Constituents of Antibacterial Extract of *Caesalpinia paraguariensis* Burk.

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The Argentinean legume *Caesalpinia paraguariensis* Burk. (Fabaceae) was selected for further fractionation work based on the strong antimicrobial activity of its CH_2Cl_2-MeOH (1:1 v/v) extract against a host of clinically significant microorganisms, including antibiotic resistant strains. 1D and 2D NMR enabled the identification of the novel benzoxecin derivative caesalpinol along with the known compounds bilobetin, stigma-5-en-3-O-β-6′-stearoylglucopyranoside, stigma-5-en-3-β-6′-palmitoylglucopyranoside, stigma-5-en-3-β-glucopyranoside, oleanolic acid, 3-O-(E)-hydroxycinnamoyl oleanolic acid, betulinic acid, 3-O-(E)-hydroxycinnamoyl betulinic acid, and lupeol from the active fractions. Oleanolic acid was found active against *Bacillus subtilis* and both methicillin-sensitive and -resistant *Staphylococcus aureus* with MICs of 8 (17.5 μM), 8 and 64 (140 μM) μg/ml, respectively. The rest of the compounds, however, did not show activity.

Key words: Caesalpinia paraguariensis, Structure Elucidation, Antibacterial Activity

Introduction

Infectious diseases remain the leading cause of death worldwide and infections due to antibiotic-resistant microorganisms have become more widespread in recent years (WHO 1999). Resistance rates among key pathogens continue to grow at an alarming rate in distinct geographic regions worldwide (Bell *et al.*, 1998; Pfaller *et al.*, 1998; Schmitz *et al.*, 1999) and the search for novel antimicrobial agents to combat such pathogens have become crucial for avoiding the threat of post-antibiotic era. As part of the International Cooperative Biodiversity Group (ICBG) program "Bioactive Agents from Dryland Biodiversity of Latin America" several plants were screened, among others, for their antibacterial activity. The CH_2Cl_2-MeOH (1:1 v/v) extract of *Caesalpinia paraguariensis* (Leguminosae) was also screened and was found to be active against *Staphylococcus aureus* and *Enterococcus faecium* and the methicillin- and vancomycin-resistant strains, respectively, of these bacteria. *C. paraguariensis* is a tree legume of the semi-arid Chaco region of southern South America. Traditionally, the plant has been used in the treatment of malaria (Kuria *et al.*, 2001), tuberculosis, diarrhea, dysentery, skin-infections, stomach aches and nervous disorders (de Padua, 1996). The tree has also been proposed as an important source of fodder for domestic livestock owing to the ideal nutritional characteristics of its seeds, seedpods and leaves (Aronson and Toledo, 1992). Chemical investigations of various species in the genus *Caesalpinia* have so far yielded primarily numerous novel cassane-type furanoditerpenes (Jiang *et al.*, 2001; Kinoshita, 2000) and to a lesser extent phenylpropanoids (Mendes *et al.*, 2000) and flavonoids (Namikoshi and Saitoh, 1987; Namikoshi *et al.*, 1987; Parmar *et al.*, 1987). These compounds were, however, not detected in the antimicrobial fractions of *C. paraguariensis.* A review of the literature indicated that this is the first report of chemical and bioactivity investigations of this plant.

Material and Methods

General experimental procedures

Optical rotation and IR (as a film on a diamond cell) were measured on a Jasco P-1020 digital polarimeter and a Thermo Nicolet Avatar 360 FT-IR spectrometer, respectively. Molar absorptivity and UV-spectra were obtained using a Beckman

DU-600 spectrophotometer. A JEOL HX110A mass spectrometer was used in recording HR-FAB mass spectra. NMR spectra (^{1}H, selective 1D-NOE, selective 1D-TOCSY, ^{13}C, DEPT-135, DEPT-90, HSQC, HSQC-TOCSY, HMBC, DQF-COSY, ROESY) were recorded using a Bruker DRX-600 spectrometer in pyridine-d_5. Chemical shifts were expressed in ppm (δ) using partially deuterated solvent chemical shifts as reference. Initial purification of the extract was done using an open column with silica gel (63–200 μm Scientific Adsorbents Inc., Atlanta, GA, USA). Further purification and isolation was carried out with the help of a centrifugal chromatography system (Analtech, Newark, DE, USA) and a 2 mm silica gel rotor (Analtech). Isolation of some compounds was effected after purification using centrifugal chromatography with a Varian ProStar semiprep HPLC system equipped with a model 230 pump, and a model 310 variable wavelength detector. HPLC columns used were silica columns in NP-HPLC (Lichrosorb, silica, 5 μm, 250 × 4 mm, at 1.3 ml/min and 200 nm, Column Engineering Inc., Ontario, Canada, or Econosphere, silica, 10 μm, 250 × 10 mm, at 5.4 ml/min and 200 nm, Alltech Associates Inc., Deerfield, IL, USA) and reversed phase column in RP-HPLC (Reliasil, C18, 10 μm, 250 × 10 mm, Column Engineering Inc., at 5.2 ml/min and 200 nm).

Plant material

The plant material was collected in December 1995, 29 km west of La Punta in the province of Santiago del Estero, Argentina (28° 14′ S; 49° 18′ W) by Renée H. Fortunato. A voucher specimen (RHF 5191) has been deposited at the Instituto Nacional de Tecnología Agropecuaria (INTA), Buenos Aires, Argentina. Intellectual Property Rights Agreements for plant collections and collaborative research have been fully executed between the University of Arizona and INTA.

Extraction and isolation

800 g powder of the aerial part of the plant was extracted with 7 × 2 l CH_2Cl_2–MeOH (1:1 v/v) at room temperature with constant stirring. The pooled extract was concentrated under reduced pressure to yield a viscous mass. This was applied onto a silica gel column and fractionated using a stepped gradient of hexane-EtOAC (100:0 to 45:55 v/v) collecting 200 ml fractions giving rise to a total of 103 fractions. Antibacterial activity was observed in fractions 47–58, 94–98 and 99–103. Purification using centrifugal chromatography (CH_2Cl_2-MeOH, 96:4) of Fractions 47–58 followed by HPLC (MeOH-water, 85:15 to 100:0 in 15 min) gave **6** (20.8 mg), **7** (8.3 mg) and **9** (3.0 mg) (R_t = 11.1, 14.3 and 14.0 min, respectively). Purification of fractions 15 and 16 using centrifugal chromatography with hexanes-*i*-PrOH (98:2) followed by NP-HPLC (hexane-*i*-PrOH-MeOH, 99:0.5:0.5 to 93:3.5:3.5 in 10 min) gave **10** (13.6 mg) while purification with centrifugal chromatography (CH_2Cl_2-MeOH, 99:1) of combined fractions 38–46 followed by NP-HPLC (hexane-*i*-PrOH-MeOH, 93:3.5:3.5 to 90:5:5 in 10 min) gave **8** (11.1 mg). Fraction 94 was purified using centrifugal chromatography with CH_2Cl_2-MeOH (98:2) followed by hexane-MeOH-*i*-PrOH (86:7:7) and compounds **4** (14.0 mg) and **5** (36.2 mg) were obtained using NP-HPLC (R_t = 6.0 and 6.4 min, respectively). On the other hand, purification with centrifugal chromatography of fractions 96–98 with hexanes-Me_2CO (45:55) followed by CH_2Cl_2-*i*-PrOH (90:10) gave **2** (4.0 mg) and **3** (20.9). Finally, centrifugal chromatography of fractions 99–102 with hexanes-Me_2CO (45:55) followed by CH_2Cl_2-MeOH (96:4) afforded **1** (1.9 mg). All compounds were identified based on analysis of their NMR, IR, MS and UV spectra.

Antimicrobial activity testing

The *in vitro* antibacterial activities were determined by the agar diffusion or microbroth dilution method as previously described (Singh, *et al.*, 2000). The agar diffusion method was used to compare the activities of antimicrobial agents against selected bacterial isolates. Assay plates (12″ × 12″ Sumilon) were prepared by pouring 125 ml of agar medium (tempered at 50 °C) inoculated with an overnight broth culture of the test organisms (adjusted to a final inoculum density of approx. 10^6 cells per ml). The medium was allowed to solidify, and 144 wells (5 mm diameter) were bored into the agar layer using an automated plate-welling machine. 10 to 20 μl volumes of antibiotic solutions diluted in a suitable solvent were dispensed into wells, and the plates were incubated at 37 °C

for 18 h. The zones of growth inhibition were measured using a hand-held digital caliper. The bacterial strains used, *Bacillus subtilis* 327*, Staphylococcus aureus* 375 (methicillin-sensitive), *Staphylococcus aureus* 310 (methiclillin-resistant), *Escherichia coli* imp 389, and *Candida albicans* 54, are laboratory cultures maintained in the Wyeth research collection. Penicillin G was used as control.

The minimum inhibitory concentrations (MICs) were determined by the microbroth dilution method using the unsupplemented Müller-Hinton broth. Briefly, microtiter plates containing 2.5 µl per well of two-fold serial dilutions of each antimicrobial agent were inoculated with 100 µl of bacterial suspension to yield the appropriate density ($1-5 \times 10^5$ CFU/ml). The plates were incubated for 18 h at 35 °C in ambient air. The MIC was defined as the lowest concentration of a compound that completely inhibited the growth of the organism as determined by the unaided eye.

Identification

Caesalpinol (**1**), yellow oil, $[\alpha]_D^{25}$ +4.3° ($CHCl_3$: *c* 0.3). IR ν_{max} (cm^{-1}) 3451, 3194, 2923, 1565, 1504, 1160, 767. UV ($CHCl_3$) λ_{max} (log ε) 272 (3.25) nm. HR-FAB$^+$ 315.3904, FAB$^-$ 313.2843, $C_{19}H_{22}O_4$. ^{13}C-NMR (150 MHz, $CDCl_3$): ^{1}H-NMR (600 MHz): see Table I.

Compounds **2–10** were identified after analysis of their NMR, MS, IR and UV spectra. NMR and other analytical data may be obtained directly from the authors upon request.

Results and Discussion

Caesalpinol (**1**) was isolated as pale-yellow powder. HR-FABMS established its molecular formula as $C_{19}H_{22}O_4$ through quasimolecular ions at *m/z* 315.3904 $[M + H]^+$ in the positive-ion mode and at *m/z* 313.2843 $[M-H]^-$ in the negative-ion mode. The IR spectrum showed absorption band at 3451 (*br*, -OH) and bands attributable to aromatic rings at 1565 and 1504 cm^{-1}. The presence of a dibenzoxy ring structure was readily apparent from the ^{13}C and ^{1}H NMR spectra. These displayed signals for three quaternary aromatic carbons at δ 160.6 (C-11), 113.0 (C-12) and 157.2 (C-9) and three tertiary aromatic carbon signals at δ 104.1 (C-10), 109.1 (C-8) and 132.8 (C-7), which were attributable to the first benzoxy group (ring A). Aromatic methine proton signals on these tertiary carbons were found at 6.94 (s, 1H, H-10), 6.91 (d, *J* = 8.3 Hz, 1H, H-8), and 7.34 (d, *J* = 8.3 Hz, 1H, H-7) in the HSQC spectrum, respectively. Two additional quaternary carbons at δ 132.4 (C-1′) and 159.0 (C-4′) together with four tertiary carbons at δ 116.3 (C-3′ and C-5′) and 128.7 (C-2′

Table I. NMR spectral data of **1** (150 MHz and 600 MHz, pyridine-d_5).

No.	^{13}C	^{1}H (J, Hz)	1D-TOCSY correlations	HMBC correlations
2	73.8	5.54, d (7.3)	H-3, H-4, H-5, H-6, H-13	C-2′, C-6′, C-13
3	35.6	2.42, dd (13.1, 7.3)	H-2, H-4, H-5, H-6, H-13	C-5, C-1′
		2.14, dd (13.1, 6.2)	H-2, H-4, H-5, H-6, H-13	C-5, C-1′
4	72.9	4.30, m	H-2, H-3, H-5, H-6, H-13	C-13, C-14
5	32.1	1.24, m	H-2, H-3, H-4, H-6, H-13	C-12, C-3, C-13
6	29.9	1.28, s	H-2, H-3, H-4, H-5, H-13	C-7, C-11, C-13
7	132.8	7.34, d (8.3)	H-8	C-9, C-11
8	109.1	6.91, d (8.3)		C-12
9	157.2			H-7
10	104.1	6.94, s		C-8, C-12
11	160.6			H-2, H-6, H-7
12	113.0			H-5, H-8, H-10
13	14.3	0.88, d (7.3)	H-2, H-3, H-4, H-5, H-6	C-4, C-5, C-6
14	55.6	3.44, s		C-4
1′	132.4			H-3, H-3′, H-5′
2′	128.7	7.52, d (8.3)	H-3′	C-2, C-4′, C-6′
3′	116.3	7.23, d (8.3)	H-2′	C-1′, C-5′
4′	159.0			H-2′, H-6′
5′	116.3	7.23, d (8.3)	H-6′	C-1′, C-3′
6′	128.7	7.52, d (8.3)	H-5′	C-2, C-2′, C-4′

and C-6′) constituted the second benzoxy ring (ring C). These two tertiary carbon signals were found to correlate with signals for aromatic methine protons at δ 7.23 (d, J = 8.3 Hz, 2H, H-3′ and H-5′) and 7.52 (d, J = 8.3 Hz, 2H, H-2′ and H-6′), respectively, in the HSQC spectrum. Also observed were three methine signals at δ 73.8 (C-2), 72.9 (C-4) and 32.1 (C-5) and two methylene signals at δ 35.6 (C-3) and 29.9 (C-6). The oxymethine proton at δ 5.54 (d, J = 7.3 Hz, 1H, H-2) showed $^3J_{CH}$ correlations with a quaternary aromatic carbon at δ 160.6 (C-11) and an aromatic methine carbon at δ 128.7 (C-2′ and C-6′) in the HMBC spectrum, establishing that the site of attachment for ring C was at C-2. On the other hand, a substructure of the eight-membered heterocyclic ring B was identified based on a series of selective 1D-TOCSY experiments whereby easily identifiable ^{1}H signals on the ring were irradiated to establish a spin system composed of H-2, H-3, H-4, H-5, H-6 and H-13. Analysis of the coupling patters of these protons and their long-range correlations in the HMBC spectrum enabled the determination of the exact position of these protons on the oxecin ring B. Finally, observation of $^3J_{CH}$ correlations between H-6 and C-11 and of the same proton with C-7 enabled the identification of all members comprising ring B. Based on the foregoing, it was assigned that caesalpinol has structure **1**.

Compounds **8** and **10** were found present in inactive fractions prepared from the active extract and, thus, were not subjected to further tests for antimicrobial activity. Compounds isolated from active fractions of *C. paraguariensis*, on the other hand, were evaluated for their antimicrobial activity. Compounds **1–5** and **7–10** were found inactive against the test organisms *B. subtilis*, methicillin-sensitive and -resistant *S. aureus*, *E. coli*, and *C. albicans* with MICs greater than 128 μg/ml. However, **6** showed moderate activity against *B. subtilis* and both methicillin-sensitive and -resistant *S. aureus* with MICs of 8 (17.5 μM), 8 and 64 (140 μM) μg/ml, respectively. The fractions from which the reported compounds were obtained all contained tannins and other high molecular weight phenolic compounds. This probably accounts for the antimicrobial activity of the fractions but not the compounds isolated from them.

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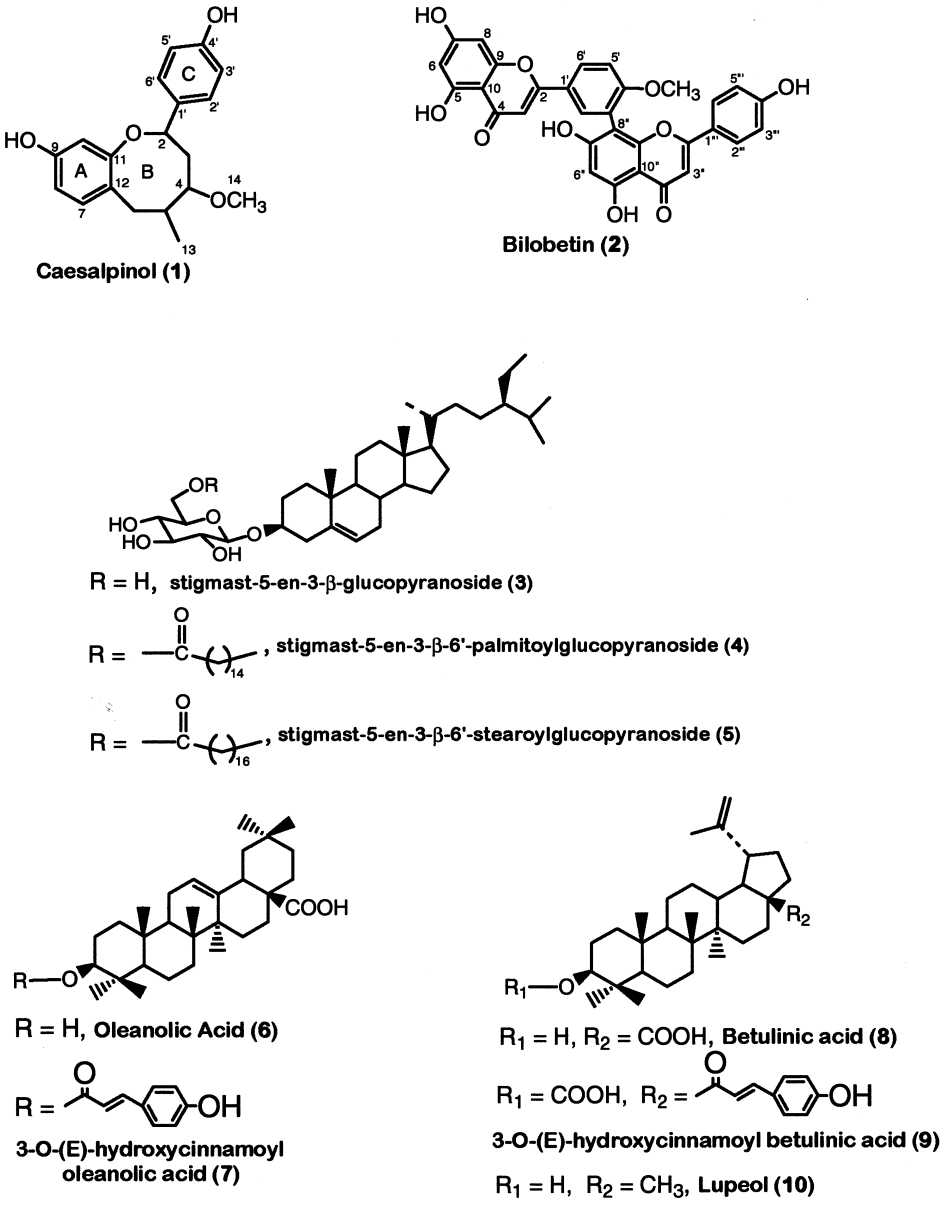

Fig. 1, Compounds isolated from antimicrobial fractions of *C. paraguariensis*.

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