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Original Article

Antigiardial activity of flavonoids from leaves of *Aphelandra scabra*

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ABSTRACT

Aphelandra scabra (Vahl) Sm., Acanthaceae, is a shrub widely used by some Mayan communities as carminative, antidote, and remedy for some infections. Bio-guided isolation of the methanol extract of leaves led us to the purification of the anti-giardial metabolites cirsimaritin and sorbifolin, along with the inactive metabolites cirsimarin, sorbifolin-6-*O*-β-glucopyranoside, and squalene. Cirsimaritin displayed high activity in the anti-giardial bioassay with an IC_{50} = 3.8 μM, being considered as outstanding when compared to previous reported metabolites, while sorbifolin showed a low activity with an IC_{50} = 75.6 μM. Additionally, both compounds proved not to be cytotoxic in an *in vitro* bioassay against HEK-293, a normal cell line. This is the first investigation on anti-giardial properties of *A. scabra* and its phytochemistry as well, thus the isolated compounds are considered as new for the plant genus and for the species.

Introduction

Giardiasis caused by the flagellated protozoan *Giardia lamblia* (*G. intestinalis*, *G. duodenalis*) is the most common parasitic disease affecting humans worldwide (Vázquez-Tsuji and Campos-Rivera, 2009). It causes an estimated 2.8×10^8 cases per year (Lane and Lloyd, 2002). In Mexico, the National System for Epidemiologic Surveillance reported 20,599 cases in 2010 (SINAVE, 2010); whereas Yucatan shows an incidence of 15.2% (Mantilla-Morales et al., 2002).

Symptoms of giardiasis include abdominal cramps and pain, and diarrhea, which can lead to malabsorption and failure of children to thrive (Tejman-Yarden and Eckmann, 2011). A variety of chemotherapeutic agents such as 5-nitroimidazole compounds, quinacrine, furazolidone, paromomycin, benzimidazole compounds, and nitazoxanide have been used in the therapy for giardiasis. Nevertheless, most drugs used have considerable adverse effects and in addition *Giardia* seems to have a great ability to resist these agents (Bussati et al., 2009). Thus, the discovery of new, effective, and safe antigiardial agents is imperative from other sources (Peraza-Sanchez et al., 2005).

Aphelandra scabra (Vahl.) Sm., Acanthaceae, a plant distributed in the southeast of Mexico, Central and South America (Flora digital: Peninsula de Yucatan, 2010), is used in Mayan *Q' eqchi'* traditional medicine to treat several conditions, including circulatory, mental, and nervous system disorders, poisoning, and infections (Treyvaud et al., 2005). In Honduras, a beverage made from mashed roots and/or leaves soaked in cool water is used as an antiflatulent (Lentz, 1993). Antimicrobial and leishmanicidal activities of the methanol extract from this plant have been reported (Peraza-Sánchez et al., 2007; Meurer-Grimes et al., 1996), therefore, a study was conducted in order to investigate the anti-giardial properties of this species.

In this study, we report the isolation of four known flavonoids and a triterpene from the leaves of *A. scabra* and their anti-giardial activity. It is worth mentioning that this is the first phytochemical investigation of *A. scabra*.

Materials and methods

General experimental procedures

Melting points were determined using a Melt-Temp II Apparatus (Laboratory Devices, USA). GC–MS data were determined on an Agilent 6890N gas chromatograph coupled to a 5975B mass spectrometer. ^{1}H NMR (400 MHz) and ^{13}C NMR (100 MHz) data were obtained on a Bruker Avance 400 spectrometer. Chemical shifts were referred to TMS (δ 0). IR spectra were recorded as KBr pellets on a Nicolet Protegé 460. UV spectra were performed on a Genesys 10 UV ThermoSpectronic. Vacuum liquid chromatography (VLC)

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Table 1
Yield and anti-giardial activity of methanol extract and fractions of *A. scabra*.

Sample	Yield [g (%)]	IC_{50} (μg/ml)
MeOH extract (ASH-1)	435.0 (14.41)[a]	11.74 ± 1.12[c]
Hx fraction (ASH-2a)	21.5 (4.94)[b]	NA[d]
DCM fraction (ASH-2b)	4.3 (0.99)[b]	2.19 ± 0.67
EtOAc fraction (ASH-2c)	4.6 (1.05)[b]	NA
Aqueous fraction (ASH-2d)	62.0 (14.25)[b]	NA
Metronidazole		0.2 ± 0.02

[a] Yield based on initial weight of dried material.
[b] Yield based on weight of methanol extract.
[c] Standard deviation.
[d] Not active.

was performed under vacuum using silica gel 60 GF_{254} (200–400 mesh, Sigma–Aldrich). Thin layer chromatography (TLC) was carried out on silica gel F_{254} (Merck) and visualized under UV light and by spraying with phosphomolybdic acid reagent followed by heating.

Plant material

Aphelandra scabra (Vahl.) Sm., Acanthaceae, leaves were collected from its natural habitat, in the locality of Piste, Yucatan (Mexico), in July 2010. A voucher specimen (PSima-3019), identified by a qualified taxonomist, was deposited at CICY's *U Najil Tikin Xiu* herbarium.

Extraction and isolation

Ground dried leaves (3018 g) were extracted with methanol for 24 h and then solvent was evaporated under vacuum. This process was repeated once again. The methanol extract (ASH-1, 435 g) was partitioned with hexane (Hx), dichloromethane (DCM) and ethyl acetate (EtOAc), for three times each one. Solvent was evaporated under vacuum. Resulting fractions including aqueous phase (ASH-2a–ASH-2d) were placed in separate vials and weighted (Table 1). The hexane fraction ASH-2a (5 g) was further fractionated in a vacuum liquid chromatography (VLC) column using mixtures of Hx, acetone (An), EtOAc, and MeOH to obtain five fractions (ASH-3a–ASH-3e). Fraction ASH-3a was purified by a chromatographic column (CC) using Hx to afford squalene (**1**, 22.5 mg). The DCM fraction ASH-2b (4 g) was further fractionated by VLC using mixtures of Hx, Hx/An, and An/MeOH to obtain eleven fractions (ASH-4a–ASH-4k). Crystallization with MeOH of fractions ASH-4d–ASH-4i yielded cirsimaritin (**2**, 465.6 mg). The EtOAc fraction ASH-2c (3.8 g) was fractionated by VLC using mixtures of same solvents used to fractionate DCM extract yielding nine fractions (ASH-6a–ASH-6i). Crystallization with MeOH of fraction ASH-6 h gave cirsimarin (**3**, 55.1 mg); meanwhile, crystallization with MeOH of fraction ASH-6c yielded sorbifolin (**4**, 11.3 mg). Fraction ASH-6i was purified by VLC using mixtures of Hx/DCM, DCM/An, and DCM/MeOH to obtain ten fractions (ASH-23a–ASH-23j). The MeOH crystallization of fraction ASH-23f led to the isolation of sorbifolin-6-*O*-β-glucopyranoside (**5**, 5.3 mg). Purities of the isolated flavonoids were verified through TLC and determination of their melting points, while purity of compound **1** was determined by means of CG-EM.

Squalene (**1**): yellow oil; GC–MS Rt = 13.168 min, *m*/*z* (%): 410 ([M+H]$^+$) (1), 341 (3), 273 (1), 137 (14), 95 (18), 41 (37), 69 (100, base peak).

Cirsimaritin (**2**): yellow needles; R_f (Hx/An; 2:1, 2×): 0.37, m.p. 253.8–256.2 °C. UV (An:H_2O; 99:1) λ_{max} nm (log ε): 209 (3.21), 330 (3.39). IR ν_{max} (KBr) cm^{-1}: 3283, 1655, 1600, 1570, 1465, 1445, 1356, 1037, 853. ^{1}H NMR (400 MHz, DMSO-d_6) δ 3.72 (3H, s, 6-OCH_3), 3.90 (3H, s, 7-OCH_3), 6.81 (1H, s, H-3), 6.87 (1H, s, H-8), 6.92 (2H, d, *J* = 8.8 Hz, H-3′, H-5′), 7.93 (2H, d, *J* = 8.8 Hz, H-2′, H-6′), 12.91 (1H, s, 5-OH). ^{13}C NMR (100 MHz, DMSO-d_6) δ 55.7 (6-OCH_3), 59.7 (7-OCH_3), 101.4 (C-8), 103.0 (C-3), 104.6 (C-10), 114.7 (C-3′), 116.3 (C-5′), 120.7 (C-1′), 127.3 (C-2′), 128.9 (C-6′), 131.4 (C-6), 151.6 (C-9), 152.2 (C-5), 158.1 (C-7), 160.8 (C-4′), 163.6 (C-2), 181.7 (C-4).

Cirsimarin (**3**): white amorphous powder; R_f (CH_2Cl_2/MeOH; 4.5:0.5, 3×): 0.50, m.p. 175.8–176.3 °C. UV (MeOH) λ_{max} nm (log ε): 213 (4.89), 277 (4.72), 323 (4.75). IR ν_{max} (KBr) cm^{-1}: 3350, 1660, 1600, 1565, 1460, 1440, 1356, 1047, 833. ^{1}H NMR (400 MHz, DMSO-d_6) δ 3.20–3.71 (6H, sugar H), 3.74 (3H, s, 6-OCH_3), 3.93 (3H, s, 7-OCH_3), 4.65 (1H, s, 6″-OH), 5.05 (1H, d, *J* = 7.1 Hz, H-1″), 5.12 (1H, s, 4″-OH), 5.20 (1H, s, 3″-OH), 5.45 (1H, s, 2″-OH), 6.97 (1H, s, H-3), 6.98 (1H, s, H-8), 7.20 (2H, d, *J* = 8.8 Hz, H-3′, H-5′), 8.08 (2H, d, *J* = 8.8 Hz, H-2′, H-6′), 12.86 (1H, s, 5-OH). ^{13}C NMR (100 MHz, DMSO-d_6) δ 55.6 (7-OCH_3), 60.1 (6-OCH_3), 60.7 (C-6″), 69.7 (C-4″), 73.2 (C-2″), 76.6 (C-5″), 77.2 (C-3″), 91.7 (C-8), 99.8 (C-1″), 103.7 (C-3), 105.2 (C-10), 116.6 (C-3′, C-5′), 123.9 (C-1′), 128.3 (C-2′, C-6′), 131.9 (C-6), 152.1 (C-5), 152.7 (C-9), 158.8 (C-7), 160.4 (C-4′), 163.4 (C-2), 182.4 (C-4).

Sorbifolin (**4**): yellow solid; R_f (Hx/An; 3:2, 2×): 0.52, m.p. 235 °C (*d*). UV (EtOH) λ_{max} nm (log ε): 206 (4.55), 281 (4.24), 338 (4.38). IR ν_{max} (KBr) cm^{-1}: 3173, 1665, 1570, 1500, 1455, 1361, 1027, 828. ^{1}H NMR (400 MHz, DMSO-d_6) δ 3.91 (3H, s, 7-OCH_3), 6.81 (1H, s, H-3), 6.91 (1H, s, H-8), 6.93 (2H, d, *J* = 8.8 Hz, H-3′, H-5′), 7.95 (2H, d, H-2′, H-6′), 8.74 (6-OH), 10.39 (4′-OH), 12.64 (5-OH). ^{13}C NMR (100 MHz, DMSO-d_6) δ 56.3 (7-OCH_3), 91.2 (C-8), 102.5 (C-3), 105.0 (C-10), 116.0 (C-3′, C-5′), 121.4 (C-1), 128.4 (C-2′, C-6′), 129.9 (C-6), 146.2 (C-5), 149.7 (C-9), 154.4 (C-7), 161.2 (C-4′), 163.8 (C-2), 182.3 (C-4).

Sorbifolin-6-*O*-β-glucopyranoside (**5**): white amorphous powder; R_f (CH_2Cl_2/MeOH; 4:1): 0.62, UV (An:H_2O; 62.5:1) λ_{max} nm (log ε): 209 (3.16), 329 (3.04). IR ν_{max} (KBr) cm^{-1}: 3500–3100, 1660, 1605, 1565, 1500, 1460, 1356, 1047. ^{1}H NMR (400 MHz, DMSO-d_6) δ 3.58 (1H, dd, *J* = 4.0, 11.1 Hz, 6b″-OH), 3.90 (3H, s, 7-OCH_3), 4.12 (1H, dd, *J* = 5.0, 10.4 Hz, 6a″-OH), 4.32 (1H, t, *J* = 5.5 Hz, 5″-OH), 4.94 (1H, d, *J* = 5.2 Hz, H-1″), 5.03 (1H, d, *J* = 4.8 Hz, 4″-OH), 5.05 (1H, s, 3″-OH), 5.17 (1H, d, *J* = 3.3 Hz, 2″-OH), 6.86 (1H, s, H-3), 6.92 (2H, d, *J* = 8.7 Hz, H-3′, H-5′), 6.93 (1H, s, H-8), 7.97 (2H, d, *J* = 8.7 Hz, H-2′, H-6′), 10.43 (1H, brs, 4′-OH), 13.06 (1H, brs, 5-OH). ^{13}C NMR (100 MHz, DMSO-d_6) δ 56.6 (7-OCH_3), 60.9 (C-6″), 69.9 (C-4″), 74.2 (C-2″), 76.6 (C-5″), 77.3 (C-3″), 91.7 (C-8), 102.0 (C-3), 102.7 (C-1″), 104.9 (C-10), 116.0 (C-3′, C-5′), 121.1 (C-1), 128.1 (C-6), 128.6 (C-2′, C-6′), 151.7 (C-5), 152.6 (C-9), 158.6 (C-7), 161.3 (C-4′), 164.0 (C-2), 182.3 (C-4).

Parasite and culture conditions

In this study, *G. lamblia* IMSS:0696:1 isolate, obtained from an individual with symptomatic giardiasis, was used (Cedillo-Rivera et al., 2003). 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 (Cedillo-Rivera et al., 1991).

Growth inhibition assay

In vitro susceptibility of *G. lamblia* was determined as previously described (Cedillo-Rivera and Muñoz, 1992). Stock solutions of extracts, fractions and pure compounds were prepared with DMSO (5 mg/ml), following serial two-fold dilutions in 1.5 ml volumes of culture medium in microcentrifuge tubes to afford concentrations of 5, 10, 20, and 50 μg/ml. The tubes were inoculated with *G. lamblia* to achieve an inoculum of 5×10^4 trophozoites/ml. As positive control, tubes with metronidazole were similarly inoculated. Tubes of culture medium with DMSO and the same inoculum were used as the negative control. After incubation for 48 h at 37 °C, trophozoites

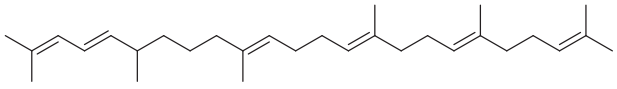
were detached by chilling and 50 μl of each culture tube was subcultured into 1.5 ml of fresh culture medium and incubated for 48 h at 37 °C. The final number of parasites was determined by counting in a haemocytometer, and the percentage of trophozoites growth inhibition was calculated by comparison with controls. The 50% inhibitory concentration (IC_{50}) was defined as the concentration of the extract, fraction or pure compound that inhibited growth by 50% as calculated by probit analysis using the Graphpad Prism 6.0 software. Each experiment was done in duplicate and was repeated at least three times.

Cytotoxic assay of active compounds

Active compounds were also tested in a cytotoxic bioassay in order to discard that the anti-giardial activity was due to toxic effects. Cytotoxicity assay was performed according to the established method of Rahman et al. (2001), where 1.5×10^4 viable cells from HEK-293 cell line were seeded in a 96-well plate (Costar) and incubated for 24–48 h. When cells reached >80% confluence, the medium was replaced and cells were incubated with compounds at successive concentrations (0.97, 1.95, 3.90, 7.81, 15.62, 31.25, 62.5, 125, 250, 500 μg/ml) and dissolved in DMSO at a maximum concentration of 0.05%. After 72 h of incubation, 10 μl of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide solution (MTT, Sigma) solution (5 mg/ml) was added to each well and incubated at 37 °C for 4 h. The medium was removed and the formazan, a product generated by the activity of dehydrogenases in cells, was dissolved in acidified isopropanol (0.4 N HCl). The amount of MTT-formazan is directly proportional to the number of living cells and was determined by measuring the optical density (OD) at 590 nm using a Bio-assay reader (BioRad, USA). Cells incubated only with 0.05% of DMSO were used as a negative control. All determinations were performed in triplicate. The CC_{50} values were calculated using the Graphpad Prism 6.0 software.

Results and discussion

The methanol extraction of the *A. scabra* leaves yielded 435 g of the crude extract ASH-1 (14.41%, w/w). Liquid–liquid partition of this extract gave fractions ASH-2a–ASH-2d (Table 1). These fractions together with the extract ASH-1 were all evaluated against *G. lamblia*. From the tested fractions, only the ASH-1 extract and ASH-2b fraction were active with an IC_{50} = 11.74 and 2.19 μg/ml, respectively (Table 1) and considered high, according to the criteria established by Amaral et al. (2006). To continue the bio-assay guided study, a VLC of the ASH-2b fraction was carried to obtain eleven subfractions which were also tested against *G. lamblia* (Table 2). The active subfractions showed a common metabolite when observed in TLC plates. Recrystallization of the abovementioned fractions led to the purification of a compound as yellow needles. Such metabolite was identified as cirsimaritin (**2**) by comparison of its spectroscopic data with those reported in the literature (Lin et al., 2006). This metabolite has been previously isolated from the species *Cirsium rhinoceros* (Yim et al., 2003), *Stizolophus balsamita* (Suleimenov et al., 2008), and *Helychrisum viscosum* (Geissman et al., 1967) belonging to the Asteraceae family; *Teucrium ramosissimum* (Ben Sghaier et al., 2011), *Dracocephalum multicaule* (Oganesyan, 2009), and *Ocimum sanctum* from Lamiaceae (Kelm et al., 2000); *Microtea debilis* (Phytolaccaceae) (Bai et al., 2011); and *Ruellia tuberosa*, a species of Acanthaceae, same family as the genus *Aphelandra* (Lin et al., 2006). According to the literature, this molecule possesses a wide range of biological activities, such as antioxidant (Ben Sghaier et al., 2011), cytotoxic against KB and GBC-SD cell lines (Lin et al., 2006; Quan et al., 2010), antiproliferative against COLO-205 cells (Bai et al., 2011), and antiprotozoal against *Leishmania donovani*, *Trypanosoma brucei rhodesiense* and *T. cruzi* (Tasdemir et al., 2006). The phytochemical study of ASH-2a fraction led us to obtain the triterpene squalene (**1**), which was identified by comparison of its fragmentation pattern with those in the NIST library provided by the GC-MS. Study of ASH-2c fraction allowed the isolation of the flavone sorbifolin (**4**) and two glycosylated flavones: cirsimarin (**3**) and sorbifolin-6-*O*-*β*-glucopyranoside (**5**), which were identified by comparison of their spectroscopic data with those reported in the literature (Fernandes et al., 2008; Lin et al., 2006; Yim et al., 2003). Sorbifolin has been isolated from the species *Astragalus annularis* (El-Hawiet et al., 2010) and *Pterogyne nitens* from Fabaceae family (Fernandes et al., 2008); *Heterotheca subaxilaris* (Morimoto et al., 2009) and *Pulicaria uliginosa* (Eshbakova et al., 1996) from Asteraceae; and *R. tuberosa* from Acanthaceae (Lin et al., 2006). Regarding its biological activities, this flavonoid has been tested in antimicrobial, antioxidant, and myeloperoxidase bioassays, resulting in moderate to null activity (El-Hawiet et al., 2010; Fernandes et al., 2008). Meanwhile, isolation of cirsimarin has been reported from species such as *R. tuberosa*, *Cirsium lineare*, *C. rhinoceros*, *Teucrium arduini* and *M. debilis* (Vukovic et al., 2011; Bai et al., 2011; Jeong et al., 2008; Lin et al., 2006; Yim et al., 2003). In the other hand, sorbifolin-6-*O*-*β*-glucopyranoside has been isolated from *Pterogyne nitens* (Fernandes et al., 2008).



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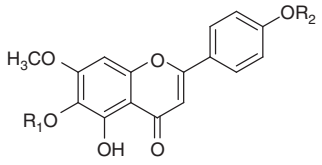

2 $R_1 = CH_3$; $R_2 = H$
3 $R_1 = CH_3$; R_2 = Glc
4 $R_1 = R_2 = H$
5 R_1 = Glc, $R_2 = H$

Compounds **2**, **3**, and **4** were tested in the anti-giardial bioassay and the results are described in Table 3. Among the tested compounds, **2** displayed high activity with an IC_{50} = 3.8 μM, being considered as outstanding, when compared with previous reported secondary metabolites, such as usambarensine, which showed an IC_{50} = 8.99 μM (Wright et al., 1994), (−)-epicatechin with and IC_{50} = 5.82 μM (Calzada et al., 2005), and tingenone having an IC_{50} = 0.74 μM (Mena-Rejon et al., 2007), while **4** showed an IC_{50} = 75.6 μM, being this activity considered low.

Table 2
Anti-giardial activity of VLC subfractions of the DCM fraction of the methanol extract of *A. scabra*.

Sample	IC_{50} (μg/ml)
ASH-4a	NA[a]
ASH-4b	NA
ASH-4c	NA
ASH-4d	2.5
ASH-4e	–[b]
ASH-4f	1.9
ASH-4g	1.1
ASH-4h	1.2
ASH-4i	NA
ASH-4j	NA
ASH-4k	NA
Metronidazole	0.2 ± 0.02

[a] Not active.
[b] Not tested.

Table 3
Anti-giardial, cytotoxic activity and selective index (SI) of pure secondary metabolites of *A. scabra* and metronidazole as positive control.

Sample	Anti-giardial activity IC_{50} (μM)	Hek 293 cell line CC_{50} (μM)	SI
Cirsimaritin (**2**)	3.8	378.68 ± 3.44[b]	310
Cirsimarin (**3**)	NA[a]	NA	–
Sorbifolin (**4**)	75.6	478.56 ± 6.78	21
Metronidazole	1.2	89.70 ± 1.25	448

[a] NA: not active.
[b] Standard deviation.

Compound **1** showed an IC_{50} = 241.28 μM in a previous study (Calzada, 2005); therefore, it was not tested in the present work, while compound **5** was purified sparingly, thus it could not be tested against *G. lamblia*.

Noteworthy is the finding that both active flavonoids had a typical flavone structure ($\Delta^{2,3}$ and C-4 keto functions) and groups such as 4′,5-OH. No activity was observed in compound **3**, where 4′-OH is substituted by glucose, despite the fact that its aglicone **2** was the most active in the present study. That tendency allows suggesting that inhibition is considerably reduced by the presence of the sugar, in accordance with Calzada and Alanís (2007).

The bioactive compounds were also tested in a cytotoxic bioassay in order to discard that the anti-giardial effect was due to cytotoxicity. In this bioassay, both metabolites proved to be not cytotoxic (Table 3). In particular, cirsimaritin showed a high SI indicating its elevated selectivity to *G. lamblia* cells. These results are in accordance with Nijveldt et al. (2001), who concluded that flavonoids are not toxic or less toxic to normal cells, and thus confirming the potential of these flavones to be used as safe anti-giardial agents, although more analyses are necessary to establish if these compounds can be used in humans.

In conclusion, we suggest that cirsimaritin and sorbifolin are the metabolites responsible for the anti-giardial activity exhibited by the methanol extract of *A. scabra* leaves, being the present study the first report on the phytochemistry of this species, thus the isolated compounds are considered as new for the genus and the species.

Authors' contribution

GIHB contributed in running the laboratory work, analysis of the data, structure elucidation of the isolated compounds, and drafted the paper. LWTT contributed to chromatographic and spectrophotometric analysis. RMP supervised biological studies and analysis of the results. SRPS designed the study, supervised the laboratory work, and contributed to critical reading of the manuscript. All the authors have read the final manuscript and approved the submission.

Conflicts of interest

The authors declare no conflicts of interest.

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