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Short communication

Antifungal activity *in vitro* and *in vivo* of extracts and lignans isolated from *Larrea divaricata* Cav. against phytopathogenic fungus

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ABSTRACT

The aim of this study was to evaluate the effectiveness of organic extracts and isolated compounds of the medicinal plant *Larrea divaricata* Cav. against phytopathogenic fungi. Chloroformic extract showed the highest antifungal activity and three compounds were identified in this extract: Apigenine-7-methylether (**1**), nordihydroguaiaretic acid (**2**) and 3,4′-dihydroxy-3′,4-dimethoxy-6,7′-cyclolignan (**3**). Compound **3** is described for the first time in the species and exhibited strong antifungal activity *in vitro* (*Fusarium graminearum* MIC = 15.6 μg/mL) and *in vivo* producing decreasing disease on glasshouse-grown wheat plants reducing damping-off (14% in pre emergency) and Severity Index from 2.6 upto 1.1. In this study it is clear that the application of botanical products from *L. divaricata* can be effective in the management of seedling blight and crown rot of wheat.

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1. Introduction

The fungal pathogen *Fusarium graminearum* causes the most economically important diseases of wheat worldwide (Reynoso et al., 2011). It is associated with the cereal damping-off complex, which causes loss of germinability, reduced emergence, and post emergence blight of seedlings, also in adult plants is the main causal agent of *Fusarium* Head Blight. The main strategy for the damage at the first development stages of the plants is the use of chemical treatments. However, it has led to accumulation of toxic residues in foodstuffs as well as an increment in environmental pollution and phytotoxicity. Therefore, the search for new strategies of bio-control to inhibit the growth of phytopathogenic microorganisms has gained importance (Dal Bello et al., 2002).

The importance of natural products from plants and their analogs in controlling different plant pathogens is well known. In this sense *Larrea divaricata* Cav. (Zygophyllacea) "jarilla" a perennial woody shrub with a wide distribution in Argentina and has long been used for its medicinal and aromatic properties. It is frequently used in traditional medicine as anti-inflammatory, antirheumatic, febrifuge, as a pest control agent and is a plant with reports of traditional antifungal use (Goleniowski et al., 2006; Svetaz et al., 2010). Recently, the water extract of *L. divaricata* was found to decrease proliferation and induced apoptosis of lymphoma cell line (Davicino et al., 2010), and the alcoholic extract has been reported to exert antibacterial activity (Zampini et al., 2007) and antifungal activity against yeast and filamentous dermatophytes (Svetaz et al., 2010). Phytochemical studies had reported the presence of lignans, essential oils, flavonoids and glycosides (Mabry et al., 1977).

In the present work we study the effects of organic extracts from *L. divaricata* Cav. on the development of four phytopathogenic fungi causing of serious diseases of vegetable crops. We also report the presence of compounds in the bioactive extract: apigenine-7-methylether (**1**), nordihydroguaiaretic acid (**2**) and 3,4′-dihydroxy-3′,4-dimethoxy-6,7′-cyclolignan (**3**) isolated for the first time in *L. divaricata* with strong inhibition of *F. graminearum* growth on *in vitro* and *in vivo* assay.

2. Material and methods

2.1. Plant material

L. divaricata Cav. was collected from their natural habitat during May 2009 in the mountainous region of San Luis (32°36′ 66°07′), in the province of San Luis, Argentina. Botanical identification was performed at the Herbarium of the Universidad Nacional de San Luis, where voucher specimens of the plant were deposited (L.A. del Vitto, N° 518).

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2.2. Preparation of extracts

The air-dried and minced material obtained from leaves and stem of *L. divaricata* (800 g) were extracted three times successively for 48 h at room temperature with *n*-hexane, chloroform and methanol. The plant extracts were filtered, evaporated in vacuum in a rotary evaporator and stored at −80 °C until tested.

2.3. Phytochemical screening

Silica Gel 60 F254 plates, 20 × 20 cm, Merck with the solvent system benzene–acetone; 9:1 (v/v), was used for the separation of the components of the chloroformic extracts by ascendent TLC. They were visualized by observation under UV light (254 and 366 nm) and qualitatively determined by staining with the following reagents: modified Dragendorff's reagent for alkaloids, methanolic potassium hydroxide for coumarins, anisaldehide/sulfuric acid for steroids and terpenes, aluminum chloride for flavonoids, methanolic ferric chloride for lignans, ethanolic sodium hydroxide for quinones and vanillin/sulfuric acid reagent for sapogenins (Spangerberg et al., 2011).

2.4. Isolation and structural identification of secondary metabolites

The chloroformic extract was subjected to flash chromatography on silica gel, eluting with *n*-hexane, *n*-hexane-EtOAc with increasing polarity mixtures and EtOAc–MeOH (97:3) to afford thirty-six fractions. The *n*-hexane-EtOAc (7:3) fraction was purified by column chromatography on Sephadex LH-20 eluting with MeOH to give twenty-three fractions. Each fraction obtained from Sephadex column was monitored by TLC (C_6H_6–dioxane-AcOH 30:5:1), and fractions 6–7 were separated and purified by TLC (C_6H_6–AcOH 8.5:1.5) to furnish compound **1** (11 mg). Fractions 10–11 were separated and purified by TLC (C_6H_6–AcOH 8.5:1.5) to furnish compound **2** (10 mg). Fractions 12–20 were separated and purified by TLC (C_6H_6–AcOH 8.5:1.5) to furnish compound **3** (9 mg). Their structures were determinate by spectroscopic methods and comparison with authentic samples (NMR spectra: Bruker-Avance-200 instrument, ^{1}H NMR:200 MHz, ^{13}C NMR: 50 MHz, $CDCl_3$ as solvent. Mass spectra: EIMS, ionization energy 70 eV, Finnigan-Mat-GCQ ion tramp instrument).

2.5. Fungi and media

Antifungal evaluations were performed against the plants pathogens of economical importance in agriculture *F. graminearum* DBM07, *Fusarium solani* DBM08, *Fusarium verticillioides* DBM09 and *Macrophomina phaseolina* DBM10. Theses fungi are part of the Laboratory of Plant–Microbe Interaction's fungal collection (Universidad Nacional de Río Cuarto), the culture were maintained on potato dextrose agar in a culture chamber at 28 °C for 7 days.

2.6. In vitro antifungal activity: microbroth dilution method

The experiment was made following the guidelines of the NCCLS for filamentous fungi (M 38 A) in 96-well microplates (CLSI, 2008). Wells containing 100 μL of RPMI 1640 medium with different extracts concentration (extract were dissolved in DMSO (100 mg/mL) were inoculated with 100 μL of inoculum suspension. The plates were incubated at 30 °C for 48 h. The minimum inhibitory concentration (MIC) was defined as the lowest concentration of extract at which no fungal growth was observed after incubation (Svetaz et al., 2010). Tests were carried out in duplicate.

2.7. Glasshouse experiments: effect of plant products on seedling stand caused by F. graminearum

To produce fungal inoculum, 200 g of ryegrass seeds were used as substrate for fungal growth. They were soaked in water, drained and autoclaved in a conical flask (30 min on 3 separate days). Each flask was inoculated with a 5 mm diameter plug of *F. graminearum* and incubated at 20 °C with 12 h photoperiod for one week. The seeds were dried and stored at 4 °C. A glasshouse potting mix was prepared by mixing soil, sand and perlite (2:1:1), the mix was sterilized at 180 °C (3 days for 2 h). Plastic pots (15 cm in diameter 20 cm high), were filled with 250 g of the potting mix. On infected pots *F. graminearum* inoculums at 1 g/kg were blended. On treated pots *L. divaricata* chloroformic extract and the most active compound on *in vitro* assay were applied to give a concentration of 10 mg/kg. Five wheat seeds were sown into the soil layer of the pots. There were four replicates, which were randomized in blocks in a growth chamber. The pots were watered every three days. Number of plant alive on each post was determined after 5 and 15 days post germination. The wheat plants were sampled after 15 days post germination. Each plant was assessed measuring aerial and radical length and weight. Severity index (SI) of crown rot was evaluated using a 0–4 scale, where 0 = symptomless, 1 = slight browning of the coleoptile and/or subcrown internode (<25%), 2 = obvious browning of the coleoptile and/or subcrown internode (25–75%), 3 = same as 2 with browning of leaf sheaths, 4 = seedlings wilted or dead (Huang and Wong, 1998).

3. Results and discussion

The extraction process of *L. divaricata* leaves yielded in hexane (7.6 g), chloroform (49.6 g) and methanol (53.5 g) extracts. The results of the *in vitro* evaluation indicates that the chloroform extract was active against all the fungi tested, *F. graminearum* and *M. phaseolina* were the most sensitive species (MIC = 250 μg/mL). *n*-Hexane was inactive against the fungi tested, and methanol extract inhibited *M. phaseolina* (Table 1). The differences in the inhibition effect of the extracts may be due to the lignans compounds present in *L. divaricata*, which had similarity to the chloroform solvent. In relation with the *n*-hexane extract, lower inhibition activity indicates, that there were interactions among non-polar inactive structures (Jasso de Rodríguez et al., 2011).

These results showed higher efficiency on *F. graminearum* inhibition than those reported by Trigui et al. (2013), who evaluated *Thymelaea hirsuta* hexane, chloroform, ethyl acetate, acetone, *n*-butanol and water extract, on *in vitro* assay. The ethyl acetate revealed antifungal activity (MIC = 312 μg/mL), the other extracts were found to be inactive.

Previously antifungal activity was described in *L. divaricata*. Svetaz et al., 2010 studied *L. divaricata* ethanolic extract against dermatophytes of high incidence in superficial infections. Author's results were similar to the inhibitions obtained with chloroform extract in this study. The filamentous fungi from the Phylum Ascomycota exhibited the highest sensitivity (MIC = 250 μg/mL) *Microsporum gypseum*, *Trichophyton mentagrophytes*, *Epidermophyton floccosum*, and *Trichophyton rubrum* present a MIC of 125 μg/mL. The MIC for *Candida albicans*, *C. tropicalis* and *Saccharomyces cerevisiae* was 500 μg/mL, mean while *Cryptococcus neoformans* present a MIC of 1000 μg/mL. On the other hand, the extract was inactive against the filamentous fungi of *Aspergillus* generous (*A. fumigates*, *A. flavus* and *A. niger*).

Ascendant TLC analysis of the chloroform extract from *L. divaricata* detected the presence of flavonoids and lignans. Among the extracts obtained the chloroform one was and compounds

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