Genotoxicity and antileishmanial activity evaluation of Physalis angulata concentrated ethanolic extract

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Abstract

Antileishmanial in vitro tests, as well as Ames and micronucleus assays were performed with a concentrated ethanolic extract of Physalis angulata (EEPA). Results: EEPA did not present mutagenic effect in Salmonella typhimurium strains at concentration reaching 3000 g/plate and did not induce mutagenic effects after two oral administrations with a 24 h interval at a dose level of 2000 mg/kg. EEPA presented antileishmanial activity and presented an IC50 value of 5.35 ± 2.50 µg/mL and 4.50 ± 1.17 µg/mL against Leishmania amazonensis and Leishmania braziliensis promastigotes, respectively. In the cytotoxicity test against macrophages, the EEPA had a LC50 of 6.14 ± 0.59 µg/mL. Importantly, the IC50 against L. amazonensis intracellular amastigotes was 1.23 ± 0.11 µg/mL. Conclusion: EEPA extract is non-mutagenic and presented a promising pharmacological effect against Leishmania parasites.

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1. Introduction

The leishmaniases comprise a broad spectrum of infectious diseases caused by parasites belonging to the Leishmania genus and transmitted by the bite of the female sandfly vector. The illness can manifest as visceral leishmaniasis (VL), lethal in 10% of the cases, and cutaneous leishmaniasis (CL), responsible for disfiguring scars (Goto and Lindoso, 2010; Drumond and Costa, 2011; Costa, 2005). Different clinical forms of CL are observed, which can vary depending on the parasite species involved in infection, as well as the genetic background and immunological status of the host. These clinical manifestations include the mild cutaneous form (localized cutaneous leishmaniasis), multiple non-ulcerative nodules (diffuse cutaneous leishmaniasis), multiple ulcerative lesions (disseminated cutaneous leishmaniasis) and the disfiguring mucosal form (muco-cutaneous leishmaniasis) (Kaye and Scott, 2011; Reithinger et al., 2007; Murray et al., 2005). Currently, CL is present in 88 countries in tropical and subtropical regions, affecting 12 million people, with significant social burden due to its deformities and disfiguring scars (WHO, 2010).
In Brazil, the main species that cause CL are L. amazonensis and L. braziliensis, which are responsible for all clinical forms of the disease (Costa, 2005).

Pentavalent antimonials are the first-line treatment of CL. The systemic use of antimonials is associated to several side effects such as hepatotoxicity and cardiotoxicity (Oliveira et al., 2011). Moreover, the use of these medicines is related to therapeutic failure caused mainly by the low drug-sensitivity of some Leishmania species and low compliance of patients to the treatment. Other second-line drugs, such as amphotericin B and pentamidine are also toxic and present additional complications as hospitalization is required for use. Therefore, development of new CL treatment, with higher efficacy and improved patient compliance is pivotal. Local treatment of cutaneous lesions would facilitate CL treatment in endemic areas, and would improve the patient treatment conformity.

New treatment options are constantly developed from natural products, exploiting the great biodiversity and numerous available biological activities (Newman and Cragg, 2012). In this context Physalis angulata L., Solanaceae, an annual herb distributed throughout the tropical and subtropical regions of the world is a promising source of bioactive natural substances. A previous phytochemical investigation of P. angulata revealed the presence of flavonoids, a complex seco steroids known as physalins and with anolides, a group of naturally occurring oxygenated ergostane type steroids (Ismail and Alam, 2001; Soares et al., 2006; Damu et al., 2007). Previous works from our group demonstrated that physalin compounds obtained from P. angulata presented antileishmanial activity against different Leishmania species (Guimarães et al., 2010). In addition, we showed that physalin F reduced lesion size, tissue damage and parasite burden in infected BALB/c mice after topical treatment (Guimarães et al., 2009). Based on our previous results and the need to develop new CL treatment strategies, a concentrated ethanolic extract from P. angulata (EEPA) was chosen for the drug development process mainly because of economic reasons: the isolation procedure of the active molecule is costly, time consuming and gives a small fraction of material. In addition, a standardized extract once it is proven to be safe and effective can be a suitable choice for a neglected disease medicine. The aim of this work was to evaluate the genotoxic potential and antileishmanial activity from the EEPA.

2. Materials and methods

2.1. Plant material

P. angulata specimens were collected and identified during the drier season (from June until November) in Belém, Pará State, Brazil (Herbarium, Neurochemistry Laboratory, Department of Physiology, University Federal of Pará, Voucher number 15). The chemical profile of the extract was controlled by HPLC-UV on each extraction process.

2.2. Extraction of P. angulata stems

Stems of P. angulata (1 kg) were dried and ground. The powder material was extracted with ethanol at 50–60 °C during 6 h. The extract was concentrated under reduced pressure, yielding 100 g (10%) of crude ethanolic extract and maintained in a desiccator until weight stabilized.

2.2.1. HPLC-UV analysis of ethanolic extract of P. angulata (EEPA)

HPLC-UV data were obtained using the Agilent HP 1100 series system consisting of an auto-sampler, high-pressure mixing pump and UV detector (Agilent Technologies, Santa Clara, CA, USA). The HPLC conditions were as follows: an Hicharlishopher C-18 column (250 mm × 4 mm i.d., Merk); solvent system: A, MeCN and B, H2O containing 0.05% trifluoroacetic acid; gradient: 3–3% of A in 0–6 min, 3–10% of A in 3–6 min, 10–10% of A in 6–9 min, 10–18% in 9–12 min, 18–18% in 12–15 min, 18 to 21% in 15–18 min, 21–21% in 18–21 min, 21–35% in 21–45 min, 35–35% in 45–55 min, 35–80% in 55–70 min, 80–80% in 70–55 min flow rate: 1 mL min−1; injection volume: 10 μL; and sample concentration: 10 mg/mL in Methanol. The UV absorbance was measured at 225 nm.

2.3. Ames test

The Ames test was carried out by TECAM Tecnologia Ambiental Ltda (São Paulo, Brazil) under the Organization for Economic Co-operation and Development (OECD) Guideline for Chemical Test Number 471 (1997a). Five Salmonella typhimurium strains were purchased from Molecular Toxicology Inc. (Annapolis, MD, USA): TA98, TA100, TA102, TA1535 and TA1537. The assay was performed with and without metabolic activation using the plate incorporation technique. The microsomal fractions (S9 fraction) prepared from Sprague Dawley male rat livers treated with the inducing agent (Aroclor 1254; Molecular Toxicology Inc.) were used in the test. The mixture S9 was freshly prepared before each test according to Maron and Ames (1983). S9 mix contained 5% (v/v) of a S9 fraction. The EEPA was dissolved in dimethyl sulfoxide (DMSO, Sigma Chemical Co., St. Louis, MO, USA) and a preliminary toxicity study was conducted (8, 40, 200, 1000 and 5000 μg/plate) in order to define non-toxic concentrations (390, 650, 1080, 1800 and 3000 μg/plate).

Cytotoxicity was characterized by inhibition of the background bacterial lawn or reduction in the number of colonies. 0.1 mL of the test substance and 0.1 mL of bacterial culture grown overnight was added to tubes containing 3.0 mL of top agar (0.6% Bacto agar, 0.5% NaCl solution and 0.5 mM histidine/biotin). After adding the test substance and the bacteria to verify the influence of metabolic activation, 0.5 mL of S9 mix was added to the top agar. Following solification, the plates were incubated for 72 h at 37 ± 1 °C. Triplicate plates were maintained for each concentration of test substance (with and without S9 mix) and negative vehicle. Positive controls were performed in duplicate.

The standard mutagens used as positive controls in the experiment without S9 mix were: sodium azide for TA100 and TA1535 (5.0 μg/plate), mitomycin C for TA102 (0.5 μg/plate), 2-nitrofluorene for TA98 (2.0 μg/plate) and ICR191-acridine for TA1537 (1.0 μg/plate). In the presence of metabolic activation, 2-aminoanthracene was used for all strains (2.5 μg/plate). The results were expressed as the number of revertant colonies per plate (mean ± SD) and the mutagenic index (MI), which...
corresponds to the ratio between the number of revertant colonies in the test plates and the number of revertant colonies on negative control plates. The colony number of spontaneous revertants for each strain of the negative control was compared to historical control data described in the literature (Maron and Ames, 1983).

2.4. Micronucleus test

The micronucleus test was carried out by TECAM under OECD Guideline for Testing of Chemicals Test 474 (1997b). One group of six Swiss male mice was treated by oral administration (gavage) at a dose selected after a tolerability test: 2000 mg/kg body weight (bw). In the tolerability test, the dosages were established using two treatments (0 and 24 h). To establish the maximum tolerated dose (MTD), fixed doses were selected according to previously published methods (Mackay and Elliott, 1992): 8, 50, 320 and 2000 mg/kg bw. A group of six mice (three male and three female) were treated with corn oil as the vehicle. Since no evidence of toxicity or mortality was observed, the study was conducted using a single dose. The 2000 mg/kg bw dose was selected for the mutagenicity test as the maximum dose used for animal identification. Slides were prepared on glass slides. Slides were air-dried, fixed, and suspended in fetal bovine serum, bone marrow smears with fetal calf serum. After centrifugation at 2000 rpm (5 min) each animal were dissected and bone marrow was washed to 24 h after the second dose administration. Femurs from animals were treated at 0 and 24 h and sampled from 18 to 24 h after the second dose administration. Femurs from each animal were dissected and bone marrow was washed with fetal calf serum. At centrifugation at 2000 rpm (5 min) and suspended in fetal bovine serum, bone marrow smears were prepared on glass slides. Slides were stained using Wright-Giemsa and coded with the same numbers used for animal identification. Slides were evaluated blindly, using an optical microscope. The polychromatic erythrocyte (PCE) and normochromatic erythrocyte (NCE) number and ratio were established for each animal by scoring a total of 1500 erythrocytes (PCE + NCE). Bone marrow preparations were evaluated for the presence of micronuclei by counting 3000 PCE for each animal.

2.5. Pharmacological studies

2.5.1. Evaluation of EEPA activity against Leishmania promastigotes

L. amazonensis (MHOM/BR88/BA-125) and L. braziliensis (MHOM/BR88/BA-3456) were cultured in Schneider's insect medium (Sigma Aldrich, St. Louis, USA) supplemented with 10% fetal bovine serum (Gibco Laboratories, Gaithersburg, MD) and 50 μg/mL gentamycin (Hipolabor, Belo Horizonte, Brazil) at 24 °C. Parasites in the stationary growth phase were plated on a 96-well plate at a density of 2 × 10^5 parasites/mL in 200 μL of Schneider's insect medium, either in absence or presence of EEPA, at concentrations ranging from 1.2 to 100 μg/mL. Amphotericin B was used as the positive control. The parasite number was evaluated using a Neubauer chamber after 72 h and the IC50 concentration was calculated based on the percent inhibition of parasite growth related to controls. The IC50 was accessed through concentration logarithm values followed by nonlinear regression curve fit.

2.5.2. Mice

Male or female, 4-8-week-old, CBA mice were obtained from the Animal Facilities of the Gonçalo Moniz Research Center-FIOCRUZ (Oswaldo Cruz Foundation, Brazil). Animals were housed in temperature-controlled rooms (22–25 °C) under a 12:12 h light–dark cycle, with rodent diet and water ad libitum. Animals were handled according to the NIH guidelines for animal experimentation. Mice were euthanized in a CO2 chamber immediately before the collection of peritoneal macrophages. All procedures described here had prior approval from the local animal Ethics Committee.

2.5.3. Cytotoxicity assays

The lethal concentration for 50% of cells (LC50) was determined in peritoneal macrophages cultures from CBA mice by Alamar Blue metabolism (Invitrogen, Carlsbad, CA) according to the manufacture's recommendations. Macrophages were collected from the mice four days after injection of 3% thiglycolate medium (Sigma Aldrich). The cells were plated in 96-well plates (5 × 10^4 cells/well in 200 μL) either in presence or absence of EEPA (1.2–100 μg/mL) for 48 h and incubated at 37 °C, 5% CO2. Alamar Blue (20 μL/well) was added and cells were incubated under the same conditions for 24 h. Colorimetric readings were performed at 570 and 600 nm.

2.5.4. Evaluation of EEPA activity against L. amazonensis amastigotes

Peritoneal macrophages were collected from the susceptible CBA mice four days after injection of 3% thiglycolate medium. The cultures were performed as described previously (Gomes et al., 2003). Briefly, 2 × 10^5 cells/mL were plated in 24-well plates containing 13 mm-diameter glass coverslips. Stationary growth phase promastigotes of L. amazonensis were added at ratio of 10:1. Cells were incubated at 37 °C, 5% CO2 for 6 h. Non-internalized parasites were removed by washing with saline solution. Cells were incubated under the same conditions for 24 h without treatment to allow differentiation into amastigotes. Next, macrophages-containing amastigotes were treated with different concentrations of EEPA (1.2–100 μg/mL) during 24 and 48 h. After the treatment, the macrophages were fixed with ethanol and stained with conventional hematoxylin and eosin (H&E). The percentage of infected cells, as well as the number of amastigotes, was determined by counting 100 cells per glass coverslip. IC50 were calculated based on the inhibition of amastigotes growth related to the non-treated control after 48 h of incubation, through a nonlinear regression curve fit. Amphotericin B was used as the positive control. The selective index (SI) was defined as the ratio between LC50 and amastigote IC50 (Bézivin et al., 2003).

2.6. Statistical analysis

The data (revertants/plate) obtained from the Ames test were assessed by analysis of variance (ANOVA), followed by linear regression to evaluate dose–response effects. For the strains TA98, TA100 and TA102, the result was considered positive when the average number of revertant colonies on the
plates-test was more than or equal to double than that observed in the negative control plates (MI ≥ 2). For strains TA1535 and TA1537, MI greater than three (MI ≥ 3) were considered. Positive results were confirmed when analysis of variance was significant (pANOVA < 0.05) and the tested concentrations indicated a clear dose-response relationship.

For the micronucleus assay, following microscopic analysis, slides were decoded and the total number of cells from each group was compared using the chi-square test modified according to Pereira (1991). The negative and positive controls were compared to establish test validity (p < 0.001). Additionally, the negative control data had to be comparable to historical control data. Finally, control for slide quality was confirmed when a clear distinction between PCE and NCE was present.

For pharmacological studies, analysis of variance (ANOVA) was used to determine the mean differences between groups, followed by Bonferroni’s multiple comparison test. The statistical analysis was performed using Graph Pad Prism version 5.01 (Graph Pad Software, San Diego, CA). Results were statistically significant when p < 0.05.

3. Results and discussion

Traditional use of physalins and extracts from *P. angulata* plant have been reported for a broad variety of diseases, such as arthritis (Brustolim et al., 2010), malaria (Sá et al., 2011), inflammation (Vieira et al., 2005; Bastos et al., 2008) and tumor metastasis (Magalhães et al., 2006; Hseu et al., 2011). In view of its promising applications in oral and topical formulation, a genotoxic risk assessment study with a standardized chemical profile was previously performed in vivo using a human lymphocyte model, and was reported to have presented genotoxic effects (Alves dos Santos et al., 2008). The results from the Ames tests with *S. typhimurium* incubation with the TA98; TA100; TA102; TA1535 and TA1537 strains, with and without metabolic activation, and expressed as the number of revertant colonies per plate (mean ± SD) and the mutagenic index (MI), are shown in Table 1. There was no significant increase in the number of revertant colonies in plates tested after treatment with EEPA, in the presence or absence of metabolic activation, for any of the concentrations tested. A dose response effect (increasing number of revertant colonies with increasing concentration of substance tested) was not observed under the criteria considered as biological relevance. The mutagenic index was inferior to 2 for strains in presence and absence of metabolic activation.

The analysis of variance (pANOVA < 0.05) did not show statistic significance for the strains tested in the presence or absence of metabolic activation, except for strains TA98 (0.001) TA102 (0.022) and TA1537 (0.003) in the absence of metabolic activation and strains TA98 (0.029), TA1535 (0.003) and TA1537 (0.001) in the presence of metabolic activation. For strains TA98 (~ S9), TA1535 (~ S9) and TA102 (~ S9) tests, an oscillation in the number of revertant colonies at different concentrations tested was observed. In the tests with strains TA98 (~ S9) and TA1537 (~ S9) toxic effects caused decrease tendency in the number of revertents with the increase of concentrations tested.

In the micronucleus test, the positive control group demonstrated a significant increase in the micronucleus number ($\chi^2 = 205.6; p < 0.001$) when compared to the negative control group. The difference between the number of micronuclei in the EEPA-treated group and the concurrent negative control was not statistically significant ($\chi^2 = 0.40; p = 0.532$). The cellular analysis showed an approximately 1:1 ratio, indicating that there was no significant toxic effect of the test substance in the bone marrow of treated animals (Table 2).

The antileishmanial activity of EEPA was first evaluated against promastigotes from two species of *Leishmania* parasites. The IC50 value of the plant extract was $5.35 \pm 2.50 \mu g/mL$ against *L. amazonensis* and $4.50 \pm 1.17 \mu g/mL$ against *L.
Table 1 – Mutagenic activity (mean number of revertant/plate ± SD) of bacterial strains TA98, TA100, TA102, TA1535 and TA1537 exposed to EEPA, at various concentrations, with (+) or without (−) metabolic activation.

<table>
<thead>
<tr>
<th>Strains</th>
<th>S9</th>
<th>Control</th>
<th>Test (µg/plate)</th>
<th>S9</th>
<th>Control</th>
<th>Positive controls (µg/plate)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>50%</td>
<td>Ethanolic extract of P. angulata (EEPA)</td>
<td>50%</td>
<td>50%</td>
<td>Sodium azide 2-Nitrofluorene 2-Aminoantracene Mitomycin C ICR191 Acridine</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>390 650 1080 1800 3000</td>
<td>5</td>
<td>2</td>
<td>2.5</td>
</tr>
<tr>
<td>TA98</td>
<td>−</td>
<td>29 ± 2</td>
<td>27 ± 1 22 ± 3 20 ± 1 22 ± 1 25 ± 2</td>
<td>2064 ± 91</td>
<td>(70.36)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>42 ± 3</td>
<td>40 ± 2 41 ± 3 43 ± 1 40 ± 2 36 ± 3</td>
<td>2429 ± 426</td>
<td>(57.83)</td>
<td></td>
</tr>
<tr>
<td>TA100</td>
<td>−</td>
<td>197 ± 8</td>
<td>199 ± 2 204 ± 0 199 ± 5 191 ± 12 187 ± 12</td>
<td></td>
<td>2009 ± 247</td>
<td>(10.18)</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>197 ± 2</td>
<td>197 ± 8 199 ± 8 197 ± 8 196 ± 11 187 ± 12</td>
<td></td>
<td>1321 ± 171</td>
<td>(6.69)</td>
</tr>
<tr>
<td>TA102</td>
<td>−</td>
<td>305 ± 5</td>
<td>307 ± 2 303 ± 2 293 ± 2 303 ± 8 308 ± 4</td>
<td>2345 ± 247</td>
<td>(7.68)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>308 ± 4</td>
<td>305 ± 6 305 ± 6 304 ± 4 307 ± 6 304 ± 4</td>
<td></td>
<td>1113 ± 10</td>
<td>(3.61)</td>
</tr>
<tr>
<td>TA1535</td>
<td>−</td>
<td>36 ± 5</td>
<td>30 ± 7 30 ± 2 26 ± 5 32 ± 1 30 ± 4</td>
<td>2037 ± 89</td>
<td>(56.58)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>28 ± 2</td>
<td>33 ± 5 38 ± 4 41 ± 2 39 ± 1 36 ± 1</td>
<td>410 ± 14</td>
<td>(8.14)</td>
<td></td>
</tr>
<tr>
<td>TA1537</td>
<td>−</td>
<td>18 ± 1</td>
<td>13 ± 3 12 ± 1 11 ± 1 13 ± 3 9 ± 2</td>
<td>1652 ± 59</td>
<td>(93.51)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>12 ± 1</td>
<td>11 ± 1 14 ± 2 11 ± 1 9 ± 1 8 ± 2</td>
<td>340 ± 57</td>
<td>(29.14)</td>
<td></td>
</tr>
</tbody>
</table>

* Negative control: DMSO 100 µL/plate. The values in brackets represent the mutagenic index.
Table 2 – Frequency of micronuclei polychromatic erythrocyte (MNPCE) and polychromatic erythrocyte/normochromatic erythrocyte (PCE/NCE) ratio in EEPA-treated, negative and positive control groups.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Body weight</th>
<th>Total number</th>
<th>Mean ± SD</th>
<th>PCE/NCE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cyclophosphamide (50 mg/kg)</td>
<td>38.2 ± 4.6</td>
<td>234</td>
<td>39.0 ± 3.3</td>
<td>0.86</td>
</tr>
<tr>
<td>Corn oil (5 mL/kg)</td>
<td>40.9 ± 2.8</td>
<td>10</td>
<td>1.6 ± 1.0</td>
<td>1.03</td>
</tr>
<tr>
<td>EEPA (2000 mg/kg)</td>
<td>39.9 ± 2.6</td>
<td>13</td>
<td>2.1 ± 1.2</td>
<td>1.01</td>
</tr>
</tbody>
</table>

Table 3 – Leishmanicidal and cytotoxic activities of EEPA.

<table>
<thead>
<tr>
<th>Sample</th>
<th>IC₅₀ L. amazonensis promastigote</th>
<th>IC₅₀ L. amazonensis amastigote</th>
<th>IC₅₀ L. braziliensis promastigote</th>
<th>LC₅₀Mφ</th>
<th>SI*</th>
</tr>
</thead>
<tbody>
<tr>
<td>EEPA</td>
<td>5.35 ± 2.50</td>
<td>1.23 ± 0.11</td>
<td>4.50 ± 1.17</td>
<td>6.14 ± 0.59</td>
<td>5</td>
</tr>
<tr>
<td>Amphotericin B</td>
<td>0.14 ± 0.04</td>
<td>0.08 ± 0.02</td>
<td>0.47 ± 0.05</td>
<td>47.98 ± 7.65</td>
<td>600</td>
</tr>
</tbody>
</table>

Values represent mean ± SEM from at least two experiments performed in triplicate. Mφ = peritoneal macrophages. IC₅₀ and LC₅₀ values are expressed in μg/mL.

* SI is the ratio between IC₅₀ L. amazonensis amastigote and LC₅₀.

Fig. 2 – Cytotoxic effect of EEPA and Amphotericin B in peritoneal macrophages. Macrophages from peritoneal exudate were collected from CBA mice and treated with either EEPA at concentrations ranging from 1.2 to 100 μg/mL or Amphotericin B (1.7–50 μg/mL) for 48 h. After the treatment, the cell viability was accessed by Alamar Blue metabolism for 24 h. *p < 0.05, one-way ANOVA followed by Bonferroni’s multiple comparison test. Results shown are mean ± SEM and represent the average from two experiments performed in sextuplicate.

Fig. 3 – Effect of EEPA against amastigotes of L. amazonensis. Macrophages from peritoneal exudate were collected from CBA mice and infected with L. amazonensis promastigotes in stationary growth phase. The cells were incubated for 24 h without any treatment to allow amastigote differentiation and parasite replication. Macrophages were treated with EEPA (3.7 and 1.2 μg/mL for 48 h). Amphotericin B was used as a positive control at 3.7 μg/mL. (a) Percentage of infected cells was evaluated by counting 100 cells, as well as (b) total parasite number. *p < 0.05, one-way ANOVA followed by Bonferroni’s multiple comparison test. Results shown are mean ± SEM and represent the average from two experiments performed in triplicate.
EEPA presented an IC50 concentration of 0.14 ± 0.04 μg/mL and 0.47 ± 0.05 μg/mL against L. amazonensis and L. braziliensis, respectively (Table 3). The in vitro evaluation showed that EEPA causes 100% cytotoxicity in peritoneal macrophages at 11 μg/mL, based on Alamar Blue metabolism. The plant stem extract induced significantly less cytotoxicity at 3.7 and 1.2 μg/mL (p < 0.001) (Fig. 2a). On the other hand, amphotericin B at concentrations close to EEPA did not present in vitro toxicity (Fig. 2b). The LC50 calculated for the EEPA following 48 h treatment of peritoneal macrophages was 6.14 ± 0.59 μg/mL (Table 3). Based on these results, infected macrophages containing amastigotes of L. amazonensis were treated with EEPA and the non-toxic treatment for the host cells was evaluated. Low concentrations of EEPA were also observed to reduce the percentage of infected cells, as well as the number of intracellular parasites (Fig. 3). After 24 h of treatment, EEPA at 3.7 μg/mL significantly reduced the percentage of infected cells and the parasitism (p < 0.05). After 48 h, the antileishmanial effect at 3.7 μg/mL increased and 91.8% of the number of intracellular parasites were reduced compared to the untreated control. At this time point, 1.2 μg/mL of EEPA also significantly reduced the parasitism (p < 0.05). EEPA presented IC50 value of 1.23 ± 0.11 μg/mL against amastigotes after 48 h of treatment, while amphotericin B, used as a positive control, had an IC50 of 0.08 ± 0.02 μg/mL (Table 3). It is noteworthy that EEPA presented no toxic effect in macrophages at 3.7 μg/mL, however significantly reduced the parasitism in infected macrophages. The SI calculated by the ratio between LC50 and IC50 (amastigote) was 5, suggesting a specific pharmacological effect of EEPA against the intracellular Leishmania (Table 3). Amphotericin B presented a SI value of 600 as a consequence of the low in vitro cytotoxicity and high antiparasitic activity. However, the known side effects related to the clinical use of amphotericin B still encourage the research for new antileishmanial medicines.

In the current study we used macrophages from susceptible CBA mice. According to Gomes et al. (2003), L. amazonensis can inactivate and become resistant to the CBA mice macrophage killing mechanisms, providing a susceptibility model to evaluate the pharmacological activity of new drug candidates in vitro. Further studies are necessary to evaluate how the EEPA exercises these pharmacological effects. Given what we know regarding in vivo EEPA toxicity, our objective is to develop an EEPA-based topical formulation and to evaluate the efficacy of this plant extract in a murine model of cutaneous leishmaniasis caused by L. amazonensis.

4. Conclusion

The results obtained from our study revealed that EEPA had no mutagenic effects in the Ames test up to 3000 μg/plate and does not induce chromosome damage in rodents. Moreover, EEPA was able to reduce the parasitism in L. amazonensis infected macrophages and showed a high selective index. We concluded that EEPA can represent a new option for the treatment of cutaneous leishmaniasis and could be commercially developed in cosmetic and pharmaceutical industries.

Conflict of interest statement

The authors declare that there are no conflicts of interests in this report.

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References


