Schistosomicidal Evaluation of *Zanthoxylum naranjillo* and its Isolated Compounds against *Schistosoma mansoni* Adult Worms

Caio G. Braguine[a], Eveline S. Costa[a], Lizandra G. Magalhães[b], Vanderlei Rodrigues[b], Ademar A. da Silva Filho[b], Jairo K. Bastos[c], Márcio L. A. Silva[a], Wilson R. Cunha[a], Ana H. Januário[a], and Patrícia M. Pauletti[a,*]

[a] Universidade de Franca, Av. Dr. Armando Salles de Oliveira, 201, CEP 14404-600, Franca, São Paulo, Brazil. E-mail: pmpauletti@unifran.br
[b] Faculdade de Medicina de Ribeirão Preto, Universidade de São Paulo, Av. do Café S/N, CEP 14040-903, Ribeirão Preto, São Paulo, Brazil
[c] Faculdade de Ciências Farmacêuticas de Ribeirão Preto, Universidade de São Paulo, Av. do Café S/N, CEP 14040-903, Ribeirão Preto, São Paulo, Brazil

* Author for correspondence and reprint requests

Z. Naturforsch. **64c**, 793–797 (2009); received July 14, 2009

Chemical investigation of the EtOAc fraction (**EF**) obtained from the ethanolic extract of *Zanthoxylum naranjillo* (Rutaceae) leaves (**EE**) by preparative HPLC resulted in the isolation of protocatechuic acid (**1**), gallic acid (**2**), *p*-hydroxybenzoic acid (**3**), and 5-*O*-caffeoylshikimic acid (**4**). This is the first time that the presence of compounds **1**–**4** in *Z. naranjillo* has been reported. Compounds **1**–**4**, the **EE**, and **EF** were tested *in vitro* against *Schistosoma mansoni* adult worms. The results showed that the *S. mansoni* daily egg production decreased by 29.8%, 13.5%, 28.4%, 17.7%, 16.3%, and 6.4%, respectively. Compounds **1** and **3** were also able to separate adult worm pairs into male and female. This activity may be correlated with the reduction in egg production, since **1** and **3** showed better inhibitory properties compared with **2** and **4**.

Key words: Zanthoxylum naranjillo, Rutaceae, Schistosomicidal Activity

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 500,000 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). 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).

In this sense, as part of our ongoing biological studies on *Z. naranjillo* (Bastos *et al.*, 1996, 1999, 2001), as well as on the antiparasitic activities of

0939–5075/2009/1100–0793 $ 06.00

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_2O (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_2O (4:6 v/v) and chromatographed over a reverse phase ODS cartridge, and subsequently submitted to preparative RP-HPLC purification using MeOH/H_2O/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_t = 7.29 min). Fractions 2 (44.8 mg, R_t = 5.80 min) and 4 (55.3 mg, R_t = 10.2 min) were purified by RP-HPLC [MeOH/H_2O/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 *Z. naranjillo* against *S. mansoni* has been reported.

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 I), 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 I), 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.

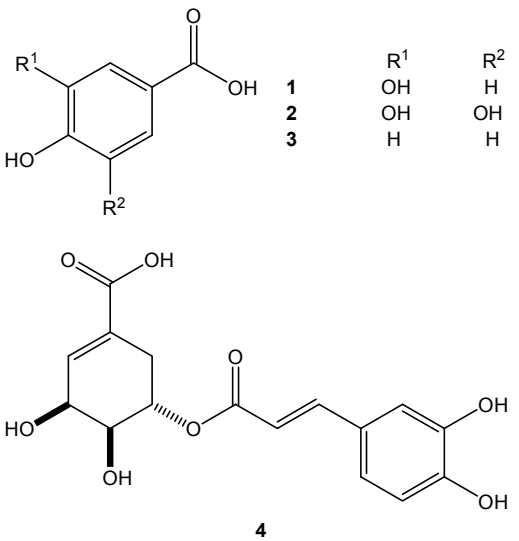

Fig. 1. Chemical structure of the isolated compounds.

Regarding egg production (Table I), 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 I). 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

Table I. *In vitro* effects of the crude extract, EtOAc fraction, and compounds from *Z. naranjillo* against *S. mansoni* adult worms.

Group	No. of worms	Incubation period [h]	No. of separated worms	Egg production	
				No. of eggs	Inhibition (%)
10% DMSO	8	24	0	135	4.2
		120	0		
Control[a]	8	24	0	141	–
		120	0		
EE[b]	8	24	0	118	16.3
		120	0		
EF[b]	8	24	0	132	6.4
		120	0		
1[c]	8	24	4	99	29.8
		120	4		
2[c]	8	24	0	122	13.5
		120	0		
3[c]	8	24	4	101	28.4
		120	4		
4[c]	8	24	0	116	17.7
		120	0		

[a] RPMI 1640 medium. [b] Tested concentration was 100 μg/ml. [c] Tested concentration was 100 μM.

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 trypomastigote forms of *Trypanosoma brucei rhodesiense* (Kirmizibekmez *et al.*, 1991).

Acknowledgement

The authors are grateful to Fundação de Amparo a Pesquisa do Estado de São Paulo (FAPESP), Coordenadoria de Aperfeiçoamento de Pessoal do Ensino Superior (CAPES), and Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) for fellowships. FAPESP is also acknowledged for financial support (Grants # 2008/01268-9; 2006/60132-4).

Adesina S. K. (2005), The Nigerian *Zanthoxylum*. Chemistry and biological values. Afr. J. Trad. CAM **2**, 282–301.

Bastos J. K., Gottlieb O. R., Sarti S. J., and Santos Filho D. (1996), The isolation of lignans and sesquiterpenoids from leaves of *Zanthoxylum naranjillo*. Nat. Prod. Lett. **9**, 65–70.

Bastos J. K., Albuquerque S., and Silva M. L. A. (1999), Evaluation of the trypanocidal activity of lignans isolated from the leaves of *Zanthoxylum naranjillo*. Planta Med. **65**, 541–544.

Bastos J. K., Carvalho J. C. T., de Souza G. H. B., Pedrazzi A. H. P., and Sarti S. J. (2001), Anti-inflammatory activity of cubebin, a lignan from the leaves of *Zanthoxylum naranjillo* Griseb. J. Ethnopharmacol. **75**, 279–282.

Botros S., William S., Hammam O., Zídek Z., and Holý A. (2003), Activity of 9-(*S*)-[3-hydroxy-2-(phosphonomethoxy)propyl]adenine against *Schistosomiasis mansoni* in mice. Antimicrob. Agents Chemother. **47**, 3853–3858.

Caffrey C. R. (2007), Chemotherapy of schistosomiasis: present and future. Curr. Opin. Chem. Biol. **11**, 433–439.

Da Silva Filho A. A., Bueno P. C. P., Gregório L. E., Silva M. L. A., Albuquerque S., and Bastos J. K.

(2004a), *In vitro* trypanocidal activity evaluation of crude extract and isolated compounds from *Baccharis dracunculifolia* D. C. (Asteraceae). J. Pharm. Pharmacol. **56**, 1195–1199.

Da Silva Filho A. A., Albuquerque S., Silva M. L. A., Eberlin M. N., Tomazela D. M., and Bastos J. K. (2004b), Tetrahydrofuran lignans from *Nectandra megapotamica* with trypanocidal activity. J. Nat. Prod. **67**, 42–45.

Da Silva Filho A. A., Costa E. S., Cunha W. R., Silva M. L. A., Nanayakkara N. P. D., and Bastos J. K. (2008), *In vitro* antileishmanial and antimalarial activities of tetrahydrofuran lignans isolated from *Nectandra megapotamica* (Lauraceae). Phytother. Res. **22**, 1307–1310.

Da Silva Filho A. A., Resende D. O., Fukui M. J., Santos F. F., Pauletti P. M., Cunha W. R., Silva M. L. A., Gregório L. E., Bastos J. K., and Nanayakkara N. P. D. (2009), *In vitro* antileishmanial, antiplasmodial and cytotoxic activities of phenolics and triterpenoids from *Baccharis dracunculifolia* (Asteraceae). Fitoterapia, doi:10.1016/j.fitote.2009.06.007.

Elford B. C., Roberts M. F., Phillipson J. D., and Wilson R. J. (1987), Potentiation of the antimalarial activity of qinghaosu by methoxylated flavones. Trans. R. Soc. Trop. Med. Hyg. **81**, 434–436.

Gohar A. A., Lahloub M. F., and Niwa M. (2003), Antibacterial polyphenol from *Erodium glaucophyllum*. Z. Naturforsch. **58c**, 670–674.

Hur J. M., Park J. G., Yang K. H., Park J. C., Park J. R., Chun S. S., Choi J. S., and Choi J. W. (2003), Effect of methanol extracts of *Zanthoxylum piperitum* leaves and of its compound, protocatechuic acid, on hepatic drug metabolizing enzymes and lipid peroxidation in rats. Biosci. Biotechnol. Biochem. **67**, 945–950.

Jullian V., Bourdy G., Georges S., Maurel S., and Sauvain M. (2006), Validation of use of a traditional antimalarial remedy from French Guiana, *Zanthoxylum rhoifolium* Lam. J. Ethnopharmacol. **106**, 348–352.

King C. H., Sturrock R. F., Kariuki H. C., and Hamburger J. (2006), Transmission control for schistosomiasis – why it matters now. Trends Parasitol. **22**, 575–582.

Kirmizibekmez H., Çalis R., Perozzo R., Brun R., Dönmez A. A., Linden A., Rüedi P., and Tasdemir D. (1991), Inhibiting activities of secondary metabolites of *Phlomis brunneogaleata* against parasitic protozoa and plasmodial enoyl-ACO reductase, a crucial enzyme in fatty acid biosynthesis. Planta Med. **70**, 711–717.

Magalhães L. G., Machado C. B., Morais E. R., Moreira E. B., Soares C. S., da Silva S. H., Da Silva Filho A. A., and Rodrigues V. (2009), *In vitro* schistosomicidal activity of curcumin against *Schistosoma mansoni* adult worms. Parasitol. Res. **104**, 1197–1201.

Pouchert C. (1992), Aldrich Library of ^{13}C and ^{1}H FT NMR Spectra, Vol. 2. Wiley, Hoboken.

Reitz P. R. (1960), Flora Ilustrada Catarinense, 1st ed. Santa Catarina, Brazil, pp. 13–14.

Souza V. A., Silva R., Pereira A. C., Royo V. A., Saraiva J., Montanheiro M., Souza G. H. B., Da Silva Filho A. A., Grando M. D., Donate P. M., Bastos J. K., Albuquerque S., and Silva M. L. A. (2005), Trypanocidal activity of (–)-cubebin derivatives against free amastigote forms of *Trypanosoma cruzi*. Bioorg. Med. Chem. **15**, 303–307.

Steinmann P., Keiser J., Bos R., Tanner M., and Utzinger J. (2006), Schistosomiasis and water resources development: systematic review, meta-analysis, and estimates of people at risk. Lancet Infect. Dis. **6**, 411–425.

Veit M., Cornelia W., Strack D., Wray V., Witte L., and Czygan F.-C. (1992), The distribution of caffeic acid conjugates in the Equisetaceae and some fearns. Phytochemistry **31**, 3483–3485.

Verotta L., DellÁgli M., Giolito A., Guerrini A., Cabalion P., and Bosisio E. (2001), *In vitro* antiplasmodial activity of extracts of *Tristaniopsis* species and identification of the active constituents: ellagic acid and 3,4,5-trimethoxyphenyl-(6'-*O*-galloyl)-*O*-D-glucopyranoside. J. Nat. Prod. **64**, 603–607.

Villalba M. A., Carmo M. I., Leite M. N., and Sousa O. V. (2007), Atividades farmacológicas dos extratos de *Zanthoxylum chiloperone* (Rutaceae). Rev. Bras. Farmacogn. **17**, 236–241.