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Activity and mode of action of *Parastrephia lepidophylla* ethanolic extracts on phytopathogenic fungus strains of lemon fruit from Argentine Northwest

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ABSTRACT

Fungal infections of fresh ripe *Citrus limon* fruit are the main cause of economic losses during their commercialization. The aim of this work was to evaluate the activity of the ethanolic extract (EE) from *Parastrephia lepidophylla* (Wedd.) Cabrera, a plant species that grows in the Argentine Northwest, on phytopatogenic fungi of lemon. Also, attempts were made to elucidate the mode of action of EE on the growth inhibition of phytopathogenic isolates of *Penicillium digitatum* Sacc. and *Geotrichum citri-aurantii* (Ferraris) E.E. Butler. The effect of the polyphenolic extracts on the conidia germination, mycelium growth and integrity of the plasma membrane was evaluated. The EE was active against both pathogens (minimal inhibitory concentration and minimal fungicidal concentration values of 150 μg of gallic acid equivalent/ml and 350 μg GAE/ml, respectively for both species). The conidia swelling and germination and the subsequent germ tube elongation was more affected by EE (100% inhibition at 200 μg GAE/ml) than the vegetative body of the fungus (50% inhibion at 400 μg GAE/ml). The fungus cell walls would not constitute a target for the EE components while the mechanism of action of the phytocomplex would be plasma membranes disruption. *In vivo* tests showed that *P. leidophylla* EE (700 μg GAE/ml) decreases the incidence of green mold disease in artificially inoculated lemons.

1. Introduction

Lemon fruit (*Citrus limon* (L.) Burm. f.) contains many important chemical components, including phenolic compounds (mainly flavonoids and phenolic acids), vitamin C, folic acid, minerals, dietary fiber, pectin, essential oils and carotenoids (Balasundram et al., 2006; Ejaz et al., 2006), that account for their health-promoting effects (Vinson et al., 2001; Proteggente et al., 2002; Wilmsen et al., 2005). It was demonstrated that they have a role in blood lipid lowering and in the prevention of diseases, such as obesity, diabetes, cardiovascular diseases, and certain types of cancer (Chasquibol et al., 2003). Industrial uses of lemon include concentrated juice, essential oil, dried peel and frozen pulp. Losses caused by postharvest diseases are among the major problems of the citrus industry worldwide. Fungal infections are the main cause of postharvest rots of fresh lemon fruit during storage and transport, and cause significant economic losses in the commercialization phase. Citrus green mold and sour rot diseases, caused by *Penicillium digitatum* Sacc. and *Geotrichum citri-aurantii* (Ferraris) E.E. Butler respectively, are some of the most frequently encountered species (Eckert and Brown 1986; Eckert and Eaks 1989; Sommer et al., 2002). Synthetic fungicides have a major role in order to reduce postharvest losses due to phytopathogenic fungi, though problems resulting from their extensive use as well as their associated health and environmental risks have promoted the search for new and safer alternatives. Furthermore, frequent findings of resistant strains due to the continuous use of fungicides are a serious risk to the effectiveness of them. Many results have been reported on the antimicrobial properties of polyphenolic extracts obtained from plant species that grow within Argentinian area of Puna (Zampini et al., 2005, 2007, 2009a,b; D'Almeida et al., 2011, 2012). In a previous work we have reported the activity of aqueous extracts of three plant species from the Andean

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phytogeographic province against fungal species causing citrus postharvest diseases (Sayago et al., 2012). Furthermore, *in vivo* activity of *Parastrephia lepidophylla* extract against *P. digitatum* was as high as those obtained by *in vitro* assays. The phytocomplex has exhibited both preventive as well as curative activities against *P. digitatum* growth inoculated in laboratory assays on lemon fresh fruit (Sayago et al., 2012). The aim of the present work was to investigate the *in vitro* and *in vivo* antifungal activity of an ethanolic extract from *P. lepidophylla* and to elucidate the mode of action on the growth inhibition of *P. digitatum* and *G. citri-aurantii* isolates.

2. Materials and methods

2.1. Plant material

Specimens of *P. lepidophylla* (Wedd.) Cab. were collected in Laguna de Vilama, Jujuy (66°55′W, 22°30′S, 4500 m above see level), a mountainous area of Puna from Northwest Argentina (Andean phytogeographic province; Cabrera 1971). The botanical identification was done by botanist Ana Soledad Cuello (Universidad Nacional de Tucumán-CONICET). The voucher specimens are deposited in Fundación Miguel Lillo Herbarium (access number 68979/LIL), Tucumán, Argentina.

2.2. Plant extracts preparation

Aerial parts of plant material were air-dried, ground and macerated in an alcoholic solution (50 g dry tissue/ 250 ml 60% ethanol) for 7 d at 30 °C with shaking (50 cycles/ min) in a water bath (Vicking, Dubnoff model). The extracts were filtered through two layers of gauze and centrifuged at 23,600 × *g* at 10 °C (Sorvall RC 5B) for 15 min. The supernatants were pooled and named *Crude Extract* (CE). The CE was concentrated at 40 °C at reduced pressure and then lyophilized. The Dry residue was first suspended in distilled water; after centrifuging (23,600 × *g*, 15 min) the supernatant was named *Aqueous Extract* (AE) and kept at −20 °C until use. The water insoluble material was then extracted with absolute ethanol (Sigma–Aldrich) and was named *Ethanolic Extract* (EE) and kept at −20 °C until use.

2.3. Chemical determinations

Total phenolic compound contents were determined by using the Folin–Ciocalteau colorimetric method (Singleton et al., 1999). Results were expressed as gallic acid equivalents (μg GAE/ml). Flavonoids were evaluated according to Woisky and Salatino (1998). Results were expressed as quercetin equivalents (QE μg/ml). Proteins were determined according to the Bradford (1976) method. The assays were performed using the micro-assay procedure of the Bio-Rad protein assay kit (Bio-Rad Laboratories). A solution of bovine serum albumin (1 mg/ml) was used as standard.

2.4. Fungal cultures

IEV 543 of *G. citri-aurantii* (Ferraris) E.E. Butler and IEV 544 of *P. digitatum* Sacc. strains were used (IEV: Culture collection of the Instituto de Estudios Vegetales, Facultad de Bioquímica, Química y Farmacia, Universidad Nacional de Tucumán, Argentina). The pathogenic fungi were isolated from lemon fruit with sour rot or green mold diseases. The identity of both fungal species was assessed according to Pitt and Hocking (1999). Stock cultures were maintained on Sabouraud Agar at 4 °C. Sabouraud Agar, Potato-Dextrose Agar and Glucose Broth (GB: 3% glucose, 1% casein peptone, pH 5.4–5.6) were used for routine fungus cultures. All reagents were purchased in Merck Chemicals Argentina, Buenos Aires.

2.5. Inoculum preparation

Conidia from 10 to 14 days cultures in PDA were suspended in sterile saline solution (0.9% NaCl) containing 0.05% Tween 80, vortexed and filtered through two layers of gauze. Conidia concentration was adjusted in a Neubauer chamber under light microscope.

2.6. Minimal inhibitory concentration and minimal fungicidal concentration determinations

Broth macrodilution tests were conducted to determine the minimal inhibitory concentration (MIC) and minimal fungicidal concentration (MFC) of the EE.

Glucose Broth containing EE (50, 100, 150, 200, 250, 300, 350, 400, 450 or 500 μg GAE/ml) was inoculated with a *P. digitatum* or *G. citri-aurantii* conidium suspension to achieve a final concentration of 0.5×10^3 conidia/ml. The incubation was carried out at 28 ± 2 °C for 72 h. The growth of fungi was assessed by direct observation of cultures. MIC was defined as the lowest extract concentration necessary to inhibit fungal development. MFC determination was performed as follows: aliquots of 100 μl of suspensions from MIC determinations (72 h of incubation at 28 °C) were subcultured on extract-free SA medium and incubated for 72 h at 28 °C. MFC was defined as the lowest concentration of extract capable to produce the irreversible loss of viability of conidia in the assay conditions (Sayago et al., 2012).

2.7. Time-kill determination

Tubes containing different amounts of EE (350 or 700 μg GAE/ml) in a final volume of 1 ml of GB were inoculated with 0.5 × 103 conidia (*P. digitatum* or *G. citri-aurantii*) and incubated at 28 °C. Aliquots of 100 μl were transferred to extract-free SA every 6 h. The fungal growth was assessed up to 72 h of incubation at 28 °C.

2.8. Effect of EE on the spore germination

a) The effect of the EE concentration on spore germination. Tubes containing increasing concentrations of EE (0–500 μg GAE/ml) were inoculated with 1×10^6 conidia/ml and incubated for 12 h at 28 °C. The percentage of germination at each EE concentration was then evaluated under light microscope by counting the germinated conidia in Neubauer chamber. The results were expressed as% inhibition of germination, % Inh. = $[(C - T)/C] \times 100$, where *T* and *C* are the amount of germinated conidia in 1 μl of the suspension with and without EE, respectively.

Table 1
Chemical analysis of extracts of *P. lepidophylla*.

Formulation	Total amount (g)[a]		
	Phenolic compounds[b]	Flavonoids[c]	Proteins[d]
Crude extract	3.202 ± 0.129	1.969 ± 0.034	0.957 ± 0.034
Aqueous extract	0.636 ± 0.021	0.402 ± 0.012	0.690 ± 0.012
Ethanolic extract	2.496 ± 0. 021	1.225 ± 0.021	0.117 ± 0.010

Total amount of phenolic compounds, flavonoids and proteins obtained from 50 g of plant material.
[a] Data represent the average of three independent assays ± standard deviation.
[b] Phenolic compounds were determined according to Singleton et al. (1999) and expressed as gallic acid equivalents (GAE).
[c] Flavonoids were assayed by the method described by Woisky and Salatino (1998) and expressed as quercetin equivalents (QE).
[d] For protein determinations (Bradford, 1976) a standard of bovine serum albumin was used.