids,essenti al oils, carotenoidic pigments
Urtica dioica	65.82	34.18	2.37	0.94	0.71	4.02	2.52	4.66	19.45	14.73	Folium (steroli, minerals elements,, ascorbic acids, pigments, phenolic acids).
Lamium album	65.40	34.60	1.78	0.66	0.56	3.00	2.64	4.35	17.15	16.46	Flavonoids, mucilage, saponins,tannin s,essential oils,glucosidevi tamins
Geum urbanum	56.28	43.72	1.14	0.64	0.38	2.16	1.78	4.68	17.69	26.03	(Tannins, essential oils, cafeic acids, starch, rafinose)
Galium verum	49.65	50.35	1.31	0.55	0.41	2..27	2.38	4.53	8.60	41.75	Tannins, organic acids, coumarin,
Tussilago farfara	80.00	20.00	1.00	0.41	0.30	1.71	2.43	4.70	16.24	3.76	Leaves (tannins, mucilage, alkaloids, pigments, steroli, mineral elements)
Consolida regalis	66.22	33.78	1.26	0.53	0.40	2.21	2.37	4.26	11.89	21.89	Flowers (glucoside,alkal oids)
Matricaria chamomilla	68.62	31.38	0.71	0.32	0.22	1.25	2.21	4.68	13.13	18.25	Fowers (essential oils, steroli, glucoside,vitam ins, pigments)

Although there is no positive relation between the quantity of the assimilatory pigments and the intensity of the photosynthesis, the quantity of pigments in heliophilous species of the tree storey is high (3.60 – 4.96 mg/g of fresh matter) which is necessary for the intense metabolic processes during summer. Consequently, in this season occurs the maximal accumulation of organic matter (81 – 86 %).

In herbaceous species, the ratios between the pigment components (a/b and (a+b)/c) are good indicators of the individual reaction to light. The value 1.78 of the a/b ratio shows a high receptivity of the chlorophyll a in *Geum urbanism*, whereas the decrease of the chlorophyll b results in high values in *Lamium album* (2.69), *Achillea millefolium* (2.60), *Hypericum perforatum* (2.59), and in *Urtica dioica* (2.52).
Regarding the ratio (a+b)/c, the individual differences among the woody species are less obvious (4.62 – 4.65), whereas in herbaceous species, despite the variability of chlorophylls (1.03 – 3.31 mg/g of fresh matter), the high quantities of the carotenoids (0.4 – 0.7 mg/g of fresh matter) significantly modifies the ratio value (4.26 – 4.95).
The knowledge of the mineral composition of the medicinal plants is particularly important for their role in the mineral nutrition of the forest plant community and for their better pharmaceutical usage.
The overall quantitative analysis revealed high values in herbaceous species (11.64 % in *Achillea millefolium* and 19.45 % in *Urtica dioica*). The substantial reduction in *Rosa canina* (5.68 %) proves an intense physiological activity in which the mineral elements are used in the synthesis of the organic matter (86 % of the dry weight).
The richness in active substances (vitamins, carotenoids) and secondary substances (phenols, volatile oils, terpens, alkaloids, and tannins) of leaves, flowers and fruits is responsible for therapeutic proprieties of these plants, which are being used in the naturist medicine.

Conclusions

Although the complex of ecological factors does not have a constant effect on the community composition, the quantitative differences regarding the contents in water, dry weight, pigments, and mineral elements of the leaves reveal the genetic variability of the investigated populations.
Depending on the light intensity, the heliophilous species display lower water contents of the cytoplasm because of the intense transpiration during summer.
In herbaceous species, the shadow maintains the higher hydration status of the leaves, because of the transpiration reduction and the quantity of the chlorophylls is strongly related with the progress of the biochemical processes in leaves, during bloom phenophase.
With regard to the investigated physiological indices and given the pedo-climatic conditions of Bălteni forest, the most valuable species are: *Tussilago farfara* (80 % water), *Galium verum* (50.35 % dry substance and 41.75 % organic matter), *Rosa canina* and *Urtica dioica* (4.96 and 4.02 mg total assimilatory pigments of which the chlorophylls have the highest proportion – 4.08 and 3.31 mg respectively), *Tilia tomentosa* and *Hypericum perforatum* (0.64 mg carrotenoids), *Urtica dioica* and *Geum urbanum* (mineral elements – 19.45 % and 17.69 % respectively).

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COMPARATIVE INVESTIGATION OF ORGANIC CHAMOMILE PRODUCTION IN DIFFERENT AGRO-ECOLOGICAL REGIONS OF GREECE AND SERBIA

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Summary

The organic model of cultivation of the Serbian chamomile (C. recutita (L.) Rausch) cv "Banatska", and essential oil yield was monitored throughout field experiments conducted in Serbia and Greece. Average yield of dry chamomile flowers obtained in Serbia was 778 kg/ha and in Greece was 767 kg/ha. The average content of essential oil in dry chamomile flowers in Serbia was 0,34 % and in Greece was 0,44 %.. The results showed that the content of essential oil appeared to be significantly higher in chamomile cultivated in Greece.

Keywords: *Chamomile, agro-ecological regions, organic fertilization*

Introduction

Chamomile [*Matricaria chamomilla* L. syn. *Matricaria recutita* (L.) Rauschert and *Chamomilla recutita* (Franz, 1993)] is a herb grown for its flowers, which are dried and used as a medicinal tea, as well as for essential oil production. It can be cultivated successfully with adequate quality of the drug in tropical areas of Ethiopia up to Central Europe (Letchamo and Vömel, 1992). Because the crop is new to Greece aspects of production require further research, especially the adoption of organic cultivation of this crop in the near future in both countries. In Italy for instance the half of chamomile production is produced through principals of organic farming (Vender, 2004).
The aim of this project was to study the performance of dry drug and oil yield of chamomile in different agro-ecological factors, as they are in Serbia and Greece, cultivated according to organic farming principals.

Material and methods

The cultivar tested was the Serbian "Banatska" (selection of the Institute for Medicinal Plant Research "Dr. Josif Pančić", Belgrade). Sowing was done in fall as chamomile needs to be sown as early as possible to ensure adequate growth before it commences flowering (Falzari, and Menary, 2003). Particularly, in Serbia was conducted on 05.10. 2004 (Graph 1), at rate 10 kg/ha and in Greece during the period 1-7.11.2004 (Graph 2), at sowing rate 6 kg/ha.
Field experiment in Serbia was conducted under dry farmyard conditions, in vicinity of Pančevo, (South Banat, Serbia), located at 81 m a. s. l., latitude $44°_{N}$, 52', 20" and longitude $20°_{E}$, 42', 25". Soil type is chernozem, on loess, of loamy-clay texture (ca. 35% clay), pH = 7.1, moderately supplied with humus and phosphorous and rich in potassium. The temperature and precipitation conditions of Pančevo are presentd in Graph 1. In the experiment the following variants of chamomile fertilization were tested:
1. Organic fertilization with cattle FYM – at 50 m^3 / ha rate
2. Mineral N at 30 kg/ha rate
3. Control treatment – without fertilization
FYM was incorporated in soil in August 2004., during plowing. Nitrogen mineral was applied as a top dressing in ammonium-nitrate form, by beginning of April 2005. The field was

previously cultivated with St. John's Wort the production of which was conducted in two years period without any application of fertilizer (period of conversion). Basic soil tillage was done in August, while the pre-sowing soil preparation took place in September, 2004. Additional care measures, except for the application of Nitrogen manure on variant 2, were not performed. A single harvest at full blossom stage was conducted on 24th May, 2005. All measurements were taken in 4 replications.

In Greece overall experimental approach to this project was a small-scale pilot field trials. In November of the year 2004 four experimental cultivations of chamomile were installed: two in Macedonia [Thermi (located at 5 m a. s. l., latitude $40°_N$, 31' and longitude $22°_E$, 58'). and Kalindria), one in Thessaly (Stefanovikio) and one in Sterea Hellas (Ag. Konstatinos). The temperature and precipitation conditions of Thermi are presentd in Graph 2. Basic soil tillage of the fields was done during summer, while the pre-sowing soil preparation took place in late October, 2004 just a week before sowing. The locations in Ag. Konstantinos, Stefanovikio and Thermi were fertilized with commercial organic fertilizer Agrobiosol (70-80% organic matter, N 6-8%, P_2O_5 0.5%, K_2O 0.5%) at rate 500 kg/ha and Acidam AVC 50 (50% element S and 10% element C) at rate 500 kg/ha. Both applied a week before sowing and they certified by official organizations, which certify organic farming in Greece. In table 1, the soil analysis of these locations are presented

Table 1. Soil texture and chemical analysis of three locations in Greece

depth (0-30cm)	Soil texture	pH 1:1	free $CaCO_3$	Organic matter %	P (Ols.) ppm	K ppm	B ppm	Ca /100g	Mg /100g
Thermi	SCL	7.73	2.64	1.43	44.57	520	0.57	2.47	0.36
Stefan.	CL	7.69	15.8	1.40	7.96	180	0.68	4.93	0.85
Ag. Kon.	SCL	7.65	15.0	1.07	8.44	260	0.57	4.28	1.21

In the cultivation of Kalindria no fertilizer or manure were applied but the field was uncultivated the previous year. A single harvest at full blossom stage was conducted in all fields during the first half of May. Particularly, on 5th May in Ag. Konstantinos 6th May in Thermi and on 13th May in Stefanovikio and Kalindria. To obtain essential oil yield, three samples of dry flowers of each locality were steam distilled for four hours.

Results and discussion

The yield of dry chamomile flowers in the first harvest (24. 05.2005) was presented in table 1.

Table 2. The yields of dry chamomile flowers under different fertilization treatments

Fertilization treatments	Yield of dry chamomile flowers	
	kg/ha	%
Cattle FYM - 50m^3/ha	778,4	178,8
Mineral N manure - 30 kg/ha	586,8	134,8
Control (no fertilization)	435,3	100
LSD 5%	*197,00*	

The highest average yield of chamomile was obtained in treatment with FYM in the first year following the application (interval of variation between replications being 725-931 kg/ha). The lowest yield was, as expected, in control treatment, while the treatment with mineral Nitrogen fertilization gave the increase of the yield half less in comparison to increase of yield obtained with FYM treatment. Since it is well-known that chamomile is not demanding crop regarding mineral nutrition (Bomme und Nast, 1998), it is obvious that the increased biological activity of the soil caused by application of FYM significantly improved

water/air/heating soil regime. This brought about noticeable better and faster plant development during cold winter months and early spring days, what consequently resulted in higher chamomile flower yields.
Content of essential oil in chamomile flowers was presented in table 2.

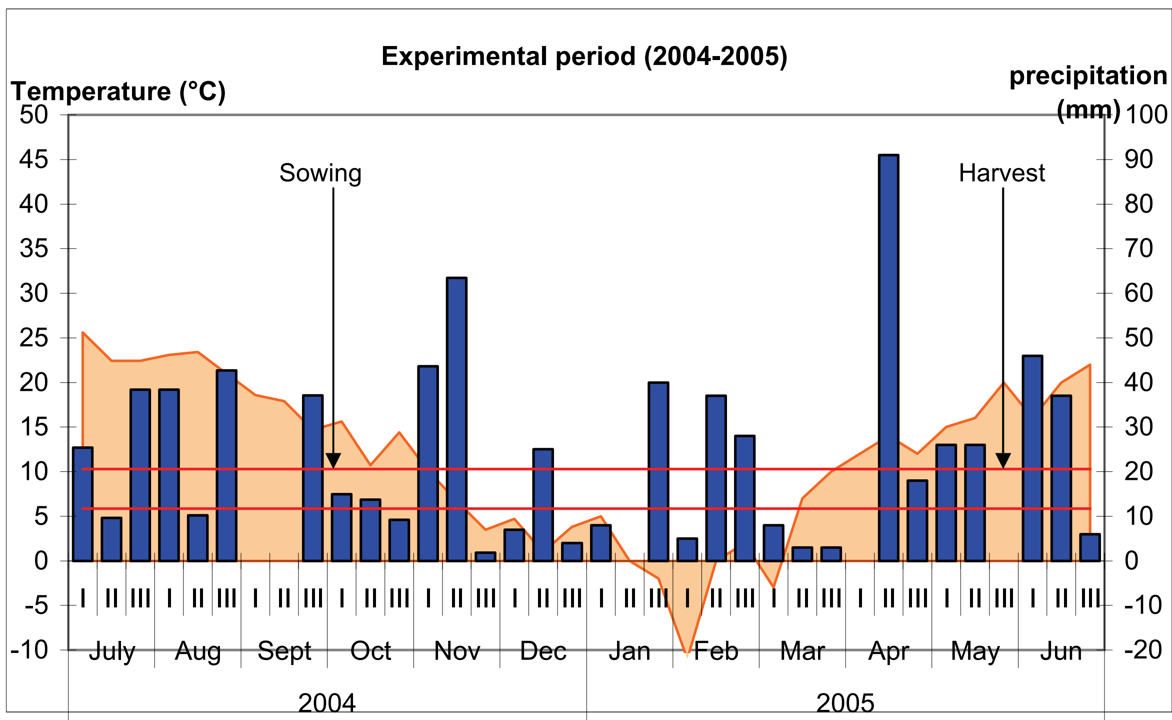
Table 3. Content of essential oil in chamomile flowers under different fertilization treatments.

Fertilization treatments	Essential oil from dry chamomile flowers (%)	
	%	Interval of variation
Cattle FYM - 50m^3/ha	0,340	0,27 – 0,41
Mineral N manure - 30 kg/ha	0,247	0,21 – 0,29
Control (no fertilization)	0,298	0,24 – 0,35

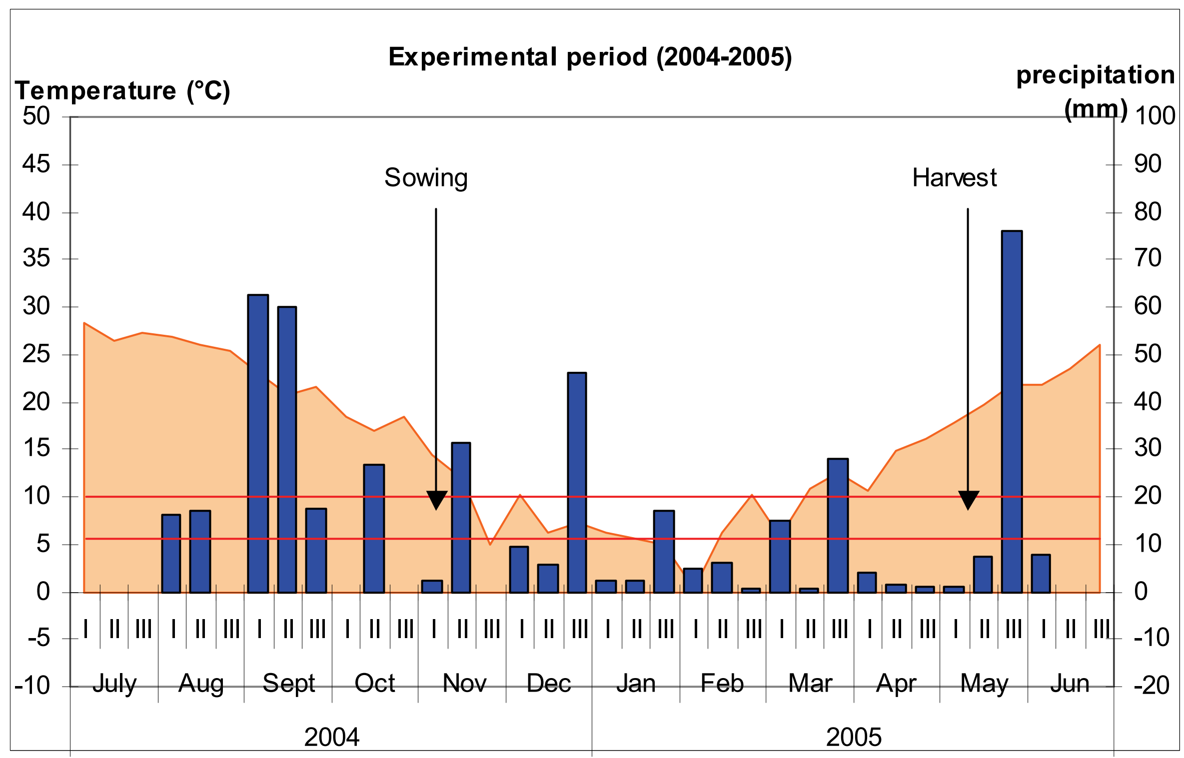
Chamomile plants in FYM treatment contained the highest percentage of essential oil, what reinforces already positive impression about the FYM treatment efficacy on chamomile yield. In treatment with mineral nitrogen, the content of essential oil in chamomile flower was the lowest, what points out on potentially harmful effects that imbalanced nutrition (nutrition with N only) may have on quality of chamomile flower.
Average yield of dry chamomile flowers obtained in Serbia was 778 kg/ha (ranging 725 – 931 kg/ha) and in Greece from 4 localities was 767 kg/ha (ranging 615 – 1027 kg/ha). The average content of essential oil in dry chamomile flowers cultivated in Serbia was 0,34 % (ranging 0,27 – 0,41%) and in Greece was 0,44 % (ranging 0,32 – 0,65%).
In Greece the average yield of dry chamomile flowers, estimated from 4 localities data, was 767 kg/ha. The yield of dry chamomile flowers and essential oil of each locality are presented in Table 4. The yield of flowers in both countries was similar except for the locality of Thermi where the yield of dry chamomile flowers was more than 1000 kg/ha. Falzari and Menary (2003) also reported yield of about a ton/ha. In Kalindria locality, where no fertilizer of any kind was applied but was uncultivated the previous year, the yield of dry chamomile flowers and essential oil obtained, pronounces the importance of a year rest field for soil fertility, which was the usual practice of traditional farming enhancing soil fertility. The differeces in essential oil yield between the two countries, even if the data defy statistical analysis, are obvious and might be attributed to climate differences. Annual temperature and precipitation data of Thermi locality (representative of Greek climate) (Graph 2) and the respective data of Pančevo (representative of Serbian climate) (Graph 1) have big differences. In Greece the winter is milder than Serbia and the annual precipitation is almost the half as much as in Serbia. It seems that these conditions are favorable for high oil yield.

Table 4. Yield of dry chamomile flowers and essential oil in four localities in Greece

Localities	Yield of dry chamomile flowers		Essential oil from dry chamomile flowers (%)
	kg/ha	fresh/dry %	%
Thermi	1027	24.00	0,35
Stefan.	615	30.00	0.33
Ag. Kon.	675	30.95	0.34
Kalindria	750	33.00	0.65



Graph. 1. Diagram of 10 days average temperature and precipitation for locality of Pančevo, South Banat, Serbia.



Graph. 2. Diagram of 10 days average temperature and precipitation for locality of Thermi Macedonia Greece.

In conclusion, the results showed that between two tested agro-ecological regions (Serbia and Greece), there are no significant differences regarding the obtained yield of dry chamomile flowers but the content of essential oil appeared to be significantly higher in chamomile cultivated in Greece.

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RESEARCH REGARDING THE CORRELATION BETWEEN TOTAL LIPIDS CONTENT AND BIOSYNTHETIC CAPACITY AT DIFFERENT ALKALOID TYPES STRAINS OF *CLAVICEPS PURPUREA* SCLEROTIA

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Summary

In this paper we present the results concerning the correlation between total lipids content and biosynthetic capacity expressed by total alkaloid content. We have analysed ten dried Claviceps purpurea sclerotia of ergotamine and ergocristine types, harvested from rye artificial infected. At ergotamine strains the total lipids amount is higher than the ergocristine strains, behaviour that is not present at the biosynthetic capacity. We find also that the ergotamine strains studied present a higher weight than the ergocristine type.

Keywords: *Claviceps purpurea, alkaloids, lipids, biosynthetic capacity,*

Introduction

The interest in ergot alkaloids is great because theirs derivatives that are potentially therapeutic agents for parkinsonian, acromegaly, amenorrhea-galactorrhea, suppression of postpartum lactation, treatment of breast cancer and possibly cancer of the prostate, migraines and the symptoms associated with them, ortostatic circulatory perturbations, senile cerebral insufficiency, hypertension as well other affections in which their antibacterial, hipolipemic and immune-regulator effects are studied (DESAI, J.D. at al. , 1983; SURDU et al., 2005).
The lipid metabolism is associated with alkaloid biosynthesis because the common precursor acetyl-CoA. The literature mentions that lipid accumulation, products of primary metabolism, parallels alkaloid yield in some cases (there is no evident competition for their synthesis) or, the amount of fatty acids increases until the beginning of alkaloid biosynthesis in other cases. In the last case, after the release of alkaloids biosynthesis process the total lipids content is approximately constant. More, it is considered that the presence of lipids inclusion is typical of strains capable to produce alkaloids (SURDU S. et al.).
Some researches results substantiate that a common regulatory moment or moments participates in the synthesis of alkaloids and lipids. The main components of the lipids are linoleic acid, palmitic acid, oleic acid and in small amounts palmitoleic acid, stearic acid and ricinoleic acid.
Generally, the strains of *Claviceps purpurea* have different biosynthetic alkaloid capabilities and predominantly produce a certain peptide alkaloid.

Materials and methods

The biological material is represented by ergotamine and ergocristine type dry sclerotia harvested from Ergo race autumn rye plants artificially infected with conidia suspension of *Claviceps purpurea* obtained in submerged cultures. There have been used five strains of each alkaloid type.
For total alkaloid amount determination, the Rumpel method has been employed (RUMPEL, W., 1955). It consists of alkaloids extraction with a methanol solution of tartaric acid and extract purification with zinc acetate. The extract reacts with the sulphuric solution of p-dimethylaminobenzaldehyde, forming a blue compound, measurable by photoelectric colorimeter.

For total lipids determination we used the method described by Soxhlet, the most commonly used example of a semicontinuous method applied to extraction of lipids. According to the Soxhlet's procedure, oil and fat from solid material are extracted by repeated washing (percolation) with an organic solvent, usually hexane petroleum ether or ethyl ether under reflux in a special glassware (ARTENIE V., TĂNASE E., 1981).
The results reported here are average values from three independent determinations and have been statistically processing.

Results and discussions

The mature sclerotia, harvested from Ergo race autumn rye plants, was dried at 50°C until constant weight. The strains utilised have been conventionally noted, after the predominant alkaloid, T1, T2, T3, T4, T5 and S1, S2, S3, S4, S5 for ergotamine (T) and respectively ergocristine (S) types.
The total alkaloid content from the first harvest of ergocristine and ergotamine sclerotia is presented in table I.

Table I. The total alkaloid content CAT) of ergosristine and ergotamine type *Claviceps purpurea* sclerotia

Crt. No.	Strain type	CAT(g%)
1	S1	0,64
2	S2	0,67
3	S3	0,69
4	S4	0,70
5	S5	0,72
6	T1	0,61
7	T2	0,61
8	T3	0,68
9	T4	0,75
10	T5	0,79

The data were statistically analysed for both alkaloid types (table II).

Table II. Statistical analysis of total alkaloid content of ergocristine and ergotamine type *Claviceps purpurea* sclerotia

Crt. No.	Statistical indicator	Ergocristine strains	Ergotamine strains
1	Mean	0,684	0,688
2	Standard error	0,013638182	
3	Median	0,69	
4	Mode	#N/A	#N/A
5	Standard deviation	0,030495901	0,081363382
6	Sample variance	0,00093	0,00662
7	Kurtosis	-0,003468609	-2,379450717
8	Skewness	+0,542995584	0,270688958
9	Range	0,08	0,18
10	Minimum	0,64	0,61
11	Maximum	0,72	0,79
12	Sum	3,42	3,44

13	Count	5	5
14	Confidence level (95%)	0,037865741	0,101026192

The standard deviation's little values indicate the fact that the data are homogenous around mean values.
We have tested the hypothesis if the total alkaloid content is the same at ergocristine and ergotamine strains in the case of a 95% confidence level (table III).

Table III. t-test for CAT at ergocristine and ergotamine strains

Crt. No.	Statistical indicator	Ergocristine strains	Ergotamine strains
1	Mean	0,684	0,688
2	Variance	0,00093	0,00662
3	Observations	5	5
4	Hypothesized Mean Difference	0	
5	df	5	
6	t Stat	**-0,102937003**	
7	P(T<=t) one-tail	0,461007024	
8	t Critical one-tail	2,015049176	
9	P(T<=t) two-tail	0,922014049	
10	t Critical two-tail	**2,570577635**	

The tested hypothesis indicates the fact that the total alkaloid content is the same indifferent the tested strain alkaloid content.
The results of total lipids determinations are presented in table IV.

Table IV. The total lipid content of ergocristine and ergotamine type *Claviceps purpurea* sclerotia

Crt. No.	Strain type	Total lipids (g%)
1	S1	31,46
2	S2	39,00
3	S3	32,41
4	S4	41,26
5	S5	32,74
6	T1	36,35
7	T2	35,84
8	T3	41,63
9	T4	41,35
10	T5	40,94

In the total lipids determination there are not errors (table V) and we tested further on the hypothesis of the 4g difference between the ergocristine and ergotamine type strains in the case of 95% confidence level (table VI)

Table V. Statistical analysis of the total lipid content of ergosristine and ergotamine type *Claviceps purpurea* sclerotia

Crt. No.	Statistical indicator	Ergocristine strains	Ergotamine strains
1	Mean	35,374	39,222
2	Standard error	1,985390642	1,283835659
3	Median	32,74	40,94
4	Mode	#N/A	#N/A
5	Standard deviation	4,439468437	2,870743806
6	Sample variance	19,70888	8,24117
7	Kurtosis	-2,422744943	-3,183416241
8	Skewness	0,701549855	-0,593565729
9	Range	9,8	5,79
10	Minimum	31,46	35,84
11	Maximum	41,26	41,63
12	Sum	176,87	196,11
13	Count	5	5
14	Confidence level (95%)	5,512339546	3,564506615

Table VI t-test for total lipid content at ergocristine and ergotamine strains

Crt. No.	Statistical indicator	Ergotamine strains	Ergocristine strains
1	Mean	39,222	35,374
2	Variance	8,24117	19,70888
3	Observations	5	5
4	Hypothesized Mean Difference	4	
5	df	7	
6	t Stat	**-0,064289093**	
7	P(T<=t) one-tail	0,475268717	
8	t Critical one-tail	1,894577508	
9	P(T<=t) two-tail	0,950537434	
10	t Critical two-tail	**2,36462256**	

The tested hypothesis indicates that total lipid content is different for the two analysed alkaloid types. The mean difference between ergotamine and ergocristine types is 4g.
To find weight average we have considered 20 sclerotia for each determination. The results are presented in table VII.

Table VII The weight of ergocristine and ergotamine type *Claviceps purpurea* sclerotia

Crt. No.	Strain type	Weight (g/sclerotium)
1	S1	0,05718
2	S2	0,04078
3	S3	0,05600
4	S4	0,07625
5	S5	0,06718
6	T1	0,09379
7	T2	0,07388
8	T3	0,09393
9	T4	0,07444
10	T5	0,07375

We have statistically analysed the results and we have concluded the homogeneousness around the mean:

Table VIII Statistical analysis about the weight of ergocristine and ergotamine type *Claviceps purpurea* sclerotia

Crt. No.	Statistical indicator	Ergocristine strains	Ergotamine strains
1	Mean	0,061268	0,081958
2	Standard error	0,00466824	0,004860405
3	Median	0,05718	0,07444
4	Mode	#N/A	#N/A
5	Standard deviation	0,10438502	0,010868195
6	Sample variance	0,000108962	0,000118118
7	Kurtosis	-0,630480519	-3,329126646
8	Skewness	0,668844587	0,606094432
9	Range	0,02647	0,02018
10	Minimum	0,04978	0,07375
11	Maximum	0,07625	0,09393
12	Sum	0,30639	0,40979
13	Count	5	5
14	Confidence level (95%)	0,012961139	0,013494675

We have tested the hypothesis that the indicator has comparable values at ergocristine and ergotamine strains with a 95% confidence level (table IX).

Table IX t-test for the weight of ergotamine and ergosristine type *Claviceps purpurea* sclerotia

Crt. No.	Statistical indicator	Ergocristine strains	Ergotamine strains
1	Mean	0,061278	0,081958
2	Variance	0,000108962	0,000118118
3	Observations	5	5
4	Hypothesized Mean Difference	0	
5	df	8	
6	t Stat	**-3,068641142**	
7	P(T<=t) one-tail	0,007689791	
8	t Critical one-tail	1,85954832	
9	P(T<=t) two-tail	0,015379583	
10	t Critical two-tail	**2,306005626**	

The test's results indicate that the ergocristine sclerotia weight is different from ergotamine sclerotia weight in favour of ergotamine type.

Conclusions

The alkaloids and lipids have common precursors and parallel biosynthetic way for these compounds. Our study praise that at comparable biosynthetic alkaloid capacity ergotamine strains has a total lipid content higher than ergocristine strains. In concordance with these results is the weight of ergotamine and ergocristine type *Claviceps purpurea* sclerotia which is also higher at ergotamine type.

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WILD AND CULTIVATED MEDICINAL PLANTS - AN IMPORTANT POTENTIAL FOR THE SUSTAINABLE ECONOMIC DEVELOPMENT OF ROMANIA

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Summary

A survey of the medicinal plants – a valuable natural resource of Romania, the tradition of their preparation, use and processing as well as the analysis of the industrial dimension of this activity is presented. Special attention was focused on the scientific results and the previous trade success, registered before the collapse of communism. New trends on the market and opportunity for business development are also reviewed.

Keywords: *medicinal plants, traditional use, industrial processing, sustainable development*

Medicinal plants – a valuable natural resource

The variety of soil, climate and relief resulted in rich and diverse vegetation in Romania. Almost 30-40% of the European flora and fauna could be found here. Out of the near 4000 species of registered higher plants, around 700 are traditionally used as medicinal (Parvu, 1991), 324 species scientifically proved to have therapeutic properties and 180 species can be used at industrial scale for plant extraction and different natural product obtaining.
Romanian flora has more than hundred species of tinctorial value, which contain very resistant and bio-degradable pigments, used in food industry as coloring agents ("digestive pigments"), cosmetic (hair care) or natural fiber dieing (especially wool and cotton). There are also 40 species interesting for their content in tannins (accumulated in bark, wood, leaves or fruits) and resins (mainly extracted from stem or buds).
Other 80 species were identified as toxic, due to their high content in pharmacologic active principles, which could have damaging – even lethal - effects on beast and people if plants are accidentally graze or eaten, respectively. Strictly controlled, these species and their chemical compounds are used in veterinary and human medicine.
Different criteria (such as botanic, chemical, geographical as well as industrial importance) could be used to classify the Romanian medicinal plants and to define the specificity of local bioresources.
Taking into account the life cycle, 51% of the medicinal plants are annual species, 31% are perennial species and 18% are biannual species. As to the life forms, we find in Romania: 36.4% hemi cryptophytes, 20.06% therophytes and 17.9 phanerophytes; the rest of ¼ is composed by geophytes (8.64%), hemitherophytes (7.01%), chamaephytaes (4.32%), hydro-helophytes (1.54%), hydrophytes (1.54%), chamaephytaes-hemicryptophytes (1.23%), hemicryptophytes-hemitherophytes (0.30%) and epiphytes (0.30%).
The medicinal species that could be found frequent in spontaneous flora belong to the Asteraceae, Labiatae and Rosaceae families (5-10%), less frequent are found the members of Ranunculaceae, Fabaceae and Aristolochiaceae families (2-5%), while not at all frequent are the members of the Poaceae and Violaceae families (less than 2%).

Traditional use of medicinal and aromatic plants

Historical and ethnographic data show a long tradition of medicinal plant use as therapeutic remedies in most of the houses, villages, monasteries and hospitals of the country. First scientific references appeared 400 years BC, but starting with the XIVth centuries a lot of

aspects started to be investigated (for example, 431 species have been listed as medicinal in the XVIIth century, together with their recommendation of use in different disease treatment-Paun, 1995).
The old activity of wild collection and manufacture transformed plants in different phyto-pharmaceutical products (such as powder, infusion, hydro-alcoholic plant extracts, volatile oils, medicinal and aromatic vinegar, syrups, creams, plant baths, etc) easy to be produced by everybody and used in most of the families (Fischer, 1999).

Scientific and economic results

The industrial dimension of this activity was reached at the beginning of XXth century, when a national network of plant processing industry (PLAFAR TRUST) was set up (it covered 25% of Romanian administrative units and has headquartered in Bucharest). In the years '80 Romania reached its best performance in the field, becoming the 5th exporter of the world (medicinal plants were exported in 20 countries); it got also an important position (the 8th place) as volume of processed medicinal plants/year (Stoianov, 2003).
As a consequence, an enhancing of phytotherapy and pharmaceutics industry interest was registered; over 500 species were the subject of different botanical and phytosociology studies, and more than 180 medicinal species were analyzed pharmacological and pharmacodynamic point of view (for example, 79 Ph. D. were written in the period 1958-1975, only in this field – Racz et al., 1986) and most of the important monographs and plant inventories (of medicinal, aromatic, toxic and tinctorial species) were published. A lot of assessment studies, cartography and biomass evaluation as well as impact studies on the environment were done (it should be noticed that in the year 1990, there were collected from spontaneous flora 150 species). All the responsible ministries created and enhanced their own networks of research and development: Education Ministry (Universities: Botany departments in all the Faculties of Biology), Health Ministry (Medicine and Pharmacy Institutes: Pharmaceutical Botany, Pharmacology and Pharmacodinamy departments), Agriculture and Forestry Ministry (Research and Production Stations, National Institutes, Academy of Agricultural and Forestry Sciences-that were involved in cultivation technologies, plant breeding and seed production, other forest products than wood, respectively), Research and Technology Ministry (Institutes, Centers of Research - that studied medicinal and aromatic plants from botanical, genetic, biochemical and ecologic point of view, different *in vitro* biotechnologies, plant protection), Romanian Academy of Science (Botanical Institute-that focused on endangered and protected species, natural reserves and protected areas). Each Botanical Garden (Iasi, Bucuresti, Craiova, Timisoara and Cluj) developed a medicinal plant sector, while in Targu Mures it was created an entire medicinal plants botanical garden, where approximately 1000 taxa could be seen. Collections of 70-200 species are found in all the important universitary cities, used for demonstrative purpose and student practice. On the other side, Natural Science Museums started to collect local medicinal flora and to create herbarium. In the same time, almost all the schools from villages were involved in wild collection activities (starting from spring up the autumn), getting thus additional funds/financial support for the education process and the poor pupil' aids.
Hundreds of research and development projects as well as the intense work aiming to identify, evaluate and manage this important natural resource of Romania, resulted in a valuable scientific support, which explain the successful economic and trade activity. The increasing of industrial needs was correlated with the rise (starting with 1925) of the agricole cooperatives (named ADONIS, CHAMOMILLA, DIGITALIS), specialized in certain plant production. Medicinal plants were cultivated on large areas (up to 41 000 ha), producing

approximately 20 000 tones/year of dry weight material (30% leaves, approximately 16% flowers, 15% herba, 13%fruits, 11% roots, less than 5% seeds, 2.5% bark, 2% buds).
The agronomist researchers have studied 52 species of cultivated medicinal and aromatic plants (Mocanu, 1999). The eco-physiological needs of the cultivated species were established (the most appropriate region for each species was designed) and the map of natural geographic distribution of medicinal plants was set up. The main cultivated species were (and still are): *Coriandrum sativum, Sinapis alba, Brassica nigra, Foeniculum vulgare, Cynara scolymus, Hyssopus officinalis, Silybum marianum, Papaver somniferum, Mentha piperita, Mentha crispa, Salvia officinalis, Calendula officinalis, Melissa officinalis.*
During the last 25 years, there were homologated 29 cultivars of 17 species *Coriandrum sativum* (Sandra/82, Omagiu/2000), *Datura innoxia* (Laura/82, Silvia/84), *Papaver somniferum* (Extaz/82, Safir/82), *Matricaria recutita* (Margaritar/82, Flora/89), *Valeriana officinalis* (Magurele/82), *Cynara scolymus* (Celesta/89, Unirea/93, Flavia/00), *Mentha piperita* (Cordial/89, Cristal/2000, Columna), *Mentha crispa* (Record/92, Mencris), *Lavandula angustifolia* (Corbeanca/92), *Thymus vulgaris* (Smarald/93), *Vinca minor* (Azur/96), *Digitalis lanata* (Tonic/00, Lanata-1), *Ocimum basilicum* (Basilica00, Geea/00), *Tagetes patula* (Tages/96), *Trigonella foenum graecum* (Robusta/00), *Foeniculum vulgare* (Hestia/01), *Artemisia dracunculus* (Armonia/01, Artemis/01). Other 24 species were domesticated (*Achillea millefolium, Acorus calamus, Angelica archangelica, Atropa belladonna, Carum carvi, Chelidonium majus, Gentiana lutea, Hypericum perforatum, Plantago lanceolata, Valeriana officinalis, Vinca minor*), 10 species were acclimatised (*Amsonia tabernaemontane, Digitalis lanata, Echinacea purpurea, Glaucium flavum, Grindelia robusta, Momordica charantia, Satureja montana, Securinega suffruticosa, Solanum laciniatum*) and 31 valuable local landraces were certified.
More than 20 technologies of cultivation (about 80 technological sequences) have been created, succeeding to establish: the preceding culture, soil preparation, fertilisation, methods of plant multiplication, sowing period, seed rate, sowing depth, raw intervals, maintenance requirements, disease prevention and cure, damaging insects control, harvesting methods, drying and storage conditions, processing techniques for fresh and dry raw material, etc.
The sustainable use of local resources (63 species mainly collected in 2000) asked the biodiversity conservation (today 297 species are *ex situ* persevered, 179 species are hold by Suceava Gene bank and 13 species, are on the National Red List (under the severe control of Romanian Academy of Science - Committee for the Nature Monument Protection). The National Catalogue of Plant Genetic Resources was published in 2000, with IPGRI'support, where the medicinal and aromatic plants cover a fascicule (Murariu et al., 2002).

Transition period and new trends on medicinal plants market

After years '90, the significant political changes resulted in the state monopole brake. This was also dramatically reflected in the food chain, which links were destroyed. In agriculture, the famous cooperatives disappeared and now, up to 84% of the farms are private. During 1990-2000 (a negative reference year for medicinal plants-Romanian statistical year book, 2004), the cultivated areas registered a significant decrease (less than 5 000 ha and a production of 1500 t dry weight, only 800 t collected from the wild, out of which 210 t were exported) and followed an unpredictable dynamics, being out of any control or national strategy. Very slow steps were made towards the organic farming of medicinal plants (in 2004 there were only 5 farms interested to certify their crop production), in spite of the existing promising prospects, due to the small quantities of chemical fertilizers used in the past (four times less than in the EU countries) and the severe decline of Romanian industry responsible by environment pollution.

As a paradox, in the same period, real progresses were registered by the processing industry. The market pressure (the increasing demands for natural products), the people trust in green pharma, the low price and the traditional use of medicinal plants influenced the development of private SME, which proved to be flexible and better adapted to the customer needs and very efficient from economic point of view. They implemented new technologies using special financial support (EU funds such as PHARE and SAPARD, WORLD BANK as well as governmental co-financing), diversified their production (40 medicines obtained from medicinal plants and near 1300 food supplements) and developed new business partnerships as well as import-export activities. SME proved to be responsible and progressive, implementing the European standards of Quality Management System (they are certified ISO 9001/2000 and 14 000), Good Manufacture Practice (for medicine production, plant cultivation, plant collection), HACCP (for food supplements), etc. They were able to follow the European trend (starting to produce organic products) and were able to sustain their own research, oriented towards the specific needs.

Regulation, monitoring and control activities

A review of the last law and regulations, show that Romania aimed to harmonize the own legislation with the international rules and directives; thus it was signed the Convention from Rio (1992) regarding the biodiversity, approved the Strategy for Plant Conservation from Haga (1992) and implemented TRAFIC Program (in 1998, as soon as CITES Convention (international trade agreement related to wild flora and fauna) was adopted. From 2003, it is currently active the Law 491, which refers specifically to medicinal, aromatic, toxic and drug plants.
The central authorities involved in rules establishment are the Government (Ministry of Agriculture, Forestry and Rural Development, Ministry of Health, Ministry of Environment and Water Management), Romanian Academy of Science and the Parliament. Some advisory boards (such as the National Council of medicinal plants and natural products, including food supplements) work near the Ministries (of Agriculture, Forestry and Rural Development, in this case), and special commissions are organized by the Senate and Chamber of Deputies.
The national and local authorities responsible for monitoring and control are: the National Drug Agency, National Agency for Veterinary Medicine and Food Safety, Environment Protection Agencies, National Agency for Customer Protection. As certification bodies for medicinal plant products there are designated Health Public Institutes and Food Bioresource Institute (for food supplement notification) and National Drug Agency (for drugs/medicine products). Aiming to join the EU, Romania had to take into account the intellectual and industrial protection (that’s why a national office, responsible for copy rights and a National Agency for Standards and Labels are active).
The civilian society started also to organize professional, scientific or economic associations. Thus, ROPAM (which is an observer member of EUROPAM) create a network of medicinal plant producers and extension service providers for agriculture, PLANTA ROMANICA put together the Romanian processors of medicinal plants, the ASSOCIATION OF IMPORTERS of medicinal plants and natural products supply the market with drug and food supplements, while different national societies (such as the SOCIETY OF PHYTOTHERAPY, ROMANIAN SOCIETY OF ETNOPHARMACOLOGY, NATIONAL SOCIETY OF HOMEOPATHY) bring together scientists and develop non-profit activities.
Interesting partnerships (public and private; scientific, economic and business development) were registered during the last years, focusing on the same subject, namely medicinal and aromatic plants. Running projects together, the public institutions in cooperation with interested private companies and other organizations (such NGO-s) started to do the first

steps to recover the product chain and to come near the previous success of the field. A real support for agriculture (that continues to be a vulnerable sector) came from the Ministry of Agriculture, which introduced medicinal plants in rural development strategy, subvention the cultures. In the mean time, the Ministry of Education and Research implements a new, modern information system, which contain a national database of large interest and could be accessed on-line.
In spite of the slow progress of society and all difficulties (especially mentality changes of the people) registered during this long period of transition, it seems that medicinal and aromatic plants have a promising future, due to the constant interest of the market for the natural products, the existing experience and development potential, and the new legal frame work which was set up.

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THE ROLE OF THE BOTANICAL GARDENS OF TARGU-MURES IN EX-SITU CONSERVATION OF RARE AND ENDANGERED SPECIES OF ROMANIA

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Summary

The unprecedented development of human civilization as an outcome of the scientific and technical innovations exercises a great pressure on Nature and its resources. This generates ecological disorders which in time will develop into a severe ecological crisis. Because of this one of the most important duties of the contemporary world is the conservation of species endangered due to different impacts and massive exploitations. We consider that due to the pertinent information obtained through our research regarding threatened, rare and vulnerable plants, they will be introduced in a crop system. The red *list of superior plants from Romania contains an inventory of **51** species of plants.*

Keywords: *conservation, rare and endangered taxa, endemic plants, medicinal species*

Introduction

Romania can be proud of a great variety of flora, but lately due to the impact people exercised upon the flora, we can assist to an unfortunate vandalism towards the exploitation of natural resources. By a judicious, limited and controlled planning of exploitation we can avoid the danger of disappearance of some medicinal plant species.
Because the harvest of plants will continue to be an economical issue in the following decays too, the initiation of some experiences which permit introduction in their culture will be implemented. This will be the task of those specialists who wish to contribute to the preservation of these plants in our landscapes.
Romania has an old established tradition in the introduction of endangered medicinal plants in the cropping system. The first experimental station of medicinal plants in the world was established in Cluj-Napoca in 1904. This was the station which provided the framework to the introduction of wide range of plants from the spontaneous flora into the cropping system.
The beginning of industrial growing of medicinal plants was realized a few decays ago by introducing *Mentha x piperita* from the Land of Bârsei (Ţara Bârsei) into the cropping system, followed by *Digitalis purpurea* from Orăştie, etc. This form of plant cultivation was extended to other renowned stations in the country (e.g. Fundulea etc.).
The botanical garden of the University of Medicine and Pharmacy, Târgu-Mureş was established in 1948 for didactic purposes, since then being a real school for training students, but at the same time an institution with an outstanding scientific value, renowned both on national and international level, also having real merits in activities meant to protect rare and endangered plants and in the introduction of some plant species in the cropping system in natural and optimal conditions. A true protection of rare and endangered medicinal plants is performed here, proving that in case of some plants cropping is more profitable and the obtained raw material is more valuable. This way the annual raw material can be ensured so that the vegetal genofond of therapeutic interest is preserved.

Material and methods

The list of endemic, rare and endangered species has been elaborated on the base of: *The World Conservation Monitoring Centre* (WCMC), *Globally threatened plants in Europe*, *The*

World Conservation Union (IUCN), *PLANTA EUROPA*, *Law. nr.13/1993* regarding Romania joining the *European Habitats* and *Wild Life Convention,* adopted at Berne on 19th September 1979 etc. [5,6,7,11,12,13,14,15,16]; *The red list of vascular plants in Romania* – elaborated by M.Oltean, et.al.(1994); *The red list of rare, vulnerable endangered, and disappeared vascular plants in Romanian flora* - N. Boşcaiu, et.al.(1994); *The red list of the Romanian plants occurring in meadow, including endemic and sub-endemic species* (*Tracheophyta*)-G. Negrean (2001) [1,2,3].
In this work the right solutions were adopted as correct in accordance with the *Code de Tokio* [4], *Flora Europaea* [8] and Flora României [9,10].

Results and discussions

Based on these information, in Botanical Garden were inventoried 51 species of rare, endangered and vulnerable plants from the spontaneous flora until now (table 1).
Vascular taxons are present in alphabetical order denominating the family and *the risk category* IUCN (World Conservation Union) they belong to [16].
The red list containing also numerous medicinal plants: *Adonis vernalis, Angelica archangelica, Bryonia cretica* ssp. *dioica, Dictamnus albus, Digitalis ferruginea, Ecballium elaterium, Galanthus nivalis, Gentiana lutea* , *Glaucium flavum, Gypsophila paniculata, Hyoscyamus niger, Paliurus spina-christi, Periploca graeca, Polemonium caeruleum, Ruta graveolens, Salvia sclarea, Taxus baccata* and *Ziziphus jujuba*.
Rush and rapacity regarding a short term profit determines the diminution of medicinal plant populations. It is known that *Romania exports huge quantities of medicinal plants.* This is the reason why some measures of protection should be instituted and rare and endangered plants should be introduced in production for their use in phytotherapy.

Table 1. Rare and endangered plants of the Romanian flora cultivated in the Botanical Garden of the University of Medicine and Pharmacy, Târgu-Mureş

IUCN Category	Species Name	Family
V/R	*Acanthus balcanicus* Heywood & I.B.K.Richardson	*Acanthaceae*
B R	*Achillea depressa* Janka	*Asteraceae*
V	*Achillea ptarmica* L.	*Asteraceae*
V	*Adonis vernalis* L.	*Ranunculaceae*
R	*Agrimonia pilosa* Ledeb.	*Rosaceae*
V	*Angelica archangelica* L.	*Apiaceae*
R	*Bryonia cretica* L. ssp. *dioica* (Jacq.) Tutin	*Cucurbitaceae*
V	*Aquilegia nigricans* Baumg.	*Ranunculaceae*
R ●●	*Aquilegia nigricans* Baumg. ssp. *subscaposa* (Borbas) Soó	*Ranunculaceae*
E/R	*Centaurea ruthenica* Lam.	*Asteraceae*
B R	*Centaurea sadleriana* Janka	*Asteraceae*
R	*Dianthus barbatus ssp. compactus* (Kit.) Heuffel	*Caryophyllaceae*
B R	*Dianthus capitatus* Balbis ex DC. ssp. *andrejowskianus* Zapal.	*Caryophyllaceae*
R	*Dianthus collinus* W. et K.	*Caryophyllaceae*
R	*Dianthus leptopetalus* Willd.	*Caryophyllaceae*
V/R	*Dictamnus albus* L.	*Rutaceae*
R	*Digitalis ferruginea* L.	*Scrophulariaceae*

E	*Ecballium elaterium* (L.) A. Rich.	*Cucurbitaceae*
R	*Echinops bannaticus* Rochel ex Schrader	*Asteraceae*
R	*Echinops ritro* L. ssp. *ruthenicus* (Bieb.) Nyman	*Asteraceae*
LR	*Fritillaria orientalis* Adams	*Liliaceae*
nt	*Galanthus nivalis* L.	*Amaryllidaceae*
R	*Gentiana lutea* L.	*Gentianaceae*
R	*Gladiolus imbricatus* L.	*Iridaceae*
V	*Glaucium flavum* Crantz	*Papaveraceae*
V	*Gypsophila paniculata* L.	*Caryophyllaceae*
LR ●●	*Hepatica transsilvanica* Fuss	*Ranunculaceae*
R	*Hyoscyamus niger* L.	*Solanaceae*
V ●	*Iris brandzae* Prodan	*Iridaceae*
R	*Iris sibirica* L.	*Iridaceae*
V	*Iris spuria* L.	*Iridaceae*
R ●	*Larix decidua* Mill.	*Pinaceae*
V	*Leucojum vernum* L.	*Amaryllidaceae*
K	*Lonicera alpigena* L.	*Caprifoliaceae*
R	*Lychnis viscaria* L. ssp. *atropurpurea* (Griseb.) Chater	*Caryophyllaceae*
R	*Muscari neglectum* Guss. Ex Ten	*Hyacinthaceae*
V/R	*Nepeta ucranica* L.	*Lamiaceae*
VU	*Paeonia tenuifolia* L.	*Paeoniaceae*
R	*Paliurus spina-christi* Miller	*Rhamnaceae*
R	*Periploca graeca* L.	*Asclepiadaceae*
R	*Polemonium caeruleum* L.	*Polemoniaceae*
R	*Potentilla astracania* Jacq.	*Rosaceae*
V ●	*Pulsatilla pratensis* Miller ssp. *hungarica* Soó	*Ranunculaceae*
R	*Pulsatilla vulgaris* Miller ssp. *grandis* (Wender.) Zamels	*Ranunculaceae*
R	*Rumex scutatus* L.	*Polygonaceae*
R	*Ruta graveolens* L.	*Rutaceae*
R	*Salvia amplexicaulis* Lam.	*Lamiaceae*
R	*Salvia sclarea* L.	*Lamiaceae*
R ●●	*Salvia transsilvanica* (Schur ex Griseb.) Schur	*Lamiaceae*
R	*Taxus baccata* L.	*Taxaceae*
V	*Ziziphus jujuba* Mill.	*Rhamnaceae*

The periclitation categories: R - Rare, **E -** Endangered**, V** - Vulnerable**, nt** – not threatened, **K** - Known insufficiently, **LR** – Less Risk,●● - Endemic, ● - Sub-endemic, **B** - Convention of Berna

Conclusions

From the flora point of view it is important to underline that 51 taxa for protection and conservation at international and national. Their protection and conservation statute is the follow:

- 1 taxa, Global red list included in the Red list IUCN, the Habitate Directive & Bern Convention: *Pulsatilla pratensis* ssp. *hungarica* Soó.

- 6 taxa, European threatened, included in the Habitate Directive & Bern Convention: *Agrimonia pilosa, Fritillaria orientalis, Galanthus nivalis, Gentiana lutea, Paeonia tenuifolia, Pulsatilla vulgaris* ssp. *grandis*.
- 44 taxa (endemic, near endemic and rare) threatened at the national level and included in the Romanian Red List.

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THE ESSENTIAL OILS OF *NEPETA* L. GENUS (*LAMIACEAE, NEPETOIDEAE*) IN ROMANIA

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Summary

The essential oils of four species of Nepeta L. genus have been examined. The oils of N. cataria and N. nuda ssp. nuda are characterized by the presence of nepetalactone isomers. The other two species represented by N. parviflora and N. ucranica ssp. ucranica have a rare status within The Vascular Plant Red List for Romania. Nepetalactone isomers are not present in the essential oils of the latest two species. All species excepting N. cataria have been studied for the first time in Romania from phytochemical point of view.

Keywords: *Nepeta, Lamiaceae, essential oil, GT-FT systems, nepetalactone.*

Introduction

The genus *Nepeta* L. (*Lamiaceae, Nepetoideae*) comprises over 250 species that are distributed over a large part of Central and Southern Europe, and West, Central, and Southern Asia. About half of the existing species are recorded in Iran [3]. The genus *Nepeta* is represented in Romania by 4 species, two of these being with a rare status within The Vascular Plant Red List for Romania [8], [9].

Nepeta species are widely used in folk medicine because of their antispasmodic, diuretic, antiseptic, antitussive, antiasthmatic or febrifuge activities. Catnip tea has been shown to have anticholinergic effects and has been used to relieve intestinal cramps; it is also beneficial for cure of colds, flu and fevers; catnip tea has been used for relief of insomnia [13]. The feline attractant properties of several *Nepeta* species have been known for a long time. Nepetalactone and its isomers are considered to be responsible for the feline attractant activity of *Nepeta* species [14].

The essential oils of four species of *Nepeta* genus have been examined. The oils of *N. cataria* L. (Sect. *Nepeta*) and *N. nuda* L. ssp. *nuda* (Sect. *Orthonepeta* Benth.) are characterized by the presence of nepetalactone isomers. The other two species represented by *N. parviflora* M. Bieb. and *N. ucranica* L. ssp. *ucranica* (Sect. *Oxynepeta* Benth.) have a rare status within The Vascular Plant Red List for Romania. Although *N. parviflora* has been studied in the literature, [4], [5], no previous data exist for *N. ucranica* ssp. *ucranica*. Is one of the objectives of this study to report the composition of *N. ucranica*.

Material and methods

Plant material: The leaves and flowering tops were collected from Cernavodă (Constanța County, July 2003) for *N. cataria*, from Frăsinei (Vâlcea County, June 2003) for *N. nuda*, from Basarabi (Fântânița-Murfatlar Natural Reserve, Constanța County, May 2005) for *N. parviflora* and Ploscoş - Valea Florilor (Cluj County, June 2000) for *N. ucranica*. Voucher specimens are kept at BUAG Herbarium, Bucharest (*N. cataria*- BUAG 23777, *N. parviflora*- BUAG 23777, *N. ucranica*- BUAG 23167). Essential oils were obtained from dried aerial parts, leaves, stems, and flowering or fruiting aerial parts of *Nepeta* species by water distillation. The material was harvested at 11-13 AM. The herbal was dried at room temperature and stored 2-7 days in paper bags in the dark.

Isolation of the essential oil: Air-dried herbal parts of the collected plant were subjected to hydrodistillation for three hours using a Clevenger-type apparatus to produce oil. The yield, the oil amounts and plant used parts are presented in Table 1.
Gas chromatography: FISIONS GC chromatograph with DB 5 column 25m length and 0,25 mm internal diameter. Carrier gas has been nitrogen, initial ramp 40° C, isothermal for 5 minutes, final temperature 280° C and 4° C/min. gradient. Using the Nicolet GC-FR-IR transfer line with MCT high sensitive nitrogen cooled detector, all peaks from GC system can be identified by infrared spectra using specific infrared gas phase flavours library. The FT-IR parameters has been: 4000-750 cm^{-1} spectral range, 8 cm^{-1} resolution and 7 scan/sec.; acquisition speed; transfer line and cell temperature 250° C. Due to the non-destructive IR analysis, the sample was conducted in a classic FID detector for quantification after the IR transfer line.
Identification of the components: Compound identification was performed using a chemical library and Kovats indices as a confirmation for the chromatographic peak position.

Results and discussions

GC and GC-MS analyses of the oils were carried out according to a procedure that has been described above. The chemical compounds of *Nepeta* chemotypes are listed in Table 1. The chromatograms with GC traces and an interesting comparison between FT-IR nepetalactone spectra are represented in Figures 1-5.

Table 1. Comparison between the compositions of the essential oils of *Nepeta* L. species growing in Romania

Taxa	Retention time	Compound name	Percentage in oil
Nepeta cataria L. (Sect. *Nepeta*)	8.62	Beta-pinene	0.31
	10.99	Ocimene	0.61
	17.34	Carvone	1.27
	18.20	Geraniol	0.12
	18.41	Geranial	0.65
	21.23	Nepetalactone I	61.96
	23.31	Nepetalactone II	9.22
	24.33	Beta-cariophylene	1.84
	27.23	D-germacrene	0.23
N. nuda L. ssp. *nuda* (Sect. *Orthonepeta* Benth.)	5.15	Alpha-pinene	1.81
	5.85	Beta-pinene	6.85
	6.90	Eucalyptol	44.87
	7.95	Ocimene	2.66
	9.71	4-terpineol	1.2
	9.99	Alpha-terpineol	4.5
	13.21	Nepetalactone I	1.02
	14.08	Nepetalactone II	14.68
	14.99	Beta-cariophylene	8.68
	15.59	Farnesol	1.08
	16.18	D-germacrene	7.76
N. parviflora M. Bieb. (Sect. *Oxynepeta* Benth.)	12.75	Eucalyptol	7.8
	24.84	Alpha-copaene	3.1
	25.22	Beta-bourbonene	1.9
	25.34	Beta-elemene	2.4
	26.28	Beta-cariophylene	6.3
	27.29	Unknown	10.2
	28.20	D-germacrene	40.5
	28.66	G-elemene	4.7
	29.42	G-cadinene	2.7
	31.05	Spathunelol	3.2

N. ucranica L. ssp. *ucranica*	5.15	Alpha-pinene	8.13
(Sect. *Oxynepeta* Benth.)	5.85	Beta-pinene	17.10
	11.28	Beta-mircene	3.72
	12.75	Eucalyptol	65.78
	17.60	4-terpineol	0.80
	18.72	Mirtenal	3.30

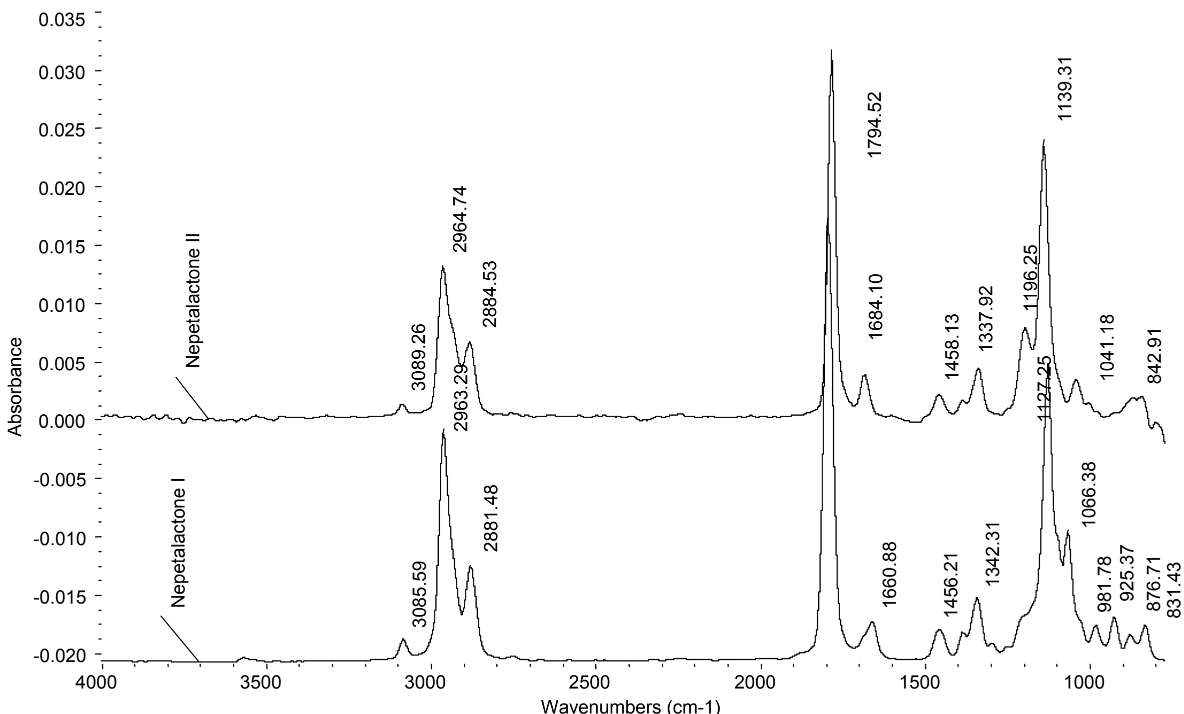

Fig. 1. FT-IR spectra of the nepetalactone isomers from *Nepeta* L.

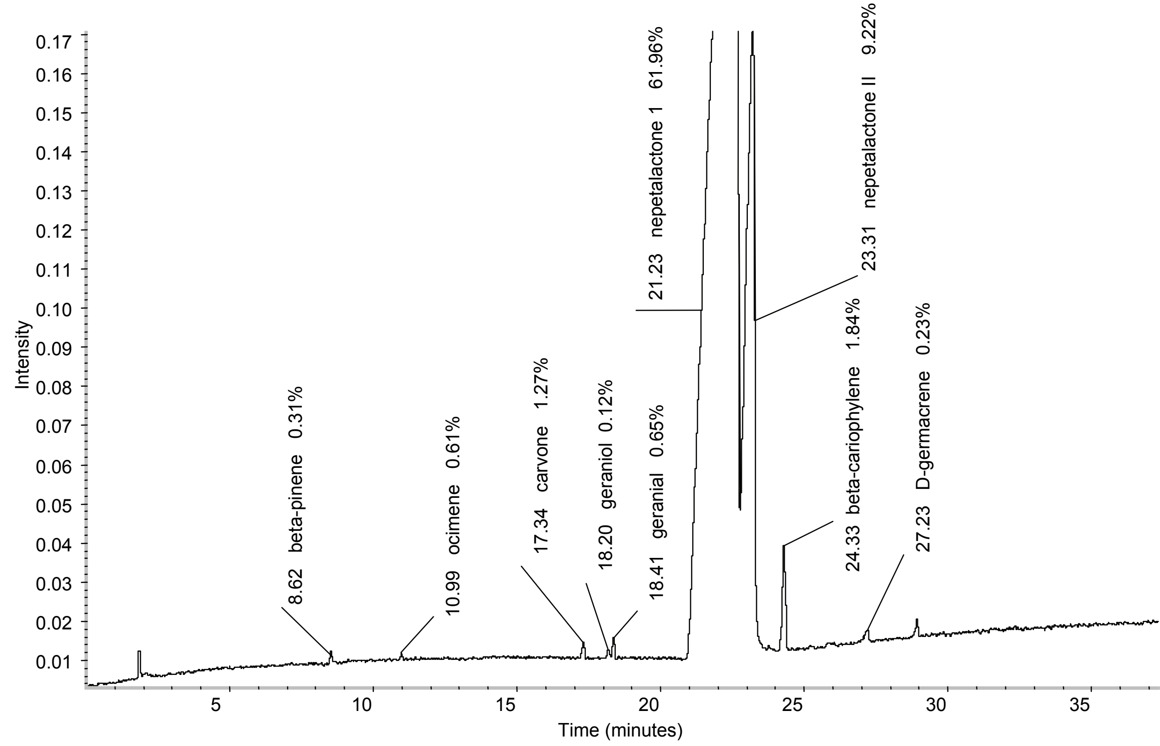

Fig. 2. The chromatogram of *N. cataria* L. - Chemotype „*Cernavodă*”

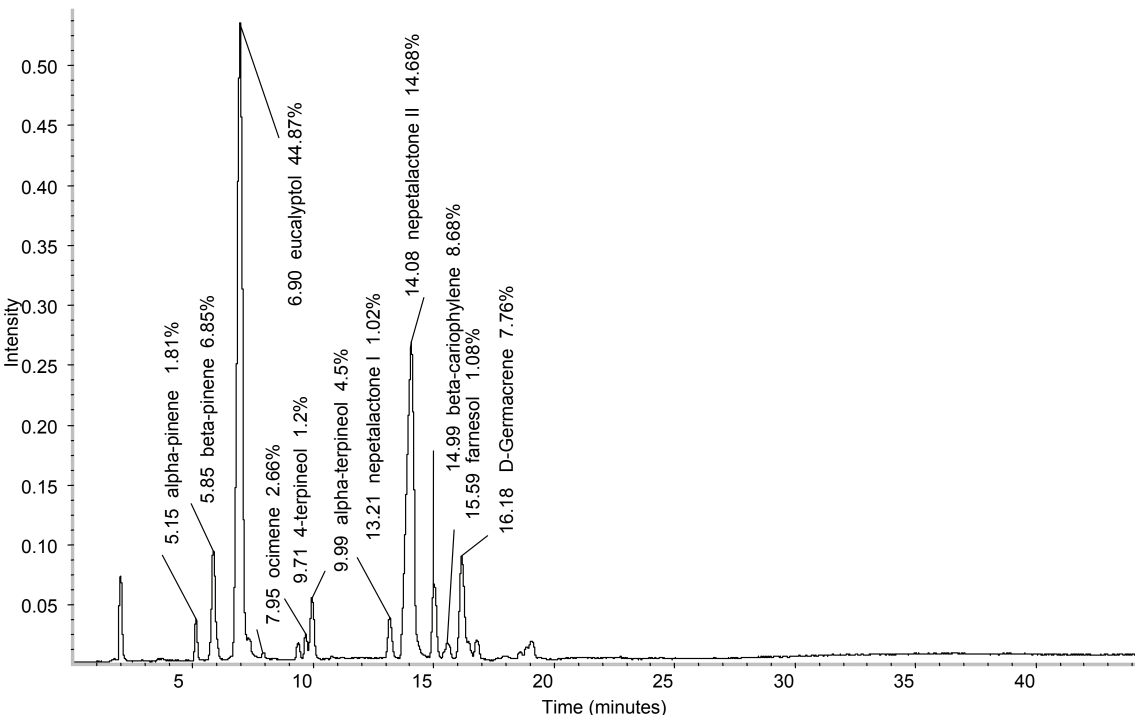

Fig. 3. The chromatogram of *N. nuda* L. ssp. *nuda* - Chemotype „*Frăsinei*"

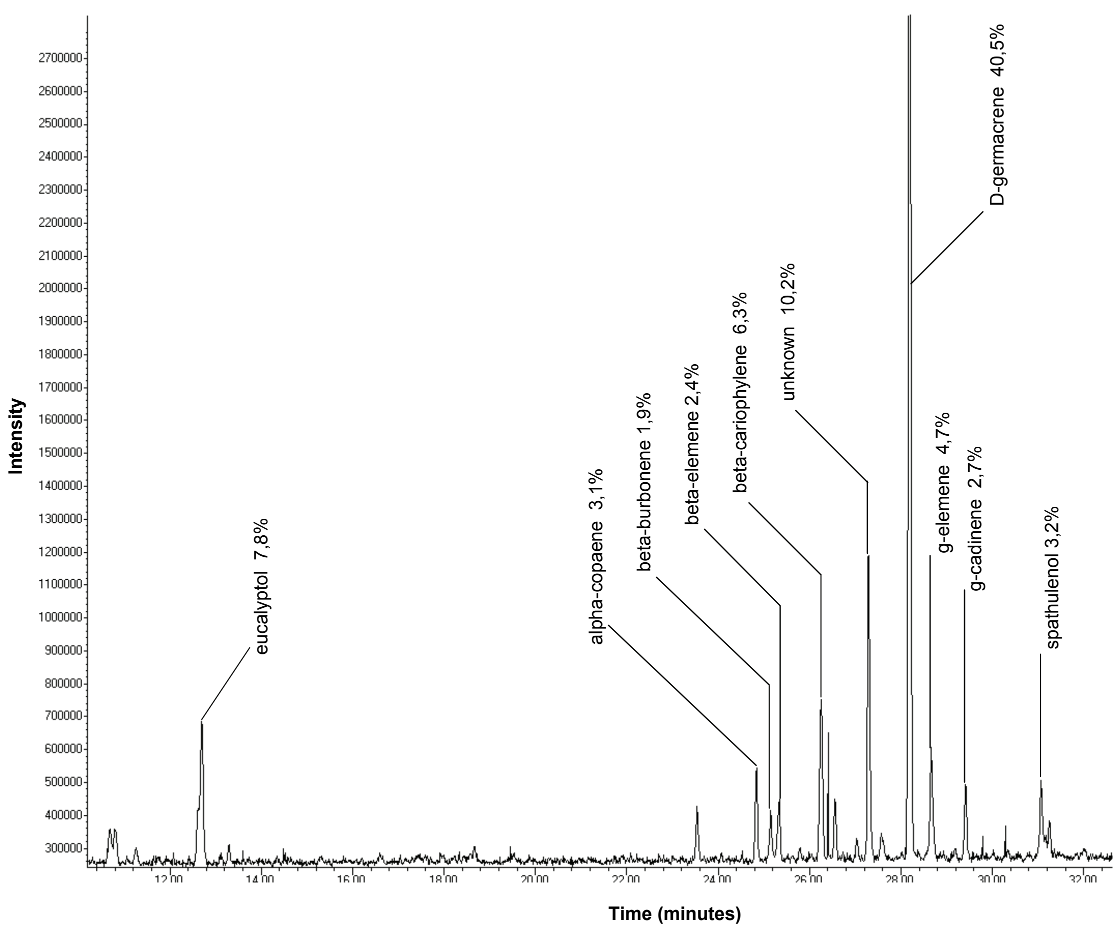

Fig. 4. The chromatogram of *N. parviflora* M. Bieb. - Chemotype „*Basarabi"*

In our literature review we found two types of *Nepeta* species which were different in oil composition. The essential oil of several species of *Nepeta* genus, for example *N. binaludensis*, *N. nepetela*, *N. racemosa*, *N. persica*, *N. crassifolia* [6] and *N. nuda* [1], [2], [7], [12] showed nepetalactone as a main component of the oil. In the second type of oil 1.8-cineol was the main component: *N. ispahanica, N. camphorata, N. glomerulosa* and *N. sulfuriflora* [6]. In the third type of oil eucalyptol was the main component in *N. nuda* (in some investigated chemotypes) [10], [11], *N. ucranica* and *N. parviflora*.

The main components of the oil of *Nepeta* species growing in Romania are represented by:

- nepetalactone I (61.96%), nepetalactone II (9.22%), beta-cariophylene (1.84%), carvone (1.27%) in *N. cataria* (Fig. 2);
- eucalyptol (44.87%), nepetalactone II (14.68%), D-germacrene (7.76%), beta-pinene (6.85%), beta-cariophylene (8.68%), nepetalactone I (1.02%) in *N. nuda* (Fig. 3);
- D-germacrene (40.5%), eucalyptol (7.8%), beta-cariophylene (6.3%), G-elemene (4.7%), spathunelol (3.2%) in *N. parviflora* (Fig. 4);
- eucalyptol (65.78%), beta-pinene (17.1%), alpha-pinene (8.13%), beta-mircen (3.27%) in *N. ucranica* (Fig. 5).

The essential oils of *N. ucranica* ssp. *ucranica* and *N. parviflora* (Sect. *Oxynepeta*) under this study did not contain neither nepetalactone isomers. These fully agree with the reported results with regard of *N. parviflora* in Hungary [4], [5] and *N. ucranica* ssp. *kopetdaghensis* in Iran [6].

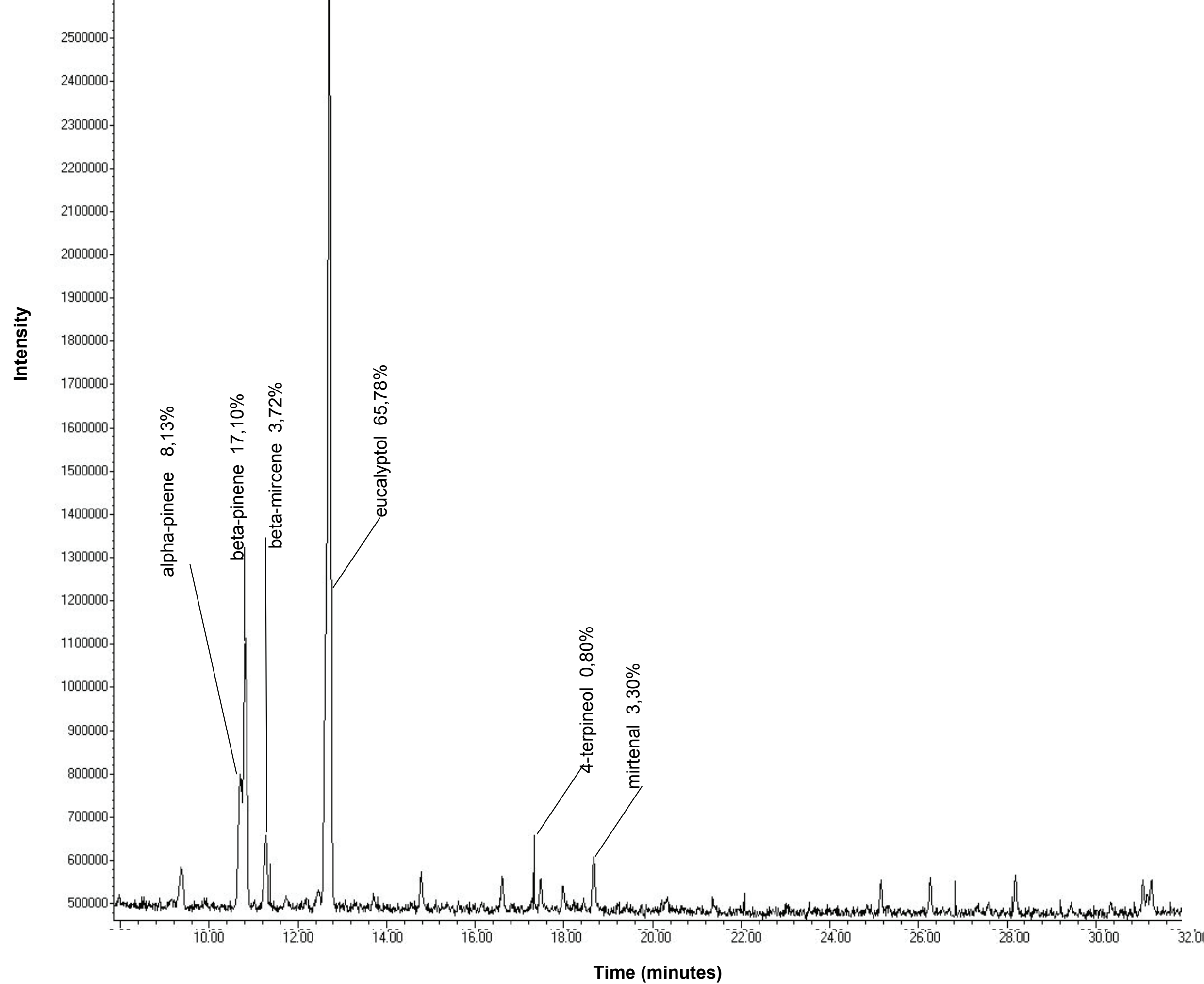

Fig. 5. The chromatogram of *N. ucranica* L. ssp. *ucranica*- Chemotype „*Ploscoş - V. Florilor”*

Conclusions

In this study we investigated the essential oil components of four species of *Nepeta* L. in Romania. All examined species excepting *N. cataria* have been studied for the first time from phytochemical point of view.
The nepetalactone isomers were present in well detectable quantities in two sections of *Nepeta* (*Nepeta* and *Orthonepeta*) while they were not regularly present in Sect. *Oxynepeta* (in *N. parviflora* and *N. ucranica* ssp. *ucranica*). These fully agree with the reported studies regarding the composition of essential oils of these rare species with restricted areal in Romania.
The mentioned chemical compounds might be used as chemotaxonomic markers between *Nepeta* species. Specific components of *Nepeta* oils representing between 77% and 98% of the total oil composition were identified. The remaining percentage consists of traces or remained unidentified by chemical searched library.
The main oil constituents of *N. cataria* and *N. nuda* are nepetalactone isomers, eucalyptol, beta-caryophylene, alpha- and beta-pinen, in different amounts. Germacrene-D (40.5%) is the main constituent of *N. parviflora* and the eucalyptol (65.8%) is the major compound of *N. ucranica* ssp. *ucranica* oil.

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FUNGI ON SAGE SEED IN SERBIA AND THEIR EFFECT TO SEED GERMINATION

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Summary

Fungi associated with garden sage (Salvia officinalis L.) seeds were studied in seeds harvested from the commercial production fields in Serbia, during five years, from 2001 till 2005, using the blotter and agar plate methods. Thirteen fungal species were identified to be associated with the seeds including Alternaria alternata (Fr.) Keissler, Fusariium oxysporum Schlechtend.,Fr. Fusarium subglutinans Wollenw. and Reinking, Fusarium equiseti (Corda) Sacc., Aspergillus flavus Link ex Fries., Aspergillus niger Van Tieghem., Aspergillus spp., Epicoccum purpurescens Ehrenb Ex Schlecht., Cladosporium cladosporioides (Snyder), Chaetomium spp. Doratomyces spp., Verticillium spp., Penicillium spp. and Rhisopus spp. The determination of fungi was accomplished on the basis of their morphological, biometrics and growing properties, having in mind the symptoms, whenever these were characteristic. The most abundant percentage of the diseased plants was attacked by the species belonging to the genus Alternaria (up to 78 %), and than to Fusarium species (up to 8%). Tests were conducted to establish the effect of the phytopathogens on the seed germination as well as germination energy. Experiment was set in the Petri dishes, on the wet filter paper, in four repetitions. While testing pathogenicity, mycelia of the selected fungi were put directly on the seeds. The control was set in the same manner, but without inoculation.

Keywords: *seed, fungi, sage, Salvia officinalis, diseases, germination*

Introduction

The sage seeds are rich in protein, carbohydrates and minerals and therefore provided a suitable substrate for the growth of microorganisms. Our literature does not contain information on the sage seed pathogens. The seed contain and transfer many fungal diseases from one year to the next and increases the parasitic flora in the soil.
As the most important factor in ensuring the high yield is the seed quality, the phytopatological analysis of the seeds determines the quality by identifying pathogens and the degree of infection.

Materials and methods

The investigations have been conducted from 2001 to 2005. The seeds samples were collected in the field collection of the Institute for the Medicinal and Aromatic Plants Research “Dr Josif Pančić” in Pančevo.
The analysis of the health status of the seeds was conducted using the standard phytopathological methods: seed incubation on wet filter paper (MATHUR et KONGSDAL, 2003) and seed incubation on the artificial medium (TEMPE, 1963).
The seeds were disinfected with the 1% solution of NaCl for 10 minutes. Morphological and developmental characteristics were investigated on the following media: potato dextrose agar (PDA), potato sucrose agar (PSA) and water agar with the sterile fragments of carnation leaves (PCA). The following morphology characteristics of obtained isolates were observed: the appearance of the air mycelia, the speed of colonial growth, the presence/luck of culture pigmentation, the shape and dimensions of conidia and conidiophores, the nature of conidia formation, production of chlamidiospores, type of conidioma formed. The size of the reproductive organs was calculated after 100 measurements. All of these characteristics as

well as the showed symptoms on the diseased seeds were used for fungi determination according taxonomic literature (MALONE and MUSKETT, 1964; ARX, 1974; BOOT, 1971; BURGESS, 1999).

Energy of germination and total germination were determined on wet filter paper in Petri plates after seven and 21 days, respectively. The seeds were cooled in the refrigerator for four days before they were incubated at alternating temperatures (12 h light/12 h darkness) of 20° and 25°C (ISTA, 2003).

Results and discussion

Fourteen species of fungi were identified on the samples of sage seeds. The intensity of their appearance is presented in the table 1.

Table 1. The percentage of seeds of sage contaminated with fungi in 2001 - 2005.

Seed pathogen	Year				
	2001	2002	2003	2004	2005
Alternaria alternata	78%	18%	30%	13%	15%
Botrytis cinerea	2%	2%	1%	2%	1%
Doratomyces spp.	1%	2%	0,5%	-	2%
Cephalosporium spp.	2%	2%	1,5%	-	2%
Epicoccum purpurascens	2%	2%	1,5%	-	2%
Fusarium oxysporum	2%	2%	2%	2%	2%
Fusarium equiseti	2%	1%	1%	-	1%
Fusarium subglutinans	3%	2,5%	0,5%	1%	2,%
Aspergillus flavus	1%	1%	1,5%	-	1%
Aspergillus niger	1%	2%	1%	1%	2%
Verticillium spp.	1%	2%	2,%	-	1%
Cladosporium cladosporioides	1%	1%	1%	-	1%
Chaetomium spp.	1%	2%	2%	2%	-
Rhizopus spp.	3%	-	-	2%	-

Alternaria spp. is the dominant species on the sage seeds. In 2001 70% of the seeds contained *Alternara* spp. The majority of contaminated seeds did not germinate at all. The literature quotes the presence of *Alternaria* on the seeds of many medicinal and aromatic herbs such as: camomile, St. John's wort, balm, valerian, feverfew (PAVLOVIC & DRAZIC, 2000; PAVLOVIĆ et al., 2000; PAVLOVIĆ, 2001; PAVLOVIĆ, 2003; KOSTIĆ et al., 2003).

Fusarium spp. is present every year but they rarely contaminates more than 5% of the seeds (7% in 2001). Fungi from the genus *Verticilium* are also common but they usually contaminate only 1% of the seeds. The rest of the fungi are common but are not serious pathogens on the sage seeds.

The importance of parasitic flora on the sage seeds in multifold. Parasitic fungi cause diseases of the seeds during their formation or storage, greatly reducing their viability and germination and, therefore, their value. Withering of the seeds is connected to *Fusarium* spp., while is caused by fungi from the genus *Botrytis*, *Epicoccum*, *Penicillium*, *Aspergillus*, *Rhisopus*, as well as many others. *Alternaria* and *Fusarium* cause spots and necrosis of the seeds.

The percentage of sage seed germination was analysed every year after the harvest (Table 2). There were no significant differences in germinative energy and germination between seed samples collected from 2001 to 2005.

Table 2. Sage seed germination in the year of harvest

Years of harvest	Energy of germination %	Germination %	Abnormal seedling %	Ungerminated seeds %
2001	42	83	4	13
2002	40	83	8	9
2003	45	84	3	13
2004	41	85	4	11
2005	47	81	1	18

The effect of storage on the seed germination was also studied (Table 3).

Table 3. The effects of seeds age on their germination

Age of seeds (years)	Years of harvest	Energy of germination %	Germination %	Abnormal seedling %	Ungerminated seeds %
0	2005	47	81	1	18
1	2004	41	74	1	25
2	2003	16	26	3	71
3	2002	0	1	0	77
4	2001	0	0	0	88

Seed samples collected in 2001-2005 were stored for under laboratory condition and in 2005 all samples were incubated under optimal condition to check their viability. The percentage germination of the seeds collected in 2005 was 81%. However, the seeds one and two years old germinated 74% and 26%, respectively. After second year of storage the seeds lost germination ability. The data show that the sage seeds are not suitable for sowing after two years of storage, as the germination and germinative energy of seeds decreased rapidly from 81 to 26% and from 41 to 16%, respectively.

Conclusion

The results obtained in this study show that the mycopopulation of sage seeds is numerous. Fourteen species of fungi have been identified: *Alternaria alternata, Fusarium oxysporum, Fusarium equiseti, Fusarium subglutinans Botrytis cinerea, Doratomyces* spp., *Cephalosporium* spp., *Epicoccum purpurascens*, *Aspergillus flavus*, *Aspergillus niger*, *Verticillium* spp., *Cladosporium cladosporioides*, *Chaetomium* spp. and *Rhizopus* spp. The highest percentage of diseased seeds was in 2001, and the lowest in 2004. Many of the fungi on the sage seed mentioned above have not been reported in Serbia until now.

Germinative energy and germination of seeds incubated in the years of collection (2001 – 2005) were satisfied and in correlation with ISTA rules of seed quality. Germination and germinative energy of seeds decreased rapidly after two years of storage.

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CURRENT STUDY ON THE PARAZITIC MICROORGANISMS OF THE MEDICINAL AND AROMATIC PLANTS IN SERBIA

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Summary

Pathogenic microorganisms on medicinal and aromatic plant species cultivated on plantation in Serbia were studied during the last ten years. The seeds of majority of these plants were contaminated with fungi from the genus Fusarium, Alternaria, Verticilium, Colletotrichum, Penicillium, Aspergillus, Rhizopus and Phoma, which significantly reduced seed germination and caused seedlings decay. On the stem and leaves the symptoms of powdery mildew rust, fleckening and wilting were manifested. The fungi from the genus Oidium, Puccinia, Coleosporium, Phragmidium, Alternaria, Fusarium, Phoma, Septoria, Sclerotinia, Verticilium and Botrytis were isolated as the causative agents. The most intensive changes were recorded on St. John's wort and coneflower, where percentage of the diseased plants was very high. In the tissue of the diseased Echinacea species with the symptoms of yellowing (witches brooms, virescence), the presence of the phytoplasma (Stolbur type) was established. In the xylem vessels of the St.John's wort diseased plants, with the symptoms of redness and remature decay, the presence of fastidious bacteria (rickettsia – like organisms – RLO) was recorded. The symptoms of redness were also registered in the cultivated oregano and lovage, as well as in the wild-growing dandelion.

Keywords: *medicinal and aromatic plants, diseases, fungi, fastidious bacteria, phytoplasma*

Introduction

Medicinal plants are among the most economically significant plants in Serbia. There are over 700 varieties of these plants on the territory of Serbia contributing 19,65% of the total flora. (Sarić M., 1989). Of the 420 registered varieties, 279 are used commercially as medicinal and aromatic plants. In order to preserve medical plants in nature from over exploitation, they are grown as commercial crops in plantation. There is a long tradition of growing medical and aromatic herbs commercially such as mint, marsh mallow, sage, St. John's wort etc. in Serbia.
The commercial cultivation led to the occurrence and spread of the plant diseases and on some hosts (St.John's wort, coneflower), the diseases were of the epidemic proportions. The parasites of the medicinal and aromatic herbs were studied over 17 years and the summary of the findings is reported in this paper.

Material and methods

The diseased plants were collected from the collection field at Institute for medicinal plant research in Pančevo, as well as from other areas in Serbia in the period of 1990 to 2005. The seeds, seedlings and adult plants are analysed for the presence of plant pathogens. The standard methods such as: seed germination on the filter paper (Mathur and Kongsdal, 2003), and isolation on the artificial medium were used. The identification of the pathogens was done according to their morphological characteristics. The pathogenicity of the most common isolated fungi was tested by Molt and Simone procedure (1967). Artificial inoculations of the test plants were done by using fragments of the mycelia or conidia suspension. Inoculated plants were kept in the wet chamber until the symptoms appeared. In some cases pathogenicity was tested by grafting fragments of the diseased plants on the healthy ones.
The differentiated centrifugation method was used to extract the pathogen from the root, stem and leaf of the St John's wort plants with the symptoms of redness The sections (50 and

100nm) of the xylem tissue of the infected plants were made by using the ultramicrotome. Preparation of the plant material was made by using Hopkins et al. procedure (1973). The sections were observed by the electron microscope. For the identification of the causers of redness of *Echinacea* sp. PCR method was used. The fragments of the diseased plants were grafted on the healthy ones.

Results and discussion

The diseases of the mint plant (*Mentha piperita*)

The numerous pathogens of the mint plant have been described, and fungi are the predominant. The leaves of the cultivated mint were severely affected by the rust (*Puccinia menthae* Pers.) on almost all location in Serbia. The leaves of such diseased plants are useless for the pharmaceutical purposes.

Among the eight species of the stolone-born fungi the most important are the ones belonging to the genus *Fusarium* and *Verticillium* which causes specific chloroses and necroses on the infected plant tissues. The following species were determined: *Fusarium equiseti*, *Fusarium semitectum*, *Fusarium subglutinans*, *Verticillium lateritum*, *Verticillium dahliae, Penicillum* spp., *Trichotecium roseum* and *Alternaria* spp. The presence of these pathogens was 1-8% only (Pavlović et al., 2000).

According to the Russian data (Dobrzrakova et al., 1956) the main pathogens of mint are *Fusarium* spp., *Septoria menthae*, *Septoria menthicola*, *Ramularia menthicola*, Eryisiphe *cichoracearum* f. *menthe* and *Peronospora sigmaticola.* The leaves attacked by those fungi could not be sold or used for further production.

The diseases of the valerian plant (*Valeriana officinalis*)

Twelve species of fungi were identified on the samples of the diseases valerian: *Alternaria alternata*, *Phoma* spp., *Peronospora valerianae*, *Fusarium oxysporum*, *Fusarium sporotrichoides*, *Fusarium solani*, *Sclerotinia sclerotiorum, Epicoccum purpirescens, Veritcillium* spp.*, Penicillium* spp.*, Mucor* spp. and *Aspergillus* spp. (Pavlović, 2003). Among the leaf and root pathogens the following fungi were prominent: *Fusarium*, *Alternaria* and *Phoma*.

The diseases of the St. John's wort (*Hypericum perforatum* and *H.barbatum*)

On the St. John's wort seeds ten species of fungi were identified: *Alternaria* spp., *Aspergillus* spp., *Fusarium oxysporum*, *Fusarium solani*, *Fusarium semitectum*, *Fusarium proliferatum*, *Penicillium* spp., *Epicoccum purpurascens, Verticillium* spp., *Mucor* spp. (Pavlović et al., 2000a). Fungi that belong to genus *Alternaria, Penicillium, Acremoniella*, *Aspergillus* and *Epicoccum* were always present in the samples, but in less intensity. Four species of the genus *Fusarium* were often present on the St.John's wort seeds, causing the death of the seeds or wilting of the seedlings.

Specific symptoms were observed on the St John's wort a few years ago (Pavlović et al., 2004). The leaves and stems of diseased plants became reddish the tissue necroted and the infected plants wilted and died before the vegetation, sometimes before flowering. On the some fields there were more than 90% of the diseased plants.

The analysis of the ultrathin vascular tissue sections of the diseased plants with the redness symptoms, the rod-shaped organisms were observed. They belonged to the group of the fastidious bacteria of the rickettia type, based on their morphology and Gram staining. Grafting fragments of the diseased plants onto the healthy ones caused successful reduction on the symptoms. A large number of leafhoppers as the potential vectors were collected and their identification is currently under way.

From the plants with the redness symptoms *Fusarium* and *Colletotrichum* were isolated (Ivanović et al., 2002). However, artificial inoculation of the St John's wort plants under the

laboratory conditions with *Fusarium* and *Colletotricum* isolates, did not produce the characteristic symptoms of the redness and drying / wilting.

The literature search shows no data on the fastidious bacteria on the aromatic herbs, therefore our study is the first one to establish the presence of this pathogen in the vascular tissue of the St John's plants (Pavlović et al., 2004, 2005). However, the phytoplasma have been described as a pathogen causing same symptoms (Bruni et al., 2005).

It is very important to study the redness disease on the aromatic plants as they spread rapidly and affect a number of hosts, causing a large reduction in yield. These symptoms have also been identified on the oregano (*Origano vulgare*), lovage (*Levisticum officinale*) and dandelion (*Taraxacum officinale*), (Pavlović et al. 2004a).

The diseases of the coneflower (*Echinacea purpurea* and *E. angustifolia*)

Echinacea species started to be grown commercially as plantations in Serbia in 1998. The investigation on the health status of the seeds of *E.purpurea* and *E.angustifolia* has shown the presence of the numerous parasites. The following fungi have been identified: *Alternaria alternata*, *Fusarium oxysporum*, *Fusarium proliferatum*, *Epicoccum purpurascens*, *Phytium* spp., *Rhizoctonia solani*, *Penicillium* spp., *Aspergillus* spp., and *Botrytis cinerea*.

The literature studied quotes these fungi *Sclerotinia sclerotiorum*, *Alternaria* spp., *Botrytis cinerea*, *Fusarium* spp. and *Phythium cichoracearum* (Sholberg et al., 1999).

Recently of type of phytoplasma has been identified as a serious and most destructive pathogen *Echinacea* spp. in the USA and Canada. (Hwang et al., 1997). The first symptoms of the disease on the cultivated *Echinacea* spp. in Pančevo and Indjija were notified after the second year of growing. The initial symptoms were yellowing and then redness of the hower leaves. The disease spread quickly, affecting 30% of the plants. The leaves of the diseased plants were smaller, wilted and dried. The redness spread on the stem, which showed smaller internodules and a reduction in growth, giving plants the appearanced of the bush. Sometimes the plants died. The flowers showed proliferation and virescence. The electromicrographs obtained from the ultrathin sections of the vascular tissue of the diseased plants, showed the presence of the phytoplasma with oval to spherical shape, without the cell wall and surrounded by a single membrane (Pavlović et al., 2004). The PCR identification at University of Udine, Italy, recently confirmed that this pathogen of the *Echinacea* belongs to the Stolbur type of the phytoplasma.

The diseases of the yellow gentian (*Gentiana lutea* L.)

The cultivation of the yellow gentian in Serbia has started recently. The pathogens cause the changes on the seeds such as colour change, withering, spots and reduced germination. The following pathogens on the gentian seeds were identified: *Alternaria alternata* (78%), *Fusarium oxysporum* (5%), *Botrytis cinerea* (3%), *Aspergillus flavus* (3%), *Penicillium* spp. (3%) and *Epicoccum purpurascens* (2%).

The economic damage is caused by the reduction in the seed germination. When the seeds were affected by the *Alternaria* they did not germinate at all. The above mentioned pathogens are also common on the seeds of balm, sage, feverfew, valerian, St. John's, conewlover, camomila. (Kostić et al., 2004).

The diseases of the balm (*Melissa officinalis* L.)

The literature studied gives little information about the pathogens affecting the seeds. A long term study of the balm seeds quality identified the presence of the diseases in the early stages of the seed development, causing regular decay of seedlings, decreasing the percentage of germination and, ultimately, the decrease of the number of plants per area unit. The following fungi were isolated: *Alternaria tenuis*, *Aspergillus niger*, *Botrytis cinerea*, *Cephalosporium* spp., *Septoria* spp. *Epicoccum purpurascens*, *Fusarium* spp., *Mucor* spp. and *Penicillium* spp. (Pavlović S., 2001).

The disease of the sage (*Salvia officinalis*)

The most common disease of the green parts of salvia is powdery mildew, and in the case of a severe attack, the diseased leaves become yellow and fall off. The leaves covered by the white-grey film lose their value as the source of aromatic oils. The following fungi were identified on the seeds and leaves of salvia: *Fusarium moniliforme* var. *subglutinans*, *Fusarium equiseti, Fusarium* spp., *Cercospora savicola*, *Alternaria* spp., *Botrytis cinerea*, *Chaetomium* spp., *Doratomyces* spp., *Epicoccum pruprurascens*, *Penicillium* spp., and *Verticillium* spp. (Kostić et al. 1999).

Pironethe (1996) cite these pathogens as well as *Pythium debaryanum, Pellicularia filamentosa, Cercospora salvicola, Ramularia salvicola, Puccinia caulicola, Puccinia farinacea, Puccinia salvicola* and *Peronospora lamii*.

Diseases of marshmallow and mallow (*Althea officinalis* and *Malva silvestris*)

Pucccinia malvacearum is a significant pathogen of the leaves of marshmallow and mallow in Serbia. The severely infected leaves were completely destroyed (Pavlović et al., 2002). The following fungi were isolated from the ungerminated seeds or the withered germ tubes of the marshmallow plants: *Fusarium* sp., *Alternaria* sp., *Epicoccum* sp., *Penicillium* sp., and *Apergillus* sp., were isolated. Three *Fusarium* species (*F. oxysporum*, *F.semitectum* and *F.sporotrichoides*) (Pavlović and Stojanović, 2002) and *Sclerotinia sclerotiorum* (Pavlović and Stojanović, 2001) were detected in the necroted tissue of the root and stalk of the marshmallow.

The diseases of camomile (*Chamomilla recutita*)

Camomile is a very important cultivated aromatic plants in Serbia. Luckily, this plant is not significantly affected by pathogens. The following fungi are detected on the seeds: *Alternaria* sp., *Penicillium* sp., *Aspergillus* sp., *Verticillium* sp., and *Fusarium moniliforme* (Pavlović and Dražić, 2000). Only *F.moniliforme* can cause the damage during the germination under the certain conditions.

Conclusion

Medicinal and aromatic herbs proved to be suitable hosts for a large number of pathogens, mainly fungi from the following genus: *Fusarium, Alternara, Penicillium, Aspergillus, Verticillium, Sclerotinia, Puccinia, Phoma, Colletotrichum, Septoria* etc. The majority of these fungi affect the seed germination and their vitality. They often cause spots, wilting and rust on the green parts of the plant.

Sclerotinia sclerotiorum is a significant pathogen on more than 400 plants, including those mentioned in this study. Considering that this fungus can survive over five years in the soil, it is important to observe the rules of rotation during the cultivation of the aromatic herbs.

The herbs mostly affected by the disease are St. John's and coneflower, where up to 90% of the plants show the symptoms. As all affected plants die before the end of vegetative season, the presence of the fastidious bacteria in St John's wort and the phytoplasma in coneflower, can significantly the cost-effectiveness of their commercial growth.

The following aspects will be subject to the future study: the identification of the molecular structure of the fastidious bacteria and phytoplasma, as well as the identification of the vectors and the host affected.

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DIGITALIS PURPUREA IN VITRO CELL CULTURES FOR BIOTRANSFORMATION OF EXOGEN HYDROQUINONE INTO ARBUTOSID (I)

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Summary

Hydroquinone biotransformation into arbutin was assessed by means of unconventional biotechnologies via Digitalis purpurea (L.) plant cell cultures. Plant cell suspensions were established from previously acquired callus cultures on MS liquid medium supplied with 2,4-D (1.0 mg/l), BA (2.0 mg/l), glutamine (0.2 g/l) and hydrolised casein (0.2 g/l). Qualitative analysis of separated components in TLC (Thin-Layer Chromatography) proved that our plant cell suspensions are capable of biotransforming exogenously added hydroquinone into arbutin.

Keywords: *hydroquinone, arbutin, Digitalis purpurea (L.), in vitro cell suspensions*

Introduction

Biotransformation refers to the technique that converts various substrates to more useful products using freely suspended, immobilized plant cells or enzymes. Much more than 150 reports of biotransformations dealing with terpenoids, steroids, phenolics, alkaloids, and other compounds [1, 2]. In this context one of the most considerable biotransformation reaction is the glycosilation of hydroquinone. Thus, there are reports of 9-18g/l arbutoside yield in plant cell cultures of *Rauwolfia serpentina* and *Catharanthus roseus* [1, 2, 3].
Digitalis pupurea (L.) it is well known for it's content in cardiotonic glycosides with great therapeutic importance. This plant's cells comprise the non-specific enzyme glycosil-transferase which is able to biotransform hydroquinone (a toxic compound) into arbutoside (a disinfecting compound used in genitourinary diseases as well as a skin whitening agent) [1, 2].
Tabata et al. (1976) [4] suggested the possibility to obtain this arbutoside through *in vitro* culture of *Digitalis purpurea* cells, yet there are no scientific data published regarding this matter. Preliminary analysis performed on *Digitalis lanata* pointed out the capacity of this plant to biotransform hydroquinone into arbutoside through *in vitro* plant cell cultures, since the qualitative analysis revealed the presence of arbutoside in concentrations between 0.17-3.1% in the dry biomass [5, 6]. These results promoted our research in the direction of testing the ability of Digitalis *purpurea* plant cell cultures to biotransform hydroquinone into arbutin.

Materials and methods

The i*n vitro* plant cell cultures of *Digitalis purpurea* were initiated from the plantlets resulting from the germination of the corresponding seeds. These seeds were germinated under aseptic conditions on a Murashige-Skoog medium (1962) (MS) ½ [7]. Furthermore explants excised from the resulting plantlets (leaves, leafstalks, roots) were transferred on four different media cultures. The basic media culture used in all cases was MS supplemented with glutamine (0.2 g/l), hydrolised casein (0.2 g/l) and agar (7.5 g/l) (pH= 5.7). Media culture variants:

1. Variant I – 1.0 mg/l IAA + 2.0 mg/l K;
2. Variant II – 1.0 mg/l IBA + 2.0 mg/l BA;

3. Variant III – 1.0 mg/l NAA + 2.0 mg/l BA;
4. Variant IV – 1.0 mg/l 2,4-D + 2.0 mg/l BA.

Plant cells were cultured in Erlenmeyer flasks of 100 ml capacity and maintained under light conditions - 30 μmol/m^2/sec photoperiod (16 hours light/8 hours darkness), as well as in darkness, at a temperature of 25±1°C.

The best development of callus culture was observed on the variant IV under light conditions, when induced from hypocotyle explants.

The primary plant cell suspension was initiated by transferring small callus fragments on liquid media culture with the same chemical composition. The primary plant cell suspensions were cultured in Erlenmeyer flasks of 200 ml and maintained on a horizontal rotary shaker at 100 rpm, under the same light conditions and temperature like the callus culture. By subculturing this suspension at every 14 days we obtained more homogenous plant cell suspensions.

Before testing the biotransformation capacity of these suspensions we determined the growth curve at different ratios of inocula:media volumes (1:20; 1:10; 1:7).

Considering that hydroquinone is an extremely toxic compound and the fact that our cell line had no induced resistance to this compound, we chose to test the biotransformation capacity of plant cell suspensions by adding this precursor in small concentrations (1mM), in the 14'th day of culture. The hydroquinone solution was added under sterile conditions by using a Millipore filter (0.45 μm). The biotransformation medium was similar to the growth medium of cell suspensions.

Biomass and media sampling was performed at each 3, 6, 24 and 48 hours after hydroquinone addition. These samples were then submitted to qualitative analysis.

Qualitative analysis of hydrochinone was performed by thin-layer chromatography (TLC) after Hörhammer and Wagner (1963) [6, 8]. Sample preparation requires dry biomass weighing, followed by extraction on a boiling water bath through an installation comprised of a ballon flask and an ascendant refrigerator. The entire operation takes 15 minutes. The extraction solvent we used consisted of a mixture of methanol:water (1:1), with a ratio biomass:solvent of 1:50. The media samples were prepared by dry evaporation of 25 ml medium followed by residue retaking in a 5 ml mixture of methanol:water (1:1). The circumstances under which chromatography was performed were as they follow:

- The stationary phase: sillicagel GF_{254} (Merck) plates of 10x20 cm and 0.25 mm thickness;
- The mobile phase: etyl acetate:methanol:water (100:16.5:13.5);
- Solutions to be analysed: dry biomass samples to be weighted and liquid media probes;
- Standard solutions: arbutoside (Fluka) solution 0.1% in methanol, hydroquinone (Suchardt) solution 0.1% in methanol.
- Migration distance: 7.5 mm;
- The amount apllied:10 μl from each probe and 5 μl from each standard solution, spotlikeness;
- Migration time: 30 minutes;
- Identification reagents: phosphomolibdenic reagent and chromatogram maintenance in ammonium vapor atmosphere; blue-grey spots will occur;

Results and discussions

Seed germination begun on the 21'st day after inoculation, the germination yield being around 80%. Callus formation varied allot with dependence on the media culture variants utilized, as well as with the illumination terms. Thus the primary callus culture was obtained after 18 days in all cases, excepting variant I maintained in light conditions. This was due to the fact that indole-

3-acetic acid (IAA) was decomposed under light conditions. The nature of explants was also critical. The best result for callus developmet was registered when using hypocotyls as explants. Generally light conditions stimulated a better development of callus culture comparatively to those maintained in darkness. The IV'th variant of media culture (2,4-D 1.0 mg/l and BA 2.0 mg/l -under light conditions) proved to be the best for callus formation (figure 1). Therefore this variant was used furtherly for massive callus culture development (figure 2). We maintained unmodified the composition of this type of media culture, vessels being held exclusively in light conditions. The callus developed on this variant was friable due to the presence of 2,4-D and therefore the most suitable for the inducement of homogenous plant cell suspension culture.

Fig. 1. Primary callus developed on variant IV under light conditions

Fig. 2. Callus culture on variant IV grown under light conditions

The primary cell suspension was obtained at 30 days after transferring small fragments of friable callus on MS liquid medium supplemented with glutamine (0.2 g/l), hydrolised casein (0.2 g/l), 2,4-D (1.0 mg/l) and BA (2.0 mg/l). The homogenous suspensions were acquired after 6 successive subcultures on a similar media culture.

The growth curves (figure 3) of plant suspensions were determined for three different quantities of inocula (2, 4 and 6 ml), but maintaining the same volume of media culture (40 ml). Consequently we performed sample harvesting once at 2 days until the 16'th day of culture for the variant with 2ml inocula, respectively in the 14'th day of culture for the variants with 4ml and 6ml inocula.

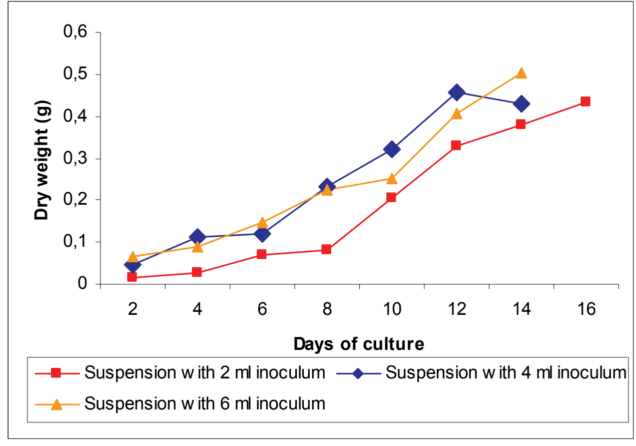


Fig. 3. Growth curve of Digitalis *purpurea* cell suspensions at different volumes of inocula (2, 4 and 6 ml)

Our results showed that the quantity of biomass has tripled in the 6'th day of culture when comparing to the 4'th day of culture in the variant with 2 ml of inoculum. The same situation was observed in the 10'th day comparatively with the 8'th day of culture. The rest of the growth periods until the 16'th day of culture was characterized by a continuous growth of biomass but with a lower intensity.

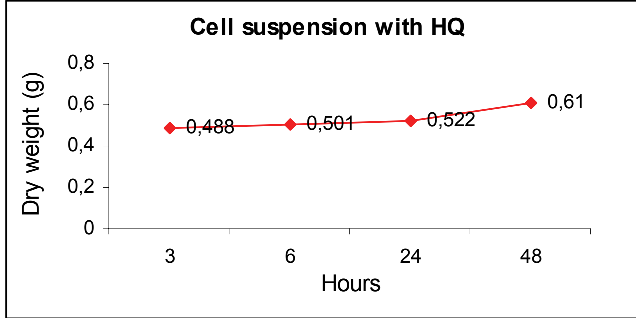
At the volume of 4 ml inoculum, we estimated in the 6'th day of culture a threefold growth in comparison with the second day of culture. Until the 12'th day of culture we registered a constant biomass growth. There is a decline in biomass growth starting with the 14'th day of culture comparatively with the case of the plant cell suspension with 2 ml of inoculum.

At 6 ml of inoculum, the quantity of biomass has doubled in the 6'th day of culture comparatively with the second day of culture as well as in the 12'th day in comparison with the 10'th day of culture. This case was similar with that of 2 ml inoculum, when considering the continuous growth of biomass until the 14'th day of culture. This evolution makes sense for a small volume of inoculum, but it was surprisingly unexpected at a volume of 6 ml inoculum. This was more improbable especially when considering the fact that at 4 ml inoculum the cell suspension registered a growth decline starting with the 14'th day of culture.

Considering these results we decide that the ration 1:10 is the most adequate (therefore it was maintained for the experiments of biotransformation) and we established the subculturing in the 14'th day of culture.

The biotransformation capacity of these plant cell suspensions was tested on variants with 5 ml inoculum/50 ml media culture. Since we didn't found any scientific publications regarding the use of *Digitalis purpurea* plant cell suspensions for the biotransformation of hydroquinone into arbutoside, we decided to use hydroquinone in small concentration, meaning 1 mM (110 mg/l media culture). We considered this concentration right as these are preliminary studies and we didn't have a hydroquinone resistant cell line. Our interest was focused especially on the capacity of our plant cell suspension to glucozilate hydroquinone.

Besides analyzing the biotransformation capacity we also studied biomass growth in the presence of hydroquinone in comparison with the controls lacking this compound. Sample harvesting was performed at 3, 6, 24 and 48 hours after hydroquinone addition. We used controls for comparison only at 24 and 48 hours of culture, considering that at a small hydroquinone concentration there would be no significant modifications in biomass growth after 3 or 6 hours of culture.



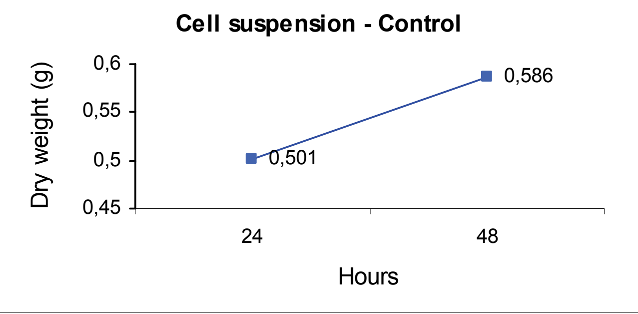
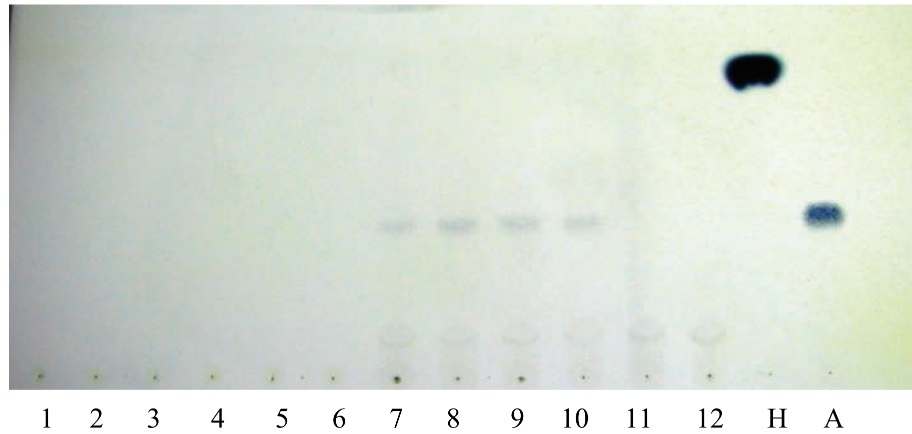


Fig. 4. Growth curve for the suspension containing hydroquinone/control

By analyzing the growth curves (figure 4) we found out that the presence of hydroquinone didn't affected cell growth to much as the quantity of biomass continued to grow until 48 hours of culture was reached. The same thing was observed for the controls.
Qualitative analysis by TLC performed out of samples consisting of dry biomass and liquid medium, clearly showed the capacity of *Digitalis purpurea* cell suspensions to biotransform hydroquinone into arbutoside. As it can be seen in the next figure (5) the arbutoside (Rf = 0.33) has been accumulated exclusively in the cells, being present only in the samples consisting of dry biomass (samples 7-10) but not in those of liquid media.



1 2 3 4 5 6 7 8 9 10 11 12 H A

Fig. 5. TLC (Thin-Layer Chromatography) from dry biomass and liquid media samples

1- medium sample harvested at 3 hours
2- medium sample harvested at 6 hours
3- medium sample harvested at 24 hours
4- medium sample harvested at 48 hours
5- control medium sample harvested at 24 hours
6- control medium sample harvested at 48 hours
7- biomass sample harvested at 3 hours
8- biomass sample harvested at 6 hours
9- biomass sample harvested at 24 hours
10- biomass sample harvested at 48 hours
11- control biomass sample harvested at 24 hours
12- control biomass sample harvested at 48 hours
H- standard hydroquinone
A- standard arbutoside

There were no traces of hydroquinone (Rf=0,6) found neither in the biomass nor in the media culture, being obvious that it was entirely biotransformed. This was clear when we found biotransformed arbutoside in the samples harvested at 3 hour after hydroquinone addition.
The efficiency of biotransformation process was due to the availability of large amounts of biomass (with correspondingly high concentrations of glycozil-transferase) as well as to the small concentration of hydroquinone that we used. In this case the toxicity level was lower as well and the cell growth was not remarkably affected, the entire quantity of hydroquinone being biotransformed into arbutoside in the first 2-3 hours after it's addition to the culture media.

Conclusions

- From all the explants tested for the inducement of the primary callus, the hypocotyle explants proved to be the most fit when cultivated in sterile environment on MS media culture with the following composition 2,4-D (1.0 mg/l) and BA (2.0 mg/l), at light;
- Massive callus culture was obtained by transferring the primary callus on the same MS media culture with 2,4-D and BA;

- The primary cell suspension resulted by transferring small fragments of friable callus on MS liquid medium supplemented with glutamine (0.2 g/l), hydrolised casein (0.2 g/l), 2,4-D (1.0 mg/l) and BA (2.0 mg/l). After 6 successive subcultivations on a similar culture medium we acquired homogenous plant cell suspensions;
- The biotransformation capacity of plant cell suspensions was tested by adding hydroquinone (1mM) to the culture medium on the 14'th day of development;
- Qualitative analysis performed by TLC (Thin-Layer Chromatography) revealed the capacity of *Digitalis purpurea* cell suspensions to biotranform hydroquinone. The samples harvested at 3 hours after hydroquinone addition had already biotransformed arbutoside.
- Hydroquinone was completely biotransformed as it couldn't be detected neither in the cells nor in the culture medium. The presence of arbutoside was noted only in the cells.

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CULTIVATION DIFFERENCES BETWEEN POT MARIGOLD (*CALENDULA OFFICINALIS* L.*)* VARIETES

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Summary

Pot Marigold, Calendula officinalis L., is an annual plant. It likes hot warm and dry climate with the lover moisture. It can be cultivated almost everywhere in Slovakian agricultural conditions. In the last five years its cultivation, collection and the sale of dry flowers became favorite and profitable activity in the Estern parts of Slovakia. The variety „PLAMEN" is allowed and recommended variety of Calendula officinalis. In the last few years Calendula officinalis is dedicated not only to seedgrowing but also tried to put into effect a variety called „PLAMEN PLUS" in the Eastern Slovakia. The new „PLAMEN PLUS" variety was bred on plenty larger flower, the advantageof which is bigger weight. According to this survey we can state that the Marigold flower drug coming from different varieties has diferent weight of fresh and dry flowers furthermore it biosynthetises diferent amount of β-carotene and a percentage of phytotherapeutic explotiable substances. In comparism of 5 groupings containing 10 flowers we can say that they are heavier under fresh and dry conditions at „PLAMEN PLUS" variety which was not affected by higher proportion shrinkage near dry raw material. As to the quality, higher content of effective substances – carotenoides, the content of β-carotene is 0,090% at "PLAMEN PLUS " variety. With the second variety it is 0,030% more (0,120%). In conclusion, we can say that the main differences between the production of flower heads and the content of active components between two Pot Marigold varieties were confirmed. We highly recommend choosing variety "PLAMEN PLUS" for production and collection. Harvest of dry flower heads can be 30% higher and the content of β-carotene can increase in 50%.

Keywords: *Pot Marigold, variety "PLAMEN PLUS", β-carotene, top cross, flower drug*

Introduction

Photosynthesis process is dependent on photoreceptors, which are biosynthesized with chlorophylls and the additive plastid pigments: β-, α-carotenes and xanthophylls. β-carotene is biosynthesized in some flowers with an orange color. The typical example is Marigold, which is one of the medicinal plants well-known for its wide therapeutic use.

Pot Marigold, *Calendula officinalis* L., is an annual plant with angular, branches, hairy stem 0.30 to 0.50 m high. The leaves are alternate, sessile, spatulate or oblanceolate, dentate with widely spaces teeth, and hairy. From June to October the plant bears large orange, terminal flower heads (Habán, 1996). β-carotene biosyntheses is carried out into the lingual flowers of the Pot Marigold heads. It is well know that hydrolysis of this carotenoide in accordance with the central double bond creates two molecules of Vitamin A. The very important attribute of both these compounds is their anti-oxidative activity; it is protection of organism against reactive oxygenic forms. β-carotene and Vitamin A influence subcutaneous tissue and appeal as anti-oxidants in regard to formation of tumorous cells result uncontrolled oxidative process. They have big value on protection of skin, mucous membrane of throat, mouth, stomach, intestines, respiratory diseases, bladder and prostate. The Pot Marigold extract properties and activities are inflammatory (Della-Logia et al., 1994), antioedematous (Zitterl-Eglsee et al, 1994) and antitumoral (Boucand-Maitre et al., 1988).

Purpose of Pot Marigold breeding was created of a variety without the disk flowers but large petal flowers around all corolla. The variety "*PLAMEN PLUS*" was introduced in a large-scale cultivation of this special crop several years ago.

In this study, several advantages of "*PLAMEN PLUS*" cultivation against an old variety "*PLAMEN*" is presented in order to a flower yield and β-carotene content and the effects of fertilization on the yield and biomass formation.

Material and methods

Growing techniques and plant material

A 3 year field experiment (2003, 2004, and 2005) was initiated with both varieties at 2 localities in Slovakia: Presov – a wet sub-mountain climatic region with sufficient amount rainfall, neutral brown fertile soil; Streda nad Bodrogom – a warm and dry climatic region of the Lowlands in East Slovakia with neutral soil having a low content of nitrogen, phosphorus and magnesium (Table 1). The fertilizer (NPK) was added in various quantities (50 kg. ha^{-1} and 100 kg. ha^{-1}) to the soil at the experimental sites.

Table 1. Environmental conditions of test locations.

	Presov			Streda nad Bodrogom		
Climate[1]	2003	2004	2005	2003	2004	2005
Temperature [^{0}C]	14.1	12.8	13.1	14.2	14.8	14.5
Rainfall [mm]	352.7	665.0	619.0	333.4	461.7	448.6
Endaphic[2]						
Fertility [$mg.kg^{-1}$]						
Nitrogen	155 – 225			28 – 87		
Phosphorus	75 – 147			21 – 34		
Potassium	74 – 87			55 – 92		
Magnesium	315 – 534			145 – 165		
Organic matter [%]	1.71 – 2.33			1.66 – 1.89		
Soil pH	7.4 – 7.5			7.0 – 7.2		

[1]For the vegetative growth period (March-August); average temperature; total rainfall,
[2]The contents of individual elements in soil are given in amounts available for plants.

Seedings were done in the spring (March and April) at 12 to 15 g per 10 m^2 in rows 0.4 m apart using seeds of Czech varieties "*PLAMEN PLUS*" (Presov) and "*PLAMEN*" (Streda nad Bodrogom) produced in the SEVA-FLORA Ltd. in Valtice. Flower heads were collected in several individual harvests, the first at the beginning of July and next 15 to 20 days later after the planting which were disturbed by the first and next harvests had regenerated. The collecting season is usual finished at the ending of August. The flower clusters were cleaned of field debris and subsequently dried for 6 to 12 days in a dark room at a 21 to 23 ^{0}C and relative humidity of 40 to 60 percent.

Chemical evaluation

Main component of Pot Marigold flowers (*Flos calendulae cum calyce*) was isolated by extraction with methanol and the β–carotene quantity was determined by spectrophotometer. 1 g of the crush dry flower heads is usual poured over 50 ml of solvent (petroether, benzene and methanol /9:1:3/ or a clear methanol), which is extracted on a Soxhlet apparatus during 2 hours. The mixture is filtered and filled by hexane. % of β–carotene is determined by spectrophotometer absorbance (1 % of β–carotene in hexane) in 445 nm.

Results and discussion

Differences between Pot Marigold clusters

The random 10 (5) Pot marigold flowers both varieties (Fig. 1) were selected in order to a comparison of their weighs and β–carotene quantity.

Fig. 1. Calendula clusters: variety „*PLAMEN* “ & variety „*PLAMEN PLUS*“.

Table 2. Yield-created characteristic and β– carotene quantity of Pot Marigold varieties.

weigh of 10 flower heads [g]		variety „*PLAMEN* “	variety „*PLAMEN PLUS*“
fresh /n = 5/	$x= x_1+...+ x_5/n$	18.28	25.78
	δ	3.68	2.72
	δ/√n	1.62	1.21
	x ± t. δ/√n	18.28 ± 4.49	25.78 ± 3.36
dry /n = 10/	$x= x_1+...+ x_{10}/n$	2.48	3.42
	δ	0.21	0.22
	δ/√n	0.06	0.07
	x ± t. δ/√n	2.48 ± 0.15	3.42 ± 0.15
ratio of parch		1 : 7.3	1 : 7.5
% extract components in 100 % methanol		25 ± 1	25 ± 2
% content of β– carotene	ethanol broth	0.090	0.120
	hexane extract	0.080	0.100

A comparison of the calendula cluster weigh from two varieties indicates the highest weigh of the variety *„PLAMEN PLUS“*(Table 2) in fresh and dry forms. In regard to a quality raw-material, the β– carotene content is 0,090 % in the variety *„PLAMEN“*, it is on 0.030 % lower than in the variety *„PLAMEN PLUS“* (0.120 %).

Yield created parameter = various variants of fertilization

The 3 year field experiments (2003, 2004, and 2005) at 2 localities with both Pot Marigold varieties were presented the obtaining dates and predictions from the part up. The experimental design was a randomnized, complete block with 4 replications. The results of plant dry biomass from various variants of fertilization in Presov and Streda nad Bodrogom are performed in tables 3 and 4.

Table 3. Quantitative changes of dry biomass from various fertilization variants in Presov

Presov, Slovakia			V_3	V_1	V_2
2005	flower heads	[g.m^{-2}]	18,7± 2,8	30,1± 4,5	30,9± 4,7
	total plant biomass	[g.m^{-2}]	371,1± 55,6	433,0± 46,1	474,7± 52,2
	underground biomass	[g.m^{-2}]	73,5± 11,0	98,4± 12,7	87,8± 13,1
	over ground biomass	[g.m^{-2}]	297,9± 33,3	334,6± 42,8	386,9± 39,1
2004	flower heads	[g.m^{-2}]	17,9± 2,6	31,7± 4,7	37,9± 5,6
	total plant biomass	[g.m^{-2}]	254,5± 38,1	419,2±42,1	466,0± 51,3
	underground biomass	[g.m^{-2}]	106,4± 15,9	94,8±12,1	106,7± 12,3
	over ground biomass	[g.m^{-2}]	148,1± 22,1	324,4± 48,6	359,3± 31,2
2003	flower heads	[g.m^{-2}]	26,4± 3,9	59,6± 8,9	65,2± 9,7
	total plant biomass	[g.m^{-2}]	359± 53,8	301± 32,1	476± 65,2
	underground biomass	[g.m^{-2}]	158,8± 23,8	80,4± 12,0	69,8± 10,4
	over ground biomass	[g.m^{-2}]	200,2± 15,5	220,6± 21,2	406,2± 47,9

Legend: V_1 - variant with a dose of 50 kg NPK. ha^{-1}, V_2 – variant with a dose of 100 kg NPK.ha^{-1}, V_3 – control without any fertilizer

Table 4. Biomass formation from various variants of fertilization in Strede nad Bodrogom.

Streda nad Bodrogom, Slovakia			V_3	V_1	V_2
2005	flower heads	[g.m^{-2}]	5,7±0,85	12,0±1,8	14,8±2,2
	total plant biomass	[g.m^{-2}]	213,5±32,0	317,4±35,7	376,4±43,9
	underground biomass	[g.m^{-2}]	79,9±11,9	91,5±13,7	99,2±14,8
	over ground biomass	[g.m^{-2}]	133,6± 20,0	225,5±33,8	277,2±32,1
2004	flower heads	[g.m^{-2}]	2,2± 0,3	9,0± 1,3	19,7±2,9
	total plant biomass	[g.m^{-2}]	51,6±7,7	107,9± 16,1	247,6± 31,1
	underground biomass	[g.m^{-2}]	4,9± 0,7	9,9± 1,4	36,4±5,4
	over ground biomass	[g.m^{-2}]	46,7±7,1	98,0±14,7	211,1± 30,1
2003	flower heads	[g.m^{-2}]	6,7± 1,2	8,2± 1,2	13,2±1,9
	total plant biomass	[g.m^{-2}]	106,7± 16,0	148,3±19,7	233,9±25,6
	underground biomass	[g.m^{-2}]	26,1±3,9	24,5± 3,6	46,4±6,9
	over ground biomass	[g.m^{-2}]	80,6± 12,0	123,8±18,5	187,5±28,1

Legend: V_1 - variant with a dose of 50 kg NPK. ha^{-1}, V_2 – variant with a dose of 100 kg NPK.ha^{-1}, V_3 – control without any fertilizer

In three test years the addition of NPK fertilizer (100 and 50 kg NPK.ha^{-1}) increases the flower yields, total, underground and over ground biomass (table 3 and 4). Very low yields and biomass quantities are presented in Streda nad Bodrogom. These results are referred in utilizing the limited soil resources and the dry climatic conditions (Šalamon et al., 2004). The NPK fertilization affected flower and biomass yield and content of the active β–carotene of Pot Marigold.

The β-carotene content

The differences in total β–carotene concentration of flower heads from two localities were presented very large differences (table 5).

Table 5. Marigold -effect of different locality cultivate conditions and β–carotene formation

Locality of cultivation	% of extract components	β–carotene content [%]
Streda nad Bodrogom	24 ± 1	0.035 ± 0.004
Prešov	17 ± 3	0.110 ± 0.055

Plant production of bioactive compounds is most strongly influenced by genetics and the growing environment (dry or wet climate). The studies reported above indicate that climate aridity has an important role as well (Vieira et al., 2002). In regard to varying soil – climatic conditions, this part of Slovakia provides heterogeneous of facilities to quantity of flower yield and secondary β-carotene component.

Conclusion

Pot Marigold, *Calendula officinalis* L., is an annual plant. It likes hot warm and dry climate with the lover moisture. It can be cultivated almost everywhere in Slovakian agricultural conditions. In this study, several advantages of the new variety "*PLAMEN PLUS*" is presented in order to a flower yield and β-carotene content and the effects of fertilization on the yield and biomass formation. The NPK fertilization affected flower and biomass formation and content of the active β–carotene of this special plant. It is confirmed, plant production of clusters and bioactive compounds is most strongly influenced by genetics and the growing environment (dry or wet climate). Increasing fertilization affected flower head yield and the enhanced the β–carotene yield was due to the higher crop yields.

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METHODS USED FOR CARAWAY (*CARUM CARVI* L.) BREEDING IN THE CZECH REPUBLIC

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Summary

The breeding of caraway in Agritec is focused on the stability of yield, good health state, the essential oil content and the non-shattering maintenance. The positive and negative selection and forced self-pollination are used for breedingof biennial caraway. The genotypes with better quantitative characters then the current varieties were obtained by recurrent phenotypic selection. The formation of dihaploid plants is new method of caraway breeding. This process accelerates the creation of the homozygous and homogenous genotype. The best results provided the dihaploid plants of winter form. The form with reduced vegetative period (winter form) is bred for the non-shattering maintenance, high essential oil content and for antifreezing.

Keywords: *caraway, Carum carvi L., breeding, dihaploid, non-shattering*

Introduction

Caraway (*Carum carvi* L.) is a famous spice plant cultivated for the achenes which contain about 2 – 7 % of essential oil. Agritec company is interested in breeding and in growing technologies of traditional biennial caraway and caraway with reduced vegetative period (winter caraway) for ten years. Caraway is one of the oldest and the most cultivated spice plant in the Czech Republic region and recently it takes up an area about 3000 hectares. Particularly, the biennial form is cultivated in pure culture or with cover crop (spring barley, triticale, poppy). Three czech varieties of non-shattering type of biennial caraway are registered in the Czech Republic (Rekord, Prochan, Kepron) and eleven varieties are included in the Common Catalogue of agricultural plant species (EU). There is no variety of winter form registered in the Czech Republic.

The breeding is focused on the stability of yield, good health state, the essential oil content and the non-shattering maintenance. The positive and negative selection and forced self-pollination are used for breeding of both caraway forms. The formation of dihaploid plants is new method of caraway breeding. This process accelerates the creation of the homozygous and homogenous genotype.

Material and methods

Caraway with standard length of vegetative period is bred by recurrent phenotypic selection for chosen characters – the yield of achenes, the essential oil content, the health state, the antifreezing, the earliness.

The other method of breeding of the biennial caraway is forced self-pollination. In this procedure, the inflorescence is separated by monofilament sacks and this is repeated during 5 – 7 generations. After that, the offspring is homogenous and homozygous. This material can provide new caraway varieties of line, in some case of hybrid type.

The tens of genotypes of winter form are developed by forced self-pollination. The advantage of this form is shorter vegetative period and it also runs out of the attack of gall mite (*Acerina carvi*). On the other hand, the disadvantage is meanwhile lower yield of achenes and lower essential oil content.

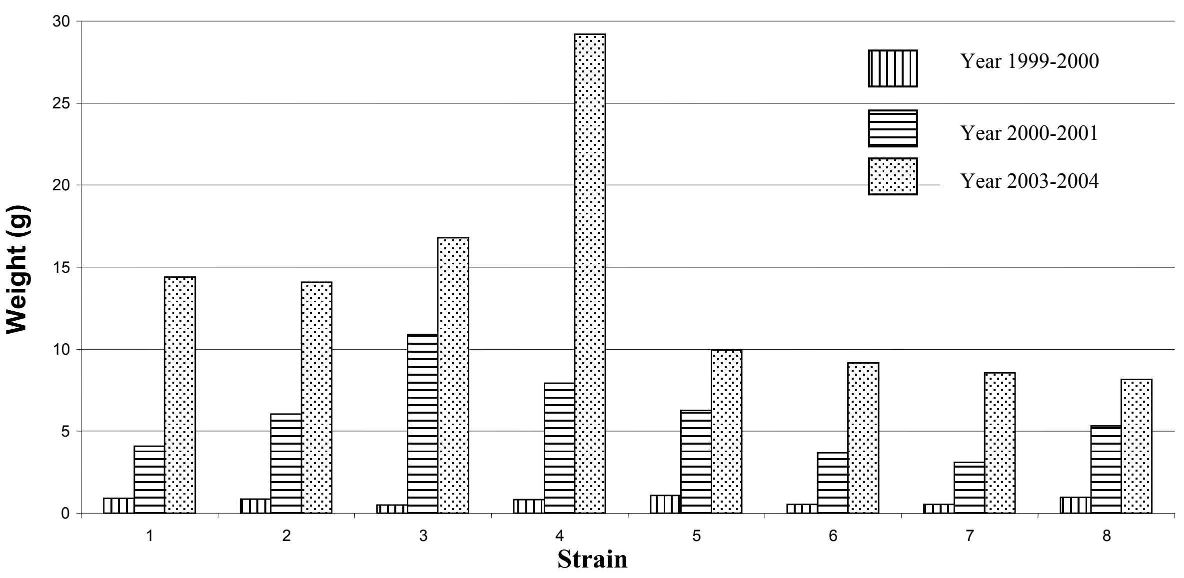
New method – the formation of dihaploid plants is used for shortening of long-term cycle of homozygotization. This process accelerates the creation of the homozygous and homogenous genotype for one vegetation period only (from sampling of pollen, till callus cultivation and growing of dihaploid plant). This method was not described for caraway yet. The procedure is finished and prepared for routine usage now in Agritec company.
The technique partially results from the knowledge about creation of the dihaploids of carrot (*Daucus carota* L.) (Andersen S. B. et al., 1990). Twenty media for cultivation of prepared anthers and for induction of embryogenal callus were tested. The pre-incubation of the donor plants proceeds in vernalisation chamber. The whole bud with pollen is took in developing stage corresponding with uninuclear microspores.
After 2 – 4 months, the cultures are removed on the media without the plant hormones and direct embryogenesis is proceeded. The plants in stage with 4 – 6 leaves are adapted for semi sterile conditions. After that the plants are removed to non-sterile conditions, they are transplanted into the substrate and watered by distilled water (Smykalova et al., 2005). When the plants strike, colchicine is applied onto the apical meristem. In this stage, the process of creation of the dihaploid plants is finished and the material can be used for further breeding activities.

Results and discussion

Table 1. The number of regenerated plants (reg.) and representation of the individual levels of ploidity (year 2005)

Genotype (the anthers resp.)	Number of reg. plants	Ploidity level		
		x	2x	4x
208a/1 (4)	37	25	12	0
210b/1 (1)	1	0	1	0
1605c/1 (4)	49	3	46	0
2908d/3 (1)	8	0	7	1
3005c/2 (1)	17	17	0	0
∑ 5 genotypes (11)	112	45	66	1

Graph 1



At the present time, the trade experiments with many genotypes of biennial caraway run. The genotypes were formatted by positive recurrent phenotypic selection. Some of these genotypes show better characters then registered varieties. Especially, the yield characters, as they are influenced by the year strongly, they depend on the genotype. After the checking of these genotypes in the trade experiments they can be given for registration as new variety of caraway.
The best results provided the dihaploid plants of winter form. When we obtain the homozygous and homogenous lines of caraway, it will be possible to think of breeding of new caraway varieties (line or hybride type).

Conclusions

The breeding is a long term process and to exceed of current varieties is not easy. The future varieties would have, except the higher yield of the achenes, their uniformity, especially the varieties created by biotechnological method (dihaploids). It is important to stick some problems which are not solved: the occurence, the spreading and the effects of gall mite (*Acerina carvi*), the changes of the growing technologies (various cover crops), the weed control by herbicides (*Asteraceae* weeds). In addition, it is necessary to solve the influence of the localities, the varieties and technology on the essential oil content in the achenes.
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RESEARCH ON TROPANE ALKALOID BIOSYNTHESIS IN *SCOPOLIA CARNIOLICA* JACQ. ADVENTITIOUS ROOT CULTURES

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Summary

Atropine (A) and scopolamine (S) biosynthesis in adventitious root cultures of S. carniolica Jacq. was analyzed. In four types of cultures, selected by root length and development, S increased in time in parallel with a slight decrease of A. Root lines selected in media containing putrescine or tropic acid were submitted to elicitation with salicylic acid (SA) (0.1 and 1.0 mM) for 24, 48 and 72 hours. The influence of SA was considered optimal for 0.1 mM concentration after 48 hours. The 1.0 mM SA acted as inhibitor.

Keywords: *Scopolia carniolica Jacq., adventitious root cultures, atropine, scopolamine, elicitors.*

Introduction

Scopolia carniolica belongs to the *Solanaceae* family, being known and used for its tropanic alkaloids, atropine and scopolamine, substances with anticholinergic activity upon acethylcholine and muscarinic receptors. The alkaloids are utilized as muscle relaxants, in preoperative medication or eye exams. Scopolamine is used in the treatment of motion sickness (Katzung, 1995). *S. carniolica* grows in Romania in wet and shady places in mountain beech woods and accumulates the two alkaloids in the rhizomes (Stefanescu *et* Tamas, 2000).

Cell and root cultures derived from other different species of *Solanaceae* have been established for the biotechnological production of tropane alkaloids (Yamada, 1997). Generally, undifferentiated cell cultures do not produce these compounds efficiently because synthesis is linked to root differentiation therefore root cultures are preferred (Robins *et al*., 1991). Our former studies on *S. carniolica* concerned the possibility of inducing *in vitro* cultures (Deliu *et al*. 2002). Also the influence of the precursors on alkaloid biosynthesis in *S. carniolica* adventitious root cultures was studied (Deliu *et al*. 2004).

The use of in vitro cultures for the production of secondary metabolites is limited by the low amounts that are biosynthesized. Therefore several strategies are followed to improve the biosynthesis capacity (Buitelaar *et* Tramper, 1992). Our present study aimed to investigate the capacity of alkaloid biosynthesis in *S. carniolica* adventitious root cultures submitted to selections (concerning root length and development) and to elicitor action.

The selections by root development concern most frequently the secondary or tertiary roots. Studies on *Hyoscyamus niger* showed that once cut, the secondary roots (become now "primary roots") could develop better than the former cultures and also the scopolamine amount found in cultures initiated from former secondary roots was higher (Woo *et al*. 1997). Taking this into consideration, in our study we compared four types of adventitious root cultures selected by cutting method applied for several times also connected to selection by root length and the report between primary and secondary roots development. The alkaloid biosynthesis capacity was investigated in the dry biomass.

In order to stimulate secondary metabolites biosynthesis for in vitro cultures several method are employed, among them being the use of elicitors. Elicitor is considered a compound that can induce accumulation of antimicrobial phytoalexins in plants (Darvill *et* Albersheim, 1984)

but that can also stimulate any type of defense response (Ebel *et* Scheel, 1997). The elicitor, when introduced in small concentrations to a living cell system, initiates or improves the biosynthesis of specific compounds, which includes secondary metabolites. Abiotic elicitors are considered stress agents which include ultraviolet radiation, heavy metal salts, chemical compounds with diverse mechanisms of action (Eilert, 1987).
In *Datura stramonium* in vitro cultures, the elicitors used to enhance atropine, scopolamine or littorine amounts were methyl jasmonate, a cell wall preparation from baker's yeast, oligogalacturonides (Zabetakis *et al*., 1999). Also jasmonic acid and aluminium chloride enhanced hyoscyamine productivity in *Brugmansia candida* root cultures after 24 hours of contact (Spollansky *et al*., 2000). The alkaloid production in *Brugmansia* root cultures was also stimulated by salicylic acid, $AgNO_3$ or yeast extract (Pitta-Alvarez *et al*., 2000).
In the present study we have monitorized the alkaloid biosynthesis capacity in adventitious root cultures submitted to salicylic acid action during 24, 48 and 72 hours. Two root lines previously selected from media containing tropic acid and putrescine were studied.

Material and methods

Secondary roots cultures. Adventitious root cultures of *Scopolia carniolica* were established from rhizogenic callus growth on Murashige and Skoog solid medium (Murashige *et* Skoog, 1962) containing 1.0 mg l^{-1} IBA (Deliu *et al*., 2002). The adventitious roots were maintained in Gamborg liquid medium (Gamborg *et al*., 1968) containing 3% sucrose and 1.0 mg l^{-1} IBA. The inoculum used was about 700 mg fresh weight per 300 ml Erlenmeyer flask with 50 ml of fresh medium. The flasks were mentained on 100 rpm rotary shaker in the dark. The root cultures were subcultured at 2-week intervals. Four lines of secondary adventitious roots were obtained. The selection of lines was made by cutting method considering the development of secondary roots and the roots length (Woo *et al*., 1997). For the first selection the inoculum consisted in cut secondary roots which led to line 1RS. The secondary roots developed by this line were selected after five months of cultivation which led to line 2RS. All cultures developed primary roots (former secondary roots) and secondary (former tertiary) roots. From the obtained cultures were selected the roots with maximal length for the principal (former secondary) root – for about 2-3 cm – and very small secondary (former tertiary) roots, which led to line 3RS. Also roots developing long secondary (former tertiary) roots – for about 1.5 cm – were selected to lead to line 4RS. The alkaloid content was analyzed in the dry biomass obtained after 14, 21 and 28 days from inoculation for each line.
Elicitation. For elicitation two adventitious root lines were utilized. The first one (RT line) was selected from solid medium with similar composition as described above, supplemented with tropic acid (2mM). The second one (RP line) was selected from similar solid medium supplemented with putrescine (2mM). Further on, the two lines were subcultured on liquid Gamborg media (Gamborg *et al*., 1968), with 1.0 mg l^{-1} IBA and without tropic acid or putrescine. Elicitation was carried out with salicylic acid added to the culture medium in two different concentrations: 0.10 mM (01RT, 01RP samples), and 1.00 mM (1RT, 1RP samples). The pH was adjusted to 5.5. The elicitor was added to 18-day-old cultures and these were incubated according to the conditions described above. Different exposure times were tested: 24 (code 24 samples), 48 (code 48 samples), and 72 (code 72 samples) hours. All the experiments were done along with controls (CRT, CRP samples) with no elicitor add. The alkaloid content in the dry biomass was determined.
Alkaloid analysis. The dry biomass obtained was thoroughly pulverized. 1.00 g powder was made alkaline with 0.5 ml 30% NH_4OH and the alkaloids were extracted three times in 5 ml $CHCl_3$ for 24 hours on continuous agitation. The reunited extracts were evaporated at the rotary evaporator at 40°C and 400 mbar. 1 mg dry extract was dissolved in 1 ml CH_3OH

(HPLC purity) and was used for injection. Extraction procedures varied slightly from those proposed by Betz *et al.* (1995). Representative LC-UV-APCI-MS analysis of the extracts containing the internal standard codeine (50 ng on column) was carried out. Column: XTerra RP_{18} (4.6 x 150 mm i.d., 3.5 μm) with a pre-column of the same material (3.9 x 20 mm i.d., 3.5 μm). Eluents: water (A) and acetonitrile (B) with 2 mM NH_4OH. Gradient: 10-32% B in 5 min, isocratic elution at 32% B for 10 min, 32-100% B in 5 min and 100% B for 5 min were applied. Flow rate: 0.8 mL/min. UV detection at 220 nm. MS conditions: capillary temperature: 150°C; vaporizer temperature: 350°C; corona needle current: 5.0 μA; sheath gas (nitrogen) pressure: 30 psi; in-source CID: –5.75 eV. Detection method varied slightly from that proposed by Zanolari *et al.* (2003).

Results and conclusions

The 1RS (fig. 1.a) and 2RS (fig. 1.b) lines showed a similar alkaloid profile, the initial atropine amount being slightly higher in 2RS in the 14th day. Initially the atropine amount was 7-9 times higher than the scopolamine amount. Up to the 28th day of culture the atropine amount decreased about three times, while the scopolamine amount followed a plateau for 1RS line or increased up to two times for 2RS line. Finally the atropine found was only 1.5-3 times higher than the scopolamine.

Lines 3RS (fig. 1.c) and 4RS (fig 1.d) also showed a similar alkaloid profile, atropine amount being 4 times higher then the scopolamine amount in the 14th day of culture. Up to the 21st day the atropine slightly increased, followed by a decreasing up to the 28th day. The decreasing of atropine was parallel with the increasing of scopolamine (2-3 times higher then in the 14th day).

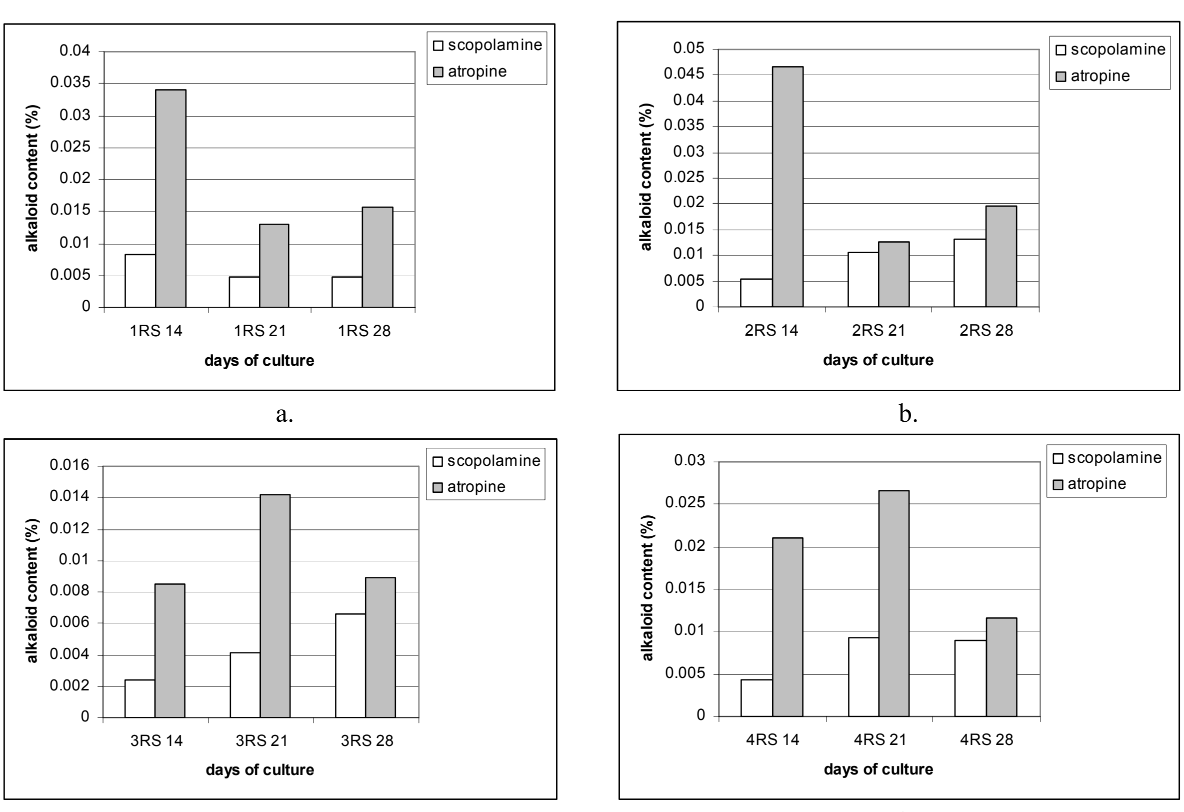

Fig. 1. Atropine and scopolamine variation during *in vitro* cultivation for *Scopolia carniolica* adventitious roots cultures: 1RS (a), 2RS (b), 3RS (c), and 4RS (d) lines

In 1RS and 2RS lines that derived from secondary roots and also in 4RS line which had well developed secondary (former tertiary) roots, the alkaloid amount was higher then in 3RS, which had long primary (former secondary) roots with small secondary (former tertiary) roots, leading to the conclusion that the biosynthesis was higher in the secondary roots. This was in agreement with the data presented by Woo *et al.* (1997). Also for the cultivation of secondary roots in the dark, the same pattern of atropine decreasing in parallel with scopolamine increasing was observed as formerly presented (Deliu *et al.* 2004).
The results obtained from the second experiment showed that from the two lines utilized, the RT line biosynthesise lower alkaloid amounts compared to line RP.
The RT line elicitation with 0.1 mM salicylic acid had as effect the increasing in both scopolamine and atropine biosynthesis, although the amount could not reach 2 times the control amount. The alkaloid amount showed an ascending line from 24 to 48 hours of contact, followed by a decrease up to 72 hours of contact. The atropine amount found after 72 hours was even smaller than the control amount.
The salicylic acid in 1 mM concentration led to the stimulation of the atropine biosynthesis only after 24 hours of contact, even more than the 0.1 mM concentration, while the scopolamine biosynthesis was inhibited. After 48 and 72 hours of contact both atropine and scopolamine biosynthesis were drastically inhibited, after 72 hours the amount being under detection limit.

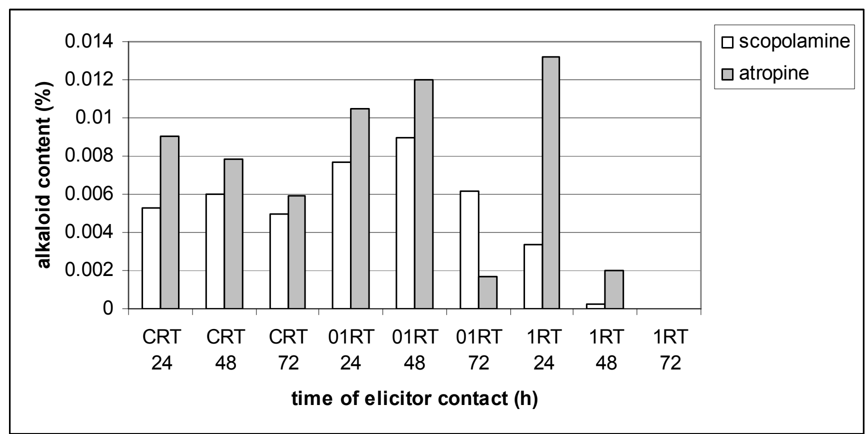

Fig. 2. The elicitor (salicylic acid) influence on atropine and scopolamine biosynthesis in RT line

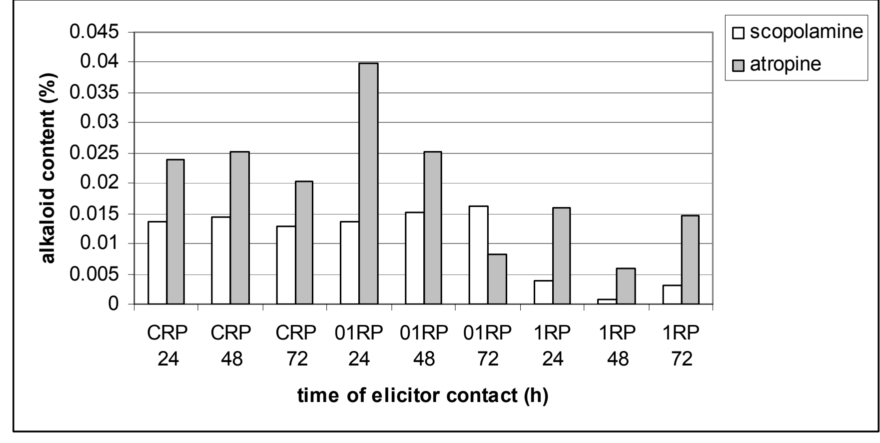

Fig. 3. The elicitor (salicylic acid) influence on atropine and scopolamine biosynthesis in RP line

Similarly, for RP line (fig. 3), the salicylic acid in 0.1 mM concentration led to the enhancing of both scopolamine and atropine biosynthesis. While the scopolamine biosynthesis was only slightly stimulated, the atropine amount was two times higher after 24 hours of contact, decreasing constantly after 48 and up to 72 hours of contact. The scopolamine amount showed a constant increase, although it was very slight. In this case also, the atropine amount found after 72 hours was even smaller than the control amount. The elicitor in 1 mM concentration inhibited scopolamine and atropine biosynthesis, the amount found being even 2-4 times smaller than the control amount.
The salicylic acid in 1.0 mM concentration inhibited more the biosynthesis in RT line than in RP line, this being correlated with the slight inhibiting action of tropic acid towards biomass development. The more favorable action after 48 hours contact in the RT samples showed the stimulation of the biosynthesis even in conditions of biomass growth inhibition. This led us to conclude that the influence of the salicylic acid in enhancing the alkaloid biosynthesis in *S. carniolica* adventitious roots was optimal for 0.1 mM concentration and after 48 hours of contact.

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MEDICINAL AND AROMATIC PLANT DECAY CAUSED BY *SCLEROTINIA SCLEROTIORUM* IN SERBIA

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Summary

The marshmallow (Althea officinalis), valerian (Valeriana officinalis), nettle (Urtica dioica), and caraway (Carum carvi) are described as the hosts of Sclerotinia sclerotiorum in Serbia. The symptoms and morphological characteristics are described. Effect of temperature and media were investigated on pathogen development and sclerotia production.

Keywords: *marsh mallow, valerian, nettle, caraway, Sclerotinia sclerotiorum*

Introduction

Sclerotinia sclerotiorum is a widespread, homothallic and necrotrophic, ascomycetous fungus (1). It is plurivorus fungus and is an important plant pathogen of over 400 species. Sclerotinia blight is a common disease of sunflower in Serbia. Recently (1999-2005), sudden wilt of marshmallow (*Althea officinalis*), valerian (*Valeriana officinalis*), nettle (*Urtica dioica*), and caraway (*Carum carvi*) was observed in the spring and summer. Diseased plants wilted and died before the end of vegetation season.

Material and methods

Diseased plants of marsh mallow, valerian, nettle and caraway were collected during spring and summer from 1999 to 2005. From symptomatic stem sections the pathogen was isolated on PDA at 22°C. Pathogenicity of obtained isolates was confirmed by inoculating seedlings of marsh mallow, valerian, nettle and caraway, which were grown in pots in the greenhouse. The inoculum was the mixture of homogenised mycelia and sclerotia in 100 ml of distilled water. The plants were kept three days in wet chamber and then transferred in a greenhouse.
Pathogen development and sclerotia formation were studied at potato dextrose agar (PDA), malt agar (MA), Sabourand dextrose agar (SDA) and water agar with fragments of carnation leaves (WCA). Effect of temperature on pathogen development "in vitro" was studied at 5-30°C, with 5°C intervals.
Vitality of sclerotia (1-5 years age) were studied by incubation on wet filter paper at 4ºC for two to four months.
Identification of pathogen was done according morphological characteristic of asci and ascospores (2).

Results and discussion

Initial symptoms if the infection started through contaminated soil included stem necrosis at the soil level (Fig. 1 a), yellowing and tan discoloration of leaves. The roots of such necroted (Fig. 1 b) and infected plants died. If infections were realised by ascospores water soaked were formed on the stems. The diseased tissue become necroted and were covered with whitish cottony mycelia (Fig. 1 c, d). As stem necrosis progressed, infected plants wilted and

died before the end of vegetation. On necrotic tissues dark, spherical to elongate spherical sclerotia, 2-8 mm diameter were produced.

Fig. 1. *Sclerotinia sclerotiorum*. Symptoms of infection basal part (a) and necrosis of the root tissue of marshmallow (b), white mycelia with sclerotia formation of pathogen on the stems of marshmallow (c) and nettle (d).

On PDA colonies were white to pale grey with abundant dark brown to black sclerotia formed (Fig. 2 a, b). Apothecia (Fig. 3 a) with characteristic asci and ascospores (Fig. 3 b) were formed from sclerotia after 2 month of incubation on wet filter paper at 4ºC. Sclerotia kept its viability for long time (over five years).

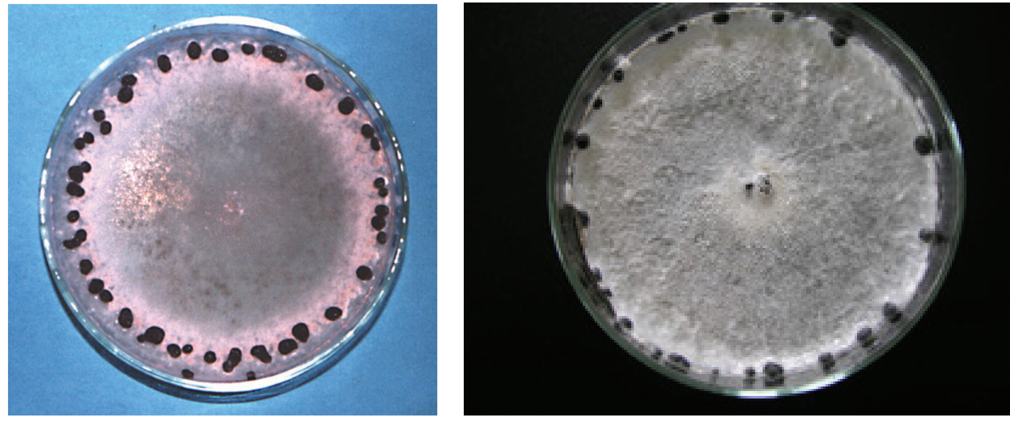

Fig. 2. *Sclerotinia sclerotiorum*. Culture appearance and sclerotia formation of isolate from caraway (left) and from valerian (right).

According to morphological characteristics of isolates obtained from symptomatic stem sections of marsh mallow, valerian, nettle and caraway pathogen was identified as *Sclerotinia sclerotiorum* (Lib). De Bary (2).
The best pathogen development *in vitro* and the most abundant sclerotia formation were at PDA. Pathogen grew well at all temperatures tested (5-30ºC) with optimum being at 20-25ºC.

Fig. 3. *Sclerotinia sclerotiorum.* Germinating sclerotia which formed apothecia (left) and ascus with ascospores (right)

Artificial inoculated plants showed similar symptoms, and characteristic mycelia and black sclerotia were formed within five and seven days, respectively. Control plants remained symptomless. The pathogen was reisolated from inoculated plants.
Sclerotinia sclerotiorum as plurivorous fungus has a wide host range (1, 2). It is generally more important as a pathogen of vegetables in the field, during transport and in store (2). This pathogen was described on some medicinal and aromatic plants such as common sage (3), *Thymus x citriodorus* (4), St. John's worth (5), coneflower (6). It has already described on marshmallow and valerian (7, 8) in Serbia, but here is reported for the first time as pathogen of nettle and caraway.

Conclusion

- Sclerotinia blight is a common and very destructive disease on many growing plants.
- It was on marshmallow and valerian in Serbia, but for the first time on nettle and caraway.
- According to morphological characteristics pathogen was identified as *Sclerotinia sclerotiorum*.
- As pathogen's sclerotia survived for a long time in the soil it could be suggested to the farmers not to grow medicinal and aromatic plants on the fields where previous crops were sensitive plants, such as sunflower.

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MEDICINAL NAD AROMATIC PLANTS RESEARCHES AT THE CZECH UNIVERSITY OF AGRICULTURE IN PRAGUE

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Summary

Research in medicinal, aromatic and spicy plants at the Czech University of Agriculture in Prague is focused on these plant species: Hypericum perforatum L., Oenthera biennis L., Matricaria recutita L., Calendula officinalis L..
In experiments with Hypericum perforatum L. we determined, e.g., hypericine content in above-ground parts of plant in different development stages in cultivar Topaz.
In research of Oenthera biennis L. we focused biology of evening primrose on long-day and short-day influence of generative parts formation.
The experiments with Matricaria recutita L. was concentrated to organic and conventional cultivation of Chamomile and how these cultivation technologies influenced content of essential oil in Chamomile.

Keywords: *Hypericum perforatum L., Calendula officinalis L., Oenthera biennsis L., Matricaria recutita L.*

Introduction

St. John's wort (*Hypericum perforatum* L.) and its quality
St. John's wort *(Hypericum perforatum L.)* was the traditional colecting medicinal plant, but today with increasing demand of pharmaceutical industry it has been cultivated as arable crop. At The Czech University of Agriculture (CUA) we cultivated Hypericum perforatum L. between 2000 to 2002 at an experimental farm in Červený Újezd. In our experiments together with agricultural engineering we mainly monitored the quality of hyperici drug as to the content of hypericin in individual above-ground parts of plants.

Evening primrose *(Oenthera biennis)* L. and its biology
At present evening primrose ranks a significant place in pharmaceutical industry, mainly the primerose oil, wchich is obtained from seeds. Evening primrose seeds contain approximately 20 % of oil with most important composition, primarily essential fatty acids: linoleic acid (18:2) 60 to 80% and acid γ – linolenic 7 to 12 %, which is antecedent eikosanoids (prostaglandins E1 and their derivatives). Cold-drawn evening primrose oil has contributed to regulation of blood pressure, has decreased cholesterol level, increased immunity. It is used for treatment of premenstruation syndrome, it helps treat serpigoes, disseminated sclerosis and other disorders.
Cultivation of Evening primrose has not been so much successful in our field conditions. Evening primrose vegetation developed from direct sowing has long and wrong stems and it should develop as a two-year plant (in the 1st year a plant can create a ground blooming leaf rosette, and in the 2nd year a bloomimg branched stalk). When giving rise to vegetation from seedlings, a part of evening primrose plants bloom in the 1st year but the seeds do not have enough time to form and ripen. Our field experiment was focused to monitor the reaction of a two-year evening primrose, how it launches generative plants bodies in long nad short days conditions. In our experiments we observed the growth and development of plants in the 1st year of vegetation.
Theme: *Calendula officinalis* L. as an oil crop for industrial use. was presented on last konference(3rd CAMAPSEEC)

Topic: *Matricaria recutita L.*- qualitative and quantitative evaluation will be presented in another contribution on this konference(4th CAMAPSEEC)

Material and methods

Agricultural engineering of experiment as follows:
Former crop – winter wheat. Variety of *Hypericum perforatum* – Topaz. The firts sowing was realized on April 19, 2000 (seeds did not germinate), the second sowing of June 19, 2000 (before the second sowing, the soil was compacted by hoop driving machine, and vegetation germinated). Inter-row distance for sowing was 50 cm and the depth of sowing was nearly above ground, sowing rate 1,5 kg/ha of seeds. Germinating capacity was 70 %.
During vegetation the growth and development of plants were followed, ranks of weed of individual variants, yield of top in flower, content of hypericin in indiivdual parts of above-ground parts of mass in dry plants.
For the experiment following variants were used:
1: undersowing of spring barley (400 grain/m2)
2: aplication of herbicide – preemergency herbicide Stomp
3: aplication of herbicide – preemergency herbicide Roundap
4: control variant – without chemical protection
Tcheduling of sampling in years 2001, 2002
I. – plants not in flower (beginnig of May)
II. – 1 st harvest – top in flower (end of June)
III. – 2 nd harvest – top in flower (1st half of September)

Chemical methods to determin the content of hypericin:
Determination of dry mass
In all samples of St. John's Wort dry mass was determined. From each sample 2.0 grams of powdered mass were weighed and closed in formely dried-up and weighed aluminium weigh-in containers. Weigh-in containers together with the content were weighed and placed in compartment kiln. The temperature of drying was 105 ^{o}C. After exsiccation the wiegh-in container was weighed again.
Extraction of samples
For the chemical analysis it was required to triturate every sample of *Hypericum perforatum L.* All of samples were grioun so that formed corresponding powdered mass. Samples were weighed into extraction crucibles.
Samples were placed into Soxhlet´s apparatus in a fume-hood and 150ml of diethylether was poured into the apparatus. The pre-extraction with diethylether in water bath (36°C, chlorophyll removement) took about 30 hours. A dried sample was extracted again using Soxhlet´s apparatus at the same temperature for 20 hours using anhydrous methanol (150ml). Each sample was filled with methanol up to volume of 250ml.
The quantity determination of total content of hypericin by means of absorbance
The experiment of quantity determination of hypericin was carried out by the Southwell metodology. This method uses ascertained absorbance of Hyperici extract. Every sample of hyperici extract was measured by spectrophotometer Specol 11 at wave length 592 nm versus methanol. Registered values were fitted to formula from technical literature - SOUTHWELL et al., 1991.

$$X = A \,.\, 3031{,}55 \text{ (ppm)}$$

X.....total content of hypericin
A.....absorbance

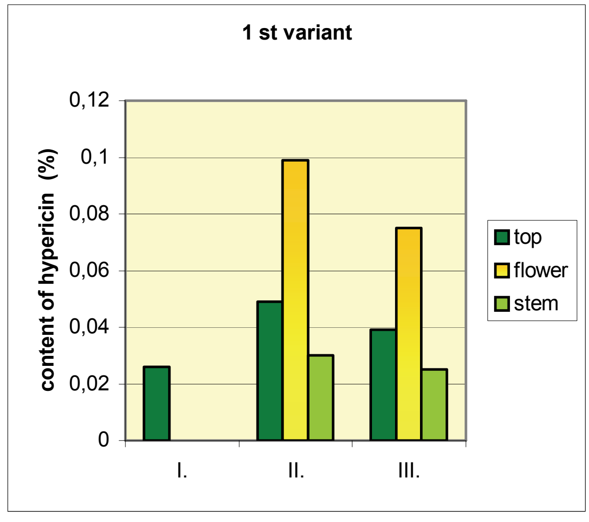
The Result presents the total content of hypericin components in individual extracts.

Pot experiments at the CZUA in Prague
Origin of Seeds: Czech origin (Libochovice) – „L“
English origin – „E“
Variants: 1st pre-cultivated microseedlings (phase of 4 original leaves, 24 DC)
2nd pre-culivated seedlings (phase of 8 original leaves, 28 DC)
Every variants had 4 frequencies
Long-day Conditions (LD)
Short-day Conditions (SD)
Monitoring the growth and development of evening primrose plants by the macrophenological scale by ŠUKOVA, 1992.

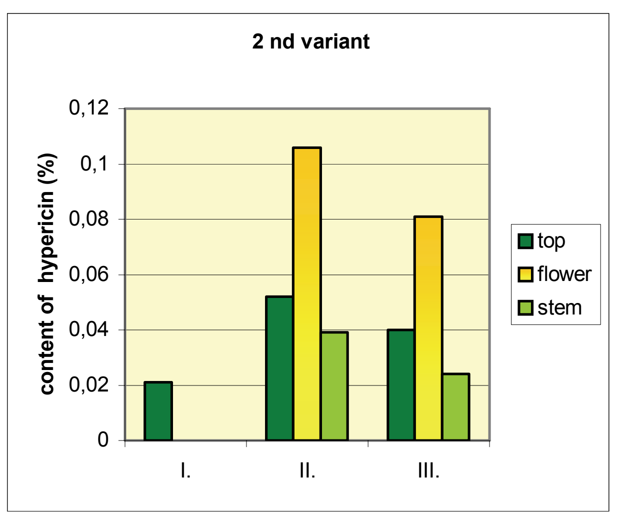
Results and discussion

When comparing individual years, no marked changes were determined. At the first term of sampling of hyperici top, which was not yet in flower, the content of hypericin was very low (cca 0,025%), which actually responds to natural patterns of production of secondary metabolism. At the second term of sampling - in the 1st harvest the highest value of content of hypericin was measured. Differences between variants were not noticeable. When comparing individual parts of hyperici plants, the highets content of hypericin was measured in flower-buds (0,11 %), then in flowers (0,106 %), in flowering top (0,054 %) and the least value of content of hypericin was measured in stems (0,039 %).
At the third term of sampling – in the 2nd harvest the highest content of hypericin was measured in flowers (0,081 %), next in flowering top (0,040 %) and the lowest content of hypericin was determined also in stems (0,025 %).
When comparing samples by dates of collecting hyperici plants contained the highest value of hypericin in the 1st harvest. The samples from the 2nd harvest were considerably lower of content of hypericin than in the hyperici plants from the 1st harvest. The lowest content of hypericin was determined in hyperici plants. which were still not in flower.

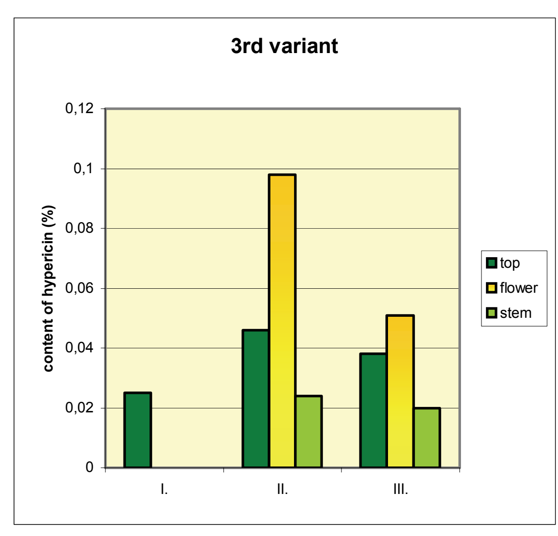
Graph 1: Average values of hypericin (%)



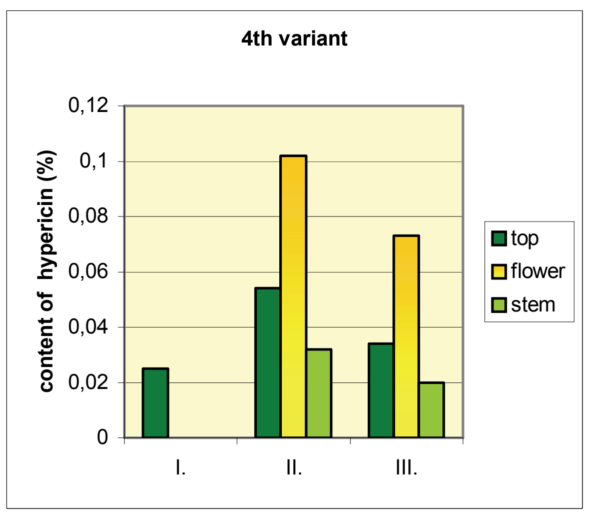
Graph 2: Average values of hypericin (%)



Graph 3: Average values of hypericin (%)



Graph 4: Average values of hypericin (%)



Final values were compared wit values, which are stated in The Czech Pharmacopoeia. Between individual variants no differences were registered. By the The Czech Pharmacopoeia, the lowest content of hypericin in hyperici herba is 0,08%. In samples from the 1st harvest only flowers and buds correspond the requirements of the quality standard as stated in The Czech Pharmacopoeia. In the 2nd harvest this quantity (0,08 %) was measured only in flowers. Tops which were not in flower and stems were less than substandard.

Southwell (1991) indicates that the content of hypericin is increasing in individual parts of hyperici plants in this order: main stem (40ppm) side stems (120ppm), lower leaves (290ppm), top leaves (380ppm), capsules (730 ppm), flowers and flower buds (2150 ppm). This order has been confirmed also in values from our experiment, even though they were lower. (graphs 1- 4).

Obtained values are given in the 1st table. Short day in seedlings and microseedlings of evening primrose plants retarded the growth, development and inhibited blooming in the both origins of seedlings, none of variants bloomed. Differences between seedlings and microseedlings and the both origins of seedlings were observed in long-day conditions. 80% of seedlings of the Czech origin bloomed. Seedlings of the English origin did not bloom. 80% of microseedlings of the Czech origin bloomed, and 60% of microseedlings of English origin bloomed. Plants grown from the seeds of the Czech origin presented better results. By SPITZOVA (1992) evening primrose can bloom already in the 1st year of vegetation, when the vegetation period is longer. SHEIDOW a ROY (1990) state, that cultivation of sufficiently big seedlings is capable of reproduction in the 1st vegetation year between 60-80 days. In our experiments plants achieved the phase of leaf rosette (28–30 DC) in this period, based on seedling length. By KOCOURKOVA (1997) seeds of English origin are selected as one-year seeds. In our research, this was not confirmed.

Table 1. Evaluation of influence of day duration, plants area and origin on flowering and tillering

growth phase	English Seedlings		Seedlings from Libochovice		Total seedlings		English microseedlings		microseedlings from Libochovice		total microseedlings	
	SD	LD	SD	LD	SD	LD	SD	LD	SD	LD	SD	LD
% plants in flower	0	0	0	80	0	40	0	60	0	80	0	80
% plants in leaf rosette	100	40	60	0	80	20	100	40	100	0	100	20

% tillering plants	0	60	40	80	20	70	0	0	0	80	0	40
% plants to tillers	0	60	40	20	20	40	0	0	0	60	0	30
% plants to tillers	0	0	0	40	0	20	0	0	0	20	0	10
% % plants to tillers	0	0	0	20	0	10	0	0	0	0	0	0
% plants with footstalk	0	0	0	80	0	40	0	60	0	80	0	80
% plants with flowering tillers	0	0	0	60	0	30	0	0	0	40	0	20
% flowering tillers	0	0	0	85	0	42	0	0	0	45	0	22

explanatory notes: **SD** – short day; **LD** - long den

Conclusion

St. John`s Wort *(Hypericum perforatum L.)* contained the most of hypericyn in the first term of the harvest, and the least at the beginning of vegetation, when plants were not still in flower. The highest content of hypericin was measured in flower buds and in blossoms, and the lowest in stalks. This can be decisive how long plant stalks may be harvested. The length between 10-20 cm appeared to be optimal, which is the length of a plant stalk in blossom. The harvest of longer stalks contains the higher stalk proportion with the lower content of hypericin. This brings down the quality of a harvested drug, and this method is not so effective.

Our results obtained from pot experiments show, that for initial cultivation of evening primrose vegetation, suitable seedlings (28 DC) are those which developed rapidly and this is a precondition of good ripening of seeds in our conditions. Microseedlings reacted positively on a day length, but their development was slower. Low ripening of seeds is still an issue, and we cannot exclude a possibility to inciate evening primrose vegetation in warmer parts in the Czech Republic (earlier field planting). In our conditions it is more advisable to iniciate vegetation from seedlings, when the vegetation period is prolonged by precultivation of seedlings in greenhouses, and field planting starts in April. Conditions of higher probability of blooming are already provided in the first vegetation year. This method of cultivation is economically more demanding, but more certain. Better results were reached by using the seeds of the Czech origin.

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MANIPULATION OF BIOMASS AND BIOSYNTHETIC POTENTIAL OF *MORUS NIGRA* AND *GLYCYRHIZA GLABRA* TISSUE CULTURE BY *AGROBACTERIUM RHIZOGENES* MEDIATED GENETIC TRANSFORMATION

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Summary

Morus nigra and Glycyrhiza glabra are very known medicinal plants used for their active principles deoxynojirimycine and glycyrrhizic acid, respectively [1]. Most of the secondary metabolites with pharmaceutical importance are synthetized by the medicinal plants in their roots. Rhizosphere proved also numerous advantages for the stability of plant tissue culture, as compared to cell culture or other plant organ culture[14]. Therefore, much attention has been focussed recently in the domain of biotechnology for the manipulation of this type of biomass and, through it, the biosynthetic potential of active principles. In vitro tissue culture and root inducing (rol) genes transfer [6], as well as molecular genetic and biochemical (HPLC) analyses are described as an experimental model for the biomass manipulation through Agrobacterium rhizogenes mediated genetic transformation in Morus nigra and Glycyrhiza glabra species.

Keywords: *rhisosphere, genetic transformation, biomass, biosynthetic potential, manipulation, Glycyrrhiza glabra, Morus nigra*

Introduction

Medicinal plants are the most exclusive source of life saving drugs for the majority of the world's population. In recent years, traditional medicine has become a topic of global importance. Although modern medicine may be available in developed countries, herbal medicines (phytopharmaceuticals) have often maintained popularly for historical and cultural reasons. The current technology is based on the capacity to introduce high biosinthetic rate in secondary metabolites [5,12]. Genetic transformation of medicinal plants has been initially tackled through *A.tumefaciens* mediations. However, because of the extensive debate focused on this GMO productive technology, an alternative, more environmental and hence bioetical friendly system has been assayed begining with the 90s. The high potential of this *A.rhizogenes* mediated genetic transformation determined the development of a new domain of medicinal plant biotechnology.

The induction of hairy roots in medicinal plants represents the main objective of such technologies and has been widely reported in the literature[7, 8]. For instance, hairy roots from 200 species of higher plants, mostly dicots, representing at least 30 plant families, have been reported, and represent a truly remarkable range of biosynthetic capabilities. The hairy roots is thus well established as an experimental system and most importantly, it has provided many insights into root specific metabolism and its regulation.

This technology is based on the genetic transformation of such plants mediated by the soil bacteria *A.rhizogenes*. Depending on the *rol* gene types and synergic interactions, there have been observed variable hairy root phenotypes, which show different branching and plagiotropic roots growing [10, 14].

The hairy roots developed at the site of the infection may serve as an efficient source of the further root cultures. Also, the hairy roots as the results of genetic transformation by *Agrobacterium rhizogenes*, have attractive properties for medical and nutritive applications. They often grow fast as or faster than plant cell cultures [3,10,14] and do not require hormones in the medium. The greatest advantages of using hairy roots system is that these

root cultures, are not pretencious as for the medium composition and hence its monitorization, then present a high genetic stability and moreover, do not demand sophysticated molecular genetic analysis.
This paper is aiming at demonstrating the effectivness of the transfered *rol* (onco)genes in the genome of two representative medicinal plants, *Glycyrrhiza glabra* and *Morus nigra,* in developing genetically transformed hairy roots with a high biosynthetic potential in corresponding active principles, glycyrrhizic acid (GA) and deoxynojirimycine (DNJ).

Materials and methods

Genetic transformation and establishment of hairy roots cultures

Hairy roots culture was established by infecting internodes and roots segments with a scalpel containing 2 day old *A.rhizogenes* LBR56 (pA4RS) liquid co-culture medium (the bacterial strain was kindly provided by Dr.Tepfer INRA, France), 10 minutes for *Glycyrrhiza glabra* and 2 day old wild-type *A.rhizogenes* (Ar 40) liquid co-culture medium (the bacterial strain was kindly provided by Faculty of Biotechnology, USAMV), 30 minutes for *Morus nigra*. The infected segments were incubated at 25 ^{0}C under 3000 lux light conditions. After 2 day of co-culture, explants were first transferred on fresh MS medium supplemented with 0,5g/l cefotaxime in order to eliminate bacteria[11]. Three-five weeks after the bacterial infection, hairy roots emerged from the infected sites (fig.1a, b). The elongating roots tips were cut off and transferred to growth regulator containing MS agar medium without cefotaxime in order to obtain hairy roots cultures on a solid medium.
Both*, Morus nigra* and *G. glabra* hairy roots were also cultured in MS liquid medium with 3% sucrose adjusted to pH = 6.8 before autoclaving. The culture flasks (200 ml) were inoculated with about 1g fresh hairy roots segments and then placed on shaker at 100 rpm at 25 ^{0}C temperature and 3000 lux (16h light/ 8h dark). After 3-4 weeks the growing hairy roots were transferred on fresh medium in order to obtain a liquid medium hairy root culture that represents a natural source for GA and DNJ content assays.

Confirmation of transformation

Phenotype analysis.The specific properties of the rapidly growing and branched roots (hairy roots) as the expression of Ri T-DNA, offered a direct visual possibility to estimate the efficiency of transformation process by the phenotype approach.
The genotype analysis. Both *G. glabra* and *M. nigra* hairy roots were used for the DNA extraction and identification of rooting locus genes (*rolB*) by polymerase chain reaction. DNA was isolated by CTAB modified method as described by Doyle and Doyle (1990)[4]. The polymerase chain reaction (PCR) was used to confirm the presence of the *rolB oncogene* in roots. The primer used [12, 13] yielded a DNA fragment of 635bp from the *rolB* oncogene.
The PCR reaction mixture (25µl) consisted of: 50mM of each dNTP, 50 ng of genomic DNA, 1mM of each primer, Taq DNA polymerase and 10X standard PCR buffer. The PCR parameters set up on a Biometra Thermal Circler used for amplification consisted of a denaturation step of 5 min at 94 ^{0}C and 35 cycles each comprising: 55s denaturation at 94 ^{0}C, 50s annealing at 55 ^{0}C for rolB and 2 min extension, followed by a final extension at 72 ^{0}C for 7 min. The PCR products were fractioned by electrophoresis on a 2% agarose gel, stained with (0,001gdm^{-3}) ethidium bromide [19, 20]. The amplicon samples were run comparatively with a DNA weight marker “PCR Low Ladder” provided by Sigma. The electrophoresis gel has been visualized and photographed in a dark chamber UVP Jencons-PLS, at 302 nm.

UV spectral analysis of GA and DNJ content in root biomass

The hairy roots were subcultured on growth-regulator-free MS liquid medium for 20-40 days, then harvested and dried to constant weight. The hydro-alcoholic root extract (250 mg dry hairy roots were grounded and extracted with 70% ethanol at 105^0C for 30 min; the extract was then filtered and the extraction procedure was repeated for two times till 10 ml total volume extract.) have been obtained and measured comparatively with the standard GA and DNJ respectively (provided by Sigma Aldrich, St. Louis MO, USA). The GA and DNJ working callibrating solutions of $1x10^{-5}$, 10^{-5}, $7,5x10^{-5}$, 10^{-4}mol L^{-1} were obtained by dissolving its in methanol and 0,1% trifluoracetic acid, and performing the appropriate dilution. The alkaloid content in hairy roots was determined by spectrophotometric quantification and HPLC, respectively and was addressed as mg GA/g or DNJ/g root biomass.

Results

The paper presents a study case for the application of transgenesis tehniques in order to obtain a high biosynthetic potential biomass with medicinal plants. The elected transformation plants were *Glycyrrhiza glabra* and *Morus nigra* which has been intensively approached in our laboratory practice [2, 9, 13]. Our experiment is using the same genetic transformation system involving *A.rhizogenes* mediation, which had been previously approached with different species (*Solanum tuberosum, Lycopersicon esculentum, Atropa belladonna*). In this case, certain peculiarities have been noticed: the hairy roots presented significant lignifications and a tendency of less branching as compared with above mentioned cases. We suspect, according with the literature reports [1,3,7] that an interesting interference may be due to the high yield of sugar and phenol compounds characteristic to the *G.glabra* tissues.

This presentation was aiming at demonstrating the benefic effect of the rol gene transfer in certain medicinal plants for obtaining a high productive biomass in pharmaceutical important compounds easy monitored further in (laboratory) bioreactors. Moreover, the *A.rhizogenes* strains used for such genetic transformation were wild type strains that did not imply other foreign genes which would raise the well known bioetic controversies linked with the modified plant release in the natural environment and its uses as food products.

The experimental model proposed demonstrated the need for simple, cheap but efficient culture and analysis techniques that enable it with advantages over the usual known.

Phenotype and genotype analysis

The phenotype and genotype analysis, proved the transformed state of the medicinal plants and the HPLC analysis of the secondary metabolites (GA and DNJ) demonstrated the effect of the *rol* gene transfer on the biosynthetic root potential.

Hairy roots emerged at the infection sites during 4-5 weeks after inoculation (fig.1a, b). The morfology of this biomass was characteristic for the „hairy root" phenotype: more uniform and displayed a typical phenotype characterized by plagiotropic growth, without high incidence of lateral branching. The variation in the characteristics of hairy roots may be ascribed to differences in the copy number and/or position of integrated T-DNA(s) into the plant genome. The further exploitation of this phenotype was directed towards: i) the initiation of a hairy root culture on liquid medium for a bioreactor culture and; ii) the regeneration of whole transformation plants for the maintaining of the germoplasm.

Integration of T-DNA into *G.glabra* and *Morus nigra* genome was confirmed by the PCR reaction with specific primers for *rolB* oncogenes (fig.2). The electrophoresis behaviour of the amplified DNA fragment, as compared with the DNA weight marker, showed an

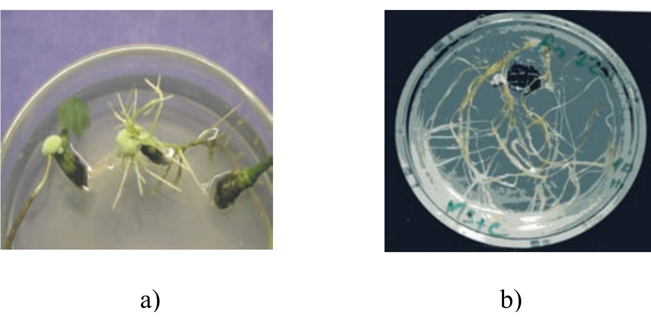
amplicon of 635bp specific for *rolB* oncogene in both root systems (*G.glabra* and *M.nigra*). These results indicated that the *rolB* from the Ri plasmid of *A.rhizogenes* LBR56 and *A.rhizogenes* 40 were integrated into the genome of both *G. Glabra* and *M.nigra,* respectively. The PCR product was absent in non-transformed tissue (control).

GA and DNJ yield

The spectral measurement at 244nm and 280nm, respectively confirmed the presence of GA and DNJ peaks in root extracts (fig.3.a, b) as compared with the standard GA and DNJ peak obtained in the same solvents. The results showed a variation when comparing the GA and DNJ peaks registered for different root biomass extracts (the hairy root and the control untransformed root extracts). We measured the growth rate of the rhizosphere biomass in the same nutritive medium and the same vessel volume and the results showed a significant (2 fold) increase during the same period of time for the hairy root biomass. Figures 4 (a, b) represent the variation in this essential parameter, which we consider to be referred to the biosynthetic rate potential of the transformed roots. The advantages of hairy root cultures may be motivated also by a greater stability of root culture, as compared with that of untransformed roots, which showed necrosis and therefore a significant active biomass loss starting after 30 days. Accordingly, the GA or DNJ concentration are increased or decreased with a coefficient linked with the biomass weight variation in the same medium volume and the same period of time.

Discussion and conclusion

An efficient transformation system was developed for medicinal plants, *G. glabra* and *M. nigra*. It enabled the introduction of the *rol* genes into the plant genome leading to the production of hairy-root cultures. The integration and expression of Ri-T-DNA containing the rol genes were confirmed by phenotype and genotype analysis. The effect of the hairy root phenotype development was the obtaining of a high productive root biomass with an increased biosynthetic potential of the medicinal compounds. Such biomass may be further maintained in laboratory bioreactors and do not interfere with the current debate on the risk of the GMO release in natural habitats.



a) b)

Fig. 1. Hairy roots phenotype: a) *Glycyrrhiza glabra;* b) *Morus nigra*

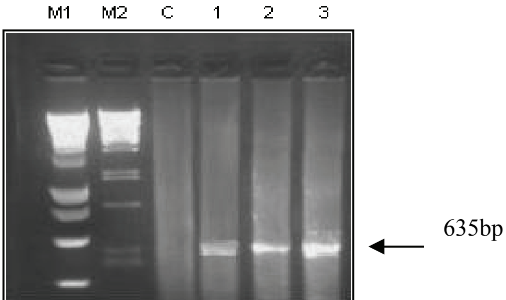
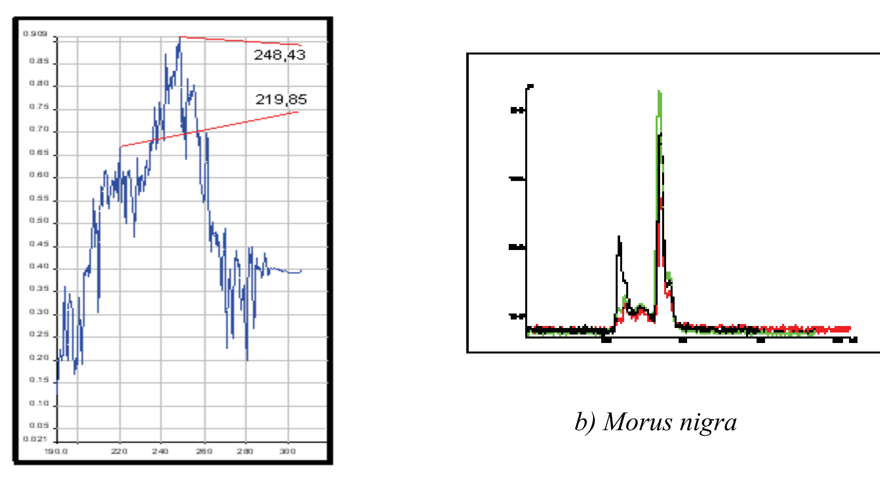


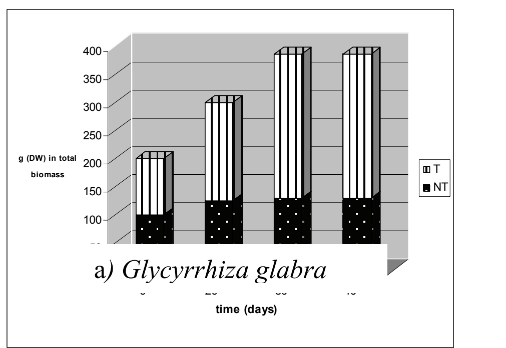
Fig. 2. The 635bp amplicons for *rolB oncogenes,* respectively, obtained by PCR amplification in transformed *Glycyrrhiza glabra* and *Morus nigra* hairy roots; (M1,2-DNA molecular weight marker, 1-2- DNA from *G.g* hairy roots; 3-DNA form *M.n* hairy roots; C-control, untransformed roots



b) Morus nigra

a) Glycyrrhiza glabra

Fig. 3.a) Direct uv spectrophotometric absorbance at 244nm of GA in alcohol extracts of Gg hairy roots; b) uv spectrum of DNJ (the 280nm peak) detected after HPLC of the alcohol extracts of *M.n* hairy roots.



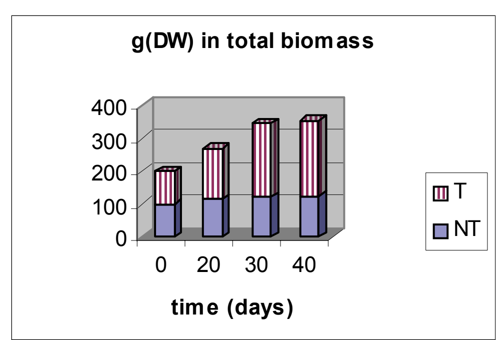


Fig. 4.The GA (a) and DNJ (b) content in T (hairy roots) compared with NT (non transformed roots) corresponding at different moments of root biomass development (0-20-30-40 days).

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THE EFFECT OF BIOFERTILIZATION ON THE INFLORESCENCE YIELD AND THE CONTENT OF CHLOROPHYLL AND CAROTENOID PIGMENTS IN MARIGOLD (*CALENDULA OFFICINALIS* L.)

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Summary

The experiments studied the effect of the microbiological compounds Azotofertil and Extrasol applied in soil and the foliar fertilizer with natural extract of plants Bionat on marigold culture. The results reveal the stimulator effect of the microbiological compounds on the inflorescence yield,photosynthetic efficency in leaves and the content of the carotenoid pigments in inflorescence.

Keywords: *biofertilization,marigold,chlorophyll,carotenoid pigments.*

Introduction

The mineral fertilization with nitrogen applied in soil may contaminate the environment, being a strong source of chemical pollution. The principles of ecological agriculture reveal the importance of some alternative efficient technologies, that may decrease soil pollution, like biofertilization applied in soil or foliar fertilization. Biofertilization is made in soil by the treatments with microbiological preparations of some free-living nitrogen fixers, like *Azotobacter chroococcum* and *Azospirillum brasilense*; the treatments stimulated some physiological processes in different agricultural species, other than leguminous (AISHWATH O. P. et al., 2003; ABU C. et al., 2005; CHOUDRY A. and KABI M. C., 2005; SAIKIA S. P. et al., 2004). Foliar fertilization is made by spraying with mineral and organic fertilization, like urea; the treatments ensure an efficient management of nitrogen in apple orchards or in other horticultural cultures(DONG S.et al.,2005 ;NESTBY R .and TAGLIAVINI M.,2005).
Biofertilization and foliar fertilization with natural plant extract are recommended especially in medicinal plant culture with a view to obtaing the unpolluated drugs. The inflorescence of marigold (*Flores Calendulae*) is a drug, a compound of different pharmaceutical preparations, the main compounds of the drug being the essential oils, saponins, sesquiterpenes, flavonic glycosides, carotenoid pigments (GRAINGER BISSET N.,1994;ROBU T.and MILICA C.,2004)
The work studies the effect of the microbiological compounds Azotofertil and Extrasol and of the foliar fertilizer with natural plant extract Bionat on the inflorescence yield and the content of chlorophyll and carotenoid pigment in marigold.

Material and methods

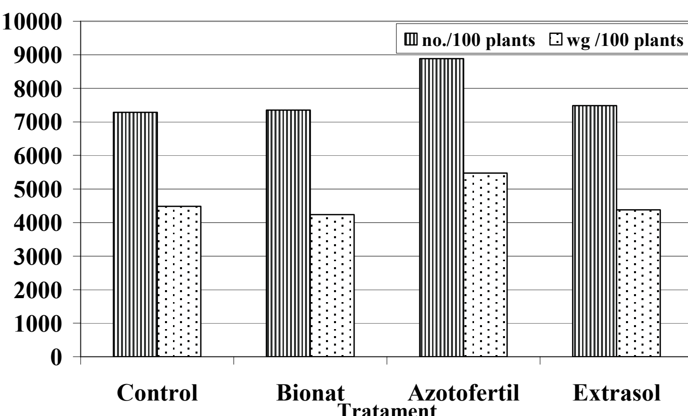
The experiments were made on marigold plants,cultivated at S.C. Biarom Comp. S. R. L., Rediu, district of Iassy. The seeds were obtained from S. C. Plafar Botoşani, being local populations. The plants were cultivated on experimental surfaces of 200 m^2, in a control, unfertilized variant, and in other three variants, with foliar fertilization and soil biofertilization. The foliar fertilization was made with Bionat, foliar fertilizer with natural plant extract,made in S.C. Panetone S.R.L.,Timişoara, but soil fertilization was made with Azotofertil, microbiological compound with free-living nitrogen fixers *Azotobacter chroococcum* and *Azospirillum lipoferum*, made in S. C. Antibiotice Iassy and with Extrasol,

microbiological compound for ecological agriculture made in Bislobi S.R.L., Bălti, Republic of Moldova. The treatments were applied by the indication of the protocols.
The dynamics of the inflorescence yield was appreciated by decadal and monthly analysis of the inflorescence number and weight in 100 plants, in each variant. The content of pigment in leaves and inflorescences was appreciated spectrofotometrically, by determination of light absorption of acetone extract in blue and red zones of the visible spectrum, that characterizes the wavelengths with maximum absorption for chlorophylls (431 - 432, 453 - 454, 616 - 617 and 662 - 663 nm) and carotenoid pigments (425 - 427 and 448 nm).

Results and discussion

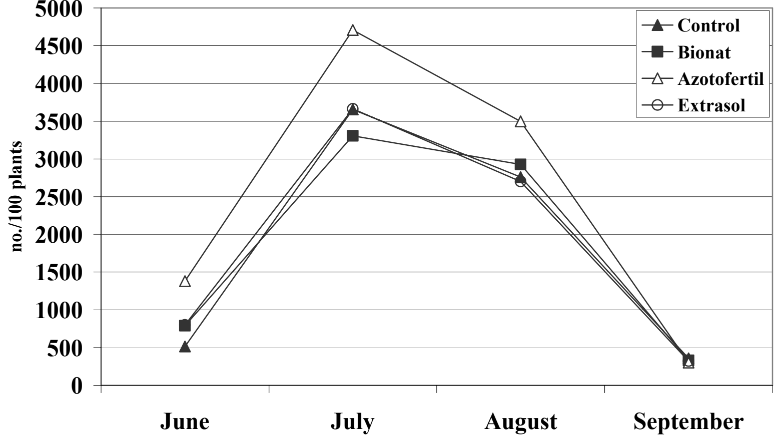
The values of total inflorescence yield are presented in fig.1. The inflorescence number and weight is higher than in unfertilized variant only in the variant biofertilized with Azotofertil, but in other variants there are the same.

Fig. 1 The values of the total inflorescence yield



The dynamics of the inflorescence number and weight determinated monthly is presented in fig.2 and 3. In all experimental variants, the inflorescence number and weight are maximum in July. The treatments stimulated flower morphogenesis and flowering in June, but the stimulation effect in July and August is higher than in unfertilized variant only in variant biofertilizated with Azotofertil.

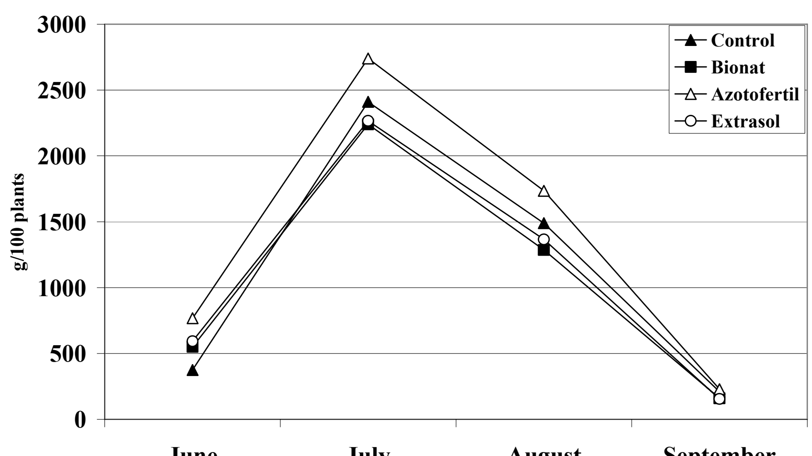
Fig. 2 Dynamics of inflorescence number harvested monthly



The analysis of the pigment content in leaves and inflorescences, appreciated by light absorption of the acetone extract reveals that in leaves there are the maximums of light absorption for chlorophyll a, 662 - 663 nm in red zone and 431 - 432 nm in blue zone, and chlorophyll b, 616 - 617 nm in red zone and 453 - 454 nm in blue zone, in all experimental variants.

All applied treatments decreased the chlorophylls content in photosynthetic systems, in the reaction centers, with maximum absorption in red zone, and in light absorption centers, with maximum absorption in blue zone of the visible spectrum (fig.4). This reaction reveals an increase of the photosynthetic efficiency in plants. The most evident results are obtained by soil biofertilization with microbiological compounds, especially Azotofertil.

Fig. 3 Dynamics of inflorescence weight harvested monthly



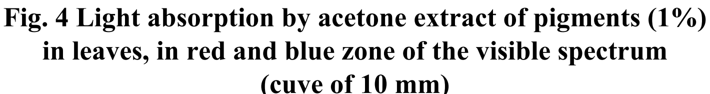
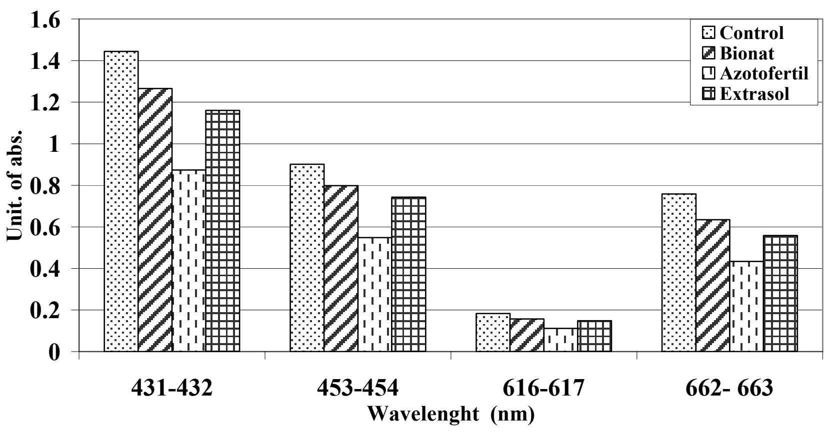


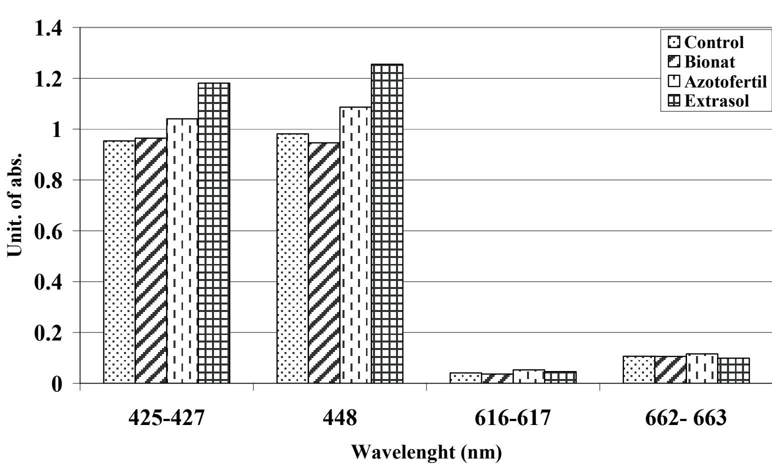
Fig. 4 Light absorption by acetone extract of pigments (1%) in leaves, in red and blue zone of the visible spectrum (cuve of 10 mm)



Recent researches reveal the stimulator effect of free-living,nitrogen-fixing bacteria on some physiological processes in different cultivated plants, other than leguminous. In wheat, biofertilization with *Azotobacter sp*.and *Azospirillum sp*.increased nitratreductase activity (AISHWATH O. P. et al., 2003); in rice stimulated nitrogen absorption and dry matter accumulation (CHOUDRY A., KABI M.C., 2005). In mays, the treatments with *Azospirillum sp*. and 2,4 D developed in plantules the paranodules on the lateral roots; the roots contain leghemoglobin pigment and nitrogenase enzyme (SAIKIA S. P. et al., 2004).

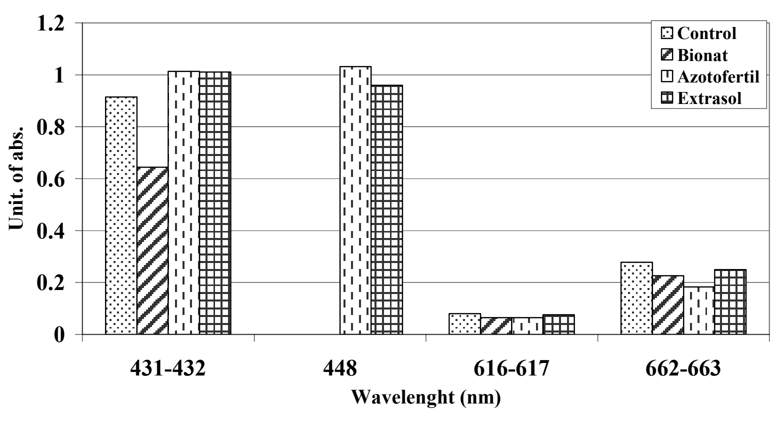
The results in mature inflorescences reveal decrease of chlorophyll and strong accumulation of the carotenoid pigments, with maximum absorption in blue zone of visible spectrum (425 - 427 and 428 nm). TAMAS V. and NEAMTU G.(1986) give these maximums for the carotenoids pigments extracted in acetone, especially alfa-carotina.
Identification of carotenoid pigments in acetone extract in marigold inflorescence was made by different authors (NAVROTESCU MIOARA TINCA et al., 2005).
In these experiments, carotenoid pigment biosynthesis is stimulated by application of microbiological compounds in soil, especially Extrasol (fig.5).

Fig. 5 Light absorption by acetone extract of pigments (1%) in mature inflorescences, in red and blue zone of the visible spectrum (cuve of 10 mm)



The results obtained in inflorescence buds reveal the decrease of chlorophyll with maximum light absorption in red zone,especially chlorophyll a 663 - 664 from the reaction center of the photosynthetic system. The content of chlorophyll a 431 - 432, a compound of light absorption center is high in variants biofertilizated in soil with microbiological compounds Azotofertil and Extrasol, at the same time with the carotenoid pigment biosynthesis (fig.6).

Fig. 6 Light absorption by acetone extract of pigments (1%) in inflorscence buds, in red and blue zone of the visible spectrum (cuve of 10 mm)



Different researches reveal the effect of nitrogen fertilization on the oil yield in marigold (SINGH M., RAO R.S.G., 2005), or on the carotenoid pigment biosynthesis(GRĂDILA MARGA, 1998).

Conclusions

1. The treatment with Azotofertil increased total inflorescence yield, stimulating the flowering and the inflorescence growth.
2. The treatments stimulated the flowering in June, but maximum inflorescences yield was in July in all variants.
3. The treatments increased photosynthesis efficiency, determining decrease of chlorophyll content in leaves, the most evident results being obtained by biofertilization with Azotofertil.
4. The treatments of biofertilization in soil with microbiological compounds increased the carotenoid pigment content in inflorescences, the most evident results being obtained with Extrasol.

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IN VITRO MICROPROPAGATION OF *HYPERICUM PERFORATUM* L. II. SAINT JOHN'S WORT CLONE SELECTION AND REGENERATION

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Summary

Micropropagation of H. perforatum, selected in experimental field in Piatra-Neamt with a view to optimising the regeneration processes (i.e caulogenesis, risogenesis), adjustment and transfer of fully adjusted plants in the experimental field. An average of 330.70 shoots were obtained at 35 flasks 14-18 days after the inoculation of callus on RM agar culture medium, supplemented with 1.0mg/l BAP and 0.5mg/l IAA or NAA, whereas on 0.5mg/l BAP, 1.0mg/l Kin and 0.5mg/l IAA supplemented medium only 136.10 shoots were obtained. In vitro risogenesis rate is of 87 - 98%, the best values (i.e., 98%) were obtained with MRI culture medium though the most vigorous shoots were obtained on MS hormone-free medium. Plant adjustment was observed in parallel, in sterile soil pots and under hydroponic system. In both cases they are covered. Under hydroponic system, restoring plant water equilibrium took 10 to 14 days whereas the adjustment rate obtained was of 89.4%. Some 57 adjusted plants were transplanted in the experimental field.

Keywords: *Hypericum perforatum, in vitro multiplication, caulogenesis, risogenesis, adjustment, setting up experimental culture*

Introduction

Hypericum perforatum L. (St. John's wort) plant is used in traditional medicine to treat various diseases. The plant contains a series of active principles relevant for their therapeutic action: the hypericin-type diantrone derivatives (hypericin and pseudo hypericin), volatile oil, which contains monoterpene and sesquiterpene, flavonoside under glycoside form and free aglycons (hyperine, rutoside, cvercetol, cvercetine, kaempferol) and biflavonoides, hyperforin, galactose, carotenes, vitamin C, vitamin PP, saponine, phenolic acids and a series of xanthones, mineral salts (Weiss, 1991; Ciulei, *et al*., 1993; Kartning, *et al*.,1996; Čellárová, 1997; Deltito and Beyer, 1998; Dias *et al*., 1998; Tămaş, 1999; Stănescu *et al*., 2004; Ayan *et al*., 2005). Currently, St John's wort is widely used as an herb remedy for the treatment of mild to moderate depression (De Smet and Nolen, 1996).
Plant cell and tissue culture were set up for a series of herbs among which St. John's wort *Hypericum perforatum* (Erzen- Vodenik and Baricevic, 1996), by using the shoot tips as explants. There were subsequent problems related to obtaining sterile explants in carrying out tissue culture of this species.
With respect to tissue cultures of species such of *Hypericum* genus information is scarce. The paper of Čellárová *et al*. (1992) on *H. perforatum* species reports on obtaining explants by sterile germination of seeds and the approach of the variability of some morphological and histological characteristics and structure of regenerants. Broderick *et al*. (1996), BEŽO and ŠTEFÚNOVÁ (2001) on *H. perforatum* species, developed a rapid technique of callus tissue propagation and of shoot multiplication and a system to produce high level hypericin in *in vitro* culture. Other authors use leaf explants for *in vitro* culture initiation (Pretto *et al*., 2003; Ayan *et al*., 2005). For *H. erectum* species – *in vitro* culture was carried out in order to trigger procianidine and to carry out quantitative analysis of specific polyphenols from procianidine (Yazaki and Okuda, 1990).
A series of papers report on the influence of some substances induced in the nutritious medium on some physiological and bioproductive parameters. Thus, supplementing medium with 0.001% (w/v) Pluronic F-68 (non-ionic surfactant), after 60 days of culture, induces a

40% growth of the mean fresh weight of regenerants and an increase of 34% of regenerated shoot number. Nonetheless, the concentration growth to 0.1% (w/v) of the same compound decreased by 15% both the number of regenerated shoots and their fresh weight (Brutovska *et al*., 1994). It was also proved that in *H. perforatum* cell suspension culture, the jasmonic-acid induced hypericin production (Travis *et al*., 2002).
As *H. perforatum* is one of the species that undergo multiple forms of reproduction in the wild, several reproductive processes were identified in wild harvested plants (Matzk *et al.*, 2004). This reproductive flexibility has led to chemical variability in field grown plants. Therefore, the in *vitro* cloning has an advantage for the multiplication of valuable individuals from a bioproductive viewpoint as well.
In the experimental series we aimed at micropropagation of *H. perforatum* plant biological material selected in the Piatra-Neamt experimental field. Considering the preliminary results obtained by cultivating *H. perforatum,* caulinary explants, the aim of this study was to focus on the optimising the regeneration processes (caulogenesis, risogenesis), the adjustment and transfer of fully adjusted plants onto the experimental field.

Material and methods

Plantlets were obtained by seed germination under aseptic conditions on agar medium and the sterile segments represented the explants used. In order to initiate and cultivate callus tissues it was necessary to use a 1:2 and 1:1 ratio, respectively, of exogenous auxines and cytokinines. MR-base medium was used containing macroelements and microelements (Linsmaier and Skoog, 1965), 20g/l glucose, 100 mg/l meso-inositol, 2 mg/l glycine and 7.5% agar.
Caulogenesis was performed by placing the callus on MS and MR variant agar media, where by inducing cytokinins (kinetin, 6 benzyl-amino purin = BA) and auxines (IAA, NAA) separated or in different combinations, 10 media variants were obtained (B02, B05, B10, K0l, K02, K05, K10, KB, RM, MR1, MR2 (Table 1). Risogenesis was performed on simple agar MS medium, simple MR and MRI (supplemented with 0.5 mg/l acid-indolilbutiric).
The cultures were maintained in culture chamber under stable semi climatic conditions, at 20°C, 16-hour light and 8-hour darkness.
After having formed roots, the new plantlets were taken out from the Erlenmeyer flasks; small agar medium was cleaned off their roots. After successive washing, they were transferred in sterile-soil pots or in hydroponic cultures. In both cases, they were covered with glasses. Throughout this adjusting period, water equilibrium was stimulated by temporary taking off the glasses and the adjusted plants were transplanted onto the experimental field.

Results and discussions

Callus was obtained on agar Murashige Skoog (MS) culture media and Linsmaier Skoog revised medium (RM), supplemented with auxines and cytokinins in different concentrations (Amariei *et al*., 2000). Despite the good semi-climatic conditions secured, the growth of the set culture cells, especially during the first stage, was slow. From a morphological viewpoint, the tissue cultures obtained displayed a wide variety. Thus, considering the phonotype aspect (colour and consistence), we obtained dark green compact callus and green callus with many growing buds, and slightly friable green-yellowish callus.
With a view to *inducing caulogenesis*, a series of studies with concern to the interaction of cytokinins concentration in the medium and the St. John’s wort callus morphogenetic response (or the sterile explants fragment). To this aim, MS-base medium was supplemented by variously concentrated BA in different concentrations (de la 0.1 mg/l up to 1 mg/l), a medium on which the inoculated callus recorded a multiple shoot differentiation (Table 1). In another series of

experiments - when a different but similarly concentrated cytokinine was used, i.e. kinetin, (0.1-1mg/l) – it was noticed that while kinetin concentrations ranging between 0.1 – 0.5 mg/l, induced shoot differentiation, the 1 mg/l concentration produced necrosis. Hence, in the case of tissue culture and *H. perforatum* plant regeneration experiments, the use of BA plant hormone is more advantageous than kinetin.

Table 1. Morphogenetic response of H. *perforatum* L. explants on different culture media variants

No.	Var.	Growth regulators (mg / l)						Morphogenetic response induced
		BAP	Kin	NAA	2,4- D	IAA	IBA	
1.	B02	0.2	-	-	-	-	-	Multiple shoot formation
2.	B05	0.5	-	-	-	-	-	Multiple shoot formation
3.	B10	1.0	-	-	-	-	-	Multiple shoot formation
								Multiple shoot formation
4.	K01	-	0.1	-	-	-	-	Shoot regeneration
5.	K02	-	0.2	-	-	-	-	Multiple shoot formation
6.	K05	-	0.5	-	-	-	-	Shoot regeneration
7.	K10	-	1.0	-	-	-	-	Significant necrosis
8.	KB	0.5	1.0	0.5	-	-	-	Multiple shoot formation
9.	RM	1.0	-	0.5	-	-	-	Multiple shoot formation
10.	RM	1.0	-	-	-	0.5	-	Abundant shooting
11.	MS	-	-	-	-	-	-	Good risogenesis
12.	MR	-	-	-	-	-	-	Good risogenesis
13.	MRI	-	-	-	-	-	0.5	Very good risogenesis

Our results are confirmed by studies conducted by Čellárová *et al.,* (1995) who, still with concern to St. John's worth, obtained similar data using RM-base medium. The authors previously cited also used 2iP, in of 0.1 – 0.5 mg/l concentrations beside BA and K – as plant hormone supplement; in this case shoot differentiation was mainly obtained as explant response.

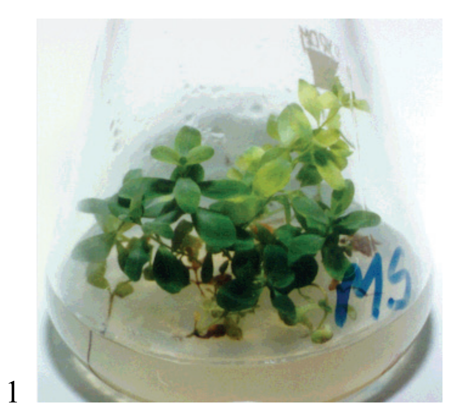
In order to determine shoot regeneration output, three culture media were chosen, e.g. MS (KB variant) and RM, and RMI on which the best caulogeneses were previously recorded. Selecting callus according to phenotypical traits manifested proved that the most rapid caulogenesis was obtained in the green callus, covered with a large number of growing buds. With a view to caulogenesis, at this stage, the callus generated by each inoculated segment was sectioned and transferred into Erlenmeyer flasks with culture media afore mentioned. Shoots formation needed 12 to 24 days. Over this period the number of shoots on the surface of inoculated callus grew as detailed in Table 2 below.

Table 2. *Hypericum perforatum* L. shoot inducing and differentiating

Culture Medium	Explants	Morphological response after 4 weeks	No. of shoots formed after 8 weeks
KB	callus	Multiple shoot formation	168
	callus	Moderate caulogenesis	79
	callus	Reduced caulogenesis	57
	callus	Shoot formation	147
	callus	Multiple shoot formation	158
	callus	Multiple shoot formation	172
	callus	Moderate caulogenesis	82
	callus	Satisfactory caulogenesis	104
	callus	Satisfactory caulogenesis	99
	callus	Multiple shoot formation	158
	callus	Multiple shoot formation	137
Mean			***136.10***

RM	callus	± Good shoot differentiation	145
	callus	Good shoot differentiation	168
	callus	Good shoot differentiation	287
	callus	Very good shoot differentiation	452
	callus	Very good shoot differentiation	351
	callus	± Good shoot differentiation	147
	callus	Very good shoot differentiation	581
	callus	Very good shoot differentiation	430
	callus	Very good shoot differentiation	342
	callus	Good shoot differentiation	195
	callus	Very good shoot differentiation	509
		Mean	***330.70***

In order to pass over the winter during shoot stage the newly formed *H. perforatum* shoots reaching 3 to 4 cm in length were separated and further transferred on identical multiplication media. At this stage, new vigorous shoots – either singular or four to six-branched ones were noticed (Photo 1 and 2).

1 

2

3

H. perforatum L. shoots – stage preliminary to rooting (Photo 1 and 2); rooting stage (Photo 3)

Regenerated shoot rooting is a very important step in all cases, especially when initiation of an experimental lot in field is pursued, for analysing regenerants from the point of view of their biology, genetic characteristics and bioproductivity. To this aim, well formed shoots were excised and transferred into Erlenmeyer flasks with agar simple MS, simple MR and MRI (supplemented with 0.5 mg/l acid-indolilbutiric). Using the three medium types in the risogenesis inducing stage (Table 1) allowed for a comparative evaluation of the process in its interaction with nutritious medium depending on mineral substance content, i.e., the lack or presence of plant hormone with a well-known stimulating role.
As a result of this experiment, single or branched *stem H. perforatum* shoots proved a good risogenesis on all media used. This process needed a longer time (6 to 7 weeks) on MR medium, a shorter one on MS (5 - 6 weeks), and the shortest period recorded (3 - 4 weeks) on MRI medium.
Moreover, culture phenotype recorded modifications during risogenesis although shoots similar in length and vigour were inoculated. Regenerated shoots recorded a significantly different development on risogene media used with a view to root generations. The most vigorous shoots were noticed on MS medium which is richer in organic substances than MR and MRI. Therefore, the media for risogenesis must be carefully chosen according to different criteria: the time factor – in which case MRI medium is more advantageous (shorter time), or the need for more vigorous plants then MS (hormone-free) medium would required a longer time (Photo 3).

The rate of St. John's wort roots inducing and formation - in *in vitro* culture ranges between 87 - 98%. The best values (98%) were recorded on MRI culture medium.
To conclude, *H. perforatum* is species endowed with a high *in vitro* regenerating capacity. A very efficient regenerating method was obtained when RM culture medium was supplemented only by 6-benzyl-amino. This regenerant system, having the same genetic origin, allowed for the study of somaclonal variation at various levels (morphological, cytogenetic and biochemical) followed by the individual selection of plants with the desired characteristics.
Adjustment of in vitro regenerated St. John's wort plants - Completely regenerated *H. perforatum L.* plants, after having undergone risogenesis, were transferred and subject to adjustment with a view to their being transplanted in the experimental field.
Two experiments were initiated with a view to plant adjustment. The former began in the second half of August while the latter at the beginning of spring the following year. As to the experiment begun in August, the new plantlets were very carefully taken out of the Erlenmeyer flasks and their *in vitro* regenerated roots were cleared of the small pieces of agar medium. After successive washing, the new plantlets were transferred into fertile soil pots. In order to avoid a massive loss of water, the plants were covered with glasses, hampering thus heavy perspiration and eventually the loss of the newly formed plants. Plant adjustment required 12 to 16 days. Throughout this adjustment period, glasses were taken out so as to stimulate water equilibrium of each new plantlet transplanted. At the end of this period, the plants adjusted their water equilibrium and resisted throughout the day under outdoors climatic conditions. In this adjusting experiment (plants transferred into sterile soil), the output reached 80.05%. During this stage, the new plantlets were transplanted into the "Stejarul" Research Centre Piatra Neamț experimental field.
During the latter experiment carried out during winter and the beginning of spring, new plantlets were taken from the agar medium similarly to the previous experiment. But this time, the new plantlets were transferred into a hydroponic system instead of sterile soil pots. Plants were also covered so as to avoid heavy loss of water via leaves, in-appropriately adjusted to the new conditions (Photo 4). In this system, restoring water equilibrium of St. John's wort plants took 10 to 14 days and then they were further kept in the hydroponic system (Photo 5 - 6). Some 57 fully adjusted plants were transplanted onto the experimental field. Hydroponic culture output reached 89.4%.

4

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6

Aspects of *ex vitro* adjustment of new plantlets obtained

Conclusions

Hypericum perforatum shoot regeneration is a more advantageous process when using MS or BA-supplemented RM, as compared to kinetin; when shoot length reached 3 to 4 cm, the most vigorous ones – either singular or 4 to 6 -branched ones were separated and transferred on rooting media.

A good risogenesis was obtained on all three media: 6 to 7 weeks on MR medium, 5 to 6 weeks on MS medium, and 3 to 4 weeks on MRI medium. The rate of St. John's wort *in vitro* inducing and root formation ranged between 87 and 98%. The highest values (i.e. 98%) were reached on MRI culture medium.

Survival rate of new plantlets over the ex *vitro* adjustment period ranged between 80.5 and 89.4% depending on the system used – sterile soil pots or hydroponic cultures; in hydroponic cultures, water equilibrium restoring time shortened by 2 to 3 days; some 50% of the plantlets transplanted in the field (in September) survived the winter; plants grew, flowered and bore fruits and they displayed a carrying characteristic to the species.

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INVESTIGATION OF OPTIMUM DRYING METHODS OF BAY LEAF (*LAURUS NOBILIS* L.)

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Summary

Bay leaf is one of the most important aromatic plants exported from Turkey. Postharvest processing, especially drying, affects essential oil content and quality of bay leaf. The aim of the present study was to determine the optimum drying method for obtaining the highest essential oil content in bay leaf. Fresh bay leaves harvested from Southern Turkey (Silifke-Mersin) were dried in three different media (under shade, under sun and in solar tunnel dryer) during 11 months, from October 2003 to August 2004. The essential oil contents were analyzed in dried leaf samples by hydrodistillation. The highest essential oil contents of bay leaf were determined under drying shade (2.89 % and in solar tunnel dryer (2.88 %) in October.

Keywords: *Bay leaf, drying methods, essential oil content, Laurus nobilis L., solar tunnel dryer*

Introduction

Laurus nobilis L.-*Lauraceae*- is an evergreen shrub or small tree (rarely up to 20 m) with Mediterranean origin, which occurs in Southern Europe, the Canary Islands, and the Azores. The main usage areas of bay tree products (leaves, leaf essential oil and fruit crude oil) are food, spice, flavoring and cosmetic industries. The large, brittle, dried leaves of bay tree are important ingredients of both sweet and savory dishes in European cuisines. They are also used in packing dried figs and licorice in order to deter weevils. The leaves have a customary usage in traditional medicine mainly for gastrointestinal complaints as well (Bown, 2001; Wyk and Wink, 2004). Essential oil content of bay leaf from different locations of Turkey was between 1.4 % to 2.6 % (Özcan and Chalchat, 2005). Main components of the essential oil are 1,8-cineole, eugenol, linalool, costunolide and deacetyllaurenobiolide (Bown, 2001; Wyk and Wink, 2004).

Bay trees naturally grow individually or in groups from north to south in Turkey. In some regions, they can reach above 600-800 m altitude. Bay products, such as leaf, fruit and their oils are considered non-forest products in Turkey (Ercan, 1983). Bay leaf and its essential oil are of the main medicinal plant products exported from Turkey. Furthermore, Turkey is the main supplier for bay products on the world. In 2003, approximately an eight million-dollar income obtained from 5099 tones of bay leaf export. In the same year, export value of bay leaf essential oil was about half million US dollars. Hong Kong, USA, Germany and Brazil were the main importers for bay leaf (Özgüven et al., 2005).

Drying has been an old traditional food conservation method in Turkey like in other countries. During the drying season, insect and mold development in harvested crops is promoted due to the high air temperature and relative humidity. In addition to these, the intensive solar radiation causes several quality reductions like vitamin losses or color changes in dried crops. When drying is performed directly on the ground, foreign material may mix into products. Thus, the conventional drying methods do not meet the particular requirements of the related standards. Agricultural products dried under controlled conditions can easily find customer and meet internal and external market request (Öztekin et al., 1999).

The aim of the present study was to determine the optimum drying method for obtaining the highest essential oil content in bay leaf harvested from Southern Turkey (Silifke-Mersin during 11 months, from October 2003 to August 2004.

Material and methods

Mediterranean region of Turkey, especially Silifke-Mersin district, is one of the main sources for bay products in Turkey. In this region, for the research years, the lowest and highest temperature values were 9.8 °C on January and 28.4 °C on July in 2004. During the study, 494.3 mm total rainfall was recorded in the region (Anonymous, 2005). The soil characteristics of the region were loamy, clay-loam and alkaline. The organic material contents of the soils were between 1-2 % (Anonymous, 1987).
Plant materials for the present study were obtained from natural bay shrubs and trees from Silifke. The samplings were started October 2003 and continued 11 months up to September 2004. Bay leaves were harvested by hand from top plant shoots. Harvested plant materials were firstly separated from the branches and then bay leaves were moved to drying places. Traditional drying methods, such as shade and sun drying and solar drying were the drying methods investigated in the present study. Fresh bay leaves were spread with 5 cm height (a loading capacity of 3 kg m^{-2}) to drying places. For sun and shade dryings, the leaves were spread out on concrete floors. Some properties of solar tunnel dryer obtained from Hohenheim University (Germany) are; high drying capacity, better moisture removal, efficient drying, high loading capacity between 1.5 kg m^{-2} for medicinal plants and 25 kg m^{-2} for grapes, easy transportation and construction, and effective solar energy use (Mastekbayeva et al., 1998). Other technical properties are given below:

Table 1. Design parameters of Hohenheim Solar Tunnel Dryer

Parameters		
Collector length	8	m
Dryer length	10	m
Total length	18	m
Width (Dryer/Collector)	2	m
Collector Area	16	m^2
Drying Area	20	m^2
Air Flow	400-1200	m^3/min
Air Temperature	30-80	°C
Power Requirement	20-40	W

Anonymous, 1996

Chemical analyses were done at Medicinal and Aromatic Plants Laboratories of Çukurova University Agricultural Faculty Field Crops Department, Adana-Turkey. Dried bay leaves (20 g) were submitted to hydrodistillation for 2 hours using a Clevenger type apparatus with three replicates. The statistical analyses were done according to split plot design by computer statistical program, MSTATC. The differences among the means were compared using LSD (1 %).

Results and discussion

The ambient minimum and maximum temperature values at the period of drying were in the range of 9.8-28.4 °C (mean 18.8 °C), while the relative humidity fluctuated between 65.5-74.3 % (mean 68.8 %). Daily sunshine period varied from 3.3 to 10.8 hours, mean of 8 h. Depending on climatic factors at drying period, the time lasted for the bay leaves to attain constant weight was quite different. While the drying periods for shade and sun were over 26 and 10 days, in solar dryer the maximum drying period was 4 days.

The results of statistical analysis showed that there were significant differences in essential oil content of bay leaves subjected to different harvest time and drying method. The highest essential oil content was recorded in October; however, there were no statistical differences between October and November harvests in terms of essential oil content. Essential oil content obtained from July harvest was the lowest, but it did not differ statistically from the August harvest. It can be seen from the Table 2, the essential oil content of the dried bay leaves are in decreasing tendency from October to July through the year. Different drying methods affected the essential oil content of bay leaf, and the highest value was obtained from shade-dried leaves, while the lowest was in sun-dried samples. Considering the harvest time and drying methods all together, the leaf samples harvested in October and November gave the highest essential oil contents for shade and solar-tunnel drying. The lowest values were found in the samples harvested in July and August for sun-drying.

Table 2. Average values of essential oil contents (%) of bay leaves for different drying methods through the drying period (in dry weight basis).

Months	Drying Methods			Month Means
	Shade	Sun	Solar Tunnel	
October	2,89 **a**	1,73 **no**	2,88 **a**	2,50 **a**
November	2,89 **a**	1,71 **o**	2,86 **a**	2,48 **a**
December	2,51 **e**	2,11 **k**	2,50 **e**	2,37 **b**
January	2,53 **de**	2,00 **l**	2,51 **de**	2,35 **b**
February	2,65 **b**	1,91 **m**	2,61 **bc**	2,39 **b**
March	2,60 **bc**	1,99 **l**	2,58 **cd**	2,39 **b**
April	2,43 **f**	1,91 **m**	2,39 **fgh**	2,25 **c**
May	2,40 **fg**	1,80 **n**	2,33 **hı**	2,18 **d**
June	2,34 **ghı**	1,59 **p**	2,29 **ıj**	2,08 **e**
July	2,29 **ıj**	1,33 **q**	2,27 **ıj**	1,96 **f**
August	2,30 **ıj**	1,39 **q**	2,26 **ıj**	1,98 **f**
Drying Method Means	2,53 **a**	1,77 **c**	2,50 **b**	
LSD (% 1): Months: 0.04; Methods: 0.02; Months x Methods: 0.07				

In the scientific literature, there are several papers indicating that the essential oil content of the aromatic plants vary seasonally during vegetation period (Buben et al., 1992; MoldaÄ o-Martins et al., 1999; Kırıcı and Inan, 2002; Yaldız et al., 2005). Acar (1987) stated that there was a relation between the essential oil content of leaves and shoot age in bay tree. He found that leaves from the younger shoots had higher essential oil content. In different studies, aiming to determine the optimum harvest time for bay leaf with high essential oil content, the best time to harvest for bay leaf found as September and October for Eastern Mediterranean region of Turkey (Müller-Riebau et al., 1997; Acar, 1988). Essential oil contents of bay leaf varied from 0,50 % to 2.19 % in other papers (Tanker and Tanker, 1976; Acar, 1987; Ceylan and Özay, 1990; Akgül, 1993). The essential oil contents found in the present study are slightly higher than that of the researchers' results. A number of factors, such as different research area, plant material, harvest time, ecological conditions, soil characteristics and climatic factor may affect essential oil contents of bay leaf.

In the present study, the highest essential oil content was obtained from shade-dried samples. The lowest value was determined in solar-dried leaf samples. There were no statistical differences between the shade-dried and solar-dried samples in terms of essential oil content in October and November harvests. Depending on having higher essential oil content and natural color, shade-dried aromatic plants are desired in the markets. Furthermore, shade-dried plant materials are less affected by solar radiation, unexpected bad climatic factors, such

as rain and storm, than sun-dried products. Contrary to these advantages, shade drying method has a number of handicaps. Depending on climatic factors longer drying time and restricted drying place are the main disadvantages of this method. Unsuitable drying place, such as stuffy warehouse, may cause some microbial damage in the product, and metals, manure, and house wastes may mix to drying material.
In the region, fall rains and strong winds may happen, and total crop loses may come out in drying period of bay leaves. Considering advantages mentioned above, solar tunnel dryer may a good alternative for drying bay leaf in this region. With this method, bay leaves can be dried under control with natural color and high essential oil content.

Conclusions

The results of the present study show that bay leaves harvested in October and November and dried under shade and in solar-tunnel dryer gave higher essential oil content compared to the sun drying. Considering advantages of solar-tunnel dryer, such as short drying time, high drying capacity, drying with natural color and high essential oil content, conservation the material from bad ambient conditions, this method may be preferable for obtaining high quality bay leaf with natural color and high essential oil content in Mediterranean region of Turkey.

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IN VITRO CULTIVATION OF *HIPPOPHAE RHAMNOIDES*

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Summary

In vitro cultivation of Hippophae rhamnoides is an unconventional alternative to conserve and perpetuate an important resource for food and health. Clonal micropropagation through adventitious and axillary shoots has begun to have impact on plants improvement.
It were obtained viable plantlets from adventitious meristematic tissue induced by the action of a cytokinin on the cotyledons of mature Hippophae rhamnoides zygotic embryos.

Keywords: *Hippophae rhamnoides, micropropagation, clone,caulogenesis, callus*

Introduction

In vitro cultivation of any species is an unconventional alternative to conserve and perpetuate the genetical resources (1), (2) (3),(6),(7). It was cultivated *Hippophae rhamnoides L.* and tested the proliferative and regenerative capacity, depended on explants origin, type and concentration of the growth regulators from the culture media.

Material and methods

The initiation of in vitro cultures at *Hippophae rhamnoides L* was achieved not only from plantlets, obtained from aseptically germination of seeds, but also from axillary buds (5). The seeds soaked in water for 24 hours were sterilized for 10 minutes in sodium hypochlorite solution 3% and rinsed with sterilized distilled water. The seeds were aseptically germinated on moistened filter paper.
The axillary buds were sterilized with ethanol 70 % and then sodium hypochlorite 0,5 % 10-15 minutes. After rinsing with sterile distilled water, the explants were transferred to MS medium containing 2mg/l BAP and 0, 2 mg/l IAA and maintained at 23° C under 16 hours light cycle.
The MS medium supplemented with 2mg/l BAP and 0, 2 mg/l IAA stimulated callus induction on young explants and shoot differentiation from axillary buds.

Results and discussions

Direct micropropagation through adventitious and axillary shoots was achieved at *Rhippophae rhamnoides L.*.
In practice stem tips and lateral buds have been the most commonly used starting point in micropropagation. The micropropagation through adventitious and axillary shoots must confer advantage in plants improvement and production (4), (5), (8) .
The must effective explants for direct „reconstruction" of plants in vitro were the axillary buds. On this level were induced the caulogenesis at *Hippophae rhamnoides L.*.
The micro-shoots achieved by excising the terminal portion of an individual shoot grown on the inductive medium and formed normal shoots, while the basal portion formed axillary shoots.
The axillary shoots developed into normal shoots when excised and transferred to fresh medium.

The MS medium supplemented with 2mg/l BAP and 0, 2 mg/l IAA stimulated shoot differentiation .
The MS medium supplemented with 2mg/l BAP and 0, 2 mg/l IAA stimulated callus induction on young explants (Photo 1, 2) and shoot differentiation from axillary buds (Photo 5, 6, 7). Organized shoot cultures were established from axillary buds. After one month of culture, many shoots emerged on the medium, containing high level of cytokinins.
It is interesting to note that in case of *Hippophae rhamnoides* different explants have shown different reactions on the same culture medium.
The plantlets of *Hippophae rhamnoides* obtained from aseptic seeds germination were cultivated on a variant of MS medium, that induced callus formation It were obtained a white-jellow colored friable callus(Photo 3 and 4).

Conclusions

- Callus cultures were obtained from plantlets of *Hippophae rhamoides* , cultivated on a MS variant with a higher concentration of cytokinin**.**
- The plantlets of ***Hippophae rhamoides L.*** have shown an intensive proliferative reaction and rapidly callus development.
- The MS medium supplemented with 2mg/l BAP and 0,2 mg/l IAA stimulated the caulogenesis at *Hippophae rhamnoides.*

Photo 1. Aseptically germination of seeds at *Hippophae rhamnoides*

Photo 2. Plantlet dedifferentiation at *Hippophae rhamnoides*

Photo 3. Callus of *Hippophae rhamnoides*

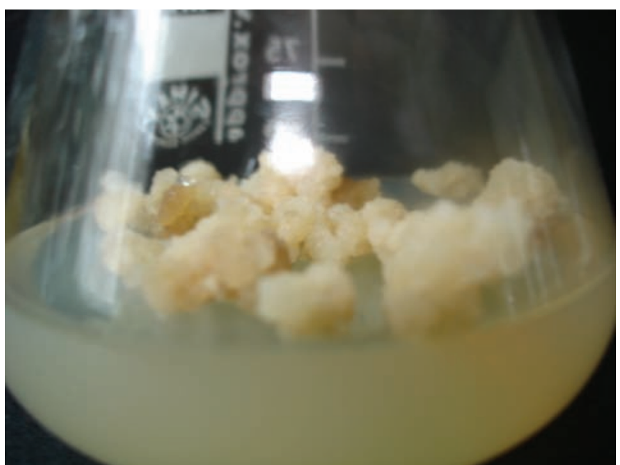

Photo 4. Callus of *Hippophae rhamnoides*

Photo 5. Axillary buds at *Hippophae rhamnoides*

Photo 6. Axillary buds at *Hippophae rhamnoides*

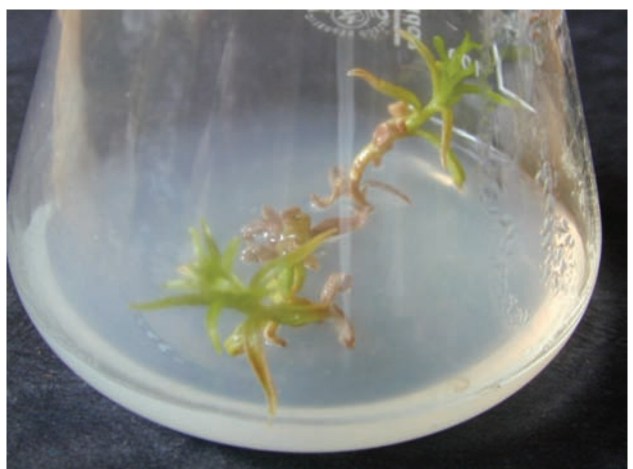

Photo 7. Caulogenesis at *Hippophae rhamnoides*

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QUALITY EVALUATION OF CHAMOMILE *(MATRICARIA RECUTITA L.)* DEPENDENCE ON CULTIVATION TECHNOLOGY

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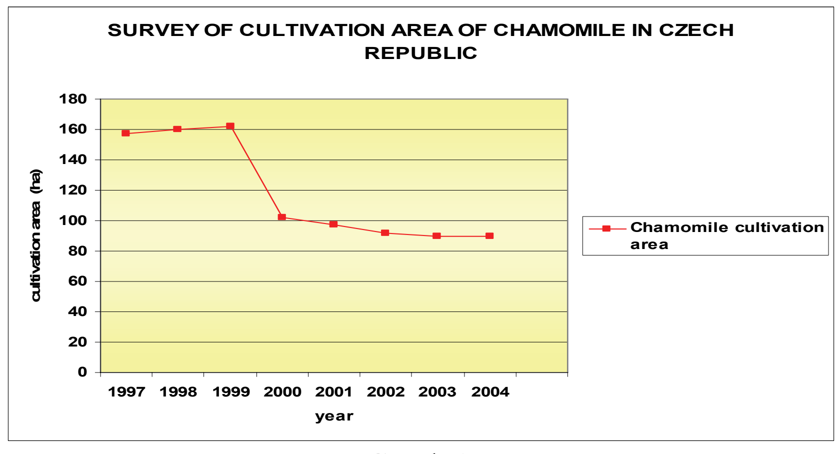
Summary

In our experiments with Chamomile (Matricaria recutita L.) we are comparing precise field experiments in conditions of both the traditional and the organic agriculture. We monitor content and the quality of essential oils, the amount of the main substances of essential oils (bisabolol, chamazulen etc.) and how these depend on growing technology. Specific field experiments in traditional agriculture are matched accurately at the experimental farm Praha-Uhrineves, which has received a licence for organic agriculture.

Keywords: *essentials oils , chamazulen, Matricaria recutita L., organic agriculture, quality evaluation*

Introduction

Chamomile *(Matricaria recutita L.)* belongs to the oldest exploited drugs, which are planted and harvested in the Czech Republic. The main substance in the floret (of medicinal Chamomile (*Matricaria recutita* L.) is 1.6-3% of essential oils. The most important substances of the essential oils are chamazulene and bisabololoxid A, along with bisabolol, cis en-in-dicykloether and bisaboloxid B. Next to essential Chamomile contains flavonoids (especially flavonoid apigenin).
At present it is grown on about 90 ha of arable lands. Decreasing of chamomile cultivation areas (Graph 1.) depend on the import of low-cost production of this commodity from abroad, especially from Bulgaria, Egypt and Turkey, represents a strong competition for our domestic production of *Matricaria recutita L.* The quantity and the quality of essential oils in the imported commodity does not respond the Czech standards, and these are markedly worse and represent onther chemocultivars than which is clear in Czech Republic – A type.



Graph 1.

The oldest variety is the variety Bohemia, which was licenced in 1952 in the Czech Republic (at that time former Czechoslovakia).
Other variety of Chamomile which we are testing is the Slovak tetraploid variety - Goral. This variety was cultivated at the University in Kosice, which was licenced in 1990. Both varieties belong to same chemocultivar A.

For this reason we focus on Chamomile with the possibility of exploitation of this medicinal plant in organic agriculture, especially these varieties - Czech diploid variety Bohemia and Slovak tetraploid variety Goral. We are testing these varieties for quality and quantity of efficient substances (e.g. Essential oils, chamazulen, flavonoids etc.) and how these depend on growing technology.

Material and methods

1. The agrotechnical part of the experiment:
Specific field experiments in traditional agriculture are matched accurately at the experimental farm Praha-Uhrineves, which has received a licence for organic agriculture.
Three variations according to an inter-row distance in four retakes formed the agrotechnical part of the experiment n (tables 1-2).
Two varieties – Bohemia and Goral. The variety of Bohemia is a diploid variety, the content of 0.47% of essential oils is standard, out of which there are over 36% of bisabololoxid A and 20% of azulen. The Goral variety belongs to the group of bisabol and bisabololoxid genotype. The Goral variety contains as standard 1-1.2 % of essential oils, over 25% of chamazulen, over of 25 % bisabolol and other substances.
The preparation of land was classical.
Dates of sowing: Spring sowing until April 10. Sowing 2g chamomile seeds on the area of 10 m^2 (2 kg/ha).
During the growing season the status of vegetation was monitored - the growth and the development of plants (phenological stages).
Harvesting in the time of technical maturity, according to the status of the varieties- 27-28.6, 18.7. and 2.8. 2005

Table 1, 2:

<table>
<tr><td colspan="3">Organic cultivation technology</td></tr>
<tr><td colspan="3">The former crop – oats (Avena sativa L.)</td></tr>
<tr><td>variant 1</td><td>variant 2</td><td>variant 3</td></tr>
<tr><td colspan="2">Inter-row distance 375 mm</td><td>Inter-row distance 250 mm</td></tr>
<tr><td colspan="2">The application of unwoven textile - supports germinating , hoeing- plants are 5cm high and weeding-plants are 15cm</td><td>The application of unwoven textile - supports germinating , hoeing- plants are 5cm high and weeding-plants are 15cm</td></tr>
<tr><td>As supplementary green manure was applied herb extract from stinging nettle (Urtica dioica L.) and comfrey (Symphytum officinale L.) (3 l/ 10m2).</td><td></td><td></td></tr>
<tr><td colspan="3">conventional cultivation technology</td></tr>
<tr><td colspan="3">the former crop - pea (Pisum speciosum Alef.)</td></tr>
<tr><td>variant 1</td><td>variant 2</td><td>variant 3</td></tr>
<tr><td colspan="2">Inter-row distance 375 mm</td><td>Inter-row distance 250 mm</td></tr>
<tr><td colspan="2">The application of unwoven textile - supports germinating , hoeing- plants are 5cm high and weeding-plants are 15cm</td><td>The application of unwoven textile - supports germinating , hoeing- plants are 5cm high and weeding-plants are 15cm</td></tr>
<tr><td></td><td>fertilisation N 20 kg/ha (♣)</td><td>aplication of herbicide Kerb - Flo (1.5 l/ ha).</td></tr>
</table>

♣: Fertilisation was not make with regard to the former crop

2. Post – harvest technology and Chemical methods to determin the content main substances in Chamomile

Oathouses were used for drying plants, the hight of the plant layer was 20cm, drying temperature was 35°C.

Isolating chamomile essential oils is conducted from 20g of flower drug (dried chamomile flower heads) by hydrodistillating method - distillation by steam as explained in The Czech Pharmacopoeia published in 2002.

As to the Czech Pharmacopoeia 2002 - a flower drug should contain at least 4 ml/1 kg drug of blue coloured essential oils. This standard is valid both for the traditional and the ecological cultivation of Chamomile .

Individual components of essential oils are determined by the methodology of the gas chromatography.

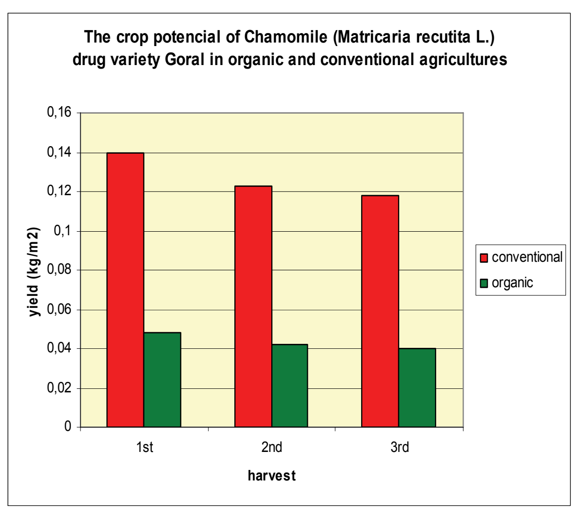
Results and discussion

1. Crop potential:

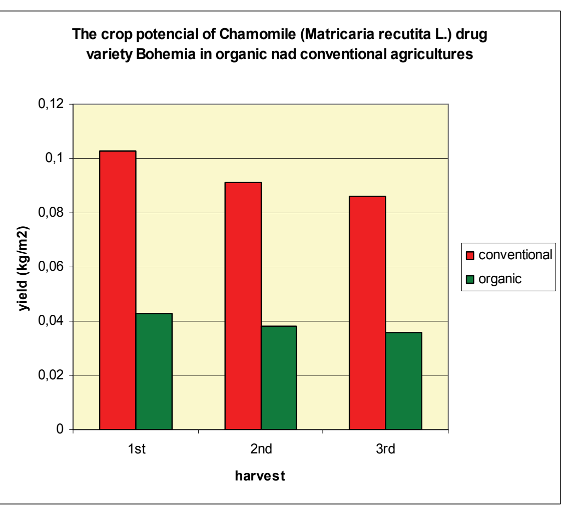
In overall yielding of chamomile drug the higher yield was acquired in the variety Goral. This variety occurred to have the higher and sustainable yield.

The organic agriculture crop was for 49% lower than that one from the same experiment of the traditional agriculture (the lower yield in organic part of experiments was probably cause by lower content of N in the soil than it was in conventional part of experiment (graph 2-3).

In the traditional part of the experiment we can see a likely noticeable dependance on the former legumen cultivation which influenced the higher crop.



Graph 2.

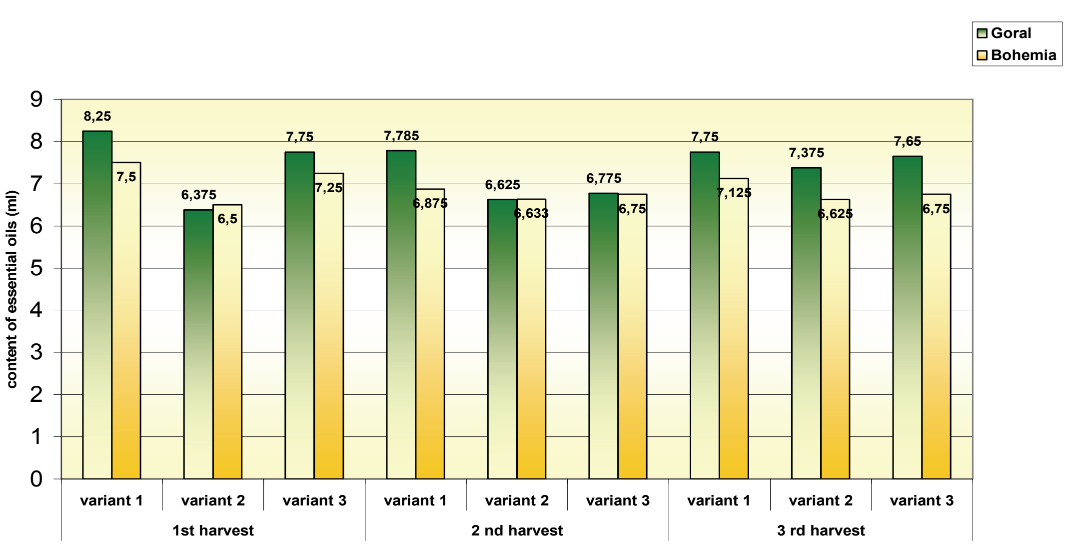


Graph 3.

2. Quality evaluation

In the experiments of both the ecological and the traditional cultivations - the content of essential oils was higher than the standard content of essential oils as stated in The Czech Pharmacopoeia. The highest measured value of the essential oils content was 8.25 ml/kg in the variety Goral at the 1st harvest in organic agriculture. In 1st variant in both varieties at the all harvests was measured the highest content of essentials oils than the other variant in same part of experiments . The highest content of essential oils in 1 st variant in 2nd harvest in organic part of experimets was probably cause by application a supplementary green manure after 1 st harvest, which is rich to nitrogen and kalium.

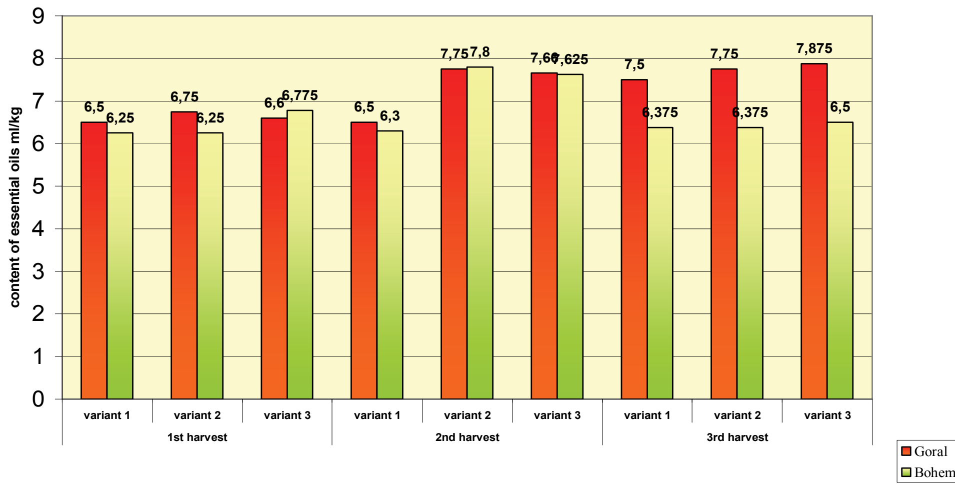
HAY 1992 published, that total content of essential oil increases with higher nitrogen rate (max. 20-60kg/ha, in three doses each 20 kg/ha).
The highest measured value in conventional part of experiment during the third harvest was found in cultivar Goral - 7,87 ml of essential oils in 1 kg of drug in variant 3 (graph 5). Slightly lower values were found in cultivar Bohemia.



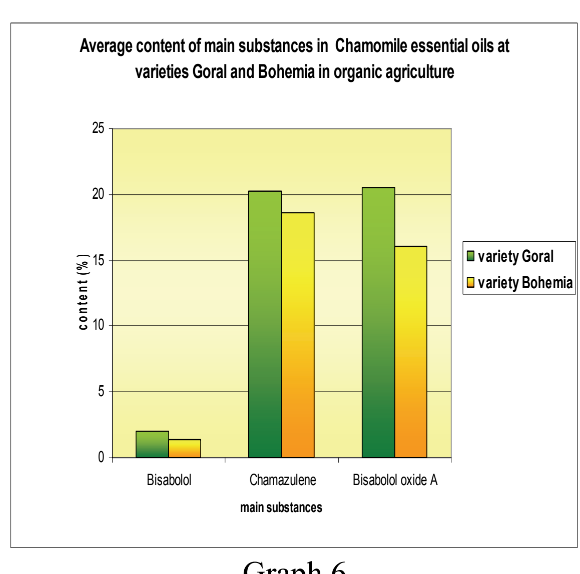
Graph 4.

The lowest content of essential oils was measured in the variant 1 of the traditional part of the experiment at the 1st harvest, nevertheless the content was always higher than the lowest content of the standard (4ml/kg.)
On the other hand the excesive content of N in the soil acquired from the former legumen cultivation caused the lower content of essential oils at the 1st harvest.
When comparing an average essential oils content of both the varieties, the tetraploid variety Goral showed a higher measured content of essential oils.
The highest measured value of main substances (chamazulene, α-bisabolol) of chamomile essential oils was also in the variety Goral at the 1st harvest in organic agriculture (graph 6) At the same time was determined decreseament of content bisaboloxide A in essentials oils than standard of this substance in both varieties in organic agricuture.
In conventional part of experiment higher average value of chamazulene was measured in cultivar Bohemia, and at the same time bisabololoxides content decreased in this cultivar compared to standard for this variety(graph 7).
The colour of essential oils differs according to the prevailing component (predominantly some bisaboloxids) from the recognized standard of the blue colour which is caused just by chamazulen (CZP, 2002).
On the contrary prevailing bisaboloxids in essential oils decrease medical action of *Matricaria recutita L.*,and their increased content is sensed by green colour of chamomile essential oils(SALAMON, 1998).

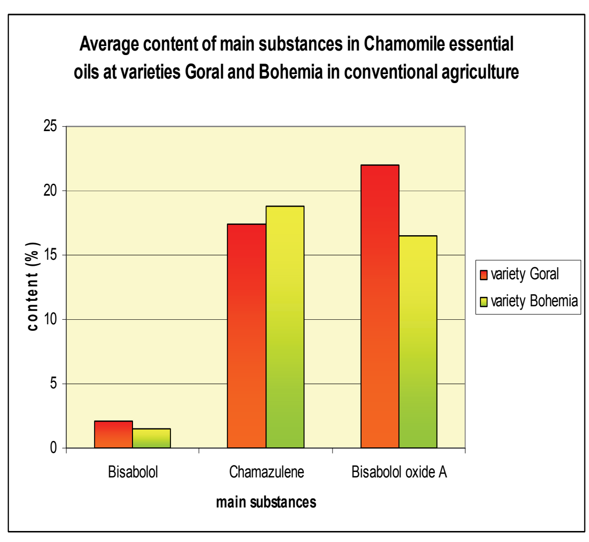
Average content of essential oils in Chamomile drug (Matricaria recutita L.) at both varieties in conventional agriculture



Graph 5.



Graph 6.



Graph 7.

Conclusions

Lower yield in organic farming does not belong to the most important criterion in medicinal plants, more important is drug quality, in our case essential oil content.
Chamomile *(Matricaria recutita L.)* yield in organic farming is lower due to more sparing farming practices, which is the same in other field crops (cereals etc.). It is regarding yield potential in organic farming steady state.
When comparing the average content of essential oils in both varieties, the higher measured value of the content of essential oils and main substances in essentail oils were present in the tetraploid variety Goral in organic and conventional parts of experiments. On the other hand also the diploid variety Bohemia in the ecological part of experiments showed a permanent high content of essential oils. We can presume, that for the organic cultivation technique both varieties are suitable.

This problematic is solved within grant agencies Grant Agency of Czech University of Agriculture (CIGA): 213133, FRVŠ 2387/G4 (Grant Agency Ministry of Education Czech Republic).

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STUDIES OF GERMINATIVE ENERGY AND FACULTY OF SOME SPECIES OF ECHINACEA (*ECHINACEA PALLIDA* NUTT. AND *ECHINACEA PURPUREA* L. MOENCH), DEPENDING ON SEEDS AGE

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Summary

Germinating energy and faculty with Echinacea pallida Nutt. increase with ageing of seeds (after eight months), and decrease after seventeen months from harvest, only to loose germination almost completely within three years from harvest. Results on germinating faculty with Echinacea purpurea (L) Moench, reveal that germinating faculty has highest values at two months after harvest, leading thus to the hypothesis that such species has a seminal pause shorter than two months.

Keywords: *Echinacea pallida,Echinacea purpurea, seed, germination*

Introduction

Medicinal vegetal products of *Echinacea* radix (*Echinacea pallida* Nutt) are to be found with those having immunomodulating and antiviral effect, by action mode and, the active principles contained are phenylpropionic (echinacosidae) compounds and, imunostimulatory polysaccharides. Thus, a new Romanian phytotherapeutical product NOVASTIN showed up (tincture, pills, gel) relying on *Echinacea pallida* Nutt., dry extract, obtained at the Faculty of Pharmacy Cluj-Napoca (*Tămaş,* 1989). Favourable results in healing lesions have led to obtaining dermatological and cosmetical products in the RO range relying on extract of *Echinacea purpurea* (L) Moench (*Hodişan,* 1994). Worthwhile mentioning that *Echinacea* species were first treated in Romanian scientific literature as late as 1990 (*Muntean,* 1990).

Material and method

The investigations were carried out between 1997 and 2000 at the Inspectorate of Seeds and Planting Material Quality Control, having in view the pursuit in time of germination in *Echinacea pallida* Nutt. and *Echinacea purpurea* (L) Moench by following the methodology in (Romanian) Standards 1634-82.
Variants under study with both species were : V1= 2 months of age (November 1997) – control ; V2= 4 months of age (January 1998); V3= 7 months of age (April 1998); V4= 8 months of age (May 1998); V5= 17 months of age (February 1998); V6= 21 months of age (June 2000); V7= 36 months of age (September 2000).
Best germination was followed, ie on which layer (TP- top of paper or BP- between paper); and at what temperature (20 or 25.5° C).
Data processing was carried out through variance analysis.

Results and discussions

1. Germinating energy and faculty in *Echinacea pallida* Nutt.

The fruits of *Echinacea pallida* Nutt. Are edged, whitish akenes having rudimentary pappus in the shape of a coronule (brown in colour), and 2-4 mm in length. MMB is between 5.6 and 6 g. Two hundred and eight seeds weigh one gram.

Fig. 1.1 shows that the germinating energy in *Echinacea pallida* Nutt. Goes up both in TP and BP layers and the two temperatures with aging of seeds (after 8 months), and comes down in 17 months after harvest in layer TP with both temperatures and there follows that in three years after harvest it looses its germination almost entirely (being considered microbiotic seeds).
Fig. 1.2 shows that germinating faculty with *Echinacea pallida* Nutt.increases both in TP and BP layers in the two temperatures, with ageing of the seeds (after 8 months), and decreases in 17 months after harvest inlayer TP, with both temperatures. There follows that in three years after harvest it looses its germination almost entirely (Vârban, 2001).
It is recommendable that on sowing *Echinacea pallida* Nutt., seeds of up to 8 months after harvest be used. Measurements on both germinating energy and faculty be performed on BP layer at 20 and 25,5°C.

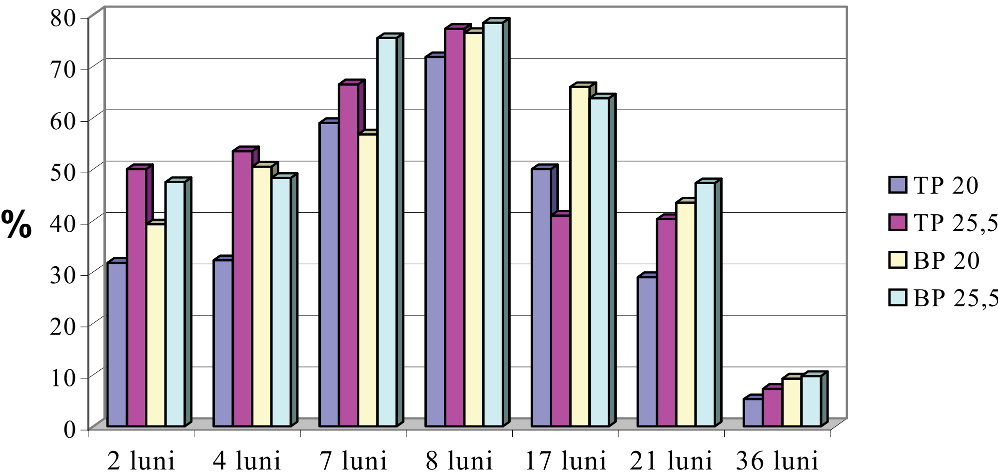

Fig. 1.1. Results regarding germinating energy in *Echinacea pallida* Nutt. depending on seed age,on two germinating stratum and at two temperatures (Cluj-Napoca,2000).

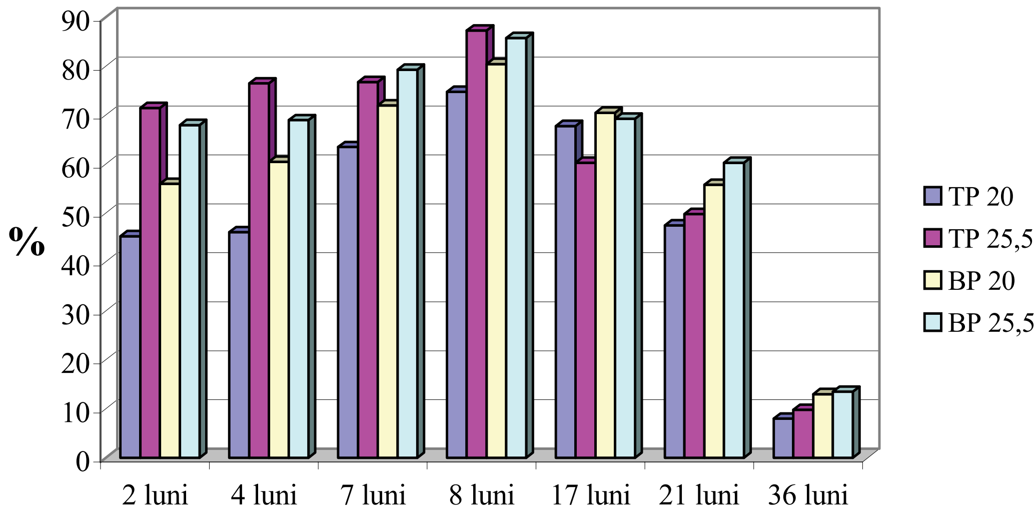

Fig. 1.2. Results regarding germinating faculty in *Echinacea pallida* Nutt. depending on seed age, on two germinating stratum and at two temperatures(Cluj-Napoca 2000)

2. Germinating energy and faculty in *Echinacea purpurea* (L) Moench

Fruits of *Echinacea purpurea* (L) Moench are edged, whitish akenes of 3-5mm in width. Their MMB is between 4-4.5 g. One gram means 223 seeds.

Fig. 2.1 shows that with *Echinacea purpurea* L.Moench the germinating energy is higher with aging of seeds (after 8 months) and lower in 17 months after harvest, on TP layer with both temperatures. There follows that in three years after harvest it looses germination to an almost complete degree.

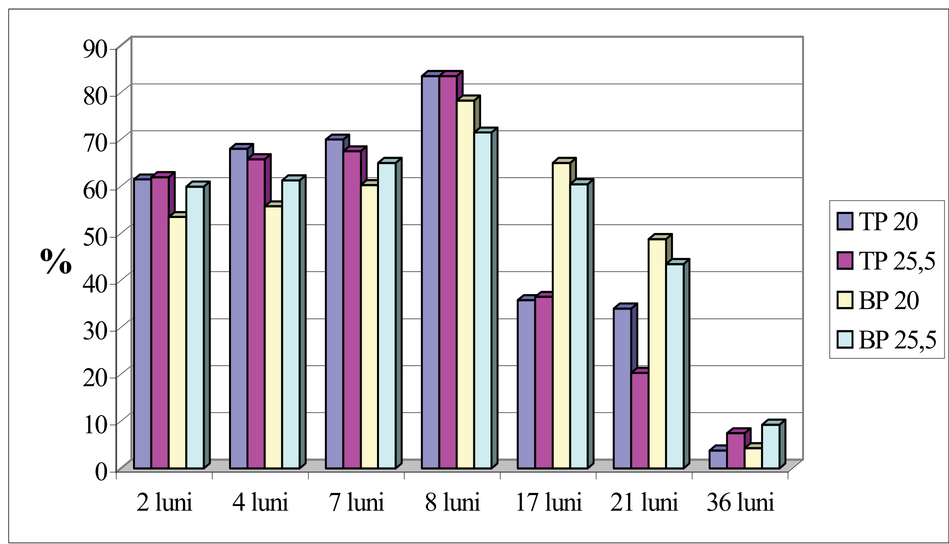

Fig. 2.1. Results regarding germinating energy in *Echinacea purpurea* (L) Moench depending on seed age,on two germinating stratum and at two temperatures (Cluj-Napoca,2000).

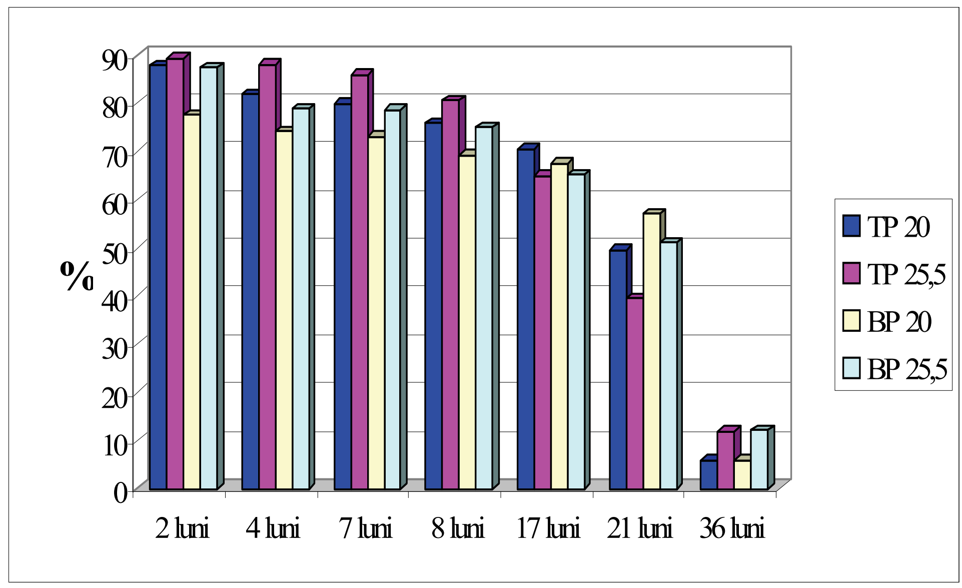

Fig. 2.2. Results regarding germinating faculty in *Echinacea purpurea* (L) Moench depending on seed age, on two germinating stratum and at two temperatures (Cluj-Napoca 2000)

On BP layer germinating energy is slow with seeds of 2 to 7 months after harvest and after 8 months it acquires highest with both temperatures and descends in 17months after harvest. Within 3 ys. From harvest germination is gone almost completely, with both temperatures.

One can notice in fig. 2.2 that germinating faculty with *Echinacea purpurea* (L) Moench (depending on seed age, on the two germination layers,ie TP or BP and, on the twotemperatures, ie 20 and 25.5 °C) has highest values at 2 months from harvest, fact leading us to the supposition that the species possesses seminal break below two months (Vârban, 2001) .
It is recommendable that seeds of *Echinacea purpurea* (L) Moench aged two to eight months be involved in sowing, and measurements on both energy and faculty of germination performed on TP layer at 20 and 25.5°C.

Conclusion

(1) MMB with *Echinacea pallida* Nutt. is comprised between 5.6 and 6g, and 1g contains 208 seeds. Germinating energy was measured at seven and that of faculty of germination at sixteen days respectively.
In *Echinacea pallida* Nutt. germinating energy and faculty, both on TP and BP layers, at the two temperatures (ie 20 and 25.5°C) go up with seed ageing (after 8 months) and come down in 17 months after harvest; there follows that within three years after harvest germination goes almost entirely (as seeds are taken to be microbiotic). It is recommendable that for sowing *Echinacea pallida* Nutt. seeds of up to eight months be used. Measurements on both energy and faculty of germination should be performed on BP stratum at 20 and 25.5 °C.
(2) MMB with *Echinacea purpurea* (L) Moench is comprised between 4 and 4.4g, numbering 233 seeds per one gram.
Results concerning germinating energy in *Echinacea purpurea* (L) Moench on TP and BP germination strata, at two temperatures (20 and 25.5°C), show that it is by 60% higher during the first eight months and has a marked fall thirty-six months after harvest.
It is to be seen that in *Echinacea purpurea* (L) Moench the two temperatures (20 and 25.5°C), on both BP and TP layer, have an impact of up to two months after harvest on germinating faculty with the ageing of seeds, fact leading to the hypothesis that this species has a seminal pause spanning less than two months.
It is recommendable that on sowing *Echinacea purpurea* (L) Moench seeds aged two to eight months be used. Both measurements on germinating energy and faculty be performed on TP stratum, at 20 and 25.5°C.

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RESEARCH REGARDING *ECHINACEA PURPUREA* (L.) MOENCH SPECIE SEEDLINGS, BIOLOGY AND OPTIMAL NUTRITION SPACE

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Summary

The average of the third experimental years show us that period of vegetation at Echinacea purpurea (L.) Moench specie was 174 days. The phenophases of vegetation lasted, in average: start of vegetation-budding 40 days (23%), budding-flowering 36 days (21%) and fowering-seed ripening 98 days (56%).
Thus the best results were obtained for the distance of 50 x 30 cm (70.000pl/ha – 462 q/ha) for the herba yield; 50 x 30 cm (70.000 pl/ha – 115 q/ha) for the radix yield.

Keywords: *Echinacea purpurea, nutrition space, density, biology.*

Introduction

Echinacea purpurea (L.) Moench were experimentally cultivated for the first time in 1982, at the University of Agricultural Sciences and Veterinary Medicine- Cluj-Napoca, Romania, with a view to obtaining fresh vegetable material, necessary to prepare homeopathic tinctures. Favourable results in its impact on wounds healing have led to obtaining certain dermatological and cosmetical products within RO-range, based on extract of *Echinacea purpurea* (L.) Moench (*Hodişan V. et al*., 1994). Also, species of Echinacea cultivated at Cluj-Napoca serve to obtain tinctures meant for homeopathic preparations. Worthwhile mentioning that *Echinacea* species were first treated in Romanian scientific literature as late as 1990 (*Muntean L,* 1990).

Material and methods

The research were carried out in the experimental field of the University of Agricultural Sciences and Veterinary Medicine Cluj-Napoca from 2001 to 2003, on a typical alluvial soil of alkaline reaction, moderate to poorly moldiferous, not too rich in nitrogen but rich in phosphorus and potassium within the limits of a sub humid climate.

1. The period and phenophases of vegetation. Planting was carried out in spring (May 2000) by spacing at 50 cm between rows and 30 cm between plants per row - thickness obtained was 70,000 plants/ha. In the experimental years (2001-2003) of my research I establish the length of vegetation period and the main phenophase.

2. The optimal nutrition space. The variants taken into consideration were: V1=planted at 50 cm (between row) x 20 cm (between plants on row), density 100 000 plants/ha (control);V2=planted at 50 x 30 cm, density 70 000 plants/ha; V3 =planted at 50 x 40 cm, density 50 000 plants/ha; V4 =planted at 50 x 50 cm, density 40 000 plants/ha; V5 =planted at 70 x 20 cm, density 70 000 plants/ha; V6 =planted at 70 x 30 cm, density 50 000 plants/ha; V7 =planted at 70 x 40 cm, density 40 000 plants/ha; V8 =planted at 70 x 50 cm, density 30 000 plants/ha. The experience was emplaced after randomized blocks method in four repetitions with the surface of plot of 3 m^2. The planting of the seedling was done in spring (May 2000) and each year we determined the total yield, herbal and radix (2001 and 2002) during the flowering period. Statistical data was interpreted through variance analysis.

Results and discussion

1. The period and phenophases of vegetation

In 1st year of its vegetation (2000), *Echinacea purpurea* (L.) Moench forms a leafy rosette and, in September and October one can witness the first floral shoots in about 40% of plants. At the end of 1st year of vegetation, of the total plant mass, the herbae represents 79%, and the underground part 21% (*Muntean L. et al*., 1990).

At *Echinacea purpurea* (L.) Moench vegetative period in the second year (2001) as result from the table 1 was 163 days and during from the 1st of may to the 13th of October. Start of vegetation – budding is 46 days and represent 28%; the phenophase budding-flowering during 23 days and represent 14%, phenophase flowering-seed ripening during 94 days and represent 58%.

In the third year (2002) period of vegetation during from 20th of April until the 14th of October and was 177 days. Start of vegetation – budding phenophase during 29 days and represent 17%; the phenophase budding-flowering during 43 days and represent 24%, phenophase flowering-seed ripening during 105 days and represent 59%.

In the fourth year (2003) period of vegetation during from 12th of April until the 9th of October and was 181 days. Start of vegetation – budding phenophase during 43 days and represent 24%; the phenophase budding-flowering during 42 days and represent 23%, phenophase flowering-seed ripening during 81 days and represent 53%.

The average of the third experimental years show us that period of vegetation at *Echinacea purpurea* (L.) Moench specie was 174 days. Phenophases of vegetation during and represents, in average: Start of vegetation – budding 40 days (23%), budding-flowering of 36 days (21%) and flowering-seed ripening of 98 days (56%) (Vârban D., 2001).

Table 1. The phenophase length in *Echinacea purpurea* (L.) Moench (Cluj-Napoca, 2001-2003)

Years of vegetation	Phenophase	Start of vegetation-Budding	Budding-flowering	Flowering-seed ripening	Total veget. period
II 2001	Data Number of day %	1.05-16.06 46 28	17.06-10.07 23 14	11.06-13.10 94 58	 163 100
III 2002	Data Number of day %	20.04-18.05 29 17	19.05-1.07 43 24	2.07-14.10 105 59	 177 100
IV 2003	Data Number of day %	12.04-24.05 43 24	25.05-5.07 42 23	6.07-9.10 81 53	 181 100
Average	Number of day %	40 23	36 21	98 56	174 100

2. The optimal nutrition space

The yield of green mass represented by leaves, stems, flowers realized by *Echinacea purpurea* (L.) Moench species at eight plantation distances and at different density is presented in table 2. The highest yield of fresh aerial parts (herbal) was realized at distances of 50 x 30 cm (462 q/ha), with a very significantly positive difference (50 x 20cm) compared to the control. Yield differences that were very significantly inferior compared to the control were registered at variants 50 x 40cm, 50 x 50cm, 70 x 30cm, 70 x 40cm and 70 x 50cm.

Table 2. Herba yield (fresh) at *Echinacea purpurea* (L.) Moench, species depending on density and planting distance (cm) (between rows/ rows) (Cluj-Napoca, average, 2001-2002)

Variant	Planting distance (cm)	Plant numbers/ m^2	Herba yield (fresh)		± Difference	Signification
			q/ha	%		
1 Control	50 x 20	10	328	100,0	-	-
2	50 x 30	7	462	140,9	+ 134	***
3	50 x 40	5	249	76,0	- 79	000
4	50 x 50	4	172	52,4	- 156	000
5	70 x 20	7	342	105,5	+ 18	-
6	70 x 30	5	178	54,2	- 150	000
7	70 x 40	4	192	58,5	- 136	000
8	70 x 50	3	134	41,0	- 194	000

LSD 5% = 30; LSD 1% = 41; LSD 0,1% = 57.

For the underground part of the plant (roots /radix), as we can observe from table 3, there were positively very significant differences compared to the control variants for the planting distance of 50 x 30cm (115 q/ha), and for the rest of variants there were negatively significant differences in comparison with the control. In our experiment fresh radix yield ranged between 31 q/ha (at a distance of 70 x 50cm) and 115 q/ha (at a distance of 50 x 30 cm).

Table 3. Fresh radix yield at *Echinacea purpurea* (L.) Moench, species depending on density and planting distance (cm) (between rows/ row) (Cluj-Napoca, average, 2001-2002)

Variant	Planting distance (cm)	Plants number/ m^2	Radix yield (fresh)		± Difference	Signification
			q/ha	%		
1 Control	50 x 20	10	89	100,0	-	-
2	50 x 30	7	115	128,7	+ 26	***
3	50 x 40	5	84	94,0	- 5	-
4	50 x 50	4	42	46,6	- 48	000
5	70 x 20	7	57	63,8	- 32	000
6	70 x 30	5	50	55,6	- 40	000
7	70 x 40	4	40	45,1	- 49	000
8	70 x 50	3	31	34,0	- 58	000

DL 5% = 9; DL 1% = 12; DL 0,1% = 17.

Between all types of green mass yield obtained at *Echinacea purpurea* (L.) Moench strong correlation was noticed (very significant) between total yield and herba yield. High value of correlation coefficient between total yield and herba yield (r = 0,995) shows that the total yield of green mass at Echinacea *purpurea* (L.) Moench is formed almost totally from herba yield and another aspect noticed from the table below is the proportional correlation between herba yield and total yield (table 4).

Table 4. Correlation coefficients between studied characteristics in the experiment with eight densities and planting distances at *Echinacea purpurea* (L.) Moench specie (Cluj-Napoca, average, 2001-2002)

Characteristically pears	Calculated correlation coefficient	Theoretical values			Statistical assurance
		r 5%	r 1%	r 0,1%	
Density – Total yield	0,759	0,707	0,834	0,925	x
Density – Herba yield	0,752				x
Density – Radix yield	0,705				x
Total yield – Herba yield	0,995				x x x
Total yield – Radix yield	0,917				x x
Herba yield – Radix yield	0,872				xx

Conclusion

The average of the third experimental years (2001-2003) show us that period of vegetation at *Echinacea purpurea* (L.) Moench specie was 174 days. Phenophases of vegetation during and represents, in average: Start of vegetation – budding 40 days (23%), budding-flowering of 36 days (21%) and flowering-seed ripening of 98 days (56%).

Considering these results we can appreciate that the highest yield of fresh matter at *Echinacea purpurea* (L.) Moench species was registered at small distance planting, which implies high density on surface unit. Thus the best results were obtained for the distance of 50 x 30 (70.000pl/ha) for the herba yield; 50 x 30 cm (70.000 pl/ha) for the radix yield.

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MORPHOLOGICAL, ANATOMICAL, BIOCHEMICAL AND PHYSIOLOGICAL RESEARCHES UPON TAXA OF *ROSA* GENUS CULTIVATED IN IASI BOTANICAL GARDEN (NOTE II)

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Summary

Three taxa of Rosa genera from the Romanian spontaneous flora have been studied. The morpho-anatomical characteristics, as well as the biochemical and physiological parameters analysed vary strictly according to the analyzed taxon, according to the phenophase cultivation. The R. rubiginosa species is the only one which has secretive hairs. Consequently, this is the only leaf origin essential oil producing species and the oil contains 56 components, among which predominant are eucalyptol (26.88 % of all the compounds identified) and borneol (15.29%); these compounds give a persistent and pleasant odor.

Keywords: *morpho-anatomical analysis, biochemical parameters, physiological parameters, essential oil*

Introduction

The genus *Rosa* is one of the spontaneous species widespread in the Holarctic region, representing a botanical entity of great fundamental as well as practical scientific interest. However, fundamental research on its biology is still scarce. The histological, anatomical and physiological research explains the behaviour of the taxa of the genus Rosa in accordance with the environment conditions and sheds light on the relations between the taxa that make up this genus. In this context, we consider that it is very useful to make a comparative study on the biology of some representatives of the genus Rosa cultivated in the "Anastasie Fatu" Botanical Garden of Iasi.

Material and research methods

Within the researches made for the present paper, we studied three species from the genus *Rosa* that exist in the Romanian spontaneous flora: *R. canina* L., *R. glauca* Pourr., *R. rubiginosa* L., cultivated in the "Anastasie Fatu" Botanical Garden of Iasi. The analyzed material was collected in specific phenophases during the summer of the year 2004 (from June to September): for the morphological and anatomical analyses, the material was collected when the first flower opened, and for the biochemical and physiological studies, in the period starting with the flowering until the formation of fruits and until senescence. The leaves analyzed were collected from the middle region of the yearly twig, and from mature plants of more than 25 years old, which excludes the behavioural variations caused by their adaptation to the environments conditions.
The anatomical analyses (made in the Laboratory of Vegetal Morphology and Anatomy of the Faculty of Biology within the "Al. I. Cuza" University of Iasi) aimed at:
a) **Investigating the anatomy of the leaf limb** (the middle vein) by the technique of hand sectioning and of the double staining. b) **Investigating the morphology of the structures that secrete volatile oils** on the fresh leaf surface, using the NOVEX microscope with a

magnifying power of 200x; the photographic images were obtained and processed with a MINOLTA digital photographic camera.

The biochemical and physiological analysis was done in the Vegetal Physiology Laboratory of the Faculty of Biology within the "Al.I.Cuza" University of Iasi The following steps were necessary:

a) Determinations on fresh material of the water and dry matter content – through gravimetric method (Boldor et al., 1983), of foliar assimilator pigments – through spectrophotometric method (Boldor et al., 1983), of the intensity of the respiratory process – through Warburg manometric method (Boldor et al., 1983), of foliar glucoses content – through Bertrand method (Artenie and Tanase, 1981) for extraction and Borel spectrophotometric method for dosage, of foliar crude lipids – through Soxhlet method (modified Artenie and Tanase, 1981).

b) Determinations on dry material of foliar crude protein content – through Kjeldahl method (Artenie and Tanase, 1981). **c) The volatile oils** were extracted from fresh leaves using hydro-distillation method and a Clevenger device; the analysis of the quality of the oil took place in the The Research Basis with Multiple Users of the Faculty of Horticulture, in Agriculture Sciences and Veterinary Medicine University of Bucharest using a GC gas chromatograph with a spectrometric mass detector Agilant 5973 and an auto sampler; the DB5 chromatographic column has a length of 25 m and an interior diameter of 0.25 m. The separated compounds were identified by means of the Nist spectra database, and the peaks' position was confirmed by the Kovats indices.

Results and discussions

Anatomical characteristics of the leaf limb (Fig. 1)

The anatomical analyses of the leaf limb aimed at identifying and describing the structures that secrete volatile oils; consequently, the descriptions presented in this paper refer to the species *Rosa rubiginosa*, which bears such structures on different segments of the leaf.

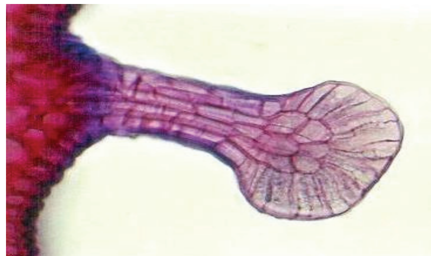

Fig. 1. *Rosa rubiginosa* L.: Pluricellular secretive hairs from the rachis of the leaf (2,5x20)

The rachis presents long tactile hairs and papillae-like structures in patches, with a headed ending, with a pluricellular leg, with cells lined up in two parallel rows and with thin and cellulosic walls that remind the pluricellular secretive hairs. The limb of the leaflet. From a frontal perspective, the epidermis is made up of polygonal cells with straight lateral walls in the upper epidermis as well as in the lower epidermis. The anomocytic stomata are present only in the lower epidermis, so the limb is hypostomatic. In both epidermises, there are long, unicellular tactile hairs, with very thick walls and with an obtuse point. At the ends of the limb, one can see pluricellular secretive headed structures, made up of cells with very thin, cellulosic walls. All these secretive structures from the petiole, rachis and leaflets are pluricellular secretive hairs. They are in much greater number on the inferior part of the limb. On the transversal section of the median vein, one can see that it is obviously prominent on the inferior part of the limb, and that the epidermis presents tactile and secretive hairs.

Biochemical and Functional Characteristics (Table 1)

The leaf assimilating pigments content. The quantity of *a chlorophyll*, the main photo-assimilating pigment within the photosynthesis process, varies between the analyzed species according to the ontogenetic moment, registered also by the taxa under investigation. For *R. canina*, the content of *a chlorophyll* remains superunitary along the whole period analyzed, while for the two other species, it becomes subunitary in the fruit phenophase. For the species *R. glauca* and *R. rubiginosa*, the quantitative evolution of the *a chlorophyll* is similar, with respect to the progression of the phenophases in the ontogenetic cycle, with maximum biosynthesis, accumulation and usage points in the flowering phenophase (2.31 mg, 1.73 mg respectively), when the biosynthesis needs of the of the test plants become maximum (it is the moment of formation of the reproductive system). For *R. canina*, the maximum biosynthesis point for *a chlorophyll* appears in the vegetative phenophase (1.62 mg), the evolution dynamics of this parameter registering a downward tendency within the time interval. In the period analyzed, the *b chlorophyll* also has a quantitative dynamics specific to the taxon under investigation, as well as to that particular ontogenetic moment. Along the entire measurement period, the *b chlorophyll* has subunitary values; the biosynthesis curve has maximum points for *R. glauca* and *R. rubiginosa* in the flowering phenophase (0.91 mg and 0.59 mg respectively). For *R. canina*, the point of maximum biosynthesis is located in the vegetative phenophase (0.58 mg).

The ratio between a chlorophyll and b chlorophyll remains constant during all the time interval, close to the value 3:1 for the *a chlorophyll*. According to these values, we can characterize the taxa studied as being amenable to light.

Carotenoidic pigments present subunitary values along the entire period analyzed. In the vegetative phenophase, all the species investigated present a content of carotenoidic pigments 10 times lower as compared to the rest of the period taken into account, and a maximum content in the flowering period.

We consider that the values registered are in accordance with the environment conditions in which the test plants lived, with a dimmer light in the first analysis interval (in spring) and with a stronger light, but also with an increase in the negative effects of solar radiations on plants in the summer stage (the flowering phenophase), when the light period and intensity increased. At a later stage, in the fruit phenophase (corresponding to the beginning of the autumn), the carotenoidic pigments content decreases again, approaching, for *R. rubiginosa*, the value registered in the spring stage (0.30 mg in the fruit phenophase, 0.32 mg in the vegetative phenophase, respectively). In the specialty literature, specialists state that there is a constant relation between the intensity of the photosynthetic process and the content of assimilating pigments. Thus, we can consider that the investigated species, of European origin, make an increased metabolic effort in order to insure an intense photosynthesis process. This process can thus produce the organic substances needed in every stage of the ontogenetic cycle of the taxa under investigation.

The water content and the leaf dry content

The water content is a parameter that varies according to the taxon analyzed, to its ontogenetic age and to the moment of the analysis. In general, for the species analyzed, the water content is never lower than 83 g% (*R. canina* in the fruit phenophase). Consequently, in an ontogenetic dynamics perspective, the water content has small variations, within the limits of 2 g% from one moment to another for *R. canina* and *R. glauca*, and with an increasing difference of about 8 g%, from the vegetative phenophase to the fruit phase for *R. rubiginosa*. This parameter does not reach values that could endanger the good functioning of the cellular enzymatic system or of the stomatic apparatus. At the same time, we consider that the values registered in practice for the test plants concerning the water content all along the year reflect the good care received by the plants from the fields of the Botanical Garden of Iasi (soil

treatment and periodical irrigations), that fought against the negative effects of the lack of rain in the period analyzed.

The dry substance content represents in fact, in the ontogenetic dynamics, the mirror image of the curve of the water content, as exact points of the graphical representation and as orientation of the curve of the graphical image. In this context, the species *R. canina* presents an obvious uniformity of the values registered in the ontogenetic dynamics for this parameter (14-16 g % all along the analyses period), with a very small decreasing tendency in the flowering phenophase). For *R. glauca*, the graphical image representing the variation of the content of dry substance has a slight decreasing tendency following the ageing of the plants. For *R. rubiginosa*, there is a greater difference as to the content of dry leaf substance when passing from the vegetative phenophase (14.9 g %) to the flowering phenophase (6.35 g %), followed by a slight value rehabilitation (6.89 g %in the fruit phenophase).

The glucoses content. The quantitative evolution of the sugar content in test plants is in strict correlation with the age of the leaves and with the general glucoses consumption for energetic needs that support the structural efforts of the ageing plants studied. The *monosaccharide* content displays an increasing tendency for *R. glauca* and *R. rubiginosa* during the entire analysis interval, while the disaccharides have a reverse, decreasing tendency. This is determined by the fact that *disaccharides* represent the main transport form in plants, migrating easily from the photosynthetising leaves to all the other consuming organs of the plants. The *soluble polysaccharides* present slightly increased values in all the period tested for *R. glauca*, followed by *R. rubiginosa* and *R. canina*, respectively. *Insoluble polysaccharides* register subunitary values in the first stage of analysis (vegetative phenophase), becoming superunitary in the flowering and fruit phenophases.

The total lipid content. Its dynamics registers an upward slope for all the three species parallel to the advance of the ontogenetic cycle, so that the maximum biosynthesis and accumulation values are registered in the fruit phenophase (10.39 g for *Rosa canina*, 12.42 g for *Rosa glauca* and 13.14 g for *R. rubiginosa*, respectively).

The total protein content varies for the test species according to the taxon analyzed and to the phenophase investigated. For all the taxa analyzed, its ontogenetic dynamics describes a curve with a maximum flowering point, when proteins are intensely used for structural purposes, for the formation of the reproducing elements.

The intensity of the respiratory process. For all the taxa investigated, the respiratory process registers maximum values in the vegetative phenophase, when young plants make great efforts for the development of the vegetative organs (including the leaves), making a special metabolic effort in order to insure its own photosynthesis processes (BURZO et al., 2004). At a later stage, the species *R. canina* and *R. glauca* register slightly higher values of the respiratory process in the fruit phenophase, when they consolidate their sexual reproductive organs (seeds) and the resistance structures for the cold period of the year. *R. rubiginosa* presents a specific respiratory dynamics, following a downward slope until the end of the vegetation period; under these conditions, in the middle of the ontogenetic cycle (the flowering stage), this taxon presents the maximum value of the respiratory process (0.1284 mg O_2). This taxon presents on its leaves numerous structures that secrete volatile oils; they have an active functioning in the flowering phenophase, determining probably an intense respiratory process as an energy source for the realization of the biosyntheses specific to this ontogenetic period.

The composition of the volatile oil (Diagram 1). The species *R. rubiginosa* presents on the leaf (on the inferior epidermis of the leaf limb, on the rachis and stipels) glandular hairs that produce a volatile oil with a scent of green apples. About 56 compounds make up this oil, among which the most important are the eucalyptol (26.88 % of all the compounds identified) and borneol (15.29 %), compounds that confer to the oil a pleasant, persistent smell. We also

mention the presence of some compounds that represent more than 1 % of all the isolated compounds: p-cymen (12.03 %), γ-terpinene (8.25 %), bornyl acetate (5.59 %), α-pinene (4.65%), camphene (3.72 %), β-pinene (3.69 %), α-humulene (3.41 %) and β-caryophillene (3.40 %). The other compounds have values close to 1% of all the identified and isolated compounds, or even smaller values.

Conclusions

- The practical data that have been obtained concerning the anatomy of the leaf for the three taxa of the genus *Rosa* analyzed are in accordance with the data presented in the specialized literature.
- The variation of the biochemical and physiological indices analyzed within the ontogenetic dynamics for the taxa under study highlight their adaptation to the vegetation conditions offered by the Botanical Garden in Iasi. The interpretation of the data is strictly related to the climatic conditions of the summer 2004, marked by an increased drought, high temperatures, which meant that the test plants had to make great metabolic efforts for survival.
- The volatile oil produced within the leaf by the species *Rosa rubiginosa*, with a specific qualitative and quantitative composition, gives the taxon a special aromatic character that increases its ornamental, aromatic and, implicitly, commercial qualities.

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Table 1. The ontogenetic dynamics of some biochemical and physiological parameters at some taxa of *Rosa* genus cultivated in Botanical Garden of Iasi

Taxon	Phenophase	a Cl.	b Cl.	Carotenoidic pigm.	a Cl/bCl	Water	Dry matter	Mono sacch. g glucse/100 g sech matter)	Disacch g glucse/100 g sech matter	Soluble polisacch g glucse /100 g sech matter	Insol. polysacch. g glucose /100 g sech matter	Total lipid content g /100 g sech matter	Total protein content g /100 g sech matter	Respiratory process cm^3 O_2/g fresh matter
		mg/g fresh matter				g%								
Rosa canina	Vegetative phenophase	1.62	0.51	0.05	3.1	85.70	14.3	0.65	1.31	1.66	0.51	4.16	9.14	0.21
Rosa glauca		1.58	0.53	0.08	2.9	92.99	7.01	0.67	0.83	2.76	0.71	4.97	3.56	0.17
Rosa rubiginosa		1.07	0.32	0.041	3.3	85.03	14.97	0.86	0.84	1.65	0.66	4.02	5.02	0.14
Rosa canina	Flowering phenophase	1.56	0.51	0.49	3.0	85.81	14.19	0.71	6.97	6.88	0.33	14.91	10.98	0.01
Rosa glauca		2.31	0.91	0.74	2.5	94.99	5.01	5.20	6.87	8.37	6.08	26.53	15.26	0.08
Rosa rubiginosa		1.73	0.59	0.55	2.9	93.64	6.36	4.79	5.20	6.43	6.32	22.75	17.77	0.12
Rosa canina	Fruit phenophase	1.37	0.58	0.49	2.3	83.44	16.56	2.76	0.48	1.01	4.99	9.26	2.75	0.01
Rosa glauca		0.88	0.33	0.29	2.6	96.35	3.65	7.74	10.19	13.67	5.56	37.17	9.40	0.08
Rosa rubiginosa		0.84	0.30	0.30	2.8	93.11	6.89	4.75	2.61	4.19	3.89	15.46	7.55	0.08

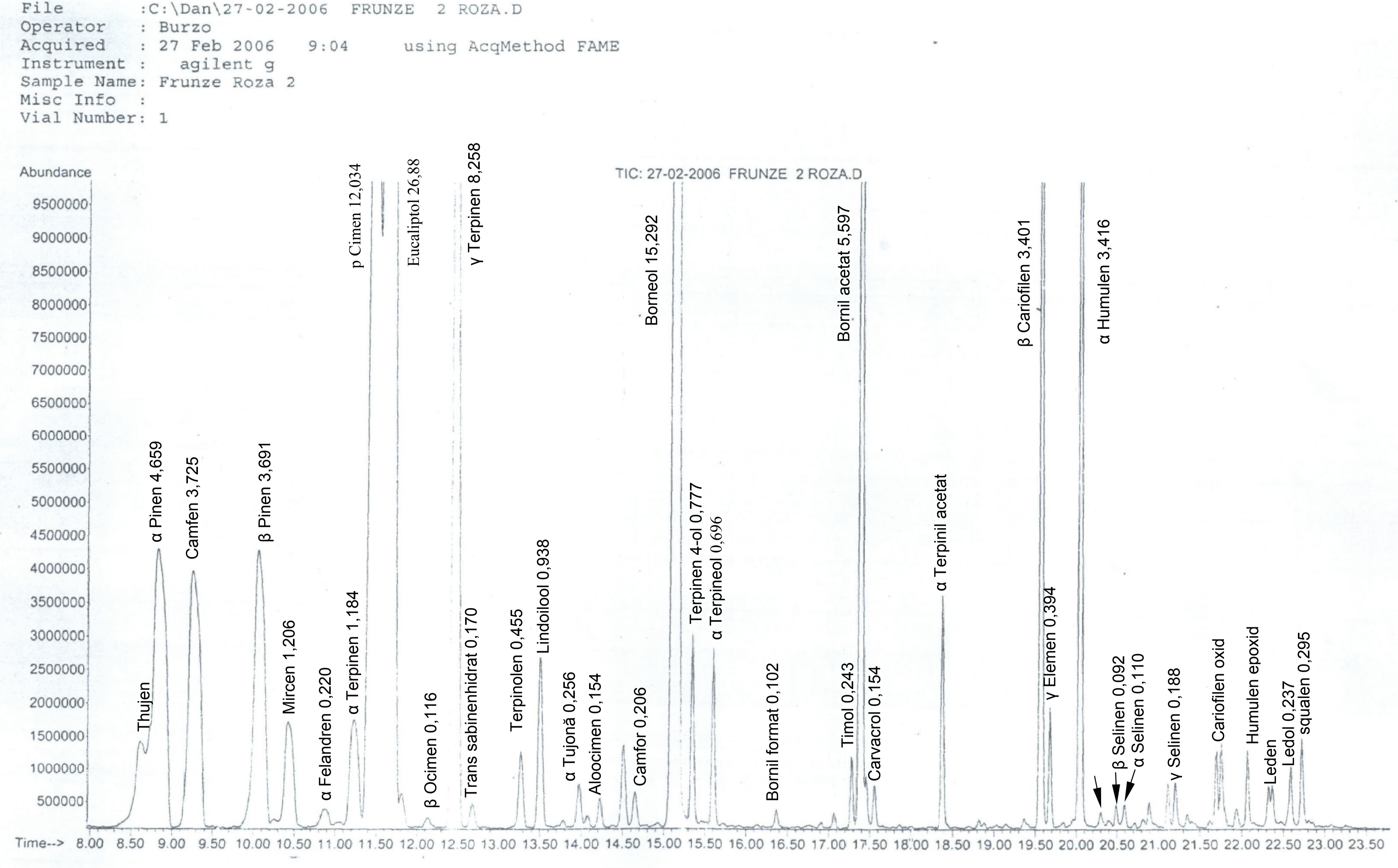

Diagram 1. The chart for the main components of volatile oils (% of all the compounds identified) extracted from *Rosa rubiginosa* L., cultivated in Iasi Botanica Garden

USE OF JASMONATES TO INDUCE TERPENES IN *PICEA ABIES* TREES AND CELL CULTURES

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Summary

Treatment of plants with jasmonates often shows that jasmonates seem to have a high potential in increasing level of biologically active compounds in plants. In this study, methyl jasmonate (MJ) was employed to manipulate levels of terpenes in intact trees and cell cultures of Norway spruce. Application of MJ caused a two to three-fold increase in mono-, sesqui-, and diterpenes in Picea abies sapwood, within a month following treatment. Although previous research have not shown terpenes in undifferentiated cell cultures, we found that P. abies cell suspension cultures constitutively biosynthesized small amounts of various monoterpene hydrocarbons de novo with a product profile similar to that of adult trees. Moreover, following application of MJ or a fungal elicitor, there was a three-fold increase in monoterpene accumulation. These results confirm that the cell suspension cultures might be a useful system in producing high-value secondary metabolites in P. abies.

Keywords: *Cell culture, elicitor, monoterpenes, Norway spruce*

Introduction

Norway spruce (*Picea abies* Karst.), one of the most important forest tree species in Europe, is often subject to attack by stem-invading pests, such as the bark beetle *Ips typographus* L. The initial penetration of the host is nearly always accompanied by inoculation with associated pathogenic fungi. The most common fungal pathogen associated with *I. typographus* is the blue-stain fungus *Ceratocystis polonica* (Siem.) (Krokene and Solheim, 1998). This fungus is a principal cause of tree death due to its ability to invade the vascular system.

To defend themselves against insect and pathogen attack, spruce and other conifers have evolved elaborate constitutive and inducible defense mechanisms (Franceschi et al. 2005). The constitutive defenses include the secretion of oleoresin (a complex mixture of monoterpenes, sesquiterpenes and diterpenoid acids) from preformed resin reservoirs, as well as lignified cells and calcium oxalate crystals, all of which may create formidable physical and chemical barriers to attack (Hudgins et al., 2003; Franceschi et al., 2005). Inducible defenses that are activated upon initial attack include the formation of a wound reaction zone (Berryman, 1972), formation of traumatic resin ducts (Nagy et al., 2000, Krokene et al., 2003), and terpene production (Martin et al., 2002).

On the other hand, conifers are difficult organisms for biochemical studies because of their large size and many types of specialized tissues which make it hard to study an individual pathway, such as terpene biosynthesis, that is confined to specialized tissue. Thus, the use of plant cell culture is an attractive alternative system for studying the production of conifer defense metabolites. Cell cultures have been established from many plants but often they do not produce sufficient amounts of the required secondary metabolites. However, in some cases the production of secondary metabolites can be enhanced by the treatment of the undifferentiated cells with elicitors such as methyl jasmonate, salicylic acid, cell wall constituents of various microorganisms, or enzymes

such as pectinase or cellulose. Plant cell suspension cultures can be useful tools for investigating terpenoid biosynthesis allowing the elucidation of the biosynthetic pathway and determination of the factors that regulate terpene formation.
Jasmonates are lipophilic, linolenic acid-derived plant hormones with roles in metabolic regulation, semiochemical communication, and defense in many plants (Beale and Ward 1998). Methyl jasmonate is known to increase terpene levels in conifers when applied to saplings (Martin et al., 2002). However, it is not known if methyl jasmonate can increase resistance to stem pathogens. In addition, no detailed information is available on the effect of MJ on the terpene content of mature trees, and the effect of this compound on other cell cultures of Norway spruce, has not been investigated. In this study, we determined the effect of MJ on the terpenoids of mature *P. abies*, and investigated if this treatment protected trees against attack by the blue-stain fungus *C. polonica.* In separate experiments, we used *P. abies* cell suspension cultures to study the regulation of induced terpene defenses in this species.

Material and methods

Methyl jasmonate treatment and sampling
Six trees from each of six clones were selected from a plantation of 40 year-old Norway spruce clones at Hogsmark, Ås, SE Norway. One tree per clone was randomly assigned to each of four different treatments with methyl jasmonate (MJ): 5, 25, 50 and 100 mM MJ in water with 0.1% Tween 20, or to a control with water and 0.1% Tween 20. Tween 20 helps to solubilize MJ in water and acts as a surfactant to help spread the solution evenly over the hydrophobic bark surface. Four weeks after MJ treatment, all trees were mass-inoculated with *C. polonica* (400 inoculations m^2, on average 276 inoculations per tree) to assess tree resistance. The inoculum consisted of actively growing mycelium of *C. polonica* (isolate no. NISK 93-208/115) cultured on malt agar (2% malt and 1.5% agar).
Samples of bark and sapwood for chemical analyses were taken from the control and MJ-treated trees on three occasions: (1) immediately before treatment with MJ or Tween 20 control, (2) immediately before fungal mass-inoculation, and (3) at the felling of the trees, 15 weeks after mass inoculation. In late July, the stems of all trees were inspected for resin on the surface of the treated stem area. Resin flow from inoculation points was visually assessed on a scale from 0 (no flow) to 5 (abundant flow from almost all points). Photos of the sections were taken to document the status.

Measurements of fungal growth, cambium necrosis, and anatomy
To quantify fungal colonization of host tissues, the proportion of the sapwood that had been blue-stained by the fungus and the proportion of dead cambium circumference were measured on the two stem discs that were removed from each tree, as described in Krokene and Solheim (1998). To quantify anatomically based defense reactions, samples containing bark and sapwood were prepared as described in Krokene et al., (2003) and examined with light microscopy.

Culture elicitation and metabolic studies measurements
Liquid cultures were started from established callus and subcultured at ten-day intervals. Cultured cells were assayed for both endogenous monoterpene production and

monoterpene synthase activity using two elicitors, 50 µM methyl jasmonate (MJ) (Sigma-Aldrich, Steinheim, Germany) or 100 ug/mL chitosan from crab shells (Sigma-Aldrich, Steinheim, Germany). Chitosan was prepared based on the protocol of Walker-Simmons et al. (1984).
To sample cultures for endogenous monoterpene production, *Picea abies* cell line *P.a. 186.3* was grown for 3 days in EDM6 suspension medium. Suspension cultures were elicited with either 50 µM methyl jasmonate or 100 ug/mL chitosan final concentration. Sterilized XAD-4 resin (Sigma-Aldrich, Steinheim, Germany) was added to a control or induced culture 1 hour after the addition of elicitors, along with isobutylbenzene (1 µg/mL final concentration) as internal standard, and grown for another 7 days. Control cells were grown for the same period of time without adding elicitors. Ten independent samples were assayed for each treatment and control. On day 10, the XAD-4 resin and cells were then harvested by filtering them onto Whatman paper to remove the medium, using a Büchner funnel with vacuum. Cells and resin were transferred to 30 mL Pyrex Culture Teflon-lined screw-capped tubes (Sigma-Aldrich, Steinheim, Germany). After pentane extraction, agitation and purification, the extract was concentrated on ice under a gentle nitrogen steam to about 200 µL and then analyzed by GC-MS.
To monitor terpene synthase activities, 7 day-old cultures were treated with either 50 µM methyl jasmonate (MJ) or 100 ug/mL chitosan or water and harvested at time of inductions, 20 minutes, 40 minutes, and 1, 2, 4, 8, 12, 24, 48, 72, or 96 hours post-induction. All time points were assayed in triplicate or quadruplicate. Harvested cells were filtered and stored at −80 °C prior to extraction. Monoterpene synthase activity was determined by published procedures (Martin et al., 2002) with minor modifications. Before assaying enzyme activity, the frozen protein extracts were placed at 37°C until just thawed. Enzyme activity was assessed with 1 mL of the desalted extracts with the addition of 10 µM GPP (with 1 µCi ^{3}H-GPP) (Biotrend, Köln, Germany) for mono-TPS activities, or 10 µM GGPP (0.5 µCi ^{3}H-GGPP) (Biotrend, Köln, Germany) as substrate for di-TPS assays. All enzyme assays were done in triplicate, overlaid with 1 mL of pentane to collect released volatiles, and incubated at 30°C for 1 hour. The conditions for all enzyme assays, including pH optimum, incubation time, substrate concentration, and temperature optimum were optimized for this system so that maximum activity was achieved in a linear range of product generation.

Chemical analyses
Terpene extractions and GC-MS analysis of terpenes were based on the procedures of Zeneli et al. (2006). The total monoterpene, sesquiterpene, or diterpene resin acid content was calculated as the sum of the individually quantified compounds.

Results and discussions

Methyl jasmonate treatment in mature trees
To quantify the induced resin response of Norway spruce after methyl jasmonate treatment, we measured traumatic resin duct formation and terpene accumulation in the sapwood, and external resin flow on the bark surface. A dose dependent response was observed in which trees treated with the highest concentration of methyl jasmonate demonstrated massive increases in traumatic resin duct formation and external resin flow compared to trees from the control group, but lower doses did generally not cause

significant changes (Figure 1). Trees treated with 100 mM methyl jasmonate produced more than 25 times as many traumatic ducts as controls, and the external resin flow on the trunk was more than 80 times higher (Figures 1A,B). There was a strong, positive correlation between these parameters ($p < 0.0001$, $R^2 = 0.80$). The total content of mono- plus sesquiterpenes in 100 mM-treated trees was 2.2 times that of untreated controls, but there were no significant differences between treatments ($F_{4,29} = 2.19$, $p = 0.11$). However, trees that survived mass-inoculation with *C. polonica* had accumulated much higher monoterpene and sesquiterpene concentrations in response to methyl jasmonate treatment than trees that were killed by the fungus. The increases in total monoterpene and sesquiterpene concentration during the first four weeks after methyl jasmonate treatment was 3.47 mg g^{-1} in surviving trees, compared with 0.36 mg g-1 in trees that eventually were killed ($p = 0.05$, $F = 4.36$).

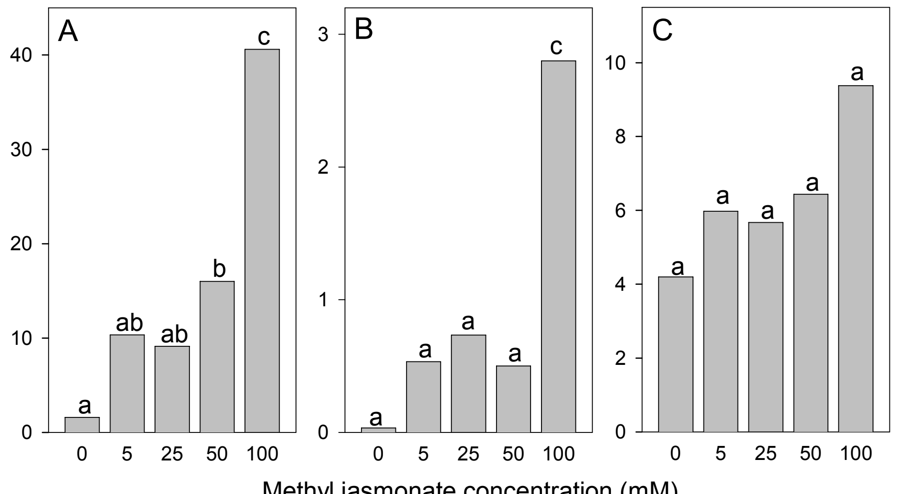

Fig. 1. Effect of various methyl jasmonate treatments on traumatic resin duct formation (A), bark resin flow (B), and terpene content (C). Values shown are averages of all clones. Traumatic ducts are expressed as the percent coverage in sample sections in a region 1.1 mm across in the tangential direction four weeks after methyl jasmonate treatment. Terpene content is presented as mg per g fresh weight four weeks after methyl jasmonate treatment. Bars with the same letter were not significantly different (LSD test a P=0.05 following ANOVA).

Within a month following methyl jasmonate treatment, traumatic resin ducts were formed in the newly developing xylem (Fig. 1A), there was a greater accumulation of monoterpenes, sesquiterpenes and diterpenes in the sapwood (Fig. 1C), and resin flow on the trunk increased (Fig. 1B). The first two responses were previously observed also in two year-old *P. abies* saplings (Martin et al., 2002). Given the generally accepted role of resin terpenes in conifer defense (Phillips and Croteau, 1999), the application of exogenous methyl jasmonate can be expected to increase the resistance of *P. abies* to attack by herbivores and pathogens.

Methyl jasmonate treatment not only increased terpene content and resin flow, but also increased resistance to the fungus *C. polonica*. The growth of this blue-staining fungus into the sapwood and the necrosis of the cambium caused by fungal invasion were both significantly reduced by methyl jasmonate application. In previous investigations of *P. abies*, similar reductions of *C. polonica* infestation were achieved by inoculation with a

sublethal dose of *C. polonica* (Krokene et al., 2003), a treatment that also may have acted by increasing the terpene content. In this study, the correlation between terpene content and fungal resistance is especially striking when one compares the different clonal lines investigated.

Monoterpene production in cultured cells

By optimizing the method of monoterpene extraction, the concentration of the elicitor, and the timing of elicitation, we could show for the first time the endogenous production of terpenoids in *Picea* cell suspension cultures, in contrast to previous reports (Lindmark-Henriksson et al., 2004). We found that suspension cultures constitutively accumulated small amounts of monoterpenes with a product profile similar to that of adult trees (Fig. 2). However, the greatest accumulation of monoterpenes occurred when elicitors were added to cultures at a final concentration of 50 µM (methyl jasmonate) or 100 µg/mL (chitosan) on day 3 of the 10 day-culture cycle. Our study revealed that *Pa.* 186.3 cells accumulated α-pinene (1), β-pinene (3) and limonene (7) in trace amounts (Fig. 2). The other monoterpenes of mature trees were not present in the culture. While this may simply reflect varietal differences, it may also underscore subtle differences in the expression of members of the spruce monoterpene synthase gene family in culture. Sesquiterpenes and diterpenes, although present in spruce saplings and trees, were also not detected in these cell suspension cultures.

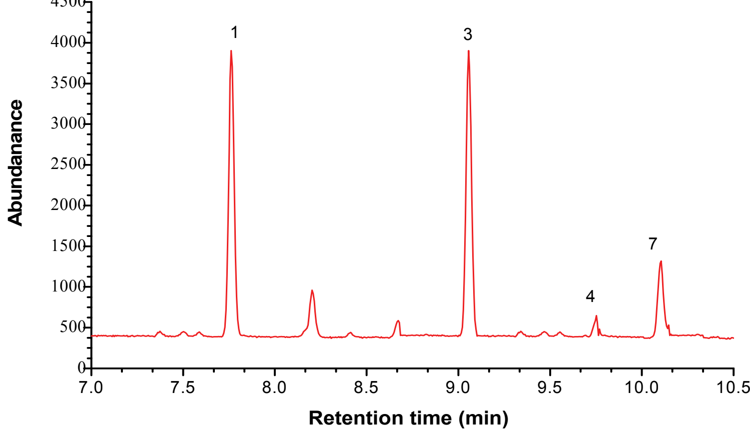

Fig. 2. Endogenous monoterpene production in spruce suspensions cultures extracted after incubation with XAD-4 resin.

Monoterpene production was induced similarly by methyl jasmonate and fungal cell walls and fragments thereof (chitosan). Elicitation with methyl jasmonate caused a 3-4 fold increase in monoterpene production compared with control (unelicited) (120 µg/g culture *vs* 35 µg/g). Chitosan elicitation was weaker than MJ, causing an increase 2-2.5 fold compared with unelicited controls (80 µg/g culture vs 35 µg/g). Culture age, relative to the most recent transfer, slightly affected the constitutive level of monoterpenes.
All of the major monoterpenes present in the elicited culture were also present in the nonelicited culture, but the relative proportion of the monoterpenes was different, indicating that elicitation does not result in the production of new types of monoterpenes, but simply affects the relative abundance of existing terpenes.

Conclusions

MJ treatment induced the formation of a new row of resin ducts in the sapwood, which was also associated with a significant increase in resin accumulation and flow onto the outer bark. MJ treatment stimulated increased accumulation of all the major terpene classes in resin. The total terpene content of sapwood increased approximately two to three-fold after 100 mM methyl jasmonate application. The growth of *C. polonica*, a blue-staining fungus, into the sapwood and the necrosis of the cambium caused by fungal invasion were both significantly reduced by methyl jasmonate application, suggesting that terpenoid oleoresin may function in defense against this pathogen.
P. abies cell suspension cultures constitutively biosynthesized small amounts of various monoterpene hydrocarbons *de novo* with a product profile similar to that of adult trees. No accumulation of sesquiterpenes or diterpenes was observed. However, following application of MJ or a fungal elicitor, there was a three-fold increase in monoterpene accumulation. Measurements of monoterpene synthase activity, the committed step in monoterpene biosynthesis, showed that this enzyme activity was significantly induced by both fungal elicitor and MJ, with MJ having the greatest effect. These results confirm that the jasmonate signaling pathway is an important endogenous regulator of induced terpene biosynthesis in spruce.

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ENEMIES AT THE GATE: TERPENOIDS AND DEFENSE AGAINST SPRUCE BARK BEETLE (*IPS TYPOGRAPHUS*)

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Summary

In the present investigation, the effect of chemical induced defense in host colonization process of the spruce bark beetle Ips typographus (L.) in mature Norway spruce were studied. We used methyl jasmonate (MJ), a well-known inducer of plant defense responses, to manipulate the biochemistry and anatomy of mature Picea abies trees and test their resistance to attack by Ips typographus. Stem sections of P. abies treated with MJ had a significantly higher concentration of mono-, sesqui- and diterpenes than untreated sections. Bark sections of P. abies treated with MJ had significantly less I. typographus colonization than bark sections in the controls and exhibited shorter parental galleries and fewer eggs deposited. The increased amount of terpenoid resin present in MJ-treated bark could be directly responsible for the observed decrease in I. typographus colonization and reproduction.

Keywords: *Conifer, defense, jasmonates, Norway spruce, terpenes.*

Introduction

The spruce bark beetle (*Ips typographus*) (Coleoptera, Scolytidae) is considered the single most destructive of the bark beetles that inhabit the coniferous forests of the Palaearctic region (Berryman, 1972; Wood, 1982). A keystone species, it causes both small-scale and large-scale disturbances, thus driving forest succession in Eurasia (Christiansen and Bakke, 1988). The last outbreak (1971–1981) killed the equivalent of 5 million m^3 of spruce timber within a 140,000 km^2 area of southeastern Norway (Christiansen and Bakke, 1988).
As the adult bark beetles enter the tree they introduce a variety of microorganisms, including phytopathogenic fungi. Bark beetles and their associated fungi represent one of the most serious threats to coniferous forests worldwide (Paine et al. 1997). Thus it is not surprising that conifers appear to have evolved sophisticated constitutive and inducible defence mechanisms to protect themselves against bark beetle colonization (Franceschi et al., 2005).
The best studied chemical defense of *P. abies* and other conifers is the oleoresin found in foliage, stems and other organs, a defense system that has existed for at least 50 million years (Labandeira et al., 2001). Oleoresin is composed largely of terpenes, the largest class of plant secondary compounds (Gershenzon and Kreis, 1999). Terpenes are formed by the fusion of C_5 isopentenoid units and classified by the number of such units present in their basic skeletons. Oleoresin has long been believed to play a crucial role in conifer defense because of its physical properties (i.e. viscosity) and repellency to many herbivores and pathogens. In *P. abies*, oleoresin is found constitutively but may also be induced by herbivore or pathogen attack (Nagy et al., 2000). Since wounding itself can cause the loss of resin, especially the volatile components, we explored the utility of a non-invasive procedure for resin induction involving the application of methyl jasmonate (MJ), an elicitor of plant defense responses in many species. In the present investigation, we have studied the host colonization process of the spruce bark beetle *Ips typographus* (L.) in mature Norway spruce *Picea abies* (L.) Karst. trees. To test if induced responses of *P. abies* to MJ also have defensive potential against bark beetles, we applied MJ to mature trees in a wild stand and challenged the treated trees with *I. typographus* attracted by pheromone dispensers.

Material and methods

Field experiment

In the spring, twelve trees were randomly selected in an open, pure stand of mature Norway spruce (60 years old, tree height ca. 28 m. On 26 May, a stem section between 1.5 and 4.5 m above ground was divided into east- and west-facing halves by two vertical lines, using a water-based latex paint. One half of each tree was treated with 100 mM MJ and the other half was left untreated to serve as a control (MJ_C). MJ was sprayed onto the stem using a small spray gun, while carefully avoiding contamination of the control side.

Three weeks after MJ application (16 June) four samples containing the bark and outermost sapwood (1.6 cm wide × 5 cm high × 1 cm deep) were removed for anatomical investigation from each tree at 1.5 and 3.5 m above ground, two on the treated side and two on the control side. At each site a smaller sample (1.6 × 1.6 × 1 cm) for analysis of terpenes and phenolics was removed, quickly frozen on liquid N_2 and transferred to a –80 ºC freezer.

On 17 June, an Ipslure pheromone dispenser (Borregaard, Sarpsborg, Norway) was placed on each tree 2 m above ground to induce attack by *I. typographus*. The dispensers were placed on the north side of the trees on the border between the MJ-treated and untreated sides. Because the beetle population in the area was relatively low and the main flight of the beetles already had taken place, an additional Ipslure dispenser was added three days later to enhance attraction. Beetle aggregation remained moderate. The pheromone dispensers remained on the stems until 25 July, when the trees were sampled to assess the beetle's attack success.

For sampling, the outer cork bark was carefully shaved away on both sides of trees at the dispenser height. A transparent plastic sheet (210 × 297 mm) was placed on the stem within the shaved area, with the long side oriented vertically, and well away from the dividing lines between the two treatments. All entrance holes covered by the sheet and penetrating into the live phloem were marked, and more developed beetle galleries were traced. On 26 August, the trees were sampled again immediately above the first sampling site, using the same method. In the laboratory we recorded the number of entrance holes and incipient galleries (i.e. tunnels longer than 10 mm), and total length of all maternal galleries on the plastic sheets. When multiple galleries extended from a single entrance hole, we recorded the sum of their lengths.

On 24 July, 2003, 12 other Norway spruce trees in the same stand (diameter at breast height 28.59 ± 3.19 cm) were treated with MJ as described above to see if MJ treatment in one year would have any effects on beetle colonization the following year. Samples for anatomical and chemical analyses were removed from the trees the following spring (12 May 2004) as described above, and two days later the trees were baited with pheromone dispensers to induce attack by *I. typographus*. At this stage, there was extensive resin flow in some trees on bark that had been treated with MJ, and resin flow was assessed qualitatively on a scale from 0 (no resin) to 4 (extensive resin flow). On 16-17 June 2004, the outer bark was removed and the outcome of the beetle attacks was assessed as described above.

Chemical analyses

Terpene extractions were based on the procedures of Zeneli et al. (2006). A Hewlett-Packard 6890 GC-MSD system, using a DB-5 MS column (30 m x 0.25 mm × 0.25 µm, J&W Scientific, Folsom, CA) was used for the GC-MS analysis of monoterpenes and sesquiterpenes. Split injections (1 µL ethereal extract) were made at a ratio of 1:5 for wood and 1:10 for bark samples with an injector temperature of 220°C. The instrument was run under the same program described by Zeneli et al. (2006). Analysis of diterpene constituents was performed on the same GC-MS instrument fitted with the same DB-5 MS column. Injections were made with 1 µL of the concentrated, derivatized ethereal extracts. GC-MS split ratios were 1:10 (for both wood and bark extracts) with an injector temperature of 220°C. The temperature programs for the instrument are described by Zeneli et al. (2006). GC-MS

generated peaks were quantified using Hewlett-Packard Chemstation software. For quantitative analysis of monoterpenes, sesquiterpenes and diterpenes, the MS detector was operated in the SIM mode. The selected ions for the internal standards, monoterpenes, sesquiterpenes and diterpene methyl esters are described by Zeneli et al. (2006). The total monoterpene, sesquiterpene, or diterpene resin acid content was calculated as the sum of the individually quantified compounds.

Statistical analyses

Data were analyzed using analysis of variance. Each variable was tested to satisfy assumptions of normality and homogeneity of variances by graphical analysis of residuals. If the variance was non-homogeneous, variables were transformed to square root, which provided distributions that satisfied these assumptions in all cases. Beetle colonization data were analyzed on a single-tree basis, by using the calculated differences between MJ treated and untreated bark within trees as the response variable. The data were subjected to one-sample t-tests using SYSTAT (SPSS Inc., Illinois, USA). A Protected LSD test was used for multiple comparisons of means.

Results and discussions

Methyl jasmonate increased the number of traumatic resin ducts and the accumulation of terpene resin constituents

Anatomical analyses showed that in both years there were significantly more traumatic resin ducts (TDs) in the xylem of MJ-treated sections of trees than in the xylem of untreated control sections (2003: 27.7% vs. 1.8% of sapwood circumference, $P = 0.001$; 2004: 14.2 vs. 5.9%, $P = 0.04$; one-sample t-test). There was no significant difference in TD abundance in MJ-treated sections between years ($P = 0.11$, t-test).

The concentrations of monoterpenes, diterpenes and total terpenes were significantly higher in MJ treated bark and sapwood than in control tissues (Fig. 1).

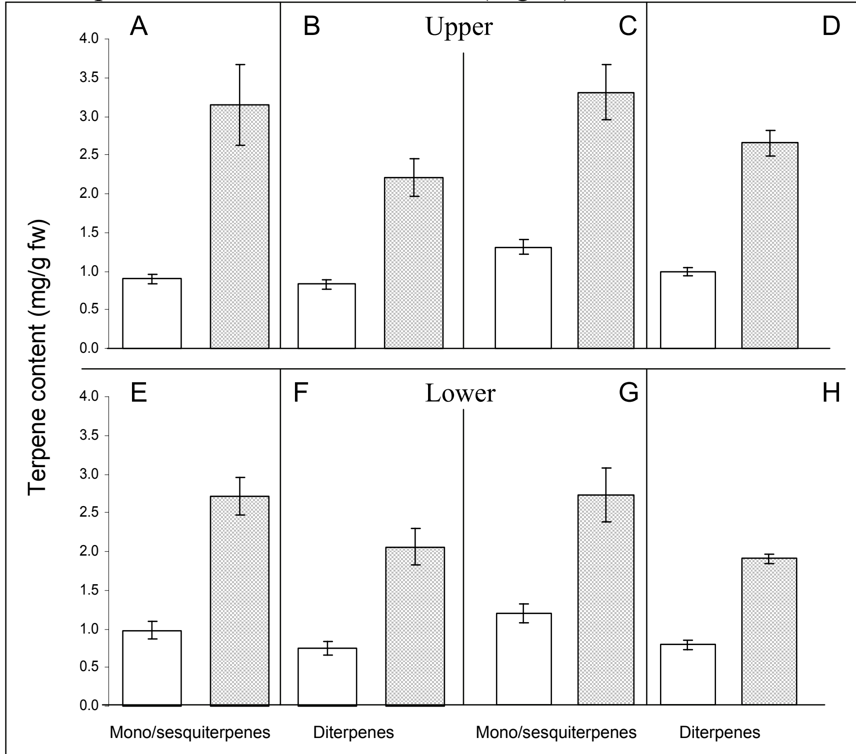

Fig. 1. Effect of methyl jasmonate (MJ) on terpenes of P. abies during the year of treatment (2003) at two different heights above the ground. Untreated controls = white bars; MJ treatment = gray bars. (A), (B), (E) and (F) = wood samples; (C), (D), (G) and (H) = bark samples.

This was true both for the year of MJ-application (2003) and the following year, but the response was much weaker in 2004, particularly in the bark. Total terpene concentration in bark was 2.5 fold higher after MJ-treatment in 2003 than in untreated portions of trees, but only 1.3 fold higher in 2004. In wood, the corresponding fold-differences were 3.0 and 2.1. There were no qualitative differences in terpene composition in MJ_C vs. MJ-treated bark or wood ($R^2 > 0.99$ for linear regression of percent composition of individual terpenes in MJ_C and MJ-treated tissues).

A total of 27 different terpenes were detected with the monoterpenes, α- and β-pinene and limonene, the sesquiterpene germacrene D, and the diterpenes, dehydroabietic acid, isopimaric acid and neoabietic acid, making up nearly three-quarters of the total terpenes. The total amount of terpenes was roughly the same in the bark and wood.

Methyl jasmonate (MJ) reduced Ips typographus colonization of Picea abies bark

After MJ treatment of portions of the bark surface of twelve 60-year-old *P. abies*, the attack of *I. typographus* was induced three weeks later by pheromone dispensers attached to the trees. Although one tree was mass-attacked and killed in 2003, attacks on the other 11 trees were all aborted. No tunnels extended more than 50 mm from the entrance hole in the eleven surviving trees and no oviposition took place; this allowed comparison of the number and length of attacks between MJ-treated and untreated bark without considering their success. The application of the pheromone was very effective and beetle attacks did not extend far from the pheromone dispenser. Nearly all were confined to the sampling area.

MJ-treated bark suffered much less bark beetle colonization than did untreated bark on the same tree (referred to as "MJ_C"), both at the lower and upper sampling positions (P= 0.0001-0.05). Treated bark had an average of 31 % fewer entrance holes and 69 % fewer galleries, and gallery length was 82 % shorter than in untreated bark. All of these differences were statistically significant. The difference between MJ_C and MJ-treated bark was significantly greater with regard to total gallery length (MJ_C:MJ ratio of 6.53) than to number of incipient galleries and entrance holes (MJ_C:MJ ratios of 2.40 and 1.66, respectively (F = 4.48, P = 0.015). This suggests that the negative impact of MJ increased as the beetles proceeded along the colonization sequence from first entry into the bark to sustained tunneling activity. Beetle colonization also varied significantly with sampling position, with more colonization on the lower position, which was closer to the pheromone source (F = 4.24-13.75, P = 0.0006-0.04 for the different colonization variables).

The anatomical and chemical changes induced by MJ application to *P. abies* in this study may be responsible for the increase in bark beetle resistance. MJ treatment has been suggested to increase resistance to herbivores and pathogens in previous studies in a similar manner (Franceschi et al., 2002; Martin et al., 2002; Hudgins and Franceschi, 2004, Zeneli et al., 2006). However, further investigation is required to confirm this conclusion since MJ could have also acted by increasing other chemical or physical defenses that were not measured. In the current study, MJ induced the formation of numerous traumatic resin ducts in the sapwood, and so can be assumed to have increased the volume of resin available for repelling biological invaders and sealing off wounds. An increase in traumatic ducts has been implicated in defense against *C. polonica*, a pathogenic fungal associate of *I. typographus* (Krokene et al., 2003; Zeneli et al., 2006).

Conclusions

This study demonstrates that MJ application to mature Norway spruce growing in a wild stand induces resistance against bark beetle attack in Norway spruce. Bark sections of *P. abies* treated with MJ had significantly less *I. typographus* colonization than control bark, with shorter parental galleries excavated and fewer eggs deposited. The numbers of beetles

that emerged and mean dry weight per beetle were also significantly lower in MJ-treated bark. The anatomical and chemical changes induced by MJ application to *Picea abies* in this study may be responsible for the increase in bark beetle resistance.

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SUPERCRITICAL FLUID EXTRACTION OF VALERIAN ROOT

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Summary

In this study supercritical carbon dioxide extraction from the root of three types of Valerian (Valeriana officinalis L) was investigated. Extractions were performed at temperatures of 313 and 323 K and pressures of 10 and 15 MPa. Effect of particle size on extraction yield was also investigated. The extracts were analyzed by analytical GC (FID) and GC-MS.

Keywords: *Valerian; Supercritical fluid extraction; Bornyl acetate; Valerenal; Isovaleric acid*

Introduction

Preparations on the basis of extracts of Valerian root (*Valeriana officinalis*, Valerianaceae) are used as mild sedatives for reduction of tension, nervousness and irritation, to help better dream and to prevent agitated sleep. In modern phytotherapy, this usage is based on the experience of traditional medicine (the root of valerian is used more then 2000 years in this purpose), as well as on the results of chemical, pharmacological and numerous clinical studies. The oil and absolute are also used as fragrance components in soaps and in moss and forest fragrances. It is used to flavor tobacco, root beer, liquors and apple flavorings [1]. Dried roots of valerian are sometimes packed in cloth bags and put around grain, fruits, berries, or vegetables, to fend off insects, rodents, and other damaging animals [2].
Drug *Valerianae radix* consists of dried underground parts according to European Pharmacopoeia should contain not less than 5 ml/kg of essential oil for the whole drug and not less than 3 ml/kg of essential oil for the cut drug, both calculated with reference to the dried drug and not less than 0,17 % of sesquiterpene acids expressed as valerenic acid. The essential oil of the Valerian root contains monoterpenes and sesquiterpenes, and after longer distillation low volatile sesquiterpenoides (valerenic, hydroxyvalerenic and acetoxyvalerenic acid). Fresh drug contains valepotriates, which are very unstable. Degradation of valepotriates leads to the formation of unsaturated iridoide aldehydes and corresponding acids, from which isovaleric one is responsible for specific unpleasant smell of older drug. Valerian root contains alkaloids and amino acids. In spite of many studies, mechanism of action of the extract of Valerian root is not completely explained. Activity is result of synergistic action of greater number of components [3-5]. Consequently, nowadays, valerian as one among 10 top-selling herbal supplements is considered to be highly respected medical plant species.
Hydrodistillation is a wide-spread method for production of essential oil of Valerian in industry and it has been studied extensively as well. Evan if the simplicity and low investment and operative costs of this method are taken into account, the fact that some of the high volatile and hydro soluble substances will be forever lost is undeniable. Alcohol extraction is often used for isolation of active components in order to preserve thermo labile and highly volatile compounds, but it requires organic solvent use. Moreover, severe legislative restrictions are currently being proposed to eliminate solvent residues in these products when used in the food, pharmaceutical and cosmetic industries. Some of these problems can be solved by employing supercritical fluid in essential oil extraction [6]. Although chemical composition of valerian oil obtain by hydrodistillation was studied

extensively, a little work has been published about isolation of active substances by supercritical fluid extraction (SFE) from valerian drug. This paper concerns with pressure, temperature and pretreatment influence on SFE yield, as well as with chemical composition of supercritical extracts.

Material and methods

Three sorts of valerian (*V. officinalis* L.) were analyzed: wild grown valerian from eastern Serbia (valerian I), wild grown valerian from central Serbia (valerian II) and cultivated sort (Arterner züctung) grown in northern Serbia (valerian III).
Extractions with SC CO_2 were carried out in the Autoclave Engineers Screening System shown in Fig. 1. The Supercritical Extraction Screening System is designed for small batch research runs using CO_2 as the supercritical medium with maximum allowable working pressure of 41.3 MPa at 511 K. Liquid CO_2 is supplied from CO_2 cylinder by a siphon tube. The CO_2 is pumped into the system by the liquid metering pump untill the required pressure is obtained. Back preassure regulators are used to set the system pressure (in extractor and separator). The extractor vessel (150 ml) is filled with the plant material from which a substance is to be extracted. Heaters are supplied on the extractor vessel for temperature elevation. The SC CO_2 flows through the extractor and enters the separator vessel. Samples of the extracted substance can be taken by opening the ball valve located at the bottom of the vessel. A flowmeter is provided to indicate the flow rate of CO_2 being passed through the system and the flow can be adjusted by micrometering valve. The CO_2 continues to flow out of the separator through the flowmeter/totalizer and out to atmosphere. Valerian root was fine milled and sieved to three fractions with average diameters: 0.4, 0.65 and 0.7 mm. Mass of the plant sample was 47g and the solvent flow rate was 0.3 kg/h in all experiments. Extractions were carried out at temperatures of 313 and 323 K and pressures of 10 and 15 MPa. The influence of particle size on SFE was investigated in the case of Valerian I, the influence of extraction conditions (T,P) was investigated in the case of Valerian II, and the SFE from the cultivated sort was performed at 313 K and 10 MPa.
Qualitative and quantutative analyses of the SFE extracts were carried out using a Hewlett-Packard GC (FID) and GC-MSD analytical systems. Model HP-5890 Series II, equipped with a split-splitless injector, HP-5 capillary column (25 m · 0.32 mm, film thickness 0.52 µm) and a flame ionization detector (FID), was employed. Hydrogen was used as carrier gas (1 ml/min). The injector was heated at 250°C, the detector at 300°C, while the column temperature was linearly programmed from 40-260°C (4°/min). GC-MSD analyses were carried out under the same analytical conditions. Model HP G 1800C Series II GCD analytical system equipped with HP-5MS column (30 m · 0.25 mm · 0.25 µm) was used. Helium was used as carrier gas. The transfer line (MSD) was heated at 260°C. The EIMS spectra (70 eV) was acquired in the scan mode in the m/z range 40-400. In each case, sample solution in hexane (1 µl) was injected in split mode (1:30).The identification of constituents was performed by matching their mass spectra and Kováts indices (I_K) with those obtained from authentic samples and/or NIST/Wiley spectra libraries, different types of search (PBM/NIST/AMDIS) and available literature data (Adams). Flame ionization detection area %, obtained by the integration of corresponding chromatograms, was used for quantification of individual components.

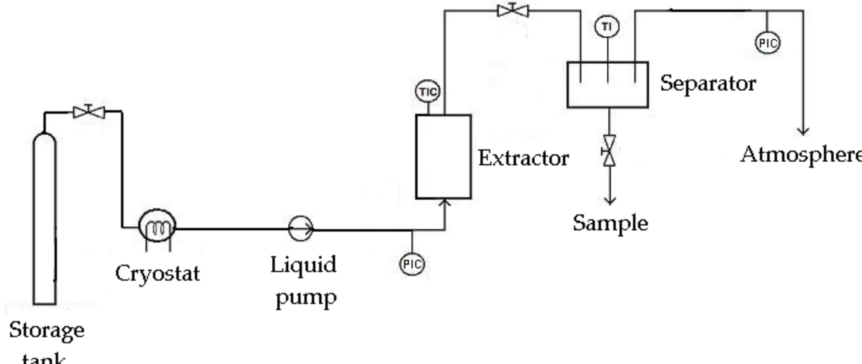

Fig. 1. Schematic presentation of The Autoclave Engineers Screening System

Results and discussion

The influence of particle size on extraction yield in the case of SFE from valerian I is presented in Fig. 2. The influence of SFE conditions (T, P) on extraction yield in the case of SFE from valerian II is presented in Fig. 3. SFE extract obtained at 10 MPa and 313 K yielded in the highest quantity of monoterpenes and sesquiterpenes. Therefore, SFE from cultivated sort (valerian III) was carried out at 313 K and 10 MPa with fraction of average particle size of 0.4 mm. Results of the analytical analyses of SFE extracts obtained at 313 K and 10 MPa are presented in Table 1. As can be seen valerenal, bornyl acetate and valerianol are dominant compounds. Supercritical extracts obtained from Valerian III contained much more isovaleric acid than wild grown sorts. Content of bornyl acetate was higher in extracts from Valerian II and III compared to Valerian I. The highest quantity of valerenal was detected in SFE extracts from Valerian III.

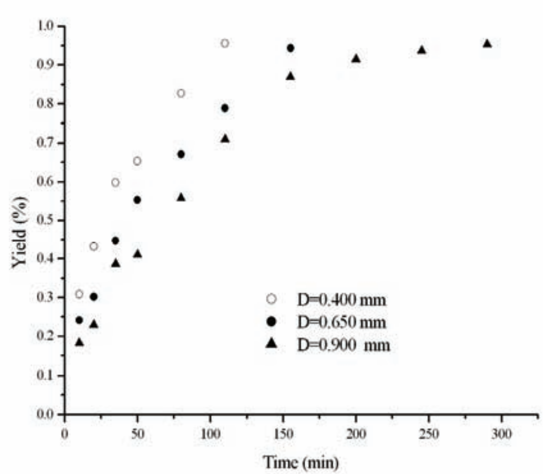

Fig. 2. Effect of particle size on extraction yield in the case of SFE from valerian I at 313 K and 10 MPa

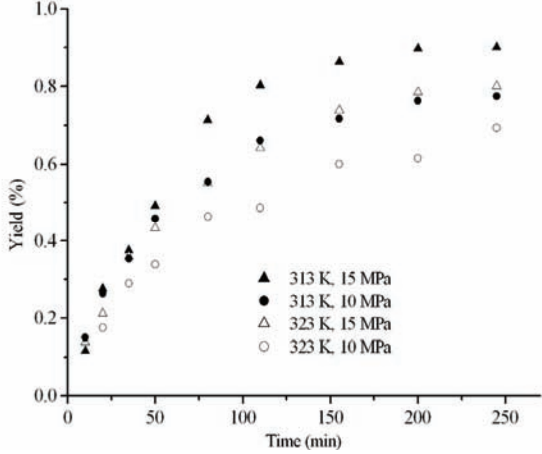

Fig. 3. Effect of SFE conditions on extraction yield in the case of SFE from valerian II

Table 1. Composition of valerian root SFE extract (%w/w) obtained at 313 K and 10 MPa ($I_{K,E}$ experimental values of Kováts indices, $I_{K,A}$ literature values of Kováts indices)

			Valerian I	Valerian II	Valerian III
	$I_{K,E}$	$I_{K,A}$	%	%	%
isovaleric acid	901,8	836	1,34	5,20	7,48
α-thujene	927,0	930		0,25	0,15
α-fenchene	940,9	953			0,14
p-menthone	1156,6	1153	0,37		0,21
borneol	1163,3	1169	4,80	1,02	0,15
terpinen-4-ol	1175,3	1177			0,19
myrtenol	1195,1	1196	0,30	0,32	0,17
bornyl acetate	1283,1	1289	4,57	7,15	6,86
menthyl acetate	1294,7	1295	0,41		
trans-pinocarvyl acetate	1296,3	1298	0,27		
carvacrol	1303,2	1299	0,37	0,41	2,16
myrtenyl acetate	1323,0	1327	0,34		1,08
δ-elemene	1333,8	1338	0,31	1,47	6,11
α-terpinyl acetate	1347,3	1349		0,22	0,14
pacifigorgia-1(9),10-dien	1383,8	1385	0,92	0,32	0,37
β-cubebene	1385,3	1390	0,27		
β-elemene	1388,0	1391		0,62	0,63
β-longipinene	1403,2	1403	0,27	0,23	0,26
pacifigorgia-1(6),10-diene	1410,2	1415	0,37		0,70
β-caryophyllene	1414,5	1419	0,45	0,64	1,35
2,5-dimetoxy-p-cymene	1421,5	1427	1,61	2,28	1,85
γ-elemene	1429,5	1437	0,38	1,16	1,72
α-humulene	1448,9	1455	0,34		0,49
valerena-4,7(11)-diene	1455,4	1456	2,97	2,06	4,26
γ-gurjunene	1472,2	1477	1,48	0,75	2,17
ar-curcumene	1479,2	1481	1,53	1,42	0,78
β-ionone	1483,3	1489	0,87	1,13	1,48
α-muurolene	1496,0	1500	0,44	1,00	0,44
β-bisabolene	1504,7	1506	0,77	1,46	2,27
bornyl isovalerate	1512,6	1512		0,65	0,16
δ-cadinene	1519,3	1523	0,33	0,27	0,21

kessane	1522,7	1533	1,16	1,06	0,52
pacifigorgiol	1540,4	1539	1,37	0,24	1,13
elemol	1545,7	1550	1,45	0,77	0,76
germacrene B	1554,4	1561	0,98	0,81	0,71
spathulenol	1573,4	1578	4,64	2,53	1,51
caryophyllene oxide	1578,7	1583	0,60	0,88	0,66
globulol	1588,0	1585	0,80	0,67	0,38
eudesm-5-en-11-ol	1589,9		0,46	0,53	0,25
viridiflorol	1597,2	1593		0,37	0,18
cis-α-copaene-8-ol	1624,3		7,93	10,08	2,77
isospathulenol	1633,8	1639	0,46	0,47	0,49
β-eudesmol	1645,0	1651	0,90	1,91	1,69
valerianol	1648,2	1652	2,47	11,29	1,37
α-cadinol	1653,7	1654	0,56	1,91	0,35
valeranone	1667,6	1675	0,44	0,92	0,48
α-bisabolol	1681,4	1686	0,67	0,70	0,26
kessyl alcohol	1690,2		0,32		0,17
valerenal	1715,7	1706	10,69	6,58	12,19
cis-nuciferol	1727,7	1727	0,34	0,37	
α-kessyl acetate	1743,9		0,56	0,38	0,16
8-acetoxyelemol isomer	1772,3		1,36	1,66	0,92
β-eudesmol acetate	1791,7	1792	2,93	3,01	
β-santalol acetate	1796,9	1800	1,37	1,28	2,08
kessanyl acetate	1806,7		9,65	6,13	3,67
(Z)-valerenyl acetate	1828,6			0,43	0,43
valerenic acid	1862,5	1843	3,09	1,78	0,75
hexadecanoic acid (palmitic acid)	1959,6	1951	0,34	0,42	1,59
(E)-valerenyl isovalerate	2052,1		1,54	0,88	2,32
hexadecyl isovalerate	2626,0		0,32	0,61	0,41
squalene	2795,3			0,41	0,49
Total			83,52	89,10	82,64

The cultivated valerian extracts were also characterized by the higher content of δ-elemene and lower contents of valerenic acid and borneol than the wild grown valerian extracts.

Conclusions

Higher extraction yield is obtained in SFE process from smaller particles, which indicates that the diffusion through the particle is the rate limiting process. SFE extract obtained at 10 MPa and 313 K yielded in the highest quantity of monoterpenes and sesquiterpenes. Valerenal, bornyl acetate and valerianol were dominant compounds in SFE extracts. The highest quantity of valerenal was detected in SFE extracts from Valerian III. Higher contents of valerenic acid were determined in extracts from wild grown species than in extracts from cultivated sort.

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SECTION II

TOPICS

- Phytochemistry
- Pharmacognosy
- Pharmacology, toxicology
- Quality control of map products
- Traditional medicine and map use

RESEARCHES CONCERNING THE IMPROVEMENT AND SIMPLIFICATION OF THE *TRITICUM* BIOASSAY

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Summary

Triticum bioassay could be improved and simplified by measuring not the absolute values of main radicle lengths, but the growth report, defined as the report between radicle lengths measured in two consecutive days and by limiting the test period to 3 days instead of 5 (however, a longer test period could be taken into account for lower concentrations, such as 0.3%).

Introduction

Biological assays or bioassays offer special advantages in the standardization and quality control of herbal products (usually heterogeneous), as well as in the activities of preliminary screening of various herbal products under investigation. Among the most used bioassays are: brine shrimp lethality test [1]; crown-gall potato disc bioassay (using *Agrobacterium tumefaciens* (Smith and Townsend) Conn) [2,3]; duckweed frond inhibition bioassay[4,5]; yellow fever mosquito bioassay [6]. Another class of bioassays often used in various science fields (plant physiology, ecology, preliminary screening in pharmaceutical research) are based on evaluation of the inhibition of germination or growth of plant embryonary radicles, using various monocotyledonous or dicotyledonous seeds (genotoxicity bioassays). Among these, the following have been used: *Triticum aestivum* L.[7,8]' *Vicia faba* L.[9,10] *Allium cepa* L.[9,10] *Pisum sativum* L.[11,12] *Brassica napus* L.[7], *Lactuca sativa* L.[13], *Eleusine coracana* (L.) Gaertn.[8], *Medicago sativa* L.[14], *Lepidium sativum* L., *Digitaria sanguinalis* L., *Pheleum pratense* L., *Lolium multiflorum* Lam.[15].
At the Faculty of Pharmacy in Bucharest there is a long tradition of a bioassay using wheat caryopses (*Triticum vulgare* or *Triticum aestivum*), bioassay known as phytobiological method Constantinescu, *Triticum* test. The classical test suppose the germination of wheat caryopses in Linhart vessels, the selection of a minimal number of caryopses with embryonary radicles of 1 cm for each solution and concentration tested, then measuring of the radicular elongation (length of main radicle) for each germinated caryopse, for 5 days. This working technique involves certain difficulties and disadvantages, especially the fact that there is a waiting period for the radicle to reach 1 cm (which could happen during daytime but equally in the most unfit hours of the night), that macroscopical measurements must be carried out for 5 days and that the inter-individual variability is relatively high. We have proposed therefore the improvement and simplification of this technique by measuring another parameter than radicle elongation and by shortening the study period to 3 days.

Materials and methods

Materials: Wheat caryopses (*Triticum vulgare* Mill, Dropia breed, kindly provided by Fundulea Institute for Agricultural Researches), Petri dishes, distilled water.
Method: The experiment used wheat caryopses germinated, arranged in 7 Petri dishes in a clockwise order (5 in each dish), so that each specimen may be uniquely and unmistakably

identified. All caryopses were simultaneously put in the dishes and all had main embryonary radicles of 1 cm long. The dishes were covered with the lid and then the karyopses were left in contact with the distilled water for 5 days. The dishes were kept in identical conditions of environment (temperature, light etc). Root elongation was evaluated for each caryopse at the same time for 5 days. Descriptive statistics were calculated through Microsoft Excel Data Analysis Pack module.

Results and discussions

The values of the main radicle length for the 35 wheat specimens in the 5 days (120 hours) are presented in Table 1.

Table 1. Main radicle length (expressed in cm) of the specimen assayed in 5 days

24h	**48h**	**72h**	**96h**	**120h**
3	6.1	9.5	11.9	13.8
2.9	5.5	8.9	13.3	13.6
2.4	4.5	5.3	9.2	11.2
2.6	4.8	7.1	10.6	13.3
2.8	5.9	8.3	10.8	11.9
2.7	4.4	7.2	10.2	11.7
2.6	5.2	8.3	11	13.7
2.4	4.5	7	9.5	11.7
2.7	5.5	8.9	12.2	14.8
2.4	4.5	7	8.5	10.3
2.2	4.9	7.8	11.6	13.4
2.8	4.9	8.1	11	12.7
2.6	4.6	7.2	10.4	13
2.9	5.9	8.6	11.8	14.5
2.3	4.4	5.3	5.9	7.2
2.6	5	8.3	11.6	14.1
2.5	3.6	6.1	9.3	11.8
2.3	4.4	6.9	9.1	11.3
2.6	5.2	8.2	11.1	11
2.6	5.3	7.9	8.2	8
2.3	4.5	7.1	11	12.8
2.8	5.2	7.9	11.1	13.8
2.2	4.5	7.7	11.1	13.2
2.3	4.7	8.1	11.7	13.4
2.8	5.9	9	11.6	13.4
2.2	4.3	6.5	9.3	11.8
2.1	3.9	6	8.7	10.3
2.7	4.9	7.5	9.8	11.9
2.6	5.2	8.3	11.2	13.9
2.3	4.5	6.8	8.9	11.1
3.1	5.9	9	12.1	12.9

2.4	5.1	7.5	10.5	13.1
2.6	4.5	7.3	10	12.3
2.6	4.5	7.2	8.7	10.4
3	6.1	9	11.3	13.1
Average				
2.6	4.9	7.6	10.4	12.3
Relative standard deviation (RSD)				
10.3%	12.7%	13.6%	14%	13.7%

The values measured show that radicle grothw is linearily time-dependent (the regression equation being: $L = 0.10375t+0.09$, where L is main radicle length and t is time expressed in hours).

Triticum test assumes that all caryopses have the same biological characteristics and behave in the same manner regarding radicle development. Therefore, when using only distilled water as a medium (instead of a test solution), as is the case for the control group, main radicle lengths for all specimens should have the same value. Because this would happen only ideally, and in reality caryopses have slightly different biological characterisics, main radicle lengths shows a certain variability, which for comparison purposes is better measured by the relative standard deviation (RSD) than the (absolute) standard deviation (SD). As Table 1 suggests, RSD had values within the range 10%-15%, increasing slightly as the time passed and the radicle length increased. But RSD values seem to have stabilized from third day somewhere around 14%.

We have thought to try using a different parameter than absolute radicle length to study the influence of various solutions on radicle growth, namely the arithmetical report between main radicle lengths in each two consecutive days. The calculated values for this report in our experiment are shown in Table 2.

Table 2. Values of the proposed report between main radicle lengths in each two consecutive days

48h/24h	72h/48h	96h/72h	120h/96h
2.033333	1.557377	1.252632	1.159664
1.896552	1.618182	1.494382	1.022556
1.875000	1.177778	1.735849	1.217391
1.846154	1.479167	1.492958	1.254717
2.107143	1.406780	1.301205	1.101852
1.629630	1.636364	1.416667	1.147059
2.000000	1.596154	1.325301	1.245455
1.875000	1.555556	1.357143	1.231579
2.037037	1.618182	1.370787	1.213115
1.875000	1.555556	1.214286	1.211765
2.227272	1.591837	1.487179	1.155172
1.750000	1.653061	1.358025	1.154545
1.769231	1.565217	1.444444	1.250000
2.034483	1.457627	1.372093	1.228814
1.913044	1.204545	1.113208	1.220339
1.923077	1.660000	1.397590	1.215517
1.440000	1.694444	1.524590	1.268817

1.913043	1.568182	1.318841	1.241758
2.000000	1.576923	1.353659	0.990991
2.038462	1.490566	1.037975	0.975610
1.956522	1.577778	1.549296	1.163636
1.857143	1.519231	1.405063	1.243243
2.045454	1.711111	1.441558	1.189189
2.043478	1.723404	1.444444	1.145299
2.107143	1.525424	1.288889	1.155172
1.954545	1.511628	1.430769	1.268817
1.857143	1.538462	1.450000	1.183908
1.814815	1.530612	1.306667	1.214286
2.000000	1.596154	1.349398	1.241071
1.956522	1.511111	1.308824	1.247191
1.903226	1.525424	1.344444	1.066116
2.125000	1.470588	1.400000	1.247619
1.730769	1.622222	1.369863	1.230000
1.730769	1.600000	1.208333	1.195402
2.033333	1.475410	1.255556	1.159292
Average			
1.9	1.5	1.4	1.2
RSD			
8%	7.4%	9.2%	6.4%

As shown by Table 2, the report betwee main radicle lengths in two consecutive days is rather constant; in all days its values show less variability than the absolute values of radicle lengths, as proved by comparing RSD values for same days (8% as compared with 12.7% for 2nd day; 7.4% as compared with 13.6% for the 3rd day; 9.2% as compared with 14% for the 4th day; and 6.4% as compared with 13.7% for the 5th day). This report expresses in fact how longer is the main radicle in the following day as compared with the previous one; for this reason and for brevity purposes, it could be named a *growth report*.

The use of the proposed growth report in *Triticum* test has two advantages: its values are more consistent between themselves (lower variability) and therefore ensure more accurate results on the one hand, and do not make measurements dependent on identical starting values of radicle lengths (1 cm as usually done) on the other hand. But it requires ensuring that each individual caryopse is unmistakebly identified and measured within the test period, which may involve using Petri dishes with a higher diameter or using a lower number of caryopses (this latter option is not desirable).

In a previously published paper [16] we proposed the use of an Inhibition Index (II), in order to quantify the growth inhibitory effect of a test solution on wheat embryonary roots. In that paper we calculated the inhibition index for a series of test solutions (infusions) at various concentrations for the 5 days as required by the classical protocol. In table 3 are presented the values for several test solutions obtained from *Cuscuta campestris* Yunck. (noted CH, CI, CP_2001 and CP_2003).

Table 3. Inhibition Index values for 4 test solutions (obtained from *Cuscuta campestris* Yunck.)

CH					
Zi/Conc	5%	3.3%	2.5%	1.7%	0.3%
Ziua 1	100%	92.31%	87.18%	8.33%	-35.26%
Ziua 2	100%	95.52%	86.87%	11.34%	-45.37%
Ziua 3	100%	96.62%	87.62%	22.51%	-35.46%
Ziua 4	100%	94.38%	86.08%	21.69%	-27.04%
Ziua 5	100%	93.76%	83.24%	21.04%	-27.05%
CI					
Zi/Conc	5%	3.3%	2.5%	1.7%	0.3%
Ziua 1	100%	99.36%	82.05%	19.23%	-1.92%
Ziua 2	100%	98.51%	92.24%	14.93%	-25.67%
Ziua 3	100%	98.12%	92.68%	17.26%	-18.39%
Ziua 4	100%	97.86%	86.21%	16.33%	-12.58%
Ziua 5	100%	97.23%	80.92%	15.61%	-12.58%
Cp_2001					
Zi/Conc	5%	3.3%	2.5%	1.7%	0.3%
Ziua 1	100%	98.72%	92.31%	67.31%	-9.62%
Ziua 2	100%	98.21%	94.93%	72.24%	-22.99%
Ziua 3	100%	98.31%	95.50%	66.79%	-10.51%
Ziua 4	100%	98.40%	94.51%	68.14%	-7.36%
Ziua 5	100%	98.61%	93.06%	49.83%	-6.94%
Cp_2003					
Zi/Conc	5%	3.3%	2.5%	1.7%	0.3%
Ziua 1	100%	100%	90.38%	42.95%	+23.72%
Ziua 2	100%	98.51%	94.33%	49.25%	+9.85%
Ziua 3	100%	98.50%	95.31%	46.72%	+2.44%
Ziua 4	100%	99.33%	95.18%	44.98%	+7.09%
Ziua 5	100%	99.08%	95.03%	42.20%	+9.02%

From table 3, it may be seen that in the classical experimental design, except for the 0.3% concentration, inhibition index values calculated in the third day are very close to those calculated in days 4 and 5. In the case of 0.3% concentration, there is a high variability in all 5 days. Therefore these data support our proposal that *Triticum* test may be performed by measuring radicle length only in the first three days, because the last two days do not provide relevant additional information (although days 4 and 5 had different values for the 0.3%, all values at this concentration have a high variability and their relevance is difficult to estimate). Anyway, for a scrupulously performed bioassay, the measurements could be continued in days 4 an 5 only for this lower concentration.

Conclusions

Triticum bioassay could be improved and simplified by measuring not the absolute values of main radicle lengths, but the *growth report*, defined as the report between radicle lengths measured in two consecutive days and by limiting the test period to 3 days instead of 5 (however, a longer test period could be taken into account for lower concentrations, such as 0.3%).

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ANTIMICROBIAL ACTIVITY OF ESSENTIAL OIL OF *HELICHRYSUM ITALICUM* L., *ASTERACEAE*

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Summary

Antimicrobial activity of essential oil of Helichrysum italicum, originated from plant material collected from three localities in Serbia, Dalmatia and Herzegovina was tested. The oils were obtained by hydrodistillation and analyzed by GC and GC/MS. The antimicrobial properties of essential oil of Helichrysum italicum from Serbia were tested against 16 different bacterial species, fungi Candida albicans and yeasts Aspergillus niger and Alternaria sp. The diffusion technique was used for testing the antimicrobial activity, and the MIC was determined by broth dilution method. The essential oil of H. italicum showed high antimicrobial activity.

Keywords: *Helichrysum italicum, essential oil, antimicrobial activity, diffusion technique, MIC.*

Introduction

Helichrysum italicum is a perennial herb grows wild in Mediterrannean regions. It can be found in regions with dry high altitudes with a lot of sun expossure. The flowers heads in the phase of blooming are considered to be the plant parts usually used for medicinal treatment. The word *Helichrysum* is derived from the Greak word 'helios' which means sun and 'chrysos' meaning gold. *Helichrysum* has been used since the time of the ancient Greeks for its great wound healing ability and was used often after battles. Common names for *Helichrysum italicum* are Immortelle and Everlasting and both names are commonly applied to the many varieties of *Helichrysum*. The whole plant is overcast with white-wooly hairs. This highly aromatic plant resembles the aroma of curry – used sparingly as a culinary spice. More significantly, this is the verified and primary source of a rather famous, expensive oil widely used in perfumery and aromatherapy. *Helichrysum* flowers often included simply to improve the appearance of various industrially – prepared herbal teas, because of their bright yellow and daysy-like in appearance [1,2]. The herb contains flavonoids (about 0.4%): isosalipurposide (a chalcone, responsible for the yellow colour of the involucral bracts), naringenin and its 5-O-diglucoside, the C(2) diastereoizomeric naringenin 5-O-glucosides helichrysin A and B (B = salipurposide), kaempferol glucosides, apigenin and its 7-O-glucoside, 3,5-dihydroxy-6,7,8-trimethoxyflavone; essential oil (about 0.4%); phthalides; bitter substances (sesquiterpene lactones); tannins; resins; small amounts of scopoletin, umbelliferone, and aesculetin; the yellow-coloured pyranone derivatives arenol and homoarenol; a so far unidentified complex of antibiotic substances known as arenarin; campesterol and β-sitosterol glucuronic acid; tannins. The flowers contain antibacterial constituents (arenarin) which are also said to promote gastric and pancreatic secretions; this may be due to the effect of the bitter substances (possibly sesquiterpene lactones) [3]. *H. italicum* is a mildly choleretic and mildly spasmolytic. It is used as an adjunct in the treatment of chronic cholecystitis and gallbladder complaints with accompanying cramps. In folk medicine, it is used as a diuretic and for jaundice, gout, rheumatism, kidney complaints and dropsy [4-6]. *Helichrysum* oil is more anti-inflammatory than *Chamomile*, more tissue regenerating than *Lavender*, more cicatrisant (helping the formation of scar tissue) than *Frankincense*. Its anti-infammatory and tissue regenerating properties also help repair swollen or weak veins. This oil is used for healing scar tissue, whether from a recent wound,

operation, or tissue that has been scarred for years. It stops bleeding, helps a scab to form and tissue to repair itself. It also has remarkable effects on sensitive and inflamed skin. It is well known antibacterial and antifungal activity of *H. italicum* essential oil against *Staphylococcus aureus, Escherichia coli, Pseudomonas aerugenosa* and *Candida albicans* [7-8]. The higher the concentrations of nerol and its esters (acetate and propionate), geraniol, eugenol, *β*-pinene and furfurole in the essential oil determining greater antimicrobial effects [9]. The main hydrocarbons compounds of the essential oil of *Helichrysum italicum* are α-pinen, *α*-cedrene, ar-curcumene, *β*-caryophyllene and limonen, while the main oxygen-containing compounds are neryl acetate and geranyl acetate, linalool, geraniol, nerol [10].

Materials and methods

Essential oil

Plant material was collected from three different locations (Serbia, Dalmatia, Herzegovina). The essential oils were obtained by hydrodistillation and analyzed by GC and GC/MS using FID and MSD.

GC and GC/MS

Qualitative and quantitative analyses of the essential oils were carried out gas chromatography, using a flame ionisation detector (FID) and mass spectrometry detector (MSD). GC analyse was carried out using a Hewlett-Packard HP-5890 Series II gas chromatograph, with a *split-splitless* injector, capillary column with HP-5 stationary phase (25 m x 0.32 mm; film thickness 0.52 µm) and flame ionisation detector (FID). Solution of essential oil and etanol was injected in split mode (split ratio 1:30). The temperature in injector was 250°C, detector 300°C, while the temperature programs were 40-240°C at a rate 4°/min. GC/MSD analyse was carried out using a HP G-1800C Series II GCD with HP-5MS capillary column (30 m x 0.25 mm; film thickness 0.25 µm) at the same regime. Helium was used as a carrier gas. Electron impact mass spectra (70 eV) were acquired in m/z range of 40-400. The identification of individual compounds was based on comparison of their relative retention times with those of authentic samples. For the components, mostly sesquiterpenes and aliphatic compounds, for which reference substances were not available, the identification was performed by matching their retention indices and mass spectra with those obtained from authentic samples and/or the NIST/NBS, Wiley libraries spectra and literature data (Adams).

Determination of antimicrobial activity

Test organisms were different species enteric pathogens bacteria : *Escherichia coli ATCC 25922, Klebsiella sp. ,Salmonella enteritidis ATCC 13076, Shigella sp., Proteus vulgaris ATCC 4307,* potential pathogens bacteria : *Pseudomonas aerugenosa ,*species ATCC 27833, DV 2739, DV 2769, DV 5999, *Staphylococcus aureus* , species ATCC 25923, DV 2678, ATCC 6538, VMA, *Corynobacterium sp.,* sporogens bacteria : *Bacillus subtilis* ATCC 6633, *Bacillus cereus,* not pathogens bacteria : *Bifidobacterium sp., Lactobacillus fermentum, Lactobacillus acidophilus* ATCC 4357, *Lactobacillus plantarum, Lactobacillus rhamnosus,* pathogens isolated from food *Listeria monocytogenes IM 2000,* yeasts: *Aspergillus niger,Alternaria sp.,* pathogenic fungi: *Candida albicans.* All of the test organisms were from the collection of the microbiology laboratory of the Faculty of the Technology and Metallurgy and from the Institute for Immunology and Virusology Torlak-Belgrade.

For determination of antimicrobial activity of essential oil from Serbia (sample HI-1) the aromatogram or well diffusion method and the broth dilution method for minimal inhibitory concentration (MIC) were used.

The aromatogram or well diffusion method: Antimicrobial tests were carried out by using suspension containing overnight culture of bacteria (~10^8 CFU/mL), yeast (~10^7 CFU/mL), and fungi (~10^4 spore/mL). The cultures were added in soft nutrient agar (NA – Torlak) for

bacteria and soft sabouraud maltose agar (SMA – Torlak) medium for yeast and fungi. The 6 ml of soft agar inoculated with 200 mL of the first dilute of the indicator culture were poured over corresponding Petri plates with previously placed tubes (7 mm diameter). After solidification of the soft agar (15 min), the tubes were removed and the obtained wells were filled with 20 μl of samples (essential oil HI-1 and ethanol). The incubation was carried out at 37 °C for the bacteria and 30 °C for the yeast and fungi. After 24 – 48 h of incubation, antimicrobial activity was evaluated by measuring the width of zone of inhibition (clear) or suppression (diffuse) of growth against the indicator organisms in comparison to a control of reference standards.

The broth dilution method for minimal inhibitory concentration (MIC) determination: The serial dilution of the tested oil samples was prepared in test tubes with 5 ml of TSB (tryptic soy broth) medium (Torlak) with 0.01% Tween 80 and 0.6% yeast extract (Torlak). The tubes were inoculated with indicator microorganisms (adjusted to about 10^6 CFU/mL) and incubated at 37 °C for the bacteria and 30 °C for the yeast and fungi. After 24 h of incubation, the results of the MIC were determined as the first tube without turbidity caused by the growth of microorganisms, but with evident growth on an agar plate without oil.
Each assay in this experiment was repeated twice.

Results and discussion

Results of qualitative and quantitative analyses of the essential oils are shown in Table 1.

Table 1. Chemical composition of the essential oil *H. italicum*

No.	Components	RI	HI-1 (%)	HI-2 (%)	HI-3 (%)
1	cyclofenchene	890	14.20	7.33	0.34
2	camphene	951	0.16	0.11	*
3	n-butyl-2-methylbutyrate	995	0.08	0.06	*
4	4-methylanisole	1012	0.01	*	*
5	cymene	1016	0.54	0.42	0.12
6	sylvestrene	1019	1.46	1.04	0.13
7	1,8-cineole	1022	0.53	0.26	0.22
8	n-octanol	1066	0.01	*	0.01
9	α-pinene oxide	1089	0.18	0.07	*
10	linalool	1093	0.49	0.50	0.38
11	cis-α-thujone	1097	0.76	*	*
12	endo-fenchol	1105	0.10	0.07	0.05
13	trans-β-thujone	1108	0.03	*	*
14	α-campholenal	1118	0.09	0.02	0.02
15	trans-pinocarveol	1130	0.20	0.11	0.14
16	camphor	1135	0.21	*	*
17	cis-verbenol	1137	0.31	0.10	*
18	borneol	1158	0.13	0.14	0.12
19	4-terpineol	1169	0.13	0.32	0.26
20	α-terpineol	1183	0.26	0.47	0.51
21	decanal	1198	*	*	0.07
22	verbenone	1202	0.07	0.02	*
23	trans-carveol	1212	0.06	0.03	0.02
24	nerol	1222	0.08	0.20	0.28
25	2-methylhexyl butyrate	1230	0.03	0.03	0.01

26	pelargonic acid	1275	*	*	0.11
27	bornyl acetate	1279	0.03	*	*
28	thymol	1290	0.21	*	0.01
29	carvacrol	1300	0.02	0.05	0.03
30	neryl acetate	1359	3.23	4.71	3.42
31	α-ylangene	1367	3.15	2.18	1.23
32	n-hexyl hexanoate	1379	0.02	0.01	*
33	isoitalicene	1390	0.14	0.10	0.06
34	cedrene	1394	3.63	2.85	1.69
35	α-farnesene	1406	0.73	0.64	0.39
36	isocaryophyllene	1410	1.25	0.04	0.76
37	β-cariofillen	1411	*	2.99	*
38	α-bergamotene	1428	0.66	0.66	0.36
39	α-humulene	1446	0.06	0.11	0.03
40	neryl propionate	1449	0.66	0.44	0.51
41	seychellene	1453	0.16	0.14	*
42	α-patchoulene	1454	*	0.15	0.06
43	α-acoradiene	1461	0.21	0.19	0.10
44	β-chamigrene	1464	1.35	1.25	0.53
45	γ-muurolene	1476	0.27	0.15	0.48
46	γ-curcumene	1476	*	1.68	*
47	ar-curcumene	1481	9.88	8.39	3.01
48	β-selinene	1483	4.60	4.88	5.89
49	α-selinene	1492	2.62	2.71	1.64
50	α-muurolene	1497	0.22	0.28	0.14
51	β-bisabolene	1505	0.08	0.07	0.07
52	γ-cadinene	1511	0.25	0.30	*
53	δ-cadinene	1521	0.32	0.75	0.62
54	6,11-oxido-acor-4-en	1532	*	0.08	0.52
55	α-cadinene	1535	*	0.04	0.02
56	α-calacorene	1540	0.05	0.16	0.16
57	nerolidol	1563	*	0.12	0.30
58	caryophyllene oxide	1581	3.54	1.65	0.13
59	lauric acid	1574	*	*	0.56
60	guaoil	1597	*	0.17	0.34
61	11-eudesmol-5-en	1606	0.06	0.31	0.78
62	γ-eudesmol	1631	*	0.05	0.15
63	cadinol	1641	*	0.12	0.44
64	β-eudesmol	1650	*	0.13	1.05
65	4-eudesm-11-en	1655	0.06	0.22	1.30
66	α-cadinol	1656	*	*	1.22
67	α-bisabolol	1685	*	*	0.29
68	xanthorrhizol	1754	0.03	0.04	0.72
69	hexahydrofarnesyl acetone	1844	*	*	0.21

HI-1 essential oil from Serbia, HI-2 essential oil from Dalmatia, HI-3 essential oil from Herzegovina

The main components of the oil from Serbia were cyclofenchene (14,20%), ar-curcumene (9,88%), β-selinene (4,60%), cedrene (3.63%), caryophyllene oxide (3,54%) and neryl acetate

(3,23%). The major components of the oil from Dalmatia were ar-curcumene (8,39%), cyclofenchene (7,33%) and *β*-selinene (4,88%), while the oil from Herzegovina contained *β*-selinene (5,89%), neryl acetate (3,42%) and ar-curcumene (3,01%), as the most abundant constituents.

Results of testing antimicrobial activity

The diffusion technique was used for testing the antimicrobial activity, and the MIC was determined by broth dilution method. Results of testing antimicrobial activity of the essential oil *Helichrysum italicum* from Serbia (sample HI-1) against bacteria, fungi and yeasts are shown in Table 2. and Table 3.

Table 2**.** Antimicrobial activity of the essential oil *Helichrysum italicum* HI-1

Indicator strain	Width of inhibition zones (mm)		
	H. italicum(HI-1)		Ethanol
	I	SG	
Bacteria			
B. cereus	∗		∗
B. subtilis	0.5		∗
E. coli ATCC 25922	∗		∗
E. coli TMF	∗	1.5	∗
P. aerugenosa DV 5999	0.5		∗
P. aerugenosa ATCC 27833	∗		∗
P. vulgaris ATCC 4307	∗		∗
S. aureus ATCC 25923	8		4
S. aureus ATCC 6538	0.5		2.5 RR
S. aureus DV 2678	1.5		∗
S. aureus VMA	1.5	2.5	∗
S. enetritidis ATCC 13076	∗		∗
Shigella sp.	∗		∗
Corynobacterium sp.	∗		∗
Klebsiella sp.	∗		∗
Bifidobacterium sp.	∗		∗
L. acidophilus ATCC 4357	∗		∗
L. fermentum	∗		∗
L. plantarum	0.5		∗
L. rhamnosus	∗		∗
Listeria monocytogenes	∗		∗
P. aerugenosa DV 2739	∗		∗
P. aerugenosa DV2769	∗		∗
Yeasts			
Candida albicans	∗		∗
Fungi			
A. niger	∗	2	∗
Alternaria sp.	∗	∗	∗

I- inhibition, SG – suppression of growth, ∗ - without zones of inhibition

Table 3. Minimal inhibiting concenration (MIC) of the essential oil *H. italicum*

Indikator strain	MIC (µl/ml)
Bakterie	
Listeria monocytogenes IM 2000	2.25
Pseudomonas aerugenosa ATCC 27833	1.5
Pseudomonas aerugenosa DV 5999	1.5
Staphylococcus aureus ATCC 25923	1.5
Staphylococcus aureus ATCC 6538	4.25
Staphylococcus aureus DV 2678	2.25
Yeasts	
Candida albicans IIVT	5
Fungi	
Aspergillus niger	2.25
Alternaria sp.	2.5

The highest sensitivity to essential oil of *H.italicum* was observed by *Pseudomonas aerugenosa* ATCC 27833, *Pseudomonas aerugenosa* DV 5999 and *Staphylococcus aureus* ATCC 25923 (MIC=1.5 µl/ml). The lowest sensitivity to essential oil of *H.italicum* was observed by *Candida albicans* (MIC=5.0 µl/ml) and *Staphylococcus aureus* ATCC 6538 (MIC=4.25 µl/ml)

Conclusion

Chemical analysis of the essential oils showed significant qualitative and quantitative differences between tested samples. Similarity in chemical composition of the essential oils is the biggest between oil samples from Serbia and Dalmatia. The essential oil of *H. italicum* showed range in antimicrobial activity from 1.5 µl/ml to 5.0 µl/ml. The highest sensitivity to essential oil of *H.italicum* was observed by *Pseudomonas aerugenosa* ATCC 27833, *Pseudomonas aerugenosa* DV 5999 and *Staphylococcus aureus* ATCC 25923 (MIC=1.5 µl/ml). The lowest sensitivity to essential oil of *H.italicum* was observed by *Candida albicans* (MIC=5.0 µl/ml) and *Staphylococcus aureus* ATCC 6538 (MIC=4.25 µl/ml). On the basis of the obtained results it may be concluded that essential oil of *H. italicum* showed significant antimicrobial activity. The obtained results confirm the justification of essential oil of *H.italicum* application in traditional medical treatment and show that besides above mentioned therapeutic effects, also has the antibacterial properties.

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THE POTASSIUM / SODIUM RATIO OF MEDICINAL SPECIES

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Summary

An adequate K: Na ratio (surpassing 100:1) in medicinal plants is considered to contribute, beside the content in various saponins and flavonoids, to their diuretic-aquaretic effect. The present study assesses this ratio in vegetal products originating from 56 medicinal species used in modern and traditional phytotherapy, and points out some products able to complete the recommended daily intake of K. Among them are situated: Malvae folium cum flos, Taraxaci herba, Violae tricoloris herba, Sambuci flos, Althaeae folium and Rubi idaei folium.

Keywords: *K/Na ratio, medicinal plants, K supplementation, ICP-AES*

Introduction

The presence of a high K content in some medicinal plants (MPs) contributes, in synergy with various saponins and flavonoids, to their diuretic – or more precisely: aquaretic effect, as K leads through osmotic mechanisms to the increased elimination of urine, without having a veritable diuretic effect [1]. The enhancement of this effect is significantly influenced by the K/Na ratio, which has to be elevated [2]. An optimum ratio is considered to be above 100:1 while a medium ratio is situated between 100:1 and 40:1 [3]. In this concept, we have proceeded to the determiantion of the K to Na ratio in vegetal products currently employed in modern and traditional Romanian phytotherapy.

The benefit of the presence of K in MPs and their aqueous extracts goes however beyond the diuretic-aquaretic effect, as this element is involved in neuro-muscular excitability, cardiac electrophysiology, glucidic metbolism, energogenesis, maintention of intracellular osmolarity and represents a cofactor for several enzymes [4]. Given the major importance of K, it is vital that its level be adequate; still, recent researches outlined that aprox. 10% of the population has a marginal deficiency in this element [5]. This situation motivated us to points out some MPs able to complete the recommended daily K intake.

Material and methods

Plant material. Eight samples of each analyzed vegetal product were gathered from the wild Romanian flora. Voucher specimens were deposited in the Herbarium of the Faculty of Pharmacy, Timisoara.

Analysis of K and Na content. The concentration of K and Na was assessed by Inductively Coupled Plasma - Atomic Emission Spectrometry (ICP-AES). Samples of 2-3 g herb were heated to 105˚C in order to establish the dry mass. Matrix destruction was done after heating at 550˚C for 6 hours, until the plant material was transformed into white ash. The ash was dissolved after boiling in hydrochloric acid 10%, followed by the quantitative transfer of the content in a test-tube. The acid solution was made up with bidistilled water to 25 mL, filtrated and measured by ICP-AES without further dilution. The apparatus employed was IRIS Interprid II ICP-AES (Thermo Electron, Dreieich, Germany). The parameters of the measurement were as follows: power 1150 W; frequency 27.12 MHz; nebulizing pressure - 2.6 bar; pumping rate 1.8 L/min; number of measurements - 3; reading wavelengths: K 766.4; Na 589.5.

As a first step, a semi-quantitative analysis was performed, allowing the estimation of the concentration ranges in the digestion solutions of the plant materials. The quantitative determinations were carried out based on a calibration curve established with ICP Multi Element Standard Solution XXI CertiPUR Merck, diluted to obtain optimal measurement range. **Accuracy of the data** has been verified by a parallel analysis of two certified reference materials: Peach Leaves 1547 and Oriental Tobacco Leaves CTA-OTL-1.

Results and discussions

The analysis of the K content in medicinal plants reveals that the aerial parts (*herba*) accumulate the most important K quantities, their average K content being 21.6 g/kg. However, the differences in K content of herba, flowers and leaves are not statistically significant (p>0.05). Plant parts like *Chelidoni herba, Lycopi herba, Taraxaci harba* and *Violae tricoloris herba* are rich in the considered element (surpassing 30 g K/kg dry matter), while *Hyperici herba, Anthyllidis vulnerariae herba* and *Genistae tinctoriae herba* have the lowest K content (beneath 10 g/kg) (Fig.1). The flowers also contain high K amounts, with an average of 19.2 g/kg (Table 1). *Sambuci flos* stand out due to an elevated K content (39 g/kg), while *Tiliae flos* contain relatively little potassium. In the category of *folium* products, there are several examples with high K concentration; interestingly two *Malvaceae* species are situated on the top: *Malva sylvestris* and *Althaea officinalis*, not known to be K-rich. Subterranean parts and fruits contain the lowest K amounts.

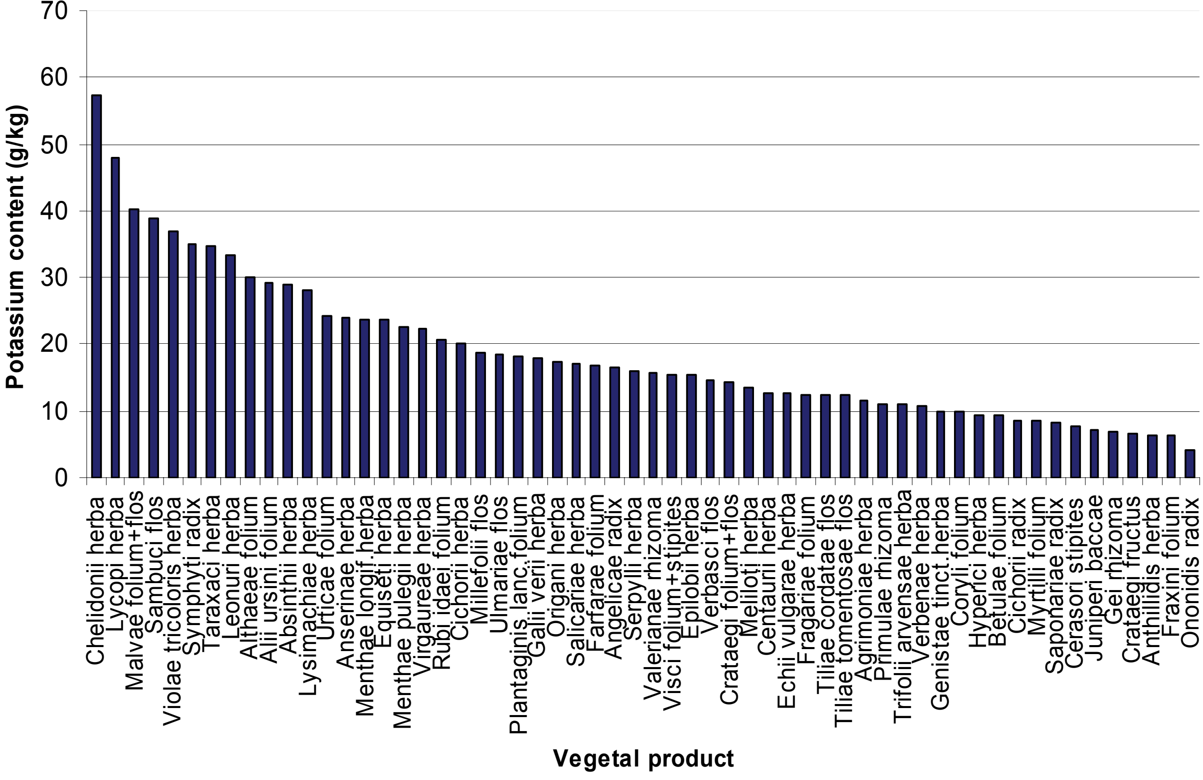

Fig. 1. The potassium content of some medicinal vegetal products

The K content of the products investigated in this study is similar to data obtained by other researchers who investigated medicinal plants [2], but significantly higher than that of various other products gathered from the wild flora (1-6 g/kg) [6].

The analysis of 56 medicinal species in our study reveals a series of MP products with high K content (above 3 g/kg), that could be employed for K supplementation. Making a selection

based on their availability, lack of toxic/very active constituents and also acceptable taste, products like *Malvae folium cum flos, Sambuci flos, Violae tricoloris herba, Taraxaci herba, Althaeae folium, Alii ursini herba, Urticae folium, Equiseti herba, Virgaureae herba and Rubi idaei folium* can be consumed in larger amounts without posing dangers to health. They can be employed either as herbal "teas", given the high extraction ratio of K through infusion and decoction [7], as salads (dandelion, wood garlic, nettle) or as encapsulated plant powder.

Table 1. The sodium and potassium content of medicinal plants (mg/kg dry matter)*

Species	**Na (mg/kg)**	**K (mg/kg)**	**K/Na**	**Species**	**Na (mg/kg)**	**K (mg/kg)**	**K/Na**
Angelica archangelica	282	16383	58	*Primula officinalis*	643	10954	17
Cichorium intybus	795	8602	11	*Saponaria officinalis*	217	8157	38
Geum urbanum	225	6788	30	*Symphytum officinale*	603	35120	58
Ononis spinosa	142	3954	28	*Valeriana officinalis*	252	15572	62
ROOTS AND RHIZOMES AVERAGE Na: 395 ± 256; K: 13191±9815; K/Na: 38±20							
Agrimonia eupatoria	26	11492	442	*Lysimachia nummularia*	42	28200	671
Anthyllis vulneraria	53	6414	121	*Lythrum salicaria*	90	17018	189
Artemisia absinthium	61	28875	473	*Melilotus officinalis*	157	13537	86
Centaurium erythraea	45	12810	285	*Mentha longifolia*	26	23660	910
Chelidonium majus	84	57233	681	*Mentha pulegium*	28	22567	806
Cichorium intybus	157	20173	128	*Origanum vulgare*	55	17302	315
Echium vulgare	23	12654	550	*Potentilla anserina*	116	23970	207
Epilobium parviflora	118	15310	130	*Solidago virgaurea*	102	22231	218
Equisetum arvense	84	23572	281	*Taraxacum officinale*	282	34809	123
Galium verum	79	17877	226	*Thymus pulegioides*	45	16013	356
Genista tinctoria	12	9810	818	*Trifolium arvense*	68	10990	162
Hypericum perforatum	40	9514	238	*Verbena officinalis*	85	10710	126
Leonurus cardiaca	88	33400	380	*Viola tricolor*	102	36823	361
Lycopus europaeus	148	47860	323				
HERBA AVERAGE Na: 82±57; K: 21660±12111; K/Na: 355±238							
Allium ursinum	330	29276	89	*Rubus idaeus*	61	20660	339
Althaea officinalis	334	30100	90	*Tussilago farfara*	52	16875	325
Betula pendula	116	9368	81	*Urtica dioica*	53	24250	458
Corylus avellana	84	9840	117	*Vaccinium myrtillus*	55	8551	155
Fragaria vesca	97	12463	128	*Viscum album*	44	15489	352
Fraxinus excelsior	29	6415	221	*Crataegus monogyna*	92	14223	155
Plantago lanceolata	208	18090	87	*Malva sylvestris*	94	40220	428
LEAVES AVERAGE Na: 118±101; K: 18273±9725; K/Na: 216±136							
Achillea millefolium	59	18772	318	*Tilia cordata*	126	12434	99
Filipendula ulmaria	103	18496	180	*Tilia tomentosa*	39	12327	316
Sambucus nigra	115	38943	339	*Verbascum phlomoides*	49	14490	296
FLOWERS AVERAGE Na: 82±37; K: 19244±10056; K/Na: 258±96							
Cerasus avium (stipites)	140	7791	56	*Juniperus communis (pseudobaccae)*	40	7265	182
Crataegus monogyna	16	6681	418				
FRUITS AVERAGE Na: 65±66; K: 7246±555; K/Na: 218±184							

* In the case of each vegetal product, eight samples from different populations were analyzed

The low Na content of MPs represents a positive aspect, given the fact that presently humans consume excessive amounts of salt in comparison to their needs. High intakes of Na are correlated by with an increased incidence of hypertension [8]. As medicinal plants contain only very small concentration of Na, their infusions do not constitute any risk for hypertensive patients; additionally herbal preparations contribute with significant K amounts, whose presence has been associated with an antihypertensive effect [9]. A good example in this regard is *Crataegi fructus*, employed in the treatment of heart conditions, and very low in Na.
The evaluation of the K/Na ratio in the analyzed medicinal plants shows that this proportion is very advantageous in case of the majority of aerial parts (herba), flowers and leaves. Remarkable in this regard are *Agrimoniae herba, Urticae folium, Origani herba, Equiseti herba, Genistae tinctoriae herba, Rubi idaei folium, Menthae longifoliae folium, Millefolii flos, Sambuci flos, Tiliae tomentosae flos cum bracteis, Verbasci flos, Crataegi fructus* (to cite only the products devoid of toxic/very active constituents). Conversely, in case of rhizomes and roots, the K/Na is constantly beneath the value of 100. Unexpectedly low are the values of the considered ratio in case of *Cerasorum stipites, Ononidis radix* and *Cichorii radix*, known in phytotherapy for their diuretic indication (in their case, the elevation of the diuresis is probably due to their flavonoid and saponin constituents).

Conclusions

Medicinal plant products with a high K content, like *Malvae folium cum flos, Sambuci flos, Violae tricoloris herba, Taraxaci herba, Althaeae folium, Alii ursini herba, Urticae folium, Equiseti herba, Virgaureae herba and Rubi idaei folium* can be employed under various forms for the completion of the recommended daily potassium intake. They could prove particular utility in case of angor pectoris, cardiac insufficiency, arrhythmias, hypertension (due to the importance of K for the optimal function of the cardio-vascular system), as well as in case of concomitant utilization of saluretic diuretics, diarrhea, emesis (increased K elimination). The high K/Na ratio ercommends aqueous extracts of *Agrimoniae herba, Lysimachiae herba, Menthae longifoliae folium, Genistae tinctoriae herba* in bacterial and inflammatory renal-urinary conditions, due to their diuretic-aquaretic effect.

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ALLIUM URSINUM L.: A POTENTIAL SOURCE FOR COMPLETING THE RECOMMENDED DAILY INTAKE OF ESSENTIAL MINERAL ELEMENTS

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Summary

The purpose of this study was to perform an extensive investigation of the inorganic components of Alii ursini folium, appreciated both as an aliment as well as a phytotherapeutical remedy. Forty-seven elements were analyzed leaves of wild-growing wood garlic. The high content in essential minerals encourages their consummation, completing the daily intake of essential mineral elements: 5 g dried leaves contribute with 58% of the normative daily intake for Ni, 51% for V, 48% for Mn, 15% for Ca, 14% of Fe, 10% for K and 7% for Cu.

Keywords: *Allium ursinum, content in mineral elements*

Introduction

Wood garlic (*Allium ursinum*) enjoys a large popularity in Romania, its leaves being consumed fresh, as salad (in spring) or dried, as a spice. Known from traditional folk medicine for its stomachic, anthelmintic, antibacterial, depurative, expectorant activities [1], several recent pharmacological investigations have documented the impressive therapeutic potential of this plant in cardio-vascular disorders. Its content in gamma-glutamyl peptides and sulfur-containing compounds is believed to be responsible for the inhibition of the angiotensin I - converting enzyme (ACE) and the interference with the nitric oxide system [2,3]. On the other hand, high levels of adenosine [4] relax smooth vascular muscles by acting upon the ATP-dependent K-channels [5], thus enhancing the blood pressure lowering effects of this plant. Additionally, cardioprotective effects were pointed out, based on the significant reduction of the incidence of ventricular fibrillation [6]. *Allium ursinum* (AU) also inhibits 5-lipoxygenase, cyclooxygenase, thrombocyte aggregation, lowers the levels of total cholesterol and increases circulating insulin [7,8]. Several authors suggest that AU has a greater therapeutic benefit compared to *Allium sativum* [3,4,8].
Although the content in organic active principles of AU has thoroughly been investigated [9], the inorganic part received less attention. However, plants are known to contain a large variety of mineral elements, according to their specific biochemical features and site of development [10]. Following the ingestion of vegetal products, human organisms are exposed to these inorganic constituents, some of them essential, some toxic to man. As AU represents a very popular vegetable and spice, it is consumed in relatively large daily amounts. Thus, the intake of inorganic compounds through AU preparations could be significant.
In this concept, the objective of the present research was to perform an extensive investigation of the inorganic constituents of AU leaves, evaluating their content in 47 elements. All samples were gathered from non-polluted sites of the wild flora, in order to assess the natural level of inorganics in the leaves.

Material and methods

Plant material. Samples of wood garlic leaves (*Allium ursinum L.* subsp. *ucrainicum* Kleopow et Oxner) were gathered from 5 populations of the wild flora of the Banatian Mountains (Romania). Voucher samples were deposited in the Herbarium of the Faculty of

Pharmacy, Timisoara. After natural drying, the vegetal products were pulverized with ceramic instruments, avoiding the contact with metals.

Analysis of mineral elements.

1) The assessment of the content in Al, B, Ca, Cr, Cu, Fe, K, Mg, Mn, Mo, Na, Ni, Pb and Zn was performed through Inductively Coupled Plasma - Atomic Emission Spectrometry (ICP-AES). Samples of 2-3 g herb were heated to 105˚C in order to establish the dry mass. Matrix destruction was done after heating at a temperature of 550˚C for 6 hours, until the plant material was transformed into white ash. The ash was dissolved after boiling in hydrochloric acid 10%, followed by the quantitative transfer of the content in a test-tube. The acid solution was made up with bidistilled water to 25 mL, filtrated and measured by ICP-AES without further dilution. The apparatus employed was IRIS Interprid II ICP-AES (Thermo Electron, Dreieich, Germany).

2) The investigation of the content in Li, Be, V, Co, As, Se, Ag, Cd, In, Sn, Sb, Te, I, Cs, Tl, lanthanides and thorium was performed through inductively coupled plasma - mass spectrometry (ICP-MS). Weighed samples of 0.3-0.4 g dried plant material were placed in Teflon crucibles and 4 ml of nitric acid (subboiled), 0.25 mL hydrochloric acid and 1 mL hydrogen peroxide were added. Mineralization was performed in a closed system with the use of microwave energy. The digestion solutions were transferred into volumetric flasks and made-up to 15 mL with water (nanopure); 1 mL of each solution was diluted 1:10 and analyzed. The apparatus used for this research was ThermoElemental X Series ICP-MS (Thermo Electron, Dreiech, Germany, 2004). As a first step, a semi-quantitative analysis was performed, allowing the estimation of the concentration ranges in the digestion solutions of the plant materials. The quantitative determinations were carried out based on a calibration curve established with ICP Multi Element Standard Solution XXI CertiPUR Merck. Internal standard was rhodium.

3) Mercury was assessed in the microwave-digested solutions by atomic fluorescence (apparatus Mercur, Analytik Jena AG, Germany, 2004).

Accuracy of the data has been verified by a parallel analysis of two certified reference materials: Peach Leaves 1547 and Oriental Tobacco Leaves CTA-OTL-1.

Results and Discussions

In the present work, 47 elements have been analyzed in wood garlic leaves. The investigated elements exist in quantities of different orders of magnitude, ranging from grams/kg dry mass (potassium, calcium, magnesium) to some micrograms/kg (lithium, mercury, arsenic, lanthanides etc). The mineral content displays a large variation interval, due to high values of standard deviations. This fact is common when evaluating plants from the wild flora, as the geogenous occurrence of the investigated element, and the nature of the weathering rocks have a high impact on the content of a mineral in plants [10]. However, in our study we could not point out a correlation between the nature of the parent rock (lime; granite) and the mineral content of the vegetal product; this situation indicates that the content of inorganics depends on many site-specific variables.

Of the investigated elements, K occurs in the highest concentration (29.3 g/kg), being followed by Ca (14.6 g/kg) and Mg (2.3 g/kg); this situation is similar in most plants [11]. When assessing the content of trace elements, aluminum is best represented, with a mean concentration of 224 mg/kg, being followed by iron, sodium, manganese, zinc, boron, barium, strontium, copper and nickel (Table 1). Ultratrace elements like vanadium (995 μg/kg), iodine (910 μg/kg), chromium (369 μg/kg), neodymium (259 μg/kg), cadmium (190 μg/kg), molybdenum (242 μg/kg) and cerium (239 μg/kg) are present in even lower amounts, of hundreds of μg/kg DM. Other constituents: thallium, mercury, uranium and certain

lanthanides (terbium, holmium, thulium, lutentium) could only be detected in very small amounts, of some µg/kg DM.
The presence of all the investigated elements in AU leaves shows that plants are able to take up various elements from their environment, according to both their physiological

Table 1. The content in d-block and p-block mineral elements of *Allium ursinum* leaves

Element		Site 1 Lime	Site 2 Lime	Site 3 Granite	Site 4 Lime	Site 5 Lime	MEAN VALUE	Strd deviat.
s-Block elements (alkaline and alkaline earths)	Li (µg/kg)	56	104	198	130	248	147	76
	Na(mg/kg)	327	123	201	125	42	164	107
	K(mg/kg)	28825	30070	30532	31180	25772	29276	2140
	Rb(mg/kg)	14.4	6.7	13.0	30.8	6.4	14.3	9.9
	Cs(µg/kg)	28.3	18.9	28.9	44.0	29.1	29.8	9.0
	Be(µg/kg)	4.2	36.5	44.2	11.3	8.6	20.9	18.1
	Mg(mg/kg)	2575	2816	1610	2618	1789	2282	543
	Ca(mg/kg)	14886	13470	7463	18684	18703	14641	4631
	Sr(mg/kg)	13.2	2.3	15.1	20.6	11.1	12.5	6.7
	Ba(mg/kg)	6.5	1.9	26.7	73.0	2.8	22.2	30.2
Lanthanides	Ce(µg/kg)	134	215	230	345	271	239	77
	Pr(µg/kg)	70	30	28	192	42	72	69
	Nd(µg/kg)	252	96	113	681	152	259	244
	Sm(µg/kg)	45.4	17.5	24.5	115.2	28.0	46.1	40.0
	Eu(µg/kg)	8.0	3.5	4.2	29.7	6.1	10.3	10.9
	Gd(µg/kg)	51.8	23.7	27.7	154.4	29.6	57.4	55.3
	Tb(µg/kg)	5.7	2.7	2.6	19.4	4	6.9	7.1
	Dy(µg/kg)	28.1	14.1	16.9	103.4	21.1	36.7	37.6
	Ho(µg/kg)	4.8	2.6	2.7	21.5	4.2	7.2	8.1
	Er(µg/kg)	11.8	7.4	7.7	51.1	11.2	17.8	18.7
	Tm(µg/kg)	1.7	1.0	1.1	6.3	2.3	2.5	2.2
	Yb(µg/kg)	6.3	6.6	8.1	30.2	9.2	12.1	10.2
	Lu(µg/kg)	1.1	0.6	0.5	3.9	1.1	1.4	1.4
Actinides	Th(µg/kg)	16.3	20.0	24.7	31.6	55.6	29.6	15.6
	U(µg/kg)	1.6	5.4	14.5	8.1	6.0	7.1	4.8
Transitional elements	V(µg/kg)	543	942	1021	1190	1280	995	286
	Cr(µg/kg)	502	561	193	335	253	369	158
	Mn(mg/kg)	66	59	175	100	72	94	48
	Fe(mg/kg)	167	136	142	235	250	186	53
	Co(µg/kg)	161	210	105	98	100	135	49
	Ni(mg/kg)	4.1	5.3	3.1	2.9	3.5	3.8	1.0
	Cu(mg/kg)	12.1	11.2	12.3	9.3	8.2	10.6	1.8
	Zn(mg/kg)	30	25	36	17	17	25	8.3
	Mo(µg/kg)	118	264	455	144	231	242	133
	Ag(µg/kg)	0.8	2.4	50.4	2.2	1.2	11.4	21.8
	W(µg/kg)	1.0	16.1	15.8	6.3	6.2	9.1	6.6
	Cd(µg/kg)	228	165	175	193	189	190	24
	Hg(µg/kg)	17.5	20.2	22.6	46.0	15.4	24.3	12.4
p-Block elements	Pb(mg/kg)	4.67	2.30	0.39	2.81	3.58	2.75	1.59
	Al(mg/kg)	196	234	314	157	217	224	58
	Tl(µg/kg)	1.0	4.1	25.3	10.3	6.0	9.3	9.5
	Sn(µg/kg)	161	317	487	63	265	259	161
	Sb(µg/kg)	9.3	41.3	17.0	25.4	27.8	24.2	12.1

	B(mg/kg)	21.4	23.1	19.9	36.2	16.9	23.5	7.5
	As(µg/kg)	163	45	43	68	112	86	51
	Se(µg/kg)	147	11	1	24	60	49	59
	Te(µg/kg)	0.1	12.2	10.4	2.3	3.2	5.6	5.3
	I(mg/kg)	0.63	0.39	1.52	0.64	1.36	0.91	0.50

requirement, as well as to the presence of the given element in their environment. In fact, it can be assumed that every natural element of the periodic system is present in higher plants. It is rather the sensibility of the employed analytical method and the ability to secure highly pure reagents / work environment that limit the identification of a given element in vegetal materials. Some of the mineral elements are essential to plants while others, termed bulky elements, have no known function in the plant organism [12]. Constituents of both categories are however passed on, to different degrees, during the consummation of the plants by animals or humans. From the viewpoint of their relevance to man, mineral constituents present in medicinal plants may contribute to the outcome of a treatment with a beneficial effect (through essential elements like Ca, Mg, K, Zn, Fe, I, V, Cr, Ni, Mn etc.) or with an undesired, toxic one (in case of an overload with Cd, Pb, Hg, Tl, Cs etc) [13].

Table 2. The average daily intake of mineral elements through 5 g dried leaves

Element	NR/day	Content in minerals	% to which the daily NR is covered
K (mg)	1500	146.4	**9.8**
Mg (mg)	200	11.4	**5.7**
Ca (mg)	500	73.2	**14.6**
Fe (µg)	6600	931.0	**14.1**
Zn (µg)	4600	125.0	**2.7**
Mn (µg)	975	468.0	**48.0**
Cu (µg)	750	53.2	**7.1**
Mo (µg)	25	1.21	**4.9**
Ni (µg)	32.5	18.9	**58.2**
I (µg)	65	4.55	**7.0**
Se (µg)	40	0.24	**0.6**
Cr (µg)	33	1.84	**5.6**
V (µg)	9.75	4.98	**51.0**
Li (µg)	97.5	0.73	**0.8**

NR – normative requirement (WHO, 1996) [14]

In order to evaluate the impact of AU leaves consummation, the average daily intake of mineral micronutrients through this product was calculated (table 2), based on the daily normative requirements (NDR) recommended by the World Health Organization [14]. Encouraging results show that AU leaves provide significant amounts of essential minerals like Ni, V, Mn, Ca, Fe, K, enhancing the beneficial effects of wood garlic in case of their prophylactic/therapeutic utilization, especially in cardio-vascular disorders. More particularly, the cardioprotective effect of AU [6] is sustained by the high Ni content of AU leaves, as researches proved that in cases of cardiac infarction the Ni content is lower in cardiac muscle compared to control persons [15]. On the other hand, vanadium enhances glucose uptake by the cells [16] and consequently has a positive impact on the nutrition of the cardiac muscle. Antioxidative micronutrients like Mn, which is the constituent / activator of several enzymes, including the mitochondrial manganese-superoxyde-dismutase, protect the cells against the

oxydative damage [17] and are believed to affect the blood pressure. The Fe supplementation has a positive effect due to its role in the distribution and utilization of oxygen; through its integration in the cytochrome C, it is implicated in oxydative phosphorylations [18]. In fact, the Fe content of AU leaves is approximately threefold than that of stinging nettle [11], considered to be one of the Fe-richest plants. The content in potassium, calcium and magnesium, vital ions in the contraction and electrophysiology of the heart [19], also have a positive impact on cardiovascular health.
Unlike other medicinal plants (*Hypericum perforatum* and *Viola tricolor* - accumulating Cd and Pb; *Equisetum arvense* – taking up large amounts of As [11]), wood garlic is not prone to accumulating toxic elements. Its average content in noxious metals is below the admitted limit for medicinal plants: 0.2 mg/kg DM for Cd, 5 mg/kg DM for Pb, 100 µg/kg DM for Hg and 1mg/kg DM for As [20,21] – table 1. Other toxic elements, like U, Cs, Be, Tl, Sn are present in extremely low quantities and do not pose any threat to human health. AU leaves also contain a series of elements (lanthanides), about which the present knowledge of the medical community is yet reduced. In comparison with the lanthanide concentration of other plants [10], the leaves of AU have a comparable content that can be considered non-toxic.

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Conclusions

The results of the present study encourage the consummation of wood garlic leaves. Besides the benefits owed to organic constituents, they complete the daily intake of essential inorganic elements. Amounts of as little as 5 g dried leaves contribute with 58% of the normative daily intake for Ni, 51% for V, 48% for Mn, 15% for Ca, 14% of Fe, 10% for K, 7% for Cu and I, and 6% for Mg.
The wide-spread character of this plant in Romania and the regenerable nature of its leaves enhance the potential of wood garlic to become a source for the completion of minerals with physiologic/pharmaceutical relevance.

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THE CHEMICAL PROFILE OF *MENTHA LONGIFOLIA* (L.) HUDS. LEAVES UNDER ANTIFUNGAL TOPSIN M TREATMENT. NOTE 3.

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Summary

The effect of Topsin M treatments on metabolism products of Mentha longifolia leaves was investigated. For this purpose, we carried out both qualitative and quantitative studies of treated variants comparative to untreated ones (control). Investigation of chemical composition of Mentha longifolia leaves showed that even in general*, both treated and control plants contain a similar array of constituents, there are quantitative changes regarding volatile oils and polyphenolic compounds at treated plants comparative to control.*

Keywords: *Mentha longifolia,Topsin M, chemical study*

Introduction

Mentha longifolia (L.) Huds. (English horsemint) is one of the most widely distributed mint species.
Its fresh or dried leaves are used as a culinary herb.
In medicinal purposes, *Mentha longifolia* is used as carminative, antiseptic and in headaches and pain, in general.
These activities are mostly dependend on its chemical constituents, in particularly on essential oil composition.
Data from literature report that pesticides or even regulators may interfere to larger or smaller extents with secondary compounds biosynthesis in medicinal plants (2).
In this context, the present study investigated the effect of antifungal treatment with Topsin M on bioactive products (essential oil, flavonoids and polyphenols of acid caffeic type) of *Menthae longifoliae* leaves. For this purpose, have carried out both qualitative and quantitative studies of treated variants comparative to untreated ones (control).

Materials and methods

Plant materials were brought from the experimental lots in "Anastasie Fatu" Botanical Garden, Iasi. In this experimental area, parallel cultures of *Mentha longifolia* (L.) Huds. have been made in 2001-2003 period.
Thus, every year there have been two experimental fields: a field which had no pesticide treatment (control field) and a field which had been treated with pesticide.
The antifungal treatment was achieved in vegetative phase by spraying a wettable powder of Topsin M 70 PU (TM) (Oltchim Rm. Valcea-Romania), as 0,1% and 0,4% aqueous solutions.
Topsin M (methylthiophanate) is a systemic foliar and radicular fungicid used as protective substance in cultures of alimentary and medicinal plants.
Investigated samples of *Mentha longifolia* are presented in table I:

Table I: Samples of *Mentha longifolia*

Nr.	Sample	Codification
1.	Control 2001	M.l. M 2001
2.	Treatment TM 0,1% 2001	M.l. TM 0,1% 2001
3.	Control 2002	M.l. M 2002
4.	Treatment TM 0,4% 2002	M.l. TM 0,4% 2002
5.	Control 2003	M.l. M 2003
6.	Treatment TM 0,1% 2003	M.l. TM 0,1% 2003
7.	Treatment TM 0,4% 2003	M.l. TM 0,4% 2003

Extraction procedure. The volatile oils were obtained from fresh plants by hydrodistillation in Clevenger apparatus (Ph. Rom., 10th edition). In this respect, we used leaves of English horsemint from both treated and untreated plants. Fresh material of *Mentha longifolia* was frozen before extracting its volatile oil.
The chemical composition of essential oil was established by GC/MS method:

GC-MS apparatus: Instrument - HP 5890 II GC and HP 5971 MSD;
GC column: HP-5 MS capillar (crosslinked 5% pH methylsiloxane)
(30 m long × 0,25 mm diameter × 0,25 μm thick);
Carrier gas flow rate: 1 ml/min (helium);
Column temperature: initial temperature 35°C, 10°C/min from 35°C at 260°C (3 minutes constant)
Injector temperature: 250°C;
Detector temperature: 250°C;
Injected volume: 0,1 μl.
The total polyphenolic content in volatile oils was quantified using a spectrophotometric method which was based on the coloured reaction of phenols with 4-aminophenasone and potassium ferricyanide in presence of disodic phosphate. The phenolic content of all volatile oils was expressed as g of thymol in 100 ml essential oil (1, 3).
Quantification of flavonoides. Flavonoidic content was determined by a spectrophotometric method (Jasco Spectrophotometer UV/VIS V-550) according to Romanian Pharmacopoeia, 10th edition. The content of flavonoides was expressed in rutoside (g rutoside in 100 g dry vegetal material).
Quantification of polyphenols. Polyphenolic content was determined by a spectrophotometric method (Jasco Spectrophotometer UV/VIS V-550) according to Romanian Pharmacopoeia, 10th edition. Results were expressed in g of caffeic acid in 100 g dry vegetal material (3,4).

Results – discussions

Figure 1 shows the percentage yield values for the essential oils obtained from treated plants comparative to control:

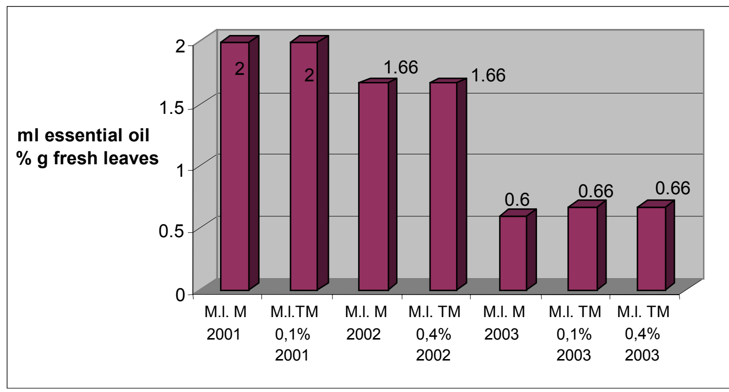

Figure 1. Percentage yield values of the essential oils

We noticed that:
- the percentage yield values of the essential oils decreases from one year to another even for the controls, indicating a pedoclimatic-induced chemical variability;
- the lowest level of volatile oil was obtained in 2003;
- there are no differences or there are insignificant differences between 0,1% and 0,4% TM treated plants and their corresponding controls (samples in 2003).

The gas-chromatographic results for essential oils from both untreated and treated TM 0,1% and TM 0,4% plants are shown in table II, III and IV:

Table II: Constituents of the volatile oils from fresh leaves of *Mentha longifolia* (2001)

Nr.	Compound	RT (min)		Area (%)	
		M.l.M 2001	M.l. TM 0,1% 2001	M.l.M 2001	M.l. TM 0,1% 2001
1.	α -thujene	5.12	5.11	1.30	1.00
2.	β-pinene	6.39	6.38	0.68	0.39
3.	sabinene	6.54	6.53	0.63	0.65
4.	β-myrcene	7.15	7.16	1.83	1.36
5.	terpinolene	7.55	7.56	0.19	0.12
6.	**1,8-cineole**	8.43	8.46	**14.42**	**14.50**
7.	γ-terpinene	8.61	8.68	0.88	0.52
8.	**cymene**	9.08	9.12	**6.80**	**5.57**
9.	terpinen-4-ol	13.89	13.88	1.95	1.80
10.	β-caryophyllene	14.02	14.07	1.35	2.79
11.	Δ-terpineol	-	14.69	-	1.53
12.	α-terpineol	15.00	14.99	1.57	2.02
13.	Δ-cadinene	15.37	-	0.54	-
14.	α-amorphene	-	15.46	-	1.01
15.	β-bisabolene	-	15.75	-	1.10
16.	(+)-carvone	15.96	16.04	1.22	1.26
17.	t-geraniol	16.75	-	0.24	-
18.	**carvacryl-acetate**	17.23	17.31	**3.82**	**4.72**
19.	γ-terpineol	-	18.61	-	0.25
20.	cis-jasmone	18.63	-	0.16	-
21.	caryophyllene-oxide	19.47	19.74	0.26	0.11

Nr.	Compound	RT (min)		Area (%)	
		M.l.M 2001	M.l. TM 0,1% 2001	M.l.M 2001	M.l. TM 0,1% 2001
22.	2,3,4,6, tetramethyl-phenol	-	19.99	-	0.09
23.	**thymol**	20.32	20.33	**17.39**	**17.61**
24.	**carvacrol**	20.77	20.80	**38.20**	**37.49**
25.	2,3,5,6-tetramethylphenol	-	28.80	-	0.07
26.	pentadecane	26.61	-	0.29	-
27.	pentacosane	-	31.16	-	0.22
28.	docosane	31.21	-	0.25	-
29.	eicosane	-	38.00	0.52	0.13

Table III: Constituents of the volatile oils from fresh leaves of *Mentha longifolia* (2002)

Nr.	Compound	RT (min)		Area (%)	
		M.l.M 2002	M.l. TM 0,4% 2002	M.l.M 2002	M.l. TM 0,4% 2002
1.	**1,8-cineole**	7.99	7.62	**4.39**	**18.65**
2.	**cymene**	10.39	9.51	**3.74**	**11.36**
3.	thymyl acetate	-	16.33	-	0.29
4.	**carvacryl acetate**	17.04	16.62	8.55	7.20
5.	**thymol**	19.47	19.31	**17.06**	**15.91**
6.	**carvacrol**	19.85	19.68	**47.33**	**37.75**

Table IV: Constituents of the volatile oils from fresh leaves of *Mentha longifolia* (2003)

Nr.	Compound	RT (min)			Area (%)		
		M.l.M	M.l. TM 0,1%	M.l. TM 0,4%	M.l.M	M.l. TM 0,1%	M.l. TM 0,4%
1.	α -thujene	8.86	8.87	8.88	1.01	1.55	1.78
2.	α-pinene	9.00	9.01	9.02	0.51	0.76	0.87
3.	camfene	-	9.32	9.32	-	0.07	0.06
4.	sabinene	9.82	9.84	9.85	0.99	1.30	1.57
5.	β-pinene	9.89	9.91	9.92	1.12	1.40	1.56
6.	myrcene	10.15	10.17	10.18	2.87	3.31	3.55
7.	3-octanol	10.28	10.31	-	0.93	0.61	-
8.	α-phellandrene	10.42	10.43	10.44	0.11	0.31	0.32
9.	α-terpinene	10.67	10.67	10.68	2.27	0.93	0.98
10.	**p-cymene**	10.86	10.90	10.86	**5.90**	**6.51**	**7.37**
11.	**1,8-cineole**	11.04	11.06	11.08	**18.17**	**18.12**	**21.70**
12.	β-ocimene	11.22	-	-	0.62	-	-
13.	Δ-3-carene	-	11.23	11.23	-	0.90	0.62
14.	γ-terpinene	-	11.48	11.50	-	2.74	3.37
15.	t-sabinene hydrate	11.62	11.63	11.64	0.59	0.70	0.70
16.	terpinolene	11.98	-	-	0.20	-	-
17.	Δ-4-carene	-	11.99	12.00	-	0.17	0.20
18.	isopinocarveol	12.92	-	-	0.22	-	-
19.	terpinen-4-ol	13.56	13.56	13.56	1.17	1.10	1.06
20.	Δ-terpineole	-	13.48	-	-	1.01	-

Nr.	Compound	RT (min)			Area (%)		
		M.l.M	M.l. TM 0,1%	M.l. TM 0,4%	M.l.M	M.l. TM 0,1%	M.l. TM 0,4%
21.	α-terpineole	13.89	13.89	13.76	1.28	0.71	0.15
22.	β-citronellol	14.40	-	-	0.39	-	-
23.	linalyl propionate	-	-	13.91	-	-	0.42
24.	nerol	14.46	14.47	14.49	0.43	0.31	0.28
25.	citral	14.69	-	-	0.20	-	-
26.	carvone	14.85	14.48	14.89	1.23	0.90	0.63
27.	**thymol**	15.35	15.36	15.37	**14.56**	**13.32**	**12.91**
28.	**carvacrol**	15.59	15.60	15.6	**26.30**	**22.28**	**21.70**
29.	thymyl acetate	-	16.26	16.27	-	0.45	0.36
30.	eugenol	16.34	16.35	-	0.14	0.15	-
31.	**carvacryl-acetate**	16.53	16.57	16.56	**1.43**	**4.42**	**3.50**
32.	β-elemene	16.89	16.90	16.90	2.04	1.71	1.50
33.	β-caryophyllene	17.38	17.90	17.39	5.44	4.41	3.91
34.	α-amorphene	17.46	-	-	0.40	-	-
35.	t-β-farnesene	17.64	17.65	17.65	0.55	0.48	0.41
36.	α-humulene	17.83	17.83	17.83	0.34	0.28	0.22
37.	germacrene D	18.21	18.21	18.21	3.40	2.73	2.21
38.	aromadendrene	18.28	18.29	18.28	0.23	0.17	0.09
39.	biciclo-germacrene	18.40	18.40	18.40	1.08	0.88	0.09
40.	Δ-cadinene	18.68	18.68	18.68	0.18	0.12	0.08
41.	spathulenol	19.47	19.48	19.47	0.34	0.29	0.19
42.	caryophyllene – oxide	19.58	-	-	0.15	-	-
43.	t-muurol	-	20.39	-	-	0.09	-

The GC-MS analysis shows that:

- all volatile oils contain ***1,8-cineole, thymol, carvacrol, carvacryl acetat and cymene***;
- ***the terpenoidic aromatic fraction is the main component*** of *Mentha longifolia* volatile oils from both untreated and TM treated plants.

The quantitative analysis of phenols in volatile oils isolated from frozen vegetal material shows an increase in phenolic content in treated plants compared with the controls (figure 2).

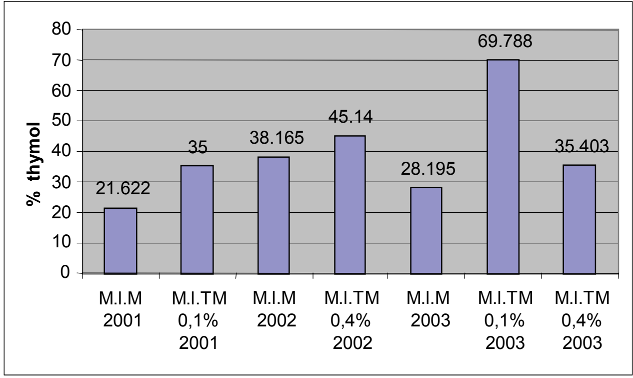

Figure 2. The phenolic content of essential oils of *Mentha longifolia*

The flavonoidic and polyphenolic content (table V) in treated plants is different from the one in corresponding controls:

Table V: The flavonoidic and polyphenolic content (%) of *Mentha longifolia* samples

Nr.	Sample	Flavonoids (g rutoside/100 g dry vegetal material)	Polyphenols (g caffeic acid/100 g dry vegetal material)
1.	M.l. M 2001	0.824	0.610
2.	M.l.TM 0,1% 2001	0.606	0.644
3.	M.l.M 2002	0.850	0.721
4.	M.l.TM 0,4% 2002	0.973	0.703

It is obvious:

- 0,1% TM treatment decreases flavonoids synthesis by 26%;
- flavonoidic synthesis increses by 14% at 0,4% TM treated plants, the controls having a relative stable flavonoidic content (0.824/0.825);
- differences registered for polyphenols were less significant than those revealed for flavonoids; on the other hand, there are significant differences in polyphenolic content between the controls in 2001 and 2002 (0.610/0.721).

Conclusions

Investigation of chemical composition of *Mentha longifolia* leaves showed that even in general, both treated and control plants contain a similar array of constituents, there are some changes regarding volatile oils and polyphenolic compounds at treated plants comparative to corresponding controls.
Thus:
- the phenolic content of volatile fraction is higher in treated plants;
- 0,1% TM treatment increases flavonoidic content in *Mentha longifolia* leaves;
- 0,4% TM treatment decreases flavonoidic content of *Mentha longifolia* leaves:
- there are lower variations in polyphenolic of acid caffeic type content than in the flavonoidic content.

Therefore, we noticed differences in volatile and polyphenolic of acid caffeic type fractions between the controls in different years; sometimes these differences are more significant than those between the sample and the corresponding control.

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BIOTECHNOLOGICAL AND PHYTOCHEMICAL RESEARCH ON *RUSCUS ACULEATUS* L.

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Summary

During the last years, Ruscus aculeatus L. has been the subject of a number of investigations due to its antiedematous, antihemorroidal and venotonic properties. This species is on the red list of the protected plants from Romanian flora. In order to obtain steroidal saponins with these important phytotherapeutical properties, in vitro tissue cultures were induced from this species. Steroidal saponins specific to Rusci rhizoma can be synthesized by in vitro plant cell and tissue culture.

Keywords: *Ruscus aculeatus L., biotechnology, steroidal saponin.*

Abbreviations*: 2,4-D: 2,4-Dichlorophenoxyacetic acid; BA: Benzyl adenine; NAA: Naphthalene acetic acid; IBA: Indole-3-butyric acid.*

Introduction

Ruscus aculeatus L. (butcher's broom), belonging to the *Liliaceae* family, is a small evergreen shrub with tough, very rigid leaves, terminated in a single sharp spine, named phylloclades that are actually an extension of the stem who have assimilatory function. The small greenish-white flowers grow from the center of the phylloclades and bloom in the early spring. The fruits are red berry with 1-2 seed [1,2].
As originating in the Mediterranean region, *Ruscus aculeatus* is a perennial species through it horizontal rhizomes and vegetates in South and Southwest part of Romania. In the present these species is on the red list of the protected plants from Romanian flora [3].
During the last years the phytochemical studies were focused on this species because it synthesizes and accumulates the active biological substances with pharmaceutical importance. The rhizomes and the roots of *Ruscus aculeatus* have steroidal saponins ruscoside and ruscine, having as aglycons, ruscogenin and neoruscogenin [4,5]. Ruscogenin is a basic sapogenin with a strong anti-inflammatory, venotonic, anti-haemorrhoidal effect leading to production of several preparates like *Mandor*[R], *Ruscorectal*[R], *Cyclo*[3R] etc [4].
Considering that in Romania, *Ruscus aculeatus* is subjected to protection law of endangered species, it led to the necessity to introduce this species in an *in vitro* cells and tissues culture in order to obtain the necessary biomass with pharmacological principles.
The main objectives of this study were: the application of *in vitro* plant biotechnology on *Ruscus aculeatus* L. species, the extraction and isolation of steroidal saponins from vegetal medicinal product (*Rusci rhizoma*); the comparative qualitative analysis of steroidal saponins from rhizomes with roots and biomass obtained by *in vitro* tissue culture (callus with roots).

Materials and methods

In vitro culture

In order to induce primary callus we used explants excised out of roots of plantlets obtained by *in vitro* aseptically germination of the *R. aculeatus* seeds. The berries with seeds were harvested in October 2004 from Dealurile Lipovei area (Arad County).

In the absence of any specific protocol regarding *in vitro* culture for this species, we tested two basal media Murashige-Skoog (1962) (MS) and Gamborg et al. (1968) (B5) [6,7,8] with sucrose (30g/l) and supplemented with glutamine (0.4g/l), hydrolyzed casein (0.4g/l) and agar (7g/l) (Table 1).

The pH was set to 5.7 for MS medium and 5.5 for B5 medium before autoclavation.

Table 1. Versions of basal media for induction of primary callus

Basal media	Variants	Growth regulators
MS		
	1	2,4-D (2.0 mg/l) + BA (0.3 mg/l)
	2	NAA (2.0 mg/l)+ BA (0.5 mg/l)
	3	IBA (2.0 mg/l) + BA (0.5 mg/l)
B5		
	4	2,4-D (2.0 mg/l) + BA (0.3 mg/l)
	5	NAA (2.0 mg/l) + BA (0.5 mg/l)
	6	IBA (2.0 mg/l) + BA (0.5 mg/l)

The cultures were incubated in a controlled environment chamber at 25±1°C under a 16 h photoperiod and a 30µm/m^{2}/sec photosynthetic active radiation (PAR), provided by cool-light fluorescent lamps.

For growth of the rhizogenic callus and roots were used the following culture media:

1. MS with sucrose (30 g/l) supplemented with glutamine (0.4 g/l), hydrolyzed casein (0.4g/l), 2,4-D (2.5 mg/l) and IBA (0.3 mg/l) and agar (7 g/l).
2. MS with sucrose (30 g/l) supplemented with glutamine (0.4 g/l), hydrolyzed casein (0.4g/l), 2,4-D (0.5 mg/l) and NAA (1.5 mg/l) and agar (7 g/l).
3. MS liquid medium with sucrose (30 g/l) supplemented with glutamine (0.4 g/l) and NAA (1.5 mg/l).

In order to induce *in vitro* roots culture, the rhizogenic callus were transferated from solid medium in flasks with liquid medium.

The flasks with liquid medium were incubated in vegetative camera under the dark, and respectively in photoperiod conditions under continuous agitation (100 rpm rotary shaker).

Steroidal saponins isolation

The vegetal material, rhizomes with roots of *R. aculeatus,* was harvested in October 2004 from Dealurile Lipovei area (Arad County).

After a drying period, the product was grinded to a fine powder and sieved (sieve VI – FR X). The powder (50 g) was degreased by extraction in Soxhlet with ethylic ether during six hours (until the liquid passed colourless). The degreased powder was extracted for two times with 500 and respectively 250 ml methanol on boiling water bath for 30 minutes after the solvent removal. The resulted methanolic solutions were mixed after filtrations and then concentrated under low pressure (rotavapor) up to aprox. 50 ml, and further precipitated in ethylic ether by agitating

them. The precipitate (steroidal saponins) was filtrated on Büchner funnel under vacuum and then introduced on $CaCl_2$ sicc. in exsiccator for 3 days until complete drying.

The TLC qualitative analysis of steroidal saponins

The TLC qualitative analysis of steroidal saponins was done in the following experimental conditions:

-the stationary phase: silica gel GF $_{254}$ Merck, plates of 10x10 cm;

-the mobile phase: chloroform-methanol-water (65:35:10), lower fase;

-samples:

1. the 1% methanolic extract out of dried liquid medium;
2. the 5% methanolic extract out of biomass obtained by *in vitro* culture;
3. 1% methanolic extract obtained from fluid extract (1:1);
4. solution of 1% isolated steroidal saponin from *R. aculeatus* rhizomes with roots in methanol;

The fluid extract (1:1) was obtained from rhizomes and roots of *R. aculeatus* by repercolation in ethylic alcohol 70° (Squib technique) [9];

Samples were traced in bands of 10 mm, 3x10 µl.

The reagents of identification:

A. Methanol – sulfuric acid (1:1) – for detection of all saponozidic fractions (brown color);
B. Ehrlich's reagent (2.7% p-dimethylaminobenzaldehyde in 95% ethanol mixed with an equal volume of 12 N HCl – for detection of all furostanol saponins (red color).

After this, the plate was oven dried at 110°C for 5 minutes [10].

The analysis of saponins isolated from rhizomes, rhizomes tincture (1:10) and biomass (1:10) [11] was done by HPLC.

Hydrolysis of steroidal saponins

A 0.5 g quantity of isolated saponin with 15 ml 2N H_2SO_4 was added to 70% iso-propanol in a 50 ml round bottom flask provided with ascendant condenser. The mixture was kept under heating boiling water at reflux for 8 hours. After cooling, filtration and adding 15 ml H_2O, the medium was extracted four times with hexane. The organic phase was then washed with 5% solution KOH and H_2O (two times). After the solvent evaporation the aglycons were dissolved in chloroform. The hydrolysis of fluid extract (1:1) (5 ml) as well as of 5% biomass methanolic extract (14 ml) and 1% methanolic extract of ingrowing medium (25 ml) were performed in the same way [10].

The qualitative analysis for steroidal aglycons

The qualitative analysis for steroidal aglycons by HPTLC was carried out under the following experimental conditions:

-the stationary phase: silica gel Merck, HPTLC plates of 10x10 cm;

-mobile phase: cyclohexan-ethyl acetate (1:1);

-samples:

1. the chloroform solution of hydrolysis product of the 1% methanolic extract out of liquid medium;
2. the chloroform solution of hydrolysis product of the 5% methanolic extract out of biomass obtained by *in vitro* culture;
3. the chloroform solution of hydrolysis product of fluid extract (1:1);
4. the chloroform solution of hydrolysis product of isolated saponin;
5. ruscogenin - reference substance;

6. neoruscogenin – reference substance;

Samples were applied in bands of 10 mm, 3x 10 µl.
The reagents of identification:

A. Solutions of p-dimethylaminobenzaldehyde (0.25 g p-dimethylaminobenzaldehyde dissolved in 0.25 ml C_2H_5OH 95% and 5 ml H_3PO_4 added with 5-7 drops of $HClO_4$).

After this the plate was oven dried at 110°C for 5 minutes [12].

Results and discussions

The primary callus was initiated from explants excised from plantlets roots obtained by aseptically *in vitro R. aculeatus* seeds germination, inoculated on two different culture media MS(1) and respectively B5(4). The callus was formed after 120 days only under illumination conditions.
After successive cultivations of primary callus on the same culture medium, on a 3rd subculture we obtained rhizogenic callus, wich subsequently developed roots, both under dark and photoperiod conditions.
In any other experiment the developed roots from this rhizogenic callus were cultivated in liquid medium.
Roots grown on the liquid medium has developed only under dark conditions.
For the phytochemical analysis we sampled roots from solid (one type) and liquid medium (Table 2).

Table 2. The *R. aculeatus* roots grown *in vitro* tissue culture

Medium type	Incubation time (weeks)	Fresh weight (g/flask)	Dry weight (g/flask)
MS solid supplemented with 2,4-D (0.5 mg/l) and NAA (1,5 mg/l)	11	1.926	0.275
MS liquid supplemented with NAA (1.5 mg/l)	4	1.023	0.140

In rhizomes and roots of *Ruscus aculeatus* harvested from Romania (Dealurile Lipovei) we found a high content of steroidal saponins (15.5%).
The isolated saponins are presented as a white-yellowish powder.
Four important saponozidic fractions have been detected by TLC, three furostanol fractions and one spirostanol fractions, the main two being also present in the *R. aculeatus* callus with roots (Rf: 0.1 and 0.14). In the culture medium the furostanol fractions are not present. In the biomass and in the fluid extract (1:1) identical spirostanol saponosides are present (Fig. 1).

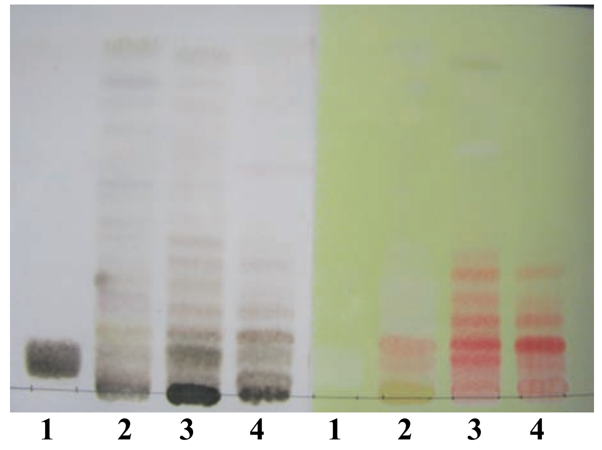

Fig. 1. TLC qualitative analysis of saponins. Reagent methanol-acid sulfuric (1:1) (left) and Ehrlich's Reagent (right); 1: 1% methanolic extract out of liquid medium; 2: 5% methanolic extract out of biomass; 3: 1% methanolic extract from fluid extract (1:1); 4: solution of 1% isolated steroidal saponins from rhizomes with roots in methanol.

HPLC analysis showed six saponosidic fractions, five being identical both in rhizomes tincture and in *in vitro* obtained biomass (Fig. 2).

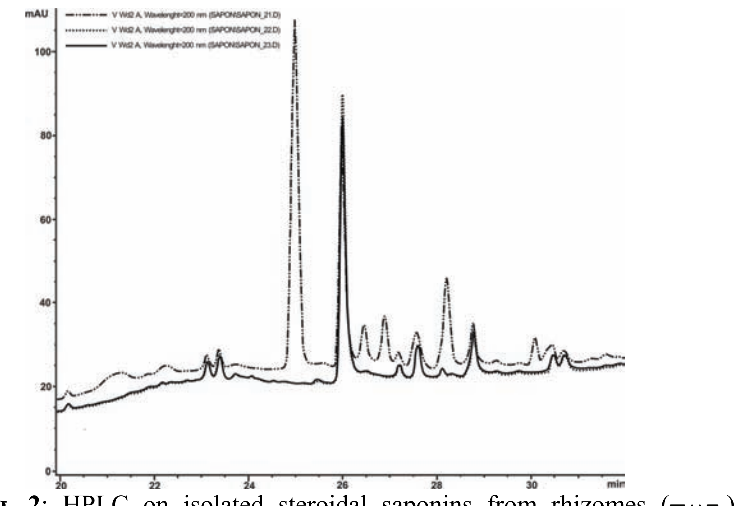

Fig. 2: HPLC on isolated steroidal saponins from rhizomes (–··–), rhizomes tincture (——) and biomass tincture (··········).

By HPTLC analysis on hydrolysis product, the aglycons ruscogenin and neoruscogenin have been detected in isolated saponin, fluid extract (1:1) and in biomass (Rf: 0.15). These couldn't be separated by TLC. In the sample from liquid medium the aglycons are absent (Fig. 3).

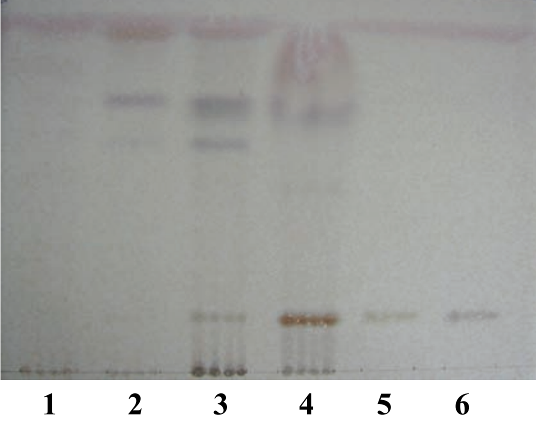

Fig. 3. HPTLC analysis of steroidal aglycons resulted after hydrolysis: 1-liquid medium; 2-biomass; 3-fluid extract (1:1); 4-isolated saponin; 5-ruscogenin; 6-neoruscogenin.

Conclusions

1. Cultures of roots as necessary biomass for comparative phytochemical analysis were obtained on culture media with high auxins concentrations starting from excised explants from plantlets developed from aseptically *R. aculeatus* seeds germination.
2. Rhizomes and roots of *R. aculeatus* from Romania (Dealurile Lipovei) have a higher content of steroidal saponins (15.5%) than the other *Ruscus* reported from different locations (6%).
3. We detected identical saponozidic fractions by TLC and HPLC both in rhizomes and in the *in vitro* biomass. Because in therapeutic practice only the extracts and not the isolated saponins are used, this opens up new perspectives in obtaining pharmacologically active extracts by using *in vitro* cell and tissue cultures.
4. The steroidal aglycons, ruscogenin and neoruscogenin were marked as result of acid hydrolysis followed by HPTLC, in isolated saponin, in fluid extract (1:1) and in biomass (rhizogenic callus and roots cultivated in liquid medium) for *R. aculeatus* species. These aglycons cannot be separated by TLC.
5. The vegetal biomass obtained by *in vitro* cell culture represents an alternative to the exploitation of *R. aculeatus* species from spontaneous flora.

Acknowledgements

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ANALYSIS OF THE POLYPHENOLIC COMPOUNDS FROM *BASILICI HERBA*

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Summary

The aim of this work was to analyze by HPLC the flavonoids and the caffeic acid derivatives from four samples of Basilici herba. The quantitative determinations made by spectrophotometric methods showed different results. We identified in samples rutoside and caffeic, chlorogenic, rosmarinic, caftaric, ferulic, coumaric acids, in different concentrations, by HPLC. After hydrolysis we emphasized the quercetol and kaempferol, like aglycons of the flavonoids.

Keywords: *Ocimum basilicum, Lamiaceae, polyphenolic compounds, TLC, HPLC*

Introduction

Ocimum basilicum L. (*Lamiaceae*), sweet basil, is an annual species, original from Asia, being cultivated in all the Mediterranean and tropical countries. In Romania is frequently cultivated in alimentary, medicinal, ornamental and religious purposes. *Basilici herba* contains: essential oil, polyphenols, triterpenic acids and phytosterols. In phytotherapy is used for their antispasmodic, antiseptic, anti-inflammatory, antioxidant, adaptogen, antiulcer, antihelmintic properties (1,2,6,7). We analysed qualitatively and quantitatively polyphenolic compounds from four samples of *Basilici herba* by HPLC and spectrophotometric methods.

Material and methods

The phytochemical research was effectated using the aerial parts harvested from on the Romanian species *Ocimum basilicum* L. We have analysed the polyphenolic compounds from four samples of *Basilici herba*. The samples are coming from the vegetal cultures initiated at the Faculty of Agronomy Cluj (sample I), south of the country (sample II) and two commercial samples of medicinal tees (III and IV).

The quantitative analysis of the polyphenolic compounds (flavonoids and caffeic acid derivatives) was made using the method described in the Romanian Pharmacopoeia [8,9]. The quantitative determinations made by spectrophotometric methods, were made using a spectrophotometer UV-VIS JASCO V-530.

HPLC determinations [3,4,5]:

Apparatus and chromatographic conditions: we used an Agilent 1100 HPLC Series (Agilent, USA) equipped with a degasser G1322A, a quaternary gradient pump G1311A, a Zorbax SB-C18 reversed-phase analytical column 100 mm x 3,0 mm i.d.(Agilent, USA), operated at 48^{0}C. The mobile phase was a binary gradient: methanol and buffer solution. The buffer solution was prepared by dissolving potassium dihydrogen phosphate (40 mM) in water and the pH was adjusted to 2,3 with 85% orthophosphoric acid. The gradient begun with a linear gradient started at 5% methanol and 42% methanol over first 35 minutes, followed by isocratic elution with 42% methanol over the next 3 minutes. The flow rate was 1 ml/min and data were collected at 330 nm. The injection volume was 5 µl.

Samples preparation: powdered herba was extracted with distillated water and ethanol, at 80°C for 30 minutes on a water bath and then they were sonicated for 5 minutes and finally heated

again for another 10 minutes at 80°C. The mixtures were centrifuged with 4000 rpm. In order to study the flavonoid aglycones that can be obtained by hydrolisis we have mixed these solutions together with hydrochloric acid 2M and ethanol and the solutions were heated at 80°C for 30 minutes on the water bath. After extraction the mixtures were centrifuged with 4000 rpm. The solutions were diluted with distillated water in a 10 ml volumetric flask.

Detection: detector UV 330 nm up to 16 min, then 370 nm up to 38 min. All compounds were identified by external standard method and by comparison of their retention times with those of the standards, in same chromatographic conditions. Quantitative determinations were performed using external standard method.

Standards: caftaric (1) acid, gentisic acid (2), caffeic acid (3), chlorogenic acid (4), p-coumaric acid (5), ferulic acid (6), sinapic acid (7), cichoric acid (8), hyperoside (9), isoquercitrin (10), rutoside (11), myricetin (12), fisetin (13), quercitrin (14), quercetol (15), patuletin (16), luteolin (17), kaempferol (18), apigenin (19). We present the HPLC chromatograms for the external standards at 330 nm (fig.1) and the retention times for all used standards (Table I, fig.1).

Table I. Retention times for standards polyphenol

No.	Polyphenolic compounds	Retention time (Rt) (min)
1.	caftaric acid	3.1
2.	gentisic acid	3.7
3.	caffeic acid	5.9
4.	chlorogenic acid	6.6
5.	p-coumaric acid	9.2
6.	ferulic acid	12.4
7.	sinapic acid	14.7
8.	cichoric acid	16.2
9.	hyperoside	19.0
10.	isoquercitrin	19.9
11.	rutoside	20.4
12.	myricetin	21.1
13.	fisetin	22.8
14.	quericitrin	23.3
15.	quercetol	26.8
16.	patuletin	28.7
17.	luteolin	29.2
18.	kaempferol	31.7
19.	apigenin	33.2

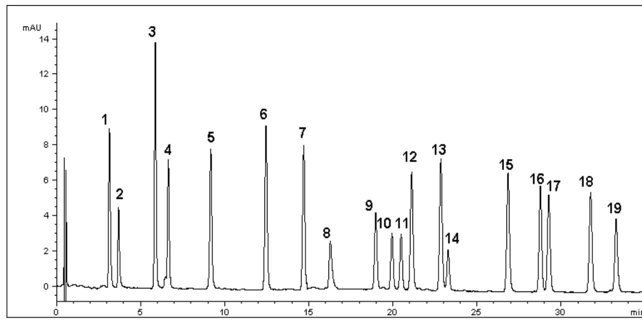

Fig.1. The HPLC chromatogram for the external standards at 330 nm: caftaric (1) acid, gentisic acid (2), caffeic acid (3), chlorogenic acid (4), p-coumaric acid (5), ferulic acid (6), sinapic acid (7), cichoric acid (8), hyperoside (9), isoquercitrin (10), rutoside (11), myricetin (12), fisetin (13), quercitrin (14), quercetol (15), patuletin (16), luteolin (17), kaempferol (18), apigenin (19).

Results and discussions

The results of quantitative spectrophotometric determinations of polyphenolic compounds from four samples of *Basilici herba* are in table II. The quantitative determinations made by spectrophotometric methods showed different results. The flavonoids and caffeic derivates are present in large quantities in the sample I (is coming from the vegetal cultures initiated at the Faculty of Agronomy Cluj) and in small quantities in the two commercial samples of medicinal tees (III and IV) (Table II).

Table II. Results of quantitative determinations of polyphenolic compounds

No.	Samples	Flavonoids (%)	Caffeic acid derivates (%)
1.	Samples I	1,10	2,28
2.	Samples II	0,60	1,19
3.	Samples III	0,45	0,71
4.	Samples IV	0,35	0,78

We have identified and measured by HPLC the following polyphenolic compounds: caftaric acid, cichoric acid, caffeic acid, chlorogenic acid, p-coumaric acid, rutoside, quercetol, kaempferol. We present the concentrations (mg polyphenolic compound /100g dried aerial parts) for these compounds before and after hydrolysis in table II. The HPLC chromatograms before hydrolysis (bh) and after hydrolysis (ah) for the four samples of *Basilici herba* analysed are shown in fig. 2-9.

Table III. The concentrations (mg polyphenolic compound /100g dried aerial parts)

Polyphenolic compound	Sample **I** bh	Sample **I** ah	Sample **II** bh	Sample **II** ah	Sample **III** bh	Sample **III** ah	Sample **IV** bh	Sample **IV** ah
caftaric acid	283.5	306.8	1595.3	1414.6	74.5	87.1	164.1	178.4
cichoric acid	21.3	-	-	-	-		-	-
caffeic acid	82.9	171.8	88.4	219.7	60.3	107.2	32.8	62.4
chlorogenic acid	-	-	-	-	-	-	30.3	29.5
p-coumaric	traces	24.3	-	-	-	11.7	traces	traces

acid								
ferulic acid	-	19.6	-	27.2	-	18.6	-	11.8
rutoside	665.05	279.9	658.4	248.6	388.1	159.2	147.1	51.4
quercetol	-	482.3	-	244.1	-	156.9	-	70.04
kaempferol	-	140.5	-	566.3	-	46.9	-	41.6

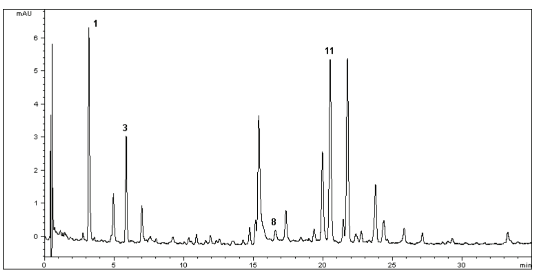

Fig.2. HPLC chromatogram of Sample I bh

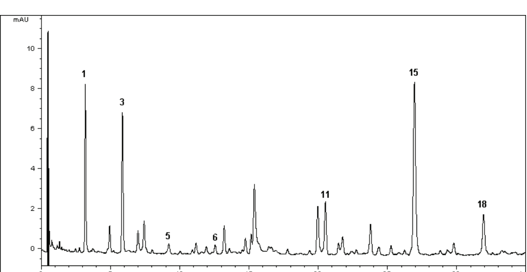

Fig.3. HPLC chromatogram of Sample I ah

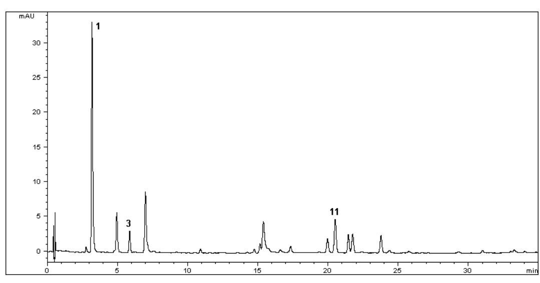

Fig.4. HPLC chromatogram of Sample II bh

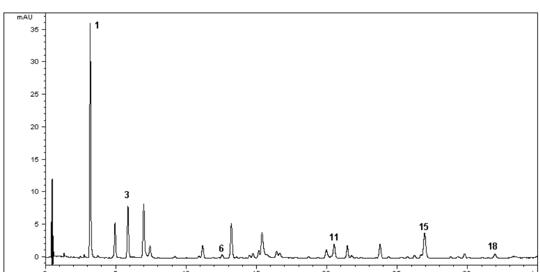

Fig.5. HPLC chromatogram of Sample II ah

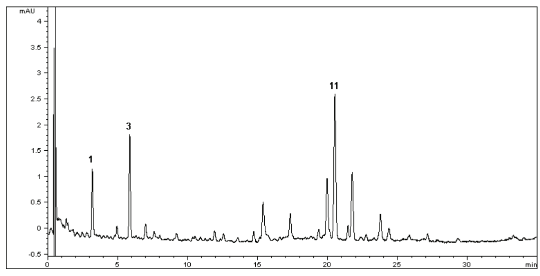

Fig.6. HPLC chromatogram of Sample III bh

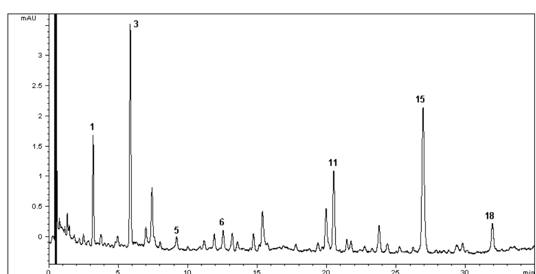

Fig.7. HPLC chromatogram of Sample III ah

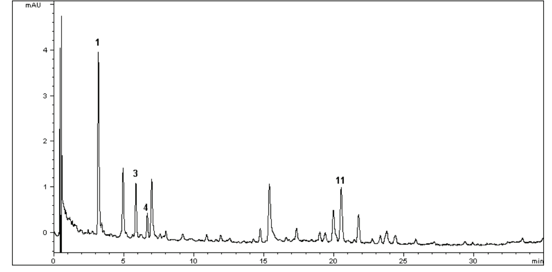

Fig.8. HPLC chromatogram of Sample IV bh

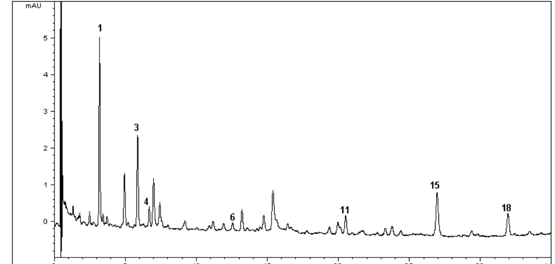

Fig.9. HPLC chromatogram of Sample IV ah

We have identified and quantified in the sample I before hydrolysis polyphenolic compounds: caftaric acid, caffeic acid, cichoric acid and rutoside. In the samples II-IV the cichoric acid is absent and in the sample IV we meet in evidence also the chlorogenic acid. We observed the caffeic acid, caftaric acid and rutoside are present in the all samples. (Table II, fig. 2-9). The flavonoids of the all samples are glycosides of quercetol and kaempferol, these aglycones being identified only after hydrolysis (fig. 2-9).

Conclusions

We have analysed the polyphenolic compounds (flavonoids and caffeic acid derivates) from four samples of *Basilici herba* by HPLC.
The quantitative determinations made by spectrophotometric methods showed different results. The flavonoids and caffeic derivates are present in large quantities in the sample I and in small quantities in the two commercial samples of medicinal tees.
We identified by HPLC in the samples before hydrolysis polyphenolic compounds: caftaric acid, caffeic acid, cichoric acid, chlorogenic, p-coumaric acid and rutoside in different concentrations. After hydrolysis we emphasized the quercetol and kaemferol, like aglycons of the flavonoids.

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FUNCTIONAL COMPONENTS OF COLD-PRESSED *AMARANTHUS* SP. SEED OIL

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Summary

The Amaranthus sp. *grain contains high levels of fat, protein and ash as compared to conventional cereal grains. Oil from Amaranthus sp. seed, obtained by Komet CA 59, Monfort Reinerss cold-press has yellow-orange color and specific taste. The content of oil and specific functional components (α-tocopherol, squalene) were compared with other oils. Amaranthus sp. may potentially serve as rich vegetable source of squalene.*

Keywords: *Amaranthus sp. seed oil, α-tocopherol, squalene.*

Introduction

Amaranthus sp. is a plant originating from America. Biological characteristics of these plants include low level of requirements towards environmental proving easy introduction in non-traditional Vojvodina growing regions (Bodroža-Solarov, 2001).
The grain contains high levels of fat, protein and dietary fiber as compared to conventional cereal grains (Saunders, 1984). The different level (5-10%) of amaranth oil in the diets induced statistically significant differences in food intake and digestibility. The fatty acid composition of amaranth oil is similar to that of wheat germ, oat, and rice brain oil in that it contains about 77% unsaturated fatty acids and rich in linoleic acid (Becker ,1989). Vitamin E contents were comparable to those of other grains crops, and tocotrenols were essentially absent (Budin et.al., 1996). *Amaranthus* may potentially serve as rich vegetable source of squalene (2.4-8%) as well as provide edible oil. Squalene (2.610.15.19.23-hexamethyl-2,6,10,14,18,22-tetracosahexaene) is an expensive terpenoid compound, derived primarily from shark (*Cantrophorus squamosus*) (Jahaniaval et. al. 2000).

Material and methods

Amarantus sp. seed was obtained from a local commercial source. The chemical composition of *Amaranthus* sp. seed was analyzed according to the current regulations. Crude fat were analyzed by the Soxlet procedure.
Amaranthus sp. oil made using the method of cold pressing by Komet CA 59, Monfort Reiners press.
The fatty acid derivatives were analyzed with Hewlett-Packard 5890 gas chromatograph by method JUS ISO 5508/2002. The content of α-tocopherol of *Amaranthus sp.* seed was detected by HPLC method JUS ISO 9936/2003 (Liquid Chromatograph HP 1090 (Hewlett-Packard)). Squalene was eluted from the C-18 column and was identified by comparing retention to pure squalene purchased from Sigma-Aldrich Co.

Results and discussion

Chemical composition of *Amaranthus sp* in comparation with cereals grain is listed in Table 1. The grain of *Amaranthus* sp. contains high levels of fat, protein, and ash as compared to conventional cereals grains.
The obtained results are in accordance with the investigations carried out in Russia and USA, where oil content of *Amaranthus sp.* seed percentage ranges from 5-10% (Kononkov,1997)

Table1. Chemical composition of *Amaranthus* sp. seed *in comparation with conventional cereals grains.*

Grain	Fat (% d.b.)	Protein (% d.b.)	Starch (% d.b.)	Ash (% d.b.)
Amaranthus sp.	6.3	16.9	64.0	2.9
Wheat [a]	2.0	14.0	60.0	1.9
Maize [a]	4.5	10.3	71.0	1.4
Rice [a]	2.2	8.5	66.0	1.4

[a] Becker

Oil from *Amaranthus* sp. seed, obtained by cold-pressing has yellow-orange color and specific taste. Color differences between cold-pressed *Amaranthus* sp. seed oil and commercial sunflower oil were representing in Fig1.

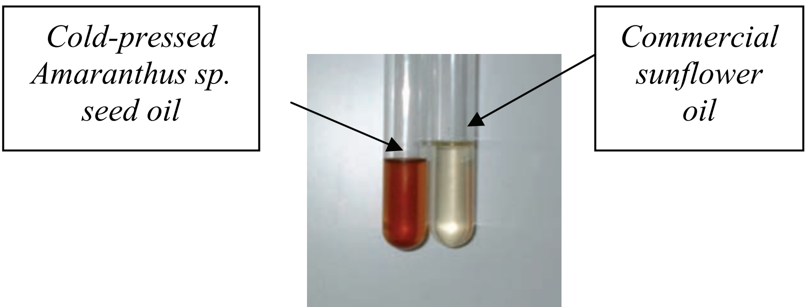

Fig.1. Cold-pressed Amaranthus sp. seed oil and commercial sunflower oil

Saturated fatty acids (palmitic (C16:0) and stearic (C18:0) represent 22,58 % of oil content (Fig 2.). Such results are in accordance with the investigations preformed by Carlsson (1979) which point at 24% of saturate fatty acids in oils of various *Amaranthus* L. species. There is the greatest content of linolic (C18:2) represent 36.58% of oil content. The content of unsaturated fatty acids is 68.2%.

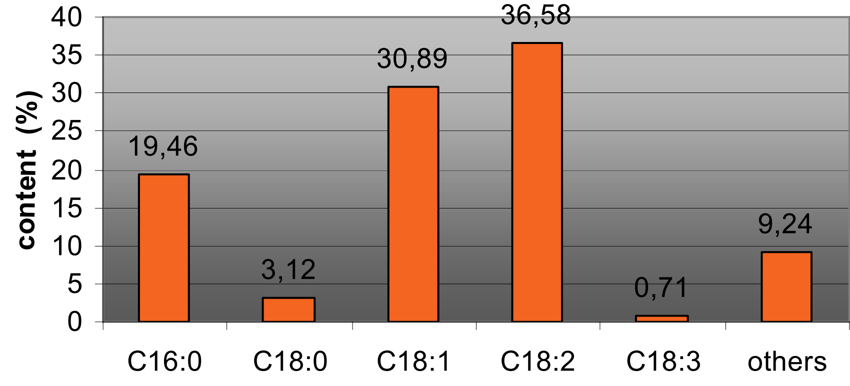

Fig. 2. The Content of oils acids (%) in Amaranthus sp seed oil

There is the greatest content of α-tocopherol (1.53 mg /100g seed) in *Amaranthus sp* seed (Fig.3). The obtained results are in accordance with the investigations carried out (Budin et.al., 1996) which point at 1.66 mg /100g seed α-tocopherol mean of various *Amaranthus* L. species.

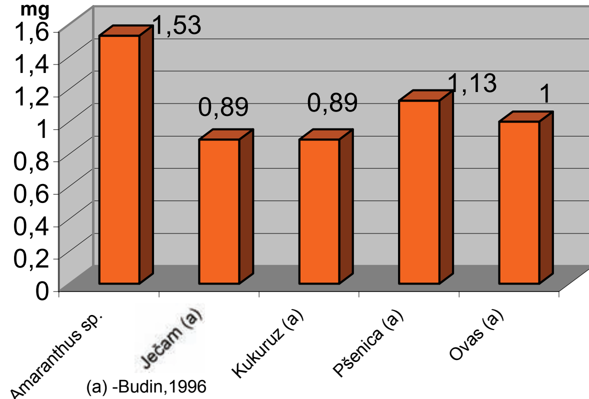

Fig. 3. The Content of α-tocopherol (mg/100g seed) in Amaranthus sp. seed and conventional cereals.

Since squalene is known obligatory biological precursors of sterols, its contribution to the nutritional ramifications of *Amaranthus* sp. grain may be important. *Amaranthus* sp. oil contains larger amounts squalene (6.4%) than other common vegetable oils (Fig.4).

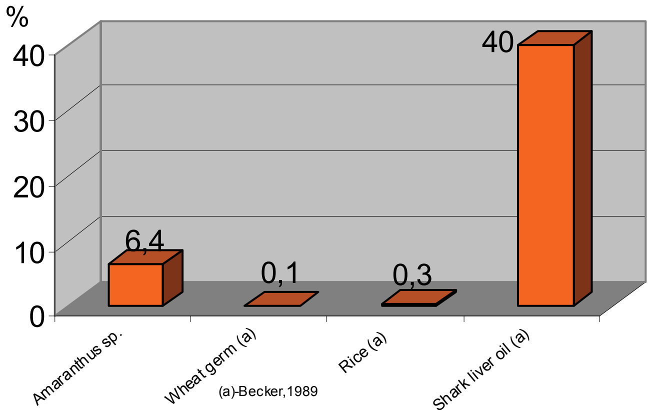

Fig. 4. The Content of squalene (%) in Amaranthus sp. seed oil, conventional cereals, and shark liver oil

Conclusion

Amaranthus sp. grain contains high levels of fat, protein, and ash as compared to conventional cereal grains. Oil from *Amaranthus* sp. seed obtained by cold-pressing method has yellow-orange color and specific taste. The results of investigation of fatty acids composition proved it to be typical plant oil. The content of unsaturated fatty acids is 68.2%. *Amaranthus* sp. seed is not unique in α-tocopherol content relative to other grains. Research has shown that *Amaranthus* sp. may potentially serve as rich vegetable source of squalene as well as provide edible oil.

Acknowledgments

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EFFICACY STUDIES OF VEGETAL EXTRACTS FROM *MAHONIA AQUIFOLIUM* AND *AESCULUS HIPPOCASTANUM* OF TOPICS FORMULATIONS

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Summary

This study investigated the effect of vegetal extract from topics formulations for treatment of psoriasis and atopic dermatitis. Clinical trials after 6 weeks of treatment on 30 patients provide an improvement of symptoms.

Keywords: *Mahonia aquifolium extract, Aesculus hippocastanum extract, topics formulations, psoriasis, atopic dermatitis*

Introduction

The topical treatment of dermatosis remains the most efficient therapy, free of risk and the most applied by the medical practice. Results of recent in vitro trials utilizing human keratinocytes, suggested that Oregon grape (*Mahonia aquifolium – Berberidaceae*) bark extract have antiproliferative, antioxidant and anti-inflammatory effects [1-3].
The horse chestnut seed and bark extract (*Aesculus hippocastanum*) have an anti-inflammatory and antioxidant effect [4]. These observations led to the preparation of two topics formulation (with Mahonia extract) for treatment of psoriasis vulgaris and two O/W cream formulas with horse chestnut extract for treatment atopic dermatitis.

Materials and methods

The studies performed on different parts of *Mahonia aquifolium* proved that the alkaloids exist in all parts of this species. However, the biggest percent of alkaloids was found in bark of root and stem. The most favorable period for harvest are May (4.5 % alkaloids) and October (5% alkaloids). Fresh plant has a big alkaloids percent (fig.1).

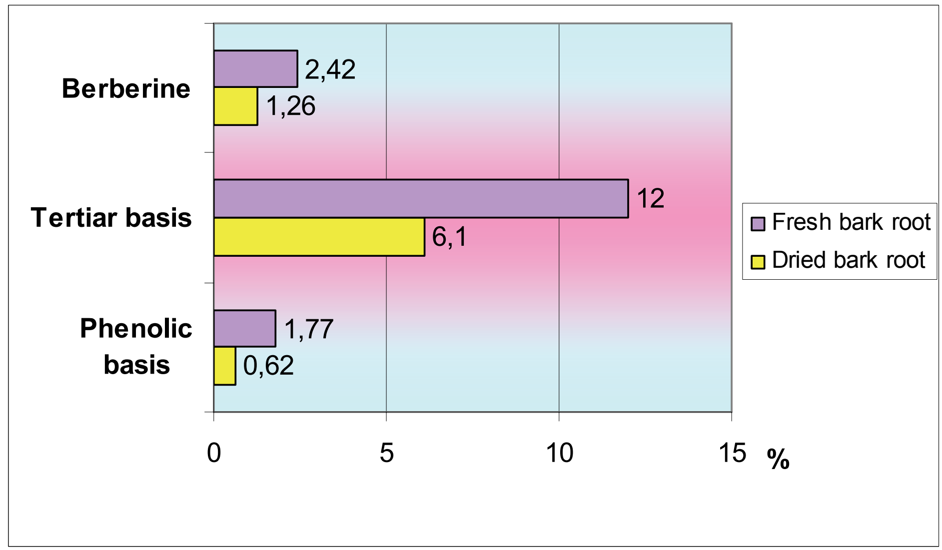


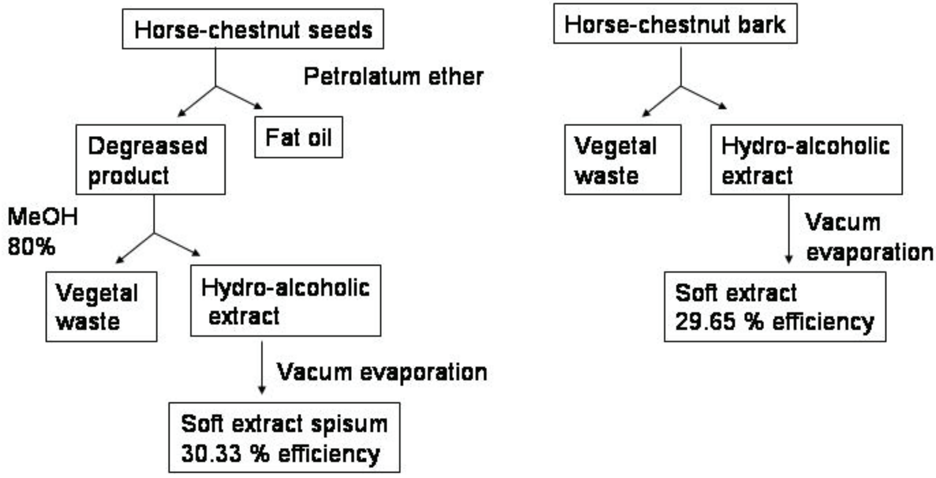
Fig. 1. Concentration of alkaloids from *Mahonia aquifolium*

For this reason it was prepared an extract from *Mahonia aquifolium* bark of root and stem, that was collected in may 2001. This ground stem and root bark were extracted at room temperature with EtOH (1:10) for 5 days and then filtred. The filtrate was concentrated at vacuum, obtaining the crude extract (spissum). The extraction efficiency was 18%.
1% Mahonia extract was incorporated in starch and tragacantha gum glycerogel in which 40% lipogel were emulsionated and associated with caffeine, urea, EDTA-disodium monocalcicum and Borago oil. (Tab. I)

Table I. The formulation of two gels

Ingredients	Quantity [g]	
	I	II
Mahonia extract	1	1
Caffeine	-	3
Urea	6	6
Benzyl alcohol	1	1
Lactic acid	1	1
EDTA-disodicum-monocalcicum	0.55	0.55
Borago oil	XI cps	XI cps
Basis to	100	100
pH	4.75	4.94

The horse chestnut seed and bark extract (*Aesculus hippocastanum*) was obtained following the scheme:



The extracts were analyzing from composition point of view using:

- thin – layer chromatography (TLC) with fertig plattes of silicogel G, Merck, 10x20 cm and as solvent a mixture of n-PrOH-AcOEt-H2O (4:4:3) and are sprayed with 20% ethanolic solution of phosphomolybdic acid (5 minutes at 100 ^{0}C);
- high pressure liquid chromatography (HPLC) using Kockar OM technique with an Shimadzu LC10AS apparatus with a Highchrom RP18 column (124x4 mmid) (5 µm); mobil phase is 35% acetonitrile with 40 ml in H3PO4/l; UV detector λ = 210 nm, flow rate 1 ml/ min, injected volume 20 µl (fig. 2-4)

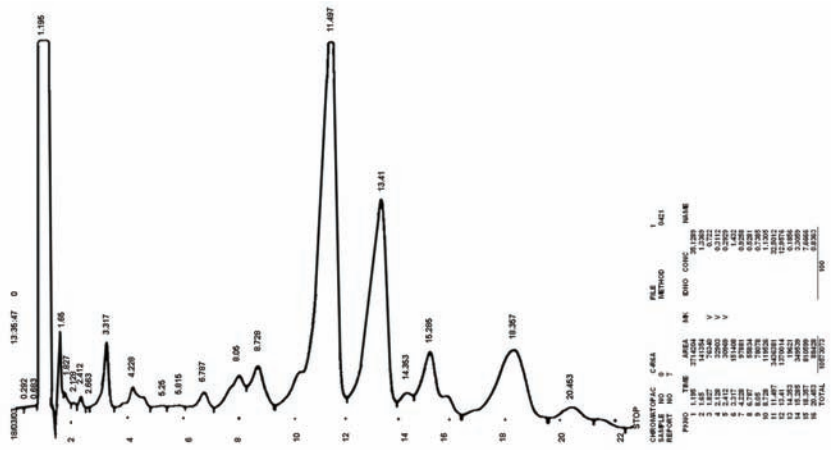


Fig. 2. HPLC for escine

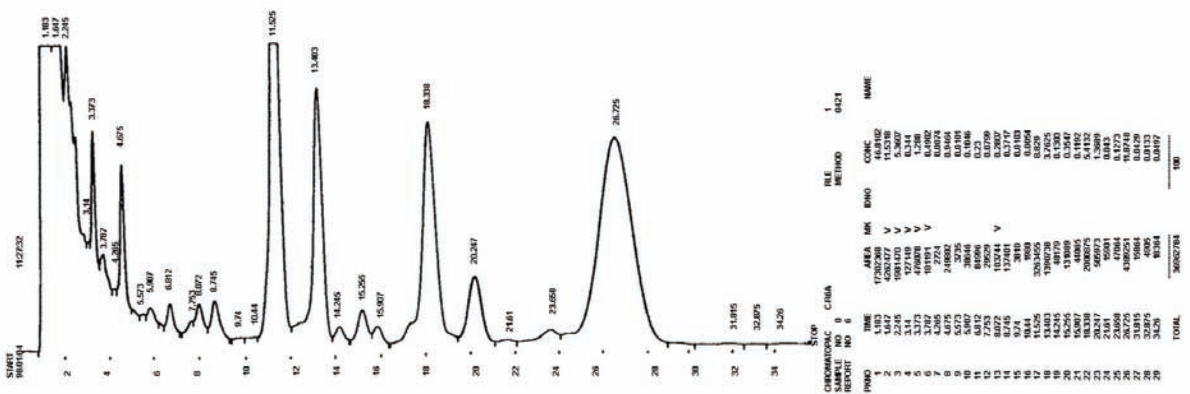


Fig. 3. HPLC for chestnut seed extract

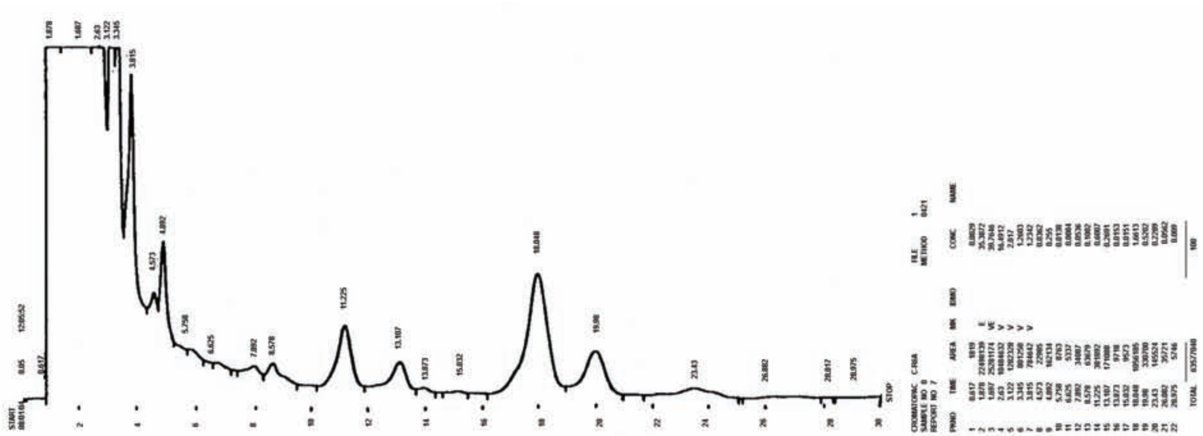


Fig. 4. HPLC for detect escine from II dermal cream

These extracts were incorporated in O/W bases – Synoderm GmbH-Germany (Table II).

Table II. The composition of topics formulas

Ingredients	**Quantity [g]**	
	I	**II**
Horse chestnut seed extract		
Horse chestnut bark extract		
Caffeine		

Allopurinol		
Sodium cromoglicate		
Kalium iodidum		
Collagen H08		
Benzyl alcohol		
Synoderm to		
Synoderm: isopropyl-palmitate, pentilenglycol, glycerilstearate, dihydroxicethyl-sodium-phosphate, cetearyl alcohol, Buxus chinesis, 5% urea, sodium loactate, lactic acid, panthenol, tocopherol acetate, water, pH = 6.46		
pH	6.60	6.48

All formulas were characterized from:

- physico-chemical point of view, determining:
    - pH (by potentiometrically methods, with IngLab pH Conductometry Level 1, at 24^0C)
    - rheological properties and viscosity using rheoviscometer Rheotest Typ RV2, at 24^oC
- the bioavailability of creams by clinical evaluation (for psoriasis) and anti-inflammatory effect on white mouse ears

Results and discussions

The efficiency of formulas with Mahonia extract was evaluated on 30 patients with different form of psoriasis: 21 patients with rounded plagues, 6 patients with guttated psoriasis and 3 patients with general psoriasis. After 6 weeks of treatment was observed symptoms amelioration in 80% of patients, by three applications per day.
We can observe:

- a good skin tolerance for both preparates
- a good skin penetrance after application
- a keratolytic effect after 2-3 weeks of treatment
- discolor of lesions after 4-5 weeks of treatment

The horse chest-nut seed extract contains 15.33 % escine (by HPLC analysis) and other poly-phenols (by TLC). The anti-irritant effect of second formula (more completely composition) was evaluated in the white mouse ears inflammation induced by turpentine oil treatment. Mouse experiences revealed that Synoderm basis reduces the irritation in 86.65% cases whiles II formula reduces the irritation in 95% cases (figure 4)

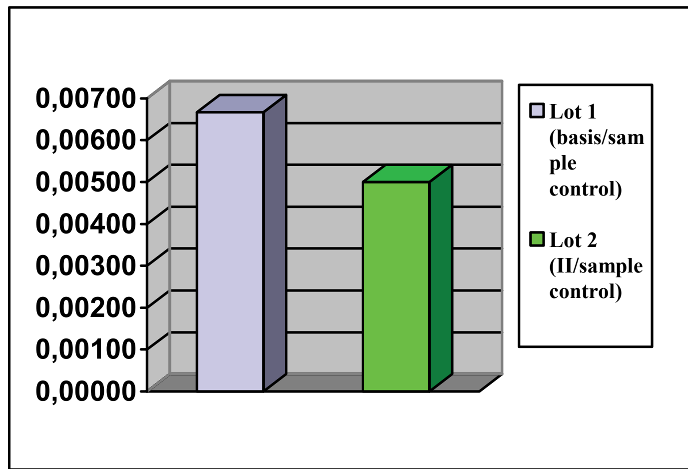


Fig. 5. Local action of formula II cream against experimental induced inflammation

Conclusions

We obtained satisfying results, especially on the patients with recent or small surface lesions of psoriasis. For psoriasis of all severity classes (mild to severe) was necessary an ointment, containing 10% Mahonia bark extract and over a period of 12 weeks. The preparates can use for a long time treatment because its have a good skin tolerance without adverse effects of the corticosteroids.
The formula with Synoderm – basis and horse chestnut seed and bark extracts can replace with success the topic treatment with corticosteroids. For a maximum effect we suggest the incorporation 3% of both extracts.

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LICHEN METABOLITES – NEW THERAPEUTIC AGENTS. *IN VITRO* STUDY OF ANTIOXIDANT AND ANTIMICROBIAL ACTIVITIES

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Summary

The antioxidant and antimicrobial activities of the chloroform and acetone extracts of 3 lichen species from Romania's spontaneous flora: Usnea barbata (L) Wigg., Parmelia physodes (L) ach. and Evernia prunastri (L) Ach. has been investigated. The extracts were found active against Gram (+) bacteria and fungi and also, exibited moderate antioxidant activity decreasing CCl_4 – induced lipid peroxidation in a concentration-dependent manner.

Keywords: *lichen metabolites, antioxidants, antimicrobial activity*

Introduction

Since ancient times some lichen extracts have been used in folk medicines to treat a variety of ailments. The studies shown that lichen metabolites exert interesting biological actions including antibiotic, antiviral, antiinflammatory, analgesic, antipyretic, antioxidant, antiproliferative and cytotoxic effects /1/.
Lichens produce a wide range of organic compounds that can be divided into two groups called primary metabolites and secondary metabolites. Primary metabolites are proteins, lipids, carbohydrates, and other organic compounds that are essential to the lichen's metabolism and structure. The chemistry of about one third af all lichen species has been studied up to now and about 350 secondary metabolites are known from lichens /2/.
Even though the manifold activities of lichen metabolites have now been recognized, their therapeutic potential has not been fully explored and thus remains pharmaceutically unexploited.
The aim of our study was to determine the potential beneficial properties of 3 lichen species from Romania's spontaneous flora: *Usnea barbata* (L) Wigg., *Parmelia physodes* (L) Ach. and *Evernia prunastri* (L) Ach., and to detect which secondary compounds are present in this species by using Thin Layer Chromatography.

Material and methods

Chemicals. Gallic acid, Folin Ciocalteu reagent, carbon tetrachloride, thiobarbituric acid, trichloracetic acid were purchased from the Sigma Chemical. All other unlabeled chemicals and reagents were analytical grade.

Lichen materials

Usnea barbata (L) Wigg*., Parmelia physodes* (L) Ach*. and Evernia prunastri* (L) Ach. were collected from the Sinaia region in 2004. Lichen species were identified by prof. dr. Nicolae Toma from Faculty of Biology, University of Bucharest.

Extraction of plant materials

100 g of lichen samples were extracted successively with chloroform and acetone using a microwave installation. The extracts were concentrated under reduced temperature and pressure using a rotary evaporator. After removing lipophilic compounds, the extracts were lyophilized and stored at –18 ˚C. Compounds were identified by TLC using methods standardized for lichen products /3,4/.

Determination of the total phenolic contents
The amount of total phenolic compounds (TPC) in the lichen extracts was determined with the Folin-Ciocalteu's reagent using gallic acid as standard. 500 µL of samples (three replicates) were introduced to test cuvettes, and then 2.5 mL of Folin-Ciocalteu reagent (diluted 1:10, v/v) and 2 mL of Na_2CO_3 (7.5%) were added. The resultswere expressed as miligrams of gallic acid equivalents (GAE) per gram of lyophylisates /5/.
Antimicrobial activity determination
In order to get general impression of the *in vitro* activity, the lichen extracts were tested against the following strains from the ICCF Type Culture Collection: *Staphylococcus aureus* ATCC 6538 P, *Bacillus subtilis* NCTC 2589, *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853 and *Candida albicans* ATCC 10231 according to the Kirby and Bauer disk diffusion method. Bacteria strains were inoculated onto nutrient agar plate (10^8 cells/ml) whereas fungus strain was inoculated onto potato dextrose agar plate (10^8 spores/ml).
For screening, 50 µL of each lichen extract was loaded onto sterile paper disks (7 mm diameter) allowing the solventto evaporate between applications and placed on the previously inoculated agar /6/. The plates were incubated for 48 hours at 37°C. Disks treated with chloroform and acetone served as negative control agents on the plates. Commercial bactericide chloramphenicol and fungicide ketoconazole were used as positive control substances.
Antioxidant activity assay
Antioxidant activity of lichen extracts was investigated by measuring TBARS (thiobarbituric acid reactive substances) formed in rat liver homogenates treated with carbon tetrachloride (CCl_4) /7/.
The amounts of peroxides formed in liver homogenates during incubation were determined specrophotometically by measuring absorbance at 535 nm. The inhibition of lipid peroxidation as percentage was calculated by following equation:

$$\% protectie = \left(1 - \frac{T - M}{I - M}\right) x100,$$

where T was the absorbance in the presence of the lichen extract sample,
M was the absorbance of the positive control reaction and
I was the absorbance of the negative control reaction .
High absorbance was an indication of a high concentration of formed peroxides.

Results and discussion

By spectroscopic methods and TLC a total of 18 known substances were identified in species examined (Table 1). These compounds were determined to be**: terpenes** - 16b, 22-dihidroxihopan-4a-oic acid (1), phlebic acid (2), **xanthones** - 4-chloro-3-O-methyl norlichexanthone (3), 2,7-dichloronorlichexanthone (4), 5-cloronorlichexanthone (5), thiomelin (6); **depsides** - 2'-methyl atranorin (7), 3-a hidroxibarbatic acid (8); **depsidones** - pannarin-methylether (9), conprotocetraric acid (10), cryptostictic acid (11), hypopsoromic acid (12), 5-dichlorovicanicin (13); **depsones** – hiperpicrolichenic acid (14), superhiperpicrolichenic acid (15); **dibenzofurans** - 9-methyl-4-hidroxipannarate (16), **usnic acids** – usnic acid (17), **antrachinones** – secalonic acid (18).

Table 1. The chemical constituents of the lichens *Usnea barbata (L) Wigg., Parmelia physodes (L) Ach. and Evernia prunastri (L) Ach.*

Lichens	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
U. barbata (chloroform extract)	*		*						*								*	
U. barbata (acetone extract)										*	*							
P. physodes (chloroform extract)		*	*	*		*	*					*	*	*	*	*	*	
P. physodes (acetone extract)					*			*			*	*						*
E. prunastri (chloroform extract)					*	*	*					*					*	*
E. prunastri (acetone extract)					*			*		*	*	*						*

In agreement with the literature data, the results, quantified by measuring the diameter of the zone of inhibition, showed that lichen extracts did not inhibit the Gram (-) bacteria but were active against Gram (+) bacteria and fungi. Both, chloroformic and acetonic extracts from *Usnea barbata* had the greatest effect on plates inoculated with *Staphylococcus aureus* ATCC 6538 P (with a mean zone of inhibition of 18 and 19.5 mm respective) and *Bacillus subtilis* NCTC 2589 (16 and 16.5 mm). Only chloroformic extracts of *Parmelia physodes* and *Evernia prunastri* had moderate effects on *Candida albicans* (13.5 and 16.5 mm).

Table 2. Antimicrobial activities of chloroform and acetone extracts of the lichens *Usnea barbata (L) Wigg., Parmelia physodes (L) Ach. and Evernia prunastri (L) Ach.*

Microorganisms	Chloroform extracts			Acetone extracts		
	Parmelia physodes	*Usnea barbata*	*Evernia prunastri*	*Parmelia physodes*	*Usnea barbata*	*Evernia prunastri*
Escherichia coli ATCC 25922	-	-	-	-	-	-
P. aeruginosa ATCC 27853	-	-	-	-	-	-
Staphylococcus aureus ATCC 6538 P	13 ± 1.00*	18 ± 1.00	17 ± 0.58	13 ± 1.00	19.5 ± 1.00	12 ± 2.00
Bacillus subtilis NCTC 2589	13 ± 0.00	16 ± 1.00	16.5 ± 1.00	12.5 ± 2.00	16.5 ± 1.00	14 ± 0.00
Candida albicans ATCC 10231	13.5 ± 1.53	-	16.5 ± 1.53	-	-	-

* Values are the mean of 3 replicate.

Although antioxidant activity (AA) of some depsides and depsidones, isolated from several lichen species, have been demonstrated, the antioxidant properties of lichens are poorly known and not many reports on the relationship between antioxidant activity and phenolic content are available /8,9/.

As seen from the Table 3, the chloroform extract of *E. prunastri* had the highest total phenolic contents (TPC), followed by the chloroform extract of *U. barbata* and acetone extracts of the same species.

In the present study, there was no linear correlation between AA and TPC values of the lichen extracts and species with similar values of TPC have distinct AA values. It can be suggested that all phenolics do not have the same antioxidant activity; some are potent while some have weak antioxidant activity. They also develop synergistic or antagonistic interactions with other types of components such as carbohydrates and proteins /8/. In addition, nonphenolic compounds may play a major role in the AA of plant material /9/.

Table 3. Antioxidant activity (AA) and total phenolic content (TPC) of the lichen species

Lichen	AA (%Inhibition)	TPC (mg GAE/g lyophylisate)
Chloroform extracts		
- *Parmelia physodes*	79% ± 1.00*	49.19 ± 4.48
- *Usnea barbata*	42% ± 1.33	60.02 ± 2.12
- *Evernia prunastri*	28% ± 2.00	64.91 ± 2.02
Acetone extracts		
- *Parmelia physodes*	48% ± 1.00	28.15 ± 1.98
- *Usnea barbata*	52% ± 0.00	55.26 ± 3.12
- *Evernia prunastri*	29% ± 3.33	58.74 ± 2.68

*The values are presented as mean ± SD.

The tested extracts showed moderate antioxidant activity. In general, the peroxidation of lipids was inhibited by the tested extracts in comparision with the control.
Parmelia physodes had a low phenolic content and showed potent antioxidant activity (79%). This difference between AA and TPC values may be attibuted to the presence of nonphenolic compounds, as well as other antioxidants.
Although chloroform and acetone extracts of E prunastri are the richest species in terms of phenolic compounds, it has low AA (28 and 29% respective). These results suggest that the AA of the some tested extracts might be attributed to the presence of non-phenolic compounds. Neverthless, it should be taken into consideration that individual phenolics may have distinct AA: there may be antagonistic or synergistic interactions between phenolic and other compounds like carbohidrates, proteins, etc.

Conclusions

Although it looks promising to use the secondary metabolites of the 3 lichen species in food and pharmaceutical industries, further investigations on the antimicrobial activity as well as the economical and fast isolation of the metabolite from the lichens are needed.

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PHYSICO-CHEMICAL STUDIES ON *ACER NEGUNDO* L. SEED OIL

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Summary

Acer negundo L. (pinnated maple) seed fatty oil was physico-chemically analysed. Fatty acids, phytosterols and monoglycerides profiles were obtained using a gas chromatographic-mass spectrometric analysis. Pinnated maple seeds were found to contain 20,76 - 21,55% oil rich in essential fatty acids: linoleic (32,44%), gamma-linolenic (8,39%) and alpha-linolenic acid (0,75%). Because of large spreading and important seeds production, pinnated maple could be a convenient source for gamma-linolenic acid, a rare compound of therapeutic interest.

Keywords: *pinnated maple, seed, gamma-linolenic acid*

Introduction

The fundamental importance of polyunsaturated fatty acids for human health is well-known. These fatty acids named essential (linoleic and linolenic) are not synthetized in organism and have to be taken from natural sources such as plant oils. They are not only a source of energy but have important biological roles because participate to the fundamental biochemical processes.
Gamma-linolenic acid was distinguished for its valuable therapeutic effects, especially with prophylactic importance. Its main actions demonstrated by medical research were: anti-inflammatory, hypocholesterolemic, protective for the vascular system and heart [1,3,6,8]. The current investigations have more purposes: to clarify its complex mechanism of action, to discover new medical applications, to establish the most efficient and safety doses. Gamma-linolenic acid is very rare, found in a few seed oils of some species from *Onagraceae*, *Saxifragaceae* and *Borraginaceae* families.
This study is an attempt to find new convenient vegetable sources for this important unsaturated fatty acid. We have chosen an arborescent species from *Aceraceae* family, *Acer negundo* L. (pinnated maple) because of its large spreading in spontaneous Romanian flora and important seeds production. According to scientific literature, pinnated maple seed oil has been summary investigated and has not been taken into account [4,5,7]. Currently, only two species of *Acer* genus have therapeutic uses: *Acer saccharinum* L. (sugar maple) as a source of sucrose and *Acer rubrum* L. (red maple) for astringent effect of tannins from its bark [1,9].
The purposes of this research were the extraction, quantitative determination and physico-chemical analysis of pinnated maple seed oil in order to establish its practical value and phytotherapeutic potential.

Material and methods

As vegetable material for this research we used the seeds of *Acer negundo* L (pinnated maple), collected from Horezu (Vâlcea district), Romania, in November 2005. Seeds were dried at room temperature under well ventilated conditions.
Acer negundo L. seeds were botanically examinated (macroscopic and microscopic study).
The oil content of seeds was determined by gravimetric method. The analytical research was carried out on fatty oil extracted with hexane by mechanical agitation at room temperature.

The physico-chemical characteristics of fatty oil (refractive index, relative density, iodine, acid and saponification values) were determined by the official methods [10].
The compositional data of the oil (free and esterified fatty acids, phytosterols and monoglycerides profiles) were obtained using a chromatographic technique (gas chromatographic -mass spectrometric analysis, GC/MS) [2,10]. Gas chromatograph (Agilent Technologies 6890N) equipped with a mass spectrometer (Agilent Technologies 5973) was used. For a differentiated analysis of the oil compounds, the samples were derivatised with two reagents. Free and esterified fatty acids were transformed in methyl esters by derivatisation with methanol in alkaline medium (hydroxide sodium) and were analysed in this form. Another sample of the oil was derivatised with bistrimethylsilyltrifluoracetamide for more volatile trimethylsilylethers derivatives of free fatty acids, phytosterols and monoglycerides analysis.

GC/MS conditions for methyl esters analysis

- capillary column: DB-WAX (30m x 0,32mm); stationary phase-polyethylene glycol (0,15 μm-film thickness); carrier gas, flow rate: helium for chromatography-1,5ml/min.; solvent delay - 6,5 min.; injection volume-1μl; split ratio-1:50; injection temperature-250^{0}C, interface temperature-280^{0}C; ionization source temperature-230^{0}C; quadruple temperature-150^{0}C.

GC/MS conditions for trimethylsilylethers derivatives analysis

- capillary column: DB5-MS (60m x 0,25mm); stationary phase-polyphenilsiloxane 5% (0,25 μm-film thickness); carrier gas, flow rate: helium for chromatography-1,2ml/min.; injection volume-1μl; split ratio-1:10; injection temperature-250^{0}C; interface temperature-280^{0}C; ionization source temperature-230^{0}C; quadruple temperature-150^{0}C.

The oil compounds were identified by the comparison of their mass spectra with Nist and Willey mass spectra data bases. The quantitative evaluation of chromatograms was made on the basis of direct proportionality of the peak area (normalization procedure). The percentages of fatty acids, sterols and monoglycerides of the total identified compounds were determined. In addition, because of its biological importance, gamma-linolenic acid was quantificated by using reference standard (gamma-linolenic acid for chromatography, Sigma) and its concentration was expressed in grams per 100 grams of oil.

Results and discussions

Polygonal cells of seed-coat with thickened and pigmented walls were established.
The pinnated maple seed fatty oil content was substantial (20,76-21,55%). The physico - chemical characteristics and the results of chemical analysis of the oil were presented in tables I – III and figures 1-2.

Table I. The results of the oil characteristics determination

Vegetal material	Water content g%	Oil content g%	Physico-chemical properties of the oil				
			n_D^{20}	d_{20}	I_A	I_I	I_S
seeds	6,61-6,85	20,76-21,55	1,4911	0,9451	20,04	100,21	190,42

Legend: n_D^{20} - refractive index; d_{20} - relative density; I_A - acid value; I_I - iodine value; I_S - saponification value

Table II. Fatty acids levels of pinnated maple seed oil

	Fatty acid	Carbon atoms Double bounds	Retention time (minutes)	% fatty acid of total
1.	palmitic acid	C16:0	15,22	3,94
2.	7-hexadecenoic acid	C16:1	15,35	0,03
3.	palmitoleic acid	C16:1	15,43	0,13
4.	heptadecanoic acid	C17:0	16,41	0,09
5.	stearic acid	C18:0	17,66	1,48
6.	oleic acid	C18:1	17,82	16,73
7.	11-octadecenoic acid	C18:1	17,90	2,34
8.	7-octadecenoic acid	C18:1	18,15	0,13
9.	linoleic acid	C18:2	18,35	32,44
10.	gamma-linolenic acid	C18:3	18,65	8,39
11.	alpha-linolenic acid	C18:3	19,11	0,75
12.	stearidonic acid	C18:4	19,46	0,06
13.	arachic acid	C20:0	20,58	0,20
14.	11-eicosenoic acid	C20:1	20,77	6,46
15.	behenic acid	C22:0	24,09	0,70
16.	erucic acid	C22:1	24,35	17,53
17.	lignoceric acid	C24:0	28,11	0,29
18.	nervonic acid	C24:1	28,45	8,15

Table III. Free fatty acids, sterols and monoglycerides levels - pinnated maple seed oil

	Compound	Retention time (minutes)	% compounds of total
1.	palmitic acid	18,79	8,34
2.	gamma-linolenic acid	20,88	5,49
3.	linoleic acid	21,18	31,80
4.	oleic acid	21,26	15,63
5.	alpha-linolenic acid	21,32	1,38
6.	11-octadecenoic acid	21,37	2,51
7.	stearic acid	21,67	1,63
8.	11-eicosenoic acid	24,89	2,49
9.	monoolein	27,49	4,36
10.	docosatrien	29,46	1,43
11.	cholest-2-eno-naphthalen	33,28	9,25
12.	stigmasterol	40,26	3,33
13.	3 beta-hydroxi-ergost-7-en	41,48	3,33
14.	3 beta-hydroxi-ergost-7,22-dien	42,25	8,96

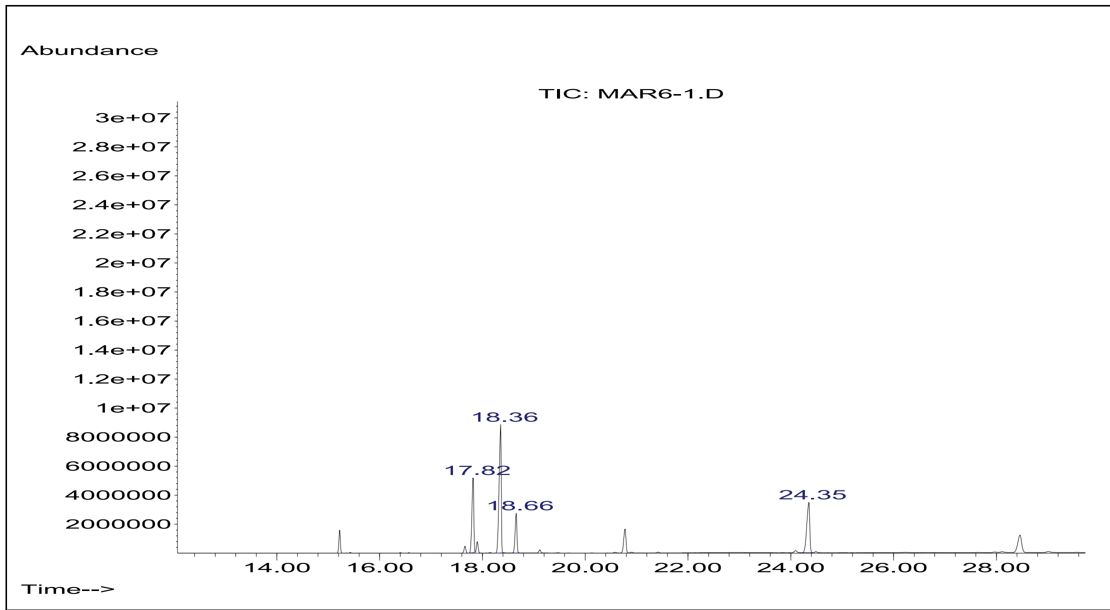

Fig. 1. Gas chromatogram of methyl esters

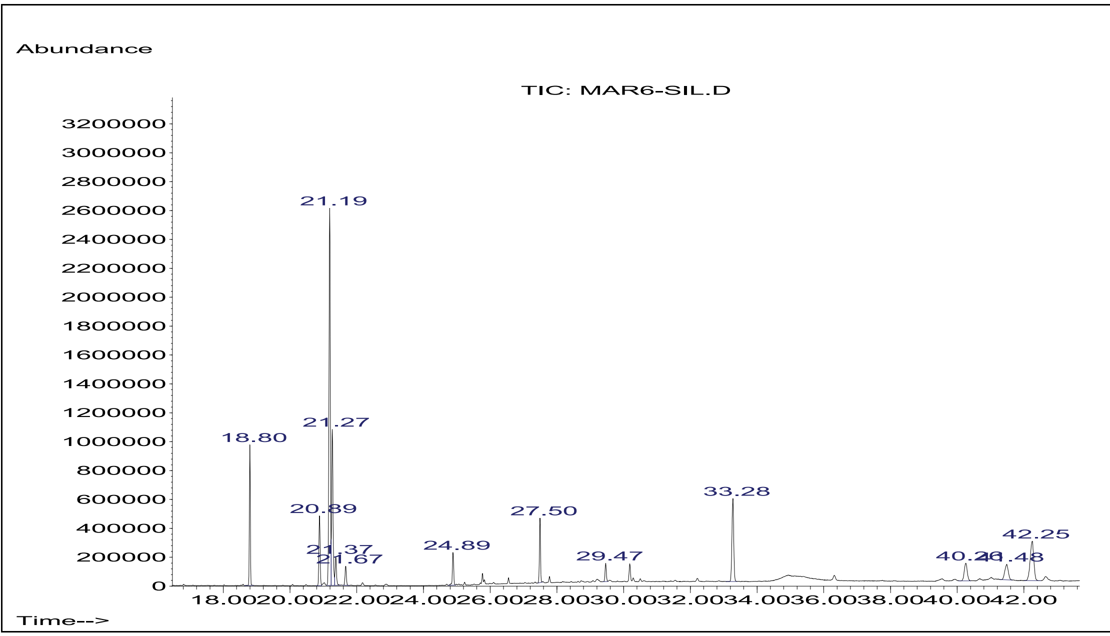

Fig. 2. Gas chromatogram of trimethylsilylether derivatives

The proportion of unsaturated fatty acids identified in pinnated maple seed oil was nearly 85% of the total fatty acids. The content of essential fatty acids (EFAs) was relative high (41,64%). Linoleic acid was the main constituent of the EFAs fraction (32,44% of the total fatty acids). The most valuable acid of EFAs fraction is gamma-linolenic that was found to be present in a significant quantity (8,39% of the total fatty acids; 4,87 g /100g oil, respectively) and alpha-linolenic acid level is very low (0,75% of the total fatty acids). The monounsaturated fatty acids identified were: erucic (17,53%), oleic (16,73%), nervonic (8,15%), 11-eicosenoic (6,46%), 11 - octadecenoic (2,34%), 7-octadecenoic (0,13%), palmitoleic (0,13%) and 7-hexadecenoic acid (0,03%).The total saturated fatty acids was 15% only (palmitic acid-3,94%, stearic acid-1,48%, arachic acid-0,20%, behenic acid-0,70%, lignoceric acid-0,29%). The principal free fatty acids identified in pinnated maple seed oil were unsaturated: linoleic, the major constituent (31,80%), oleic (15,63%), gamma-linolenic

(5,49%), alpha-linolenic (1,38%), 11-octadecenoic (2,51%) and 11-eicosenoic acid (2,49%). One monoglyceride, monoolein (4,36%) and one usual phytosterol, stigmasterol (3,33%) were identified. The presence of gamma-linolenic acid, stearidonic acid, monoolein and phytosterols in pinnated maple seed oil was not reported by scientific literature that we consulted. The others usual fatty acids identified in pinnated maple seed oil were quoted in literature, in percentages little different from the results of this study because of various pedoclimatic conditions.

Conclusion

Pinnated maple seeds were found to have significant therapeutic potential because of important oil content (20,76-21,55%) and essential fatty acids level (41,64%). Because modest gamma-linolenic oil level (8,39%) comparative to *Oenothera* species (13,75-15,70%), was compensated by abundance in nature, pinnated maple seeds could be a precious source for this rare and very important polyunsaturated fatty acid.

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ULTRASTRUCTURAL EFFECTS OF *NIGELLA SATIVA* TOTAL ALKALOIDS EXTRACT AT LIVER LEVEL (*MUS MUSCULUS*)

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Summary

The single or combined effect of a total acid alkaloid extract obtained from Nigella sativa seeds, administered in distilled water or in deuterium-depleted water (DDW), against a stress factor (the whole body X-irradiation), on the liver ultrastructure of Mus musculus, was analysed. The single action of X-rays induced alteration of the hepatocyte structure. DDW induced some minor's modifications, especially the increase of the lysosome numbers, and the cell metabolic activity. The total alkaloid extract in DDW had not a protective effect at the lever level.

Keywords: *alkaloid, Nigella sativa, radioprotection, liver.*

Introduction

Deuterium-depleted water (DDW) is distilled water, with a smaller isotopic deuterium concentration (120 - 30 ppm), than its concentration in natural water (144 ppm D/D+H; STEFANESCU et al., 2004). SOMLYAI et al. (2004), suggest that some cells can modify the D/H ratio through some molecular mechanisms, probably implied in the cell cycle regulation. Also, SOMLYAI et al. point out that DDW causes the tumour regression in the xenotransplanted mice. Nigella sativa (Fam. Ranunculaceae), is a specie of great medicinal importance, used for edible and medicinal purposes in Arab or Mussulman countries (India, Pakistan, Saudi Arabia, Syria, Iran, Egypt), or other countries (AHMAD et al., 2004). Pharmacological activity of different extracts from seeds, are multiple: anti-inflammatory action (sore throat), anti-arthritic action (rheumatoid arthritis), analgesic properties (toothache, migraines), hormonal activity (regulates period pains, increase milk flow and is beneficial against hot flushes), anti-septic properties (mouth sores), anti-viral (cold and flu, a/o), dermatological activity (acne and eczema), urinary tract activity (kidney stones), immune system activity (increases resistance to illness), gastro-intestinal tract activity (nausea, diarrhoea, flatulence, colic, constipation and piles), circulatory system activity (improves efficiency of the heart), nematocidal activity (effective against tapeworms), respiratory tract activity (short breath), a/o (AHMAD et al., 2004). From the *Nigella sativa* seeds, were isolated three alkaloids: *nigellicine*, *nigellidine* and *nigellamine-N-oxide*. Nigellamine-n-oxide is structurally related to quinolone, being used in the treatment of malaria and for the prophylactic treatment of the cardiac arrhythmias. Also they haven vasodilator effects and can therefore be used in treatment of migraine and also tend to have anti-psychotic effects (AHMAD et al., 2004). The alkaloids are capable of affecting human physiology, are the alkaloids role from *Nigella sativa*, and are incomplete studied until now.

Material and methods

Biological material

Nigella sativa (Fam. *Ranunculaceae*) has been used since ancient times for different purposes in health, alimentation or cosmetics. Many researches point out the anticarcinogenic activity

of the different seeds constituents (MADENICA et al., 1997; CORNEANU et al., 2005, a/o). In this experiment, seeds of *Nigella sativa* L., Craiova population were used, from which a total acid alkaloid extract was obtained.
The experiment was performed on a young white mouse population (*Mus musculus* L.), of 20-25 g each, 3-5 exemplars per experimental variant.

Work method
Total alkaloid extract. 100 g seeds broken into small pieces of 1 mm diameter have subject to extraction with 400 ml 15% NaOH for 2 hours under continuous agitation, for alkaloid liberation from cells. The alkaloid separation from the obtained total basic extract with 400 ml n-butane solution (400 ml), for 30 minutes, by strong agitation, the alkaloids passed in the acid phase. In this experiment, a dilution of 1% from the crude total acid alkaloid extract obtained, in distilled water or in DDW with 30 ppp deuterium, were used. The animals were peritoneal injected, every other day, with 0.5 ml solution per animal (five injections).
Stress factor. Half of experimental animals were irradiated (entire body) one day after the thirds injection, with an X-ray source (an RUP 150/130 apparatus), at the following parameters: 250 kV, 5 mA, DF = 50 mm, 1 mm filter diameter, dose output 0.528 Gy/min, in an unique dose of 5.28 Gy.
Electron microscopy investigation. Experimental animals were sacrificed through the jugular vein section and then the biological material was harvested for investigations. For electron microscopy analysis, pieces of about 1 mm^3 of liver were prefixed in glutaraldehyde, postfixed in Milloning fixation and included in EPON 812. The serrated sections of about 90 nm thick were contrasted with uranyl acetate and analysed at a TEM Philips CM 120 microscope, in *Ovidius* University from Constanta.

Results and discussions

Control variant. The structural features in the control variant are characteristic for this organ and specie. The hepatic lobule present a centrolobular vein covered with an epithelium. Around the centrolobular vein, are disposed the sinusoid capillary. The hepatocytes of polygonal shape are disposed in rows. Every hepatocyte present one (two) nuclei of oval-spherical shape. In cytoplasm are present numerous mitochondria, normal structured, with an electron-dense matrix (Fig. 1). Among mitochondria are dispersed a rugous endoplasmic reticule formed from narrow profiles, usually disposed around the mitochondria (Fig. 1).
At the vascular pole, the hepatocytes present microvillus evaginated in the Disse space. In sinusoid capillary, there are Kupffer cells with numerous lysosomes and with a normal activity. Smooth endoplasmic reticule, as well as the dictyosomes, is poor represented. The lipids drops are present in a small quantity, being represented through small drops, disposed with predilection toward the vascular pole (in transit; Fig. 2).
Both in Control variant, and in other experimental variants, in hepatocytes were observed some parasite entities (viruses), and some filamentous structures as result of the metabolic activity (CORNEANU et al., 2006).
X-irradiation effect. Comparatively with the Control variant, under action of the X-rays irradiation, were induced some adulterations. The hepatocytes nuclei present an unregulated shape outline (Fig. 3). In some of them, the nuclei are hypertrophied. In nuclei, the vacuole and pars amorphous components are also hypertrophied. The smooth endoplasmic reticule is proliferated, as reaction at the destructive action of the X-rays. Rugous endoplasmic reticule presented dilated cisterns, and little ribosome's associated, because of the diminished of the metabolic activity and of the protein synthesis (Fig. 3). Also, was emphasised a depletion of the glycogen. In some hepatocytes, in which are present small focuses of cytoplasm lyses, the

nuclei are pycnotic and hyperchrome, as well as a lipid accumulations under shape of drops of different size. As result of the advanced of the adulteration in the hepatocytes, the plasmalemma was destroyed the cellular compounds being free in sinusoid capillary. In some hepatocytes, the perinuclear space is dilated and the chromatin is uniformly (a degradation process). The Kupffer cell is inactive (Fig. 4) and in the Disse space is present accumulation of glycogen.

DDW effect. In comparison with Control variant, the hepatocytes present some slight adulterations which not affect significant the cell metabolism. The nuclei present a structure almost normally with a spherical-oval shape and with heterochromatin disposed in the electron dense blocks at their periphery. In cytoplasm are present numerous mitochondria which present their matrix slightly electron-dense in comparison with the Control variant. The rough endoplasmic reticule is in an intense activity, in comparison with the Control variant, being disposed in parallel profiles, apt for the protein synthesis, but present some slightly dilatations (Fig. 5). Also, the smooth endoplasmic reticulum is hypertrophied in comparison with the Control variant, being implicate in the detoxification process (Fig. 5). At the vascular pole, the hepatocytes present numerous microvilli, which denote an intense activity of absorption of the metabolites. In some hepatocytes, is observed a slightly lipids retention. Not are present the collagen accumulation in the Disse spaces.

The Kupffer cells, present a normal activity, having many lysosomes (Fig. 6). In the macrophages are present the primary lysosomes and the cellular residues.

The irradiation in the DDW presence effect. On the basis of the slightly adulteration induced by the DDW presence in hepatocytes, the X-irradiation accentuated the adulterations effect of the hepatocyte ultrastructure. This suggests that DDW not present a radioprotective effect. In this variant, the amount of rough endoplasmic reticule is bigger represented in the cell, in comparison with the case of the irradiated animals. Also, in the case of combined action of the DDW and a stress factor (X-rays), the drops lipids is in a smaller number in the cells, in comparison with the Control, unirradiated or irradiated. The nuclei present an irregular contour, and in generally the chromatin is rarefied. The nucleolus is hypertrophied and the vacuole component and pars amorphous are enhanced quantitatively, or with an adulterated structure (Fig. 7). The cytoplasm matrix is rarefied, with many lyses area, of small size. Also, was observed a reduction of the number of cytosol ribosomes. Mitochondria are present in a smaller number, being polymorphous as size and shape. As effect of the X-irradiation, in hepatocytes are maintained a high quantity of rough endoplasmic reticule.

At the vascular pole of the hepatocytes, the plasmalemma of some cells is dense, many compounds migrates in sinusoids (Fig. 8).

Alkaloid extracts effect. The application of the alkaloid extract in DDW, not affect the normal structure of the hepatocyte. The nucleus present a polymorphism regarding their shape and the stage of cellular cycle. In some cells, the chromosomes are well structured, the cells being in an intense metabolic activity (Fig. 9). In the cells are present an accumulation of lipid drops. The rough endoplasmic reticule is disposed in parallel profiles, having numerous ribosome's, propitious for the protein synthesis (Fig. 10). The mitochondria present an electron dense matrix and cristas poor represented. The vascular pole of the hepatocytes, present a different structure, depending on the hepatocytes position. At the hepatocytes situated toward the periphery of the hepatic lobule, the microvillus are evaginated in the Disse space, while at the hepatocytes situated toward the centro-lobular vein, the microvillus are absent.

X-irradiation in the alkaloid extracts presence. The ultrastructural modification induced by the X-rays, in the presence of the DDW, were major in comparison with the adulteration recorded under the action of the X-rays alone. In some cells, the nucleus is of normal shape, with heterochromatin disposed in blocks in its inner as well as on the inner part of the nuclear

envelope (Fig. 11). The smooth endoplasmic reticule was in a bigger quantity, and rough endoplasmic reticule presented the slightly the dilated profiles (Fig. 12). In other cells, the cytoplasm present lyses areas, rough endoplasmic reticule cistern dilated and nucleus with rarefied chromatin. The mitochondria presented a slight polymorphism, in comparison with the alkaloid extract application alone. Their matrix is electron-dense and cristas are poor represented. At the vascular pole, the microvilli are rarefied. The Kupffer cells are inactive, without lysosomes. The collagen fibbers are well represented.

Conclusions

Under action of a source of X-rays of sublethal value (5.28 Gy), the hepatocyte ultrastructure is adulterated, especially the nucleus, mitochondria and endoplasmic reticule. Also, are affected the quantity of the lipids drops and glycogen amount from the cells, as well as the Kupffer cell ultrastructure.
Under action of the DDW, are established some slightly adulteration of the hepatic cells, which not significant affect the cell metabolism.
The DDW not present a radioprotection effect, because at the combined application of the two factors (DDW and X-rays), the adulteration effect of the hepatocytes induced by X-rays alone applied, was enhanced.
The application of the alkaloid extract in DDW, affect the normal structure of the hepatocyte. The irradiation in the presence of the alkaloid extract diluted in DDW, induced major modifications, in comparison with the X-rays action alone. The alkaloid extract dilute in distilled water, or in DDW, not manifest a protector effect at the lever level, in comparison with the protector effect at the spleen level, manifest by the same alkaloid, in same species.

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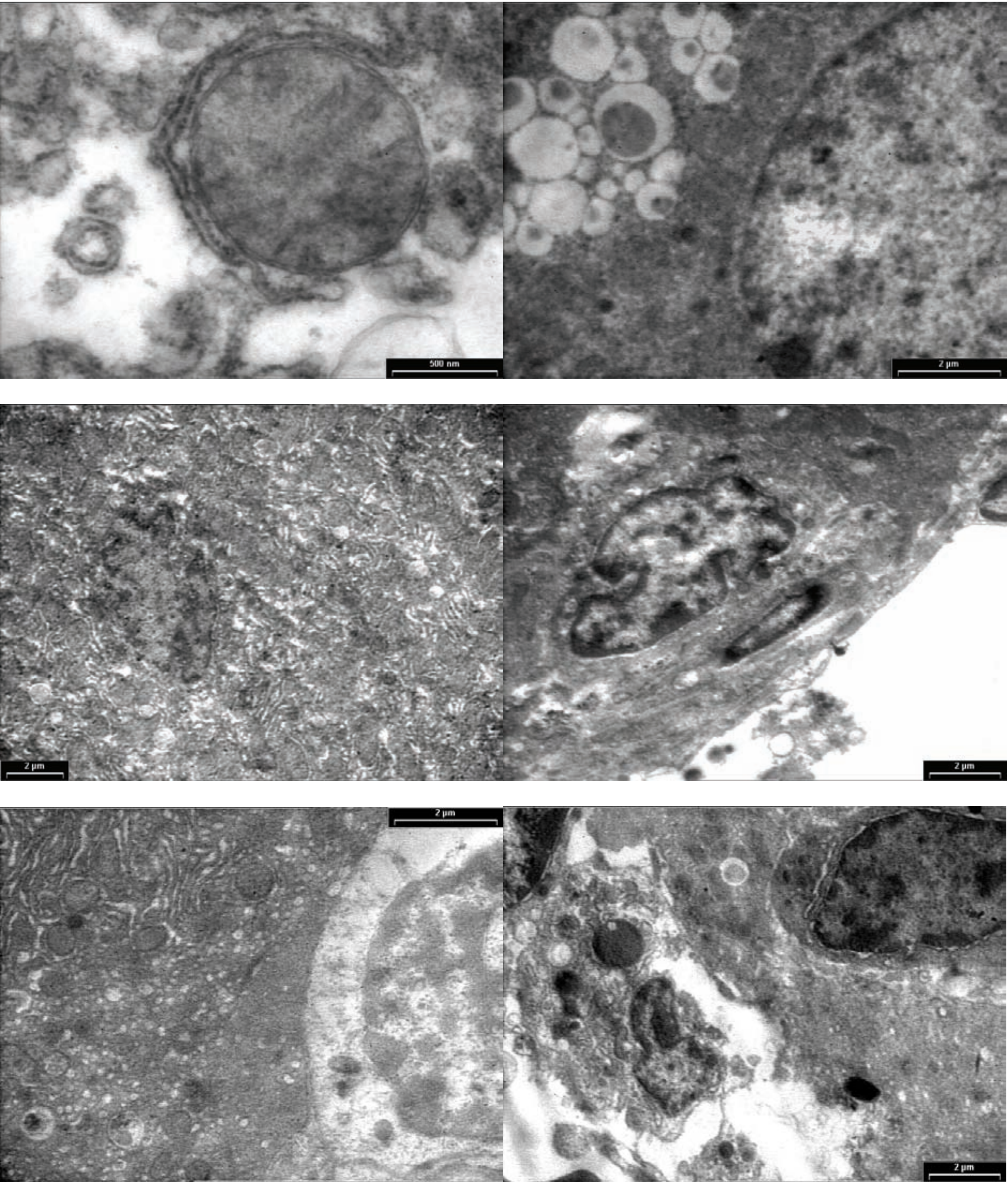

Plate 1. Liver ultrastructure in *Mus musculus*.

Fig. 1. Control. Mitochondria and endoplasmic reticule. Fig. 2. Control. Nucleus structure and lipid drops in the vascular pole of the hepatocyte. Fig. 3. X-rays. Nucleus with unregulated shape and endoplasmic reticule with dilated cisterns. Fig. 4. X-rays. An inactive

Kupffer cell. Fig. 5. DDW effect. Endoplasmic reticule hypertrophied. Fig. 6. DDW effect. The Kupffer cell in metabolic activity.

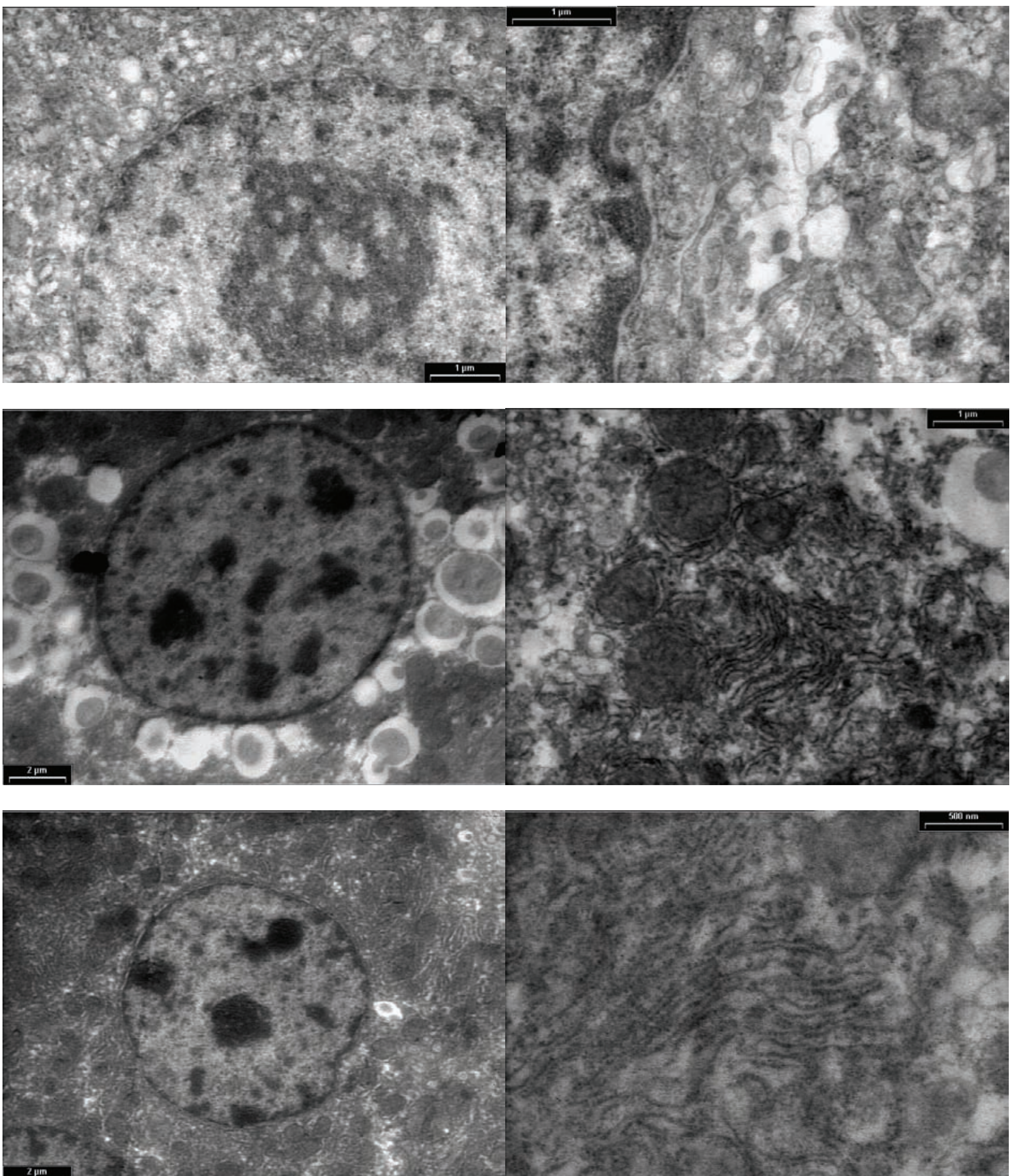

Plate 2. Liver ultrastructure in *Mus musculus*.

Fig. 7. DDW and X-rays effect. The nucleus and nucleolus ultrastructure. Fig. 8. DDW and X-rays action. The vascular pole of the hepatocyte. Fig. 9. Alkaloid in DDW. Nucleus ultrastructure with structured chromosome. Fig. 10. Alkaloid in DDW. Rugous endoplasmic reticule. Fig. 11. X-rays and alkaloids in DDW. The nucleus ultrastructure. Fig. 12. X-rays and alkaloids in DDW. Endoplasmic reticule with slightly dilated profiles.

IN VITRO RADIOPROTECTIVE EFFECT OF A TOPICAL GEL WITH COLLAGEN-GREEN TEA MIXTURE

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Summary

Mixtures of a green tea (GT) extract and collagen (COL), in different ratios (GT:COL 1:50, 1:100, 1:200) were prepared. In vitro biological tests regarding the degree of cell adhesion to these mixtures, conditioned as culture substrates, and the morphology of adhered cells were performed. Results indicated that the GT:COL mixtures were biocompatible and 1:50 GT:COL mixture was conditioned as hydrogel. Its UV radioprotective efficacy was demonstrated using a stabilized epithelial cell line.

Keywords: *green tea, collagen, antioxidant, radiation, hydrogel*

Introduction

Solar ultraviolet (UV) irradiation of human skin may cause sunburn, immunosuppression, oxidative stress, skin cancers as well as premature aging (photoaging) through yet unclear mechanisms that lead to overexpression of matrix metalloproteinases and denaturation of collagen and elastin [1-6]. Among the three regions of sunlight UV radiation, UV-B is particularly known to affect skin by altering cellular function via DNA damage [7], generation of ROS [8], and the resultant alterations in a large variety of signaling events [9, 10].

Green tea (*Camellia sinensis*) (GT) contains polyphenolic compounds, which gain great interest due to their significant antioxidant and anti-inflammatory activities [11]. It has been shown that GT have remarkable preventive effects against phototoxicity in hairless mouse models [12] as well as in humans [13].

Collagen (COL) is the most abundant protein in animals and the major component of connective tissues. COL isolated from natural sources it is increasingly used for surface engineering of biomaterials to accelerate receptor-mediated cell adhesion [14] and for composites intended to regenerative medicine [15-18].

Although GT extracts have become popular additions to topical skincare products, no studies investigating GT-COL mixtures and their effects on ageing skin have been published to date. A new conditioning formula for the GT-COL product, as hydrogel, is proposed in this work,because of its ability to absorb and retain water [19], an important requirement for skin wound dressings [20].

The aims of this paper were:

- to establish the best GT:COL ratio, in order to obtain a biologic active mixture which combines the antioxidative properties of GT with COL's biocompatibility and
- to investigate the *in vitro* UV radioprotective efficacy of a GT-COL-based hydrogel using a stabilized epithelial cell line.

Material and methods

GT-COL mixture preparation. A known quantity of GT was boiled, in bidistilled demineralized water (1:10), for 3 min. COL was prepared from bovine tendons by pepsin treatment and was dissolved in acetic acid at 5 mg/mL [21]. Mixtures of GT-COL were prepared in 1:50, 1:100, and 1:200 (w/w) ratios. Small quantities from these mixtures were

placed in 12-well culture plates and incubated for 8h, at 37^0C, resulting in biologic substrates for cell culture.

GT-COL hydrogel preparation. The GT:COL 1:50 mixture was added in a 1% (w/v) Carbopol gel to final concentrations of 10%, 15% and 20% (w/w), respectively, resulting in colourless, solid hydrogels.

Cell adhesion. Human dermal fibroblasts ($4,3x10^4$ cells/mL) from 7 passage were cultivated on GT-COL substrates. After 4h of cultivation, the adhesion degree was determined by cell trypsinization and counting on a THOMA haemocytometer.

Cell morphology. Dermal fibroblast cells ($4,3x10^4$ cells/mL) were cultivated, for 3 days, on GT-COL substrates. In order to analyze their morphology, after 72h of cultivation, cells were stained and photographed at a Nikon inversed-phase microscope.

In vitro model for GT-COL hydrogel radioprotection evaluation. A desired quantity of GT-COL hydrogel was placed on the bottom of several Petri dishes and sterilized. Epithelial cells from a stabilized line (VERO) were placed on this substrate, at a cell density of $1x10^5$ and cultivated in DMEM containing 10% fetal bovine serum and 1% antibiotic mixture, in a humidified atmosphere with 5% CO_2, at 37^0C, for 24 h. A Petri dish with cells cultivated on plastic, in the same conditions, was used as control. UV-B irradiation, in a minimal dose, was performed using a fluorescent lamp that emitted an energy peak at 312nm, in both cells and hydrogel+cells. After irradiation, the Petri dishes were incubated for 48h, in the same described culture conditions.

Cytotoxicity assay. After 48h, the drug- and radiation-induced cytotoxicity in untreated and irradiated cells+hydrogel, respectively, was measured using the 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) test [22]. Briefly, the medium was discarded and replaced with MTT solution. After incubation at 37^0C, the formed formazan crystals were dissolved and absorbance at 570 nm was registered on an UV-VIS spectrophotometer (Jasco V-530). Results were expressed as percentage from the control (untreated cells) considered as 100 %. The experiments were performed in triplicate.

Results and discussion

Previous experiments in our lab have showed a high content and a complex distribution of polyphenols in the GT extract [23]. Also, its antioxidant activity, assessed by two different methods, as TEAC (Trolox equivalent antioxidant activity) against DPPH (2,2-diphenyl-1-picrylhydrazyl) free radical [24] and as ferric reducing power [25], was higher than that of Trolox, a synthetic analog of vitamin E and of BHT, a synthetic standard antioxidant. GT extract was also found to possess significant metal chelation activity in the 1-50 µg/mL range of tested concentrations (data not shown). These molecular defense mechanisms against radiation-induced oxidative stress were mimicked in a cell-free system, in the presence of a GT extract and demonstrated its potential as radioprotective product.

GT-COL mixture in vitro biocompatibility evaluation

COL is known as a good substrate for the adhesion and growth of different cell types [26, 27]. In order to assess the biocompatibility of various GT-COL mixtures, they were dried on the bottom of culture plates and used, in this experiment, as cell substrates. For *in vitro* tests of cell adhesion degree and cell morphology it was used a stabilized epithelial cell line (VERO). The results, presented in Table 1, showed that the adhesion percent of dermal fibroblasts to biologic substrates was similar to control (plastic) for the GT:COL mixtures in 1:50 and 1:100 ratios. It was also observed a smaller degree of cell adhesion to 1:200 GT:COL mixture as substrate.

Table 1. Percentage of adhered cells

Substrate	Adhered cells (%)
Plastic (control)	88,8
GT-COL 1:50	87,30
GT-COL 1:100	85,30
GT-COL 1:200	75,00

After three days of cultivation, the morphology of the adhered cells to the GT-COL mixtures was observed by light microscopy (fig. 1).

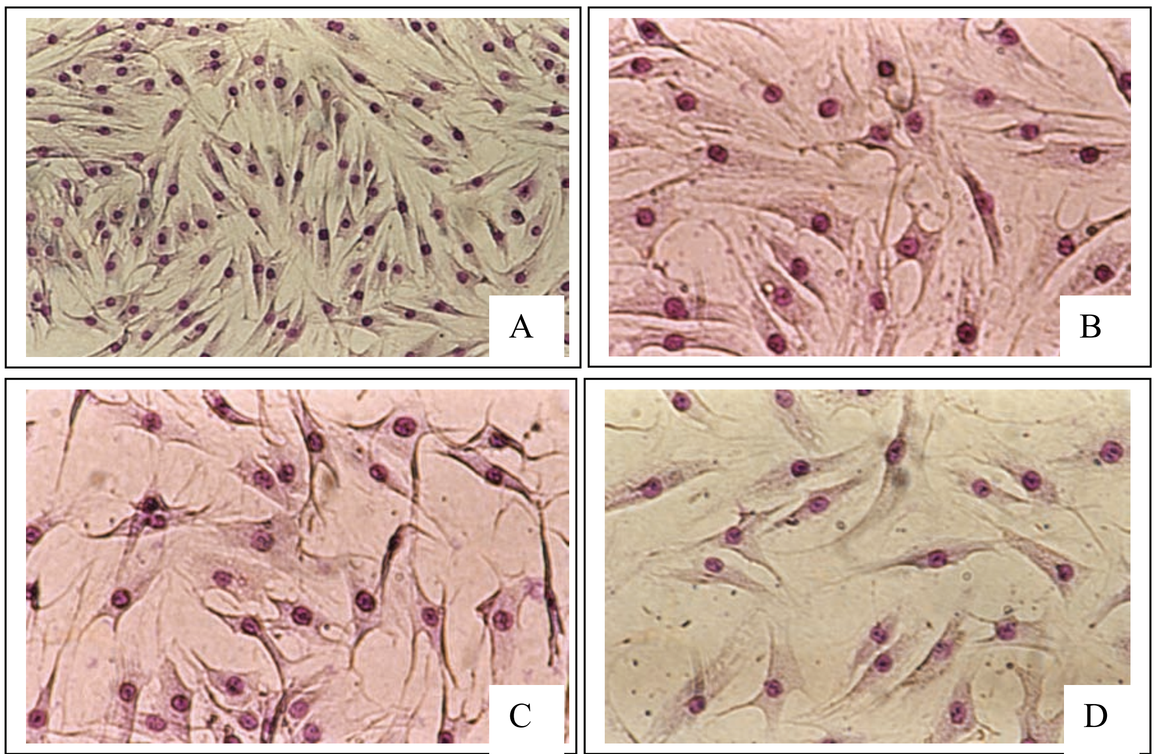

Fig. 1 - Light micrographs showing the morphology of dermal fibroblasts cultivated on plastic (A) and on GT-COL mixtures in 1:50 ratio (B), 1:100 (C), and 1:200 (D). (Giemsa staining)

It was noticed that the cells had, in majority, a polygonal, fusiform morphology, with long, finely branched cytoplasmic projections, firmly anchored into the matrix (fig. 1 B-D). Cell spreading as a network on the entire surface of the biologic substrates demonstrated their *in vitro* biocompatibility.

Biological tests indicated that the GT-COL mixtures were biocompatible. The best cell growth and adhesion were observed on GT:COL in 1:50 and 1:100 ratios. Because GT:COL 1:50 had the highest quantity of antioxidant (GT), this mixture was selected in order to be used, as biologic active complex, in different concentrations, in the composition of a Carbopol-based hydrogel.

***In vitro* testing of the GT-COL hydrogel as an UV protective screen for epithelial cells**

In order to establish the UV-B radiation dose which allowed maintaining a high cell viability (more than 80%), epithelial cells were irradiated with an UV lamp, for various exposure periods. After 48h of incubation in standard culture conditions (air with 5% CO_2), it was assessed their viability using the MTT test which was based on the ability of NAD and

NADH dehydrogenases from viable cells to catalyze the reduction of soluble tetrazolium salts to dark blue formazan crystals [28].
The UV-B irradiation time which induced a 85% cell viability corresponded to 0,5 minutes exposure (fig. 2).

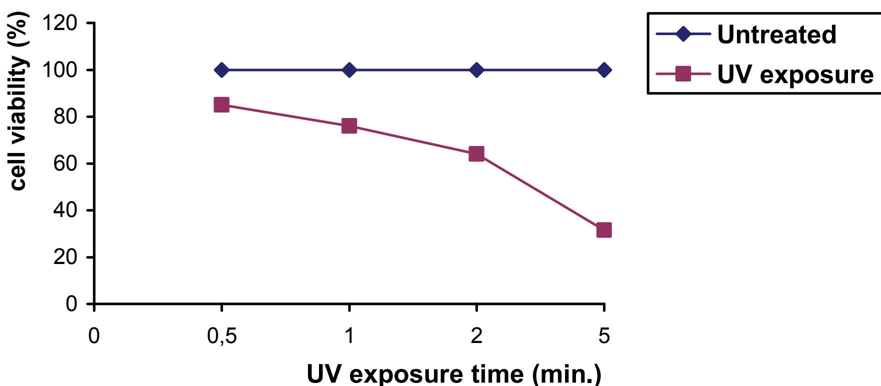

Fig. 2. Effect of ultraviolet exposure time on cell viability.
Untreated control was considered as 100% viable cells.

When cell irradiation took place in the presence of various hydrogels, it was registered an increase of cell viability in a GT-COL concentration-dependent manner (fig. 3).

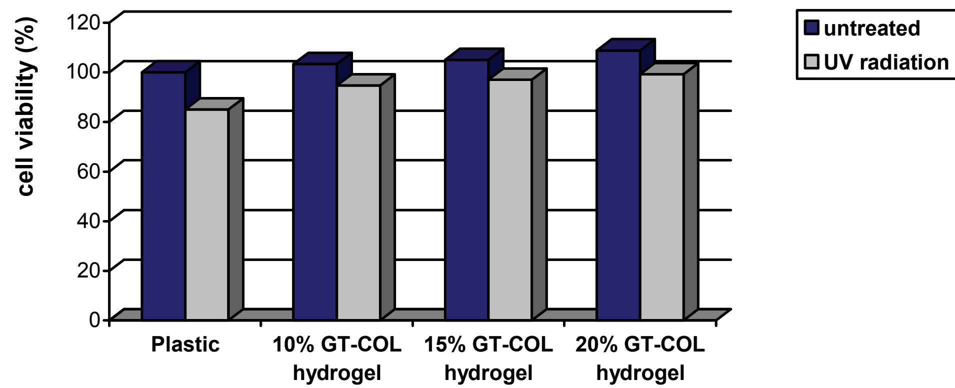

Fig. 3. Effect of GT-COL hydrogels on cell viability, at 48 h after UV irradiation.

All hydrogel types rendered UV-B protection, in comparison to the unprotected irradiated cells and the best UV protection was observed for 20% GT-COL hydrogel.
Another experiment group was represented by cells cultivated in presence of hydrogels, without UV treatment. The percent of cell survival was also higher with increasing quantities of GT-COL, when 5 mg hydrogel/well was used (fig. 3). This indicated a high *in vitro* biocompatibility of the GT-COL hydrogels.

Conclusions

- Mixtures of GT-COL were good substrates for epithelial cells in culture. The GT-COL mixture with the highest concentration of GT, as antioxidant (1:50), exhibited also a good biocompatibility.

- The new conditioning formula for GT-COL mixtures, as hydrogel, was easy-to-made and cheap and could help maintaining the skin hydration.
- *In vitro* tests demonstrated that 20% GT-COL hydrogel rendered the best UV protection, probably through various mechanisms exerted by different constituents present in the GT extract and COL that acted synergistically.

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RESEARCHES TO OBTAIN SOME PHYTOTHERAPEUTICAL PRODUCTS FROM FLAVONOIDIC COMPOUNDS TO TREAT MENOPAUSAL OSTEOPOROSIS NOTE 2. STUDIES REGARDING FLAVONOIDS EXTRACTION

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Summary

The aim of our study was to establish the optimum extraction parameters (extraction type, extraction solvent, the ratio vegetal product/ solvent, extraction time) and to obtain selective extracts which are standardized, enriched with flavonoid compounds (flavones, polyphenols, isoflavones) and amino acids.
The vegetal extracts from Medicago herba, Glycine semen and Trifolii rubri flos were quantitatively and qualitatively analyzed by phytochemical methods.

Keywords: *extraction, flavonoid compounds, osteoporosis*

Introduction

Osteoporosis is a chronic, progressive condition associated with microarhitectural deterioration of bone tissue that results in low bone mass. Conventional therapies for treating osteoporosis in women have emphasized agents that inhibit bone resorption and include hormone replacement therapies, either estrogen alone (ERT) or combination of estrogen and progestogens (HRT) [3]. Despite its potential benefits, HRT has significant risks, the disastrous being breast and ovarian cancers. Therefore, there is a need for alternative treatments to HRT that provide benefits to bone without adverse effects.
Some natural compounds of vegetal origin represent an efficient alternative to HRT [7]. These are represented by flavonoid compounds like flavones, isoflavones, and polyphenols in *Glycine semen* (soybean), *Medicago herba* (alfalfa) and *Trifolii rubri flos* (red clover). Flavones and polyphenols have an antioxidant action which is very important in reduction of bone mass lost in menopausal women. Isoflavones protect against bone mass lost due to their estrogen- like mechanism of action [4, 7-8].
Our purpose was to realize a technology for obtaining complex extracts (with flavones, isoflavones, polyphenols and amino acids) from soybean, alfalfa and red clover.

Materials and methods

We used the following vegetal products as sources of flavonoid compounds and polyphenols: red clover (*Trifolii rubri flos*), alfalfa (*Medicago herba*) and soybean (*Glycine semen*). The extractive solvents used were different concentration- hydroalcoholic solutions and water. Standards including rutin, caffeic acid, chlorogenic acid, arginine, leucine, glutamic acid, genistein and daidzein were purchased from Sigma- Aldrich. All reagents used were of analytical grade.

Extraction

The experimentation of extraction technology was realized at pilot level and consisted in two types of extraction tests for each above- mentioned vegetal species. The extraction variants of vegetal matters differ per extraction methods and some technological parameters (the ratio vegetal product / solvent, extraction time) (table 1).

Table 1 – The content in extractive substances (dry matter) and bioactive principles (flavones, polyphenols and amino acids) in different manufacturing stages in function of technological extraction parameters.

Vegetal matter		**Soybean**		**Alfalfa**		**Red clover**	
Stage /equipment	Parameter	**TEST 1**	**TEST 2**	**TEST 1**	**TEST 2**	**TEST 1**	**TEST 2**
Extraction	*Total rate raw vegetal matter/solvent*	*1/16*	*1/16*	*1/12*	*1/12,72*	*1/16*	*1/10*
	Number of extractors	*1*	*1*	*1*	*1*	*1*	*1*
	Number of extractions	*2*	*2*	*2*	*2*	*2*	*1*
E1	Rate raw vegetal matter/solvent	1/10	1/10	1/6	1/7,6	1/10	1/10
	Solvent	Water	Alcohol 70^0	Alcohol 95^0	Alcohol 70^0	Alcohol 95^0	Alcohol 70^0
	Extraction time	6 h	6 h	6 h	6 h	6 h	6 h
	Temperature	70^0C	50^0C	50^0C	50^0C	50^0C	50^0C
	Total dry matter (g%)/ 100 ml solution	0.64	2.05	2.70	2.38	2.60	2.86
	Flavones (as rutosid) g% /d. m.)	0.059	0.87	0.55	1.21	4.38	4.27
	Polyphenols (as caffeic acid) g%/d. m.)	-	-	-	-	17.23	12.5
	Amino acids as glutamic acid) g%/d. m.)	2.64	0.94	8.59	9.51	7.15	4.38
E2	Rate raw vegetal matter/solvent	1/6	1/6	1/6	1/5,12	1/6	-
	Solvent	Alcohol 80^0	Water	Alcohol 80^0	Water	Alcohol 80^0	-
	Extraction time	6 h	6 h	6 h	6 h	6 h	-
	Temperature	50^0C	70^0C	50^0C	70^0C	50^0C	-
	Total dry matter (g%)/100 ml solution	0.77	0.99	0.99	0.83	1.43	-
	Flavones (as rutosid) g%/d. m.)	0.48	0.47	1.35	0.66	4.75	-
	Polyphenols (as caffeic acid) g%/d. m.)	-	-	-	-	10.24	-
	Amino acids as glutamic acid) g%/d. m.)	1.99	1.18	5.96	4.33	4.63	-

The optimum variants of extraction for each vegetal product were selected depending on the quantity of total extractive substances in correlation with flavonoid compounds, polyphenols and amino acids. The selected fluid extracts were concentrated and spray dried.

Phytochemical analysis

The extractive solutions were qualitative and quantitative analyzed.

For HPTLC analysis of flavonoids and polyphenols we used the silica gel plates (60 F254) (Merck, Germany). The developing system (mobile phase) was ethyl- acetate: formic acid: glacial acetic acid: water. Flavonoids and polyphenols were visualized under 254 nm and 366 nm after spraying the plate with 1% NP (Sigma, Germany) followed by 5%PEG 4000 (Sigma, Germany). For HPTLC analysis of amino acids each sample was spotted on silica gel plates (60 F254) (Merck, Germany). The developing system (mobile phase) was propanol: water. Amino acids were visualized under 254 nm and 366 nm after spraying the plate with 0, 25% nynhidrin in acetone [1-2, 5-6].

We have determined the total flavonoid content calculated as rutin, using the aluminum chloride colorimetric method. Rutin was used to make the calibration curve. Total polyphenols content was calculated as caffeic acid, using the method with Arnow reactive. Total amino acids content was calculated as glutamic acid, using the nynhidrin reaction [1-2, 5-6].

Results

The extraction tests were compared by the quantification of dry matter, flavones, polyphenols and amino acids variations (fig 1, 2, 3).

The best alternative for soybean extraction is test 2 (multiple extraction, two extraction dynamic steps, and two extraction solvents= 70^c hydroalcoholic solvent and water, partial ratio vegetal matter/solvent= 10/1; 6/1, total ratio= 16/1, extraction temperature= 50-70^0C depending on the solvent nature, extraction time= 6 h for each extractor and each extraction step.

The best alternative for alfalfa extraction is test 2 (multiple extraction, two extraction dynamic steps, and two extraction solvents= 70^c hydroalcoholic solvent and water, partial ratio vegetal matter/solvent= 7, 6/1; 5, 12/1, total ratio= 12, 72/1, extraction temperature= 50-70^0C depending on the solvent nature, extraction time= 6 h for each extractor and each extraction step.

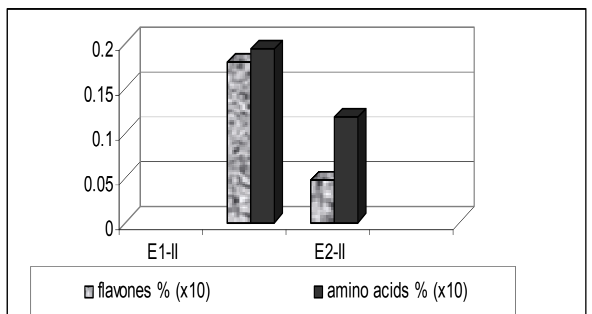

Fig. 1. Variation of flavones (g%/100 ml solution) and amino acids (g%/100 ml solution) contents during soybean extraction in test 2.

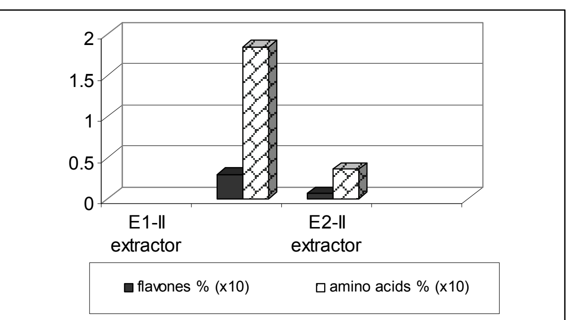

Fig. 2. Variation of flavones (g%/100 ml solution) and amino acids (g%/100 ml solution) contents during alfalfa extraction in test 2.

The best alternative for red clover extraction is test 1 (multiple extraction, two extraction dynamic steps, and two extraction solvents= 95^c alcoholic solvent and 80^c hydroalcoholic solvent, partial ratio vegetal matter/solvent= 10/1; 6/1, total ratio= 16/1, extraction temperature= 50^0C, extraction time= 6 h for each extractor and each extraction step.

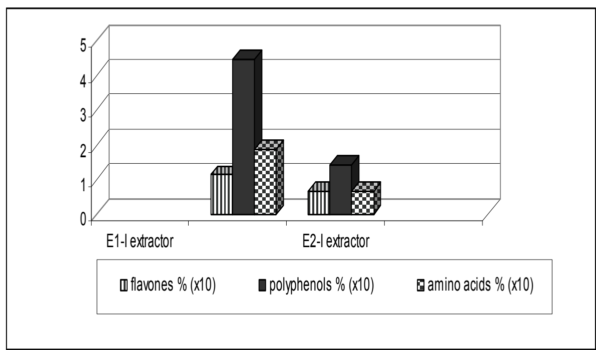

Fig. 3. Variation of flavones (g%/100 ml solution), polyphenols (g%/100 ml solution) and amino acids (g%/100 ml solution) contents during red clover extraction in test 1.

The HPTLC qualitative study showed the presence of flavonoid, polyphenolic compounds and amino acids.

Conclusions

The main purpose in our study was to propose a method for extraction of soybean, alfalfa and red clover recognized for their content in flavones, isoflavones, polyphenols and amino acids important in menopausal osteoporosis, for obtaining complex extracts in order to condition them in form of oral tablets.
The combination of these extracts assures the necessary amount of active principles for an adjuvant treatment of osteoporosis.

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RESEARCHES TO OBTAIN SOME PHYTOTHERAPEUTICAL PRODUCTS FROM FLAVONOIDIC COMPOUNDS TO TREAT MENOPAUSAL OSTEOPOROSIS NOTE 3. FLAVONOID – PHYTOMEDICINES TO TREAT MENOPAUSAL OSTEOPOROSIS

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Summary

The aim of our study was to obtain phytotherapeutical products comprising flavonoids- containing plant extracts from Medicago herba, Glycine semen and Trifolii rubri flos, and vegetal powders from Spirulina platensis, Equiseti herba and Medicago herba, and methods of manufacturing them.
We have made the phytochemical quantitative and qualitative analysis by HPTLC, UV-VIS spectrophotometry and atomic absorption spectrophotometry in order to determine the active principles (flavonoids compounds, amino acids and minerals) from the phytotherapeutical products with an important impact on the treatment of menopausal osteoporosis.

Keywords: *flavonoids compound, phytotherapeutical products, osteoporosis.*

Introduction

Osteoporosis is a chronic, progressive condition associated with microarhitectural deterioration of bone tissue that results in low bone mass. Conventional therapies for treating osteoporosis in women have emphasized agents that inhibit bone resorption and include hormone replacement therapies, either estrogen alone (ERT) or combination of estrogen and progestogens (HRT) [3]. Despite its potential benefits, HRT has significant risks, the disastrous being breast and ovarian cancers. Therefore, there is a need for alternative treatments to HRT that provide benefits to bone without adverse effects.
Some natural compounds of vegetal origin represent an efficient alternative to HRT [7]. These are represented by flavonoid compounds like flavones, isoflavones, polyphenols, amino acids and minerals in *Glycine semen* (soybean), *Medicago herba* (alfalfa) and *Trifolii rubri flos* (red clover). Flavones and polyphenols have an antioxidant action which is very important in reduction of bone mass lost in menopausal women. Isoflavones protect against bone mass lost due to their estrogen- like mechanism of action [4, 7-8]. Minerals and amino acids in the above- mentioned vegetal products can be used in combination with flavonoid compounds as adjuvants for menopausal osteoporosis treatment. Calcium represents the main element of bones. Magnesium is the determinant factor of bone strength, imperative for calcium crystals formation and of vitamin D active form. It has been suggested that consumption of vegetal origin- amino acids results in less urinary calcium excretion.
Our purpose was to establish associations of vegetal extract from *Medicago herba*, *Glycine semen* and *Trifolii rubri flos*, and vegetal powder from *Spirulina platensis*, *Equisetii herba* and *Medicago herba* in order to obtain some phytotherapeutical products with an important impact on the treatment of menopausal osteoporosis.

Materials and methods

We used vegetal extracts from red clover (*Trifolii rubri flos*), alfalfa (*Medicago herba*) and soybean (*Glycine semen*), and the following vegetal powders from *Spirulina platensis*,

Equiseti herba (horsetail) and *Medicago herba* (alfalfa) as sources of flavonoid compounds, polyphenols, amino acids and minerals. Standards including rutin, caffeic acid, chlorogenic acid, arginine, leucine, glutamic acid, genistein and daidzein were purchased from Sigma-Aldrich. All reagents used were of analytical grade.

Conditioning

We have obtained three types of tablet: tablets (T1) based on vegetal powders of spirulina, horsetail and alfalfa; tablets (T2) based on vegetal powders of spirulina and horsetail; tablets (T3) based on extracts of soybean, alfalfa and red clover;

All these are important sources of flavonoid compounds, polyphenols, amino acids and minerals and can be used in the treatment of osteoporosis and also in case of demineralization and hypo- and avitaminosis.

All tablets were obtained by direct compression, using compression excipients (maltodextrine, cellulose microcrystalline, magnesium stearate, croscarmelose). The tablets were tested for hardness, disintegration and friability.

Phytochemical analysis

The tablet formulas were qualitative and quantitative analyzed.

For HPTLC analysis of flavonoids and polyphenols we used the silica gel plates (60 F254) (Merck, Germany). The developing system (mobile phase) was ethyl- acetate: formic acid: glacial acetic acid: water. Flavonoids and polyphenols were visualized under 254 nm and 366 nm after spraying the plate with 1% NP (Sigma, Germany) followed by 5%PEG 4000 (Sigma, Germany). For HPTLC analysis of amino acids each sample was spotted on silica gel plates (60 F254) (Merck, Germany). The developing system (mobile phase) was propanol: water. Amino acids were visualized under 254 nm and 366 nm after spraying the plate with 0, 25% nynhidrin in acetone [1-2, 5-6].

We have determined the total flavonoid content calculated as rutin, using the aluminum chloride colorimetric method. Rutin was used to make the calibration curve. Total polyphenols content was calculated as cynarin, using the method with Folin reactive. Total amino acids content was calculated as glutamic acid, using the nynhidrin reaction [1-2, 5-6].

Tablet formulas were processed through mineral digestion with a Gerhardt SMA-8M digestion block. About 0.5 + 0.0005 g of every product were weighted into labeled 100 ml digestion tubes. We used concentrated nitric acid (HNO_3) and 30% hydrogen peroxide (H_2O_2) as reagents. After mineral digestion, minerals were determined using an atomic absorption spectrophotometry (Shimadzu- Japan) [6].

Results

The final products obtained by vegetal powders and by vegetal extracts in form of tablets were qualitative and quantitative characterized (table 1, fig. 1-7).

Table 1. Characteristics of final products.

Characteristics	T1	T2	T3
Disintegration, min.	8	10	15
Friability, %	120.8	131.5	193
Hardness, N	0,3	0,15	0,22

All final products were tested for phytotoxicity on *Triticum aestivum* L. They have no toxic effects.

By quantitative analysis was estimated the amount of active principles important for the menopausal treatment.

Fig. 1. Ca (ppm) variation content in tablet formulas

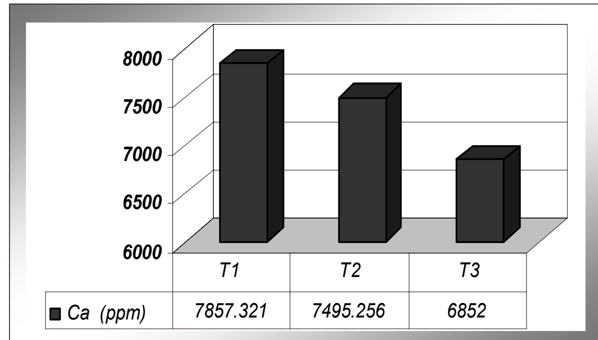


Fig. 2. Mg (ppm) variation content in tablet formulas

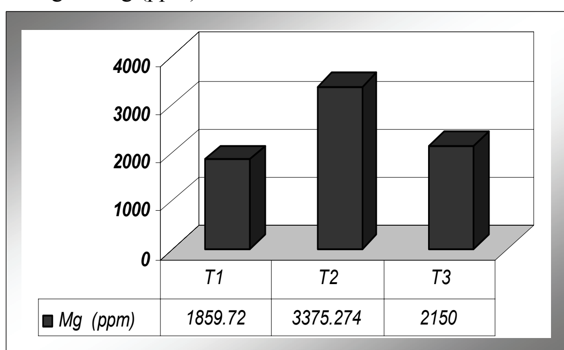


Fig. 3. Na (ppm) variation content in tablet formulas

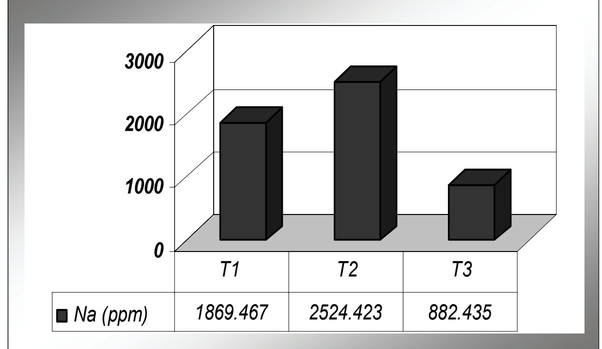


Fig. 4. K(ppm) variation content in tablet formulas

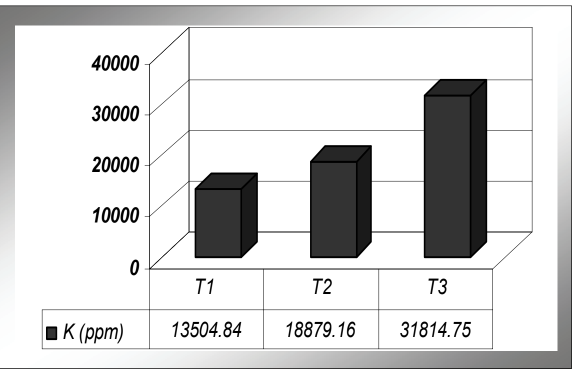


Fig. 5. Flavones (g %) variation content in tablet formulas

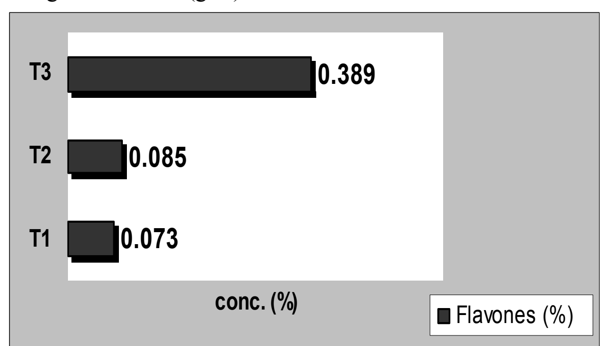


Fig. 6. Polyphenols (g %) variation content in tablet formulas

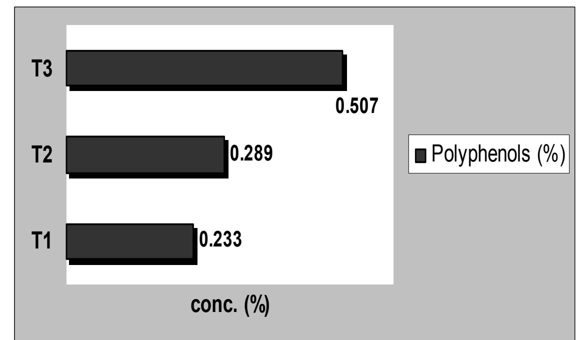
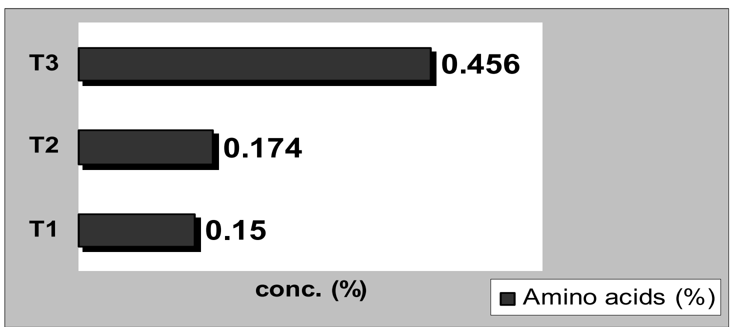


Fig. 7. Amino acids (g %) variation content in tablet formulas



The higher contents of flavonoids and polyphenols are present in T3 tablets: approximately 0.389 g % and 0.507 g% respectively (fig.5, fig. 6). The higher amino acid contents are present in T3 tablets: approximately 0.456 g% (fig. 7). The higher amount of Ca is present in T1 tablets (7857.321 ppm) (fig. 1), the higher amount of Mg is present in T2 tablets

(3375.274 ppm) (fig. 2), of Na in T2 tablets (2524.423 ppm) (fig. 3) and of K in T3 tablets (31814.75 ppm) (fig. 4).
HPTLC method was used for tablet investigation for flavonoids, polyphenols and amino acids presence. The chromatograms revealed the presence of rutin, caffeic acid, chlorogenic acid, luteolin, arginine, glutamic acid at characteristic Rf values.

Conclusions

We have obtained three phytotherapeutical products comprising flavonoids- containing plant extracts from *Medicago herba*, *Glycine semen* and *Trifolii rubri flos*, and vegetal powders from *Spirulina platensis*, *Equiseti herba* and *Medicago herba*.
These products can be used in the treatment of menopausal osteoporosis, especially those with extracts and for organism mineralization, especially those with vegetal powders.

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THE ANALYSIS OF SOME SEMISOLID PHARMACEUTICAL FORMULA WITH EXTRACTS FROM BUDS AND LEAVES OF *BETULA PENDULA* ROTH

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Summary

The aim of the study was the formulation and the quality and stability analysis of some semisolid type (creams) pharmaceutical preparations with vegetal active principles proceed from birch tree. The final forms applicability is important in dermo-cosmetical field.
The extracts were made from dry vegetal material (leaves, buds), by extraction with Soxhlet device and for the buds concordant with French Pharmacopoeia, X-th Edition, by maceration, as hydro-glycero-alcoholic forms (1:1:1). These were incorporated in formula with polymers and anionic surfactant (sodium lauryl sulphate). The tests for the final formula were: TEWA-metrics, moistening parameters, texture, etc., on 15 human volunteers. The stability was tested by microscopy, with Nikon Eclipse E60 microscope and Coolpix digital camera.
The main conclusion of the study was that the analysed preparations have presented the quality and stability demands which were satisfied and that indicates future application as cosmetical and therapeutical formulations.

Keywords: *birch tree, leaves, buds, semisolid formulations.*

Introduction

The vegetal sources with saponines and flavones are very helpful in dermatological and cosmetical area (cellulite, etc.). Other therapeutical indications are the skin protection and treatment after UV exposure damages [1]. The semisolid cutaneous formulations are applied for the preliminary treatment of skin aging and tumoral incipient processes in this area [1,2]. In a preliminary analysis the semisolid formulations have to be stable, homogenous with a proper spreadability and washability [2,3]. All this laboratory parameters are evaluated by microscopy technique, water washing test, etc [1,4]. The obligatory test for to use in practice the semisolid formula like gels or creams are: moistening degree, pH, SELS parameters [5]. The aim of the study was to analyse the behaviour of the proposed formulations as quality aspects and efficiency/noxiousness effects, which are very important parameters even the product will by external applied on human body [1,5]. For the quality objective the study included rheological and microscopical behaviour of the formula [4]. The skin hydration level and transepidermal waterloss link to skin topography are the most important parameters for the clinical part of the study [6,7,8].

Materials and metods

The extracts were prepared from dry vegetal material (leaves, buds), by extraction with Soxhlet device, 2 hours and for the buds concordant with French Pharmacopoeia, X-th Edition, by maceration, as hydro-glycero-alcoholic forms (1:1:1). The leaves extract was a dry type obtained with a rotavapor, also from the macerate (buds) was gentle remove all the possible evaporated quantity. These final forms of extracts were incorporate in formula with polymers and anionic surfactant (sodium dodecil sulphate). The materials for the two formula were: gelling agent, carbomer (Carbopol 940, BF Goodrich), hydrophilic emulsifier sodium dodecilsulphate (Fluka), fatty substances, cethyl alcohol (Merck), cacao butter (Magnesia GmbH), paraffin oil and vaseline (Merkur Vaseline); antimicrobial preservatives, methyl-p-

hydroxybenzoate and propyl-p-hidroxybenzoate (Merk), glycerol (Chimopar), ethanol (Chimopar), triethanolamine (Fluka), distillated water (Ph. Eur.).
The gel and cream formula are presented in the table 1 and 2.
The corbopol 940 gel was prepared in two steps. In the first step, the Carbopol 940 powder was slowly added in 2/3 of the total preservative solution under stirring. The dispersion was strongly agitated for 15 minutes, followed by 24 h of dark storage rest (necessary for the total hydration of the powder). A diluted aqueous solution of triethanolamine (obtained by mixing the organic base with half of the remaining 1/3 of preservative solution) was then added, slowly stirring with a rod to avoid introducing air bubbles and securing a homogeneous neutralizer distribution. Through the neutralisation of the dispersion with this alkaline substance, a transparent dense mass is obtained.
In the second step, the dry extract of *Birch tree* leaves was dispersed in the rest of preservative solution. The obtained hydrogel was progressively added to aqueous dispersion of the dry extract of *Birch tree* leaves under continuous agitation (500 rpm) until a homogeneous preparation was obtaining.
The aqueous phase of the hydrophilic cream containing 1% extract of *Birch tree* buds was prepared by mixing the dispersion of the extract of *Birch tree* in the mixture of glycerol and ethanol with the sodium dodecilsulphate solution (prepared by dissolving the emulsifier in 1/3 of preservative solution). The fatty substances (cetyl alcohol, cacao butter, paraffin oil, vaseline) were mixed; then both the oily and aqueous phases were separately heated to 70ºC to 80ºC and finally, the oily phase was added to the aqueous phase with continuous stirring until cooled to room temperature. The obtained W/O emulsion was mixed with the carbopol 940 hydrogel, beforehand prepared, with gentle stirring to obtain the hydrophilic cream. The prepared semisolid formulations were analysed visually for their colour, homogeneity and phase separation. The pH values of 1% aqueous solutions of the obtained preparations were determined potentiometric using a pH-meter (Jenway 3030) at room temperature (25ºC). The viscosity of the two semisolid preparations was determined at 25ºC using a rotational viscosimeter (Brookfield RV – DV I) with spindle SC4 28 and connected to a thermostatically controlled circulating water bath (Brookfield Thermosel accesory). The viscosity values measured were graphically represented obtaining the rheograms, which are shown in figures 2 and 3.
The stability was tested by microscopy, with Nikon Eclipse E60 microscope and Coolpix digital camera, objective x40 (figure 1).
The clinical measurements consist in initial data (before the application of the product) and after 6 hours from the first application. Before the application of the creams or gels the skin was treated with a SDS 10% solution, a surfactant which imitate the epidermal physiological stress conditions. As it is mentioned in the literature the body region accepted for all these measurements was forearm [8,9]. The applications were made simultaneous. For each evaluation, the moisturizing level was monitored with CM 820 Corneometer, transepidermal water loss with a TM 210 Tewameter and the SELS parameters with a VC 98 Visioscan. The measurements were made in constant environment conditions such as: humidity 40-60%, temperature 20-22˚C.
The volunteers' selection and test development was based on ethical rules of European legislation. The inclusion criteria were: a healthy epidermis, the approving for the test conditions. The exclusion criteria were: excess of hair on the skin, not agree with the testing conditions, developing treatments. The number of subjects was a minimum number, 15 volunteers, female, clinical healthy with ages between 35-55 years.
The efficiency of the formulations was established by comparing the final values with the initial ones for each type of them.

All the formulations were microbiologically tested before their skin application. The cutaneous tolerance consisted in occlusive patches applied for 24 hour was evaluated. (COLIPA method).
The efficiency criteria for a short time action could be characterised by the following rules: moisturizing level is essential; transepidermal water loss indicates the integrity of the skin protection layer and its barrier function, SELS (Surface Evaluation on Living Skin) parameters with the specific ones (roughness, smoothness, desquamation).

Results and discussions

All the prepared formulations were viscous, creamy preparations with a smooth and homogeneous appearance. The tests were correlated with EU demands [10]. The hydrophilic gels have had a brown – reddish colour while the hydrophilic cream was white. They were easily spreadable on the skin with acceptable bioadhesion properties. The pH values of the obtained semisolid preparations ranged from 5.3 to 5.5, corresponding to a high tolerance after application to the skin. All the semisolid preparations containing 1% extract of *Birch tree* buds or leaves were stable upon storage for three months, where no changes were observed in their physical appearance, pH and rheological properties.
The microscopic aspect of the O/W emulsion (hydrophilic cream) studied through the optical microscopy proved the O/W emulsion type and the spherical form of the dispersed drops, which mean diameter was 33,4 μm. Also, the homogenous distribution of the emulsion particles was observed (figure 1). The consistency of semisolid preparations, especially gels, depends on the ratio of solid fraction, which produces the structure, to liquid fraction. Differences in concentration and kind of the gelling agents result in changes in the occurring structure consistency. As seen in the figures 2,3,4 all semisolid preparations exhibited a shear – thinning behaviour since the viscosity decreased with increasing the shear rate. As the shear stress is increased the particles of gelling agent is forced to align their long axes in the direction of flow; such orientation reduces the internal resistance and hence decreases the viscosity. The figures also indicate that both of the preparations possessed only a pseudoplastic behaviour without thixotropy because the structural network formed is very stable and it is very difficult to break it by agitation. The extensometry studies show that the carbopol 940 1% hydrogel and the hydrophilic cream spreadabilities are very close (figure 6). The values of penetrometric determination are presented in figure 5, which shows that penetration capacity of carbopol 940 hydrogel and has a better penetration capacity than O/W cream for high weight values. In the weight values range from 10g to 40g, both of the two preparations presented a similarly penetration capacity.
The Carbopol 940 hydrogel showed considerably higher viscosity values than hydrophilic cream. The shape of the rheograms indicates that Carbopol 940 hydrogel is easier to apply on the skin than a hydrophilic cream.
The viscosity curves linearisation indicated a practically linear relationship between the structural viscosity of the studied preparations and the shear rate. This linearity is described by following equation:

$$\log \eta = \log \eta^{+} - m \log \gamma$$

where η = structural viscosity (Pa.s), η^{+} = viscosity extrapolate to shear rate, m = tangent of regression line, γ = shear rate (s^{-1}).
Concerning the effects of gel and cream both products were well tolerated. – (patch-test evaluation). The gel and the cream cannot cancel the noxiousness consequences of the SDS applied for 6 hours on the hydrolypidic layer (figure 8). – (TEWL measurements)
The moisturizing increased with 28.3% for the cream and 16.3% for the gel results that indicated a proper hydration in a short-term exposure (figure 7).

Smoothness (depth of horizontal and vertical lines), affected following the treatment with SDS, is restored only in the presence of the gel containing birch tree extract, the cream being not efficacious in one application, after 6 hours of action.
Both cream and gel are efficacious in decreasing exfoliation, attaining values even lower than the initial ones, in one application, after 6 hours of action through they act on an epidermis disturbed by the SDS effect.
Roughness (number of horizontal and vertical lines), affected by the treatment with SDS, is improved following the gel action, tending towards the initial value in 6 hours after the application of products. The cream containing *Betula pendula* Roth buds extract fails to counterbalance the effect of SDS in one application, after 6 hours of action.

Conclusions

The gel and hydrophilic cream with birch tree extracts obtained from leaves and buds were stable and with a proper rheological behaviour. The extracts were well incorporated and don't change the physical properties (stability, spreadability, penetrometry) of the final formula. The pH of the final formulations was acceptable for the skin require. The formulations didn't present noxiousness effects and were well tolerated.
The products didn't restore the integrity of hydrolipidic layer of the epidermis, but it is possible that the time of investigation (6h.) is too short for this type of action. The moisturizing effect and efficacy in decreasing exfoliation of both cream and gel, restoring of smoothness by gel treatment, and improvement of roughness after gel application indicated the good efficiency of the two products, better for gel formulation.

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Table 1. The gel formula
Carbopol 940 gel

Components	g%
Extract of *Birch tree* leaves	1
Carbopol 940	1
Triethanolamine	1
Preservative solution	97

Table 2. The cream formula
Hydrophilic cream

Components	g%
Extract of *Birch tree* buds	1
Sodium dodecilsulphate	1
Cetyl alcohol	10
Cacao butter	9
Vaseline	15
Paraffin oil	5
Carbopol 940	0,3
Triethanolamine	0,3
Glycerol	10
Ethanol	10
Preservative solution	38,4

Table 3. Regression straight line equations and correlation coefficient of viscosity curves corresponding to studied semisolid preparations

Semisolid preparation	Straight line equations	Correlation coefficient (r)
Hydrophilic cream	y = 1.5436 – 0.6276x	0.9985
Carbopol 940 1% hydrogel	y = 2.3758 – 0.7894x	0.9983

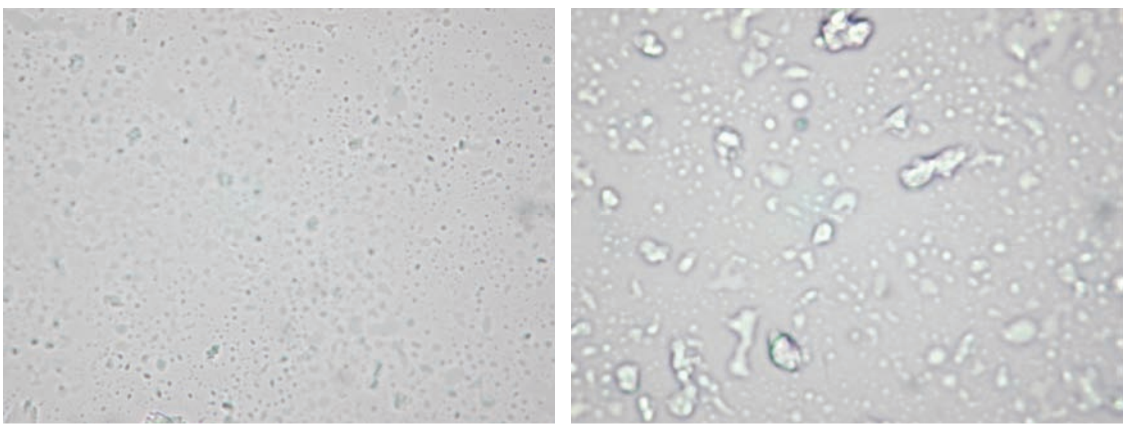


Fig. 1. Birch tree cream at the preparation moment non-diluted (a) and diluted (b)

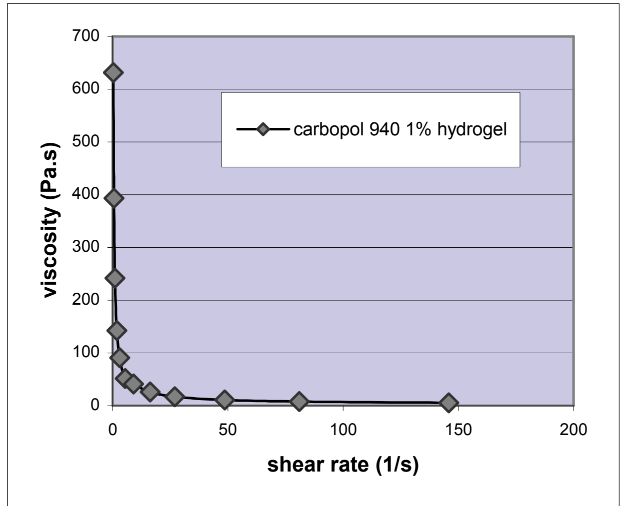


Fig. 2. The rheogram for the carbopol 940 gel

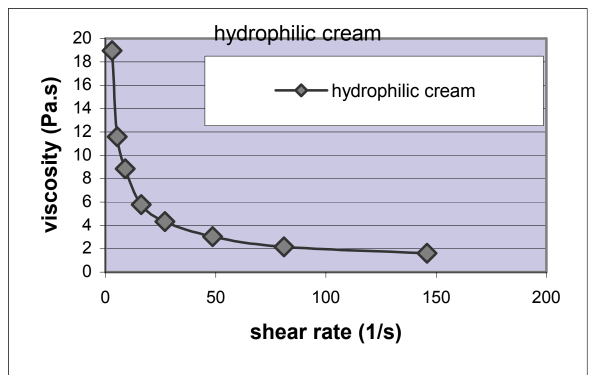


Fig. 3. The rheogram for hydrophilic cream

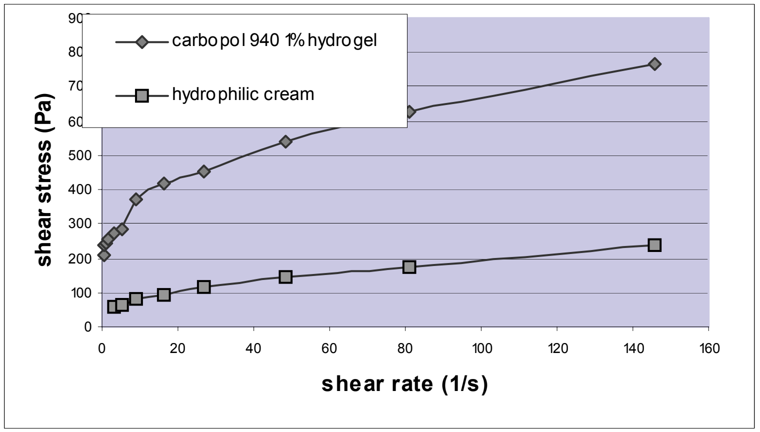


Fig. 4. Flow curves of the two semsiolid preparations

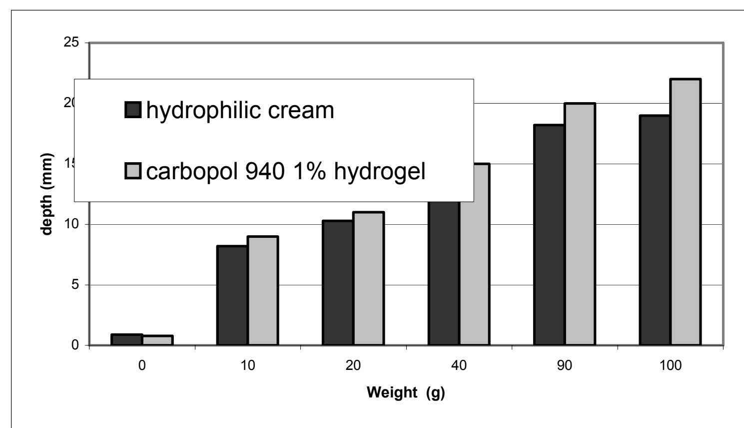


Fig. 5. Penetration capacity of semisolid preparations containing 1% extracts of *Birch tree*

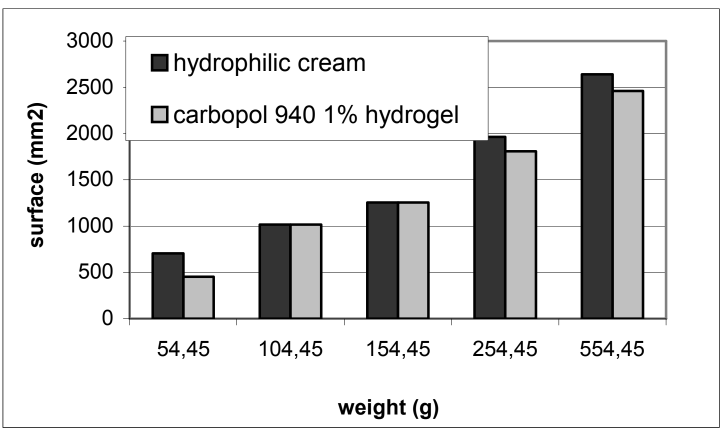


Fig. 6. Spreadability of semisolid preparations containing 1% extracts of *Birch tree*

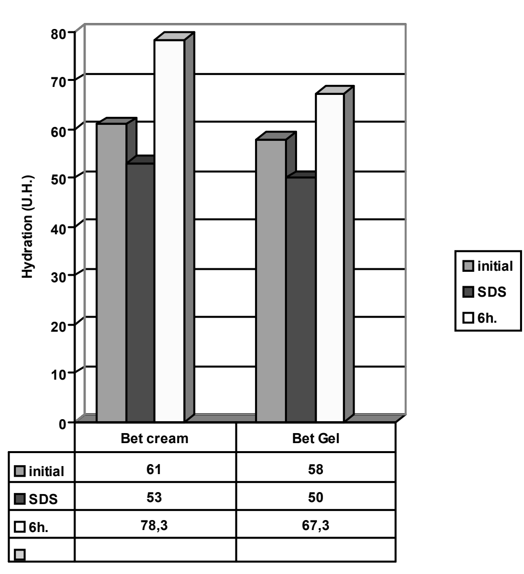
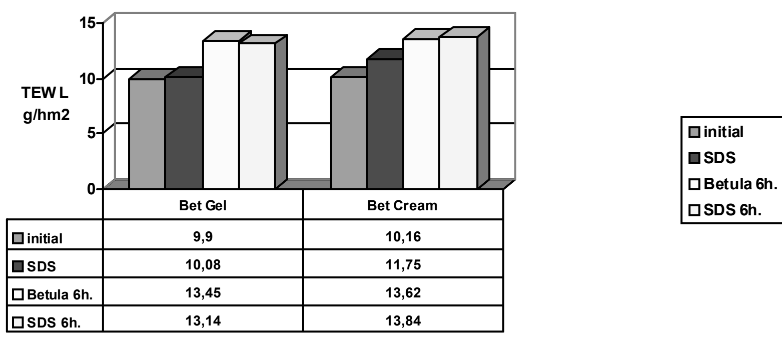


Fig. 7. The hydration level on skin after the application of the semisolid formula

The transepidermal waterloss after the *Betula pendula* **Roth buds/leaves cream/gel application**



	Bet Gel	Bet Cream
initial	9,9	10,16
SDS	10,08	11,75
Betula 6h.	13,45	13,62
SDS 6h.	13,14	13,84

Fig. 8. The TEWA-metry parameters for the tested formula

ESSENTIAL FATTY ACIDS AND NUTRITIVE VALUE OF COLD PRESSED HEMPSEED OIL, *CANNABIS SATIVA* L.

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Summary

The fatty acid composition, nutritive value and stability of cold pressed oil, obtained from seven different hemp cultivars, were investigated. The results show that the content of gama-linolenic acid in the oil depends on the cultivar, ranging from 0.80 to 2.46%. The ratio between essential omega-6 and omega-3 fatty acids in the oil was 3.9:1 to 4.2:1, satisfying the demands of modern healthy nutrition regarding lipids. Due to high content of polyunsaturated fatty acids, the oxidative stability of hemp oil is poor. The induction period at 100 °C, determined by accelerated oxidation method, Rancimat test – was 6.4 to 7.6 hours.

Keywords: *hempseed oil, fatty acids, gama-linolenic acid, nutritive value, shelf life*

Introduction

Hemp (*Cannabis sativa* L.), an annual herbaceous plant, has been cultivated many centuries for its fibre and oil. The relatively recent "antidrug" ban on hemp cultivation in many countries has prevented food scientists from more serious investigation of a wide range potential uses of this seed. However, recently a cultivation of a low THC (the phytochemical drug component, δ-9-tetrahydrocannabinol) form of industrial hemp (less than 1%) has been legalized in Canada, while a 0.3% THC standard has been established by the European Union (1, 2). Hempseed contains 20-25% protein, 20-30% carbohydrates, 25-35% oil and 10-15% insoluble fiber and a whole range of minerals (3). Several Australian states, including Queensland, have introduced legislation in recent years that permits studying the commercial potential of producing low THC hemp for fiber, fabric, food and other products.

Hempseed oil is an exceptionally rich source of unsaturated fatty acids, especially the essential fatty acids (EFAs), like linoleic acid (LA) and α-linolenic acid (LNA), that the human body cannot produce and, therefore, must be taken from dietary sources (4, 5, 6).

LA (C 18:2) is an ω-6 fatty acid. In other words, the first of its two double bonds is positioned at the sixth carbon atom counted from the methyl end of the fatty acid molecule. Once in the body, various desaturase and elongase enzymes set to work, creating downstream metabolites γ-linolenic, dihomo-γ-linolenic and arachidonic acid, the major members of the ω-6 family. LNA is an ω-3 fatty acid, with a double bond positioned at the third carbon atom from the methyl end. It is the precursor for two additional ω-3 long-chain polyunsaturated fatty acids: EPA (C 20:5 ω-3) and DHA (C 2:6 ω-3) (6). In contrast to the shorter-chain and more saturated fatty acids, EFA serves not as energy source, but as a row material for cell structure and precursor for biosynthesis of many regulatory biochemicals in the human body (7). Hempseed oil provides a favorable ratio between LA and LNA essential fatty acids required for proper human nutrition, besides a significant contribution of γ-linolenic - GLA (C 18:3 ω-6) acid with a potential therapeutic efficacy (8,9). By Callaway et al. (10) hempseed oil is endowed with significant quantities of GLA and also it contains stearidonic acid - SDA (C 18:4 ω-3), particularly some special varieties.

Several nutritional disorders resulting from low EFA levels in the human diet are treated with dietary supplements containing LA, LNA and/or GLA. Tuberculosis and AIDS-related wasting syndromes, skin conditions such as psoriasis, atopic eczema and mastalgia, stress and hypertension and aging-included (as well as diabetes-induced) low GLA levels are all treated

with EFAs. GLA also acts on the circulatory and nervous systems and assists in the repair of damaged myelin sheath tissue surronding nerves. Also, it has been proven as a very useful aid in the treatment of diabetes nerve deadening, which if left untreated results in a gangrene of the fingers and toes. A wide range of GLA experimental uses include the treatment of cardiovascular, psychiatric and immunological disorders (5, 8 , 11).
The availability of hempseed is expected to be increased due to the renewed demand for hemp fibre used for paper and clothing production. The versatility of the seed lends itself to the development of numerous products for the food, cosmetic, therapeutic, functional food and nutraceutical industry. An ideal hemp seed variety would produce a high yield, normally 0.5-1.0 t/ha by Deferne et al. (8) and 0.8-1.6 t/ha by Schuster (12), with a seed containing a high percentage of good quality oil.
Nowdays, the extraction of oil from hemp seed is carried out by cold pressing process. The mentioned process does not allow an extraction yield equal to that of techniques employing solvents or high temperatures, but it has an advantage of minimizing degradative changes in the oil (13). Cold pressed seed oils retain more naturally beneficial components of the seeds, including natural antioxidants. Also, they are free of chemical treatments, and nowdays they are becomming a more and more interesting substitute for conventional practices because of consumers' desire for natural and safe food products. Cold pressed oils, as novel dietary sources, are desired by consumers and food manufacturers to benefit human health through improving nutrition. This is the reason for establishing many small oil factories in Central and Southeast European Countries lately. Specialty oils are produced in these factories applying cold pressing process on small capacity screw presses. Hempseed is a very suitable raw material for pressing (to be pressed) in these factories because of its composition and technical characteristics (14). Therefore, the present study was conducted to investigate the fatty acid profile, nutritive value and oxidative stability of different varieties of hempseed oils obtained by cold pressing on a laboratory screw press.

Material and methods

Cold pressed hempseed oil was obtained from several hemp varieties cultivated under the same agroecological conditions on an experimental field. Following hempseed cultivars were investigated: Novosadska (Serbia and Montenegro), Secuieni (Romania), Beniko (Poland), Felina 34 (France), Futura 75 (France), Tiborszállási (Hungary) and Carmagnola Seleccionata (Italy).
In compliance with the elementary principle of hemp cultivation, considering it as an oilseed (15), the distance between two rows was 70 cm, and the distance between plants is 50-60 cm. Each hemp variety was grown in two rows of 50 m length. The seed was collected in a maximum ripening stage, cleaned and dried to 10% moisture content. A cold pressed oil was obtained on a laboratory screw press »Komet«. The final temperature of pressed oil was 40-45 ^{o}C. After the pressing, oil was kept two days at room temperature (22°C) to precipitate, after which a decantation and filtration through laboratory filter paper followed. Until the further analyses samples were stored in a refrigerator at 8 ^{o}C.
Gas chromatographic analysis of fatty acids, ISO 5508, was performed on HP 5890 Series II System equipped with Flame Ionization Detector (FID) and capillary column Omegawax 320 30m x 0,32mm x 0.25 µm. Flow rate of carrier gas (Helium) was 1 mL/min, and pressure was 100 bar. For the preparation of fatty acid methilesters TMSH reagent was used (trimethylsulfonium hydroxide solution in 0.25 mol. methanol).
Peroxide value was determined by standard method ISO 3969.
The oxidative stability of oil at 100 ^{o}C was determined by Rancimat 617 apparatus, using the accelerated oxidative method, ISO 6886 (2.5 g of sample, air flow 18-20 l/h).

Results and discussion

The most important criteria for oil or fat quality is the fatty acid composition. Hempseed oil samples contain a variety of fatty acids, Table 1, among which the unsaturated linoleic and α-linolenic acids are predominant, which is in a compliance with literature (8, 16, 17).

Table 1. Fatty acid composition of cold pressed hempseed oil from different varieties

Fatty acid (%)	Novo-sadska	Secuieni	Beniko	Felina 34	Futura 75	Tibor-szállási	Carma-gnola S.
C 10:0	1.02	0.88	0.96	1.02	1.09	0.58	1.02
C 16:0	7.32	6.91	7.00	8.04	7.59	7.20	7.22
C 18:0	2.97	2.57	2.66	3.14	3.28	2.88	2.47
C 18:1	14.55	16.17	13.74	15.24	16.37	15.22	14.21
C 18:2	55.50	55.05	55.74	53.01	51.94	53.79	54.75
C 18:3 n-3	14.35	13.00	14.23	12.98	12.35	15.36	15.39
C 18:3 n-6	0.80	1.87	2.46	1.91	1.73	1.30	1.38
C 20:0	0.87	0.88	0.86	1.04	0.98	0.92	0.84
Total saturated	12.18	11.24	11.48	13.24	12.94	11.58	11.55
Total unsaturated	85.20	86.09	86.17	83.14	82.39	85.67	85.73
Ratio LN:LNA	3.87:1	4.23:1	3.92:1	4.08:1	4.21:1	3.50:1	3.65:1

The total content of unsaturated fatty acids in analized samples is 82 – 86%, where the linoleic acid presents 51.94 - 55.74 %, and the share of α-linolenic acid is 12.35 - 15.39 %. This polyunsaturated oils are similar to soybean or walnut oil (18). Mediavilla et al. (19) in different field experiments carried out in Switzerland in year 1996-1998 important characteristics of 29 hemp varieties were studied. According to their results, the content of linoleic acid in all samples was very similar, from 54.2 to 56.8%, while the content of LNA varied from 15.2 to 22.6%. Rumyantseva and Lemeskev (20) analizing 100 hemp varieties from all parts of the world found a slightly higher amount of linoleic acid (55.5-59.5%) as well as the amount α-linolenic acid (16.2-24.3%) in samples in comparison with our results. Nadeem et al. (21) the hempseed oil indigenous to Pakistan was found to contain high levels of LA, up to 61.50%, followed by LNA, oleic, palmitic and stearic acids up to level 17.4, 12.4, 5.6 and 2.1 respectively. By Mölleken and Theimer (22) origin of the seed seems to influence fatty acid composition. According to the literature data the composition of fatty acids also depends on the maturity of hemp fruits (23).

All cultivars, as it can be seen from Table 1, contained varying quantities of GLA, from 0.8 % in domestic, Novosadski cultivar, to 2.46 % in Beniko. It is well known that varieties from regions with mild or warm climate contain smaller amount of this acid, while varieties from regions that are temperate or even cold in the summer have a large amount (22). However, the content of GLA is obviously a variety characteristic, since all oil samples were obtained from different hemp cultivars cultivated under the same agroecological conditions. According to Mölleken and Theimer (22) the content of GLA in hempseed cultivated in our region is 3%. Acoording to Mediavilla et al. (19) the content of GLA in Beniko cultivar is 3.2%, in Feline 34 cultivar 3.0% and in Novosadska cultivar 1.8%. The potential physiological effects of GLA have been extensively investigated. In the body, GLA is normally derived from LA and serves as an intermediary for the formation of longer-chain fatty acids and short-lived hormone-like substances, *i.e.* eicosanoids. The metabolic conversion of LA to GLA is slow in

mammals. Further, it has been suggested that due to stress, ageing or pathology (*e.g.,* by hypertension, diabetes, *etc*.), formation of a sufficient amount or balance of eicosanoids may be impaired. This problem may be relieved by direct GLA supplementation (8).
According to the healthy nutrition principle, beside the fatty acid composition, their ratio *i.d.* the ratio between saturated and unsaturated fatty acids, as well as the ratio between ω-6 and ω-3 acids is very important. According to the results shown in Table 1, it can be seen that the content of unsaturated fatty acids is quite high in all samples, ranging from 82.39 to 86.17%, and the ratio between ω-6 and ω-3 fatty acid is very favourable, ranging between 3.50:1 to 4.2:1. According to Mediavilla et al. (19) the mentioned ratio is between 2.46:1 and 3.65:1. In samples analized in this study the determined ratio is 3.02:1 in Novosadska cultivar, 3.10:1 in Felina 34 cultivar and 3.36:1 in Beniko cultivar. The ratio between ω-6 and ω-3 fatty acids in the average Western diet is now estimated to be from 10:1 to 30:1. Researchers disagree about what the optimum ratio should be, but the most would agree it should be somewhere between 1:1 to 4:1 (6). Balance of 3:1 has been claimed optimal for human nutrition by Erasmus (24).
In Table 2 the average content of fatty acids determined in cold pressed hempseed oil samples is compared with the literature data for fatty acid profile of different nonrefined oils. According to results the hempoil has an ideal fatty acid composition regarding nutrition claims.

Table 2. Fatty acid composition of nonrefined edible oils (%)

	Essential fatty acid content	LA	LNA	GLA	Oleic fatty acid	Saturated fatty acids**	Ratio between ω-6:ω-3
Hempseed oil*	68.20	54.25	13.95	1.64	15.07	10.18	3.9 : 1
By literature date (23, 24)							
Hempoil	80	55-60	18-23	up to 2	12	8	3-4 : 1
Flax	72	14	58	-	19	9	1 : 4
Sunflower	65	65	traces	-	23	12	65 : 1
Canola	37	30	7	-	54	7	4.3 : 1
Olive	9	8.5	0.5	-	75	16	18 : 1
Evening primrose (25)	70.83	70.83	-	8.83	12.55	7.89	8.02 : 1

*the average values of analyzed samples
**saturated fatty acids: palmitic and stearic

The extreme sensitivity of hemp oil to oxidative rancidity can be explained by a high degree of unsaturation, since unsaturated chemical bonds are highly vulnerable to attack by atmospheric oxygen. Table 3 shows the data for oxidative stability of analyzed cold pressed hempseed oil samples in corellation with peroxide value, as well as the induction period at high temperature, while Figure 1 shows the corellation between the induction period and a total content of unsaturated fatty acids.

Table 3. Peroxide value and induction period by Rancimat test of cold pressed hemp oils

	Novo-sadski	Secuieni	Beniko	Felina 34	Futura 75	Tibor-szállási	Carma-gnola S.
Peroxide value (mmol/kg)	6.42	6.43	4.51	4.26	4.24	4.10	5.20
Induction period* (h)	6.8	6.4	6.4	7.0	7.6	6.8	6.6

*temperature 100 ^{o}C, air flow 18-20 l/h

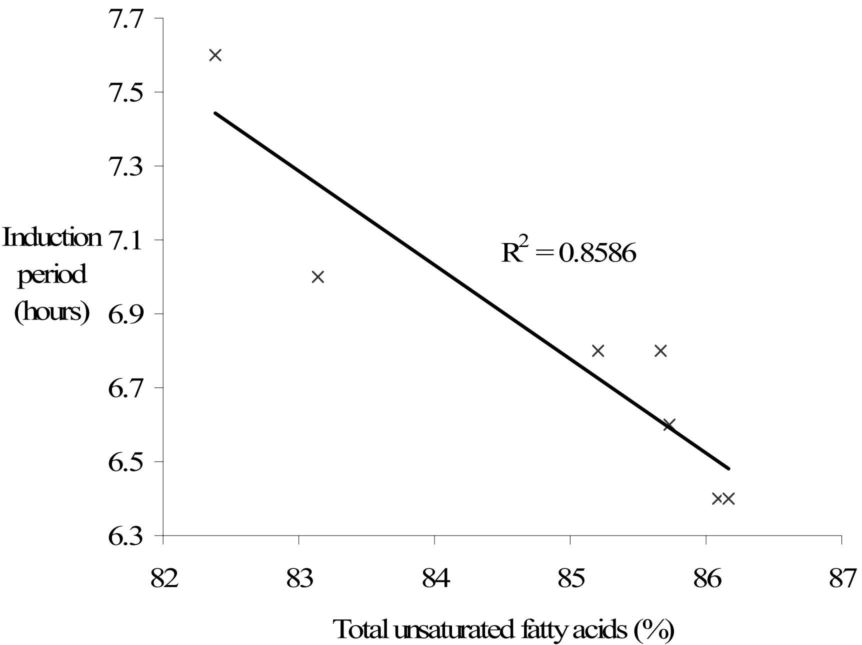

Fig. 1. Correlation between induction period and content of total unsaturated fatty acids in cold pressed hemp oil samples

The induction period, ranging between 6.4 and 7.6 hours, is the indicator of poor oxidative stability of this oil at high temperature. It can be also noticed that the decrease of oxidative stability of oils is in corellation with the unsaturation, with a coefficient of corellation 0.8586. For this reason, the cold pressed hempoil is not recommended for frying or baking. The best way of use is to consume it as a table oil, on salad or as a butter/margarine substitute for dipping bread, similar in use to olive oil. As the peroxide value is concerned, with values ranging between 4.10 and 6.43 mmol/kg, the oil's high sensibility toward rancidity is confirmed.

Conclusions

Analyzed cold pressed oils obtained from different hemp cultivars possess an ideal fatty acid profile regarding nutrition claims, and contrary to the majority of edible oils, also possess GLA. Due to a high content of unsaturated fatty acids, oxidative stability of analyzed samples at 100 ^{o}C is quite poor, with values ranging from 6.4 to 7.6 hours. For that reason special protective measures are necessary to prevent oxidation in order to preserve the high nutritive value of this kind of oil.

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RESEARCHES ON A NEW ROMANIAN CHEMOVAR OF *ARTEMISIA DRACUNCULUS* L. *METHYLEUGENOLIFERUM* FOR ITS STANDARDIZATION AND USE IN AROMATHERAPY SECOND NOTE: STUDIES ABOUT CHEMOVAR'S STABILITY

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Summary

In order to find a local source of methyleugenol, we have studied Artemisia dracunculus L. methyleugenoliferum, a chemotype that have been created at Fundulea Medicinal and Aromatic Plants Researches Resort. We were interested in chemotype's stability, in morph-anatomical characters and in the quantity and chemical composition of the essential oil obtained through water steam distillation of aerial parts of plants. Through GC/MS we noticed little quantity variations of methyleugenol during 3 years.

Keywords: *methyleugenol, Artemisia dracunculus L. methyleugenoliferum, GC/MS method*

Introduction

The phenol methyl ethers are well known as essential oils components with remarkable spasmolytic properties. Among them, methyleugenol is the most valuable. It has higher spasmolytic activity than papaverine and has no adverse effects as anethole (estrogenic) and estragole (potential carcinogenic)[1].
Because there are few sources of methyleugenol (oil of *Ocimum basilicum* L. var. *minimum* = 55 – 60%, *Melaleuca leucadendron* L. *methyleugenoliferum* = 99%)[1], we wanted to find through the species created at Fundulea Medicinal and Aromatic Plants Researches Resort, an eventual source which can be used in aromatherapy. Among the chemotypes that have been created here, we were interested in *Artemisia dracunculus* L., tarragon (*Asteraceae*), which was cultivated on many lots (8, 10 and 16)[2, 3].
We discovered in *Artemisia dracunculus* L. an important quantity of methyleugenol; we named it *Artemisia dracunculus* L. *methyleugenoliferum* and we supposesed that it can be used as a local source of methyleugenol. We wanted to assure that the content of the essential oil remains unchanged from the chemical composition point of view. The aim of our study is asses the chemical composition's stability over three consecutive years (2003 – 2005).

Material and method

From the genotypes found in the amelioration field, with an interesting essential oil composition, we have chosen to study the clones: $C_{33/99}$ (lot 8), $C_{10/95}$ (lot 10) and $C_{23/93}$ (lot 16). The vegetable material (aerial parts) was harvested a full blooming. The mentioned genotypes (the lots 8, 10, 16) were noticed among three different years. First year (2003) was very droughty and with high temperatures (35 - 40°C), while the second and the third year (2004 and 2005) were full of rainfalls and with moderate temperatures (22 - 30°C). The determination of characteristic morph-anatomic elements and of chemotypes quality was made by pharmacognostic analysis [4], with micro- and macroscopic exam, and by chemical quality and quantity exam. For the microscopic study we have made pictures of leaves and stem transversal sections, with an optical microscopic Labophot 2 - Nikon (with 10X ocular, objectives of 4X, 10X, and 40 X).

The essential oil was obtained through distillation with waters teams in a closed circuit with a Neo-Clevenger apparatus [5]. This method is used also for volumetrically measurement. The identification and measurement of chemical compounds from essential oil of tarragon were made with a gas-chromatograph Shimadzu with split injection GC-17A, connected on a mass spectrometer QP 5000. The specifications were: Macrogol column 20000, with length of 30 m and diameter of 0,25 mm; injector's temperature of 220° C; oven temperature in growing from 65° C to 200° C; the injected volume between 0,25 µl and 0.6 µl; split ratio = 1/100; carrier gas: helium, flow rate, 0.6 ml/min. Compounds were identified by computer matching of mass spectral data with data in the Shimadzu NIST62 mass spectral database and by comparing the retention times and mass spectra to those of standard compounds. Quantification was performed by integrating the peak areas of total ion chromatograms with the Shimadzu Class 500 software and using the appropriate standard curves [6].

Results and discussions

The main macroscopic features of the studied tarragon's genotypes are: plants of a 90 – 160 cm high, with a bush aspect, with many upward leafy stems, fully branched; simple leaves, undivided, of a spear shape, alternative, with complete edge, of 4,3 – 6,5 cm length and 0,3 – 0,51 cm width, color depending on the lot: yellow-greenish (lot 8), grey-greenish (lot 10) and pea greenish (lot 16); globular anthodia inflorescence of yellow or green- yellow color; sterile yellowish central flowers, those on the edges fertile.

After examination of the transversal stem cuts we noticed a secondary structure with 10 libberian-ligneous circularly arranged fascicules (5 primary and 5 secondary), separated by little developed medullar rays. The fascicles are protected by a fibber, sclerified pericycle with a crown shape. The medullar parenchyma is very good developed and includes cells with thickened walls, pierced by canalliculas. Along the slashes we found secretory canals with essential oil. Over a comparative analysis of the 3 lots stems, we noticed few differences: a tendency of diminishing the pentagonal shape at the lot 8 (fig 1A) and more secretory canals on lot 16 (has a bigger quantity of essential oil) (fig1D) than in lot 8 or 10 (fig 1B). The transversal section through leaf (fig1E) is characterized by a heterogenic asymmetrical structure with collateral libberian-ligneous fascicule straight to the vein, collenchimatous pericycle and glandular biseriates trichomes (fig1 F), characteristic to the family and secretory canals (fig 1 G), also. On the epidermis there are stomata with 3-4 annexed undifferentiated cells (anisocytic), and cuticle striations (fig 1 H). The epidermis among the nervures presents moniliform thickenings.

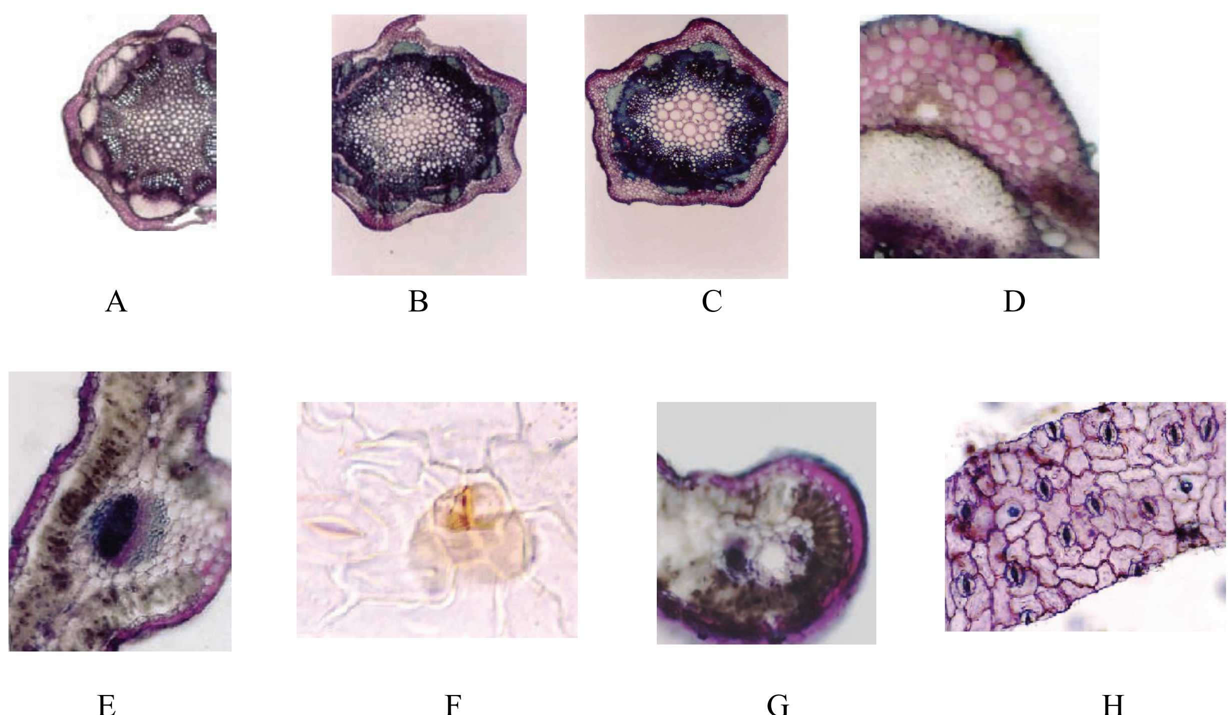

Fig. 1. Transversal sections through stem and leaf of *Artemisia dracunculus* L. *methyleugenoliferum*

We have obtained an essential oil quantity of 1.1 – 2.55 ml reported to the dried substance. The results are inserted in the table I. The GC/SM chromatographic analysis results are shown in the table II

Table I. The *Artemisia dracunculus* L. *methyleugenoliferum* essential oil content

Nr.	Lot nr.	Vegetal product mass (g)	Humidity %	Essential oil volume (ml)	Essential oil volume ml %
1	8/2003	400	59.62	2.3	1.1
2	8/2004	100	60.26	0.98	2.46
3	8/2005	1000	61.89	9	2.36
4	10/2003	400	46.98	2.7	1.27
5	10/2004	100	59.96	0.92	2.29
6	10/2005	520	63.29	4.54	2.37
7	16/2003	200	45.21	1.5	1.36
8	16/2004	94	43.23	1.04	2.55
9	16/2005	666	63.98	6.18	2.57

Table II**.** Components from essential oils of *A. dracunculus* L. *methyleugenoliferum* L.

Nr.	Essential oil components	Lot 8			Lot 10			Lot 16		
		2003 %	2004 %	2005 %	2003 %	2004 %	2005 %	2003 %	2004 %	2005 %
1	Methyl eugenol	59.71	51.91	57.47	61.06	51.15	51.12	69.73	55.33	55.04

2	Methyl isoeugenol	2.4	2.33	1.68	2.21	2.18	1.73	3.49	4.06	5.04
3	estragole	-	0.78	0.49	-	0.79	0.65	0.22	0.68	0.77
4	citronelole	0.6	-	-	2.64	-	-	-	-	2.31
5	terpinen-1ol-4	2.3	0.38	5.08	1.32	4.07	5.52	1.65	4.5	6.75
6	felandrene	-	17.07	-	-	18.56	-	-	20.86	-
7	β-pinene	28.11	0.97	20.61	25.67	1.64	20.94	19.16	0.46	17.88
8	β-myrcene	0.61	0.96	1.14	1.18	1.13	1.24	0.52	1.24	1.37
9	limonene	-	1.11	1.24	0.13	1.23	1.10	0.08	-	0.27
10	ocimene	1.64	0.68	-	0.59	7.29	5.80	0.95	3.08	-

Analyzing the data from the tables I and II we see that in the essential oil of *Artemisia dracunculus* L. *methyleugenoliferum*we the main component is methyleugenol, while the quantity of estragole is very low (lot 16 /2003 and 2005, lots 8, 10, 16/2004) or in undetectable quantities (lot 8, 10/2003).
We noticed little quantity variations of methyleugenol during these 3 years (59.71/2003, 51.91/2004, 57.47/2005 – the lot 8; 61.06/2003, 51.15/2004, 51.12/2005 - the lot 10 and 69.73/2003, 55.33/2004, 55.04/2005 - the lot 16). Though, there is a little decreasing of the quantity of the methyleugenol in the last 2 years.

Conclusions

We think that some of the modifications of the chromatographic profile are attributed to the climate conditions (the year 2003 – warm and droughty, while last 2 years – colder and with much more rainfalls, especially in 2005). We don't exclude the possibility of some modifications in the plant metabolism due to a transformation of plant from cultivated specie to a sub spontaneous one.
In order to elucidate these aspects, the researches have to continue.

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CONTRIBUTIONS TO THE STUDY OF *TAMUS COMMUNIS*

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Summary

To the purpose of turning to good account a species so little investigated, a morphostructural and chemical study was carried out, completed with a test for anti-inflammatory potential of the plant and the formulation of a pharmaceutical form to exhibit the anti-inflammatory properties indicated. The tests performed showed a reduced toxicity and significant anti-inflammatory action. These prompted the preparation of an ointment with anti-inflammatory properties.

Keywords: *blak bryony, calcium oxalate raphides, anti-inflammatory agent, steroid sapogenins*

Introduction

Tamus communis L., commonly named black bryony, is a member of the *Dioscoreaceae* family. In our country, the plant can be found particularly in Transylvania and Banat, less in Wallachia, and occasionally in Moldavia. It is a dioecious species, with a 2 to 4 m high voluble, twining stem. Underground, it has a cylindrical, fleshy tuber rhizome, while its stem leaves are heart-shaped, petiolated and of a vivid and glossy green. Another characteristic feature is the fruit on the stem, at various stages of ripening, of green, orange or red color (when fully ripe). It thrives in shady forests, woodland edges, shrubs, alongside hedges, in vineyards, winding around support plants [5, 12, 14].
The plant has been used in the traditional medicine since ancient times in the treatment of contusions and ecchymoses (hence its French name of *herbe à la femme battue*), of rheumatic pains, as a purgative, in arteritis, or cancer [4, 9, 10, 14].
At present, it is indicated particularly in the therapy of rheumatic disorders (arthralgias) and as an anti-inflammatory agent primarily for external and less internal use, due to its toxicity [7,15,16]. The literature reports irritation of mucous membranes and digestive tract, as well as long-term depression. Intoxications with the berries of the species have been reported in children, manifested by colic, vomiting or even led to death. Still internally, *Tamus communis* is used as a diuretic and purgative. It is used as a 4 – 6% decocted tincture or macerated preparation by steeping scrapped rhizome into alcohol or mixing with a fatty ointment base. The vegetable product used, *Tami radix*, is the rhizome together with the roots.
In terms of chemical composition, the plant contains alkaloids, different glycosides, tannins, saponins, mucilages, diosgenin (small quantities), histamine and calcium oxalate, sterols [1, 2, 3, 6].
Taken internally or externally in high doses, it can cause accidents such as irritation of the pharyngoesophageal gastric mucosa, contact dermatitis. Responsible for the irritative action are the calcium oxalate raphides (in large number) and histamine [11, 13, 16].
The purpose of this paper was the investigation of the *Tamus communis* rhizomes, a vegetable material very little subjected to research, but frequently used in the treatment of various forms of rheumatism.

Material and method

The fresh vegetable material, i.e. the *Tamus communis* L. rhizomes, was first cut in round slices and dried in a thermostat at 50°C for 48 hours. Then, the material was powdered in a

mortar, the resulting powder being used to obtain the microscopic preparations using an 80% solution of chloral hydrate according to the routine procedures [8]. The microscopic examination and field photographing was carried out with a Nikon Eclipse E400 microscope equipped with a semiautomatic micro-photographing device (Nikon FM2).
The qualitative chemical analysis was carried out following the routine methodology. The chromatographic examination was carried out by thin layer chromatography (TLC) on SiO_2, Al_2O_3 and cellulose adsorbents, and paper chromatography (PC) using W_1 and W_3 chromatographic paper as support. The following mixtures were used as solvent systems: nBuOH-AcO-H_2O (60:10:30), $CHCl_3$-CH_3OH-H_2O (65:40:10), and nPrOH-AcOEt-H_2O (40:30:30) for TLC using SiO_2 adsorbent and the Partridge system (nBuOH-AcO-H_2O 4:1:5) for PC. The identification of saponins was carried out using phosphomolybdic acid, while the separation of diosgenin by PC was carried out using sulphanilic acid and a 10% solution of sodium carbonate, and respectively ferric chloride.
The acute toxicity of the alcohol extract was determined by administering it *per os* to mice (20-25g) according to the routine methodology. The anti-inflammatory action was demonstrated using carrageenan-induced paw inflammation edema model in rats.

Results and discussions

Upon microscopic examination of the preparations obtained using *Tamus communis* L. rhizome powder clarified with an 80% solution of chloral hydrate, the following characteristic elements were noticed:

- numerous calcium oxalate raphides in the form of large or small bunches (see fig.1, 2), most often wrapped in a mucilaginous shell (see fig. 3) and located in idioblasts (see fig. 4); other times, the raphides are scattered, long needle-like crystals sharply pointed at both ends, either oriented in the same direction or superposed (see fig. 5);

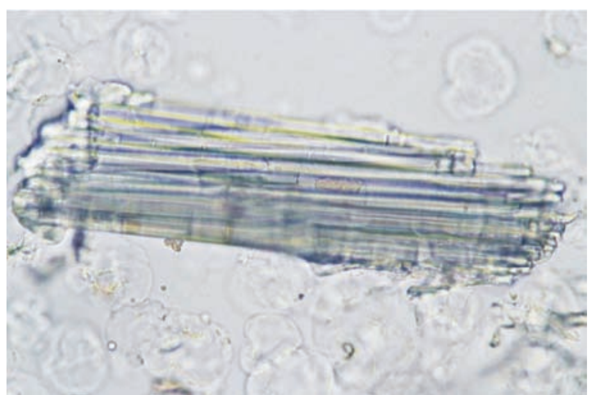

Fig. 1 – Large calcium oxalat raphide

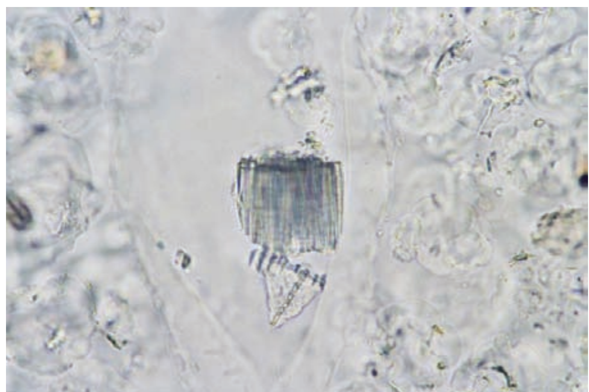

Fig. 2 - Small calcium oxalat raphide

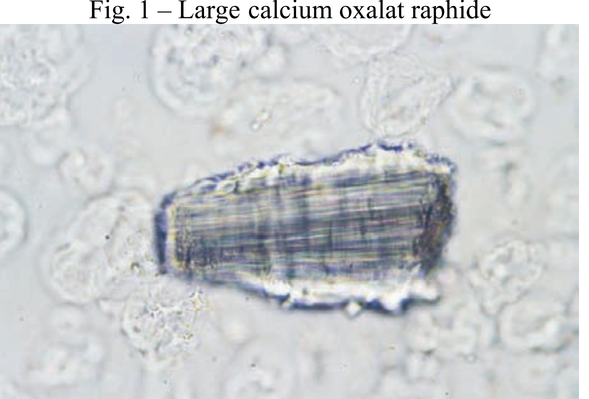

Fig. 3 - Calcium oxalat raphide with mucilaginous shell

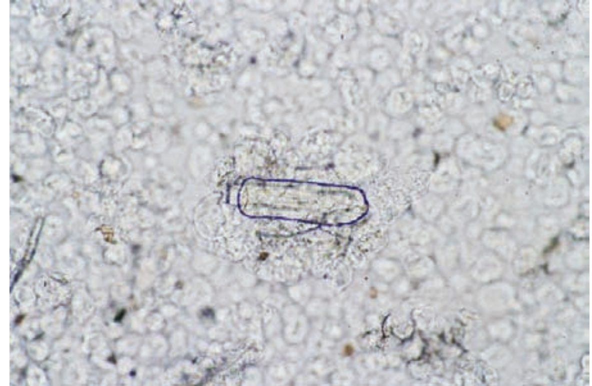

Fig. 4 – Idioblast with calcium oxalat raphide

- abundant starch in nearly spherical stromata, present in all the microscopic field (see fig. 3, 4);

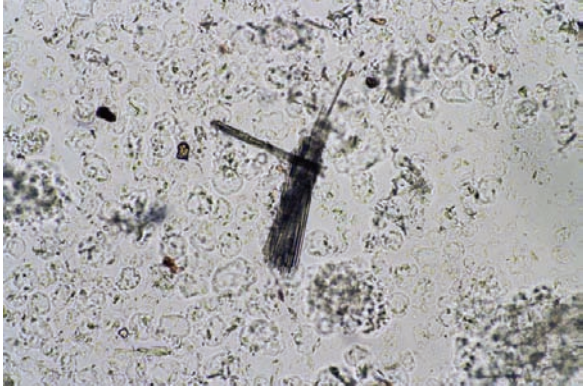

Fig. 5 - Calcium oxalat raphide

- large, reticulate woody vessels (see fig. 6, 7, 8);
- brown colored fragments of suberized tissue (see fig. 9);

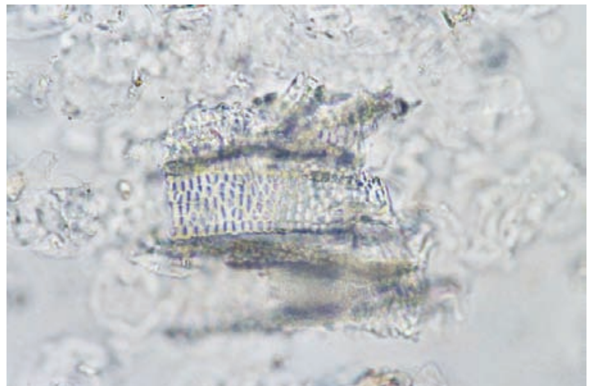

Fig. 6 - Reticulate woody vessels

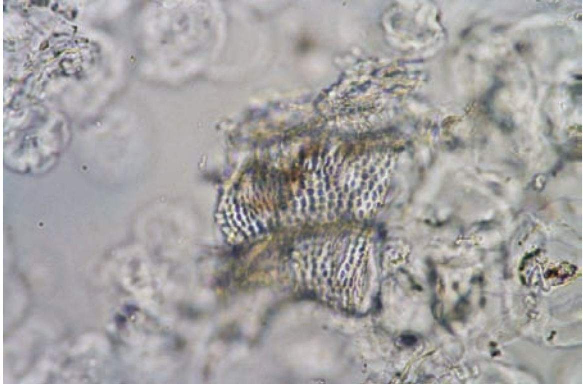

Fig. 7 - Reticulate woody vessels

Fig. 8 – Large reticulate woody vessels

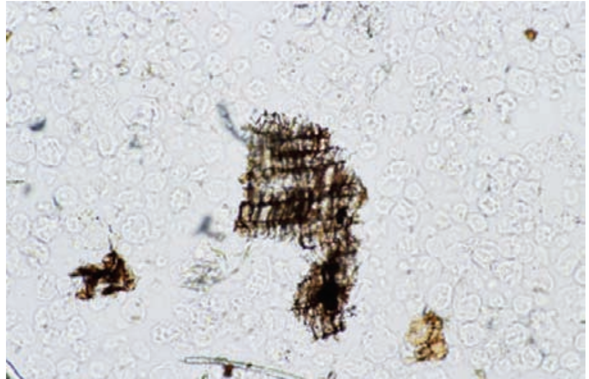

Fig. 9 - Suberized tissue

- thin, articulated sclerenchymatous fibers (see fig. 10, 11, 12);
- distinctive associations of fibers and reticulate woody vessels covered by brown suberized tissue (see fig. 13).

Fig. 10 - Sclerenchymatous fiber

Fig. 11 - Sclerenchymatous fiber

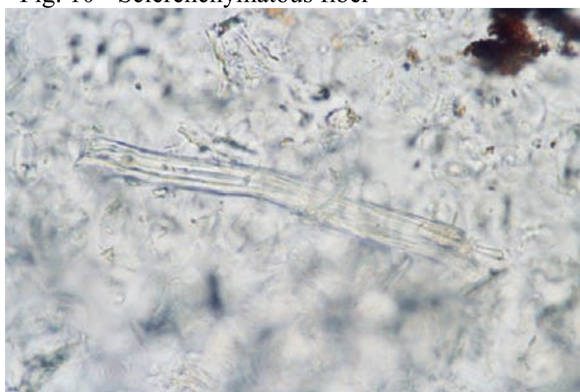
Fig. 12 - Sclerenchymatous fiber

Fig. 13 - Associations of fibers, reticulate woody vessels and suberized tissue

As it is referred in the literature, the calcium oxalate raphides are the characteristic element of the black bryony rhizomes, being spread practically in the entire peel and in the general parenchyma in the central cylinder.

The qualitative chemical analysis carried out by selective extraction with solvents of different polarity (ethyl ether, alcohol, and water), and then the examination of every extract in turn detected several groups of active ingredients such as: phytosterols, fatty acids, resinic acids, tannins, reducing simple sugars, sapogenins, mucilages.

The presence of saponins was confirmed both by the hemolysis test and by TLC comparing with the total contents of saponins - **steroidal** (as those found in the *Balanites aegyptiaca* fruit) and **triterpenoid** (*Hedera helix* and *Aesculus hippocastanum*). The analyses showed a complex saponin composition, with 8 spots in the case of the methanol extract (MeOH 70°) and 11 spots in the case of the ethanol one (EtOH 80°). In addition, diosgenin, the distinctive aglycon (sapogenol) of the steroidal saponins, was identified in the dichloromethane extract both by TLC on cellulose support and by PC.

The tests for anti-inflammatory action of the extracts of black bryony rhizomes were based on their frequent use in the traditional medicine to treat rheumatic conditions, bruises, and ecchymoses.

The experiment was conducted after determining the acute toxicity of the alcohol extract (EtOH 80°). The toxicity test showed that the extract was practically non-toxic, the maximum allowable dose exceeding 7g/Kg *per os* in mice.

The anti-inflammatory action of the alcohol extract from the black bryony rhizomes was determined comparatively with phenylbutazone, both administered *per os* in suspension with CMC 0.25%. The results are indicated in the Table I.

Table I. Results of anti-inflammatory test

Product to be examined	Dose (mg/Kg)	Experimental inflammatory edema (ml) Average ± S.D.	P	Inhibition %	R.A.I.
Controls	-	0.67 ± 0.23	-	-	-
Phenylbutazone	100	0.14 ± 0.06	< 0.01	79.1	1
Tamus extract	100	0.37 ± 0.16	< 0.05	44.8	0.57

R.A.I. - Relativ Activity Index

The *Tamus* extract exhibited a superior, statistically significant anti-inflammatory activity (inhibition 44.8%; P <0.05). Compared to phenylbutazone, the test showed an activity of over 50% of the reference product.
Based on the anti-inflammatory properties demonstrated and on the fact that the species was extensively sued in the popular medicine, we pursued the obtaining of an ointment based on alcohol extract of black bryony rhizomes. The formula of the ointment prepared is given below:

Tamus extract……………………... 31g
Oleum Hyperici…………………… 5.5g
Oleum Hippophaes……………….. 5g
Calendula extract…………………. 1g
Aetheroleum Lavandulae…………. 0.3g
Aetheroleum Pini…………………. 0.4g
Ointment base………….……..….. ad 100g

A lipophil base, highly emulsifying and penetrating was used in formulating the ointment.
The ointment obtained is intended to be used as anti-inflammatory agent in sports medicine.

Conclusions

The study conducted emphasized the importance of the *Tamus communis* L. species in therapeutics.
The microscopic examination identified the characteristics of the powder of black bryony rhizomes as follows:

- numerous raphides of calcium oxalate
- fragments of suberized tissue
- abounding starch
- isolated fibers or associations with suberized tissue and reticulate woody vessels
- reticulate woody vessels

The qualitative chemical analyses and the TLC examination resulted in the identification of many groups of active ingredients among which steroid sapogenins with diosgenin as the main aglycon moiety.
The tests performed showed a reduced toxicity of the alcohol extract and a significant anti-inflammatory action. This prompted the formulation of an ointment with anti-inflammatory properties.

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AQUAPLANT- LINE- BALNEARY USE PRODUCTS BASED ON A PHYTOCOMPLEX FROM MEDICINAL AND AROMATIC PLANTS

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Summary

*The aim of our study was to bring up the scientific and applicative research results of our specialists, with an important application in the elaboration of some balneary use products of medicinal and aromatic plants (**AQUAPLANT-line)**. Their main purpose is a systemic effect which can lead to amelioration of some chronical affections, and to improve the tonus of the organism. We have combined the phytocomplex with essential oils as potentiating agents.*

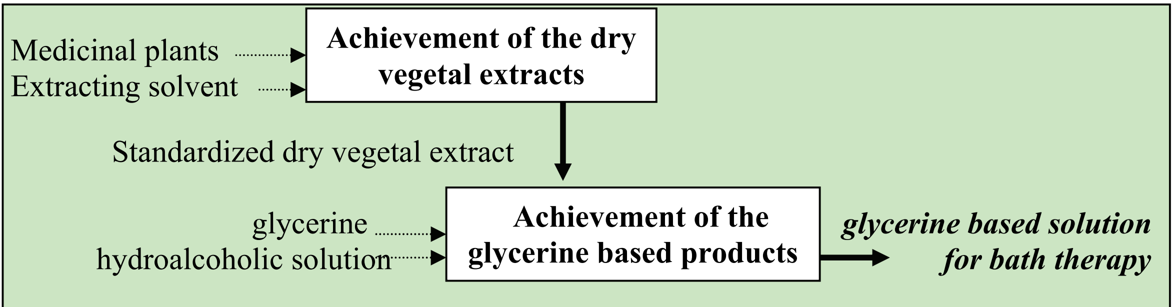
Keywords: *balneary products, phytocomplex, potentiating agents*

Introduction

The elaborated products aim a systemic effect, adjuvant in the basic treatment, intended to ameliorate some chronical affections and to generally fortify the organism. Being standardized, the preparations have the advantage to assure the treatment in conditions to be reproduced along its whole duration. In formulating each product we considered the association of the active phytocomplex working torwards the aimed affection with the potentiating agent, the volatile oil, respectively. The efficiency of the phytocomplex has at its basis of the synergic action of the active principles forming it. The potentiating agent has its input both by composing its specific action with that of the phytocomplex and by the influence on the pharmacokynetics of the product, favouring the penetration through the skin of the active principles.[1-6]

Materials and method

Obtaining the formulas was based on work steps as follows:



The standardisation of the products were done according to the therapeutic action, their qualitative and quantitative characterization heaving at its basis physical-chemical and microbiological methods (The X-th Romanian Pharmacopeia , European Pharmacopeia , quantitative methods elaborated and approved of by the company laboratories). Taking into account the topical use , we performed tests of biocompatibility (PATCH- TEST, HET-CAM, estimating hypoallergenicity)

The elaborated formulas as a result of associating medicinal and aromatic herbs are in table1.

Table 1 Association Formulas

Product	Composition	Active phytocomplex	Action
Aquaplant-ALGIN	***Vegetal extract*** of : *Fraxinus excelsior* (ashtree), *Salix alba* (wite willow), *Thymus serpyllum* (shepard's thyme), *Galium verum* (fairy), *Equisetum arvense* (horse tail) And *Pini montanae aetheroleum* (mountain pine essential oil).	***Association of***: Volatile oils, phenolic acids, methylsalicylate compounds, tannins, saponins, gums, flavonoids and microelements	Degenerative joint rheumatisme, abarticular rheumatic diseases, posttraumatic problems, muscle cramps
Aquaplant-SEDIN	***Vegetal extract*** of : *Humulus lupulus* (hops), *Tilia species* (lime tree), *Crataegus oxyacantha* (hawthorn), *Melissa officinalis* (balm mint), *Valeriana officinalis* (valerian) and *Lavandulae aetheroleum* (lavander oil)	***Association of***: Farnesol acetate, valepotriates, flavonoids, cumarin derivates, triterpenes, volatile oils	Sensorial and cerebral nervous disfunctions, minor or major sleeplessness, sexual neurosis, hysteria, nervous irritability, stress, anxiety
Aquaplant-FEMINA	***Vegetal extract*** of : *Calendula officinalis* (crawfoot), *Lythrum salicaria* (loosestrife), *Matricaria chamomilla* (chamomile), *Salvia officinalis* (common sage), *Lamium album* (white deadnettle), *Potentilla anserina* (silverweed)	***Association of***: Tannins, saponins, flavonoids, volatile oils, heterosides, naphtoquinones	In affections of the genital apparatus: vaginitis, leucorrhoea, vulvar itching, dysmenorrhea / natural support for feminin hygiene
Aquaplant-VENOL	***Vegetal extract*** of : *Plantago species* (plantain), *Salvia officinalis* (sage), *Mellilotus officinalis* (melilot), *Urtica dioica* (nettle), *Thymus vulgaris* (savory), *Lythrum salicaria* (loosestrife), and *Thymi aetheroleum* (savory essential oil)	***Association of***: Volatile oils (especially thymol), flavonic derivates, tannins	To prevent affection of the circulatory system, pre-varicose syndromes, to prevent pains and oedemas in varicose veins and hemorrhoids during pregnancy, varicose veins complications : superficial thrombophlebitis, trophic disorders, varicose ulcer.
Aquaplant-RENAL	***Vegetal extract*** of : *Equisetum arvense* (horse tail), *Agropyron repens* (coach grass), *Mentha piperita* (mint), *Polygonum aviculare* (Knot grass) ,and *Pini motanae aetheroleum* (pini volatile oil).	***Association of***: Saponosides, equisetonin, flavonoids, derivated from quercitrin, kaempferol and rutin, volatile oils, potassium salts and silicium dioxide.	Assures the hygiene of the inferior urinary tract and of the mucous membranes of the urogenital tract by its antibacterial and antiinflamatory effects. Offers a natural support of the renal function.

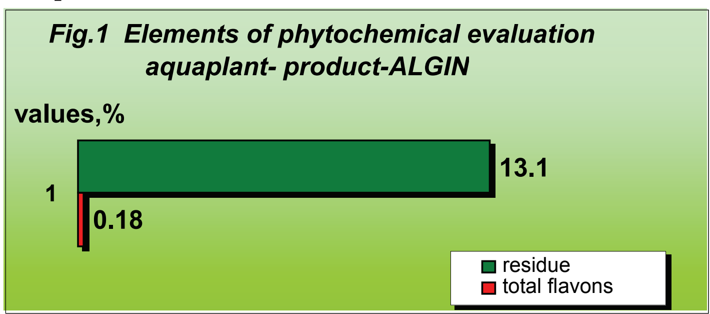
Results and Discussions

1. The glycerine based product for rheumatic diseases - ALGIN

The association of the *Pini montanae aetheroleum* with that of *Thymus serpyllum* and the methyl-salicylate compounds from *Salix alba*, determines an important anti-inflamatory antalgic effect in the degenerative or inflammatory lesions of the connective tissue. The silicates and the silicic acid from *Equisetum arvense* associated with the flavones of *Thymus serpyllum* contribute to its healing, makeing it more elastic and vigurous.

The complex of flavonoids and volatil oils from *Galim verum*, determines an antispasmodic action inducing an effect of relaxation of the striated muscles followed by a normalization of the general tonus.

The *Pini montanae aetheroleum* **,** having in its composition α and β pinene, has a hyperemisant action as well as one of stimulating the capacity to penetrate the skin with the active principles that are part of the phytocomplex being in the same time anti-inflammatory and slightly antalgic . [1,2,5]



The product was standardized in total flavones expressed as rutin. . The product testing for the evaluation of human risk has not shown any hypersensitivity reactions during administration.

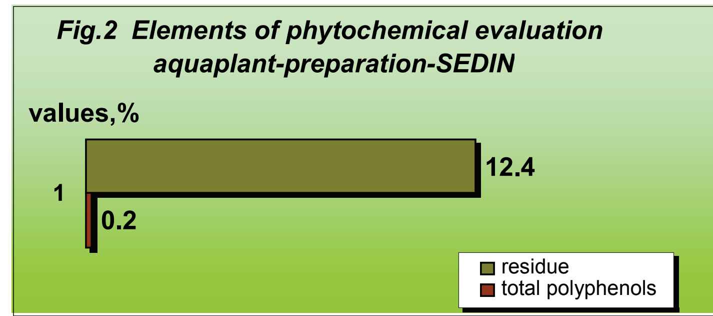
2. The glycerine based product for the nervous system affections - SEDIN

The presence of the farnesol acetate from *Tilia species* ,with that of methylbutenol from *Humulus lupulus*, and with the volatile oil and the valepotriates from *Valeriana officinalis* , determines a slight sedative and hypnotic action on the central nervous system.

The flavonoids (derivates of quercetol and kaempferol), the coumarin derivates, the pentacyclic triterpenes, the triterpenic acids, the volatile oil of *Crataegus oxyacantha* and *Melissa officinalis* , have a synergic action with the other components, potentiating the product.

The heart function normalizing by regulating the excitability of some internal receptors and by protecting the capillares. It also assures a venotonic protection.

Lavandulae aetheroleum , having as main components linalol and linalyl acetate, has a tonic and relaxing action and slight antidepressive effect. It is a heart tonic calming the heart beats, completing and strengthening the action of vegetal phytocomplex.[5,6]



The product was standardized in total polyphenols expressed as caffeic acid. The product testing for the evaluation of human risk has not shown any hypersensitivity reactions during administration.

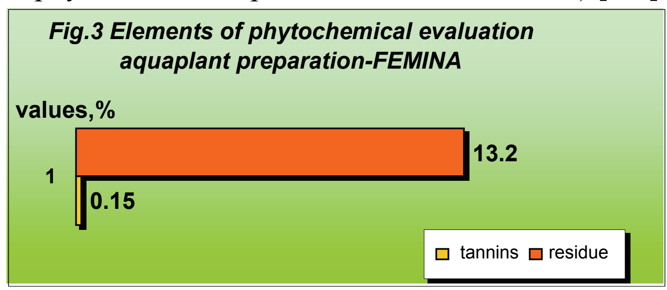
3. The glycerine based product for the genital apparatus affections - FEMINA

The tannins present in *Lythrum salicaria* , *Salvia officinalis* and *Potentilla anserina* are responsible for the astringent , hemostatic, antiseptic action and for the impermeabilization of the superficial skin and mucous membranes strata, protecting, as such, the inferior strata favouring cicatrization.

The tannins also have vasoconstrictor effects on the small vessels, favouring the recovering of the harmed tissues. The saponins from *Calendula officinalis* and *Lamium album* , have an antibacterial, anti-inflamatory and fungicidal action.

The association of the chinones from *Salvia officinalis* with the naftochinones from *Lamium album* and the tannins from *Potentilla anserina* confers the product a hemostatic, antibacterial, fungicidal and analgesic action.

As such, the preparation assures the calming of the pains at the level of the pelvis which might be caused by the existence of some genital malformations (vulvar inflamations, ovarian or of the uterine cervix) which in their turn, may be produced by bacterial, fungic or viral infections; the disappearing of the mucopurulent secretions and the sensation of local pruritus in leucorrhoea, trichomoniasis; the interruption of the infectious vaginal secretions (candidosis, infections with staphylococcus, streptococcus and colibacillus).[1-6]



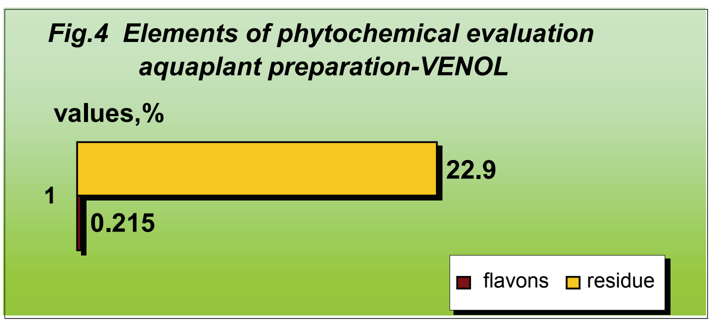
The product was standardized in total tannins expressed as tannic acid . The product testing for the evaluation of human risk has not shown any hypersensitivity reactions during administration.

4. The glycerine based product for the circulatory disfunctions – VENOL

The active substances from the selected medicinal plants raise the resistancy of the capillary walls decreasing their permeability having in the same time, an antiseptic, anti-inflamatory, hemostatic and cicatrizing action especially in the treatment of the varicose ulcers and of the piles.

The volatile oil of *Thymus vulgaris* has tymol as its main constituent and may assure up to 0.6% of the vegetal product along with carvacrol, terpinen, cineol, pinene, linalool, borneol. It has antiseptic properties as well as favorable effects on the blood vessels..

The flavone derivates contained by *Salvia officinalis*), *Mellilotus officinalis* , *Urtica dioica* , have a vasoconstrictor action straightly on the capillaries. Taking into account the chemical structure, we may consider that the flavonoids strengthen the intercellular cement of the capillary walls filling the existing pores. It is a process of chelatation to which the calcium ions contribute.
The tannins existing in *Lythrum salicaria* have an astringent, antiseptic and antibacterial action..



The product was standardized in total flavones expressed as rutin . The product testing for the evaluation of human risk has not shown any hypersensitivity reactions during administration.

5. The glycerine based product for the renal affections of the urinary tracts and bladder - RENAl

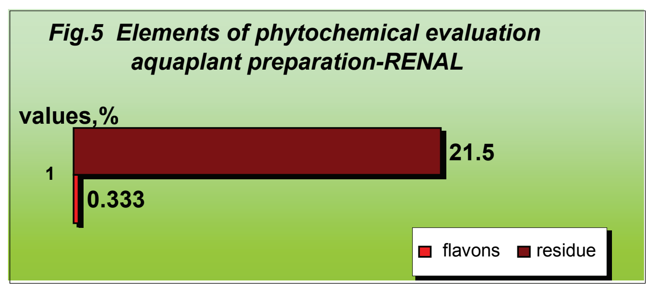
Associating the saponosides (equisetonine), flavonoids (quercetol derivates , kaempferol and rutoside) volatile oils, potassium salts and silicic dioxide from *Equisetum arvense* , *Agropyron repens, Mentha piperita and Polygonum aviculare* determine a slight diuretic, anti-inflamatory and antiedematous activity.

The simple cumarins (melittoside, melitonin, melilotic acid, fraxoside) and the furanocumarins in association with the pentacyclic triterpenic saponosides (ursolic acid), the flavonosides and the allantoin originating from *Mentha piperita* intensify the lymph. Besides this they also have an anti-inflamatory, antiseptic action and the diuretic ,antiedematous determining the raise of the venous discharge and the antispastic effect.

The anti-inflammatory , analgesic and antispastic action is also sustained by the association of pentacyclic triterpenes (ursolic acid) with the volatile oils, the iridoidic glucosides (asperuloside) and the salicilic acid from *Agropyron repens* , *Mentha piperita* and *Polygonum aviculare*

The preparation also contains inuline, tricine, tanin with antiseptic, slightly emolient at the tegument level. .

The volatil oil of *Pini montanae* containing α şi β pinene, carene, cuminaldehyde and other terpenes has an antiseptic, antispastic, analgesic and slightly sedative effect [3-6]



The product was standardized in total flavones expressed as rutin . The product testing for the evaluation of human risk has not shown any hypersensitivity reactions during administration.

Conclusions

Inspired by the well know Antique Romei therme, the modern world redescovered the beneficial effects of herbal bath therapy for which the whole range of balneotherapeutic preparations under the form of glycerine solutions was created. These herbal formulas have a therapeutic and prophylactic / preventive action for a series of affections that may beneficiate of bath therapy and constitutes an optimum modality , accessible and with proved therapeutic efficiency proved by clinical speciality tests.

In table 2 the main elements are synthetized by a characterization of the herbal formulas.

Table 2 Characteristics of the Bath Preparation

No. Crt.	Characteristics / value/ evaluation				
	ALGIN	**SEDIN**	**FEMINA**	**RENAL**	**VENOL**
Evaporation residue ,%	13.1	12.4	13.2	22.9	21.5
The total flavonic content, expressed in rutin,%	0.18	-	-	0.215	0.333
The total polyphenol content, expressed in caffeic acid,%	-	0.2	-	-	-
The tannin content, expressed in tannic acid,%	-	-	0.15	-	-
Total numbers of germs, 10^2/ml	80	100	50	50	90
Moulds, 10^2/ml	absent	absent	absent	absent	absent
Pseudomonas aeruginosa, ml	absent	absent	absent	absent	absent
Staphylococcus aureus, ml	absent	absent	absent	absent	absent
Enterobacteria, 10^1/ml	absent	absent	absent	absent	absent
Estimating compatibility / mucous membrane	Non-iritant	Slightly iritant	Non-iritant	Non-iritant	Non-iritant
Hypoalergenicity estimation	Minim risk	Minim risk	Without reaction	Without reaction	Without reaction

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A PRELIMINARY TRIAL CONCERNING THE "STAR" PHYTOIATRIC REMEDIES WITH PSYCHOTHERAPEUTICAL EFFECTS: ASHWAGANDA AND POTENT POWER

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Summary

The phytoiatric treatments of patients suffering of psychic complaints belonging to its various nosological entities are the subject of this communication.
It concerns a trial carried out in The Clinical Psychiatric Hospital "Prof. Obregia" Bucharest. The treatments were carried out with the ayurvedic phytoiatric "Star" remedies Ashwaganda and Potent Power with very good results.

Keywords: *phytoiatry, ayurveda, "Star" programme, Ashwaganda, Potent Power.*

Introduction

The work was carried out in the aim to apply the phytoiatric ayurvedic remedies in the tratment of psychiatric complaints with neurotic character and intensity (6). The motivation of the clinical research, carried out in The Psychiatric Clinical Hospital "Prof. Dr. Obregia" in Bucharest, by a medical team led by Prof. Dr. George Ionescu (4), president of The Commision of Psychiatry belonging to The College of Physicians in Romania, was the purpose of the treatment with iatrobotanic remedies was also to offer a complementary treatment with phytobioactive compounds susceptible to be administrated without any risks of side effects (7), in long lasting, chronic treatments and in the same time to protect by antitoxic and even detoxifying factors the patients'organisms submitted to the inherent chemical therapy. This reserch was also a target of the manysided phytoiatric "Star" Programme of The Ayurvedic Medical Centre in Bucharest, in order to implement in the medical practice the harmless ayurvedic phytoiatric remedies, already tested in India by chemical, analitical, phytopharmacological and clinical way too, accomplishing also by such a conribution the hippocratic ideal: "primum non nocere, deinde curare" and in the same time in the spirit of the aphorism: "The medicine based on proofs is the single possible and admissible", this aphorism beeing also the slogan of The "Star" Programme (6).

SUBJECTS AND METHODS

The clinical ayurvedic phytoiatric trial compriezed 52 patients hospitalized in The Psychiatric Clinical Hospital "Prof. Dr. Obregia", aged 40 – 65, suffering of neurotic troubles almost reactive to existential complaints (in family, professional, enviromental stresses) not surpassing the neurotic intensity and in the same time cooperative patients too, susceptible to follow the medical recommendations.
The patients' complaints were: depressive reactive states, anxiety, disadaptative states towards any inconvinience in the behaviour of the environmental human milieu and any deleterious events having, in almost of the cases, a psychic fragile, vulnerable structure. Almost of the patients especially those aged after fifty and already subjected to climacteric troubles, or beyond the climacterium elicited a premature ageing in the psychophysiological

functions and all the subjects had psychoemotional erotic and sexual functional disabilities. The patients' were 5 women and the rest were males. (3)

The treatment lasted one month (6 patients had to leave one week earlier the hospital) and consisted in an association of two ayurvedic phytoiatric remedies Ashwaganda Rasayan tablets (6), 1 – 2 tablets three times a day, and Potent Power tablets, with the same posology.

Ashwaganda is a remedy whose sanscrit name evokes the ancestral source of the bioactive ingredients obtained by conservative extraction from the indian medicinal species *Ashwaganda mool*, fam. *Solanaceae*, *Asgandh* in hindi, *Winter cherry* in english (*Withania somnifera* Dunal) and the species *Chlorophytum arundinaceum*, the first species name means in sanscrit "smelling like a horse or mare", in traditionally ayurvedic treatments it is known as a geriatric fortifying remedy, having also an aphrodisiac effect. It is used also against rheumatic complaints in consumptive states, fatigue. *Theophrastus* describes this plant mentioned also in The Arab Phytoiatric Compendium *"Kaknaj El Manoum"*. Two other classical authors, *Dr. Trebut* (1880) and *P. L. Simmonds* (1891) (10), mention the sedative and hypnotic effects of this species, from where also its specific name, "illo tempore" used in the Civil Hospital from Alger for these properties. Extensively spread in the drier areas of India, especially in the indo-gangetic area, *Withania somnifera* is also cultivated. (10), (11) It is an evergreen shrub. The active molecules of this species belong to the class of alkaloids, as: cuscohygrine, anahygrine, anaferine etc. besides the neurotic relatively mild nosological entities as reactive depressions, stress induced astenic neurosis, anxiety. (6)

The bioactive factors yelded from the plants of the species *Chlorophytum arundinaceum* have also, since historic times, a stimulant effect on the libidinal impulses.

The *Gumacacia* is an excipient ingredient having the role of a biological link of the tablets' particles, produced and yelded from various species of *Acacia* belongingto the indian flora or even imported. (10), (11)

Ashwaganda remedy is recommended as an energizing psychic and somatic factor and erotic stimulant in both sexes; its tonic effect is obvious in the slow down of the ageing process and the accompanying involutive pathology, in convalescent patients, as well as in persons exposed to a hard psychic and/or physical labor. As many other ayurvedic "Star" products, Ashwaganda induces besides the above mentioned effects also a lot of other adjacent beneficent effects like the increase of immunocompetence, an antiinflammatory and implicitly an analgesic effect. Very probably the sterolic (phytosterolic) compounds of Ashwaganda could be involved as precursors in the anabolic synthesis of steroidic hormones; it is known also that Ashwaganda improves the immunocompetence and the adaptive capacity against stress. (10), (11)

The remedy Potent Power belonging also to The "Star" Phytoiatric Programme containing bioactive factors extracted from about 14 species of medicinal plants belonging to the "green pharmacy" of India, as for instance *Sida cordifolia* Linnaeus, fam. *Malvaceae*, a small downy erect shrub containing phytosterols, but efedrine too. It stimulates the metabolism of the periferal nervous system. The gametogenesis, in both sexes, is stimulated by the bioactive factors from *Mucuna prurita* Hook, fam. *Fabaceae*, as mucunine, pruriendine, and improves the neurotransmitter metabolism and the synaptic and nervous circulation's physiology. Other species like *Asparagus racemosus* Willd (fam. *Liliaceae*), *Chlorophytum arundinaceum*, have an aphrodisiac and galactogenic (in women) effect; many other eutrophic mineral and organic compounds of the Potent Power, explain its tonic – an interesting example in this order of ideas is the ingredient *Coral bhasm*, prepared from the skeleton of corals, containing calcium compounds and other biological valuable minerals, with high degree of biodisponibility and assimilability – effect including the sexual functions, the libido, the erectile function, by improving the blood penetration into the cavernous penian system. Potent Power has also an aphrodisiac effect following an psychobiocatalitical enzymatic way (cf. *Prof. Dr. Farm.*

Seneca Bergheanu). Potent Power exerts also other beneficial effects, as: control of fatigue, depressive states and anxiety, of sterility and of light cardio-vascular troubles, as well it improves the diuresis. (10), (11)

Results and discussions

The improvment of the state of all the patients treated with the associated remedies, occured 8 till 10 days after the beginning of the treatment with Ashwaganda and Potent Power and concerned in first line the nosological entities like neurotic states, manifested by asthenia, psychic hypo or even adynamia, bradypsychic states, dissomnia, cephalalgia without lesional back-ground, depressive (especially reactive) states. A second nosological fields with possibilities for successful treatments with the two “Star” remedies are depressive states marked by the withdrawal from existential various fields namely professional activities, former hobbies, love, artistic events, journeys, culture and reading, family life, even resignation, surrendering, discouragement...

The ageing burden with its progressive darkness and decrepitude induced by the “tragic senectogenic time”, can also have the benefit of the slowing down by these remedies, of the involutive processes (3).

The sexual and sentimental erotic life improved after 12 – 15 days of treatment obviously, especially in patients with less than 50 years at the normal level for their age, and even for the “seniors” over 55 (14 patients) some erotic activities compatible with their age occured too.

In 6 patients suspected to have some liver complaints, probably because the intolerance towards the chemical, synthetic, hepatic treatments, with biochemical changes (increased transaminases, a modified proteinogramme), was administrated also also the liver protective phytoiatric “Star” remedy Livecom during the basic treatment, showed an improvement of these biochemical indicatives.

Two patients aged 22 and 24, hospitalised for a treatment against dependency towards stupefying drugs, followed the treatment with Ashwaganda and Potent Power, with an obvious improvement of their psychic state.

On the back-ground of deontological reasons, several more severe cases belonging to the phytoiatric clinical test were not deprived from the classical chemotherapeutical psychiatric therapy applied in synergism with the phytoiatric treatment, with also good results.

Conclusions

The phytoiatric ayurvedic treatments with remedies of The “Star” Programme demonstrated their efficacy in the treatment of neurosis, with anxiety, depressive states, psychic asthenia, disadaptive states, ageing psychic complaints, troubles of sexual and erotic-emotional functions. The strategy, the schedule, of the treatment, has to follow the rules of a clinical research, but has also to consider the reality of the psychopathology unavoidably heterogenous with a high degree of many sided individuality, also inherent. The capacity of the reserchers to manage, by an adequate strategy of the test, and a very analytical understanding of the results, are without any doubt the single way susceptible to lead to the theoretical and praxiological success of the phytoiatric research, especially in the so complicated field as psychopathological one and its many fold determinants.

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CORELATION BETWEEN BIOCHEMICAL CHARACTERISTICS AND SPECIFIC ACTIONS OF AROMATIC AND MEDICINAL PLANTS USED IN SKIN THERAPEUTICS

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Summary

The phytotherapeutical treatments are based on the utilization of vegetal species with antibiotic, disinfectant, cicatrizing, antiseptic and anti-inflammatory characteristics. Aromatic species, which contain mainly oils composed of terpenes, aldehydes, cetones, phenols, hydrocarbons, alcohols, etc., at various proportions, have an important role in these treatments, giving specificity to fragrance, physical, chemical and therapeutic characteristics.

Key words: *skin, therapeutics, aromatic plant, medicinal plant*

Skin can be considered as a monumental side of human body. Like a monument, which can be damaged by external factors, skin is similarly aggressed by influences causing various diseases within the population. Concerns in the field of naturist cosmetics existed since the appearance of human civilization.
In order to maintain skin health, man has used many efficient remedies; among them, medicinal plants had a special place.
Each stage of human culture has created proper conceptions on skin beauty and health, which had to be reached by cosmetic means.
The medical practice in the last decades has found out that many preparations of chemical synthesis can result in adverse secondary reactions and unpredictable intoxications. Active principles from plants are synthesized metabolically by enzyme reactions, at the following complex forms: alkaloids, tannins, saponoside, glycoside, aliphatic hydrocarbons, organic acids, phenols, cetones, terpenoides, etc. They are easily metabolised and assimilated by human cell and lack undesirable secondary effects.
The use of medicinal plants in cosmetics has at the base the utilization of active principles from different vegetal organs, which give some therapeutic characteristics: emollient, cicatrizing, antiseptic and antibacterial, revulsive and anti-inflammatory, hydrating, nutritive tonic and revitalizing ones.
Due to the complex chemical composition, many aromatic and medicinal plants are used in cosmetics and dermatology as such or in combination with other natural products, under the shape of lotions, creams and shampoos, etc.
From the multitude of medicinal plants cultivated in Romania, this paper presents a few species of two botanic families: *Compositae* and *Labiatae.*

Materials and methods

From the group of aromatic plants, efficiently used in dermatologic treatments, we have studied the therapeutic characteristics and the actions, typical for controlling different diseases of the following species: pot marigold (*Calendula officinalis),* lavender (*Lavandula angustifolia),* rosemary *(Rosmarinus officinalis),* and common sage *(Salvia officinalis).*
For the elaboration of research methods, we have correlated the utilization forms in external treatments, to plant chemical composition and biological active principles connected to therapeutic characteristics.

We have used cultivated plants from the experimental field, by help of which we have done preparations (oils, tinctures and face waters) by maceration at cold, in alcohol and vegetal oils.
The alcohol maceration is done by adding alcohol of 60-70^0 on plants, in a glass or porcelain vessel. The mixture is kept for 8-10 days in a hermetically closed vessel, at dark and is stirred periodically, then it is filtered and kept in dark-coloured bottles.
Medicinal oils have been obtained by oil maceration of plants, during 4-6 weeks, at sun or next to a heating source, stirring daily the preparation.

Results

Calendula officinalis
Family *Compositae*

Calendula officinalis is an annual or biannual grass, rustic or cultivated, which becomes seldom wide (A. Laza et al., 1975, E. Fisher, 2000).
Origin and spreading
Originated from the Mediterranean regions, western Asia and Canaries Island, the species spread all over the Europe as ornamental plant. It is also found as sub-spontaneous species.
It is cultivated as medicinal plant in Germany, Czech Republic, Slovakia, Poland, Bulgaria, Hungary, Austria, Syria, Egypt (C. Pârvu, 2000).
In Romania, it is cultivated in all farming regions.
Organs used: flowers;
Characteristics: cicatrizing, astringent, emollient, anti-inflammatory, decongestive, haemostatic (C. I. Milică et al., 2005);
Chemical composition: saponine and triterpenic saponoside, flavonoids, carotenoids, fatty acids, organic acids, mucilage, vitamins, essential oil, etc. –(V. Ceauşescu et al., 1988);
Utilization: acne, irritated, sensitive, dry and faded complexions, eye diseases, hair conditioning;
Means of utilization: infusion, powder mixture, concentrated decoct, lotion, tincture, disinfectant, cream (C. I. Milică et al., 2005, C. Pârvu, 2000).

Lavandula angustifolia
Family *Labiatae*

Origin and spreading
Lavender has originated from Europe, its spreading centre being the western side of the Mediterranean basin.
The main growing countries are France, Spain, Bulgaria, Yugoslavia, Italy, Hungary, etc. (C. Pârvu, 2000, E. Fischer , 2000, C. I. Milică et al., 2005).
In Romania, this crop was cultivated since 1950, became more important after 1970 and, in 1988, it was extended at almost 420 ha, and, in 1990, the crop reached 3000 ha.
Besides the species *L. angustifolia* Mill., there are also known the following species*: L. latifolia* Vill. (*L. spica* L.), *L. stoechas* L., *L. dentata* L., *L. pedunculata* Cav., *L. burmanii* Benth, the hybrid *L. hybrid* R. For Romania, *L. angustifolia* Mill. presents a great economic significance (A. Laza et al., 1975, Ş. Mocanu et al., 1983)
Organs used: flowers;
Therapeutic characteristics: antibacterial, sedative, cicatrizing, anti-allergic, astringent, activating of blood circulation (C. I. Milică et al., 2005);
Chemical composition : essential oil, (linalil acetate, linallol, geraniol, camphor, etc.) tannins, organic acids, mineral salts (Eliu-V. Ceauşescu et al., 1988).
Utilization: burns, infected plagues, wounds, contusions, regeneration of damaged skin, rosaceous complexion.
Means of utilization: mask, lotion, powder mixture, infusion, tincture, decoct, aromatic vinegar (C.I. Milică et al. 2005)

Rosmarinus officinalis
Family *Labiatae*

Rosemary is an aromatic and medicinal plant known and used since Antiquity. The name given by Romans was kept until today (*ros*=dew, *marinus*=sea, therefore, moistened by sea dew).
Origin and spreading
A bush, which is typical to southern Europe and growths as spontaneous plant in the Mediterranean region; it is spread in the Dalmatian Islands of the Adriatic Sea, in the southern France, on the Spanish Coast, in the Balearic Islands (E. Fisher, 2000, C. Pârvu, 2000).
In Romania, it is cultivated in the south-western regions of Romania, in sweet climate regions, without excessive temperatures during winter.
Organs used : aerial part with leaves and flowers;
Therapeutic characteristics: it has good effects in scurf controlling and hair growth, in complexion smoothing; it also has a revitalizing effect in case of tired complexion, with dilated pores and bad blood circulation (C. I. Milică et al., 2005);
Chemical composition: monoterpenic hydrocarbons (pinene, camphene, limonene, etc.), terpineol, linalool, camphor (Eliu-V. Ceauşescu et al., 1988);
Utilization: tired impure complexes
Means of utilization: aromatic vinegar, plant mixture, macerate, infusion, tincture, cleaning milk, face lotions (C. I. Milică et al., 2005)

Salvia officinalis
Family *Labiatae*

Origin and spreading
The sage originated from south-eastern Europe, from the Dalmatian Coast until Macedonia. It is spread in spontaneous flora or, as cultivated species, in all the Mediterranean region, until Spain, France, Italy, Greece and islands from the Adriatic Sea (E. Fischer, 2000, C. Pârvu, 2000).
In our country, it growths under favourable cropping conditions in south and south-eastern Romania.
Organs used: leaves and non-lignified aerial part;
Chemical composition: essential oil (oxygenated monoterpene compounds, monoterpene hydrocarbons, sesquiterpenoide), tannins, saponine, mucilage, flavonoids, organic acids, vitamins, mineral salts, etc. (Eliu -V. Ceauşescu et al., 1988);
Therapeutic characteristics: astringent, antiseptic, antibiotic, bacteriostatic, anti-inflammatory, cicatrizing (C. I. Milică et al., 2005);
Utilization: skin ulcerations, wounds, contusions, varix, seborrhoea, scurf, acne, dry, irritated and flushed complexes;
Means of application: tincture, aromatic vinegar, infusion, compresses, etc. (C. I. Milică et al., 2005)

Conclusions

The studied plants, prepared under different forms (infusions, tinctures, decocts, creams, cleaning lotions, unguents, unction, face waters, shampoos, emulsions, etc.) are efficient in the treatment of dermatologic affections, such as: acne, plagues, wounds, burns, chilblains, ulcerations, eczemas, itch, moles, skin cancer, etc.
The dry, sensitive, wrinkled and tired complexions, with dilated pores and bad blood circulation, can benefit by 100% naturist treatments, based on utilization of aromatic plants:
- sensitive and dry skin gets a shining aspect;
- oily complexions with dilated pores become smooth and pores close and impure complexes become fresh and rosy;
- dry, tired and aged complexions become fresh.

Diseases of head skin and hair (seborrhoea, scurf, alopecia) benefit by treatments with special shampoos, which give vitality to hair, due to the effects of deep cleaning and disinfection.
Calendula officinalis, an annual species well adapted to ecologic conditions of the Moldavian central area (with high flower yields), has many therapeutic characteristics (antibiotic, cicatrizing, anti-inflammatory, emollient, sedative); these effects are tested by curing many dermatologic diseases, respectively, skin plagues and wounds, furuncles, eruptions, and tegument stains, ulcerations, and eczemas. In cosmetics, it is used in the treatment of acne, irritated, dry and pale complexions.
Lavandula angustifolia, a species cultivated on great areas in the Mediterranean basin, is used in perfume industry for compositions of fougère-type and eau-de-Cologne, included in cold

creams, deodorants, and simple or amber tinctures. Due to antiseptic, cicatrizing, analgesic characteristics, it has many effects in controlling some skin diseases: infected plagues, wounds, eczemas, insect stinging and alopecia.
Rosmarinus officinalis, an aromatic and medicinal plant, used since Antiquity, has high essential oil contents, with therapeutic actions on tired complexions; it is also used for face smoothing, scurf controlling and hair growth stimulant.
Salvia officinalis, considered in Antiquity as universal remedy in medicine, has bacteriostatic, antiseptic, anti-inflammatory, cicatrizing characteristics; it is used externally in skin ulcerations, bleeding or infected wounds, acne, seborrhoea, scurf and stimulation of hair growth; it is also used in maintaining an intense hair colour.

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ESSENTIAL OIL OF WILD GROWING PISTACIA SPECIES FROM MONTENEGRO

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Summary

Steam distilled essential oil obtained from the leaves and branches of Pistacia lentiscus and Pistacia therebinthus, grown in Montenegro, were analyzed by capillary GC and GC/MS. The most abundant constituents of P. lentiscus leaves oil were α-pinene (24.8 %), β-pinene (10.3 %) and δ-cadinene (5.8 %). The oil obtained from the branches was reach in α-pinene (32.1 %), β-pinene (10.9 %) and sabinene (4.8 %). The most abundant constituents of P. therebinthus leaves oil were caryophylla-3,8(13)-dien-5-β-ol (10.9 %), τ-cadinol (7.1 %) and δ-cadinene (6.4 %). Oil obtained from the branches were reach in limonene (10.1 %), caryophyllene oxide (6.5 %), α-pinene (5.5 %) and trans-β-ocimene (5.5 %).

Keywords: *Pistacia lentiscus, Pistacia therebinthus, leaves, branches, essential oil, composition.*

Introduction

The members of the genus *Pistacia* are shrubs or little trees, very characteristic for the maquia type of vegetation in the Mediterranean region. Those species belong to the sumac family (Anacardiaceae) and they are the main sources of mastic resin. *Pistacia lentiscus* and *P. therebinthus* are present in the vegetation of the peninsula Luštica at the Adriatic coast (Montenegro). There is no data about composition of the constituents of mastic resin or essential oil obtained from the plants growing in this area. This paper deals with the essential oil analysis.

Material and methods

Plant material

Plant materials were collected in the July of 2004, at the peninsula Lustica.

Oil isolation and analyses

The air-dried plant material was cat and the essential oil was obtained by hydrodistillation using Clevenger type apparatus. Diluted oil solutions in n-hexane (1%) were analysed by analytical gas chromatography (GC/FID) and combination of gas chromatography and mass spectrometry (GC/MS).

Gas chromatography

A Hewlett Packard, HP-5890 gas chromatograph, equipped with a split-splitless injector, fused silica capillary column HP-5 (25 m x 0.32 mm; 0.5 μm film thickness), and FID was employed. Oil solutions in hexane (~ 1%) were injected in split mode (1:30). Injector was heated at 250°C, detector (FID) at 300°C, while the column temperature was linearly programmed from 40°C-240°C (4°C/min.).

GC/MS

Analyses were carried out on a Hewlett Packard, HP G1800C Series II GCD analytical system equipped with split-splitless injector and fitted with HP-5MS capillary column (30 m x 0.25 mm; 0,25 μm film thickness). The chromatographic conditions were as above. Injector was heated at 250°C, transfer line (MSD) at 280°C, while the column temperature was linearly programmed from 40°C-240°C (4°C/min.). EIMS spectra (70 eV) were obtained in scan mode in m/e range 40-450.

Component identification and quantification

The components of the oil were identified by comparison of their mass spectra to those from Adams (1), Wiley and NIST/NBS libraries. The experimental values for retention indices were determined by the use of calibrated Automated Mass Spectral Deconvolution and Identification System software (AMDIS ver.2.1., DTRA/NIST, 2002). Results obtained were correlated with retention indices with data available in common literature (1) as well as from other sources available on Internet (for instance: www.flavornet.org; www.pherobase.com). For quantification purpose, area percent reports obtained by FID were used.

Results and discussion

Essential oil was isolated by steam distillation of the air-dried plant material. Obtained oil was dissolved in n-hexane (around 1% solution) and analysed by GC/FID and GC/MS. Chemical composition of investigated oils is presented in the Table 1.

The main constituents present in the essential oils isolated from the leaves and branches of *P. lentiscus* were: α-pinene, β-pinene, α-phellandrene, α-terpinene, p-cymene, limonene, γ-terpinene, trans-pinocarveol, cis-verbenol, pinocarvone, terpinene-4-ol, myrtenol, β-caryophyllene, α-humulene, γ-muurolene, germacrene D, epi-bicyclosesquiphellandrene, δ-cadinene, spathulenol, caryophyllene oxide, humulen-epoxide II, 1-epicubenol, τ-cadinol, α-cadinol and 10-peroxi-muurolan-3,9(11)-diene. The most abundant constituents of the oil from leaves were: α-pinene (24.8 %), β-pinene (10.3 %) and δ-cadinene (5.8 %). The oil obtained from the branches was rich in α-pinene (32.1 %), β-pinene (10.9 %) and sabinene (4.8 %).

The main constituents present in the essential oils isolated from the leaves and branches of *P. therebinthus* were: α-pinene, β-pinene, α-terpinene, p-cymene, limonene, 1,8-cineol, trans-β-ocimene, γ-terpinene, linalool, trans-pinocarveol, pinocarvone, terpinene-4-ol, α-terpineol, myrtenol, thymol, α-copaene, α-guaiene, α-humulene, alloaromadendrene, γ-muurolene, germacrene D, α-muurolene, β-bisabolene, δ-cadinene, α-cadinene, elemol, elemicin, spathulenol, caryophyllene oxide, humulene-epoxide II, γ-eudesmol, τ-cadinol, caryophylla-3,8(13)-dien-5-β-ol, caryophyllenol II, cuzinol, hexahydrofarnesylacetone and farnesylacetone. The most abundant constituents of the leaf oil were: caryophylla-3,8(13)-dien-5-β-ol (10.9%), τ-cadinol (7.1%) and δ-cadinene (6.4%). Oil obtained from the branches were rich in limonene (10.1%), caryophyllene oxide (6.5 %), α-pinene (5.5%) and trans-β-ocymene (5.5%).

Table 1. Chemical composition of the essential oil of wild growing *P. lentiscus* and *P. therebinthus* collected in Montenegro

Components	RIA	RIE	*P. lentiscus*		*P. therebinthus*	
			leaves % m/m[r]	branches % m/m	branches % m/m	leaves % m/m[r]
trans-2-hexenal	855	870.5	0.06	-	-	-
tricyclene	927	920.4	0.54	-	-	0.42
α-thujene	930	921.3	0.28	0.26	-	0.24
α-pinene	939	938.3	24.76	5.53	-	32.05
camphene	954	946.8	2.24	0.50	-	1.56
verbenen	968	952.0	0.16	0.46	-	0.35
sabinene	975	974.4	5.48	0.52	-	4.75
β-pinene	979	978.4	10.27	3.07	-	10.89
β-myrcene	991	994.8	0.39	0.63	0.42	0.26
α-phellandrene	1003	1004.5	1.00	0.37	0.89	0.52
α-terpinene	1017	1016.9	1.00	0.63	0.30	0.64

p-cymene	1025	-	2.68	2.60	-	4.44
limonene	1029	1038.5	3.87	10.07	1.18	4.14
1,8-cineole	1031	-	-	2.47	-	-
cis-β-ocimene	1037	-	-	-	0.35	-
trans-β-ocimene	1050	1049.1	0.05	5.50	3.14	-
isoamylbutyrate*	1060	1057.4	0.15	1.24	0.50	-
γ-terpinene	1060	1058.3	2.02	0.15	0.26	1.13
α-terpinolene	1089	1087.2	0.78	0.81	0.35	0.55
2-nonanone	1090	1092.8	0.09	-	-	0.11
linalool	1097	-	-	0.77	1.18	-
α-pinene-oxide	1099	1096.8	0.15	-	-	0.23
nonanal	1101	-	-	0.52	0.95	-
cis-p-ment-2-en-1-ol	1122	1121.1	0.23	-	-	0.19
α-campholenal	1126	1124.9	0.24	-	-	0.81
trans-pinocarveol	1139	1138.0	0.85	1.45		2.35
cis-verbenol	1141	1144.9	0.08	0.67	-	1.44
camphene-hydrate	1150	1146.5	0.12	-	-	0.29
β-pinene-oxide	1159	-	-	0.42	0.26	-
trans-pinocamphone	1163	1154.9	0.11	-	-	0.19
pinocarvone	1165	1161.1	0.48	1.37	0.32	1.85
terpinene-4-ol	1177	1177.5	5.62	3.08	0.27	3.31
p-cymene-8-ol	1182	1184.1	0.15	0.18	0.56	0.20
α-terpineol	1189	1194.7	0.94	0.71	3.12	0.95
myrtenal	1196	-	-	0.23	0.63	-
myrtenol	1196	1198.1	0.88	1.82	0.91	2.12
α-phellandrene-epoxide		1201.4		0.41	0.30	0.27
verbenone	1205	1210.0	0.07	-	-	0.25
trans-carveol	1217	1220.1	0.10	0.24	0.26	0.36
thymolmethyl ether	1235	-	-	0.36	-	-
cuminal	1242	-	-	0.28	0.55	-
isoamylcaproate		1251.6	0.30	-	-	0.19
piperitone	1253	1251.7	-	-	-	0.20
trans-2-decenal	1264	1261.7	-	-	0.33	0.14
bornyl acetate	1289	1285.3	0.12	0.19	-	0.20
thymol	1290	-	-	1.95	2.39	-
2-undecanone	1294	1293.9	0.15	-	-	-
carvacrol	1299	1304.4	-	-		0.30
trans,trans-2,4-decadienal	1314	-	-	0.18	0.23	-
myrtenyl acetate	1327	-	-	-	0.37	-
α-terpinyl acetate	1349	1348.7	0.22	-	-	-
α-cubebene	1351	-	-	0.26	-	-
2-methylundecanal	1368	-	-	0.10	0.85	-
α-ylangene	1375	-	-	0.20	-	-
α-copaene	1377	1373.5	-	1.71	0.26	-
β-bourbonene	1388	1381.8	0.98	0.47	0.56	0.28
β-cubeben	1388		0.97	0.13	0.39	0.36
methyleugenol	1404	-	-	0.15	-	-
β-caryophyllene	1419	1420.4	2.89	10.70	7.58	0.51
β-gurjunene (calarene)	1434	1426.9	0.22	0.21	0.63	0.09
α-guajene	1440	-	-	0.35	5.26	-
α-humulene	1455	1452.3	1.21	1.64	2.09	0.22
trans-β-farnesene	1457	1457.2	0.63	-	-	0.25
alloaromadendrene	1460	-	-	0.26	1.31	-

γ-muurolene	1480	1477.5	2.37	1.59	1.84	0.83
germacrene D	1485	1482.0	3.72	0.57	2.43	0.59
Epi-bicyclosesquiphelandrene		-	2.34	0.20		0.94
α-murolen	1500	-	-	1.17	2.13	-
β-bisabolen	1506	-	-	0.12	1.22	-
γ-cadinen	1514	1512.4	0.60	1.10		0.94
δ-cadinen	1523	1523.0	5.76	4.43	6.44	1.96
Cadina-1,4-dien	1532	-	-	0.16	-	-
α-cadinene	1539	-	-	0.26	1.38	-
α-calacoren*	1542	1540.4	0.27	0.70	0.66	0.12
Phenilmethylhexanoat	-	-	0.17	-	-	0.11
Elemol	1550	1549.0	-	0.96	1.35	0.18
Elemicin	1557	1556.4	-	0.67	1.27	-
Germacrene B*	1556	1553.4	0.36	-	-	0.48
cis-3-hexenilbenzoat	1570	1568.4	-	0.29	0.37	
Spathulenol	1578	1576.9	0.71	1.46	3.15	1.30
Caryophylene oxide	1583	1581.5	1.79	6.45	1.60	1.72
Salvial-4(14)-en-1-on, viridiflorol	1595	1591.0	0.13	0.55	0.36	0.15
Humulen-epoxide II	1608	1615.2	0.96	0.77	1.21	1.17
1-epi-cubenol	1629	1625.9	1.35	0.09	0.59	0.70
γ-eudesmol	1632	1630.5	-	0.42	2.43	-
τ-cadinol	1640	1640.1	2.89	2.81	7.12	2.17
α-cadinol	1654	1655.3	1.66	-	-	2.39
caryophylla-3,8(13)-dien-5-β -ol	-	1655.8	-	4.49	10.86	-
caryophyllenol II	-	-	-	1.97	3.75	-
14-hydroxi-9-epi-trans-caryophyllene	1670	1670.4	0.32	-	-	0.65
cuzinol	1680	1684.1	-	0.14	1.35	-
eudesma-4(15),7-dien-1-b-ol	1688	1683.0	0.31	-	-	0.71
copaen-15-ol	-	-	-	-	0.65	-
leden-oxide (II)/ledenol*	-	1701.2	0.17	-	-	0.75
aristol-9-en-3-ol	-	1716.1	-	-	-	0.31
n.i.*	-	1718.8	-	-	0.63	0.36
10-peroxi-muurolan-3,9(11)-dien	1760	1763.0	-	-	0.33	-
sclareol-oxide	-	1797.2	0.08	-	-	1.50
hexahydrofarnezyl acetone	1843	1842.7		0.16	2.59	
n.i.*		1858.9		0.12	1.04	
n-hexadecanol	1876	1878.2	0.12	0.65		
n.i.*		1896.2		0.13	0.27	
farnezyl acetone		1915.0		0.21	3.24	
phytol	1940	1944.4		0.21	0.29	
octadecanol	2078	2083.0		0.31		

RIE – Retention index (experimentaly obtained data); RIL – Retention index (literature data); *- tentative identification (EIMS, 70 eV)

Acknowledgements

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LIPOPHILIC SUBSTANCES FROM FRUITS OF *PHYSALIS ALKEKENGI* L.

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Summary

This paper constitutes a part of our chemical investigation into lipids from fruits of Physalis alkekengi L. The chromatographic analysis (TLC, HPLC) of the petroleum ether extract obtained from dried and powdered fruits revealed the presence of tocopherols (alfa- beta- and delta-tocopherol), phytosterols (beta-sitosterol), along with acylglycerols and carotenoids.

Keywords: *Physalis alkekengi, acylglycerols, phytosterols, tocopherols, carotenoids.*

Introduction

Physalis alkekengi (Bladder Cherry, Winter Cherry) is rather known as an ornamental plant due to its bright orange to red papery calyces which resemble Chinese lanterns and which in winter become skeletonized showing an orange berry within. The fruits are edible and taste sour. In folk medicine of several East-European countries they are used as a diuretic, to eliminate the excess of uric acid in gout and as an anti-rheumatic. They have also been a major source of vitamin C. [1,5]

"Alkekengi fructus" (Baccae Alkekengi) was an officinal drug in the French and Venezuelan pharmacopoeias and an ingredient of the syrup "Chicoree compose" from the Pharmacopée Française 1937. [1,8]

Excepting fruits, the whole plant is toxic due to alcaloids of the sterol (fisalones) [3], pyrroline (cuskhigrine) [11] and nortropane (calystegines) classes [13]. Previous phytochemical studies on fruits showed the presence of carotenoids [10], bitter principles (fisalin A, B, C etc), tannins, mucilages, vitamin C, sugars, organic acids (citric acid), minerals, pectins, rezins and fatty oil in seeds [1,2,5,7].

In continuation of our investigations of the chemical composition of Alkekengi fructus, we now report the chemical composition of the petroleum ether extract.

Materials and methods

Reagents

HPLC-grade methanol, isopropanol and acetonitrile (ACN) were purchased from Merck (Darmstadt, Germany). The chemicals used for extraction, column chromatography and TLC such as petroleum ether, acetone, diethyl ether, glacial acetic acid and ethanol were purchased from Chimopar (Bucharest, Romania). Methanol used for saponification, ethyl acetate and anhydrous sodium sulphate from Reanal (Hungary), potassium hydroxide, ascorbic acid and sulphuric acid from Merck (Darmstadt, Germany). TLC reagents such as rhodamine 6G, 2',7'-dichlorofluorescein and diphenylboric acid aminoethyl ester (Neu, NP) from Sigma (Steinheim, Germany), phosphomolybdic acid from Merck (Darmstadt, Germany), anisaldehyde from Merck (Hohenbrunn, Germany). Sitosterol used for TLC determinations was purchased from Koch – Light Laboratories Ltd (England), beta-sitosterol 95%, alfa-tocopherol, beta-tocopherol and delta-tocopherol used for HPLC determinations from Sigma (Steinheim, Germany). "Vitamina E - capsule moi" (alfa-tocopheril acetate in sunflower oil) was purchased from Biofarm – Bucharest. Virgin olive oil and sunflower oil for TLC were purchased from a shop.

Sample preparation

Ripe fruits of *Physalis alkekengi* collected from wild plants growing in the Mureş district were dried at room temperature. Approximately 30 g powdered fruits were exhaustively extracted with petroleum ether in a Soxhlet apparatus, concentrated in a rotavapor (RV 05-ST, Janke & Kunkel IKA Labortechnik) and stored in a refrigerator till further analysis (TLC, C.C., HPLC).

Petroleum ether solutions of each fraction were applied to TLC-plates.

Saponification of fractions I-IV preceded HPLC analysis. It was performed as follows: 5 mL KOH 30% and 20 mL MeOH and 0,50 g ascorbic acid were added to each fraction and heated under reflux for 35 minutes. After cooling at room temperature, 15 mL of 20% NaCl solution was added and extracted twice with 50 mL ethyl acetate in a separation funnel. The ethyl acetate solution was washed tree times with 20 mL water, dried on anhydrous sodium sulphate and evaporated under reduced pressure. Fractions I and II were disolved in 3 mL MeOH-HPLC grade, fractions III and IV in a mixture of 3 ml eluent (isopropanol/acetonitrile /methanol - 55:35:10) and 2 mL MeOH-HPLC grade.

Sunflower oil and Vitamin E were saponified as decribed above. The dried residues were disolved in methanol.

Standard solutions were prepared with methanol in case of sitosterol and with petroleum ether in case of vegetable oils and Vitamin E.

Column chromatography (CC)

The petroleum ether extract was chromatographed on a column (25/1,5 cm) packed with alumina (63-200 mesh, III) from Merck (Darmstadt, Germany). Elution was performed with 2% (v/v) acetone/petroleum ether followed by acetone 100%. Fractions were monitored by TLC and reunited to give four fractions.

Thin layer chromatography (TLC)

Precoated TLC plates of silica gel 60, layer 0.25 mm (Alugram® Sil G, MN, Germany) were used for analytical TLC. The following mobile phases were used: S1, petroleum ether-diethyl ether-glacial acetic acid (65:15:1); S2, petroleum ether-diethyl ether-glacial acetic acid (65:15:5); S3, petroleum ether-diethyl ether-glacial acetic acid (70:30:10); S4, petroleum ether-benzene-acetone (14:2:6); S5, petroleum ether. Plates were developed in presaturated chambers and dried. Lipids were visualized after spraying with rhodamine 6G and 2',7'-dichlorofluorescein and viewed in daylight and UV_{366} light, with p-anisaldehyde-sulfuric acid or phosphomolybdic acid and heating at 105°C for 5 minutes and viewed in daylight. [4, 9] To avoid omitting methoxylated flavonoids, which might be present in the extract, plates were also sprayed with diphenylboric acid aminoethyl ester and viewed in daylight and under UV_{366} light.[9]

High Performance Liquid Chromatography (HPLC)

HPLC separation was performed using a Waters Alliance 2695 Separation Module, autosampler with a 20 µL loop, with a Waters 2996 Photodiode Array Detector. Operation and data aquisition Empower Pro Software.

The first separation was performed on a Nucleodur 100-5 C18 ec, 250x4.6 mm (MN, USA) column with gradient elution at a flow rate of 0.9mL/min. Mobile phase was a mixture of methanol (A) and isopropanol : acetonitrile : methanol (55:35:10) (B). Gradient elution was performed as follows: A 100% for 5 minutes; A 10% and B 90% for 20 minutes; B 100% for 5 minutes.

The second separation was performed on a Nucleosil 50 – 5 C8 ec, 250x4.6 mm (MN, Germany) column with acetonitrile : water (95:5) as mobile phase. The isocratic elution was performed at a flow rate of 0.8 mL/min. [4, 6, 12]

The column effluent was monitored at 208 nm (sterols) and 295 nm (tocopherols).

Results and discussion

In order to identify the lipophylic compounds of the fractions collected from the column, TLC separation was performed with several solvent systems (S1,S2, S3, S4, S5). Best separation in case of fractions I, II and III was obtained with double development, first with solvent system S5, followed by S2. The separated compounds were identified by comparison with standards and literature data. Olive oil was used as a standard solution to reveal the relativ order of mobilities of hydrocarbons, sterol esters, triacylglycerols, free fatty acids, 1,3-diacylglycerols, 1,2-diacylglycerols and free sterols on TLC-plates. Saponified sunflower oil was developed along with saponified Vitamin E (disolved in sunflower) to reveal its hydrolised compounds.
Fraction I contains sterol esters (Rf 0,85), triacylglycerols (Rf 0,7) and diacylglycerols (Rf 0,3). Spots in the range of Rf values 0,5-0,6 might be free fatty acids. Fraction II resembles fraction I but sterol esters are missing, triacylglycerols are predominantly and diacylglycerols are present in traces. Fraction III, after spraying with Rhodamine 6G exhibit seven spots. Two of them (Rf 0,50 and 0,58) are deep red-violet in daylight and quench fluorescence under UV_{366} light. Their chromatographic behaviour corresponds to that of tocopherols. The other fife spots are pink in daylight and exhibit pale yellow fluorescence under UV_{366} light. Plates sprayed with phosphomolybdic acid or anisaldehyde show that spot Rf 0,42 (free sterols) is partially overlapped with carotenoids; spot Rf 0,30 corresponds to diacylglycerols. (Fig. 1,2,3,4,5)
Separation of fraction IV was performed with solvent system S3, revealing four lipid spots: traces of tocopherols and one spot at the level of free sterols (Rf 0,14).
Spraying plates with diphenylboric acid aminoethyl ester (Neu, NP) revealed that none of the fractions contains lipophylic flavonoids.
While monitoring fractions collected from the column, carotenoids could be observed due to their own colour. This class of lipophylic substances constitutes the subject of further investigations.
HPLC separation carried out on a Nucleodur 100-5 C18 ec column with gradient elution did not allow a simultaneous separation of sterols and tocopherols.
HPLC separation performed on a Nucleosil 50 – 5 C8 ec column with isocratic elution (acetonitrile/water, 95:5) revealed in fractions I, II and IV small amounts of free sterols which could not be identified due to lack of standards. Fraction III contains beta-sitosterol and remarkable quantities of beta- and delta-tocopherol and less quantities of alfa-tocopherol. Small quantities of tocopherols were also identified in fraction IV. (Fig. 6,7,8)

Conclusions

The chromatographic conditions choosed for CC, TLC and HPLC allowed the separation of several simple lipids. According to standards and literature data we identified sterol esters and free sterols, triacyl- and diacylglycerols, alfa-, beta- and delta-tocopherol, sitosterol and carotenoids.

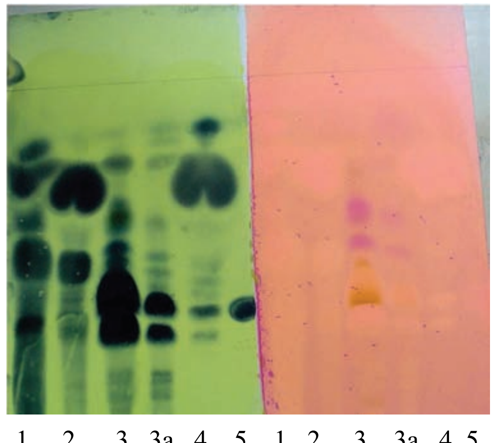

Fig. 1. TLC of fractions I-III in daylight after spraying with phosphomolybdic acid (left) and rhodamine 6G (right). (1-fr.I, 2-fr.II, 3-fr.III, 3a-fr.III purified with chloroform, 4-olive oil, 5-sitosterol)

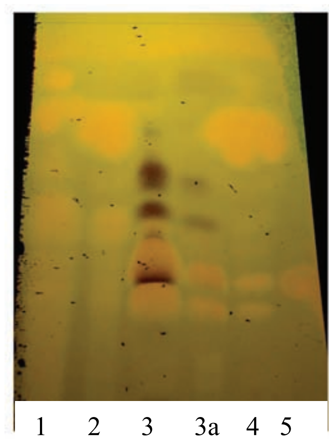

Fig. 2. TLC of fractions I-III under UV light after spraying with rhodamine 6G.

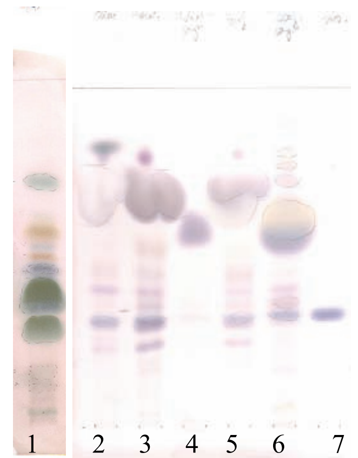

Fig. 3. TLC of fr.III (1) compared with standards (2-olive oil, 3-sunflower oil, 4-sunflower oil after saponification, 5-vitamin E, 6-vitamin E after saponification, 7-sitosterol) after spraying with anisaldehide reagent and heating

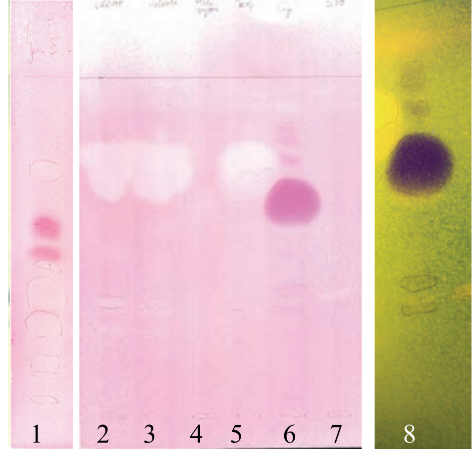

Fig. 4. TLC of fr.III in daylight (1) compared with standards (2-olive oil, 3-sunflower oil, 4-sunflower oil after saponification, 5-vitamin E, 6-vitamin E after saponification, 7-sitosterol, 8- vitamine E after saponification under UV light) after spraying with rhodamine 6G

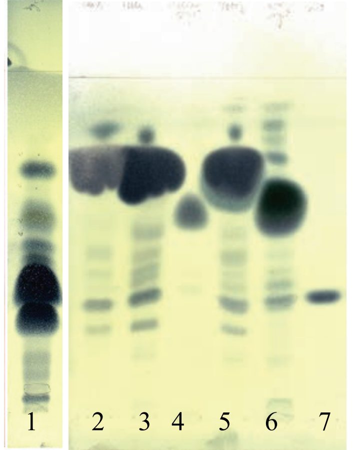

Fig. 5. TLC of fr.III (1) compared with standards (2-olive oil, 3-sunflower oil, 4-sunflower oil after saponification, 5-vitamin E, 6-vitamin E after saponification, 7-sitosterol) after spraying with phosphomolybdic acid and heating

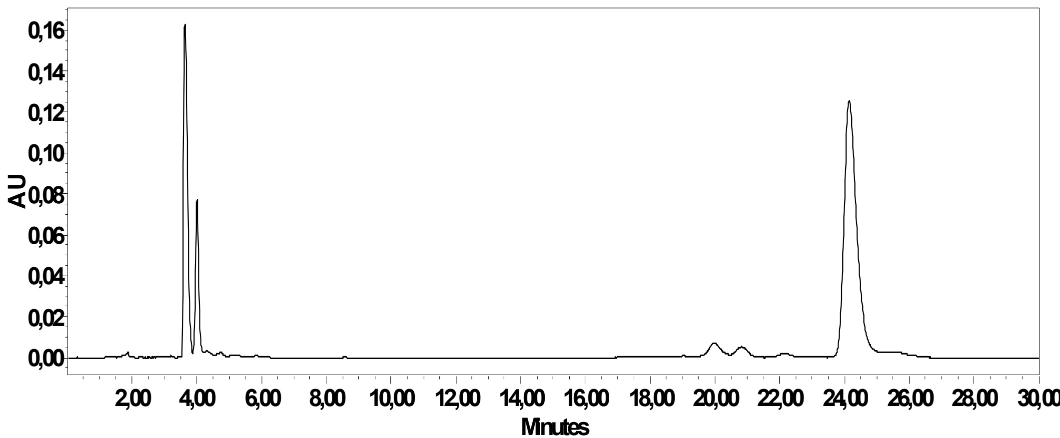

Fig. 6: Beta-sitosterol standard

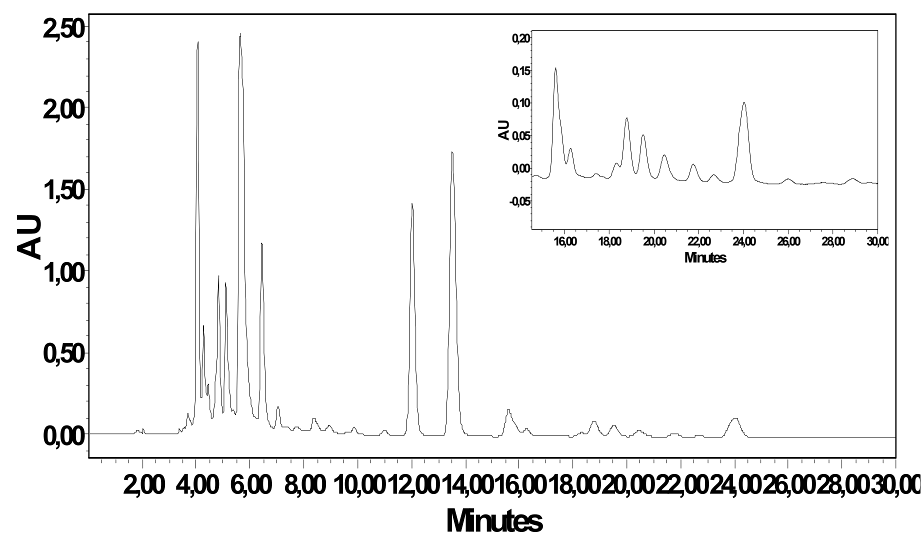

Fig. 7: Fraction III, 208 nm

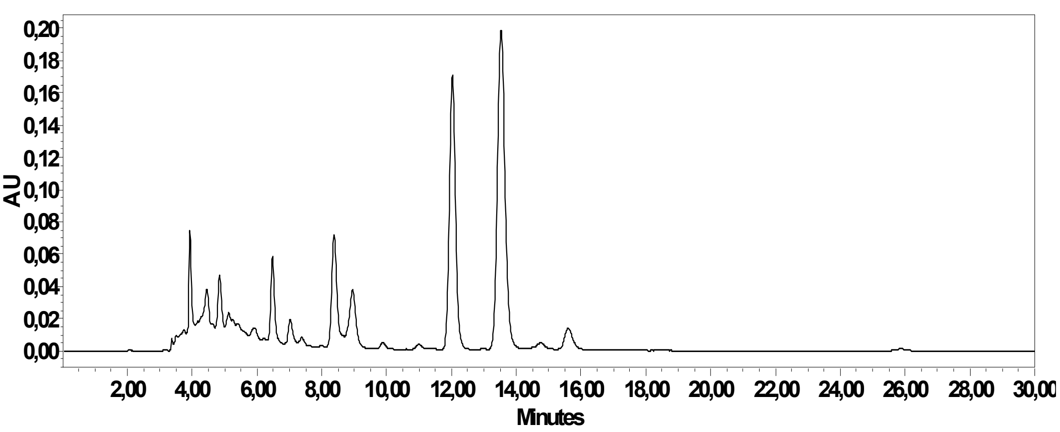

Fig. 8: Fraction III, 295 nm

Acknowledgments

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OXYGEN SPECIES SCAVENGING ACTIVITY OF *BASILICI HERBA* EXTRACT

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Summary

Dried and powdered aerial parts of Ocimum basilicum L. were extracted using doubled distilled water. The extract was concentrated and lyophilized. The phytochemical analysis of the extract revealed the presence of flavonoids, phenolic acids, tannins, sterols, saponins, proteins, sugars. Flavonoids and phenolic acids were quantified according to Romanian Pharmacopoeia, 10th edition. Chemiluminescence method was used to evaluate the integral antioxidant capacity of the aqueous Basilici herba extract.

Keywords: *Ocimum basilicum L., polyphenolic compounds, scavenger, reactive oxygen species.*

Introduction

In the past years interest in search for radioprotectors has been intensively growing. Exposure to ionizing radiation (radiation therapy, radiological accidents) causes severe tissue injury. Numerous studies have shown that radiation injury occurs through the formation of reactive oxygen species from the water molecules. Hydroxyl and superoxide radicals, hydrogen peroxide cause cell membrane disintegration, membrane protein damage and DNA mutations. Therefore, antioxidants with free radical scavenging activity may play a relevant role in the prevention of radiation – induced oxidative injury. Polyphenolic compounds, like flavonoids and phenolic acids, have been reported to have antioxidant activity (1, 2).
Aerial parts of *Ocimum basilicum* L. (*Lamiaceae*) are used in folk medicine to prepare teas with stomahic, carminative, spasmolitic, diuretic, antiseptic, antiulcerous and sedative effects. Phytochemical studies of this drug revealed the presence of flavonoids and phenolic acids (3). Since phenolic compounds occur in this species, they will be present in the aqueous extract. Therefore, this work reports the phytochemical analysis of *Basilici herba* aqueous extract and its antioxidant potential using chemiluminescence method.

Material and methods

Aerial parts of *Ocimum basilicum* L. were purchased from PLAFAR - CLUJ. Rutin and caffeic acid were from Merck, trolox and horseradish peroxidase were from Sigma, while luminol, ammonium peroxide and p-iodphenol were obtained from Perkin Elmer. All other chemicals were of reagent grade quality.
Extract preparation. 100 g of *Ocimum basilicum* L. dried and powdered aerial parts were extracted with water (three changes) on the water bath at 50° C. The resulting extract was concentrated under reduced pressure and lyophilized to get 25,19 g dry residue.
Phytochemical investigation. Phytochemical screening of *Basilici herba* aqueous extract was carried out according to the methods described in Ciulei et al. (4).
The extract was examined by thin layer chromatography on Silicagel (Merck). Polyphenols were separated in ethyl acetate – formic acid – acetic acid – water (100:11:11:27, v/v/v/v) and detected at 365 nm by spraying with 1 % 2 – aminoethyldiphenylborate methanolic solution (5).
Both flavonoids and phenolic acids were quantified in *Basilici herba* aqueous extract. For this purpose, 0,2008 g lyophilized extract were solved in 50 ml doubled distilled water (solution

S). The quantification of flavonoids and phenolic acids was carried out as described in *Cynarae folium* monograph (6).

Quantification of flavonoids. 5 ml solution S were diluted with methanol to 25 ml. The mixture was filtered after 10 minutes. 5 ml sodium acetate (100 g/l) and 3 ml aluminium chloride (25 g/l) were added to 5 ml filtered solution. After 15 minutes the absorbance of the yellow coloured solution was determined at 430 nm using a Jasco V-550 UV-VIS spectrophotometer (Japan). The blank was prepared by mixing 5 ml filtered solution, 8 ml double distilled water and methanol to 25 ml.

Flavonoidic content was expressed as g of rutin per 100 g *Basilici herba* extract.

Quantification of phenolic acids. 5 ml phosphowolframic acid were added to 5 ml solution S. The mixture was filtered. 0,5 ml filtered solution were diluted with sodium carbonate (200 g/l) to 10 ml. After 1 minute the absorbance of the blue coloured solution was determined at 660 nm using a Jasco V-550 UV-VIS spectrophotometer (Japan). The blank was prepared by mixing 0,25 ml phosphowolframic acid and double distilled water to 10 ml.

Phenolic acids content was expressed as g of caffeic acid per 100 g *Basilici herba* extract.

Determination of total antioxidant activity. Total antioxidant capacity of *Basilici herba* extract was measured by chemiluminescence method (7, 8). In this respect, the lyophilized extract was solved in double distilled water and solutions with different concentrations were prepared (5 mg/ml, 4 mg/ml, 3 mg/ml, 2 mg/ml). A Bertold – Lumat LB 9507 apparatus was used. 20 µl luminol, 20 µl prooxidant mixture (ammonium peroxide and p-iodphenol), 20 µl horseradish peroxidase and 140 µl doubled distilled water were mixed. The prooxidant light generating system was adjusted to 5 milion RLU (relative light units). Addition of the sample (20 µl) induced a significant decrease of the signal for a period of time which was proportional with the antioxidant capacity of the sample. A standard curve of trolox was plotted and the results were expressed as trolox equivalents (µmol/l).

Results and discussions

The phytochemical investigation of *Basilici herba* extract revealed the presence of flavonoids, phenolic acids, tannins, saponins, proteins and sugars.

The quantitative analysis demonstrated that *Basilici herba* extract contained 0,79 % phenolic acids (expressed as g of caffeic acid per 100 g extract) and 1,12 % flavonoids (expressed as g of rutin per 100 g extract).

The antioxidant potential of *Basilici herba* extract was evaluated by measuring the inhibition of the chemiluminescence reaction of luminol in presence of different extract concentrations. Ammonium peroxide and horseradish peroxidase induce a reaction of decomposition of luminol; this process is accompanied by chemiluminescence. Antioxidants which are added to the system decrease the intensity of chemiluminescence; the inhibition is dependent on their capacity to inhibit oxygen species generation and to scavenge them.

The standard curve of trolox is shown in figure 1 and the total antioxidant capacity of sample solutions quantified by the ability to quench chemiluminescence is given in table I.

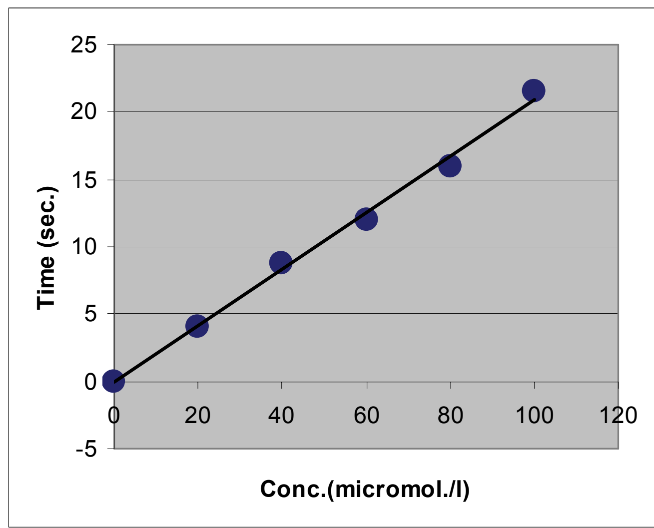

Fig. 1. Standard curve of trolox

Table I. Antioxidant activity of sample solutions expressed as trolox equivalents

Sample solution (mg/ml)	Trolox equivalents
5	693,5
4	437
3	280,25
2	23,75

In literature, natural compounds as flavonoids and phenolic acids are reported to have a high antioxidant potential. Tea polyphenols, rutin, quercetin, luteolin, genistein, orientin reduced oxidative injury induced by ionizing radiations in different experimental models (1). Polyphenols, mainly flavonoids can express their antioxidant potential by:

- suppressing oxygen reactive species formation by inhibiting some enzymes or chelating trace elements involved in free radical production;
- scavenging reactive oxygen species. In a scavenging reaction a hydrogen atom is added to the radical. Since this process involves the breaking of O – H bond and a hydrogen atom consists of an electron and a proton, it is very likely that the oxidizability of a compound reflects its ability to scavenge radicals;
- up-regulating or protecting antioxidant defence (9).

In this study we evaluated the antioxidant capacity of an aqueous extract obtained from the aerial parts of *Ocimum basilicum* L. We have shown that the antioxidant potential of the extract (expressed as trolox equivalents) depended on concentration. Numerous studies have demonstrated that compounds of medium and high polarity are the most potent antioxidants even if their total amount in the plant extract is small (2).

We can conclude that polyphenols (phenolic acids, flavonoids) are the main compounds responsable for the scavenging activity of *Basilici herba* aqueous extract.

Conclusions

Low concentrations of *Basilici herba* aqueous extract scavenged *in vitro* reactive oxygen species. Besides, it is well known that *Basilici herba* has no oral toxicity. Therefore, it would be interesting to evaluate *in vivo* protective potential of the extract towards radiation – induced oxidative injury.

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DETERMINATION OF IRIDOIDE FROM *MELAMPYRUM CRISTATUM* L. SPECIES BY CHROMATOGRAPHYC METHODS

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Summary

The study consists of qualitative and quantitative analysis through methods of thin layer chromatography coupled with photodensitometry as well as high performance liquid chromatography coupled with mass spectrometry (LC/MS). In CSS case, the chromatographic plate was scanned with Shimadzu CS9000 photodensitometer after pulverization with iron clorure, and for HPLC it is used analytical column Atlantis HILIC 100 mm x 3.0 mm i.d., 3.5 µm (Waters), and source of ions ESI (electrospray ionisation). After CSS analysis the iridoides concentation expressed in aucubin is 0.23 mg/ml in flowers and 0.29 mg/ml in leaves, and through LC/MS method the concentration is 1.0928 mg/ml in flowers and 0.8675 mg/ml in leaves.

Keywords: *Melampyrum cristatum L. , iridoides, aucubin, identification, CSS, HPLC-MS*

Introduction

Until now the *Melampyrum* genus, respectively *Melampyrum cristatum L.* species, was not studied in Romania, but few data that were found show the existence aucubin in other species of this genus.[1] Another supposition that in this species there might be iridoides is the one due to the black colour, the so called " black bread" which was obtained when the flour was impurified with *Melampyrum* seeds.

The iridoides represent a monoterpene pentamethylene-pyrinic group, but their presentce in the plant was signalles even in 1846. The phisiological role of this substance is little studied, but the bitter taste and the antibiotic properties of some of then lead to the hypotesis of a possible role in protection of plants.

Experimental part

The vegetal sample was taken from Mures country, in Băla , Ercea village ,in 24th June 2004. For analysis there were used leaves and flowers from *Melampyrum cristatum L.*species. Extraction method : the starting point is 10 g vegetable product which were extracted with10 ml methilic alcool after which it is evaporated completely.

CSS was performed in the following condition: solution to analyze extract from Melampyrum cristatum L. flowers and leaves; standard solution :methanolic solution from acubin(Roth) 1,18 mg/ml - 10 ml applied ;stationary phase: kieselgel 60F_{254} (Merck); mobil face n-propanol (Merck) - toluen (Merck) – acid acetic (Merck) – water (25: 20:10:10, vol.), [2]; migration distance 10 cm ; migration time : 40 min. The application of solution in layers was carried out in bands of 1 cm at 1.5 cm distance from the lower border of the plate.

The chromatographic plate was scanned with a Shimadzu CS9000 photodensitometer after pulverization with iron clorure. The photodensitometer parameters are : way of functioning in reflexion, wolframe lamp , scanning method : zig zag, λ = 550 nm.

HPLC coupled with mass spectrometer made binary pump**;** autosampler; thermostat HP 1100 Series**;** detector UV HP 1100 Series; mass spectrometer Agilent Ion Trap 1100 VL .

MS working condition : source of ione- ESI (electrospray ionisation); ionization manner-positive; nebulizer-nitrogen , pression 60 Psi ; drying gas-nitrogen , flow rate 12 L per minute, temperature 300 C , potential capillary : 4000 V ; method of analysis-monitoring M/Z 369.

HPLC working condition : analytical column Atlantis HILIC 100 mm x 3.0 mm i.d., 3.5 µm (Waters); on-line filter 0,2 microns(Agilent); mobil phase : mixture water-acetonitril, elution ingredient after the following program :

No.	Time(min.)	% Acetonitril
1	0.00	95.0
2	2.30	81.1
3	2.31	10.0
4	2.70	10.0
5	2.71	95.0

Flow rate: 1 ml per minute ,temperature 40 C, post column addition of 1% acetic acid solution in water, also containing sodium acetate 200 micron, flow rate of 0,07 ml per minute.
Detection: ultaviolet, 208 nanometer (only for visualization of the chromatographic profile) MS-monitoring of ione with M/Z 369 corresponding to the adduct formed aucubin with sodium ,the injection volume : 2 µl .

HO H O HO H O–β-D-glucose

Chart 1 Aucubin formula

Results and discussions

After the photodensitometrie evaluation, there have been obtained densitograms which show the presence of aucubin, in different concentrations.

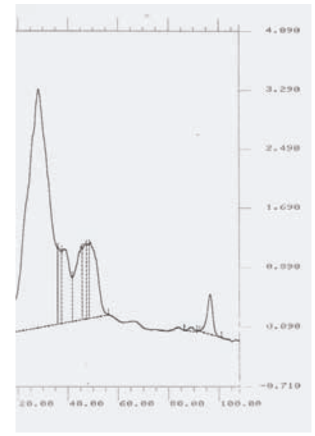


Chart 2. Densitogram of *Melampyrum* flowers extract

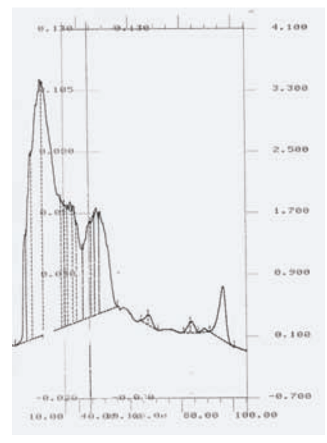
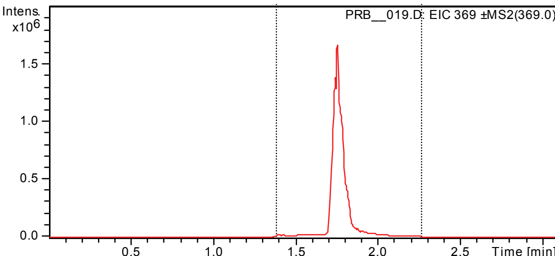


Chart 3. Densitogram of *Melampyrum* leaves extract

Tabel 1 Aucubin concentration through CSS

Sample	Area	Concentration mg/ml
M. cristatum flowers	92634.4	0.23
M. cristatum leaves	116763.4	0.29

After mass spectrum was realized, there was performed a phase of elimination of interferences (a " cleaning" of the spectrum). This phase leads to an analithical method whose sensitivity is doubled. Besides MS signal, there has also been monitored the absorbance in UV at 208 nm (corresponding to the maximum aucubin absorbtion) and it is noticed that the MS detection is considerably more specific and sensitive than the UV detection in case of aucubin.



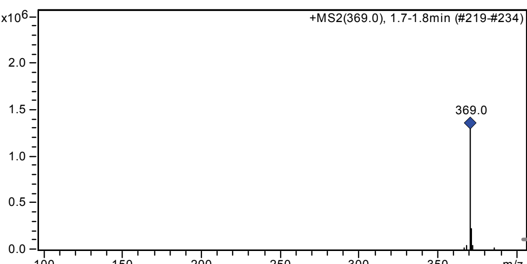


Chart 4 Chromatogram and mass spectrum of *Melampyrum cristatum* L. flowers extract

Tabel 2. Theaucubin concentration of extracts, determined by HPLC

Sample	Concentration found (mg/ml)	Extract concentration (mg/ml)	Plant quantity ug/g
MC flowers	1.0928	218.56	1092.8
MC leaves	0.8675	173.49	867.5

Conclusion

By thin layer chromatography , the presence of accubin was signalled in the two samples studied. CSS coupled with photodensitrometry also allowed a quantitative determination of aucubin . The aucubin concentation 0.23 mg/ml in flowers and 0.29 mg/ml in leaves by CSS, and by LC/MS method the concentration is 1.0928 mg/ml in flowers and 0.8675 mg/ml in leaves.

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CHEMICAL RESEARCH ON SOME POLYPHENOLIC COMPOUNDS FROM *SALVIA SP.* (*LAMIACEAE*)

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Summary

The polyphenolic compounds (flavonoids and caffeic acid derivatives) from Salvia sp. (Lamiaceae) were analysed, quantitatively by a spectrophotometric method and qualitatively by HPLC, before and after hydrolysis. We studied 7 indigenous species of Salvia: S. officinalis L., S. pratensis L., S. austriaca L., S. nemorosa L., S. sclarea L., S. verticilata L., S. glutinosa L.. The richest species in caffeic acid derivatives (>3%) *and flavonoids (>2,5%) were S. officinalis, S. verticilata and S. glutinosa.*

Keywords: *Salvia species, polyphenolic compounds*

Introduction

Salvia officinalis (sage) is a known medicinal plant, used for its carminative, stomachic, antispasmodic, antimicrobial, anti-inflammatory, antioxidant, antiseptic, antihidrotic and astringent properties. The leaves of sage contain phenolic acids (caffeic acid derivatives), tannins, flavonoids, essential oil. The sage is traditionally used to treat the symptoms of gastrointestinal disturbances (epigastric bloating, eructation, flatulence), for excessive perspiration and topically, in mouthwashes for oral hygiene (1, 4). The main antioxidative effect of sage has been reported to relate to the presence of rosmarinic acid and carnosic acid /2, 7/. In contrast to sage, its aqueous preparations and its hydroalcoholic extracts, which seem to have little toxicity, the essential oil is neurotoxic, this action been linked to the ketones (thujones) /5/.
In order to compare the polyphenolic compounds of some *Salvia species*, we studied 7 indigenous species of *Salvia: S. officinalis L., S. pratensis L., S. austriaca L., S. nemorosa L., S. sclarea L., S. verticilata L., S. glutinosa L.*.

Material and methods

Quantitative analysis of flavonoids and caffeic acid derivatives from *Salvia sp.* (*Lamiaceae*) were performed spectrophotometrically and results were expressed in rutoside (flavonoids) and in caffeic acid (caffeic acid derivatives) /6, 9/. Quantitative analyses were performed using a UV-VIS Spectrophotometer Jasco V530.
Polyphenolic compounds were analysed by HPLC, in the following conditions /3,8/: separation was performed with an Agilent 1100 HPLC Series; Zorbax SB-C18 reverse-phase analytical column 100 mm × 3,0 mm, 3,5 µm particle; T^0 = 48^0C; mobile phase: a binary gradient - methanol and buffer solution: KH_2PO_4 40mM, pH=2,3 adjusted with 85% ortophosphoric acid. The gradient begun with a linear gradient started at 5% methanol to 42% methanol over the first 35 min., followed by isocratic elution with 42% methanol over the next 3 min. The flow rate was 1ml/min and data were collected at 330 nm. Injection volume was 10µl. Detection: UV Detector at 330-370nm, identification by external standard method.
Samples preparation
Dried vegetal product was extracted in Soxhlet with chloroform, then with methanol; the residue was dissolved in hot water; successive extractions in separation flask with ethylic

ether, ethyl acetate and n-buthanol were performed. The methanolic solutions of the reuniting residues were analysed by HPLC.
In order to study the flavonoidic aglycons, a hydrolysis with HCl 2N was performed.
For quantification of rosmarinic acid in *Salvia officinalis*, a HPLC method coupled with mass spectrometry (LC/MS/MS) was used. The mobile phase consisted of acetonitrile and 1mM ammonium acetate in water, gradient elution: start 5% acetonitrile, at 33,3min. 25% acetonitrile. The mass spectrometer operated using ESI source in negative mode and was set for isolation and fragmentation of deprotonated rosmarinic acid ion with m/z = 359. Quantification of rosmarinic acid was based on the sum of ions with m/z = 160.7, 178.6 and 196.7 from the MS spectrum of parent ion.

Results and discussion

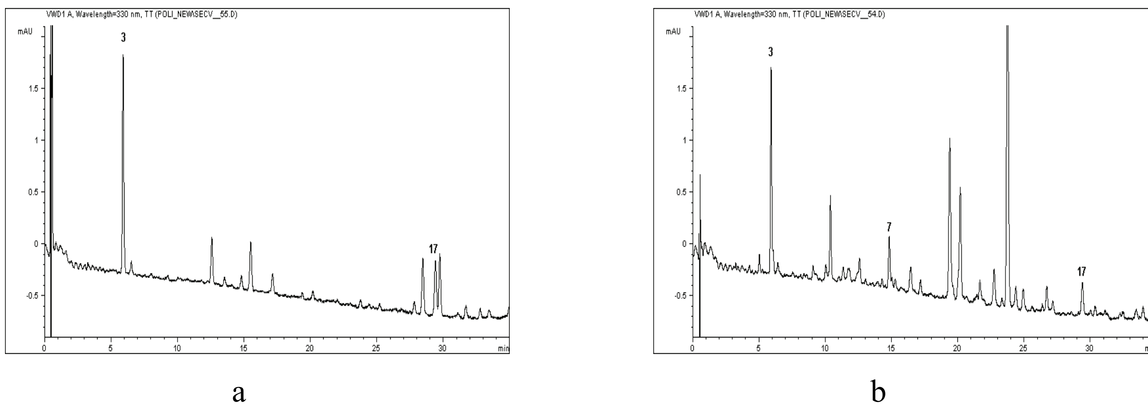
The flavonoids concentrations expressed in rutoside and the caffeic acid derivatives concentrations expressed in caffeic acid are presented in the table I and the identified compounds by HPLC, in table II.

Table I. Content of flavonoids and caffeic acid derivatives from *Salvia sp*.

Nr.crt.	*Salvia sp.*	Flavonoids (rutoside) %	Caffeic acid derivatives (caffeic acid) %
1	*S. officinalis*	2,82	3,40
2	*S. sclarea*	1,44	1,.06
3	*S. pratensis*	0,85	2,03
4	*S. austriaca*	0,78	1,75
5	*S. nemorosa*	1,52	2,15
6	*S. verticilata*	2,54	3,21
7	*S. glutinosa*	2,75	3,76

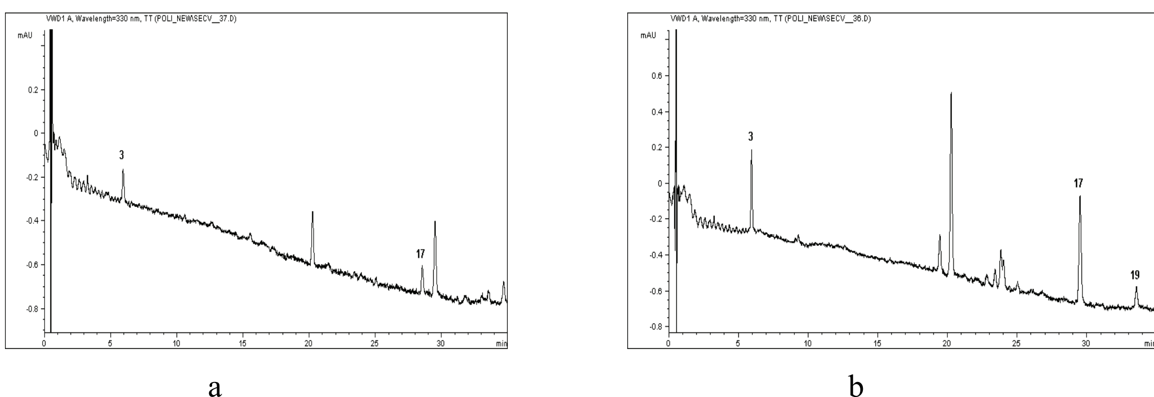
Table II. Identified compounds by HPLC

No	Compounds Rt (min.)	*S.officinalis* (mg/100g) before / after hydrolisis	*S.pratensis* (mg/100g) before / after hydrolisis	*S.austriaca* (mg/100g) before / after hydrolisis	*S.nemorosa* (mg/100g) before / after hydrolisis	*S.sclarea* (mg/100g) before / after hydrolisis	*S.verticilata* (mg/100g) before / after hydrolisis	*S.glutinosa* (mg/100g) before / after hydrolisis
1	caftaric acid (3,1)	10,3/12,3	-	-	-	-	-	-
2	caffeic acid (5,9)	41,0/65,2	5,3/7,3	4,6/8,4	8,2/21,6	7,1/39,1	11,9/35,5	30,8/52,7
3	p-coumaric acid (9,2)	2,5/6,3	-	-	-	1,0/12,0	urme/1,0	-
4	ferulic acid (12.4)	2,6/16,0	-	-	-	2,6/5,0	-	-
5	sinapic acid (14,7)	9,2/31,4	-	-	-	0,6/3,4	-	-
6	luteolin (29.2)	11,9/13,4	5,9/20,5	14,9/25,3	10,2/11,4	8,2/45,5	10,8/97,6	40,6/85,0
7	apigenin (33.2)	12,3/18,8	26,1/28,4	urme/10,4	10,6/21,9	7,0/38,3	36,8/68,7	17,7/17,9



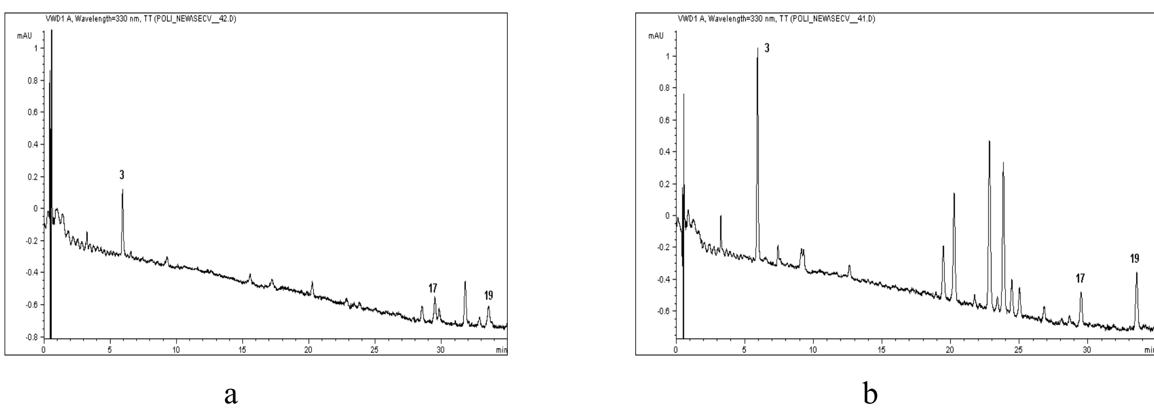
a b

Fig. 1. HPLC chromatograms for *S. officinalis* extracts before hydrolysis (a) and after hydrolysis (b)



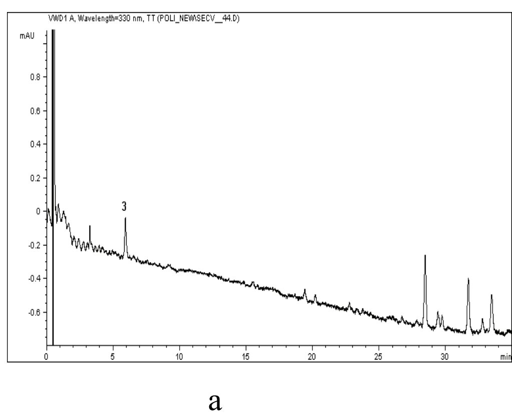
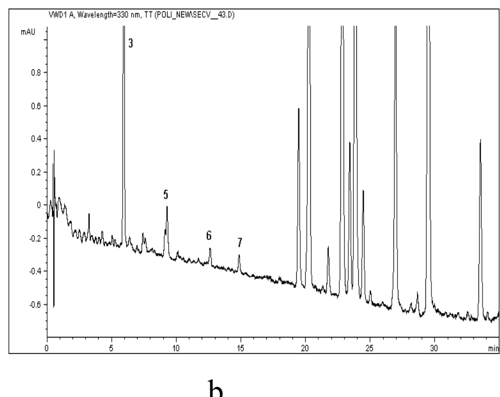
a b

Fig. 2. HPLC chromatograms for *S. austriaca* extracts before hydrolysis (a) and after hydrolysis (b)



a b

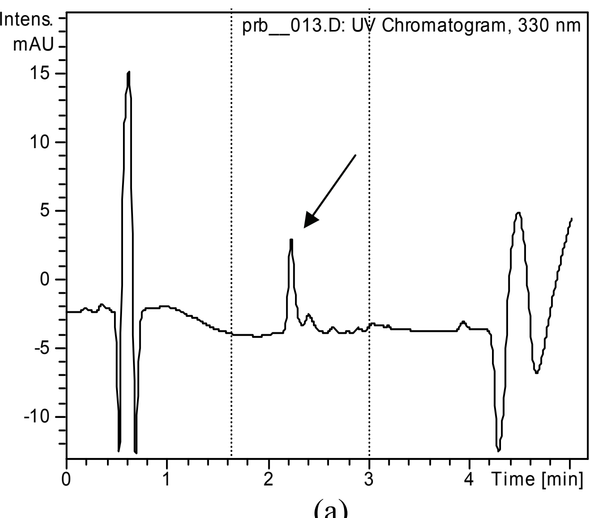
Fig. 3. HPLC chromatograms for *S. nemorosa* extracts before hydrolysis (a) and after hydrolysis (b)

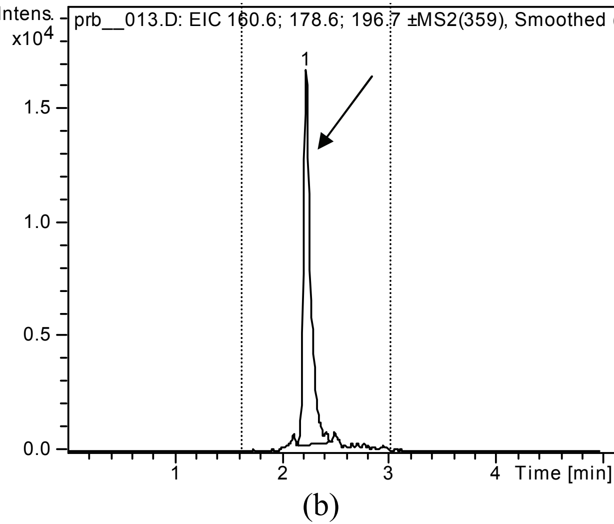



a b

Fig. 4. HPLC chromatograms for *S. sclarea* extracts before (a) and after hydrolysis (b)

The quantitative analysis showed us that the richest species in caffeic acid derivatives (>3%) and flavonoids (>2,5%) were *S. officinalis, S. verticilata* and *S. glutinosa*.
The following compounds: caffeic acid, luteolin and apigenin in all species, and coumaric acid, ferulic acid, sinapic acid, only in *S. officinalis* and *S. sclarea,* were identified by HPLC, using extracts of the leaves of plants in ethylic ether, ethylic acetate and n-buthanol. The higher concentrations of these compounds after hydrolysis showed us that in plants they had formed glycosides or esters.
Rosmarinic acid was detected in *S. officinalis* by HPLC coupled with mass spectrometry (LC/MS/MS) in three extracts, as follows: 6,80mg/g in ethanolic 70º extract (tº=60ºC), 5,63mg/g in methanolic extract (tº=60ºC) and 5,67mg/g in tincture (1:10). The best solvent for the extraction of rosmarinic acid is ethanol 70º.
A sample chromatogram of rosmarinic acid from *S. officinalis* extract are presented in fig. 5a (the UV trace at 330 nm) and fig. 5b (the MS signal).
The retention time for rosmarinic acid is 2.2 min.





(a) (b)

Fig. 5. Chromatograms of rosmarinic acid from *S. officinalis* extract (a) UV signal at 330 nm; (b) MS/MS signal.

Conclusions

- 7 indigenous species of *Salvia* were analysed quantitatively and qualitatively by HPLC, before and after hydrolysis.

- Caffeic acid, luteolin and apigenin were identified in all species and coumaric acid, ferulic acid and sinapic acid, only in *S. officinalis* and *S. sclarea*. The higher amounts of these compounds after hydrolysis showed us that in plant they are linked in glycosides or esters.
- The quantification of rosmarinic acid in *S. officinalis* by LC/MS/MS emphasized that the better way of extraction uses the ethanol 70º (tº=60ºC).
- The obtained results complete the existing information on chemical composition of different *Salvia species*.

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RECENT BIOACTIVITY ASPECTS ON TURKISH *PISTACIA VERA* L.

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Summary

The nuts of Pistacia vera L. (Anacardiaceae), commonly referred to pistachio, are quite popular flavoring foodstuff and also have been used to treat asthma in Turkish folk medicine. Besides, it has a significant contribution to the major agricultural exports of Turkey. In this presentation, results of a number of in vitro and in vivo biological activities including antimicrobial, antiviral, antiprotozoal, anti-inflammatory and antinociceptive activities of P. vera of Turkish origin are reviewed.

Keywords: *Pistacia vera, Anacardiaceae, pistachio, biological activity*

Introduction

Pistacia vera L. (Anacardiaceae) is a small tree grown in southern Europe and Asia minor, being the only species within the 11 species belonging to the genus *Pistacia* that produces edible nuts. In addition to its economical value, kernels of *P. vera* are remarkably rich in linoleic and linolenic acids, the fatty acids vital for human health (Maskan *et al.,* 1998; Aslan *et al.*, 2002; Satil *et al.*, 2003). On the other hand, *Pistacia* species were reported to have various biological activities such as anti-atherogenic, hypoglycemic, antioxidant, anti-inflammatory, and anti-insect activities (Dedoussis *et al.,* 2004; Hamdan *et al.*, 2004; Demo *et al.,* 1998; Giner-Larza *et al.*, 2000). Antifungal activity of the essential oils and the leaf extracts of three *Pistacia* species including *P. vera* were also studied (Kordali *et al.* 2003; Duru *et al.* 2003).
The recent results of several biological activities such as antibacterial, antifungal, antiviral, antileshmanial, antitryponasomoal, antiplasmodial, anti-inflammatory, and antinociceptive activities of the different extracts of *Pistacia vera* L. growing in Turkey are outlined herein.

Material and methods

Plant material was collected from Nizip, Gaziantep, Turkey in September, 2003 and was separated into the leaves, branches, stem, shell skins, and kernels. A voucher specimen is preserved at the Herbarium, Department of Pharmacognosy, Faculty of Pharmacy, Gazi University, Ankara, Turkey (GUE 2381).
The plant parts used in this study were classified as follows: Fresh leaves **(PV-FL)**, stem **(PV-ST)**, branches **(PV-BR)**, fresh skin of natural-woody shell (non-processed) **(PV-FSN)**, dried skin of natural-woody shell (non-processed) **(PV-DSN)**, fresh kernel **(PV-FK)** and skin of processed-woody shell **(PV-SPS)**.

Extraction
The plant parts used for anti-inflammatory and analgesic activities were classified as fruits, leaves, branches and peduncles, while the oleoresin occurring naturally on trunk of the plant was exuded. Each of the above cited plant part was dried under shade and powdered to a fine grade by using a laboratory scale mill. 10 g of the each plant part was weighed accurately and, except for the oleoresin, two kinds of the extract were prepared with 96% ethanol and water, separately, at room temperature macerating for two times (x 200 ml). Following the filtration, the ethanol and aqueous extracts were evaporated to dryness *in vacuo* to give crude extracts (percent yields for the ethanolic extracts; fruit 34.87%, leaf 27.9%, branch 8.7%, peduncle

16%; and for the aqueous extracts; fruit 24.1%, leaf 21.7%, branch 22.5%, peduncle 9.4%, w/w).
The organic solvent extraction of the chopped parts of *P. vera* (12-80 g dry weight) was carried out in a conventional Soxhlet device using *n*-hexane as solvent, in the presence of anhydrous Na_2SO_4. The cartridges were introduced in the Soxhlet apparatus containing 300 ml of solvent. The total reflux time was 5 h for each sample. After cooling, the solvent was separated from the solute using an evaporator with a vacuum controller 40°C. The resulting extracts were weighed in an analytical scale (Shimadzu, Libror AEG-120).

Antibacterial and antifungal activities
Standard and the isolated strains of the following bacteria, namely *Escherichia coli* (ATCC 35218), *Pseudomonas aeroginosa* (ATCC 10145), *Enterococcus faecalis* (ATCC 29212), and *Staphylococcus aureus* (ATCC 25923) for the determination of antibacterial activity, and standard strains of *Candida albicans* (ATCC 10231) and *Candida parapsilosis* (ATCC 22019) for the determination of antifungal activity were used. Microorganisms were obtained from Department of Microbiology and Clinical Microbiology, Faculty of Medicine, Karadeniz Technical University (Trabzon-Turkey). The isolated strains of *E. coli*, *P. aeruginosa*, *E. faecalis* and *S. aureus* were obtained from Department of Medical Microbiology, Faculty of Medicine, Gazi University, Ankara (Turkey). The lipophylic extracts of *P. vera* were dissolved in ethanol:hexane (1:1) by using 1% Tween 80 solution at a final concentration of 1024, 512 and 256 µg/ml and sterilized by filtration using 0.22 µm Millipore (MA 01730, USA) and used as the stock solutions. Mueller-Hinton Broth (Difco) and Mueller-Hinton Agar (Oxoid) were applied for growing and diluting of the microorganism suspensions. Before the test, all strains of fungi were cultured on Sabouraud dextrose agar (SDA) (Oxoid) and passaged at least twice to ensure purity and viability at 35^oC for 24 to 48 hs. Five colonies were used from each culture. The synthetic medium RPMI-1640 with L-glutamine was buffered pH:7 with 3-[*N*-morpholino]-propansulfonic acid] (MOPS) and culture suspensions were prepared according to the NCCLS M27-A. Also, standard and isolated strains of bacteria were grown to exponential phase in medium (MHB) at 37^oC overnight with aeration. 20µl of the culture was then plated on MHA. After overnight incubation at 37^oC with aeration, approximately five colonies of cultures were plated in MHB. The bacterial suspensions used for inoculation were prepared at 10^5 cfu/ml by diluting fresh cultures at McFarland 0.5 density (10^8 cfu/ml). The fungi suspension was prepared by the spectrophotometric method of inoculum preparation at a final culture suspension of $2.5x10^3$ cfu/ml (National Committee, 2002, Baran *et al.*, 1994). The microdilution method was employed for antibacterial and antifungal activity tests.

Antiviral activity
In order to determine the antiviral activity of the extracts, *Herpes simplex virus* (HSV) and *Parainfluenza virus* (PIV) were used. The test viruses were obtained from Department of Virology, Faculty of Veterinary, Ankara University. Media (EMEM) were placed into each 96 wells of the microplates (Greiner[R], Germany). Stock solutions of the extracts were added into first raw of each microplate and two-fold dilutions of the extracts (512-0.00006µg/ml) were made by dispensing the solutions to the remaining wells. Two-fold dilution of each material was obtained according to Log_2 on the microplates. Acyclovir (Biofarma) and oseltamivir (Roche) were used as the control agents. Strains of HSV and PIV titers were calculated by the Frey and Liess method as $TCID_{50}$ (1971). They were inoculated into all the wells. The sealed microplates were incubated in 5% CO_2 at 37°C for 2 hs to detect the possible antiviral activities of the samples. After incubation, 50µl of the cell suspension of 300.000 cells/ml which were prepared in EMEM + 5 % fetal bovine serum was put in each

well and the plates were incubated in 5% CO_2 at 37°C for 48 hs. After the end of this time, the cells were evaluated using cell culture microscope (X 400), comparing with treated-untreated control cultures and with acyclovir and oseltamivir as the control agents. Consequently, maximum cytopathogenic effect (CPE) concentrations as the indicator of antiviral activities of the extracts were determined (Esquenazi *et al.*, 2002).

Cytotoxicity
The maximum non-toxic concentration (MNTC) of each sample was determined by the method described previously by Uysal-Gökçe *et al.* (2004) based on cellular morphologic alteration. Several concentrations of each sample were placed in contact with confluent cell monolayers and incubated in 5% CO_2 at 37°C for 48 hs. MNTC values were determined by comparing treated and controlling untreated cultures.

Antitrypanosomal activity test against Trypanosoma brucei rhodesiense
Minimum Essential medium (50 µl) supplemented according to Baltz et al. (1985) with 2-mercaptoethanol and 15% heat-activated horse serum was added to each well of a 96-well microtiter plate. Serial drug dilutions were prepared covering a range from 90 to 0.123 µg/ml. Then 10^4 bloodstream forms of *T. brucei rhodesiense* STIB 900 in 50 µl were added to each well and the plate incubated at 37ºC under a 5% CO_2 atmosphere for 72 hs. 10 µl of Alamar Blue (12.5 mg rezasurin dissolved in 1 L distilled water) were then added to each well and incubation continued for a further 2-4 hours. The plate was then read in a Spectramax Gemini XS microplate fluorometer (Molecular Devices Cooperation, Sunnyvale, CA, USA) using an excitation wavelength of 536 nm and emission wavelength of 588 nm (Räz *et al.*, 1997). Fluorescence development was expressed as percentage of the control, and IC_{50} values were determined. Cytotoxicity was assessed using the same assay and rat skeletal muscle myoblasts (L6 cells).

Antitrypanosomal activity against Trypanosoma cruzi
Rat skeletal muscle myoblasts (L6 cells) were seeded in 96-well microtiter plates at 2000 cells/well in 100 µl RPMI 1640 medium with 10% FBS and 2 mM L-glutamine. After 24 hs, the medium was removed and replaced by 100 µl per well containing 5000 trypomastigote forms of *T. cruzi* Tulahuen strain C2C4 containing the -galactosidase (Lac Z) gene. 48 hours later, the medium was removed from the wells and replaced by 100 µl of fresh medium with or without a serial drug dilution. Seven 3-fold dilutions were used covering a range from 90 µl/ml to 0.123 µl/ml. After 96 hs of incubation, the plates were inspected under an inverted microscope to assure growth of the controls and sterility. Then the substrate CPRG/Nonidet (50 µl) was added to all wells. A color reaction developed within 2-6 hours and could be read photometrically at 540 nm. Data were transferred into a graphic program (e.g. EXCEL), sigmoidal inhibition curves were determined and IC_{50} values calculated.

Antileishmanial activity
Amastigotes of *Leishmania donovani* strain MHOM/ET/67/L82 were grown in axenic culture at 37°C in SM medium at pH 5.4 supplemented with 10% heat-inactivated fetal bovine serum under an atmosphere of 5% $C0_2$ in air. 100 µl of culture medium with 10^5 amastigotes from axenic culture with or without a serial drug dilution were seeded in 96-well microtiter plates. Seven 3-fold dilutions were used covering a range from 30 µg/ml to 0.041 µg/ml. After 72 hs of incubation the plates were inspected under an inverted microscope to assure growth of the controls and sterile conditions. 10 µl of Alamar Blue (12.5 mg resazurin dissolved in 100 mL distilled water) were then added to each well and the plates incubated for another 2 hs. Then the plates were read with a Spectramax Gemini XS microplate fluorometer (Molecular Devices Cooperation, Sunnyvale, CA, USA) using an excitation wavelength of 536 nm and an emission

wavelength of 588 nm. Data were analysed using the software Softmax Pro (Molecular Devices Cooperation, Sunnyvale, CA, USA). Decrease of fluorescence (=inhibition) was expressed as percentage of the fluorescence of control cultures and plotted against the drug concentrations. From the sigmoidal inhibition curves the IC_{50} values were calculated.

Antiplasmodial activity

Antiplasmodial activity was determined using the K_1 strain of *Plasmodium falciparum* (resistant to chloroquine and pyrimethamine). A modification of the [^{3}H]-hypoxanthine incorporation assay was used (Matile and Pink, 1990). Briefly, infected human red blood cells in RPMI 1640 medium with 5% Albumax were exposed to serial drug dilutions in microtiter plates. After 48 hs of incubation at 37°C in a reduced oxygen atmosphere, 0.5 µCi ^{3}H-hypoxanthine was added to each well. Cultures were incubated for a further 24 hs before they were harvested onto glass-fiber filters and washed with distilled water. The radioactivity was counted using a Betaplate^{TM} liquid scintillation counter (Wallac, Zurich, Switzerland). The results were recorded as counts per minute (CPM) per well at each drug concentration and expressed as percentage of the untreated controls. From the sigmoidal inhibition curves IC_{50} values were calculated.

Standard drugs

Artemisinin, chloroquine (*P. falciparum*), benznidazole (*T. cruzi*), melarsoprol (*T. brucei rhodesiense*), miltefosine (*L. donovani*) and podophyllotoxin (L6 cell cytotoxicity) were used as positive controls.

p-Benzoquinone-induced abdominal constriction test in mice for antinociceptive activity

In accordance with the method of Okun et al. (1963), 60 min after the oral administration of test samples, the mice were intraperitoneally injected with 0.1 ml/10 g body weight of 2.5% (v/v) *p*-benzoquinone (PBQ; Merck) solution in distilled H_2O. Control animals received an appropriate volume of dosing vehicle. The mice were then kept individually for observation and the total number of abdominal contractions (writhing movements) was counted for the next 15 min, starting on the 5th min after the PBQ injection. The data represent average of the total number of writhes observed. The antinociceptive activity was expressed as percentage change from writhing controls. 100 mg/kg and 200 mg/kg aspirin (ASA) was used as a reference drug.

Carrageenan-induced hind paw edema for anti-inflammatory activity

60 min after the oral administration of test sample or dosing vehicle, each mouse was injected with freshly prepared (0.5 mg/25 µl) suspension of carrageenan (Sigma, St.Louis, Missouri, USA) in physiological saline (154 nM NaCl) into subplantar tissue of the right hind paw (Yeşilada and Küpeli, 2002). As to the control, 25 µl saline solutions were injected into that of the left hind paw. Paw edema was measured in every 90 min during 6 h after induction of inflammation. The difference in footpad thickness was measured by a gauge calipers (Ozaki Co., Tokyo, Japan). Mean values of treated groups were compared with mean values of a control group and analyzed using statistical methods. Indomethacin (10 mg/kg) was used as reference drug.

Gastric-ulcerogenic effect

After the antinociceptive activity experiment, mice were killed under deep ether anesthesia and their stomachs were removed. Then the abdomen of each mouse was opened through the greater curvature and examined under dissecting microscope for lesions or bleedings (Yeşilada and Küpeli, 2002).

Statistical analysis of data
Data obtained from animal experiments were expressed as mean standard error (±SEM). Statistical differences between the treatments and the control were evaluated by ANOVA and Students-Newman-Keuls post-hoc tests. $p<0.05$ was considered to be significant [* $p<0.05$; ** $p<0.01$; *** $p<0.001$].

Results and discussion

Fifteen lipophylic extracts of *P. vera* were screened for their antibacterial activities against both standard and the isolated strains of *E. coli*, *P. aeruginosa*, *E. faecalis* and *S. aureus*. All of the extracts had greater potent antibacterial activity against Gram-positive bacteria than Gram-negative in this assay. The extracts also displayed remarkable antifungal activity against *C. albicans* and *C. parapsilosis* at 16 µg/ml concentration, which was close to the effective concentrations of the references.
The data obtained from antiviral activity screening showed that **PV-SPS** (16-<0.00006 µg/ml), **PV-FK** (8-<0.00006 µg/ml), and **PV-GU** (8-<0.00006 µg/ml) had significant activity against DNA virus same as the acyclovir. The other three extracts, namely **PV-GR** (32-0.0039 µg/ml), **PV-SR** (32-0.125 µg/ml) and **PV-DSN** (16-1 µg/ml), were less active than the extracts given above. The rest of the extracts did not possess any antiviral activity against DNA virus (Table 2). On the other hand, **PV-GR** (32-16 µg/ml), **PV-GP** (32-16 µg/ml), **PV-IP** (32-16 µg/ml) and **PV-FSN** (32-4 µg/ml) showed outstanding activity against RNA virus. Besides, **PV-ST** (16-8 µg/ml), **PV-BR** (16-8 µg/ml), **PV-IU** (8-8 µg/ml), and **PV-SR** (8-8 µg/ml) had also lower activity compared to the extracts above. Remaining eight extracts showed no activity.
In this study, we also employed a medium-throughput screening strategy and evaluated the *in vitro* antiprotozoal activities of thirteen lipophylic extracts prepared from various parts of Turkish *Pistacia vera* L. at two different concentrations (0.8 and 4.8 µg/ml, or at 1.6 and 9.7 µg/ml).
According to these results, none of the extracts displayed appreciable inhibitory activity against *T. brucei rhodesiense* at both 0.8 and 4.8 µg/ml concentrations as compared to melarsoprol (IC_{50} 0.002 µg/ml). The extracts also lacked any significant inhibitory potential against *T. cruzi* even at high concentrations (9.7 µg/ml), therefore not further investigated. The reference compound, benznidazole, strongly inhibited the growth of the parasite with an IC_{50} value of 0.315 µg/ml.
All extracts, except for the branch extract of *P. vera* (**PV-BR**), failed to inhibit the growth of *L. donovani* amastigotes over 50% at high concentrations (Table 1). The leishmanicidal activity of **PV-BR** extract at 4.8 µg/ml concentration was remarkable (77.3% inhibition) and its IC_{50} value was determined as 2.3 µg/ml. Miltefosine, a reference drug used in treatment of leishmaniasis, had an IC_{50} value of 0.14 µg/ml. The **PV-BR** extract possessed very weak cytotoxicity towards L6 cells (IC_{50} 44.5 µg/ml), whereas the IC_{50} value of podophyllotoxin on the same cell line was 0.005 µg/ml.
As for the antiplasmodial activity, only the dried leaf extract of *P. vera* (**PV-DL**) displayed notable activity, causing 60.6 % growth inhibition against *P. falciparum* at 4.8 µg/ml. The IC_{50} value of this extract was determined to be 3.65 µg/ml, as compared to that of chloroquine (IC_{50} 0.045 µg/ml) and artemisinin (IC_{50} 0.002 µg/ml). This extract did not exert any cytotoxic effect on mammalian L6 cells even at very high concentrations (IC_{50} >90 µg/ml).
Through the results we obtained, only the oleoresin, out of the all extracts screened herein, exhibited a dose-dependent anti-inflammatory activity on carrageenan-induced hind paw edema model in mice ranging between 27.4-34.7% at 250 mg/kg and 32.6-38.7% at 500 mg/kg doses without inducing any gastric damage (Table 1). Moreover, the oleoresin had a

very close inhibition value to that of indomethacin at 500 mg/kg dose whereas the rest of the extracts were found to be inactive in the same assay. As for antinociceptive activity, the oleoresin also displayed 32.1% inhibition at 500 mg/kg dose while it showed 21.7% inhibition at 250 mg/kg (Table 2). However, the ethanolic and aqueous extracts of the fruits, leaves, branches and peduncles of *P. vera* did not show any perceptible antinociceptive effect on *p*-benzoquinone induced abdominal contractions in mice. These experimental results have supported the folkloric utilization of the oleoresin as remedy.

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THE ANTIFUNGAL ACTIVITY OF *ALOË ARBORESCENS* FRESH LEAVES HYDROALCOHOLIC EXTRACT

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Summary

The hydroalcoholic extract obtained from Aloë arborescens fresh leaves and Fluka aloine were comparatively tested against the mycelial growth of Aspergillus niger, Botrytis cinerea, B. gladiolorum, Fusarium oxysporum f. sp. gladioli and Penicillium gladioli on Czapek-agar medium and they manifested inhibitory activity. The plant extract's minimum fungicidal concentration (MFC) varied between 60-100 µl/ml and aloine's between 100-180 µl/ml, depending on the fungal species.

Keywords: *antifungal activity, Aloë arborescens, aloine, chromatogram, phytopathogenic fungi*

Introduction

Literature data show that plant extracts from *Aloë* leaves have antifungal properties. There was obtained plant extract from fresh and dry leaves of *Aloë eru, A. vera* and *A. arborescens*, which had a significant inhibitory action on the germination and growth of *Aspergillus niger, Cladosporium herbarum* and *Fusarium moniliforme* (Ali et al., 1999). The main active constituent of *Aloë* plant extract is aloine, an anthraquinone heteroside (Bruneton, 1993).
The results obtained in testing the activity of *Aloë arborescens* fresh leaves total hydroalcoholic plant extract against the mycelial growth of *Aspergillus niger, Botrytis cinerea, B. gladiolorum, Fusarium oxysporum* f. sp. *gladioli* and *Penicillium gladioli* on Czapek-agar medium, compared to Fluka aloine, are presented in this communication.

Materials and methods

The total hydroalcoholic plant extract was obtained from *Aloë arborescens* fresh leaves, cultivated in "Alexandru Borza" Botanical Garden greenhouses from Cluj-Napoca, by the method described in the literature (Anonymous, 1993).
93.9 g *Aloë arborescens* fresh leaf fragments of about 0.5-1 cm were immersed in 220 ml 70 % ethylic alcohol, kept in the dark for cold maceration, for 24 hours. 250 ml of *Aloë arborescens* total plant extract was obtained by filtering the content.
There was determined the quantity of aloine from *Aloë arborescens* plant extract, compared to Fluka aloine by a high-performance liquid chromatography method coupled with mass spectrometry (LC/MS/MS), according to literature (Wu et al., 2005). The LC/MS system was an Agilent 1100 Series HPLC system (Agilent Technology Co., Ltd.) consisting of a binary pump, degasser, autosampler, thermostat operating at 48 ºC, VL Ion Trap detector and UV detector. Chromatographic separation was performed on a Zorbax SB-C18 column (100mm×3.0mm i.d., 3.5µm) (Agilent) preceded by a 0.5 µm online filter. The mobile phase consisted of acetonitrile and 0.4 % (V/V) formic acid in water, in the ratio 20:80 (V/V) and was delivered at a flow rate of 1 ml/min. The autosampler injection volume was set at 50 µl. The UV detection was performed at 354 nm.
In vitro antifungal activity of *Aloë arborescens* total plant extract against the mycelial growth of *Aspergillus niger* isolated from *Allium cepa, Botrytis gladiolorum* and *Penicillium gladioli*

isolated from *Gladiolus hybridus, Botrytis cinerea* isolated from *Rosa* spp. flowers, and *Fusarium oxysporum* f. sp. *gladioli* isolated from *Freesia hybrida* was determined by agar dilution method, compared to aloine and the control, 5 days after inoculation (Bhandari, 2000). The minimum fungicidal concentration (MFC) of plant extract and aloine was determined for each pathogenic species.
The nutritive medium from Petri plates (70 mm in diameter) was inoculated in the central point with fungal inoculum obtained by the dilution method (1×10^{-5}/ml), and the fungal colonies were incubated at optimal temperature (22-25 ºC), according to literature (Constantinescu, 1974; Samson and van Reenen-Hoekstra, 1988).
Total hydroalcoholic plant extract and/or aloine were introduced in the nutritive medium and different concentrations were obtained, according to each experimental variant, which was repeated 6 times. A quantity of 70 % ethylic alcohol equal to that from the plant extract was added to the control variant.
The percentage of mycelial growth inhibition (P) at each concentration of plant extract was calculated by the formula $P = (C-T)/C \times 100$, where C is the diameter of the control and T that of the treated ones, according to literature (Nidiry and Babu, 2005).
The statistical analysis of data, compared to the control, was performed with ANOVA test and the results for which $p<0.01$ were considered significant.

Results and discussion

The quantity of aloine (0.07263 mg aloine/ml plant extract) in *Aloë arborescens* plant extract, obtained from fresh leaves, was determined by HPLC method, compared to Fluka aloine (Figs. 1, 2).

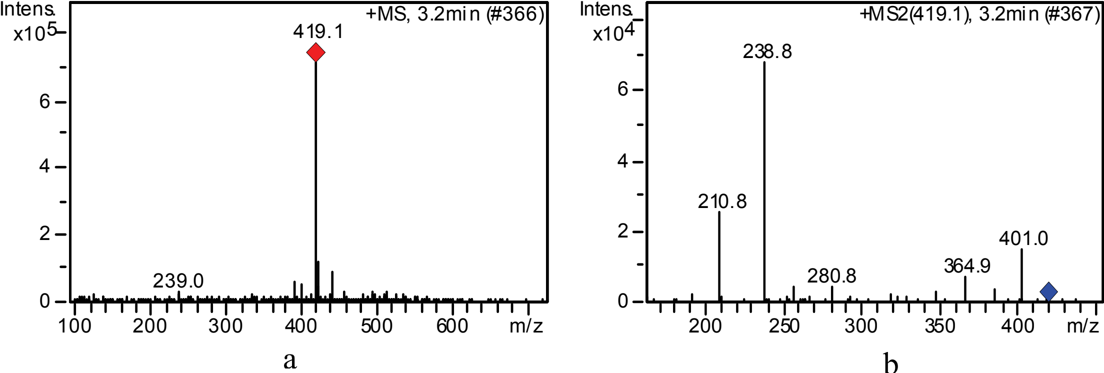

Fig. 1. a) Full-scan ESI-MS spectra of aloine in mobile phase; b) MS/MS spectra of aloine in mobile phase

Aloine quantity in *Aloë* plant extracts is different depending on species. Thus, a quantity of 0.017705 mg aloine/ml was determined in *Aloë vera* plant extract and 0.0003112 mg aloine/ml in *Aloë saponaria* plant extract (Article not published).
The mass spectrometer operated using ESI source in pozitive mode and was set for isolation and fragmentation of aloine adduct with H^+ with m/z = 419 (Fig. 1a). Quantification of aloine was based on the ion with m/z = 238.8 from the MS spectrum of parent ion (Fig. 1b). Calibration curve was linear in the range of 0.38-3.8 µg/ml, with a correlation coefficient of 0.9997.
Sample chromatograms of aloine from *Aloë vera* plant extract are presented in Fig. 1c (the UV trace at 354 nm) and Fig. 1d (the MS signal).

The retention time for aloine was 3.15 min. Due to enhanced sensitivity and selectivity of MS/MS over the UV detection, we have chosen to use it for quantification of aloine in *Aloë arborescens* plant extract.
However, in MS chromatogram (Fig. 1d) another compound having a retention time of 2.5 min can be seen, which is, most probable, an isomer of aloine (has the same molecular mass and the same MS spectra with it).

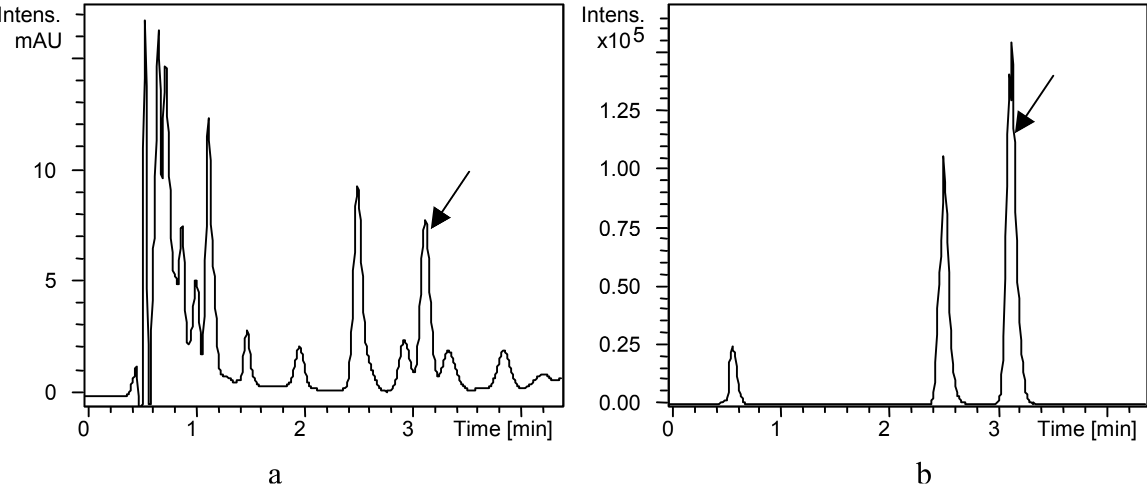

Fig. 2. Chromatogram of aloine from *Aloë arborescens* plant extract: a) UV signal at 354 nm; b) MS/MS signal. The retention time of aloine is 3.15 min (peak marked with an arrow)

In our experiments, the antifungal activity of *Aloë arborescens* was determined against the mycelial growth of *Aspergillus niger, Botrytis cinerea, B. gladiolorum, Fusarium oxysporum* f. sp. *gladioli* and *Penicillium gladioli* on Czapek-agar medium, compared to the control.
Different plant extract concentrations were done, depending on the fungal species, and the MFC was established for each one. Thus, for *Aspergillus niger* there were done concentrations between 40-100 µl/ml, for *Botrytis cinerea* between 20-60 µl/ml, for *B. gladiolorum* between 20-70 µl/ml, for *Fusarium oxysporum* f. sp. *gladioli* between 20-100 µl/ml, and for *Penicillium gladioli* between 40-90 µl/ml (Tab. 1).
The antifungal activity of *Aloë arborescens* plant extract was proportional to its concentration in the nutritive medium, in case of all fungal species, compared to the control.

Table 1. The antifungal activity of *Aloë arborescens* total hydroalcoholic plant extract against the mycelial growth of some phytopathogenic fungi

Fungal species	Concentration of total plant extract in the nutritive medium (µl/ml)	Colony's diameter (mm), after 5 days	P (%)	Standard error
Aspergillus niger	M	24	-	±0.258
	40	23	4.16	±0.341
	60	15	37.5	±0.210
	80	6	75	±0.258
	90	4	83.33	±0.166
	100	0	100	0
Botrytis cinerea	M	65	-	±0.447
	20	60	7.69	±0.210
	40	20	69.23	±0.542
	60	0	100	0

Botrytis gladiolorum	M 20 40 60 70	67 60 25 2 0	- 10.44 62.68 97.01 100	±0.447 ±0.341 ±0.210 ±0.258 0
Fusarium oxysporum f.sp. *gladioli*	M 20 40 60 80 90 100	60 45 25 7 3 2 0	- 25 58.33 88.33 95 96.66 100	±0.957 ±0.614 ±0.654 ±0.258 ±0.258 ±0.307 0
Penicillium gladioli	M 40 60 80 90	10 9 4 2 0	- 10 60 80 100	±0.333 ±0.341 ±0.341 ±0.210 0

Legend: P = Mycelial growth inhibition
C = Control
\- = Absent

Aspergillus niger control colony had 24 mm, *Botrytis cinerea* had 65 mm, and *Botrytis gladiolorum* 67 mm, 5 days from inoculation. *Fusarium oxysporum* f.sp. *gladioli* control colony had 60 mm, and *Penicillium gladioli* had 10 mm (Tab. 1).
The total inhibition of mycelial growth, determined by the plant extract, was recorded at different concentrations, according to the fungal species. Thus, for *Aspergillus niger* and *Fusarium oxysporum* f. sp. *gladioli* the MFC was recorded at 100 µl/ml, for *Botrytis cinerea* at 60 µl/ml, for *B. gladiolorum* at 70 µl/ml, and for *Penicillium gladioli* at 90 µl/ml (Tab. 1).
The results were significant from the statistical point of view ($p<0.01$).
There were also tested different concentrations of 70% hydroalcoholic aloine solution against the mycelial growth of *Aspergillus niger, Botrytis cinerea, B. gladiolorum, Fusarium oxysporum* f. sp. *gladioli* and *Penicillium gladioli* on Czapek-agar medium.
In case of *Aspergillus niger* there were done concentrations between 40-180 µl/ml, for *Botrytis cinerea* between 40-120 µl/ml, for *B. gladiolorum* between 20-100 µl/ml, for *Fusarium oxysporum* f. sp. *gladioli* between 40-180 µl/ml, and for *Penicillium gladioli* between 20-120 µl/ml (Tab. 2).
The total inhibition of mycelial growth, determined by aloine, was recorded at different concentrations, according to fungal species. Thus for *Aspergillus niger* and *Fusarium oxysporum* f. sp. *gladioli* the total inhibition was recorded at 180 µl/ml, for *Botrytis cinerea* and *Penicillium gladioli* at 120 µl/ml, and for *B. gladiolorum* at 100 µl/ml (Tab. 2).
Aloë arborescens total plant extract had a more powerful inhibitory activity than aloine, in case of all fungal species, at the same concentration (Tab. 1, 2).
The more powerful inhibitory action of the plant extract can be explained by the presence, besides aloine, of other active substances.
The obtained results were statistically significant ($p< 0.01$), mostly for the higher concentration values.

Table 2. The antifungal activity of aloine against the mycelial growth of some phytopathogenic fungi

Fungal species	Concentration of total plant extract in the nutritive medium (µl/ml)	Colony's diameter (mm), after 5 days	P (%)	Standard error
Aspergillus niger	M	24	-	±0.258
	40	22	8.33	±0.258
	80	13	45.83	±0.223
	120	6	75	±0.210
	160	3	87.5	0
	180	0	100	0
Botrytis cinerea	M	65	-	±0.447
	40	54	16.92	±0.258
	60	25	61.54	±0.223
	80	14	78.46	±0.333
	100	5	92.31	±0.166
	120	0	100	0
Botrytis gladiolorum	M	67	-	±0.447
	20	65	1.53	±0.341
	40	53	20.89	±0.333
	80	15	77.61	±0.341
	100	0	100	0
Fusarium oxysporum f.sp. *gladioli*	M	60	-	±0.957
	40	53	11.66	±0.307
	100	10	83.33	±0.210
	150	4	93.33	±0.210
	180	0	100	0
Penicillium gladioli	M	10	-	±0.333
	20	9	10	±0.258
	40	8	20	±0.210
	100	4	60	±0.258
	120	0	100	0

Legend: see Table 1

Conclusions

The results we obtained showed the antifungal activity of *Aloë arborescens* total hydroalcoholic plant extract, obtained from fresh leaves, against the mycelial growth of *Aspergillus niger, Botrytis cinerea, B. gladiolorum, Fusarium oxysporum* f. sp. *gladioli* and *Penicillium gladioli* on nutritive medium.
Aloë arborescens plant extract and aloine had an inhibitory activity against all fungal species, proportional to their concentration in the nutritive medium.
The minimum fungicidal concentration (MFC) of plant extract varied between 60-100 µl/ml, and of aloine between 100-180 µl/ml, depending on the fungal species.

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FOLIAR BUD EXTRACTS: CUTANEOUS TESTING UNCOVERS THEIR POTENTIAL IN DERMATOLOGY

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Summary

The present research evaluates the possibility to extend the therapeutic application of gemmotherapy in the field of local cutaneous therapies. Characterized by an intense metabolic activity, buds are rich in microelements, vitamins, enzymes, vegetal hormones, free amino acids, polyphenols etc. The presence of these substances argues in favor of their beneficial employment in dermatologic disorders and cosmetology. In order to evaluate the potential of buds in this regard, hydro-glycero-alcoholic extracts (1:1:1) were prepared from fresh buds of *Abies sp*., *Tilia sp*., *Populus sp*., according to the provisions of the Xth French Pharmacopoeia. After the evaporation of ethanol, the fractions were incorporated in ointment basis of O/W type. The stability of the obtained ointments was assessed microscopically (Nikon Eclipse E60). Subsequently, dermatologic tests (corneometry, TEWA metry) were performed, using the ointment basis as control. The degree to which the preparations influence skin barrier function, skin texture, moisture, rugosity was evaluated. All analyzed parameters were positively influenced after the application of ointments containing bud extracts, uncovering novel possibilities for their utilization in dermo-cosmetology.

Key words: foliar buds, cutaneous therapy, O/W basis, TEWA metry, corneometry

Introduction

Gemmotherapy is a new concept applied in cosmetology and therapy [1,2,3]. The large area of biological activity is close to their chemical composition consists in important compounds like polyphenols, saponines, flavones, vitamins, etc [1,4,5,6,7]. Cosmetic area included buds in practice but the developing of this concept is still new [1,8,9]. Semisolid formulations with moisturizing effect are important in maintain the healthy skin [8,10]. The formulations with cosmetic application have to be without any noxiousness effect for short and long time utilization [8,11]. The semisolid formula have to be stable and with proper rheological behavior [10]. The spreadability pH and washability are other demands for these formulations [8,10]. The SELS parameters are important in the appreciation and evaluation of efficiency/noxiousness balance of cosmetic product [12,13].

Materials and methods

The extracts were prepared from vegetal material (buds), by concordant with French Pharmacopoeia, X-th Edition, by maceration, as hydro-glycero-alcoholic forms (1:1:1). Same types of extracts going to dryness were incorporated in a hydrophilic cream. The materials for the formula were: hydrophilic emulsifier sodium dodecilsulphate (Fluka), fatty substances, cethyl alcohol (Merck), cacao butter (Magnesia GmbH), paraffin oil and vaseline (Merkur Vaseline); antimicrobial preservatives, methyl-p-hydroxybenzoate and propyl-p-hidroxybenzoate (Merk), glycerol (Chimopar), ethanol (Chimopar), distillated water (Ph. Eur.).
The cream formula is presented in the table 1.
The aqueous phase of the hydrophilic cream containing 1% dry macerate was prepared by mixing the glycerol and ethanol with the sodium dodecilsulphate solution (prepared by dissolving the emulsifier in 1/3 of preservative solution). The fatty substances (cetyl alcohol, cacao butter, paraffin oil, vaseline) were mixed; then both the oily and aqueous phases were separately heated to 70ºC to 80ºC and finally, the oily phase was added to the aqueous phase with continuous stirring until cooled to room temperature. The obtained W/O emulsion was mixed with the carbopol 940 hydrogel, beforehand prepared, with gentle stirring to obtain the hydrophilic cream. The prepared semisolid formulations were analysed visually for their colour, homogeneity and phase separation. The pH values of 1% aqueous solutions of the obtained preparations were determined potentiometric using a pH-meter (Jenway 3030) at room temperature (25ºC). The viscosity of the two semisolid preparations was determined at 25ºC using a rotational viscosimeter (Brookfield RV – DV I) with spindle SC4 28 and connected to a thermostatically controlled circulating water bath (Brookfield Thermosel accesory). The viscosity values measured were graphically represented obtaining the rheogram, which is shown in figure 2.
The stability was tested by microscopy, with Nikon Eclipse E60 microscope and Coolpix digital camera (figure 1).
The clinical measurements consist in initial data (before the application of the product) and after 6 hours from the first application. Before the application of the creams or extracts the skin was treated with a SDS 10% solution, a surfactant which imitate the epidermal physiological stress conditions [12,13]. As it is mentioned in the literature the body region accepted for all this measurement was forearm. The applications were made simultaneous. For each evaluation the hydration level was monitored with CM 820 Corneometer, transepidermal waterloss with a TM 210 Tewameter and the SELS parameters with a VC 98 Visioscan. The measurements were made in constant environment conditions such as: humidity 40-60%, temperature 20-22˚C.
The volunteers' selection was the criteria selection type and consists in inclusion and exclusion rules. The inclusion criteria were: a healthy epidermis, the approving for the test conditions. The exclusion criteria were: excess of hair on the skin, not agree with the testing conditions, developing treatments. In conclusion the number of subjects was a minimum number, 15 volunteers, female, clinical healthy with ages between 35-55 years.

The type of the test was that the efficiency of the formulations. It was establish by comparing the final values with the initial ones for each type of them.
All the formulations were microbiologically tested. The cutaneous tolerance consisted in occlusive patches have been applied for 24 hour (COLIPA method).
The efficiency criteria for a short time action could be characterise by the following rules: hydration level is essential, transepidermal waterloss indicates the integrity of the skin protection layer and its barrier function, SELS (Surface Evaluation on Living Skin) parameters with the specific ones (roughness, smoothness, desquamation).
All the formulations were microbiologically tested before their skin application.

Results and discussions

The prepared formulation was viscous, creamy preparations with a smooth and homogeneous appearance. The tests were correlated with EU demands [14]. The hydrophilic cream was white. The semisolid formula was easily spreadable on the skin with acceptable bioadhesion properties (figure 4). The pH values of the obtained semisolid preparation ranged from 5.3 to 5.4. The semisolid preparations containing 1% vegetal extract were stable upon storage for three months.
The microscopic aspect of the O/W emulsion (hydrophilic cream) studied through the optical microscopy proved the O/W emulsion type and the spherical form of the dispersed drops, which mean diameter was 33,2 μm. Also, the homogenous distribution of the emulsion particles was observed (figure 1). Figure 3 also indicated that preparations possessed a pseudoplastic behaviour without thixotropy. The values of penetrometric determination are presented in figure 4, which shows that penetration capacity of O/W cream had high weight values.
Concerning the effects of extracts and cream can cancel the noxiousness consequences of the SDS applied for 6 hours on the hydrolypidic layer in different proportion and the best were Tilia sp. products (figure 8,9 and for *Tilia sp*. 10).
All products were well tolerated and the SELS parameters were acceptable. The hydration degree was: *Abies sp*. extract: 53.5%, *Tilia sp.* cream 38.6% - extract 53.6% *Populus sp.* cream 35% - gel 43% figures 5,6,7. Abies sp. extract recovered the skin layer after SDS application, in 6 h with 12.5% figure 8. The extracts were more efficacies in transepidemal waterloss recovery than creams. The best from all, concerning the recovery activity was *Tilia sp.* products, the extract and the cream, too (figure 10).
Smoothness (depth of horizontal and vertical lines), affected by application of SDS, is restored only in presence of the cream containing Populus extract, Tilia cream and extract and Abies extract, the extract alone of Populus being not quite efficacious.
Both the cream and Populus extract are efficacious in decreasing exfoliation, by 17.6% and 15.3% respectively, as compared with the initial level. The results achieved are more eloquent, the products acting on a damaged skin by the treatment with SDS (the detergent induced an increase in exfoliation by 24% as against the initial level). Both the cream and Tilia extract are efficacious in reducing desquamation, re-establishing the initial level, disturbing by the action of SDS, in one application, after 6 h. of action. Abies extract is efficacious in decreasing exfoliation, establishing values even lower than the initial ones, in one application, aftert 6 hours of action, even though it acts on an epidermis severely damaged by SDS effect.

Conclusions

The hydrophilic creams with bud's extracts were stable and with a proper rheological behaviour. The extracts were well incorporated and don't change the physical properties (stability, spreadability, penetrometry) of the final formula. The pH of the final formulations was acceptable for the skin require. The formulations didn't present noxiousness effects and were well tolerated. The hydration degree between 35% and 53.6% obtained after the extracts and creams application on volunteers' skin indicated the moisturizing effect of the products. The most efficient hydrating agent was *Abies sp.* extract. The most efficient forms concerning the TEWA-metry positive results were the *Tilia sp.* ones.

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TABLE 1 The cream formula

Hydrophilic cream

Components	**g%**
Dry extract of *Birch tree*	1
Sodium dodecilsulphate	1
Cetyl alcohol	10
Cacao butter	9
Vaseline	15
Paraffin oil	5
Carbopol 940	0,3
Triethanolamine	0,3
Glycerol	10
Ethanol	10
Preservative solution	38,4

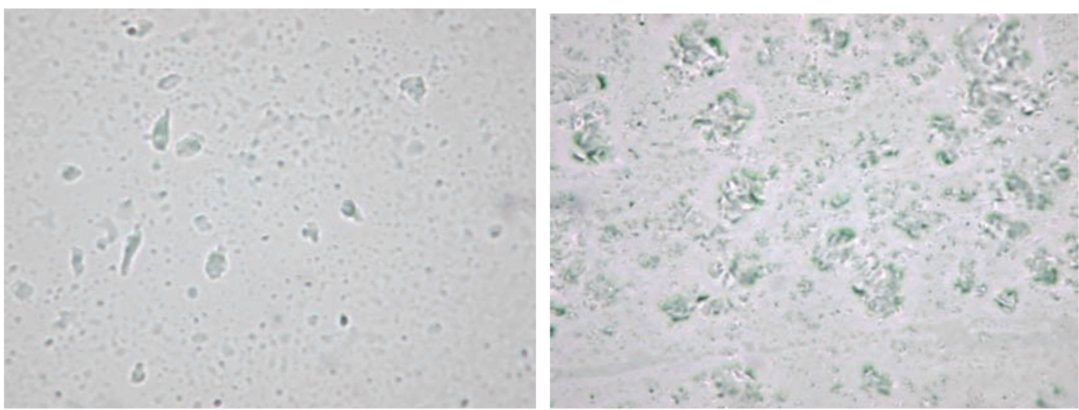

Figure 1. The cream image at the moment of preparation in two dimensions

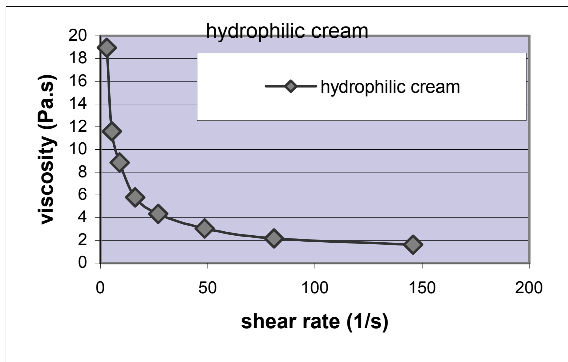

Figure 2. The rheogram for hydrophilic cream

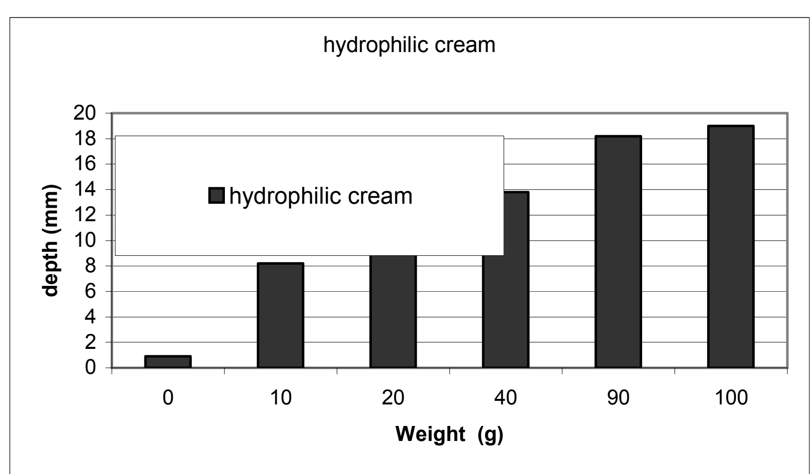

Figure 3. Penetration capacity of semisolid preparation for to incorporate bud's extracts

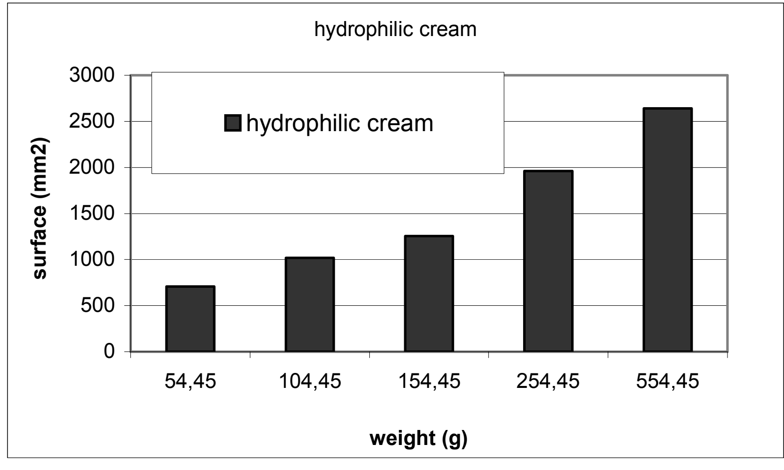

Figure 4. Spreadability of semisolid preparation for to incorporate bud's extracts

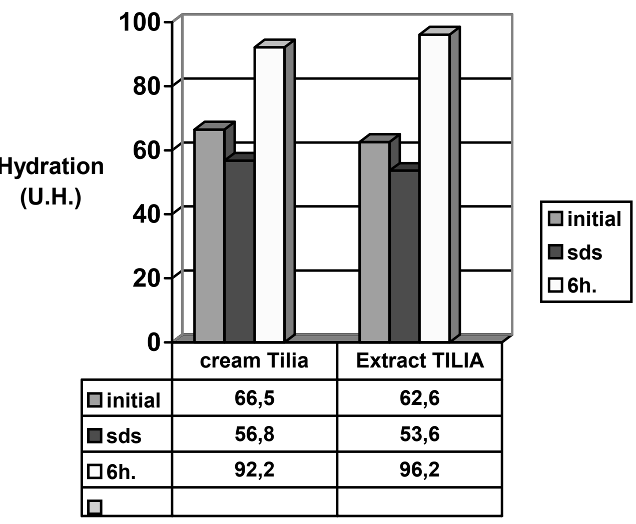

Figure 5. Tilia sp. forms and their hydration capacity

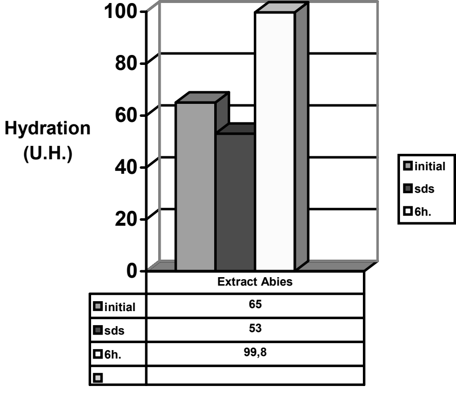

Figure 6. Abies bud's extract hydration

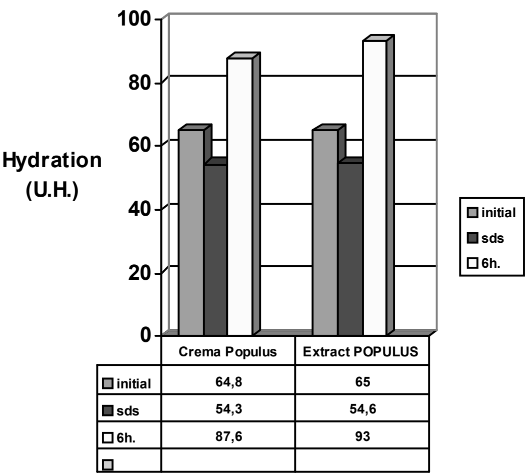

Figure 7. Moisturizing degree for *Populus sp.* forms

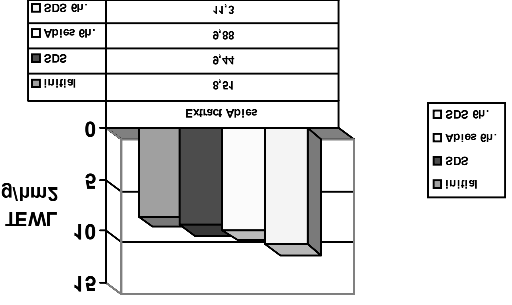

Figure 8. TEWL measurements for Abies sp. extract

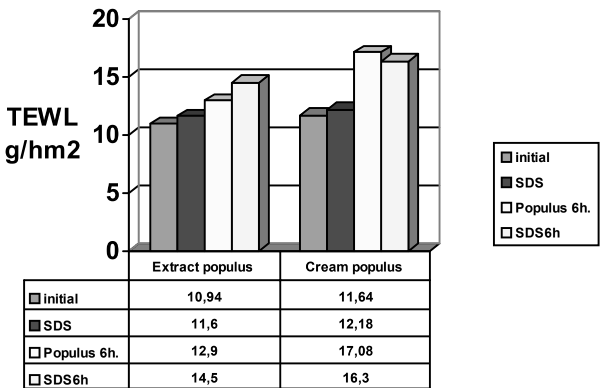

Figure 9. Populus product's TEWL measurements

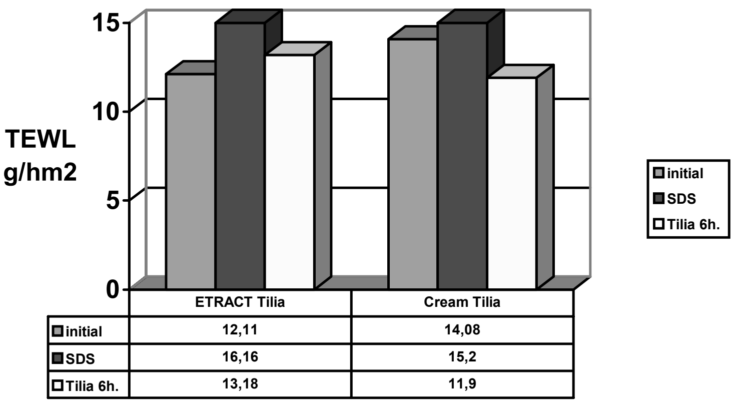

Figure 10. TEWL measurements for Tilia sp. buds

ANTIBACTERIAL ACTIVITY OF THE OIL FROM *MENTHA VIRIDIS L.* AND *MENTHA PIPERITA L.*

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Summary

We have been tested for in vitro antibacterial activity the volatile oils derived from the Mentha piperita L and Mentha viridis L (originate in vitro cultures). The sample of M. viridis II is superior in antimicrobial activity, amongst the tested oils, with a MIC values between 0,7- 47 mg/l against bacteria. The antifungal activity tested against C. albicans showed a pronounced activity with MIC of 0,4 mg/l for both M. viridis II and M. piperita samples and 1,5 mg/l for M. viridis I. The results of antibacterial assay indicate that the oil from M. viridis II was generally superior against tested strains when compared to the M. viridis I and M. piperita.

Keywords: *Antibacterial activity, Mentha viridis L., Mentha piperita L.*

Introduction

Natural essential oils (mixtures of fragrant chemical) obtained from various parts of plants are efficient active antimicrobial agents.
In traditional medicine the different parts of plants (flowers, fruits, seeds, herb, leaves, wood, roots) are used as treatments for infectious diseases. The widespread use of antimicrobial agents selects resistant bacterial strains, which seriously compromise the effectiveness of antibiotic treatment.
The use of herbal medicines might be a precautionary measure to prevent the development of lack of susceptibility to synthetic antibiotics that is associated with therapeutic failures.
The purpose of this study was to evaluate the in vitro antibacterial activity of the volatile oils derived from the *Mentha piperita* L and *Mentha viridis* L (originate in vitro cultures).

Materials and methods

Plant material. *Mentha piperita* L and *Mentha viridis* L, aerial parts, were collected in the flowering stage from Piatra Neamt (Easter region of Romania) in June 2004. The air-dried samples were crushed and hydrodistilleed for 3 h, according to the European Pharmacopoeia (2002) recommendation.
We obtained three volatile oil samples codified as following:

- *Mentha viridis* I- the oil from the same vegetal material but in the first vegetative year;
- *Mentha viridis* II- oil obtained from the plant material in their second vegetative year;
- *Mentha piperita* oil

The isolated oils have a relative density of 0.9473.

Antibacterial activity

The antimicrobial activity was evaluated against eight Gram positive and Gram negative bacteria: *Staphylococcus aureus* ATCC 25923, *S. epidermidis*, *Sarcina lutea* ATCC 9341, *Bacillus cereus*, *Escherichia coli* ATCC 25922, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Candida albicans*. The qualitative antibacterial determination of the oils was carried by the disc diffusion method (Brown and Blowers, 1978). The diameters of inhibition zones of bacteria growth were measured after 24 h of incubation at 37^0C.

Minimum inhibitory concentrations (MICs) and minimum bactericidal concentrations MBCs) were evaluated by the macrodilution method in broth (NCCLS, 2001).
In order to achieve a stable emulsion of the oil in broth the addition of the emulsifier Tween 80 is necessary at concentrations of 0.5% to minimize adverse effect on bacteria (Carson et all, 1995, Griffin 2000). Double dilutions of each oil sample (ranging from 0.09 μg/ml to 100 μg/ml) were tested. The tubes with a volume of 0.05 ml bacterial inoculum containing approximately 10^6CFU. The last tube resulting in complete inhibition of visible growth of the tested bacteria after 20 h incubation at 37^0C, represent the minimum inhibitory concentration. The minimum bactericidal concentration was determined by transferring 0.1 ml from each of the tubes showing no growth on the surface of agar plate.

Results and disscusion

The data obtained in the qualitative antimicrobial activity determination are presented in Table I. In evaluating the qualitative antimicrobial activity of mint oils we made comparisons with the ampicillin (25 μg/disc), respectively nystatin (100 μg/disc). The discs of Whatman paper No 3 contained amounts of 5 μl sample.

Table I. Susceptibility to mint oils of Gram positive and Gram negative bacteria

Oil sample	Diameter of zone inhibition (mm)						
	S. aureus ATCC 25923	*S. epidermidis*	*Sarcina lutea* ATCC 9341	*B. cereus*	*E. coli* ATCC 25922	*Klebsiella* spp.	*Candida albicans*
M. viridis I	14	19	21	26	16	14	28
M. viridis II	21	26	23	31	16	12	21
M. piperita	15	16	23	23	11	16	27
Ampicillin (25 μg/disc)	24	28	34	22	25	15	-
Nystatin (100 μg/disc)	-	-	-	-	-	-	21

The tested strains were less susceptible to mint oil samples than to ampicillin.
In contrast, significant increase in *C. albicans* susceptibility to mint oils was observed by comparison with nystatin 100 μg/disc.
The antimicrobial activity of mint oils is attributed mainly to menthol, one of the main components. The mint oils in vitro exerted a different inhibitory effect on the bacterial growth.
Five organisms have been tested for their susceptibilities to mint oils and data are summarized in Table II.
All bacteria excepted *P. aeruginosa* are susceptible to mint oils at concentrations ranging between 0.4 and 6.25 mg/ml.
The oil obtained from *Mentha viridis* II has generally MIC and MBC values lower than these of *M. viridis* I.
The lowest minimum inhibitory concentration was 0.2 mg/ml *M. viridis* II oil against *B. cereus*.
The oil of *M. piperita* was generally less active than *M. viridis* oil samples. The MIC and MBC values are similarly to these of *M. viridis* II against *C. albicans* and 2 times below than the MIC of *M. viridis* I.

Table II. Susceptibility data for bacteria tested against mint oil samples

Samples	Bacterial species									
	S. aureus ATCC 25923		*B. cereus*		*E. coli* ATCC 25922		*P. aeruginosa*		*C. albicans*	
	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
Mentha viridis I	1.6	6.25	0.8	1.6	0.8	1.6	50	100	1.6	3.2
Mentha viridis II	1.6	3.12	0.2	0.4	0.8	1.6	25	50	0.4	0.8
Mentha piperita	1.6	3.12	3.12	12.5	0.8	1.6	50	100	0.4	0.8

The fungicidal concentrations range from 0.4 mg/ml to 1.6 mg/ml. Concerning antifungal activity, the both oil samples obtained from mint are comparable MIC values to these published by Carson et al. (2006) for the tea tree oil. This volatile essential oil is derived from plant *Melaleuca alternifolia* that has MICs range between 0.03 and 0.5 %. The tea tree oil has a broad- spectrum activity, antibacterial, antifungal, antiviral, antiprotozoal action, and it is incorporated in many topical formulations.

All oil samples exhibited the same activity against *E. coli*. *M. viridis* II oil exceeds the activity of *M. viridis* I and *M. piperita* against *P. aeruginosa* to 2-fold. The antistaphylococcal efficacy of these oil samples is identical.

Regarding the antibacterial activity of other essential oils, such as oil from *Achillea ageratum* L., described in the literature, our data show that mint oil possess a better action (Puerta et al, 1996).

Kalemba and Kunicka (2003) investigated the activity of different essential oils and demonstrate strong antimicrobial properties of mint oil.

In conclusions, comparing the antibacterial activity of all mint oils tested it becomes obvious that the most active was *M. viridis* II sample.

Our study suggest the efficacy of essential mint oils preparations against bacterial and fungal infections. These essential oils have an importance as pharmaceuticals and preservatives.

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INFRASPECIFIC CHEMICAL TAXA OF *ACHILLEA DISTANS* W. ET K.

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Summary

Starting with chemical analyse of the essential oil from three subspecies of Achillea distans W et K (mountain yarrow) we established that the taxa of sub alpine zone are missing of azulene, whereas the taxa of sub mountain has a very low azulene (2.44%) while in Achillea millefolium L the content of azulene is 25.26%.

Keywords: *Achillea distans, Achillea millefolium, azulenes, essential oil.*

Introduction

In Romania’s spontaneous flora 23 species of *Achillea* (*Asteraceae*) vegetates (1), but for medical purposes are used only the flowers of *Achillea millefolium L. (Millefolii flos),* official in Romanian Pharmacopeia 10th edition (2). These flowers contain essential oil, flavonoids, tannins, triterpenes (3,4).
Achillea millefolium L. (yarrow) is one of the oldest medicinal plants used for its anti-inflamatory, spasmolytic, carminative, antimicrobial, diuretic, expectorant, haemostatic and antireumatic properties. Due to these properties, *Achillea millefolium* is used in digestive, respirators, genito-urinary and dermatologic diseases; it can be administrated both internally and externally (3,4).
One of the species that can adulterated *Achillea millefolium* is *Achillea distans* W et K (mountain yarrow), which vegetates in mountain and hill zones. Many times *Achillea distans* vegetates together with *Achillea millefolium* in sub mountain zone. These two species are similar regarding flowers and inflorescences, but *Achillea distans* is taller and presents longer and wider leaves (5).
The essential oil, being the most important active principle from the flowers composition, our research focused on the extraction and analysis of the essential oils from the two species of *Achillea*.
Another research on different species of *Achillea* from Romania showed the presence of prochamazulenes just in *Achillea millefolium*, while in *Achillea distans* they weren’t found (6).

Materials and methods

The vegetal material studied was obtained from the following species:
- *Achillea distans ssp. alpina* (pink flowers), harvested from Rodnei Mountains (1800 m)
- *Achillea distans ssp. distans* (white flowers), harvested from Rodnei Mountains (1750 m)
- *Achillea distans ssp. distans,* harvested from Tarnita Lake, Cluj district (700 m)
- *Achillea millefolium,* harvested from Cluj district

The inflorescences were harvested from the spontaneous flora, in july-august, during blooming and were dried on the ambient temperature.
The essential oils were obtained by water steam distillation with Neo-Clevenger apparatus using inflorescences harvested from the two *Achillea species* (2).
Apparatus and chromatographic conditions: we used a Hewlett-Packard 5890 GC coupled with a Hewlett-Packard 5972 SM. A capillary column (30.0 m/0.25 mm) and helium as

carrier gas (with a flow rate of 1 ml/min) were used. 0.2 μl of oil was injected into the column. The GC owen temperature was programmed from 50 to 240ºC. Quantification of the essential oil was conducted by gas-chromatography with a flame ionisation detector (GC-FID).

Results and discussion

The content of essential oils for the four samples is showed in table I.

Table I. The characteristics of the isolated essential oils

No.	Taxa	Selection	Essential oil (ml/100g vegetal product)	Content of azulenes (%)
1.	*Achillea distans* ssp. *alpina* with pink flowers	Rodnei Mountains, Iezer (1800 m)	0.25 colorless	-
2.	*Achillea distans* ssp *distans* with white flowers	Rodnei Mountains, Iezer (1750 m)	0.40 colorless	-
3.	*Achillea distans* ssp *distans* with white flowers	Apuseni Mountains, Tarnița (750 m)	0.24 light blue	2.41
4.	*Achillea millefolium*	Cluj-Napoca (320 m)	0.40 dark blue	25.26

By gascromatography there were identified a variable number of compounds for each essential oil; 10 in essential oil of *Achillea distans ssp. alpina*, 8 in essential oil of *Achillea distans ssp. distans* from sub alpine zone, 9 in *Achillea distans ssp. distans* from sub mountain zone and 14 in essential oil of *Achillea millefolium* (table II). The identified compounds from the analyzed essential oils showed in table II.

Table II. The identified compounds from the essential oils

No	Retention time (min)	Compound	*A. distans ssp. alpina* (sub alpine zone) %	*A. distans ssp. distans* (sub alpine zone) %	*A. distans ssp. distans* (sub mountain zone) %	*A. millefolium* %
1	5.49	α-pinene	1.15		4.25	3.24
2	6.50	sabinene	6.37	15.60		
3	8.34	1,8-cineol	20.97			
4	9.31	γ-terpinene	1.01	1.71		1.32
5	12.60	camphor	4.94		11.41	
6	18.68	bornyl acetate	2.61		11.11	
7	24.50	βcaryophyllene	3.00		4.25	9.15
8	25.96	α- humulene	0.43			1.16
9	27.10	germacrene-d	2.35		0.64	5.59
10	34.85	valeranone	2.30			
11	11.07	α-thujone		33.31		
12	11.51	β-thujone		25.52		
13	27.76	zingiberene		0.83		0.76
14	24.38	trans-caryophyllene		0.79	4.25	
15	28.80	δ-cadinene		0.87	0.59	0.32
16	34.13	naphtalene		1.53		

17	6.60	β-pinene			13.51	32.11
18	7.88	α-terpinene				0.45
19	8.95	trans-β-ocimene				0.45
20	23.11	β-bourbonene				0.17
21	24.93	β-cubebene				0.50
22	33.69	α- amorphene				0.16
23	37.50	azulene			2.41	25.26

In *Achillea distans ssp. alpina* essential oil, the predominant compounds are 1,8-cineol (20.97%) and the sabinene (6.37%) and the rest of the identified compounds are below 1% (table II.).
The essential oil of *Achillea distans ssp. distans* (sub alpine zone) contains large quantities of α-thujone (33.31%) and β-thujone (25.52%). Besides these two compounds there is also the sabinen (15.60%), the rest of the identified compounds are in concentration of below 2% (table II.).
The main compounds of essential oil extracted from *Achillea distans, ssp.distans* (sub mountain zone) are β-pinene (13.51%), camphor (11.14%) and bornyl acetate (11.11%), the rest of the compound being below 5% (table II.).
The azulenes (25.26%) and β-pinene (32.11%) are the main compounds of essential oil from *Achillea millefolium,* but in larger quantities there are also β-caryophyllene (9.15%) and germacrene-d (5.59%) (table II.).
Note that the azulenes are presents in significant quantities (over 25%) just in essential oil from *Achillea millefolium,* thus confirming the dates presented in the reference materials.
Regarding the presence and the quantities of azulenes from essential oil of the two subspecies of *Achillea distans* there were established major differences between the two subspecies. If in the two subspecies of *Achillea distans* from sub alpine zone were not identified azulenes, in *Achillea distans* from sub mountain zone the azulenes are in low quantities (below 3%); as a results we can assume the existence of two taxa depending on altitude. There is possible that ecological factors, like altitude, to influence the proazulenes biosynthesis, but it also must taken into consideration that in the sub mountain zone *Achillea millefolium* vegetates together with *Achillea distans*, so there is possibility of hybridization between the two species, which could influence the chemical composition of essential oil.
The mixture ingathering of the inflorescences of the two species may produce a decrease of the contain of azulenes from essential oils extracted, due to the absence of azulenes from the essential oil of *Achillea distans*. This it not admitted the substitution of *Achillea millefolium* flowers with *Achillea distans* flowers because the essential oil obtained is qualitative inferior due to the low content of azulenes, knowing that the azulenes are the active principle responsible for anti-inflammatory action of the product.

Conclusions

- Were studied the chemical composition of essentials oils extracted from 2 species: *Achillea distans* (with 2 subspecies ingathered from different altitudes) and *Achillea millefolium.*
- The main difference between the two species is the large quantities of azulene in *Achillea millefolium* essential oil, while in the *Achillea distans* essential oil the azulenes are absent or are in very low quantitie.
- In case of *Achillea distans ssp. distans*, were observed differences of azulenes concentration determining by area of spreading as following: the azulenes are absent from

essential oil extracted from taxa situated in sub alpine zone and are in very low quantities in essential oil extracted from taxa situated in sub mountain zone.

- The chemical composition differences of essential oil from *Achillea distans* subspecies are leading to the idea of the existence of two chemical taxa depending on the altitude, our research in this field will be continued.
- Due to the absence or the low concentration of azulenes from *Achillea distans* essential oil, the ingathering of inflorescences of this species instead of *Achillea millefolium* inflorescences' is considered an adulteration which depreciates the medicinal product.

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THE INFLUENCE OF *ELEUTHEROCOCCUS SENTICOSUS* MAXIM. ON EGGS PRODUCTION OF THE LAYING HENS HISEX BRAUN

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Summary

There were examined changes in hen eggs production after administration of dried extract of Eleutherococcus senticosus MAXIM. The control group (CG) consisted of 13 birds. In the 1. experimental group (1.EG, n=10) was administered extract of Eleutherococcus senticosus in dose of 0.1% concentration in food. In experimental group (2.EG, n=12) was administered extract of Eleutherococcus senticosus in dose of 0.5 % concentration in food. The laying of eggs were observed every week during all experiment - 8 weeks. Concerning eggs production the results of this study indicate that addition of Eleutherococcus senticosus extract were not statistically significant. Despite of this the higher eggs production in the 2. Experimental group during all experiment was discovered.

Keywords: *Eleutherococcus senticosus, adaptogene, poultry, eggs production, laying hens*

Introduction

Adaptogenes have historical, biological, pharmacological and medical relevance. Eleuterokok tŕnistý (*Eleutherococcus senticosus* Maxim.) is a plant with a specific pharmacological activity. It also has adaptogene characteristics with antioxidant, imunemodulative, hypocholesterol effects and causes a hypoglycaemia. Eleuterokok stimulates the activity of macrophages and has antibacterial and antiradiation effects, increases the T-lymphocytes proliferation (Miyanomae, Frindel, 1988) and prevents from the influence of toxic substances. Pharmacological studies have shown an immunosuppressed effect of the some adaptogene substances on humoral immunity and a stimulation effect on the cell-mediated immunity response.

Application of the phytoadditives can provide a suitable alternative to further embrace of the animal health and production often effected by the polluted environment, different supplements in feed mixture, heavy metals and pesticides hangovers e.g. (Kimáková et al., 2004; Kottferová et al., 2003; Koréneková et al., 2002; Šutiak et al., 2001).

The presented study observes the influence of *Eleutherococcus senticosus* Maxim. on the egg-laying capacity of the Hisex braun hens during the eight weeks period.

Material and methods

The study was carried out on 35 laying hens of a Hisex braun breed and that were divide into 3 groups – a control group and 2 experimental groups. The control group with 13 hens (CG, n =13) was without addition of the dried extract. The dried extract of *Eleutherococcus senticosus* was supplemented into the feed mixture of the 1. experimental group (1. EG, n =10) in a dose of 0.1% and of the 2. experimental group (2. EG, n =12) in a dose of 0.5 %. During the two months period we observed the health conditions and the egg-laying capacity in three groups of birds.

The hens were fed with HYD–6 feed mixture at the beginning of the experiment and HYD-10 feed mixture from the beginning of an egg accumulation and throughout the rest of the experimental period. Feed mixture and water were administered *ad libitum*.. The experimental conditions met the ethic and microclimatic requirements and poultry farming welfare.

The obtained results were evaluated using Unistat 4.53 software, statistical methods to calculate median (Me) and variation (Vr) and the Mann-Whitney U-test for comparison of two independent data groups (Hendl, 2004).

Results and discussion

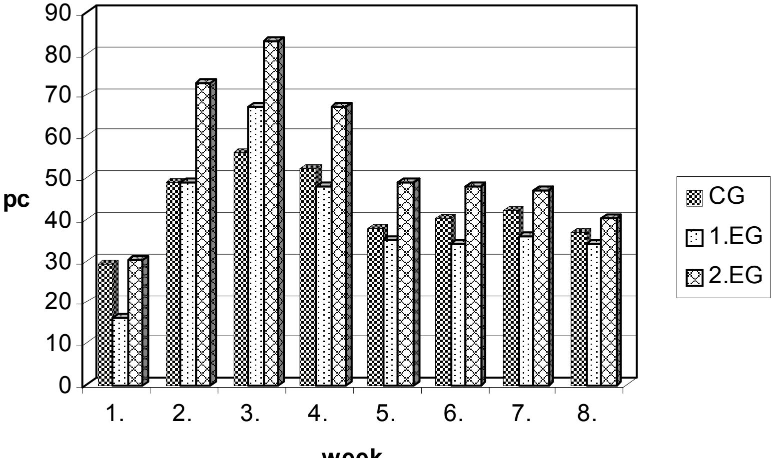
Table 1 presents the egg-laying capacity of the control group and both experimental groups after the application of the dried extract of *Eleutherococcus senticosus* throughout the 8 weeks of experiment. Graf 1 shows the dynamic changes in the egg-laying capacity of the control and two experimental groups of hens.

Table 1. The egg-laying capacity of the control group (CG), 1st experimental group (1. EG) and 2nd experimental group (2. EG) after the application of the dried extract of *Eleutherococcus senticosus* Maxim

Week	Control group (CG) (n=13)	1st experimental group (1.EG) (n=10)	2nd experimental group (2. EG) (n=12)
1.	29	16	30
Me	4	3	4
Vr	2	2	2
2.	49	49	73
Me	7	7	10
Vr	3	2	1
3.	56	67	83
Me	7	7	11
Vr	2	2	1
4.	52	48	67
Me	8	7	10
Vr	2	2	3
5.	38	35	49
Me	5	5	7
Vr	1	0	0
6.	40	34	48
Me	6	5	7
Vr	1	1	1
7.	42	36	47
Me	6	5	7
Vr	0	1	1
8.	37	34	40
Me	5	5	6
Vr	1	1	1

Me – Median; Vr – Variation

The differences in the egg production between the control group and two experimental groups were not statistically significant. In spite of that the results presented in Table 1 and Graf 1 show the positive changes in the egg-laying capacity in the 2nd experimental group with the supplemented dried extract of *Eleutherococcus senticosus* in a dose of 0.5 %. in the feed mixture during the whole experiment. Throughout the experiment we did not observe any mortality and diseases of the studied animals.



Graf 1. The dynamic changes in the egg-laying capacity of the control and two experimental groups after the application of the dried extract from *Eleutherococcus senticosus* Maxim, CG – control group, 1. EG – 1st experimental group, 2. EG – 2nd experimental group, pc – total number of the laid eggs

Conclusion

The dried extract of *Eleutherococcus senticosus* Maxim. that was supplemented in different doses to the feed mixture of the hens did not make statistically significant differences in the egg laying capacity between control and two experimental groups. But the increase of the egg production in the 2nd experimental group during the whole experiment suggests that more investigation into the problem needs to be done. In the next part of the experiment we will concentrate on a longer period observation of the commercial characteristics and health status of animals studying the biochemical and haematological parameters.
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EFFECT OF HERBAL MIXTURE "GASTROHERB®" ON THE QUALITY OF PIZZA CRUSTS

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Summary

In order to obtain novel bakery products with specific biological attributes, medicinal and aromatic herb mixtures have been included in product formulations. Researches from the Institute for Medicinal Plant Research "Dr Josif Pančić", Belgrade have formulated herbal mixture "Gastroherb®" in pulverized form and extract that enhances the functionality of bakery products. Medicinal herbs and aromatic components of the mixture (sage, artichoke, coriander and oregan) contain a complex of biologically active components exhibiting numerous activities (choleretic, carminative, antioxidative, antimycotic) that positively influence digestion.
Herbal mixture "Gastroherb®" doses varried in dough formulations depending on the form: 2% (flour basis) for pulverized mixture and 8% (flour basis) for extract. The addition of herbal mixture to pizza crust contributed to the development of new sensory profile and functional properties (improves digestion). In the paper, characterization of herbal raw materials and the blend, process technology as well as the sensory properties of pizza crusts have been accented.

Keywords: *medicinal and aromatic herbs, bakery products, sensory properties*

Introduction

Medicinal herbs have been considered as the most usable resources for the development of nutraceutics - functional foods regarding food industry, (Arsić, 2003). According to T.P. Shukla (Shukla, 1998), herbs and spices were being used at a rate of 860 million lb per year in the United States alone in 1998. Baked foods are objective points to nutraceutical development because of their nutraceutical value and their value as nutraceutical carriers. When consumed at the daily recommended intake levels, fortified bread and bakery products as mass consumed food items can easily serve as an effective medium for adminstering a daily dose of nutraceuticals. Medicinal and aromatic herbs not just influence the taste and flavour of the product but have a beneficial effect on human organism. Herbal extracts contain aetheric oils and other active substances such as tannins, organic acids, enzymes, resins, pigments, vitamins, minerals, mucous substances, etc. that enhance aroma and improve nutritive value of a product (Willbrandt, 1989).
During the last decade, the production of pizza supplemented with proteins, minerals, vitamins and dietary fibers has become very popular. Varying the composition of filling and pizza crust, an expansion of the product range with endless combinations of products could be achieved.
Associates from the Institute for Medicinal Plant "Dr Josif Pančić", Center for Cereal Technology and "Kikinda" Bakery have developed a new category of pizza crusts supplementing them with medicinal herbs (Brkić et al., 2001, Šimurina et al., 2000). Untill now, herbs and spices were not being used as direct supplements in bakery products except for decorations (Neumann, 1999).
The aim of the paper was to define an appropiate formulation and dose of herbal blend for metabolism enhancement and to incorporate it to pizza crust. The impact of herbal blends on the technological quality of pizza crusts was also examined.

Materials and methods

Preparation and characteristics of herbal blend

Herbal mixture Gastroherb® was formulated in two forms: as a powder and liquid extract. Medicinal raw material used for the production of the herbal mixture was provided partially from spontaneous flora and cultivation, depending upon the species. According to the principles of Good Manufacturing Practice (GMP), which are applying in production of medicines and herbal preparations, herbal drugs were tested according to directions supplied by actual regulation in this field (Ph Jug 2000). Quality control of all used herbal drugs covered: confirmation of identity, determination of sensory characteristics, determination of purity, determination of moisture content, determination of ash content as well as determination of active substances, and additional testing (microbial quality).

Table 1. Qualitative characteristics of herbal drug constituents of Gastroherb® blend

Herbal drug	Weight loss after drying, %	Ash content, %	Level of grinding	Content of active substance (aetheric oils)	Microbiological quality
Coriandri fructi pulvis (73621204)	3,58	4,51 (Jus. max.7)	1	0,311%	Appropriate
Cynarae folii pulvis (47911005)	4,40	17,67	Not uniform	-	Appropriate
Origani heracleoticae herbae pulvis	5,36	10,65	1	3,197%	Appropriate
Herbal blend 3B	5,08	8,35	1	1,525%	Appropriate

Pulverisation and sieving of single herbal drug was accomplished by the use of appropriate equipment (mills, sieves). This way the processed pulverized plant drugs (<3 mm) were mixed in proportions given by defined prescription, giving final herbal mixture with trade name Gastroherb® pulvis.

Plant extract was prepared by double percolation method, using 45% propylene glycol (PG) as solvent. Ratio of herbal drug to solvent was 1:2

Baking procedure

Pizza crusts were produced using no-time dough procedure.The basic dough formulation and procedure is described in Table 2.

Table 2. Basic dough formulation and processing parameters in the production of pizza crusts

Ingredients and parameters	Thin cracker-like crust (type 1)	Thick bread-type crust (type 2)
	%	
Basic dough formulation		
Wheat flour, type 500	100	100
Salt	1.8	1.8
Sugar	3.5	2.0
Oil	3.0	-
Baker's yeast	5.0	5.0

Water	According to water absorption	According to water absorption
Processing parameters		
Mixing time, min.	2+8	
Dough temperature, °C	24-25	
Bulk fermentation	-	
Dough make-up	Manual dividing and rounding	
Dough weight, g	150	
Dough sheeting	Manual	
Thickness of dough sheet, mm	4	
Final fermentation, min	30	
Bake temperature, °C	250	
Bake time, min.	8	

A commercial bread improver was also included in dough formulation in doses according to the recommendation of manufacturer. The quality attributes of flour was analyzed according to the current regulations (Kaluđerski, Filipović, 1998, Pravilnik 74/1988, Pravilnik 52/1995). Processing quality of used wheat flour is reported in Table 3.

Table 3. Processing quality of wheat flour

Quality parameter	**Value**
Water content, %	14.7
Ash content, % d.b.	0.51
Wet gluten content, %	28.4
Farinogram	
Water absorption, %	54.3
Departure, min.	4.5
15-min drop, B.U,	120
Quality number	14.1
Quality group	51.2
Extensigram	
Extensigraph area, cm^2	13.1
Resistance, B.U.	80
Extensibility, mm	126
Ratio	0.63

Results from preliminary trials were used to select doses of herbal blends that do not disturb the taste characteristics and acceptance of bread. Gastroherb® herbal blend in powdered form was added in amount of 2% flour basis while the same herbal blend in liquid form was added in amount of 8% flour basis.

Sensory evaluation
Sensory properties of pizza crusts with herbal blends were compared to standard pizza crust with respect to the following parameters: dough thickness, crumb grain structure, crumb colour, mastication, melting-in-the-mouth, cutting ability, taste, odour, having importance mainly for consumer's acceptance (Bojat and Vukobratović, 1993.). Sensory panel consisted of 3 trained judges.

Results and discussion

The sensory properties of pizza crusts supplemented with herbal blends compared to that of the control standard pizza crust are shown in Tab 4.

Table 4. Sensory properties of pizza crusts supplemented with herbal blends

Quality parameters	Control sample		Crust with powdered herbal blend		Crust with liquid herbal extract	
	type 1	type 2	type 1	type 2	type 1	type 2
Dough thickness, mm	5	6	4,5	5.5	4	5
Cutting ability	very good	very good	very good	very good	good	good
Porosity	fine	spongy	fine	fine	fine	almost fine
Texture	crispy	crispy	crispy	crispy	crispy	firm
Crumb colour	light yellow	light	yellow with visible particles of herbs	yellow with visible particles of herbs	yellow-greyish	yellow-greyish
Taste/Odour	characte ristic	characte ristic	palatable, expressive herbal taste	palatable, expressive herbal taste	palatable, aromatic	palatable, aromatic
Melting-in-the-mouth	excellent	excellent	excellent	excellent	good	very good
Mastication	very good	very good	very good	good	almost good	good

The results confirmed that pizza crust supplementation with powdered herbal blend provided good quality pizza crusts with specific sensory attributes. The taste charasteristics of the supplemened crusts are different from those of the control sample but well accepted by the panelists. The samples have well developed, porous and elastic crumb. Colour of the crumb is specific and visually very accepted. Excellent elasticity and porosity of the crumb for bread-like pizza crusts with extended shelf-life properties was observed. Pizza crusts supplemented with liquid herbal extract showed poorer sensory properties.

Conclusion

Important step in creating a formulation for bakery products supplemented with medicinal herbs is deriving an optimal ratio of ingredients that does not negatively affect the sensory properties of the product as well as a suitable preparation mode of herbal extracts. The optimum doses of herbal blends reported (2 % flour basis for powder and 8 % flour basis for liquid extract) were found not to disturb the sensory properties and acceptance of the product but are sensorily registered. Pizza crusts supplemented with mixture of herbal extracts have specific taste and odour, coloured crumb and an acceptable sensory score.

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BUILDING OF DATABASE FOR THE FAST SCREENING OF FLAVOURS AND FRAGRANCES BY LS/MS TECHNIQUE

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Summary

The usage of LS/MS technique for the fast screening of commercial flavours and fragrances was tested. For this purpose, 49 samples of flavours and fragrances of different origin were analysed by LS/MS. Acquired mass spectra were processed and stored in MS library, whose usability for differentiation of samples was tested by common search engines. Further, MS data from created library were transformed, imported, and statistically processed to get better insight into differentiation power of LS/MS technique applied to selected samples.

Keywords: *LS/MS technique, flavours, fragrances, HCA.*

Introduction

In our first contribution in this field, the concept of development of a new analytical technique for characterisation of essential oils has been described. The concept of liquid sampling mass spectrometry (LS/MS), developed in the first instance for the characterisation of essential oils, consisted of following main items: selection of hardware components for LS/MS analytical system (a), selection and LS/MS working conditions (b), selection of software for essential oil mass spectra processing (c), testing and optimisation of items a to c (d), acquisition of essential oil mass spectra and preparation of appropriate MS libraries (e). At that time LS/MS technique was tested on a wide range of essential oil samples and promising results opened wide range of possibilities in the application of LS/MS technique in the field of characterisation of these products [1].
In the next phase, our efforts were focussed to comparison of similarity of LS/MS spectra of pure samples, to those which could be obtained from the analysis of diluted samples and/or already acquired appropriate standard GC/MS data files, as well as enlargement of initially prepared spectral libraries for available essential oil samples. It was concluded that similarity between the native, composite and extracted LS/MS spectra were enormous, which could elevate expansion of newly created spectral libraries with already acquired data by common GC/MS [2,3].
Furthermore, LS/MS technique was tested for the fast chemical screening of essential oils of several *Thymus* species [4], as well as for fast quantification of certain major constituents in selected essential oils [5].
Although compositions of flavours and fragrances are used for quite different purposes, there are few details where these could be compared from the point of view of analysts dealing with their characterisation. The most important one is that both mentioned group of product are in essence formulations, containing often very complex mixtures of several ingredients. In the case of flavours, for example, natural, nature-identical, or synthetic active (and other) ingredients could be used in formulation of finished product, where named origin can be detrimental for its price and acceptability on the market [6,7].
The aim of the present work was to check usability of LS/MS technique for the fast screening of compositions of different flavours and fragrances used in pharmaceutical, cosmetic and food industry.

Material and methods

Samples selection

Among 21 samples supplied by company Drom (Germany), 19 were belonging to the group of fragrance compositions [d_459730 (vita-jogurt), d_459729 (jogurt), d_460650 (bambi baby), d_460652 (babes), d_460651 (lola), d_460653 (baby balance), d_460654 (baby care), d_414259 (od italie), d_460656 (hand & cream), d_460657 (hand & nail), d_459731 (jogurt & vanila), d_459732 (cerealy jogurt), d_459733 (jogi-balance), d_414492 (naturella), d_414493 (gino), d_414494 (foot spray), d_460785 (colorviva), d_460786 (repair), d_460782 (voodoo lounge), and remaining 2 to the group of flavours [d_0030.139 (strawberry flavour), and d_0035.061 (mint flavour)]. Further 6 aroma compositions, supplied by company Firmenich (Germany) and designated as dentifrice series (for dentistry), were f_52.723, f_52.722T, f_52.721T, f_52.720T, f_52.739T, and f_52.737T. Next 10 mainly fruit aroma compositions (orange, mandarin, walnut, almond, strawberry, caramel, chocolate, vanilla, lemon and coconut), were products of Lachema aromatica Co. (Belgrade). Milk aroma was produced by Quest (The Netherlands), lemon, strawberry, and raspberry flavours from Eterika (Serbia and Montenegro), and latest 5 fruit aroma compositions [wild strawberry (02-601 PiA, 011897), bilberry (18350/279), raspberry (186040031), black currant (01300122) and red currant (186490002)] from other suppliers. Three additional perfume compositions [s_102480 (musk S), s_108046 (carat), and s_ 108032 (birch)] were obtained by Symrise Co. (UK).
Approximately a half of selected samples (49) were fragrance compositions (24), and other half – flavour compositions (25).

LS/MS analytical system and working conditions

Apparatus has been built on the platform of HP G-1800C GCD analytical system, whose mainframe, split-splitless injector equipped with automatic liquid sampler (ALS), detector and data station were used without any modification as the backbone of the new system. Standard capillary GC column has been replaced with fused silica capillary (i.d.=100 µm, l=5 m), which was used as the transfer line between injector and MS detector. Injector, transfer line (oven) and detector were heated at 250 °C, 260 °C and 260 °C, respectively. To enable normal ALS operation duration of analytical runs was extended to 3 minutes. Pure samples of selected flavour and fragrance compositions (200 nl) were injected in split mode (1:60) by ALS equipped with nanoliter adapter in 3 repetitions. Carrier gas was helium. Electron impact (EI) mass spectra of samples were acquired in the m/z range 40-400.

Selection of software for processing of essential oil mass spectra

For processing of the acquired mass spectra, two types of software were used. Probability merge search (PBM), revision B0.01, and NIST MS Search 2.0, as well as additional software for conversion of data between two mentioned data formats. Further analysis of normalised mass spectral data was achieved by hierarchical cluster analysis (HCA), where SPSS software (ver. 10.0.1) was efficiently used.

Results and discussion

The average retention time of peak appearing in virtual "columnless chromatograms" obtained by LS/MS analysis was very short (about 30 seconds). However, peaks mainly were of irregular shape, asymmetric, and far away of those expected theoretically. The example of such a chromatogram is shown in Figure 1.

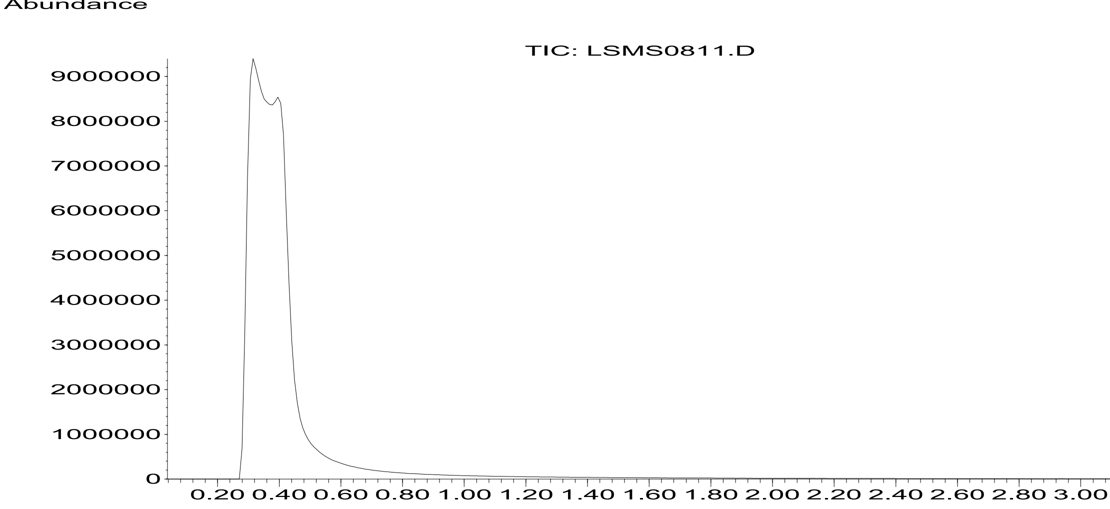

Fig. 1. LS/MS "columnless" chromatogram of Dentifrice 52.723 flavour (Firmenich)

Response of detector is obviously too high, although capillary with higher restriction was used, as wall as injection volume of only 200 nl. In spite of this, the structure of corresponding mass spectrum appeared acceptable (Figure 2).

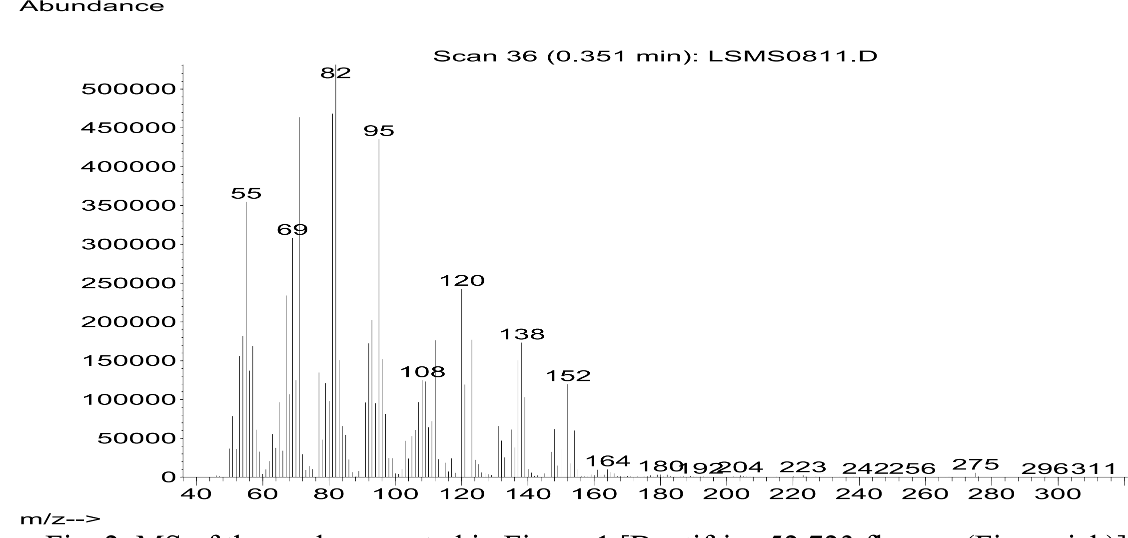

Fig. 2. MS of the peak presented in Figure 1 [Dentifrice 52.723 flavour (Firmenich)]

From data files obtained by multiple injections of all samples, the library of composition of flavour and fragrance mass spectra (ff_2005.l), consisting of 49 records has been created. Evaluation of possibilities of its use for differentiation of flavour and fragrance compositions, according their mass spectra was conducted using two search engines. PBM search was applied directly, giving excellent differentiation between examined samples in the majority of cases. It should be noticed that it was expected from the search engine to recognise analysed sample in the library, and to put that sample as the first on the list of possible hits. One such result is given in Figure 3, where hit list obtained from created database for example mass spectrum (Figure 2) is presented.

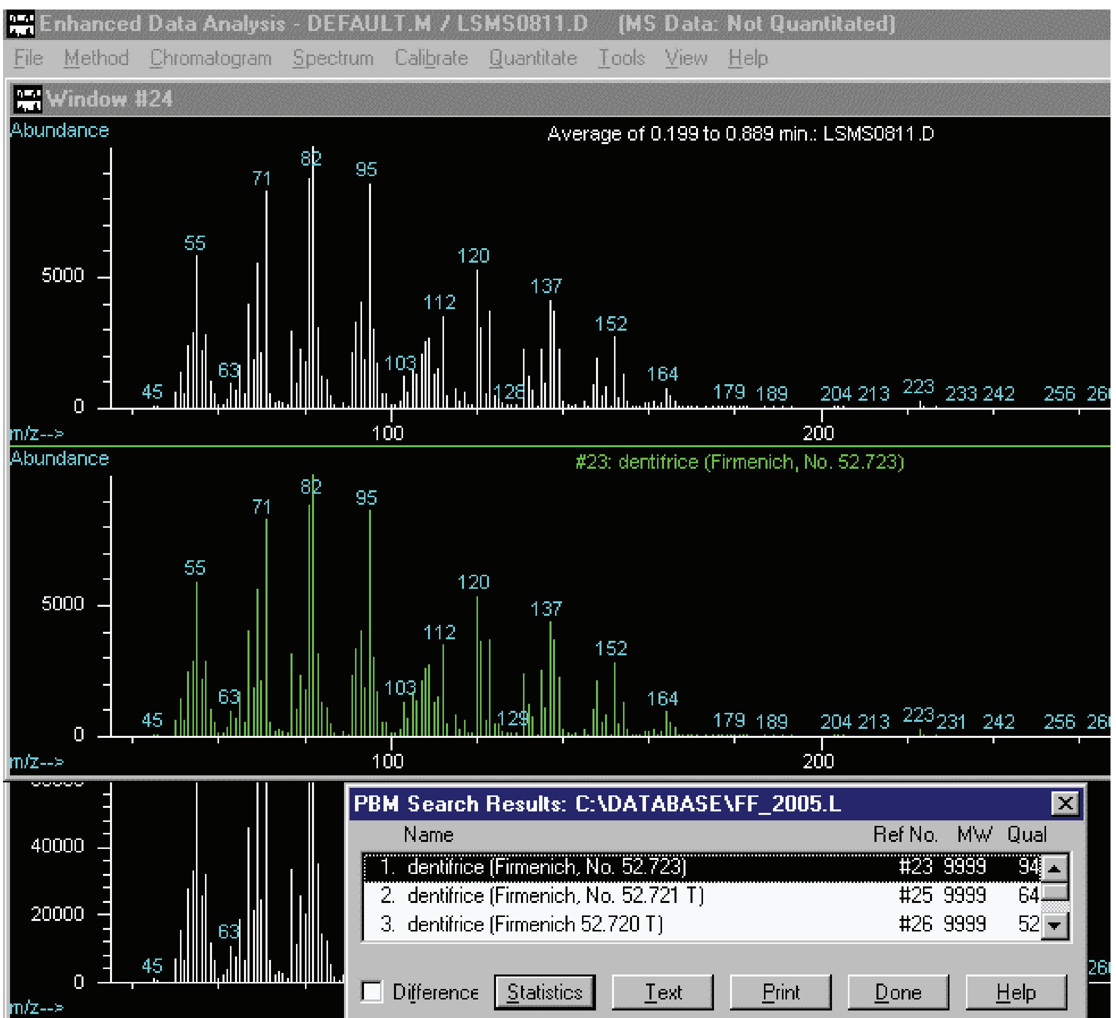

Fig. 3. Hit list for MS of the peak given in Figure 1 [Dentifrice 52.723 flavour (Firmenich)]

It could be seen that PBM search result for all three records from the MS library, which appears in the appropriate window presented in Figure 3, point at the better match quality (94) for the first record. The first suggested result is the right one, other two results are numerically far from that, indicating clear differentiation between these spectra.

Knowing that PBM search algorithm uses selected number of peaks from mass spectrum of library record, with the biggest value of specificity, and compares them with spectra of unknown, we have decided to evaluate NIST search engine, in order to achieve better differentiation between mass spectra. Subsequently, library of the mass spectra of flavour and fragrance compositions was translated from HP to NIST format, necessary for putting NIST MS Search 2.0 software into operation. From the difference of PBM search algorithm, NIST search algorithm takes into account and operates with all peaks from the mass spectrum of the sample.

In the case of comparison of mass spectra of different commercial composition of flavours and fragrances, NIST search engine gave better results than the PBM one.

At the end, hierarchical cluster analysis (HCA) was implemented on mass spectra from the library ff_2005. It showed grouping of clusters, which approves usage of total mass spectra of composition of flavours and fragrances for their characterisation and differentiation (Figure 4).

```
Dendrogram using Ward Method (Rescaled Distance Cluster Combine)
    C A S E      0         5        10        15        20        25
  Label     Num  +---------+---------+---------+---------+---------+
  D_460652   32   ⇩⬂
  D_460653   34   ⇩▫
  D_460650   31   ⇩▫
  D_459730   29   ⇩▫
  D_460654   35   ⇩▫
  D_414259   36   ⇩▫
  D_459733   41   ⇩▫
  BIRCH_SY   22   ⇩⇳⇩⬂
  D_460651   33   ⇩▫ ▫⇩⇩⇩⬂
  D_460656   37   ⇩⬀ ⇔   ▫⇩⇩⇩⇩⇩⇩⇩⇩⇩⇩⇩⇩⇩⇩⬂
  D_459729   30   ⇩⇩⇩⬀   ⇔             ⇔
  D_459731   39   ⇩×⇩⇩⇩⇩⇩⬀             ⇔
  D_414492   42   ⇩⬀                  ⇔
  MUSK_SY    20   ⇩×⇩⬂                ▫⇩⇩⇩⇩⇩⇩⇩⇩⇩⇩⇩⇩⇩⇩⇩⇩⇩⬂
  D_460782   49   ⇩⬀ ▫⇩⬂              ⇔                ⇔
  CARAT_SY   21   ⇩⬂ ⇔ ⇔              ⇔                ⇔
  D_460785   47   ⇩⇳⇩⬀ ▫⇩⇩⇩⬂          ⇔                ⇔
  D_460657   38   ⇩⬀   ⇔   ⇔          ⇔                ⇔
  RED_CRR     1   ⇩⇩⇩⇩⇩⬀   ▫⇩⇩⇩⇩⇩⇩⇩⇩⇩⇩⇩⇩⇩⬀               ⇔
  D_414493   43   ⇩⇩⇩×⇩⬂   ⇔                           ⇔
  D_460786   48   ⇩⇩⇩⬀ ▫⇩⬂ ⇔                           ⇔
  LEMON_LA   14   ⇩⇩⇩×⇩⬀ ▫⇩⬀                           ⇔
  MINT_DRM   46   ⇩⇩⇩⬀   ⇔                             ▫⇩⇩⇩⇩⇩⇩⇩⇩⇩⇩⇩⇩⬂
  D_459732   40   ⇩⇩⇩⇩⇩⇩⇩⬀                             ⇔            ⇔
  MNDRN_LA    7   ⇩⇩⇩⇩⇩×⇩⇩⇩⬂                           ⇔            ⇔
  ALMND_LA    9   ⇩⇩⇩⇩⇩⬀   ▫⇩⬂                         ⇔            ⇔
  RSP_1860    3   ⇩×⇩⇩⇩⇩⇩⬂ ⇔ ⇔                         ⇔            ⇔
  RSP_ET     18   ⇩⬀      ▫⇩⬀ ⇔                        ⇔            ⇔
  STRW_LA    10   ⇩⇩⇩×⇩⬂ ⇔   ⇔                         ⇔            ⇔
  D_414494   44   ⇩⇩⇩⬀ ▫⇩⬀   ⇔                         ⇔            ⇔
  MILK_QST   19   ⇩⇩⇩⇩⇩⬀     ⇔                         ⇔            ⇔
  ORNG_LA     6   ⇩⬂         ⇔                         ⇔            ⇔
  LEMON_ET   16   ⇩▫         ▫⇩⇩⇩⇩⇩⇩⇩⇩⇩⇩⇩⇩⇩⇩⇩⇩⇩⇩⇩⇩⇩⇩⇩⇩⇩⇩⬀           ⇔
  WALNT_LA    8   ⇩▫         ⇔                                      ⇔
  CHOC_LA    12   ⇩▫         ⇔                                      ⇔
  B_BERRY     4   ⇩▫         ⇔                                      ⇔
  STRW_ET    17   ⇩⇳⇩⇩⇩⬂     ⇔                                      ⇔
  CARML_LA   11   ⇩▫   ⇔     ⇔                                      ⇔
  WLD_STRW    5   ⇩▫   ▫⇩⇩⇩⇩⇩⬂                                      ⇔
  STRW_DRM   45   ⇩▫   ⇔     ⇔                                      ⇔
  COCON_LA   15   ⇩⬀   ⇔     ⇔                                      ⇔
  VNLLA_LA   13   ⇩⇩⇩⇩⇩⬀     ⇔                                      ⇔
  BLACK_CR    2   ⇩⇩⇩⇩⇩⇩⇩⇩⇩⇩⇩⇩⬀                                     ⇔
  F_52721T   25   ⇩⬂                                                ⇔
  F_52720T   26   ⇩⇳⇩⬂                                              ⇔
  F_52737T   28   ⇩⬀ ▫⇩⬂                                            ⇔
  F_52723    23   ⇩⇩⇩⬀ ▫⇩⇩⇩⇩⇩⇩⇩⇩⇩⇩⇩⇩⇩⇩⇩⇩⇩⇩⇩⇩⇩⇩⇩⇩⇩⇩⇩⇩⇩⇩⇩⇩⇩⇩⇩⇩⇩⇩⇩⇩⇩⇩⇩⬀
  F_52722T   24   ⇩⇩⇩×⇩⬀
  F_52739T   27   ⇩⇩⇩⬀
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Fig. 4. HCA of total mass spectra from the library ff_2005 (49 records)

It should be noticed that sample, whose basic characterisation has been given as an example in Fig. 1-3 [Dentifrice 52.723 flavour (Firmenich)=F_52723], at the end of cluster presented in Fig. 4, belongs to the sub-cluster containing six similar flavour compositions of the same origin. Although distinction, which could be estimated from mentioned sub-cluster (visually) seemed foggy, results obtained by LS/MS were perfect and quite clean prove of certain differences, approving huge differentiation potential of LS/MS technique.

Conclusions

Clear differentiation of samples belonging to different groups of products (fruit flavours, flavours used in dentistry, and fragrances), as well as those within the same group was

achieved by the use of LS/MS technique. However, screening of different compositions of flavours and fragrances such the way, requires work with large MS-libraries, covering whole spectrum of these products. Moreover, data obtained by LS/MS should be correlated with those obtained by GC/FID, GC/MS, and others, actually required. Subsequently, our further efforts in the application of LS/MS in the field of flavours and fragrances will be focussed in this direction.

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GAS CHROMATOGRAPHIC ESTIMATION OF AROMATIC HERBAL DRUG CONTENT IN THEIR MIXTURES

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Summary

Gas chromatographic method was developed for estimation of aromatic herbal drug content in their mixtures subjected to application in bakery industry. Method includes isolation of essential oil from single herbal drugs and their mixtures, GC analysis of isolated oils, selection of reference constituent in GC profile of single oils, and calculation of the contents of constitutive herbal drugs in their mixtures. With certain precautions this approach could be successfully applied in current quality control practice of such and similar products.

Keywords: *herbal drug, mixtures, determination, essential oils, gas chromatography*

Introduction

When certain product consists of the mixture of several cut or pulverised herbal drugs, or appropriate plant extracts, the most often is very difficult to accomplish required quantitative analysis. Quantification in this case assumes estimation (or determination) of contents of constitutive single herbal drugs (or appropriate extracts). Although modest direction for the quantification, which can be found in the most common reliable sources, offering and pointing at procedures that includes work with certain herbal drug fingerprints, it is not quite clear how to achieve this goal.
From the other side, mentioned fingerprints of medicinal and aromatic plants, the most often obtained by certain chromatographic techniques, such as, thin-layer chromatography (TLC), gas-liquid chromatography (GC), or high performance liquid chromatography (HPLC), are variable. This variability is closely related with the chemical composition of analysed herbal drugs (or extracts), which can vary in very wide proportions. Subsequently, it is quite clear that virtual analytical standards in targeted quantification could be only constitutive original single herbal drugs (or extracts), used for preparation of finished product.
Furthermore, in each of fingerprints (chromatographic profiles) of single herbal drugs (or extracts), appropriate marker constituent should be selected (as a reference) for quantification purposes. All these markers must present specific constituents for selected drugs (extracts) for targeted mixture, and should be easily identified, and quantified in it. Then, after conducting sharply defined procedures for obtaining certain fingerprints (chromatographic profiles) of all standards and samples, and properly applying basic quantification rules for processing of raw chromatographic data, successive estimation of constitutive herbal drugs (or extracts) in their mixtures could be expected.
In this article, results of determination essential oils in homogeneous mixtures of pulverised herbal drugs and starting (original) pulverised single herbal drugs were used, along with those coming out form accompanied GC analyses, for single drug estimation in their mixtures.
Proposed procedure consisted of the following steps: isolation of essential oil from single herbal drugs and their mixtures (1°), GC analysis of isolated oils (2°), selection of reference (marker) constituent in GC profile of each single oil (3°), and calculation of the contents of constitutive herbal drugs in their mixtures (4°).

Material and methods

Samples selection

Subjects of the characterisation were two herbal mixtures, which contained, along with one non-aromatic drug, two or three pulverised aromatic herbal drugs, as constitutive (single) herbal drugs. The first of these contained 35% of *Frangulae cortex pulvis*, 20% of pulverised mint leaf (*Menthae piperitae foilum*), 20% of caraway fruit (*Carvi fructus*) and 25% of parsley fruit (*Petroselini fructus*). The second one contained of 15% of *Cynarae folium pulvis*, 55% of pulverised oregano leaf (*Origani heracleotici folium*) and 30% of coriander fruit (*Coriandri fructus*).

Isolation of essential oils

Essential oils were isolated and quantified in three repetitions in a Clevenger type apparatus, according to Ph. Jug. IV.

Analytical gas chromatography (GC/FID)

GC/FID analysis of the oils was carried out on a Hewlett-Packard HP-5890 Series II GC apparatus, equipped with split-splitless inlet and automatic liquid sampler (ALS), attached to HP-5 column (25 m · 0.32 mm, 0.52 µm film thickness) and fitted to flame ionisation detector (FID). Carrier gas flow rate (H_2) was 1 ml/min, split ratio 1:30, injector temperature was 250°C, detector temperature 300°C, while column temperature was linearly programmed from 40-260°C (at rate of 4°/min). Solutions of essential oil samples in ethanol (¨1%) were consecutively injected by ALS (1 µl, split mode) in triplicate. Area percent reports, obtained as result of standard processing of chromatograms, were used as base for the quantification purposes.

Gas chromatography - mass spectrometry (GC/MS)

The same analytical conditions (as those mentioned for GC/FID) were employed for GC/MS analysis, along with column HP-5MS (30 m · 0.25 mm, 0.25 µm film thickness), using Hewlett-Packard HP G 1800C Series II GCD system. Instead of hydrogen, helium was used as carrier gas. Transfer line was heated at 260°C. Mass spectra were acquired in EI mode (70 eV), in m/z range 40-450. Sample solutions in ethanol (¨1 %) were injected by ALS (200 nl, split mode).

Selection of marker constituents

While selecting marker constituents in each of single oils, the aim was to select the most abundant and the most specific components, whenever it was possible. Although the final calculation could be theoretically accomplished taking from GC profile of selected oil component of free choice, for this purpose the major oil constituents were typically selected. Marker constituents for selected herbal drugs and related oils, in the case of this study, were menthol for mint, carvone for caraway, myristicin for parsley, carvacrol for oregano and linalool for coriander.

Results and discussion

Results on the essential oil content, as well as the contents of selected marker constituents are presented in Table 1. Simultaneously, normalised chromatograms (GC) of essential oils isolated from test mixtures and their constitutive herbal drugs are given in Figures 1 and 2.

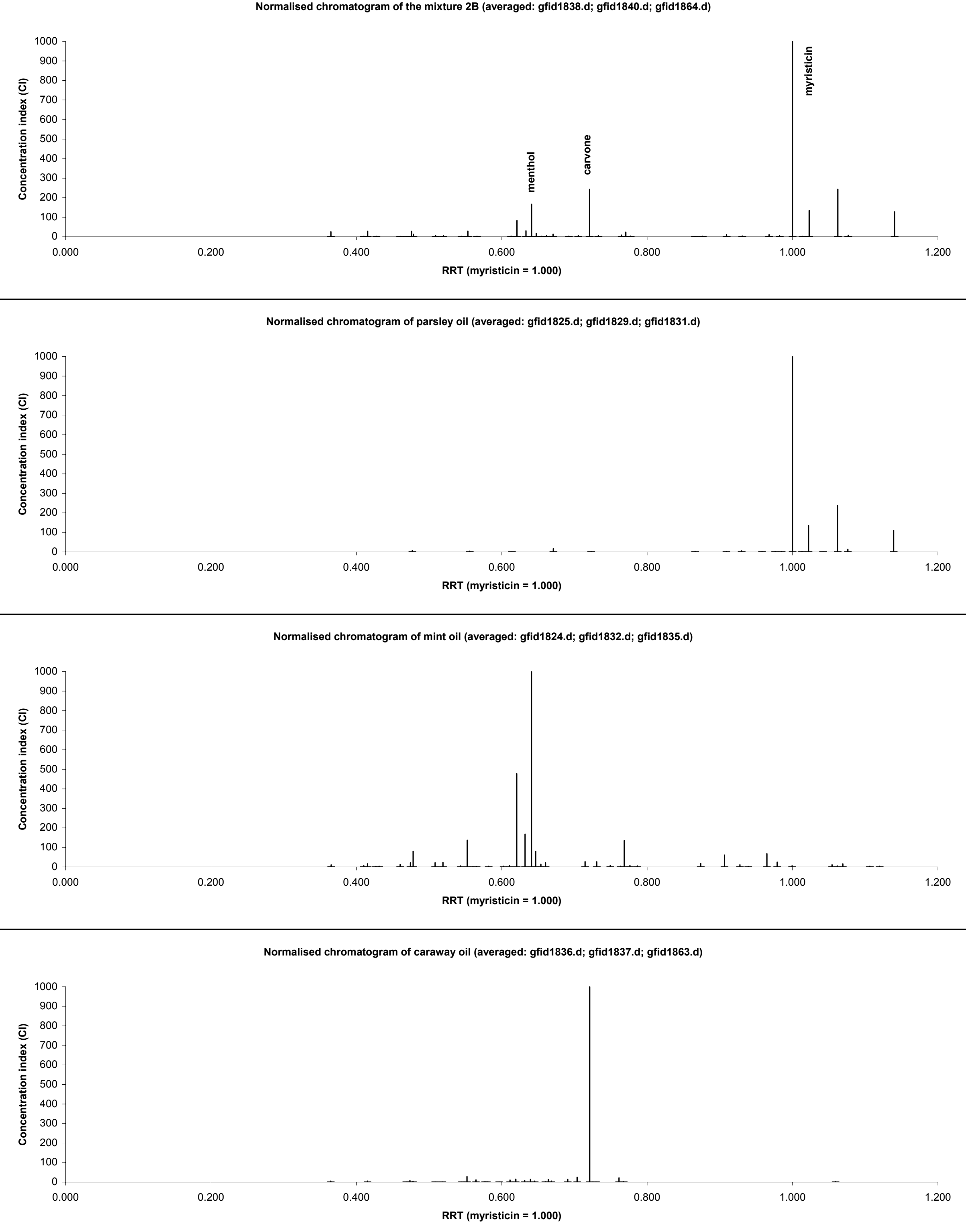

Fig. 1. Normalised chromatograms of the essential oils of mixture 2B and its constitutive aromatic herbal drugs (mint leaves, caraway seeds and parsley seeds)

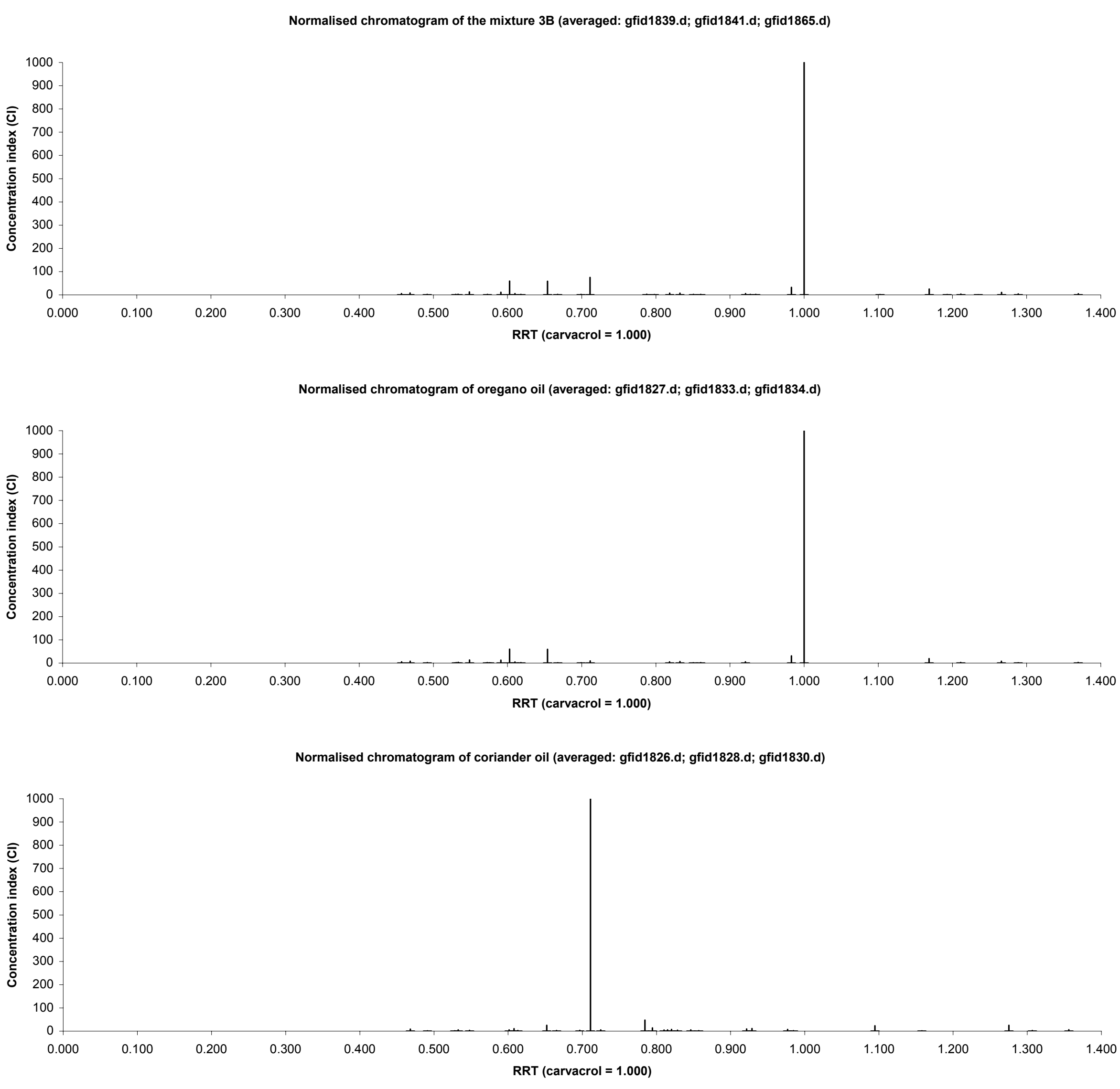

Fig. 2. Normalised chromatograms of the essential oils of mixture 3B and its constitutive aromatic herbal drugs (oregano leaves and coriander seeds)

Table 1. Average contents of essential oils and related marker constituents in test samples

Sample	Content of the oil (%)	Marker content (%)	Remark
Coriander	0.31	79.97	linalool
Parsley	0.64	64.60	myristicin
Mint	0.78	39.04	menthol
Oregano	3.20	78.83	carvacrol
Caraway	0.39	82.70	carvone
Mixture 2B	0.88	7.19 10.50 43.29	menthol carvone myristicin
Mixture 3B	1.52	5.53 73.49	linalool carvacrol

Tentative calculation procedure assumed following approximations: content of the essential oil in non-aromatic drugs coming into composition of herbal mixtures (*Frangulae cortex* and *Cynarae folium*) is zero (AP1), and relative density of all essential oils isolated from different single herbal drugs and mixtures is identical and equal to 1 (AP2).
Content of essential oils of single herbal drugs ($\%EO_x$) in the essential oils of herbal mixtures was calculated according to the equation [Eq.1], where M_m and M_x are concentrations of selected marker constituent (expressed in percents) in the oil from mixture and corresponding pure oil, respectively.

$$\%EO_x=100\cdot M_m/M_x \qquad [Eq.1]$$

Content (% m/m) of single herbal drugs (%DX) in appropriate mixtures could be calculated from the equations [Eq.2], or [Eq.2a], where $\%EO_m$ is the content of oil in herbal mixture and $\%ED_x$ - content of the oil in the single herbal drug (X).

$$\%DX=100\cdot(\%EO_m/\%ED_x)\cdot(M_m/M_x) \qquad [Eq.2], e.g.$$

$$\%DX=100\cdot(\%EO_m\cdot M_m)/(\%ED_x\cdot M_x) \qquad [Eq.2a]$$

Simultaneously, sum of contents of all constitutive herbal drugs in their mixture ($\%DX_1$, $\%DX_2$, ..., $\%DX_n$), should be 100, as is presented in equation [Eq.3].

$$\%DX_1+\%DX_2+ ...+\%DX_n=100 \qquad [Eq.3]$$

Table 2. Content of the oils of mint, caraway and parsley in mixture 2b essential oil

Constituents	Content (% m/m)	
	Expected*	Found**
Menthae piperitae aetheroleum	39.59	18.42
Carvi fructi aetheroleum	19.80	12.70
Petroselini fructi aetheroleum	40.61	67.01
Total:	100.00	98.13

*According to determined content of essential oil in single herbal drugs.
** According to calculation using equation [Eq.1] (uncorrected).

Table 3. Content of the oils of oregano and coriander in mixture 3b essential oil

Constituents	Content (% m/m)	
	Expected*	Found**
Origani aetheroleum	95.08	93.23
Coriandri fructi aetheroleum	4.92	6.91
Total:	100.00	100.14

*According to determined content of essential oil in single herbal drugs.
** According to calculation using equation [Eq.1] (uncorrected).

Table 4. Content of mint, caraway and parsley in mixture 2b

Constituents	Content (% m/m)		
	Declared	Found*	Found**
Frangulae cortex (pulvis)	35.00	-	36.49
Menthae pip. folium (pulvis)	20.00	20.77	9.30
Carvi fructus (pulvis)	20.00	28.64	12.83
Petroselini fructus (pulvis)	25.00	92.43	41.38
Total:	100.00	141.84	100.00

*EO_m is taken from Table 1 and %DX is calculated from [Eq.2], without correction in [Eq.3].
**EO_m is calculated as corrected (proportional) sum of constitutive oils and %DX is calculated from [Eq.2].

Table 5. Content of oregano and coriander in mixture 3b

Constituents	Content (% m/m)		
	Declared	Found*	Found**
Origani folium (pulvis)	55.00	44.28	53.93
Coriandri fructus (pulvis)	30.00	33.88	41.29
Cynarae folium (pulvis)	15.00	21.84	4.78
Total:	100.00	100.00	100.00

*EO_m is taken from Table 1 and %DX is calculated from equation [Eq.2].
**EO_m is calculated as corrected (proportional) sum of constitutive oils and %DX is calculated from [Eq.2].

Discussion about above presented results should take into account the aim of developed procedure, for quality control of herbal mixtures suggested for use in bakery industry in relatively low concentrations (up to 2-3%).
The first approximation in our calculation (AP1) assumes that non-aromatic drugs (*Frangulae cortex* and *Cynarae folium*) which come into composition of herbal mixtures do not contain essential oils at all. In opposite, certain deviation of obtained results to those expected, could be occurred. According to the second approximation (AP2), relative densities of all essential oils coming into account are identical and equal to 1, what is surely not true. Typical values for relative densities of oils taken into account ranging from a 0.900-0.916 for mint, 901-920 for caraway, 1.043-1.083 for parsley, 0.935-0.960 for oregano, and 0.862-0.878 for coriander, what means that density could vary (roughly) from 0.86-1.08, or about 20%. Subsequently, it is quite clear that AP2 itself could be the source of rather significant deviations.
Furthermore, procedure for isolation and determination of the essential oil of parsley (gravimetric), differed from that applied in the case of all other samples (volumetric). Because of nature of procedure applied in the case of parsley, there is a certain suspicion that composition of oil is somewhat changed, due to possible decrease of contents of low volatile constituents, during "drying" of oil.
Cause of deviations could be also found in non-uniformity of samples tested (first of all herbal mixtures), imprecision in standard procedure applied for isolation and quantification of essential oils, errors coming out from GC analyses, as well as poor selection of marker constituents.

Conclusions

Results obtained approved our assumption that this approach could be successfully applied in current quality control practice of such and similar products. However, certain precautions should be applied especially in the part dealing with the essential oil quantification and isolation.

Acknowledgements

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SOME ASPECTS CONCERNING THE USE OF CERTAIN TOXIC PLANTS IN PHYTOTHERAPY

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Summary

In the spontaneous flora of Romania, there are numerous species featuring phytoterapeutical properties.
Some of them are regarded as toxic and some their use in phytotherapy needs quite careful precautions, especially when they are empirically used.
Poisonous species could be used generally for infusion, decoct, tinctures, forms etc are being used either alone or alone or mixtures, but the quantity must be very few for avoid intoxication. The paper aims to present the most important herbs that could have toxic effect, the poisoning symptoms and their medicinal use.

Keywords: *Herbs, phytotherapy, toxic, intoxication.*

Introduction

In the worldwide and Romanian flora there are countless species belonging to different families having higher or lower toxicity degree (Dihoru Gh, 1984). Some herbs owe their phyto-therapeutic properties to certain substances that are giving a toxic character to the said species. This is mainly due to the presence of some alkaloids in their cells. The main toxic substances but being used in therapeutics are the glycosids and alkaloids. Nevertheless, numerous species are used in phyto-therapy, or in the industrial production of medicines.

Material and method

For achieving this work, they have been used Romanians and foreign references. Speciality literature has been reviewed to which the related experience of the authors is added. For the identification of the toxic species, from Romania and especially from Moldavia have been taken over the speciality literature bibliography from the systematic botanic field was used, then the phyto-therapeutic literature was consulted, thus establishing the therapeutic properties for each plant apart. The toxic species with healing properties have been identified in the literature that dwells on medicinal plants (Pharmacognosy, Phytotherapy etc) (Ciulei I, Grigorescu Em. StanescuUrsula 1993). Out of the various toxic species identified, the most known by people and having numerous and important uses both empiricaly and especially in the phyto-therapeutic industry have been selected. For all species presented, a brief description, the organs used and phyto-therapeutic properties are given. For some species, a brief description regarding the symptoms of poisoning and treatment means are presented. (Grig W, Podlech D., 1996)

Results and disscusion

***Atropa belladonna (LINN.)* – Belladonna -** Family*: Solanaceae*
Parts Used: Root, leaves, tops.
Habitat: Cultivated in England, France and North America. Widely distributed over Central and Southern Europe, Algeria and South Western Asia.
Bio-active substances: The root is the basis of the principal forms of use of Belladonna. The medicinal properties of Belladonna depend on the presence of Hyoscyamine and Atropine.

The total alkaloid existing in the root varies between 0.4 and 0.6 percent, but as much as 1 percent has been found, consisting of Hyoscyamine and its isomer Atropine, 0.1 to 0.6 percent; Belladonnine and occasionally, Atropamine. Starch and Atrosin, a red coloring principle, are also present in the root. Scopolamine (hyoscine) is also found in traces, as is a fluorescent substance similar to that found in horse-chestnut bark and widely distributed through the natural order Solanaceae. The greater portion of the alkaloid matter consists of Hyoscyamine, and it is possible that any Atropine found is produced during extraction. The amount of alkaloids present in the leaves varies somewhat in wild or cultivated plants, and depending on the methods of drying and storing adopted, as well as on the conditions of growth, soil, weather, etc. The proportion of the total alkaloid present in the dried leaves varies from 0.3 to 0.7 percent. The greater part consists of Hyoscyamine, the Atropine being produced during extraction, as in root. Belladonnine and Apotropine may also be formed during extraction from the drug.
Phyto-therapeutic action and Uses: Antispasmodic, diuretic, mydriatic, narcotic, sedative. Atropine, obtained during extraction, being its most important constituent on account of its power of dilating the pupil. Scarcely any operation on the eye can safely be performed without the aid of this valuable drug. It is a strong poison, the amount given internally being very minute, 1/200 to 1/100 grain. For its action on the circulation, it is given in the collapse of pneumonia, typhoid fever and other acute diseases. It is of value in acute sore throat, and relieves local inflammation and congestion. As an antidote to Opium, Atropine may be injected subcutaneously, and it has also been used in poisoning by Calabar bean and in Chloroform poisoning. It has no action on the voluntary muscles, but the nerve endings in involuntary muscles are paralyzed by large doses, the paralysis finally affecting the central nervous system, causing excitement and delirium. Small doses relieve cardiac palpitation, and the plaster is applied to the cardiac region for the same purpose, removing pain and distress.
It is a strong antispasmodic in intestinal colic and spasmodic asthma.
Forms of use: Powdered leaves, powdered root, fluid extract leaves, fluid extract root, tincture, alkaloid Atropine, alcoholic extract, green extract, juice, liniment, plaster, ointment.

***Adonis autumnalis, Adonis vernalis* - Hellebore, False -**Family: *Ranunculaceae*
It is a graceful plant, growing about a foot high, with finely cut leaves and terminal flowers like small scarlet buttercups.
Bio-active substances: A. vernalis contains a glucoside Adonidin and has an action almost exactly like that of digitalin, but is much stronger and it is said not to be cumulative. It appears to be about ten times as powerful as digitoxin.
Phyto-therapeutic action and Uses: It has been prescribed instead of digitalis, and sometimes succeeds where digitalis fails, especially where there is kidney disease. It is, however, less certainly beneficial in valvular disease than digitalis, and should be used only where digitalis fails. It produces vomiting and diarrhea more readily than digitalis. It is given under the form of infusion.
Forms of use: Fluid extract, glucoside adonidin, infusion.

***Aconitum napellus* (LINN.) - Aconite –** Family*:Ranunculaciae*
Habitat: From Himalayas through Europe to Great Britain. Lower mountain slopes of North portion of Eastern Hemisphere.
Bio-active substances: Aconite root contains from 0.3 to 1 per cent alkaloid matter, consisting of Aconitine - crystalline, acrid and highly toxic - with the alkaloids Benzaconine (Picraconitine), Aconine, Aconite acid, starch, etc. On incineration, the root yields about 3 per cent ash.

A. Napellus is the Aconite that contains the best alkaloid. All varieties of Aconite are useful, but this kind with the close set in helmet to the flower is the most valuable.
Phyto-therapeutic action and Uses: Anodyne, diuretic and diaphoretic. The value of Aconite as a medicine has been more fully realized in modern times, and it is now ranked as one of our most useful drugs. It is used much in homeopathy. Forms of use of Aconitic are employed for outward application locally to the skin to diminish the pain of neuralgia, lumbago and rheumatism. The official tincture taken internally diminishes the rate and force of the pulse in the early stages of fevers and slight local inflammations, such as feverish cold, laryngitis, first stages of pneumonia and erysipelas; it relieves the pain of neuralgia, pleurisy and aneurysm.
Forms of use: tincture liniment as such or mixed with chloroform.

***Bryonia dioica (LINN.)*- Bryony, White -** Family: *Cucurbitaceae*
Part Used: Root.
Habitat: It is of frequent occurrence in central and southern Europe.
Part Used: The root is collected in the autumn and used both in the fresh and dry state.
Phyto-therapeutic action and Uses: Irritating, hydragogue, and cathartic. Its chief use was as a hydragogue cathartic, but is now superseded by Jalap. Its use as a purgative has been discontinued as dangerous, on account of its powerful and highly irritant nature.
It has been used for cataplasms, and praised as a remedy for sciatica, rheumatism and lumbago.
It is still considered useful in small doses for cough, influenza, bronchitis and pneumonia, and has also been recommended for pleurisy and whooping-cough, relieving the pain and allaying the cough.
In case of poisoning by Bryony, the stomach must be evacuated and demulcent drinks given.
Forms of use: cataplasms

***Colchicum autumnale (LINN.)* - Saffron, Meadow- -**Family: *Liliaceae*
Habitat: Grows wild in meadows, especially on limestone.
Phyto-therapeutic action and Uses: The Colchicum is valued for its medicinal properties. The parts used are the root and seeds, these being anti-rheumatic, cathartic, and emetic. Its reputation rests largely upon its value in acute gouty and rheumatic complaints. It is mostly used in connection with some alkaline diuretic; also in pill form.
Bio-active substances: the active principle is said to be an alkaline substance of a very poisonous nature called Colchinine. It is acrid, sedative, and acts upon all the secreting organs, particularly the bowels and kidneys. It is apt to cause undue depression, and in large doses acts as an irritant poison.
Forms of use: Powdered root, extract, fluid extract (root), fluid extract (seed), tincture, wine, acetic solid extract.

***Datura stramonium (LINN.)* - Stramonium** – *Family:* Solanaceae
Habitat: Throughout the world, except the colder or Arctic regions.
Bio-active substances: Stramonium leaves contain the same alkaloids as Belladonna, but in thorn apple somewhat smaller proportion, the average of commercial samples. The alkaloid consists chiefly of hyoscyamine, associated with atropine and hyoscine (scopolamine), malic acid also being present. The Daturin formerly described as a constituent is now known to be a mixture of hyoscyamine and atropine. The leaves also yield 17 to 20 percent of ash, and are rich in potassium nitrate, to which, doubtless, part of the antispasmodic effects are due, and they contain also a trace of volatile oil, gum, resin, starch, and other unimportant substances.
Phyto-therapeutic action and Uses: Antispasmodic, anodyne and narcotic. Its properties are virtually those of hyoscyamine. It acts similarly to belladonna, though without constipating, and is used for purposes similar to those for which belladonna is employed, dilating the pupil

of the eyes in like manner. It is considered slightly more sedative to the central nervous system than is belladonna. Applied locally, in ointment, plasters or fomentation, Stramonium will palliate the pain of muscular rheumatism, neuralgia, and also pain due to hemorrhoids, fistula, abscesses and similar inflammation.
Forms of use and Dosages: Powdered leaves, fluid extract leaves, fluid extract seeds, tincture leaves, powdered extract, solid extract.

Digitalis purpurea (LINN.) - **Foxglove -** Family: Scrophulariaceae
Part Used: Leaves.
Habitat: Needing little soil, it is found often in the crevices of granite walls, as well as in dry hilly pastures, rocky places and by roadsides.
Bio-active substances: Digitalis contains four important glucosides of which three arecardiac stimulants. The most powerful is Digitoxin, an extremely poisonous and cumulative drug, insoluble in water, Digitalin, Digitalein, and Digitonin, which is a cardiac depressant, containing none of the physiological action peculiar to Digitalis, and is identical with Saponin, the chief constituent of Senega root. Other bio-active substances are volatile oil, fatty matter, starch, gum, sugar, etc. (Milică si colab. 2004)
Phyto-therapeutic action and Uses: Digitalis has been used from early times in heart cases. It increases the activity of all forms of muscle tissue, but more especially that of the heart and arterioles, the all-important property of the drug being its action on the circulation. The first consequence of its absorption is a contraction of the heart and arteries, causing a very high rise in the blood pressure. It has also been employed in the treatment of internal hemorrhage, in inflammatory diseases, in delirium tremens, in epilepsy, in acute mania and various other diseases, with real or supposed benefits. (SCHENK A., 1998) Digitalis is an excellent antidote in Aconite poisoning, given as a hypodermic injection.
Forms of use and Dosages: Tincture, Infusion, Powdered leaves, Fluid extract, Solid extract.

Euphorbia sp. - **Spurge,** ***Officinal*** **-**Family: *Euphorbiaceae*
Genera more than 200, species more than 3,000, representing almost all habits of growth and exhibiting a high degree of adaptability to varying environments. The genus Euphorbia comprises nearly a thousand species, and a large number of these species yield a milky juice. Some are herbaceous or shrubby, with or without leaves, the leafless varieties flourishing on African deserts like the cactus, having spiny stems.
Phyto-therapeutic action and Uses: For external use it is of service in chronic rheumatism and paralysis as a counter-irritant, alone, or combined with cantharides, merezeon bark, etc. It is a violent irritant and caustic poison.
Bio-active substances: The milky juice of the stem coagulates on exposure to air, forming a resinous mass which is generally marketed in form of tears. The chief constituent is resin, and it also contains bassorin, volatile oil, wax, potassium malate, lignin, calcium malate, and water, with no soluble gum. Another analysis gives euphorbo-resene, euphorbic acid, calcium malate, euphorbone, a very acrid substance not yet isolated and vegetable debris. The roots of some species contains oil, starch, glucose, and various salts, also resin.
Preparation: Tincture of Euphorbia, Fluid extract, Decoction. At the Cape, the capsules are used for destroying animals. It may produce delirium Compound Elixir of Euphorbia.

Papaver somniferum **(LINN.) - Poppy, White -**Family: Papaveraceae
Habitat: The Opium Poppy (*Papaver somniferum*, var. *album*) is indigenous to Asia Minor, and is cultivated largely in European and Asiatic Turkey, Persia, India and China for the production of Opium.
Bio-active substances: The most important bio-active substances of opium are the alkaloids, which constitute in good opium about one-fifth of the weight of the drug. No fewer than

twenty-one have been reported. The principal alkaloid, both as regards its medicinal importance, and the quantity in which it exists, is Morphine. Next to this, Narcotine and Codeine are of secondary importance. Among the numerous remaining alkaloids, amounting in all to about 1 per cent of the drug, are Thebaine, Narceine, Papaverine, Codamine and Rhoeadine. Meconic acid exists to the extent of about 5 per cent combined with morphine.
Phyto-therapeutic action and Uses: Hypnotic, sedative, astringent, expectorant, diaphoretic, antispasmodic. Opium is one of the most valuable of drugs, Morphine and Codeine, the two principal alkaloids, being largely used in medicine. It is unexcelled as a hypnotic and sedative, and is frequently administered to relieve pain and calm excitement. For its astringent properties, it is employed in diarrhea and dysentery, and on account of its expectorant, diaphoretic, sedative and antispasmodic properties, in certain forms of cough, etc.
Small doses of opium and morphine are nerve stimulants.
Forms of use: Morphine and Codeine, Syrup of Poppy, Syrup Papav. alba. Capsules, etc.

***Paris quadrifolia* (LINN.) - Paris, Herb -**Family: Trilliaceae
Habitat: Europe, Russian Asia, and fairly abundant in Britain, but confined to certain places.
Bio-active substances: A glucoside called Paradin.
Phyto-therapeutic action and Uses: Narcotic, in large doses producing nausea, vomiting, vertigo, delirium convulsions, profuse sweating and dry throat. The drug should be used with great caution; overdoses have proved fatal to children and poultry. In small doses it has been found of benefit in bronchitis; spasmodic coughs, rheumatism; relieves cramp, colic, and palpitation of the heart; the juice of the berries cures inflammation of the eyes. A cooling ointment is made from the seeds and the juice of the leaves for green wounds and for outward application for tumors and inflammations. It has been used as an aphrodisiac - the seeds and berries have something of the nature of opium. In Russia the leaves are prescribed for madness. The leaves and berries are more actively poisonous than the root.
Herb Paris is useful as an antidote against mercurial sublimate and arsenic.
Forms of use: A tincture is prepared from the fresh plant.

***Ta xus baccata* - Yew -** Family: *Taxaceae (Coniferae)*
Habitat: Europe, North Africa, Western Asia.
Bio-active substances: The most poisonous parts of the tree to be the fruit and seeds seem. An alkaloid taxine has been obtained from the seeds; this is a poisonous, white, crystalline powder, only slightly soluble in water; another principle, Milossin, has also been found.
Phyto-therapeutic action and Uses: The wood was formerly much valued in archery for the making of long bows. The wood is said to resist the action of water and is very hard, and, before the use of iron became general, was greatly valued. (In homeopathy a tincture of the young shoots and also of the berries is used in a variety of diseases: cystitis, eruptions, headache and neuralgia, dimness of vision, gout, rheumatism, affections of the heart and kidneys.
*Form of use: t*incture.

***Veratrum album* - Hellebore, White -** Family: *Lilaceae*
Parts Used: Rhizome, root.
Bio-active substances: Authorities differ as to the presence or absence of the veratria of cevadilla. It contains jervine, pseudo-jer-vine, rubijervine, veratralbine and veratrine. Cevadine is stated to be absent. There is fatty matter, composed of olein, stearin and a volatile acid, supergallate of Veratia, yellow colouring matter, starch ligneous matter, and gum; the ashes contain much phosphate and carbonate of lime, carbonate of potassa and some traces of silica, and sulfate of lime.

Phyto-therapeutic action and Uses: A violent, irritant poison. When snuffed up the nose it occasions profuse running of the nose; when swallowed, severe vomiting and profuse diarrhea. It was formerly used in cerebral affections, such as mania, epilepsy, etc., and for gout, as a substitute for colchicum. (Wichtl M. 1993)
Forms of use: The principal use of the plant is in veterinary medicine, powder, vinous tincture. In Romania is used to prepare a very active product for rheumatism (Boicil).
Other toxic species: *Aesculus hippocastanum (Castan salbatec), Asarum europaeum(Pochivnicum), Berberis vulgaris (Dracila), Canabis sativa (Cânepa) , Chelidonium majus Rostopasca) Convalaria majalis (Lăcrămioare), Consolida regalis (Nemţişori), Dryopteris fix-mas (Feriga), Hedera helix (Iedera), Hyosciamus niger (Maselarita), Iris pseudacorus,(Sanjenel de balta), Lycopodium clavatum (Pedicuta), Sambucus nigra (Soc), Scopolia carniolica (Mutulica), Solanum dulcamara (Lăsniciorul), Viburnum opulus (Călinul), Viscum album (Vâscul),*

Conclusions

- In the flora of Romania there are numerous toxic species having therapeutic properties as well.
- The toxic species are recommended to be used with much cautiousness as for their therapeutic properties and only by persons specialized in phyto-therapy.
- In is recommended the use of toxic species on therapeutic purpose only under medicine form (either in allopathic or homeopathic form) and only under careful surveillance of the doctor.
- In cases where certain species are also used in a traditional manner, it is desirable to be administrated as for external use.
- The waste resulted from the industrial processing of the toxic herbs must be carefully stored in specially arranged spaces.
- It is prohibited the disposal of the toxic herbs waste in empty spaces, outdoors, nearby waters or in low areas where water from precipitation might gather. Such situations can lead to the pollution of the running waters, various static waters and soil.
- Some

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THE FUNGAL AUTOCHTHONOUS BIOPREPARATION ERGO-1, A NEW POTENTIAL ANTITUMORAL CHEMOTHERAPEUTIC AGENT

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Summary:
The in vivo successive and interdependent investigations of the effect of an autochthonous fungal extract, Ergo 1, of ergolinic nature, upon the development of Guérin T-8 lymphotropic epithelioma and Walker 256 carcinosarcoma – highlighting the tumorsuppressor pharmacodynamic action, its reproducibility, the dose-effect relationship existence, its therapeutic significance comparatively with the antitumoral effectiveness of some standard cytostatics of clinical use – have revealed its antineoplastic chemotherapeutic property.

Keywords: *ergolinic biopreparation, solid experimental tumoral systems, appropriate pharmacodynamic models, qualitative and quantitative antitumoral evaluation indices, reference preclinical screening programs.*

Introduction

In the fight against cancerous diseases, chemotherapy holds pride of place, but it is still of small effectiveness, fact explained and by its negative impact on the normal cells of the organism under neoplasm aggression and by the development of a resistance phenomenon of the tumoral cells to the cytostatic drugs action.
The improvement of the oncochemotherapy effectiveness represents a major biomedical desideratum, which imposes the broadening of the chemicopharmaceutical researches, on the basis of the last information from molecular and cellular biology, structural and functional genomics, proteomics, and respectively, metabolomics, as well as pharmacogenomics and toxicogenomics (2, 7, 16, 20, 21). The main directions of investigations are: the discovery and design of new oncolytic agents that should specifically target the tumoral cells; the identification of new therapeutic ways of action upon carcinogenesis process; the conceiving of new strategies and programs of anticancerous chemotherapy; the use of different drug monithorized delivery and transport systems; the discovery of agents which can potentiate the antitumoral effect of the oncochemoterapeutic drugs (1, 2, 4 – 9, 18, 19).
In this context we can mentioned that the our previous *in vitro* investigations – performed in adequate cellular models – have pointed out the negative impact of an original ergolinic alkaloid biopreparation, Ergo 1, upon protein synthesis, cell proliferation and viability, membrane phenomena, metabolic events of the HeLa and HEp-2p tumoral cells, with inhibitory consequences on cellular cultures development (11, 15). These effects, which argued a significant *in vitro* cytostatic and/or cytotoxic property of this ergolinic extract Ergo 1, have imposed its investigation into the *in vivo* screening circuit, on animals bearing various experimental tumoral systems, for the preclinical pharmacological evaluation of the antineoplastic activity.
The present paper describes and examines the results of the successive and interdependent tests, on the animals with various experimentally induced tumoral systems, of the Ergo 1 effects upon the tumoral development process, for the qualitative and quantative pharmacodynamic assessment of the ergolinic biopreparation tumorsuppressor action.

Material and Methods

The original bioactive agent – used in the treatment of various experimental tumoral systems of solid type for the preclinical pharmacological evaluation of its oncostatic action – namely Ergo 1 – is an extract of ergolinic nature, which was specifically obtained from submerged cultures of *Claviceps purpurea* fungus, its pH being readjusted to 7.3.
The standard cytostatics, included in the reference experimental antitumoral therapy, were: methotrexate (MThX), cyclophosphamide (CPhS) and melphalan (MLPh).
The *in vivo* tests were performed on experimental models adequated to preclinical pharmacological trial, using white, female Wistar rats – pure line – of 125-150 g b.w, either with Guérin T-8 lymphotropic epithelioma or with Walker 256 carcinosarcoma. The animals were housed in individual cages having free access to water and standard food in a normal light/dark cycle and at 22^0 C ambient temperature.
The tumoral transplant was performed by the subcutaneous injecting of 0.2 ml of cell suspension, obtained by homogenizing cancerous tissue in saline solution containing streptomycin and penicillin (14).
The experimental antitumoral treatment – which consisted in daily intraperitoneal (i.p.) administration of the bioactive agents in various doses (µg or mg/kg. b. w.) – was initiated 24 hours after the tumoral transplant and was continued for 16 and 19 days, respectively, in the case of Guérin tumor and Walker 256 line, respectively. An equal volume of 0,9% saline solution was injected to the control animals.
The oncostatic impact was assessed by determining the rate of mean tumor regression (%M.T.R.), as well as by calculating the T/C ratio (where T = M.T.W. for the treated groups and C = M.T.W. for the control group) and the statistic significance by means of Student's “t” test (3, 10, 12, 13, 17).
The demonstration of the pharmacodynamic effect reproducibility has also involved the assessment of some specific indices: T/C x 100 value of the retests; the superior and inferior limits of the admissible variability range, established on the basis of the formulas T/C x 100 x 1.82 and T/C x 100 / 1.82 (the T/C x 100 value corresponds to the first test); the products of the T/C values obtained in the first two test and in all tests.
The qualitative and quantative pharmacodinamic evaluation of the Ergo 1 antitumoral activity has been performed by comparing the values of our evaluation indices with those imposed by the selection criteria for cytostatic agents. Those criteria were established by the preclinical screening programs of the National Cancer Institute in the U.S.A. (3, 13), and of the Institute for Microbiology and Experimental Therapy in Germany (12) for the preliminary steps of pharmacological preclinical evaluation.

Results and Discussions

It can be seen – from the results included in Table 1 – that the i.p. daily therapeutical administration of the bioactive Ergo 1 agent to the rats bearing one of the solid tumours induced a significant decrease of the mean tumoral weight by comparison with the control.

Table 1. Antineoplastic effect of the Ergo 1 biopreparation, administered daily i.p., in a dose of 50 µg/kg b.w., to the rats with Guérin T-8 lymphotropic epithelioma or Walker 256 carcinosarcoma. Figures in brackets indicate the number of experimental animals.

Group/ Treatment	Guérin T-8				Walker 256			
	M.T.W. (g) X ± S. E.	% M.T.R	T/C value	p	M.T.W (g) X ± S.E.	% M.T.R.	T/C value	p
Control	28.9 ± 3.9 (15)	–	–	–	19.6 ± 2.9(15)	–	–	–
Ergo 1	14.2 ± 3.1 (10)	50.9	0.49	<0.01	11.7 ± 2.7(10)	40.3	0.59	NS

The reference values of the evaluation indices, established by the german and american screening programs for a first testing step are: a M.T.R. of minimum 35%. (12); a T/C value of 0.54 – 0.64 (3, 13), registered in at least one solid tumoral system.

The comparative analysis of our evaluation indices values (M.T.R. 50.9% and 40.3%, respectively; T/C ratio 0.49 and 0.59, respectively, corresponding to Guérin T-8 and Walker 256 tumour, respectively**)** with the standard ones, has revealed that the ergolinic treatment has induced an inhibitory effect upon the tumoral development process, in comparison with the control groups, this antitumoral pharmacodynamic action having a different intensity of the effect in relation to the type of experimental tumour.

The higher amplitudes of the induced mean tumoral regressions, than the stipulated minimum level (35.0%), have imposed thoroughgoing research in order to demonstrate the reproducibility and stability of the oncostatic property of this biosynthetic preparation. Thus, we have performed a series of three successive tests in identical experimental conditions with those of the primary testing, which revealed its *in vivo* cytostatic property (Table 2).

Thus, in the initial experiment on the lymphotropic epithelioma and respectively on the carcinosarcoma, one can notice that therapy with Ergo 1 has induced significant decreases of M.T.W., this antitumoral activity being expressed both by the M.T.R. rate (53.2% and respectively 41.5%) and by the T/C value (0.47 and respectively 0.58). The T/C x 100 values of the opening test were 47% and respectively 58%. These values were necessary in order to define the admissible variability ranges. Their upper and lower limits are 85.5% or 105.6% and 25.8% or 31.9%, in the case of the Guerin T-8 or Walker 256 tumors.

The values of evaluation indices in the first retesting confirm the significant antitumoral potential of Ergo 1, its T/C x 100 value being 48% or 56%.

The T/C values of the first two experiments allow us to estimate a product of 0.22 or 0.32.

Table 2. Successive testing of the daily antitumoral therapy with Ergo 1 ergolinic agent (50 µg/kg b.w./i.p.) in rats bearing Guérin T-8 lymphotropic epithelioma and respectively Walker 256 carcinosarcoma. Figures in brackets indicate the number of experimental animals.

Group/ Treatment	Guérin T-8				Walker 256			
	M.T.W. (g) X ± S.E.	% M.T.R.	T/C value	p	M.T.W (g) X ± S. E.	% M.T.R.	T/C value	p
Control	23.5 ± 3.2(10)	–	–	–	20.5 ± 2.3(10)	–	–	–
Ergo 1	11.0 ± 2.3 (7))	53.2	0.47	<0.01	12.0 ± 1.9 (7))	41.5	0.58	<0.02
Control	26.3 ± 2.9(10)	–	–	–	18.7 ± 2.0(10)	–	–	–
Ergo 1	12.6 ± 2.1 (7)	52.1	0.48	<0.002	10.5 ± 1.4 (7)	43.9	0.56	<0.01
Control	28.5 ± 3.2(10)	–	–	–	21.3 ± 3.0(10)	–	–	–
Ergo 1	13.0 ± 1.9 (7)	54.4	0.46	<0.001	12.1 ± 2.6 (7)	43.2	0.57	<0.05

Finally, the second retesting has also pointed to the anticancerous action of Ergo 1 bioactive agent, the evaluation indices (M.T.R. = 54.4% or 43.2%; T/C ratio = 0.46 or 0.57) being close to those in the previous experiments. In that case the T/C x 100 values were: 46% or 57%.

The product of the T/C values in the three successive tests is 0.10, in the case of the Guerin T-8, or 0.18, in the case of the Walker 256.
The chemotherapeutic programs of *in vivo* preclinical screening – used for the analysis and interpretation of the significance of the obtained data – have established specific criteria for assessing the reproducibility and stability of the induced antitumoral action. Thus, for this step, the German program (12) requires closed M.T.R. values, and the American screening (3,13) imposes, on the one hand, the framing of the T/C x 100 values of the second and third retests in the admissible variability range, estimated on the basis of the first retest T/C value, and the other hand, the value proximity of the successive T/C products – obtained by multiplying the T/C ratios of the first two tests (0.20 – 0.24) as well as of all three tests (0.08 – 0.09) – with those of reference
In the light of the above standard evaluation indices – established for the proving the reproducible character, of the pharmacodynamic effect – it can be observed: the value proximity of the induced mean tumoral regressions; the framing of the retests T/C x 100 values between the lower and upper limits of the corresponding admissible variability ranges; the concordance of the T/C products – estimated by multiplying the T/C ratios, both of the first two tests, and of all three tests – with the standard values.
This comparative analysis of our values with those stipulated by the preclinical screening programs, for the first and second steps of the qualitative pharmacodynamic evaluation, certifies the antineoplastic property of the Ergo 1 ergolinic extract and its reproducible and stable character. Thus, has been also created the premises for the establishment of the oncochemotherapeutic effectiveness significance of the ergolinic biopreparation by the quantitative evaluation of this pharmacodynamic action.
The interference of daily antitumoral therapy, performed by administration of Ergo 1 different doses, with the development process of Guérin T-8 lymphotropic epithelioma and respectively Walker 256 carcinosarcoma, can be followed from Table 3.

Table 3 Experimental oncochemotherapeutic potential of various doses of Ergo 1 ergolinic biopreparation (µg/kg b.w./day) upon Guérin T-8 lymphotropic epithelioma and Walker 256 carcinosarcoma. Figures in brackets indicate the number of experimental animals.

Group/ treatment	Guérin T-8				Walker 256			
	M.T.W. (g) X±S.E.	% M.T.R.	T/C Value	Statistical significance	M.T.W. (g) X±S.E.	% M.T.R.	T/C Value	Statistical significance
Control	28.9 ± 3.9 (12)	-	-	-	19.6 ± 2.4 (12)	-	-	-
25 µg/ kg b.w.	21.2 ± 3.3 (8)	26.7	0.73	NS	15.0 ± 2.5 (8)	23.5	0.76	N.S.
50 µg/ kg b.w.	14.2 ± 3.1 (8)	50.9	0.49	<0.01	11.7 ± 2.0 (8)	40.3	0.59	=0.02
100 µg/ kg b.w.	10.5 ± 4.1 (8)	63.7	0.36	<0.01	8.8 ± 1.8 (8)	55.1	0.45	<0.002
150 µg/ kg b.w.	8.7 ± 2.7 (8)	69.9	0.30	<0.001	6.9 ± 2.1 (8)	64.8	0.35	<0.001

One can observe that the differentiated antitumoral treatment of the rats bearing lymphotropic epithelioma or carcinosarcoma with Ergo 1 was followed by progressive and significant decreases of M.T.W as compared with the control value. The dynamic of the M.T.R. and T/C values has highlighted that the experimental manipulation of the therapeutical doses – in the sense of the progressive increase of the dose – was correlated with an optimization of the antitumoral effectiveness. Therefore, we appreciate that the ergolinic anticancerous treatment has inhibited the development of tumors in relation to the dose employed.
Appreciation of the results, obtained in the first stage of the quantitative evaluation of the antitumoral effect requires their analysis according to the stipulations of the reference screening programs imposed for this preclinical investigation stage. According to the German and American programs, the dose-response relationship is confirmed if: M.T.R. values have progressively increased in relation to the raising of the therapeutic dose; at least one of the

T/C ratios, obtained after the dose differentiated treatment, is within the limits of the admitted range (0.42-0.54).
In the light of the above criteria we conclude the existence of a dose-response relationship, which allows the improvement of the tumorsuppressor potential by the experimental manipulation of the therapeutical doses. This criterion for the estimation of the therapeutical effectiveness of the studied agent has conditioned the evaluation of the experimental antitumoral efficacy of the ergolinic compund in the context of the experimental pharmacodynamic potential of some standard oncochemotherapeutic agents upon carcinogenesis in laboratory conditions.
Table 4 includes the evaluation indices values of the antitumoral impact induced by Ergo 1, methotrexate, melphalan and cyclophosphamide, respectively, on the development of the solid Guérin T-8 and Walker 256 tumor .

Table 4. The antitumoral effect of the experimental ergolinic treatment, compared with that of some standard cytostatics, upon lymphotropic epithelioma and Walker 256. Figures in brackets indicate the therapeutic doses (µg or mg/kg b.w./daily) and the number of experimental animals, respectively.

Group/ treatment	Guérin T-8				Walker 256			
	M.T.W. (g) X±S.E.	% M.T.R.	T/C Value	p	M.T.W. (g) X±S.E.	% M.T.R.	T/C Value	p
Control	27.5 ± 3.5 (12)	–	-	–	20.3 ± 2.7 (12)	-	-	-
Ergo 1 (50µg)	13.3 ± 2.8 (8)	51.7	0.48	<0.01	11.7 ± 1.9 (8)	42.4	0.57	<0.01
MThX(0,15mg)	14.5 ± 2.5 (8)	47.3	0.53	<0.01	4.7 ± 1.6 (8)	76.9	0.23	<0.001
MLPh (0,30mg)	13.6 ± 2.7 (8)	50.6	0.49	<0.01	13.5 ± 1.8 (8)	33.5	0.66	<0.05
CPhS (1,6mg)	11.8 ± 2.1 (8)	57.1	0.43	<0.002	16.0 ± 2.0 (8)	21.2	0.79	NS

Here are the effects of the antitumoral treatment compared with the data in the control group: Ergo 1 agent has significantly inhibited ($p<0.01$) the evolution of the experimental tumoral systems; this tumorsuppressor effect being expressed by characteristic M.T.W., M.T.R. and T/C values; methotrexate, melphalan and respectively cylophosphamide have induced a significant cancerostatic effect, this being illustrated by corresponding M.T.W., M.T.R. and T/C values.

The experimental results obtained in this preclinical screening stage enables the assessment of the antitumoral effectiveness of Ergo 1 agent in comparison with that of the standard cytostatic agents. The comparative analysis of evaluation indices values of the anticancerous activity reveals a significant experimental therapeutic effectiveness of the autochthonous alkaloid ergolinic agent. This is close as compared with the antitumoral potential of the methotrexate, melphalan and cyclophosphamide.

The possibility to improve the cytostatic effectiveness by manipulating the therapeutical doses, as well as the antitumoral potential significance of the Ergo 1 agent, established by comparison with the standard oncostatic drugs, have made possible the quantitative evaluation of its antineoplastic pharmacodynamic effect.

Conclusions

The entire set of experiments, performed on models adequated to qualitative pharmacological evaluation, have provided a bulk of results which highlights the antitumoral pharmacodynamic property of the Ergo 1 autochthonous ergolinic biopreparation and its reproducible and stable character.

Testing of the alkaloid ergolinic bioproduct effect upon the Guérin T-8 lymphotropic epithelioma and Walker 256 carcinosarcoma development has revealed a directly proportional correlation between the therapeutical dose and the antineoplastic potential of the Ergo 1 agent.

The comparative analysis of the experimental antitumoral impact of the fungal ergolinic extract and of some standard cytostatics, respectively, was relevant for the appreciation of a significant oncostatic effectiveness of this natural bioactive ergolinic biopreparation.

The positive answers obtained to these major problems of the preclinical quantitative pharmacodynamics evaluation require further toxicology and cell-molecular biology investigations for the assessment of the ergolinic chemotherapy biocompatibility and for establishment of the Ergo 1 action mechanisms at cellular, subcellular and molecular level in the expression of the overall pharmacodynamic effect.

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THE AYURVEDIC PHYTOIATRY AND ITS LONG WAY, FROM THE VEDDIC CIVILIZATION TO THE ACTUAL SCIENTIFIC ACHIEVMENTS

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Summary

*This proceeding is a presentation of the sofiatric ayurvedic conception, as well theoretical as praxiological, in its hypostasis of medical expression of the high culture of the veddic history. As "**science of life**", **Ayurveda** is marked from its start by the empirical traditional knowledge about the botanical medicinal patrimony of India and by the works of some outstanding scholars , and it became a genuine scientific domain of medicine, as a result of the manysided accomplishments of the medical phytoiatric indian school, in the last decades, having as slogan: "**The medicine based on facts, the single possible and acceptable**". Using the knowledge accumulated during several milleniums about the indian medicinal species of plants, the indian phytoiatry developed an exhaustive phytotechnical, phytochemical, phytopharmaceutical research, and also a phytopharmacological, experimental and clinical too.*

Keywords: *Ayurveda, sophiatry, phytoiatry, indian iatrobotany, academic phytoiatric research.*

Foreword

The use of medicinal plants is one of the eldest medical practices of traditional empirical medicine. Owing its folkloric characteristics, this practice has been called also ethnoiatry, iatrofolklore and when we refer only to the botanical compound of this practice iatro-ethno-botany.
Everywhere in the world the iatro-ethno-botanical remedies were the unique really efficient possibility to heal or at least to alleviate the sufferings produced by desease or other injury, of course inside the limits of the possibilities of the time's knowledge and means.
Phytoiatric practices were contemporary also with other non medical practices but with "medical" assumption, exercised by magicians, sorcerers, various impostors so called "healers" (unfortunately, nowadays, such imposture occurs frequently, everywhere in the world bringing much harm). The phytoiatry, while almost empirical, followed a successful continuous way, even in its ancestral age. In India phytoiatry developed in the frame of the bright *veddic* civilization. (1), (3) Present even in the previous civilization belonging to *Mohenjo Daro* and *Harappa* periods, phytoiatry in India appears also in the *Rikveda*, but flourishes theoretically and praxiologically in the last period: *Vedanta*, of the veddic era, under the name of **Ayurveda**, what means in sanscritic language **"the science of life"**. So that the philosophical and cultural trends evolved in conjunction with medical trends of ayurvedic phytoiatry; so, during the thousands of years of existence, the phytoiatry in India accumulated a huge experience fertilized by a high, lofty thinking.
Illustrious scientists were involved in ayurvedic science, before Jesus Christ, and after too, namely ***Caraka*** and later ***Susrutra***. The actual academic back-ground, is in India the most manysided developed and scientifically directed system in favour of the traditional medicine. Nowadays, owing the fact that the chimiosynthetic therapies induce very often many adverse, sometimes even deleterious side effects, the acute and subacute are well known, the chronical less, many medical specialists scientists, as well as practitioners focus their attention towards the phytomedicine.

Many initiatives involved in the medical and pharmaceutical fields, show a big continually increasing interest towards the phytomedicinal resources and methods, and their more and more extensive use, so that it is worth to affirm, that besides their application in complementarity with synthetic or half-synthetic pharmaceuticals, in some diseases – following the physician's indications – the phytoiatric remedies could be applied, in monotherapic longterm therapy, of course on the back-ground of a very careful judgement.
The problem of scientific registration of the side effects of various pharmaceutical drugs was very well monitorized by a national pharmacovigilence commission and its journal, of course "*mutatis mutandis*" the activity of this valuable commission, that has to be revived, can extend also to phytoiatric remedies. Never less it is important to retain that the botanical medical resources are not – in a scientific based conception – a substitute of the chemical synthetic medical means, but an ally in the fight against diseases, for sanogenesis, this being the endeavour: an integrative medicine.
This endeavour concerns ayurvedic phytomedicine too, as well as the ayurvedic products belonging to the **"Star"** manysided programme of **The Ayurvedic Medical Centre** in Bucharest, this short writing presents in the following pages the wide field of application of these products, in the integrative thinking and practicing, of modern medicine.

From the history premises to actuality…

An important aspect demonstrated by the history of the botanical medicine and pharmacy is the fact, that both evolved from a traditional concept, in theory and practice, to a contemporary academic condition; therefore the botanical medicine became in the actual trend of the medical sciences an entity in the family of medical specialities: in the pharmaceutical specialities this hypostasis being since a longtime a well known fact, namely, the existence in the didactic disciplines teached in the pharmaceutical schools of the pharmacognosy as well as the important place in the pharmaceutical research, of the botanical medicinal resources. It is actually possible therefore, to consider the botanical based medicine, as a distinct domain: the phytoiatry (from the greek "phyton" = plant, and "iatreia" = medicine).
The ayurvedic phytoiatric's history is characteristic for the evolution of this so old kind of medicine and in the same time so compatible with actual progresses in biology, pharmaceutics, medicine. The parallel history of phytoiatric and "classical" medicine demonstrates that already since the antiquity, the two domains were closely related, an example being the phytoiatric works of Theophrastos and the very sophisticated and complex medicament: the "*Theriac*" considered a genuine panacea. It seems that the *Theriac* was conceived by the personal physician of Nero Emperor, Andromacus, from 70 ingredients (following other opinions the author of the *Theriac* could be Mitridates – King of Pont). The first edition of the "Pharmacopea Germanica" mentions the ingredients of the *Theriac*, a big number of them belonging to botanical inventory. Herewith follws a list of the above mentioned sources, concerning the compounds of *Theriac*: "1 teil Opium, 3 teilen Spanisher wein, 6 teilen Angelikawurze, 4 teilen radix Serpentariae, 2 teilen Baldrian wurzel, 2 teilen Meerzwiebel, 2 teilen Zitwer wurzel, 2 teilen Zimt, 1 teil Kardamom, 1 teil Myrrhe, 1 teil Eisenvitriol (SO4Fe?), 72 teilen Gereinigtemhonig". The first professor of pharmacnosy from the University of Padua follwed very soon, in 1540, in Germany by Valerius Gordus from the University of Wittenberg. The Portuguese physician Garcia de Orta that lived and carried out his research in India, named in Goa, elaborated in 1560 a very interesting, probably the first work with this target, about the Indian medicinal flora and remedies, belonging to the ayurvedic traditional botanical patrimony too; pharmacognosy is defined also as a scientific field concerned with the study and use of remedies with zoological origin, like for instance the ayurvedic *Coral bhasm* and *Coral pisti*, present in the "**Star" Programme** remedies, like:

Potent Power and **Kamaiany**, coming from the corals from Indo-Pacific areas (7) very rich in calcium compounds and other minerals easily assimilable.
Therefore the expectations of the medical staff and of so many patients, that have already benefited and are susceptible to have in the future too, the benefit of the ayurvedic phytoiatry, have to be informed and helped by the specialists that themselves need an access to all the theoretical and practical principles and methods of the ayurvedic phytomedicine, this being the main purpose of this writing, elaborated as a document of "**Star" Programme**.
In Romania the ayurvedic phytomedicine, as a medicine based only on scientifically demonstrated facts, was introduced by **The Ayurvedic Medical Centre** (**A.M.C.**) in Bucharest since an important number of years. **A.M.C.** brought in Romania, only the ayurvedic phytoremedies already well tested in India as well experimentally and evaluated clinically in the specialized clinics of this country, as already registered in the Indian pharmacopoeias. About all these trends the following pages will inform the reader.

THE AYURVEDIC PHYTOMEDICINE AND IT'S RELATIVES, SIDDHA AND UNANI MEDICINES, FROM TRADITION TO CONTEMPORARY SCIENTIFIC BACK-GROUND

The flourishing civilization that evolved in India after the establishment of the indo-european vedic tribes reverberated not only in the social, economic, artistic fields, but also in the scientific, including medical sciences; probably also during the former civilizations: Mohenjo-Daro and Harappa, a medical use of the plants from India, of the sub-continent having an area of 3,287,782 km2, occured.
The big variety of climates, geomorphology and soil structures of India correspond to a big variety of the composition of the Indian flora.
The vedic culture in the medical field, especially in the last period of the vedic era, the Vedanta, generated the ayurvedic medicine that had as back-ground an existential philosophy, namely a healthy relation of the human being with environment (the natural and the social too) as well from physical point of view. While the ayurvedic medicine (in Sanskrit language, **Ayurveda** means ***"the science of life"***) deals with various methods, physiotherapy, aromatheraphy, the ayurvedic phytotherapy using the very rich botanical treasure of the medicinal species of plants from India, accomplished a perennial successful work since about 5000 years, accumulating a very valuable experience. The empirism of the younger history of Ayurveda brought in modern times the knowledge of it's practical accomplishments, validated by the scientific contemporary research – chemical, medical – that observated by evidencing the bioactive molecules anabolised, in practical, all the organs of the ayurvedic medicinal plants, the importance of these plants for health, demonstrating by experimental research their positive effects, and in this frame, by clinical application and conclusive appreciation the prophylactic and therapeutic opportunities the ayurvedic phytotheraphy offers. Therefore it was possible for the ayurvedic products, native or at a higher phytopharmacological level, as remedies (processed from the ayurvedic species of plants) to penetrate in modern medicine as an important compound of the entity of medical sciences, the phytoiatry.
Before presenting the contemporary trend of ayurvedic phytoiatry, it is worth to retrospect the first steps of the ayurvedic phytoiatric history.
As everywhere in the world, the beginning of the ayurvedic phytoiatry was linked with religious rituals, magic ceremonial, sorcery, folkloric, phytoiatric knowledge, named also etnoiatry, hygienic traditional empiric habits where medicinal plants find always a place with ablutions (very much practiced before vedic era in Harappa civilization; however in India the

vedic scholars, of course "illo tempore", at the level of their life times gave like scolars from ancient Greece and Rome an academic-like content to the ayurvedic concepts and practice.
Two examples are in this order of ideas relevant: the physician, scientist - Caraka (Tşaraka) – and philosopher personal physician of the Kushan sovereign Kanishka (1-2 century), a bright protector of culture and science; this circumstance offered him the opportunity to study beside other domains of ayurvedic botanical treasure and to put in practice this knowledge in the 8 volumes of his works: *Caraka Samhita*, much later one of his disciples the Indian outstanding physician Susruta elaborated a magistral medical ayurvedic monograph, basic opus for many years of the Indian medical sciences: *Susruta Samhita*. This magistral work of Susruta, called also "Materia Medica", comprises 37 chapters and mentions and describes 700 remedies of ayurvedic species of medicinal plants.
From the conceptual point of view, the Ayurveda considers in a holistic kind of thinking, the humoral compounds of the organism, the "*tridosha*" as the "*nervum rerum*" of life comprising the movement the dynamic humoral factor, the "*vata dosha*", the "*pitta dosha*" that suggests what was called later the metabolism and the integrative systems. It is possible to identify some common peculiarities with the Hippocratic concepts and also with the traditional Chinese concepts in the medical field that attributes an important pathogenic role to the disbalance in the energetic state of the organism.
This back-ground of ayurvedic medicine partially traditional, partially conceptual, allowed it's maintenance and it's vigorous survival during the many changes in the medical kind of thinking and acting, in India and everywhere while some other complementary medical methods did not resist in time and a lot of them are actually subjected to controversial considerations. The junction of traditionalindian ethnoiatry, ayuvedic phytoiatry and the academic actual ayurvedic medicine in India and throughout the whole world was possible just because Ayurveda has accumulated a huge millenary thoughtful history of Indian phylosiphy and scientific axiology that, of course, in the limits of it's epoch was a high, a lofty, lighting guide towards truth, an truth in action .
Actually in India, the ayurvedic medicine has a very extensive system of scientific research, medical assistance high leveled teaching institutions whose slogan is: ***"The only possible and acceptable medicine is the medicine based on facts, science and worship for deontological ideas!"***
The **Unani** medicine was introduced in India by the arab civilization, via Persia, and brought some influences of the hellenic antiquity, more exactly the hippcratic scientific thinking. **Unani** flourished in India, toghether with islamic philosophy, in the period of The Mogul Empire, but is still an interesting actual practice and medical kind of thinking.
In **Unani** as well as in **Siddha**, medical fields, the phytoiatrical domain has an important place and in the next future, the **"Star" Programme** will develop in Romania, also **Siddha** and **Unani** activities.

GENERAL PRINCIPLES OF THEORETICAL AND PRAXIOLOGICAL AYURVEDIC MEDICAL ASSISTANCE

Any ayurvedic practioner has – before any diagnosis and before prescribing any treatment – to be completely aware about patient's internal and environmental status, that means the patient's condition in all it's morphophysiological systems, the holistic understanding of relations between all these systems and especially about their balance or disbalance, in the spirit of the tridosha philosophy, adapted of course to the modern trends of medicine, the same kind of thinking has to be applied to the patient's relations with his ecological system as a compound of this system. The Indian philosophy of Panoha Mahabootas, of course in contempory version. The ayurvedic practitioner has to identify the belonging of the patient

(more or less pronounced “uttered”) to such a structural psychic and biological, structural type and expectable activity, of course such an anamnestic record is necessary for any other kind of compulsory owing the basis philosophical ayurvedic thinking that constitutes the conceptual and logic infrastructure of ayurvedic medicine.
The harmony normal back-ground of health or disharmony as mainsource of disease of the patient’s state is in the above mentioned order of ideas, an important target of the anamnestic check-up carried-out by the ayurvedic medicine practitioner.
Actually, the ayurvedic medicine works in the following eight directions:
- general medicine;
- oto-rhino-laringology & oftalmology & stomatology;
- geriatrics and biostimulating and psychostimulating medicine;
- obstretics, paediatrics;
- sexology and sexual deficiencies with various origins;
- surgery;
- toxicology.

All this branches of the ayurvedic medicine are known in ayurvedic scientific semantics under the name of ayurvedic origin: Ashtanga, having a thoughtful semantics.
It could seem surprising (even, may be, astonishing) the fact that the ayurvedic phytoiatry is not mentioned like a special branch of ayurvedic medicine, but if we analyse carefully, why this omission it’s reason becomes obvious, namely, because the ayurvedic phymedicine is applicable and applied in all the eight classical branches of ayurvedic medicine so that the omission is only apparent. However the fields of medicine, the medical specialities where ayurvedic medicine is applicable, became more and more numerous, whereas a chapter of this writing concerns this many sided possibilities of ayurvedic medicine based on the iatrobotanical wealth of India.

Table I. The Ayurvedic Institutions in India (short overview – adapted by the authors)

Name	Functions	Remarks	Conected units
Regulatory Council	- ayurvedic education - deontology of medical ayurvedic practice - maintains the code of conduct in ethics and deontology	Institution settled by an Act of The Indian Parliament	Ministry for Health and Family – Department for Traditional Medicine
Back-ground of Ayurveda and similar fields	- ayurvedic teaching activities and their infrastructure	Universities with ayurvedic involvment: - Banars Nindu - Gujarat Ayurveda - The National Institute of Ayurveda	- 194 undergraduate colleges (7200 capacity) - 55 postgraduate colleges - 2.258 hospitals - 14.416 dispensaries
Pharmacopoeial standards	- on the back-ground of the: Drugs and Cosmetic Acts and Rules	Related and cowork fields with: - Pharmacopoeial Laboratory for Indian Medicine - manufacturing of Drugs Regulation system in India	- The system for integra- tion of Ayurveda, with modern concepts and practice - The Indian Medicine Pharmaceutical Corpora- tion. - 4 important

			phytomedical Journals
Central Council for Research in Ayurveda and Siddha	- basic, clinical, drug documentary, family welfare research - editorial activities	- 80 monographs and proceeding volumes - Obtenance of numero- us patents for isolation and identification of bioactive molecules extracted from indian ayurvedic plants	

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CONSIDERATIONS CONCERNING THE MANYSIDED TARGETS OF THE "STAR" AYURVEDIC PROGRAMME

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Summary

*The build up of the Ayurvedic Medical Center in Romania was followed soon by the establishment of a multidisciplinary and manysided targeted "**Star**" phytoiatric ayurvedic programme whose endeavour was, from the beginning, based on the promotion of several domains.*

Keywords: *"Star" programme, information, education, prophylaxy, therapy.*

Foreword

The ayurvedic phytoiatry developed since a long time in Romania, as a result of the settlement in Bucharest of The Ayurvedic Medical Centre (A.M.C.) the creator of this medical establishment, *Dr. Shawki Salman*, passioned since his childhood, for the "green pharmacy" of the nature; namely the phytomedicine or iatrobotany, was amazed especially by so rich and diversified medicinal flora of India, that he studied in the huge indian country, actively participating at important scientific events, one of the most important beeing The Phytoiatric Congress in Cochin (Southern indian, Kerala). Funding during his universitarian studies a family in Romania, he became also the founder of the A.M.C. and elaborated the manysided phytoiatric "Star" Programme.

CONSIDERATIONS CONCERNING THE MANYSIDED TARGETS OF THE "STAR" AYURVEDIC PROGRAMME

Methods and targets of the "Star" Programme

In Romania the ayurvedic phytoiatriy penetraded since a long time, the company **A.M.C.** (2), (7) from Bucharest is pioneer in this domain: it introduced many phyto-remedies since a long time already successfully experienced in India; in basic and applied research research, in experimental "*in vivo*" and "*in vitro*" tests as well as in ambulatory and clinical medical assistance.
A.M.C. has acknowledged its offer in the medical and pharmaceutical circles and to any body to whom it may concern including the mass-media (press, radio, television, conferences for the wide public, courses for physicians etc.), proceedings in the frame of symposiums, congresses, other scientific events too, exhibitions.
A.M.C. obtained the legal approval from **The Ministry of Health** for the use of it's offer in Romania and initiated also a clinical research with the universitarian staff in the frame of a clinic of psychiatry, belonging also to **The State University of Medicine and Pharmacy "Carol Davila"** from Bucharest, under the aegis of a high consultative and deliberative medical forum **The Medical College – Department of Psychiatry**, and in co-work with The President, ***Prof. Dr. George Ionescu***, of this forum.
Paralelly the **"Star" Programme** has diversified it's activities, namely:

- permanent informative courses and contacts with the family physicians and other medical specialists;
- co-work with the police (*Department for the fight against organized criminality and control of stupefying drug consumption*), for treatment of persons under desintoxication treatment in which this company products proved to be useful, and also with the co-work with the educational authorities, and information especially of youngsters about. This programme started in Ploieşti, in co-work with the "Argus" Journal of the Police;
- buid-up of programmes with ayurvedic remedies applied in professional pathology;
- build-up of programmes specialized for geriatric care (in homes for elderlies and geriatric hospitals, as with organization of elderly and retired people in ambulant condition;
- treatments with mentally diseased and also retarded children;
- stimulative treatments system for persons (including students, pupils) to submitted to an intensive – in certain periods – phychic or/and physical efforts;
- immunostimulative treatments before and after surgical treatments – for instance in the prophylaxy of nosocomial infections.

The development of foreign trade of Romania, in the frame of private initiative of private economic agents. In this respect the **"Star" Programme** has answered to some requirement in foreigh countries as: Serbia-Muntenegru, Bulgaria, and now in the frame of the build-up of an important economic forum Romania – Iraq.

In this respect the **"Star" Programme** develops a co-work also with the system of Chambers Of Commerce from Romania (for instance the C.C.I. of the Prahova County) and it's monthly press and weekly journals and daily news-papers.

In the frame the **"Star" Programme** exists an activity for phytoiatric counseilling for phytomedical patients that includes also indications for hygienic and hygienic behaviour and measures that have to be taken, applie and also respected in order to improve the effects of the phytoiatric treatments and to avoid mistakes in the general behavioral and especially dietetic field to hinder the effects of the treatment with STAR's ayurverdic remedies, completly deprived of adverse side effects. (3), (6)

A monograph entitled :"***Ayurvedic Phytotheraphy Now In Romania***" elaborated by the outstanding scientist ***Prof.Dr. Eugeniu Rotaru***, first scientific counsellor of the **"Star" Programme**.

Many proceedings and scientific as well as educational articles elaborated by ***Prof. Univ. Dr. Farm. Ing. Sandrin-Seneca Bergheanu***, scientific counsellor, published in the phytoiatric journals, and ***Prof. Dr. Vladimir – Jules Gusic***.

The **"Star" Programme** is on the way to elaborate a monograph concerning Indian ayurvedic phytoiatry and phytoiuatric resources of India and the scientific back-ground of phytoiatry. (9), (10)

The endeavor to introduce new ayurvedic products determined the **"Star" Programme** to develop also it's own research. Such a programme opens new doors for "Star" products for dermatology and medical cosmetology, to a continuous improvement of remedies's conditioning, for new products for oral or transdermal or physiotherapic and aromatherapic application, whose psychophysiological limbic reactive emotional back-ground gives a scientific basis to the aromatherapy. The development of a programme: ":the food as medicament and the medicament as food", involving also food additives (spices, aromatisants, conservants, tonifiants etc.).

The **"Star" Programme** continues to participate as we have already mentioned to various scientific and educational meetings and specialized exhibitions and is a permanent active participant to various exhibitions of the greatest and most prestigious expositional and fair centres from Romania: **ROMEXPO** – member of **The international union for Fairs** - , including to the collateral events where the company organizes lectures, symposiums,

presentation of it's offer and its endeavour is to settle on permanent back-ground a national network of competent co-workers of the **"Star" Programme** everywhere in Romania.

THE RANGE OF AYURVEDIC "STAR" REMEDIES ISSUED FROM THE BOTANICAL INDIAN UNIVERSE

General considerations

The 17 remedies of the **"Star" Programme** ayurvedic offer for phytoiatric purposes in Romania, is pharmaceutically conditioned like non coated pills resulting from a mixture of medicinal Indian plants powdered and agglutinated with the gum resulting from species of *Accacia* belonging to the indian flora (*Gumacacia*). Besides its technopharmaceutical role, the gum by its chemical structure bears some substances that have themselves bioactive functions. Each ayurvedic "**Star"** remedy contains compounds issued from several medicinal species of Indian plants, sometimes about 20; every species containing a number of bioactive principles. By this structure, the remedies offer to the patient's organism a variety of medicinal opportunities, but every remedy is nevertheless characterized by a main therapeutic effect that defines its first line specific phytoiatricidentity. (comparable with the specific role of a solist accompanied in a musical play by a choir. ///in order to continue). The role of the other compounds of the remedy is just to corroborate the main medicinal effect of the main specific compound of the respective remedy .(to express this relation in a metaphoric kind we have to think to a plant with a main stem and root with adventive branches, stems and roots every with its role.
The application of the **"Star"** remedies is based on some other principles too: being practically deprived by adverse side effects, this remedies allow sustained treatments, namely, long lasting cures.
In some ailments they can be used as unique therapeutic factor like lassitude (psychical or/and physical), fatigue during or after a longer and/or heavier, solicitous, period of work (see **Ashwaganda**, **Memo On**, **Potent Power**). In other complaints, more severe, like, for instance, a chronic hepatitis the respective remedies have to be associated with other remedies, belonging to medical fields other than the phytoiatrics that mens, in a complementry way can be used in phytoiatry including the phytoiatric "**Star**" remedies and food supplements. (1)
In any case, the treatments with any phytoiatric remedy has to be applied only with the following conditions: only a physician can decide about the treatments (alternative or complementary, in more or less close intricacy, shorter or longer time lasting) and, of course, in co-work with a phytoiatrician (physician with phytoiatric competences, pharmacist, psychologist or specialist in medical biology with phytoiatric competence too).
Of course this is also the back-ground of the **"Star" Programme** conceptuality and methodology to which it is necessary to associate an interactive communication and information with the patient and a compliant behaviour with the patient's psychism. These aspects are also a stringent endeavour of the **"Star" Programme**.

Results and conclusions

The many sided **"Star" Programme** made possible a scientific and deontological based phytoiatric practice. By such means, the**"Star" Programme** contributes to ascertain the **phytoiatry** what it deserves to be a new medical speciality, an entity among medical sciences based on the slogan **"The medicine based on proofs is the single possible and admissible"**, slogan belongs also to the**"Star" Programme**.

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INFLUENCE OF *AJUGA GENEVENSIS Jacq.* (*LAMIACEAE*) ALCOHOLIC EXTRACT ON RESERPINE'S GASTRIC EFFECT

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Summary

Ajuga genevensis L. alcoholic extract at high dilutions doesn't act upon incidence, number and severity of reserpine-induced gastric ulcerations. At high concentration, Ajuga extract reduces number and severity of gastric ulcerations and diminishes muscular tonus of the stomach. Alcoholic Ajuga extract does not influence body weight and morphometric parameters of rumen and glandular stomach.

Key words*: Ajuga genevensis, gastric ulcers, reserpine, rat*

Introduction

Most species of Ajuga grow spontaneously, others are of attractive horticultural value but they also show multiple therapeutic virtues [1]. In the European medical folklore the preparations of Ajuga are used in the treatment of cough, tuberculosis, as cardiotonics and diuretics. They change iodine metabolism and were used in hyperthyroidism. Cataplasms with Ajuga leaves hasten the healing of skin damages. By internal route, Ajuga extracts have epithelising properties [2]. In the area of the Western Carpathians they constitute a treatment for dyspepsia [3]. In experimental medicine, extracts of various species of Ajuga present vasoconstrictor [4] but also vasodilator [5], analgesic [2,6,7], hypoglycaemic[8], choleretic and hepatoprotective [9], nephroprotective effects [10], erythropoiesis stimulating [11] and anticarcinogenic effects [12,13]. Antibacterial and antiparasitic effects were also revealed: antibacterial effects on staphylococci, E. coli and pyocianic bacilli [14,15], on mycobacteria [16], antifungal [17], antimalarial [18] and insecticide [19] effects.

The plants from the genus Ajuga contain iridoids [2], anthocyanins [20], clerodane diterpenoids [21,22], phytoecdysteroids [23].

The presumptive trophic actions of the extracts of Ajuga on the epithelia has been determined in the initiation of the present experiment. The effect of an alcoholic extract of *Ajuga genevensis Jacq.* was studied on a model of experimental ulcer in rats [24].

Materials and methods

The experiments were made on male Wistar-Bratislava albino rats weighing 126 - 202 g. The animals were kept in a natural light-dark regimen, under a standard diet at the Experimental Animal Farm of "Iuliu Hatieganu" University. The rats were randomly distributed in 5 groups of 10 animals each. Group I was gavaged with 1:2 alcoholic extract of Ajuga genevensis (AGE), 1 ml/100 g body weight, at 12 hours intervals, in 5 doses. Group II received by the same route distilled water in the same volume and according to the same schedule as group I. Groups III, IV and V were gavaged with AGE in concentrations of 1:2, 1:4 and 1:8 respectively. The gavage was made one hour before

feeding the animals. Forty-five minutes after the last gavage with AGE or distilled water (group II) the animals of groups II, III, IV and V were injected intraperitoneally (i.p.) with reserpine, 5 mg/kg. The animals were killed 8 hours after reserpine administration. The stomach was removed and the mucosa was examined in order to evaluate the gastric lesions. The mean of the total gastric ulcerations larger than 1 mm and confluent was calculated. The severity of the gastric ulcerations was appreciated on a nonparametric scale from 0 to 4, the mean representing the ulcer index (UI). Also, the rumen and the glandular part were weighed separately immediately after being removed and 24 hours after drying at 110°C. The weight index for the two gastric regions was calculated by comparing the weight of the tissues to the body weight (mg tissue/100 g body weight).

Statistical analysis

The incidence of the ulcerations is shown as percentage and the comparison between the groups was made by 2 x 2 tables. All the other results were expressed by the arithmetical mean and standard error ($x \pm s.e.$). The statistical analysis used Student "t" test, the significance level being considered values of $p<0.05$.

Drugs used

* Ajuga genevensis extract obtained from the Department of Pharmaceutic Botany of the Faculty of Pharmacy, "Iuliu Hatieganu" University. An alcohol extract in amount of 175 g was obtained from 32 g dry material. Ethanol concentration was 50%. From the original extract three dilutions were obtained: 1:2, 1:4 and 1:8.
* Reserpine phosphate (Raunervil®) 2 ml vials; 5 mg/ml

Results

The body weight did not differ among the groups (Table III).

The incidence of the gastric ulcerations in the animals receiving reserpine was 100%. The animals previously treated with AGE extract in the 3 concentrations presented an incidence of the gastric ulcerations of 60, 70 and 80% respectively. These percentages did not differ significantly from the percentage of 100%. The number of the gastric ulcerations had the highest mean in the group treated with reserpine and the lowest AGE dilution. The differences between the mean large and confluent total ulcerations did not differ between the reserpine treated group and the groups receiving the 3 AE dilutions together with reserpine (table III). The severity index (UI) was significantly decreased at the highest AGE concentration but it was not significantly changed after lower AG concentrations (table II).

The weight indexes of the rumen had close values in all the 5 groups. On the other hand, the glandular stomach had a lower weight index in the group treated with reserpine as compared with the group treated with the highest AGE concentration. On the background of reserpine administration AGE slightly in crease the weight index of the glandular stomach (table III).

Discussion

Until now, *Ajuga genevensis* preparations have not been tested in gastric ulcer model crease.

AGE does not have a noxious action on the gastric mucosa. The small erosions noted after gavage of the animals with AGE 1:2 are sometimes produced by simple gavage of the animals with aqueous solution. Reserpine produced gastric ulcerations in all treated

animals. The 1:2 concentrated extract reduced the incidence of the ulcerations to 60% (table I) and it also reduced the number of the gastric ulcerations and the severity index (table II). The high dilution, 1.8, increased the number of gastric ulcerations and their severity as compared with the group treated with reserpine only (table II).
AG administered as such and in reserpine treated animals showed the tendency to slow down the gastric emptying. In these conditions, in the animals kept under fasting conditions and treated with AGE, food residues were found in the gastric rumen. The phenomenon was more marked following AGE administration in high concentrations. The protecting action of these foods by their buffer effect against the acidity of the gastric juice is not precluded. The evolution of the gastric ulcerations in the present experiment was also influenced by the ethanol present in the extracts. At the highest concentration of 25% the damaging of the gastric mucosa is less probable. In fact, the protective effect of the extract was diminished as the concentration of ethanol decreased.

Conclusions

1. Reserpine produces reproducible acute hemorrhagic ulcerations in rats
2. Ajuga genevensis alcohol extract administered orally has various effects on ulcerogenesis, depending on the concentration. In high concentrations (1:3 dilution) the protecting effect is statistically significant
3. The 1:4 and 1:8 dilutions of the extract of Ajuga genevensis do not influence the evolution of reserpine induced ulcerogenesis
4. The concentrated extract of Ajuga genevensis increases the weight index of the glandular gastric mucosa
5. The anti-ulcer action on this model is probably also accounted for by the inhibition of the gastric motility at higher concentrations of the Ajuga reptans extract.

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PRELIMINARY STUDIES ON THE *CRATAEGUS MONOGYNA* AND *CRATAEGUS OXYACANTHA* (L) SPECIES AND THEIR IMPORTANCE IN THE TREATMENT OF CARDIOVASCULAR DISEASES

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Abstract

Our goal is to conduct studies on Crataegus monogyna specie used in treatment of cardiovascular affections in many countries. The indigene species have been studied from a phyto-chemical point of view, by using techniques such as thin-layer chromatography, gas-chromatography and HPLC (High Performance Liquid Chromatography) combined with mass spectrometry (LC/MS/MS). In the next step, we created an original Romanian product, standardized in flavonoids, called CRATECORD, which was the subject to pharmacological and preliminary clinical tests. The phytopreparation was tested in the next step on different patients diagnosed as cardiac insufficiency level II and III (NYHA), using 3x2 pills of 0.37 g dry extract/day during 8 weeks associated to the previous treatment for cardio-vascular disease. The results consist of improvement of the clinical parameters, electro-cardiogram, eco-cardiogram and the decrease of Pro-BNP.

Keywords: *Crataegus, dry extract, cardiac insufficiency*

Introduction

Since in our country the therapeutic possibilities of medicinal plants for the treatment of cardio-vascular diseases are not enough studied we focused our research on the possibilities to prepare some pharmaceutical products based on standardized *Crataegus* extracts.
The phytopreparation was tested in preliminary clinical studies to establish the therapeutic efficiency. The benefic effect of *Crataegus* on different diseases is well described in literature, but most of the research focused on cardio-vascular diseases. The *Crataegus* phytopreparation was used since the antiquity (China – in Tang-Beng-Cao 659 B.C.) for the cardiac stimulation action and for coronary-dilatation proprieties. During this period, the toxicological studies emphasize the lack of toxicity.
There are known many studies focused on the proprieties of this plant (WISSO study, with special extract of *Crataegus* WS (R) 1442), focused mainly on production of new medicines to be used for the treatment of cardio-vascular diseases.[1]
The plant material used in our study is *Crataegi folium cum flore* and *Crataegi fructus*, represented by inflorescences with attached leaves before the maturity and dry grown-up fruits, collected from *Crataegus monogyna Jacq. (Lindmann*) hawthorn, (*Rosaceae)*.
Flowers contain at least 0.6% flavonoids expressed as hiperoside(by FR X).
There are well known from literature the following actions: minor cardiotonic, improve the blood flow in necrosis tissues after myocardium infarct, antispasmodic and central sedative, improve the oxygenation capacity of the brain, decrease the cardiac rhythm, ameliorate the systolic ejection. [2,3,4,5,6,7]
The pharmacokinetics studies on alcoholic extract emphasize the following effects: positive inotropic, negative chronotropic and β-blocking, antihypertensive, increase the blood flow (coronary vasodilatation).

Materials and methods

The plant material (flowers, leaves and fruits) has been bought from Cluj Napoca 2004. The following measurements were done: qualitative analysis of flavonoids by TLC and quantitative determination of flavonias by TLC, flavanoids using FR X method from *Crataegi folium cum flore* and *Crataegi fructus* and quantitative measurements of proantocyanidins from *Crataegi folium cum flore* and fluid extract of *Crataegus*.

Method used to obtain the fluid extract from *Crataegi folium cum flore*
The fluid extract (1:1) was obtained using the repercolation technique (Squibb). Plant material was grinded and sieve through sieve no. VI (F.R.X). As a solvent we used ethylic alcohol 70^0 in a ratio of 1:2.7 (plant material:solvent). We obtained fluid extract (1:1) with the following characteristics:

Aspect	Clear liquid
Color	Brown- green
Taste	Bitter-astringent
Density	0.9849-0.9890
% of alcohol	min 60%
Residual content after evaporation	min 10%
Flavonoids content	min 0.5%
Proantocyans content	min 0.25%

Method used to obtain the fresh fruits tincture of *Crataegus*
Fresh fruits tincture of *Crataegus* was obtained using the steeping procedure according to the German Homeopathic Pharmacopeia [Deutshes Homoopathisches Arzneibuch]. Fresh fruit collected in September were cut in small pieces. We added alcohol 90^0 in a quantity equal to the humidity percentage of fruits. For example, if the humidity value is 60% than for 1000g fresh fruits we added 600g alcohol 90^0. At the beginning we added alcohol 90^0, in a quantity equally to 50% of dry weight fruits and after the measurement of the humidity (3 hours) we added the rest of the alcohol.
The steeping process lasts 10 days, mixing everyday. After 10 days, the tincture separates from fruits by decantation. The fruits are squeezed out in gauze and the two fractions are mixed.
The *Crataegi fructus* tincture has the following characteristics:

Aspect	Clear liquid
Color	Light red – brown
Taste	A little bit astringent
pH	4.9-5
Density	0.9749-0.9790
% of alcohol	45±2.5
Loss on drying	7.5%
Flavonoids content	0.148-0.579 g% hiperoside
Proantocyanidins content	0.43%

Method used to obtain the dry extract of *Crataegus* and the phyto-preparation CRATECORD

The dry extract of *Crataegus* was obtained mixing fresh fruits tincture (T) (3p) and fluid extract 1:1 (E) of *Crataegi folium cum flore* (1p) and lay down on microcrystalline lactose-cellulose PVP + talc. From 1kg mixture tincture:fluid extract (3:1) results 350g dryextract, dried using the Aeromatic-Fielder from Niro Pharma Systems. A quantity of 0.35 g dry extract was introduced in capsules, each capsule being equivalent to 1g mixture T:E (3:1).
In order to realize a standardized product for the plant material collected from Cluj County wild flora, we realized qualitative and quantitative measurements of flavonoids, flavonioidic antocyanidins and proantocyanidins from *Crategi folium cum flore* and fluid extract of *Crataegus*.

Clinical studies

For clinical studied we used capsules with 0.37g dry extract each. The phyto-preparation was produced at Faculty of Pharmacy.
During the study we were monitoring two groups of patients suffering of cardio-vascular diseases: patients treated with CRATECORD pills (*Crataegus* extract) besides the previous treatment and control group, patients without CRATECORD treatment, only with previous treatment.
The measurements of proBNP, electrocardiogram, and echocardiograph for selected patients were done at CMDTA Ploiesti in association to the cardiology specialist.
We presented below for exemplification one of the study cases:
E.C., 71 old, rural area, Prahova County.
Diagnosis: dilative ischemic cardiomyopathy, class II heart failure, atrial fibrillation with ventricular medium frequency, mitral insufficiency, myocardial stroke, HTA with medium risk.
EKG: FiA with AV 102/min, QRS ax left deviation -29^0, Q in V2-V4 and in II, III, aVF, ST-T under the level in I, aVL, V5, V6.
Echocardiograph: VS (D) 70 (normal 39-57), VS (S) 64 (normal 25-45), AS 47 (normal 21-37), VD 38 (normal 30), AD 40 (normal 29-42), i.v. sept. 10 (normal 7-11), p.p. 10 (normal 7-11), Ao – ring 21 (normal 18-23) Ao ascd. 28 (normal 35), FE 30-35% (normal 60-70%), free pericardium, class II mitral insufficiency with central jet (spectral and color Doppler). Global hypokinesis wall left ventricle, globular VS, V_{max}Ao=1.5m/s; no thrombus in AS/VS.
Conclusions: global grown cavities, altered systolic function, wall global hipokinesis left ventricle, mitral flow back level II.
proBNP measurement = 6602pg/ml (normal <80pg/ml)
After the below treatment (the first 5 medicines were part of the initial treatment, before the treatment with CRATECORD) applied during a period of 8 weeks:

1. Digoxin 1/day, 5 days/week
2. Diurex 50, 1 / 2 days, in the morning
3. Isodinit 10, 2/day
4. Trombo Ass, 1/day
5. Enalapril 10mg/day
6. CRATECORD 6/day

We obtained the following results:
Echocardiograph: VS (D) 58 (normal 39-57), VS (S) 49 (normal 25-45), AS 45 (normal 21-37), FE 40-45% (normal 60-70%), free pericardium, free cavities, class I mitral insufficiency

(spectral and color Doppler), inferior and posterior wall hypokinesia, akinesia of the inferior wall's basal third

proBNP measurement=1472 pg/ml

We emphasize the real improvement of the clinical parameters (effort dyspnea, fatigability etc) after first 4 weeks of treatment associated to CRATECORD.

Results and discussions

- The qualitative analysis of plant material used to prepare the CRATECORD underline the presence of the flavonoids compounds, flavonoidic aglycans and proantocyanidins.
- The quantitative measurements of flavonoids and proantocyanidins emphasize the values are close to the values from literature.
- The fresh fruits tincture was prepared according to the methods from literature.
- The phytopreparation CRATECORD, prepared from a mixture of fresh fruits tincture and fluid extract of flowers and leaves of *Crataegus,* laid down on microcrystalline lactose-cellulose PVP + talc, is a modern one, stabile, safety and easy to be used in treatments as capsule.
- No preclinical tests were done to establish the toxicity level since the data from literature mention no toxicity effects of *Crataegus* and DL_{50} is 1000 bigger than the dose used by us.
- The preliminary preclinical studies were realized on an insufficient number of patients for a statistical analysis but enough to prove the efficiency of CRATECORD for the treatment of the ischemic cardiovascular diseases and cardiac insufficiency level II and III NYHA.
- The improvement of the clinical parameters (dyspnea, fatigability, shanks edema, and anginous pains), echocardiography (the decrease of VS dimensions and the increase of FE) besides the serious decrease of pro-BNP (the most important marker for cardiac insufficiency) emphasize the benefic effects of the CRATECORD product.

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PRELIMINARY RESULTS OF CLINICAL TRIALS WITH *ROSMARINI FOLIUM PULVIS* 0,5G/CAPSULE IN THE TREATMENT OF CHRONIC LOW BLOOD PRESSURE

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Abstract

In medical literature, Rosemary is noted to efficiently increase blood pressure in chronic low blood pressure patients. Our goal was to create a set of standardized pharmaceutical products made from Rosemary, which were subjected to preliminary clinical testing in order to prove their therapeutic efficiency in chronic low blood pressure. Species of rosemary was cultivated in Cluj-Napoca were used during the trial.

Keywords: *Rosmarini, chronic low blood pressure*

Introduction

The benefic effect of Rosmarini is well known in literature especially in:

- low blood pressure;
- stimulation of the body immunity
- antioxidants
- tissue regeneration
- hair growth;
- antibacterial
- antidispeptic
- rheumatic diseases
- stimulation of peripheral blood circulation [2,3,4]

Materials and methods

Leaves of Rosmarini (*Rosmarini folium)* are grinded into small size particles and sieved using no. VI sieve. A quantity of 0.5 g plant material is introduced in capsulated pills. Each pill contains 0.125 mg of flavonoids as rutoside and 3.9 mg rosmarinic acid.
The purpose of the work is to study the effect of *Rosmarini* leaves on low blood pressure in different clinical studies since is known from literature the benefic effect on this type of diseases.
It is important to mention that there is no commercial medication produced by pharmaceutical industry in Romania recommended for chronic low blood pressure patients. Therefore, we consider that this medication can be used by a large number of patients with low values of TA or as a secondary effect of some diseases (suprarenal insufficiency etc).
There is important to mention that the phytoproduct is recommended only for chronic low blood pressure patients but not for emergency cases (shock, collapse etc).
Capsulated pills containing 0.5 g leaves of Rosmarini were prepared by micro production laboratory of the Faculty of Pharmacy, Cluj-Napoca. All the patients were carefully selected after a 2-4 weeks monitoring period of TA values. Only the patients with systolic TA values lowers or equals to 85 mmHg and low blood pressure clinical symptoms were selected for our study. We received the approval of all the patients to be included in our study and they had the possibility to measure the TA value at home by themselves. The paper presents the preliminary results for one group of 20 patients, the research going on for the rest of them.

Since in the literature it is mention that quantities of 1 g dry weigh produced no toxic effects or other adverse reactions we escape the preliminary phase of preclinical studies and toxicological effects of the phytoproduct.[5,6]
The patients selected from both, the CMDTA – MAI Ploiesti office and the private room was grouped in two: group no. 1 treated with 2 pills of *Rosmarini folium pulvis* per day and group no. 2 no treatment (control group).
The patients have been trained before the study regarding the treatment, monitoring TA values and reporting any health modification due to the new treatment. For patients suffering from other associated diseases we recommended to continue the treatment.
For both groups, the study last 8 weeks. During this period we measure TA twice per month and each time they considered as necessary at home by themselves.
They received 2 pills/day of 0.5 g plant material, in the morning and in the evening, before table. A complete clinical and echographic investigation was done at the beginning and at the end of the study for all the patients. The treatment was done ambulatory.

[1]

patient no.	σA [mmHg]		MSAP	σA [mmHg]		MSAP	σA [mmHg]		MSAP	σA [mmHg]		MSAP	σA [mmHg]		MSAP
	first checkup			2 week checkup			4 week checkup			6 week checkup			8 week checkup		
	SP	DP		SP	DP		SP	DP		SP	DP		SP	DP	
01	80	55	63.33	90	60	70.00	110	60	76.67	115	60	78.33	115	60	78.33
02	75	50	58.33	85	60	68.33	120	65	83.33	125	65	85.00	125	70	88.33
03	70	50	56.67	105	60	75.00	110	60	76.67	110	60	76.67	105	65	78.33
04	85	55	65.00	120	60	80.00	120	60	80.00	125	60	81.67	125	65	85.00
05	80	60	66.67	105	55	71.67	110	60	76.67	115	60	78.33	115	60	78.33
06	80	55	63.33	110	60	76.67	110	65	80.00	120	65	83.33	120	70	86.67
07	75	60	65.00	125	60	81.67	120	65	83.33	120	65	83.33	125	70	88.33
08	80	60	66.67	120	65	83.33	120	65	83.33	115	60	78.33	115	65	81.67
09	80	60	66.67	115	60	78.33	115	65	81.67	120	65	83.33	120	65	83.33
10	75	60	65.00	120	70	86.67	120	70	86.67	125	70	88.33	125	70	88.33
11	85	65	71.67	105	60	75.00	105	65	78.33	115	65	81.67	115	65	81.67
12	80	60	66.67	110	60	76.67	115	60	78.33	120	70	86.67	120	75	90.00
13	75	65	68.33	120	65	83.33	120	65	83.33	120	65	83.33	120	70	86.67
14	80	60	66.67	105	60	75.00	115	70	85.00	115	70	85.00	120	70	86.67
15	80	60	66.67	100	60	73.33	120	65	83.33	120	60	80.00	120	65	83.33
16	85	55	65.00	110	65	80.00	115	70	85.00	125	70	88.33	115	70	85.00
17	75	55	61.67	120	55	76.67	120	65	83.33	120	65	83.33	120	60	80.00
18	80	65	70.00	110	80	90.00	115	65	81.67	120	65	83.33	120	60	80.00
19	70	50	56.67	120	60	80.00	120	60	80.00	120	65	83.33	115	65	81.67
20	80	55	63.33	105	60	75.00	110	60	76.67	120	65	83.33	115	65	81.67

Table 1 - First study lot (2x1 capsules/day, each containing 0,5g Rosmarini pulvis)

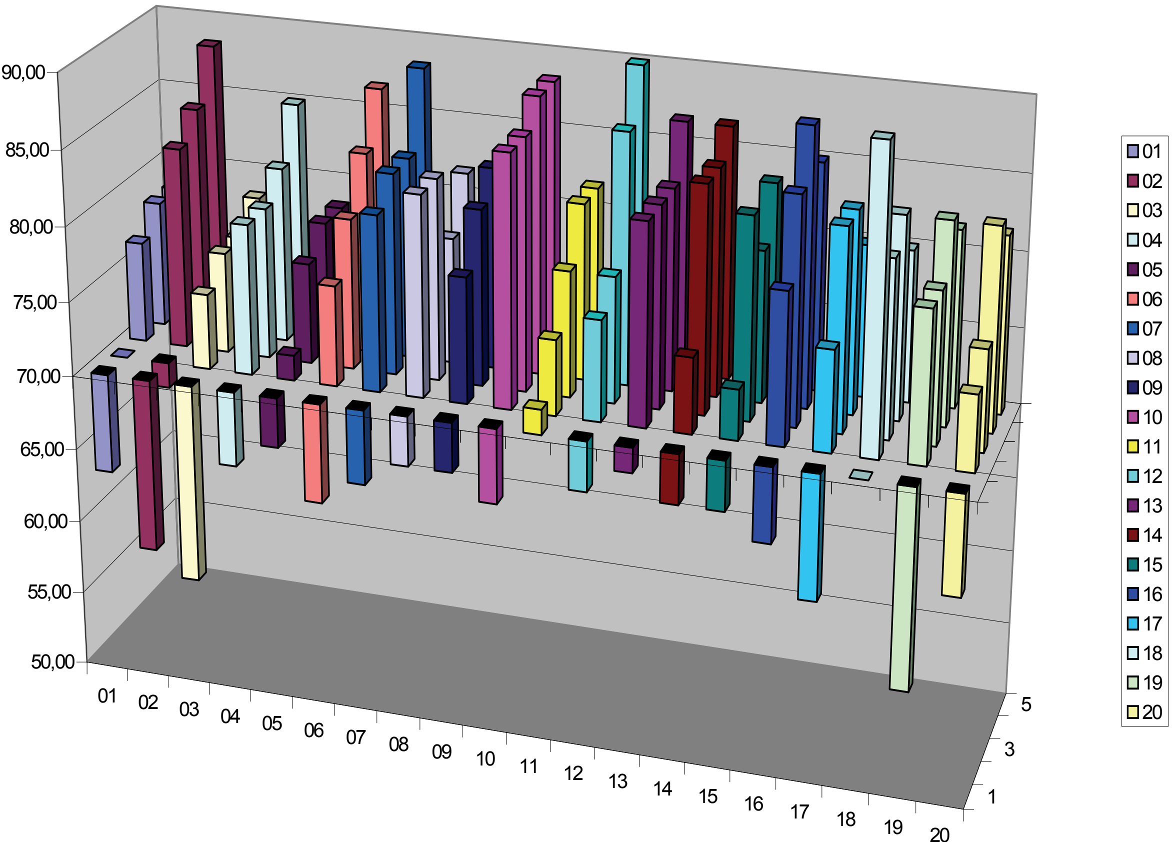
σA = Arterial pressure
SP = systolic systemic blood pressure
DP = dyastolic systemic blood pressure
PP = SP - DP
MSAP = mean systemic arterial pressure = DP+PP/3 = (SP+2DP)/3

Before each checkup, the patients filled out a survey about the primary symptoms that they had before beginning the treatment. The survey allowed the gradual description of the symptoms:

- feeling of weakness
  - strong
  - medium
  - vague
  - absent
- blurry eyesight
  - severe

- medium
- minimum
- absent
- prelipothymies
- lipothymies

We rejected from our study the patients did not respect the treatment, they interrupt it, they took only one pill per day or they did not present at the private room for control. Two more patients were rejected from the study after they accused digestive problems.



Results and discussion

1. The 20 patients treated to 2x1 pills of *Rosmarini folium pulvis* 0.5 g / pill did not manifested adverse or allergic reactions during the study;
2. All the patients manifested an improvement of the clinical state (no more weakness sensations, prelipothymies etc.) even during the summer when due to the high temperatures they used to manifest prelipothymies or lipothymies.
3. The measurement of TA values proved a slow increasing especially systolic, and mainly after 2 weeks of treatment for most of the patients.
4. The increase of systolic TA range between 20 and 40 mmHg for all the patients, the maximum systolic TA value being 125 mmHg and the minimum 105 mmHg.
5. There was no improvement of TA values for group no. 2 (control, no treatment) or occasional values higher than 90 mmHg during the study.
6. A preliminary conclusion is the *Rosmarini* can be use to treat the chronic low blood pressure diseases using a dose of 1 g/day grinded *Rosmarini* leaves.

There are necessary further studies to clarify the mechanism through which the blood pressures increases and also the prescription and contraindications of the phytoproduct.

patient no.	σA [mmHg]		MSAP	σA [mmHg]		MSAP	σA [mmHg]		MSAP	σA [mmHg]		MSAP	σA [mmHg]		MSAP
	first checkup			2 week checkup			4 week checkup			6 week checkup			8 week checkup		
	SP	**DP**		**SP**	**DP**		**SP**	**DP**		**SP**	**DP**		**SP**	**DP**	
001	80	55	63,33	80	55	63,33	75	50	58,33	85	50	61,67	80	60	66,67
002	75	50	58,33	85	55	65,00	75	60	65,00	85	50	61,67	75	55	61,67
003	55	50	51,67	75	50	58,33	80	55	63,33	75	60	65,00	55	60	58,33
004	85	55	65,00	85	55	65,00	85	55	65,00	85	65	71,67	85	50	61,67
005	80	60	66,67	75	60	65,00	80	55	63,33	85	60	68,33	75	60	65,00
006	80	55	63,33	80	55	63,33	80	65	70,00	85	55	65,00	85	55	65,00
007	75	60	65,00	85	55	65,00	75	60	65,00	85	50	61,67	80	55	63,33
008	80	60	66,67	85	65	71,67	85	65	71,67	75	60	65,00	75	60	65,00
009	80	60	66,67	75	55	61,67	75	50	58,33	85	55	65,00	75	50	58,33
010	75	60	65,00	75	60	65,00	85	55	65,00	85	60	68,33	85	65	71,67
011	85	65	71,67	80	55	63,33	80	60	66,67	80	50	60,00	80	50	60,00
012	80	60	66,67	75	50	58,33	75	55	61,67	85	55	65,00	75	60	65,00
013	75	65	68,33	85	65	71,67	85	60	68,33	85	50	61,67	85	55	65,00
014	80	60	66,67	55	55	55,00	75	55	61,67	85	60	68,33	80	55	63,33
015	80	60	66,67	75	55	61,67	80	60	66,67	85	60	68,33	85	60	68,33
016	85	55	65,00	80	65	70,00	75	55	61,67	75	55	61,67	75	55	61,67
017	75	55	61,67	85	55	65,00	80	60	66,67	85	50	61,67	85	60	68,33
018	80	65	70,00	80	60	66,67	75	60	65,00	85	50	61,67	80	60	66,67
019	55	50	51,67	85	55	65,00	75	55	61,67	85	55	65,00	75	50	58,33
020	80	55	63,33	80	55	63,33	80	55	63,33	85	50	61,67	75	50	58,33

Table 2 - Placebo group

σA = Arterial pressure
SP = systolic systemic blood pressure
DP = dyastolic systemic blood pressure
PP = SP - DP
MSAP = mean systemic arterial pressure = DP+PP/3 = (SP+2DP)/3

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THE EFFECTS OF THE ALCOHOLIC EXTRACT FROM CRATAEGUS MONOGYNA L (ROSACEAE) ON ISOPRENALINE-INDUCED CARDIAC HYPERTROPHY IN MICE

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Summary

We have studied the effect of an alcoholic extract from Crataegus monogyna L. in cardiac hypertrophy produced by isoprenaline in mice. In animals treated with isoprenaline, the 1:10 and 1:50 dilutions of this extract don't alter the weight parameter of the heart

Keywords*: Crataegus, cardiac hypertrophy, isoprenaline, rats*

Introduction

History tells us that various Crataegus species have been used for medical purposes since the first century AD.[1] Nowadays, preparations made from Crataegus are strongly recommended in cardiovascular pathology [2,3,4,5], primarily in the treatment of cardiac failure [6,7,8,9,10].
In most cases, it's associated with conventional therapy [11,12,13]. Both preliminary clinical studies [8,14] and recent, multi-center randomized studies [7,11,12,15] prove the therapeutic benefits of Crataegus extracts in mild cases of NYHA II cardiac failure. The preparations are standardized based on their contents in flavones or procyanidins [11,14,15,16]
Crataegus extracts have benefic effects in patients with heart failure through their positive inotropic actions. [17,18]. They increase the maximum capacity of the heart when solicited and relieve shortness of breath and fatigue.[12,14,15]. They also increase cardiac output and tissue perfusion. These extracts also have dilating effects on the blood vessels [19] and are known to improve the symptoms of ischemia and arrhythmia [8]. They're also proficient as hypolypemiants [8], antioxidants [20,21] and anti-inflammatory. [7,21]
In this study we have used a model of cardiac hypertrophy induced by isoprenaline [22] in order to analyze the effects of an alcoholic Crataegus extracts (ACE) on the morphometric parameters in mice.

Materials and methods

We worked with albino, male Swiss mice, weighing 22-33g, from the Biological base of the "Iuliu Hațieganu" University in Cluj-Napoca. The test animals were accustomed to the environment of the lab, in natural lighting, with standard food and water *ad libitum*.
We randomized the animals in 4 groups, each containing 10 mice. The control group received the alcoholic solvent intraperitonealy (i.p.). The second group received, along with the solvent**,** 50 µmoles/kg isoprenaline i.p. We injected a total of 6 doses of isoprenaline, 12 hours apart from each other. The 3rd and 4th group received isoprenaline and an alcoholic extract of Crataegus, diluted 1:10, and 1:50 respectively. 12 hours after

the last injection, the animals were sacrificed and their hearts, submandibular salivary glands and spline were removed for study. The organs were weighed immediately and after 24 hours of drying at 110° C. The organ mass indices were calculated by comparing the tissue weight to body weight (1mg organ/10g body weight). We obtained 3 types of organ indices: total, dry and, by comparison, water contents (tables I-III).
We also calculated the percentage of dry residue.
The results were expressed as an arithmetic mean and a standard error (x ± s.e.) and compared using the "t" Student test. The correlation between the morphometric parameters was established through the Bravais test. The statistical significance had a threshold of $p< 0.05$.

Drugs used

1. Isoprenaline sulphate (Terapia SA, Cluj-Napoca) a dose of 50 micromoles/kg/20ml. In this way the volume of the solution injected was 0.2 ml on 10 g body weight.1 micromole isoprenaline sulphate = 0.27835mg. A solution of isoprenaline sulphate of 2.5×10^{-3} M was prepared. There are 50 micromoles of isoprenaline sulphate in 20 ml of this solution.
2. An alcoholic extract from Crataegus monogyna L., 1:3, produced at the Warehouse of Pharmaceutical Botany of the "Iuliu Hațieganu" University in Cluj-Napoca. The dilutions used were 1:10 and 1:50.

Results

Body weight is similar in the 4 groups (table IV). Isoprenaline significantly increases the 3 components of the heart index (table I). The heart weight indices of the groups treated with 2 concentrations of ACE are close to the isoprenaline-treated group (table I). The animals that received ACE in the 1:50 dilution have the dry residue percentage lower than the placebo group and the isoprenaline group (table IV).
The modifications produced by isoprenaline on the salivary glands are less obvious. The increase in mass index is statistically irrelevant when compared to the control group. The two dosages of ACE tend to lower the morphometric parameters of the salivary glands (table II) and increase the dry residue ratio of salivary glands (table IV). The effects of the isoprenaline and the alcoholic Crataegus extract on the spline are insignificant (table III, table IV).
The correlation between the morphometric parameters was obvious when analyzing the body weight, the weight of the dried heart and the three types of weight of each organ (table V).

Discussion

When using the specified doses, isoprenaline induces a noticeable increase of the weight of the heart (table I), with similar, yet limited statistic-wise, effects on the salivary glands (table II). Alcoholic Crataegus extracts don't alter isoprenaline's hypetrophic effects on the myocardium, but tend to lower the increase in weight of the salivary glands that isoprenaline normally produces.

Literature references of the influence of Crataegus extracts on the effects of isoprenaline on the heart are scarce [23,24,25,26]
The water extract from Crataegus oxyacantha L. reduces the unfavorable effects of isoprenaline on the antioxidant enzymes of the heart and increases the rate at which oxygen is captured, under ATP stimulation. The protective effect also extends onto the lesions that the isoprenaline inflicted upon the mice's hearts [24]. When given as preventive treatment, Crataegus extracts limit the increase of hepatic (and possibly myocardial too) cytolitic enzymes and reduces the toxic effects of isoprenaline on the heart, blood vessels and liver [23, 26]. Crataegus extracts alleviate the alterations of the ST segment and the T wave produced by isoprenaline [25].
Crataegus extracts have obvious cardioprotective effects on other experimental models [27,28]. It reduces the effects of myocardial ischemia produced by the ligature of the coronary arteries in rats [21.25.29] and lowers the occurrences and the harshness of consecutive ventricular arrhythmias [21, 29]. The antiarrhythmic effects may be produced by effective refractory period prolongation. The effect was observed on adult rat myocytes [30], guinea pig papillary muscles [31] and isolated perfused guinea pig hearts [32]. Crataegus extracts increase the duration of the action potential and of the effective refractory period by blocking the delayed and the inward rectifier potassium current [31].
In cardiac failure, Crataegus extracts can reestablish the potentially compromised function of the vascular endothelium [21,33,34].
In this experiment, Crataegus extracts don't cancel the cardiac hypertrophy induced by isoprenaline. The interaction between Crataegus extracts and isoprenaline remains to be studied further in other experiments. The lack of efficiency of the Crataegus extract in this case doesn't necessarily exclude the extracts' cardioprotective effects, proven in various other experiments and clinical studies.

Conclusions

1. In mice, in sub-acute administration isoprenaline induces the hypertrophy of the heart and salivary glands.
2. The alcoholic extract from Crataegus monogyna doesn't alter the effects of isoprenaline on the heart and salivary glands.
3. Crataegus extracts have proven their cardioprotective effects in various other experimental models and also in patients with cardiac failure.

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GENOTOXIC AND ANTIGENOTOXIC POTENTIAL OF BASIL (*OCIMUM BASILICUM* L.)

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Summary

Essential oil (EO) of basil and its major constituent Linalool showed no mutagenic effect in S. typhimurium TA100 strain. They also significantly inhibited spontaneous and t-BOOH-induced mutagenesis in E. coli strain deficient in induction of antioxidative enzymes. In S. cerevisiae, EO and Linalool showed co-mutagenic effect with t-BOOH, but exhibited protective capacity against H_2O_2-induced DNA strand breaks. Obtained results indicate genotoxic and antigenotoxic effects of basil, depending on the dose and the test system.

Keywords: *basil, E. coli, S. cerevisiae, reversion tests, Comet assay*

Introduction

Numerous experimental data suggest that oxidative DNA damage, from both endogenous and exogenous sources are of great importance in the etiology of many human diseases such as cancer, atherosclerosis, diabetes and neurodegenerative diseases (Marnett, 2000; Olinski *et al.,* 2002). Natural antioxidants contained in medicinal and aromatic plants, fruits and vegetables may be useful in preventing the deleterious consequences of oxidative damage caused by ROS and therefore they are considered as possible chemopreventive agents. They can possess a variety of biological activities e.g. anti-mutagenic, anti-proliferative, scavenging of free radicals or activated mutagens/carcinogens, they can modulate DNA repair and other enzyme activities or even regulate gene expression (Brigelius–Flohe and Traber 1999; Craig 1999; Heo *et al.* 2001; Kris-Etherton *et al.,* 2002; Nikolić *et al.* 2004).
Basil (*Ocimum basilicum* L.), the common medicinal and culinary herb, is widely used in many traditional medicines. Numerous laboratory studies have shown various protective effects of *Ocimum* sp. (Dasgupta *et al.,* 2004). In this work we examined antigenotoxic potential of EO of basil and its major constituent, terpenoid alcohol Linalool. In preliminary experiments, EO and Linalool were pre-screened for mutagenic effect in *Salmonella*/microsome mutagenicity assay in strain TA100. The investigation of antigenotoxic effect included detection of antimutagenic potential of test substances using prokaryotic and eukaryotic reversion tests, as well as, the examination of protective effect of Linalool against oxidative DNA damage by comet assay on *S. cerevisiae* (Miloshev *et al.*, 2002).

Materials and methods

Table 1. Tester strains

	Strain	Relevant marker	References/source
S. typhimurium	TA100	*hisG46 rfa ΔuvrB bio-*/pKM101	(Maron and Ames, 1983)
E. coli B/r WP2	IC185	*lon11 sulA1 trpE65 lamB*$^+$	(E. M. Witkin, 1956)
	IC202	*lon11 sulA1 trpE65 oxyR lamB*$^+$/pKM101	(Blanco *et al.,* 1998)
S. cerevisiae	D7	*ade2-40/119 trp5-12/27 ilv1-92/92*	(Zimmermann *et al*., 1975)
	3A	*a/α gal1 leu2 ura3-52*	G. Miloshev

Plant material. Basil (*Ocimum basilicum* L.) was cultivated in the experimental field of the Institute for Medicinal Plant Research ”Dr. Josif Pančic” in Pančevo, Serbia and Montenegro.
Tester strains. The tester strains used in this study are listed in Table 1. The *Saccharomyces cerevisae* strain A3 was kindly provided by George Miloshev.
Preparation of essential oil of basil. The EO was prepared according to Ph. Jug. IV, by distillation of dried aerial part (*Basilicii herba*) in $2m^3$ steam distiller (Hromil) for 2 hours, at the pressure 3-4 bars and temperature 135-145°C.
Chemicals. Stock solutions of Vitamin E (DL α-Tocopherol acetate, Galenika a.d.), Linalool (Aldrich) and EO of basil were freshly dissolved in 96% ethanol (1:9). *t*-Butyl hydroperoxide (*t*-BOOH, Aldrich) and hydrogen peroxide (H_2O_2, Sigma-Aldrich) were freshly dissolved in distilled water.
Media and growth conditions. All bacterial strains were grown overnight at $37^{\circ}C$ in LB medium and *S. cerevisiae* strains in YPD medium at $30^{\circ}C$ with aeration. Media for reversion assays were as described by: Maron and Ames (1983), Blanco *et al.* (1998) and Zimmermann *et al.* (1975). S9 fraction was isolated from the liver of Albino Wister male rats (170-180 g) induced with pheno-barbital/β-naphtho flavone or Aroclor 1254 (Ong *et al*., 1980).
Detection of mutagenic potential (Ames test). Samples (0.1 ml) of overnight culture of *S. typhimurium* TA100 strain were added to 3 ml of molten top agar with and without S9 mixture (0.3 ml), mixed with different concentrations of EO or Linalool and poured in duplicates onto minimal glucose agar plates. Ethanol was used as a negative control. After incubation at 37°C for 48h the number of His^+ revertants was determined and the presence of the bacterial background lawn on all plates was inspected.
Detection of antimutagenic potential. E. coli WP2 reversion test was performed by mixing 0.1ml of overnight cultures of WP2 tester strain, water dilution of *t*-BOOH, appropriate volume of EO or Linalool solution and 3 ml of molten top agar (45°C). The mixture was poured onto minimal medium supplemented with 0.5µg/ml tryptophan (Blanco *et al.,* 1998). The number of Trp^+ revertants was determined after incubation at 37°C for 48^h. Simultaneously, we examined the influence of EO or Linalool on spontaneous mutagenesis and viability of treated and untreated cells by plating appropriate diluted overnight cultures on LA plates. Ethanol was used as a negative control and Vitamin E (1.5 mM) as a positive control.
S. cerevisiae D7 reversion test was performed by mixing 0.1ml of yeast overnight cultures with water dilution of *t*-BOOH, appropriate volume of EO or Linalool solution and 3 ml of molten top agar (45°C). The mixture was poured onto minimal medium without isoleucine to determine the number of Ilv^+ revertants. Plates were incubated at 30°C for 72h. Simultaneously, we examined the influence of EO or Linalool on spontaneous mutagenesis. Cell survival of treated and untreated cells was determined by plating appropriate diluted overnight cultures on YPD plates. Ethanol was used as negative control and Vitamin E (3 mM) as a positive control.
Detection of DNA strand breaks (Yeast Comet assay). S. cerevisiae 3A was grown to middle logarithmic phase (5 x 10^7 cells/ml) and than pre-treated with Linalool for 15 min at room temperature. After washing, cells were treated with H_2O_2 for 10 min at 4°C. In the case of co-treatment H_2O_2 and Linalool were applied simultaneously for 10 min at 4 °C. As a negative control we used ethanol and as a positive control Vitamin E (0.05 µM). The spheroplasting with zymolyase was carried out in the gel. The cells were mixed with low-melting agarose, spread on microscope slides pre-coated with 0.5% agarose and submersed into lysing solution for 1h. After unwinding the DNA in alkaline conditions, we subjected the slides to electrophoresis. Following electrophoresis, the micro-gels were dehydrated and dried, the DNA was stained and comets were visualized under the fluorescent microscope. The comets

and spheroplasts were scored visually at fifty randomly selected fields for each slide and percentage of comets was calculated.
Statistical analysis. The Student's *t*-test was employed for statistical analysis. The significance was tested at the $P < 0.05$ level. The results presented in figures and tables are expressed as the means obtained from three independent experiments, with the standard error of the mean.

Results and Discussion

Reversion tests
Mutagenic effects of EO of basil and its main constituent Linalool were tested in the *Salmonella*/microsome mutagenicity assay, in TA100 tester strain, with 4NQO and B[a]P as positive controls. No mutagenic effect of EO and Linalool was detected (data not shown). Results obtained for Linalool confirm previous findings (Rockwell and Raw, 1979; Ishidate *et al*., 1984; Heck *et al*., 1989).
Antimutagenic potential of basil derivatives against spontaneous and induced oxidative mutagenesis were tested in strain IC202 of *E. coli* WP2, recommended for detection of oxidative mutagens (Blanco *et al*., 1998). Mutagenesis monitored was *trpE65* → Trp^+ reversions. *trpE65* mutation is an ochre mutation and can revert by base substitutions at the AT base pairs in the *trpE65* site or at extragenic ochre suppressor loci (Martinez *et al*., 2001). The oxidative mutagen, *t*-BOOH, is latent donor of ROS, particularly alkoxyl radicals ($RO^{\bullet}$), that cause both transitions and transversions of AT base pairs and increase *trpE65* → Trp^+ reversions (Urios and Blanco, 1996). Vitamin E, a strong antioxidant possessing high potential to reduce *t*-BOOH-induced mutagenesis, was used as positive control. Strain IC202 is an *oxyR* mutant deficient in removing ROS, and thus more sensitive for detection of the antimutagenic potential of antioxidants. The OxyR protein is a redox-sensitive transcriptional activator of genes encoding antioxidative enzymes: catalase-hydroperoxidase I, alkyl hydroperoxide reductase and glutatione reductase. The strain IC185, proficient in antioxidative defense, was used as a control.
Concentration of 0.5 µl/p of EO showed reduction of *t*-BOOH-induced mutagenesis (35%) while higher concentration exhibited stronger antimutagenic effect but also the decline of viability (Table 2). Antimutagenic potential of Linalool against *t*-BOOH-induced mutagenesis was shifted towards higher concentrations (58% of inhibition for 1 µl/p) with lower lethality effects and was almost the same as that obtained with Vitamin E (61% of inhibition). Considering that IC202 strain is deficient in induction of antioxidative enzymes, obtained results could indicate that antimutagenic potential of basil derivatives is probably attributed to their antioxidative properties (ROS scavenging mechanism). This is consistent with previously reported data about antioxidative activities of basil and Linalool (Celik and Özkaya, 2002; Javanmardi *et al*., 2003; www.ncl.ac.uk/medplant).
Both EO and Linalool exhibited antimutagenic potential against spontaneous mutagenesis. Maximum of spontaneous mutagenesis inhibition obtained with nontoxic concentrations was 32% for the oil and 43% for Linalool (Table 2). Inhibition of spontaneous mutagenesis indicates that other mechanisms of antimutagenesis, except scavenging of ROS, are also involved.
Toxic effect of EO and Linalool observed at the same concentrations in both $OxyR^-$ and control $OxyR^+$ strain (data not shown) indicate that lethal effect is not a consequence of oxidative DNA damage. The most probable explanation for cytotoxicity of Linalool and EO is destruction of cell membranes, effect which is shown for many plant volatiles in bacterial model systems (Stammati *et al*., 1999).

Table 2. Antimutagenic effect of basil derivatives against spontaneous and *t*-BOOH-induced mutagenesis in IC202 (*oxyR*)

	- *t*-BOOH				+ *t*-BOOH			
Substance	Viable cells/p[a]	S (%)[b]	Trp^+ reverts/p[c]	M (%)[d]	Viable cells/p[a]	S (%)[b]	Trp^+ reverts/p[c]	M (%)[d]
0	110±4		148±8		117±5		578±7	
Ethanol	123±6	**100**	138±7	**100**	124±9	**100**	561±10	**100**
EO (µl/p)								
0.25	116±3	**94**	135±5	**98**	106±7	**85**	427±20*	**76**
0.5	116±4	**94**	94±3*	**68**	106±3	**85**	365±16*	**65**
1.0	73±5*	**59**	94±4*	**68**	70±3*	**56**	282±3*	**50**
Lin (µl/p)[e]								
0.25	118±5	**96**	119±6	**86**	120±7	**97**	434±7*	**77**
0.5	119±2	**97**	104±3*	**75**	120±3	**97**	360±18*	**64**
1.0	116±3	**94**	79±5*	**57**	96±5	**77**	234±13*	**42**
Vit E (mM)								
1.5	108±3	**88**	116±7	**84**	115±6	**93**	217±6	**39**

[a]Viable cells/ml = viable cells/p x 10^7; [b]S – survival (%S = Nt/Nc; Nt-sample with tested substances, Nc-control sample (ethanol)); [c]Trp^+ reverts/ml = Trp^+ reverts/p x 10; [d]M – mutagenesis (%M = Nt/Nc; Nt-sample with tested substances, Nc-control sample (ethanol)); [e] Linalool 1 µl/p ~ 0.2 mM. Concentration of *t*-BOOH was 25 µg/p (10 µM). The results presented in figures and tables are expressed as the means obtained from three independent experiments, with the standard error of the mean. *p<0.05

In eukaryotic reversion test on *S. cerevisiae* D7 EO and Linalol showed no effect on spontaneous mutagenesis but co-mutagenic effect with *t*-BOOH. *t*-BOOH-induced mutagenesis was increased up to 1.6 fold in the presence of Linalool and up to 2.7 fold in the presence of EO (Table 3).

Table 3. Antimutagenic effect of basil derivatives against *t*-BOOH-induced mutagenesis in *S. cerevisiae* D7

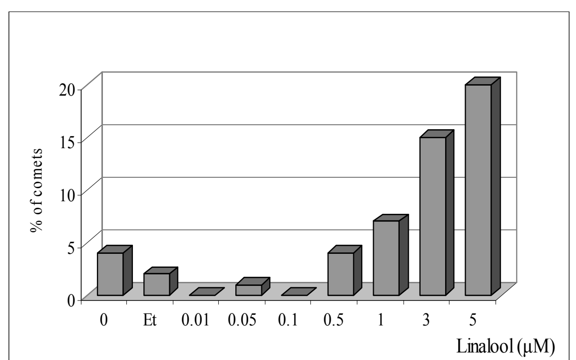
Substance		Viable cells/p[a]	S (%)[b]	Ilv^+ reverts/p[c]	M (%)[d]
0		145±4		60±5	
Ethanol		146±4	**100**	62±4	**100**
EO (µl/p)	0.5	144±3	**99**	78±3*	**126**
	1.0	155±9	**100**	113±8*	**182**
	2.0	146±1	**100**	169±1*	**273**
Linalool (µl/p)[e]	0.5	144±2	**99**	74±3	**119**
	1.0	116±7*	**79**	93±13*	**150**
	2.0	112±3*	**77**	99±7*	**160**
Vit E (mM)	3	135±1	**93**	43±6	**69**

[a]Viable cells/ml = viable cells/p x 10^6; [b]S – survival (%S = Nt/Nc; Nt-sample with tested substances, Nc-control sample (ethanol)); [c]Ilv^+ reverts/ml = Ilv^+ reverts/p x 10; [d]M – mutagenesis (%M = Nt/Nc; Nt-sample with tested substances, Nc-control sample (ethanol); [e]1 µl/p ~ 0.2 mM. Concentration of *t*-BOOH was 500 µg/p (200 µM). The results presented in figures and tables are expressed as the means obtained from three independent experiments, with the standard error of the mean. *p<0.05

Co-mutagenic effect with *t*-BOOH is most probably a consequence of their synergistic effect in releasing of ROS due to the damage of mitochondrial membranes. This is consistent with previously reported data about damaging effects of some essential oils on mitochondrial and cell membranes in yeast (Bakkali *et al.,* 2005).

Comet assay

To test protective effects of Linalool against oxidative DNA damage we used alkaline yeast comet assay, performed on *S. cerevisiae* cells (Miloshev *et al*., 2002). Hydrogen peroxide was used to induce DNA strand breaks. By testing increasing concentration of H_2O_2 we established that concentration of 250 μM of H_2O_2 induced the highest number of comets without effect on cell viability (data not shown). Knowing that antioxidants may exhibit pro-oxidative effect (Labieniec *et al*., 2003; Azam *et al*., 2004) in preliminary experiments we tested different concentrations of Linalool (concentration range 0.01-5 μM). Increased concentrations of Linalool increased the number of comets relative to the control, presumably due to its pro-oxidative potential (Fig. 1, left).



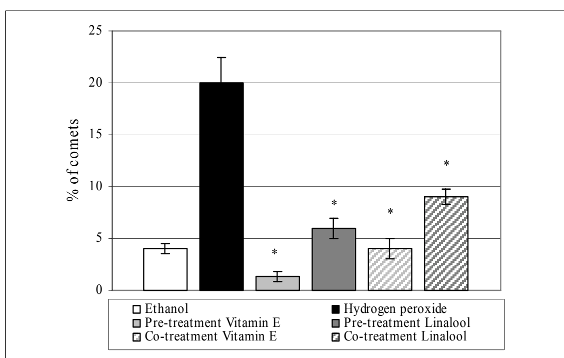


Fig. 1. Effect of Linalool on spontaneous (left) and H_2O_2-induced (right) oxidative DNA damage in yeast cells. Applied concentrations: H_2O_2 (250 μM), Vitamin E (0.05 μM), Linalool (0.5 μM). The presented values are averages of three independent experiments. $^{*}p<0.05$.

The highest concentration of Linalool that did not induce strand breaks (0.5 μM) was used in antioxidant pre-treatment as well as co-treatment experiments. Co-treatment predominantly detects scavenging of ROS, while pre-treatment could point at additional beneficial effects of antioxidant cell pool together with scavenging action. As in reversion tests, Vitamin E (0.05 μM) was used as a positive control. Applied concentration of Linalool significantly decreased the number of H_2O_2-induced comets. In comparison with Vitamin E, Linalool was slightly less effective (Figure 1, right). Stronger inhibitory effect of Linalool against H_2O_2-induced damage in pre-treatment in comparison with co-treatment, indicates that except scavenging of ROS, there is an additional effect, which is probably related to the increase of antioxidant cell pool and/or induction of antioxidative and DNA repair enzymes.

Conclusions

Considering the obtained results, it could be concluded that basil derivatives possess antimutagenic potential, based predominantly on their antioxidative properties, comparable with that obtained with Vitamin E. Nevertheless, they could also cause oxidative damage and mutagenic effect, depending on the dose and test system.

The inhibitory effect of tested compounds was shown in *E. coli* WP2 reversion test against spontaneous and *t*-BOOH-induced mutagenesis, as well as against H_2O_2-induced oxidative DNA damage in comet assay. Inhibition of spontaneous mutagenesis by both derivatives indicates that other mechanisms of antimutagenesis, except scavenging of ROS, are also involved. Stronger protective effect of Linalool against H_2O_2-induced damage in pre-treatment variant of comet test pointed out the additional effect, which is probably related to

the increase of induction of the genes involved in antioxidative defense. However, higher concentrations of Linalool might saturate antioxidative defense mechanisms, resulting in genotoxic injury. Co-mutagenic effect of both derivatives with *t*-BOOH is probably a consequence of their synergistic effect in damaging yeast mitochondrial membranes and releasing of ROS, which could increase oxidative stress.
Regarding the detected antimutagenic potential against oxidative mutagenesis and protective effect against induced oxidative DNA damage, EO of basil and Linalool could be recommended for further testing on higher organisms. Considering the observed adverse effects in eukaryotic tests, detailed *in vivo* study on mammals and human cultured cells are necessary.

Acknowledgements

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ALKALOID PLANTS AS INDICATORS OF THE SOIL CONTAMINATION

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Summary

In six samples of three plant species, which could be used as indicators of the anthropogenic soil contamination, tropane alkaloids were determined. Different parts of thorn apple (Datura stramonium L.), belladonna (Atropa belladonna L.), and henbane (Hyosciamus niger L.) were collected at two locations of Southern Serbia, and subjected to determination of atropine and scopolamine contents. These contents in different plant parts of tested wild growing plants were determinated by GC/MS and considered as reference for non-contaminated soil.

Keywords: *thorn apple, belladonna, henbane, soil contamination, tropane alkaloids.*

Introduction

Realisation of the right of the man to live in adequate life environment and in wellness starts from the system of the environment protection, where environment means the sum of the natural and created values, where their complex mutual relations makes the surroundings, i.e. the space and conditions for life. The system of environment protection makes measures, conditions and instruments for holding control of natural values and preventing, controlling, reducing and restoring against all forms of its pollution.
Special place in protection of the natural values is protection of biosphere and protection of biodiversity. Biodiversity is diversity of the organisms in scope of the species, between the species and between ecosystems and comprise the complete diversity of genes, types and ecosystems at the local, national, regional and global level. Protection of biosphere comprises of protection of the organisms, their communities and habitats including preservation of the natural processes and natural balance inside the ecosystems, with providing of their survival. Biodiversity and biological resources are protected and used in a way that makes possible their survival, diversity, renewal and advancement.
Monitoring is the integral part of the unique information system of the environment management. Monitoring is done by the systematic measuring, analysing and evaluation of the condition indicators and pollution of the life surroundings that consists of the following of the natural factors, i.e. condition changes and characteristics of the environment, cross-border monitoring of air, waters, land, forests, biodiversity of flora and fauna, climate elements, ozone layer, ionising and non-ionising radiation, noise, waste, early announcement of accident with following and evaluation of pollution development of the life surroundings, and also overtaken obligations from international contracts.
One of the basic conditions of environment quality control is good set and efficacy of monitoring system. The best indicator of damaging biological diversity is disappearance of the organic species and natural ecosystems. It should be understand that the natural replacement by the adjusted forms and systems, or final extinction of some of them, is developed over the long-term evolution and in accordance with the laws of nature. On the contrary, man by his side influenced on course and speed of the changes in nature. Fastest and the most direct changes in the environment i.e. ecosystems, resulting in disappearance of the numerous plant species. The right understanding of biodiversity became the main motive and reason of preservation, measure of behaviour of the modern man and only possibility for the complete protection of nature on the planet.

Indicator plant species

Specialised plant species of the particular metabolites, i.e. physiological forms, and characteristic morpho-anatomical adaptation, inhabiting the land with the specific or common characteristics. Among the numerous plants species there are certain species, which are adjusted during the period of evolution and survived at the certain habitats on very specific foundation and soil.

German phytoecologist Elenberg 1974 first ranked the plants according to degree of correlation for the certain type of soils, pH of soil, their needs toward the certain mineral elements, light, humidity, as well as for other factors that affects the habitats. Therefore, some of plant species are very useful indicators of the specific geological foundation, quality and soil characteristics, underground waters or some chemical compounds and even elements in traces and toxic substances in certain soil. Consequently, those bio-indicator plant species are used to follow up the changes at soils in relation with the presence of the heavy metals, organic waste, radionuclides, etc.

Certain plants that verifiably and specifically indicates by its presence general appearance and adaptive characteristics, on edaphically as well as to general climate conditions of the area where they are, representing the indicator species. In anthropologically changed areas, plants can be very powerful indicators of the endangered ecological relations on the given area. In accordance with its specific needs and genetically determined possibilities, some plants, in the polluted areas or anthropologically disturbed areas, could react by alternative changes in main functional and structural characteristics, or accumulating certain specific metabolites. According to this, indicator organisms could survive in specific way, by changing environmental conditions, or gradually disappear from the certain area. The change in floristic composition and disappearance of the plant species from the certain localities extremely precisely indicates to long term changes in the ecosystem.

Plants on organic waste rich soil

On the soils enriched with the organic waste, resulting from the intensive human activities, at the waste lands around the cities, or other permanent of temporary human or domestic animal residential areas, along the railroads, roads or on the dams, there are ruderal plants. From the certain point of view, ruderal plants belongs to weeds, considering that they are growing at the urban places under the close care of man (parks, gardens and playgrounds). These plants are almost greenish, rarely bushy type, fast developing in different period of year and efficacy take over anthropogenic changes regions. They have intensive affinity toward a large quantity of nitrite, phosphorus, sodium and potassium salts, substances originated from the organic waste and residues of different organisms on such damaged, unstable or initial, so called ruderal soils. Therefore they are also called nitrophylus plants. Ruderal plants belong to the numerous families and species, *Chenopodium*, *Atriplex*, *Amaranthus*, *Cirsium*, *Rumex*, *Polygonum*, *Urtica*, *Parietaria*, *Setaria*, *Solanaceae* and others.

Biochemical indicators

In scope of the biological monitoring, biological indicators represents the base for the evaluation of the quality of the environment. These indicate the presence of the polluted substances, which expresses the ecological changes in terrestrial systems, giving a cumulative picture of the pollution effects, i.e. environment quality.

As the reaction of the certain organisms and populations, resistance toward certain pollutants can be very important. The lack of the sensitive or presence of the resistible plants may be clear indication of pollution, not only the cumulative, but also a specific one. In terrestrial ecosystems, some plants are important as bioindicators because of clear response and

symptoms to certain pollutants. Chemical analyses are used in cases when there is a positive correlation between concentration of pollutants in the environment and marker organisms. Usually after biochemical changes, in the cases of intensive pollution and longer exposition, there are structural anatomical-morphological changes and disturbance of physiological processes. It is known that physiological manifestations are useful for biological monitoring and that some modification in metabolism can give specific reaction to some pollutants, which can be recorded before manifestation of the visible damages.

Research topic

Soil pollution appears when the large quantities of the waste material, which cannot be easily degraded, is distributed over the soil surface. The excessive soil pollution by the organic and inorganic compounds in unique ecosystems leads to disturbance of the normal processes in soil and its degradation. Such the soil is usually enriched in nitrogen and humus, and could be good base for its inhabitation with certain ruderal plants, and among them some of the members of the family *Solenaceae*.

This study is continuation of our investigation of anthropogenic polluted soil effects on the yield of tropane alkaloids with widely spread species *Datura stramonium* L. It was found that this plant is a good bioindicator of the anthropogenic-polluted soil. The subject of this study was also the other ruderal plant, *Hyosciamus niger* that grows on deserted nitrogen enriched soil around the cities. It is not so abundant as *D. stramonium* and contains 0,05-0,14% of the total tropane alkaloids.

Atropa belladonna, one of the most poisonous plants, contains about 0,3% of the total tropane alkaloids, where hyosciamin is the most abundant one. It grows on bare mountainous terrain, appearing after the beech forest illegal cut.

The aim of this contribution was the determination of tropane alkaloids (atropine and scopolamine) in three ruderal plant species, which could be used as specific indicators for detection of contaminated soil by organic waste.

Materials and methods

Plant material

Samples of targeted indicator plant species, thorn apple (*Datura stramonium* L.), belladonna (*Atropa belladonna* L.) and henbane (*Hyosciamus niger* L.), were collected at two localities of Southern Serbia, Vratna and Ploče, in the phase of full maturity. After drying properly at the room temperature, selected plant parts were carefully separated, cut and processes, according to alkaloids extraction procedure, described bellow.

Isolation of alkaloids

Into the round-bottom flask of 1000 ml, 20 g of sample (dried herbal drug) and 500 ml of chloroform-ammonium hydroxide mixture (24:1, V/V) is transferred and extracted under reflux for 1 hour at the boiling temperature. After removing condenser, content of the flask was cooled for 15 minutes, putting the flask in tank with cool water. Furthermore, extract was filtrated, and remaining solid residue extracted once again, under the same conditions. After filtration, and combining of both extracts, solvent was removed by evaporation, and resulting residue was dried in vacuum, at 40 °C, till constant mass.

Determination of scopolamine and atropine

Calibration

For the preparation of reference solutions, analytical standards of atropine (Roth, Germany) and scopolamine hydrobromide (Roth, Germany), were used. Whilst for the preparation of

atropine stock solution appropriate analytical standard was just measured and dissolved in chloroform, scopolamine was previously isolated from its salt by multiple chloroform extraction from alkaline solution. From stock solution of atropine (107.1 mg/10 ml) and scopolamine (105.6 mg/10/ml) in chloroform, a series of five reference solutions containing atropine and scopolamine in concentrations ranging between about 20 µg/ml and about 2000 µg/ml, were prepared. Exact concentrations of atropine and scopolamine in these solutions were 24.42 µg/ml and 21.12 µg/ml (1°), 42.84 µg/ml and 42.24 µg/ml (2°), 214.2 µg/ml and 211.2 µg/ml (3°), 428.4 µg/ml and 422.4 µg/ml (4°), and 2142 µg/ml and 2112 µg/ml (5°), respectively.

Preparation of sample solution

Sample solutions for GC determination of tropane alkaloids were prepared just by dissolving crude measured extracts of tested plants in chloroform. Concentrations of prepared sample solutions were about 2.5-3.5 mg of extract per millilitre.

Gas Chromatography

Reference and sample solutions were analysed by combination of gas chromatography and mass spectrometry (GC/MS). For this purpose HP G1800C Series II GCD (EID), equipped with split-splitless injector and automatic liquid sampler/injector (ALS), HP-5MS fused silica capillary column (30 m · 0.25 mm · 0.25 µm film tickness), and mass selective detector (MSD) operating in standard electron impact mode (70 eV) in m/z range 40-450 was employed. Chromatographic conditions were as follows. Injector temperature was 250°C, detector temperature 280°C, while column temperature was linearly programmed from 40-220°C, at the rate of 8°/min. Carrier gas was helium (1 ml/min). Injections of reference and sample solutions were carried out by ALS (1 µl) in splitless mode in three repetitions.

Results and discussion

Through the processing of raw chromatographic data for calibration runs, two calibration curves, the first for determination of atropine, and the second for determination of scopolamine (presented in Figures 1 and 2) were obtained. Both of these curves showed good linearity in the range of concentrations of about 20-2000 µg/ml, and should be considered as suitable for determination of targeted alkaloids.

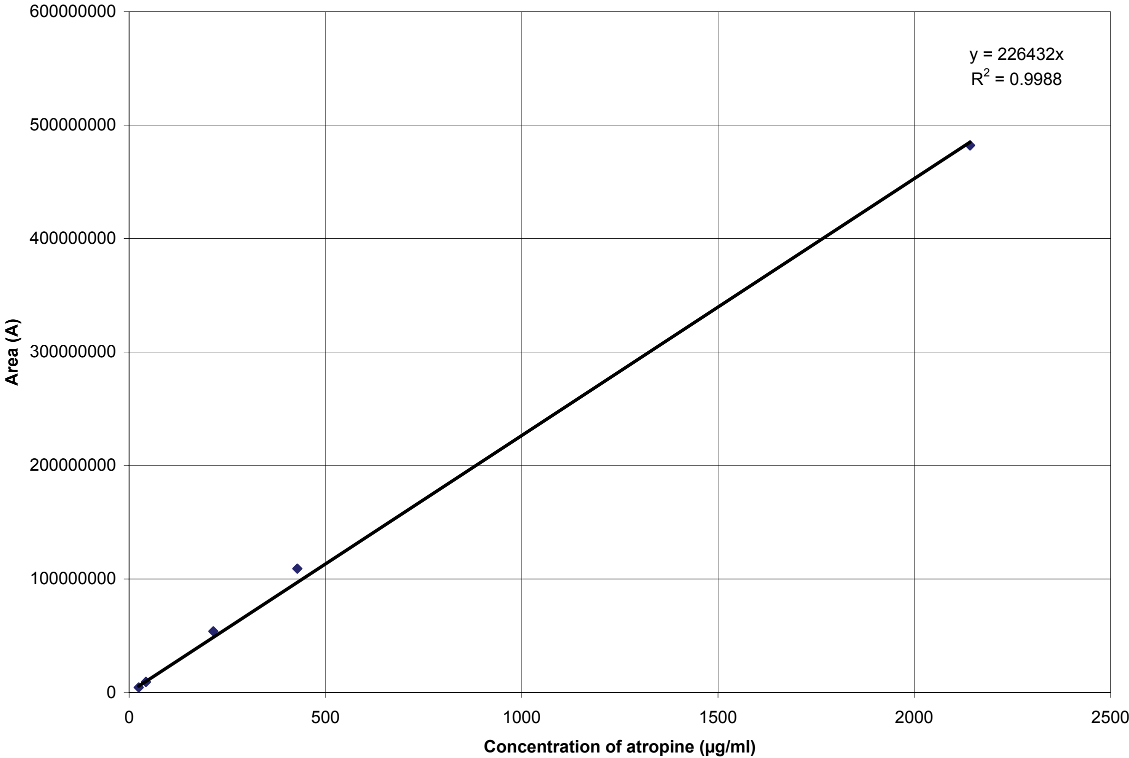

Fig. 1. Calibration curve for determination of atropine [GC/MS (TIC)-ESD]

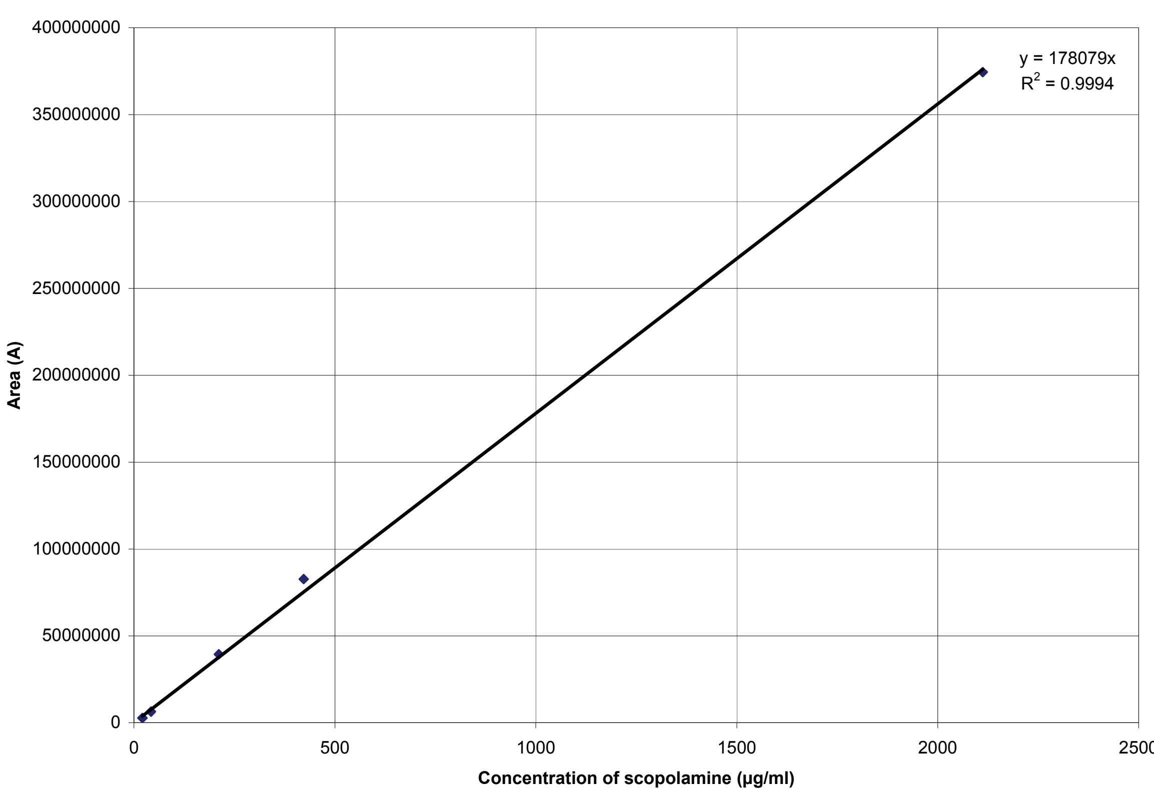

Fig. 2. Calibration curve for determination of scopolamine [GC/MS (TIC)-ESD]

Further, after processing of data from sample chromatograms, results on the contents of atropine and scopolamine in tested samples listed in Table 1 were obtained.

Table 1. Results of determination of tropane alkaloids in tested samples

Sample	Plant part	Locality	Extract (%)	Atropine (%)	Scopolamine (%)
Atropa belladonna	*folium+fructus*	Vratna	5.9	1.72 (29.16)*	0.09 (1.51)*
Datura stramonium	*semen*	Vratna	12.6	0.27 (2.15)*	0.05 (0.38)*
Datura stramonium	*folium+fructus*	Vratna	4.1	0.24 (5.75)*	0.06 (1.35)*
Datura stramonium	*fructus*	Vratna	1.9	0.41 (21.60)*	0.13 (7.09)*
Hyosciamus niger	*fructus+flores*	Ploča	5.3	0.05 (0.96)*	0.08 (1.44)*
Hyosciamus niger	*folium*	Ploča	4.6	0.05 (1.15)*	0.06 (1.30)*

*Content of appropriate alkaloid in the extract in given in the brackets.

Conclusion

In six samples of indicator plant species, thorn apple (*Datura stramonium* L.), belladonna (*Atropa belladonna* L.) and henbane (*Hyosciamus niger* L.), in different plant parts, content of major constitutive alkaloids (atropine and scopolamine) was determined by GC/MS using ESD calibration method.

The biggest content of atropine (1.72%) was recorded in belladonna (leaves and fruits), and the lowest (0,05%) in both henbane samples. Simultaneously, in three of thorn apple samples, atropine content varied from 0.24% (leaves and fruits) till 0.41% (fruits).

Sample of scopolamine in the all tested samples was rather uniform (0.05-0.13%), exceeding 0.1% (0.13%) only in the case of thorn apple fruits.

Based on the results of the chemical analyses of alkaloids, targeted plant species could be accepted as appreciable indicators of anthropogenic polluted soil.

COMPARATIVE PHYTOCHEMICAL STUDY ON *ERYNGIUM* SP. FROM ROMANIA

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Summary

The aim of the present paper was to highlight the similarities and differences among the aerial parts of indigenous Eryngium sp.: Eryngium planum L., E. campestre L. and E. maritimum L. By using the officinal spectrophotometric methods, we determined the total content of: flavonoids, polyphenols as caffeic acid type and tannins. The contents of pectins and triterpene saponins were determined by gravimetric method.

Keywords: *Eryngium sp., Apiaceae, polyphenols, triterpene saponins, pectins*

Introduction

Eryngium genus (Apiaceae) is represented by 317 species, subspecies and varieties (1). In the flora of Romania, there are only 3 species belonging to *Eryngium* genus: *Eryngium planum* L. (also known in Romanian folk-medicine as "scai vânăt"), *E. campestre* L. (known as "scaiul dracului") and *E. maritimum* L. (known in Romania as "vitrigon") (2). Infusions and decocts of the aerial and root parts of *Eryngium* sp. are used in the folk medicine as antitussives, expectorants and diuretics (3). Among the 3 species, *Eryngium planum* is the only one recognized by the phytotherapy of Romania as medicinal plant. Drug *"Eryngii plani herba"* is a popular remedy used for treating whooping cough due to the calming, cough-relieving, and antispasmodic effects (4). Previous data on this plant confirmed that the triterpene saponins are the active principles responsible for the diaphoretic, expectorant, depurative and diuretic effects (5).

Eryngium campestre and *E. maritimum* were in the past popular medicinal herbs used in W. European folk medicine for its anti-scorbutic, diaphoretic, diuretic, expectorant, carminative, mild stimulant, antitussive, anti-inflammatory and aphrodisiac properties. Contemporary European herbal medicine recommends the aerial and root parts of these 2 species strictly as diuretics in renal disorders (cystitis, urethritis, chronic prostatitis) and for preventing kidney stones formation (6).

The folk use and the relatively few scientific data on these species, prompted us to study the chemical composition of *Eryngium planum*, *E. campestre* and *E. maritimum* from Romania.

Material and methods

Plant material

Aerial parts of *Eryngium planum* and *E. campestre* were collected in July 2004 from Jucu, Sălaj, Romania while the aerial parts of *E. maritimum* were collected in June 2004 from Eforie Nord, Romania.

Total content of flavonoids

Total content of flavonoids expressed as rutin was determined by spectrophotometry at 430 nm, according to IX th Romanian Pharmacopoeia officinal technique described to *"Cynarae folium"* monography (7).

HPLC method for screening and quantification of flavonoids (glycosides and aglycones) and phenolic acids

A HPLC method is available for qualitative and quantitative determination using 18 phenolic compounds as reference standards: caftaric acid, gentisic acid, caffeic acid, chlorogenic acid, p-coumaric acid, ferulic acid, sinapic acid, cichoric acid, hyperoside, ellagic acid, isoquercitrin, rutin, quercitrin, quercetol, patuletine, luteolin, kaempferol and apigenin (8).

HPLC was carried out on an Agilent 1100 HPLC Series (Agilent, USA) equipped with a degasser G1322A, a quaternary gradient pump G1311A, an autosampler G1311A, a column oven G1316A, a Zorbax SB-C18 reversed-phase analytical column 100 mm x 3.0 mm i.d., 3.5 µm particle (Agilent, USA) and an UV HP 1100 Series detector. We operated at 48^{o}C and the UV detector was set at 330 nm. The mobile phase was a binary gradient: methanol and buffer solution. Buffer solution consisted in 40 mM KH_2PO_4 aqueous solution adjusted to pH 2.3 with 85% phosphoric acid. The gradient begun with a linear gradient started at 5% methanol and 42% methanol over the first 35 minutes, followed by isocratic elution with 42% methanol over the next 3 minutes. The flow rate was 1 mL/min and data were collected at 330 nm. The injection volume was 10 µL. Methanol extracts partitioned successively with ethyl ether, ethyl acetate and n-butanol were used as samples (8). All compounds were identified by external standard method by comparing theirs retention times with those of standards. Quantitative determinations were performed using the external standard method, as well.

Total content of polyphenols as caffeic acid type

The officinal spectrophometric method at 500 nm with Arnow reagent was employed (7).

Tannin content

Tannin content was calculated by the officinal technique described in the Xth Romanian Ph. (9).

Extraction and isolation of crude saponins

The method elaborated by Grecu and Cucu was employed (10). Briefly, plant material (25 g), previously defatted with chloroform was extracted with methanol (2 x 250 ml) under reflux for 1h. The combined extracts were concentrated in a rotavapory, at 35^{o}C to a syrupy liquid, which was dropped, while stirring, in 500 ml acetone when saponin precipitated. After filtration *in vacuo,* crude saponin was dried in an exicatory over anhydrous $CaCl_2$ followed by gravimetric determination. Purification of saponins employed dissolving of crude saponins in hot methanol, followed by re-precipitation in acetone.

TLC-densitometry of saponins

TLC-densitometry for quantitative analysis of triterpene saponins was developed (11, 12).

TLC-densitometry employed precoated silica gel plates $60F_{254}$ (Merck). The TLC solvent system $CHCl_3$-MeOH: H_2O (70:44:10) was used (13). The spray reagent for saponins was Liebermann-Burchard reagent (14). For detection and quantification, scanning densitometry was carried out on a Desaga CD60 densitometer (Sarstedt, Germany) in reflectance mode at 666 nm representing the absorption maximum of the resulted compounds after spraying, with a slit of 0.2 x 6 mm. 0.5% ethanol (50^{0}) solutions of *Eryngium* saponins were spotted as samples.

Extraction of sapogenins

Crude saponins (0.04 g) were refluxed with 25 mL HCl 2N, 1 mL dioxan and 3 mL benzene, on a boiling steam bath, for 4 h (13). After cooling, 3 successive extractions were carried out in a separating funnel (3x10 mL $CHCl_3$). The organic layers were filtered through anhydrous Na_2SO_4 and then were concentrated to 2mL, approximately. This last solution was further subjected to TLC analysis.

TLC analysis of sapogenins

Qualitative TLC for sapogenins employed precoated silica gel plates $60F_{254}$ (Merck). For sapogenins, the TLC solvent system $CHCl_3$-MeOH (10:1) was used (13). The spray reagent

was 20% phosphowolframic acid in ethanol (15). The $CHCl_3$ solutions of the *Eryngium*-sapogenins and 0.1% ursolic acid (Roth) in ethanol were used as samples and standard solution, respectively.

Extraction and isolation of pectins

The repeated water extraction method was employed (16). Briefly, plant material (previously defatted with chloroform and extracted with methanol) was extracted with water (1: 10 parts) under reflux, on a boiling steam bath, for 30 min. The combined filtrates were concentrated to 1: 5. This last solution was dropped, while stirring, in 500 mL 1% acetic acid ethanol when pectins precipitated. After filtration *in vacuo,* pectins were dried in an exicatory over anhydrous $CaCl_2$ and pectic content was determined by gravimetric method.

Acid hydrolysis of pectins

50 mg pectin and 5 mL H_2SO_4 4 % introduced in a glass ampoule were maintained in a boiling steam bath for 2h (16). After cooling, ampoule's content was neutralized by adding $BaCO_3$. $BaSO_4$ precipitate was removed by filtering. The filtrate was concentrated to dryness, and the residue was dissolved in 2 mL of an H_2O-MeOH mixture (9: 1) and was then centrifuged for 10 minutes (3000 rpm). The supernatant was used for TLC.

TLC Analysis of the compounds resulted after acid hydrolysis of pectins

TLC employed precoated silica gel plates $60F_{254}$ plates (Merck). For sugars, the TLC solvent system n-BuOH-acetone-Na_2HPO_4 1.6% solution (4: 5: 1) upper layer was used (17). For uronic acids, solvent system n-BuOH: acetic acid: H_2O (50: 25: 25) was used (18). The spray reagent was timol/H_2SO_4 (14).

Results and discussions

Total content of flavonoids expressed as rutin varied from 0.325% in *E. maritimum* and 0.328% in *E. campestre*, respectively to 0.562% in *E. planum*.

Important differences were observed in the total content of polyphenols calculated as caffeic acid among the 3 *Eryngium* sp.: 0.27% in *E. planum*, 0.53% in *E. maritimum*, being highest in *E. campestre* (1.39%).

Content of tannins was 0.85% in *E. planum*, 0.975% in *E. maritimum*, being highest in *E. campestre* (1.34%).

Total content of crude saponins determined by gravimetric method was 3.7% in *E. campestre*, 4.2% in *E. planum*, being highest in *E. maritimum* (10.1%).

Pectic content determined by gravimetric assay varied considerably among the 3 species: 1.7% in *E. campestre*, 4.65% in *E. planum*, being highest in *E. maritimum* (8.8%).

14 polyphenol compounds were identified and quantified in indigenous *Eryngium* sp. (Table 1): caffeic acid, chlorogenic acid, p-coumaric acid, ferulic acid, cichoric acid, caftaric acid, isoquercitin, quercitrin, hyperoside, rutin, quercetol, kaempferol, apigenin and luteolin. HPLC chromatogram of standards mixture was presented in Figure 1. HPLC chromatogram of the ethyl acetate fraction from *E. maritimum* was presented in Figure 2. Results confirmed that caffeic acid, quercitrin and rutin are common constituents to all tested *Eryngium* sp. Considerable differences were observed among *Eryngium* sp. in the content of these 3 polyphenols. Thus, cafeic acid content varied from 1.44 to 10.28%, being highest in *E. maritimum.* Quercitrin content varied from 8.09 to 270.675%, being highest in *E. maritimum*, while rutin content varied from 6.137 to 14.56%, being highest in *E. campestre* (Figure 3).

TLC-densitometry for saponins yielded 4 saponosides in *E. planum*, 3 in *E. campestre* and 5 in *E. maritimum*; 2 fractions were common to all three species (Table 2). By comparing these results, we observed some similarities among the tested 3 *Eryngium* saponins. Thus, the presence of 2 common saponosides to all 3 tested *Eryngium* saponins (Rf-value: 0.61, 0.67) was confirmed. In addition, saponoside fraction (Rf 0.50) proved to be common to *E.*

campestre and *E. maritimum*, while saponoside (Rf 0.73) was common to *E. planum* and *E. maritimum*. TLC-densitogram of a 0.5% solution of *E. maritimum* saponin was presented in Figure 4.

TLC for sapogenins yielded 4 pink fractions in *E. planum* (Rf: 0.43, 0.50, 0.56, 0.68), 1 pink fraction in *E. campestre* (Rf 0.43) and 7 fractions in *E. maritimum* (Rf-value: 0.37, 0.43, 0.50, 0.56, 0.68; 0.81, 0.87). Ursolic acid (Roth) showed a characteristic pink spot (Rf 0.62) to triterpene framework.

TLC for compounds resulted after acid hydrolysis of pectins yielded the same 4 characteristic red spots for sugars attributed to galactose (Rf 0.26), glucose (0.33), xylose (0.54) and rhamnose (0.69) in all 3 tested *Eryngium* sp. Galacturonic acid (brown spot Rf 0.48) was also detected in all *Eryngium* sp.

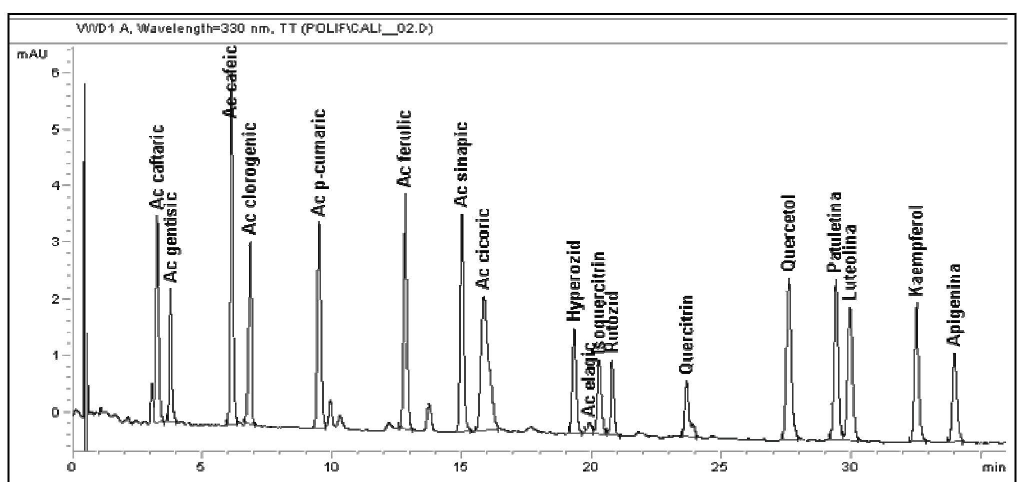

Fig. 1. HPLC chromatogram of standards mixture (1µg/mL standard)

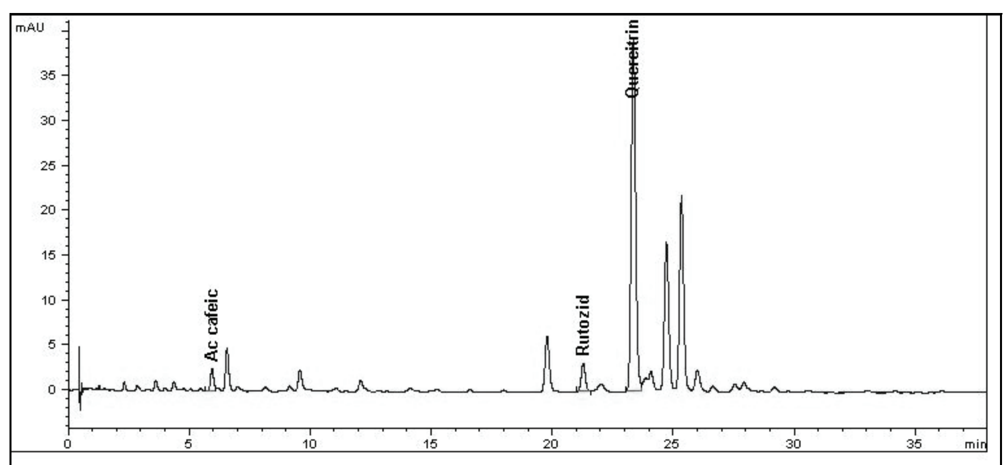

Fig. 2. HPLC chromatogram of the ethyl acetate fraction from *E. maritimum*

Table 1. Distribution of identified polyphenols (%) in *Eryngium* sp. by HPLC means

Identified compound (g/100 g plant material)	*E. planum*	*E. campestre*	*E. maritimum*
Caffeic acid	1,52	1,44	10,28
Chlorogenic acid	27,575	11,757	-
p-coumaric acid	1,63	-	-
Ferulic acid	7,865	-	-
Cichoric acid	0,48	-	-
Caftaric acid	-	0,959	-

Isoquercitrin	83,83	20,116	-
Quercitrin	8,09	80,275	270,675
Hyperoside	2,42	7,961	-
Rutin	6,137	24,902	14,56
Quercetol	-	1,587	-
Luteolin	-	2,407	-
Kaempferol	2,17	-	-
Apigenin	3,535	2,555	-

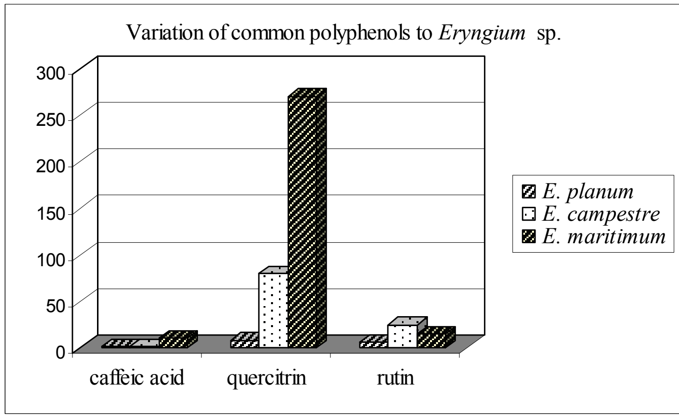

Fig. 3. Distribution of common polyphenols (%) to *Eryngium* sp.

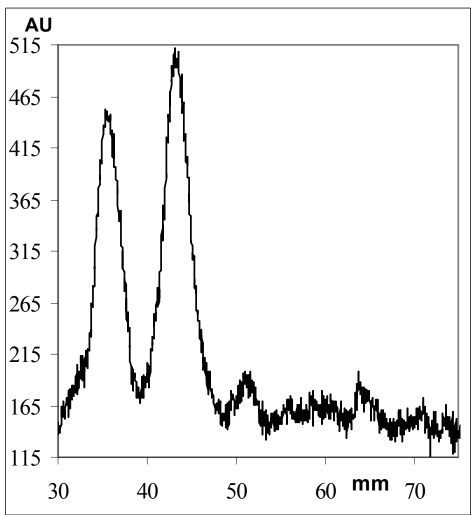

Fig. 4. TLC-densitogram of a 0.5% solution of saponin isolated from *Eryngii maritimi herba* in etanol 50^0

Table 2. Saponosides from *Eryngium* saponins as determined by TLC – densitometry

0,5% *E. planum* saponin in EtOH 50^0				0,5% *E. campestre* saponin in EtOH 50^0			0,5% *E. maritimum* saponin in EtOH 50^0		
Nr.	R_f	Area	%	R_f	Area	%	R_f	Area	%
1	0,36	578,1	20,5	0,51	319,6	11,15	0,50	739,3	30,17
2	0,62*	1929,6	68,9	0,61*	199,0	6,7	0,60*	1237,6	50,51
3	0,65*	278,6	9,9	0,67*	2346,3	81,9	0,67*	97,4	4
4	0,73	31,6	1,1				0,75	62,3	2,54
5							0,83	201,4	8,22
6							0,91	111,9	4,5

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THE ANALYSIS OF *ARNICAE FLOS* FROM ROMANIA ACCORDING TO THE EUROPEAN PHARMACOPOEA

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Summary

Arnicae flos is an important drug, harvested from Apuseni Mountains (Cluj and Alba Counties) and exported to the western European countries. That is the reason why we performed an analysis of Arnicae flos, according to the stipulations of European Pharmacopoeia. We determined by HPLC that the content of total sesquiterpene lactones expressed in tiglate of helenaline, varied between 0,74% (Plafar, Cluj) and 1,08 (WWF Garda de Sus), thus a higher content than Eu. Ph. Stipulations (0,4%).

Keywords: *Arnica montana, Arnicae flos, sesquiterpene lactones, HPLC*

Introduction

The blossoms of *Arnica montana* L. (Asteraceae) are one of the most important drug in phytotherapy. The richest natural basins from Romania are located in Apuseni Mountains in Alba and Cluj Counties. The drug is exported to the western European countries. That is why we analyzed the Romanian drug by the stipulations of European Pharmacopoeia (2002).
The blossoms of *Arnica montana* consisted in peduncles (2-3 cm), the involucre of two ranges of bracts, the receptacle, 2-20 ligulate florets and the tubular florets. The ovary bear hairs that generate the pappus by drying [1].
In the chemical composition of *Arnicae flos* were identified 0.2-0.8% sesquiterpene lactones of pseudoguaianolide type with considerable variation depending of the origin; 0.4-0.6% flavonoids; 0.2-0.35% essential oil with a buttery conistency; cinamic acid derivatives (chlorogenic acid, cynarine, caffeic acid); coumarines, polyacetylens, choline, carotenoids [1,2,3]. Helenaline is considered the main active principle [5].
Arnicae flos and the pharmaceutical specialties prepared from this drug has wound healing properties but also antiseptic, antiphlogistic, antirheumatic and analgesic properties. It is indicated for the treatment of sprains, bruises, dislocations, haematoma, edema associated with fractures, phlebitis, thrombosis, furuncles and insect bites [1,2,3].
The most frequently used form of *Arnicae flos* is the tincture preparated from 1 p drug and 10 p ethanol 70%. The oral use must be carefully controlled, and also large and frequent external applications must be avoided, since the product possesses sensitizing capabilities. Over 200 specialties containing *Arnicae flos* extracts are traded in Europe as allopathic and homeopathic remedies, half of them being for external use only [1].
Arnicae flos may be adultered with flower heads of Mexican Arnica (*Heterotheca inuloides* Cass. Asteraceae (ligulate florets without pappus and tubular florets with a double pappus and V-shaped stigma, the fruit short and ovoid without phytomelan in the fruit wall), which lacks the sesquiterpene. The other adulterations may be with *Calendula officinalis* or *Doronicum sp*, which can be easily recognized macro and microscopically [1].
Our objective was to verify if Romanian *Arnicae flos* complies with the European Pharmacopoeia stipulations for this drug and to determine the content of sesquiterpene lactones by Eu.Ph. techniques [4].

Materials and methods

Two samples of *Arnicae flos* were analyzed:

1. Plafar Cluj and
2. WWF Garda de Sus (Alba County) harvested in 2005 and dried.

The following characteristics were determined: the odor, macroscopic and microscopic aspect, TLC analysis for flavonoids and HPLC analysis for total sesquiterpene lactone content, loss on drying and total ash.

Characteristic	Eu.Ph. stipulations	Sample 1	Sample 2
Odor	Aromatic (flavoured)	In accordance with Eu.Ph.	In accordance with Eu.Ph.
Macroscopic assay		In accordance with Eu.Ph.	In accordance with Eu.Ph.
Microscopic assay		In accordance with Eu.Ph.	In accordance with Eu.Ph.
Foreign matter	Max 5%	1%	0.5%
Flowers of Callendula officinalis and Heterotheca inuloides	absent	absent	absent
Loss on drying	Under 10%	9.62%	9.44%
Total ash	Under 10%	7.65%	8.26%

<u>TLC assay for flavonoids</u>
<u>Test solution</u>: 2.00 g *Arnicae flos* powder (355 µm) with 10 ml methanol, warmed and stirred on water bath at 60^0 C for 5 min, cooled and filtered.
Reference compounds: 2.0 mg caffeic acid, 2.00 mg chlorogenic acid and 5.0 mg rutoside dissolved in 30 ml methanol.
Loading: 15 µl each of the test and reference solutions as bands on silica gel.
Solvent system: formic acid (anhydrous):water:methyl-ethyl-ketone:ethyl acetate (10:10:10:50). After 15 minutes the plate is dried on air for a few minutes and than sprayed with a detection reagent.
Detection reagent: amino ethanol diphenylborate 10g/l in methanol, than with a macrogol 400 solution 50 g/l in methanol and heated at $100\text{-}105^0$ C and than dried on air and observed in UV at 365 nm.
Evaluation: For the reference solution, a band with yellow-orange fluorescence (rutoside) appeared in the lowest part, a band with a blue fluorescence corresponding to the chlorogenic acid appeared in the middle part, and a band with a blue fluorescence corresponding to caffeic acid appeared in the upper part.

<u>Determination of sesquiterpene lactones expressed in tiglates of helenaline [4]</u>
Standard solution: freshly prepared solution of 0.01g santonine in 10 ml methanol.
Test solution: 1.00 g *Arnicae flos* (powdered at 355 µm) is introduced in a 250 ml round bottom flask and then added 50 ml of methanol-water (1:1), warmed and stirred on a water bath for 30 min. After cooling, the solution is filtered. The paper, cut in small pieces together with the residue is introduced in the flask and extracted once again with 50 ml methanol-water (1:1) in the same conditions. The operation is repeated two times. The reunited solution is added 3 ml of standard solution of santonine and then is concentrated at low pressure to a volume of 18 ml. The flask is rinsed with 2 ml water to 20 ml. The solution is transferred in a

chromatographic column with a length of 15 cm and a 3 cm interior diameter, which contained 15 g kieselgur, rested for 20 minutes and then was eluted with 200 ml ethylacetate-methylenchloride. The elute is completely evaporated into a 250 ml round flask. The residue is dissolved in 10 ml methanol then added 7.0 g aluminium chloride, stirred 120 second, centrifuged at 5000 r.p.m. for 10 minutes, and filtered through paper. 10 ml of the solution are evaporated, and the residue is dissolved in 3,0 ml methanol-water (1:1) and filtered. This solution is subjected to the HPLC analysis (Eu.Ph.), the content of total sesquiterpene lactones being expressed in tiglates of helenaline with the following formula:

$$S_{LS} \cdot C \cdot V \cdot 1.187 \cdot 100 / S_S \cdot m \cdot 1000, \text{ where}$$

S_{LS}- total areas of picks corresponding to sesquiterpene lactone that appear after the pick of santonine from HPLC
S_S- area of santonine in HPLC
m- weight submitted in work (in grams)
C- concentration of santonine in the standard solution (in mg/ml)
V- volume of internal standard introduced in the test sample (in ml)
1.187- correction factor between helenaline tiglate and santonine

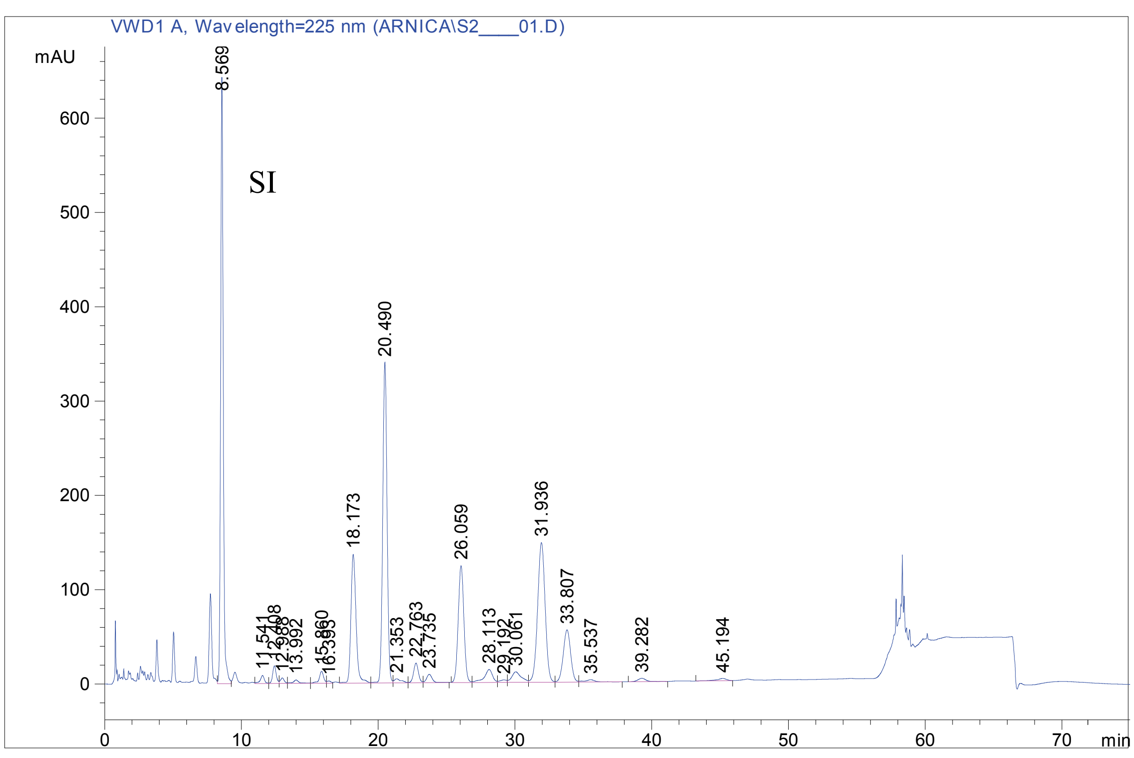

Fig. 1. HPL Chromatogram of sample 1; SI: internal standard (santonine).

Results and discussions

The results for the content in sesquiterpene lactones are: 0,74% for sample 1 (Plafar, Cluj) and 1,08% for sample 2 (WWF Garda de Sus, Alba). Area for internal standard was of 8578.1 whereas area of lactone sesquiterpene of sample 1 was 26183.2.

The analysis of two samples of *Arnicae flos* from Romania, demonstrated the accordance with the stipulations of Eu.Ph. concerning the macro and microscopic characteristics. The foreign matter content is even lower than the stipulations of Eu.Ph., the flowers of *Callendula* and other species are absent. The loss on drying is in accordance with Eu.Ph., and also the content of total ash.

The TLC assay for flavonoids and polyphenolcarboxylic acids demonstrated that the flowers of *Arnica montana* from Romania present three bands characteristic for flavonoids, and two for polyphenolic acids, whereas the band for rutoside is absent.
The content of sesquiterpene lactones determined by HPLC is higher that the content stipulated by Eu.Ph., thus the flowers of *Arnica montana* harvested in Romania is rich in the main active principle.

Conclusions

1. Based on the analysis of *Arnicae flos* from Romania we can conclude that this drug is in accordance with the Eu.Ph. stipulations.
2. The content of total sesquiterpene lactone was determined for the first time in Romania.

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THE IDENTIFICATION AND QUANTITATIVE DETERMINATION OF ROSMARINIC ACID BY TLC AND HPLC-MS FROM MEDICINAL *LAMIACEAE* SPECIES

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Summary

A qualitative and quantitative analysis of rosmarinic acid (RA) from 7 medicinal species of Lamiaceae (Origanim vulgare, Rosmarinus officinalis, Melissa officinalis, Ocimum basilicum, Hyssopus officinalis, Salvia officinalis and Ajuga genevensis) was performed by TLC and HPLC/MS.
The highest content of RA was determined in Origanum vulgare (1.24%) and the lowest in Ajuga genevensis (0.02%).

Keywords: *rosmarinic acid, Lamiaceae, TLC, HPLC/MS*

Introduction

The rosmarinic acid (RA) is a depside, the ester of caffeic acid with α-hyroxydihidrocaffeic acid, named also as "Lamiaceae tannin" being a substance characteristic for *Lamiaceae* family [1,2,3].

ROSMARINIC ACID

In the last ten years a lot of important biological activities of this substance were discovered, which have increased the interest for this study. Thus the mechanism of antioxidant activities was discovered [4,5,6], the effects against aflatoxin B1 in human hepatoma cell lines [7], the chemo protective effect against anthracycline – induced toxicity [8], inhibition of the formation of reactive oxygen and hydrogen species in macrophages [9,8], antiapoptotic and antioxidant effects in astrocytes [10], anti-inflammatory and anti-allergic effect in seasonal allergic rhino-conjunctivitis [11], antimicrobial and antioxidant activities [12], inhibition of angiogenesis [13].
In Romania literature the data about the content or the presence of RA in medicinal species of *Lamiaceae* and also for other species are absent.
Therefore, we initiated a qualitative and quantitative research on seven species of *Lamiaceae* for RA (see Tab. 1).

Material and methods

1 g dried vegetal products Tab. 1 (pulvis) (sieve VI, RPh. X) were extracted with 20 ml of methanol, on boiling bath, in a flask with a vertical condenser for 30 min. After filtration the solution was brought to 20 ml with methanol (sample 1-7).
The qualitative TLC analyse [14,15] was performed in the following technical condition:

- stationary phase: silicagel GF254 (Merck) plate of 20x15 cm.
- mobile phase: toluene - ethyl-acetate – formic acid – water (5:100:10:10) [14].
- distance of elution: 12 cm.
- standard solution 0.01% in methanol of RA (Fluka).
- quantity applied: 10 µl from standard solution and 20 µl for samples 1-7 in liniar spots 3 x 10 mm.
- reagent and identification: R. Neu-PEG, UV 365 nm [15].

HPLC assay

The identification and quantification of RA from the extracts eas performed by high-performance liquid chromatography method coupled with mass spectrometry (LC/MS/MS). The LC/MS system was an Agilent 1100 Series HPLC system (Agilent Technology Co., Ltd.) consisting of a binary pump, degasser, autosampler, thermostat operating at 48 ◦C, VL Ion Trap detector and UV detector). Chromatographic separation was performed on a Zorbax SB-C18 column (100mm×3.0mm i.d., 3.5um) (Agilent) preceded by a 0.5 um online filter. The mobile phase consisted of acetonitrile and 1 mM ammonium acetate in water, gradient elution: start 5% acetonitrile, at 3.3 min 25% acetonitrile. The mobile phase was delivered at a flow rate of 1 ml/min. The autosampler injection volume was set at 50 ul. UV detection was performed at 330 nm. The mass spectrometer operated using ESI source in negative mode and was set for isolation and fragmentation of deprotonated rosmarinic acid ion with m/z = 359 (Fig. 1a). Quantification of rosmarinic acid was based on the sum of ions with m/z = 160.7, 178.6 and 196.7 from the MS spectrum of parent ion (Fig1b). Calibration curve was linear in the range of 80-640 ng/ml, with a correlation coefficient of 0.999.

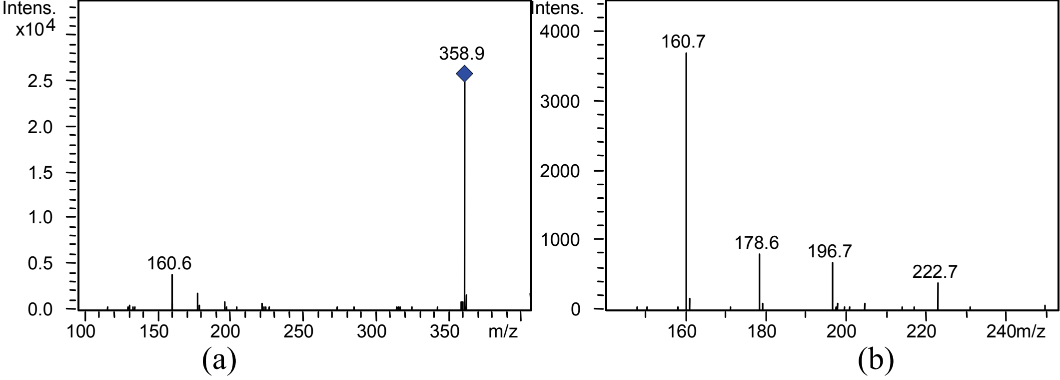
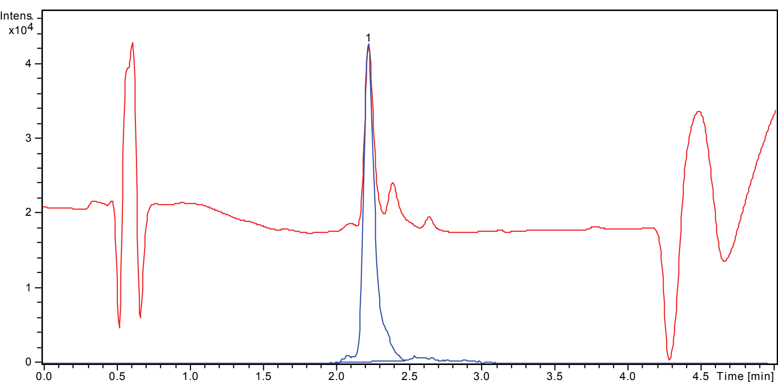


Fig. 1. (a) full-scan ESI-MS spectra of rosmarinic acid in mobile phase; (b) MS/MS spectra of rosmarinic acid in mobile phase.

A sample chromatogram of rosmarinic acid from *Origanum vulgare* extract is presented in Fig. 2a (the UV trace at 330 nm) and Fig. 2b (the MS signal). The retention time for rosmarinic acid was 2.2 min.



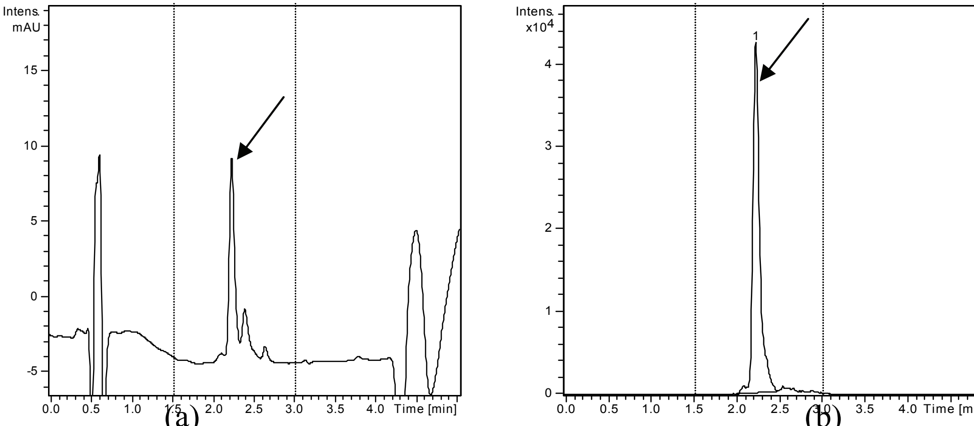


Fig. 2. Chromatograms of rosmarinic acid from *Origanum vulgare* extract (a) UV signal at 330 nm; (b) MS/MS signal. The retention time of rosmarinic acid is 2.2 min (peak marked with an arrow).

The back-calculated concentration of rosmarinic acid in samples is presented in Table 1.

Results and discutions

In TLC analysis the RA appear at 0.78 Rf value with bright-blue fluorescence in UV365 nm. By the intensity and size of spots a relative evaluation of RA in sample is possible. So the biggest and brightest is those of *Origanum vulgare*, *Ocimum basilicum*, *Rosmarinus* and *Melissa officinalis*. The spots appear in addition in chromatogram of other phenolic compounds in blue (phenyl-propanoids) and yellow-orange (flavonoides).

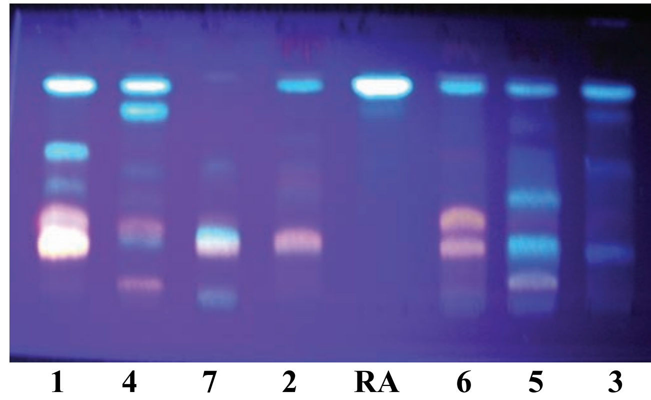


Fig. 3. TLC for RA

Table 1.

Nr.	Species	Used part	Station/month of harvested	Concentration of RA	
				µg/ml sol. 5%	% g/g of dried material
1	*Origanum vulgare L.*	Herba	Vl. Draganului (Cj) (07)	620.20	1.24
2	*Rosmarinus officinalis*	Folim	Cluj-Napoca (09)	392.60	0.78
3	*Melissa officinalis*	Folium	Cluj-Napoca (07)	392.39	0.78
4	*Ocimum basilicum*	Herba	Cluj-Napoca (08)	179.52	0.35
5	*Hyssopus officinalis*	Herba	Cluj-Napoca (09)	142.58	0.28
6	*Salvia officinalis*	Folium	Cluj-Napoca (07)	106.20	0.21
7	*Ajuga genevensis*	Herba	Ciucea (Cj) (06)	1.48	0.02

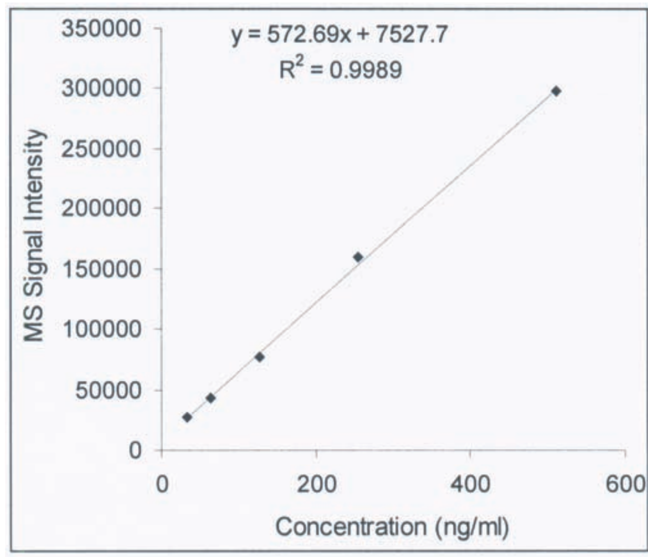


Fig. 4. The calibration curve of RA, MS/MS detection

In HPL Chromatography the retention time of RA is 2.2 min. (arrow), the same with RA from the extracts. The identity of RA is confirmed by MS analysis.
The content of RA in the extracts and plants are showed in tab.1. The highest content was recorded for *Origani herba* (1.24%) followed by *Rosmarini folim* and *Melissa officinalis*. All the medicinal plants contained over 0.20% RA, except *Ajuga genevensis,* where RA is in traces.
These results are in accordance with TLC chromatogram.

Conclusions

- The presence of RA in 7 species of *Lamiaceae* from Romania was identified by TLC and HPLC-MS.
- The content of RA varies by species, the high content was recorded in *Origanum vulgare* (1.24), *Rosmarinus officinalis* and *Melissa officinalis* whereas in *Ajuga genevensis* the content is very low (0.02%).

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IMMUNOPHARMACOLOGICAL STUDIES OF TWO EXTRACTS PREPARED FROM *ALLIUM CEPA* L. BULBS

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Introduction

The *Allium* genus includes approximately 500 species, the most widely used being onion (*Allium cepa*) and garlic (*Allium sativum*).
Onion is widely cultivated and consumed worldwide, and its beneficial effects have been known for thousands of years. According to the traditional medicine, onion is antiseptic and diuretic. The World Health Organization notes that *Allii cepae bulbus* is used for treatment of age-dependent changes in blood vessels and loss of appetite. Recent investigations suggest that onion lowers glucose and lipid levels, is active on platelet aggregation and thromboxane synthesis. Onion has been reported to protect against different types of cancers: stomach, colorectal, lung, bladder, breast, ovarian and brain.This protective effect appears to be related to the presence of organosulfur compounds and flavonoidic derivates.
In a series of previously communicated studies, it has been prepared two extracts of *Allii cepae bulbus*, codified EC1(hidro-alcoholic) and EC2 (aqueous); they were investigated from theirs content in flavonoids, polyphenols, pyruvic acid, aminoacids and sulf point of view, using spectrophotometric methods.
In this paper, there were continued the respective researches on EC1 and EC2 extracts, in order to detect the possible immunomodulatory activity: influence on some components of specific (lymfocites T and B) and unspecific (macrophages, leucocytes, PMN) immunitary mechanisms.

Material and method

In order to accomplish the immuno-pharmacological study, there were used 5 lots of white mice, who have been treated intraperitoneal during 14 days according to the following protocol: lot I: saline isotonic solution (SI) (0.5 ml/20g/day);lot II: prednisone (PDN)(5 mg/kg/day);lot III: levamisole (10 mg/kg/day) (LEV); lot IV: EC1 extract (DEV=5:2); lot V: EC2 extract (DEV=1:2). There were evaluated the following parameters: serum opsonic capacity (OC), phagocytic and bactericidal capacity of peritoneal macrophages (PC and BC), phagocytic capacity of peripheral neutrophils (NBT test), splenic T lymfocites with rosseting capacity.

Results and discussions

The obtained results regarding the effects of EC1 and EC2 extracts on the OC, PC and BC are presented in table no. I.

Table I. Influence of EC1/EC2 extracts on OC, PC and BC

Tested substance	OC	PC	BC
SI	816.67	700	641.67
PDN	1575	1200	1037.5
LEV	558.33	333.33	300
EC1	457.5	262.5	225
EC2	471.67	355	330

From the data analyse, we can conclude that EC1 stimulates the phagocytic capacity of peritoneal macrophages in a higher degree than the control lot treated with isotonic saline solution. The stimulation effect on the investigated parameters is more powerful comparing to the one induced by LEV.
For EC2 extract, the stimulation of CO is stronger then LEV; but, the stimulation effect on PC and BC is significantly reduced comparing to the one determined by LEV.
The influence study on splenic T lymfocites with rosseting capacity, suggested that EC1 and EC2 extracts determine a significantly increase of this parameter within the control lot, this effect being even more intensively compared to the one induced by LEV (Fig. 1).

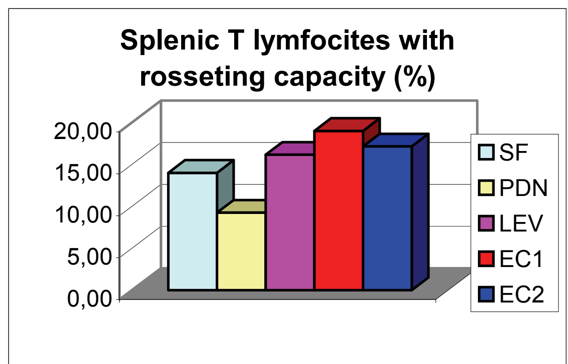

Fig. 1. % of splenic T lymfocites with rosseting capacity

EC1 and EC2 extracts determine an important increase on NBT test values. Also, the effect on phagocytic capacity of peripheral neutrophils is more intense than the one determined by LEV; EC1 significantly stimulates this parameter, comparing with EC2, as it resulted from statistic data. The effects of the tested extracts on the studied parameter, compared on experimental lots, were decreasing in intensity as it follows: EC1>EC2>LEV.

Conclusions

The resulted data suggest that EC1 and EC2 extracts obtained from *Allii cepae bulbus* have a immunostimulatory action on cellular components of unspecific and specific immunitary protection system.
The test's results justify the possibility of including EC1 and EC2 extracts in immunomodulatory products.

COMPARATIVE PHYTOCHEMICAL RESEARCH ON SOME INDIGENOUS SPECIES OF *VIOLA* (*VIOLACEAE*) FROM ROMANIA

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Summary

We have initiated a comparative phytochemical study and we have analysed the polyphenolic compounds (flavonoids, polyphenol carboxylic acids, anthocyanins, proanthocyanins) from three Viola species: V. tricolor L., V. arvensis Murray and V. declinata Waldst. et Kit. The qualitative analysis was performed by TLC and HPLC, whereas the quantitative determinations by spectrophotometric methods. We have identified quercetol and kaempferol as flavonoid aglycones and delphinidin and cyanidin as anthocyanin aglycones.

Keywords: *Viola sp., polyphenolic compounds, HPLC, flavonoids, anthocyanins*

Introduction

The *Viola* genus contains many species, native in the temperate zones of Europe and Asia. *Viola tricolor* L. (wild pansy) is widely spread in Romania's spontaneous flora. In traditional medicine, the aerial parts are used for their anti-inflammatory, expectorant, diuretic properties, to treat skin conditions, bronchitis, cystitis, rheumatism. Its properties are ascribed to the presence of the following active principles: saponins, flavonoids, mucilages, salicylic derivatives, carotenoids, coumarins [1, 2, 3]. *Viola arvensis* Murray and *Viola declinata* Waldst. et Kit. are closely related to *V. tricolor* L., but their chemical composition is less studied [4, 5]. Recently, in *Viola species* were identified the cyclic polypeptides with diverse biological activities (including anti-HIV, cytotoxic, antimicrobial), called the cyclotides, found so far only in *Violaceae*, *Rubiaceae* and *Cucurbitaceae* plant families [6, 7].
We have initiated a comparative phytochemical study and we have analysed the polyphenolic compounds (flavonoids, polyphenol carboxylic acids, anthocyanins, proanthocyanins) from the three *Viola species* by TLC, HPLC and by spectrophotometric methods.

Materials and methods

We have analysed the aerial parts harvested from different regions of Romania at varied periods of the following species: *Viola tricolor* L. (May 2004, Cluj and May 2004, Brasov), *V. arvensis* Murray (May 2003, Cluj and May 2005, Cluj) and *V. declinata* Waldst. et Kit. (July 2002, Cluj and July 2005, Alba) for the polyphenolic compounds' study. The aerial parts were air dried and then pulverised.
Spectrophotometric determinations were made using a spectrophotometer UV-VIS JASCO V-530.
The quantitative analysis of flavonoids was made using the method described in the Romanian Pharmacopoeia Xth Edition for the drug *Cynarae folium* [8].
The quantitative analysis of polyphenol carboxylic acids (caffeic acid derivatives) was made using the method described in the Romanian Pharmacopoeia IXth Edition for the drug *Cynarae folium* [9].
The quantitative analysis of anthocyanins was made using the technique described by Markakis [10].
The quantitative analysis of proanthocyanins was made using the Lebreton technique [11].

***HPLC determinations* [12]:**

Apparatus and chromatographic conditions: We used an Agilent 1100 HPLC Series (Agilent, USA) equipped with a degasser G1322A, a quaternary gradient pump G1311A, a Zorbax SB-C18 reversed-phase analytical column 100 mm x 3,0 mm i.d., (Agilent, USA), operated at 48°C. The mobile phase was a binary gradient: methanol and buffer solution (KH_2PO_4 40 mM) and the pH was adjusted to 2,3 with 85% orthophosphoric acid. The gradient begun with a linear gradient started at 5% methanol to 42% methanol over first 35 minutes, followed by isocratic elution with 42% methanol over the next 3 minutes. The flow rate was 1 ml/min and the injection volume was 5 µl.

Samples preparation: Powdered herba was extracted with distillated water and ethanol, at 80°C for 30 minutes on a water bath, then they were sonicated for 5 minutes and finally heated again for another 10 minutes at 80°C. The mixtures were centrifuged with 4000 rpm. In order to study the flavonoid aglycones that can be obtained by hydrolisis we have mixed these solutions together with hydrochloric acid 2M and ethanol and the solutions were heated at 80°C for 30 minutes on the water bath. After extraction the mixtures were centrifuged with 4000 rpm. The solutions were diluted with distillated water in a 10 ml volumetric flask.

Detection: detector UV 330 nm up to 16 min, then 370 nm up to 38 min. All compounds were identified and quantified by external standard method and by comparison of their retention times with those of the standards, in same chromatographic conditions.

Standards: caftaric acid, gentisic acid, caffeic acid, chlorogenic acid, p-coumaric acid, ferulic acid, sinapic acid, cichoric acid, hyperoside, isoquercitrin, rutoside, myricetin, fisetin, quercitrin, quercetol, patuletin, luteolin, kaempferol, apigenin.

We present the HPLC chromatogram for standards (fig. 1) and their retention times (table I).

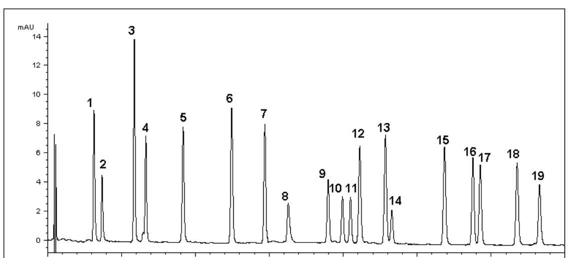

Fig. 1. The HPLC chromatogram for standards: 1-caftaric acid; 2-gentisic acid; 3-caffeic acid; 4-chlorogenic acid; 5- p-coumaric acid; 6-ferulic acid; 7-sinapic acid; 8-cichoric acid; 9-hyperoside; 10-isoquercitrin; 11-rutoside; 12-myricetin; 13-fisetin, 14-quercitrin; 15-quercetol; 16-patuletin; 17-luteolin; 18-kaempferol; 19-apigenin.

Table I: Retention times for all standards

Polyphenolic compound	Retention time (min)	Polyphenolic compound	Retention time (min)
caftaric acid	3,1	rutoside	20,4
gentisic acid	3,7	myricetin	21,1
caffeic acid	5,9	fisetin	22,8
chlorogenic acid	6,6	quercitrin	23,3
p-coumaric acid	9,2	quercetol	26,8
ferulic acid	12,4	patuletin	28,7
sinapic acid	14,7	luteolin	29,2
cichoric acid	16,2	kaempferol	31,7
hyperoside	19,0	apigenin	33,2
isoquercitrin	19,9		

TLC for flavonoids and polyphenol carboxylic acids was performed using Kieselgel 60 (DC-Plastikfolien Merck) as stationary phase, ethyl acetate-formic acid-acetic acid-water: 100-11-11-26 as solvent system and observation under UV at 365 nm after spraying with NEU 1% and PEG 6000 5% (methanolic solutions). We analysed methanolic 5% solutions from the three *Viola species*, prepared on a water bath by heating at 60^{o}C for 30 minutes [13, 14].
Anthocyanins' aglycones were analysed after acid hydrolysis, by TLC (cellulose AVICEL Merck) using acetic acid-chlorhidric acid-water: 30-3-10 as solvent system and observation in VIS. UV-VIS spectra of anthocyanins' aglycones isolated by preparative-TLC (using the same conditions) were recorded with a spectrophotometer UV-VIS JASCO V-530 [10, 15].

Results and Discussion

The results of quantitative spectrophotometric determinations of polyphenolic compounds from the three *Viola species* are shown in table II.

Table II: Results of quantitative determinations of polyphenolic compounds

Species	Flavonoids (%)	Polyphenol carboxylic acids (%)	Anthocyanins (mg%)	Pronthocyanins (%)
V. tricolor L.	1,812-1,992	0,597-0,599	26,002-28,643	0,1015-0,1278
V. arvensis Murr.	1,289-1,564	0,178-0,314	14,786-20,909	0,0389-0,0623
V. declinata Waldst. et Kit.	1,454-1,495	0,157-0,160	54,771-55,136	0,2634-0,3379

The flavonoids are the major polyphenolic compounds in all lots of *Viola species*, the richest being *V. tricolor* L. Caffeic acid derivatives are present in small quantities, as well as anthocyanins and proanthocyanins. The smaller quantity of polyphenolic compounds was found in *Viola arvensis* Murray. Anthocyanins and proanthocyanins are in bigger quantities in *V. declinata* Waldst. et Kit.
Preliminary TLC analysis of flavonoids and polyhenol carboxylic acids showed small differences between all lots of *Viola* studied, especially quantitatively ones. Comparing the R_f values and the fluorescence of spots with the ones of reference substances, we identified by TLC in all lots rutoside and caffeic acid. In *V. declinata* we identified by TLC delphinidin and cyanidin as anthocyanin aglycones, by comparison of the R_f values of spots with the ones of an hydrolysed extract of *Myrtilli fructus* (which contains delphinidin, cyanidin and other aglycones). In order to confirm their presence, we effectuated a preparative TLC followed by spectrophotometrical analysis. UV-VIS spectra of anthocyanins' aglycones isolated are presented in fig. 2 and 3. Delphinidin (R_f=0,25) has λ_{max} at 550 nm, whereas cyanidin (R_f=0,40) has λ_{max} at 540 nm.

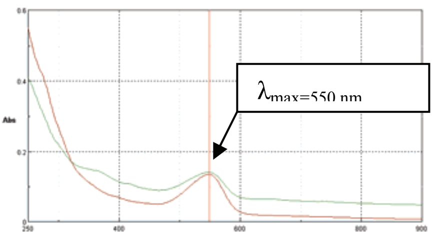

Fig. 2. UV-VIS spectra of delphinidin

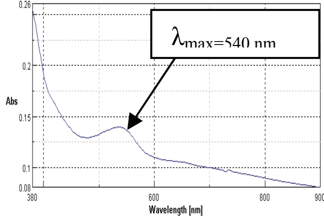

Fig. 3. UV-VIS spectra of cyanidin from *V. declinatae herba*

We have identified and measured by HPLC the following polyphenolic compounds: caftaric acid, gentisic acid, caffeic acid, chlorogenic acid, p-coumaric acid, quercetol, kaempferol. We

present the concentrations (mg polyphenolic compound / 100g dried aerial parts) for these compounds before and after hydrolysis in table III.
The HPLC chromatograms before hydrolysis and after hydrolysis for the three *Viola species* analysed are shown in fig. 4-9.

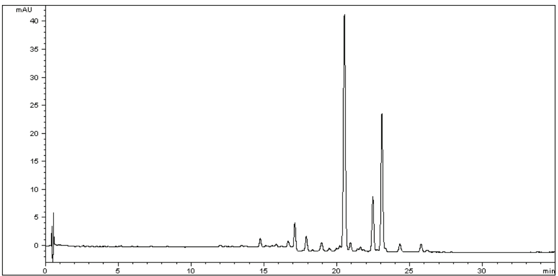

Fig. 4: HPLC chromatogram of *V. tricolor* before hydrolysis

Fig. 5: HPLC chromatogram of *V. tricolor* after hydrolysis

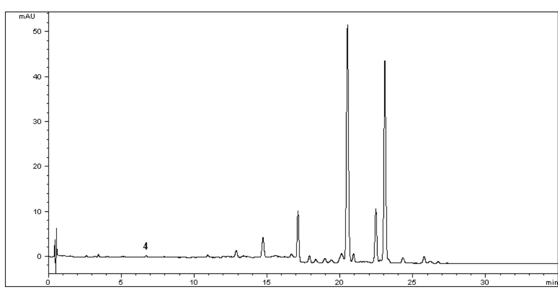

Fig. 6: HPLC chromatogram of *V. arvensis* before hydrolysis

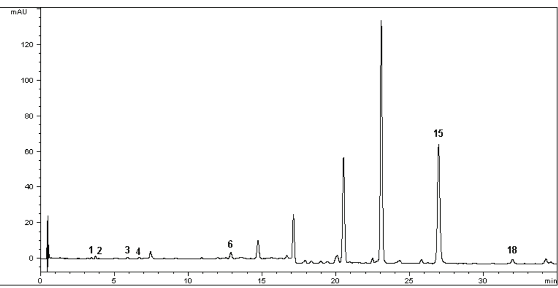

Fig. 7: HPLC chromatogram of *V. arvensis* after hydrolysis

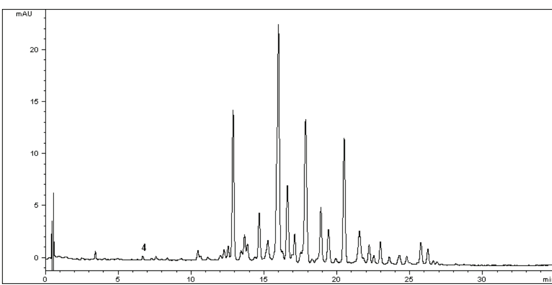

Fig. 8: HPLC chromatogram of *V. declinata* before hydrolysis

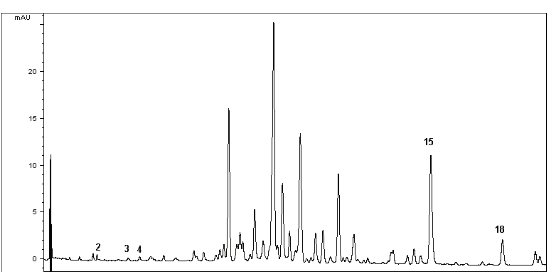

Fig. 9: HPLC chromatogram of *V.declinata* after hydrolysis

Table III: The concentrations (mg polyphenolic compound / 100g dried aerial parts)

Species / Polyphenolic compound	**Retention time** (min)	***V. tricolor*** Before / After hydrolysis	***V. arvensis*** Before / After hydrolysis	***V. declinata*** Before / After hydrolysis
caftaric acid	3,1	- / -	- / 19,55	- / -
gentisic acid	3,7	- / 44,65	- / 66,73	- / 40,2
caffeic acid	5,9	- / -	- / 22,67	- / 14,75
chlorogenic acid	6,6	- / -	21,11 / 29,45	26,92 / 27,47
p-coumaric acid	9,2	- / traces	- / -	- / -
quercetol	26,8	- / 1938,35	- / 2599,28	- / 738,95
kaempferol	31,7	- / 110,25	- / 138,8	- / 200,82

The HPLC chromatograms before and after hydrolysis resemble very much, after hydrolysis we identified and quantified two flavonoid aglycones in all studied lots. Because the quantity of other polyphenolic compounds didn't changed (didn't decreased) after acid hydrolysis we can suppose that they are C-flavonoids. In previous studies, in *Viola sp.* were identified various flavonoids, both O-glycosides (rutoside, luteolin-7-O-glucoside) and C-flavonoids (violanthin, violarvensin, vitexin, orientin) [2, 4, 5, 16, 17, 18]. Although rutoside was previous identified in *Viola sp.* and the compound with retention time (RT) 20,4 is a majoritary compound in all HPLC chromatograms presented (fig. 4-9) and rutoside as a reference substance has RT=20,4, the fact that it still remains in a great quantity (almost half) after acid hydrolysis made us think that it could be a C-flavonoid (with the same RT in these chromatographic conditions) present, as well as rutoside (which is hydrolysed to quercetol). The flavonoids of *Viola sp.* are mostly glycosides of quercetol and kaempferol, these aglycones being identified only after hydrolysis and they are not present as free compounds.
Concerning the polyphenolic acids, chlorogenic acid was identified and measured in all tested lots, before and after hydrolysis. The fact that after hydrolysis the quantity increases indicates that it could be present in plants in glycosidated forms. Caffeic acid was identified only after hydrolysis, so it exists as caffeic acid derivatives in plants. Caftaric acid was identified only in *V. arvensis* after hydrolysis and gentisic acid in all lots, only after hydrolysis.

Conclusions

We have initiated a comparative phytochemical study and we have analysed the polyphenolic compounds from three *Viola species*. We have analysed for the first time the chemical composition of *V. declinata*, as well as anthocyanins and proanthocyanins from the three species. We completed the literature data with the new ones, concerning qualitative and quantitative determinations of different classes of polyphenolic compounds by spectrophotometrical methods as well as for individual active principles by HPLC study. Our phytochemical study of the polyphenolic compounds (flavonoids, polyphenol carboxylic acids, anthocyanins, proanthocyanins) showed small differences between the three *Viola species*, especially quantitatively ones. Our results are closely related to the methods that we have used, they represent basic data for future phytochemical determinations by more performant other methods (HPLC-SM).

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PIGMENTS FROM THE AERIAL PARTS OF THE *NIGELLA DAMASCENA* L. AND *NIGELLA SATIVA* L. SPECIES *(RANUNCULACEAE*)

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Summary

Nigella sativa L. and Nigella damascena L. are two herbal species from the Ranunculaceae family, original in the Magreb's: Morocco, Algeria, Tunisia and Egypt and also in the Middle Orient: Syria. In the originated countries the plants are very frequently used for their seeds, Nigellae sativae (damascenae) semen, venerated by all the Muslim peoples and thus quoted in the Koran for the multiple therapeutic effects. Thus the black caraway's seeds, the popular name of Nigella L. (Ranunculaceae), are appreciated for their anti-inflammatory, carminative, antihelmintec, ocitocic virtues and as one of the best Muslim traditional spices used for bread and cheese products.
The object of this study is to investigate the presence of carotenoids pigments in the stems of above-mentioned species. The investigating technique used was Very High-Pressure Chromatography (HPLC), after the extracting and saponification operations. Another important objective was to determine the quantity of the carotenoid pigments.
After using this techniques proved the presence of the pigments: lutein, zeaxantin, β-criptoxantin, α-caroten, β-caroten, neoxantin şi violaxantin, and the percentage of these carotenoids. This were also determined the percentage concentrations of the carotenoids in the vegetal product Nigellae herba.

Keywords: *carotenoids, Nigellae sativae herba, Nigellae damascenae herba, Ranunculaceae, HPLC*

Introduction

The carotenoids are terpenoidic pigments made of isoprenic units,yellow, orange, red, or even violet colors, found in unsaturated hydrocarbonates' plants or their derivated oxygenation and form the main vitamin A vegetal source for animal world.
Nowadays, the whole carotenoids group is reconsidered from biological importance point of view, being well known the fact that, many components expand exceptional antioxidant processes, sometimes having a distinct specificity for certain organs or tissues.
The carotenoids are biosynthesized only by plants. The animals cannot synthesized them, reason why they acquire these pigments from food [1,2].
In the literature there are a lot of studies about carotenoids, but the most importants are about alimentar using pigments or very familiar plants with large contents in carotenoids [3 – 10].
The present research deals with a qualitative and quantitative research on carotenoid pigments from the aerial parts of the *Nigella sativa* L. and *Nigella damascena* L. species(*Ranunculaceaea*), cultivated in Romania.

Material and method

The two plants *Nigella sativa* L. and *Nigella damascena* L. (*Ranunculaceae*) were collected from Vladimirescu village, Arad County in June 2003, the aerial parts of the plants being tested. The analysed plants have been cultivated and then harvested in Romania, Vladimirescu locality, Arad County, starting from seeds originated in North Africa (Morocco and Tunisia).

In order to accomplish the analysis of carotenoid fraction from the aerial parts of *Nigella* L. (*Ranunculaceae*) species, the extraction was performed as follows:
The material was grinded, using an Ultraturax apparatus, then was subject to repeatedly extraction procedure with acetate of ethyl:methanol:petrol ether (1:1:1 v/v/v). The extraction was made under continuous succussion, in shadower light, using butilhydroxytoluen as an antioxidant and sodium bicarbonate (added to prevent epoxydic rearrangements which may take place in acid environment). After filtration, the reunited extracts were dried evaporated using a rotation machine at 35 ºC under low pressure. The obtained residue was retested in a well known ethylic ether volume for spectrophotometric dosage of carotenoids [2 – 4].
The two unfinished extracts, containing carotenoidic pigments, were subjected to saponification, in order to release carotenoids, shaped as esters, and to remove saponificable lipids.
For doing this, the extracts were redissolved in ethylic ether, adding an equal volume of KOH 30% in methanol. The saponification was made in a closed balloon, in perfect dark and under continuous succussion for 8 hours. The result extract was funnelled with 1% NaCl and washed until the waters had a neutral pH (comparing to phenolphthalein). The etheric superior phase, containing the carotenoidic pigments, was separated, dried evaporated and kept at –20 °C under inert gas.
For carotenoids quantitative determination was applied the spectrophotometrical method in UV-VIS under following experimental conditions:
Equipment: spectrophotometer UV-VIS M 40
Wavelength: 450 nm
Vats: 1 cm side
The quantity of carotenoidic pigments was calculated using the next relation [5,6]:
X (mg carotenoids) = (AxVx1000)/(2500x1x100)
in which:

A = read probe absorption at λ_{max} =450 nm
V = probe volume (ml)
2500 = carotenoids specific absorption = $A^{1\%}_{1cm}$
1 = 1 cm- spectrophotometer vat length (optic way)

Cromathographic identification through HPLC (High Performance Liquid Cromathography) of carotenoidic pigments was done using liquids cromatography with a machine having:

- a system of Kontron 322 pumps,
- cromathographic pillar in reversed phase Discovery C18, 250 mm length and 4,6 mm diameter, and 5um diameter of particles;
- detection was made with a photodiode array detector Waters 990 [5,6].

The following system was used:
Solvent A: Acetonitrile : Water 9:1, (v/v) +0,5% EPA (ethylisopropilamine)
Solvent B: Ethyl acetate + 0,5% EPA

The used program was:
System A
At minute 0: 0% B in A
16: 60% B in A
25: 60% B in A
27: 0% B in A
The solvent debit was 1 ml/minute.
The carotenoids retention times were compared to free standards retention times, separated in same conditions (Fig. 1-3).

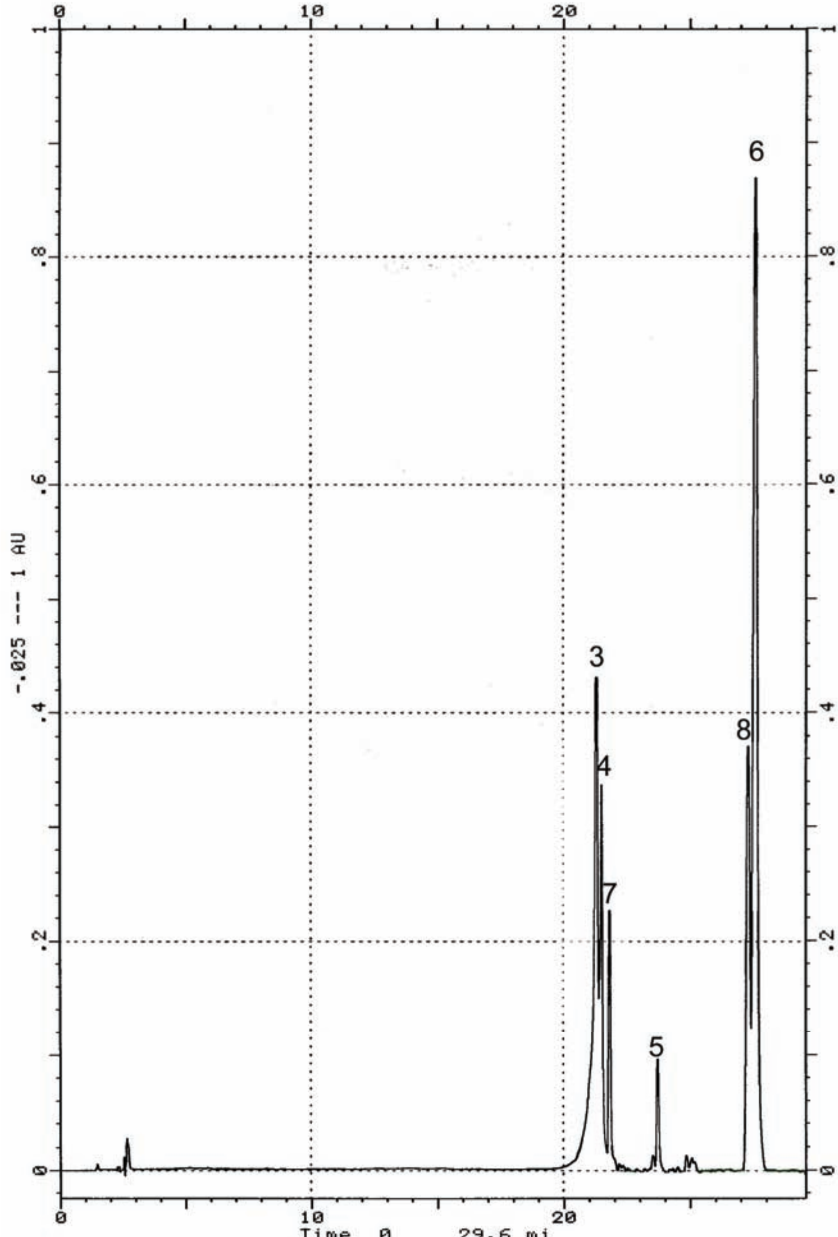

Fig.1. HPLC cromathograme of a mixture of standards. 3-Lutein, 4-Ziaxanthine, 5-β-cryptoxanthine, 8-α-carothen, 6-β-carothen, 7-Cantaxanthine.

The other components were identified on the correlation of cromathographic behaviour and absorption spectrums in UV-VIS., being carotenoids characteristics (Fig.1-3) [1,5,6].

Results and discussions

The following results were obtained when we determined quantitatively the total carotenoids:
Nigellae damascenae herba: 8,82 mg %
Nigellae sativae herba: 8,97 mg %, which means that the two species are quite similar, taking into account the content.
Analysing the total carotenoidic of the two species of *Nigella* (*Ranunculceaea*), the aerial part, after saponification and comparing HPLC diagrames obtained for lutein, ziaxanthine, cantaxanthine, Β-cryptoxanthine, α and β-caroten (Fig.1), we come to the conclusion that *Nigella damascena* sampling consists of two unidentified components which have retention

times, under 10 minutes, besides four more components, lutein, ziaxanthine, β-cryptoxanthine and β-caroten, which appear in 20-30 minutes period of time, the most concludent ones being the lutein followed by ziaxanthine (Fig.2).

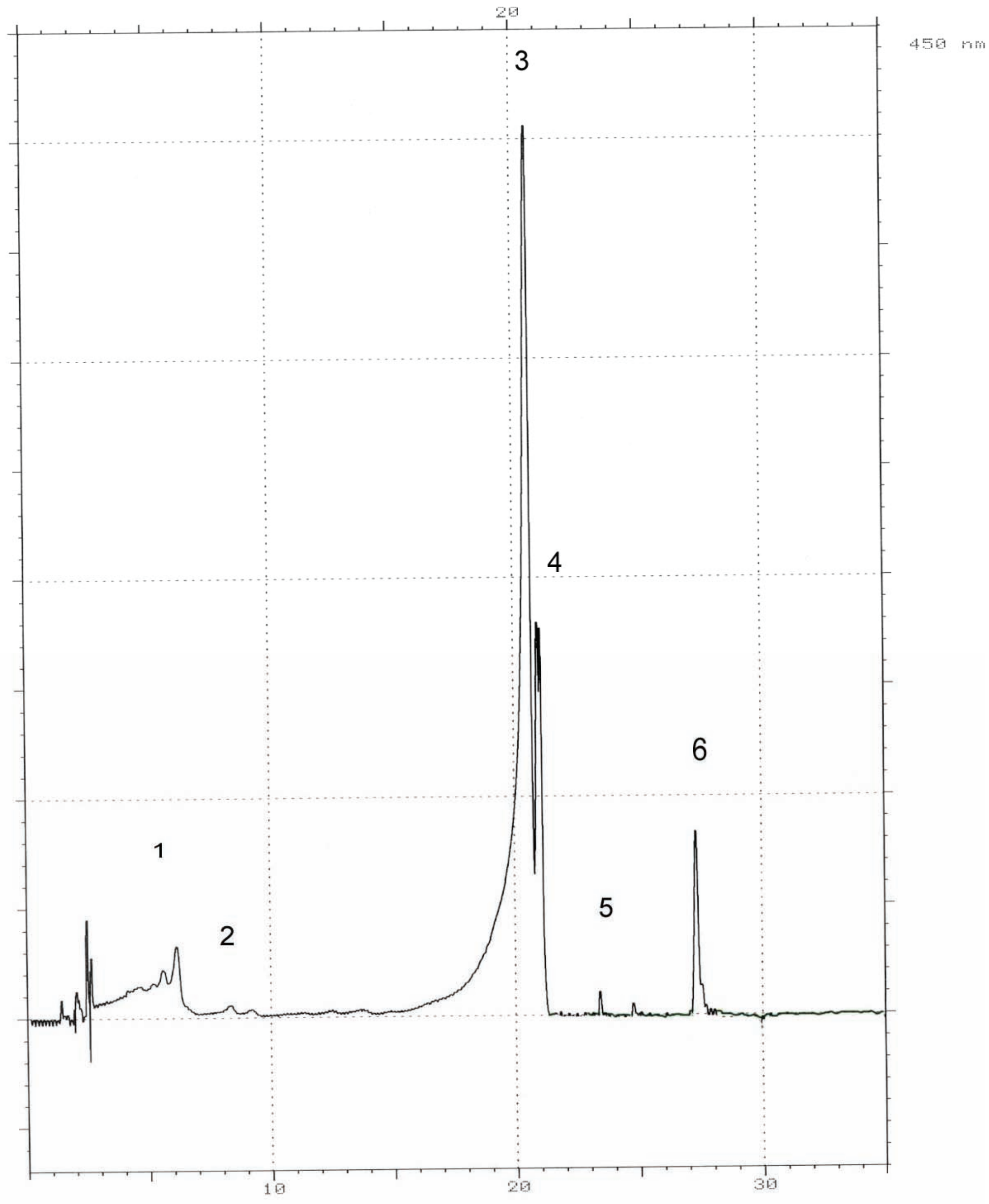

Fig. 2. HPLC cromathograme of *Nigellae damascenae herba* (*Ranunculaceae*) saponificate extract

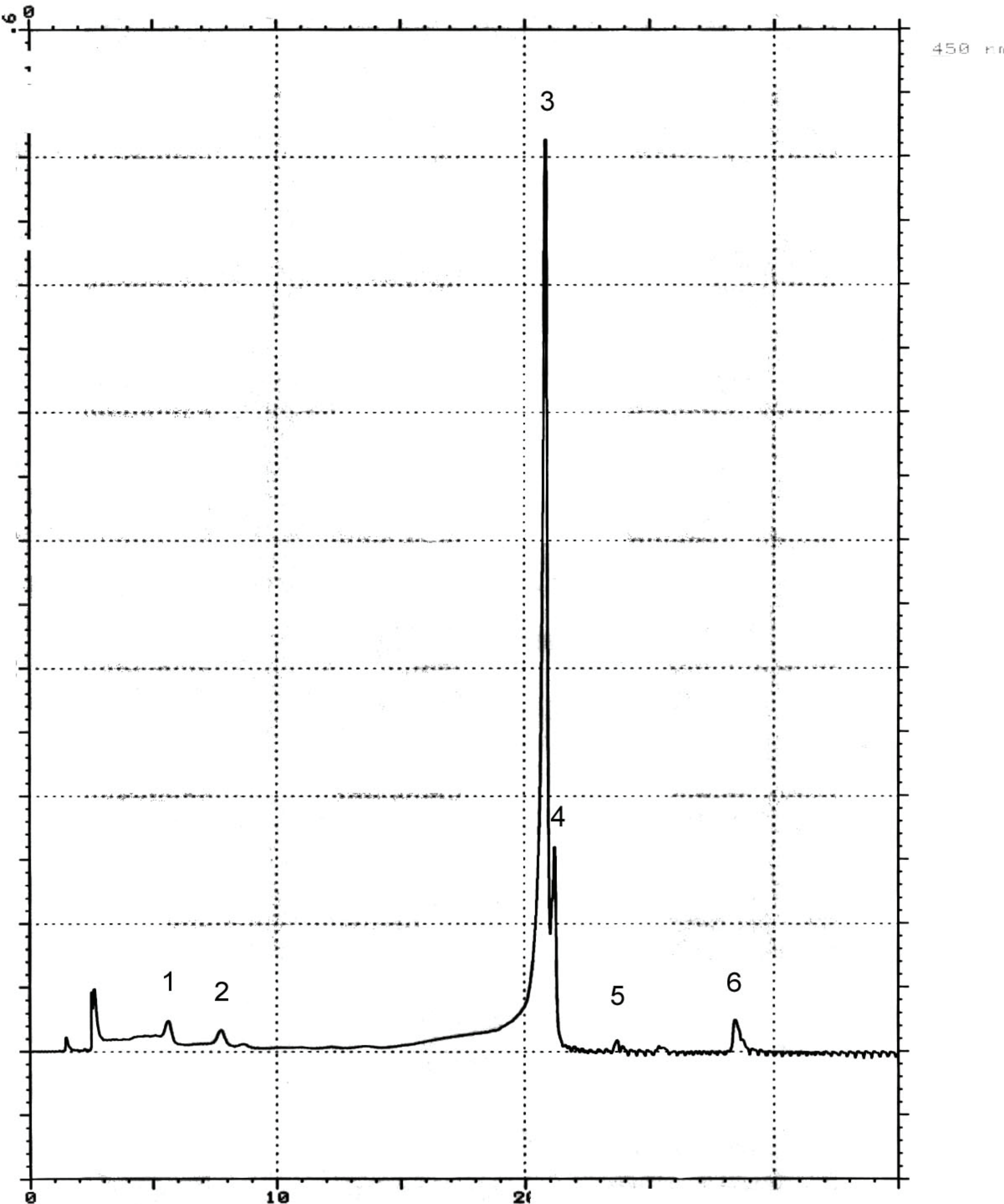

Fig. 3. HPLC cromathograme of *Nigellae sativae herba* (*Ranunculaceaea*) saponificate extract

The HPLC curve for the carotenoids from *Nigella sativa* (Fig.3) identifies between the same retention time of 10 minutes, two unidentified drips followed by lutein, ziaxanthine, β-cryptoxanthine and β-caroten. Lutein also prevails in this case followed by ziaxanthine.
According to the reference catalogue in between 10 minutes time, separates noexanthine (6,10 minutes) and violaxanthine (7,78 minutes).
Trying to conclude/deduce the identified carotenoidic pigments' percentage in each of the two saponificate carotenoidic factions, taking into account the drips area, we achieved the results from Table 1.

Table 1. The percentage composition of the identified carotenoidic pigments using HPLC regarding the two species of *Nigella* L. (*Ranunculaceaea*)

Pigment	Nr.pigment on cromathograme	Retention time (HPLC)	*Nigella sativa* (*herba*)	*Nigella damascena* (*herba*)
Lutein	3	20.70	78.32	74.42
Ziaxanthine	4	21.08	10.94	14.16
β-cryptoxanthine	5	23.60	0.30	0.28
α-Caroten	5	27.50	Signs	Signs
β-Caroten	6	28.03	3.40	7.78
Neoxanthine	1	6.10	1.42	1.70
Violaxanthine	2	7.78	1.41	0.82

By separating each drip through HPLC and recording their absorption spectra we acquired the diagrams having absorption maxima recorded in Table 2.

Taking into account the values indicated in the table, the correspondence between absorption maxima recorded in case of separated carotenoids from our tests and the ones indicated in our line is quite large, so that we came to the conclusion by which the extracts subjected to testing have very important carotenoids, judging from therapeutic point of view, lutein and ziaxanthine being the prevailed ones. (Table 2)

The most representative carotenoidic derived structures that have been found in our tested extracts from aerial parts of *Nigella* L. (*Ranunculaceaea*) are presented below.

Table 2. The identified pigments in *Nigella species* L.(*Ranunculaceaea*) extracts.

Carotenoids	Number pigments on cromathograme	Retention time (HPLC)	Maxime of absorbtion	Maxime of absorbtion Standard	Raport III/II%
Lutein	3	20.70	422, 444, 473	422,445, 473**	55
Ziaxanthin	4	21.08	428, 450, 474	424,449,476**	25
B-cryptoxanthin	5	23.60	428, 450, 474	424,449,476**	25
α-Caroten	5	27.50	422, 444, 473	422,445, 473**	55
β-Caroten	6	28.03	425, 452, 479	425,450, 477**	25
Neoxanthin	1	6.10	418, 438, 467	416,438,467*	87
Violaxanthin	2	7.78	418,439,466	416,440,465*	100

OH

HO

Lutein

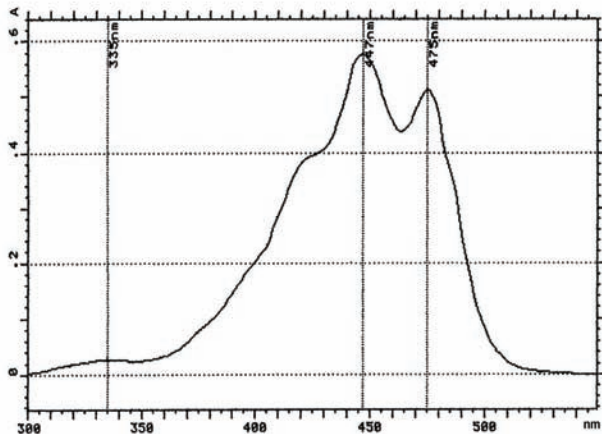

Fig.4. Lutein absorption spectrum and chemical formula.

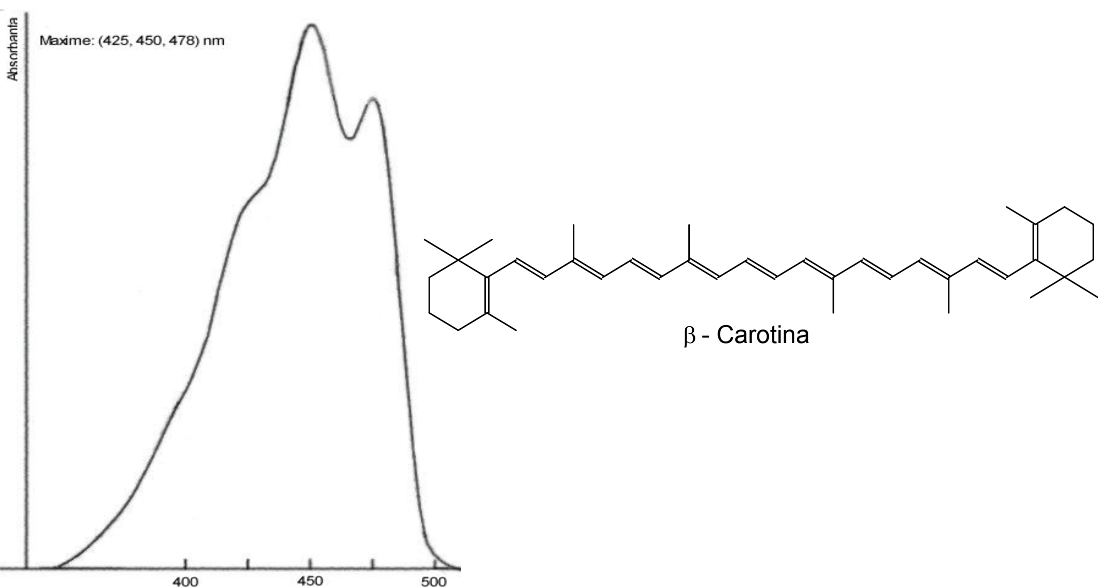

Fig. 5. β-caroten absorption spectrum and chemical formula.

Conclusions

It was for the first time when the *Nigella sativa* L. (*Ranunculaceaea*) aerial part of mucilages had been studied, establishing the saturation items for plant varied organs, and after their isolation from aerial parts of the species, glucidic monometers were qualitatively and quantitatively determined.

It was established that quantitatively the galacturonic acid had a preponderence of 26, 08%, followed by arabynose and xylose.

The carotenoidic fraction study on aerial parts of *Nigella damascena* and *Nigella sativa* species, which has been done for the first time in this thesis, proved that in both cases these are made of lutein, ziaxanthine, β-caroten, β-cryptoxanthine, besides neoxanthine and violaxanthine (small quantities) and few traces of α-caroten.

Quantitatively determined, lutein prevails, representing 74,42% from carotenoidic factin of *Nigella damascena* and 78,32% from *Nigella sativa*.

Nigella damascenae herba vegetable product is richer in β-caroten (7,78%) being over double comparing to *Nigella sativae herba* product (3,40%).

This component has already a well-known demonstrated effect as a major antioxidant and pro-vitamine A, having very useful therapeutic valencies.

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COMPLETE SCREENING OF THE CATIONS PRESENTS IN THE SPECIES OF *ADONIS, HELLEBORUS, RANUNCULUS* FROM *RANUNCULACEAE* FAMILY

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Summary

The species of Adonis, Helleborus, Ranunculus are herbal plants of the Ranunculaceae family with therapeutic actions well determined, but little investigated from the cations presence in the different morphological categories point of view [1,2,3].
The object of this study is the investigation and the comparing of the content of different cations in the roots, stems, leaves, flowers and fruit in the above mentioned species, this being able to point the part of the plant most useful to obtain the diuretic effect [4,5].
By wet disintegration followed by spectroscopy techniques of atomic absorption, K^+, Na^+, Mg^{2+}, Ca^{2+}, $Fe^{2+/3+}$, Mn^{2+}, Zn^{2+}, Cu^{2+}, Pb^{2+} cations were investigated.
As a common characteristic of the above mentioned types is their diuretic effect. From this point of view the presence of the Potassium cations become very important, and the utilisation spectrum is considerably increased.
This study actually presents a complete screening of the main cations present in the different parts of the many species of the Ranunculaceae family and the statistic analysis of a great number of experimental data have concluded to very precise results.

Keywords: *cations, Ranunculaceae, diuretic effect, atomic absorption spectroscopy.*

Introduction

The ***Ranunculaceae*** **family** include about 1500 species, spread all over the world, especially in the temperate regions. They are perennial herbs, biennial or annual but, also, lianas with adventitious roots, with rhizomes, bulbs and tubers, and a little ligneous underground stems. The leaves are complete or divided, alternately disposed, generally simple, rarely compound, without stipels. Due to the indefinite great number of floral elements, as well as to their spiro-cyclic arrangement, most of them actinomorphous, but, sporadically, some of them zygomorphous, the flower has a character of inferiority. The flowers are solitary or grouped in bisexed cimaous inflorescence. The perianth is usually simple, petalled, with 4-5 pieces. Usually, the gynaecium is an apocarp polymer, and the ovary is superior. As a rule the fruits are multiple, follicle or nut-like, rarely berries or capsules [1,2].
Many species from this family are interesting from phytotherapeutical point of view due to the content of the active principles which can be either of alkaloidic or saponozidic nature, or of heterosido-cardiotonic one. From the species with therapeutical application, the aerial parts or the underground stems are used which, in most cases, are toxic [2].
This analysis is intended to the study of *Adonis, Helleborus* and *Ranunculus* species evincing the content of different elements in them, as an argumentation for the therapeutical use (e.g. diuretic effect) of the previously mentioned vegetal products.
In case of the ***Adonidis vernalis herba***, the therapeutical action is due to the presence of the cardiotonic glycosides, which are of digitaline type. This has the advantage that like the digitalines they do not accumulate in the body [2,3].
Due to the presence of flavones, the same vegetal product has a diuretic effect [2], as well as a sedative and a slight hypertensive one. Good results are also obtained in the treatment of tachycardias and extrasystoles of nervous nature, and in neurovegetative disorders, too. For its

diuretic action it is recommended in the treatment of pleuresy, ascites of hepatic nature, and cardiac and renal insufficiency.
Helleborus species contain stereoidic heterosides of buphanolidic type (hellebroside) from which, by hydrolysis, hellebrigenine, glucose and ramnose result. It also contains two saponosides: helleborine and helleboreine, as well as an unsaturated lactone: protoanemonine.
The therapeutical action is of cardiotonic type, with rapid elimination, but it is also diuretic, antirheumatic and antiinflammatory of the articulations.
Therapeutical indications: cardiac insufficiency (only as standardized preparations in cardiotonic glycosides); articular diseases, rheumatism, myalgia, neuralgia.

Material and methods

Adonis, Helleborus and *Ranunculus* were taken from a place called Baciu, Cluj County. All of them were gathered in June 2004, dried adequately at room temperature, protected against sunbeams, then dried in an oven until constant weight.
The following parts (or morphological categories) of a plant were used: roots, leaves (from different heights), stems (from different heights), seeds, involucres, receptacles, petals (of different colours), capsules with immature fruit.
The amount of samples varied between 0.5 and 2 grams of dry substance, prepared according to the described technique.
It should be mentioned that the values given in Table 1 represent the arithmetical mean obtained after weighing 5 distinct samples for each part of the plant. All of them were then processed according to the described technique for wet disintegration, and, afterwards, were subjected to spectrophotometrical analysis [5,6,7,9].

Table 1. The weights of the samples (g)

Morphological category	Mass of the sample to be considered (g)
Adonis roots	0,5261 ± 0,0005
Adonis leaves	0,2771 ± 0,0002
Adonis stalk (1/3 inferior)	0,3190 ± 0,0003
Adonis stalk (1/3 medium)	0,2859 ± 0,0002
Adonis stalk (1/3 superior)	0,1958 ± 0,0001
Adonis yellow petals	0,2148 ± 0,0002
Adonis buds	0,2209 ± 0,0002
Adonis flowers	0,3846 ± 0,0003
Heleborus roots	1,0356 ± 0,0006
Heleborus stalks	0,9472 ± 0,0005
Heleborus leaves	0,3809 ± 0,0003
Heleborus flowers	0,4026 ± 0,0004
Heleborus fruits	0,2340 ± 0,0002
Ranunculus roots	0,2323 ± 0,0002
Ranunculus stalks	0,1989 ± 0,0001
Ranunculus leaves	0,1676 ± 0,0001
Soil from the cultivation area of species	1,1025 ± 0,0006

Preparation of the Samples

For the determination of the amount of oligoelements by means of atomic absorption spectrophotometry, an amount of the vegetal mass (buds, leaves, roots, petals, flowers) is previously treated in a porcelain dish with a solution of 1% HNO_3, which is removed after the sample is washed, and dried in an oven at about 105 ^{o}C until constant weight. The well dried

vegetal substance is ground in a mortar. The fine powder obtained thus is subjected to mineralization.
Mineralization consists in the treatment of the weighed samples with about 15-30 ml of 65% Nitric acid extra pure (Merck) in a crucible or a porcelain dish. Then, the samples are let to macerate for at least 24 hours, and afterwards dried out on a sand bath.The resulted residues were washed again, more times, using about 10-20 ml of 65% Nitric acid (Merck), or Nitromuriatic acid (1 part of concentrated HNO_3 and 3 parts of concentrated HCl), then dried out on a sand bath until the resulted residues became colourless [6,9].
The final residues were put in a warm solution of 1% HNO_3, filtered on filter paper by suction, and after the washing of the filter, the solutions were collected in a flask of 25 ml, and completed to the sign with the same solvent (1% Nitric acid).
The obtained solutions were analysed by atomic absorption sprectrophotometry (AAS), flame procedure, using a SPECTR AA device, produced by the Varian firm. For the determination of the Pb, a previous calcination was performed in a GTA 110 oven, followed by spectrophotometry [8].
The investigated elements were: K, Na, Mg, Ca, Fe, Mn, Zn, Cu, Pb.

Parameters of the spectrophotometry:
Device: SPECTR AA (Varian)
Process: with flame
Tank: 1 cm in side
Analysed cations: K^{+}, Na^{+}, Mg^{2+}, Ca^{2+}, $Fe^{2+/3+}$, Mn^{2+}, Zn^{2+}, Cu^{2+}, Pb^{2+}.

Results and discussion

In Table 2 are presented the values obtained by spectrophotometry. It should be mentioned that they are expressed in mg element per gram of dry sample, for the analysed elements.
The mathematical calculation of the obtained values was performed according to the formula given below: $C(mg/g) = [c(mg/l) \times V_b(l)]/m_{probă}(g) = [c(mg/l) \times 0,025]/ m(g)$
To obtain the value expressed as ppm (μg/g) the following calculation formula is used:

$$C(\mu g/g) \text{ or ppm} = c(mg/g) \times 1000$$

Table 2. The presence of the elements in the samples in mg/g

Morphological category	K	Na	Mg	Ca	Fe	Mn	Zn	Cu	Pb
Adonis roots	33,7 ± 0,3	0,395 ± 0,003	5,46 ± 0,03	95,6 ± 0,7	2,24 ± 0,02	0,0121 ± 0,0001	1,96 ± 0,02	0,0211 ± 0,0001	0,0076 ± 0,0001
Adonis stalk (1/3 inferior)	44,6 ± 0,3	0,492 ± 0,004	1,42 ± 0,01	21,5 ± 0,2	1,22 ± 0,01	0,0031 ± 0,0002	1,62 ± 0,01	0,0176 ± 0,0001	0,0188 ± 0,0001
Adonis stalk (1/3 medium)	102 ± 1	0,867 ± 0,008	7,73 ± 0,05	59,6 ± 0,5	2,83 ± 0,02	0,0032 ± 0,0002	1,04 ± 0,01	0,0173 ± 0,0001	0,0297 ± 0,0002
Adonis stalk (1/3 superior)	56,6 ± 0,4	1,02 ± 0,01	6,60 ± 0,05	46,8 ± 0,4	2,26 ± 0,01	0,0019 ± 0,0002	0,272 ± 0,003	0,0254 ± 0,0002	0,0370 ± 0,0002
Adonis leaves	16,8 ± 0,2	0,586 ± 0,004	6,95 ± 0,06	24,2 ± 0,2	1,86 ± 0,01	0,0109 ± 0,0001	1,84 ± 0,01	0,0101 ± 0,0001	0,0217 ± 0,0001
Adonis yellow petals	42,5 ± 0,3	0,865 ± 0,006	5,82 ± 0,04	25,4 ± 0,2	2,92 ± 0,02	0,0141 ± 0,0001	1,93 ± 0,02	0,0296 ± 0,0002	0,0268 ± 0,0002
Adonis buds	41,4	0,787	0,702	13,5	3,06	0,0052	1,81	0,0317	0,0272

	± 0,3	± 0,006	± 0,005	± 0,1	± 0,02	± 0,0001	± 0,01	± 0,0002	± 0,0002
Adonis flowers	33,2 ± 0,2	0,409 ± 0,003	4,86 ± 0,03	19,3 ± 0,2	2,11 ± 0,02	0,0127 ± 0,0001	1,96 ± 0,01	0,0298 ± 0,0002	0,0176 ± 0,0001
Heleborus roots	1,04 ± 0,01	0,0545 ± 0,000	4,56 ± 0,05	1,24 ± 0,01	0,739 ± 0,008	0,0433 ± 0,0004	0,661 ± 0,004	0,0050 ± 0,0001	0,0054 ± 0,0001
Heleborus stalks	34,5 ± 0,2	0,402 ± 0,005	2,84 ± 0,03	5,24 ± 0,04	0,478 ± 0,005	0,0109 ± 0,0001	0,217 ± 0,002	0,0091 ± 0,0001	0,0045 ± 0,0001
Heleborus leaves	25,2 ± 0,2	0,672 ± 0,005	6,17 ± 0,04	22,1 ± 0,1	1,02 ± 0,01	0,0436 ± 0,0003	0,181 ± 0,002	0,0099 ± 0,0001	0,0118 ± 0,0001
Heleborus flowers	26,3 ± 0,1	0,604± 0,004	4,94 ± 0,04	25,2 ± 0,2	1,12 ± 0,01	0,0189 ± 0,0001	0,050 ± 0,003	0,0101 ± 0,0002	0,0118 ± 0,0001
Heleborus fruits	33,4 ± 0,3	1,26 ± 0,01	5,85 ± 0,05	33,6 ± 0,2	1,51 ± 0,01	0,0255 ± 0,0002	0,662 ± 0,007	0,0200 ± 0,0002	0,0224 ± 0,0002
Ranunculus roots	11,5 ± 0,1	1,97 ± 0,01	7,83 ± 0,07	21,8 ± 0,1	9,64 ± 0,08	0,200 ± 0,002	6,27 ± 0,07	0,0666 ± 0,0004	0,0301 ± 0,0003
Ranunculus stalks	78,9 ± 0,5	41,7 ± 0,3	4,79 ± 0,04	25,1 ± 0,2	11,5 ± 0,2	0,0171 ± 0,0001	2,65 ± 0,02	0,0429 ± 0,0003	0,0302 ± 0,0003
Ranunculus leaves	42,9 ± 0,4	28,2 ± 0,3	11,2 ± 0,1	33,9 ± 0,3	5,79 ± 0,04	0,102 ± 0,001	6,17 ± 0,05	0,0275 ± 0,0002	0,0373 ± 0,0004
Soil from the cultivation area	29,5 ± 0,4	0,290 ± 0,007	33,8 ± 0,4	65,8 ± 1,1	69,4 ± 1,2	5,03 ± 0,03	1.483 ± 0,01	0,0408 ± 0,0010	0

After the investigation of the cations there resulted that K is the best represented microelement in all the types investigated species; an exception is the 1,04 mg/g in the roots of *Helleborus,* and of 29,5 mg/g in the cultivation soil.

The Na^+ cation is found in different morphological categories with values of 0,054 mg/g in the *Helleborus* roots, and of 41,7 mg/g in the *Ranunculus* stems, while in the cultivation soil there is an amount of only 0,290 mg/g Na. This shows a marked tendency of Na accumulation in the stems and leaves of *Ranunculus*, indicating them as being the main source in the alkaline-earth metals for the *Ranunculaceae* family.

It has been proved that Magnesium is accumulated in the investigated plants in amounts between 0,702 mg/g in the floral buds of *Adonis* while in the cultivation soil the amount of magnesium is of 33,8 mg/g. As an element with curative value, the presence of magnesium is very important, so it is worth studying and investigating its accumulation in higher amounts in the soil.

Calcium is another significant element, it is found in the studied *Ranunculaceae* types in amounts of 1,24 mg/g in the same *Helleborus* root, and of 95,6 mg/g in the *Adonis* root. The very high variation of the calcium content is explained by its different accumulation in the species of the family.

As far as iron is concerned, in the soil was found an amount of 69,4 mg/g Fe, and in the studied plants the amount was of 0,478 mg/g in the *Helleborus* stems, and of 11,5 mg/g in the *Ranunculus* stems. This data show that iron has a very low tendency of accumulation in *Helleborus,* while in *Ranunculus* this tendency is about 20 times higher.

Mn^{2+}, considered in many sources of information as an indicator of the environment pollution, didn't show a tendency of accumulation in the studied plants. Therefore, this could be a special indication for the use of the mentioned plants as diuretic remedies of vegetal origin.
The accumulation tendency of zinc and copper in the *Ranunculaceaee* is, also, very low.
Lead was identified as being present in all the investigated types. However, its content is in a range of permitted values.
This reasoning shows that the leaves of the plants are very sensitive, so they could become real markers of the environment pollution in the cultivation area.
A general conclusion can be drawn after the investigation of the before mentioned cations, namely, that neither of them which have a toxicologic potential (Pb^{2+}, Mn^{2+}) are present in high amounts. At the same time, the *Helleborus* roots are the poorest in microelements.

Conclusions

For the first time a complete screening of the main elements present in the different morphological categories of the *Adonis, Helleborus, Ranunculus L. (Ranunculaceae)* species, has been carried out.
The high amount of potassiun found in them can be explained by the general tendency of all the plants to accumulate it.
High amounts of Ca and Mg are also remarked, an aspect which is extremly interesting for the therapeutical availability of the medicinal products obtained from the studied species.
Concerning the content of Fe, Mn and Cu it can be concluded that there is a low degree of danger, since their amounts are small.
The presence of lead at different levels of the plant reveals the proximity of a source of elimination of the tetraethyl lead, used as antiexplosive for fuels.
Also considerable amounts of Ca^{2+}, Mg^{2+} were found, as the presence of Pb^{2+} is minor, especially at the higher leaves, but the determined values are not even reaching the maximum admitted limit (5mg/kg of dried vegetal mass).

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SECONDARY METABOLITES FROM *ASPERULA LUTEA* SUBSP. *RIGIDULA*

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Summary

From the aerial parts of Asperula lutea subsp. rigidula (Halacsy) Ehrend., an endemic of south eastern Greece, 9 known compounds, β-sitosterol, chlorogenic acid, geniposidic acid, deacetyl-asperulosidic acid, scandoside, quercetin, hyperi, isoquercitrin and rutin, were isolated. The structures of the compounds were elucidated by application of one- and two-dimensional NMR spectroscopy. From the analysis of a non polar fraction of the methanolic extract by means of GC-MS nineteen compounds were identified, mainly esters of fatty acids. The major components were ethyl ester of palmitic acid (32.7%), ethyl ester of linolenic acid (20%) and ethyl ester of linoleic acid (10.5%)

Keywords: *Asperula lutea subsp. rigidula; Rubiaceae; flavonoids; iridoids; fatty acids.*

Introduction

The genus *Asperula* (Rubiaceae) includes c. 90 species, 66 of which growing wild in Europe (Mabberley 1997). *A. odorata* L. (syn. *Galium odoratum*) is used in folk medicine as a diuretic, tonic and against diarrhea. Iridoid glycosides, cardenolides, flavonoids and anthraquinone glycosides have been reported from several *Asperula* species. However, no work has been reported on the chemical constituents of *A. lutea* subsp. *rigidula* (Halacsy) Ehrend. an endemic of south eastern Greece (Ehrendorfer and Krendl 1976).

Materials and methods

Aerial parts of *A. lutea* subsp. *rigidula* were collected in Megara (Prefecture Attiki), during the flowering period, in June 2000. Air-dried aerial parts (530 g) were extracted with methanol in room temperature; the solvent was removed under reduced pressure to afford 33.53 g of residue. VCC separation was performed with Kieselgel 60H (Merck), TLC were performed with Kieselgel 60 F_{254} (Merck aluminum support plates) and spots detected with 15% H_2SO_4 in MeOH reagent, and with Cellulose (Merck) and spots detected with Neu. Preparative HPLC were carried on Waters 2487 Dual λ Absorbency Detector equipped with a reversed phase column Supercosil, SPLC-18, 58368, Col: 10799-006, 25 cm x 10 mm, 5μm. The NMR spectra were recorded on Bruker AC 200 MHz and Bruker DRX 400 MHz spectrometers. The UV spectra were taken with a Shimadzu UV model 160A spectrometer.
The GC-MS analysis was carried out on a Hewlett Packard 5973-6890 GC-MS system, operating in the EI mode at 70 eV, equipped with a split/splitless injector (200^o C). The transfer line temperature was 250^o C. Helium was the carrier gas and the capillary column used was HP-5 MS (30m × 0.25mm, film thickness 0.25μm). The temperature program was 60^o C to 280^o C at a rate 3^o C /min; split ratio 1:10. The injected volume was 1.0 μl. The compounds were identified by using Wiley and NIST/NBS MS libraries, by comparison of their retention times with those of authentic compound (Sigma Chemical) and literature (Adams 2001). The relative amounts of the components were calculated from the peak areas.

Results and discussion

The composition of a non polar fraction (Asp 1) from the VCC of the methanolic extract of *A. lutea* subsp. *rigidula* is presented in Table 1. Nineteen components were identified, representing

98.8% of the total composition. Constituents were mainly esters of fatty acids. The major components were ethyl ester of palmitic acid (32.7%), ethyl ester of linolenic acid (20.1%) and ethyl ester of linoleic acid (10.5%). The natural presence of methyl and ethyl esters of fatty acids was particularly interesting because they are related with the biosynthesis of fatty substances of the plants and it shows that methyl esters in higher plants are not as rare as it was thought to be.
Furthermore from the methanolic extract were isolated nine components; one sterol: *β*-sitosterol, one hydroxycinnamoylquinic acid: chlorogenic acid, three iridoids: geniposidic acid, deacetyl-asperulosidic acid, scandoside and four flavonoids: quercetin, hyperin, isoquercitrin and rutin. The structures of the isolated compounds were identified on the basis of their chromatographic behavior, spectral characteristics and with the aid of one and two-dimensional NMR experiment, as well as by comparison with literature data.
The isolated flavonoids are derivatives of quercetin and have been reported previously from various *Asperula* species. From the isolated iridoids, deacetyl-asperuloside has been reported from several *Asperula* species and the other isolated iridoids belong to the asperuloside type.
Iridoids have been used as chemotaxonomic markers in the closely related genus *Galium*. Further studies on other *Asperula* species may lead in the discovery of metabolites that could be used to verify the taxonomic position of the genus.

Table 1. Constituents identified in fraction Asp 1

Component	RRI	%
Thymol	1291	7.1
n-Tetradecane ($C_{14:0}$)	1400	1.3
Ethyl laurate ($C_{12:0}$)	1573	0.9
n-Hexadecane ($C_{16:0}$)	1600	2.4
Ethyl myristate ($C_{14:0}$)	1780	1.3
n-Octadecane ($C_{18:0}$)	1800	1.4
Hexahydrofarnesyl acetate	1833	1.7
Ethyl pentadecanoate ($C_{15:0}$)	1883	0.7
Methyl palmitate ($C_{16:0}$)	1919	4.6
Ethyl 9-hexadecenoate	1984	1.3
Ethyl palmitate ($C_{16:0}$)	1994	32.7
Linoleic acid methyl ester ($C_{18:2}$)	2084	3.2
Linolenic acid ($C_{18:3}$) methyl ester	2091	4.7
Linoleic acid ($C_{18:2}$) ethyl ester	2156	10.5
Linolenic acid ($C_{18:3}$) ethyl ester	2164	20.1
Oleic acid ($C_{18:1}$) ethyl ester	2168	1.0
Ethyl stearate ($C_{18:0}$)	2188	2.2
Neophytadiene	2217	0.6
Arachidic acid ethyl ester	2333	1.1

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PHARMACOLOGICAL RESEARCH ON DIURETIC, SALURETIC AND URICOSURIC ACTIVITY OF SOME EXTRACTS FROM *HIERACIUM PILOSELLA* L. *(ASTERACEAE)*

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Summary

Hieracium pilosella, known as mouse-ear hawkweed, is a perennial plant, of little size, with a horizontal rhizome with numerous overground stolons. In order to test the diuretic, saluretic and uricosuric properties of two extracts from Hieracium pilosella, four lots of Wistar rats, with a medium weight of 150g were used. All the extracts prepared from Hieracium pilosella showed a diuretic and saluretic activity superior to the wittness lot, but inferior to furosemide.

Keywords: *Hieracium pilosella, diuretic, saluretic, uricosuric*

Introduction

Hieracium pilosella L. (Asteraceae) known as mouse-ear hawkweed, is a perennial plant, of little size, with numerous and long over ground stolons. The basal rosette has leaves white below, green with long white hairs. A single erect stem arises from the rosette, carrying a single anthodium. The flowers are exclusively ligulated, pale yellow. The plant vegetates on pastures, dry fields and sunny hills, where it forms dense patches (1,2).
The phytochemical analysis, performed by TLC and UV spectrometry, identified the main active principles of the plant: flavonoids (glycosides of apigenine and luteoline), coumarins (umbelipherone), phenyl-propane compounds (chlorogenic acid) (3).
The aerial part of the plant (*Pilosellae herba*) is used in traditional medicine as diuretic, uricosuric and anti-inflammatory, in inflamed kidneys or respiratory problems, but the pharmacological properties of the plant were not studied before in Romania.

Materials and method

The aerial part of *Hieracium pilosella* was harvested during the flowering period (August), from Cluj area. After drying, the vegetal product was grinded to a fine powder, from which the following were prepared:

- -1:6 alcoholic extract with 70^0 alcohol, after Squibb technique (4)
- -16 % aqueous extract according to FR X regulations (5)

Diuretic, saluretic and uricosuric activity of the extracts was tested on male Wistar rats, with a medium weight of 150 g. The animals were placed in groups of five in specially designed diuretic cages. The access to food was prohibited 24 h prior to the experiment. After 24h, each animal was given orally 2,5 ml/100 g normal saline solution for hydration, and after 1 h the following substances were administered orally:
Group I (witness): normal saline solution 1ml, group II (control): 30 mg/kg furosemide, group III: 1 ml 1:6 alcoholic extract from *Pilosellae herba*, group IV: 1 ml 16 % aqueous extract from *Pilosellae herba*.

The animals' urine was collected after 24h from each individual diuretic cage, the obtained volume being expressed in ml/kg/24 h. Diuretic index (I.D.) was calculated as a ratio between the diuresis of the treated animals compared with the witness group (6,7).
The concentration of sodium and potassium ions was determined in urine by a potentiometric method, using a VITROS 250 Chemistry System autoanalyser (Johnson and Johnson Clinical Diagnostic), and was expressed in mmol/kg/24h. Saluretic index was calculated for sodium and potassium as a ratio between their concentrations in the urine of the treated groups compared to the witness group.
Also, uric acid concentration was determined in urine, using a colorimetric method at λ=670 nm, using an enzymatic reaction which transforms uric acid in a colored compound, and finally was expressed in mg/kg/24 h.

Results

The animals that were given furosemide (group II) excreted an urine volume of 48,326 ml/kg/24h (ID=2,038), far superior to the witness group, which eliminated 23,703 ml/kg/24h. Among the extracts from *Pilosellae herba*, the1:6 alcoholic extract presented an urine volum of 29,700 ml/kg/24h, with ID=1,371), while the animals treated with the 16 % aqueous extract eliminated an urine volume of 29,962 ml/kg/24h, with ID=1,089).
Animals treated with furosemide excreted an amount of sodium ions of 6,856 mM/kg/24h (I_{Na}=2,146), and 3,149 mM/kgcorp/24h potassium ions, respectively (I_K=2,703). The group treated with the 1:6 alcoholic extract from *Pilosellae herba* excreted an amount of 4,346 mM/kg/24h sodium ions (I_{Na}=1,360), and 1,947 mM/kg/24h potassium ions (I_K=1,671), while the aqueous extract group presented values of 5,347 mM/kg/24h sodium ions (I_{Na}=1,674), and 2,044 mM/kg/24h potassium ions (I_K=1,754). As for uricosuric effect, the urinary elimination of uric acid was 3,471 mg/kg/24h for the group that was given the 1:6 extract, and 2,231 mg/kg/24h for the group treated with the 16% aqueous extract. Thus, uricosuric activity was superior to the witness group that eliminated 2,895 mg/kg/24h, only in the animals treated with the 1:6 alcoholic extract.
The results are presented in tables 1 and 2. Statistical interpretation was performed by „t" Student test

Table 1. Diuretic activity of the extracts from *Pilosellae herba*:

No.	Treatment	Administered volume (ml)	Diuresis (ml/kg/24h) X±es	Diuretic index
I	Saline solution	1	23,703±2,656	-
II	Furosemide	1	48,326±1,854 p=0,002*	2,038
III	1:6 alcoholic extract	1	29,700±4,194 p=0,05*	1,253
IV	16% aqueous extract	1	29,962±3,697 p=0,02*	1,264

*Statistically significant (p<0,05)

Table 2. Saluretic activity of the extracts from *Pilosellae herba*:

No.	Treatment	Saluresis Na^+ (mmol/kg/24h) X±es	Saluretic index Na^+	Saluresis K^+ (mmol/kg/24h) X±es	Saluretic index K^+
I	Saline solution	3,194±0,071	-	1,165±0,025	-
II	Furosemide	6,856±0,540 p=0,003*	2,146	3,149±0,181 p=0,0002*	2,703
III	1:6 alcoholic extract	4,346±0,145 p=0,0006*	1,360	1,947±0,065 p=0,0001*	1,671
IV	16% aqueous extract	5,347±0,241 p=0,0005*	1,674	2,044±0,092 p=0,0003*	1,754

* Statistically significant ($p<0,05$)

Discussion

All the tested vegetal extracts produced a diuresis superior to the witness group (table 1), but inferior to furosemide, the intensity of the diuretic effect being very similar for the two extracts. (I.D. =1,253-1,264)
As for the saluretic activity (table 2), vegetal extracts from *Pilosellae herba* have produced an increased urinary elimination of Na^+ ions, presenting saluretic indexes between 1,360-1,674, and also an increased elimination of K^+ ions, saluretic index varying between 1,671-1,754. The most intense saluretic effect was produced by the 16% aqueous extract from *Pilosellae herba*. As for the uric acid excretion, only the 1:6 alcoholic extract presented a relatively weak uricosuric effect.

Conclusion

Diuretic, saluretic and uricosuric acivity of the 1:6 alcoholic extract, and 16% aqueous extract from *Hieracium pilosella* (L.) (Asteraceae) was studied.
The diuretic activity was superior to the witness group for all the tested extracts. Saluretic effect was superior to the witness group for both Na^+ and K^+ for all vegetal extracts.
Uricosuric activity was present only in the 1:6 alcoholic extract from *Hieracium pilosella*.
The results confirmed the diuretic, saluretic and uricosuric effects of *Hieracium pilosella* L. (Asteraceae).

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SPRUCE TERPENES AND OXIDATIVE STRESS: A REALITY CHECK

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Summary

The chemical composition and basic physiology of conifer resin has been studied for years, but the functions of resin in the life of the tree itself are still only poorly understood. While the non-volatile resin components have often been suggested to have a role in protecting the tree against herbivores and pathogens, the function of the volatile portion of the resin is less clear. Based on the fact that certain monoterpenes and sesquiterpenes react readily with Active Oxygen Species (AOS) in laboratory experiments, it has been hypothesized that they could protect plant cells against oxidation in vivo. However, this hypothesis has never been studied. A series of experiments was set up to evaluate the role of volatile plant terpenes in protecting conifers against AOS. The experiments were designed to measure the quantity of volatile terpenes emitted from Norway spruce after different treatment and meanwhile to measure the extent of oxidative damage. Treatments caused a dramatic increase in terpene emission as well as reduction on MDA which support the hypothesis of the protective role of volatile terpenes against oxidative stress, but more experiments are needed to confirm it.

Keywords: *Active oxygen species, AOS, Lipid-peroxidation, spruce, volatile terpenes.*

Introduction

The concept that plant secondary compounds, especially terpenes evolved for defence against herbivores and other parasites is now well established in the ecological literature. Terpenoids in conifers are usually stored in complex multicellular compartments, such as resin ducts (Franceschi et al., 2005). It has therefore been suggested that the production of terpenoids may generally be limited more by the number and size of storage compartments than by the availability of carbon for terpenoid synthesis (Gershenzon, 1994). While the non-volatile resin components have often been suggested to have a role in protecting the tree against herbivores and pathogens, the function of the volatile portion of the resin is less clear. Volatile organic compounds (VOCs) emitted by plants show a puzzling diversity and Norway spruce (*Picea abies*) is the most important terpenoid emitter in the north European boreal forest and that due to its much higher biomass compared to that of the deciduous emitters (Geron et al., 2000). The volatile resin substances may also function in defense against enemies, but recently it has been proposed that plant chemical defence could be primarily aimed at abiotic stresses (Peñuelas and Llusià, 2004). Most of this evidence comes from their chemical reactivity in atmosphere with certain reactive oxygen species (Lerdau et al., 1997).
Among these VOCs, isoprene is the most frequntly emitted (Geron et al., 2000) and its possible function one of the most studied (Sharkey and Yeh 2001). One potential role of the isoprene is thought to be the thermal protection i.e. the stabilization and protection of plant membranes against high temperatures (Sharkey and Singsaas, 1995). A second hypothesis for a protective role of isoprene and monoterpenes is that they serve as an antioxidant in leaves (Loreto and Velikova 2001). Isoprene may confer protection against singlet oxygen in leaves (Affeck and Yakir, 2002) but the antioxidant effect may be a general hydrocarbon effect and related to the double bonds in the isoprene molecule. However, the role of other terpenes rather than isoprene in plants is still unclear. Several experiments have shown that terpenes may have a role in protecting plants from thermal damage (Delfine et al., 2000; Loreto et al., 2004). However, such enhancement of thermotolerance has not always been found and the amount of terpenes fumigated to increase the thermotolerance has been very high compare with normal emitting values (Delfine et al., 2000).

In the present study, we used the herbicide paraquat to stimulate the oxidativee stress, and tested the antioxidative role of monoterpenes from Norway spruce. Paraquat (methyl viologen, 1,1'-dimethyl-4,4'-bipyridinium dichloride) exerts its phytotoxic effects by catalyzing the transfer of electrons from Photosystem I to molecular oxygen. Resulting accumulation of superoxide radicals in chloroplasts cause lipid peroxidation and membrane destruction.

Material and methods

Plant growth and treatment

Norway spruce seedlings were grown in pots with 0.3 kg perlite and fertilized every five days. The experiments were carried out in a growth chamber with a light/dark regime of 16 h/22° C and 8 h/16° C. Light intensity was 240 µE m^{-2} s^{-1}.

Forty days old-seedlings were used. Twelve pots with ca 10 seedlings were spayed (pretreated) with 100 µM Methyl Jasmonate, six pot with paraquat while six other pots were sprayed only with water and used as Control). 24 hours later, six pot previously pretreated with MJ were sprayed with Paraquat 4 mg/mL. The sublethal doses of both MJ and paraquat had been determined in previous experiments. Samples for both terpene analysis and MDA analysis were collected 8, 24, 36, 48, 72 and 96 hours after treatment with paraquat. 100 mg of fresh tissue for Malondialdehyde (MDA) and 75 mg for terpene analysis were harvested at every sampling time. Volatile terpenes were collected using a closed-loop stripping system (Fig. 1)(for more details on the system see Tholl et al., 2006).

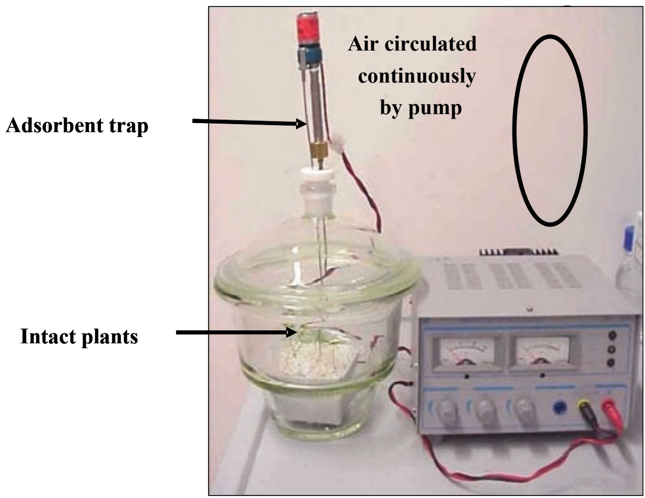

Fig. 1. Schematic presentation of the Close-Loop Stripping system

A charcoal filter was used to absorb the released volatiles. The filter was eluted with 100µL of dichloromethane and the Isobutylbenzene (IBB) was added as internal standard for quantification of the monoterpenes. The non-emitted terpenes were extracted based on the procedure of Zeneli et al. (2006). GC-MS analysis of terpenes were run as described by Zeneli et al. (2006). The total monoterpene content was calculated as the sum of the individually quantified compounds.

Estimation of lipid peroxidation

The extent of lipid peroxidation in needles was estimated by measuring the amount of MDA by the method described by Hodges et al. (1999), which takes into account the possible influence of interfering compounds in the assay for thiobarbituric acid (TBA)-reactive substances. Briefly, samples were repeatedly extracted with 80:20 (v/v) ethanol :water containing 1 p.p.m. butylated hydroxytoluene (BHT) using sonication. After centrifugation,

supernatants were pooled and an aliquot of appropriately diluted sample was added to a test tube with an equal volume of either: (1) –TBA solution containing 20% (w/v) trichloroacetic acid and 0.01% (w/v) BHT; or (2) +TBA solution containing the above plus 0.65% (w/v) TBA. Samples were heated at 95°C for 25 min and, after cooling, absorbancewas read at 440, 532 and 600 nm. MDA equivalents (nmol ml^{-1}) were calculated as 10^6 x [(A – B)/157000, where A = [(Abs 532_{+TBA}) - (Abs 600_{+TBA})] - [(Abs 532_{-TBA} - Abs 600_{-TBA})] and B = [(Abs 440_{+TBA} - Abs 600_{+TBA})] x 0.571

Results and discussions

Oxidative stress has no effect on biomass

Monoterpene protection against effects of oxidative stress was investigated in Norway spruce seedling. Paraquat induced oxidative stress and this stress was also visible. Seedling experienced paraquat alone loosed they green colour and turned to brown. However no biomass reduction was observed. This because of the relative short experimental time.

Methyl jasmonate increases terpene content of both non-emitted and emitted volatile fractions

The non-emitted fraction of the terpene from seedling was dominated by the presence of α-pinene and β-pinene, relatively lower amount of limonene and β-phellandrene, and the great variability of Δ-3-carene among individuals. Some other monoterpenes were found only as traces. Methyl jasmonate treatment caused a more than 2 fold increase in monoterpenes present in the needles. The first increase was recorded as early as 12 hours after treatment and this increase was recorded up to 72 hours post-treatment. However, as previously reported for *Pinus* species (Heijari et al. 2005) methyl jasmonate treatment did not have any substantial effect on the terpene composition of *Picea abies* seedlings. In contrast, terpene composition has been demonstrated to be significantly altered by mechanical wounding, herbivory or pathogen infestation in other conifers, such as *Abies grandis*.

The emitted fraction of the terpene from seedling was an order of the magnitude lower than non-emitted volatile fraction but yet was dominated by the presence of α-pinene and β-pinene, relatively lower amount of limonene and β-phellandrene. Methyl jasmonate treatment caused a more than 3 fold increase in monoterpenes present in the needles. In contrary to the non-emitted terpene fraction, methyl jasmonate treatment caused substantial effect on the terpene composition of *Picea abies* seedlings. Unlike in the non-volatile fractions where no sesquiterpenes were recorded, a relatively high presence of the trans-β-farnesene was measured in the volatile fraction of methyl jasmonate treated seedling. Similar result has been reported also in 4-year old spruce saplings after methyl jasmonate treatment (Martin et al., 2003).

Paraquat treated seedlings showed monoterpene emission rates showed similar to that of control. No qualitative or quantitative differences were recorded between contol and paraquat treated seedlings.

Exogenous Methyl Jasmonate slightly prevented paraquat induced lipid peroxidation

In Norway spruce seedlings not protected by methyl jasmonate pre-treatment of the paraquat induced oxidative stress compared to control and the pretreated seedlings. Paraquat treatment resulted increasingly in the formation of malondialdehyde (MDA) as an indicator of membrane lipid peroxidation. Compared with control samples, MDA levels increased three fold (80 μmol/g FW in paraquat stressed seedlings *vs* 27 μmol/g FW in control seedlings).

In contrary, pre-treatment with methyl jasmoante uncoupled the formation of MDA from the intensity of paraquat stress. MDA levels measured in methyl jasmonate pretreated seedlings were ca. 40% lower than in paraquat stressed seedlings (55 μmol/g FW). However, even these seedlings also turned brownish at the end of the experiment.

Conclusions

In Norway spruce seedlings, paraquat treatment caused oxidative stress. Formation of Active Oxygen Species (AOS) produced in the photosynthetic membranes lead to production of peroxidation of lipids as showed by the production of Malondialdehyhe. Terpene production and emission was increased by application of methyl jasmonate and this was correlated with slightly decrease in MDA productions. However it might be highly speculative to draw a conclusion on the antioxidative effect of monoterpenes emitted from spruce seedlings. Other antioxidant systems already established in the plants might be involved. We are in the progress of evaluating other enzymatic and non-enzymatic antioxidant that might be responsible.

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