SYNERGISTIC EFFECTS OF EXTRACTS OF *ALOE SECUNDIFLORA* ENG L. AND *CALLISTEMON CITRINUS* WILLIAM C. IN *LEISHMANIA MAJOR* INFECTED BALB/c MICE

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Synergistic Effects of Extracts of *Aloe secundiflora* Eng L. and *Callistemon citrinus* William C. in *Leishmania major* Infected BALB/c Mice

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DECLARATION

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

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This thesis has been submitted for examination with our approval as university supervisors.

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DEDICATION

To my wife Cecilia Minoo and our sons John Muuo and Antony Mwendwa, thank you for your prayers and being there for me when the journey was rough. Finally, to my parents Late Jackson Ndeti Wambua (1954-2015) and mum Mrs. Theresia Ndeti for their inspiration throughout my academic life.

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ABBREVIATIONS AND ACRONYMS

µL	Microlitre
^{0}C	Degree Celsius
ACUC	Animal Care and Use Committee
ANOVA	Analysis of Variance
BALB/c	Inbred laboratory mice susceptible to *Leishmania major*
CBRD	Centre for Biotechnology Research and Development
CL	Cutaneous leishmaniasis
CO_2	Carbon dioxide
Conc.	Concentration
CTMDR	Centre for Traditional Medicine and Drug Research
DMEM	Dulbecco's modification of Eagle Medium
DMSO	Dimethyl-sulfoxide
FBS	Fetal Bovine Serum
IC_{50}	Inhibition Concentration 50%
IFN-γ	Interferon gamma
IL-4	Interleukin-4
IL-5	Interleukin-5

IP	Intraperitoneal
IR	Infection rate
KEMRI	Kenya Medical Research Institute
LDU	Leishmania Donovani units
MCL	Mucocutaneous leishmaniasis
MEM	Minimum Essential Medium.
MI	Multiplication index
MIC	Minimum Inhibition Concentration
mM	Millimoles
Mt.	Mount
MTT	3-(4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide
Mw.	Molecular weight
PBS	Phosphate Buffered Saline
SIM	Shneider's Insect Media
VL	Visceral leishmaniasis

ABSTRACT

Leishmania major causes painful skin sores in humans medically known as cutaneous leishmaniasis. The current drugs against the disease required prolonged use, is toxic and expensive. The prolonged use has led to emergence of drug resistant by the parasite. Studies have reported antileishmanial activity of several plants with low toxicity and thus such plants can be used as alternative medicine. For instance, *Aloe secundiflora* and *Callistemon citrinus* individual aqueous extracts of have been shown to have antileishmanial activities. Combination therapy using different plant extracts can potentially reduce drug resistance. Efficacy of *A. secundiflora* and *C. citrinus* combination therapy (1:1 ratio) against BALB/c mice *L. major* infected and treated intraperitoneally and orally was evaluated. Positive and negative controls were Pentostam administered intraperitoneally, and phosphate buffered saline intraperitoneally and orally treated respectively. Dry ground test materials were soaked in H_2O at 80^0 C for 1 hour, filtered, and freeze dried. Data was analyzed using student *t*-test and ANOVA with significance level of *p* value < 0.05. *A. secundiflora* had of MIC 2mg/ml while C. *citrinus* had 5mg/ml with IC_{50} levels 467.09µg/ml and 457.88µg/ml respectively. The MIC for combination therapy was at 1: 1 ratio with 58.45µg/ml of IC_{50}. The Infection rate (IR) and multiplication index percentage (MI) of the combination therapy was at 19% and 52.81% respectively. Combination therapy cell viability was at 58.45 µg/ml compared to 467.09µg/ml and 457.88µg/ml for *A. secundiflora* and *C. citrinus* respectively. No statistically significant difference (*t = 2.481*, *p = 0.089*) observed even though combination therapy had high cytotoxicity. The IR of combination therapy (1:1 ratio) at 125*µ*g/ml concentration was 19% compared to 46% for *C. citrinus* and 23% for *A. secundiflora*. ANOVA comparison of the IR% showed no statistically significant difference ($F_{(1, 3)} = 2.242$ and *p = 0.446*). The MI% for *A. secundiflora*, *C. citrinus* and combination therapy (1:1) were 49%, 189% and 127% respectively. There was statistically significant difference (*p* = 0.05) among the MI% using ANOVA analysis. The Nitric Oxide could not be determined since the OB produced was less than 0.1 and therefore antileishmanial activity in these extracts is not related to NO production. Lesions sizes decreased after treatment with combination therapy and there was statistically significant difference ($F_{(4,16)} = 9.127$ and *p* = 0.001) in decrease of the lesion sizes among the treatments. Post hoc analysis using Games-Howell for multiple comparison of lesion sizes showed statistically significant difference (*p* = 0.042 and *p* = 0.044) between oral combination treated and both PBS oral and ip treated groups respectively. The average total parasite load in BALB/c mice treated with the combination therapy reduced and there was significant statistical ($F_{(4, 12)} = 113$, *p = 0.0001*) when comparing with controls. The spleen parasite load reduced significantly (*t* = 2.653, *p = 0.057*) after treatment with combination therapy. In conclusion, combination therapy was effective against *L. major* parasites, significantly reduced both lesion size and showed possibility of synergistic effects both *in vitro* and *in vivo*. This study recommends further research on natural products of test plants to support use of their extracts in management of VL in poverty stricken leishmaniases endemic areas of Kenya.

CHAPTER ONE

INTRODUCTION

1.1 Background Information

Leishmaniases disease is caused by protozoa parasite called *Leishmania*. There are over 20 species of *Leishmania* causing the disease of genus *Leishmania* (Kinetoplastida: Trypanosomatidae) (WHO, 2014). These species are predominant in tropics, subtropics, and southern Europe in about 88 countries (Dujardin *et al*., 2008). Leishmaniasis is classified into 4 main types namely cutaneous (CL), diffuse cutaneous (DCL), mucocutaneous (MCL and visceral (VL). A report by WHO, (2014), estimates the disease risk to over 350 million people while 14 million people are already infected globally. Annually, 1.3 million new cases and 20,000 to 30,000 deaths are reported worldwide (WHO, 2014). The VL accounts for an estimated 200 – 400, 000 of these new cases. About 90% of these VL new cases occur in South American, Asian, and African countries. They include but not limited to Brazil, Asia, Sudan, Ethiopia, South Sudan, Kenya, and Somalia (Alvar *et al*., 2012).

Visceral leishmaniasis also known as kalaazar is characterized by various symptoms. They include high fever, substantial weight loss, hepatosplenomegaly, leucopenia, thrombocytopenia, lymphadenopathy, and anemia. This type is usually fatal if left untreated especially within 2 years of infection. Its fatality rate is high as 100% within the same period if left intreated (WHO, 2014). In addition, oedema, jaundice, co-morbidities, and bleeding are associated signs of poor VL prognosis. VL attack mostly internal organs resulting to their failure which result to majority of the fatal cases. The predisposing factors include malnutrition, young age, organ transplantation, malignancies, and immunosuppressive diseases. Other risks such as civil unrest, poor health systems, underlying diseases, poor diagnostic tests and lack of first-line drugs can lead to large-scale epidemics with higher death rates.

Current treatments are either monotherapeutic or combined drugs and each presents different drawbacks. Treatment of VL in Eastern Africa has been by sodium

stibogluconate (SSG) monotherapy for 30 days. However, WHO in 2010 revised the treatment to combination of SSG and paramomycin for 17 days (WHO, 2010). This was as result of a phase III trial conducted in the region (WHO, 2010). Despite the new treatment been administered for shorter period than the previous one, several drawbacks are reported. First, the treatment must be administered via 2 separate painful injections while patient is hospitalized for the 17 days. Secondly, life-threatening toxicities associated with SSG which is antimony-based treatment such as cardiotoxicity, pancreatitis and hepatotoxicity being reported in some patients. The second line drug for VL is liposomal amphotecirin B (AmBisome®) and is basically used as rescue treatment for specific target populations such as pregnant women, severe disease, or HIV co-infection (Alvar *et al*., 1997; Desjeux *et al*., 2000; Desjeux & Alvar, 2003; Cruz *et al*., 2006; Mishra *et al*., 2011a; Mishra *et al*., 2011b; Roy *et al*., 2012). Its general use is however limited in East Africa due to high cost, need for cold chain storage and should be administered by high skilled health personnel.

There is insufficient and inefficient control mechanisms for the spread Leishmania parasite because it depends on the type of cycle exhibited by the it and the behavior of the animal reservoir (Singh & Sundar, 2015). It involves testing and culling all seropositive animals, indoor spraying, use of insecticide treated bed nets (ITNs), dipping of dogs and topical application of insecticides. Other strategies include use of Deltamethrin treated collars and application of biolarvicides in dwelling places. Each of these methods have one or more associated limitations and drawbacks that limit their general use. Some of the reported limitations include time consuming, high cost, limited efficacy, environmental pollution and uncomfortable to use especially during warmer climates.

Therefore, there is need to upscale pre-clinical research that may result in the identification of new chemical entities that are efficacious, safe, and have short treatment period. In addition, the ideal treatment should be also affordable as well as suitable to be used and stored in remote areas especially of high temperature and poor transport network.

There is sufficient data that shows that new potential and selective agents to treat most tropical diseases caused by protozoans do exists in natural products (Ferreira *et al*., 2002). This study aims at combining the crude extracts of *C. citrinus* and *A. secundiflora* which individually have shown antileishmania activity to exploit the benefit of synergy or additive that can be of greater value to treat cutaneous leishmaniasis caused by *L. major.*

Aloe secundiflora is commonly found in East African countries namely Kenya, Rwanda, Tanzania, Sudan, and Ethiopia. It is evergreen with more than 20 succulent spear-shaped leaves (Tropical Plants Database, 2022). The leaves can be 30 – 75 cm long with a width of 8 – 30cm at base. It can have a stem of about 30 cm long or be stemless. It has been locally used as traditional medicine for both human and livestock and as a fermenting agent (Tropical Plants Database, 2022). Crude extracts from different part of *Aloe secundiflora* have shown inhibition activity against different microbes globally. A study by Msoffe and Mbilu, (2009) reported crude extracts from mature leaves of *A. secundiflora* can inhibit growth of *Candida albicans in vitro*. *Candida albicans* causes candidiasis which causes vaginal irritation and infect male genital organs. It also causes fungemias particularly in immunocompromised patients. Crude extracts from *A. secundiflora* have also been found effective against several bacteria species including *Streptococcus* species, *Pseudomonas*, species, *Pasteurella* species, *Escherichia coli*, Proteus species and *Staphylococcus aureus* (Waihenya, 2002). A study by Kaingu *et al*., (2013) reported inhibitory effects of *A. secundiflora* extracts against larval development of *Ascaridia galli in vitro.* Ethno-veterinary use of *A. secundiflora* leaf exudates have been reported against bacterial and viral diseases. For instance, Newcastle disease, fowl typhoid, coccidiosis among other enteric conditions has been effectively treated using the leaf exudates (Waihenya *et al*., 2005). A study blending crude aqueous extracts of *M. stenopetala*, *C. citrinus* and *A. sativum* at different ratios reported *in vitro* and *in vivo* antileishmanial activity against *L. major* parasites (Kinuthia *et al*., 2013).

Callistemon citrinus is an erect, evergreen shrub and straggly that may grow 1 to 4.5 meters tall. It is mainly known for its ornamental use especially in some parts of Australia (Tropical Plants Database, 2022). *C. citrinus* grows in warm temperate to subtropical and even found in tropical countries such as Kenya. It has variable leave shape that when pressed, emit a refreshing lemon scent (Tropical Plants Database, 2022). Other than its ornamental use, pre-clinical investigations have reported antimicrobial activity against several microorganisms. Single leave and flower extracts have demonstrated to produce strong antioxidant effects (Radulović *et al*., 2015). A study by Fayemi *et al*., (2019) suggested that the antioxidant activity can be used in treatment of metabolic disorders such as type-2 diabetes. Tests of leave and flower extracts from *C. citrinus* have shown antimicrobial activity against *S. typhimurium, E. coli, L. plantarum* and *L. sakei*. Further exciting inhibitory effects have been reported against both gram-negative bacteria including *Y. enterocolitica, S. typhimurium* and *E. coli*) and gram-positive bacterium such *B. cereus, S. aureus, L. monocytogenes* (Fayemi *et al*., 2019). Single flower extracts of *C. citrinus* demonstrated antileishmanial activity at low toxicity (Kinuthia *et al*., 2013).

It is evident that monotherapy of leaves from *A. secundiflora*, flower and leaves extract from *C. citrinus* have extensively been studied and shown to have bioactivity against several bacteria, viruses, and protozoans. Therefore, there is need to explore synergistic effects of these sources against *L. major* parasites both *in vitro* and *in vivo.* There is no doubt if well investigated and maximally exploited, they are likely to produce novel chemical entities which can lower high cost of treatment and reduce resistance to drugs. They will also reduce the use of insecticides thereby reducing environmental pollution.

1.2 Problem Statement

Leishmaniases is major public health issue worldwide and is predominant within the poorest people within the population. The annual global estimate of new cases is 700,000 to 1.3 million. The most affected countries are India, Brazil, and East Africa. Visceral leishmaniasis also known as kala-azar accounts to an estimated 50,000 to 90,000 cases yearly. The main signs and symptoms of VL included irregular bouts of

fever, anemia, enlargement of both spleen and liver and weight loss. Mortality rate can be as high as 95% in the reported case if left untreated. Neglected Tropical diseases in 2017 Global Burden of Disease study was responsible for 62 million disability adjusted life years (DALYs). An estimate of 774,000 DALYs were contributed by leishmaniasis alone with VL contributing to 90% of the DALYs in 2017 survey. Further, the household economic burden contributed by VL is significant. The cost of treatment varies across the affected countries. An average cost of treatment per patient is estimated at £760, £128, £197, £220 in Sudan, Nepal, India and Bangladesh respectively. With an estimated annual 700,000 cases, the economic loss is more than £89.6m yearly. Indirect costs such as lost working days, loss of wages for both patients and caregivers further magnify the problem.

1.3 Justification

Currently, very few antileishmanial treatment options exist. The available treatments as recommended by WHO include sodium stibogluconate mono-therapy for 30 days, combinations of stibogluconate and paramomycin for 17 days and recently miltefosine and stibogluconate. These treatments present several drawbacks and limitations that leads to prolonged suffering of the patients. The reported limitations include life-threatening toxicities such as cardio-toxicity, hepatotoxicity and pancreatitis, expensive, long treatment period or invasive administration which require prolonged hospitalization. Moreover, lack of human vaccine combined with vector control deficiencies has resulted to leishmaniasis management over reliance on prompt and effective diagnosis and treatment.

Studies have reported that *C. citrinus* and *A. secundiflora* extracts exhibit *in vitro* antileishmania activity. This is attributed to natural compounds such as alkaloids, terpenes, and phenolic derivatives in these plants. Methanol and water extracts of *A. secundiflora* showed low toxicity against healthy Vero cells. Water extracts of *C. citrinus* showed low toxicity as compared to Pentostam. Combination of *C. citrinus* and *A. secundiflors* have shown to reduce the footpad lesion sizes. The low cytotoxic potential of the extracts in this study is of great significance for their traditional usefulness in the treatment of leishmaniasis. *A. secundiflora* and *C. citrinus* herbal

products are environmentally friendly and should be encouraged due to their safety. In Kenya and other many cultural systems, monotherapy and combined herbal product use has existed for centuries.

Therefore, there is need for continued scientific research in antileishmania herbal products whose combined therapy can be of great breakthrough to treatment of *Leishmania*. An upstream of pre-clinical research that may result to alternatives which are efficacious, safe, ideally of short duration, affordable is thus important. The rationale of combining the plant extracts in this study is to determine whether the combinations could have synergistic or additive effects when used against *L. major in vivo.*

1.4 Research Questions

1. What is the toxicity of combined plant extracts of *C. citrinus* and *A. secundiflora?*
2. Is treatment with combined plant extracts of *C. citrinus* and *A. secundiflora* against *L. major* better than convectional drugs (Pentostam) in reducing lesion development?
3. Are combined plant extracts of *C. citrinus* and *A. secundiflora* have synergistic effect in the treatment of *L. major*?

1.5 Null Hypothesis

There is no difference in the antileishmanial activity of combined therapy of *C. citrinus*, *A. secundiflora* extracts and Pentostam (synthetic drug) against *L. major.*

1.6 Objectives

1.6.1 General Objective

To evaluate synergistic effects of combined *C. citrinus* and *A. secundiflora* plant extracts in *Leishmania major* infected BALB/c mice.

1.6.2 Specific Objectives

1. To determine toxicity levels of combined and single plant extracts of *C. citrinus* and *A. secundiflora*?
2. To compare lesion sizes in *L. major* infected BALB/c mice treated with a combined plant extracts of *C. citrinus* and *A. secundiflora* with Pentostam.
3. To determine synergistic effect in antileishmania activity of combined *C. citrinus* and *A. secundiflora* plant extracts in treatment of *L. major* infected BALB/c mice.

CHAPTER TWO

LITERATURE REVIEW

2.1 The leishmaniases

Leishmaniasis is a complex parasitic disease manifested in various forms. These forms are established by different strains which determine pathogenesis and immune response by the host (Bari, 2012). The disease caused by range from mild self-limiting cutaneous lesion to fatal visceral diseases affecting the spleen, liver and lymph nodes (Bari, 2012). Currently, leishmaniasis exist in four forms namely: visceral (VL), cutaneous (CL), diffuse cutaneous (DCL) and mucocutaneous (MCL) (WHO, 2014).

2.1.1 Life cycle of Leishmania parasite

The life of *Leishmania* parasite consists of two major stages: promastigotes and amastigote (Freitas-Junior *et al*., 2012). It is a digenetic life cycle found in an insect vector and a mammal host (Freitas-Junior *et al*., 2012). In the vector, which is female phlebotomine sand fly, the parasite undergoes extracellular development stage while in the host it undergoes intracellular development (Freitas-Junior *et al*., 2012). This occurs in the alimentary canal and macrophages respectively (Freitas-Junior *et al*., 2012). In the former, the parasites exist as promastigote which is a motile, flagellated and elongated form (Freitas-Junior *et al*., 2012). Maturation of the parasite into an infective metacyclic promastigote occurs in the mid gut of the vector (Freitas-Junior *et al*., 2012). As the sand fly feeds on the mammalian host, it inoculates the parasite leading to oviposition (Freitas-Junior *et al*., 2012).

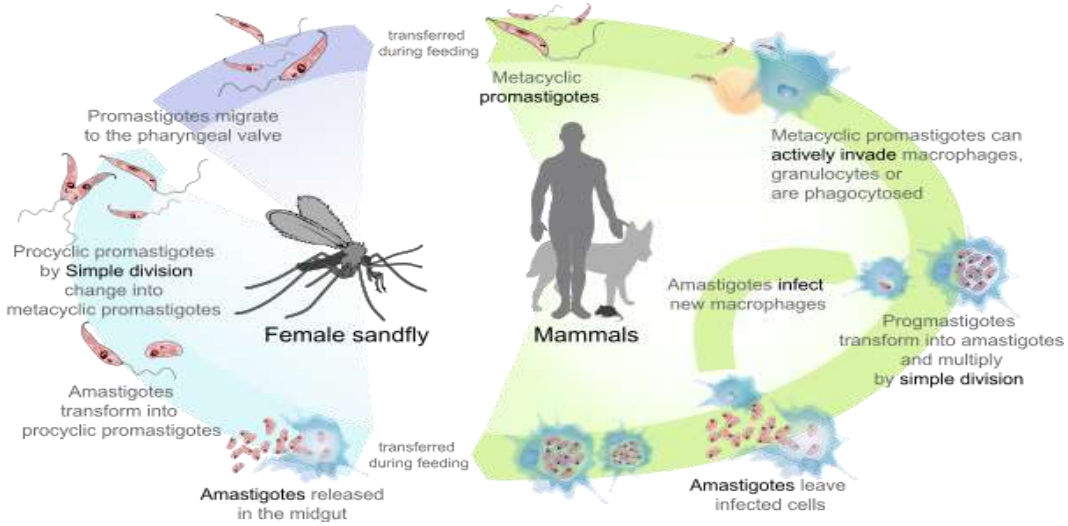

Figure 2.1: The life cycle of Leishmania (www.pathobiol.sdu.edu.cn, accessed, 2014)

A round 100 – 1000 metacyclic promastigotes are contained in each inoculation (Iborra *et al*., 2005). Once introduced in the body, they are quickly engulfed by leukocytes especially neutrophils, macrophages, and dendritic cells (Bates *et al*., 1992). In these cells, the promastigotes undergo transformation into amastigotes form (Roberts *et al*., 2006). The parasite undergoes both morphological and metabolic changes. The former results to an ovoid shaped parasite known as amastigote while the latter leads to a switch from aerobic to anaerobic metabolism (Roberts *et al*., 2006). These happen in the phagolysosome compartment where the conditions are acidic as the host fights to eliminate the parasites (Roberts *et al*., 2006). *Leishmania* parasite metabolism is remarkably complex and despite recent advances it remains poorly understood. In a bid to enhance research on the parasite dynamics, tissue biopsies and animal infections have been used as a source of amastigotes (Torres-Guerrero *et al*., 2017).

Amastigotes are usually transformed into promastigotes in the vector but now are axenicaly cultured (Bates *et al*., 1992). This effort has been necessary due to the potential implications of *Leishmania* parasite to human health due to its role in causing diseases (Bates *et al*., 1992). Promastigote culture has been a routine

procedure and parasites produced in this way have been intensively used in studies on the biochemistry and molecular biology of *Leishmania* (Grogl *et al*., 1992). For that reason, they have acted as alternative models for leishmanial studies hence alteration or loss of biological properties is a compromise made for experimental convenience and reproductivity (Grogl *et al*., 1992).

2.2 Form of leishmaniases

2.2.1 Cutaneous leishmaniasis

This is the most common type resulting from infected sand fly bites (WHO, 2022). It manifests by development of skin sores and also swelling of lymph nodes after a few weeks or months of the bites which can progress in size and appearance (Bravo & Sanchez, 2003). These lesions and sores appear on arms, face, legs and lower limbs (David & Craft, 2009). The sores develop a raised red border with a central depression over time and a scar is left upon healing (David & Craft, 2009). Cutaneous leishmaniasis is self-healing though it may lead to serious disability and permanent depression (David & Craft, 2009). The disease has been classified as old world (the Eastern Hemisphere versus new world (American) cutaneous leishmaniasis depending on the species responsible. For instance, *L. major, L. tropica and L. aethiopica* are etiologic agents in the old world cutaneous leishmaniases while *L. panamensis, L. Mexicana and L. braziliensis* are responsible for CL in the new world (David & Craft, 2009).

2.2.2 Mucocutaneous leishmaniasis (MCL)

This is a lesser form, and its pathogenesis is still unclear. It affects mucosa of different parts including nose, pharynx, mouth, and ear causing disfiguring destruction which leads to severe mutilation of the face (Sundar *et al*., 2007). Mucocutaneous leishmaniasis infection may worsen if a fungal or bacterial infection is present in the host (Sundar *et al*., 2007). It can result from a sequel (consequence) of some species that causes cutaneous leishmaniasis (Strazzulla *et al*., 2013). This happens when the parasite penetrates to the mucous membrane from the skin where

they cause sores. The most affected mucosa is that of the mouth and throat (Strazzulla *et al*., 2013). Severe deformity may occur if the lesions multiply rapidly and increases in size (Paredes *et al*., 2003). The parasites may also invade the respiratory tract mucosa leading to numerous respiratory problems. Malnutrition and pneumonia may result from MCL (Paredes *et al*., 2003). The main causative agents are *L. aethiopica* in the old world and *L. braziliensis* in the new world (Paredes *et al*., 2003). However, a few cases have been reported from *L. donovani*, *L. major* and *L. infantum* infection (Paredes *et al*., 2003).

2.2.3 Diffuse cutaneous leishmaniasis

This is a polar form characterized by disseminated nodules and abundance of the parasite during the period of disease infection (WHO, 2009). In addition, specific cell-mediated immune response against the parasite and poor antimonials treatment response is also absent (WHO, 2009). Diffuse cutaneous leishmaniasis usually starts as a primary lesion on the skin which spreads across the other areas of the skin including face, limbs and buttocks. The progress of the lesion formation is slowly and usually become chronic (Bari, 2012). Upon treatment, the patient improves gradually but relapse is always a must (Bari, 2012). However, there is no systemic involvement and macrophages loaded with the amastigotes are shown upon histology tests (Bari, 2012; Craig, 2011). The known causative agents in the new world are *L. amazonensis*, *L. Mexicana* and *L. pifanol* and *L. aethiopica* in the old world (Desjeux, 2004). However, DCL in Central and South America is predominantly caused by *L. amazonensis* (Desjeux, 2004).

2.2.4 Visceral leishmaniasis (VL)

It is also known as Kala-azar and is systemic disease caused by different species across the globe (Bari, 2012). For instance, *L. donovani* and *L. infantumi* are responsible for the disease in Europe, India, Africa and Middle East while *L. chagasi* causes the disease in Central and South America (Bari, 2012). These parasites invade the spleen and liver causing a condition known as splenomegaly (Craig, 2011). The disease is manifested by abdominal swelling as the main clinical feature. In addition,

irregular fever, weight loss, skin pigmentation, leukopenia and anaemia do characterize the VL (Craig, 2011). Lastly, parasite invasion of the spleen, small intestines, lungs, liver, larynx, bone marrow, stomach, skin, oral mucosa, sex cells and esophagus render the patients weak (Gilles, 1999; Craig, 2011).

2.3 Etiology of the Leishmaniases

The causative agent of Leishmaniases is *Leishmania* parasite (Arfan & Simeen, 2008). This is an obligatory intracellular parasite which exists in two distinctive forms namely amastigote and promastigote (Arfan & Simeen, 2008). The former exists in man and other vertebrate hosts while the latter occurs in culture and gut of phlebotomine (Diptera: Psychodidae) (Arfan & Simeen, 2008). In the old and new world, the female sand flies belong to the Phlebotomus and Lutzomyia genera respectively (WHO, 2014). They are mainly transmitted to humans through biting while feeding on blood and to a lesser extent via blood transfusion, sharing of contaminated needles or from pregnant mother to their fetus (Salam, 2004). The major causative agent of CL in Africa, Europe and Asia are *L. tropica*, *L. major*, *L. killicki*, *L. ethiopica* and *L. infantumi* (Reitinger *et al*., 2007; Aoun *et al.*, 2008).

2.4 Control of the leishmaniases

The control of different types of the leishmaniases depends on the type of cycle exhibited by the parasite and the behavior of the animal reservoir (Singh & Sundar, 2015). The readily available control measures target animal reservoir and transmission vector (Singh & Sundar, 2015).

2.4.1 Control in animal reservoir

This control targets the vector which harbors part of life cycle of the parasite (Davies *et al*., 2003). Basically, it involves testing and culling of seropositive animals such as dogs especially in areas where zoonotic VL is endemic (Davies *et al*., 2003). Topical application of repellents such as Deltamethrin in impregnated collars has been used to break zoonotic visceral leishmaniasis foci (Davies *et al*., 2003). In addition,

rodents are controlled using poison baits and environmental management thereby controlling CL (Davies *et al*., 2003).

2.4.2 Control of vector

There are more than 500 species of *phlebotomine* sand flies but only 30 or so known currently to transmit *Leishmania* parasite in the old world (Claborn, 2010). Majority of these species are susceptible to insecticides including those used to control *Anopheles* mosquitoes (Alexander & Maroli, 2003). Sand fly population drastically reduces during control of *Anopheles* mosquitoes hence interrupting leishmaniasis transmission. Therefore, indoor residual spraying is the commonly used intervention to control endophilic sand flies hence reducing CL cases (Alexander & Maroli, 2003). Despite efficiency of insecticides to control CL, the spraying programs are often unsustainable. Insecticide treated bed nets (ITNs) are effective in the control of endophagic sand fly bites in areas of high population especially where people sleep near their livestock (Davies *et al*., 2003). These bed nets are however reported to be uncomfortable during warmer climates. In addition, they require high cost to sustain the insecticide treatment and hence limited efficiency (Alexander & Maroli, 2003). In addition, most of the leishmaniasis causing species in Kenya are exophilic and exophagous and thus reduce the efficacy of ITNs (Davies *et al*., 2003). Another method of VL vector control is the application of biolarvicides in their dwelling places (Davies *et al*., 2003). However, the main limitation of this method is the diverse breeding habitat of sand fly hence reducing its efficiency in the control of VL (Davies *et al*., 2003). According to Davies *et al*., (2003), targeting animal reservoir is a feasible strategy particularly in areas where VL is primarily zoonotic. This is especially in regions such as Mediterranean basin, Central and Southwestern Asia and Latin America (Davies *et al*., 2003). Culling of domestic dogs is also used as a method of control of VL vector and has been practiced in Brazil (Davies *et al*., 2003). However, a study by Davies *et al*., (2003) reported its limitation including incomplete coverage, delays in sample collection and diagnosis and high turnover rate of dog population. Other novel strategies in control of leishmaniases include dipping of dogs, topical application of insecticides lotions and the use of

Deltamethrin treated collars (Davies *et al*., 2003). These methods can effectively reduce sand fly bites from animals such as dogs and subsequent human infection (Davies *et al*., 2003). However, they pose environmental pollution concern because these chemicals eventually end up in the soil, rivers and in the atmosphere (Davies *et al*., 2003).

Despite the availability of diverse VL vector control strategies, each has limited efficiency due to one or more shortcomings. It is thus necessary to continue research to discover better, effective and environmentally friendly methods. The use of crude extracts from *A. secundiflora* and *C. citrinus* either monotherapy or combination may provide a better solution to this problem.

2.5 Treatment of Leishmaniasis

2.5.1 Chemotherapy

The main leishmaniasis control strategy over time is chemotherapy (Haldar *et al*., 2011). It is used to decrease both the disease morbidity and mortality (Haldar *et al*., 2011). The drug category used as first line of treatment for both visceral and cutaneous is pentavalent antimonials (Haldar *et al*., 2011). They include Amphotericin B, sodium stibogluconate (Pentostam®) and meglumine antimoniate (Glucantinme®) (Haldar *et al*., 2011). The former is specifically used as rescue drug in patients with low or no improvement while under pentavalent antimony treatment (Corft *et al*., 2006). These drugs are administered slowly through parenteral intravenous. Several challenges are associated with this treatment and hence need for further research to realize better treatment (Singh & Sivakumar, 2003). It is estimated an average of about two hours is required daily to administer a full dose (Singh & Sivakumar, 2003). Therefore, patients will have to be monitored closely for any side effect after treatment (Singh & Sivakumar, 2003).

A study by Tasdemir, (2008) also reported other shorting comings of the available anti-protozoal drugs. He reported drug resistance, increased toxicity, and low efficacy thus unable to clear all parasite stages from the infected animal. This finding

further justifies the necessity for more research on leishmaniasis treatment. Nevertheless, other approved drugs with more efficacies do exist. They include pentamidine, paramomycin and miltefosine but similarly are toxic and require long treatment course of parenteral administration (Vacchina & Morales, 2014). The cost of treatment is too high to afford for most of the infected population, because those infected are mostly from remote areas (Corft *et al*., 2006). Unfortunately, there is no protective vaccine available at the moment hence research in the same is also need (Corft *et al*., 2006).

Systemic administration of pentavalent antimonials has remained the treatment of choice; however, in the recent years paramomycin has been used as topical formulations (Guerin *et al*., 2002). Though pentavalent antimonials have recorded resistance to parasites, they still remain the drugs of choice (Guerin *et al*., 2002). Research on VL and CL drugs for oral treatment is ongoing particularly of Sitamaquine in experimental models. Sitamaquine is an 8-aminoquinolone and no positive results reported for CL this further (Guerin *et al*., 2002). The available data explains how the drug accumulates in *leishmania* parasites, but its molecular targets are yet to be identified (Guerin *et al*., 2002). Promising results on its short elimination half-life and prevention of emergent rapid resistance have been published (Guerin *et al*., 2002). However, the antileishmanial effects of its metabolites are still not known (Guerin *et al*., 2002). Increased use of herbal medicine has been reported due to increased drug failure and resistance of the parasites to the available treatment (Guerin *et al*., 2002). Most of the studies have been carried out using animal models for parenteral administration than orals which could be cheap and easy in administration (Kinuthia *et al*., 2013). Ketoconazole which is an imidazole based antifungal drug has been found to be effective against *L. major* without any side effects (Berman, 1988). The other drug that has been tested with limited success against *L. major* is the antibiotic rifampicin, (Selim *et al*., 2007)**.**

2.5.2 Combination of standard drugs and extracts as a treatment for leishmaniases

Drug combination therapy has several advantages as compared to single treatment. For instance, increased activity has been reported due to additive or synergistic interaction of the active ingredients from each treatment (Chakravarty & Sundar, 2010). This has shown to reduce or eliminate drug resistance in several indications. Furthermore, drug combination may require lower doses of individual drugs hence minimizing the level of toxicity and costs (Chakravarty & Sundar, 2010). Combination therapy may have several disadvantages which may include antagonistic effects, increased risk of drug interactions, increased toxicity and increased costs (Tahany *et al*., 2010). Positive results have been reported using drug combinations to treat leishmaniasis (Sundar *et al*., 2011). Any successful combination treatment, when approved may provide a solution to minimize or prevent resistance while increasing efficacy and reducing treatment course (Sundar *et al*., 2011). For instance, clinical trials in India combining miltefosine with either Paramomycin or Amphotericin have reported increased efficacy (Freitas-Junior *et al*., 2012). This treatment may be a solution to treat VL infections resistance to antimonials in India and worldwide if approved (Freitas-Junior *et al*., 2012). Clinical trials in Sudan combining Paramomycin and Pentostam showed increased efficacy compared to standard treatment (Pentostam) alone (Melaku *et al*., 2007). A study by Barragan *et al*., (2010) reported increased response in HIV-infected patient case when treated with combination of antimonials/paramomycin drugs followed by itraconazole/miltefosine drugs. The patient had several relapses when treated with standard therapy (Pentostam) that stopped after treatment with the said combination (Barragan *et al*., 2010).

Combination of herbal or traditional medicine has been in use for the longest time in several cultural systems to treat various infectious disease. For instance, fever has been treated with combination of *Artemisia annua* extracts with other traditional herbs in China (Gathirwa *et al*., 2008). A study by Ghanzafari *et al*., (2000) reported decreased lesion size in *L. major* infected mice treated glucantime combined with

garlic extract compared to single treatment with either garlic or glucantime alone. A 1:1 (10 µg:10 µg) combination ratio of crude extracts from *Peganum harmala* and *Alkana tincturia* at low dose shown better *in vitro* efficacy against *L. major* compared to their single treatment (Kinuthia *et al*., 2013). Similarly, different combinations comprising of three different (*C. citrinus, M. stenopetala and A. sativum*) aqueous and methanolic extracts showed better in vitro efficacy against *L. major* parasites. A combination extract of 125µg/mL and at 2:2:1 ratio inhibited *L. major* amastigotes *in vitro* more when compared to two combined extracts at the same concentration and at 1:1 ratio (Kinuthia *et al*., 2013). Another study reported synergic effect from essential oils of *Chenopodium ambrosioides* L. (Mexican tea) when incubated in pentamidine treated *L. amazonensis* promastigotes (Monzote *et al*., 2007).

2.6 Challenges in treatment and control of leishmaniases

Many times, the leishmaniasis infection is misdiagnosed and even where diagnoses are made, treatment is expensive and associated with numerous side effects (Singh and Sundar, 2015). The method to administer the standard antimonials especially for localized leishmaniasis is not perfect and they are also continuously excreted from the body (Singh & Sundar, 2015). The mode of action of these antimonial is poorly understood. It is assumed that they may be working directly against the parasites in the amastigote or even through activation of macrophages or other immune systems (Singh & Sundar, 2015). However, available evidence suggests induction of high levels of certain cytokines which stimulate high population of macrophages (Ashford, 2000). In response, the *Leishmania* parasites try to exert a negative counter effect (Ashford, 2000). This therefore reduces treatment efficacy while increasing survival chances of the parasites. There is enough evidence that the infective metacyclic promastigotes injected by sand fly into host blood stream manipulates complement system (Ashford, 2000). As such, they are silently taken up by macrophages and no oxidative burst reactions due to cross linking (Beach *et al*., 1984). They produce downward regulation of expression of MHC class II molecules on the surface of macrophages while inside (Beach *et al*., 1984). This enables them

to hide from the immune system effect hence are free. The communication will make any attempts of standard therapies to booster immune system difficult hence their survival. Therefore, most chemotherapies have proven to be unsatisfactory, poor clinical agents and barriers to treatment of leishmaniasis (Beach *et al*., 1984).

Phlebotomus duboscqi which is most known vector for *L. major* in Kenya has many breeding sites (Beach *et al*., 1984). Some of the identified and reported breeding sites include animal burrows, termite mounds and holes in trees. However, the latter two harbor the vector to a lesser extent (Ngumbi *et al*., 1992). The accessibility to these sites during spraying is difficult which makes the control difficult. *Leishmania* parasite has many sources of food (blood) which means there is sufficient source of food all throughout the year. As a matter of fact, it feeds on carnivores, ruminants, rodents and human (Le Blancq & Peters, 1986). Mongoose, *Helogale spp* and chicken are also reported to provide extra feeding options (Mutinga *et al*., 1986). Two rodent species Nile grass rat (*Arvicanthis niloticus*) and gerbil (*Tatera robusta*) are known to host *L. major* (Githure *et al*., 1984). These rodents live in burrows which also are living areas for *P. duboscqi* and thus allow feeding of sand flies (Kasili *et al*., 2009). Many breeding sites and burrows for these rodents promote the life cycle of *Leishmania* parasite and hence difficult to eradicate the disease. All these challenges suggest that there is a need and urge to search for alternative natural plant products therapy for the treatment of leishmaniasis (Berman & Lee, 1984).

2.7 Investigated herbal plants against leishmaniasis

2.7.1 Overview

The earth is estimated to host an approximate of 2.5 million species of higher plants (Joy *et al*., 2001). More than 80,000 of the 2.5 million species are reported to be medicinal with 5,000 species identified to be of therapeutic value (Joy *et al*., 2001). Resistance and non-responsiveness to drugs used to treat the leishmaniases has led to the need to develop several new drugs (Ganguly *et al*., 2006). It has been suggested that emergence of disease resistance can be limited through use of simultaneous or sequential combination regimens (Chakravarty & Sundar, 2010). Methanol extracts

of *Aloe nyeriensis*, *Albizia coriaria* and *Acacia tortilis* have shown reasonable anti-leishmanicidal activity (Kigondu *et al*., 2009). Comparable *L. major* promastigotes anti-leishmanial activity of hexane extracts from *Warbugia ugandanensis* and leishmaniasis standard drugs in particular Pentostam (Sodium stibogluconate) and Amphotericin B have been reported (Ngure *et al*., 2009). *A. sativum* has been recognized as an antimicrobial agent (Singh & Singh, 2008). *A. sativum* has multi-medicinal properties activities including but not limited to antimicrobial, antitumor, antithrombotic and hypolipidemic (Augusti, 1996). On the other hand, *M. stenopetala* barks, roots and leaves have been used to treat diabetes, hypertension, malaria and asthma in Ethiopia. *M. stenopetala* extracts have also been reported expel retained placenta as well as reducing stomach problems (Mekonnen *et al*., 1999). Exponential-phase promastigotes from six leishmania strains were used to test leishmanial activity of ethanolic leaves extracts of *Artemisia Indica* Willd (Asterales: Asteraceae). The results were promising as the extracts were non-toxic with IC_{50} values ranging from 0.21 to 0.58 mg/mL (Ganguly *et al*., 2006).

2.7.2 Aloe secundiflora Engl. Order: Liliales, Family: Aloaceae

Aloe secundiflora is widespread especially across East Africa in countries like Tanzania, Sudan and Kenya (Ogeto *et al*., 2013). It does well in altitudes from 1,350 to 1,550 m above sea level and on well drained soils. It grows in Acacia bush land as well as in open grassland. It contains diverse chemical properties in gel and exudates. The former has high concentration of polysaccharides while the later consists of a mixture of phenolic compounds (Ogeto *et al*., 2013). These include phenyl pyrones, anthrones and chrones. Extracts from *A. secundiflora* leaves have shown to prohibit survival of different viruses, bacteria, fungi and helminths (Ogeto *et al*., 2013).

An *in vitro* study investigating efficacy of *A. secundiflora* methanolic extracts against most prevalent chicken nematode, *Ascaridia galli*, reported significant activity (Kaingu *et al*., 2013). The methanolic extracts of *A. secundiflora* have been shown to be active against all the mycobacterial strains used (Zero GUs against *Mycobacterium kansasii*, *Mycobacterium fortuitum* and *Mycobacterium smegmatis* and 157 GUs against *M. tuberculosis* at minimal concentration of 0.5 mg/mL and

diarrhea-causing bacteria salmonella (Richard *et al*., 2011). An antifungal study of crude sap from fives leaves of *A. secundiflora* against *Candida albicans* exhibited clear zones of inhibition. This indicated the potency of the chemical compounds in these leaves to inhibit growth of this fungus (Waihenya, 2002). In another study of Salmonella gallinarum infected chicken, decreased clinical signs and reduced disease (fowl typhoid) severity were reported in the interventional group than in control group (untreated group) (Waihenya *et al*., 2005). Crude aqueous extract of *A. secundiflora* and three HPLC separated fractions mainly comprising of phenolic compounds were tested against Newcastle Disease Virus (NDV). The test was done against NDV in chicken eggs with embryos free of any specific pathogen. The findings were reduced virus growth which provide the justification on the ethno-veterinary use of the *A. secundiflora* exudates in control of Newcastle Disease Virus among chicken (Waihenya *et al*., 2005). A study by Ogeto *et al*., (2013) investigating antileishmanial activity of both aqueous and methanolic leaf extracts from *A. secundiflora* reported growth inhibition of *L. major* parasites. This further provides evidence of medical activity of extracts from *A. secundiflora* and its potential to be used as a therapy for different types of leishmaniasis.

2.7.3 Callistemon citrinus William Curtis Order: Myrtales Family: Myrtaceae

Callistemon citrinus is a native shrub of Southeast Austria where it is locally named as Crimson bottlebrush (Viridans Biological Databases, 2009). It grows to a height of about 1 and 3 meters with leaves of length approximately 3 to 7 cm and width of 5 to 8 mm. The leaves have visible veins on both sides. The flowers are in form of spikes of an estimated length and diameter of 6 to 10 cm and 4 to 7 cm respectively. The flower male parts (stamen) are lilac, red or purple red with dark-colored anthers. It has woody, cup-shaped seed capsules clustered along stems of about 7 mm wide (Viridans Biological Databases, 2009).

Several studies done on *C. citrinus* have shown that the plant has therapeutic properties on several microbes (Kinuthia *et al*., 2013). The water and methanolic extracts have antileishmania effects against *L. major*, with water extracts showing better activity (Kinuthia *et al*., 2013). These extracts administered either orally or

intraperitoneally in BALB/c mice infected with *L. major*, have been shown to inhibit lesion development (Kinuthia *et al*., 2013). The same study reported marked *in vitro* synergistic and additive interactions at various fixed ratios of blends of aqueous crude extracts against *L. major* promastigotes. These blends studied are *C. citrinus* combined with *A. sativum* or with *M. stenopetala* separately. Another study by Abdelhady & Aly, (2012) reported *Callistemon* genus to possess anti-bronchitis, anti-cough and insecticidal agents and as a treatment of gastro-enteritis, diarrhea and skin infections. Studies on *Callistemon citrinus* and *C. salignus* flower extracts revealed significant growth inhibition within 1-hour exposure against *Aeromonas hydrophilia, Bacillus subtilis, Pseudomonas fluorescens* and *Bacillus cereus* cultures (Ian, 2012). However, when tested against *Artemia francis cananauplii* bioassay there was low toxicity observed (Ian, 2012).

In summary, complex life cycle, ability of the *L. major* parasite to evade immune system and multiple treatment drawbacks present a need for continuous research to come with new treatment options. From the literature analysis, current treatment options have shown to have toxicity to internal organs, long treatment period, long hospitalization, environmental pollution concerns, increased drug resistance, expensive and storage challenges especially in remote areas. Further, the availability of data on the test extracts to inhibit growth and multiplication of *L. major* both *in vivo* and *in vitro* supports the need to conduct the current study.

CHAPTER THREE

MATERIAL 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 analyzed using machines while others were manually calculated, and the final data was analyzed using the SPSS software programme version 20. The study conceptual framework is presented in figure 3.1 below.

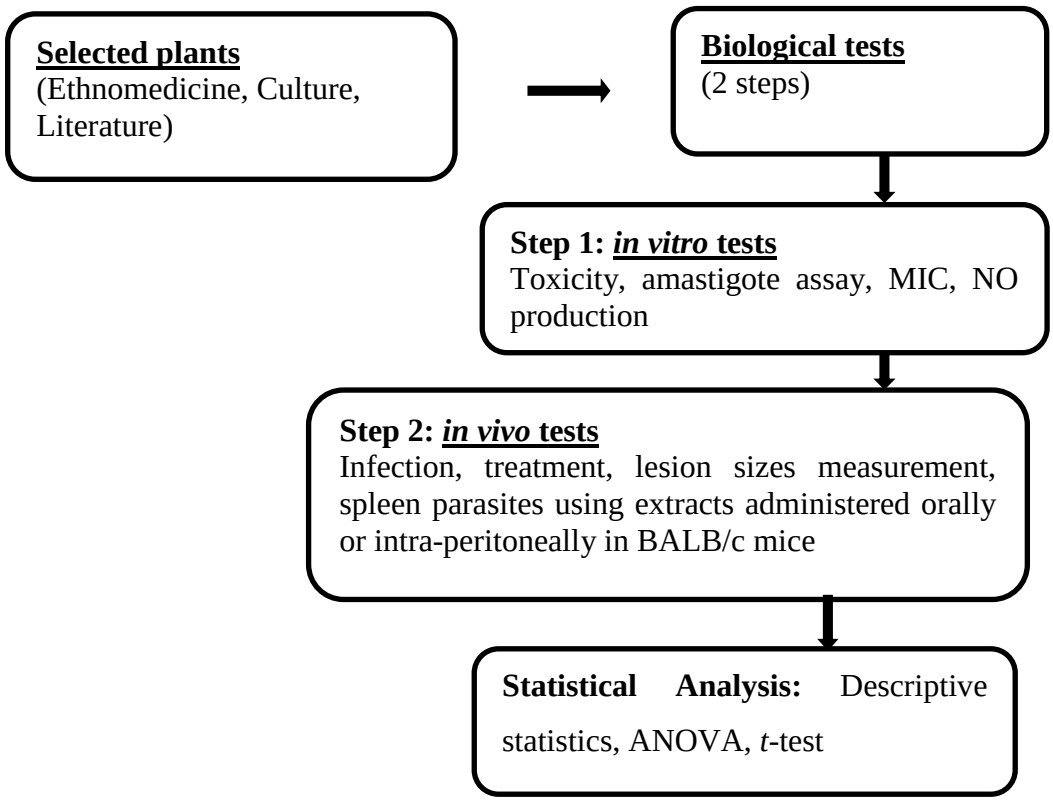

Figure 3.1: Conceptual framework flow

3.2 Study site

Flowers of *C. citrinus* were harvested from previously identified area in Upper Hill, Nairobi (1.2984° S, 36.8132° E). On the other hand, *A. secundiflora* leaves were harvested from a selected home in Ruai, Nairobi (1.2559° S, 36.9860° E). The locations were preferred because the landowners had been previously approached and agreed for free harvesting of leaves. The plants samples were then presented to the herbarium of National Museums of Kenya for scientific identification by experts from Botany Department. After positive identification, a voucher number 001499 was issued and subsequently deposited at the same institution. The identified plants samples were then taken to KEMRI Leishmaniasis section, Nairobi for processing. The age of the study plants was not determined due to limited resources. They were dried until brittle with no further loss of weight at room temperature was observed. The final dried plants samples were packaged separately and labeled appropriately.

Plate 3.1: C. citrinus flowers (www.noodlesnacks.com, accessed, 2014)

Plate 3.2: A. secundiflora photograph in a selected homestead in Ruai, Kiambu County (Researcher, 2014)

3.3 Sample size determination

The sample size was calculated using Mead's Resource Equation, E = N-B-T.

Where:

- *N* = total number of mice in study - 1.
- *B* = blocking component -1
- *T* = (Number of experimental groups + controls) – 1
- *E* = degrees of freedom of the error component.

Assuming 2 blocking factors of weight and environment (B = 2-1=1), with 5 groups of experiment, the degree of freedom of the error component is 4 and T is (5 - 1 = 4). The study consisted of 5 groups: A, B, C, D and E. Groups A and B consisted of 7 mice each. However, groups C, D and E had 3 each because results from previous studies conducted by Kinuthia *et al*., (2013) and Ogeto *et al*., (2013) reported no significant difference or effect in increasing the number in controls. This was also

considered as per animal care and use committee recommendations of 3Rs (reduce pain, reduce number and replace). Therefore, the sample size of the current study was 23 mice.

3.4 Experimental Mice

Inbred BALB/c mice were obtained from KEMRI, Kenya. They were handled according to standard operating procedures developed by Animal Care and Use Committee (ACUC-KEMRI) for handling experimental animals. Briefly, they were fed with pellet standard commercial diet, provided with tap water *ad libitum* in animal house maintained at 25^0C (room temperature). Temperature monitoring was done using min/max thermometer. Male mice aged four (4) weeks were used for both *in vitro* and *in vivo* assays. Male mice were preferred because of the risk of pregnancy among the female. Age of weeks was used because mice usually attain sexual maturity at 4-7 weeks of age.

3.5 Plant extracts preparation

Dried leaves of *A. secundiflora* and flowers of *C. citrinus* were moved to CTMDR for extraction. Each plant was ground separately into powder using Gibbons electric mill (Christy and Norris Limited, England). Water extraction followed as previous described by Delahaye *et al*., (2009). Water extraction was chosen because of the safety, resource limitation and based of previous study by Kinuthia *et al*., (2013). Briefly, 200g powder of each plant material was weighed and 1000 mL of distilled water added. The mixture was then gently warmed for 1 hour in water bath at 80 ^{0}C. Filtration of the solution then followed using filter papers of Whatman NO. 1. The filtrate was coated in a conical flask in dry ice enhanced with acetone and then freeze-dried using Edwards freeze dryer then weighed. The coating was done since dry ice sublimes at -78 ^{0}C and a mixture of acetone/dry ice maintains -78 ^{0}C. The end products were coded as A and B for *A. secundiflora* and C. *citrinus* respectively and stored at -4 ^{0}C until when needed for *in vitro* and *in vivo* assays.

3.6 Preparation of the stock solutions test extracts

Preparation of the stock solution was done aseptically in laminar flow hood (Biological Safety Cabinet). For single solution extracts, ten (10) mg of each plant powder was dissolved in 10 mL of sterile PBS to make an initial concentration of 1mg/mL crude extract. Single extract crude solutions were used for *in vitro tests* such as MIC and cell viability. Combined crude extracts on the other hand was prepared by mixing 2mg and 5mg of *A. secundiflora* and *C. citrinus* respectively and dissolving them in 1 mL. The selection of the doses was based on the previous study by Kinuthia *et al*., (2013). Each crude extract was aseptically filtered using 0.22 μM filter flasks and stored - 4^0C for *in vitro* and *in vivo* anti-leishmanial bioassays.

3.7 Cytotoxicity assay using Vero cells (in vitro)

Vero cells were used to perform *in vitro* cytotoxicity assay for both single extracts and combination therapy as previously described by Wabwoba *et al*., (2010). Briefly, minimum essential medium (MEM) supplemented with 10% FBS was used to grow the cells in 25 mL culture flasks. Added also in the MEM was 100 μg/mL of streptomycin and 100 IU/mL) of penicillin to prevent growth of microorganisms. The mixture was incubated for 24 hours at 37 ^{0}C in a humidified 5% CO_2 atmosphere. After the incubation period, the cells were harvested by trypsinization and pooled in 50 ml centrifuge tubes. A volume of 100 μL with a concentration of 1 × 10^6 cells per ml of the culture medium was put into 2 wells of row a A-H (Figure 3.5) in a 96-well flat-bottomed microtiter plate per well. The plate was incubated in 5% CO_2 at 37 ^{0}C for the cells to attach and MEM aspirated off. The tests extracts (A, B and C) at a volume of 150 μL at highest concentration of 1000 μg/mL) were added in wells of row H and diluted 3-fold upwards to a concentration of 1.37 μg/mL in wells of row B. No extracts were put in wells of row A and therefore were used as positive control. In every third row after the row with cells and extracts was left blank; only medium was put and hence was used as negative control. The prepared microtiter plate was then incubated for 48 hours in a humidified 5% CO_2 atmosphere at 37^0C. The set up was removed from incubator and 10 μL of MTT reagent added into each plate well. Further incubation between 2 to 4 hours followed until

Formazan (purple precipitate) was visible under microscope. The MTT reagent and media were gently aspirated off and 100 µL of DMSO added. Vigorous shaking of the mixture was done for 5 minutes to dissolve formazan. Micro-titer plate reader was used to measure optical density (absorbance) of each well at 690 nm wavelength. The formula of Mosmann, (1983) was used to calculate cell viability at each concentration while Chemosen software automatically calculated IC_{50} values of the test extracts. The percentage of cell viability was manually calculated using the following formula:

Cell viability (%) = [(Average absorbance in duplicate drug wells - average blank wells)/ Average absorbance in control wells] *100

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 & Wright, 1979), and 250 µg/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 Minimum inhibitory concentration (MIC) evaluation

A procedure previously used by Kinuthia *et al*., (2013) was used in this study to determine MICs. Briefly, Shneider's Insect Media (SIM) was used to grow $1*10^6$ cell/ml of the *L. major* promastigotes in 24 well micro titer plate with the test extracts (A, B, and C). The concentration of the test extracts ranged from 0.5 to 5mg/ml as previously done by Kinuthia *et al*., (2013). For combination therapy, five (5) different fixed ratios (1:1, 1:2, 3:1, 3:1, 2:1), arbitrary determined, were blended to prepare the combination therapy. Promastigotes were then exposed to each of

these combinations. The survival of the promastigotes was determined in all the test extracts. In single extracts test, the lowest concentration that support the least survival and growth of promastigotes was recorded as MIC. For combination therapy, the MIC was taken as the ratio where minimum growth of the promastigotes was observed.

3.10 Anti-Amastigote assay of test extracts

Macrophages cells harvested from 3 BALB/c were used to perform anti-amastigote test. The mice were induced for 24 hours to produce macrophages cells by intraperitoneal injection of 2% starch. A previous procedure described by Delorenzi *et al*., (2001) was used to carry out the anti-amastigote assay. Briefly, the mice were anaesthetized using 100 µL pentabarbitone sodium (Sagatal®). The mice body surface was disinfected with 70% ethanol in order to obtain peritoneal macrophages. The peritoneum was then exposed by shearing the mice abdominal skin dorsoventrally. A volume of 10 mL of sterile RPMI media injected into the peritoneum using a sterile syringe and needle. The mice were shaken and the injected RPMI withdrawn to harvest peritoneal macrophages and the contents put into a sterile 50ml centrifuge tube. The contents were centrifuged at 2000 rpm (Hitachi, 05PR-22) for 10 minutes to wash the cells. A complete RPMI 1640 medium was the used to re-suspend the pellets. A 24-well plate were used to adsorb the macrophage cells and incubated for 4 hours at 37 ^{0}C in a 5% CO_2 to adhere. Cold PBS was used to wash off non-adherent cells after which the macrophages were further incubated overnight in RPMI. Infection of adherent macrophages with parasite at a ratio of 6:1 was done and incubated for 4 hours at 37^{0}C in the 5% CO_2. Extensive washing with PBS to remove unattached promastigote followed and the cultures incubated for 24 hours in RPMI. The infected macrophages were then treated once with the test extracts. Pentostam treated infected macrophages were used as positive control to compare inhibition of the parasites. Daily replenishing of medium and drug was done for 3 days, and the monolayers washed with PBS 37^{0}C after the 5th day. It was then fixed in methanol, stained with 10% Giemsa and amastigotes counted. Counting was done in at least 100 macrophages in duplicate cultures. The number of infected

macrophages in 100 macrophages (IR = Infection rate) and multiplication index (MI) were used to present the results (Delorenzi *et al*., 2001). The percentage MI was calculated using the formulae:

MI% = [(Number of amastigotes in experimental culture/100 macrophages) / (Number of amastigotes in 100 control culture/100 macrophages)] * 100

3.11 In vitro bioassay to measure production of nitric oxide production

Greiss reaction for nitrites was used to measure nitric oxide (NO) released by macrophage culture. This test was carried out to explore the possibility of the combined extracts stimulating macrophages to produce NO as a mode of action against *L. major* and of their synergistice effect. Test drugs/ extracts were introduced to microphage culture and 100 µL of supernatants harvested after 48 hours of incubation. Triplicate assay of 96-well micro-litre plates were prepared from the supernatants. Sixty (60) mL of Greiss reagent A (1% Sulphonilmide in 1.2 M HCL) and Greiss reagent B (0.35 N[1-naphthy] ethylenediamine) were added respectively. An Enzyme Linked Immunosorbent Assay (ELISA) reader was used to were read the plates at 540nm. A standard curve was constructed using sodium nitrites in RPMI and the amount produced in test plates extrapolated.

3.12 Infection of BALB/c mice with L. major parasites and monitoring of disease development

The study BALB/c mice were infected as previously described by Wabwoba *et al*., (2010). Briefly, 40 µL of PBS containing at $1* 10^6$ *L. major* stationary phase culture was intradermally inoculated at the left hind pad of each BALB/c mice at room temperature. The right foot was not infected for comparison purposes (contra lateral

control). Four (4) cages were used to randomize the infected mice each comprising of 6 mice and left for 4 weeks for lesions to develop. Monitoring of the lesions were done weekly using Vernier caliper to measure the thickness of infected footpad (Nolan & Farrell, 1987).

3.13 Treatment of L. major leishmaniasis diseased BALB/c mice with study extracts

Treatment with single, combination therapy and controls commenced 4 weeks after infection when the lesions had developed. The mice were randomized into 5 cages according to number of treatment groups (Table 3.1). The necessary dosage for combination therapy was calculated using the MICs established and a ratio of 1:1 of the extracts used to treat mice. Briefly, 2 mg of *A. secundiflora* and 5mg of *C. citrinus* was weighed and mixed then dissolved in 1 mL of PBS. Treatment was done to 7 mice of group A by injecting IP 100 µL of the combination of test extracts per mouse per day using 1ml 30-gauge insulin needle. Group B of 7 mice were orally treated with the same dose daily using a cannula for 5 weeks while group C of 3 mice was treated with the standard dose of 20mg/kg daily of Pentostam for 30 days intraperitoneally. Group D of 3 mice were treated with 100 µL PBS while group E of 3 mice treated with 100µl PBS using a cannula for the same period. The survival rate was 100% as no mice was reported to have died during the treatment period.

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

Group	Treatment	Route	No. of mice	Mice marking
A	A. secundiflora/C. citrinus	IP	7	Neck marked
B	A. secundiflora/C. citrinus	Oral	7	Neck and tail marked
C	Pentostam	IP	3	Neck, tail, and rump marked
D	PBS	IP	3	Back marked
E	PBS	Oral	3	Ear marked

3.14 Lesion size measurements

After infection, the footpad was monitored weekly to establish development and progression of the lesion size. Once a swelling was observable in the infected left footpad, vernier caliper was used to measure the thickness by comparing with the non-infected right footpad. Direct reading was recorded for all mice in each group and weekly means calculated for comparison. The lesion size was calculated as the difference between the size (mm) of infected footpad less the size (mm) of the uninfected footpad (Nolan & Farrell, 1987). Left foot pad paracetamia not done due to resource limitation.

3.15 Estimation of splenic impression smears parasite load

After 5 weeks of treatment, mice were killed by inoculating 60mg/kg body weight of penta barbitone sodium. Clean microscopic slides were used to prepare spleen impression smear. A freshly acquired spleen was cut vertically using a scalpel, gently tapped on the slide, and left to dry for 15 minutes. Slides were then dipped in an absolute methanol to fix the smears. Parasite loads were estimated using Bradley & Kirkley, (1984) method by immersing the fixed slides in 5% freshly prepared solution of Giemsa stain for 20 minutes. They were then flushed with tap water and dried at room temperature (25^0C). Compound microscope set at a high-power magnification was used to examine and the number of amastigotes per 1000 host nuclei was enumerated. The number of parasites in the spleen was calculated as Relative and total Leishman-Donova Units using the formula developed by Bradley & Kirkley, (1984). Comparison between relative and total LDU values for all the groups was done.

Bradley and Kirkley formulae for estimating splenic parasite load:

i. Relative LDU = No. of parasite/1000 host nuclei
ii. Total LDU = LDU*Organ Weight*2*10^5

3.16 Statistical analysis

Statistical Package for Social Sciences (SPSS) software was used for all data analysis. Comparison of data from all groups was done utilizing student's *t*-tests and one-way analysis of variance (ANOVA) tools at $p \leq 0.05$ significance. ANOVA, a *post hoc* analysis where applicable was done with Tukey and Games-howell test. Statistic and descriptive statistics were used appropriately. Figures, tables, line and bar graphs were used for visual presentation of the data.

3.17 Ethical considerations

The study was approved by KEMRI's Scientific Steering Committee (SSC), ACUC, and Ethical Review Committee (ERC) through the Centre of Biotechnology Research and Development (CBRD). The processing (sacrifice and dispose) of the mice were in accordance with SOPs set by ACUC-KEMRI.

CHAPTER FOUR

RESULTS

4.1 Plant materials yields

Collection of the study plants were done between August and September 2014. They were identified and air dried under shade until a constant weight was attained. A laboratory mill was used to grind them into a powder.

Table 4.1: The percentage yields of the plant extracts obtained from the study plants

Plant species	Part used	Type of extraction	Code	Initial weight (g)	Yield (g)	Yield (%)
Aloe secundiflora	Leaves	Aqueous	A	200	18.44	18.44
Callistemon citrinus	Flowers	Aqueous	B	200	17.24	17.24

4.2 In vitro bioassays

These assays were meant to determine the effects of the plant extracts on Vero cells (cytotoxicity) and Minimum inhibition concentration (MIC) of the extracts on *L. major* promastigotes. Thirdly, they were used to estimate the infection rates (IR) and multiplication indices (MI) of the *L. major* amastigotes in the host's macrophages which were exposed to the study plant extracts. Finally, to determine production of nitric oxide (NO) by macrophages infected with *L. major* parasites and treated with the study plant extracts.

4.2.1 Minimum inhibitory Concentration (MIC) Evaluation

The survival of *L. major* promastigotes in varying concentrations was estimated with the use of a + for presence of life parasites or – for no life at all.

Table 4.2: L. major promastigotes survival in different single concentrations and changing ratios of the plant extracts combinations (MICs)

Test plant extract	Code	Concentrations of the extracts (μg/mL)					
		5000	4000	3000	2000	1000	500
A. secundiflora	A	-	-	-	+	++	+++
C. citrinus	*B*	+	++	+++	++++	++++	++++
	Code	Ratio of the two combinations based on MIC's					
		1:1	1:2	2:3	3:1	2:1	1:1
Combination drug (A/B)	C	+	-	-	-	-	+
Controls		Concentrations of the Pentostam (μg/ml)					
		100	50	25	12.5	6.25	3.125
Pentostam (+ve control)		-	-	-	+	++	+++
SIM (-ve control)		++++	++++	++++	++++	++++	++++

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

Table 4.2 shows MICs of *A. secundiflora* was 2000 µg/mL (2mg/mL) and *C. citrinus* was 5000 µg/mL (5 mg/mL). For combination therapy, MIC was observed at the ration or 1:1 and no life observed for combination of more than one part of either extract. The MIC for positive control (Pentostam) was 12.5 µg/mL while negative control (SIM) supported life of *L. major* promastigotes in all levels.

4.2.2 Cytotoxicity test using Vero cells and Cell viability (%)

When aqueous extracts were screened for cytotoxicity in health Vero cells (P27), water extracts of *A. secundiflora*, and *C. citrinus* showed low toxicity (IC_{50}). The toxicity level observed was 467.09µg/ml and 457.88µg/ml for *A. secundiflora*, and *C. citrinus* respectively. High toxicity was reported in the combination therapy (58.45 µg/ml) compared to monotherapy. However, the difference was not significant when compared using one sample test ($t = 2.481$, $p = 0.089$). The low cytotoxic potential of the extracts in this study is of great significance for their traditional usefulness in the treatment of leishmaniasis. The cell viability (%) increased as the test extracts concentration reduced showing that they affected cell growth in an inverse manner (Tables 4.3 and 4.4).

Table 4.3: The IC_{50} (µg/mL) of the test extracts and for Pentostam

Drug	Code	IC_{50} (µg/mL)	$-\log_{10}IC_{50}$
A. secundiflora	A	467.09	-2.67
C. citrinus	B	457.88	-2.66
A. sec/C. cit (1:1)	C	58.45	-1.77
Pentostam	Pento	0.26	0.59

Note: Serial dilution of factor 3 was done with an initial concentration of 1000 µg/mL (test extracts) and 100 µg/mL for Pentostam.

The higher the cytotoxicity, the higher was the negative log base 10 IC_{50} values ($-\log_{10}IC_{50}$), for instance, combination of A and B was highly toxic to Vero cells with a $-\log_{10}IC_{50}$ value of -1.77 while aqueous *A. secundiflora* was lowly toxic with a $-\log_{10}IC_{50}$ value of -2.77.

Table 4.4: Vero cells viability (%) at various concentrations (µg/mL) of the experimental drugs

Test extracts/ Control drugs	**Vero cells viability (%) at specific concentrations (µg/mL)**							
	1000.00	**333.33**	**111.11**	**37.04**	**12.35**	**4.12**	**1.37**	**0.46**
A. secundiflora	10	68	112	110	111	113	106	100
C. citrinus	-70	79	57	93	118	108	112	100
A. sec/C. cit	76	40	10	79	85	93	100	100
Positive control	**Vero cells viability (%) at specific concentrations (*µ*g/ml)**							
	100	**50**	**25**	**12.5**	**6.25**	**3.125**	**1.563**	
Pentostam	74.83	83.74	103.17	103.79	104.65	105.65	110.92	
Negative control* (MEM)	Average viability (%) of Vero cells ±S.D = 92.86± 0.16							

*The % viability in the negative control was the average for viabilities of Vero cells for MEM in microtitre plates A and B.

4.2.3 Macrophage assay (testing Anti-amastigote activity)

When microphages were incubated in RPMI and infected with *L. major* promastigote, the parasites infected the microphages. This was evidenced as engulfed amastigotes could be seen under microscopy in the cells (macrophages).

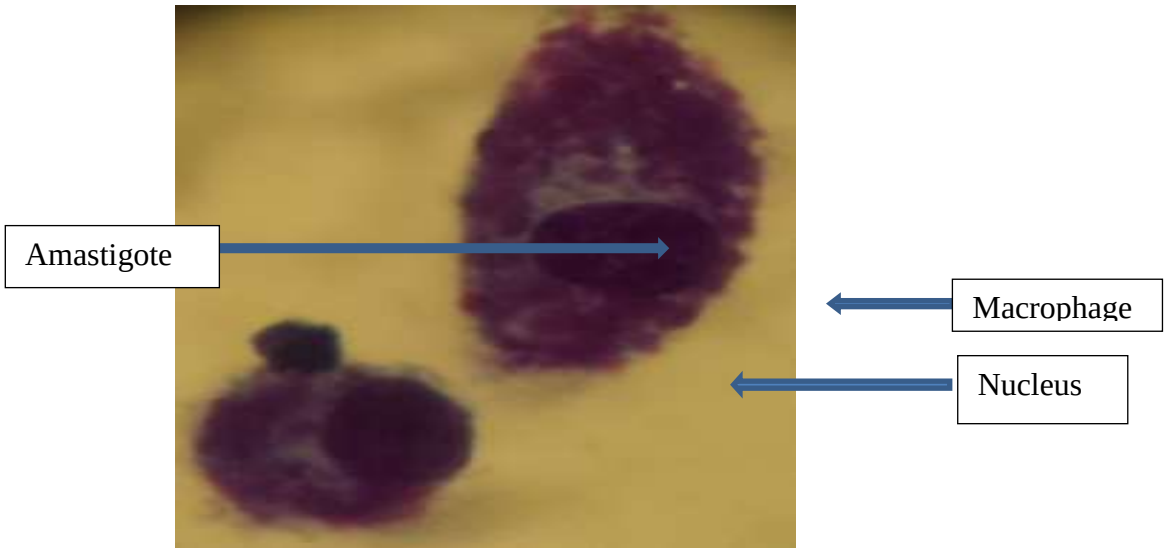

Plate 4.1: L. major amastigotes engulfed in peritoneal BALB/c mice harvested macrophages after 5 days of infection in RPMI-1640 medium culture

RPMI 1640 reported the highest Infection rate (IR) of peritoneal macrophages by *L. major* amastigotes at 71.5± 2.12% due to lack of active ingredient or drug. *C. citrinus* reported the second highest IR (46%) while *A. secundiflora* reported IR of 23%. The combination therapy (1:1 ratio) at 125µg/mL concentration recorded IR of 19%. As expected, Pentostam inhibited infection of BALB/c mice peritoneal macrophages greatly (IR = 21%) at lower concentration of 50μg/ml. This shows that *A. secundiflora* combined with *C. citrinus* has improved antileishmanial activity compared to single extracts. This means there could be presence of synergistic or additive potential in the combined therapy (Table 4.5).

Table 4.5: L. major amastigotes in vitro IR and MI per 100 BALB/c peritoneal macrophages treated with single and combination test extracts at different concentrations

Test extracts and controls	Conc. (*μ*g/mL)	IR (%)	Amastigotes per 100 cells	MI (%)
A	125.00	23	49	20.37
	62.5	30	187	77.75
	31.25	57	360	149.69
B	125.00	46	189	78.59
	62.5	50	196	81.49
	31.25	51	243	101.04
C (A/B)	1:1	19	127	52.81
RPMI (Control)				
	1st	70	230	n/a
	2nd	73	251	n/a
	Average	71.5± 2.12(SE)	240.5±10.5	100
Pento (Control)	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. A = *A. secundiflora*,
B = *C. citrinus*; C = Combination of A and B, Pento = Pentostam (+ve control).

Table 4.5 shows infection rates (%) of *A. secundiflora* at concentration of 125μg/ml and combination of A/B at ratio of 1:1 was 23% and 19% respectively. This compared very closely to that of Pentostam (21%) at a low concentration of 50μg/ml. Using One-way ANOVA to compare these infection rates, no statistically significant difference ($F_{(1, 3)} = 2.242$ and $p = 0.446$) observed. At concentration of 31.25 μg/ml for A, B and C, the IR% recorded was 57%, 51% and 19% respectively. A comparison was done using One-way ANOVA and no statistically significant difference ($F_{(1, 3)} = 21.348$, $p = 0.157$) observed. The +ve (concentration of 12.5μg/ml) and -ve controls recorded IR% of 37% and 71.5% respectively.

The MI% for *A. secundiflora*, *C. citrinus* and combination therapy (1:1) were 49%, 189% and 127% respectively. Using One-way ANOVA to compare the MI%, there was statistically significant difference ($F_{(1, 3)} = 5.308$, $p = 0.05$). A post hoc analysis

using Tukey comparing aqueous extracts MI% against pento, there statistically significant difference ($F_{(1, 3)} = 5.487$, $p = 0.015$) observed. A statistical difference ($p = 0.032$) was also observed in the group treated with *C. citrinus* compared to that treated with the combination. The shows that precisely blended combination therapy can be used a potential treatment for *L. major* infection. The observed improved efficacy in the combination therapy of the test extracts shows possibility of additive or synergistic effects. There was no statistical difference ($F_{(1, 3)} = 6.821$, $p = 0.602$ and $F_{(1, 3)} = 6.348$, $p = 0.225$) between combination vs pento and A vs pento respectively.

4.2.4 *In vitro* nitric oxide assay

A study by Gamboa-leon *et al*., (2007) reported that Nitric oxide (NO) as a mechanism used to kill Leishmania parasites within host macrophages. In this regard, it was important to investigate whether the test extracts stimulated the production of NO by macrophages. Notably, NO is broken down to release nitrite (NO_2^-) as one of the products and therefore quantifying the NO_2^- content would estimate the amount of NO produced.

Table 4.6 shows optical densities (OD) readings for the three study aqueous extracts (A, B, C) of less than 0.1 compared to the positive control. The test extracts were dilu ted 3-fold starting with high concentration of 100 µg/ml. At highest concentration of 100 µg/ml, OD recorded were 0.066, 0.045 and 0.092 for A, B and C respectively. The RPMI-1640 medium (-ve control) produced a high OD of 0.079 that was close to those of the test extracts. This implies that the antileishmanial activity expressed by the test extracts is as a result of other mechanisms rather that stimulation of production of nitric oxide. The test extracts could be acting on the cell membrane, on enzymes or DNA of the parasites. This therefore requires more research to determine the mode of action by the active ingredients in the study extracts.

Table 4.6: Estimation of Nitric Oxide production by Macrophages treated with varying concentrations of the experimental tests

Conc. (μM)	Standard Nitrite (+ve control)	RPMI (-ve control)	*A. secundiflora* (A)	*C. citrinus* (B)	Combination (C)
0.0	0.054± 0.011	0.021	0.051	0.032	0.038
20	0.153± 0.013	0.030	0.053	0.035	0.039
40	0.178±0.001	0.038	0.061	0.036	0.044
60	0.331± 0.000	0.048	0.051	0.040	0.051
80	0.599± 0.004	0.055	0.059	0.042	0.087
100	0.983± 0.010	0.079	0.066	0.045	0.092

Figure 4.6 shows absorbance against nitrite concentration used to estimate the amount of NO produced by mice peritoneal macrophages at 3-fold dilution of initial concentration of 100 μg/mL. The standard nitrite produced high levels of NO with increasing concentrations. The RPMI and test extracts produced similar levels of NO which are negligible compared to the standard nitrite levels. Therefore, the estimates levels of NO could not be determined since the OB produced was less than 0.1 and thus the antileishmanial activity in these extracts is not related to NO production.

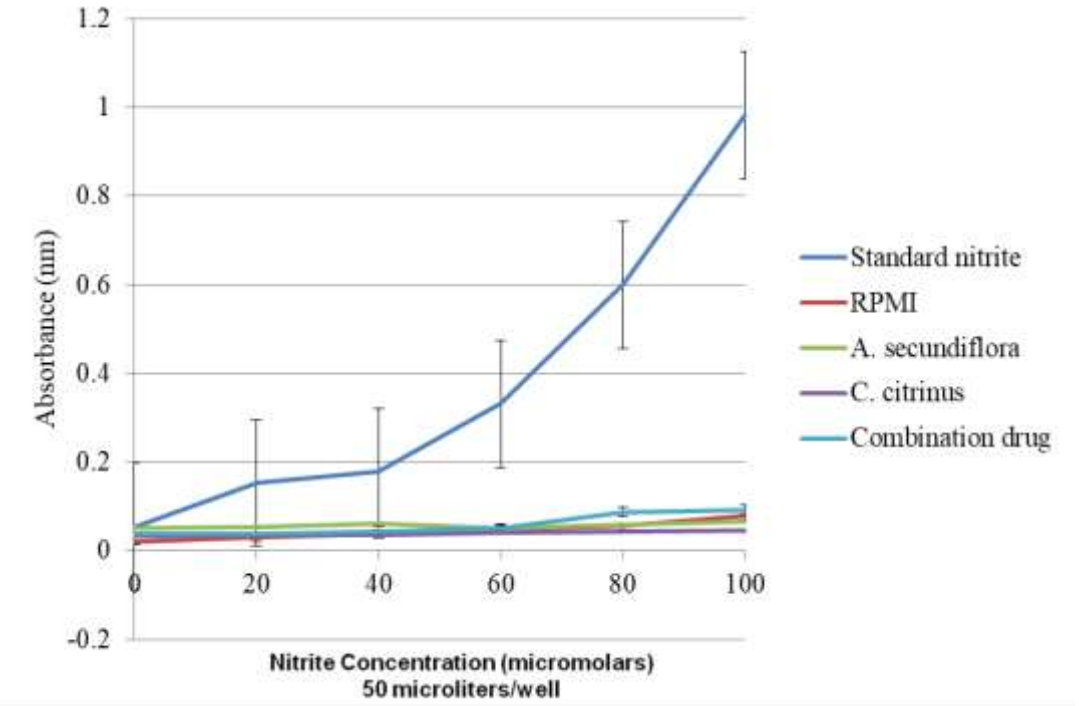

Figure 4.1: Amount of Nitric Oxide (µM) produced by peritoneal macrophages of BALB/c mice treated with study extracts/drugs

4.3 In vivo bioassays

This was done for combination therapy and the controls since for the single extracts was done in the previous studies; *A. secundiflora* by Ogeto *et al*., 2013 and C. citrinus by Kinuthia *et al*., 2013.

4.3.1 L. major infection of BALB/c mice and treatment with study extracts or drugs

The figure 4.3 shows one the infected mice with lesion after subcutaneous infection with infective *L. major* amastigotes. The left hind leg is swollen compared to the non-infected contra-lateral right footpad. This shows that the parasites were viable and produced the study disease as expected during this study. The lesions developed after 4 weeks of initial infection on the infected hind leg.

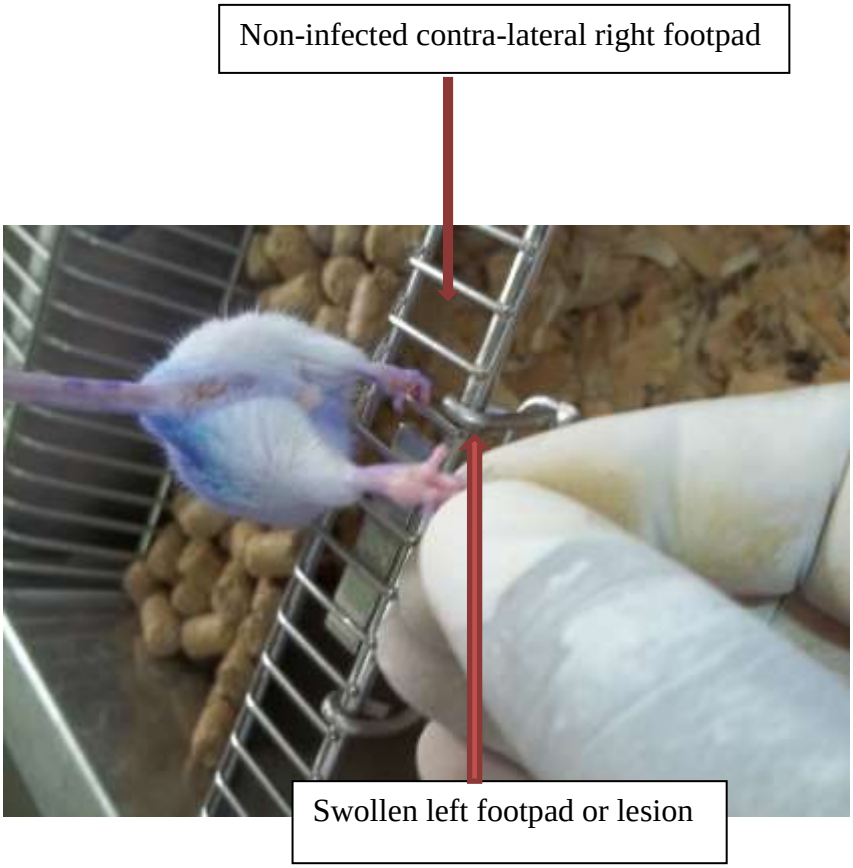

Plate 4.2: *L. major* disease swelling or lesion on left footpad after 4 weeks post infection

4.3.2 Combination therapy treatment outcome on body weights

There were different changes in body weights of the BALB/c mice study groups. The group treated combination therapy administered intraperitoneally, the weight increased while that administered orally decrease slightly. All mice survived during the experiment until humanely sacrificed.

Table 4.7: Mean body weights of L. major infected BALB/c mice 5 weeks post treatment test drugs

Paired t- test for the weight (g) Mean body					
Test drug	**Initial weight ±SE**	**Final weight ±SE**	**Difference(g)**	**Calculated *t***	**p value**
A. secundiflora	19.80±1.02	22.28±2.04	+2.48	2.457	0.311
C. citrinus	20.57±2.03	21.00±1.24	+0.43	1.402	0.457
Combination (IP)	19.71±1.02	21.43±1.04	+1.72	1.353	0.225
Combination (oral)	23.43±0.84	23.14±0.74	-0.29	0.420	0.689
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 (ns)

Table 4.7 shows average body weights (g) in BALB/c mice infected with *L. major* and treated with combination, single aqueous test extracts and controls administered orally or intra-peritoneal (ip) over 5 weeks (weeks) period. Using paired *t* test to compare the weight before and after treatment, there was no significant statistical difference among all the groups. The calculated *p* value ranged from 0.073 in PBS oral treated group to 1.000 for Pentostam ip treated group. This shows that infecting the mice with the *L. major* parasites did not automatically affect body weights. Other factors such as age, nutrition, diseased state, and stress contributed to change in body weights. This therefore implies that the combination therapy did not impact the body weight of the treated BALB/c mice and could be a potential treatment for leishmania.

4.3.3 Combination therapy treatment outcome on lesion sizes

Treatment for the first two weeks led to decreased foot pad lesion sizes in all BALB/c *L. major* infected mice. However, further treatment for 3 weeks did not have any effect as the lesion sizes remained constant. Table 4.8 shows mean footpad lesion/swelling 5 weeks post ip and oral treatment with combination therapy, +ve and -ve controls. One-way ANOVA comparison of the mean lesion sizes with and between the groups, there was statistically significant difference ($F_{(4,16)} = 9.127$ and

p = 0.001) in decrease of the lesion sizes. This shows that the combination therapy contains active ingredients that reduced disease progression. Surprisingly, administration through oral route seemed more effective than through intraperitoneally.

Table 4.8: Mean *L. major* disease footpad swelling (mm) over 5 weeks of oral and ip treatment with combination therapy and controls

			Mean footpad swelling (mm) [a]/Mean lesion size					**Drug vs PBS oral**
Drugs	Code	Route	Wk1	Wk2	Wk3	Wk4	Wk5	P-value
A.sec/C.cit	C	IP	0.60	0.40	0.20	0.20	0.20	0.792
A.sec/C.cit	C	Oral	0.20	0.38	0.24	0.16	0.10	0.044*
Controls:								
Pentostam	Pento	IP	0.40	0.30	0.20	0.10	0.10	0.047*
PBS	-	Oral	0.29	0.57	0.79	0.79	0.79	N/A
PBS	-	IP	0.38	0.68	1.12	1.02	0.79	0.878

*Shows a significant difference against PBS oral

Table 4.9 shows mean lesion reduction post 5 weeks of treatment with combination therapy (Oral and ip) and control drugs. Post hoc analysis using Games-Howell for multiple comparison showed statistically significant difference (p = 0.042 and p = 0.044) between oral combination treated and both PBS oral and ip treated groups respectively. Similar results were observed in positive control group against both oral (p = 0.042) and ip PBS (p = 0.047) treated groups. However, no statistically significant difference (ip vs oral, p = 0.792) observed between the two routes combination therapy treatment. This shows the efficacy of the combination therapy is not affected by the route of administration.

Table 4.9: Mean footpad swelling Post hoc tests (Games-Howell) comparison among combination and control treated groups

Drugs compared	Mean lesion difference	S.E	*p* value	95% CI	
				Lower	Upper
C ip vs C oral	0.104	0.093	0.792	-0.236	0.444
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

Figure 4.4 shows footpad lesion outcome within 5 weeks of treatment. In combination (oral and ip) and Pentostam treated groups, the lesion increased for the for two weeks and started to decrease. For negative controls, the lesions increased drastically up to week 3 and started a slight decline. The decline in negative control could be attributed to other reasons such as experimental errors or immunological response of the BALB/c mice to the *L. major* parasites. However, the decrease in the combination extracts and Pentostam treated groups is because of the active ingredients. This therefore shows combination therapy of the test extracts had antileishmanial agents leading to reduction of the lesion.

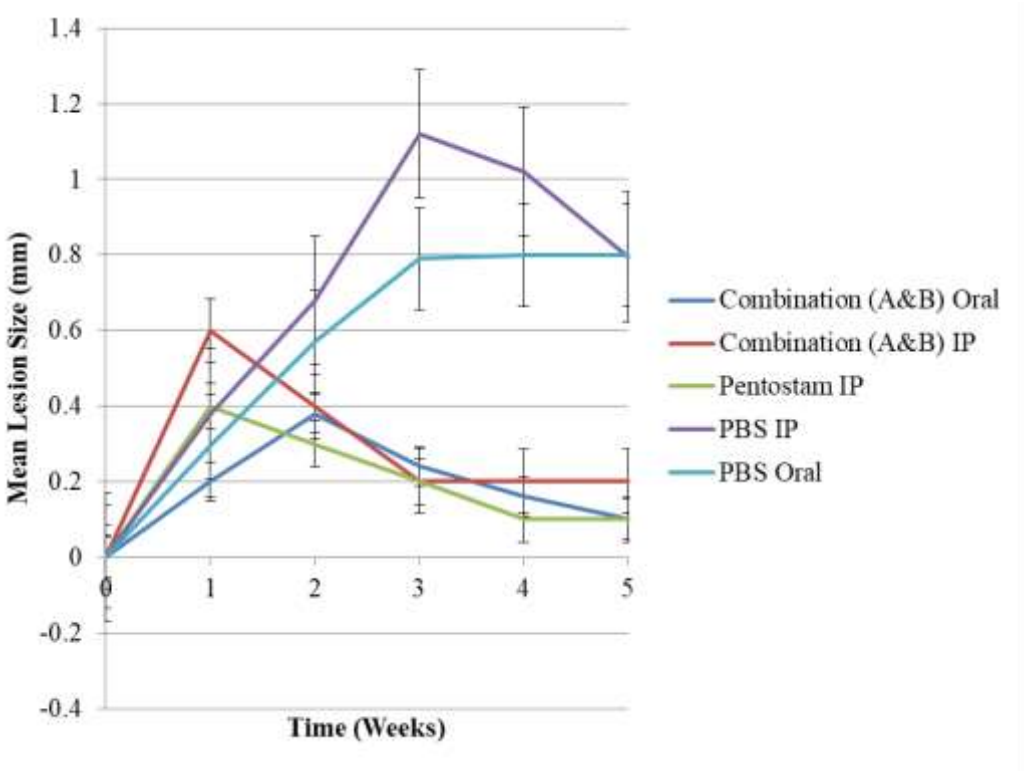

Figure 4.2: Footpad lesion outcome post 5 weeks oral and ip treatment with combination therapy and controls

4.3.4 Combination therapy treated BALB/c mice splenocytes impression spears infected with L. major parasites

The mice were sacrificed and at necropsy, the spleens were weighed, and their impression smears made. The slides smears are those of spleen from BALB/c mice treated with combination drug both administered orally and ip.

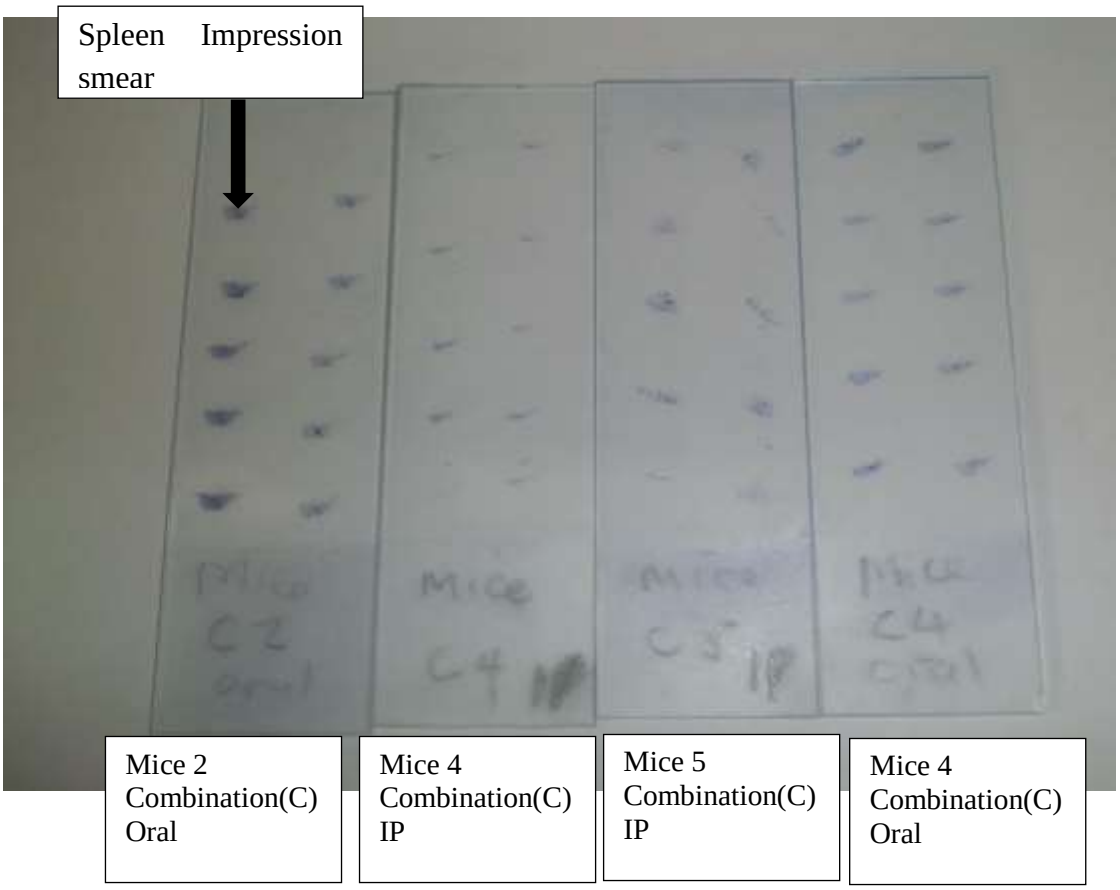

Plate 4.3: Giemsa-stained impression smears made from the spleen tissues

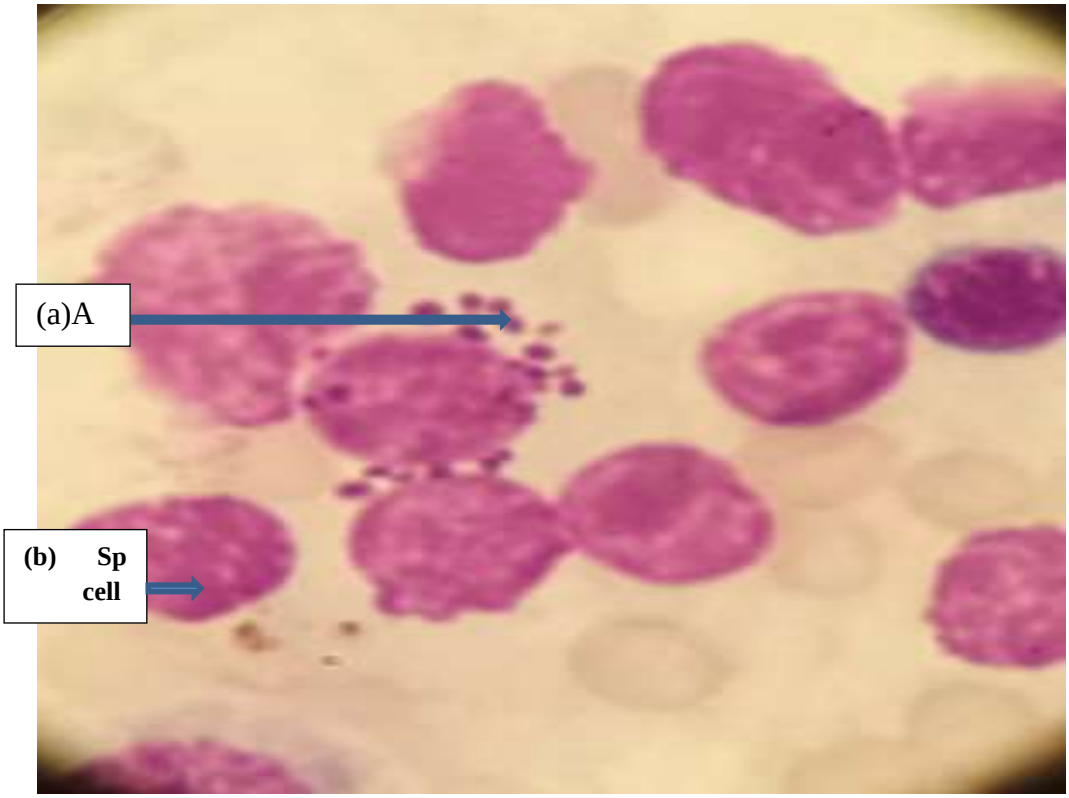

Plate 4.4: Spleen smears from an infected BALB/c mouse that had been treated with oral PBS for five weeks

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

Table 4.10: Number of Leishmania parasites in the infected BALB/c mice splenocytes

Treatment	Route	Ave spleen weight ±SE	Ave spleen Index (%) ±SE	Ave LDU ±SE	Ave total LDU ±SE (× 1000)
A. sec/C. cit	IP	0.13± 0.02	0.67 ± 0.42	0.35± 0.02	8.96 ± 0.82
A. sec/C. cit	Oral	0.13± 0.01	0.53 ± 0.45	0.42± 0.02	10.87±0.64
Pentostam	IP	0.10±0.003	0.45 ± 0.19	0.11±0.003	2.22 ± 0.13
PBS	IP	0.20±0.010	1.01 ± 0.05	0.93±0.012	37.15±1.09
PBS	Oral	0.23±0.020	1.10 ± 0.11	0.93±0.001	40.95±3.75

Table 4.10 shows average total parasite load in BALB/c mice treated with the study extracts and controls. One-way ANOVA comparison of total LDU showed there was significant statistical difference of the combination therapy ($F_{(4, 12)} = 113$, $p = 0.0001$) and controls. Comparing the parasites in both combination routes (ip and oral), there was no statistical difference in average total LDU ($p = 0.886$). However, comparison of total LDU in Pentostam treated group and of PBS oral and PBS ip showed significant statistical difference ($p = 0.001$). There was no significant statistical difference ($p = 0.536$) in average total LDU in PBS orally treated group against PBS ip treated group.

Table 4.11 shows the efficacy of combination therapy in reducing *L. major* parasites load in the spleen of BALB/c mice. Combination therapy administered intraperitoneally had higher reduction rate (78.12%) compared to oral route (73.46%). However, the difference (4.66%) was not too large and could be due to experimental errors. The observed parasite load clearance is further evidence that when used in combination, the aqueous extracts of the study plants could be providing synergistic or additive effects. As expected, the standard drug had the highest parasite clearance (94.58%) at low concentration. Some reduction of 9.28% was observed in negative control PBS ip treatment which could be attributed to experimental errors.

Table 4.11: Calculation of BALB/c spleen L. major parasite load clearance after treatment with combined (1:1) test aqueous and controls

Treatment	Route	Ave total LDU ±SE (× 1000)	% Parasite reduction
A. sec/C.citri	IP	8.96 ± 0.82	78.12
A. sec/C.citri	Oral	10.87±0.64	73.46
Pentostam	IP	2.22 ± 0.13	94.58
PBS	IP	37.15±1.09	9.28
PBS	Oral	40.95±3.75	0.00(100% LDU)

NB: PBS (Oral) had 0.00% parasite clearance and was arbitrary assigned 100% parasite load and used as total LDU from with others were calculated.

Table 4.12 show paired differences of spleen parasite load clearance (%) after treatment with combination therapy. Using paired *t*-test compare parasite clearance before and treatment with combination therapy, there was a near significant statistical difference ($t = 2.653$, $p = 0.057$). This shows that the combination therapy when precisely mixed can lead to significant treatment of leishmaniasis caused by *L. major* parasites.

Table 4.12: Paired t-test analysis of spleen parasite load clearance % before and after treatment with drugs

Mean	SD	SE	95% CI		*t*	*p* value
			Lower	Upper		
51.15	43.12	19.28	-2.39	104.70	2.653	0.057

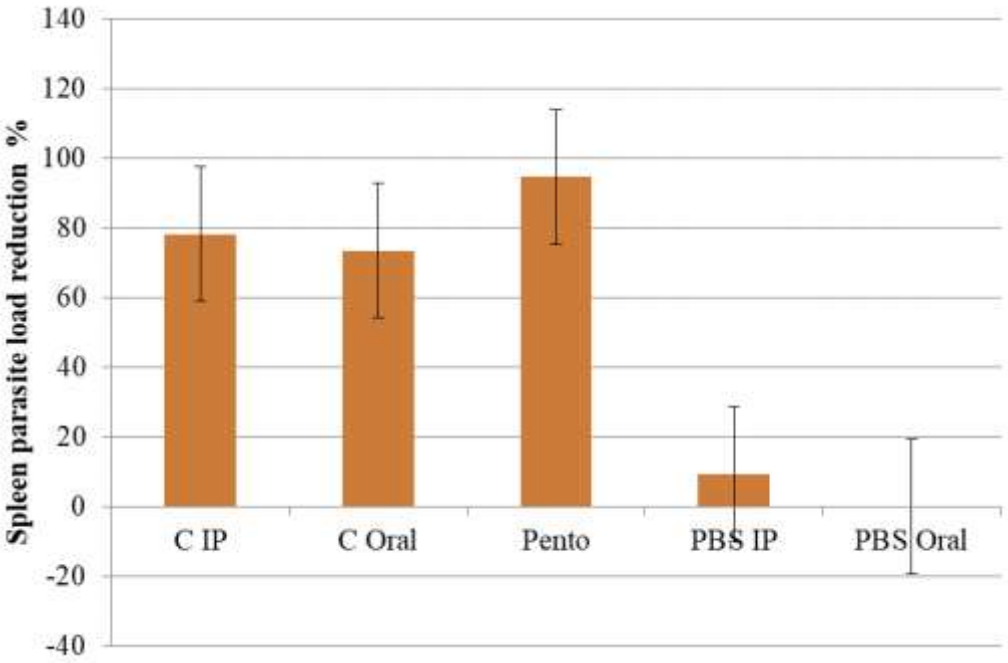

Figure 4.3: Spleen parasite load reduction/clearance % after treatment with the experimental drugs. Data represent mean lesion size ±S.E, (n = 5), five weeks after treatment

CHAPTER FIVE

DISCUSSION, CONCLUSSIONS AND RECOMENDATIONS

5.1 Discussion

Various experimental studies have shown that several plants in different genera contain compounds that have antileishmanial activity and can be used as alternative therapies although their effectiveness differs according to the active compounds present in the individual plants (Maobe *et al*., 2013). The main advantage of using plant-based products has been shown to reduce development of drug resistance by parasites (Polonio & Effert, 2008). In this study ant-leishmanial activity of combination therapy of aqueous leaf extracts from *A. secundiflora* plant and aqueous flower extracts of *C. citrinus* was assessed for the first time utilizing *in vitro* and *in-vivo* tests.

The leaf exudate of *A. secundiflora* has been confirmed to contain a mixture of phenolic compounds. Some of identified compounds include anthrones such as aloenin, aloenin B, barbaloin, isobarbaloin and other aloin derivatives (Weihenya *et al*., 2003). The *Callistemon* family on the other hand have shown to contain secondary metabolites such as tannins (Hanaa & Mohamed, 2002), C-methyl flavonoids (Huq & Misra, 1997) and triterpenoids (Cuong *et al*., 2016). Ismail *et al*., (2021) also reported the existence of Phloroglucinol derivatives in *Callistemon* family. This chapter discusses the results obtained when the aqueous crude extracts of combining *A. secundiflora* and *C. citrinus* 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.

The current study reported low toxicity (IC_{50}) against healthy Vero cells of 467.09 µg/ml and 457.88 µg/ml for single extracts of *A. secundiflora* and *C. citrinus* respectively. Previous studies reported IC_{50} of 547.88µg/mL for *A. secundiflora* (Ogeto *et al*., 2013) while *C. citrinus* showed 467 µg/mL (Kinuthia *et al*., 2013). A slight variation of *A. secundiflora* toxicity reported in current could be due to method

of extraction, calibration of software used to generate the (IC_{50}) among other factors. The combination treatment of the extracts showed high toxicity of 58.45µg/ml which could suggest presence of synergistic effect since individual extracts had low toxicity. However, it was not comparable to that of Pentostam at 0.26µg/ml. This therefore means the combination therapy can provide safer treatment to treat *L. major* if properly blended. According to Santos *et al*., (2008), most antileishmanial drugs are highly toxic and thus the studied combination could be used to produce safer treatment. It means that if the combination of *A. secundiflora* and *C. citrinus* is well studied and prepared, it can be used as an alternative medicine for visceral leishmaniasis. Further, cell viability was shown to be affected by IC_{50} where single extracts reported % viability levels of 68% and 79% for *A. secundiflora* and *C. citrinus* respectively at a concentration of 333.33 µg/ml. Their combination at the same concentration showed cell viability of 40% which is lesser than that of individual extracts. This is further evidence that, the 2 extracts could be working differently to produce an additive outcome. Pentostam reported cell viability of 74.83% at a concentration of 100 µg/mL This showed that combination extracts were less toxic as compared to standard drug, Pentostam.

Bioactivity investigation studies to detect leishmanicidal activity in plant extract has mainly used promastigotes form of the *Leishmania parasite*. This form is easy to culture, subculture and maintain with laboratory conditions. There is enough evidence and hence the promise to discover and derive plant-based leishmaniasis therapeutics (Dupouy-Camet, 2004). The current study reported MICs of 2000µg/mL for *A. secundiflora* and 5000µg/mL for *C. citrinus* like that reported by Kinuthia *et al*. (2013). Several combinations of MICs for the single extracts were prepared and minimum growth of *L. major* promastigotes were observed at ratio of 1:1. No life of *L. major* promastigotes was observed when more that one part was used. This provided the basis for *in vivo* treatment of BALB/c infected *L. major* parasites as it was assumed that the combination would inhibit the growth and multiplication of parasites.

The *L. major* parasites infect mononuclear phagocytes involved in provision of human immunity. Therefore, molecules that would increase activation of the infected macrophages would be ideal for control of infection. This is because the activation would escalate the killing of the parasite hence inhibiting their replication (James, 1995). According to Chan-Bacab *et al*., (2001), complementing the promastigote *in vitro* experiments with intracellular amastigote-infected macrophages would provide reliable leishmanicidal activity data. Gamboa-Leon *et al*., (2007) reported nitric oxide production by infected macrophages as mechanisms of killing the parasites. It was interesting to note that *A. secundiflora* extracts reduced the level of production of nitric oxide in the macrophages. This suggested that the possibility of direct action of the extract to the parasites could be high compared to immune system activation to deal with the amastigotes (Ogeto *et al*., 2013). According to Kinuthia *et al.,* (2013), aqueous *C. citrinus* crude extracts were however observed to induce negligible production of NO by peritoneal murine macrophages yet the extracts had inhibitory effects against the amastigotes *in vitro*. The current study agreed with the previous studies that none of the single extracts stimulated the production of enough levels of nitric oxide that could be used in the lysis of the *L. major* parasites. This study did not report significant production of nitric oxide by the infected microphages as means to fight the parasites. This therefore suggests that other mechanisms were used. The compounds in combination extract might have formed complexes with the parasites cell wall hence interfering with structure, caused DNA polymerase inhibition, or even inhibiting other enzymes necessary for the parasite replication and growth among other modes of action. This is an area of further investigation given that the study provided positive results related to killing of *L. major* parasites by the combination therapy of *A. secundiflora* and *C. citrinus*.

The combination inhibited the infection of macrophages more effectively than the single extracts. The combination and control drug were effective at concentrations of 125 μg/mL and 50μg/mL respectively. The study reported no significant difference ($p < 0.05$) in efficacy between the two treatments. This shows the possibility of additive effects of the antileishmanial compounds in the two aqueous extracts when used in combination. The combination extracts considerably reduced infection rates

(IR) of macrophages by the *L. major* amastigotes almost 3 times (19%) compared to that of the negative control (RPMI 1640 = 71.5± 2.12 %). This is consistence with the findings of Berman & Wyler (1980). As a matter of fact, they reported three-fold multiplication of *L. tropica* and *L. donovani* amastigotes in human macrophages after 6 days when grown in RPMI-1640 free of any antileishmanial agents. In addition, the IR of the current study also compared very closely to that reported by Kinuthia *et al*. (2013) of 84.67±2.96%.

In vivo investigation reported commendable reduction of foot pad lesion sizes in *L. major* infected BALB/c mice. Both oral ($p = 0.042$) and intraperitoneally ($p = 0.047$) treated BALB/c mice with the combination test extracts at ratio of 1:1 had their lesions reduced by the end of treatment period of 5 weeks. However, the group treated with the standard drug (Pentostam) reported the highest lesion size reduction. These findings were comparable to those reported by Nasimiyu *et al*., (2016). In their study, they reported a *p* value = 0.158 in treatment of *A. secundiflora* combined with *A. sativum*. *A. secundiflora* is known to possess strong antibacterial agents which could have contributed to inhibition of several bacteria responsible for exacerbation of wounds thus promoting the healing of the lesions (Ogeto *et al*., 2013). Similarly, *C. citrinus* extracts have been reported to have anti-inflammatory properties that would have enhanced lesion or wound healing (Shinde *et al*., 2012). It is therefore evident that the combination extracts led to lesion reduction even though not similar to that in Pentostam treated group.

The body weights for the combination therapy increased for intraperitoneal, decreased slightly for oral and for the negative controls. In the Pentostam group, the weights were unaffected. This was analyzed using paired *t*-test to determine the level of significance at $p < 0.05$ and there was no significant difference. The change observed could not be solely attributed to the study treatment because other factors were not studied which have been reported to affect weight. They include factors like nutrition, disease state, stress, and age among others. However, the mice were handled with great care to avoid stress, properly fed, and provided with enough water hence these confounders were well minimized.

The study reported a total LDU of 8960, 10870, 2220, 37150 and 40950 parasites in the spleens of Combination ip, Combination oral, Pentostam, PBS ip and PBS oral respectively after necroscopy. Arithmetically, 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 as treatment, PBS was arbitrary assigned 100% load that had no reduction because it contained no drug or antileishmanial agent. The parasite reduction percentage for the other tests were calculated using PBS oral as the standard measure. The outcomes were Combination ip (78.12%), Combination oral (73.46%), Pentostam ip (94.58%) and PBS ip (9.28%). This shows there was substantial parasites load reduction in the combination therapy and for Pentostam compared to the negative controls. Pentostam ip cleared most parasite load compared to the combination therapy but there was no significant difference. Nevertheless, the route of administration did not affect the reduction parasite load for the combination therapy.

The synergistic effect of the combination extracts of *A. secundiflora* and *C. citrinus* in treatment of *L. major* is presumed or exhibited in both *in vitro* and *in vivo* tests. First, it was evident that combination inhibited the infection of macrophages more effectively than the single extracts. Secondly, failure of the combination extracts to stimulate production of NO indicated possibility of direct action of these extracts to the *L. major* parasites. Further, increased ability to reduce substantially spleen parasite load suggested a possibility of these extracts working synergistically. However, there is need for further investigation to establish the kind of synergy that may exist between *A. secundiflora* and *C. citrinus* extracts when used in combination.

5.2 Conclusion

This study showed that treatment with a combination of extracts of *A. secundiflora* and *C. citrinus* against *L. major* parasites can be used to minimize pathology caused by the parasite. Considering that the extracts exhibited very low toxicity, this makes them better alternatives for disease control. The marked high antileishmanial activity in the extracts studied, though not of comparative concentrations than that of the

standard reference drug (Pentostam), suggests that it is possible that if fortified and/or used together with other drugs improve leishmaniasis treatment. These extracts could provide synergistic or additive effects in the treatment and control of different *Leishmania* species.

5.3 Recommendations

1. Further studies are required to establish the *in-vivo* activity of the crude extract, the active ingredient, dosage, and safety of combination of extracts of *Aloe secundiflora* and *C. citrinus*.
2. Further pre-clinical research of *A. secundiflora* and *C. citrinus* combination extracts on *L. major* parasitemia in different internal organs should be done to establish the level of parasite elimination in these organs.
3. Further studies to determine the efficacy and synergistic effects of these combined extracts should be carried out in non-human primates.

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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/103308 24th October, 2014

Cosmas Ndeti

Thro'
Director, CBRD
NAIROBI

REF: SSC No. 2920 (Revised) – Efficacy of Combination Therapy Using Extracts of *Aloe secundiflora* and *Callistemon citrinus* in *Leishmania major* Infected Balb/C Mice

Thank you for your letter dated 14th October, 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

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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/ 01.10.14

15th October, 2014

Cosmas Muia Ndeti
P. O. Box 440 – 90137,
Kibwezi, Kenya.
Email:cosmasmuia@gmail.com

Ndeti,

RE: Animal use approval for SSC 2920 - "Efficacy of combination therapy using extracts of *aloe secundiflora* and *callistemon citrinus* in *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.

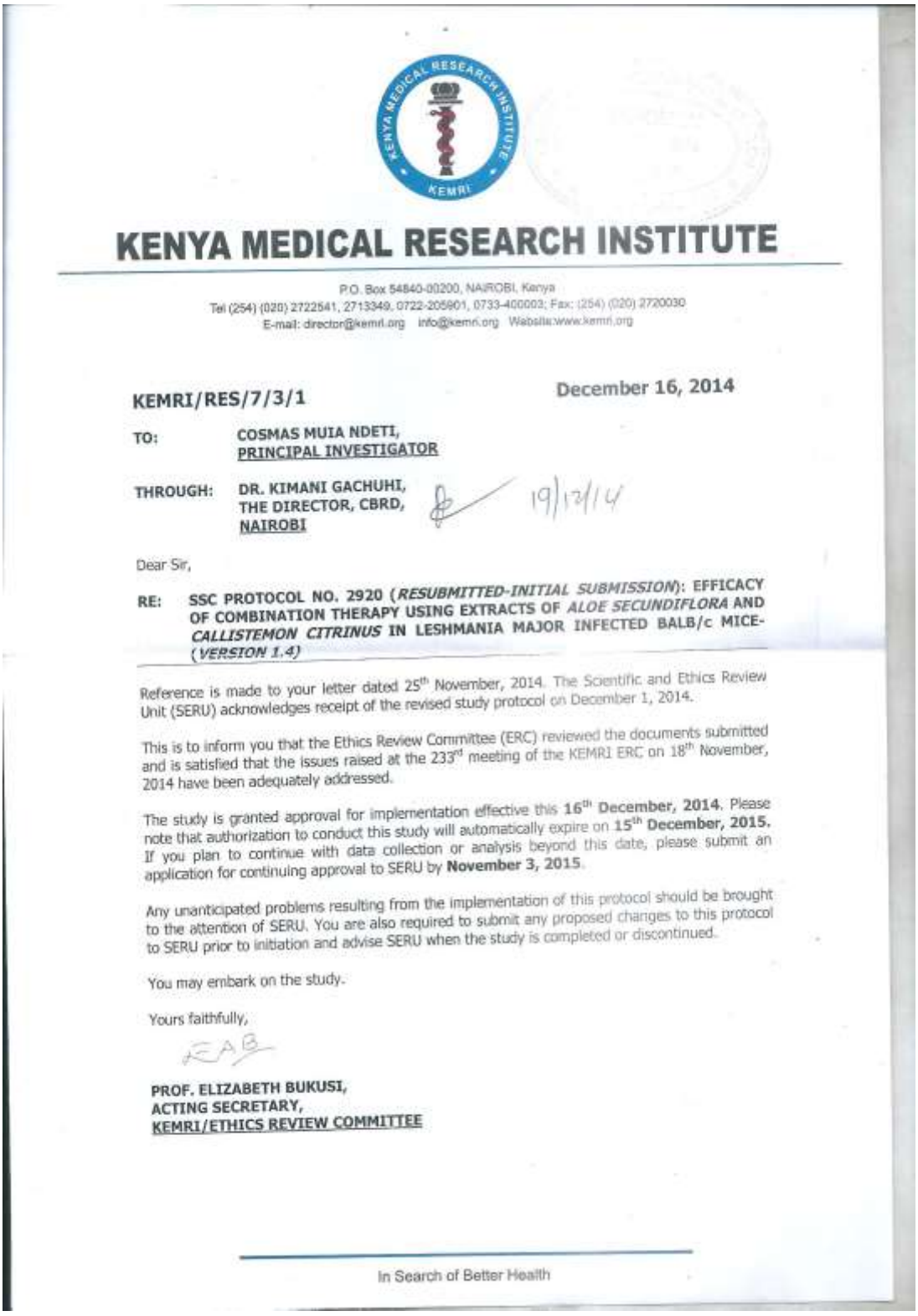
Yours sincerely,

Dr. Kenongoi Limbaso
Chairperson KEMRI ACUC

KENYA MEDICAL RESEARCH INSTITUTE
★ 15 OCT 2014 ★
ANIMAL CARE AND USE COMMITTEE
Signature: .....

In Search of Better Health

Appendix III: Ethical Approval



KENYA MEDICAL RESEARCH INSTITUTE

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E-mail: director@kemri.org info@kemri.org Website:www.kemri.org

KEMRI/RES/7/3/1 December 16, 2014

TO: COSMAS MUIA NDETI,
PRINCIPAL INVESTIGATOR

THROUGH: DR. KIMANI GACHUHI,
THE DIRECTOR, CBRD,
NAIROBI 19/12/14

Dear Sir,

RE: SSC PROTOCOL NO. 2920 (*RESUBMITTED-INITIAL SUBMISSION*): EFFICACY OF COMBINATION THERAPY USING EXTRACTS OF *ALOE SECUNDIFLORA* AND *CALLISTEMON CITRINUS* IN LESHMANIA MAJOR INFECTED BALB/c MICE-(*VERSION 1.4*)

Reference is made to your letter dated 25th November, 2014. The Scientific and Ethics Review Unit (SERU) acknowledges receipt of the revised study protocol on December 1, 2014.

This is to inform you that the Ethics Review Committee (ERC) reviewed the documents submitted and is satisfied that the issues raised at the 233rd meeting of the KEMRI ERC on 18th November, 2014 have been adequately addressed.

The study is granted approval for implementation effective this **16th December, 2014**. Please note that authorization to conduct this study will automatically expire on **15th December, 2015.** If you plan to continue with data collection or analysis beyond this date, please submit an application for continuing approval to SERU by **November 3, 2015**.

Any unanticipated problems resulting from the implementation of this protocol should be brought to the attention of SERU. You are also required to submit any proposed changes to this protocol to SERU prior to initiation and advise SERU when the study is completed or discontinued.

You may embark on the study.

Yours faithfully,

EAB

PROF. ELIZABETH BUKUSI,
ACTING SECRETARY,
KEMRI/ETHICS REVIEW COMMITTEE

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Appendix IV: List of publications arising from the study

1. Ndeti C.M., Kituyi, C., Ndirangu, M., Ingonga, J., Chimbevo, M. L., Ochieng, J.O., Barasa, M., Kinuthia, G., Maina, E.M., Nyambati, V.C.S. & Anjili, C.O. (2016). Efficacy of combination therapy using extracts of *Aloe secundiflora* Eng L. and *Callistemon citrinus* William C. in *Leishmania major* infected Balb/c mice. ***East African Medical Journal*. 93(2); 72-79.**