BIOACTIVITY OF *Ricinus communis* AND *Azadirachta indica* EXTRACTS ON *Leishmania major* INFECTION IN BALB/c MICE

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2014

DECLARATION

Declaration by the candidate

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DEDICATION

This thesis is dedicated to my wife Mercie and three sons Stuart, Patrice and Justin for their patience

ABSTRACT

Leishmaniasis is endemic in over 88 countries in the world and 350 million people are considered at risk. The standard drugs for treatment of leishmaniases are toxic, expensive and resistance against them is increasing. They require protracted administration, and currently, there is no vaccine. This study examined both *in vivo* and *in vitro* response of *Leishmania major* infection to combined therapy of *Ricinus communis* and *Azadirachta indica* crude extracts in BALB/c mice. BALB/c mice were inoculated intradermally on the left hind footpad (LHFD) with 10^6 *L. major* infective promastigotes. The mice were treated intraperitoneally with different drug combinations of daily doses for 28 days. Pentostam and amphotericin B were used as the reference drugs under the same experimental conditions. Negative controls were treated with Phosphate Buffered Saline (PBS) and Roswell Park Memorial Institute 1640 Medium (RPMI) *in vivo* and *in vitro* respectively. All experiments were performed in triplicate, the mean standard deviation of at least three experiments were determined, statistical analysis of the differences between mean values obtained for the experimental groups was done by the students t-test. P. values of 0.05 or less ($p \leq 0.05$) were considered to be significant. BALB/c mice, treated with combination therapy resulted in significantly ($P < 0.0003$) larger reduction of lesions than those treated with monotherapies. The spleen and spleno-somatic index was found to be significantly low with combination therapy than monotherapies. However, standard drugs had better efficacy for reduction of parasite load. Antiparasitic effect of *A. indica* and *R. communis* on amastigote with a 50% inhibitory concentration (IC_{50}) was of $11.5 \mu gml^{-1}$ and $16.5 \mu gml^{-1}$ respectively while combination therapy gave $9.0 \mu gml^{-1}$ compared to the standard drugs, pentostam and amphotericin B which had an IC_{50} of $6.5 \mu gml^{-1}$ and $4.5 \mu gml^{-1}$, respectively. Optimal efficacy of *A. indica* and *R communis* was 72% and 59.5%, respectively, combination therapy gave 88%, while pentostam and amphotericin B had 98% and 92%, against amastigotes respectively. Against promastigotes *A. indica* and *R. communis* gave an IC_{50} of $10.1 \mu gml^{-1}$, $25.5 \mu gml^{-1}$ respectively, while combination therapy was $12.2 \mu g\, ml^{-1}$ against $4.1 \mu g\, ml^{-1}$ and $5.0 \mu g\, ml^{-1}$ for pentostam and amphotericin B, respectively. The optimal efficacy of the compounds against promastigotes was 78.0%, 61.5% and 91.2% (*A. indica, R. communis* and *A. indica* + *R. communis* respectively) against 96.5% and 98% for pentostam and amphotericin B respectively. The concentrations at optimal efficacy were significantly different ($p<0.002$) among the test compounds. Toxicity level for combined therapy was lower than standard drugs. The Leishman Donovan Units (LDU) of combination therapy was not significantly different ($P<0.001$) from that of pentostam though amphotericin B was better. These results showed that combination therapy of *A. indica* and *R. communis* had better antileishmanial activity than the monotherapies. It was also better than the standard drugs since it had lower toxicity to body cells. Further tests on the two plant extracts need to be done using non-human primates that are susceptible to leishmaniasis such as vervet monkey, *Cercopithecus aethiops*.

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LIST OF ABBREVIATIONS/ACRONYMS

µg/ml	microgram/ milliliter
ANOVA	Analysis of Variance
ACUC	Animal Care and Use Committee
BALB/c	Inbred mice very susceptible to *L. major*
CBRD	Centre for Biotechnology Research and Development
CC_{90}	90% cytotoxic concentration
CC_{50}	50% cytotoxic concentration
CL	Cutaneous Leishmaniasis
CTMDR	Centre for Traditional and Medicine and Drugs Research
DDT	Dicloro-diphenyl-trichloroethane
DEET	N, N -Diethyl- meta -toluamide
DMSO	Dimethyl sulfoxide
ELISA	Enzyme Linked Immunosorbent Assay
FBS	Foetal Bovine Serum
HIV	Human Immonodeficiency Virus
IC_{50}	Concentration that kills 50% of parasite population

IC_{90}	Concentration that kills 90% of parasite population
INFγ	Interferon gamma
IR	Infection rate
KEMRI	Kenya Medical Research Institute
LDU	Leishman Donovan Unit
LHFP	Left Hind Foot Pad
MCL	Muco-Cutaneous Leishmaniasis
MEM	Minimum Essential Medium
MI	Multiplication Index
MIC	Minimum Inhibitory Concentration
MTT	Methyl Thiazol Tetrazolium

NFE	Non Feed Effect
NK	Natural Killer cells
NNN	Novy-Nicolle-McNeal Medium
NO	Nitric oxide
PBS	Phosphate Buffered Saline
PKDL	Post-Kala-azar Dermal Leishmaniasis
RHFP	Right Hind Foot Pad
RPMI	Roswell Park Memorial Institute 1640 Medium
SbV	Pentavalent antimony
SOPs	Standard Operating Procedures
Th1/2	T helper-1, 2 cells
TNF	Tumor Necrosis Factor
VL	Visceral Leismaniasis
WHO	World Health Organization
ZCL	Zoonotic Cutaneous Leishmaniasis

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CHAPTER ONE

INTRODUCTION

1.1. Background of the study

In many tropical and subtropical developing countries protozoan parasites are amongst the most common infectious agents and have serious consequences for socio-economic development (Alvar *et al.*, 2006). The World Health Organization (WHO) considers leishmaniasis to be one of the most serious parasitic diseases and the World Health Assembly has advocated a concertation for its control (WHO, 2007). Approximately 350 million people, who are often impoverished, are at risk of contracting the disease (Alvar *et al.*, 2006). Currently, the disease appears to be underestimated and on the rise in several countries.

The Leishmaniases, which are vector-borne diseases that are caused by obligate intra-macrophage protozoa, are endemic in large areas of the tropics, subtropics and the Mediterranean basin (Chappuis *et al.*, 2007). Leishmaniases consist four main clinical syndromes: cutaneous leishmaniasis; muco-cutaneous leishmaniasis (also known as espundia); visceral leishmaniasis (VL; also known as kala-azar); and post-kala-azar dermal leishmaniasis (PKDL). In cutaneous leishmaniasis, the patient generally presents with one or several ulcer(s) or nodule(s) in the skin. Due to species differences in tissue tropism, virulence and their interaction with the host's immune system, infection by leishmaniasis can result in a variety of clinical manifestations ranging from single self-healing ulcers in cutaneous forms to life threatening visceral infections (Desjeux, 2004). The ulcers heal spontaneously although slowly in immunocompetent individuals, but cause disfiguring scars. In muco-cutaneous leishmaniasis, patients suffer from

progressively destructive ulcerations of the mucosa, extending from the nose and mouth to the pharynx and larynx. These lesions are not self-healing and are usually seen months or years after a first episode of cutaneous leishmaniasis, when the macrophages of the naso-oropharyngeal mucosa become colonized.

During its life cycle, *Leishmania* spp. presents two forms: promastigotes, and amastigotes. Promastigotes are flagellated, motile forms that are found in the alimentary tract of phlebotomine sand flies. Amastigotes are non-motile forms found inside mononuclear phagocytes of the mammalian host. Metacyclic infective promastigotes are transmitted by the female sand flies to mammals, where they invade and multiply as amastigotes within macrophages. One of the earliest events after promastigotes have entered the mammalian host is their contact with plasma proteins. It has been shown that fresh normal human serum (f-NHS) can cause the lyses of *Leishmania* spp. *in vitro* (Franke *et al.*, 1985) through the alternative pathway of complement (Mosser, 2007). Therefore, they must escape the lytic effect of serum before they can invade macrophages (Jokiranta *et al.*, 2005). In fact, it has been shown that non-infective promastigotes from logarithmic stage of growth, which are susceptible to complement (Franke *et al.*, 1985), differentiate into stationary phase promastigotes with an increased resistance to complement and higher infectivity(Franke *et al.*, 1985; Barral-Netto *et al.*, 1987). Differences in the pattern of promastigotes sensitivity to complement, lead to the hypothesis that complement resistance of *Leishmania* spp. is species-specific (Franke *et al.*, 1985), and that their resistance could be related to the severity of the disease caused

by each species (Barral-Netto *et al.*, 1987). Therefore treatment of leishmaniasis should involve targeting the parasites species.

In traditional medicine, many plants have already provided valuable clues for potentially antiparasitic compounds, especially simple quinones, quassinoids, and related naphthoquinones (Iwu *et al.*, 1994). Alternative plants are still required that contain these compounds necessary for control of *Leishmania* protozoans.

The castor oil plant, *R. communis* is a species of flowering plant in the spurge family, *Euphorbiaceae*. It belongs to a monotypic genus, *Ricinus*, and sub-tribe, *Ricininae*. Its seed is the castor bean which, despite its name, is not a true bean. Castor seed is the source of castor oil, which has a wide variety of uses. Alcoholic extract of the leaf has been shown to be hepatoprotective in rats (Gupta *et al.*, 2004). Methanolic extracts of the leaves of *R. communis* have shown antimicrobial properties (Oyeewole, 2010). The pericarp of castor bean showed central nervous system effects in mice at low doses. Antihistamine and anti-inflammatory properties were found in ethanolic extract of *R. communis* root bark (Lomash *et al.*, 2010).

The neem tree, *A. indica,* A. Juss (*Meliaceae*) is an Indian tree that has many useful compounds that act as insecticide. However, no study is available that has tested the efficacy of combination therapy of *A. indica* and *R. communis*. In light of the scanty data on efficacy of the herbal medicines especially in the tropical regions where there are large forested land under these plants, the aim of this study was to assess response of *L. major*

to combined therapy of *R. communis* and *A. indica* crude extracts in BALB/c mice

1.2 Problem Statement

Cutaneous leishmaniasis is rarely fatal but may result in disfiguring scars. Untreated, severe cases of visceral leishmaniasis are almost always fatal. Death can result directly from the disease through organ failure or wasting syndromes. It may also occur as a result of a secondary bacterial infection such as pneumonia. Despite the existence of antiparasitic agents to control parasites, the problems of developing a single drug or formulation for all forms of leishmaniasis revolve around factors that include: that the visceral and cutaneous sites of infection impose differing pharmacokinetic requirements on the drugs to be used; the intrinsic variation in drug sensitivity of the 17 *Leishmania* species known to infect humans and increased efficacy in immunosuppressed patients, in particular due to HIV co-infection. In the latter case, where there is exacerbation of disease or emergence from latent infection, the depleted immune capability means that standard chemotherapy is frequently unsuccessful. There is also a relatively high cost and constraints due to the length of the modern treatment to control the leishmaniasis in developing countries like Kenya; therefore, there is a need to search for alternatives. Fortunately, many rural communities in Kenya know a lot about medicinal plants that can cure many diseases but due to a number of protocols required, their effective use remain speculative. Thousands of plants have been screened for anti-parasite activities using a standard WHO procedure. However, there is still lack of vital information including anti-parasite efficacy of most plant species in Kenya, and therefore research on the plants species in Kenya is still deemed to be limited.

There are no toxicity data or studies available that have so far been conducted in this field proving the anti-parasite properties of many plant extracts, more significantly are the lack of information on the combined therapies on various parasites. Furthermore, efficacies of plant extracts are known to be affected by among other things; location, amount of active compounds in the plants, extraction procedure and species of organism under study, which makes it very difficult to generalize the anti-parasite properties of many plant species. Lack of such information has limited the use of plant biocides against *Leishmania* in Kenya.

1.3 Objectives of the study

1.3.1 General objective

To investigate the antileishmanial activity of combined therapy of *R. communis* and *A. indica* crude extracts to infected *L. major* BALB/c mice.

1.3.2 Specific objectives

(i) To compare parasite loads in spleens of *L. major* infected BALB/c mice treated with mono- and combination therapy of *R. communis* and *A. indica* crude extracts.

(ii) To determine nitric oxide production in treated *L. major* infected macrophages treated with mono- and combined therapy of *R. communis* and *A. indica* crude extracts.

(iii)To determine the cytotoxicity of *R. communis* and *A. indica* crude extracts to Vero-E6 cells.

1.4 Hypotheses

H_o: There is no significant difference between treatment with standard drugs and mono or combination therapy of *R. communis* and *A. indica* crude extracts on parasite loads in spleens of *L. major* infected BALB/c mice.

H_o: Treatment with mono or combination therapy of *R. communis* and *A. indica* crude extracts does not significantly differ with standard drugs on nitric oxide production in *L. major* infection.

H_o: Treatment with mono or combination therapy of *R. communis* and *A. indica* crude extracts does not significantly differ with the standard drugs in toxicity to Vero-E6 cells

1.5 Significance of the study

The leishmaniasis burden will increase, as a result of: i) increasing migration ii) regional climate change and iii) impaired immunity, resulting from malnutrition and/or HIV. Despite this, efforts to control the disease are either nonexistent or remain sub-optimal, and are usually conducted in response to epidemic outbreaks. One of the rational approaches to therapy is to test compounds that are known to have anti-parasitic effects within the kinetoplastid parasites.

Plant extracts have been tested for anti-protozoal activities with varied results. However, many antimicrobial antibiotics are also important anti-protozoal drugs or have provided important leads in disease control. Existing information confirms that *A. indica* and *R. communis* has many antimicrobial, dermatological and cytological activities against protozoan parasites. *R. communis* also shows activity when applied as an ointment on *L. amazonensis* lesions on BALB/c mice.

CHAPTER TWO

LITERATURE REVIEW

2.1 The Leishmaniases

Leishmaniases are major vector-borne diseases caused by obligate intra-macrophage protozoa of the genus *Leishmania* parasites infecting numerous mammal species, including humans. Leishmaniases are transmitted by the bite of phlebotomine sand flies and its species are widespread on all continents except Antarctica (Banuls *et al.*, 2007). Leishmaniases are endemic in areas of the tropics, subtropics, and southern Europe specifically, is endemic in 88 countries and is the only tropical vector-borne disease that has been endemic to southern Europe for decades (Dujardin *et al.*, 2008). Currently, leishmaniases have a wider geographical distribution pattern than before and it is considered to be a growing public health concern for several countries. The increase in leishmaniases worldwide is mainly attributed to the increase of several risk factors that are clearly man-made and include massive migration, deforestation, urbanization, immunosuppression, malnutrition and treatment failure (Desjeux, 2001). Man-made changes to the environment, as well as the population movements, may lead to alterations in the range and density of the vectors and reservoirs and consequently may increase human exposure to infected sand flies. People who live in many tropical and subtropical countries are likely to be infected with leishmaniases. This ranges from rainforests in Central and southern America to deserts in West Asia and Middle East. It affects 12 million people worldwide with 1.5-2 million new cases yearly (WHO, 2007). WHO yearly reports indicate that, countries from the four major continents: Africa, South America, Asia and Europe are affected by leishmaniases. The Geographical distribution

of leishmaniasis is limited by the distribution of the sand fly, the main vector of this disease (Dey and Singh, 2006).

2.2 Life cycle of *Leishmania*

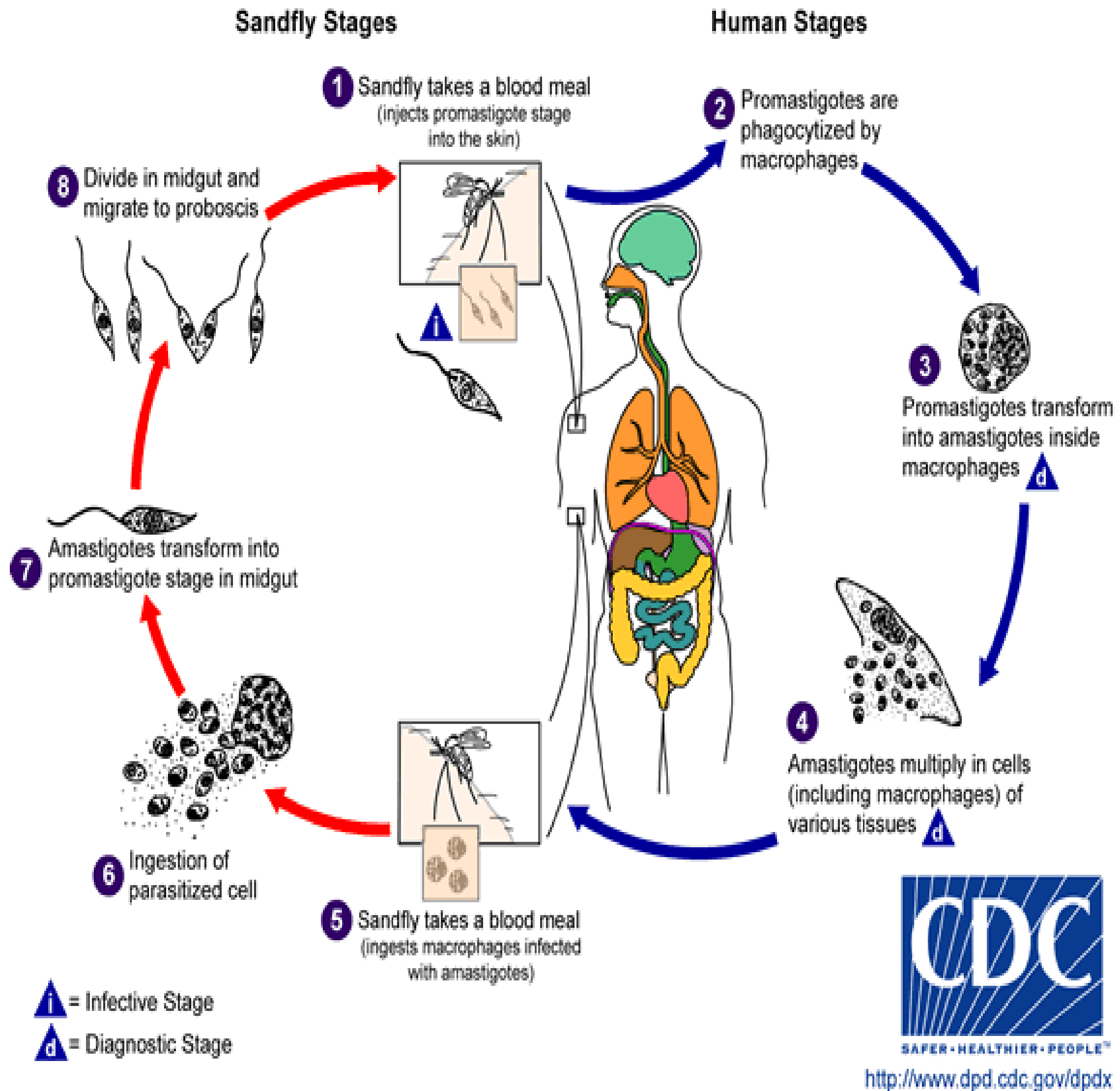


Figure 2.1: Life cycle of *Leishmania* spp.

(CDC, 2013)

The life cycle of leishmania is shown in Figure 2.1. Leishmaniasis is transmitted by the bite of female phlebotomine sand flies. The sand flies inject the infective stage,

metacyclic promastigotes, during blood meals. Metacyclic promastigotes that reach the puncture wound are phagocytisized by macrophages and transform into amastigotes. Amastigotes multiply in infected cells and affect different tissues, depending, in part on which *Leishmania* species is involved. These various tissue specificities cause the different clinical manifestations of the various forms of leishmaniasis. Sand flies become infected during blood meals on infected hosts when they ingest macrophages infected with amastigotes. In the sand fly's midgut, the parasites differentiate into promastigotes, which multiply, differentiate into metacyclic promastigotes, and migrate to the proboscis.See Figure 2.1.

2.3 Disease transmission, clinical and epidemiological features

Leishmaniasis is a protozoan disease caused by a parasite member of the *Leishmania* genus and presents high morbidity and mortality rates. The disease is transmitted to its vertebrate host by the female infected sand fly. The female needs a blood meal for egg maturation, hence, like mosquitoes, only the female sand fly is haematophagous. The invertebrate hosts are small insects of the order Diptera, belonging to the *Phlebotominae* subfamily and only two of the six genera described are of medical importance: *Phlebotomus* of the "Old World" (Africa, Asia, and Europe) and *Lutzomyia* of the "New World" (the Americas) (Killick-Kendrick, 1990, 1999). Some phlebotomine species such as *Phlebotomus papatasi* and *P.sergenti* can support the growth of only those species of *Leishmania* with which they are infected in nature, whereas other species such as *Lutzomyia longipalpis* and *P. argentipes* can develop mature transmissible infections when infected with several *Leishmania* species (Rogers *et al.*, 2004). The potential

diseases' reservoirs include many different orders of mammals such as rodents, canids, edentates, marsupials, procyonids, primitive ungulates and primates (Lainson *et al.*, 1987). On the other hand, humans are considered to be accidental hosts of these parasites. The establishment of the disease depends on the success of the *Leishmania* parasite to differentiate into the amastigote form (Bogdan *et al.*, 1990; Sereno *et al.*, 2005). It has been established that visceral leishmaniasis, a clinical form of the disease could be directly transmitted via blood (needle sharing, transfusion, transplacental spread) or organ transplantation (Morillas-Marquez *et al.*, 2002; Pagliano *et al.*, 2005). The *Leishmania* parasite is involved in different pathologies that range from the cutaneous to the visceral forms, depending on the species of *Leishmania* and the host immune response (Barral *et al.*, 1991). In humans, infection with leishmania parasites could also result in asymptomatic forms. It is well documented that asymptomatic human hosts could contribute in the maintenance of the leishmaniasis foci (Banuls *et al.*, 2007). Leishmaniasis development depends on several risk factors such as malnutrition, immunosuppression, age, immunological status and genetic factors. Several investigators have reported the negative effect of malnutrition on leishmaniasis clinical course. The leishmaniases are characterized by a spectrum of clinical manifestations: ulcerative skin lesions, as shown in plate 2.1 and appendix 5, which they develop at the site of the sand fly bite (localized cutaneous leishmaniasis); multiple non-ulcerative nodules (diffuse cutaneous leishmaniasis);destructive mucosal inflammation (mucosal leishmaniasis) and disseminated visceral leishmaniasis (Desjeux, 2004). Cutaneous leishmaniasis is frequently self-healing in the "Old World"; cutaneous infection can remain subclinical or become clinically apparent after a variable incubation period that averages several weeks.

Leishmania and HIV co-infections have been reported in 35 out of 88 countries in which leishmaniases are endemic and are a growing concern in Brazil, Eastern Africa and the Indian subcontinent, where both diseases overlap geographically (Cruz *et al.*, 2006). *Leishmania* spp. resistance in some regions to antimonial drugs (the first-line drugs in the majority of developing countries) could be a novel risk factor for the disease's incidence increase (Croft *et al.*, 2006).

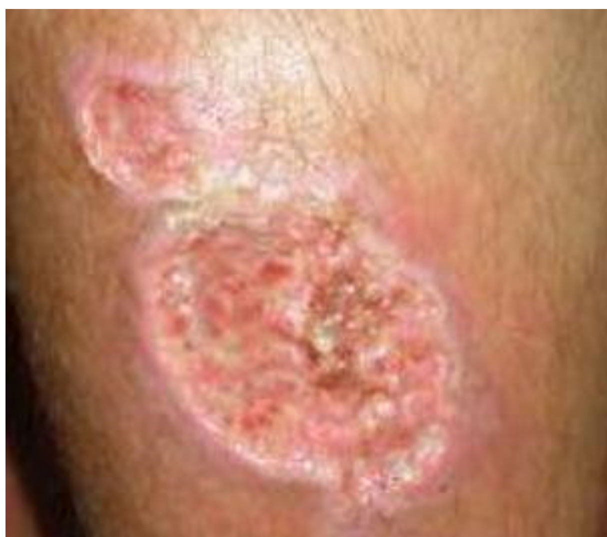


Plate 2.1 Skin lesions of a *Leishmania* patient (Source: Author, 2014)

2.4 Control strategies and prevention measures

Since anti-leishmanial vaccines are still being developed, the current control strategies for leishmaniasis rely on case management (case detection and treatment), vector and reservoir control. Case management that includes early diagnosis and treatment is essential for both individual patients and for the community. However, case management is difficult to be conducted and inefficient if feasible; this practice is mainly restricted by several factors such as the lack of access to affordable, active drugs, the incorrect

prescriptions and the poor compliance. Diagnosis for cutaneous leishmaniasis relied on is skin smears and treatment is based on pentavalent antimonials.

Sand flies are highly susceptible to insecticides and they are indeed susceptible to the same insecticides as used against anopheles mosquitoes, the malaria vector. It is encouraging that even though sand flies possess the essential biochemical mechanisms for resistance development to various insecticides, the reports on resistance are few. The only insecticide resistance for sand flies is for the organo-chlorine DDT (dichloro-diphenyl-trichloroethane) (Davies *et al.*, 2000). Sand fly control is now mostly dependent on pyrethroids. House spraying is focused on the control of endophilic sand flies (that rest mostly indoors after feeding). However, residual spraying is much more effective in urban situations when every house and animal shelter is treated than in rural areas where relatively few dispersed houses are sprayed and the sand flies represent a small proportion of the vector population. The synthetic pyrethroids used for nets' treatment combine the properties of low to moderate mammalian toxicity, low volatility and high insecticidal activity (Alexander and Maroli, 2003). The use of insect repellents (such as DEET, Diethyl-polyamide) or protective clothing has also been suggested as a prophylactic measure against leishmaniasis. The above are being suggested for people who are at risk for *Leishmania* transmission such as travelers and soldiers in maneuvers or hunters. DEET can be effective for over 4 hours (Alexander and Maroli, 2003). Repellants applied to clothing rather than skin has been proposed as an alternative approach to personal protection against sand fly vectors. However, several studies have concluded that impregnated clothing to protect humans from sand fly vectors may be impractical (Alexander and Maroli, 2003).

Where leishmaniasis is primarily zoonotic (Latin America, Mediterranean region, central and southeast Asia) *Leishmania* transmission to humans may be reduced by targeting the animal reservoir. The current leishmaniasis control programs have largely failed mainly because of the insufficient regional health delivery systems and due to the limited local resources. This has resulted to classification of leishmaniasis as an emerging and uncontrolled disease (belonging to category 1 of the diseases) by W.H.O. There is also hope that the first leishmaniasis vaccine will become available within a decade. Until an efficient vaccine becomes commercially available, the identification of risk factors could greatly help in designing prevention strategies

2.5.1 Treatment of Leishmaniasis

Cutaneous leishmaniasis is not always treated. Cases with few lesions that are small and appear to be healing are sometimes simply monitored. Possible treatments for cases arriving in the U.S. include oral ketoconazole (Nizoral, Extina, Xolegel, and Kuric), intravenous pentamidine, or liposomal amphotericin B. An antimonite called stibogluconate (pentostam) is available under an investigational new drug protocol through the CDC. Because treatment must be individualized according to the country of acquisition and the species, consultation with public-health officials, infectious-disease consultants, and the CDC is strongly recommended. Mucocutaneous leishmaniasis is less common, and there is no clear consensus on treatment; as such, consultation with the CDC and an infectious-diseases consultant is again recommended. Pentostam resistant *Leishmania*s have been detected and the compound has been shown to have low activity against CL infections and they have to be administered intravenously requiring

hospitalization. Quite often, chemotherapy treatment may lead to the tendency for patients to have a relapse (Gamboa-Leon *et al.*, 2006). More effective and less toxic therapeutic drugs are urgently needed, especially for cutaneous leishmaniasis.

2.5.2 Treatment of *Leishmania* with alternative medicine

Due to the aforementioned factors that influence the treatment of leishmaniases and considering the circumstance that the sufferers are forced to find themselves in, there is growing interest in alternative and complementary medicine. In recent years, there has been growing interest in alternative therapies comprising of natural products especially those derived from plants (Tonui, 2006; Dutta *et al.*, 2007. Studies in several countries including Brazil, Argentina, Bolivia, Mexico, Kenya, Sudan and Columbia have shown activity of plant extracts against Leishmania {Dutta, 2007 #132). Nephthoquinones such as plumbagin from *Plumbago Zeylanica* and biplumbagin isolated from stem and root extracts of *Pera benesis* (*Euphobiaceae*) have been used in folk medicine in Bolivia as treatment of CL caused by *L. braziliensis* when fresh stalks are applied directly on the lesion (Fournet *et al.*, 1992). Extracts of 11 plants used in Nigerian traditional medicine have been evaluated for possible antileishmanial activity using a radiorespirometric microtest technique based on *in vitro* inhibition of catabolism of 14 CO_2 from a battery of 14C-substrates by promastigotes, of the 13 methanol extracts tested, 5 from *Gongronema latifolia, Dorstenia multiradiata, picralima nitida, Cola altiensis* and *Desmodium gangeticum* have shown activity at concentrations of 50mg/ml or less against a visceral *Leishmania* isolate (Iwu *et al.*, 1992) .

Recent *in vitro* studies in Kenya using crude extracts have indicated that plant extracts

that include *Aloe kedogensis* (*Liliaceae*), *Albizia coriaria* (*Febaceae*), *Maytenus putterlickoides* (Celastracea) *Acasis melliflera* (leguminoseae) and *W ugandensis* have antileishmanial activities (Tonui, 2006). More recently it has been shown that hexane extracts of *W. ugandensis* have good antileshmanial activity (Ngure *et al.*, 2009).

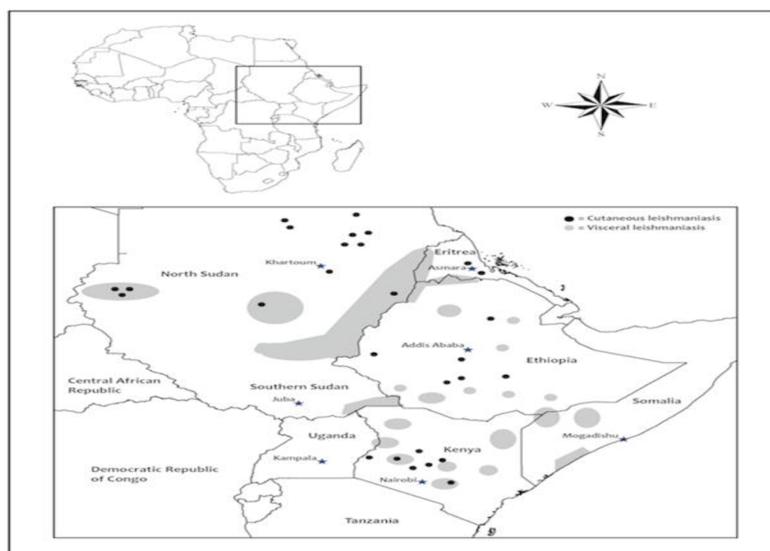
The extract of *Ricinus communis* has also been used and found to have antileishmanial effect (Iwu *et al.*, 1992; Okech, 2006). Studies have been carried out in experimental models but least in humans. Studies carried out, that have examined the activity of *A. indica* on trypanosomatids, including *L amezonensis* (Khalid *et al.*, 1998; Mbaya *et al.*, 2010). In all these studies, there is evidence of antileishmanial activity from the plant extracts.

2.6 Distribution of leishmaniases in East Africa

In Kenya, leishmaniasis affected areas include Mount Elgon (Mutinga *et al.*, 1990) Machakos (Wijers and Kiilu, 1984), kitui (Southgate and Oriedo, 1967), and West pokot (Mutinga *et al.*, 1990). Visceral leishmaniasis, caused by *L. donovani* and cutaneous leishmaniasis caused by *L. major, L. tropica* and *L. aethiopica* are endemic in Kenya (Lawyer *et al.*, 1991). Visceral leishmaniases in Kenya is thought to be anthroponotic (Perkins *et al.*, 1988). Zoonotic leishmaniasis due to *L. major* was reported by Heisch in Baringo district, Kenya in 1963 but the human cutaneous leishmaniasis due to *L. major* from the same region was not reported until 1987 (Muigai *et al.*, 1987). The vectors for *L. major* and *L. donovani* are *P. duboscqi* (Beach *et al.*, 1984), and *P.martini*, respectively. Other sand fly vectors of leishmaniasis in Kenya include *P. pedifer* Lewis, Mutinga & Ashford (Sang *et al.*, 1993) and *P. guggisbergi* (Lawyer *et al.*, 1991)

Cutaneous leishmaniasis due to *L. tropica* has been suggested to have a wider distribution

in Kenya than originally thought (Johnson *et al.*, 1993). Unlike the distribution of visceral leishmaniasis which is found in arid and semi-arid lowland regions of Kenya, cutaneous leishmaniasis is found in highland regions of Kenya at approximate rangers of 1,500m – 2,400m above sea level (Lawyer *et al.*, 1991). Figure 2.1 below shows where leishmaniasis is endemic in East Africa



Legend: the stars represent the major cities and the dots the CL and VL

Figure 2.2: Map of leishmaniases foci in Eastern Africa(Osman *et al.*, 2000)

2.7 *Azadirachta indica* and its medicinal uses

It is a fast growing, long leaved tree with unpleasant smelling wood and it grows to about 12m. It has ever green pinnate leaves and small yellow white flowers followed by green yellow berries as shown in Plate 2.2

Plate 2.2 *A. Indica* (Neem twigs) (source: Author, 2014)

Neem has two closely related species: *A. indica* A. Juss and *M. azedarac*, the former is popularly known as Indian neem (margosa tree) or Indian lilac, and the latter as the Persian lilac. Neem has been extensively used in India. The importance of the neem tree has been recognized by US National Academy of Sciences, which published a report in 1992 entitled 'Neem, a tree for solving global problems'.

Azadirachta indica (A. Juss), known as the Indian neem tree, is a species of Meliaceae, abundantly prevalent in tropical countries. Leaves of the Meliaceae species *A. indica* (neem) have been reported to exhibit immunomodulatory, anti-inflammatory, anti-hyperglycemic, anticarcinogenic, nematicidal, antiparasitic, antiviral, insecticidal and antioxidant properties (Khan *et al.*, 2001; Wandscheer *et al.*, 2004). A few studies have been carried out that have examined the activity of *A. Indica* on trypanosomatids, including *L. major* (Mbaya *et al.*, 2010). This information motivated this study to discover this plant's activity against *L. major*, an important causative agent of cutaneous leishmaniasis.

2.7.1 Bioactivity of *A. indica*

The active substances of *A. indica* appear to be terpenic in origin. This class includes an important constituent of neem, azadirachtin, a substance present in all parts of the plant, but in higher concentration in the seed (Forim *et al.*, 2010). Azadirachtin has induced resistance to the insect vector against re-infection by *T. cruzi* (Garcia *et al.*, 1991), and has shown activity against *Plasmodium falciparum* (El Tahir *et al.*, 1999), however, other terpenes may be responsible for the anti-leishmanial activity of *A. indica*. There are reports that *A. indica* is capable of activating the immune system to induce the production of interferon-gamma (IFN-γ) and tumour necrosis factor-alpha (TNF-α) (Mukherjee *et al.*, 1999), cytokines that act in synergy in the activation of macrophages to produce nitric oxide and control the infection by *Leishmania* (Bogdan *et al.*, 1996). Species *A. indica* has a number of biological and toxic effects, many of them associated with the presence of salanin, melzatriol, nimbin, cardiac glycosides, tannins, alkaloids and saponins {Mondal, 2002 #137, but there are no previous reports of cytotoxicity for macrophages.

2.8 *Ricinus communis* (Euphorbiaceae)

It belongs to family Euphorbiaceae. Castor plant (*R. communis*) from which castor beans and oil are subsequently derived grows naturally over a wide range of geographical regions and may be activating under a variety of physical and climatic regimes. Believed to be native to Africa; *R. communis* has been introduced and is cultivated in many tropical and subtropical areas of the world, frequently appearing spontaneously. See Plate 2.3.

2.8.1 Bioactivity of *R. communis*

The plant contents are said to have ricin which is poisonous, however this fraction is concentrated in the fruit than the leaves. The leaves are only mildly poisonous. Methanolic extracts of the leaves of *R. communis* have been used in antimicrobial testing against eight pathogenic bacteria in rats and showed antimicrobial properties. From past studies *R. communis* has been found to confer antimicrobial and the cytotoxic effects due to their essential oil which has synergistic effect in their compositions (Edris, 2007). The essential oils from it also do not enhance the "antibiotic resistance", a phenomenon caused by long-term use of synthetic antibiotics. *R. communis* is well known for its biological activities; most important of which are hepatoprotective, laxative, anti-diabetic, and anti-fertility activities. The following Plate 2.3 is the twig for *R. communis* plant

Plate 2.3.*R. communis* (Euphorbiaceae)(Source: Author, 2014)

CHAPTER THREE

MATERIALS AND METHODS

3.1 Study site

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

3.2 Experimental design

The *in vivo* and *in vitro* studies were carried out using a comparative study design. Efficacy and toxicity of samples were compared with those of pentostam and amphotericin B. Shneider's Drosophila culture Media was used as a negative control in *in vitro* experimental chemotherapeutic studies while PBS was used in *vivo* studies. *In vivo* studies were carried out using a complete randomized block design. Initially, BALB/c mice aged eight weeks were randomly divided into six groups of 8 each treated using the Independent and combined therapies of *A. indica, R. communis* extract, and pentostam, amphotericin B as positive controls. PBS-treated mice were the negative controls. Results were compared to determine the efficacy of the test samples against the known standard drugs for treating leishmaniasis.

3.3.1 Sources of plant extracts

Plant extracts were obtained from *A. indica* and *R. communis*. Leaves of *A. indica* and seeds of *R. communis* were collected from western region of Kenya, Vihiga County, and Sabatia subcounty. Plant extractions were done in KEMRI at the Centre for Traditional Medicine and Drugs Research (CTMDR) laboratory.

3.3.2 Plant collection and extract preparation

Voucher specimens were taken to the herbarium of the Museums of Kenya in Nairobi for authentication by taxonomists. Plant extracts were then taken to the CTMDR at the KEMRI laboratory Nairobi for methanolic extraction. They were then kept in the fridge at 20^0C at the CBRD laboratory where the study was done.

Plant extracts were processed according to the method of Kigondu (2009). Plant parts were chopped into small pieces; air dried at room temperature (25^0C) for 14 days and then ground into powder form using laboratory blender. 1kg of each powder was soaked in methanol for 3 days to extract compounds. The extract was filtered, dried with Na_2SO_4 and the solvent removed under vacuum in a rotary evaporator at 30-35°C. For aqueous extraction, 100 g of ground material in 600 ml of water was placed in a water bath and maintained at 60°C for 2 hours. This filtrate was freeze dried, weighed and stored at -20°C until required for use. DMSO was used in all the drug formulations because it has been reported to increase drug penetration (Idson, 1975).

3.4.1 Experimental animals (mice)

BALB/c mice aged 8 weeks used in the study were obtained from the KEMRI's animal house facility. Experiments were done in compliance with Animal Care and Use Committee (ACUC) guidelines of KEMRI. Standard Operating procedures (SOPs) observed at *Leishmania* laboratory at CBRD included infecting the animals using standard 21G needles, anaesthetisizing and killing them using painless method approved by ACUC(100µl of Sagatal).

3.4.2 *Leishmania* parasites

Leishmania major (strain IDUB/KE/83=NLB-144) which was originally isolated in 1983

from a female *P. duboscqi* collected near Marigat Baringo County Kenya were used (Beach *et al.*, 1984). These parasites were cultivated in Schneider's Insect Medium supplemented with 20% heat inactivated foetal bovine serum (FBS), 100µg/ml penicillin and 50µg/ml streptomycin (Hendricks and Wright, 1979) and 250µg/ml 5-fluorocytosine arabinioside (Kimber *et al.*, 1981). This strain has been maintained by cryopreservation *in vitro* culture and periodic passage in BALB/c mice at KEMRI Nairobi. Plates 3.1 and 3.2 show the amastigotes and promastigotes respectively.

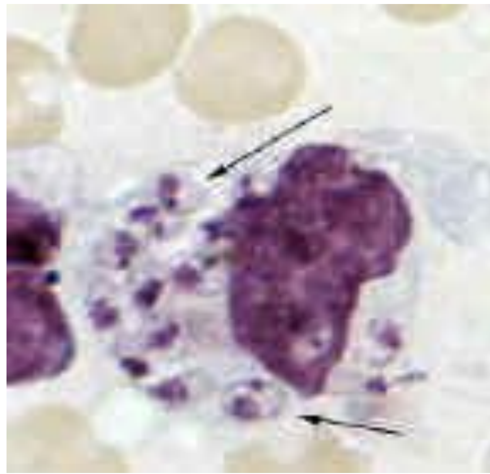


Plate 3.1:Amastigotes (Source: Author, 2014)

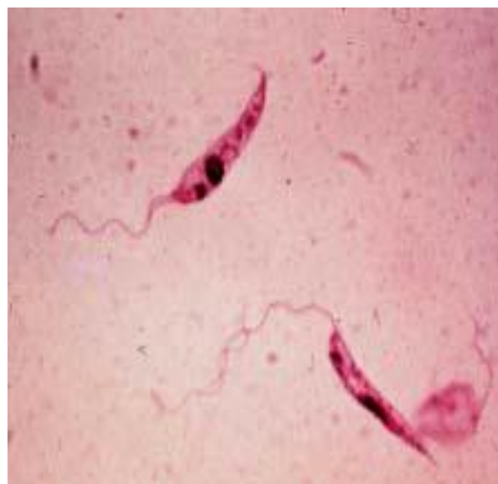


Plate 3.2: Promastigotes (Source: Author, 2014)

3.5 *In vitro* studies

3.5.1 Cytotoxicity studies

Vero cells were cultured and maintained in Minimum Essential Medium (MEM) supplemented with 10% FBS. The cells were cultured at 37^oC in 55% CO_2 for 24 hours, harvested by trypsinization, pooled in a 50ml vial and 100µl cell suspension (1x 10^6cells/ml) put into 2 wells of rows A-H in a 96-well micro titer plate, the medium aspirated off and 150µl of the highest concentration (1000µg/ml) of the *A .indica* and *R. communis* added into the same row and serial dilution carried out. The experimental plates were incubated further at 37^oC for 48 hours. The controls used were cells with no extract and medium alone. MTT reagent (10µl) was added into each well and the cells incubated for 4 hours until a purple precipitate clearly visible under a microscope was formed. The medium together with MTT were aspirated off from the wells. Dimethylsulfoxide (DMSO) (100µl) was added and the plates shaken for 5 minutes. The absorbance was measured for each well at 562nm using a micro-titre plate reader (Wang *et al.*, 2006). Cell viability (%) at each concentration was calculated as described by (Wang *et al.*, 2006) using the formula:

Cell viability for vero-E6 cells (%) =

$$\frac{\text{Average absorbance in duplicate drug wells - average blank wells x100}}{\text{Average absorbance control wells}}$$

3.5.2 MIC (Minimum Inhibitory Concentration) Evaluation

L. major promastigotes (10^6 parasites/ml) was incubated at 26^0C for 120hr in fresh media (brain heart infusion medium), Supplemented with 10% FBS in the absence or presence of several concentrations (10µg/mL to 100µg/mL) of the extracts (cell growth was

determined daily by assessment of visible turbidity). The MIC was considered as the lowest concentration of each substance used that inhibited more than 99% of *L. major* growth *in vitro.*

3.5.3 Determination of 50% inhibitory concentration

Parasite viability was determined using MTT assays (Tada *et al.*, 1986), see Appendix 2. The anti-leishmanial activity against intracellular amastigote was determined with infected macrophage (Tempone *et al.*, 2004) using pentostam as standard drug. Parasite burden was defined as number of infected macrophages in total of 400 cells. Each assay was performed in triplicate, 50% inhibitory concentration for suppression of parasite infection in macrophage and calculated using sigmoid regression analysis.

3.5.4 Anti-promastigote assay

L. major promastigotes were incubated in 24-well plates in the presence of different concentrations of the extracts. After 5 days of cultivation, aliquots of parasites were transferred to a 96-well micro-titre plate. The parasites were then incubated at 27°C in 5% CO_2 for 24 hours; 200µl of highest concentration of extract was added and diluted. The experimental plates were incubated further at 27°C for 48 hours. The controls used were promastigotes with no extracts and RPMI medium alone. The media together with MTT (3-4, 5-dimethgylthiaol-2-yl-2, 5-diphenyltetrazolium bromide) was aspirated off the wells. In each well, 100µl of DMSO was added and the plates shaken for 5 minutes. Absorbance was measured for each well at 562nm using a micro titre reader (Mosmann, 1983) and the 50% inhibitory concentration (IC_{50}) values generated. Percentage promastigotes viability was calculated as follows using the formula by (Mosmann, 1983)

3.5.5 Anti-amastigote assay

Anti-amastigote assay was carried out as described by (Delorenzi *et al.*, 2001). Briefly, peritoneal macrophages were obtained from BALB/c mice. Mice were anaesthetized using 100μl pentobarbital sodium (sagatal). The body surface was then disinfected with 70% ethanol. The torso skin was torn dorsoventrally to expose the peritoneum. Using a sterile syringe and needle, 10ml of sterile cold phosphate-buffered saline (PBS) was injected into the peritoneum. After shaking the mouse, peritoneal macrophages were harvested by withdrawing the PBS. The contents were transferred into a sterile 50 ml centrifuge tube. The suspension was centrifugally washed at 2000 rpm for 10 minutes and the pellet re-suspended in complete RPMI 1640 medium.

Macrophages were adsorbed in 24-well plates and allowed to adhere for 4 hours at 37°C in 5% CO_2 non-adherent cells were washed with cold PBS and incubated overnight in RPMI. Adherent macrophages then infected with parasite/macrophage ratio of 6:1 and further incubated at 37°C in 5% CO_2 for 4 hours. Free macrophages were removed by extensive washing with PBS and the cultures incubated in RPMI for 24hours.Treatment of infected macrophages with the samples was done once. Pentostam and amphotericin B were used as a positive control drugs for comparison of parasite inhibition. The medium and drug were replenished daily for 3 days. After 5days, the monolayers were washed with PBS at 37°C, fixed in methanol and stained. With Giemsa, The number of amastigotes was determined by counting at least 100 macrophages in duplicate cultures, and the results used together with those of the weight to calculate the LDU values.

3.5.6 Nitric oxide production assay

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

3.6 *In vivo* studies

3.6.1 Parasite inoculation and treatment

In the whole study BALB/c mice were used. They are highly susceptible to *L. major* parasites. Inoculation of mice with parasites was done intradermally. Inoculation with *L. major* was done on the left hind footpad (LHFP) and the right hind footpad (RHFP) served as an uninfected control (see Appendix 6). Lesion measurement was done using a direct reading vernier caliper using the method of (Nolan and Farrell, 1987)

The mice were divided as follows: set of 48 mice were inoculated intradermally with 1 million of *L. major* promastigotes on the LHFP and left for 4 weeks, then divided into 6, group 1 treated with *A. indica* extracts, group 2 with *R. communis,* group 3 with combination of *A. indica* and *R. communis,* group 4 with sterile PBS group 5 pentostam and group 6 amphotericin B as shown in the Table 3.1. Treatment commenced on the

week 5 till week 10. Lesion measurements were done using a direct reading vernier caliper using the method of (Nolan and Farrell, 1987). The infected (LHFP) and uninfected footpad (RHFP) (see Appendix 6, 8) were measured and data tabulated to establish lesion development. Lesion size calculation was done as follows:

Lesion size = (size of infected footpad - size of uninfected control footpad) mm

On termination of the experiment, sample mice were picked from each group, their spleens were removed (see Appendix 7). Impression smears were made from the spleens and the smears stained with Giemsa and examined microscopically. Parasite count was recorded using the standard World Health Organization method (WHO, 2002). The parasite load determined using the Leishman Donovan Units (LDU) method of Bradley and Kirkley (1977) as follows:

$$\text{LDU} = \frac{\text{amastigotes in nucleated cells x weight of organ (grams) x } 2\text{x}10^{5}}{1000}$$

3.6.2 Disposal of animals

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

3.7 Data Analysis

The data collected on lesion sizes, parasite loads and absorbance were analyzed using the SPSS software. All experiments were performed in triplicate, the mean standard deviation of at least three experiments were determined, statistical analysis of the differences between mean values obtained for the experimental groups was done by the students t-test. P. values of 0.05 or less ($p \leq 0.05$) were considered to be significant.

CHAPTER FOUR

RESULTS

4.1 Effect of test mono and combination therapy of *A. Indica* and *R. communis* on *L. major* lesion development in BALB/c mice

There was a uniform development of lesion sizes in BALB/c mice during the first five weeks post-infection with *L. major*. Differences in lesion sizes between weeks 5 to week 10 were subjected to repeated measure ANOVA, which indicated that there was significant difference in lesion sizes among different treatments ($p<0.05$). The lesion sizes of the untreated controls of BALB/c mice increased steadily after infection without decrease in lesion sizes until 5 weeks post-infection. Among the extracts used, the largest reduction in lesion size after 5 weeks post-infection occurred in BALB/c treated with pentostam which was significantly ($P < 0.05$) similar to lesion size of mice treated with amphotericin B. However, in BALB/c mice, treated with *A. indica* in combination with *R. communis* resulted in significantly ($P < 0.05$) larger reduction of lesion than BALB/c mice treated with monotherapies of *A. indica* or *R. communis*. Treatment of BALB/c mice with *R. communis* resulted in the least reduction in lesion sizes among all the tested drugs after 4 weeks of treatment. There was no significant difference between the combined therapy and pentostam (P>.0.05); however the monotherapies showed lower activity than standard drugs. The lesion sizes of BALB/c mice at the start of infection, and at the end of the experiment with test compounds and controls are shown in table 4.1. There was no significant difference in the lesion sizes at the start of the experiment ($P=0.0001$). However, there were significant differences in the lesion sizes post-infection ($P=0.003$), 5 weeks post-treatment. Smallest lesion sizes occurred in BALB/c mice

treated with combination therapy and amphotericin B, which was slightly lower than BALB/c mice, treated pentostam, BALB/c mice treated with monotherapies of *A. indica* or *R. communis* had larger lesion sizes. The overall mean lesion sizes of *L. major* infected BALB/c mice exposed to various treatments are shown in Table 4.1.

Table 4.1: The overall mean lesion sizes of BALB/c mice treated with test compounds

			Mean lesion size	
Treatment agent	Duration (days)	Number of mice	Pre-treatment	Post treatment
Az	28	8	1.13 ± 0.17	0.38 ± 0.07^{b}
Az + Rc	28	8	1.05 ± 0.09	0.23 ± 0.09^{a}
Rc	28	8	1.15 ± 0.10	0.47 ± 0.20^{d}
Amphotericin B	28	8	1.12 ± 0.05	0.21 ± 0.04^{a}
Pentostam	28	8	1.09 ± 0.05	0.22 ± 0.09^{a}
PBS	28	8	1.19 ± 0.12	3.14 ± 0.07^{c}
	ANOVA	F	12.525	29.312
		Df	5	5
		P	0.0001	0.0003

Legend: Means in the same column followed by the same superscript show no significant difference between them

Az: *A. indica*

Rc: *R. communis*

Az+Rc: *A. indica* and *R. communis* (combination therapy)

PBS: Phosphate Buffered Saline (negative control)

Pentostam: standard drug (positive control)

Amphotericin B: standard drug (positive control)

4.2 Efficacy of mono and combination therapies on promastigotes and amastigote of *L. major*

The amastigote growth was significantly affected by the various test compounds (P=0.002) after 24hr. of exposure. There were significant differences in the optimal efficacy of the test drugs ($P < 0.05$). The optimal efficacy of the standard drugs was 72%, 59% and 88% *A. indica*, *R. communis* and combination of *A. indica* and *R.communis* respectively, compared to standard drugs that had 92% and 98% for pentostam and amphotericin B respectively, combined therapy of *A. indica* + *R. communis* was found to be the most effective of the test drugs against amastigote followed by *A. indica* while *R. communis* was the least effective. None of the known standard test compound achieved IC_{90} except the combined therapy of *A. indica* + *R. communis* (35µg/mL) compared to the test drugs which had 34µg/ml, 25.5µg/ml, for pentostam and ampotericin B respectively. There was significant (P=0.003) 0.05) difference in the IC_{50} with the lowest IC_{50} occurring in *A. indica* + *R. communis*, followed by *A. indica* and least in *R. communis* among the known standard test drugs.Table 4.3 describes the optimal efficacy, concentration at optimal efficacy, IC_{90}, IC_{50} of the test drugs against amastigote forms of the parasites.

Table 4.3: Optimal efficacy, IC90 and IC50 of test compounds against amastigote form of the parasites for 24 hr period

	Test drugs					Parameter and statistics	
Concentration (µg/mL)	Az	Rc	Az + Rc	Pento.	AMB	F-value	P-value
Optimal efficacy (%)	72	59.5	88	98	92	17.311	0.002
Concentration at optimal efficacy(µg/ml)	25.5	28.2	35.1	25,	34.5	9.212	0.001
IC_{90}	-	-	34.5	15.5	24.5	19.221	0.001
IC_{50}	11.5	16.5	9.0	6.5	4.5	12.489	0.000

Legend

Az: *A.indica*

Rc: *R. communis*

Az+Rc: Combination of *A. indica* and *R. communis*

Pento: pentostam

AMB: amphotericin B

There were significant differences in the optimal efficacy of the test drugs. The optimal efficacy of the standard drugs was 96.5% and 98% for amphotericin B and pentostam respectively. The test drugs did not achieve this efficacy levels against promastigotes. Among the test drugs, combined therapy of *A.indica* and *R. communis* was the most effective against promastigote followed by *A. indica* while *R. communis* was the least effective. None of the test compound achieved IC_{90} except the combined therapy of *A. indica* + *R. communis* (42µg/mL). There was significant difference in the IC_{50} with the lowest IC_{50} occurring in *A. indica* + *R. communis*, followed by *A. indica* and least in *R. communis* among the known standard test drugs. Table 4.5 describes the optimal efficacy, concentration at optimal efficacy, IC_{90}, IC_{50} of the test drugs against promastigote forms of theparasite.

Table 4.5: Optimal efficacy, IC_{90} and IC_{50} of test compounds against promastigote form of the parasites for 24 hr period

	Test drugs			**Controls**		**Parameter and statistics**	
Concentration (µg/mL)	Az	Rc	Az+ Rc	Pent	AMB	F-value	P-value
Optimal efficacy (%)	78	61.5	91.2	96.5	98	26.654	0.002
Concentration at optimal efficacy(µg/mL)	43.5	69.5	55.2	30.4	40.2	9.257	0.012
IC_{90}	-	-	42	16.2	25.1	15.226	0.003
IC_{50}	10.1	25.5	12.2	4.1	5.0	15.456	0.000

4.3 Parasite loads in spleen of BALB/c mice treated with mono and combination therapies.

There were significant differences in the weight of spleen, spleno-somatic index and number of parasites among treatments ($P \leq 0.05$). In *L. major* infected BALB/c mice, the spleen and spleno-somatic index was found to be significantly high when treatment was done using *R. communis* followed by *A. indica.* There was no significant difference between that of combination therapy of *A. indica* + *R. communis* and pentostam. The index was highest in the untreated controls. BALB/c mice treated with pentostam and those treated with amphotericin B, the differences in spleen weight were not significant ($P \geq 0.05$). The number of parasites was also high in untreated controls, followed by those treated with *R. communis* and *A. indica* while combination treatment with *A. indica* + *R. communis* was lower. Nevertheless, treatment using amphotericin B and pentostam resulted in the lowest numbers of parasites. Body weights, weight of spleen, spleen-somatic index and number of parasites in BALB/c infected with *L. major* under various treatments is shown in Table 4.6.

Table 4.6: Body weight, weight of spleen, spleno-somatic index and number of parasites in BALB/c mice following various treatments

Treatment	Body weight(g)	Weight of spleen(g)	Spleno-somatic index	No of parasites
Az	22.11 ± 0.54	0.18 ± 0.021[c]	0.84 ± 0.10[c]	37.3 ± 6.4[c]
Rc	21.78 ± 0.89	0.23 ± 0.021[b]	1.04 ± 0.08[b]	58.4 ± 9.8[b]
Az + Rc	20.50 ± 0.45	0.15 ± 0.006[a]	0.73 ± 0.02[a]	26.4 ± 0.7[a]
Amphotericin B	21.00 ± 1.00	0.13 ± 0.005[a]	0.74 ± 0.06[a]	25.7 ± 0.5[a]
Pentostam	21.00 ± 0.58	0.14 ± 0.010[a]	0.75 ± 0.07[a]	26.1 ± 0.4[a]
PBS	21.00 ± 1.73	0.37 ± 0.014[d]	1.83 ± 0.21[d]	145.7 ± 6.7[d]
ANOVA				
F	2.1332	35.255	71.214	46.987
Df	5	5	5	5
P	0.3245	0.0001	0.0000	0.0000

Legend

Means followed by the same letter superscript in the same column are not significantly different

Az: *A. indica*

Rc: *R. communis*

Az+Rc: *A. indica* and *R. communis* (combination therapy)

PBS: Phosphate Buffered Saline (negative control)

Pentostam: standard drug (positive control)

Amphotericin B: standard drug (positive control)

There were significant differences in the LDU of *L. major* parasites in BALB/c mice treated with monotherapies of *A. indica*, *R. communis* and a combination of *A. indica* + *R. communis* (ANOVA; (P ≤0.05). In *L. major* infected BALB/c mice, treatment with pentostam, amphotericin B and combination therapy resulted in the lowest LDU. The LDU decreased in order of: *R. communis*>*A. indica*>*A. indica* + *R. communis*. There were no significant differences between LDU of Balb/c mice treated with pentostam and that treated with combination therapy (p<0.05) The LDU of *L. major* parasite in BALB/c mice infected with *L. major* receiving various treatments is shown in Figure 4.4

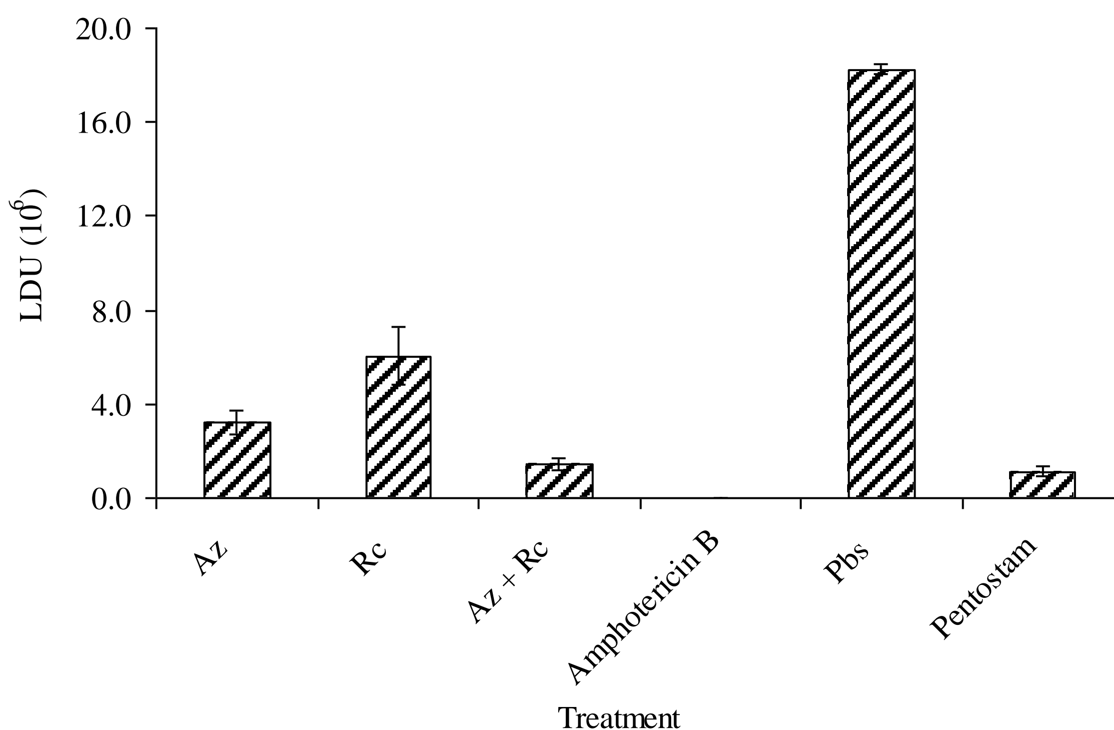


Figure 4.2: The LDU of *L. major* parasite in spleen of BALB/c mice infected with *L. major* receiving various treatments

4.4 Nitric oxide production in *L. major*-infected macrophages treated with the plant extracts.

The production of Nitric Oxide decreased in the order *R. communis*> *A. indica* + *R.*

communis>*A. indica*, hence treatment with combination therapy leads to more production of NO by the macrophages than that from standard drugs and monotherapy of *A. indica*. Nitric Oxide produced by macrophages treated with pentostam and Amphotericin B were the lowest during the experiment. Infected macrophages treated with RPMI produced high amounts of Nitric Oxide. The nitric oxide production in macrophages of BALB/c mice infected with *L. major* amastigotes and subjected to various drugs is shown in Figure 4.5.

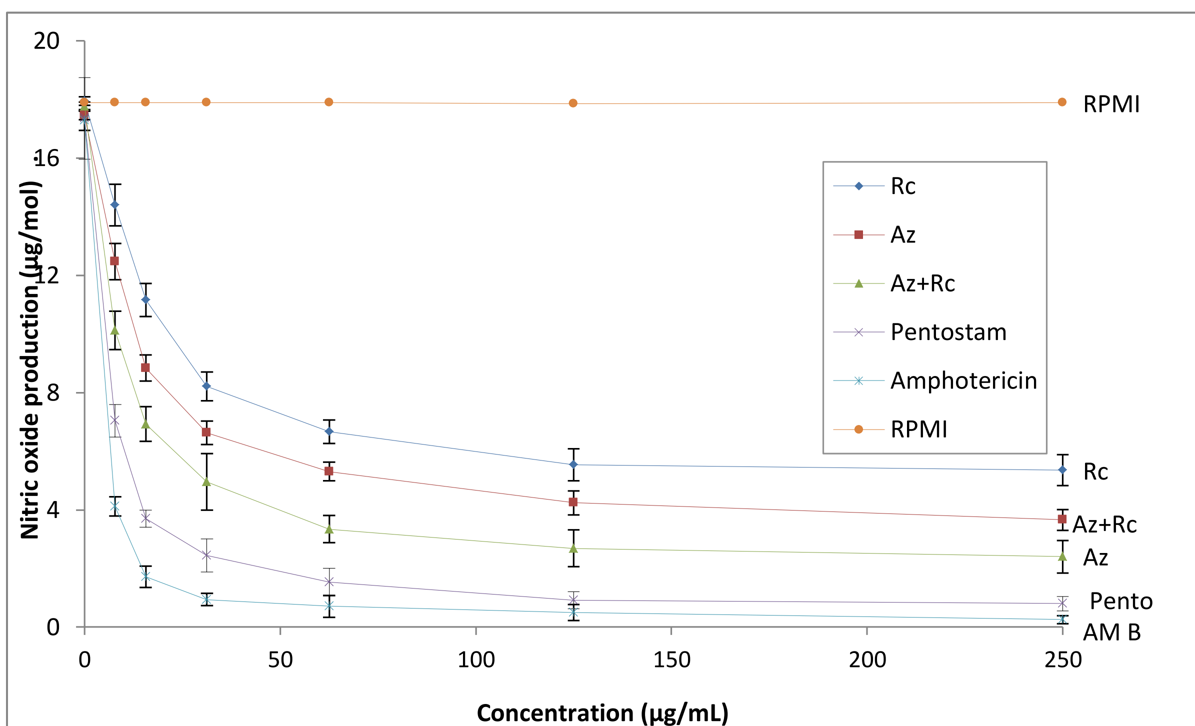


Figure 4.3: Nitric oxide production in the macrophages of BALB/c infected mice infected with *L. major* and subjected to different treatments by various test compounds

4.5 Cytotoxicity assay of plant extracts on Vero-E6 cells

Vero-E6 cells were significantly affected by treatment using the test drugs of *A. indica*,

R. communis and *A. indica* + *R. communis* (P ≤ 0.05). The concentration of the test drug required to destroy 50% of the mammalian cell was significantly low in *R. communis* (92 µg/ml) followed by *A. indica* + *R. communis* (101 µg/ml) and highest in treatment using *A. indica* (149 µg/ml). Results indicating the cell viability of the Vero-E6 cells subjected to the test drugs are shown in Figure 4.6.

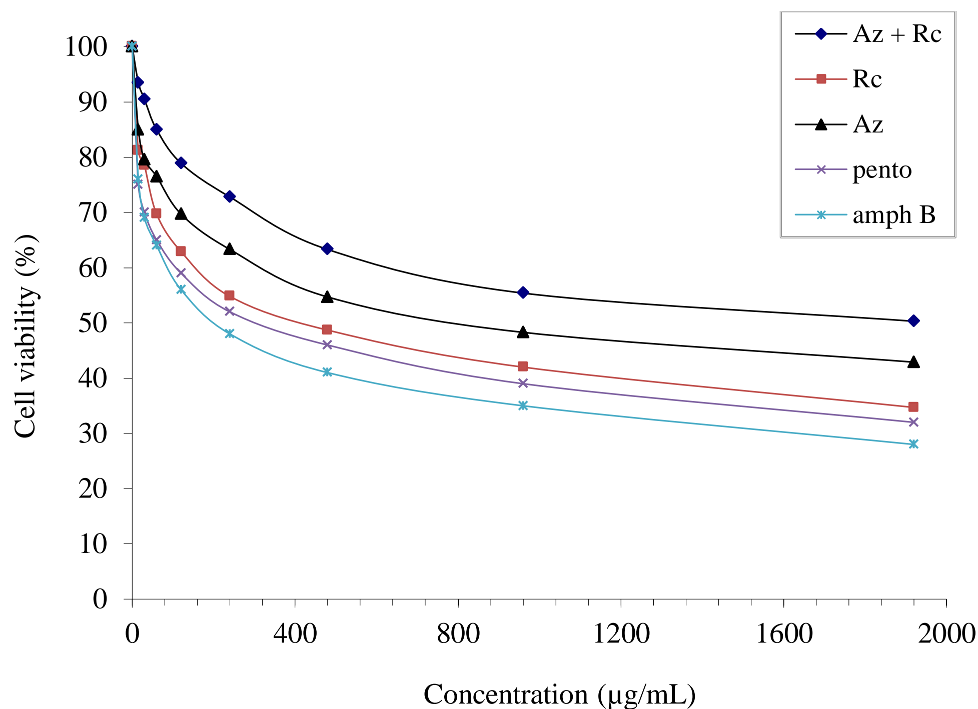


Figure 4.4: cell viability of the Vero-E6 cells subjected to the test compounds

CHAPTER FIVE

DISCUSSION, CONCLUSION AND RECOMMENDATIONS

5.1 Discussion

This study set out to determine the antileishmanial activities of *A. indica* and *R. communis* used in combination and independently against *L. major* infection in susceptible Balb/c mice. The study further investigated whether there is any difference in the toxicity level of the combination therapy and the standard drugs. Additionally, the study set out to determine whether nitric oxide production in the amastigotes is induced by the plant extracts. The combination therapy of *A. indica* and *R. communis* was very efficient in decreasing parasite loads. This antileishmanial activity was not significantly different from that of pentostam. Though the monotherapies were active against the *L. major* infection, the combination therapy surpassed their efficacy showing that there might have been a way in which combining them improved the performance of the extracts.The level of toxicity of the combination theapy was also lower than the standard drugs indicating that further tests of the extracts may lead to novel treatment of *L. major* infection.

One of the major evasion strategies of leishmania parasite is rapid transition of the parasite from the intracellular fluid to the macrophages (Wenzel *et al.,* 2012). A drug that could target the amastigotes would hence offer relief to the search for treatment of this infection. The present study shows that combination therapy caused marked reduction of the parasite load in the mice during the treatment period.However, this therapy was better on the promastigotes than the amastigotes. Most previous studies have been done on

monotherapies (Okech, 2006; Kigondu *et al.*, 2009; Wabwoba and Kigondu, 2010) The monotherapy treatments are in tandem with earlier studies (Okech, 2006; Sabrina, 2012; Zarai *et al.*, 2012) that show potential antimicrobial effect of *R. communis* and *A. indica* with evidence of *in vivo* and *in vitro* activities of the plant extracts. However these studies have not been as efficacious as the combination therapy employed in this study. Furthermore, other studies have also shown that combination thearapy is a more effective option to the monotherapy (Khayeka-Wandabwa *et al.*, 2013; Makwali *et al.*, 2012). These results suggest the extracts were successful in inhibiting *L. major* parasite growth as monotherapies.The improvement observed during treatment with combination therapy may be attributed to the synergism between the two plant extracts. This may be indicative of the existence of the different modes of action by the two, *R. communis* inducing production of nitric oxide by the macrophages while *A. indica* failing to induce as much. In some study, isolation of the active ingredients from the plant extracts has been know to improve potency(Carneiro *et al.*, 2012). However, the use of crude extracts may have increased the efficacy of the extracts. Maybe further studies on the same would offer more insights.

Nitric oxide has been found to be upregulated in macrophages during infection with *L. major* infection (Marina, 2012). This is one of the ways of parasite killing by the macrophages. Macrophages, the target cells in therapy of leishmaniasis play an important role in the immunological control of intracellular parasites through the production of cytokines and oxygen metabolites (Balaraman *et al.*, 2004). This is one of the main mechanisms in the up-regulation of nitric oxide inside the macrophages, which is an effective mediator in killing amastigotes (Manuel, 1997). Previous studies have reported

that *A. indica* is capable of activating the immune system to induce production of interferon-gamma (IFN-γ) and tumor necrosis factor-alpha (TNF-α) (Mukherjee *et al.*, 1999), cytokines that act in synergy in the activation of macrophages to produce nitric oxide and control the infection by *Leishmania* (Bogdan *et al.*, 1996). *A. indica* is cytotoxic to promastigotes than amastigotes and causes production of very low levels of NO from macrophages than that produced by *R. communis*. This indicates its low induction activity on peritoneal macrophages for production of NO, than that observed in *R. communis* treatment. The combination therapy has a higher induction potential suggesting that the mode of action against the amastigotes may be through NO cytolytic activity. In this study combination therapy caused a slight increase in the NO produced by the macrophages, a slight improvement from that produced by the monotherapies which suggests that it may be one of the modes of action of the extracts but not the only mechanism involved in parasite elimination. However in this study *R.communis* caused higher induction of NO than *A. indica.* This may imply that, induction of NO may not be the only way in which the extract kills parasite in the mcrophages (Sabrina, 2012). The combined therapy however, has a synergy of action leading to its activity, which causes immunomodulatory action.

The effect of the drugs on the VeroE6 cells *in vitro* shows that the toxicity of the two extracts is in the manageable range, this also improves in combination. This was also observed in the *in vivo* experiments for none of the mice died during the experimental period and there was no marked weight reduction or ill health among the treated mice. This may be due to the fact that for *R. communis*, the leaves are less toxic, have lesser ricin , than the fruits which has been documented to be very toxic (Okech, 2006;

Manpreet, 2012; Sabrina, 2012). *Azadirachta indica* too was only cytotoxic to the parasites but less toxic to the veroE6 cells and the macrophages. The species *A. indica* has a number of biological and toxic effects, many of them associated with the presence of salanin, melzatriol, nimbin, cardiac glycosides, tannins, alkaloids and saponins (Biswas, 2002), but there are no previous reports of cytotoxicity for macrophages. The extracts studied showed excellent antileishmanial activity that was unrelated to toxicity, which guarantees safety to the macrophages and specificity to the parasite which is consistent with studies by (Nwaka and Hudson, 2006). Combining the two drugs does not seem to increase toxicity to veroE6 cells and peritoneal macrophages but improves their potency against the amastigotes and promastigotes. This gives a good base for further investigation to assess whether these extracts could become valid candidates for drug development

5.2 Conclusion

This study showed that combination therapy has better antileishmanial activity than the monotherapies of *R. communis* and *A. indica*. Combination therapy is less toxic than the standard drugs used in treatment of *L. major* infection. The level of cytotoxicity of the combination therapy on the host cells was comparatively lower than that of pentostam and amphotericin B. This further compounds the merits of this chemotherapy when compared with the standard drugs. It is also notable that inducton of NO production by the macrophages is one of the mechanisms by which the plant extracts carry out parasite elimination intracellularly.

5.3 Recommendations.

Further followup *in vivo* studies using a primate model, like the vervet monkeys (*Cercopithecus aethiops*)is recommended to find out whether the results from such studies will give comparable results to the ones obtained in this study.

It would be important to do further studies on a more purified extract of the two study plants to investigate whether the efficacy of the combination therapy could improve.

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APPENDICES

APPENDIX I :Determination of nitric oxide concentration in supernatants

Requirements:

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

Preparation of a Nitrite Refernce curve

I. Prepare 1µmL of a 100µM solution by diluting the provided 0.1M Nitrite standard 1:1000 in the RPMI 1640

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

III. Add 100L of the 100µM nitrite solution to the remaining 3 wells in row A.

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

Nitric oxide measurement (Greis reaction)

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

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

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

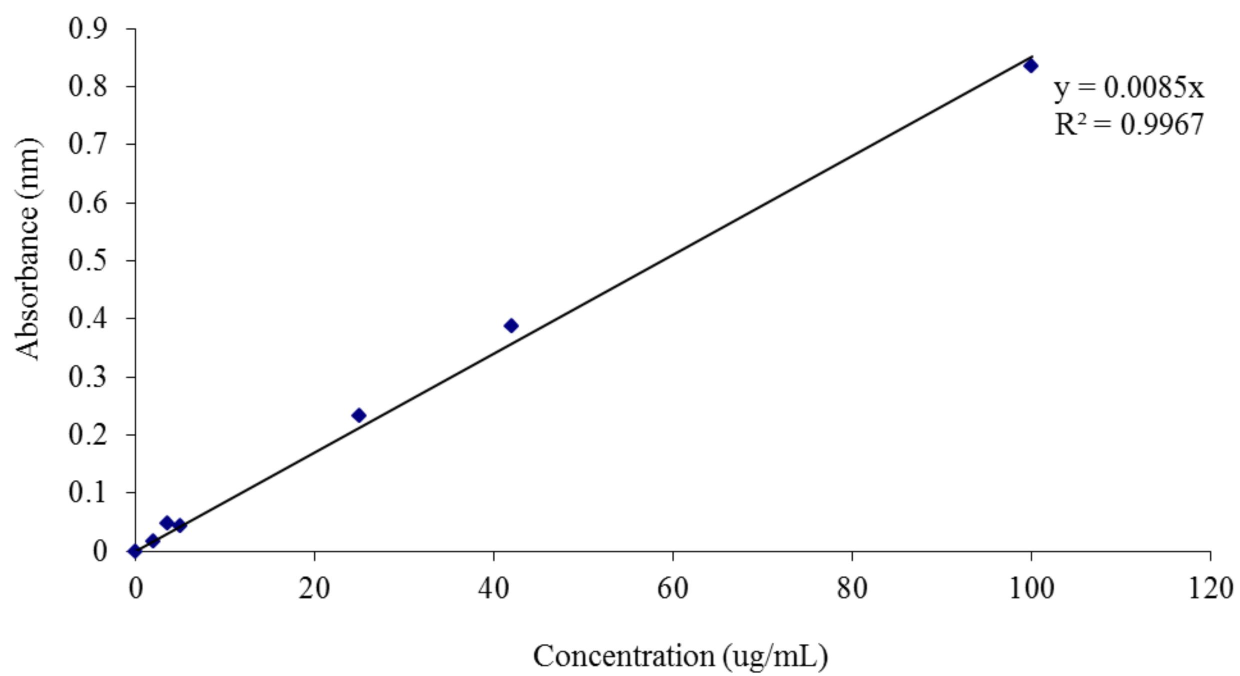
iii. Incubate 5-10 minutes at room temperature, protected from light

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

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

vi. Measure absorbance within 30minutes in a plate reader with a filter between 520-550nm(Holzmuller *et al.,* 2002)

NO standard curve used to determine NO production by macrophage treated with fractions



0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
Absorbance (nm)
y = 0.0085x
R² = 0.9967
0
20
40
60
80
100
120
Concentration (ug/mL)

APPENDIX II: *In vitro* MTT cytotoxicity assay

Requirements

- Standard cell lines: VeroE6 cells
- Standard drug: pentostam and amphotericin B
- Standard conditions: Minimum Essential Medium (MEM), !0% foetal Bovine
- Serum (FBS), 96-well mlicrotitre plate, $37^{o}C/5CO_2$ incubator

Drug preparation

5mg of plant is dissolved in 1ml of DMSO and topped with double distilled water to give stock solutions of 100mg/ml

MTT solution

Prepare 5mg/ml of MTT in PBS

Procedure

i. Add 100µL of cell suspension ($1x10^5$) in wells of rows B-H and row A wells
ii. Add media without cells 9-12 of row A. leave overnight to attach
iii. The next day remove media from row H
iv. Add 15µ0L of the drug concentration in duplicate. Carry out serial dilutions using a multichannel pipette by removing 50L from wells of row H and adding to row

G. after mixing, another 50µL is transferred to wells of row F and mixed well; this is continued up to row B discarding the last 50µL of this row F(a three fold dilution is achieved)

v. Incubate at 37° C 5%CO_2 for 48 hours to allow the drug to take effect

Evaluation

i. After 48hours, check the growth of the cells in the plate under an inverted microscope to ensure normal growth

ii. Add 10µL of MTT reagent to each well including controls

iii. Return cells to incubator for 2-4 hours. Periodically view the cells under an inverted microscope, dump off the media from the cells

iv. Add 200µL of DMSO; shake for five minutes to mix.

v. Measure the absorbance in each well at 562 in a microtitre plate reader (Absorbance can be read with any filter in the wavelength range of 550-600nm. The reference wavelength should be greater than 650nm.The blanks should give value close to zero (± o.1)

vi. Determine the average value for the duplicate and subtract the average value for the blank

vii. Determine the % cell viability as follows:

% cell viability = Absorbance in treated wells- absorbance of blanks x 100

Absorbance of control wells-absorbance of blanks

(Source: http://www.bruschwig-ch.com)

Phosphate buffered saline (PBs) 0.15pH 7.2 preparation)

Sodium chloride (NaCl)……………………………………8.0g/L

Potassium chloride (KCl)…………………………………..0.2g/L

Di-Sodium hydrogen phosphate (Na_2HPO_4) (0.008)……...1.15g/L

Potassium dihydrogen phosphate (KH_2PO_4………………0.2g/L

Procedure

Dissolve in 1000mL of distilled waster. It is convenient to make a x10 solution for storage and dilute as required

APPENDIX III: Species of *Leishmania* and their geographical distribution

Subgenus	complex	Species	Main geographic locations	Main clinical manifestations
Old World				
	L. donovani	*L. donovani*	India, sub-Saharan Africa, China, Pakistan, Sudan Uganda	Visceral leishmaniasis
		L. infantum	Mediterranean, Middle East, North and Sub-Saharan Africa, Balkans & China	Visceral leishmaniasis
	L. major	*L. major*	Middle East, Africa, china	Cutaneous leishmaniasis ('wet ulcer')
	L. tropica	*L. tropica*	Middle East, India ,Southern Europe,	Cutaneous leishmaniasis ('Dry ulcer')
	L. aethiopica	*L. aethiopica*	Ethiopia, Kenya, Yemen	Cutaneous leishmaniasis

Leishmania				
New world				
Leishmania	*L. donovani*	*L. chagasi*	Latin America, India and Central Asia	Visceral leishmaniasis (‘post-kala-Azar’)
	L. mexicana	*L.venezuelensis*	Venezuela	Cutaneous Leishmaniasis
		L. Mexicana	Mexico, Central America, Texas, Oklahoma	Cutaneous Leishmaniasis
		L. amezonensis	Amazon basin, Brazil	Cutaneous Leishmaniasis
Viania	*L. brazilenzis*	*L. brazilensis*	Latin America	Muco-cutaneous leishmaniasis
		L. peruviana	Peru and Argentina(highlands)	Cutaneous leishmaniasis
	L. guyanensis	*L. guyanensis*	North Amazon basin, Guyanas	Coetaneous leishmaniasis

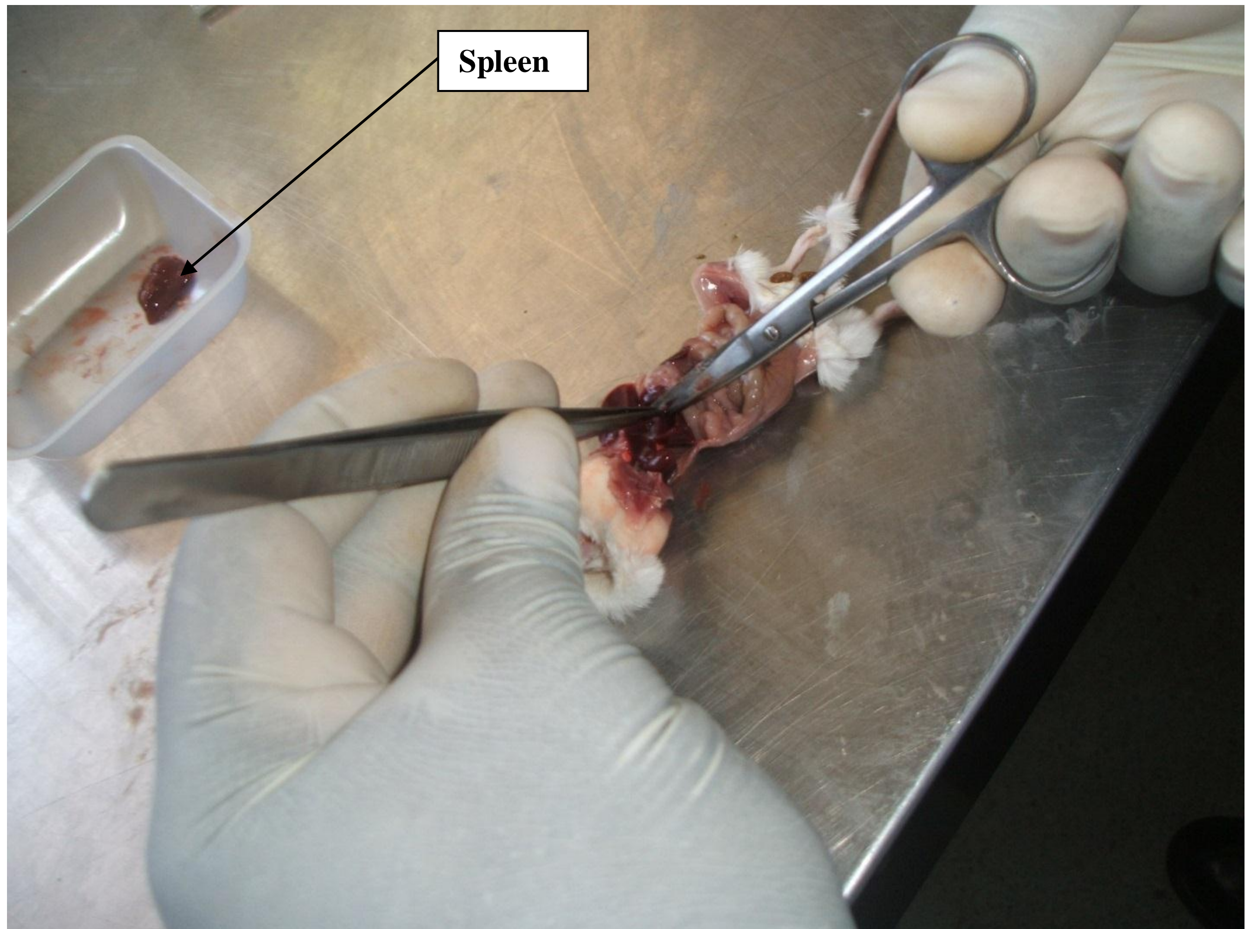
		L. panamensis	Panama, Costa Rica, Columbia	Cutaneous leishmaniasis
(Source: *www.utdol.com*)				

APPENDIX IV: A lesion on the LHFP of a Balb/c mouse post-infection

The arrow shows the lesion on the untreated LHFP post infection

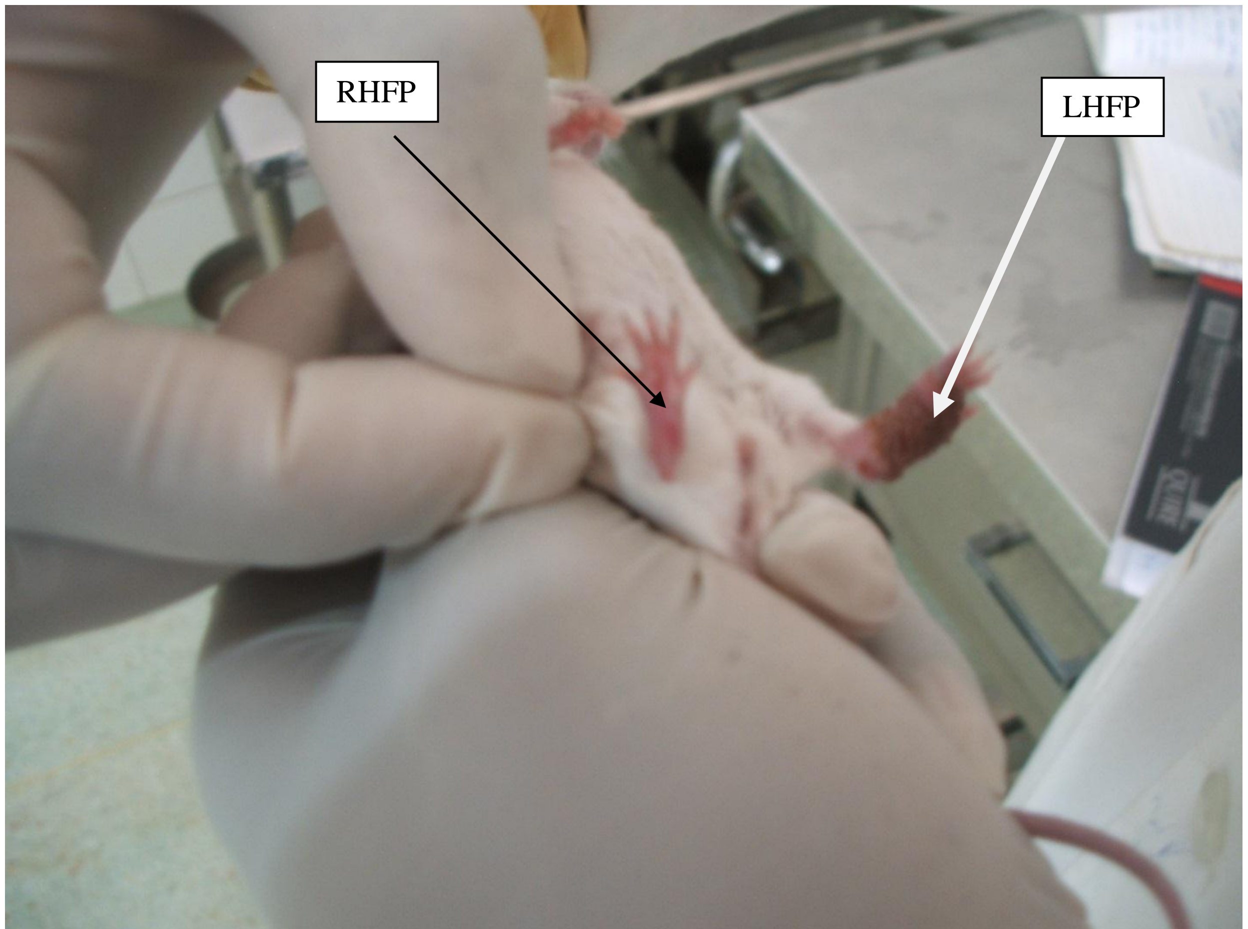
(Source: Author, 2014)

APPENDIX VI: Removal of the spleen after for parasite load determination.



(Source: Author,2014)

APPENDIX VII: The LHFP and RHFP post- infection



The two footpads showing the infected one with a lesion (LHFP) and the uninfected footpad (RHFP) (Source: Author, 2014)