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Antifungal Chalcones and New Caffeic Acid Esters from *Zuccagnia punctata* Acting against Soybean Infecting Fungi

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The crude methanolic extract of *Zuccagnia punctata* was active toward the fungal pathogens of soybean *Phomopsis longicolla* and *Colletotrichum truncatum*. Assay guided fractionation led to the isolation of two chalcones, one flavanone and a new caffeoyl ester derivative as the compounds responsible for the antifungal activity. Another new caffeoyl ester derivative was isolated from the antifungal chloroform extract but proved to be inactive against the soybean infecting fungi up to 50 μg/mL

KEYWORDS: Antifungal compounds; soybean; phytopathogenic fungi; *Zuccagnia punctata*

INTRODUCTION

Soybean (*Glycine max* L. Merr.) is a primary source of edible oil and proteins and an important component of nutritionally balanced meals. Unfortunately, it is usually attacked by fungal infections during cultivation, or post harvest (in transit or in storage), highly affecting its productivity (*1*). Seeds and infected harvest debris (*2*) are the main source of primary infections, and their damage level depends on environmental conditions (*3*) such as high relative humidity, dew, and temperatures above 25 °C.

Although there is an extensive list of available agricultural fungicides, the appearance of resistant strains and the water and field pollution when using long-lived fungicides have emerged as significant problems (*4*). New antifungals that overpass these disadvantages and are environmentally safe are still needed to control soybean diseases (*1*).

In the course of our ongoing project aimed at the detection of antifungal compounds from natural sources (*5*−*7*), we decided to study *Zuccagnia punctata* Cav. (Fabaceae, Caesalpinoideae, or Caesalpinieae), a monotypic species occurring in central and western Argentina (*8*, *9*), for its capacity of inhibiting the growth of soybean pathogenic fungi. This native shrub is known as "jarilla macho", "jarilla de la puna", "laca", or "pus-pus" and is currently used in traditional medicine as an antiseptic (*9*). Earlier studies showed that *Z. punctata* possesses antifungal properties against phytopathogenic fungi (*10*).

Regarding its chemical composition, flavanones, flavones, and chalcones have been previously isolated from its methanolic extract (*11*, *12*). Among them, 2′,4′-dihydroxy-3′-methoxychalcone showed antibacterial properties against *Staphylococcus aureus* and *Escherichia coli* (*13*).

We report here the isolation and identification of antifungal compounds of *Z. punctata* acting against fungi which were isolated from infected soybean plants, growing in the most important soybean-producer regions of Argentina.

MATERIAL AND METHODS

General Experimental Procedures. Melting points were determined using an Electrothermal apparatus and are uncorrected. NMR spectra were recorded at 400 MHz for ^{1}H and at 100 MHz for ^{13}C in the Fourier transform mode in $CDCl_3$ or MeOH-d_4 solutions in a Bruker spectrometer. Carbon chemical shifts are expressed in the δ scale using $CDCl_3$ as a reference signal at 76.9 ppm. Silica Gel 60 (Merck 230−400 mesh) was used for column chromatography, while TLC analysis was carried out in Silica Gel GF254 precoated plates. IR spectra were measured with a Bruker IFS 25 Spectrophotometer. Mass spectra were measured at 70 eV for electron impact (EIMS) in a Varian unit.

Plant Material. Leaves and twigs of *Zuccagnia punctata* Cav. were collected near San Antonio, Belgrano Department, San Luis Province, Argentina, in April 2001. The plant material was identified by Luis A. Del Vitto and Elisa Petenatti and a voucher specimen was deposited in the Herbarium of the National University of San Luis (Del Vitto and Petenatti #9230, UNSL).

Extraction and Isolation. The air-dried aerial parts (655 g) were ground and extracted repeatedly with EtOH at room temperature (3 ×

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800 mL, 24 h each). The extracts were combined, and the solvent was evaporated under reduced pressure at 40 °C, giving 168 g of a semisolid EtOH extract (25% w/w in terms of dry starting material).

A 40-g sample of EtOH extract were dissolved in 260 mL of EtOH/H_2O 78:22 and successively partitioned with *n*-Hexane (3 × 130 mL), $CHCl_3$ (3 × 130 mL) and *n*-BuOH (4 × 50 mL) to afford after concentration under reduced pressure 3.7 g of *n*-hexane extract, 14.57 g of $CHCl_3$ extract, and 1.94 g of *n*-BuOH extract.

A 1-g sample of the $CHCl_3$ extract was chromatographed on Silica Gel 60H and eluted successively with $CHCl_3$, $CHCl_3$/EtOAc, EtOAc, MeOH, MeOH/H_2O, and H_2O. Fractions were analyzed by TLC, with different mobile phases and using UV light or sulfuric *p*-anisaldehyde as revealing agents. Each fraction was concentrated to dryness in vacuo and assayed for antifungal activity. Repeated column chromatography of the active fractions led to the isolation of three known compounds (**1**, **2**, and **3**) and two new ones (**4** and **5**). Chalcones **1** (41.9 mg, 0.38% w/w in terms of dry starting material) and **2** (16.4 mg, 0.15% w/w in terms of dry starting material), and flavanone **3** (6.6 mg, 0.06% w/w in terms of dry starting material) were identified by comparison of their spectroscopic data with those reported in the literature (*14*).

1-Methyl-3-(4′-hydroxyphenyl)-propyl Caffeate (4). Yield: 19.3 mg (0.17% in terms of dry starting material). Yellowish solid. NMR data in the text. MS *m*/*z* (rel int. %): 328.131569 (6) (calcd for $C_{19}H_{20}O_5$, 328.131074), 286 (30), 272 (21), 180 (7), 166 (21), 164 (12), 148 (100), 133 (70), 107 (28). FT-IR (KBr, cm^{-1}): 3423, 3312, 1686, 1612, 1514, 1448, 1364, 1274, 1194. $[\alpha]^D$: −27.0 (c = 0.392%; MeOH).

1-Methyl-3-(3′, 4′-dihydroxyphenyl)-propyl Caffeate (5). Yield: 17.9 mg (0,16% in terms of dry starting material). Yellowish solid. NMR data in the text. MS *m*/*z* (rel int. %) 344.122719 (10) (calcd for $C_{19}H_{20}O_6$, 344.125989); 288 (10); 272 (11); 240 (8); 182 (9), 180 (20); 164 (100), 151 (36), 136 (25), 123 (54). FT-IR (KBr, cm^{-1}): 3387, 1677, 1603, 1518, 1446, 1364, 1282, 1189, 1114, 1060. $[\alpha]^D$: −3.65 (c = 0.246%; MeOH).

Microorganisms and Media. Fungi were isolated from soybean plants showing typical symptoms of fungal diseases during the 2000−2001 growing season, from different places of the Santa Fe Province (Bigand, Casilda, Classon, and Barrancas) and Buenos Aires Province (Pergamino, San Pedro), Argentina.

Carpels, stems, and seeds were superficially disinfected with NaClO 2% for one minute. Pathogens were isolated using the incubation test (*15*) in the following conditions: potato glucose agar 2% as the culture medium; 7 days at 25 ± 2 °C alternating 12 h periods under near-ultraviolet light with 12 h periods in the dark. The isolated pathogens were cultured on acidified potato glucose agar, with 1.6% glucose, 2% lactic acid, and on water-agar, with 2% agar at 25 ± 2 °C to promote fructification (*16*).

Fungi were characterized by the morphology of their colonies, fruiting bodies, and spores. Colonies and fruiting bodies were examined in a stereoscopic microscope (×40, CETI, Belgium) and spores were examined using a light microscope (×400, ×600, and ×1000, CETI, Belgium). Fungal identities were verified according to Barnett and Hunter (*17*), Domsch (*18*), and Nelson et al. (*19*), and named according to Rossman et al. (*20*) and Hawksworth et al. (*21*).

The collected pathogens were deposited in the Center of Mycological Reference (CEREMIC, (CE)). The following fungal isolates were obtained from soybean carpels and/or seeds: *Phomopsis longicolla* Hobbs (CE117), *Alternaria alternata* (Fr.) Keissler (CE172); *Fusarium equiseti* (Corda) Sacc (CE181), *Colletotrichum truncatum* (Schw.) Andrus and W. D.Moore (CE175). One isolate of *Sclerotium bataticola* Taub. (CE173) was obtained from stem. Four isolates of *Fusarium graminearum* (Schw.) (CE170, CE171, CE169, and CE135) were also evaluated.

Antifungal Assays. To carry out the antifungal evaluation with agar dilution assays, concentrations of extracts and pure compounds up to 1000 and 50 μg/mL respectively were incorporated into growth media according to reported procedures (*22, 23*). Extracts and pure compounds with MIC values > 1000 or >50 μg/mL respectively were considered inactive.

Spore suspensions were obtained according to reported procedures (*24*) and adjusted to 10^6 spores with colony forming ability/mL. Stock solutions of extracts in DMSO were diluted to give serial dilutions that were added to each medium resulting in concentrations ranging from 0.24 to 1000 μg/mL (or 0.20−50 μg/mL for pure compounds). The final concentration of DMSO in the assay did not exceed 2%. Using a micropipet, an inoculum of 5 μL of spore or mycelial suspension was added to each Sabouraud-dextrose agar test tube containing 500 μL media. The antifungal strobirulines Pyraclostrobin (MC Comet, BASF, Buenos Aires, Argentina) and Azoxystrobin (Amistar, Syngenta, Buenos Aires, Argentina), as well as the azoles methyl benzimidazol-2-ylcarbamate (Carbendazim, Agar Cross, Rosario, Argentina) and ketoconazole (Sigma, MO) were included in the assay as positive controls. Drug-free medium was used as a negative control. Plates were incubated for 48 or 72 h at 28 °C (according to the control fungus growth). MIC was defined as the lowest extract or compound concentration showing no visible fungal growth after incubation time.

Table 1. MIC Values of Different Extracts from *Zuccagnia punctata*

	extracts				controls			
test organism	EtOH	*n*-Hexane	$CHCl_3$	n-BuOH	ket[a]	pyr[b]	carb[c]	azo[d]
P. longicolla	100	250	62.5	>1000	0.5	0.01	0.01	0.05
A. alternata	500	1000	250	>1000	5	0.4	75	2.5
S. bataticola	250	750	250	>1000	50	2.5	0.25	2.5
F. equiseti	500	1000	250	>1000	50	12.5	0.75	12.5
F. graminearum[e]	100	125	>1000	>1000	40	0.05	0.75	0.25
F. graminearum[f]	100	125	>1000	>1000	40	0.05	0.4	0.4
F. graminearum[g]	100	125	>1000	>1000	40	1	0.75	5
F. graminearum[h]	100	250	>1000	>1000	40	20	1	7.5
C. truncatum	500	1000	250	>1000	1.5	0.04	0.01	0.05

[a] Ketoconazole. [b] Pyraclostrobin. [c] Carbendazim. [d] Azoxystrobin. [e] CE170. [f] CE171. [g] CE135. [h] CE169.

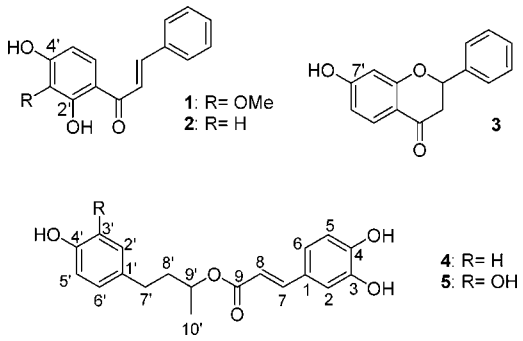

Figure 1. Compounds isolated from the chloroform extract of *Zuccagnia punctata*.

RESULTS AND DISCUSSION

The EtOH extract of aerial parts of *Z. punctata* showed activity against all of the fungi tested with MIC values between 100 and 500 μg/mL (**Table 1**). This extract was successively partitioned between *n*-hexane, $CHCl_3$, and *n*-BuOH, showing the chloroform extract as the lowest MIC value against the most relevant pathogenic fungus on soybean seed (**Table 1**).

Repeated bioassay-guided chromatographies of the chloroform extract led to the isolation of 2′,4′-dihydroxy-3′-methoxychalcone **1**, 2′,4′-dihydroxychalcone **2** and 7-hydroxyflavanone **3**. In addition, two new caffeic acid esters were isolated: 1-methyl-3-(4′-hydroxyphenyl)-propyl caffeate **4** and 1-methyl-3-(3′,4′-dihydroxyphenyl)-propyl caffeate **5** (**Figure 1**).

Compounds **1**−**3** were identified by their spectroscopic data, which are in agreement with those reported in the literature (*11*, *12*, *25*).

The ^1H and ^{13}C NMR data of compounds **4** and **5** are summarized in **Table 2**. The ^1H NMR spectrum of the new compound **4** showed two aromatic moieties, two *trans*-olefinic protons and an aliphatic carbon chain. Rings A and B presented a 1,2,4-trisubstituted and a 1,4-disubstituted patterns, respec-

Table 2. ^{1}H- and ^{13}C NMR Data of Compounds 4 and 5 (MeOH-d_4), 400 MHz for ^{1}H and 100 MHz for ^{13}C

	compounds			compounds	
H	4	5	C	4	5
			1	126.64 s	126.76 s
2	7.07 d (2)	6.95 d (1.9)	2	113.99 d	114.80 d
			3	144.85 s	145.87 s
			4	148.32 s	148.51 s
5	6.80 d (8.2)	6.73 d (8.1)	5	115.07 d	114.80 d
6	6.97 dd (8.2, 2)	6.82 dd (8.2; 1.9)	6	121.81 d	121.97 d
7	7.54 d (15.9)	7.48 d (15.9)	7	145.88 d	145.87 d
8	6.27 d (15.9)	6.18 d (15.9)	8	114.89 d	114.82 d
			9	167.94 s	167.94 s
			1′	132.55 s	133.67 s
2′	7.00 d (8.5)	6.59 d (1.9)	2′	129.31 d	115.54 d
3′	6.71 d (8.5)		3′	115.44 d	143.39 s
			4′	155.54 s	145.01 s
5′	6.71 d (8.5)	6.64 d (8.0)	5′	115.44 d	115.54 d
6′	7.00 d (8.5)	6.46 dd (8.0; 2.0)	6′	129.31 d	119.50 d
7′	2.59 m (2H)	2.49 m (2H)	7′	30.84 t	30.49 t
8′	1.96 m	1.95 m	8′	38.27 t	38.24 t
	1.83 m	1.85 m			
9′	4.96 m	4.91 m	9′	70.71 d	70.69 d
10′	1.29 d (6.3)	1.30 d (6.2)	10′	19.42 q	19.39 q

tively, pointing out a caffeoyl moiety. An AB system at δ 7.54 (d, $J = 15.9$ Hz) and δ 6.27 (d, $J = 15.9$ Hz) can be assigned to the caffeoyl unit, while a clear sequence indicates a $-CH_2-CH_2-CH-CH_3$ chain attached to the benzene ring. The *m* at δ 4.96 (1 H) due to a methine proton coupled with the *d* at δ 1.29 ($J = 6.3$ Hz, 3H), assignable to a CH_3 group, and the *m* at δ 2.59 (2 H), which in turn coupled with the methylene group (δ 1.96 m and δ 1.83 m) adjacent to the benzyl group. The ^{13}C NMR spectrum showed typical signals for a caffeoyl and a 4-(4-hydroxyphenyl)-2-butanol unit. In the HMBC spectrum, clear relationships were observed between the signals at δ 167.94 with 7.54, 6.27, and 4.96; δ 155.54 with 7.00 and 6.71; δ 132.68 with 7.00; and 70.71 with 2.54, 1.29, 1.96, and 1.83. The MS of **4** presented a molecular ion at *m*/*z* 328 and two fragments derived by a McLafferty rearrangement at *m*/*z* 148 and 180. The alcohol moiety was confirmed by the fragments at *m*/*z* 166 [$C_{10}H_{14}O_2$], 148 [$C_{10}H_{12}O$], 133 [C_9H_9O], and 107 [C_7H_7O], supporting the proposed structure.

The ^{1}H NMR spectrum of compound **5** was close of that of **4** differing only in an additional OH function in the benzene ring. The spectrum indicates two 1,3,4-trisubstituted aromatic moieties, which in addition to the signals for the olefinic protons and aliphatic side chain pointed out to a caffeoyl and a 4-(3,4-dihydroxyphenyl)-2-butanol moieties. The assignation of the NMR signals was confirmed by the HMBC spectra where clear relationships were observed between the s at δ 167.94 with 7.48, 6.18, and 4.91; δ 133.67 with 6.64, 2.49, 1.94 and 1.85; δ 30.49 with 6.46 and 6.59. The MS of **5** presented a molecular ion at *m*/*z* 344 and two fragments derived by a McLafferty rearrangement at *m*/*z* 164 and 180. The alcohol moiety was confirmed by the fragments at *m*/*z* 182 [$C_{10}H_{14}O_3$], 164 [$C_{10}H_{12}O_2$] and 123 [$C_7H_7O_2$] and is in agreement with the proposed structure.

The caffeic acid esters **4** and **5** are reported for the first time. The close related compounds 1-methyl-2-phenylethyl isoferulate and 1-methyl-3-(3′-methoxy-4′-hydroxyphenyl)-propyl caffeate have been isolated from *Onosma heterophylla* Griseb. (Boraginaceae) (*26*).

Table 3 shows the antifungal activities (MIC values in μg/mL) of compounds **1**−**5** isolated from *Z. punctata*, against all isolated fungi. Chalcones **1** and **2** have potent antifungal activities against all fungi tested with MIC values ranging from 6.25 and 3.12 to 50 μg/mL, respectively. 7-Hydroxyflavanone **3** and 1-methyl-3-(4′-hydroxyphenyl)-propyl caffeate **4** show very interesting activities only against *P. longicolla* (MIC = 6.25 μg/mL), while 1-methyl-3-(3′,4′-dihydroxyphenyl)-propyl caffeate **5** did not show any activity up to 50 μg/mL.

Table 3. MIC Values (μG/mL) of Compounds Isolated from the Chloroform Extract of *Zuccagnia punctata*

	compounds					controls			
test organism	1	2	3	4	5	ket[a]	pyr[b]	carb[c]	azo[d]
P. longicolla	6.25	3.12	6.25	6.25	>50	0.5	0.01	0.01	0.05
A. alternata	25	25	>50	>50	>50	5	0.4	75	2.5
S. bataticola	50	50	>50	>50	>50	50	2.5	0.25	2.5
F. equiseti	25	25	>50	>50	>50	50	12.5	0.75	12.5
F. graminearum[e]	25	25	>50	>50	>50	40	0.05	0.75	0.25
F. graminearum[f]	12.5	25	>50	>50	>50	40	0.05	0.4	0.4
F. graminearum[g]	12.5	25	>50	>50	>50	40	1	0.75	5
F. graminearum[h]	12.5	25	>50	>50	>50	40	20	1	7.5
C. truncatum	6.25	6.25	>50	>50	>50	1.5	0.04	0.01	0.05

[a] Ketoconazole. [b] Pyraclostrobin. [c] Carbendazim. [d] Azoxystrobin. [e] CE170. [f] CE171. [g] CE135. [h] CE169.

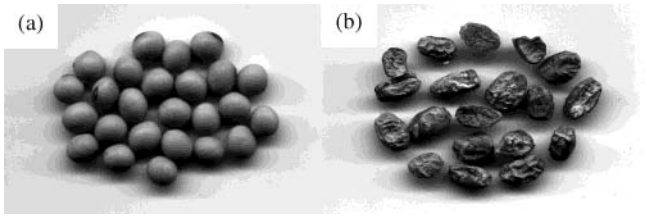

Figure 2. Soybean seeds decay caused by *Phomopsis longicolla*: (a) healthy seeds; (b) diseased seeds.

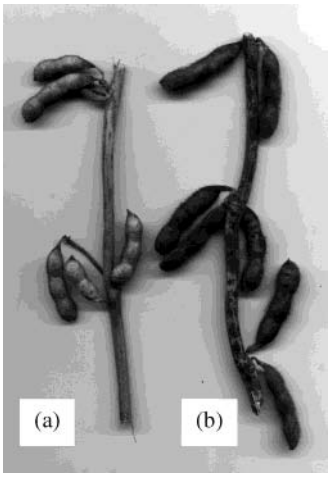

Figure 3. Soybean stem Anthracnose produced by *Colletotrichum truncatum*: (a) healthy stem; (b) diseased stem.

It is interesting to note that four of the five compounds isolated from the antifungal chloroform extract of *Z. punctata* displayed very good activities (MIC $\leq$ 6.25 μg/mL) against *P. longicolla*. This fungus is a primary agent of seed decay (**Figure 2**), a highly severe pathology that affects soybean seed quality and yield, and it is present in almost every region of soybean production in the world (*27*). In Argentina, this pathogen was isolated and quantified in carpels and seeds of industrial and green soybean (fresh consume), from different localities of core producing area (*28*, *29*). Infected seeds are shrivelled and cracked and appear white and chalky. When soybean matures in warm and wet weather and harvest is delayed, seed decay associated with pod and stem blight may also lower seed viability (*28*, *30*).

Chalcones **1** and **2** showed strong activities against *Colletotrichum truncatum* (MIC = 6.25 μg/mL).

C. truncatum is among the most common soybean pathogens. It is the causal agent of soybean anthracnose, a disease acquired mainly in the last growing step that affects stems and pods diminishing the number of seeds and their weight (**Figure 3**). *C. truncatum* and *P. longicolla* are the cause of the most serious soybean diseases due to their high incidence and persistence, causing reduction of seed quality and yields (*16*, *31*).

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