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Bioassay-guided *in vitro* study of the antileishmanial and cytotoxic properties of *Bixa orellana* seed extract

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PEER REVIEW

Peer reviewer

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Comments

The paper is within the journal's scope, presents a certain relevance to the field of research and is sufficiently interesting to warrant publication.
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ABSTRACT

Objective: To investigate the leishmanicidal effect of the *Bixa orellana* crude seed extract and its fractions against *Leishmania amazonensis*.
Methods: Four main fractions (BO-A, BO-B, BO-C and BO-D) were obtained by exhaustion with solvent with increased polarity from the *Bixa orellana* crude seed extract and 28 sub-fractions. The antileishmanial activity was evaluated in intracellular amastigotes and the cytotoxicity was assessed in murine intraperitoneal macrophages.
Results: The BO-A and BO-B fractions showed a good antileishmanial activity with IC_{50} values of (12.9±4.1) and (12.4±0.3) μg/mL, respectively. The sub-fractions BO-B1 (IC_{50}=(11.8±3.8) μg/mL) and BO-B3 [IC_{50}=(13.6±4.7) μg/mL] also proved to have a good leishmanicidal effect. In general, the sub-fractions showed a lower toxicity than the crude extract. A selectivity index of 9 indicated a moderate selectivity of the BO-A, BO-B and BO-C fractions and BO-B1 sub-fraction.
Conclusions: Potential of this plant against cutaneous leishmaniasis should be further investigated.

KEYWORDS

Bixa orellana, Traditional medicine, Bioassay-guided fractionation, *Leishmania amazonensis*

1. Introduction

Leishmaniasis is a neglected tropical disease with important health consequences for millions of people worldwide[1]. Currently, there are no vaccines available and the treatment depends on a limited number of drugs, which are toxic, expensive, associated with resistance or require parenteral administration[2,3]. Therefore, there is a desperate need for safer and cheaper antileishmanials, which may be found in medicinal plants that are used as traditional remedies for various diseases[4].

Bixa orellana L. (Bixaceae) (*B. orellana*), a native plant of tropical America, can be found in divergent regions spanning the globe. It grows from seeds or cuttings, performs well in lowlands and mountainous regions and requires full sunlight and protection from the wind[5,6]. The plant is used in different countries for diverse purposes[7,8]. For example, the pulp from the seeds is used as a safe natural colorant in the food and cosmetic industry worldwide[9]. Furthermore, the effect of the plant against bacteria, yeasts and parasites has been evaluated[10-13]. Several reports indicate the use of *B. orellana* for the traditional treatment of leishmaniasis[14-16].

Previously, the laboratory investigated the *in vitro* and *in vivo* performance of the hydroalcoholic extract of *B. orellana* against *Leishmania amazonensis* (*L. amazonensis*). A significant activity against promastigotes (IC_{50}=22 μg/

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mL) and amastigotes (IC_{50}=9 μg/mL) was found. The extract also had an antileishmanial effect in *L. amazonensis*-infected BALB/c mice without death or weight loss after 15 d of intraperitoneal treatment with 100 mg/kg[17]. To support and expand these promising findings, this paper describes a bioassay-guided study of the antileishmanial effect and the cytotoxicity of the *B. orellana* seed.

2. Materials and methods

2.1. Plant material

The plant sample was collected in February 2008 in Havana (Cuba) and authenticated by MSc. Ramón Scull (Institute of Pharmacy and Food) according to the Cuban Flora. Their voucher specimen or collector's numbers were assigned 9600288 and a sample of each was deposited in the herbarium of the National Botanical Garden (NBG), Havana, Cuba.

2.2. Preparation of extract/fractions/subfractions

The seeds of *B. orellana* were dried in an oven with a ventilation system at 30 °C and crushed. The fluid extracts were prepared by maceration for 7 d using an ethanol: water solution (80:20, v/v)[18]. The solvent was evaporated and the residue lyophilized. A first step of fractionation by exhaustion was performed using different solvents of increasing polarity (*n*-hexane, ethyl acetate and methanol) to obtain four fractions: BO-A, BO-B, BO-C, and BO-D. The solvent was evaporated and each fraction was submitted to a second step of sub-fractionation in a Sephadex LH-20 column of 2 cm×30 cm using methanol as the mobile phase to obtain 10, 5, 4 and 9 sub-fractions from BO-A, BO-B, BO-C, BO-D, respectively (Figure 1). The solvent was evaporated and the extract was lyophilized. A stock solution of 20 mg/mL in dimethyl sulfoxide (DMSO) was used for the biological assays.

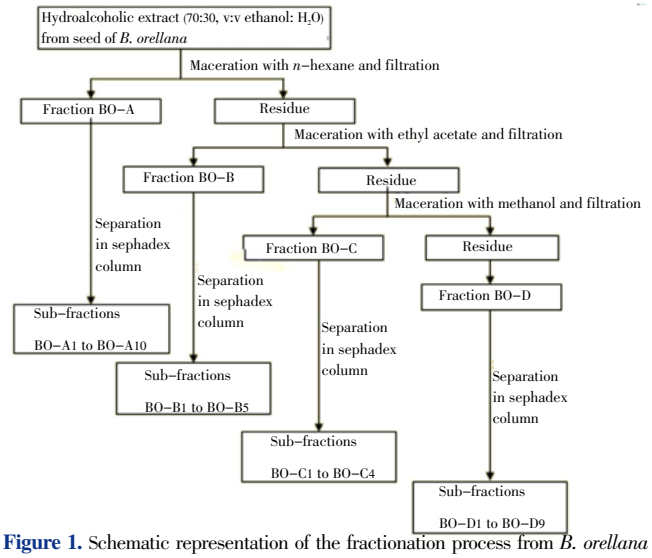

Figure 1. Schematic representation of the fractionation process from *B. orellana* seed extract.

Fraction obtained with *n*-hexane (BO-A) and sub-fractions (BO-A1 to BO-A10); fraction obtained with ethyl acetate (BO-B) and sub-fractions (BO-B1 to BO-B5); fraction obtained with methanol (BO-C) and sub-fractions (BO-C1 to BO-C4); D: residue (BO-D) and sub-fractions (BO-D1 to BO-D9).

2.3. Chemical analysis

Nuclear magnetic resonance (NMR) analysis was performed using about 50 mg of sub-fraction B0-B1, which was dissolved employing $CDCl_3$ (ca. 0.5 mL). A Bruker DRX-600 spectrometer operating at 599.19 MHz for ^1H, using the UXNMR software package, was used for the NMR experiments.

2.4. Cytotoxicity assay

The 50% inhibitory concentration (IC_{50}) of the crude seed extract, fractions and subfractions on peritoneal macrophages from BALB/c mice was determined. Resident macrophages from the peritoneal cavity were collected in RPMI 1640 medium (Sigma) supplemented with antibiotics (penicillin 200 IU, streptomycin 200 mg/mL). Then, 10^6 macrophages/mL were seeded in a 96-well Lab-Tek (Costar®, USA) and left to adhere for 2 h at 37 °C and 5% CO_2. The non-adherent cells were removed by washing with phosphate buffer solution. Then, 198 μL medium with 10% heat-inactivated fetal bovine serum (HFBS) and antibiotics (penicillin 200 IU, streptomycin 200 mg/mL) was added to each well. Macrophages were treated with 2 μL of the *B. orellana* products (12.5 to 200 μg/mL in medium) for 72 h. DMSO was included as control treatment. The cytotoxicity was determined using the colorimetric 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) (15 μL at 5 mg/mL in phosphate buffer solution, Sigma) assay[19]. Linear dose-response curves indicated the IC_{50}. Three replicates were performed and the results were expressed as the average and standard deviation.

2.5. Anti-amastigote activity

The peritoneal macrophages from BALB/c mice were collected, seeded with a density of 10^6/mL in 24-well Lab-Tek (Costar®, USA) and incubated for 2 h at 37 °C and 5% CO_2. Non-adherent cells were removed and stationary-phase of *L. amazonensis* (MHOM/77BR/LTB0016) promastigotes in Schneider's medium (Sigma-Aldrich, St. Louis, MO, USA) containing 10% HFBS (Sigma-Aldrich), 100 μg of streptomycin/mL, and 100 IU of penicillin/mL were added in a 4:1 parasite/macrophage ratio. After 4 h, the cell monolayers were washed to remove the free parasites and 1 990 μL RPMI medium and 10 μL *B. orellana* fractions or sub-fractions (12.5 to 100 μg/mL) were added. DMSO-treated control cultures were included. Plates were incubated for 48 h[20]. Then, cultures were fixed with absolute methanol, stained with Giemsa, and light microscopically evaluated. For each sample, the number of intracellular amastigotes was determined by counting the amastigotes in 100 macrophages. The results were expressed as the percentage of the reduction of the infection rate (IR%) in comparison with the positive controls. The infection rates were obtained by multiplying the percentage of infected macrophages by the number of amastigotes per infected macrophages[21]. The IC_{50} values were calculated by linear regression analysis. Each experiment was performed in duplicate and the results were expressed as the average and standard

deviation. Pentamidine (stock solution 10 mg/mL, Richet, Buenos Aires, Argentina) was used as positive control.

2.6. Selectivity index (SI)

The SI, calculated as the ratio of the IC_{50} for the macrophages over the IC_{50} for the amastigotes, was used to analyze the toxicity versus the activity of the *B. orellana* fractions and sub-fractions.

2.7. Integrated antimicrobial screening

Test plate production was performed in 96-well plates (Greiner, Germany) at fourfold dilutions in a dose-titration range of 64 μg/mL to 0.25 μg/mL. Dilutions were carried out by a programmable precision robotic station (BIOMEK 2000, Beckman, USA). Each plate also contained medium-controls (blanks: 0% growth), infected untreated controls (negative control: 100% growth) and reference controls (positive control). All tests were running in duplicate. The integrated panel of microbial screens for the present study and the standard screening methods were adopted as have been described previously[22]. Activity of the products against protozoa *Plasmodium falciparum*[23], trypomastigotes of *Trypanosoma brucei brucei* Squib-427[24], intracellular amastigotes of *Trypanosoma cruzi* Tulahuen CL2[25] and *Leishmania infantum* (*L. infantum*) MHOM/MA(BE)/67 were perform. In parallel, inhibition on *Escherichia coli* ATCC8739, *Staphylococcus aureus* ATCC6538, *Trichophyton rubrum* B68183, *Candida albicans* B59630 was assay. The IC_{50} value was determined from the linear regression of the concentration-response curves and the results were express as the mean±SD of at least two independent experiments.

2.8. Statistical analysis

The data were analysed using STATISTICA for Windows, Version 4.5, 1993. Mann-Whitney tests were performed to compare the IC_{50} values of the *B. orellana* products. *P*-values less than 0.05 were considered statistically significant.

3. Results

3.1. Activity of B. orellana extracts on intracellular amastigotes

Figure 2 shows the antileishmanial activity and cytotoxicity of the *B. orellana* seeds extracts, fractions and sub-fractions. The BO-A and BO-B fractions showed the highest activity with IC_{50} values of (12.9±4.1) and (12.4±0.3) μg/mL, respectively. These IC_{50} values were similar to that of the crude extract (P>0.05). However, the four fractions had a higher selectivity than the crude extract. The BO-A, BO-B and BO-C fractions showed a SI of 9.

The BO-B1 and BO-B3 sub-fractions had the same activity (P>0.05) as the original fraction with IC_{50} values of (11.8±3.8) and (13.6±4.7) μg/mL, respectively. Some sub-fractions

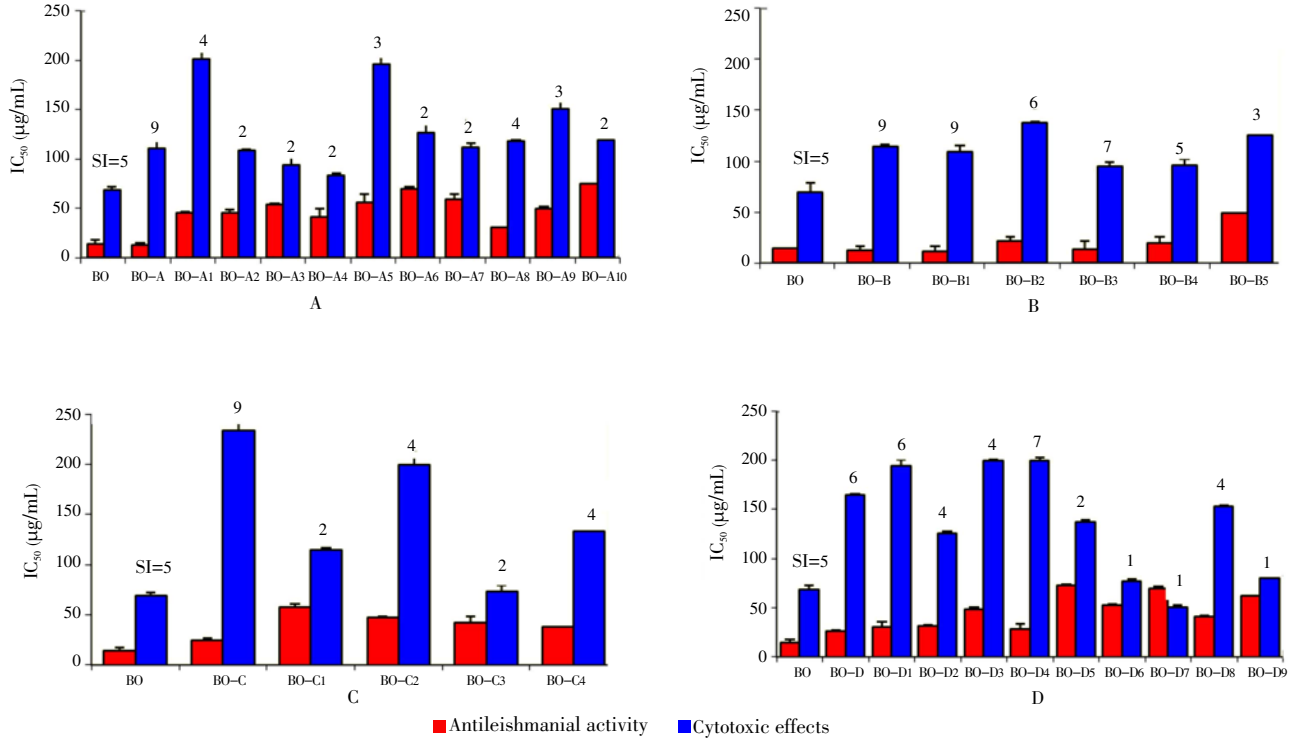

Figure 2. Antileishmanial activity and cytotoxic effects of fraction and sub-fractions from *B. orellana*.
A: fraction obtained with *n*-hexane (BO-A) and sub-fractions (BO-A1 to BO-A10); B: fraction obtained with ethyl acetate (BO-B) and sub-fractions (BO-B1 to BO-B5); C: fraction obtained with metanol (BO-C) and sub-fractions (BO-C1 to BO-C4); D: residue (BO-D) and sub-fractions (BO-D1 to BO-D9). SI: selectivity index appears in each extract, fraction and sub-fraction evaluated (ratio of the IC_{50} for the macrophages over the IC_{50} for the amastigotes).

showed the highest SI with values between 7 and 9, including BO-B1, BO-B2, BO-B3, BO-C2 and BO-D4.

3.2. Integrated antimicrobial screening

In addition, the BO-B1 sub-fraction was evaluated in a wide panel of microorganisms, including protozoa (*L. infantum*, *Trypanosoma cruzi*, *Trypanosoma brucei brucei* and *Plasmodium falciparum*), bacteria (*Escherichia coli* and *Staphylococcus aureus*) and fungi (*Trichophyton rubrum* and *Candida albicans*). No activity against the tested micro-organisms was observed, with IC_{50} values >64 μg/mL.

3.3. Chemical analysis

Sub-fraction B0-B1, one of the most active against *L. amazonensis*, displayed the main signals in the aliphatic region of the ^{1}H NMR spectrum. A complex region between 1.3 and 2.1 mg/L rich in very intense singlet signals was appreciated. Some signals at greater chemical shifts (between 4-6 mg/L) were also distinguished. Examination of the aromatic region (7-9.5 mg/L) didn't evidence the presence of aromatic residues.

4. Discussion

In developing countries, people are almost completely dependent on traditional medicines for their primary health care and higher plants remain the main source of drugs. The recognition and validation of traditional medical practices and the search for plant-derived compounds could lead to new strategies for the treatment and control of leishmaniasis[26-28].

The present study was part of a systematic ethno-medical approach to search for antileishmanial agents based on *B. orellana* seeds, which previously demonstrated *in vitro* and *in vivo* activity[17]. For example, the hydroalcoholic extract was fractionated yielding four fractions, which were tested for their effects on *L. amazonensis*. The results indicated that the *n*-hexane (BO-A) and ethyl acetate (BO-B) fractions showed the best activities with IC_{50} values [(12.9±4.1) and (12.4±0.3) μg/mL, respectively] similar (P>0.05) to that of the extract [(14.3±1.0) μg/mL]. However, the BO-C and BO-D fractions showed a loss of activity.

Several other studies evaluated the activity of crude plant extracts against *Leishmania* species, often with a lower activity than the *B. orellana* crude seed extract. The methanolic crude extract of *Jacaranda puberula* Cham. showed an IC_{50} value of 359 μg/mL against *L. amazonensis* amastigotes[29]. Similarly, Takahashi *et al*. proved that crude *Porophyllum ruderale* (Jacq.) Cass. extract had an IC_{50} value of 77.7 μg/mL against *L. amazonensis* amastigotes[30]. Ghosh *et al.* presented the excellent activity of *Valeriana wallichii* DC (Valerianaceae) root extracts. The chloroform extract was the most active with an IC_{50} of 0.8 μg/mL against *Leishmania major* amastigotes[31].

Also the antileishmanial activity of plant fractions has been reported. Several papers mention similar IC_{50} values as this study. The methanolic fraction of *Jacaranda puberula* Cham. had an IC_{50} of 14.0 μg/mL against *L. amazonensis* amastigotes[29]. Hexane-chloroform, ethyl acetate-methanol and methanol fractions of *Musa paradisiaca* L. showed IC_{50} values of 15.1, 14.2 and 16.5 μg/mL, respectively, against *Leishmania chagasi* amastigotes. The chloroform-ethyl acetate fraction of *Spondias mombis* L. revealed a similar antileishmanial activity (IC_{50} of 17.1 μg/mL)[32]. The ethyl acetate and chloroform fractions of *Ricinus communis* L. revealed IC_{50} values of 17.3 μg/mL against *L. infantum*. Several others plants fractions showed a lower activity, such as the ethyl acetate fraction of *Coriandun sativum* L. (IC_{50} of 27.3 μg/mL) and the chloroform and methanol fractions of *Aloe vera* (L.) Burm.f. (IC_{50} values of 96.6 and 79.8 μg/mL, respectively) against *L. infantum* amastigotes[33].

With regard to the cytotoxicity of the fractions, coherent results were found. For the peritoneal macrophages, the BO-A and BO-B fractions showed lower IC_{50} values compared to the BO-C and BO-D fractions. These results suggest that the components responsible for the antileishmanial activity could be the same than those that cause the cytotoxic effects. Another explanation could be that the non-polar characteristics of *n*-hexane and ethyl acetate solvents enabled the extraction of a high number of components, including active and toxic compounds. Nevertheless, if SIs are calculated, the BO-A and BO-B fractions will show higher values (SI=9).

The four fractions were sub-fractionated. The BO-B1 and BO-B3 sub-fractions were the most active with IC_{50} values of (11.8±3.8) and (13.6±4.7) μg/mL, respectively. These activities were similar (P>0.05) to those of the corresponding fractions [(12.4±0.3) μg/mL] and the crude extract [(14.3±1.0) μg/mL].

The other sub-fractions showed a lower activity than the corresponding fractions. This could be explained by two reasons: (i) the active compounds were separated in different sub-fractions, in which their concentration decreased and consequently their biological activity or (ii) a synergistic activity could result from the interaction of the diverse compounds that were separated in the different sub-fractions. The chemical characterization and comparison between sub-fractions could elucidate the exact reason for the loss of activity.

Sub-fractions from different plants have been studied against *Leishmania* species. For example, the F4 and F5 sub-fractions from a methanolic extract of *Polyalthia suaveolens* Engl. & Diels showed interesting activity against the intracellular *L. infantum* amastigotes with IC_{50} values of 5.6 and 12.4 μg/mL, respectively[34].

The analysis of the cytotoxicity on mouse peritoneal macrophages and the selectivity of the sub-fractions showed that BO-B1 had an SI of 9. The BO-B2, BO-B3 and BO-C2 sub-fractions had an SI of 7. These results indicate the presence of compounds with reasonable antileishmanial potency. In general, the CC_{50} values were around 100 μg/mL. Interestingly, the BO-D3 and BO-D4 sub-fractions did not

cause toxicity at the maximum concentration tested (200 μg/mL), which could suggest that the components present in these fractions have a specific antileishmanial activity and could be safe to mammalian cells.

In previous studies, main compounds isolated from *B. orellana* seeds have been unambiguously identified as bixin, norbixin and many other related compounds. All of them contain a large aliphatic chain between 8-9 double bonds and 4-5 methyl groups, generally[35,36]. All these compounds originate very similar ^{1}H NMR spectra to that obtained using sub-fraction B0-B1. The methyl groups in bixin and its analogues that usually appear in the ranges of δ_H 1.4-2.1 mg/L, and originate the most intense signals of their ^{1}H NMR spectra, were clearly visible in ^{1}H NMR spectrum of sub-fraction B0-B1. The presence of vinyl protons was supported by chemical shifts appreciated between 4-6 mg/L and these are also in agreement with chemical characteristics of the compounds mentioned above, in which some protons (CH group) are bonded to sp^2 carbon atoms. The absence of aromatic signals was also in concordance with our hypothesis: sub-fraction B0-B1 seems to be rich in aliphatic compounds similar to bixin and theirs analogues. However, ^{1}H NMR spectrum exhibited about 9 signals of methyl groups indicating the presence of 203 different compounds at least in sub-fraction B0-B1. These results suggest that sub-fraction B0-B1 seems to contain a mixture of compounds very closely related.

In addition, the BO-B1 sub-fraction was evaluated in a wide panel of micro-organisms and no activity was observed, which could indicate that the BO-B1 sub-fraction contains specific antileishmanial compounds. It is interesting that the BO-B1 fraction did not show activity against *L. infantum*, the causal agent of visceral leishmaniasis. Braga *et al*. reported similar results in which they showed that *B. orellana* seed extract has activity against *L. amazonensis*, the causal agent of cutaneous leishmaniasis, while 250 μg/mL had no effect against *Leishmania chagasi*, the causal agent of visceral leishmaniasis[13]. These results could suggest that the therapeutic properties of *B. orellana* are highly specific against cutaneous species of the *Leishmania* parasite. Further experiments against other agents responsible of cutaneous leishmaniasis can be performing.

In conclusion, the results support the continuation of the study of *B. orellana* as a potential source of new drugs against cutaneous leishmaniasis. The BO-B1 sub-fraction could constitute a potential lead for further purification, identification and evaluation of its active principles.

Conflict of interest statement

We declare that we have no conflict of interest.

Acknowledgements

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Comments

Background

Leishmaniasis is a neglected tropical disease whose treatment depends on a limited number of drugs. Some antileishmanials found in medicinal plants are used as traditional remedies; among them, *B. orellana* is a native plant of tropical America.

Research frontiers

Studies are being performed to support and expand some previous promising findings of the authors. They describe a study regarding the leishmanicidal and cytotoxic effects of *B. orellana* crude seed extract and its different fractions. The results show that some fractions had an activity on the growth of *L. amazonensis* amastigotes in macrophage cultures. No activity was demonstrated on *L. infantum*, *Trypanosoma* spp., some bacteria and fungi.

Related reports

Different authors report the antileishmanial activity of different plants linked to the presence of different compounds. Many of these extracts have been used for centuries and are a source of new drugs. It can be envisaged that in the next years some different molecules discovered by screening programs and obtained from different plant oils and extracts will become useful therapeutic tools.

Innovations and breakthroughs

This study has showed the selective activity of some fractions obtained from seed extract of *B. orellana* on *L. amazonensis*.

Applications

The results of the present study suggest the interest in the purification and evaluation of the active compounds present in particular fractions of the extract responsible of the leishmanicidal activity.

Peer review

The paper is within the journal's scope, presents a certain relevance to the field of research and is sufficiently interesting to warrant publication.

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