Activity of Neolignans Isolated from *Piper regnellii* (Miq.) C. DC. var. *pallescens* (C. DC.) Yunck against Trypanosoma cruzi

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The in vitro antiproliferative effects of 4 neolignans purified from the ethyl-acetate extract from leaves of *Piper regnellii* (Miq.) C. DC. var. *pallescens* (C. DC.) Yunck against Trypanosoma cruzi were investigated. These isolated compounds were identified through spectral analyses of UV, EI-MS, 1H-, 13C-NMR, gNOE, HETCOR, and HMBC. The compounds eupomatenoid-5, eupomatenoid-6, and conocarpan showed considerable activity against epimastigote forms of *T. cruzi*, with 50% inhibition concentrations (IC50) of 7.8, 7.5, and 8.0 μg/ml respectively. After methylation, these compounds showed a lessened inhibitory activity to the growth of the protozoan, suggesting that loss of the hydroxyl group from their molecules reduces the activity. The compound eupomatenoid-3 showed lower activity than the hexane fraction. Eupomatenoid-5 was significantly more active than benznidazole, the antiparasitic drug of choice for treatment of Chagas’ disease. The crude extract, hexane fraction, and eupomatenoid-5 caused no lysis in sheep blood at concentrations which inhibit the growth of epimastigote forms. The compound eupomatenoid-5 showed low cytotoxic effects against Vero cells. These results provide new perspectives on the development of novel drugs obtained from natural products with trypanocidal activity. However, the extracts and active compound isolated from *P. regnellii* var. *pallescens* should be further studied in animal models for in vivo efficacy.

Key words anti-trypanosoma; neolignan; *Piper regnellii*; Trypanosoma cruzi

Chagas’ disease is an important medical problem in Latin America, affecting about 18 million people and causing the deaths of 45000 people annually. The etiologic agent *Trypanosoma cruzi* is transmitted to the vertebrate host by a hematophagous reduvid bug vector. The initial acute phase has a low mortality (<10%) and is often asymptomatic, whereas the chronic phase typically occurs 10 to 20 years after the parasite is contracted, and affects 30 to 40% of those infected. Cardiac and gastrointestinal pathology are the most common manifestations of chronic disease.15) Currently available chemotherapy, based on benznidazole and nifurtimox, is unsatisfactory because of limited efficacy in the prevalent chronic stage of the disease and because of the frequent toxic side effects.22) It is necessary to develop alternative, powerful trypanocidal drugs with no side effects.

In spite of the great advances in modern medicine in recent decades, plants still make an important contribution to health care. Marked growth in the worldwide phytotherapeutic market has occurred over the last 15 years.23) Plants provide chemical diversity and bioactivity, which has led to the development of hundreds of pharmaceutical drugs. Natural products represent an unparalleled source of molecular diversity for drug discovery and development.24) Several studies have demonstrated that plants show activity against *T. cruzi*.5—7) The idea that herbal drugs are safe and free of side effects is false. Plants contain hundreds of constituents and some of them are very toxic.25) Medicinal plants are distributed worldwide, but they are most numerous in tropical countries.

*Piper regnellii* (Miq.) C. DC. var. *pallescens* (C. DC.) Yunck (Piperaceae) is an herbaceous plant popularly known in Brazil as “Pariparoba” and grows in tropical and subtropical regions of the world.5) The leaves and roots are used in the forms of crude extracts, infusions, or plasters to treat wounds and reduce swelling and skin irritations.9—11) Holetz et al.12) screened 13 Brazilian medicinal plants, and reported antimicrobial activity of the hydroethanolic extract of leaves of *P. regnellii* against the bacteria *Staphylococcus aureus* and *Bacillus subtilis*, and against the yeasts *Candida krusei* and *Candida tropicalis*.

Various species of *Piper* have been shown to have interesting biological activities.13) Phytochemical study of *P. regnellii* roots has demonstrated the accumulation of phenylpropanoids and dihydrobenzofuran neolignans.14) Several compounds of this class (benzofuran neolignans) have been isolated from species of Piperaceae, and show a variety of biological activity including anti-PAF, antifungal, and insecticidal.15) In the present study, we describe the anti-*Trypanosoma cruzi* activity of extracts and neolignans isolated from *P. regnellii* var. *pallescens*.

MATERIALS AND METHODS

Plant Material The leaves of *P. regnellii* var. *pallescens* were collected in July 2002 in the Prof.ª Irene Silva Garden of Medicinal Plants on the campus of the State University of Maringá. The plant material was identified through morphological analysis, by Marília Borgo of the Botanical Department of the Federal University of Paraná, and a voucher specimen (Nº HUM 8392) is deposited at the Herbarium of the State University of Maringá, Paraná, Brazil.

General Experimental Procedures The NMR spectra were obtained in a BRUKER ARX400 (9.4 T) and VARIAN GEMINI 300 (7.05 T), using deuterated solvent for field homogeneity, TMS as the internal standard, and a constant temperature of 298 K. IR: film NaCl plates; ES-MS were
forms of Trypanosoma cruzi Y strain was used as test organisms. The protozoans were grown in liver-infusion tryptone broth-LIT \cite{17} supplemented with 10% fetal bovine serum (Gibco Invitrogen Corporation, New York, U.S.A.).

**Trypanocidal Activity in Vitro** The epimastigote form of *T. cruzi* was assayed with crude extract ethyl-acetate phase, hexane fraction, and isolated compounds. The extracts to be tested were dissolved in dimethyl sulfoxide (DMSO). The final concentration of DMSO did not exceed 1% and for each experiment there was a growth control with and without DMSO. \cite{13} The experiments were performed in 24-well plates containing 1 ml of diluted compound at different concentrations. The starting inoculum consisted of 10^6 parasites per well in logarithmic growth phase. The cells were incubated at 28°C and the growth was determined by counting the parasites with a Neubauer haemocytometer every 24 h over a 7-d period. Benznidazole (N-phenyl-2-nitro-1-imidazolacetamide, Roche Pharmaceuticals, Rio de Janeiro, Brazil) was used as the reference drug. The assays were performed in duplicate on separate occasions.

**Red Blood Cell (RBC) Lysis Assay** A 4% suspension of freshly defibrinated sheep blood was prepared by adding 2.0 ml of blood to 48 ml of 5% glucose solution. Stock solutions of crude extract ethyl-acetate phase, hexane fraction, and eumatomenoid-5 solubilised in DMSO were further di-
luted in 5% glucose at different concentrations. One millilitre of RBC suspension was added and gently mixed, and the tubes were incubated at 37 °C. Samples were centrifuged at 1000 g for 10 min. Absorbance of the supernatant was determined at 540 nm. The inhibition of haemolysis was calculated according to the equation: haemolysis inhibition (%)
\[
= \left(\frac{Ap - As}{Ap - Ac}\right) \times 100;
\]
where Ap, As, and Ac are the absorbance of the positive control (Triton X-114), test sample, and negative control respectively. These tests were performed in duplicate on separate occasions.

**Cellular Toxicity**

The Vero cells were seeded onto 96-well microtitre plates at a concentration of 2.5×10^4 cells per well, and allowed to proliferate for 24 h in DMEM containing 5% FBS. Different concentrations of eupomatenoid-5 were applied to the cell monolayer in triplicate. DMSO was used as a negative control. After incubation at 37 °C with 5% CO₂ for 72 h, the cell growth was evaluated by the sulforhodamine B assay. Data were calculated as the percentage of inhibition. The concentration of 50% cellular toxicity (CC₅₀) was defined as the concentration which reduces the OD 530 of treated cells to 50% of that of untreated cells.

**RESULTS AND DISCUSSION**

The 50% inhibition concentration (IC₅₀) for *T. cruzi*, of the crude extract ethyl-acetate phase obtained from leaves of *P. regnellii* var. *pallescens*, was 54 μg/ml, whereas the aqueous phase was inactive (IC₅₀ >500 μg/ml). On the basis of this finding, the ethyl-acetate extract was fractionated on silica gel into nine fractions (F1—F9). The F1, F2, F3, and F4 fractions at a concentration of 50 μg/ml, showed a growth inhibition of 92.2, 86.9, 75.0, and 64.0% respectively (Fig. 1). The other fractions (F5 to F9) showed an activity lower than that of the crude extract ethyl-acetate phase in the growth of *T. cruzi*.

The pure compounds isolated from the hexane fraction (F1) were assayed against the epimastigote forms of *T. cruzi* (Fig. 2). As illustrated in Figs. 2B, C, and D, *T. cruzi* showed similar sensitivity when eupomatenoid-5, eupomatenoid-6, and conocarpan were assayed at different concentrations. At 50 μg/ml these compounds inhibited 100% of the growth of *T. cruzi* after 96 h of incubation. Eupomatenoid-3 showed less activity than the hexane fraction (Fig. 2A). Compounds 1, 2, 3, and 4 were identified as eupomatenoid-3, eupomatenoid-5, eupomatenoid-6, and conocarpan respectively (Flowchart). Their ¹H-, ¹³C-NMR spectral data were identical to those of published values.⁴,¹⁶

Eupomatenoid-5, eupomatenoid-6, and conocarpan were methylated and their antiproliferative effects were estimated. Table 1 shows the values of IC₅₀ of isolated and methylated compounds obtained from the hexane fraction of *P. regnellii* var. *pallescens* against the epimastigote forms of *T. cruzi*. While the IC₅₀ of eupomatenoid-3 against *T. cruzi* epimastigotes was 26.3 μg/ml, eupomatenoid-5, eupomatenoid-6 and conocarpan showed similar IC₅₀ levels of 7.0, 7.5, and 8.0 μg/ml, respectively. When these purified compounds were methylated, the IC₅₀ increased to 29.2, 21.8, and 24.5 μg/ml, respectively. After methylation, these compounds showed lowered inhibitory activity on the growth of the protozoans, suggesting that the loss of the hydroxyl group from their molecules reduces the activity. Eupomatenoid-3 possesses no phenolic hydroxyl group. The phenolic hydroxyl present in the active structure is apparently able, through hydrogen-bond links with the specific molecular structures on the cell surface, to inactivate essential metabolic pathways.²⁰

![Fig. 1. Effect of Crude Extract Ethyl-Acetate Phase (CE) and Fractions (F1—F9) at 50 μg/ml Obtained from *Piper regnellii* var. *pallescens* on Growth of the Epimastigote Form of *Trypanosoma cruzi* after 96 h of Incubation](image1)

![Fig. 2. Effect of Compounds Isolated from *Piper regnellii* var. *pallescens* on Growth of the Epimastigote Form of *Trypanosoma cruzi*: (A) Eupomatenoid-3; (B) Eupomatenoid-5; (C) Eupomatenoid-6; (D) Conocarpan](image2)
which showed 78% growth inhibition. The medium containing 1% DMSO did not affect the growth of the protozoans (data not shown). Benznidazole, the most important antiparasitic drug for treatment of Chagas’ disease, has limited efficacy in the chronic phase of the disease. Disparities in the susceptibility of *T. cruzi* strains to drugs may explain, in part, differences in drug efficacy in the treatment of vertebrate hosts. Yong et al. showed that the values of IC<sub>50</sub> of benznidazole against *T. cruzi* Dm28 strains resistant and sensitive to synthetic inhibitors of cysteine-protease, were 1.56 and 1.30 μg/ml respectively.

An important criterion in the search for compounds active against *T. cruzi* with therapeutic potential is that they have no toxic effects on mammalian host cells. For this purpose, a test of cytotoxicity to sheep erythrocytes was performed in order to determine the ratio of selectivity to biological activity. Figure 4 shows the effect of the crude extract ethyl-acetate phase, hexane fraction, and eupomatenoid-5 isolated from *P. regnellii* var. *pallescens* and amphotericin B in sheep blood after 2 h of incubation at 37 °C. Neither the crude extract, hexane fraction, nor eupomatenoid-5 affected red blood cell integrity at concentrations which inhibit the growth of epimastigote forms of *T. cruzi*. At 100 μg/ml the percentage of haemolysis varied from 1 to 10%. On the other hand, the drug amphotericin B, which has a potent effect against *T. cruzi* but is toxic to host cells, shows 78% haemolysis. As reported by Lee et al., amphotericin B showed strong haemolytic activity with a minimum lytic concentration (MLC) of 6.25 μg/ml within 60 min, whereas 1% DMSO did not cause lysis.

In the experiment used to evaluate cytotoxicity for mammalian cells, Vero cells were treated with the pure isolated compound, and after 72 h the viability was checked by the sulforhodamine B assay. When Vero cells were treated with eupomatenoid-5, the 50% cytotoxic concentration (CC<sub>50</sub>) was 250 μg/ml. The toxicity for Vero cells and the activity against the protozoans were compared by using the selectivity index (SI) ratio (CC<sub>50</sub> for Vero cells/IC<sub>50</sub> for protozoans). A value greater than 1 is considered more selective for activity against the parasite. Eupomatenoid-5 was more selective against the parasites than the Vero cells, with an SI ratio of 35.7.

These data revealed that leaves of *P. regnellii* var. *pallescens* contain compounds with anti-trypanosomal activity, clearly indicating that our results provide preliminary scientific validation for the medicinal use of this plant. However, the extracts and active compounds isolated from *P. reg-
nelli var. pallescens should be further studied in animal models for in vivo efficacy.

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