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Artemisia thuscula Cav.*:* antibacterial, antifungal activity of the plant extracts and associated endophytes

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Abstract In this paper we are presenting preliminary results for the antifungal and antibacterian activity of the *Artemisia thuscula* Cav. all together with the endophytic communities encountered in symbiosis with this specie. This plant is endemic for the Canary Islands and it is recognised for its traditional medicinal use (like other species of the same genus in the rest of the world) and for being a functional repellent of insects.
The ethanol extracts tested showed an interesting activity against the phytopathogenic fungi *Fusarium monilforme*, *F. solani* and *F. oxysporum* and antibiotic activity against 2 Gram-positive bacteria*: Bacillus cereus* and *Streptomyces griseus*, in an primary screening.
The diversity of endophytes found in this plant, especially in the roots, showed promising results supporting further work on this species.

Key words

Artemisia thuscula, antifungal activity, antibacterial activity, endophytes

The study of biodiversity related to agricultural landscapes has an increasing importance. Taking this into consideration, innovative tools are being used to manage diverse types of 'eco agriculture' spaces to increase production, biodiversity and benefit local people.
Overuse and mismanagement of pesticides polluted water and soil [17], developed pest resistance, decreased natural enemies, and created human health related problems [22]. Agricultural pesticides may also kill non-target insects and weeds that constitute the food base for insect- and grain-eating species [14].
An interesting way of searching for bio rational pesticides is screening naturally occurring compounds in plants [12]. Botanical insecticides are generally pest specific and, although natural products cannot automatically be assumed to be without risk, are relatively harmless against non-target organisms including humans, are also biodegradable and less harmful to the environment. Moreover, unlike conventional insecticides that are based on a single ingredient, plant-derived insecticides include an array of compounds that decrease the chance of pests to develop resistance. The interaction between plants and insects is chemically mediated by secondary metabolites. Among the 500,000 estimated plant secondary metabolites (PSMs), only few have been characterized. The main groups of PSMs are phenylpropanoids and phenolics, terpenoids and steroids, alkaloids and nitrogen compounds [10].
Artemisia genus has species reported to have insecticidal properties and repellent activity. The application has been made in the form of maceration, decoction or infusion, essential oils and extracts
In this context, the interest in the study of endophytic organisms in increasing. These organisms live inside plants without causing any symptoms throughout their development, and participating actively in plant protection against phytopatogenic agents[18]. They are important because their presence influences the development of the plant on which they live, which can benefit in different ways and also constitute an interesting source of new bioactive products (alkaloids, coumarins, cytochalasines, quinones, peptides, phenols, phenolic acids, semiquinones, steroids, terpenoids, xanthones and lactones).
The term endophyte refers to a complex interaction (sometimes applied to a particular moment in which the host did not manifest reaction), where considered endophytic fungi belonging to different species, are more or less ubiquitous, more or less specific of their hosts and more or less selective in the plant organ on which they live [9]).
In relation to its role as a potential source of active compounds, the study of fungal endophytes of plants has uncovered a range of metabolites with pharmacological interest [11], antimalarial [21], antifungal [23], antibiotics [20], antitumor [15].
An example of high pharmacological interest compounds produced by endophytes is taxol, a diterpenoid very effective as an antitumor agent produced by the bark of *Taxus brevifolia* L., but which is also produced by one of its endophytes, *Taxomyces andreanae* [1]. In the species *Taxus wallichiana*

Zuccarini the endophyte *Pestalotiopsis microspora* Spegazzini also produce this substance [16]. Further examples of plant secondary metabolites detected in endophytic fungi include naphthodianthrones, such as hypericin. *Hypericum* species have been used for centuries against mild forms of depression and anxiety. An endophytic fungus from *H. perforatum* was found to produce hypericin in culture [1].

Presence of endophytes in the plant can help increase the resistance to diseases caused by other fungal pathogens. Recent studies with the genus *Phoma*, shows their great activity against phytopathogenic fungi widely distributed such as *Fusarium oxysporum* Schltdl, *Rhizoctonia solani* Kühn or *Colletotrichum gloeosporioides* Penz [23].

Therefore, endophytes represent a very interesting line of work for application in agriculture. The high probability that the metabolites of endophytic fungi have medicinal and industrial uses is an additional incentive to study this group [19]. Gunatilaka [11] made a review in which he presents nearly 230 metabolites isolated from different endophytes, which gives us an idea of both structural diversity of these compounds have activity and reinforces the interest in the study of these organisms.

Our research group has studied for several years endophytic fungal communities present in various plant species in the Canary Islands, particularly in the formation known as Laurel forest, present only in the Macaronesian archipelagos of the Canaries, Azores and Madeira. We have isolated many species belonging to genera also present in tropical forests with very interesting properties, against several target organisms (phytopathogenic fungi, chewing insects, aphids and human parasites) [9].

Therefore, we selected the plants species *Artemisia thuscula* Cav. (Asteraceae), a canarian endemism to study its endophytic fungal community. Our previous work has shown that ethanol extracts of this species are repellent against some insect pests of great economic interest, such as the banana weevil *Cosmopolites sordidus* Germar (unpublished data).

The Asteraceae family, consisting of more than 800 species that are widespread all over the world. Many species of this family have been known in traditional medicine as being anti malarial, anti cancer, antiviral, antitumoral etc [5, 3].

Materials and Methods

Samples of *Artemisia thuscula* were collected from four different locations in the north of the island of Tenerife: Taganana, La Laguna and La Matanza, located 600 meters above sea level, and Las Aguas, located at the coast. Plant vouchers were prepared for authentication and conservation the Department of Phytopathology, University of La Laguna, Tenerife. The plant organs were separated (root, stem and leaves) and dried at room temperature. After 48 hours of maceration in ethanol, the extracts were filtered and the solvent removed in a rotary vacuum evaporator.

The target organisms were phytopathogenic fungi that cause severe damages in crops: three species of *Fusarium* (*F. moniliforme* Sheldon, *F. oxysporum* fs. *lycopersici* Scheldt and *F. solani* Mart). We also tested on four species of bacteria: *Bacillus cereus* Frankland & Frankland and *Streptomyces griseus* Waksman and Henrici as Gram-positive and *Escherichia coli* Migula and *Pseudomonas aeruginosa* Migula as Gram-negative; provided by the Microbiology Lab of ULL

The antifungal bioassays were carried out by means of the biometric agar dilution method. The extracts obtained from the plant (Stock solution prepared at a concentration of 40mg/ml ethanol) were incorporated into the culture medium at 3 different concentrations (0.05mg, 0.1mg, 0.5mg, 1 mg/ml), prior to be poured in the Petri plates.

The final concentration of ethanol in the medium was 2%. In parallel, blank Petri dishes were prepared. Plates with 5 ml of medium were spot-inoculated at 8 equidistant points and then incubated in darkness at 27°C for 48 hours. The diameter of the scanned colony images was measured with an image processing program. The parameter used to determine the activity of the extracts is the percentage of growth inhibition, which is calculated by comparing the diameter of the treated colonies with the control ones. The results are computed as IC_{50}, the effective dose of an agonist that half maximally inhibits the pathogen. IC_{50} was calculated with GraphPad Software by means of a regression curve (see table **1)**.

The Kirky-Bauer [2] disc diffusion method was used to screen the antibacterial activity of plant extracts and performed by using LB medium [4]adjusting the pH to 7 using a concentrated NaOH solution (1-5 M), prior to autoclaving. Broth cultures of 0,5 McFarland standards were made from each strain. 100 µl of bacterial suspension were uniformly swabbed on the LB medium (5ml) surface, and the inoculum was allowed to dry for 5 minutes. Sterilized filter paper discs (Whatman, 6mm in diameter) were placed on the LB surface previously treated with different concentrations of extracts (0,1-0,5-1-2-3-4-5 mg/disc). Two controls were used: one with chloranphenicol (1mg/disc) and ethanol (50µl/disc). The inoculated plates were stored at 25 ºC for *S. griseus*, *P. aeruginosa*, *B. cereus* and 28 ºC for *E.coli*, during 24h in inverted position.

The average area of inhibition was measured by a modified Kirby-Bauer protocol, by counting the distance between the margin of the disc and the end of the inhibition zone.

Results

The ethanol extract of *Artemisia thuscula* shows antifungal and antibacterial activity (Table 2). Only *Streptomyces griseus* and *Bacillus cereus*, both gram

positive, were affected by the extract. In the case of the Gram-negative species, the inflorescences extract and leaves at 3, 4 and 5mg/ml were active (Table 2).

Table 1

IC_{50} of *A. thuscula* extracts in agar difussion bioassays against fungal plant pathogens

Extract	Phytopathongens	IC50 mg/ml (conf. Levels)
Roots extract 181	*F. moniliforme*	2,79 (0 – 6,03)
Branches extract 182	*F. moniliforme*	1,2 (0,79-1,6)
Branches extract 182	*F. oxysporum*	2,36 (0-13)
Leaves extract 183	*F. solani*	1,2 (0-1,84)
Leaves extract 183	*F. oxysporum*	1,2 (0,9-1,4)
Leaves extract 777	*F. oxysporum*	1,57 (0 – 6,5)

The strongest antifungal activity was shown by the leaf extract at 4 and 5mg/ml, with an IC_{50} under 2mg/ml. (Table 1).

The most active extract was from the leaves, which are a renewable biomass that can be harvested yearly. Further research is needed in order to isolate the active compounds

Table 2

***A. Thuscula* extracts antibacterial activity**

		Doses: mg/disc						
Extract	Bacteria	0,1	0,5	1	2	3	4	5
Leaves Taganana	ST	+	+	++	++	++	++	++
	PS	-	-	-	-	+	+	+
	EC	-	-	-	+	+	+	+
	BC	-	+	++	++	+	++	++
Leaves Las Aguas	ST	-	-	-	++	++	++	+++
	PS	-	-	-	-	-	-	+
	EC	-	-	-	+	+	-	-
	BC	nt	nt	Nt	+	+	+	++
Leaves La Laguna	ST	nt	nt	-	-	-	+	+
	PS	nt	nt	-	-	-	-	-
	EC	nt	nt	-	-	-	+	-
	BC	nt	nt	-	-	-	+	+++
Inflores cences Las Aguas	ST	nt	nt	-	+	+	+	++
	PS	-	-	-	+	+	+	+
	EC	nt	nt	-	-	-	+	+
	BC	nt	nt	+	+	++	+	+
Roots La Laguna	ST	nt	nt	+	+	+	+	+
	PS	nt	nt	-	-	-	-	+
	EC	nt	nt	-	-	-	-	-
	BC	nt	nt	+	+	+	+	+
Stem La Laguna	ST	nt	nt	-	-	-	-	-
	PS	nt	nt	-	-	-	-	-
	EC	nt	nt	-	-	-	-	-
	BC	nt	nt	-	+	+	+	++

ST =*Streptomyces griseus*; PS=*Pseudomonas aeruginosa*; EC=*Escherichia coli*; BC=*Bacillus cereus*

(-) = no activity. + = 0-2mm ++ = 2-4 , +++ = plus 4 mm.
(nt)= not tested

From this plant species we have isolated 29 isolates: 20 from roots, 7 from stems and 2 from leaves. 17 of the isolates were obtained in PDA culture medium, and the rest on YMA medium.

Based on morphological characteristics of the mycelium of the different isolates, we can establish three groups corresponding to each of the organs under study, which appears to confirm that endophytes can be host, tissue or organ specific.

In the isolates obtained from root and leaf samples, whitish and cottony mycelia predominated, while the mycelia of the endophytes isolated from the stem presented hard consistency and crust appearance.

Few isolates have reproductive structures that facilitate their identification, therefore we have to rely on molecular techniques, which are in process.

The antibacterial and antifungal effects shown by this plant together with the diversity of the endophyte community encountered gives an added value to the study of plant and fungal biodiversity.

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