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Antigiardial Activity of *Cupania dentata* Bark and its Constituents

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Abstract. The MeOH extract of *Cupania dentata* bark (Sapindaceae) as well as its hexane, CH_2Cl_2, EtOAc, and BuOH fractions showed high activity against *Giardia lamblia* trophozoites (IC_{50} = 2.12-9.52 μg/mL). The phytochemical study of fractions resulted in the isolation of taraxerone (**1**), taraxerol (**2**), scopoletin (**3**), and two mixtures of steroidal compounds. Taraxerone was the metabolite with the highest giardicidal activity (IC_{50} = 11.33 μg/mL).
Key words: *Cupania dentata*, Sapindaceae, giardicidal activity, taraxerone, taraxerol, scopoletin, sterols.

Resumen. El extracto MeOH de *Cupania dentata* corteza (Sapindaceae) así como sus fracciones de hexano, CH_2Cl_2, AcOEt y BuOH mostraron gran actividad contra los trofozoítos de *Giardia lamblia* (CI_{50} = 2.12-9.52 μg/mL). El estudio fitoquímico de estas fracciones resultó en el aislamiento de taraxerona (**1**), taraxerol (**2**), escopoletina (**3**) y dos mezclas esteroidales. Taraxerona tuvo la más alta actividad giardicida (CI_{50} = 11.33 μg/mL).
Palabras clave: *Cupania dentata*, Sapindaceae, actividad giardicida, taraxerona, taraxerol, escopoletina, esteroles.

Introduction

The protozoo *Giardia lamblia* is the most frequently isolated intestinal protozoan parasite around the world and it is the causal agent of the disease known as giardiasis [1]. In Latin America the prevalence of giardiasis is 3.7-22.3% [2] and a recent seroepidemiologic study in Mexico found an seroprevalence of 55.3%, with no significant differences among geographic regions according to their economic development [3]. The pharmacological treatment of giardiasis is based mainly in the use of nitroimidazoles, benzimidazoles, and nitrofurans; nevertheless, these drugs produce severe side effects and their indiscriminate use has generated a selection of resistant strains to these drugs [4, 5]. Due to this situation, the search for new drugs becomes necessary.

The *Cupania* L. genus comprises 45 species growing around warm places of the American continent [6]. *C. belizensis* is useful in treating diarrhea [7], *C. americana* leaves and seeds are used to treat pain and diarrhea, respectively [8], while *C. vernalis* leaves show antileishmanial activity [9]. Chemical compounds have been reported from some species, such as vernanolide, a glycosyl diterpene isolated from *C. vernalis* [9], a long-chain fatty alcohol glycoside named cupanioside from *C. glabra* [7], and a polyprenol named cupaniol from *C. latifolia* [8].

Taking advantage of the ancestral knowledge on the medical use of some plant species growing in the Yucatan peninsula and the giardicidal activity described for the MeOH extract of *Cupania dentata* bark [10], we studied the hexane, CH_2Cl_2, EtOAc, and BuOH fractions of the MeOH extract of *C. dentata* bark in order to obtain those metabolites responsible of the giardicidal activity of the plant. Not any phytochemical or biological study has been reported for this species.

Results and Discussion

The methanol extract as well as the hexane, CH_2Cl_2, EtOAc, and BuOH fractions obtained by chromatographic partitioning were all evaluated against *G. lamblia* trophozoites (Table 1). The biological activity of the MeOH extract (IC_{50} = 8.17 μg/mL) was similar to that obtained previously (IC_{50} = 7.59 μg/mL) [10] and can be considered highly active according to the criteria established by Amaral *et al*. [11], who established extracts with $IC_{50} \leq 100$ μg/mL as highly actives. The biological activity of *C. dentata* fractions remained similar to that of the crude MeOH extract (Table 1), in some cases stronger, such as the hexane and CH_2Cl_2 fractions (IC_{50} = 4.43 and 2.12 μg/mL, respectively).

Compounds **1** and **2** were isolated from the hexane fraction and were identified as taraxerone and taraxerol, respectively, by comparison of their spectroscopic data with those reported in the literature [12-14]. Both compounds are ubiquitous metabolites found in a large number of plants. Compounds **1** and **2** have been reported with different biological activities, such as allelopathic and antifungal [15, 16]; taraxerol has been reported with analgesic and anti-inflammatory activities [17, 18].

Also, from the hexane fraction a mixture (**A**) of stigmasterol and β-sitosterol in a ratio 2:1 was obtained. The sterols were identified by comparison their MS data with the database of the equipment and to those reported in the literature [19]. The ratio was determined by inspection of the gas chromatogram. Both are ubiquitous compounds of plants. β-Sitosterol possesses different activities, such as antibacterial, antimicotic, and as an inhibitor of the carcinogenesis [20], while stigmasterol was found to inhibit tumor promotion in two-stage carcinogenesis in mice [21]. The mixture of both sterols has shown anti-inflammatory activity in topical applications [22].

Table 1. Antigiardial activity of methanol extract of *C. dentata* bark, its fractions and pure isolates.

Sample	IC_{50} (μg/mL)	IC_{90} (μg/mL)
MeOH extract	8.17 (8.17-8.22)[a]	1,573.26 (1,514.86-1,634.63)
Hexane fraction	4.43 (4.42-4.44)	76.20 (75.43-77.01)
CH_2Cl_2 fraction	2.12 (2.11-2.13)	135.52 (133.02-138.06)
EtOAc fraction	9.52 (9.48-9.56)	311.39 (305.25-317.72)
BuOH fraction	6.50 (6.48-6.52)	102.31 (101.12-103.52)
Taraxerone (**1**)	11.33 (11.30-11.36)	63.31 (62.90-63.72)
Taraxerol (**2**)	16.11 (16.05-16.17)	102.40 (101.51-103.31)
Scopoletin (**3**)	33.60 (33.4-33.8)	282.55 (279.2-286.0)
Taraxerol acetate (**4**)	NA[b]	NA
Mixture **A**	5.23 (5.22-5.24)	32.74 (32.57-32.91)
Mixture **B**	26.77 (26.69-26.86)	386.63 (383.47-389.88)
Metronidazole	0.21 (0.20-0.22)	

[a] 95% Confidence interval in parentheses.
[b] NA = Not active.

Compound **3** was isolated from the CH_2Cl_2 fraction and was identified as scopoletin, a 6,7-dioxygenated coumarin, by comparison of its spectroscopic data with those reported in the literature [23, 24]. Scopoletin exhibited a potent inhibitory effect on rabbit platelet aggregation [25] and showed activity as inhibitor of eicosanoid-release from ionophore-stimulated mouse peritoneal macrophages [26].

The mixture **B** was isolated from the BuOH fraction and was identified as a combination of β-sitosterol-3-*O*-β-D-glucopyranoside and stigmasterol-3-*O*-β-D-glucopyranoside (1:1.5). The ratio was determined by inspection of the ^{1}H NMR spectrum. The components of this mixture were identified by comparison of their spectroscopic data, mainly ^{13}C NMR, with those reported in the literature [27].

None of the metabolites mentioned above has been reported in the literature as having antigiardial activity, except for two reports on β-sitosterol and its glucoside [28, 29] with an IC_{50} = 71.1 and 61.5 μg/mL, respectively, but the mixture of stigmasterol-β-sitosterol (2:1) has not been reported with this activity. In the present work, the two triterpenes taraxerone (**1**) and taraxerol (**2**) showed an IC_{50} = 11.33 and 16.11 μg/mL, respectively, while the coumarin scopoletin (**3**) had an IC_{50} = 33.60 μg/mL in the growth inhibition bioassay. In the literature there are 31 metabolites reported as having antigiardial activity with an $IC_{50} \leq 25.0$ μg/mL [11], then the antigiardial activity of **1** and **2**, in comparison, is considered outstanding on this aspect, but moderate with respect to the drug of election, metronidazole, which showed an IC_{50} = 0.2 μg/mL. Taraxerol acetate did not show antigiardial activity in the same model. Scopoletin (**3**) showed only a week activity against *G. lamblia*, but this is the first report of scopoletin as having this activity. The mixture of sterols (**A**) showed a stronger activity, with an IC_{50} = 5.23 μg/mL, which, perhaps, depends on the synergism that there might be within stigmasterol and β-sitosterol together, but more studies are necessary to confirme this hypothesis. The mixture (**B**) of stigmasterol and β-sitosterol glucosides showed less activity (IC_{50} = 26.77 μg/mL) than the mixture (**A**). In other bioassay models, some works have demonstrated activity of various mixtures, such as that formed of the stilbene pinosylvin and the flavonoid galangin that worked in concert to provide antifeedant activity, while they were not active individually [30].

General Experimental Procedures

EIMS data were determined on an Agilent Technologies 6890N chromatograph connected to a mass detector 5975B. The ^{1}H and ^{13}C NMR data were obtained on a Bruker Avance 400 instrument (400 MHz). Chemical shifts were referred to TMS (δ 0) as internal standard. Vacuum liquid chromatography (VLC) separations were carried out using TLC-grade silica gel (Merck); open-column chromatography separations were run using silica gel 60 (70-230 mesh, Merck); and flash columns were run using silica gel 60 (230-400 mesh, Merck). Sephadex

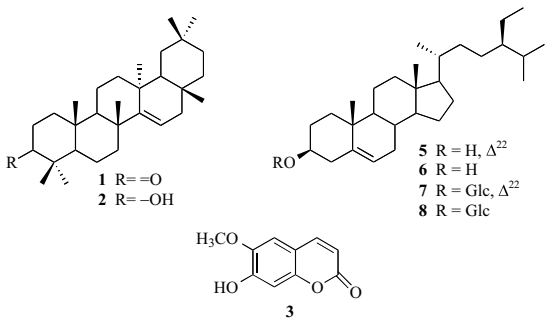

Fig. 1. Structures of the metabolites isolated from *C. dentata*. Taraxerone (**1**), taraxerol (**2**), scopoletin (**3**), stigmasterol (**5**), β-sitosterol (**6**), stigmasterol glucoside (**7**) and β-sitosterol glucoside (**8**).

LH-20 (GE Healthcare) was used for gel permeation column chromatography. Preparative TLC (PTLC) separations were performed on glass-coated (1 mm thickness) 20 × 20 cm plates (Aldrich). TLC analyses were carried out using aluminum-backed silica gel 60 F_{254} plates (0.20 mm thickness, Merck); spots on TLC plates were first visualized under a UV lamp (254 and 365 nm) and then by spraying with 4% phosphomolibdic acid containing a trace of ceric sulfate in 5% H_2SO_4, followed by heating at 100 °C.

Plant Material

The bark of *Cupania dentata* DC. (Sapindaceae) was collected 22 Km west of Bacalar on the road to Carrillo Puerto, Quintana Roo, Mexico, and identified by the experienced taxonomist Paulino Simá Polanco. A voucher specimen (PSimá 2587) was deposited on the herbarium of the Unidad de Recursos Naturales of the Centro de Investigación Científica de Yucatán.

Extraction and Isolation

Dried-ground bark (983 g) was extracted with methanol three times at room temperature, for 48 h each time. After filtration, the extracts were combined and the solvent was evaporated under reduced pressure to give 166.4 g of organic extract. The extract was suspended in a mixture of methanol/water (1:3, 500 mL) and the resulting aqueous suspension was successively partitioned between hexane (2:1, 3×), CH_2Cl_2 (2:1, 3×), EtOAc (2:1, 3×), and BuOH (2:1, 3×), to afford the hexane (6.1 g), CH_2Cl_2 (11.0 g), EtOAc (59.2 g), and BuOH (30.2 g) fractions.

The hexane fraction was purified by VLC using gradient elutions of hexane/ethyl acetate, ethyl acetate/acetone and acetone/methanol to produce nine fractions (3A-3I) and a precipitate, which was washed with methanol and then recrystallized from chloroform to give 51.9 mg of the mixture (**A**) of stigmasterol and β-sitosterol. The fractions 3B and 3C presented two different precipitates, both were washed with methanol and then recrystallized from chloroform, to give taraxerone (**1**, 16.0 mg), with m.p. = 241-243 °C and $[\alpha]_D$ = + 11.9° ($CHCl_3$) [31], and taraxerol (**2**, 52.2 mg), with m.p. = 279-280 °C and $[\alpha]_D$ = + 2.9° ($CHCl_3$) [31].

The CH_2Cl_2 fraction was purified by VLC, using gradient elutions of hexane/dichloromethane and dichloromethane/methanol to produce seven fractions (4A-4G). Open-column chromatography purification of fraction 4D (538.1 mg) eluting with dichloromethane and gradient mixtures of dichloromethane/methanol produced 11 fractions (5A-5K). Further purification of fraction 5C (136.9 mg), using an open chromatography column with chloroform/methanol (98:2), afforded fractions 6D and 6E, which were washed with hexane and then recrystallized from methanol to give 37.7 mg of compound **3**, with m.p. = 199-201 °C [32].

The BuOH fraction was purified by a Sephadex-LH-20 column using gradient elutions of methanol/butanol/water to produce nine fractions (7A-7I). Fraction 7B was further purified using a Sephadex-LH-20 column in methanol to obtained 58.7 mg of crystals of mixture (**B**), constituted by stigmasterol glucoside and β-sitosterol glucoside.

Taraxerol acetate (**4**). A mixture of taraxerol (10 mg), acetic anhydride (1 mL) and pyridine (0.5 mL) was allowed to stir at room temperature for 72 h. After the usual work up 7.3 mg (66.4%) of crude acetylated product, identified as taraxerol acetate (**4**), were obtained, with m.p. = 296-298 °C [31].

Taraxerone (1). White crystals: mp 241-243 °C; $[\alpha]_D$ +11.9 (*c* 0.360, $CHCl_3$); ^{1}H NMR ($CDCl_3$, 400 MHz) δ 5.56 (1H, dd, *J* = 3.2, 8.2 Hz, H-15), 2.58 (1H, m, H-2a), 2.33 (1H, m, H-2b), 2.07 (1H, dt, *J* = 3.3, 12.9 Hz, H-7a), 1.92 (1H, dd, *J* = 3.1, 15.1 Hz, H-16a), 1.88 (1H, m), 1.14 (3H, s, H-25), 1.09 (3H, s, H-26), 1.08 (3H, s, H-23), 1.07 (3H, s, H-24), 0.95 (3H, s, H-29), 0.92 (3H, s, H-27), 0.91 (3H, s, H-30), 0.83 (3H, s, H-28); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 217.7 (C-3), 157.6 (C-14), 117.2 (C-15), 55.8 (C-5), 48.7 (C-18), 48.7 (C-9), 47.6 (C-4), 40.6 (C-19), 38.9 (C-8), 38.3 (C-1), 37.7 (C-13), 37.7 (C-17), 37.5 (C-10), 36.7 (C-16), 35.8 (C-12), 35.1 (C-7), 34.1 (C-2), 33.5 (C-21), 33.3 (C-29), 33.1 (C-22), 29.8 (C-26), 29.9 (C-28), 28.8 (C-20), 26.1 (C-23), 25.6 (C-27), 21.5 (C-24), 21.3 (C-30), 19.9 (C-6), 17.4 (C-11), 14.8 (C-25); EIMS *m*/*z* (rel. int.): 424 $[M]^+$ (25), 300 (87), 285 (70), 204 (100), 133 (67).

Taraxerol (2). White crystals: mp 279-280 °C; $[\alpha]_D$ +2.9 (*c* 0.490, $CHCl_3$); ^{1}H NMR ($CDCl_3$, 400 MHz) δ 5.53 (1H, dd, *J* = 3.2, 8.2 Hz, H-15), 3.24 (1H, dd, *J* = 4.7, 11.0 Hz, H-3), 2.03 (1H, dt, *J* = 3.1, 12.6 Hz, H-7a), 1.92 (1H, dd, *J* = 3.0, 14.6 Hz, H-16a), 1.09 (3H, s, H-26), 0.98 (3H, s, H-23), 0.95 (3H, s, H-29), 0.93 (3H, s, H-25), 0.91 (3H, s, H-27), 0.90 (3H, s, H-30), 0.82 (3H, s, H-28), 0.80 (3H, s, H-24); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 158.1 (C-14), 116.9 (C-15), 79.1 (C-3), 55.5 (C-5), 49.2 (C-18), 48.7 (C-9), 41.3 (C-19), 39.0 (C-4), 38.7 (C-8), 38.0 (C-1), 37.7 (C-17), 37.6 (C-13), 37.5 (C-10), 36.6 (C-16), 35.8 (C-12), 35.1 (C-7), 33.7 (C-21), 33.3 (C-29), 33.1 (C-22), 29.9 (C-28), 29.8 (C-26), 28.8 (C-20), 28.0 (C-23), 27.1 (C-2), 25.9 (C-27), 21.3 (C-30), 18.8 (C-6), 17.5 (C-11), 15.4 (C-24), 15.4 (C-25); EIMS *m*/*z* (rel. int.): 426 $[M]^+$ (9), 302 (25), 287 (25), 204 (100), 133 (40).

Scopoletin (3). Yellow crystals: mp 199-201 °C; ^{1}H NMR (CD_3OD, 400 MHz) δ 7.86 (1H, d, *J* = 9.5 Hz, H-4), 7.13 (1H, s, H-5), 6.78 (1H, s, H-8), 6.21 (1H, d, *J* = 9.6 Hz, H-3), 3.81 (3H, s, 6-OCH_3); ^{13}C NMR (CD_3OD, 100 MHz): δ 164.1 (C-2), 152.9 (C-7), 151.4 (C-9), 147.1 (C-6), 146.1 (C-4), 112.6 (C-3), 112.6 (C-10), 109.9 (C-5), 103.9 (C-8), 56.8 (6-OCH_3); EIMS *m*/*z* (rel. int.): 192.1 $[M]^+$ (100), 177 (64), 164 (29), 149 (60), 121 (27), 69 (43), 79 (21).

Biological evaluation

Giardia lamblia trophozoites

Giardia lamblia IMSS:0696:1 isolate, obtained from an individual with symptomatic giardiasis, was used [33]. Trophozoites were cultured in TYI-S-33 modified medium, supplemented with 10% calf serum, and subcultured twice a week; for the assay, trophozoites were tested in their log phase of growth [34].

Antiprotozoal assay

The assay has been described in the literature [35-37]. Stock solutions of extracts, fractions, or pure compounds were prepared with DMSO (5 mg/mL), from which, by means of two-folded serial dilutions with TYI-S-33 modified medium, four final solutions in a range of 1-50 μg/mL were obtained. Each solution was inoculated with *G. lamblia* to achieve an inoculum of 5×10^4 trophozoites/mL. The test included metronidazole (Sigma-Aldrich) as the drug of reference, a control (culture medium with trophozoites and DMSO), and a blank (culture medium). After 48 h at 37 °C, parasites were detached by chilling, and 50 μL of each culture tube were subcultured in fresh medium without extracts or drug and incubated for 48 h at 37 °C. Cell proliferation was measured with a hemocytometer, and the percentage of trophozoite growth inhibition was calculated by comparison with the controls. The percentage of inhibition calculated for each concentration was transformed into Probit units. The plot of Probit against log concentration was made; the best straight line was determined by regression analysis, and the 50% inhibitory concentration (IC_{50}) values were calculated. The experiments were done in duplicate and repeated at least three times.

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