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In vitro antileishmanial activity of leaf and stem extracts of seven Brazilian plant species

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Abstract

Ethnopharmacological relevance

Leishmaniasis is a parasitic disease that affects people all over the world. The number of cases of leishmaniasis is increasing and the drugs used for its treatment are toxic and not always effective. The recognition of the global nature of this disease and its direct or indirect effects on health economics and actions focuses attention on the development of new therapeutic options. In Brazil, this parasitic disease is endemic in many regions. The plants used by the population against leishmaniasis can be good starting points in the search of new lead compounds for antileishmanial drugs.

Aim of the study

The aim of the present study was to investigate the antileishmanial activity of extracts from leaves and stems of seven Brazilian plant species used by the population to treat leishmaniasis, and symptoms that might be related to Leishmania infections.

Materials and methods

Twenty two extracts from seven plants belonging to five different botanical families were prepared by different methods and evaluated for their effect on the viability of promastigote forms of Leishmania infantum (MHOM/BR/1967/BH46) using the resazurin-based colorimetric assay. The extracts were considered active when they inhibited the growth of promastigotes in a percentage greater than or equal to 50 % at 100 and 200 µg/mL. The active samples were further investigated to determine IC₅₀, CC₅₀ and SI values against promastigote forms of L. infantum. The active and non-cytotoxic extracts (SI> 10) were evaluated against amastigote forms of L. infantum. In addition, the active extracts against the amastigote forms were analyzed by TLC and HPLC, while the EtOAc extract of stems from Aspidosperma tomentosum was also evaluated by GC/MS.

Results
Among the twenty two extracts evaluated, two were considered active against *L. infantum*. The EtOH extract of leaves from *Dyospiros hispida* (IC$_{50}$ 55.48 ± 2.77 µg/mL and IC$_{50}$ 80.63 ± 13.17 µg/mL, respectively) and the EtOAc extract of stems from *Aspidosperma tomentosum* (IC$_{50}$ 9.70 ± 2.82 µg/mL and IC$_{50}$ 15.88 ± 1.53 µg/mL, respectively) inhibited significantly the growth of promastigote and amastigote forms of *L. infantum*. Some extracts, although active in the initial screening, were considered toxic since the SI was lower than 10. In TLC and HPLC analysis the leaf extract of *Dyospiros hispida* showed the presence of antraquinones, terpenes and saponins, and in the EtOAc extract of stems from *Aspidosperma tomentosum* alkaloids and flavonoids were detected. In addition, in the latter extract the indole alkaloids uleine and dasycarpidone could be identified by GC/MS.

**Conclusions**

The ethnopharmacological data of *Aspidosperma tomentosum* and *Dyospiros hispida* in part support the results found in the biological models used. Extracts of *Aspidosperma tomentosum* and *Dyospiros hispida* presented promising results against *L. infantum*.

**Keywords**

*Leishmania infantum*, promastigotes forms, amastigotes forms, plant extracts, Brazilian species
1- Introduction

Leishmaniasis is a neglected infectious disease that affects nearly 98 countries around the world and represents a serious health problem worldwide (Souza et al., 2018). In Brazil, this infection is endemic in many regions. According to Brazil’s Ministry of Health in 2016 the Northeast region registered the highest number of cases of visceral leishmaniasis (VL) with 1523 cases followed by the southeast with 592 cases (Brasil, 2017a, Brasil, 2017c). Considering tegumentary leishmaniasis (TL), the highest number of cases occurred in the north region with 5075 cases registered followed by the Northwest with 3176 cases (Brasil, 2017a; Brasil, 2017c). From the period of 2005 to 2016, the number of leishmaniasis cases in Brazil has decreased. In the worldwide, 1.5 to 2 million people are affected annually by this infection and 350 million are at risk of contracting the disease (Torres-Guerrero et al., 2017; Souza et al., 2018). One the reasons for the increase in the number of new cases is the growing resistance of parasites to conventional chemotherapy, plus the difficulties in controlling the vectors and environmental changes (Shaw, 2007; Freitas–Junior et al., 2012). In addition, this disease can present itself in serious and lethal forms associated with malnutrition and co-infections (Gontijo and Melo, 2004; Luz et al., 2018). On the other hand, it has received little or no attention from the pharmaceutical industries (Micheletti and Beatriz, 2012). There are a limited number of drugs that are clinically employed and the treatment is based on pentavalent antimonial compounds (sodium stibogluconate and meglumine antimonate), amphotericin B, miltefosine, paromomycin and pentamidine. Their effectiveness depends on the clinical form of the disease, the age of the patient, and region of residence. All the available drugs have many drawbacks such as severe side effects, long treatment cycles, poor efficacy and an increasing resistance of the parasites to the drugs (Hotez et al., 2007).

Natural products (NP) continue to be a good source of new lead compounds despite the development in drug discovery technology and the reduced interest in NP by industries (Newman
and Cragg, 2016). The antileishmanial activity exhibited by plant extracts has been attributed to compounds belonging to different groups of secondary metabolites (Chan-Bacab and Pena-Rodrigues, 2001, Rocha et al., 2005, Tastemir et al., 2006; Singh et al., 2014) such as quinones, alkaloids, iridoids and flavonoids (Singh et al., 2014).

Our group has been researching into native species of the Brazilian Cerrado with the aim of discovering new bioactive compounds with the therapeutic potential to treat several diseases, including neglected diseases. The aim of this study was to investigate the antileishmanial activity of extracts from leaves and stems of seven Brazilian species against the promastigote and amastigote forms of Leishmania infantum (BH46 strain).

2- Material and methods

2.1 Plant material

The species (Table 1) were selected based on their ethnopharmacological use to treat symptoms that might be related with leishmaniasis such as fever and wound healing. The plant materials were collected, identified and voucher specimens were deposited at the Herbarium PAMG of Empresa de Pesquisa Agropecuária de Minas Gerais. The access and remittance of components of the genetic heritage were performed according to the authorization for scientific research from CNPq (Nº 010310/2013-4). Stems and leaves of plant material were dried at 40 °C in an air circulating oven and powdered.
**Table 1**- Brazilian plant species studied for their activity against the promastigote and amastigote forms of *Leishmania infantum* (strain BH46)

<table>
<thead>
<tr>
<th>Species / Family</th>
<th>Voucher</th>
<th>Vernacular name</th>
<th>Traditional uses</th>
<th>Biological activity</th>
<th>Date / Collection site</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Aspidosperma tomentosum</em> Mart. Apocynaceae</td>
<td>PAMG 57593</td>
<td>Peroba-do-campo</td>
<td>Malaria, leishmaniasis, cancer, inflammation, fever, rheumatism</td>
<td>Anticholinesterase, anti-hypertensive, antiinociceptive, anti-inflammatory, analgesic activity against <em>Plasmodium falciparum</em> 3D7 strain and W2 strain</td>
<td>27/01/15 São José de Almeida – Jaboticatubas - S 19° 25' 00.7&quot; WO 43° 47' 06.2&quot;</td>
<td>Kohn et al., 2006; Albernaz et al., 2010; Dolabela et al., 2012; Aquino et al., 2013.</td>
</tr>
<tr>
<td><em>Aspidosperma subincanum</em> Mart. ex A. DC. Apocynaceae</td>
<td>PAMG 57595</td>
<td>Pau-pereira, guatambu</td>
<td>Fever, cardiovascular diseases, hypertension and erectile dysfunction</td>
<td>Activity against <em>Plasmodium falciparum</em>, increase in diuresis and electrolyte excretion of Na⁺ and K⁺ in rats, acetylcholinesterase inhibitory activity, antioxidant and antimicrobial against <em>Staphylococcus aureus</em></td>
<td>27/01/15 São José de Almeida – Jaboticatubas-S 19° 25' 48.0&quot; WO 43° 48' 29.2&quot;</td>
<td>Gilbert et al., 1965; Kobayashi et al., 2002; Ribeiro et al., 2015; Rocha et al., 2018 Almeida et al., 2018; Bernardes et al., 2013;</td>
</tr>
<tr>
<td><em>Piptocarpha rotundifolia</em> (Less.) Baker Asteraceae</td>
<td>PAMG 52485</td>
<td>Infalível, coração-de-negro, paratudo</td>
<td>Anti-syphilis, wound healing</td>
<td>Larvicidal activity against 3rd. stage <em>Aedes aegypti</em> larvae, molluscicidal activity</td>
<td>27/05/08 São José de Almeida – Jaboticatubas Cerrado</td>
<td>Mendes et al., 1999; Rodrigues et al., 2006; Mesquita et al., 2007;</td>
</tr>
<tr>
<td><em>Myrsine umbellata</em> Mart. Primulaceae</td>
<td>PAMG 52500</td>
<td>Pororoca, capororoca</td>
<td>To treat snake bites, tumors and wounds</td>
<td>Not reported</td>
<td>16/06/08 São José de Almeida - Jaboticatubas S 19° 26' 080&quot; WO 43° 48' 668&quot;</td>
<td>Rodrigues and Carvalho, 2001; Watzlawick, 2005; Rodrigues and Carvalho, 2010a; Rodrigues and Carvalho, 2010b</td>
</tr>
<tr>
<td><em>Diospyros hispida</em> A. DC. Ebenaceae</td>
<td>PAMG 56316</td>
<td>Olho de boi, fruta-de-jacú, fruta-de-boi, caqui-do-mato, caqui-do-cerrado, caqui-bravo</td>
<td>To treat infectious diseases, malaria, leprosy, skin eruptions, eye infections and fever</td>
<td>Activity against <em>Plasmodium falciparum</em> FcB1 strain, <em>Leishmania</em> (L.) <em>chagasi</em>, <em>Trypanosoma cruzi</em> and NIH-3T3 mammalian cells</td>
<td>28/06/11 São José de Almeida – Jaboticatubas S 20° 04’ 193&quot; WO 43° 48’ 399&quot;</td>
<td>Albernaz et al., 2010; Ribeiro et al., 2014</td>
</tr>
<tr>
<td><em>Zanthoxylum riedelianum</em> Engl. Rutaceae</td>
<td>PAMG 52459</td>
<td>Mamica-de-porca</td>
<td>To treat inflammation, rheumatism and skin rashes</td>
<td>Anti-inflammatory and analgesic activities of the ethanolic extracts from leaves and stem barks</td>
<td>23/04/08 São José de Almeida – Jaboticatubas S 19° 25’ 834&quot; WO 43° 48’ 506&quot;</td>
<td>Ferreira et al., 2002; Lima et al., 2007; Fernandes et al., 2009; Beirigo et al., 2016</td>
</tr>
<tr>
<td><strong>Esenbeckia febrifuga</strong> (A. St. Hill) Juss ex. Mart.</td>
<td><strong>PAMG 56829</strong></td>
<td><strong>Jasmim do mato, quina-do-mato, três folhas</strong></td>
<td><strong>Fever and/or malaria</strong></td>
<td><strong>Active against <em>Leishmania major</em> Friedlin promastigotes, activity against <em>Plasmodium falciparum</em> W-2 strain and 3 D7 strain</strong></td>
<td><strong>25/06/2012</strong> Fazenda Experimental de Pitangui S 19° 42' 54,8&quot; W 44° 54' 04,6&quot;</td>
<td><strong>Napolitano et al., 2004; Dolabela et al., 2008</strong></td>
</tr>
</tbody>
</table>
2.2 Extract preparation by sonication

The extracts of *Aspidosperma subincanum*, *Diospyros hispida*, *Zanthoxylum riedelianum* and *Esenbeckia febrifuga* (Table 1) were prepared by sonication in an ultrasonic bath at 40 KHz (Unique® Maxiclean 1400 model) for 40 min with ethanol 95 °GL (Vetec Química Fina, Brazil). The obtained extracts were filtered and concentrated using a rotary evaporator (Buchi Waterbath B-480 model) at temperatures below 40 °C. The individual yields (%, w/w) were calculated.

2.3 Alkaloid enriched extracts

Powdered stems and leaves of *Aspidosperma subincanum* and *Esenbeckia febrifuga* were mixed thoroughly with a 10% ammonia solution and then extracted with dichloromethane (Vetec Química Fina, Brazil) under Soxhlet reflux. The extracts were filtered and then concentrated using the rotary evaporator (Buchi Waterbath B-480 model) at temperatures below 40 °C.

2.4 Extracts preparation by percolation

Dried powdered stems and leaves of *Aspidosperma tomentosum*, *Piptocarpha rotundifolia* and *Myrsine umbellata* were percolated using sequentially hexane (Vetec Química Fina, Brazil), dichloromethane (Vetec Química Fina, Brazil), ethyl acetate (Vetec Química Fina, Brazil) and methanol (Vetec Química Fina, Brazil) until exhaustion. The obtained percolates were then concentrated using the rotary evaporator (Buchi Waterbath B-480 model) at temperatures below 40 °C.

2.5 Biological assays

2.5.1 In vitro activity of extracts on the viability of *Leishmania infantum* promastigotes
Initially, the extracts were solubilized in DMSO at 100 mg/mL. The susceptibility tests on promastigotes of WHO reference strain of *Leishmania infantum* (MHOM/BR/1967/BH46) were performed for all the extracts at two concentrations 100 and 200 µg/mL by resazurin-based colorimetric assay (Corral et al., 2013). Log-phase *Leishmania infantum* promastigotes (2.5 x 10⁵ parasites/well) were seeded in flat-bottomed 96-well cell culture plates with lid in a complete RPMI 1640 medium and incubated at 26°C with an 5% CO₂ atmosphere. The extracts were tested in triplicate at each concentration. Non-treated parasites were used for viability comparison. The extracts were then incubated for 24h. After the incubation period, 10% v/v of a resazurin solution (0.15 mg/mL in DPBS) was added to the wells and plates and incubated for a further 4h. The fluorescence was measured at 550 nm excitation and 590 nm emission wavelength. Fluorescence intensity was expressed as arbitrary unit s.

The extracts were considered active when they inhibited the growth of the parasite by 50% or more in both concentrations (100 and 200 µg/mL). The inhibitory concentration (IC₅₀) was determined for the active samples using the aforementioned conditions using a resazurin-based colorimetric assay (Corral et al., 2013). The extracts were 2-fold serially diluted over six concentrations (200 to 6.25 mg/mL) in a complete RPMI 1640 medium, and tested in triplicate at each concentration. The results were expressed as mean ± standard deviation and evaluated with origin 8.5 program with a dose-response curve with a sigmoidal fit.

### 2.5.2 Cytotoxicity in LLC cells

Mouse lung cells LLC-1 were maintained at 37 °C, 5 % CO₂ in 75 cm² sterile culture flasks (Corning®) with RPMI 1640 culture medium supplemented with 5% FBS, penicillin (10 U mL⁻¹), and streptomycin (100 g mL⁻¹), with changes of medium twice a week. When confluent, the monolayers were trypsinized (0.05% trypsin/0.5 mM EDTA), washed, counted, diluted in a complete medium,
distributed in 96-well microplates (4 x 10³ cells per well), then incubated for 24 h at 37 °C. After 24h the test samples and controls were diluted to a final concentration of 0.02% DMSO in a culture medium to yield four concentrations in serial dilutions starting at 1,000 mg mL⁻¹ and then added to 96-well microplate, incubated for 24 h at 37 °C and then added 18 µL/well of MTT solution (5mg mL⁻¹ in PBS) followed by another 1h30min at 37°C. The culture plates were read in a spectrophotometer with a 570 nm filter. Each test was performed in duplicate; the concentration that killed 50% of the cells (CC₅₀) was determined. The selectivity index (SI) for the antileishmania activity was then calculated based on the rate between CC₅₀ and IC₅₀ for the in vitro activity against L. infantum (BH46 strain).

2.5.3 In vitro activity of extracts on the viability of Leishmania infantum intracellular amastigotes

Immortalized murine macrophages RAW 264.7 were cultured in complete RPMI 1640 medium and maintained at 37 °C, 5% CO₂ and 95% humidity. The macrophages were seeded (5x10⁴/well) on a 24-well tissue culture plate containing a circular coverslip per well and incubated (37 °C, 5% CO₂) for 4h cell adherence. Then Leishmania infantum promastigotes in late stationary growth phase were added to interact with the macrophages in a proportion of 10 promastigotes/macrophage/well for 24 h. Then test extracts and amphotericin B were added to the wells in two fold dilutions. For the extracts, the highest concentration tested in the intracellular amastigote assays corresponded, on average, to five fold the IC₅₀ obtained for the extract in the promastigote assays and 500 to 31.25 ng/mL for amphotericin B. After 48 h the coverslips were removed and stained with rapid panoptic, mounted in glass slides using Canada balsam and analyzed under a light microscope in order to determine the infection rate of macrophages. The values obtained for each concentration were used to obtain the amastigote intracellular IC₅₀ value for extracts and amphotericin B.
2.6 Phytochemical screening and HPLC analysis

The active extracts against amastigote forms of *Leishmania infantum* (Table 3) were subjected to phytochemical screening by thin-layer chromatography (TLC) while their profiles were obtained by RP-HPLC-DAD. The screening was performed using methods described by Wagner and Bladt (2001) on silica gel 60 F254 aluminium plates (Merck, German) which were carried out according to the conditions described in Table 2. The solvents used for the plant extraction of the plant material were of analytical grade. The extracts and standards were prepared at 1 mg/mL.

<table>
<thead>
<tr>
<th>Class of secondary metabolites</th>
<th>Eluent system (v:v)</th>
<th>Spray reagent</th>
<th>Reference sample</th>
</tr>
</thead>
<tbody>
<tr>
<td>Coumarins</td>
<td>Toluene - chloroform- acetone (11:4:4)</td>
<td>KOH solution 5% + UV 365 nm</td>
<td>Coumarin (Sigma-Aldrich)</td>
</tr>
<tr>
<td>Tannins</td>
<td>Ethyl acetate: methanol: water: (20:3:2)</td>
<td>K$_3$Fe(CN)$_6$ 1%: FeCl$_3$ 2% (1:1) (+) catechin hydrate (Sigma-Aldrich)</td>
<td></td>
</tr>
<tr>
<td>Flavonoids</td>
<td>Ethyl acetate: formic acid: acetic acid: water (55:5:5:12)</td>
<td>AlCl$_3$ 2% in methanol + UV 365 nm</td>
<td>Rutin (Sigma-Aldrich)</td>
</tr>
<tr>
<td>Anthraquinones</td>
<td>Ethyl acetate: methanol: water (81:11:8)</td>
<td>KOH solution 5% + UV 365 nm</td>
<td>Anthraquinone</td>
</tr>
<tr>
<td>Terpenes and steroids</td>
<td>Hexane: ethyl acetate (7:3)</td>
<td>Anisaldehyde-sulfuric acid + UV 365 nm</td>
<td>Lupeol (Sigma-Aldrich)</td>
</tr>
<tr>
<td>Cardiotonic glycosides</td>
<td>Ethyl acetate: methanol: water (81:11:8)</td>
<td>Kedde reagent</td>
<td>Digitoxin (Merck)</td>
</tr>
<tr>
<td>Alkaloids</td>
<td>Ethyl acetate: methanol: water: (95:15:11)</td>
<td>Dragendorff reagent</td>
<td>Quinine sulphate (Extrasynthese)</td>
</tr>
<tr>
<td>Saponins</td>
<td>Ethyl acetate: methanol: water: (20:3:2)</td>
<td>Anisaldehyde-sulfuric acid + 105 °C/5 min</td>
<td>β-escine (Sigma-Aldrich)</td>
</tr>
</tbody>
</table>

In order to obtain RP-HPLC-DAD profiles of the active extracts, the samples were prepared at 10 mg/mL in acetonitrile: water (8:2). HPLC-grade organic solvents (Sigma-Aldrich, China) and ultrapure water (Milli-Q system, Millipore) were employed. After dissolution, samples were submitted to centrifugation at 10000 rpm for 10 min (Eppendorf, model 5415D), and the supernatant was injected in an Agilent Technologies 1200 series HPLC system equipped with diode array detector and a ChemStation data handling system (Agilent Technologies, USA). The separation
was achieved on a reversed phase column Hypersil C-18 column (250 × 4.6 mm, 5 µm). Elution was carried out with water (A) and ACN (B), both acidified with 0.1% formic acid in a linear gradient elution from 5 to 95% of B over 60 min. The peaks were detected using UV absorbance at λ 254 and 280 nm, flow rate of 1.0 mL/min, 40°C and injection volume of 10 µL. The UV spectra of the major peaks were included in the chromatograms.

2.7 Gas chromatography analysis

The ethyl acetate extract of *Aspidosperma tomentosum* stems (AtS-A, 10 mg/mL) was analyzed by GC-MS. The GC–MS analysis was carried out using the GCMS-QP 2010 Ultra (Shimadzu) system with a SH-RTx-5MS capillary column (crossbond 5% diphenyl/95% dimethyl polysiloxane, 30 m × 0.25 mm ID × 0.25 µm, Shimadzu). The instrument was set to an initial temperature of 60°C and maintained at this temperature for 3 min. Then, the oven temperature was increased to 290°C at an increasing rate of 10°C/min and kept at 290°C for 30 min. Injection port temperature was ensured at 220°C and helium flow rate was ensured at 0.92 mL/min. The ionization voltage was 70 eV. The samples were injected in split less mode. Mass spectral scan range was set at 20-500 (m/z). Comparing the obtained data with NIST Library some compounds present in these plant extracts were identified. AtS-A were analysed again by Single Ion Monitoring (SIM) mode in the same conditions as in GC method and MS parameters adjusted to monitoring ions of interest.

3- Results and discussions

Different criteria can be used to select plant species to be investigated aiming to identify bioactive compounds which could became novel drug leads. In this study, the species were selected according to ethnopharmacological criteria.
In the literature, there are many successful examples of drug discovery using the ethnopharmacological criterium, such as the discovery of quinidine and quinine isolated from the bark of Cinchona trees used by the Peruvian Indians to stop their shivering when they were working in cold mines (Meshnick and Dobson, 2001). In the present work, the selection of plant species was based on the literature data reporting their medicinal use mainly in the treatment of symptoms that might be related to Leishmania infections. According to Mohanna (2015), some symptoms of visceral leishmaniasis are fever, pallor, hepatosplenomegaly, coughing, diarrhea, weight loss as well as deterioration in the individual’s general condition. Others symptoms such as wound healing and inflammation can be associated with cutaneous leishmaniasis. The symptoms of malaria could be similar to the aforementioned ones. In order to have a correct diagnosis of leishmaniasis it is necessary to perform laboratorial exams such as the traditional serological tests and the detection of circulating antibody to the leishmanial antigen K39 (Sundar et al., 1998). In addition, a conclusive diagnosis cannot always be obtained in endemic areas of leishmaniasis in Brazil due to their geographical isolation and the difficulties of the population to get access to the health centers.

For this study, it was selected seven plants belonging to five different botanical families, and their vernacular name, traditional use and biological activity described in the literature are presented in the Table 1. Twenty two extracts from the selected plants were prepared by distinct methods and evaluated at two concentrations, 100 and 200 µg/mL, against promastigote forms of Leishmania infantum (Table 3). The extracts were considered actives when they inhibited the growth of the parasite by 50 % or more in both concentrations (100 and 200 µg/mL). Eight extracts were considered actives in this primary screening. They were further investigated to determine IC_{50}, CC_{50} and SI values (Table 3) against promastigote forms of Leishmania infantum. Finally, active and non-cytotoxic extracts in the aforementioned assays (SI higher than 10) were tested for their
efficacy against intracellular amastigotes of *Leishmania infantum* (Table 3). Amphotericin B was used as a positive control.

Among the samples evaluated, the most effective extract against promastigote and amastigotes forms of *Leishmania infantum* was the EtOAc extract from *Aspidosperma tomentosum* stems (AtS-A). This extract showed CC\textsubscript{50} >1000.00 µg/mL for the mouse lung cells (LLC-1), with an IC\textsubscript{50} 9.70 ± 2.82 µg/mL and 15.88 ± 1.53 to promastigote and amastigote forms of *Leishmania infantum*, respectively, resulting in a selectivity index (SI) above 104.49 (promastigote form) and 62.97 (amastigote form), hence being considered non-toxic. Extracts with CC\textsubscript{50} values on mammalian cells above 250 µg/mL and SI greater than 10 are considered safe for an initial evaluation (Albernaz et al., 2010). The MeOH extract from *Aspidosperma tomentosum* stems (AtS-M) showed an IC\textsubscript{50} 28.14 µg/mL against *Leishmania infantum* promastigote forms, CC\textsubscript{50} >1000.00 µg/mL against the LLC-1 cells, resulting in a selectivity index (SI) of 35.53. However, this extract was inactive against intracellular amastigotes. The EtOAc extract from *Aspidosperma tomentosum* leaves (AtL-A) was considered toxic since its SI was <10. This extract showed an IC\textsubscript{50} 20.0 ± 0.4 µg/mL and CC\textsubscript{50} 50.48 ± 4.86, resulting in a SI of 2.96. The extracts obtained with hexane and DCM from leaves and stems of *Aspidosperma tomentosum* were not tested due to their low solubility in the test conditions. From the leaves and barks of *Aspidosperma tomentosum* many indole alkaloids and flavonoids have been isolated (Epifânio et al., 2007, Pereira et al., 2007; Aquino et al., 2013). The presence of these groups of metabolites were confirmed in this study by both TLC screening and the HPLC exploratory profile. At last, the chromatogram obtained at λ 254 nm for the EtOAc extract of stems of *Aspidosperma tomentosum* (AtS-A) by RP-HPLC (Figure 1A) presented two major peaks with retention time (rt) 15.525 and 19.378 min. The UV spectra obtained online for the first peak showed absorption bands with λ\textsubscript{max} at 245, 270 and ca 360 nm while the UV spectra obtained for
the second peak showed absorption bands with $\lambda_{\text{max}}$ at 220, 255 and 370 nm. These absorption profile is typical of flavonoids (Harborne et al., 1975).

In the GC-MS analysis of EtOAc extract of *Aspidosperma tomentosum* stems (AtS-A) the presence of indole alkaloids was observed. Uleine (rt 23.88 min, $m/z$ 266) and dasycarpidone (rt 24.24 min, $m/z$ 268) were identified (Figure 2A and B) by comparing the data from GC-MS with the NIST Library. Both alkaloids have shown antiplasmodial activity (Dolabela et al., 2015; Rocha e Silva et al., 2012; Andrade-Neto et al., 2007) and the AtS-A antileishmanial activity can be possibly associated with this class of compounds.

The mass spectra of uleine (2C) showed the molecular ion peak at $m/z$ 266. Additional characteristic signals were detected at $m/z$ 237, 223, 209, 194, 180 and 167. The major fragmentation pathways proposed for uleine are shown in Figure 3. The breaking of the bond between C-14/15 leads to the formation of an allylic radical with the subsequent loss of the ethyl radical and formation of fragment $m/z$ 237. The loss of $\text{C}_2\text{H}_4\text{NHCH}_3$ neutral from the fragment at $m/z$ 237 affords the fragment at $m/z$ 181. The formation of the fragment at $m/z$ 209 (base peak) could be explained by breakage of the bond between C-21/N4 and loss of a $\text{C}_2\text{H}_4\text{NHCH}_3$ radical from the molecular ion peak at $m/z$ 266 forming an allylic radical. Another way proposed for the formation of the fragment at $m/z$ 209 is a neutral loss of $\text{C}_2\text{H}_4\text{NHCH}_3$. Despite the compatibility between the mass spectra of uleine (Aquino, 2006) and the obtained spectrum, there is a possibility that the compound with rt 23.88 min ($m/z$ 266) is its epimer, *epi*-uleine (Figure 2). Both diastereoisomeric compounds have been described in *Aspidosperma tomentosum* but they cannot be distinguished using only mass spectrometry.

The mass spectra of dasycarpidone (2D) showed its molecular ion peak at $m/z$ 268. Additional characteristic signals were detected at $m/z$ 211 (base peak), 198 and 168 with a fragmentation pattern similar to that observed for uleine (Figure 3). Likewise as for uleine, there is
a possibility that the compound with rt 23.88 min (m/z 266) could be epi-dasyccarpidone which is an epimer of 2b (Figure 2).

Both extracts of *Aspidosperma subincanum* were considered active and non-toxic. The EtOH extract of leaves showed an IC50 of $18.23 \pm 2.64 \mu g/mL$ and CC50 of $215.66 \pm 38.93 \mu g/mL$ for the LLC-1 cells, resulting in an SI of 11.82. However, this extract promoted lysis in macrophages during the tests with intracellular amastigotes, suggesting its cytotoxicity for this cell line. The alkaloidic extract of leaves presented IC50 of $20.29 \pm 0.88 \mu g/mL$ against *Leishmania infantum* promastigote forms and CC50 $>1000.00 \mu g/mL$ for the LLC-1 cells, resulting in a SI of 49.28, being considered non-toxic. Nevertheless, it was not active against intracellular amastigotes at the concentrations tested (IC50 $>100\mu g/ml$).

The ethanolic extracts of leaves (DhL-E) and stems (DhS-E) of *Diospyros hispida* were also considered active in the preliminary assays. The DhL-E showed IC50 of $55.48 \pm 2.77 \mu g/mL$ and DhS-E showed IC50 of $42.65 \pm 7.44 \mu g/mL$ against *Leishmania infantum* promastigote. Both extracts showed CC50 $>1000.00 \mu g/mL$ for the LLC-1 cells, resulting in a selectivity index (SI) of 18.2 and 23.44, respectively. The extracts were, thus, considered non-toxic.

The *in vitro* assays against intracellular amastigotes demonstrated that only the extract DhL-E was active. The DhS-E extract presented cytotoxicity for the RAW 264.7 macrophage cell line, although, it was well tolerated by the LLC-1 cell line.

The species *Diospyros hispida* is traditionally used to treat infectious diseases. In studies carried out by Albernaz and co-workers (2010), an EtOAc extract of roots of *D. hispida* showed activity against chloroquine-resistant *Plasmodium falciparum* FcB1/Colombia strain (IC50 1 \mu g/mL), *Cryptococcus neoformans* LMGO 02 with (MIC 15.62 \mu g/mL) and promastigotes of *Leishmania* (*Leishmania*) chagasi (MCER/BR/79/M6445) (IC50 18.9 \mu g/mL). However, in a study by Ribeiro and co-workers (2014), *D. hispida* did not show activity against *Leishmania amazonensis*
In the TLC screening of the ethanolic extract from leaves of this specie, quinones, terpenes, steroids, tanins and saponins were detected. These results are in accordance with the data reported in the literature, which describe the presence of steroids, terpenoides and naphthoquinones in this genus (Mallavadhani et al., 1998; Nematollahi et al., 2012). In the HPLC profile of DhL-E (Figure 1B) were observed three peaks with rt 1.161, 10.588 and 12.573 min. UV spectra obtained online for the first peak presented two absorption bands with $\lambda_{\text{max}}$ at 225 and 280nm, characteristic of naphthoquinones. The other two peaks presented UV spectra with two absorption bands with $\lambda_{\text{max}}$ at 265 and 330 nm.

Although the alkaloid-enriched extract from the leaves of *Esenbeckia febrifuga* (EfL-Al) showed activity in the initial antileishmanial screening, this extract was considered toxic since it presented a SI of only 4.56 ($\text{IC}_{50}$ 46.75 ± 1.60 µg/mL for promastigote forms of *Leishmania infantum* and $\text{CC}_{50}$ 213.37 ± 9.85 µg/mL for LLC-1 cells). In a previous work, *Esenbeckia febrifuga* showed significant activity in the *Plasmodium berghei* infected mice, at a dose of 1.0 g/kg, causing 43% inhibition of parasite multiplication (Carvalho et al., 1991; Brandão et al., 1992). Napolitano and co-workers (2004) isolated from leaves of *Esenbeckia febrifuga* the 7-geranyloxycoumarin, that shows significant growth inhibition on the *Leishmania major* Friedlin promastigotes ($\text{LD}_{50}$ 30 µM).
Table 3 - Percentage growth inhibition of promastigotes forms of *Leishmania infantum* (B46 strains) treated with plant extract at 200 µg/mL and 100 µg/mL, IC₅₀ (µg/mL) against promastigote and amastigote forms of *Leishmania infantum* (B46 strains), CC₅₀ (µg/mL) in LLC cells and selectivity t indices (SI) and yield (% w/w)

<table>
<thead>
<tr>
<th>Species</th>
<th>Part of plant/solvent/extraction method</th>
<th>Extract</th>
<th>Percentage growth inhibition</th>
<th>Antileishmania activity IC₅₀ (µg/mL) against L. infantum (BH46 strains)</th>
<th>CC₅₀ (µg/mL) LLC-1 cells</th>
<th>SI (CC₅₀/IC₅₀)</th>
<th>Yield (% w/w)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Aspidosperma tomentosum</em> Mart.</td>
<td>stems/ EtOAc/ percolation</td>
<td>AtS-A</td>
<td>86.35 83.28 9.57 ± 2.82 15.88 ± 1.53</td>
<td>&gt; 1000 &gt; 104.49 &gt; 62.97</td>
<td>9.6</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>stems/ MeOH/ percolation</td>
<td>AtS-M</td>
<td>84.82 79.25 28.14 ± 1.95 Inactive</td>
<td>&gt; 1000 &gt; 35.53 ND</td>
<td>14.3</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>leaves/ EtOAc / percolation</td>
<td>AtL-A</td>
<td>91.78 90.30 20.04 ± 0.83 ND*</td>
<td>50.48 ± 4.86 2.96 ND*</td>
<td>6.0</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>leaves/ MeOH/ percolation</td>
<td>AtL-M</td>
<td>39.45 24.75 ND ND ND</td>
<td>ND ND ND</td>
<td>7.0</td>
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<tr>
<td><em>Aspidosperma subincanum</em> Mart. ex A. DC.</td>
<td>stems/EtOH/ sonication</td>
<td>AsS-E</td>
<td>55.16 30.55 ND ND ND</td>
<td>ND ND ND</td>
<td>2.4</td>
<td></td>
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<tr>
<td></td>
<td>leaves/EtOH/ sonication</td>
<td>AsL-E</td>
<td>85.93 74.21 18.23 ± 2.64 Cytotoxic**</td>
<td>215.66 ± 38.93 11.82 Cytotoxic**</td>
<td>7.1</td>
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<td>stems/alkaloids enriched extract</td>
<td>AsS-Al</td>
<td>72.23 47.64 ND ND ND</td>
<td>ND ND ND</td>
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<td>leaves/alkaloids enriched extract</td>
<td>AsL-Al</td>
<td>85.22 68.44 20.29 ± 0.88 Inactive</td>
<td>&gt; 1000 &gt; 49.28 ND</td>
<td>5.7</td>
<td></td>
<td></td>
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<tr>
<td><em>Piptocarpha rotundifolia</em> (Less.) Baker</td>
<td>leaves/ EtOAc/percolation</td>
<td>PrL-A</td>
<td>64.20 49.08 ND ND ND</td>
<td>ND ND ND</td>
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<td></td>
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<td>leaves/ MeOH/ percolation</td>
<td>PrL-M</td>
<td>55.84 32.60 ND ND ND</td>
<td>ND ND ND</td>
<td>3.5</td>
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<td>stems/ EtOAc/percolation</td>
<td>PrS-A</td>
<td>64.62 40.96 ND ND ND</td>
<td>ND ND ND</td>
<td>0.9</td>
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<td>stems/ MeOH/ percolation</td>
<td>PrS-M</td>
<td>32.84 21.16 ND ND ND</td>
<td>ND ND ND</td>
<td>6.6</td>
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<tr>
<td><em>Myrsine umbellata</em> Mart.</td>
<td>stems/ EtOAc/percolation</td>
<td>MuS-A</td>
<td>44.41 26.15 ND ND ND</td>
<td>ND ND ND</td>
<td>0.6</td>
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<td>stems/ MeOH/ percolation</td>
<td>MuS-M</td>
<td>49.06 45.37 ND ND ND</td>
<td>ND ND ND</td>
<td>13.3</td>
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<td></td>
<td>Extract Method</td>
<td>Concentration</td>
<td>% Reduction</td>
<td>% Selectivity</td>
<td>Cytotoxicity</td>
<td>Amphotericin B</td>
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<td>--------------------------------</td>
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<tr>
<td><strong>Diospyros hispida A. DC.</strong></td>
<td>leaves/MeOH/percolation</td>
<td>MuL-M</td>
<td>53.38</td>
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<td>ND</td>
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<td>leaves/MeOH/percolation</td>
<td>MuL-A</td>
<td>46.34</td>
<td>26.54</td>
<td>ND</td>
<td>ND</td>
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<td>leaves/EtOH/percolation</td>
<td>DhS-E</td>
<td>90.91</td>
<td>89.72</td>
<td>42.65 ± 7.44</td>
<td>&gt; 1000</td>
<td></td>
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<tr>
<td></td>
<td>leaves/EtOH/percolation</td>
<td>DhL-E</td>
<td>87.29</td>
<td>80.27</td>
<td>55.48 ± 2.77</td>
<td>&gt; 1000</td>
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<td><strong>Zanthoxylum riedelianum Engl.</strong></td>
<td>leaves/EtOH/sonication</td>
<td>ZruL-E</td>
<td>41.64</td>
<td>29.33</td>
<td>ND</td>
<td>ND</td>
<td></td>
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<td>leaves/EtOH/sonication</td>
<td>DhS-E</td>
<td>90.91</td>
<td>89.72</td>
<td>42.65 ± 7.44</td>
<td>&gt; 1000</td>
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<td>DhL-E</td>
<td>87.29</td>
<td>80.27</td>
<td>55.48 ± 2.77</td>
<td>&gt; 1000</td>
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<tr>
<td><strong>Esenbeckia febrifuga (A. St. Hill) Juss ex. Mart.</strong></td>
<td>leaves/EtOH/sonication</td>
<td>Efs-E</td>
<td>48.74</td>
<td>30.32</td>
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<td>ND</td>
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<td>stems/EtOH/sonication</td>
<td>Efs-AI</td>
<td>61.60</td>
<td>44.17</td>
<td>ND</td>
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<td>Stems/alkaloids enriched extract</td>
<td>Efs-AI</td>
<td>61.60</td>
<td>44.17</td>
<td>ND</td>
<td>ND</td>
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<tr>
<td></td>
<td>leaves/alkaloids enriched extract</td>
<td>Efs-AI</td>
<td>61.60</td>
<td>44.17</td>
<td>ND</td>
<td>ND</td>
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<td><strong>Amphotericin B</strong></td>
<td>-</td>
<td>Positive control</td>
<td>ND</td>
<td>ND</td>
<td>0.05</td>
<td>0.09</td>
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</tbody>
</table>

**Legend:** EtOAc: ethyl acetate, MeOH: methanol, EtOH: ethanol, ND: percentage of parasite reduction of less than 50% in the highest concentration tested; ND*: selectivity index more than 10; **: cytotoxic for macrophages line cell.
Figure 1 - RP-HPLC-DAD profiles and UV spectra of selected peaks obtained for the active extracts against promastigotes forms of *Leishmania infantum*. (A) Extract obtained with acetyl acetate of stems of *Aspidosperma tomentosum* Mart. (B) Ethanolic extract obtained of leaves of *Diospyros hispida*. **Conditions:**
Mobile phase water (A) and ACN (B) with 0.1% formic acid; gradient elution from 5 to 95% of B in 60 min; column Zorbax XBD (50 cm x 4.6 µm x 1.8 µm); flow 1.0 mL/min; 40°C and injection volume of 10 µL of samples at 10 mg/mL.
Figure 2 - Structures of indole alkaloids and their CG-MS chromatographic profiles. (A) 20-epi-dasycarpidone, dasycarpidone, 20-epi-uleine and uleine structures. (B) Chromatographic analysis by gas chromatography coupled to mass (CG-MS) of *Aspidosperma tomentosum* stems. (C) Mass-spectrum of GC-MS of uleine (rt 23.88 min). (D) Mass-spectrum of GC-MS of dasycarpidone (rt 24.24 min).
Figure 3 - Major fragmentation patterns proposed for uleine obtained from GC-MS (Adapted from Aquino, 2006).

4- Conclusions

Overall, among the twenty two extracts evaluated only two of them were considered active (36.3 %) against promastigote and amastigote forms of *Leishmania infantum* (MHOM/BR/1967/BH46). The species *Aspidosperma tomentosum* and *Diospyros hispida* presented promising results both against promastigotes and intracellular amastigotes and should be further investigated. Additional experiments with these species are in progress aiming to isolate and identify the active constituents. Moreover, the extracts and purified compounds need to be evaluated *in vitro* against other strains of *Leishmania*, and also by in vivo assays.
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Authors’ contributions

CGS, VLA, LP, KF designed the research. AFS was responsible for collecting and identifying the plant species; RCP, KFF, MPR, CPSM carried out the study; SMS e FF designed the biological experiments. SMS, CPSM, PRVC, CGS, JCDL and VLA analyzed the data and wrote the manuscript. VLA supervised the work and provided the facilities for the studies; the authors read and approved the manuscript.

References


