ANTILEISHMANIAL ACTIVITY AND TOXICITY OF *Momordica foetida* SCHUMACH AND THONN EXTRACTS AGAINST *Leishmania major*

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DECLARATION

Declaration by the Student

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DEDICATION

I dedicate this thesis to my mother Mrs Milka Koech and the entire family of Mr. John Kipsugut Koech.

ABSRACT

Human leishmaniasis is a spectrum of diseases caused by protozoan parasites of the genus *Leishmania.* About 350 million people are at risk of infection, 15 million clinical cases, 1.5million annual incidence and 20- 30 thousand deaths occur annually. Treatment of leishmaniasis has traditionally relied on pentavalent antimonials which are toxic, expensive and are complicated by the variation in sensitivity of the *Leishmania* species and increasing levels of antimonial resistance. They also require protracted administration, and there is no vaccine at the moment, therefore, there is need for research for alternative and cheaper remedies including plant based drugs. The current study was to evaluate methanolic and aqueous extracts of *Momordica foetida* for antileishmanial activities and toxicity in *Leishmania major* infected BALB/c mice and in cell culture plates. The extracts were dried and re-dissolved in dimethyl sulfoxide (DMSO) 1% solvent before subjecting to *L. major* infected mice and cultures. Parasites inhibitions were then tested with serial concentration (0.07 to 19.9 mg ml-1) of the extracts. The lesion progression and body weight measurements were recorded weekly. Mice were sacrificed by injecting pentabarbitone sodium, the spleen of each mouse was weighed and splenic impression smears made on slides for microscopy evaluation of parasites. Data was analyzed using Statgraphic software and antileishmanial activities within and between all groups, the t-test and ANOVA being used respectively. The aqueous and methanolic extracts of *M. foetida* inhibited the parasites after 48 hrs incubation against *L. major* Amastigotes *and* promastigotes, demonstrating MIC of 125 ± 0.01 and 250 ± 0.03 mg/ ml and IC_{50} of 15.6±0.05 and 23.4±0.53mg/ml, respectively. The MIC of Pentostam and Amphotericin B was at the concentration of 62.5 ± 0.02 and 31.3 ± 0.01µg/ml with IC_{50} of 11.7 ± 0.054 and 7.8±0.053 mg/ml respectively. The Minimum inhibition concentration for aqueous extracts (125µg/ml) demonstrated higher inhibitory factor than that of methanolic extracts (250µg/ml) by 125 units. Increased concentration of *M. foetida* extracts did not stimulate the macrophages to produce sufficient amount of nitric oxide, hence the extracts could be having active compounds that acted directly on parasites. Considering *M. foetida* with no toxicity effects in BALB/c mice and vero cells it's an indication that it's safe for use in the chemotherapy of *L. major*. By showing antileishmanial activities with no toxicity, *M. foetida* extracts, therefore, supports its traditional use as an antileishmanial remedy and it should also be tested against other species of the parasite such as *L. donovani*, *L. tropica* and *L. aethiopica.*

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LIST OF ABBREVIATIONS

A	Amastigotes
a / b	Intercept/ slope
AI	Association index
ACT	Artemesinin-derived Combination Therapy
APG III	Angiosperm Phylogeny Group III system
AmB	Amphotericin B
ANOVA	Analysis of Variance
ATP	Adenosine triphosphate
BALB/c	Inbred Laboratory mice very susceptible to *Leishmania major*
CBRD	Centre for Biotechnology Research and Development at KEMRI
CTMDR	Centre of Traditional Medicine and Drug Development Research
CL	Cutaneous Leishmaniasis
DMSO	Dimethyl Sulfoxide
ELISA	Enzyme Linked Immunosorbent Assay
Exts	Extracts
FBS	Foetal Bovine Serum
IC_{50}	Concentration that kills 50% of parasite population
INFγ	Interferon gamma
ITNs	Insecticide Treated Bed Nets
IP	Intra Peritoneal
IR	Infection Rate

KEMRI	Kenya Medical Research Institute
LDU	Leishman Donovan Units
ln	Natural logarithm
LPG	Lipophosphoglycan
MQS	Machrophages
MCL	Mucocutaneous Leishmaniasis
MEM	Minimum Essential Medium
Met- Exts	Methanolic Extracts
MI	Multiplication Index
Mg	Milligram
ML	Millimeter
MIC	Minimum Inhibitory Concentration
ML	Mucosal leishmaniasis
MTT	3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrozolium bromide
NADP	Nicotinamide Adenine Dinucleotide Phosphate
NADPH	Reducing form of NADP
$NaSO_4$	Sodium Sulphate
NIAID	National Institude of Allergy of Infectious Diseases
NNN	NovyMacneal Nicolle medium
NK	Natural Killer cells
NO	Nitric Oxide
PBS	Phosphate Buffered Saline
PKDL	Post-Kala-zar Dermal Leishmaniasis

Pento	Pentostam
RPMI	Roswell Park Memorial Institute Medium
SE	Standard Error
Sb	Sodium Stibogluconate
SD	Standard deviation
SIM	Schneider's Insect Media
STA	Slug Test Analysis
TNF	Tumour Necrosis Factor
Trts	Treatments
Try- R	Trypanothine Reductase
µ	Micron
µg	Microgram
VL	Visceral Leishmaniasis
VP	Viable parasites
Wk	Week
Wt	Weight
WHO	World Health Organisaton
ZCL	Zoonotic Cutaneous Leishmaniasis

DEFINITION OF OPERATIONAL TERMS

Amastigote: An ovoid/rounded stage of *Leishmania* parasites, as found in mammals.

Antioxidant: A molecule that inhibits oxidation of other molecules.

Bioassay: The determination of the potency of a drug by testing it upon a living organism.

Cryopreservation: Process where cells, tissues or any substance susceptible to damage caused by chemicals or enzymatic activity are preserved by cooling to sub-zero temperature without causing additional damage caused by ice formed during freezings.

Cutaneous leishmaniasis: Form of leishmaniasis disease normally produces skin ulcers on exposed parts of the body such as the face, arms and legs.

Cytotoxicity: The ability of substance to damage cells.

Endemic: Disease peculiar to a particular region or locality

Epidemic: Disease affecting large numbers of a population at the same time.

Exacerbate: To increase the serverity of a disease.

Incubation period: The period of an infectious disease between infection and appearance of the first symptoms.

Incubation: Artificially maintaining conditions of warmth and moisture favourable for the development of parasites in culture.

Inoculation: Introduction of a parasite into a suitable medium for growth.

Inflammation: The complex biological response of body tissues to harmful stimuli.

Leishmaniasis: Pathological condition caused by a flagellate protozoan of the genus *Leishmania*.

Machrophage: A phagocytic cell of the reticuloendothelial system which has the ability to engulf particulate matter and store vital dyes, as a histiocyte of connective tissue or Kupffer cell of the liver.

Mucocutaneous leishmaniasis: Form of leishmaniasis disease where lesions developed lead to partial or total destruction of the mucous membranes of nose, mouth and throat cavities and surrounding tissues.

Oxidation: Chemical reaction involving the loss of electrons or an increase in oxidation state. **Parasite:** An organism which lives in or on another organism for nourishment.

Promastigote: Pear shaped form of *Leishmania*, as found in the sand fly and in artificial culture.

Thawing: Leaving a culture at room temperature after many hours of incubation.

Trypsinization: Process of making cell form monolayer by adding trypsin on culture

Vaccine: A suspension of killed or living, attenuated organisms which, when inoculated or taken into the body, acts as an antigen causing the development of antibodies that render the body immune against infection by the specific organism.

Vector: Any agent that carries and transmits an infectious pathogen into another living organism

Vero cells: Lineage of cells isolated from kidney epithelial cells of an African Green monkey.

Visceral leishmaniasis: Form of leishmaniasis characterised by irregular bouts of fever, substantial weight loss, swelling of the spleen and liver, and anaemia, also known as Kala azar.

Zoonosis: Disease of animals which is transmissible to man.

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CHAPTER ONE

INTRODUCTION

1.1. Background Information

Leishmaniasis is a vector-borne disease caused by obligate intra-macrophage protozoan parasites of the genus *Leishmania* and is the world's most neglected disease (Yamey & Torrele, 2007), and is considered endemic in large areas of the tropics, subtropics and the Mediterranean basin (Chappius *et al*., 2007). A total of 350 million people are at risk of infection,15 million clinical cases, 1.5million annual incidence & 20- 30 thousand deaths occurs annually (WHO, 2006).

Leishmaniases consist of four main clinical syndromes: cutaneous leishmaniasis (CL); muco-cutaneous leishmaniasis (MCL), visceral leishmaniasis and post-kala-azar dermal leishmaniasis (PKDL) (Zijlstra *et al*., 2003). Different species of *Leishmania* can infect the macrophages in the dermis, with variable clinical presentations and prognoses (Arevalo *et al*., 2008). *Leishmania* spp. presents basically the following stages of development: promastigotes, amastigotes, paramastigotes and nectomonads. Infective metacyclic promastigotes are transmitted by female sand flies to healthy mammals, where they invade and multiply as amastigotes within macrophages (Killick-Kendrich, 1990). Risk factors of leishmaniasis are Socioeconomic, Malnutrition, Population mobility and Climate changes.

Treatment of leishmaniasis is difficult because the parasites infect the macrophages and thus patients present with immune deficiency and are not able to eliminate the parasites through natural mechanisms of defence (Rocha *et al*., 2005; Mondal, 2014). Infection

with diseases such as malaria increases the fatality of the illness if it is not diagnosed and treated in time (Carvalho *et al*., 2008). Leishmaniasis has been exacerbated by the evolution of acquired immune deficiency syndrome (AIDS) due to parallel infections in HIV patients, as well as by the development of drug-resistance by the parasites (Croft *et al*., 2006).

Pentavalent antimonials and meglumine were the first-line treatment for leishmaniases (Frezard *et al*., 2009), but only cheaper, generic forms of these drugs are available that have been shown to be equivalent to the branded products (Croft *et al*., 2007)**.** Antimonial drugs are toxic and sometimes have life-threatening, adverse effects, including cardiac arrhythmia and acute pancreatitis (Chappuis *et al*., 2007; Cook, 1995). Patients under the age of 2 or aged greater than 45 with signs of advanced disease and/or severe malnutrition are at higher risk of death during antimonial therapy owing to drug toxicity, slowness of drug action, and leishmaniasis complications or a combination of these factors (Seaman *et al*., 2006)**.**

Amphotericin B has replaced antimonials in the treatment for leishmaniasis, but its also has side effects such as shortness of breath, skin rash, fever and rigor (Chappius *et al*., 2007), it is costly and requires slow infusions on alternate days showing a need for more research and development to improve the drugs for leishmaniases (Guerin *et al*., 2002; Van Griensen & Diro, 2012) that will be available, safe, cheap and for oral or topical administration in shorter treatment cycles. This calls for alternative approach in the treatment of leishmaniasis.

Several plant extracts such as *Allium sativum* (Kinuthia *et al*., 2013), *Plumbago capensis* (Padhye *et al*., 2013), *Albizia coriara* (Kigondu *et al*., 2009), *Acacia tortilis* (Tiuman *et al*., 2011), *Warbugia ugandensis* (Maobe & Nyarango, 2013), *Asparagus racemosus* (Sachdeva *et al*., 2014) and *Delonez ovate* (Katalinic *et al*., 2006) have been screened and found to possess some antileishmanial activity. The aim of this study was to determine the antileishmanial effects of *M. foetida* aqueous and methanolic extracts *in vitro* and *in vivo* in *L.major* experimentally infected BALB/c mice.

1.2 Statement of the Problem

Over 15 million people are infected with leishmaniasis in the world and 2 million new cases are reported annually (Hotez *et al*., 2004). Currently the drugs of choice in the treatment of leishmaniasis include pentavalent antimony, sodium stibogluconate (Pentostam) and Amphotericin B. They are expensive and toxic with persistent side effects due to the protracted regime of administration that is recommended for inpatients. In addition parasites are developing resistance caused by the prolonged exposure (Benedict, 2003). The drugs are not readily available to most people in endemic areas (Hailu, 2005).

This calls for alternative approach to research in medicinal plants for treatment of leishmaniasis in terms of their availability, acceptability, compatibility, and cost effectiveness. The present study investigated the effect of crude methanolic and aqueous extracts of *M. foetida* against *L. major* in experimentally infected BALB/c mice. Since no new drug formulations are currently available to substitute the old toxic drugs, antileishmanial activity of *M. foetida* can be used to fill this gap.

1.3 Justification of the Study

Leishmaniasis is one of the tropical diseases that have been neglected in the world inspite of affecting millions of people. Available antileishmanial drugs such as pentavalent -antimonials are extremely toxic with terrible side effects.

Cutaneous leishmaniasis which forms the bulk of the infection has a self healing nature (Hailu, 2005) and as a result, people do not seek medical attention despites the associated chronic sufferings. There is need for new treatment options that are cheaper, non toxic, non parasite resistant, accessible and available in endemic areas.

Sitamaquine and Miltefosine, which are also anticancer agents have been shown to have gastrointestinal toxicity and teratogenicity, and therefore cannot be administered to women of childbearing age (Croft *et al*., 2006). It is essential that new treatment options become truly accessible and available in endemic areas.

The choice of *M. foetida* plant in the present study emanated from the need to validate the traditional use of the plant against *Leishmania* parasites. *M. foetida* is found in many parts of Kenya including areas like Baringo, Elgeyo Marakwet, West Pokot, Mt. Elgon, Meru, Machakos and Kitui where *L. major* infection is also endemic (Kigondu, 2009), It is anticipated that extracts from *M. foetida* may solve the problem of drug availability, oral administration and early treatment for people who wait for self healing. Findings of this study will be useful in providing new insights on the potential of plant extracts as chemotherapeutic agents in the treatment of leishmaniases.

1.4 Objectives of the Study

1.4.1 General Objective

To investigate the *in vivo* and *in vitro* antileishmanial activities and toxicity of methanolic and aqueous extracts of *M. foetida* against *L. major*.

1.4.2 Specific Objectives

i. To determine the *in vivo* and *in vitro* antileishmanial activity of *M. foetida* extracts administered intraperitoneally and orally in *L. major* infected BALB/c mice.

ii. To determine the levels of nitric oxide production in infected macrophages treated with *M. foetida* extracts.

iii. To assess the toxicity of methanolic and aqueous extracts of *M. foetida* on vero cells and BALB/c mice.

1.5 Hypotheses of the Study

H_0: Methanolic and aqueous extracts of *M. foetida* extracts have no *in vivo* and *in vitro* antileishmanial activities.

H_0: Methanolic and aqueous extracts of *M. foetida* do not stimulate macrophages to produce nitric oxide.

H_0: Methanolic and aqueous extracts of *M. foetida* have no toxic effects on vero cells and BALB/c mice.

CHAPTER TWO

LITERATURE REVIEW

2.1 Leishmaniasis

Leishmaniasis is a parasitic disease resulting from infection by protozoans of the genus *Leishmania* and are transmitted through the bite of infected female sand flies (Diptera: Psychodidae), mainly *Lutzomyia* Franca and *Phlebotomus* Rondani (Rogers *et al*., 2004). The disease affects internal organs of the body, mucous membranes and the skin and is found in three forms: Cutaneous, Visceral and Mucocutanious leishmaniasis (Liaison & Shaw, 1987).

Human leishmaniasis is caused by about 21 of 30 species of genus *Leishmania* that infect mammals. These include *L. donovani complex* with 3 species (*L. donovani, L. infuntum*, and *L. chagasi*); *L. Mexicana complex* with 3 main species (*L. mexicana, L. amanzonensis*, and *L. venezuelensis*); *L. tropica*; *L. major*; *L. aethiopica*; and the subgenus *viannia* (*V*) with 4 main species (*L.* (*V.*) *braziliensis, L.* (*V.*) *guyanensis, L.* (*V.*) *panamensis, and L.* (*V.*) *peruviana*) (Croft *et a*l., 2006).

The different species are morphologically indistinguishable, but they can be differentiated by isoenzyme analysis, molecular methods, or monoclonal antibodies (Dohm *et al*., 2000). Due to species differences in tissue tropism, virulence and their interaction with the host's immune system, infection by *leishmania* parasites can result in a variety of clinical manifestations ranging from single self-healing ulcers in cutaneous forms to life threatening visceral infections (Alexander *et al*., 2004).

2.2 Types of Leishmaniases in Kenya

2.2.1 Cutaneous leishmaniasis

Cutaneous leishmaniasis (CL is the most common form, which causes skin sores that typically develop within a few weeks or months of the infected female sand fly bite and can change in size and appearance over time (Hundson *et al*., 2004). The sores may start out as papules (bumps) or nodules (lumps) and may end up as ulcers. Some people have swollen glands near the sores marked by swelling of the lymph nodes on arms, legs or face which develops as a red raised border and a depression in the middle (Chappius *et al*., 2007). It is commonly caused by *L. major, L. braziliensis, L. mexicana* and *L. panamensis* (James *et al*., 2006). Natural transmission of *Leishmania* parasites is carried out by infected female phlebotomine sand flies of the genera *Phlebotomus* and *Lutzomyia* (Beach, 1984)**.** Most forms of the disease are zoonoses, transmissible only from animals but can be spread between humans congenitally (Pearson, 1996; Ashford, 2000) and also from human to the sand fly and then to the human in an anthroponotic cycle (Fong & Chang, 1981).

In Africa CL is caused mainly by *L. major, L. tropica* and *L. aethiopica* (Ashford, 2000), while in Kenya, infections are caused by four species, *L. donovani*, *L. tropica*, *L. aethiopica* and *L. major* (Beach, 1984). Within Rift Valley province, CL caused by *L. tropica* is endemic in two foci. The active foci for disease were found to be Muruku location in Laikipia district (Mebrahtu *et al*., 1987; Desjeux, 1991), where the vector was identified as *P. guggisbergi* at Utut reserve in Njoro in Nakuru district, (Dohm *et al*., 2000), the probable vector was identified as *P. aculeatus* (Morillas-Marquez *et al*., 2002).

Cutaneous leishmaniasis is endemic around the Mt. Elgon area of Western Kenya, *L. aethiopica* has been incriminated as the parasite that causes it in this area. The parasites have been isolated from rock hyraxes and the parasite has been found to be zoonotic and transmitted by pedifer (Mutinga & Kamau, 1987).

Zoonotic cutaneous leishmaniasis caused by *L. major* is endemic in Marigat and Baringo district in Rift Valley province, where the sandfly *P. Phlebotomus duboscqui* has been incriminated as vector (Mutinga *et al*., 1986; Koech, 1994) and is mainly associated with rodent burrows where it is thought to both breed and rest (Basimike & Mutinga, 1995). This sand fly species is rarely found in termite mounds in which other sand flies species are thought to breed and rest (Minter, 1964; Darlington, 1994). The incubation period ranges from 2 to 6 weeks and the initial lesions are often multiple and located to lower extremities (Santana *et al*., 2008).

2.2.2 Visceral leishmaniasis

Visceral leishmaniasis (VL) is a systemic disease that is fatal if left untreated and is caused by the *L. donovani complex, L. donovani sensu stricto* in East Africa and the Indian subcontinent and *L. infantum* in Europe, North Africa and Latin America (Luke, 2007).

It is characterized by its effect on the internal organs, particularly the liver, the spleen and the bone marrow and the effects can be life threatening (Van-Grienven & Diro, 2012).

The illness develops within months (sometimes as long as years) of the sand fly bite (Murray *et al*., 2005). Affected people usually have fever, weight loss, enlargement

(swelling) of the spleen and liver and low blood count-a low red blood cell count (anemia), a low white blood cell count (leukopenia), and a low platelet count (thrombocytopenia) (Kolaczinski *et al*., 2008).

There are two types of VL, which differ in their transmission characteristics. Zoonotic VL is transmitted from animal to vector to human and anthroponotic VL is transmitted from human to vector to human (Gramiccia & Gradoni, 2005). Species of *Viannia* and *Leishmania* subgenus are involved in human leishmaniases diseases (Marfurt *et al*., 2003). These diseases is characterized by both diversity and complexity and it is caused by more than 20 leishmanial species and transmitted to humans by 30 different species of phlebotomine sand flies (Liainson & Shaw, 1987; Pearson & Sousa, 1996; Grimaldi & Tesh, 2012).

The major known foci of VL are in Turkana, Baringo and West Pokot Counties in Rift Valley Province and also in Machakos, Kitui and Meru Counties (Mbui *et al*., 2003). In these foci, the disease is believed to be anthroponotic with a man- sandfly-man cycle, with *P. martini* as the incriminated vector (Tonui, 2008). After successful treatment with Amphotericin B, 3 to 10% of the cases of VL develop post kala azar dermal leishmaniasis (PKLDL) wart like nodules over the face and extensor surface of the limbs (Awasthi *et al*., 2004). In the Indian disease, PKDL appears after a latent period of 1 to 2 years and may last for several years (Carvalho *et al*., 2008).

2.2.3 Mucocutaneous leishmaniasis (MCL)

Mucosal leishmaniasis is a less common form of leishmaniasis. The pathogenesis of Mucocutenious leishmaniasis (MCL) is still unclear. However, it is believed that host genetic factors are important in the advancement of the disease (Desjeux, 2004).

This form can be a sequela (consequence) of infection with some of the species (types) of th ACKNOWLEDGEMENT

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Certain types of the parasite might spread from the skin and cause sores in the mucous membranes of the nose (most common location), mouth, or throat (Strazzulla *et al*., 2013). Lesions may multiply and increase in size, which can contribute to severe deformity. Respiratory tract mucosal invasion may also occur, causing numerous respiratory problems, and can result in malnutrition and pneumonia (Gill & Beeching, 2011). Secondary infection is responsible for most deaths (Ameen, 2010).

2.3 Developmental Stages of *Leishmania* Parasite

2.3.1 Promastigotes Stage

The promastigote stage of *Leishmania* exists in the sand fly. It is flagellated and spindle-like in shape (Sacks, 1989). The amastigotes transform to promastigotes in the posterior midgut of a sand fly within hours of the sanfly bite while the promastigotes transform to amastigotes inside macrophages (Singh, 2006). In this form, nucleus is situated at the center and kinetoplast transversely towards the anterior end (Assafa *et al*., 2006). It also exhibits a single and delicate flagellum. Morphologically, it is similar to those grown in culture (Grimm *et al*., 2011).

2.3.2 Amastigote Stage

The amastigote form exists in the macrophages of the reticuloendothelial system of vertebrates such as: the spleen, liver, bone- marrow and lymph node (Rittig & Bogdan, 2000). They are ovoid and non–flagellated forms of *Leishmania*. The centrally located

round/oval nucleus and adjacent but smaller round/ rod shaped kinetoplast are distinguishable. The flagellum is not functional in amastigotes and does not extend beyond the cell body but with 'Flagellar pocket' which serves as a site for endocytosis and exocytosis.

Its cytoplasm contains mitochondria, neutral red vacuoles and basophilic, and volutin granules containing RNA.The organism is surrounded by a double membrane below which is a row of 130-200 hollow fibrils (Holzmuller *et al*., 2006).

2.4 Epidemiology of leishmaniasis

Leishmaniases occurs in 88 countries with a population of 350 million people (Nalan & Farrel, 1987; WHO, 2006). More than 90% of the global cases of visceral leishmaniasis (VL) occur in India, Bangladesh, Nepal, Brazil and Africa-East and North Africa including Sudan (Alvar *et al*., 2012). Globally, the number of new cases of cutaneous leishmaniasis (CL) and visceral leishmaniasis (VL) was estimated at 1.5 million with an annual incidence of 500,000 respectively (Singh & Sivakumar, 2003). People of all ages are at risk of infection with leishmaniasis if they live or travel where the disease is endemic.

In Kenya, the disease has been reported in Baringo, Kitui, Machakos, Meru, West Pokot, Elgeyo Marakwet and Turkana County, which are known to be endemic foci for kala-azar (VL) (Nolan & Farrel, 1987). *L. major* (Kinetoplastida: Trypasomatidae) that causes rodent and human cutaneous leishamiasis is endemic in a small focus in Baringo county, Rift Valley, Kenya, where it is transmitted by *P. duboscqiu* Neveu-Lemaire (Diptera: Psychodidae) (Muigai, 1987; Mutinga & Kamau, 1987). In this focus, the

disease is a zoonosis with rodents such as *Tatera robusta* Cretzschmar, *Arvicanthis niloticus* Geoffrey, **Taterillus emini* Thomas and *Mastomys natalensis* Smith (Rodentia: Muridae) as the proven reservoir hosts (Mutunga *et al*., 1986). The burrows these murines dig and dwell in, are also the breeding sites for the sand fly (Maroli *et al*., 2013).

2.5 Pathogenesis and Life Cycle of Leishmaniasis

Leishmaniases are transmitted by phlebotomine sand flies (ref).The life cycle of the leishmaniases is shown in Figure 2.1

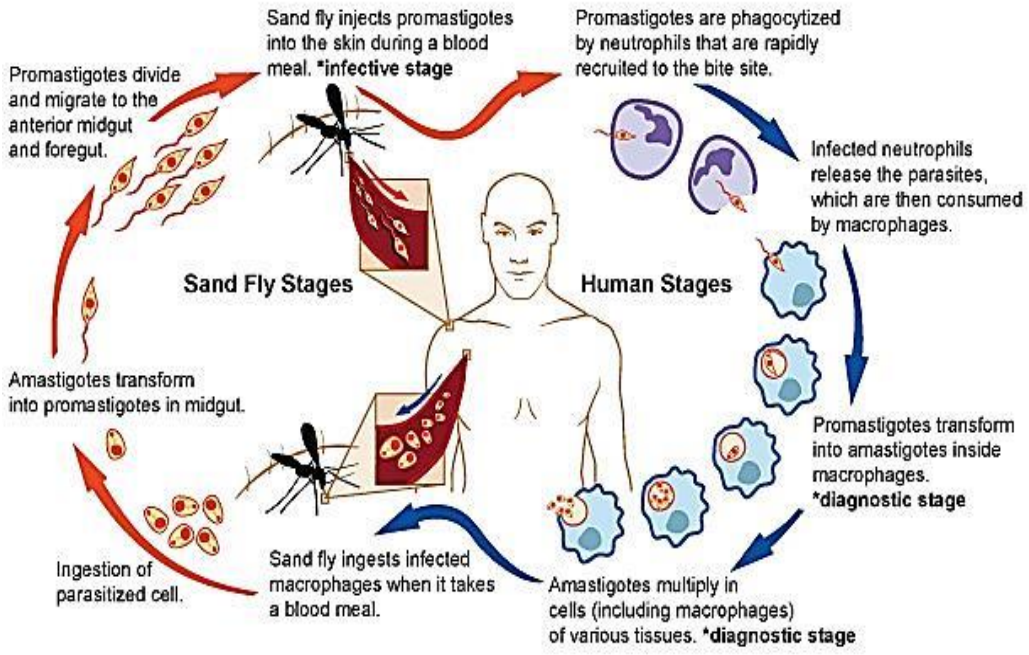

Figure 2.1: Life cycle of *Leishmania* species (Source: Jochim *et al*., 2008)

2.6 Mode of Transmission

All species are transmitted by small blood-sucking sand flies of genus *Phlebotomus* in the old world (Middle-East and Africa). Infections are confined to tropical and sub

tropical areas and only females feed on blood (Beach, 1984). Amastigotes ingested during feeding are normally transformed in the midgut or hindgut of insect vectors into promastigotes which multiply by binary fission (Kato, 2010).

Parasites migrate forward to the foregut and proboscis where some become swept away by saliva into the bite site when the sand fly feeds and are transmitted via the bite to the tissues of vertebrate hosts. The reservoir hosts for *Leishmania* include domestic animals such as dogs, cats and horses. Peridomestic animals like mice and rats and wild animals including rodents, Hyraxes, Sloths, Bat, Oppsuumes, Kangaroos, Wolves and Foxes also serves as reservoirs (Bates, 2007;Grondalen *et al*., 2008).

2.7 Control of the Leishmaniases

The control of different types of the leishmaniases depends on the type of cycle exhibited by the parasite and the behaviour of the animal reservoir (Bates, 2007). Measures to check the spread of leishmaniases include treatment of infected individuals, active case detection, and chemotherapy to eradicate the parasite reservoirs in anthroponotic transmission cycles and control strategies against the vector and zoonotic reservoirs in order to break transmission in zoonotic cycles (Lacerda, 2004).

2.7.1 Animal Reservoir Control

Reservoir control usually involves the testing and culling of seropositive dogs in zoonotic visceral leishmaniasis endemic areas (Davies *et al*., 2009). Impregnated collars, a novel method of topical application repellents such as Deltamethrin, have been used in breaking the transmission of leishmaniasis in zoonotic visceral leishmaniasis (ZVL) foci (Peterson & Esch, 2014). Animals' reservoir control for CL is based on the

use of poison baits and environmental management to control rodents (Moncaz *et al*., 2003).

2.7.2 Vector Control

The only proven vector of the *Leishmania* parasite is the blood-sucking female sand flies of the genus *Phlebotomus* in the Old World and *Lutzomyia* in the New World (Murray *et al*., 2005). The insects are 2-3 mm long and are found throughout the tropical and temperate parts of the world (Slappendel, 1988). Only 30 or so of over 500 species of *Phlebotomine* sand flies are known to transmit *Leishmania* parasites in the old world (Sharma & Singh, 2008).

The phlebotomine sand flies are very susceptible to insecticides and their populations have been observed to drop drastically during control of *Anopheles* mosquitoes, resulting in interruption of leishmaniases transmission (Kaul *et al*., 1994). Indoor residual spraying with insecticide is the most widely used intervention for controlling sand flies that are endophilic and can considerably reduce CL cases (Davies *et al*., 2000; Reyburn *et al*., 2000). However, spraying programmes are often unsustainable (Murray *et al*., 2005).

Where sand flies are endophagic and are active when people are asleep, insecticide bed nets impregnated with the synthetic pyrethroids permethrin, deltamethrin and lambda-cyhalothrin provide considerable protection (Bern *et al*., 2008). The limitations associated with the use of bed nets include the discomfort generated by smaller mesh

nets in warmer climates, the requirement for periodic re-impregnation of the nets and the high cost of long-lasting insecticide-treated bed nets (Murray *et al*., 2005).

Application of biolarvicides in field conditions is difficult due to the diverse breeding habitats of sand flies and their practical application appears to be of limited use in the control of VL (Kishore *et al*., 2006). Satellite remote sensing for early prediction of disease by identifying the sand fly flying conditions and the use of pheromones should be exploited in the control of leishmaniasis (Palit *et al*., 2005; Kishore *et al*., 2006).

In regions such as Latin America, Mediterranean Basin, Central and Southwestern Asia where VL is primarily zoonotic, reducing transmission to human beings by targeting the animal reservoir is a feasible strategy (Davies *et al*., 2009). However, culling infected domestic dogs in Brazil to reduce human VL was not been effective because of incomplete coverage; delays between taking blood samples, diagnosis, culling and the high dog population turnover rate (Courtenary *et al*., 2002). In view of the above shortcomings, dipping dogs in insecticides, applying topical insecticide lotions and the use of deltamethrin treated collars are the novel strategies that can substantially reduce sand fly bites on dogs and the subsequent human infection.

The use of insecticide treated bed nets (ITNs) can offer good protection against transmission of leishmaniasis by endophagic sand fly vectors. In Kenya, most vector species such as *P. duboscqiu, P.guggisberg* Kirk and Lewis, *P. martini* Parrot, *P. pedifer* Mutinga and Ashford, *P. longipes* Parrot and Martin are exophilic and exophagous, thus negating common strategies such as ITN use (Davies *et al*., 2009). Both male and female sand flies feed on plant juices and sugary secretions. Females

also feed on blood to produce eggs. Sand flies use their mouthparts to probe exposed skin, leading to the formation of a pool of blood from which they feed.

Sand fly saliva contains pharmacologically active components that aid in the feeding process (Kamhawn, 2000). Feeding activity is influenced by temperature, humidity and air movement. Sand flies are weak fliers so even light wind can inhibit flight and reduce biting. Most species feed at dusk and during the night, when temperature falls and humidity rises (Lawyer & Perkins, 2000). The majority of species feed outdoors, although daytime biting can occur indoors in darkened rooms or among shaded vegetation/trees, especially if disturbed by human activity. An adult sandfly feeding is shown in Figure 2.2.

Figure 2.2: Adult female sand fly feeding (Schlein *et al.*, 2001)

2.8 Treatment of Leishmaniasis

2.8.1 Chemotherapy

Chemotherapy has been the main method of leishmaniasis control. It is aimed at minimizing morbidity and mortality associated with the disease. The recommended first line drugs currently used for both visceral and cutaneous leishmaniasis are pentavalent antimonials: sodium stibogluconate (Pentostam®) and meglumine antimoniate (Glucanthine®) and, Amphotericin B (Benedict, 2003; Berman, 2011). These drugs require long courses of parenteral administration that takes about two hours of intravenous therapy per day administered in hospitals, hence the patients must have close medical follow-up to monitor side effects (Guerin *et al*., 2002: Singh & Sivakumar, 2003).

Currently, anti-protozoal drugs are inadequate due to parasite resistance to drugs, toxicity, lack of efficacy and inability to eliminate all stages of parasites from the host (Santos *et al*., 2008; Tasdemir, 2008). With the absence of an operational vaccine for malaria or leishmaniasis in the immediate horizon, chemotherapy and chemoprophylaxis still remain the main methods for disease control.

Treatment of leishmaniasis is difficult because the parasites infect the macrophages and thus patients present with immune deficiency and are not able to eliminate the parasites through natural mechanisms of defence (Rocha *et al*., 2005; Mondal, 2014).

Infection with diseases such as malaria increases the fatality of the illness if it is not diagnosed and treated in time (Carvalho *et al*., 2008). Leishmaniasis has been exacerbated by the evolution of acquired immune deficiency syndrome (AIDS) due to

parallel infections in HIV patients, as well as by the development of drug-resistance by the parasites (Croft *et al*., 2006).

Pentavalent antimonials and meglumine were the first-line treatment for leishmaniases (Frezard *et al*., 2009), but only cheaper, generic forms of these drugs are available that have been shown to be equivalent to the branded products (Croft *et al*., 2007)**.** Antimonial drugs are toxic and sometimes have life-threatening, adverse effects, including cardiac arrhythmia and acute pancreatitis (Chappuis *et al*., 2007; Cook, 1995). Patients under the age of 2 or aged greater than 45 with signs of advanced disease and/or severe malnutrition are at higher risk of death during antimonial therapy owing to drug toxicity, slowness of drug action, and leishmaniasis complications or a combination of these factors (Seaman *et al*., 2006)**.**

Amphotericin B has replaced antimonials in the treatment for leishmaniasis, but its also has side effects such as shortness of breath, skin rash, fever and rigor (Chappius *et al*., 2007), it is costly and requires slow infusions on alternate days showing a need for more research and development to improve the drugs for leishmaniases (Guerin *et al*., 2002; Van Griensen & Diro, 2012) that will be available, safe, cheap and for oral or topical administration in shorter treatment cycles. This calls for alternative approach in the treatment of leishmaniasis.

Other drugs approved for treatment are pentamidine, paromomycin and miltefosine (Vacchina & Morales, 2014). Although these drugs are effective against leishmaniasis, they also require long courses of parenteral administration and have toxic side effects (Kayser *et al*., 2003).

The costs of treatment are too high to afford for the large majority of the infected population, because those infected are mostly from remote areas (Balasegaram *et al*., 2012). Unfortunately, there is no protective vaccine available at the moment but Amphotercin B is used in those patients who do not respond to pentavalent antimony (Croft *et al*., 2006). The current standard treatment regimes for cutaneous leishmaniasis all involve monotherapy (Mitropoulos *et al*., 2010).

Systemic administration of pentavalent antimonials has remained the treatment of choice. However in the recent years Paromomycin has been used as topical formulations (Blum *et al*., 2004). Although parasites have recorded resistance to pentavalent antimonials, it still remains the drug of choice (Guerin *et al*., 2002). So far in the treatment of leishmaniasis only monotherapy is being used. Little has been done on combination therapy which can improve the activity through use of compounds that have synergistic or additive activity hence preventing drug resistance (Charkravarty & Sundar, 2010)**.**

Sitamaquine is an 8-aminoquinoline in development for the treatment of visceral leishmaniasis (Guerin *et al*., 2002) and cutaneous leishmaniasis (Sindermann & Engel, 2006; Soto & Toledo, 2007) by oral route, no activity being observed on experimental cutaneous leishmaniasis models. Recent data explain how sitamaquine accumulate in *Leishmania parasites*. However its molecular targets remain to be identified though it has advantage of short elimination half-life, preventing a rapid resistance emergence (Guerin *et al*., 2002).

The selection of a Sitamaquine-resistant clone of *L. donovani* in laboratory and the phase II clinical trials pointing out some adverse effects such as methemoglobinemia and nephrotoxicity are considered for a further development decision (Loiseau & Schrével, 2011). With the increase in cases of drug resistance and their failure, there has been an increase in the use of herbal medicine. Most of the studies have been carried out using animal models for parenteral administration than oral which could be cheap and easy in administration (Kinuthia *et al*., 2013).

2.8.2 Antimonials Resistance

Although the selection of resistant *Leishmania* has long been a part of laboratory studies, it is only in the past 20 years that acquired resistance has become a clinical threat. The first indication of drug resistance came from North Bihar, in the early 80s, of about 30% patients not responding to the prevailing regimen of Sodium stibogluconate, which was a small daily dose (10 mg/kg; 600 mg maximum) for short duration (6 to 10 day). Then two 10-day courses with a 10-day interval therapy with sodium antimony gluconate were recommended by an expert committee leading to a marked improvement in the cure rates up to 99%. However, in1984, it was seen that with 20 mg/kg (maximum 600 mg) for 20 days, 86% of patients were cured and cure rate with 10mg/kg was quite low (Awasthi *et a*l., 2004).

In the same year, the WHO expert committee recommended that pentavalent antimony be used in doses of 20 mg/kg up to a maximum of 850 mg for 20 days, and a repetition of similar regimen for 20 days in cases of treatment failures.

The WHO recommendations was evaluated a few years later by Thakur *et al*. and it was reported that only 81% of patients were cured by this regimen, although with an extension of the treatment for 40 days, 97% of patients could be cured. Three years later, the same group noted a further decline in cure rate to 71% after 20 days of treatment, and recommended extended duration of treatment in non responders. However, by early 90s, extending the therapy to 30 days could cure only 64% of patients in a hyperendemic district of Bihar (Cook, 1995).

In two studies carried out under strictly supervised treatment schedules, it was observed that only about one-third of the patients could be cured with the currently prevailing regimen. The incidence of primary unresponsiveness was 52%, whereas 8% of the patients relapsed. Incidentally, only 2% of the patients from the neighboring state of (Eastern) Uttar Pradesh (UP) failed treatment (Charlab *et al*., 1999). There are reports of antimony resistance spreading to the Terai regions of Nepal, especially from the district adjoining the hyperendemic areas of Bihar, where up to 30% of the patients seem to be unresponsive, though in Eastern Nepal a 90% cure rate has been reported. These studies confirmed that a high level of antimony resistance existed in Bihar, whereas it was still effective in surrounding areas (Nalon, 1987).

There had been speculations whether Indian *Leshmania donovani* had become truly refractory to Sb^v or resistance occurred because of the inadequate doses being used in Bihar. The reasons for the emergence of resistance were the widespread misuse of the drug. Sb^v was freely available in India, both qualified medical practitioners and unqualified quacks used the drug and this unrestricted availability of the drug led to

rampant misuse. Almost 73% patients consulted unqualified practitioners first; most of them did not use the drug appropriately. It was a common practice to start with a small dose and gradually build up to the full dose over a week; it was also advocated to have drug free periods to minimize the toxicity, especially renal toxicity and physicians split the daily dose in two injections (Pearson & Queriroz, 1996).

Almost half of the patients, receiving pentamidine as a second-line drug, had not received adequate antimony treatment before being labeled as refractory to Sb^v. These facts indicated large-scale misuse of antileishmanial drugs in Bihar, contributing to development of drug resistance. There were several manufacturers of Sb^v in India, and quality of products were inconsistent, resulting in occasional batches being substandard and toxic, this added to the problems associated with Sb^v therapy causing serious toxicity and deaths related to the drug (Loomis & Hayes, 1996).

Another reason for the growing resistance to Sb^v in India while it still remained sensitive all over the world could be due to the fact that leishmaniasis usually has zoonotic transmission except in the Indian subcontinent and East Africa where the transmission is largely anthroponotic. In an anthroponotic cycle once Sb^v resistance gets established, it spreads exponentially and organisms sensitive to the drug get eliminated quickly, whereas the drug-resistant parasites continue to circulate in the community (Ritting & Bogdan, 2000).

HIV/VL coinfected patients is another subset who respond poorly to Sb^v, as the drug needs an intact immune system to be effective, and the response is not as good as in immunocompetent patients.

Initial parasitological cure with Sb^{v} could be as low as 37%, and eventually most of the initially cured patients tend to relapse. Thus, they are a potential source for emergence of drug resistance (Salotra & Singh, 2006).

In CL the response is not as predictable, because there is considerable variation in sensitivity to Sb^{v} among primary isolates from untreated patients with cutaneous leishmaniasis, which correlates with patients' response to treatment (Assafa *et al*., 2006). Except Bihar, primary resistance is quite uncommon, but resistance develops in patients with VL, CL, and MCL who have relapsed. Chances of response to further courses of antimonials diminish once there is a relapse after the initial Sb^{v} treatment (Blum *et al*., 2007). In *L. infantum* isolates taken from VL patients in France drug-sensitive strains (ED50 of<40 μg/ml) were isolated from patients who responded quickly to the meglumine treatment, whereas all the strains which were resistant under *in vitro* conditions (ED50 of>70 μg/ml) corresponded to clinical failures and *in vitro* sensitivity of strains decreased progressively in relapsing patients treated with meglumine (Desjeux, 2004).

2.8.3 Mechanism of Antimonials Resistance

The mechanisms of action of antimonials are still unclear. The unique thiol metabolism of *Leishmania* is thought to play a pivotal role in the mechanism of action of antimonial drugs. In these parasites, the major low-molecular-mass thiol is trypanothione ($T[SH]_2$). Antimonial drugs are administered as pentavalent antimony ($Sb^{[V]}$), a prodrug requiring conversion to the trivalent form [Sb(III)], before becoming biologically active.

However, the site of reduction (host macrophage, amastigote, or both) and mechanism of reduction (enzymatic or nonenzymatic) remain unclear (Ritting & Bogdan, 2002). Sb(III) interferes directly with thiol metabolism, decreasing thiol-buffering capacity in drug-sensitive *Leishmania donovani* by inducing rapid efflux of intracellular $T[SH]_2$ and GSH (Ameen, 2010). Sb(III) also inhibits $T[SH]_2$ reductase in intact cells, resulting in the accumulation of the disulfide forms of both $T[SH]_2$ ($T[S]_2$) and GSH. These two mechanisms act synergistically against Leishmania parasites, leading to a lethal imbalance in thiol homeostasis (Soto & Toledo, 2007).

Some studies have reported apoptosis in Sb (III)-treated amastigotes involving DNA fragmentation and externalization of phosphatidylserine on the outer surface of the plasma membrane. However, these effects do not involve the classical caspase mediated pathway. and do not meet the more recent stringent definition of apoptosis (Charkravarty & Sundar, 2010).

Diminished biological reduction of Sb^v to Sb (III) has been demonstrated in *L. donovani* amastigotes resistant to sodium stibogluconate (Alvar *et al*., 2006). It is not known whether this mechanism occurs in clinical isolates at present. Although recently an arsenate reductase gene *(LmACR2)* and a thiol-dependent reductase (TDRl) from *L. major* has been identified their role in drug resistance is not known (Kollar, 2009)

In prokaryotes and eukaryotes (yeast and mammalian), aquaglyceroporins (AQPs) are known to transport trivalent metalloids. Aquaglyceroporins from *L. major* (LmAQPl) have recently been demonstrated to mediate uptake of Sb(III) in *Leishmania* spp. and

overexpression of aquaglycoporin 1 in drug resistant parasites is seen to render them hypersensitive to Sb(III) (Awasthi *et al*., 2004).

Increased levels of trypanothione(TSH) have been observed in some lines selected for resistance to Sb(III) or arsenite is due to increased levels of the rate-limiting enzymes involved in the synthesis of glutathione (glutamylcysteine synthetase, GCS) and polyamines (ornithine decarboxylase, ODC) the two precursor metabolites to trypanothione. The modulation of TSH levels by using specific inhibitors of γ-GCS or ODC could reverse the resistance in mutants (Blum *et al*., 2007).

The ATP-binding cassette (ABC) protein PGPA (renamed as MRPA) has been assumed to play a major role on metal resistance in *Leishmania* (Ritting & Bogdan, 2000). PGPA is a member of the multidrug-resistance protein (MRP) family, a large family of ABC transporters, several of which are implicated in drug resistance (Berman, 2011). The *PGPA* gene has been shown to be frequently amplified in *Leishmania* cells that are selected for resistance to arsenite- or antimony-containing drugs (Awasthi *et al*., 2004) Legare *et al*. observed that PGPA is localized in small vesicles near flagellar pocket and these are responsible for intracellular sequestration of arsenic/antimony-thiol conjugates, thereby conferring arsenite and antimonite resistance (Ameen, 2010).

In a study on *Leishmania infantum* amastigote parasites selected for resistance to Sb(III) the expression of three genes coding for the ABC transporter MRPA (PGPA), S-adenosylhomocysteine hydrolase, and folylpolyglutamate synthase were found to be consistently increased. Transfection of the *MRPA* gene was shown to confer sodium stibogluconate resistance in intracellular parasites which could be reverted by using the

glutathione biosynthesis-specific inhibitor buthionine sulfoximine (Aswasthi *et al*., 2004).

However, in an isolate from Sb^v refractory patients no amplified *PGPA* sequence could be detected, instead a novel 1.254-kb gene whose locus is on chromosome 9 involved in protein phosphorylation was identified (Cook, 1995). Transfection experiments established that this isolated fragment confers antimony resistance to wild-type *Leishmania* species. It remains to be established whether this recently identified gene sequence can be used as a probe in the clinic to identify antimony-resistant clinical isolates on the Indian subcontinent (Croft *et al*., 2006).

Pentamidine is another antileishmanial which suffered the same fate as Sb^v in North Bihar. It was the first drug to be used in patients refractory to Sb^v and cured 99% of these patients initially however in the next two decades its efficacy dwindled to approximately 70% of patients (Grimaldi & Tresh, 2012).

Its use in VL was ultimately abandoned due to its decreased efficacy and serious toxicities. However, it has been used to good effect in treatment of both Old and New World CL and MCL. Fewer injections over short periods result in a high cure rate with minimum toxicity. In CL caused by *L. guyanensis*, 89% of cases were cured with two injections (4 mg/kg) given 48 h apart, and 80% of remaining patients were cured by a second course with minimum adverse effects (Murray *et al*., 2005).

The antileishmanial mechanism of action of pentamidine, are still not clearly known, however possible mechanism include inhibition of polyamine biosynthesis, DNA minor groove binding, and effect on mitochondrial inner membrane potential.

Pentamidine-resistant promastigote clones of *L. donovani* and *L.amazonensis* were shown to have 18- and 75-fold reduced uptakes, respectively, and increased efflux. Specific transporters for pentamidine uptake have been characterized and might have a role in resistance (Ritting & Bogban, 2000).

Amphotericin B a polyene antibiotic is now being used as a first line therapy in areas with Sb^v resistance. It has excellent cure rates (~100%) at doses of 0.75–1.00 mg/kg for 15 infusions on daily or alternate days. It has been used extensively in Bihar with uniformly good results (Balasegaram *et al*., 2012).

Lipid-associated amphotericin preparations are as effective as conventional amphotericin B, and have negligible adverse reactions. The dose requirement of liposomal amphotericin B varies from region to region; while in the Indian subcontinent a small dose induces high cure rates a higher dose in needed for Eastern Africa, the Mediterranean region and Brazil. This higher efficacy of liposomal amphotericin B against *L. donovani* than *L. infantum/L. chagasi* infections is probably related more to parasite load and host immune status pathology than species sensitivity (Croft *et al*., 2006).

To determine the mechanism of resistance, a resistant clone of *L. donovani* promastigotes was selected through a stepwise increase in amphotericin B concentration

in culture. Resistant promastigotes showed a significant change in plasma membrane sterol profile by gas chromatography-mass spectrometry, ergosterol being replaced by a precursor, cholesta-5, 7, 24-trien-3β-ol. This probably results from a defect in C-24 transmethylation due to loss of function of S-adenosyl-L-methionine-C24-Δ-sterolmethyltransferase (SCMT). In *L. donovani* promastigotes two transcripts of the enzyme have now been characterized, one of which was absent in the amphotericin B-resistant clone, the other overexpressed but without a splice leader sequence which would prevent translation (Chippius *et al*., 2007).

Clinical resistance to amphotericin B is rare. Nevertheless, with the increasing use of amphotericin B, especially in lipid formulations which have longer half life, the possibility of resistance cannot be ignored. There are two small inconclusive studies on the emergence of amphotericin B resistance in *L. infantum*/HIV-infected cases in France. One study failed to find a change in sensitivity in promastigotes derived from isolates taken before and after the treatment of one patient. In contrast, a decrease in sensitivity was observed in isolates taken over several relapses from another patient (Benedict, 2003).

Miltefosine, an alkyl phospholipid is the first oral agent approved for the treatment of leishmaniasis. At the recommended doses (100mg daily for patients weighing ≥25 kg and 50mg daily for those weighing ≤25 kg for 4 weeks) cure rates were 94% for VL. It has a long-terminal half-life, which ranges between 150 and 200 h. About four half-lives (25–33 days) are required to reach more than 90% clearance of the plateau levels (at steady-state). Thus, subtherapeutic levels may remain for some weeks after a

standard course of treatment. This characteristic might encourage the emergence of resistance (Eissa *et al*., 2012)

In vitro studies shows variation in the sensitivities of both promastigote and amastigote stages of *L. donovani, L. major, L. tropica, L. aethiopica, L. mexicana*, and *L. panamensis* to miltefosine (Frezard *et al*., 2009).

In all assays *L.donovani* was the most sensitive species and *L. major* was the least sensitive species. Studies on clinical isolates using a murine macrophage-amastigote model have confirmed the high sensitivity of *L. donovani* from both Sb-sensitive and Sb resistant patients from Nepal and lack of sensitivity of *L. braziliensis* and *L. guyanensis* isolates from patients in Peru (Charkravaty & Sunder, 2010).

This variability in sensitivity reflects differences in intrinsic susceptibility however it could have an important impact on clinical outcome. The greatest clinical significance is seen in Central and South America where distribution of *L. mexicana, L. amazonensis, L. panamensis, L. braziliensis* overlap. The clinical relevance of this finding was observed for CL by Soto *et al*. in Colombia, where *L. panamensis* is common, the cure rate was 91%, whereas in Guatemala, where *L. braziliensis* and *L. mexicana* are common, the cure rate was 53% (Eissa *et al*., 2012).

Paromomycin, an aminoglycoside-aminocyclitol antibiotic, has been used for the treatment of VL in a parenteral formulation and CL in both topical and parenteral formulations. In the phase III trial of Paromomycin in the Indian subcontinent, it was shown to be noninferior to amphotericin B and was approved by the Indian government

in August 2006 for the treatment of patients with visceral leishmaniasis (Riiting & Bogdan, 2000). Topical preparations of paromomycin, a soft paraffin-based ointment containing 15% of paromomycin and 12% methyl-benzethonium chloride (MBCL), are effective against both Old World as well as New World CL (Charkravaty & Sunder, 2010).

Variation in sensitivity has been seen in both experimental models and clinical cases of CL, as lesions caused by *L. major* treated with paromomycin ointment resolved faster and more completely than lesions caused by *L. amazonensis* and *L. panamensis* (Hailu, 2005).

A more in depth *in vitro* analysis on the sensitivity of amastigotes in a murine macrophage model showed that *L. major* and *L. tropica* were more sensitive than *L. braziliensis* and *L. mexicana* isolates and *L. donovani* showed intermediate sensitivity (Clinical resistance with this drug in VL is not known as it has not been used extensively. However, following a 60-day parenteral course for treatment of CL in two *L. aethiopica* cases, isolates taken from relapsed patients were three- to fivefold less sensitive to the drug after treatment than isolates taken before treatment in an amastigote-macrophage assay (Alvar *et al*., 2006).

The mechanisms of action of paromomycin in *Leishmania* spp. is exactly not known however mitochondrial ribosomes and induction of respiratory dysfunction and mitochondrial membrane depolarization have been implicated. In studies on selected populations of promastigotes, resistance was related to decreased drug uptake in *L. donovani.* In a recent study, the mitochondrial membrane potential was significantly

decreased after 72 hours of exposure to paromomycin indicating that this organelle might be the ultimate target of the drug. Both cytoplasmic and mitochondrial protein synthesis were inhibited, however, the drug induced reduction in membrane potential and inhibition of protein synthesis were less pronounced in the resistant strain as compared to the wild-type. A line selected for resistance to the drug showed reduced paromomycin accumulation associated with a significant reduction in the initial binding to the cell surface (Assafa *et al*., 2004).

Sitamaquine, a 4-methyl-6-methoxy-8-aminoquinoline has limited clinical use and no reported resistance. Relatively poor efficacy compounded with nephrotoxicity suggests that this drug cannot be used as monotherapy in VL (Loiseau & Schrevel, 2011).

Azole-like ketaconazole and triazoles, intraconazole, and fluconazole have antileishmanial effects (Frezard *et al.,* 2009). One placebo-controlled trial on the treatment of CL showed that *L. mexicana* infections (89%) were more responsive than *L. braziliensis* infections (30%) to ketoconazole indicating intrinsic differences in sensitivity of *Leishmania* species to azoles. These drugs have limited clinical use and clinical resistance is not known (Milroppoulos *et al*., 2010).

2.9 Kenyan Plants with Antileishmanial Activities

Pentostam and Amphotericin B have not been very sucessful in the control of the leishmaniasis because of their prohibitive cost, emergence of drug resistance and high toxicity (Croft *et al*., 2007; Charkravarty & Sundar, 2010).

These diseases are endemic in some rural parts of Kenya where the patients have no access to adequate treatment (Alvar *et al*., 2006).

Use of plant-derived medicine that is readily available in the rural areas can be useful in the control of leishmaniasis. Herbal products are increasingly becoming important because they symbolize safety in contrast to the synthetic drugs (Joy *et al*., 2001). Natural compounds that include terpenes, triterpenes, alkaloids, and phenolic derivatives obtained from a variety of plants have been reported to show antileishmanial activities (Kayser *et al*., 2003; Palit *et al*., 2005; Makwali *et al*., 2012; Wabwoba *et al*., 2013). These natural plant compounds have always been a common practice in Kenya, and have been used by various cultural systems for centuries (Farnsworth, 1982).

The following are the orders, families and plant species that have been shown to have anti-leishmanial activity either *in vitro, in vivo* or in both systems. In Asparagales: Amaryllidaceae, (Garlic), *Allium sativum L*. have been shown to possess very low toxicity against vero cells and anti-leishmanial activities against CL caused by *L. major in vitro* and *in vivo* and *L. donovani in vitro* (Chase *et al*., 2009; Kinuthia *et al*., 2013). The advantage of using *A. sativum* extracts is that it can be used to treat both orally and by injection (Kinuthia *et al.,* 2013).

Its treatment on BALB/c mice infected with *L. major* reduced lesion sizes. *A. sativum* was able to up-regulate a ten-fold production of nitric oxide by macrophages.

Aqueous extracts of garlic have also been shown to have potential in the treatment of human and animal pathogenic protozoa (Bahmani *et al*., 2013). Some of the bioactive ingredients in *A. sativum* have been identified as allicin, diallyl disulphide, S-allylcysteine and diallyl trisulfide (Kimbaris *et al*., 2006).

Warburgia ugandensis Sprague (Canellales: Canellaceae) extracts have been shown to have antleishmanial, antimalarial, antifungal and antibacterial properties both *in vitro* and in animal models (Olila *et al*., 2001; Were *et al*., 2010). Ngure *et al.* (2009) and Githinji *et al*., (2010), also showed that hexane extracts of the same plant have better activity against *L. major* promastigotes *in vitro*. In Fourier Transformer Infra-Red Spectrophotometer Analysis, isocyanides, phenols, carboxylic acid anhydrides, esters and lactones, alkanes and alkenes were some of the bioactive chemicals found in leaves of *W. ugandensis* (Moebe & Nyarango, 2013).

Within the order Brassicales, family Moringaceae, *Moringa stenopetala* has been shown to have antiparasitic properties against a variety of organisms (Mekonnen *et al*., 1999; Anwar & Rashid,2007). Tested leaf and root extracts of *M. stenopetala* against *Trypanosoma* were found to be effective against *T. brucei*. *M. stenopetala* has been used in Ethiopian folk medicine as an antimalarial agent (Ghebreselassie *et al*., 2011) while their leaf extracts from Baringo County, Kenya were tested against *L. major* (Olson, 2006).

Some of the active ingredients that have been isolated from *Moringa* preparations and have been reported to have hypo-tensive, anticancer, and antibacterial activity are glucosinolates, particularly 4-(4'-*O*-acetyl-α-L-rhamnopyranosyloxy)benzyl isothiocy-anate**,** 4-(α-L-rhamnopyranosyloxy)benzyl isothiocy-anate**,** niazimicin, pterygospermin**,** benzyl isothiocyanate**,** and 4-(α-L-rhamnopyranosyloxy (Mekonnen *et al*., 1999; Fahey, 2005). Extractives of the bottlebrush tree, *Callistemon citrinus* have been reported to exhibit antileishmanial and antioxidant activities and were found to be effective against *L. major* and *L. donovani* promastigotes and amastigotes (Haque *et al*., 2012).

When administered orally in *L. major*-infected BALB/c mice, a blend of *M. stenopetala* and *C. citrinus* caused a reduction of footpad lesions (Kinuthia *et al*., 2013).Within the order Caryophyllales: Plumbaginaceae, *Plumbago zeylenica* L, *P. capensis* and *P. auriculata* have been shown to have antileishmanial effects both *in vitro* and *in vivo*. Plumbagin or 5-hydroxy-2-methyl-1, 4-naphthoquinone is an organic compound with the chemical formula $C_{11}H_8O_3$. It is regarded as one of the main active compounds against parasitic protozoa (Padhye *et al*., 2013).

Others are juglone, $C_{10}H_6O_3$ also chemically called 5-hydroxy-1, 4-naphthalenedione (Yang *et al*., 2009), and lawsone also known as hennotannic acid, $C_{10}H_6O_3$**,** 2-hydroxy-1, 4-naphthoquinone. Trypanothione and trypanothione reductase (TryR)-based redox metabolism found in *Leishmania* and other trypanosomatids exemplify the unique features of this group of organisms.

Trypanothione reductase is an NADPH-dependent flavoprotein unique to protozoans of the genera *Leishmania* and *Trypanosoma* and has long constituted an attractive target for chemotherapeutic research in relation to diseases caused by parasites in the two genera since there are numerous pieces of evidence indicating that the enzyme is essential for parasite survival as well as specific in *Leishmania* (Gundampati & Jagannadham, 2012). Its absence in mammalian hosts, together with the sensitivity of trypanosomes against oxidative stress, makes this enzyme a unique target for exploitation for potential antileishmanial chemotherapeutics (Mekonnen *et al*., 1999). Plumbagin, a plant-derived naphthoquinone, is reported to possess antileishmanial properties by inhibiting Trypanothione (TryR), (Sharma *et al*., 2012).

Plants of the genus *Aloe* within the Family Xanthorrhoeaceae are known to have a variety of activities against insects, human and veterinary parasitic diseases (Lans *et al*., 2007; Omwenga *et al*., 2007). In different studies, sap from leaves of *A.* s*ecundiflora* ahas been used to treat wounds, pimples and ringworm (Njoroge & Bussmann, 2007). Infusions of leaves in water have also been used to treat malaria (Muthaura *et al*., 2007).

Methanolic and aqueous extracts were effective in suppressing the development of *L. major in vitro* (Ogeto *et al.*, 2001).*A. secundiflora* contains several major groups of chemical compounds namely tannins, saponins, alkaloids, cardiac glycosides, flavonoids and terpenidoes (Omwenga *et al*., 2007). Extracts of *A. nyeriensis* Christian**,** *Albizia coriara*, *Acacia tortilis*, *Asparagus racemousus* and *Suregada zanzibariensis* have shown reasonable leishmanicidal activities (Kigondu, 2009).

The methanol extracts of *Acacia toritilis* and *A. coriara* were reported to be effective in killing promastigotes at lower concentrations and with active ingredients being phenolics and tannins (Ogungbe *et al*., 2012). It has also been used successfully by some Ayurvedic practitioners for nervous disorders, inflammation, liver diseases and certain infectious diseases (Innocent *et al*., 2010).

The methanolic extracts of *Delonix elata* has been shown to possess an array of biological and pharmacological properties which include antibacterial activity (Chitravadivu *et al*., 2009), anti-inflammatory activity (Lawal *et al*., 2010) and antioxidant activities (Doss *et al*., 2009). The active ingredients in the plant include sterculic and malvalic acids, beta-amyrin, hesperitin and neohesperidin (Khan & Abourashed, 2011).

Studies have shown that plant extracts of *Callistemon citrinus* and *Allium sativum* L. have antileishmanial activities against *L. major* (Kinuthia *et al*., 2013). *A. sativum* alone was shown to have antileisthmanial activities (Wabwoba *et al*., 2013; Makwali *et al*., 2012). Makwali *et al*, (2012) also reported that extracts from *Plumbago capensis* (Plumbaginaceae) had antileishmanil activities. In a study that was carried out by Ngure *et al*, (2009), it was shown that *Warburgia urgandensis* Sprague (Canellales: Canallaceae) had antileishmanial activity when used against *L. major*. Other plants incude: *A. secundiflora* (Omwenga, 2009), *Albizia coriara* (Kigondu *et al*., 2009), *Acacia tortilis* (Tiuman *et al*., 2011), *Asparagus racemosus* (Sachdeva *et al*., 2014) *S. zanzibariensis* (Rehman *et al*., 2014), *Delonez ovate* (Katalinic *et al*., 2006), *M. stenopetala* (Olson, 2006).

2.9.1 *Momordica foetida*

The plant *M. foetida* Schumach & Thonn (Cucurbitaceae family), growing in most parts of tropical Africa, has been shown to have chemotherapeutic value (Oliver-Beyer, 1986). Mostly, in the kalenjin communities, aerial parts of the plant are used for treating various diseases like skin rashes, wounds, swelling joints of the body, cancer and inflammation related ailments (Pokot, Nandi and Keiyo herbalists).

Scientific studies conducted before, has validated that *M. foetida* extracts can be used for the treatment of malaria (Froelich *et al*., 2007) and diabeties (Bailey *et al*., 1989). It is a perennial climbing vine with tendrils. The flowers are cream often with a reddish or orange centre. Male and female flowers are found on the same plant (Schaefer &Renner, 2010). The characteristic fruit is bright orange with prickles. This species with its beautiful habit but a strong and unpleasant smell belongs to the Cucurbitaceae

family. *M. foetida* is closely related to the bitter melon, *M. charantia* L and balsam apple (*M. balsamina* L.).

The Cucurbitaceae consist of about 120 genera and 850 species that are widely distributed (Froelich *et al*., 2007). Many species are commercially grown for their nutritive value (Nesamvuni *et al*., 2001) and in some cases they are used for medicinal purposes (Afolayan & Sunmonu, 2010). On focusing the attention on *M. foetida plant*, some studies using modern techniques have authenticated its use in diabetes and its complications (nephropathy, cataract and insulin resistance), antibacterial as well as antiviral agent (Chitemerere & Mukanganyama, 2011). Leaves from *M. foetida* are traditionally used to treat symptoms of malaria in parts of East Africa (Waako, 2007; Froelich *et al*, 2007).

The leaves are boiled and eaten as vegetables; plants are grazed by cattle in Sudan while fresh leaves are used as fodder in Kenya and Tanzania where it was reported to be suitable for fattening rabbits (Nesamvuni *et al*., 2001).

In West Africa it was reported that traditional medicinal uses of *M. foetida* are numerous and shared with other *Momordica* species. The juice of crushed leaves is used to relieve cough, stomach -ache, intestinal disorders, headache, earache, toothache and as an antidote for snakebites. Skin problems caused by smallpox, boils, spitting cobra poison and malaria are also treated with crushed leaves (Oliver, 1986; Yineger *et al*., 2008). The crushed seeds are used in East Africa to cure constipation while fruits pulp is said to be poisonous to weevils, moths and ants, and is used as an insect repellent in Tanzania (Ruffo *et al*., 2002). Rescent experimental studies have exhibited the potential

of *M. foetida* against Helicobacter pylori and cancer (Desai *et al*., 2008; Koller, 2009) and Helicobacteriaceae (Nerurkar *et al*., 2010).

Most importantly, the studies of plants from the same order have shown its positive effects in various cancers treatments such as lymphoid leukemia, lymphoma, choriocarcinoma, melanoma, breast cancer, skin tumor, prostatic cancer, squamous carcinoma of tongue and larynx, human bladder carcinomas and Hodgkin's disease (Kintzios, 2006; Desai *et al*., 2008; Nerurkar *et al*., 2010).

The plant within the same order, *M. cymbalaria* has been shown to possess alkaloids, saponins, triterpins, flavonoids, tripenes which are known to have antiparasitic activities (Jeyadevi *et al*., 2013).

Extracts from *M. foetida* have also been shown to be a potential laxative when granules are used as capsules (Odusote & Awaraka, 2008). *M. charantia*, from the same genus has been shown to treat several ailments (antidiabetic, abortifacient, anthelmintic, contraceptive, dysmenorrhea, eczema, emmenagogue, antimalarial, galactagogue, gout, jaundice, abdominal pain, kidney stone, laxative, leprosy, leucorrhea, piles, pneumonia, psoriasis, purgative, rheumatism, fever and scabies) in various systems of traditional medicine (Afolayan & Sunmonu, 2010; Hakizamungu *et al*., 1992; Grover & Yadav, 2004).

Therefore, considering that *M. foetida* has been shown to have antiparasitic activities (Afolayan & Sunmonu, 2010; Hakizamungu *et al*., 1992) and it contains compounds that have been shown to have activities against various protozoan parasites (Jeyadevi, 2013), it is for this reason that this study aimed at testing methanolic and water extracts

of *M. foetida* plant against *L. major* parasite.The picture of *M. foetida is shown in* Plate 2.1 and some of its active ingredients are shown in Figures 2.3, 2.4 and 2.5.

Plate 2.1: Picture of *M. foetida* plant (Source: Author, 2015)

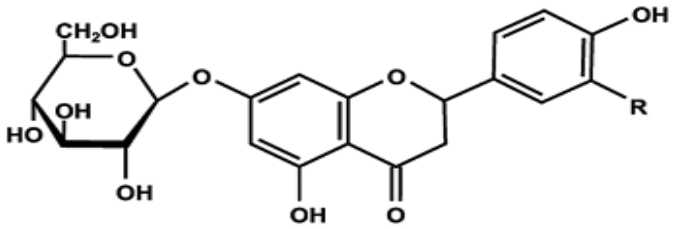

Figure 2.3: Structure of flavanone glycosides (Prunin) - $C_{21}H_{20}O_{10}$ isolated from *M. foetida* (Froelich *et al* 2007)

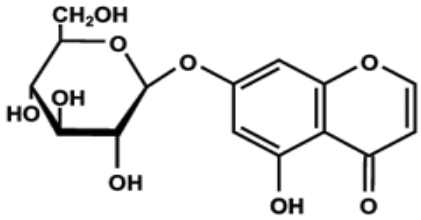

Figure 2.4: Structure of 5,7-Dihydroxychromone–7– *O-β*-D-glucopyranoside (Froelich *et al* 2007)

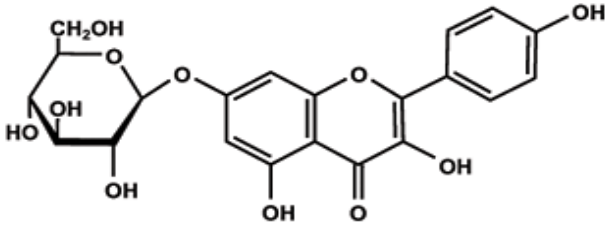

Figure 2.5: Structure of Kaempferol– 7 – *O-β*-D-glucopyranoside (Froelich *et al.*, 2007)

CHAPTER THREE

MATERIALS AND METHODS

3.1 Study Design

The study involved laboratory experimental testing of the antileishmanial activity of methanol and aqueous extracts of *M. foetida* against *L. major* in BALB/c mice (*in vivo*) and *in vitro* in micro well plate culture in Carbon dioxide incubator.

In *in vivo* Bioassays, 56 female BALB/c mice of 8 weeks old, weighing between 12g and 15g were randomly put in eight different metal cages in groups of seven mice each. All mice were infected with *L. major* metacyclic promastigotes on the left foot to stimulate cutaneous lesion development at the site of inoculation and systemic parasites in blood circulation.

Some groups of infected mice were then treated each with *M. foedita* extracts daily after lesion appearance 32 days after infection date by IP and oral administrations. Positive controls involved treating a group of mice with the standard drugs (Pentostam® and Amphotericin B®) while a negative control group of mice was treated with sterile phosphate buffered saline (PBS).

The lesion size progression and weight of each mouse were recorded weekly for five weeks. The lesions size measurements were by venear calliper where the difference (D) was worked out by subtracting measurements in the left and right foot diameters and the weight got using analytical balance. At the end of the experiment, all animals were sacrificed complying with ethical guidelines governing Animal Care and Use Committee (ACUC) of KEMRI.

Parasite burden from the spleen and livers of the mice were quantified using Leishman-Donovani Units (LDU) and total Leishman-Donovani Unit (totalLDU) as described by Veerareddy *et al*., (2004).

In *in vitro* bioassays antipromastigote and antiamastigote effects of *M. foetida* extract were tested. The *L. major* parasites were exposed to different concentrations of *M. foetida* extracts to determine the minimum inhibition concentration (MIC), inhibitory concentration (IC_{50}), access the viability of the promastigotes and amastigotes and to determine amoumt of nitric oxide production labels. Cytotoxicity of the extracts was tested using vero cells *in vitro* in plate culture in carbon dioxide incubator for 72hrs.

3.2 Collection and Processing of Plant Material

The plant *M. foetida* was collected from Trans Nzoia County. It was identified by a plant taxonomist in the University of Eldoret and deposited at the Herbarium in Biological science department with voucher number, M/23/14. The pecimen plant with all parts was preserved.

The aerial parts of the plant were collected very early in the morning before the sun rose to avoid photoreaction and deactiactivation of its active ingredients then transported while fresh to KEMRI, Centre for Traditional Medicine and Drug Development Research (CTMDR) where they were air dried under a shade for 21days and shred into powder using an aluminium blender. The aerial part of the plant was chosen because of its availability. Prior studies had indicated its effectiveness in inflammatory and anti-malaria (Waako *et al*., 2007).

3.3 Extraction of Plant Material

3.3.1 Extraction using Methanol

Quantities of 200grams of dried and powdered M. foetida aerial parts were soaked in 1litre of absolute methanol for 48 hours with intermittent shaking using a mechanical shaker. The solvent was filtered using a Buchner funnel and Whatman No. 1 filter paper and distilled on a water bath. The extract was dried in a vacuum drier, weighed and the required doses prepared and stored at 4 ^{0}C in air tight bottles. Extracts were only dispensed when required for bioassay investigations following the techniques described by Koduru *et al.* (2006).

3.3.2 Extraction using Water

200grams of dried and powdered *M. foetida* aerial parts were soaked in 1000ml of distilled water in conical flask, plugged with a cork and placed in a water bath maintained at 60^{0}C for 48 hrs. The filtrate from Buchner funnel and watman No.1 filter paper was freeze-dried using Edwards freeze dryer, weighed and required dose was prepared and stored at 4^{0}C in air tight bottles. Extracts were only dispensed when required for bioassays following the techniques described by Barbosa *et al*., (2008).

3.3.3 Preparation of Plant Extracts for *in vivo* and *in vitro* Bioassays

3.5.3.1 Formulation of Methanol Extracts

Methanol extracts were not readily soluble in water hence 10mg of the extract was first dissolved in 1ml of dimethyl sulfoxide (DMSO, Sigma Chemical CO., St Lovis, MO, USA) followed by subsequent dilution to lower concentration of DMSO, to ≤ 1% to

avoid carry over (solvent) effect (Elueze *et al*., 1996). The final concentration was 100µg/ml for the *in vitro* assays. For *in vivo* assay 2.0g of the extract was weighed and dissolved in 2ml of distilled water to give a final concentration of 1mg/kg/day.

3.3.3.2 Formulation of Aqueous Extracts

Water extracts of plant samples that had been prepared earlier, were retrieved from 4^0C fridge and diluted in distilled water to make the highest concentration in the microtitre plates of 100µg/ml for *in vitro* assay and 1mg/kg/day for the *in vivo* assay. For the *in vitro* experiments, 0.05g of the extract was diluted to a final volume of 5ml (stock solution of 10,000µg/ml). Each quantity of the extracts was then filter sterilized with syringe adaptable to 0.22µ filter into sterile Bijoux bottles in a Laminar Flow Hood (Bello Glasses Inc. USA) and stored at -20^0C. This was later diluted using distilled water to make 1mg/ml. For the *in vivo* assay, 2.0mg of the extract was weighed and diluted in 2ml of distilled water. This gave a final concentration of 1mg/kg/day for each mouse.

3.4 Experimental Animals

In *in vivo* study, 56 BALB/c mice of 8 weeks old females, weighing between 12g to15g were used. They were obtained from the animal house facility of Kenya Medical Research Institute (KEMRI) were housed in clean cages placed in well ventilated experimental laboratory for the whole period of study (6 months). The mice were provided with food and water daily and libitum following the ethical considerations of the committee on Animal Care and Use Committee (ACUC) of KEMRI.

3.5 Culture of *Leishmania* Parasites

Metacyclic promastigotes of *L. major* (Strain IDUB/KE/94=NLB-144) were used. The promastigotes assays were done and maintained as previously described by Githinji *et al*., (2010). Parasite aspirates were taken from infected mouse footpad after injecting biphasic NovyMacNeal Nicolle medium (NNN medium) into footpad then cultivated in Schneider's *Drosophila* medium (SIM) supplemented with 25% foetal bovine serum (FBS), penicillin G (100 µg/ml), and streptomycin (100µg/ml) at 35 ^{0}C for 5days. The pH of the culture medium was maintained at 3.5. Stationary-phase metacyclic promastigotes (SPMP) of *L. major* (1x10^{6} /ml) were isolated from 7 day-old cultures washed and purified three times in phosphate buffered solution (PBS) then centrifuged at 1500 rpm for 15 minutes before assays.

3.6 *In vivo* Bioassays

3.6.1 Determination of Lesion Progression and Body Weight of *L. major* Infected BALB/c Mice

The left hind footpads of BALB/c mice were inoculated with 10^{6} cells/ml stationary phase culture of *L. major* promastigotes in 40µl phosphate buffered saline (PBS) intradermally. Lesion development was monitored weekly by measuring the thickness of the infected footpad using a venear calliper. Treatment with the extracts and standard reference drugs started 32 days after date of infection, and groups of mice were treated for four weeks by oral administration of 100mg/kg /day of test compound using oropharyngeal cannula and intra-peritoneal injections in the morning before mice became hyper active. To determine the lesion sizes the difference in thickness between

the infected left hind footpad and the uninfected right hind footpad diameter measured and calculated.

The weights of mice were also measured weekly using an analytical balance. All observations and measurements were recorded in a laboratory note book and later transferred to excel spreadsheet protected by a password.

3.6.2 Quantification of Parasite Load in Spleen of Infected BALB/c Treated with *M. foetida* Extracts and Controls

At the end of each experiment, all mice were sacrificed by ether anaesthesia. At necropsy, the spleens of all treated animals were weighed and impression smears made as described by Chulay & Bryceson, (1983). Briefly, impression smears were fixed in methanol and stained with Giemsa to evaluate visceralization of *L. major* parasites after treatment of mice. The slides were examined under a compound microscope and an oil immersion lens to enumerate the number of amastigotes per 1000 host nuclei. At least, 100 microscopic fields were examined before an imprint was reported negative (Eissa *et al*., 2012).

The relative and total numbers of parasites in the spleen, named Leishman-Donovan Units (LDU) and the total Leishman-Donovan Units (total LDU) respectively were calculated according to the following formulae described by Bradley & Kirkley (1997) and Veerareddy *et al*., (2004).

$$LDU = \frac{Number\ of\ parasites\ (amastigotes)}{1000\ host\ nuclei\ \ x\ 100}$$

$$Total\ LDU = \frac{Number\ of\ parasites \quad x\ weight\ of\ organ\ (grams)\ x\ (2\ x\ 10^5)}{1000\ host\ nuclei}$$

3.6.3 Disposal of Animals

All sacrificed animals were disposed according to the regulations of the Animal Use and Care Committee (ACUC), KEMRI. At sacrificing, all animals were injected with 100µl of pentabarbitone sodium (Salotra & Singh, 2006). Disposal form was filled at least 3 days before disposal date to disposal department where they were incinerated at high temperatures.

3.7 *In Vitro* Bioassays

3.7.1 Anti-promastigote Assay

Anti-promastigote assay was carried out as described by Evance *et al*., 1981. *L. major* promastigotes were cultured in Schneider's Insect Medium (SIM) supplemented with 20% foetal bovine serum, 100µg/ml streptomycin and 100µg/ml penicillin-G and 5-fluorocytosine for 48hrs in culture flasks then transferred to 24-well plates containing Roswell park Memorial Institute Medium (RPMI) at concentration of $1x10^6$ promasigotes per mililiter then subjected to test compounds at various concencentrations ranging from 2mg/ml to 0.0609µg/ml at 25^0C and incubated further for 24hrs after which aliquots of the parasite were transferred to a 96-well micro-titer plate, at density of 10^6 of parasites/ml.

The plates were incubated further at 27°C for 48hours. The negative control used was RPMI medium while positive controls were Pentostam and Amphotericin B. Blank

wells contained 3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyltetrozolium bromide (MTT) and RPMI. Ten microlitres of MTT reagent were added into each well and the plates were futher incubated for 4 hours.The medium together with MTT was aspirated off from the wells, 100µl of DMSO (0.2%) added and the plates shaken for 5 mins.

Absorbance was measured for each well at 540 nm using a micro-titer plate reader and 50% Inhibitory Concentration (IC_{50}) values generated. MTT calorimetric assay was used to measure reduction of MTT dye from tetrazolium into formazan by mitochondrial enzymes in viable promastigotes.

Percentage promastigote viability values were calculated using the following formula described by Jevprasesphant *et al*., (2003).

$$Cell\ viability\ (\%) = \frac{(Average\ absorbance\ of\ test\ samples\ wells - \ average\ blank\ wells)\ \times 100}{Average\ absorbance\ control\ wells}$$

3.7.2 Evaluation of Minimum Inhibitory Concentration (MIC)

The MIC was determined and evaluated as described by Evans *et al*., (1981). Briefly, *Leishmania* promastigotes ($1x10^6$cells/ml) were grown and maintained in culture in the presence of several concentrations (1mg/ml to 1µg/ml) of test compounds. Cell growth was evaluated daily by assessment of visibility and turbidity in order to evaluate MIC. The lowest concentration of the samples that prevented the growth of *Leishmania* parasites *in vitro* was considered as the MIC.

3.7.3 Anti-amastigote Assay

The anti-amastigote assay was carried out as described by Delorenzi *et al.,* (2001). Peritoneal macrophages were obtained from infected BALB/c mice. The body surface of the mouse was disinfected with 70% ethanol, the skin torn dorso-ventrally to expose the peritoneum and a sterile syringe used to inject 10 ml of PBS into the peritoneum.

After 24hrs, mouse peritoneal macrophages were harvested by withdrawing the fluid into sterile centrifuge tubes. Cell suspensions were centrifuged at 2000 rotations per minute (rpm) for 10 minutes and the pellet re-suspended in 5ml of complete RPMI-1640 medium. Macrophages were absorbed in 24-well plates and allowed to adhere for 4 hours at 37^0C in 5% CO_2.

Non-adherent cells were washed off with PBS, and the macrophages incubated overnight in RPMI 1640 media. Adherent macrophages were infected with *L. major* metacyclic promastigotes at a parasite/macrophage ratio of 6:1 and incubated at 37^0C in 5% CO_2 for 4 hrs. There after free promastigotes were removed by extensive washing with PBS and the cultures further incubated in RPMI-1640 medium for 24 hours.

Treatment of infected macrophages with each sample was done once. Pentostam ® and Amphotericin B were used as positive controls for parasite growth inhibition. The medium and drug were replenished daily for 3 days. After 5 days the monolayers were washed with PBS at 37^0C, fixed in methanol and stained with 10% Giemsa. The numbers of amastigotes was determined by counting at least 100 macrophages in duplicate cultures, and results expressed as infection rate (IR) and multiplication index (MI) as described by Evans *et al*., 1981).

% IR = Number of infected macrophages per 100 macrophages

$$MI = \frac{Number\ of\ amastigotes\ in\ experimental\ culture\ per\ 100\ macrophages}{Number\ of\ amastigotes\ in\ control\ culture\ per\ 100\ macrophages}$$

Hence, % MI = % IR of experimental test culture x 100

3.7.4 Nitric Oxide Production Determination

Nitric oxide (NO) released from the supernatants of macrophage cultures was measured by the Griess reaction for nitrites as described by Tsikas, (2007). 100 µl of the supernatants was collected 48 hours after introducing the tested compounds into the culture medium. The assay was done in triplicate wells in a 96-well micro-titer plate. To achieve this, 60ul of Griess reagent A (1% sulphanilamide in 1.2 M HCI) was added, followed by 60ul of Griess reagent B (0.3% N-[1-naphthyl] ethylenediamine). The plates were read at 540 nm in an Enzyme Linked Immunosorbent Assay (ELISA) reader. Sodium nitrite in RPMI was used to construct a standard curve for each plate reading.

3.7.5 Cytotoxicity Assay

Vero cells were cultured and maintained in Minimum Essential Medium (MEM), supplemented with 10% Foetal Bovine Serum (FBS) as described by Mosmann, (1983). The cells were cultured at 37^0C in 5% CO_2 incubator, harvested by trypsinization, pooled in a 50 ml vial and 100 µl cell suspensions ($1x10^6$cell/ml) put into wells of rows

A-H in a 96-well micro-titer plate for each sample of tested extracts and standard reference drugs.

The cells were incubated at 37^0C in 5% CO_2 for 24 h to attach, the medium aspirated off and 150µl of the highest concentration of each of the test samples seriallly diluted. The experimental plates were incubated further at 37^0C for 48 hours. The control experiments used were cells in media with no test compounds. MTT reagent (10µl) was added to each well and the cells incubated for 4 hours. The medium together with MTT was aspirated off from the wells, after which 100µl of DMSO was added and the plates shaken for 5 minutes. The Absorbance was measured for each well at 540 nm using a micro-titer plate reader. Cell viability (C.V) (%) was calculated at each concentration as described by Patel *et al*., (2009) using the formula:

$$CV\ (\%) = \frac{Average\ absorbance\ in\ duplicate\ drug\ wells - \ Average\ blank\ wells)\ x100}{Average\ absorbance\ control\ wells}$$

3.8 Data Management and Statistical Analyses

All experiments were done in triplicates in all *in vitro* assays and in in vitro. Data collected were recorded in a hard cover note book and later tranfered to Ms. Excel spread sheets protected by password. Data was analyzed using Stat graphic software. Lesion sizes and body weights were compared between groups using multiple regressions analysis. Student t- test and ANOVA were used respectively to estimate significant difference within and between the samples. Data considered to be significant at $P \leq 0.05$.

CHAPTER FOUR

RESULTS

4.1 Effects of Intraperitoneally Administered *M. foetida* Extracts on Lesions Development in *L. major* Infected BALB/c Mice

The results of lesions size progressions in intraperitoneally administered *M. foetida* extracts are shown in Table 4.1. Resuts showed that methanolic extracts of *M. foetida* and Pentostam reduced lesions size from 0.4mm to 0.02mm (92.5%). *M. foetida* aqueous extracts and Amphotericin B reduced 97.5% and 98.75% of the lesions size respectively.

M. foetida aqueous extract showed higher antileishmanial activity than methanolic extract in reduction of parasites load and lesion healings. Amphotericin B treatment reduced lesion to mean of 0.005 ± 0.1mm), resulted in the smallest lesion size followed by aqueous extracts (0.01 ± 0.1mm) then Pentostam (0.02 ± 0.3mm), and Methanolic (0.02 ± 0.2mm) extracts respectively. Untreated mice subjected to intraperitoneal PBS led to prominent enlargement of lesion size (5.8 ± 0.5) with high proliferation of parasites.

The results for lesions size progression in IP experiments significantly ($P < 0.05$) increased uniformly in all mice during pre-treatment period. After treatment, *M. foetida* extracts and standard reference drugs significantly ($P < 0.05$) showed reduction in lesions size progression compared to PBS.

Table 4.1: Means of Lesion Size (mm) Progression in *L. major* Infected BALB/c Mice Treated Intraperitoneally with *M. foetida* Extracts, Pentostam, Amphotericin B and PBS.

Trts	Met exts	H2O exts	Pento	AmB	PBS
Wk 1	0.4 ± 0.3	0.4 ± 0.2	0.4 ± 0.1	0.4 ± 0.2	0.4 ± 0.2
Wk 2	0.68 ± 0.3	0.67 ± 0.2	0.67 ± 0.3	0.69 ± 0.2	0.67 ± 0.1
Wk 3	1.38 ± 0.5	1.37 ± 0.4	1.39 ± 0.4	1.37 ± 0.4	1.39 ± 0.3
Wk 4	1.74 ± 0.5	1.73 ± 0.3	1.75 ± 0.5	1.74 ± 0.5	1.71 ± 0.3
Wk 5	1.24 ± 0.7	1.22 ± 0.4	1.27 ± 0.5	1.21 ± 0.5	2.43 ± 0.5
Wk 6	0.84 ± 0.5	0.72 ± 0.4	0.81 ± 0.4	0.72 ± 0.4	3.00 ± 0.5
Wk 7	0.163 ± 0.4	0.158 ± 0.5	0.164 ± 0.3	0.154 ± 0.3	3.50 ± 0.3
Wk 8	0.039 ± 0.3	0.025 ± 0.3	0.038 ± 0.1	0.010 ± 0.2	4.04 ± 0.5
Wk 9	0.020 ± 0.2	0.010 ± 0.1	0.020 ± 0.3	0.005 ± 0.1	5.80 ± 0.5
%LR	95%	97.5%	95%	98.75%	0
P value	0.0225	0.0111	0.0435	0.0271	0.1962

Legend: **LR** = Lesions reduction, **Trts** = Treatments, **Met exts** =Methanol extracts, **H_2O exts** = Aqueous extracts, **Pento** = Pentostam, **AmB** = Amphotericin B, **PBS** = Phosphate buffered saline, **P value** = Probability values and **Wk**= Week

4.2 Effect of Orally Administered *M. foetida* Extracts on Lesions Development in *L. major* Infected BALB/c mice

The results of lesion size (mm) progression in orally administered *M. foetida* extracts are shown in Table 4.2. There were no difference in lesion reduction between *M. foetida* extracts and standard drugs compared to PBS. Methanolic extracts and Pentostam demonstrated equal effectiveness on *L. major* parasites by reducing lesion size from 0.4mm to 0.03 ± 0.07mm and 0.03 ± 0.03mm respectively. The results showed that

aqueous extracts (0.01± 0.01mm) and Amphotericin B (0.005 ± 0.01mm) are more effective than the methanolic extract and Pentostam in reducing *L. major* parasites proliferations preventing lesion progressions. PBS showed no reduction effects on *Leishmania* parasites but incresed to the largest lesion size (5.6 ± 2.0mm).

Table 4.2: Means of Lesion Size (mm) Progression in *L. major* Infected BALB/c Mice Treated Orally with *M. foetida* Extract, Pentostam, Amphoterin B and PBS

Trts	**Met- exts**	**H_2O exts**	**Pento**	**AmB**	**PBS**
Wk 1	0.40 ± 0.05	0.40 ± 0.10	0.40 ±0.02	0.40 ± 0.10	0.40 ± 0.30
Wk 2	0.67 ± 0.03	0.65 ± 0.30	0.65 ±0.04	0.60 ± 0.30	0.64 ± 0.52
Wk 3	1.27 ± 0.06	1.26 ± 0.40	1.27 ± 0.02	1.28 ± 0.70	1.24 ± 0.50
Wk 4	1.71 ± 0.06	1.73 ± 0.50	1.71 ± 0.06	1.72 ± 0.50	1.70 ± 0.90
Wk 5	1.42 ± 0.08	1.40 ± 0.10	1.41 ± 0.04	1.40 ± 0.50	1.98 ± 0.83
Wk 6	0.55 ± 0.04	0.50 ± 0.10	0.6 ± 0.05	0.40 ± 0.50	2.17 ± 1.11
Wk 7	0.30 ± 0.05	0.25 ± 0.05	0.30 ± 0.05	0.20 ± 0.20	3.31 ± 1.0
Wk 8	0.12 ± 0.05	0.10 ± 0.05	0.15 ± 0.05	0.07 ±0 .030	4.04 ± 1.54
Wk 9	0.03 ± 0.07	0.01 ± 0.01	0.03 ±0.03	0.005 ± 0.01	5.60 ± 2.0
%LR	92.5%	97.5%	92.5%	98.75%	0
DF	7	7	7	7	7
P value	0.0299	0.0144	0.0422	0.0410	0.1202

Legend: **LR** = Lesions reduction, **Trts** = Treatments, **Met exts** =Methanol extracts, **H_2O exts** = Aqueous extracts, **Pento** = Pentostam, **AmB** = Amphotericin B, **DF** = Degree of freedom **RPMI** = Roswell Park Memorial Institute Medium, **PBS** = Phosphate buffered saline and **Wk**- Week

4.3 Effects of Intraperitoneally Administered Extracts of *M. foetida* on Body Weight of *L. major* Infected BALB/c Mice

The results of IP treatments are shown in Figure 4.1. Mice treated with Amphotericin B displayed the highest increase in weight from 13 ± 2g to 20 ± 2.5g, followed by aqueous extract, Pentostam and methanol extract with 18.5g ± 3g, 18 ± 2g and 16 ±2.5g respectively. PBS treatment showed the lowest mean weight increment from 12.5g to 13.5 ± 3.5g at the end of treatments. The results also showed that *M. foetida* aqueous extracts (P= 0.0211) significantly increased weight of mice compared to methanolic extracts (P= 0.03477), Pentostam (P=0.0459) and PBS (P= 1.999). While Amphotericin B showed the highest significance at P= 0.019778).

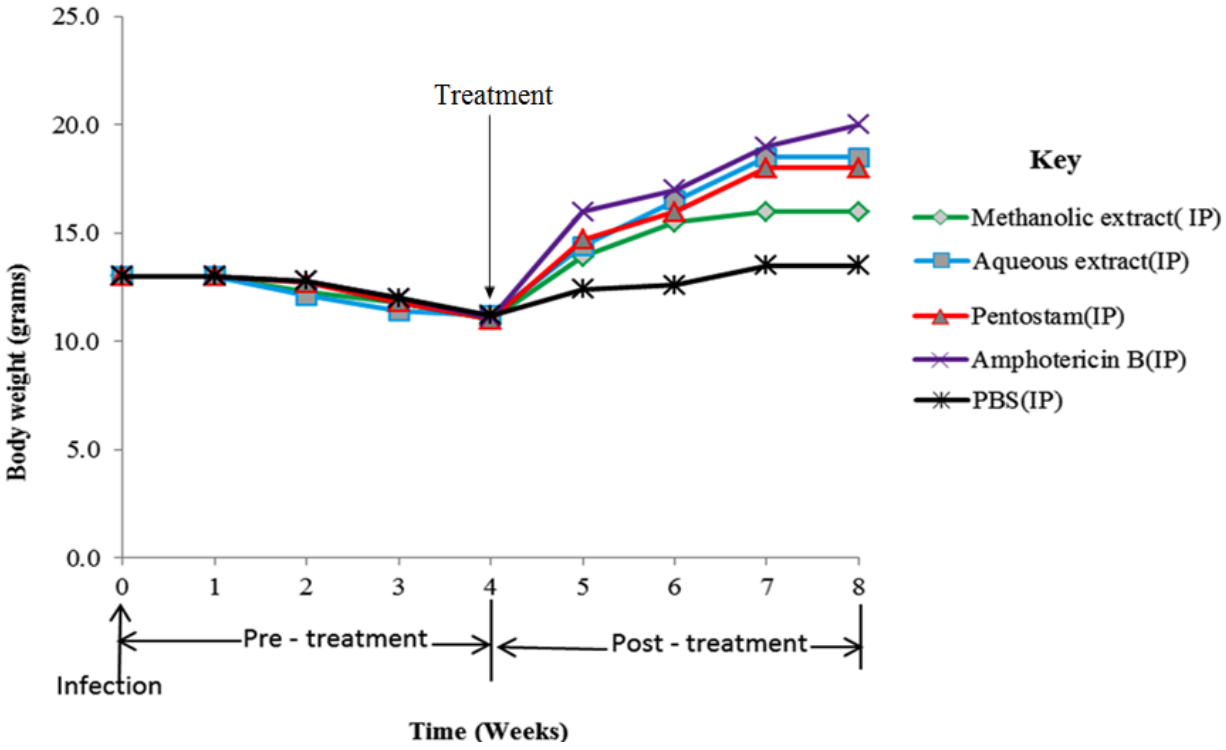

Figure 4.1: Effects of Interperitoneally Administered *M. foetida* Extracts and Controls on Body Weight of *L. major* Infected BALB/c Mice

4.4 Effects of Orally Administered *M. foetida* Extracts on Body Weight of *L. major* Infected BALB/c Mice

The trend in Figure 4.2 showed increase in all test compounds. The results also showed that aqueous extracts significantly increased the body weight of mice than Pentostam and methanolic extracts. Amphotericin B showed the highest weight increment from 13±3.5g to 21.5±1.5 while Pentostam, aqueous, methanolic and PBS increased weights from 13±5g to 20±2g, 21±3.5g, 19.5±3g and 14±5g respectively. There was no significant difference (P = 0.111) in body weight increase in all mice treated with *M. foetida* extracts and standard reference drugs. PBS showed minimum increase of weight from 10±3g in 4th week to 13±5g in the 8th week demonstrating significant difference (P = 2.567) with other test compounds.

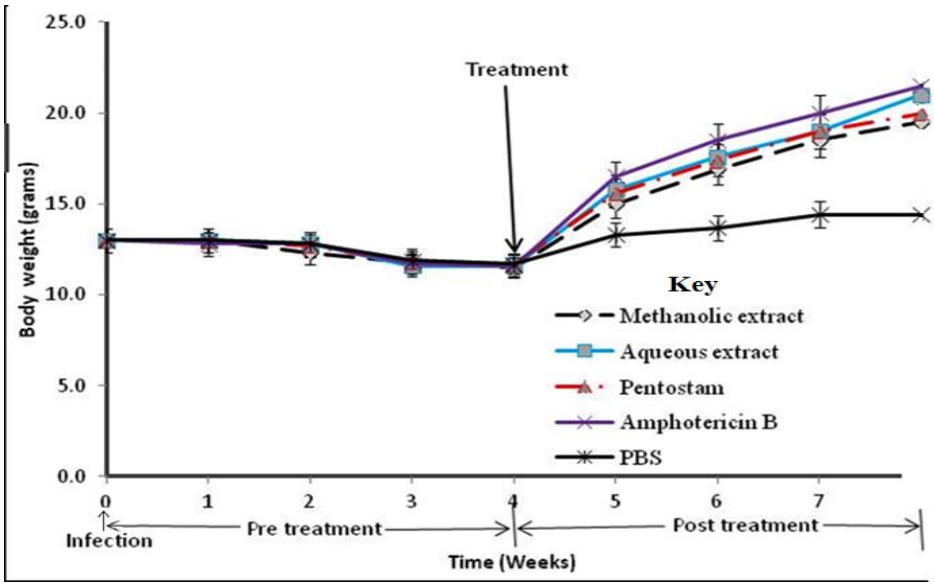

Figure 4.2: Effects of Orally Administered *M. foetida* Extracts, Pentostam, Amphotericin B and PBS on Body Weights of Infected BALB/c Mice.

4.5 Effect of *M. foetida* Extracts on Parasite Load in the Spleen of *L. major* Infected BALB/c Mice

The results of effects of *M. foetida* extracts on parasites load compared to controls treatments are shown in Table 4.3. Both extracts of *M. foetida* and standard drugs reduced the parasites load in the spleen of BALB/c mice compared to PBS.

The observation showed that aqueous extract subjected orally and intraperitoneally (P = 0.000) significantly reduced macrophages *L. major* parasites infections (LDU) from 100% (10^3 cells) to 21.5% (215 cells) and 26% (260cells) with total LDU values of 7 and 6 respectivelly while orally (P= 0.010) and intraperitoneally (P = 0.019) subjected methanolic extracts significantly reduced macrophages infections (LDU) to 31.2% (312 cells) and 32.9% (329 cells) to give total LDU value of 7.

Amphotericin B (P = 0.001) and Pentostam (P = 0.010) treatments resulted to no different significance with *M. foetida* (P = 0.000) extracts by demonstrating the smallest percentage of macrophages infections (LDU) of 10.2 % (102 cells) and 14.3% (143 cells) with total LDU values of 3 and 4 respectivelly. PBS showed the highest percentage of macrophages infections (LDU) of 80% (800 cells) and total LDU value of 9.

Table 4.3: Parasite Load in the Spleen of Infected BALB/c Mice Treated with *M. foetida* Extracts and Controls

Treaments	df	LDU (%)	T. LDU	Spln.wt(g)	P value
Methanolic-oral	3	31.2 ± 0.86	8 ± 0.86^{c}	0.18c ± 0.006	0.010
Methanolic-IP	3	32.9 ± 0.99	8 ± 0.86^{c}	0.19c ± 0.009	0.019
Aqueous-oral	3	21.5 ± 0.155	7 ± 0.56^{b}	0.14b ± 0.004	0.000
Aqueous -IP	3	26.8 ± 0.149	6 ± 0.6^{b}	0.15b ± 0.005	0.000
Pentostam IP	3	14.3 ± 1.0	4 ± 0.9^{a}	0.13a ± 0.010	0.010
Amphotericin B IP	3	10.2 ± 0.1	3 ± 0.4^{a}	0.12a ± 0.012	0.001
PBS- oral	3	78.4 ± 0.99	9 ± 1^{d}	0.21d ± 0.026	0.090
PBS -IP	3	80.9 ±1.0	10 ± 0.5^{d}	0.22d ± 0.028	0.095
Total	24				

Legend: The letters **a, b, c** and **d**= homogeneous groups, **df** =Degree of freedom, **LDU**= Leishman Donovan unit, **T. LDU**= Total Leishman Donovan unit, **Spln.wt** = Spleen weight, **P values** = Probability values, **PBS** = Phosphate buffer saline.

4.6 Effect of *M. foetida* Extracts on Promastigotes and Minimum Inhibition Concentration

The antipromastigotes effects of *M. foetida* are shown in Table 4.4 and Figure 4.3**.** The results indicated that *M. foetida* methanolic extracts (70%), aqueous extracts (76), Pentostam (76%) and Amphotericin B (80%) significantly ($P < 0.05$) inhibited promastigotes of *Leishmania* parasite *in vitro* culture after 72 hours of incubation compared to RPMI which reduced by 10%. Methanolic extracts exhibited the highest inhibition concentration value (IC_{50}) of 23.5µg/ml followed by aqueous extracts,

Pentostam and Amphotericin B with 15.6µg/m,11.7µg/ml and 7.8µg respectively by inhibiting 50% of 10^6 promastigotes.

In addition, the study showed that *M. foetida* methanol extracts also had the highest (MIC) value of 250 ± 0.003µg/ml compared to other tested coumpounds. The MIC values for aqueous extracts, Pentostam and Amphotericin B were 125 ± 0.001µg/ml, 62.5 ± 0.002ug/ml 31.3 ± 0.001µg/ml and respectively. The inhibition effects of *M. foetida* extracts and standard drugs increased with increase in concentrations compared to RPMI. Smaller the IC_{50} and MIC values, the higher antileishmanial activity of the test compounds.

Table 4.4: Mean IC_{50}, MIC and % Inhibition of Parasites by *M. foetida* Extracts

Trts	**RPMI**	**H_2O exts**	**Met- exts**	**Pentostam**	**AmB**
IC_{50} (µg/ml)	0 ± 0.01	15.6 ± 0.054	23.4 ± 0.053	11.7 ±0.054	7.8 ± 0.053
MIC (µg/ml)	0 ± 0.09	125 ± 0.001	250 ± 0.03	62.5 ± 0.002	31.3 ± 0.001
PI (%)	10%	76 %	70 %	76%	88%
VP%	90%	24%	30%	24%	12%
SE	0.15	0.054	0.073	0.095	0.099
T statistic	106.82	-11.88	-16.21	-9.22	-2.24
P value	1.000	0.000	0.000	0.014	0.001

Legend: **SD** = Standard deviation, **SE** = Standard Error, **IP** =Parasites inhibition, **VP** = Viable parasites, **AmB** = Amphotericin B, **Trts** = Treatments, **Met exts** =Methanol extracts, **H_2O exts** = Aqueous extracts, **Pento** = Pentostam, **RPMI** = Roswell Park Memorial Institute Medium and **PBS** = Phosphate buffered saline

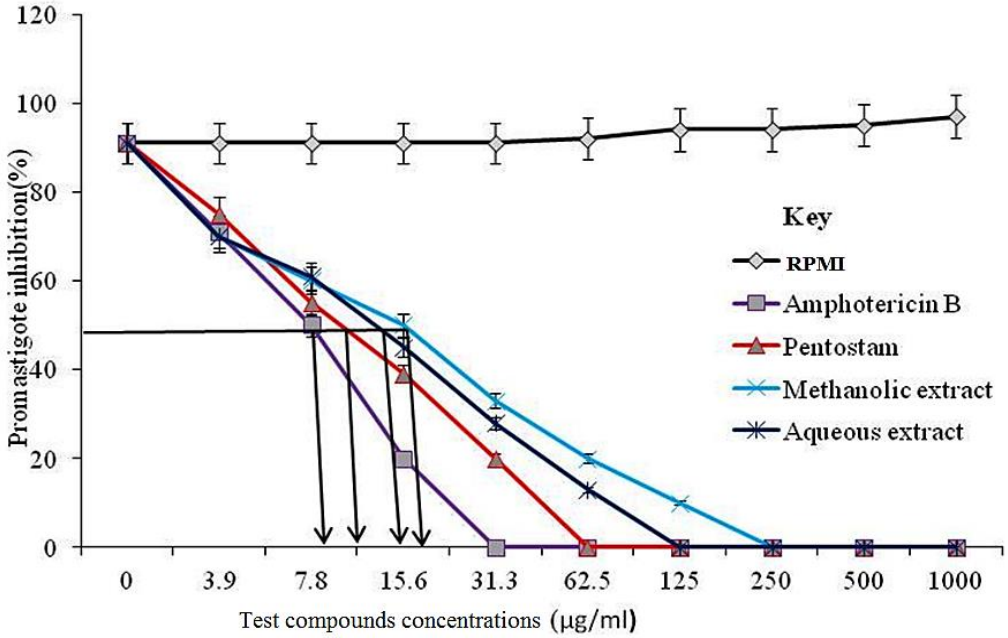

Figure 4.3: Trend Showing Percentage Promastigote Inhibition by *M. foetida* Extracts and Control Treatments

4.7 Effects of *M. foetida* Extract Treatments on *L. major* Infected Macrophages from BALB/c Mice.

The results presented in figure 4.4 shows that *M. foetida* extracts and the standard reference drugs significantly demonstrated antileishmanial activity by inhibiting amastigotes of *L. major* parasites in infected macrophages compared to RPMI.

Methanolic extract showed slightly higher macrophages infection rates by eliminating 30% parasites at 200µg/ml, 45% at 100µg/ml and 60% at 200µg/ml than aqueous extract which eliminated 35% at 50µg/ml, 45% at 100µg/ml and 58% at 200µg/ml. Pentostam protected 64% macrophages from *L. major* infection at 50µg/ml, 75% at 100µg/ml and 82 at 200µg/ml while Amphotericin B exhibited higher antileishmanial

activity at all prepared concentrations by inhibiting >85% of *L. major* parasites protecting 80% macrophages.

The slopes of the trend dropped with increase of concentrations of *M. foetida* extracts and standard reference drugs from 50µg/ml to 100µg to 200µg/ml reducing number of infected macrophages and parasites.

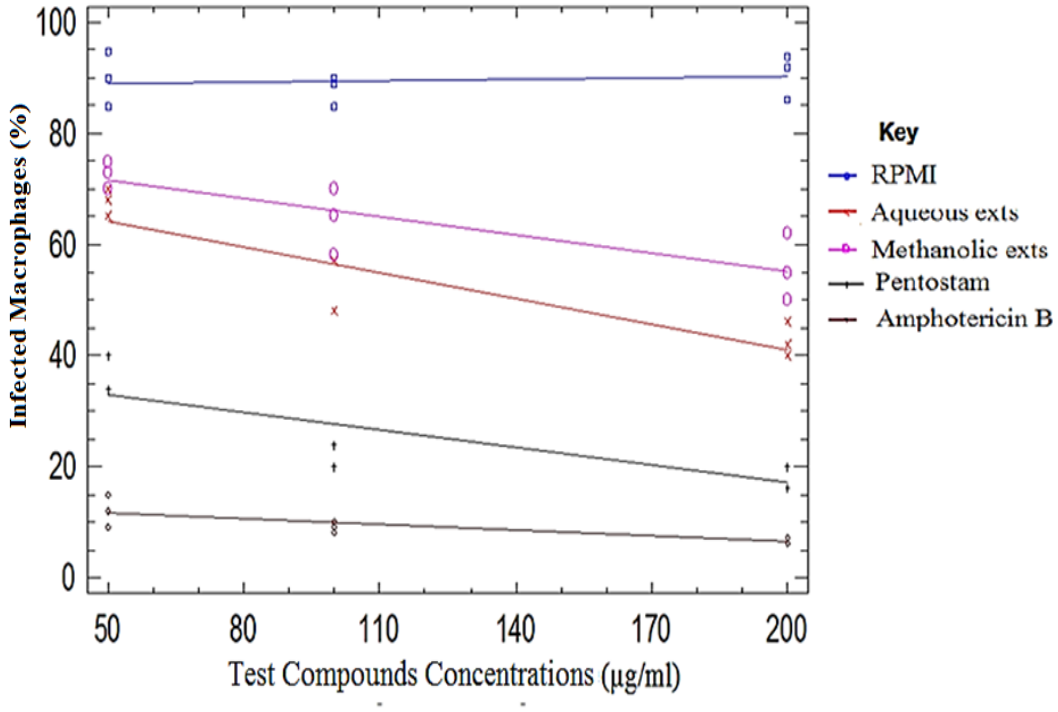

Figure 4.4: Macrophages Infected by *L. major* Amastigotes after Treatment with *M. foetida* Extracts compared with Controls at Concentration of 50µg/ml, 100µg/ml and 200µg/ml

4.8 Effect of *M. foetida* Extract Treatments and Controls on *L. major* Amastigotes Infection on Macrophages Extracted from BALB/c Mice

The results of amasigotes viability are shown in Figure 4.5. It showed that *M. foetida* extracts and standard drugs inhibited *L. major* amastigotes reducing macrophages infection compared to RPMI that showed the highest value of infection of LN 6.6 after converting the number of parasites to percentages then to natural logaritims and was the same for the concentration of 50µg/ml, 100µg/ml and 200µg/ml followed by methanolic extracts, LN of 6.5 at 50ug/ml, 6.2 at 100ug/ml and 6.0 at 200ug/ml and aqueous extracts with LN 5.8 at 50ug/ml, 5.4 at 100ug/ml and 50ug/ml.

Pentostam reduced to LN 4.6 at 50ug/ml, 4.4 at 100ug/ml and 4.2ug/ml while Amphotericin B reduced the highest number of parasites to LN 3.5 at 50ug/ml, 3.2 at 100ug/ml and 3.0ug/ml. The results also showed that increase in *M. foetida* extracts and standard reference drugs concentrations increased inhibition of amastigotes growth therefore reducing infections.

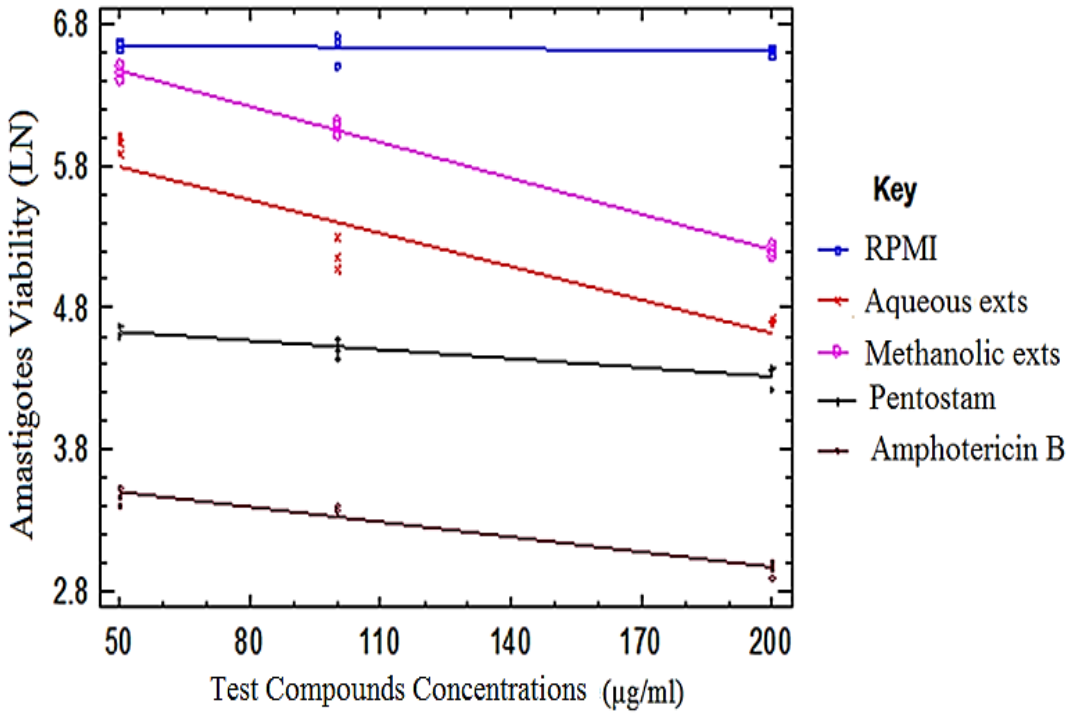

Figure 4.5: Effect of *M. foetida* Extracts, Pentostam and Amphotericin B on *L. major* Amastigotes Viability

4.9 Infection Rates (IR) and Multiplication Index (MI)

Results of infection rates (IRs) and multiplication indices (MIs) of parasites in macrophage infections under different test compound treatment are shown in Table 4.5. The results showed that increase in *M. foetida* extracts and standard reference drugs concentrations reduced macrophage infection by amastigotes compared to steady parasite load in the negative control (RPMI).

Methanol extracts reduced the parasites load reducing infection rate (IR) from 100 to 61%, 26% and 18% while aqueous extracts reduced from 100 to 36%, 16% and 11% at

concentrations of 50 µg/ml, 100µg/ml and 200µg/ml respectively. Pentostam and Amphotericin B in the same concentrations reduced from 17% to 50%, 9% and 7% and 100% to 11%, 7% and 3% respectively compared with RPMI whose IR of parasites load was high (>90%) with MI of 1.

The extracts of *M. foetida* and standard drugs showed reduction of amastigotes infections to macrophages compared to RPMI. The multiplication indices (MIs) of Methanolic extracts and aqueous extracts were 0.65 (50µg/ml), 0.28 (100µg/ml), 0.18 (200µg/ml) and 0.38 (50µg/ml), 0.17 (100µg/ml) and 1.11 (200µg/ml) respectively compared to Pentostam and Amphotericin B with MI of 0.18 (50µg/ml), 0.1 (100µg/ml), 0.07(200µg/ml) and 0.11 (50µg/ml), 0.07 (100µg/ml), 0.03 (200µg/ml) respectively. RPMI which showed the highest number of amastigotes in infected macrophages.

Table 4.5: Infection Rates and Multiplication Indices of *L. major* Amastigotes in Infected Macrophages after Treatment with *M. foetida* Extracts and Controls Compounds

Trts/Conc	Infected MQs	No. of amasigotes	%IR	MI	P value
Met exts					
50µg/ml	61 ± 8	249 ± 7	61%	0.65	0.047
100µg/ml	26 ± 10	127 ± 8	26%	0.28	0.033
200µg/ml	18 ± 5	60 ± 8	18%	0.19	0.000
H_2O exts					
50µg/ml	36 ± 5	144 ± 10	36%	0.38	0.011
100µg/ml	16 ± 9	80 ± 9	16%	0.17	0.000
200µg/ml	11 ± 9	30 ± 4	11%	0.11	0.000
Pento					
50µg/ml	17 ± 5	72 ± 10	17%	0.18	0.006
100µg/ml	9 ± 3	37 ± 8	9%	0.10	0.013
200µg/ml	7 ± 8	28 ± 8	7%	0.07	0.000
AmB					
50µg/ml	11 ± 5	46 ± 4	11%	0.12	0.000
100µg/ml	7 ± 5	30 ± 5	7%	0.07	0.000
200µg/ml	3 ± 5	17 ± 5	3%	0. 03	0.000
RPMI					
50µg/ml	94 ± 10	1400 ± 86	94%	1	1.912
100µg/ml	94 ± 8	1566 ± 58	94%	1	1.766
200µg/ml	96 ± 15	1847 ±11 9	96%	1	1.999

Legend: **Trts** = Treatments, **Conc** = Concentrations, **IR** = Infection rate, **MI** = Multiplication index, **MQs** = Macrophages, **AmB** =Amphotericin B, **Pento**= Pentostam, **Met exts** = Methanolic extracts, **H_2O exts** = Aqueous extracts, **PBS** = Phosphate buffer saline, **RPMI** = Roswell Park Memorial Institute Medium

4.10 Effect of *M. foetida* Extracts and Control Treatment on Nitric Oxide Production on *L. major* Infected Macrophages Extracted from BALB/c Mice

Results of nitric oxide production by infected macrophages treated with *M. foetida* extracts and controls are shown in Figure 4.6. Production of Nitric oxide (NO) by different test compounds was shown to be dependent on concentrations with RPMI showing increase of NO production from 75% at 50µg/ml to 90% at 200 µg/ml. Methanolic and water extracts showed significant ($P < 0.05$) amount of NO production from 56% at 50µg/ml to 68% at 200µg/ml and 65% at 50µg/ml to 64% at 200µg/ml respectively.

Amphotericin B and Pentostam had nitric oxide production in macrophages infected with *L. major* ranging from 73% at 50µg/ml to 54% at 200µg/ml and 70% at 50µg/ml to and 57% at 200µg/ml respectively. All the test compounds showed no significant production of NO when compared with that of standard nitric oxide production which was 100.

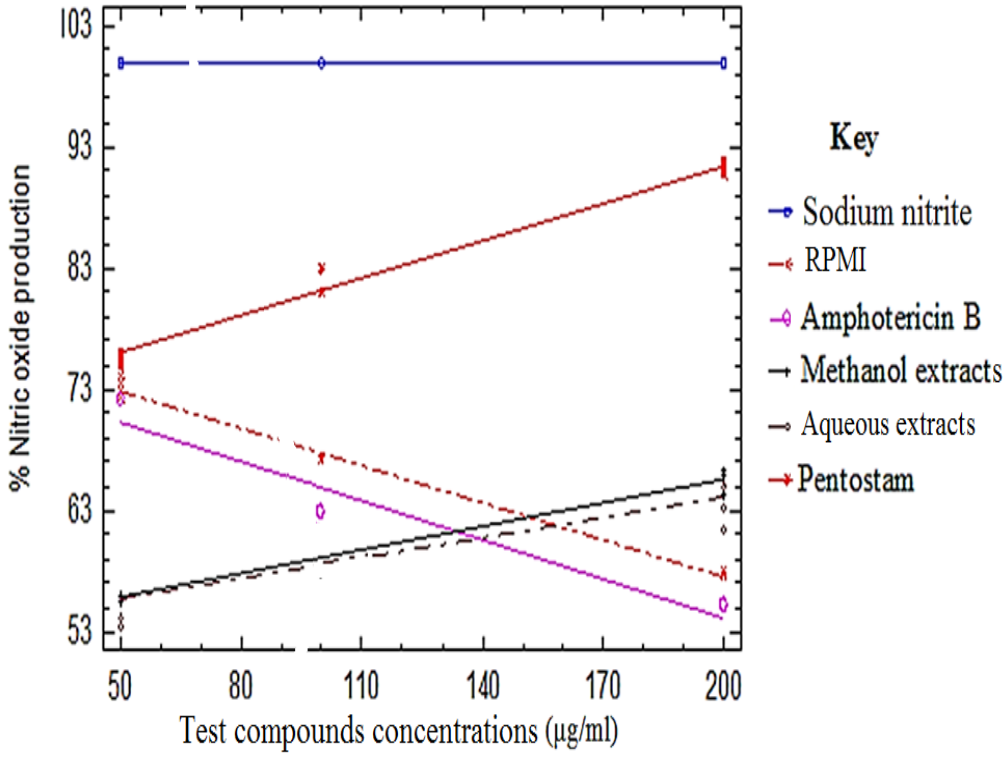

Figure 4.6: Nitric oxide production by *L. major* infected macrophages after treatment with *M. foetida* extracts and controls

4.11 Cytotoxicity of *M. foetda* Extracts

The cytotoxicity of *M. foetida* extracts compared to controls are shown in Table 4.6. Cytotoxicity of methanolic and aqueous extracts of *M. foetida* against vero cells were compared with those of Pentostam and Amphotericin B and the resuls showed that *M. foetida* extracts are toxic based on scale values of classification of toxicity levels; cytotoxic at CC_{50} < 2µg/ml, moderate at CC_{50} between 2µg/ml-99µg/ml and not cytotoxic at CC_{50} > 100µ/ml (Loomis & Hayes, 1996). Methanolic and aqueous extract had CC_{50} of 104µg/ml and 122.5µg/ml respectively indicating no toxic while Pentostam

(CC_{50} of 76.2ug/ml) and Amphotericin B (IC_{50} of 88.6ug/ml) showed moderate cytotoxicity.

The IC_{50} values and percentage parasites inhibition by methanol extracts, aqueous extract, Pentostam and Amphotericin B were 0.22ug/ml (72 ± 0.08%), 0.26ug/ml (80 ± 0.02%), 0.46ug/ml (80 ± 0.65%) and 0.68ug/ml (88 ± 0.95%) respectively demonstrating inhibition of > 70% of *L. major* parasites.

Despite the difference in IC_{50} values of aqueous extracts (80 ± 0.02%) and Pentostam (80 ± 0.65%), both inhibited the same percentage of parasites. Methanolic extracts (72 ± 0.08%) showed the lowest inhibition of parasites while Amphotericin B (88 ± 0.95%) showed the highest inhibition.

Table 4.6: Table for Cytotoxicity Test and% Inhibition of Parasites by Test Compounds

Treatments	CC_{50}µg/ml	%PI	IC_{50}	a/ b	P value
RPMI	0.000	0.00	-	4.51 ± 0.05	0.000
Methanol extracts	104 ± 0.1	72 0 ± 08	0.220.0 ± 5	-0.87 ± 0.4	0.010
Aqueous extracts	122.5 0 ± 07	80. 0 ± 02	0.26 0 ±.01	-0.64 ± 0.5	0.001
Pentostam	76.2 0 ±.12	80.6 ± 5	0.46 0 ±14	-0.49 ± 1	0.074
Amphotercin B	88.6 0 ± 19	88 0 ± 95	0.68 0 ±11	-0.28 ± 0.9	0.062

Legend: **CC_{50}** = Cytotoxic concentration (CC < 2ug/ml = toxic, CC > 2ug/ml and < 99ug/ml = Moderate toxic and CC > 99ug/ml = non toxic), **PI** = Parasites inhibition, **IC_{50}** = Inhibition concentration, **a/b** = Slope/interval and **P value** = Probability value.

CHAPTER FIVE

DISCUSSION

The currently available drugs for leissmaniasis are of high costs and are associated with many adverse effects. Therefore new strategies are required for treatments of leishmaniasis that will be available, cheap and non toxic to human. Plants have been used by all communities in Kenya as source of medicine (Farnworth, 1982). The biologically active natural products developed from the plants are being used as commercial drugs (Mourice *et al*., 1999). Various experimental studies have shown that several plants in different genera containts compounds that have antileishmanial activities (Kokwaro, 1993) and can be used as alternative therapies, even though their effectiveness differs per group of compounds (Rates, 2000). The advantages of using of using plant based products have been shown to be as a result of lack of easy development of resistance by parasites (Croft *et al*., 2006).

The study showed that *M. foetida* methanolic and aqueous extracts has both *in vivo* and *in vitro* antileishmanial activities. Observed reduction of lesion size and healing of ulcerated *L. major* infected foot, increase in mice weight, inhibition of amastigotes in macrophages in spleen and cultures as well as promastigotes inhibition following intraperitoneal and oral administration of *M. foetida* extracts in treatment of infected BALB/c mice could be due to presence of active ingredients such as flavonoid, momordine, foetin & Kaemferol as repoted by Froelich *at al.,* (2007) that hence the killing of *L. major* parasites reducing parasite load.

Aqueous extracts of *M. foetida* significantly reduced disease progression in the BALB/c mice in comparison to methanol and pentostam with one fold strength in comparison to Amphotericin B by reducing mean lesion sizes is an indication that the therapeutic effect of *M. foetida* can also be considered when targeting cutaneous leishmaniasis. These results are in agreement with an earlier study which demonstrated the plant presence of active compounds which could be contributing to the medicinal property of the plant. This observation made from the study may be partly due to different modes of action of *M. foetida* which may effectively reduce parasite resistance. Extracts of *M. foetida* compound being effective in reducing parasite burden in BALB/c mice is consistent with the *in vitro* activity of *M. foetida* on amastigotes with general agreement that they were still more effective against amastigotes.

Aqueous extracts showed better antileishmanial than methanolic, this could be attributed to the non-toxicity of water. Oral administration which showed better results could be as a result of direct absorption, enzymatic action and the PH changes through the alimentary canal hence supporting oral administration of *M. foetida* as antileishmanial by traditional practitioners among Kalenjin community and its ethinobotanical uses (Olover - Bever, 1986).

The increase in body weight in the orally and intraperitoneally treated mice could also mean that *M. foetida* has nutritional values, since it was intermediate between Pentostam and Amphotericin B. Nutrional values of *M. foetida* have long been documented and the leaves are reported to be eaten after boiling as a vegetable in Gabon, Sudan, Uganda, Tanzania and Malawi (Nesamvuni *et al*., 2001; Frézard *et al*., 2009 Strazzulla *et al*., 2013). The pulb of ripe fruits is eated by people in Africa and the

leaves are also used as fodder in Kenya and Tanzania and are reported to be suitable for fattening rabbits (Fleuret, 1979, Ruffo *et al*., 2002; Nesamvuni *et al*., 2001). The increase of weight was also observed in mice subjected to PBS and it could be due to hepato-splenomecally due to visceralization of *L. major* parasites in liver and spleen of mice.

The antileishmanial activities of *M. foetida* extracts as demonstrated in both *in vivo* and *in vitro* treatments could be due to its presence of active ingredients present like flavonoid (prunin), and Kaemferol (populnin) (Froelich *at al* 2007) with ability to kill *L. major* parasites in infected BALB/c mice. The progression of the lesion in leishmaniasis could also be associated with secondary microbial infections.

Besides, it has been reported that *M. foetida* bears antimicrobial (Kareru *et al*., 2011) and wound healing activities (Hamill *et al*. 2000).

This could explain why *M. foetida* extracts significantly ($P < 0.05$) decreased the diameter of lesions as for standard drugs in comparison with PBS control groups. For intraperitoneally treated mice, *L. major* inhibition could be enhanced by the direct action of the *M. foetida* on extracellular forms of the parasites present at the site of infection (Sontos *et al*. 2008). Therefore, *in vivo* antileishmanial effect of *M. foetida* may be due to a combination of its immunostimulatory, antimicrobial, and anti-inflammatory activities, as well as its antileishmanial effects on promastigote and amastigote forms of *L. major* (Hamill *et al*. 2000; Wiart, 2007).

Flavonoids are known to inhibit cell enzyme activities (Molehin, *et al*., 2014). *M. foetida* has been reported to contain triterpenoids such as foetins, cucurbitacins,

kuguacins and momordicine (Mulholland *et al*., 1997), polyphenolic compounds and antioxidants (Molehin *et al.*, 2014) which are known for disrupting the cell membrane of *L. major* accounting for its inhibitory activity (Wong *et al*., 2014).

The leishmanial inhibitory activity of *M. foetida* could also be attributed due to presence of triterpenoids together with three known analogues, 3beta, 7beta, 25-trihydroxycucurbita - 5,(23E) – diene - 19 - al, 3beta, 25 - dihydroxy-5beta,19-epoxycucurbita - 6,(23E)-diene, and momordicine (Mulholland *et al*., 1997) polyphenolic compounds, flavonoids and antioxidants (Molehin *et al.*, 2014). These compounds have shown to have broad antiparasitic properties including antileishmanial activities by interrupting the cell membaranes of the *L. major* (Wong *et al*., 2014). *M. foetida* has been observed to play a role in the control of some protozoan's parasites (Waako, 2005) which could be the possibility of its efficacy in inhibiting *L. major*.

Since the plant grows in the semi-arid areas that are also endemic for the leishmaniases within the country, and sand flies are known to feed on plants for carbohydrates for energy, the antpromastigote activity of *M. foetida* could make it possible that if the the plant is grown near the known and suspected sand fly breeding and resting sites, it can help minimize parasite development in the vector and hence reduce transmission of the *Leishmania* parasite. Leishmaniases are rural diseases, it is possible that the population at risk where standard drugs are not available, *M. foetida* demonstrated antileishmanial activity can solve the problem hence, incidence and the prevalence of the leishmaniases can be reduced.

Lack of significant increase of nitric oxide production by extracts compared to the standard drugs suggests that *M. foetida* could be using different pathways in killing the parasites as is the case reported by Roy *et al*., (2012) and Iwu, (1994) who reported that catechins-flavonoids act by inhibiting the actions of deoxyribonucleic acid (DNA) polymerase and supported Acquaviva *et al*., (2013) who reported *M. foetida* as antioxidant. Considering that there was no toxicity of the extracts on Vero cells that was recorded, this is also an indication that *M. foetida* is a safe plant for use in the chemotherapy of *L. major.*

There was no apparent toxicity or mortality observed throughout the *in vivo* experiments with *M. foetida* extracts treatments. There were no signs of toxicity observed in *L. major* infected and treated BALB/c mice such as hypo-active, pilo erection, low appetite, crowding together in the cage and the weight reduction as observed.

Methanolic (CC_{50} of 104µg/ml) and aqueous (122.5µg/ml) extracts of *M. foetida* were not toxic compared with standard drugs based on scale values of classification of toxicity levels as; cytotoxic at $CC_{50} \leq$ 2µg/ml, moderate at CC_{50} between > 2µg/ml and ≤99µg/ml and not cytotoxic at $CC_{50} \geq$ 100µ/ml (Loomis & Hayes, 1996). Pentostam (CC_{50} of 76.2ug/ml) and Amphotericin B (IC_{50} of 88.6ug/ml) showed moderate cytotoxicity. Lack of toxicity of *M. foetida* on Vero cells is an indicator that it is safe to be use in the chemotherapy of leishmaniases.

CHAPTER SIX

CONCLUSIONS AND RECOMMENDATIONS

6.1 Conclusion

Methanolic and aqueous extracts of *M. foetida* showed both *in vivo* and *in vitro* antileishmanial potency by inhibiting *L. major* promastigotes and amastigotes stages of the parasites. The results also showed that the *M. foetida* extracts were effective in suppressing development of lesions caused by *L. major* parasites and increased in body weight of experimented mice.

Both *M. foetida* aqueous and methanolic extracts did not stimulate the macrophages to produce sufficient amount of nitric oxide, hence the extracts could be acting directly on the parasite rather than stimulating nitric oxide production to kill the parasites.

Extracts showed no toxicity against BALB/c mice and Vero cells as compared to the standard drugs. Effectiveness of water extracts administered orally and lack of toxicity to the host are the main indications that they are safe for use in the treatment of the *L. major*.

This study could provide some scientific basis on the plant being used as antileishmanial therapeutic agent, and therefore it can be used as alternative treatments for the cuteneous *L. major* instead of the standard drugs.

6.2 Recommendations

The study recommended on the following:-

Water extracts o *M. foetida* which were shown to be very effective against the *L. major* parasites *in vivo* and *in vitro* can be converted into tablets and/or capsules for oral leishmaniasis treatment in order to avoid the invasive injection method used in administering Pentostam.

Extracts of *M. foetida* were effective in treating cuteneous *L. major* leishmaniasis. The plant should also be tested against other forms of the parasite such as the visceralizing *L. donovani*, cutaneous *L. tropica* and *L. aethiopica.*

Extracts of *M. foetida* were shown to inhibit *L. major* parasites without sufficient production of nitri oxide; therefore further investation of plant on mechanism of inhibition against *L. major* should be done.

Active ingredients and toxicity of parts of *M. foetida* extracts need to be isolated, formulated and be tested for possible oral and topical treatment of cutaneous leishmaniasis.

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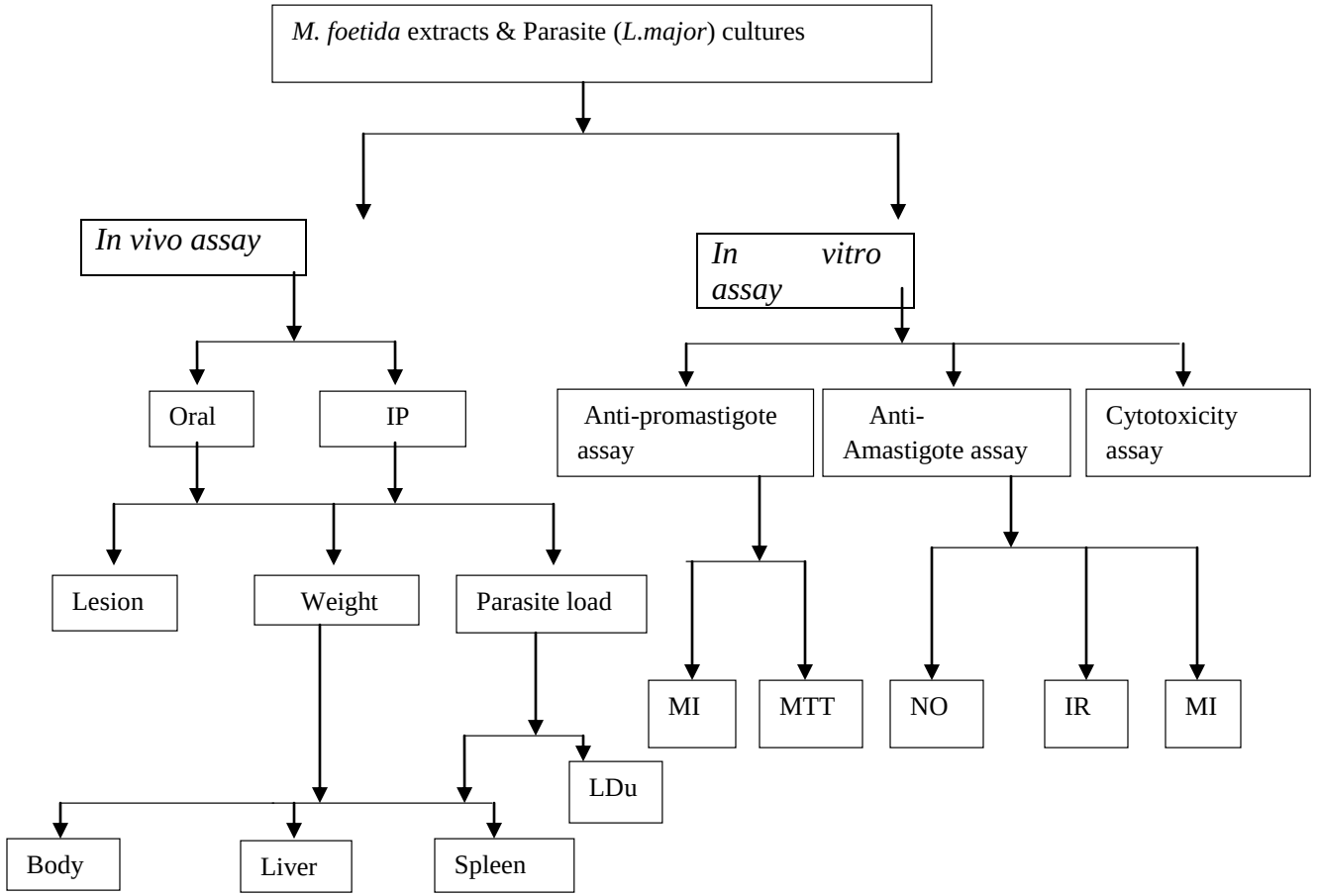
APPENDICES

Appendix I: Approval of Ethical Review and Institute's Scientific Steering Committee

Appendix II: Cost of Medicines in Current use for the Treatment of Leishmaniases

Compound and commercial name	**Manufacturer**	**Price information**
Liposomal amphotericin B AmBisome®	WHO negotiatioed price GlaxoSmithKline	US$ 18 per 50mg (Kshs.1,548.00)
Sodium stibogluconate (SSG), Pentostam®	GlaxoSmithKline	66.43 GBP per vial 100ml, 100mg/ml (Kshs. 9,545,99)
Paromomycin	Gland Pharma, India WHO- negotiated prices	Approximate price, US$ 15 Per adult course of days (Kshs.(1,290.00)
Miltefosine Impavido®	Paladin, Canada	For adults: €45.28-54.92 for 56 (50-mg) capsules (10mg) Capsules (Kshs. 4,680.00)
Meglumine-antimoniate Glucantime®	Aventis WHO- negotiated price	US$ 1.2 per 5ml vial pf 81mg/ml (Kshs. 1,671.84)

Appendix III: The flow chart of experimental design for in vivo and in vitro antileishmanial activity of *M. foetida* extracts



Legends: LDU = Leishman Donovan Unit, **MIC** = Minimum Inhibitory Concentration, **NO** =Nitric Oxide Production, **IR** = Infection Rates, **MI** = Multiplication Index, **IP** = Intraperitoneal and **MTT** = 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrozolium bromide

Appendix IV: Estimated means of promastigotes from *M. foetida* extracts and controls treatments

Treatments	Intercept and slopes	T statistic	P value
RPMI verses Extracts	29.779 ± 0.0592	178.455	0.089
Standard/ Extracts	459.417 ± 0.021	22052	0.027
Methanolic / Aqueous	312.5 ± 0.030	10606.6	0.000
Pentostam / Aqueous	6.33333 ± 0.030	1214.96	0.000
Pentostam / Methanolic	16.5555 ± 0.043	22948	0.012
Amphotericin B / Aqueous	478.083 ±0.021	1814.66	0.010
Amphotericin B Methanolic	458.566 ± 1.004	1594.738	0.626

Appendix V: Statistical table showing *L. major* Infected Macrophages treated with *M. foetida* extracts and controls after arcsine transformation

Treatments	Sum of squares	Df	F ratio	P value
Standard drugs / Extracts	1.96 ± 0.6	1	60.11	0.0001
RPMI / Extracts	2.60 ± 0.5	1	7.98	0.223
Methanolic / Aqueous	425.04± 100.2	1	0.01	0.9119
Pentostam/ Extracts	1.69 ± 0.5	1	48.6	0.0000
Amphotericin B	1.05 ± 0.5	1	62.6	0.0007
Total (n)		5		

Appendix VI: Table showing means in order fitted for amastigotes inhibition after *M. foetida* extracts and control treatments

Treatments	a/b	SE	T statistic	P value
RPMI	62.9	2.57126	24.4627	0.000
Concentration	0.009238	0.019437	0.475286	0.6375
Pentostam	-17.6	3.63632	-4.84006	0.000
Amphotericin B	-13.15	3.63632	-3.6163	0.0009
Aqueous extract	-40.5833	3.63632	-11.1606	0.000
Methanol extract	-55.3167	3.63632	-15.2123	0.000
Concentration/Pentostam	-0.11591	0.027488	-4.21656	0.0002
Concentration/Amphotericin B	-0.0909	0.027488	-3.30707	0.0022
Concentration/Aqueous extract	-0.07205	0.027488	-2.62106	0.0129
Concentration/Methanol extract	-0.0289	0.027488	-1.05154	0.3002

Appendix VII: Statistical table showing Nitric oxide production by macrophages treated with *M. foetida* extracts and control treatments

Treatments	a / b	SE	T statistic	P value
Nitric Oxide reference	100.00	1.22374	81.7168	0.2100
Methanol extracts	-0.10235	0.013082	-7.82372	0.0011
Aqueous extracts	-0.10725	0.013082	-8.19791	0.0000
Pentostam	0.064562	0.013082	4.93505	0.0020
Amphotericin B	0.056395	0.013082	4.3108	0.0001
RPMI	0.102838	0.013082	7.86085	0.0006

Appendix VIII: Table showing Physical Paramers of *L. major* infected BALB/c Mice Subjected to *M. foetida* and Controls Treatments

Treatments	Physical parameters observations
Methanol oral	-Active -Pilo erection of far -Low appetite (Low food and water intake) -formed without mucus faecal matter -Reduction of weight -Dull coat and far looked greyish -Eyes were watery - No mortality observed - Iching nose and mouth
Methanol IP	-Hyper active at initial stage then become restless at the 6th week -Pilo erection of far -Low appetite (Low intake of food and water) -Semi formed with mucus faecal matter -Reduction in weight -Dull coat (Fur changes from white to grey) -Eyes were watery and redish - No mortality observed
Aqueous oral	-Active -No pilo erection of fur - Normal intake of food and water -Formed without mucus faecal matter -Increase in weight -Dull coat (White fur) -Eyes were dry -No mortality observed - No signs of iching nose and mouth
Aqueous IP	-Active -No pilo erection of far -Have appetite -Semi formed with mucus faecal matter -Increse in weight -Dull coat - Eyes were dry - No mortality observed

Pentostam	Hypo active -Pilo erection of fur -Low appetite (Low intake of food and water) -Few semi formed with mucus faecal matter -Increase in weight -Dull coat (White fur) -Eyes were watery - No mortality observed
Amphotericin B	-Active -No pilo erection of fur - Poor appetite -Semi formed with mucus faecal matter -Increase in weight - Shiny coat (White fur) -Eyes were watery - No mortality observed
PBS Oral	-Hypo active -Pilo erection of fur -No appetite -Semi formed with mucus faecal matter -Reduction in mice weight -Dull coat (grey fur) -Eyes were watery -No crowding in the cage - Mortality observed
PBS IP	-Hypo active -Pilo erection of far -No appetite -Few semi solid with mucus faecal matter -Reduction of weight -Dull coat -Eyes were watery - Crowding in the cage - Mortality observed
Untreated/uninfected	-Active -No pilo erection of fur -Have appetite -Semi formed with mucus faecal matter -Increase in weight -Shiny coat (White fur) -Eyes were dry -No crowding - No mortality observed