Journal of Applied Microbiology

Journal of Applied Microbiology ISSN 1364-5072

ORIGINAL ARTICLE

Antifungal and antimycotoxigenic metabolites in Anacardiaceae species from northwest Argentina: isolation, identification and potential for control of *Fusarium* species

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Keywords
alkylcatechols, deoxynivalenol, fumonisins, *Fusarium*, lupeol.

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2013/2491: received 11 December 2013, revised 1 January 2014 and accepted 4 January 2014

doi:10.1111/jam.12436

Abstract

Aims: The purpose of this research was to identify antifungal compounds from leaves of *Schinus* and *Schinopsis* species useful for the control of toxigenic *Fusarium* species responsible of ear rot diseases.

Methods and Results: Leaves of *Schinopsis* (*S. lorentzii* and *S. haenkeana*) and *Schinus* (*S. areira*, *S. gracilipes* and *S. fasciculatus*) were sequentially extracted with dichloromethane, ethyl acetate and methanol. The antifungal activity of the fraction soluble in methanol of these extracts (fCH_2Cl_2, fAcEt and fMeOH, respectively) was determined by the broth microdilution method and the disc-diffusion method. The minimum inhibitory dose (MID), the diameter of growth inhibition (DGI) and the minimum concentration for 50% inhibition of fungal growth (MIC_{50}) were calculated. The fCH_2Cl_2 and fAcEt of the *Schinopsis* species had the lowest MID and MIC_{50} values and the highest DGI. The antifungal compounds were identified as lupeol and a mix of phenolic lipids. The last one had the highest antifungal activity with MIC_{50} 31–28 $\mu g\ g^{-1}$ and 165–150 $\mu g\ g^{-1}$ on *Fusarium graminearum* and *Fusarium verticillioides*, respectively. The identified metabolites completely inhibited fumonisin and deoxynivalenol production at lower concentrations than ferulic acid, a natural antimycotoxigenic compound.

Conclusions: It was proven that lupeol and phenolic lipids were inhibitors of both fungal growth and mycotoxin production of toxigenic *Fusarium* species. This fact is specially interesting in the control of the toxigenic *Fusarium* species because several commercial antifungals showed to stimulate mycotoxin biosynthesis at sublethal concentrations.

Significance and Impact of the Study: Control of toxigenic *Fusarium* species requires compounds able to inhibit both fungal growth and mycotoxin production. Our results suggest that the use of lupeol as food preservative and the phenolic lipids as fungal growth inhibitors of *F. verticillioides* and *F. graminearum* did not imply an increase in mycotoxin accumulation.

Introduction

Fusarium graminearum (teleomorph *Gibberella zeae*) and *Fusarium verticillioides* (teleomorph *G. moniliformis*) cause ear rot diseases in cereals from the subtropical and temperate regions of Argentina (Presello *et al.* 2008; Sampietro *et al.* 2009).They not only reduce cereal yield but also contaminate infected grains with mycotoxins noxious for human and animal health. The repertory of mycotoxins produced by *Fusarium* spp. includes a wide spectrum of structurally very different compounds. Trichothecenes, fumonisins, zearalenone and emerging toxins such as fusaproliferin, enniatins, beauvericin, and moniliformin (Marin *et al.* 2013). Exposure to fumonisins mainly

produced by *F. verticillioides* has been associated with several diseases in animals including leucoencephalomalacia in equines (Marasas *et al.* 2004), pulmonary oedema in swine (Harrison *et al.* 1990), liver cancer in rats (Norred *et al.* 1992) and immunosuppression in poultry (Keck and Bodine 2006). Epidemiological studies suggest that fumonisins increase the incidence of human oesophageal cancer in Africa, Brazil, China and Italy (Marasas *et al.* 2004; Gelineau-van Waes *et al.* 2005). *Fusarium graminearum* produces mainly trichothecenes. This structural group of trichothecenes all bear a common tricyclic 12,13-epoxytrichothec-9-ene core structure. Type A, B, C and D trichothecenes can be distinguished based on substitutions at position C-4, C-7, C-8 and/or C15. Deoxynivalenol (DON), a type B trichothecene, is the predominant mycotoxin in small grain cereals. Animal intake of DON is associated with feed refusal, vomiting and suppressed immune functions (Girardet *et al.* 2011). This mycotoxin is also acutely phytotoxic and acts as a virulence factor on cereal hosts (Jansen *et al.* 2005). Several public institutions, such as the FAO/WHO Expert Committee on Food Additives, the U.S. Food and Drug Administration and the European Commission, have established maximum permissible contents for fumonisins and DON in cereals and derived products for human intake (JECFA 2001; FDA 2002). There is currently no a single robust control measure to manage the ear rot disease caused by *F. verticillioides* and *F. graminearum* or the concomitant mycotoxin presence. Fungicide application is one strategy to reduce the mycotoxigenic risk, but the spectrum of fungicides against *Fusarium* is quite narrow comprising the azoles amongst others. However, it was shown that ineffective applications of azole fungicides can lead to sublethal exposure of *Fusarium* species which in turn can increase mycotoxin production in the field (Audenaert *et al.* 2010, 2011). This finding was corroborated pursuing a qRT-PCR approach which showed that the expression of *Tri4*, *Tri5* and *Tri11,* and genes crucial for DON biosynthesis was higher in cultures of *F. graminearum* isolates supplemented with sublethal concentrations of the fungicides tebuconazole and propiconazole than that in nontreated controls (Kulik *et al.* 2012).Moreover, the intensive use of azole fungicides has increased fungal resistance to these xenobiotics (Becher *et al.* 2010).

Several species of *Schinus* and *Schinopsis* are endemic of northwest Argentina (Pell *et al.* 2011). The berries of *S. areira*, *S. fasciculatus* and *S. gracilipes* have been used to spice food in south American countries for centuries. The stems and leaves are used in folk medicine as antiseptic, antimicrobial or as insect repellents (Brack Egg 2003). In the case of *Schinopsis*, the trees *S. lorentzii* and *S. haenkeana* are appreciated for their very hard durable timber. Tannins extracted from the woods are known antimicrobials. The leaves and bark of these species have wound healing properties and are anti-asthmatic, features usually attributed to tannins (De Fátima Agra *et al.* 2007). Some of the compounds responsible for these activities are defence metabolites against pests and diseases and are a potential source of antifungal compounds. In this context, our present work has focused on the screening of antifungal compounds from leaf extracts of *Schinus* and *Schinopsis* species native from northwest Argentina on toxigenic isolates of *F. verticillioides* and *F. graminearum*. The bioactive compounds of the most active extracts were structurally characterized as well as their effects on fungal growth and mycotoxin biosynthesis.

Materials and methods

Plant material

Leaves of the native Anacardiaceae were collected during February–March 2010. *Schinus* species (*S. gracilipes*, *S. fasciculatus* and *S. areira*) were found in the west of Tucuman province (Tafi del Valle department, Tucuman, Argentina), while *Schinopsis* species (*S. lorentzii* and *S. haenkeana*) were located in the north of the province (Trancas department, Tucuman, Argentina). The botanical identity of the plant materials was confirmed by Dr. Nora Muruaga (Laboratory of taxonomy of fanerogamic plants, Miguel Lillo Foundation, Tucuman, Argentina) by comparison with the voucher specimens already deposited in the Herbarium of the Miguel Lillo Foundation. The leaves were dried in the dark for several weeks and then stored in paper bags at room temperature.

Micro-organisms

Strains used in the antifungal assays belonged to *F. verticillioides* isolated from maize (P364 from Pergamino, Buenos Aires province; LABI4 isolated from Cruz Alta, Tucuman province; both are fumonisin-producer strains) and *F. graminearum* isolated from wheat (GVI-II-3 from La Plata, Buenos Aires province; LABI2 isolated from maize in Trancas, Tucumán province). Using an *in vitro* assay with rice medium, the selected strains of *F. verticillioides* and *F. graminearum* were shown to produce fumonisins and DON, respectively (Sampietro *et al.* 2010, 2011).

Extraction of plant metabolites

Leaf extracts of each plant species were obtained according to the protocol shown in Fig. 1. Leaves of each

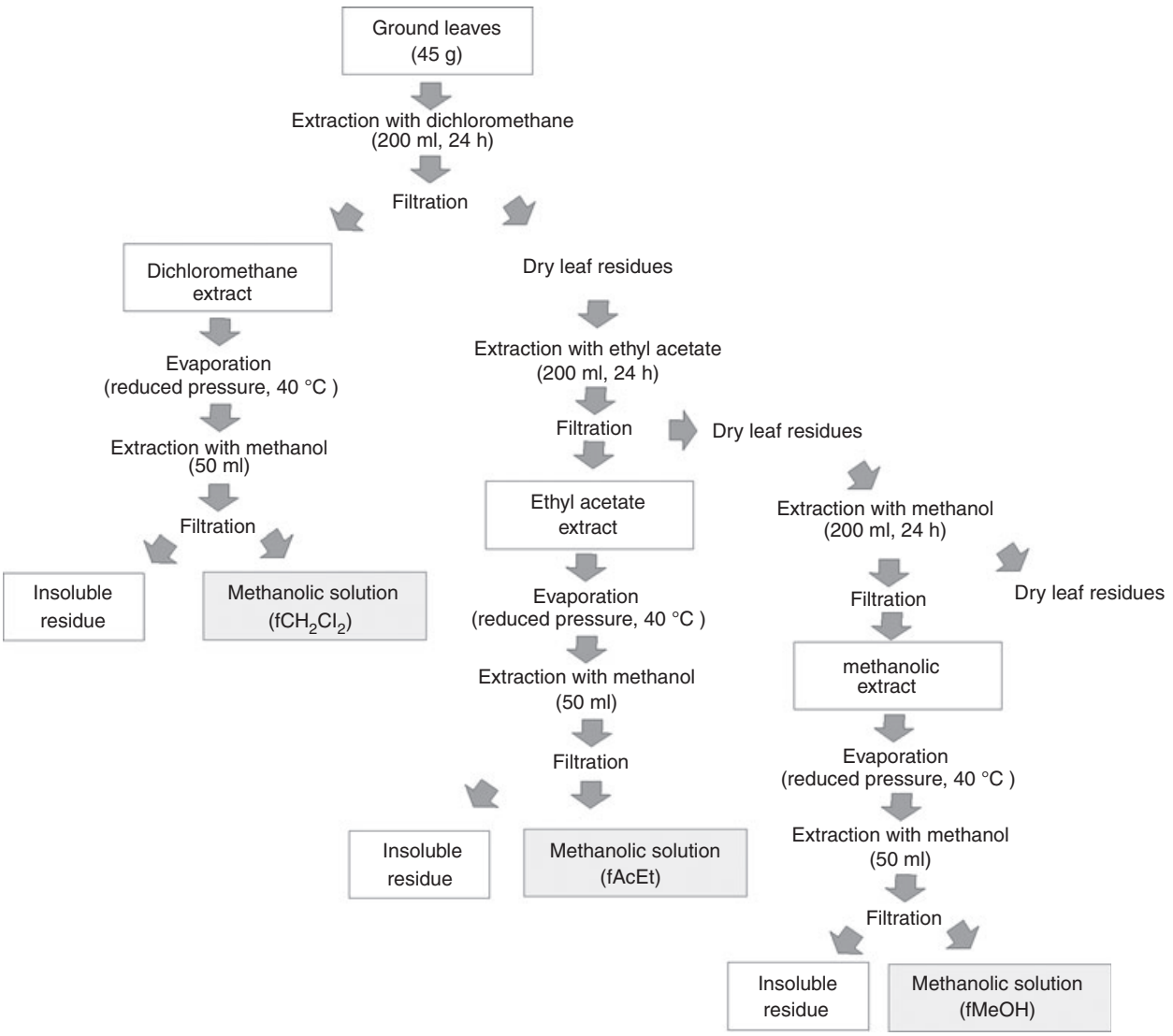

Figure 1 Protocol for extraction of secondary metabolites investigated in this work. Dry ground leaves from each Anacardiaceae species were extracted following this procedure.

species were powdered in a wiley mill. Then, a portion of the powdered material (45 g) was sequentially extracted with solvents of increasing polarity (dicloromethane, ethyl acetate and methanol). The powdered plant material was exposed 24 h to each organic solvent (1 : 4·4, w/v), subsequently trapped in a filter paper and left to dry at room temperature before immersion in the next organic solvent. This sequential extraction allowed a first fractionation of the leaf components. The organic fractions obtained were evaporated under reduced pressure, individually resuspended in 50 ml of methanol and filtered through a filter paper. The solutions obtained after filtration are hereafter referred as fCH_2Cl_2, fAcEt and fMeOH, which correspond to the dichloromethane, ethyl acetate and methanol extracts readily soluble in methanol. The methanolic fCH_2Cl_2, fAcEt and fMeOH fractions from leaves of the five Anacardiaceae species were further used in the antifungal assays.

Disc-diffusion and microdilution methods

The antifungal activity of the methanolic fractions (fCH_2Cl_2, fAcEt and fMeOH) was evaluated by the disc-diffusion method in agar and the broth microdilution method proposed by the National Committee for Clinical Laboratory Standards reference method M38-A (Wayne 2002) with some modifications. In the disc-diffusion method, 4-mm-diameter sterile discs of Whatman #1

paper were impregnated with different volumes of the methanolic fractions to obtain 125, 250, 500, 700, 1000 and 2000 μg dry matter per disc. Positive controls consisted of discs impregnated with the azole fungicide ketoconazol (2 μg per disc), while negative controls were prepared by adding 100 μl of methanol, which was the highest volume of the methanolic fraction added to a disc. Petri dishes containing 15 ml of peptone–malt–agar (5 g l^{-1} peptone, 15 g l^{-1} malt, 20 g l^{-1} agar and 20 g l^{-1} sucrose) were inoculated in the centre with a 5 mm plug obtained from the edge of a 3-day-old fungal colony grown in the same medium. After inoculation, the fungal diameter was allowed to reach 1 cm before being exposed to the antimicrobial fractions. Afterwards, the discs containing the doses of the methanolic fraction, and the negative and positive controls were placed on the surface of the medium, at 1·5 cm from the edge of the fungal colony. The Petri dishes were incubated 72 h at 25°C. Afterwards, the minimum inhibitory dose (MID) and the diameter of the associated growth-inhibitory zone were determined. The MID was defined as the minimum dose of a methanolic fraction included in a paper disc able to show visual inhibition of mycelial growth.

Fungal strains were also grown in Petri dishes containing Spezieller Nährstoffarmer Agar (SNA) at 25°C in the dark (*F. verticillioides*) or under a photoperiod of 12 h of black light (*F. graminearum*) for 7 days. Then, they were washed with 2 ml of sterile saline solution (0·9%). The spores in the obtained suspensions were counted in a Neubauer chamber and then diluted to 2×10^4 CFU ml^{-1} (hereafter referred as the working suspension) in YES medium (20 g l^{-1} yeast extract, 0·5 g l^{-1} $MgSO_4$, 150 g l^{-1} sucrose) supplemented with 0·125% agar. The broth microdilution method was performed according to documents M38-A and M38-P (NCCLS, 2002) with some modifications, using plastic microplates with 96 flat-bottomed wells. Each methanolic fraction was evaporated under reduced pressure and then dissolved in DMSO to obtain a stock solution of 200 mg dry matter ml^{-1}. This stock solution was used to prepare a 2-fold dilutions in the medium mentioned. In each well, 100 μl of working suspension was added to 100 μl of each dilution, and the final concentrations were compressed between 1000 and 7·8 μg of dry matter of a methanolic fraction per millilitre of medium, with 1% (v/v) of DMSO. Growth controls contained 100 μl of YES medium with 1% DMSO instead of a dilution. Epoxiconazole, pyraclostrobin and ketoconazole were also assayed for comparative purposes at final concentrations ranging from 64 to 0·25 μg ml^{-1} of medium in 1% DMSO. Each concentration of extract assayed as well as the positive and negative controls were replicated 4 times, and the experiments were repeated twice. Absorbance in the well microplates was measured at 630 nm after incubation of 72 h at 25°C. Means of the absorbance readings were calculated and corrected with controls of colour. The lowest concentration of dry matter required to inhibit 50% fungal growth (MIC_{50}), with ±95% confidence limits, was calculated by Probit analysis (Finney 1971), using the software package XLSTAT ver. 7.5.2 (Addinsoft, New York, NY, USA).

Bioautographic method

Components of the methanolic fractions were separated on thin layer chromatography (TLC) plates (silica gel G60 F_{254}, Merck). After development, the chromatograms were left to dry under sterile conditions in a laminar flow. Then, 5 ml of peptone–malt–agar medium with 0·8% agar containing 10^5 microconidia of *F. verticillioides* or macroconidia of *F. graminearum* was applied on each TLC plate, and the plates were incubated for 3 days at 25°C. Absence of mycelial growth on the TLC plates indicated the presence of antifungal compounds. Antifungal bands were visualized in TLC plates after spraying with 0·5% $FeCl_3$ in methanol or with 1% acid *p*-anisaldehyde (0·5 ml *p*-anisaldehyde in 50 ml glacial acetic acid and 1 ml of concentrated sulphuric acid) and further exposure to 105°C. Each bioautography was performed in triplicate.

Separation of the antifungal compounds from the methanolic fractions of *Schinopsis*

The TLC bands with antifungal activity identified in fractions fAcEt and fCH_2Cl_2 of *Schinopsis* were separated by column chromatography. Each column containing 23 g of silica gel (0·04–0·063 nm) as stationary phase and was eluted with hexane/ethyl acetate (7 : 1, v/v). Elution of the bands was monitored by TLC as previously indicated. Eluted fractions containing the antifungals were combined and evaporated. Fractions recovered were named M1 from fCH_2Cl_2 of *S. lorentzii*; M3 from fAcEt of *S. lorentzii*; M2 from fCH_2Cl_2 of *S. haenkeana*; M4 from fAcEt of *S. haenkeana*. These fractions were dissolved in ethyl acetate and injected in a gas chromatograph Hewlett-Packard 6890 equipped with a Perkin Elmer Elite 5MS column (5% phenylmethylsiloxane; 30 m × 0·25 mm × 0·25 μm thickness as stationary phase). Helium was used as mobile phase at a flow of 1 ml min^{-1}; split: 80 : 1; interface temperature 280°C; ion source temperature: 230°C; temperature of the mass detector (quadrupole): 150°C; injector temperature: 250°C; injection volume: 1 μl; temperature program: 170°C (0 min), 170–320°C (ramp 5°C min^{-1}), 320°C (10 min). The constituents of each fraction were identified by matching of their mass spectra fragmentation patterns with those stored in the

Wiley/NIST database and also with published data (Zhao *et al.* 2009; Ma *et al.* 2012). Retention index of each identified compound was calculated in relation to n-alkanes (Kovats 1965).

Antifungal activity of components partitioned from M1

Fraction M1 was dissolved in 1·8 ml of hexane and partitioned three times with 1·44 ml of acetonitrile. Two fractions were recovered: ALK which was composed of the acetonitrile volumes recovered, and LUPf consisting of the remaining hexatic phase. GC-MS analysis indicated that 92% of ALK was a mixture of phenolic lipids, while 96% of LUPf contained soley lupeol. These fractions were assayed by the microdilution method, in the same way as indicated previously, on both *F. verticillioides* and *F. graminearum*. A commercial source of ferulic acid (99% purity, Sigma, St. Louis, MO, USA) and lupeol (96% of purity, Sigma) was included as positive controls. Subsequently, the inhibitory effect of ALK, LUPf and commercial lupeol and ferulic acid on fumonisin and trichothecene accumulation was tested in liquid cultures. Strains of *F. verticillioides* were grown in a fumonisin-inducing medium (López-Errasquín *et al.* 2007) that contained malt extract (0·5 g l^{-1}), yeast extract (1 g l^{-1}), peptone (1 g l^{-1}), KH_2PO_4 (1 g l^{-1}), $MgSO_4{\cdot}7H_2O$ (0·3 g l^{-1}), KCl (0·3 g l^{-1}), $ZnSO_4{\cdot}7H_2O$ (0·05 g l^{-1}), $CuSO_4{\cdot}5H_2O$ (0·01 g l^{-1}) and fructose (20 g l^{-1}). Strains of *F. graminearum* were grown in trichothecene-inducing medium that contained yeast extract (1 g l^{-1}), peptone (1 g l^{-1}) and glucose (50 g l^{-1}). Aliquots of 8 ml of the media supplemented with ALK, LUPf, commercial lupeol or commercial ferulic acid dissolved in ethanol were placed in 125 ml Erlenmeyer flasks. Each flask was inoculated with 10^4 microconidia (*F. verticillioides*) or macroconidia (*F. graminearum*) in 1 ml of medium in triplicate. The percentage of solvent used (2·5% of the volume of the initial medium) did not affect fungal growth. Concentrations assayed ranged from 35 to 250 μg ml^{-1} of liquid medium. The flasks were then incubated in the darkness at 25°C in an orbital shaker at 150 rev min^{-1} for 7 days. Following incubation, cultures were centrifuged at 3000 ***g*** for 10 min. Supernatants were stored at −20°C before quantification of mycotoxins. Fungal biomass was measured by weighing the mycelial pellet after 48 h of freeze-drying. The initial pH of the culture medium was not affected by the supplementation, and the final pH conditions did not vary between treatments. Contents of mycotoxins were measured in the thawed supernatants with ELISA kits (Ridascreen Fast fumonisin or Fast DON, Biopharm, Germany), and expressed as μg g^{-1} of dry mycelial biomass. The obtained data were subjected to ANOVA and a subsequent post hoc Dunnet T3 test. All statistical analyses were conducted at a significance level of $P = 0{\cdot}05$.

Results

Effectiveness of plant extracts assessed using the disc-diffusion test

The fractions fCH_2Cl_2, fAcEt and fMeOH of the Anacardiaceae species were assayed by the disc-diffusion method on strains of *F. verticillioides* and *F. graminearum*. Fractions of the *Schinus* species had MIDs in the range of 500–800 μg dry matter per disc on the strains of *F. graminearum* (Table 1). Except for fCH_2Cl_2 and fAcEt of *S. areira*, the same fractions did not produce any visual inhibition on the mycelial growth of strains of *F. verticillioides* at all doses assayed (125–2000 μg dry matter per disc). The fCH_2Cl_2 and fAcEt of the *Schinopsis* species had DIMs of 125 μg dry matter per disc on the strains of *F. graminearum* and 250 μg dry matter per disc on those of *F. verticillioides*. They generated the largest zones of inhibition recorded for the strains of both *Fusarium* species, with larger zones for strains of *F. graminearum* than for *F. verticillioides*. The MIDs of fMeOH from the *Schinopsis* species were in the range of 500–1000 μg dry matter per disc on strains of *F. graminearum* and were not reached at any dose on strains of *F. verticillioides*.

Effectiveness of plant extracts using the microdilution method

The presence of antifungals in fCH_2Cl_2, fAcEt and fMeOH of the Anacardiaceae species was also evaluated by the microdilution method (Table 2). The MIC_{50S} obtained on strains of *F. graminearum* for fCH_2Cl_2 and fAcEt of the *Schinopsis* species (range 334–670 μg dry matter ml^{-1}) were lower than those obtained for *Schinus* species (range 195–228 μg dry matter ml^{-1}). The MIC_{50S} on strains of *F. verticillioides* were only obtained for fCH_2Cl_2 and fAcEt of *S. areira* (range 725–950 μg dry matter ml^{-1}) and the *Schinopsis* species (range 252–490 μg dry matter ml^{-1}). The fractions fMeOH of the Anacardiaceae species showed no antifungal activity on strains of both *F. verticillioides* and *F. graminearum*.

Antifungal activity visualized by bioautography

In order to characterize the active compounds responsible of the observed antifungal activity in fCH_2Cl_2 and fAcEt of the *Schinopsis* species, a TLC approach was pursued. Bioautographic and TLC analyses indicated that the bands with Rf = 0·65 (M1 and M2 obtained from

Table 1 Minimum inhibitory dose (MID) and its corresponding diameter of inhibition (ID) zone generated by fractions fCH_2Cl_2, fAcEt and fMeOH on *Fusarium graminearum* (GVI-II-3, LABI2) and *Fusarium verticillioides* (P364, LABI4)

	fCH_2Cl_2				fAcEt				fMeOH			
	GVI-II-3		LABI2		GVI-II-3		LABI2		GVI-II-3		LABI2	
Plant species	MID†	ID§	MID	ID	MID	ID	MID	ID	MID	ID	MID	ID
Schinus												
S. fasciculatus	NI*	NI	NI	NI	500	10 ± 2a	500	11 ± 3a	NI	NI	NI	NI
S. gracilipes	500	10 ± 2a‡	500	11 ± 3a	700	8 ± 1a	800	6 ± 2c	500	11 ± 3a	500	11 ± 3a
S. areira	500	10 ± 2a	500	11 ± 2a	700	8 ± 2a	800	6 ± 2c	700	8 ± 2a	700	6 ± 2c
Schinopsis												
S. haenkeana	125	15 ± 3b	125	16 ± 1b	125	15 ± 4b	125	16 ± 1b	1000	8 ± 2a	700	6 ± 1c
S. lorentzii	125	15 ± 2b	125	16 ± 2b	125	15 ± 3b	125	16 ± 2b	500	10 ± 2a	500	10 ± 2a

	P364		LABI4		P364		LABI4		P364		LABI4	
	MID	ID	MID	ID	MID	ID	MID	ID	MID	ID	MID	ID
Schinus												
S. fasciculatus	NI	NI	NI	NI	NI	NI	NI	NI	NI	NI	NI	NI
S. gracilipes	NI	NI	NI	NI	NI	NI	NI	NI	NI	NI	NI	NI
S. areira	1000	4 ± 1d	1000	4 ± 1d	500	4 ± 1d	500	4 ± 1d	NI	NI	NI	NI
Schinopsis												
S. haenkeana	250	6 ± 1c	250	6 ± 2c	250	4 ± 1c	250	4 ± 1c	NI	NI	NI	NI
S. lorentzii	250	6 ± 1c	250	6 ± 1c	250	4 ± 1c	250	4 ± 1c	NI	NI	NI	NI

*NI = Absence of growth inhibition.

†MID = minimum inhibitory dose expressed in μg dry mass per disc.

‡Means in the same column with the same letter are not significantly different (Dunnet T3 test, $P = 0{\cdot}05$).

§Data of inhibition diameters (ID) are expressed in millimetre and are reported as mean values ± standard deviation, based on two experiments where each treatment had three replications.

Table 2 Concentrations of fractions fCH_2Cl_2, fAcEt and fMeOH needed to inhibit 50% (MIC_{50}) the growth of *Fusarium graminearum* (GVI-II-3, LABI2) and *Fusarium verticillioides* (P364, LABI4)

	fCH_2Cl_2		fAcEt		fMeOH	
Plant species	GVI-II-3	LABI2	GVI-II-3	LABI2	GVI-II-3	LABI2
Schinus						
S. fasciculatus	376 (263–383)*	390 (370–410)	355 (347–368)	365 (350–378)	>1000	>1000
S. gracilipes	358 (325–386)	370 (350–380)	334 (313–362)	345 (330–358)	>1000	>1000
S. areira	686 (639–709)	670 (626–685)	499 (439–585)	510 (500–525)	>1000	>1000
Schinopsis						
S. haenkeana	212 (198–223)	200 (185–211)	228 (185–210)	215 (200–230)	>1000	>1000
S. lorentzii	215 (202–233)	195 (178–210)	221 (217–228)	220 (210–230)	>1000	>1000

	P364	LABI4	P364	LABI4	P364	LABI4
Schinus						
S. fasciculatus	>1000	>1000	>1000	>1000	>1000	>1000
S. gracilipes	>1000	>1000	>1000	>1000	>1000	>1000
S. areira	906 (822–952)	950 (867–1080)	751 (717–797)	725 (710–736)	>1000	>1000
Schinopsis						
S. haenkeana	374 (327–435)	350 (310–380)	400 (356–413)	490 (480–510)	>1000	>1000
S. lorentzii	252 (235–284)	295 (210–300)	435 (383–451)	395 (380–410)	>1000	>1000

*Values are presented as MIC_{50} followed by lower and upper limits of 95% confidence intervals within parenthesis. Values are expressed as dry matter per ml.

fCH_2Cl_2 extracts) and Rf = 0·80 (M3 and M4 obtained from fAcEt extracts) caused the observed antifungal activity (Fig. 2). The GC-MS indicated that all bands contained lupeol, stigmasterol and urushiol homologues with different length and degree of saturation in the side-chain bound to the catechol moiety at carbon 3 (Table 3). A 3-trialkenyl phenol and a 3-pentadecyl catechol were solely detected in bands isolated from leaf extracts of *S. lorentzii*, while two isomers of 3-heptadecenyl catechol were unique of M3 and M4 of *S. haenkeana*.

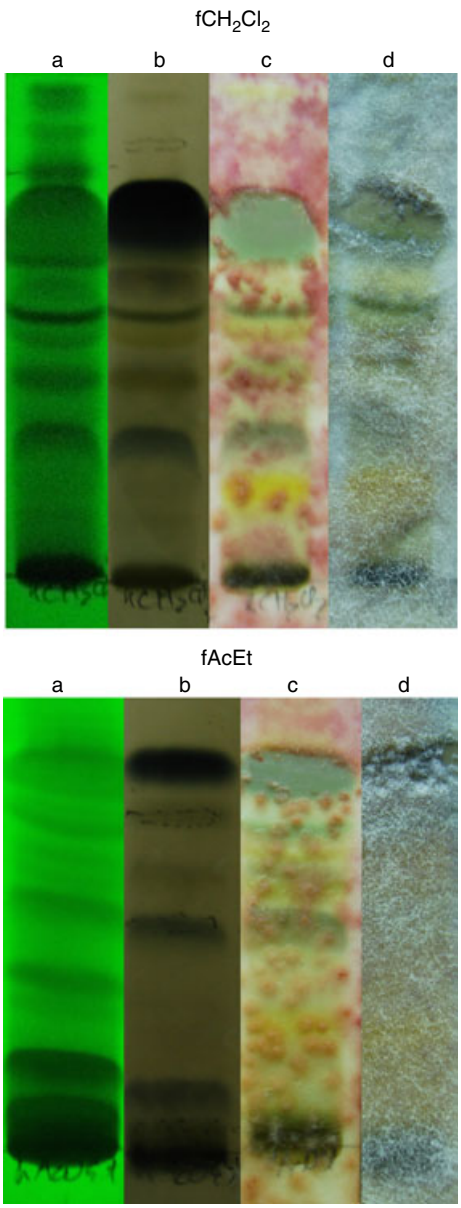

Figure 2 Silica gel chromatograms of fractions fCH_2Cl_2 and fAcEt representative of both *Schinopsis* species. They were developed in hexane: ethyl acetate 7 : 3 and 6·5 : 3·5 (v/v), respectively. Chromatograms were (a) observed under UV 254 nm, (b) revealed with 0·5% $FeCl_3$, and (c) bioautographed against *Fusarium graminearum* and (d) *Fusarium verticillioides*.

The MIC_{50} of M1 and its partitioned fractions enriched in lupeol (LUPf) and phenolic lipids (ALK) was determined by the microdilution method (Table 4). The three fractions showed values of MIC_{50} on strains of *F. graminearum* lower than those recorded for strains of *F. verticillioides*. ALK was the most inhibitory fraction on fungal growth. On average, it was 20 and 15 times more inhibitory than ferulic acid on *F. graminearum* and *F. verticillioides*, respectively. The MIC_{50} values of LUP and LUPf were lower than the MIC_{50} of ferulic acid on *F. graminearum* and higher than MIC_{50} of the same acid on *F. verticillioides*. The antifungal activity of ALK, LUP and LUPf was in one to three orders of magnitude lower than that of the commercial fungicides assayed.

Effect of the partitioned fractions, lupeol and ferulic acid on DON and fumonisin production

DON production was completely inhibited at concentrations higher than 38 $\mu g\ ml^{-1}$ by M1, ALK and LUPf and higher than 75 $\mu g\ ml^{-1}$ by LUP (Table 5). Fumonisin production was not detected at concentrations higher than 75 $\mu g\ ml^{-1}$ for LUPf, and 125 $\mu g\ ml^{-1}$ for ALK, M1 and LUP. Ferulic acid did not provide complete inhibition of fumonisins and DON biosynthesis at any concentration assayed.

Discussion

Disc-diffusion and microdilution assays indicated that the antifungal compounds in fractions fAcEt and fCH_2Cl_2 from leaves of *Schinopsis* were the most inhibitory on growth of both *F. graminearum* and *F. verticillioides*. In the performed assays, the differences in the degree of fungal inhibition were evident between both species assayed but not between strains belonging to the same *Fusarium* species. The bioautographic assays showed that only one band inhibited fungal growth in each of these fractions. The GC-MS analysis of each bioactive band indicated the presence of phenolic lipids comprising a mixture of 3-n-alk(en)ylcatechols, known as urushiols, and minor amounts of an alkenylphenol. These phenolic lipids are also predominantly present in the sap of other Anacardiaceae species such as the oriental lacquer trees (i.e. *Toxicodendron succedaneum* and *Toxicodendron vernicifluum*), American poisonous weeds (i.e. *Toxicodendron radicans* and *Toxicodendron pubescens*) and species including *Semecarpus*, *Lithrea* and *Metopium* genera (Rivero Cruz *et al.* 1997; Mohan *et al.* 2006; Zhao *et al.* 2009; Urzua *et al.* 2011; Ma *et al.* 2012). The molecular structure of

Table 3 GC-MS of antifungal bands identified by bioautography as M1, M3, M2 and M4

Retention Index*	Compound	Relative areas (%)† *Schinopsis lorentzii* M1	*Schinopsis lorentzii* M3	*Schinopsis haenkeana* M2	*Schinopsis haenkeana* M4	[M$^+$], m/z (%)	Major fragment ions, m/z (%)
1200	3-pentadecylcatechol	5·8	2·9	ND	ND	320 (27)	43 (18), 55 (11), 67 (6), 79 (3), 91 (5), 107 (9), 123 (100), 136 (7), 147 (3), 163 (1)
1201	3-heptadec-8,11,14-trienyl phenol	0·7	ND	ND	ND	326 (4)	41 (44), 55 (33), 67 (42), 79 (67), 95 (39), 107 (100), 123 (38), 135 (16), 147 (21), 161 (5), 193 (2), 205 (1), 320 (10)
1299	3-heptadec-8,11-dienyl catechol	17·6	16·1	6·2	3·1	344 (6)	41 (18), 55 (21), 67 (25), 81 (17), 95 (18), 109 (8), 123 (100), 136 (23), 149 (8), 163 (9)
1300	3-heptadec-8-enyl catechol	18·8	36·9	8·6	13·1	346 (1)	41 (19), 55 (19), 67 (25), 79 (26), 95 (22), 107 (9),
1301	Double bond position isomer of 3-heptadecenyl catechol (probably Δ^{11})	ND	ND	9·5	15·2	346 (11)	41 (16), 55 (22), 67 (7), 81 (4), 95 (5), 110 (3), 123 (100), 136 (25), 149 (6), 163 (6)
1302	Double bond position of 3-heptadecenyl catechol (probably Δ^{14})	ND	ND	16·6	27·2	346 (12)	41 (18), 55 (18), 69 (8), 83 (4), 106 (4), 123 (100), 136 (24), 149 (5), 163 (5)
1401	Stigmasterol	0·9	13·1	5·8	7·2	414 (33)	41 (44), 43 (100), 55 (67), 69 (31), 81 (43), 95 (42), 107 (46), 119 (32), 133 (30), 145 (40), 159 (29), 173 (13), 187 (8), 199 (10), 213 (22), 231 (12), 243 (1), 255 (15), 273 (12), 288 (2), 303 (20), 315 (1), 329 (20), 354 (4), 381 (16), 396 (15)
1402	alfa-amirine	ND	ND	ND	2·3	426 (3)	43 (28), 55 (32), 69 (30), 81 (24), 95 (26), 107 (20), 119 (19), 135 (20), 147 (13), 161 (8), 175 (8), 189 (16), 203 (51), 218 (100), 257 (2), 281 (3), 341 (1), 402 (2)
1500	Lupeol	55·7	22·9	44·2	25	426 (17)	41 (58), 43 (74), 55 (91), 69 (76), 81 (88), 95 (100), 107 (88), 121 (83), 135 (85), 147 (44), 161 (35), 175 (28), 189 (68), 203 (32), 207 (49), 218 (59), 234 (7), 257 (6), 272 (3), 315 (6), 370 (2), 411 (7)

*Retention index calculated in relation to n-alkanes (Kovats 1965).
†Values are means of $n = 2$.
ND, not detected.

Table 4 Minimum concentrations of fractions LUPf, ALK and M1 needed to inhibit 50% the mycelial growth (MIC_{50}) of the *Fusarium* strains. LUPf and ALK had 96% lupeol and 92% phenolic lipids, respectively. GVI-II-3 and LABI2: strains of *Fusarium graminearum*. P364 and LABI4: strains of *Fusarium verticillioides*. Commercial sources of ferulic acid (FA) and lupeol (LUP) were included as controls

	GVI-II-3	LABI2	P364	LABI4
ALK	31 (24–38)*	28 (23–34)	165 (143–170)	150 (133–169)
LUPf	76 (66–87)	66 (56–77)	2452 (1398–2800)	2562 (1299–3342)
LUP	85 (76–104)	90 (74–114)	2600 (2569–2650)	2670 (2645–2695)
M1	54 (49–58)	45 (39–54)	305 (260–337)	292 (265–326)
FA	600 (592–610)	587 (579–604)	1110 (1084–1180)	1200 (1180–1216)
Ketoconazol	7 (6–8)	7 (6–8)	3 (2·5–4)	4 (3–4·5)
Epoxyconazol	1 (0·5–1·5)	1 (0·5–1·2)	0·5 (0·2–0·8)	0·5 (0·1–0·6)
Pyraclostrobin	2 (1·5–3)	2 (1–3)	27 (24–29)	25 (23–28)

*Values are presented as MIC_{50} followed by lower and upper limits of 95% confidence intervals within parenthesis. Values are expressed as dry matter per ml.

phenolic lipids usually shows interspecific variations in Anacardiaceae specially in the length (15 or 17 carbon atoms) and degree of saturation of the side-chain joint to the aromatic moiety. For example, side chains of urushiols from *T. radicans* and *T. pubescens* have C15 and C17 carbon atoms, respectively, with 0, 1, 2 or 3 double

Table 5 Antimycotoxigenic activity of LUPf, ALK and M1 on *Fusarium* strains. LUPf and ALK had 96% lupeol and 92% phenolic lipids, respectively. GVI-II-3 and LABI2: strains of *Fusarium graminearum*. P364 and LABI4: strains of *Fusarium verticillioides*. Commercial sources of ferulic acid (FA) and lupeol (LUP) were included as controls

	GVI-II-3		LABI2		P364		LABI4	
	DON (μg mg^{-1}*)	Biomass (ref control)	DON (μg mg^{-1})	Biomass (ref control)	Fumonisins (μg mg^{-1})	Biomass (ref control)	Fumonisins (μg mg^{-1})	Biomass (ref control)
Growth Control	0·71 ± 0·02a	1·00 ± 0·01†	0·90 ± 0·03a	1·00 ± 0·02	4·20 ± 0·03a	1·00 ± 0·02	4·00 ± 0·02a	1·00 ± 0·01
ALK (μg ml^{-1})								
38	0·15 ± 0·01c‡	0·60 ± 0·02	0·16 ± 0·02c	0·60 ± 0·01	4·20 ± 0·02a	1·10 ± 0·01	3·90 ± 0·02a	1·00 ± 0·01
75	ND	0·50 ± 0·01	ND	0·50 ± 0·01	4·30 ± 0·02a	1·00 ± 0·01	3·80 ± 0·01a	1·00 ± 0·01
125	ND	0·33 ± 0·03	ND	0·33 ± 0·03	3·37 ± 0·04e	0·80 ± 0·02	3·20 ± 0·02d	0·70 ± 0·03
250	ND	0·17 ± 0·02	ND	0·17 ± 0·02	ND	0·60 ± 0·01	ND	0·55 ± 0·02
LUPf (μg ml^{-1})								
38	0·13 ± 0·01c	1·05 ± 0·01	0·10 ± 0·02c	1·00 ± 0·01	1·70 ± 0·02b	1·12 ± 0·01	1·05 ± 0·02b	1·10 ± 0·02
75	ND	1·11 ± 0·01	ND	0·98 ± 0·01	1·69 ± 0·04b	1·16 ± 0·01	1·10 ± 0·02b	1·15 ± 0·02
125	ND	1·00 ± 0·01	ND	1·00 ± 0·01	ND	1·00 ± 0·02	ND	1·00 ± 0·03
250	ND	0·95 ± 0·02	ND	0·95 ± 0·02	ND	0·85 ± 0·01	ND	0·80 ± 0·01
LUP (μg ml^{-1})								
38	0·25 ± 0·01b	1·00 ± 0·01	0·21 ± 0·01b	1·00 ± 0·01	1·00 ± 0·02c	1·00 ± 0·01	1·10 ± 0·02b	1·00 ± 0·01
75	0·19 ± 0·01c	1·00 ± 0·01	0·20 ± 0·02bc	1·00 ± 0·01	0·96 ± 0·03c	1·00 ± 0·03	1·00 ± 0·01b	1·00 ± 0·01
125	ND	1·00 ± 0·02	ND	0·95 ± 0·02	1·00 ± 0·02c	1·00 ± 0·02	1·20 ± 0·02b	1·00 ± 0·03
250	ND	0·85 ± 0·03	ND	0·85 ± 0·03	ND	1·00 ± 0·01	ND	0·95 ± 0·02
M1 (μg ml^{-1})								
38	0·10 ± 0·02c	0·60 ± 0·01	0·14 ± 0·01c	0·56 ± 0·01	0·70 ± 0·01f	1·00 ± 0·01	0·90 ± 0·01b	1·00 ± 0·01
75	ND	0·40 ± 0·02	ND	0·45 ± 0·02	0·66 ± 0·01f	1·00 ± 0·01	0·80 ± 0·02b	1·00 ± 0·01
125	ND	0·30 ± 0·01	ND	0·28 ± 0·01	0·38 ± 0·02 g	0·90 ± 0·02	0·34 ± 0·01 h	0·95 ± 0·02
250	ND	0·20 ± 0·02	ND	0·17 ± 0·02	ND	0·90 ± 0·01	ND	0·91 ± 0·01
FA (μg ml^{-1})								
38	0·25 ± 0·02b	1·00 ± 0·01	0·30 ± 0·01b	1·00 ± 0·01	3·00 ± 0·03a	1·00 ± 0·01	3·10 ± 0·02d	1·00 ± 0·01
75	0·17 ± 0·01c	1·00 ± 0·01	0·17 ± 0·01c	1·00 ± 0·01	2·95 ± 0·03a	1·00 ± 0·03	3·05 ± 0·03d	1·00 ± 0·03
125	0·14 ± 0·02c	0·90 ± 0·02	0·10 ± 0·01c	0·80 ± 0·02	2·80 ± 0·04a	0·94 ± 0·01	2·90 ± 0·03d	0·90 ± 0·01
250	0·10 ± 0·01c	0·80 ± 0·01	0·07 ± 0·02c	0·67 ± 0·01	2·24 ± 0·05 h	0·86 ± 0·01	2·30 ± 0·02f	0·80 ± 0·01

*μg of mycotoxin per mg of dry fungal biomass.

†60 mg of dry weight/Petri dish.

‡Means in the same column with the same letter are not significantly different (Dunnet T3 test, $P = 0·05$).

ND, Not detected.

Data are reported as mean values ± standard deviation, based on two experiments where each treatment had three replications.

bonds in the side chain (Hsu *et al.* 2013). In this regard, the *Schinopsis* species showed interspecific differences in saturation and length of the side chain as well as in the hydroxyl substitution of the aromatic ring. The impact of these findings on chemosystematics, however, requires the analyses of *Schinopsis* populations representative of the dispersion area of both species. As in the case with other phenolic lipids, the identified alk(en)ylcatechols and alkenylphenols might be phytoanticipins synthesized before attack of phytopathogenic organisms (Sampietro *et al.* 2013). They usually generate contact dermatitis and are likely responsible for the allergic reactions reported in rural workers exposed to the native *Schinopsis* trees (Luna 2012). This feature hampers the use of these compounds as preservatives in foods and feeds. Urushiol-like compounds have been identified as antifungal, cytotoxic, antitumoral, antifeedant, phytotoxic and antibiotic compounds (Zhao *et al.* 2009).

The antifungal bands isolated from fCH_2Cl_2 and fAcEt also contained important amounts of lupeol. This pentacyclic triterpene is often found in the surface waxes of several plants and is a common component of the human diet incorporated in edible plant organs such as the fruits of olive, mango, strawberry and grapes (Gallo and Sarachine 2009). 4Lupeol is known for its health promoting properties such as strong antioxidant, anti-inflammatory, anti-arthritic, antimutagenic, cardioprotective, cancer preventive and antimalarial activities in *in vitro* and *in vivo* systems (Nigam *et al.* 2007; Lira *et al.* 2008). It did not show any systemic toxicity effect in animals when tested at doses 40–200 mg kg^{-1} under various protocols in long- and short-term treatments (Saleem 2009). These

doses are several times higher than those showing fungal growth inhibition in this work and indicate that lupeol should be investigated as a food preservative.

Both fractions ALK and LUPf inhibited the fumonisin and the deoxynivalenol produced by *F. verticillioides* and *F. graminearum,* respectively. The antimycotoxigenic effect of both fractions was stronger than that observed for ferulic acid, a natural antimycotoxigenic and fungistatic compound found in high levels in the pericarp of cereal grains (Boutigny *et al.* 2009; Sampietro *et al.* 2012). The reasons for the observed inhibitions, however, could not be elucidated. Previous studies report that the increase in lipophilicity enhances the fungistatic (Guiraud *et al.* 1995), and antifumonisin activities of phenolic compounds (Dambolena *et al.* 2011) and other natural products (Picot *et al.* 2013). The high lipophilicity of a natural product favours its entry into the fungal cell where it might act on physiological modulators of mycotoxin biosynthesis or on signal transduction pathways upstream of the biosynthetic pathway (Holmes *et al.* 2008). As mycotoxin production is often linked to fungal stress, the antioxidant activity of natural products has been suggested to avoid the redox balance required for mycotoxin biosynthesis (Reverberi *et al.* 2010). The mycotoxin production in response to these and other molecular properties, however, depends on how they interact with several environmental factors such as substrate, water activity and temperature available for fungal growth. This situation might explain conflicting observations that avoid a general interpretation of the antimycotoxigenic activity of natural products (Ponts *et al.* 2011; Ferrochio *et al.* 2013). For this reason, further research is needed in order to characterize the effect of the identified metabolites of the *Schinopsis* species under different environmental conditions and substrates important for the control of *F. verticillioides* and *F. graminearum*.

Acknowledgements

Kris Audenaert thanks to the Research Fund of University College Ghent for a postdoctoral grant.

Conflict of Interest

Authors have no conflict of interests of any nature to state in this section.

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