Parasitology

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Phloroglucinol derivatives from *Hypericum* species trigger mitochondrial dysfunction in *Leishmania amazonensis*

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Research Article

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Abstract

Bioactive molecules isolated from plants are promising sources for the development of new therapies against leishmaniasis. We investigated the leishmanicidal activity of cariphenone A (1), isouliginosin B (2) and uliginosin B (3) isolated from *Hypericum* species. Promastigotes and amastigotes of *Leishmania amazonensis* were incubated with compounds 1–3 at concentrations 1–100 μM for 48 h. The anti-promastigote effect of compounds was also tested in combinations. The cytotoxicity against macrophages and human erythrocytes were determined using the 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) method and hemolysis assay, respectively. The compounds 1–3 showed high leishmanicidal activity against promastigotes, IC_{50} values of 10.5, 17.5 and 11.3 μM, respectively. Synergistic interactions were found to the associations of compounds 1 and 2 [Σ fractional inhibitory concentration (FIC) = 0.41], and 2 and 3 (ΣFIC = 0.28) on promastigotes. All *Hypericum* compounds induced mitochondrial hyperpolarization and reactive oxygen species production in promastigotes. The compounds showed low cytotoxicity toward mammalian cells, high selectivity index and killed intracellular amastigotes probably mediated by oxidative stress. These results indicate that these compounds are promising candidates for the development of drugs against leishmaniasis.

Introduction

There is no effective vaccine to prevent human leishmaniasis and the drugs available to chemotherapy have several limitations. Thus, the development of new drugs is a challenge because of the variety of *Leishmania* species, different clinical forms of the disease and the profile of the immune response. Therefore, new therapeutic alternatives are urgently needed and, in this context, natural products are an important source of potential molecules.

Plants from the genus *Hypericum* (Hypericaceae) presented many biological actions, including antiparasitic (Cargnin *et al.* 2013), antifungal (Fenner *et al.* 2005) and antiviral activities (Fritz *et al.* 2007). Considering that some phloroglucinol derivatives, including acylphloroglucinols and benzophenones, have shown leishmanicidal activity (Bharate and Singh, 2011; Sidana *et al.* 2011; Socolsky *et al.* 2016) and that *Hypericum* species are a rich source of these compounds (Ccana-Ccapatinta *et al.* 2015), in a previous study, our group investigated and demonstrated that the lipophilic extracts of *Hypericum polyanthemum* Klotzsch ex Reichardt, *Hypericum carinatum* Griseb. and *Hypericum linoides* A. St.-Hil. containing phloroglucinols were able to kill infective forms of *Leishmania amazonensis* (Dagnino *et al.* 2015). The purpose of this study was to evaluate the anti-*Leishmania* action of two acylphloroglucinols and a benzophenone isolated from *Hypericum* species against promastigote and amastigote forms of *L. amazonensis*. Our data exhibit for the first time that isouliginosin B, uliginosin B and cariphenone A isolated from *Hypericum* species present potent activity against both promastigote and amastigote forms of *L. amazonensis*.

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Materials and methods

Plant material

Flowering aerial parts of *H. carinatum* Griseb were collected in the State of Rio Grande do Sul, in Southern Brazil, between October and December 2011. Flowering aerial parts of *H. andinum* Gleason were collected in the State of Cuzco, in Southern Peru, in May 2008. The plants were identified by Dr Sérgio Bordignon (UNILASSALE, Brazil) and voucher specimens were deposited in the UFRGS herbarium (ICN) (Bordignon and Ferraz 2309, and Ccana-Ccapatinta *et al.* 05 respectively). The collection of plants was authorized by IBAMA (no. 003/2008; Protocol 02000.001717/2008-60) and Dirección General Forestal y de Fauna Silvestre of the Republic of Peru (0147-2010-AG-DGFFS-DGEFFS).

Isolation procedures of Hypericum compounds

The air-dried and powdered plant materials were subjected to extraction with *n*-hexane over 24 h (plant-solvent ratio 1:5) until exhaustion. The *n*-hexane extracts were pooled and evaporated to dryness under reduced pressure and then treated with cold acetone to obtain extracts without fatty residues. The obtained extracts were submitted to chromatographic procedures, as described previously, to isolate the phloroglucinol derivatives 1–3 (Fig. 1). Cariphenone A (1) and uliginosin B (3) were isolated from *H. carinatum* as described by Bernardi *et al.* (2005). Isouliginosin B (2) was isolated from *H. andinum* as described by Ccana-Ccapatinta *et al.* (2014, 2015). The identity and purity of the isolated compounds were confirmed by ^{1}H and ^{13}C nuclear magnetic resonance spectroscopy.

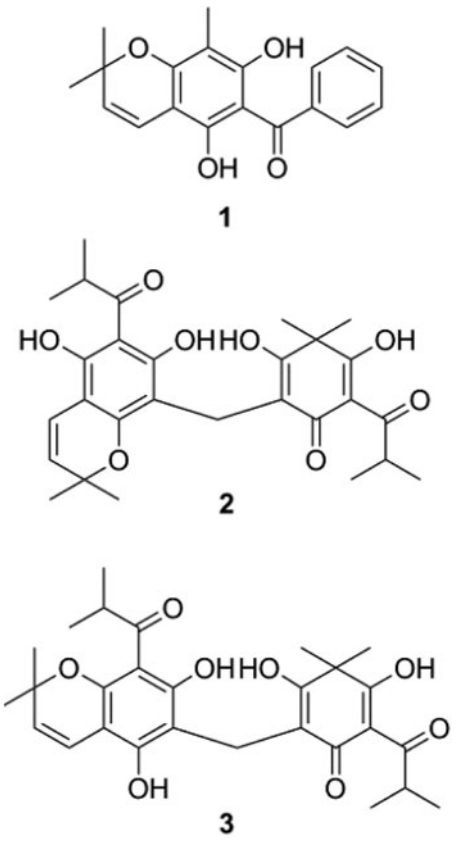

Fig. 1. Chemical structures of the phloroglucinol derivatives screened for anti-*L. amazonensis* activity.

L. (Leishmania) amazonensis culture

The MHOM/BR/73/M2269 strain of *Leishmania (Leishmania) amazonensis* was cultured as previously described elsewhere (Dagnino *et al.* 2015). All experimental procedures were performed in accordance with the guidelines of the National Institute of Health and the Brazilian Society for Science on Laboratory Animals, with the approval of the local Ethics Committee from Federal University of Health Sciences of Porto Alegre (process number 11-061).

Effects of isolated compounds of Hypericum on L. amazonensis viability in vitro

Promastigotes of *L. amazonensis* (3×10^6 on stationary phase) were plated in 96 well microplates and incubated with M199 medium with 10% fetal bovine serum (FBS) in the presence of the isolated compounds 1–3 at concentrations of 0 to 100 μM for 48 h. The activity of each compound was compared with control samples incubated with M199 medium (100% of viability) or with amphotericin B (Sigma, USA) at 1 μM as a standard antileishmanial drug (100% of mortality). Control cells were incubated with M199 medium containing less than 0.05% of polysorbate 80 (Dagnino *et al.* 2015). To determinate the kinetics of the leishmanicidal activity, the viability of parasites treated with compounds at 50 μM was evaluated at different time points (6, 12, 24 and 48 h) by counting the viable promastigotes using a hemocytometer (Antonello *et al.* 2017). Survival rate was calculated according to the formula: percentage of survival = (average number of viable parasites in treated group/average number of viable untreated parasites) × 100. The 50% inhibitory concentration (IC_{50}) value for each isolated compound was determined by nonlinear regression analysis using the GraphPad Software.

Field emission scanning electron microscopy

To evaluate parasite ultrastructural alterations by scanning electron microscopy (S.E.M.), *L. amazonensis* promastigotes were incubated with M199, M199 containing *Hypericum* compounds (IC_{50} value) or treated with amphotericin B at 0.13 μM (IC_{50}) for 6 h, washed with phosphate buffered saline (PBS) and fixed with 25% glutaraldehyde, distilled water in 0.2 M phosphate buffer, pH 7.4. After being washed three times in the same buffer, the parasites were adhered to glass slides previously coated with 0.1% aqueous poly-L-lysine for 30 min at room temperature. Subsequently, the slides were washed three times with 0.2 M phosphate buffer pH 7.4, post-fixed in a solution of 2% OsO_4 with 0.2 M phosphate buffer (1:1) for 45 min at room temperature. All samples were dehydrated in a graded series of acetone (30–100%), critical point dried using CO_2, mounted on metal stubs, and coated with gold for observation in a Field Emission Scanning Electron Microscope (Inspect F50, FEI).

Evaluation of cell membrane integrity

Leishmania amazonensis promastigote (3×10^6) were untreated or treated with 25 μM of cariphenone A, isouliginosin B and uliginosin B for 12 h. Hydrogen peroxide (2 mM) and amphotericin B (0.20 μM) were used as positive control. After that, the parasites were harvested and washed twice with PBS, resuspended and incubated with 100 μL of propidium iodide (PI–Sigma–Aldrich®) at 50 μg mL^{-1} for 15 min at 25 °C. Following incubation, each sample was completed with 400 μL of PBS. A total of 20 000 events were acquired and analysed on BD *FACScalibur flow cytometer and CellQuest Pro software*. PI fluorescence intensity was quantified as the percentage of the fluorescence compared

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with the untreated promastigotes. Results were obtained from three independent experiments performed in triplicate.

Leishmania *DNA fragmentation analysis*

Qualitative analysis of DNA fragmentation was performed by agarose gel electrophoresis of DNA extracted from 5×10^7 promastigotes of *L. amazonensis* (Sereno *et al.* 2001; Rotureau *et al.* 2005). *Leishmania amazonensis* promastigote was untreated or treated with 25 μM of cariphenone A, isouliginosin B, uliginosin B or amphotericin B at 0.2 μM for 24 h. Cell pellets were incubated in 200 μL lysis buffer [10 mM Tris, 5 mM EDTA, 0.5% SDS (sodium dodecyl sulfate), 200 mM NaCl, 100 μL mL^{-1} proteinase K pH 8] for 1 h at 65 °C. DNA was precipitated with 400 μL absolute ethanol. After a centrifugation step, supernatants were discarded and the pellets were allowed to dry for 30 min at 50 °C followed by resuspension in 20 μL recovery buffer (10 mM Tris, 0.1 mM EDTA pH 7.5). Following this, 10 μg of DNA from each sample was submitted to electrophoresis in the presence of migration buffer [40 mM Tris, 20 mM sodium acetate, 1 mM EDTA, pH 8.5 (Tris-borate-EDTA), 50% glycerol] on a 2% agarose gel in Tris-borate-EDTA buffer for 2.5 h at 120 V. DNA was then visualized under UV light after gel staining with ethidium bromide.

Determination of the mitochondrial membrane potential ($\Delta\Psi m$)

The mitochondrial membrane potential was quantified according to Ferlini and Scambia (2007) using rhodamine 123 (Rh 123, R8004, Sigma–Aldrich, St. Louis, MO, USA). Promastigotes of *L. amazonensis* (3×10^6 on log phase) were either treated or not with isolated compounds at 25 and 50 μM (about 2.5- and 5-fold the IC_{50} value found at 48 h of incubation) for 1, 6 or 12 h. Then the cells were washed with PBS and incubated with rhodamine 123 1 μg mL^{-1} for 10 min at 37°C. Hydrogen peroxide (H_2O_2 2 mM) and amphotericin B (0.2 μM) were used as positive control. A total of 20 000 events were acquired on BD *FACScalibur flow cytometer* (Becton–Dickinson, Rutherford, NJ, USA) and analysis was performed using *CellQuest Pro software* (Joseph Trotter, Scripps Research Institute, La Jolla, CA, USA). Alterations in Rh 123 fluorescence were quantified using an index of variation (IV) obtained from the equation IV = (Mt – Mc)/Mc, in which Mt is the median fluorescence for the treated parasites, and Mc is the median fluorescence for the untreated parasites. Negative IV values correspond to depolarization and positive values, hyperpolarization of the mitochondrial membrane.

Determination of intracellular reactive oxygen species (ROS) production

ROS production was evaluated using the reagent 2′,7′dichlorofluorescein diacetate (DCF-DA), that becomes fluorescent when oxidated by ROS (Wu and Yotnda, 2011). Promastigotes of *L. amazonensis* (3×10^6 on log phase) were either treated or not with *Hypericum* compounds at 25 and 50 μM for 1 or 6 h. Then the cells were centrifuged and incubated with 10 μM DCF-DA for 30 min at 37 °C, followed by two washes with PBS. H_2O_2 2 mM and amphotericin B (0.2 μM) were used as positive controls. A total of 20 000 events were acquired on BD *FACScalibur flow cytometer* (Becton–Dickinson, Rutherford, NJ, USA) and the fluorescence of the probe, indicating ROS levels were analysed using a *CellQuestPro softwar*e. Data are demonstrated in percentage relative to control.

Macrophage viability

Macrophage viability was measured using 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT). RAW 264.7 cell line macrophages were plated at 1×10^5 cells per well (in 96-well microplate) in DMEM plus 10% FBS and were incubated overnight at 37 °C and 5% CO_2. Then, the plates were washed with PBS and cultured for 44 h in DMEM or medium plus different isolated compounds (100–1000 μM). Subsequently, MTT was added and incubations were continued for 4 h after that. The purple formazan was solubilized and the absorbance at 570 nm determined using a Spectramax M2 microplate reader (Molecular Devices). The concentration of isolated compounds that causes 50% of macrophage cytotoxicity (CC_{50}) was determined by nonlinear regression analysis. The selectivity index (SI) of compounds was determined by the equation: CC_{50} against mammalian cells/IC_{50} against *L. amazonensis*.

Hemolytic assay

The hemolytic assay was performed according to Gauthier *et al.* (2009) with some modifications. Fresh human blood was obtained from healthy voluntary donors and the human erythrocytes were washed three times with PBS pH 7.0 and resuspended to obtain a 1% (v/v) erythrocytic suspension. The erythrocytes were incubated into 96-well microplates with *Hypericum* isolated compounds at concentrations ranging from 125 to 2000 μM. The microplate was incubated at 37 °C under agitation (90 rpm) for 60 min and the absorbance of the supernatant was measured at 540 nm using a Spectramax M2 microplate reader (Molecular Devices). SDS 0.01% (100% haemolysis) and PBS were used as positive and negative controls, respectively. Each experiment was performed in quadruplicate and repeated three times.

Leishmanicidal activity of macrophages treated with isolated compounds of Hypericum and nitric oxide determination

The anti-amastigote assay was performed as previously described (Antonello *et al.* 2017). RAW 264.7 cell line macrophages (1×10^5 cells per well in 96 well microplates) were cultivated for 12–16 h at 37 °C and atmosphere of 5% CO_2. Non-adherent cells were removed and the adherent cells were washed three-times with previously warmed DMEM medium. Then, the macrophages were infected with *L. amazonensis* promastigotes (5 parasites per cell) and 4 h later washed with PBS to remove not internalized parasites. Then, macrophages were incubated with medium, *Hypericum* compounds at 100 μM or with lipopolysaccharide (LPS, 10 ng mL^{-1}) plus interferon-gamma (IFN-γ, 1 ng mL^{-1}) in the presence or absence of aminoguanidine 300 μM, cariphenone A, isouliginosin B or uliginosin B for 48 h. Initially, we used *Hypericum* compounds at 100 μM since this concentration did not cause any cytotoxicity to macrophages or erythrocytes (data not shown). Following that, the supernatant was removed for nitric oxide (NO) determination and adherent cells incubated with 100 μL of SDS solution 0.01% (w/v) in M199 serum-free medium for 20 min. Then, the cells were supplemented with 100 μL of M199 30% FBS and incubated at 26 °C until parasite releasing to determine the number of promastigotes recovered once only viable amastigotes are capable to differentiate into motile forms. The leishmanicidal activity of macrophages was analysed by determining the number of viable parasites using a hemocytometer.

The concentration of NO in the supernatants of *L. amazonensis*-infected macrophages was quantified using Griess's reaction (Romao *et al.* 1999). Briefly, Griess reagent [NEED (0.1% w/v) plus sulphanilamide (1% w/v in H_3PO_4 5% v/v)] at room temperature (100 μL) was incubated with an equal volume of

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supernatants. The absorbance at 540 nm was measured and NO_2^- calculated from a standard curve of 1–200 μM $NaNO_2$.

In another experimental set, we evaluated the activity of *Hypericum* compounds against *intramacrophage amastigotes* using concentrations ranging from 1 to 20 μM. The IC_{50} value for each isolated compound was determined after 48 h incubation as described before for the anti-promastigote activity. The activity of each compound was compared with M199 control (100% of viability) and with amphotericin B at 0.2 μM as a standard antileishmanial drug (100% of mortality).

Assessment of the interaction between Hypericum compounds and amphotericin B on L. amazonensis

The leishmanicidal activity of combinations of *Hypericum* compounds was determined as described above. The interaction between compounds was evaluated by the fractional inhibitory concentration index (FIC) method and isobologram construction (Seifert and Croft, 2006; Wagenpfeil *et al.* 2006). For this, promastigotes (3×10^6 on stationary phase) were exposed to five different concentrations of isouliginosin B (1–20 μM) plus cariphenone A or uliginosin B, both at 1 or 5 μM. In another experimental set, parasites were exposed to uliginosin B (1–20 μM) plus cariphenone A (1 or 5 μM) or amphotericin B at concentrations equivalent to −5 or −10-fold the IC_{50} value, i.e. 0.026 or 0.013 μM. On the other hand, infected macrophages were exposed to 4 different concentrations of isouliginosin B (1–20 μM) plus cariphenone A or uliginosin B, both at 5 μM. In yet another experimental set, parasites were exposed to uliginosin B (1–20 μM) plus cariphenone A at 5 μM or amphotericin B at concentrations equivalent to −10-fold the IC_{50} value, i.e. 0.013.

Each point in the isobologram (a, b) indicates the IC_{50} value against promastigote obtained for the combination. The FIC of each isolated compounds was calculated according to Equation: FIC = (IC_{50} of combination)/(IC_{50} of isolated compound). The sum FICs (ΣFICs) were calculated as FIC of isouliginosin B + FIC of cariphenone A, as FIC of isouliginosin B + FIC of uliginosin B, FIC of cariphenone A + FIC of uliginosin B, and FIC of uliginosin B + FIC of amphotericin B. The mean ΣFIC was calculated for each combination and then compared with the reference values and reported as synergistic interactions when $\Sigma FIC \leqslant 0.5$, indifferent when $0.5 < \Sigma FIC < 4.0$ or antagonistic when $\Sigma FIC \geqslant 4$ (Seifert and Croft, 2006). The interaction of the isolated compounds was also analysed by constructing an isobologram using the IC_{50} values of compounds isolated, which were plotted on the *x*- and *y*-axes. The line joining these two points is the additivity line. They were subsequently plotted the concentrations of associated compounds required to cause 50% death of the parasites (IC_{50} combinations). Antagonistic interactions were defined as points above the additivity line and as synergistic when the points below the line (Wagenpfeil *et al.* 2006).

Effect of Hypericum compounds on the expression of CD80 and CD86 costimulatory molecules on macrophages

To better investigate the immunomodulatory activity of *Hypericum* compounds on macrophages we assessed the expression of surface costimulatory molecules CD80 and CD86 on *L. amazonensis*-infected macrophages. For this, BALB/c mice ($n = 3$) were euthanized under lidocaine (10 mg kg^{-1} i.p.) and thiopental (100 mg kg^{-1} i.p.) and the peritoneal cells were harvested by washing the peritoneal cavity with 3 mL of PBS. Cells (2×10^5 per well) were distributed in 96-well microplate and incubated overnight in RPMI medium at 37 °C in an atmosphere of 5% CO_2. Then, the cells were washed with PBS to remove non-adherent cells, and adherent macrophages were infected with *L. amazonensis* (5 parasites per cell). Four hours later, the cultures were washed to remove not internalized *Leishmania* and cells were incubated with RPMI medium or with cariphenone A, isouliginosin B or uliginosin B at concentration of 10 μM. After that, cells were stained with monoclonal antibodies conjugated with anti-mouse CD80-fluorescein isothiocyanate (FITC) (clone 16-10A1; BIOGEMS, USA) or anti-mouse CD86-phycoerythrin (PE) (clone GL1; BIOGEMS, USA), anti-CD14-FITC (clone Sa2-8; eBioscience). Thirty minutes after incubation, cells were resuspended in 0.4 mL of 1% BSA (bovine serum albumin) in PBS and analysed by flow cytometry. Fluorescent signals were collected in logarithmic mode (six-decade logarithmic amplifier). Macrophages were identified and gated according to *their forward* scatter (*FSC*) and side scatter (SSC) profiles related to the CD14 expression. The expression of CD80 and CD86 were evaluated in CD14 + macrophages, based on fluorescence-1 (FL1-FITC) *vs* fluorescence 2 (FL2-PE) dot plots. A minimal of 20 000 events of gated cells were acquired for analysis using FACScalibur flow cytometer (Becton-Dickson, Rutherford, NJ, USA). The analysis was performed using software FlowJo 7.6.3 (TreeStar, Ashland, Oregon, USA).

Ethical considerations

All of the experimental procedures with mice were performed in accordance with the guidelines of the National Institute of Health and the Brazilian Society for Science on Laboratory Animals with the approval of local Ethics Committee (CEUA UFCSPA number 505/17). The hemolytic assay using human blood erythrocytes obtained from healthy voluntary donors was approved by the Research Ethical Committee from the Federal University of Health Sciences of Porto Alegre (project under authorization CAAE 63282416.6.0000.5345).

Statistical analysis

The results were expressed as mean ± S.E. of the mean from the average of four replicates in each experiment. Comparisons of parameters between different experimental groups were performed by one-way ANOVA test followed by Bonferroni's post-test. Differences were considered statistically significant when $P < 0.05$ (*) and $P < 0.01$ (**). All statistical tests were performed using GraphPad Prism 6 (GraphPad Software, San Diego, CA, USA).

Results

Dimeric acylphloroglucinols and benzophenone derivatives (Fig. 1) isolated from *Hypericum* species killed the promastigote forms of *L. amazonensis* in a concentration and time-dependent manner (Fig. 2A and B). When parasites were incubated with cariphenone A, isouliginosin B and uliginosin B for 48 h, all tested concentrations caused significant mortality (almost 100% of viability inhibition at 100 μM) (Fig. 2A). Moreover, significant differences were observed in the viability of parasites treated at 50 μM in all time points evaluated, indicating that all compounds act early, reducing the viability already in the first 6 h of exposure (Fig. 2B). The IC_{50} values of cariphenone A, isouliginosin B and uliginosin B on promastigote forms of *L. amazonensis* were calculated to be 10.55, 17.48 and 11.27 μM, respectively (Table 1), while the reference antileishmanial drug, amphotericin B presented an IC_{50} 48 h = 0.13 μM (data not shown).

One strategy for the optimization of therapies, aiming for greater therapeutic efficacy, is the combination of two or more drugs. The isobologram analysis demonstrated that the association between different concentrations of isouliginosin B and uliginosin B at 1 μM or 5 μM (Fig. 3B) was the most promising

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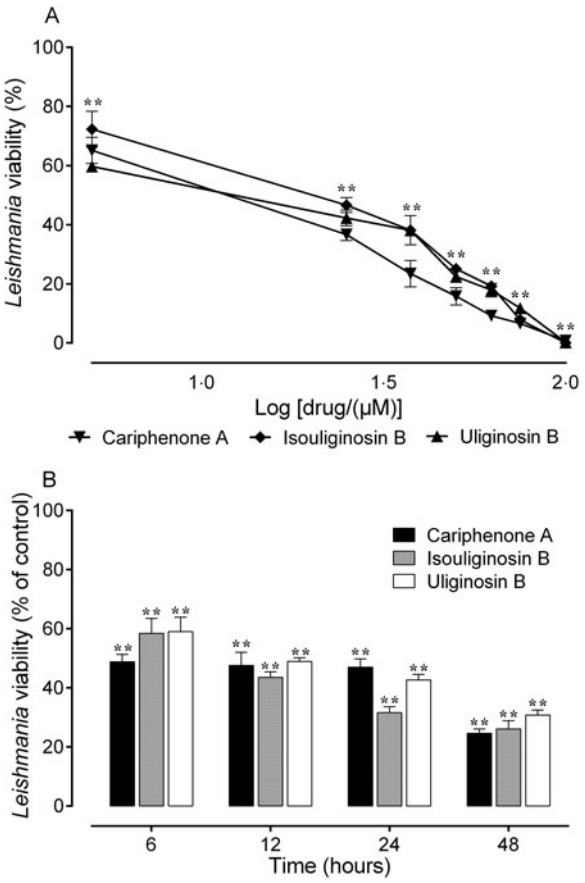

Fig. 2. Leishmanicidal activity of isolated compounds of *Hypericum* on promastigote forms of *L. amazonensis*. (A) Promastigote forms (3×10^6) in M199 medium were incubated with cariphenone A, isouliginosin B or uliginosin B at concentrations of 5–100 μM for 48 h and the viability determined using a hemocytometer, (B) Time course effect of *Hypericum* compounds at 50 μM on *L. amazonensis*. Data are expressed as means ± S.E.M. of four replicates and are representative of three independent experiments. $^{**}P < 0.01$ compared with control group (M199 = 100% viability).

association with an overall mean ΣFIC value of 0.284, when compared with the association between isouliginosin B and cariphenone A with mean ΣFIC value = 0.408 (Fig. 3A), indicating synergistic effects. However, the association between uliginosin B (1–20 μM) and cariphenone A at 1 μM or 5 μM (Fig. 3C) did not decrease the IC_{50} values calculated to the individual compounds, generating a mean ΣFIC value = 1.458 (Fig. 3C), suggesting an antagonistic effect. With respect the association between uliginosin B (1–20 μM) and amphotericin B at 0.013 μM or 0.026 μM, the isobologram analysis demonstrated an indifferent effect (ΣFIC value = 0.9545) (Fig. 3D).

As illustrated in Fig. 4, when promastigotes were treated with IC_{50} *Hypericum* compounds values, the parasites showed striking morphological alterations. While untreated parasites exhibited elongated body and an unique flagellum, promastigotes treated with all *Hypericum* compounds evidenced the presence of membrane holes, flagellum duplication, rounded shape and multi-septation of the cell body.

To investigate whether the leishmanicidal activity of *Hypericum* compounds involves the necrotic death, we evaluated the plasma membrane integrity of promastigotes treated with compounds. It was observed that compared with untreated control, PI fluorescence was significantly increased in parasites treated with 25 μM of cariphenone A, isouliginosin B or uliginosin, as well as in parasites treated with H_2O_2 and amphotericin B (Fig. 5A–F). In addition, these compounds also induced DNA fragmentation after 24 h of incubation (data not shown). In relation to the mitochondrial effect, we observed that promastigotes treated with cariphenone A, isouliginosin B and uliginosin B at 25 μM showed a marked increase in Rh 123 fluorescence after 12 h of incubation compared with the control group, indicating mitochondrial hyperpolarization [Fig. 6B–D). Moreover, according to the flowing formula IV = (Mt – Mc)/Mc], the Rh 123 fluorescence variation between treated and untreated parasites found positive IV values (cariphenone A: IV = 0.70, isouliginosin B: IV = 0.82 and uliginosin B, IV = 0.69) confirming ΔΨm hyperpolarization. Similar patterns of hyperpolarization were detected after 6 h of incubation with *Hypericum* compounds at 25 and 50 μM (Fig. 6E). However, no mitochondrial membrane potential alterations were triggered by the compounds at 25 and 50 μM after 1 h of incubation (data not shown). As expected H_2O_2 induced depolarization of ΔΨm (IV = −0,16) (Fig. 6A and E). Moreover, promastigotes treated with cariphenone A, isouliginosin B and uliginosin B at 25 and 50 μM for 6 h, but not 1 h, presented significant increase in ROS production (Fig. 6F). The incubation of *L. amazonensis* with H_2O_2 and amphotericin B induced significant increase in ROS at 1 h of treatment.

Table 1. Leishmanicidal activity, macrophage cytotoxicity and haemolytic effect of *Hypericum* compounds at 48 h

Hypericum isolated compounds	IC_{50}[a], μM (CI 95%[b]) promastigote	IC_{50}[a], μM (CI 95%[b]) amastigote	CC_{50}[c], μM (CI 95%[b])	SI[d] promastigote	SI[d] amastigote	RBC_{50}[e], μM
Cariphenone A	10.55 (6.76–16.45)	21.19 (12.63–35.56)	160.8 (110.1–235)	15.2	7.6	>1000
Isouliginosin B	17.48 (9.94–30.75)	1.09 (0.49–2.39)	183.7 (115.2–292.9)	10.5	168.5	>1000
+Cariphenone A 1 μM	2.67 (1.10–6.45)		–	–		–
+Cariphenone A 5 μM	1.66 (0.71–3.85)	1.04 (0.61–1.78)	–	–		–
+Uliginosin B 1 μM	0.57 (0.21–1.55)		–	–		–
+Uliginosin B 5 μM	0.05 (0.01–0.31)	1.18 (0.58–2.40)	–	–		–
Uliginosin B	11.27 (4.91–25.89)	5.25 (2.28–12.07)	234.7 (183.3–300.5)	20.8	44.7	>1000
+Cariphenone A 1 μM	11.47 (2.88–45.70)		–	–		–
+Cariphenone A 5 μM	14.33 (8.09–25.38)	2.15 (0.91–5.08)	–	–		–

[a]IC_{50}: concentration of the isolated compound that causes 50% of mortality *of L. amazonensis.*
[b]CI 95%: 95% confidence interval.
[c]CC_{50}: concentration of the isolated compound that causes 50% of macrophage cytotoxicity.
[d]SI: selectivity index, calculated as ratio of CC_{50} for macrophage/IC_{50} for *L. amazonensis.*
[e]RBC_{50}: concentration of the isolated compound that causes 50% of red blood cell haemolysis.

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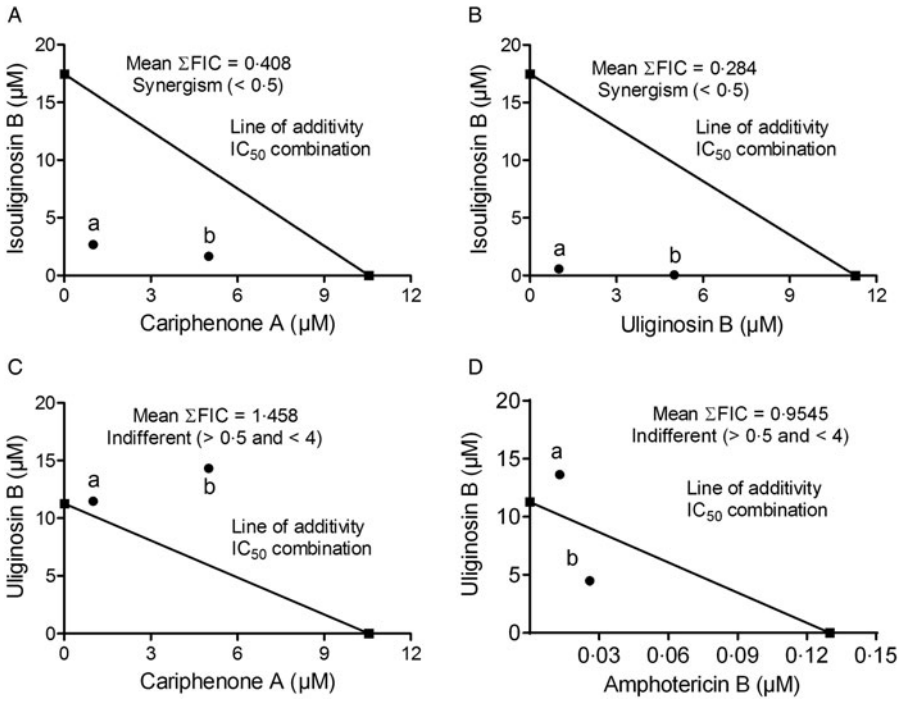

Fig. 3. Effects of combinations of isolated compounds of *Hypericum* on the viability of *L. amazonensis* promastigotes (A–D). Percentage of promastigote killing was determined by counting the number of viable parasites after 48 h of incubation as described in materials and methods. (A) Isobolograms for associations between isouliginosin B + cariphenone A, (B) isouliginosin B + uliginosin B, (C) uliginosin B + cariphenone A and (D) association between uliginosin B and amphotericin B. The points (a and b) refer to IC_{50} of compounds in combination (a and b). The line indicates synergy, additivity or antagonism when the points are located below, on or above the line, respectively. FIC, fractional inhibitory concentration. Experiments were performed in four replicates and are representative of two independent experiments.

The effect of *Hypericum* compounds on intracellular amastigotes was first evaluated using compounds at 100 μM. It was found that unlike the leishmanicidal activity of macrophages stimulated by LPS plus IFN-γ, cariphenone A, isouliginosin B and uliginosin B drastically reduced the viability of intracellular amastigotes by a mechanism independent of NO production (Fig. 7). Moreover, the anti-amastigote activity induced by *Hypericum* compounds was not associated with the increase on costimulatory CD80 (control macrophages: mean fluorescence intensity = 691.3 ± 4.43; *L. amazonensis* infected-macrophages: MFI = 689.7 ± 7.57; *L. amazonensis*-infected macrophages treated with cariphenone A: MFI = 705.33 ± 6.42; *L. amazonensis*-infected macrophages treated with isouliginosin B:

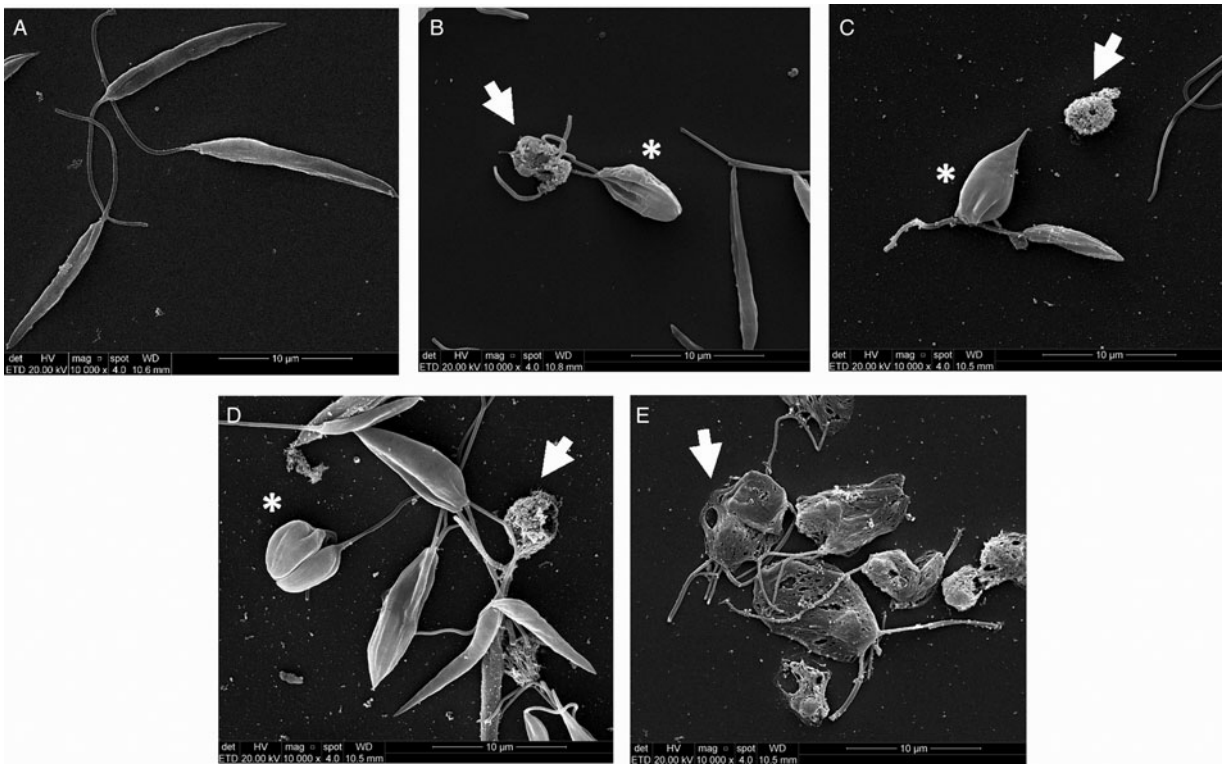

Fig. 4. Scanning electron micrographs of *L. amazonensis* promastigotes exposed to the isolated compounds of *Hypericum* for 6 h. (A) untreated parasites showing the typical elongated shape (parasite body and anterior flagella), (B) parasites treated with cariphenone A (IC_{50} = 10.55 μM), (C) isouliginosin B (IC_{50} = 17.48 μM), (D) uliginosin B (IC_{50} = 11.27 μM) or (E) amphotericin B (IC_{50} = 0.13 μM) showing morphological alterations such as membrane ruffles, membrane holes, flagellum loss, round-shaped forms and complete loss of typical promastigote morphology after 6 h of treatment. Note membrane disruption (arrows) and multi-septation of the cell body (asterisks). Bars: (A–E) 10 μm.

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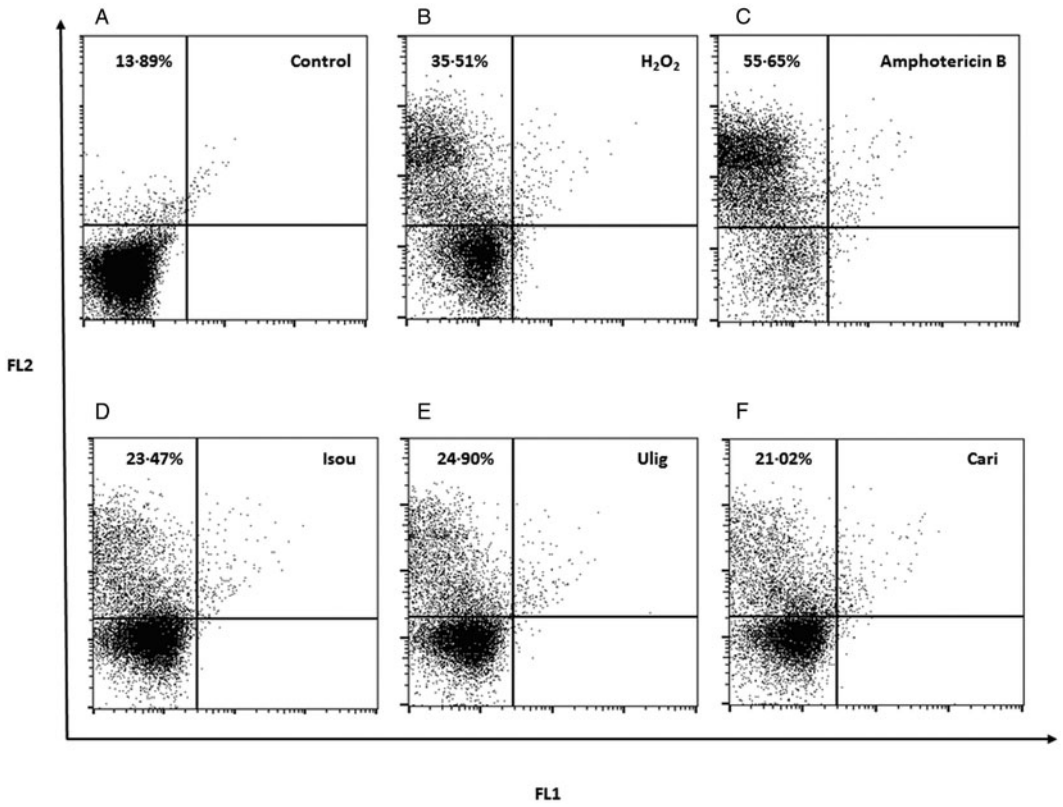

Fig. 5. Determination of membrane integrity of *L. amazonensis* using propidium iodide-PI. Acquisition dot plot of control promastigotes (A), promastigotes in the presence of H_2O_2 2 mM (B), in the presence of amphotericin B 0.20 μM (C), in the presence of isouliginosin B 25 μM (D), in the presence of uliginosin B 25 μM (E) and in the presence of cariphenone A 25 μM for 12 h (F). Upper left = PI stained cells. Experiments were performed in three replicates and are representative of two independent experiments.

MFI = 694.66 ± 5.50; *L. amazonensis*-infected macrophages treated with uliginosin B: MFI = 689.33 ± 1.15), and CD86 (control macrophages: MFI = 696.10 ± 8.71; *L. amazonensis* infected-macrophages: MFI = 690.33 ± 6.62; *L. amazonensis*-infected macrophages treated with cariphenone A: MFI = 693.93 ± 7.23; *L. amazonensis*-infected macrophages treated with isouliginosin B: MFI = 691.33 ± 6.02; *L. amazonensis*-infected macrophages treated with uliginosin B: MFI = 695.66 ± 0.57).

In another set of experiments, it was observed that, even at low concentrations, *Hypericum* compounds caused potent decrease in the intramacrophage amastigote viability (isouliginosin B: 100% amastigote mortality in concentrations ⩾5 μM, uliginosin B: 100% mortality in concentrations ⩾10 μM and cariphenone A: 46% mortality at 20 μM) (Fig. 8A). The IC_{50} values calculated for these compounds against amastigotes indicated better or similar activities compared with promastigote activity (Table 1).

Regarding the effect of *Hypericum* compounds and amphotericin B combinations on amastigotes, it was observed that the association between isouliginosin B plus cariphenone A, as well as isouliginosin B plus uliginosin B both at 5 μM was able to caused 100% of amastigote mortality (Fig. 8B). In addition the combination between uliginosin B plus cariphenone A at the same concentration of 5 μM caused 72% of amastigote mortality (Fig. 8B). Moreover, the association between uliginosin B at 5 μM and amphotericin B at 0.013 μM resulted in 100% amastigote mortality (Fig. 8B). All combinations were more potent when compared with the effect of individual drugs.

In relation to cytotoxic effect of *Hypericum* compounds, cariphenone A, isouliginosin B and uliginosin B showed low rate of haemolysis (Table 1). Moreover, they also present low cytotoxicity against macrophages and high selectivity toward parasites (Table 1).

Discussion

To the best of our knowledge, this is the first study reporting the leishmanicidal activity of phloroglucinol derivatives from *Hypericum* species against *L. amazonensis*. Our data showed that these compounds presented potent leishmanicidal activity against both promastigote and amastigote stages of parasites and synergistic interactions when tested in association (isouliginosin B plus cariphenone A and isouliginosin B plus uliginosin B) against promastigotes. Moreover, these *Hypericum* compounds induced ROS production and mitochondrial dysfunction in promastigotes of *L. amazonensis*, as well as presented low toxicity towards macrophages and red blood human cells, high selectivity index and killed the amastigote intracellular forms by a mechanism independent of nitric oxide.

In a previous study, our group has demonstrated that the lipophilic extracts of *H. polyanthemum*, *H. carinatum* and *H. linoides* containing phloroglucinols were able to kill infective forms of *L. amazonensis* (Dagnino *et al.* 2015). Regarding the antiprotozoal activity of phloroglucinol derivatives, it was demonstrated that uliginosin B, and also isouliginosin B decreased the viability of trophozoites of *Trichomonas vaginalis* with IC_{50} values of 121.96 and 59 μM, respectively (Cargnin *et al.* 2013; Menezes *et al.* 2017). In our study, the IC_{50} values obtained for uliginosin B, and isouliginosin B against *L. amazonensis* promastigotes were 11.27 and 17.48 μM, respectively. Moreover, uliginosin B showed antimicrobial activity against *Staphylococcus aureus* and *Staphylococcus epidermidis* with minimum inhibitory concentration (MIC) of 3.0 $\mu g\ mL^{-1}$, while isouliginosin B presented MIC value of 1.5 and 3.0 $\mu g\ mL^{-1}$ for *S. aureus* and *S. epidermidis*, respectively (França *et al.* 2009).

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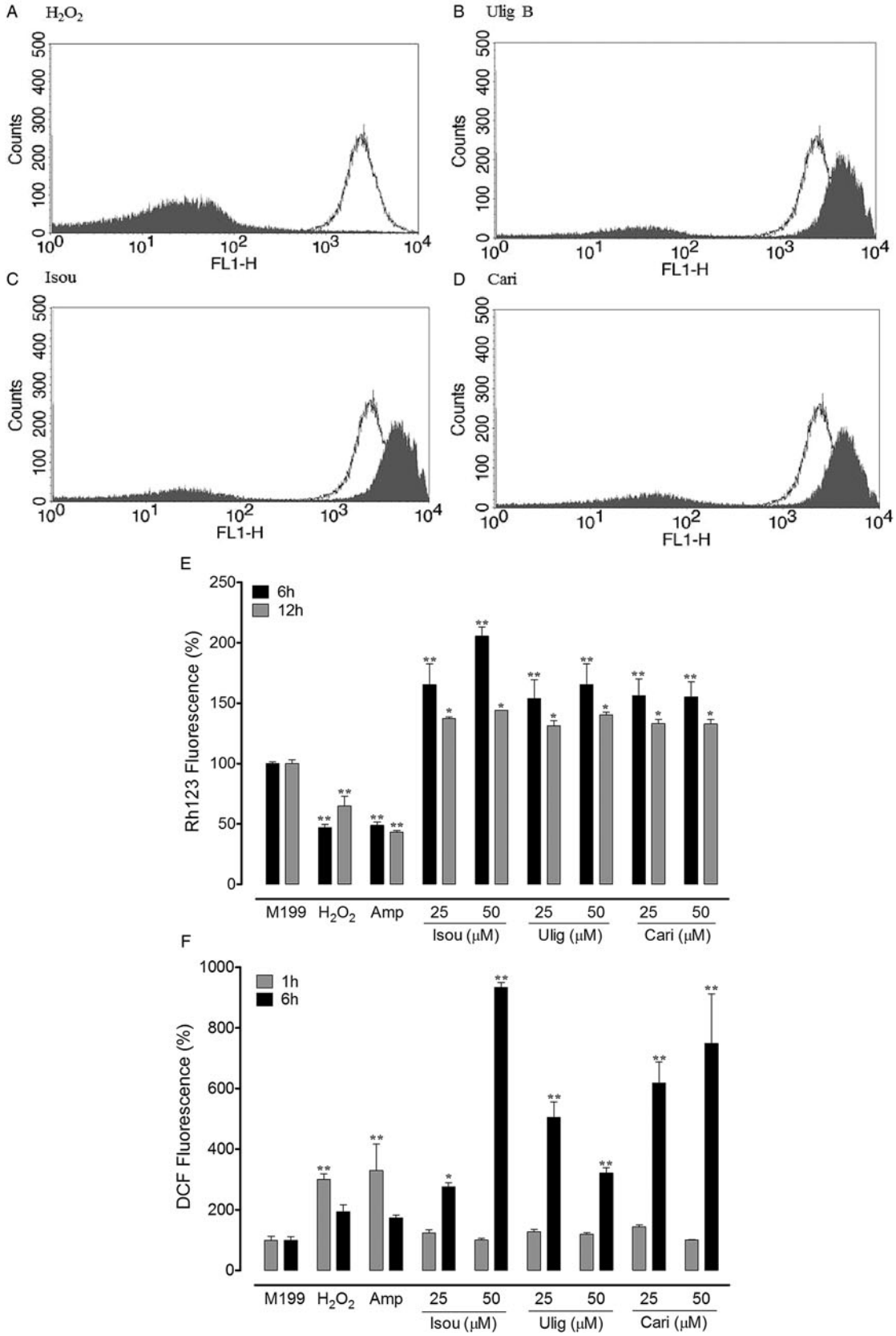

Fig. 6. Mitochondrial membrane potencial ($\Delta\Psi$m) and intracellular reactive oxygen species (ROS) in promastigotes of *L. amazonensis* exposed to *Hypericum* compounds. *L. amazonensis* promastigotes (1×10^6) were treated with H_2O_2 2 mM, amphotericin B 0.2 μM, cariphenone A, isouliginosin B or uliginosin B (25 and 50 μM for 1, 6 or 12 h) to $\Delta\Psi$m or by 1 or 6 h for ROS evaluation. $\Delta\Psi$m was determined using rhodamine 123 (A– E) and ROS using 2′,7′dichlorofluorescein diacetate (DCFH-DA) (F). (A–D) Demonstrative histograms of $\Delta\Psi$m of *L. amazonensis* untreated control (panel A white), with H_2O_2 (panel A filled), uliginosin B (panel B filled), isouliginosin B (panel C filled) or cariphnone A (panel D filled). (E) Graphic representation of Rh 123 mean fluorescence intensity in percentage relative to control of different treatment groups. (F) Graphic representation of DCF mean fluorescence intensity in percentage relative to control of different treatment groups. Data represent mean ± S.E.M. of four replicates and are representative of three independent experiments. *$P < 0.05$ *vs* M199; **$P < 0.01$ *vs* M199.

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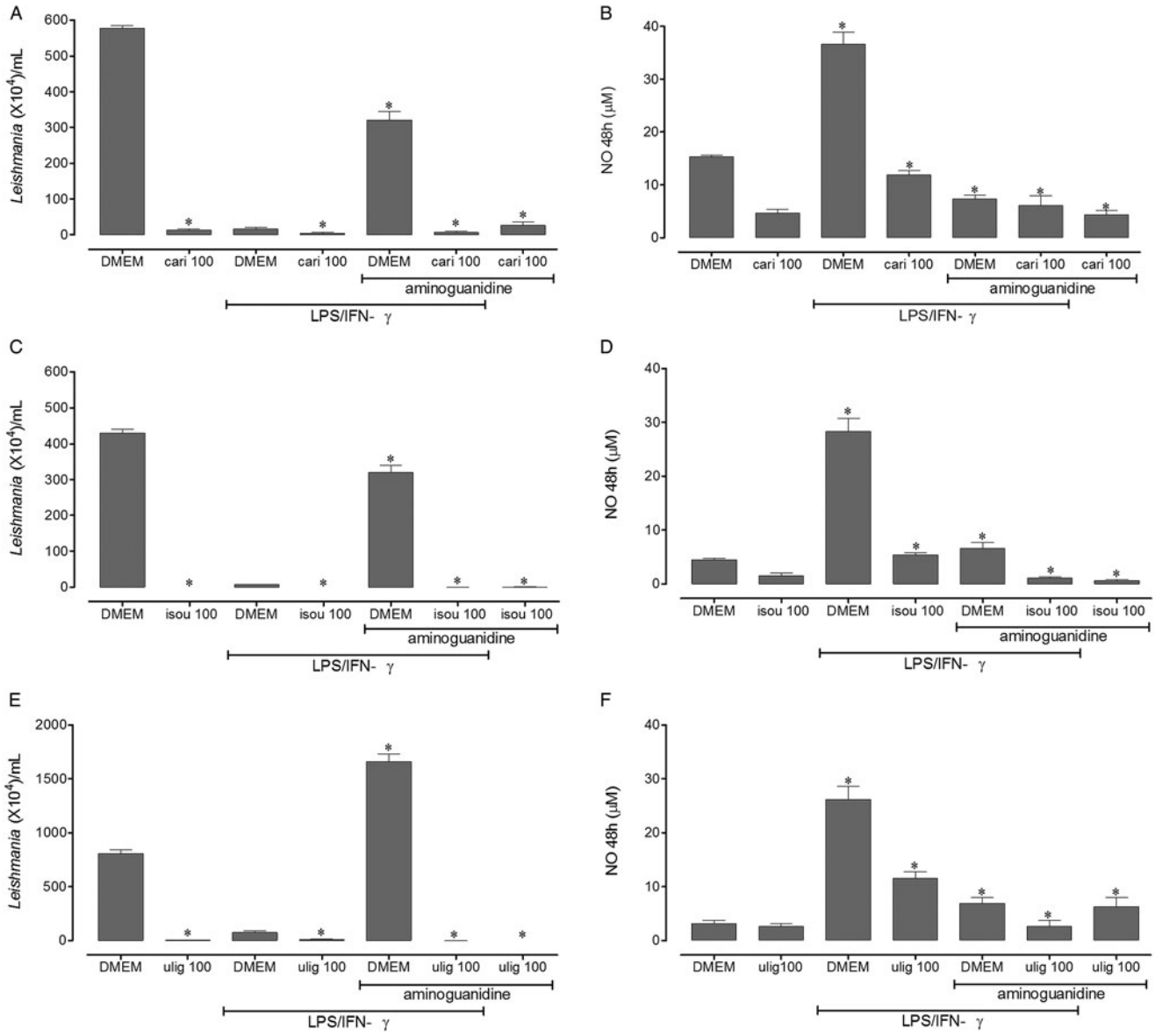

Fig. 7. Effects of isolated compounds of *Hypericum* on the viability of amastigotes of *L. amazonensis* and nitric oxide production *in vitro*. Macrophages RAW 264.7 strain were incubated at 1×10^5 cells/well and infected with *L. amazonensis* promastigotes. Subsequently, they were treated with cariphenone A (A and B), isouliginosin B (C and D) and uliginosin B (E and F) at 100 μM in the presence or absence of LPS (10 ng mL^{-1}) plus IFN-γ (1 ng mL^{-1}) or 300 μM of aminoguanidine. Data are expressed as means ± S.E.M. of four replicates and are representative of three independent experiments. $^*P < 0.05$, $^{**}P < 0.01$ compared with control group (macrophages incubated with DMEM).

The leishmanicidal activity of other phloroglucinols has been reported. Formylated phloroglucinols presented activity against promastigotes of *L. donovani* (IC_{50} = 88.24 to 244 μM) (Sidana *et al.* 2011), prenylated acylphloroglucinols lindbergins E – I against promastigotes of *L. braziliensis* (IC_{50} = 13.6–87.7 μM) and *L. amazonensis* (IC_{50} = 16.3–151.3 μM) (Socolsky *et al.* 2016) and phloroglucinol-terpene adducts against *L. donovani* promastigotes (IC_{50} = 0.62–7.68 μM) (Bharate and Singh, 2011). In addition, nemorosone and guttiferone A induced significant mortality of *L. tarentolae* promastigotes with IC_{50} values of 0.67 and 6.2 μM, respectively (Monzote *et al.* 2015). Moreover, prenylated benzophenone garcinielliptone FC from *Platonia insignis* showed an IC_{50} value of 42.8 μM against *L. amazonensis* promastigotes (Costa Junior *et al.* 2013). In agreement, it was shown that benzophenone-derived bisphosphonium salts with intermediate hydrophobicity inhibited *Leishmania* proliferation at low micromolar concentrations (Luque-Ortega *et al.* 2010). In fact, synthetic benzophenones containing more lipophilic radicals are studied in order to increase their anti-*Leishmania* activity (Maciel-Rezende *et al.* 2013).

Here, the S.E.M. observations revealed that the treatment with *Hypericum* compounds induces swelling and overall rounding, multi-septation of the cell body, with significant loss in body length and flagellum, corroborating evidences of the action of *Hypericum* extracts and isolated compounds from different plant species on *L. amazonensis* (Brenzan *et al.* 2012; Dagnino *et al.* 2015; da Silva *et al.* 2015). On the other hand, it was demonstrated that the treatment of promastigotes with *Hypericum* compounds led to significant increase in PI staining in parasites, indicating the loss of membrane integrity. It is known that necrosis process is characterized by swelling of organelles, volume augments of the cell and membrane rupture (Laster *et al.* 1988). Although we did not deeply evaluate apoptosis, the DNA analysis by agarose gel electrophoresis revealed DNA fragmentation in parasites treated with *Hypericum* compounds as well as with amphotericin B or H_2O_2, suggesting

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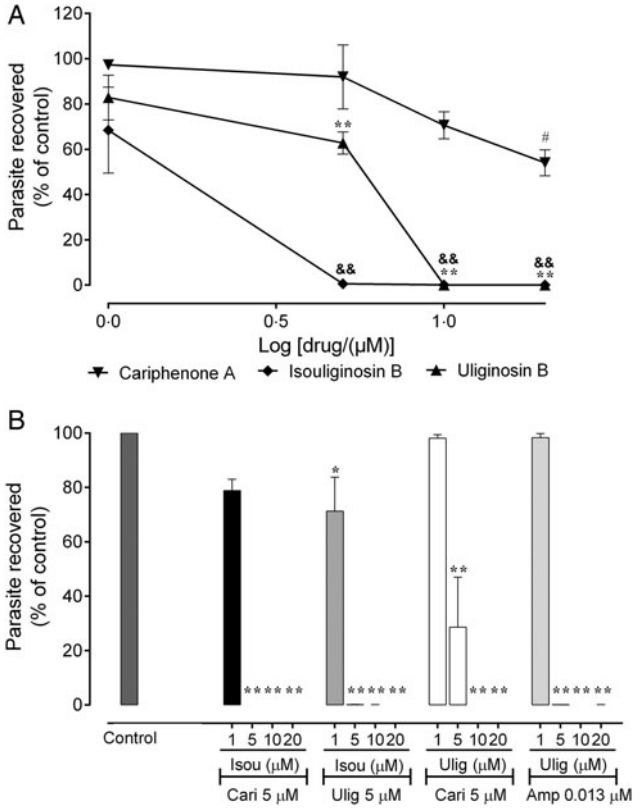

Fig. 8. Effects of individual or combinations of isolated *Hypericum* compounds on intracellular amastigote forms of *L. amazonensis*. Macrophages 1×10^5 cells per well were infected with *L. amazonensis* promastigotes (five parasites per cell) and incubated with DMEM medium, cariphenone A, isouliginosin B, uliginosin B (1 to 20 μM) for 48 h. The leishmanicidal activity of treated-macrophages was determined as described in materials and methods (A). Effects of associations between isouliginosin B + cariphenone A, isouliginosin B + uliginosin B, uliginosin B + cariphenone A, and uliginosin B + amphotericin B (B). Data are expressed as means ± S.E.M. of four replicates and are representative of two independent experiments. $^{\#}P < 0.05$ *vs* M199; $^{**}P < 0.01$ *vs* M199; $^{\&\&}P < 0.01$ *vs* M199.

induction of apoptosis. Further analysis should clarify this point.

Mitochondria play a pivotal role for parasite viability in *Leishmania*, since they are the essential source for ATP production during the mitochondrial respiratory chain (Van Hellemond and Tielens, 1997; Van Hellemond *et al.* 1997). Then, irreversible damage and dysfunction of this vital organelle caused by drugs will have disastrous consequences on parasites. In this context, it was demonstrated that the antileishmanial drug pentamidine accumulates in the *Leishmania* mitochondrion and that the resistance phenotype is accompanied by lack of drug mitochondrial accumulation and its exclusion from the parasites (Basselin *et al.* 2002; Mukherjee *et al.* 2006). On the other hand, amphotericin B causes a significant decrease in mitochondrial membrane potential (Lee *et al.* 2002). In our study, cariphenone A, isouliginosin B and uliginosin B induced hyperpolarization of the mitochondrial transmembrane potential of *L. amazonensis*. Hyperpolarization is considered to be less common than mitochondrial depolarization, arising mainly from dysfunctions in Fo/F1 ATPase, and it is considered a preapoptotic event (Sen *et al.* 2004). Indeed, both hyperpolarization and loss of $\Delta\Psi m$ can result in apoptotic death of promastigotes forms, demonstrating that an unstable mitochondrial membrane potential and redox transitions can have negative consequences for mitochondrial integrity and parasite survival.

Based on that, intracellular oxidative stress was investigated in *L. amazonensis* promastigotes exposed to the different *Hypericum* compounds. In *Leishmania* spp., the deviation of electrons from the mitochondrial complexes is the primary source of endogenous ROS (Gille and Nohl, 2001). In our study, we observed a significant increase in ROS production in promastigotes treated with cariphenone A, isouliginosin B and uliginosin B at the same time of mitochondrial membrane hyperpolarization was detected. Similarly to our results, *Leishmania donovani* promastigotes treated with anthraquinone derivative obtained from *Hypericum perforatum* called hypericin, also triggered ROS production (Singh *et al.* 2015).

Because *Leishmania* possesses a single mitochondrion, mitochondrial dysfunction and energetic metabolic plasticity in *Leishmania* are more limited than in other eukaryotic cells with multiple mitochondria, such as macrophages. In this respect, *Hypericum* compounds presented low cytotoxicity against macrophages. Thus, uliginosin B exhibited greater selectivity (SI) and best biological profile, with a SI = 20.8, followed by cariphenone A (SI = 15.2) and isouliginosin B (SI = 10.5). Literature data refer that the effectiveness of a compound is indicated by selectivity indices $\geqslant$10 (Nava-Zuazo *et al.* 2010). Moreover, SI > 20 is considered adequate for subsequent *in vivo* studies following the drug discovery pipeline (Don and Ioset, 2014).

In our study, the treatment with cariphenone A, isouliginosin B and uliginosin B drastically reduced the viability of intracellular amastigotes by a mechanism independent of NO, suggesting the involvement of other mechanisms including the interference with essential metabolic pathways of parasites or indirectly by the induction of other oxidants products such as oxygen reactive species that was not determined in this study. For example, de Almeida *et al.* (2015) showed that the synthetic benzophenone 2-hydroxy-4-O-(3,3-dimethyl)-allylbenzophenone presented antiamastigote activity (IC_{50} *L. amazonensis* = 74.4 μM) and caused a potent inhibitory effect on trypanosomatids cysteine proteases. On the other hand, hypericin isolated from *H. perforatum* inhibited the spermidine synthase in *L. donovani* promastigotes, leading to spermidine starvation and parasite death (Singh *et al.* 2015).

Our data exhibit for the first time that benzophenone cariphenone A and acylphloroglucinols isouliginosin B and uliginosin B isolated from *Hypericum* species (alone or in association) present potent activity against both promastigote (infective forms) and amastigote forms (intracellular stage) of *L. amazonensis* by a mechanism dependent on ROS generation and mitochondrial dysfunction. These results indicate that cariphenone A, isouliginosin B and uliginosin B are promising candidates for the development of drugs against *L. amazonensis*.

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Conflict of interest. The authors declare that this article content has no conflict of interest.

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