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Study on antifungal activity of medicinal plant

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Abstract
Pathogenic fungi and the main infectious agents in plants, causing alterations during developmental stages including post-harvest. The aim of the study was to evaluate the antifungal activity of extracts of 10 plant species used in traditional Uruguayan medicine against the phytopathogenic fungus Alternaria spp. The plants were selected on the basis of their reported ethnobotanical uses. We conclude from this that these extracts exhibit amazing fungicidal properties that support their traditional use as antiseptics.

Keywords: Antifungal and Medicinal Plant

Introduction
In fruit and vegetables, there is a wide variety of fungal genera causing quality problems related to aspect, nutritional value, organoleptic characteristics, and limited shelf life In addition, in some cases fungi are indirectly responsible for allergic or toxic disorders among consumers because of the production of mycotoxins or allergens. Generally, phytopathogenic fungi are controlled by synthetic fungicides; however, the use of these is increasingly restricted due to the harmful effects of pesticides on human health and the environment (Harris). The increasing demand of production and regulations on the use of agrochemicals and the emergence of pathogens resistant to the products employed, justifies the search for novel active molecules and new control strategies.
Since antiquity, the plant kingdom has provided a variety of compounds of known therapeutic properties, like analgesics, anti-inflammatories, medicines for asthma, and others. In recent years, antimicrobial properties of plant extracts have been reported with increasing frequency from different parts of the world. For example, a large proportion of the South American population use plant extracts obtained from traditional medicinal plants as medicine for many infectious diseases. Plants from the genus Pterocaulon, known as "quitoco", are commonly used in veterinary medicine in southern Brazil to treat animal problems popularly diagnosed as "mycoses" (Demo and Oliva). Several works have demonstrated in laboratory trials that different plant tissues, such as roots, leaves, seeds and flowers possess inhibitory properties against bacteria, fungi and insects (Davicino et al.,). Currently, there is little evidence on the antimicrobial properties of the medicinal plants under investigation against phytopathogen fungi.
The aim of this work was to evaluate in vitro the potential antifungal activity of medicinal Uruguayan plant extracts against Alternaria spp., in order to verify possible inhibition activity. As well, the smallest concentration capable of inhibiting or preventing growth was determined among the species and extracts that demonstrated inhibitory properties.

Materials and methods
Different plant tissues from plant species used in traditional medicine (Table 1) were collected in 2008 in their natural habitat in Uruguay.

Table 1: list of medicinal plants used in this work with the popular uses in Uruguy

Species (family)	Local name	Popular uses	Organ used
Lonicera japonica Thunb. Madreselva (Caprifoliaceae)	Madreselva	Antibacterial, antiviral, diuretic, anti-inflammatory, febrifue, antispasmodic.	Flowers
Baccharis trimera (Less.) DC. (Asteraceae)	Carqueja	Hepatoprotective, anti-acid, anti-inflammatory, digestive, carminative, diuretic, antihelmintic.	Leaves
Zea mays L. (Poaceae)	Maíz dulce	Diuretic, anti-inflammatory, analgesic, against colds	Seeds
Cynara scolymus L. (Le) (Asteraceae)	Alcaucil	Digestive, diuretic, cardiotonic, hypotensive, anticholesterolemic.	Seeds
Salvia sclarea L. (Lamiaceae)	Salvia moscatel	Antiseptic, sedative, antidepressant and hypotensive.	Seeds
Salvia officinalis L. (Le) (Lamiaceae)	Salvia	Antiseptic, facilitator of digestion. External use: antiseptic, anti-inflammatory and healing properties.	Leaves
Rosmarinus officinalis L. (Le) (Lamiaceae)	Romero	Antiseptic, antispasmodic, diuretic. The essence is nervous stimulant, carminative. In external use for rheumatism, muscle aches, skin problems.	Leaves
Schinus molle L. (Anacardiaceae)	Anacahuita of also pimiento	Analgesic, antidepressant, antispasmodic, antimicrobial, astringent, diuretic, stimulant.	Leaves
Aloe vera (L.) Burm. f. (Asteraceae)	Aloe	Laxative, hepatic diseases. In external use for inflammatory disorders, burns, eczema. Very effective in fighting infections and healing wounds.	Seeds
Lippia alba (Mill.) N.E. Br. ex Britton & P. Wilson (Verbenaceae)	Salvia trepadora	Sedative, expectorant, digestive, antispasmodic.	Leaves

Seed and flower samples were thoroughly washed and ground to a fine powder in liquid nitrogen, using a mortar and pestle. Leaf samples were dried under forced circulation of heated air at 40 °C to reduce deterioration of the plant drug material and ground to powder in liquid nitrogen, using a mortar and pestle. The powder from all the samples was carefully stored a -20 °C.

Determination of extraction yield (% yield)

The yield (%, w/w) from all the dried extracts was calculated as:

Yield (%) = $(W_1 * 100)/W_2$

where W_1 is the weight of the extract after lyophilisation of solvent, and W_2 is the weight of the plant powder.

Fungal strain

Strains of *Alternaria* spp. were obtained from the Plant Protection Department of the Instituto Nacional de Investigación Agropecuaria (INIA Las Brujas). The fungus was grown at 27 °C on potato dextrose agar (PDA) (OXOID, Hampshire, England). Spores of the fungus were collected from cultures on agar plates after 7 d as described by Broekaert *et al.* (1990). The sporangial suspension concentration was estimated using a cellcounting chamber and adjusted to 2 × 106 spores mL-1 (Abril *et al.*, 2008) [2]. The fungal spore suspensions were stored in 20% glycerol at -40 °C.

Determination of antifungal activity

Antifungal activity was measured by a quantitative microspectrophotometric assay (Broekaert *et al.*, 1990). Growth inhibition was measured in 96-well microtiter plates at 595 nm. Routinely, tests were performed with 20 µL of the extract to be assayed, 10 µL of a spore suspension and 70 µL of potato dextrose broth (PDB) (HiMedia, Mumbai, India). Microcultures containing 20 µL of sterile distilled water instead of test solution were used as a negative control. The commercial fungicide captan (*N*-(trichloromethylthio)cyclohex-4-ene-1,2- dicarboximide) at 0.2 mg mL-1 (Satish *et al.*, 2007) was used as a positive control. The plates were left standing for 30 min at 27 °C to allow the spores to sediment, after which absorbance was measured at 595 nm in an ELISA plate reader. After 48 h of incubation at 27 °C, growth was recorded by measuring absorbance. All assays for antifungal activity were carried out at least in triplicate. Growth inhibition (Broekaert *et al.*, 1990) was determined based on the equation $[(\Delta C - \Delta T)/\Delta C] \times 100$, where ΔC is the corrected absorbance of the control microculture at 595 nm and ΔT is the corrected absorbance of the test microculture. The corrected absorbance values equal the absorbance at 595 nm of the culture measured after 48 h minus the absorbance at 595 nm measured after 30 min.

Minimal inhibitory concentration (MIC) and minimum fungicidal concentration (MFC)

A microplate method, as previously described (Eloff, 1998), was used with slight modifications to determine minimal inhibitory concentration (MIC) values of plant extracts. Plant extracts were serially diluted, ranging from 1/2 up to a 1/100 dilution from the crude extract. In each well, 100 µL of each extract dilution was mixed with 100µL of the fungal spore suspension (2×10^6 spores mL^{-1} in fresh PDB). The microplates were incubated for 2-3 d at 27 °C with daily monitoring. All experiments were done in triplicate. The MIC readings were performed spectrophotometrically with a microplate reader at 595 nm. MICs values were calculated by comparing growth in control wells and the extract blank, which consisted of ninoculated plates. The MIC of the extracts was defined as the lowest concentration of plant extract that caused growth inhibition of more than 90% at 48 h, as compared to the control.

Results and Discussion

The present study tested the antifungal activity of crude extracts and their respective dilutions from medicinal plants belonging to seven plant families against *Alternaria* sp. These medicinal plants were chosen based on either traditional usage (Table 1), suggestive of antimicrobial activity, or previous studies that have demonstrated antifungal properties using different kinds of extracts (Guo *et al.*, Wilson *et al.*, Zhu *et al.*). Of the 29 extracts evaluated, 31%, from nine plants, exhibited *in vitro* antifungal activity, with inhibition values of over 90%. The species with the

most pronounced antifungal activity were buffer extract of *C. scolymus*, acid extracts of *S. sclarea*, *S. officinalis* and buffer and acid extracts of *Lippia alba*, with 98% growth inhibition of *Alternaria*. Table 2 shows the extraction yields for the aqueous and acid plant extracts. The extraction yields varied from 8.6% to 41.9% and 0.7% to 42.2%, respectively. A wide range of the yields among extracts was observed depending on the extraction solvent and plant material used. *Schinus molle* exhibited the lowest extraction yield of 0.7% (acid extraction). The maximum extraction yield was obtained from seeds of *S. sclarea*, with 42.2% (acid extraction). Aqueous, buffer and acid extracts of 10 plant species were screened *in vitro* for their antifungal activity against *Alternaria* sp. The growth inhibition for the crude extracts was measured (Figure 1).

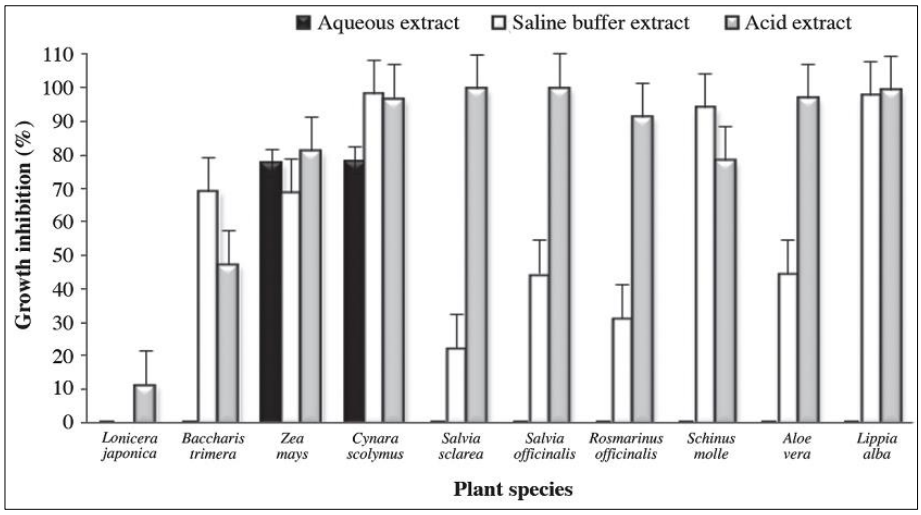

Fig 1: Growth inhibition of Alternaria sp. by crude extracts of traditional Uruguayan medicinal plants

Among the 29 extracts evaluated, 13 (45%), belonging to eight plant species, Showed percentages of inhibition above 75%. Nine extracts (31%), belonging to seven plant species, showed similar inhibition values as those of the commercial fungicide used as a positive control, with inhibition rates of over 90%.

Table 2: Percentage extract yield of medicinal plants used

Plant species	Extracta	Extract yield (%)
Lonicera japonica	A	8.6
	C	2.6
Baccharis trimera	A	28.7
	C	23.3
Zea mays	A	25.4
	C	28.2
Cynara scolymus	A	29.0
	C	9.7
Salvia sclarea	A	41.9
	C	42.2
Salvia officinalis	A	18.6
	C	29.4
Rosmarinus officinalis	A	20.4
	C	23.5
Schinus molle	A	34.5
	C	0.5
Aloe vera	A	41.1
	C	39.0
Lippia alba	A	17.4
	C	28.1

Extract: A: aqueous; C: acidic

Table 3 shows MICs and MFCs of active plant extracts. From the total extracts evaluated, 12 showed fungistatic activity and four showed fungicidal activity. The MIC values ranged from 1.25 to 25.0 µg mL^{-1} and MFCs values ranged from 1.25 to 10.0 µg mL^{-1}. Acid extracts of *S. sclarea*, *S. officinalis* and *R. officinalis* had the lowest MIC (1.25 µg mL^{-1}), while buffer extracts of *Schinus molle* and *L. alba*, and acid extracts of *Aloe vera* and *L. alba* had the highest MIC (25.0 µg mL^{-1}). The MIC of aqueous, buffer and acid extracts of *C. scolymus* were 5.0, 10.0 and 10.0 µg mL^{-1}, respectively, and amazingly it was the only species whose three extracts showed fungistatic activity. The minimum fungicidal concentration of the acid extract of *R. officinalis* proved to posses the highest fungicidal action against *Alternaria* sp. as indicated by the low value (1.25 µg mL^{-1}, Table 4). According to the results obtained, the extraction yields of water and acid extracts are quite similar. However, greater efficiency in the extraction of solutes is not directly related to greater inhibition. In this regard, acid extracts proved to have more activity against *Alternaria* than aqueous extracts. Thus, it can be concluded that acetic acid was more efficient in the extraction of water-soluble biomolecules with antifungal activity. The aqueous extracts that demonstrated the least activity against *Alternaria* spp. could be explained by the fact that when plant materials are ground in water, some phenolases and hydrolases are released and could have modulating effects on the activity of the compounds in the extracts. It could also be due to incomplete extraction of the active principles (El-Mahmood *et al.*).

Our results are in accordance with Pinelo *et al.*, who suggested the that chemical characteristics of the solvent, the method used during the extraction process and diverse structural and compositional aspects of the natural products result in each materialsolvent system showing distinct behaviour. Differences in polarity among various solvents have been reported to account for the differences in solubility of active plant active properties, hence variations in the degree of activity. For example, Itako *et al.* reported 60% inhibition of *Alternaria solani* germination when using *R. officinalis aq*ueous extract, whereas in the present work *R. officinalis* aqueous extract did not inhibit the growth of *Alternaria* spp. On the other hand, *R. officinalis* acid extract showed 91.2% of growth inhibition.

Table 3: Minimal inhibitory concentration (MIC) and minimum fungicidal concentration (MFC) of crude extracts from plant species against Alternaria sp.

Plant Species	MIC			MFC		
	Aqueous extract	Saline buffer extract	Acid extract	Aqueous extract	Saline buffer extract	Acid extract
	——————— µg mL^{-1} ———————					
Lonicera Japonica						
Baccharis trimera	(–)	(–)	(–)	Nf	Nf	Nf
Zea mays	5.0	(–)	(–)	Nf	Nf	Nf
Cynara scolymus	5.0	10	10	NF	10	Nf
Salvia sclarea	(–)	5.0	1.25	NF	NF	2.5
Salvia officinalis	(–)	(–)	1.25	NF	NF	2.5
Rosmarinus officinalis	(–)	(–)	1.25	NF	NF	1.25
Schinus mole	(–)	25	(–)	NF	NF	NF
Aloe vere	(–)	(–)	25	NF	NF	NF
Lippia alba	(–)	25	25	NF	NF	NF

Conclusion

Studies are needed to determine the chemical identity of the bioactive compounds responsible for the observed antifungal activity. Natural plant-derived fungicides may be a source of new alternative active compounds, in particular with antifungal activity. The high proportion of active extracts in the assayed species, selected according to available ethnobotanical data, corroborates the validity of this approach for the selection of plant species in the search for a specific activity.

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