Antileishmanial activity of amides from *Piper amalago* and synthetic analogs

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Article

Abstract: Two natural amides isolated from the chloroform extract of *Piper amalago* L., Piperaceae, leaves, a hydrogenated derivative and seven synthetic analogs were tested against the promastigote and intracellular amastigote forms of *Leishmania amazonensis*. The antileishmanial activity was evaluated in terms of growth inhibitory concentration for 50% of protozoa (IC50). The cytotoxicity toward the J774A1 macrophages was evaluated in terms of the cytotoxic concentrations for 50% of macrophages (CC50). The ability to induce nitric oxide production was also investigated for all compounds. The saturated amide 7-(1,3-benzodioxol-5-yl)-1-(1-pyrrolidinyl)-1-heptanone was obtained by hydrogenation of the natural compound *N*-[7-(3',4'-methylenedioxyphenyl)-2(*Z*),4(*Z*)-heptadienoyl]pyrrolidine. Synthetic amides were prepared by addition of the appropriate amine to the corresponding acyl chloride. The natural compound, *N*-[7-(3',4'-methylenedioxyphenyl)-2(*E*),4(*E*)-heptadienoyl] pyrrolidine, was the most active of all tested compounds against the promastigote and intracellular amastigote forms with IC50 values of 15 μM and 14.5 μM, respectively. None of the compounds modulated the production of nitric oxide.

Introduction

Leishmaniases are a group of diseases caused by several intracellular protozoan parasites belonging to the genus *Leishmania*. Studies report that around 350 million women, men and children are at the risk in 88 tropical and subtropical countries (Singh & Sivakumar, 2004). The therapy in most countries is still restricted to drugs, which are painful, toxic, have a high cost and are administrated by intravenous injection. New alkylphosphocholine derivatives with amides have shown antileishmanial activity (Obando et al., 2007). Therefore, all these facts reveal the urgency associated with need for the development of new cheaper and safer drugs. In this context, plants have been researched as sources of new compounds with potential activity and lower toxicity (Bero et al., 2011; Brenzan et al., 2012).

Species of the genus *Piper* L. (Piperaceae), such as, *P. chaba, P. claussenianum, P. longum, P. sanguineispicum* and *P. tuberculatum*, have shown antileishmanial activity. These plants have been studied chemically and the following compounds have been identified: propanoic acid, esters, lignans, terpenes and amides (Cabanillas et al., 2010; Ferreira et al., 2010; Marques et al., 2011; Ghosal et al., 2012; Naz et al., 2012).

Piper amalago L. has been used in folk medicine as an anti-inflammatory, analgesic, antipyretic, therapy for stomach problems, and vermifuge. The phytochemical composition of *P. amalago* L. roots consists mainly of sesquiterpenes, pyrrolidines and isobutylamides (Heckel, 1897; Achenbach et al., 1984; 1986; Domínguez & Alcorn, 1985; Domínguez et al., 1986). The supercritical extract of the leaves rich in amide **1** showed significant antileishmanial activity (Carrara et al., 2012).

The purpose of this study was to screen the amides isolated from the leaves of *P. amalago,* a hydrogenated derivative, and synthetic analogs and evaluate the activity of these compounds against *L. amazonensis*. The derivative and synthetic analogs were synthesized with the aim of investigating whether they could significantly improve the antileishmanial activity, as has already been demonstrated for their

antifungal and insecticidal activity (Pagnocca et al., 2006; Sangwan et al., 2008; Castral et al., 2011). All compounds were analyzed in terms of the activity against the promastigote and amastigote forms of the protozoa, the cytotoxicity toward the macrophages, and the ability to induce nitric oxide production.

Materials and Methods

General experimental procedures

Compounds **1-3** were identified on a mass spectrometer (EI) QP DSQ II (Thermo Electron Corporation®, USA). ^{1}H and ^{13}C NMR were recorded on a Varian Gemini 2000 BB (300 and 75 MHz, respectively) (Varian®, USA). Column chromatography was performed to purify the compounds, using silica gel 60 (70-230 and 230-430 mesh).

For the synthesis of compounds **4-10**, unless otherwise noted, all commercially available reagents were purchased from Aldrich® Chemical Co. Reagents and solvents were purified when necessary according to the usual procedures described in the literature. 1H and ^{13}C NMR spectra were recorded on a Bruker Avance® III spectrometer (400 and 100 MHz respectively). The IR spectra refer to films and were measured on a Bomem® M102 spectrometer. Mass Spectra were recorded on a Shimadzu® GCMS-QP5000. Analytical thin-layer chromatography was performed on a 0.25 µm film of silica gel containing fluorescent indicator UV_{254} supported on an aluminum sheet (Sigma-Aldrich®). Flash column chromatography was performed using silica gel (Kieselgel 60, 230-400 mesh, E. Merck®). Gas chromatography was performed with a Shimadzu® GC-17A with H_2 as carrier and using a DB-5 column. Melting points were performed in Microquimica® MQAPF - 301. Reactions were irradiated in a focused microwave oven CEM Discover.

Plant material

Leaves of *Piper amalago* L., Piperaceae, were collected in the forestry garden belonging to "Dr. Luís Teixeira Mendes" in Maringá, Paraná, Brazil. The voucher specimen is deposited in the Herbarium of Universidade Estadual de Maringá (number HUEM 9885).

Extraction and isolation

The isolation of the compounds *N*-[7-(3',4'-methylenedioxyphenyl)-2(*Z*),4(*Z*)-heptadienoyl]pyrrolidine (**1**) and *N*-[7-(3',4'-methylenedioxyphenyl)-2(*E*),4(*E*)-heptadienoyl]pyrrolidine (**2**) from the leaves of *P. amalago* and their spectral data were described in a previous study (Carrara et al., 2012).

Hydrogenation product of *1*

Considering that compound **1** was the major compound isolated, it was chosen for preparing the derivative. A mixture of the methanol solution of compound **1** (36 mg) and 5% Pd/C (10 mg) was hydrogenated at 40 psi for 4 h and then filtered, washed with methanol (5 x 10 mL) and concentrated in a rotary evaporator to give compound **3** (22 mg) in a yield of 95.3% (Sangwan et al., 2008). This compound was identified by EIMS, ^{1}H and ^{13}C NMR spectral data.

7-(1,3-benzodioxol-5-yl)-1-(1-pyrrolidinyl)-1-heptanone (**3**): ^{1}H NMR (300 MHz-CDCl$_3$) δ: 6.71 (d, *J* = 7.8 Hz, H5'; 1H); 6.66 (d, *J* = 1.8 Hz, H2'; 1H); 6.60 (dd, *J* = 1.4, 7.9 Hz, H6'; 1H); 5.90 (s, H1'''; 2H); 3.46 (t, *J* = 6.6 Hz, H1''; 2H); 3.39 (t, *J* = 6.6 Hz, H4''; 2H); 2.51 (t, *J* = 7.8 Hz, H7; 2H); 2.25 (t, *J* = 7.8 Hz, H2; 2H); 1.92-1.99 (m, H2''; 2H); 1.79-1.90 (m, H3''; 2H); 1.64 (t, *J* = 7.5 Hz, H3, 2H); 1.57 (t, *J* = 7.5 Hz, H6; 2H); 1.23-1.41 (m, H4, H5; 4H). ^{13}C NMR (75 MHz-CDCl$_3$) δ: 24.55 (C3''); 25.01 (C3); 26.25 (C2''); 29.07 (C4); 29.47 (C5); 31.73 (C6); 34.93 (C2); 35.74 (C7); 45.83 (C1''); 46.84 (C4''); 100.82 (C1'''); 108.15 (C2'); 108.98 (C5'); 121.17 (C6'); 136.78 (C1'); 145.51 (C3'); 147.56 (C4'); 172.17 (C1). EIMS: *m/z*: 304 (6); 303 (M⁺, 36); 168 (30); 147 (10); 126 (58); 135 (32); 113 (100); 112 (12); 72 (10); 71 (15); 70 (24); 55 (16).

Synthetic procedures

Analogs **4-6**

Compounds *N*-[3-(3',4'-methylenedioxyphenyl)-2-(*E*)-propenoyl]pyrrolidine (**4**), *N*-[3-(3',4'-methylenedioxyphenyl)-2-(*E*)-propenoyl]benzylamide (**5**) and *N*-[3-(3',4'-methylenedioxyphenyl)-2-(*E*)-propenoyl]piperidine (**6**) were prepared as described by Corrêa and workers (Pagnocca et al., 2006).

Analogs **7-10**

Appropriate amounts of cinnamic acid (4.5 g; 23.4 mmol) and thionyl chloride (8 mL) were added to a 50 mL round-bottomed flask under nitrogen atmosphere, equipped with a magnetic stir bar and a condenser. The system was maintained at 50 °C for 4 h, and anhydrous hexane (30 mL) was then added and the solvent was removed in a rotary evaporator. The reaction afforded the corresponding cinnamoyl chloride as a solid. This compound was diluted in anhydrous dichloromethane (36 mL) under nitrogen atmosphere and the appropriate amine (25.7 mmol) was added in order to obtain the corresponding amide. A saturated solution of sodium bicarbonate (3 mL) was added after 12 h, and the extraction was carried out

with dichloromethane (3 x 3 mL). The organic phase was washed with distilled water (2 mL) followed by brine (2 mL), and dried with anhydrous sodium sulfate. After removing the solvent, the crude product was purified by silica gel column chromatography (230-400 mesh) using hexane-ethyl acetate (1:2 v/v) as the eluent. Compounds **7-10** were obtained in 64, 93, 54 and 52% yield, respectively. The amides were analyzed through IR, EIMS, ^{1}H and ^{13}C NMR spectral data and by comparison with data available in the literature (Castral et al., 2011).

N-[3-(2'-fluorophenyl)-2-(*E*)-propenoyl]piperidine (**7**): ^{1}H NMR (200 MHz-CDCl$_3$) δ: 1.54-1.75 (m, H2", H3", H4"; 6H); 3.58-3.67 (m, H1", H5"; 4H); 7.04 (d, *J* = 16 Hz, H2; 1H); 7.13-7.50 (m, H3', H5', H6'; 4H); 7.69 (d, *J* = 16 Hz, H4'; 1H). ^{13}C NMR (100 MHz-CDCl3) δ: 24.67 (C3"); 25.62 (C2"); 26.76 (C4"); 43.37 (C1"); 47.05 (C5"); 116.00 (C3'); 116.22 (C2); 121.00 (C1'); 123.48 (C5'); 124.33 (C6'); 129.63 (C4'); 135.08 (C3); 160.01 (C2'); 165.34 (C1). EIMS: *m/z*: 233(M$^+$, 59), 149 (100), 138 (48), 121 (46), 101 (62), 84 (30), 75 (14). IR (v$_{max}$, film): 2937, 2854, 1645, 1610, 1487, 1440, 1274, 1218, 1137, 1018, 756 cm^{-1}.

N-[3-(4'-fluorophenyl)-2-(*E*)-propenoyl]piperidine (**8**): ^{1}H NMR (400 MHz-CDCl$_3$) δ: 1.63-1.86 (m, H2", H3", H4"; 6H); 3.57-3.61 (m, H1", H5"; 4H); 6.80 (d, *J* = 16 Hz, H2, 1H); 6.99-7.07 (m, H2', H6'; 2H); 7.44-7.54 (m, H3, H3', H5'; 3H). ^{13}C NMR (100 MHz-CDCl3) δ: 25.55 (C3"); 26.88 (C2"); 27.92 (C4"); 44.65 (C1", C5"); 116.62, 116.91 (C2); 118.52, 118.55 (C3', C5'); 131.01, 131.12 (C2', C6'); 133.05, 133.10 (C1'); 142.55 (C3); 167.47, 163.34 (C4'); 166.64 (C1).
EIMS: *m/z*: 233(M+, 48), 149 (100), 138 (29), 121 (50), 101 (44), 84 (48). IR (v$_{max}$, film): 3041, 2935, 2850, 1647, 1598, 1510, 1442, 1280, 1222, 1162, 101, 993, 833, 511 cm-1.

N-[3-(4´-difluoromethoxyphenyl)-2-(*E*)-propenoyl]piperidine (**9**): ^{1}H NMR (200 MHz, CDCl$_3$) δ: 1.59-1.77 (m, H2", H3", H4"; 6H); 3.56-3.84 (m, H1", H5"; 4H); 6.51 (t, *J* = 16 Hz, CHF$_2$O; 1H); 6.83 (d, *J* = 16 Hz, H2; 1H); 7.09 (d, *J* = 4 Hz, H3', H5'; 2H); 7.50 (d, *J* = 4, H2´, H6´; 2H); 7.59 (d, *J* = 8 Hz, H3, 1H). ^{13}C NMR (100 MHz, CDCl$_3$): 24.61 (C3"); 26.73 (C4"); 43.34 (C1"); 47.01 (C5"); 113.07; 115.65 (C2) 117.96; 119.59 (C3´,C5´); 129.12 (C1'); 140.74 (C3); 151.79 (CHF$_2$O); 165.07 (C1). EIMS *m/z*: 281 (M$^+$), 279, 197 (100), 169, 138, 119, 84. IR(v$_{max}$, film): 2920, 2856, 2362, 2345, 1647, 1604, 1514, 1446, 1243, 1216, 1116, 1037, 981, 823 cm^{-1}.

N-[3-(4'-trifluoromethoxyphenyl)-2-(*E*)-propenoyl]piperidine (**10**): Compound **10** was prepared as described by Corrêa and workers (Castral et al., 2011).

Determination of the antileishmanial activity

Parasites

A strain of *Leishmania amazonensis* (MHOM/BR/77/LTB0016) was used. Parasites isolated from infected mice were maintained as promastigotes through weekly passages in Schneider medium (Sigma®) supplemented with 10% of fetal bovine serum, penicillin (100 UI/mL), and streptomycin (100 µg/mL) at 26 ºC.

Cells

J774A1 macrophages were cultured in RPMI medium (pH 7.2) supplemented with 10% of fetal bovine serum, and incubated at 37 ºC under an atmosphere of 5% CO$_2$. Macrophage cultures were maintained by passages every two or three days, according to ATCC.

Animals

Male BALB/c mice (25-30 g) were kept in a 12 h light/dark cycle in a temperature-controlled room with free access to water and food. The study reported in this manuscript was carried out in accordance with the Fiocruz Ethical Committee on Animal Use (CEUA-Fiocruz protocol number LW-7/10).

Stock solutions

Stock solutions of 10 mM of the compounds (1-10) were prepared in dimethyl sulfoxide (DMSO) (Sigma®, Sant Louis, USA). Assay concentrations were prepared with culture medium used in the experiments, as indicated in each case.

Antileishmanial activity against promastigotes

The method used to evaluate the anti-promastigote activity was adapted from Denizot & Lang (1986). Promastigotes were adjusted to a concentration of 1 x 10^6 cells/mL in Schneider medium (supplemented with 10% of fetal bovine serum, penicillin (100 UI/mL), and streptomycin (100 µg/mL). Parasites were incubated with the compounds (3.125-200 µM) (0.5% DMSO was used to dissolve the highest concentration of the samples) at 26 ºC for 72 h. Promastigotes in culture medium supplemented without test compounds, were used as the negative control. Pentamidine isotionate (0.3125-20 µM) was used as the reference. The antileishmanial activity was evaluated by adding 22 µL of MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] at 5 mg/mL (Sigma®, Sant Louis, USA) to each well. After 2 h, 80 µL of DMSO was added and the optical density was determined at a wavelength of 570 nm in a microplate reader (µQuant

Bio-Tek Instruments®, Winooski). The assays were carried out in triplicate in 96-well plates (Costar®, New York, USA). The inhibition percentage was estimated by the comparison with the negative control. Logarithm regression analysis was performed in order to obtain IC50 values (concentrations that inhibit the growth of promastigotes by 50%).

Cytotoxicity evaluation

The cytotoxicity test used was an adaption of that described in Mendez et al. (2009). A continuous J774A1 macrophage lineage was used in order to analyze the cytotoxicity of all compounds. The macrophages (2 x 10^6 cells/well) in RPMI culture medium at pH 7.2 (supplemented with 10% of fetal bovine serum) were incubated with compounds (50-400 μM) for 72 h at 37 °C under 5% CO_2 in 96-well plates. Cells in culture medium plus DMSO (0.5%) were used as control of viability. The supernatant was removed and viable cells were quantified by adding 200 μL of MTT at 5 mg/mL in phosphate buffer saline (PBS). The supernatant was removed again after 2 h, and 100 μL DMSO were added to each well. The optical density was determined at a wavelength of 570 nm in the microplate reader. The tests were carried out in triplicate. The percentage of viable cells was calculated relative to the control cells. Logarithm regression analysis was performed in order to obtain CC50.

Antileishmanial activity against intracellular amastigotes

BALB/c mice macrophages were obtained by peritoneal lavage with 5 mL of cold RPMI medium (Sigma®, Saint Loius, USA). The cell suspension (2 x 106 macrophages/mL) was applied in a Labtek chamber (Nunc®, New York, USA) and incubated for 1 h at 37 °C in 5% CO_2. The cultures were then washed with PBS at 37 oC for the removal of non-adherent cells. The remaining cells were incubated with the stationary phase of promastigotes of *L. amazonensis* at a ratio of 3:1 at 37 °C in 5% CO_2. After 3 h, the chambers were washed to remove free parasites and incubated with the compounds (12.5-200 μM) at 37 °C in 5% CO_2 for 72 h. Infected cells with DMSO (0.5%) were used as the negative control. Pentamidine isethionate (1.25-10 μM) was used as the reference. The cells were stained with the haematological system Instant Prov (New Prov®, Curitiba, Brazil). The anti-amastigote activity was analyzed by microscopy counting at least 100 macrophages per sample. The experiments were performed twice in duplicate (Torres-Santos et al., 1999). Logarithm regression analysis was performed in order to obtain IC50 values (concentrations that inhibit the growth of amastigotes by 50%). Results were expressed as the ratio of infection (IF) using the following formula:

$$IF = (\% \text{ infected cells} \times \text{number of amastigotes})/\text{total number of macrophages}$$

Assay for nitric oxide production

The supernatants of infected macrophages were collected to quantify the secreted nitric oxide by determining the nitrite concentration using the Griess assay. Griess reagents (1% sulfanilamide/0.1% naphthylethylenediamine dihydrochloride/3% phosphoric acid) were added to the supernatant (1:1 v/v), and left to stand for 5 min at room temperature. The absorbance was determined at 470 nm in a microplate reader. The nitrite concentration was calculated from a standard curve of sodium nitrite (10 to 50 μM). The experiments were performed twice in duplicate (Roach et al., 1991; Ding et al., 1998).

Statistical analysis

The results obtained from two or three independent experiments were presented as the mean+SD. The data were evaluated by analysis of variance and the Student´s t-test using GraphPad Prism 5.0 software (San Diego, CA, USA). Differences were considered significant when the p value was <0.05.

Results and Discussion

Chemistry

The known amides **1** and **2** were isolated from the extract of *Piper amalago* L., Piperaceae, leaves, and their spectral data were compared with those available in the literature (Alécio et al., 1998, Jacobs et al., 1999). Compound **3** was obtained by hydrogenation of compound **1**, the major compound in *P. amalago*, in order to improve the antileishmanial activity. The reduction of the double bound was confirmed by the ^{1}H NMR and ^{13}C NMR data of the compound **3**. The mass spectra of the compound **3** showed the molecular ion at *m/z* 303 ($C_{18}H_{25}NO_3.^+$). In addition, the fragments at *m/z* 126 ($C_7H_{12}NO^+$) and *m/z* 168 ($C_{10}H_{18}NO^+$) indicated the saturated lateral chain of the amide.

Ribeiro et al. (2004) described the trypanocidal effects of the natural alkaloid piperine and twelve synthetic derivatives, including compound **6**, against epimastigote and amastigote forms of the protozoan parasite *Trypanosoma cruzi*, the causative agent of the Chagas' disease. In order to contribute to the structure-activity relationship study of this class of compounds, we decided to test amides containing a shorter side chain, different amines and substituents in the aromatic ring, including fluorine (Hou & Xu, 2001).

Although fluorine does not have the same valency

as hydrogen, it is often considered an isostere of that atom, since it is virtually the same size. Replacement of a hydrogen atom with a fluorine atom will have little steric effect, but since the fluorine is strongly electronegative, the electronic effect may be dramatic. The use of fluorine as an isostere for hydrogen has been highly successful in the development of new drugs (Patrick, 1995).

Amides **4-10** were prepared as described in the literature by conversion of the appropriate cinnamic acid to the corresponding acyl chloride followed by the addition of the amine (Pagnocca et al., 2006; Castral et al., 2011). The synthetic compounds were characterized by ^{1}H NMR spectroscopy. The vinylic hydrogens of the *trans* double bond presented coupling constants in the range of 16 Hz (Ribeiro et al., 2004). Furthermore, the presence of shielded signal corresponding to ten hydrogens of the piperidinic ring confirmed the structures of the synthetic products **6-10.** All ^{13}C NMR spectra confirmed the presence of the amidic carbonyl, in the range of 160 ppm. For compounds **7-10** it was also observed heteronuclear coupling ^{13}C-^{19}F. The IR spectra displayed C=O stretching bands in the region of 1650 cm^{-1}, and thus confirmed the presence of amide carbonyl groups. From the mass spectra it was possible to obtain the mass of the molecular ion, besides the main fragmentation of amides to the complete characterization of each synthesized compound (Castral et al., 2011).

Antileishmanial activity of the amides against the promastigotes and intracellular amastigotes of L. amazonensis

Piperamides with significant antileishmanial activity are characterized by a phenyl group, with a side chain with at least one insaturation ending in a carbonyl group, and a piperidine, pyrrolidine or isobutyl groups

containing nitrogen (Bodiwala et al., 2007; da Silva et al., 2009; Regasini et al., 2009). These compounds have been investigated by some researchers as a model for the development of new compounds with potential biological activities (Pagnocca et al., 2006; Sangwan et al., 2008; Castral et al., 2011).

Natural compounds **1** and **2**, the hydrogenation product **3**, and the synthetic analogs **4-10** were evaluated against the promastigote and intracellular amastigote forms of *L. amazonensis*. Promastigote forms were treated for 72 h with increasing concentrations of the compounds (3.125-200 μM). In order to evaluate the anti-amastigote activity, macrophages obtained from the peritoneal cavity of mice were infected with promastigotes and treated with the compounds (12.5-200 μM) for 72 h. Pentamidine isethionate was used as the reference and the IC50 values were 2.2 μM, and 1.5 μM against the promastigote and amastigote forms, respectively, with a selectivity index (SI) towards the latter of 46.67. The cytotoxicity was evaluated using J774A1 macrophages treated for 72 h with the compounds at concentrations of 50-400 μM. The selectivity index (SI) for the protozoan was calculated according to the following formula: CC50 for J774A1 macrophages/ IC50 for the intracellular amastigotes. The compound was considered to be more selective for the amastigote forms than for the macrophages when the SI value was greater than 1. All compounds were compared according to their antileishmanial activity, cytotoxicity and SI (Table 1). Natural compounds **1** and **2** showed the best antileishmanial activity with IC50 values of around 20 μM and 15 μM respectively, compound **2** being more active and selective than compound **1** with a SI ratio of 12.89. The isolated amides inhibited significantly both promastigote and intracellular amastigote forms. Inhibitory action of a compound for all parasite forms of *Leishmania* is more desirable, considering that promastigotes are the
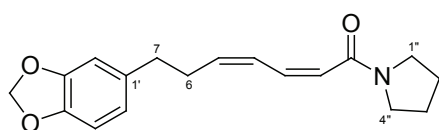
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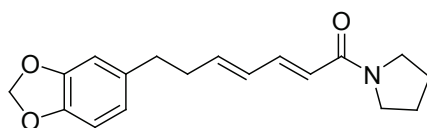
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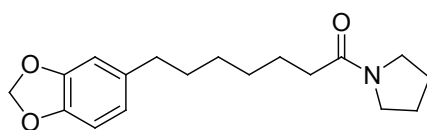
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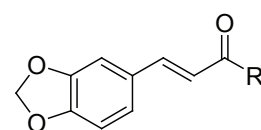
4 R$_1$=1-pyrrolidinyl
5 R$_1$=benzylaminyl
6 R$_1$=1-piperidinyl
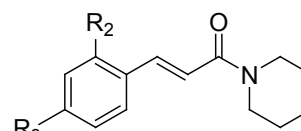
7 R$_2$=F; R$_3$=H
8 R$_2$=H; R$_3$=F
9 R$_2$=H; R$_3$=CHF$_2$O
10 R$_2$=H; R$_3$=CF$_3$O

forms which enter to the human bloodstream and finally infect the macrophages, where there is the change to the amastigote forms (Genaro, 1998). The derivative **3** was less active than compounds **1** and **2**, because it showed lesser antipromastigote activity, as indicated by the IC50 value of around 70 μM. All synthetic analogs showed lower antileishmanial activity than the natural compounds. Compound **5** showed growth inhibition of *Leishmania* forms with an IC50 value of around 25 μM. Compound **6** was the most selective compound for the amastigote forms, (SI ratio of 13.11) and showed low activity against promastigote forms. Compounds **4, 7, 8** and **9** were the least active compounds against all forms of Leishmania with IC50>100 μM. Compound **10** showed low antileishmanial activity with IC50>54 μM.

The results obtained for the compound **4** were not statistically different from those of the negative control (*p*>0.05) in the anti-promastigote experiment, according to the Student t-test. The rest of the results for the anti-promastigote and anti-amastigote assays were significantly different to those of the control group (*p*<0.05), according to the Student´s t-test.

Table 1. Anti-promastigote and anti-amastigote activity as well as cytotoxicity toward the J774A1 macrophages, and SI of the compounds.

Samples	Promastigote IC50 (μM)[a]	Cytotoxicity CC50 (μM)[a]	Intracellular Amastigote IC50a	SI
1	20±0.88*	101±0.95*	20.5±0.78*	4.93
2	15±0.25*	187±2.65*	14.5±1.05*	12.89
3	70±0.7*	182±1.98*	20.5±1.76*	8.88
4	> 200	178±4.0*	200±0.7*	0.89
5	25±2.5*	213±16.0*	20±4.0*	10.65
6	135±2.5*	354±11.15*	27±3.25*	13.11
7	> 200	205±16.0*	153±0.14*	1.34
8	> 200	325±43.30*	172±0.35*	1.89
9	100±1.65*	175±45*	112±2.96*	1.56
10	74±0.98*	150±24*	54.5±1.35*	2.75
Pentamidine Isethionate	2.2±0.15*	70±5*	1.5±0.2*	46.67

[a]Values represent the mean±SD of experiments performed in triplicate for anti-promastigote activity and evaluation of cytotoxicity, and mean±difference between the two experiments for anti-amastigote activity. SI = CC50 for J774A1 macrophages/IC50 for the intracellular amastigotes. *Significant difference from the negative control for the promastigotes and intracellular amastigotes, and from the viable cells for the macrophages (*p*<0.05).

Further experiments will be necessary to clarify the structure-activity relationships of the amides. However, it was possible to observe some tendencies:
1. The double bonds in the side chain having a (*E,E*) configuration in compound **2**, led to a slight

increasing in the antileishmanial activity;
2. The substituents of the compounds **7-10** with deactivating electron-withdrawing groups (*e.g.*, fluorine, difluoromethoxy, trifluoromethoxy) have a negative effect on the activity, which was shown by their lesser activity than compounds **1-3, 5** and **6**. This observation is in agreement with the results reported for the antileishmanial activity of imidothiocarbamates and imidoselenocarbamates described by Moreno et al. (2011).

The effects of the compounds were evaluated in relation to nitric oxide production, in order to determine whether anti-amastigote activity resulted from activation of this antileishmanial mechanism. None of the compounds promoted significant nitric oxide production, suggesting a direct and selective action of the compounds on the intracellular amastigotes. Moreover, all amides were less cytotoxic than pentamidine isothionate.

In summary, natural compounds **1** and **2** showed higher antileishmanial activity than the derivative and the synthetic analogs, with the compound **2** being the most active. Moreover, this compound was more selective for the amastigotes than macrophages and may act directly on the parasite, demonstrating a significant potential for the treatment of the cutaneous leishmaniasis (McConville & Handman, 2007). Compound **2** showed an interesting action and low cytotoxicity compared with other alkaloids with antileishmanial activity, especially pyrrolidine amides recognized for the growth inhibition of the intracellular amastigotes forms reported in the literature (Bodiwala et al., 2007; Mishra et al., 2009; Ghosal et al., 2012). These data could be important to further studies using natural compounds from *P. amalago* L. leaves in order to establish their mechanism of action. The present study showed the antileishmanial activity of the major compounds of *P. amalago* L., which might become useful for the development of a new medicine to the benefit of people afflicted by leishmaniasis.

Authors' contributions

VSC contributed in collecting plant sample and identification, confection of herbarium, running the laboratory work, analysis of the data and drafted the paper. EFCJ and ECTS contributed to the biological studies. AGC and JLM contributed in synthesizing the analogs. EFCJ, ECTS, AGC, JLM, IGD and MVCL contributed to critical reading of the manuscript. DAGC designed the study, supervised the laboratory work and contributed to

critical reading of the manuscript. All the authors have read the final manuscript and approved the submission.

References

Achenbach H, Grob J, Portecop J 1984. Ishwarol, the main sesquiterpene in *Piper amalago*. *Planta Med 50*: 528-529.

Achenbach H, Fietez W, Wörth J, Waibel R, Portecop J 1986. Constituents of tropical medicinal plants IXX. GC/MS-investigations of the constituents of *Piper amalago* - 30 new amides of the piperine-type. *Planta Med 52*: 12-18.

Alécio AC, Bolzani VS, Young MCM, Kato MJ, Furlan M 1998. Antifungal amide from leaves of *Piper hispidum. J Nat Prod 61*: 637-639.

Bero J, Hannaert V, Chataigné G, Hérent MF, Quetin-Leclercq J 2011. *In vitro* antitrypanosomal and antileishmanial activity of plants used in Benin in tradicional medicine and bio-guided fractionation of the most active extract. *J Ethnopharmacol 137*: 998-1002.

Bodiwala HS, Singh G, Singh R, Dey CS, Sharma SS, Bhutani KK, Singh IP 2007. Antileishmanial amides and lignans from *Piper cubeba* and *Piper retrofractum. J Nat Prod 61*: 418-421.

Brenzan MA, Santos AO, Nakamura CV, Filho BPD, Ueda-Nakamura T, Young MCM, Corrêa AG, Junior JA, Morgado-Días JA, Cortez DAG 2012. Effects of (-) mammea A/BB isolated from *Calophyllum brasiliense* leaves and derivatives on mitochondrial membrane of *Leishmania amazonensis. Phytomedicine 19*: 223-230.

Carrara VS, Serra LZ, Cardozo-Filho L, Cunha-Júnior EF, Torres-Santos EC, Cortez DAG 2012. HPLC analysis of supercritical carbon dioxide and compressed propane extracts from *Piper amalago* L. with antileishmanial activity. *Molecules 17*: 15-33.

Cabanillas BJ, Le Lamerm AC, Castillo D, Arevalo J, Rojas R, Odonne G, Bourdy G, Moukarzel B, Sauvain M, Fabre N 2010. Caffeic acid esters and lignans from *Piper sanguineispicum. J Nat Prod 73*: 1884-1890.

Castral TC, Matos AP, Monteiro JL, Araujo FM, Bondância TM, Pereira LGB, Fernandes JB, Vieira PC, Silva MFGF da, Corrêa AG 2011. Synthesis of a combinatorial library of amides and its evaluation against the fall armyworm, *Spodoptera frugiperda. J Agric Food Chem 59*: 4822-4827.

Da Silva DR, Nakamura CV, Dias-Filho BP, Ueda-Nakamura T, Cortez DAG 2009. *In vitro* antileishmanial activity of hydroethanolic extract, fractions, and compounds isolated from leaves of *Piper ovatum* Vahl against *Leishmania amazonensis. Acta Protozool 48*: 73-81.

Denizot F, Lang R 1986. Rapid colorimetric assay for cell growth and survival. Modifications to the tetrazolium dye procedure giving improved sensitivity and reliability. *J Immunol Methods 89*: 271-277.

Ding AH, Nathan CF, Stuher DJ 1988. Release of reactive nitrogen intermediates and reactive oxygen intermediantes from mouse peritoneal macrophages. Comparison of activating cytokines and evidence for independent production. *J Immunol 141*: 2407-2412.

Domínguez XA, Verde J, Sugar S, Trevino R 1986. Two amides from *Piper amalago. Phytochemistry 25*: 239-240.

Domínguez XA, Alcorn JB 1985. Screening of medicinal plants used by Huastec Mayans of Northeastern Mexico. *J Ethnophamacol 13*: 139-156.

Ferreira MGPR, Kayano AM, Silva-Jardim I, da Silva TO, Zuliani JP, Facundo VA, Calderon LA, de Almeida-e-Silva A, Ciancaglini P, Stabeli RG 2010. Antileishmanial activity of 3-(3,4,5-trimethoxyphenyl) propanoic acid purified from Amazonian *Piper tuberculatum* Jacq., Piperaceae, fruits. *Rev Bras Farmacogn 20*: 1003-1006.

Genaro O 1998. Leishmaniose Tegumentar Americana. In: D.P. Neves (Ed.), Parasitologia humana. Atheneu, São Paulo, p. 64-81.

Ghosal S, Deb A, Mishra P, Vishwakarma, R 2012. Leishmanicidal compounds from the fruits of *Piper longum. Planta Med 78*: 906-908.

Heckel E 1897. Les Plantes Médicinales et Toxiques de la Guyane Française. Macon, France: Protat Freres, 93 pp.

Hou T, Xu X 2001. Three dimensional quantitative structure-activity relationship analyses of a series of cinnamamides. *Chemom Intell Lab Syst 56*: 123-132.

Jacobs H, Seeram NP, Nair MG, Reynolds WF, McLean S 1999. Amides of *Piper amalago* var. *nigrinodum. J Indian Chem Soc 76*: 713-717.

Marques AM, Barreto ALS, Curvelo JAR, Romanos MTV, Soares RMA, Kaplan MAC 2011. Amtileishmanial activity of nerolidol-rich essential oil from *Piper claussenianum. Rev Bras Farmacogn 21*: 908-914.

McConville MJ, Handman E 2007. The molecular basis of *Leishmania pathogenesis. Int J Parasitol 37*: 1047-1051.

Mendez S, Traslavina R, Hinchman M, Huang L, Green P, Cynamon MH, Welch JT 2009. The antituberculosis drug pyrazinamide affects the course of cutaneous leishmaniasis *in vivo* and increases activation of macrophages and dentritic cells. *Antimicrob Ag Chemother 53*: 5114-5121.

Mishra BB, Kale RR, Singh RK, Tiwari VK 2009. Alkaloids: Future prospective to combat leishmaniasis. *Fitoterapia 80*: 81-90.

Moreno D, Plano D, Baquedano Y, Jiménez-Ruiz A, Palop JA, Sanmartin C 2011. Antileishmanial activity of imidothiocarbamates and imidoselenocarbamates. *Parasitol Res 108*: 233-239.

Naz T, Mosaddik A, Rahman MM, Muhammad I, Haque ME, Cho SK 2012. Antimicrobial, antileishmanial and cytotoxic compounds from *Piper chaba. Nat Prod Res 26*: 979-986.

Obando D, Widmer F, Wright LC, Sorrell TC, Jolliffe KA 2007. Synthesis, antifungal and antimicrobial activity of

alkylphospholipids. *Bioorg Med Chem 15*: 5158-5165.

Pagnocca FC, Victor SR, Bueno FC, Crisóstomo FR, Castral TC, Fernandes JB, Corrêa AG, Bueno OC, Bacci M, Hebling MJA, Vieira PC, Silva MFGF 2006. Synthetic amides toxic to the leaf-cutting ant *Atta sexdens rubro-pilosa* L. and its symbiotic fungus. *Agric Forest Entomol 8*: 17-23.

Patrick GL 1995. *An Introduction to Medicinal Chemistry.* Oxford Univ. Press: Oxford.

Regasini LO, Cotinguiba F, Passerini GD, Bolzani V da S, Cicarelli RMB, Kato MJ, Furlan M 2009. Trypanocidal activity of *Piper arboreum* and *Piper tuberculatum* (Piperaceae). *Rev Bras Farmacogn 19*: 199-203.

Ribeiro TS, Mendonca-Previato L, Freire-de-Lima L, Heise N, Previato JO, Lima MEF 2004. Toxic effects of natural piperine and its derivatives on epimastigotes and amastigotes of *Trypanosoma cruzi. Bioorg Med Chem Lett 14*: 3555-3558.

Roach TI, Kiderlen AF, Blackwell JM 1991. Role of inorganic nitrogen oxides and tumor necrosis factor alpha in killing *Leishmania donovani* amastigotes in gamma interferon-lipopolysaccharide-activated macrophages from Lshs and Lshr congenic mouse strains. *Infect Immun 59*: 3935-3944.

Sangwan PL, Koul JL, Reddy MV, Thota N, Khan IA, Kalia NP, Qazi GN 2008. Piperine analogs as potent *Staphylococcus aureus* NorA efflux pump inhibitors. *Bioorgan Med Chem 16*: 9847-9857.

Singh S, Sivakumar R 2004. Challenges and new discoveries in the treatment of leishmaniasis. *J Infect Chemother 1*0: 307-315.

Torres-Santos EC, Moreira DL, Kaplan MAC, Meirelles MN, Rossi-Bergaman B 1999. Selective effect of 2´,6´-dihydroxy-4´methoxychalcone isolated from *Piper aduncum* on *Leishmania amazonensis. Antimicrob Ag Chemother 43*: 1234-1241.

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