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ORIGINAL RESEARCH

In vitro Anti-Leishmanial Activities of Methanol Extract of *Brucea antidysenterica* J.F. Mill Seeds and Its Solvent Fractions

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Introduction: Leishmaniasis is one of the neglected tropical diseases, threatening lives of about 350 million people globally. *Brucea antidysenterica* seeds are used for the treatment of cutaneous leishmaniasis in the traditional medicine in Ethiopia.

Objective: This study aimed to evaluate *Brucea antidysenterica* seeds' anti-leishmanial activity in vitro.

Methods: The crude (80% methanol) extract of *Brucea antidysenterica* seeds and its fractions were evaluated for their anti-leishmanial activities against promastigotes and intracellular amastigotes of *Leishmania donovani and Leishmania aethiopica*, and for their cytotoxic effects against mammalian cells. The quantitative estimations of total phenolic compounds (TPCs), flavonoids (TFCs) and alkaloids (TACs) were determined, spectrophotometrically. Median inhibitory concentration (IC_{50}) and median cytotoxic concentration (CC_{50}) of the extract and its solvent fractions were calculated using GraphPad Prism 9.1.0 computer software. Data was presented as mean ± standard error of the mean (SEM).

Results: The crude extract and its hexane, ethyl acetate and butanol fractions showed anti-leishmanial activities, with IC_{50} values of 4.14–60.12 µg/mL against promastigotes, and 6.16–40.12 µg/mL against amastigotes of both *Leishmania* species. They showed moderate cytotoxicity against Vero cell lines and peritoneal mice macrophages, with CC_{50} values of 100–500 µg/mL, but >1600 µg/mL against red blood cells. Selectivity indices ranged from 7.97 to 30.97. The crude extract, and its ethyl acetate and hexane fractions possessed 54.78–127.72 mg of gallic acid equivalent TPC, 18.30–79.21 mg of quercetin equivalent TFC, and 27.62–97.22 mg of atropine equivalent TAC per gram of extracts.

Conclusion: The seeds of the plant possessed anti-leishmanial activities against *L. aethiopica* and *L. donovani* that might provide a scientific justification for its use in the treatment of leishmaniasis by traditional healers. Future works are recommended to isolate, purify and identify the possible secondary metabolites attributed to the anti-leishmanial activity.

Keywords: anti-leishmanial activity, *Brucea antidysenterica* seeds, *Leishmania aethiopica*, *Leishmania donovani*, promastigote, amastigote

Introduction

Over 28.96 million and 3.2 million people in Ethiopia inhabit areas with risk of cutaneous leishmaniasis (CL) and visceral leishmaniasis (VL) infections, respectively.[1,2] Ethiopia has the third largest number of VL cases per year in sub-Saharan Africa, next to South Sudan and Sudan.[3] Therapeutic options for leishmaniasis are limited. It comprises amphotericin B,[4] pentavalent antimonials,[5] paromomycin,[6] miltefosine[7] and pentamidine[8] – most of which have drawbacks in terms of variable efficacy and safety. Limited supply of the existing drugs in resource-limited settings, their high costs[9] and the emergence of resistance[10] are also other challenges in fighting leishmaniasis, which calls for looking for viable options.

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Brucea antidysenterica J.F. Mill (genus: *Brucea*; family: Simaroubaceae) is one of the medicinal plants used in Ethiopian folk medicine for the treatment of leishmaniasis. It is an evergreen shrub 10–15 meter high, growing in the altitude range from 1400 to 2800 meters high.[11] It is widely distributed in tropical African countries such as Nigeria, Ethiopia, Cameroon, Burundi, Sudan, Guinea, Congo, Angola, Zambia and Malawi.[11] The plant got its name in honor of a Scottish traveler, James Bruce, who stayed in Ethiopia from 1769 to 1771.[12]

Ethnobotanical studies conducted in various parts of Ethiopia indicated that leaves, roots, bark, stems and seeds of *B. antidysenterica* J.F. Mill have been used for the treatment of numerous human medical disorders in folk medicine. For instance, the paste of leaf powder is applied topically to treat skin cancer,[13] leprosy,[14,15] eczema,[16] scabies,[16] and taken orally with water for the treatments of helminthiasis, anthrax, and malaria.[17] The seeds of the plant are also used for the treatment of different diseases: the paste of seeds powder is used in wound healing and venereal diseases,[18] and its powder is applied topically in the infected area for the treatment of cutaneous leishmaniasis.[15] In addition to reports on the use of *B. antidysenterica* J.F. Mill in folk medicines, the in vitro and in vivo study indicated that the plant extract and its compound isolates exhibited activities against *Mycobacterium tuberculosis*,[19] *Entamoeba histolytica*,[20] *Plasmodium berghei*[21] and against different strains of bacteria.[22] The aim of the present study was to evaluate anti-leishmanial activity of 80% methanol extract of *Brucea antidysenterica* J. F. Mill seeds against *L. donovani* and *L. aethiopica* using in vitro models.

Materials and Methods

Leishmania Parasites Test Strains, Cell Lines and Laboratory Animals

Clinical isolates of *L. aethiopica* (CL-027/20) and *L. donovani* (VL-139/19) were obtained from Leishmaniasis Research and Diagnostic Laboratory (LRDL), Addis Ababa University. The strain of *L. aethiopica* (CL-027/20) was isolated from a 13-year-old male patient living in Guna Woreda, Arsi Zone, Oromia region, Ethiopia, while the strain of *L. donovani* (VL-139/19) was isolated from a 27-year-old male patient residing in Kolme Woreda, Konso Zone, Southern Nations, Nationalities, and People's Region, Ethiopia. Red blood cells were collected from a 32-year -old health volunteer with no underlying chronic disease; Vero cell line (Vero ATCC CCL-81) was obtained from National Animal Health Diagnostic and Investigation Center, Sebeta, Ethiopia that was provided previously by the National Veterinary Institute (Bishoftu) previously; while Swiss Albino mice were obtained from Laboratory Animal House, Department of Pharmacology and Clinical Pharmacy, School of Pharmacy, College of Health Science, Addis Ababa University, Ethiopia. *Leishmania* parasite test strains, cell lines and laboratory animals were used after getting ethical clearance from approved Ethics Review Board of the School of Pharmacy, as stated in the ethical clearance section of this paper.

Collection and Authentication of Plant Materials

The ripened seeds of *Brucea antidysenterica* J.F. Mill were collected from Dega Damot district 399 km away from capital city, Addis Ababa, in the month of January, 2020. The plant was identified and authenticated by Mr. Melaku Wondafrash, a botanist at the College of Natural and Computational Sciences, Addis Ababa University, Ethiopia. The voucher number TK-004 was given to *B. antidysenterica* J.F. Mill specimen and kept in Addis Ababa University National Herbarium for future reference.

Preparation of Plant Extract

The seeds of *B. antidysenterica* J.F. Mill were air-dried under shade for 3 weeks and grounded to coarse powder using mortar and pestle. Then, 400 gram of the powder was macerated with 1.2 liter of 80% methanol (1:3) in volumetric flask. The macerate was shaken occasionally to increase solvent penetration and solubility of bioactive agents. Post 72 h of maceration, the extract was filtered with muslin gauze, followed with Whatman No. 1 filter paper. By adding fresh solvents, the mark was re-macerated twice and filtered again to maximize the yield. The combined filtrates were concentrated using a rotary evaporator at 40°C, then frozen at −20°C overnight and dried using a lyophilizer.[23]

Finally, a gold-colored solid extract of *B. antidysenterica* J.F. Mill having sticky nature was obtained and its percentage yields were calculated from its dried mass using the following formula:

$$Yeild\ \% = \frac{Weight\ of\ extract\ obtained}{Dry\ weight\ of\ plant\ material}$$

Preparation of Solvent Fractions

The dried 80% methanol extract was fractionated to n-hexane, ethyl acetate, n-butanol and aqueous fractions, respectively, based on increasing polarity of solvents according to a method described by Toma et al.[24] The 80% methanol extract (16 gm) was transferred to a separatory funnel and dissolved in distilled water (150 mL). The aqueous solution of the extract was washed with 150 mL of n-hexane (3 times) followed by equal volumes of ethyl acetate (3 times) and n-butanol (4 times), respectively, until the extracting solvent became colorless. Fractions of organic solvents were concentrated using a rotary evaporator under reduced pressure at temperatures of 40°C and dried in an oven at temperatures of 30°C, while the aqueous fraction was dried using freeze dryer. The dried crude extract and fractions were weighed and stored in freezer until used for the procedure.

The yield of 80% methanol extract of *B. antidysenterica* J.F. Mill was 32.68 gm (8.17%). The yield of n-hexane, ethyl acetate, butanol and aqueous fractions of 80% methanol extract of *B. antidysenterica* were 1.38 gm, 3.12 gm, 4.37 gm and 5.82 gm, respectively.

Promastigote Cultures

The logarithmic stages of the clinical isolates of *L. aethiopica* and *L. donovani*, after being grown in Novy–MacNeal–Nicolle media, were seeded and grown in 25 mL tissue culture flasks containing M199 medium supplemented with 20% heat-inactivated fetal calf serum, 25 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 2 mM L-glutamine and 100 IU/mL penicillin and 100 µg/mL streptomycin solution at 22°C for *L. aethiopica* and 26°C for *L. donovani* following the method described by Tariku et al[25] with minor modification.

Vero Cell Line Culture

The Vero cells (the green African monkey kidney cell lines) were cultured in Roswell Park Memorial Institute (RPMI-1640) with L-glutamine and sodium bicarbonate medium supplemented with 10% heat inactivated newborn calf serum (HINBCS), 100U/mL penicillin, and 100µg/mL streptomycin in humidified 5% CO_2 incubator at 37°C, as described by Ammerman and colleagues.[26] The cells' growth was monitored under inverted microscope and the media was changed every 3 days. The cells were sub-cultured when they reached >70% confluent monolayer.

Mice Peritoneal Macrophage Collection and Culture

The peritoneal macrophages were harvested from pathogen free, typically 6- to 8-week-old either sex of Swiss Albino mice according to the protocol previously described by Zhang et al,[27] with minor modification. Each mouse was injected with 2 mL of 2% potato starch solution, intraperitoneally, to induce inflammatory response. Post 48 h of injection, each mouse was euthanized with chloroform, its abdomen was swabbed with 70% ethanol and the skin underlying the peritoneal cavity was opened. Then, 10 mL of sterile ice-cold phosphate buffered saline solution (PBS) supplemented with 3% HINBCS was injected into the peritoneal cavity of each animal, its abdomen was massaged for about 10–15 seconds and the macrophage cells were collected by drawing 6–8 mL of peritoneal exudates of PBS from each mouse. The peritoneal exudates were centrifuged at 450 × g (1500 rpm) for 10 min at 4 °C. The supernatant was discarded, and the resulting pellets were re-suspended in cold Minimum Essential Media (MEM) medium supplemented with 10% HINBCS, 25mM HEPES, 2mM L-glutamine and 100 U penicillin and 100 µg streptomycin/mL. The cells were counted using hemocytometer and adjusted to 3.5×10^6 cells/mL in complete MEM medium.

Hemolysis Assay

Hemolytic effect of 80% methanol extract of *B. antidysenterica* J.F. Mill and its solvent fractions were conducted using methods described by Abeje et al[28] and Zohra et al.[29] From a healthy volunteer person, about 4 mL of blood samples were

collected using sterile syringe and transferred to a heparinized test tube. Two milliliter of the blood was added to 8 mL of PBS solution (PH 7.2) already transferred to 15 mL Eppendorf tube. The mixture was mixed well and centrifuged at 1000 × gm for 10 minutes. The supernatant was pipetted out using serological pipettes. The resulting pellet (1mL) was then transferred to 49 mL PBS solution (PH 7.2) to obtain 50 mL of 2% red blood cell suspension required for hemolytic tests.[29] Two hundred microliter of the suspension was transferred to Eppendorf tubes containing 200 µL of serially diluted concentrations of extracts (1600–50 µg/mL) of standard drugs: amphotericin B (AMB) (40–1.25 µg/mL) and pentamidine (120–3.75 µg/mL) prepared in two fold dilutions with each test concentration in triplicates. PBS solution was used as a diluting medium for the preparation of serial dilutions. Triton X-114 (5 µL/mL) in PBS solution and 1% DMSO in PBS solution were used as positive and negative controls, respectively. The contents of Eppendorf tubes were mixed gently and incubated at 37°C in the incubator for 2 h, except for Triton X-114, which was incubated for 30 min. After incubation, the tubes were centrifuged at 1000 × g for 10 min. From each tube, 100 µL of supernatant was transferred to a separate well of a 96-well microplate. Finally, absorbance of the supernatant was measured at 540 nm using Victor Multilabel Reader.[28] The hemolytic effect of each test substance was expressed as a percentage as per the following formula:[29]

$$Hemolysis\ (\%) = \frac{Absorbance\ of\ test\ drug\ wells - Aveage\ blank\ wells}{TritonX - 114\ Average\ absorbance\ in\ wells - Aveage\ blank\ wells} \times 100$$

Vero Cell Lines Cytotoxicity Assay

Vero cells at a density of 5×10^5 cells/mL were seeded in 96 microtiter plates. After incubation at 37°C in a 5% CO_2 incubator for 24 h, 100µL of serially diluted concentration of extract (50–1600 µg/mL), standard drugs, AMB (1.25–40 µg/mL) and pentamidine (3.75–120 µg/mL) in complete RPMI-1640 medium were added into 96-well microtiter plates in triplicates. The microplate was then incubated at 37°C, 5% CO_2 for 72 h. Post 68 h of incubation, 20 µL of 10% resazurin solution was added to each well and allowed to rest for 4 h. Finally, the viability of the cells was measured fluorometrically using multilabel plate reader at excitation and emission wavelengths of 544 nm and 590 nm, respectively, as described by Nigussie et al and Chan et al.[30,31] Standard anti-leishmanial drugs and negative controls (medium alone and 1% DMSO) were used in this assay to provide reference values:

$$Cell\ Viability\ (\%) = \frac{Absorbance\ in\ test\ drug\ wells - Average\ blank\ wells}{Average\ absorbance\ control\ wells - Average\ blank\ wells} * 100$$

Mouse Macrophage Viability Assay

Cytotoxicity of each test substance against mouse peritoneal macrophage isolates was determined using methods described previously by Afrin et al,[32] with minor modifications. Hundred microliter of mice peritoneal macrophages, suspended in a complete MEM medium (5×10^5 cells/mL), was seeded in 96-well microtiter plates. Serially diluted extracts (50–1600 µg/mL) and positive controls [AMB (1.25–40 µg/mL) and pentamidine (3.75–120 µg/mL)] and negative control (1% DMSO + Medium) in 100 µL of complete MEM medium were added to 96-well microtiter plates in triplicates and incubated in CO_2 incubator for 72 h at 37°C, 5% CO_2 and 95% humidity. After 68 h of incubation, 20µL of 10% resazurin solution (0.125 mg/mL) was added to each well and left for 4 h. Then, the fluorescence intensity of each well was measured using a Multilabel Reader at excitation and emission wavelengths of 544 nm and 590 nm, respectively.

The cytotoxic effects of test substances against mouse peritoneal macrophage isolates were expressed in terms of percentage of macrophage viability, which was determined using the following formula:

$$Cell\ Viability\ (\%) = \frac{Absorbance\ in\ test\ drug\ wells - Aveage\ blank\ wells}{Average\ absorbance\ control\ wells - Aveage\ blank\ wells} \times 100$$

Anti-Promastigote Assay

The anti-promastigote activities of the extracts were ascertained using methods previously described by Tariku et al,[25] with minor modifications. Serially diluted extract (400–6.25 µg/mL) contained in 100 µL of complete culture medium (M199 medium) was added to 96-well microtiter plates in triplicates. Then, 100 µL of logarithmic growth phase of parasite suspensions (3.00×10^6 promastigote cells/mL of *L. aethiopica* or *L. donovani*) were added to each well containing test substances. The contents of the microtiter plates were incubated for 72 h at 22°C (for *L. aethiopica*) and 26°C (for *L. donovani*). After 68 h of incubation, 20 µL of 10% fluorochrome resazurin solution (0.125 mg/mL, pH=7.2) was added to each well and allowed to rest for 4 h. Then, the fluorescence intensity was measured using a Multilabel Reader at an excitation wavelength of 544 nm and emission wavelength of 590 nm. Assays with standard anti-leishmanial drugs, AMB (10–0.156 µgm/mL), pentamidine (15–0.234 µg/mL) and medium with 1% DMSO were conducted as positive and negative controls, respectively.

The anti-promastigote activity of the extracts was expressed as a percentage of parasite growth inhibition, and it was determined using the following formula:[33]

$$Inhibition\ (\%) = 100 - \frac{Absorbance\ in\ test\ drug\ wells - Average\ blank\ wells}{Average\ absorbance\ control\ wells - Average\ blank\ wells} * 100$$

Anti-Amastigote Assay

Intracellular anti-amastigote activity of the extracts and controls were assayed using methods previously described by Afrin et al[32] and Utaile et al,[34] with minor modifications. Mouse peritoneal macrophages suspended in a complete MEM medium (3×10^5 cells/mL, 200 µL) were seeded in 16-well chamber slides and incubated for 24 h at 37°C in a humidified 5% CO_2 incubator. After 24 h, the chamber slides were washed with warm complete medium to remove non-adherent macrophages. The stationary stages of *L. donovani* and *L. aethiopica* promastigotes (parasite: macrophage: 10:1) were seeded in the chamber slides containing adherent macrophages and maintained at 31°C (*L. aethiopica*) or 37°C (*L. donovani*), 5% CO_2 and 95% relative humidity for further 24 h. Then, non-internalized promastigotes were removed by extensive washing with warmed MEM medium and serially diluted concentrations of extracts (400–6.25 µg/mL) were added and incubated for a further 48 h in CO_2 incubator. The culture medium was aspirated, and the slides were washed with PBS, fixated with methanol for 30 seconds and stained with Giemsa (10%) for 15 min. Finally, 50 macrophages per slide were counted and the number of amastigotes in each macrophage were enumerated. The assay was repeated with standard anti-leishmanial drugs: AMB (10–0.156 µgm/mL), pentamidine (15–0.234 µg/mL) and medium with 1% DMSO (negative standard). Serial concentrations of each test were prepared in two-fold dilutions with each test concentration in triplicates.

The anti-amastigote activity of the extracts per well was expressed as infection index, and it was calculated using the following formula:[35]

$$\text{Infection Index} = \frac{Infected\ macrophages\ (\%) \times Number\ of\ amastigotes}{\text{Total number of counted macrophages}}$$

Selectivity Index

The selectivity index (SI) of each test substance was determined from its CC_{50} against mammalian cells (Vero cell line and mice macrophage) and their corresponding IC_{50} against *Leishmania* parasites (IC_{50} of amastigotes). The SI of the extracts and standards in killing parasites as opposed to mammalian cells was estimated using the following formula:[31]

$$Selectivity\ index\ (SI) = \frac{CC50\ of\ Vero\ cell\ lines\ or\ mice\ macrophage\ cells}{IC50\ of\ antiamastigote\ parasites}$$

Quantitative Determination of Total Phenolic Compounds, Flavonoids and Alkaloids

The total phenol, flavonoid and alkaloid contents of 80% methanol extract of the seeds of *B. antidysenterica* J.F. Mill and its ethyl acetate and hexane fractions were determined using UV-spectrophotometry as per the following protocols.

Determination of Total Phenolic Compounds Content

The total phenolic contents of 80% methanol extract of the plant and its fractions were determined using Folin–Ciocalteu's method, as described by Nigatu et al.[36] Serial concentrations of gallic acid (100–6.25 µL/mL) as a standard solution were prepared in methanol. From each aliquot of this solution, 1 mL was transferred into a separate 10mL tube. Five milliliters of methanol, 0.5 mL of Folin–Ciocalteu's reagent and, 5 minutes later, 1.5 mL of 20% Na_2CO_3 were added into each Eppendorf tube. Each tube content was mixed gently, its final volume adjusted to 10 mL with methanol and incubated at room temperature. After 90 min incubation, the absorbance of each solution was measured at a wavelength of 760 nm using UV-Vis spectrophotometer. The solutions of the crude extract and its fractions at concentration of 100 µg/mL were prepared in methanol. One mL of each solution of 80% methanol extract, ethyl acetate fraction, hexane fraction and methanol (blank) were transferred to separate Eppendorf tubes. The total phenol estimates were expressed as mg of gallic acid equivalent per g of dry extract/fractions (mg of GAE/g).

Determination of Total Flavonoids Content

The total flavonoids content of the 80% methanol extract and its fractions were determined using a complex formed by aluminum chloride as per methods previously described by Nigatu et al.[36] Serial concentrations of standard, quercetin solution, (1–0.065 mg/mL) were prepared in methanol to establish calibration curve. One mL of each aliquot of standard solution, followed by 0.3 mL of 5% $NaNO_2$, was transferred to separate 15 mL tubes and left for 5 min. Another 0.3 mL of 10% $AlCl_3$ was added to the contents, mixed with the solution and left to stand for 5 min once again. Then, 2 mL of 1M NaOH solution was added, and the final concentration of the complex was adjusted to 10 mL with distilled water. Each complex was incubated for 30 min at room temperature. Finally, the absorbance of the complex was measured at a wavelength of 510 nm using UV-Vis spectrophotometer. The crude extract and its fractions were also dissolved in methanol to obtain 1 mg/mL. Then, 1 mL of each solution of 80% methanol extract, its fractions and methanol (blank) were transferred into separate Eppendorf tubes and the same procedure was followed as standard solution. Serial concentrations of solution were prepared in two-fold dilutions, and all tests were performed in triplicate. The total flavonoid estimates were expressed as mg of quercetin equivalent per g of dry extract/fractions (mg of QE/g).

Determinations of Total Alkaloid

Total alkaloid content of the 80% methanol extract and its fractions were determined, using the method previously described by Ajanal et al.[37] Two mL solutions of each 80% methanol extract and its fractions (1 mg/mL) in methanol were mixed with 2 mL of 2N HCl solution in separate 50 mL tubes and filtered. One mL of the filtrate was transferred to a separatory funnel and washed with 5 mL of chloroform 2 times. The chloroform extract was discarded, and the pH of the rest of the solution was adjusted to neutral with 0.1 M NaOH solution. To the neutralized solution, 5 mL of bromocresol green solution and 5 mL of buffer solution (PH, 4.7) were added and shaken. The complex was extracted with 4 mL of chloroform, and repeated once with 4 mL of chloroform upon vigorous shaking. The extract was collected in separate 50 mL tubes and its final volume was adjusted to 10 mL with chloroform. The absorbance of the chloroform extract was measured at a wavelength of 470 nm using UV-spectrophotometer.

The average absorbance of blank solution was subtracted from standard and crude extract/fraction solutions and the total alkaloid content was determined using standard curve of Atropine. The total alkaloidal estimates were expressed as mg of atropine equivalent per g of dry extract/fractions (mg of AE/g).

Statistical Analysis

Anti-promastigote activity (IC_{50}) and anti-amastigote activity (IC_{50}) were calculated from sigmoidal dose–response curves of percentage inhibition and infection index, respectively. The cytotoxic effect (CC_{50}) of the extract against

mammalian cells was calculated from sigmoidal dose–response curves of percentage viability of mammalian cells. Data was presented as mean ± standard error of the mean (SEM). The total content of each secondary metabolite in the crude extract and its fractions was calculated using a calibration curve of absorbance of standards. GraphPad Prism 9.1.0 computer software (GraphPad Software, Inc., CA, USA) was used to estimate IC_{50} and CC_{50}. Selectivity index (SI) was also obtained from the ratio between CC_{50} to IC_{50}.

Ethical Clearance

All procedures conducted in this research work were reviewed and approved by Ethics Review Board of the School of Pharmacy, College of Health Sciences, Addis Ababa University, with a letter number ERB/SOP/177/13/2020 dated September 2020. This Ethics Review Board reviews basic research involving experimental animals and clinical research involving patients and human volunteers. Informed consent was obtained from the patients to use the parasite isolates and from the volunteer human to use the collected red blood cells for research purposes. Experimental animal rearing and handling were in compliance with the European Directive 2010/63/UE.

Results

Anti-Leishmanial Activity

The 80% methanol extract of *B. antidysenterica* J.F. Mill seeds and its solvent fractions showed activities against clinical isolates of promastigotes and intracellular amastigotes of *L. aethiopica* and *L. donovani* with varying IC_{50}. The ethyl acetate fraction revealed the highest anti-leishmanial activity, with $4.14 \pm 0.62 \leq IC_{50} \leq 6.85 \pm 1.46$ µg/mL against promastigotes and amastigotes of test strains (Table 1 and Table 2).

The aqueous fraction ($189.3 \pm 8.70 \leq IC_{50} \leq 208.9 \pm 20.2$ µg/mL) and the butanol fraction ($36.29 \pm 6.00 \leq IC_{50} \leq 40.12 \pm 5.30$ µg/mL) exhibited the lowest activities against the promastigotes and amastigotes of leishmania species, respectively (Table 1 and Table 2).

Cytotoxicity Effects

The cytotoxic effects (CC_{50}) of 80% methanol extract of the plant and three other solvent fractions against human red blood cells, Vero cell lines and peritoneal mice macrophages were also presented. All solvent fractions and crude extract showed $CC_{50} > 1600$ µg/mL against red blood cells. The crude extract and its hexane, ethyl acetate and butanol fractions revealed 8.28 ± 0.01, 7.76 ± 0.23, 7.63 ± 0.47 and 4.57 ± 0.42% of hemolysis at 1600 µg/mL, respectively. On the other hand, the hexane fraction revealed the highest cytotoxic effect, with CC_{50} values of 126.75 ± 6.55 and 134.35±12.95 µg/mL against Vero cell lines and macrophages, respectively (Table 3). The finding also exhibited that the 80% methanol

Table 1 The IC_{50} of Test Substances Against Promastigotes of *Leishmania aethiopica*

Test Substance	*Leishmania aethiopica*				*Leishmania donovani*			
	Experiment-1		Experiment-2		Experiment-1		Experiment-2	
	IC_{50} (µg/mL) [a]	R^2	IC_{50} (µg/mL) [a]	R^2	IC_{50} (µg/mL) [a]	R^2	IC_{50} (µg/mL) [a]	R^2
MET-BA	25.63 ± 1.07	0.9968	27.83 ± 1.06	0.9941	20.77 ± 1.55	0.9911	22.15 ± 3.31	0.9721
HEX-BA	8.54 ± 0.29	0.9977	9.35 ± 0.76	0.9911	6.84 ± 1.18	0.9619	7.19 ± 1.00	0.9755
ETAC-BA	6.53 ± 0.57	0.9897	6.77 ± 0.47	0.9930	4.14 ± 0.62	0.9706	4.74 ± 0.42	0.9896
BUT-BA	43.81 ± 4.14	0.9855	46.35 ± 3.00	0.9930	60.12 ± 6.95	0.9725	56.07 ± 5.07	0.9869
AQU-BA	208.9 ± 20.2	0.9561	200.5 ± 16.8	0.9642	196.85 ± 15.05	0.9751	189.3 ± 8.70	0.9897
PEN	0.82 ± 0.13	0.9740	0.9 ± 0.10	0.9849	0.87 ± 0.11	0.9635	0.91 ± 0.14	0.9747
AMB	0.17 ± 0.08	0.9497	0.18 ± 0.04	0.9796	0.15 ± 0.01	0.9879	0.18 ± 0.03	0.9850

Notes: The values are expressed as mean ± SEM; n=3. [a]Effective concentration required to achieve 50% growth inhibition in µg/mL.

Abbreviations: MET-BA, 80% methanol extract of seeds of *Brucea antidysenterica* J.F. Mill; HEX-BA, ETAC-BA, BUT-BA, AQU-BA, hexane, ethyl acetate, butanol and aqueous fractions of 80% methanol extract of seeds of *Brucea antidysenterica* J.F. Mill, respectively; R^2, Regression coefficient; AMB, Amphotericin B; PEN, Pentamidine isethionate.

Table 2 The IC_{50} of Test Substances Against Intracellular Amastigotes of *Leishmania aethiopica* and *Leishmania donovani*

Test Substance	Against *L. aethiopica*		Against *L. donovani*	
	IC_{50} (μg/mL) [a]	R^2	IC_{50} (μg/mL) [a]	R^2
MET-BA	18.55 ± 3.72	0.9533	16.47 ± 2.39	0.9742
HEX-BA	8.99 ± 2.02	0.9439	7.89 ± 1.37	0.9608
ETAC-BA	6.16 ± 1.12	0.9584	6.85 ± 1.46	0.9456
BUT-BA	36.29 ± 6.00	0.9638	40.12 ± 5.30	0.9742
PEN (Reference drug)	0.69 ± 0.05	0.9731	0.64 ± 0.08	0.9802
AMB (Reference drug)	0.089 ± 0.01	0.9978	0.079 ± 0.02	0.9901

Notes: The values are expressed as mean ± SEM; n=3. [a]Effective concentration required to achieve 50% growth inhibition in μg/mL.
Abbreviations: MET-BA, 80% methanol extract of seeds of *Brucea antidysenterica* J.F. Mill; HEX-BA, ETAC-BA, BUT-BA, hexane, ethyl acetate and butanol and fractions of 80% methanol extract of seeds of *Brucea antidysenterica* J.F. Mill, respectively; R^2, Regression coefficient; AMB, Amphotericin B; PEN, Pentamidine isethionate.

Table 3 CC_{50} of Test Substance Against Vero Cell Lines and Peritoneal Mice Macrophage Isolates

Test Substances	Against Vero Cell Lines		Against Macrophage Cells	
	CC_{50} (μg/mL) [a]	R^2	CC_{50} (μg/mL) [a]	R^2
MET-BA	221.50 ± 19.60	0.9850	232.5 ± 21.00	0.9788
HEX-BA	126.75 ± 6.55	0.9958	134.35 ± 12.95	0.9849
ETAC-BA	187.00 ± 15.40	0.9880	190.80 ± 23.20	0.9784
BUT-BA	323.45 ± 15.95	0.9952	319.90 ± 27.60	0.9856
PEN (Reference drug)	8.28 ± 2.22	0.9068	9.16 ± 2.02	0.9348
AMB (Reference drug)	9.35 ± 1.34	0.9623	12.77 ± 0.85	0.9891

Notes: The values are expressed as mean ± SEM; n=3. [a]Effective concentration required to kill 50% animal cell lines in μg/mL.
Abbreviations: MET-BA, 80% methanol extract of seeds of *Brucea antidysenterica* J.F. Mill; HEX-BA, ETAC-BA, BUT-BA, hexane, ethyl acetate and butanol fractions of 80% methanol extract of seeds of *Brucea antidysenterica* J.F. Mill, respectively; NC, negative control; DMSO, dimethyl sulfoxide; R^2, regression coefficient; AMB, Amphotericin B; PEN, Pentamidine isethionate.

extract of the plant and its solvent fractions ($7.97 \leq SI \leq 30.97$) showed selective toxicities toward leishmania parasites (Table 4).

Total Contents of Phenolic Compounds, Flavonoids and Alkaloids

The TPC and TFC of 80% methanol extract of seeds of *B. antidysenterica* and its ethyl acetate and hexane fractions are presented in Table 5. The TPC of the 80% methanol extract and its fractions were obtained from the equation of the calibration curve of gallic acid: $Y = 0.006352*X + 0.08988$, $R^2 = 0.9778$. On the other hand, their TFCs were calculated from the standard curve of quercetin: $Y = 0.6187*X - 0.01021$, $R^2 = 0.9952$. Additionally, the total alkaloid contents were extrapolated from the calibration curve of atropine: $Y = 0.001312*X + 0.001750$, $R^2 = 0.9934$.

Discussion

The highest and the lowest anti-promastigote activities were recorded by ethyl acetate and aqueous fractions, respectively. The IC_{50} of the crude extract and its solvent fractions against promastigotes of *L. donovani* were ranging from 27 to 1170, and 5 to 217 times those of amphotericin B and pentamidine isethionate, respectively. Similarly, their IC_{50} against promastigotes of *L. aethiopica* were ranging from 38 to 1170 and 8 to 238 times the IC_{50} of amphotericin B and

Table 4 Selectivity Indices of Test Substances Between Parasite and Animal Cell Lines

Test Substances	Selectivity Index (SI)			
	Vero Cell Lines (CC_{50} of Cell /IC_{50} PARASITE)[a]		Peritoneal Mice Macrophage Cells (CC_{50} of Cell /IC_{50} Parasite)[a]	
	L. aethiopica	*L. donovani*	*L. aethiopica*	*L. donovani*
MET-BA	11.94	13.45	12.53	14.12
HEX-BA	14.10	16.06	14.94	17.03
ETAC-BA	30.36	27.30	30.97	27.85
BUT-BA	8.91	8.06	8.82	7.97
PEN (Reference)	12.00	12.94	13.28	14.31
AMB (Reference)	105.06	118.35	143.48	161.65

Notes: [a]Value selectivity index calculated as ratio of CC_{50} of mammalian cell to its respective IC50 against parasite.
Abbreviations: MET-BA, 80% methanol extract of seeds of *Brucea antidysenterica* J.F. Mill; HEX-BA, ETAC-BA, BUT-BA, hexane, ethyl acetate and butanol fractions of 80% methanol extract of seeds of *Brucea antidysenterica* J.F. Mill, respectively; AMB, Amphotericin B; PEN, Pentamidine isethionate.

Table 5 Total Phenolic, Flavonoid and Alkaloid Content of 80% Methanol Extract of Seeds of *Brucea antidysenterica* J.F. Mill and Its Fractions

Types of Extract/Fractions	Total Phenol (Mg of GAE/g)	Total Flavonoid (Mg of QE/g)	Total Alkaloid (Mg of AE/g)
80% Methanol extract	127.72 ± 1.82	79.21 ± 0.19	27.62 ± 0.27
Ethyl acetate fraction	70.78 ± 0.95	46.67 ± 0.14	97.22 ± 0.25
Hexane fraction	54.78 ± 1.39	18.30 ± 0.07	38.04 ± 0.25

Notes: The values are expressed as mean ± SEM; n = 3.
Abbreviations: GAE/g, milligram of gallic acid equivalent per gram of dry extract/fraction; QE/g, milligram of quercetin equivalent per gram of dry extract/fraction; AE/g, milligram of atropine equivalent per gram of dry extract/fraction.

pentamidine isethionate, respectively, suggesting that the anti-promastigote activities of the crude extract and its solvent fractions were much lower than the activities shown by reference drugs (Table 1).

The level of in vitro activities of the reference drugs, crude extract and its solvent fractions were appreciated based on the following criteria: $IC_{50} \leq 5$ µg/mL pronounced activity; $5 < IC_{50} \leq 20$ µg/mL good activity; $20 < IC_{50} \leq 30$ µg/mL moderate activity; $30 < IC_{50} \leq 64$ µg/mL low activity; and $IC_{50} > 64$ µg/mL inactive.[38] Accordingly, the crude extract, n-hexane fraction and n-butanol fraction possessed moderate, good and low anti-promastigote activity, respectively, against both *L. aethiopica* and *L. donovani* isolates. The ethyl acetate fraction demonstrated good anti-leishmanial activity against *L. aethiopica* and pronounced activity against *L. donovani,* while aqueous fraction was found to be inactive against both *L. donovani* and *L. aethiopica* promastigotes (Table 1).

The in vitro promastigote parasites culture in cell free media is simple and reproducible,[40] even though the ecology, metabolism, morphology, composition of the surface glycocalyx of the promastigote differ from those of amastigote.[40,41] Hence, the results obtained from anti-promastigote screening may not be correlated with ex vivo intracellular amastigotes or in vivo animal models. In the current study, the potential anti-amastigote activities, clinically relevant stages of *Leishmania*, of the crude extract and hexane, ethyl acetate and butanol fractions were evaluated as confirmatory tests of their anti-promastigote activities.

Eighty percent methanol extract, hexane and ethyl acetate fractions were found to have good activity against the intracellular amastigotes of both *L. donovani* and *L. aethiopica,* whereas butanol fraction was found to have low anti-amastigotes activity against both species of *Leishmania* (Table 2). Like anti-promastigotes assay, ethyl acetate fractions exhibited the highest activity against intracellular amastigotes (Table 2).

The toxicity level of bioactive agents was classified previously based on the following criteria: <10μg/mL very strong cytotoxicity, 10–100 μg/mL strong cytotoxicity, 100–500 μg/mL moderate cytotoxicity.[42] Accordingly, the crude (80% methanol) extract and its fractions are non-toxic to RBC. On the other hand, the crude extract and its solvent fractions exhibited a moderate cytotoxic effect against both Vero cells and mice macrophages (Table 3). However, extrapolating how the in vitro cytotoxicity test results relate to the in vivo results is problematic as pharmacokinetics of a bioactive compound in the tissue is not considered in the environment of in vitro study.[39]

A selectivity index (SI) value >1 is considered to be selective against the *Leishmania* parasites and a value <1 is considered as selective against mammalian host cells.[42] In view of that, all test groups were selective against amastigotes of both strains of *Leishmania*. But the crude extract and its fractions showed less selectivity than amphotericin B. Conversely, they showed comparable selectivity against *Leishmania* spps as pentamidine isethionate except ethyl acetate fraction, which is more selective against the parasites (Table 4).

Preliminary phytochemical screening conducted previously by Tessema et al indicated the presence of alkaloids, tannins, flavonoids, triterpenoids, phenols, steroids and glycosides in 80% methanol extract of seeds of *B. antidysenterica* J.F. Mill.[23] Phenolic compounds include simple phenols, cinnamic acid derivatives, coumarins, benzoic acid derivatives, tannins, flavonoids, lignins and lignans, among others.[43] Phenolic compounds obtained from various plants having inhibitory activities against protozoan parasites including *Leishmania* parasites were reported. Cinnamic acid derivatives (eg *o*-coumaric acid and *p*-coumaric acid), flavonol derivatives (morin and rutin), hydroxybenzoic acid derivatives (gallic acid, gentisic acid, vanillic acid) and ellagic acid were reported for their in vitro activities against *L. amazonensis*. Moreover, gentisic acid and *p*-coumaric acid were also reported to have in vivo activity against *L. amazonensis* in BALB/c mouse model.[44] Treatment of *L. donovani* promastigotes and intracellular amastigotes with rosmarinic acid also led to alteration of membrane integrity of mitochondrial and other cells as a result of its iron chelation capability.[45] The phenolic compounds contained in 80% methanol extract and its ethyl acetate and hexane fractions might be attributable to their anti-leishmanial activities (Table 5).

Furthermore, flavonoids which include chalcones, flavones, flavonols, and isoflavones are also largely known for their wide spectrum of activities against leishmaniasis.[46] Quercetin, another flavonoid compound, was also found to chelate iron and inhibit topoisomerase II, the enzymes used in the replication of parasites within the phagolysosomes of macrophage.[47] In the current study, TFC of 80% methanol extract and its ethyl acetate and hexane fractions might be responsible for their ant-leishmanial activities (Table 5).

Canthine alkaloids (canthin-6-one and 5-methoxycanthin-6-one) isolated from stem bark of *Zanthoxylum chiloperone*[49] also previously isolated from *B. antidysenterica*[50] demonstrated in vitro anti-leishmanial activity against *L. braziliensis, L. amazonensis*, and *L. donovani*. Canthin-6-one also displayed an interesting leishmanicidal activity against *L. amazonensis*-infected BALB/c mice when administered intralesionally, even though it did not show any activity against the parasites when administered orally.[49] It might be due to inactivation of the compound in gastro-intestinal tract of the mice as a result of digestive enzymes or gastric acid contents. Another alkaloid isolate, β-Carboline -1-propionic acid (β-CPA), obtained from stem bark of *Quassia amara* L. (Simaroubaceae)[51] and previously isolated from *B. antidysenterica,*[52] displayed potent anti-leishmanial activity of *L. amazonensis* and *L. infantum* against promastigotes and intracellular amastigotes, respectively.[51] The alkaloids contained in 80% methanol extract and its ethyl acetate and hexane fractions might be responsible for their anti-leishmanial activities (Table 5).

The crude extracts and compound isolates obtained from other members of genus *Brucea* also revealed activities against *Leishmania and Trypanosoma*. Aqueous, and 80% methanol extracts of seeds and leaves of *Brucea sumatrana* and their fractions revealed anti-leishmanial activities against *L. infantum*.[38,53] The plant was also found to be active against two *Trypanosoma* (*T. cruzi* and *T. brucei brucei*), the parasites related to *Leishmania* species.[38,53]

This finding indicated that the high concentrations of phenolic compounds and flavonoids were detected in 80% methanol extract while high concentration of alkaloids was detected in its ethyl acetate fraction, respectively. Conversely, the lowest concentrations of TPC, TAC and TFC were found in hexane fractions (Table 5). This suggested that the anti-leishmanial activities of 80% methanol extract of the seeds of *B. antidysenterica* and its ethyl acetate fraction were attributed to phenols and alkaloids, respectively, or it might be the combined effects of phenols, flavonoids, alkaloids and other secondary metabolites contained in the plant.

Conclusion

Ethyl acetate and hexane fractions were found to exhibit high anti-leishmanial activities against *L. donovani and L. aethiopica* isolates. The report validates use of *B. antidysenterica* J.F. Mill seeds in the treatment of leishmaniasis by traditional healers. Isolation and identification of specific compounds from the active fractions are required to find hits and lead compounds that can be an input in anti-leishmanial product development.

Abbreviations

AMB, Amphotericin B; AE/g, Atropine equivalent per gram of extract/fraction; ATCC, American Type Culture Collection; CC50, Cytotoxic Concentrations that kill 50% of the cells; CL, Cutaneous leishmaniasis; DMSO, Dimethylsulfoxide; GAE/g, Gallic acid equivalent per gram of extract/fraction; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; HINBCS, Heat Inactivated New Born Calf Serum; GAE/g, Gallic acid equivalent per gram of extract/fraction; IC50, Effective Concentration that inhibits 50% of the cells; LRDL, Leishmaniasis Research and Diagnostic Laboratory; M-199, Medium −199 with Earle's salts; MEM, Minimum Essential Media; QE/g, Quercetin equivalent per gram of extract/fraction; RPMI, Roswell Park Memorial Institute; SI, Selectivity Index; TAC, Total alkaloidal content; TFC, Total flavonoid content; TPC, Total phenolic content; VL, Visceral leishmaniasis; WHO, World Health Organization.

Data Sharing Statement

Data supporting the results reported in the manuscript will be available upon request.

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Author Contributions

All authors made a significant contribution to the work reported, whether that is in the conception, study design, execution, acquisition of data, analysis and interpretation, or in all these areas; took part in drafting, revising or critically reviewing the article; gave final approval of the version to be published; have agreed on the journal to which the article has been submitted; and agree to be accountable for all aspects of the work.

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