Introduction

There is growing awareness of the huge public health significance of the so-called neglected tropical diseases. Schistosomiasis, caused by trematode flatworms of the genus *Schistosoma*, is one of the most significant neglected tropical diseases in the world. Even after great advances in treatment and prevention, the disease still shows significant values of prevalence and morbidity, affecting more than 200 million people worldwide and resulting in as many as 200,000 deaths each year with over 750 million people at risk (Steinmann et al., 2006; Caffrey, 2007). Praziquantel (PZQ) and oxamniquine are the currently available drugs for the treatment of schistosomiasis. Reduced cure rates in patients and failure in treatment after PZQ administration have been reported (Botros et al., 2003). In addition, the existence of resistant strains reinforces the need for the development of novel safe and effective schistosomicidal drugs (Magalhães et al., 2009; King et al., 2006).

The literature describing the indigenous medicine of central and South America contains references on the use of various plant species belonging to the genus *Zanthoxylum* (Rutaceae). They have been employed in the treatment of inflammatory diseases, malaria, snakebite, toothache, tuberculosis, not to mention their antimicrobial activity (Jullian et al., 2006; Villalba et al., 2007). *Zanthoxylum naranjillo* Griseb., known in Brazil as ‘juva’, ‘naranjillo’, ‘tembetari’, and ‘espinilho’, is used in Brazil as a folk medicine against illnesses associated with inflammatory processes (Reitz, 1960). Phytochemical investigations of this genus have reported the presence of alkaloids, amides, flavonoids, coumarins, terpenes, and lignans (Adesina, 2005; Jullian et al., 2006; Bastos et al., 1996, 1999, 2001). In addition, previous studies have shown that some lignans from *Z. naranjillo* and their derivatives display significant trypanocidal and anti-inflammatory activities (Souza et al., 2005; Bastos et al., 1996, 1999, 2001), as well as on the antiparasitic activities of...
natural products (Da Silva Filho et al., 2004a, b, 2008, 2009; Souza et al., 2005), we now report the isolation and structural identification of chemical constituents from the polar fraction of Z. naranjillo leaves, as well as their schistosomicidal activities against Schistosoma mansoni adult worms, which have not yet been described.

**Experimental**

**General**

$^1$H and $^{13}$C NMR spectra were recorded in DMSO-$d_6$ on a Varian Unity 500 NMR or Bruker Ac-200 spectrometer, using TMS as internal standard. 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 Reodyne manual injector. Separations of the micromolecules were carried out on a Shimadzu Shim-pack ODS (particle diameter 5 μm, 250 × 4.60 mm and 250 × 20 mm) columns equipped with a pre-column of the same material. The methanol used in the experiments was of HPLC grade (J. T. Baker). Ultrapure water was obtained by passing redistilled water through a Direct-Q UV3 system (Millipore). Solid phase extraction (SPE) was carried out with a reverse phase ODS Agilent cartridge.

**Plant material**

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

**Extraction and isolation**

The air-dried leaves (1.6 kg) of Z. naranjillo were powdered and exhaustively extracted by maceration with methylene chloride and EtOH at room temperature. After filtration, the solvents were removed under reduced pressure, to yield 110 g and 80 g, respectively. The ethanolic extract (40 g) was then dissolved in MeOH/H$_2$O (9:1 v/v) and successively partitioned with n-hexane, methylene chloride, EtOAc, and n-butanol. After solvent removal using a rotaevaporator, each partition phase yielded 7.6, 2.5, 2.2, and 7.1 g, respectively. The EtOAc residue (0.94 g) was dissolved in MeOH/H$_2$O (4:6 v/v) and chromatographed over a reverse phase ODS cartridge, and subsequently submitted to preparative RP-HPLC purification using MeOH/H$_2$O/AcOH (40:59.9:0.1 v/v/v, UV detection at 254 nm, flow rate 10 ml/min) leading to eight fractions. Fraction 3 afforded compound 1 (74 mg, $R_f$ = 7.29 min). Fractions 2 (44.8 mg, $R_f$ = 5.80 min) and 4 (55.3 mg, $R_f$ = 10.2 min) were purified by RP-HPLC [MeOH/ H$_2$O/AcOH (30:69.9:0.1 v/v/v), UV detection at 254 nm, flow rate 9 ml/min], affording compounds 1 (9 mg), 2 (5 mg), 3 (5 mg), and 4 (13 mg), respectively.

**In vitro schistosomicidal assays**

Parasite culture and maintenance

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 (male and female) were recovered under aseptic conditions from mice previously infected with 200 cercariae by perfusion of the livers and mesenteric veins (Magalhães et al., 2009). The worms were washed in RPMI 1640 medium (Invitrogen), kept at pH 7.5 with 20 mM HEPES (N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid) and supplemented with penicillin (100 UI/ml), streptomycin (100 μg/ml), and 10% bovine fetal serum (Gibco). After washing, one pair 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. 24 h after incubation, the crude ethanolic extract (EE), the EtOAc fraction (EF), and compounds 1–4 were dissolved in 10% DMSO and added to the RPMI 1640 medium, to give final concentrations of 100 μg/ml (crude extract and fractions) and 100 μM (isolated compounds). The effects of the EE, EF, and 1–4 on S. mansoni were assessed by observing the viability of the worms, as well as pairing, egg production, and egg development (Magalhães et al., 2009). All experiments were authorized by the Ethical Committee for Animal Care of the University of Franca and University of São Paulo, Brazil and they were in accordance with the national and international accepted principles for laboratory animal use and care.
In vitro assay

For the in vitro test with *S. mansoni*, samples were dissolved in 10% DMSO and added to the medium containing one pair of adult worms after a period of 24 h of adaptation to the culture medium. The parasites were kept for 120 h, to evaluate their general condition in terms of: motor activity, alterations in the tegument, and mortality rate. Changes in pairing were also evaluated by means of an inverted microscope (Leitz) (Magalhães et al., 2009). The control worms were treated with 10% DMSO in RPMI 1640 medium. All experiments were carried out in quadruplicate using RPMI 1640 medium and RPMI 1640 medium with 10% DMSO as negative control groups, and 10 μM PZQ as positive control group.

Results and Discussion

A previous antiparasitic study with *Z. naranjillo* had revealed that some lignans display significant trypanocidal activity (Bastos et al., 1999), which encouraged us to continue the study of the biological and chemical investigations of polar metabolites from this plant, which has not been performed to date. Also, no study with metabolites from this plant, which has not been

The spectral data of all the isolated compounds (Fig. 1) were in agreement with previously published data, which allowed the identification of protocatechuic acid (1), gallic acid (2), p-hydroxybenzoic acid (3), and 5-O-caffeoylshikimic acid (4) (Hur et al., 2003; Gohar et al., 2003; Pouchert, 1992; Veit et al., 1992). Previous phytochemical studies on the aerial parts of this plant have described the isolation of lignans and sesquiterpenoids (Bastos et al., 1996), but, to the best of our knowledge, this is the first report of compounds 1–4 in *Z. naranjillo*.

Regarding the schistosomicidal assay (Table 1), pairs of coupled adult worms, incubated with the ethanolic extract (EE, 100 μg/ml) and EtOAc fraction (EF, 100 μg/ml), demonstrated decreased motor activity. However, both the EE (100 μg/ml) and EF (100 μg/ml) did not cause death, separation, or tegumental alterations of the *S. mansoni* adult worms.

Considering the schistosomicidal activity of the isolated compounds (Table 1), worms incubated with compounds 1 and 3 (100 μM) showed significantly decreased motor activity. In addition, two pairs of coupled adult worms were separated into individual males and females, without tegumental alterations, after 120 h of incubation with compounds 1 (100 μM) and 3 (100 μM). On the other hand, compounds 2 and 4 (100 μM) did not display any activity against *S. mansoni* adult worms. The appearance and motor activity of the worms in the 10% DMSO group were similar to those in the negative control group. PZQ (10 μM), used as positive control, caused death of the parasites and tegumental alterations without separation of worms.

Regarding egg production (Table 1), the EE (100 μg/ml) and EF (100 μg/ml) inhibited this production by 16.3% and 6.4%, respectively, in comparison with the negative control group treated with RPMI 1640 medium. Additionally, compounds 1–4 (100 μM) showed a significant decrease in the number of eggs, by 29.8%, 13.5%, 28.4%, and 17.7%, respectively, after 120 h of incubation (Table 1). However, the effect of compounds 1 and 3 on egg production may be correlated with their ability to separate adult worms into males and females. On the other hand, the effects of the EE, EF, 2, and 4 did not result from the separation of the coupled adult worms, but they may be related to the effect of these samples against the pairs of coupled adult worms.

Furthermore, it can be observed that compounds 1, 2, and 3 are quite similar, differing mainly in the presence of the number of hydroxy
groups in the benzoic acid moiety. Therefore, considering egg production, it is suggested that the presence of one or two hydroxy groups in the aromatic ring, as in the case of compounds 3 and 1, may improve the activity of the benzoic acid derivatives 1–3 since compounds 1 and 3 were more active than 2.

The mechanism by which the benzoic acid derivatives 1 and 3 exert their in vitro schistosomicidal effect is not clear. However, considering their antiparasitic activities, the benzoic acid derivatives 1–3 have been shown to possess antimalarial activity (Verotta et al., 2001). In addition, the interest in plant phenols as potential complementary antimalarial agents has arisen because they have been shown to enhance the in vitro effect of artemisinin (Elford et al., 1987). Moreover, compound 4 exhibits a pronounced effect against Leishmania donovani amastigotes, as well as appreciable trypanocidal activity against trypanostigote forms of Trypanosoma brucei rhodesiense (Kirmizibekmez et al., 1991).

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