Addis Ababa University

College of Natural and Computational Sciences

Department of Microbial, Cellular and Molecular Biology

In vitro antipromastigote activity of decocted and hydrodistilled leaf extracts of *Clematis hirsuta* against *Leishmania donovani* and *Leishmania aethiopica*

By: Etsegenet Abebe

Advisors: Prof. Gurja Belay

Dr. Tegenu Gelana

Dr. Solomon Mequanente

Dr. Yehenew Asmamaw

A thesis paper presented to the Department of Microbial, Cellular and Molecular Biology of Addis Ababa University in partial fulfillment of the requirements for degree of MSc. in Infection Biology

January, 2024

Addis Ababa, Ethiopia

ACKNOWLEDGEMENT

I would like to thank my advisors Prof. Gurja Belay, Dr. Tegenu Gelana, Dr. Solomon Mequanente and Dr. Yehenew Asmamaw for their motivation and boost on this thesis work. I specially appreciate their readily availability for any questions and their comprehensible advisement. I would also like to thank them for their comments and encouragements throughout this process. I would also like to thank Prof Asrat Hailu for his help in providing the necessary facility for me to do my research.

Secondly, I would also like to thank Mr. Dawit Araya and Mr. Mulugeta Gichile for their technical support during media preparation and parasite culture. I appreciate their guidance throughout the laboratory experiment process. I would also like to thank Ms. Betelhem Sirak for her help in preparing plant extracts. I would also like to show my appreciation to Mr. Markos Tadele for his immense help during the data analysis process.

I would also like to thank Rebecca Tewodros, Yeabtsega Tilahun and Bethel Sitotaw for their immense help during the preparation of this paper and for their motivation.

Finally, my special thanks goes to my mother Migib Getahun and my brother Yohannes Abebe for their amazing belief in me.

Above all, I am thankful to God for giving me the strength and patience.

Table of contents

ACKNOWLEDGEMENT i
LIST OF FIGURES iv
LIST OF TABLES v
LIST OF ACRONYMS AND ABBREVIATIONS vi
ABSTRACT vii
1. INTRODUCTION 1
1.1. Statement of the problem 3
1.2. Research Questions 4
1.3. Objectives 5
1.3.1. General objective 5
1.3.2. Specific objectives 5
2. LITERATURE REVIEW 6
2.1 Clinical forms of leishmaniasis 6
2.2 Life cycle of leishmaniasis 8
2.3 Epidemiology of leishmaniasis 10
2.4 Diagnosis of leishmaniasis 13
2.5 Treatments of leishmaniasis 13
2.5.1 Treatment of leishmaniasis using medicinal plants 13
2.5.2 Modern drugs used in treatment of leishmaniasis 18
2.5.2.1 Pentavalent antimonials (SbV) 18
2.5.2.2 Amphotericin B (Amp B) 18
2.5.2.3 Paromomycin 19
2.5.2.4 CO_2 laser and thermotherapy 19
2.5.2.5 Therapeutic electricity 20
2.5.2.6 Combination therapy 20
2.6 Overview of *Clematis hirsuta* 21
3. MATERIAL AND METHODS 25
3.1. Materials 25
3.1.1. Test strains 25

3.1.2. Reference drug 25
3.2. Methods 25
3.2.1. Plant material collection and extraction 25
3.2.1.1 Decoction extraction 25
3.2.1.2 Hydrodistillation extraction 26
3.2.2 Selection of test strains 26
3.2.3 Antipromastigote assay 27
3.2.4 Cytotoxicity test 27
3.2.4.1 Hemolysis assay 27
3.2.5 Selectivity Index (SI) 28
3.3. Statistical analysis 29
3.4. Ethical Clearance 29
4. RESULTS 30
4.1 Growth inhibition screening 30
4.2 Antipromastigote assay 31
4.3 Hemolysis assay 32
4.4 Cytotoxic effect of *Clematis hirsuta* extracts 33
4.5 Selectivity index of *Clematis hirsuta* extracts 34
5. DISCUSSION 35
6. CONCLUSION 40
7. RECOMMENDATION 41
REFERENCES 42
Appendix 56
Annex: Ethical clearance letter 59

LIST OF FIGURES

Figure 1: Cutaneous leishmaniasis papule 6
Figure 2: Mucocutaneous leishmaniasis 7
Figure 3: Visceral Leishmaniasis 8
Figure 4: Leishmaniasis life cycle 10
Figure 5: *Euphorbia abyssinica* 15
Figure 6: *Phytolacca dodecandra* 15
Figure 7: *Sphaeranthus Steetzii* 16
Figure 8: *Otostegia integrifolia* 17
Figure 9: *Ferula communis* 17
Figure 10: *Clematis hirsuta* 21
Figure 11: Percentage of growth inhibition of extracts and standard drug. 31
Figure 12: Hemolysis percentage graph 33

LIST OF TABLES

Table 1: Percentage of inhibition against *Leishmania* promastigotes 30
Table 2: The IC_{50} of the extracts against *Leishmania* promastigotes. 32
Table 3: Hemolysis percentage 32
Table 4: CC_{50} result against red blood cells 33
Table 5: Selectivity index of extracts 34

LIST OF ACRONYMS AND ABBREVIATIONS

Amp B	Amphotericin B
CHD	Decoction extract
CHH	Hydro distilled extract
CL	Cutaneous leishmaniasis
FBS	Fetal bovine serum
FDA	Food and drug administration
L- Amp B	Liposomal amphotericin B
MCL	Mucocutaneous leishmaniasis
NNN	Novy–MacNeal–Nicolle (NNN) media
PBS	Phosphate buffer saline
PKDL	Post kala-azar dermal leishmaniasis
RPMI	Roswell Park Memorial Institute (RPMI-1640 medium)
Sbv	Pentavalent antimonials
SSG	Sodium stibogluconate
VL	Visceral leishmaniasis

ABSTRACT

Leishmaniasis is one of the six most notable neglected tropical illnesses. The protozoan parasite known as *Leishmania*, which belongs to the family *Trypanosomatidae*, causes all three types of leishmaniasis in both the Old and New Worlds. The Food and drug administration approved liposomal amphotericin B (L-Amp B) as medication. *Clematis hirsuta* leaves and stem are used to treat leishmaniasis as locally known by the people of Debre Libanos monastery. This traditional claim is still untested, thus taking this evidence into consideration the current study was aimed at scientifically validating the antileishmanial potential of *Clematis hirsuta* by testing the *in vitro* antipromastigote activity of *Clematis hirsuta* decoction and hydrodistilled leaf extracts and to test for cytotoxicity. The extracts of *Clematis hirsuta* antipromastigote activities against the promastigotes of *Leishmania aethiopica* and *Leishmania donovani* at 100 µg/ml and their cytotoxic effects against human red blood cells were evaluated. The decoction extract of *C. hirsuta* showed 75.36 ± 1.47 % and 87.37 ± 0.39 % growth inhibition on *L. aethiopica* and *L. donovani*, respectively at 100 µg/ml. While the hydrodistilled extract of *C. hirsuta* showed 97.22 ± 0.02 % and 97.54 ± 0.07 % growth inhibition on *L. aethiopica* and *L. donovani*, respectively. The IC_{50} values of decocted extract were 0.01 µg/ml and 0.002 µg/ml against *L. aethopica* and *L. donovani*, respectively. While the IC_{50} values of hydrodistilled extracts were 0.39 µg/ml and 0.06 µg/ml against *L. aethopica* and *L. donovani*, respectively. On hemolysis assay, the decoction extract showed 18.18 ± 2.14 % hemolysis on red blood cells. While the hydrodistilled extract showed 57.57 ± 4.28 % hemolysis on red blood cells. The CC_{50} values of decocted extract and hydrodistilled extracts were >1000 µg/ml and 881.0 µg/ml on red blood cells respectively. On selectivity index values, the decocted extract had a SI >1000 on both *Leishmania* promastigotes. While the hydrodistilled extract had SI of 2258.97 on *L. aethiopica* and 14,683.3 on *L. donovani*. In conclusion, the decocted extract showed above 75% activity while the hydrodistilled extract showed above 97% activity against both *Leishmania* promastigotes. The extracts were less toxic against RBC as well. Both extracts were also selective against both *Leishmania* promastigotes. This suggested that these extracts were active against the parasite and validate the claims of its use against leishmaniasis. Future works are recommended to test antiamastigote activity.

Key Words and Phrases: Antipromastigote, antileishmania, *Clematis hirsuta*, *Leishmania aethopica*, *Leishmania donovani*

1. INTRODUCTION

The protozoan parasite known as *Leishmania*, which belongs to the family *Trypanosomatidae*, causes all three types of leishmaniasis (cutaneous, mucocutaneous and visceral leishmaniasis) in both the Old and New Worlds. It can be found across Asia, Africa, the Middle East, and Central and South America. Over 90 countries that are poverty stricken are affected. It is a complicated disease caused by different species with different clinical symptoms and locations of occurrence, which causes confusion even among experts (Mann *et al.,* 2021).

Leishmaniasis is one of the six most notable neglected tropical illnesses, according to the World Health Organization, and its effects on human health have been poorly underestimated for a long time (WHO, 2010). The occurrence of cutaneous leishmaniasis (CL) ranges from 600,000 to 1 million cases per year, 95 percent of these cases are reported from the Americas, Mediterranean basin, Middle East, and Central Asia (WHO, 2023). Yearly the occurrence of visceral leishmaniasis (VL) is currently estimated at 50,000 to 90,000 cases, with Brazil, east Arica and India accounting for majority of cases (WHO, 2023). Furthermore, mucocutaneous leishmaniasis (MCL) is estimated to account for 90% occurrence in Bolivia, Brazil, Ethiopia and Peru. Poverty, population migration, malnutrition, poor cleanliness, and an immunocompromised health status are all conditions that increase the likelihood for leishmaniasis (WHO, 2023). In Ethiopia, where the disease is endemic and very frequent, people in the highlands are more likely to get cutaneous leishmaniasis than those in the lowlands, where visceral leishmaniasis is more common (Aklilu Lemma *et al.,* 1969; Alvar *et al.,* 2008; Ahmed Seid *et al.,* 2014; Abrha Bsrat *et al.,* 2015; Endalamaw Gadisa *et al.,* 2015).

The disease leishmaniasis is transmitted by the insect vector known as the sand fly. Seventy distinct kinds of phlebotomine sand flies of the *Diptera* Family *Psychodidae* genera are subdivided into *Phlebotomus* in the Old World and *Lutzomyia* in the New World, transmit the parasite *Leishmania* (Boelaert and Sundar, 2014). Because of the wide range of species, leishmaniasis has been classified as either Old World or New World based on location. Asia, the Middle East, Africa, and Southern Europe are all part of the Old World, which refers to the Eastern Hemisphere. The New World, on the other hand, refers to the Western Hemisphere, specifically Mexico, Central America, South America, and the United States (Mann *et al.,* 2021).

These flies can also transmit viruses such the Changuinola and Chandipura viruses, sand fly fever, and vesicular stomatitis. A bacterial infection called bartonellosis is also spread by a number of high Andean phlebotomines. Although research into phlebotomines in the Old World's more desert areas gave rise to the common name "sand fly," the Americas contain an equally diversified species population as well (Mullen and Durden, 2019).

The majority of leishmaniasis strains are zoonoses. From region to region human interaction differs. Sand fly species, ecology and behavior heavily influence the epidemiology, also the accessibility of a diverse scope of non-human hosts, and the species and genetic variant of *Leishmania* parasites can determine distribution. Sand flies in some areas interact entirely among wild or tamed animals, with zero human interaction, whereas in others, livestock may be an important reservoir host of infection for humans. In India, without the presence of animals infections can be transmitted between people. As such leishmaniasis epidemiology is complicated (Service, 2012).

Pentavalent antimonials have historically been employed to treat leishmaniasis; nevertheless, patients have experienced significant adverse effects, including arthralgias, myalgias, pancreatitis, leukopenia, and cardiotoxicity (Berman, 2003; Croft and Coombs, 2003; Oliveira *et al.,* 2011). Despite their high cost, liposomal amphotericin B (Amp B) formulations are believed to be effective (Mondal *et al.,* 2010).

In an attempt to find new antileishmanial medications, secondary chemicals isolated from plants have received a lot of interest recently (Croft and Coombs, 2003; Tiuman *et al.,* 2005; Khaliq *et al.,* 2009; Vendrametto *et al.,* 2010). Investigations using substances taken from plant tissue and/or pure molecules with antileishmanial activity have been conducted (Lage *et al.,* 2013). The economical, and eco-friendly qualities of medicinal plants make them a more appealing alternative to chemotherapy than other treatments. Moreover, natural compounds made from plants are thought to be a secure and successful leishmaniasis treatment (Cheuka *et al.,* 2017).

Clematis hirsuta is used as an analgesic, anti-rheumatic, and anti-inflammatory in herbal medicine in Asia (Al-Taweel *et al.,* 2007). However, it is widely prevalent over tropical Africa and areas with intermediate altitudes, such as Ethiopia. Known locally by some as "Hidda Feetii", it is among the medicinal plants used in Ethiopia to treat a variety of illnesses (Zelalem Abdisa and Fekede Kenea, 2021). It's also locally known by the people of Debre Libanos monstery as "Azo Hareg"

and its leaves and stem are used to treat leishmaniasis, while its leaves also treat 'Yeshererit Beshita' (Herpes zoster) and Haemorrhoids . When it comes to its veterinary use, its leaf is used to treat 'Yeferes Ekeke' (Lymphangitis) (Tilahun Teklehaymanot *et al.,* 2007).

Thus the aim of this study was to assess the *in vitro* antipromastigote activity of the decocted and hydrodistilled leaf extracts of *Clematis hirsuta* against *Leishmania donovani* and *Leishmania aethiopica* and to test its cytotoxic activity to RBC.

1.1. Statement of the problem

Leishmaniasis is an ever growing problem. Tropical leishmaniasis is brought on by parasitic protozoans of the genus *Leishmania* (Grimaldi and Tesh, 1993). It is a disregarded illness and isn't even on Ethiopia's list of priority tropical diseases (Hotez *et al.,* 2004). Like other neglected tropics illnesses, leishmaniasis has a focused distribution and is prevalent in remote locations (Bern *et al.,* 2008).

Annually, there are thought to be 700,000 to 1 million new cases of leishmaniasis. Brazil, east Africa, and India account for the majority of VL instances. Only 25–45% of the estimated 50–90,000 new cases of VL that are reported to WHO each year are actually seen worldwide. There is a chance of outbreak and fatality in this instance. Only about 200,000 CL new cases are reported to WHO each year, out of an estimated 600,000 to 1 million worldwide (WHO, 2023).

There are 98 nations where the disease is native, the majority of them are developing nations. (Didwania *et al.,* 2017). In Ethiopia, an estimated 4500–5000 new instances of VL are disclosed every year, where the disease burden is quite high and the nation is severely afflicted by both MCL and VL (Samson Leta *et al.,* 2014). In addition, *L. aethiopica*, *L. donovani*, *L. major*, and *L. tropica* are all prevalent in the nation (Ayalew Assefa, 2018).

Since there is yet no human-useable vaccine, chemotherapy is the mainstay of leishmaniasis treatment. However, there has been a growing resistance to first-line medications. In locations with low resources, the second-line medications can have serious side effects and are expensive (Ghorbani and Farhoudi, 2017). The idea behind multidrug therapy was introduced to counteract these problems. Its aim was to boost activity by using drugs that act at various sites in a complementary or additive manner (Chakravarty and Sundar, 2019). Moreover, in order to find

novel main substances and/or new medications against the disease that merit examination, researchers' attention is being attracted to naturally produced chemicals utilized to combat parasitic diseases, including leishmaniasis (Hiwot Ayalew *et al.,* 2018). This increases the need for additional antileishmanial drugs from natural resources to stop and treat this illness.

90% of the livestock in Ethiopia and about 80% of the population rely on traditional medicine as a form of treatment, while more than 95% of traditional medicine preparations are created from plant sources. Similar to this, the demand for traditional medicines has been steadily increasing both globally and in the health care systems of many developing nations (Yeneayehu Fentahun *et al.,* 2017).

1.2. Research Questions

The basic research questions are as follows

- Does the leaf extracts of *C. hirsuta* have antipromastigote activity?
- What are the median inhibitory concentration and median cytotoxicity concentration of the leaf extracts of *C. hirsuta*?
- Can the leaf extracts of *C. hirsuta* cause cell destruction in an *in vitro* environment?
- Is the decocted leaf extracts of *C. hirsuta in vitro* method active against the promastigote?
- Is the hydrodistilled leaf extracts of *C. hirsuta in vitro* method active against the promastigote?
- Which *C. hirsuta* leaf extract is more active against the promastigotes parasites?
- Can the decocted leaf extracts of *C. hirsuta* be cytotoxic to RBC?
- Can the hydrodistilled leaf extracts of *C. hirsuta* be cytotoxic to RBC?

1.3. Objectives

1.3.1. General objective

The general objective of the study was to evaluate the antipromastigote activity of the decocted and hydrodistilled leaf extracts of *Clematis hirsuta* and to test their cytotoxicity activity.

1.3.2. Specific objectives

The specific objectives were:-

- ✓ To evaluate anti-promastigote activity of the decocted and hydrodistilled leaf extracts of *Clematis hirsuta* using *in vitro* method against *Leishmania donovani* and *Leishmania aethiopica* isolates.
- ✓ To evaluate *in vitro* cytotoxicity of the decocted and hydrodistilled leaf extracts of *Clematis hirsuta* to RBC.

2. LITERATURE REVIEW

2.1 Clinical forms of leishmaniasis

Leishmaniasis has three clinical forms: cutaneous leishmaniasis (CL), visceral leishmaniasis (VL) and mucocutaneous leishmaniasis (MCL). Some *Leishmania* species can produce both CL and VL, these being *Leishmania amazonensis, Leishmania infantum,* and *Leishmania guyanensis* in the New World and *Leishmania donovani, Leishmania infantum,* and *Leishmania tropica* in the Old World. However, most *Leishmania* species produce only one clinical form (Mullen and Durden, 2019).

When it comes to cutaneous leishmaniasis multiple lesions can occur. CL ulcers can be seen as one non-suppurative pimple at where the sand fly took a bite (on uncovered areas of the body). It is found in both the Old World (*L. tropica, L. major, L. aethiopica,* and less commonly *L. infantum and L. donovani*) and the New World (*L. mexicana, L. amazonesis, L. venezuelensis,* and *L. viannia* subgenus, which includes *L. V. braziliensis, panamensis*, and *guyanesis*) (Herwaldt, 1999a; Bennett *et al.,* 2015). The dapple (macule) is first seen at the beginning on the site of inoculation which progress into a papule. The papule (Figure 1) increases in size and eventually becomes a slow-growing, painless ulcer. The lesion could be single or multiple, or it could be non-ulcerative and diffuse (Mullen and Durden, 2019). It can heal in the spur of the moment within months or years but can also cause disfiguration and malformation (Berman, 1997).

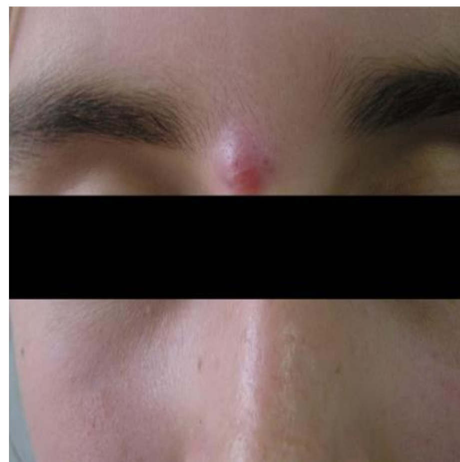

Figure 1: Cutaneous leishmaniasis papule

(Bilgic-Temel *et al.,* 2019)

MCL is the most deforming form of disease, resulting facial changes years after the earliest indications have set on. The parasite, which is frequently caused by the *L. Viannia* subgenus, spreads from amastigotes on the skin to the nasal mucosa via the hemopoietic and lymphatic systems (Herwaldt, 1999a). It involves lesions of the nose, mouth, and pharynx and can develop after the primary lesion has healed or in the absence of a recognized primary lesion (Mullen and Durden, 2019). This is seen below on figure 2.

Dermal signs and mucosal lesions may occur together, though this is less common. Patients frequently report chronic nasal symptoms such as secretions, epistaxis, and pain; physical examination regularly reveals ulceration, bleeding, and inflammation (Weller *et al.,* 2005). Initially, mucosal activity throughout the nostrils and mouth take place, followed by oropharynx and larynx involvement later in the disease's progression. MCL does not heal on its own like cutaneous disease does (Berman, 1997; Scope *et al.,* 2003). This form is usually produced when leishmania species are heavily infected with an RNA virus. These viruses are able to divert host immune responses from duplicating leishmania, resulting in less controlled parasite take over (Ives *et al.,* 2011).

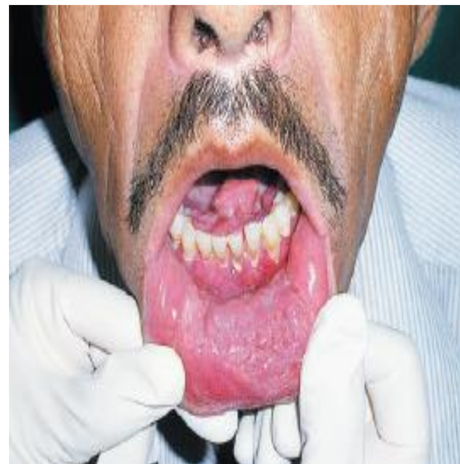

Figure 2: Mucocutaneous leishmaniasis

(Sethuraman *et al.,* 2008)

VL (also known as kala-azar) is the most lethal form of leishmaniasis and can cause systemic infection affecting the liver, spleen, hemopoietic and lymphatic systems. *L. donovani, L. infantum* (in the old World), and *L. chagasi* (same as *L. infantum* but found in the New World) are the species most commonly associated with visceral disease (Herwaldt, 1999a).

A tiny cutaneous lesion at the location of the infectious sand fly bite serves as the initial sign of VL. From the site of inoculation the parasites travel through the bloodstream and cause chronic fever, expansion of the lymph nodes, liver, and spleen, a lack in platelets, red and white blood cells. The symptoms at an early stage differ. For example, viscerotropic leishmaniasis refers to discreet and undetectable (subclinical) infections caused by *Leishmania tropica*. If untreated, clinically evident VL is usually fatal. In most cases, the intrinsic gestation period lasts between 2 and 4 months. After apparent recovery or cure cutaneous lesions can be seen and can be around for up to 20 years if not treated. Post kala-azar cutaneous leishmaniasis or post kala-azar dermal leishmaniasis (PKDL) are the names given to such lesions (Mullen and Durden, 2019). VL signs can be seen on figure 3.

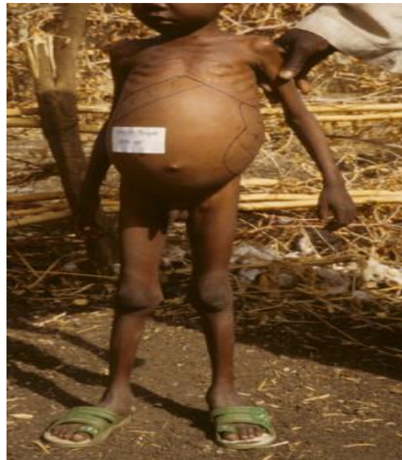

Figure 3: Visceral Leishmaniasis

(Zijlstra, 2016)

2.2 Life cycle of leishmaniasis

Leishmania has to go through two important hosts in its life cycle, which means it goes from mammalian to insect and vice versa. The sandfly from the *Phlebotomus* genus and *Lutzomyia* genus, in Old World *leishmania* species and in New World *Leishmania* species respectively, are the insect vectors. The parasite undergoes morphological differentiation known as the amastigotes and promastigotes whenever it moves between the host and the vector (Sasidharan and Saudagar, 2021).

The female phlebotomine sand fly exposes humans or other animal reservoirs to the leishmaniasis parasite during the night ("from dusk till dawn"). Promastigote and amastigote are the two separate life stages of *Leishmania* sp. (Organization PAHO, 2019; CDC, 2020). The sandfly becomes infected after consuming blood from an infected host.

Female sand fly blood meals include amastigote parasites, which mature into promastigotes, which are elongate, have a flagellum, attach to the mid-gut or hind-gut wall, and reproduce quickly. The procyclic promastigotes which are flagellated and motile parasites have slender bodies that measure 15-20 m in length and 1.5-3.5 m in width (Service, 2012; Sasidharan and Saudagar, 2021).

The non-dividing nectomonad promastigotes are formed when the promastigote multiply in the sandfly's stomach, midgut. From the abdomen to the anterior midgut, these nectomonad promastigotes move before transforming into leptomonad promastigotes. The metacyclic promastigotes from the leptomonad promastigotes move to the insect's proboscis where they are then delivered to a mammalian host by a bite. This process is known as metacyclogenesis, lasts 7 to 10 days (Gossage *et al.,* 2003).

Many, on the other hand, are void when the fly defecates. The ones that survive this defecation go toward the anterior (front) mid-gut and then to the foregut after development to a greater extent. After the fly took an infected blood meal the metacyclic forms will be found for 4 - 25 days in the sand fly's mouthparts. (Service, 2012).

Once the metacyclic promastigotes is introduced they initiate begin the phagocytic activity by adhering to the plasma membrane (Lodge and Descoteaux, 2008). In this way, the macrophage's parasitophorous vacuole is infected by the promastigotes that have entered it. Once inside the parasitophorous vacuole the promastigotes differentiate becoming ovoid amastigotes with a diameter of 2-4 m (Herwaldt, 1999b).

The amastigotes grow and increase in number until the macrophage lysed open, thus the mature amastigotes become released (Sasidharan and Saudagar, 2021). According to underlying host and parasite species parameters, the amastigotes multiply and grow within the host's reticulo-endothelial system, resulting in either asymptomatic or symptomatic illness. Amastigotes can spread hematogenously and lymphatically, resulting in visceral and mucosal illness (Organization

PAHO, 2019). Finally, these released amastigotes will start a reaction of infection, eventually leading to one of the clinical manifestations (Sasidharan and Saudagar, 2021).

The parasite has been known to infect mammalian hosts such as hyraxes, anteaters, rodents, dogs, sloths and opossums. *L. infantum* has been found in jackals, raccoon dogs, cats and foxes, but more research is needed to establish it as a reservoir (WHO, 2010).

Infective flies probe more frequently than uninfected flies, maximizing parasite transmission during blood feeding (Service, 2012). Thus the life cycle continues as seen in figure 4 below.

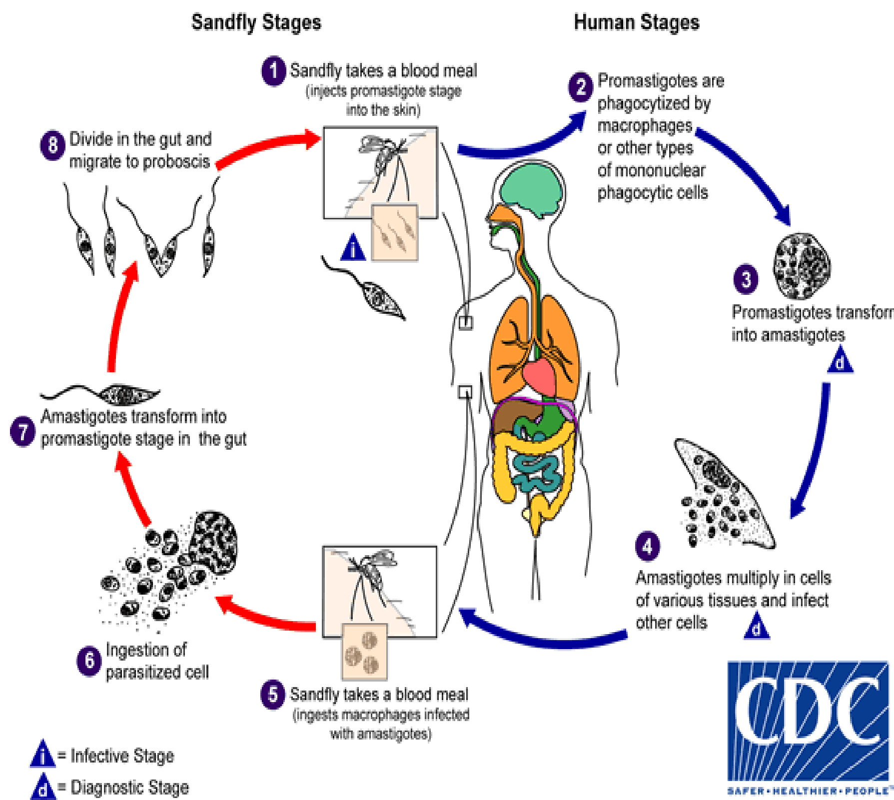

Figure 4: Leishmaniasis life cycle

(CDC, 2020b).

2.3 Epidemiology of leishmaniasis

Throughout the decades the distribution of leishmaniasis differs. According to WHO data, 22,233 new VL cases and 200,918 new CL cases were reported in 2016. 90% of all VL cases worldwide were accounted by Ethiopia, Brazil, India, Somalia, Kenya, Sudan and South Sudan. While 90%

of the global burden of CL cases were accounted for by Algeria, Afghanistan, Colombia, Brazil, the Syrian Arab Republic and Pakistan in 2016, Nicaragua, Morocco, Sudan, Peru, Yemen and Tunisia accounted for the remaining 10% (WHO, 2018).

As of 2018 also, according to the World Health Organization, Kenya, Brazil, Somalia, India, South Sudan, Ethiopia, and Sudan have 94% of all new cases. The disease is endemic in 88 countries, 72 of which are classified as developing countries. India, Nepal, Sudan, Brazil, and Bangladesh have more VL occurrence, whereas Afghanistan, Iran, Syria, Brazil, Saudi Arabia, and Peru account for 91% of CL. 90% of MCL cases are from the Plurinational State of Bolivia, Peru, and Brazil (Banuls *et al.,* 2007).

As of late like stated before, there are between 600,000 and 1 million cases of CL annually reported from the Americas, Mediterranean basin, Middle East, and Central Asia, while there are 50,000 to 90,000 occurrences of VL per year with Brazil, east Africa and India. Furthermore, mucocutaneous leishmaniasis (MCL) is estimated to account for 90% occurrence in Bolivia, Brazil, Ethiopia and Peru (WHO, 2023).

In Africa, leishmaniasis affects different countries in different ways throughout the years. Algeria is one among the eight nations in northern Africa that make up 90% of all CL worldwide (Desjeux, 2004). The Mediterranean region, where leishmaniasis is common, includes Morocco. There have been reports of both CL and VL as well. CL has been a significant danger to public health in Morocco over the past 20 years (Rhajaoui *et al.,* 2007).

Approximately two thirds of all VL cases in East Africa that are reported each year are from Sudan (Al-Salem *et al.,* 2016). Despite having incidence rates that were lower than those for Ethiopia in 2015 (Malaria Consortium, 2010), around 93% of the population of Sudan is currently thought to be at risk of CL and 25% of the population is thought to be at risk of VL (WHO, 2017a).

In other African countries like Kenya, due to *L. donovani*, VL is endemic in Kenya's semi-arid and arid regions, including the Rift Valley and provinces in the country's east and north east throughout the years (Ngure *et al.,* 2009; Okindo *et al.,* 2017). An estimated 4,000 VL cases are anticipated per year recently in 2020 (Kanyina, 2020). With an incidence rate of 2.96 per 10,000 persons for VL but no information on CL, almost 5 million people in Kenya were thought to be at risk of leishmaniasis exposure since 2017 to 2020 (WHO, 2017b; Kanyina, 2020). According to the WHO

information on Somalia, the incidence of VL, but not CL, is endemic, where it is 4.98 per 10,000 people (WHO, 2015).

Ethiopia also has its fair share of leishmaniasis as well. For both CL and VL, Ethiopia is an endemic country (Aysheshm Kassahun *et al.,* 2015). VL cases ranging from 3,700–7,400 and 30,000–50,000 cases of CL, respectively, are thought to be surplus in each year (Helina Fikre *et al.,* 2019; Yibeltal Aschale *et al.,* 2019). Ethiopia, which borders Sudan in East Africa, is ranked second for the annual rate of VL cases (Asrat Hailu *et al.,* 2009). In endemic locations, 6.28 cases of VL and 1.05 cases of CL occur for per 10,000 people (WHO, 2017c). On a national scale, it is estimated that between 28 and 30 million individuals reside in places where leishmaniasis is a possibility (Ahmed Seid *et al.,* 2014; Dawit Gebremichael, 2018). *L. donovani* and *L. aethiopica* are the main causal species in this area for VL and CL, respectively (Asrat Hailu *et al.,* 2006; Alvar *et al.,* 2012; Van Henten *et al.,* 2018). The neighboring areas of the Afder and Liben zones in the southeast of the Somali state, the Awash Valley in the north of the state of Afar, and Moyale and Genale river basins in the south of Oromia have also reported occurrences of VL (Asrat Hailu *et al.,* 2009; Samson Leta *et al.,* 2014; Ayalew Assefa, 2018).

The cutaneous type of the disease, in contrary to VL, is closely related to Ethiopia's highland regions, where elevations exceed 1,400m (Dawit Gebremichael *et al.,* 2018; Helina Fikre *et al.,* 2019). The presence of CL is anticipated to be widespread, but certain areas have been identified as foci, such as the Kutaber region in Amhara, Aleku, Sebeta, Ochollo in the Rift Valley, the Adigrat and Saesie Tsaedaemba districts of Tigray and the Bale and Sidamo highlands of Oromia (Dawit Gebremichael *et al.,* 2013; Gessessew Bugssa *et al.,* 2014; Abrha Bsrat *et al.,* 2015; Aysheshm Kassahun *et al.,* 2015; Pareyn *et al.,* 2019). While VL brought on by *L. donovani* is common in the northern Ethiopian lowlands of Humera and Metema, with an incidence of 1000–2000 cases per year. Of those 20–40% are also co-infected with HIV (WHO, 1996; Ritmeijer, *et al.,* 2006).

Thus leishmaniasis is an overgrowing case throughout the world. It has been affecting diiferent parts of the world throughout decades and so far based on recent epidemiology reports it is still an overgrowing problem. It affects millions each year and its distribution can become complicated based on the type of hosts it shelters in as stated before. It affects different parts of Ethiopia as well.

2.4 Diagnosis of leishmaniasis

Leishmaniasis can be diagnosed in a variety of laboratory settings by identifying the *Leishmania* species and detecting the parasite. Using light-microscopic examination of stained slides, molecular methods, and specialized culture techniques, leishmaniasis is diagnosed by detecting *Leishmania* parasites (or DNA). This parasite can be found at different parts of the body depending on the type of leishmaniasis the patient has. In the case of cutaneous leishmaniasis a tissue specimens, such as from skin lesions is where it is taken. While in the case of visceral leishmaniasis, it is taken from the bone marrow. The patients information of where they lived or traveled can be used as a diagnostic method of predictively assuming what type of strains are present in those areas. It will help in determining if theses strains are present based on the symptoms seen on the affected patient. The species can be further identified using a variety of laboratorial ways, which include biochemical techniques (isoenzyme analysis of cultured parasites) and molecular methods (such as PCR). Serologic tests may be able to confirm the diagnosis of visceral leishmaniasis. Geographical location and host characteristics can affect how well different serologic assays function (CDC, 2023).

2.5 Treatments of leishmaniasis

2.5.1 Treatment of leishmaniasis using medicinal plants

Locals use medicinal herbs as part of their traditional healing regimens. Nowadays, medicinal plants are a major source of pharmaceuticals. The majority of medications are made from plants with various bioactivities. Usually, information on these kinds of plants comes from observation and evaluation of practice, attitude, and knowledge of the local population (Bereket Alemayehu *et al.,* 2023). Thus developing native knowledge in the process.

In all of Ethiopia, medicinal plants and knowledge on how to use them serve a vital part in meeting the requirements of the people and the animals in terms of healthcare. Ninety percent of the cattle and eighty percent of Ethiopia human population use traditional healthcare (Gidey Yirga, 2010).

In Ethiopia, traditional medicine is very well known and practiced. Most Ethiopians prefer traditional medicine than modern medicine. For medicinal use plant materials have been used for thousands of years (Cortés *et al.,* 1998). The majority of the medicinal herbs used in traditional

healthcare are gathered in natural settings. Despite this, both man-made and natural disasters are reducing the biodiversity of medicinal plants. Additionally, the traditional knowledge related to the preservation and utilization of medicinal plants is also rapidly vanishing. Due to cultural acceptance, physical accessibility, potential efficacy against specific ailments and relative affordability to modern medication, traditional medicine is widely used in Ethiopia among both urban and rural populations. Given this, advancements in traditional medicine and their eventual incorporation into the modern system are anticipated to have a substantial impact on the expansion of health care coverage (Reta Regassa, 2013).

According to estimates from the World Health Organization (WHO, 2023), up to 80% of people in under developed nations rely on herbal medicines and other traditional treatments to cure illnesses linked to poverty (Tripathi and Tripathi, 2003). Many of these medicinal plants have not yet received formal recognition since little study has been done on their effectiveness, safety, and quality (Nguyen Hoai, 2009).

Early humans' discovery of medicinal plants had to have been a trial and error process driven by the need to alleviate sickness symptoms. Such knowledge was passed down from generation to generation before the advent of writing and documenting history (Thomford *et al.,* 2018). Traditional treatment is an important part of Ethiopia's medical history. Eighty percent of its population uses traditional medicine and ninety five percent of its source is from plants. This can be due to economic status, lack of accessibility of medical facilities, cultural acceptability and such (Dawit Abebe *et al.,* 2001).

For example, different plants are used for treatment of leishmaniasis in different places in Ethiopia. *Euphorbia abyssinica* is one of the plants known to treat leishmaniasis (Figure 5). It is a tree from the family *Euphorbiaceae* and locally known as "Kulkual". Crushed leaves from it are combined with butter to treat CL (Muluken Wubetu *et al.,* 2017), while its latex are applied to the wound after it is contacted with a heated thread to treat VL (Yayesh Limenih *et al.,* 2015).

Figure 5*: Euphorbia abyssinica*

(Berhan Mengiste *et al.,* 2014)

Phytolacca dodecandra is a shrub from the family *Phytolaccaceae* also known to treat leishmaniasis (Figure 6). It is locally known as endode and in this case its roots are effective against leishmaniasis. The root is smeared with butter after being pulverized. Finally, it is applied topically or externally. This plant is most effective in treating CL (Sultan Suleman and Tamirat Alemu, 2012).

Figure 6: *Phytolacca dodecandra*

(Qwarse *et al.,* 2017)

Sphaeranthus Steetzii is also another traditional leishmaniasis treatment. It is a shrub from the family *Asteraceae* that has purple buds on the top. It is locally known as "Qoricha –Cheffe", and

its bark and leaves are used in the treatment of CL (Figure 7). The freshly pulverized leaves and bark mixture is applied to the skin's surface where the wound is found. The wound is then bandaged (Mesfin Tadesse *et al.,* 2005).

Figure 7*: Sphaeranthus Steetzii*

(West African plants, 2023)

In a recent investigation it was found that extracts of *Otostegia integrifolia* (Figure 8) and *Ferula communis* (Figure 9) in 80% methanol showed potential in vitro antileishmanial activity. *Ferula communis* showed to have the highest antipromastigote activity, while *Otostegia integrifolia* had the highest effect against VL (*Leishmania dononvani*) (Hulubanchi Nigatu *et al.,* 2021). This study showed traditional treatments of these medicinal plants living up to their effectiveness against leishmaniasis within laboratory settings as well.

Figure 8*: Otostegia integrifolia*

(Sisay Alemu and Jelalu Kemal, 2015)

Figure 9*: Ferula communis*

(Lahmar *et al.,* 2018)

Plants such as these have been used by local people to treat leishmaniasis. Taking into the consideration of the above uses of these plants, we can conclude that these plants are medicinal in which they have the activity to treat leishmaniasis and probably many more illnesses. The last two plants mentioned can be an indication of traditional treatment turning into a more modern scientific treatment.

In other countries like Cuba, studies have shown that plant extracts from *Hura crepitans*, *Bambusa vulgaris*, and *Simarouba glauca* demonstrated encouraging antileishmanial action against *Leishmania amazonensis* life cycle stages (García *et al.,* 2012). In Brazil antileishmania tests were carried out as well. They found some plants of being active against *Leishmania amazonensis*. They found out that plant extracts *Chenopodium ambrosioides* and *Pfaffia glomerata* demonstrated direct efficacy against the parasite at 87.4% and 88.7% suppressing growth respectively (De Queiroz *et al.,* 2014). This also shows how there are different types of plants around the world that can be effective in treating leishmaniasis.

2.5.2 Modern drugs used in treatment of leishmaniasis

2.5.2.1 Pentavalent antimonials (SbV)

There are different types of modern drugs used to treat leishmaniasis, one of them being pentavalent antimonials. The two forms, sodium stibogluconate (SSG) and meglumine antimoniate (MA), are where pentavalent antimonials (SbV) comes from (Chakravarty and Sundar, 2019). Before it became ineffective in the Indian subcontinent due to resistance, antimonials at a dose of 20 mg/kg body weight for 28–30 days had been the conventional treatment for VL for many years (Sundar *et al.,* 2000; Rijal *et al.,* 2003). SSG and a drug known as paromomycin have been suggested as the primary treatment for VL in eastern Africa (Musa *et al.,* 2012; Kimutai *et al.,* 2017). Despite being beneficial in other regions of the world, especially in Africa, it has a significant level of toxicity, particularly cardiotoxicity (cardiac arrhythmias and ventricular premature beats), is a major deterrent (Chakravarty and Sundar, 2019). Local inflammation, renal changes, anorexia, nausea, and vomiting are some of the adverse effects of Sbv as well (Sundar and Chakravarty, 2015). Patients who are getting Sbv medication while also having HIV and leishmaniasis show notable side effects, high failure rates, and high rates of recurrence (Cota *et al.,* 2013). These patients experience not only severe side effects, but also decreased efficacy, and increased mortality during SSG therapy (Ritmeijer *et al.,* 2006; Ermias Diro *et al.,* 2014).

2.5.2.2 Amphotericin B (Amp B)

Perhaps the most well-known drug for leishmaniasis treatment is amphotericin B. A broad-spectrum antifungal, amphotericin B deoxycholate (Amp B) is effective against protozoan

leishmania species (Roatt *et al.,* 2020). With cure rates of 100% at a dose of 0.75-1.0 mg/kg for 15-20 intravenous infusions, this polyene antibiotic was frequently used in India for VL materials to antimonials (Mishra *et al.,* 1992; Thakur *et al.,* 1999). Additionally, it is advised for use in the Indian subcontinent to treat PKDL at a dose of 1 mg/kg/day for a total of 60-80 doses spread out over 4 months (WHO, 2010). Even with its efficiency, the harmful impact, which include hypokalemia, myocarditis, nephrotoxicity, and infusion reactions, necessitate monitoring and hospitalization of the patient, raising the cost of therapeutic interventions (Chakravarty and Sundar, 2019).

To reduce these side effects of Amp B, lipid formulations, in which deoxycholate was substituted with different lipids, were created. These formulations quickly enter the body and concentrate in the reticuloendothelial tissue, the diseased area in VL, in organs including the liver and spleen. It stays there for a long time, making it possible to administer high quantities of the medication quickly. Nephrotoxicity is reduced because it spares organs such as the kidney. For leishmaniasis, especially VL, amphotericin B lipid complex, amphotericin B cholesterol dispersion, and liposomal amphotericin B (L-Amp B) have all undergone thorough testing. The only Food and Drug Administration (FDA) approved medication is L-Amp B (Chakravarty and Sundar, 2019).

2.5.2.3 Paromomycin

A topical and parenteral formulation of a drug known as paromomycin, which has been used to treat CL and VL respectively and is an aminoglycoside-aminocyclitol (Sundar and Chakravarty, 2008). In some parts of India, it is used as a systemic treatment for VL and a topical and systemic treatment for CL (Sundar and Chakravarty, 2008; Jhingran *et al.,* 2009). However, in East Africa, paromomycin has limited efficacy as a monotherapy (Asrat Hailu *et al.,* 2010; Musa *et al.,* 2012). It was utilized to treat dysentery in VL patients during the southern Sudanese region's 1989 VL outbreak. It was noted that it was synergistic with SbV in the treatment of VL and not only reduced the incidence of dysentery (Sundar and Chakravarty, 2008). In Africa, it was subsequently combined with SbV (Chakravarty and Sundar, 2019).

2.5.2.4 CO_2 laser and thermotherapy

The introduction of drug-resistant strains (Sundar, 2000; Mueller *et al.,* 2007), severe toxicity, concurrent infections with *Leishmania* spp. and HIV, and limited therapeutic equipment's

availability to treat the illness, and the decreased expenditure in drug discovery/development pushes scholars and global health groups to look for fresh approaches, such as the following for dealing with and regulate this disease (Roatt *et al.,* 2020).

Based on the theory of immediately destroying *Leishmania* parasites, CO_2 laser and thermotherapy are perhaps a more recent modern form of treatment. They treat in a simple way in which a delivery of heat from the outside on afflicted tissues, causing parasitism in specific locations (Asilian *et al.,* 2004; Valencia *et al.,* 2013). Skin lesions that have been subjected to direct heat may heal more quickly (Navin *et al.,* 1990). Over a 2-year follow-up period, a single session in Cuba completely healed all 10 patients, with no relapses (Rodriguez *et al.,* 1990). Hyperpigmentation, prolonged redness, and hypertrophic scarring were the most frequent side effects of CO_2 laser treatment (Chakravarty and Sundar, 2019).

2.5.2.5 Therapeutic electricity

Another modern leishmaniasis treatment is the application of therapeutic electricity on *Leishmania major* infected mice which resulted in substantial parasite death on the wounded areas (Hejazi *et al.,* 2004). Latest evidence has shown that electric fields have an impact on the motility, clumping, and survivability of *Leishmania tarentolae* promastigote *in vitro* (Dorsey *et al.,* 2018).

2.5.2.6 Combination therapy

Another therapy well recommended is combination therapy. The idea behind multidrug therapy was to boost activity by using drugs that act at various sites in a complementary or additive manner (Chakravarty and Sundar, 2019). Combination therapy aims to reduces the amount of time needed for treatment and the dosage needed, which lowers the risk of adverse effects, improving patient adherence to the regimen, lowers cost, encourage cure while also delaying and controlling parasite resistance development to the drug, particularly in complex cases of VL (Monge-Maillo and López-Vélez, 2013; Sundar and Chakravarty, 2013). It is primarily advised for those who have not improved after using Sbv monotherapy. The majority of the research was specifically on *Leishmania donovani* patients done in India (Monge-Maillo and López-Vélez, 2013). Investigations conducted in Eastern Africa showed that combining paromomycin with SSG enhanced the recovery rate reaction when compared to SSG monotherapy (Yosef Melaku *et al.,* 2007).

2.6 Overview of *Clematis hirsuta*

It has been reported that several traditional medicine utilizes plant parts to treat various infection kinds (Ogbulie *et al.,* 2007). *Clematis hirsuta* belongs to the *Ranunculaceae* family. It is woody and perennial climber that can grow to be up to 4 meters long. It has complex leaves with opposing arrangements (Figure 10). Blooming occurs in panicles that are either white or yellow (Asmamaw Habtamu and Yalemtsehay Mekonnen, 2017).

Ye dega woratura (የደጋ ወራቱራ) and Ye kola woratura (የቆላ ወራቱራ) is its local name from the highland of Ochollo village, Gamo Zone, south Ethiopia from where it was collected for the experiment.

According to Hao *et al.,* (2013), several medicinal chemicals found in *Clematis* species include glycosides, saponins, alkaloids, xanthones, and anthocyanidins. As a result, plants in the *Clematis* genus are utilized as medicines. These intricate chemical compounds with various compositions are present in one or more of these plants as secondary plant metabolites and are beneficial to humankind (Okigbo *et al.,* 2008). According to reports, it is used to treat a variety of conditions, including edema, leishmaniasis, herpes, hemorrhoids, and a neck tumor in Ethiopia (Tilahun Teklehaymanot *et al.,* 2007).

Figure 10*: Clematis hirsuta*

(Photo credit: Betelhem Sirak)

There are different uses for it in different places. To treat earaches, fresh leaves of *C. hirsuta* are smashed, compressed, and a tiny quantity of the fluid put via the ear canal over the course of two days (Haile Yineger and Delenasaw Yewhalaw, 2007). Various *Clematis*, such as *Clematis brachiata* Thunb. leaves as well as the roots of *C. hirsuta* and *Clematis indica Lour* are used as an antimalarial medicine in Africa (Kokwaro, 1976; Chhabra *et al.,* 1991). It has also been known to have ethnomedicinal properties in treating kidney problems in Blue Nile State, Sudan (Musa *et al.,* 2011).

Other uses for *C. hirsuta* have been reported through the years. It is among the Rwanda therapeutic herbs utilized to traditionally heal urinary illness and abortifacient (Baerts and Lehmann, 1989; Rwangabo, 1993). According to Cos *et al.,* (2002b), *C. hirsuta* ethanolic extract had an activity in complement assay and lymphocyte proliferation, which later also showed its anti-inflammatory activity. It has also been reported as a medicinal plant to treat diarrhea (Gahamanyi *et al.,* 2021). Its leaves are also typically utilized to cure leprosy, diarrhea and yaws (Baerts and Lehmann, 1989; Rwangabo, 1993).

According to traditional health practitioners, *C. hirsuta* has also been known to have a side effect of abortion hence a danger for children and pregnant women if high doses are administered (Mukazayire *et al.,* 2011). At concentrations greater than or equivalent to 46.9 mg/ml, its leaves when tested on the three dermatophytes, *E. floccosum*, *T. rubrum* and *M. canis* as well as the yeast *C. albicans*, displayed strong antifungal activity (Cos *et al.,* 2002a).

It accounts for 36.73% of all medicinal plants taken into consideration when researching how to manage various animal maladies in Bale Districts, and *Sida schimperiana* and *C. hirsuta* roots (*Malvaceae*) are pulverized, smashed then combined with water to be both nasal and oral delivered to treat blackleg (Haile Yineger *et al.,* 2007). In the southern region of Ethiopia, among the Meinit ethnic group, *C. hirsuta* is also utilized to treat cataract and respiratory tract issues (Mirutse Giday *et al.,* 2009).

According to Asmamaw Habtamu and Yalemtsehay Mekonnen (2017) research, by using agar disk diffusion methods, the crude chloroform extract of *C. hirsuta* leaves at the concentration of 200 mg/ml on *P. aeruginosa* demonstrated the highest inhibition zone (12.33 ± 0.50 mm). In contrast, *C. hirsuta* leaves methanol extract at a dosage of 200 mg/ml inhibited *P. aeruginosa* at 8.5 ± 0.50 mm. As a result, at the same concentration, the chloroform extract of the leaves had a

much greater inhibitory zone than the 80% methanol. This study supported the claims of conventional healers that *P. aeruginosa* and other germs that can infect and harm the skin are prevented from growing by the two plant extracts. The 80% methanol extract of *C. hirsuta* used in this study (200 mg/kg) on mice showed no signs of toxicity or mortality. Both extracts of *C. hirsuta* leaves demonstrated a moderate inhibitory zone on several pathogenic bacteria.

In order to treat syphilis, sore throats, and gonorrhea, an infusion made from the leaves of *C. hirsuta* is consumed. On impacted tumor locations, a powder produced from the plant's bark, leaves, and stems is administered. Leprosy, fever, and other skin conditions were all treated with the leaves. Sneezing results from sniffing the leaf extracts, which are used to unclog clogged noses. Additionally, it treats headaches and a common cold (Pankhurst, 2001). Sterols and triterpenes were found in *C. hirsuta* petroleum ether and butanol extracts and demonstrated the plant's anti-inflammatory effects in a rat model (Abdel-Kader *et al.,* 2008).

In addition, *C. hirsuta* leaves were found to have antifungal activity in another research as well (Gruenwald *et al.,* 2000). Previous research on the several species in this genus has shown that the plant's various sections each have unique antibacterial properties. Triterpenoid, saponin, and alkaloid phytochemicals that have been extracted from various portions of the genus' species have been shown to have cytotoxic (Yan *et al.,* 2009), antibacterial (Ding *et al.,* 2009), and antifungal (Chen *et al.,* 2009) properties. The leaves and stem bark of *C. papuasica* showed a broad range of antibacterial activity (Khan et al., 2001). Concentration-dependent antibacterial and antifungal activity was detected in the methanol and petroleum ether extracts of the leaves of *Clematis longicauda* and *Clematis burgensis*, respectively (Hawaze *et al.,* 2012).

The plant's root could potentially provide strong natural antioxidants according to Zelalem Abdisa and Fekede Kenea, (2021). With a concentration of 2.000 mg/mL and a percentage DPPH inhibition of 98.2%, the root extract of *C. hirsuta* at the same dose concentrations demonstrated high radical scavenging activity as the standard ascorbic acid (95.8%). The root extract's %DPPH inhibition, however was lower at lower doses than ascorbic acid's at comparable concentrations. The *C. hirsuta* root's methanol extract shown some antioxidant properties. This plant's traditional use for the treatment of numerous illnesses is supported by the antioxidant activities that have been observed.

During the COVID-19 pandemic Kole district in Northern Uganda used different medicinal plants. Locally known as “Adwe”, *C. hirsuta* was the main plant used to treat the flu by crushing the flower of the plant and sniffing it. A cough, breathing difficulties and also sore throat can be treated by it but at a lower percentage compared to its treatment of the flu (Nakaziba *et al.,* 2021).

Plants such as *C. hirsuta* have been utilized by local people to cure leishmaniasis. Taking into the consideration of the above characteristics of the plants, the goal of this experiment is to test the antipromastigote activity of this plant decoction and hydrodistilled extracts by *in vitro* methods and to establish the toxicity level of the extracts for safer dosages.

3. MATERIAL AND METHODS

3.1. Materials

3.1.1. Test strains

Clinical isolates *Leishmania donovani* (VL: GR1140), *Leishmania aethiopica* (CL: 584/17) and human blood sample were provided by Leishmaniasis Research and Diagnostic Laboratory, Department of Immunology, Microbiology and Parasitology of School of Medicine, Addis Ababa University. *Leishmania aethiopica* (CL: 584/17) strain was acquired from a patient from Gurage zone, Tiya woreda. *Leishmania donovani* (VL: GR1140) strain was acquired from a patient from Amhara region, Gondar. Human blood sample was taken from a 24 year old healthy woman.

3.1.2. Reference drug

Amphotericin B (Gibco, United States) was used as positive control in antipromastigote activity experiment.

3.2. Methods

3.2.1. Plant material collection and extraction

The plant *Clematis hirsuta* leaves were collected and extracted from the highlands of Ochollo village, Gamo Zone, South Ethiopia (520 km southwest of Addis Ababa, Ethiopia, at latitude 6°25'09.0"N and longitude 37°48'00.8"E) located in the Rift Valley above the west shore of Lake Abaya. The plant material was authenticated by Mr. Melaku Wondafrash, an expert in National Herbarium, Department of Biology, College of Natural and Computational Sciences, Addis Ababa University (AAU), where a botanical specimen with the code BS006 was given and deposited for future reference.

3.2.1.1 Decoction extraction

Fresh leaves of *C. hirsuta* (600g) were cleaned with water to remove dirt. The plant material was then socked in enough amount of distilled water and subjected to decoction on a hot plate for 1 hour. After the decoction cooled down it was filtered first with sterile gauze and then using Whatman no. 1 filter paper. The filtrate was then concentrated in an oven at 40 °C. The dried

extract (0.44% yield) was finally labelled as CHD and transferred to an amber-coloured bottle and stored in a refrigerator at 4 °C until use as described previously by Zintchem *et al.,* (2013).

3.2.1.2 Hydrodistillation extraction

Fresh leaves of *C. hirsuta* (600g) were chopped into small pieces and subjected to hydrodistillation (boiling the plant in distilled water) for 3 hours using a distillation apparatus (Clevenger apparatus). The condensate was then collected and extracted with chloroform (3x) using a separatory funnel. The organic solvent was concentrated in a rotavapor at temperature not exceeding 35 ℃. The oil (1.86% yield) was then transferred into vial and labelled as CHH and stored in a refrigerator at 4 °C for further experiment as described earlier by Abdellatif and Hassani, (2015).

3.2.2 Selection of test strains

Test strains provided by the diagnostic laboratory were taken for observation. Small drops of different samples (test strains) were put on the different slides and spread. The samples were observed under the microscope for active motility, cylindrical body, less cluster and a good amount of number of CL/VL parasite.

Through a series of observing using an inverted light microscope different samples from different places and times, sample numbers 584/17 and GR1140 were selected for CL and VL respectively. The slanted NNN media made earlier was opened and its cap sterilized when opening. Two milliliter of lockes solution and a small drop of the parasite was added into this new culture flask. The cap was heated after closing to sterilize and the flask was labelled. After two days or so the flask was checked for growth. Later they were sub cultured into two new culture flasks containing NNN media and locke's solution for better growth. Once they were able to grow very well, they were transferred to liquid media hence complete liquid media. Every once in a while their volume was increased by adding media in small amounts. A small drop of the parasite was added on a haemocytometer (counting chamber) to make sure the count is from 1 x 10^6 promastigote/ml to 3 x 10^6 promastigote/ml (if less than 1 x 10^6 promastigote/ml then we would let the parasite grow a bit more for a couple of days). It was checked for clear and non-clustered presence while observing it again under a microscope. When it was confirmed about its suitability for the assay, the antipromastigote assay was proceeded to.

3.2.3 Antipromastigote assay

The assay was performed as previously described by Yitagesu Tewabe *et al.,* (2019). Our CL and VL assays were performed in triplicate with the following protocol. A uniform amount of 100 µl was added throughout the 96 well plate. Except for the first row where 120 µl of complete media was added along with 80 µl of extracts CHD (100 - 0.78125 µg/ml), CHH (100 - 0.78125 µg/ml) and positive control (amphotericin B) (10 - 0.078125 µg/ml) in triplicate form. One hundred microliter was then serial diluted from these wells and discarded on the last row. Thus a two-fold serial dilution was done. One hundred microliter of selected parasite (584/17 for CL; GR 1140 for VL) was added on all wells except for the blank well which contained only the media. Thus one column well in the plate then contained media and parasite which acted as a negative control. Finally, the cap of 96 well plate was cleaned using a guaze and closed. It was then covered with an aluminum foil and labelled (name, date of experiment, CL, reading date and time). It was incubated for 72 hours and on the 68 hrs, 20 µl alamar blue was added in each well. After 4 hours it was read on Fluoroskan Ascent^{TM}, Thermo Scientific at an excitation wavelength of 544 nm and an emission wavelength of 590 nm.

The median inhibitory concentration (IC_{50}) to parasites was obtained directly from linear equation of dose-response curves as well as the table format provided by fluoroskan ascent readings. IC_{50} value was expressed in number form. The antipromastigote activity of the extract was expressed in percentage of inhibition by this formula (Markos Tadele *et al.,* 2020):

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

3.2.4 Cytotoxicity test

3.2.4.1 Hemolysis assay

Hemolysis assay was performed as previously described by Fetene Abeje *et al.,* (2014). The assay was performed using the human blood sample provided by the laboratory. PBS was made first by mixing 1000 ml of distilled water and one phosphate buffer powder packet in a beaker. Four milliliter of the blood was taken from the EDTA tube that it was stored in by using a syringe. Forty

eight milliliter PBS and 2ml of the human blood was added into a 50ml falcon tube. A cold centrifuge at 4°C was used to centrifuge the mixture at 3500 rpm for 10 minutes. The pellet was saved and the supernatant was discarded. A drop of PBS was added and centrifuged at the same calibration again. This process was repeated three times. The pellet volume reached 1 ml through this process after third round of centrifugation repetition. Forty nine milliliter of PBS was added on the 1 ml pellet and mixed, making a red blood cell suspension. This experiment was performed in duplicate. In similarity to the antipromastigote assay, 100 µl of the red blood cell suspension was mixed with 100 µl of the serially diluted extracts (1000 – 7.8125 µg/ml) in an eppendorf tube. 2% Triton X-114 and 1% DMSO were used as positive and negative control, respectively. The Eppendorf tubes was incubated at 37°C for 2 hours, except for Triton which was for 30 min. It was then centrifuged at 1000 rpm for 10 mins. Seventy five microliter of the supernatant was transferred to a 96 well plate. It was 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 haemolytic effects were expressed in percentage by the formula below (Zohra and Fawzia, 2014).

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

3.2.5 Selectivity Index (SI)

The CC_{50} against red blood cells and their corresponding IC_{50} of against *Leishmania* promastigote were used to determine the selectivity index (SI) of each extract. The following formula was used to calculate selectivity of the extracts and the standards of killing parasites as opposed to mammalian cells (Dereje Nigussie *et al.,* 2015).

$$Selectivity\ index = \frac{CC50\ of\ red\ blood\ cells}{IC50\ of\ antipromastigote\ assay}$$

3.3. Statistical analysis

The IC_{50} of antipromastigote 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 hemolysis. GraphPad Prism 8.4.2 (GraphPad Software, LLC, CA, USA) computer software was used for data analysis. As well as Microsoft excel in expressing the values in mean ± standard error of the mean. The ratio of CC50 to IC50 was used to determine selectivity index (SI) (Tasisa Ketema *et al.,* 2023).

3.4. Ethical Clearance

All research experiments done in this study were approved by the Institutional Review Board, School of Pharmacy, College of Health Sciences, Addis Ababa University with the protocol number 092/21/SOP dated January 2022. The Review Board reviews basic research involving patients and human volunteers. The ethical clearance letter is therefore stated at the end of this thesis document.

4. RESULTS

4.1 Growth inhibition screening

The purpose of this screening was to assess the plant extracts of *C. hirsuta* ability to inhibit the *Leishmania* promastigotes. At 100 µg/ml, the decoction extract of *C. hirsuta* showed 75.36 ± 1.47 % and 87.37 ± 0.39 % growth inhibition on *L. aethiopica* and *L. donovani* respectively. While the hydrodistilled extract (100 µg/ml) of *C. hirsuta* showed 97.22 ± 0.02 % and 97.54 ± 0.07 % growth inhibition on *L. aethiopica* and *L. donovani* respectively. The hydrodistilled extract showed a higher percentage inhibition compared to the decoction extract in both cases. The positive control (Amp B) showed 98.73 ± 0.12 % and 98.31 ± 0.17 % growth inhibition on *L. aethiopica* and *L. donovani* respectively (Table 1). At different concentration each extract along with the Amp B had an increasing sigmoid curve when tested against both *Leishmania* promastigotes (Figure 11).

Table 1: Percentage of inhibition against *Leishmania* promastigotes

Types of extracts and Concentration	% of inhibition of extracts against *Leishmania* promastigotes	
	Against *L. aethiopica*	**Against *L. donovani***
CHD (100 µg/ml)	75.36 ± 1.47	87.37 ± 0.39
CHH (100 µg/ml)	97.22 ± 0.02	97.54 ± 0.07
Amp B (10 µg/ml)	98.73 ± 0.12	98.31 ± 0.17

The values are expressed as mean ± SEM; CHD: decoction extract of leaves of *C. hirsuta*; CHH: hydrodistilled extract of leaves of *C. hirsuta*; Amp B: Amphotericin B (positive control); 100 µg/ml indicates the concentrations of the extracts; 10 µg/ml indicates the concentrations of the Amp B.

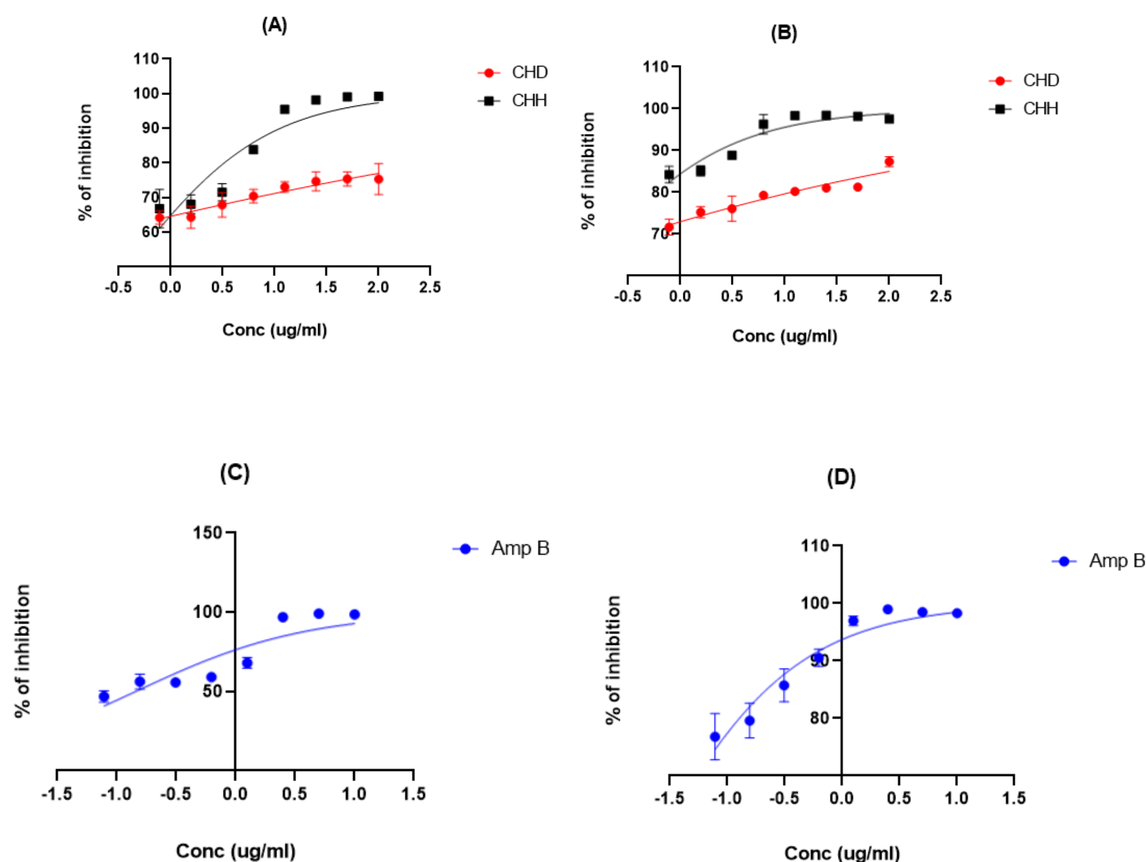

Figure 11: Percentage of growth inhibition of extracts and standard drug.

(A): Extracts against *L. aethiopica*; (B): Extracts against *L. donovani*; (C): Amp B against *L. aethiopica*; (D): Amp B against *L. donovani*; CHD: decoction extract of leaves of *C. hirsuta*; CHH: hydrodistilled extract of leaves of *C. hirsuta*; Amp B: Amphotericin B (positive control); Conc (ug/ml): Concentration was expressed/transformed to log ^{x}.

4.2 Antipromastigote assay

The purpose of this assay was to assess the concentration of the extracts along with Amp B at which 50% of *Leishmania* promastigotes are destroyed hence IC_{50} values are determined. The IC_{50} values of decocted extract were 0.01 µg/ml and 0.002 µg/ml against *L. aethiopica* and *L. donovani* respectively. While the IC_{50} values of hydrodistilled extracts were 0.39 µg/ml and 0.06 µg/ml against *L. aethiopica* and *L. donovani* respectively. The IC_{50} value of the positive control (Amp B) were 0.14 µg/ml and 0.01 µg/ml against *L. aethiopica* and *L. donovani* respectively. The IC_{50} value

of the decocted extract was lower than the hydrodistilled extract of *C. hirsuta* in both cases (Table 2).

Table 2: The IC_{50} of the extracts against *Leishmania* promastigotes.

Types of extracts	Against *L. aethiopica*		Against *L. donovani*	
	IC_{50} (µg/ml) (95% CI)	**R^2**	**IC_{50} (µg/ml) (95% CI)**	**R^2**
CHD	0.01 (0.001 - 0.04)*	0.74	0.002 (0.0003 – 0.007)*	0.85
CHH	0.39 (0.23 – 0.56)*	0.87	0.06 (0.02 – 0.11)*	0.86
Amp B	0.14 (0.08 – 0.21)*	0.80	0.01 (0.008 – 0.02)*	0.908

The values are expressed as mean; Calculated at 95% CI; CHD: decoction extract of leaves of *C. hirsuta*; CHH: hydrodistilled extract of leaves of *C. hirsuta*; Amp B: Amphotericin B; R^2 is measurement of fitness (regression coefficient); 95% CI: 95% confidence interval; Differences with *P* value < 0.05 (*) were considered significant.

4.3 Hemolysis assay

The purpose of this assay was to assess the percentage of red blood cell destruction that the extracts can cause in an *in vitro* environment at specific concentrations. The decoction extract of *C. hirsuta* showed 18.18 ± 2.14 % hemolysis on red blood cells. While the hydrodistilled extract of *C. hirsuta* showed 57.57 ± 4.28 % hemolysis on red blood cells. The hydrodistilled extract showed a higher hemolysis percentage compared to the decoction extract. The positive control hemolysis percentage was 100 % (Table 3). At different concentration each extract had an increasing curve (Figure 12).

Table 3: Hemolysis percentage

Types of extracts	% of hemolysis of extracts against human red blood cells
CHD (1000 µg/ml)	18.18 ± 2.14
CHH (1000 µg/ml)	57.57 ± 4.28
Triton X-114	100

The values are expressed as mean ± SEM; CHD: decoction extract of leaves of *C. hirsuta*; CHH: hydrodistilled extract of leaves of *C. hirsuta;* Trtion X-114: positive control; 1000 µg/ml indicates the concentrations of the extracts.

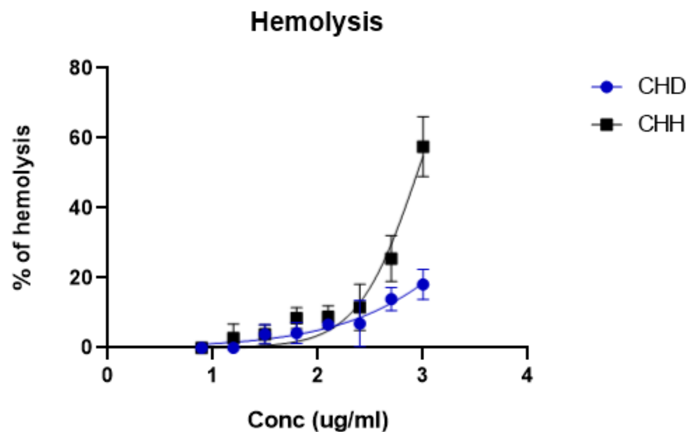

Figure 12: Hemolysis percentage graph

CHD: decoction extract of leaves of *C. hirsuta*; CHH: hydrodistilled extract of leaves of *C. hirsuta*; Conc (ug/ml): Concentration was expressed/transformed to log ^{x}.

4.4 Cytotoxic effect of *Clematis hirsuta* extracts

The purpose of this assay was to assess the concentration at which 50% of red blood cell destruction that the extracts can cause in an *in vitro* environment. The decocted extract had CC_{50} value >1000 µg/ml on red blood cells (erythrocyte). While the CC_{50} values of hydrodistilled extract was 881.0 µg/ml on red blood cells. The CC_{50} value of the hydrodistilled extract was lower than the decocted extract of *C. hirsuta* (Table 4).

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

Types of extracts	Against red blood cells	
	CC_{50} (µg/ml) (95% CI)	**R^2**
CHD	>1000 *	-
CHH	881.0 (748.9 – 1118) *	0.92

The values are expressed as mean; CHD: decoction extract of leaves of *C. hirsuta*; CHH: hydrodistilled extract of leaves of *C. hirsuta*; R^2 is measurement of fitness (regression coefficient); 95% CI: 95% confidence interval; Differences with *P* value < 0.05 (*) were considered significant.

4.5 Selectivity index of *Clematis hirsuta* extracts

The purpose of selectivity index determination was to evaluate the capability of the extracts to selectively destroy either the *Leishmania* promastigotes or the red blood cells. The selectivity index of the decocted extract showed a SI >1000 on both *Leishmania* promastigotes. While the hydrodistilled extract showed an SI of 2258.97 and 14,683.3 on *L. aethiopica* and *L. donovani* respectively. The SI value of the decocted extracts was much higher than the hydrodistilled extracts (Table 5).

Table 5: Selectivity index of extracts

Types of extracts	Against red blood cells (CC_{50} of cell/IC_{50} of parasite)	
	L. aethiopica	*L. donovani*
CHD	>1000	>1000
CHH	2258.97	14683.3

The values are expressed as ratio of CC_{50} of red blood cells and IC_{50} of *Leishmania* promastigote; CHD: decoction extract of leaves of *C. hirsuta*; CHH: hydrodistilled extract of leaves of *C. hirsuta.*

5. DISCUSSION

In this study, the plant *C. hirsuta* extracts (decocted and hydrodistilled extracts) were tested for their activity against *L. aethiopica* and *L. donovani*. This was accomplished by the determination of percentage of inhibition. Following that, the 50% concentration at which the extracts can have an inhibition effect (IC_{50}) was determined. The hemolysis percentage and CC_{50} were also calculated to test the toxicity of the extracts on red blood cells. Finally, selectivity index was determined.

The percentage of growth inhibition of the promastigotes *L. aethiopica* and *L. donovani* shown by the decocted extract was 75.36 ± 1.47 % and 87.37 ± 0.39 % respectively at the concentration of 100 µg/ml. While the percentage of inhibition of *L. aethiopica* and *L. donovani* shown by the hydrodistilled extract were 97.22 ± 0.02 % and 97.54 ± 0.07 % respectively at concentrations of 100 µg/ml (Table 1). This showed that the hydrodistilled extract have a higher inhibition compared to the decocted extract at the concentration of 100 µg/ml. In Brazil antileishmania tests were carried out and they found some plants of being active against *Leishmania amazonensis*. They found out that plant extracts *Chenopodium ambrosioides* and *Pfaffia glomerata* demonstrated direct efficacy against the parasite at 87.4% and 88.7% suppressing growth respectively. Along with the extract *Ruta graveolens* which dramatically reduced the quantity of the promastigotes in this investigation, showing 74.4% suppression at 100 *µ*g/mL as well (De Queiroz *et al.,* 2014). This showed that decocted and hydrodistilled extracts are very ideal inhibitor of the *Leishmania* promastigotes in this study when compared to the Brazilian plants seen in the other study.

Firstly, the hydrodistilled extract had a similar inhibition against *L. donovani* and *L. aethiopica.* This reached the conclusion that CHH is a good inhibitor against *L. donovani* and *L. aethiopica*. Secondly, this extract showed a very high inhibition for both *Leishmania* promastigotes compared to the decocted extract and a slightly lower inhibition compared to the positive control (Amp B). This suggested that CHH is a better inhibitor on both *L. aethiopica* and *L. donovani* than CHD.

While the decocted extract had a better inhibition against *L. donovani* than *L. aethiopica.* This confirms that CHD acts better on *L. donovani* than *L. aethiopica*. The decocted extract also showed that it had the lowest percentage of inhibition compared to the hydrodistilled extract. However,

against *L. donovani* the decocted extract had a much closer inhibition percentage to CHH. This suggested that CHD is more or less a good inhibitor against *L. donovani* as much as CHH.

This confirms the plants leaves activity in the traditional treatment of leishmaniasis as it is known to be used by the people of Debre Libanos monstery (Tilahun Teklehaymanot *et al.,* 2007).

Even though there aren't studies on antileishmania activity of *C. hirsuta*, there are other studies showing its effectiveness in antibacterial and antioxidant tests. One of these studies showed that the root methanol extract has a strong inhibition against microorganisms like *E. coli*. Its chloroform extract also showed a somewhat inhibition against *S. aureus* and *P. aeruginosa* (Zelalem Abdisa and Fekede Kenea, 2021).

In another study conducted by Gemechu Ameya *et al.,* (2022) it showed that the leaf extract of *C. hirsuta* had the highest zone of inhibition on the bacterium *E. coli*. Lower concentrations of the leaf extracts were able to inhibit the bacteria and fungi in this study.

This shows that it's a medicinal plant with the ability of inhibiting microorganisms. Thus antipromastigote results of its extracts also proved that its activity against the promastigote life form of the leishmania parasite.

As presented in Figure 11, the decocted and hydrodistilled extracts did not make an S-shaped graph. However the uphill graph suggested that CHD and CHH had high percentages of inhibition at concentrations ranging from 100 – 0.78125 µg/ml. The graph shows that the curve at the highest peak is the highest inhibitor. The opposite is true for the lowest peak on the graph.

The antipromastigote activity of the decocted extracts were presented to be lower than the positive controls (Amp B) activities, while the hydrodistilled extracts was higher than Amp B. The IC_{50} value represents the concentration at which the extracts can destroy 50% of the test strains. Based on this, the lower the IC_{50} value the better activity the extract has. The IC_{50} values of decocted extract were lower against both *L. aethiopica* and *L. donovani* than when compared to CHH. Thus at a very low concentration it is very active against both of the test strains. While the IC_{50} values of hydrodistilled extracts were also low but not as low as CHD against both *L. aethiopica* and *L. donovani*. The IC_{50} value of the positive control (Amp B) were also low against *L. aethiopica* and *L. donovani* (Table 2).

The antipromastigote activity of the extracts was determined based on the screening (ranges). An IC_{50} less than or equal to 5 µg/mL has strong activity; IC_{50} between 5 and/or equal to 20 µg/mL has good activity; IC_{50} between 20 and/or equal to 30 µg/mL has mild activity; IC_{50} between 30 and/or equal to 64 µg/mL has poor activity; and IC_{50} greater than 64 µg/mL has no activity (Ehata *et al.,* 2012). Based on this, both decocted and hydrodistilled extracts have strong activity against both *L. aethiopica* and *L. donovani*. This also proves its activity against leishmania promastigotes. When comparing CHH and Amp B IC_{50} value, the closer IC_{50} value was the one against *L. donovani.* This meant that 0.06 µg/mL of CHH destroyed 50% of *L. donovani* while 0.01 µg/mL of Amp B destroyed 50% of *L. donovani*. This suggested that CHH and Amp B had very strong activities when against *L. donovani*. However, Amp B had a better activity compared to CHH. The IC_{50} value of CHH and Amp B when tested against *L. aethiopica* were further apart than when tested on *L. donovani*. Hence it showed that Amp B had a much stronger activity than CHH.

The decocted extracts IC_{50} value were the lower in this study. When CHD was against *L. donovani* it had a lower IC_{50} value compared to when it was against *L. aethiopica.* This meant that 0.002 µg/mL of CHD destroyed 50% of *L. donovani*. This suggests it is more active against *L. donovani.* When comparing CHD and Amp B IC_{50} value, they had further values from each other. In both cases, when CHD was tested on *L. aethiopica* and *L. donovani*, it had a better activity than Amp B and CHH. However, in both cases of test strains it had a closer activity to Amp B than CHH, suggesting it had higher activity than CHH.

Other plant extracts such as *Brucea antidysenterica* J.F. Mill Seeds and its solvent fractions were tested for antileishmania activity in which the ethyl acetate fraction exhibited high antipromastigote activity with $4.14 \pm 0.62 \leq IC_{50} \leq 6.77 \pm 0.47$ µg/mL while the aqueous fractions had the lowest activity with $189.3 \pm 8.70 \leq IC_{50} \leq 208.9 \pm 20.2$ µg/mL. Its crude extract (80% methanol) showed $20.77 \pm 1.55 \leq IC_{50} \leq 27.83 \pm 1.06$ µg/mL of antipromastigote activity (Tasisa Ketema *et al.,* 2023). This study compared to *C. hirsuta* antipromastigote activity shows that the crude leaf extracts of *C. hirsuta* has better antipromastigote activity than that of the methanol extract and fractions of *Brucea antidysenterica.*

After the activity of the plants extracts being known, next the toxicity level of the extracts was determined. The decocted extract had 18.18 ± 2.14 % hemolysis while hydrodistilled extract had 57.57 ± 4.28 % hemolysis at concentrations of 1000 µg/ml. Triton X-114 had 100 % hemolysis

as well (Table 3). Hydrodistilled extract had a higher hemolytic activity compared to decocted extract. While the decocted extract had the lowest hemolytic activity. Both the decocted and hydrodistilled extracts had less hemolytic activity than positive control. Thus the hydrodistilled extract can destroy more than 50 % of red blood cells while Triton X-114 can assure a 100 % destruction.

As presented in Figure 12, the decocted and hydrodistilled extracts made a uphill graph. This graph suggests that CHH has higher percentages of hemolytic activity than CHD at concentrations ranging from 1000 – 7.8125 µg/ml. On the graph, it can be seen that the curve at the highest peak was the highest hemolytic, in this case CHH and the one at the lowest peak is CHD.

With the use of hemolysis percentage lead to the determination of CC_{50} value of the plant extracts. This was the determination of the half concentration at which hemolysis can occur by these plant extracts. The toxicity criteria falls at different ranges; <10µg/mL very strong cytotoxicity, 10–100 µg/mL strong cytotoxicity and 100–500 µg/mL moderate cytotoxicity (Indrayanto *et al.,* 2020). The decocted extracts and hydrodistilled extract had a CC_{50} of >1000 µg/ml and 881.0 µg/ml respectively (Table 4). The CC_{50} value of the decocted extract was higher than the hydrodistilled extract. This meant that the decocted extract and the hydrodistilled extract destroyed 50% of red blood cells at >1000 µg/ml and 881.0 µg/ml concentrations respectively with the range of concentration being 1000 – 7.8125 µg/ml. This showed that the hydrodistilled was closer to be moderately cytotoxic compared to that of the decocted extract, which was less toxic. Over all, the extracts were less cytotoxic based on the range. Since Triton X-114 had 100 % hemolysis, it is the most cytotoxic compared to CHH and CHD.

Once the CC_{50} values and IC_{50} values were determined, the selectivity index was calculated. *Leishmania* parasites are believed to be selectively destroyed by an extract with selective index (SI) >1, while mammalian host cells are believed to be selectively destroyed by a selective index (SI) <1 (Indrayanto *et al.,* 2020). The decocted extract had a SI >1000 on both *Leishmania* promastigotes species. While the hydrodistilled extract had SI of 2258.97 on *L. aethiopica* and 14,683.3 on *L. donovani* (Table 5). According to this, both extracts are selective against both *Leishmania* promastigotes. The hydrodistilled extract SI against *L. aethiopica* has less selectivity of the parasite compared to SI against *L. donovani*. It also had less selectivity compared to

decocted extract. This shows that the hydrodistilled extract had a small chance of selectivity against red blood cells compared to the decocted extract.

The limitation during this study were the difficulty in culturing the parasites strains to a certain volume for assay capabilities. They can be problematic if not monitiored regularly and subcultured frequently. Even with proper maintenance some parasites strains were able to not grow as much as they were needed to. Creating a proper environment along with proper selection of ideal test strains helped in overcoming this problem but even then the ideal volume of strains for the assay were hard to grow.

This research can be significant in way that it shows that the plant *C. hirsuta* has antipromastigote activity which opens a doorway to further studies. In my suggestion, drug manufacturing from this plant may provide a better alternative to the current drugs for the treatment of leishmaniasis. This may in turn make the drug affordable as well. This study will act as a reference for future studies.

6. CONCLUSION

The current study showed that the leaves of *C. hirsuta* extracts (decocted and hydrodistilled) have different levels of antipromastigote activity at different concentrations against both *L. aethiopica* and *L. donovani*. The hydrodistilled extract was found to be the most active against both *L. aethiopica* and *L. donovani* than the decocted extract. It was also almost as active as Amp B, which is a significant result since Amp B is a known drug used in treatment of leishmaniasis. The decocted extract showed above 75 % inhibition while the hydrodistilled extract showed above 97 % inhibition against both *L. aethiopica* and *L. donovani*. The extracts were also found less toxic against human red blood cells as well. This study validated the traditional claims of *C. hirsuta* use in the treatments of leishmaniasis as it has proven the antipromastigote activity of the plant.

7. RECOMMENDATION

Given that the leaf extracts of *Clematis hirsuta* have demonstrated promising potential against *L. aethiopica* and *L. donovani*, this investigation suggests the need for additional research to address the followings:

- Isolation and testing on its different fractions.
- Further evaluation on its antiamastigote activity potential using animal model.
- Testing its toxicity further on Vero cell lines and mouse macrophages.
- Isolation of the specific compound responsible for its antipromastigote activity.
- Lastly, I would recommend to users of traditional medicine to use *C. hirsuta* for leishmaniasis treatment in small amounts but to seek a more modern treatment for safety.

REFERENCES

Abdel-Kader, M. S., Al-Taweel, A. M., and El-Deeb, K. S. (2008). Bioactivity guided phytochemical study of *Clematis hirsuta* growing in Saudi Arabia. *Nat. Prod. Sci*. **14**(1): 56.

Abdellatif, F. and Hassani, A. (2015). Chemical composition of the essential oils from leaves of *Melissa officinalis* extracted by hydrodistillation, steam distillation, organic solvent and microwave hydrodistillation. *J. Mater. Environ. Sci.* **6**(1): 207-213.

Abrha Bsrat, Nega Berhe, Meshesha Balkew, Mekonnen Yohannes, Tsigemariam Teklu, *et al.,* (2015). Epidemiological study of cutaneous leishmaniasis in Saesie Tsaeda-emba district, eastern Tigray, northern Ethiopia. *Parasit. Vectors*. **8**: 149.

Ahmed Seid, Endalamaw Gadisa, Teshome Tsegaw, Adugna Abera, Aklilu Teshome, *et al.,* (2014). Risk map for cutaneous leishmaniasis in Ethiopia based on environmental factors as revealed by geographical information systems and statistics. *Geospat. Health*. **8**: 377–87.

Aklilu Lemma, Foster, W.A., Teferi Gemetchu, Preston, P.M., Bryceson, A. and Minter, D.M. (1969). Studies on leishmaniasis in Ethiopia: I. Preliminary investigations into the epidemiology of cutaneous leishmaniasis in the highlands. *Ann. Trop. Med. Parasitol.* **63**: 455–472.

Alsaad, R.K.A. and Kawan, M.A. (2021). *In vitro* cultivation of *Leishmania* spp. Using blood from human and dog in Novy-MacNeal-Nicolle (N.N.N.) modified medium. *Ann. Romanian Soc. Cell Biol*. **25**(6): 17753–17762.

Al-Salem, W., Herricks, J.R. and Hotez, P.J. (2016). A review of visceral leishmaniasis during the conflict in South Sudan and the consequences for East African countries. *Parasit. Vectors*. **9**: 460.

Al-Taweel, A. M., El-Deeb, K. S., Abdel-Kader, M. S., and Mossa, J. S. (2007). GC/MS analysis of the fatty acids of three *Clematis* species growing in Saudi Arabia and their anti-inflammatory activity. *Saudi Pharma. J.* **15**(3/4): 224.

Alvar, J., Aparicio, P., Abraham Aseffa, Den Boer, M., Canavate, C., *et al.,* (2008). The relationship between leishmaniasis and AIDS: The second 10 years. *Clin. Microbiol. Rev*. **21**(2): 334–359.

Alvar, J., Velez, I.D., Bern, C., Herrero, M., Desjeux, P., *et al.,* (2012). Leishmaniasis worldwide and global estimates of its incidence. *PLoS One*. **7**: e35671.

Asilian, A., Sharif, A., Faghihi, G., Enshaeieh, S., Shariati, F. and Siadat, A.H. (2004). Evaluation of CO_2 laser efficacy in the treatment of cutaneous leishmaniasis. *Int. J. Dermatol.* **43**: 736–738.

Asmamaw Habtamu and Yalemtsehay Mekonnen (2017). Antibacterial potential of the 80% methanol and chloroform extracts of *Clematis hirsuta. Afr. J. Pharm. Pharmacol.* **11**(16): 204-208.

Asrat Hailu, Di Muccio, T., Tamrat Abebe, Mesfin Hunegnaw, Kager, P.A. and Gramiccia, M. (2006). Isolation of *Leishmania tropica* from an Ethiopian cutaneous leishmaniasis patient. *Trans. R. Soc. Trop. Med. Hyg.* **100**: 53–8.

Asrat Hailu, Gramiccia, M. and Kager, P.A. (2009).Visceral leishmaniasis in Aba-Roba, south-western Ethiopia: prevalence and incidence of active and subclinical infections. *Ann. Trop. Med. Parasitol.* **103**: 659–70.

Asrat Hailu, Musa A, Wasunna M, Balasegaram M, Sisay Yifru, Getahun Mengistu, *et al.,* (2010). Geographical variation in the response of visceral leishmaniasis to paromomycin in East Africa: a multicentre, open-label, randomized trial. *PLoS Negl. Trop. Dis*. **4**(10): 709.

Ayalew Assefa. (2018). Leishmaniasis in Ethiopia: A systematic review and meta-analysis of prevalence in animals and humans. *Heliyon*. **4**(8):e00723.

Aysheshm Kassahun, Sadlova, J., Benda, P., Kostalova, T., Warburg, A., Asrat Hailu, *et al.,* (2015). Natural infection of bats with Leishmania in Ethiopia. *Acta Trop*. **150**: 166–70.

Baerts, M. and Lehmann, J. (1989). *Guérisseurs et plantes médicinales de la région des crètes Zaire-Nil au Burundi*. In: Annalen economische wetenschappen. Ed. Koninklijk museum voor Midden-Afrika, Tervuren, Belgium, Vol. **18**.

Banuls, A-L., Hide, M. and Prugnolle, F. (2007). Leishmania and the leishmaniasis: a parasite genetic update and advances in taxonomy, epidemiology and pathogenicity in humans. *Adv. Parasitol.* **64**: 1–458.

Bennett, J.E., Dolin, R. and Blaser, M. J. (2015). *Mandell, Douglas, and Bennett's principles and practice of infectious diseases* (8th edition). Elsevier Saunders, Philadelphia.

Bereket Alemayehu, Abraham Getachew Kelbore, Mihiretu Alemayehu, Chimdesa Adugna, Tessema Bibo, Aberham Megaze and Leirs, H. (2023). Knowledge, attitude and practice of the rural community about cutaneous leishmaniasis in Wolaita zone, southern Ethiopia. *Plos one.* **18**(3): 1-13.

Berhan Mengiste, Abebe Mekuria, Haileleul Aleme, Berihun Afera and Guesh Negash. (2014). Treatment of Skin Disease Using Ointment of Latex of *Euphorbia abyssinica* Medicinal Plant on Animal Model. *World Appl. Sci. J*. **32** (9): 1913-1917.

Berman, J.D. (1997). Human leishmaniasis: clinical, diagnostic, and chemotherapeutic developments in the last 10 years. *Clin. Infect. Dis*. **24**(4): 684–703.

Berman, J. (2003). Current treatment approaches to leishmaniasis. *Curr. Opin. Infect. Dis.* **16**(5): 397–401.

Bern, C., Maguire, J.H. and Alvar, J. (2008) Complexities of assessing the disease burden attributable to leishmaniasis. *PLoS Negl. Trop. Dis*. **10**: e313.

Bilgic-Temel, A., Murrell, D.F. and Uzun, S. (2019). Cutaneous leishmaniasis: A neglected disfiguring disease for women. *Int. J. Women's Dermatol*. **5**(3): 158-165.

Boelaert, M. and Sundar, S. (2014). Leishmaniasis. In *Manson's tropical diseases. 23rd ed.* Elsevier Saunders, Philadelphia, pp. 631-651.

Castells-Sala, C., Martorell, J. and Balcells, M. (2017). A human plasma derived supplement preserves function of human vascular cells in absence of fetal bovine serum. *Cell Biosci.* **7**: 41.

CDC. (2020). Epidemiology and risk factors. [Available from: https://www.cdc.gov/parasites/leishmaniasis/epi.html]

CDC. (2020b). Biology. [Available from: https://www.cdc.gov/parasites/leishmaniasis/biology.html]

CDC. (2023). Parasites-Leishmaniasis-Resources for health professionals. [Available from: https://www.cdc.gov/parasites/leishmaniasis/health_professionals]

Chakravarty, J. and Sundar, S. (2019). Current and emerging medications for the treatment of leishmaniasis. *Expert Opin. Pharmacother.* **20**(10): 1251-1265.

Chelladurai, K.S., Christyraj, J.D.S., Rajagopalan, K., Yesudhason, B.V., Venkatachalam, S., Mohan, M., Vasantha, N.C. and Christyraj, J.R.S.S. (2021). Alternative to FBS in animal cell culture: An overview and future perspective. *Heliyon.* **7**: e07686.

Chen, J. H., Du, Z. Z., Shen, Y. M., and Yang, Y. P. (2009). Aporphine alkaloids from *Clematis parviloba* and their antifungal activity. *Arch. Pharm. Res*. **32**(1): 3–5.

Cheuka, P., Mayoka, G., Mutai, P. and Chibale, K. (2017). The role of natural products in drug discovery and development against neglected tropical diseases. *Molecules*. **22**: 58.

Chhabra, S.C., Mahunnah, R.L.A. and Mshiu, E.N. (1991). Plants used in traditional medicine in eastern Tanzania. V. Angiosperms (Passifloraceae to Sapindaceae). *J. Ethnopharmacol.* **33**: 143–157.

Chouihi, E., Amir, F., Bouslimi, N., Siala, E., Selmi, K., *et al.,* (2009). Cultures on NNN medium for the diagnosis of leishmaniasis. *Pathol. Biol.* (Paris). **57**(3): 219-24.

Cortés, A., Gutiérrez, L. I. and Aoki, M. K. (1998). Effect of *Backebergia militaris* Cactus Extract on Intestinal Smooth Muscle Contractility. *Phytother. Res*. **12**: 480-483.

Cos, P., Hermans, N., De Bruyne, T., Apers, S., Sindambiwe, J.B., Vanden, B.D., Pieters, L. and Vlietinck, A.J. (2002a). Further evaluation of Rwandan medicinal plant extracts for their antimicrobial and antiviral activities. *J. Ethnopharmacol*. **79**: 155-163.

Cos, P., Hermans, N., Van Poel, B., De Bruyne, T., Apers, S., Sindambiwe, J.B. Vanden Berghe, D., Pieters, L. and Vlietinck, A.J. (2002b). Complement modulating activity of Rwandan medicinal plants. *Phytomedicine.* **9**: 56-61.

Cota, G.F., de Sousa, M.R., Fereguetti, T.O. and Rabello, A. (2013). Efficacy of antileishmania therapy in visceral leishmaniasis among HIV infected patients: a systematic review with indirect comparison. *PLoS Negl. Trop. Dis.* **7**(5): 2195.

Croft, S. L. and Coombs, G. H. (2003). Leishmaniasis—current chemotherapy and recent advances in the search for novel drugs. *Trends Parasitol.* **19**(11): 502–508.

Dawit Abebe, Medhin Zewdu, and Abebe Demisie (2001). "The role of medicinal plants in health care coverage of Ethiopia, the possible integration," in Conservation and Sustainable Use of Medicinal Plants in Ethiopia, proceeding of the national work shop, pp. 6–21.

Dawit Gebremichael. (2018). Zoonotic impact and epidemiological changes of leishmaniasis in Ethiopia. *Open Vet. J.* **8**: 432–40.

Dawit Gebremichael, Bariagabr, F.H. and Hagos Hadgu. (2018). Incidence and trends of leishmaniasis and its risk factors in Humera, Western Tigray. *J. Parasitol. Res*. **2018**: 8463097.

Dawit Gebremichael, Girma Zewde and Simenew Keskes. (2013). A review on biology, epidemiology and public health significance of leishmaniasis. *J. Bacteriol. Parasitol*. **4**: 2.

De Queiroz, A.C., Dias, T.de L., Da Matta, C.B., Silva, L.H.C., *et al.,* (2014). Antileishmanial activity of medicinal plants used in endemic areas in northeastern Brazil. *Evid. Based Complement Alternat. Med.* **2014**: 478290.

Dereje Nigussie, Geremew Tasew, Eyasu Makonnen, Asfaw Debella, *et al.,* (2015). *In-vitro* investigation of fractionated extracts of *Albizia gummifera* Seed against *Leishmania donovani* amastigote stage. *J. Clin. Cell. Immunol*. **6**(6): 373.

Desjeux, P. (2004). Leishmaniasis: current situation and new perspectives. *Comp. Immunol. Microbiol. Infect. Dis.* **27:** 305– 318.

Didwania, N., Shadab, M., Sabur, A., Ali N. Shadab, M., Sabur, A. and Ali, N. (2017). Alternative to Chemotherapy—The Unmet Demand against Leishmaniasis. *Front Immunol*. **8**: 1779.

Ding, Q., Yang, L. X., Yang, H. W., Jiang, C., Wang, Y. F., and Wang, S. (2009). Cytotoxic and antibacterial triterpenoid derivatives from *Clematis ganpiniana*. *J. Ethnopharmacol.* **126**(3): 382–385.

Dorsey, B.M., Cass, C.L., Cedeño, D.L., Vallejo, R. and Jones, M.A. (2018) Effects of specific electric field stimulation on the release and activity of secreted acid phosphatases from *Leishmania tarentolae* and implications for therapy. *Pathogens.* **7**(4): 77.

Ehata, M.T., Phuati, A.M., Lumpu, S.N., *et al.,* (2012). In vitro antiprotozoal and cytotoxic activity of the aqueous extract, the 80 % methanol extract and its fractions from the seeds of *Brucea sumatrana* Roxb. (Simaroubaceae) growing in democratic Republic of Congo. *Chin Med*. **3**(1): 65–71.

Endalamaw Gadisa, Teshome Tsegaw, Adugna Abera, Elnaiem, D.E., Den Boer, M., Abraham Aseffa and Jorge, A. (2015). Eco-epidemiology of visceral leishmaniasis in Ethiopia. *Parasit. Vectors.* **8**: 381.

Ermias Diro, Lynen, L., Mohammed, R., *et al.,* (2014). High parasitological failure rate of visceral leishmaniasis to sodium stibogluconate among HIV co-infected adults in Ethiopia. *PLoS Negl. Trop. Dis.* **8**(5): 2875.

Fetene Abeje, Daniel Bisrat, Asrat Hailu and Kaleab Asres. (2014). Phytochemistry and Antileishmanial Activity of the Leaf Latex of *Aloe calidophila* Reynolds. *Phytother. Res*. **28**(12): 1801-1805.

Gahamanyi, N., Munyaneza, E., Dukuzimana, E., Tuyiringire, N., Pan, C.-H. and Komba, E.V.G. (2021). Ethnobotany, Ethnopharmacology, and Phytochemistry of Medicinal Plants Used for Treating Human Diarrheal Cases in Rwanda: A Review. *Antibiotics*. **10**: 1231.

García, M., Monzote, L., Scull, R. and Herrera, P. (2012). Activity of Cuban Plants Extracts against *Leishmania amazonensis*. *ISRN Pharmacol.* **2012**: 104540.

Gemechu Ameya, Manilal, A., Sabu, K.R. and Solomon Aragie. (2022). Bioassay-Guided Phytochemical Analyses and Antimicrobial Potentials of the Leaf Extract of *Clematis hirsuta* Perr. and Guill. Against Some Pathogenic Bacteria and Fungi. *Infect. Drug Resist*. pp 6577-6588.

Gessessew Bugssa, Asrat Hailu and Balem Demtsu. (2014). The current status of cutaneous leishmaniasis and the pattern of lesions in Ochollo Primary School Students, Ochollo, Southwestern Ethiopia. *Sci. J. Clin. Med*. **3**(6):111-116.

Ghorbani, M. and Farhoudi, R. (2017). Leishmaniasis in humans: drug or vaccine therapy? *Drug Des. Devel. Ther*. **12**:25.

Gidey Yirga. (2010). Assessment of indigenous knowledge of medicinal plants in Central Zone of Tigray, Northern Ethiopia. *Afr. J. Plant Sci*. **4**(1): 6-11.

Gossage, S.M., Rogers, M.E. and Bates, P.A. (2003). Two separate growth phases during the development of Leishmania in sand flies: implications for understanding the life cycle. *Int. J. Parasitol.* **33**:1027–1034.

Grimaldi, G. and Tesh, R. (1993) Leishmaniasis of the New World: current concepts and implications for future research. *Clin. Microbiol. Rev.* **6**: 230-250.

Gruenwald, J., Brendler, T., and Jaenicke, C. (2000). *PDR for herbal medicines.* Medicinal Economics Company. Montvale, New Jersey.

Haile Yineger and Delenasaw Yewhalaw (2007). Traditional medicinal plant knowledge and use by local healers in Sekoru District, Jimma Zone, Southwestern Ethiopia. *J. Ethnobiol. Ethnomed.* **3**: 24.

Haile Yineger, Ensermu Kelbessa, Tamrat Bekele and Ermias Lulekal (2007). Ethnoveterinary medicinal plants at Bale Mountains National Park, Ethiopia. *J. Ethnopharmacol*. **112**(1): 55-70.

Hao, D., Gu, X. and Peng, Y. (2013). Chemical and biological research of *Clematis* medicinal resources. *Chin. Sci. Bull.* **58**(10): 1120-1129.

Hawaze, S., Deti, H., and Suleman, S. (2012). In vitro antimicrobial activity and phytochemical screening of clematis species indigenous to Ethiopia. *Indian J. Pharm. Sci.* **74**(1): 29–35.

Hejazi, H., Eslami, G. and Dalimi, A. (2004). The parasiticidal effect of electricity on *Leishmania major*, both in vitro and in vivo. *Ann. Trop. Med. Parasitol.* **98**(1): 37–42.

Helina Fikre, Mashalla, Y.J., Mohammed, R. and Tshweneagae, G.T. (2019). Cutaneous leishmaniasis a neglected tropical disease: community knowledge, attitude and practices in an endemic area, Northwest Ethiopia. *BMC Infect Dis*. **19**: 855.

Herwaldt, B.L. (1999a). Leishmaniasis. *Lancet*. **354**(9185): 1191–9.

Herwaldt, B.L. (1999b). Miltefosine—the long-awaited therapy for visceral leishmaniasis? Massachusetts Medical Society.

Hiwot Ayalew, Solomon Tadesse, Daniel Bisrat, Lindemann P, Yitagesu Tewabe, Asrat Hailu and Kaleab Asres. (2018). Chemical Composition and *In Vitro* Antipromastigotes Activity of Essential Oil of the Leaves of *Discopodium pennnervium* Hochst. *EPJ*. **34**(2): 75–80.

Hotez, P.J., Remme, J.H., Buss, P., George, G., Morel, C., *et al.,* (2004) Combating tropical infectious diseases: report of the Disease Control Priorities in Developing Countries Project. *Clin. Infect. Dis*. **6**: 871-878.

Hulubanchi Nigatu, Alemnesh Belay, Hiwot Ayalew, Besufekad Abebe, Alemu Tadesse, Yitagesu Tewabe and Abel Degu (2021). In Vitro Antileishmanial Activity of Some Ethiopian Medicinal Plants. *J. Exp. Pharmacol.* **13**: 15-22.

Indrayanto, G., Putra, G.S. and Suhud, F. (2020). *Profiles of Drug Substances, Excipients, and Related Methodology:* Validation of in-vitro Bioassay Methods: Application in Herbal Drug Research (1st edition). Elsevier Inc.

Ives, A., Ronet, C., Prevel, F., Ruzzante, G., Fuertes-Marraco, S., Schutz, F., *et al.,* (2011). Leishmania virus controls severity of mucocutaneous leishmaniasis. *Science*. **331**(6018): 775-778.

Jhingran, A., Chawla, B., Saxena, S., Barrett, M.P. and Madhubala, R. (2009). Paromomycin: uptake and resistance in *Leishmania donovani*. *Mol. Biochem. Parasitol*. **164**(2): 111–117.

Kanyina, E.W. (2020). Characterization of visceral leishmaniasis outbreak, Marsabit County, Kenya, 2014. *BMC Public Health*. **20**: 446.

Khaliq, T., Misra, P., Gupta, S. *et al.,* (2009). Peganine hydrochloride dihydrate an orally active antileishmanial agent. *Bioorg. Med. Chem. Lett*. **19**(9): 2585–2586.

Khan, M. R., Kihara, M., and Omoloso, A. D. (2001). Antimicrobial activity of *Clematis papuasica* and *Nauclea obversifolia*. *Fitoterapia*. **72**(5): 575–578.

Kimutai, R., Musa, A.M., Njoroge, S., Omollo, R., Alves, F., *et al.,* (2017). Safety and effectiveness of sodium stibogluconate and paromomycin combination for the treatment of visceral leishmaniasis in Eastern Africa: results from a pharmacovigilance programme. *Clin. Drug Investig*. **37**(3): 259–272.

Kokwaro, J.O. (1976). *Medicinal plants of East Africa*. East african literature Bureau, Nairobi. Department of Botany, University of Nairobi, Nairobi, Kenya.

Lage, P.S., de Andrade, P.H.R, Lopez, A.S., Fumagalli, M.A.C. *et al.,* (2013). *Strychnos pseudoquina* and Its Purified Compounds Present an Effective *Invitro* Antileishmanial Activity. *Evid. Based Complement Alternat. Med.* **2013**: 304354.

Lahmar, R., Mahjoub, T., Fourel, I., Gault, G. and Grancher, D., (2018). Giant fennel (*Ferula communis* L) intoxication in goats in Tunisia. *Vet. Rec. Case Rep*. **6** (4): e000738.

Lobanovska, M. and Pilla, G. (2017). Penicillin's Discovery and Antibiotic Resistance: Lessons for the Future? *Yale J. Biol. Med.* **90**(1): 135–145.

Lodge, R. and Descoteaux, A. (2008). Leishmania invasion and phagosome biogenesis. In: Molecular mechanisms of parasite invasion. Springer. pp. 174-181.

Luzzatto, L., Apirion, D. and Schlessinger, D. (1968). Mechanism of action of streptomycin in *E. coli*: interruption of the ribosome cycle at the initiation of protein synthesis. *Proc. Natl. Acad. Sci.* **60**(3): 873–880.

Malaria Consortium. (2010). Leishmaniasis Control in Eastern Africa: Past and Present Efforts and Fututre Needs. Situation and Gap Analysis.

Mann, S., Frasca, K., Scherrer, S., *et al.,* (2021). A Review of Leishmaniasis: Current Knowledge and Future Directions. *Curr. Trop. Med. Rep*. **8**: 121-132.

Markos Tadele, Solomon Mequanate Abay, Eyasu Makonnen and Asrat Hailu. (2020). *Leishmania donovani* Growth Inhibitors from Pathogen Box Compounds of Medicine for Malaria Venture. *Drug Des. Devel. Ther.* **14**: 1307-17.

Menberework Chanyalew, Abraham Aseffa, Harboe, M., Okao, J.O. and Asrat Hailu (2009). Experimental infections with *Leishmania aethiopica* and immune response analysis of *Cercopithecus aethiops. Ethiop. J. Health Biomed. Sci*. **2**(1): 1–8.

Mesfin Tadesse, Debela Hunde and Yehenew Getachew (2005). Survey of medicinal plants used to treat human diseases in Seka Chekorsa, Jimma Zone, Ethiopia. *Ethiop. J. Health Sci.* **15**: 2.

Mirutse Giday, Zemede Asfaw and Zerihun Woldu (2009). Medicinal plants of the Meinit ethnic group of Ethiopia: An ethnobotanical study. *J. Ethnopharmacol*. **124**: 513–521.

Mishra, M., Biswas, U.K., Jha, D.N., *et al.,* (1992). Amphotericin versus pentamidine in antimony-unresponsive kala-azar. *Lancet.* **340**(8830): 1256–1257.

Mondal, S., Bhattacharya, P. and Ali, N. (2010). Current diagnosis and treatment of visceral leishmaniasis. *Expert Rev. Anti. Infect. Ther*. **8(**8): 919–944.

Monge-Maillo, B. and López-Vélez, R. (2013). Therapeutic options for visceral leishmaniasis. *Drugs.* **73**(17): 1863–1888.

Mueller, M., Ritmeijer, K., Balasegaram, M., Koummuki, Y., Santana, M.R. and Davidson, R. (2007). Unresponsiveness to AmBisome in some Sudanese patients with kala-azar. *Trans. R. Soc. Trop. Med. Hyg.* **101**(1): 19–24.

Mukazayire, M-J., Minani, V., Ruffo, C.K., Bizuru, E., Stévigny, C. and Duez, P. (2011). Traditional phytotherapy remedies used in Southern Rwanda for the treatment of liver diseases. *J. Ethnopharmacol.* **138**(2011): 415-431.

Mullen, G.R and Durden, L.A. (2019). *Medical and Veterinary Entomology* (3rd edition). Academic Press. USA.

Muluken Wubetu, Tefera Abula and Getye Dejenu (2017). Ethnopharmacologic survey of medicinal plants used to treat human diseases by traditional medical practitioners in Dega Damot district, Amhara, Northwestern Ethiopia. *BMC Res. Notes*. **1**: 157.

Musa, A., Khalil, E., Asrat Hailu, Olobo, J., Balasegaram, M., Omollo, R., *et al.,* (2012). Sodium stibogluconate (SSG) & paromomycin combination compared to SSG for visceral leishmaniasis in East Africa: a randomised controlled trial. *PLoS Negl. Trop. Dis.* **6**(6): 1674.

Musa, M.S., Abdelrasool, F.E., Elsheikh, E.A., Ahmed, L.A.M.N., Mahmoud, A.L.E. and Yagi, S.M. (2011). Ethnobotanical study of medicinal plants in the Blue Nile State, South-eastern Sudan. *J. Med. Plant Res*. **5**(17): 4287-4297.

Nakaziba, R., Anyolitho, M.K. and Kabunga, A. (2021). Traditional herbal remedies for managing COVID-19 major symptoms: A case study of Kole district, Northern Uganda. *TMR Pharmacol Res*. **1**(4): 22.

Navin, T.R., Arana, B.A., Arana, F.E., de Merida, A.M., Castillo, A.L. and Pozuelos, J.L. (1990). Placebo-controlled clinical trial of meglumine antimoniate (glucantime) vs. localized controlled heat in the treatment of cutaneous leishmaniasis in Guatemala. *Am. J. Trop. Med. Hyg*. **42**: 43–50.

Ngure, P.K., Kimutai, A., Ng'ang'a, Z.W., Rukunga, G. and Tonui, W.K. (2009) A review of Leishmaniasis in Eastern Africa. *J. Nanjing Med. Univ*. **23** (2): 79–86.

Nguyen Hoai, N., Dejaegher, B., Tistaert, C., Nguyen Thi Hong, V., Rivière, C., Chataigné, G., *et al.,* (2009). Development of HPLC fingerprints for *Mallotus* species extracts and evaluation of the peaks responsible for their antioxidant activity. *J. Pharm. Biomed. Anal*. **50**: 753-763.

Ogbulie, J.N., Ogueke, C.C. and Nwanebu, F.C. (2007). Antibacterial properties of *Uvaria chamae, Congronema latifolium, Garcinia kola, Vemonia amygdalina* and *Aframomium melegueta*. *Afr. J. Biotechnol*. **6**(13): 1549-1553.

Okigbo, R. N., Eme, U. E., and Ogbogu, S. (2008). Biodiversity and conservation of medicinal and aromatic plants in Africa. *Biotechnol. Mol. Biol. Rev*. **3**(6), 127–134.

Okindo, E.G., Kutima, H.L., Mutai, J. and Kasili, S. (2017). Attitude and practices of household heads towards leishmaniasis infections in Marigat sub-county, Baringo County, Kenya. *East Afr. Med. J.* **94**: 86–94.

Oliveira, L. F., Schubach, A.O., Martins, M. M. *et al.,* (2011). Systematic review of the adverse effects of cutaneous leishmaniasis treatment in the New World. *Acta. Trop*. **118**(2): 87–96.

Organization PAHO. (2019). Manual of procedures for leishmaniasis surveillance and control in the Americas. [Available from: https://iris.paho.org/handle/10665.2/51838.]

Pankhurst, R. (2001). The status and Availability of oral and written knowledge on traditional health care in Ethiopia. In *Proceedings of the National Workshop on biodiversity conservation and sustainable use of medicinal plants in Ethiopia.* **28**: 92–106.

Pareyn, M., Van Den Bosch, E., Nigatu Girma, Van Houtte, N., Van Dongen, S., Van der Auwera, G., *et al.,* (2019). Ecology and seasonality of sandflies and potential reservoirs of cutaneous leishmaniasis in Ochollo, a hotspot in southern Ethiopia. *PLoS Negl. Trop. Dis*. **13**: e0007667.

Qwarse, M., Mihale, M.J., Henry, L., Sempombe, J., Mugoyela, V. and Sung'hwa, F. (2017). Effects of *Phytolacca dodecandra* Extracts on the Quality Parameters of Stored Maize Grains. *J. Appl. Biosci.* **112**: 11005-11015.

Rani, U., Singh, M., Saith, A., Jain, S.L., Aggarwal, A. and Aggarwal, S. (2022). Evaluation of use of RPMI Medium to preserve cell morphology for pleural/peritoneal fluid cytology. *J. Cytol*. **39**(1):26-29.

Reta Regassa. (2013). Assessment of indigenous knowledge of medicinal plant practice and mode of service delivery in Hawassa city, southern Ethiopia. *J. Med. Plant Res.* **7**(9): 517-535.

Rhajaoui, M., Nasereddin, A., Fellah, H., Azmi, K., Amarir, F., Al-Jawabreh, A., Ereqat S., Planer, J. and Abdeen Z. (2007). New clinico-epidemiologic profile of cutaneous Leishmaniasis, Morocco. *Emerging. Infect. Dis*. **13:** 1358- 1360.

Rijal, S., Chappuis, F., Singh, R., *et al.,* (2003). Treatment of visceral leishmaniasis in south-eastern Nepal: decreasing efficacy of sodium stibogluconate and need for a policy to limit further decline. *Trans. R. Soc. Trop. Med. Hyg.* **97**(3): 350–354.

Ritmeijer, K., Abren Dejenie, Yibeltal Assefa, *et al.,* (2006). A comparison of miltefosine and sodium stibogluconate for treatment of visceral leishmaniasis in an Ethiopian population with high prevalence of HIV infection. *Clin. Infect. Dis.* **43**(3): 357–364.

Roatt, B.M., de Oliveira Cardoso, J.M., De Brito, R.C.F., Coura-Vital, W. *et al.,* (2020). Recent advances and new strategies on leishmaniasis treatment. *Appl. Microbiol. Biotechnol.* **104**(21): 8965-8977.

Rodriguez, M.E., Inguanzo, P., Ramos, A., *et al.,* (1990). Treatment of cutaneous leishmaniasis with CO_2 laser radiation. *Rev. Cubana Med. Trop.* **42**(2): 197–202.

Rwangabo, P.C. (1993). La médecine traditionnelle au Rwanda. Paris: Editions Karthala et ACCT.

Samson Leta, Dao, T.H.T., Frehiwot Mesele and Gezahegn Alemayehu. (2014). Visceral Leishmaniasis in Ethiopia: an Evolving Disease. *PLoS Negl. Trop. Dis*. **8**(9):e3131.

Sasidharan, S. and Saudagar, P. (2021). Leishmaniasis: where are we and where are we heading? *Parasitol. Res.* **120**: 1541-1554.

Scope, A., Trau, H., Bakon, M., Yarom, N., Nasereddin, A. and Schwartz, E. (2003). Imported mucosal leishmaniasis in a traveler. *Clin. Infect. Dis*. **37**(6): 83–7.

Service, M.W. (2012). *Medical Entomology for Students* (5th edition). Cambridge University Press. UK.

Sethuraman, G., Sharma, V. K. and Salotra, P. (2008). Indian Mucosal Leishmaniasis Due to *Leishmania donovani* Infection. *N. Engl. J. Med.* **358**(3): 313–315.

Sisay Alemu and Jelalu Kemal. (2015). The Properties of Selected Medicinal Plants Against *Bovicola ovis* and *Amblyomma varigatum*: A Review. *Eur. J. Appl. Sci*. **7** (6): 277-290.

Sultan Suleman and Tamirat Alemu (2012). A survey on utilization of ethnomedicinal plants in Nekemte town, East Wellega (Oromia), Ethiopia. *J. Herbs Spices Med. Plants*. **1**: 34-57.

Sundar, S. (2000). Drug resistance in Indian visceral leishmaniasis. *Trop. Med. Int. Health*. **6**: 849–854.

Sundar, S. and Chakravarty, J. (2008). Paromomycin in the treatment of leishmaniasis. *Expert Opin. Inv. Drug*. **17**(5): 787–794.

Sundar, S. and Chakravarty, J. (2013). Leishmaniasis: an update of current pharmacotherapy. *Expert Opin. Pharmacother.* **14**(1): 53–63.

Sundar, S. and Chakravarty, J. (2015). An update on pharmacotherapy for leishmaniasis. *Expert Opin. Pharmacother.* **16**(2): 237–225.

Sundar, S., More, D.K., Singh, M.K., *et al.,* (2000). Failure of pentavalent antimony in visceral leishmaniasis in India: report from the center of the Indian epidemic. *Clin. Infect. Dis.* **31**(4): 1104–1107.

Sundar, S. and Rai, M. (2002). Laboratory diagnosis of visceral leishmaniasis. *Clin. Diagn. Lab. Immunol*. **9**(5): 951–958.

Tasisa Ketema, Markos Tadele, Zewdie Gebrie, Eyasu Makonnen, Asrat Hailu and Solomon M Abay. (2023). *In vitro* Anti-Leishmanial Activities of Methanol Extract of *Brucea antidysenterica* J.F. Mill Seeds and Its Solvent Fractions. *J. Exp. Pharmacol.* **15**: 123-135.

Thakur, C.P., Singh, R.K., Hassan, S.M., *et al.,* (1999). Amphotericin B deoxycholate treatment of visceral leishmaniasis with newer modes of administration and precautions: a study of 938 cases. *Trans. R. Soc. Trop. Med. Hyg.* **93**(3): 319–323.

Thomford, N. K., Senthebane, D. A., Rowe, A. Munro, D., *et al.,* (2018). Natural Products for Drug Discovery in the 21st Century: Innovations for Novel Drug Discovery. *Int. J. Mol. Sci*. **19**: 1578.

Tilahun Teklehaymanot, Mirutse Giday, Girmay Medhin and Yalemtsehay Mekonnen (2007). Knowledge and use of medicinal plants by people around Debra Libanos monastery in Ethiopia. *J. EthnoPharmacol*. **111**(2): 271–283.

Tiuman, T. S., Ueda-Nakamura, T., Cortez, D.A.G., *et al.,* (2005). Antileishmanial activity of parthenolide, a sesquiterpene lactone isolated from *Tanacetum parthenium*. *Antimicrob. Agents Chemother.* **49**(1): 176–182.

Triparthi L. and Triparthi N.J. (2003). Role of biotechnology in medicinal plants. *Trop. J. Pharm. Res*. **2**(2): 243-253.

Valencia, B.M., Miller, D., Witzig, R.S., Boggild, A.K. and Llanos-Cuentas, A. (2013). Novel low-cost thermotherapy for cutaneous leishmaniasis in Peru. *PLoS Neg. Trop. Dis.* **7**: 2196.

Van Henten, S., Adriaensen, W., Helina Fikre, Akuffo, H., Ermias Diro, Asrat Hailu, *et al.,* (2018). Cutaneous leishmaniasis due to *Leishmania aethiopica*. *EClinicalMedicine*. **6**: 69–81.

Vendrametto, M. C., Santos, A. O. D., Nakamura, C. V., Filho, B. P. D., Cortez, D. A. G. and Ueda-Nakamura, T. (2010). Evaluation of antileishmanial activity of eupomatenoid-5, a compound isolated from leaves of *Piper regnellii* var. *pallescens*. *Parasitol. Int.* **59**(2): 154–158.

Weller, P.F., Durand, M.L. and Pilch, B.Z. (2005). Case 4-2005: A 35-Year-Old Man with Nasal Congestion, Swelling and Pain. *N. Engl. J. Med*. **352**(6): 609–15.

West African plants (2023). *Sphaeranthus Steetzii*. Available from (http://www.westafricanplants.senckenberg.de/root/index.php?page_id=14&id=3846#image=80318).

WHO. (1996). *Manual on Visceral Leishmaniasis Control*. WHO, Geneva.

WHO (2010). Control of the leishmaniasis: Report of a Meeting of the WHO Expert Committee on the Control of Leishmaniasis. Geneva: World Health Organization. *Tech. Rep. Ser*. **949**: 1–186.

WHO (2015). WHO Leishmaniasis Country Profile- Somalia 2015.

WHO. (2017a). WHO Leishmaniasis Country File – Sudan 2015.

WHO. (2017b). Leishmaniasis Country Profile - Kenya 2015.

WHO. (2017c). WHO - Leishmaniasis- Ethiopia-Country File 2015.

WHO. (2018). Weekly Epidemiological Record. **93**(48): 649–660. Available from: http://www.who.int/wer/2018/wer9348/en/

WHO. (2023). Leishmaniasis. (https://www.who.int/news-room/fact-sheets/detail/leishmaniasis)

Yan, L. H., Xu, L. Z., Lin, J., Yang, S. L., and Feng, Y. L. (2009). Triterpenoid saponins from the stems of *Clematis parviloba*. *J. Asian Nat. Prod. Res*. **11**(4): 332–338.

Yayesh Limenih, Shemsu Umer and Messay Wolde-Mariam (2015). Ethnobotanical study on traditional medicinal plants in Dega damot woreda, Amhara region, North Ethiopia. Inter. J. Res. Pharm. Chem. **5**: 258-73.

Yeneayehu Fentahun, Girma Eshetu, Abebe Worku, Taher Abdella. (2017). A survey on medicinal plants used by traditional healers in Harari regional State, East Ethiopia. *J. Med. Plant Res.* **1**: 85-90.

Yibeltal Aschale, Animen Ayehu, Ligabaw Worku, Habtie Tesfa, Meseret Birhanie and Wossenseged Lemma. (2019). Malaria-visceral leishmaniasis co-infection and associated factors among migrant laborers in West Armachiho district, North West Ethiopia: Community based cross-sectional study. *BMC Infect Dis*. **19**: 239.

Yitagesu Tewabe, Belete Kefarge, Habtamu Belay, Daniel Bisrat, Asrat Hailu and Kaleab Asres. (2019). Antipromastigotes Evaluation of the Leaf Latex of *Aloe macrocarpa*, Aloin A/B, and Its Semisynthetic Derivatives against Two Leishmania Species. *Evid. Based Complement Altern. Med*. **2019**: 4736181.

Yosef Melaku, Collin, S.M., Keus, K., Gatluak, F., Ritmeijer, K. and Davidson, R.N. (2007). Treatment of kala-azar in Southern Sudan using a 17-day regimen of sodium stibogluconate combined with paromomycin: a retrospective comparison with 30-day sodium stibogluconate monotherapy. *Am. J. Trop. Med. Hyg*. **77**: 89–94.

Zelalem Abdisa and Fekede Kenea (2021). Phytochemical screening, antibacterial and antioxidant activity studies on the crude root extract of *Clematis hirsuta*. *Cogent Chem.* **6**: 1862389.

Zijlstra, E. E. (2016). Visceral leishmaniasis: a forgotten epidemic. *Arch. Dis. Child.* **101**(6): 561–567.

Zintchem, R., Njinkio, B., Kamgang, R., Fokunang, C. *et al.,* (2013). Antioxidative properties of *Mallotus oppositifolium* decoction leaves extract using *in vitro* models. *Int. J. Biol. Chem. Sci*. **7**(6): 2396-2408.

Zohra, M. and Fawzia, A. (2014). Hemolytic activity of different herbal extracts used in Algeria. *Int. J. Pharm. Sci. Res*. **5**(8): 495-500.

Appendix

Chemicals and materials

Sterile flask, magnetic stirrer, aluminum foil, hot plate, autoclave, water bath, hood, rotavaporator, culture flask, Bunsen burner, yellow tips, slides, micropipettes (multichannel), microscope, haemocytometer (counting chamber), falcon tubes, beaker, 96 well plates, sterile guaze, Whatman no. 1 filter paper, amber-coloured bottle, oven, separatory funnel, fluoroskan ascent, Victor[3] Multilabel reader, EDTA tube, syringe and cold centrifuge are the materials used in this research.

Glucose, Nacl, Distilled water, chloroform, Nutrient agar, Sheep blood, $CaCl_2.2H_2O$, $NaHCO_3$, KCl, L-glutamine (Gibco, United States) Pen strep (Gibco, United States), Roswell Park Memorial Institute (RPMI-1640 medium) (Sigma-Aldrich, UK), FBS (fetal bovine serum) (Gibco, United States), 100% DMSO (Sigma-Aldrich, UK), Amphotericin B (Gibco, United States), Alamar blue, Triton X-114 (Scintran, Poole, England) and PBS (Phosphate buffer saline) are the chemicals used to do this research.

Media preparation

Novy–MacNeal–Nicolle (NNN) media preparation

It is thought that NNN medium, which is often made with the blood of sheep or rabbits, works well for isolating parasites from biological materials (Sundar and Rai, 2002). When cultivating *Leishmania*, NNN medium is employed for both subculturing and initial isolation of the suspected parasite from expelled fluids (Alsaad and Kawan, 2021). Thus the need for this medium in the culturing of the test strains. In this study, the preparation of NNN media was based on Chouihi *et al.,* (2009). A sterile flask first was taken and 4.6 g of nutrient agar, 0.3 g of glucose and 1.2 g sodium chloride were added. Two hundered milliliter of distilled water was added into the sterile flask. A magnetic stirrer was added in the flask and covered with an aluminum foil. On a hot plate it was boiled at the heat temperature of 150°C. The autoclave was opened and checked if there is enough water beneath to start the autoclaving process. The boiled media was put into the autoclave at 121°C and 100 kpa for 20 minutes.

Next the water bath was turned on and let to reach 56°C. Fifty milliliter of sheep blood was taken out from the refrigerator and put at room temperature. After 20 minutes, the autoclaved media was taken out and put in water bath along with the sheep blood for another 20 minutes or so. The media and blood was then taken to the hood. Fifty milliliter of the sheep blood was mixed with the media and 3-4ml of the mixture was poured into culture flasks. The flasks were slanted immediately on the desk at a 45 degree angle.

Locke's solution (500 ml)

For the maintenance and cultivation of parasites, Locke's solution was frequently employed as an overlay on the NNN blood base agar medium (Menberework Chanyalew *et al.,* 2009). Thus as per the laboratories preparation, a sterile 500 ml flask was taken and 4.6 g of sodium chloride, 0.1 g of $CaCl_2.2H_2O$, 0.075 g of $NaHCO_3$, 0.2 g of KCl , 1.25 g of D-glucose and 500 ml distilled water were added. It was then covered with aluminum foil and autoclaved for 15 minutes. After the autoclave, 2 ml of glutamine was added and stored at 4°C.

Complete media preparation

This media containing different components help in parasite culture. It is an environment made with a well-known cell culture medium known as RPMI medium (Rani *et al.,* 2022) and has antibiotics to prevent bacterial contamination (Luzzatto *et al.,* 1968; Lobanovska and Pilla, 2017). Growth factors, hormones, antibodies, vitamins and proteins are also incorporated into this media with the addition of fetal bovine serum (FBS) (Castells-Sala *et al.,* 2017; Chelladuraj *et al.,* 2021). Thus the need for this media for culturing. As per the laboratories preparation, a sterile 50 ml corning flask was taken and 40 ml of RPMI medium, 2 ml of L-glutamine (if the RPMI medium is without L-glutamine), 1 ml of pen strep (penicillin) and 10 ml of FBS were added. The mixture was mixed and stored at 4°C.

Stock preparation

DMSO preparation (3%)

A sterile 15 ml falcon tube was taken. A DMSO stock was made by adding 9.7 ml of distilled water and 0.3 ml of 100% DMSO (Sigma-Aldrich, UK) in the falcon tube and mixed. It was then labelled.

Crude extract stock preparation

Two sterile 15 ml falcon tube were taken and 0.01 g of each crude extracts were measured, and mixed with 1ml of 3% DMSO in each falcon tube and shook until both tubes became homogenous mixtures. Finally labeled as CHD and CHH.

Temporary stock preparation

Sterile 15 ml falcon tubes were taken and 50 µl of the crude extract stock was added, along with 950 µl of RPMI and mixed. This was done for both CHD and CHH.

Triton X-114 and DMSO stock preparation for hemolysis assay

Two percent Triton X-114 was made by mixing 200 µl of 100% Triton X-114 in 19.8 ml of PBS. Similarly, 1% DMSO was made by mixing 100 µl of 100% DMSO in 9.9 ml of distilled water.

Annex: Ethical clearance letter

ADDIS ABABA UNIVERSITY, COLLEGE OF HEALTH SCIENCES (IRB)
አዲስ አበባ ዩኒቨርስቲ ጤናሳይንስ ኮሌጅ
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