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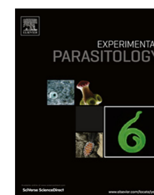

Research Brief

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*.

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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, ethyl-acetate 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 $1 \times 10^5\ mL^{-1}$ 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.

2.8. Chemical, apparatus and general procedures

All chemical were of analytical grade. Methanol, acetic acid, gallic acid, chlorogenic acid and caffeic acid purchased from Merck (Darmstadt, Germany). Quercetine, rutine and kaempferol were acquired from Sigma Chemical Co. (St. Louis, MO, USA). High performance liquid chromatography (HPLC-DAD) was performed with the HPLC system (Shimadzu, Kyoto, Japan), Prominence Auto Sampler (SIL-20A), equipped with Shimadzu LC-20AT reciprocating pumps connected to the degasser DGU 20A5 with integrator CBM 20A, UV–VIS detector DAD (diode) SPD-M20A and Software LC solution 1.22 SP1.

2.9. Quantification of compounds by HPLC-DAD

Reverse phase chromatographic analyses were carried out under gradient conditions using C_{18} column (4.6 × 250 mm) packed with 5 μm diameter particles; the mobile phase was water containing 2% acetic acid (A) and methanol (B), and the composition gradient was: 5% of B until 2 min and changed to obtain 25%, 40%, 50%, 60%, 70% and 100% B at 10, 20, 30, 40, 50 and 80 min, respectively, following the method described by Laghari et al. (2011) with slight modifications. The samples of the fern were analyzed, dissolved in ethanol at a concentration of 3 mg/mL. The presence of six phenolics compounds was investigated, namely, gallic, chlorogenic and caffeic acids and the flavonoids quercetin, rutin and kaempferol. Identification of these compounds was performed by comparing their retention time and UV absorption spectrum with those of the commercial standards. The flow rate was 0.6 mL/min, injection volume 40 μL and the wavelength were 254 nm for gallic acid, 325 nm for caffeic and chlorogenic acids, and 365 nm for quercetine, rutine and kaempferol. All the samples and mobile phase were filtered through 0.45 μm membrane filter (Millipore) and then degassed by ultrasonic bath prior to use. Stock solutions of standards references were prepared in the HPLC mobile phase at a concentration range of 0.020–0.200 mg/mL for kaempferol, quercetin and rutin; and 0.050–0.250 mg/mL for gallic, caffeic and chlorogenic acids. The chromatography peaks were confirmed by comparing its retention time with those of reference standards and by DAD spectra (200 to 400 nm). Calibration curve for caffeic acid: $Y = 10523x + 1478.8$ ($r = 0.9999$); caffeic acid: $Y = 12765x + 1381.7$ ($r = 0.9995$); rutin: $Y = 12691 - 1165.0$ ($r = 0.9998$); quercetin: $Y = 13495x - 1092.6$ ($r = 0.9999$) and kaempferol: $Y = 15692x - 1218.1$ ($r = 0.9997$). All chromatography operations were carried out at ambient temperature and in triplicate.

3. Results and discussion

Several researches use *in vitro* and *in vivo* biological assays against parasites from the Trypanosomatidae family in mammal cell lines or in animal models. According Castilhos (2008), studies against *Leishmania* spp. are focused in the extracellular form of this parasite, called promastigote form due the facility to keep *in vitro* cultures and the possibility of not use other cell lines as macrophages. All these assays are preliminary, but very importance to determine the antiparasitic potential of drugs and natural products to be tested using now *in vivo* assays. The same reason explains the use of epimastigote forms of *T. cruzi*. This cell form is present inside the macrophage of the vertebrate host (Michalick, 2005).

Cytotoxic *in vitro* assay is the first test to evaluate the biocompatibility of any material for use in biomedical devices (International Standard, 1992). In most cases, the results are not extrapolated directly to the animals, because if a product causes damage to cells *in vitro*, may also affect animals exposed (Carvalho, 1996).

The most widely used parameter to assess cytotoxic activity is the cell viability, which can be detected by the use of dyes as neutral red, which allows the distinction between living cells and damaged or dead (Ciapetti et al., 1996).

Different products have been tested to evaluate the cytotoxic potential, allowing the possibility of discovering new drug candidates, whose side effects are lower. Among plants, the angiosperms are the most studied. Different cells can be used in these assays, as astrocytes, macrophages and human or murine fibroblasts, or brine

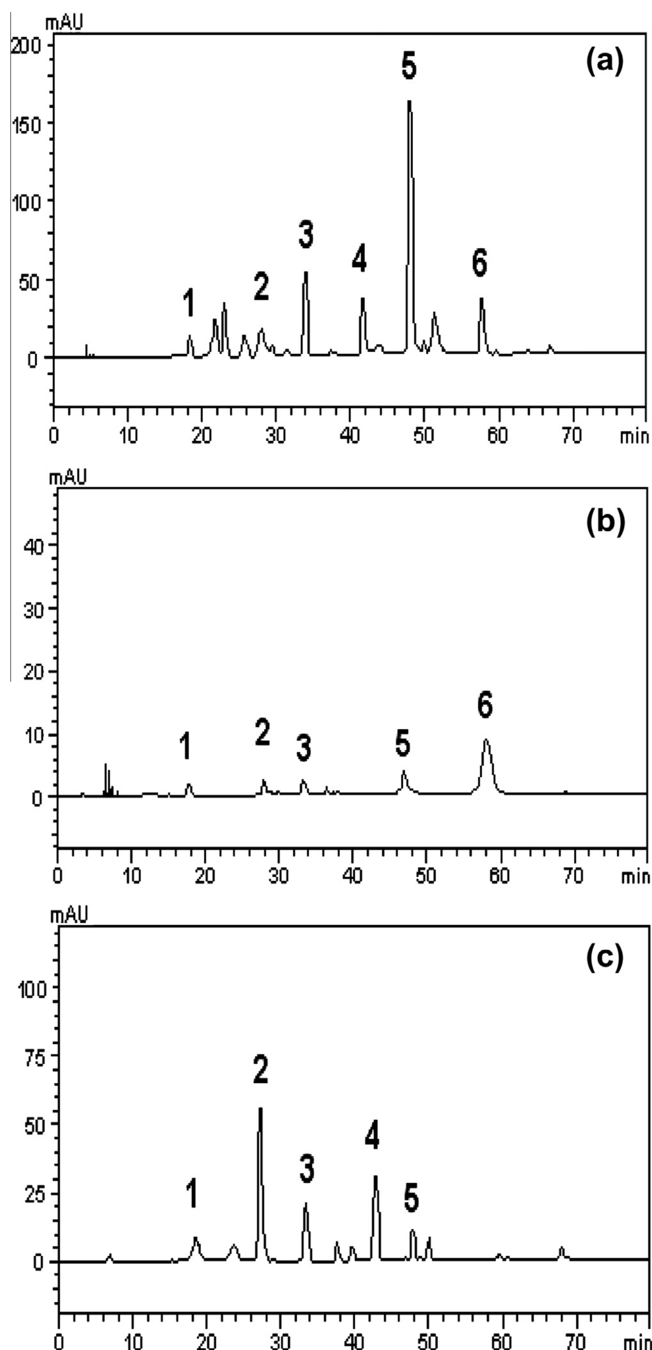

Fig. 1. Representative high performance liquid chromatography profile of ethyl acetate fraction (a), dichloromethane fraction (b) and methanol fraction (c) detection UV was at 327 nm. Gallic acid (peak 1), chlorogenic acid (peak 2), caffeic acid (peak 3), rutin (peak 4), quercetin (peak 5) and kaempferol (peak 6).

Table 1
In vitro activity of fractions of ethanol extract of leaves of *L. venustum* against *L. brasiliensis* and *T. cruzi* and cytotoxicity.

Fractions	Concentrations (μg/mL)	(%C)	Antiparasitic activity	
			(%EA)	(%PA)
Dichloromethane	500	0	34	6
	100	0	0	0
Ethylacetate	500	67	45	0
	100	30	45	0
Methanol	500	53	63	68
	100	0	28	31

%C: percentage of killing fibroblasts; %EA: percentage of killing epimastigotes of *T. cruzi*; %PA: percentage of killing promastigotes of *L. braziliensis*.

shrimp as *Artemia salina* (Martins et al., 2009; Nakamura et al., 2006; Hughes et al., 2006; Lima et al., 2009).

A qualitative chemical screening using the methodology of Matos (1997) showed that the ethanol extract of *L. venustum* presents in its composition of secondary metabolites (data not shown), such as flobabenic tannins, flavones, xanthones, chalcones, flavonoids, alkaloids and flavonones and some of these compounds have already demonstrated antiparasitic potential (Torres-Santos et al., 1999; Kam et al., 1999; Leite et al., 2010).

HPLC fingerprinting of *L. venustum* fractions revealed the presence of the gallic acid (t_R = 17.83 min; peak 1), chlorogenic acid (t_R = 28.14 min; peak 2), caffeic acid (t_R = 34.09 min; peak 3), rutin (t_R = 42.11 min; peak 4), quercetin (t_R = 49.78 min; peak 5) and kaempferol (t_R = 58.96 min; peak 6) (Fig. 1). The HPLC analysis revealed that flavonoids (quercetin, rutin and kaempferol) and phenolics acids (gallic, chlorogenic and caffeic acids) are present in the fern (Table 2).

Amongst the evaluated fractions, the dichloromethane fraction had not demonstrated antiparasitic activity against both parasites at 100 μg/mL and a low activity against *T. cruzi* at 500 μg/mL. The main phenolic compound present in this fraction is the kaempferol (0.39%), indicating a low level of phenolic compounds. Kaempferol and its acethylated derived had demonstrated an *in vitro* anti-*Trypanosoma* activity using mammal cell lines, affecting the metacyclic forms of this parasite, the replications and the transformation to trypomastigote forms (Boutaleb-Charki et al., 2011). However, this effect was observed when the kaempferol was assayed alone. When assayed mixed with other phytocompounds, its activity can be inhibited or not, being necessary a high concentrations to be observed the same effect. Te cytotoxic assay indicates that the phytocompounds extracted using non-polar solvents demonstrated a low toxicity against fibroblasts (Table 1).

The ethylacetate fraction is well known about the efficiency to extract flavonoids (Simões et al., 2010). This fraction had not any effect against *L. brasiliensis* in the assayed concentrations, but was observed 45% of inhibition against the epimastigote form. These results suggest that epimastigote form is more sensitive than the amastigote form to the phenolic compounds in this fraction. Besides, according Turnock and Ferguson (2007), the glycoconjugates in the cell surface of the trypanosomatidae parasites are involved in the surveillance, virulence and infectivity of the parasites on yours vectors and mammal hosts. These authors identified monosaccharides present also in *T. cruzy* and *L. major* (GDP-α-D-mannose, UDP-α-D-*N*-acetylglucosamine, UDP-α-D-glucose, UDP-α-galactopyranose, GDP-ß-L-fucose and UDP-α-D-galactofuranose), present only in *T. cruzi* (UDP-ß-L-rhamnopyranose, UDP-α-D-xylose and UDP-α-D-glucuronic acid) and GPD-α-D-arabinopyranose, present only in *L. major*. The presence or absence of these compounds in the parasites cells can determine the selective uptake of determined phytocompounds.

The main compound of the ethylacetate fraction of *L. venustum* is quercetine (5.98%). Several reports indicate that this flavonoid present an remarkable antiparasite activity against *Leishmania donovani* (EC_{50} 1 mg/mL), but not against *T. cruzi* (Tasdemir et al., 2006). In this study, the activity of quercetine apparently was antagonized by other phytocompounds of this fraction. However, was observed an expressive cytotoxic activity, killing 67% of fibroblasts at 500 μg/mL (Table 1).

The best results against the promastigote and epimastigote forms of *L. brasiliensis* and *T. cruzi* respectively were observed in the assays with the methanol fraction, killing 68% and 63% of the parasites cell forms at 500 μg/mL (Table 1). The main phenolic compound detected in this fraction was the Chlorogenic acid (1.13%). A moderate leishmanicidal activity of the chologenic acid was detected in the study of Kirmizibekmez et al. (2004), being similar with our results. By other side, the antiparasitic activity of this fraction can be attributed to the other phytocompounds due the fact of using a polar solvent as methanol, the extraction of heterosides and alkaloids can be enhanced (Simões et al., 2010). Other studies had demonstrated that these other secondary metabolites present a relevant antiparasitary activity against *Leishmania* and *Tripanosoma* (Bravo et al., 2001; Batista et al., 2007; Fournet et al., 2007). The cytotoxic activity demonstrated by this fraction was considered moderate (53%).

The analysis of biochemical factors involved in *Leishmania*- and *Trypanosoma*-host interactions shows that these parasites have metabolic pathways similar to those found in host cells. This condition makes the treatment of the diseases caused by these protozoa is difficult (Soares-Bezerra et al., 2004). So, the research of new drugs with antiparasitic activity and low toxicity is very important, mainly to be used as therapeutic alternatives against these neglected diseases.

4. Conclusion

Until this study, no species of the family Lygodiaceae had been tested against parasites of the genus *Leishmania* and *Trypanosoma*, as well as never before had their cytotoxic effects observed. So, this is the first report on bioactivity and cytotoxicity against parasites by a species of this pteridophytes group. This fact indicates the necessity of study these plants as a future source of natural products with antiparasitic and other activities.

Table 2
Phenolics and flavonoids composition of *Lygodium venustum* fractions.

Natural products	Gallic acid	Clorogenic acid	Caffeic acid	Rutin	Quercetin	Kaempferol
EAFLV mg/g	3.07 ± 0.01a	6.65 ± 0.03b	13.02 ± 0.04c	10.74 ± 0.02d	59.83 ± 0.01e	11.09 ± 0.01 cd
EAFLV%	0.30	0.66	1.30	1.07	5.98	1.10
DFLV mg/g	0.62 ± 0.03a	0.57 ± 0.01b	0.71 ± 0.01ab	–	0.80 ± 0.02b	3.93 ± 0.01c
DFLV%	0.06	0.05	0.07	–	0.08	0.39
MFLV mg/g	2.02 ± 0.03a	11.39 ± 0.02b	3.91 ± 0.03c	6.16 ± 0.01c	1.88 ± 0.01a	–
MFLV%	0.20	1.13	0.39	0.61	0.18	–

Results are expressed as mean ± standard deviations (SD) of three determinations. Averages followed by different letters differ by Tukey test at $p < 0.005$. EAFLV: ethyl acetate fraction of *L. venustum*; DFLV: dichloromethane fraction of *L. venustum*; MFLV: methanol fraction of *L. venustum*.

Conflict of interest

The authors declare that they have no conflict of interests.

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