In vitro and in vivo antiparasitic activity of Physalis angulata L. concentrated ethanolic extract against Trypanosoma cruzi

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ABSTRACT

Background: The current treatment of Chagas disease, endemic in Latin America and emerging in several countries, is limited by the frequent side effects and variable efficacy of benznidazole. Natural products are an important source for the search for new drugs.

Aim/hypothesis: Considering the great potential of natural products as antiparasitic agents, we investigated the anti-Trypanosoma cruzi activity of a concentrated ethanolic extract of Physalis angulata (EEPA).

Methods: Cytotoxicity to mammalian cells was determined using mouse peritoneal macrophages. The antiparasitic activity was evaluated against axenic epimastigote and bloodstream trypanomastigote forms of T. cruzi, and against amastigote forms using T. cruzi-infected macrophages. Cell death mechanism was determined in trypanomastigotes by flow cytometry analysis after annexin V and propidium iodide staining. The efficacy of EEPA was examined in vivo in an acute model of infection by monitoring blood parasitaemia and survival rate 30 days after treatment. The effect against trypanomastigotes of EEPA and benznidazole acting in combination was evaluated.

Results: EEPA effectively inhibits the epimastigote growth (IC50 2.9 ± 0.1 μM) and reduces bloodstream trypanomastigote viability (EC50 1.7 ± 0.5 μM). It causes parasite cell death by necrosis. EEPA impairs parasite infectivity as well as amastigote development in concentrations noncytotoxic to mammalian cells. In mice acutely-infected with T. cruzi, EEPA reduced the blood parasitaemia in 72.7%. When combined with benznidazole, EEPA showed a synergistic anti-T. cruzi activity, displaying CI values of 0.8 ± 0.07 at EC50 and 0.83 ± 0.1 at EC90.

Conclusion: EEPA has antiparasitic activity against T. cruzi, causing cell death by necrosis and showing synergistic activity with benznidazole. These findings were reinforced by the observed efficacy of EEPA in reducing parasite load in T. cruzi-mice. Therefore, this represents an important source of antiparasitic natural products.

Introduction

Chagas disease, a zoonosis caused by the hemoflagellate protozoan Trypanosoma cruzi, remains a serious health problem in many Latin American countries, where it is an endemic disease that affects approximately 10 million people (Rassi et al. 2010). It is estimated that over 400,000 individuals are infected in non-endemic areas, mainly in the USA and in European countries (Coura and Viñas 2010). Once the individual has been infected by T. cruzi, there is no effective treatment and the development of a vaccine is still in experimental stage (Gupta et al. 2013; Maya et al. 2007). The current treatment is based on the 2-nitroimidazole benznidazole (LAFEPE, Brazil), which...
is recommended for all acute, early chronic and reactivated cases (Da Silva et al. 2011). Its inefficacy against the chronic phase of the disease, allied to many side effects which frequently lead to treatment interruption (Bahia et al. 2012; Urbina 2009) encourages the search for alternative compounds for a better treatment for Chagas disease.

In the continuous search for new drugs, natural products are an important source of compounds to a large number of illnesses (Newman and Cragg 2012). A considerable number of plant-derived compounds are nowadays recognized for their antiparasitic properties (Negi et al. 2014; Rocha et al. 2005; Singh et al. 2014; Wink 2012). An example of plant species with active compounds is the Solanaceae Physalis angulata L., an annual herb widespread in many tropical countries and widely used in folk medicine (Soares et al. 2003; Tomassini et al. 2000). Previously, our research group demonstrated the antiparasitic properties of seco-steroids physalins isolated from P. angulata against Plasmodium falciparum and different Leishmania species (Guimaraes et al. 2009, 2010; Sâ et al. 2011). More recently, it was also reported that physalins, specifically B and F, are endowed with a strong anti-T. cruzi activity against different evolutive forms of T. cruzi (Meia et al. 2013). Isolating physalins is quite costly, time consuming and affords low yields, aspects which hamper the use of isolated physalins for a neglected disease. To overcome this, a feasible alternative is the use of a Physalis angulata extract. In fact, the concentrated ethanolic extract from P. angulata (EEPA) is nonmutagenic, presents low toxicity in mice and is effective against different Leishmania species (Nogueira et al. 2013). Therefore, in the present work the anti-T. cruzi activity in vitro and in vivo of EEPA was evaluated.

Material and methods

Plant material

P. angulata specimens were collected and identified during the drier season (from June until November) in Belém, Pará State, Brazil. A voucher specimen was deposited in the Herbarium of the Department of Physiology from the University Federal of Pará (Voucher number 15).

Extraction and phytochemical analysis of P. angulata

Stems of P. angulata (1 kg) were dried and crushed. Then, extraction was carried out with ethanol at 50–60 °C during 6 h. The extract was concentrated under reduced pressure, yielding 100 g (10%) of crude ethanolic extract. It was maintained in a desiccator under vacuum until weight stabilized. HPLC-UV analysis was determined in an Agilent HP 1100 series system consisting of an auto-sampler, high-pressure mixing pump and UV detector (Agilent Technologies, Santa Clara, CA). Samples were run in a reverse phase C-18 column 5 μm (250 mm × 4 mm, Hibar® LiChrosorb®, Merk, Germany) and UV absorbance was measured at 225 nm by injecting 10 μl of EEPA dissolved in 10 mg/ml in MeOH at flow rate 1 ml/min. Physalins in the EEPA were confirmed by comparing with standard isolated physalin previously characterized. These procedures were performed as described by Nogueira et al. (2013) and executed in accordance with European Medicines Agency guidelines for herbal products.

Drugs

Benznidazole (LAFEPE, Recife, Brazil) was used as reference drug in the anti-T. cruzi assays. Gentian violet (Synth, São Paulo, Brazil) was used as positive control in the cytotoxicity assays. All compounds were dissolved in DMSO (Sigma–Aldrich, St. Louis, MO) and diluted in cell culture medium for use in the assays. The final concentration of DMSO was less than 1% in all in vitro experiments.

Parasites

The T. cruzi strains Y (DTU II; Silva and Nussenzweig 1953) and Colombian (DTU I; Federici et al. 1964) were used in this study. T. cruzi epimastigotes were grown in liver infusion tryptose (LIT) medium supplemented with 10% fetal bovine serum (FBS; Culttub, Campinas, Brazil), 1% hemin (Sigma–Aldrich), 1% R9 medium (Sigma–Aldrich) and 50 μg/ml of gentamycin (Novafarma, Anápolis, Brazil) at 26 °C. Tissue culture trypomastigotes were obtained from the supernatants of 5- to 6-day-old infected LLC-MK2 cells maintained in RPMI-1640 medium supplemented with 10% FBS and 50 μg/ml gentamycin at 37 °C in a 5% humidified CO2 atmosphere.

Animals

Female BALB/c mice (18–22 g) were provided by the animal breeding facility and maintained in sterilized cages under a controlled environment, water ad libitum and receiving a balanced diet for rodent at Centro de Pesquisa Gênico Moniz (Fundação Oswaldo Cruz, Bahia, Brazil). All experiments were carried out in accordance with the recommendations of Ethical Issues Guidelines, and were approved by the local Animal Ethics Committee.

Assessment of cytotoxicity to macrophages

Peritoneal exudate macrophages were obtained by washing, with cold RPMI medium, the peritoneal cavity of BALB/c mice 4–5 days after injection of 3% thioglycolate in saline (1.5 ml per mouse). Then, cells were seeded into 96-well plates at a cell number 1 × 105 cells/well in RPMI-1640 medium supplemented with 10% FBS and 50 μg/ml of gentamycin and incubated for 24 h at 37 °C and 5% CO2. After that time each sample was added at eight concentrations (0.02–50 μg/ml) in triplicate and incubated for 72 h. Twenty microliters/well of AlamarBlue (Invitrogen, Carlsbad, CA) were added to the plates during 10 h. Colorimetric readings were performed at 570 and 600 nm. CC50 values were calculated using data-points gathered from three independent experiments. Gentian violet was used as positive control.

Antiparasitic activity

Epimastigotes (1 × 106 cells/well) were seeded in fresh medium in LIT medium in the absence or presence of the EEPA at eight concentrations (0.02–50 μg/ml) in triplicate. Cell growth was determined after culture for 5 days by counting viable forms in a Neubauer chamber. Bloodstream trypomastigotes forms of T. cruzi from the supernatants of previously infected LLC-MK2 cells were dispensed into 96-well plates (4 × 105 cell/well) in RPMI medium supplemented with 10% FBS and 50 μg/ml of gentamycin in the absence or presence of different concentrations (0.02–50 μg/ml) of the plant extract, in triplicate. Viable parasites were counted in a Neubauer chamber 24 h after incubation. The percentage of inhibition was calculated in relation to untreated cultures. To determine the inhibitory concentration of 50% (IC50) and the effective concentration at 50% (EC50) for epimastigote and trypomastigote forms of T. cruzi, respectively, a nonlinear regression on Prism 5.02 GraphPad software was employed. Experiments were performed in both Y and Colombian strains and benznidazole was used as anti-T. cruzi reference drug. For in vitro drug combinations, doubling dilutions of each drug (EEPA and benznidazole), used alone or in fixed combinations were incubated with 4 × 105 cells/well trypomastigotes (Y strain) for 24 h. The analysis of the combined effects was performed by determining the combination index (CI) by using Chou-Talalay CI method and the CompuSyn software version 1.0 (ComboSyn Inc., Paramus, NJ), as described previously (Chou and Talalay 2005). CI values were used as cutoff to determine synergism.
**T. cruzi-infected macrophages**

Peritoneal exudate macrophages (2 × 10^5 cells/well) obtained from BALB/c mice were seeded in a 24 well-plate with rounded coverslips on the bottom in RPMI supplemented with 10% FBS and incubated for 24 h. Cells were then infected with trypomastigotes (10:1) for 2 h. Free trypomastigotes were removed by successive washes using saline solution and the cells were incubated for 24 h to allow full internalization and differentiation of trypomastigotes to amastigotes. Next, cultures were incubated in complete medium alone or with the EEPA or benznidazole (0.1–2.5 μg/ml) for 72 h. Cells were fixed in absolute alcohol and the percentage of infected macrophages and the number of amastigotes/100 macrophages was determined by manual counting after hematoxylin and eosin staining using an optical microscope (Olympus, Tokyo, Japan). The percentage of infected macrophages and the number of amastigotes per 100 macrophages was determined by counting 100 cells per slide. The one-way ANOVA and Bonferroni for multiple comparisons were used to determine the statistical significance of the group comparisons. Experiment was done with both strains (Y and Colombian) and benznidazole was used as positive control.

**Propidium iodide and annexin V staining**

*T. cruzi* trypomastigotes from Y strain (1 × 10^7) were incubated for 24 and 72 h at 37 °C in the absence or presence of EEPA (1.7, 3.4 or 5 μg/ml). After incubation, the parasites were labeled for propidium iodide (PI) and annexin V using the annexin V–FITC apoptosis detection kit (Sigma–Aldrich), according to the manufacturer's instructions. Acquisition and analyses was performed using a FACs Calibur flow cytometer (Becton Dickinson, San Diego, CA), with FlowJo software (Tree Star, Ashland, OR). A total of 30,000 events were acquired in the region previously established as that corresponding to trypomastigotes forms of *T. cruzi*.

**Infection in mice**

Female BALB/c mice (18–22 g) were infected with bloodstream trypomastigotes by intraperitoneal inoculation of 10^4 parasites in 100 μl of saline solution and then mice were randomly divided in groups (six animals per group). After the day 5 of infection, treatment with 50 or 100 mg/kg weight of EEPA was given orally for 5 consecutive days. For the control group, benznidazole was given orally at dose of 100 mg/kg weight. Saline containing 20% DMSO was used as a vehicle and administrated on untreated and infected group. Animal infection was monitored daily by counting the number of motile parasites in 5 μl of fresh blood sample drawn from the lateral tail veins, as recommended by standard protocol (Brener 1962). Survival was monitored for 30 days after treatment. The one-way ANOVA and Bonferroni for multiple comparisons were used to determine the statistical significance of the group comparisons.

**Results**

Extraction and phytochemical analysis of EEPA revealed that this extract is rich in seco-steroids, mainly physalins B, D, F and G. By HPLC–UV, it was estimated that each 100 mg of EEPA contains 0.84% of physalin B, 0.90% of physalin D, 0.37% of physalin F and 0.36% of physalin G. This is in agreement with the literature showing that this plant contains a number of steroids (Nogueira et al. 2013; Tomassini et al. 2000).

The trypomocidal activity of EEPA was initially evaluated against epimastigotes and trypomastigotes (Y and Colombian strains) through the determination of IC_{50} and EC_{50}, respectively. The plant extract showed a potent anti-*T. cruzi* activity, being able to inhibit epimastigote proliferation and lyse trypomastigotes. The IC_{50} of EEPA treatment was 2.9 and 7.4 μg/ml for epimastigotes of Y and Colombian strains, respectively, while benznidazole exhibited an IC_{50} of 2.8 and 4.1 μg/ml, respectively. For trypomastigotes, the EC_{50} calculated of EEPA was 1.7 and 2.8 μg/ml for Y and Colombian strains, respectively. Under the same assay conditions, the standard drug benznidazole presented an EC_{50} of 2.8 and 3.3 μg/ml for Y and Colombian strains, respectively. Next, the cytotoxicity of EEPA was determined in mammalian cells. The plant extract exhibited a CC_{50} value of 6.1 μg/ml, being several times less cytotoxicity than the reference drug, gentian violet (CC_{50} = 0.21 μg/ml) (Table 1).

The activity of EEPA against intracellular amastigotes (Y strain) was also investigated. The plant extract induced a concentration-dependent reduction in the analyzed parameters: percentage of infected macrophages (Fig. 1A) and the mean number of intracellular amastigotes per infected macrophage (Fig. 1B). Under the same conditions, a similar reduction was observed on treated-benznidazole macrophages. No signs of cytotoxicity were observed on macrophage cultures (data not shown).

After confirming that EEPA was able to decrease parasite viability, the mechanism of EEPA-induced cell death was investigated. In untreated cultures, most cells were negative for annexin V and PI staining, demonstrating cell viability (Fig. 2A). Cultures treated with EEPA showed a concentration and time-dependent increase in the number of PI-positive parasites when compared with untreated parasites (Fig. 2B–D). Treatment with 3.4 μg/ml of EEPA for 72 h resulted in 11.5 and 41.3% of cells positive for PI and PI + annexin V, respectively, whereas 1.1% cells were stained only for annexin V. These results indicate that EEPA treatment increases the number of PI staining, which is characteristic of a parasite cell death caused by a necrotic process.

Next, *in vivo* studies to evaluate the effects of EEPA against *T. cruzi* infection in mice (*acute phase*) were performed. As shown in Fig. 3, EEPA treatment significantly (*P < 0.001*) reduced blood parasitaemia when compared with mice treated with vehicle (Fig. 3). At doses of 50 and 100 mg/kg, EEPA administration caused a reduction in blood parasitaemia of 42.6 and 72.7%, respectively (Table 2). In the group treated with benznidazole, it was observed >99% of inhibition of blood parasitaemia, indicating that eradication of infection was achieved. Treatment with EEPA, similar to the treatment with benznidazole, had a protective effect on mortality. Mice from benznidazole or EEPA treated groups did not show any behavioral alteration or signs of toxicity (data not shown).

Finally, the antiparasitic effect of EEPA and benznidazole in combination was investigated against trypomastigotes. In comparison to drug alone, the combination of EEPA and benznidazole reduced both EC_{50} and EC_{90} values. In fact, the EC_{50} and EC_{90} of benznidazole decreased in average by 59% when combined with EEPA (Table 3). The combination index values revealed that EEPA and benznidazole have synergistic effects.

### Table 1

Antiparasitic activity of EEPA on extracellular forms of *T. cruzi* and host cell cytotoxicity.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Epimastigotes IC_{50} ± S.D. (μg/ml)</th>
<th>Trypomastigotes EC_{50} ± S.D. (μg/ml)</th>
<th>MØ CC_{50} ± S.D. (μg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Y strain</td>
<td>Colombian strain</td>
<td>Y strain Colombian strain</td>
</tr>
<tr>
<td>EEPA</td>
<td>2.9 ± 0.1</td>
<td>7.4 ± 0.2</td>
<td>2.3 ± 0.2</td>
</tr>
<tr>
<td>BDZ</td>
<td>2.8 ± 0.7</td>
<td>4.1 ± 0.1</td>
<td>3.3 ± 0.1</td>
</tr>
<tr>
<td>GV</td>
<td>0.1</td>
<td></td>
<td>0.21 ± 0.02</td>
</tr>
</tbody>
</table>

Values were calculated using concentrations in triplicate and three independent experiments were performed. IC_{50} = inhibitory concentration at 50%. EC_{50} = effective concentration at 50%. CC_{50} = cytotoxic concentration at 50%. S.D = standard deviation.

a Determined 24 h after incubation with compounds.

b Determined 24 h after incubation with compounds.

c Cell viability of BALB/c mouse macrophages determined 72 h after treatment.
Fig. 1. EEPA inhibited amastigote proliferation in *T. cruzi*-infected macrophages. Mouse peritoneal exudate macrophages were infected with Y strain trypomastigotes for 2 h and treated with different concentrations of EEPA (2.5, 1, 0.5 or 0.25 μg/ml) or benznidazole (2.5 μg/ml), a standard drug. Cells were stained with hematoxylin and eosin and analyzed by optical microscopy. The percentage of infected macrophages (A) and the relative number of amastigotes per 100 macrophages (B) were determined by counting hematoxylin and eosin-stained cultures. BDZ is benznidazole. Values represent the mean ± SEM of triplicates. *P < 0.05; **P < 0.01; ***P < 0.001 compared to the control group.

Fig. 2. Flow cytometry analysis of trypomastigotes treated with EEPA for 24 or 72 h and stained with propidium iodide (PI) and annexin V. (A) Representative untreated trypomastigotes (24 h); (B) trypomastigotes treated with EEPA (5 μg/ml) for 24 h; (C) trypomastigotes treated with EEPA (1.7 μg/ml) for 72 h; (D) trypomastigotes treated with EEPA (3.4 μg/ml) for 72 h.

Fig. 3. Parasitemia of BALB/c mice infected with *T. cruzi* and treated with EEPA. Female BALB/c mice were infected with 10⁴ Y strain trypomastigotes. Five days after infection, mice were treated orally with EEPA (50 or 100 mg/kg) or benznidazole (100 mg/kg) once a day during 5 consecutive days. Parasitemia was monitored by counting the number of trypomastigotes in fresh blood samples. Values represent the mean ± SEM of 6 mice per group. Results are from one experiment of two replicates performed. *P < 0.05; ***P < 0.001 compared to untreated-infected group (vehicle).

Discussion

In this study, the potent anti-*T. cruzi* activity of concentrated ethanolic extract from *P. angulata* (EEPA) rich in physalins was demonstrated *in vitro* on different strains of *T. cruzi* and *in vivo* on an acute model of infection. EEPA was chosen mainly because the isolation of the active molecule is costly, time consuming and gives a small fraction of material. On the other hand, the extract is easily obtained, has low cytotoxicity and high stability (Nogueira et al. 2013).

EEPA effectively inhibits the growth of epimastigotes and was toxic against bloodstream trypomastigotes of *T. cruzi*. More importantly, has potent activity against amastigotes inside infected...
et al. 2013). In this study, EEPA demonstrates synergistic effects with benznidazole for the treatment of parasitic diseases (Vivas et al. 2008; Diniz et al. 2013). Moreover, high selectivity index was observed, pointing out the great efficacy against L. amazonensis (Nogueira et al. 2013).

To identify the mechanism of cell death induced by EEPA treatment, analyses of the classical necrosis/apoptosis processes were performed. From the literature, it is well known that activation of cell death pathway depends on type, time, and intensity of the stimulus (Kessler et al. 2013). Necrosis is usually defined as a process of cell collapse that involves oncosis (increase in cell volume), rupture of plasma membrane and the unorganized dismantling of swollen organelles. Apart from the presence of plasma membrane permeabilization, necrosis lacks specific biochemical markers (Kroemer et al. 2009). In this study, EEPA treatment caused an increase time-dependent in the number of PI-positive parasites, suggestive of cell death by necrosis. PI-positive parasites also were observed after 24 h of treatment with physalin B (Meira et al. 2013). In addition, ultrastructural analyses showed remarkable alterations in the morphology of the Golgi complex, kinetoplast, endoplasmic reticulum and plasma membrane of trypanosomatids, confirming a pattern consistent with necrotic cell death.

Combination therapies can be a valuable tool to improve treatment efficacy and reduce dose levels and toxicity, as well as to prevent the potential development of resistance, which may be advantageous for the treatment of parasitic diseases (Vivas et al. 2008; Diniz et al. 2013). In this study, EEPA demonstrates synergistic effects with benznidazole against the bloodstream parasites in vitro, showing a promising profile for drug combination.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Dose (mg/kg)</th>
<th>% Blood parasitemia reduction in mice&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Mortality&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
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<tbody>
<tr>
<td></td>
<td></td>
<td>8 dpi</td>
<td>10 dpi</td>
</tr>
<tr>
<td>EEPA</td>
<td>50</td>
<td>42.6</td>
<td>49.7</td>
</tr>
<tr>
<td>EEPA</td>
<td>100</td>
<td>72.7</td>
<td>71.7</td>
</tr>
<tr>
<td>BDZ</td>
<td>100</td>
<td>&gt;99</td>
<td>&gt;99</td>
</tr>
<tr>
<td>Vehicle</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
</tbody>
</table>

Dpi = days post-infection. BDZ = benznidazole. Vehicle = untreated and infected group.

<sup>a</sup> Calculated as \( \frac{([\text{average vehicle group} - \text{average treated group}] \times 100)}{\text{average vehicle group}} \).

<sup>b</sup> Mortality was monitored until 30 days after treatment.

### Table 3

Antiparasitic activity of EEPA or benznidazole in Y strain trypomastigotes alone or in combination.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Drug alone</th>
<th>Combination</th>
<th>Drug alone</th>
<th>Combination</th>
<th>EC&lt;sub&gt;50&lt;/sub&gt; ± S.D. (μg/ml)&lt;sup&gt;a&lt;/sup&gt;</th>
<th>CI at&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>EEPA</td>
<td>1.7 ± 0.5</td>
<td>3.1 ± 0.2</td>
<td>1.3 ± 0.1</td>
<td>0.8 ± 0.07</td>
<td>0.83 ± 0.1</td>
<td></td>
</tr>
<tr>
<td>BDZ</td>
<td>2.8 ± 0.7</td>
<td>6.7 ± 0.2</td>
<td>2.7 ± 0.2</td>
<td>0.1</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

S.D. = standard deviation. BDZ = benznidazole.

<sup>a</sup> EC<sub>50</sub> and EC<sub>90</sub> values were calculated using concentrations in triplicates and two independent experiments were performed.

<sup>b</sup> Combination index (CI). Cutoff: CI value of 0.3–0.7, synergism; 0.7–0.85, moderate synergism; 0.85–0.9, slight synergism; 0.9–1.1, additivity; >1.1, antagonism.

### Conflict of interest

All authors have no conflict of interest to disclose.

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