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Artigo

# ANTI-TRYPANOSOMAL ACTIVITY OF 1,2,3,4,6-PENTA-O-GALLOYL-β-D-GLUCOSE ISOLATED FROM *Plectranthus barbatus* Andrews (Lamiaceae)#

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MeOH extract from the leaves of *Plectranthus barbatus* Andrews (Lamiaceae), showed *in vitro* anti-trypanosomal activity. The bioassay-guided fractionation resulted in the isolation of a gallic acid derivative, identified as 1,2,3,4,6-penta-O-galloyl-β-D-glucose (PGG), after thorough NMR and MS spectral analysis. Finally, this compound was tested against trypomastigote forms of *T. cruzi* and displayed an $EC_{50}$ value of 67 μM, at least 6.6-fold more effective than the standard drug benznidazole. This is the first occurrence of PGG in the *Plectranthus* genus and the first anti-parasitic activity described for PGG in the literature.

Keywords: *Plectranthus barbatus*; 1,2,3,4,6-penta-O-galloyl-β-D-glucose; anti-trypanosomal activity.

## INTRODUCTION

The genus *Plectranthus*, synonym *Coleus* (Lamiaceae), contains about 350 species with occurrence in Africa, Asia and Australia.[1] In Brazil, it is an introduced genus, whose occurrence has been described in Northeast and Southeast regions.[2] Due to its ethnobotanical uses, *Plectranthus* can be classified as an important genus from Lamiaceae,[1] affording wide biologically active substances.[3-6]

*P. barbatus*, popularly known as "falso boldo", is one of the most relevant specie of the *Plectranthus* genus and has been used in traditional medicine for the treatment of digestive and respiratory disorders as well as for heart and central nervous diseases.[7-9] Pharmacological studies have been conducted with extracts of *P. barbatus*, in which antioxidant,[10] antimicrobial,[11] antifungal[12] and antiacetylcholinesterase potentials were detected.[13]

Chemically, this specie has been characterized by the presence of diterpenoids[14], especially modified abietanoids.[8,9,15] Some typical abietane derivatives isolated from leaves of *P. barbatus*, classified according to their structural characteristics, are barbatusin, ciclobutatusin, plectrinone A, and coleun U.[5,16,17] Other described constituents include the phenolic derivatives rosmarinic acid and 7-O-glucoronide 4'-methyl ether.[6]

Chagas disease is one of most neglected diseases in several underdeveloped and developing countries and affects approximately ten million people worldwide, mostly in Latin America.[18] It is caused by the protozoan parasite *Trypanosoma cruzi* (*T. cruzi*), which is transmitted to humans by triatomine insects primarily through posterior transmission from fecal matter during feeding, by blood transfusion, and even via congenital transmission.[19] The therapeutic arsenal is very limited. At present, there are only two effective drugs for the treatment of Chagas disease: nifurtimox and benznidazol (nitroheterocycle derivatives).[20] These drugs show high cytotoxicity and the main side effects for nifurtimox are anorexia, loss of weight, psychological changes, besides digestive manifestations such as nausea, vomiting and diarrhoea. Benznidazol is also highly toxic and the symptoms of adverse reactions are hypersensitivity, dermatitis with cutaneous eruptions, besides articular and muscular pain.[21] Also, these drugs are ineffective against the chronic phase of the disease demonstrating the urgent need for new drugs.[20] In our continuing search for anti-parasitic natural products from Brazilian plants,[22-24] the crude MeOH extract from leaves of *P. barbatus* displayed *in vitro* anti-trypanosomal activity and was subjected to bioactivity-guided fractionation. Using several chromatographic procedures, the compound 1,2,3,4,6-penta-O-galloyl-β-D-glucose (PGG) was isolated for the first time in the *Plectranthus* genus, identified after NMR and LRESIMS analysis.

## RESULTS AND DISCUSSION

Preliminary testing of crude MeOH extract obtained from leaves of *P. barbatus* revealed considerable anti-trypanosomal activity. With the aim of isolating active compounds, this extract was partitioned using EtOAc and organic active phase was subjected to several steps of bioactivity-guided fractionation using $SiO_2$ and Sephadex LH-20. An amorphous white solid was thus isolated, found to be composed of a pure substance after TLC and HPLC analysis.

This compound showed a molecular formula of $C_{41}H_{32}O_{26}$ evidenced by a *pseudo*-molecular ion peak at *m/z* 938.6 $[M-H]^-$ observed in the LRESIMS (negative mode) spectrum. The $^1H$ NMR spectrum showed signals attributed to seven protons of the glucopyranosyl unit at δ 6.14 (*d*, 1H, *J* = 8.4 Hz, H-1), δ 5.52 (*dd*, 1H, *J* = 8.4; 9.9 Hz, H-2), δ 5.81 (*t*, 1H, *J* = 9.9, H-3), δ 5.49 (*t*, 1H, *J* = 9.6 Hz, H-4), δ 4.42 (*d*, 1H, *J* = 10.5 Hz, H-5), δ 4.32 (*m*, 2H, H-6). This spectrum also showed singlets at δ 7.01, 6.95, 6.88, 6.85, 6.80 which could be assigned to H-2'/H-6' of five tetrasubstituted aromatic rings (Gal 1-Gal 5), suggesting that the glucopyranosyl unit could be esterified with five galloyl units. The

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$^{13}$C NMR and DEPT 135° spectra showed signals of methynic carbons at δ 93.9 (C-1), δ 72.4 (C-2), δ 74.2 (C-3), 69.9 (C-4), 74.5 (C-5), and methylenic carbon at δ 63.3 (C-6), characteristic of a glucopyranosyl unit.[25] Additionally, were observed peaks were observed at the δ 110-168 range, assigned to galloyl units.[26] The complete structure of the bioactive compound was determined by analysis of the HMBC spectrum, which showed cross peaks between the signals at δ 7.01 (H-2´ and H-6´) and δ 166.4 (C-7') as well as between the peaks at δ 6.14 (H-1) and δ 166.4 (C-7'). Finally, the comparison of NMR data with those described in the literature[25-27] (Table 1) allowed the identification of 1,2,3,4,6-penta-O-galloyl-β-D-glucose (PGG) Figure 1, whose occurrence has been described for the first time in the *Plectranthus* genus. This compound was previously identified in various medicinal plants, including species of *Rhus*, *Schinus*, *Galla* and others.[27,28] Pharmacologically, this substance showed diverse biological activities, such as antioxidant,[25] anticancer,[29] and inhibitory action against the invasion of mouse melanoma.[30] Additionally, an inhibitory effect of the α-glycosidase of PGG was described, suggested by the presence of galloyl substituents at the glucose unit[26].

Ethnopharmacological studies have revealed anti-parasitic activity of the *Plectranthus* species, especially an anti-leishmanial effect. *P. amboinicus* has been used in popular medicine to treat cutaneous leishmaniasis in a rural endemic area of Bahia state, Brazil.[31] Its activity against *L. chagasi* promastigotes has also been described from MeOH extract of *P. barbatus*.[32]

In our assays, trypomastigotes of *T. cruzi* showed susceptibility to the isolated PGG, with an $EC_{50}$ value of 66.66 μM (Table 2). Considering the activity against the parasite, it could also be shown that PGG was 6.6-fold more effective than the standard drug benznidazole, although the cytotoxicity against mammalian cells, resulted in a $CC_{50}$ of 39.52 μM (Table 2). In summary, our results demonstrated that PGG displayed activity against *T. cruzi* parasites and suggested the performance of additional studies on reducing its cytotoxicity with the aim of using this compound as a prototype for the development of new drugs to treat Chagas disease.

## EXPERIMENTAL

### General procedures

$^{1}$H and $^{13}$C NMR spectra were recorded on a Bruker DPX-300 spectrometer operating at 300 and 75 MHz, respectively, using $CD_3OD$ (Tedia Brazil) as the solvent and internal standard ($\delta_H$ 3.21 and $\delta_C$ 49.2). LRESIMS (negative mode) was performed on an Esquire 3000 Plus spectrometer. Analytical HPLC analysis was performed on a Dionex Ultimate 3000 chromatograph, using a Luna Phenomenex $C_{18}$ column (3 μm, 150 × 5 mm) and an UVD-DAD detector. Silica gel (Merck, 230-400 mesh) and Sephadex LH-20 (Amersham Biosciences) were used for column chromatographic separation, while silica gel 60 $PF_{254}$ (Merck) was used for analytical (0.25 mm) TLC.

### Plant material

Leaves of *P. barbatus* were collected at the Instituto Plantarum de Estudos da Flora, Brazil, in Nova Odessa/São Paulo state, in October 2010. The studied specie was identified by MSc. H. Lorenzi and a voucher specimen has been deposited in the Herbarium Plantarum under number H. Lorenzi 771.

### Extraction and isolation

Leaves of *P. barbatus* (227 g) were dried, milled and extracted at room temperature with 3 X 1 L of MeOH. Concentration *in vacuo*

**Table 1**. $^{1}$H, $^{13}$C and HMBC data of 1,2,3,4,6,-penta-O-galloyl-β-D-glucose ($CD_3OD$, 300 and 75 MHz)

| Position | $\delta_H$ | $\delta_C$ | HMBC (H → C) |
|---|---|---|---|
| 1 | 6.14 (1H, *d*, 8.4 Hz) | 93.4 | $C_2$, $C_3$, $C_{7'}$ (Gal 1), |
| 2 | 5.52 (1H, *dd J* = 8.4, 9.9 Hz) | 72.4 | $C_1$, $C_3$, $C_4$, $C_{7'}$ (Gal 2) |
| 3 | 5.81 (1H, *t,* 9.9 Hz) | 74.2 | $C_1$, $C_2$, $C_4$, $C_5$, $C_{7'}$ (Gal 3) |
| 4 | 5.49 (1H, *t,* 9.6 Hz) | 69.9 | $C_2$, $C_3$, $C_5$, $C_6$, $C_{7'}$ (Gal 4) |
| 5 | 4.42, (1H, *d*, 10.5 Hz) | 74.5 | $C_3$, $C_4$, $C_6$ |
| 6a/6b | 4.32 (2H, *m*) | 63.3 | $C_4$, $C_5$, $C_{7'}$ (Gal 5) |
| Gal 1 | | | |
| 1’ | - | 119.8 | $EC_{50}$ |
| 2’/6’ | 6.95 (2H, *s*) | 110.7 | $C_{1'}$,$C_{3'}$, $C_{4'}$, $C_{5'}$, $C_{6'}$, $C_{7'}$ |
| 3’/5’ | - | 141.0 | - |
| 4’ | - | 146.7 | - |
| 7’ | - | 166.4 | - |
| Gal 2 | | | |
| 1’ | - | 120.3 | - |
| 2’/6’ | 6.85 (2H, *s*) | 110.5 | $C_{1'}$, $C_{3'}$, $C_{4'}$, $C_{5'}$, $C_{6'}$, $C_{7'}$ |
| 3’/5’ | - | 140.5 | - |
| 4’ | - | 146.5 | - |
| 7’ | - | 167.2 | - |
| Gal 3 | | | |
| 1’ | - | 120.5 | - |
| 2’/6’ | 6.80 (2H, *s*) | 110.5 | $C_{1'}$, $C_{3'}$, $C_{4'}$, $C_{5'}$, $C_{6'}$, $C_{7'}$ |
| 3’/5’ | - | 140.3 | - |
| 4’ | - | 146.4 | - |
| 7’ | - | 167.4 | - |
| Gal 4 | | | |
| 1’ | - | 120.3 | - |
| 2’/6’ | 6.88 (2H, *s*) | 110.6 | $C_{1'}$, $C_{3'}$, $C_{4'}$, $C_{5'}$, $C_{6'}$, $C_{7'}$ |
| 3’/5’ | - | 140.6 | - |
| 4’ | - | 146.6 | - |
| 7’ | - | 167.1 | - |
| Gal 5 | | | |
| 1’ | - | 121.1 | - |
| 2’/6’ | 7.01 (2H, *s*) | 110.4 | $C_{1'}$, $C_{3'}$, $C_{4'}$, $C_{5'}$, $C_{6'}$, $C_7$ |
| 3’/5’ | - | 140.2 | - |
| 4’ | - | 146.6 | - |
| 7’ | - | 168.1 | - |

**Table 2.** Anti-trypanosomal activity and cytotoxicity of PGG and benznidazole

| Compound | $EC_{50}$ μM *T. cruzi* tripomastigotes (95% C.I.) | $CC_{50}$ μM NCTC (95% C.I.) |
|---|---|---|
| PGG | 66.66 (57.00-77.96) | 39.52 (33.10-47.17) |
| Benznidazole | 441.15 (406.54-478.85) | 470.38 (415.38-532.69) |

$EC_{50}$: 50% Effective Concentration; $CC_{50}$: 50% Cytotoxic Concentration; 95% C.I. : 95% confidence interval. For $EC_{50}$ values was determined % *T. cruzi* survival: 159.57 μM: 15.5 ± 1.92; 79.79 μM: 27.53 ± 0; 39.89 μM: 95.14 ± 2.85; 19.95 μM: 100 ± 0.42. (Values expressed as mean ± SEM of two determinations)

yielded 9.1 g of crude extract. Part of this material (9 g) was dissolved in MeOH:$H_2O$ 8:2 and subsequently extracted using hexane, EtOAc and $CHCl_3$. After evaluation of trypanocidal potential, the active EtOAc phase (4.0 g) was subjected to silica gel column chromatography eluted with increasing amounts of EtOAc in hexane to give 77 fractions (10 mL). After TLC analysis, these fractions were combined into 23 groups (A1-A23), which displayed activity against *T. cruzi* trypomastigote forms. As bioactivity was detected at group A18, part of this material (700 mg) was introduced to a Sephadex LH-20 column which was eluted with MeOH. This procedure afforded 40 fractions (5 mL each) which were pooled into 11 groups (A18/1-A18/11) after TLC analysis. As the activity was detected at the A18/1 group, this was analyzed by HPLC (MeOH:$H_2O$ 7:3) to afford 48 mg of pure 1,2,3,4,6-penta-O-galloyl-β-D-glucose.

*1,2,3,4,6-penta-O-galloyl-β-D-glucose*

White amorphous solid. NMR data: see Table 1. LRESIMS *m/z* 938.6 $[M - H]^-$.

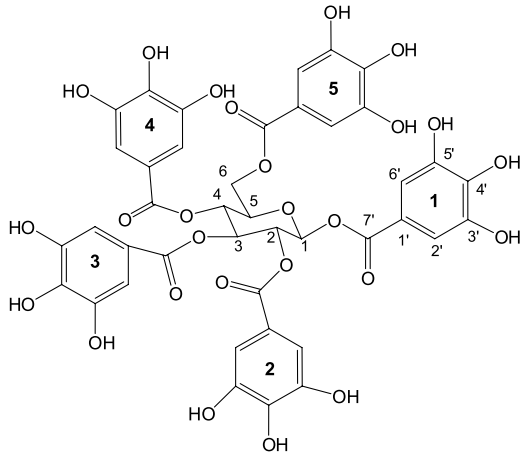


***Figure 1.*** *Structure of 1,2,3,4,6- penta-O-galloyl-β-D-glucose (PGG)*

**Animals**

BALB/c mice and Golden hamsters (*Mesocricetus auratus*) were supplied by the animal breeding facility at the Adolfo Lutz Institute of São Paulo and maintained in sterilized cages under a controlled environment, receiving water and food *ad libitum*. Animal procedures were performed with the approval of the Research Ethics Commission.

**Anti-trypanosomal activity**

Trypomastigotes of *T. cruzi* (Y strain) were obtained from LLC-MK2 culture (ATCC CCL 7), counted on a Neubauer hemocytometer and applied to 1 x $10^6$/wells in 96-well plates. The substance was dissolved in DMSO and further incubated in various concentrations ranging from 150 to 0.073 µg/mL for 24 h at 37 °C with 5% $CO_2$ in a humidified incubator, using benznidazole as the standard drug. The DMSO concentration did not exceed 0.5% (v/v) of the final volume of wells to avoid damage to parasites. The viability of parasites was measured by the trypomastigote cellular conversion of MTT solution - bromide 3-(4,5-dimethylthiazol-2-yl-2,5-diphenyltetrazolium bromide) - in insoluble formazan by mitochondrial enzymes.[33] The extraction of formazan was performed with 10% (v/v) SDS for 18 h (100 µL/well) at 24 °C.[34] MTT and the test compound (without parasites) was added to an internal control to investigate possible oxidative reaction.

**Determination of cytotoxicity against mammalian cells**

Mouse subcutaneous connective tissue cells, NCTC clone 929, (ATCC CCL-1™) were seeded at 4 x $10^4$ cells/well in 96-well microplates and incubated with compounds to the highest concentration of 200 µg/mL for 48 h at 37 ºC in a 5% $CO_2$ humidified incubator. The viability of cells was determined by the MTT assay.[35] Benznidazole was used as the standard drug. Control cells were incubated in the presence of DMSO and without drugs.

**Statistical analysis**

Data represent the mean and standard deviation of duplicate samples from two independent assays. The IC50 values were calculated using sigmoidal dose-response curves in Graph Pad Prism 5.0 software. The Mann-Whitney t-test (unpaired two-tailed) was used for significance testing ($p < 0.05$).

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