

Antifungal Saponins from the Maya Medicinal Plant *Cestrum schlechtendahliae* G. Don (Solanaceae)

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Bioassay-guided fractionation of the crude extract (80% EtOH) of the leaves of *Cestrum schlechtendahliae*, a plant used by Q'eqchi' Maya healers for treatment of athlete's foot, resulted in the isolation and identification of two spirostanol saponins (1 and 2). Structure elucidation by MS, 1D-NMR, and 2D-NMR spectroscopic methods identified them to be the known saponin (25R)-1 β ,2 α -dihydroxy-5 α -spirostan-3- β -yl-O- α -L-rhamnopyranosyl-(1 \rightarrow 2)- β -D-galactopyranoside (1) and new saponin (25R)-1 β ,2 α -dihydroxy-5 α -spirostan-3- β -yl-O- β -D-galactopyranoside (2). While 2 showed little or no antifungal activity at the highest concentration tested, 1 inhibited growth of *Saccharomyces cerevisiae* (minimum inhibitory concentration (MIC) of 15–25 μ M), *Candida albicans*, *Cryptococcus neoformans*, and *Fusarium graminearum* (MIC of 132–198 μ M). Copyright © 2015 John Wiley & Sons, Ltd.

Keywords: *Cestrum schlechtendahliae*; saponin; antifungal; Q'eqchi' Maya; traditional medicine; Solanaceae.

INTRODUCTION

The Mesoamerican region of Central America is a biodiversity hotspot of semi-evergreen tropical trees (Myers *et al.*, 2000). Indigenous Maya cultures in this region have strong traditions of healer apprenticeship and continue to use traditional medicines derived from local plants for primary health care. Ethnobotanical studies show that the Maya have an extensive knowledge of useful flora, and quantitative methodologies show that there is a high degree of consensus for many usage categories (Ankli *et al.*, 1999; Amiguet *et al.*, 2005; Bourbonnais-Spear *et al.*, 2005).

The use of plants for infections is one area that shows a high degree of consensus. For dermatological infections, Heinrich (2000) reported an informant consensus factor of 0.52 among Yucatec Maya community. For the Q'eqchi, our own research calculated a consensus factor of 0.68 for treating infections using 96 plant species (Amiguet *et al.*, 2005). The informant consensus factor is calculated by dividing the number of healers who use a plant for a specific ailment by the total number of healers (Amiguet *et al.*, 2005). Laboratory studies have also shown that there is often a sound pharmacological

basis for the use of many of these plants. In a lab study of 38 plant species used by the Tzeltal and Tzotzil Maya communities from Chiapas (Mexico), 65% of plants showed antimicrobial activity against Gram-positive *Staphylococcus aureus*, Gram-negative *Escherichia coli* bacteria, and the fungal pathogen *Candida albicans* (Meckes *et al.*, 1995).

The Solanaceae family is of particular interest in the Maya traditional medicine for treatment of mycoses. For example, the Yucatec Maya community healers from San Jose Succotz Belize use *Solanum torvum* Sw. and *Solanum mammosum* L. for the treatment of athlete's foot (Arnason *et al.*, 1980). Furthermore, leaves of *Cestrum dumetorum* Schlecht. are used by the Huastec Maya to treat warts and infected wounds (Alcorn, 1984). A pilot blinded clinical study on tinea pedis infections was undertaken by Lozoya *et al.* (1992) with *Solanum chrysotrichum* Schltdl., a plant widely used by Maya communities in Chiapas for severe athlete's foot. A cream containing a 5% MeOH extract of the leaves provided a statistically better response rate than the miconazole control with complete remission in 45% of cases. Further information on the active constituents and mode of action of these antimicrobial plants is required.

In the present study, we examined the antifungal activity of a Q'eqchi' Maya plant *Cestrum schlechtendahliae* G. Don. This plant is known as ik che (pepper tree) or ik kejen (pepper plant), and crushed leaves are used by the Q'eqchi' healers of Belize for fungal infections such as athlete's foot and thrush. Calderón *et al.* (2006) reported

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weak antileishmanial activity of *C. schlechtendahlia* leaves extract; however, no phytochemical work has been presented on this species. This is the first study to report the antifungal activity of this plant and its phytochemical constituents.

MATERIALS AND METHODS

General experimental procedures. Infrared spectra were recorded on a Shimadzu 8400-S FT/IR spectrometer. Optical rotations were registered on a Perkin-Elmer 241 digital polarimeter. NMR spectra were recorded on a Bruker Avance 500 spectrometer in CD₃OD at 500 (¹H) and 125 MHz (¹³C) using tetramethylsilane as an internal standard. High resolution electrospray ionization mass spectrometry (HRESIMS) was carried out using a Waters Xevo G2 UPLC-QTOF-MS/MS system. Electrospray ionization mass spectrometry (ESI-MS) was carried out also using a Shimadzu LCMS 2020 Series system. Open column chromatography was carried out with silica gel 60 (70–230 mesh, Merck). Thin layer chromatography (TLC) analyses were performed on silica gel 60F254 plates (Merck) with visualization using a ceric sulfate (10%) solution in H₂SO₄. For sugar analyses, silica gel 0.25 mm plates (Merck) were used, and visualization was carried out with an anisaldehyde reagent (5% *p*-anisaldehyde, 5% concentrated H₂SO₄ in EtOH).

Plant material. Leaves of *C. schlechtendahlia* were collected in Jalacte, Belize in May 2010 under ethical approval (permits #H11-11-09, #H03-070-01). Collecting and export permits, as well as phytosanitary certificates, were obtained from the Belize Forest Department (ref. no. CD/60/3/08(33)). Plant material was preserved in 70% ethanol immediately after collection. Voucher specimens (UOH19776) have been deposited at the University of Ottawa Herbarium, the Herbario Juvenal Valerio Rodriguez, and the Belize Forest Department.

Extraction and isolation. Leaves (600 g) were ground using a blender (Waring commercial LR 8992) and extracted with 80% EtOH in a 1:10 biomass to solvent (w/v) ratio. The plant material/ethanol mixture was

shaken at room temperature overnight at 200 rpm and vacuum filtered. A second extraction was performed with the plant residue using a 1:5 w/v ratio, and the mixture was again shaken and filtered. The two extracts were combined, dried *in vacuo* using a rotary evaporator at 45 °C, lyophilized to remove any residual water using a freeze dryer (EC Super Modulyo, ~ -55 °C, 10⁻² mbar), and stored at -20 °C in the dark until needed. The crude leaf extract (79 g) was then fractionated using silica gel chromatography (column size 80 × 10 cm ID) with elution gradients hexane–EtOAc (1:0 → 0:1), EtOAc, and EtOAc–MeOH (1:0 → 0:1), and 26 primary fractions were collected (CSE-I to CSE-XXVI). Active fraction CSE-XVIII (10 g) eluted with 80:20 EtOAc–MeOH was then chromatographed using another silica gel column (180 × 5 cm ID) using the same solvent systems to result in 39 secondary fractions (CSE-XVIII-1 to CSE-XVIII-39). Compound **1** (800 mg) was eluted from fraction CSE-XVIII-13 with 85:15 EtOAc–MeOH and crystallized via spontaneous precipitation. Fraction CSE-XVIII-19 was further chromatographed with a Sephadex LH-20 column (60 × 2 cm ID) with MeOH eluted isocratically to yield compound **2** (3.4 mg).

(25R)-1β,2α-dihydroxy-5α-spirostan-3-β-yl-O-α-L-rhamnopyranosyl-(1→2)-β-D-galactopyranoside (1). Amorphous white powder (MeOH–EtOAc 80:20); [α]_D²⁵ -53.5 (c 0.001, MeOH); IR (KBr) ν_{max} 3360, 2905, 1235, and 1050 cm⁻¹; ¹H-NMR (CD₃OD, 500 MHz) and ¹³C-NMR (CD₃OD, 500 MHz), see Table 3; HRESIMS (negative mode) *m/z* 755.3107 [M - H]⁻ (calculated for C₃₉H₆₃O₁₄: 755.4296). ESI-MS *m/z* 779 [M + Na]⁺ (80), 757 [M + H]⁺ (40), 595 [M - C₆O₅H₁₁]⁺ (40), 449 [M - C₆H₁₁O₅ - C₆H₁₁O₄]⁺ (55), 431 [M - C₆H₁₁O₅ - C₆H₁₁O₄ - H₂O]⁺ (30), 413 [M - C₆H₁₁O₅ - C₆H₁₁O₄ - 2H₂O]⁺ (100).

(25R)-1β,2α-dihydroxy-5α-spirostan-3-β-yl-O-β-D-galactopyranoside (2). Amorphous white powder (MeOH). [α]_D²⁵ -50.5; (c 0.001, MeOH); IR (KBr) ν_{max} 3365, 2900, 1240, and 1065 cm⁻¹; ¹H-NMR (CD₃OD, 500 MHz) and ¹³C-NMR (CD₃OD, 500 MHz), see Table 3; HRESIMS (negative mode) *m/z* 609.2575 [M - H]⁻ (calculated for C₃₃H₅₃O₁₀: 609.3768); ESI-MS *m/z*

Table 1. Antifungal activity of *Cestrum schlechtendahlia* G. Don leaves extract (2 mg/disk), fractions (1 mg/disk), and compound **1** (0.5 mg/disk) against yeast-like fungi (disk diameter = 7 mm; N = 3) compared with berberine (0.5 mg/disk) and ketoconazole (90 μg/disk)

Fraction or compound	Zone of inhibition (mm)		
	<i>Saccharomyces cerevisiae</i> S288C	<i>Candida albicans</i> D10	<i>Cryptococcus neoformans</i>
Crude extract	15.1 ± 0.2	11.4 ± 0.5	11.7 ± 0.8
CSE-XVII	15.8 ± 0.2	10.4 ± 0.2	10.2 ± 0.2
CSE-XVIII	20.7 ± 0.2	17.2 ± 0.3	17.3 ± 0.2
CSE-XVIX	19.6 ± 0.8	10.2 ± 0.4	10.9 ± 0.8
CSE-XX	17.6 ± 0.8	11.1 ± 1.0	10.7 ± 0.6
CSE-XXI	16.3 ± 0.4	12.6 ± 0.7	14.3 ± 0.1
CSE-XXII	14.3 ± 0.2	14.0 ± 0.4	12.7 ± 0.4
1	21.4 ± 0.7	16.2 ± 0.6	17.3 ± 0.3
Berberine	23.2 ± 0.4	25.3 ± 1.2	22.4 ± 0.7
Ketoconazole	22.6 ± 0.4	15.3 ± 0.3	28.1 ± 0.3

633 $[M+Na]^+$ (100), 611 $[M+H]^+$ (40), 449 $[M-C_6O_5H_{11}]^+$ (25), 431 $[M-C_6H_{11}O_5-H_2O]^+$ (25), 413 $[M-C_6H_{11}O_5-2H_2O]^+$ (70).

Acid hydrolysis of compounds 1 and 2. Samples of **1** (10 mg) and **2** (1 mg) were dissolved separately in 5 mL of 2 M HCl (dioxane–H₂O 1:1). The solutions were refluxed for 4 h at 100 °C. The reaction mixture was diluted with H₂O and extracted three times with dichloromethane (10 mL each). The organic layer from compound **1** hydrolysis was passed through anhydrous magnesium sulfate and dried *in vacuo* to give saponin **1A** (6 mg). The structure of **1A** was confirmed by ¹H-NMR and ESI-MS (see Supporting Information). The aqueous phases from the hydrolysis of compounds **1** and **2** were neutralized by passing through an Amberlite IRA-93ZU column (Organo, Japan) and concentrated by lyophilization. The sugar identities were confirmed by TLC methods [EtOAc:MeOH:CH₃COOH:H₂O (11:2:2:2)] using authentic monosaccharide standards (Sigma-Aldrich, St. Louis, MO, USA). TLC plates were visualized with an anisaldehyde solution. This confirmation of sugar identity by TLC has been used by previous phytochemical studies involving saponins (Ahmad *et al.*, 1995; González *et al.*, 2004; Lu *et al.*, 2009). In support of the TLC results, optical rotational measurements confirmed the presence of L-rhamnose ($[\alpha_D] -13.6^\circ$) on **1** and D-galactose on both **1** and **2** ($[\alpha_D] +18.5^\circ$ and $[\alpha_D] +17.9^\circ$, respectively).

Phytochemical analyses. Chromatographic analyses of the crude extract and fractions were performed on an Agilent 1100 series HPLC system comprising a quaternary pump, a degasser, an autosampler with 100 μ L loop, a column thermostat, and a diode array detector (DAD). The identification of the phenolics was corroborated by comparing the retention time and maximum UV absorption values with authentic commercial standards (Sigma-Aldrich, St. Louis, MO, USA). The analyses were performed using a Luna C18 column (250 mm \times 4.6 mm, 5 μ m particle size) with column temperature set at 55 °C and a flow rate of 1.5 mL/min. The mobile phase A was acetonitrile containing 0.05% trifluoroacetic acid, and B was water containing 0.05% trifluoroacetic acid. Optimized separation was achieved with the following method: initial conditions of 5% A and 95% B with an increasing gradient to 100% A in 25 min; the column was flushed with 100% A for 5 min and then set back to the initial conditions. DAD was set to monitor wavelengths 254, 280, and 330 nm.

The active fractions were analyzed using a Shimadzu UPLC-PDA-MS system (Mandel Scientific Company Inc, Guelph, ON, Canada), which consisted of LC30AD pumps, a CTO20A column oven, a SIL-30 AC autosampler, and an LCMS-2020 mass spectrometer with an electrospray ionization source. Briefly, 1 μ L of each fraction was injected through the autosampler to an Acquity CSH C18 column (100 \times 2.1 mm, 1.7 μ m particle size; Waters, Mississauga, ON, Canada) with an Acquity CSH C18 VanGuard Pre-column (5 \times 2.1 mm). The mobile phases were H₂O (A) and acetonitrile (B). The gradient elution method initiated with 5% B then increased to 95% B in 5 min. The column was then washed

with 90% B for 3 min and changed back to the initial condition in 1 min. The flow rate was set at 0.8 mL/min with the column temperature set at 50 °C. The photodiode array detector was set to monitoring wavelengths from 190 to 400 nm. The mass spectrometer with ESI interface was operating in positive and negative scan modes; the nebulizing gas flow was set at 1.5 L/min, and drying gas flow was at 10 L/min. The desolvation line temperature and heat block temperature were set at 300 and 450 °C, respectively. The *m/z* range of both positive and negative scan is from 150 to 600 with 938 u/s scan speed.

Isolated saponins were analyzed using a Waters Xevo G2 UPLC-QTOF-MS/MS system to confirm their masses. UPLC conditions: Acquity BEH C18 1.7 μ m 2.1 \times 100 mm column connected with a VanGuard Pre-column 2.1 \times 5 mm. Mobile phases were water + 0.1% formic acid for A and acetonitrile + 0.1% formic acid for B (Fisher Optima LC-MS); the flow rate was 0.5 mL/min, column temperature set at 50 °C, and sample temperature at 10 °C. Mobile phase composition was as follows: 0–1 min 5% A isocratic, 1–6 min linear gradient 5–50% B, 6–8 min 50–95% B, 8.01–10 min 5% A isocratic (total run time 10 min). Sample injection conditions: 1- μ L injection followed by a strong wash 200 μ L (90% acetonitrile + 10% water) and weak wash 600 μ L (10% acetonitrile + 90% water). QTOF analysis conditions: MassLynx software, MSe ESI+ mode, lock mass Leucine Enkephalin 12C 556.2615, source temperature 120 °C, desolvation 304, temperature 400 °C, cone gas (N₂) flow 50 L/h, desolvation gas (N₂) flow 1195 L/h. MSe conditions: mass range 100–1500 Da, F1 CE, 6 V, F2 CER 10–30 V, cone voltage 20 V, scan time 1 s. Calibration was done using 50–1000 Da sodium formate.

Fungal strains. Three yeast-like fungal strains, *Saccharomyces cerevisiae* S288C, *Cryptococcus neoformans*, and *C. albicans* D10, were used for disk diffusion assays. The strains used to determine minimum inhibitory concentrations (MICs) in liquid culture were as follows: *S. cerevisiae* BY4741 (haploid), *S. cerevisiae* BY4743 (diploid), and *Fusarium graminearum* [teleomorph *Gibberella zeae* (Schwein.) Petch]. *F. graminearum* ZTE-2A (DAOM 227650), a gift from Robert Proctor (USDA), constitutively expresses green fluorescent protein (Skadsen and Hohn, 2004).

Antifungal disk diffusion assay. *Saccharomyces cerevisiae* S288C, *C. neoformans*, and *C. albicans* D10 were cultured in Sabouraud dextrose broth (Difco) at 30 °C. Berberine (95%, Sigma-Aldrich, St. Louis, MO, USA) and ketoconazole (>98%, Sigma-Aldrich, St. Louis, MO, USA) were used as antifungal positive controls and methanol as a negative control. Overnight cultures were grown to an optical density of 1.0 ($\sim 1.0 \times 10^7$ CFU/mL) at 600 nm and diluted 1:100. Aliquots (100 μ L) of this inoculum were spread over the surface of Sabouraud agar plates. Paper disks (7.0 mm diameter) were loaded with crude extract (2 mg/disk), berberine (0.5 mg/disk), ketoconazole (90 μ g/disk), fractions (0.5 mg/disk), saponin (0.5 mg/disk), or methanol (carrier solvent) and allowed to air dry. The amended disks were placed treated side down on the prepared medium and incubated in the dark at 30 °C for 48 h, prior to measurement of inhibition zones

with a ruler. All experiments were repeated three times, with three technical repetitions per biological repetition.

Yeast growth assay. This microdilution method was modified from CLSI M07-A9 (CLSI, 2012) using *S. cerevisiae* BY4741 and BY4743 cultured overnight in YPD broth (1% yeast extract, 2% peptone, 2% dextrose w/v) at 30 °C and distributed into wells of a 96-well flat bottom plate (Costar 3596) at an inoculum size of $5\text{--}7 \times 10^3$ CFU/mL. Test compounds were dissolved at varying concentrations and serially diluted 1:1 across the plate. Wells containing berberine (95%, Sigma-Aldrich, St. Louis, MO, USA) or appropriate quantities of the MeOH carrier solvent were used as positive and negative controls, respectively. Hygromycin B (92%, VWR, Mississauga, ON, Canada) and geneticin (>98%, Sigma-Aldrich, St. Louis, MO, USA) were also used as positive controls. The plate was incubated at 30 °C shaking at 600 rpm and absorbance readings taken at 600 nm every 10 min for 24 h (Biotek Powerwave XS2 microplate reader, Winooski, VT, USA). All experiments were repeated three times, with three technical repetitions per biological repetition.

Serial dot dilution assay. Overnight cultures of *S. cerevisiae* BY4741 and BY4743 grown in YPD medium at 30 °C were adjusted to 1.5×10^6 CFU/mL and dilutions of 1:10, 1:100, and 1:1000 produced. In a six-well flat bottom plate (Falcon 3046), compound **1** at chosen concentrations was dissolved in YPD agar before solidification. Separate plates were prepared with berberine as a positive control. Once the media had set, 1 μ L of each diluted inoculum was separately spotted twice into each well, corresponding to 1.5×10^5 , 150, 15, and 1.5 CFU/mL. The plate was then incubated in the dark at 30 °C for up to 72 h. The plates were inspected for growth every 24 h.

All experiments were repeated three times, with three technical repetitions per biological repetition.

Fusarium growth assay. *Fusarium graminearum* was grown in 100 mL of CMC broth (Cappellini and Peterson, 1965) at 28 °C with shaking for 3 to 5 days to generate conidia. Mycelium was separated from conidia by filtration through one layer of sterile miracloth. Conidia were then washed with sterile water twice by centrifugation at 4000 rpm for 15 min at room temperature and resuspended in sterile water for storage at 4 °C. Conidia were inspected and counted with a hemocytometer prior to use. Conidia were diluted in GYEP broth to ~ 1000 CFU/well in a 96-well flat bottom white plate (Costar 3632) and allowed to germinate for 24 h before compounds were added at varying concentrations (Nasmith *et al.*, 2011). Wells containing berberine or appropriate quantities of the MeOH carrier solvent were also included to act as positive and negative controls, respectively. Hygromycin B and geneticin were also used as positive controls. Growth was monitored via fluorescence using a Polarstar Optima Microplate Reader (BMG Labtech, GmbH, Offenberg, Germany) running FLUOstar OPTIMA Ver. 2.20R2. The plate was incubated at 28 °C shaking at 600 rpm; readings (excitation/emission at 485 nm/520 nm) were taken every 23 min for 72 h. All experiments were repeated three times, with three technical repetitions per biological repetition.

RESULTS

The leaf extract of *C. schlechtendahlia* (CSE) inhibited growth of all three yeast-like fungi (*S. cerevisiae* S288C, *C. albicans* D10, and *C. neoformans*) in the disk diffusion assay (Table 1). HPLC–DAD analysis of the crude extract identified the presence of caffeic acid (**3**),

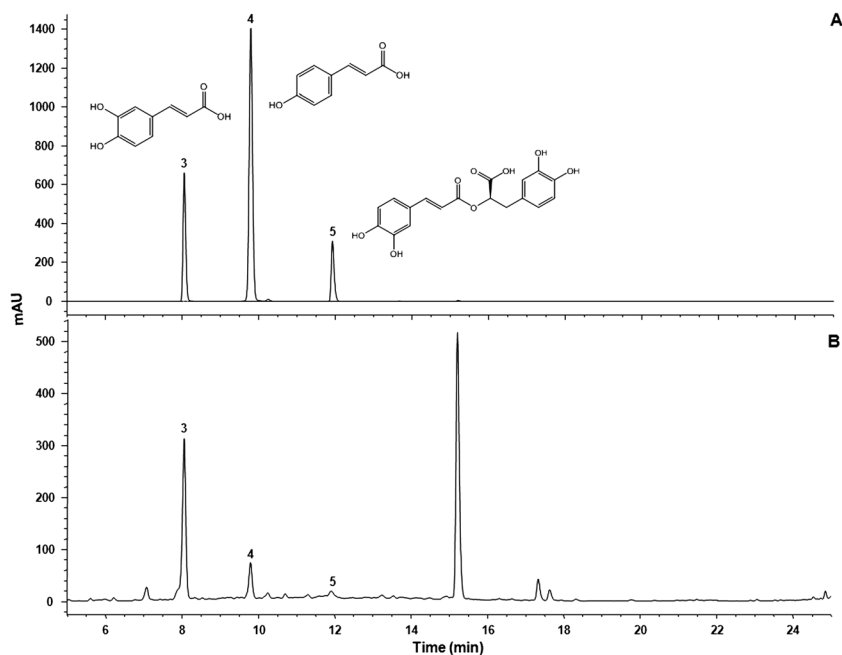


Figure 1. HPLC–DAD analysis for the identification of caffeic acid (**3**), *p*-coumaric acid (**4**), and rosmarinic acid (**5**) in the crude extract of *Cestrum schlechtendahlia* leaves (**B**) compared with the standard mix (**A**).

p-coumaric acid (**4**), and rosmarinic acid (**5**) (Fig. 1), none of which showed antifungal activity in our assays. The crude extract (yield of ~13%) was then fractionated using silica gel open glass column chromatography and fractions CSE-XVII to CSE-XXII had antifungal activity (Table 1). Because CSE-XVIII (13.5 g) eluted with 80:20 EtOAc–MeOH was most active, this fraction was chromatographed using another silica gel column resulting in 39 secondary fractions (CSE-XVIII-1 to CSE-XVIII-39). From sub-fraction CSE-XVIII-13 eluted with 85:15 EtOAc–MeOH, compound **1** (800 mg) was obtained. All secondary fractions and **1** were tested in antifungal disk assays. CSE-XVIII-19 was further chromatographed with a Sephadex LH-20 column to yield compound **2** (3.4 mg). Because of the low yield of **2**, this compound was not tested in the disk diffusion assay. A small set of MICs was carried out subsequently with compounds **1** and **2** (Table 2).

The chemical composition of the active fraction CSE-XVIII was initially assessed by TLC on silica gel using as an eluent EtOAc:MeOH (80:20). The results of this analysis indicated the absence of UV active chromophores and the presence of oxidizable groups in the components of CSE-XVIII, shown by the formation of a broad band after the development of the plate with a ceric sulfate solution. Moreover, CSE-XVIII composition was also analyzed by HPLC-DAD. During the analysis, no signal was detected using any of the chosen monitoring UV wavelengths (210, 254, 280, and 330 nm), confirming that the compound has no UV absorption and supporting the initial TLC findings. This fraction was then analyzed using UPLC–MS, which showed the presence of three structurally related saponins, a major (pk 2) and two minor (pk 1 and pk 3) with masses of 756, 754, and 610, respectively (Fig. 2). Pk 2 and pk 3 were isolated and identified as compounds **1** and **2**, respectively; their structures were verified via NMR. The structure of pk1 was tentatively identified; however, this compound was not isolated because of its low concentration in the plant extract. The purity of compounds **1** and **2** was estimated to be >98% via NMR and UPLC–MS.

Compound **1** was obtained as an amorphous white powder. HRESIMS (negative mode) showed a quasimolecular ion $[M - H]^-$ at a mass charge ratio (m/z) of 755.3107 ($C_{39}H_{63}O_{14}$), $[\alpha]_D -53.5$. The analysis of the MS fragments along with the strong IR absorptions values (3360, 2905, 1235, and 1050 cm^{-1}) allowed preliminary identification of the amphipathic (glycosidic and aliphatic) nature of **1** (see Supporting Information). ESI–MS showed that compound **1** contained two sugar moieties, one hexopyranose [m/z 593 ($M - C_6H_{11}O_5$)] and one deoxyhexopyranose [m/z 449 ($M - C_6H_{11}O_5 - C_6H_{11}O_4$)] (Supporting Information).

Moreover, the analysis of the 1D-NMR (1H , ^{13}C , and Distortionless Enhancement by Polarisation Transfer (DEPT)) and 2D-NMR (Heteronuclear Multiple Quantum Coherence (HMQC), Correlation Spectroscopy (COSY), Nuclear Overhauser Effect Spectroscopy (NOESY), and Heteronuclear Multiple Bond Coherence (HMBC)) experiments led to the identification of the major compound

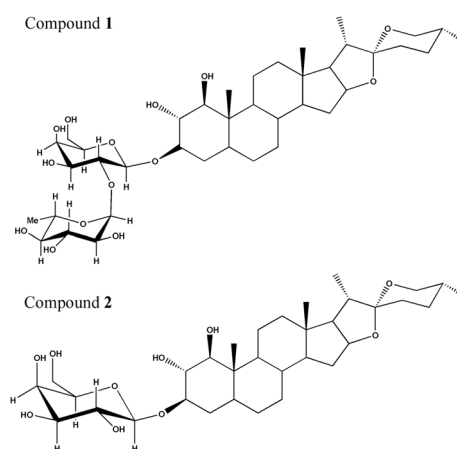


Figure 3. Structures of isolated saponins from the leaves of *Cestrum schlechtendahlilii*: (25*R*)-1β,2α-dihydroxy-5α-spirostan-3-β-yl-*O*-α-*L*-rhamnopyranosyl-(1→2)-β-*D*-galactopyranoside (**1**) and (25*R*)-1β,2α-dihydroxy-5α-spirostan-3-β-yl-*O*-β-*D*-galactopyranoside (**2**).

Table 2. Minimum inhibitory concentrations (μM) of saponins **1** and **2**, sapogenin **1A**, and positive controls berberine, geneticin, and hygromycin B against *S. cerevisiae* strains and *F. graminearum* ZTE-2A

Fungal strain (culture type)	1	2	1A	Berberine	Geneticin	Hygromycin B
<i>S. cerevisiae</i> BY4741 (liquid)	15–25	>95	>105	600–900	57–231	45–54
<i>S. cerevisiae</i> BY4743 (liquid)	20–35	>95	>105	600–900	57–231	45–54
<i>F. graminearum</i> ZTE-2A (liquid)	132–198	NT	NT	744–807	27–54	118–284
<i>S. cerevisiae</i> BY4741 (1.5% agar)	25–35	NT	NT	223–372	57–231	95–379
<i>S. cerevisiae</i> BY4743 (1.5% agar)	25–35	NT	NT	223–372	57–231	95–379

MICs are reported as the range of concentrations from the highest concentration tested at which there is growth to the lowest concentration where no growth was observed. NT, not tested.

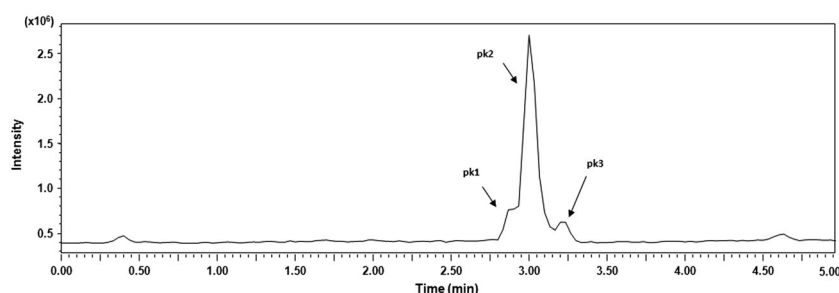


Figure 2. UPLC–MS analysis of the active fraction CSE-XVIII showing the presence of three saponins (pk 1 to pk 3).

1 as a known spirostanol saponin (25*R*)-1 β ,2 α -dihydroxy-5 α -spirostan-3- β -yl-*O*- α -L-rhamnopyranosyl-(1 \rightarrow 2)- β -D-galactopyranoside (Fig. 3, Supporting Information). The spectroscopic values (Table 3) were in agreement with those previously reported by Haraguchi *et al.* (2000), and the identities of sugar moieties were confirmed by comparison with authentic monosaccharide standards

(Sigma-Aldrich, St. Louis, MO, USA) using acid hydrolysis and TLC methods to be L-rhamnose and D-galactose (see Experimental Part). Chemical structure of the aglycone portion (1A) was confirmed by ¹H-NMR and ESI-MS (Supporting Information).

Compound **2** was obtained as an amorphous white powder. HRESIMS (negative mode) showed a

Table 3. ¹H-NMR and ¹³C-NMR spectroscopic data for compounds **1** and **2**

Position	1		2	
	δ_C^1	δ_H^2	δ_C^1	δ_H^2
1	82.6	3.12, d (9.0)	82.5	3.13, d (9.2)
2	77.2	3.38, t (9.2)	77.0	3.39, t (9.0)
3	81.1	3.59, dd (6.0, 9.0)	81.6	3.52, dd (6.2, 9.0)
4	33.6	H-4a, 1.69, m H-4b, 1.49, m	34.0	H-4a, 1.71, m H-4b, 1.45, m
5	43.0	1.08, m	42.9	1.11, m
6	32.5	H-6a, 1.67, m H-6b, 1.58, m	32.5	H-6a, 1.64, m H-6b, 1.59, m
7	33.3	H-7a, 1.69, m H-7b, 0.92, m	33.3	H-7a, 1.70, m H-7b, 0.90, m
8	36.6	1.54, m	37.0	1.54, m
9	56.7	0.89, m	56.7	0.85, m
10	42.7			
11	29.2	H-11a, 1.49, m H-11b, 1.42, m	29.3	H-11a, 1.40, m H-11b, 1.40, m
12	41.6	H-12a, 1.67, m, H-12b, 1.13, m	41.3	H-12a, 1.70, m H-12b, 1.13, m
13	41.3			
14	57.7	1.17, dd (3.5, 12.5)	57.6	1.12, m
15	32.9	H-15a, 1.97, m H-15b, 1.23, m	33.0	H-15a, 1.97, m H-15b, 1.25, m
16	82.1	4.36, dd (7.5, 14.5)	82.2	4.37, dd (7.8, 14.6)
17	64.0	1.72, dd (7.0, 8.5)	64.0	1.71, m
18	17.5	0.78, s	17.5	0.79, s
19	8.7	0.90, s	8.7	0.90, s
20	43.0	1.89, qui (7.0)	42.8	1.90, qui (6.8)
21	14.9	0.95, d (7.0)	14.9	0.95, d (7.2)
22	110.6		110.6	
23	29.9	H-23a, 1.62, m H-23b, 1.40, m	29.9	H-23a, 1.60, m H-23b, 1.42, m
24	25.1	H-24a, 2.32, dd (3.5, 14.5) H-24b, 1.44, m	25.1	H-24a, 2.31, dd (2.8, 14.4) H-24b, 1.42, m
25	31.5	1.62, m	31.5	1.57, m
26	67.9	H-26a, 3.44, m H-26b, 3.30, m	67.9	H-26a, 3.45, m H-26b, 3.32, m
27	17.0	0.79, d (6.0)	17.0	0.79, d (5.0)
1'	101.1	4.42, d (8.0)	103.2	4.31, d (7.6)
2'	77.1	3.65, dd (5.2, 7.2)	76.9	3.49, m
3'	76.1	3.60, dd (3.5, 9.5)	72.4	3.52, dd (4.0, 8.5)
4'	76.7	3.51, dd (4.6, 7.5)	74.5	3.48, dd (4.0, 7.0)
5'	71.0	3.75, brd (3.0)	70.4	3.80, brd (2.0)
6'	62.7	H-6'a, 3.77, dd (7.0, 11.5) H-6'b, 3.67, dd (5.0, 11.0)	62.7	H-6'a, 3.80, dd (8.0, 11.6) H-6'b, 3.67, dd (4.4, 11.0)
1''	102.4	5.16, d (1.5)		
2''	72.2	3.91, dd (1.7, 3.2)		
3''	72.4	3.64, dd (1.7, 4.0)		
4''	74.0	3.38, t (9.2)		
5''	69.8	4.08, dd (6.0, 9.5)		
6''	18.0	1.24, d (6.0)		

¹Measured in 500 MHz for ¹H and 125 MHz for ¹³C, in CD₃OD.

²Coupling constants values (*J*) in Hz in parentheses.

quasimolecular ion $[M-H]^-$ at m/z of 609.2575 ($C_{33}H_{53}O_{10}$), $[\alpha]_D -50.5$. IR, optical rotation, and MS data of compound **2** were closely related to those displayed by compound **1**. The analysis of ESI-MS fragments showed that **2** contained the same aglycone portion and hexopyranose moiety present in **1** [m/z 449 ($M-162$)] (Supporting Information). The absence of the deoxyhexopyranosyl moiety in **2** was further confirmed by the analysis of 1D-NMR spectral data (Table 3). The ^{13}H -NMR and ^{13}C -NMR of compound **2** displayed again characteristic signals for a spiro-steroidal saponin: one acetalic carbon at δ_C 110.6 (C-22); two quaternary methyl groups at δ_H/δ_C 0.79/17.5 (C-18) and 0.9/8.7 (C-19); two tertiary methyl groups at δ_H/δ_C 0.95/14.9 (C-21) and 0.79/17.0 (C-27); four hydroxymethine of the aglycone skeleton at δ_H/δ_C 3.13/82.5 (C-1), 3.39/77.0 (C-2), 3.52/81.6 (C-3), and 4.37/82.2 (C-16); four hydroxymethine groups assignable to a hexopyranosyl moiety at δ_H/δ_C 3.49/76.9 (C-2'), 3.52/72.4 (C-3'), 3.48/74.5 (C-4'), and 3.80/70.4 (C-5'); one anomeric group at δ_H/δ_C 4.31/103.2 (C₁) and two hydroxymethylene groups, one belonging to the aglycone portion [δ_H/δ_C 3.45 (26a), 3.32 Hb/67.9 (C-26)] and the second to the sugar moiety [δ_H/δ_C 3.80 (Ha), 3.67 (Hb)/62.7 (C-6')] (Supporting Information). In addition, detailed interpretation of 2D-NMR experiments such as COSY, NOESY, and HMQC and the key long range correlations displayed in the HMBC (Fig. 4) led to the determination that compound **2** is (25*R*)-1 β ,2 α -dihydroxy-5 α -spirostan-3- β -yl-*O*- β -D-galactopyranoside, a monosaccharide derivative of **1** and a novel natural product (Fig. 2). The sugar moiety in **2** was assessed again using acidic hydrolysis, TLC and the measurement of its optical rotation, which identified it to be D-galactose.

Compound **1** had greater inhibitory activity against both yeast-like and filamentous fungi than the positive control berberine, an alkaloid with broad spectrum antifungal activity that is found in various plant species in the Berberidaceae as well as other families (Rabbani *et al.*, 1987; Mahady and Chadwick, 2001; Ficker *et al.*, 2003; Amiguet *et al.*, 2006; Quan *et al.*, 2006). With *S. cerevisiae* strains, the MIC of **1** in liquid culture is at least 15-fold lower than that of berberine and six-fold lower in solid culture. Likewise, the MIC of **1** against *F. graminearum* ZTE-2A was lower than that of berberine. Compound **2** and **1A** (sapogenin) showed little or no activity against *S. cerevisiae*, even at the highest concentration tested (Table 2). In comparison with the microbial-derived antibiotics, compound **1** had a lower MIC range than both geneticin and hygromycin B against *S. cerevisiae*. For *F. graminearum*, the inhibitory activity of compound **1** was comparable to that of hygromycin B; geneticin was more inhibitory toward *F. graminearum* as its MIC was at least four-fold lower than that of compound **1**.

DISCUSSION

Initial antifungal assays using disk diffusion assays revealed pronounced inhibition of *S. cerevisiae*, *C. neoformans*, and *C. albicans*. While not quantitative, the disk diffusion assay is an excellent technique for the preliminary identification of antimicrobials as it can detect inhibition by compounds of varying polarity and solubility (Meazza *et al.*, 2003; Galván *et al.*, 2008). The observed antifungal activity of compound **1** validates the traditional use of *C. schlechtendahl* by the Q'eqchi' Maya healers of Belize. *C. schlechtendahl* (in addition to *S. torvum*, *S. chrysotrichum*, and *C. dumetorum*) is used by Maya healers in the treatment of fungal infections. All of these Solanaceae species contain spirostanol saponins that may have potential applications as antifungal agents. Even though **1** has been isolated from another species in the Solanaceae by Haraguchi *et al.* (2000), this is the first report of antifungal activity by this compound. Other spirostanol saponins isolated from the Solanaceae as well as from other plant families have also been reported to have antifungal activities (Alvarez *et al.*, 2001; Haraguchi *et al.*, 2000; Shen *et al.*, 2003; González *et al.*, 2004; Yang *et al.*, 2006; Lu *et al.*, 2009). Our observation that compound **1** inhibits growth of the human pathogens *C. albicans* and *C. neoformans* suggests it has potential applications in treating mycoses such as ringworm, athlete's foot, and onychomycoses fungal infections of the nails. The inhibition by compound **1** of *C. albicans* D10 is of particular interest because this clinical isolate is resistant to commercial antifungals such as amphotericin B and ketoconazole (Ficker *et al.* 2003). This indicates that the mode of action is not related to ergosterol biosynthesis and that **1** may be useful in treating recalcitrant mycoses.

Because **1** is also active against *F. graminearum*, a major cereal pathogen, further experiments could show potential agricultural applications as well. In support of this, the crude extract of *Cestrum nocturnum* L. leaves was previously reported by Hernández-Albíter *et al.* (2007) to inhibit the germination of conidiospores of the plant pathogenic fungus *Colletotrichum gloeosporioides*. Phytochemical analyses of *C. nocturnum* identified spirostanol saponins with three or more sugar moieties (Ahmad *et al.*, 1995; Mimaki *et al.*, 2001).

Since **1**, **1A**, and **2** have the same sapogenin, and compound **2** and **1A** (sapogenin) showed little or no activity against *S. cerevisiae*, the presence of the two sugar moieties must be important for the antifungal activity of **1**. Although **1A** has not been tested previously, its relatively low antifungal activity is consistent with studies of diosgenin (a structurally similar sapogenin) that showed little or no antifungal activity (Imai *et al.*, 1967; Chalenko *et al.*, 1977). In a structure-activity relationship study by

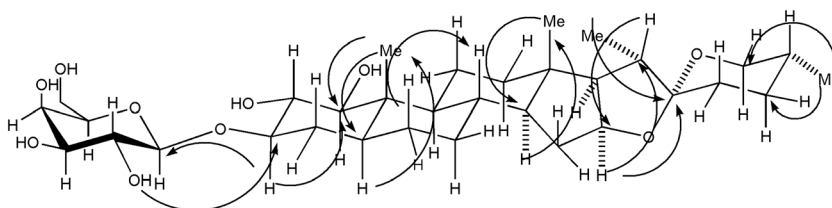


Figure 4. Key HMBC correlations in compound **2**.

Yang *et al.* (2006), spirostanol saponins containing two or more sugar moieties exhibited inhibition of various pathogenic fungi, suggesting that the number of sugar residues present is important to the antifungal activity.

These results show that extracts of *C. schlechtendahl* have antifungal activity and provide pharmacological validation for the traditional use of this plant by Q'eqchi' Maya healers of Belize to treat athlete's foot and other fungal infections. Further in-depth studies using other fungal species and mechanistic experiments would contribute to the assessment of the usefulness of saponins in the treatment of phytopathogenic infections and human mycoses.

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Conflict of Interest

The authors declare no conflict of interest.

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