In vitro fungitoxic activity of *Larrea divaricata* cav. extracts

E.N. Quiroga, A.R. Sampietro and M.A. Vattuone

Cátedra de Fitoquímica, Instituto de Estudios Vegetales 'Dr Antonio R. Sampietro' Facultad de Bioquímica, Química y Farmacia, Universidad Nacional de Tucumán, San Miguel de Tucumán, Argentina

ABSTRACT

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Aims: To evaluate the fungitoxic activity of *Larrea divaricata* Cav. extract and one of its components against yeasts and fungi. This activity was compared with the action of ketoconazole, a known synthetic antimycotic.

Methods and Results: Antifungal activity of *Larrea divaricata* extract and of a fraction (Fr. B) purified by thin layer chromatography, was investigated using different methodologies. Both exhibited strong activity against the majority of the assayed fungi. Only *Fusarium oxysporum* and *Schizophyllum commune* growth was not affected with the assayed conditions. The fungitoxic and cytotoxic activity of the ethanolic extract and ketoconazole were compared.

Conclusions: Ethanolic extracts of *L. divaricata* Cav. produce growth inhibition of several fungi. One of its constituents with the same activity was purified and identified as a glycoside of a flavanone. A comparison with the action of ketoconazole, which is currently used as antimycotic and can cause adverse health effects was made.

Significance and Impact of the Study: Our data suggest that *L. divaricata* extract contains, at least, one compound of phenolic nature, with fungitoxic potency against yeasts and fungi.

Keywords: antifungal activity, cytotoxicity, fungitoxic activity, *Larrea divaricata* Cav.

INTRODUCTION

As plants are sessile, they synthesize a vast array of secondary metabolites as defence mechanisms for protecting themselves against pathogen infections (bacteria, fungi and viruses) (Osbourn 1996; Mendoza *et al.* 1997; Skadhange *et al.* 1997; Tereschuk *et al.* 1997; Bois *et al.* 1999; Hou and Forman 2000; Rauha *et al.* 2000). Although there are several natural and synthetic products available to ameliorate fungal infections, it is recognized that infections induced by fungi are in a continuous increase (Wu 1994; Walsh *et al.* 1996). Consequently, there are needs to detect new sources of antifungal compounds with potential application in medicine and also as additives in food and feed preservatives.

Previously, we reported a systematic biological investigation of the *in vitro* antimicrobial activity of ethanolic extracts of some Argentine plants used in traditional medicine (Quiroga *et al.* 2001). The exploration of new antifungal compounds has lead to a joint interest of agriculture, chemistry and medicine in fundamental research. Furthermore, the identification of naturally occurring fungicidal compounds without toxicity for cells would be beneficial.

The purpose of this work was to demonstrate the presence of antifungal compounds in *Larrea divaricata* Cav. extracts and the partial purification of bioactive compounds. We also determined antifungal and cell toxicity of the extract in comparison with ketoconazole.

MATERIALS AND METHODS

Plant material

Aerial parts of *L. divaricata* Cav. (family Zygophylaceae) were collected in Amaicha del Valle, Tucumán, Argentina. This plant is popularly known as 'jarilla' or 'jarilla hembra'.

Correspondence to: Marta A. Vattuone, Cátedra de Fitoquímica, Instituto de Estudios Vegetales 'Dr Antonio R. Sampietro' Facultad de Bioquímica, Química y Farmacia, Universidad Nacional de Tucumán, San Miguel de Tucumán, Argentina (e-mail: sampietro@tucbbs.com.ar).

Plants were identified and taxonomically characterized by Dr A.R. Sampietro (Instituto de Estudios Vegetales, Facultad de Bioquímica, Química y Farmacia, Universidad Nacional de Tucumán, Argentina). Voucher specimens were preserved in this chair and in the Instituto 'Miguel Lillo' (Tucumán, Argentina). Plant material was dried in the shade in a well-ventilated chamber, ground to a coarse powder and stored in the dark at room temperature.

Chemicals

All chemicals and reagents used were of the highest grade commercially available. Ketoconazole (Parafarm, Droguería Saporiti, Argentina) was used as an antifungal drug. Stock ethanolic solution were prepared (0.1–10 mg ml^{-1}) and stored at $-18°C$ until it was used.

Micro-organisms

The micro-organisms used included four xylophagous fungi: *Pycnoporus sanguineus* (E. ex Fr.) Murr. (IEV 006), *Lenzites elegans* Spreng. Ex Fr. (IEV 012), *Schizophyllum commune* Fr. (IEV 009) and *Ganoderma applanatum* (Pers. Ex Walls) Pat (IEV 017). These fungi were isolated from decaying wood and classified in the Cátedra de Fitoquímica above mentioned. The yeasts *Rhodotorula* sp. (IEV 001) and *Saccharomyces carlsbergensis* (IEV 002) were a kind gift from the Laboratorio de Micología of the Instituto de Microbiología (UNT, Argentina), and the phytopathogenic fungi *Fusarium oxysporum* Schlect, *Penicillium notatum* Westling *Trichoderma* spp. and *Aspergillus niger* Thom and Church were obtained from the Instituto Nacional de Tecnología Agraria (Tucumán, Argentina). All the strains were maintained in solid culture medium (SM: 15 g l^{-1} malt extract, 5 g l^{-1} peptone and 15 g l^{-1} agar). Other media used were soft medium (SSM: 15 g l^{-1} malt extract, 5 g l^{-1} peptone and 6 g l^{-1} agar) and liquid medium (LM: 15 g l^{-1} malt extract, 5 g l^{-1} peptone).

Assays of antifungal activity

Several techniques were applied according with the nature of the assayed fungus:

Bioautographic analysis was made on Silica gel 60 plates (Merck, Darmstadt, Germany) by dot blot or after thin layer chromatography (TLC) of the plant extract in different solvents (Homans and Fuchs 1970). After plates (6 × 8 cm) were dried under sterile conditions, they were covered with 3 ml of SSM containing 0·3 ml of a spore suspension of *A. niger*, *P. notatum* or *Trichoderma* spp. ($1 × 10^4$ spores per millilitre). Fungitoxic activity was macroscopically visualized after incubation at 30°C in a moist chamber for 48–72 h.

Hyphal radial growth assay: different concentrations of *L. divaricata* extracts, ranging from 0 to 3·6 μg of phenolic compounds per millilitre of the culture medium were evaluated. A 3 mm diameter plug of an actively growing mycelium of filamentous fungi harvested from an 8–10 days of culture or 10 μl of spore suspensions ($2·5 × 10^4$ spores ml^{-1}) were placed onto the centre of Petri dishes (60 × 15 mm) containing 5 ml of SM plus increasing extract concentrations. Plates were incubated at 30°C for 4–5 days in a moist chamber and the percentage of growth inhibition was calculated as (Reyes Chilpa *et al.* 1997):

$$\% \text{ inhibition} = \frac{\text{Mycelial growth in control} - \text{mycelial growth in extracts plant}}{\text{Mycelial growth in control}} \times 100.$$

Well-plate diffusion method: aliquots (50 μl) of a spore suspension ($1 × 10^4$ spore ml^{-1}) of *A. niger* or *Trichoderma* spp. were overlaid on Petri dishes (90 mm in diameter) containing 15 ml of SM. Six equidistant wells were made in the medium using a sterile cork borer (4 mm in diameter). A 10 μl volume of various extract concentrations (0–24 μg of phenolic compounds) or ethanolic ketoconazole solution (0–10 μg) were placed in the wells. Ethanol was added as control. The diameter of the inhibition zones were measured after incubation at 30°C for 48 h. The effect was calculated as a mean of six tests.

Paper disc diffusion assay: paper discs (4 mm diameter; Whatman no. 4) containing different quantities of plant extract were placed on SM (15 ml per plate) containing $1 × 10^6$ yeast cells per millilitre. Dishes with 96% ethanol were used as control. Plates were incubated at 30°C for 48 h and the diameter of the inhibition zones were measured in four different directions and the mean value and the S.D. were calculated for each concentration.

Broth dilution assays were performed in a total volume of 0·2 ml in sterile polystyrene 96-well plates containing LM and fixed concentrations of *L. divaricata* extract (2·7 μg phenolic compounds 0·2 ml^{-1}). Wells were inoculated with 10 μl of a spore suspension ($1 × 10^4$ spore per millilitre). Controls were run simultaneously. Each experiment was made eight times. Growth was quantified by $A_{550 \text{ nm}}$ using an automatic microplate reader Model 550 (Bio-Rad Laboratories, Richmond, CA, USA) (Piecková and Roeijmans 1999) after incubation for 72 h at 37°C and percentage of growth inhibition was calculated.

Cell cytotoxicity assay

In vivo lethality assay of *Artemia salina* Leach was used (Meyer *et al.* 1982). Brine shrimp eggs were placed in sea water (3·8% w/v sea salt in distilled water) and incubated at

22–24°C in front of a lamp. Eggs hatch and mature within 48 h providing large number of larvae (nauplii). A convenient number of nauplii were placed in vials containing 5 ml of sea water plus increasing concentrations of plant extract or ketoconazole (1–1000 ppm) for comparison purpose (Espinel-Ingroff *et al.* 2001). Controls were made with 96% ethanol. Alive nauplii were counted after 16 h at 22°C and the lethal dose 50 (DL_{50}) was calculated. Six replicates were made.

Data and statistical analysis

Data were expressed as mean ± S.D. Lethality assays were evaluated with Finney computer program to determine the DL_{50} values and 95% confidence intervals. A copy of this program for IBM PC's is available with Dr J.L. McLaughlin (Department of Medicinal Chemistry and Pharmacognosy, School of Pharmacy, Purdue University, West Lafayette, IN, USA).

Extract preparation

Extracts were prepared according to the Farmacopea Nacional Argentina 6ta. Ed.. Briefly: powdered aerial parts (10 g) were extracted with 100 ml of 96% ethanol by maceration with stirring (40 cycles min^{-1}) at 37°C for 48 h and filtered through Whatman no. 4 paper. The solvent of the filtrate was removed by evaporation to dryness under reduced pressure at 30°C. The residue was dissolved in 96% ethanol ($28 \cdot 1$ mg ml^{-1}) and used as stock solution. This solution contained $12 \cdot 92$ mg of phenolic compounds per millilitre.

Phytochemical screening

Silica Gel 60 F_{254} plates, 20 × 20 cm, Merck (Camm *et al.* 1975) with the solvent system toluene-ethyl acetate-formic acid; 4 : 5 : 1, v/v, was used for the separation of the components of the ethanolic extracts by ascendent TLC. They were visualized by observation under u.v. light (254 and 366 nm) and qualitatively determined by staining with the following reagents: modified Dragendorff's reagent for alkaloids, Folin Ciocalteau reagent for total phenolics, methanolic potassium hydroxide for coumarins, aluminium chloride for flavonoids and anisaldehide/sulphuric acid for steroids and terpenes (Wagner *et al.* 1984).

Analytical methods

Total phenolic content was quantitatively determined by the method of Folin Ciocalteau (Lowry *et al.* 1951) and expressed as micrograms of coumarin and neutral sugars by the phenol–sulphuric acid method (Dubois *et al.* 1956).

Fr. B was subjected to acid hydrolysis, briefly: $0 \cdot 2$ ml of Fr. B were added to $0 \cdot 2$ ml of 2 N H_2SO_4 and heated at 100°C for 1 h. The preparation was neutralized with solid $CaCO_3$ and centrifuged at 830 g for 5 min. The supernatant was analysed by descendent paper chromatography on Whatman no. 4 paper with the solvent system butanol–pyridine–water (6 : 4 : 3, v/v). Sugars were detected by the method of Trevelyan *et al.* (1950).

RESULTS

The ethanolic extracts of aerial parts of *L. divaricata* inhibit the growth of several xylophagous and phytopathogenic fungi and yeasts. Bioautographic assays on TLC showed inhibition of spore germination of *A. niger*, *Thichoderma* spp, and *P. notatum* (not shown). The hyphal radial growth inhibition effectiveness varied from $41 \cdot 3$ to $77 \cdot 6\%$ for the tested filamentous fungi when the phenolic compound concentration was 368 μg ml^{-1} in the culture medium (Table 1). Yeast growth, analysed by paper disc assay showed that *Rhodotorula* spp. and *S. carlsbergensis* growth was inhibited by $11 \cdot 5$ and $15 \cdot 2$ μg of phenolic compounds, respectively, in our assay conditions. The cytotoxicity of the plant extract was compared with ketoconazole, a well-known synthetic fungitoxic imidazole derivative, by means of the brine shrimp lethality bioassay. The LD_{50} obtained for the *L. divaricata* extract and ketoconazole was 851 and 73 ppm, respectively (Fig. 1). The fungitoxic effect of the plant extract and ketoconazole on fungus growth was analysed by monitoring the development of inhibition zones in the culture media inoculated with spores of *A. niger* or *Trichoderma* spp. As shown in Fig. 2 the antifungal potency of ketoconazole was around $7 \cdot 36$–$7 \cdot 80$ and $7 \cdot 33$–$7 \cdot 58$ times

Table 1 Hyphal radial growth inhibition of filamentous fungi by the ethanolic extract of *Larrea divaricata*. The *L. divaricata* ethanolic extract concentration was 368 μg of phenolic compounds per millilitre of culture medium (μg) of coumarin ($0 \cdot 8$ mg of dry extract per millilitre)

Fungi	Growth inhibition (%)	Antifungal activity**
L. elegans	$77 \cdot 6 \pm 0 \cdot 09$	++
S. commune	$57 \cdot 9 \pm 0 \cdot 05$	++
P. sanguineus	$40 \cdot 4 \pm 0 \cdot 03$	++
G. applanatum	$41 \cdot 3 \pm 0 \cdot 05$	++
F. oxysporum	$56 \cdot 2 \pm 0 \cdot 07$	++
P. notatum	$89 \cdot 0 \pm 0 \cdot 05$	+++
A. niger	$42 \cdot 8 \pm 0 \cdot 03$	++
Trichoderma spp.	$48 \cdot 7 \pm 0 \cdot 07$	++

$n = 12$.
*The percentage of inhibition was calculated according to Reyes Chilpa *et al.* (1997).
**The antifungal activity was expressed as: (+) 20 to 40; (++) 40 to 80 and (+++) >80% growth inhibition.
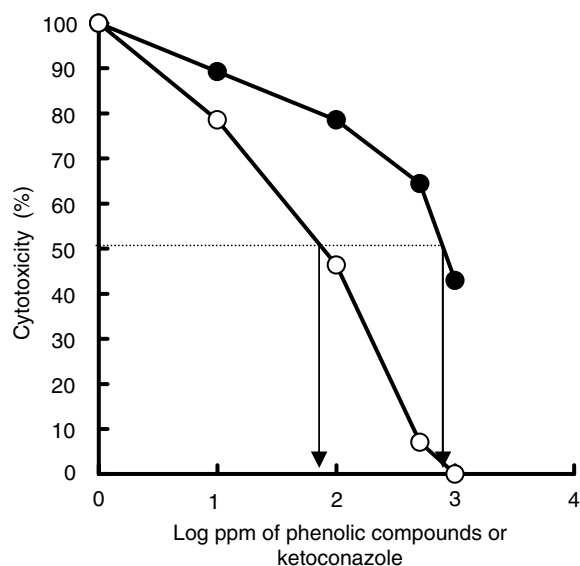
Fig. 1 Cytotoxicity of the ethanolic extract of *Larrea divaricata* and of ketoconazole determined by means of the brine shrimp lethality bioassay. *Larrea divaricata* (-●-) and of ketoconazole (-○-). The LD_{50} was calculated with Finney computer program

higher than the plant extract for *A. niger* or *Trichoderma* spp., respectively.

Components of the ethanolic extract from *L. divaricata* were analysed by ascendent TLC in analytical scale. Three main fractions (Fr. A, Fr. B and Fr. C) were revealed with reagents for phenolic compounds. Otherwise, the presence of detectable quantities of alkaloids, coumarins, steroids and terpenes was discarded by spraying the developed TLC with different reagents. The separated fractions were scraped out from the plate, eluted and adjusted to 90 μg phenolic compounds per millilitre with methanol. Only Fr. B prevented fungal growth at concentrations of phenolic compounds that were half than that determined for the crude extract. *Pycnoporus sanguineus*, *L. elegans* and *G. applanatum* were assayed by hyphal radial growth test showing that Fr. B (3·6 μg of phenolic compounds per millilitre of culture medium) produced a growth inhibition of 21, 30 and 54%, respectively (Fig. 2a,b and Table 2). Otherwise, *P. notatum*, *A. niger*, *Trichoderma* spp., *Rhodotorula* spp., *F. oxysporum* and *S. commune* were tested by broth microdilution method. In this case Fr. B (2·7 μg of phenolic compounds in 0·2 ml) produced 49, 42, 21 and 20% of growth inhibition, respectively, but had not effect on *F. oxysporum* or *S. commune* growth (Table 2). Fr. B showed a bluish fluorescence under u.v. light (366 nm) and its u.v. spectrum (200–450 nm) exhibited a maximum at around 282 nm. Furthermore, in u.v. light (366 nm) an orange fluorescence was observed following NP/PEG treatment that is an indicator of two adjacent hydroxyl groups in ring
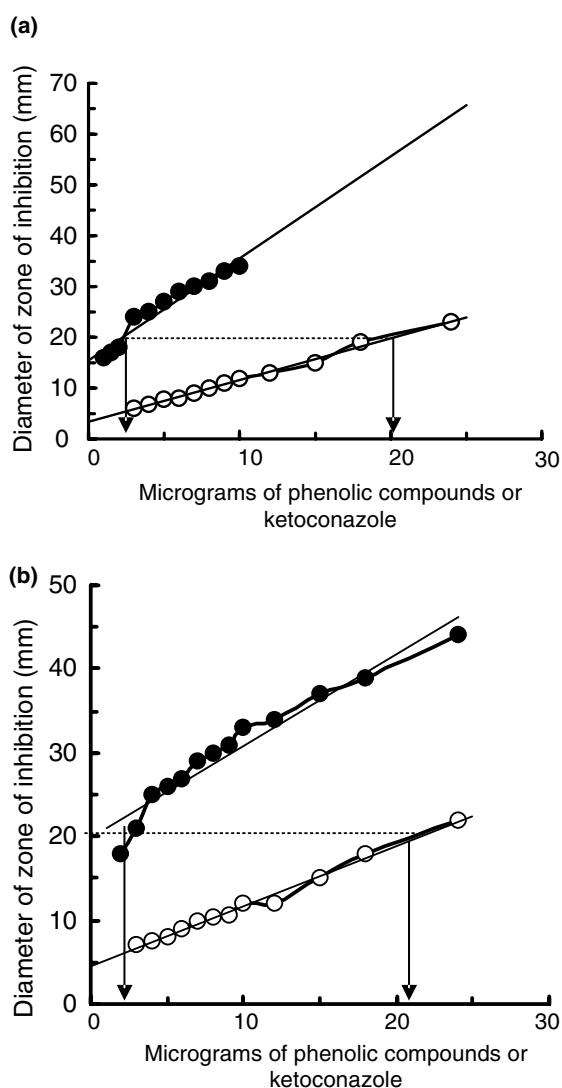
Fig. 2 Antifungal potency of the ethanolic extract of *Larrea divaricata* (-○-) and of ketoconazole (-●-) on *Aspergillus niger* (a) and *Trichoderma* spp. (b) growth. Arrows indicate the amount of phenolic compounds and of ketoconazole necessary to attain an inhibition zone of 20 mm in diameter, respectively. The tendence line is indicated ($n = 6$)

B and the pale orange colour under u.v. 366 nm after treatment with ammonium vapours a hydroxyl free group in 4′ position. Otherwise, acid hydrolysis of Fr. B with subsequent paper chromatography allowed separating glucose and two pentoses. Moreover, a strong positive reaction was observed after TLC treatment with $FeCl_3$.

DISCUSSION

Previously we reported the *in vitro* antifungal activity of ethanolic extracts of 10 higher plants used in folk medicine (Quiroga *et al.* 2001). Among them *L. divaricata* was

Table 2 Antifungal activity of Fr. B assayed by hyphal radial growth and broth microdilution methods

Method	Phenolic compound concentration	Fungi	Growth inhibition (%)
Hyphal radial growth	$3.6\ \mu g\ ml^{-1}$ of culture medium	*P. sanguineus*	21 ± 0.3
		L. elegans	30 ± 0.4
		G. applanatum	54 ± 0.3
Broth microdilution	$2.7\ \mu g\ (0.2\ ml)^{-1}$	*P. notatum*	21 ± 0.1
		A. niger	42 ± 0.05
		Trichoderma spp.	49 ± 0.1
		Rhodotorula spp.	20 ± 0.1
		F. oxysporum	0.0
		S. commune	0.0

selected for this study because it showed the broadest spectrum of activity. The fungal growth inhibition was demonstrated in the raw ethanolic extract by bioautographic techniques and broth microdilution assay for sporulating fungi, by inhibition of hyphal growth for filamentous fungi, and by paper disc and well plate diffusion assays for yeasts. Otherwise, the comparison of the antifungal potency of the extract with ketoconazole, a well-known imidazole derivative, revealed that the extract fungitoxic strength is lower than that of the standard drug though ketoconazole is several times more cytotoxic than the extract. It worth to take into account that despite the clinical utility of antifungal azoles, several subjects require investigations including the emergence of polyazole-resistant isolates after long-term treatment with azoles (Hitchcook 1993; Johnson *et al.* 1995), and to decrease toxicity and side-effects of the drug. Consequently, the search of new plant sources linked with bioassays of the corresponding crude extracts would contribute to the discovery of new biologically active compounds. The extract fractionation gave rise to the isolation of a fraction (Fr. B) with fungitoxic activity and the different staining reagents applied to TLC suggested the phenolic nature of this fraction. The fluorescence under u.v. light and its maximum of absorption at 282 nm induced to think the presence of a flavanone structure. Moreover, the staining of the TLC with several reagents confirmed this result and suggested the presence of two adjacent hydroxyl groups in the B ring being one of them in position 4′. Structural analysis allowed to demonstrate that the isolated compound is an O-glycoside with glucose and two pentoses in the glycosidic fraction. Up to the present only one flavanone aglycone, dihydromyricetin 3′, 5′-dimethyl ether (dihydrosyringetin) was detected as component of the genus *Larrea* during studies on the taxonomic relationships between

L. tridentate and *L. divaricata* (Sakakibara *et al.* 1976) but its structure is different from the flavanone purified in our study and its biological activity unknown.

Our data suggest that *L. divaricata* extract contains, at least, one compound (a glycoside of a flavanone) with high fungitoxic potency against yeasts and filamentous fungi. This is the first step in a long process toward the examination and structural elucidation of compounds with antifungal ability in ethanolic extracts of *L. divaricata* Cav. Moreover, this work underlines the importance of the screening of biological activities of tropical ethno-medicinal plants. Further testing *in vivo* and studies on the structure-function relationships are now in progress.

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