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ORIGINAL ARTICLE

Antifungal activity of *Parastrephia quadrangularis* (Meyen) Cabrera extracts against *Fusarium verticillioides*

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Significance and Impact of the Study: In Argentina, *Fusarium verticillioides* causes 'ear rot', a disease that produces important yield and nutritional quality losses in the maize producing region. This study suggests that *Parastrephia quadrangularis* extracts have potential for the growth inhibition against *F. verticillioides* M7075, and the bioactivity is reported for the first time. The results obtained will provide a starting point for discover new antimicotic candidate in natural products.

Keywords
antifungal activity, *Fusarium verticillioides*, HPLC–UV–ESI MS/MS analyses, *Parastrephia quadrangularis*, *p*-coumaroyloxitremetone, plant extracts.

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Abstract

Fungi are cosmopolitan organisms that grow in and adapt to a vast number of substrates and environments, and that can cause diseases in humans and animals, as well as in crops. The vast area and diverse geographical characteristics of Argentina, with the consequent climatic diversity make the country an important source of biological resources suitable for the search of new compounds. The aim of the present study was to describe the antifungal activity of extracts of *Parastrephia quadrangularis*, a species from northern Argentina, against *Fusarium verticillioides* M7075. Bio-guided fractionation and MS/MS studies were conducted to elucidate the chemical structure of active compounds. The extracts exhibited a minimum inhibitory concentration among 118·74 and 250 μg ml^{-1} and the differences between the treatments and the inoculum control was 12·5–16·5 mm, respectively, in colony growth. Moreover, hyphae treated with the extracts stained blue with Evans blue, showed alterations in permeability of plasma membranes. HLPC-MS analysis of active fractions revealed the presence of *p*-coumaroyloxitremetone, and a derivate structure for another compound is proposed.

Introduction

In the Puna grassland, a phytogeographic region of northern Argentina, at more than 3200 m a.s.l., many xerophytic plants are known for the complex arrays of natural compounds they produce as adaptations to the prevailing extreme heat, desiccation, ultraviolet radiation and herbivory conditions (Timmermann and Hoffmann 1985; Maatooq *et al.* 1996). This environment is characterized by the presence of particular ecosystems on slopes or high plains named 'tolares' (local name of the site where *Parastrephia* species grow). According to ethnobotany reports, the species was used to treat diseases of muscles, tumours, superficial injuries, etc. (Ríos 2015, personal comm).

Different bioactivity evaluations have shown that the species has antimicrobial, antinflammatory, antioxidant and acaricide properties (Ayma *et al.* 1995; Zampini *et al.* 2008, 2009; Alberto *et al.* 2009; D'Almeida *et al.* 2012; Echiburu-Chau *et al.* 2017), as well as antifungal activity (Salvat 2010; Sayago *et al.* 2012; Palavecino Ruiz *et al.* 2016). Some phytochemical studies conducted in

Parastrephia lucida identified 42 metabolites including several phenolic acids, flavonoids, coumarins, tremetones and entclerodane diterpenes (Echiburu-Chau *et al.* 2017). Bohlmann *et al.* (1979) mentioned tremetone, metoxytremetone, *p*-coumaroylmethyl ester, *p*-coumaroyloxytremetone, umbeliferone, isofraxidin, etc, in *Parastrephia quadrangularis* (under the name of *P. lepidophylla* (Wedd.) Cabrera); moreover, Loyola *et al.* (1985) reported the isolation of 5,7-dihydroxy-3,8,30,40-tetramethoxyflavone in this species, as well as the presence of scopoletin, umbelliferone and *p*-coumaroyloxytremetone.

In Argentina, *Fusarium verticillioides* causes 'ear rot', a disease that produces important yield and nutritional quality losses in the maize producing region (Martínez *et al.* 2010; Garrido *et al.* 2012); in some cases, it can also synthesize mycotoxins known as fumonisins. The toxicity of these fumonisins is due to the inhibition of ceramide synthase, an essential enzyme for the production of ceramide and sphingolipids, which causes disruptions in the metabolism, producing the syndromes known as equine leucoencephalomalacia, and porcine pulmonary oedema (Riley *et al.* 2001). They also have adverse effects on the immune system in chicken, (Voss *et al.* 2007). In humans, they can cause oesophageal cancer, neural tube development and may affect child growth (IARC, 2015).

Damage caused by these fungi is prevented with the application of increasing amounts of fungicides, which accumulate in the environment, causing selection mechanisms with the consequent development of resistant populations. Since 2001 we are bioprospecting tests that have been conducted in the different phytogeographic regions of Argentina, (Salvat *et al.* 2001, 2004; Salvat 2010). The aim of the present study was to describe the antifungal activity of *P. quadrangularis* extracts from northern Argentina against *F. verticillioides* M7075; bioguided fractionation and MS/MS studies were conducted to elucidate the chemical structure of active compounds.

Results and Discussion

Microdilution method

The extract of *P. quadrangularis A* had a significant activity against *F. verticillioides* M7075 (minimum inhibitory concentration (MIC) = 118·74 μg ml^{-1}), whereas MIC of extract B was 250 μg ml^{-1}. Previous studies determined activity of this genus against the plant pathogenic fungus *F. graminearum* (Salvat 2010); other authors also reported antifungal activity against the fungi *Penicillium digitatum* and *Geotrichum citri-aurantii* (Palavecino Ruiz *et al.* 2016). The differences in MIC values observed between extracts *A* and *B* can be attributed to differences between years (2003–2016), phenological stages and sample collection sites.

Hyphal radial growth test

The *P. quadrangularis* extracts *A* and *B* inhibited growth of *F. verticillioides* M7075 after 7 days of incubation at 28°C (Fig. 1). Reduction in growth ratio with respect to control inoculum was 12·5 mm in extract *A* and 16·5 mm in extract *B*. Differences between treatments, caused by a delay in growth (mm) of mycelium, were noticed after 4 days of incubation. At the end of the assay, growth delay was sustained by both extracts. These results suggest the fungistatic rather than fungicidal nature of antifungal action of the extracts.

Visualization of hyphal alterations

Figure 2 shows blue staining of hyphae of *F. verticillioides* M7075 treated with *P. quadrangularis* extracts; in contrast, control hyphae maintained their natural coloration (translucent). This staining allows us to determine cell viability and consists of the exclusion of the stain when there is no alteration in the fungus membrane (viable cells), and the inclusion of the stain when there is some modification (nonviable cells) (Smith *et al.* 1982).

Isolation of bioactive fractions

Contact autobiography of *P. quadrangularis* extracts *A* and *B* showed inhibition bands (Fig. 3). In extract *A*, a blue fluorescent band was observed, at a reaction front (Rf) = 0·68, and an orange fluorescence one, at Rf = 0·95, which coincided with the inhibition zones. Extract *B* also showed fluorescent bands at the same Rf, but its inhibition zone covered a greater area at Rf = 0·95.

The dichloromethane (DCM) and ethyl acetate (EtOAc) extracts and fractions exhibited antifungal activity. All active samples were resuspended in methanol (MeOH) and analysed by high performance liquid chromatography (HPLC)-MS for tentative identification of active principles. Total ion chromatograms of all samples exhibit four peaks: at 18 min (1), 18·5 min (2), 19·2 min (3) and 20·7 min (4) (Table 1, Fig. 4). MS analysis of principal ions showed 363 *m*/*z* as the principal ion of peak 1, 405 *m*/*z* of peaks 2 and 3, and 525 *m*/*z* of peak 4. The MS/MS analysis of 363 *m*/*z* ion in peak 1 identified this compound as *p*-coumaroyloxytremetone (MW: 364), a toxin that was also reported in *Parastrephia* species (Bohlmann *et al.* 1979; Loyola *et al.* 1985; D'Almeida *et al.* 2012). The MS/MS fragmentation of peak 2 produced 363 *m*/*z* ion, suggesting acetate derivative of the same compound that was identified in peak 1; however, peak 3 did not produce 363 *m*/*z* fragments. In this case, 405 *m*/*z* might represent a pseudomolecular ion $[M+H-120]^-$, a fragmentation behaviour typical of C-glycosides. In contrast to O-glycosides, C-

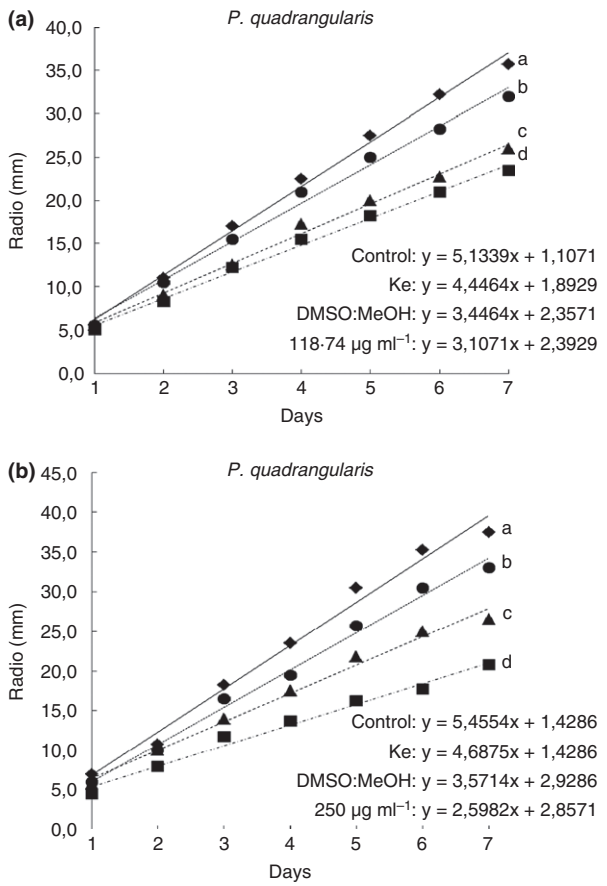


Figure 1 Hyphal radial growth test. Data are shown as average values of fungal growth rate for each treatment. Different letters indicate significant differences ($P < 0{\cdot}05$) among treatments (*Parastrephia quadrangularis* extract *A*: ◆, control; ■, 118·74 µg ml^{-1}; (1a) and 250 µg ml^{-1} (1b) ▲, DMSO : MeOH; ●, Ke; ——, lineal (control); - - -, lineal (118·74 µg ml^{-1}); (1a) and 250 µg ml^{-1} (1b) – – –, lineal (DMSO : MeOH); ·····, lineal (Ke).

glycosides do not generate abundant aglycone ions (de Rijke *et al.* 2006). At peak 4, the ion of 525 *m*/*z* suggests the presence of a hexose or a coumaroyl derivative (162 amu, atom mass units) of the same compound. Further analyses are needed to confirm these structures. Another peak (5), at 17 min, 285 *m*/*z*, was present in DCM fractions and subextracts; this compound was identified as kaempferol, a flavonol also reported in *P. lucida* (Echiburu-Chau *et al.* 2017).

Previous studies using plant extracts with antifungal activity they found that also has antimycotoxigenica activity (Aristimuño Ficoseco *et al.* 2014). In this regard, it would have been expected that extracts of *P. quadrangularis* present a similar effect. Anyway, further analysis is needed, such as the characterization of its metabolites and their possible application in crops. These aspects are being contemplated for future publications of our group.

The antifungal activity of *P. quadrangularis* extract against *F. verticillioides* M7075 is reported and described. Active samples were identified by bio-guided fractioning and thin layer chromatography (TLC) contact bioautography. Finally, some structures were suggested by high performance liquid chromatography-tandem mass spectrometry (HPLC-MS/MS). *p*-coumaroyloxytremetone and its derivative are potential candidates to be responsible for its antifungal activity.

Materials and methods

Plant material and extract preparation

The plant specimens used were collected and identified by R.H Fortunato. The voucher specimens were deposited at BAB Herbarium: http://sciweb.nybg.org/Science2/IndexHerbariorum.asp: BAB

Parastrephia quadrangularis A: Aerial parts with capitulum in ripe fruits, voucher: Fortunato 8151 (BAB), collection site: Argentina, Jujuy province: Yavi department, 4 km N of Abra del Azoite. March 2003.

Parastrephia quadrangularis B: Aerial parts with capitulum in flowering, voucher: D. Rios s/n° (BAB 92631), collection site: Argentina, Jujuy province: Cochinoca department, Paraje esquina Katari.12 km S of Abra Pampa. November 2016.

Parastrephia quadrangularis A and *B* were dried, finely ground and extracted with MeOH (Merck, Buenos Aires, Argentina) (10 g of dry plant material per 100 ml) at room temperature in total darkness for 48 h. The extracts were filtered, dried under reduced pressure at 40°C, and weighed. This crude methanolic extract was dissolved in MeOH at a concentration of 80 mg dry matter per ml. For extract conservation, 1 ml of each extract was diluted with 9 ml of dimethyl sulfoxide (DMSO; Biopack, Buenos Aires, Argentina) until a final concentration of 8000 µg dry matter per ml was obtained. This solution was sterilized by passing through a 0·45-µm cellulose acetate membrane (Minisart, Sartorius, Buenos Aires, Argentina). All extracts were maintained in cryovials at −35°C until analysis.

Micro-organism

The *F. verticillioides* strain M7075, fumonisin B_1 producer, was isolated from maize in Argentina and deposited in the Fusarium Research Center Collection (Pennsylvania State University, University Park, PA), was kindly provided by the University of Río Cuarto, Córdoba, Argentina. This strain was cultured in Synthetischer Nährstoffärmer Agar (SNA) tubes at 28°C for 7 days. Then, peptone water + Tween 80 was added to each tube. Conidial

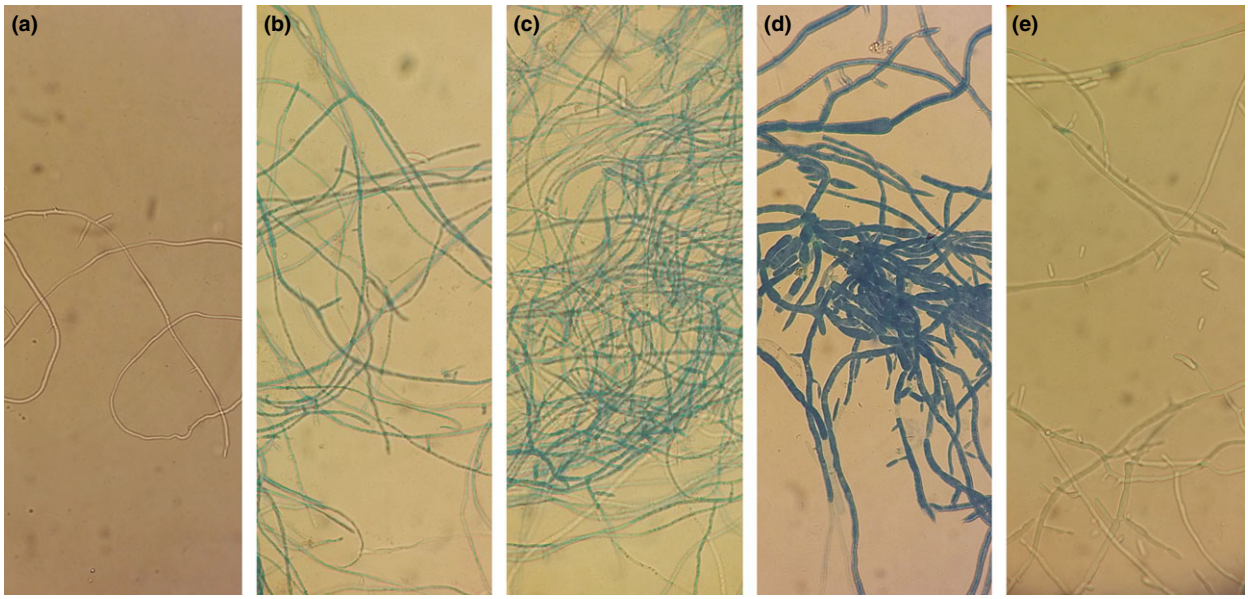


Figure 2 Visualization of hyphal alterations after Evans blue staining, by light microscopy. Effect of different treatments on *Fusarium verticillioides* M7075: (a) Control (live hyphae). (b) Ke. (c) *Parastrephia quadrangularis* extract *A* (118·74 μg ml^{-1}). (d) *P. quadrangularis* extract *B* (250 μg ml^{-1}). (e) DMSO : MeOH (9 : 1). [Colour figure can be viewed at wileyonlinelibrary.com]

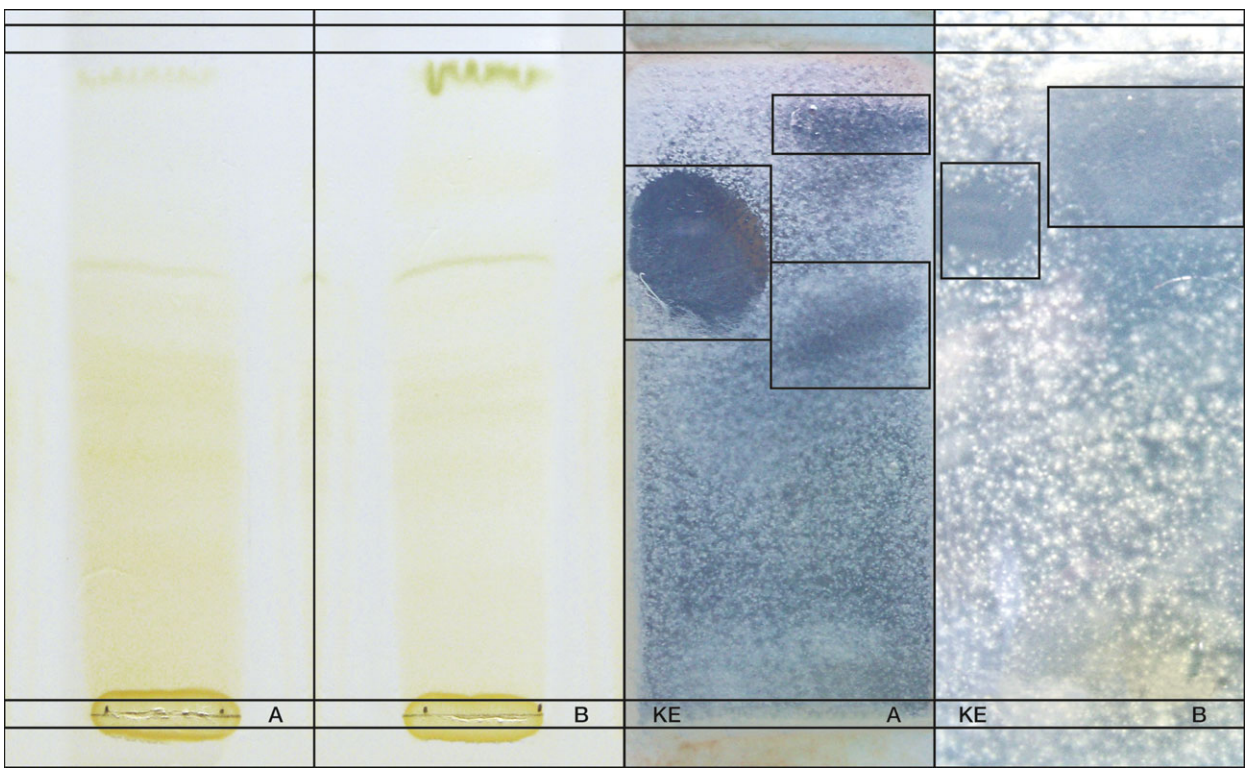
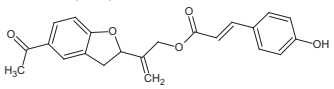


Figure 3 Contact bioautography (from left to right): TLC of *Parastrephia quadrangularis* extract *A*. TLC of *P. quadrangularis* extract *B*. Contact bioautography of *P. quadrangularis* extract *A* and Ke. Contact bioautography of *P quadrangularis B* and Ke. The inhibition zones of the extracts and Ke are highlighted in the figure. [Colour figure can be viewed at wileyonlinelibrary.com]

Table 1 *Parastrephia quadrangularis* extracts, subextracts and active fractions. Retention time, UV, MS, MS/MS and suggested compounds

Peak	Rt (min)	UVmax (mn)	$[M-H]^-$, *m/z*	MS/MS, *m/z*	Suggested compound
1	18·0	224, 298	363·3	162·0,144·8,117·9	Coumaroyloxytremetone 
2	18·5	224, 284	405·3	363·3, 297	Coumaroyloxytremetone acetate
3	19·2	228, 294	405·3 $[M-H-120]^-$	297, 177	Coumaroyloxytremetone—C—hexoside
4	20·7	229, 296	525·4		Coumaroyloxytremetone—hexoside/ coumaroyl
5	17·3	225, 290	285	163,135,151,243	Kaempferol

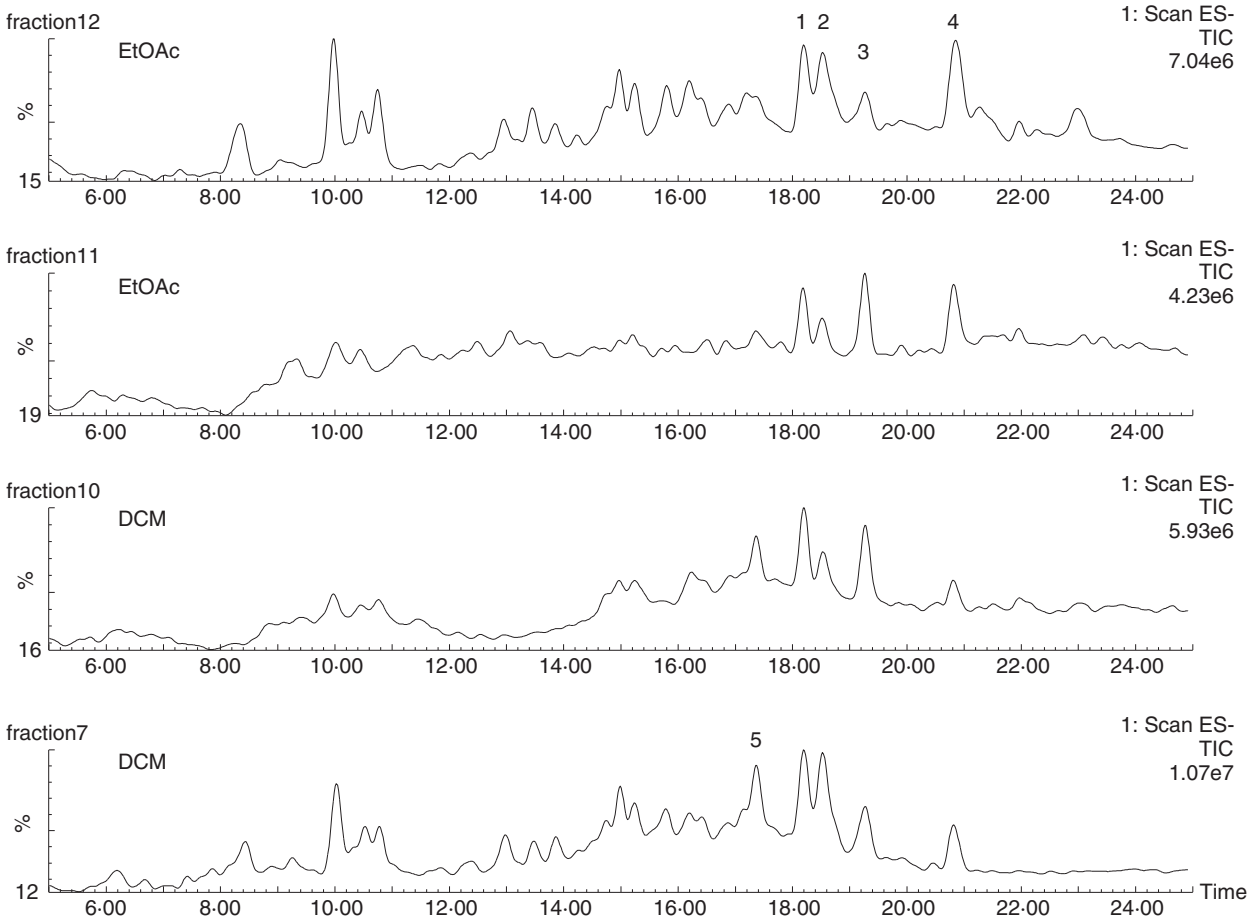


Figure 4 Chromatogram of the active fractions. (fractions of extract *B*). From top to bottom, total ion chromatogram of EtOAc fractions 12 and 11, DCM fraction 10 and 7. Peaks 1, 2, 3 and 4 are visualized in all the active chromatograms; however, peak 5 is present in DCM fraction 10 and smaller in the first fraction of DCM ones (fraction 7).

suspension was filtered through a sterile gauze inside a glass jar. Conidia were counted with a Neubauer chamber and the suspension was then adjusted to a concentration ranging 1–3 × $10^{4/5}$ conidia per ml, depending on the assay to be performed.

Media and antifungal agent

Media

The following media were used: Roswell Park Memorial Institute 1640 (RPMI 1640) synthetic medium (Gibco by

Life Technologies, Grand Island, NY, USA) 1x with L-glutamine without phenol red. SNA, Oxoid PDA medium (potato dextrose agar). Resazurin as dye (0·01% aqueous solution), Sigma-Aldrich (St. Louis, MO).

Antifungal agent

Ketoconazole (Ke) at a concentration of 2 μg ml^{-1} for colorimetric assay for antifungal susceptibility testing, Evans blue staining and hyphal radial growth test at 50 μg ml^{-1} for contact autobiography were used. All the solutions were sterilized using sterile filters with 0·45-μm cellulose acetate membrane and preserved in cryovials at −35°C.

Antifungal assays

Microdilution method

This assay was conducted following the procedure indicated in M38-A (NCCLS, 2002), with some modifications for antifungal activity (Salvat 2010). This technique was conducted in sterile 96-well microplates Biofil (Guangzhou, China). Then, 200 μl of the MeOH : DMSO solutions (1 : 9) of the plant extracts was added to each of the wells in row A, and 100 μl of RPMI 1640 was added to the remaining wells (rows B–H). For preparing dilutions, 100 μl was removed from row A wells and added to wells in row B; the same procedure was repeated until reaching row H (in vertical direction). The excess dilution (100 μl) of row H was discarded. Thus, the highest and the lowest extract concentrations corresponded to wells in row A and in row H respectively. Then, 100 μl of the inoculum, which contained between 1–3 × 10^4 conidia per ml in all the wells, was added to all the wells, except for the RPMI 1640 medium control. Finally, 20 μl of Resazurin was added to all the wells (Espinel-Ingroff *et al.* 1995). A number of wells in each plate were reserved for control of RPMI 1640 sterility, inoculum viability, antifungal agent (Ke) at the before mentioned concentration, and MeOH : DMSO (1 : 9) solvent effect. Microplates were incubated at 28°C in the dark, for 48 h. The colour change in Resazurin from blue to pink indicates fungal growth. The MIC was considered the highest concentration (in μg of dry matter per ml of medium) at which no colour change in Resazurin was detected. An extract was considered active at a MIC <500 μg ml^{-1} (Salvat *et al.* 2001; Derita *et al.* 2009). This assay was performed in triplicate.

Hyphal radial growth test

The antifungal activity of the extracts *A* and *B* was evaluated using this test, at 7-day culture of *F. verticillioides*. Discs (5 mm) of the culture were taken and placed in the centre of Petri dishes containing 20 ml of PDA with the extracts at their respective MICs. In addition, the respective controls were performed, corresponding to the untreated inoculum, the inoculum with a Ke and the control of the extract solvent. All the plates were incubated at 28°C for 7 days. Colony radio (mm) was measured every day and treatment efficiency was evaluated (Mier *et al.* 2002; Quiroga *et al.* 2009). This assay was performed in triplicate.

Statistical analysis

Data on fungal colony growth were analysed by analysis of variance followed by the Bonferroni post-test. The analysis was expressed as mean ± SD and differences were statistically significant at P<0·05.

Evans blue staining: visualization of hyphal alterations

Spores of *F. verticillioides* were incubated in RPMI 1640 liquid medium at 28°C for 24 h. After that period, 1 ml of the medium containing the hyphae was transferred to 1·5-ml Eppendorf tubes. Then, the extracts *P. quadrangularis A* and *B* at their respective MICs were individually added to the tubes; in addition, a tube as positive control (Ke) and another one as inoculum control were included. All the tubes were incubated at 28°C for 24 h and then centrifuged; the culture media were removed and two drops of Evans blue (0·05%) were added for 5 min. Finally, the hyphae were washed with sterile distilled water to remove excess stain. The difference in staining was observed under light microscope at 400x and evaluated following Semighini and Harris (2010) and Savi *et al.* (2013).

Contact bioautography

The components of extracts *A* and *B* were separated by TLC. The stationary phase consisted of silica gel 60 TLC plates (9 × 5 cm); the mobile phase consisted of chloroform : methanol (9 : 1) (Moreno López 2011). PDA was inoculated with 1–3 × 10^5 conidia per ml of *F. verticillioides* and then placed in glass plates. Methanolic extracts were used at a concentration of 400 mg ml^{-1} and Ke positive control. Fluorescent bands in TLC were observed under UV light and placed on inoculated agar at 28°C for 24 h. Then they were removed and incubation continued for 24 h. Finally, inhibition zones were read and the Rf was calculated (Zacchino and Gupta 2007).

Extraction and isolation of bioactive fractions

The ground dry plant material was sequentially macerated in hexane (Hx), DCM, EtOAc and MeOH (100 ml each) at room temperature in total darkness for 24 h; were filtered, dried under reduced pressure at 40°C and weighed (Aristimuño Ficoseco *et al.* 2014; Babajide *et al.* 2015). They were then redissolved in each solvent at a concentration of 80 mg dry matter per ml.

Crude methanolic extracts (1 ml) was also fractionated by silica gel 60 (0·063–0·200 mm), column 20 cm length ×

1 cm internal diameter, and eluted with 5 ml Hx, 5 ml DCM, 5 ml EtOAc and 5 ml MeOH (Franco *et al.* 2007).

All extracts and fractions were tested by microdilution method and active samples analysed by HPLC-MS/MS.

HPLC-UV-ESI MS/MS analyses

The samples were analysed using HPLC-MS/MS. The studies were performed on a Waters Quattro Premier XE spectrometer (Waters, Milford, MA) equipped with a Waters 2695 binary pump plus auto sampler. The HPLC separation was performed on Kinetex C18 column (250 mm × 4·5 mm, 5 μm). The mobile phase consisted of (A) water/0·1% formic acid and (B) acetonitrile/0·1% formic acid. An increasing linear gradient (v/v) of solvent B was used (*t* (min), %B): (0–2, 10), (20–25, 80), (27–33, 10) with a flow rate of 0·9 ml min^{-1} and a column temperature of 35°C. The gradient and the sample concentration were optimized in order to minimize the ion suppression caused by the plant extract matrix. The mass instrument was operated in electrospray negative ion mode; the capillary voltage was kept at 3·0 kV, the cone voltage at 30 V and the source temperature at 150°C. Nitrogen was used as desolvation gas and cone gas. MS/MS experiments were performed in order to identify the most abundant unknown ions in the samples. Argon was used as collision gas at a flow rate of 0·35 ml min^{-1}. The collision energy and the cone voltage were optimized for each unknown ion by varying the parameters in subsequent chromatographic runs.

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Conflict of Interest

No conflict of interest declared.

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