In vitro evaluation of the leishmanicidal potential of selected plant-derived extracts against Leishmania (Leishmania) amazonensis

Abstract

Background: Leishmaniasis is a potentially fatal, neglected parasitic disease caused by different species of Leishmania sp. Natural products, especially from plants; represent a rich source for the screening of potential antiparasitic compounds.

Purpose and study design: In this study, we evaluated the leishmanicidal activity of thirteen plant extracts against the parasite Leishmania (Leishmania) amazonensis in vitro, the cytotoxic and hemolytic activity. The extracts with activity against the parasite, was determined the chemical constituents.

Results: The hexane extracts of Bidens sulphurea and Plectranthus neochilus were the most effective extracts against promastigote forms at 24h and 48h. The EC_{50} (50% effective concentration) value obtained for these extracts against promastigote forms were calculated to be 84.26µg/mL and 46.32µg/mL in 24h, respectively. The EC_{50} values against intracellular amastigotes were higher than 100µg/mL after 48h of incubation for both extracts. Regarding cytotoxicity in peritoneal macrophages, extracts of B. sulphurea showed CC_{50} values (cytotoxicity concentration of 50% of cells) of 103.9 and 80.30µg/mL at 24 and 48h, respectively, whereas the CC_{50} values for the P. neochilus extract were 66.95 and 34.39µg/mL at 24 and 48h, respectively. The extracts showed no significant hemolysis at the concentrations evaluated, and the CH_{50} values were higher than 100µg/mL. The chemical constituent of the hexane extracts of B. sulphurea and P. neochilus and their activity against L. amazonensis has not been previously described.

Conclusion: Despite the unsatisfactory results against amastigotes forms, this study shows extracts obtained from botanical sources merit further study for their leishmanicidal properties.

Keywords: Leishmania (Leishmania) amazonensis, natural products, hexane plant extracts, leishmanicidal activity

Introduction

Leishmaniasis, one of the most important neglected tropical diseases, is endemic in 98 countries, with more than 12million cases and 350million people living in areas at risk of infection.\(^1,2\) This disease is caused by an obligate intracellular protozoan of the genus Leishmania,\(^3\) and is broadly classified into three different forms: visceral leishmaniasis (VL), cutaneous leishmaniasis (CL) and mucocutaneous leishmaniasis.\(^4\) In Latin America, Leishmania (Leishmania) amazonensis is responsible for the cutaneous diffuse form of the disease,\(^5\) that in some cases may also result in visceral leishmaniasis.\(^6,7\) According to the Brazilian Ministry of Health, since 2005, the presence of L. (L.) amazonensis has been present in almost all Brazilian regions,\(^8\) thus raising concern about this infection. The first-line drugs for leishmaniasis treatment are sodium stibogluconate (Pentostan) and meglumine antimoniate (Glucantime); amphotericin B and pentamidine are second-line drugs.\(^9\) However, the current standard-of-care is unsatisfactory due to are expensive, potentially toxic and long-term treatment requirements, resulting in patient non-compliance.\(^2\) Also, there are significant differences in the sensitivity of these species to standard drugs.\(^8,9\)

In the last decade, the scientific investigation of medicinal plants has received considerable attention in drug development against protozoan diseases.\(^10-12\) In this context, the evaluation of plant-derived extracts and isolated natural compounds can result in potential leads for use against infectious diseases. Recently, it was demonstrated that the hexane extracts derived from plants of Asteraceae, Lamiaceae, Myrtaceae, and Verbenaceae families showed promising activity against cariogenic bacteria.\(^13\) As part of our ongoing interest in the antiparasitic activity of natural products and their derivatives, we evaluate here the leishmanicidal potential of thirteen selected plant-derived hexane extracts from the leaves of herbaceous or arbustive plant species (Table 1) against the parasite L. (L.) amazonensis.

Material and methods

Plant material and extraction

Specimens of thirteen species (Table 1) were collected in May 2010 at "Sítio 13 de Maio" (20°26’S 47°27’W 977m), localized near the city of Franca, State of São Paulo, Brazil and identified by Prof. Dr. Milton Groppo. Voucher specimens were deposited at the Herbarium of Departamento de Biologia, Faculdade de Filosofia, Ciências e Letras de Ribeirão Preto, Universidade de São Paulo, São Paulo, Brazil. Leaves of each species were dried carefully in a
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The powdered leaves were extracted with hexane, as previously reported. Three extractions per species were retained, with each extraction lasting 15min. The samples were concentrated using a rotary evaporator under reduced pressure to provide the respective hexane extracts.

**Gas Chromatography (GC) and gas chromatography mass spectrometry (GC-MS) analyses**

Gas chromatography–mass spectrometry (GC–MS) analyses was carried out as previously reported. The chemical components of the hexane extract of Bidens sulphurea were identified on the basis of their retention indices relative to a homologous series of n-alkanes (C<sub>₅</sub>-C<sub>₉</sub>) on a Rtx-5MS capillary column under the same operating conditions and computer matching with the Wiley 7, NIST 08 and FFNSC 1.2 spectral libraries of the GC-MS system.

**Animals**

Male BALB/c mice were maintained under controlled conditions of temperature (22±2°C), humidity (50±10%), and light–dark cycle. All the experiments were authorized by the University of Franca’s Ethics Committee for Animal Care (Approval number: 046/15). All animals were handled using good animal practice as defined by the University of Franca in concordance with Brazilian legislation.

**Parasites**

*L. (L.) amazonensis* (MHOM/BR/PH8) was routinely in M199 and amphotericin B (0.002-1.56µg/mL), 1650. The hexane extracts, as the negative control. The number of amastigotes was determined for 48h at the same conditions described above. Parasites incubated (6.25-100 and 3.12-50μg/mL) and amphotericin B (0.18-3μg/mL) macrophages were incubated with the cells were removed, and the cells were infected with promastigote forms of these species were selected on the basis of previous reports in the literature or on their use as antimicrobial and antiparasitic activities in folk medicine. A preliminary screening of hexane extracts was performed at 100µg/mL against promastigote forms of *L. (L.) amazonensis* to identify the most active extracts at higher concentrations. Five hexane extracts of these species were selected on the basis of previous reports in the literature or on their use as antimicrobial and antiparasitic activities in folk medicine.

**Results and discussion**

Several extracts and compounds isolated from plants have been investigated for their biological properties, including their leishmanicidal activity. In the present study, thirteen hexane extracts from leaves of cultivable herbaceous or arbustive plant species (Table 1) were evaluated against *L. (L.) amazonensis*. The hexane extracts of these species were selected on the basis of previous reports in the literature or on their use as antimicrobial and antiparasitic activities in folk medicine. A preliminary screening of hexane extracts was performed at 100µg/mL against promastigote forms of *L. (L.) amazonensis* to identify the most active extracts at higher concentrations. Five hexane extracts of *Artemisia camphorata, Arrabidaea chica, Eclipta alba, Foeniculum vulgare, Lippia alba* showed no activity after 24h and activity lower than 25% in 48 h of treatment. Six extracts (*Alternanthera brasiliana, Bidens sulphurea, Pluchea capillifolia, Plectranthus neochilus, and Alternanthera brasiliana,* as the negative control). The 50% effective concentration (EC<sub>50</sub>) was calculated as described below. All tests were conducted in triplicate, and three independent assays were performed.

**Cytotoxicity against peritoneal macrophages**

The toxicity to red blood cells was determined as previously described with some modifications. Briefly, erythrocytes were incubated with *B. sulphurea, P. neochilus* and amphotericin B at room temperature for 30 min and hemolysis was determined by the hemoglobin release, quantitated by the absorbance of the supernatants at 415nm. The percentage of lysis was calculated in relation to total lysis. The negative control used erythrocytes with NaCl solution 0.9%, while the positive control used erythrocytes with water. The 50% hemolytic concentration (HC<sub>50</sub>) was calculated as described below. All tests were conducted in triplicate, and three independent assays were performed.

**Statistical analysis**

Data represent the mean number (±SD) of three independent experiments performed in triplicate. The results were compared by analysis of variance, one-way ANOVA, followed by Dunnett’s test to determine significance between the negative control group and treated groups. The EC<sub>50</sub>, CC<sub>50</sub> and HC<sub>50</sub> were calculated using dose–response curves using GraphPad Prism 5 (GraphPad Software, San Diego, California, USA). The SI was calculated using the ratio of CC<sub>50</sub>/EC<sub>50</sub>.
Coreopsis lanceolata, Pelargonium graveolens, Stachytarpheta cayennensis, Senna occidentalis and Tagetes erecta) showed a percentage of inhibition of cell growth of less than 50% at 24 and 48h (Table 2). On the other hand, the hexane extracts of Bidens sulphurea and Plectranthus neochilus were the most effective extracts against promastigote forms of L. (L.) amazonensis at 24h and 48h; they showed a percentage of inhibition of cell growth higher than 90% after 48h (Table 2).

**Table 1** Classification and characteristics of the plant species selected for this study and their respective voucher number

| Family                | Botanical name                        | Voucher number | Biological activities                    | References                                      |
|-----------------------|---------------------------------------|----------------|------------------------------------------|------------------------------------------------|
| Amaranthaceae         | Alternanthera brasiliana (L.) Kuntze   | 10018          | Anti-inflammatory; analgesic              | Moraes et al., 1994; Souza et al., 1998        |
| Apiaceae              | Foeniculum vulgare Mill.              | 12024          | Diuretic; analgesic; antipyretic; antioxidant | Forster et al., 1980; Tanja et al., 1996; Oktay et al., 2003; Itako et al., 2008; Franzener et al., 2003. |
| Asteraceae            | Artemisia camphorata Vill.            | 10006          | Antibacterial; Anti-fungal                |                                                |
|                       | Bidens sulphurea(Capr.) Sch. Bip.      | 12020          | Anti-fungal; eliminating free radicals    | Botsaris 2007                                  |
|                       | Coreopsis lanceolata L.               | 10007          | Antioxidant; Analgesic                    | Crotti et al., 2013; Tanimoto et al., 2009     |
|                       | Eclipta alba (L.) Hassk                | 10008          | Anti-fungal; antiepileptic; antimicrobial | Shaikh et al., 2013; Karthikumar et al., 2007   |
|                       | Tagetes erecta L.                     | 10009          | Antioxidant; eliminating free radicals    | Lorenzi & Souza, 2001; Bashir & Gilani, 2008.  |
| Bignoniaceae          | Arrabidaea chica(Humb.& Bonpl.) B. Verl. | 10013          | Collagen production; antimicrobial; leishmanicidal | Aro et al., 2013; Mafioleti et al., 2013; Rodrigues et al., 2014 |
| Fabaceae              | Senna occidentalis (L.) Link           | 10012          | Toxicity                                 | Barbosa-Ferreira et al., 2011; Barros et al., 1999 |
| Geraniaceae           | Pelargonium graveolens L’ Hér.         | 12023          | Antioxidant; anti-fungal.                | Cάvar et al., 2012; Singh et al., 2008          |
| Lamiaceae             | Plectranthus neochilus Schltr.         | 12323          | schistosomicidal; Antioxidant             | Caixeta et al., 2011; Viana et al., 2011       |
| Verbenaceae           | Lippia alba (Mill.) N.E.Br             | 12022          | Antioxidant; Antimicrobial               | Stashenko et al., 2004; Aguiar et al., 2008    |
|                       | Stachytarpheta cayennensis(Rich.) Vahl.| 10005          | Leishmanicidal; Antimicrobial; Antispasmodic | Moreira et al., 2007; Okoye et al., 2010       |

NR: Not Reporte

**Table 2** Screening in vitro of leishmanicidal activity against L.(L.) amazonensis promastigotes after 24 and 48h of incubation with hexane plant extracts

| Species                             | % Inhibition of cell growth±SD |
|-------------------------------------|--------------------------------|
|                                     | 24 h                           | 48 h                           |
| Alternanthera brasiliana            | 4.12±1.71                      | 6.51±7.05                      |
| Artemisia camphorata                | 0±0                            | 19.13±3.93                     |
| Arrabidaea chica                    | 0±0                            | 16.60±2.80                     |
| Bidens sulphurea                    | 57.34±1.48                     | 92.72±8.05                     |
| Coreopsis lanceolata                | 0.11±0.16                      | 7.63±2.32                      |
| Eclipta alba                        | 0±0                            | 0.69±0.97                      |
| Foeniculum vulgare                  | 0±0                            | 5.66±4.38                      |
| Lippia alba                         | 0±0                            | 21.20±0.96                     |
| Pelargonium graveolens              | 25.88±4.29                     | 41.58±2.67                     |
| Plectranthus neochilus              | 80.23±2.39                     | 92.19±2.62                     |
| Stachytarpheta cayennensis          | 39.38±1.29                     | 40.28±10.90                    |
| Senna occidentalis                  | 12.64±2.04                     | 45.12±3.30                     |
| Tagetes erecta                      | 2.10±2.97                      | 27.08±4.04                     |
| Anfotericina B(2µg/mL)              | 100±0                          | 100±0                          |

Percentage of inhibition cell growth was calculated relative to the negative control (0.1% DMSO).

Each experiment was performed in triplicate and repeated three times.

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Another study demonstrated that ethanolic extracts from Artemisia kalbadicica, Artemisia cininomnes and Artemisia santolina had an EC₅₀ of 25.25 and 80μg/mL, respectively, against promastigotes forms of L. (L.) major after 24h of incubation.²² However, no study has demonstrated the effect of A. camphorata extracts against L. (L.) amazonensis. As described previously in the literature, the hexane extract of A. chica showed EC₅₀ values of 31.8μg/mL and 14.7μg/mL against L. (L.) amazonensis e L. (L.) infantum at 120h, respectively.²³

In another study, the aqueous and ethanolic extract of E. alba inhibited 100% growth of L. (L.) donovani promastigotes at concentration of 0.5mg/mL.²⁴ Besides, study demonstrated that essential oils obtained from the L. alba species collected at different locations in Colombia showed different leishmanicidal activities against L. (L.) chagasi promastigotes, which suggest that different location may show changes in the chemical composition of the plant.²⁵ Maquaveli and co-workers also reported that butanol fraction of the aqueous extract of S. cayennensis showed EC₅₀ values of 51.0 (72h) and 32.0 (24h) μg/mL against promastigote and amastigote forms, respectively.²⁶

To determine the EC₅₀ values of the hexane extracts of P. neochilus and B. sulphurea, promastigote forms of L. (L.) amazonensis were incubated with the hexane extracts for 24 and 48h. The activity of extracts has been classified as follows in the literature: highly active (EC₅₀ value <10μg/mL); active, (10<EC₅₀<50μg/mL), moderately active (50 < EC₅₀<100μg/mL) and non-active (EC₅₀>100 μg/mL).²⁷ Our results revealed that in 24h the hexane extract of B. sulphurea showed an EC₅₀ value of 84.26 μg/mL (95% Confidence Interval 95% CI) 81.23-87.56μg/mL) (moderately active), while hexane extract of P. neochilus showed a value of 46.32μg/mL (95% CI- 38.42-57.54μg/mL) to considerate as moderate activity. In 48h, both extracts were considerate active, with EC₅₀ values of 40.37 (95% CI-29.64-55.64μg/mL) and 43.20μg/mL (95% CI-39.57-50.87μg/mL) for hexane extracts of B. sulphurea and P. neochilus, respectively. Amphotericin B showed an EC₅₀ value of 0.011μg/mL at 24 (95% CI-0.0058-0.019μg/mL) and 0.012μg/mL 48h (95% CI-0.0063-0.022μg/mL) (Table 3).

According to Tempone and co-workers, the methanol extracts of Aristolochia cymbifera, Plectranthes ambionicus, Plectranthus barbatus and Lippia alba showed EC₅₀ values of 45.14; 89.17; 54.46 and 62.67 μg/mL, respectively against L. (L.) chagasi at 48h.²⁸ In addition, the methanol extract of P. neochilus was inactive against Leishmania species.²⁹ Another study, Antinarelli and co-workers demonstrated that the methanolic extract of P. neochilus showed active against L. (L.) chagasi, but it did not show activity against L.(L.) amazonensis, L.(L.) major and Leishmania (Vianna) braziliensis.²⁰ Despite these results, it is interesting to notice that extracts methanolic and/or hexanic from the genus Plectranthus has showed values of EC₅₀ considered active or moderately active, because of that this genus should be better investigated about its antiparasitic activity.

Although promastigotes can be used for fast screenings of potential compounds, the clinically relevant form of the parasite is the amastigote form, which shows metabolic differences from the extracellular forms.²⁹,³⁰ When the hexane extracts were evaluated against intracellular amastigotes, it was observed that after 48h of incubation with B. sulphurea the EC₅₀ value were 371μg/mL (95% CI-254-487μg/mL) and when incubated with P. neochilus the EC₅₀ were 141μg/mL (95% CI-90.09-192.4μg/mL), demonstrating that the hexane extracts have no activity against these parasitic forms. The EC₅₀ obtained after incubation with amphotericin B were 0.095 μg/mL (95% CI-0.07-0.12μg/mL) (Table 3).

An important criterion in the research of active compounds and extracts is to determine the absence of toxic effects on the host cells. In this study, the toxicity of hexane extracts of B. sulphurea and P. neochilus was evaluated on peritoneal macrophage. The hexane extract of B. sulphurea showed CC₅₀ value (50% cytotoxic concentration) of 103.9μg/mL and 80.30μg/mL (95% CI-99.46-128.98μg/mL and 73.15-88.15μg/mL, respectively) after 24 and 48h of incubation. Moreover, the hexane extract of P. neochilus showed CC₅₀ value of 66.95μg/mL and 34.39μg/mL (95% CI-59.55-75.27μg/mL and 28.21-41.93) at 24 and 48h, respectively. Amphotericin B was more toxic to mammalian cell than hexane extracts, showing CC₅₀ values of 4.29 and 2.98μg/mL (95% CI-3.25-6.77μg/mL and 1.41-3.87) in 24 and 48h, respectively (Table 4). However, the methanolic extract of P. neochilus presented a CC₅₀ value of 111μg/mL when incubated with peritoneal macrophages after 72hours of incubation.³¹

According to one study, a selectivity index (SI) value greater than 10 can suggest better safety of the product for use in mammals.³¹ The hexane extract of B. sulphurea showed a SI of 1.23 and 1.98 in 24h and 48h, respectively. In addition, the hexane extract of P. neochilus showed a SI of 1.44 and 0.79 in 24h and 48h, respectively. Despite the low SI, the hexane extracts showed values close to those obtained by amphotericin B, with a SI of 390 and 248.3 in 24h and 48 h, respectively (Table 4).

One of the main treatments for leishmaniasis is the use of pentavalent antimony as a first-line and amphotericin B as a second-line. One of the biggest problems associated with this regimen is the need for intravenous or intramuscular administration for both medicines.³² Thus, there is concern about the effect of these or other proposed compounds or extracts with antileishmanial activity with respect to hemolytic activity. In determining the hemolytic activity of the hexane extracts from B. sulphurea and P. neochilus, we observed that the extracts showed no hemolytic activity at the concentrations evaluated, and the HC₅₀ values were higher than 100μg/mL for B. sulphurea and P. neochilus (Table 4).

Recently, the chemical composition of the hexane extract of P. neochilus was determined by gas chromatography–mass spectrometry (GC-MS). A total of thirteen compounds were detected, with predominance of sesquiterpenes (88.8%). The major constituents were identified as being spathulenol (46.1%), trans-caryophyllene (19.0 %), caryophyllene oxide (10.7%) and germacrene D (7.8%).³³ According to Acebey and co-workers, the sesquiarterpen spathulenol, isolated from an ethyl acetate extract of the bark of Hedosyosnum angustifolium, did not show activity against L. (L.) infantum in vitro.³⁴ This could indicate that some other compounds may be responsible for the activity of the extract. The other major constituents were already described in extracts or essential oils, but their isolated activity was not been described.³³,³⁴ Thus, the activity of the isolates should be better investigated against parasites from genus Leishmania sp.

In our study, a total of fifteen compounds were detected on the B. sulphurea hexane extract and the major constituents were identified as being 2,4-bis(dimethylbenzyl)phenol (54.1%), (3-tert-butyl-5-hydroxy methyl-cyclohex-2-enyl)-methanol (8.1%), pulegol (7.3%) and (2-Dodecen-1-yl-succinic anhydride (7.2%) (Table 5). This is the first study describing the effects of the major constituents of B. sulphurea extract on protozoa.

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**Table 3** Effective concentration of 50% against promastigotes and amastigotes after 24 and 48h of incubation with the extracts *B. sulphurea*, *P. neochilus* and amphotericin B

| Compound          | EC₅₀ values against promastigotes(µg/mL)(95% CI) | EC₅₀ values against amastigotes(µg/mL)(95% CI) |
|-------------------|-----------------------------------------------|-----------------------------------------------|
|                   | 24h                                          | 48h                                          | 48h                                          |
| *B. sulphurea*    | 84.26(81.23-87.56)                           | 40.37(29.64-55.64)                           | 371.00(254.00-487.00)                       |
| *P. neochilus*    | 46.32(38.42-57.54)                           | 43.20(36.57-50.87)                           | 141.00(90.09-192.4)                        |
| Amphotericin B    | 0.011(0.0058-0.019)                           | 0.012(0.0063-0.022)                           | 0.095(0.07-0.012)                           |

CI: Confidence Interval of 95%

**Table 4** Cytotoxic Concentration of 50%, Hemolytic Concentration of 50% and Selectivity Index obtained after 24 and 48 h of incubation with the extracts *B. sulphurea*, *P. neochilus* and amphotericin B

| Compound          | CC₅₀ values against murine macrophages(µg/mL)(95% CI) | HC₅₀(µg/mL)(95% CI) | Selectivity Index(SI)* |
|-------------------|------------------------------------------------------|--------------------|------------------------|
|                   | 24h                                                   | 48h                | 48h                    | 24h                    | 48h                    |
| *B. sulphurea*    | 103.9(99.46-128.98)                                   | 80.30(73.15-88.15) | >100                   | 1.23                   | 1.98                   |
| *P. neochilus*    | 66.95(59.55-75.27)                                    | 34.39(28.21-41.93) | >100                   | 1.44                   | 0.79                   |
| Amphotericin B    | 4.29(3.25-6.77)                                       | 2.98(1.41-3.87)    | 40.42(36.69-44.15)     | 390                    | 248.3                  |

CI: Confidence Interval of 95%

*Value obtained using the EC₅₀ from promastigotes assays as described by Londero and co-workers.*

**Table 5** Chemical composition of the hexane extract of *Bidens sulphurea*

| Compound                                           | RT(min) | RI  | %RA |
|----------------------------------------------------|---------|-----|-----|
| 3,3-Dimethoxy-2-butanone                           | 3.23    | 826 | 0.8 |
| 1-Methyl-2-(3-methylpentyl)cyclopropane             | 5.86    | 914 | 0.7 |
| 2-ethyl-1,3-Dioxolane-4-methanol                   | 9.45    | 1036| 1.2 |
| Pulegol                                            | 13.25   | 1141| 7.6 |
| Citronellyl propionate                             | 25.11   | 1447| 0.4 |
| (3-tert-Butyl-5-hydroxymethyl-cyclohex-2-enyl)-methanol | 31.16  | 1614| 8.4 |
| 10,10-Dimethoxy-3,7-dimethyl-deca-2,6-dien-1-ol    | 34.58   | 1714| 0.2 |
| Palmitaldehyde                                     | 37.96   | 1818| 4.9 |
| Neophytadiene                                      | 38.31   | 1829| 0.9 |
| 1-methyl-spiro[2.3]hexane-5-carboxylic acid menthol| 38.70  | 1842| 0.2 |
| Phtyone                                            | 38.88   | 1847| 4.5 |
| E-phytol                                           | 41.83   | 1946| 4.2 |
| 2-Dodecen-1-y1-succinic anhydride                  | 42.36   | 1961| 7.5 |
| Oxalic acid. docecyl isohexyl ester                | 48.51   | 2276| 0.8 |
| 2,4-Bis(1-methyl-1-phenylethyl)phenol              | 51.58   | 2491| 56.3|

Total: 98.7

RT, retention time(min); RA, relative content calculated from the peak area relative to the total peak area in the GC-FID chromatogram; values are averages of three replicates; Compound identification, Comparison of the SI(Similarity Index) and retention index(RI) with those from mass spectra Wiley 7, NIST 08, and FFNSC 1.2 spectral libraries.

**Conclusion**

The hexane extracts were evaluated in vitro in relation to the protozoan *L. (L.) amazonensis* and the results demonstrate a moderate leishmanicidal activity after 24 and 48 h of incubation. Despite the unsatisfactory results against amastigotes forms, this study shows extracts obtained from botanical sources merit further study for their leishmanicidal properties.

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Conflicts of interest

The authors have declared that there are no conflicts of interest.

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