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Screening of Yucatecan Plant Extracts To Control *Colletotrichum gloeosporioides* and Isolation of a New Pimarene from *Acacia pennatula*

SERGIO R. PERAZA-SÁNCHEZ,*,† ERNESTO O. CHAN-CHE,§ AND ESAU RUIZ-SÁNCHEZ§

Centro de Investigación Científica de Yucatán (CICY), 43 #130 Chuburná, 97200 Mérida, Yucatán, Mexico, and Instituto Tecnológico Agropecuario #2 (ITA), Conkal, Yucatán, Mexico

Screening of seven Yucatecan plant extracts to look for fungicidal activity for the control of *Colletotrichum gloeosporioides* was carried out. Bioassay-directed purification of the root extract of one of the most active plants, *Acacia pennatula*, resulted in the isolation of the new compound 15,-16-dihydroxypimar-8(14)-en-3-one (**1**), which in the in vitro bioassay "agar dilution" was shown to have growth, sporulation, and germination inhibition activity. Nuclear magnetic resonance spectroscopic techniques were used to elucidate its structure.

KEYWORDS: Plant extracts; *Acacia pennatula*; *Petiveria alliacea*; *Piscidia piscipula*; *Pithecellobium albicans*; *Pithecellobium dulce*; *Tribulus cistoides*; *Viguiera dentata*; *Colletotrichum gloeosporioides*; pimarene; diterpene

INTRODUCTION

Colletotrichum gloeosporioides (Penz.) Penz. & Sacc. is the causal agent of anthracnose in diverse tropical fruit trees, which causes low yield and poor quality of the fruits (*1*−*4*), the damage being caused mainly after harvest (*5*). This pathogen has developed resistance to some synthetic chemical fungicides, principally those in the group of benzimidazoles and quinones (*6*). For that reason, and to find new strategies to combat this phytopathogen, we carried out the screening of seven Yucatecan plants with potential fungal activity (**Table 1**). In addition, chromatographic purification of the methanolic extract of one of the most active plants, *Acacia pennatula* (Schltdl. & Cham.) Benth., was undertaken.

A. pennatula, known in the Náhuatl language as *algarrobo* and *huizache* and as *ch'imay* and *k'ambk'i'lixché* in the Mayan language, belongs to the Fabaceae family, which comprises 700 genera and 18860 species, and to the Mimosoideae subfamily (*7*). The genus *Acacia* includes 500 species (*8*). *A. pennatula* grows in the states of Chihuahua, Durango, Jalisco, Oaxaca, Veracruz, and Yucatan in Mexico and is also found in countries such as El Salvador, Guatemala, Honduras, and Nicaragua, in Central America, and Colombia and Venezuela in South America. It is a tree 2−6 m high with branches containing thorns 2−4 mm long; its leaves are pubescent, bipinnately compound, 5−10 cm long, spirally disposed, with a dark green surface and a light green back; its flowers are arranged as yellow or orange heads; and its fruit is a reddish brown, flattened pod, 8−10 cm long (*9*). This plant is a good supply of tanning agents, firewood, and building materials for the construction of rural houses and fences. Its small leaves serve as soil fertilizer, and its pods are a favorite food source for free-ranging cattle (*10*). Medicinally, it is used to alleviate stomach- and toothache (9). Methanolic and ethanolic extracts of its bark present cytotoxic activity (*11*). No chemical compounds isolated from this species have been reported.

Table 1. Plant Species Studied and Their Parts Used To Obtain the Methanolic Extracts

species	common name	family	part used
Acacia pennatula (Schltdl. & Cham.) Benth.	Ch'imay	Fabaceae	roots
Petiveria alliacea L.	Payché	Phytolaccaceae	leaves
Piscidia piscipula (L.) Sarg.	Habín	Fabaceae	leaves
Pithecellobium albicans (Kunth) Benth.	Chukum	Fabaceae	leaves, roots
Pithecellobium dulce (Roxb.) Benth.	Ts'iuché	Fabaceae	leaves, roots
Viguiera dentata var. *helianthoides* (Kunth) S.F. Blake	Tajonal	Asteraceae	leaves
Tribulus cistoides L.	Abrojo	Zygophyllaceae	leaves, roots

Herein we report the isolation from *A. pennatula* of the new compound 15,16-dihydroxypimar-8(14)-en-3-one (**1**), which was shown to have growth, sporulation, and germination inhibition activity in the in vitro bioassay agar dilution against *C. gloeosporioides*. Nuclear magnetic resonance (NMR) spectroscopic techniques were used to elucidate its structure.

MATERIALS AND METHODS

General Experimental Procedures. Samples for infrared (IR) spectra were dissolved in $CHCl_3$ (Merck, Uvasol), and spectra were

* Author to whom correspondence should be addressed [telephone (999) 981-3923; fax (999) 981-3900; e-mail speraza@cicy.mx].
† Centro de Investigación Científica de Yucatán (CICY).
§ Instituto Tecnológico Agropecuario #2 (ITA).

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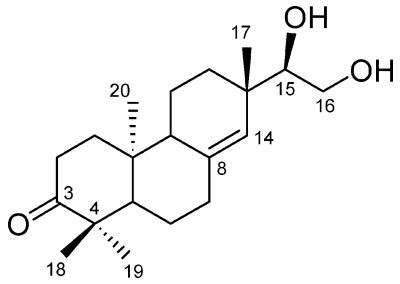

Figure 1. Molecular structure of compound **1**.

recorded as a film using a Nicolet Magna Protégé 460 FT-IR instrument. ^{1}H and ^{13}C NMR spectra were obtained on a Bruker AMX-400 (400 MHz), using $CDCl_3$ as solvent and TMS as a standard reference. Mass spectra were recorded at 70 eV on a high-resolution mass spectrometer (Autospec, Micromass Inc.). Gas chromatography−mass spectrometry (GC-MS) analyses were performed on a Hewlett-Packard 5890 gas chromatograph coupled to a Hewlett-Packard 5971A mass selective detector (Ultra 2 column: cross-linked 5% Ph Me silicone, 25 m × 0.32 mm × 0.52 mm film thickness; oven temperature = 290 °C). Analytical thin-layer chromatography (TLC) was carried out on precoated silica gel aluminum plates (Merck, 60 F_{254}, 0.2 mm thickness). TLC plates were analyzed under UV light using a viewing cabinet (Spectroline, model CX-20) and by spraying a solution prepared with 20 g of phosphomolybdic acid ($MoO_3H_3PO_4 \cdot XH_2O$) and 2.5 g of ceric sulfate in 500 mL of 5% aqueous H_2SO_4. Purifications using vacuum layer chromatography (VLC) were run using TLC silica gel 60 F_{254} (Merck) and a mixture of hexane/$CHCl_3$/acetone as the solvent system, increasing the polarity to elute the fractions. Open-column chromatography was performed using silica gel 60 (0.063−0.200 mm, Merck) packed in a 2.0 cm diameter column, eluting fractions with a mixture of hexane/$CHCl_3$/acetone (55:20:25).

Plant Material. Samples of plant material were collected in Telchac Puerto and Yokdzonot, Yucatan, in October 2001. The plant material was authenticated by qualified personnel at the Unidad de Recursos Naturales at Centro de Investigación Científica de Yucatán (CICY), and voucher specimens are deposited at CICY's herbarium. The plant material was air-dried for 3 days, then dried in an oven at 50 °C for 5 more days, and, after that, finely ground (**Table 1**). A re-collection of *A. pennatula* roots was made at 3 km from Komchén, Yucatan, in June 2002. A voucher specimen (no. 2208) was deposited at CICY's herbarium. This material was also dried and ground as described before, yielding 1.75 kg.

Plant Extract Preparation. For preliminary testing, samples of 10 g of dried material of each plant part were extracted consecutively three times with 100 mL of MeOH. The methanolic extracts were filtered, combined, and dried in a rotary evaporator to give oily solids, each weighting ∼10% of the initial plant material. Extracts were then tested at 2.0 mg/mL in the agar dilution antifungal bioassay.

Extraction and Isolation of Antifungal Compound. Dried and ground roots (1.75 kg) of *A. pennatula* were extracted with MeOH (5.0 L) three times at room temperature until 116 g of a crude extract (1A) was obtained. This extract was dissolved in a mixture of MeOH/H_2O (1:3, 1.2 L) and submitted to a chromatographic partitioning three times using consecutively hexane, chloroform, and ethyl acetate (1:1) to obtain, respectively, low (2A, 30 g), medium (2B, 28 g), medium-high (2C, 3.0 g), and high (MeOH/H_2O, 1 g/50 mL) polar fractions. Each fraction, previously dissolved in dimethyl sulfoxide (DMSO), was tested to identify its antifungal activity. Active fraction 2A was purified by means of a VLC, using hexane/$CHCl_3$/acetone (199.4:4.0:0.2) as the solvent system and then mixtures of increasing polarity, obtaining 16 fractions (3A−3O) that were also tested for antifungal activity. Active fraction 3M was further purified by means of a gravity column using hexane/$CHCl_3$/acetone (55:20:25) as the solvent system to obtain 11 fractions (4A−4K). Bioassay results showed fraction 4F to be the most active of all the fractions tested, which, after analysis by gas chromatography, was shown to be constituted of a sole compound (**1**, 22 mg). The molecular structure of compound **1** (**Figure 1**) was elucidated by means of mass, IR, and 1D and 2D NMR spectroscopic techniques as the new diterpene 15,16-dihydroxypimar-8(14)-en-3-one.

Table 2. ^{13}C and ^{1}H NMR Data for **1**[a]

C	δ (^{13}C)	δ (^{1}H)	HMBC[b]
1	38.1 t	1.51 m (H-1α)	
		2.01 ddd (J = 3.2, 5.7, 13.3 Hz, H-1β)	3
2	35.2 *t*	2.30 ddd (J = 3.2, 4.0, 14.8 Hz, H-2α)	3
		2.66 ddd (J = 5.7, 14.6, 14.8 Hz, H-2β)	1, 3, 10
3	217.3 s		
4	48.3 s		
5	55.6 d	1.48 m	
6	23.4 t	1.53 m (H-6α)	
		1.53 m (H-6β)	
7	35.8 t	2.11 m (H-7α)	
		2.36 m (H-7β)	
8	138.6 s		
9	50.2 d	1.79 br t (7.9)	
10	38.2 s		
11	18.9 t	1.51 m (H-11β)	
		1.67 m (H-11α)	
12	29.4 t	1.33 m (H-12β)	
		1.51 m (H-12α)	
13	38.5 s		
14	128.5 d	5.31 br s	7, 9, 12, 13
15	79.6 d	3.46 dd (J = 2.1, 9.1 Hz)	16
16	63.1 t	3.52 dd (J = 9.1, 10.6 Hz, H-16β)	15
		3.75 dd (J = 2.1, 10.6 Hz, H-16α)	
17	23.2 q	1.00 s	12, 15
18	26.1 q	1.09 s	4, 5, 19
19	22.8 q	1.07 s	4, 5, 18
20	14.9 q	0.99 s	5, 9, 10

[a] Run in $CDCl_3$, 100 MHz for ^{13}C and 400 MHz for ^{1}H NMR; chemical shifts are shown with multiplicities; J values are shown in parentheses. [b] Protons showing long-range correlation with indicated carbon.

*15,16-Dihydroxipimar-8(14)-en-3-one (**1**):* dark yellow wax (22 mg), not visible under UV light; R_f 0.32 [hexane/$CHCl_3$/acetone (55:20:25)]; IR (film) ν_{max} 3395, 2934, 2868, 1700, 1465, 1383, 1029, 871 cm^{-1}; ^{1}H and ^{13}C NMR (see **Table 2**); LREIMS, *m*/*z* 320 $[M]^+$ (1), 289 (1), 271 (2), 259 (100), 241 (7), 229 (2), 217 (3), 199 (2), 185 (2), 173 (3), 161 (3), 149 (5), 135 (7), 121 (35), 107 (43), 95 (20), 81 (25), 67 (7), 55 (8), 43 (10), 31 (1); HREIMS, *m*/*z* 259.2062 $[M - C_2H_5O_2]^+$ (calcd for $C_{18}H_{27}O$, 259.2055).

***C. gloeosporioides* Isolation.** *C. gloeosporioides* was isolated from infected papaya fruits (*Carica papaya* L.) according to a procedure published in the literature (*12*). Fruits showing lesions and characteristic symptoms of fungal infection were collected from commercial orchards located at Conkal, Yucatan. These fruits were washed with tap water, and small portions (0.5 cm^2) of contaminated epidermis were cut off and then disinfected with a 1% solution of sodium hypochlorite (trade name Cloralex) for 1 min. After disinfection, tissue portions were washed thoroughly three times with distilled water to eliminate chloride residues. Each portion cut was placed in Petri dishes (100 × 15 mm) containing PDA medium (trade name Bioxon), and the dishes were stored in the dark in an oven at 28 °C for 5−8 days until fungal growth was observed. Fungus structure (conidia and mycelia) were observed with an optical microscope (Leica Gallen III), and identification was according to published taxonomic key (*13*). Once *C. gloeosporioides* was isolated, an aqueous solution containing 2500 spores/mL was prepared from the fungal growth to purify it, and by means of a capillary tube, spores were transferred to the center of another Petri dish containing PDA medium, which was incubated in the dark for 8 days at 28 °C.

In Vitro Antifungal Assay. Extracts, fractions, and pure compound **1** were evaluated in the in vitro agar dilution bioassay to test the inhibition activity. Plant extracts (40 mg) were dissolved individually in DMSO (100 μL), and an aliquot (25 μL) was added to 5.0 mL of sterilized PDA medium to obtain a final concentration of 2 mg/mL. Once homogenized, this solution was allowed to gel in a Petri dish (50 × 15 mm). The maximum concentration of DMSO was 0.5%. PDA disks of 1.0 cm in diameter cut from the edge of the Petri dish by means of a hollow punch and containing 8-day-old pure culture of *C.*

Table 3. Effect of Various Plant Extracts on Mycelial Growth of *C. gloeosporioides* after 5 Days of Incubation at 28 °C[a]

plant species	mycelial growth[b] (mm)	% inhibition[c]
Acacia pennatula, roots	7.3 cd (2.49)[d]	54.4
Petiveria alliacea, leaves	15.0 ab (0.00)	6.2
Piscidia piscipula, leaves	7.0 cd (2.16)	56.2
Pithecellobium albicans, leaves	15.3 ab (0.94)	4.4
Pithecellobium albicans, roots	12.3 bc (0.47)	23.1
Pithecellobium dulce, leaves	3.0 de (1.41)	81.2
Pithecellobium dulce, roots	12.6 bc (2.62)	21.2
Tribulus cistoides, leaves	12.3 bc (1.24)	23.1
Tribulus cistoides, roots	12.3 bc (3.09)	23.1
Viguiera dentata, leaves	9.0 c (1.41)	43.7
positive control (Benlate)[e]	0.0 e (0.00)	
negative control (DMSO)	16.0 ab (0.00)	

[a] Average of three experiments. [b] Means followed by the same letter are not significantly different according to Tukey's multiple test ($p < 0.05$). [c] Micelial inhibition based on inhibition of radial growth compared to the negative control (DMSO). [d] Standard deviation in parentheses. [e] Concentration = 0.4 mg/mL.

gloeosporioides (1.65×10^6 conidia/mL) were transferred to the center of a Petri dish containing an extract and incubated at 28 °C for 5 days in the dark. Benomyl (trade name Benlate 50 PH) dissolved in water (0.4 mg/mL) was used as a positive control. Mycelial growth inhibition, expressed as percent inhibition, was evaluated by measuring the radial colony growing in each Petri dish every 24 h for 5 days. Inhibition of sporulation was determined as follows: spore suspensions were prepared from Petri dishes (treatments and controls) with 8-day-old colonies by transferring a PDA disk of 1.0 cm in diameter to a test tube containing 2.0 mL of distilled water. From this suspension, an aliquot of 20 μL was taken, and the spores were counted using a Neubauer hemocytometer (Boeco) under the microscope. Finally, to evaluate the inhibition of spore germination, an aliquot (10 μL) of a spore suspension containing 1×10^5 conidia/mL was added to a Petri dish containing a sample of the isolated pure compound (5 mg/25 μL of DMSO) dissolved in PDA (1.0 mg/mL) and covered with a cover slide. Visual inspection under the microscope (40×) of 50 conidia was carried out every hour to observe germinal tube elongation (*3*).

RESULTS AND DISCUSSION

Plant material for this study was chosen on the basis of previous reports in the literature in which these genera or species were regarded as having antifungal activity (*14*, *15*). When all 10 methanolic extracts of the seven plant species were evaluated in the in vitro agar dilution bioassay against *C. gloeosporioides*, it was observed that the extract of leaves of *Pithecellobium dulce* was the one which showed the strongest effect toward mycelial growth (81.2% inhibition), followed by *Piscidia piscipula* leaves (56.2%) and *Acacia pennatula* roots (54.4%), whereas the rest of the plant species evaluated showed an inhibition activity of <44% compared to the negative control (DMSO) (**Table 3**). Results observed for *P. dulce* were in agreement with those reported by Bautista-Baños et al. (*16*), who found that leaf extracts of this species were very effective in controlling *Fusarium* sp., *Alternaria* sp., *Rhizopus* sp., and *Pestalotiopsis* sp. In another study, Barrera-Necha et al. (*17*) reported that seed, leaf, and fruit powders of *P. dulce* showed some inhibitory effect when tested against *C. gloeosporioides* at concentrations of 0.5, 2, and 5.0 mg/mL, suggesting that this species possesses antifungal activity. With regard to *Acacia farnesiana* (L.) Willd., Montes-Belmont et al. (*18*) reported that fruit extracts of this species showed 76% growth inhibition of *Aspergillus flavus* Link. at a concentration of 20 mg/mL.

Even though *P. dulce* roots and *P. piscipula* leaves were the extracts with the greatest effect, we chose to study *A. pennatula* roots due to the lack of information in the literature on its chemical constituents. *A. pennatula* root extract was submitted to a bioassay-directed chromatographic purification as described under Materials and Methods, resulting in the isolation of the active metabolite **1**. Compound **1** showed a mycelial growth inhibition of 80% at 1.0 mg/mL compared to the negative control. Previous papers have shown that natural metabolites isolated from plants have great activities against *C. gloeosporioides*; for example, a quinolone alkaloid derivative isolated from leaves of *Ruta graveolens* L. is reported as causing 67.7% growth inhibition of this fungus at a concentration of 36.3 μg/mL (*19*), and another publication reports that capillarin, a compound isolated from aerial parts of *Artemisia dracunculus* L., inhibited 30% the growth of *C. gloeosporioides* at 6.0 μg/mL (*20*). In comparison, in our study the observed activity for compound **1** can be regarded as moderate. Compound **1** also inhibited spore production by 76% at 1 mg/mL, the spore density being 3.8×10^5 conidia/mL compared to the negative control, which was 1.6×10^6 conidia/mL, whereas the positive control (benomyl) totally inhibited sporulation. Complete inhibition of spore germination was observed for compound **1** and the positive control when tested at 1 mg/mL for a period of 10 h, in which the negative control showed 100% germination (**Table 4**). In this regard, Domergue et al. (*21*) have reported that compound *Z,Z,E*-1-acetoxy-2-hydroxy-4-oxoheneicosa-5,12,15-triene, isolated from fruits of avocado (*Persea americana* L.), inhibited 50% the germination of *C. gloeosporioides* at 0.5 mg/mL. The activity of this compound was determined by measuring the percent of spore germination and germ tube elongation, the latter considered to be inhibited when its length was less than twice the length of the spore (*22*). Other findings have already pointed out the antifungal activity of pimarane diterpenes; for example, Evidente et al. (*23*) found that three compounds termed sphaeropsidins A, B, and C had antimicrobial activity against some pathogenic fungi, particularly against *Seiridium cardinale* and *Seiridium cupressi*, two microorganisms that cause canker to Italian cypress. Similarly, Dettrakul et al. (*24*) reported a new pimarane diterpene termed diaporthein B, which had strong antimycobacterial activity with the minimum inhibitory concentration value of 3.1 μg/mL.

Table 4. Effect of Compound **1** at 1.0 mg/mL on in Vitro Development of *C. gloeosporioides* at 28 °C[a]

	mycelial growth		sporulation		
	mm	inhibition[b] (%)	conidia/mL[c]	inhibition (%)	germination inhibition[d] (%)
compound **1**	4.0 b[e] (0.81)[f]	80.0 b	3.8×10^5 b (3.89×10^4)	76.0 b	100.0
Benlate[g]	0.0 c (0.00)	100.0 a	0.0 c (0.00)	100.0 a	100.0
negative control	20.0 a (0.00)	0.0 c	1.6×10^6 a (3.67×10^4)	0.0 c	0.0

[a] Average of three experiments. [b] Mycelial inhibition based on inhibition of radial growth compared to control. [c] Number of conidia harvested from a single Petri dish. [d] Observed at 10 h. [e] Means followed by the same letter are not significantly different according to Tukey's multiple test ($p < 0.05$). [f] Standard deviation in parentheses. [g] Concentration = 0.4 mg/mL.

Compound **1** was obtained as a dark yellow wax. When run on a TLC with a mixture of hexane/$CHCl_3$/acetone (55:20:25), **1** was shown to be a sole spot not visible under UV light, but visible after spraying with a phosphomolybdic acid solution (R_f = 0.32). Furthermore, the purity of this isolate was also proven by obtaining a gas chromatogram in which only one peak was observed at t_R = 15.60 min. Analysis of the IR spectrum of **1** showed strong bands due to the presence of hydroxyl (3395 cm^{-1}) and carbonyl (1700 cm^{-1}) groups in the molecule, functionalities that were also confirmed by detailed examination of the ^{13}C NMR spectrum, which showed signals at δ 63.1

(−CH_2OH), 79.6 (−CHOH), and 217.3 (C═O). Additional inspection of this spectrum demonstrated the presence of four tertiary methyl groups at δ 14.9 (C-20), 22.8 (C-19), 23.2 (C-17), and 26.1 (C-18), and two olefinic carbons (δ 138.6, 128.5). Furthermore, the ^{13}C NMR and DEPT 135 experiment exhibited the existence of seven methylenes and three methines, one of which as an oxymethine. Accounting for the number of carbons present in the ^{13}C NMR spectrum and inspection of the HREIMS, compound **1** was assigned the formula $C_{20}H_{32}O_3$. The base peak at *m*/*z* 259 in the mass spectrum was consistent with the loss of the fragment −$CHOHCH_2OH$, corresponding to the side chain. Finally, the chemical shift values of the olefinic carbons (C-8 and C-14), along with the data presented above, suggested a pimar-8(14)-ene skeleton. The stereochemistry at C-15 was inferred by comparison of the corresponding ^1H and ^{13}C NMR shift values with those reported in the literature for other pimaranes (*25*−*28*). Therefore, the structure of **1** was deduced as the new compound 15,16-dihydroxypimar-8(14)-en-3-one.

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