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The Antibacterial Metabolites and Proacacipetalin from *Acacia cochliacantha*

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Abstract. Chromatographic purification of the antibacterial hexane, ethyl acetate and methanol extracts from the aerial parts of *Acacia cochliacantha* provided twelve known antibacterial compounds: β-sitosterol, stigmasterol, β-sitosterol 3-*O*-β-D-glucoside, stigmasterol 3-*O*-β-D-glucoside, lupenone, taraxerone, apigenin, luteolin, quercetin, gallic acid, methyl gallate and salicylic acid whose MIC values were determined. Additionally, proacacipetalin, squalene, (+)-pinitol, and palmitic, linoleic, oleic, stearic and myristic acids were isolated. These substances were fully characterized by 1D and 2D NMR spectroscopy as well as by their physical properties. This is the first time that stigmasterol 3-*O*-β-D-glucoside and taraxerone are isolated from *Acacia*.
Key words: *Acacia cochliacantha*, antibacterial activity, triterpene glucosides, triterpenes, flavonoids, proacacipetalin.

Resumen. La separación cromatográfica de los extractos antibacterianos de hexano, acetato de etilo y metanol de la parte aérea de *Acacia cochliacantha* condujo a la obtención de doce compuestos antibacterianos conocidos: β-sitosterol, estigmasterol, 3-*O*-β-D-glucósido de β-sitosterol, 3-*O*-β-D-glucósido de estigmasterol, lupenona, taraxerona, apigenina, luteolina, quercetina, ácido gálico, galato de metilo y ácido salicílico cuyos valores de MIC fueron determinados. Adicionalmente, se aislaron proacacipetalin, escualeno, (+)-pinitol y los ácidos palmítico, linoleico, oleico, esteárico y mirístico. Estos compuestos se caracterizaron completamente mediante espectroscopía de RMN en 1D y 2D, así como por sus propiedades físicas. Esta es la primera vez que el 3-*O*-β-D-glucósido de estigmasterol y la taraxerona se aíslan de *Acacia*.
Palabras clave: *Acacia cochliacantha*, actividad antibacteriana, glicósidos triterpénicos, triterpenos, flavonoides, proacacipetalin.

Introduction

The genus *Acacia* is widely distributed in México with approximately 70 species, 30 of which are endemic of the country [1]. These plants grow abundantly in the states of Jalisco, Michoacán, Guerrero and Oaxaca, where they are common in mountains and plains. Currently, they are considered as weeds and they have been attacked with herbicides in order to clean crop fields. The Mexican species of *Acacia* have received little scientific attention concerning their biological properties and chemical composition. To the best of our knowledge, only *A. farnesiana* [2], *A. cedilloi* [3] and *A. gaumeri* [3] have been chemically explored. Interestingly, a qualitative study in five mexican species of *Acacia* showed the presence of cyanogenic glycosides [4]. On the other hand, the chemical and biological studies carried out in other *Acacia* species in the world (mainly Australian and African species) have revealed that this genus is an important producer of secondary metabolites with significant biological activity such as antitumor, anti-inflammatory, antibacterial and anti-parasitic [5-8]. Chemical studies have showed that the structures of the main active principles are triterpenoid saponins, flavonoids and tannins. In addition, a number of alkaloids, cyanogenic glycosides, cyclitols, fatty acids, fluoroacetate, gums and non-protein amino acids are also present [9].

Acacia species have had a long history of medicinal use in the treatment of diarrhea, urinary infections, throat inflammation, gastritis, tuberculosis and headaches [2, 10]. This paper describes the principal antibacterial metabolites of *A. cochliacantha* Humb. & Bonpl. ex Willd. (Leguminosae) as well as their main cyanogenic glycoside, proacacipetalin.

Results and discussion

The dry aerial parts of *A. cochliacantha* were successively extracted with hexane, ethyl acetate and methanol by maceration. These extracts showed relevant antibacterial activity against Gram positive and Gram negative bacteria; the ethyl acetate and hexane residues of the flowers and leaves presented the best activity. Standard column chromatographic separation over silica gel of the hexane, ethyl acetate and methanol extracts allowed the isolation of twelve known antibacterial metabolites: β-sitosterol [11], stigmasterol [11], β-sitosterol 3-*O*-β-D-glucopiranoside [11-13], stigmasterol 3-*O*-β-D-glucopiranoside [11,12], lupenone (Fig. 1) [14], taraxerone (Fig. 1) [15], apigenin [16], luteolin [16], quercetin [16], gallic acid [17], methyl gallate [17] and salicylic acid [18]. The minimal inhibitory concentration (MIC) values of all these compounds were determined (Table 1). Surprisingly, the highest antibacterial activity was presented by taraxerone, which together with stigmasterol 3-*O*-β-D-glucopiranoside were isolated from *Acacia* for the first time. The triterpene lupenone has been isolated in some *Acacia* species [3, 19]. In addition to these antibacterial substances, proacacipetalin (Fig. 1), squalene [20], (+)-pinitol [21] and palmitic, linoleic, oleic, stearic and

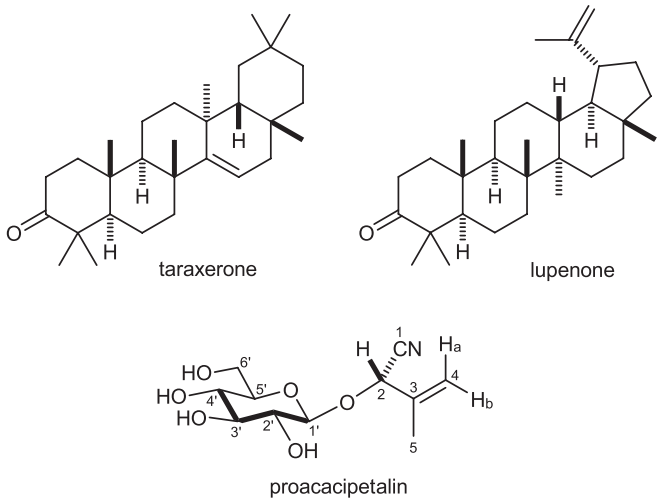

Fig. 1. The structures of taraxerone, lupenone and proacacipetalin isolated from *Acacia cochliacantha*.

myristic acids were obtained, although none of them presented antibacterial activity.

Proacacipetalin, incorrectly designated as acacipetalin in the literature [22], has been described as the principal cyanogenic glycoside in mexican species of *Acacia*, including *A. cochliacantha* [4, 23]. Despite that the structure of this cyanogenic glycoside has been revised [22], its absolute configuration has not been unambiguously established, both *R* [24] and *S* [22] configurations for the aglycone moiety have been proposed based on comparison with the NMR data of substances of known stereochemistry. Although NMR data of proacaciptelin in D_2O [22] and CD_3OD [25] have been reported, the ^1H signals for the glucose moiety remain ambiguous. To avoid these ambiguities, in this work we present two-dimensional NMR spectroscopy measurements, including COSY and HETCOR experiments in D_2O, to establish the correct ^1H and ^{13}C NMR assignment of the compound found in *A. cochliacantha.* The results confirm that this compound is the same as the one previously described as proacacipetalin [22].

The metabolites isolated from *A. cochliacantha* do not show antibacterial activity sufficiently potent to be used exclusively for the control of specific bacteria, but their antibacterial activity is broad enough so they can be used as cosmetic preservatives.

Experimental

General experimental procedures. IR spectra were measured in $CHCl_3$ on a Perkin Elmer 2000 FT-IR spectrophotometer. Optical rotations were determined in $CHCl_3$ and MeOH on a Perkin Elmer 341 polarimeter. NMR measurements were performed at 400 MHz for ^1H and 100 MHz for ^{13}C on a Jeol Eclipse 400 spectrometer from $CDCl_3$, D_2O, DMSO-d_6 or piridine-d_5 solutions. Mass spectra were recorded at 70 eV on a Hewlett Packard 5890 Series II spectrometer. Column chromatography was carried out on Merck silica gel 60 (230-400 mesh ASTM).

Plant material. Whole plants were collected in the municipality of Tepalcatepec, in Michoacán state, México, during June 2004. A voucher specimen (J. M. Torres Valencia 57) is preserved in the Herbarium of the Biological Research Center,

Table 1. Minimum inhibitory concentration (MIC) values of the antibacterial metabolites from *A. cochliacantha*.

Compound	MIC values (mg mL^{-1})							
	S. aureus	*B. subtilis*	*E. faecium*	*L. plantarum*	*E. coli*	*S. typhimurium*	*K. pneumoniae*	*Ps. aeruginosa*
β-sitosterol + stigmasterol	16.5	—	8.3	—	—	—	—	—
β-sitosterol 3-*O*-β-D-glucopiranose + stigmasterol 3-*O*-β-D-glucopiranose	10.5	—	21.0	10.5	—	—	—	—
lupenone	2.8	1.4	5.6	11.3	2.8	22.5	22.5	—
taraxerone	0.4	1.4	—	1.4	0.2	2.8	1.4	2.8
apigenin	0.8	—	—	—	—	—	—	—
luteolin	0.8	3.4	3.4	6.9	6.9	13.8	13.8	6.9
quercetin	0.4	2.8	2.8	5.6	5.6	5.6	—	5.6
gallic acid	12.5	25.0	25.0	200.0	12.5	50.0	50.0	100.0
methyl gallate	12.5	12.5	12.5	50.0	12.5	12.5	12.5	12.5
salicylic acid	7.8	3.9	3.9	7.8	3.9	7.8	7.8	7.8

— : Did not show inhibition.

Universidad Autónoma del Estado de Hidalgo, Pachuca, Hidalgo, México, and was identified by Professor Manuel Gonzalez Ledesma of that Institute.

Extraction and isolation. Dried, ground flowers and leaves (3 kg) of *A. cochliacantha* were extracted at room temperature by maceration with hexane, EtOAc and MeOH successively for six days to give 4.1 g, 7.3 g and 50 g of residue, respectively. The stems (1.9 kg) were extracted in the same way with MeOH giving 52 g of residue. The hexane extract of the flowers and leaves was fractionated by column chromatography on silica gel using $CHCl_3$ and $CHCl_3$-MeOH mixtures of increasing polarity to give four fractions (A-D). Fraction A (1.4 g) was subjected to column chromatography on silica gel using hexane-acetone (1:0.01 v/v) as eluent, to afford squalene (15 mg). Fraction B (340 mg) was fractionated using hexane-$CHCl_3$ mixtures of increasing polarity to yield lupenone (1.8 mg), taraxerone (12 mg) and a mixture 1:1 of β-sitosterol and stigmasterol (14 mg). Similarly, fraction C (1.6 g) was chromatographed using hexane-$CHCl_3$ mixtures of increasing polarity to give β-sitosterol and stigmasterol (34 mg). NMR and GC-MS analysis of fraction D (700 mg) indicated the presence of palmitic, linoleic, oleic, stearic and myristic acids, with a relative ratio of 35.5%, 32.6%, 15.2%, 13.0% and 3.6%, respectively. Addition of acetone to the EtOAc residue of the flowers and leaves allowed to obtain a precipitate, which was filtrated to afford a pale yellow powder (525 mg) which contains principally (+)-pinitol and a small quantity of β-sitosterol 3-*O*-β-D-glucoside and stigmasterol 3-*O*-β-D-glucoside. The filtrate was concentrated and fractionated by column chromatography on silica gel using $CHCl_3$-MeOH to give a mixture of β-sitosterol and stigmasterol, and a 1:1 mixture of β-sitosterol 3-*O*-β-D-glucoside and stigmasterol 3-*O*-β-D-glucoside (14.5 mg). Column chromatography over silica gel of the MeOH residue of the flowers and leaves using hexane, hexane-EtOAc (1:1 v/v), EtOAc, EtOAc-acetone (1:1 v/v) and acetone, and collecting fractions of each polarity, afforded frations A-E. On fraction A fatty materials were identified. Fraction B (150 mg) was fractionated by column chromatography on silica, with EtOAc-MeOH mixtures of increasing polarity to yield (+)-pinitol (23 mg). Fractions C, D and E were combined (3.1 g) and chromatographed on silica gel, using $CHCl_3$, $CHCl_3$-acetone (1:1 v/v), acetone, acetone-MeOH (1:1 v/v), MeOH and water, to obtain gallic acid (5 mg) and proacacipelin (85 mg). The MeOH residue of stems was dissolved in water and sequentially extracted with hexane, $CHCl_3$, EtOAc and *n*-BuOH. Column chromatography on silica gel of the AcOEt extract (5.5 g), using mixtures of $CHCl_3$-MeOH (9:1, 8:2, 7:3, 6:4, 1:1, 4:6, 3:7, 2:8, 1:9 v/v), and collecting fractions of each polarity, afforded fractions A-I. On fractions A and B (0.5 g) fatty materials were obtained, while salicylic acid (24 mg) was obtained from fraction C. Fraction D (32 mg) was fractionated by column chromatography on silica gel using $CHCl_3$-MeOH (1:0.05 v/v) yielding apigenin (4.2 mg). Fraction E (22 mg) and F (38 mg) were combined and chromatographed on silica gel, with $CHCl_3$-MeOH (95:5 v/v) as eluent, to give luteolin (11 mg) and methyl gallate (2.4 mg). Fraction H (32 mg) and I (24 mg) were also combined and fractioned by column chromatography on silica gel using $CHCl_3$-MeOH (1:0.05 v/v) to afford quercetin (2.5 mg).

Proacacipetalin. Colourless oil; $[\alpha]_D$ –26.2 (*c* 1.16, MeOH); ^{1}H NMR (D_2O, 400 MHz, solvent residual peak referenced at 4.80 ppm) δ 5.42 (1H, br s, H-4a), 5.40 (1H, s, H-2); 5.28 (1H, br s, H-4b), 4.69 (1H, d, *J* = 7.7 Hz, H-1'), 3.92 (1H, dd, *J* = 12.4, 2.2 Hz, H-6'a), 3.73 (1H, dd, *J* = 12.4, 5.5 Hz, H-6'b), 3.53 (1H, dd, *J* = 9.5, 9.1 Hz, H-3'), 3.50 (1H, ddd, *J* = 9.5, 5.5, 2.2 Hz. H-5'), 3.41 (1H, dd, *J* = 9.5, 9.1 Hz, H-4'), 3.34 (1H, dd, *J* = 9.1, 7.7 Hz, H-2'), 1.89 (3H, br s, CH_3-5); ^{13}C NMR (D_2O, 100 MHz, assignments by APT and HETCOR) δ 137.1 (C-3), 118.1 (C-4), 117.5 (C-1), 100.3 (C-1'), 76.4 (C-5'), 75.7 (C-3'), 72.8 (C-2'), 70.4 (C-2), 69.6 (C-4'), 60.7 (C-6'), 17.8 (C-5).

Bacterial cultures and growth conditions. Bacterial strains used in this study as test organisms were *Staphylococcus aureus* ATCC 25923, *Bacillus subtilis* ATCC 6633, *Enterococcus faecium* ATCC 10541, *Lactobacillus plantarum* ATCC 8014, *Escherichia coli* ATCC 25922, *Salmonella typhimurium* ATCC 14028, *Klebsiella pneumoniae* ATCC 10031 and *Pseudomonas aeruginosa* ATCC 27853. Cultures of bacteria were grown for 18 h in soy tripticase broth at 37 °C and a stock culture was maintained on soy nutrient agar at 4 °C.

Antibacterial activity assay. An agar well diffusion method [26] was used to determine the antibacterial properties of the extracts. 20 mL of Mueller-Hinton agar were seeded with 0.5 mL of test bacterial culture (ca. 10^8 cells/mL) [27] for 18 h, then uniform and equidistant wells of 6 mm diameter were cut in the agar into which 100 μL quantities of known concentration of extracts were poured. The bacterial seeded plates were incubated at 37 °C for 24 h, and then diameters of zones of inhibition were measured. Extracts that showed an inhibition zone greater than 9 mm by the disc diffusion method were selected for the agar dilution test to determine the MIC, defined as the lowest concentration of the sample required to inhibit the growth of the test bacteria.

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