Leishmanicidal activity of an alkenylphenol from *Piper malacophyllum* is related to plasma membrane disruption

Alberto de Oliveira [a,*], Juliana T. Mesquita [b], André G. Tempone [b], João Henrique G. Lago [c], Elsie F. Guimarães [d], Massuo J. Kato [e]

[a] Instituto de Química, Universidade Federal de Uberlândia, Uberlândia, MG, Brazil
[b] Departamento de Parasitologia, Instituto Adolfo Lutz, São Paulo, SP, Brazil
[c] Instituto de Ciências Ambientais, Químicas e Farmacêuticas, Universidade Federal de São Paulo, Diadema, SP, Brazil
[d] Instituto de Pesquisas Jardim Botânico do Rio de Janeiro, Rio de Janeiro, RJ, Brazil
[e] Instituto de Química, Universidade de São Paulo, São Paulo, SP, Brazil

HIGHLIGHTS

▶ Alkenylphenol gibbilimbol B was isolated from *Piper malacophyllum*.
▶ Gibbilimbol B was evaluated *in vitro* against *Leishmania* spp. and *Trypanosoma cruzi*.
▶ *L.* (*L.*) *infantum chagasi* and *T. trypomastigotes* were susceptible to gibbilimbol B.
▶ The alkenylphenol suggested the disruption of the *Leishmania* plasma membrane at initial incubation time.

GRAPHICAL ABSTRACT
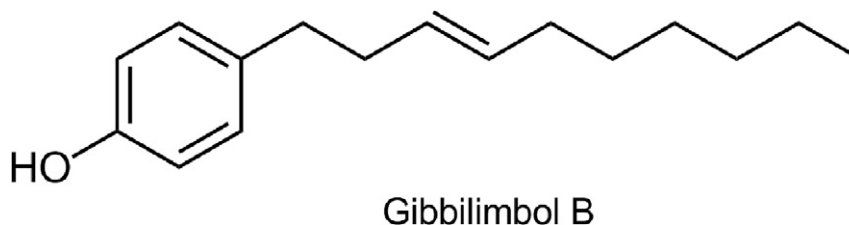
Gibbilimbol B

ARTICLE INFO

ABSTRACT

Leishmaniasis and Chagas disease are parasitic protozoan infections that affect the poorest population in the world, causing high mortality and morbidity. As a result of highly toxic and long-duration treatments, novel, safe and more efficacious drugs are essential. In this work, the methanol (MeOH) extract from the leaves of *Piper malacophyllum* (Piperaceae) was fractioned to afford one alkenylphenol, which was characterized as 4-[(3′E)-decenyl]phenol (gibbilimbol B) by spectroscopic methods. Anti-protozoan *in vitro* assays demonstrated for the first time that *Leishmania* (*L.*) *infantum chagasi* was susceptible to gibbilimbol B, with an *in vitro* EC_{50} of 23 µg/mL against axenic promastigotes and an EC_{50} of 22 µg/mL against intracellular amastigotes. Gibbilimbol B was also tested for anti-trypanosomal activity (*Trypanosoma cruzi*) and showed an EC_{50} value of 17 µg/mL against trypomastigotes. To evaluate the cytotoxic parameters, this alkenylphenol was tested *in vitro* against NCTC cells, showing a CC_{50} of 59 µg/mL and absent hemolytic activity at the highest concentration of 75 µg/mL. Using the fluorescent probe SYTOX Green suggested that the alkenylphenol disrupted the *Leishmania* plasma membrane upon initial incubation. Further drug design studies aiming at derivatives could be a promising tool for the development of new therapeutic agents for leishmaniasis and Chagas disease.

1. Introduction

The Piperaceae family comprises 14 genera and ca. 4000 species (Monteiro and Guimarães, 2009). Among these, *Piper* and *Peperomia* are the most abundant with approximately 700 and 600 species, respectively (Jaramillo et al., 2004). Phytochemical investigations carried out on *Piper* species have revealed many bioactive compounds such as amides, alkaloids, lignans, benzoic acids, and chromenes (Sengupta and Ray, 1987; Ruangrungsi et al., 1992; Wu et al., 1997; Parmar et al., 1998; Alécio et al., 1998; Navickiene et al., 2000; Silva et al., 2002; Lago et al., 2004; Freitas et al., 2009;

* Corresponding author.
 E-mail address: alberto@iqufu.ufu.br (A. de Oliveira).

Lago et al., 2009). Several of these compounds isolated from *Piper* species display antileishmanial (Torres-Santos et al., 1999; Hermoso et al., 2003; Bodiwala et al., 2007; Silva et al., 2009; Vendrametto et al., 2010) and trypanocidal activities (Martins et al., 2003; Ribeiro et al., 2004; Luize et al., 2006; Felippe et al., 2008; Regasini et al., 2009; Flores et al., 2009; Mota et al., 2009; Cotinguiba et al., 2009; Pelizzaro-Rocha et al., 2010; Batista et al., 2011). Thus, considering the availability of Piperaceae species in Brazil, our bioprospecting studies have been directed towards the discovery of new antiparasitic agents in plants belonging to this family, including *Piper malacophyllum*.

P. malacophyllum is a shrub commonly known as "pariparaba-murta", with occurrence restricted to the Southeastern and South regions of Brazil (Guimarães and Monteiro, 2006). Previous studies have described that the bioactivity-guided fractionation of a leaf extract from *P. malacophyllum* afforded two piperolides with anti-fungal activity against *Cladosporium* sp. (Lago et al., 2005) and its leaf essential oil showed broad spectrum antimicrobial activity, especially antifungal (Santos et al., 2012). Additionally, another report described the characterization of piperolides from a leaf extract using capillary electrophoresis (Oliveira et al., 2010). In this study, a continuation of the *P. malacophyllum* investigation, the MeOH extract from leaves was subjected to partition procedures as well as chromatographic separation to afford 4-[(3′E)-decenyl]phenol (gibbilimbol B), which was characterized by spectroscopic analysis, mainly nuclear magnetic resonance (NMR) and mass spectrometry (MS). This compound displayed *in vitro* antileishmanial activity against the promastigotes and amastigotes of *Leishmania* (*L.*) *infantum chagasi,* the etiologic agent of visceral leishmaniasis, as well as against the trypomastigotes of *Trypanosoma cruzi*, with disruption of the membrane of *Leishmania* parasites.

2. Material and methods

2.1. General experimental procedures

^{1}H NMR and ^{13}C spectra were recorded, respectively, at 200 and 50 MHz in a Bruker AC-200 spectrometer. CDCl$_3$ (Aldrich) was used as the solvent and tetramethylsilane (TMS, Aldrich) as the internal standard. Chemical shifts are reported in δ units (ppm) and coupling constants (J) in Hz. Low resolution electronic impact mass spectrometry (LREIMS) was performed using an HP 5990/5988A mass spectrometer. Silica gel (Merck, 230–400 mesh) was used for column chromatographic separation, while silica gel 60 PF$_{254}$ (Merck) was used for analytical thin layer chromatography (TLC, 0.25 mm).

2.2. Plant material

Piper malacophyllum leaves were collected in the Parque Estadual Intervales (Sisbio 15780-2), SP, Brazil, in November 2004 and were identified by Dr. Elsie F. Guimarães. A voucher specimen (K-447) has been deposited at the herbarium of Instituto de Pesquisa do Jardim Botânico, Rio de Janeiro, RJ, Brazil.

2.3. Extraction and isolation

Dried and powdered leaves of *P. malacophyllum* (93 g) were extracted with MeOH (3 × 300 mL at room temperature), affording 10.5 g of a syrupy green extract after solvent removal under reduced pressure. This extract was resuspended using MeOH:H$_2$O (4:1) and subjected to Celite column chromatography eluted with MeOH:H$_2$O 4:1 (fractions A1 and A2), MeOH (fraction A3) and acetone (fraction A4). Fraction A1 was extracted with CH$_2$Cl$_2$

(3 × 200 mL), and the organic phase, after being dried using Na$_2$SO$_4$, was subjected to a silica gel column chromatography eluted with increasing amounts of ethyl acetate in *n*-hexane (up to 100%) to afford six fractions (B1–B6). Fraction B6 (264 mg) was subjected to preparative TLC eluted with *n*-hexane:CH$_2$Cl$_2$ to give 159.8 mg of 4-[(3′E)-decenyl]phenol (gibbilimbol B).

2.4. 4-[(3′E)-decenyl]phenol (gibbilimbol B)

^{1}H NMR (200 MHz, CDCl$_3$) δ_H: 7.04 (d, J = 8.3 Hz, H-3/H-5), 6.75 (d, J = 8.3 Hz, H-2/H-6), 5.43 (m, H-3′/H-4′), 4.96 (br s, OH), 2.60 (t, J = 7.9, H-1′), 2.26 (m, H-2′), 1.95 (m, H-5′), 1.38–1.19 (m, H-6′ to H-9′), 0.89 (t, J = 7.0 Hz, H-10′). ^{13}C NMR (50 MHz, CDCl$_3$) δ_C: 153.5 (C-1), 134.4 (C-4), 131.2 (C-4′), 129.5 (C-3 and C-5), 129.3 (C-3′), 115.0 (C-2 and C-6), 35.2 (C-1′), 34.7 (C-2′), 32.6 (C-5′), 31.7 (C-8′), 29.5 (C-6′), 28.8 (C-7′), 22.6 (C-9′), 14.8 (C-10′). IR (film, cm^{-1}): 3379 (OH), 1609 (C=C), 1514 (aromatic C=C), 966 (Csp^2-H opp). LREIMS (70 eV) *m/z* (int. rel.): 232 (2), 108 (8), 107 (100), 91 (1), 77 (6), 55 (3), 43 (3), 41 (7).

2.5. Bioassay procedures

BALB/c mice and golden hamsters were supplied by the animal breeding facility at the Instituto Adolfo Lutz, 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, in agreement with the Guide for the Care and Use of Laboratory Animals from the National Academy of Sciences.

2.6. Parasite maintenance

Promastigotes *L.* (*L.*) *infantum chagasi* (MHOM/BR/1972/LD) were maintained in golden hamsters while the amastigotes were obtained from the spleen by differential centrifugation at the 60–70th day post-infection (Grecco et al., 2010). *T. cruzi* trypomastigotes (Y strain) were maintained in LLC-MK2 (ATCC CCL 7) cells using RPMI-1640 medium supplemented with 2% calf serum at 37 °C.

2.7. Mammalian cells

Peritoneal macrophages were collected from the peritoneal cavity of female BALB/c mice by washing with RPMI-1640 without phenol red, supplemented with 10% fetal bovine serum. NCTC clone 929 cells were maintained in RPMI-1640 medium without phenol red and supplemented with 10% fetal bovine serum at 37 °C in a 5% CO$_2$ humidified incubator.

2.8. Trypanosoma cruzi trypomastigotes

4-[(3′E)-Decenyl]phenol (gibbilimbol B) was dissolved in MeOH and diluted in RPMI-1640 medium to determine the 50% effective concentration (EC$_{50}$ value) as described above for the antileishmanial assay. Free trypomastigotes obtained from LLC-MK2 cultures were counted in a Neubauer hemocytometer and seeded at 1 × 10^6/well in 96-well microplates. 4-[(3′E)-Decenyl]phenol (gibbilimbol B) was added at the highest concentration of 150 μg/mL for 24 h at 37 °C in a 5% CO$_2$ humidified incubator. Benznidazole was used as the standard drug. The viability of trypomastigotes was based on the cellular conversion of the soluble tetrazolium salt MTT (20 μL/well) into insoluble formazan by mitochondrial enzymes. Formazan extraction was carried out using 10% SDS for 18 h (80 μL/well) at 24 °C as described previously (Lane et al., 1996). The number of living trypomastigotes was determined indirectly by the OD at 550 nm (Grecco et al., 2010).

2.9. Determination of the 50% effective concentration against Leishmania spp. promastigotes

To determine the 50% effective concentration (EC_{50} value) against *L.* (*L.*) *infantum chagasi* promastigotes, 4-[(3′E)-decenyl] phenol (gibbilimbol B) was previously dissolved in MeOH and diluted with M-199 medium in 96-well microplates to the highest concentration of 150 µg/mL (based on dry weight). Each compound was tested twice at eight concentrations prepared at twofold dilutions. Promastigotes were counted in a Neubauer hemocytometer and seeded at 1×10^6/well with a final volume of 150 µL. Controls with MeOH and without drugs were also performed. Amphotericin B was used as a standard drug. The plate was incubated for 24 h at 24 °C and the viability of promastigotes was verified by morphology using light microscopy and the diphenyltetrazolium (MTT) assay (Tempone et al., 2009). 100% viability was expressed based on the OD of control promastigotes, after normalization.

2.10. Intracellular amastigotes

Peritoneal macrophages were obtained as described previously and seeded for 24 h at 1×10^5/well in 16-well slide chambers (NUNC) before infection with *L.* (*L.*) *infantum chagasi* amastigotes, which were prepared at a 1:10 ratio (macrophage/amastigotes) for 18 h at 37 °C in a 5% CO_2 humidified incubator. After 18 h of incubation, infected macrophages were incubated at 37 °C with 4[(3E)-dec-3-en-1-yl]phenol (gibbilimbol B) for 120 h under the same conditions described above. Macrophages incubated without drugs were used for control (100% infected). Glucantime was used as a standard drug. At the end of the assay, macrophages were fixed with MeOH, stained with Giemsa and observed using a light microscope (1000 × magnification). The parasite burden was determined by the number of infected macrophages out of 400 macrophages (Tempone et al., 2010).

2.11. Determination of the cytotoxicity against mammalian cells

NCTC-clone 929 cells were seeded at 6×10^4 cells/well in 96-well microplates and incubated with gibbilimbol B and the standard drugs amphotericin B, glucantime and benznidazole for 48 h at 37 °C in a 5% CO_2 humidified incubator. The viability of cells was determined by the MTT assay as described above and the 50% cytotoxic concentration (CC_{50}) was calculated.

2.12. Spectrofluorimetric detection of disruption of the Leishmania plasma membrane

Late growth-phase (non-stationary) promastigotes of *L.* (*L.*) *infantum chagasi* were washed in phosphate-buffered saline, seeded at 2×10^6/well and incubated for 15 min with 1 µM SYTOX Green (Molecular Probes®) in M199 medium without phenol red, supplemented with fetal bovine serum and hemin (Mangoni et al., 2005). Gibbilimbol B was added at the previously determined EC_{50} and fluorescence was measured every 30 min for a total period of 180 min. Maximum fluorescence was determined by the addition of 0.5% Triton X-100. The control group consisted of promastigotes incubated with 0.5% MeOH. Fluorescence was measured using a microplate reader (FilterMax F5-Molecular Devices, USA) with excitation and emission wavelengths of 485 and 520 nm, respectively (Kulkarni et al., 2009).

2.13. Hemolytic activity

The hemolytic activity of gibbilimbol B was evaluated using BALB/c erythrocytes. A 3% suspension of mouse erythrocytes was incubated for 2 h with gibbilimbol B in a 96-well U-shaped microplate at 25 °C. The supernatant was assessed at 550 nm using a Multiskan reader (Morais et al., 2012; Sartorelli et al., 2010).

2.14. Statistical analysis

Results are represented by the mean and standard deviation of triplicate samples from two independent assays. The EC_{50} values were calculated using sigmoid dose–response curves in Graph Pad Prism 5.0 software, and the 95% confidence intervals are included in parentheses.

3. Results and discussion

As part of a continuous search to discover new anti-protozoal derivatives from Brazilian flora (Grecco et al., 2010; Corrêa et al., 2011; Morais et al., 2012; Grecco et al., 2012), this study reports the isolation of one active alkenylphenol from *P. malacophyllum* which was characterized by the analysis of its spectroscopic data. Analysis of the ^{1}H, ^{13}C, DEPT 135°, and DQF-COSY NMR spectra along with comparison of spectroscopic data reported in the literature (Orjala et al., 1998) allowed the identification of the isolated compound as 4-[(3′E)-decenyl]phenol (gibbilimbol B) (Fig. 1).

Gibbilimbol B was tested *in vitro* against the visceral species of *Leishmania* (*L.* (*L.*) *infantum chagasi*). According to the lack of mitochondrial oxidation (MTT assay), this compound showed a leishmanicidal activity against promastigotes, with an EC_{50} of 23.32 µg/mL (Table 1). Amphotericin B was used as the standard and was about 167-fold more effective than the tested compound (EC_{50} of 0.137 µg/mL). Although gibbilimbol was less effective than amphotericin B, it could also be observed that it was not more toxic to mammalian cells than the standard drug, resulting in a similar CC_{50} value. Considering the clinical importance of the intracellular amastigotes, additional studies were performed using macrophages as host mammalian cells. Gibbilimbol B was effective in eliminating amastigotes with an EC_{50} of 22.06 µg/mL, which was similar to that observed for the standard drug glucantime (EC_{50} of 21 µg/mL) (Table 1).

The possible action of gibbilimbol B on *Leishmania* membranes was studied using the fluorescent probe SYTOX Green (Fig. 2). Gibbilimbol B exposure led to a gradual increase in SYTOX Green permeability, with the increase in fluorescence beginning at 30 min to a maximal intensity at 150 min. SYTOX® Green (Molecular Probes) is a high-affinity nucleic acid stain that penetrates cells with compromised plasma membranes and enhances its fluorescence by more than 500-fold upon nucleic acid binding. The non-ionic surfactant Triton X-100 was used as the positive control, resulting in the maximal intensity of fluorescence. Thus, increased plasma membrane permeability in the early minutes of incubation with gibbilimbol B could be ascribed to the disruption of the plasma membrane of *Leishmania*, leading to the leakage of ions and cellular death. Another compound in clinical use for leishmaniasis (amphotericin B) has been shown to induce pore formation in the plasma membrane of *Leishmania*, especially due to its high affinity for ergosterol (Saha et al., 1986; Olliaro and Bryceson, 1993; Urbina, 1997). Further studies with gibbilimbol B should be performed to
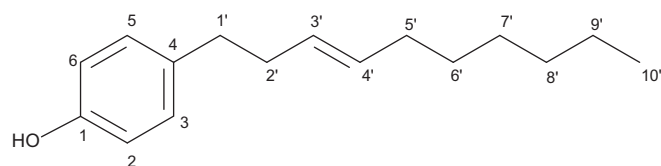

Fig. 1. Chemical structure of 4-[(3′E)-decenyl]phenol (gibbilimbol B).

Table 1
Antiparasitic (anti-leishmanial and anti-trypanosomal) and cytotoxic effects of 4-[(3′E)-decenyl]phenol (gibbilimbol B) and standard drugs.

Cell	EC_{50} µg/mL (95% CI)	
	Gibbilimbol B	Standard drugs
L. (L.) infantum chagasi promastigotes	23.32 (19.20–28.31)	0.137[a] (0.071–0.254)
L. (L.) infantum chagasi amastigotes	22.06 (17.01–28.62)	21.01[b] (20.34–24.37)
T. cruzi trypomastigotes	17.49 (14.49–21.12)	187.5[c] (129.9–273.8)
Cytotoxicity (NCTC cells)	59.04 (50.64–68.84)	59.33[a] (41.93–67.55)
		>100[b]
		124.13[c] (111.08–128.25)

EC_{50} – 50% effective concentration; 95 CI – 95% confidence interval; nd – not determined.
  [a] Amphotericin B.
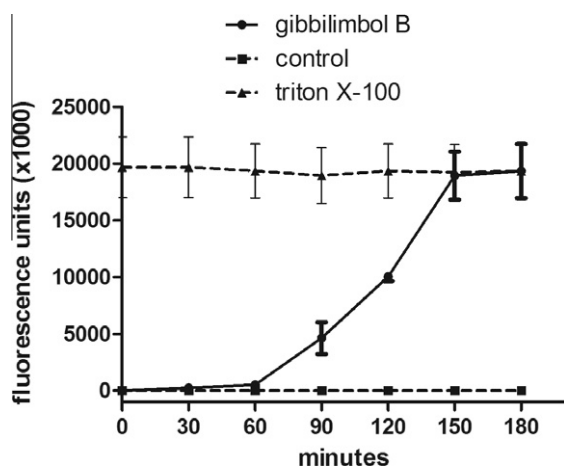 [b] Glucantime.
  [c] Benznidazole.

Fig. 2. Effect of gibbilimbol B on the membrane of *Leishmania* promastigotes using the fluorescent probe SYTOX Green. Promastigotes of the control group were treated with 0.5% MeOH. Fluorescence was measured in a microplate reader with excitation and emission wavelengths of 485 and 520 nm, respectively.

elucidate other mechanisms of action and its possible binding to 24-ergosterol in *Leishmania*.

This is the first report describing the anti-protozoan activity of gibbilimbol B. Gibbilimbols A–D were isolated from *Piper gibbilimbum* (Piperaceae), demonstrating anti-bacterial activity against *Staphylococcus epidermidis* and *Bacillus cereus* (Orjala et al., 1998). In our assays, gibbilimbol B showed promising anti-trypanosomal activity, with an EC_{50} of 17.49 µg/mL, which was about 10-fold more effective than the standard drug benznidazole (EC_{50} of 187 µg/mL). By the MTT assay and light microscopy, it was also possible to confirm its trypanocidal activity (Table 1).

In order to study the possible cytotoxicity of gibbilimbol B, NCTC cells were incubated for 48 h, resulting in a CC_{50} value of 59.04 µg/mL. According to its anti-parasitic activity, the selectivity index (EC_{50} parasite/CC_{50} mammalian cells) was calculated and resulted in 3.3 for *T. cruzi* and 2.7 for *Leishmania*. Despite the toxicity against the NCTC cells, no hemolytic activity could be detected for gibbilimbol B at 75 µg/mL.

4. Conclusion

The alkenylphenol gibbilimbol B was tested *in vitro* against *Leishmania* spp. promastigotes and amastigotes and *Trypanosoma*

cruzi trypomastigotes. These *in vitro* assays demonstrated for the first time that *Leishmania* (*L.*) *infantum chagasi* and *Trypanosoma cruzi* trypomastigotes are susceptible to gibbilimbol B. The straightforward synthesis of gibbilimbols A–D reported in the literature (Abe et al., 2001; Vyvyan et al., 2002) associated with the novel anti-protozoal activity of gibbilimbol B suggests that this compound could be used as a novel prototype for further drug design studies for neglected diseases such as Chagas disease and leishmaniasis.

References

Abe, Y., Takikawa, H., Mori, K., 2001. Synthesis of Gibbilimbols A–D, cytotoxic and antibacterial alkenylphenols isolated from *Piper gibbilimbum*. Biosci. Biotechnol. Biochem. 65, 732–735.
Alécio, A.C., Bolzani, V.S., Young, M.C.M., Kato, M.J., Furlan, M., 1998. Antifungal amides from *Piper hispidum*. J. Nat. Prod. 61, 637–639.
Batista, J.M., Batista, A.N.L., Rinaldo, D., Vilegas, W., Ambrosio, D.L., Cicarelli, R.M.B., Bolzani, V.S., Kato, M.J., Nafie, L.A., Lopez, S.N., Furlan, M., 2011. Absolute configuration and selective trypanocidal activity of gaudichaudianic acid enantiomers. J. Nat. Prod. 74, 1154–1160.
Bodiwala, H.S., Singh, G., Singh, R., Dey, C.S., Sharma, S.S., Bhutani, K.K., Singh, I.P., 2007. Antileishmanial amides and lignans from *Piper cubeba* and Piper retrofractum. J. Nat. Med. 61, 418–421.
Corrêa, D.S., Tempone, A.G., Reimão, J.Q., Taniwaki, N.N., Romoff, P., Fávero, O.A., Sartorelli, P., Mecchi, M.C., Lago, J.H.G., 2011. Anti-leishmanial and anti-trypanosomal potential of polygodial isolated from stem barks of *Drimys brasiliensis* Miers (Winteraceae). Parasitol. Res. 109, 231–236.
Cotinguiba, F., Regasini, L.O., Bolzani, V.D., Debonsi, H.M., Passerini, G.D., Cicarelli, R.M.B., Kato, M.J., Furlan, M., 2009. Piperamides and their derivatives as potential anti-trypanosomal agents. Med. Chem. Res. 18, 703–711.
Felippe, L.G., Baldoqui, D.C., Kato, M.J., Bolzani, V.D., Guimaraes, E.F., Cicarelli, R.M.B., Furlan, M., 2008. Trypanocidal tetrahydrofuran lignans from *Peperomia blanda*. Phytochemistry 69, 445–450.
Freitas, G.C., Kitamura, R.O.S., Lago, J.H.G., Young, M.C.M., Guimarães, E.F., Kato, M.J., 2009. Caldensinic acid, a prenylated benzoic acid derivative from *Piper caldense*. Phytochem. Lett. 2, 119–122.
Flores, N., Jimenez, I.A., Gimenez, A., Ruiz, G., Gutierrez, D., Bourdy, G., Bazzocchi, I.L., 2009. Antiparasitic activity of prenylated benzoic acid derivatives from *Piper* species. Phytochemistry 70, 621–627.
Guimarães, E.F., Monteiro, D., 2006. Piperaceae na Reserva Biológica de Poço das Antas, Silva Jardim, Rio de Janeiro, Brasil. Rodriguésia 57, 567–587.
Grecco, S.S., Reimão, J.Q., Tempone, A.G., Sartorelli, P., Romoff, P., Ferreira, M.J., Fávero, O.A., Lago, J.H.G., 2010. Isolation of an antileishmanial and antitrypanosomal flavanone from the leaves of *Baccharis retusa* DC (Asteraceae). Parasitol. Res. 106, 1245–1248.
Grecco, S.S., Sartorelli, P., Reimão, J.Q., Tempone, A.G., Cunha, R.L.O.R., Romoff, P., Ferreira, M.J.P., Favero, O.A., Lago, J.H.G., 2012. In vitro antileishmanial and antitrypanosomal activities of flavanones from *Baccharis retusa* DC (Asteraceae). Exp. Parasitol. 130, 141–145.
Hermoso, A., Jimenez, I.A., Mamani, Z.A., Bazzocchi, I.L., Pinero, J.E., Ravelo, A.G., Valladares, B., 2003. Antileishmanial activities of dihydrochalcones from *Piper elongatum* and synthetic related compounds: structural requirements for activity. Bioorg. Med. Chem. 11, 3975–3980.
Jaramillo, M.A., Manos, P.S., Zimmer, E.A., 2004. Phylogenetic relationships of the perianthless Piperales: reconstructing the evolution of floral development. Int. J. Plant Sci. 165, 403–416.
Kulkarni, M.M., Mcmaster, R.W., Kamysz, W., Mcgwire, B.S., 2009. Antimicrobial peptide-induced apoptotic death of *Leishmania* results from calcium-dependent, caspase-independent mitochondrial toxicity. J. Biol. Chem. 284, 15496–15504.
Lago, J.H.G., Ramos, C.S., Casanova, D.C.C., Morandim, A.A., Bergamo, D.C.B., Cavalheiro, A.J., Bolzani, V.S., Furlan, M., Guimarães, E.F., Young, M.C.M., Kato, M.J., 2004. Benzoic acid derivatives from *Piper* species and their fungitoxic activity against *Cladosporium cladosporioides* and *C. sphaerospermum*. J. Nat. Prod. 67, 1783–1788.
Lago, J.H.G., Tanizaki, T.M., Young, M.C.M., Guimarães, E.F., Kato, M.J., 2005. Antifungal piperolides from *Piper malacophyllum* (Prels) C. DC. J. Braz. Chem. Soc. 16, 153–156.
Lago, J.H.G., Chen, A., Young, M.C.M., Guimarães, E.F., Oliveira, A., Kato, M.J., 2009. Prenylated benzoic acid derivatives from *Piper aduncum* L. and *P. hostmannianum* C. DC (Piperaceae). Phytochem. Lett. 2, 96–98.

Lane, J.E., Ribeiro-Rodrigues, R., Suarez, C.C., Bogitsh, B.J., Jones, M.M., Singh, P.K., Carter, C.E., 1996. In vitro trypanocidal activity of tetraethylthiuram disulfide and sodium diethylamine-N-carbodithioate on Trypanosoma cruzi. Am. J. Trop. Med. Hyg. 55, 263–266.

Luize, P.S., Ueda-Nakamura, T., Dias, B.P., Cortez, D.A.G., Nakamura, C.V., 2006. Activity of neolignans isolated from Piper regnellii (Miq.) C. DC. var. pallescens (C. DC.) Yunck against Trypanosoma cruzi. Biol. Pharm. Bull. 29, 2126–2130.

Mangoni, M.L., Saugar, J.M., Dellisanti, M., Barra, D., Simmaco, M., Rivas, L., 2005. Temporins, small antimicrobial peptides with Leishmanicidal activity. J. Biol. Chem. 280, 984–990.

Martins, R.C.C., Lago, J.H.G., Albuquerque, S., Kato, M.J., 2003. Trypanocidal tetrahydrofuran lignans from inflorescences of Piper solmsianum. Phytochemistry 64, 667–670.

Monteiro, D., Guimarães, E.F., 2009. Flora do Parque Nacional do Itatiaia – Brasil: Manekia e Piper (Piperaceae). Rodriguésia 60, 999–1024.

Morais, T.R., Romoff, P., Fávero, O.A., Reimão, J.Q., Lourenço, W.C., Tempone, A.G., Hristov, A.D., Di Santi, S.M., Lago, J.H.G., Sartorelli, P., Ferreira, M.J.P., 2012. Anti-malarial, anti-trypanosomal, and anti-leishmanial activities of jacaranone isolated from Pentacalia desiderabilis (Vell.) Cuatrec. (Asteraceae). Parasitol. Res. 110, 95–101.

Mota, J.D., Leite, A.C., Batista, J.M., Lopez, S.N., Ambrosio, D.L., Passerini, G.D., Kato, M.J., Bolzani, V.S., Cicarelli, R.M.B., Furlan, M., 2009. In vitro trypanocidal activity of phenolic derivatives from Peperomia obtusifolia. Planta Med. 75, 620–623.

Navickiene, H.M.D., Alécio, A.C., Kato, M.J., Bolzani, V.S., Young, M.C.M., Cavalheiro, A.J., Furlan, M., 2000. Antifungal amides from Piper hispidum and Piper tuberculatum. Phytochemistry 55, 621–626.

Oliveira, A., Silva, C.A., Silva, A.M., Tavares, M.F.M., Kato, M.J., 2010. Development and validation of a micellar electrokinetic chromatography method for quantitative determination of butenolides in Piper malacophyllum (C. Presl) C. DC. Phytochem. Anal. 21, 428–433.

Olliaro, P.L., Bryceson, A.D.M., 1993. Pratical progress and new drugs for changing patterns of leishmaniasis. Parasitol. Today 9, 323–328.

Orjala, J., Mian, P., Rali, T., Sticher, O., 1998. Gibbilimbols A–D, cytotoxic and antibacterial alkenylphenols from Piper gibbilimbum. J. Nat. Prod. 61, 939–941.

Parmar, V.S., Jain, S.C., Gupta, S., Talwar, S., Rajwanshi, V.K., Kumar, R., Azim, A., Malhotra, S., Kumar, N., Jain, R., Sharma, N.K., Tyagi, O.D., Lawrie, S.J., Errington, W., Howarth, O.W., Olsen, C.E., Singh, S.K., Wengel, J., 1998. Polyphenols and alkaloids from Piper species. Phytochemistry 49, 1069–1078.

Pelizzaro-Rocha, K., Veiga-Santos, P., Rocha, K., Silva, S., Ueda-Nakamura, T., Dias, B., Ximenes, V., Nakamura, C., 2010. The trypanocidal action of eupomatenoid-5 isolated from Piper regnellii var. pallescens may be related to an imbalance between the antioxidant system and ROS. Planta Med. 76, 479. Abstract from 58th International Congress and Annual Meeting of the Society for Medicinal Plant and Natural Product Research.

Regasini, L.O., Cotinguiba, F., Passerini, G.D., Bolzani, V.S., Cicarelli, R.M.B., Kato, M.J., Furlan, M., 2009. Trypanocidal activity of Piper arboreum and Piper tuberculatum (Piperaceae). Braz. J. Pharmacogn. 19, 199–203.

Ribeiro, T.S., Freire-de-Lima, L., Previato, J.O., Mendonça-Previato, L., Heise, N., de Lima, M.E.F., 2004. Toxic effects of natural piperine and its derivatives on epimastigotes and amastigotes of Trypanosoma cruzi. Bioorg. Med. Chem. Lett. 14, 3555–3558.

Ruangrungsi, N., Prathanturarug, S., Lange, G., Organ, M., 1992. An N-methyl aristolactam and an oxygenated cyclohexane derivative from Piper ribesioides. Phytochemistry 31, 2397–2400.

Saha, A.K., Mukherjee, J., Bhaduri, A., 1986. Mechanism of action of amphotericin B on Leishmania donovani promastigotes. Mol. Biochem. Parasit. 19, 195–200.

Santos, T.G., Rebelo, R.A., Dalmarco, E.M., Guedes, A., Gasper, A.L., Cruz, A.B., Schmit, A.P., Cruz, R.C.B., Steindel, M., Nunes, R.K., 2012. Composição química e avaliação da atividade antimicrobiana do óleo essencial das folhas de Piper malacophyllum (C. Presl.) C. DC. Quim. Nova 35, 477–481.

Sartorelli, P., Salomone Carvalho, C., Quero Reimão, J., Lorenzi, H., Tempone, A.G., 2010. Antitrypanosomal activity of a diterpene and lignans isolated from Aristolochia cymbifera. Planta Med. 76, 1454–1456.

Silva, R.V., Navickiene, H.M.D., Kato, M.J., Bolzani, V.D., Méda, C.I., Young, M.C.M., Furlan, M., 2002. Antifungal amides from Piper arboreum and Piper tuberculatum. Phytochemistry 59, 521–527.

Sengupta, S., Ray, A.B., 1987. The chemistry of Piper species: a review. Fitoterapia LVIII 3, 147–165.

Silva, D.R., Nakamura, C.V., Dias Filho, B.P., Ueda-Nakamura, T., Cortez, D.A.G., 2009. In vitro antileishmanial activity of hydroalcoholic extract, fractions, and compounds isolated from leaves of Piper ovatum Vahl against Leishmania amazonensis. Acta Protozool. 48, 73–81.

Tempone, A.G., Taniwaki, N.N., Reimão, J.Q., 2009. Antileishmanial activity and ultrastructural alterations of Leishmania (L.) chagasi treated with the calcium channel blocker nimodipine. Parasitol. Res. 105, 499–505.

Tempone, A.G., Mortara, R.A., de Andrade Jr., H.F., Reimão, J.Q., 2010. Therapeutic evaluation of free and liposome-loaded furazolidone in experimental visceral leishmaniasis. Int. J. Antimicrob. Agents 36, 159–163.

Torres-Santos, E.C., Moreira, D.L., Kaplan, M.A.C., Meirelles, M.N., Rossi-Bergmann, B., 1999. Selective effect of 2′,6′-dihydroxy-4′-methoxychalcone isolated from Piper aduncum on Leishmania amazonensis. Antimicrob. Agents Chemother. 43, 1234–1241.

Urbina, J., 1997. Lipid biosynthesis pathway as chemotherapeutic targets in kinetoplastid parasites. Parasitology 114, S91–S99.

Vendrametto, M.C., dos Santos, A.O., Nakamura, C.V., Dias, B.P., Cortez, D.A.G., Ueda-Nakamura, T., 2010. Evaluation of antileishmanial activity of eupomatenoid-5, a compound isolated from leaves of Piper regnellii var. pallescens. Parasitol. Int. 59, 154–158.

Vyvyan, J.R., Holst, C.L., Johnson, A.J., Schwenk, C.M., 2002. Total synthesis of gibbilimbols A–D. J. Org. Chem. 67, 2263–2265.

Wu, Q., Wang, S., Tu, G., Feng, Y., Yang, J., 1997. Alkaloids from Piper puberullum. Phytochemistry 44, 727–730.