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Research Brief

Phenol composition, cytotoxic and anti-kinetoplastidae activities of *Lygodium venustum* SW. (Lygodiaceae)

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HIGHLIGHTS

- The better antiparasitary activity was observed in the methanol fraction.
- The main compound in the methanol fraction was Chlorogenic acid.
- The methanol fraction was the low toxic fraction.

GRAPHICAL ABSTRACT

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ABSTRACT

The search for new therapeutic agents has been a constant for the treatment of diseases such as leishmaniasis and Chagas disease. Most drugs used have side effects, justifying the need to evaluate the cytotoxicity of the tested products for candidates to new drugs. In this study, the bioactivity of *Lygodium venustum*, a cosmopolitan fern of Lygodiaceae, was assessed about their leishmanicidal and trypanocidal potential. The better activity was observed using methanol fraction, with inhibition percentage of 63% and 68% for promastigotes and epimastigotes, respectively, at a concentration of 500 μg/mL. The ethyl acetate and methanol fractions demonstrated a higher cytotoxic potential. This was the first report of leishmanicidal, trypanocidal and cytotoxic activities to *L. venustum*.

1. Introduction

The Leishmaniasis is a polymorphic group of diseases caused by protozoan parasites of the genus *Leishmania* (Kinetoplastida, Trypanosomatidae). The parasite is transmitted by female sandflies of the genus *Phlebotomus* in the Old World and *Lutzomyia* in the New World (Berman, 1998). The disease has diverse clinical presentations and can affect the skin, mucous membranes and internal organs. The parasite *Leishmania brasiliensis* is one of those responsible for the cutaneous form (CL) and mucocutaneous (LMC) of the disease, being understood as American Cutaneous Leishmaniasis (Genaro and Michalick, 2005; Genaro and Reis, 2005). For the treatment of Leishmaniasis are used pentavalent antimonials, amphotericin B, pentamidine and miltefosine, all demonstrating high toxicity, high cost and problems during the

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use, beside the possibility to cause resistance of parasite (Rath et al., 2003; Croft and Coombs, 2003).

Chagas disease or American trypanosomiasis is a disease caused by *Trypanosoma cruzi*, transmitted by haematophagous insects from the genus *Triatoma*, (Reduviidae) (Lana and Tafuri, 2005). Several tests were performed seeking a therapeutic solution against this infectious disease. Benzonidazole and nifurtimox are the main compounds used as antiparasitic drugs for American trypanosomiasis, but these drugs are not consistently effective and have serious side effects, including cardiac and renal toxicity (Veloso et al., 2001; Ruiz et al., 2004).

Due the difficulty to discover drugs effective in the therapy and non toxic to humans, natural products have been tested. Many species of plants have been investigated for their antileishmanial and trypanocidal activities (Mesquita et al., 2005; Coro et al., 2005). Isolated semi-synthetic or synthetic compounds are also studied to discover new therapeutic agents against these diseases (Roldós et al., 2008; Saraiva et al., 2007; Moran, 1995).

Lygodium venustum (Lygodiaceae) is a fern with worldwide distribution and lianescent habit (Klaus, 2006). This fern is used as a bioindicator of environmental degradation and as a remedy in the folk medicine by South American populations (Duke, 2008; Argueta et al., 1994; Brasileiro et al., 2006).

The objective of this study to was evaluate, through *in vitro* assays, the trypanocidal and leishmanicidal and cytotoxic activity of ethanol extract and dichloromethane, ethyl acetate and methanol fractions of leaves from *L. venustum*.

2. Material and methods

2.1. Plant material

Leaves of *L. venustum* were collected in may 2010 in the city of Crato, Ceará State, Brazil. The plant material was identified by Dr. Antonio Álamo Feitosa Saraiva, and voucher specimen have been deposited with the identification number 5569 HCDAL at the Herbarium Caririense Dárdano de Andrade-Lima, of the University of the Region of Cariri – URCA.

2.2. Preparation of ethanol extract (EELV) and dichloromethane, ethylacetate and methanol fractions (DFLV, EAFLV, MFLV) of L. venustum

Leaves were collected and 211.18 g were weightened, dried and keeped at room temperature. This material was powdered and extracted by maceration using 1L of 95% ethanol solvent at room temperature. The mixture was allowed to stand for 72 h at room temperature. The extract was filtered and concentrated under vacuum in rotary evaporator at 60 °C and 760 mm/Hg temperature and pressure, respectively (Buckner et al., 1996), obtaining 12.42 g of ethanol extract. Fractionation was performed using the ethanol extract, resulting in the fractions used in the tests (dichloromethane, ethyl acetate and methanol to yield 0.39 g, 0.52 g and 3 g, respectively). The extract and fractions were diluted to 0.01 mg each using DMSO before the assays.

2.3. Cell lines used

For *in vitro* studies of *T. cruzi*, the clone CL-B5 was used (Le Senne et al., 2002). Parasites were stably transfected with the *Escherichia coli* β-galactosidase gene (*lacZ*), were provided by Dr. F. Buckner through Instituto Conmemorativo Gorgas (Panama). Epimastigotes were grown at 28 °C in liver infusion tryptose broth (Difco, Detroit, MI) with 10% fetal bovine serum (FBS) (Gibco, Carlsbad, CA), penicillin (Ern, S.A., Barcelona, Spain) and streptomycin (Reig Jofré S.A., Barcelona, Spain), as described previously (Roldós et al., 2008), and harvested during the exponential growth phase. Culture of *L. brasiliensis* was obtained from the Instituto de Investigaciones en Ciencias de la Salud, Asunción, Paraguay - IICS. The maintenance of strain, the form of cultivation and isolation shape promatigota followed the procedures described by Roldós et al. (2008). The inhibition assays of promastigotes were performed using the strain of *L. braziliensis* (MHOM/BR/75/M2903), grown at 22 °C in Schneider's *Drosophila* medium supplemented with 20% FBS. For the cytotoxic assays, was used the fibroblast cell line NCTC929 grown in Minimal Essential Medium (Sigma). The culture medium was supplemented with heat-inactivated FBS (10%), penicillin G (100 U/ml) and streptomycin (100 μg/mL). Cultures were maintained at 37 °C in humid atmosphere with 5% CO_2. The viability of these strains was assessed according to Roldós et al. (2008), through the use of resazurin as a colorimetric method.

2.4. Reagents

Resazurin sodium salt was obtained from Sigma–Aldrich (St. Louis, MO) and stored at 4 °C protected from light. A solution of resazurin was prepared in 1% phosphate buffer, pH 7, and filter sterilized prior to use. Chlorophenol red-β-D-galactopyranoside (CPRG; Roche, Indianapolis, IN) was dissolved in 0.9% Triton X-100 (pH 7.4). Penicillin G (Ern, S.A., Barcelona, Spain), streptomycin (Reig Jofré S.A., Barcelona, Spain) and dimethylsulfate were also used.

2.5. In vitro epimastigote susceptibility assay

The screening assay was performed in 96-well microplates with cultures that had not reached the stationary phase (Vega et al., 2005). Briefly, epimastigotes were seeded at 1×10^5 mL^{-1} in 200 μL of liver tryptose broth medium. The plates were then incubated with the drugs (0.1–50 μg/mL) at 28 °C for 72 h, at which time 50 μL of CPRG solution was added to give a final concentration of 200 μM. The plates were incubated at 37 °C for an additional 6 h and were then read at 595 nm. Each experiment was performed twice and independently, each concentration was tested in triplicate in each experiment. The efficacy of each compound was estimated by calculating the anti epimastigotes percentual (AE%).

2.6. In vitro leishmanicidal assay

The assay was performed using a modification of a previous method. Cultures of promastigotes of *L. brasiliensis* were grown to a concentration of 10^6 cells/mL and then transferred to the test. The compounds were dissolved in DMSO to the concentrations to be tested and were transferred to microplates. Each test was performed in triplicate. The activity of compounds was evaluated after 72 h by direct counting of cells after serial dilutions and compared with an untreated control.

2.7. Cytotoxic assays

NCTC929 fibroblasts were plated in 96-well microplates at a final concentration of 3×10^4 cells/well. The cells were grown at 37 °C in an atmosphere of 5% CO_2. After that, the culture medium was removed and the compounds were added to 200 μL, and performed a new culture for 24 h. After this incubation, 20 μL of a 2 mM solution of resazurin was added to each well. The plates were incubated for 3 h and the reduction of resazurin was measured using dual absorbance at wavelengths of 490 and 595 nm. The value of the control (blank) was subtracted. Each concentration was tested in triplicate.