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Cytotoxic Activity of Casearborin C Isolated from *Casearia corymbosa*

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Abstract. Casearborin c (**1**), syringic acid (**2**), *ent*-3β-hydroxy-(−)-13-*epi*-manoyl oxide (**3**), *ent*-(−)-13-*epi*-manoyl oxide (**4**), *ent*-(−)-kaur-16-en-19-oic acid (**5**), and γ-sitosterol (**6**) were isolated from *Casearia corymbosa* stem bark. Only casearborin c showed cytotoxic activity on HeLa and SiHa cancer cell lines. This work contributes to the description of three compounds (**1**-**3**) newly isolated from *C. corymbosa* and highlights that casearborin c, a clerodane-type diterpene, is responsible for the cytotoxic activity shown in the original methanol extract of this species.
Keywords: Salicaceae; *Casearia corymbosa*; cytotoxic activity; diterpenes; clerodanes; casearborin c.

Resumen. Casearborina c (**1**), ácido siríngico (**2**), óxido de *ent*-3β-hidroxi-(−)-13-*epi*-manoilo (**3**), óxido de *ent*-(−)-13-*epi*-manoilo (**4**), ácido *ent*-(−)-kaur-16-en-19-oico (**5**) y γ-sitosterol (**6**) se aislaron de la corteza del tallo de *Casearia corymbosa*. Sólo casearborina c mostró actividad citotóxica en las líneas de células cancerígenas HeLa y SiHa. Este trabajo describe tres compuestos (**1**-**3**) aislados por primera vez de *C. corymbosa* y destaca que casearborina c, un diterpeno de tipo clerodano, es responsable de la actividad citotóxica del extracto metanólico de esta especie.
Palabras clave: Salicaceae; *Casearia corymbosa*; actividad citotóxica; diterpenos; clerodanos; casearborina c.

Introduction

Cancer is one of the major health problems in the world that urgently requires of an effective cure. Plants play a very important role in finding new treatments for this disease, since about 50% of the drugs available to treat cancer come from natural products or their derivatives [1,2]. The genus *Casearia* includes 180 species [3] and belongs to Salicaceae family. This family is constituted of 54 genera and 1,200 species, composed of trees or bushes of cosmopolitan distribution [4], and considered as source of clerodane-type diterpenes [5-7]. In the Yucatan peninsula there are four species of the genus *Casearia* [8], including *Casearia corymbosa* Kunth. This species is used in Mayan traditional medicine as anti-inflammatory, anti-syphilitic, carminative, antitussive, against asthma, erysipelas and skin wounds [9]. There are very few chemical studies on this species [10,11]. Previously, we evaluated the cytotoxic activity of a MeOH extract of *C. corymbosa* stem bark, showing activity against HeLa and SiHa human cancer cell lines [12]. In a continuing investigation on bioactive molecules from medicinal plants of the Yucatan peninsula, we have undertaken the isolation of metabolites from a cytotoxic MeOH extract of *C. corymbosa* stem bark.

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Experimental procedures

IR spectra were taken with a Nicolet 8700 (FTIR) Thermo Scientific spectrometer using KBr disks. NMR spectra (^{1}H, ^{13}C) were acquired on a Bruker Avance Ultrashield 400 (400 MHz) spectrometer and on a Varian/Agilent Premium Compact 600 MHz spectrometer. Low resolution mass spectra (MS) were taken on an Agilent Technologies 6890N gas chromatograph coupled to a 5975B mass spectrometer (GC-MS). Column chromatography was carried out either over silica gel (60 Å, 0.75 cm^3/g, 2-25 μm, Sigma-Aldrich; 60 Å, 0.75 cm^3/g, 70-230 mesh, Merck) or Sephadex LH-20 (Sigma-Aldrich). TLC was performed on precoated silica gel plates (60 Å, F_{254}, Merck). Spots on TLC were visualized under UV light in a Chromato-Vue® C-75 cabinet and by spraying phosphomolybdic acid or oleum reagents followed by heating. The optical density of the developed plates with sulforhodamine B (SRB) was read in a ThermoSpectronic spectrophotometer at 560 nm.

Plant material. Stem bark of *C. corymbosa* was collected in November 2013 at Othón P. Blanco, Quintana Roo (Mexico). The plant material was identified by the taxonomist Paulino Simá-Polanco from the Unit of Natural Resources of the Scientific Research Center of Yucatan (CICY). A specimen was deposited at CICY's *U Najil Tikin Xiw* herbarium with the voucher number P.Simá 3562.

Extraction and isolation. The stem bark (1.327 Kg) of *C. corymbosa* was dried, grounded, and extracted by maceration with MeOH (4L, 3×) at room temperature for 24 h. The MeOH extract (1A, 112.5 g) was then diluted with MeOH-H_2O (3:1) and partitioned successively with hexane (Hx), CH_2Cl_2, and EtOAc (3× each) to afford the corresponding fractions 2A (30.7 g), 2B (6.6 g), and 2C (16.3 g). Fractions 2A and 2B were cytotoxic. The active fraction 2B was subjected to vacuum liquid chromatography (VLC) using mixtures of Hx/EtOAc of increasing polarity, obtaining nine fractions (3A-3I). Fraction 3D (0.717 g) was subjected to Sephadex LH-20 chromatography with CH_2Cl_2/MeOH (1:1), to obtain three fractions (4A-4C). Fraction 4B (0.497 g) was purified in an open chromatographic column (CC) with CH_2Cl_2/EtOAc (9.5:0.5, 9:1, and 8:2), obtaining eight fractions (5A-5H). Fraction 5E (0.042 g) was subjected to two consecutive CC with CH_2Cl_2/EtOAc (8:2) and (7:3) until compound **1** (8 mg) was isolated. Fraction 3E (0.383 g) was subjected to Sephadex LH-20 with CH_2Cl_2/MeOH (1:1), yielding six fractions (6A-6F). Fraction 6C (0.061 g) was subjected to CC with CH_2Cl_2/EtOAc (8:2), obtaining compound **2** (6.7 mg). The active fraction 2A was subjected to VLC using Hx/CH_2Cl_2 and CH_2Cl_2/EtOAc of increasing polarity, yielding eight fractions (7A-7H). Fraction 7F (10.6 g) was subjected to VLC using Hx/acetone (An) of increasing polarity, obtaining six fractions (8A-8F). Fraction 8A (3.08 g) was subjected to Sephadex LH-20 eluted with Hx/CH_2Cl_2/MeOH (2:1:1) until six fractions were obtained (9A-9F). Fraction 9D (1.65 g) was subjected to CC with Hx/An (9:1) to obtain compound **3** (467.1 mg). Fraction 9B (0.623 g) was subjected to three consecutive CC with Hx/An (9:1) and Hx/EtOAc (9:1) to obtain compound **6** (12.5 mg). Fraction 7B (3.87 g) was subjected to VLC using consecutively Hx/CH_2Cl_2 and CH_2Cl_2/EtOAc of increasing polarity to yield seven fractions (10A-10G). Fraction 10B (0.298 g) was subjected to two consecutive CC with Hx/EtOAc (9.9:0.1) and Hx/CH_2Cl_2 (7.8:2.2) to obtain compound **4** (81.5 mg). Fraction 7D (6.55 g) was subjected to VLC using Hx/CH_2Cl_2 and Hx/EtOAc of increasing polarity, giving six fractions (11A-11F). Fraction 11A (1.07 g) was subjected to Sephadex LH-20 with CH_2Cl_2/MeOH (1:1), yielding compound **5** (189.1 mg).

Biological activities

Cell culture. Cell lines of cervix adenocarcinoma (HeLa, ATCC-CCL-2), cervix squamous carcinoma (SiHa, ATCC-HTB-35), and normal green monkey kidney cell line (Vero, ATCC-CCL-81), from the American Type Culture Collection (ATCC) were maintained in DMEM medium (Gibco), supplemented with fetal bovine serum (10%, v/v), 10,000 U/mL penicillin G, 100 mg/mL streptomycin, and 2.5 mg/mL amphotericin B in an atmosphere with 95% of humidity and 5% CO_2 at 37 °C.

Cytotoxicity assay. Sulforhodamine B (SRB) colorimetric assay was used to estimate cell number by staining total cellular protein with SRB dye [25]. Cells were seeded in 96-well plates (Costar) at a concentration of 5×10^4 cells per well and incubated for 24 h to 48 h; when cells reached 100% confluence, the medium was replaced with new medium without fetal bovine serum (FBS) and the cells were treated with either the organic extract or compound. Previously, a stock of the extract or compound dissolved in dimethylsulfoxide was prepared at a concentration of 10 mg/mL before being added to the culture medium at concentrations of 50, 25, 12.5, and 6.25 μg/mL. After 48 h of incubation, cells were fixed with 100 μL of trichloroacetic acid (TCA, Aldrich) for 30 min at 4 °C and then 100 μL SRB (0.1% SRB in 1% acetic acid,

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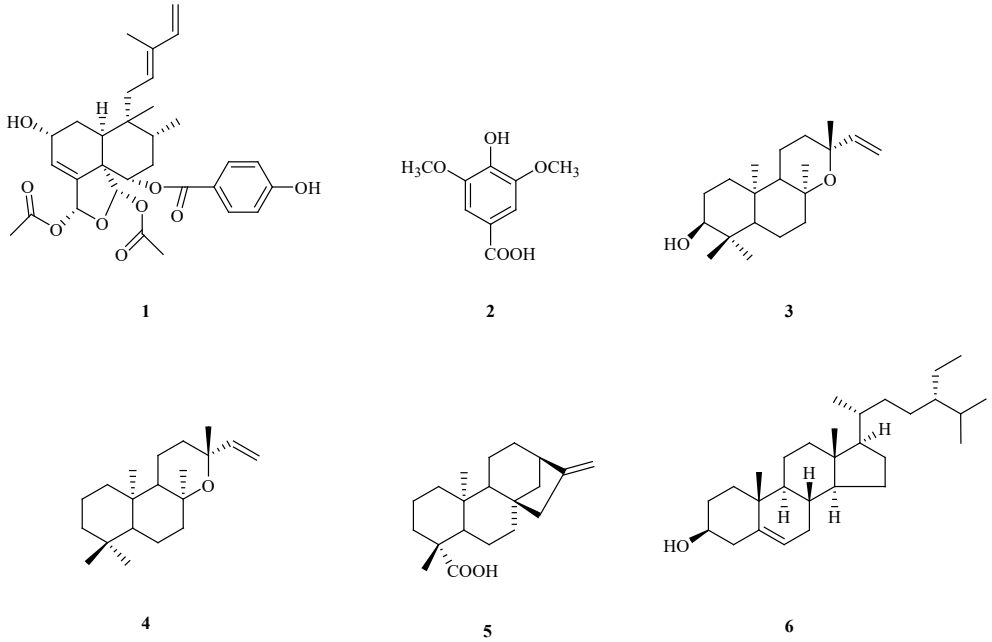
Sigma) were added to each well and incubated at room temperature for 15 min; later the supernatant was retired and plates were washed twice with acetic acid (1%). Subsequently, 100 µL of Tris-base (10 mM) were added to solubilize the dye and optical density was read on a spectrophotometer at 560 nm using an ELISA reader (model 450, Bio-Rad, USA). Untreated cells were used as negative control and docetaxel as positive control. The CC_{50} values were obtained by nonlinear regression using GraphPad-Prism 4.00 software. We also carried out an antiproliferative assay using SRB; the method is the same as that for the cytotoxicity assay, except that when cells reached 70-80% confluence the medium was replaced with DMEM 10% FBS to induce cellular proliferation during extract treatments.

Results and Discussion

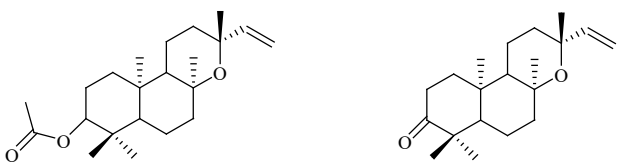
The crude MeOH extract was subjected to a bioguided fractionation using a HeLa cancer cell line in the *in vitro* assay to evaluate fractions and pure compounds. Then, the MeOH extract was submitted to liquid-liquid partition, being the hexane and dichloromethane fractions the more active, from which casearborin c (**1**), syringic acid (**2**), *ent*-3β-hydroxy-(−)-13-*epi*-manoyl oxide (**3**), *ent*-(−)-13-*epi*-manoyl oxide (**4**), *ent*-(−)-kaur-16-en-19-oic acid (**5**), and γ-sitosterol (**6**) were isolated, being **1** and **3**-**5** diterpenes. Due to its high yield and having an OH group in its structure, **3** was subjected to acetylation and oxidation derivatization to obtained **7** and **8**, respectively. Structures of **1**-**6** were confirmed by comparison of their RMN spectral data to those published in the literature (Fig. 1), while structures of **7** and **8** (Fig. 2) were confirmed by comparison of their IR and NMR spectral data with those of the original sample (compound **3**).



1 **2** **3**

4 **5** **6**

Fig. 1. Compounds isolated from *C. corymbosa* stem bark.



7 **8**

Fig. 2. Acetylated and oxidized derivatives of compound **3**.

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Casearborin c (**1**), a clerodane-type diterpene, was isolated for the first time from *Casearia arborea* roots [13]. Syringic acid (**2**), a phenolic acid, is characterized by being a simple molecule [14]. *Ent*-3β-hydroxy-(−)-13-*epi*-manoyl oxide (**3**) is a labdane-type diterpene derived from manoyl oxide [15,16]. The chemical shift value of H-3 at δ 3.22 (1H, dd, J = 4.5, 11.6 Hz) of **3** confirmed that the OH is axial and made possible to establish that the structure of **3** actually corresponds to the isomer of *ent*-3α-hydroxy-(−)-13-*epi*-manoyl oxide [17], formerly isolated from *C. corymbosa* [10]. This is the first report of compounds **1**-**3** from *C. corymbosa*. Compounds **4**, a labdane-type diterpene [18,19], and **5**, a kaurene-type diterpene [20,21], have previously been isolated from *C. corymbosa* [10]. γ-Sitosterol (**6**), a common sterol in plants [22], was identified by comparison of its GC-MS mass spectrum with that from the NIST database.

Casearborin c (**1**) exhibited high cytotoxic activity on HeLa cancer cell line and low cytotoxic activity on SiHa cell line and normal Vero cell line, with mean cytotoxic concentration values of 13.44, 77.36, and 50.26 μM, respectively, displaying low selectivity on HeLa line (3.7) and no selectivity on SiHa line (0.6) (Table 1). This compound has been evaluated on melanoma (LOX) and glioblastoma (SF539) cell lines, reporting a cytotoxic effect [13]. The isolated compounds had no antiproliferative activity at the concentrations evaluated (data not included). Syringic acid (**2**) has displayed cytotoxic activity on two colorectal cancer cell lines (SW1116 and SW837) [23]. *Ent*-3β-hydroxy-(−)-13-*epi*-manoyl oxide (**3**) has been evaluated on HeLa cell line by Su *et al*. [24], showing low cytotoxic activity. In the present study we also evaluated **3** against HeLa, but no activity was obtained; however, we tested lower concentrations than those used by Su *et al*. Compounds **5** and **7** showed cytotoxic activities only at the highest concentration (50 μg/mL) on the cell lines evaluated, while **4**, **6**, and **8** presented no effect.

Table 1. Cytotoxic activity of casearborin c in different cell lines.

Compound	**CC_{50} μM[a] (SI)[b]**		
	Vero	**HeLa**	**SiHa**
Casearborin c	50.26	13.44 (3.7)	77.36 (0.6)
Docetaxel	1.36	0.25 (5.5)	0.22 (6.1)

[a]50% cytotoxic concentration. [b]SI: selective index.

Conclusions

The genus *Casearia* is considered a rich source of diterpenes of the clerodane type, which are suggested as the main responsible for the cytotoxic activity reported in different cancer cell lines [5,13]. Among the isolated metabolites from *C. corymbosa* in the present study, there were diterpenes of clerodane, labdane, and kaurene types, as well as a phenolic acid and a sterol. This study highlights the fact that the clerodane-type diterpenes are the only ones responsible for the observed cytotoxic activity in the crude MeOH extract, since casearborin c was exclusively the one that showed activity; also, this work contributes to the phytochemical knowledge of *C. corymbosa*, since casearborin c, syringic acid, and e*nt*-3β-hydroxy-(−)-13-*epi*-manoyl are being reported for the first time from this species.

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