EVALUATION OF ANTILEISHMANIAL ACTIVITY OF PYRETHRIN EXTRACTS OF *CHRYSANTHEMUM CINERARIAEFOLIUM*TREVIR (ASTERALES: ASTERACEAE) PLANT

RUTTOH REUBEN KIPYEGON

MASTER OF SCIENCE

(Medical Microbiology)

JOMO KENYATTA UNIVERSITY OF

AGRICULTURE AND TECHNOLOGY

2019

Evaluation of antileishmanial activity of pyrethrin extracts of *Chrysanthemum cinerariaefolium*Trevir (Asterales: Asteraceae) plant

Ruttoh Reuben Kipyegon

A Thesis Submitted in Partial Fulfillment for the Degree of Master of Science in Medical Microbiology in the Jomo Kenyatta University of Agriculture and Technology

2019

DECLARATION

This thesis is my original work and has not been presented for a degree in any other University.

Signature …….Date……

Ruttoh Reuben Kipyegon

This thesis has been submitted for examination with our approval as University Supervisors.

Signature…….Date …….

Dr. Mourine Kangogo, PhD

JKUAT. Kenya.

Signature ……Date ……

Dr. Amos Mbugua, PhD

JKUAT, Kenya.

DEDICATION

This thesis is dedicated to my family especially my wife Catherine Chebet and my daughters and son for their overwhelming support and prayers

ACKNOWLEDGEMENT

I take this opportunity to thank the almighty God for the gift of life, care, intellect, wisdom and grace since I was born and during my time of carrying out this project. I am sincerely grateful to my supervisors Dr Mourine Kangogo, department of Medical Microbiology and Dr Amos Mbugua department of Medical laboratory sciences both from Jomo Kenyatta University of Agriculture and Technology (JKUAT) and Dr Christopher Anjili Centre for Biotechnology Research and Development (KEMRI) for their guidance throughout the study period. Their ideas, comments and corrections during the proposal development and implementation provided the means through which the results were realized. I also thank Dr. Festus Tolo and Dr. Peter Mwitari of CTMDR at Kenya Medical Research Institute for having believed in me and given me the opportunity to carry out this study. I wish to thank both the director KEMRI and CBRD for allowing me to use the KEMRI facilities to carry out this study. Also thank the staff of *Leishmania* section for their unselfish support during this study period. My special thanks to Mr. Bernard Osero and Johnstone Ingonga of Leishmania laboratory for their enormous guidance in laboratory activities and data analysis.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF APPENDICES

ACRONYMS AND ABBREVIATIONS

ACUC	Animal Care and Use Committee
ANOVA	Analysis of variance
CBRD	Centre for Biotechnology Research and Development
CL	Cutaneous leishmaniasis
CTMDR	Centre for Traditional Medicine and Drugs Research
DMEM	Defined Minimum Essential Medium
DMSO	Dimethyl Sulphoxide
HCL	Hydrochloric Acid
ELISA	Enzyme-linked immunosorbent assay
ERC	Ethical Review Committee
HPLC	High Performance Liquid Chromatography
IC_{50}	Inhibitory concentration at 50%
IFN-ɣ	Interferon gamma
IL4	Interleukin 4
iNOS	inducible nitric oxide
IR	Infection rate
KEMRI	Kenya Medical Research Institute

LDU	Leishman-donovan units
MCL	Mucocutaneous leishmaniasis
MI	Multiplication index
MIC	Minimum inhibition concentration
MTT	Thiazolyl blue tetrazolium bromide
NO	Nitric oxide
PKDL	Post-kalaazar dermal leishmaniasis
PBS	Phosphate buffered saline
RPMI	Roswell Park memorial Institute Medium
SD	Standard deviation
SIM	Schneider's insect medium
SERU	Scientific & Ethics Review Unit
TNF-α	Tumor Necrosis factor alpha
WHO	World Health Organization

ABSTRACT

Treatments of leishmaniases have relied on antimonials drugs which are toxic, expensive and resistance has been reported. The pyrethrum plant, *Chrysanthemum cinerariaefolium* has been shown to have antiplasmodial, antitrypanosomal and insecticidal activity but little has been studied about its potential on anti-*Leishmania* effects. The objective of this study was to investigate the antileishmanial activity of water and methanol extracts of *Chrysanthemum cinerariaefolium* both *in vitro* and *in vivo*. In this study concentration between 31.25 to 1000µg/ml of methanolic and aqueous extracts of *C. cinerariaefolium* were tested for anti- parasitic effects against *Leishmania major* in *in vitro* cell culture and in BALB/c mice. The inhibitory concetrations (IC_{50}) and Minimum inhibition concentration (MIC) on *L. major* promastigotes, percentage rates of macrophages infected by amastigotes and cytotoxicity on vero cells were determined. In vivo bioassays BALB/c mice were infected subcutaneously with 1×10^6 promastiogetes and kept for 4 weeks to allow disease manifestation. They were then treated with 250µg/ml of water extracts and 125µg/ml of methanol extracts, 20mg/kg/body weigth of pentostam and amphotericin B and Phosphate buffered saline (PBS) were subjected orally and intraperitoneally for four weeks. The lesions were measured weekly and at the 4^{th} week mice were sacrificed and impression smears from spleen were made on slide and examined microscopically. Cytokine analysis was done from serum and nitric oxide production by infected macrophages treated with extracts in vitro was determined. The MIC of water extracts was was not significantly high (250µg/ml) than that of methanol extracts (125µg/ml). IC_{50} for both methanol (8.3µg/ml) and water extracts (13.6µg/ml) were lower than those of pentostam (14.1µg/ml) and Amphotericin B (8.623µg/ml) but not significant ($P> 0.05$). Water (238µg/ml) and methanol (121µg/ml) extracts had non significant toxicity ($P>0.05$) against vero cells compared to Pentostam and Amphotericin B (106.8 and 68.6µg/ml respectively) and NO produced by macrophages stimulation by extracts was minimal. Treatment with both extracts significantly reduced the number of infected macrophages compared to untreated ($P=0.042$). The mean difference in multiplication index (number of amastigotes) in macrophages treated with methanolic extract was significantly reduced compared to water extract treatment ($t =7.9994$, $P=0.0076$). The infected macrophages treated with pentostam and amphotericin B had significantly reduced number of amastigotes than those treated with both the extracts ($P<0.05$). Reduction in lesion sizes, low parasite burden in the spleen and low toxicity exhibited by water and methanol of *C. cinerariaefolium* extracts makes them good candidate compounds for tests in the treatment of various *Leishmania* species. The study observed that IFN gamma cytokine produced by methanolic and water extracts (at concentration of 125µg/ml and 250µg/ml respectively) treated BALB/c mice were not significantly different from those treated with pentostam and Amphotericin B at 20mg/kg/body weigth. The anti-leishmanial activity and immunological modulation by water and methanol extracts of *C. cinerariaefolium* suggests that they can be a good candidate for management of leishmaniasis, therefore more studies is required before a conclusive policy on the use of these extracts of treatment of leishmaniasis.

CHAPTER ONE

INTRODUCTION

1.1 Background Information

The leishmaniases are a group of intra- celluler protozoan diseases caused by leishmania species that are transmitted to humans and other mammals by female sand flies (Diptera: Psychodidae), mainly *Lutzomyia* Franca and *Phlebotomus* Rondani (Murray *et al*.,2005. WHO, 2010). The leishmaniases rank as the leading neglected tropical disease (NTD) in terms of mortality and morbidity with an estimated 50,000 deaths in 2010 (Lozano *et al*., 2012) and 3.3 million disability adjusted life years (Murray *et al*., 2012). Leishmaniasis is endemic in 98 countries, 72 of which are developing nations and 13 correspond to the least developed ones (Sinha *et al*., 2005, WHO, 2010). Over 350 million people reside in areas with active parasite transmission (Murray *et al*., 2005). Annually, an estimated 1.5–2 million develop symptomatic disease, and approximately 50,000 die, mostly children (Desjeux, 2004; WHO, 2010). The disease is normally divided into three main categories: Cutaneous, Mucocutaneous, and Visceral. Cutaneous leishmaniasis (CL) is the most extensively studied form of the disease, usually appearing as a self-healing skin ulcer or dermal granuloma that may need several months or years to heal (Monge-Maillo and López-Vélez, 2013). In some cases, these ulcers can become chronic (Kroidl *et al*., 2014). While most *Leishmania* species cause lesions confined to small areas of the skin, a few, such as *L. braziliensis*, cause diffuse lesions that may even spread to mucosal tissues leading to the muco-cutaneous form of the disease that leads to partial or total destruction of mucous membranes of the nose, mouth and throat. (Strazzulla *et al*., 2013). Visceral leishmaniasis is the most severe form of the disease, caused by *Leishmania donovani, Leishmania infantum* and *Leishmania chagasi*. It is characterized by fever, cachexia, hepatosplenomegaly and hypergamaglobulinemia and, when untreated, can be fatal (Ready, 2014).

There is no human vaccine available at the moment. Treatment options are limited to antimonials that present significant toxicity and require parenteral administration (Van Griensven *et al*., 2010). Drug resistance is also a growing limitation of some antileishmanial therapies (Yasinzai *et al*., 2013). Therefore, it is essential to develop alternative treatment options and vaccine strategies.

In many instances, traditional medicines are the alternative for accessible treatments against parasitic diseases (Calla-Magarinos *et al*., 2009). Unfortunately, most of them are hardly explored and their mechanisms of action are mainly unknown. Plants possess a large repertoire of secondary metabolites that display a wide variety of pharmacological activities. Indeed, numerous plant derived bioactive compounds have been described, such as terpenoids, flavonoids, alkynes, alkaloids, saponins, sterols, phenylpropanoyl esters, lactones, tannins, and coumarins (Mazid *et al*., 2011; Cragg and Newman, 2013; Ma *et al*., 2013). Natural pyrethrins have shown antiplasmodial and antitrypanosomal activities but there is no existing data on antileishmanial activity (Yoshie *et al*., 2011).

1.2 Statement of the problem

Some parasitic infections, such as malaria, trypanosomiasis or leishmaniasis can be deadly if the patients are not treated with adequate therapeutics. In most endemic areas humans usually live in close proximity and the transmission of parasites within the human population is often facilitated.

Leishmaniasis is a reportable disease in 88 countries where it is known to be present (http://www.who.int/tdr/diseases/leish/diseaseinfo.htm). A global burden of leishmaniasis is estimated at 2.4 million disability adjusted years.

Chemotherapy of leishmaniasis relies on the use of pentavalent antimonials, amphotericin B, paromomycin, miltefosin and liposomal amphotericin B. However, the application of these drugs is limited due to low efficacy, life-threatening side effects, high toxicity, induction of parasite resistance, length of treatment and high cost (Machado *et al*., 2012;

Wiwanitkit, 2012; Lage *et al*., 2013). Serious side effects including arthralgias, myalgias, leukopenia, pancreatitis, liver problems, cardiotoxicity and cardiac arrhythmia in the patients, prolonged treatment time, and increased parasite resistance (Chakravarty and Sundar, 2010; Lage *et al*., 2013; Diro *et al*., 2014). In two studies carried out, it was observed that only about one-third of the patients could be cured with the antimonial regimen (Sundar *et al*., 2000). In addition there is no effective vaccine against leishmaniasis (Santos *et al*., 2008). HIV coinfected patients is another subset who respond poorly to Sbv, 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 Sbv could be as low as 37% (Laguna *et al*., 2003). New, safer and more efficacious drugs are therefore urgently required (Croft *et al*., 2005).

Since victims of leishmaniasis are generally poor, lengthy treatment using expensive drugs with related costs is far beyond the means of such families (Oryan et al., 2014). Therefore, many patients seek for herbal therapy which is cheaper and readily available. However, most of the herbs have still not been evaluated scientifically (Alviano *et al*., 2012; Garcia *et al*., 2012; Ogeto *et al*., 2013; Chouhan *et al*., 2014).

Medicinal plants are noted to be a good source of bioactive agents which are useful against many diseases including leishmaniasis. The natural pyrethrins have shown antiplasmodial activity with IC50 between 4 and 12 μM, and antitrypanosomal activity with IC_{50} from 7 to 31 μM. The pyrethroids have been shown to exhibit weaker antiplasmodial and antitrypanosomal activity than the pyrethrins. Both pyrethrins and pyrethroids have shown moderate cytotoxicity against L6 cells (Yoshie *et al*., 2011) but no existing data on antileishmanial activity. Pyrethrum plant is rich in secondary metabolites like alkaloids, gallic and cathechic, tannins, flavonoids and triterpenes which have shown antibacterial activity (Hanane *et al*., 2014). This fact could justify their extraction and use especially in the prevention of diseases and the management of many infections.

1.3 Justification

Leishmaniasesis is one of the tropical diseases that have been neglected in the world 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 and as a result, people do not seek medical attention despite the associated chronic sufferings (Hailu, 2005). There is need for new treatment options that are cheaper, non-toxic, non-parasite resistant, accessible and available in endemic areas. Two oral drugs used are Sitamaquine and miltefosine, which are also anticancers 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 *C. cinerarariefolium* plant for the present study emanated from the need to validate the traditional use of the plant against *Leishmania* parasites. *C. cinerariaefolium* plants are found in many parts of Kenya including, Baringo, Elgeyo Marakwet, West Pokot, Mt. Elgon, Meru, Machakos and Kitui where *L. major* infection is endemic (Kigondu *et al.,* 2009). It is anticipated that extracts from *C. cinerariaefolium* extracts may solve this problem of drug unavailability, oral administration and early treatment for people who wait for self-healing. Finding of this study are useful in providing new insights on the potential use of this plant extracts as chemotherapeutic agents in the leishmaniases.

1.4 Research questions

1. What are the minimum inhibition concentration and IC_{50} of water and methanol extracts of *Chrysanthemum cinerariaefolium* on *L. major* promastigote?
2. Is water and methanol extracts of *Chrysanthemum cinerariaefolium* toxic to vero cells?
3. What are the levels of nitric oxide produced by infected macrophages treated with water and methanol extracts of *Chrysanthemum cinerariaefolium*?

4. What is the disease phenotype and parasite burden in *L.major* infected BALB/c mice after treatment with water and methanol extracts of *Chrysanthemum cinerariaefolium*?
5. Do the water and methanol extract of *Chrysanthemum cinerariaefolium* elicit immune response in *L.major* infected BALB/c mice upon treatment?

1.5 Objectives

1.5.1 General Objective

To evaluate the antileishmanial activity of water and methanol pyrethrin extracts of *Chrysanthemum cinerariaefolium.*

1.5.2 Specific Objectives

1. To determine the minimum inhibition concentration, parasite growth and infection rates *in vitro* upon treatment of *Leishmania major* with *Chrysanthemum cinerariaefolium* extracts.
2. To assess the toxicity of pyrethrin extracts of *Chrysanthemum cinerariaefolium* on vero cells.
3. To determine the lesion size on *Leishmania major* infected BALB/c mice treated with Chrysanthemum cineraraiefolium extracts.
4. To determine the levels of immunological responses (IFN-γ, IL-4 in vivo and NO in vitro) cytokines after treatment of infected BALB/c mice and macrophages respectively with extracts of pyrethrins

1.6 Hypothesis

H0: Pyrethrin extracts of C. cinerariaefolium have no leishmaniacidal activities on *L. major* parasites.

CHAPTER TWO

LITERATURE REVIEW

2.1 Introduction to *Leishmania* species

Leishmania are intracellular parasites that are transmitted through bites of infected females 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 (MCL) (WHO, 2017). Human leishmaniases infections are caused by about 21 of 30 species of genus *Leishmania* that infect mammals (Croft *et al*., 2006). These include the *L. donovani, L. infuntum*, and *L. chagasi* that causes visceral leishmaniasis in the old world including the East Africa.The other species like *L. Mexicana, L. amanzonensis*, and *L. venezuelensis* causes visceral leishmaniasis in the new world including South America. *L. tropica*; *L. major* and *L. aethiopica* species causes cutaneus leishmaniasis in East Africa including Kenya, where as *L. braziliensis, L. guyanensis, L. panamensis, and L. peruviana* are the main causes of cutaneus leishmaniasis in the new world. The different species are morphologically indistinguishable, but they can be differentiated by isoenzyme analysis, molecular methods, or monoclonal antibodies (Dohm *et al*., 2000).

2.2 Global burden and distribution of leishmaniasis

The leishmaniases rank as the leading NTD in terms of mortality and morbidity with an estimated 50,000 deaths in 2010 (Lozano *et al*., 2012) and 3.3 million disability adjusted life years (Murray *et al*., 2012).

Of the 10 countries (Afghanistan, Colombia, Brazil, Algeria, Peru, Costa Rica, Iran, Syria, Ethiopia, and Sudan) that contribute 75% of the global estimated CL incidence (Alvar *et al*., 2012), only Algeria did not have regions of complete evidence consensus on presence

due to incomplete and non-contemporary case data. Similarly, of the six countries (Brazil, Ethiopia, Sudan, South Sudan, India, and Bangladesh) that report 90% of all VL cases (Alvar *et al*., 2012), all six had regions of complete consensus on VL.

In the Americas, VL is present in 12 countries (PAHO, 2017), with 96% of the cases being reported in Brazil (4,200 to 6,300 cases per year) (Alvar *et al*., 2012) while CML occurs in 20 American countries, being endemic in 18 of them, albeit with different transmission intensities (PAHO, 2017). In Brazil, CML affects 72,800 to 119,600 people annually (Alvar *et al*., 2012). From1990 to 2016, 84,922 cases of VL were confirmed in Brazil, with the case-fatality rate reaching 7.4% in 2016 (MS, 2016a). In the same period, 687,780 cases of CML have been reported, albeit with low mortality (MS, 2016b). Expected annual cases in Kenya average 600 annually. Case fatality rate of up to 7% is seen in out break situation. Cases rise to over 1,000 in an epidemic year (Tonui, 2006)

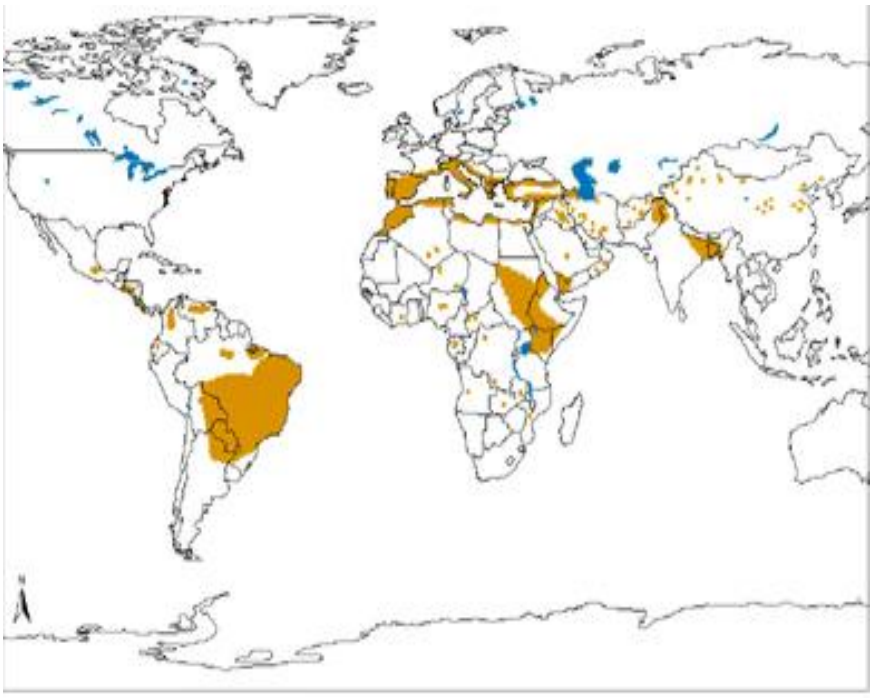

Figure 2.1: Geographical distribution of visceral and cutaneous leishmaniasis in the Old and New world (Data source & Map production, 2010)

2.3 Types of Leishmaniases

2.3.1 Cutaneous leishmaniasis

Cutaneous leishmaniasis is the most common form, which causes skin sores that typically develop within a few weeks or months after infection with 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 mainly caused by *L. major, L. tropica* and *L. aethiopica* in Kenya and most of African countries (James *et al*., 2006). Natural transmission of *Leishmania* parasite is carried out by the infected female phlebotomine sandflies of the

genera *Phlebotomus* and *Lutzomyia* (Killick-Kendrick, 2002)**.** Most forms of the disease are zoonoses transmissible only from animals but can be spread between humans congenitally (Ready, 2013)) and from human to the sand fly and then to the human in an anthroponotic cycle (Alvar *et al*., 2012). The aetiological agents for CL include *L. major, and L.tropica* in the Middle East, Central Asia and Africa (Kenya, South Sudan and Ethiopia). Where as *Leishmania braziliensis complex* and *Leishmania Mexicana* causes cutaneous *Leishmaniasis* in South America. In Kenya CL is endemic in areas like Baringo, Laikipia, Samburu, Isiolo, Nakuru and Nyandarua districts where *L.tropica* is the causative agent while in Mt Elgon *L. aethiopica* is the cause of the disease. *P. duboscqi*, *P. guggisbergi* have been shown to be the vectors of *L. major* and *L. tropica*, respectively, while *P. pediffer*, *P. longipes* and *P. elgonensis* have been implicated as vectors of *L. aethiopica* (Tonui, 2 006) and in Utut reserve, Njoro in Nakuru county, the probable vector was identified as *P. aculeatus* ((Dohm *et al*., 2000 and Morillas-Marquez *et al*., 2002).

The incubation period of CL ranges from 2 to 6weeks 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 *Leishmania 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; effects can be life threatening (Van-Grienven & Diro, 2012). The illness develops within months (sometimes as long as years) of the sandfly bite (Murray *et al*., 2005). Infected people usually have fever, weight loss, enlargement (swelling) of the spleen and liver and low blood count, a low red blood cell count (anaemia), a low white blood cell count (leukopenia), and a low platelet count (thrombocytopenia) (Kolaczinski *et al*., 2008).

These diseases are characterized by both diversity and complexity; they are caused by more than 20 leishmanial species and are transmitted to humans by 30 different species of phlebotomine sand flies (Grimaldi & Tesh, 2012).The major known foci of VL are in Turkana, Baringo and West Pokot districts in Rift Valley Province and also in Machakos, Kitui and Meru districts (Mbui *et al*., 2003). In these foci, the disease is believed to be anthroponotic with a man- sand fly-man cycle, with *Phlebotomus martini* as the incriminated vector (Tonui, 2006). After successful treatments with Amphotericin B, 3 to 10% of the cases of VL develop post kala-azar dermal leishmaniasis (PKDL) wart like nodules over the face and extent to 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 years (Carvalho *et al*., 2008).

2.2.3 Mucocutaneous leishmaniasis

Mucocutaneous leishmaniasis (MCL) is less common form of leishmaniasis; the pathogenesis of MCL is still unclear. However, it is believed that host genetic factors are important in advancement of the disease (Desjeux, 2004). This form of leishmaniasis is caused by *L. major* and *L. tropica* parasites (Desjeux, 2001) characterized by spreading from the skin and cause sores in the mucous membranes of the nose, 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 and Beaching, 2011). Secondary infection is responsible for most deaths (Ameen, 2010).

2.3 Parasite morphology, sandfly and life cycle

2.3.1 Introduction

Like many protozoan parasites, *Leishmania* have a digenetic life cycle involving both a mammalian host and an insect vector. Leishmania parasites exhibit two different cell morphologies i.e promastigotes and amastigotes and a number of cell types (developmental forms) that are adapted to either the host or the vector. Some of these developmental forms are proliferative, whereas others are quiescent and pre-adapted for transmission to the next host (Matthews, 2011).

The basic cellular architecture of *leishmania* parasite is however conserved cross-linked sub-pellicular corset microtubules that is housed within the cell are the nucleus and a set of single-copy organelles such as the mitochondrion and the Golgi apparatus. Anterior of the nucleus is the kinetoplast, the mass of concatenated mitochondrial DNA which is directly connected to the basal body from which the flagellum extends (Ogbadoyi *et al*., 2003; Wheeler *et al*., 2016). At the base of the flagellum is an invagination of the cell membrane forming a vase-like structure called the flagellar pocket, which is important in these parasites as it is the only site of endocytosis and exocytosis and is hence a critical interface between the parasite and its host environment (Lacomble *et al* ., 2009).

The *Leishmania* cell is constructed from a series of modular units such as the flagellum, basal body–mitochondrial kinetoplast unit and a Golgi–flagellar pocket neck unit (Ogbadoyi *et al*., 2003). These modular units are then positioned relative to each other to give rise to the different cell morphologies observed (Hayes *et al*., 2014; Sunter and Gull, 2016).

Sand flies are grouped in the Suborder Nematocera of the Order Diptera (two-winged flies). Below that, the classification is not universally agreed, but they are generally put in the Family Psychodidae, subfamily Phlebotominae. Lane (Killick-Kendrick, 2002) recognises six genera of which only two are of medical importance, namely *Phlebotomus*

of the Old World, divided into 12 subgenera, and *Lutzomyia* of the New World, divided into 25 subgenera and species groups.

2.3.2 Promastigotes stage

The promastigotes stage of *Leishmania* that exhibit a single flagellated and spindle-like in shape that exists in the sand fly, when in their mature state; metacyclic forms ther transform into amastigotes forms after inoculated into human host and get engulfed by macrophages (Sighn, 2006). In this form, nucleus is situated at the center and kinetoplast transversely towards the anterior end (Assafa *et al*., 2006). Morphologically, they are similar to those grown in culture (Grimm *et al*., 2011). (Figure 2.1)

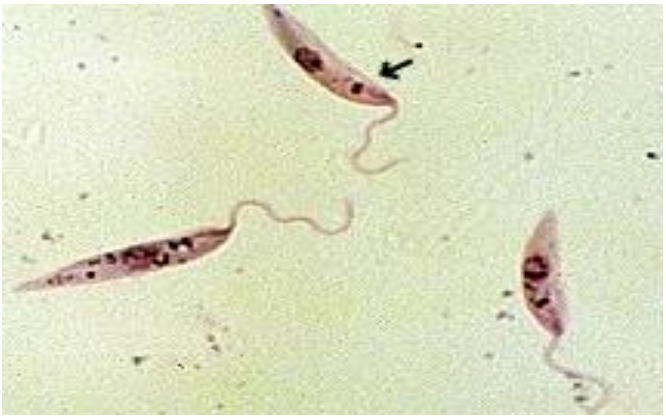

Figure 2.2: Promastigote flagellates

2.3.3 Amastigote Stage

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

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

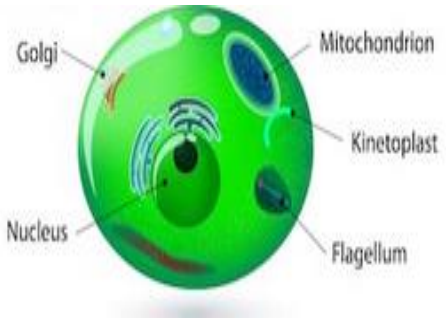

Figure 2.3: Amastigote

2.3.4 Sand fly

Official publications have revealed the existence of 48 species of sand flies in the country, 30 species belonging to the genus *Phlebotomus*, and 18 speicies of the genus *Sergentomyia* (Yaghoobi-Ershadi, 2012, Zahraei-Ramazani *et al*., 2013, 2015). The practical application of sand fly control measures has been appraised in most Old World situations (Alexander and Maroli, 2003). Indoor Residual Spraying (IRS) with DDT (dichlorodiphenyltrichloroethane) against malaria vectors in India during the 1950s and again in the 1970s was effective in reducing the density of *P. argentipes* and Visceral Leishmaniasis cases, but after the cessation of IRS, sand fly population and incidence of VL increased (Kishore *et al*., 2006, Thakur, 2007).

Both male and female sand flies feed on plant juices and sugary secretions. Female 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 (Figure 2.2).

Figure 2.4: Adult female sand fly feeding (Schlein et al., 2001)

2.3.5 Life cycle

Leishmaniasis is transmitted by the bite of female phlebotomine sandflies (Chatterjee, 2009). The sandflies inject the infective stage, promastigotes, during blood meals. Promastigotes that reach the puncture wound are phagocytized by macrophages and transform into amastigotes (UCLA, 2014). Amastigotes multiply in infected cells and affect different tissues, depending in part on the *Leishmania* species. This originates the clinical manifestations of leishmaniasis. Sandflies become infected during blood meals on an infected host when they ingest macrophages infected with amastigotes. In the sandfly's midgut, the parasites differentiate into promastigotes, which multiply and migrate to the proboscis (Richard *et al*., 2001).

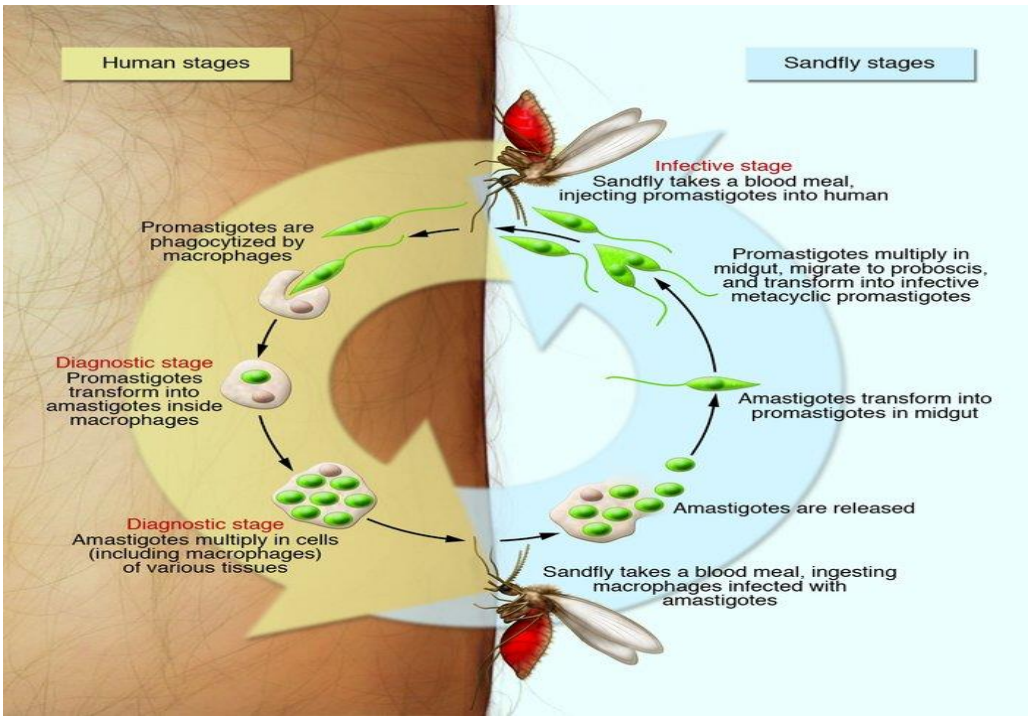

Figure 2.5: Lifecycle of *Leishmania* parasite (source: CDC. 2016)

2.4 Treatment and control

2.4.1 Chemotherapy

2.4.1.1 Pentavalent Antimonials

Systemic administration of pentavalent antimonials has remained the treatment of choice however in the recent years Paromycin has been used as topical formulations (Charkravarty and Sundar, 2010). Pentavalent antimonials (SbV) become the drug of choice for the treatment of all types of leishmaniases. Pentostam, sodium stibogluconate, manufactured by Wellcome Foundation was first used. Glucantime, meglumine antimoniate, manufactured by Rhone Poulenc in France can also be used. (Tracy and Webster, 2001).

Recent studies have suggested the participation of an parasite-specific enzyme in the process of reduction of Sb (V) to Sb (III) antimoniate reductase (ACR2) (Zhou *et al*., 2004). Evidence was obtained that Sb (III) enters Leishmania cells primarily though an aquaglyceroporin named AQP1 and that the level of expression of this transporter may modulate the resistance of the parasite to Sb (III) (Gourbal *et al*., 2004). Increase in total thiols (Cys, GSH and T (SH) 2) and/or overexpression of ABC transporter are often observed in metalresistant Leishmania (Ouellette *et al*., 2004). The thiol increase is mediated by the overexpression or amplification of a number of different genes involved in the synthesis of GSH or polyamines, which are the two building blocks of T(SH)2 (Ouellette *et al*., 2004). Among the potential molecular targets of Sb (III), evidence was obtained that trypanothione reductase (TR) or zinc-finger protein may be involved. Although TR shares structural and mechanistic similarity with GR, differences in the disulfide binding site between TR and GR draw the interest for selective inhibition. Fairlamb and co-workers recently reported that trivalent antimonials interfere with T (SH)2 metabolism by inhibiting TR and inducing rapid efflux of intracellular T(SH)2 and GSH in intact Leishmania cells (Wyllie *et al*., 2004). In another recent study, the ability of Sb(III) to bind to a CCHC zinc finger peptide model and to promote the ejection of Zn(II) (Demicheli *et al*., 2008). Another recent study revealed that meglumine antimoniate increased the phagocytic capacity of monocytes and neutrophils and enhanced superoxide anion production by phagocytes, which represent the first line of defense against the parasite (Muniz-Junqueira and Paula-Coelho, 2008)

It has also been reported to cause non specific blocking of SH groups of amastigote proteins and cause inhibition of DNA topoisomerase I (Walker and Saravia, 2004). More recently, it was demonstrated that antimony can alter the thiol-redox potential in both forms of parasite by actively promoting efflux of thiols, glutathione and trypanotione, thus rendering the parasite more susceptible to oxidative stress (Ameen, 2007). The recommended dose is 15-20 mg SbV/Kg of body weight per day for 21-28 days by intramuscular or intravenous route. The long course treatment allows antileishmanial

levels of the drug to accumulate in tissues, particularly in liver and spleen. The treatment with antimonials causes several side effects, such as: nausea, abdominal pain, myalgia, pancreatic inflammation, cardiac arrhythmia and hepatitis, leading to the reduction or cessation of treatment (Thakur and Narayan, 2004).

The variable efficacy against CL and VL, as well as the emergence of significant resistance has been increased (Croft and Coombs, 2003). Second, new generic of Pentostam have been produced with the aim to decrease the high cost of the treatment. However, caution must be exercised before using SbV from new manufactures as bad batches causes fatal cardiotoxicity (Croft and Coombs, 2003). Intralesional administration can be a choice but each lesion has to be injected individually and do not prevents the potential dissemination of infection (Singh & Sivakumar, 2004). Combinations of hexadecylphosphocholine with AmB, paromomycin, or pentavalent antimonials have been evaluated in an in vivo model, which revealed that the combinations of hexadecylphosphocholine with AmB or paromomycin were efficacious (Seifert and Croft, 2006)

Historically, the chemotherapy of leishmaniasis has been based on the use of toxic heavy metals, particularly antimony compounds. When this kind of treatment is not effective, other medications used include pentamidine and amphotericin B. All these pharmaceuticals require administration by injection and clinical supervision or hospitalization during treatment due to the severity of the possible secondary effects (Anke *et al*., 2018).

Currently, these molecules have two major limitations: first, side effects are frequent and can be serious; second, parasite resistance is emerging in some endemic areas, causing an increase in treatment failure (Hadighi *et al*., 2006). Currently, in the most heavily affected areas of India, resistance to antimonials may reach more than 60% of the cases, thus representing a public health problem (Sundar *et al*., 2000; Abdo *et al*., 2003)

2.4.1.2 Amphotericin B

Amphotericin B is a macrolide polyene antifungal antibiotic agent, discovered in 1956, from a bacterium: *Streptomyces nodusus*, actinomycetes obtained from the soil of Orinoco River in Venezuela. Its mechanism of action consists in binding to the ergosterol fraction of the cell membrane of the parasite, thus increasing its permeability. However, this pharmaceutical product is also associated with an important number of side effects, including the alteration of the renal function in approximately 80% of treated patients (Srivastava *et al*., 2011). The drug is poorly absorbed by gastrointestinal tract.

Amphotericin B exhibits multi-compartmental distribution and is found to be present in low concentrations in aqueous humor, pleural, pericardial, peritoneal and synovial fluids. The elimination in adult is approximately 24 hours and can be found in blood for up to 4 weeks and in urine for 4-8 weeks in case of discontinuation of therapy (Tracy & Webester, 2001). The antileishmanial activity of amphotericin B is attributable to its selectivity for 24 substituted sterols, namely ergosterol *vis-a-vis* cholesterol, the primary sterol counterpart in mammalian cells eventually helping to increase drug selectivity towards the microorganism. Therapeutic doses of amphotericin B deoxycholate of 0.5 to 1mg/Kg body weight by endovenous bolus daily for 20 days can be administered or alternate days and with a total dosage between 1.5 to 2.0 g (Requena *et al*., 2004).

Serious adverse reactions have been displayed by the treatment with amphotericin B, including fever with rigor and chills, thrombophlebitis and occasional serious toxicities like myocarditis, severe hypokalaemia, renal dysfunction and even death. Its use requires prolonged hospitalization and close monitoring (González *et al*., 2004)).

Amphotericin B has excellent leishmanicidal activity and constitutes an option in patients that showed resistance to treatment with antimonials. The major limiting factor about the use of this drug is due to their toxicity. Currently, toxic effects of amphotericin B have been largely ameliorated with the advent of lipid formulations. In these formulations,

deoxycholate has been replaced by other lipids that mask amphotericin B from susceptible tissues, thus reducing toxicity, and facilitating its preferential uptake by reticuloendothelial cells. Thus, this drug delivery result in increasing efficacy and reduced toxicity. Three lipid associated formulations of amphotericin are commercially available: liposomal amphotericin B (AmBisome), amphotericin B lipid complex (Abelcet) and amphotericin B colloidal dispersion (Amphocil). These compounds have been considered between the most striking advances in leishmaniasis therapy (Veerareddy and Vobalaboina, 2004).

Among the lipid formulations, AmBisome is the best tested and some studies demonstrated the successful in patients with CL and VL, particularly in areas where antimonials resistance has been detected. AmBisome have been considered as a high effective, non-toxic form of treatment for VL when administered in a short course (Mishra and Singh, 2007; Solomon *et al*., 2007).

2.4.1.3 Miltefosine

Miltefosine is effective *in vitro* against both promastigotes and amastigotes of various species of *Leishmania* (Escobar *et al*., 2002), and also other kinetoplastidae (*Trypanosoma cruzi*, *T. brucei*) and other protozoan parasites (*Entamoeba histolytica*, Acanthamoeba). Evidence of apoptosis-like death has been shown in *L. donovani* promastigotes treated with miltefosine (Paris *et al* 2004; Verma and Dey 2004).

For drug binding, the phospholipid composition of cell membranes seems critical: miltefosine resistant *L. donovani* have altered fatty acid elongation and unsaturation, and the C-24-alkylation of sterols (Rakotomanga *et al*., 2005). For transport across the membrane, a putative transporter was recently identified; a Leishmania P-type ATPase gene, belonging to the aminophospholipid translocase (APT) subfamily termed LdMT (*L. donovani* Miltefosine Transporter) has been cloned. LdMT is expressed in the plasma membrane where it mediates the translocation of phospholipids across the plasma

membrane in Leishmania parasites (Perez-Victoria and Gamarro *et al*., 2003). As Leishmania amastigotes reside inside macrophages, membrane binding and flip-flopping will have to occur multiple times across the various membranes until equilibrium is reached. Leishmania parasites do not seem to have the ability to metabolize miltefosine, but can extrude via either exocytosis or protein-dependent flop across the plasma membrane (possibly by proteins of the ABC transporters family, such as P-glycoprotein (mdr1) (Perez-Victoria *et al*., 2001).

2.4.1.4 Pentamidine

Pentamidine therapeutic regimen varies considerably in different countries (Neves *et al*., 2011). Roussel *et al* (2006) compared 1 and 2 IM injections of pentamidine (7mg/kg) in patients from French Guiana with CL and obtained cure rates of 78.8%.

Mild adverse events were reported by 17 (85%) patients, mainly transient pain at the site of applications (85%), while nausea (5%), malaise (5%) and dizziness (5%) were reported in one patient (Gadelha *et al*., 2015).

The mitochondrion is the target organelle for the pharmacological action of pentamidine (Mehta and Shaha, 2004)). Through the use of in vitro-generated pentamidine-resistant *Leishmania mexicana* parasites, Basselin and colleagues demonstrated that the fluorescent analog of pentamidine, 4′,6-diamidino-2-phenylindole (DAPI), is excluded from the kinetoplast of the resistant phenotype, again indicating that the site of action for the diamidine-type inhibitors pentamidine and DAPI is highly associated with mitochondria of *Leishmania* parasites (Basselin *et al*., 2002). More recently, a series of diamidines with DNA binding properties was synthesized and evaluated for antileishmanial activities. A correlation between DNA binding strength and activity against *L. donovani* axenic amastigotes has been observed. Using the same parasite form, researchers next demonstrated that the most potent analog, with a 50% inhibitory concentration (IC_{50}) of 0.27 μM, significantly altered the structure of mitochondria; as observed by electron

microscopy, treated mitochondria exhibited dramatic dilation as well as disintegration of kinetoplasts (Hu *et al*., 2008).

2.4.2 Prospect of vaccine development

Over the years, several vaccine targets and vaccination routes have been proposed and tested (Jain *et al*., 2015). Some of them have shown great potential in protecting animals against leishmaniasis (Chappius *et al*., 2007). Remarkably few have been able to proceed to a clinical trial. This lack of progress is partly due to lack of a small-animal model that reflects human disease, and to the fact that many vaccines are tested with cutaneous strains where the testing has been undertaken by injections instead of sand fly bites (Duthie *et al*., 2012). It takes great effort to develop vaccines. So far, vaccine development has been carried out with limited information on the pathophysiological and immunological complexity of *Leishmania* infection.

First generation vaccines include killed or live attenuated pathogens. Such vaccines have the potential to closely mimic natural infection. In the Middle East, so-called leishmanisation has been practised, whereby pus from cutaneous lesions and parasites from culture were used to induce a local infection (Noazin *et al*., 2008).

Second generation vaccines are purified or recombinant proteins expressed in bacteria or eukaryotic cells. Such vaccines usually require an adjuvant to induce a good T-cell response (Duthie *et al*., 2012). The Infectious Disease Research Institute in Seattle has developed a vaccine where several proteins conserved across several species of Leishmania are expressed in a single vaccine using a toll-like-receptor agonist as an adjuvant to induce a strong T-cell response (Llanos-cuentas *et al*., 2010; Duthie *et al*., 2012). This vaccine has shown promising results (Llanos-cuentas *et al*., 2010).

Third generation vaccines are nucleic acid vaccines where expression vectors encoding for a protein antigen are introduced. Here not the antigen itself, but the information required to produce it, is introduced. The recipient's cells produce and present the antigens

to immune cells (DeFrancisco *et al*., 2017). This vaccine strategy has obvious attractions, such as the fact that the vaccine can be rapidly and cheaply produced. The DNA-vaccine approach has been tested with several antigens against Leishmania-infected mice, but its effect remains unconvincing. Also, to the best of our knowledge, there are currently no pre-clinical or clinical trials using RNA-based vaccine technology for Leishmania. Vectors such as Adenovirus, expressing recombinant antigens, can also be used as vaccines. A novel vaccine uses this approach by expressing a gene encoding for two Leishmania proteins, and initial results are promising (Osman *et al*., 2017). But this is only in the phase I clinical trial stage, and there are significant challenges ahead.

2.5 Management of Leishmaniases

2.5.1 Introduction

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 reservoir in anthroponotic transmission cycles and control strategies against the vectors and zoonotic reservoirs in order to break transmission in zoonotic cycles (Lacerda, 2004).

2.5.2 Animal Reservoir Control

Animal 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 foci. Animals' reservoir control for CL is based on the use of poison baits and environmental management to control rodents (Moncaz *et al*., 2003).

2.5.3 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). 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. 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).

The application of biolarvicides in the field condition is difficult due to diverse breeding habitat of sand fly 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 pheromons 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 south western 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 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 insecticide, applying topical insecticide lotions and the use of deltamethrin treated collars are novel strategies that can substantially reduce sand fly bites on dogs and subsequent human infection. The use of insecticide treated bed nets (ITNs) can offer good protection against transmission of leishmaniasis by endophagic sand fly vector. In Kenya, most vector species such as *P. duboscqi, P. guggisberg* Kirk and Lewis, *P. martini* Parrot, *P. pedifer, P. longipes* Parrot are exophilic and exophagous, thus negating common strategies such as insecticide treated nets (Davies *et al*., 2009).

2.6 Antileishmania activity of natural products

Traditional herbal medicines are gaining increased attention as they can reduce the risk of chronic diseases and act as antibiotics, antioxidants, and/or immunomodulators. Several studies have described the effects of plant extracts or isolated compounds in immune cells and cytokine production (Cragg and Newman, 2013) Thus, the study of active compounds obtained from plants used in traditional medicine plays a pivotal role in the search for new antileishmanial molecules (Dey *et al*., 2015; Calla-Magarinos *et al*., 2009). Several raw extracts from different plants have been shown to exhibit antileishmanial activity, which may not only be due to their direct action on the parasite, but also due to a concomitant effect on the host immune response (Calla-Magarinos *et al*., 2009). Therefore, the search for plant extracts with a wide spectrum of antileishmanial and immunomodulatory activities may enable the discovery of substances suitable for the disease control. Some studies have focused on the effects of leishmanicidal essential oils and plant extracts in the production of pro- and anti-inflammatory soluble mediators. Altogether, these studies suggest that the induction or inhibition of cytokine production is a critical factor for effective parasite destruction without producing excessive tissue damage.

Many people in rural areas depend largely on popular treatments to alleviate the symptoms, particularly the use of medicinal plants (Fournet and Muñoz, 2002). The natural products are potential sources of wide chemistry with a remarkable diversity and accessibility in nature. Recently, the Tropical Diseases Program of the World Health Organization with the Drug Discovery Research Program has considered a priority the pharmacological investigation of plants (Chan-Bacab and Peña-Rodríguez, 2001).

Extensive studies of activity of natural products against *Leishmania* during the last years have been accumulated. Recently, the most advances in this field have been excellent reviewed which have listed plants and natural product derived that showed some level of antileishmanial activity, (Chan-Bacab & Peña-Rodríguez, 2001; Fournet & Muñoz, 2002: Polonio & Efferth, 2008).

Some studies revealed the search of new products in microorganism or marine sources, such as a glycoprotein isolated from the sponge *Pachymatisma johnstonii*, which showed a high activity *in vitro* against *L. donovani, L. braziliensis* and *L. mexicana* at IC_{50} of 1µg protein/ml (Le Pape *et al*., 2000), and aphidicolin a fungal metabolite isolated from *Nigrospora sphaerica*, which inhibited the growing of promastigotes and amastigotes of *L. donovani* at a dosage of EC_{50} between 0.02-1.83µg/ml (Kayser *et al*., 2001). Nevertheless, studies on the evaluation of plants extract from different geographic areas have been reported like in Brazilian, Mexican, Colombian and Peruvian (Kvist *et al*., 2006; Peraza-Sánchez *et al*., 2007; Tempone *et al*., 2008) flora extracts showed the antileishmanial activity of plants used by people from endemic areas of Latin America. The antileishmanial activities of essential oil have been evaluated and details have been reviewed by Antony and col (Antony *et al*., 2005). The oil of *Croton cajucara*, a plant used in folk Brazilian medicine, causes the inhibition of *L. amazonensis* and increased the nitric oxide production (Rosa *et al*., 2003). Nerolidol is a compound present in the essential oils of some plants and inhibits the *in vitro* growing of *L. amazonensis, L. braziliensis* and *L. chagasi* at a concentration of 85, 74,75 µM respectively. Their

mechanism of action could be the inhibition of earlier steps of ergosterol synthesis (Arruda *et al*., 2005).

Other advanced studies have been done to evaluate potential compounds isolated from both synthetic and natural source, which displayed some of the most promising antileishmanial activity, table 2.1 and 2.2 below.

Table 2.1: Synthetic Products that Showed Antileishmanial Activity

Compound	Drug Design	Antileishmanial Activity	References
3-substituted quinolones	Potential activators of macrophages	Antileishmanial *in vitro* effects against *L. chagasi* promastigotes and amastigotes was observed at IC_{50} of 0.8µg/ml.	Tempone *et al*., 2005
9,9dimethylxanthene tricyclics	Inhibitors of trypanotione reductase	Caused *in vitro* inhibition of amastigotes of *L. donovani* with an ED50 values of 0.32 µM	Chibale *et al*., 2000
Edelfosine and Ilmofosine	New alkyl-lysophospholipid derivatives	Demonstrated high *in vitro* activity against *L. donovani* promastigotes and amastigotes with ED50 between the range of 26.73–33.31 µM against promastigotes and 16.46–23.16 µM against amastigotes.	Azzouz *et al*., 2005
N-acetyl-l-cysteine	Precursor of glutathione	Showed *in vivo* activity against *L. amazonensis* in BALB/c mice at a dosage of 200mg/kg daily	Chaga *et al*., 2018
Nicotinamide	Inhibitor of certain III Nicotinamide adenine dinucleotide-dependent Deacetylase	Caused *in vitro* inhibition of *L. infantum* promastigotes and amastigotes at concentration of 5mM.	Sereno *et al*., 2005

Table 2.2: Natural Products that Showed Antileishmanial Activity

Compound	Natural Source	Antileishmanial Activity	References
Canthin-6-one alkaloids	*Zanthoxylum chiloperone*	Demonstrated *in vivo* activity in BALB/c mice infected with *L. amazonensis* at concentration of 100mg/ml	Ferreira *et al*., 2002
Coronaridine	*Peschiera australis*	Showed *in vitro* activity against promastigotes and amastigotes of *L. amazonensis.* A 97% inhibition of promastigote growth was obtained with 12.5 mg of coronaridine per ml, while CLF at the same concentration inhibited 65% of growth	Delorenzi *et al.*, 2001.
Maesabalide III	*Maesa balansae*	Caused *in vitro* and *in vivo* activity against *L. donovani* at dosage of 0.2 mg/kg	Maes *et al*., 2004
Parthenolide	*Tanacetum parthenium*	Displayed activity against promastigotes and amastigotes of *L. amazonensis* at a concentration of 0.37 µg/ml.	Tiuman *et al*.,2005

2.7 Chrysanthemum cinerariaefolium (pyrethrum)

2.7.1 Introduction

Chrysanthemum is a cosmopolitan genus, comprising about 300 species of herbs and shrubs, among which a few yield the commercial insecticide known as Pyrethrin derived from their large, showy, multi-coloured flowers (Figure 2.6). Pyrethrum has been under cultivation around the world for nearly150 years with Kenya accounting for about 83% of the present world production (Kenya Agricultural Research Institute, 2008). *Chrysanthemum cinerariaefolium* is shown in the plate.

Figure 2.6: Chrysanthemum cinerariaefolium flower (Kenya Agricultural Research Institute, 2008)

2.7.2 Uses of Chrysanthemum cinerariaefolium

Pyrethrum has various uses in the world, some which are as old as human kind for instance, the yellow or white chrysanthemum flowers of the species *C. morifolium* are boiled to make a sweet drink in some parts of Asia. The resulting beverage is known simply as *chrysanthemum* tea. In Korea, a rice wine flavored with *chrysanthemum* flowers is called *gukhwaju*. *Chrysanthemum* leaves are steamed or boiled and used as greens, especially in Chinese cuisine (Regnault-Roger *et al*., 2005). The flowers may be added to thick snake-meat soup to enhance the aroma. Small *chrysanthemums* are used in Japan as a sashimi garnish (Philogene *et al*., 2008). *Chrysanthemum cinerariaefolium* is economically important as a natural source of insecticide. The flowers are pulverized, and the active components, called pyrethrins, which occur in the achenes, are extracted and sold in the form of an oleoresin. This is applied as a suspension in water or oil, or as a powder. Pyrethrins attack the nervous systems of all insects, and inhibit female mosquitoes from biting (https://en.wikipedia/ chrysanthemum). In sub-lethal doses they

have an insect repellent effect. They are harmful to fish, but are far less toxic to mammals and birds than many synthetic insecticides. They are not persistent, being biodegradable, and also decompose easily on exposure to light (Regnault-Roger *et al*., 2005).

2.7.3 Chemical constituents of pyrethrum

Search for the concerned active compounds has led to the isolation of several sesquiterpenoids, flavonoids, coumarins, triterpenoids, steroids, phenolics, purines, lipids, aliphatic compounds and monoterpenoids from different plant parts of *Chrysanthemum.* Aqueous extract of leaves and stem of *C. cinerariaefolium* produced a concentration-dependent contraction of the rat duodenum, which was abolished by atropine. Preliminary phytochemical screening revealed the presence of glycosides, flavanoids, tannins and alkaloids in the extract (Amos *et al*., 2000).

Chrysanthemum coronarium extracts showed high cytotoxicity. The essential oil from *C. sibiricum* exhibited cytotoxic properties along with mild antioxidant activity (IC50 = 97.2 mg/ml) (Lee *et al*., 2002).The essential oil of *C. viscidehirtum* exhibited strong activity against *Salmonella typhi* and *Proteus mirabilis* at a concentration of 1.25µg/ml. The activity was investigated by agar dilution method (Khallouki *et al*., 2000). The essential oil of *C. boreale* exhibited the activity against six Gram+ve bacteria and 8 Gram –ve bacteria (Kim *et al*., 2003). The activity of *C. Coronarium* was evaluated against 12 fungal species. In agar diffusion plate assay, the growth of *Alternaria* sp., *Aspergillusflavus* and *Pythiumultimum* was highly reduced (> 80%) at the third day (Kim *et al*., 2003). Activity was also shown by essential oil from *C. viscidehirtum* against four fungi at concentration of 150 ppm (Bouchra *et al*., 2003).

2.8 Inflammatory Response to *Leishmania* Infection

Parasite-host interaction is a complex process that modulates *Leishmania* infection and the immunological response to it, including inflammation. Several molecules are involved in inflammation during leishmaniasis, such as cytokines and the lipidmediator leukotriene

B4. Many of the molecules that promote inflammation also activate phagocytes leading to the production of nitric oxide, the main effector molecule in parasite killing. However, an exacerbated production of these molecules may also lead to tissue damage (Morato *et al*., 2014).

IL-10 is an important anti-inflammatory cytokine responsible for peripheral tolerance to self-antigens and preventing exacerbated immune responses to foreign antigens. However, when expressed in large quantities, IL-10 may have deleterious effects during leishmaniasis, leading to an early suppression of innate and acquired immune responses, pathogen proliferation, and aggravation of the disease (Saraiva and O'Garra, 2010). In leishmaniasis, phagocytes are stimulated to produce IL-10, which leads to a reduced production of cytokines related to the Th1 profile, such as interleukin 12 (IL-12) and IFN-γ (Nandan and De Oliveira, 2012). This causes a reduction in NO production that consequently reduces the microbicidal capacity of macrophages. Macrophages are the main host cell for *Leishmania* spp. When infected with these parasites, macrophages sustain *Leishmania* spp. growth. However, when activated to produce nitric oxide, macrophages can kill *L. major* parasites (Maria *et al*., 2012).

Interleukin 10 (IL-10) may be secreted by numerous cells, including macrophages, T cells, and B cells. The cytokines IL-12 and IL-4 also play an important role during *Leishmania* infection. They define the cell profile through the polarization of CD4+ T cells and modulate the response from other cells (Liew, 2002). IL-12 activates natural killer cells and CD8+ T cells, leading to IFN-γ production (Sun and Lanier, 2011). In addition, IL-12 induces the differentiation of CD4+ T cells to the Th1 profile, which also produces IFN-γ, a potent inducer of NO production in macrophages. Thus, IL-12 possesses an indirect microbicidal action. In contrast, IL-4 induces the differentiation of CD4+ T cells to a Th2 profile, which produces IL-4, IL-5, and IL-13. This profile suppresses NO production and leads to an increase in eosinophils (Rodr´ıguez and Wilson, 2014).

The importance of the type of immune response, if Th1 or Th2, lies in the fact that Th1 immune response characterizes the resistance mechanism to *Leishmania* infection, while Th2 response has been associated with susceptibility to parasite infection. The Th1 immune response is associated with production of proinflammatory cytokines such as IFN-γ, tumor necrosis factor alpha (TNF- α), and IL-12, while the susceptibility profile of Th2 response is characterized by anti-inflammatory cytokines expression such as IL-10 and IL-4 (Kumar and Nyl´en, 2012).

In humans, protection against VL is mediated by Th1 immune response whereas pathogenesis is associated with Th2 response. Some studies have demonstrated the importance of proinflammatory cytokines IFN-γ, TNF-α, and IL-12 in *L. donovani* infection. Depletion of these cytokines aggravated the disease progression or made hosts susceptible to infection by *L. donovani* (Bhattacharjee *et al*., 2012)

CHAPTER THREE

MATERIALS AND METHODS

3.1 Study site

This was a laboratory experimental study. The study was carried out in *Leishmania* laboratory at Centre for Biotechnology Research and Development, Kenya Medical Research Institute. Pyrethrum plant was collected from Baragoi, West Pokot County and transported to CTMDR for extraction of pyrethrin.

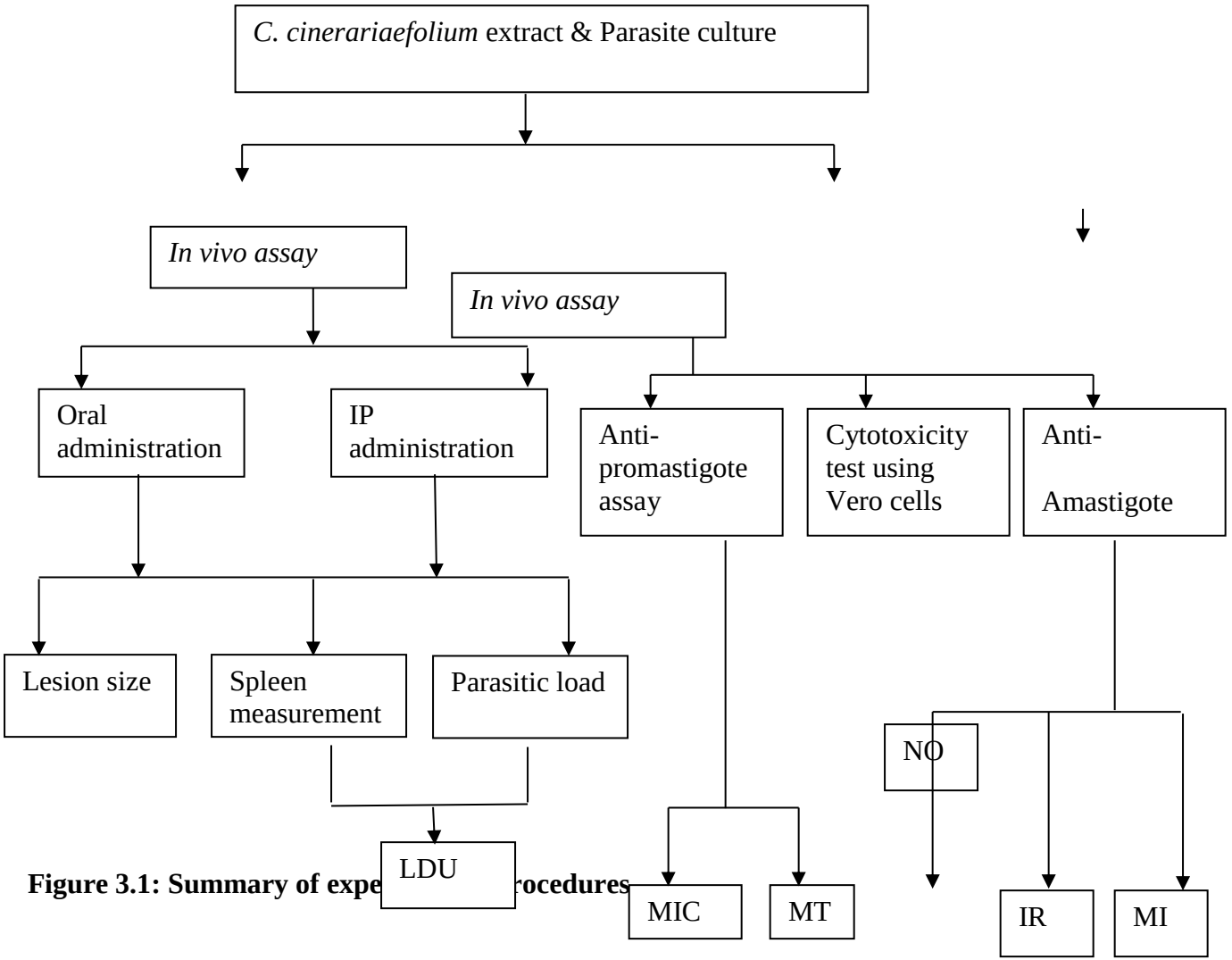

Figure 3.1: Summary of expe rocedures

Key: LDU= Leishmania 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

3.2 Extraction of pyrethrins

Pyrethrins were extracted from pyrethrum flowers as described by Shawkat *et al*., 2011. Pyrethrin is naturally known to occur in Chrysanthenum flowers. Briefly the flowers were extracted with petroleum ether (v/v) (analytical grade) at room temperature and filtered. The filtrate was further partitioned with water and methanol at a ratio of 4:1 (filtrate: methanol) vigorously shaken and allowed to settle. Yellowish layer containing pyrethrins were carefully removed and dried in a vacuum using a Buchi rotary evaporator R-200, Swizaland.

3.3 Quantification of Pyrethrins

For quantitative analysis of pyrethrins, method described by (Kasaj *et al*., 1999) with some modification was adopted. Briefly, extracted pyrethrins were dissolved in acetonitrile and filtered using a 0.22 μm watman number 1 filter. They were analyzed using a Beckman Coulter system Gold 126P solvent module high performance liquid chromatography. Separations was done on a C18 column (150 x 3.9 mm) using a solvent mixture of acetonitrile and water at a flow rate of 1.4 ml/minute. Absorbance was monitored at a wavelength of 225 nm. Pyrethrins in the extract were quantified on the basis of their retention time in comparison to the reference standard. Since individual pyrethrins were not available as pure compounds, quantification method was used, a commercial pyrethrum mixture with an estimated amount of 25% total pyrethrins as the

reference solution. This was obtained from the Pyrethrum Board of Kenya. The amount of total pyrethrins in the assayed sample was estimated by calculating the sum of measured peak areas of individual pyrethrins (Kasaj *et al*., 1999).

3.4 Parasites, cells and media

Leishmania major obtained from the spleen of infected BALB/c mice, maintained in the KEMRI animal house, were cultivated *in* vitro in Schneider's insect media (SIM) supplemented with 15% heat-inactivated fetal bovine serum (Githinji *et al*., 2010). The human normal vero cells were cultivated *in vitro* in defined minimum essential media containing 4 mM l-glutamine, 1 mM sodium pyruvate supplemented with 10% heat-inactivated foetal bovine serum and penicillin–streptomycin (100 UI/ml to100 µg/ml). All cells were incubated in a humidified atmosphere with 5% CO_2 at 37^{o}C except the *Leishmania* promastigotes which were incubated at 28 ˚C.

3.5 Evaluation of minimum inhibitory concentration (MIC)

MIC is defined as the lowest antimicrobial concentration that prevents visible growth of micro-organisms. MIC methods are widely used in the comparative testing of new agents, or when a more accurate result is required for clinical management (Andrews, 2001). MICs are used by diagnostic laboratories mainly to confirm resistance, but most often as a research tool to determine the *in vitro* activity of new antimicrobials.

To assess the anti-*Leishmania* activity of water and methanol extracts of pyrethrins, Leishmania promastigotes (1×10^6 parasites/ml) were 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 turbidity in order to evaluate MIC (Appendix 1). The lowest concentration of the samples that prevented the growth of *Leishmania* parasites *in vitro* were considered as the MIC.

3.6 *In vitro* test for antileishmanial activity (IC50).

The *in vitro* test was performed as described by Hoet *et al*. (2004). Amphotericin B (VESTAR, INC, USA) and Pentostam (The Wellcome Foundation, London) were used as positive controls. *Leishmania major* promastigotes were incubated in 24-wellplates. After

five days of cultivation, aliquots of parasites were transferred to 96-well micro-titer plate. The parasites were incubated at 27°C for 24 h and 200 µl of the highest concentration of each of the test samples were added and serial dilution carried out. The experimental plates were incubated further at 27°C for 48 h. The negative controls used were promastigotes with no drugs and media alone. Ten microlitres of MTT reagent was added into each well and the cells incubated for 2 - 4 hrs until a purple precipitate is clearly visible under a microscope. The medium together with MTT was aspirated off from the wells, a hundred microlitres of Dimethyl sulfoxide (DMSO) added and the plates shaken for 5 minutes. Absorbance was measured for each well at 562 nm using a microtiter plate reader and the 50% inhibitory concentration (IC_{50}) values generated. The assays were repeated in triplicates and were independent of each other. Percentage promastigote viability was calculated as follows:

$$Promastigote\ viability\ (\%) = \frac{(Average\ absorbance\ in\ drug\ wells - average\ blank\ wells) \times 100}{Average\ absorbance\ control\ wells}$$

3.7 Cytotoxicity

Toxicity of water and methanol extracts of pyrethrin on vero cells, cell line E6 (obtained from Polio laboratory, Centre for Virus Research, KEMRI) was determined using 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrozolium bromide (MTT).

The MTT assay is a colorimetric assay for assessing cell metabolic activity. NAD (P) H-dependent cellular oxidoreductase enzymes may, under defined conditions, reflect the number of viable cells present. These enzymes are capable of reducing the tetrazolium dye to its insoluble formazan, which has a purple color. MTT, a yellow tetrazole, is reduced to purple formazan in living cells (Mosmann, 1983) (Appendix 3). A solubilization solution (usually either dimethyl sulfoxide, an acidified ethanol solution, or a solution of the detergent sodium dodecyl sulfate in diluted hydrochloric acid) is added to dissolve the insoluble purple formazan product into a colored solution.

An important criterion in the search for compounds active against protozoans with therapeutic potential, is to determine whether they show toxic effects on mammalian host cells. For this purpose, a test of cytotoxicity to Vero cells was performed in order to determine the ratio of selectivity to biological activity.

Vero cells were cultured and maintained in Minimum Essential Medium MEM, supplemented with 10% FBS as described (Ngure *et al*,. 2009; Wabwoba *et al*., 2010). The cells were cultured at 37 ºC in 5% CO_2, harvested by trypsinization, pooled in a 50 ml vial and 100 µl cell suspension (1 x 10^5 cell/ml) put into 2 wells of rows A-H in a 96-well micro-titer plate for one sample. The cells were incubated at 37 ºC in 5% CO_2 for 24 hrs to attach, the medium aspirated off and 150µl of 1000µg/ml of each of the test samples and serial diluted to 31.25µg/ml. The experimental plates were incubated further at 37ºC for 48 hrs. The controls used was medium alone (no drugs and no cells). MTT reagent (10 µl) was added into each well and the cells incubated for 2 - 4 hrs. The medium together with MTT were aspirated off from the wells, after which 100µl of DMSO was added and the plates shaken for 5 minutes. The absorbance was measured at wavelength 562 nm using a ELX800 micro-titer plate reader (BioTek, Canada) (Patel *et al*., 2009). The assay were in triplicates. Cell viability (%) was calculated at each concentration as described by Huq *et al*., 2004 using the formula:

$$Cell\ Viability\ (\%) = \frac{(Average\ absorbance\ in\ duplicate\ drug\ wells - average\ blank\ wells)\ x\ 100}{\text{Average absorbance control wells}}$$

3.8 Determination of Nitric oxide production

Nitric Oxide released in macrophages culture was measured using the Greiss reaction for nitrites (Hollzmuller *et al*., 2002). Five hundred microlitres of the supernatants were collected 8 hours after treating cultured macrophages with extracts, Amphotericin B and untreated one. The assay was done in duplicate wells in the 96-well micro-titre plates. To do this, 60 ml of Greiss reagent A (1% Sulphonilmide in 1.2 M HCL) was added followed by 60 ml of Greiss reagent B (0.35 N [1-naphthy] ethlenediamine). The plates were read

at 540 nm in the enzyme linked immunosorbent assay (ELISA) reader. Sodium nitrite in RPMI was used to construct a standard curve for each plate reading.

3.9 Anti-amastigote assay

Adherent macrophages were infected with promastigotes at a parasite/macrophage ratio of 6:1 and further incubated at 37ºC in 5% CO_2 for 4 hours. Free promastigotes were removed by extensive washing with PBS and the cultures incubated in RPMI for 24 hours. Treatment of infected macrophages with the extract dissolved in RPMI was done once. Pentostam® and amphotericin B were used as positive control drug for comparison of parasite inhibition. The cells were incubated for 48hrs, washed with PBS, dried, fixed in methanol and stained with Geimsa stain. The numbers of amastigotes were determined by counting 100 macrophages in duplicate cultures, and the results expressed as infection rate (IR) and multiplication index (MI) (Berman and Lee, 1984). The infection rate was used in calculations of the Association Index (AI). The association indices were determined by multiplying the percentages of infected macrophages by the number of parasites per infected cell. Association indices were interpreted as the number of parasites that actually infected the macrophages.

IR = No. of infected macrophages in 100 macrophages

$$MI = \frac{(No.\,of\ amastigotes\ in\ experimental\ culture\ per\ 100\ macrophages)\ x\ 100}{\text{No. of amastigotes in control culture per 100 macrophages}}$$

3.10 Determination of the sample size

The sample size of this study was calculated using the resource equation method that depends on the law of diminishing returns in which , adding one experimental unit to a small experiment gives good returns, while adding it to a large experiment does not do so.

It depends on the size of the whole experiment and the number of treatment groups, not the individual group sizes. Mead suggests that if the error degrees of freedom (E) is

between about 10 and 20, then the experiment will probably be of an appropriate size. However, these limits should not be applied too rigidly. A good case can be made for E being 25 – 30 or more to ensure equal group sizes, and it can go even higher when the experimental units are not expensive. The value of E should lie between 10 and 20. If E is less than 10 then adding more animals will increase the chance of getting more significant result, but if it is more than 20 then adding more animals will not increase the chance of getting significant results.

E= (total number of animals used – the number of treatment groups)

E= N –T

Where:

- ***N*** is the total number of individuals or units in the study
- ***T*** is the treatment component, corresponding to the number of treatment groups (including control group) being used, or the number of questions being asked
- ***E*** is the degrees of freedom of the error component, and should be somewhere between 10 and 20 or more if units are cheap.

For three treatment groups each with 15 mice;

Hence;

N=45, T=3 hence, E= (45 – 3) = 42

The experiment was done in triplicate hence a total of 5×3 × 3= 45 mice, which is within acceptable limit as per the equation.

3.11 Infection of mice, treatment and determination of parasite numbers in cutaneous lesions

Fourty five BALB/c mice (6-8weeks old) (Appendix 2) were used for this experiment and divided into three groups of 15 each. Group 1 were experimental, treated with pyrethrin extracts, group 2 were positive controls treated with Pentostam and amphotericin B respectively. Group 3 were negative controls (treated with PBS). BALB/c mice were infected in the hind left footpad with 1 $\times 10^6$ *L. major* metacyclic promastigotes. In all experiments, treatment was initiated 4 weeks after infection had established as determined by the presence of lesions. Infected mice treated with PBS were used as negative controls. Experimental mice were treated with the standard drug, pentostam and Amphotericin B in regimens of 20mg per body weight daily for 30 days intraperitoneally. 250 µg/ml water and 125µg/ml of methanolic extracts were administered both orally (using a cannula) and intraperitoneally daily for 30 days. The untreated group received PBS. Lesion development was followed by measuring thickness of the infected footpad as compared to the thickness of the contralateral footpad prior to infection using a vernier caliper and expressed as follows:

Lesion size = Size of infected footpad-Contralateral uninfected footpad (mm)

Means of weekly readings were calculated to facilitate comparison of lesion progression.

3.12 Determination of visceralization (Leishman Donovan Units)

After 4 weeks of treatment, mice were sacrificed by inoculation with 100 µl of pentabarbitone sodium. Spleen impression smear were made on clean microscopic slides. They were left to dry followed by fixation using absolute methanol. The method of Bradley and Kirkley (1977) was used to quantitate the parasite loads**.** Briefly fixed slides

were immersed in a freshly prepared 5% giemsa stain solution for 20 minutes then flushed with tap water and left to dry. The slides were examined under a compound microscope for enumerating the number of amastigotes per 1000 host nuclei. The relative and total numbers of parasites in the spleen, named Leishman-Donovan Units (total LDU) respectively were calculated as follows:

LDU = No. of parasites/1000 host nuclei

Total LDU = LDU x organ weight x 2 x 10^5

Comparison between LDU values for all the groups was done.

3.13 Serum preparation

Blood was collected from the BALB/c mice tail into 2ml tube. Blood was allowed to clot for 30 minutes at 25°C and centrifuged at 2,000 x g for 15 minutes at 4°C using an Eppendorf micro centrifuge model 5424 (Thermo Fisher Scientific, USA). Top yellow serum layer was pipetted off without disturbing the white buffy layer. Serum was stored on ice. Serum was diluted 1:5 with diluted Assay Buffer before assaying.

3.14 Serum interferon-γ and IL-4 immunoassay

Serum interferon-γ immunoactivity was determined using a commercial single analyte ELISArray kit (SABiosciences) exactly according to the manufacturer's instructions (Appendix 4). Outcomes were quantified by optical density at 450 nm, with background correction at 570 nm, using ELX 800 a universal microplate reader. The detection levels of interferon-γ and IL-4 was 39pg/ml and 32.8 pg/ml respectively as per the kit insert guidelines.

3.15 Ethical considerations

This study was approved by Scientific & Ethics Review Unit (SERU) and Animal Care and Use committee (ACUC) of KEMRI (Appendix 7).

3.16 Statistical analysis

Data were recorded in Microsoft Excel and imported into SPSS 13.0 for analysis. All experiments were carried out in triplicate. The mean and standard deviation of at least three experiments were determined. Statistical analysis of the differences between mean values obtained for the experimental groups were done by Student's t test. P values of ≤ 0.05 or less were considered significant.

CHAPTER FOUR

RESULTS

4.1 Anti-promastigote assay

4.1.1 Determination of minimum inhibition concentration (MIC)

Water and methanol extracts had similar MIC as the reference drugs pentostam and amphotericin B (250µg/ml and 125µg/ml respectively). This therefore implies that the anti-Leishmania activity of the methanolic extracts was similar to that of the amphotericin B and same case for water extracts and pentostam. However results shows that the methanolic extracts (MIC=125 µg/ml) exhibited stronger activity against *L. major* parasites than the water extracts, but not significantly different (P>0.05) (Table: 4.1)

Table 4.1: MIC and IC_{50} values of methanol and water plant extracts on promastigotes

Test samples	Water extracts	Methanol extracts	Pentostam	Amphotericin B
MIC(µg/ml)	250±2.45	125±0.85	250±5.25	125±7.12
IC_{50}(µg/ml)	13.6±0.56	8.3±1.54	14.1±0.75	8.62±0.92

4.1.2 Determination of IC_{50} (inhibition concentration at 50%) values of methanol, reference drugs and water extracts on promastigotes

Methanolic extracts exhibited the lowest inhibition concentration value (IC_{50}) of 8.3µg/ml followed by, Amphotericin B, water extracts and pentostam with 8.62µg/m, 13.6µg/ml and 14.1µg/ml respectively that killed 50% of 1×10^6 promastigotes per milliliter. This

implies that methanolic extracts had stronger activity at a lower concentration, followed by Amphotericin B, water extracts and pentostam respectively. (Table.4.1)

4.1.3 Determination of cytotoxicity of methanol and water plant extracts on Vero cells

The methanolic and aqueous extracts of *Chrysanthemum cinerariaefolium* were screened for cytotoxic activity against vero cells using MTT assay. The vero cells were treated with 1000µg/ml of extracts in first well and serial diluted to 31.25 µg/ml. Methanol and water extracts showed low toxicity against healthy vero cells, which is manifested by the high concentration required to achieve the 50% inhibition concentration (IC_{50} 238 and 124 µg/ml) respectively for water and methanol. The low cytotoxic potential of the extract is of great significance for the management of Leishmaniasis (Table 4.2, Figure 4.1).

Table 4.2: Shows the toxicity levels of extracts, pentostam and amphotericin B on vero cells. The higher the IC_{50} the less toxic an extract.

Test samples	**Water extracts**	**Methanol extracts**	**Pentostam**	**Amphotericin B**
Toxicity IC_{50} (µg/ml)	238±3.1	124±1.5	106.8±0.6	68.6±0.09

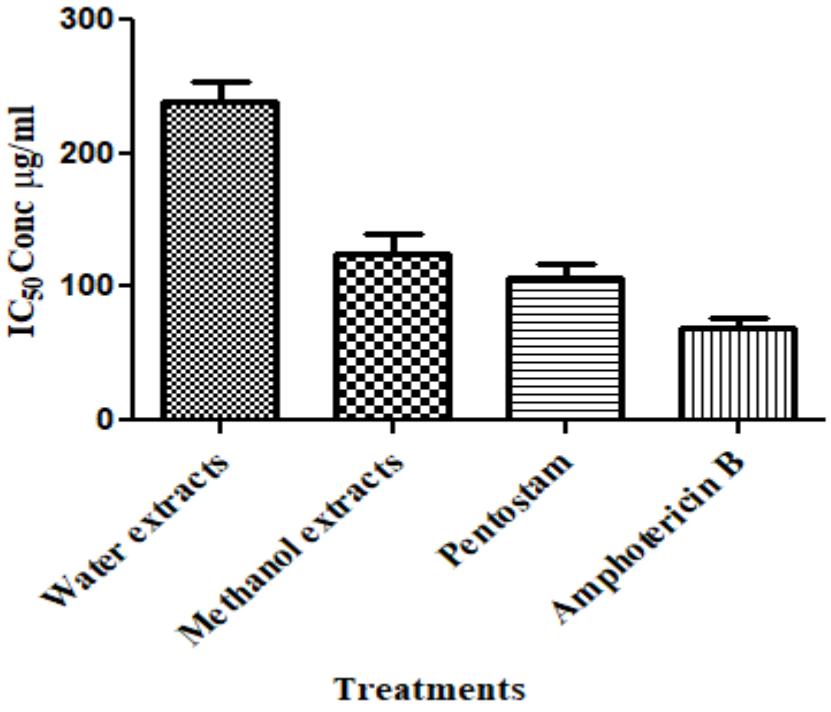

Figure 4.1: IC_{50} concetration of test samples compared to reference drugs.

4.1.4 Determination of Nitric Oxide production by stimulation of macrophages by water and methanol extracts

The nitric oxide production in supernatants from macrophages culture treated with test samples were determined and compared with sodium nitrite standard curve for test samples at concentration between 31.25 and 1000µg/ml. Nitric oxide production by macrophages was higher for methanolic treatment (37µM) as compared to water extracts, pentostam and amphotericin B (30µM) but was not significant (Mean= -5.99±5.62, t=-1.0662, P=0.1609). However the mean nitric oxide production for macrophages stimulated by treatment with water extract was more than the two reference drugs, pentostam and Amphotericin B. but was not significant (Mean=0.305±2.6, t = 0.1173, P=0.455) and (Mean 1.715±2.734, t=0.6273, P=0.2752) respectively.

The mean nitric oxide production for macrophages stimulated by treatment with both water and methanolic extract was significantly higher than that of RPMI (Mean 19.67± 2.52, t =7.7969, P<0.0001) and (Mean 25.66 ±5.33, t =4.8155, P=.001) respectively (Figure 4.2).

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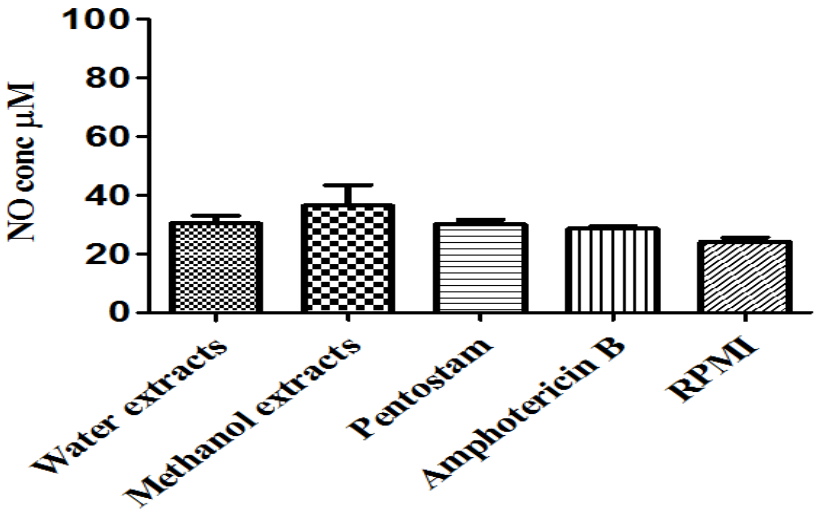


Figure 4.2: Nitric oxide production by macrophages after treatment with water, methanol extracts and reference drugs.

4.1.5 Comparing infection rates for water and methanolic extract treatment on infected macrophages with reference drugs and untreated macrophages

Infected macrophages were treated with test samples at concetrations between 31.25 µg/ml to 1000µg/ml and the proportion of infected macrophages counted and the means from the triplicate experiments calculated and compared to the positive and negative controls as shown in figure 4.3. The proportion of infected macrophages after treatment

with water extracts was significantly higher as compared to treatment with methanol extracts (19±4.16, t = 4 .5637, P=0.0224). , pentostam (44.67±5.8, t = 7.6854, P=0.0083) and amphotericin B (Mean= 39.67±6.39, t=6.2117, P= 0.0125) respectively. The proportion of infected macrophages after treatment with both water and methanolic extracts were significantly lower as compared to those treated with RPMI 1640 (negative control) (Mean= -16.67±3.84, t =-4.3355, P=0.0246).and (Mean= -35.67±1.856, t = -19.22, P= 0.0013) respectively.

However the proportion of infected macrophages after treatment with methanol extracts was significantly higher compared to both reference tratment pentostam (Mean= 25.67±4.67, t =5.5, P=0.0158) and amphotericin B (Mean= 20.67±5.04, t =4.0971, P=0.0274) respectively (figure.4.3)

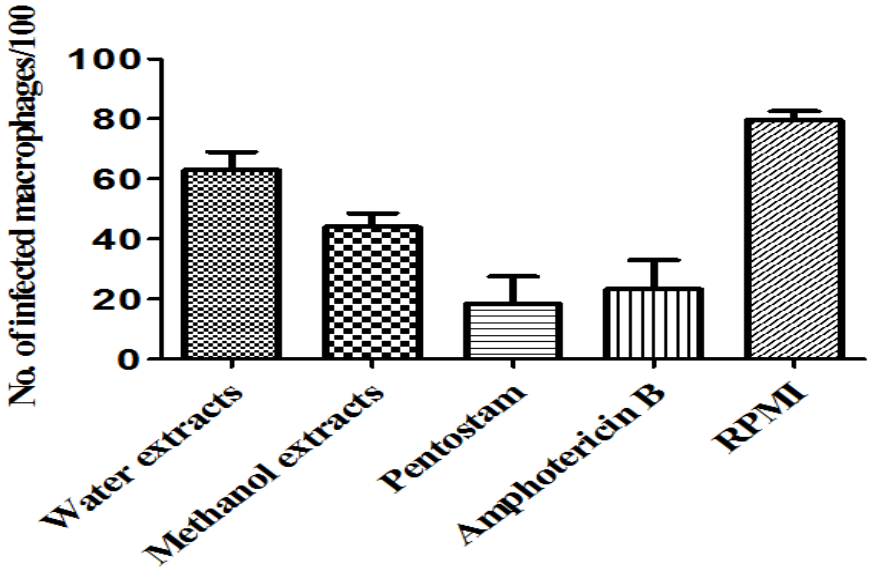


Figure 4.3: Infection rates

4.1.6 Evaluation of parasite growth in macrophages after treatment with the extracts and reference drugs.

Infected macrophages were treated with test samples at concetrations between 31.25 µg/ml to 1000µg/ml and the number of amastigotes in 100 infected macrophages counted and the means from the triplicate experiments calculated and compared to the positive controls as shown in figure 4.4.The mean difference in multiplication index (number of amastigotes) in macrophages treated with water extract was significantly higher than that treated with methanol extract (Mean18.8±2.35, t=7.9994, P=0.0076), pentostam (Mean =39.07±1.22, t=32.0599, P=0.0005) and Amphotericin B (Mean=35.4±2.05, t =17.266, P=0.0017) respectively. The mean multiplication index of macrophages treated with methanol extracts was significantly higher than the two reference drugs pentostam (Mean= 20.27±2.34, t=8.6698, P=0.0065) and Amphotericin B (Mean= 16.6±3.82, t = 4.3504, P=0.0245) (Figure: 4.4)

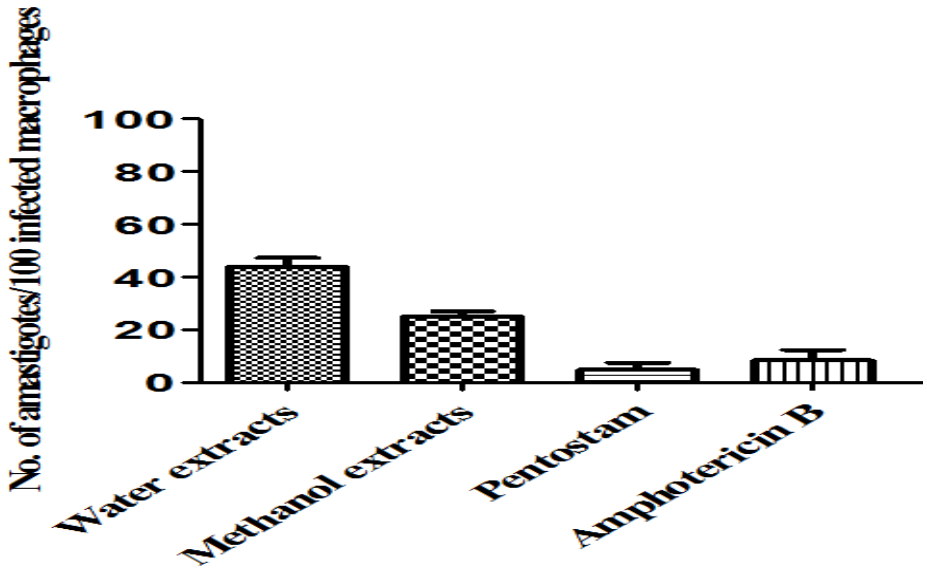


Figure 4.4: Comparing mean multiplication index after treatments

4.2 *In vivo* determination of antileishmanial activity of pyrethrum plant extracts

4.2.1 Determination of disease progression by measurement of lesion sizes and visceralization.

BALB/c mice infected on the left hind footpad with *L. major* promastigotes showed skin lesions as a single nodule with or without ulceration one month after inoculation. Efficacy of plant extract treatments on the development of lesion sizes in BALB/c mice was done by comparing the left hind infected foot pad with uninfected right foot pad. Treatment was started after four weeks of infection and 4 weeks post-treatment lesion sizes were determined. The PBS treated controls both for oral and intraperitneal route displayed the highest increase in lesion size. However, low rates of increase in lesion were recorded in water; methanol extracts and reference drugs treated BALB/c mice but not significant (P> 0.05) (Figure: 4.5)

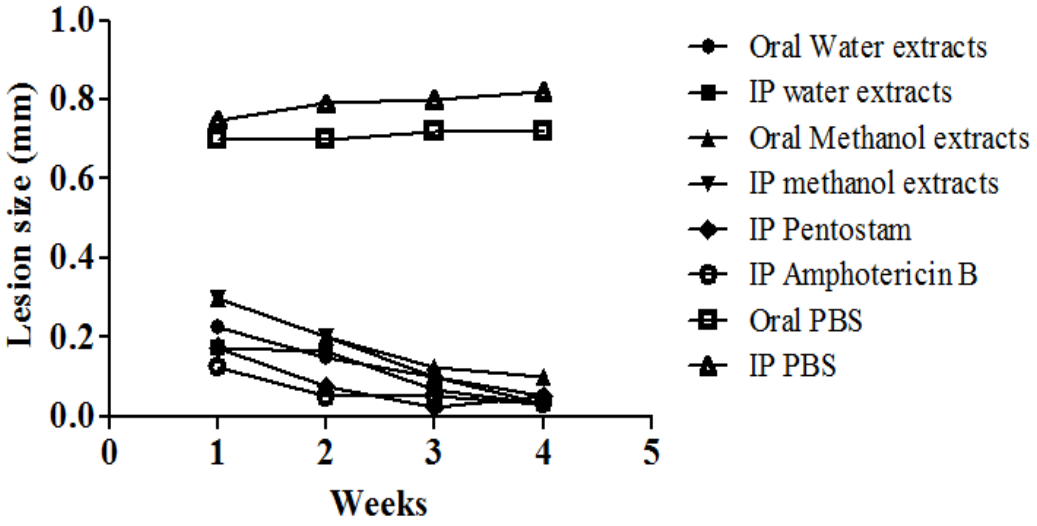


Figure 4.5: Comparison of lesion sizes in *L. major* infected BALB/c mice during treatments

4.2.2 Determination of visceralization after treatment

The study showed that both extracts and reference drugs significantly ($P < 0.05$) reduced the parasites load in the spleen of BALB/c mice compared to PBS treatment in both intraperitoneal and oral administration. The observation showed that methanolic extract administered orally and intraperitoneally had a better activity against leishmania infections when compared to water extracts with the same administration routes but not significant ($P>0.05$).However the reference drugs showed a better activity against leishmania infection when compared to both water and methanolic extracts but was not significant ($P>0.05$). Both oral and intraperitoneally treated mice with PBS had a significantly higher parasite burden compared to test samples and reference drugs ($P<0.05$) (Figure: 4.6)

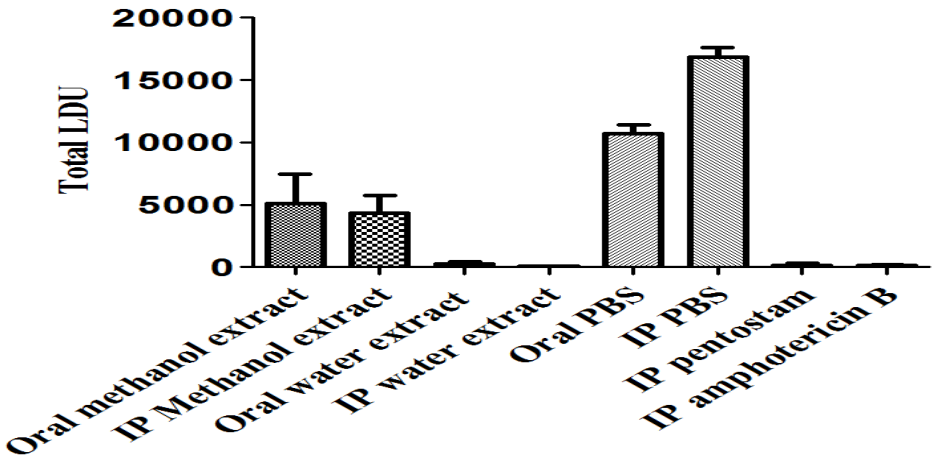


Figure 4.6: LDU in BALB/c mice following treatment with water, methanol extracts, reference drugs and PBS.

4.2.3 Serum levels of IFN-γ and IL-4 cytokines in L. major infected BALB/c mice treated with water, methanol extracts , PBS and reference drugs.

4.2.3.1 Comparison of the levels of IFN-γ and IL-4 cytokines after oral treatment of L. major infected BALB/c mice

BALB mice were treated with 125µg/ml methanolic and 250µg/ml water extracts of pyrethrin as per MIC concentration from the *invitro* experiments. BALB/c mice treated orally with 125µg/ml methanolic extracts of pyrethrin stimulated more production of IFN gamma levels than those treated with 250µg/ml water extracts but the difference was not significantly different (Mean= 11.5±42.31, t=0.2718, P=0.40). It was observed that both methanol and water extracts treatment stimulated significantly high production of IFN-γ than PBS treated mice, however water and methanol extracts treated BALB/c mice had significantly low levels of IL-4 cytokines as compared to PBS treated mice (Table 4.3).

Table 4.3: Levels of IFN-γ and IL-4 cytokines after oral treatment

IFN-γ						**IL-4**				
Pyrethrum extracts	**Mean diff**	**SDV**	**SEM**	**t**	**p**	**MeanDiff**	**SDV**	**SEM**	**t**	**p**
Methanol/water	11.5	84.61	42.31	0.272	0.40	-33.25	23.33	11.66	-2.85	0.032
Methanol/PBS	197.25	88.61	44.31	4.46	0.01	-70.5	47.89	23.95	-2.94	0.033
Water/PBS	185.75	36.49	18.24	10.18	0.001	-37.25	27.34	13.67	-2.72	0.036

4.2.3.2 Comparison of the levels of IFN-γ and IL-4 cytokines after intraperitoneal treatment of *L. major* infected BALB/c mice

BALB mice were treated with 125µg/ml methanolic and 250µg/ml water extracts of pyrethrin as per MIC concentration from the *invitro* experiments. It was observed that methanol extracts of pyrethrum administered intraperitoneally stimulates high production

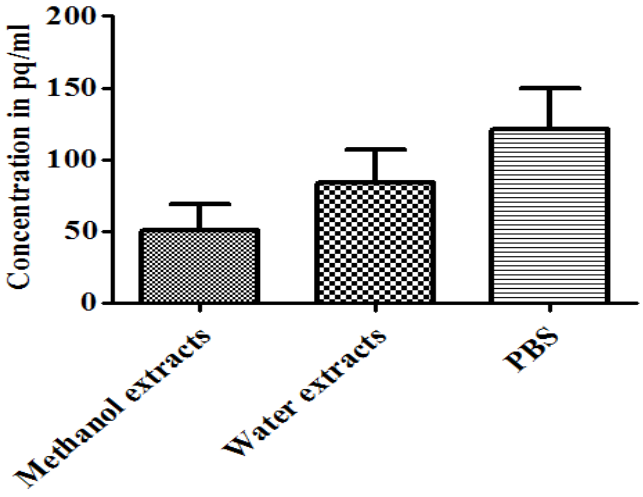
of IFN-γ than water extracts but not significant (P> 0.05) and vice versa for IL-4 production. BALB/c mice treated intraperitoneally with pentostam stimulated more production of IFN gamma than the methanolic extracts of pyrethrin treated mice but the difference was not significant.(P=0.2088), however methanolic extracts stimulated less amounts of IL-4 compared to pentostam treated mice but the difference was not significant (P=0.1178).

This study also found out that BALB/c mice treated intraperitoneally with amphotericin B stimulated more production of IFN gamma than the methanolic extracts of pyrethrin treated mice but the mean difference was not significant (P=0.3818), but BALB/c mice treated intraperitoneally with methanolic extracts of pyrethrin stimulated significantly less amounts of IL-4 than the amphotericin B treated mice (P=0.05). BALB/c mice treated intraperitoneally with methanolic extracts of pyrethrin significantly stimulated more production of IFN gamma than the PBS treated mice (P=0.05), however methanolic extracts of pyrethrin stimulated significantly less amounts of IL-4 than PBS treated mice (P=0.03).(Table 4.4, Figure 4.7)

Table: 4.4: Levels of IFN-γ and IL-4 cytokines after IP treatment

IFN-γ						IL-4				
	Mean diff	**Std-dev.**	**Std-err.**	**t-value**	**p-value**	**Mean Diff.**	**Std-dev.**	**Std-err.**	**t-value**	**p-value**
Methanol/water	43	61.49	35.5	1.21	0.17	-4.67	20.4	11.78	-0.40	0.37
Methanol/pento.	-31	53	3060	-1.01	0.21	-28	28.93	16.7	-1.67	0.12
Methanol/AmpB	-10	50.31	2905	-0.34	0.38	-15.3	7.57	4.37	-2.84	0.07
Methanol/PBS	115	74.48	43	2.67	0.06	-142	63.5	36.7	-3.87	0.03
Water/pento	-74	113.3	65.4	-1.13	0.19	-23.3	16	9.1	-2.52	0.06
Water/Amp B	-53	19.9	11.5	-4.6	0.02	-7.7	22.2	12.84	-0.60	0.31
Water/PBS	72	21.4	12.34	5.84	0.01	-137.3	76.1	43.9	-3.13	0.04

A



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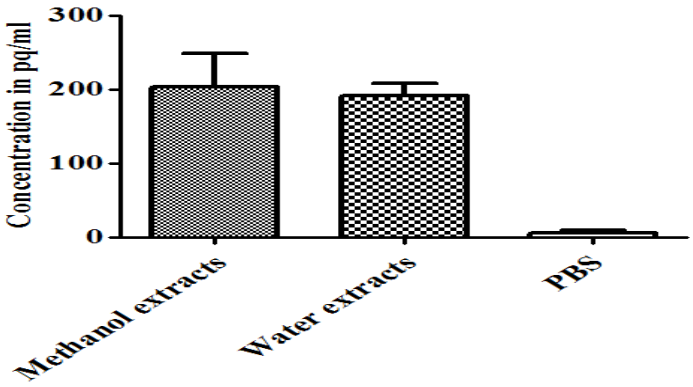
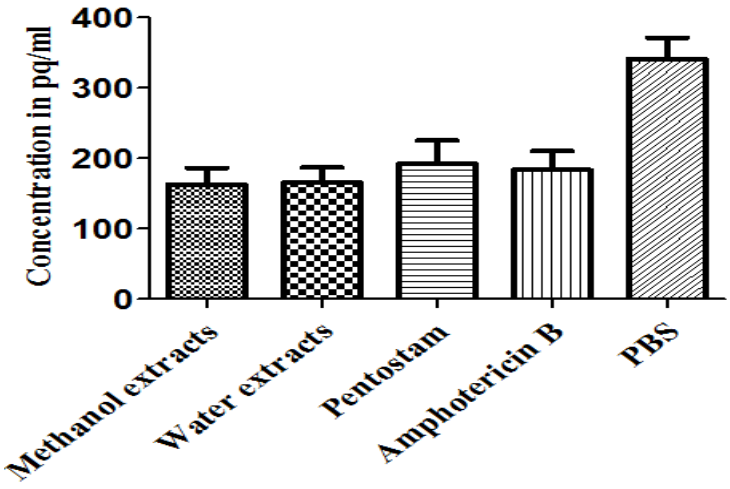


Figure 4.7: IFN-γ and IL-4 cytokine levels after oral treatment. A=IL-4 levels after oral treatment and B=IFN-ɣ levels after oral treatment

The reference drugs Amph.B and pentostam intraperitoneal treatment on *L.Major* infected BALB/c mice were able to stimulate higher production of IFN-γ compared to water extracts treatment, however the difference between Amph.B and water extracts was significant (P=0.022) where as the difference between pentostam and water extracts treatment was not significant (P=0.3055).

Treatment of BALB/c mice intraperitineally with water extracts of pyrethrin produced significantly low levels of IL-4 when compared to Amph.B, but the difference between water and pentostam treatment IL-4 production was not significant (Table 4.4, Figure 4.8)

A



B

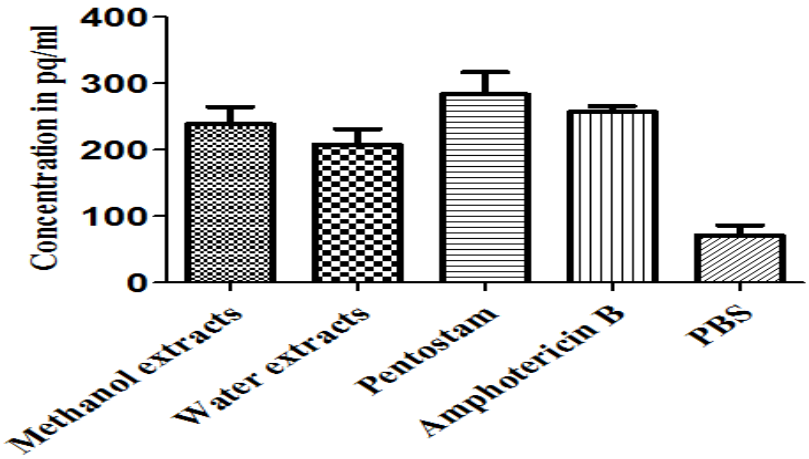


Figure 4.8: IFN-γ and IL-4 cytokine levels after IP treatment. A=IL-4 levels after lP treatment And B=IFN-ɣ levels after lP treatment

IFN-γ production by infected BALB/c mice after intraperitoneal treatment with water extracts was significantly higher than stimulation by PBS (control) treatment (P= 0.0141).

The IL-4 produced by infected BALB/c mice after intraperitoneal treatment with water extracts was significantly lower than that produced by the same treated with PBS(P=0.0445)(Table 4.4, Figure 4.8).

CHAPTER FIVE

DISCUSSION

The treatment of leishmaniasis in Kenya today with sodium stibogluconate is unsatisfactory as it is expensive, resistance, relapses and major adverse effects have been reported and is a serious issue in *Leishmania* control (Croft and Seifert, 2005). The two treatments i.e amphotericin B and miltefosine are being effectively used but their high cost and therapeutic complications limit their use in endemic areas (Berman, 1996). Traditional herbal medicines are gaining increased attention as they can reduce the risk of chronic diseases and act as antibiotics, antioxidants, and/or immunomodulators. Several studies have described the effects of plant extracts or isolated compounds in immune cells and cytokine production (Cragg and Newman, 2013). Thus, the study of active compounds obtained from plants used in traditional medicine plays a pivotal role in the search for new antileishmanial molecules (Calla-Magarinos *et al*., 2009; Dey *et al*., 2015).

The methanolic extract of the *C. cinerariaefolium* exhibited antileishmanial activity with adequate selectivity (IC_{50} 8.3µg/mL), which matches with the previously study by Yoshie *et al*., (2011) showing antiplasmodial activity with IC_{50}s between 4 and 12µg /ml, and antitrypanosomal activity with IC_{50}s from 7 to 31µg. In the current study methanolic extracts exhibited lower MIC (125µg/ml) as compared to water extracts (250µg/ml). This suggests that the methanolic extract has more antileishmanial activity against promastigotes *in vitro* than the water extracts. However both methanolic and water extracts had the same MIC as that of pentostam and amphotericin B respectively (Figure 4.1). This study therefore suggests that the activity of methanolic extracts was similar to that of pentostam and that of water extracts similar to that of amphotericin B in an *in vitro* assay.

Previous studies carried out on phytochemical screening revealed the presence of glycosides, flavanoids, tannins, alkaloids, sesquiterpenoids, coumarins, triterpenoids,

steroids, phenolics, purines, lipids, aliphatic compounds and monoterpenoids in the plant extracts (Amos *et al*., 2000). The individual activities of these compounds have been proven (Cowan, 1999). Moreover, different studies have shown potent antileishmanial activities of these compounds such as terpenic derivatives, carvacrol, p-cymene, thymol, carvone, limonene, and terpinene (de Melo *et al*., 2013; Monzote *et al*., 2014). Therefore, the presence of these phytoconstituents in *C. cinerariefolium* extract could be responsible for their antileishmanial effect though their exact mode of action is not clear. This action was attributed to iron-dependent enzymes and membrane lysis of the parasite due to the presence of flavonoids, alkaloids in pyrethrins (Amos *et al*., 2000). Flavonoids have shown to be active against *L. donovani* intramacrophage amastigotes with IC_{50} range from 1.7-3.6 µM (Lewin *et al*., 2014), supporting this current study. Methanolic and water extracts of *C. cinerariefolium* were efficacious against amastigotes with significant difference with amphotericine B and pentostam however the water and methanol extracts at a concentration between 31.25µg/ml and 1000µg/ml showed low toxicity against vero cells. Thus, it can be suggested that *C. cinerariefolium* methanolic and water extracts are safe for mammalian cells. The antileishmanial activity of some plant extracts has been attributed to flavonoids (Manjolin *et al*., 2013; Wong *et al*., 2014). Flavonoids such as catechins are able to form complexes with the parasite cell wall to influence processes requiring cell linking, and hence inhibit the parasite growth (Ogeto *et al.,* 2013). In a research work carried out by Manjolin *et al*. (2013), it has been shown that dietary flavonoids such as fisetin (the most potent alkaloid), quercetin, luteolin and 7,8-hydroxyflavone with low cytotoxicity characteristics was able to inhibit arginase enzyme from *L. amazonensis*. Arginase plays a central role in the biosynthesis of polyamine which is very important and essential for protecting the parasite against oxidative stress and ROS produced by the host's defense system. Therefore the proposed mechanism of action for water and methanolic extracts of *C. cinerariaefolium* may have been due to inhibition of arginase enzyme.

A review conducted by Mishra *et al*. (2009) has reported the leishmanicidal activity of various alkaloids including indole, quinoline, isoquinoline, pyrimidine-carboline, steroidal and diterpene derived from various plants by interfering with the macromolecular biosynthesis and inhibit respiration of amastigotes, modulation of mitogen activated protein kinase, a regulatory enzymes for apoptosis and inflammation, induction apoptotic-like death cell mediated by free radicals and Nitric oxide production (Sen and Chatterjee, 2011). The methanolic and water extracts of *C. cinerariefolium* efficacy on *L. major* parasites may be attributed to the presence of alkaloids since in this study a significant amount of Nitric oxide was produced.

The previous studies have shown that extracts of *C. cinerariefolium* contain triterpenoids which have also been extracted from roots of *Plumbago capensis* and have shown to have leishmacidal activity against *L. major* parasites (Makwali *et al*., 2012), therefore this current study suggests that water and methanol extracts of *C. cinerariefolium* may contain triterpenoids. Their potential in killing *L. major* parasites may be attributed to increased production of NO, induced apoptosis via inhibition of parasite DNA topoisomerase, and inhibit the parasitic growth (Sen and Chatterjee, 2011).

C. cinerariefolium has been shown to contain tannins (Moss *et al*., 2000) that have shown antleishmanial activity through increased production of NO in infected macrophages and enhance the expression of cytokines including tumor necrosis factor-α (TNF-α) and IFN-γ. It has been shown that acquired immunity in murine cutaneous leishmaniasis caused by *L. major* is mediated by parasite induced production of IFN-γ by CD4 T cells. This study suggests that *C. cinerariefolium* extracts may have induced production of TNF-α that contributed to protective immunity by synergizing with IFN-γ to activate macrophages for NO synthesis (Melby and Anstead, 2001).

Methanolic and water extracts of *C. cinerariefolium* on *L. major* infected BALB/c mice exhibited decreased lesion sizes and parasite burden in the spleen. This may be attributed to exhibited high amounts of IFN-γ production by CD4+T cells which has been associated

with healing of *L. major*-infected C57BL/6 mice, while low levels of IL-4 production associated with susceptibility in the BALB/c mice. Macrophages have been identified as inducible nitric oxide synthase (iNOS)-producing cells within the skin lesions, and regions where iNOS levels were high had decreased parasite loads and small skin lesions (Stenger *et al*., 1994). In the current study the levels of NO production in macrophages (in vitro) after stimulation with water and methanolic extracts were similar as those of pentostam and amphotericin B. this suggests that the plant extracts had same potential in stimulating macrophages to produce NO. This observation correlates with the exhibited low skin lesion sizes and parasite loads in the spleen in BALB/c mice.

Routes of administration greatly affect bioavailability by changing the number of biologic barriers a drug must cross or by changing the exposure of drug to pumping and metabolic mechanisms (Stan *et al*., 2011). From the current study oral and intraperitoneal administration routes were compared in respect to treatment with water and metrhanolic extracts of pyrethrum flowers. Both oral and IP routes of administration of methanol extracts were more effective against leishmania parasites compared to water extracts using same routes, however the difference was not significant. The same scenario was depicted in reference drugs where the infected mice treated with reference drugs through the IP routes had lower lesion sizes and parasite burdens compared to mice treated intraperitoneally with water and methanolic extracts.

CHAPTER SIX

CONCLUSION AND RECOMMENDATIONS

6.1 Conclusions

1. This study demonstrates the antileishmanial activity of *C. cinerariefolium* suggesting that water and methanol extracts contain active compounds against *L. major* promastigotes and amastigotes, therefore they could serve as an alternative agent in the control of leishmaniasis.
2. Reduction of lesions sizes, healing of lesion ulcerations and inhibiton of amastigotes in macrophages and spleen by extracts could be attributed to high amounts of IFN-γ production by CD4+T as a result of immunomodulatory effects by the extracts which has been associated with healing of *L. major*-infected C57BL/6 mice.
3. Water and methanolic extracts of *C. cinerariefolium* have high activity against *Leishmania major* parasites as shown by low MIC values (250µg/ml and 125µg/ml respectively), IR, MI and LDUs. However methanol extracts was more active than water extracts because it had low MIC, IC_{50} (8.3µg/ml), infection rates and parasite burden as compared to water extracts (IC_{50}=13.6µg/ml).
4. Water and methanol extracts of *C. cinerariefolium* were less toxic on vero cells as compared to pentostam and amphotericin B.
5. Water and methanolic extracts of *C. cinerariaefolium* stimulated the macrophages to produce NO but the levels were not significant compared to those of pentostam and Amphotericin B.
6. Water and methanol extracts of *C. cinerariaefolium* stimulated T cells to produce high levels of IFN-γ and low IL-4 cytokines in *Leishmania major* infected BALB/c mice, suggesting a positive immunomodulators activity.
7. This study shows that both oral and intraperitoneal route of administration of water and methanol extracts into mice exhibited similar antileishmanial activity.

Therefore the route of administration does not influence the efficacy of the two extracts.

6.2 Recommendations

The anti-inflammatory activity of these extracts witnessed in this study forms basis for further studies.

The single dosage of the water and methanolic extracts of *C. cinerariaefolium* used *in vivo* studies was derived from MIC experiments, therefore there is need for more studies to be carried out using different dosages.

Further studies on phytochemical characterization should be done to determine the bioactive ingredients present in water and methanolic extracts.

Further studies on comnbination of water and methanolic extracts of *C. cinerariaefolium* should be done to determine if there is synergism

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Data source & Map production: WHO/NTD/IDM HIV/AIDS, Tuberculosis and Malaria (HTM) World Health Organization, October 2010

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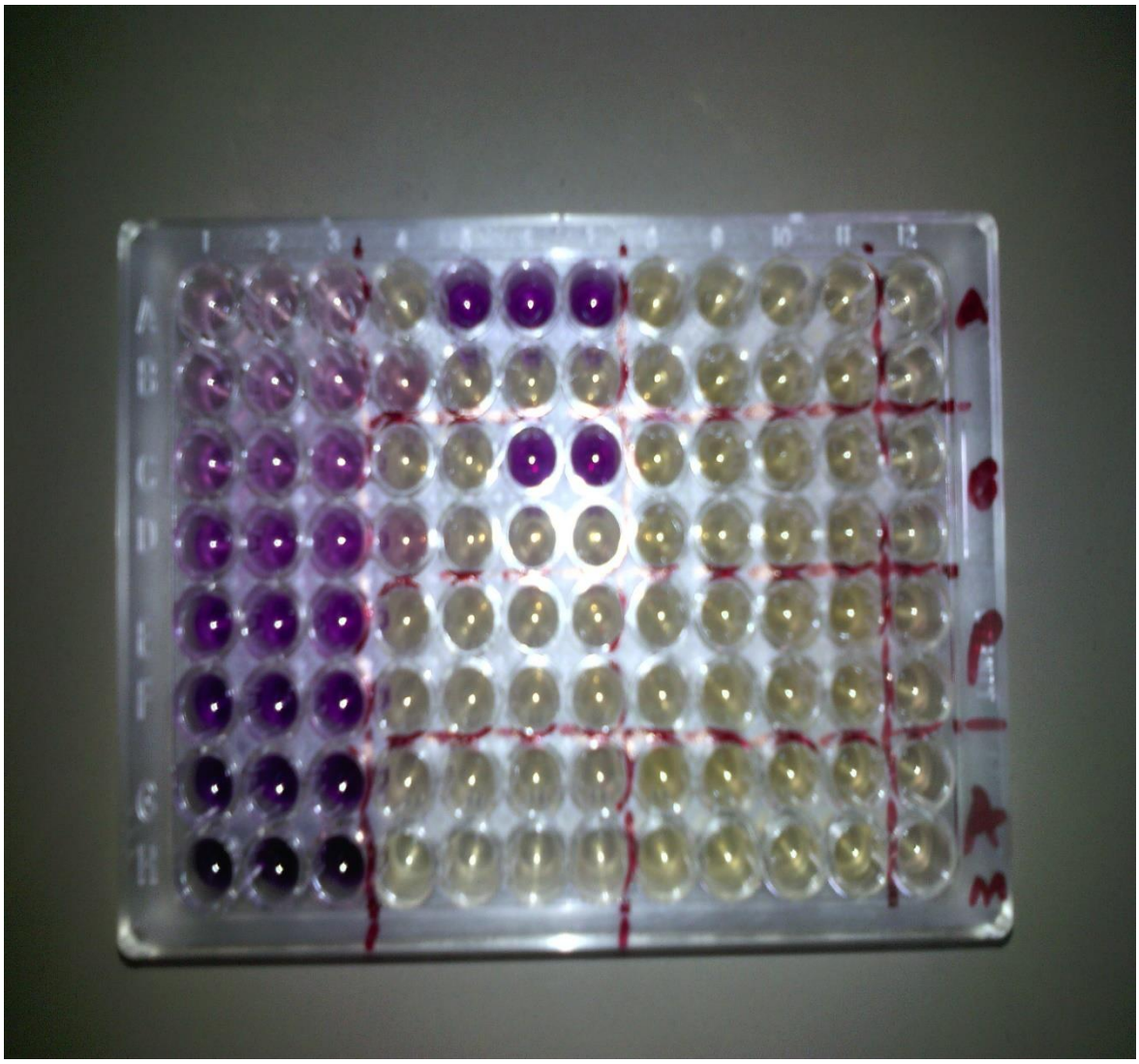
APPENDICES

Appendix 1: Determination of MIC using microscopy

Appendix II: 6-8 weeks old BALB/c mice

Appendix III: 96 well plate showing change of color during MTT assay



Appendix IV: Single Analyte ELISArray Kit

Mouse IFNγ

Catalog Number	Contents
SEM03121A	One Plate of Twelve 8-well Strips, pre-coated with capture antibody, and Reagents

Description

The Mouse IFNγ Single Analyte ELISArray Kit is designed to quantitatively measure the amount of this cytokine in cell culture supernatant, serum or plasma using a conventional enzyme-linked immunosorbent assay (ELISA). Each 96-well plate (8 wells/strip, 12 strips) is coated with the protein-specific capture antibody. We screened all commercially available antibodies to identify the best capture and detection antibodies. The high sensitivity, good linearity and low background of the Single Analyte ELISArray Kits provide reliable and reproducible results for your cytokine and chemokine analyses.

More information on IFNγ from Wikipedia:

"In contrast to interferon-α and interferon-β which can be expressed by all cells, IFN-γ is secreted by T lymphocytes and NK cells only. Also known as immune interferon, IFN-γ is the only Type II interferon. It is serologically distinct from Type I interferons and it is acid-labile, while the type I variants are acid-stable. IFN-γ has antiviral, immunoregulatory, and anti-tumour properties. It alters transcription in up to 30 genes producing a variety of physiological and cellular responses. Activation by IFN-γ is achieved by its interaction with a heterodimeric receptor consisting of IFNGR1 & IFNGR2 (interferon gamma receptors). IFN-γ binding to the receptor activates the JAK-STAT pathway. In addition, IFN-γ activates APCs and promotes Th1 differentiation by upregulating the transcription factor T-bet. IFN-γ is the hallmark cytokine of Th1 cells (Th2 cells produce IL-4). NK cells and CD8+ cytotoxic T cells also produce IFN-γ. IFN-γ suppresses osteoclast formation by rapidly degrading the RANK adaptor protein TRAF6 in the RANK-RANKL signaling pathway, which otherwise stimulates the production of NFκB."

Kit Contents / Packing List / Storage Conditions

Please check the kit components immediately after you receive this package. SABiosciences is not responsible for any missing items not reported within two (2) business days upon receipt.

Shelf Life: Do not use kit beyond the expiration date printed on the label.

Enough reagents are provided to process the included plate of 12 ELISA strips.

Component / Description	Quantity
BOX 1	
Antigen Standard (1 ug/ml)	1.5-ml tube
Detection Antibody	1.5-ml tube
Avidin-HRP Conjugate	1.5-ml tube
10% BSA	15 ml bottle
Donkey Serum*	15 ml bottle
BOX 2	
Pre-coated Capture Antibody 8-well strips	One plate of 12 strips in a pouch
Sample Dilution Buffer Stock	60 ml bottle
Assay Buffer Stock	60 ml bottle
Wash Buffer (10× Concentrate)	125 ml bottle
Development Solution	25 ml bottle
Stop Solution	60 ml bottle

Box 1 is shipped on dry ice or blue ice packs and should be stored at -20 °C upon receipt.
Box 2 is shipped at ambient temperature and should be store at 4 °C upon receipt.

* Customs Agent: To comply with USDA regulations, Donkey (*Equus asinus*) Serum is not contained in cross-border shipments of this kit. This component is packed separately into the kit upon arrival at the distributor in Japan.

Brief Protocol

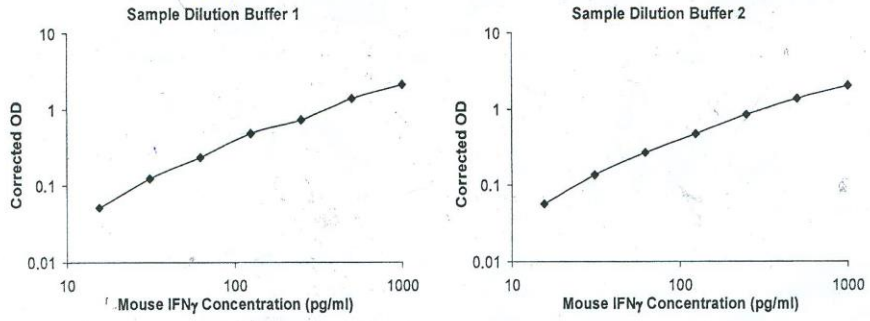
The Brief Protocol is meant for experience users only.
First-time users should completely read the User Manual.

1. Prepare replicate serial dilutions of the Antigen Standard and your experimental samples.
2. Pipette 50 µl of Assay Buffer into each well of the 8-well ELISArray strips.
3. Transfer 50 µl samples and/or standards to the appropriate wells of the ELISArray strips.
4. Gently shake or tap plate for 10 seconds. Incubate for 2 hours at room temperature.
5. Washing ELISArray Wells:
   Decant or aspirate well contents. Add 350 µl 1× Washing Buffer. Gently shake or tap plate for 10 seconds. Decant or aspirate. Blot array upside down on absorbent paper to remove any residual buffer. Repeat wash twice more.
6. Pipette 100 µl of Detection Antibody solution. Incubate 1 hour at room temperature.
7. Wash ELISA wells as described above.
8. Add 100 µl Avidin-HRP solution to all wells. Incubate for 30 minutes at room temperature.
9. Wash ELISA wells for a total of 4 washes.
10. Add 100 µl of Development Solution to each well. Incubate the plate for 15 minutes at room temperature in the dark.
11. Add 100 µl of Stop Solution to each well. The color changes from blue to yellow.
12. Read absorbance at 450 nm within 30 minutes of stopping the reaction. If wavelength correction is available, subtract readings at 570 nm from the reading at 450 nm.

Lot-Specific Performance Characteristics:

Standard Curves

	Sample Dilution Buffer 1					Sample Dilution Buffer 2				
[IFNγ] (pg/ml)	OD450 1	OD450 2	OD450 3	Average OD450	Corrected OD450	OD450 1	OD450 2	OD450 3	Average OD450	Corrected OD450
0	0.090	0.071	0.068	0.077	0.000	0.081	0.079	0.065	0.075	0.000
16	0.145	0.120	0.120	0.128	0.052	0.143	0.125	0.126	0.131	0.057
31	0.216	0.189	0.193	0.200	0.123	0.222	0.198	0.206	0.208	0.134
63	0.331	0.298	0.298	0.309	0.232	0.327	0.332	0.356	0.338	0.264
125	0.581	0.531	0.580	0.564	0.488	0.562	0.577	0.501	0.547	0.472
250	0.861	0.803	0.781	0.815	0.739	0.923	0.901	0.934	0.919	0.844
500	1.474	1.414	1.520	1.469	1.393	1.376	1.446	1.519	1.447	1.372
1000	2.352	1.981	2.266	2.200	2.123	2.035	2.059	2.213	2.102	2.028



Sensitivity

Minimal Detectable Dose (LOD)	39.0 pg/ml IFNγ

Limit of Detection (LOD) = mean optical density zero antigen control + two standard deviations (n =16)

Mouse IL4

Catalog Number	Contents
SEM03013A	One Plate of Twelve 8-well Strips, pre-coated with capture antibody, and Reagents

Description

The Mouse IL4 Single Analyte ELISArray Kit is designed to quantitatively measure the amount of this cytokine in cell culture supernatant, serum or plasma using a conventional enzyme-linked immunosorbent assay (ELISA). Each 96-well plate (8 wells/strip, 12 strips) is coated with the protein-specific capture antibody. We screened all commercially available antibodies to identify the best capture and detection antibodies. The high sensitivity, good linearity and low background of the Single Analyte ELISArray Kits provide reliable and reproducible results for your cytokine and chemokine analyses.

More information on IL4 from NCBI:
"The protein encoded by this gene is a pleiotropic cytokine produced by activated T cells. This cytokine is a ligand for interleukin 4 receptor. The interleukin 4 receptor also binds to IL13, which may contribute to many overlapping functions of this cytokine and IL13. STAT6, a signal transducer and activator of transcription, has been shown to play a central role in mediating the immune regulatory signal of this cytokine. This gene, IL3, IL5, IL13, and CSF2 form a cytokine gene cluster on chromosome 5q, with this gene particularly close to IL13. This gene, IL13 and IL5 are found to be regulated coordinately by several long-range regulatory elements in an over 120 kilobase range on the chromosome. Two alternatively spliced transcript variants of this gene encoding distinct isoforms have been reported."

Kit Contents / Packing List / Storage Conditions

Please check the kit components immediately after you receive this package. SABiosciences is not responsible for any missing items not reported within two (2) business days upon receipt.

Shelf Life: Do not use kit beyond the expiration date printed on the label.

Enough reagents are provided to process the included plate of 12 ELISA strips.

Component / Description	Quantity
BOX 1	
Antigen Standard (1 µg/ml)	1.5-ml tube
Detection Antibody	1.5-ml tube
Avidin-HRP Conjugate	1.5-ml tube
10% BSA	15 ml bottle
Donkey Serum*	15 ml bottle
BOX 2	
Pre-coated Capture Antibody 8-well strips	One plate of 12 strips in a pouch
Sample Dilution Buffer Stock	60 ml bottle
Assay Buffer Stock	60 ml bottle
Wash Buffer (10× Concentrate)	125 ml bottle
Development Solution	25 ml bottle
Stop Solution	60 ml bottle

Box 1 is shipped on dry ice or blue ice packs and should be stored at -20 °C upon receipt.
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Brief Protocol

The Brief Protocol is meant for experience users only.
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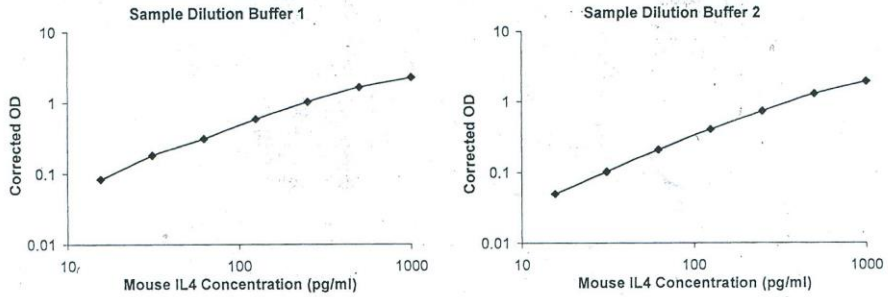
1. Prepare replicate serial dilutions of the Antigen Standard and your experimental samples.
2. Pipette 50 µl of Assay Buffer into each well of the 8-well ELISArray strips.
3. Transfer 50 µl samples and/or standards to the appropriate wells of the ELISArray strips.
4. Gently shake or tap plate for 10 seconds. Incubate for 2 hours at room temperature.
5. Washing ELISArray Wells:
   Decant or aspirate well contents. Add 350 µl 1× Washing Buffer. Gently shake or tap plate for 10 seconds. Decant or aspirate. Blot array upside down on absorbent paper to remove any residual buffer. Repeat wash twice more.
6. Pipette 100 µl of Detection Antibody solution. Incubate 1 hour at room temperature.
7. Wash ELISA wells as described above.
8. Add 100 µl Avidin-HRP solution to all wells. Incubate for 30 minutes at room temperature.
9. Wash ELISA wells for a total of 4 washes.
10. Add 100 µl of Development Solution to each well. Incubate the plate for 15 minutes at room temperature in the dark.
11. Add 100 µl of Stop Solution to each well. The color changes from blue to yellow.
12. Read absorbance at 450 nm within 30 minutes of stopping the reaction. If wavelength correction is available, subtract readings at 570 nm from the reading at 450 nm.

Lot-Specific Performance Characteristics:

Standard Curves

	Sample Dilution Buffer 1					Sample Dilution Buffer 2				
[IL4] (pg/ml)	OD450 1	OD450 2	OD450 3	Average OD450	Corrected OD450	OD450 1	OD450 2	OD450 3	Average OD450	Corrected OD450
1000	2.481	2.257	2.218	2.319	2.266	1.986	2.001	1.956	1.981	1.915
500	1.689	1.715	1.709	1.704	1.652	1.356	1.322	1.375	1.351	1.285
250	1.094	1.095	1.064	1.084	1.032	0.813	0.791	0.778	0.794	0.728
125	0.665	0.639	0.617	0.640	0.588	0.468	0.474	0.461	0.468	0.402
63	0.381	0.349	0.350	0.360	0.308	0.266	0.272	0.277	0.272	0.206
31	0.260	0.219	0.213	0.231	0.178	0.166	0.167	0.169	0.168	0.102
16	0.149	0.125	0.133	0.135	0.083	0.114	0.120	0.111	0.115	0.049
0	0.049	0.059	0.050	0.052	0.000	0.062	0.067	0.068	0.066	0.000



Sensitivity

Minimal Detectable Dose (LOD)	32.8 pg/ml IL4

Limit of Detection (LOD) = mean optical density zero antigen control + two standard deviations (n =16)

Appendix V: Raw data for IFN-gamma ELISA results

Software Version	2.05.5
Experiment File Path:	C:\Users\CBRD\Desktop\INFg.xpt
Protocol File Path:	
Plate Number	Plate 1
Date	11/30/2006
Time	12:12:57 AM
Reader Type:	ELx800
Reader Serial Number:	Unknown
Reading Type	Reader
Procedure Details	
Plate Type	96 WELL PLATE
Read	Absorbance Endpoint
	Full Plate
	Wavelengths: 450
	Read Speed: Normal

Results

	1	2	3	4	5	6	7	8	9	10	11	12	
A	1.148	1.128	1.272	0.908	0.765	0.6	0.641	0.68	0.724	0.855	0.74	0.833	450
B	0.743	0.782	0.863	0.66	0.681	0.553	0.562	0.674	0.622	0.673	0.602	0.677	450
C	0.573	0.56	0.587	0.533	0.482	0.445	0.513	0.598	0.522	0.727	0.676	0.776	450
D	0.407	0.47	0.557	0.455	0.45	0.361	0.442	0.535	0.507	0.648	0.719	0.762	450
E	0.284	0.369	1.031	0.324	0.274	0.313	0.356	0.446	0.391	0.588	0.648	0.681	450
F	0.235	0.357	0.338	0.292	0.272	0.299	0.337	0.369	0.334	0.464	0.501	0.519	450
G	0.272	0.353	0.354	0.299	0.314	0.324	0.415	0.367	0.524	0.456	0.494	0.457	450
H	0.231	0.265	0.299	0.233	0.261	0.229	0.264	0.269	0.273	0.31	0.359	0.408	450

Appendix VI: Raw data for IL-4 ELISA results

Software Version	2.05.5
Experiment File Path:	C:\Users\CBRD\Desktop\IL4.xpt
Protocol File Path:	
Plate Number	Plate 1
Date	11/30/2006
Time	12:17:09 AM
Reader Type:	ELx800
Reader Serial Number:	Unknown
Reading Type	Reader
Procedure Details	
Plate Type	96 WELL PLATE
Read	Absorbance Endpoint
	Full Plate
	Wavelengths: 450
	Read Speed: Normal
Results	

	1	2	3	4	5	6	7	8	9	10	11	12	
A	0.783	1.005	0.846	0.721	0.639	0.769	0.732	0.578	0.735	0.57	0.769	0.552	450
B	0.608	0.944	0.798	0.817	0.654	0.711	0.482	0.528	0.78	0.568	0.482	1.178	450
C	0.946	0.727	0.723	0.822	0.696	0.761	0.613	0.552	0.524	0.476	0.584	0.81	450
D	0.494	0.706	0.799	0.695	0.545	0.63	0.586	0.492	0.697	0.477	0.562	0.391	450
E	0.602	0.675	0.744	0.625	0.709	0.712	0.562	0.553	0.518	0.557	1.712	0.48	450
F	0.703	0.678	0.619	0.587	0.538	0.628	0.529	0.777	0.579	0.454	0.488	0.418	450
G	0.68	0.665	0.804	0.691	0.787	0.79	0.561	0.621	OVRFLW	0.496	0.475	0.425	450
H	0.467	0.621	0.683	0.654	0.571	1.315	0.548	0.504	0.511	0.541	0.442	0.509	450

Appendix VII: Scientific Ethics and Review Unit certificate

KENYA MEDICAL RESEARCH INSTITUTE

P.O. Box 54840-00200 NAIROBI - Kenya
Tel: (254) (020) 2722541, 254 (020) 2713349, 0722-205901, 0733-460003 Fax (254) (020) 2720036
Email: director@kemri.org , info@kemri.org Website: www.kemri.org

KEMRI/RES/7/3/1 June 24, 2016

TO: REUBEN RUTTOH,
PRINCIPAL INVESTIGATOR

THROUGH: DR. JOSEPH MWATHA,
ACTING DIRECTOR, CBRD,
NAIROBI

Dear Sir,

RE: PROTOCOL NO. KEMRI/SERU/CBRD/0150/3259 (*RESUBMISSION OF INITIAL SUBMISSION*): LABORATORY EVALUATION OF ANTILEISHMANIAL ACTIVITY OF PYRETHRUM EXTRACTS OF *CHRYSANTHEMUM CINERARIAEFOLIUM* TREVIR (ASTERALES: ASTERACEAE) PLANT--(*VERSION 4.0 DATED 14TH JUNE, 2016*)

Reference is made to your letter 15th June, 2016. The KEMRI/Scientific and Ethics Review Unit (SERU) acknowledges receipt of the revised study documents on the same day.

This is to inform you that the Committee notes that the issues raised during the 249th Committee B meeting of the KEMRI/SERU held on 16th March, 2016 have been adequately addressed.

Consequently, the study is granted approval for implementation effective this day, **24th June, 2016** for a period of one year. Please note that authorization to conduct this study will automatically expire on **June 23, 2017.** If you plan to continue data collection or analysis beyond this date, please submit an application for continuation approval to SERU by **May 12, 2017.**

You are required to submit any proposed changes to this study to SERU for review and the changes should not be initiated until written approval from SERU is received. Please note that any unanticipated problems resulting from the implementation of this study should be brought to the attention of SERU and you should advise SERU when the study is completed or discontinued.

You may embark on the study.

Yours faithfully,

DR. EVANS AMUKOYE,
ACTING HEAD,
KEMRI/SCIENTIFIC AND ETHICS REVIEW UNIT

In Search of Better Health