In vitro anti-leishmanial activities of 80% methanol extract of *Brucea antidysenterica* J.F. Mill seeds and its solvent fractions

BY:

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This is to certify that the thesis prepared by Tasisa Ketema titled "*In vitro* anti-leishmanial activities of 80% methanol extract of *Brucea antidysenterica* J.F. Mill seeds and its solvent fractions" and submitted in partial fulfillment of the requirements for the Degree of Master of Science in Pharmacology complies with the regulations of the University and meets the accepted standards concerning originality and quality.

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ABSTRACT

***In vitro* anti-leishmanial activities of 80% methanol extract of *Brucea antidysenterica* J.F. Mill seeds and its solvent fractions**

Tasisa Ketema

Addis Ababa University, 2022

Leishmaniasis is a neglected tropical disease threatening lives of about 350 million people, globally. However, the currently available treatment options are limited and have drawbacks including variable efficacy, toxicity and unaffordability. Seeds of *Brucea antidysenterica* is used for the treatment of cutaneous leishmaniasis in traditional medicine. This study was aimed to evaluate its anti-leishmanial activity *in vitro*. Following preliminary anti-leishmanial activity studies, crude (80% methanol) extract of *Brucea antidysenterica* 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 (TPC), flavonoids (TFC) and alkaloids (TAC) were determined, spectrophotometrically. The crude extract and its hexane, ethyl acetate and butanol fractions showed anti-leishmanial activities with IC_{50} values of ($4.14 \pm 0.62 \leq IC_{50} \leq 60.12 \pm 6.95$ µg/ml) against promastigotes, and ($6.16 \pm 1.12 \leq IC_{50} \leq 40.12 \pm 5.30$ 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 $100 \leq CC_{50} \leq 500$ µg/ml but > 1600 µg/ml against red blood cells. Having the selectivity index of ($7.97 \leq SI \leq 30.97$), the crude extract and its fractions showed selectivity toward *Leishmania* parasites. The crude extract, and its ethyl acetate and hexane fractions possessed ($54.78 \pm 1.39 \leq TPC \leq 127.72 \pm 1.82$ mg of gallic acid equivalent), ($18.30 \pm 0.07 \leq TFC \leq 79.21 \pm 0.19$ mg of quercetin equivalent) and ($27.62 \pm 0.27 \leq TAC \leq 97.22 \pm 0.25$ mg of atropine equivalent) per gram of extracts. 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 of seeds of *B. antidysenterica*.

KEY WORDS: Anti-amastigotes, anti-leishmanial, anti-promastigotes, *Brucea antidysenterica*, *Leishmania aethiopica*, *Leishmania donovani*

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LIST OF ABBREVIATIONS AND ACRONYMS

AE/g	Atropine equivalent per gram of extract/fraction
AMB	Amphotericin B
ATCC	American type culture collection)
CC_{50}	Cytotoxic concentrations that kills 50% of the cells
CL	Cutaneous leishmaniasis
DMSO	Dimethylsulfoxide
GAE/g	Gallic acid equivalent per gram of extract/fraction
GP63	Glycoprotein (zink metallopeptidase)
HEPES	(4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid)
HINBCS	Heat inactivated new borne calf serum
IC_{50}	Effective concentration that which inhibits 50% of the cells
IFN-γ	Interferon gamma
IL-12	Interleukin 12
iNOS	*Inducible* nitric oxide synthase
JAK	Janus kinase
JAK-STAT	Janus kinase and signal transducer and activator of transcription
LPG	Lipophosphoglycan
LRDL	Leishmaniasis research and diagnostic laboratory
M-199	Medium -199 with earle's salts (M199)
MAPK	Mitogen-activated protein kinase
MCL	Mucocutaneous leishmaniasis
MEM	Minimum essential medium
NF-kB	Nuclear factor kappa
NNN	Novy-MacNeal-Nicolle
PBS	Phosphate buffered saline
PG	Phosphoglycan
PKC	Protein kinase C
PKDL	Post- kala-azar dermal leishmaniasis
PSG	Promastigote secretory gel
QE/g	Quercetin equivalent per gram of extract/fraction

ROS/RNS	Reactive oxygen species/ reactive nitrogen species.
RPMI-1640	Roswell park memorial institute 1640
SHP-1	Protein tyrosine phosphatases
SI	Selectivity index
SNNPR	Southern nations and nationalities people's region
SSG	Sodium stibogluconate
TAC	Total alkaloidal content
TFC	Total flavonoid content
TGF-β	Transforming growth factor β
THP-1	Human leukemia monocyte THP-1 cells
TPC	Total phenolic content
U-937	Human myeloid leukaemia cell line
UV	Ultraviolet
VL	Visceral leishmaniasis
WHO	World Health Organization

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1. INTRODUCTION

1.1. Aetiology and Clinical Forms of Leishmaniasis

Leishmaniasis is a vector borne neglected tropical disease caused by parasites in the genus *Leishmania*. It is transmitted by bite of infected female sand fly (1,2). There are nearly 21 *Leishmania* species responsible for *Leishmania* infections (Table 1.1) (3). Depending on the *Leishmania* species and the immunological response of the patients toward the disease, leishmaniasis has a broad range of clinical manifestations: cutaneous, mucocutaneous, visceral, post-kala-azar dermal and diffuse cutaneous leishmaniasis (4).

Visceral Leishmaniasis

Visceral leishmaniasis (VL) is mainly caused by *Leishmania donovani* and *Leishmania infantum.* Exceptionally, in some immuno-compromised patients dermatotropic *Leishmania* species may cause visceral leishmaniasis (5). Clinical VL is a severe medical condition that results in death in the absence of treatment. In most infections parasites are controlled by self-limiting granulomatous tissue response but it might not be removed completely (6). Based on their epidemiology and clinical features, there are two main forms of visceral leishmaniasis. The first one is a zoonotic visceral leishmaniasis which is caused by *L. infantum.* It is prevalent in China, the Middle East, South America and Mediterranean basin. As we can understand from its name the reservoirs of the zoonotic VL are animals mainly, dogs, and it usually affects young children and dogs. The second form is an anthroponotic visceral leishmaniasis which is caused by *L. donovani* and occurs in India, Bangladesh, and Nepal and East African countries, and it affects people of all age groups. Unlike zoonotic VL, anthroponotic VL is transmitted from man to man through bites of infected sand flies and in this sense it doesn't affect other animals (7). VL is characterized by enlarged liver (hepatomegaly), markedly enlarged spleen (splenomegaly) (Fig.1.1. H), pancytopenia (which is caused by hypersplenism, bone marrow infiltration, and autoimmune mechanisms), anemia and hypergammaglobinemia (Fig.1.1. I), prolonged fever, abdominal pain, fatigue, and unintentional weight loss (8,9).

Table 1. 1. Types of *Leishmania* Species, Distribution and Clinical Forms of Leishmaniasis (3).

Geographical distribution	**Subgenus and Species**			
	L.(Leishmania)	**L.(Leishmania)**	**L.(Viannia)**	**L.(Viannia)**
Old World	*L. donovani* *L. infantum*	*L. major* *L. tropica* *L. killicki* *L. aethiopica* *L. infantum*		
New World	*L. infantum*	*L. infantum* *L. mexicana* *L. pifanoi* *L. venezuelensis* *L. garnhami* *L. amazonensis*	*L. braziliensis* *L. guyanensis* *L. panamensis* *L. shawi* *L. naiffi* *L. lainsoni* *L. lindenbergi* *L. peruviana* *L. colombiensis*	*L. braziliensis* *L. panamensis*
Principal tropism	Viscerotropic	Dermotropic	Dermotropic	Mucotropic

Post–Kala-azar Dermal Leishmaniasis

Post- Kala-azar Dermal Leishmaniasis (PKDL) is a complication of visceral leishmaniasis which may occur before full recovery from its primary infection (VL). It is mainly limited to areas endemic for VL caused by *L. donovani*. It is prevalent in Africa particularly in Sudan and to a lesser extent in Ethiopia, Kenya or Uganda and Asia such as Bangladesh and to a lesser extent India. It may also occur occasionally in *L. infantum* or *L. chagasi* endemic areas such as Latin America and Mediterranean countries (10).

PKDL is characterized by maculopapular, macular and nodular rashes (Fig.1.1. A - C) and usually start around mouth and spread to other body parts depending on its severity. These phenomena is mainly seen in Sudan and India with degree of 50% and 5-10%, respectively, following VL therapy (11). There are, however, important clinical and epidemiological

difference between African and Asian PKDL. The first difference is in terms of its clinical presentations of skin complications (90% papular rash seen in Africa while 90% macular rash in Asia). The second difference is the time interval between VL and PKDL development (0-13 months elapsed in Africa while 2-3 years in Asia). In addition, in Africa, PKDL is most common in Sudan; where in most patients (about 85%) the disease is self-limiting that heal within 12 months and only severe or chronic cases observe chemotherapies. In Asia all patients require chemotherapies even though there are some self-limiting case reports (12). In addition, PKDL is also thought to be a reservoir for visceral leishmaniasis, thus, adequate control of PKDL is required to eliminate VL (13). Although the risk factors for the likelihood of development of PKDL is not fully understood, incomplete or inadequate treatments of VL (in terms of duration, drug type), being young, HIV infection, parasite strain (emergence of resistance), malnutrition and genetic backgrounds of the patients are some examples of risk factor for PKDL development (12,13).

Mucocutaneous Leishmaniasis

Mucosal (Mucocutaneous) leishmaniasis (MCL) is defined as a destructive form of leishmaniasis that causes mucosal inflammation or ulceration of mucus membranes such as mouth, nose and throat (Fig.1.1. F - G) (14). It has been mainly prevalent in the new world countries such as Bolivia, Brazil and Peru and caused by mucotropic *Leishmania* species such as *L. braziliensis* and *L. panamensis* (3). It may also develops from the complications of dermotropic leishmaniasis in the circumstance where parasites are transported to upper respiratory tract mucosal membranes from its primary site, cutaneous, via either lymphatic system or blood. Once it reach there, it causes partial or total destruction of nasal mucosal membranes, pharynx, larynx and upper lips (3,14). It is a potentially life threatening *Leishmania* infection leading to permanent disfigurements of larynx which may leads to aspiration pneumonia that needs to be diagnosed and treated promptly. Immuno-suppression, multiple or large primary lesions, a primary lesions above the waistline or delayed healing from primary lesions are risk factors for mucocutaneous leishmaniasis (15).

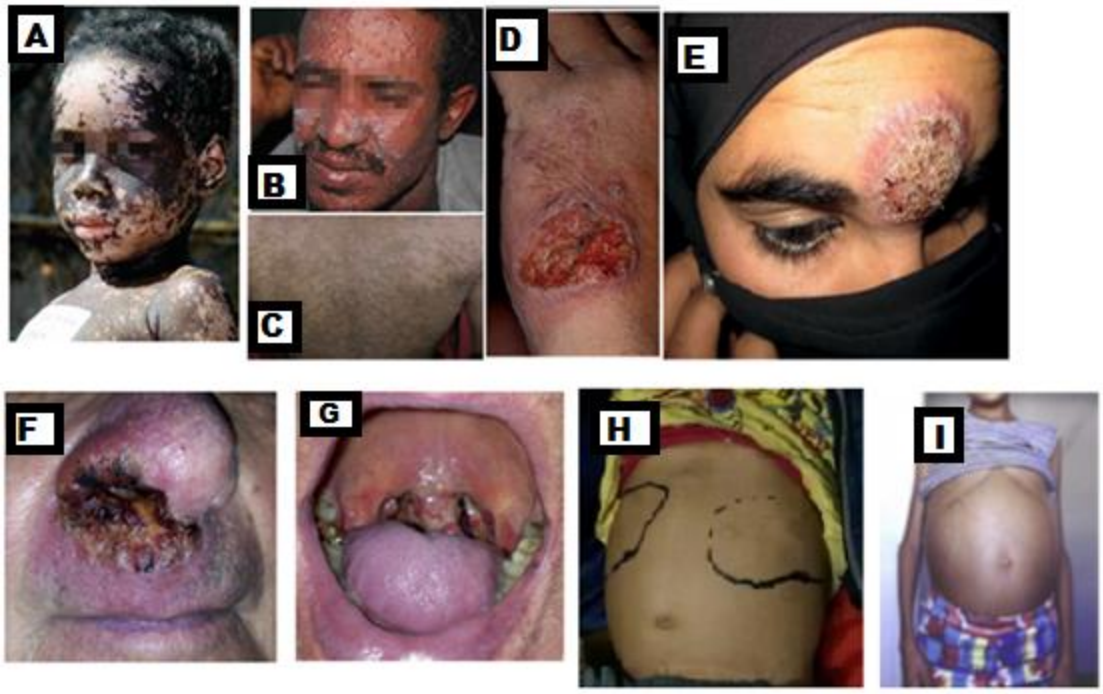

Figure 1. 1.Various clinical forms Leishmaniasis. (A-C) Post–Kala-azar Dermal Leishmaniasis (10); (D-E) Cutaneous Leishmaniasis (15); (F-G) ML (15) and (H-I) Visceral Leishmaniasis (16,17)

Cutaneous Leishmaniasis

Cutaneous leishmaniasis (CL) is a skin diseases (lesions) caused by various *Leishmania* parasites (18). The lesions are characterized by the presence of papules, nodules and ulcers (Fig.1.1. D - E) which heal with scarring (4) and lymphadenitis may be seen especially in CL caused by Vianna subgenus (3). About 70-75% worldwide cases of cutaneous leishmaniasis are mainly from ten countries such as Afghanistan, Iran, Syria, Algeria, North Sudan, Colombia, Brazil, Costa Rica, Peru and Ethiopia (18). Based on types of causative *Leishmania* species and host immune responses, CL are presented with various clinical features which may vary between and within regions (19). For instance, *L. tropica, L. major* and *L. aethiopica* are the major causative agents for cutaneous leishmaniasis in the old world. The CL caused by *L. aethiopica* which is mainly found in Ethiopia and Kenya is characterized by small skin lesions of face with absence of ulceration. In rare cases it may affect mucous membranes of nose and lips and distort nostrils and lips (3,18). Painless dry ulcers of the skin of face, legs, feet and arms is the clinical manifestations of CL caused by *L. tropica*. Another CL caused by *L. tropica*, which is known as leishmaniasis recidivans , lupoid or tuberculoid leishmaniasis, is slowly progressive skin lesion of the face (3). In the new world the *Leishmania* species belong to *Leishmania* or *Viannia*

subgenera such as *Leishmania amazonensis*, *Leishmania mexicana*, *Leishmania braziliensis*, and *Leishmania guyanensis* (15). Its clinical forms are localized, disseminated, diffuse and atypical cutaneous and mucocutaneous leishmaniasis (3).

1.2. Life Cycles of Leishmaniasis

Leishmania, a protozoan parasite of professional phagocytes, has a digenetic lifecycle stages as exemplified by the extracellular motile flagellated promastigotes present in the invertebrate vector (sand fly) and intracellular non-motile aflagellated amastigotes present in mammalian host (2). In the invertebrate hosts, the leishmania life cycle is restricted to the digestive tract of the insect. Most *Leishmania* species of subgenus leishmanial are suprapylarian parasites; meaning their development is confined to the midgut whereas members of Viannia subgenus are peripylarian parasites; meaning they enter the hindgut before travelling onward into the midgut. Therefore, the natural infection is commenced when sand flies feed on the blood meal that contains macrophages infected with amastigotes (Fig.1.2. 5 - 6), followed by the release of amastigotes to the gut of the insect where it begins its extracellular development (Fig.1.2. 7 - 8) (20). Furthermore, the promastigotes have different developmental stages including procyclic, nectomonad, haptomonad and metacyclic promastigotes each with distinctive morphology and functions (21). The amastigotes transform into procyclic promastigote, the first stage of promastigote in vector, which is characterized by small, weakly motile (with short flagella), inactive (non-infective), actively multiplies in blood meal. The ingested blood meal, and initial blood meal phase of promastigote is enclosed by a chitin and protein mesh known as peritrophic matrix (PM) which is secreted by the midgut epithelium of sand flies. After a few days, some promastigotes escape from the peritrophic matrix and transform into elongate, strongly motile nectomonad promastigotes that adhere to the midgut of the insect by inserting its flagellum in between villi of the gut until they reach stomodeal valve. The parasite secretory chitinase and probably the action of endogenous sand flies chitinase is responsible for the breakage of the peritrophic matrix and escape of nectomonad promastigotes from blood meal (22). Once the nectonomad promastigotes reach the stomodeal valve they differentiate into leptomonad promastigotes (slender and replicative form) which are responsible for the secretion of promastigote secretory gel (PSG). PSG forms a plug that prevents the influx of the blood into the midgut and also plays a key role in the transmission of leishmania by facilitating regurgitation (Fig.1.2 –1) (23). Either nectomonads or leptomonads promastigote differentiate into either

small, highly specialized leaf-like form haptomonad promastigotes which attach to the stomodeal or metacyclic promastigotes which is highly infective stage that can be found in the anterior part of the midgut and in the mouthparts of the sand fly, so that ready for successful transmission to the next vertebrate host (Fig.1.2. 2 - 4) (24).

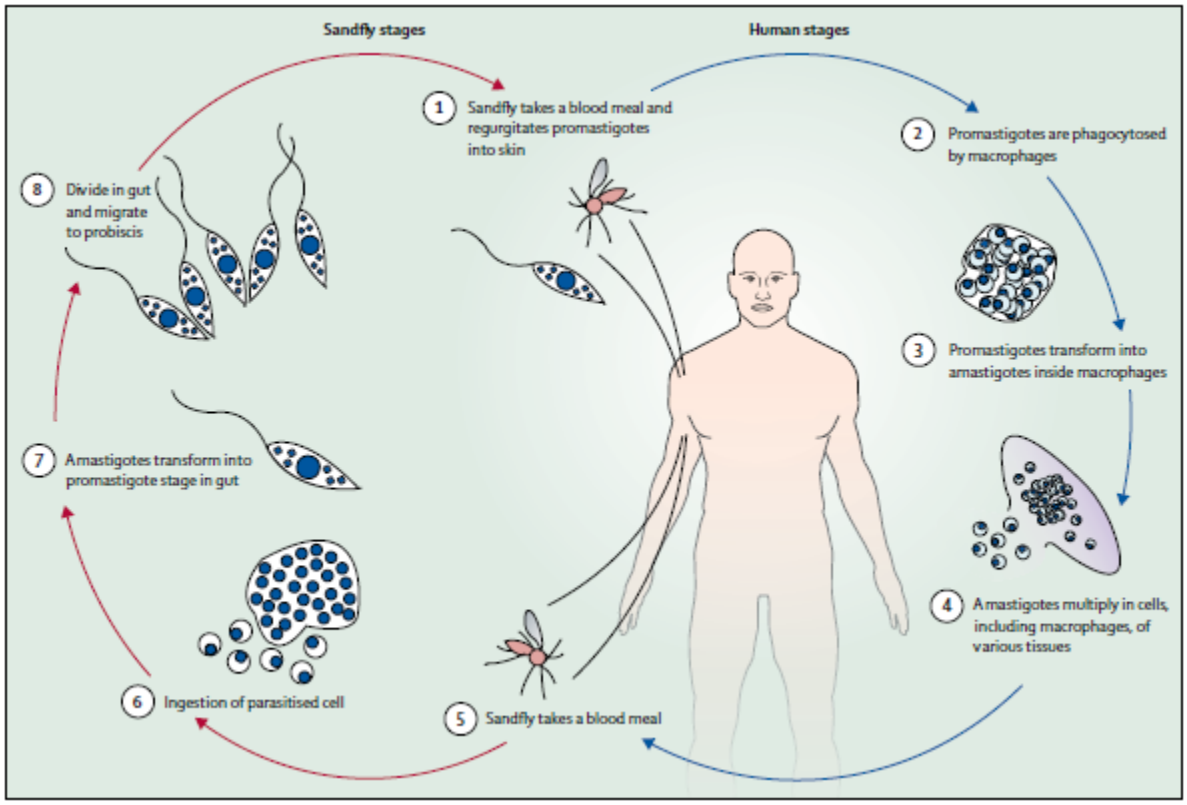

Figure 1. 2. Life Cycles of Leishmaniasis (15).

1.3. Pathogenesis of Leishmaniasis

Macrophages are important effector cells of innate immune system that contact with, recognize and eradicate pathogens and damaged tissues. Using pattern recognition receptors (PRRs) expressed on their surfaces, macrophages and other immune cells recognize danger signals from pathogens or damaged tissues (25). Once danger signals are recognized, macrophages and other professional phagocyte cells like neutrophils, monocytes and dendritic cells phagocytosis and destroy the invading pathogens. Macrophages and other phagocyte cells degrade engulfed microbes through generation of reactive oxygen species, reactive nitrogen species and secretion of hydrolytic enzymes such as lysosomes, proteases, lipases and others (26).

For host cells entry, promastigote stages of *Leishmania* species uses manose-fucose, fibronectin, Toll-like receptors and complement receptors (CR_1 and CR_3) expressed on macrophage cell surfaces. As the parasites enter the host cells, *Leishmania* cell surface molecule particularly metallopeptidase GP63 blocks NADPH oxidase assembly, so that, acidity and phagosome degradative properties of the macrophages phagolysosome is altered (27). This in turn favors the promastigotes to survive in the macrophages until it transformed to amastigotes, a more acidic environment resistant stage (23,24). Additionally, for successful establishment of infection the parasite devised several strategies to suppress innate immune responses of host. The parasite either inhibit proteins that activate immune cells or conversely activating regulatory molecules that suppress immune cells activation. Protein kinase C (PKC), Janus kinase 2 (JAK2), mitogen-activated protein kinases (MAPKs) are some examples of proteins that activates the host immunity but are affected by *Leishmania* infection. *Leishmania* parasites also activates protein phosphatases, a regulatory proteins, that have the ability to suppress host immunity through dephosphorylation of aforementioned proteins (28).

The protein Kinase C (PKC), a protein family composing serine/threonine protein kinases, are involved in variety of pathways that regulates cell growth, differentiation, and immune cell functions (29). PKC signaling pathway activates cytokines such as IFN-γ and TNF-α , both having important roles in driving macrophage mediated generation of reactive oxygen species (ROS) and reactive nitrogen species (RNS) (28). The PKC also activates nicotinamide adenine dinucleotide phosphate (NADPH) oxidase enzyme which in turn generate ROS and RNS (29).

Mitogen-activated protein kinase (MAPK) families such as extracellular signal-regulated kinase (ERK), Jun kinase / stress activated protein kinase (JNK/SAPK) and p38 MAPK (30) play an important role in regulating macrophage activation and proliferation. Once activated in response to environmental stress, MAPK regulates macrophage activities such as proliferation and differentiation of macrophage, inflammatory responses, and its polarization between microphage 1 and macrophage-2. It enhances the production of inflammatory cytokines such as IL-12 and IFN-γ but downregulate the expression of anti-inflammatory cytokines such as TGFβ (31). Furthermore activated JNK MAPK enhanced the activation of NF-kB and AP-1 in mouse peritoneal macrophages. The activation of NF-kB and AP-1 related to increased expression of iNOS gene that synthesize iNOS enzyme responsible for production of NO (32).

The Janus kinase (JAK) families such as three JAKs (JAK1-3) and tyrosine kinase 2 (TYK2) are found in human that are activated upon binding of cytokines to extracellular receptors (33). Following binding of ligands to their receptors, receptor-associated JAKs undergo phosphorylation which subsequently forms docking sites for signal transducers and activators of transcription (STATs) proteins where they are phosphorylated. Phosphorylated STAT proteins translocated to the nucleus to activate or suppress gene transcriptions (34). Inflammatory cytokines, such as interleukins (ILs), interferons (IFNs), and multiple growth factors transcriptions, growth and differentiations are among the roles of JAK-STAT signaling pathway (35), so that, it has a critical role in the regulations of immune cells. The initiation, duration and termination of this signaling pathway is determined by regulatory proteins such suppressors of cytokine signaling (SOCS), and protein tyrosine phosphatases (PTPs) and protein inhibitors of activated STATs (PIAS) (33).

In general, inactivation of PKC, MAPK and or JAK-STAT signaling pathway or activations of regulatory proteins such as phosphatase, an enzyme that inactivate aforementioned signaling pathways, favors the survival of the parasite and establishment of *Leishmania* infection. For instance, *L. donovani* showed impair tyrosine phosphorylation and thereby activation of JAK-STATs in response to IFN-gamma activation, possibly by cellular activation of protein tyrosine protein tyrosine phosphatases (SHP-1) and similarly activation of SHP-1 also responsible for dephosphorylation and inactivation of MAPK pathways (36).

Molecules of *Leishmania* parasite such as Leishmania major surface protease (GP63), secreted acid phosphatases (SAPs), lypophosphoglycan (LPG), proteophosphoglycans (PPGs), cysteine proteases (CPs), peroxiredoxins and exosome are responsible for subversion of host immunity. Because they lack the catalase enzymes, the trypanosomatids use a set of proteins called peroxiredoxins to neutralize ROS generated by host immune cells (37). LPG, a major surface molecule component of promastigote, may scavenge ROS directly or inhibit its synthesis by inhibiting NADPH oxidase assembly at the phagosome even though *L. mexicana* promastigotes do not require LPG for virulence suggesting the presence of the virulence factors other than LPG (38). It also binds to Toll –Like receptor (TLR) 2 and 4 on macrophage and natural killer cell and then inhibit the production of ROS, NOS, secretion of pro-inflammatory cytokines (39) and blocks the PKC signaling pathways (40). *Leishmania* parasite zinc-metalloprotease (GP63) on

the hand activates protein tyrosine phosphatase (SHP-1) enzymes that negatively regulate the host signaling pathways such as PKC, JAK-STATs, and MAPK pathaways (41). Secreted acid phosphatases (SAPs), another *Leishmania* molecule, also prevent the parasite against low pH and proteolytic activities of macrophage phagolysosome (37).

1.4. Epidemiology of Leishmaniasis

Leishmaniasis is a worldwide public health problem endemic in more than 98 countries in which about 350 million people are at risk of infection (42) and about 20,000-30,000 people die annually (43). Even though under estimated, the annual incidence of cutaneous leishmaniasis ranges from 0.7 to 1.2 million cases with approximately 95% of the cases being from America, the Mediterranean basin, the Middle East, and Central Asia. On the other hand, the annual incidence of visceral leishmaniasis is thought to be less than 100,000 cases with approximately 95% of the cases are from Brazil, China, Ethiopia, India, Kenya, Nepal, Somalia, and Sudan (44). About 5-10% and 50-60% of people treated for visceral leishmaniasis develop post-kala-azar dermal leishmaniasis (PKDL) in South Asia and East Africa, respectively (43).

Both VL and CL are also endemic to Ethiopia with the first scientific reports of CL and VL dating back to 1913 and 1942, respectively (45). Nationally, over 28.96 million and 3.2 million people inhabit in areas with risk of CL and VL infections, respectively (46,47). It is estimated that Ethiopia accounts for more than 3,700 – 7,400 cases of VL and 20,000 – 50,000 cases of CL, annually (48). Ethiopia has the third largest numbers of VL cases per a year in the sub-Saharan Africa, next to South Sudan and Sudan (49). *L. donovani* for VL and *L. aethiopica* for CL are the major causative agents of leishmaniasis infection in these regions though *L. infantum* for VL as well as *L. major*, *L. tropica* and sporadically *L. infantum and L. donovani* for CL have been also reported less frequently as the causative agents of the diseases (50,51). Even though Ethiopia does not have an official overall estimation of the prevalence of leishmaniasis for either form of infections at country level, the systemic review conducted by Assefa *et al* reported a national pooled prevalence of 19% (95% CI = 14-25%) (52).

1.5. Treatments of Leishmaniasis

Treatment alternatives for leishmaniasis are limited. Drugs for such a disease are not attractive targets for the profit-driven pharmaceutical industry to invest research & development efforts because most of the patients are from poor countries (53). Currently, chemotherapeutic agents such as pentavalent antimonials (54), amphotericin B formulations, miltefosine (55), paromomycin (56) and pentamidine isethionate (57) are in use for the treatments of leishmaniasis.

Physical therapy which include thermotherapy and cryotherapy is also used for the treatment of CL (58). It is considered as a therapeutic alternatives for clinically defined simple cutaneous leishmaniasis in the old world regions, for localized leishmaniasis caused by *Leishmania* species unlikely develop into ML in the new world regions. It is also considered alternative therapy to CL for pregnant women and for patients contraindicated to systemic therapy (59). Simple CL is defined as absence of mucosal lesion involvement, single or few skin lesions with small size (<1 cm), if site of lesion is not cosmetically important and feasible for physical therapy, if the causative *Leishmania* species are unlikely to be associated with mucosal leishmaniasis and the treated host is immunocompetent (58). The amastigotes of dermatotropic species of *Leishmania* are thermosensitive. One to two time's applications of heat on lesion under local anesthesia with thermo-device is used in treating CL as high temperature suppress replications of the parasites (55). Like in thermotherapy, *Leishmania* parasites are also sensitive to freezing (cryotherapy) as too low temperature resulted in break apart of *Leishmania* parasites available in connective tissues of dermis (60).

1.6. Overview of Medicinal Plants

1.6.1. Medicinal plants with reported anti-leishmanial activities

Several researches have been carried out on anti-leishmanial activity of crude extracts or chemically defined compounds derived from plants *in vitro* against promastigotes and amastigotes or *in vivo* against *Leishmania* infected animals. Some of these works are reviewed as follows:

The ethanolic extract of fruits of *Picramnia gracilis* revealed anti-leishmanial activity against CL caused by *L.(V.) panamensis* both *in vitro* and *in vivo*. Its major metabolite, 5, 3'-hydroxy-7, 4'-dimethoxyflavanone, demonstrated anti-leishmanial activity against *L.(V.) panamensis* with (IC_{50} of 17.0 + 2.8μg/ml *in vitro*. Furthermore, at concentration of 2mg/kg/day or cream (2%) of it showed clinical improvement and no toxicity to hamsters in *in vivo* study (61).

The latex of Ethiopian plant, *Aloe calidophila* Reynolds, showed moderate activity against *L. aethiopica* and *L. major* strains with IC_{50} values of 64.05 and 82.29 µg/ml, respectively. Aloinoside, aloin, and microdontin are three anthrones isolated from latex of *Aloe calidophila* Reynolds. The anthrone isolates showed better anti-leishmanial activity against both strains than its crude counterparts with IC_{50} ranging from 2.09 to 8.85 µg/ml against *L. major* and 1.76 to 6.32 µg/ml against *L. aethiopica*. The ant-leishmanial activities of the isolated compounds were also higher than standard drug (amphotericin B) where its IC_{50} is 0.109 and 0.067 µg/mL against *L. aethiopica* and *L.* major, respectively (62).

Another leaf latex of *Aloe macrocarpa*, Aloin A/B, and its semisynthetic derivatives (aloe-emodin and rhein) showed potent *in vitro* antileishmanial activities against *L. aethiopica* and *L. donovani* promastigotes and axenically cultured amastigotes but showed lower activity (IC_{50} = 6.7 to 12.1 μM for promastigotes and IC_{50} = 3.6 to 10.2 μM for axenic amastigotes) than the standard drug amphotericin B (IC_{50} = 1.3 to 2.7 μM). The test compounds also revealed that better selective toxicity towards parasites than the reference drug. Amphotericin B (CC_{50} = 11.1 μM) was much more toxic to human monocytic cell line (THP-1) than the test compounds (LC_{50} = 369.2 – 611.6 μM) (63).

The solvent fractions of the ethanolic seeds extract of *Albizia gummifera* demonstrated MIC values of 11.2 µg/ml, 33.5 µg/ml, >89 µg/ml and 1.32 µg/ml and IC_{50} value of 2.16 µg/ml, 2.27 µg/ml, 4.85 µg/ml and 1.10 µg/ml for butanol, aqueous and dichloromethane fractions and standard drug (amphotericin B) respectively against *L. donovani*. The aqueous and n-butanol

fractions inhibited the growth of intracellular *L. donovani* amastigote significantly compared to amphotericin B. In addition, n-buthanol and aqueous fractions were also found to nontoxic to mammalian cell line, Vero cell line, having selectivity index of 2.43 and 6.87, respectably (64).

The *in vitro* anti-leishmanial activity of three plants against *L. major* revealed that the methanol extracts of flowers of *Calendula officinalis*, seeds of *Datura stramonium* and leaves of *Salvia officinalis* inhibited the growth of promastigotes and intracellular amastigotes of *Leishmania* parasites. The IC_{50} of the methanol extracts of S. *officinalis* leaves, *D. stramonium* seeds, *C. officinalis* flowers and reference drug miltefosine for promastogote assay were 184.32 µg/ml, 155.15 µg/ml, 108.19 µg/ml and 5.3 µg/ml, respectively. The extracts also reduced the numbers of amastigote parasites internalized in macrophage cell lines from 264 for negative control groups to 88-102 for the plant extracts. Even though the anti-leishmanial activities of these plant extracts were incomparable with reference drug, miltefosine, they exhibited significant activity in reducing the number of intracellular amastigotes in comparison to negative control and they are less toxic to mammalian cell lines than miltefosine (65).

The *in vivo* anti-leishmanial activity of *Warburgia ugandensis* Spraque (Canellaceae, a known traditional medicine in Kenya) against *L. major* was assayed in animal study. Oral administration of solvent fractions of stem bark of the plant (hexane, dichloromethane, ethyl acetate fractions) to infected BALB/c mice resulted in a significant reduction of size of lesions as compared to phosphate buffer saline, a negative control. In addition, administration of solvent fractions intraperitoneally to infected mice resulted in an increase in lesion size initially for hexane, dichloromethane and ethyl acetate extracts but gradually decreased and healed at day 42 which showed that the anti-leishmanial potential of extracts of *W. ugandensis* (66).

1.6.2. Overview of the Experimental Plants

Brucea antidysentrica J.F. Mill (Genus: *Brucea*; Family: Simaroubaceae) is an evergreen shrub 10-15 meter high, grow in the altitude ranges from 1400 to 2800 meter high (67). It is widely distributed in tropical African countries such as Nigeria, Ethiopia, Cameroon, Burundi, Sudan, Guinea, Congo, Angola Zambia and Malawi (67).

The plant was named after an honor of a Scottish traveler James Bruce who stayed in Ethiopia from 1769 to 1771. When he was about to return to Europe he was infected by deadly dysentery and unable to regain his health with the help of medications he had brought along from Europe.

Then, by order of the Chief of Ganjar of Shanquilla, James Bruce took two teaspoonful of the powder of root bark of *B. antidysentrica* ('Waginos' in Geez and 'Yedega Abalo' in Amharic), a well-established local remedy in Northern Ethiopia, with camel's milk. After 6^{th} or 7^{th} days of treatment, he recovered and was able to continue his journey to England. On his way back to Ethiopia, he took the seeds of '*Wagnos'* and brought it to British Museum of Natural History and it was planted in several British gardens. In the honor of James Bruce and medicinal value of the plant, antidysentery, Miller later named the plant *Brucea antidysenterica* (68). In addition, the president of royal society, Sir Joseph Banks, employed Mr. Miller to take drawing from the shrub of *Brucea antidysenterica* as it had grown at garden of Kew (district of London). Mr. Miller's drawing was as elegant as it could be wished and finally Sir Joseph Banks renamed the plant as *Brucea antidysebtrica* J.F.Mill (69).

The Simaroubaceae family comprises of 32 genera and greater than 170 species of trees and shrubs (70) of which 10 species are belongs to genus *Brucea* (71). They are mainly distributed to Asia (Malaysia), tropical America, extending to West Africa, Madagascar, and around pacific basin of Australia. Many findings indicated that numerous types of biologically active chemical constituents were isolated from Simaroubaceae family. The quassinoids, alkaloids, steroids, flavonoids, triterpenes, coumarins, anthraquinones and other secondary metabolites responsible for their biological activities are among the isolated components. Quassinoids are considered as a taxonomic markers of this family as a result of their abundance in members of this family (70).

Quassinoids, the degraded products of triterpenes, are responsible for wide ranges of biological activities such as antimalarial, antitrypanosomal, anti-inflammatory, anti-ulcerogenic, antipyretic, insecticide, feeding deterrent, amebicide, antifertility, anticancer, antileishmanial, antiviral and other biological activities (72,73). In addition, alkaloids such as various canthin alkaloids have been isolated and reported to display numerous biological activities (73).
The preliminary phytochemical screening of 80% methanol extract of seeds of the plant showed the presence of various secondary metabolites such as alkaloids, tannins, flavonoids, triterpenoids, phenols, steroids and glycosides (74). Additionally, various quassionids and canthin alkaloids were isolated from different parts of the plant since 1973. 1,11-dimethoxycanthin-6- one, 11-hydroxy-1-methoxycanthin-6-one, canthin-6-ne (75), 11-hydroxycanthin-6-one, 1-hydroxy-11-methoxycanthin-6-one and 1-methoxycanthin-6-one (76)

were among canthin alkaloids isolates. Non-glycosidic quassinoids isolates such as bruceanic acid A-D (77), bruceanol A-C (78,79), bruceanol D-F (80), bruceanol G-H (81), bruceantarin, bruceantin, bruceine B (82), dehydrobruceantarin, dehydrobruceantin, dehydrobruceantol, dehydrobruceine B, bruceantarin, bruceantinol and, isobruceine B (83) were also isolated from the plant. Glycosidic quassinoids including bruceantinoside A-B (84), bruceantinoside C, yadanzioside G, yadanzioside N (85), yadanzioside K (78), yadanzioside M and yadanzioside P (86) are also isolated previously from the plant.

Figure 1. 3. *B. antidysenterica* J.F.Mill (Photo credit by Dejene Hailu)

Ethnobotanical studies conducted at various parts of Ethiopia indicated that leaves, roots, barks, stems and seeds of *B. antidysentrica* J.F.Mill has been used for the treatment of numerous human medical disorders in folk medicine. For instance the paste of leaves powder is applied topically to treat skin cancer (87), leprosy (88,89), eczema (90), scabies (90), and taken orally with water for the treatments of helminthiasis, anthrax, and malaria (91). The seeds of the plant is also used for the treatment of different diseases: the paste of seeds powder is used in wound healing and

venereal diseases (92), its powder is applied topically in the infected area for the treatments of cutaneous leishmaniasis (89).The barks powder is macerated and drunk before meal for the treatment of tinea corporis, amoebiasis (93), malaria (93,94) and cancer (94). The juice of roots and stem barks powder is taken orally for the treatment of dysentery (bloody diarrhea), stomach-ache (95), cancer and malaria (94).

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 is active against various infectious and non-infectious diseases. The quassinoid compound isolates such as bruceantin, brucenol-F and dihydrobruceantinol exhibited antituberculosis activities against *Mycobacterium tuberculosis* strain H37Rv *in vitro* (96). Bruceantin, a purified isolated from *B. antidysenterica* J.F.Mill showed amoebicidal activity *in vitro*. The potency of bruceantin (IC_{50} = 0.018 µg/ml) was higher than positive standard drug metronidazole (IC_{50} = 2.15 µg/ml) (97). A study reported antimalarial activities of aqueous, chloroform and methanol seeds extract against *P. berghei* (98). Another study exhibited that the root extract has antidiarrheal and antimicrobial activity in animal (*in vivo)* and in *vitro* study, respectively. The 80% methanol root extract showed dose dependent response in reducing the average number and weight of fecal output, gastrointestinal motility and in prolonging the onset of diarrhea significantly in mice (71). The root extract also possessed antibacterial activities against some diarrhea causing bacterial ATCC strain of *Shigella flexneri, Salmonella typhi, E. coli, P. aeruginosa*. Better zone of inhibition was reported for *P. aeruginosa* even though smaller zone of inhibition for others compared to positive standard drug (71). The chloroform leaf extract of the plant had also antibacterial activities against ATCC strain of food born bacterial infections such as *S.aureus, S. typhimurium, P. aeriginosa* and *S. sonnei* with highest zones of zone of inhibition was seen for *P. aeriginosa* (99). This showed that both the root and leaf extracts of the plant has promising activities against multidrug resistant strains of *P. aeruginosa*. Compound isolates obtained from *B. antidysenterica* J.F.Mill such as bruceanic acid D (77), bruceanol D, E and F (80), bruceanol C (78), bruceanols G and H (81), 11-hydroxy-1-methoxycanthin-6-one and1-methoxycanthin-6-one (76) showed cytotoxic effects against different cancer cell lines *in vitro*.

1.7. Rationale of the Study

Leishmaniasis is an infectious disease affecting 0.7-1 million people in which about 350 million people are at risk of infection (100), and about 20-30 thousand people die annually, globally (43). Poverty, malnutrition, immigration, poor hygiene and an immuno-incompetence state are risk factors for leishmaniasis (44). It affects developing countries as the aforementioned risk factors are common in these countries. In Ethiopia, over 28.96 million and 3.2 million people live in areas with risk of CL and VL infections, respectively and about 50,000 and 7,000 new cases of CL and VL per year, respectively (46,101).

There are limited therapeutic options for the treatment of leishmaniasis as there are only a few developed for this purpose. It comprises amphotericin B (101), pentavalent antimonials (102), paromomycin (103), miltefosine (53) and pentamidine (57) – all of which have drawbacks in terms of variable efficacy and adverse effect. For VL which demands systemic treatments, the routes of administration for all drugs are through parenteral routes except miltefosine which is administered orally. Limited supply of the existing drugs and its high costs are also the challenges in fighting leishmaniasis (104). The emergence of resistance against the existing drugs is also another problem that complicates leishmaniasis managements (105) which calls upon for looking for viable options. The use of natural products such as animals, plants, microorganisms and marine organisms, as medicines in treatment of diseases was started at least 60,000 years ago (106). Powder of seeds of *B. antidysenterica* (89) and fresh roots or leaves of *R. nepalensis* (95) are some examples of plants used for the treatments of leishmaniasis when applied to the affected area in Dega Damot district and Dek Island, respectively.

In the current study, the potential ant-leishmanial activities of 80% methanol extracts of seeds of *B. antidysenterica* J.F.Mill and its solvent fractions against *L. aethiopica* and *L. donovoni* was evaluated to challenge its claimed use in traditional medicine.

2. OBJECTIVES

2.1. General Objective

The general objective of this study was to evaluate the anti-leishmanial activities of extracts of seeds of *Brucea antidysenterica* J. F. Mill against *Leishmania donovani* and *Leishmania aethiopica in vitro*.

2.2. Specific Objectives

- ✓ To evaluate anti-promastigote activity of 80% methanol extract of *B. antidysenterica* and its fractions
- ✓ To evaluate anti-amastigote activity of 80% methanol extract of *B. antidysenterica* and its fractions
- ✓ To evaluate cytotoxic effect of 80% methanol extract of *B. antidysenterica* and its fractions against mammalian cells: Vero cell lines, red blood cells and peritoneal mice macrophages isolates
- ✓ To determine selectivity index of 80% methanol extract of seeds of *B. antidysenterica* and its fractions
- ✓ To determine the quantitative estimation of secondary metabolites found in of 80% methanol extract of seeds of *B. antidysenterica* and its fractions

3. MATERIALS AND METHODS

3.1. Materials

3.1.1. Chemicals and Reagents

The chemical and reagents used in the study include: butanol (Loba Chemie, India), chloroform (Carlo Erba, Franc), distilled water (LRDL, Leishmania laboratory), ethanol (Iso Lab Chemicals, India), ethyl acetate (Lobe Chemie, India, Geimsa solution (Fisher scientific, UK), phosphate buffer saline (Sigma - Aldrich USA), hexane, methanol (Sisco research laboratories, India), potato starch (Sigma-Aldrich, Germany), trypthan blue stain (Grand island, USA), trypsin-EDTA (Gibco, UK), resazurin sodium salt (Sigma-Aldrich, Germany), dimethyl sulfoxide (laborchemikalien GmbH, Germany), Triton X- 114 (Sigma-Aldrich, Germany), NaOH (Ranchem Industry, Turkey) and HCl (Loba Chemie, India), Na_2CO_3 (Loba Chemie - India), gallic acid (MerCK, Germany), Folin–Ciocalteu's solution, quercetin (Sigma Aldrich - Germany), $AlCl_3$ (Loba Chemie - India), $NaNO_2$, Bromocresol green (Sisco research laboratories, India), Na_2HPO_4 (BDH Chemicals, England), citric acid (Avonchem, UK), and atropine

3.1.2. Culture Medium and Supplements

The culture medium and supplements include: minimum essential medium (MEM)**,** heat inactivated new born calf serum (HINBCS), penicillin streptomycin solution, (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) (HEPES), medium -199 with Earle's salts (M199) (All from Sigma-Aldrich, USA) and Roswell Park emorial institute (RPMI-1640) (Sigma-Aldrich, UK) were used to prepare complete medium. D (+) glucose (anhydrous), nutrient agar, calcium chloride, potassium chloride, sodium bicarbonate, sodium chloride (all from sigma –Aldrich USA) and defibrinated and heat inactivated sheep blood were used to prepare NNN (Novy-MacNeal-Nicolle) media and Locke's solution.

3.1.3. Equipment and Supplies

The equipment used in the study include: polystyrene sterile tissue culture flasks (25, 50 and 75 ml) (Corning incorporated, USA), Chamber slides (8 and 16 wells) (Nalge Nunc international, USA), 96 well Microtiter plates (Nalge Nunc international, USA), analytical balance (Mettler Toledo, Switzerland), Autoclave machine (Timo, Italy), Biosafety cabinet with UV (Laboculture ESCO class IIA), Centrifuges (eppendorf 5804R), carbon dioxide incubator (Thermo Electron),

Digital water bath (Julabo TW20), Eppendorf tube (1.5ml, 15 ml, 50 ml) (Corning incorporated, USA), Flouroscan Ascent (Thermo electron), Hemocytometer (Improved Double Neubauer type), Compound microscope and Inverted microscope (Olympus and Labomed), Lyophilizer (OPERON, Korea), table-top vacuum filter (micropore, Brazil), Micro pipettes (Pipetman ultra), multichannel pipettes (Hamilton), Microplate reader (victor^3 Perkin Elmer), pipette tips and its rack (eppendrof), Rotary evaporator (BÜCHI Rota vapor R-200), heating bath (BÜCHI Heating Bath B- 490), deep freezer, filter paper (Whatman number one), gauze (Nylon clothes), Mortar and pestle, Magnetic stirrer, vortex (whirl VIB2), Separatory funnels, Serological pipettes, PH meter, Aluminum foils, Parafilm, Oven drier, Hot plate, Bunsen burner, UV spectrophotometer (Jenway Model 6500, England)

3.1.4. Reference Drugs

Pentamidine isethionate, amphotericin B (both from Sigma-Aldrich, USA) were drugs used as positive controls.

3.1.5. *Leishmania* Parasite Strains and Mammalian Cells

Clinical isolates of *L. aethiopica* (CL-027/20) and *L. donovani* (VL-139/19) were acquired from LRDL, Microbiology, Immunology and Parasitology department, Addis Ababa University, Ethiopia. The parasite strain of *L. aethiopica* (CL-027/20) was isolated from 13 years old male patient who lives in Guna Woreda, Arsi Zone, Oromia region, Ethiopia. *L. donovani* strain (VL-139/19) was isolated from 27 years old male patient who lives in Kolme Woreda, Konso Zone, SNNPs region, Ethiopia. Red Blood Cells were collected from healthy 32 years old volunteer with no underlying chronic disease. Vero cell line was obtained from National Animal Health Diagnostic and Investigation Center (NAHDIC), Sebeta, Ethiopia, while white 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.

3.2. Methods

3.2.1. Collection of Plant Material

Both plant parts were collected from West Gojjam zone, Amhara regional state, Ethiopia in January 2020. The seeds of *Brucea antidysenterica* J.F.Mill was collected from Dega Damot district 399 km away from Capital city, Addis Ababa. The roots of *Rumex nepalensis* spreng was also collected from Dek Island found on Lake Tana, located in Bahir Dar Zuria woreda 600 km away from Addis Ababa. The plants were identified and authenticated by Mr. Melaku Wondafrash, a botanist at College of Natural and Computational Sciences, Addis Ababa University, Ethiopia. The voucher numbers TK-002 and TK-004 were given to *R. nepalensis* spreng and *B. antidysenterica* J.F.Mill specimens, respectively and kept in National Herbarium of Addis Ababa University for future reference.

3.2.2. Preparation of Crude Extracts

The seeds of *B. antidysenterica* J.F.Mill was air-dried under shade for 3 weeks and then grounded to coarse powder using mortar and pestle. Similarly, the roots of *R. nepalensis* spreng was washed with tap water to remove any foreign bodies, sliced into pieces using knife. It was dried under shade for 1 month and then milled with mortar and pestle. Then, 400 gm of *B. antidysenterica* seeds powder and 400 gm of *R. nepalensis* roots powder were macerated with 1.2 liter of 80% methanol (1:3) in separate volumetric flasks. The macerates were shaken occasionally to maximize solvent penetration and bioactive solubility. Post 72 hours of maceration, each extract was filtered with muslin gauze for several times and then filtered using Whatman NO-1filter paper. The mark was re-macerated twice in order to maximize the yield by adding fresh solvents and then filtered in similar manner. The combined filtrates were concentrated using a rotary evaporator at 40^{o}C. The concentrated extracts were frozen in refrigerator at -20^{o}C overnight and then dried using a lyophilizer (74). Finally, a gold colored solid extract of *B. antidysenterica* J.F.Mill having sticky nature and brown colored solid extract of *R. nepalensis* spreng were obtained and their percent yields were calculated from their dried mass using the following formula:

$$Yeild\ \% = \frac{Weight\ of\ extract\ obtained}{Weight\ of\ extracted\ plant\ sample}$$

3.2.3. Preparation of Solvent Fractions

After having activity test using crude extracts of the two plant materials, the dried 80% methanol extract of *B. antidysenterica* J.F.Mill was fractionated to n-hexane, ethyl acetate, n-butanol and aqueous fractions, respectively based on the increasing polarity of solvents as methods described previously by Toma *et al* (107). The 80% methanol extract (16 gm) was transferred to separatory funnel and dissolved in distilled water (150 ml). Then, 150 ml of n-hexane was added to the solution, mixed well and allowed to rest until stable layer was formed. Then, the hexane fraction was collected to a separate container. The remaining aqueous solution was washed with 150 ml of n-hexane twice again. Next, the remaining aqueous solution was washed with 150 ml of ethyl acetate (3 times), followed by with 150 ml of n-butanol (4 times) until the extracting solvent became colorless. The remaining portion left after successive fractionation was aqueous fraction. Fractions of organic solvents were concentrated using rotary evaporator under reduced pressure at temperatures of 40^{o}C, followed by dried in oven drier at temperatures of 30^{o}C while the aqueous fraction was dried by freeze drier (lyophilizer). The dried crude extracts and fractions were weighed and stored in deep freezer until used for the procedure.

The yield of 80% methanol extracts of *B. antidysenterica* J.F.Mill and *R. nepalensis* spreng were 32.68gm (8.17%) and 69.76gm (17.44%), respectively. The yield of n-hexane, ethyl acetate, butanol and aqueous fractions of 80% methanol extract of *B. antidysenterica* J.F.Mill were 1.38 gm, 3.12 gm, 4.37 gm and 5.82 gm, respectively.

3.2.4. Culture Conditions

3.2.4.1. Promastigote Cultures

The logarithmic stages of clinical isolates of *L. aethiopica* and *L. donovani*, after grown in NNN media, were seeded and grown in 25 ml tissue culture flasks containing M199 medium supplemented with 20% heat-inactivated fetal calf serum (HIFCS), 25 mM HEPES, 2 mM L-glutamine and 100 IU/ml penicillin and 100 µg/ml streptomycin solution at 22^{o}C for *L. aethiopica* and 26^{o}C for *L. donovani* following the method described by Tariku and colleagues (108).

3.2.4.2.Vero Cell Line Culture

The Vero cells (the green African monkey kidney cell lines) were cultured in RPMI-1640 with L-glutamine and sodium bicarbonate medium supplemented with 10% Heat Inactivated New Born Calf Serum (HIFCS), 100 U/ml penicillin, and 100 µg/ml streptomycin in humidified, 5% CO_2 incubator at 37℃ as described by Ammerman *et al* (109). The cells growth was monitored under inverted microscope and the media was changed every 3 days. When cells reached >70% confluent monolayer, they were sub-cultured.

3.2.4.3. Mice Peritoneal Macrophage Collection and Culture

The peritoneal macrophages were harvested from pathogen free, typically 6 to 8 weeks old Swiss Albino mice according to protocol previously described by Zhang *et al* (110) with minor modification. Each mouse was injected with 2 ml of 2% potato starch solution intraperitoneally to allow inflammatory response to proceed. Post 48 hours of administration of starch solution, each mouse was euthanized with chloroform, its abdomen was swabbed with 70% ethanol and the skin underlying the peritoneal cavity was removed. 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. Then the peritoneal exudates were centrifuged in cold centrifuge (4^{o}C) for 10 minutes at 450 × g (1500 rpm). The supernatant was discarded, and the resulting pellets were re-suspended in cold 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.

3.2.5. Biological Assay

3.2.5.1. Preliminary Anti-leishmanial Activity Screening

The preliminary anti-promastigote activities of 80% methanol extracts of *B. antidysenterica* J.F.Mill and *R. nepalensis* Spreng. were done following the methods previously described by Tadele *et al* (111). In 96-well microtiter plate containing 100 µl of complete M199 medium, each extract at concentration of 100 µg/ml were added in separate wells with each test substances in triplicate. Then 100 µl of suspension of parasites (3.00 x 10^6 promastigotes/ml of *L. aethiopica* and *L. donovani*) in a logarithmic phase was added to each well and contents of the plates were

then maintained at 22 ºC (for *L. aethiopica*) and 26 ºC (for *L. donovani*) for 72 hours. After 68 hrs of incubation, 20 µL of 10% fluorochrome resazurin solution (0.125mg/ml, pH=7.2) was added to each well and allowed to rest for 4 hrs. Then the fluorescence intensity was measured using a Multilabel Reader at an excitation wavelength of 544 nm and emission wavelength of 590 nm. Following preliminary screening, extract with higher of percentage of inhibition of the growth of *Leishmania* spps was selected for IC_{50} determination and cytotoxicity studies. The anti-promastigote activity of the extracts were expressed as percent of parasite inhibition and it was determined using the following formula.

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

3.2.5.2. Cytotoxicity Assay

3.2.5.2.1. Hemolysis Assay

The hemolytic effects of 80% methanol extract of *B. antidysenterica* J.F.Mill and its solvent fractions were conducted as methods described by Abeje *et al* (62) and Zohra *et al* (112). From healthy volunteer man about 4 ml of blood samples were collected using sterile syringe and transferred to heparinized test tube. Two milliliter of the blood was added to 10 ml of PBS solution (PH 7.2) already transferred to 15 ml Eppendorf tube. The mixture was mixed well and then centrifuged at 1000×gm for 10 minutes. The supernatant was pipetted out using serological pipettes. The resulting pellets (1ml) was added and mixed with 49 ml PBS solution (PH 7.2) to obtain 50 ml of 2 % red blood cell suspension required for hemolytic tests (112). Two hundred microliter of 2% RBC suspension was added to 1.5 ml Eppendorf tubes containing 200 µl of serially diluted concentrations of extracts (1600 -50 µg/ml), standard drugs; AMB (40-1.25 µg/ml) and pentamidine (120-0.3.75 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 +PBS solution) was used as positive and negative controls, respectively. The Eppendorf tubes content were mixed gently and incubated at 37ºC in the incubator for 2 hours except Triton X-114 which was incubated for 30 minutes. After elapsed time of incubation the content of Eppendorf tubes were centrifuged at 1,000 × g for 10 minutes. Then, from each tube 100 µl of the supernatant was transferred to 96 well microtiter plates without affecting the sediment. Finally, the absorbance of

the supernatants were measured at 540 nm using Victor^3 Multilabel Reader (62).The hemolytic effects of each test substances were expressed in percentage as per the following formula (112).

$$\text{Hemolysis (\%)} = \frac{Absorbance of\ sample's\ in\ wells - Aveage\ blank\ wells}{Triton\ X - 114\ Average\ absorbance\ \ in\ wells - Aveage\ blank\ wells} \times 100$$

3.2.5.2.2. Vero Cells Viability Assay

A confluent Vero cell lines monolayer with the cell density of 3×10^5cells/ml were seeded in 96 microtiter plates. After incubation at 37 °C in a 5 % CO_2 incubator for 24 hours, 100µl of serially diluted concentrations of extracts (1600-50 µg/ml), standard drugs, AMB (40-1.25 µg/ml) and pentamidine (120-0.3.75 µg/ml) in complete RPMI-1640 medium were added into 96-well microtiter plates. The 96-well content was incubated at 37ºC, 5% CO_2 for 72hrs. Post 68 hrs of incubation, 20 µl of 10% resazurin solution was added to each well and allowed to rest for 4 hours. Finally, the viability of the cells were measured fluorometrically using multi-label plate reader at excitation wavelength of 544 nm and emission wavelength of 590 nm as previously described by Nigussie *et.al* and Chan *et al* (64,113). Standard antileishmanial drugs and negative controls (medium alone and 1% DMSO) were used in this assay to have reference values. Serial dilutions were prepared in two fold dilutions with each test concentrations in triplicates.

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

3.2.5.2.3. Mouse Macrophage Viability Assay

The cytotoxicity of each test substances against mouse peritoneal macrophage isolates was determined as methods described previously by Afrin *et al* (114). Hundred microliter of mice peritoneal macrophages suspended in complete MEM medium (3×10^5cells/ml) was seeded in 96 well microtiter plates. Then serially diluted concentrations extracts (1600-50 µg/ml) and positive controls; AMB (40-1.25 µg/ml), pentamidine (120-0.3.75 µg/ml) and negative control (1%DMSO + Medium) contained in 100 µl of complete MEM medium were added to 96-well microtiter plates content which were then incubated in CO_2 incubator for 72 hrs at 37ºC, 5% CO_2 and 95% humidity. After 68 hrs of incubations 20µl of 10% resazurin solution (0.125mg/ml) was added to each well and left for 4 hours and then the fluorescence intensity of each well was

measured using a Multilabel Reader at an excitation wavelength of 544 nm and emission wavelength of 590 nm. Serial dilutions were prepared in two fold dilutions with each test concentrations in triplicates.

The cytotoxic effects of test substances against mouse peritoneal macrophage isolates were expressed as percentage of macrophage viability and it was determined as the following formula.

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

3.2.5.3. Anti-promastigote Assay

The antipromastigote activities of the extracts were done as methods previously described by Tariku *et al* (108). Serially diluted concentrations of each extract (400-6.25 µg/ml) contained in 100 µl of complete culture medium (M199 medium) were added to 96 well microtiter plates. 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 hours at 22 ^{o}C (for *L. aethiopica*) and 26 ^{o}C (for *L. donovani*). After 68 hrs of incubation, 20 µL of 10% fluorochrome resazurin solution (0.125mg/ml, pH=7.2) was added to each well and allowed to rest for 4 hrs. 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 antileishmanial 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. Blank wells containing complete M199 medium was used to monitor background fluorescence activity of resazurin; and the average was subtracted from every well. Serial concentrations of all test substances and controls were prepared in two fold dilutions with each test concentrations in triplicates.

The anti-promastigote activity of the extracts were expressed as percent of parasite inhibition and it was determined using the following formula (111).

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

3.2.5.4. Anti-amastigote Assay

An intracellular anti-amastigote activities of the extracts and controls were assayed as methods previously described by Afrin *et al* (114) and Utaile *et al* (115). Mouse peritoneal macrophages suspended in complete MEM medium (3×10^5 cells/ml, 200 µl) were seeded in 16 well chamber slides and incubated for 24 hours at 37ºC in 5% CO_2. After 24 hours the chamber slides were washed with pre-warmed complete medium to remove non-adherent macrophages. The stationary stages of *L. donovani* and *L. aethiopica* promastigotes (*Leishmania*: macrophage; 10:1) were seeded in the chamber slides containing adherent macrophages and maintained at 31ºC (*L. aethiopica*) or 37ºC (*L. donovani*), 5% CO_2 and 95% relative humidity for further 24 hours. Then, non-internalized promastigotes were removed by extensive washing with pre-warmed MEM medium and serially diluted concentrations of extracts (400 - 6.25 µg/ml) were added and incubated for further 48 hours 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 minutes. Finally, 50 macrophage nuclei per slide were counted and the number of resident amastigotes in each macrophages were enumerated. The assay was repeated with standard antileishmanial 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 was prepared in two fold dilutions with each test concentrations in triplicates.

The anti-amastigote activity of the extracts per well was expressed as infection index, and it was calculated using the following (116).

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

3.2.5.5. Selectivity Index/ SI

The selectivity index (SI) of each extract was determined from their CC_{50} against mammalian cells (Vero cell line and mice macrophage) and their corresponding IC_{50} of against *Leishmania* parasites (IC_{50} of amastigotes). The selectivity of the extract and standards in killing parasites as opposed to mammalian cells was assessed using the following formula (64):

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

3.2.6. Quantitative Determination of Total Phenolic Compounds, Flavonoids and Alkaloids

The total phenols, flavonoids and alkaloids 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.

3.2.6.1. Determination of Total Phenolic Compounds Content

The total phenolic contents of 80% methanol extract of the plant and its fractions was determined by Folin–Ciocalteu's method as per methods previously described by Nigatu *et al* (117). Serial concentrations of standard solution, gallic acid (100 – 6.25 µl/ml) were prepared in methanol. From each aliquots of standard solution, 1 ml was transferred into separate Eppendorf tubes. Then 5 ml of methanol and 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 tubes contents and mixed gently. The final volumes were adjusted to 10 ml with methanol and incubated at room temperature. After 90 minutes incubation, the absorbance of the solutions were measured at wavelength of 760 nm using UV 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. Similar procedures were followed for crude extract, its fractions and blank solutions. The serial concentrations of standard solution was prepared in two fold dilution and all tests were conducted in triplicates.

The average absorbance of blank solution was subtracted from standard, crude extract and its fractions solutions and the total phenolic content was determined using a standard curve of gallic acid. The calculated total phenols result were expressed as mg of gallic acid equivalent per g of dry extract/ fractions (mg of GAE/g).

3.2.6.2. Determination of Total Flavonoids Content

The total flavonoids content of the 80% methanol extract and its fractions was determined using complex formed by Aluminum chloride as per methods previously described by Nigatu *et al* (117). Serial concentrations of standard, Quercetin solution (1-0.065 mg/ml) was prepared in methanol to establish calibration curve. One ml each aliquots of standard solution and then 0.3 ml of 5% $NaNO_2$ was transferred to separate 15 ml Eppendorf tubes and left for 5 minutes. Another 0.3 ml of 10 % $AlCl_3$ was added to the contents, mixed with the solution and left to stand for 5 minutes 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. Then, each complex was incubated for 30 minutes at room temperature. Finally, the absorbance of the complex was measured at wavelength of 510 nm using UV 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) was transferred into separate Eppendorf tubes and the same procedure was followed as standard solution. The serial concentrations of standard solution were prepared in two fold dilutions, and all tests were performed in triplicates.

The average absorbance of blank solution was subtracted from standard and extract/fractions solutions, and the total flavonoid content was obtained using standard curve of quercetin. The calculated total flavonoids content results were expressed as mg of quercetin equivalent per g of dry extract/fractions (mg of QE/g).

3.2.6.3. Determinations of Total Alkaloid

Total alkaloid content of the 80% methanol extract and its fractions was determined as method previously described by Ajanal *et al* (118).

Preparation of reagents: - Bromocresol green (BCG) solution reagent's and phosphate buffer solution were prepared as follows.

BCG solution: - To 34.9 mg of bromocresol green, 1.5 ml of 2N NaOH and 2.5 ml of distilled water was added and heated until completely dissolved and the volume was made up to 500 ml with distilled water.

Phosphate buffer solution: - 35.8 gm of sodium phosphate (Na_2HPO_4) and 21.01 gm of 0.2 M citric acid were dissolved in distilled water separately in different volumetric flasks and each solution's final volume was adjusted to 500 ml with distilled water. Finally, the two solutions were mixed up to prepare phosphate buffer solution (PH, 4.7).

Preparation of Standard curve

Serial concentrations of standard solution, atropine (120, 60, 30, 15 and 7.5 µg/ml) were prepared in methanol to establish a calibration curve. One ml of each aliquots of atropine solution was transferred to different separatory funnel. Then 5 ml of phosphate buffer (PH, 4.7) and 5 ml of BCG solution were further added to separatory funnel contents. To the mixture, 4 ml of chloroform was added with vigorous shaking and then extracted. The extraction was repeated with 4 ml of chloroform once again. The chloroform extracts were collected in separate 50 ml Eppendorf tube and their final volume were adjusted to 10 ml with chloroform. The absorbance of the each chloroform extracts of the Atropine was measured at wavelength of 470 nm using UV-Spectrophotometer. All procedures were conducted in triplicates.

Separation of Alkaloid

Two ml of solutions of each 80% methanol extract and its fractions (1 mg/ml) prepared in the methanol was mixed with 2ml of 2N HCl solution in separate 50 ml Eppendorf 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 solution was adjusted to neutral with 0.1 M NaOH solution. To the neutralized solution, 5 ml of BCG and 5 ml of buffer solution (PH, 4.7) were added and shaken. The complex was extracted with 4 ml of chloroform, and once repeated with 4 ml of chloroform upon vigorous shaking. The extract was then collected in separate 50 ml Eppendorf tube and its final volume was adjusted to 10 ml with chloroform. The absorbance of the chloroform extract was measured at wavelength of 470 nm using UV-Spectrophotometer. The same procedure was followed for blank solution, methanol. All procedures were conducted in triplicates.

The average absorbance of blank solution was subtracted from standard and crude extract/fractions solutions and the total alkaloid content was determined using standard curve of Atropine.

The calculated total alkaloidal content results were expressed as mg of atropine equivalent per g of dry extract/fractions (mg of AE/g).

The TPC of the 80% methanol extract and its fractions were calculated from the equation of calibration curve of gallic acid: Y = 0.006352*X + 0.08988, R^2 = 0.9778 (Fig.3.1A). On the other hand, their TFC were calculated from the standard curve of quercetin: Y = 0.6187*X - 0.01021, R^2 = 0.9952 (Fig. 3.1B). Additionally, the total alkaloid contents were extrapolated from the calibration curve of atropine: Y = 0.001312*X + 0.001750, R^2 = 0.9934 (Fig 3.1C) and were presented.

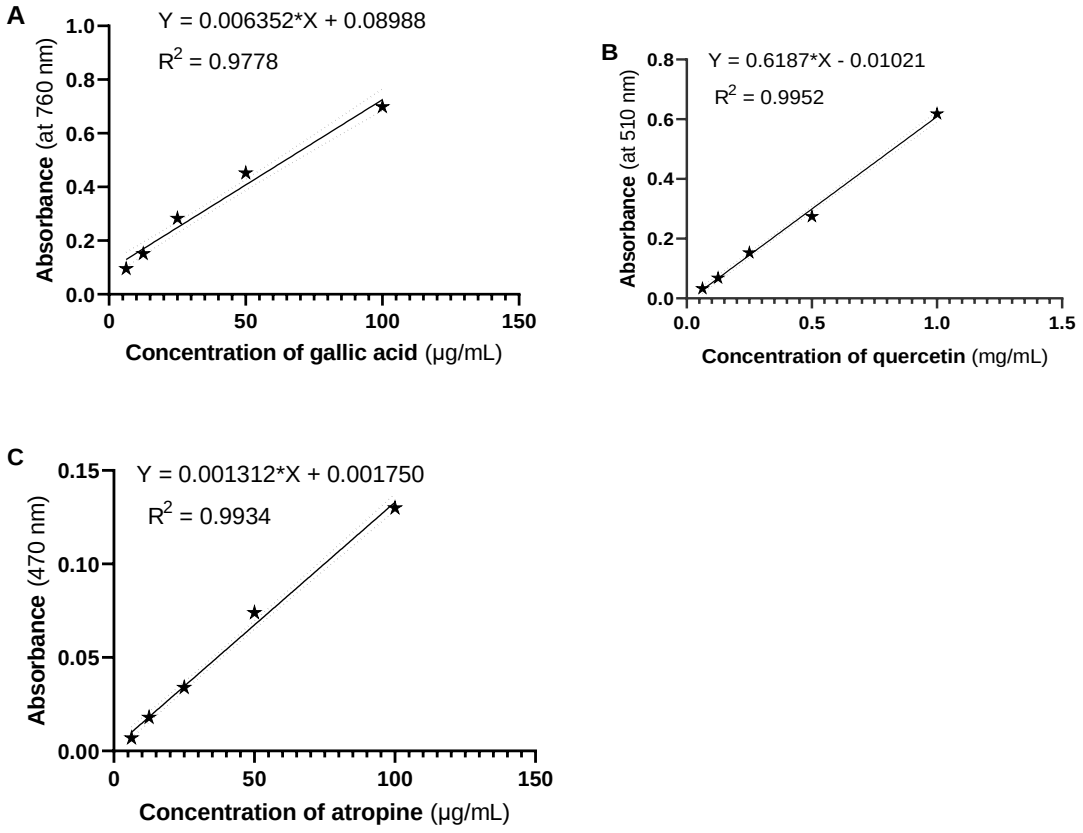

Figure 3. 1.Titration curves of Phenols, Flavonoids and Alkaloids: Phenol (A), Flavonoid (B) and Alkaloid (C)

3.2.7. Statistical Analysis

The IC_{50} of anti-promastigotes and anti-amastigotes study assays were calculated from sigmoidal dose-response curves of percent inhibition and infection index, respectively. The cytotoxic effect (CC_{50}) of the extract against animal cells and red blood cells was calculated from sigmoidal dose-response curves of percent viability of cells. Data was presented as mean ± standard error of the mean (SEM). The quantitative estimations of TPC, TFC and TAC of 80% methanol extract and its fractions were calculated from linear dose-response curves of the corresponding absorbance of their solution. For all data analysis GraphPad Prism 9.1.0 computer software (GraphPad Software, Inc., CA, USA) was used (108). Furthermore, the selectivity index (SI) was obtained from the ratio between CC_{50} and IC_{50}. All the analyses were carried out at 5% level of significance (64).

3.2.8. Ethical Clearance

All procedures conducted in this research work, including use of Vero cell lines were reviewed and approved by Ethical Review Committee, School of Pharmacy, College of Health Sciences, Addis Ababa University with letter number ERB/SOP/177/13/2020 dated September, 2020. Informed consent was obtained from the patients after diagnosis in order to use the parasite isolates and from volunteer man to use the collected red blood cells for research purpose.

4. RESULTS

4.1. Preliminary Anti-leishmanial Activity

The preliminary anti-leishmanial activity study of 80% methanol extracts of seeds of *B. antidysenterica* and roots of *R. nepalensis* against logarithmic growth phases of promastigote of *L. aethiopica* and *L. donovani* were determined and expressed as percentage of inhibition (%I) (Table 4.1). Eighty percent methanol extract of *B. antidysenterica* (%I ≥ 74.46 ± 2.86%,) showed higher percentage of growth inhibition of *Leishmania* parasites than 80% methanol extract of *R. nepalensis* (%I ≥ 39.43 ± 0.59%) at concentration of 100 µg/ml. The anti-leishmanial activities (IC_{50}) and cytotoxic effects (CC_{50}) of the 80% methanol extract of *B. antidysenterica* and its solvent fractions were continued.

Table 4. 1. Percentage inhibition of 80% Methanol extracts of *B. antidysenterica* and *R. nepalensis*

Types of extract and its conc. (µg/ml)	**% Inhibition of extracts against *Leishmania* promastigotes**			
	Against *L. aethiopica*		**Against *L. donovani***	
	Experiment-1	**Experiment-2**	**Experiment-1**	**Experiment-2**
BA – 100 µg/ml[a]	74.46 ± 2.86	77.85 ± 2.21	78.36 ± 1.02	79.64 ± 1.13
RN – 100 µg/ml[a]	50.05 ± 1.71	46.26 ± 1.17	42.41 ± 1.17	39.43 ± 0.59
Medium alone (NC)	0.00[b]	0.00[b]	0.00[b]	0.00[b]
1% DMSO + Medium (NC)	0.00[b]	0.00[b]	0.00[b]	0.00[b]

The values are expressed as mean ± SEM: $n = 3$; BA: 80% methanol extract of seeds of *B. antidysenterica* J.F Mill; RN: 80% methanol extract of roots of *R. nepalensis* spreng.; NC: negative control; DMSO: dimethyl sulfoxide. 100 µg/ml indicates the concentrations of the extracts

[a]Effective concentration that inhibit parasite growth. [b]No effect

4.2. Anti-promastigote Activity

The IC_{50} of the crude extract (80% methanol) of seeds of *B. antidysenterica* and its four solvent fractions against promastigotes of *L. aethiopica* and *L. donovani* isolates were determined in two separate experiments (Table 4.2 - 4.3). The IC_{50} of the crude extract and its solvent fractions were much higher than that of the reference drugs. The IC_{50} of the crude extract, ethyl acetate, hexane, butanol and aqueous fractions against promastigotes of *L. aethiopica* were 25.63 ± 1.07, 6.53 ± 0.57, 8.54 ± 0.29, 43.81 ± 4.14 and 208.9 ± 20.2 1 µg/ml, respectively (Table 4.2).

Similarly, the IC_{50} of the crude extract, ethyl acetate, hexane, butanol and aqueous fractions against promastigotes *L. donovani* were 20.77 ± 1.55, 4.14 ± 0.62, 6.84 ± 1.18, 60.12 ± 6.95 and 196.85 ± 15.05 µg/ml, respectively (Table 4.3). But the IC_{50} of the reference drugs against both strains of *Leishmania* were < 1 µg/ml (Table 4.2 – 4.3).

The anti-promastigote activities of all test groups in experiment-1 against *L. aethiopica* and *L. donovani* isolates were comparable to their corresponding activities displayed in experiment-2 (Table 4.2 – 4.3).

Table 4. 2. The IC_{50} of test substances against promastigotes of *Leishmania aethiopica*

Test substance	Experiment-1		Experiment-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
HEX-BA	8.54 ± 0.29	0.9977	9.35 ± 0.76	0.9911
ETH-BA	6.53 ± 0.57	0.9897	6.77 ± 0.47	0.9930
BUT-BA	43.81 ± 4.14	0.9855	46.35 ± 3.00	0.9930
AQU-BA	208.9 ± 20.2	0.9561	200.5 ± 16.8	0.9642
PEN (Reference drug)	0.82 ± 0.13	0.9740	0.9 ± 0.10	0.9849
AMB (Reference drug)	0.17 ± 0.08	0.9497	0.18 ± 0.04	0.9796
Medium alone (NC)	0.00[b]		0.00[b]	
1%DMSO + Medium (NC)	0.00[b]		0.00[b]	

The values are expressed as mean ± SEM; $n = 3$; MET-BA: 80% methanol extract of seeds of *Brucea antidysenterica* J.F Mill; HEX-BA, ETH-BA, BUT-BA, AQU-BA: are hexane, ethyl acetate, butanol and aqueous 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
[a]Effective concentration required to achieve 50% growth inhibition in μg/mL; [b]No effect

Table 4. 3. The IC_{50} of test substances against promastigotes of *Leishmania donovani*

Test substance	Experiment-1		Experiment-2	
	IC_{50} (µg/ml)[a]	**R^2**	**IC_{50} (µg/ml)[a]**	**R^2**
MET-BA	20.77 ± 1.55	0.9911	22.15 ± 3.31	0.9721
HEX-BA	6.84 ± 1.18	0.9619	7.19 ± 1.00	0.9755
ETH-BA	4.14 ± 0.62	0.9706	4.74 ± 0.42	0.9896
BUT-BA	60.12 ± 6.95	0.9725	56.07 ± 5.07	0.9869
AQU-BA	196.85 ± 15.05	0.9751	189.3 ± 8.70	0.9897
PEN (Reference drug)	0.87 ± 0.11	0.9635	0.91 ± 0.14	0.9747
AMB (Reference drug)	0.15 ± 0.01	0.9879	0.18 ± 0.03	0.9850
Medium alone (NC)	0.00[b]		0.00[b]	
1%DMSO+ Medium (NC)	0.00[b]		0.00[b]	

The values are expressed as mean ± SEM; $n = 3$; MET-BA: 80% methanol extract of seeds of *Brucea antidysenterica* J.F Mill; HEX-BA, ETH-BA, BUT-BA, AQU-BA: are hexane, ethyl acetate, butanol and aqueous 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
[a]Effective concentration required to achieve 50% growth inhibition in μg/mL; [b]No effect

4.3. Anti-amastigotes Activity

The IC_{50} of 80% methanol extract of *B. antidysenterica* and other three solvent fractions against intracellular amastigotes of *L. donovani* and *L. aethiopica* determined from their corresponding infectivity index were shown (Table 4.4). The ethyl acetate fraction exhibited the highest anti-amastigote activity against *L. aethiopica* (IC_{50} = 6.16 ± 1.12 µg/ml) and *L. donovani* (IC_{50} = 6.85 ± 1.46 µg/ml) among crude extract and solvent fractions. In contrary, the butanol fraction displayed the lowest anti-amastigote activity against *L. aethiopica* (IC_{50} = 36.29 ± 6.00 µg/ml) and *L. donovani* (IC_{50} = 40.12 ± 5.30 µg/ml). The three solvent fractions and crude extract revealed lower anti-amastigote activities against both test strains than the activities revealed by reference drugs; amphotericin B and pentamidine isethionate (Table 4.4).

Table 4. 4. 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
ETH-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
Medium alone (NC)	0.00[b]		0.00[b]	
1% DMSO + Medium (NC)	0.00[b]		0.00[b]	

The values are expressed as mean ± SEM; $n = 3$; MET-BA: 80% methanol extract of seeds of *Brucea antidysenterica* J.F Mill; HEX-BA, ETH-BA, BUT-BA: are hexane, ethyl acetate and butanol and 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

[a]Effective concentration required to achieve 50% growth inhibition in μg/mL. [b]No effect

4.4. Cytotoxicity Effects

The cytotoxic effects (CC_{50}) of 80% methanol extract of the plant and other three solvent fractions against human red blood cells were determined based on Alamar Blue® reduction assay. All solvent fractions and crude extract showed CC_{50} > 1600 µg/ml. They showed < 10% RBC hemolysis at maximum test concentration, 1600 µg/ml. Eighty percent methanol, hexane, ethyl acetate and butanol fractions resulted in 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, their cytotoxic effects against other mammalian cell lines: Vero cell lines and peritoneal mice macrophages were also presented (Table 4.5). 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. In contrast, the lowest cytotoxic effect was revealed by butanol fraction with CC_{50} values of

323.45 ± 15.95 and 319.90 ± 27.60 µg/ml against Vero Cell lines and peritoneal mice macrophage cells, respectively. The crude extract and other three solvent fractions showed lower cytotoxic effect against Vero cell lines and peritoneal mice macrophage isolates than reference drugs (Table 4.5).

Table 4. 5. CC_{50} of test substance against Vero cell lines and peritoneal mice macrophages 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
ETH-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
Medium alone (NC)	0.00[b]		0.00[b]	
1% DMSO + Medium (NC)	0.00[b]		0.00[b]	

The values are expressed as mean ± SEM; $n = 3$; MET-BA: 80% methanol extract of seeds of *Brucea antidysenterica* J.F Mill; HEX-BA, ETH-BA, BUT-BA: are 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

[a]Effective concentration required to kill 50% animal cell lines in μg/mL; [b]No effect

4.5. Selectivity Index of Test Substances

The selectivity indices (SI) of each test groups, calculated as the ratio of CC_{50} of the test group against mammalian cell lines to its corresponding IC_{50} value against intracellular amastigote *Leishmanial* species, were displayed (Table 4.6). The finding exhibited that the 80% methanol extract of the plant and other three solvent fractions (SI values ranging from 7.97 to 30.97) showed comparable selective toxicity to *Leishmania* parasites as pentamidine isethionate (12.00

≤SI ≤ 14.31) except ethyl acetate which showed more selectivity toward the parasites. In contrary, they exhibited less selectivity to *Leishmania* spps than Amphotericin B (105.06 ≤ SI ≤ 161.65).

Table 4. 6. 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
ETH-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

MET-BA: 80% methanol extract of seeds of *Brucea antidysenterica* J.F Mill; HEX-BA, ETH-BA, BUT-BA: are 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

[a]The value of selectivity index calculated as ratio of CC50 of mammalian cell to its respective IC50 against parasite

4.6. Total Contents of Phenolic compounds, Flavonoids and Alkaloids

The quantitative estimations of the TPC, TFC and TFC of crude extract of *B. antidysenterica* and its hexane and ethyl acetate fractions calculated from their corresponding calibration curves were presented (Table 4.7). Higher concentrations of both TPC (127.72 ± 1.82 mg of GAE/g) and TFC (79.21 ± 0.19 mg of QE/g) were contained in 80% methanol extract than its ethyl acetate and fractions. Higher concentration of TAC (97.22 ± 0.25 mg of AE/g) was also found to present in ethyl acetate fraction.

Table 4. 7. Total Phenolic, Flavonoid and Alkaloid Content of 80% Methanol Extract of seeds of *Brucea antidysenterica* J.F.Mill and its Fractions

Types of extract/ fraction	**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

The values are expressed as mean ± SEM; n = 3; GEA/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

5. DISCUSSION

The percent inhibition of the growth of promastigotes of *L. donovani* and *L. aethiopica* revealed by 80% methanol extract of *B. antidysenterica* was ≥ 74.46 ± 2.86% while ≥ 39.43 ± 0.59% for 80% methanol extract of *R. nepalensis* at concentrations of 100 µg/ml (Table 4.1). The plant extract with higher percentage of inhibition, *B. antidysenterica,* was fractionated to hexane, ethyl acetate, butanol and aqueous solvents for further study while the study of the less active extract, *R. nepalensis,* was not continued. Naphthalenes, flavonoids and phenols compounds isolated from root extracts of *R. nepalensis* possessed anti-inflammatory activities (119) that in turn accelerates wound healing. This suggested that use of this plant for the treatment of cutaneous leishmaniasis in traditional medicine is probably due to its indirect activity in wound healing process in even though it showed low direct anti-leishmanial activity against the parasites.

The crude extract of *B. antidysenterica* and its solvent fractions revealed anti-leishmanial activities with varying level of activities against *L. donovani* and *L. aethiopica* and non-toxic to mammalian cells: Vero cell lines, mice macrophages isolates and red blood cells. But they showed lower anti-leishmanial activities and less toxic to mammalian cells as compared to reference drugs; amphotericin B and pentamidine isethionate.

The anti-promastigotes activity of the crude extract of the plant was higher than the activities exhibited by butanol and aqueous fractions but lower than activities exhibited by ethyl acetate and hexane fractions against both *L. donovani* and *L. aethiopica*. The highest and the lowest anti-promastigotes activities were recorded by ethyl acetate and aqueous fractions, respectively against *Leishmania* test strains. 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 that 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 than the IC_{50} of Amphotericin B and pentamidine isethionate, respectively, suggesting that the anti-promastigotes activities of the plant extract and its solvent fractions were much lower than the activities showed by reference drugs (Table 4.2, Table 4.3).

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} ≤ 30 μg/ml: moderate activity, 30 < IC_{50} ≤ 64 μg/ml: low activity, IC_{50}> 64 μg/ml: inactive (120). Accordingly, the crude extract of *B. antidysenterica* J.F.Mill (20 < IC_{50} ≤ 30 μg/ml), n-hexane fraction (5 < IC_{50} ≤ 20 μg/ml) and n-butanol fraction (30 < IC_{50} ≤ 64 μg/ml) possessed moderate, good and low anti-promastigote activity, respectively against both *L. aethiopica* and *L. donovani* isolates. The ethyl acetate fraction possessed good activity against *L. aethiopica* and pronounced activity against *L. donovani* while aqueous fraction with IC_{50} > 64 μg/ml was found to be inactive against both *L. donovani* and *L. aethiopica* promastigotes (Table 4.2, Table 4.3).

Anti-promastigotes screening of bioactive agents is rapid, reproducible, and inexpensive and easily quantified using different methods, so that, suitable for high-throughput screening (121,122). The *in vitro* promastigote parasites culture in cell free media is also simple (123) even though the ecology, metabolism, morphology, composition of the surface glycocalyx of the promastigote is quit differ from those of amastigote (123,124). Therefore, the results obtained from anti-promastigotes screening may not be correlated with *ex vivo* intracellular amastigotes or *in vivo* animal models.

In the current study, the potential anti-amastigotes activities, a clinically relevant stages of *Leishmania*, of the crude extract and its fractions were evaluated as confirmatory to their anti-promastigotes 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* with 5 < IC_{50} ≤ 20 μg/ml whereas butanol fraction (30 < IC_{50} ≤ 64 μg/ml) was found to have low anti-amastigotes activity against both species of *Leishmania* (Table 4.4). Like in anti-promastigotes assay, ethyl acetate fractions exhibited the highest activity against intracellular amastigotes while lowest activity was recorded by butanol fraction. The anti-amastigotes activities of the reference drugs were much higher than the plant extract and its fractions as seen in anti-promastigotes study (Table 4.4).

Even though *Leishmania* spps are able to infect and reside in the dendritic cells, neutrophils, macrophages, monocytes and other cells of immune system, they commonly infect, multiply in and reside in macrophage cells (122) where they are transformed to amastigotes. The promastigotes transformed to the most clinically relevant stages, amastigotes, through 2 ways. The first method is by infecting macrophage cells with metacyclic promastigotes. Primary

peritoneal or bone marrow mouse macrophages isolates or animal cell lines such as J774.1, U937, THP-1, and RAW264.7 can be infected with the parasites *in vitro*. Subsequently, they are transformed to amastigotes which is termed as intracellular amastigotes (122). The second method is creating intra-macrophage – like environment in the promastigotes culture. Increasing the temperature and lowering the pH of host cell free promastigotes culture (37℃ and pH 5.5 in 5% CO2) can induce the transformation of promastigote to amastigote-like forms which is termed as axenic amastigotes (125). Like promastigotes, use of axenic amastigotes as biological model in the screening of bioactive agents for their anti – leishmanial effects have the advantages. The axenic promastigotes culture is easily manipulated, quantification of the bioactive agent's effect is rapid and cheap and the model does not require laboratory animal. Therefore, these model is preferable for the evaluation of anti-leishmanial activities of large numbers of compounds (126). However, due to the absence of host-parasite interaction such screen doesn't reflect the physiological situation as intracellular amastigote in that: axenic amastigotes differ from intracellular amastigotes in several cellular processes such as metabolism, response to oxidative stress and intracellular transport (121,127). Therefore, compounds active against axenic amastigotes might be not active against intracellular amastigotes and *vice versa* (121). The present study used intracellular amastigote assay to mimic the real biological environment to screen the test extract and its fractions and represent the human disease model.

Safety of a given bioactive agent is the most critical factor in the development of every medicament which necessitate an inclusion of a wide toxicity screening of both active pharmaceutical ingredients and excipients at the early stages of formulation development (128). These agents might be toxic to cells through different mechanisms such as protein synthesis inhibition, cell membrane damage, irreversibly binding to receptor, enzymatic reactions, and prevention of nucleotide elongation (129). In the present study, the toxic effect of 80% methanol extract of *B. antidysenterica* and its solvent fraction were evaluated against three mammalian cells: Vero cell lines, peritoneal mice macrophage cells and human red blood cells *in vitro*. The toxicity level of bioactive agents was classified based on the following criteria: <10µg/ml: very strong cytotoxicity, 10–100 µg/ml strong cytotoxicity, 100–500 µg/ml moderate cytotoxicity (130). Having CC_{50} values of ($10 \leq CC_{50} \leq 500$ µg/ml), the crude extract and its hexane, ethyl acetate and butanol fractions demonstrated moderate cytotoxic effect against both Vero cells and

mice macrophages (Table 4.5). However, both reference drugs possessed very strong cytotoxic effect against both cells except Amphotericin B possessed strong cytotoxic effect activity against peritoneal mice macrophage cells (Table 4.5). The hexane, ethyl acetate and butanol fractions, and the crude extract of the plant were, therefore, much less toxic to mammalian cells than reference drugs. However, extrapolating the *in vitro* cytotoxicity test results to *in vivo* results is a problematic as pharmacokinetics of a bioactive compound in the tissue is not considered in *in vitro* study (121). If a given bioactive agent proves to be non – toxic against animal cell lines *in vitro*, it only means that don't start the next toxicity study in animal model with smallest concentration, but from medium or high concentration to determine LD_{50} (128). 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 (130). Therefore, all test groups were selective against amastigotes of both strains of *Leishmania*. But the crude extract and its fractions ($7.97 \leq SI \leq 30.97$) were showed less selective than Amphotericin B ($105.06 \leq SI \leq 161.65$). Conversely, they showed comparable selectivity against *Leishmania* spps as pentamidine isethionate ($12.00 \leq SI \leq 14.31$) except ethyl acetate fraction which showed more selectivity against the parasites (Table 4.6).

The preliminary phytochemical screening conducted previously indicated the presence of alkaloids, tannins, flavonoids, triterpenoid, phenols, steroids and glycosides in 80% methanol extract of seeds of *B. antidysentrica* (74). Phenolic compounds include simple phenols, cinnamic acid derivatives, coumarins, benzoic acid derivatives, tannins, flavonoids, lignins and lignans among others (131). Phenolic compounds obtained from various plants having inhibitory activities against protozoan parasites including *Leishmania* parasites were reported. Cinnamic acid derivatives (*o*-coumaric acid, *p*-coumaric acid, cinapic acid, *trans*-cinnamic acid), flavonol derivatives (morin and rutin), hydroxybenzoic acid derivatives (gallic acid, gentisic acid, vanillic acid) ellagic acid were reported for their *in vitro* activities against *L. amazonensis*. Moreover, gentisic acid and *p*-coumaric acid were also showed significant *in vivo* activity against *L. amazonensis* in infected BALB/c mice (132). 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 (133). The phenolic compounds contained in 80% methanol extract and its ethyl acetate and hexane fractions with

TPC of 127.72 ± 1.82, 70.78 ± 0.95 and 54.78 ± 1.39 mg of GAE/g of extract/fraction, respectively might be attribute to their anti-leishmanial activities (Table 4.7).

Furthermore, flavonoids which include chalcones, flavones, flavonols, and isoflavones are also largely known for their wide spectrum of activities against leishmaniasis (134). Gossypetin 3, 7, 8, 4'-tetra-O-methyl ether and Kaempferol 3, 7-di-O-methyl ether are flavonoids that showed anti-leishmanial activities against *L. amazonensis* through arresting cell cycles. In addition to arresting cell cycle, the later compound also induced autophagy which resulted in death of the parasites (135). Quercetin, other 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 (136). In the current study, TFC of 80% methanol extract and its ethyl acetate and hexane fractions were 79.21 ± 0.19, 46.67 ± 0.14 and 18.30 ± 0.07 mg of QE/g of extract/fraction and might be responsible for their ant-leishmanial activities (Table 4.7).

The inducible nitric oxide synthase (iNOS) isoform is an enzymes that originally expressed in activated macrophages and functions as a component of the innate immune system through increasing the synthesis of nitric oxide (NO) (137). The quassinoid compound, quassin, isolated from the bark of *Q. amara* was found to be induce the expression of inducible nitric oxide synthase subtype-2 (iNOS2) at protein and mRNA level in *L. donovani* infected murine peritoneal macrophages. It also found to increases release and expression of inflammatory cytokines such as TNF-α and IL-12 and decreases the release and expression of anti-inflammatory cytokines such as TGF-β and IL-10 in *L. donovani* infected macrophages (138). Release of NO and the associated inflammatory mediators, TNF-α and IL-12, are responsible for the eradication of microbes including *Leishmania* parasites (139,140). Simalikalactone D, another quassinoid, obtained from the root bark of *Simaba orinocensis* (Simaroubaceae) showed pronounced activity against *L. donovani* with IC_{50} of 0.035 µg/ml which was more than 46 and 31-fold potent than pentamidine (IC_{50} = 1.6 µg/ml) and Amphotericin B (IC_{50} =1.1 µg/ml), respectively (141). Bruceanic acid A-D, bruceanol A-H, bruceantarin, bruceantin, bruceine B, yadanzioside G, K, M, N, P are amongst quassinoid isolates found in *B. antidysenterica* (142), suggesting that one or more of the quassinoid found in the plant might be responsible for its anti-leishmanial activities.

Two canthine alkaloids: canthin-6-one and 5-methoxycanthin-6-one isolated from stem bark of *Zanthoxylum chiloperone* (143) also previously isolated from *B. antidysentrica* (75) demonstrated *in vitro* anti-leishmanial activity against *L. braziliensis*, *L. amazonensis*, and *L. donovani*. Canthin-6-one, when administered intralesionally, also displayed an interesting leishmaniacidal activity against *L. amazonensis* infected BALB/c mice. (143). Another alkaloid isolate, β-Carboline-1-propionic acid, (β-CPA) obtained from stem bark of *Quassia amara* L. (Simaroubaceae) (144) also previously isolated from *B. antidysenterica* (145) displayed a potent anti-leishmanial activity against *L. amazonensis* and *L. infantum* against promastigotes and intracellular amastigotes (144). The alkaloids found in 80% methanol extract (TAC = 27.62 ± 0.27) and its ethyl acetate (TAC = 97.22 ± 0.25) and hexane fractions (TAC = 38.04 ± 0.25) mg of AE/g of extract /fraction as per current finding might be responsible for their anti-leishmanial activities.

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* (120,146). The plant was also found to be active against two *Trypanosoma* (*T. cruzi* and *T. brucei brucei*), the parasites related to *Leishmania* species (120,146).

This finding suggested that, the anti-leishmanial activities of 80% methanol extract of the seeds of *B. antidysenterica* and its fractions were attributed to phenolic compounds, alkaloids, flavonoids and/ or other secondary metabolites.

6. CONCLUSION

The current study showed that 80% methanol extract of seeds of *B. antidysenterica* and its ethyl acetate, hexane and butanol fractions possessed a varying level of anti-leishmanial activities against both *L. aethiopica* and *L. donovani* and showed selective cytotoxic effect to *Leishmania* parasites. Ethyl acetate and hexane fractions were found to exhibit higher anti-leishmanial activities against both *Leishmania* strains. The result might provide a scientific justification for use of *B. antidysenterica* J. F.Mill seeds in the treatments of leishmaniasis by traditional healers.

7. RECOMMENDATION

Since the crude extract of seeds of *B. antidysenterica* J.F. Mill and its ethyl acetate, hexane and butanol fractions showed potential *in vitro* anti-leishmanial activity against *L. donovani* and *L. aethiopica* in the current study, further studies including the following are recommended:

- To isolate, purify and identify specific secondary metabolites responsible for anti-leishmanial activity
- Mechanism of anti-leishmanial activity
- The *in vivo* anti-leishmanial activity of the plant extracts

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