ACTIVITY OF *TEPHROSIA VOGELII* CRUDE EXTRACT AGAINST *LEISMANIA MAJOR* IN EXPERIMENTALLY INFECTED BALB/c MICE

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DECLARATION

Declaration by the Candidate

This thesis is my original work and has not been presented for a degree in any other university. No part of this thesis can be reproduced without the prior permission of the author and/or University of Eldoret.

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Approval by the supervisors

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DEDICATION

This thesis is dedicated to Mr. and Mrs. Wanyama Marango's family because you have made me who I am and for the love and support you have given and continue giving me.

ABSTRACT

Leishmaniasis, a vector-borne disease caused by obligate intra-macrophage protozoa, is endemic in large areas of the Tropics, Subtropics and the Mediterranean basin and 350 million people are considered at risk. The drugs currently used in the treatment of leishmaniases are pentavalent antimonials such as Sodium stibogluconate (Pentostam) which are known to be expensive, toxic and resistance has been widely reported. Moreover, there is no vaccine that has been developed against leishmaniasis. Therefore, a need exists to investigate the activity of medicinal plants that may have anti-parasitic properties for the treatment of the leishmaniases. This study investigated the activity of *Tephrosia vogelii* against cutaneous leishmaniasis caused by *Leishmania major* using the BALB/c mouse model *in vivo* and *in vitro*. Pentostam and Amphotericin B were used as positive controls while Phosphate Buffered Saline and Roswell Park Memorial Institute 1640 Medium were used as negative controls *in vivo* and *in vitro* respectively. Five groups of eight female mice were used in the study. They were inoculated intradermally on the left hind footpad with 10^6 *L. major* infective promastigotes. The mice were treated intraperitoneally with the different drugs at daily doses for 28 days except for *T. vogelii* which was administered both orally and intraperitonealy. All experiments were performed in triplicate. The mean standard deviation of at least three experiments was determined and statistical analysis of the differences between mean values obtained for the experimental groups was done by the students't-test. P. values of equal to or less than 0.05 ($P \leq 0.05$) were considered significant. Toxicity level for *T. vogelii* therapy was lower than standard drugs. There was significant difference in the test drugs against promastigotes ($P<0.05$). *T. vogelii* had an IC_{50} and IC_{90} of 12µg/ml and 68.5µg/ml respectively, while the positive controls had IC_{50} of 5.5µg/ml and 7.8 µg/ml for pentostam and amphotericin B respectively and IC_{90} of 18µg/ml and 25.5 µg/ml for pentostam and amphotericin B respectively. In the amastigote assay, the infection rates decreased with increase in concentration. There was significant difference between the standard drugs and *T. vogelii* in the infection rates ($P=0.01$). The Multiplication indices for *L. major* amastigotes in macrophages treated with 200µg/ml of the test drugs were significantly different ($P<0.05$). 200µg/ml of *T.vogelii* extract showed a multiplication index of 20.57%, 5.65% for Amphotericin B and 9.56% for pentostam. *T. vogelii* administered intraperitonealy resulted in larger lesion size reduction than the oral administration. The parasite loads and the Leishman Donovan Units in the spleens and liver of the treated mice were significantly low in *T. vogelii* administered intraperitonealy than those administered orally. However, standard drugs had better efficacy for reduction of parasite load ($P=0.001$). There was also significant levels of Nitric oxide produced in the macrophages ($P<0.05$). *T. vogelii* extract has anti-leishmanial activity and further tests should be done on the extract to establish the active compounds responsible for antileishmanial activity.

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LIST OF ABBREVIATIONS/ACRONYMS

µg	Microgram
°C	Degrees Celcius
ACUC	Animal Care and Use Committee
ANOVA	Analysis of Variance
Ave.	Average
BALB/c	Inbred strain of mice very susceptible to *Leishmania Major*
CBRD	Centre for Biotechnology Research and Development
CDC	Centre for Disease Control
CL	Cuteneous Leishmaniasis
CTMDR	Centre for Traditional Medicine and Drugs Research
DCL	Diffuse Cutaneous Leishmaniasis
DDT	Dichloro-diphenyl-trichloroethane
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic Acid
ELISA	Enzyme Linked Immunosorbent Assay
FBS	Foetal Bovine Serum
F-NHS	Fresh Normal Human Serum
Hcl	Hydrochloric Acid
IC_{50}	Concentration that kills 50% of parasite population
IC_{90}	Concentration that kills 90% of parasite population
IFA	Indirect Flourescent Antibody
IFN-α	Interferon Alpha
IFN-γ	Interferon Gamma
IL	Interleukin

IHLFP	Infected Hind Left Foot Pad
IR	Infection Rate
IP	Intraperitoneal administration of the drug
KEMRI	Kenya Medical Research Institute
PKDL	Post-kala-azar Dermal Leishmaniasis
LDU	Leishman Donovan Unit
LSD	Least Significant Difference
MCL	Muco-cutaneous Leishmaniasis
MEM	Minimum Essential Medium
MI	Multiplication Index
MIC	Minimum Inhibition Concentration
MTT	Methyl-Thiazol-Tetrazolium
NO	Nitric Oxide
OR	Oral administration of the drug
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
RPMI	Rosewell Park Memorial Institute 1640 Medium
SOPs	Standard Operating Procedures
SPSS	Statistical Package for Social Sciences
T-cells	A type of white blood cells that is of key importance to adaptive immunity
Th1 and Th2 cells	T helper cells one and two respectively
UHRFP	Uninfected Hind Right Foot Pad
VL	Visceral Leishmaniasis
WHO	World Health Organization

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Above all, I thank God for giving me life this far.

CHAPTER ONE

INTRODUCTION

1.1 Background of the study

Leishmaniasis, a vector-borne disease that is caused by obligate intra-macrophage protozoa, is endemic in large areas of the tropics, subtropics and the Mediterranean basin (Alvar *et al*., 2012) and 350 million people are considered at risk (Chappuis *et al*., 2007). The disease is caused by more than 20 leishmanial parasites of subgenera *Leishmania* with variable clinical presentations and prognoses (Pratlong *et al.*, 2012). The disease is zoonotic and transmitted to humans by approximately 30 different species of phlebotomine sandflies (Pearson and Sousa, 2009).

Due to species differences in tissue tropism, virulence and their interaction with the host's immune system, infection by leishmaniasis can result in a variety of clinical manifestations ranging from single self-healing ulcers in cutaneous forms to life threatening visceral infections (Desjeux, 2004). Leishmaniasis is characterized by four main clinical syndromes namely, Cutaneous Leishmaniasis (CL), Muco-Cutaneous Leishmaniasis (MCL; also known as espundia), Visceral Leishmaniasis (VL; also known as kala-azar) and Post-Kala-azar Dermal Leishmaniasis (PKDL).

In cutaneous leishmaniasis, the patient generally presents with one or several ulcers in the skin. In the Old World, human cutaneous leishmaniasis is caused by at least three main species of *Leishmania*: *L. aethiopica*, *L. major* and *L. tropica*. During its life cycle, *Leishmania sp*. presents basically three forms: promastigotes, paramastigotes and amastigotes. Promastigotes and paramastigotes are flagellated, motile forms that are found in the alimentary tract of phlebotomine sandflies. Amastigotes are non-

motile forms, found inside mononuclear phagocytes of the mammalian host (Bari and Rahman, 2008).

Metacyclic infective promastigotes are transmitted by female sandflies to mammals, where they invade and multiply as amastigotes within macrophages (Bari and Rahman, 2008). One of the earliest events after promastigotes have entered the mammalian host is their contact with plasma proteins. It has been shown that fresh normal human serum (f-NHS) can cause the lysis of *Leishmania spp*. *in vitro* through the alternative pathway of complement (Mosser, 2007). Therefore, they must escape the lytic effect of serum before they can invade macrophages (Jokiranta *et al*., 2005). In fact, it has been shown that non-infective promastigotes from logarithmic stage of growth, which are susceptible to complement (Bandyopadhyay *et al*., 2001), differentiate into stationary phase promastigotes with an increased resistance to complement and higher infectivity (Sacks and Kamhawi, 2001).

Differences in the pattern of promastigotes sensitivity to complement, lead to the hypothesis that complement resistance of *Leishmania* spp. is species-specific (Mosser, 2007), and that their resistance could be related to the severity of the disease caused by each species (Sacks and Kamhawi, 2001). Therefore treatment of leishmaniasis should involve targeting the *Leishmania* parasite species.

In elimination of the *Leishmania* parasites, the pentavalent antimonials and meglumine antimoniate have been the first-line treatment in many areas for more than 70 years (Croft *et al*., 2006). Yet, antimonials are toxic drugs with frequent, sometimes life-threatening, adverse side effects, including cardiac arrhythmia and

acute pancreatitis. As such there is ongoing research aimed at using alternative and cheap drugs for oral, parenteral or topical administration (Croft and Coombs, 2003).

In traditional medicine, many plants have already provided valuable clues for potentially antiparasitic compounds, especially simple quinones, quassinoids, and related naphthoquinones (Fournet *et al*., 1992). The antiprotozoal activities of plant-derived phenolics have attracted renewed attention since simple naphthoquinones such as plumbagin and its dimers were isolated by bioassay guided fractionation of *Pera benensis* and used in the treatment of cutaneous leishmanaisis (CL) in Amazonian Bolivia (Fournet *et al*., 1992). The antiprotozoal activity of hydroxynaphthoquinones has long been established. Attention has re-focused on this group of compounds, since a pronounced activity against a range of protozoan parasites, including *Plasmodium* sp., *Theileria, Toxoplasma, Eimeria* spp. and against *Pneumocystis carinii* was documented (Hudson *et al*., 1991). Many naphthoquinones have been identified as possible lead regimens against *Leishmania*, *Trypanosoma* or *Plasmodium* parasites. Therefore, alternative plants are still required that contain these compounds necessary for control of leishmania protozoans.

Tephrosia vogelii is a multipurpose leguminous plant, native to the tropical countries and has been introduced to Kenya and previously used as antiparasitic agent (Dzenda *et al*., 2007). The plant is a potential source of rotenone, an important non-residual insecticide.

In light of the scanty data on efficacy of plant extracts against parasites in the tropical regions, the aim of this study was to establish antiparasitic efficacy of crude extracts of *Tephrosia vogelii* against *Leishmania major* in BALB/c mice.

1.2 Statement of the problem

Cutaneous leishmaniasis is not usually fatal but may result in disfiguring scars. However, if untreated, severe cases of cutaneous leishmaniasis are almost always fatal. Despite existence of drugs that treat *Leishmania* parasites, these drugs are expensive and toxic. There is also increasing evidence of parasite resistance to conventional antiparasitic therapies (Ashutosh *et al*., 2007) and high cost and constraints due to the length of the modern treatment to control the leishmaniasis in developing countries like Kenya (Gupta and Nishi, 2011). Moreover, vaccines against *Leishmania species* are still under development hence no vaccine exist yet (Handman, 2013). Therefore, there is need to search for alternatives.

Patients, in particular, the resource-poor people still use traditional medicine for the treatment of various diseases of parasite and and non-parasite origin. Fortunately, many rural communities in Kenya know a lot about medicinal plants that can cure diseases of the skin, hair and nails. In recent years, as a consequence of the constraints to chemical parasitoid use and of the encouraging results obtained from plant extracts, interest in plants with antiparasite properties has increased (Ngure *et al*., 2009; Wabwoba *et al*., 2010).

Thousands of plants have been screened for antiparasitic activities using a standard WHO procedure (WHO, 2000). However, there is still lack of vital information including antiparasitic efficacy of most plant species in Kenya and therefore research on the plants species in Kenya is still deemed to be limited. Moreover, there is limited data on their efficacy on the target parasites. Furthermore, efficacy of plant extracts is known to be affected factors like location, amount of active compounds in

the plants, extraction procedure and species of organism under study among others, and they make it very difficult to generalize the antiparasite properties of many plant species. Lack of such information has limited the use of plant biocides against leishmaniasis in Kenya.

1.3 Justification of the study

Leishmaniases are developing resistance to the present drugs of choice which are expensive and toxic, and an urgent need therefore exists for alternative chemotherapeutic regimens. The known activities of crude plant extracts targeting the kinetoplastid parasites may make them appropriate for investigation, both *in vitro* and *in vivo* systems. Therefore this study will provide additional information necessary to determine the antileishmanial activity of *T. vogelii* that will move towards enhancing leishmaniasis treatment in Kenya.

Plant extracts have been tested for anti-protozoal activities with varied results. Extracts of *T. vogelii* vary widely in the kind and intensity of biological activity. Some of the more important reported activities include haemolytic, local irritant, inflammatory, cytotoxic and antimicrobial activity. Existing information confirms that *T. vogelii* has many antimicrobial, dermatological and cytological activities against protozoan parasites. *T. vogelii* also shows activity when applied as an ointment on *L. amazonensis* lesions on BALB/c mice and hence this study could give a wider understing on the antileishmanial activity of *T. vogelii* both *in vivo* and *in vtro* when used against *L. majo*r. The known activities of *T. vogelii* against many parasites could lead to the development of a less expensive, less toxic approach to the elimination or control of leishmaniasis.

1.4 Objectives of the study

1.4.1 General objective

The broad objective of the study was to investigate the activity of *T. vogelii* crude extracts on *L. major* infected BALB/c mice.

1.4.2 Specific objectives

1. To test the efficacy of *T. vogelii* crude extracts on promastigotes and amastigotes of *L. major*
2. To determine the effect of *T. vogelii* crude extracts on *L. major* lesion development in BALB/c mice.
3. To determine the cytotoxicity of *T. vogelii* crude extracts on vero cells.

1.5 Hypotheses

1. H_0: *T. vogelii* crude extracts have no signicant effect on promastigotes and amastigotes of *L. major.*

   H_A: *T. vogelii* crude extracts have signicant effect on promastigotes and amastigotes of *L. major.*
2. H_0: *T. vogelii* crude extracts have no significant effects on *L. major* lesion development in BALB/c mice.

   H_A: *T. vogelii* crude extracts have significant effects on *L. major* lesion development in BALB/c mice.
3. H_0: *T. vogelii* crude extract are significantly toxic on vero cells when compared to the standard drugs.

   H_A: *T. vogelii* crude extract are not significantly toxic on vero cells when compared to the standard drugs.

CHAPTER TWO

LITERATURE REVIEW

2.1 Leishmaniasis

Leishmaniasis is a parasitic disease caused by various species of *Leishmania*, unicellular kinetoplastid protozoan flagellates. *Leishmania* parasites infect numerous mammal species including humans. They are stransmitted by the bite of infected phlebotomine sand flies whose species are widespread on all continents except Antarctica (Alvar *et al.*, 2012).

Leishmaniasis is endemic in the subtropics, tropics and southern Europe; in 98 countries with more than 350 million people being at risk. There is an indication of an estimated incidence of 2 million new cases per year; 0.5 million of visceral leishmaniasis and l.5 million of cutaneous leishmaniasis (Chappuis *et al*., 2007).

WHO, (2007) indicates that countries from the four major continents: Africa, South America, Asia and Europe that are affected by leishmaniases are those whose geographical distribution is limited by the distribution of the sandfly (Dey and Singh, 2006). Many endemic areas show large fluctuations in incidence over time, which are sometimes attributable to specific events, such as population displacement or climate factors. Climatic, socioeconomic and other environmental changes could expand the geographical range of the vectors and reservoirs and consequently increase human exposure to infected sand flies. There is worldwide increase in leishmaniasis incidence which is attributed to increase in risk factors that include malnutrition, immunosuppresion and lack of treatment (Desjeux, 2004). Leishmaniasis is one of

the most neglected diseases in developing countries including Kenya and it largely affects the poor.

2.2. Distribution of leishmaniases

The WHO reports indicate that more than 90% of visceral cases appear in India, Nepal, Bangladesh, Sudan and Brazil, 90% of all cases of mucocutaneous leishmaniasis occur in Bolivia, Brazil and Peru and 90% cases of cutaneous leishmaniasis (CL) occur in Afghanistan, Brazil, Peru, Iran, Saudi Arabia and Syria (WHO, 2009). This disease is characterized by both diversity and complexity and is caused by a large number of *Leishmania* species as shown in Appendix I.

2.2.1 Leishmaniasis in Africa

In southern Africa, the distribution of CL appears to be confined in Namibia. In this region, CL has a topographical association with rocky outcrops and mountain cliffs, which provide shelters for the rock hyrax, *Procavia capensis*; reservoir host in the region. Leishmaniasis is also endemic in West Africa and only *L. major* has been reported as the causative species in Ghana, Gambia, Senegal, Burkina Faso and Mali (Boakye *et al.,* 2006). Leishmaniasis is also endemic in East Africa.

2.2.2 Distribution of leishmaniases in East Africa

In East Africa, leishmaniasis is endemic in Ethiopia, Kenya, South Sudan, Uganda, Somalia and Sudan. The leishmaniases in this region are caused by *L. major, L. aethiopica, L. tropica and L. donovani.* The distribution of leishmaniasis in East Africa is shown in Appendix II (Lawyer *et al.*, 1991).

2.2.3 Distribution of leishmaniasis in Kenya

In Kenya, leishmaniasis affected areas include Mount Elgon, Machakos, West Pokot, kitui. In Kenya.visceral leishmaniasis is caused by *L. donovani* and cutaneous leishmaniasis caused is by *L. major, L. tropica* and *L. aethiopica.* These two types of leishmaniasis are endemic in Kenya (Lawyer *et al.*, 1991).

Post Kala-azar dermal leishmaniasis has also been shown to be endemic in Turkana, Baringo, Kitui, West Pokot, MachakoS, Mwingi, Meru, Wajir, Mandera, Keiyo and Marakwet. Baringo district is the only focus reported where both VL and CL are known to occur in Kenya (Tonui, 2006).

Visceral leishmaniasis is endemic in Kitui, Meru, Kajiado and Baringo. Visceral leishmaniasis sero prevalence in Kenya is unknown because of the lack of a practical and accurate diagnostic test or surveillance system (Ryan *et al.*, 2006). Moreover, most VL infections occur in remote geographical areas where health facilities are not well established and where the infections often co-exist with malaria and other debilitating parasitic infections (Ryan *et al.*, 2006).

Cutaneous leishmaniasis in Kenya is caused by *L. major* and *L. aethiopica*. These species are transmitrted by *P. duboscqi* mainly found in animal burrows where it feeds on rodents, which are frequently infected (Githure *et. al*., 1996).

Diffuse cutaneous leishmaniasis (DCL) is found in Mount Elgon area. *Leishmania aethiopica* has been identified as the etiological agent, rodents as the animal reservoirs and *P. pedifer* as the vector of DCL in the region (Sang and Chance, 1993).

Although various aspects of the transmission and control of leishmaniases have been studied in Kenya, the impact of the disease and particularly VL is still enormous (Tonui, 2006).

2.3 Transmission and Clinical Presentation of Leishmaniasis

Leishmaniasis is a disease caused by parasites of the genus *Leishmania* and transmitted by the bite of infected female phlebotomine sand fly to the vertebrate host. The female sandfly is haematophagous and needs a blood meal for egg maturation. The life cycle of *Leishmania* parasite has two stages: one within the invertebrate host (phlebotomine sand fly) and the other one within a vertebrate host. The parasites exist in two main morphological forms; the amastigotes and promastigotes, which are found in vertebrate and invertebrate hosts, respectively (Koutis, 2007).

The invertebrate hosts are small insects of the order Diptera, belonging to the *Phlebotominae* subfamily and only two of the six genera described are of medical importance: *Phlebotomus* of the "Old World" (Africa, Asia, and Europe) and *Lutzomyia* of the "New World" (the Americas) (Alvar *et al*., 2012). Some phlebotomine species such as *Phlebotomus papatasi* and *P. sergenti* can support the growth of only those species of *Leishmania* with which they are infected in nature, whereas other species such as *Lutzomyia longipalpis* and *P. argentipes* can develop mature transmissible infections when infected with several *Leishmania* species (Koutis, 2007). The potential diseases' reservoirs include many different orders of mammals such as rodents, canids, edentates, marsupials, procyonids, primitive ungulates and primates. On the other hand, humans are considered to be accidental hosts of these parasites.

The life cycle begins when an infected female sand fly takes a blood meal from the vertebrate host. During the obtaining of the blood meal, the sand fly pierces the skin tearing tissues and the salivary gland content is injected together with *Leishmania* promastigotes into the host's skin (Bañuls *et al*., 2007). The metacyclic promastigotes that reach the punctured wound are phagocytisized by macrophages and transform into amastigotes. Amastigotes multiply in infected cells and affect different tissues, depending, in part on which *Leishmania* species is involved. These various tissue specificities cause the different clinical manifestations of the various forms of leishmaniasis. Sand flies become infected during blood meals on infected hosts when they ingest macrophages infected with amastigotes. In the sand fly's midgut, the parasites differentiate into promastigotes and multiply. They then differentiate into metacyclic promastigotes and migrate to the proboscis. The life cycle is shown in appendix III.

The establishment of the disease depends on the success of the *Leishmania* parasite to differentiate into the amastigote form (Bañuls *et al*., 2007). It has been established that visceral leishmaniasis could be directly transmitted via blood (needle sharing, transfusion, transplacental spread) or organ transplantation. Moreover, it has been shown that cutaneous infection can also be developed in case of needle injection of contaminated with Leishmanial material (Bañuls *et al*., 2007).

The *Leishmania* parasite is involved in different pathologies that range from the cutaneous to the visceral forms, depending on the species of *Leishmania* and the host immune response (Sacks and Kamhawi, 2001). In humans, infection with leishmaniasis parasites could also result in asymptomatic forms. It is well documented

that asymptomatic human hosts could contribute to the maintenance of the leishmaniasis foci (Bañuls *et al*., 2007).

It has been shown through several epidemiological studies that the majority human infections by *Leishmania* parasites remain asymptomatic (Bucheton *et al.*, 2003). These asymptomatic human subjects are able to clear the infection or remain asymptomatic carriers for years. Thus, leishmaniasis development depends on several risk factors such as malnutrition, immunosuppression, age, immunological status and genetic factors. Several investigators have reported on the negative effect of malnutrition on leishmaniasis clinical course. In this regard, it has been shown that malnutrition in *Leishmania donovani* infected subjects is able to alter the immune response and consequently increases the risk of clinical leishmaniasis (Desjeux, 2004).

Furthermore, it has been observed that there are ethnic differences in the ratio of asymptomatic to symptomatic infections (Bucheton *et al.*, 2003); suggesting that human susceptibility to the clinical expression of leishmaniasis depends on host's genetic risk factors.

Leishmaniases are characterized by a spectrum of clinical manifestations which include: ulcerative skin lesions which develop at the site of the sand fly bite (localized cutaneous leishmaniasis); multiple non-ulcerative nodules (diffuse cutaneous leishmaniasis); destructive mucosal inflammation (mucosal leishmaniasis) and disseminated visceral leishmaniasis (Desjeux, 2004). Cutaneous leishmaniasis is frequently self-healing and can remain subclinical or become clinically apparent after

a variable incubation period that averages several weeks. The first sign of an infection is a small erythema that develops at the site of the sand fly bite after a variable period of time. The erythema develops into a papule, then into a nodule; the nodule ulcerates over a period of 2 weeks to six months to become a lesion that is characteristic of the cutaneous leishmaniasis: see appendix IV. The lesions vary in severity (size), clinical appearance and time to cure (Reithinger *et al.,* 2007).

Spontaneous self-healing usually results in lifelong protection from the disease. In the case of cutaneous leishmaniasis, different *Leishmania* species can infect the macrophages in the dermis with variable clinical presentations and prognoses (Arevalo *et al.,* 2007). In diffuse cutaneous leishmaniasis, the lesions are multiple, long-lasting, disseminated and cannot be spontaneously healed (Bañuls *et al.,* 2007). This form of leishmaniasis is considered to be a severe public health problem since it has devastating consequences to the patient. In the case of mucosal leishmaniasis, the most affected area is the nasal mucosa although lesions may be found in the lips, mouth, pharynx and larynx (Lessa *et al.*, 2007). These lesions are not self-healing and are usually seen months or years after the first episode of cutaneous leishmaniasis.

It is established that mucosal involvement is usually secondary to cutaneous lesions, although there are some cases where the mucosa is the primary site. Disease progression, from cutaneous to mucosal lesions, is mediated through the lymphatic system and rarely by direct contact between the mucosa and the cutaneous lesion. Although mucosal leishmaniasis can be caused by *L. panamensis, L. amazonensis, L. major L. tropica* and *L. infantum*, it seems that *Leishmania braziliensis* is responsible for the majority of mucosal leishmaniasis cases (Aliaga *et al.,* 2003).

Visceral leishmaniasis (Kala-zar) is the most severe form and is a systemic disease that is fatal if left untreated. Visceral leishmaniasis is caused by *L. donovani* (in East Africa and in India) and *L. infantum* (in Europe, Latin America and North Africa). Visceral leishmaniasis patients present symptoms and signs of persistent systemic infection including undulating fever, fatigue, weakness, loss of appetite and weight loss. Furthermore as the disease progresses it is manifested by lymph node, spleen, liver enlargement, lymphadenopathies, and anaemia. After recovery, patients may develop a chronic cutaneous form called post kala-zar dermal leishmaniasis that usually appears within two years after apparent clinical cure of the visceral infection (Salotra *et. al.*, 2001). The post-kala-zar dermal leishmaniasis is characterized by skin lesions that can be of various types and initially are most prominent on the facial area.

Leishmaniasis includes two major epidemiological entities: zoonotic which includes animal reservoir hosts in the transmission cycle of the disease, and anthroponotic in which humans are considered to be the sole source of infection for the sandfly vector. From a point of view of *Leishmania* control, priority has been given to anthroponotic foci, since these foci have been proven to be the source of visceral leishmaniasis epidemics (Desjeux, 2004).

2.4 Control Strategies and prevention measures

Current control strategies for leishmaniasis rely on case management, vector and reservoir control. Case management includes early diagnosis and treatment and is essential for both individual patients and the community. However, case management is difficult to be conducted because it is mainly restricted by several factors such as the lack of access to affordable, active drugs, incorrect prescribing and poor

compliance (Reithinger *et al.*, 2007). For visceral leishmaniasis, serological diagnosis is mainly based on ELISA, IFA and Direct agglutination test. Parasitological diagnosis can also be conducted using spleen, bone marrow and lymph node aspirates (Singh and Sivakumar, 2003). Diagnosis for cutaneous leishmaniasis is mainly conducted on skin smears.

Usually, pentavalent antimonials are used as first line drugs against leishmaniasis while Amphotericin B are used as second line drugs. Unfortunately due to their high cost they are restricted to developed areas (Koutis, 2007; Reithinger *et al.*, 2007).

Vector control is usually conducted on sandflies (Warburg and Faiman, 2011). Even though sandflies possess the essential biochemical mechanisms for resistance, they are highly susceptible to insecticides (Aghaei *et al*., 2011). In India, reports have shown that sandflies have shown resistance on organochlorine DDT (dichloro-diphenyl-trichloroethane) (Alexander and Maroli, 2003). Sandfly control is now mostly dependent on pyrethroids. House spraying is focused on the control of endophilic sandflies that rest mostly indoors after feeding (Kumar *et al.,* 2009). House spraying with the pyrethroid lambdacyhalothrin resulted in a risk reduction of cutaneous leishmaniasis by 60% and 54% in Kabul and in the Peruvian Andes, respectively (Kumar *et al.,* 2009). However, residual spraying is much more effective in urban situations when every house and animal shelter is treated than in rural areas where relatively few dispersed houses are sprayed and the sandflies represent a small proportion of the vector population.

Several studies have shown that pyrethroid-treated bednets provided 50-65% protection against leishmaniasis (Alexander and Maroli 2003; Picado *et al*., 2010). The synthetic pyrethroids used for net treatment combine the properties of low to moderate mammalian toxicity, low volatility and high insecticidal activity (Picado *et al*., 2010). The use of insect repellents or protective clothing has also been suggested as a prophylactic measure against leishmaniasis (Ritmeijer *et al.*, 2007). The above have been suggested for people who are at risk for leishmaniasis transmission such as travelers and soldiers in manoeuvres or hunters. Repellents applied to clothing rather than skin has been proposed as an alternative approach to personal protection against sandfly vectors. However, several studies have concluded that impregnated clothing for protecting humans from sandfly vectors may be impractical (Alexander and Maroli, 2003).

Reservoir control has mostly been employed where leishmaniasis is primarily zoonotic (Latin America, Mediterranean region, central and southeast Asia). In this case, screening and treatment of domestic animals, especially of dogs has been proposed. Nevertheless, a recent study showed poor specificity of the diagnostic assay (75%) used for diagnosing the infected dogs (Reithinger *et al.*, 2002). Treating infected dogs is however not an effective control strategy as relapses are frequent and dogs can regain infectivity weeks after treatment, even if they are clinically cured (Reithinger *et al.*, 2002). Besides, the widespread veterinary use of leishmaniasis drugs can lead to parasite resistance. On the other hand, killing of the seropositive animals has also been proposed, but the efficiency and acceptability of this control method is increasingly debated (Tesh, 1995). Several canine vaccine candidates are under study and one of them has recently been registered in Brazil for veterinary use

against symptomatic canine leishmaniasis with 80% efficacy (Queiroz *et al.*, 2009). Treating dogs with topical insecticide lotions can significantly reduce sandfly bites on dogs and consequently protect them from infection (Reithinger *et al.*, 2002). In this regard, insecticidal effect is long term and requires regular applications.

Another control method for protecting dogs from the disease is to fit them with collars impregnated with insecticides, such as deltamethrin. The use of treated collars reduced sandfly bloodfeeding by up to 90% and reduced the risk of *L. infantum* incidence rates in domestic dogs in southern Italy (Maroli *et al.*, 2001).

Leishmaniasis control remains a difficult issue and eradication of the disease is even more difficult. The current leishmaniasis control programs have largely failed mainly because of the insufficient regional health delivery systems and due to the limited local resources. Hence leishmaniasis has been classified as an emerging and uncontrolled disease by the World Health Organisation (WHO, 2010). Until an efficient vaccine becomes commercially available, the identification of risk factors could greatly help in designing prevention strategies

2.5 Current Leishmaniasis Chemotherapy

Treatment of leishmaniasis is difficult because of the intra-macrophagic location of the infectious form (Bañuls *et al*., 2007). In the absence of a potent vaccine against human leishmaniasis, the control of this disease relies primarily on chemotherapy (Handman, 2013). Current chemotherapy against leishmaniasis includes pentavalent antimonials, Amphotericin B, Pentamidine, miltefosine and paramomycin.

2.5.1 Pentavalent antimonials

Pentavalent antimonials are one of the drugs of choice for the treatment of all types of leishmaniasis. Pentostam and sodium stibogluconate can be administered intramuscularly or intravenously, which is distributed in high concentration in the plasma, liver and spleen. The recommended dose is 15-20mg/kg of body weight per day for 28 to 30 days by intramuscular or intravenous route. Intralesional administration of the drug has shown promising results by injection of 0.2-1 mL of pentavalent antimonial (Moore *et al.*, 2001).

The long course treatment allows antileishmanial levels of the drug to accumulate in tissues, particularly in liver and spleen. The treatment with antimonials has led to several side effects, such as: nausea, abdominal pain, myalgia, pancreatic inflammation, cardiac arrhythmia and hepatitis, leading to the reduction or cessation of treatment (Thakur *et. al*., 2004).

Currently, several limitations have decreased the use of antimonials. The variable efficacy against cutaneous leishmaniasis and visceral leishmaniasis, as well as the emergence of significant resistance has been increased. The recommendations have replaced the antimonials by amphotericin B (Murray, 2004).

2.5.2 Amphotericin B

Amphotericin B is poorly absorbed by gastrointestinal tract. Amphotericin B exhibits multicompartmental distribution and is found to be present in low concentrations in aqueous humour, pleural, pericardial, peritoneal and synovial fluids. Therapeutic doses of amphotericin B deoxycholate of 0.5 to 1mg/Kg by endovenous bolus daily

for 21 to 28 days can be administered or alternate days and with a total dosage between 1.5 to 2.0 g (Requena *et. al*., 2004). Serious adverse reactions have been displayed by the treatment with amphotericin B, including fever with rigor and chills, thrombophlebitis and occasional serious toxicities like myocarditis, severe hypokalaemia, renal dysfunction and even death. Its' use requires prolonged hospitalization and close monitoring (Croft *et* al., 2006).

Amphotericin B has excellent leishmanicidal activity and constitutes an option in patients that showed resistance to treatment with antimonials. The major limiting factor about the use of this drug is due to their toxicity. Three lipid associated formulations of amphotericin liposomal amphotericin B (AmBisome), amphotericin B lipid complex (Abelcet) and amphotericin B colloidal dispersion (Amphocil). These compounds have been considered the most striking advances in leishmaniasis therapy. Among the lipid formulations, AmBisome is the best tested and some studies demonstrated the successful in patients with cutaneous leishmaniasis and visceral leishmaniasis, particularly in areas where antimonials resistance has been detected (Solomon *et. al*., 2007).

2.5.3 Pentamidine

Pentamidine can be administered parenterally, by intramuscular or intravenous route. The regimen consists of 4 mg/Kg three times a week for 3-4 weeks (Tracy and Webester, 2001). Commonly, the treatment with pentamidine causes myalgias, pain at the injection site, nausea, headache and less frequently result in a metallic taste, a burning sensation, numbness and hypotension. Reversible hypoglycemia occurs in

about 2% of cases. It causes irreversible insulin dependent diabetes mellitus and death (Sundar and Chatterjee, 2006).

Pentamidine is one of the drugs for clinical use in all forms of leishmaniasis. However, a low cure rate for pentamidine (35%) in patients infected with *L. braziliensis* in Peru has been reported (Andersen *et. al*.2005). The cure rate associated with low dose of pentamidine, given for a short period, makes it an attractive alternative for cutaneous leishmaniasis in antimonies treatment failure cases. In general, the use of this drug has declined due to their low efficacy and toxicity (Sundar and Chatterjee, 2006).

2.5.4 Miltefosine

Miltefosine has been in the treatment of visceral leishmaniasis cases in immunocompetent patients from India and Germany (Berman, 2006). Depending on the individual weight, the recommended therapeutic regimen for patients weighing less than 25 Kg is a single oral dose of 50 mg for 28 days by oral route, whereas individuals weighing more than 25 Kg require a twice daily dose of 50 mg for 28 days (Sundar and Chatterjee, 2006). Adverse effects of miltefosine include gastrointestinal disturbances and renal toxicity. Fortunately, these symptoms are reversible and they are not a major cause for concern.

2.5.5 Paromomycin

Paromomycin is poorly absorbed into systemic circulation after oral administration, but rapidly absorbed from intramuscular sites of injection. Peak concentration in

plasma occurs in 30-90 min and its apparent volume of distribution is 25 % of body weight.

Three preparations of paromomycin ointments have been used for cuteneos leishmaniasis: paromomycin 15% plus methylbenzethonium chloride 12%, paromomycin 15% with urea 10% and paromomycin plus gentamicin 0.5%. These formulations have shown variable results according to the species of *Leishmania* involved and the epidemiologic situation (Sundar and Chakravarty, 2008).

2.6 Immunomodulation

Previous studies which have divulged that leishmanicidal plants and plant secondary metabolites can potentiate key cellular immune responses, have proven to be coherent in the quest for more efficacious and less toxic antileishmanial drugs (Chouhan *et. al*., 2014). Since elevation of host immunity is critical in parallel to the drug-mediated killing of *Leishmania* parasites, the antileishmanial arsenal may be benefited by antileishmanials that can prong a bifurcated attack, by eliminating the parasites and restoration of Cell Mediated Immunity.

The potential of immunomodulators in treating experimental leishmaniasis gained momentum with the discovery of antileishmanial activity of imiquimod, a receptor which is present on macrophages and dendritic cells and promotes the development of Th1 immune response (Arevalo, *et. al*., 2001).

Various herbal formulations and plant secondary metabolites such as flavonoids, isoflavonoids, saponins, alkaloids, sesquiterpenes, polysaccharides, tannins, indoles, and glucans are known to be immunomodulatory in *Leishmania* infections (Chouhan

et. al., 2014). Plant extracts contain biomolecules that can naturally kill *Leishmania* parasites and also exert immunostimulatory properties on otherwise depressed immune system during the diseased state.

Studies examining immunomodulatory effect of bioactive plant extracts or compounds have reported skewing of immune response from Th2 (diseased state) to Th1 (cure) by causing the upregulation or downregulation of pro-inflammatory (activating Th1) and anti-inflammatory (promoting Th2) cytokines, respectively.

The most commonly assessed immunomodulatory parameter for parasite clearance is stimulation of Nitric Oxide (NO). NO is the principle effector molecule in killing of *Leishmania* amastigotes and is either estimated directly as nitrite concentration in culture supernatant or indirectly by the changes in nitric oxide synthase gene expression levels (Ukil *et. al*., 2005).

IL-12 is the central cytokine produced by dendritic cells, natural killer cells and T cells, which activates macrophages to produce IFN-γ and TNF-α. Different plant secondary metabolites and extracts have induced IL-12 up-regulation, indicating the worthy potential of natural resources. Macrophages also produce IL-18, which in synergism with IL-12 stimulates IFN-γ production and aids in parasite clearance (Trun *et. al*., 2006).

2.7 Treatment of Leishmaniasis using medicinal plants

There is growing interest in alternative medicine due to the factors that influence the treatment of leishmaniases. These alternative medicines are from natural products derived from plants (Calixto, 2000). Previous studies using natural plant extracts have

shown activity against *Leishmania*. A range of species of plants have been used in these studies including *Allium sativum* (Wabwoba *et al.,* 2010), *Ricinus communis* (Okech *et al.,* 2006), *Aloe vera* (Dutta *et al*., 2007), Waburgia ugandensis (Ngure *et al*., 2009) and *Moringa stenopetala* (Kinuthia *et al*., 2014) among others.

Plant derivatives have also been used against *Leishmania spp*. These include naphthoquinones like plumbagin from *Plumbago zeylanica* and biplumbagin isolated from stem and root extracts of *Pera benesis* (*Euphobiaceae*) have been used in folk medicine in Bolivia as treatment of CL caused by *L. braziliensis* when fresh stalks are applied directly on the lesion (Fournet *et al.,* 1992). Lichochalcone, derived from a Chinese Lichorice plant roots has been shown to inhibit growth of *L. major, L. donovani* and *Plasmodium falciparum* (Chen *et al*., 1993). Other plant constituents shown to have antileishmanial activites are saponin, sequiterpenes, anthocynadins, alkylamines, iridoid glycosides, lignin, diospyrin and aromatic polysulphur compounds (Tiuman *et al*., 2005; Makwali *et al*., 2012).

2.8 Tephrosia species

The genus *Tephrosia* is a legume found in the tropical and subtropical regions of the world, some of which have been used for many beneficial purposes. These include *T. densiflora, T. purpurea, T. uniflora, T. vogelii* and *T. nana* (Burkill, 1995). These species are used as fish poison beans, abortifacient, against skin diseases, for treating diarrhoea and whooping cough in children; vaginal douches, spasmodic coughing, fevers, sterility, rickets, bone disease caused by a deficiency of vitamin D or calcium (Touqeer *et al*., 2013). *T. purpurea* is so much related to *T. vogelii* and it has been used against *L. donovani* infection in hamsters (Sharma *et al*., 2003).

In Nigeria *Tephrosia spp.* is used as a diuretic, blood purifier, a gargle, laxative, diuretic and for deobostruant properties, for bronchitis and bilious febrile attacks, the treatment of boils, pimples and bleeding piles, for coughs and kidney disorders (Oliver-Bever, 1982). The seed oil from these species is applied to scabies, itch, eczema and other eruptions of the skin (Rastogi and Mechrottra, 1990).

T. uniflora has been shown to be toxic to *Bulinus globulus*, a fresh water snail vector of schistosomiasis (Adewunmi, 1980). Some other *Tephrosia* species of less ethnomedical importance include *T. deflexa*, *T. gracilipes*, *T. humilis*, *T. mossiensis* and *T. radicans*. The plant species *T. vogelii* has several ethnomedical uses. The leaf is ichthyotoxic and has been used as an insecticide, rodenticide and antihelminthic. The leaf sap and root scrapings are used as ear and tooth ache remedies respectively. Extracts from the root have been used as a molluscicide. The plant extracts have also been used in the treatment of tuberculosis, typhoid fever and localised fungal infections (Ekpendu, 1998b). *T. vogelii* is widely distributed and readily available locally. The species is reviewed in detail below.

2.8.1 Morphology and taxonomy of *T. vogelii*

T. vogelii is a leguminous plant belonging to the class Magnoliopsida (dicotyledons), subclass Rosidae order Fabales, family Fabaceae, genus *Tephrosia* and species vogelii. It is a much branched shrub and is always under cultivation, and ubiquitous in tropical Africa and India. There are two morphologically alike forms differing only in flower colour, red or bluish purple commonly in West Africa, and white in East Africa (Burkill, 1995).

The plant is easy to propagate by seed, seeding at 6-7 months, but taking about 3 years to reach maturity (Burkill, 1995). *T. vogelii* is a shrub, 1.83-3.05 m high, clothed with dense yellowish or rusty tomentum. The stems are more or less erect and the leaflets are five or more pairs. Fruits of *T. vogelii* are large and 2-12 cm long, very densely villous or tomentose. The shrub may grow as rapidly as 2-3 m in 7 months: see appendix V.

According to Gaskins *et al.,* (1992), the flower of *T. vogelii* is typically papilionaceous, about 2.5 cm across and purple with white markings or white. The flowers are borne on compact racemes that bloom over a 3 to 6 week period. There may be 20 to 30 flowers per raceme with up to 200 flowers per plant. Pods usually contain 8 to 16 seeds. The flowers have a faint but definite pleasant aroma, and bees visit them freely for both nectar and pollen. Flowering occurs on decreasing day lengths. *T. vogelii* is native to West Africa, including Nigeria, and other regions of tropical Africa in general, but is now found in India, Asia and other tropical regions (Lambert, 1993). According to Burkill, (1995) the exact origin of the plant is uncertain. It is cultivated throughout tropical Africa, particularly in West Africa and chiefly in the forest regions, but also in the Savannah zones (Lambert, 1993). *T. vogelii* is cultivated around many villages, either casually or by riverine people in fields for use in stupefying fish (Lambert, 1993). The principal use from which the very wide dispersal of the plant has arisen is as a fish poison (Burkill, 1995).

2.8.2 Ethnomedical uses of *T. vogelii*

Many ethnomedical uses have been advocated for this plant. Dzenda *et al*., (2007) reviewed the ethnomedical and veterinary uses of *T. vogelii* Hook F (Fabaceae) in

Nigeria. Apart from its wide spread use as a fish poison (Ibrahim *et al.*, 2009.), it is cultivated to a lesser extent as part of a medicament for bone setting (Ekpendu, 1998a).

Ground leaves and stem bark are mixed with vegetable oil and rubbed on the skin around the fractured limb; pieces of cut stem are used to hold the broken limb in position. Roots are boiled in water and when warm, feet with localised fungal infections are immersed therein for some minutes (Ekpendu, 1998b). In East Africa the hot water leaf extract is used as an abortifacient and for induction of menses (Haaf, 1971).

The leaf macerate is purgative and emetic (Burkill, 1995). The crude methanolic extract of *T. vogelii* leaves has been shown to induce contraction of isolated rabbit jejunum (Dzenda, 2004a) and guinea pig ileum (Dzenda, 2004b) in a concentration dependent manner, supporting its use as purgative. The sap is added to palm wine to treat diarrhea (Burkill, 1995).

In Angola, it is one of the medicinal plants used as a piscicide, anthelminthic, insecticide and for treating tuberculosis and as a bactericide. Its' anthelminthic property is dose dependent (Bossard, 1993). The roots are used by natives to treat typhoid fever. It is used in China as a botanical insecticide and fly repellent (Chiu, 1989). *T. vogelii* has been shown to have toxic and repellent effects against certain insect pests of stored grains (Koona and Dorn, 2005) supporting the widespread use of the plant by local farmers as a grain protectant. *Tephrosia* has been used as a rat poison by compounding with ground nut (Nwude, 1997).

The powder from *T. vogelii* is effectively used against the stored ground nut pest *Caryedon serratus* and also as a seed dresser for cereals and legumes (Nwude, 1997). It is also applied directly to treat head lice, fleas, scabies and other ectoparasites (Nwude, 1997). The leaf extract has been observed to be highly toxic to ticks. Cattle sprayed with the extract had a residual protection period of ten days from reinfection by ticks in Zambia (Kaposhi, 1992). Fresh water snails have been found to be susceptible to extracts of crushed, unboiled root, and this could have some bearing in combating schistosomiasis (Burkill, 1995).

2.8.3 Biological activities of *T. vogelii* extracts

Extracts of the leaf, bark, root, seed and/or flower of *T. vogelii* possess numerous biological activities when tested in the laboratory. *T. vogelii* was found to possess antimicrobial activity (Wanga *et al*., 2006). The dichloromethane extracts from the roots and leaves were tested against *S. aureus*, *E. coli* and *F. phoseolida*. Hu *et al*., (2011) also studied the antimicrobial and bactereostatic activity of ethanol and aqueous extract from *T. vogelii* seeds on *E. coli*, *S. aureus* and *S. paratyphi* and proved the antibacterial efficacy of the plant to be significant at high doses (Hu *et al*., 2011).

T. vogelii has also demonstrated anti-inflammatory activity. The methanolic extract of *T. vogelii* showed significant analgesic and anti-inflammatory activity in mice and rats using hot plate method and egg albumin induced oedema respectively (Adaudi *et al*., 2009). The extracts also possess larvicidal activity. Acetone extract of the leaf showed feeding deterrent activity against the insect *Pieris rapae* and the acetone extract of the seed showed larvicidal activity against *Aedes aegypti* (Matovu and Olila, 2007).

T. vogelii leaf extract was found to be effective in controlling ticks, an important insect and ectoparasite (Gadzirayi *et al*., 2010). Acaricidal activity of leaf extracts of *T. vogelii* on the tick *Rhipicephalus appendiculatus* (Kalume *et al*., 2012) and *R. sanguineus* (Jacques *et al*., 2015) has been reported.

The leaf extract of *T. vogelii* was found to possess significant anthelmintic activity against *Ascaridia galli*, a parasite in chicken (Siamba *et al*., 2007). *T. vogelii* also showed molluscidal activities. The water extract of the dried flowers, stem and leaves showed molluscidal activity against *Physopsis globosa* while the water extract of the dried leaf showed activity against *Bulinus globosus* (Chiotha and Msonthi, 1986). The flower and flower bud of *T. vogelii* have been reported to be toxic to *Bulinus (Physopsis) globosus* (Adewunmi, 1980). The petroleum ether extract of the plant demonstrated molluscidal activity against *Biomphalaria glabrata* (Marston *et al*., 1984). Ethanol (80%) extract of the dried leaf showed weak molluscidal activity against *Biomphalaria pfeifferi* and *Bulinus truncatus* (Abdel-Aziz *et al*., 1990). Water extract of oven dried stem, leaf and seed showed weak molluscidal activity against *Biomphalaria pfeifferi* (Kloos *et al*., 1987). The plant is also used against parasitic skin diseases (Oliver-Bever, 1982).

CHAPTER THREE

MATERIALS AND METHODS

3.1 Study site

The study was carried out in the *Leishmania* laboratory of the Centre for Biotechnology and Research Development (CBRD), at the Kenya Medical Research Institute (KEMRI), Nairobi.

3.2 Study design

The *in vitro* studies were carried out using a comparative study design. The efficacy and toxicity of test samples were compared with those of pentostam and Amphotericin B. Rosewell Park Memorial Institute 1640 Medium (RPMI) was used as a negative control in experimental chemotherapeutic studies. *In vivo* studies were carried out using a completely randomized design. Initially, *L. major* infected BALB/c mice aged eight weeks were randomly divided into five groups of 8 mice each treated using the *T. vogelii* extract orally, *T. vogelii* extract intraperitoneally, PBS, Amphotericin B and Pentostam.

3.3 Source of plant and collection of the leaves

Identified and dried *T. vogelii* leaves originally collected form Karura forest in Nariobi were obtained from Kenya Medical Research Institute (KEMRI) herbarium.

3.4 Preparation of Methanolic plant extracts

Extraction was done in KEMRI at the Centre for Traditonal Medicine and Drug Research (CTMDR). The dried leaves were processed (to obtain the extract) according to the method of Kigondu *et al*., (2009).

The dry leaves were chopped into small pieces and then ground into powder form using a laboratory blender. 1 kg of each powder was soaked in absolute methanol for three days to extract compounds. The extract was filtered, dried with Sodium Sulphate and the solvent removed under vacuum in a rotary evaporator at 30-35°C and stored at 20°C until required for use. This methanolic extracts were used for anti leishmanial tests.

3.5 Mice

In this study, BALB/c mice were used because they are susceptible to *Leishmania* parasite. BALB/c mice aged 8 weeks were used for the study and were obtained from the KEMRI's animal house facility. The experiments were done in compliance with Animal Care and Use Committee (ACUC) guidelines of KEMRI. Standard Operating procedures (SOPs) available at *Leishmania* laboratory at CBRD including immunizing the animals using standard 21G needles, anaesthetizing and killing them using painless method approved by ACUC(100 µl of Sagatal) were adhered to. Dead mice were sterilized by dipping in appropriate biohazard bags before transfer for incineration.

3.6 *Leishmania* parasites

Leishmania major (strain IDUB/KE/83=NLB-144) which was originally isolated in 1983 from a female *P. dubosqi* collected near marigat Baringo County Kenya were used (Beach *et al*., 1984). These parasites were cultivated in Schneider's Insect Medium supplemented with 20% heat inactivated foetal bovine serum, 100 µg/ml penicillin and 50µg/ml streptomycin (Hendricks and Wright, 1979) and 250 µg/ml 5-fluorocytosine arabinioside (Kimber *et al*., 1981). This strain has been maintained by

cyropreservation and *in vitro* culture and periodic passage in BALB/c mice at KEMRI, Nairobi.

3.7 *In vitro* studies

3.7.1 Cytotoxicity studies

In this assay, vero cells were used. Vero cells were used to determine the toxic levels of the plant extract on mammalian cells. The vero cells were cultured and maintained in Minimum Essential Medium (MEM) supplemented with 10% Foetal Bovine Serum. The cells were cultured at 37^{o}C in 55% CO_2 for 24 hours and harvested by trypsinization. The cells were then pooled in a 50ml vial and 100µl cell suspension (1x 10^{6}cells/ml) and put into 2 wells of rows A-H in a 96-well micro titer plate. The medium was aspirated off and 150µl of the highest concentration (1200µg/ml) of the *T. vogelii* added into the first row and serial dilution carried out.

The experimental plates were incubated further at 37^{0}C for 48 hours. The controls used were cells with no extract but medium alone. MTT reagent (10µl) was added into each well and the cells incubated for 4 hours until a purple precipitate clearly visible under a microscope was formed. The medium together with MTT was aspirated off from the wells. Dimethylsulfoxide (DMSO) (100µl) was added and the plates shaken for 5 minutes. The absorbance was measured for each well at 562nm using a micro-titre plate reader. Cell viability (%) at each concentration was calculated as described by Wang *et al.*, (2006) using the formula:

$$\text{Cell viability (\%)} = \frac{\text{Ave. absorbance in duplicate drug wells - Ave. blank wells x100}}{\text{Average absorbance control wells}}$$

3.7.2 Minimum Inhibitory Concentration (MIC) Evaluation

L. major promastigotes (10^6 parasites/ml) were incubated at 26^0C for 120hr in fresh media (brain heart infusion medium), supplemented with 10% FBS in the absence or presence of several concentrations ((10µg/ml to 100µg/ml) of the extracts (cell growth was determined daily by assessment of visible turbidity). The MIC was considered as the lowest concentration of each substance used that inhibited more than 99% of *L. major* growth *in vitro.*

3.7.3 Determination of 50% inhibitory concentration (IC_{50})

The anti-leishmanial activity against promastigotes was determined as explained by Tempone *et al.*, (2004). Parasite viability was determined using MTT assays (Tada *et al.*, 1986) The antileishmanial activity against intracellular amastigote was determined with infected macrophage (Tempone *et al.*, 2004) using pentostam and Amphotericin B as standard drugs. Parasite burden was defined as number of infected macrophages in total of 400 cells. Each assay was performed in triplicate.

3.7.4 Anti-promastigote assay

L. major promastigotes were incubated in 24-well plates in the presence of different concentrations of the extract. After 4-5 days of cultivation, aliquots of parasites were transfered to a 96-well micro-titre plate.

The parasites were then incubated at 27^oC in 5% CO_2 for 24 hours; 200µl of highest concentration of extract was added and diluted. The experimental plates were incubated further at 27^oC for 48 hours. The controls used were promastigotes with no extracts and medium alone. The medium together with MTT (3-(4, 5-dimethylthiaol-

2-yl)-2, 5-diphenyltetrazolium bromide) was aspirated off the wells. In each well, 100µl of DMSO was added and the plates shaken for 5 minutes.

Absorbance was measured for each well at 562nm using a micro titre reader and the 50% inhibitory concentration (IC_{50}) values generated. Percentage promastigotes viability was calculated as follows using the formula by Mosmann, (1983) for each concentration

$$\text{Cell viability (\%)} = \frac{\text{Ave. absorbance in duplicate drug wells} - \text{Ave. blank wells} \times 100}{\text{Average absorbance control wells}}$$

3.7.5 Anti-amastigote assay

The anti-amasigote assay was carried out as described by Delorenzi *et al.,* (2001) using peritoneal macrophages which were obtained from BALB/c mice. Mice were anaesthesized using 100µl pentobarbital sodium (sagaatal). The body surface was then disinfected with 70% ethanol. The torso skin was torn dorsoventrally to expose the peritoneum. Using a sterile syringe and needle, 10ml of sterile cold phosphate-buffered saline (PBS) was injected into the peritoneal cavity.

After shaking the mouse, peritoneal macrophages were harvested by withdrawing the PBS. The contents were transferred into a sterile 50 ml centrifuge tube. The suspension was centrifugally washed at 2000 rpm for 10 minutes and the pellet resuspended in complete RPMI 1640 medium.

Macrophages were adsorbed in 24-well plates and allowed to adhere for 4 hours at 37^oC in 5% CO_2. Non-adherent cells were washed with cold PBS and macrophages incubated overnight in RPMI. Adherent macrophages were then infected with parasite/macrophage ratio of 6:1 and further incubated at 37^oC in 5% CO_2 for 4 hours.

Free macrophages were removed by extensive washing with PBS and the cultures incubated in RPMI for 24hours. Treatment of infected macrophages with the samples was done once. Pentostam and Amphotericin B were used as a positive control drugs for comparison of parasite inhibition. The medium and drug were replenished daily for 3 days. After 5days, the monolayers were washed with PBS at 37^{o}C, fixed in methanol and stained with Giemsa. The number of amastigotes was determined by counting at least 100 macrophages in duplicate cultures, and the results expressed as infection rate (IR) and multiplication index (MI) (Berman & Lee, 1984) as follows;

IR = No. of infected macrophages in 100macrophages

$$MI = \frac{\text{No. of macrophages in experimental culture/100macrophages x100}}{\text{No. of macrophages in 100 control cultures/1000 macrophages}}$$

3.7.6 Nitric oxide production assay

Nitric oxide release in macrophage cultures was measured using the Griess reaction for nitrites (Hollzmuller *et al.*, 2002); see appendix VIII. Briefly, 100µl of the supernatants were collected 48 hours after introducing the test drug into the culture medium. The assay was done in triplicate wells in 96-well micro-titre plates. To this, 60µl of Griess Reagent A (1% sulphanilmide in 1.2M HCL) was added followed by 60µl of Griess Reagent B (0.3% N(1-naphthyl)Ethylenediamine). The plates were read at 540nm in enzyme-linked immune absorbent assay (ELISA) reader. Sodium nitrite in RPMI was used to construct a standard curve for each plate reading.

3.8 *In vivo* studies

3.8.1 Mice and parasite inoculation

In the whole study BALB/c mice were used. At the beginning, eight week old female BALB/c mice were used. They are highly susceptible to *L. major*. Inoculation of mice

with parasites was done intradermally on the left hind footpads while the right hind footpad served as an uninfected control. Lesion measurement was done using a direct reading vernier caliper using the method of Nolan and Farrell, (1987); See appendix VI.

Lesion size = Size of infected footpad - Size uninfected control footpad

3.8.2. Experimental protocol

The mice were divided as follows: set of 40 mice were inoculated intradermally with 10^6 of *L. major* culture and left for 4 weeks. They were then divided into 5 groups of 8 mice each. Group 1 was treated with *T. vogelii* extracts orally, group 2 with *T. vogelii* extracts intraperitoneally, group 3 with pentostam intraperitoneally, group 4 with Amphotericin B intraperitoneally and group 5 with PBS intraperitoneally.

3.8.3. Quantifying parasite burden from spleens and liver

All the BALB/c mice that were infected with 1×10^6 *L. major* stationary phase promastigotes and treated (experimental and positive control and negative controls) were euthanized and the spleens and liver removed and weighed; ten weeks post infection as shown in appendix VII. Impression smears were made by making transverse section of the spleens and the liver on grease free microscope slides. The impression smears were dried, methano-fixed and Giemsa-stained and examined microscopically.

Parasite counts were then recorded using the standard World Health Organization method (WHO, 2004). The parasite loads were determined using the Leishman Donovan Units (LDU) method of Bradley and Kirkley, (1977) as follows:

$$LDU = \frac{\text{Amastigotes in nucleated cells x weight of organ (grams) x } 2x10^5}{1000}$$

3.8.4. Disposal of animals

All sacrificed animals were disposed according to the regulations of the Animal Use and Care Commtitte (ACUC), KEMRI. At sacrifing, all animals were injected with 100 ul pentabarbitone sodium (Sagatal).

3.9 Data Analysis

SPSS software was used in the analysis of lesion sizes, parasite loads and absorbance. The mean and standard errors of the lesions of each treatment group were compared using students't-test and analysis of variance (ANOVA). Students't-test was used in the analysis of differences in means obtained for the experimental groups. Chi-square test was used in the analysis of infection rates and multiplication indices. P values of equal or less than 0.05 ($P \leq 0.05$) were considered significant.

CHAPTER FOUR

RESULTS

4.1 Cytotoxicity assay

Safety of the test compounds was tested by treating vero cells with the test compounds and their viability was checked. Results indicating the cell viability of vero cells subjected to the test compounds are shown in Figure 4.1.

Crude extracts of *T. vogelii* caused no significant adverse effects on the vero cells (P=0.125). Concentration of the test drug required to destroy 50% of the mammalian cell was significantly low in Amphotericin B (200µg/ml), followed by Pentostam (250 µg/ml) then *T. vogelii* (1200 µg/ml). RPMI was a negative control hence did not have any effect on the vero cells. Minimum Inhibitory concentration for all the test drugs did not result in significant vero cell destruction. 1% of the cells were inhibited by 10 µg/ml concentration of amphortericin B, 12 µg/ml concentration of pentostam and 25 µg/ml concentration of *T. vogelii* respectively at the end of the treatments.

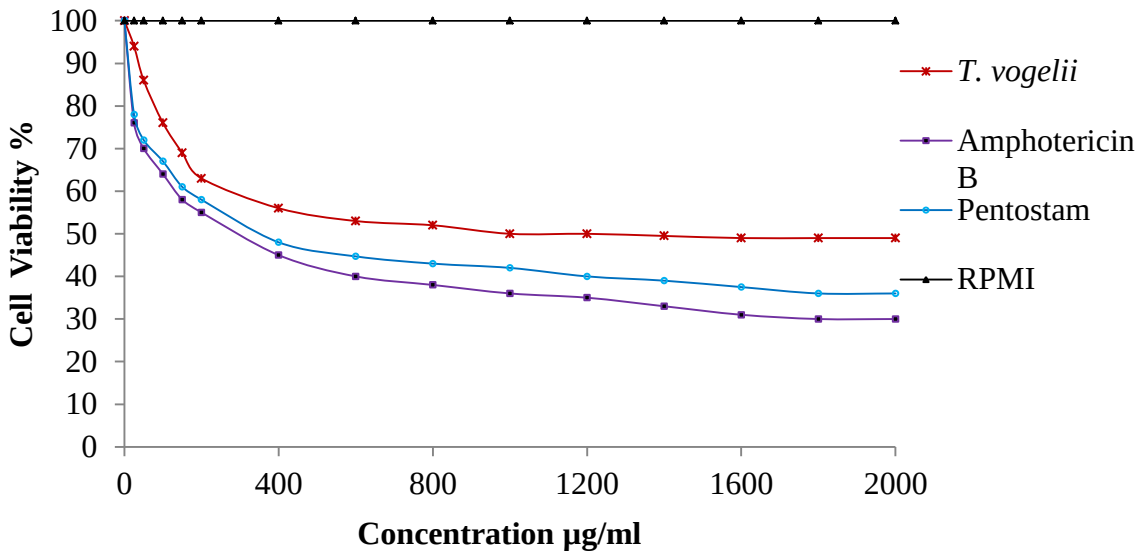

Figure 4.1: Cell Viability of the vero cells subjected to the different test drugs

4.2 Efficacy of *T. vogelii* crude extracts on promastigotes of *L. major*

Table 4.1 describes the optimal efficacy, IC_{90} and IC_{50} of the test drugs against promastigote forms of the parasites.

The growth of promastigotes were significantly inhibited by the various test compounds (P=0.003) after 24 h of exposure. There were significant differences in the optimal efficacy of the test drugs (P = 0.0001). The optimal efficacy of the standard drugs was 97.5% and 98.9% for Amphotericin B and Pentostam respectively. *T.vogelii* achieved an optimal efficacy of 91.4% against promatigotes. There was a significant difference in the IC_{50} and IC_{90} of the test compounds (P < 0.05). The standard drugs were more effective against promastigote as compared to *T. vogelii* with Pentostam having an IC_{50} of 5.5µg/ml, Amphotericin B with 7.8µg/ml while that of *T. vogelii* being 12µg/ml. There was significant (P =0.001) difference in the IC_{90} with the lowest IC_{90} occuring in Pentostam followed by Amphotericin B then by *T. vogelii* with 18µg/ml, 25.5µg/ml and 64µg/ml respectively.

Table 4.1: Optimal efficacy, IC_{90} and IC_{50} of test drugs against promastigote form of *L. major*

	Test drugs			**Statistics**	
Concentration	***T. vogelii***	**Pentostam**	**Amphotericin B**	**F-value**	**P-value**
Optimal efficacy (%)	92.0	98.9	97.5	36.654	0.0001
Concentration at optimal efficacy	65	30	32	9.012	0.001
IC_{90}	64	18.0	25.5	65.226	0.0002
IC_{50}	12.0	5.5	7.8	85.456	0.001

4.3: Efficacy of *T. vogelii* crude methanolic extracts on amastigotes of *L. major*

To test the efficacy of the test drugs against *L.major* amastigotes, the Infection Rates (IR) and Multiplication Indices (MI) were determined.

4.3.1 Infection rates of the amastigotes in peritoneal macrophages

The infection rates are shown in Figure 4.2. There was a general decrease in infection rate with increase in concentration of the treatments applied across the groups with significant difference between *T. vogelii* and the standard drugs (P=0.01). However, Pentostam and Amphotericin treatments were not significantly different (P = 0.32). At the least concentration of 25µg/ml, the macrophages were infected by amastigotes at 80% with *T.vogelii* treatment. At the same concentration Pentostam and Amphotericin B had 22% and 18% infection rates respectively. The infection rates reduced with increase in concentration with Amphotericin B and Pentostam falling from 18% to 2% and 22% to 3% respectively; the same trend is observed in *T. vogelii* reducing from 80% to 20% when the concentration is increased to 200µ/ml.

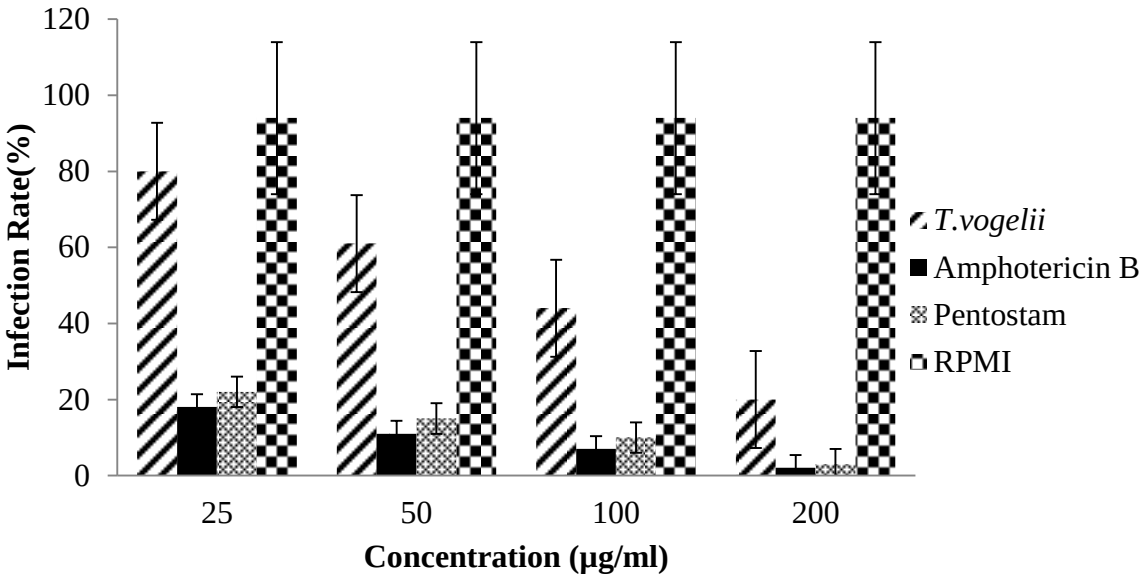

Figure 4.2: The infection rates of amastigote in peritoneal macrophages following treatments with the different test drugs

4.3.2: Multiplication index of *L. Major* amastigotes in the peritoneal macrophages

The multiplication indices are shown in Figure 4.3. The Multiplication index for *L. major* amastigotes in peritoneal macrophages treated with 200µg/ml of *T. vogelii* was significantly different when compared with the standard drugs (P=0.031). However, there was no significance difference between Amphotericin B and Pentostam (P=0.075). At the highest concentration (200µg/ml), *T. vogelii* extract treatment showed a multiplication index of 20.57%, 5.65% for amphotericin B and 9.56% for pentostam. At a lower concentration of 25µg/ml *L. major* amastigotes showed multiplication indices of 75.86% in *T. vogeli*i extract, 47.5% in Amphotericin B and 51.07% in Pentostam.

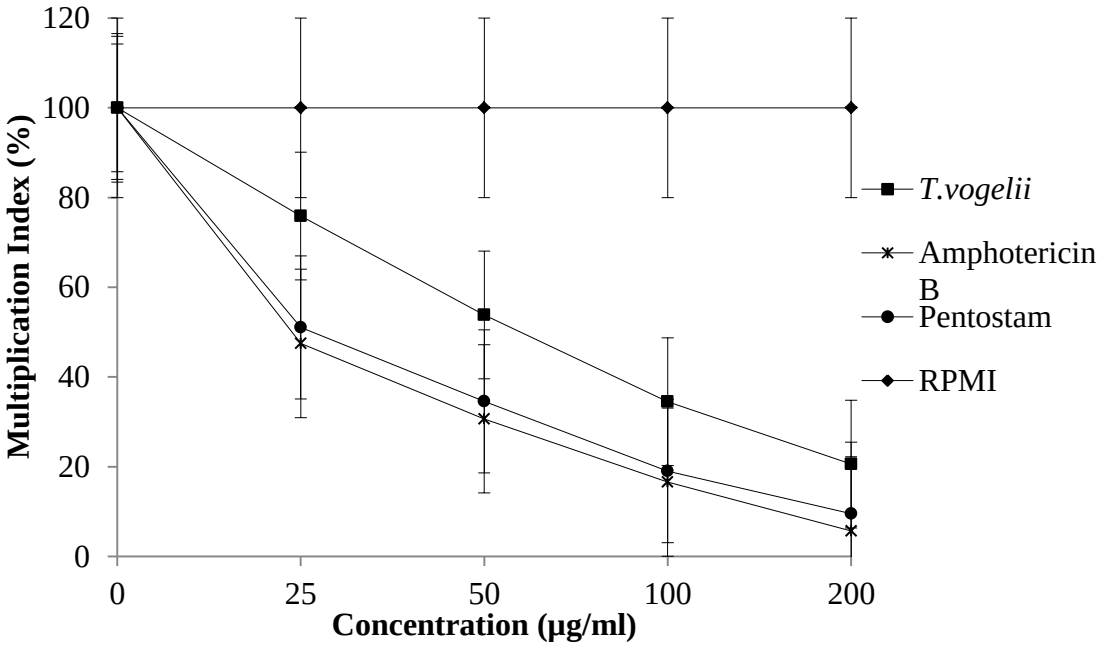

Figure 4.3: Multiplication indices (growth rates) of amastigotes in peritoneal macrophages after treatment with the different test drugs.

4.4: Effect of *T. vogelii* crude methanolic extracts on *L. major* lesion development in BALB/c mice

The lesion sizes of BALB/c mice at the start of infection and in the course of treatment with the different test drugs are as shown in Figure 4.4.

There were no significant differences in development of lesion sizes in BALB/c mice during the first 4 weeks post-infection with *L. major* (P=0.34). Between week 5 and 10, the differences in lesion sizes progression were subjected to repeated measure ANOVA, which indicated that there were significant differences in lesion sizes among different treatment groups (P = 0.004). The lesion sizes of the untreated control BALB/c mice increased steadily after infection. Smallest lesion sizes occurred in BALB/c mice treated with amphotericin B, which was slightly lower than BALB/c mice treated with Pentostam but there was no significance difference between the two (P=0.35). BALB/c mice treated with *T. vogelii* intraperitoneally resulted in significantly (P= 0.01) larger reduction of lesion sizes than the BALB/c mice treated orally with *T. vogelii* treatment as shown in Figure 4.4.

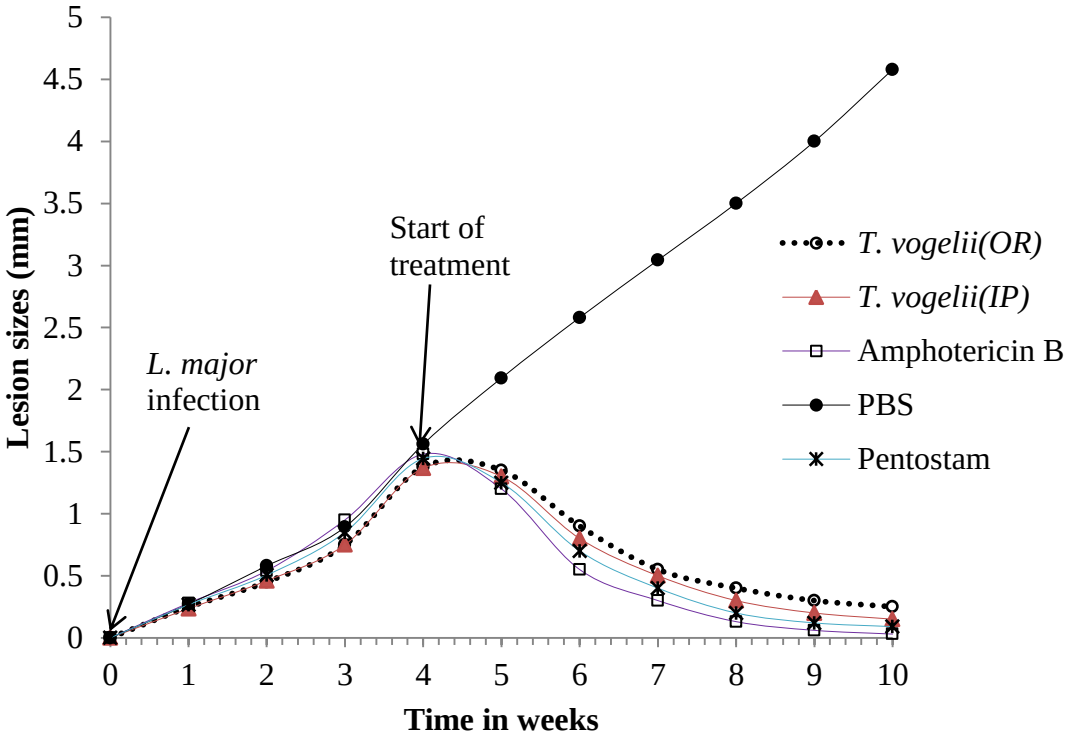

Figure 4.4: Lesion sizes of BALB/c mice at the start of infection, at the start of treatment and during the treatment with the test drugs

4.5: Body weights, organs weight, organo-somatic indices and parasite loads in spleen and liver of BALB/c mice treated with different test drugs

Body weights, weight of spleen, liver and organo-somatic indices in BALB/c infected with *L. major* infection under various treatments are as shown in Table 4.2.

There were significant differences in the weight of spleen, liver, spleno-somatic and hepato-somatic indices among treatment groups ($P<0.05$). Body weights of the mice were not significantly different after the various treatments ($P=0.058$). The untreated control group had significantly large spleen and liver weights alongside spleno-somatic and hepato-somatic indices accounting for the spleno and hepato megaly observed.

Pentostam and Amphotericin B treated groups had no significant difference between them producing the least spleen and liver weights and respective spleno-somatic and hepato-somatic indices (P=0.0002). *T. vogelii* treatment produced an intermediate between the untreated control and the standard drugs treated groups. However, intraperitoneal administration of the test extract resulted in lower spleen/liver weights and organo- somatic indices than the oral administration mode (P=0.01).

Table 4.2: Body weight, organ weight and organo-somatic indices in BALB/c mice after the different treatments

Treatment	Body weights (g)	Weight of spleen (g)	Spleno-somatic index	Weight of liver (g)	Hepato-somatic index
***T. vogelii* (IP)**	21.10 ± 0.52	0.17 ± 0.021[b]	0.85 ± 0.10[b]	0.18 ± 0.021[b]	0.86 ± 0.01[b]
***T. vogelii* (OR)**	21.02 ± 1.00	0.21 ± 0.005[c]	0.99 ± 0.06[c]	0.20 ± 0.05[c]	0.98 ± 0.07[c]
Amphotericin B	21.05 ± 0.54	0.14 ± 0.010[a]	0.74 ± 0.05[a]	0.15 ± 0.015[a]	0.73 ± 0.05[a]
Pentostam	21.06 ± 0.58	0.15 ± 0.010[a]	0.75 ± 0.05[a]	0.16 ± 0.010[a]	0.75 ± 0.05[a]
PBS	21.06 ± 1.72	0.38 ± 0.014[d]	1.83 ± 0.21[d]	0.37 ± 0.014[d]	1.83 ± 0.51[d]
F	2.21	32.25	69.23	29.1498	65.72
Df	4	4	4	4	4
P	0.053	0.0001	0.0003	0.001	0.000

Legend:

T. vogelii (IP): *T. vogelii* given through intraperitonial administration

T. vogelii (OR): *T. vogelii* given orallys

The means in the same column having the same letter superscript are not significantly different; by LSD (Scheffe method).

4.6: Parasite loads in spleens of *L. major* infected BALB/c mice after treatment with the different test drugs

The LDU of *L. major* parasites in the spleens of BALB/c mice infected with *L. major* and treated with *T. vogelii*, Amphotericin B, Pentostam and PBS are shown in Figure 4.5.

The LDU in the spleens of BALB/c mice subjected to oral and intraperitoneal administration of *T. vogelii* were significantly different (P = 0.0014). Treatment with *T. vogelii* intraperitoneally resulted in significantly lower LDU (3.21×10^6) than those observed when *T. vogelii* was administered orally 5.05×10^6 (P=0.025). Infected BALB/c mice treated with Amphotericin B and Pentostam resulted in the lowest LDU; 0.24×10^6 and 0.45×10^6 respectively but there was no significant difference between Pentostam and Amphotericin B treated groups (P=0.0832).

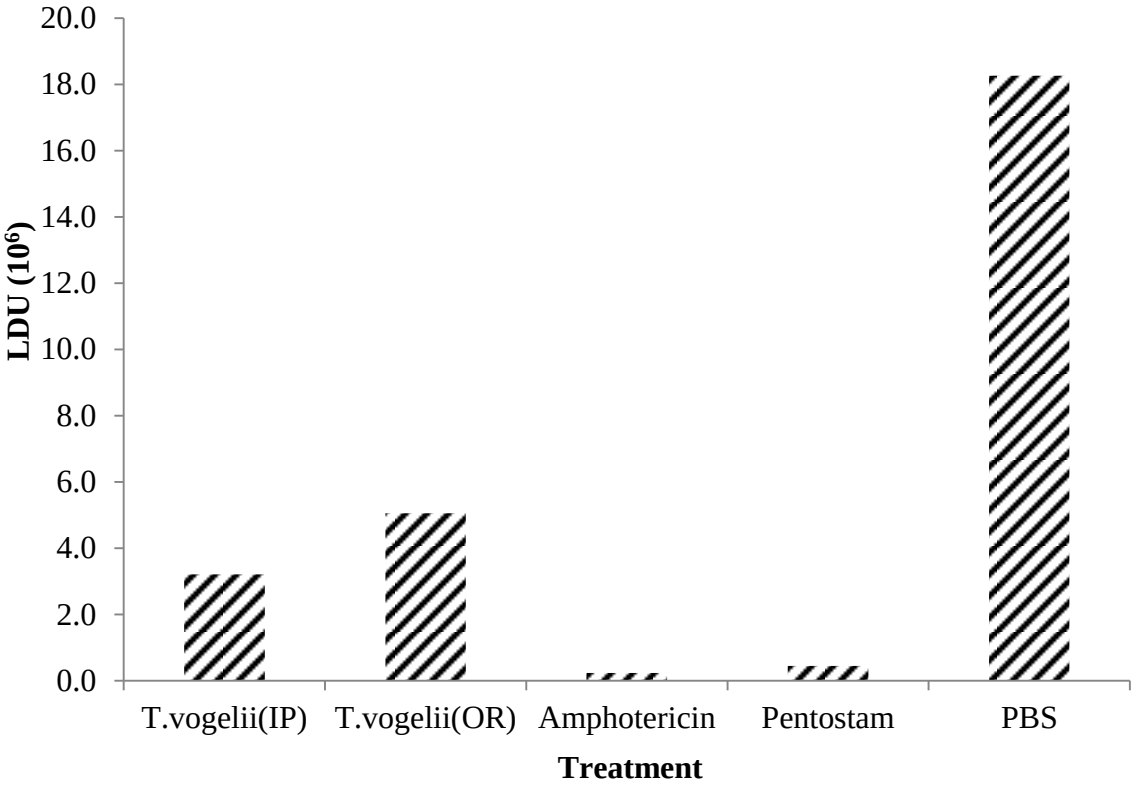

Figure 4.5: *L.major* LDUs in spleens of BALB/c mice treated with the different test drugs

4.7: Parasite loads in liver of *L. major* infected BALB/c mice after treatment with the different test drugs

The LDU of *L. major* parasite in the liver of BALB/c mice infected with *L. major* and treated with *T. vogelii*, Amphotericin B, Pentostam and PBS treatments is shown in Figure 4.6.

Intraperitoneal and oral administration of *T. vogelii* produced significantly different LDU trends (P= 0.002) in the liver. Pentostam and Amphotericin B resulted in significantly the lowest LDU; 0.18 $\times 10^6$ and 0.32 $\times$ 10^6 respectively. However, there was no significant difference between Pentostam and Amphotericin B (P= 0.06) treated groups.

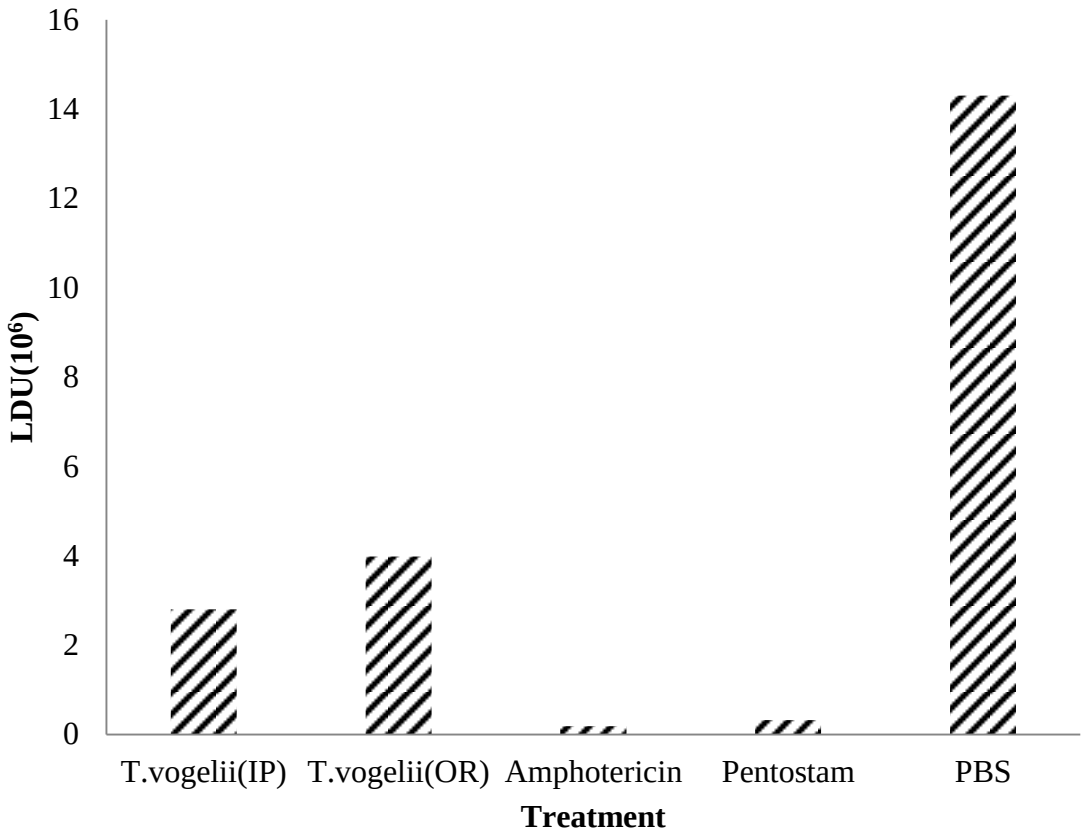

Figure 4.6: *L. major* LDUs in liver of BALB/c mice treated with the different test drugs

4.8 Nitric oxide production in treated *L. major*-infected macrophages treated with different test drugs

Nitric oxide production in the macrophages of *L. major* infected BALB/c mice and treated with different test drugs is shown in Figure 4.7

There was significant difference in nitric oxide production by macrophages of BALB/c mice that were infected with *L. major* amastigotes and subjected to the different test drugs (P=0.0052).

RPMI treatment produced the highest nitric oxide maintaining a constant in all the concentrations. Amphotericin B treatment produced the lowest nitric oxide followed by Pentostam however, there was no significant difference between the two (P=0.058). *T. vogelii* treatment produced higher amounts of nitric oxide compared to the standard drugs treated groups.

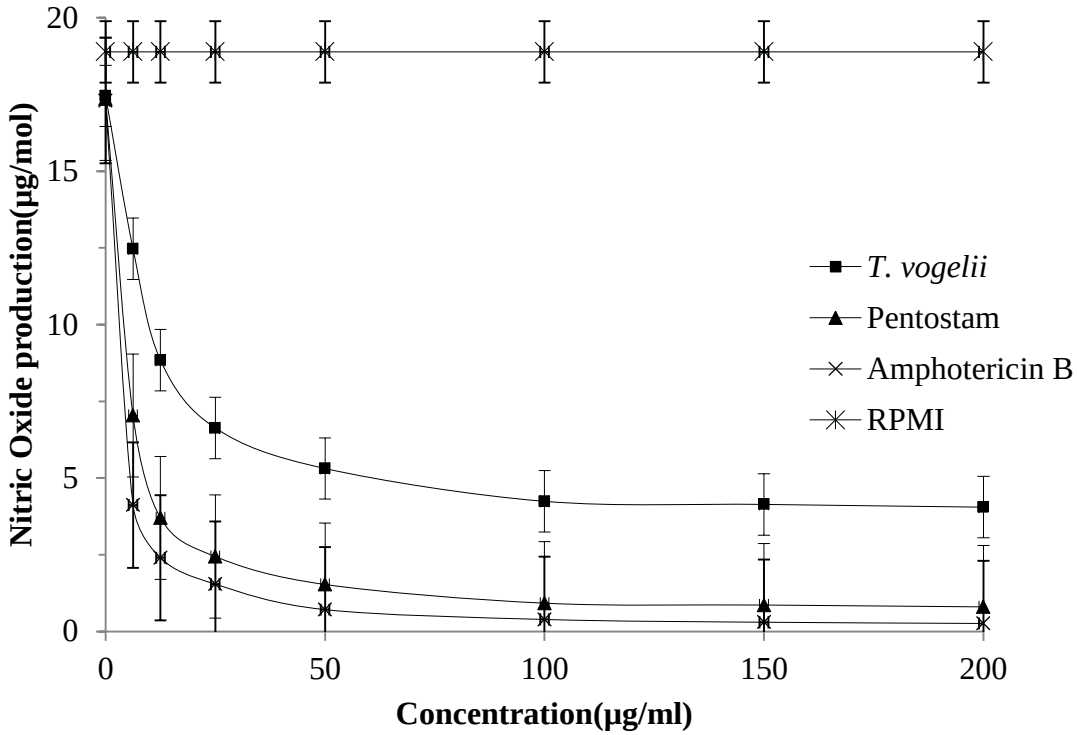

Figure 4.7: Nitric oxide production in the macrophages of *L. major* infected BALB/c mice and treated with the different test drugs

CHAPTER FIVE

DISCUSSION, CONCLUSION AND RECOMMENDATIONS

5.1 Discussion

Studies on medicinal plants and their chemotherapeutic activities against *Leishmania species* have been on the rise. These plants have demonstrated effectiveness against the parasite; making a contribution as alternative therapies (Croft and Coombs, 2003). Several plants have shown anti-leishmanial activities as reported by Dutta *et al*., (2007); Gamboa-Leon *et al* ., (2007); Ngure *et al*., (2009) and Kinuthia *et al*., (2014) who carried out antileishmanial activity tests on *Allium sativum, Aloe vera,Wurburgia ugandensis* and *Moringa stenopetala* respectively. The advantages of using plant based products have been shown to be as a result of lack of easy development of resistance by parasites (Polonio and Effert, 2008).

This study was carried out for the first time at KEMRI to investigate activity of *T. vogelii* against *L. major*. The study was set to determine both the *in vitro* and the *in vivo* experimental studies. In the *in vitro* studies the cytotoxicity assay was set up to determine the toxicity levels of the test drugs on vero cells. The cytotoxicity assay on the crude extracts of *T. vogelii* caused no significant adverse effects on the vero cells. This was further supported when BALB/c mice peritoneal macrophages were exposed to the extracts *in vitro* in the amastigote assay.

The non-toxicity of *T. vogelii* is also reflected in the *in vivo* treatment using the extract, in which no mice died and none had a marked weight reduction. Moreover, there was no marked change on the appearance of the skin of the mice suggesting that the plant extract was tolerable. Compared to amphotericin B and pentostam, *T. vogelii*

was significantly less toxic to vero cells hence can lead to novel treatment for *L. major* infection.

T. vogelii extracts demonstrated remarkable *in vitro* antileishmanial activity against *L. major* promastigotes and amastigotes. In both assays, the activity of *T.vogelii* extracts against *L. major* showed that the plants contained some pharmacologically active substances that could prevent growth and proliferation of *L. major* promastigotes and amastigotes in the macrophage.

Compounds including flavonoids, glycosides and tannins have been isolated from *T. vogelii* (Chen *et al*., 2014). Antileishmanial activity of *T. vogelii* could be attributed to the presence of tannins and flavonoids which have antimicrobial properties (Wang *et al*., 2006). Tannins disrupt the cell membranes of organisms (Dharmananda, 2003). Therefore, the inhibitory activity of *T. vogelii* against promastigoes and amastigotes may be attributed to the tannins disrupting the cell membranes of the *L. major* parasite. Flavonoids destroy organism through oxidative damage of membranes, proteins and DNA (Ferguson, 2001). Flavonoids have shown to exhibit antitrypanosomal and antileishmanial activities (Tasmedir *et al*., 2006).

Apart from flavonoids and tannins, *T. vogelii* contains rotenone as the primary compound as far as its insecticidal property is concerned but it is usually found in combination with several related rotenoids, the principal one being deguelin (Chen *et al*., 2014). Deguelin mediates antiproliferation properties in a variety of cell types. It also exerts growth inhibitory effects via induction of apoptosis and cell cycle arrest (Murillo *et al*., 2002). In summary, therefore, the inhibitory action of *T. vogelii*

against *L. mojor* may be attributed to tannins, flavonoids and deguelin either single or in combination.

Leishmania parasites evade the host's immune system by transforming from the flagellated promastigote to non-flagellated amastigote in macrophages of the mammalian host. This stage is critical for establishment of the infection (Wenzel *et al*., 2012) and therefore needs a drug that will target the parasite in the amastigote form while inside the macrophages. A reduction in infection rates was evident following treatment with *T. vogelii* which is an indication that the extract suppressed either the establishment or the development process of *L. major* amastigotes in BALB/c mice peritoneal macrophages. The multiplication indices observed implied that the higher the concentrations the higher the inhibition rate of replication of amastigotes in the macrophages.

This study also indicated that *T. vogelii* extract caused marked reduction in the parasite loads in the mice during treatment period. There were significant differences in lesion sizes among different treatments. *T. vogelii* resulted in significantly smaller lesion sizes than the negative control. *T. vogelii* extracts administered intraperitonially gave better results than those administered orally. This was due to the fact that intraperitoneal treatments usually act faster as the drugs/extracts are absorbed faster than those given orally.

Nitric Oxide is known to mediate macrophage cytotoxicity against microbes and tumor cells. It is usually upregulated in macrophages during *L. major* infection (Marina *et al*., 2012). Nitric Oxide is an active mediator in killing amastigotes and is

upregulated by immunological production of cytokines and oxygen metabolites (Oliver *et al.,* 2005). Apart from the normal nitric oxide produced by *Leishmania* infected macrophages naturally, the crude extract stimulated the macrophages *in vitro* to produce more nitric oxide to kill the parasite indirectly. *T. vogelii* caused relatively higher induction of nitric oxide compared to the controls. This suggested that the plant extract employed the mechanism of nitric oxide cytolytic activity to eliminate the amastigotes which could be due to the different compounds present in *T. vogelii* extract.

5.2 Conclusion

1. Based on the findings of this study, *T. vogelii* extract had anti-leishmanial activity with low toxicity levels as demonstrated in the cytotoxicity assay compared to the standard drugs. *T. vogelii* produced inhibitory activity both in promastigotes and amastigotes.
2. The plant extract also demonstrated significant activity when administered orally, hence could be administered both orally and intraperitonealy unlike the standard drugs that could only be administered intraperitonealy.
3. The plant extract stimulated macrophages to produce significant amount of nitric oxide, which contributed to the elimination of parasites intracellularly.
4. It is therefore possible that if fortified and/or used together with other drugs, the extracts could provide additive or synergistic effects in the control of *Leishmania* parasite.

5.3 Recommendations

Further studies should be done on the other *Leishmania* parasites and *in vivo* studies on non-human primate to find out whether the results from such studies will give comparable results to the ones obtained in this study.

Further studies should also be done on isolating and testing individual active compounds which may give better anti-leishmanial activities.

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APPENDICES

Appendix I: Distrubution of Leishmania species in the world

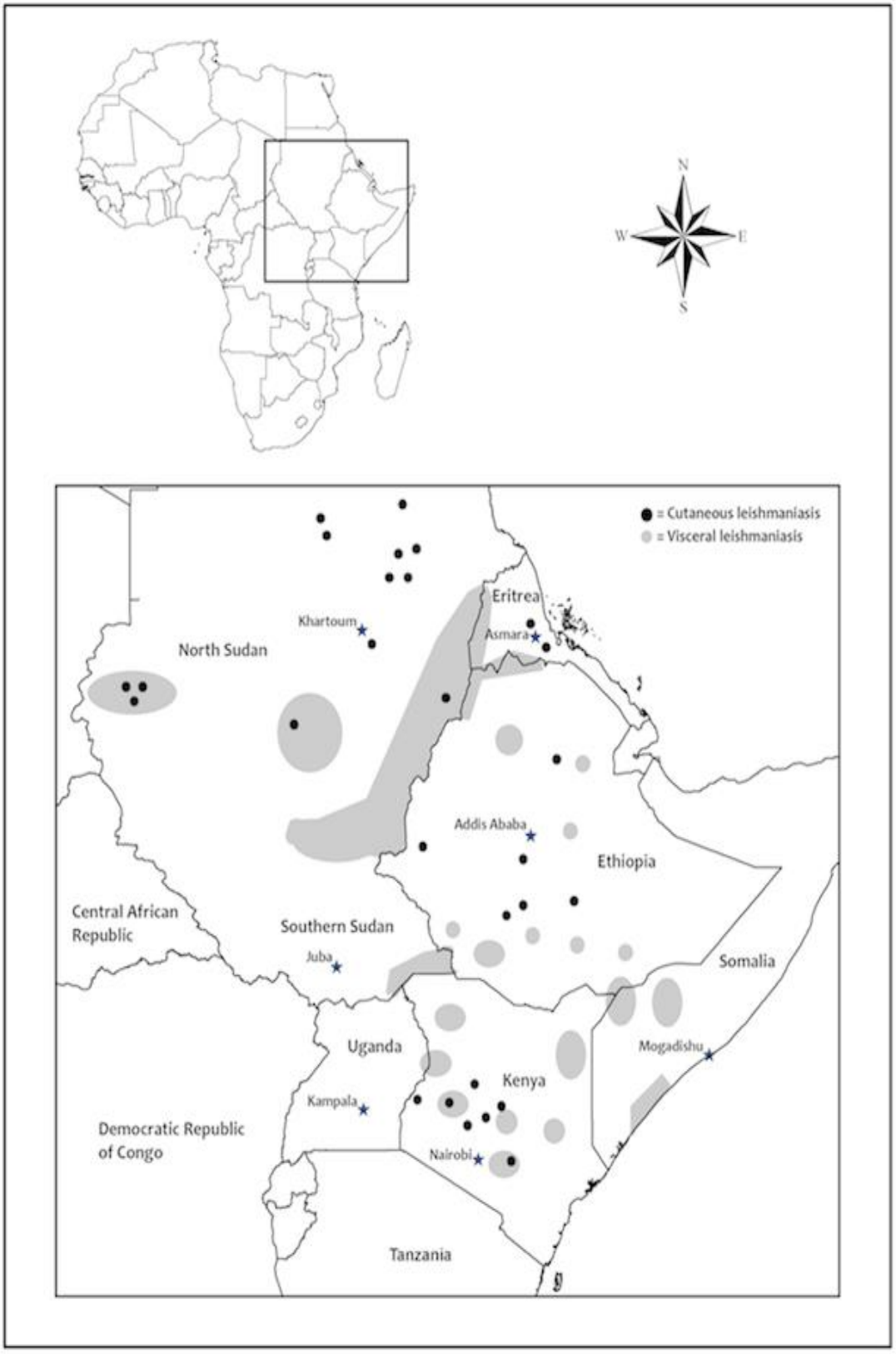
Table 5.1 **Species and Subspecies of *Leishmania***

Parasite	Locality
Subgenus *Leishmania* (Ross, 1903)	
L. donovani phenetic complex	
L. donovani (Laveran and Mesnil, 1903)	India, China, Bangladesh
L. archibaldi (Castellani and Chalmers, 1919)	Sudan, Ethiopia
L. infantum phenetic complex	
L. infantum (Nicolle, 1908)	North central Asia, northwest China, Middle East, southern Europe, northwest Africa
L. chagasi (Cunha and Chagas, 1937)	South and Central America
L. tropica phenetic complex	
L. tropica (Wright, 1903)	Urban areas of Middle East and India
L. killicki (Rioux, Lanotte, and Pratlong, 1986)	Tunisia
L. major phenetic complex	
L. major	Africa, Middle East, Soviet Asia
L. gerbilli phenetic complex	
L. gerbilli (Wang, Qu, and Guan, 1973)	China, Mongolia
L. arabica phenetic complex	
L. arabica (Peters, Elbihari, and Evans, 1986)	Saudi Arabia
L. aethiopica phenetic complex	
L. aethiopica (Bray, Ashford, and Bray, 1973)	Ethiopia, Kenya
L. mexicana phenetic complex	
L. mexicana (Biagi, 1953)	Mexico, Belize, Guatemala, South central United States
L. amazonensis (Lainson and Shaw, 1972)	Amazon Basin, Brazil
L. venezuelensis (Bonfante-Garrido, 1980)	Venezuela
L. enrietti phenetic complex	
L. enrietti (Muniz and Medina, 1948)	Brazil
L. hertigi phenetic complex	
L. hertigi (Herrer, 1971)	Panama, Costa Rica
L. deanei (Lainson and Shaw, 1977)	Brazil
Subgenus *Viannia* (Lainson and Shaw, 1987)	
L. braziliensis phenetic complex	
L. braziliensis (Viannia, 1911)	Brazil
L. peruviana (Velez, 1913)	Western Andes
L. guyanensis phenetic complex	
L. guyanensis (Floch, 1954)	French Guiana, Guyana, Surinam
L. panamensis (Lainson and Shaw, 1972)	Panama, Costa Rica

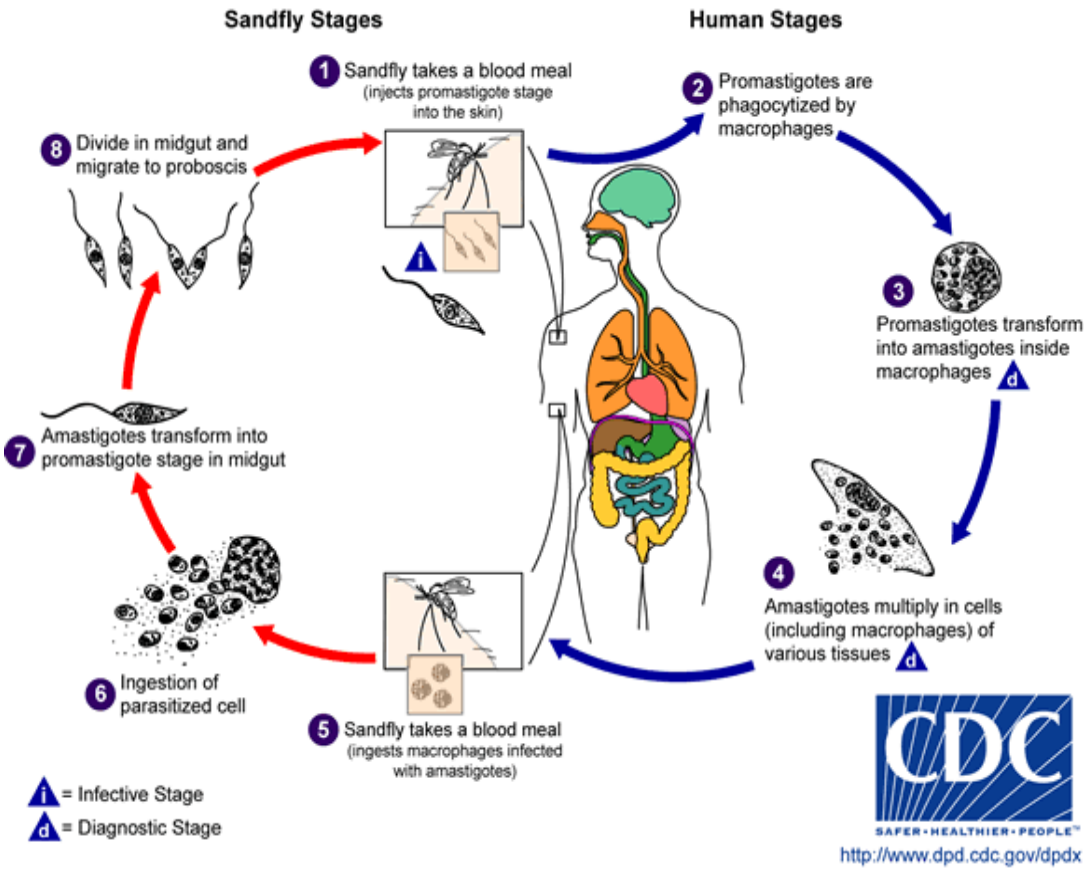
In other classifications, subspecies of *L. mexicana* have been recognized, and these names—*L. mexicana aristedesi, L. m. garnhami,* and *L. m. pifanoi*—do appear in the literature, with the subspecific name sometimes used as a specific epithet. The groupings in the table are those of Rioux et al.[89] and are based on extensive isozyme and cladistic analysis. The term *phenetic complex* refers to zymodemes revealed by cluster analysis.

Distribution of Leishmania (Source: Peters and Gilles, 1977).

Appendix II: Distribution of Leishmaniasis in East Africa

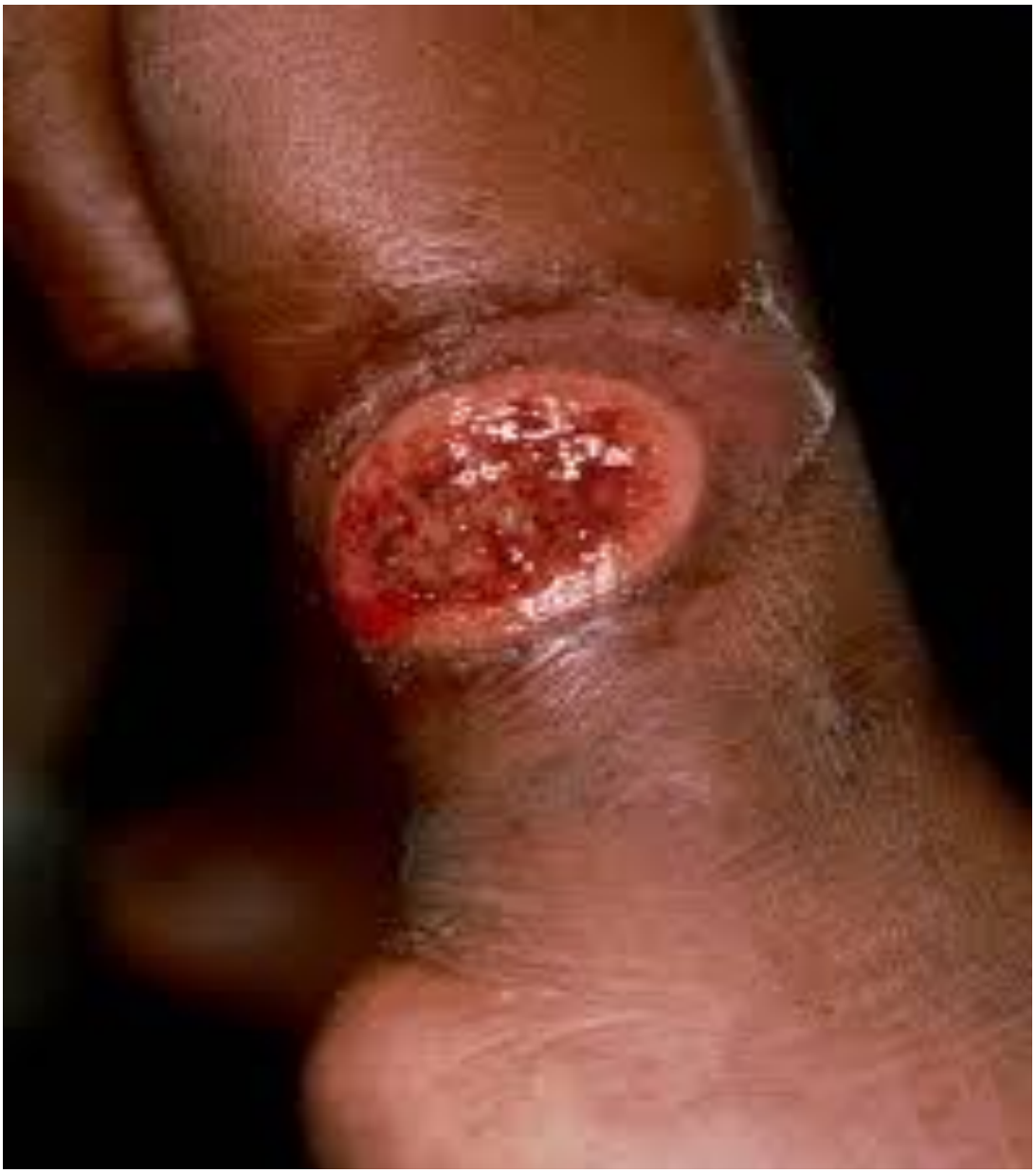


Appendix III: The Lifecycle of Leishmania Parasite



The sandflies inject the infective stage, metacyclic promastigotes, during blood meals (1). Metacyclic promastigotes that reach the puncture wound are phagocytized by macrophages (2) and transform into amastigotes (3). Amastigotes multiply in infected cells and affect different tissues, depending on the part in which *Leishmania* species is involved (4). These differing tissue specificities cause the differing clinical manifestations of the various forms of leishmaniasis. Sandflies become infected during blood meals on infected hosts when they ingest macrophages infected with amastigotes (5, 6). In the sandfly's midgut, the parasites differentiate into promastigotes (7), which then multiply, differentiate into metacyclic promastigotes, and migrate to the proboscis (8).

Appendix IV: Skin Lesion of a Leishmania patient

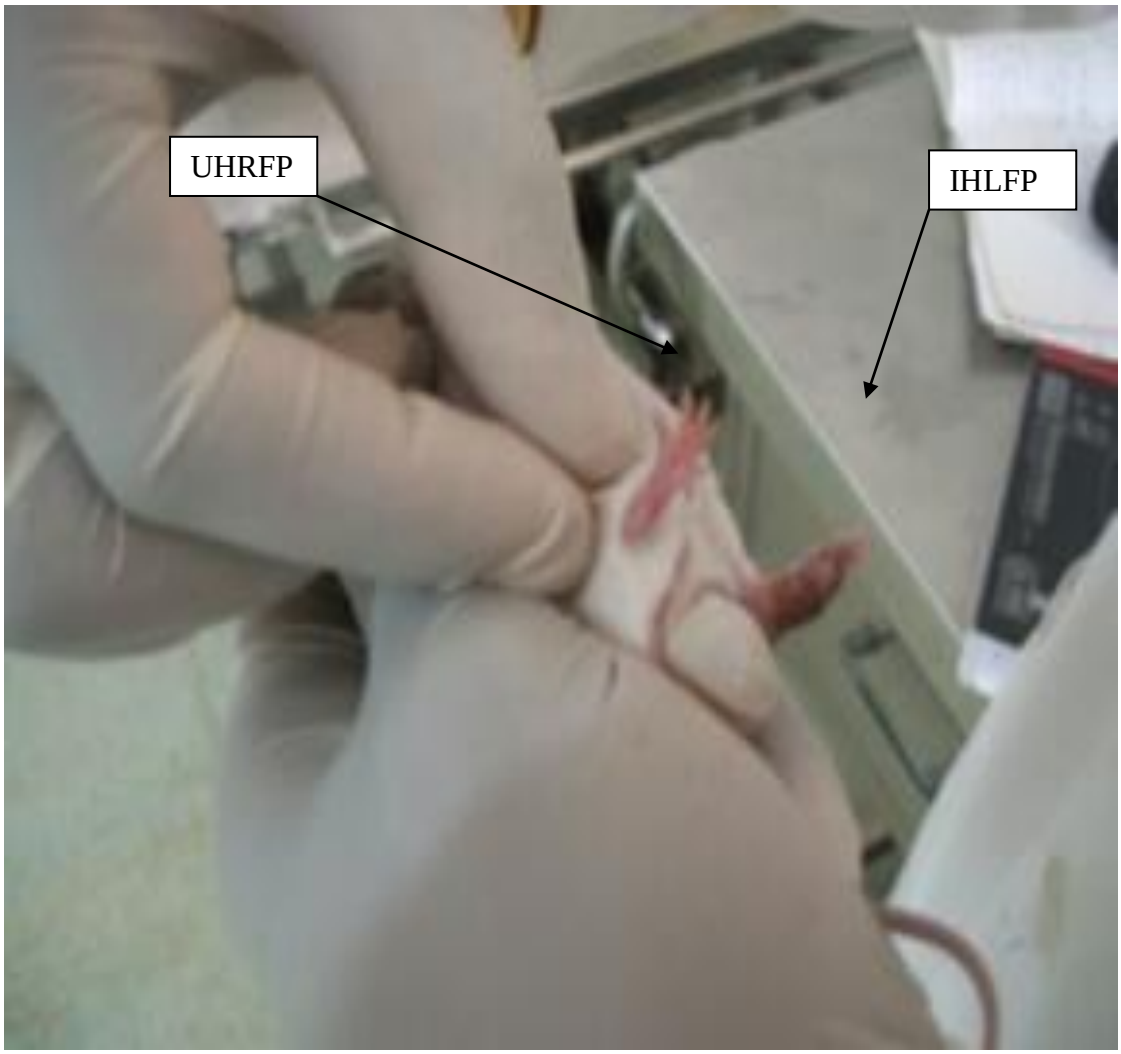


Skin Lesion of a Leishmania patient (Source: Sarah R., 2015).

Appendix V: Tephrosia vogelii plant

T.vogelii **(Fish poison bean) plant. (Source: Author)**

Appendix VI: BALB/c mouse showing the right and the left foot pad post infection

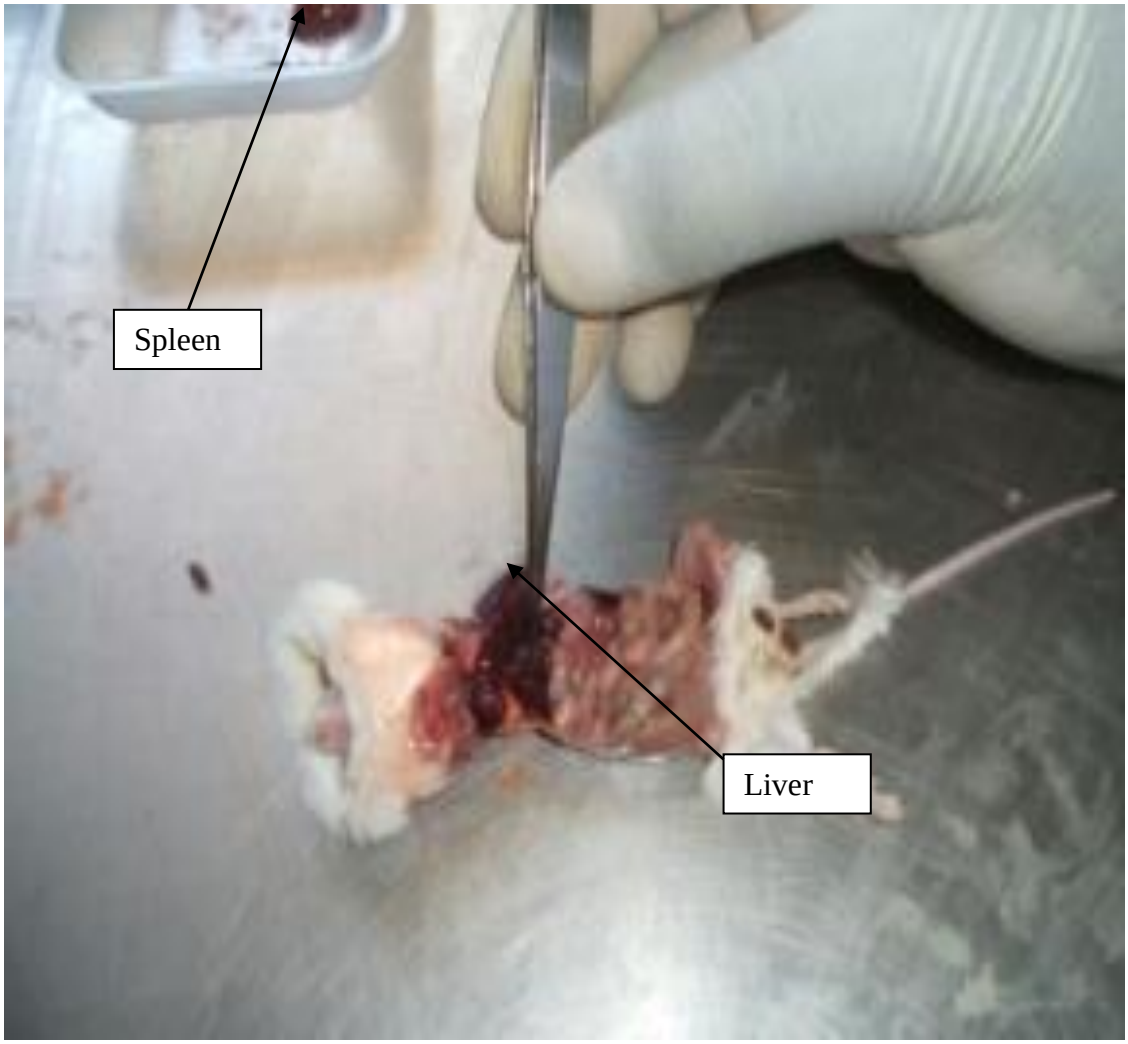


(Source: Author, 2015)

Legend: IHLFP- The infected Hind Left foot pad

URFP-The Uninfected Hind right foot pad

Appendix VII: Removal of spleen and liver from a treated BALB/c mouse



(Source: Author, 2015)

Appendix VIII: Determination of nitric oxide concentration in supernatants

Requirements:

- Reagent reservoirs and multi-channel pippettor
- 96 well flat bottomed enzymatic assay plates
- Plate reader with 520-550 filter

Preparation of a Nitrite Standard Reference curve

i. Prepare 1μmL of a 100μM solution by diluting the provided 0.1M Nitrite standard 1:1,000 in the RPMI 1640.

ii. Designate 3 columns (24 wells) in the 96 well plate for the nitrite standard reference curve. Dispense 50μL of the appropriate matrix of buffer into the wells in rows B-H.

iii. Add 100μL of the 100μM nitrite solution to the remaining 3 wells in row A.

iv. Immediately perform 6 serial 2-fold dilutions (50μL/well) in triplicate down the plate to generate the nitrite standard reference curve (100, 50, 25, 12.5, 6.25, 3.13 and 1.56μM) discarding 50μM from the 1.56μM set of wells. Do not add any nitrite solution to the last set of wells (0μM). Note: The final volume in each well is 50μL and the nitrite concentration range is 0-100μM

Nitric oxide measurement (Greis reaction)

i. Allow the suphanimide solution and NED solution to equilibrate to room temperature (15- 30minutes).

ii. Add 50 μL of each experimental sample to wells in duplicate or triplicate.

iii. Using multichannel pipette, dispense 50μL of sulphanilamide solution to all experimental samples and wells containing the dilution series for the nitrite standard reference curve.

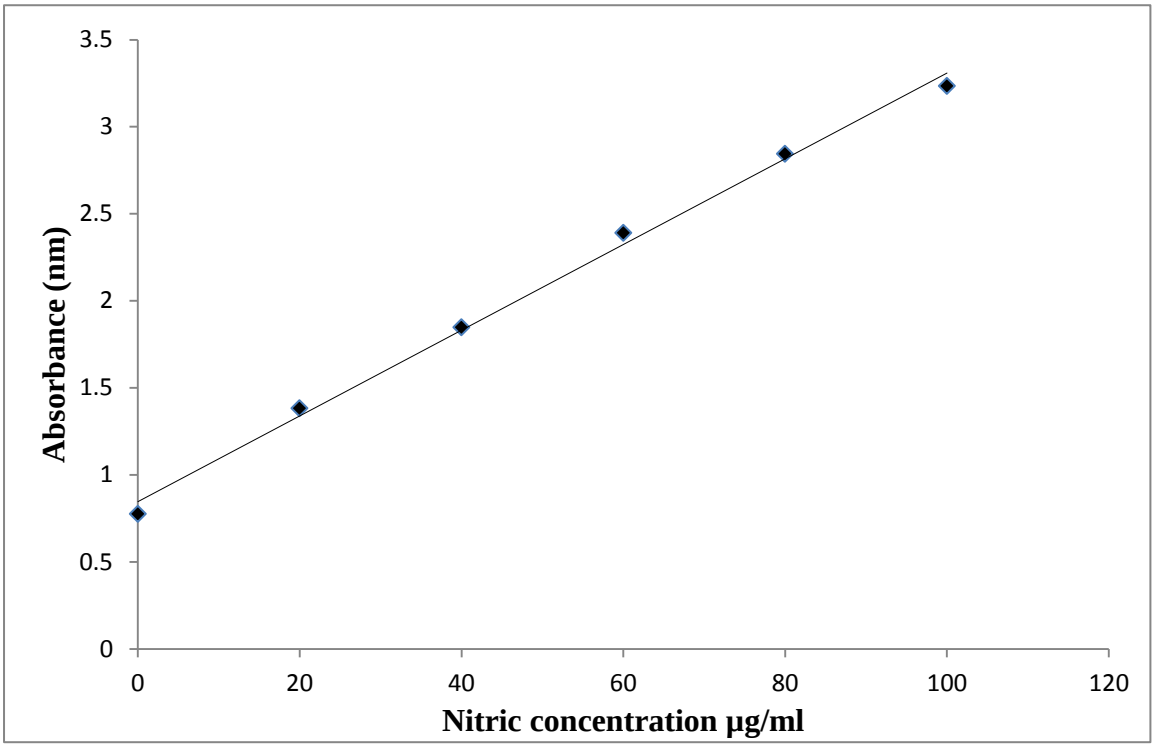
iv. Incubate 5-10 minutes at room temperature, protected from light.

v. Using a multichannel pipette, dispense 50µL of the NED solution to all wells.

vi. Incubate 5-10 minutes at room temperature protected from light. A purple colour/magenta will begin to form immediately.

vii. Measure absorbance within 30minutes in a plate reader with a filter between 520- 550nm

The standard curve is shown below



Nitric Oxide standard curve for determinining Nitric Oxide production by macrophage treated with fractions