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Full Length Research Paper

Flavonoids and an alkaloid from *Zanthoxylum naranjillo* and their *in vitro* evaluation on the reproductive fitness of *Schistosoma mansoni*

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The chemical investigation of *n*-butanol fraction (BF) from the leaves of *Zanthoxylum naranjillo* (Rutaceae) by open column and preparative HPLC resulted in the isolation of magnoflorine (1), apigenin-6-*C*-β-D-glucopyranoside (2) and apigenin-8-*C*-β-D-glucopyranoside (3). This is the first time that the presence of compounds 1 to 3 in *Z. naranjillo* has been reported. Compounds 1 to 3 and BF were screened *in vitro* against *Schistosoma mansoni* adult worms. The results indicated that BF and compound 3 were able to separate coupled *S. mansoni* adult worms.

Key words: *Zanthoxylum naranjillo*, Rutaceae, antischistosomal activity.

INTRODUCTION

Zanthoxylum naranjillo Griseb (Rutaceae) is popularly known in Brazil as 'juva', 'naranjillo', 'tembetari', and 'espinilho'. In Brazilian folk medicine, it is used against illnesses associated with inflammatory process (Reitz, 1960). Phytochemical studies on the leaves of this plant have described the isolation of lignans, sesquiterpenoids, and phenolic compounds (Bastos et al., 1996). The lignans from *Z. naranjillo* and their derivatives displayed significant trypanocidal and anti-inflammatory activities (Bastos et al., 1999, 2001; Souza et al., 2005). Additionally, recent investigation carried on the EtOAc fraction of *Z. naranjillo* leaves has led to isolation of phenolic compounds, which have been tested *in vitro* against *Schistosoma mansoni* adult worms. However, only protocatechuic and *p*-hydroxybenzoic acids were able to decrease daily egg production by *S. mansoni* and to separate adult worm pairs into male and female (Braguine et al., 2009).

Schistosomiasis is one of the major health problems worldwide that is caused by trematode flatworms of the genus *Schistosoma*. This disease shows significant values of prevalence and morbidity, affecting more than 200 million people around the world and resulting in as many as 280,000 deaths each year with over 779 million people at risk of infection (Zhang et al., 2010; Steinmann et al., 2006). The treatment of schistosomiasis includes praziquantel (PZQ) and oxamniquine, although reduced cure rates and treatment failure have been reported for patients receiving PZQ (Caffrey, 2007). This reinforces the need for the development of novel safe and effective schistosomicidal drugs. We report the isolation of two flavonoids and one alkaloid from the *n*-butanol fraction

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from the leaves of *Z. naranjillo*, and their antischistosomal evaluation against *S. mansoni* adult worms.

MATERIALS AND METHODS

Nuclear magnetic resonance (NMR) spectra were recorded in dimethyl sulfoxide-d_6 (DMSO-d_6) on a Varian Unity 500 NMR spectrometer, using tetramethylsilane (TMS) as internal standard. Both analytical and preparative high performance liquid chromatography (HPLC) separation analyses were carried out on a Shimadzu LC-6AD system equipped with a degasser DGU-20A5, a ultraviolet visible (UV-VIS) detector SPD-20A series, a communication bus module CBM-20A, and a Reodyne manual injector. Separations of the compounds were accomplished on SHIMADZU Shim-pack C18 (Octadecylsilane) (particle diameter 5 µm, 250 × 4.60 mm, and 250 × 20 mm) columns equipped with pre-columns of the same material. Thin layer chromatography (TLC) analyses were performed on glass plates precoated with silica gel 60 F_{254} nm (Fluka). Octadecyl functionalized silica gel (230 to 400 mesh, Sigma-Aldrich) and Sephadex LH-20 (Sigma-Aldrich) were employed for purification. The methanol used in the experiments was HPLC grade and had been purchased from J. T. Baker. Ultrapure water was obtained by passing redistilled water through a Direct-Q UV3 system, Millipore. Solid phase extraction (SPE) was conducted by using a reverse phase C18 Agilent cartridge.

Leaves of *Z. naranjillo* Griseb were collected in the campus of the University of Campinas (UNICAMP), Campinas, SP, Brazil, in March 2004. The material was identified by Professor Hermogenes de Freitas Leitão Filho, and a voucher specimen (4EC13627) was deposited in the Botany Institute Herbarium of the university.

The air-dried leaves (1.6 kg) of *Z. naranjillo* were powdered and exhaustively extracted by maceration with CH_2Cl_2 and EtOH at room temperature. After filtration, the solvents were removed under reduced pressure, to yield 110 and 80 g, respectively. The ethanolic extract (20 g) was then dissolved in CH_3OH-H_2O (9:1 v/v, 500 ml) and successively partitioned with hexane, CH_2Cl_2, EtOAc, and *n*-butanol. After solvent removal using a rotary evaporator, each partition phase yielded 7.6, 2.5, 2.2, and 7.1 g, respectively. The *n*-butanol residue (4 g) was dissolved in CH_3OH and was chromatographed over Sephadex LH-20 using this same solvent for elution, which yielded sixteen fractions. The obtained fractions were analyzed by TLC using $CHCl_3$-CH_3OH-H_2O (43:37:20 v/v/v). Purification of fraction 5 (842 mg) by column chromatography over C18 silica gel using a CH_3OH-H_2O gradient resulted in isolation of compound 1 (26 mg). Fraction 6 (533 mg) was dissolved in CH_3OH-H_2O (35:65 v/v), chromatographed over reverse phase C18 cartridge, and subsequently submitted to preparative reverse phase- high performance liquid chromatography (RP-HPLC) [CH_3OH-H_2O-AcOH (35:64.9:0.1 v/v/v), UV detection at 254 nm; flow rate 9 ml/min], which furnished compounds 2 (82 mg) and 3 (10 mg).

(+)-Magnoflorine (1)

White powder; ^{1}H-NMR (300 MHz, DMSO-d_6) δ (ppm): 6.73 (1H, d, *J*=7.8 Hz, H-9), 6.56 (1H, d, *J*=7.8 Hz, H-8), 6.65 (1H, s, H-3), 4.38 (1H, d, *J*=12.8, H-6a), 3.73 (3H, s, -OC$\underline{H}_3$), 3.70 (3H, s, -OC$\underline{H}_3$), 3.67 (1H, dl, *J*=6.1, H-5), 3.61 (1H, dd, *J*=13.0 and 4.8, H-5), 3.30 (3H, s, -NC$\underline{H}_3$), 3.19 m (2H, H-4 and H-7), 2.89 (3H, s, -NC$\underline{H}_3$), 2.84 m (1H, H-4), 2.61 (1H, t, *J*=12.8, H-7); ^{13}C-NMR (125 MHz, DMSO-d_6) δ (ppm): 151.5 (C-2), 150.7 (C-10), 149.8 (C-11), 149.2 (C-1), 126.3 (C-7a), 122.8 (C-11a), 122.6 (C-11b), 121.2 (C-11c), 115.9 (C-8), 115.6 (C-3a), 111,6 (C-9), 110.5 (C-3), 69.8 (C-6a), 61.3 (C-5), 56.8 and 56.4 (O$\underline{C}H_3$), 53.7 and 43.5 (N-$\underline{C}H_3$), 31.1 (C-7), 24.1 (C-4).

Apigenin-6-*C*-β-D-glucopyranoside (2)

Yellow powder; ^{1}H-NMR (300 MHz, DMSO-d_6) δ (ppm): 13.54 (1H, s, 5-O$\underline{H}$), 7.90 (2H, d, *J*=8.7 Hz, H-2' and H-6'), 6,92 (2H, d, *J*= 8.7 Hz, H-3' and H-5'), 6,75 (1H, s, H-3), 6.50 (1H, s, H-8), 4.58 (1H, d, *J*= 9.8 Hz, H-1''), 4.04 (1H, t, *J*= 9.8 Hz, H-2''), 3.10 to 3.20 (3H, m, H-3'', H-4'' and H-5''), 3.68 (1H, dl, *J*= 12.0, H-6''), 3.41 (1H, dd, *J*= 12.0 and 6.0, H-6''); ^{13}C-NMR (125 MHz, DMSO-d_6) δ (ppm): 182.0 (C-4), 164.2 (C-2), 164.0 (C-7), 162.0 (C-5 and C-4'), 157.1 (C-9), 129.3 (C-2' and C-6'), 121.9 (C-1'), 116.8 (C-3' and C-5'), 109.5 (C-6), 104.2 (C-10), 103.6 (C-3), 94.5 (C-8), 82.3 (C-5''),79.8 (C-3''), 73.9 (C-1''), 71.4 (C-4''), 71.3 (C-2''), 62.3 (C-6'').

Apigenin-8-C-β-D-glucopyranoside (3)

Yellow powder; ^{1}H-NMR (300 MHz, DMSO-d_6) δ (ppm): 13.05 (1H, s, 5-O$\underline{H}$), 7.90 (2H, d, *J*=8.7 Hz, H-2' and H-6'), 6,78 (2H, d, *J*=8.7 Hz, H-3' and H-5'), 6,65 (1H, s, H-3), 6.14 (1H, s, H-6), 4.59 (1H, d, *J*=8.9 Hz, H-1''), 3.73 (1H, t, *J*=8.9 Hz, H-2''), 3.14 to 3.18 (3H, m, H-3'', H-4'' and H-5''), 3.65 (1H, dl, *J*=10.6, H-6''), 3.41 (1H, dd, *J*=10.6 and 5.0 H-6''); ^{13}C-NMR (125 MHz, DMSO-d_6) δ (ppm): 181.9 (C-4), 163.8 (C-2 and C-7), 161.1 (C-9), 160.4 (C-4'), 156.5 (C-5), 128.8 (C-2' and C-6'), 121.6 (C-1'), 115.7 (C-3' and C-5'), 104.6 (C-10), 104.2 (C-8), 102.3 (C-3), 98.1 (C-6), 81.7 (C-5''), 78.5 (C-3''), 73.3 (C-1''), 70.7 (C-2''), 70.4 (C-4''), 61.2 (C-6'').

The Luis Evangelista (LE) strain of *S. mansoni* was maintained by passage through *Biomphalaria glabrata* snails and Balb/c mice. Eight weeks after infection, *S. mansoni* adult worms were recovered under aseptic conditions from mice previously infected with 200 cercariae by perfusion of the livers and mesenteric veins (Smithers and Terry, 1965). 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 (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 the same medium and was incubated at 37°C in a humid atmosphere containing 5% CO_2 prior to use. At 24 h after incubation, fraction (BF) and the isolated compounds (1 to 3) were dissolved in DMSO and were added to RPMI 1640 medium, to give final concentrations of 100 µg/ml or 100 µM. The parasites were kept for 5 days and were monitored every 24 h, for evaluation of their general condition. The effects of BF and 1 to 3 on *S. mansoni* were assessed by observing the viability of the worms, as well as pairing, motor activity, and tegument alteration, which were evaluated by means of an inverted microscope (Leitz). 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 (Magalhães et al., 2009). Quadruplicate measurements were accomplished for each employed concentration (10, 25, 50, and 100 µM for compounds 1 to 3 and 100 µg/ml for fraction), and two independent experiments were performed. The concentration of drug giving 50% separation of coupled worms (IC_{50}), for compound 3 was calculated by non-linear regression analysis of percentage of separation versus concentration at 120 h. RPMI 1640 medium and RPMI 1640 with 1% DMSO (the highest concentration of drug solvent) were used as negative control groups. PZQ (10 µM) was used as positive control group. All experiments were authorized by the Ethics Committee for Animal Care of the University of Franca and University of São Paulo, and they were in accordance with the national and international accepted principles for laboratory animal use and care.

RESULTS AND DISCUSSION

The spectral data of the isolated compounds (Figure 1)

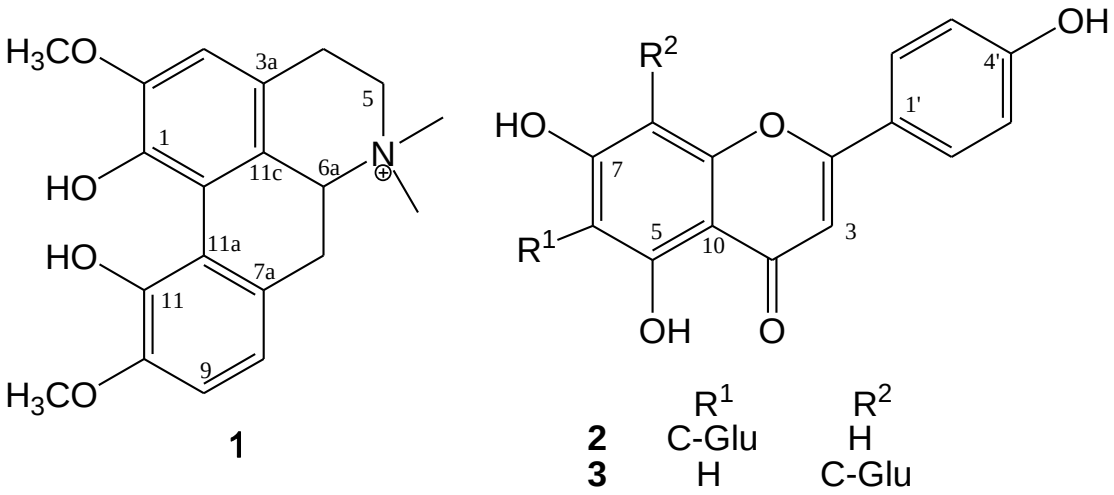

Figure 1. Chemical structures of the isolated compounds.

were in agreement with previously published data and allowed identification of (+)-magnoflorine (1), apigenin-6-*C*-β-D-glucopyranoside (2), and apigenin-8-*C*-β-D-glucopyranoside (3) (Barbosa-Filho et al., 1997; Agrawal, 1989). To the best of our knowledge, this is the first report of compounds 1 to 3 in *Z. naranjillo* (Figure 1).

Regarding the antischistosomal assay, incubation of *S. mansoni* coupled adult worms with the *n*-butanol fraction (BF, 100 µg/ml) did not result in death, decreased motor activity, or extensive tegumental alterations. However, it prompted separation of the coupled worms (25%) at 24 and 120 h. This result indicates improved activity for this fraction as compared to our previously published data (Braguine et al., 2009) on crude ethanolic extract and EtOAc fraction, which were both unable to separate the adult worms. This parameter is important since females are completely dependent upon male contact for growth, feeding, and egg production (Fitzpatrick and Hoffmann, 2006).

Considering the antischistosomal activity of the isolated compounds (Table 1), worms incubated with compound 1 (100 µM) exhibited significantly reduced motor activity, without tegumental alterations. On the other hand, compounds 2 and 3 (100 µM) did not elicit any tegumental alterations, and only compound 2 led to diminished motor activity. Compound 3 was able to separate adult worms into male and female (100%), which occurred at 120 h. Compound 3 exhibits an IC_{50} value of 15.8 µM at 120 h regarding the paring of *S. mansoni*. However, compounds 1 to 3 were unable to cause death of the worms at 100 µM. The appearance and motor activity of the worms in the 1% DMSO group were similar to those observed in the negative control group. In the two latter groups, neither death, separation, decreased motor activity, or extensive tegumental alterations of the *S. mansoni* adult worms observed. PZQ (10 µM), used as positive control, caused death of the parasites and tegumental alterations without separation of worms.

Compounds 2 and 3 differ mainly in terms of the position of the *C*-β-D-glucopyranoside group in the flavone moiety (C-6 and C-8, respectively). Therefore, considering the separation of adult worms, it can be suggested that the location of the glucose group at C-8 in the flavone ring, as in the case of compound 3, may improve the activity of flavone derivatives, since the latter compound was able to separate the male and female worms, while compound 2 was not.

The mechanism by which flavone derivatives, particularly compound 3, exert their *in vitro* effect against *S. mansoni* is not clear. However, the flavone luteolin has been identified as a non-selective inhibitor of the *S. mansoni* NAD+ catabolizing enzyme (SmNACE), which is localized in the outer surface (tegument) of the adult parasite (Kuhn et al., 2010). Moreover, luteolin exhibits pronounced effect against chloroquine-sensitive (NF-54) *Plasmodium falciparum* strains and inhibits the *P. falciparum* enoyl-ACP-reductase (FabI) protein (Gupta et al., 2010). Considering their antiparasitic activities, some flavones have been shown to possess antimalarial activity (Kaur et al., 2009). Additionally, magnoflorine has also been demonstrated to display weak antimalarial activity (Likhitwitayawuid et al., 1993).

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Table 1. *In vitro* effects of the *n*-butanol fraction (BF) and compounds (1 to 3) isolated from *Z. naranjillo* against *S. mansoni* adult worms.

Group	Incubation period (h)	Separated worms[a] (%)	Dead worms[a] (%)
Control[b]	24	0	0
	120	0	0
1% DMSO	24	0	0
	120	0	0
BF[c]	24	25	0
	120	25	0
1[d]	24	0	0
	120	0	0
2[d]	24	0	0
	120	0	0
3[d]	24	0	0
	120	100	0
PZQ[e]	24	0	100
	120	0	100

[a]Eight worms; [b]RPMI 1640; [c]Tested concentration 100 µg/ml; [d]Tested concentration 100 µM; [e]Tested concentration 10 µM.

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