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Artigo

# Trypanocidal activity of *Piper arboreum* and *Piper tuberculatum* (Piperaceae)

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**RESUMO: "Atividade tripanocida de *Piper arboreum* e *Piper tuberculatum* (Piperaceae)".** No escopo de nossas pesquisas sobre agentes bioativos da flora brasileira, vinte e quatro extratos e frações de *Piper arboreum* Aub. e *Piper tuberculatum* Jacq. (Piperaceae) tiveram sua atividade tripanocida avaliada através do ensaio colorimétrico com MTT. As atividades mais potentes foram manifestadas pelas frações hexânicas das folhas de *P. arboreum* ($CI_{50}$ = 13,3 µg/mL) e *P. tuberculatum* ($CI_{50}$ = 17,2 µg/mL). As frações hexânicas dos frutos de *P. tuberculatum* e *P. arboreum* também apresentaram efeito tóxico potente contra as formas epimastigotas de *Trypanosoma cruzi*, com valores de $CI_{50}$ (µg/mL) de 32,2 e 31,3, respectivamente. Adicionalmente, o estudo fitoquímico da fração hexânica das folhas de *P. arboreum* forneceu duas amidas pirrolidínicas, piperilina (**1**) e 4,5-diidropiperilina (**2**), que podem ser responsáveis pela atividade antiprotozoária desta fração.

**Unitermos:** Antiprotozoário, tripanocida, *Piper arboreum*, *Piper tuberculatum*, Piperaceae, *Trypanosoma cruzi.*

**ABSTRACT:** In the scope of our ongoing research on bioactive agents from Brazilian flora, twenty-four extracts and fractions obtained from *Piper arboreum* Aub. and *Piper tuberculatum* Jacq. (Piperaceae) were screened for trypanocidal activity by using MTT colorimetric assay. The strongest activity was found in hexane fractions from the leaves of *P. arboreum* ($IC_{50}$ = 13.3 µg/mL) and *P. tuberculatum* ($IC_{50}$ = 17.2 µg/mL). Hexane fractions of the fruits of *P. tuberculatum* and *P. arboreum* showed potent toxic effects on epimastigote forms of *Trypanosoma cruzi*, with values of $IC_{50}$ (µg/mL) of 32.2 and 31.3, respectively. Additionally, the phytochemical study of the hexane fraction of *P. arboreum* leaves furnished two pyrrolidine amides, piperyline (**1**) and 4,5-dihydropiperyline (**2**), which could be responsible, at least in part for the observed antiprotozoal activity.

**Keywords:** Antiprotozoal**,** trypanocidal, *Piper arboreum*, *Piper tuberculatum*, Piperaceae, *Trypanosoma cruzi.*

## INTRODUCTION

Piperaceae species have been extensively investigated as a source of secondary metabolites with anti-*Trypanosoma cruzi*, antileishmanial, anxiolytic, anticonvulsant and anti-inflammatory activities (Barbosa-Filho et al., 2006; Nakamura et al., 2006; Amorim et al., 2007; Saúde-Guimarães & Faria, 2007; Quintans-Júnior et al., 2008). Phytochemical investigations of *Piper* species have led to the identification of typical classes of compounds such as amides, terpenes, benzoic acid derivatives, carotenes, and hydroquinones in addition to lignans, neolignans and a few alkaloids (Navickiene et al., 2000; Silva et al., 2002; Lago et al., 2004; Navickiene et al., 2006; Potzernheim et al., 2006; Barbosa-Filho et al., 2008; Regasini et al., 2008; Continguiba et al., 2009).

As part of our research aiming to uncover new trypanocidal compounds in Brazilian Piperaceae species, we have previously reported the occurrence of chromenes in *P. aduncum* and *P. gaudichaudianum* (Batista-Júnior et al., 2008). Additionally, hydroquinones and flavanones from leaves of *P. crassinervium* have been described as well (Lopes et al., 2008). In this context, we have screened various plants of *Piper* genus collected in the State of São Paulo (Brazil), *P. arboreum* Aub. and *P. tuberculatum* Jacq. (Piperaceae) were chosen

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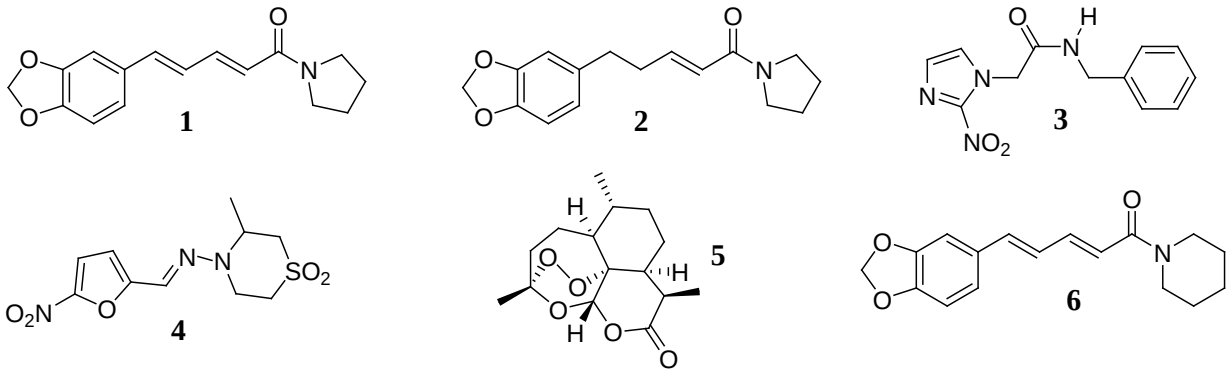


**Figure 1.** Molecular structures of piperyline (**1**), 4,5-dihydropiperyline (**2**), benznidazole (**3**), nifurtimox (**4**), artemisinin (**5**), piperine (**6**). Pyrrolidine alkylamides **1** and **2** were isolated from *Piper arboreum* leaves.

for biological and chemical investigation, and to our knowledge there are no previous reports on antiprotozoal activity of these species.

In some Northeastern Brazil communities, *P. tuberculatum*, popularly known as "pimenta-d'arta" and "pimenta-longa" has been used as antidote for snake bite and sedative (Felipe et al., 2007). On the other hand, a decoction of *P. arboreum* (vernacular names: "fruto-de-morcego", "alecrim-de-angola", "pau-de-angola", and "beto-preto") has been used against venereal diseases and infections of the urinary throat (Agra et al., 2007, 2008).

Thus, the major aim of the current study was to evaluate the extracts and fractions of leaves, stems, fruits and compounds from *P. arboreum* and *P. tuberculatum* for an anti-trypanosomal effect against epimastigote forms of *Trypanosoma cruzi*.

## MATERIAL AND METHODS

### Plant material

Specimens of *P. arboreum* and *P. tuberculatum* were cultivated from seeds under greenhouse conditions at the Institute of Chemistry, Universidade Estadual Paulista, Araraquara-SP, Brazil. Plant material was collected in May, 2006, and identified by Dr. Guillermo E. D. Paredes (Universidad Pedro Ruiz Gallo, Lambayeque, Peru). The vouchers specimens Kato-163 and Cordeiro-1936 were deposited at the herbarium of the Institute of Biosciences, Universidade de São Paulo, São Paulo-SP, Brazil.

### Extraction

Shade-dried and powdered plant material (leaves, fruits or stems) of *P. arboreum* and *P. tuberculatum* (30.0 g) were extracted with ethanol (5 x 350 mL), for three weeks at room temperature. After filtering, the solvent was evaporated under reduced pressure to yield a thick syrup, which was dispersed in methanol:water (4:1) and then successively partitioned with hexane and ethyl acetate. Samples of the ethanol extracts and the hexane, ethyl acetate, and lyophilized hydromethanol fractions were tested for potential trypanocidal activity.

### Isolation and identification of amides 1 and 2

The hexane fraction of the leaves of *P. arboreum* (880 mg) was subjected to column chromatography with silica gel (18 x 3.3 cm i.d.) and eluted with hexane:ethyl acetate (4:1). Twenty-five fractions (10 mL) were collected and checked by TLC on silica gel $F_{254}$ plates developed with hexane:ethyl acetate (6:4), and revealed with Dragendorff reagent. Fractions 10-13 (520 mg) were purified by preparative TLC [hexane:dichlorometh ane:acetone:acetic acid (6:3:1:0.1), four elution] to yield piperyline (**1**; 310 mg) and 4,5-dihydropiperyline (**2**; 135 mg). The molecular structures of these compounds were identified by comparison with literature data, mainly $^{1}H$ and $^{13}C$ NMR δ values (Alecio et al., 1998; Navickiene et al., 2000).

### Trypanocidal activity *in vitro*

All experiments were performed with the Y-strain epimastigote forms of *T. cruzi*. They were grown axenically at 28 °C in liver-infusion Tryptose (LIT) medium supplemented with 10% fetal calf serum and harvested during the exponential phase of growth (7 day-old culture forms). Extracts, fractions and compounds were dissolved in DMSO and further added to a 96-well tissue culture plate (TPP) in different final concentrations. *T. cruzi* (1 x $10^7$ parasites/mL) were added into each well and the same quantity of LIT medium was added into the controls wells. These plates were maintained at 28 °C for 72 h 10 µL of a 2.5 mg/mL MTT-PMS solution was added to each well and the plates were incubated for 75 min in the dark at 28 °C. A solution of 10% (100 µL) of sodium dodecyl sulfate (SDS) was added to the anterior solution and maintained at room temperature and in the

dark at 30 min. The absorbance of the samples had read at 595 nm. The 50 % inhibitory concentration ($IC_{50}$) values were determined by linear regression analysis after a 72 h incubation period. The $IC_{50}$ values of samples and benznidazole (positive control) were determined. For the statistical analysis, probit's method was employed (Muelas-Serrano et al., 2000).

## RESULTS AND DISCUSSION

Chagas' disease or American trypanosomiasis, caused by the protozoan flagellate *T. cruzi,* is an important public health problem in Latin America, affecting millions peoples annually (Sanchez-Burgos et al., 2003). The most common treatment for this disease involves two drugs, benznidazole (**3**) and nifurtimox (**4**) (Figure 1), which are active only during the acute and short-term chronic phases. Beznidazole (Rochagan®/ Brazil and Radanil®/Argentina) is now the only drug still available since the production of nifurtimox was stopped. Besides presenting severe side effects, narrow therapeutic windows, and variable drug susceptibilities among *T. cruzi* strains result in low clinical efficacies for these 2-nitroimidazole (Coura & Castro, 2002). For this reason, there is thus a distinct need for new, safer and more effective trypanocidal drugs.

Natural products have long been used as templates for the development of new lead compounds, which may be useful against parasitic diseases, such as artemisinin (**5**) (Figure 1), an antimalarial sesquiterpene isolated from *Artemisia annua*, popularly known as "quinhaosu" (Renslo & McKerrow, 2006). In this context, several secondary metabolites of different structural patterns have proven active against *T. cruzi* (Saúde-Guimarães & Faria, 2007), and screening of plant extracts is a valid strategy being exploited to discover trypanocidal agents (Leite et al., 2001; Takahashi et al., 2002; Luize et al., 2006; Pizzolatti et al., 2008).

In the present work, the ethanol extract and three fractions (hexane, ethyl acetate and hydromethanol) of the fruits, leaves and stems of *P. arboreum* and *P. tuberculatum* were evaluated for trypanocidal activity and the results are shown in Table 1.

**Table 1.** Toxic effects of *Piper arboreum* and *Piper tuberculatum* on epimastigote forms of *Trypanosoma cruzi* expressed by $IC_{50}$ ($\mu g.mL^{-1}$).

| Plant part or compounds | Extract or fractions tested | *Piper arboreum* | *Piper tuberculatum* |
|---|---|---|---|
| Fruits | | | |
| | ethanol | 79.3 | 82.0 |
| | hexane | 31.3 | 32.2 |
| | ethyl acetate | 94.7 | 65.5 |
| | hydromethanol | >100 | >100 |
| Leaves | | | |
| | ethanol | 66.1 | 44.6 |
| | hexane | 13.3 | 17.2 |
| | ethyl acetate | 47.6 | 49.0 |
| | hydromethanol | >100 | >100 |
| Stems | | | |
| | ethanol | >100 | >100 |
| | hexane | >100 | 79.3 |
| | ethyl acetate | >100 | >100 |
| | hydromethanol | >100 | >100 |
| Piperyline (**1**) | — | 3.57 (6.57 μM) | — |
| 4,5-Dihydropiperyline (**2**) | — | 57.3 (210 μM) | — |
| Benznidazole (**3**) | — | 8.74 (33.6 μM) | — |

In general, ethanol extracts (crude extracts) obtained from leaves and fruits exhibited stronger anti-trypanosomal activity than did those from stems. Hexane fractions were more effective than ethyl acetate and hydromethanol fractions, suggesting that the potential antichagasic compounds were in the low-polarity fractions. The hexane fractions of the leaves of *P. arboreum* and *P. tuberculatum* exhibited the best activities against epimastigote form of *T. cruzi*, with values of $IC_{50}$ (μg/mL) of 13.3 and 17.2 μg/mL, respectively, and most hexane fractions of the fruits showed an activity considered potent ($IC_{50}$ < 33 μg/mL). Ethyl acetate fractions of the leaves of *P. tuberculatum* and *P. arboreum* showed moderate trypanocidal activity, with values of $IC_{50}$ (μg/mL) of 49.0 and 47.6, respectively. Hydromethanol fractions proved to be inactive, since the concentration at which these samples showed activity was over 100 μg/mL.

Additionally, phytochemical fractionation of *P. arboreum* leaves furnished two amides responsible for the observed anti-trypanosomal effect of hexane fraction. Piperyline (amide **1**) showed higher activity which an $IC_{50}$ of 6.57 μM, almost five times more potent than positive control, benznidazole ($IC_{50}$ = 33.6 μM). The hydrogenated analogue of piperyline (amide **2**) was also assayed, exhibiting an $IC_{50}$ of 210 μM. These data suggest that reduction of the double bond at positions 4 and 5 interferes with the anti-*Trypanosoma cruzi* activity.

Compounds **1** and **2** are pyrrolidine alkylamides of remarkable occurrence in *Piper* species whose biological properties have been extensively demonstrated in the literature (Alecio et al., 1998; Koul et al., 2000; Wei et al., 2004; Venkatasami et al., 2004). Other amides from *Piper* species are among the natural products of interest since many of it compounds display a broad range of antiprotozoal activities (Raay et al., 1999; Bodiwala et al., 2007; Freire-de-Lima et al., 2008). Kapil (1993) reported an investigation of piperine (amide **6**) activity against promastigote forms of *Leishmania donovani*. More recently, Ribeiro et al. (2004) reported the highly significant trypanocidal activity of piperine and its synthetic derivatives on epimastigotes and amastigotes of *T. cruzi*.

## CONCLUSION

It may be concluded from the study that *P. arboreum* and *P. tuberculatum* have potential anti-trypanosomal activity based on toxic effect against epimastigote form of *T. cruzi*. Furthermore, hexane fractions of the leaves of *P. arboreum* and *P. tuberculatum* could be an important source of promising antiprotozoal compounds, useful for developing of novel anti-*Trypanosoma cruzi* agents. Two pyrrolidine alkylamides, piperyline (**1**) and 4,5-dihydropiperyline (**2**), have been isolated from *P. arboreum* leaves, which could be responsible, at least in part for the observed trypanocidal effect. In view of these findings, further chemical and pharmacological investigations to identify others secondary metabolites and to evaluate the potential of these *Piper* species as antichagasic agents *in vivo* are recommended.

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