

In Vitro Antischistosomal Activity and Cytotoxicity of 5-Methoxylated Flavones from *Vochysia divergens*, a Flood-Adapted Species from Brazilian Pantanal

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Abstract

Background: *Vochysia divergens* is native to the Amazon Basin and considered an invasive species in the Brazilian Pantanal. In this work, the ethanolic extract of *V. divergens* leaves was chemically investigated and evaluated *in vitro* against *Schistosoma mansoni* adult worms together with the isolated compounds.

Methods: The samples were available at concentrations of 12.5, 25, 50 and 100 μM . The parasites were kept for 4 days and monitored every 24 h to evaluate their viability and motor activity comparing with the positive control praziquantel. The cell viability of isolated flavones as determined by XTT assays in V79 cells.

Results: The flavones 3',5-dimethoxy luteolin-7-O- β -glucopyranoside, 5-methoxy luteolin and 3',5-dimethoxy luteolin were isolated from *V. divergens*. Flavone 5-methoxy luteolin caused the death of 25% of male and female adult *S. mansoni* worms in 24 h at 100 μM and also reduced their motor activity. Flavones 3',5-dimethoxy luteolin-7-O- β -glucopyranoside and 3',5-dimethoxy luteolin were able to slightly reduce the motor activity to 25% within 24 h at 25 $\mu\text{g/mL}$ and 12.5 $\mu\text{g/mL}$, respectively, but only in male worms. The results were compared with the flavone luteolin. For XTT assay in V79 cells the IC₅₀ values were 1468.4 \pm 10.5 μM , 5946.6 \pm 25.1 μM , > 7960.8 μM and 270.6 \pm 9.3 μM for compounds 3',5-dimethoxy luteolin-7-O- β -glucopyranoside, 5-methoxy luteolin, 3',5-dimethoxy luteolin and luteolin, respectively.

Conclusion: To the best of our knowledge, flavones 5-methoxy luteolin and 3',5-dimethoxy luteolin are reported for the first time as constituents of *V. divergens* and the Vochysiaceae family.

Keywords: *Vochysia divergens*; Vochysiaceae; Flavones; Schistosomicidal activity

Introduction

Vochysia divergens Pohl (Vochysiaceae), commonly known as Cambará, is native to the Amazon Basin and considered an invasive species in the wetlands of the Brazilian Pantanal. *Vochysia divergens* has wide tolerance for seasonal variations in hydrology, allowing it to endure seasonal drought during the dry season and seasonal flooding during the wet season in the Pantanal [1].

The chemical constituents of *Vochysia* genus include ellagic acid derivatives, flavonoids, steroids and acid triterpenoids [2].

V. divergens is used in folk medicine against infectious diseases and asthma. The antibacterial activity of the ethanolic extract of *V. divergens* stem bark has been evaluated against *Escherichia coli* and *Staphylococcus aureus* with MIC >5 and 1.5 mg/mL, respectively [3]; however, the potential schistosomicidal of this species remains unknown.

Schistosomiasis, which is caused by trematode flatworms of the genus *Schistosoma*, is one of the most significant and neglected tropical diseases in the world. This disease has significant prevalence and morbidity, affecting more than 207 million people worldwide and resulting in as many as 280,000 deaths each year. Moreover, there are almost 800 million people who are at risk of infection despite the great advances in its treatment and prevention [4-6].

Praziquantel and oxamniquine are the drugs that are currently available for the treatment of schistosomiasis. Praziquantel does not prevent re-infection and is inactive against juvenile schistosomes. Furthermore, it has a limited effect on existing lesions in the liver and spleen [7].

Low cure rates and treatment failure following praziquantel administration together with the existence of resistant strains reinforces the need to develop new safe and effective schistosomicidal agents [8,9]. As part of our advancing investigation on the schistosomicidal activity of Brazilian species from "Cerrado" and "Pantanal" [10-12], the present work reports on the *in vitro* schistosomicidal activity of the ethanolic extract and three isolated 5-methoxy-flavones from *V. divergens* leaves.

Materials and Methods

General

¹H and ¹³C NMR spectra were recorded in methanol-*d*₄ for compounds **1** and **2** and in pyridine-*d*₅ for compound **3** on a Bruker® DRX-400 and Bruker® DRX-500 spectrometer using TMS as the internal standard.

The analytical HPLC separation analyses were carried out on a Shimadzu Prominence LC-20AD binary system equipped with a DGU-20A5 degasser, a SPD-20A series diode array detector, a CBM-

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20A communication bus module, an SIL-20A HT autosampler, and a CTO-20A column oven.

The preparative HPLC separation step was carried out on a Shimadzu LC-6AD system equipped with a DGU-20A5 degasser, a SPD-20A series UV-VIS detector, a CBM-20A communication bus module, and a Reodyne manual injector. Separation of the micromolecules was accomplished on a Shimadzu Shim-pack ODS columns (particle diameter 5 μm , 250 \times 4.6 mm, and 250 \times 20 mm) equipped with a pre-column of the same material.

The MeOH used in the experiments was HPLC grade and was obtained from J. T. Baker. Ultrapure water was obtained by passing redistilled water through a Direct-Q UV3 system from Millipore. Luteolin L9283 ($\geq 98.9\%$) was purchased from Sigma-Aldrich (St. Louis, MO).

Plant material

Vochysia divergens was collected in the Pantanal Mato-grossense region (S16°35'22, 90" and W56°47'83,40") in October 2012. A voucher specimen, UFMT 39559, was deposited in the Herbarium of Federal University of Mato Grosso, UFMT, Brazil (Herbarium UFMT).

Extraction and isolation of flavones

The air-dried leaves of *V. divergens* (1.27 kg) were powdered and exhaustively extracted by maceration at room temperature using EtOH. After filtration, the solvent was removed under reduced pressure, yielding 82.83 g of crude extract VdE. A total of 3 g of obtained ethanolic extract was chromatographically separated over Sephadex LH-20[®] and eluted with methanol to afford seven fractions based on their TLC behavior. Fraction 3 (80 mg) was chromatographed over a reverse-phase ODS cartridge and then purified by preparative RP-HPLC [$\text{CH}_3\text{OH}-\text{H}_2\text{O}-\text{CH}_3\text{COOH}$ (50:49.9:0.1 v/v/v)] to yield fractions 1 (10 mg) and 3 (8.0 mg). Fraction 5 (30 mg) was also purified by preparative RP-HPLC [$\text{CH}_3\text{OH}-\text{H}_2\text{O}-\text{CH}_3\text{COOH}$ (50:49.9:0.1 v/v/v)] to yield fraction 2 (6 mg). The spectral data of all isolated flavones are in agreement with previously published data and allowed for the identification of 3',5'-dimethoxy luteolin-7-O- β -glucopyranoside (1), 5-methoxy luteolin (2) and 3',5'-dimethoxy luteolin (3) [13-15].

3',5'-Dimethoxy luteolin-7-O- β -glucopyranoside (1): ¹H NMR (400 MHz, δ , CD_3OD): 3.92 (s, 3H, OCH_3), 3.95 (s, 3H, OCH_3), 6.61 (s, 1H, H-3), 6.71 (d, $J = 2.3$, 1H, H-6), 6.92 (d, $J = 8.4$, 1H, H-5'), 6.96 (d, $J = 2.3$, 1H, H-8), 7.47 (d, $J = 2.1$, 1H, H-2'), 7.51 (dd, $J = 2.1$ and 8.4, 1H, H-6'), 7-O-Glu 5.1, (d, $J = 7.7$, H-1), 3.49-3.52 m; 3.55-3.60 m, 3.65-3.79 m). ¹³C NMR (100 MHz, δ , CD_3OD): 56.6 (3'- OCH_3), 56.6 (5- OCH_3), 62.6 (C-6''), 71.5 (C-4''), 74.8 (C-2''), 77.9 (C-5''), 97.7 (C-8), 98.6 (C-6), 100.4 (C-1''), 107.0 (C-3), 110.4 (C-2'), 116.8 (C-5''), 122.0 (C-1'), 149.6 (C-3'), 152.3 (C-4'), 160.8 (C-9), 162.0 (C-2), 164.2 (C-7), 173.2 (C-4).

5-Methoxy luteolin (2): ¹H NMR (500 MHz, δ , CD_3OD): 3.85 (s, 3H, 5- OCH_3), 6.39 (d, $J = 1.75$, 1H, H-6), 6.43 (s, 1H, H-3), 6.48 (d, 1H, $J = 1.75$, H-8), 6.86 (d, $J = 8.5$, 1H, H-5'), 7.27 (d, 1H, $J = 2.0$, H-6'), 7.29 br s, 1H, H-2').

3',5'-Dimethoxy luteolin (3) ¹H NMR (400 MHz, δ , Pyridine-*d*₅): 3.79 (s, 3H, OCH_3), 3.85 (s, 3H, OCH_3), 6.74 (d, $J = 2.0$, 1H, H-6), 6.98 (d, $J = 2.0$, 1H, H-8), 6.98 (s, 1H, H-3), 7.25 (d, $J = 8.3$, 1H, H-5'), 7.57 (m, 1H, H-2'), 7.62 (dd, $J = 2.1$ and 8.3, 1H, H-6'). ¹³C NMR (100 MHz, δ , CD_3OD): 56.1 (OCH_3), 56.2 (OCH_3), 96.6 (C-8), 107.8 (C-3), 110.1 (C-2'), 117.0 (C-5'), 120.7 (C-1'), 151.7 (C-4'), 160.6 (C-2), 161.9 (C-5).

Luteolin (4) ¹H NMR (500 MHz, δ , $\text{DMSO}-d_6$): 6.24 (d, $J = 2.0$, 1H,

H-6): 6.47 (d, $J = 2.0$, 1H, H-8), 6.57 (s, 1H, H-3), 6.89 (d, $J = 9.0$, 1H, H-5'), 7.40 (dd, $J = 3.0$, and 9.0, 1H, H-6'), 7.43 (d, $J = 3.0$, 1H, H-2').

In vitro schistosomicidal assay

The LE (Luis Evangelista) strain of *S. mansoni* was maintained by passage through *Biomphalaria glabrata* snails and Balb/c mice at the Parasitology Research Laboratory, the University of Franca [16]. After eight weeks, adult *S. mansoni* worms were recovered under aseptic conditions from mice previously infected with 200 cercariae by perfusion of the livers and mesenteric veins [17].

The worms were washed in Roswell Park Memorial Institute (RPMI) 1640 medium (Invitrogen), maintained at pH 7.5 with 20 mM HEPES, and supplemented with penicillin (100 UI/mL), streptomycin (100 $\mu\text{g}/\text{mL}$), and 10% bovine fetal serum (Gibco). After washing, two adult worms were 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, the ethanolic extract (VdE), isolated compounds 1-3 and luteolin 4 were dissolved in dimethyl sulfoxide (DMSO) and added to the RPMI 1640 medium to yield respective final concentrations of 12.5, 25, 50 and 100 $\mu\text{g}/\text{mL}$ or μM . The parasites were kept for 4 days and monitored every 24 h to evaluate their general condition.

The movement was examined by visual inspection using an inverted microscopy and the worms were considered dead when no movement was observed for at least 2 min of examination and no movement at the other observation time points was detected [18]. After the observation, the culture medium was removed and added fresh culture medium without substance and the motility re-examined up to 24 horas to confirm the mortality. Quadruplicate measurements were recorded for each concentration, and three independent experiments were performed. RPMI 1640 medium and RPMI 1640 with 1% DMSO (the highest concentration of drug solvent) were used as negative control groups. Praziquantel (PZQ) at 12.5 μM was used as a positive control group.

Data were statistically analysed by one-way analysis of variance followed by Dunnett's comparison. The statistical tests were performed with the aid of the Graphpad Prism (version 5.0) software.

All experiments were authorized by the Ethics Committee for Animal Care of the University of Franca (protocol number 028/12), and they were carried out in accordance with the national and international accepted principles for laboratory animal handling and care.

XTT-based cytotoxicity assay

Cytotoxicity was measured using an *in vitro* Toxicology Colorimetric Assay Kit (XTT; Roche Diagnostics) according to the manufacturer's instructions. XTT reduction occurs extracellularly on the surface of the plasma membrane via transmembrane electron transport [19] and was originally described for the evaluation of tumor cell line sensitivity to chemicals.

The method is widely used for the quantitative determination of cell proliferation or the cytotoxic effects of chemicals [20]. Chinese hamster lung fibroblasts (V79 cells) were used for the cytotoxicity experiments. The cell line was cultured in HAM-F10 (Sigma-Aldrich) and DMEM (Sigma-Aldrich) (1:1) culture medium supplemented with 10% fetal bovine serum (Nutricell), antibiotics (0.01 mg/mL streptomycin and 0.005 mg/mL penicillin; Sigma-Aldrich), and 2.38 mg/mL HEPES (Sigma-Aldrich) at 37°C and 5% CO_2 . For these experiments, the cells (10^3 cells/well) were plated onto 96-well microplates, and each

well received 100 μ L of culture medium. Twenty-four hours after seeding the cells, culture media containing the tested concentrations of compounds 1-4 dissolved in 0.5% DMSO were added.

The concentrations tested ranged from 2.6 to 8741.3 μ M. The negative (without treatment), solvent (0.5% DMSO) and positive (25% DMSO) controls were included.

After incubation at 37°C for 24 h, the medium was removed and the cells were washed with 100 μ L PBS (phosphate buffered-saline). The cells were then exposed to 100 μ L of HAM-F10 medium without phenol red. Then, 20 μ L of XTT was added to each well, and the microplates were covered and incubated at 37°C for 17 h. The absorbance of each sample was determined using a multi-plate reader (ELISA –Asys – UVM 340 / Microwin 2000) at a test wavelength of 450 nm and a reference wavelength of 620 nm.

Cell viability was expressed as a percentage of untreated cells, which served as the negative control group and was designated as 100%. Therefore, the results are expressed as a percentage of the negative control. The 50% inhibition of cell lines growth (IC_{50}) was used as analysis parameter calculated using Prism Graphpad Programme and One-way ANOVA was used for the comparison of the mean ($P < 0.05$). The experiments were performed in triplicate.

Results and Discussion

The chemical study of *V. divergens* successfully isolated the flavones 3',5-dimethoxy luteolin-7-O- β -glucopyranoside (1), 5-methoxy luteolin (2) and 3',5-dimethoxy luteolin (3) (Figure 1). Although the flavone luteolin and its derivative luteolin-7-O- β -glucoside have a wide distribution in the vegetal kingdom, the methoxylated derivatives 1 and 3 are rare. To the best of our knowledge, this report is the first to isolate flavones 2 and 3 from *V. divergens* and from the Vochysiaceae family in general.

Regarding the schistosomicidal assay (Table 1), the incubation of male and female adult *S. mansoni* worms with the ethanolic extract of *V. divergens* at a concentration of 50 μ g/mL resulted in a 100% reduction in motor activity within 24 h regardless of the parasite gender, but the treatment did not induce mortality at the two assayed concentrations. However, 5-methoxy luteolin (2) caused the death of 25% of male and female adult *S. mansoni* worms in 24 h at 100 μ M and also reduced the

motor activity by 25% in 24 h at 25 μ M and 50% at 100 μ M. In addition, luteolin (4) was able to kill 75% of male and female parasites within 72 h at 100 μ M and reduced the motor activity by 75% under these conditions for male and female adult *S. mansoni* worms.

The other two isolated flavones, 3',5-dimethoxy luteolin-7-O- β -glucopyranoside (1) and 3',5-dimethoxy luteolin (3), showed a slight reduction in their motor activity and the absence of worm mortality. Flavone 3 was more active than 1 because it was able to reduce mobility by 25% only for male worms at a concentration of 12.5 μ g/mL at 24 h, whereas flavone 1 presented the same result at 25 μ g/mL.

To ascertain the cytotoxicity, XTT assays were performed in V79 cells during 24 h of treatment at concentrations ranging from 2.6 to 8741.3 μ M for compounds 1-4. The viability of the cultures was determined, establishing a relationship between the absorbance obtained in the treated and untreated (control) groups as shown in Figure 2.

The treatment of V79 cells with compound 1 resulted in a statistically significant reduction in cell viability at concentrations higher than 656.5 μ M when compared to the negative control, whereas treatment with compound 2 showed a statistically significant reduction in cell viability at concentrations above 4166.7 μ M. Conversely, treatment with the compound 3 did not result in a statistically significant reduction in cell viability. In contrast, compound 4 showed a statistically significant reduction in cell viability at concentrations higher than 136.4 μ M. The IC_{50} values were 1468.4 ± 10.5 μ M, 5946.6 ± 2.1 μ M, > 7960.8 μ M and 270.6 ± 9.3 μ M for compounds 1-4, respectively.

Considering the schistosomicidal activity results of flavonoids 1-3 which share the 2-phenyl-1,4-benzopyrone scaffold, the results related to the isolated 5-methoxy flavones suggest that the exchange of methoxyl group at positions 3' and 7' by hydroxyl groups appears to be important to increase the schistosomicidal activity as observed in more active flavones 2 and 4. Conversely, the glycosylation at the C-7 position in ring B does not appear to be relevant to the reduction in motor activity in *S. mansoni* adult worms. Some *in vitro* studies with natural products have reported that male *S. mansoni* worms are often more susceptible than female worms [21,22], whereas other studies have not reported any differences between male and female worms [18,23].

In the present study, the flavones 1 and 3 appear to affect more male than female, this fact can be related to the pairing of the male and female which male schistosome ensures the survival of the female by providing protection physical transportation within the vasculature, musculature to aid feeding and other chemo- or thigmatic-maturation factors as well as providing sperm to fertilize the oocyte [24].

Shalaby and co-authors studied the effects of *Daucus carota* var. boissieri extracts, which have a high content of flavones, including luteolin, on the immune responses of *Schistosoma mansoni*-infected mice. The anthelmintic activities of the extracts were evidenced via the percent protection, the humoral and cellular immune responses of the mice, as well as the mechanism of immunomodulation [25]. In addition, Kellenberger and collaborators reported that the flavone luteolin is an inhibitor of human enzyme CD38 and its homologous enzyme *Schistosoma mansoni* NAD(+) catabolizing enzyme (*SmNACE*) expressed in *Schistosoma mansoni* with an IC_{50} of 8.2 ± 0.2 μ M and 8.4 ± 0.4 μ M, respectively [26]. According to Michels *et al.*, 3',5-dimethoxy luteolin (3) and luteolin (4) showed cytotoxic potential in H4IIE rat hepatoma cells (30% reduction after 24 hr at 250 mM). The differences in sensitivity between several cell lines may be due several

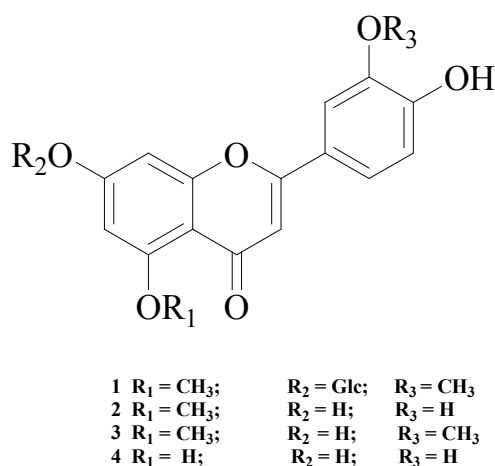
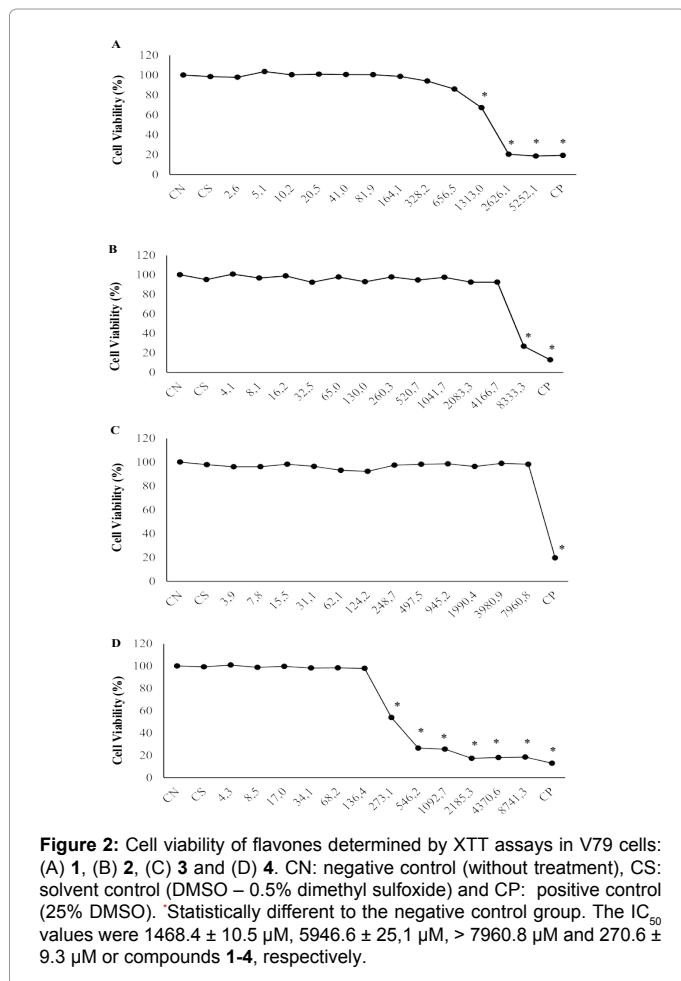


Figure 1: Structures of compounds 1-3 from *V. divergens* and luteolin (4).



factors, among them differences in cellular uptake, export mechanisms or metabolizing enzymes [27].

Conclusions

In conclusion *V. divergens* is a promising species with which to carry out chemical and biological investigations. The schistosomicidal potential of *V. divergens* has not yet been described in the literature, and further biological studies are required to clarify its schistosomicidal action mechanisms.

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Sample	Incubation period (h)	% Reduction in motor activity at a given concentration (µg/mL or µM)							
		12.5		25		50		100	
		M	F	M	F	M	F	M	F
VdE ^b (µg/mL)	24	n.t. ^a	n.t.	n.t.	n.t.	100 ^e	100 ^e	100 ^e	100 ^e
	72	n.t.	n.t.	n.t.	n.t.	100 ^e	100 ^e	100 ^e	100 ^e
1 ^c	24	0	0	25 ^e	0	25 ^e	0	50 ^e	0
	72	0	0	25 ^e	0	25 ^e	0	50 ^e	0
2 ^c	24	0	0	25 ^e	25 ^e	25 ^e	25 ^e	50 ^e	50 ^e
	72	0	0	25	25 ^e	25 ^e	25 ^e	50 ^e	50 ^e
3 ^c	24	25	0	25 ^e	0	25 ^e	0	25 ^e	0
	72	25	0	25 ^e	0	25 ^e	0	25 ^e	0
4 ^c	24	0	0	0	0	50 ^e	50 ^e	75 ^e	0
	72	0	0	25 ^e	25 ^e	50 ^e	50 ^e	75 ^e	75 ^e
Control ^d	24	0		0		0		0	
	72	0		0		0		0	
1% DMSO	24	0		0		0		0	
	72	0		0		0		0	

^a n.t.: not tested.

^b µg/mL

^c µM

^dRPMI 1640

PZQ at 12.5 µM = 100% parasite death after 24 h of incubation.

^eAsterisk indicates statistically significant differences compared with the negative control group (RPMI 1640 medium only) (p<0.001).

Table 1: *In vitro* effects of VdE ethanolic extract and flavones 1, 2, 3 and 4 on the motor activity of adult *S. mansoni* worms.

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