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Full Length Research Paper

Identification and Comparison of Volatile Constituents of Himalayan Phytofood; *Allium auriculatum* Kunth and its Antimicrobial Activity

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Abstract

Allium auriculatum yielded 0.1% pale yellow oil with a pleasant aroma. The GC-MS analysis of the essential oil of Allium auriculatum shows rich in phthalates contributing 79.80% and headspace GC-MS analysis of Allium auriculatum revealed the dominant component are 60.05% limonene, 17.33% isovaleraldehyde. Allium auriculatum oil extracts also revealed a broad spectrum activity.

Key words: *Allium auriculatum Kunth, GC-MS, Headspace GC-MS, Minimum Inhibitory Concentration (MIC)*

Introduction

Allium is the onion genus, with 860 known species making it one of the largest plant genera in the world. *Allium* was classified in family Alliaceae. However, in the classification of Angiosperm Phylogeny Group (2009), Alliaceae is now the subfamily Allioideae of the family Amaryllidaceae. Previously some botanical authorities have included it in the lily family (Liliaceae).(Eric Block., 2010; James *et al*., 2008; Dilys Davies, 1992;Haim D. Rabinowitch, *et al*., 2002)

Allium auriculatum Kunth which is distributed in Uttarakhand (Dharchula and Munsiyari), Himachal Pradesh, Jammu & Kashmir in India. It is found abundantly on rocky slopes at 3350-5480 m. Flowering and fruiting occurs between July – September (Anjula Pandey, 2008) , a herb which is 12-35 cm high; bulbs cylindrical, 5-8 x 1-1.5 cm, coats fibrous, reticulate; root c. 1 mm in diameter. Leaves 3 or 4, arising from basal half of stem, linear, 12-20 x 0.3-0.5 cm, flat, obtuse; internodes 1-5 cm long, Umbels hemispherical and 1.5-2.5 cm in diameter. It is used as spice and tempering in food in traditional dishes of hill area of Uttarakhand. It is known that the volatiles in *Allium* species are produced by enzymatic splitting of the non-volatile precursors, *S*-alk(en)yl-l-cysteine sulfoxides, when the plants are crushed. The alk (en) yl groups are mainly a combination of propyl, 1-propenyl, allyl, and methyl groups, depending on the species (Fenwick, G. R.,1985; Carson, J. F., 1987).

Saghir *et.al.* found that volatile compounds found in *Allium* species may be divided into the following groups: sulphides, disulphides, trisulphides, tetrasulphides, pentasulphides, oxygenated compounds, and terpenes (Saghir, A. R., 1964). May-chien *et.al.* reported that sulphur-containing compounds account for 85 % and 77 *5%* of the total volatiles in the distilled oils of welsh onions and scallions, respectively. Carbonyl compounds (aldehydes, ketones, and 3-furanones) are 3.3% and 7.2% of the total volatiles of welsh onions and scallion oils, respectively (May-Chien Kuo, 1990). Epidemiological evidence indicates that a high dietary intake of plants of the Allium family decreases the risk of cancer in humans. Munday *et.al* suggested that this effect is due to the ability of the aliphatic mono-, di-, tri-, and tetra sulphides derived from these vegetables to increase tissue activities of Phase 2 detoxification enzymes (Munday-Rex, 2003). The literature survey revealed that no work has been reported on the chemical analysis and biological activities of *Allium auriculatum* of Kumaun and Garhwal Himalayas

Materials and Methods

Allium auriculatum leaves collected from Dharchula (Uttarakhand) which is about 3000 m amsl and Both regions lies between 29°44' - 31°28' N latitudes and 77° - 81° E longitudes along the central and south eastern parts of the Central Himalaya. The plants were identified by Botany Department of Kumaun University, Nainital and National Bureau of Plant Genetic Resource (NGPR), Niglat, Dist. Nainital.

Chemical Investigation

Extraction of Essential Oil

The essential oil was obtained by steam distillation of fresh plant material (500 g) using a copper still fitted with spiral glass condensers. The distillate was saturated with NaCl and extracted with hexane. The hexane extract was dried using anhydrous sodium sulphate and the solvent was removed with a rotovap at reduced pressure at 25 °C to yield oil.

GC-MS Condition

GC-MS analysis was performed in Agilent 6890 GC equipped with MSD-5975 system fitted with HP-5MS column (19091S-433). GC was conditioned for flow rate of 1.0 ml/min, inlet temperature 250 ºC, oven temperature starting from 50 ºC hold 1 min, 250 ºC at rate of 4 ºC per min. Helium was used as carrier gas and the mode was split less.MSD was conditioned for EI mode (70ev), scan acquisition performed from 40.0amu to 550.0amu, quadrupole at 150 ºC, mass source at 230 ºC. Sample components were identified by matching their mass spectra with those in NIST 05 MS library search and by comparing with literature reports.

Headspace Condition

1.0 gm of sample is taken in 20 ml headspace vial. Headspace is created and these vapours are injected in GC equipped with MSD. The headspace was conditioned at vial temperature 120 ºC, loop temperature 130 ºC, transfer line 150 ºC and the sample was equilibrated for 15 min. Headspace components were identified by matching their mass spectra with those in NIST 05 MS library search and by comparing with literature reports.

Determination of Antimicrobial Activity (Minimum Inhibitory Concentration MIC) Of Oil Extracts by Two-Fold Serial Dilution Method

Materials

Nutrient agar, nutrient broth, and sabouraud Dextrose agar and sabouraud Dextrose broth were purchased from Sisco Research Lab Pvt. Ltd., The bacterial, fungal incubator and cell homogeniser were from Macro Scientific Works. The test organisms; bacterial and fungal cultures (*Staphylococcus aureus* MTCC - 96, *Escherichia coli* MTCC - 40, *Candida albicans* MTCC – 227 and *Cryptococcus neoformans* ATCC-32045 were obtained from Central Drug Research Institute, Lucknow and maintained in the Microbiology laboratory , Department of Biotechnology, M.B. Govt. P.G. College, Haldwani for further use on suitable growth promoting media.

Preparation of Nutrient agar slants for the maintenance of bacterial culture

Suitable quantity of nutrient agar medium, (peptone 5.0 g, NaCl 5.0g, yeast extract 2.0g, Beef extract 1.0 g/lit. pH 7.4±0.2 Agar 15.0 g) weighed and suspended in suitable amount of distilled water. The agar was dissolved by heating the medium in a water bath the melted agar was transferred of 6" x ¾" glass tubes containing approximately 10-12 ml in each tube. After autoclave the medium containing were incubated in a bacteriological incubator at 37 ºC for 24 hrs for the test of sterility of the nutrient medium.

Preparation of Sabouraud Dextrose agar slant for the maintenance of fungal strains

Suitable quantity of Sabouraud Dextrose agar medium (Glucose 20.0, Neopeptoone g/lit pH 5.6±0.2) was suspended in suitable amount of distilled water. The SDA slants were also prepared and autoclaved. The sterility of the SDA slants was also checked by incubating the tubes in a fungal incubator at 28 ºC for at least 72 hrs. These slants were stored in the refrigerator for periodical sub-culturing of bacterial and fungal strains.

Sub-culturing of bacterial and fungal strains in their maintenance media

A lapful culture from the stock of different strains was introduced into fresh agar slants to their respective media aseptically in the laminar air flow with the half of a sterile inoculating needle the inoculated agar slants were then incubated at the optimum temperature i.e 37º±1 ºC in case of bacteria and at 28º±1 ºC in case of fungal strains for 48 hrs. The purity of the strains was checked by staining and observation under microscope. Gram's staining procedure was adopted to stain the bacterial cultures and cotton blue stain was used to stain the fungal cultures.The bacterial and fungal cultures were stored in refrigerator for further use.

Preparation of nutrient broth and Sabouraud's dextrose broth for the determination of minimum inhibitory concentration

100 ml each of nutrient broth (Peptone 5.0, NaCl 5.0, yeast extract 2.0, Beef extract 1.0g/lit pH 7.4±0.2,) and Sabouraud's dextrose broth(Glucose 20.0, peptone 10g/Lit, pH 5.6±0.2) was prepared in 250 ml conical flask by dissolving suitable quantity of each broth in distilled water. The flasks containing the media were cotton plugged and sterilized as described earlier. Microbiological glass pipettes and assay tubes thoroughly washed and dried, cotton plugged with non absorbent cotton were sterilized in a hot air oven at 160 ºC for 2hrs.

Preparation of inoculums of test organisms

For the preparation of inoculums nutrient broth and sabourauds Dextrose broth were prepared in 6" x ¾" glass tubes. The tubes containing broth were sterilized. Bacterial cultures (*Staphylococcus aureus* and *Escherichia coli*, yeast cultures *Candida albicans* and *Cryptococcus neoformans* were inoculated in the respective broth from the stock cultures stored in the refrigerator. The inoculated broth was incubated at optimum temperature for optimum period in the incubators. The fully grown cultures were diluted to such an extent with sterile medium prepared in 250 ml conical flask containing 100 ml nutrient broth/Sabouraud's dextrose broth that a cell concentration of 10^5 cells/ml (10^5/ml colony forming unit (CFU) was achieved. The cell counting was done by haemocitometer.

Preparation of stock solution of oil extracts

The oil extracts were accurately weighed and a stock solution of 1 mg/ml concentration was prepared in dimethyl sulphoxide (DMSO). DMSO was chosen as a carrier solvent because it is water miscible and does not inhibit the growth of the microbes under test a concentration of 200 µl/ml. Furthermore all the oil extracts were soluble in this organic solvent.

For the detection of minimum inhibitory concentration of a single oil extract against a single test organism at least six 4" x ½" assay tubes were arranged in a rack. The bulk medium containing 10^5 CFU/ml of test organism was dispensed in the assay tubes aseptically in such a way that the first tube contained 1.8 ml broth and rest tubes 1.0 ml each with the help of sterile microbiological glass pipettes 0.2 ml or 200 µl of sample from stock solution (1 mg/ml) of oil extract was added to the first tube in the row containing 1.8 ml inoculated broth and mixed thoroughly; 1.0 ml from this tube was taken out and added to the next tube. This process of dilution was repeated till the last or the sixth tube. In this way the concentration of the compound was reduced to its half in each tube. Culture control and solvent control (DMSO) controls were also run simultaneously all the experimental tubes and control tubes were incubated at optimum temperature for optimum period of time i.e. at 37º±1ºC in case of detection of minimum inhibitory concentration against bacterial cultures for 24 hrs and at 28º±1ºC for fungal strains for 48 hrs. After the completion of incubation period the tubes were removed from the incubator and MIC was noted by judging the turbidity or comparing the growth with respective culture controls with unaided eye. In the same manner all the extracts were tested and biological activity was evaluated. The lowest concentration of a compound preventing appearance of turbidity or growth of test organism in the tube under test was considered to be the minimum inhibitory concentration of that compound. The MIC of oil extracts is given in the tabular form.

Results and Discussion

Chemical Investigation

Allium auriculatum essential oil GC-MS

The whole aerial parts of *Allium auriculatum* yielded 0.1% pale yellow oil with a pleasant aroma. The GC and GC-MS analysis of the essential oil of *Allium auriculatum* revealed the presence of 9 compounds and all of them were identified by comparing their mass spectra with NIST05 MS library. The oil is rich in phthalates contributing 79.80%, while the monoterpenoids contributed 4.07%. The major constituents are 79.8% bis (2-ethylhexyl) phthalate, 7.45% palmidrol, 6.83% octanamide and 2.65% d-limonine. The complete analysis of *Allium auriculatum* is being reported for the first time. (Table: 1, Figure: 1)

Table 1: Chemical composition (%) of essential oil of *Allium auriculatum* by GC-MS

Pk	RT	Area %	Common Name
1	12.305	0.96	o-cymene
2	12.404	2.65	d-limonene
3	13.453	0.46	γ-terpinene
4	19.305	1.34	(s)-(+)-6,8-p-menthadien-2-one
5	39.210	7.45	palmidrol
6	42.713	6.83	octanamide
7	44.934	0.24	2-(4-methoxy-2,6-dimethylphenyl)-3-methyl-2h-benzo[g]indazole
8	46.094	79.80	bis(2-ethylhexyl) phthalate
9	47.079	0.27	9-hexacosene

Note: On basis of NIST05 MS Library

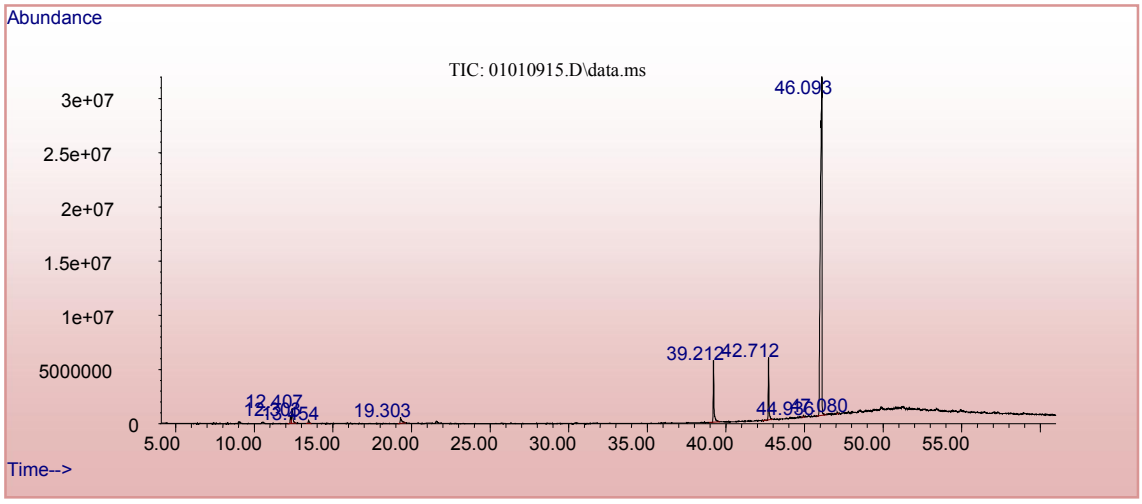

Fig 1: GC/MS of Essential oil of *Allium auriculatum.*

Allium auriculatum Headspace GC-MS

The whole aerial parts of fresh *Allium auriculatum* were shade dried and crushed to powder. One gram of powdered material was taken for dynamic headspace GC-MS analysis. The headspace GC-MS of *Allium auriculatum* revealed the presence of twenty one volatile organic components and all of them were identified by comparing their mass spectra with ms library except one. The dominant component of the oil is a monoterpene hydrocarbon. The monoterpene hydrocarbon, aldehyde, sulphur containing compounds, oxygenated monoterpenes, hytrocyclic compounds, organic acids, fatty acids, sulphur and nitrogen containing compounds and aromatic compounds amounted to 61.39%, 17.33%, 5.54%, 4.02%, 3.44%, 2.36%, 1.68%, 1.14% and 0.46%

respectively. The major constituents are 60.05% limonene, 17.33% isovaleraldehyde, 4.02% (s)-carvone, 2.67% furfural, 2.36% acetic acid, 1.88% thiophene, 2,5-dimethyl, 1.68% l-proline, n-allyloxycarbonyl-, dodecyl ester, 1.12% disulphide dipropyl and 1.05% β-pinene. (Table: 2, Figure: 2)

Table 2: Chemical composition (%) of *Allium auriculatum* by HS/GC-MS

Pk	RT	Area %	Common Name
1.	2.617	17.33	isovaleraldehyde
2.	5.858	2.67	furfural
3.	7.711	1.88	thiophene, 2,5-dimethyl
4.	8.370	2.36	acetic acid
5.	8.726	0.15	1-butanamine, 4-methoxy
6.	9.961	0.94	dimethyl trisulphide
7.	10.171	0.77	furfural, 5-methyl
8.	10.736	1.05	β-pinene
9.	12.281	60.05	limonene
10.	12.654	0.46	hyacinthin
11.	12.951	0.29	α-pinene
12.	14.519	1.12	disulphide, dipropyl
13.	14.793	0.95	rhodanine, 3-amino-
14.	15.877	1.68	l-proline, n-allyloxycarbonyl-, do decyl ester
15.	16.221	0.23	1-propene-1-methylthio
16.	16.384	0.33	sulphide, allyl methyl
17.	16.536	0.33	1,3-dithiolane
18.	18.774	4.02	(S)-carvone
19.	20.937	0.99	unidentified
20.	21.350	0.71	cis-3,5-diethyl-1,2,4-trithiolane
Note: On basis of NIST05 MS Library			

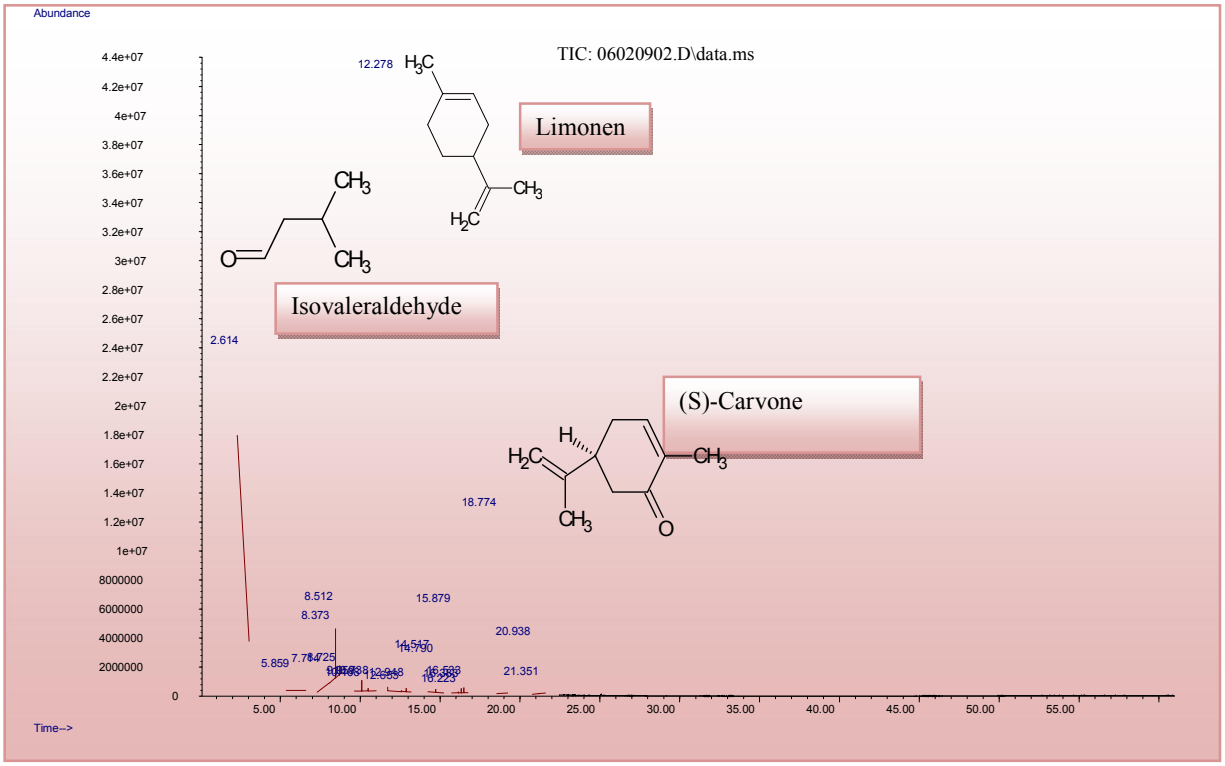

Fig 2: Headspace GC/MS of *Allium auriculatum.*

Discussion

Kameoka et al. reported that allium species contains 40-80 % sulphur containg volitales compounds (Kameoka, H., 1974). . The main compounds are s-allylcysteine sulphoxide (alliin), s-propenylcysteine sulphoxide (lacrimatory precursor), s-propylcysteine, sulphoxide, s-methylcysteine sulphoxide, llicin, ajoene, diallylsulphide, diallyldisulphide, diallyltrisulphide, allylmethyldisulphide, allylmethyltrisulphide, dipropylsulphide, dipropyldisulphide, dipropyltrisulphide, propylmethylsulphide, propylmethyldisulphide and propylmethyltrisulphide (MacKenzie, J. A., 1977). Chemical analysis of *allium auriculatum* is being

reported for the first time. The main constituent's bis (2-ethylhexyl) phthalate also investigated for the first time in *allium* species. Tsing Hua et al. had reported the antitumor activity (Tsing, H., 2005). Lee, K. H. *et al.* also reported the anti leukemic effects of bis (2-ethylhexyl) phthalate (Lee, K.H., 2000). Present study also shows the presence of o-cymene, d-limonene and γ-terpinene for the first time in this plant.

The dynamic headspace gc-ms of *allium auriculatum* are being reported first time. It shows the presence of seven sulphur containing and three monotepnoids hydrocarbon compounds, which makes it a remarkable phytofood. Isovaleraldehyde was found to be one of the most abundant headspace components constituting 17.33% the total gc peak area. The compound isovaleraldehyde is used in perfumes, pharmaceuticals and for flavouring. Yamanishi Tei et al. stated that isovaleraldehyde reacts with alcohols, aldehydes and thiols in food, consequently various kinds of flavoring substances are formed in food. (Yamanishi, T., 1956)

Antimicrobial activity

Evaluation of antimicrobial activity by determination of minimum inhibitory concentration of the *Allium auriculatum* oil extracts revealed a broad spectrum activity. The MIC of the samples ranged from 25 µg/ml to 100 µg/ml for bactericidal and fungicidal activity (Table 3).

Oil extracts from *Allium auriculatum* were found inactive against the gram positive bacterium *Staphylococcus aureus and Candida albicans*. Going through the literature no biological activity has been reported for *Allium auriculatum* oil extract, therefore this is a new finding of present work.

Table 3: Minimum Inhibitory concentration of different Allium oil extracts (MIC)

S.No.	Name of Oil extracts from	Test Organisms			
		Bacterial strains		Fungal strains	
		Staphylococcus aureus	***Escheridria coli***	***Candida albicans***	***Cryptococcus neoformans***
1	*Allium auriculatum*	Nil	100 µg/ml	Nil	100 µg/ml

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