IN VITRO AND *IN VIVO* EFFICACY OF COMBINATION THERAPY USING *ALLIUM SATIVUM* AND *ALOE SECUNDIFLORA* AGAINS *LEISHMANIA MAJOR* INFECTED BALB/c MICE

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In vitro and *in vivo* efficacy of combination therapy using *allium sativum* and *aloe secundiflora* against *leishmania major* infected balb/c mice

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DECLARATION

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

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

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DEDICATION

I dedicate this work to my lovely husband Kenneth Otieno and my sweet daughters Marleen Hope Otieno and Malliah Tabitha Otieno, thank you for your support and being there for me.

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ABBREVIATIONS AND ACRONYMS

ACUC	Animal Care and Use Committee
AIDS	Acquired Immunodeficiency Syndrome
ANOVA	Analysis of variance
APG	Angiosperm Phylogen Group III
AS	*Allium sativum*
AF	*Aloe secundiflora*
CBRD	Centre for biotechnology research and development
CD4	Cluster of differentiation
CDC	Centre for disease control
CL	Cutaneous leishmaniases
CR	Complement receptors
CTMDR	Centre for traditional medicine and drug research
DALYs	Disability adjusted life years
DDT	Dichlorodiphenyltrichloroethane
DMSO	Dimethyl sulfoxide
ELISA	Enzyme linked immunosorbent assay
ERC	Ethics review committee
FBS	Fetal bovine serum
FML	Vaccine formulations
HIV	Human immunodeficieny virus
ID	Intradermal

IFN-gamma	Interferon gamma
IL	Interleukin
IP	Intraperitoneal
IR	Infection rate
ITNs	Insecticide treated nets
KEMRI	Kenya medical research institute
Kg	Kilograms
LD-bodies	Leishman donovan bodies
LDU	Leishman donovan units
MCL	Muco-cutaneous leishmaniasis
MIC	Minimum inhibitory concentration
MI	Multiplication index
MTT	3-(4, 5-dimethylthiazol-2-yl)-2 5-diphenyltetrazolium bromide)
NTDs	Neglected tropical diseases
NO	Nitric oxide
PBS	Phosphate buffered saline
PKDL	Post kala-azar dermal leishmaniasis
RPMI	Roswell park memorial institute
SOPs	Standard operating procedures
SSC	Scientific steering committee
Th-	T helper cells
TNF	Tumor necrosis factor
VL	Visceral leishmaniasis

ZVL Zoonotic visceral leishmania

ABSTRACT

Cutaneous leishmaniases (CL) is endemic in more than 88 countries worldwide. Zoonotic cutaneous leishmaniasis in Kenya is caused by *Leishmania major*. Currently used drugs like pentostam, Amphotericin B are expensive, toxic and require prolonged use. Combination therapy prevents drug resistance and reduces toxicity. In addition, herbal extracts can be safe and cheaper. *Allium sativum* and *Aloe secundiflora* water extracts have shown to have antileishmanial activities. In this study, the efficacy of combination therapy using *A. sativum* and *A. secundiflora* against *L. major* in BALB/c mice was studied using both intraperitoneal and oral routes of administration. The standard drug pentostam and phosphate buffered saline were used as positive and negative controls respectively. T-test and ANOVA were used for data analysis and P-value of < 0.05 was considered significant. Plant materials were dried, ground, soaked in water at 75^{o}C for 1 hour, filtered then freeze dried. The minimum inhibitory concentrations (MICs) of aqueous extracts of *A. secundiflora* (AF) and *A. sativum (*AS) were 2000 μg/ml and 5000 μg/ml and IC_{50} were 467.09μg/ml and 457.88μg/ml respectively while the IC_{50} for their combination at ratio (1:1) was 391.79 μg/ml as compared to MICs of 12.5μg/ml and IC_{50} of 108.58μg/ml for pentostam. The combination therapy had Infection rate (IR) of 17% and multiplication index (MI) of 48.65% compared to pentostam with an IR=21% and MI=11.64%. The combination therapy reduced the footpad lesion size significantly ($P < 0.05$) like the pentostam control drug and no significant nitric oxide stimulated. The oral and intraperitoneal combination treatment reduced spleen amastigotes in mice by 55.48% and 64.13% corresponding to total Leishman Donovan Units (LDU) of 18.23 ± 0.90 and 14.69 ± 1.33 respectively compared to pentostam 94.58% and LDU of 2.22±0.13. In summary, the combination therapy was active against *L. major* parasite, by reducing spleen parasite load significantly but did not prevent visceralization as amastigotes were seen in spleen smears. This study recommends the health sector to consider developing therapeutic products from the test plants for the treatment of CL in poverty stricken leishmaniases endemic areas of Kenya.

CHAPTER ONE

INTRODUCTION

1.1 Background Information

Leishmaniases are one of the major tropical neglected zoonotic parasitic diseases caused by obligate intracellular protozoan parasites of the genus *Leishmania* (Conjivaram & Ruchir, 2007). This infection was named after Leishman who first described it in London in May 1903. The infection is transmitted through bites by infected female sandflies of the genus *Phlebotomus* and *Lutzomyia* in the old world and new world, respectively (Piscopo & Mallia, 2006).

The global burden of leishmaniasis has remained stable for some years causing morbidity and mortality loss of 2.4 million disability adjusted life years (DALYS) and approximately 70,000 deaths (Ngure *et al.*, 2009). Leishmaniases are associated with poverty because treatment is expensive and unaffordable (WHO, 2007). Leishmaniasis is characterized by three forms: visceral leishmaniasis (VL), cutaneous leishmaniasis (CL), and mucocutaneous leishmaniasis (MCL) (Molynex & Ashford, 1983). The type of the disease expressed depends on type of *Leishmania* species. Clinical manifestations depend on the host's specific immune responses to parasite antigens and species (Roberts, 2006).

Several studies have shown that natural products are potential sources of new and selective agents for the treatment of important tropical diseases caused by protozoans

(Kinuthia *et al.*, 2013). There is need to explore maximally these sources because they are likely to lower the high cost of treatment, reduce resistance of drugs and reduce environmental pollution. This study aimed at combining the crude extracts of *A. sativum* and *A. secundiflora* which individually have shown antileishmanial activities in order to exploit the potential synergistic or additive benefits of these plant extracts in treating cutaneous leishmaniasis caused by *L. major* (Kinuthia *et al.*, 2013).

1.2 Statement of the problem

The leishmaniases affect about 12 million people worldwide and are one of the neglected tropical infectious diseases (NTDs) that are common in poverty stricken areas. It is prevalent in the Middle East, South America and Sub-Saharan Africa including Kenya. With the rise of HIV pandemic, the leishmaniases are rapidly becoming opportunistic protozoan infections in HIV and AIDS patients (WHO, 1994).

Chemotherapy is the main treatment for leishmaniasis. This is costly, highly toxic and requires long hospitalization time and therefore burdensome to poor economies. Pentavalent antimonial drugs are known to accumulate in the body tissues causing health complications like cardiac arrhythmia, pancreatitis and liver problems (Martinez & Marr, 1992). *Leishmania spp* have been reported showing resistance to the commonly used pentavalent antimonials thus there is a need for alternative chemotherapy for leishmaniasis. Additionally, many patients therefore seek for herbal therapies which are cheaper and readily available. These challenges associated with leishmaniases treatment calls for an intensive search for alternative cheaper natural treatment especially in

developing countries where medicinal plants are relatively abundant and present alternative affordable remedies if their efficacies and chemotherapeutic applications are evaluated.

1.3 Justification of the study

Aloe secundiflora (family Aloaceae), extracts contains natural compounds like alkaloids, terpenes and phenolic derivatives that have shown antileishmania activity (Ogeto *et al*., 2013). In addition, *Allium sativum* contains antiprotozoal aromatic polysulphur compounds that include allicin, alliin and diallyl disulphide (Kinuthia *et al*., 2013). Previous studies showed that water extracts of *A. secundiflora* and *A. sativum* showed low toxicity to Vero cells than Pentostam when used singly (Kinuthia *et al*., 2013, Ogeto *et al*., 2013). The low cytotoxic potential of the extracts in this study is of great significance for their traditional usefulness in the treatment of leishmaniasis. Previous studies have also shown that water extracts of this plants when tested singly were efficacious against *L. major* (Ogeto *et al*., 2013 and Kinuthia *et al*., 2013). Combination therapy using herbal products is a common practice in Kenya and has existed in many cultural systems for centuries (Gathirwa *et al.*, 2008). Most of these herbal products are environment friendly and safe. There is need for continued scientific researches in antileishmania herbal products whose combined therapy can be of great breakthrough to treatment of *Leishmania*. Possible synergistic and additive effects of the candidate extracts can be easily achieved by using combined therapy as it has shown to be effective against leishmaniases. In this study, aqueous extracts obtained from *A. secundiflora* and

A. sativum plant materials were tested in combination against *L. major* in BALB/c infected mice.

1.4 Research Questions

1. Does the combination therapy using *A. sativum* and *A. secundiflora* extract against *L. major* better than the standard drug (pentostam) in reducing lesion development in BALB/c infected mice?
2. What are the parasite burdens in the spleen of BALB/c mice after treatment with aqueous extracts of *A. sativum* and *A. secundiflora* compared to pentostam?
3. Does the combination therapy using *A. sativum* and *A. secundiflora* extracts lead to decreased or increased *in vitro* toxicity?
4. Does the combination therapy using *A. sativum* and *A. secundiflora* extracts lead to increased nitric oxide production?

1.5 Null Hypothesis

The aqueous crude extracts from *A. sativum* and *A. secundiflora* combination are not efficacious against *L. major* infection as compared to the standard drug pentostam.

1.6 Objectives

1.6.1 Main Objective

To determine and compare the efficacy of combined therapy of *A. sativum* and *A. secundiflora* extracts in *Leishmania major* infected BALB/c mice against the standard drug Pentostam.

1.6.2 Specific Objectives

a) To determine *in vitro* toxicity levels of combined therapy using *A. sativum* and *A. secundiflora* vero cells

b) To determine lesion sizes in *L. major* infected BALB/c mice treated with a combination of *A. sativum* and *A. secundiflora*.

c) To determine the spleen parasite burden in L. major infected BALB/c mice treated with a combination of *A. sativum* and *A. secundiflora.*

d) To quantify the levels of *in vitro* amount of nitric oxide produced in combined therapy using *A. sativum* and *A. secundiflora* in infected macrophages.

CHAPTER TWO

LITERATURE REVIEW

2.1 Global distribution of Leishmaniases

Leishmaniases causes substantial clinical, public health and socioeconomic problems in endemic regions in more than 88 countries in the Indian sub-continent, South Western Asia, Southern Europe, Africa, Central and South America (Desjeux, 2004). There is a remarkable increase in risk factors for leishmaniasis world-wide and the disease burden is increasing (Reithinger *et al.*, 2007). The global estimate for new cases of visceral leishmaniasis is 500, 000 cases per year out of which 90% of the reported cases occur in five countries namely Bangladesh, Brazil, India, Nepal and Sudan (Desjeux, 2004). Each year, there are 1.5 million cases reported in Afghanistan, Algeria, Brazil, Islamic Republic of Iran, Peru, Saudi Arabia and Syria (Ghalib & Modabber, 2007). Ninety percent of all cases of mucocutaneous leishmaniasis cases occur in Bolivia, Brazil and Peru (Desjeux, 2004).

Geographical distribution of leishmaniasis is restricted to tropical and temperate regions (Conjivaram & Ruchir, 2007). VL is a particular problem in Kenya, Sudan, Ethiopia and Eritrea (Wasunna *et al.,* 2005). Sudan is the most affected country, being one of the five countries that constitute 90% of all global cases of VL (Guerin *et al*., 2002). VL has been been in Sudan since 1904 to be endemic along the Blue Nile where it enters Ethiopia and its tributaries (Wasunna *et al.,* 2005).

Leishmaniasis is also endemic in West Africa although it is one of the poorly studied parasitic infections in this region (Niamba *et al*., 2007). Cases of leishmaniasis have been reported in Niger, Mali, Nigeria, Senegal, Cameroon, Burkina Faso, Gambia and Guinea. There is high prevalence of both HIV and *Leishmania* co-infection have been reported in Burkina Faso (Niamba *et al*., 2007).

2.2 Leishmaniases in Kenya

Leishmaniases are endemic in Kenya for a long time (Wassuna *et al*., 2005). The most prevalent forms of leishmaniases are the cutaneous and visceral forms. In addition, post kalazaar-dermal lesions (PKDL) has been reported (Ngure *et al*., 2009). VL is endemic in Baringo, Koibatek, Turkana, West Pokot, Kitui, Meru, Keiyo, Marakwet, Mwingi and Machakos regions (Wassuna *et al*., 2005). Baringo district is the only foci where both VL and CL are known to occur in Kenya. VL was first reported in Kenya among King's African Rifles troops in Lake Turkana (Ngure *et al*., 2009). The disease is caused by *L. donovani* and transmitted by *Phlebotomus martini*, though other vectors including *P. orientalis* have been reported (Ngure et al., 2009). In 2001, there was an outbreak of VL in Wajir and Mandera regions of north Eastern Kenya with 904 patients diagnosed between May 2000 and August 2001 (Marlet *et al*., 2003).

In Kenya, CL is caused by *L. major, L. aethiopica* and *L. tropica* (Ngure *et al*., 2009). *L. tropica* infection is often referred to as dry urban oriental sore. Dry painless ulcers are produced which self are healing usually after 1-2 years but often leave disfiguring scars. The patient is immune to the infection thereafter (Mebrahtu *et al*., 1992).

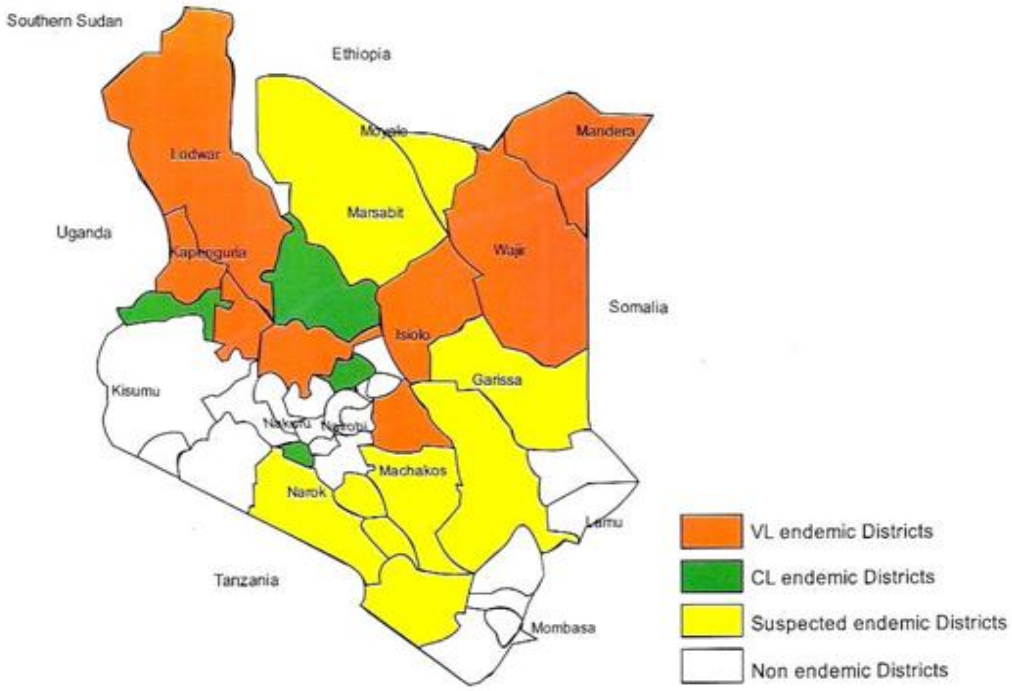

Figure 2.1: Leishmaniasis Endemic Districts in Kenya

(Source: National Multi-Year Strategic Plan for control of NTDs 2011-2015).

2.3 The Leishmaniases

2.3.1 Visceral leishmaniasis

Visceral leishmaniasis (VL), also known as Kala-zar is caused by obligate intracellular protozoan parasites, particularly by the species *Leishmania donovani* and *L. infantum*/*L. chagasi* (Conjivaram & Ruchir, 2007). VL ranges from asymptomatic infection to severe life threatening infection. It is the most severe form of leishmaniasis

and usually fatal within 2 years if left untreated (Conjivaram & Ruchir, 2007). Post kala-azar dermal leishmaniasis (PKDL) is a complication of visceral leishmaniasis characterized by rashes ranging from papular or nodular, maculopapular, micropapular to macular rashes (Conjivaram & Ruchir, 2007). VL mostly affects some of the internal organs of the body like the spleen, liver, and bone marrow). VL is predominantly transmitted through the bite of an infected female phlebotomine sand fly, although congenital and blood transfusions and needle sharing have been reported (Murray, 2012).

In the Old World, VL is found in parts of Asia particularly Indian and southwest and central Asia, Africa especially East Africa, and southern Europe, East Africa (Sudan, South Sudan, and Ethiopia), and Brazil; none of the affected areas in these 7 countries are common tourist destinations (Pavli & Maltezou, 2010).

The geographic distribution of cases of VL evaluated in countries such as the United States reflects travel and immigration patterns. VL is uncommon in US travelers and expatriates. Occasional cases have been diagnosed in short-term travelers (tourists) to southern Europe and also in longer-term travelers (such as expatriates and deployed soldiers) to the Mediterranean region and other areas where VL is found (Pavli & Maltezou, 2010).

The incubation period for VL typically ranges from weeks to months. The onset of illness can be abrupt or gradual. Stereotypical manifestations of VL include fever, weight loss, hepatosplenomegaly especially splenomegaly as shown in figure 2.2, and pancytopenia i.e anemia, leukopenia, and thrombocytopenia. If untreated, severe and advanced cases of

VL typically are fatal. Latent infection can become clinically manifest years to decades after exposure in people who become immunocompromised for other medical reasons such as HIV infection (Murray *et al.*, 2005).

Diagnosis of VL should be considered in people with a relevant travel history and a persistent, unexplained febrile illness, especially if accompanied by other suggestive manifestations such as splenomegaly and pancytopenia. Laboratory confirmation of the diagnosis is achieved by microscopical examination of stained specimens to detect *Leishmania* parasites or DNA in infected tissue such as in bone marrow, liver, lymph node, or blood, culture techniques, or molecular methods are also used. Serologic testing can provide supportive evidence for the diagnosis (Myles *et al*., 2007).

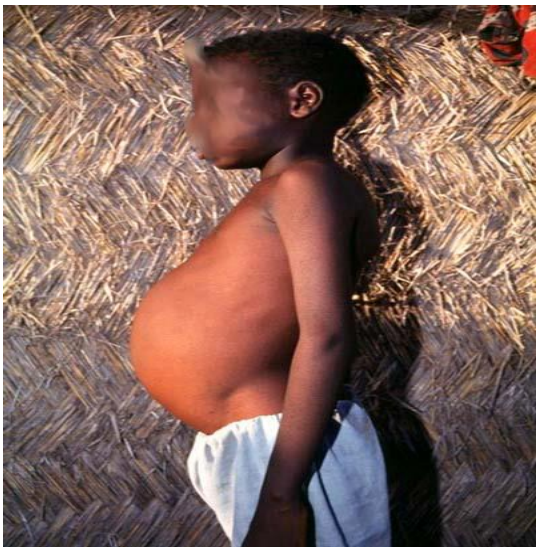

Figure 2.2: Visceral leishmaniasis (splenomegaly) (murray, 2012)

2.3.2 Cutaneous Leishmaniasis

Cutaneous leishmaniasis (CL) is the most common form both in general and in travelers. CL is approximately caused by 20 *Leishmania* species. CL is transmitted through the bite of an infected female phlebotomine sand fly. After accidental occupational (laboratory) exposures to leishmania parasites, might also cause CL (Murray *et al.,* 2005).

CL causes skin sores. The sores normally develop within a few weeks or months after the sand fly bite. Mostly, the sores change in size and appearance with time. The sores may start out as papules or nodules (lumps) and may end up as ulcers (like a volcano, with a raised edge and central crater); skin ulcers might be covered by scab or crust as shown in figure 2.3. The sores usually are painless but can be painful. Some people have swollen glands near the sores (for example, under the arm, if the sores are on the arm or hand) (Conjivaram & Ruchir, 2007).

Clinicians should consider CL in people with chronic (non healing) skin lesions who have been in areas where leishmaniasis is found. Laboratory confirmation of the diagnosis is achieved by detecting *Leishmania* parasites (or DNA) in infected tissue, through light-microscopic examination of stained specimens, culture techniques, or molecular methods (Myles *et al*., 2007).

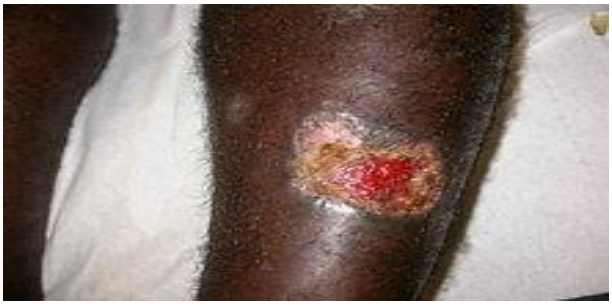

Figure 2.3: Cutaneous leishmaniasis (L. major) lesion (Piscopo and Malia, 2006)

2.3.3 Mucocutaneous leishmaniasis (MCL)

Mucosal leishmaniasis is less common forms of leishmaniases; the pathogenesis of mucocutaneous leishmaniasis (MCL) is still unclear. This disease leads to partial or total destruction of mucous membranes of the nose, mouth and throat leading to a severe mutilation of the face (WHO, 2007). The disease may get worse if secondary bacterial or fungal infection occurs (Sundar *et al*., 2007). This form can be a sequel (consequence) of infection with some of the species (types) of the parasite that cause cutaneous leishmaniasis: certain types of the parasite might spread from the skin and cause sores in the mucous membrane of the nose (most common location as shown in figure 2.4), 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 & Beaching, 2011). MCL is caused by *L. aethiopica* in the Old world and *L. braziliensis* complex in the

new world. Cases due to *L. donovani*, *L. major* and *L. infantum* have been reported (Paredes *et al*., 2003).

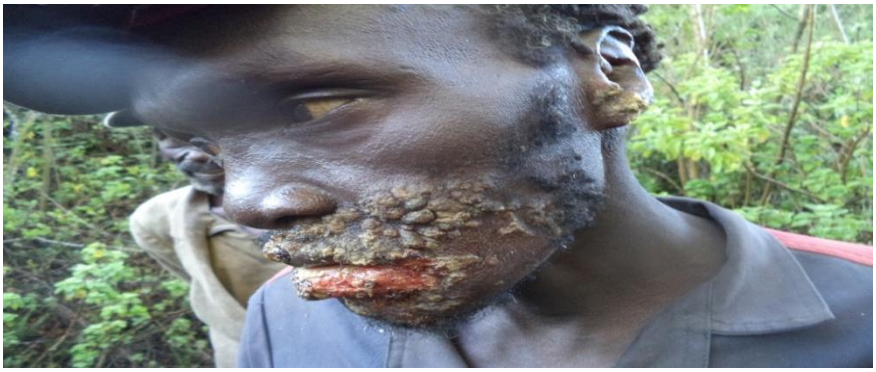

Figure 2.4: Mucocutaneous leishmaniasis (Gilgil, Kenya).

2.4 Transmission and life cycle of *Leishmania*

Leishmaniases are transmitted through bites of the hematophagus Phlebotomine sand flies (Diptera: Psychodidae) (figure 2.6), endemic in many tropical areas of Africa, Asia and Europe and through *Lutzomyia* sandflies in South America and the Carribean region (Santos *et al.,* 2008).The *Leishmania* parasite has 2 forms: the amastigote form which occurs in humans and the promastigote form which occurs in the sandfly and in artificial culture (Conjivaram & Ruchir, 2007). In the vertebrate host, *Leishmania* parasites survive and multiply intracellularly in mononuclear phagocytes as tiny, ovoid to round, non motile amastigotes about 3-5µm in diameter (Singh, 2006). Female sand flies need a blood meal to start laying eggs and they will seek this food from birds, amphibians, reptiles and mammals. When a sand fly feeds on an infected host during a blood meal, the

fly ingests parasitized macrophages which are then released into the gut of the sand fly. The released amastigotes transforms into the motile, elongated (10-20 µm) and flagellated promastogotes form (Roberts, 2006).

The promastigotes then attach to the alimentary tract of the fly, where they live extracellulary and multiply by binary fission and subsequently differentiate into the metacyclic promastigote stage that is infectious to the vertebrate host (Roberts, 2006). By the time the sand fly takes a new blood meal; the metacyclic promastigotes will have affected the feeding mechanism of the sand fly in such a way that they cause regurgitation of the midgut content containing the parasites, into the feeding wound thereby ensuring efficient transmission to the vertebrate host (Roberts, 2006).

Although most promastigotes will be rapidly eliminated by the host via complement-mediated killing, some are opsonized and taken up by macrophages, neutrophils and dendritic cells where they rapidly revert to the amastigote form (Olivier *et al*., 2005). *Leishmania* are able to resist the microbiocidal action of the acid hydrolases released from the lysozymes hence survive and multiply inside the macrophages, eventually leading to the lysis of the macrophages (Olivier *et al*., 2005). Released amastigotes are taken up by additional macrophages and the cycle continues. Ultimately all the organs containing macrophages and phagocytes are infected, especially the spleen, liver and bone marrow. The complete life cycle of leishmania parasites is summarized in figure 2.2 below.

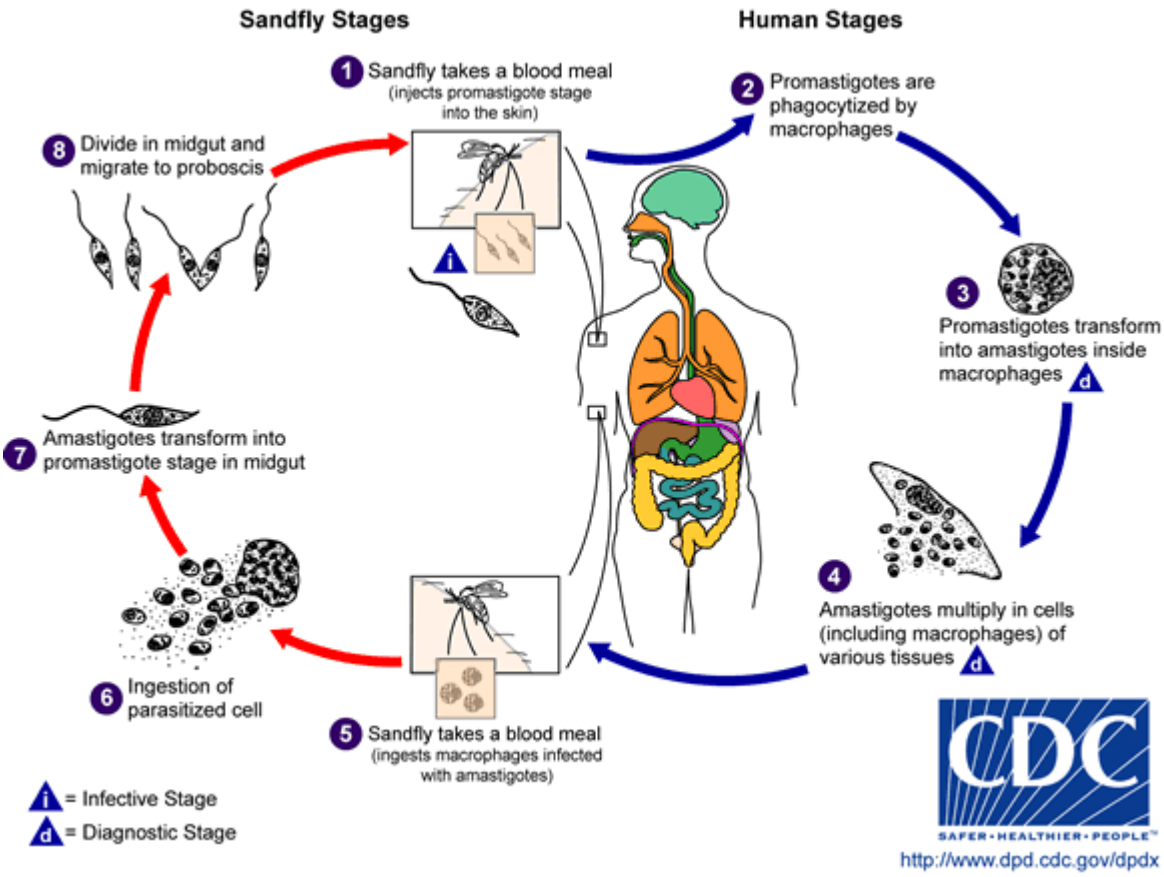

Figure 2.5: Life cycle of Leishmania spp (htt://www.dpd.cdc.gov/dpdx 2015)

Figure 2.6: Phlebotomus species sandfly in the insectary, kemri.

2.5 The biology of *Leishmania* parasites

Leishmania is a protozoan that is capable of infecting animals, humans and sandflies (Piscopo & Mallia, 2006). There are at least 20 species of *Leishmania* each capable of causing a disease depending on the species and the host response (Piscopo & Mallia, 2006). *Leishmania* parasites have a digenetic life cycle with an extracellular developmental stage in the female *Phlebotomine* sandfly and a developmental stage in mammals, which is mostly intracellular (Roberts, 2006). In 1903, Leishman and Donovan separately described this protozoan now called *Leishmania donovani* in splenic tissue from VL patients in India Singh, 2006). As a result, the amastigote stage seen in clinical samples is commonly known as Leishman-Donovan (LD) bodies (Singh, 2006). A typical leishmania promastigotes is shown in figure 2.7.

Figure 2.7: A typical leishmania parasite (science photo library).

2.6 Development of *L. major* in mice

Different strains of mice have shown variability in their susceptibility to *Leishmania* parasites (Bradley & Kirkley, 1977). BALB/c mice for instance have shown to be highly susceptible to *L. major*. They show signs of slow breeding, are small in size and delicate to breed (Bradley & Kirkley, 1977). The Swiss Albino mice on the other hand are easy to breed, virtually all are the same in appearance and they are quite resilient but are known to show resistance to both *L. major* and *L. donovani* (Santos *et al*., 2003). Therefore BALB/c mice have been selected for use in this study due to their susceptibility to infection.

2.7 Immunology of Leishmaniases

Studies with the experimental laboratory mice models have demonstrated that protective immunity to *L. major* in resistant strains such as C57 BL/6, C3H and CBA is dependent on the ability to mount CD4+ type 1 helper T cell response with interferon- Gamma as the key cytokine to activate macrophages to kill intracellular parasites (Noben-Trouth *et al.*, 2003). In contrast, susceptibility to *L. major* as demonstrated in BALB/c mice has been attributed to a CD4+ type 2 helper T cell response associated with the production of interleukin 4 (IL-4) and interleukin 5 (IL-5) (Mahmoodi *et al.*, 2003). These implications of immune responses against *Leishmania* parasites show that induction of immunity against leishmaniasis by vaccination is therefore a feasible strategy for control of the disease (Mahmoodi *et al.,* 2003). This has been supported by recent evidence which suggest immune response to leishmaniasis is not only as a result of Th1/Th2 cytokine responses (Tonui & Titus, 2006). Several vaccine studies have been studied previously

despite lack of definite breakthrough. These include FML vaccine formulation and other trials involving uses of immunodilations with immunotherapy (Barroso *et al.,* 2007) while others have employed use of synthetic compounds like levamisoleametidine, Tufsin and imiquimod. Amastigotes are first observed in liver Kupffer cells, where they survive without killing. Early parasite replication in the liver and spleen occurs when there is little IFNγ and few IL-12 producing cells (Buates & Matlashewski, 1999).

Upon entering the mammalian bloodstream, *L. major* meets the focal point of infection, the macrophage. Promastigotes are able to bind to several macrophage receptors as a result of two surface molecules, the protease gp63 and a lipophosphoglycan, (King *et al.,* 1987). Promastigote attachment to macrophages is facilitated by a number of receptors, including complement receptors CR1 and CR3, and the receptor for advanced glycosylation end products (Guerrant *et al.,* 2006). Activation of complements occurs far from the cell membrane, and insertion of the membrane attack complex does not occur. This action is what allows the parasite to evade lysis, and to persist within the host's macrophages (Guerrant *et al*., 2006).

2.8 Pathology of Leishmaniases

Cutaneous leishmaniasis (CL) is the most common form of leishmaniasis (Desjeux, 2004). Multiple species produce CL in children and adults, primarily *L. major*, *L. tropica, L. aethiopica*, *L. infantum* and *L. chagasi* (Murray *et al*., 2005). The disease produces skin lesions mainly on the face, arms and legs. Cutaneous leishmaniasis starts as a papule

at the site of sand fly bite, which then increases in size, crusts and eventually ulcerates (Plate 2.1) (Piscopo & Mallia, 2006).

Upon becoming infected with *L. major*, patients usually present with lesions at the site of the sand fly bite. The infection is acute, and usually has duration of about 3–6 months (Markell *et al.,* 1992). As more and more phagocytic cells engulf promastigotes, prompting the production of amastigotes, nodules form on the skin (Guerrant *et al.,* 2006). These nodules then ulcerate, although due to the variable characteristics of the lesions, species specific identification of the pathogen is impossible (Guerrant *et al.,* 2006). Generally though, lesions appear moist and have raised outer borders, a granulating base, an overlying layer of white purulent exudate, and have been described as "pizza-like." (Markell *et al.,* 1992, Guerrant *et al.,* 2006). Biopsies of these lesions usually reveal a number of findings including numerous macrophages containing intracellular amastigotes as well as lymphocytes with observed granuloma formation and few parasites (Ridley, 1979).

Post kala-azar dermal leishmaniasis is a disease that appears after treatment of VL (Ghalib & Modabber, 2007), and it requires lengthy and costly treatment (WHO, 2007). This is usually due to infection by the *L. donovani sensustricto* cluster (Piscopo & Mallia, 2006). The skin lesions are macular, maculopapular or nodular, and usually spread from the peri-oral area to other areas of the body. The symptoms first appear around the mouth; those which do not heal spontaneously become denser and spread over the entire body.

2.9 Diagnosis of Leishmaniases

L. major should be considered in the diagnosis of chronic lesions of people who have spent time in areas where it is endemic. However, other pathogens can cause similar lesions and therefore infections like histoplasmosis, sporotrichosis, lobomycosis, lupus vulgaris, *Mycobacterium ulcerans*, syphilis, cutaneous sarcoidosis, and leprosy should all be considered as well (Guerrant *et al.,* 2006). The most common ways of diagnosing leishmaniasis is by identifying amastigotes in a Giemsa-stained touch preparation or through isolation of the parasites in cultures (Guerrant *et al.,* 2006).

Diagnosis of visceral leishmaniasis is usually based on microscopic detection of amastigotes in smears of tissue of bone marrow (Piscopo & Mallia, 2006). The parasite can also be detected through direct examination of peripheral blood, bone marrow or splenic aspirates. The smears are stained with Leishman, Giemsa or Wright stains and examined under oil immersion objective lens (Conjivaram & Ruchir, 2007). The clinical signs and epidemiological manifestations of VL and CL cannot be used in diagnosis (Singh, 2006). They can mimic several other conditions like misdiagnosis of malaria, tropical splenomegally, schistosomiasis, cirrhosis, lymphoma and leukemia hence a laboratory diagnosis is necessary to confirm the infection (Singh, 2006).

2.10 Control of the leishmaniases

Prevention and control of leishmaniasis require a combination of intervention strategies because transmission occurs in a complex biological system involving the human host, parasite, sandfly vector and in some cases an animal reservoir hosts such

as forest rodents, hamsters, opossums, peri-domestic dogs and hydrax. Measures to check the spread of leishmaniases include early diagnosis and effective case management, vector control, effective disease surveillance and control of reservoir hosts by eliminating the host (WHO, 2007).

2.10.1 Treatment of leishmaniases

Treatment is basically by chemotherapy. The standard drugs for leishmaniases are pentavalent antimonials, mainly sodium stibogluconate (Pentostam) and meglumineantimoniate (Glucantime) (Berman & Lee, 1984). These drugs are expensive and are known to exert severe toxic effects in treated individuals (Haidaris & Bonventre, 1983). *Leishmania* strains that are resistant to Pentavalent antimonials have been detected and furthermore the compounds have low activity against CL infections and they are administered intravenously which calls for hospitalization (Berman & Lee, 1984). Pentavalent antimonials have been associated with prolonged healing time of 3 to 4 months and serious side effects which include pancreatitis, liver enzyme abnormalities and cardiac arrhythmia (Almeida *et al.*, 2005). Amphotercin B and Pentamidine are alternative leishmaniasis drugs which are of greater toxicity; however, liposomal amphotericin B is less toxic, more effective but expensive (Grogyl *et al.*, 1992).

2.10.2 Vector control

The only proven vector of the *Leishmania* parasite is the blood-sucking female sand flies of the genus *Phlebotomus* in the Old world and *Lutzomyia* in the New World (Murray *et al.*, 2005). The insects are 2-3 mm long and are found throughout the tropical and

temperate zones of the world (Murray *et al*., 2005). About 30 out of over 500 species of phlebotomine species 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 (Chavasse *et al*., 1999). Indoor residual spraying using DDT is a simple and cost effective method of controlling endophilic vectors (vectors that rest indoors) (sand fly) and DDT remains the insecticide of choice for the control of leishmaniasis. However resistance to insecticide is likely to become more widespread in the population especially in those areas in which insecticide has been applied over long duration (Kishore *et al*., 2006). Spraying programmes are often unsustainable where sand flies are endophagic and are active when people Deltamethrin and labda-cyhalothrin provide considerable protection (Murray *et al*., 2005).

The use of ITNs is an effective, cheaper and sustainable method of malaria control (Curtis *et al*., 1990; The synthetic pyrethroids used for the treatment of the nets combine the properties of low to moderate toxicity in mammalian host, low volatility and high insecticidal activity (Davies *et al.*, 200). ITNs have also been evaluated against leishmania vectors in several countries like Italy, Syria, Sudan, Kenya, Colombia, Venezuela and Burkina (Davies *et al*., 2000). The use of ITNs may represent the most sustainable method of reducing transmission of leishmania in communities surrounded by forest, where the diurnal resting sites of vectors are unknown or inaccessible (Curtis *et al*., 1990).

The application of bio-larvicides 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 leishmaniasis (Kishore *et al*., 2006). Satellite remote sensing for early prediction of disease by identifying the sand fly flying conditions and the use of pheromones should be exploited in the control of leishmaniases (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 (Alexander & Maroli, 2003). The use of insecticide treated bed nets (ITNs) can offer good protection against transmission of leishmaniasis by endophagic sand fly vector. In Kenya most vectors species such as *P. duboscqi, P. guggisberg*, *P. martini, P. pedifer* and *P. longipes* are exophilic and exophagous, thus negating common strategies such as ITNs (Davies *et al*., 2009).

2.10.3 Animal reservoir control

Reservoir control usually involves the testing and culling of seropositive dogs in zoonotic visceral leishmaniasis endemic areas (Murray *et al*., 2005). Impregnated collars, a novel method of topical application repellents such as Deltamethrin, have been used in breaking the transmission cycle of leishmaniasis in zoonotic visceral leishmaniasis (ZVL) foci. Animals' reservoir control for CL is based on control of animal reservoir and the use of poison baits and environmental management to control rodents (Alexander *et al.,* 2003).

2.11 Selected medicinal plant with antileishmanial activity

2.11.1 *Allium sativum*: (family: Amaryllidaceae)

Allium sativum, commonly called garlic, is a perennial plant that originated from Central Asia and is now available throughout the world. *A. sativum* is a member of the amaryllidaceae family and has been found to be a crucial antimicrobial agent (Singh &Singh, 2008). According to the new classification system by APG III (Angiosperm Phylogeny Group III) in 2009, the family Alliceae has been replaced by family Amaryllidaceae (Salgado *et al.,* 2011; Islam *et al.,* 2011). *A. sativum* has been used as food, spice and medicine for thousands of years (Singh and Singh, 2008). Medicinal properties of this plant are many and they range from antimicrobial, hypolipidemic, antithrombotic to antitumor property (Augusti, 1996). The compound bulb is the most important part of the garlic plant for medicinal purposes which is made up of 4 to 20 cloves, each weighing about one gram (Figure 2.8a). *A. sativum* contains organosulfur compounds that have been attributed to medicinal properties present in the bulbs (Islam *et al*., 2011).

(a) (b)

Figure 2.8: (a) The bulb of *A. sativum (*b) cloves retrieved from the bulb.

2.11.2 *Aloe secundiflora:* (Family: Aloaceae)

The APG III system places the genus in the family Aloaceae. A. secundiflora is a medium sized solitary stemless. Aloe found in Kenya and Tanzania.it has curved stiff succulent leaves with margins with sharp teeth. There may be a few white spots on the leave surfaces (Muthaura *et al*., 2007). In the past the aloe species was assigned to families Aloaceae and Liliaceae (Chase *et al*., 2009). Plants of the genus *Aloe* within the Family Aloaceae are known to have a variety of activities against insects, human and veterinary parasitic diseases (Cheney, 1970). Some of the species that have been studied include *A. vera, A. turkanensis, A. ngongensis, A. fibrosa* and *A. secundiflora.* The efficacy of *Aloe vera* leaf exudates against promastigotes of *L. braziliensis, L. Mexicana, L. tropica, L. major* and *L. infantum* have been reported to show leishmanicidal activity (Dutta *et al*., 2007). The leaf exudates of *Aloe vera* and triterpenoid saponin of Careyaarborea (Mandal 2006) have been reported to be leishmanicidal. Extracts of *A. turkanensis, A. ngongensis* and *A. fibrosa* have been shown to exhibit larvicidal effects against *Anopheles gambiae* (Diptera: Anophelinae) (Matasyoh *et al*., 2008). In different

studies, sap from leaves of *A.* s*ecundiflora* (figure 2.9) has been used to treat wound, pimples and ringworm (Njoroge & Bussman, 2007). Infusions of leaves in water have also been used to treat internal body pains (Kokwaro, 1976) and paludism (Muthaura *et al*., 2007). *Aloe secundiflora* has also been shown to have antimicrobial activities against *Staphylococcus aureus*, *Mycobacterium tuberculosis*, *Salmonella typhi* and *Escherichia coli* (Richard *et al.,* 2011). *Aloe secundiflora* contains several major groups of chemical compounds namely tannins, saponins, alkaloids, cardiac glycosides, flavonoids and terpenes (Omwenga *et al*., 2009).

Figure 2.9: A. secundiflora plant

The *A. secundiflora* plant shown above was collected from a homestead in Ruai in Nairobi County from a plantation where they are maintained.

2.12 Combination therapy

Combination therapy or polytherapy refers to using multiple therapies to treat a single disease and it can also involve non-medical therapy, such as the combination of medications and talk therapy to treat depression. Conditions treated with combination therapy include tuberculosis, leprosy, cancer, malaria, and HIV/AIDS (Allen *et al.*, 2013).. One major benefit of combination therapies is that they reduce development of drug resistance, since a pathogen is less likely to have resistance to multiple drugs simultaneously (Allen *et al.*, 2013).

Combination therapy may seem costlier than monotherapy in the short term, but when used appropriately, it causes significant savings: lower treatment failure rate, lower case-fatality ratios, and fewer side-effects than monotherapy, slower development of resistance and consequently, less money needed for the development of new drugs (Allen *et al.*, 2013)

CHAPTER THREE

MATERIALS AND METHODS

3.1 Study design

This was laboratory based study that combined both quantitative and qualitative methods. Research plants were collected from the field. Meads resource equation was used to calculate the sample size of the mice. Random sampling design was used and only male mice were used. Two experimental groups were designed and three control groups (positive and negative controls). Some samples were analysed using machines while others were manually calculated and the final data was analysed using the SPSS software programme version 20. The study conceptual framework is presented in figure 3.1 below.

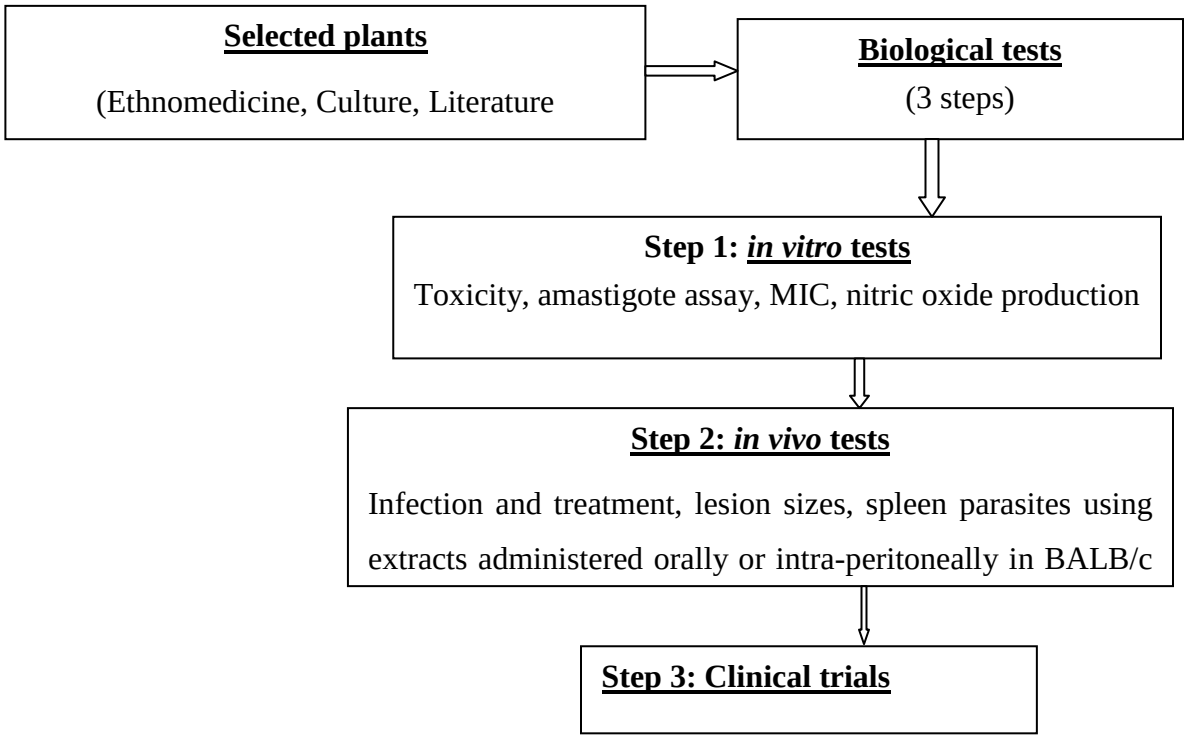

Figure 3.1: Conceptual framework flow

Table 3.1: Design of the *in vivo* protocol for testing extracts using BALB/c mice

Group	**Treatment (route)**	**Dosage**	**Frequency of treatment**	**Marking**
Experimental grp 1 (8 mice)	Intraperitoneal combined extracts	100µl	daily	back-black
Experimental grp 2 (8 mice)	Oral combined extracts	100µl	daily	back-blue
Group 3 positive control (3 mice)	Intraperitoneal pentostam	100µl	daily	back-green
Group 4 Negative control (3 mice)	Intraperitoneal PBS	100µl	daily	back-red
Group 5 Negative control (3 mice)	Oral PBS	100µl	daily	tail-red

3.2 Study Site

This study was carried out in Kenya Medical Research Institute (KEMRI), Centre for Biotechnology Research and Development (CBRD) in the *Leishmania* laboratory and Centre for Traditional Medicine and Drugs Research (CTMDR) laboratory where extraction and cytotoxicity assays using Vero cells was done. The *A. sativum* bulbs were purchased from Marikiti market, Nairobi Kenya where they had been obtained before for previous studies. Leaves of *A. secundiflora* were harvested from a selected homestead in Ruai, Nairobi County, Kenya. The plants were properly identified at National Museums of Kenya, in the Botany Department. The cloves of *A. sativum* were removed from the bulbs then sliced into small pieces, were then dried for 17 days at room temperature. Leaves of A. secundiflora were also sliced into small pieces and dried for 14 days at room temperature. Drying of the plant materials was done at Kenya Medical research Institute (KEMRI). The dried plants were well and separately packaged in different small carton boxes and labeled appropriately.

3.3 Ethical considerations

The approvals were acquired from Scientific Steering Committee (SSC), Animal Care and Use Committee (ACUC) and Ethical Review Committee (ERC), all of Kenya Medical Research Institute, (KEMRI). All the mice were sacrificed and disposed off in accordance with the regulations that have been set by Kenya Medical Research Institute (KEMRI)'s Animal Care and Use Committee (ACUC).

3.4 Experimental Mice

Inbred eight weeks old BALB/c male mice were obtained from KEMRI, Kenya. They were housed at the animal house at 25°C and were fed on mice pencils which is a commercial diet and given tap water *ad libitum*. The mice were handled in accordance with the regulations that have been set by Animal Care and Use Committee (ACUC) at KEMRI.

3.5 Sample Size Determination

Sample size determination was by the Mead's resource equation (Kirkwood & James, 2010).

$$n = N – B – T$$

Where:

- *N* is the total number of individuals or units in the study (minus 1)
- *B* is the blocking component, representing environmental effects allowed for in the design (minus 1)
- *T* is the treatment component, corresponding to the number of treatment groups (including control group)
- P is the sample size

In this study, 36 mice were to be used with 5 treatment groups. Therefore actual animals used in the study = 35-0-4=31 .

3.6 Plant extracts preparation

The dried plants were transferred to the Centre of Traditional Medicine and Drug Research (CTMDR) where they were separately ground into fine powder using Gibbons

electric mill (Christy and Norris Limited, England). This powder was used for aqueous extraction. Extraction was done as described by Delahaye *et al.* (2009). 100g of the dried ground plant material in 1000ml of distilled water was placed in a water bath at 75^0C for 1.5 hours. The extract was then filtered using Whatman NO. 1 filter papers and the filtrate was freeze dried and weighed. The final extracts of *A. sativum* and *A. secundiflora* were coded AS and AF respectively. The two extracts were then stored at 4ºC until needed for bioassays.

3.7 Preparation of the stock solutions of test extracts

Stock solutions of 1mg/ml concentration of crude extracts were made by weighing 10mg of single test extracts and dissolving in 10ml of PBS for each extract separately for *in vitro* anti-leishmanial assays. The stock solution was then filtered through 0.22μm filter flasks in a laminar flow hood (Biological Safety Cabinet). The combination drug (C) was made by weighing 2mg of *A. secundiflora* and dissolved in 1ml PBS and 5mg of *A. sativum* was also dissolved in 1ml PBS separately. For use, appropriate ratios were mixed for *in vivo* work. The stock solutions were stored at 4ºC and retrieved when needed for *in vitro* and *in vivo* assays.

3.8 *Leishmania* parasites

The *Leishmania major* strain (IDUB/KE/94=NLB-144) was obtained from CBRD, Leishmania laboratory, KEMRI, (Kenya) where it had been cryopreserved in liquid nitrogen. The parasites were grown to stationary phase at 25ºC for 15 minutes in Schneider's *Drosophila* medium supplemented with 20% heat inactivated fetal bovine

serum (FBS)-HYCLONE® USA), 100 U/ml penicillin and 500μg/ml streptomycin (Hendricks and Wright, 1979), and 250μg/ml 5-fluorocytosine arabinoside (Kimber *et al*., 1981). The stationary-phase metacyclic stage promastigotes were then harvested by centrifugation at 1500 rpm. The metacyclic promastigotes were used for the *in vitro* and *in vivo* assays.

3.9 *In vitro* bioassays

These assays were carried out to determine the effects of the plant extracts on monkey kidney cells (Vero cells), minimum concentration (MIC) on *L. major* promastigotes, determine the infection and multiplication rates of the *L. major* amastigotes in their host macrophages that were exposed to the plant extracts. Lastly to determine the nitric oxide (NO) production by infected macrophages which were treated with the test plant extracts.

3.9.1 Cytotoxicity assay using vero cells

The assay was used to test the cytotoxicity of the individual extracts and combination therapy against Vero cells. The assay was carried out as described by Wabwoba *et al*. (2010). Vero cells were grown in minimum essential medium (MEM) supplemented with 10% FBS, penicillin (100 IU/ml) and streptomycin (100μg/ml) in 25ml cell culture flasks incubated at 37^{o}C in a humidified 5% CO_2atmosphere for 24 hours. The Vero cells were harvested by trypsinization, and pooled in 50 ml centrifuge tubes from where 100μl of the cell suspension were put into 2 wells of rows A-H in a 96-well flat bottomed microtitre plate at a concentration of 1×10^6 cells per ml of the culture medium per well and incubated at 37^{o}C in 5% CO_2 in order to attach (figure 3.2). The MEM was gently

aspirated off and 150 µl of the highest concentration (1000µg/ml) of the test extracts (AS, AF and AS/AF) was added and serially diluted by a factor of 3 up to a concentration of 1.37μg/ml at wells of row B. The microtitre plates containing the Vero cells and test extracts were further incubated at 37^0C for 48 hours in a humidified 5% CO_2 atmosphere. The control wells comprised of Vero cells and medium while the blank wells had the medium only. 10μl of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reagent was added into each well and incubated further for 2 to 4 hours until a purple precipitate (Formazan) was visible under the microscope (dead cells don't metabolize MTT). The media together with MTT reagent were gently aspirated off, after which 100 μl of DMSO was added, and vigorously shaken for 5 minutes in order to dissolve formazan. The absorbance (optical density) was measured for each well plate using a micro-titer plate reader at wavelength of 562 nm. Cell viability was calculated at each concentration as previously described by Mosmann (1983) formula. The IC_{50}values of the extracts were determined automatically using the Chemosen software program. The design of the 96 wells plate is as illustrated in Figure 3.2. Plate design was used for the single and combined plant extracts cytotoxicity assay.

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

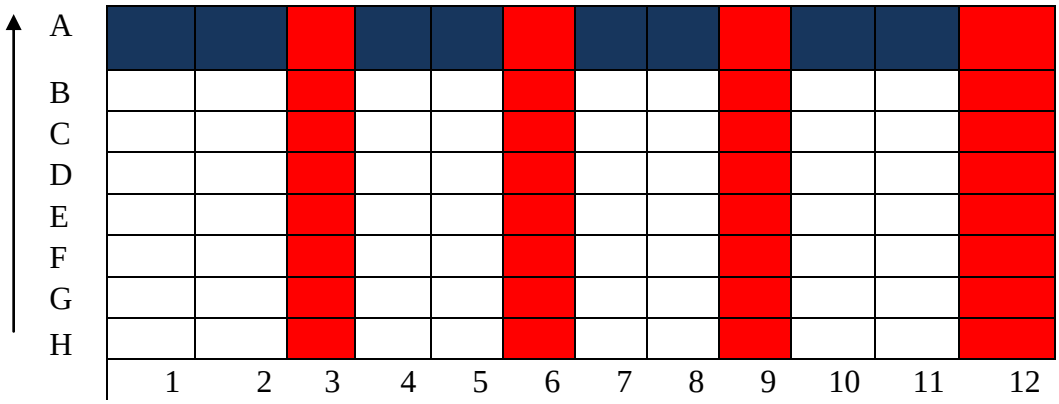

Figure 3.2: The 96 well plate design for the cytotoxicity assay of the extracts using vero cells

Key

Wells with Vero cells maintenance media and MMT

Wells with vero cells, maintenance media and plant extracts where concentration decreased from H to B

Blank wells had maintenance media and plant extracts only (Negative control)

→ The direction of extracts' dilution decreased upwards from H to B in the order. Concentration of extracts (drugs) were in the order of 1000, 333.33, 111.11, 37.04, 12.35, 4.12 and 1.37μg/ml, A had no drug (Positive control).

3.9.2 Evaluation of Minimum Inhibitory Concentration (MIC)

The MICs were determined as described Wabwoba *et al*. (2010). The *L. major* promastigotes at a concentration of 1×10^6 per ml were grown in Schneider's insect medium (SIM) in 24 well micro titer plate containing the test aqueous extracts (AF, AS and C) in concentrations of 5mg/ml for *A. sativum* and 2mg/ml for *A. secundiflora* and from which different ratios were made for combination therapy. Survival of the promastigotes upon exposure to five different fixed ratios like 1:1, 1:2, 3:1, 3:1, 2:1 for blends of AF/AS was determined. The combination ratio that supported the least survival of promastigotes growth was noted (Kinuthia *et al*., 2013).

3.9.3 Anti-amastigote assay

The anti-amastigote assay was carried out as described by Delorenzi *et al*. (2001). BALB/c mouse was induced by injecting 2% starch intra peritoneally and left for 24 hours. The mouse was anaesthetized using 100μl pentobarbitone sodium (Sagatal®). The body surface of the mouse was disinfected with 70% ethanol after which it was torn dorso-ventrally to expose the peritoneum. Ten milliliters of sterile cold PBS was injected into the peritoneum. Peritoneum was gently massaged for 2 minutes to dislodge and release macrophages into the PBS. The peritoneal macrophages were then harvested by withdrawing the PBS. The macrophages were washed by centrifuging at 2,000 rpm for 10 minutes and the pellet obtained was re-suspended in 1ml of RPMI culture medium. 250µl of macrophages were adsorbed in 24-well plates for 4 hours at 37ºC in 5% CO^2. Non-

adherent cells were washed off with cold sterile PBS and the adherent macrophages were incubated overnight in RPMI culture medium. Adherent macrophages were then infected with 1×10^6 of *L. major* promastigotes and were further incubated at 37ºC in 5% CO^2 for 4 hours after which they were washed with sterile PBS to remove the free promastigotes, that were not engulfed by the macrophages. This was followed by incubation of the preparation for 24 hours in RPMI 1640 culture medium. The infected macrophages were then treated with combined aqueous extracts of *A. secundiflora*/*A. sativum* MIC based concentrations in fixed ratios of 1:1. Pentostam was used as positive control drugs to compare the parasite inhibition with that of the plant extracts. The medium and test extracts or drug were administered daily for 3 days. After 5 days, the macrophages were washed with sterile PBS at 37ºC, fixed in methanol and stained with 10% Giemsa. The number of amastigotes was determined by counting at least 100 macrophages in duplicate cultures, and the count was expressed as infection rate (IR) and multiplication index (MI) as described by Berman & Lee (1984) in the following formulae ;

$$IR = \text{Number of infected macrophages in 100 macrophages}$$

$$MI = \frac{\text{Number of amastigotes in experimental culture (extracts)/100 macrophages}}{\text{Number of amastigotes in control culture (RPMI)/100 macrophages}} \times 100$$

3.9.4 Determination of Nitric oxide production

Nitric oxide was quantified to determine whether the extracts function by stimulating tne macrophages to synthesize nitric oxide involved in the killing of the parasite. Nitric Oxide (NO) release in macrophages culture was measured as described by Gamboa-Leon

et al., (2007). BALB/c peritoneal macrophages were placed in individual wells in 96-well plates and allowed to adhere at 37ºC in 5% CO_2 in humidified atmosphere for 2 hours. Then RPMI-1640 with 10% FBS was added to the wells containing the cells and finally the test drugs (AF, AS, AF/AS) and controls were added and the mixtures were further incubated for 48 hours. 100 ml of the supernatants were collected for NO measurements. The assay was done in triplicate. Measuring of NO was done as described by Hollzmuller e*t al*., 2002. A volume of 60 ml of Greiss reagent A (1% Sulphonilmide in 1.2 M HCL) was added to the wells followed by 60ml of Greiss reagent B (0.35 N[1-naphthy] ethlenediamine). The plates were read at 540nm in the enzyme linked immunosorbent assay (ELISA) reader. Sodium nitrite in RPMI was used to construct a standard curve for each plate reading.

3.10 *In vivo* Assays

3.10.1 Infection and treatment of BALB/c mice

Inoculations were done using fine 1ml, 30 gauge insulin needles. The infection of BALB/c mice with *L. major* was carried out as described by Gamboa-Leon *et al*. (2007). Briefly, the thicknesses of hind footpads of the mice were measured using a reading vernier caliper prior to infection. The left hind footpads of the mice were subcutaneously inoculated with 1×10^{6} stationary phase infective metacyclic promastigotes of *L. major* in 40μl sterile PBS. The right footpad was left as a contra lateral control. Lesions were left to develop for 4 weeks (Nolan and Farrell, 1987).

After four weeks, treatment with test extracts and controls commenced after the lesions had developed. The mice were grouped into 8 and marked differently according to treatments as shown in table 3.1. The necessary dosage for combination therapy was calculated using the MICs established and a ratio of 1:1 of the extracts was used to treat the mice. Briefly, 2mg of *A. secundiflora* and 5mg of *A. sativum* was weighed and then dissolved in 1ml of PBS. Treatment was done to each group that comprised of 8 mice. Group 1 were injected 100µl (50µl of AF and 50µl of AS) of the combination drug per mouse per day using 1ml, 30 gauge insulin needle intraperitoneally for 5 weeks, Group 2 were orally treated with the same dose daily using a cannula for 5weeks. Group 3 (Positive control) were treated with the standard dose of 20mg/kg daily of pentostam for 30 days intraperitoneally, Group 4 were treated with 100µl PBS intraperitoneally (negative control group) for 5 weeks and group 5 were treated with 100µl PBS (negative control group) orally using a cannula for 5 weeks.

3.10.2 Lesion size measurements

Lesions development and progression was monitored weekly by using a direct reading vernier caliper to measure the thickness of the infected left hind foot pad and comparing it with that of non-infected right hind foot pad. The infected footpads of all mice groups in different treatment groups were measured using a direct reading vernier caliper and lesion size calculations was done using the method described by (Nolan & Farrell, 1987) as follows:

Lesion size = Size of infected footpad-Contra lateral uninfected footpad (mm).

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

3.10.3 Determination of spleen parasite loads in splenic impression smears

After 5 weeks of treatment, mice were killed by inoculating 60mg/kg body weight of penta barbitone sodium. Spleen impression smear were prepared on clean microscope slides. They were left to dry for 15 minutes followed by fixation using absolute methanol. The method of Bradley & 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 in order to enumerate the number of amastigotes per 1000 host nuclei at a high power magnification. The relative and total numbers of parasites in the spleen, named Leishman-Donovan Units (LDU) and total LDU respectively were calculated according to the formula by Bradley & Kirkley (1977) as follows:

$$\text{LDU} = \frac{\text{No. of parasites}}{\text{1000 host nuclei}}$$

$$\text{Total LDU} = \text{LDU} \times \text{organ weight} \times 2 \times 10^5$$

Comparison between LDU values for all the groups was done.

3.11 Statistical analysis

Data were analyzed using Statistical package for social sciences (SPSS) software programme version 20 utilizing t-test and one way analysis of variance (ANOVA) with Tukey and Games-howell test statistic as *Post hoc* tests where applicable. Descriptive statistics were used where appropriate. *P* value of less than or equal to 0.05 was considered statistically significant. Data were presented in form of tables, line graphs or bar graphs.

CHAPTER FOUR

RESULTS

4.1 Plant materials yields

The initial weight of the ground powder was taken before extraction. 100g was used and the yield was also weighed after extraction and the percentage yield was calculated as a percentage of the initial weight. The results are shown in the table 4.1 below.

Table 4.1: Yields of the plant extracts obtained from 100g of plant material

Plant species	Part used	Type of extraction	Code	Initial weight (g)	Yield(g)	Yield (%)
Aloe secundiflora	Leaves	Aqueous	AF	100	18.44	18.44
Allium sativam	Bulbs	Aqueous	AS	100	25.30	25.30

4.2 *In vitro* bioassays

4.2.1 Cytotoxicity test using Vero cells

Cytotoxicity of the aqueous single plant extracts on Vero cells were 467.09µg/ml for *A. secundiflora* and 540.14µg/ml for *A. sativum* (table 4.2). The combination drug showed a

cytotoxicity level of 391.79µg/ml. The combination therapy showed high toxicity of 391.79µg/ml compared to the single plant extracts.

Table 4.2: The IC_{50} (μg/ml) of the test extracts and viability of treated vero cells

Drug	Code	IC_{50} (μg/ml)	$-\log_{10}IC_{50}$	Cell viability (%)
A. secundiflora	AF	467.09	-2.67	98.15
A. sativum	AS	540.14	-2.73	99.4
A. sec/ A. sativum (1:1)	C	391.79	-2.59	90.9
Pentostam	Pento	108.58	-2.04	79.10

Note: The initial concentration of the test extracts was 1000μg/ml, while that of control drug was 100 μg/ml and both were serially diluted by a factor of 3.

The results shows that the higher the cytotoxicity, the higher the negative log base $\log_{10}IC_{50}$ values (- $\log_{10}IC_{50}$). The results above shows that the combination of aqueous AF/AS was more toxic to Vero cells with a $\log_{10}IC_{50}$ value of -2.59, followed by *A. secundiflora* with a $\log_{10}IC_{50}$ value of -2.67 and *A. sativum* was the least with a $\log_{10}IC_{50}$ value of -2.73. The cell viabilities (%) of the Vero cells following treatment with the same extracts and the control drug also showed the same pattern. For instance, Vero cells treated with the slightly toxic combination ASF/AS, had the lower viability of 90.9% compared to the less toxic extract of *A. secundiflora* with a viability of 98.15% and the least toxic *A. sativum* 99.4%. One sample t-test showed a significant difference in the ability of cells to grow in these conditions (t = 4.008, P = 0.028). The low cytotoxic

potential of the extracts in this study is of great significance for their traditional usefulness in the treatment of leishmaniasis.

4.2.2 Evaluation of Minimum inhibitory Concentration (MIC)

When promastigotes were exposed to different concentrations of single plant extracts, combination of the extracts and the control pentostam drug, *A. secundiflora* (AF) showed MIC of 2000 μg/ml or 2 mg/ml and *A. sativum* (AS) inhibited the survival of *L. major* promastigotes *in vitro* at concentration of 5000 μg/ml or 5 mg/ml. The combination therapy using *A. secundiflora and A. sativum* had a minimum growth of promastigotes at ratio of 1:1. In comparison pentostam (+ve control) was able to inhibit the growth of *L. major* promastigotes *in vitro* at a concentration of 12.5μg/ml. The Schneider's *Drosophila* medium, on the other hand, supported the maximum survival of the *L. major* **promastigotes as indicated by the four pluses (++++) in Table 4.3.**

Table 4.3: Survival of the *L. major* promastigotes in varying concentrations of single and combination plant extracts (MICs).

Test plant extract	**Code**	**Concentrations of the extracts (μg/ml)**					
		5000	**4000**	**3000**	**2000**	**1000**	**500**
A. secundiflora	AF	-	-	-	+	++	+++
A. sativum	AS	+	++	+++	++++	++++	++++
		Ratio of the two extracts based on MIC's					
		1:1	1:2	2:3	3:1	2:1	1:1
Combination drug (AF/AS)	C	+	-	-	-	-	+
Controls		Concentrations of the pentostam (μg/ml)					
		100	50	25	12.5	6.25	3.125
Pentostam (+ve)		-	-	-	+	++	+++
Schneider's Insect Medium(-ve		++++	++++	++++	++++	++++	++++

Key: - no live parasite seen, + minimum parasite seen, ++ more than minimum parasites seen

4.2.3 Anti-amastigotes assay (Macrophage assay)

RPMI 1640 mediumwhich had no drug supported the growth of L. major amastigotes in peritoneal macrophages (figure 4.1b) and this was indicated by an infection rate of 71.5%. The leishmaniasis drug pentostam inhibited the *in vitro* survival of *L. major* amastigotes in BALB/c mice peritoneal macrophages more effectively at IR of 21% at a concentration of 50μg/ml which was very close to drug AF and combination drug (AF/AS) while RPMI 1640 medium which had no drug in-cooperated supported the growth of *L. major* amastigotes in peritoneal macrophages more effectively and this was indicated by a high infection rate (IR) of 71.5± 2.12 % (Table 4.4). *A. secundiflora, A. sativum,* and their combination (1:1) had IR of 23%, 53% and 17% respectively at a concentration of 125μg/ml as shown in table 4.4 below.

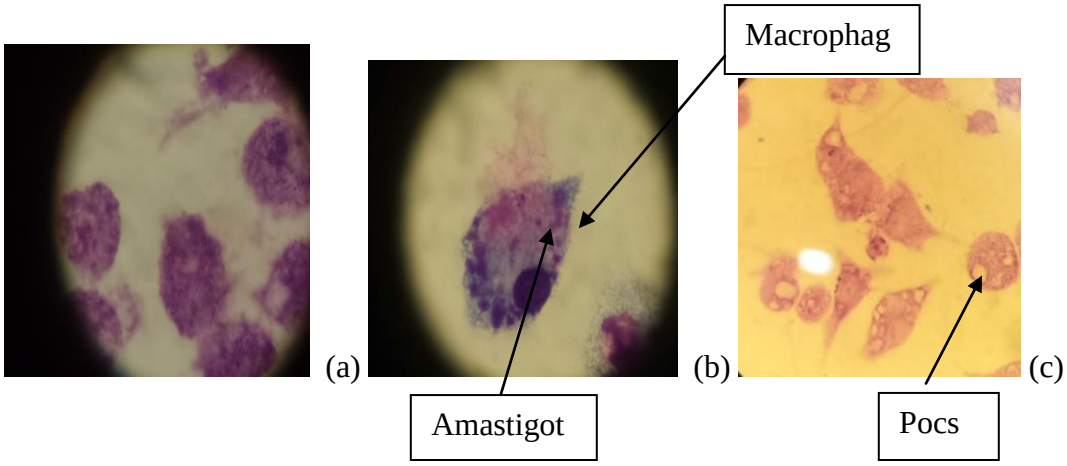

Figure 4.1: (a) BALB/c mice peritoneal macrophage without amastigotes. (b) BALB/c mice peritoneal macrophage (m) having engulfed *L. major* amastigotes (Am) in RPMI-1640 medium culture. (c) BALB/c mice peritoneal macrophages with pocs after escape of the L. major amastigotes after treatment with combined extracts.

Table 4.4: The *in vitro* infection rates (IR) and multiplication indices (MI) of L. major amastigotes per 100 peritoneal macrophages following treatment with single and combination test extracts at concentrations that ranged between 125 to 31.25µg/ml.

Test extracts and controls	**Concentration. (*µ*g/ml)**	**Infection rate (%)**	**Amastigotes per 100 cells (multiplication)**	**Multiplication index (%)**
A. secundiflora	125.00	23	49	20.37
	62.5	30	187	77.75
	31.25	57	360	149.69
A. sativum	125.00	53	231	96.05
	62.5	82	364	151.35
	31.25	88	487	202.49
C(AF/AS)	1:1	17	117	48.65
RPMI	1st	70	230	n/a
	2nd	73	251	n/a
	Average	71.5± 2.12(SE)	240.5±10.5	100
Pentostam	50.00	21	28	11.64
	25.00	33	30	12.47
	12.50	37	49	20.39

RPMI: Negative control in which the amastigotes multiplied maximally. AF= *A. secundiflora,* AS = *A. sativum*; C= Combination of AF and AS, Pento= pentostam (+ve control).

Infection rate was used to determine the number of infected cells per 100 macrophages. The infection rate (%) after treatment with *A. secundiflora* at concentration of 125μg/ml and combination of AF/AS at ratio of 1:1 was 23% and 17% respectively which compared closely with that of control drug (pentostam) at 21% at a low concentration of 50μg/ml shows that their activity was relatively higher. High concentration of test extracts and the control drug resulted to low IRs and MIs of *L. major* amastigotes. However, One way ANOVA analysis of the IR% of AF, AS at 125μg/ml and C (1:1) compared to pentostam at concentration of 50μg/ml and RPMI showed no significant difference with $F_{(1,3)} = 1.369$ and $P = 0.544$. Comparison of IR% of the drugs at 31.25μg/ml (57%, 88%) and combination, C, (17%) with the controls, pentostam at 12.5μg/ml (37%) and RPMI (71.5%) showed no significant difference ($F_{(1, 3)} = 1.837$, $P = 0.486$).

When the MIs of amastigotes in peritoneal macrophages that were treated with 125μg/ml to 31.25μg/ml of test aqueous extracts (AF, AS, and Combination, C) were compared with those treated with 50μg/ml of pentostam and RPMI, using one way ANOVA, there was a significant difference ($P = 0.05$) observed in some groups. A Tukey *post hoc* test revealed that the MI% of aqueous *A. sativum* (AS) was significantly different from that of RPMI ($P = 0.015$) and pentostam compared to RPMI was significantly different with a $P = 0.003$. The comparison of MIs% of drug C and AS (*A. sativum*), drug C and pentostam revealed no significant difference ($P = 0.07$ and 0.631) respectively. However, comparison between drug C and RPMI was significantly different with a $P= 0.020$.

4.2.4 *In vitro* nitric oxide assay

Nitric oxide (NO) plays a key role as a leishmanicidal effector molecule in host macrophages (Gamboa-leon *et al*., 2007). Therefore the effect of the single aqueous plant extracts and their 1:1 combination in NO production was evaluated *in vitro*. Since the breakdown of NO occurs in the macrophages to release nitrite (NO_2^-) as one of the products, NO produced was estimated by quantifying the NO_2^- content. The representative nitrite standard reference curve in RPMI medium was prepared. The optical densities (OD) readings of the aqueous single plant extracts and their combination were all less than 0.10, ranging between 0.030-0.092 (Table 4.5) at concentrations of the extracts that ranged between 3.125 to 100µg/ml and this implied that, negligible amount of NO was produced (≤20µm of NO) as estimated from the standard nitrite curve. RPMI-1640 medium produced similar negligible levels of NO (negative control). These findings shows that both the test extracts and the control drug did not stimulate the macrophages to produce NO which is an effector molecule in the parasite killing. Test drugs and the drug of choice, pentostam used other mechanisms in the killing of the parasites.

Table 4.5: Absorbance (OD) units ± SD of the standard nitrite (positive control), RPMI- 1640 (negative control), the single aqueous extracts and combination from the study plants.

Conc (µM)	Standard Nitrite (-ve Control)	RPMI	*A. secundiflora* (AF)	*A. sativum* (AS)	Combination (C)
0.0	0.054± 0.011	0.021	0.051	0.030	0.038
20	0.153± 0.013	0.030	0.053	0.033	0.039
40	0.178±0.001	0.038	0.061	0.036	0.044
60	0.331± 0.000	0.048	0.051	0.041	0.051
80	0.599± 0.004	0.055	0.059	0.043	0.087
100	0.983± 0.010	0.079	0.066	0.044	0.092

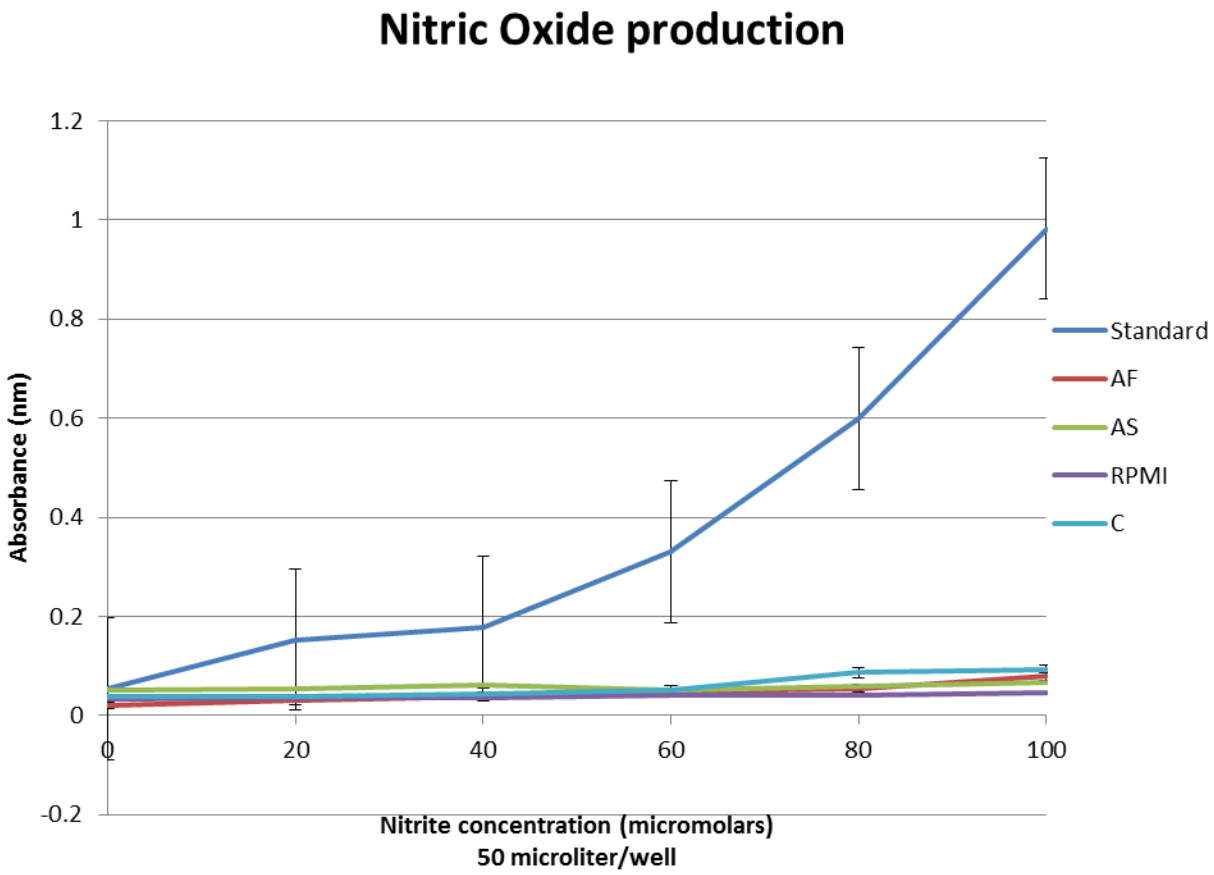

Figure 4.2: production of nitric oxide (µM) by BALB/c mouse derived macrophages following treatment with different concentrations of single aqueous test extracts, combination and RPMI

4.3 *In vivo* bioassays

4.3.1 Effects of combination therapy (C) on lesion sizes and body weights

Figure 4.3 shows the swollen foot pad that was measured for 5 weeks during treatment. When combined extracts of *A. secundiflora* and *A. sativum* (C) were administered intra-peritoneally and orally to treat *L. major* infected BALB/c mice, the foot pad lesion sizes tended to decrease during the first two weeks and there after remained constant for the

next 3 weeks subsequently despite continued treatment. One way ANOVA ($P < 0.05$) analysis showed there was a significant difference of the mean lesion sizes within and between the groups with $F = 9.613$ and $P = 0.0001$. Post hoc tests of multiple comparisons (Games-Howell) showed there was a significant difference between combination (C) IP treatment compared to PBS oral with a $P=0.008$ in lesion sizes while there was no significant difference with combination (C) oral and PBS oral with a $P=0.609$ in lesion sizes. Pentostam also produced a significant difference against PBS oral with a $P=0.017$. Post hoc tests also showed that there was no significant difference in the mean lesion sizes between the mice treated intraperitoneally and the drug of choice pentostam ($p=0.844$) which was also same to mice treated orally $p=1.000$ as shown in (table 4.7).

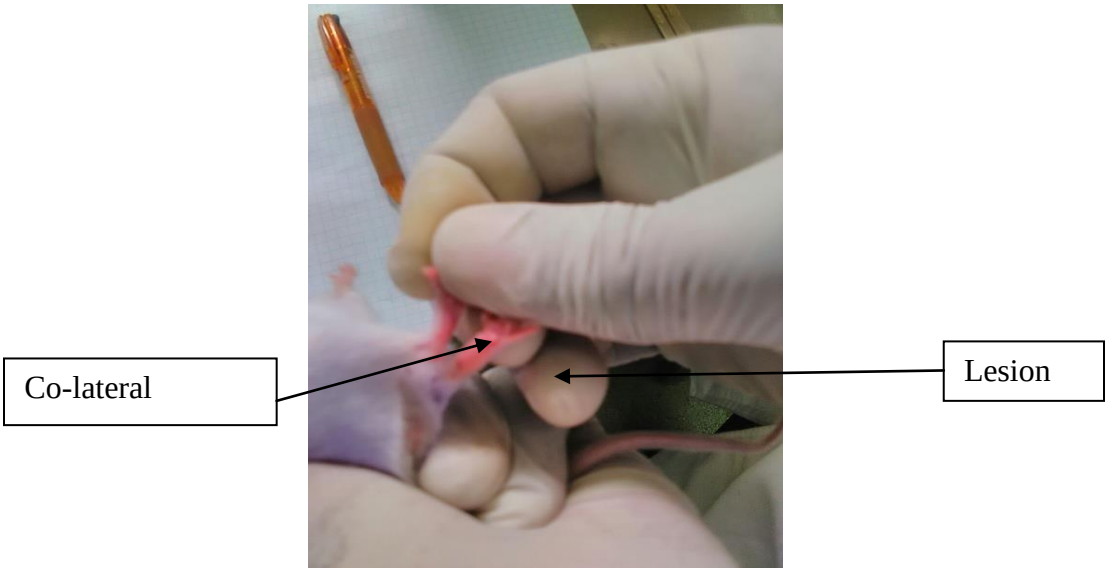

Figure 4.3: Swollen left foot pad or lesion of a BALB/c mouse after infection with metacyclic *L. major* promastigotes compared to non infected contra-lateral right footpad.

Table 4.6: Average lesion size (mm) of BALB/c mice infected with *L. major* treated with combination aqueous test extracts and controls administered orally or intra-peritoneal (ip) for 5 weeks.

Drugs	Code	Route	Mean f/pad swelling (mm) [a] Wk1	Wk2	Wk3	Wk4	Wk5	Drug vs PBS sig. value
A.sec/A. sat oral	C	IP	0.24	0.23	0.16	0.13	0.13	0.008*
A.sec/ A. sat	C	Oral	0.7	0.6	0.45	0.3	0.3	0.609
Controls:								
PBS	-	IP	0.38	0.68	1.12	1.02	0.79	0.725
PBS	-	Oral	0.29	0.57	0.79	0.79	0.79	N/A
Pentostam	-	IP	0.40	0.30	0.20	0.10	0.10	0.017*

[a]Average foot pad swelling was determined by getting the difference in thickness between the infected left hind footpad and the contra- lateral non infected right hind footpad; *shows significant difference against PBS oral.

Table 4.7: Post hoc tests (Games-Howell) of multiple comparison of mean lesion size in combination therapy, positive control and the negative control groups.

Drugs compared	Mean lesion difference	SE	P value	95% CI	
				Lower	Upper
C ip vs C oral	-0.292	0.122	0.158	-0.657	0.073
C ip vs PBS ip	-0.478	0.153	0.093	-1.035	0.079
C ip vs PBS oral	-0.326	0.127	0.171	-0.769	0.118
C ip vs Pento	0.100	0.099	0.844	-0.249	0.449
C oral vs PBS ip	-0.582*	0.139	0.042*	-1.138	-0.026
C oral vs PBS oral	0.430*	0.109	0.044*	-0.847	-0.013
C oral vs Pento	-0.004	0.075	1.000	-0.266	0.258
PBS oral vs Pento	0.578*	0.143	0.042*	0.026	1.130
PBS oral vs Pento	0.426*	0.115	0.047*	0.007	0.845

*shows significant difference between the two drugs, SE=Standard error of the mean, CI= Confidence Interval, C ip=Combination therapy of *A. secundiflora/A.sativum* intraperitoneal,

C oral=Combination therapy of *A. secundiflora/A. sativum* oral, PBS=Potassium buffered Saline, Pento=Pentostam.

Table 4.8 Body weights (g) of BALB/c mice infected with *L. major* and treated with combination test extracts and controls administered orally or intra-peritoneal (ip) over 5 weeks**.**

Paired t- test for the body weights					
Test drug	**Initial wgt ±SE**	**Final wgt ±SE**	**Difference(g)**	**Calculated t**	**p value**
Combination(IP)	21.67±2.03	20.67±2.33	-1.00	1.168	0.296
Combination(oral)	30.25±1.16	29.38±0.82	-0.87	1.369	0.213
PBS (IP)	20.07±0.52	19.33±0.33	-0.74	2.750	0.111
PBS(Oral)	21.00±0.56	18.67±0.33	-2.33	3.500	0.073
Pentostam(IP)	21.67±0.33	21.67±0.33	0.00	0.001	1.000

Key:, SE=Standard error of the mean.

The body weights of infected mice decreased during treatments with plant extracts under combination therapy. Similar findings were observed in the negative control group injected with PBS buffer. The weights of the mice in the positive control group (pentostam) were not affected. The t-test showed that there was no significant loss of body weights at $P < 0.05$ for infected BALB/c mice that were treated both orally and

intraperitoneally with the combined drug as shown in table 4.8. However, the effect of the body weight is also affected by factors like nutrition, age, stress and the diseased state of the mice hence did not depend on the infection alone.

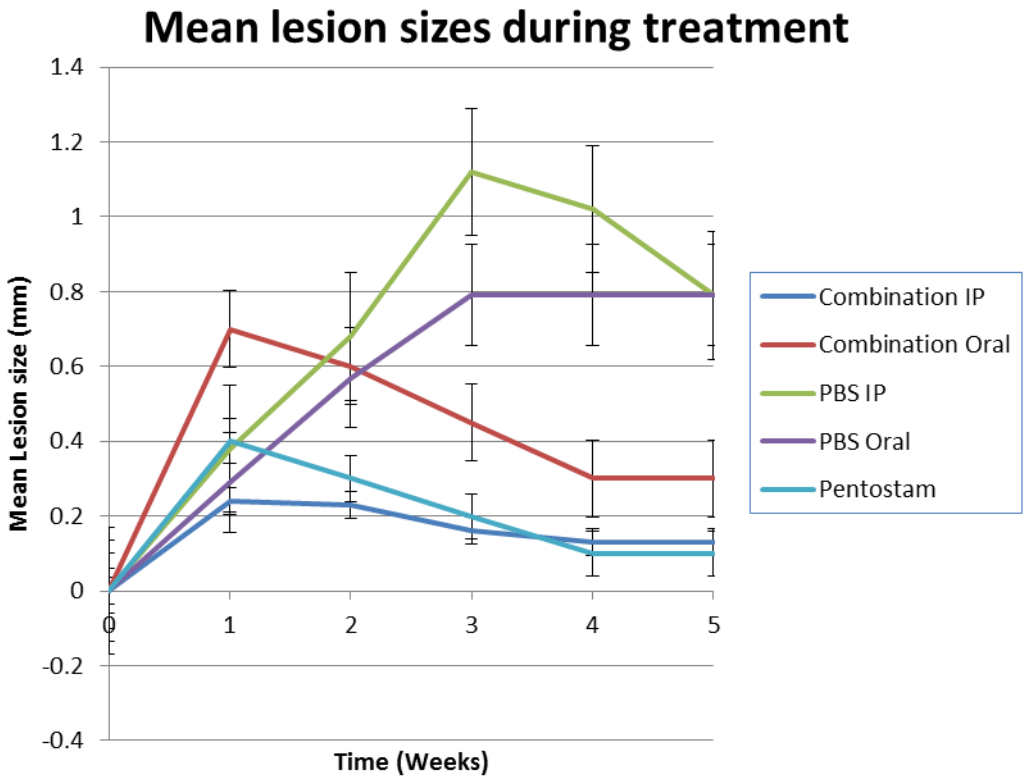

Figure 4.4: The foot pad swelling after oral treatment of L. major infected BALB/c mice (combination therapy (C) oral and intraperitoneally, Pento = Pentostam; PBS oral = phosphate buffered saline administered orally and intraperitoneally)

Combination ip led to reduction in mean lesion size relatively faster than combination oral suggesting that ip administration is the better route of extract delivery than oral as shown in figure 4.4. This is because intraperitoneal administration the extracts reached

the body systems faster than oral as it has to be absorbed fast hence delay in reaching the systems.

4.3.2 Estimation of number of *Leishmania* parasites in the infected BALB/c mice splenocytes

At the end of the experiment, the mice were sacrificed, the spleens were weighed and their impression smears made and the numbers of amastigotes per 1000 nucleated splenocytes (Figure 4.4) were counted (figure 4.6). The spleen index (%), Leishman-Donovan Unit (LDU), total Leishman-Donovani Unit (total LDU) and percentage parasite reduction were determined

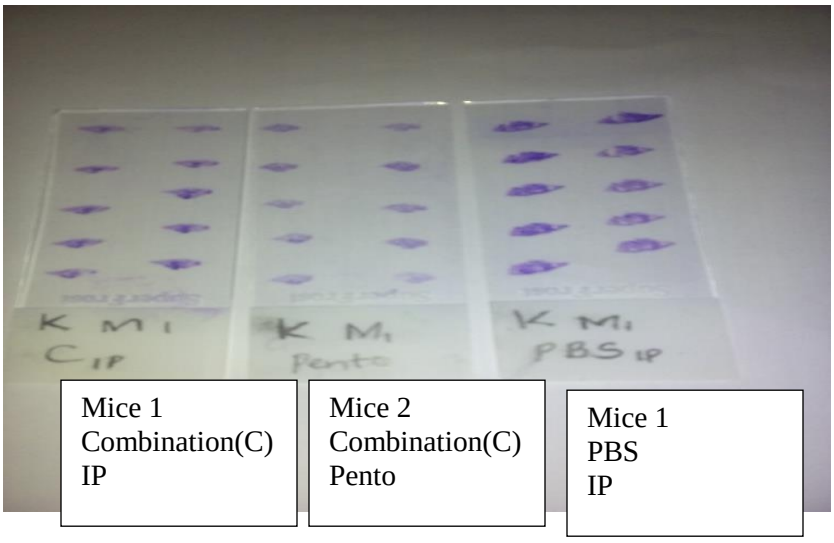

Figure 4.5: Giemsa-stained impression smears made from the spleen tissues obtained from *L. major* infected BALB/c mice.

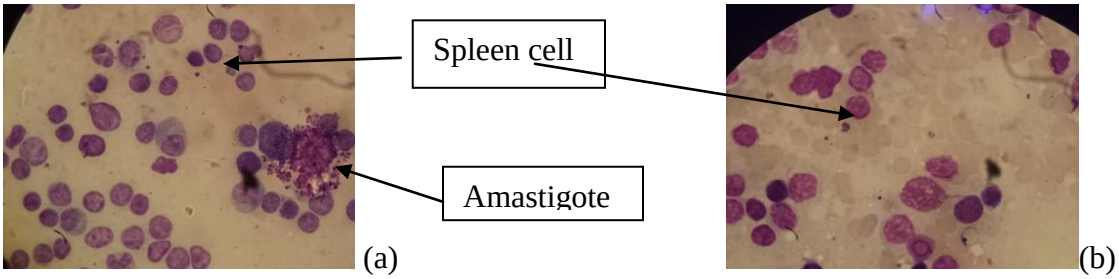

Figure 4.6: (a) Amastigotes which are interspersed among the splenocytes (smear from an infected BALB/c mouse that had been treated with oral PBS for five weeks. (b) Splenocytes (that lacked amastigotes in a spleen smear of an infected BALB/c mouse which was treated with oral combination therapy (C) for five weeks).

The PBS oral treatment was used to calculate the % parasite reduction and was arbitrary assigned 0.00% parasite reduction as shown in table 4.9. The ip combination therapy had the highest parasite reduction percentage of 64.13% as compared to oral combination therapy with 55.48%. Pentostam, the positive control, had the highest parasite reduction percentage of 94.58% as expected as it's the drug of choice for leishmaniasis. A small percentage was observed in the negative control (PBS). It was observed that mice treated intraperitoneally had a higher % parasite reduction in their spleens which corresponds to a lower total LDU value as compared to those treated orally. Anova analysis of average total LDU for both test drugs and the controls showed significant difference with P=0.033. However, combination, C, ip and combination, C, oral had no significant difference in total LDU (P= 0.199). Comparison of total LDU of pentostam and of PBS oral and PBS ip showed significant difference (P=0.001).

Table 4.9 Average spleen weight, index, LDU, total LDU and %parasite reduction for groups of L. major infected BALB/c mice that were treated with combined test aqueous and controls.

Drug/ Controls	Route	Ave spleen weight ±SE	Ave spleen Index (%) ±SE	Ave LDU ±SE	Ave total LDU ±SE	% parasite reduction[a] (× 1000)
Combination ratio 1:1						
A. sec/A. sat	ip	0.125± 0.014	0.53 ± 0.07	0.59 ± 0.018	14.69 ± 1.33	64.13
A. sec/ A. sat	oral	0.15± 0.01	0.50 ± 0.05	0.61 ± 0.03	18.23 ± 0.90	55.48
Control						
Pentostam	ip	0.10± 0.003	0.45 ± 0.19	0.11 ± 0.003	2.22 ± 0.13	94.58
PBS	ip	0.20± 0.010	1.01 ± 0.05	0.93 ± 0.012	37.15 ±1.09	9.28
PBS	oral	0.23± 0.020	1.10 ± 0.11	0.93 ± 0.001	40.95 ± 3.75	0.00

[a] means that the % was calculated in reference to total LDU for PBS oral which was taken to represent 100% parasite burden.

Table 4.10: ANOVA analysis of parasite reduction

ANOVA

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	18632.892	4	4658.223	141558.644	.0001
Within Groups	.329	10	.033		
Total	18633.222	14			

Analysis of parasite reduction percentage using ANOVA showed there was a statistical significance in the parasite reduction between the test drugs, positive control (pentostam) and the negative control (PBS) P=0.001 (table 4.10).

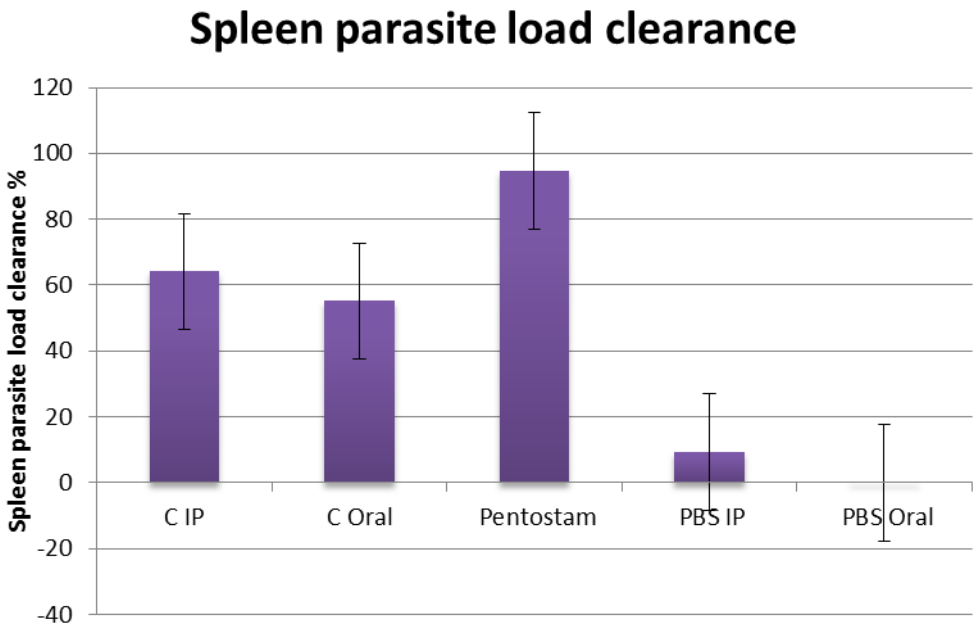

Figure 4.7: Showing spleen parasite load clearance after treatment

This shows there was significant parasites load reduction in the combination therapy and for pentostam compared to the negative control. Pentostam ip cleared most parasite load being the drug of choice for leishmaniases compared to the combination therapy but there was no significant difference. The ip administration showed a more reduction percentage than the oral combination therapy but there was no significant difference (figure 4.7).

CHAPTER FIVE

DISCUSSION, CONCLUSIONS AND RECOMMENDATIONS

5.1 Discussion

Research is taking place on various plant extracts with the hope of developing natural products with substantial antimicrobial activity. These plants contain various compounds that posses' antimicrobial activities whose effectiveness and modes of action differs with the group of compounds involved (Cohen *et al.,* 2001). The alarming arise in the development of drug resistant infections, emerging and reemerging infections have all contributed to intensive research for alternative therapy. Using plant based products are more advantageous as development of resistance by parasites is limited (Cohen *et al.,* 2001). Many plants have been documented to have antimicrobial activity. *A. secundiflora* leaf extracts has shown to contain a mixture of phenolic compounds, mainly anthrones, aloenin, aloenin B, isobarbaloin, barbaloin and other aloin derivatives (Rabecca *et al*., 2003). On the other hand, *A. sativum* bulbs are known to contain organosulphur compounds present in the bulbs (Kinuthia *et al.,* 2013). This chapter discusses the results obtained when the aqueous crude extracts of combining *A. secundiflora* and *A. sativum* were tested against *L. major in vitro* and *in vivo.* The efficacy of combination therapy carried out in this study is presumed to the synergistic interaction of water soluble components of these plants.

Previous studies showed that both water and methanolic extracts of *A. secundiflora* and *A. sativum* have efficacy in reducing *L. major* lesion development in experimental BALB/c mice (Kinuthia *et al.*, 2013 ; Ogeto *et al.*, 2013). This study sought to determine whether combining the two plant extracts and using them to treat *L. major* infection revealed that when administered either orally or intraperitoneally, combination therapy can be used in partial control of the leishmaniases.

Aloe secundiflora and *A. sativum* water extracts showed low toxicity (IC_{50}) against healthy Vero cells of 467.09µg/ml and 540.14µg/ml, respectively. The IC_{50} from a previous study on *A. secundiflora* reported a level of 547.88µg/ml (Ogeto *et al.*, 2013), while *A. sativum* showed 1306.68µg/ml (Kinuthia *et al.*, 2013). This variation of the IC_{50} could be due to method of extraction, source of the plants, calibration of the software used to generate the $IC_{50,}$ among other factors. The combination treatment of the extracts showed high toxicity of 391.79µg/ml which could suggest presence of synergistic effect since individual extracts had low toxicity. The combination had a lower toxicity than pentostam at 108.58µg/ml but the difference was not significant. The IC_{50} affected the cell viability with the combination closely comparing with that of pentostam at 79.1% and 90.9%, respectively. This showed that the combination therapy was less toxic as compared to the drug of choice, pentostam.

This study reported a minimum inhibition concentration of 2000µg/ml and 5000µg/ml for *A. secundiflora* and *A. sativum,* while Ogeto *et al*. (2013) and Kinuthia *et al*. (2013) reported the same MICs respectively. The MIC for the single extracts was combined into

several ratios and the 1:1 ratio supported the minimum growth of the *L. major* promastigotes. When more than one part of MIC of any single extracts was combined with one part of the other no life *L. major* promastigotes was observed and this provided the basis for *in vivo* treatment of BALB/c infected *Leishmania major* parasites with assumption it would inhibit the growth and multiplication of parasites.

Since the extracts were not toxic to Vero cells and macrophages and, did not stimulate the macrophages to produce sufficient amounts of nitric oxide, the actual mode of action by the extracts is not fully known. Presumably, the activity of *A. secundiflora* aqueous and methanolic extracts against *L. major* strains could be due to the ability of flavonoids to form complexes with the parasite cell wall and inhibiting the action of DNA polymerase (Ogeto *et al*., 2013). The plant also contains terpenoids, flavonoids and tannins which are known for disrupting the cell membranes of the *L. major*. This could also account for the inhibitory activity of *A. secundiflora* extracts (Ogeto *et al.*, 2013).

The efficacy of the test aqueous extracts and combination therapy in inhibiting the replication of amastigotes in infected BALB/c peritoneal macrophages was quantified by calculating their infection rate (IR) and multiplication indices (MI). The study reported IR of 23% and MI of 20.37% for *A. secundiflora*, an IR of 53% and MI of 96.05% for *A. sativum* at concentration of 125µg/ml, and an IR of 17% and MI of 48.65% for their combination (1:1) respectively. In comparison, the control drug, pentostam showed an IR% of 21% and MI% of 11.64% at a concentration of 50µg/ml. it can be noted from the results above the combination (C) inhibited the infection of macrophages more effectively as compared to the single extracts at a concentration of 125µg/ml and the

control drug at concentration of 50µg/ml. There was a significant difference between the efficacy of the test crude extracts, their combination and that of the *Leishmania* drug ($P < 0.05$). This shows that the antileishmanial compounds present in aqueous extracts of *A. secundiflora* and *A. sativum* have additive effects. When the test extracts and their combinations were compared with negative control, IR of macrophages by *L. major* amastigotes in plain RPMI - 1640 medium was 71.5± 2.12%. This agrees with Berman and Wyler (1980) who observed that *L. tropica* and *L. donovani* amastigotes in human macrophages multiplied about three fold in six days when grown in RPMI - 1640 medium in absence of antileishmanial agents. When comparing the IR of the negative control of the current study is very close to that reported by Kinuthia *et al*. (2013) of 84.67±2.96%.

In vivo studies indicated that there was a significant reduction of foot pad lesion sizes in *L. major* infected BALB/c mice that were treated intraperitoneally and orally with the aqueous extracts of *A. secundiflora* and *A. sativum* in a ratio of 1:1 (AF/AS), when compared to the mice treated with the controls PBS, negative control and pentostam, positive control. All the lesion size of all the mice decreased with time in the groups treated with the combination therapy either intraperitoneally or orally and pentostam, but increased in the negative control (pbs oral and ip). By the end of the fifth week of treatment, the lesions were not fully cleared. There was no significant difference in lesion size for the combination treatment of the oral and ip group. All of the infected mice treated orally or intraperitoneally with the combined aqueous extracts of *A. secundiflora* and *A. sativum* extracts had their lesion sizes reduce significantly when compared to those treated

with PBS both orally and intraperitoneally. *A. secundiflora* extracts have been reported to be strong antibacterial agents and this would have contributed a lot in inhibiting the bacterial effects in the lesion wounds hence accelerating their healing (Ogeto *et al*., 2013). Similarly, Garlic extracts have previously been reported to cause re-epithelialization of exposed wounds and they increase the number of loosely packed collagen and maturation of collagen bundles (Ejaz *et al*., 2009). Kinuthia *et al*., (2013) observed that combination therapy has been reported to be effective against leishmaniasis using the combination of *C. citrinus, A. sativum* and *M. stenopetala.*

L. major parasites are known to cause visceralization that is, invade the liver and spleen which are known as visceral organs (Conjivaram & Ruchir, 2007). This research aimed also to determine if the combination therapy of *A. secundiflora* and *A. sativum* would prevent visceralization because this could be a great innovation since the herbal plants could prevent the damage of these important internal organs. Visceralization was done by preparing the impression smears to check for the presence of the amastigotes in the splenocytes of the spleen. Amastigotes were present in the spleen after examination of smears suggesting that the combination therapy could not prevent visceralization. However, upon counting of the parasites in the experimental groups (combination treatment) followed by comparison with controls, there was a significant reduction which showed that the treatment with combination was effective. The study reported a total LDU of 14688, 18226, 2220, 37200 and 40950 parasites in the spleens of Combination treatment intraperitoneally, Combination orally, pentostam, PBS ip and PBS oral respectively after necroscopy. PBS oral and ip showed high parasites load compared to

the other treatment which was significantly different after ANOVA analysis. To determine the parasites reduction, PBS was arbitrary assigned 100% that is no parasite reduction. Parasite reduction for the other treatments was calculated using PBS oral as the standard measure. The findings showed significant reduction in parasite load in the combination therapy and pentostam compared to the negative control. Pentostam cleared high number of parasites being the drug of choice for leishmaniases compared to the combination therapy but there was no significant difference. The ip administration showed a more reduction percentage than the oral combination therapy although there was no statistical difference. Analysis of parasite reduction percentage using ANOVA showed there was a statistical significance in the parasite reduction between the test drugs, positive control (pentostam) and the negative control (PBS).

5.2 Conclusion

The aqueous extracts of *A. secundiflora*, *A. sativum* and their combination were relatively less toxic to Vero cells *in vitro* with a cell viability of 98.15%, 99.4% and 90.9% respectively as compared to pentostam 79.1%. Therefore, the combination extract was less toxic to Vero cells and BALB/c mice than the standard drug pentostam and due to this; they can provide better alternatives as drug for the control of leishmaniases. Intraperitoneally and orally administered combined aqueous of *A. secundiflora* and *A. sativum* extracts both reduced the lesion sizes of *L. major* infected BALB/c mice, but ip had a higher *in vivo* efficacy in reducing amastigotes burden in the spleens of *L. major* infected BALB/c mice with percentage parasite reduction of 64.13%, than oral with

55.48% respectively. Aqueous extracts of *A. secundiflora, A. sativum* and combination did not stimulate production of significant NO in peritoneal BALB/c macrophages, therefore, these extracts used other modes of action in killing the parasites as the NO produced was negligible (less than 0.1) to stimulate the host macrophages to kill the amastigotes hence if used together with other drugs the extracts could provide additive or synergistic effects in the control of different leishmania species. In summary, the findings suggest that the combination therapy using aqueous extracts of *A. secundiflora* and *A. sativum* at a ratio of 1:1, showed high *in vitro* anti promastigote activity, anti-amastigote activity, less toxic to Vero cells as compared to the standard drug pentostam, reduced lesions significantly but did not prevent visceralization (parasite burdens).

5.3 Recommendations

1. Further research should be carried out to determine the active ingredients and their mode of actions of *A. secundiflora* and *A. sativum*.
2. It is important to determine the efficacy of combined extracts of *A. secundiflora* and *A. sativum* in non human primates.
3. People living in leishmaniases endemic areas are advised to use *A. sativum* in their diet due to its antileishmanial activity.

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APPENDICES

Appendix I: Scientific Steering Committee Approval

KENYA MEDICAL RESEARCH INSTITUTE

P.O. Box 54840-00200, NAIROBI, Kenya
Tel (254) (020) 2722541, 2713349, 0722-205901, 0733-400003; Fax: (254) (020) 2720030
E-mail: director@kemri.org info@kemri.org Website:www.kemri.org

KEMRI/SSC/103273 **6th October, 2014**

Christine Kituyi

Thro'
Director, CBRD
NAIROBI

6/10/14

REF: SSC No. 2930 (Revised) – In Vitro and In Vivo Efficacy of Combination Therapy using *Allium sativum* and *Aloe secundiflora* extracts against *Leishmania major* infected Balb/C Mice

Thank you for your letter dated 29th September, 2014 responding to the comments raised by the KEMRI SSC.

I am pleased to inform you that your protocol now has formal scientific approval from SSC.

The SSC however, advises that work on the proposed study can only start after ERC approval.

Sammy Njenga, PhD
SECRETARY, SSC

Appendix II: Animal Care and Use Committee Approval

KENYA MEDICAL RESEARCH INSTITUTE

Centre for Virus Research, P.O.Box 54628 - 00200 NAIROBI - Kenya
Tel: (254) (020) 2722541, 254 02 2713349, 0722-205901, 0733-400003 Fax (254) (020) 2726115
Email: cvr@kemri.org

KEMRI/ACUC/ 03.03.15 **24th March, 2015**

Kituyi Christine
P. O. Box 62000-00200
Nairobi, Kenya.

Kituyi,

RE: Animal use approval for SSC 2930 (Revised) – "In vitro and in vivo efficacy of combination therapy using *allium sativum* and *aloe secundiflora* against leishmania major infected balb/c mice" protocol

The KEMRI ACUC committee acknowledges the resubmission of the above mentioned protocol. It has been confirmed that all the issues raised earlier have been addressed appropriately.

The committee grants you the approval to use laboratory mice in your study but recommends that you proceed after obtaining all the other necessary approvals that may be required.

Approval is granted for a period of one year starting from when the final ethical approval will be obtained. The committee expects you to adhere to all the animal handling procedures as described in the protocol.

The committee wishes you all the best in your work.

KENYA MEDICAL RESEARCH INSTITUTE
★ 24 MAR 2015 ★
ANIMAL CARE AND USE COMMITTEE
Signature: .....

Yours sincerely,

Dr. Konongoi Limbaso
Chairperson KEMRI ACUC

Appendix III: Ethical and Review Committee Approval

KENYA MEDICAL RESEARCH INSTITUTE

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KEMRI/RES/7/3/1 **March 30, 2015**

TO: **KITUYI CHRISTINE (PRINCIPAL INVESTIGATOR)**

THROUGH: **DR. KIMANI GACHUHI,**
THE DIRECTOR, CBRD,
NAIROBI

2/4/15

Dear Madam,

RE: **SSC PROTOCOL No. 2930 (*RESUBMISSION 3 OF INITIAL*): IN VITRO AND INVIVO EFFICACY OF COMBINATION THERAPY USING *Allium sativum* AND *Aloe* secundiflora AGAINST LEISHMANIA MAJOR INFECTED BALB/C MICE (*VERSION 1.5 DATED 24TH MARCH 2015*)**

Reference is made to your letter dated 24th March 2015 and received at the KEMRI Scientific and Ethics Review Unit on 27th March 2015.

This is to inform you that the Committee notes that the issues raised at the 233rd C meeting of the KEMRI SERU held on 18th November, 2014 have been adequately addressed.

Consequently, the study is granted approval for implementation effective this **30th March 2015** for a period of one year. Please note that authorization to conduct this study will automatically expire on **March 29, 2016.** If you plan to continue data collection or analysis beyond this date, please submit an application for continuation approval to SERU by **February 16, 2016**.

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,

EAB

PROF. ELIZABETH BUKUSI,
ACTING HEAD,
KEMRI/SCIENTIFIC AND ETHICS REVIEW UNIT

Appendix IV: Dissection of the rat to obtain the spleen