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Trypanocidal activity of polysaccharide extract from *Genipa americana* leaves

Racquel Oliveira da Silva Souza[a,*], Paloma Leão Sousa[a], Ramon Róseo Paula Pessoa Bezerra de Menezes[b], Tiago Lima Sampaio[b], Louise Donadello Tessarolo[a], Francisca Crislandia Oliveira Silva[c], Maria Gonçalves Pereira[c], Alice Maria Costa Martins[a]

[a] Departamento de Análises Clínicas e Toxicológicas, Universidade Federal do Ceará, Fortaleza, Ceará, Brasil
[b] Departamento de Fisiologia e Farmacologia, Universidade Federal do Ceará, Fortaleza, Ceará, Brasil
[c] Faculdade de Educação, Ciências e Letras do Sertão Central, Universidade Estadual do Ceará, Quixadá, Ceará, Brasil

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ABSTRACT

Ethnopharmacological relevance: The parts of the *Genipa americana* (Rubiaceae) tree, also known as "jenipapo" or "jenipapeiro", has been used in traditional Medicine in parasitic and bacterial infections. Thus, the experimental evolution of the antiparasitic activity of polysaccharide extracts from *Genipa americana* leaves, and correlation with antiparasitic and popular use is important.

Aim of the study: To evaluate the effect of polysaccharide extract obtained from *Genipa americana* leaves on all *Trypanosoma cruzi* (Y strain: benznidazole-resistant) developmental forms, a protozoan that causes Chagas' disease.

Materials and methods: An extract rich in polysaccharides was obtained from the leaves of *Genipa americana* (GaEPL) by associating depigmentation in methanol followed by extraction of polysaccharides in NaOH and precipitation with ethanol. Cytotoxicity to mammalian cells (LLC-MK2) was determined using an MTT assay. Antiparasitic activity was evaluated against epimastigote, trypomastigote and amastigote forms of *T. cruzi*. Cell-death mechanism was determined in epimastigote forms by flow cytometry analysis after FITC-annexin V (Ax), 7-AAD, and H2DCFDA staining. Striking morphological changes were observed by scanning electron microscope.

Results: GaEPL (6.5% yield; 54.6% total carbohydrate; 21.1% uronic acid and 12% protein), inhibited all *T. cruzi* developmental forms, epimastigotes after periods of 24 h (IC_{50} = 740 ± 0.075 μg/mL), 48 h (IC_{50} = 710 ± 0.053 μg/mL) and 72 h (IC_{50} = 870 ± 0.052 μg/mL) of incubation; trypomastigotes (IC_{50} = 470 ± 0.082 μg/mL) after periods of 24 h and intracellular amastigotes ($IC_{50/2}$ = 235 or IC_{50} = 470 μg/mL) after periods of 24 and 48 h of incubation, with no toxicity on LLC-MK2 cells at the used concentrations. Analysis of the possible action mechanism in the parasites suggested cell death by necrosis with the involvement of reactive oxygen species (ROS). The scanning electron microscopy (SEM) confirmed *T. cruzi* death by necrosis.

Conclusions: GaEPL showed significant activity against the epimastigote, trypomastigote and amastigote forms of *T. cruzi*, strain Y, suggesting cell death by necrosis with involvement of reactive oxygen species.

1. Introduction

American trypanosomiasis, also called Chagas disease, is considered one of the main public health problems in Latin America, where it causes more than 7000 deaths per year (WHO, 2015). In Brazil, benznidazole (BZ) is the drug of choice to treat the disease, but it has limited therapeutic action and several side effects, which frequently lead to treatment interruption (Cerecetto and González, 2002; Bahia et al., 2012).

Natural plant products are a source of compounds that could be potentially active against protozoa (Croft et al., 2005; Salem and Werbovetz, 2006). Mesquita et al. (2005) identified antileishmanial and antitrypanosomal activities of ethanolic extract of *Caesaria sylvestris* leaves from medicinal plants of the Brazilian Cerrado (tropical savanna ecoregion). A previous study demonstrated that methanolic extracts of plants used in Mexican folk medicine exhibited the highest trypanocidal activity (Molina-Garza et al., 2014).

Genipa americana (Rubiaceae), a plant popularly known as

* Corresponding author.
E-mail addresses: racquel_oliveira2@hotmail.com (R.O.d.S. Souza), martinsalice@gmail.com (A.M.C. Martins).

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"jenipapo" or "jenipapeiro", is found in Brazil from Amapá to São Paulo and Mato Grosso states (Corrêa, 1984; Delprete et al., 2005). In Brazil, several medicinal uses have traditionally been attributed to this species. The leaves decoction has been used as antidiarrheal and anti-syphilitic therapy (Corrêa, 1984), whereas the leaves macerated, has been used to treat fever by some native tribes (Delprete et al., 2005). Based on ethnobotanical surveys, the traditional use of this species is for the treatment of cough, anemia, contusions, dislocations; as depurative and associated with popular beliefs (Souza et al., 2013). In addition, the barks decoction (*ad libitum*) is used in the treatment of malaria by indigenous of the Upper Rio Negro in Amazonas (Kffuri et al., 2016).

The phytochemical analysis of *G. americana* leaves extracts revealed the presence of iridoids, mono and sesquiterpenes and of triterpenes and steroids. Therefore, also revealed the presence of hydrolyzable tannis, proanthocyanidins, cinnamic derivatives, phenylpropanoglycosides, geniposidic acid, genipatriol and flavonoids (Guarnaccia et al., 1972; Hossain et al., 2003; Alves et al., 2017; Vasconcelos et al., 2017).

Experimental studies performed with the extracts of leaves from *G. americana* had demonstrated antimalarial activity *in vitro* on *Plasmodium falciparum* and *in vivo* on *Plasmodium berghei* (Deharo et al., 2001), as well as the anti-parasitic and antimicrobial effects (Oliveira et al., 2012; Nogueira et al., 2014; Tallent, 1964).

Although the literature has several studies on extracts and pure compounds obtained from plants with good potential for the trypanocidal action (Paveto et al., 2004; Veiga-Santos et al., 2010), little is known about their mechanism of action. However, experimental reports of the antitrypanosomal effect of *Genipa americana* polysaccharide extracts were not found. Thus, this study focuses on evaluation of antitrypanosomal activity of polysaccharide extracts from *Genipa americana* leaves on all *Trypanosoma cruzi* developmental forms and the type of cell death that was triggered.

2. Materials and methods

2.1. Plant material

Samples of *G. americana* leaves were collected in nature in the District of Custodio-Quixadá, state of Ceará, Brazil and identified by Ms. Vanecia dos S. Gomes (State University of Ceará). A voucher specimen was deposited at Prisco Bezerra Herbarium (Federal University of Ceará, n. 4683).

2.2. Chemicals

Fetal bovine serum (FBS) was obtained from Invitrogen (Grand Island, NY, USA). LIT medium (HiMedia Laboratories, Mumbai, IND), DMEM medium (VITROCELL, São Paulo, BR), Catalase (CAT; Sigma-Aldrich, Brazil). Benznidazole (BZ; Lafepe, Brazil), Sodium dodecyl sulfate (SDS - Vetec, São Paulo, Brazil), (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenytetrazolium bromide formazan – MTT) were obtained from Amresco (Ohio, USA), FITC-conjugated to annexin V/7-AAD from BD Pharmingen, California, USA, and H_2DCFDA from Sigma-Aldrich™, St. Louis, USA.

2.3. Extraction of polysaccharides from Genipa americana leaves

G. americana leaves were washed with distilled water, dried at 40 °C, grounded into powder (5 g), suspended in methanol (1:50 w/v, 76 °C, 2 h) and the filtered for removal of methanol-soluble material; the procedure repeated twice. The insoluble material was extracted in 0.1 M NaOH (1:50 w/v, 97 °C, the procedure was repeated three times) resulting in alkaline extracts that were pooled (supernatants 2 and 3), neutralized with 1 M HCl, precipitated with 4 volumes of ethanol and centrifuged. The supernatant was dialyzed against running water for 72 h, centrifuged (1445 × *g*, 30 min, r.t.) and the final supernatant was lyophilized and named polysaccharide extract of *G. americana* - GaEPL (Souza et al., 2015).

2.4. Cells and parasites

LLC-MK2 (epithelial cells from the kidney of the monkey *Macaca mulatta*) were maintained in DMEM medium (Vitrocell, São Paulo, Brazil) supplemented with 1% antibiotic solution and 10% of FBS in T-25/75 cm^2 flasks maintained at 37 °C in a humidified atmosphere with 5% CO_2. These cells were split each time they reached 80–90% confluence after being harvested with a solution containing 0.25% trypsin and 2.21 mM EDTA.

The *Trypanosoma cruzi* Y (benznidazole-resistant) strain was isolated in the Laboratory of Parasite Biochemistry, University of São Paulo (São Paulo – Brazil) and donated in epimastigote and trypomastigote forms. Epimastigote forms of *T. cruzi* (Y strain) were maintained at 28 °C with weekly transfers in liver infusion tryptose (LIT) supplemented with 10% FBS. Trypomastigote forms were obtained by infecting LLC-MK2 cell monolayers in DMEM supplemented with 2% FBS at 37 °C in a 5% CO_2 atmosphere.

2.5. Genipa americana polysaccharide extract activity assay against T. cruzi epimastigote forms

Epimastigote forms of *T. cruzi* (10^6 cells/mL) were grown in 96-well plates at 28 °C in LIT medium supplemented with 10% FBS, incubated in triplicate in the absence or presence of the GaEPL (9–1500 μg/mL) or the positive control BZ (1.56–200 μg/mL) for 24, 48 and 72 h. Subsequently, parasite growth inhibition was estimated by counting the parasites in a Neubauer chamber, and the concentration that inhibited growth by 50% (IC_{50}) was determined by a nonlinear regression in relation to the reference drug (Camargo, 1964).

2.6. Genipa americana polysaccharide extract activity assay against T. cruzi trypomastigote forms

Trypomastigote forms (10^6 cells/mL) were obtained from the supernatant of infected LLC-MK_2 cells that were dispensed into 96-well plates in DMEM medium supplemented with 2% FBS in the absence or presence of different concentrations of GaEPL (9–1500 μg/mL) or BZ (1.56–200 μg/mL), in triplicate. The parasites were incubated for 24 h at 37 °C in a 5% CO_2 atmosphere. After incubation, parasite viability was determined by assessing mobility under an Olympus CX31 light microscope. An aliquot (10 μL) of each sample was placed on slides. The slides were cover-slipped and the parasites were counted in a Neubauer chamber, and the concentration that inhibited growth by 50% (IC_{50}) was determined by regression analysis of the data (Adade et al., 2014).

2.7. Cytotoxicity to mammalian cells

LLC-MK_2 cells were assayed for cell viability determination using MTT assay (Mosmann, 1983). A suspension of 10^5 cells was seeded in a 96-well microplate and maintained at 37 °C in a 5% CO_2 atmosphere for 24 h to obtain confluent cell monolayers. Subsequently, the medium was removed, and the monolayers were then treated with different concentrations of GaEPL (9–3000 μg/mL), BZ (1.56–200 μg/mL) or phosphate-buffered saline (PBS) in triplicates for 24 h. After the treatment, 100 μL of the culture medium were removed and 10 μL of MTT (Amresco, Ohio, USA; 5 mg/mL) solution was added. The microplate was incubated for 4 h in a 5% CO_2 atmosphere at 37 °C, and 90 μL of 10% SDS (sodium dodecyl sulfate) was added. After 17 h of incubation, absorbance was read at 570 nm on a microplate reader (Biochrom® Asys Expert Plus). The percentage of viable cells was calculated by comparison with controls. The IC_{50} was determined by regression analysis of the data. The Selectivity index (SI) was calculated by the ratio of cytotoxic/trypanocidal activity (Nwaka and Hudson, 2006).