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In vitro activity of compounds isolated from *Piper crassinervium* against *Trypanosoma cruzi*

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This study describes the antichagasic potential of five compounds isolated from leaves of *Piper crassinervium* (Piperaceae). Two prenylated benzoic acid derivatives, one prenylated hydroquinone and two flavanones, were evaluated. The *in vitro* trypanocidal activity was determined against epimastigote forms of *Trypanosoma cruzi* (Y strain), the etiologic agent of Chagas disease. The most active compound was the prenylated hydroquinone [1,4-dihydroxy-2-(3^0,7^0-dimethyl-1^0-*oxo*-2^0-*E*,6^0-octadienyl)benzene] with an IC_{50} value of $6.10\,\mu g\,mL^{-1}$, which was in the same order of activity if compared with the positive control benznidazole ($IC_{50} = 1.60\,\mu g\,mL^{-1}$). This is the first report of trypanocidal activity for prenylated hydroquinone and benzoic acid derivatives.

Keywords: prenylated benzoic acid; prenylated hydroquinone; flavanone; *Trypanosoma cruzi*; *Piper crassinervium*

1. Introduction

American trypanosomiasis or Chagas disease is a zoonosis caused by the hemoflagellate protozoan *Trypanosoma cruzi*. In Latin and South America millions of people (estimated at 16–18 million in the decade of the 1990s) have been infected, and more than 25% of the population is at risk of being contaminated by the parasite (Grael, Albuquerque, & Lopes, 2005). However, over the last 15 years, prevalence estimates have been reduced, largely due to intensive large-scale programs designed to halt transmission by eliminating domestic populations of the insect vectors and to improve the screening of blood donors (Jannin & Villa, 2007). Nevertheless, transmission of Chagas disease is still a major health problem because of the transfusion of infected blood. Moreover, a recent case report in Brazil suggested the oral transmission of parasites, renewing the discussions about this problem (Pérez-Gutiérrez, Agrelo Salvatella, & Figueroa, 2006). Currently available chemotherapy, based on nifurtimox and benznidazole, is accepted for treating acute infections, but it is unsatisfactory in the prevalent chronic stage because of limited efficacy, besides the

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frequent toxic effects (Luize, Ueda-Nakamura, Dias Filho, Garcia Cortez, & Nakamura, 2006). At present, the only trypanocidal compound used to prevent infection in blood banks is gentian violet, but it shows toxic effects and its use is limited (Rosas et al., 2007). The lack of effective medicines against acute and chronic infection is the critical reason to find more efficient and less toxic drugs than those currently used on the way to a cure for this pan-Latin-American disease. In spite of the great advances in modern medicine in recent decades, plants still make an important contribution to health care, providing chemical diversity and bioactivity, as well as to the development of hundreds of pharmaceutical drugs (Luize et al., 2006). Piperaceae family, belonging to the largest basal Angiosperm order, includes two rich genera, *Piper* (ca. 2000 species) and *Peperomia* (ca. 1500–1700 species) (Wanke et al., 2007). Several *Piper* species have been well studied, in which *Piper nigrum* produce piperine, the active pungent principle of black pepper; from *P. hispidum, P. arboreum* and *P. tuberculatum* were isolated antifungal and insecticide amides (Navickiene et al., 2000; Silva et al., 2002). Additionally, chromenes and polyketides with significant antifungal activity have also been isolated from *P. aduncum, P. hostmanianum*, *P. crassinervium* and *P. gaudichaudianum* (Baldoqui et al., 1999; Danelutte, Lago, Young, & Kato, 2003; Kato & Furlan, 2007).

Hydroquinones bearing prenyl moieties are natural products uncommon to land plants, but highly frequent in *Piper* species (Lago et al., 2004). Reported biological activities for such compounds include antitumoral (Danelutte et al., 2003), analgesic (De Pasquale, Circosta, Occhiuto, De Rosa, & De Stefano, 1991), antioxidant (Yamaguchi, Lago, Tanizaki, Di Mascio, & Kato, 2006) and antimicrobial (Lago et al., 2004). Prenylated benzoic acid derivatives were also isolated from *Piper* species, including *P. crassinervium*, and their bioactivity spectra are similar to prenylated hydroquinones. Here we report the evaluation of trypanocidal activity for five known compounds, **1** [4-hydroxy-(3′,7′-dimethyl-1′-*oxo*-octa-2′-*E*-6′-dienyl)benzoic acid] (Lago et al., 2004), **2** [4-hydroxy-3-(3′,7′-dimethyl-3′-hydroxy-1′-*oxo*-6′-octenyl)benzoic acid] (Lago et al., 2004), **3** [1,4-dihydroxy-2-(3′,7′-dimethyl-1′-*oxo*-2′-*E*-6′-octadienyl)benzene] (Danelutte et al., 2003), **4** isosakuranetin (5,7-dihydroxy-4′-methoxyflavanone) (Agrawal & Bansal, 1989) and **5** sakuranetin (5,4′-dihydroxy-7-methoxyflavanone) (Agrawal & Bansal, 1989; Danelutte et al., 2003), (Table 1) isolated from leaves of *P. crassinervium*, as a part of our program devoted to discover new hits in the development of drugs for treatment of Chagas disease. This constitutes the first report of trypanocidal activity for prenylated hydroquinone and benzoic acid derivatives.

2. Materials and methods

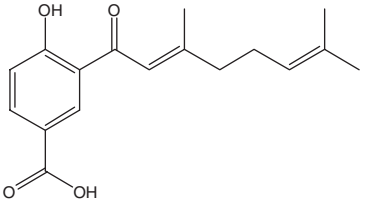
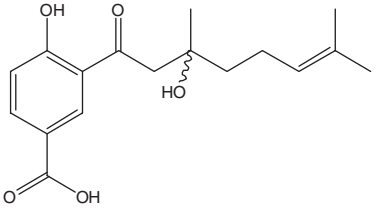
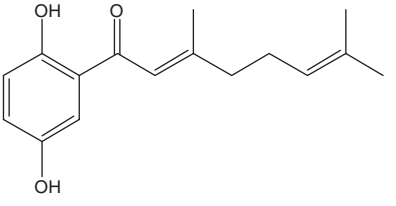
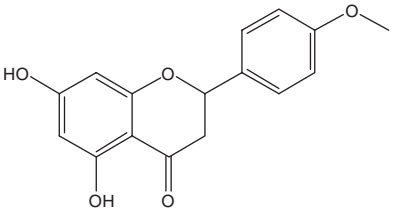
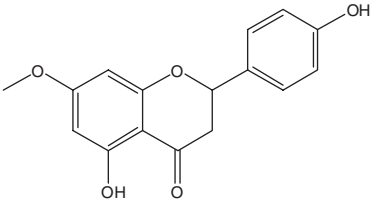
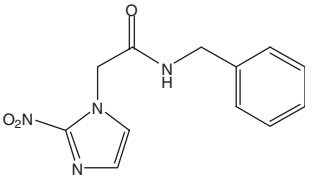
2.1. *Plant material*

Leaves of *P. crassinervium* Kunth were collected in the region of Vale do Ribeira, Atlantic Forest (São Paulo State, Brazil) and identified by Dr. Guillermo E.D. Paredes (Universidad Nacional Pedro Ruiz Gallo, Peru). A voucher specimen (KATO-0084) was deposited at the Herbarium of Instituto de Botânica, São Paulo, SP, Brazil.

2.2. *Extraction and isolation of the constituents*

Dried leaves (350 g) were extracted with EtOAc and concentrated to dryness under vacuum. The crude extract (20 g) was resuspended in MeOH : H_2O (8 : 2) and partitioned

Table 1. Inhibition of epimastigote forms of *T. cruzi* by compounds **1**–**5** isolated from *P. crassinervium*. IC_{50} values are given in $\mu g\,mL^{-1}$.

Compounds	Chemical structure	IC_{50} ($\mu g\,mL^{-1}$)
1		16.3
2		15.8
3		6.1
4		119.6
5		111.7
Benznidazole[a]		1.6

Note: [a]Positive control.

with hexane and EtOAc. The hexane soluble portion (6.0 g) was fractioned using column chromatography over silica gel (70–230 mesh; Merck, column size 3.0 × 25 cm), which was eluted with hexane containing increasing amounts of EtOAc (up to 30%) to yield 11 fractions. Fraction 4 (0.284 g) was further separated by column chromatography over reversed phase ODS (Merck; C-18; 1.0 × 4.5 cm column) with MeOH–H_2O (4 : 1) to yield eight fractions; the first fraction (0.221 g) was submitted to column chromatography over silica gel (Merck; 230–400 mesh; 1.0 × 3.0 cm column) eluted with hexane containing increasing amounts of EtOAc (up to 30%) to yield six fractions, the third fraction yielded compound **3** (0.023 g). Fraction 6 (0.015 g) was applied to a column chromatography over reversed phase ODS (Merck; C-18; 1.0 × 4.5 cm column) with MeOH–H_2O (4 : 1) to yield compound **1** (0.011 g).

The portion soluble in EtOAc (3.0 g) was fractioned by column chromatography over LH-20 (Merck, column size 3.0 × 100 cm) and eluted with MeOH to yield 16 fractions. Fraction 3 (0.050 g) was submitted to preparative TLC over silica gel (230–400 mesh; Merck), which was developed with hexane–acetone (6 : 4) to yield compound **2** (0.009 g). Fraction 11 (0.100 g) was chromatographed on preparative HPLC using hexyl-phenyl phase, which was eluted with MeOH–H_2O (7 : 2), to yield **4** (0.030 g) and **5** (0.010 g). Compounds **1–5** were identified by comparison of their physical properties with literature values.

2.3. *Trypanocidal assay*

The assays of the anti-parasitic activities of the isolates were conducted by screening their effects on the proliferation of epimastigote forms of *T. cruzi* Y-strain, grown in axenic culture in LIT medium. Stock solutions (containing 1.0 mg mL^{-1}) of all tested compounds were prepared in dimethylsulphoxide and serially diluted (1 : 3) in liver infusion tryptose medium to yield sample solutions with concentrations of 0.41, 1.23, 3.70, 11.1, 33.3, 100.0 and 300.0 μg mL^{-1}. All assays were conducted in triplicate. The number of remaining viable protozoa was established by counting in a Neubauer chamber following incubation at 28°C for 72 h with the test sample. The 50% inhibitory concentration (IC_{50}) values and statistical data were determined by using Probit analysis statistical method with GW-BASIC 2.33 (Microsoft Software, 1987). The IC_{50} values for compounds **1–5** and for benznidazole (employed as positive control) are shown in Table 1.

3. Results and discussion

In this work, we have tested the inhibitory effects of five known compounds isolated from *P. crassinervium* against epimastigote forms of *T. cruzi*. Results are presented in Table 1. Three groups of molecules were tested here, two benzoic acid derivatives (**1** and **2**), one hydroquinone derivative (**3**), and two flavanones (**4** and **5**). Moreover, compounds **1–3** are *C*-geranylated molecules.

The results showed that geranylated compounds were more active than the flavanones. Within the active compounds, the best activity was observed for **3** (IC_{50} value of 6.10 μg mL^{-1}) followed by geranylated *p*-hydroxybenzoic acid derivatives **1** and **2**. The IC_{50} value observed for compound **3** [1,4-dihydroxy-2-(3′,7′-dimethyl-1′-*oxo*-2′-*E*-6′-octadienyl)benzene], a *p*-hydroquinone derivative, represents an interesting hit

if compared with positive control benznidazole (IC_{50} value 1.6 μg mL^{-1}, Table 1). This molecule has two phenolic hydroxyl groups, one of them with a chelated function, which is also present at compounds **2** and **3**. The change of one phenolic hydroxyl group (compound **3**) by a carboxylic acid one (compound **1**) produced a loss of activity greater than 50% (IC_{50} value from 6.1 to 16.3 μg mL^{-1}, Table 1), suggesting its crucial role in trypanocidal activity. This observation is in agreement with Luize, P.S. et al., who proposed that the loss of the hydroxyl group in neolignans isolated from *P. regnellii* resulted in the complete loss of activity against epimastigote forms of *T. cruzi* (Luize et al., 2006). In addition, the phenolic hydroxyl group present in the active molecule is apparently able, through hydrogen-bond links with the specific molecules on the cell surface, to inactivate essential metabolic pathways within the parasite (Patrick, 1995). The decrease in the activity observed for compound **2** (IC_{50} value 15.8 μg mL^{-1}, Table 1), is also in agreement.

Isosakuranetin (**4**) and sakuranetin (**5**) (Agrawal & Bansal, 1989; Danelutte et al., 2003), showed weak antichagasic activity with IC_{50} values of 119.6 and 111.7 μg mL^{-1}, respectively (Table 1). Several types of flavonoids have been identified as antiprotozoal principles of plant extracts (Camacho et al., 2002; Grael et al., 2005; Jordão, Vichnewski, Souza, Albuquerque, & Lopes, 2004). However, there are few reports about the activity of flavonoids against epimastigotes of *T. cruzi*. Our results are in agreement with such observations, suggesting that the molecular topology is very important for adequate interactions at receptor level to exert trypanocidal effect. Flavanone skeleton was less active than geranylated hydroquinone derivatives, even with phenolic hydroxyl groups in the molecule.

The presence of lipophilic geranyl moiety oxygenated in benzylic position, in compounds **1**–**3** could be important to trypanocidal activity; this fact was also considered crucial to exert antioxidant activity, because of their better insertion into lipophilic surfaces (Kato & Furlan, 2007). Several studies were reported about the antileishmaniasic activity of natural hydroquinone and related structures, however there are few results concerning their antichagasic potential. In this context, a prenylated dihydroquinone isolated from *Peperomia galioides* (Piperaceae) displayed a promising toxicity against *Leishmania brasiliensis*, *L. donovani* and *L. amazonensis*, but it was inactive on trypomastigote forms of *T. cruzi* (Mahiou et al., 1995; Mahiou et al., 1996).

The present investigation revealed that 3-geranylated hydroquinone and benzoic acid derivatives, isolated from leaves of *P. crassinervium*, showed potent trypanocidal activity. More extensive and detailed studies on the trypanocidal activity of natural and synthetic geranylated derivatives from Piperaceae species will be the target of future studies focusing on the understanding of the structural requirements of this group of natural products. Also, *in vitro* and *in vivo* unspecific toxicity should be investigated in order to evaluate their potential as new leader structures for the development of antichagasic drugs.

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