Evaluation of *Cuspidaria pulchra* and its Isolated Compounds Against *Schistosoma mansoni* Adult Worms

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Abstract: The present study has investigated the chemical composition of the bioactive EtOAc fraction of *Cuspidaria pulchra* aerial parts, as well as its schistosomicidal activities against *Schistosoma mansoni* adult worms *in vitro*. To this end, the crude ethanol extract obtained from the aerial parts of *Cuspidaria pulchra* (Bignoniaceae) was partitioned with *n*-hexane, EtOAc, and *n*-BuOH. The EtOAc fraction was purified by preparative HPLC, which afforded 3,4-dihydroxybenzaldehyde (**1**), *p*-coumaric acid (**2**), *p*-hydroxybenzoic acid (**3**), ursolic acid (**4**), and oleanolic acid (**5**). The bioassay results indicated that the crude ethanol extract and the EtOAc fraction at 100 μg/mL killed the adult schistosomes *in vitro*. Compounds **1** and **3** at 100 μM were only able to separate coupled *S. mansoni* adult worms.

Keywords: *Cuspidaria pulchra*, Bignoniaceae, Schistosomicidal activity, Phenolic compounds.

INTRODUCTION

Schistosomiasis, an infectious disease caused by parasitic trematodes (schistosomes) dwelling in the host's mesenteric portal system, is a major public health problem in tropical and subtropical regions [1]. Approximately 207 million people are infected, 120 million suffer from the clinical disease, and 20 million exhibit severe morbidity, not to mention that 800 million are at risk of being infected in 76 endemic countries worldwide [2-3]. Praziquantel (PZQ) has been used as first-line drug for the chemotherapy of schistosomiasis since 1984. Besides PZQ, artemether and artesunate have also been employed for the control of this infectious disease since the late 1990s [1]. The emerging resistance to the currently available drugs, mainly in the case of PZQ, has led to the urgent need for the discovery of new therapeutic agents that can act against this disease [4]. In this context, the search for new schistosomicidal compounds from natural products can afford hits for the development of drugs for neglected tropical diseases.

Cuspidaria pulchra (Cham.) L.G. Lohmann, synonym *Arrabidaea pulchra* (Cham.) Sandwith, belongs to the tribe Bignonieae (Bignoniaceae), which comprises nearly half of the genera and more than one third of the species in Bignoniaceae [5]. This group is widely distributed in the neotropics and occurs in Central America, Amazonia, the Atlantic forests of eastern Brazil, and the open dry forests and savannas of Argentina, Bolivia, Brazil, and Paraguay [6]. Previous phytochemical studies of this taxon have resulted in the isolation of *C*-glucosylxanthones, phenylpropanoids, flavonoids, anthocyanidins, allantoins, triterpenes, and C-26 keto fatty acid [7-13]. Regarding biological investigations, it has been found that extracts from *A. brachypoda*, *A. craterophora*, *A. formosa*, *A. pulchra*, and *A. sceptrum* exhibit antiviral activity, and that the ethanol extract from the leaves of *A. triplinervia* display *in vitro* action against trypomastigotes of *Trypanosoma cruzi*, having ursolic and oleanolic acids been identified as the trypanocidal agents [12, 14]. Additionally, the ethanol extract from *C. argentea* has potential molluscicide activity against *Biomphalaria glabrata* [15].

As part of our continuing investigations on the schistosomicidal activity of natural products [16-19], the present work has evaluated the schistosomicidal activities of the extract, fractions, and compounds isolated from the aerial parts of *C. pulchra* against *Schistosoma mansoni* adult worms.

MATERIALS AND METHODS

General Experimental Procedures

^{1}H and ^{13}C NMR spectra were recorded in DMSO-d_6 and CDCl$_3$ on a Varian Unity 300 and 500 NMR or

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Bruker AC200 NMR spectrometers, using TMS as internal standard. A negative-ion mode HRESIMS analysis was conducted on a Bruker Daltonics HRMS ultrOTOF-Q-ESI-TOF employing electrospray ionization. Both analytical and preparative HPLC separation analyses were carried out on a Shimadzu LC-6AD system equipped with a degasser DGU-20A5, a UV-VIS detector SPD-20A series, a communication bus module CBM-20A, and a Rheodyne manual injector. Separation of the compounds was accomplished on SHIMADZU Shim-pack ODS (particle diameter 5 µm, 250 x 4.60 mm, and 250 x 20 mm) columns equipped with pre-columns of the same material. The MeOH used in the experiments was HPLC grade and was purchased from J. T. Baker. Ultrapure water was obtained by passing redistilled water through a Direct-Q UV3 system from Millipore. Silica gel 90 reverse-phase ODS (Fluka, 230-400 mesh) was utilized for column chromatography, as well as silica on TLC Alu foils containing a fluorescent indicator (254 nm, Sigma-Aldrich).

Plant Material

The aerial parts of *Cuspidaria pulchra* (Cham.) L.G. Lohmann were collected from the Brazilan Cerrado area located in the city of Luis Antonio (21°33′ - 21°37′ S and 47°45′ - 47°57′ W), state of São Paulo, in October 2008. The materials were identified by Prof. V. M. M. Gimenez and Prof. M. Groppo and voucher specimens (SPFR12599) were deposited in the Herbarium of the Department of Biology, Laboratory of Plant Systematics, Faculdade de Filosofia Ciências e Letras de Ribeirão Preto, University of São Paulo, Brazil (Herbarium SPFR).

Extraction and Isolation

The air-dried, powdered aerial parts (288.2 g) were extracted with EtOH. After filtration, the solvents were removed under reduced pressure, to yield 32 g of the extract. The ethanol extract (20 g) was then dissolved in MeOH/H_2O (2:8, v/v) and successively partitioned with *n*-hexane, EtOAc, and *n*-butanol. Each liquid phase were evaporated and resulted in *n*-hexane (2.4 g), EtOAc (5.5 g), *n*-butanol (7.9 g), and hydromethanol (3.2 g) fractions. The EtOAc residue was submitted to Solid Phase Extraction by using silica gel 90 reverse-phase ODS and employing MeOH/H_2O as eluent, which afforded ten fractions. Fraction 1 (325 mg) and Fraction 2 (95 mg) were subjected to preparative RP-HPLC purification using an isocratic condition: MeOH/H_2O/AcOH (35:64.9:0.1, v/v/v), UV detection at

254 nm, and a flow rate of 9 mL/min, which gave eight (Fractions 1-1 to 1-8) and six (Fractions 2-1 to 2-6) fractions, respectively. Fractions 1-5 and 2-5 afforded compounds **1** (15.4 mg, t_R= 12.7 min) and **2** (4.0 mg, t_R= 26.43 min), respectively. Fraction 2-3 (5.9 mg, t_R=10.42 min) was purified by RP-HPLC using MeOH/H_2O/AcOH (30:69.9:0.1, v/v/v), UV detection at 254 nm, and a flow rate of 9 mL/min, which gave rise to compounds **1** (2.6 mg, t_R= 16.31 min) and **3** (2.7 mg, t_R= 21.03 min). Fraction 9 (164,2 mg) yielded a mixture of compounds **4** and **5**.

3,4-dihydroxybenzaldehyde (1)

White crystalline powder; ^{1}H-NMR (300 MHz, DMSO-d_6) and ^{13}C-NMR (125 MHz, DMSO-d_6): see Table **1**.

p-coumaric Acid (2)

White crystals; ^{1}H-NMR (300 MHz, DMSO-d_6) δ (ppm): see Table **1**. HR-ES-MS *m/z* 163.0407 (Calcd for $C_9H_7O_3$ [M-H]⁻, 163.0401), 119 [M-H-CO_2]⁻.

p-hydroxybenzoic Acid (3)

White crystalline powder; ^{1}H-NMR (300 MHz, DMSO-d_6) and ^{13}C-NMR (125 MHz, DMSO-d_6): see Table **1**.

Ursolic Acid (4)

White powder; ^{1}H-NMR (200 MHz, CDCl$_3$ and DMSO-d_6) δ (ppm): 5.15 (m, H-12), 3.04 (m, H-3), 2.14 (d, *J* = 9.7, H-18), 1.10 - 2.11 (m), 0.74 - 1.23 (m), 0.70 - 1.06 (m), 0.65 - 1.01 (m); ^{13}C-NMR (50 MHz, CDCl$_3$ and DMSO-d_6): see Table **1**.

Oleanolic Acid (5)

White powder; ^{1}H-NMR (200 MHz, CDCl$_3$ and DMSO-d_6) δ (ppm): 5.15 (m, H-12), 3.04 (m, H-3), 1.10 - 2.11 (m), 0.74 - 1.23 (m), 0.70 - 1.06 (m), 0.65 - 1.01 (m); ^{13}C-NMR (50 MHz, CDCl$_3$ and DMSO-d_6): see Table **1**.

In Vitro Schistosomicidal Assay

The LE strain of *S. mansoni* was maintained by passage through *Biomphalaria glabrata* snails and Balb/c mice. After eight weeks, *S. mansoni* adult worms were recovered under aseptic conditions from mice previously infected with 200 cercariae by perfusion of the livers and mesenteric veins [20]. The worms were washed in Roswell Park Memorial Institute (RPMI) 1640 medium (Invitrogen), kept at pH 7.5 with HEPES 20 mM, and supplemented with penicillin

**Table 1:   Summary of Spectral Data of ^{1}H-NMR and ^{13}C-NMR**

#	1 δ_H, m (J in Hz)	1 δ_C, m[b]	2 δ_H, m (J in Hz)	3 δ_H, m (J in Hz)	3 δ_C, m[b]	4 δ_C, m[b]	5 δ_C, m[b]
1	-	129.7, s	-	-	122.0, s	38.8, t	38.8, t
2	7.22, d (1.7)	115.2, d	7.48, d (8.4)	7.77, d (8.6)	131.7, d	27.3, t	27.6, t
3	-	146.7, s	6.77, d (8.4)	6.80, d (8.6)	115.3, d	77.1, d	77.1, d
4	-	153.0, s	-	-	161.7, s	38.9, s	38.9, s
5	6.89, d (8.0)	116.4, d	6.77, d (8.4)	6.80, d (8.6)	115.3, d	55.2, d	55.2, d
6	7.26, dd (8.0, 1.7)	125.3, d	7.48, d (8.4)	7.77, d (8.6)	131.7, d	18.4, t	18.4, t
7	9.68, s	191.9, d	7.46, d (15.9)	-	167.6, s	32.0, t	33.1, t
8	-	-	6.27, d (15.9)	-	-	39.2, s	39.0, s
9	-	-	-	-	-	47.5, d	47.6, d
10	-	-	-	-	-	36.9, s	37.0, s
11	-	-	-	-	-	23.6, t	23.2, t
12	-	-	-	-	-	125.1, d	122.0, d
13	-	-	-	-	-	138.5, s	144.2, s
14	-	-	-	-	-	42.0, s	42.0, s
15	-	-	-	-	-	28.5, t	27.9, t
16	-	-	-	-	-	24.3, t	23.2, t
17	-	-	-	-	-	47.3, s	46.0, s
18	-	-	-	-	-	52.8, d	41.7, d
19	-	-	-	-	-	39.3, d	45.9, t
20	-	-	-	-	-	39.3, d	30.7, s
21	-	-	-	-	-	30.7, t	33.3, t
22	-	-	-	-	-	36.7, t	32.5, t
23	-	-	-	-	-	28.5, q	28.5, q
24	-	-	-	-	-	15.6, q	15.6, q
25	-	-	-	-	-	15.4, q	16.2, q
26	-	-	-	-	-	17.2, q	16.3, q
27	-	-	-	-	-	23.6, q	25.9, q
28	-	-	-	-	-	179.1, s	179.1, s
29	-	-	-	-	-	17.3, q	32.8, q
30	-	-	-	-	-	21.4, q	23.6, q

[a]NMR spectra were acquired in DMSO-d_6 for **1-3** and in CDCl$_3$ and DMSO-d_6 for **4-5**.
[b]Carbon multiplicities were determined by DEPT 135°.

(100 UI/mL), streptomycin (100 µg/mL), and 10% bovine fetal serum (Gibco). After washing, one couple of adult worms was transferred to each well of a 24-well culture plate containing 2 mL of the same medium and incubated at 37 °C in a humid atmosphere containing 5% CO_2 prior to use. At 24 h after incubation, extract, fractions, and the isolated compounds (**1-3**) were dissolved in DMSO and added to RPMI 1640 medium, to give final concentrations of 100 µg/mL or µM. The parasites were kept for 5 days

and monitored every 24 h, for evaluation of their general condition. The effects of extract, fractions and of compounds **1-3** on *S. mansoni* were assessed by observing the viability of the worms, as well as their pairing, motor activity, and tegument alteration. The worms were considered dead when no movement was observed for at least 2 min of examination and when no movement was detected at the other observation time points [21]. Quadruplicate measurements were accomplished for each employed concentration, and
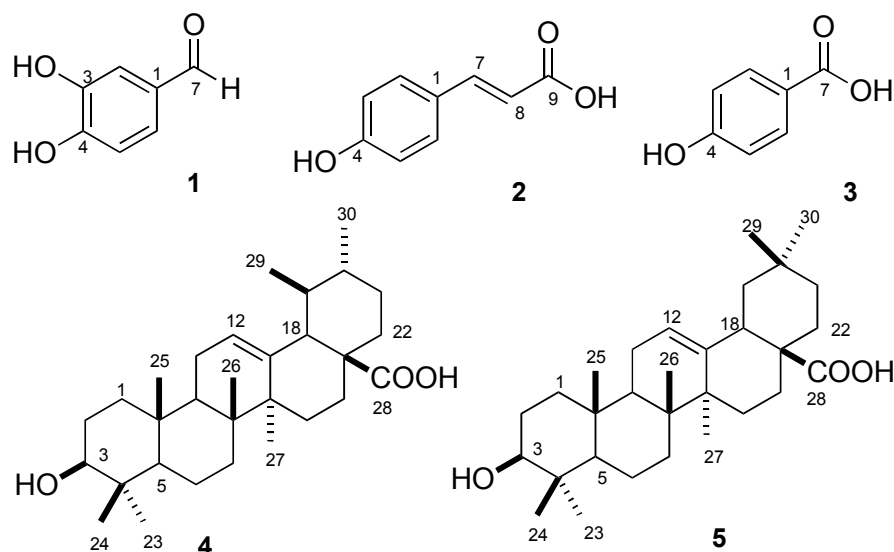
Figure 1: Structures of compounds **1**-**5** from *C. pulchra*.

two independent experiments were performed. RPMI 1640 medium and RPMI 1640 with 1% DMSO (the highest concentration of drug solvent) were used as negative controls. Praziquantel (PZQ) was utilized as positive control. All experiments were authorized by the Ethics Committee for Animal Care of University of Franca (São Paulo, Brazil) and University of São Paulo (São Paulo, Brazil), and they were in accordance with the nationally and internationally accepted principles for laboratory animal use and care.

RESULTS AND DISCUSSION

The incubation of adult worms of *S. mansoni* with the ethanol extract of *C. pulchra* (100 μg/mL) induced death of 25 % of the worms at 120 h as well as decreased motor activity, but there were no extensive tegumental alterations. The fractionation of the bioactive extract yielded four major fractions that were also screened against *S. mansoni*. The EtOAc fraction (100 μg/mL) exhibited the highest activity: it caused death of 50 % of the worms at 120 h as well as reduced motor activity, without extensive tegumental alterations. However, the *n*-hexane, *n*-butanol, and hydromethanol fractions were inactive.

The purification of the EtOAc fraction led to the isolation of five compounds. The chemical structures of the isolated compounds (Figure **1**) were established using NMR and MS spectra and are in agreement with previously published data (Table **1**). The isolated compounds were identified as being 3,4-dihydroxybenzaldehyde (**1**), *p*-coumaric acid (**2**), *p*-hydroxybenzoic acid (**3**), ursolic acid (**4**), and oleanolic

acid (**5**) [22-23]. This is the first time that compounds **1**-**3** and **5** have been identified in *C. pulchra*.

The schistosomicidal assay results of the isolated compounds are summarized in Table **2**. Worms incubated with compound **1** (100 μM) exhibited moderately diminished motor activity, with moderate tegumental alterations. On the other hand, compounds **2** and **3** (100 μM) promoted weak and moderate tegumental alterations, respectively, and moderate reduction in the motor activity. Additionally, compounds **1** and **3** were able to separate adult worms into male and female (100 and 75 %); separation occurred at 24 h and reached 100 % at 120 h. The appearance and motor activity of the worms in the 1 % DMSO group were similar to those observed in the case of the negative control. In these groups, there was no detection of death, separation, decreased motor activity, or extensive tegumental alterations of the *S. mansoni* adult worms. PZQ (10 μM), used as positive control, elicited death of the parasites and tegumental alterations without separation of worms.

It is noteworthy that compounds **1**, **2**, and **3** are quite similar; the main difference lies on the substituent present in the *p*-hydroxybenzene ring. Therefore, considering the separation of adult worms, it can be suggested that the presence of an acid and aldehyde at C-1 in the benzene ring, as in the case of compounds **1** and **3**, may improve the activity of phenolic derivatives (**1-3**), since compounds **1** and **3** were able to separate the male and female worms. Compound **2** is structurally analogous; it contains a 2-propenoic acid moiety at C-1. Thus, on the basis of the

Table 2:   *In Vitro* Effects of the Isolated Compounds from *C. pulchra* Against *S. mansoni* Adult Worms

Group	Incubation period (h)	% of separated worms	% of dead worms
DMSO 1%	24 h	0	0
	120 h	0	0
Control[a]	24 h	0	0
	120 h	0	0
PZQ[b]	24 h	0	100
	120 h	0	100
1[c]	24 h	100	0
	120 h	100	0
2[c]	24 h	0	0
	120 h	0	0
3[c]	24 h	75	0
	120 h	100	0

[a]RPMI 1640. [b]Tested concentration 10 μM. [c]Tested concentration 100 μM.

obtained results, it can be implied that the presence of this extended chain culminates in no activity regarding the separation of worm couples.

The mechanism through which *p*-hydroxybenzene ring derivatives (**1** and **3**) exert their *in vitro* effect on *S. mansoni* worms is not known. Nevertheless, considering their antiparasitic activities, benzoic acid derivatives have been shown to possess antimalarial action, while compound **3** has been demonstrated to have the ability to separate adult *S. mansoni* worm pairs [16-24].

Ursolic and oleanolic acids are multipotential molecules with anti-inflammatory, antitumoral, antihyperlipidemic, hepatoprotective, antimicrobial, anti-ulcer, hypoglycemic, and anti-cariogenic activities [25]. The antileishmanial and schistosomicidal potential of these molecules have been equally established by previous studies. These compounds previously isolated from the aerial parts of the *Miconia langsdorffii* Cogn. (Melastomataceae), have been screened for their *in vitro* antileishmanial activity against the promastigote forms of *Leishmania amazonensis*, with IC_{50} of 360.3 and 439.5 μM, respectively [26]. The schistosomicidal properties of compounds **4** and **5** isolated from *M. langsdorffii* have also been evaluated, and it has been found that they do not have lethal effects on *S. mansoni* adult worms (data under consideration for publication).

CONCLUSION

The present study indicated that the ethanol extract induced death and decreased motor activity of adult worms of *S. mansoni.* The crude extract fractionation resulted in the bioative EtOAc fraction, which caused death and reduced motor activity of the worms. Chemical investigations of this fraction resulted in the isolation and identification of compounds **1**-**5**. Additionally, biological results indicated that compounds **1** and **3** were able to separate adult worms into male and female. Moreover, the assayed compounds did not kill the adult schistosomes *in vitro*. Although, **1-3** exhibited moderate reduction in the motor activity. Further investigations are in progress to disclose other important biological effects of this plant, whereas *C. pulchra* have great potential as a source of active compounds.

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