Addis Ababa University

College of Natural and Computational Sciences

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In vitro antipromastigote activity of methanol extracts of *Malva parviflora* leaves and roots against *Leishmania donovani* and *Leishmania aethiopica*.

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College of Natural and computational studies

This is to certify that the thesis prepared by Yeabtsega Tilahun titled "*In vitro* antipromastigote activity of methanol extracts of *Malva parviflora* leaves and roots against *Leishmania donovani* and *Leishmania aethiopica*." and submitted in partial fulfillment of the requirements for the Degree of Master of Science in Infection Biology complies with the regulations of the University and meets the accepted standards concerning originality and quality.

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ACRONYMS AND ABBREVIATIONS

AmB	Amphotericin B
CC_{50}	Minimal cytotoxic concentration
CDC	Center for Disease Control and Prevention
CL	Cutaneous Leishmaniasis
DMSO	Dimethyl Sulfoxide
FBS	Fetal Bovine Serum
IC_{50}	Half Maximal Inhibitory Concentration
NNN	Novy-McNeal Nicolle
PKDL	Post kala-azar dermal leishmaniasis
PBS	Phosphate Buffer Saline
RPMI	Rose Park Memorial Institute
VL	Visceral Leishmaniasis
WHO	World Health Organization

ABSTRACT

Leishmaniasis is a neglected tropical disease threatening the lives of about 350 million people, globally. There is a small selection of medications on the market that can be used to treat leishmaniasis. However, the currently available drugs are limited in number and have drawbacks including variable efficacy, toxicity and unaffordability which call upon search for viable options. The leaf and root of *Malva parviflora* are used for the treatment of leishmaniasis in traditional medicine in Ethiopia. The aim of this study was to evaluate the antipromastigote activities of methanol extracts of *Malva parviflora* against *Leishmania* promastigotes**.** Fresh leaves and roots of *Malva parviflora* were chopped and then macerated with sufficient amount of 100% methanol at room temperature. Clinical isolates of *L. donovani* and *L. aethiopica* were obtained and the isolates were grown in tissue culture flasks containing RPMI 1640 medium supplemented with fetal bovine serum. *L. aethiopica* and *L. donovani* at the logarithmic stages were inoculated in to liquid media for the assay. The percent inhibition of the growth of promastigotes of *Leishmania* species of the methanol extracts of leaf and root of *M. Parviflora* evaluated *in vitro,* at concentrations of 100 µg/ml and investigated for their anti-leishmanial activities against promastigotes of *L. aethiopica* and *L. donovani* and for their cytotoxicity. The percent of inhibition of leaf of *Malva parviflora* was 55.89 ± 2.19% and 79.84 ± 1.57% and the root of *M. parviflora* was 74.21 ± 2.39% and 78.81 ±1.88% respectively. The leaf extract has an IC_{50} of 1.73 and an IC_{50} of 0.002 against both promastigotes of *L. aethiopica* and *L. donovani* respectively. The IC_{50} of the root extract against *L. aethiopica* and *L. donovani* are 0.05 and 0.002 respectively. The leaf extract had a hemolysis percentage of 21.05 ± 11.54% and the root extract had 0 ± 26.54% percentage hemolysis of the extracts against human red blood cells. The leaf extracts had a selective index of 75.2 against *L. aethiopica* and a selective index of 260,200 against *L. donovani* isolates. The root extracts exhibited a selectivity index of >1000 against both *L. aethiopica* and *L. donovani*. The findings of this study conclude that the root extracts of *Malva parviflora* exhibit higher anti-leishmanial activities against both *Leishmania* strains and was found to be less toxic against human red blood cells when compared with the leaf extract. Further investigations on the bioactive compounds and fractions of *M. Parviflora* are recommended.

Key Words and Phrases: Leishmaniasis*, Leishmania aethiopica, Leishmania donovani, in vitro,* anti-promastigotes*, M. Parviflora*., Ethiopia

1. INTRODUCTION

Leishmaniasis is a group of diseases spread by sand flies and are prevalent in tropical and subtropical regions of North and South America, Europe, Asia, and Africa. Leishmaniasis is brought on by parasitic protozoans of the genus Leishmania, which is closely linked to flagellated trypanosomes. On the basis of genetic and/or immunologic criteria, about 20 species have been identified. Visceral leishmaniasis and cutaneous leishmaniasis are the two main clinical types of leishmaniasis (Mullen and Durden, 2019).

Leishmaniasis, according to the World Health Organization, is one of the seven most significant tropical diseases. It is a critical global health issue that has a wide range of clinical symptoms and a potentially deadly consequence. Except for Oceania, it can be found on every continent. It is native to specific regions of Northeastern Africa, Southern Europe, the Middle East, Southeastern Mexico, and Central and South America (Andrade-Narváez, *et al.,* 2001).

Leishmaniasis is a significant cause of illness and mortality. The illness manifests in at least four different ways: cutaneous, diffuse cutaneous, mucocutaneous, and visceral. Visceral leishmaniasis (VL), a mutilating illness, muco-cutaneous leishmaniasis (MCL), and diffuse cutaneous leishmaniasis (DCL), a long-lasting disease, are just a few of the clinical manifestations of leishmaniasis that are exceptionally variable and represent a complex of disorders. Cutaneous leishmaniasis (CL), a disease caused by a poor cellular-mediated immune response, is paralyzing when the lesions are numerous (Desjeux, 2004).

Despite the fact that leishmanial infections have been ignored for many years, recent research has shown promising targets for treatment that could be used to stop the spread of parasites. The substantial antiparasitic potential of numerous substances derived from natural sources has recently also been shown; however, the majority of these studies are restricted to primary evaluation, and only a few have progressed to the clinical stages. Few medications, such as pentamidine (antimicrobial), amphotericin B (antifungal), or miltefosine (antitumor), are being used but have significant side effects due to the rising prevalence of antimony resistant parasites and the lack of real antileishmanial substances (Tiwari *et al.,* 2017).

Although *M. parviflora* plays a significant role by being used in traditional medicine across many countries including Ethiopia, only a limited study has been done to scientifically explore its antibacterial activity and almost none have been done on its antileishmanial activities, and there have been no previous reports on the isolation and structural characterization of the bioactive compounds present in this plant. Thus, the aim of this study was to evaluate the antipromastigote activities of methanol extracts of *Malva parviflora* leaves and roots against *Leishmania donovani* and *Leishmania aethiopica in vitro*.

1.1. Statement of the problem

Leishmaniasis is endemic to Ethiopia. Visceral Leishmaniasis (VL) and Cutaneous Leishmaniasis (CL) are both serious health issues in the nation (Ayele and Ali, 1984). *Leishmania aethiopica*, which is primarily prevalent in the country's highlands, is the primary source of the three clinical forms of the disease, that includes diffuse cutaneous leishmaniasis (DCL), mucosal leishmaniasis, and localized CL, which account for an estimated 50,000 cases annually (Ngure *et al*., 2009).

According to the systematic review and meta-analysis of (Assefa, 2018) it is indicated that visceral leishmaniasis is the most prevalent kind of leishmaniasis in Ethiopia. However, northern Ethiopia, particularly the Libokemkem, Metema, and Humera areas, was found to be far more devastated (Ferede *et al.,* 2017; Mengesha *et al.,* 2014; Welay *et al.,* 2016). Most significantly, Cutaneous Leishmniasis is extremely common in the rift valley area of the southern part of the country. It was primarily reported in Kutaber districts of northern Ethiopia (Lemma *et al.,* 1969; Negera *et al.,* 2008).

There is a small selection of medications on the market that can be used to treat leishmaniasis. It consists of miltefosine (MF), pentavalent antimonials (Sbv), amphotericin B deoxycholate (AB), lipid formulations of amphotericin B, and paromomycin (PM), all of which have toxicity and effectiveness restrictions. Except for MF, which is delivered orally, all medications for visceral leishmaniasis, which requires systemic therapy, are administered by parenteral routes (AB and its lipid formulations by vein infusion, Sbv and PM by intramuscular injection) (Hendrickx, *et al.,* 2018).

For instance, the most commonly used medication (Sbv) is now only marginally effective in northern Bihar, India, which alone is responsible for 50% of the global burden of *Leishmania*

donovani caused visceral leishmaniasis. The difficulties in treating leishmaniasis include difficult treatment schedules, a lack of available medications in developing nations, Drug side effects, high costs because the disease requires long-term management and the emergence of parasite resistance to drugs. It is challenging to just rely on the current or conventional pharmaceuticals due to the issues mentioned above as well as the relentless urge and endeavor of mankind to match the continuous demand, and this forces us to search for workable alternatives. (Kooti, *et al.,* 2016).

1.2. Research questions

- Does the methanol extract of *M. parviflora* leaves and roots have anti leishmanial activity?
- What is the half maximal inhibitory concentration and median cytotoxic concentration of the leaf and root extract of *M. parviflora?*
- What is the median inhibitory concentration and median cytotoxicity concentration of the leaf and root extract of *M. parviflora?*
- Is the leaf extract more effective against *L. donovani* or *L. aethiopica* than the root extract?
- Is the root extract more effective against *L. donovani* or *L. aethiopica* than the leaf extract?
- Are the methanol extracts of *M. parviflora* leaves cytotoxcic to human red blood cells?
- Are the methanol extracts of *M. parviflora* roots cytotoxcic human red blood cells?

1.3. Significance of the study

This study aims to evaluate the antileishmanial activity of *M. parviflora* which has been proven to have many significant roles in the treatment of many ailments using traditional methods. The importance of this study is to indicate whether *M. parviflora'*s anti leishmanial activity are viable so that they can processed and manufactured into a safe treatment which will be available for fair distribution across Ethiopia and Africa.

1.4. Objectives

1.4.1 General Objective

The general objective of this study was to evaluate the anti-promastigote activities on methanol extracts of *M. Parviflora* leaves and roots against *Leishmania donovani* and *Leishmania aethiopica in vitro*.

1.4.2 Specific objectives

- Evaluate the *in vitro* anti-promastigote activities on methanol extracts of *M. parviflora* leaves against *Leishmania donovani* and *Leishmania aethiopica*.
- Evaluate the *in vitro* anti-promastigote activities on methanol extracts of *M. parviflora* roots against *Leishmania donovani* and *Leishmania aethiopica*.
- To evaluate cytotoxicity of methanol extracts of *M. parviflora* leaves *in vitro* via hemolysis assay.
- To evaluate cytotoxicity of methanol extracts of *M. parviflora* roots *in vitro* via hemolysis assay.

2. LITERATURE REVIEW

2.1. Cutaneous leishmaniasis

The leishmaniases are characterized by a variety of clinical symptoms, including several non-ulcerative nodules (diffuse cutaneous leishmaniasis, DCL) and ulcerative skin lesions that appear at the site of the sandfly bite (localized cutaneous leishmaniasis, LCL) (Reithinger *et al.,* 2007). As a result of its many clinical manifestations, cutaneous leishmaniasis has a wide range of differential diagnoses. The majority of instances will begin with a skin ulcer that won't heal, which is often mistaken for an infected bug bite or a true tropical ulcer (Magill, 2005). *Leishmania aethiopica*, *Leishmania mexicana*, and *Leishmania. amazonensis* have the potential to produce a diffuse form of the disease under specific circumstances, which is defined by the development of nonulcerative lesions far from the initial site of infection (Reithinger *et al.,* 2007).

The most prevalent type of leishmaniasis is called LCL and is also referred to as "Oriental sore." Typical erythema usually characterizes lesions when an infected sandfly has bitten the host (Figure 1). An erythema turned into papules, which ultimately turned into skin ulcers. Most lesions are limited to and occur on exposed body parts including the face, arms, and legs (Chappuis *et al.,* 2007), this lesion grows with time. The incubation period for LCL lesions can run from a few days to many months, and LCL lesions vary in size, clinical presentations, and duration to cure (Reithinger *et al.,* 2007).

Particularly in people who are immunosuppressed, cutaneous leishmaniasis can spread and become diffuse (diffuse cutaneous leishmaniasis). This condition cannot resolve on its own and might last for years. Infected individuals with the human immunodeficiency virus (HIV) are especially vulnerable. Leishmaniasis recidivans, in which small nodules form around a healed scar, and post-kalaazar dermal leishmaniasis, in which broad cutaneous lesions appear after a visceral infection, are two more rare forms of cutaneous disease (Markle and Makhoul, 2004).

LCL is typically brought on by *Leishmania major*, *Leishmania tropica*, and *Leishmania aethiopica* in the Old World (regions of North and East Africa, Central Asia, and the Middle East). The species that cause the various forms of CL in the New World (i.e., Central and South America, the USA, and Mexico) include *Leishmania mexicana*, *Leishmania venezuelensis*, *Leishmania amazonensis*, *Leishmania braziliensis*, *Leishmania panamensis*, *Leishmania guyanensis*, and *Leishmania peruviana* (Reithinger *et al.,* 2007).

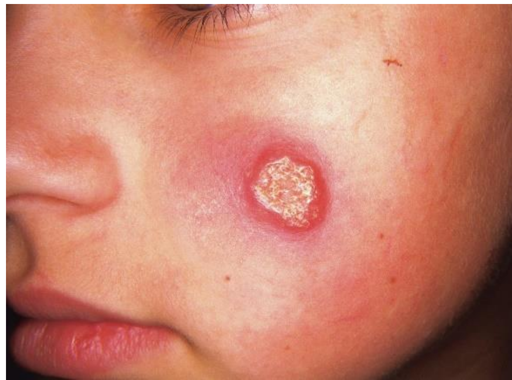


Figure 1: Cutaneous leishmaniasis (Altmeyer, 2022).

2.2 Mucocutaneous leishmaniasis

Mucocutaneous leishmaniasis (MCL), or disfiguring mucosal lesions, are caused by the lymphatic or haematogenous spread of parasites to oropharyngeal mucosal locations. *Leishmania aethiopica* (rare) is the causative agent of MCL in the Old World, whereas *Leishmania braziliensis, Leishmania guyanensis*, *Leishmania mexicana, Leishmania amazonensis*, and *Leishmania panamensis* are the causative agents in the New World. MCL brought on by *Leishmania braziliensis* and *Leishmania panamensis* is marked by severe facial mutilation, significant damage of the naso-oral and pharyngeal cavities, and horrifying disfiguring lesions (Figure 2) Another name for MCL is Espundia. After the initial oriental sore appears to have fully healed, MCL may develop into metastatic lesions on the buccal or nasal mucosa (Chappuis *et al.,* 2007, Reithinger *et al.,* 2007).

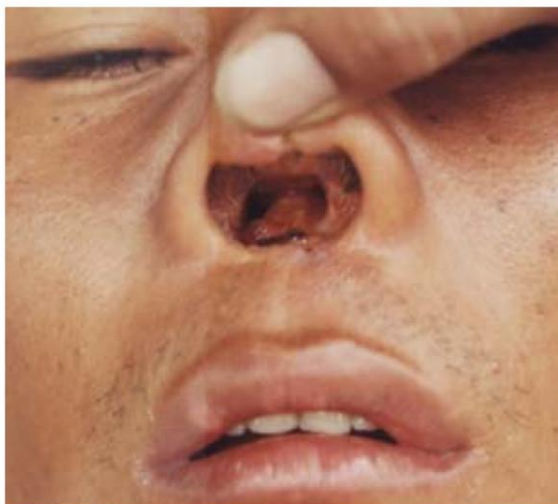


Figure 2: Mucocutaneous leishmaniasis (Calvopiña *et al.,* 2006).

2.3 Visceral Leishmaniasis

Human Visceral Leishmaniasis **(**VL) caused by *Leishmania donovani* or *Leishmania infantum* is a severe disease characterized by the parasites spreading generally to the reticuloendothelial system, such as spleen, liver and bone marrow (Kumar, 2013). The *Leishmania donovani* complex, which includes *Leishmania infantum* in Europe, North Africa, and Latin America and *Leishmania donovani* in East Africa and the Indian subcontinent, is what causes VL, the most dreaded and devastating amongst the various forms of leishmaniasis, a deadly systemic disease if left untreated (Lukeš *et al.,* 2007, Mauricio *et al.,* 2000).

There are two forms of VL, zoonotic and anthroponotic, which differ in how they are spread. Zoonotic VL is spread from animal to vector to human, and anthroponotic VL is spread from human to vector to human. In the former, dogs are the major host species while humans only serve occasionally hosts for the parasite (Alvar *et al.,* 2004). While anthroponotic VL is found in locations where *L. donovani* is transmitted, zoonotic VL is found in areas where *L. infantum* is present. After therapy, post kala-azar dermal leishmaniasis (PKDL), an East African and Indian subcontinent complication of VL characterized by a macular, maculo-papular, or nodular rash, is frequently seen in Sudan but less frequently in other East African nations (Zijlstra, *et al.,* 2003).

In regions where *L. infantum* is endemic, it can also happen to immunocompromised people. Because the nodular lesions of PKDL cases contain a large number of parasites, these cases are

thought to serve as a reservoir for anthroponotic VL in between epidemic cycles (Addy *et al.,*1992).Patients with VL display symptoms and indicators of chronic systemic infection, such as fever, weakness, fatigue, lack of appetite, and weight loss, as well as parasitic invasion of the blood and reticulo-endothelial system, or the general phagocytic system, such as enlargement of the lymph nodes, spleen, and liver (Figure 3) (Chappuis *et al.,* 2007).

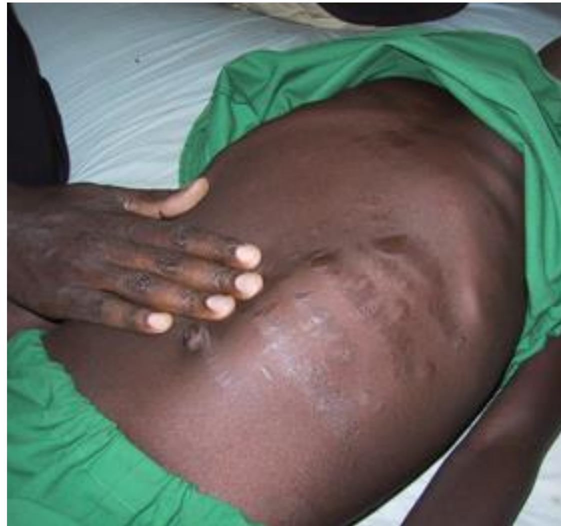


Figure 3: Person affected with visceral leishmaniasis (WHO,2016).

2.4 Etiology of leishmaniasis

2.4.1 Vectors

Leishmaniasis is spread by the sand fly, which serves as its vector. The subfamily Phlebotominae includes sand flies. There are between 70 and 80 species that are known to spread the illness (CDC, 2013). *Phlebotomus* and *Lutzomyia* sand flies transmit the disease in the Old World and new world, respectively. The tropics and subtropics are home to a large population of phlebotomine sand flies. The favored habitats and breeding locations of these species differ greatly from one another (EL Sawaf *et al.,* 2016).

There are more than 30 species of *Phlebotomus* known to transmit the disease, from which *Phlebotomus orientalis*, *Phlebotomus martini*, and *Phlebotomus celiae* have been confirmed to be

present in Ethiopia (Leta *et al.,* 2014, Gebresilassie *et al.,* 2015). Adult phlebotomine sand flies can be recognized by their very small size (usually less than 5mm long), hairy appearance, large black eyes and long and stilt-like legs (Service, 2012).

2.4.2 Reservoir hosts

If a host can pass the parasite onto the next stage, which is the vector, then it serves as a reservoir host for the parasite. Therefore, one requirement for any mammal to be a leishmania reservoir is that it has to be infected host for the sand fly to feed on (Roque and Jansen, 2014). In various regions of the world, a number of species of wild, domesticated, and synanthropic mammals have been found to be hosts or reservoirs for *Leishmania* spp. Rats, rock Hyraxes, Mongoose, Dogs, Cats, Foxes, Jackals, Bats, Monkeys, Armadillos, and other domestic animals are some of the multi-host reservoirs that help to keep leishmaniasis transmitted in many places (Rohousova *et al.,* 2015, Dereure *et al.,* 2000).

2.5 Life cycle of the Parasites

Amastigotes in vertebrate hosts and promastigotes in invertebrate hosts are the two primary morphological types of the parasites. promastigotes are extracellular, elongated, motile cells with flagella that are between 10 µm and 20 µm in size. Amastigotes, on the other hand, range in size from 2 to 5µm, are intracellular, spherical to oval, do not have flagella, and are not mobile (Dawit *et al.,* 2013).

The sand fly's proboscis releases the infectious stage (infective promastigotes) during a blood meal (Figure 4), which initiates the life cycle. Because the adult female sandfly, unlike the male, is a bloodsucker, amastigotes are consumed along with the blood meal when it bites an infected person or, in the case of zoonotic disease, an infected animal zoonotic reservoir (CDC,2013). Some of the flagellates that enter the bloodstream after being bitten by a sandfly are killed, while others penetrate the reticuloendothelial system cells (Handman,2000). After infection, parasites enter macrophages after spending an undetermined amount of time inside neutrophils (Hurrell *et al.,* 2013). The amastigotes that are phagocytosed by the host's macrophages and other mononuclear phagocytic cells undergo continuous binary division and change from promastigotes to amastigotes. The process of reproduction continues until the host cell becomes overloaded with parasites and bursts, releasing the amastigotes into the bloodstream (Handman, 2000, Dawit *et al.,*

2013). Additional macrophages take up the released amastigotes, and the process of amastigote proliferation is then repeated. All organs that contain macrophages afterwards get infected, including the lymph nodes, liver, spleen, and bone marrow (Handman, 2000).

Amastigotes in the sandfly mature into promastigotes and move to the midgut (Leishmania) or the hindgut (Viannia), where they change into flagellated promastigotes and divide. When the sandfly injects the extracellular promastigotes into the host's skin during a blood meal, some of them convert into metacyclic-promastigotes, the infectious form. From there, they move to the proboscis and the cycle is restarted (CDC, 2013).

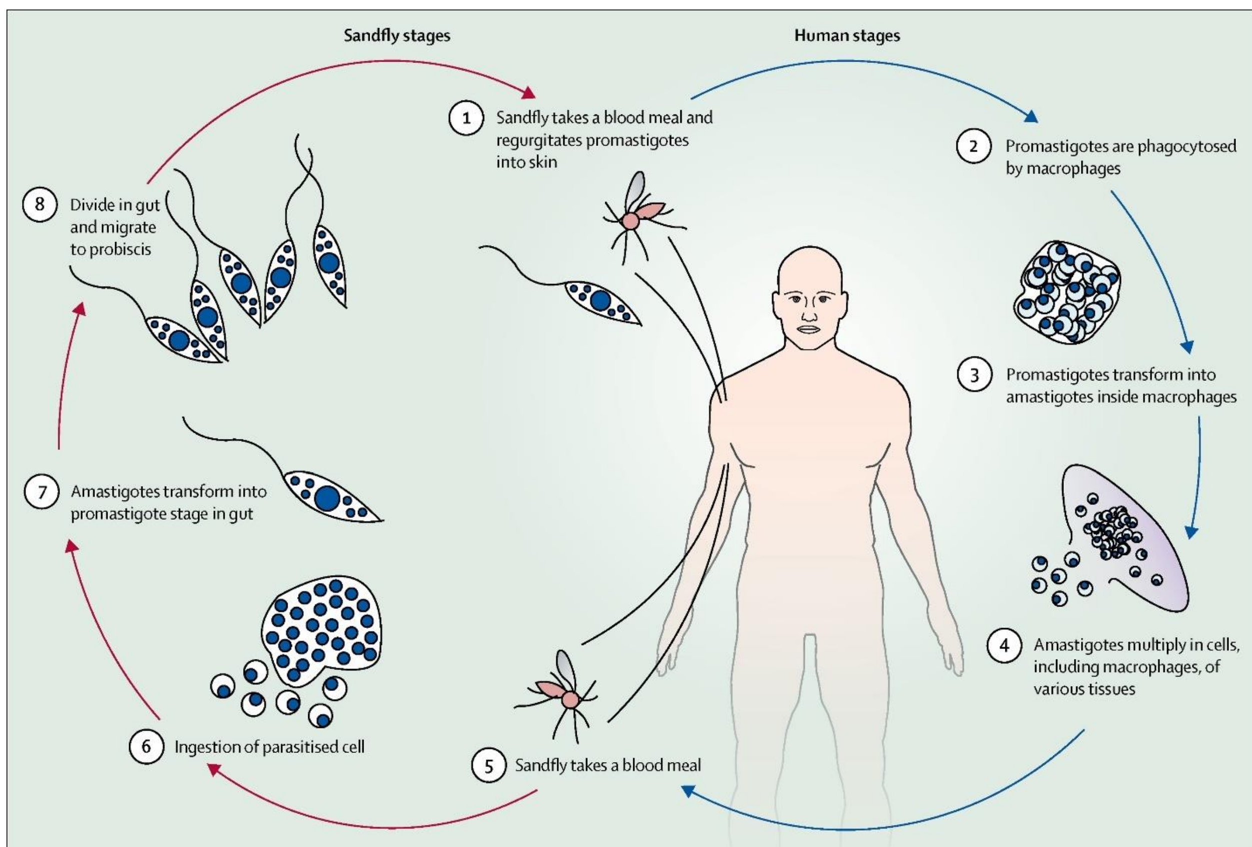


Figure 4: Life cycle of *Leishmania* parasites (Burza *et al.,* 2018).

2.6 Epidemiology of Leishmaniasis

Leishmaniasis is brought on by more than 20 *Leishmania* parasites. Leishmaniasis is spread by about 30 species of sandflies from the genera *Lutzomyia* and *Phlebotomus*, respectively, in the new and old worlds. Humans contract the parasite Leishmania from sandfly bites. Rarely, VL can be spread by intravenous drug use, organ transplantation, and blood transfusion (Georgiadou *et al.,* 2015).

An estimated 700 000 to 1 million new cases of leishmaniasis occur annually. Only a small fraction of those infected by parasites causing leishmaniasis will eventually develop the disease. with approximately 95% of cases occurring in the Americas, the Mediterranean basin, the Middle East, Central Asia, Brazil, China, Ethiopia, India, Kenya, Nepal, Somalia, and Sudan (WHO, 2023)

With a frequency of 19% overall, cutaneous leishmaniasis and VL are rising health issues that are endemic to Ethiopia. Except for Harari, every region in Ethiopia has documented cases of visceral leishmaniasis (Assefa, 2018). These include Metema and Humera in northern Ethiopia; the Weyto River Valley, the Omo lowlands, and the Aba Roba emphasis in South Nations, Nationalities and Peoples; the Moyale area and Genale river basin in Oromia; Afder and Liban in Somalia; and the Awash Valley in Afar. A further estimate places the annual incidence of VL cases between 2,000 and 4,500. The average age of those who get visceral leishmaniasis is 23. Conducive habitats, as those populated by Red Acacia and Balanites trees in the north and termite hills in the south, are linked to the increased prevalence and incidence of VL (Assefa, 2018).

With a prevalence of 0.01 to 10.8% active infections, cutaneous leishmaniasis in Ethiopia is a disease that mostly affects highland and lowland residents and poses a risk to 30 million people's lives. Dembidollo, Dessie, Wolega, Shewa, Meta Abo, Kutaber, Ochollo, Silti, Addis Abeba, and SaesieTsaeda-Emba are the regions that reported having active CL cases in Ethiopia. Furthermore, the disease primarily affects children and young people and has no preference for sex (Henten et al., 2018).

2.7 Treatment of leishmaniasis

The preferred first-line and second-line treatments vary depending on the type of disease and are frequently determined by regional practice in light of what is currently most available and effective. There are several types of medications for different forms of leishmaniasis (Natarajan *et al*., 2014)

2.7.1 Traditional treatment

Euphorbia abyssinica and *Brucea antidysenterica* (Wubetu *et al.,* 2017), *Ficusvasta forssk, Rehamnus prinoides*, and *Rumex abyssinicus* (Limenih *et al.,*2015), *Clematis hirsute* (Teklehaymanot *et al.,*2007), *Phytolacca dodecandra* (Suleman and Alemu, 2012), *Commicarpus pedunculosus* (Teklay *et al.,*2013) and *Datura stramonium* (Araya *et al.*2015*)* are some of the plants used as a traditional medicine as treatment for leishmaniasis in Ethiopia.

2.7.2 Chemotherapy

Pentavalent antimony

First-line medications for treating the neglected tropical illness leishmaniasis include pentavalent antimonials. These outdated medications' significant adverse effects, requirement for daily parenteral administration, and therapeutic failures, however, place restrictions on their use (Grumezescu, 2017). Leishmaniasis has been treated with two main pentavalent antimonial compounds since 1940: meglumine antimoniate and sodium stibogluconate, complexes of Sb^{5+} with N-methyl-D-glucamine and sodium gluconate, respectively (Frézard *et al.*, 2009).

Pentavalent antimonials have some drawbacks despite their effectiveness as antileishmanial agents. Musculoskeletal discomfort, , pancreatitis, hepatitis, gastrointestinal disturbances, and headache are the most often reported adverse effects following pentavalent antimonials treatment. Rarely, after receiving antimonial medication, patients may experience hemolytic anemia, renal impairment, shock, or sudden death (Oliveira *et al*., 2011).

Amphotericin B

Amphotericin B, an antifungal medication, is a polyene antibiotic that was first discovered from the bacteria species *Streptomyces nodosus* in 1955 (Nagle *et al.*, 2014). The drug's antifungal activities and anti-leishmanial effects are assumed to have a similar mechanism. As an ergosterol precursor in the parasite lanosterol, it binds to it permanently, causing the parasite cell membranes to rupture. As soon as the parasite membrane is broken, electrolytes begin to flow out through holes, which lead to the parasite's eventual demise (Sundar and Chakravarty, 2010).

One of the main problems with AmB use is its high cost. Mild renal impairment and urticarial rash were among the side effects of AmB that went away with management. Because of its smaller toxicity range, the treatment is well tolerated. The drug's potential for treating leishmaniasis has been demonstrated by its remarkable cure rates. Also, because the term of this medication is very short—just six days—it is far more convenient for patients (Solomon *et al.*, 2010).

Miltefosine

It was the first oral drug registered for the treatments of patients with visceral leishmaniasis and more recently for cutaneous leishmaniasis (Croft and Engel, 2006) and PKDL with greater accessibility and lower toxicity than pentavalent antimonials (Nagle *et al.*, 2014). The recommended oral dose is 50 mg per day for 28 days for patients weighing below 25 kg and 100 mg per day for 28 days for adults weighing above 25 kg while 2.5 mg/kg daily for 28 days for children <12 years of ages (Boelaert and Sundar, 2013). High product cost, inconsistent supply and lack of consolidated coordination among stakeholders in the public and private sectors to ensure miltefosine supply were the key problems that hinder the accessibility of miltefosine to the patients in need (Boelaert and Sundar, 2013).

The main side effects of this medication are hepatotoxicity, renal failure, and gastrointestinal problems like mild to moderate diarrhea and vomiting. It is prohibited during pregnancy due to its teratogenic effects. Miltefosine has a long half-life, so women of childbearing age should closely follow contraception during medication and for three months after therapy (Boelaert and Sundar, 2013). Miltefosine monotherapy is susceptible to resistance due to the difficulties of adhering to a twice-daily dosing schedule, long-term therapy, a long half-life, accessibility issues, and high cost (Sunyoto *et al.*, 2018).

Paromomycin

The anti-leishmanial activity of the paromomycin was discovered in 1960s but introduced as anti-leishmanial drug against visceral leishmaniasis in 2006 when administered parenterally (Boelaert and Sundar, 2013) and cutaneous leishmaniasis when administered topically (Sykes, 2013). Unlike other ant-leishmanial drugs it is affordable, and it is also used as first line alternative in case of resistance to other classical anti-leishmanial drugs (Wiwanitkit,2012). Because of this drug's unsatisfactory efficacy in treating VL patients in Africa, monotherapy is not advised. However, a combination regimen of paromomycin and pentavalent antimonials for 17 days was a more effective and safer choice (Boelaert and Sundar, 2013).

Pentamidine

Pentamidine is an aromatic diamidine derivative having significant anti-parasitic activity. It was synthesized in the early 1940s. It has been widely utilized to treat *Pneumocystis jirovenci* and cutaneous leishmaniasis in addition to trypanosomiasis and visceral leishmaniasis (Gadelha, 2015). Early in the 1980s, it was also applied to the treatment of refractory VL in India. However, due to its decreased efficacy and significant toxicities, including insulin dependent diabetic mellitus (Nagle *et al.*, 2014), it was withdrawn from use and replaced with amphotericin B. Along with sterile abscess and indurations at the injection site, anaphylactic shock (hypotension), syncope, dizziness, myalgia, nausea, and vomiting are other frequent side effects (Sundar and Singh, 2016). The most prevalent side effects of tis medication include vesicles, skin irritation, erythema, local discomfort and ototoxicity (Nassif, *et al.*, 2017).

2.7.3 Physical therapy

Cryotherapy

In order to treat Old World CL brought on by *L. tropica, L. aethiopica*, and *L. infantum*, liquid nitrogen administration directly to CL lesions has been utilized once or multiple times, up to five times, every 3–7 days, or in conjunction with intralesional antimony for *L. major* (Desta *et al.*,2005). This therapy acts destroying infected tissues and showed effectiveness specially

combined with meglumine antimoniate. The side effects of this therapy include erythema, hypo- or hyperpigmentation secondary infections and burning (Nassif, *et al.*, 2017).

Heat therapy

Old and New World CL lesions can be heated at 50 °C for 30 seconds up to three times to expedite clearance. HIV-positive patients who do not respond to antimonial therapy have also found this treatment to be beneficial against CL. The outcome is comparable to systemic or intralesional antimony therapy. (Prasad *et al.*, 2011). Hyperpigmentation and secondary infections are side effects reported for this therapy (Nassif, *et al.*, 2017).

2.8 Overview of Malva parviflora

A perennial herbaceous plant belonging to the Malvaceae family, *Malva parviflora*, is mostly found in tropical, subtropical, and temperate regions of Asia, Africa, and Europe (Naser *et al.,* 2022). The Xhosa people of South Africa use the leaf of this plant to heal boils (Grierson and Afolayan, 1999) and inflamed, purulent wounds (Watt and Breyer Brandwijk, 1962). Its leaves are often used to make a poultice to treat swellings and wounds. The plant is used in Lesotho as part of a lotion to cure bruises and broken limbs, and herbalists clean wounds and sores with dried powder or an infusion derived from the plant's leaves and roots (Shale *et al.,* 1999).

A place in France called La Réunion uses a heated leaf poultice to cure wounds and swellings, and leaf tea is consumed as a nervine tonic, taenicide, and treatment for heavy menstruation (Sharma and Ali 1999). According to Ishtiaq *et al.* (2012), bronchitis and a dry, irritating cough are treated with leaf tea. In addition to being used as a hair rinse to get rid of dandruff and soften hair, the leaves' decoction is also made into a tea that is used to cleanse the mother's system after childbirth (Mukul 2012). Seeds are demulcent, used to treat cough and ulcers in the bladder (Sharma and Ali 1999). In Ethiopia, *M. parviflora*'s fresh root bark is chopped into small pieces and applied on the damaged skin surfaces to treat furuncles, carbuncles, wound infections and other related ailments (Ododo *et al.,* 2016).

In Ethiopia, *M. parviflora* is also referred to as Uka (in Wolaita). It has a wide range of naturalized areas outside of its native Asia, Northern Africa, and Europe. In waste areas and agricultural farmlands, the plant frequently grows up to 40 inches. a herb plant known as *M. parviflora* can be

annual, biennial, or perennial. Dark green leaves, blooms that appear at the bases of the leaf stalks, and fruits that are divided into lobes are all features of this plant (Ododo *et al.,* 2016).

Figure 5: *Malva parviflora* (Chisale, 2016).

M. Parviflora (Figure 5) is a member of the Malvaceae family. The plants of this family play a significant role in the treatment of stomach and bowel irritations as well as coughs, throat infections, and other bronchial issues. Emollient in nature, the flowers and leaves are used to soothe sensitive skin. To lessen swelling and pull out toxins, it is placed as a poultice. The laxative properties of the leaves assist in reducing intestinal irritability. It is effective when combined with eucalyptus as a treatment for cough and other chest conditions (Benzie and Galor, 2011).

The root of *M. parviflora* is used to treat wounds and asthma in Ethiopia (Abate and Demissew,1989).Additionally, *M. parviflora* has been utilized to treat different stomach issues, fever, ulcers, and headaches. In order to get rid of dandruff and soften the hair, a decoction of roots or leaves has also been applied as a hair rinse (Duncanson, 2012, Sobek, 2012, Abdel-Galil, 2010 and Garret, 2011).

M. parviflora had an inhibitory impact on some fungi but was ineffective against some kinds of bacteria, according to Grierson and Afolayan's research from 1999. In contrast, Shale *et al*. (1999) discovered that the hexane and methanol extracts of the roots had antibacterial action but that the methanol leaf extract had very moderate antibacterial activity (Afolayan *et al.,*2010).

Further research revealed that entire *M. parviflora* extracts in hexane, methanol, and water demonstrated potent antibacterial properties against a variety of Gram positive and Gram negative bacteria (Arellanes *et al.,* 2013). Additionally, the whole herb's hexane extract demonstrated anti-inflammatory activity (Abdel-Galil *et al.,* 2010). Investigations of the entire herb of *M. parviflora*'s wound-healing abilities were also conducted (Lans *et al.,* 2007) Traditional herbalists all around the world have employed herbal plants or botanical medicines to treat and prevent liver disease (Watson and Preedy, 2012).

There are numerous pharmacological effects of *M. parviflora* in treating various illnesses. The control of edema and wounds is helped by leaves. Broken limbs and bruises can be effectively treated using a lotion made from leaves. Antioxidant, anti-aging, anti-mutagenic, antibacterial, anti-irritant, antidiabetic, neuroprotective, antifungal, anti-ulcerogenic, hepatoprotective, and analgesic properties are all present in *M. parviflora* (Naser *et al.,* 2022).

3. MATERIAL AND METHODS

3.1. Plant material collection, authentication and extraction

3.1.1. Plant Material collection

Fresh leaves and roots of *Malva parviflora*, were collected (Figure 6) from Ochollo village located in Chencha woreda, at latitude 6°25'09.0'N and longitude 37°48'00.8"E, Gamo zone. The Gamo zone is located in the southern part of Ethiopia, approximately 520 km southwest of Addis Ababa. The village is situated in the Rift Valley above the west shore of Lake Abaya, one of the largest lakes in Ethiopia. The fresh leaves and roots were carefully harvested from areas where *Malva parviflora* was commonly found and transported to the laboratory for further analysis by wrapping them carefully in newspaper and secured with a plastic bag.

Authentication

The plant material was authenticated by Mr. Melaku Wondafrash, National Herbarium, Department of Biology, College of Natural and Computational Sciences, Addis Ababa University (AAU), where a botanical specimen was deposited for future reference with the code BS007.

Figure 6. The collection of *Malva parviflora* leaves and roots (Photo credit: Betelhem Sirak).

3.1.2 Plant material Extraction

Maceration

Fresh leaves (500g) and roots (400g) of *M. parviflora* were rinsed with water to remove dirt. The fresh plant materials were chopped into small pieces and then macerated with sufficient amount of 100% methanol at room temperature for 24 h with continuous agitation and was filtered first with sterile gauze and then using Whatman no. 1 filter paper. This was repeated twice and the combined filtrate was concentrated in a Rotavapor at 40°C. The dried extract was labeled as MP-L (*Malva parviflora* leaves) and MP-R (*Malva parviflora* Roots) respectively, and then they were transferred to an amber-colored bottle and stored in a refrigerator until use.

3.1.3. Promastigote selection and culture

Clinical isolates of *L. donovani* and *L. aethiopica* strains were obtained from leishmaniasis Research and Diagnostic Laboratory (LRDL) in Tikur Anbessa specialized hospital, Department of Immunology, Microbiology and Parasitology, School of medicine, Addis Ababa University. The isolates were kept in transparent glass vials with enough NNN media to keep the parasites growing until they are ready for subculturing.

The strains were checked under a microscope if they had active motility, cylindrical shape of a promastigote, formed no cluster around each other and enough amount of the parasite to reproduce. After the strongest suitable strains were chosen based on how susceptible they were to liquid media, they were subcultured into two new culture flasks containing NNN media and Locke's solution for better growth. To check if the parasites were ready for the assay, a small drop of the parasite was added on a hemocytometer to make sure the count is from 1×10^6 promastigote/ml to 3×10^6 promastigote/ml.

Parasites at the logarithmic stage were inoculated into liquid media for the assay. The *L. aethiopica* and *L. donovani* isolates were grown in tissue culture flasks containing RPMI 1640 medium supplemented with fetal Bovine serum (FBS) following method described by Tariku *et al,* 2010. To promote healthy development, the culture was closely watched every day. then a small drop of the parasite was added on a hemocytometer (counting chamber) to make sure that the total count of parasites is from 1×10^6 promastigote/ml to 3×10^6 promastigote/ml. Later, promastigotes were

extracted from the liquid media that reached their logarithmic stage for each anti-promastigotes biological assay.

L. donovani isolates were acquired from a patient that was from Gondar Amhara region, labelled VL Gr 1140 while *L. aethiopica* strains were acquired from a patient that was CL 584/17, from Tiya woreda, Gurage zone.

The human red blood cells used in the cytotoxicity test were obtained from Tikur Anbessa Leishmaniasis Research and Diagnostic Laboratory given by a healthy 24year old volunteer with no underlying chronic disease.

Comment note ; . a penicillin strip was added during the media preparation to avoid contamination.

3.2 Biological Assay

3.2.1 Anti-promastigotes assay

This assay was performed in a triplicate both for CL and VL using two different plates but using the process as described by Tariku *et al.,*2010.

In a 96 well microtiter plate 120 µl of complete media was then added on the row of the first wells of every row. Eighty microliter of the plant leaf extract was added to the first three wells and Eighty microliter of root extract on the next three wells and then 80 µl of AmB was added on the last three wells of the plate. Two hundred microliter of complete media was added on an entire column of the plate. Each well containing the plant extract and the positive control was serially diluted until the final test concentrations is 100 µl. One column well of the plate was left empty while another column well was the negative control containing the media and the parasite alone. Empty wells and wells containing complete medium were used to monitor background fluorescence activity of Alamar blue solution and the average value was subtracted from every well (Abeje *et al.,* 2014). Then 100 µl of suspensions containing the promastigotes in a logarithmic phase was added to each well to make a total of 200 µl. After the initial 68 hrs. of incubation, 20 µl of fluorochrome Alamar blue solution was added to each well. Then after 4hrs the fluorescence intensity was measured using a flouroskan ascent after a total incubation time of 72 hrs. the result obtained from this reading was used to calculate the percentage of inhibition and the median inhibitory concentration (IC_{50}).

The percentage of inhibition of antipromastigote activity of the extract was expressed in by this formula (Tariku *et al.,* 2010),

$$\% \, of \, inhibition = \left(100 - \left(\frac{\text{Absorbance of each well} - \text{Average of blank well}}{\text{Average of negative control} - \text{Average of blank well}}\right)\right) * 100$$

3.2.2 Hemolysis assay

The hemolytic effects of the methanol extract of *M. parviflora* leaves and roots were conducted in duplicate as described by Abeje *et al*., 2014 and Zohra and Fawzia, 2014. First 1000 ml of distilled water was added on phosphate buffer powder packet to make phosphate buffer saline (PBS). Then 4 ml of blood was added to an EDTA tube using a syringe.

A 50 ml falcon tube was acquired to add 48 ml of PBS and 2 ml of human blood and put into a cold centrifuge at 4°C and centrifuged at 3500 rpm for 10 minutes. The supernatant was discarded using pipettes. The resulting pellets was mixed with PBS solution and centrifuged at the same speed again. This process was repeated three times until the pellet volume reached 1ml. next, 49 ml of pbs was added on the 1ml pellet containing RBC and was mixed together. One hundred microliter of the red blood cell suspension was mixed with 100 µl of the leaf and root extracts (1000 – 7.8125µg/ml) in an Eppendorf tube. Next 2% triton X-114 and 1% DMSO were used as positive and negative controls respectively. The next step was to incubate the tube at 37°C for 2 hours, then centrifuged at 1000 rpm for 10 minutes. Seventy-five microliter of the supernatant was poured on to a 96 well plate and then read spectrophotometrically using Victor^3 Multilabel reader at 530nm wavelength.The median cytotoxic concentration (CC_{50}) to monocyte from a human blood was obtained directly from linear equation of dose-response curves.

The percentage of haemolytic effects was expressed by the formula below (Zohra and Fawzia, 2014).

$$hemolysis(\%) = \frac{\text{Absorbance of test drug} - \text{Absorbance of blank}}{\text{Absorbance of positive control} - \text{Absorbance of blank}} * 100$$

3.3 Selectivity index/ SI

The selectivity index (SI) of each extract was determined from their CC_{50} against red blood cells and their corresponding IC_{50} of against the *Leishmania* parasites. The selectivity of the plant extracts and the standard drug in selectively killing parasites as opposed to mammalian cells was assessed using the following formula (Woldemichael *et al*., 2015).

$$Selectivity\ index\ = \frac{\text{CC50 of red blood cells}}{\text{IC50 of anti promastigote assay}}$$

3.4 Data Analysis

Antipromastigote IC_{50} value was calculated from sigmoidal dose response curve of the percentage of inhibition. The cytotoxicity was calculated from the sigmoidal dose response curve of the percentage of cell viability. All experimental data will be expressed as mean values ±SEM and was subjected to bio statistical analysis by GraphPad Prism 9.0.0.121 (GraphPad Software, Inc., CA, USA).

3.5 Ethical clearance

All procedures conducted in this research work were approved by the Institutional Review Board, College of Health Sciences, Addis Ababa University with the protocol number 092/21/SOP dated January 2022 (ANNEX 1).

4. RESULTS

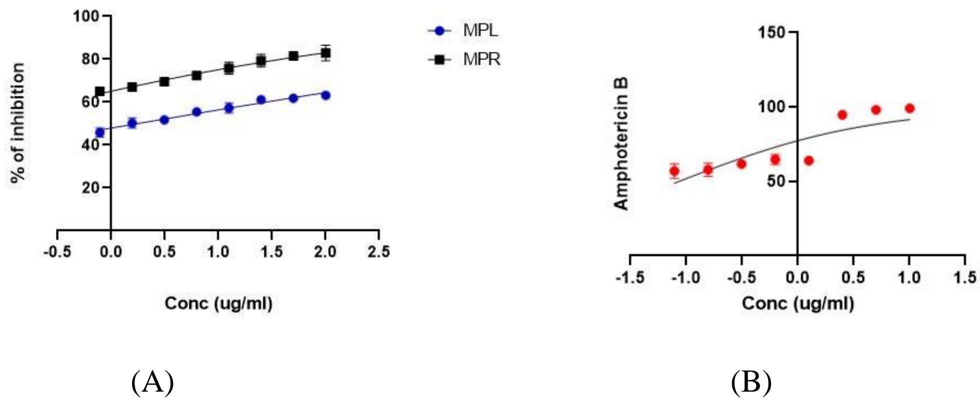
4.1. Preliminary Anti-leishmanial Activity Screening Results

The preliminary anti-leishmanial activity study of methanol extracts of leaf and root of *M. Parviflora* against logarithmic growth phases of promastigote of *L. aethiopica* and *L. donovani* were determined and expressed as percentage of inhibition. The leaf extract showed a percentage of growth inhibition of 55.89 ± 2.19% against *L. aethiopica* and 79.84 ± 1.57% against *L. donovani.* The root extract of *M. parviflora* exhibited a percentage of inhibition of 74.21 ± 2.39% and 78.81 ±1.88% against *L. aethiopica* and *L. donovani* respectively (Table 1). The standard drug used, AmB, exhibited a percentage of inhibition of 74.73 ± 6.71 and 86.81± 4.56 against *L. aethiopica* and *L. donovani* respectively. Figure 7 and Figure 8 illustrate the increasing sigmoid curve of the percentage of inhibition of the leaf and root extracts at different concentrations compared with AmB against both *Leishmania* promastigotes.

Table 1. Percentage inhibition of the extracts against *Leishmania* promastigotes.

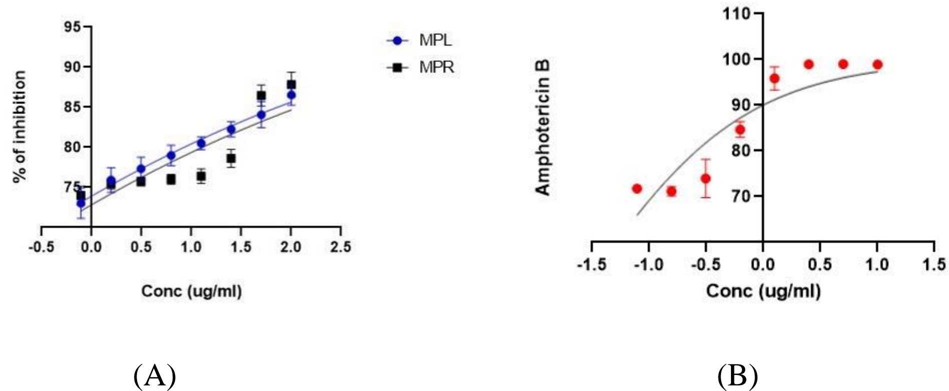
Types of extract and its conc. (µg/ml)	**% Inhibition of extracts against *Leishmania* promastigotes**	
	Against *L. aethiopica*	**Against *L. donovani***
MPL 100µg/ml	55.89 ± 2.19	79.84 ± 1.57
MPR 100µg/ml	74.21 ± 2.39	78.81 ±1.88
AmB 10 µg/ml	74.73 ± 6.71	86.81± 4.56

The values are expressed as mean ± SEM, MPL: leaf extracts of *M. parviflora.* MPR: Root extracts of *M. parviflora*, AmB: Amphotericin B, 100µg/ml indicates the concentration of the extracts; 10 µg/ml indicates the concentration of AmB



(A) (B)

Figure 7: Graphical representation of percentage inhibition of the extracts (A): Leaf and root extracts of *M. parviflora* against *L. aethiopica.* (B): Amphotericin B against *L. aethiopica.* MPL: leaf extracts of *M. parviflora.* MPR: Root extracts of *M. parviflora.* Conc (ug/ml): Concentration is expressed in log x.



(A) (B)

Figure 8: Graphical representation of percentage inhibition of the extracts: (A): Leaf and root extracts of *M. parviflora* against *L. donovani.* (B): Amphotericin B against *L. donovani.* MPL: leaf extracts of *M. parviflora.* MPR: Root extracts of *M. parviflora.* Conc (ug/ml): Concentration is expressed in log x.

4.2. Anti-promastigote Assay Results

The IC_{50} of methanol extracts of leaf and root of *M. parviflora* against promastigotes of *L. aethiopica* and *L. donovani* isolates were determined as indicated in the table below. The leaf extract showed an IC_{50} of 1.73 and 0.002 against *L. aethiopica* and *L. donovani* respectively. The

root extract showed an IC_{50} of 0.05 and 0.002 against *L. aethiopica* and *L. donovani* respectively while the IC_{50} values of the positive control, amphotericin B, against *L. aethiopica* and *L. donovani* are 0.09 and 0.03 respectively (Table 2).

Table 2. IC_{50} of the *M. parviflora* against *Leishmania* promastigotes.

Types of extract used	**Against *L. aethiopica***		**Against *L. donovani***	
	IC_{50} (µg/ml) (95% CI)	**R^2**	**IC_{50} (µg/ml) (95% CI)**	**R^2**
MPL	1.73 (1.2 – 2.3)	0.90	0.002 (0.0004-0.004)	0.92
MPR	0.05 (0.02 – 0.09)	0.92	0.002 (0.0003 - 0.05)	0.72
AMB	0.09 (0.036 – 0.15)	0.73	0.03 (0.012-0.04)	0.84

The values are expressed as at 95% confidence interval, MPL: leaf extracts of *M. parviflora.* MPR: Root extracts of *M. parviflora.*, AmB: Amphotericin B, R^2: is a measurement of fitness (regression coefficient). 95% CI: 95% confidence interval; Differences with *P* value < 0.05 (*) were considered significant.

4.3. Hemolysis Assay Results

The methanol extract of leaf and root of *M. parviflora* against human red blood cells were determined and Triton X-114 was used as a positive control. As seen in Table 3 below, the leaf extract of *M. parviflora* showed 21.05 ± 11.54% hemolysis against human red blood cells, while the root extract of *M. parviflora* showed 0 ± 26.54% hemolysis against red blood cells. Triton X-114, the positive control, had a 100% hemolysis percentage against human red blood cells (Table 3). Figure 9 illustrates an increasing curve that shows the percentage of hemolysis of the extracts at different concentrations up to 1000µg/ml.

Comment note: Triton was used instead of AmB as it does kill 100% of RBC's but since AmB is a drug approved for use by the food and drug authorities there was no need to

prove its ability to kill RBC's or compare it to the extracts and therefore wasn't included in hemolysis, cytotoxicity and selectivity index.

Table 3. Percentage of hemolysis of the extracts against human red blood cells.

Types of extract and its conc. (µg/ml)	Percentage of hemolysis of extracts against human red blood cells
MPL 1000µg/ml	89.47 ± 1.86
MPR 1000µg/ml	18.42 ± 3.72
Triton X-114	100

The values are expressed as mean ± SEM, MPL: leaf extracts of *M. parviflora.* MPR: Root extracts of *M. parviflora.*, Triton X-114: as a positive control, 1000µg/ml indicates the concentration of the extracts.

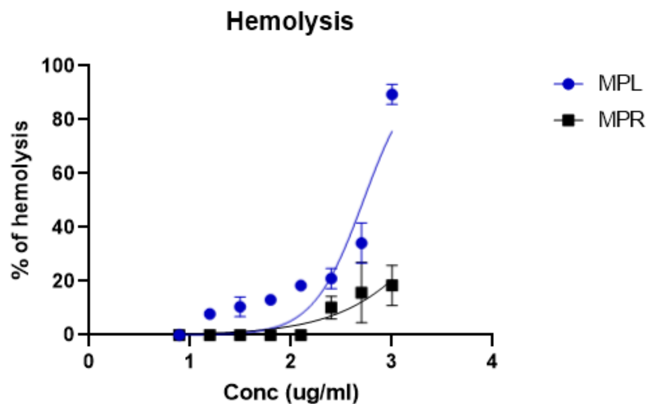


Figure 9. Graphical representation of percentage of hemolysis of the extracts MPL: leaf extracts of *M. parviflora.* MPR: Root extracts of *M. parviflora.* Conc (ug/ml): Concentration is expressed in log x.

4.4. Cytotoxicity effect result

Based on the percentage of hemolysis acquired, the cytotoxic effects (CC_{50}) of methanol extract of leaf and root extracts of *M. parviflora* were determined as described in Table 4. The leaf extracts have a CC_{50} value of 520.4 µg/ml while the root extracts have a CC_{50} value of >1000 µg/ml on red blood cells.

Table 4. CC_{50} result against red blood cells

Types of extracts	Against red blood cells	
	CC_{50} (µg/ml) (95% CI)	**R^2**
MPL	520.4 (390.5 – 675.9)	0.9
MPR	>1000	-

The values are expressed as mean at 95% confidence interval, MPL: leaf extracts of *M. parviflora.* MPR: Root extracts of *M. parviflora.*, Triton X-114: as a positive control, R^2: is a measurement of fitness (regression coefficient). 95% CI: 95% confidence interval; Differences with *P* value < 0.05 (*) were considered significant.

4.5. Selectivity index

The selectivity index of methanol extract of leaf and root of *M. parviflora* were determined as described in Table 5. The leaf extracts had a selective index of 300.8 against *L. aethiopica* and a selective index of 260,200 against *L. donovani* isolates. While the root extracts exhibited a selectivity index of >1000 (Table 5).

Table 5. Selectivity Index of the plant extracts

Types of extract	**Against red blood cells (CC_{50} / IC_{50})**	
	Against *L. aethiopica*	**Against *L. donovani***
MPL	300.8	260,000
MPR	>1000	>1000

The values are expressed as ratio of CC_{50} of red blood cells and IC_{50} of Leishmania promastigote, MPL: leaf extracts of *M. parviflora.* MPR: Root extracts of *M. parviflora.*

5. DISCUSSION

The *in vitro* promastigote parasites culture in cell free media is simple and reproducible (Gupta, 2011). This experiment has evaluated the effectiveness of methanol extracts of the leaf and root of *M. parviflora* against promastigotes of *L. aethiopica* and *L. donovani in vitro.*

The percent inhibition of the growth of promastigotes of *L. aethiopica* and *L. donovani* revealed by methanol extract of the leaf of *M. parviflora* was 55.89 ± 2.19% and 79.84 ± 1.57% (Table 1), respectively at concentrations of 100µg/ml. The anti-promastigote activity of *M. parviflora* leaves had a higher percentage of inhibition in *L. donovani* than in *L. aethiopica.* The percentage of growth inhibition of the promastigotes *L. aethiopica* and *L. donovani* shown by methanol extract of the root of *M. parviflora* was 74.21 ± 2.39% and 78.81 ±1.88% (Table 1) respectively, at concentrations of 100µg/ml.

The root extracts showed a significantly higher rate of inhibition against *L. aethiopica* than the leaf extracts of the plant and a slightly similar percentage of inhibition when compared to AmB (Figure 7). This shows that the root extracts have better inhibition against *L. aethiopica* isolates when compared with the leaf extracts. On the contrary, the root extracts of *M. parviflora* exhibited a percentage of inhibition against *L. donovani* that is a bit closer to the positive control and is less than the percentage of inhibition of the leaf extracts at 95% confidence interval and differences with P value < 0.05 (*) were considered significant (Figure 7). This indicates that the leaf extract is better suited at inhibiting *L. donovani* isolates when compared to the root extracts.

The leaf extracts of *M. parviflora* presented a higher percentage of inhibition against *L. donovani* isolates and a closer percentage of inhibition when compared to AmB making the leaf extracts effective by comparison. However, the leaf extracts displayed a lower percentage of inhibition against the clinical isolates of *L. aethiopica* when compared against the percentages of both *M. parviflora* root extract and AmB.

The antipromastigote activity of the extracts inhibiting the parasites was determined by the IC_{50} value. According to Ehata *et al.,* (2012), an IC_{50} value less than or equal to 5 µg/mL has strong activity, an IC_{50} between 5µg/mL and or equal to 20 µg/mL has good activity, an IC_{50} between 20 and/or equal to 30 µg/mL has mild activity, an IC_{50} between 30 and/or equal to 64µg/mL has poor activity and an IC_{50} greater than 64 µg/mL has no activity at all. By the standards of this scale, the

leaf extract has a good activity against *L. aethiopica* with an IC_{50} of 1.73 while it has a strong activity against isolates of *L. donovani* with an IC_{50} of 0.002 (Table 2). The leaf extract against *L. donovani* has a closer IC_{50} to the effects of AmB against *L. donovani*, 0.03, making it more effective against the parasite. On the other hand, the root extract has a strong activity against both promastigotes of *L. aethiopica* and *L. donovani* with an IC_{50} of 0.05 and 0.002 (Table 2) respectively. When compared with the leaf extract and the positive control, the root extract has stronger activity against *L. aethiopica* but the leaf extract has a stronger IC_{50} value than the root extract against *L. donovani.* Both the leaf and root extracts presented a higher IC_{50} value than the positive control against both promastigotes of *L. aethiopica* and *L. donovani.*

In this evaluation, the cytotoxic effect of methanol extracts of the leaf and root of *M. parviflora* against were assessed against human red blood cells *in vitro*. This was achieved by first calculating the percentage hemolysis of the extracts against human red blood cells. the leaf extract had a hemolysis percentage of 89.47 ± 1.86%, the root extract had 18.42 ± 3.72% and Triton X-114 had a hemolysis percentage of 100% (Table 3). This reveals that the leaf extract exhibits a slight hemolytic activity causing harm to only a limited amount of red blood cells when compared to the root extract which showed an even smaller percentage of hemolytic activity. On the other hand, Triton X-114 can destroy all of the red blood cells and was hence used as a positive control (Figure 8).

The toxicity level of bioactive agents was classified previously based on the following criteria: <10µg/mL very strong cytotoxicity, 10–100 µg/mL strong cytotoxicity, 100–500 µg/mL moderate cytotoxicity (Indrayanto G *et al.,*2020). The hemolysis assay of the red blood cells was also used as a basis to determine the CC_{50} result of the leaf and root extracts. The leaf extracts had a CC_{50} value of 520.4 µg/ml while the root extracts have a CC_{50} value of >1000 µg/ml on red blood cells (Table 4). These were the respective concentrations at which the plant extracts were 50% toxic. The total concentration in the well for this assay ranged from >1000 - 7.8125 µg/ml. Although the leaf extract isn't considered toxic with its presented CC_{50} value, if it's compared with its root extract counterpart, the root extract has a higher CC_{50} value and can be deemed less toxic. For an even bigger comparison, Triton X-114 had a 100% hemolysis which is completely toxic to the red blood cells, and is more toxic to red blood cells than the leaf and root extracts of *M. parviflora*. We can conclude that the leaf extracts showed a closer range to moderate cytotoxicity if we

consider the difference of about 20 μg/ml while the root extracts showed value greater than 1000 which means it has low toxicity.

The findings of Bouriche *et al.,* (2011) state that the anti-inflammatory properties of *M. parviflora* may be due in part to the plant's capacity to scavenge free radicals and chelate ions. The health benefits of *M. parviflora* leaf extracts are most likely brought on by flavonoids and phenolic acids. in various *in vivo* and *in vitro* cases, these compounds demonstrate anti-inflammatory properties.

The selectivity index of each plant extract was determined from its CC_{50} against human red blood cells and their corresponding IC_{50} against *Leishmania* parasites. When the selectivity index (SI) value is greater than 1, it is thought to be selective against *Leishmania* parasites, and when it is less than 1, it is thought to be selective towards mammalian host cells (Indrayanto G *et al.,* 2020). Both of the leaf and the root extracts were found to have a selectivity index values greater than 1, which means the respective plant extracts are selective in targeting the leishmania parasites (Table 5).

The methanol extracts from *M. parviflora* leaves and roots exhibit anti-leishmanial properties as seen in the above findings against both *L. aethiopica* and *L. donovani*. Higher levels of the anti-leishmanial activity were exhibited by the root extract in contrast to the leaf extract. Isolation and identification of specific fractions from the leaf and extract will give further insight into their anti-leishmanial activities.

The limitation during this study was the difficulty culturing the *Leishmania* promastigotes to reach a volume required for the assay and it was challenging to obtain sufficient number of parasites. After multiple attempts of selecting strong promastigotes and improved laboratory conditions the volume needed to conduct the assay was reached.

6. CONCLUSION

This study has demonstrated that the methanol extract of the leaf and root of *M. parviflora in vitro* possessed different levels of anti-leishmanial activities against both *L. aethiopica* and *L. donovani* and showed selective cytotoxic effect to the *Leishmania* isolates. Both the leaf and root extract showed a higher IC_{50} value than AmB against both promastigotes of *L. aethiopica* and *L. donovani*. Although the leaf extract is just as effective, the root extract was found to exhibit higher anti-leishmanial activities against both *Leishmania* strains and was found to be less toxic against human red blood cells. This result provides a scientific justification as to why the leaves and roots of *M. parviflora* have potential for the treatment of leishmaniasis by traditional healers.

7. RECOMMENDATIONS

Since the leaf and root extracts of *M. parviflora* 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 bioactive compounds and fractions responsible for their anti-leishmanial activity.
- To identify the specific mechanism of anti-leishmanial activity.
- To test the anti-amastigote activity of the plant extract and its fractions against other *Leishmania* species.
- To evaluate cytotoxic effect of methanol extracts of the leaf and root extracts of *M. parviflora* and its fractions against other mammalian cells, Vero cell lines and peritoneal mice macrophages isolates.
- The continued use of *M. parviflora* in traditional medicine as the leaf and root has been effective in the treatment of leishmaniasis.

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Appendix

Chemical and reagents

Reagents and chemicals used in this work includes; methanol, Nacl, dimethyl sulfoxide (100% DMSO), absolute ethanol, $CaCl_2.2H_2O$, $NaHCO_3$, KCl, distilled water, Giemsa powder, Alamar blue, Triton X-114, potato starch powder, penicillin, phosphate buffer saline. Amphotericin B were used as reference drugs (positive control).

Culture medium

Roswell Park Memorial Institute (RPMI-1640), chloroform, FBS (fetal bovine serum) was used to make complete culture medium. D (+)-glucose, calcium chloride dihydrate, potassium chloride, sodium bicarbonate and potassium dihydrogen phosphate, sodium chloride, Sheep's blood, were used to prepare NNN (Novy-MacNeal-Nicolle) media and Locke's solution.

Equipment

The following equipment's were used: sterile culture flasks, Corning flask, falcon tubes, 96 well plates, gauze, micro pipettes, multichannel pipettes, pipette tips, pipette tips rack, hemocytometer (counting chamber), Eppendorf tube (generic), compound microscope and inverted microscope, autoclave, biosafety cabinet with UV and analytical balance, hot plate, EDTA tube (Ethylenediaminetetraacetic acid), Bunsen burner, cold centrifuge, Flouroskan Ascent, Victor^3 Multilabel reader, centrifuge, digital water bath, Rotavapor, vortex, , microscope slides, syringes, aluminum foil, parafilm, transparent glass vials and clamps.

Preparation of Novy–MacNeal–Nicolle (NNN) media

NNN media is designed to provide the necessary nutrients for the growth of the promastigotes. 4.6 g Nutrient agar, 0.3 g D-(+) Glucose and 1.2 g sodium chloride were all weighed, mixed and dissolved in 200ml of distilled water by boiling in hot plate with repeated shaking until clear solution obtained. Then it was autoclaved for 30 minutes. Sheep's blood was collected with a

sterile bottle containing glass beads was defibrinated by shaking and heat inactivated by keeping it in 37°C water bath for 50 minutes and then transformed into 56°C water bath for 20 minutes. The autoclaved ingredients (400 ml) and of heat inactivated blood (100 ml) were mixed at 50°C. Two milliliters of the mix is dispensed among culture vials, allowed to settle slanted at a 45degree angle. and stored at 4°C until use.

Complete Media Preparation

A sterile 50 ml corning flask was acquired and 40ml of RPMI media was added with 2ml of L-glutamine since the RPMI is without L-glutamine, 1ml of pen strip (penicillin) to avoid microbial contamination and 10ml of FBS (fetal bovine serum). The mixture was mixed and stored at 4°C.

Preparation of Locke's solution

The ingredients 4.6 g sodium chloride, 0.2 g potassium chloride, 0.1 g of $CaCl_2.2H_2O$, 75mg sodium bicarbonate and 1.25 g D-(+)-glucose all were mixed and dissolved in 500ml distilled water. The mixture was covered with aluminum foil and autoclaved for 15 minutes, and added 2ml of glutamine and stored at 4°C until use.

Preparation of stock solutions of chemicals

- A stock solution was prepared to prevent contamination to the plant sample by taking and mixing 950µl RPMI and 50 µl of each plant leaf and root extract into two separates in sterile 15ml falcon tubes and added 1ml of 3% DMSO in each falcon tube that was prepared by mixing 7ml of distilled water and 3ml of 100% DMSO and shaken until it was a homogenous mixture.
- A solution of amphotericin B was prepared in phosphate-buffered saline (PBS). The prepared stock solutions of plant extracts were stored at -20°C but amphotericin B (AmB) at 4°C.
- A 2% Triton X-114 solution was obtained by taking 20 ml of PBS and in a falcon tube and taking out 200 µl and replacing it by 200 µl of PBS and adding 200 µl of 100% Triton X-114.

- To get 1% DMSO, 10ml of distilled water, taking out 100 µl and adding 100 µl of 100% DMSO and mixing it.

ANNEX 1

ADDIS ABABA UNIVERSITY, COLLEGE OF HEALTH SCIENCES (IRB)
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Institutional Review Board

ANNEX 3
Form AAUMF 03-008

IRB's Decision

Meeting No: 01/2022 Meeting Date: January 12,2022
Protocol number: 092/21/SoP

Protocol Title: Investigating some Ethiopian medicinal plants against malaria and neglected tropical diseases: Translating discoveries into health interventions (PHASE ONE)		
Principal Investigator:	Dr. Solomon M.Abay	
Institute:	College of Health Sciences, AAU	
Elements Reviewed (AAUMF 01-008)	☑ Attached	☐ Not attached
Review of Revised Application ☐ Yes ☐ No	Date of Previous review:	
Decision of the meeting:	☑Approved ☐ Approved with Recommendation ☐Resubmission ☐ Disapproved	

I. Elements approved- 1. Protocol Version No: 2
2. Protocol Version Date:
3. Informed consent Version No. 2
4. Informed Consent Version Date:

II. Obligations of the PI-
1. Should comply with the standard international & national scientific and ethical guidelines
2. All amendments and changes made in protocol and consent form needs IRB approval
3. The PI should report SAE within 10 days of the event
4. End of the study, including manuscripts and thesis works should be reported to the IRB
5. The PI should report non-compliance and unanticipated events

III. TO NERC ☐

Institution Review Board (IRB) Approval: Period from: April 18, 2022 to April 17, 2023
Follow up report expected in 3 Months ___6 months ✓ 9 months_____one year____

Chairperson, IRB
Dr. Adamu Addissie

Signature
Date: April 18, 2022