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Original Research Article

In vitro antileishmanial evaluation of Vernonia Brachycalyx leaf latex extract against two leishmania species

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ABSTRACT

Background: Leishmaniasis is a major public health problem, and the alarming spread of parasite resistance has increased the importance of discovering new therapeutic products. In the present study, the antileishmanial activity of the methanolic extract of the leaf latex obtained from the Ethiopian plant Vernonia brachycalyx O. H. (family Asteraceae) was evaluated by in vitro testing against Leishmania aethiopica and *L. donovani*.

Materials and Methods: Antileishmanial activity test was carried out using the Alamar Blue assay on promastigotes and axenic cultured amastigotes of *L. aethiopica* and *L. donovani* clinical isolates, and cell viability was fluorometrically determined. Amphotericin B was used as a positive control, and 1% dimethyl sulfoxide (DMSO) and the media were employed as a negative control.

Moreover, preliminary phytochemical analysis of the extracts was performed.

Results: Results of the study indicated that the latex possesses good activity against both parasites, with IC_{50} values of 6.82 ± 0.18 and 6.34 ± 0.20μg/ml against promastigotes and 3.53 ± 0.33 and 2.61 ± 0.907μg/ml against axenically cultured amastigotes of *L. aethiopica* and *L. donovani*, respectively. The latex demonstrated selectivity indices (SIs) of 15.27 and 16.42 against promastigotes and 29.50 and 39.90 against axenically cultivated amastigotes of *L. aethiopica* and *L. donovani*. While, amphotericin B demonstrated SIs of 7.91 and 8.23 against promastigotes and 7.45 and 7.73 against axenically cultured amastigotes of and *L. donovani*, respectively. Phytochemical screening demonstrated that the latex contains flavonoids, tannins, cardiac glycosides, terpenoids, saponins, alkaloids, and steroids.

Conclusion: The findings of this investigation attest that the latex of V. brachycalyx possesses promising antileishmanial activity against *L. aethiopica* and *L. donovani*, warranting further investigations into the active constituents.

1. Introduction

Leishmania is a disease caused by a single-celled parasite of the genus Leishmania, which is spread by the bite of several species of fireflies (subfamily Phlebotomine). Although not bearing the same country name as malaria, Leishmaniasis continues to have a major impact on a significant portion of the world's population and is currently considered an emerging disease with high morbidity and mortality in the tropics and subtropics (Ornellas-Garcia et al., 2023). Leishmania is characterized by both its diversity and its complexity. Depending on the parasite strain(s) involved in the pathogenesis and host immune response, it can cause clinical symptoms ranging from mild, self-limiting skin lesions to visceral disease. (Loría-cervera et al., 2014; Tariku, 2008). There are several different forms of leishmaniasis in humans. The three main clinical forms are cutaneous, mucocutaneous, and visceral leishmaniasis. A

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fourth, less common form is diffuse cutaneous leishmaniasis (Pagheh et al., 2023).According to the World Health Organization, the incidence of leishmaniasis has increased worldwide in recent decades. Leishmania is endemic in 200 countries in Asia, Africa, the Americas, and the Mediterranean region (WHO, 2021). Leishmaniasis causes 2 million disability-adjusted life years in 90 countries, mostly in the developing world (Desjeux, 2004). In Ethiopia, cutaneous leishmaniasis is mainly caused by the endemic species *L. aethiopica* and rarely by L. tropica and L. major. It is found almost everywhere in the country at altitudes between 1500 and 2700 meters (Hailu et al., 2007). Visceral leishmaniasis (VL) is distributed in lowlands and semi-deserts (below 1500 m altitude) with varying degrees of prevalence. To date, cases of VL have been reported in at least 40 localities, with an estimated annual burden of 2000–4500 cases in Ethiopia (Ministry of Health, 2013). Treatments for human leishmaniasis include pentavalent antimonial, amphotericin B, pentamidine, miltefosine, and paromomycin. Due to frequent side effects and increasing drug resistance, these drugs are not effective. Therefore, the need for better, safer, and more effective drugs is of utmost importance (Feroche et al., 2022). This poses a need for new antileishmanial drugs to stop the spread of the disease.In addition, to find new lead compounds and/or disease-fighting drugs to investigate, researchers are focusing on natural compounds used to treat parasitic infections, including leishmaniasis (Ayalew et al. 2018). The use of plants as alternative medicine in Ethiopia has been an ancient practice for centuries. In fact, in Ethiopia, for 70–80% of the population and about 90% of livestock, traditional medicines are used as the main source of treatment (Chekole et al., 2017). Therefore, in this study, the in vitro antileishmanial activity, phytochemical analysis, and in vitro cytotoxicity studies of the 80% methanolic Vernonia brachycalyx latex extract were investigated. [1–3]

2. Materials and Methods

2.1. Plant material collection

Fresh latex of V. brachycalyx was collected from the Bale Mountains, Oromia Regional State, Ethiopia, in June 2021. The identification and authenticity of the plant material were confirmed by Mr. Melkamu Wondafrash, National Herbarium, Department of Plant Biology and Biodiversity Management, College of Natural and Computational Sciences, Addis Ababa University. The resulting latex is left to dry in the shade. The dried latex is then ground into a coarse powder and stored in a desiccator at room temperature until further use.

2.2. Preparation of latex extract

The latex is collected from the leaves of V. brachycalyx by cutting the leaves at the bottom and letting the latex drip onto a plastic sheet. The latex is then allowed to air dry for 3 days to form a light powder.[4–11]The dry material was then weighed and stored in a refrigerator at 4°C until use. Powdered dry latex (30 g) was soaked with 80% methanol (3 × 0.5 L, 72 h each). The extract was then filtered, and the methanol in the filtrate was removed by a rotary evaporator to obtain 19 g of brown powder.

2.3. Cell culture promastigote culture

The *L. aethiopica* and *L. donovani* were grown in tissue culture flasks containing RPMI 1640 medium (Gibco, Invitrogen Co., UK) supplemented with 10% heat-inactivated fetal bovine serum (HIFCS) (Gibco, Invitrogen Co., United Kingdom) and 100 IU penicillin/ml -100μg/ml streptomycin solution (Sigma Chemical Co., St. Louis, USA) at 22^0C for *L. aethiopica* and 26^0C *L. donovani* (Nigatu et al., 2021). Cell-free medium was used to culture the parasites in vitro and set up a test system to determine the IC_{50} value of the extract.

2.4. Axenic cultured amastigote

Axenically cultured amastigotes were cultured according to the methods described for *L. aethiopica* and *L. donovani* (Nigatu et al., 2021) with minor modifications. Late stationary phase promastigotes were centrifuged and resuspended in Hank's balanced salt 199 medium, supplemented with 20% heat-inactivated fetal bovine serum (HIFCS), 2 mM L-glutamine, penicillin 50 IU/mL and streptomycin 50μg/mL; pH was adjusted to 5.5 (for both strains) with 0.10N HCl. The cells were then incubated at 31°C (for *L. aethiopica*) or 37°C (for *L. donovani*) in a humidified 5% CO_2 incubator for 7 days.

2.5. In vitro antileishmanial test

All experiments were performed in triplicate. Briefly, serial dilutions of Vernonia brachycalyx were prepared. Final concentrations of 100, 50, 25, 12.5, and 6.25 μg/mL were performed to establish adequate dose titration and to determine the IC_{50} value. Plant latex was added to a 96-well microtiter plate containing 100 μL of complete culture medium to achieve a final concentration of 100 μg/mL of each. Then, a 100μL suspension of the parasite (3.5 × 106 promastigotes of *L. aethiopica* and *L. donovani*) obtained from previous cultures were added to each well. The parasites were then incubated for 72 h at room temperature for the promastigotes of the two strains, at 31°C and 37°C for axenic amastigote cultures of *L. aethiopica* and *L. donovani*, respectively, in the presence of different concentrations of the extract. Then, resazurin (0.125 mg/mL) was added to the 20 μL suspension (10% of the total volume of each well). The mixture is covered with aluminum foil and left at the above temperature. Fluorescence intensity was measured using a Victor 3

multilabel counter (PerkinElmer, MA, USA) at an excitation wavelength of 544 nm and an emission wavelength of 590 nm. The trial was performed in triplicate and compared with negative controls (1% DMSO and medium alone) and a reference drug (amphotericin B, Sigma-Aldrich, Germany). During the assay, cell viability was monitored by measuring the fluorescence signal. The fluorescence intensity produced is proportional to the number of viable cells (Tewabe et al., 2019).

2.6. Study of cytotoxicity *in* THP-1 monocytes

THP-1 cells were cultured in RPMI 1640 medium (Sigma-Aldrich, Co., St. Louis, USA) supplemented with 10% HINBCS, 100 IU/ml penicillin, and 100 μg/ml streptomycin at 37°C in a 5% CO_2 humidified incubator (Thermo Scientific, USA). In 96-well plates, THP-1 monocytes were plated at a density of 4 × 104 cells per well (in a volume of 200 μl) with or without latex extract, and the plates were incubated at 37 °C with 5% CO_2 for 72 h. Then Alamar Blue was added.[12–22] During the final 3 h of incubation, cell viability was measured by fluorescence measurement as described previously (A. T. Feroche et al., 2021).

2.7. Phytochemical screening

V. brachycalyx leaf latex extract in methanol has been used to evaluate the presence or absence of plant components such as alkaloids, saponins, tannins, anthraquinones, flavonoids, steroids, cardiac glycosides, resins, phenolic compounds, phlobatannin, comarin, and terpenoid. Standard methods for preliminary phytochemical analysis were used (Alamzeb et al., 2013; Thusa and Mulmi, 2017).[23–29]

2.8. Data analysis

Antileishmanial activity (IC_{50}) values were calculated from sigmoid inhibitor dose-response curves using the computer software GraphPad Prism 8.0.1.244 (GraphPad Software, Inc., CA, USA) and Microsoft Excel. Values are expressed as the mean standard deviation of triplicate experiments.

3. Result and Discussion

3.1. Antileishmanial activity

Current alternative treatment options for leishmaniasis are considered inadequate because these drugs are associated with high toxicity and high cost, and parasite resistance to these drugs is increasing (Tasdemir et al., 2006). In this context, strategies to identify a new compound with lower toxicity and better efficacy may be useful, as they are considered highly desirable. As an alternative to chemotherapy in the treatment of parasitic diseases, herbal medicines have received much attention. Furthermore, natural products have potential advantages as novel and selective agents for the treatment of other tropical diseases caused by protozoa (Ribeiro TG. et al., 2014).

Table 1: Antipromastigote activity of V. brachycalyx against Leishmania aethiopica and Leishmania donovani.

Test substance	Antipromastigote activityIC_{50} (μg/mL) [x]	
	L. aethiopica	*L. donovani*
Latex extract	6.82 ± 0.18	6.34 ± 0.20
AmB (reference)	1.29 ± 0.08	1.24 ± 0.01
DMSO (NC)	0.00[y]	0.00[y]
Media alone (NC)	0.00[y]	0.00[y]

Notes: Values are expressed as mean ± SD; n = 3. [x] Effective concentration required to achieve 50% growth inhibition in μg/mL; [y] no effect.
Abbreviations: NC, negative control; DMSO, dimethyl sulphoxide. AmB, Amphotericin B

Table 2: Effects of V. brachycalyx on axenically cultured amastigotes of Leishmania aethiopica and Leishmania donovani and THP-1 monocytes.

Test substance	Antipromastigote activityIC_{50} (μg/mL) [x]		Cytotoxic Effect in THP-1 LC_{50} (μg/mL)
	L. aethiopica	*L. donovani*	
Latex extract	3.53 ± 0.33	2.61 ± 0.907	104.13 ± 0.94
AmB (reference)	1.37 ± 0.05	1.32 ± 0.15	10.21 ± 0.25
DMSO (NC)	0.00y	0.00y	00
Media alone (NC)	0.00y	0.00y	00

Notes: Values are expressed as mean ± SD; n = 3. [x] Effective concentration required to achieve 50% growth inhibition in μg/mL; [y] no effect.

Table 3: Phytochemical analysis of an 80% methanol latex extract of *V. brachycalyx*

Phytochemicals	*V. brachycalyx* latex extract
Alkaloid	+++
Cardiac glycosides	+++
Flavonoids	+++
Saponins	+++
Steroids	+++
Tannins	+++
Terpenoids	+++
Cardiac glycosides	+++

Therefore, this study was conducted with the aim of finding compounds that inhibit the in vitro growth of two Leishmania parasites, *L. aethiopica* and *L. donovani*. First, the leaf latex of V. brachycalyx was tested for its growth inhibition against *L. aethiopica* and *L. donovani*, as several plants of the genus Vernonia have been reported to have antiprotozoal activity (C.N. Muthaura et al., 2007). Indeed,

plant latex showed strong inhibitory activity on the flagellate and extracellular premature stages of *L. aethiopica* and *L. donovani*, with IC_{50} values of 6.82 ± 0.18 and 6.34 ± 0.20 respectively (Table 1). However, these results are considered preliminary, as Leishmania promastigote is more sensitive to drug-induced effects than amastigotes (Y. Baquedano et al., 2016). Furthermore, the clinical manifestations of the disease in humans are related to intracellular amastigotes, which are the evolved form of vertebrate parasites, and not to extracellular promastigotes. Therefore, the latex was further tested against axenic amastigotes of both pathogenic parasites.Tests showed that the latex had better activity than that observed for the promastigotes of *L. aethiopica* and *L. donovani*, with IC_{50} values of 3.53 ± 0.33 and 2.61 ± 0.907μg/mL respectively (Table 2).At higher concentrations, leaf latex showed the highest inhibitory activity against promastigote and amastigote of *L. aethiopica* and *L. donovani.* This indicates that there is a direct correlation between the concentration of the latex extract and the percentage inhibition.The antileishmanial effect of the reference drug, amphotericin B, was also determined for comparison purposes in clinical isolates and strains of *L. donovani* and *L. aethiopica*.For comparison, the antileishmanial effect of the reference drug, amphotericin B, was determined both in clinical isolates of *L. aethiopica* and *L. donovani*.Even so, the leaf latex extract was less active than amphotericin B, having IC_{50} values of 1.29 ± 0.08 μg/mL and 1.24 ± 0.01 μg/mL on promastigotes (Table 1) and IC_{50} values of 1.37 ± 0.05μg/mL and 1.32 ± 0.15 μg/mL on axenically cultured amastigote of *L. aethiopica* and *L. Donovani*, respectively (Table 2).

4. Phytochemical Analysis

The results of various phytochemical screening tests obtained during the experiment were flavonoids, tannins, cardiac glycosides, terpenoids, saponins, quinones, alkaloids, and steroids (Table 3). Numerous studies performed on several plant secondary metabolites have reported their potential growth inhibitory effects on Leishmania spp. These include alkaloids (Henriques et al., 2001), steroids (Sartorelli et al., 2007), polyphenols (Bodiwala et al., 2007), tannins (Kolodziej et al., 2001), flavonoids (Tasdemir et al., 2006), anthraquinones (Chan-Bacab et al., 2001), saponins (Maes et al., 2004), and terpenoids (Kayser et al., 2003). The biological activities observed in this V. brachycalyx may be due to individual groups of compounds present in the plant or to a synergistic effect caused by individual compounds.

4.1. In vitro cytotoxicity studies

A large proportion of the world's population does not have access to standard medicines and is dependent on traditional natural medicines. To complement this growing interest in alternative treatment plans and ensure safe therapeutic use of herbal medicines, their toxicity needs to be investigated. Toxicity testing indicates how safe the herbal medicine is, and the results of these tests are very important for further in vivo studies. In this study, cytotoxicity tests were performed in vitro to evaluate the safety of the latex extract.The selectivity index (SI) is an important tool for describing the safety of biologically active compounds. The SI determined for the tested substances is expressed as the ratio between cytotoxicity (LC_{50} value on THP-1 cells) and activity (IC_{50} value on *L. aethiopica* or *L. donovani* amastigote). The latex exhibited a selectivity index (SI) of 15.27 and 16.42 for promastigote and 29.50 and 39.90 for axenically cultured amastigote of *L. aethiopica* and *L. donovani*. While amphotericin B exhibited SIs of 7.91 and 8.23 for promastigotes and 7.45 and 7.73 for axenically cultured amastigotes of L. aethiopica and *L. donovani*, respectively. The latex extract showed a much higher SI than the reference drug, amphotericin B, indicating its high selectivity for Leishmania parasites.

In the present study, the latex extract was twice as selective as amphotericin B on the promastigotes of *L. aethiopica* or *L. donovani* and four times more selective than amphotericin B on the amastigotes of *L. aethiopica* or *L. donovani*. The results obtained in this study are much higher than the results of other similar studies conducted with the same genus of Vernonia on Leishmania aethiopica (Azeb T. et al., 1993). Furthermore, it was higher than that reported for latex extracts of A. rugosifolia (IC50 = 31.21 ± 0.01 μg/ml and IC_{50} = 24.5 ± 0.24 μg/ml) (Chemeda et al., 2022) on the promastigote and amastigote stages of the parasite.Although the potency of the tested substances against intracellular amastigotes was lower than that of the reference drug, their low toxicity to THP-1 cells and their high SI represent a great advantage over the reference drug. Compounds with SI values above 20 are ideal candidates for further development of antileishmanial agents (Nwaka S. and Hudson A., 2006).For axenically cultured amastigotes in the present work, the latex extract exhibited a significantly greater SI value than 20. According to this criterion, the latex extract of V. brachycalyx may serve as a model for developing safer and more effective antileishmanial agents. In addition, latex extracts have the added advantage of being used therapeutically in their own right as they are active components of many common herbal medicines that have long been used as antimalarial agents (C.N. Muthaura et al., 2007).

5. Conclusion

The results of this study demonstrated that the methanol extract of the leaf latex of V. brachycalyx showed promising activity, with a direct correlation between the latex extract concentration and the percentage inhibition against promastigotes and axenically cultured amastigotes

of *L. aethiopica* and *L. donovani*, respectively. This could be explained by the existence of secondary metabolites such as flavonoids and terpenoids, which have previously been reported to have true antileishmanial activity. Furthermore, the extract showed much higher SI values than the reference drug, amphotericin B, which showed high selectivity for Leishmania parasites. However, the leaf latex extract was less active than amphotericin B on promastigotes and amastigotes of *L. aethiopica* and *L. donovani*, respectively.The promising activity profile of the methanol extract of V. brachycalyx leaf latex, together with its relative safety margin, requires further study of the plant's phytochemicals to isolate the compounds responsible for its antileishmanial activity. Plant bioactive compounds can be isolated and used as effective anti-leishmanial agents or as starting points for the development of safer, more effective, and more economical leishmaniasis treatment options.Latex extracts have also been tested for phytochemicals. The plant components found were flavonoids, tannins, cardiac glycosides, terpenoids, saponins, quinones, alkaloids, and steroids.

6. Data Sharing Statement

The corresponding author (Alemu Tadesse) will make the data used to support the findings of this study available upon reasonable request from the school of pharmacy at Addis Ababa University. Email: donganegn@gmail.com

7. Author Contributions

Mr. Alemu Tadesse contributed to the research's design, synthesis, evaluation, and laboratory work, as well as the analysis of results and manuscript writing.

8. Abbreviations

CL, Cutaneous Leishmaniasis; DMSO, Dimethyl Sulfoxide; IC_{50}, the half maximal inhibitory concentration; MCL, Mucocutaneous Leishmaniasis; VL, Visceral Leishmaniasis; DCL, Diffuse Cutaneous Leishmaniasis; PKDL, Post kala-azar Dermal Leishmaniasis; USA, United States of America; HIFCS, Heat Inactivated Fetal Calf Serum.

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11. Declaration of Interest

The author declares that there is no conflict of interest.

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