ANTI-LEISHMANIAL ACTIVITY OF *ACACIA MELLIFERA* (LEGUMINOSAE: MIMOSOIDEAE) AGAINST *LEISHMANIA MAJOR.*

MBURU, FAITH WAMBUI (BSc).

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DECLARATION

Candidate

I, Faith Wambui Mburu, do hereby acknowledge that this is my original work and has not been submitted for a degree in any other university.

Signature: _____. Date: 12th March 2007.

Supervisors

This thesis has been presented with our approval as University Supervisors:

Dr. Ephantus W. Kabiru

Signature: _____. Date. 15/03/07.

Department of Pathology, Kenyatta University.

Dr. Willy Kiprotich Tonui

Signature: _____. Date. 12/3/07.

Center for Biotechnology and Research Development (CBRD),

Kenya Medical Research Institute (KEMRI), Nairobi, Kenya.

Dr. Charles Mutai

Signature: _____. Date. 13/3/2007

Center for Traditional Medicine and Drug Research (CTMDR),

Kenya Medical Research Institute (KEMRI), Nairobi, Kenya.

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DEDICATION

I would like to dedicate this work to my parents, Mr. David Mburu and Mrs. Judy Mburu; my dear brothers, Wakaimba and Kamunya Mburu; my niece, little Chirû, and her dear mum, Anne. Most of all, I dedicate it to my dearly beloved Renee, my crown and my joy.

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LIST OF ABBREVIATIONS

ACL	Anthroponotic cutaneous leishmaniasis
ANOVA	Analysis of variance
AVL	Anthroponotic visceral leishmaniasis
BCG	Bacille Calmette Guerin
CBRD	Center for Biotechnology and Research Development
CC	Column chromatography
CDC	Center for Disease Control
CD4+	Cluster of differentiation molecules that bind to MHC class II molecules
CD8+	Cluster of differentiation molecules that bind to MHC class I molecules
CD40	A receptor molecule on the cell surface of mature B cells and epithelial cells
CD40L	Molecule that binds to CD40
CL	Cutaneous leishmaniasis
COONO	Alkyl peroxynitrates
C_6H_6	Cyclohexane
C3b	Larger fragment of complement molecule C3 of 195kD
C3bi	Hydrolysed C3b molecule
DALYs	Disability adjusted life years
DCL	Diffuse cutaneous leishmaniasis
DDT	Dichlorodiphenyl trichloromethane
DEET	Diethyl metatoluamide
DMSO	Dimethyl sulfoxide

DNA	Deoxyribonucleic acid
ELISA	Enzyme linked immunosorbent assay
EtOAc	Ethylacetate
FBS	Fetal bovine serum
GM-CSF	Granulocyte macrophage colony stimulating factor
Gp63	Glycoprotein that weighs 63 kilo daltons
HBSS	Hanks balanced salt solution
HCH	Hexachlorocyclohexane
HGPTRase	Hypoxanthine-guanine phosphoribosyl transferase
HIV	Human immunodeficiency virus
HSV	Herpes simplex virus
$H_2O_2^-$	Hydrogen peroxide
IAEA	International Atomic Energy Agency
IFN-γ	Interferon gamma
IL	Interleukin
iNOS	Inducible nitric oxide synthase
IRS	Indoor residual spraying
KEMRI	Kenya Medical Research Institute
LPG	Lipophosphoglycan
LR	Leishmaniasis recidivans
MCL	Mucocutaneous leishmaniasis
MeOH	Methanol
MHC	Major histocompatibility complex

mRNA	Messenger ribonucleic acid
NNN	Novy Nicolle McNeal medium
NO	Nitric oxide
O_2^-	Superoxide anion
PBS	Phosphate buffered saline
PKDL	Post kala-azar dermal leishmaniasis
RNA	Ribonucleic acid
RPMI	Roswell Park Memorial Institute
SOPs	Standard operating procedures
SSG	Generic sodium stibogluconate
TGF	Tumor growth factor
TLC	Thin layer chromatography
TNF	Tumor necrosis factor
Th	Helper T cell
VL	Visceral leishmaniasis
WHO	World Health Organization
ZCL	Zoonotic cutaneous leishmaniasis
ZVL	Zoonotic visceral leishmaniasis
α	Alpha
γ	Gamma

DEFINITION OF OPERATIONAL TERMS

Cell free media Media that does not contain cells parasitized by the parasites, in this case, media without macrophages.

Cervical dislocation A physical method of euthanasia by separation of cervical tissues.

Chromatography Separation of a mixture of compounds by distribution between stationary and mobile phases.

Cryopreservation The process of freezing tissue at a slow freezing rate for preservation to enable stocks to be stored.

DALYs The disability adjusted life year (DALY) provides a means to measure disease burden that is more informative than crude measures of mortality and prevalence.

Diazotization A reaction that converts an $-NH_2$ group connected to a phenyl ring to a diazonium salt, which is unstable and explosive in dry form, and are used to manufacture azo dyes.

IC_{50} The concentration of a drug that is required for 50% inhibition of parasite replication *in vitro*.

In vacuo	In vacuum or in isolation.
In vivo	Analysis carried out inside a living body.
In vitro	Maintenance of parasites outside the body, in culture.
Intraperitoneal	Injecting into the peritoneal cavity.
Oviposition	Egg-laying
Parenteral	Routes that involve introduction of material into the body through injections into various compartments of the body.
Phytochemistry	The examination of plants to find the chemical constituents responsible for their biological activity.

ABSTRACT

Leishmaniasis is a major group of parasitic diseases in the tropical regions. Their public health importance requires integrated measures in order to ensure effective control of the disease. Chemotherapy is the mainstay of control with pentavalent antimonial agents still widely used as the drugs of choice. However, these drugs are associated with many undesired effects, which include renal and cardiac toxicity, myalgia, anthralgia, pancreatitis, gastrointestinal problems, cardiac arrhythmia and sudden death. The antimonials are also very expensive, and require a long period of administration. Subsequently, the search for compounds with possible antileishmanial activity is growing. A lot of focus is currently being given to natural products. It has long been known that plants are a rich source of compounds with potential medicinal uses. *Acacia mellifera* subsp. *mellifera* is a recognized medicinal plant used for conditions such as malaria, pneumonia, sterility, stomach problems and primary stages of syphilis. However, limited information is available on its isolated metabolites. The main objective of this study was to determine whether *A. mellifera* has any antileishmanial activities. To achieve this, the stem bark and leaves of *A. mellifera* were extracted using methanol and dichloromethane then extracts evaluated against *Leishmania major* promastigotes and amastigotes *in vitro*. Further fractionation on dichloromethane extracts yielded nine fractions designated as fractions I-IX. The effects of extracts as well as fractions were evaluated both in cell free media and using infected macrophages. To evaluate the immunostimulatory activities of the extracts, production of nitric oxide in supernatants from macrophages stimulated with extracts was detected by Griess test. All tests were done in triplicate. Statistical analyses of the results obtained were done by analysis of variance, chi-square and Kruskal-Wallis test. Results revealed that extracts containing a combination of methanol and dichloromethane had the highest activity against *L. major* promastigotes in cell-free culture. 100% mortality was observed on promastigotes cultured with 5mg/ml of the methanol and dichloromethane crude extracts. The methanol and dichloromethane stem bark extracts, however, did not inhibit the transformation of *L. major* amastigotes into promastigotes ($P>0.05$). The stem bark methanol extract had the lowest infection rate of 41.35%. Infection rates of the extracts compared to a positive control had a difference ($\chi^2 =13.89$; $df=9$; $P>0.05$). The fractions of dichloromethane had a statistically significant difference in their infection rates ($F=6.827$; $df=4,15$; $P<0.01$). Fraction VII had the lowest infection rates of 30% at 250µg/ml and also the lowest IC_{50} of 54.19µg/ml. Close in its activity were fractions VIII and IX. Results for immunostimulatory activity among the fractions of dichloromethane indicated that there was no significant production of nitric oxide ($P>0.05$). These results suggest another possible mechanism of action of the fractions other than through stimulation of nitric oxide production. A comparison of the immunostimulatory effects between the methanol and dichloromethane indicated production of nitric oxide by the methanol extract. There was a difference ($H_{4,4,4,4}= 14.04$; $P<0.05$). The results from this study demonstrate that *Acacia mellifera* indeed has activity against *Leishmania major* parasites. This is a major contribution toward attainment of the Millenium Development Goal that seeks to combat disease.

CHAPTER 1: INTRODUCTION

1.1 Background

Leishmaniasis is a parasitic disease caused by protozoan flagellates of the Genus *Leishmania* Ross; Family Trypanosomatidae and Order Kinetoplastida (Dedet and Pratlong, 2003). They are obligatory intracellular parasites found in mononuclear phagocytes, primarily macrophages, cells designed to phagocytose and destroy invading organisms. The parasites produce a spectrum of disease ranging from simple cutaneous, diffuse cutaneous, mucocutaneous, and visceral forms of the disease, all of which may have devastating consequences (WHO, 2000). Parasites responsible for cutaneous forms of the disease establish their infections on the skin, while those responsible for visceral leishmaniasis disseminate and establish their infections in the viscera (Croft and Yardley, 2002). The pathological manifestations depend on complex interactions between the host's immune responses and the parasite's invasiveness, tropism, and pathogenicity (Wilson and Pearson, 1990). Species causing visceral leishmaniasis are able to grow at core temperatures, while those responsible for cutaneous leishmaniasis grow best at lower temperatures (Niknam *et al.*, 1996).

Approaches to effective leishmaniasis control consist a combination of chemotherapy, integrated vector management, reservoir control, and health education. TDR, a partner of the visceral leishmaniasis eradication initiative is supporting research on the most efficient integrated vector intervention (WHO, 2007). The main control measure taken against leishmaniasis is chemotherapy. For many decades treatment has been based on antimonial compounds. These are founded upon the use of a toxic heavy metal, antimony.

They include sodium stibogluconate (Pentostam®), and meglumine antimoniate (Glucantime®). Nevertheless, these are the leishmanicidal agents with the most favourable therapeutic index. Treatment requires intramuscular or intravenous administration for about a month. Patients are usually hospitalized in order to monitor them closely for any side reactions.

Other drugs such as amphotericin B (Fungizone®), liposomal amphotericin B (AmBisome), Pentamidine isethionate, Paromomycin (Aminosidine) and miltefosine are also available for treatment of leishmaniasis. These are advocated for in cases of resistance to antimonials. They are also described for unresponsive mucocutaneous leishmaniasis, diffuse cutaneous leishmaniasis or *Leishmania*/ HIV co-infections (Croft and Yardley, 2002). Lipid formulations of Amphotericin B relate to lower toxicity but their commercial use is limited by their high cost (Hailu *et al.*, 2005).

1.2 Statement of problem

Leishmaniases still account for high proportions of morbidity and mortality. To date no effective vaccine is available for use against any form of leishmaniases and neither are there any prophylatic drugs (reviewed by Handman, 2001). Treatment of the various forms of leishmaniases with antimonials is faced with several challenges. The drugs of choice have side effects often reflected as renal or cardiac toxicity, pancreatitis, myalgia, anthralgia, and bone marrow suppression. The routes of administration, which include, intramuscularly or intravenously, are often painful at the site of injection and thereby less preferred. Increasing resistance, up to 65%, has been reported especially in areas in

Northern Bihar, India (Desjeux and Karbwang, 2003). The antimonials are very expensive thus unaffordable to many as the disease affects mainly the poor. It is estimated that 80% of the people living in endemic areas earn less than $2 a day (Davies *et al.*, 2003), whereas the drug costs US$150 (WHO, 2004). As such there is need for continuous research for new drugs that can overcome the resistance problem, are available, affordable, and can be administered through less painful routes such as orally or topically. In Africa, the use of native indigenous plants still plays an important role in many diseases including leishmaniasis. Traditionally, treatment consists of oral administration of crude plant extracts for the systemic form of the disease and as topical preparations for cutaneous forms. These plants may be sources of novel compounds with anti-leishmanial activity. Despite the effective use of traditional crude plant extracts the laboratory evaluations of their anti-leishmanial activity has not been thoroughly done.

1.3 Justification

The standard regimen for leishmaniasis is often unaffordable to many patients in endemic areas. These areas mainly in developing countries have a low-income population. Therefore, alternative cheaper methods of treating the disease need to be sought. Natural compounds are often safe, available, and affordable. This study aims at evaluating extracts from *Acacia mellifera* for activity against *Leishmania major in vitro*. Though the plant is recognized for its medicinal properties, limited information is available on its isolated metabolites. Earlier claims by traditional healers about the efficacy of herbal preparations have led to discovery of compounds such as Licochalcone A, a lead plant product from a Chinese liquorice plant, *Glycyrrhiza* spp., with antileishmanial activity

(Croft and Yardley, 2002). Natural products structures have high chemical diversity, biochemical specificity, and other molecular properties that make them favorable as lead structures for drug discovery (Basso *et al.*, 2005). Lead compounds ensure a sustainable global pipeline for innovative products (WHO, 2007). Natural products also contain some non-pharmacologically active compounds, which give synergy to the active moieties. Reviews indicate that quinones, alkaloids, terpenes, saponins, and phenolic derivatives found in plants have good anti-leishmanial activity (Chan-Bacab and Peña-Rodríguez, 2001).

1.4 Research question

What is the activity of extracts from *Acacia mellifera* on *Leishmania major* parasites *in vitro*?

1.5 Null hypothesis

Extracts from *Acacia mellifera* do not have anti-leishmanial activities.

1.6 Objectives

1.6.1 Main objective

To investigate the anti-leishmanial activity of extracts from *Acacia mellifera* against *Leishmania major*.

1.6.2 Specific objectives

1.6.2.1 To determine the effects of *Acacia mellifera* extracts on *Leishmania major* promastigotes in cell free culture.

1.6.2.2 To determine the effects of *Acacia mellifera* extracts on *Leishmania major* amastigotes in cell free culture.

1.6.2.3 To establish the infection rates and multiplication indices of *Leishmania major* infected macrophages treated with *Acacia mellifera* extracts and fractions of dichloromethane extract.

1.6.2.4 To determine the immunostimulatory effects of *Acacia mellifera* extracts and fractions of dichloromethane extract on infected macrophages.

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CHAPTER 2: LITERATURE REVIEW

2.1 Global distribution of leishmaniasis

Leishmaniasis is found in many areas of the world, both in the tropics and subtropics. It is endemic in 88 countries, 22 of which are in the New World and the other 66 in the Old World (Dedet and Pratlong, 2003). It is estimated that 12 million people are infected while 350 million are at risk of contracting the disease with annual mortality of about 60,000 (WHO, 2004). Annual incidence is estimated at 1-1.5 million cases of cutaneous leishmaniasis (CL), and 500,000 cases of visceral leishmaniasis (VL) (WHO, 2004). Of the 500,000 new cases of VL that occur annually, 90 % are in five countries, namely Bangladesh, Brazil, India, Nepal and Sudan (WHO, 2000). It is estimated that 90 % of all cases of muco-cutaneous leishmaniasis (MCL) occur in Bolivia, Brazil, and Peru, while 90 % of all cases of CL occur in Afghanistan, Brazil, Iran, Saudi Arabia, and Syria (WHO, 2000). The disease burden is often heavy as reviews indicate that disability adjusted life years (DALYs) lost are close to 2.4 million (WHO, 2004). Australia, South Pacific and South Asia are some of the areas in the world thought to be free of leishmaniasis (CDC, 2005-2006). This has recently been questioned on finding cutaneous *Leishmania* infections in red kangaroos, *Macropus rufus* (Rose *et al.*, 2004).

During the past decade, there have been epidemics of VL in the Sudan, Northeast Brazil, Bangladesh and the states of Patna and Bengal in India (Croft and Yardley, 2002). A major epidemic reported in the Sudan from 1989 to 1993 was responsible for the deaths of approximately 10% of that country's population (Seaman *et al.*, 1996). A dramatic upsurge in cases of visceral leishmaniasis in the Horn of Africa was reported in the last

months of 1997, and the beginning of 1998 (WHO, 1998). The high mortality was mainly due to the absence of diagnostic facilities and the non-availability of first line drugs at the local level (WHO, 1998). More recently, a sharp increase in the overlapping of HIV infection and visceral leishmaniasis has been observed (WHO, 2004). *Leishmania*/ HIV co-infection is rising as an extremely serious new condition and it is increasingly frequent (WHO, 2004). It is considered a real threat in Southwestern Europe, especially in countries such as Italy, Portugal, Spain and France (WHO, 2000; Martin-Sanchez *et al.*, 2004). The condition is probably still largely underestimated.

2.2 Leishmaniases in Kenya

In Kenya, cases of visceral leishmaniasis were reported as far back as 1935 (Ashford and Bettini, 1987). Numerous outbreaks of visceral leishmaniasis (Kala-azar) have been recorded in districts of Baringo, West Pokot, Machakos, Kitui, Meru and parts of North Eastern Kenya (WHO, 1984). In March 2000, an outbreak with a number of cases of VL were noted in Garissa, Wajir and Mandera districts, North Eastern Kenya, thus indicating a situation of re-emergence of visceral leishmaniasis reported (Marlet *et al.*, 2003). Currently, the disease is widespread throughout Baringo (Ryan *et al.*, 2006). The *Synphlebotomus* species are incriminated for transmission of *Leishmania donovani* in Kenya. These include *Phlebotomus martini,* which is most widespread vector in Kenya, and *P. celiae* and *P. vansomerenae,* which are found in limited areas in Eastern Kenya such as Meru and Kitui (Minter, 1989).

Cutaneous leishmaniasis (CL) caused by *Leishmania major* is endemic in Baringo District (Muigai *et al.*, 1987). This district is the only one in Kenya where both VL and CL have been found to co-exist (Ryan *et al.*, 2006). *Leishmania major* is transmitted by *Phlebotomus duboscqi* Neveu-Lemaire (Beach *et al.*, 1984). Cutaneous leishmaniasis by *Leishmania aethiopica* is restricted to foci in Bungoma district, Mt. Elgon, and the Aberdare range (Mebrahtu, 1987). The vectors incriminated for transmission of this parasite are *Phlebotomus longipes* in the endemic areas. *Leishmania tropica* parasites are transmitted by *Phlebotomus pedifer* and *P. guggisbergi*, which are found mainly in caves in highland areas such as Njoro, Nyandarua, Nakuru, Narok and Laikipia (Mebrahtu *et al.*, 1987; Lawyer *et al.*, 1991; Sang *et al.*, 1994).

2.3 Transmission of leishmaniases

2.3.1 Vectors of leishmaniasis

Leishmania species are spread by the bite of an infected phlebotomine sandfly (Diptera: Psychodidae, subfamily Phlebotominae), belonging to the genus *Phlebotomus* in the Old world (Africa, Asia and Europe), and the genus *Lutzomyia* in the new world (the Americas) (Cheesbrough, 1998; Lainson and Rangel, 2005). Distribution of leishmaniasis is often limited to the geographic locations where the sandflies are found (WHO, 2000). These vectors are associated with warm and humid climates (Mishra, 2005). Sandflies usually rest during the day in dark and sheltered places, but are active at dusk or during the night while the host is resting (Reithinger *et al.*, 2001). Adults can also be found in cracks and crevices in dark corners of a house. They are weak fliers, taking a series of short erratic hops (Rozendaal, 1997). They breed in wet soil rich in organic matter, in

ventilation shafts of termite hills, in animal burrows, in caves, in tree holes, and even between buttresses of large trees (Dedet and Pratlong, 2003).

Both sexes feed on plant juices and sugary secretions. Their mouthparts are short and inconspicuous but are adapted for blood sucking, consisting of a labrum, paired mandibles and maxillae, a hypopharnx and a fleshy labium (Service, 1980). At their base is a pair of five-segmented maxillary palps. The females in particular need a blood meal in order to develop eggs (Rozendaal, 1997). The dermal-epidermal interface is reached by proboscis. Their salivary glands have become specialized to deliver anti-coagulants. Sandfly saliva is documented to contain vasodilatory agents, which enable blood capillaries to be reached. These agents include, maxadilan, adenosyne, apyrase and prostaglandin E2 (de Almeida *et al.*, 2003). A sandfly bite is generally not felt and leaves a small round, reddish bump that starts itching hours or days later.

Out of 500 known phlebotomine species, only some 30 of these have been incriminated as vectors of the disease (WHO, 2004). A natural restriction of individual species of *Leishmania* to specific sandflies has been observed (Rotureau, 2006). In the Indian subcontinent *Phlebotomus argentipes* is the vector responsible for transmission of *Leishmania donovani* (Bora, 1999). *Phlebotomus orientalis* is an established vector in the Sudan region (Seaman *et al.*, 1996). One species of the *Synphlebotomus* genus, *Phlebotomus martini* is the most widespread in Kenya, contiguous areas of Uganda and probably of Somalia and Ethiopia. In the new world, *Lutzomyia longipalpis* is the most apparent vector of *Leishmania chagasi* (Lainson and Rangel, 2005; Rotureau, 2006).

2.3.2 Anthroponotic leishmaniasis

Anthroponotic leishmaniasis refers to transmission cycles where humans are considered sole reservoirs of infection (Desjeux, 2001). Transmission of anthroponotic cutaneous leishmaniasis (ACL) is found in well-established settlements, often in urban conditions; there is no known reservoir and transmission is peridomestic such as for *Leishmania tropica* (Minter, 1989). Anthroponotic visceral leishmaniasis (AVL), caused by *Leishmania donovani*, is primarily restricted to East Africa and the Indian subcontinent (Dedet and Pratlong, 2003). Since humans are the sole reservoirs, untreated cases and especially post kala-azar dermal leishmaniasis (PKDL) cases are those who harbour and disseminate the parasite. Patients with *Leishmania*/HIV co-infections also harbour numerous *Leishmania* parasites and therefore become sources of infection for the vector (Schmidt and Roberts, 2005). Anthroponotic visceral leishmaniasis epidemics are frequent with high death rates (Desjeux, 2001).

2.3.3 Zoonotic leishmaniasis

Most leishmaniases are zoonotic with various mammalian reservoir hosts responsible for the long-term maintenance of *Leishmania* in nature (Mandell *et al.*, 2000). The animal reservoir for zoonotic cutaneous leishmaniasis (ZCL) is mainly located in forests and less frequently, in peridomestic areas. In the Old World rodents and hyraxes are reservoirs of wild zoonotic cutaneous leishmaniasis due to *Leishmania major* and *L. aethiopica.* Zoonotic visceral leishmaniasis (ZVL) occurs mainly in Latin America, the Mediterranean basin and Asia (Reithinger *et al.*, 2001). The dog is the major domestic

animal reservoir of *Leishmania infantum* in the Mediterranean region, and *Leishmania chagasi* in South America (Martin-Sanchez *et al.*, 2004). Foxes, jackals, sloths and armadillos are the sylvatic animal reservoirs (Rotureau, 2006). The main risk factors related to ZCL include urbanization, deforestation and new settlements, and agricultural development (Desjeux, 2001).

2.3.4 Factors associated with increase of leishmaniasis

Increase in cases of leishmaniasis is related to economic development and to behavioral and environmental changes. These include, new settlements, intrusion into primary forests, deforestation, massive rural to urban migration, unplanned urbanization and agricultural development (WHO, 2004). Population movements such as rural to urban migration in North Eastern Brazil expose thousands of non-immune individuals to the risk of infection (Dedet and Pratlong, 2003). Risk factors associated include malnutrition and immunosuppression before onset of infection (Wilson and Pearson, 1990). *Leishmania*/HIV co-infections have increased the importance of leishmaniasis due to the reinforcement that the two infections have on each other resulting in much suffering of the patients and quicker deaths for those co-infected. More recently, it has been reported that intravenous drug use, tattooing, piercing and cocaine snorting with shared devices are predisposing factors to leishmaniasis (Martin-Sanchez *et al.*, 2004). Individuals with frequent exposures to agents spread through the parenteral route show a high prevalence of *Leishmania* seropositivity in Spain (Martin-Sanchez *et al.*, 2004). Studies have shown that homes with cow dung provide ideal conditions for the sandfly's breeding sites, and

result in a sharp increase in anthroponotic leishmaniasis cases (Desjeux, 2001). Incomplete therapy of initial disease is a risk factor for recurrence of leishmaniasis.

2.4 Life cycle of *Leishmania* parasites

Leishmania parasites undergo a digenetic life cycle with the parasite migrating between sandflies and mammalian hosts (de Almeida, 2003). Figure 1 shows the lifecycle.

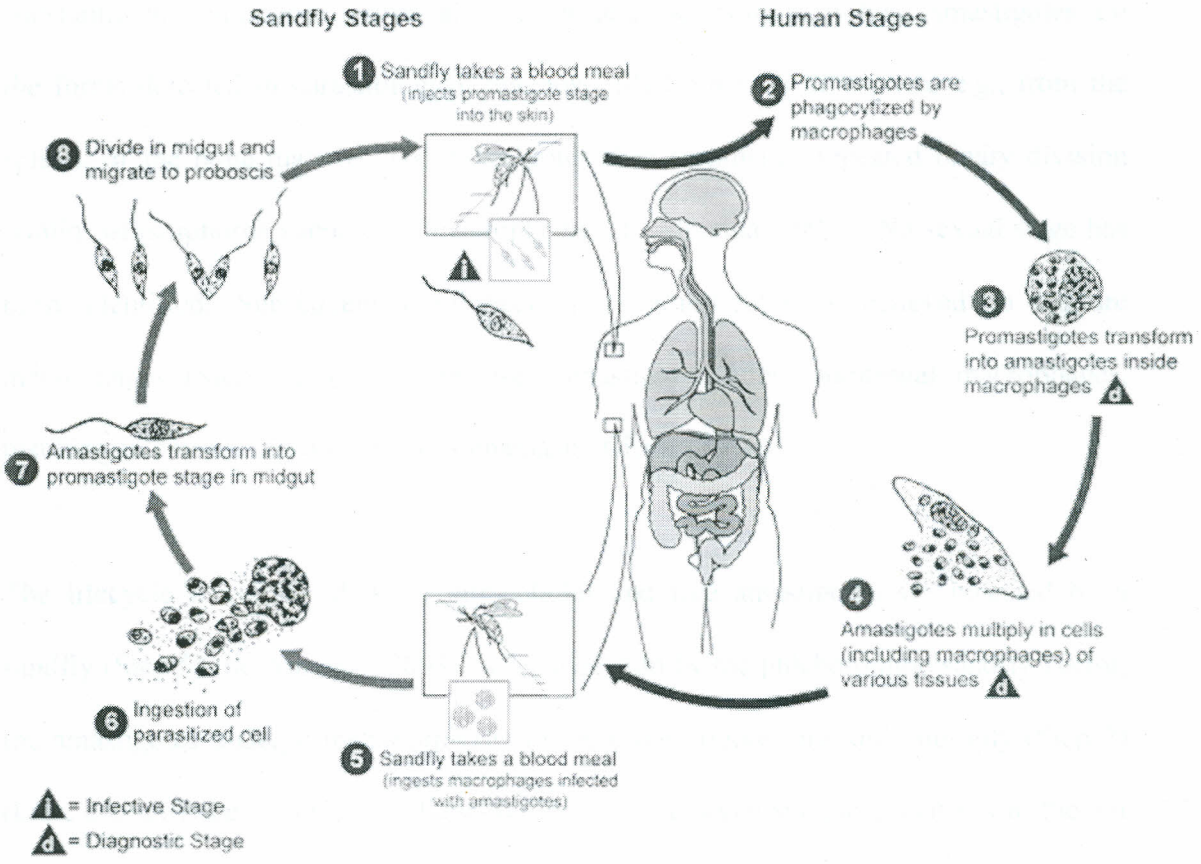

Figure1. *Leishmania* digenetic life cycle (adapted from, www.uni.tuebingen.de/modelling/Mod_Leish_Cycle_en.html, 2004).

Leishmania parasites have two major developmental forms in their life cycle, namely, extracellular flagellated promastigotes found within the sandfly (15-25μm), and non-motile amastigotes (2-3μm) residing intracellularly in mammalian cells (Niknam *et al.*, 1996). Infections start when flies carrying infective metacyclic promastigotes inject these into a vertebrate host as they feed (Step 1) (Chang, 1990). The promastigotes are taken up by macrophages of the host and lodge in phagosomes to which lysosomes fuse creating phagolysosomes. Profound morphological and biochemical changes accompany leishmanial differentiation into amastigotes. These include loss of flagella, and a substantional diminution in size, growth rate and metabolic activities. Amastigotes are the forms detected in parasitological diagnosis (d) from a tissue aspirate e.g., from the spleen, or the bone marrow. The amastigotes then commence repeated binary division within parasitiphorus vacuoles of macrophages (de Almeida, 2003). No sexual stage has been identified. Subsequent multiplication of amastigotes is believed to rupture macrophages (Step 4), and the released amastigotes infect additional macrophages, resulting in chronic diseases, the leishmaniasis (Chang, 1990).

The lifecycle is continued when intracellular and free amastigotes are ingested by a sandfly (Step 5) (de Almeida, 2003). After ingestion by the phlebotomine sandfly vector, the amastigotes undergo metamorphosis to the promastigote form and multiply (Step 7) (Lane and Crosskey, 1993). The flagellum propels the organism and attaches it to the gut wall or salivary gland epithelium. Further metamorphosis takes place within the digestive tract of a sandfly. The first developmental forms are multiplicative procyclics (short ellipsoid promastigotes), which later transform into nectomonads (migratory),

haptomonads and finally metacyclics (Lawyer *et al.*, 1990; Rotureau, 2006). Metacyclic promastigotes, which are infective (i), are highly motile and immunologically prepared for invasion of the vertebrate host (Lane and Crosskey, 1993). The cycle is made complete on successful inoculation of metacyclic promastigotes into a new susceptible host.

2.5 Cutaneous leishmaniasis

Cutaneous leishmaniasis is a dermatological infection of the skin known since earliest antiquity, with parasites living in the skin macrophages (histiocytes). It has symptoms that could be mild or severe, differing with regions, depending on the species of the parasite and the immune response of the patient (Rozendaal, 1997). Often the infection starts as an erythematous papule at the site of bite, which regularly enlarges. Lesions appear on exposed parts of the body, either singly or multiple (El Hassan, 2001). A thin crust usually develops but hides a spreading ulcer underneath (Dedet and Pratlong, 2003). In uncomplicated cases, the ulcer will heal within two months to a year, leaving a depressed scar (Schmidt and Roberts, 2005).

2.5.1 Old world cutaneous leishmaniasis

Old world cutaneous leishmaniasis is caused by *Leishmania tropica, Leishmania major*, and *Leishmania aethiopica* (Cheesbrough, 1998). Zoonotic cutaneous leishmaniasis due to *Leishmania major* is the most extensive and is characteristic of lowland steppe and semi-desert zones, which are sparsely populated (Minter, 1989; Schmidt and Roberts,

2005). It is distributed through West, North and East Africa, Middle East and parts of Central Asia (Dedet and Pratlong, 2003). *Leishmania major* causes infections often referred to as wet oriental sore. An inflammatory reaction is elicited by the parasite antigen that results in infiltration of the dermis by lymphocytes, macrophages, and plasma cells (El Hassan, 2001). It gives rise to localized lesions, 5-10mm in diameter, that resolve spontaneously and leaves the host with a strong immunity to re-infection (Cheesbrough, 1998; Mandell *et al.*, 2000). Nodulo-ulcerative lesions have a volcanic appearance with the major part being in the subcutaneous tissue (iceberg concept). *Leishmania major* infections can protect against infection with *Leishmania tropica.*

Leishmania tropica causes infections referred to as "dry urban oriental sore", with painless ulcers, 25-70mm in diameter, which are self-healing in 1-2 years (WHO, 1999). It is mainly found in more densely populated areas (Schmidt and Roberts, 2005). Rarely there may develop multiple unhealing lesions, a condition known as leishmaniasis recidivans (LR) or relapsing leishmaniasis that can last many years (Handman, 2001). *Leishmania tropica* can also become viscerotropic, resulting in enlarged spleen and inflammation of lymph glands as reported among military personnel (Schmidt and Roberts, 2005).

Leishmania aethiopica causes cutaneous lesions that are similar to typical oriental sore with healing in 1-3 years, but however can cause diffuse cutaneous leishmaniasis (DCL) in patients with little or no cell-mediated immunity against the parasite (Cheesbrough, 1998). Diffuse cutaneous leishmaniasis is a severe form of cutaneous leishmaniasis where

nodules are numerous, at first isolated, then joining to form large patches, disseminated to the whole body via the lymphatic system (Dedet and Pratlong, 2003). These nodules do not ulcerate. This condition does not heal spontaneously. *Leishmania aethiopica* can also cause muco-cutaneous leishmaniasis, which affects oronasal tissues. *Leishmania aethiopica* is a sylvatic species found only at high altitudes, around 2000m above sea level, mainly in the Ethiopian highland plateau, but also in Kenya around Mt Elgon region.

2.5.2 New world cutaneous leishmaniasis

New world CL is caused by *Leishmania mexicana, L. peruviana, L. guyanensis,* and *L. panamensis* (Dedet and Pratlong, 2003; Rotureau, 2006). This variety tends to be more severe and slower to heal (WHO, 2002). Muco-cutaneous leishmaniasis (MCL), also known as espundia in South America, is caused mainly by *Leishmania braziliensis* and *L. panamensis*, and occasionally, *L. guyanensis* (Rozendaal, 1997; Rotureau, 2006). It is the most severe and destructive form of CL in South America. It is sequelae of new world CL and results from direct extension or hematogenous or lymphatic metastasis to the nasal or oral mucosa (Singh and Sivakumar, 2003). Nasal lesion appears as a small sized inflammatory granuloma evolving to an ulcer. The soft tissues and cartilage in these areas are then progressively destroyed by the parasites (Rozendaal, 1997). Tissue necrosis and disfigurement in advanced stages result in disfiguring mutilations. Mutilations are severe and occasionally result in death due to malnutrition and bronchopneumonia (Dedet and Pratlong, 2003). Muco-cutaneous leishmaniasis is often progressive and non-healing, but recovery can occur with strong immunity to re-infection (Mandell *et al.*, 2000).

2.6 Visceral leishmaniasis

Visceral leishmaniasis (VL), also called kala-azar, is a human systemic disease and the most severe clinical form of leishmaniasis (Dedet and Pratlong, 2003). It can be fatal if not treated. It is caused by *Leishmania donovani* (in the Indian subcontinent and at the Horn of Africa), *Leishmania infantum* (Southern Europe, Central Asia and the Mediterranean region), and *Leishmania chagasi* in the Americas (Rotureau, 2006).

Clinical features ensue gradually in a period of weeks or even months. These are characterized by hepatosplenomegaly, recurring and irregular bouts of fever, anaemia, pancytopenia, weight loss, and weakness (Seaman *et al.*, 1996; Hailu *et al.*, 2005). Anaemia is thought to result from crowding of bone marrow by parasites, antibody formation against red blood cells and iron deficiency caused by haemorrhages (Mishra, 2005). Affected patients become progressively weak, cachetic, and susceptible to intercurrent infections (Hailu *et al.*, 2005). Skin changes are common as the skin becomes greyish, hence the name "black sickness," or, kala-azar in Hindu (Cheesbrough, 1998). Dark pigmentation is attributed to disturbed tyrosine formation (Mishra, 2005). Jaundice is unusual and occurs late in the disease.

In India and occasionally in East Africa, a cutaneous form of leishmaniasis can occur after treatment and recovery from visceral leishmaniasis (Dedet and Pratlong, 2003). This is referred to as post kala-azar dermal leishmaniasis (PKDL). Hypopigmented and raised erythematous patches can be found on the face, trunk and limbs that may develop into

nodules (Cheesbrough, 1998). Post kala-azar mucosal leishmaniasis has been described as another phenomenon that may follow treatment of kala-azar (El-Hassan *et al.*, 1995).

2.7 Immune responses to leishmaniases

Immunity to *Leishmania major* is T-cell mediated, with important interactions occurring between T lymphocytes and macrophages. Both $CD4^+$ and $CD8^+$ cells contribute to this immunity. Macrophages present antigen in the context of major histocompatibility complex (MHC) class II molecules to T cells that together with interleukin (IL-1) leads to T-cell activation (Bogdan *et al.*, 1996). Protective immunity depends on the induction of T cells producing Th1 cytokines, primarily, interferon gamma (IFN-γ), and tumor necrosis factor alpha (TNF-α), which activate macrophages to kill the intracellular organisms primarily through a nitric oxide-mediated mechanism (Jones *et al.*, 1998, Handman, 2001). Presence of IL-12 early in infection promotes Th1 responses. Th 2 subset exacerbate disease largely because cytokines produced both shut off the potential for IFN-γ release (IL-4), and promote production of immature bone marrow derived macrophages (IL-3 and GM-CSF) which contribute to disease progression (Bogdan *et al.*, 1996). Transforming growth factor (TGF) β and IL10 also stimulate a Th2 response.

Nitric oxide (NO) is a primary effector molecule synthesized through the L-arginine metabolic pathway used by the macrophage to kill the parasite (Holzmuller *et al.*, 2002). In the mouse model, NO is produced in macrophages via the upregulation of the enzyme inducible nitric oxide synthase (iNOS), which generates NO from precursor molecules (Jones *et al.*, 1998). The CD40-CD40L receptor/ligand system is a critical co-stimulatory

pathway for the efficient induction on iNOS and leishmanicidal activity in macrophages (Bogdan *et al.*, 1996). The nitric oxide molecule is reactive because of its free radical structure. It can complex with super oxides such as lipophilic peroxyl radicals, important propagating molecule in the biological chain reaction of lipid peroxidation, to generate alkyl peroxynitrates (COONO) (non-radical), which may be a major cytotoxic agent (Lancaster and Hibbs, 1990). Nitric oxide produced in large quantities inhibits the proliferation of intracellular pathogens and tumor cells. These cytotoxic effects are thought to result from the reactivity of NO with iron in the [Fe-S] centers of critical proteins, resulting in iron mobilization from tumor target cells which inhibits DNA synthesis and mitochondrial respiration (Watts and Richardson, 2002). Studies indicate that both axenically grown and intracellular *Leishmania amazonensis* amastigotes, when submitted to NO action, die through apoptosis (Holzmuller *et al.*, 2002).

2.7.1 Intracellular survival of *Leishmania*

Humoral factors play a role in control of infection, through complement membrane attack complex present in human serum, activated through the alternative pathway (Wilson and Pearson, 1990). However, though the complement cascade is activated, lysis does not occur because the long and complex molecules on the parasites surface coat (lipophosphoglycan- LPG) prevent access of the membrane attack complex to the plasma membrane. Amastigotes of *Leishmania major* are sensitive to lysis and as a result are restricted to dermal lesions. On the contrary, amastigotes of *Leishmania donovani* are resistant to complement, therefore the ability to spread to visceral organs (Mandell *et al.*, 2000). They are phagocytized by macrophages, in which the process of lysosome-

phagosome fusion occurs. Intracellular amastigotes have been shown to be resistant to respiratory burst killing, mainly mediated by lymphokine activation (El-On *et al.*, 1990).

Usually, phagocytosis triggers a respiratory burst by mononuclear phagocytes, resulting in the generation of microbicidal oxygen radicals such as hydrogen peroxide, hydroxyl radicals, singlet oxygen, and iron-containing superoxide dismutase. *Leishmania* parasites may rely on trypanothione peroxidase as the scavenger for the detoxification of hydrogen peroxide. An acid phosphatase has been found from the plasma membrane of *Leishmania donovani* to diminish the respiratory burst by blocking the production of O_2^- and $H_2O_2^-$ by phagocytes (Remaley *et al.*, 1985). *Leishmania* parasites may be protected by a surface layer of molecules resistant to acid hydrolases, enabling them to survive in the acidic environment of the macrophage phagolysosomes (Wilson and Pearson, 1990). The surface layer molecules may also play a role in detoxification of microbicidal factors in this environment. Gp63 (leishmanolysin) has been identified as a protease active at pH 4.0, and conceivably could destroy host enzymes that normally have microbicidal properties (Wilson and Pearson, 1990; Joshi *et al.*, 1998). Gp63 has also been shown to be a major acceptor of activated complement fragments of the third component (C3b, C3bi), thus enhancing phagocytosis by host macrophages (Joshi *et al.*, 1998). In this way, parasite proteins are protected from phagolysosomal degradation. The LPG is capable of inhibiting the lysosomal β-galactosidase of macrophages (El-On *et al.*, 1990).

2.8 Control of leishmaniasis

2.8.1 Chemotherapy

Treatment of infected individuals involves use of pentavalent antimony (Sb^{V}) compounds, namely sodium stibogluconate (Pentostam ®, GSK) or meglumine antimoniate (Glucantime ®, Aventis) as the first line drugs for leishmaniases (WHO, 2002). These are given parenterally (intramuscularly or intravenously) since there is poor oral absorption (WHO, 2002). The recommended dose of Sb^{V} is 20mg/kg body weight per day for 20-28 days depending on the infecting *Leishmania* species and the clinical syndrome (Mandell *et al.*, 2000). In resistant cases, the dose may be extended to 40 days. The long course of administration allows antileishmanial levels of the drug to accumulate in the tissues, namely the liver and spleen (Croft and Yardley, 2002). Nutritional deficiencies should be corrected where possible and a protein rich diet provided as precaution (WHO, 2002). This regimen is contraindicated in case of severe kidney disorders and breastfeeding (WHO, 2002). Infections with *Leishmania major* are responsive to intralesional injections with antimonial compounds (WHO, 2002).

The antimonials are absorbed quickly and excreted rapidly from the body with a half-life of approximately two hours and the remainder about 766hours later (Mandell *et al.*, 2000). Pentavalent antimony is converted to trivalent antimony that is thought to responsible for the slow terminal elimination; toxicity resulting with long-term, high dose therapy (Mandell *et al.*, 2000). The precise mechanisms of action have not been understood. A number of potential targets have been indicated including glycolysis, DNA topoisomerase I, and trypanothione (Croft and Yardley, 2002). It is also documented that

the antimonials interact with the immune response to have a complete curative antileishmanial effect (Croft and Yardley, 2002).

The pentavalent antimonials are relatively well tolerated and still effective in most endemic countries, with 95% cure rates (Hailu *et al.*, 2005). However, they have many limitations. Treatment involves a long course of administration, about 4 weeks or more. This lengthy duration often causes drug levels to accumulate in the tissues resulting in side effects such as myalgia, pancreatitis, anthralgia, abdominal symptoms, liver enzyme elevation, bone marrow suppression, neuropathy and sudden death (Alrajhi, 2003). Patients therefore need to be under a doctor's supervision for close monitoring (WHO, 2002). Systemic administration of the treatment requires multiple injections, which often are painful. The cost of the antimonials ranges between $120 and $150, a cost that is usually prohibitively expensive hence, unaffordable by many.

High drug resistance has been reported especially in Northern Bihar, India, where it is up to 65% (Desjeux and Karbwang, 2003). Development of drug resistance is thought to result from irregular and incomplete treatment especially among the rural poor where health facilities are inadequate (Bhattacharya *et al.*, 2002). Toxicity to the renal and cardiac systems has been recorded. Relapses are common. In some cases, treatment is followed by the appearance of dermal nodules containing parasitized cells (post kala-azar dermal leishmaniasis- PKDL) (Handman, 2001). Generic sodium stibogluconate (SSG) has been advocated because of its affordability. It costs about fourteen times cheaper than

the GlaxoSmithKline product (Pentostam®). However, it has the same efficacy and toxicity as the antimonials (Rijal *et al.*, 2003).

Treatment of mucocutaneous leishmaniasis may be complicated by immune responses following administration of antimonials. Emergency use of corticosteroids may be needed to control pharyngeal or tracheal oedema produced by severe inflammation resulting from antigens liberated from dead parasites during the early phase of treatment (WHO, 2002). Antibiotics may also be needed to treat secondary infections. Plastic surgery offers the only means of ameliorating disfiguring scars (WHO, 2002).

Pentamidine isethionate and amphotericin B are second-line drugs used in recalcitrant cases of leishmaniases, but these are more toxic alternatives (WHO, 2002). Pentamidine is an aromatic diamidine that is administered parenterally either by deep intramuscular injection or by intravenous infusion (WHO, 2002). It is the recommended drug when *Leishmania aethiopica, L. braziliensis* or *L. guyanensis* is responsible for infection. Relapse is unusual. Toxicity is a limitation on use with reports of hypoglycemia, nephrotoxicity, tachycardia, hypotension, severe headaches and pain at site of injection (Chan-Bacab and Péna-Rodriguez, 2001).

Amphotericin B deoxycholate (Fungizone®) is a polyene antibiotic that is predominantly an antifungal. It binds to leishmanial membrane sterols and phospholipids forming aqueous pore in the membranes of cells thus increasing its permeability (Croft and Yardley, 2002). Lipid formulations of the drug have been prepared to target the

parasitized cells and to reduce toxicity of the drugs. Liposomal amphotericin B is a formulation that is currently effective, though prohibitively expensive and has to be administered intravenously (Hailu *et al.*, 2005). It is also successful in treatment of antimonial-resistant post kala-azar dermal leishmaniasis (Alrajhi, 2003).

Azoles are also sterol biosynthesis inhibitors. Imidazoles, which include ketoconazole, fluconazole, clotrimazole, miconazole, and itraconazole are reported to inhibit the sterol synthesis pathway of *Leishmania* (Dedet and Pratlong, 2003).

Miltefosine® or hexa-decylphosphocholine (HePc), an alkylphosphocholine analogue, is a potential oral drug against VL (Desjeux and Karbwang, 2002). It is an anti-cancer drug that has been shown to be active against intracellular *Leishmania donovani* in animal models and, in a small dose-escalation clinical trial in India, was shown to be effective against VL (WHO, 1999). It is thought to inhibit cell-signaling pathways and induce apoptosis (Croft and Yardley, 2002). Miltefosine is registered in India for first-line treatment of VL, and in Europe for treatment of VL in patients co-infected with HIV (Hailu *et al.*, 2005). It is, however, expensive and teratogenic (Hailu *et al.*, 2005).

The purine analogue, allopurinol®, is an oral agent that halts protein synthesis in *Leishmania* and appears to potentiate the activity of antimonials (Greenwood, 1995; Croft and Yardley, 2002). The analogue is incorporated in *Leishmania* RNA halting protein synthesis (Mandell *et al.*, 2000). Allopurinol is a drug used in the treatment of gout. It is thought to function as an alternative substrate for the enzyme hypoxanthine-guanine

phosphoribosyl transferase (HGPRTase), so allowing the incorporation of allopurinol riboside into RNA that leads to inhibition of protein synthesis.

Several topical applications are being tried for treatment of cutaneous leishmaniasis. Aminosidine (paromomycin®) could become the first-line treatment for uncomplicated CL in *Leishmania major* foci (Asilian *et al.*, 2003). Leshcutan®, a topical drug constituting 15% paromomycin sulphate and 12% methybenzethonium chloride, in soft white paraffin, is effective against a variety of leishmanial strains (El-On *et al.*, 1984). It is applied twice daily for 10 days in the lesion. Paromomycin is an antibiotic originally developed as an antibacterial agent that acts by binding to the ribosome small sub unit leading to misreading of mRNA (Croft and Yardley, 2002).

2.8.2. Immunotherapy

Immunotherapy refers to modulation of the immune response to overcome the negative control systems and to boost the positive killing responses (Croft and Yardley, 2002). Immunomodulators like BCG, muramyl dipeptide, trehalose dimycolate, glucan, tuftsin and protein A that have a direct effect on macrophages have been studied. Tucaresol is a compound that enhances Th1 responses and the production of IL-2 and IFNγ (Croft and Yardley, 2002). Imiquimod has been shown to induce nitric oxide production in macrophages *in vitro* killing *Leishmania donovani* amastigotes (Croft and Yardley, 2002). Immunotherapy with interferon gamma (IFNγ), in antimony-treated patients with newly diagnosed VL achieves an accelerated parasitologic response evident within 10 days after initiation of combination therapy and more rapid induction of apparent cure

(Sundar *et al.*, 1995). IFNγ enhances macrophage accumulation of antimony thus shortening the duration of conventional therapy required for ultimate cure.

2.8.3 Cryotherapy

Cryotherapy has been used successfully in treatment of cutaneous leishmaniasis. This procedure refers to application of extreme cold to destroy diseased tissue. It involves treatment of individual lesions by use of liquid nitrogen alone or in combination with intralesional administration of antimony compounds (Alrajhi, 2003). It eliminates parasites but the ensuing scar may be larger than that of *Leishmania*. This method, however, is labor intensive and not suitable for complicated lesions (Alrajhi, 2003).

2.8.4 Herbal medicine

Communities living in leishmaniasis endemic regions have used herbal remedies in control of leishmaniases. For instance, among the Tugen people, living in Rift valley province of Kenya, are plants such as *Ajuga remota, Ziziphus mucronata* and *Myrsine africana* that have been used (Munguti, 1994). There is concern on patient safety since unregulated and inappropriate use of traditional medicines can have negative effects (WHO, 2003). Some of the plants have been studied and, so far, anti-leishmanial activity has been found in a number of plant species (Rono *et al.*, 2003). Interesting novel metabolites have been isolated with potential leishmanicidal or immunostimulant activities (Chan-Bacab and Péna-Rodriguez, 2001).

Araujo *et al.* (1998) attributed anti-leishmanial activity of plant extracts to compounds belonging to diverse chemical groups such as isoquinolone alkaloids, indole alkaloids, quinolones and terpenes. Berberine, a quaternary isoquinolinic alkaloid is one of the alkaloids with highest leishmanicidal activity (Chan-Bacab and Péna-Rodriguez, 2001; Croft and Yardley, 2002). This metabolite is the main constituent in various told remedies used in the treatment of leishmaniasis, malaria and amoebiasis (Chan-Bacab and Péna-Rodriguez, 2001). It has been used clinically for over 50 years with significant activity. Harmaline, an indole alkaloid, is a major constituent of plants utilized in traditional medicine (Chan-Bacab and Péna-Rodriguez, 2001).

Terpenes consist a large family that contains numerous medicinal metabolites such as iridoids, monoterpenes, diterpenes, triterpenes, sesquiterpenes, limonoids and saponins (Kayser *et al.*, 2003). Several triterpenes have been found to have antileishmanial activity such as betulin-aldehyde. However, these metabolites are often toxic to macrophages. Among the saponins with antileishmanial activity include α-hederin, β-hederin, hederagenin, and mimengoside. These inhibit the growth of promastigote forms by inducing a drop in membrane potential of the parasite (Chan-Bacab and Péna-Rodriguez, 2001).

Other metabolites found to have anti-leishmanial activity include phenols such as chalcones, aurones, coumarins, lignans, flavonoids, naphthoquinones and tetralones (Akendengue *et al.*, 1999; Napolilano *et al.*, 2004). Licochalcone A, an oxygenated chalcone from the Chinese licorice plant (*Glycyrrhiza* spp.) has been found to have anti-

protozoal (anti-leishmanial included) activity (Croft and Yardley, 2002). The proposed mechanism of action for licochalcone A involves the alteration of the ultrastructure and the function of mitochondria (Zhai *et al.*, 1995). Aurones share structural similarities with chalcones and thereby similar antiparasitic activities (Kayser *et al.*, 2003).

2.8.4.1 *Acacia*

The genus *Acacia* comprises a dominant element of the tree flora in the vegetation of the warm sub arid and arid areas of the world (Seigler, 2003). *Acacia* belong to the Order Fabales, Family Leguminosae, subfamily Mimosoideae, and Tribe Acacieae (Seigler, 2003). This subfamily has sometimes been treated as Family Mimosacea. Recent reviews however suggest that this genus belongs to Family Fabaceae. The people of Africa have used the leaves, pods, bark, and gum of many *Acacia* species for medicinal purposes for generations (Coe and Beentje, 1991). The gums produced must have evolved as a means of protecting the trees from damage, for a wound is quickly filled with these sticky exudates, which harden rapidly on exposure to air.

Phytochemical studies of the genus *Acacia* have yielded several secondary metabolites. These include amines and alkaloids, cyanogenic glycosides, cyclitols, fatty acids, and seed oils, fluoroacetate, gums, non-protein aminoacids, terpenes (including essential oils, diterpenes, phytosterol and triterpene genins and saponins), hydrolysable tannins, flavonoids and condensed tannins (Seigler, 2003).

2.8.4.2. Acacia mellifera subsp. *mellifera*

Acacia mellifera, also known as the "black thorn", is a much branched tree or shrub (Appendix 2) that is recognized as a medicinal plant and thus used for treatment of conditions such as malaria, pneumonia, sterility, primary infection with syphilis, and stomach problems (Kokwaro, 1976; Gunner *et al.*, 1992). There are cases where it has been reported as used against leishmaniasis. Some of the local names here in Kenya include *Kezia* (Taveta), *Muthia/Kithia* (Kamba), *Kikwata* (Swahili), *Ebunyu* (Turkana), *Sabansa-Gurach* (Boran) (Dale and Greenway, 1961; Kokwaro, 1976).

Acacia mellifera is found in Africa, Asia, Australia and the Middle East (ILDIS, 1994). In Africa, it is found in Angola, Botswana, Egypt, Ethiopia, Kenya, Mozambique, Namibia, Somalia, South Africa, Sudan and Tanzania, Zambia and Zimbabwe. Dale and Greenway (1961) indicate that it is found in Northern, Southern and Coast parts of Kenya and is often gregarious.

Rutin and vicenin are flavonoids found in the seeds and leaves of *A. mellifera* (Thieme and Khogali, 1974). Rutin (Quercetin 3-O-rutinoside) may have anti-oxidant activities (Gao *et al.*, 2002). Vicenin (Apigenin-6,8-bis-C-β-D-glucopyranoside), on the other hand has been found to protect mice against radiation injury (Uma Devi *et al.*, 2003). Vernolic acid (cis-12,13-epoxyoctadec-cis-9-enoic) is found in the seeds of *A. mellifera* (Seigler, 2003). Other fatty acids found are oleic and linoleic acid (Seigler, 2003). Previous studies indicate that the aqueous extracts of *Acacia mellifera* are safe and have shown significant anti-HSV activity (Tolo, 2004). Dichloromethane extracts of this

plant are rich in lupane-type triterpenoids, some of which with anti-tumor activity (Mutai *et al.*, 2004).

2.8.5 Vector control

2.8.5.1 Personal protective measures

Use of insecticide treated bed nets, window screens with very fine mesh, and use of repellents reduce man-fly contact especially where sandflies mainly feed indoors (Davies *et al.*, 2003). To prevent transmission outdoors, avoidance of outdoor activities at dawn or at dusk and use of repellents should be done (Lane and Crosskey, 1993). Repellents currently useful include permethrin, and diethyl metatoluamide (DEET®). DEET has been found to be very effective in repelling most biting insects including sandflies. New settlements in forests should preferably be surrounded by a forest-free belt about 300-400m in diameter (Rozendaal, 1997).

2.8.5.2 Use of insecticides

Indoor residual spraying (IRS) of houses and farm buildings with insecticide is the most widely used intervention for controlling sandflies that are endophilic (Davies *et al.*, 2003). Targeted insecticidal applications to resting habitats are necessary to enhance control (Ryan *et al.*, 2006). Large scale spraying is recommended during epidemics (Mishra, 2005). The groups of chemical insecticides used against sand flies include organochlorines, organophosphates, carbamates and pyrethroids.

Organochlorines such as dichloro diphenyl trichloromethane (DDT) and lindane act by contact with the insects. DDT has been used extensively in the past with high efficacy, low cost and long residual action. However, environmental side effects and human health hazards are militating against it since they are very persistent in soil and in tissues of plants and animals (Lainson and Rangel, 2005). Organophosphorus compounds, carbamates and pyrethroids are less persistent and breakdown quickly in the environment. Organophosphorus compounds such as temephos, fenthion and pirimiphos methyl; carbamates such as propoxur; and pyrethroids such as permethrin, deltamethrin, lambda cyhalothrin and cyfluthrin have commendable activities (Rozendaal, 1997). Deltamethrin, permethrin, and fenthion have been shown to reduce blood-feeding rates and to increase sandfly deaths (Reithinger *et al.*, 2001). Synthetic pyrethroids are quick acting and highly toxic to insects (Rozendaal, 1997). They are considered generally safe to humans at recommended doses.

Limitations facing vector control are diversity and inaccessibility of breeding grounds and insecticide resistance. Spraying houses regularly is not sustainable due to high costs (WHO, 2004). Cessation of spraying campaigns invariably leads to the re-emergence of leishmaniasis to pre-control levels (Davies *et al.*, 2003). There have been reports of *Phlebotomus argentipes* developing resistance (Bora, 1999).

2.8.5.3 Environmental Management.

Environmental management strategies are key in control of leishmaniasis (WHO, 2007). People living in endemic areas are encouraged to eliminate termite mounds near their

houses since these provide breeding places for the sandflies. They are also encouraged to fill up animal burrows around their compounds to keep away possible animal reservoirs such as rodents.

2.8.6 Control of reservoir hosts

Case detection and treatment of asymptomatic seropositive patients in anthroponotic foci reduce transmission by reducing human reservoir (Desjeux, 2001). Previous findings have shown that subclinical and treated cases remain potential reservoirs for long periods (Ryan *et al.*, 2006). The diagnostic tool may vary for each leishmanial syndrome, but the gold standard is demonstration of parasites from tissues (Singh and Sivakumar, 2003). *Leishmania* parasites are demonstrated from a splenic or bone marrow aspirate. Splenic aspirate, though associated with risk of fatal hemorrhage in inexperienced hands, is the most valuable method for diagnosis of kala-azar, with a sensitivity exceeding 95% (Sundar and Rai, 2002).

Reservoir hosts serve to maintain parasite population in nature. These often have no clinical symptoms but can maintain a prolonged infection. The primary source of infection for vectors is usually a wild mammal. Numerous species of rodents, marsupials and carnivores have been reported as natural hosts in Brazil (Rotureau, 2006). Domestic dogs are known to be the most important reservoir hosts of *Leishmania infantum* and *L. chagasi.* Spraying in and around kennels was reported to reduce risk of infection (Killick-Kendrick and Baneth, 2002). Deltamethrin-impregnated collars are effective in killing sandflies that feed on dogs, and have strong anti-feeding effect on the flies (Killick-

Kendrick and Baneth, 2002). The collars contain a formulation that permits slow release of insecticide to the lipids of the dog's skin through the complete body of the animal and retains the activity for many months. The implementation of a topical insecticide treatment such as topical lotions to protect dogs has also been advocated for it is likely to have consent of a population at risk (Reithinger *et al.*, 2001).

Treating infected dogs with antileishmanial agents is not a practical approach because of the prohibitive cost involved and the high relapse rates among treated and clinically cured dogs (Reithinger *et al.*, 2001). Destruction of serologically and parasitologically positive dogs temporarily affects the cumulative incidence of seroconversion in these animals and diminishes incidence of human cases of visceral leishmaniasis. The efficacy of dog culling is however questionable (WHO, 2004).

2.8.7 Health Education

Health education incorporated with other control measures which include rapid diagnosis, complete treatment, spraying of residual insecticide and elimination of seropositive dogs was found to have a great impact in an emergency control plan (1994) in Brazil (Arias *et al.*, 1996).

CHAPTER 3: MATERIALS AND METHODS

3.1. Study area

The study area was Machakos District located in Eastern Province, Kenya (Appendix 3). Machakos is one of the endemic areas for visceral leishmaniasis. Plant material was collected from Central and Kalama divisions (areas shaded in the map). The district stretches from latitudes 0 ° 45' South to 1 ° 31' North, and longitudes 36° 45'East to 37 ° 45'East (CBS, 2002-2008). Most of the district is semi-arid, and receives very little and erratic rainfall. Vegetation in the district varies with altitude. The plains, which receive less rainfall, are characterized by open grassland with scattered *Acacia* trees. The high altitude areas that receive high rainfall have dense vegetation.

3.2 Materials

3.2.1 *Acacia mellifera*

The stem bark of *Acacia mellifera* subsp. *mellifera* were collected and identified at the East African Herbarium-Museum. A voucher specimen was deposited at the Museum with voucher number ChM-1. These were dried at room temperature and ground to powder in preparation for extraction.

3.2.2 Parasites

Leishmania major parasites (Strain IDUB/KE/83=NLB-144) were used. These were originally isolated from a female *Phlebotomus duboscqi* Neveu-Lemaire collected near Marigat, Baringo district, Rift Valley province, Kenya (Beach *et al.*, 1984) and have been

maintained by cryopreservation, *in vitro* culture, and periodic passage in BALB/c mice at KEMRI. BALB/c mice are highly susceptible to *Leishmania major*; upon infection they develop large skin ulcers, which expand and metastasize, leading to death (Handman, 2001).

3.2.3 Macrophages

These were obtained according to methods described by Shaw and Anderson (1984). Briefly, BALB/c mice were injected intraperitoneally with 2% starch solution to stimulate macrophage proliferation, and after three days, they were killed. This was done by placing mice, one at a time, in a jar with chloroform. The body surface was disinfected, and the skin torn dorso-ventrally to expose the peritoneum. A sterile syringe was used to inject 10 ml of chilled incomplete RPMI 1640 (Gibco®) into the stimulated peritoneum. Mouse peritoneal macrophages were harvested by withdrawing the lavage and placing it in sterile centrifuge tubes. The cell suspension was centrifuged at 755g for 10 minutes at 4°C. A pellet which formed in the centrifuging tube was re-suspended in 2ml of complete RPMI 1640 medium. This pellet contained macrophages. These were kept in a jar of ice briefly to keep them from being active before culturing them.

3.3 Plant preparation

3.3.1 Extraction of the plant material

The powdered stem barks (2.25kg) of *A. mellifera* were extracted, in succession, with dichloromethane (100%), dichloromethane (in 50% methanol) and methanol (100%).

The mixture was kept at room temperature and occasionally agitated to facilitate the extraction process. After 24 hours, the sample was decanted and filtered through paper funnel. The extracts were evaporated *in vacuo* to dryness in rotary evaporator at 40°C and TLC analysis was done. The TLC analysis of the dichloromethane extracts revealed number of spots after spraying with concentrated sulphuric acid. The dichloromethane (100%) and dichloromethane (in 50% methanol) showed related TLC profile.

3.3.2 Fractionation of dichloromethane extract.

The dichloromethane residue (6.6482g) was separated on a silica gel column. Elution was done using solvents with increasing polarity down the column (cyclohexane, ethylacetate, then methanol). Increasing solvent polarity forces compounds of greater polarity to come down the column (Pavia *et al.*, 1976). The column was eluted with cyclohexane (C_6H_6) - ethylacetate (EtOAc) (gradient) as shown in Table 1. Cyclohexane is a highly non-polar solvent and was expected to elute non-polar compounds. Small percentages of ethylacetate were mixed slowly with cyclohexane since a rapid change from one solvent to another can bring about cracking of column packing. The column was further eluted later with ethylacetate (EtOAc) containing 1% methanol (MeOH), 2% MeOH, 5% MeOH, 10% MeOH, 25% MeOH, and finally with pure MeOH. Methanol, a more polar solvent, was expected to move more compounds down the column.

Table 1: The following table shows gradient elution of dichloromethane extract using cyclohexane and ethylacetate mixture in order of increasing polarity.

Cyclohexane (C_6H_6) in%	Ethyl acetate (EtOAc) in %	Total amount in ml
100	0	300
95	5	285/15
90	10	270/30
85	15	255/45
80	20	240/60
75	25	225/75
70	30	210/90
65	35	195/105
60	40	180/120
55	45	165/135
50	50	150/150
45	55	135/165
40	60	120/180
35	65	105/195
30	70	90/210
25	75	75/225
20	80	60/240
15	85	45/255
10	90	30/270
5	95	15/285
0	100	300

Fractions (50ml) were collected, analyzed by TLC and combined where necessary depending on the results. And each fraction was further purified by column chromatography (CC) and/or preparative thin layer chromatography (TLC).

Fractions 1-19 yielded an oily substance, which appeared as a single spot on TLC using the cyclohexane/ ethyl acetate (50:50) solvent system. It was concentrated by evaporation and was labeled **AM-I**. Fractions 20-23 were a mixture, showing three spots when analyzed by TLC using the same solvent system as for AM-I. The fraction 20-23 was labeled **AM-II.** Fraction 24-25 was labeled **AM-III** and TLC profile showed 4 spots on using cyclohexane/ ethyl acetate (95:5) solvent system. Fraction 26-28 was labeled **AM-IV,** fractions 29-36 was labeled **AM-V** and fraction 37-39 (labeled **AM-VI**). Fraction 40-43 was labeled **AM-VII** and fraction 44-55 was labeled **AM-VIII.** Fractions 56-61 (labeled **AM-IX**) showed four spots on TLC.

3.3.3 Drug reconstitution

The extracts were weighed and dissolved in culture media. 1% v/v dimethyl sulphoxide (DMSO) was used to increase solubility of extracts during reconstitution (Hoet *et al.*, 2004; Ferreira *et al.*, 2004). These were then passed through 0.45 and 0.2 μm pore size membrane filters (Nalgene®), before being introduced into tissue culture flasks or culture plates. Serial dilutions were performed to obtain a range of concentrations. Assays in cell-free media were performed using doubling concentrations with the maximum concentration being 5mg/ml. Macrophage assays were performed using a maximum concentration of 250μg/ml due to the toxicity of higher concentrations on macrophages.

3.4 Cell free assays

3.4.1 Anti-promastigote assay

The anti-promastigote assay was performed according to the methods used by Berman and Lee (1984). Procedures used to prepare media are included in Appendix I. Briefly, *Leishmania major* parasites were obtained from lesions on hind footpads of infected BALB/c mice. These parasites were cultured in Novy Nicolle McNeal (NNN) media overlaid with 2 ml Schneider's *Drosophila* medium (Sigma®), supplemented with 20% fetal bovine serum (FBS) (Sigma®), 100µg/ml streptomycin and 100U/ml G-penicillin (Sigma®), and 5-fluorocytosine (Sigma®). The cultures were made in 25 cm^2 sterile disposable culture flasks (Corning®) and incubated at 25°C as recommended by Evans *et al.* (1989), to stationary metacyclic stage. Promastigotes were then counted at x400 magnification using an improved neubauer chamber, using the middle square. Parasite population was estimated using the formula: Counts x dilution factor x 5 x 10^4.

Promastigotes were seeded in microtiter well plates in the presence (experimental group) and absence of the plant extracts (control group). After four days of cultivation, promastigotes were immobilized in 2% formalin in PBS and counted in a haemocytometer. Trypan blue dye was used to distinguish between the live and the dead promastigotes.

3.4.2 Anti-amastigote assay

Amastigotes were harvested from infected mice with well-swollen lesions on their hindfeet. Footpads were excised and ground using a homogenizer and the homogenate

centrifuged at 755g for 15minutes at 4° C. Parasite counts were taken and the numbers adjusted to 1 x 10^6 amastigotes/ml. These were seeded and incubated in complete Schneider's medium in microtiter well plates (Nunc™) for 10 days. Experimental cultures were incubated in the presence of doubling concentrations of the plant extracts (156, 312.5, 625, 1250, 2500, and 5000µg/ml), whereas control culture in the absence. Thereafter, the transformed parasites were counted using a hemocytometer, and inhibition concentration fifty (IC_{50}) determined according to the formula used by Sixsmith *et al.* (1984), as illustrated in section 3.5.

3.5 Macrophage assay

3.5.1 Activity of *Acacia mellifera* on macrophages

Activity of extracts of *Acacia mellifera* was determined as described by Berman and Lee (1984). Briefly, macrophage viability was confirmed using trypan blue dye. Dead cells took up the dye due to breakdown of the cellular permeability barrier whereas live cells remained bright. Parasite concentrations were determined by counting using an improved neubaeur chamber. The average count was multiplied by the dilution factor used and by 10^4, and then macrophage numbers adjusted to 1x10^5 live cells per ml. These were adsorbed to microwell plates (Nunc™) and incubated for 24 hours at 37°C, 95% humidity and 5% CO_2. They were infected with amastigotes, and 4 hours later, cultures were washed with Hank's Balanced Salt Solution (HBSS) (Gibco®) to remove non-phagocytized parasites. Infected macrophages were incubated further for 24 hours in culture medium consisting of complete RPMI 1640 (Gibco®). Thereafter, plant extracts were introduced to the cultures in different concentrations (31.25, 62, 125, 250, 500 and

1000µg/ml). These continued to incubate in 95% air, and 5% CO_2 at 37°C for six days in the absence (control) and in presence of *Acacia mellifera*. All tests were done in triplicate. The medium and extracts were replenished after three days. The cultures were then fixed with absolute methanol, stained with 10% Giemsa stain. The bases of culture wells were carefully cut out and stuck on slides for examination at x 1,000 magnification, and parasite counts taken.

3.5.2 Measurement of nitric oxide production

Nitrite oxide release in supernatants of macrophage culture was measured by the Griess diazotization reaction for nitrites as described by Holzmuller *et al.* (2002). Briefly, supernatants were collected (100µl), 48 hours after introducing plant extracts into the culture medium. These were put in triplicate wells in a 96-well microtiter plate. To this, 60µl of Griess Reagent A (sulphanilic acid) was added, then 60µl of Griess Reagent B (0.3% N-[-naphthyl]ethylene diamine). The mixture was incubated for 30 minutes at room temperature. A photometric reference sample was prepared by mixing 120µl of Griess reagents with 100µl of culture media. The absorbance of the samples was measured relative to the reference sample at 490 nm in an enzyme-linked immunosorbent assay plate reader. Sodium nitrite ($NaNO_2$) in RPMI with concentrations between 1-100µM was used to construct a standard curve for each plate reading. Absorbance readings were converted to nitrite concentrations.

3.5.3 Cytotoxicity assays

Effects of the plant extract were determined by observation of morphological changes on mouse peritoneal macrophages resulting from introduction of fractions of the dichloromethane extract of *Acacia mellifera*. Briefly, cells were maintained in complete RPMI Medium. They were cultivated in microtiter plates at 37°C in a humidified 5% CO_2 atmosphere. After 24 hours, extracts were introduced in concentrations of 31.25, 62, 125, 250, 500, 1000µg/ml, and then incubated for 48 hours. Cytotoxicity was determined by the destruction on the macrophages that were treated.

3.6 Data analysis

The data collected consisted of the effects of different extracts and different concentrations. Means of parasite numbers were analyzed by using chi-square and Kruskal-Wallis test. Percentages were used in the search for inhibition concentrations of *Acacia mellifera* on *L. major*. The following formulae were used in analysis of data:

Mortality rate = (Number of parasites dead/ Total number of parasites counted) x 100%.

$$\mathbf{IC}_{50} = \text{Antilog}\left\{\log X_1 + \left\{ \frac{[\log Y_{50} - \log Y_1]}{[\log Y_2 - \log Y_1]} [\log X_2 - \log X_1] \right\}\right\}$$

Where Y_{50} is half of the average of controls = $\frac{\Sigma \text{controls}}{\text{No of wells}} \times \frac{1}{2}$

X_1 is the lower concentration, to the Y_{50} value; X_2 is the higher concentration, to the Y_{50} value; Y_1 is the counts correlating to X_1 and, Y_2 is the counts correlating to X_2.

Infection rate = No of infected macrophages /100 macrophages x 100.

Percentage of amastigotes surviving

$$= \left[\frac{\text{No. of amastigotes / 100 macrophages in experimental culture}}{\text{No. of amastigotes/ 100 macrophages in control cultures}} \right] \times 100.$$

CHAPTER 4: RESULTS.

4.1 Activity of *Acacia mellifera* against *Leishmania major* promastigotes

From the anti-promastigote assays, there was inhibition of promastigote population with 100% mortality at a concentration of 5mg/ml for all the extracts tested as determined using the trypan blue exclusion test (table 2). At a lower concentration of 2.5mg/ml, live parasites were observed indicating lower inhibition of *Leishmania major* promastigotes. Methanol extracts (CH_3OH) had very low level of inhibition with a mortality rate of 0.725% only. Similarly, the stem bark dichloromethane extract (CH_2Cl_2) had mortality rate of 4%. Higher mortality rates were however recorded with the stem bark dichloromethane/methanol extract where 47.6% was recorded. This result indicates that the dichloromethane/ methanol extract had a higher inhibitory capacity than the other two extracts. For the methanol and dichloromethane extracts, their inhibition is likely to be between the concentration of 2.5mg/ml and 5mg/ml since 100% inhibition was recorded at the higher concentration of 5mg/ml.

Table 2. Mortality rates determined from parasites grown in the presence of 2.5mg/ml and 5mg/ml *Acacia mellifera* extracts by the fourth day.

Extract	2.5mg/ml	5mg/ml
Stem bark, dichloromethane (CH_2Cl_2)	4%	100%
Stem bark, dichloromethane in methanol	47.6%	100%
Stem bark, methanol (CH_3OH)	0.725%	100%

4.2. Parasite transformation following exposure to *Acacia mellifera* extracts

In this experiment, *Leishmania major* amastigotes had been seeded into 96 well plates and exposed to *Acacia mellifera* extracts. Transformation of the parasites was not inhibited, as they were able to transform to the promastigote stages. Promastigote forms were observed in all the cultures that had been done. However, very few parasites had fully transformed to the promastigote metacyclic stage; most were still in the intermediate forms, especially the nectomonad stage. Their development was slowed down by the presence of the extracts. Figure 2 shows the parasite populations for doubling concentrations between 156µg/ml and 5000µg/ml.

The parasite numbers from cultures treated with dichloromethane extracts were much higher than the numbers in the controls. Methanolic extract recorded the lowest parasite population at 5000 µg/ml. Comparison of the extracts showed that there was a significant difference in the promastigote numbers for the different extracts ($F= 7.169$; $df =3, 20$; $P<0.05$). Tukey's multiple comparison test then showed that there was no significant difference between controls and methanol or dichloromethane/methanol extract. However, there was a significant difference in the activity of dichloromethane extract ($P<0.05$). A steady rise of parasite numbers with increase of the concentration was evident from the culture in dichloromethane extract. This was a complete contrast to the expectation of inhibition where parasite numbers would decrease with increase of the extract. This observation indicated that the methanol and dichloromethane extracts of *Acacia mellifera* did not inhibit the transformation or development of amastigotes.

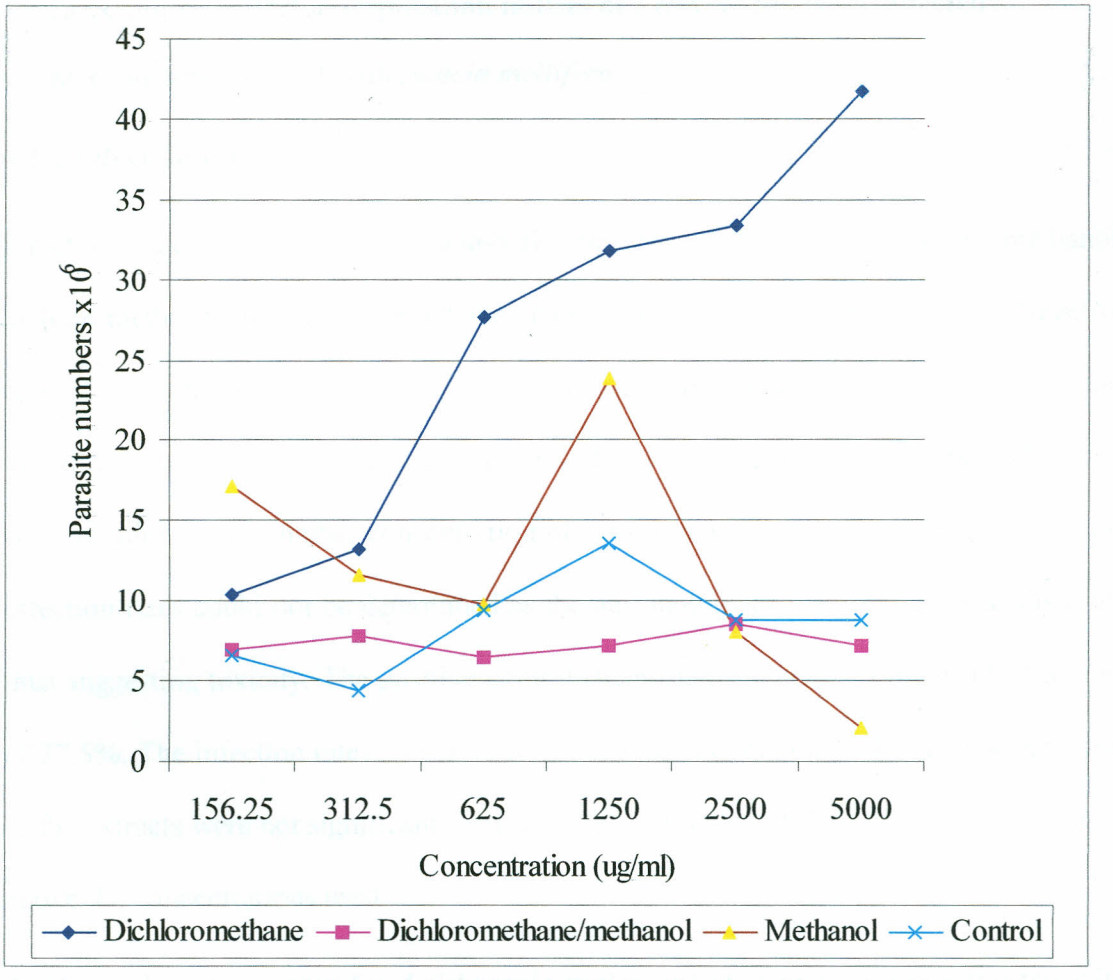

Figure 2. Parasite numbers of *Leishmania major* promastigote forms with increase of concentration of stem bark extracts of *Acacia mellifera*.

4.3 Infection rates and multiplication indices of *Leishmania major*-infected macrophages treated with *Acacia mellifera.*

4.3.1 Infection rates

Infection rates of the three stem-bark extracts of *Acacia mellifera*, methanol, dichloromethane, and dichloromethane/ methanol extracts were determined. Negative controls, which had RPMI medium, only, had an average infection rate of 73%. Results in Table 3 show the percentage infection rate from 31.25µg/ml to 250µg/ml in doubling concentrations. At a higher concentration of 1000µg/ml, many cells were destroyed and infection rates could not be determined as the morphology of macrophages was distorted thus suggesting toxicity. The positive control (Pentostam®) had the lowest infection rate of 27.5%. The infection rates increased as the drug concentrations reduced. The activities of the extracts were not significantly different (χ^2 =13.89; df=9; P>0.05). Their IC_{50}s were above the concentrations used.

Table 3: Infection rates for *Leishmania major*- infected macrophages treated with extracts of *Acacia mellifera* extracts at varying concentrations.

Concentration of extract (µg/ml)	Extracts of *Acacia mellifera*			Positive Control, Pentostam®
	Methanol	Dichloromethane	Dichloromethane in methanol	
250	41.4%	52.5%	51.2%	27.5%
125	60.7%	51.5%	58.7%	52.0%
62.5	64.4%	61.8%	61.5%	69.0%
31.25	69.0%	78.5%	63.0%	82.3%

Activity of fractions of dichloromethane was tested and infection rates also determined. Table 4 below shows the infection rates at different concentrations of the fractions. Four out of nine fractions were tested. Activities of fractions I-V could not be determined as the quantities obtained were too small. There was a difference among the fractions used ($F= 6.827$; $df =4,15$; $P<0.05$). Tukey's multiple comparison test revealed that effects of fractions VII, VIII, and IX were very similar. Effects of fractions VIII and IX had similarities to the effects of Pentostam®. However, effect of fraction VII did not equal that of Pentostam®, thus suggesting that this activity was higher than that of its close rivals i.e., fractions VIII and IX. Effects of fraction VI did not equal that of any other fraction. However, it was close to that of Pentostam®.

Table 4: Infection rates for the *Leishmania major*- infected macrophages treated with four fractions of dichloromethane extract of *Acacia mellifera* at varying concentrations.

Parameter	Concentration of fractions (µg/ml)	Fractions of dichloromethane extract				Positive Control (Pentostam®)
		VI	VII	VIII	IX	
Infection Rates (%)	250	63.0%	30.0%	37.7%	37.0%	27.5%
	125	70.0%	26.0%	33.3%	47.2%	52.0%
	62.5	75.5%	33.3%	51.0%	40.5%	69.0%
	31.25	74.5%	52.0%	59.0%	40.0%	82.3%
IC50s (µg/ml)		>500	54.19	107.67	263.9	91.8

From the infection rates, IC_{50}s were determined. Among the fractions tested, VII had the highest activity (where the IC_{50} was lowest) with a reading of 54.19µg/ml (Table 4). This was followed by fraction VIII then fraction IX that had 107.67 and 263.9µg/ml respectively. Fraction VI had the lowest activity since a very high concentration of the drug was needed to inhibit amastigotes. The positive control Pentostam® had an IC_{50} of 91.8µg/ml.

4.3.2 Multiplication indices

4.3.2.1. Multiplication indices for macrophages treated with extracts of *Acacia mellifera.*

Multiplication indices showed the percentage of amastigotes surviving in macrophages after subjection to treatments with extracts of *Acacia mellifera.* Results showed that multiplication indices increased with the lowering of the concentration of a plant extract. Treatment with extracts showed that there was a difference in the indices of the treatments (χ^2=38.72; df=6; P<0.05). The positive control had considerable inhibition of amastigotes at 250µg/ml. Low concentrations of Pentostam, however, (between 125 and 31.25µg/ml) did not show activity against *Leishmania major* amastigotes. This is demonstrated by the high indices recorded. A summary of the percentage multiplication indices for extracts of *A. mellifera* is shown in Table 5.

Table 5: Multiplication indices for *Leishmania major*- infected macrophages treated with extracts of *Acacia mellifera*.

Concentration of treatment (µg/ml)	Methanol	Dichloromethane	Pentostam®, Positive control.
250	53.8%	44.9%	46.0%
125	22.7%	48.1%	105.3%
62.5	35.7%	53.0%	96.1%
31.25	47.9%	61.8%	134.4%

4.3.2.2 Multiplication indices for macrophages treated with fractions of the dichloromethane extracts of *Acacia mellifera*.

Among the fractions of dichloromethane, fraction IX had the lowest multiplication indices indicating the lowest numbers of amastigotes surviving after treatment (Table 6). This indicated that it had the highest activity when compared to the activities of the other fractions. Fraction VII had relatively similar activity recorded for concentrations of 250 and 125µg/ml. Lower concentrations, that is, 62.5 and 31.25µg/ml had increasing multiplication indices showing lower activity. Fraction VI had the highest number of amastigotes surviving. This observation relates with the results on infection rates where fraction VI also had the highest infection rates. Chi-square analysis showed that there was a difference in the activities of the different fractions (χ^2=68.32; df=12; $p<0.05$).

Table 6. Multiplication indices after treatment of infected macrophages with fractions of dichloromethane extract of *Acacia mellifera* using varying concentrations.

Concentration of fractions in µg/ml	Fractions of dichloromethane extract				Positive control, Pentostam
	VI	VII	VIII	IX	
250	111.35%	33.19%	46.51%	31.11%	46.0%
125	144.9%	35.74%	35.02%	40.38%	105.3%
62.5	112.9%	51.62%	60.7%	34.98%	96.1%
31.25	99.7%	93.19%	55.87%	39.79%	134.4%

4.4 Immunostimulatory effects of *Acacia mellifera* on infected macrophages.

4.4.1 Immunostimulatory effects among extracts of *Acacia mellifera.*

From the results, methanol extract had a steady increase in nitrite levels with increase of the concentration (figure 3). Kruskal-Wallis test was used to compare the means of the nitrite levels obtained. This showed that there was a difference among the means ($H_{4,4,4,4}$ = 14.04; $P<0.05$). Activity of the methanol extract was higher than that of dichloromethane extract, the negative control (RPMI) and of the positive control (Pentostam®). This extract stimulated production of significant levels of nitric oxide.

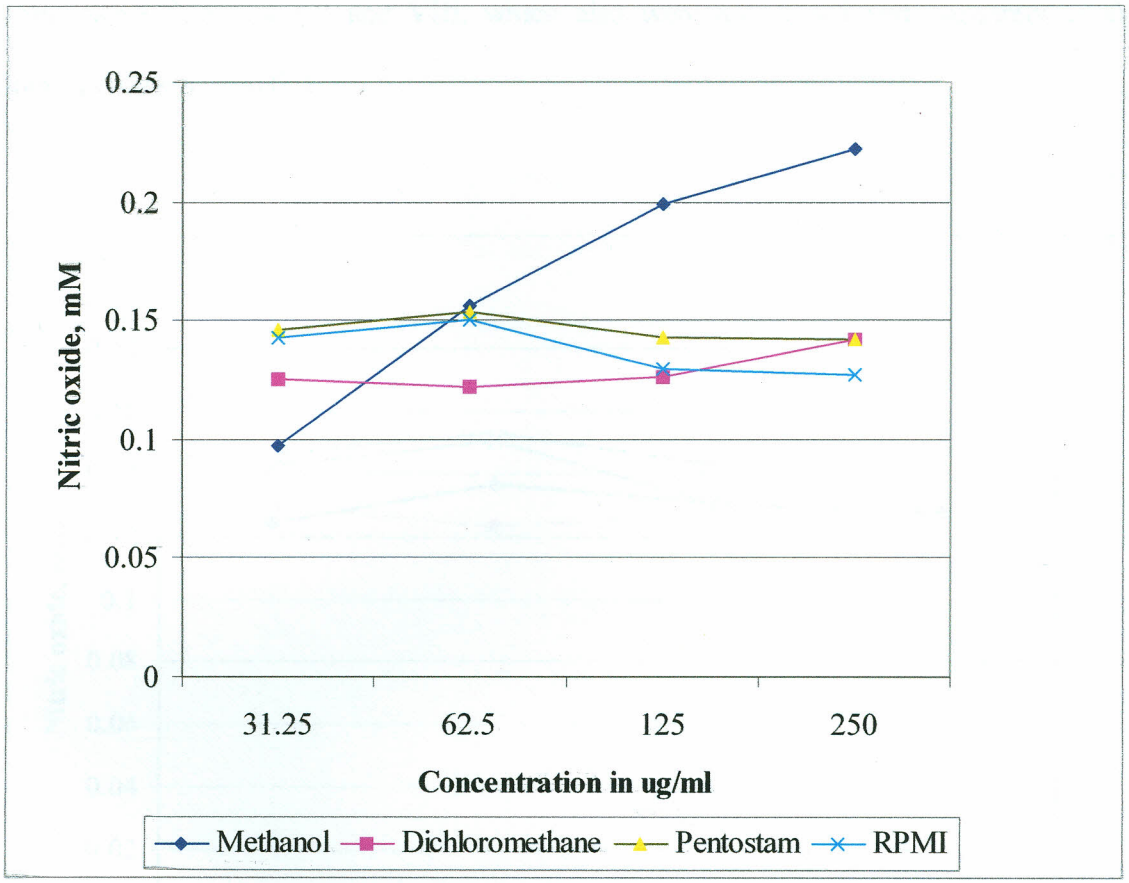

Figure 3. A graph of nitric oxide levels from the treatment of *Leishmania major*-infected macrophages with extracts of *Acacia mellifera* for the concentrations between 31.25- 250 µg/ml.

4.4.2 Nitric oxide production among fractions of dichloromethane extract of *Acacia mellifera*.

Fractions of dichloromethane extract of *Acacia mellifera* were also tested for immunostimulatory activities. Figure 4 shows the results for nitric oxide production between fractions VI and IX. A statistical analysis of these results showed that there was no significant difference in production of nitric oxide among the treatments ($P>0.05$). Two other fractions of dichloromethane extract were tested for production of nitric oxide.

These were fractions VII and VIII, which also were not significantly different from control cultures (P>0.05).

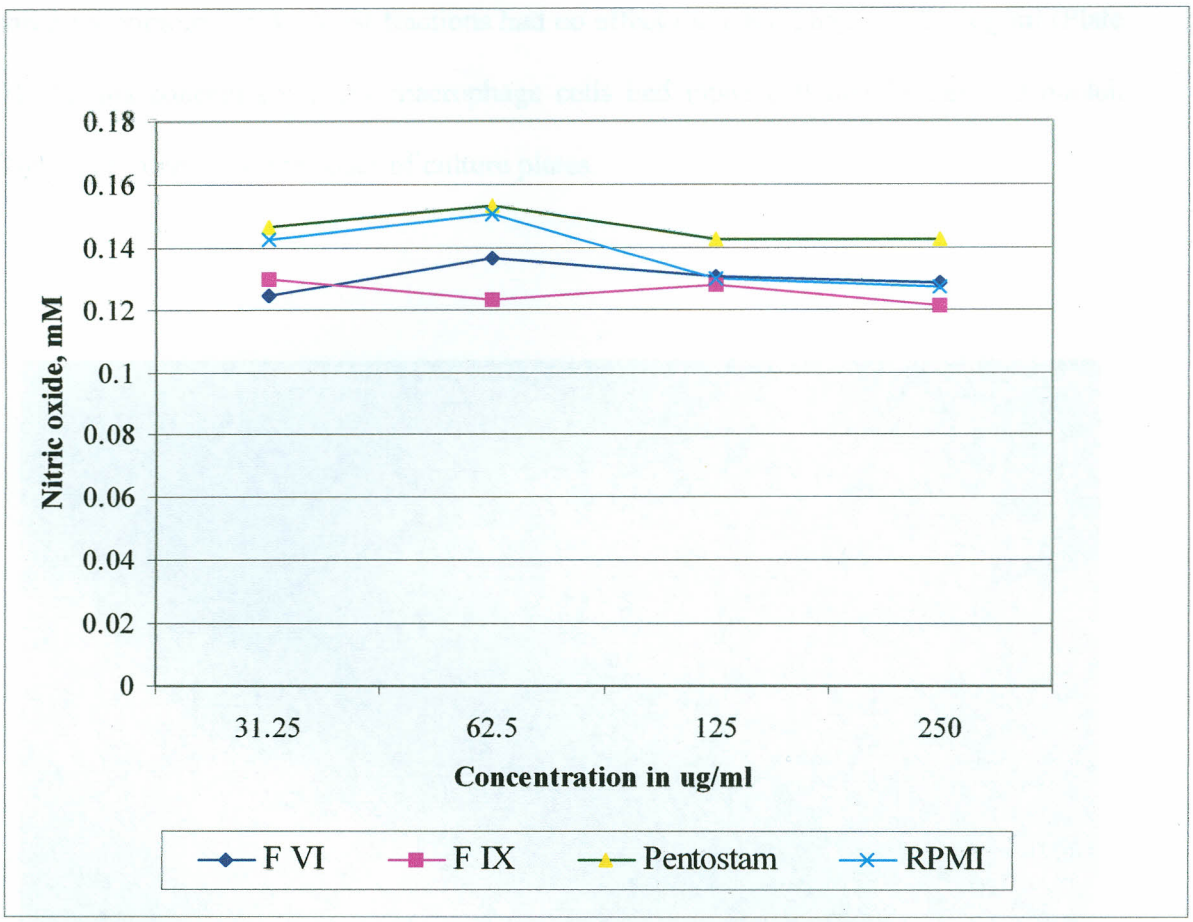

Figure 4. Comparison of nitric oxide production levels for fractions VI and IX of stem bark dichloromethane extract of *Acacia mellifera* using concentrations between 31.25µg/ml and 250µg/ml.

4.5 Effect of fractions of dichloromethane on uninfected macrophages

The extracts and fractions of *Acacia mellifera* had effect on mouse peritoneal macrophages at 1000 µg/ml. The cells observed were found to have their cell membrane integrity compromised. Most fractions had no effect on macrophages at 250µg/ml (Plate 1). At this concentration, the macrophage cells had intact cell membranes and nuclei. They also adhered to the bases of culture plates.

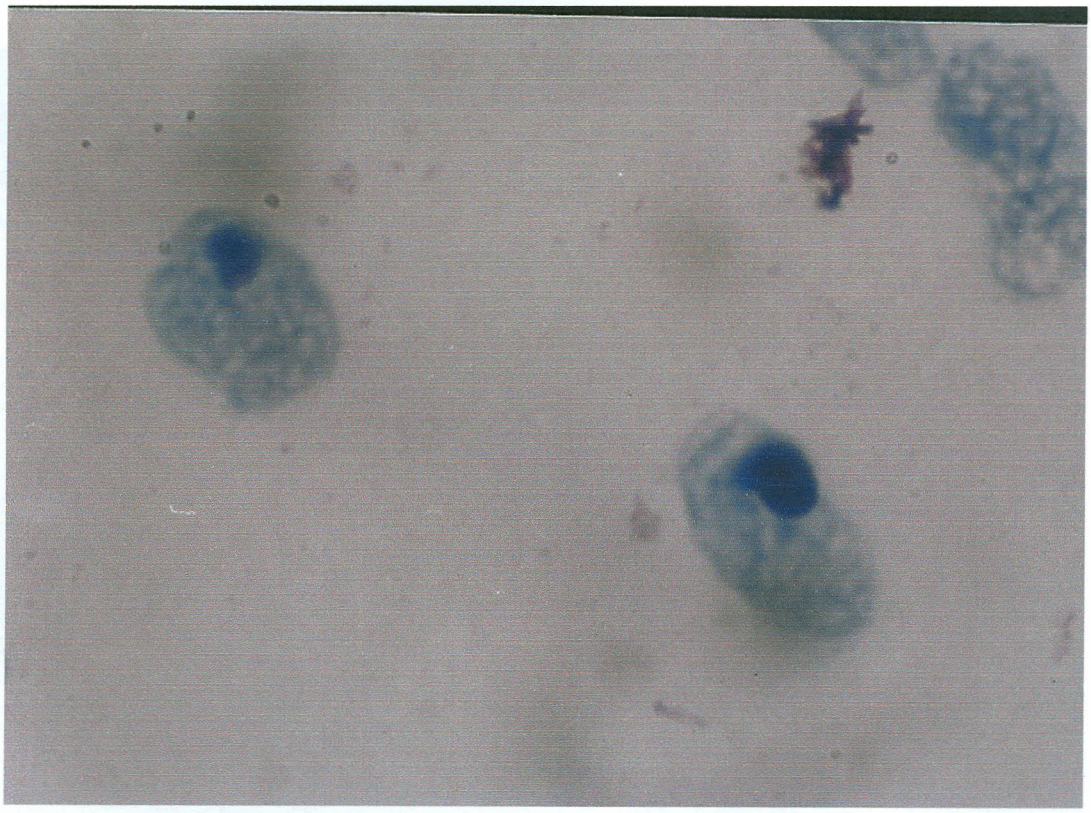

Plate 1. A photograph of mouse peritoneal macrophages treated with fraction IX at 250µg/ml.

Fraction V was very potent as most cells begun to disintegrate only minutes after introduction of the drug. Many cells were detached from the bases of the culture well plates. This activity was observed at even lower concentrations such as 125µg/ml. Concentrations lower than 100µg/ml did not have similar activity. The fraction destroyed by lysing the cells (Plate 2). It is likely to have had trysinizing ability as it broke the enzymes that allow normal macrophages to adhere to culture plate bases. Most of the cells did not have definite nuclei. This seemed to be destroyed. The cell membranes of the macrophages were shown to be rugged.

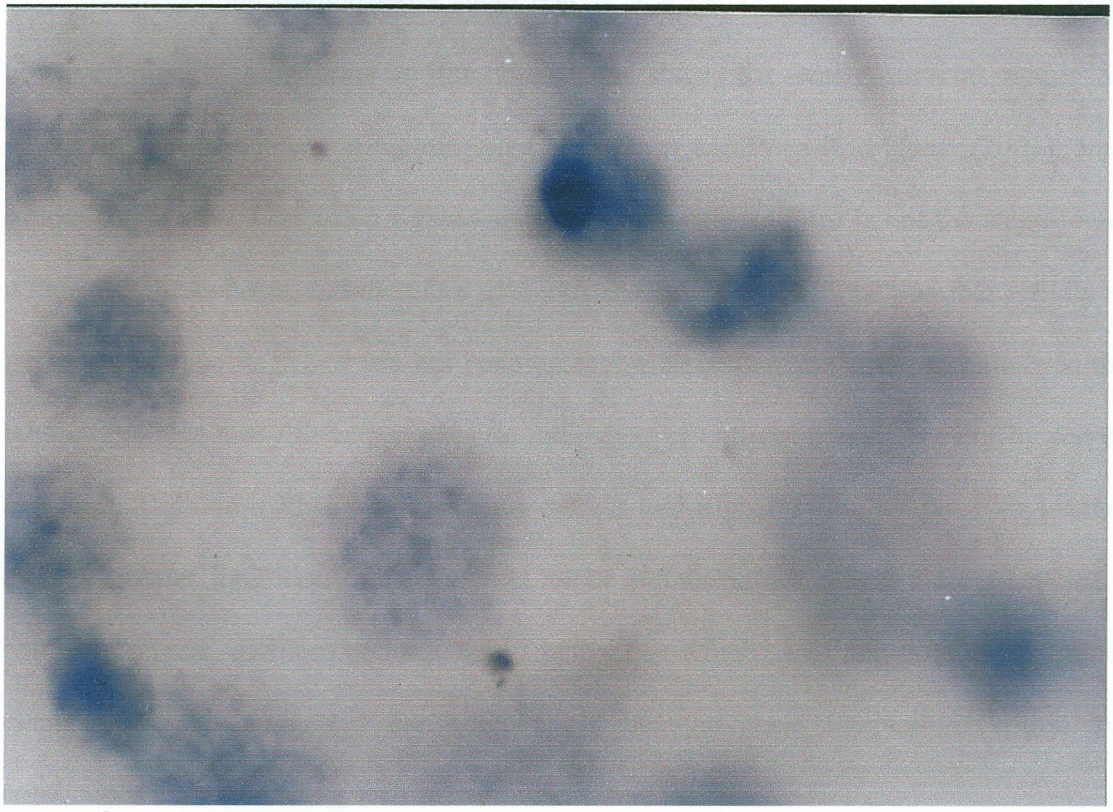

Plate 2. A photograph of mouse peritoneal macrophages treated with fraction V at 1000µg/ml.

CHAPTER 5: DISCUSSION

5.1 Effects of *Acacia mellifera* extracts against *Leishmania major* promastigotes.

Results from cell free assays showed that treatment of *Leishmania major* promastigotes with *Acacia mellifera* extracts led to variable mortality rates. The concentrations with inhibitory activity were in the range between 2.5mg/ml and 5mg/ml. Anti-promastigote assays are among the widely performed antileishmanial assays. A dose-dependent anti-promastigote effect from a chloroform extract of *Peschiera australis* was reported by Delorenzi *et al.* (2001). During an infection through the bite of an infected sandfly vector, it is the metacyclic promastigote stage that is injected into the new host. This stage however quickly transforms to the amastigote stage, for easier survival within an intracellular environment. According to Chan-Bacab and Péna-Rodriguez, (2001), anti-promastigote assays are often limited since the promastigote form is not the stage found within vertebrate hosts, and therefore give only indicative value of the possible activity of the tested metabolite. Their use has been extensive since they are easier to culture in the laboratory. This assay therefore gave an indication of antileishmanial activity in *Acacia mellifera*. Dichloromethane in methanol extract had higher mortality rates suggesting thereby greater inhibition of *Leishmania major* promastigotes. Since the extract had a combination of dichloromethane and methanol, which are non-polar and polar, respectively, this suggested that there could be synergistic interactions within the compounds extracted using each solvent.

5.2 Effects of *Acacia mellifera* extracts against *Leishmania major* amastigotes.

The dichloromethane, methanol and dichloromethane in methanol extracts did not inhibit development of amastigotes to promastigote stages. This was evident as promastigote stages of *Leishmania major* parasites were found after culturing amastigotes in the presence of the extracts. In the natural environment, the transformation of amastigotes to promastigote stage occurs within a *Phlebotomus duboscqi* sandfly. The development is usually suprapylarian characterized with forward migration of parasites. Studies carried out by Lawyer *et al.* (1990), indicated that the first evidence of transformation of ingested amastigotes to promastigotes occurred 12-18 hours post-infection. The development was observed to occur in three major stages. The first stage involved 0-2 days post-feeding where parasite development occurred in the bloodmeal within the peritrophic space of the abdominal midgut. This was characterized by development and multiplication of procyclics. In the second stage, 2-5 days post-feeding, the peritrophic membrane ruptures. Intense multiplication of procyclics occurs accompanied by development to nectomonads, many becoming attached to the microvillar lining by their flagella. In the third stage, the gut clears of the bloodmeal and a massive infection of dividing haptomonads, paramastigotes and nectomonads is established in the stomodeal valve. Paramastigotes and haptomonads begin to migrate anteriorly as they develop to metacyclic promastigotes spreading to the pharynx, cibarium and proboscis (Lawyer *et al.,* 1990). By this time, the fly is ready to seek another bloodmeal. These results may suggest that the extracts of *Acacia mellifera* would not prevent development of *Leishmania major* promastigotes in the vector. Consequently, these extracts would not block transmission of *Leishmania major* parasites. Earlier work on transformation assay

was performed by Tonui, (1998), who co-cultured *Leishmania major* amastigotes with lipophosphoglycan monoclonal antibodies. From his work, inhibition of parasite development was observed. Grimm (1999) suggests a tiered approach for drug development that proposes screening of amastigotes forms *in vitro* before co-culturing them with macrophages as the initial step. In his study he exposed axenic amastigotes forms to various drug concentrations for 48hours then determined anti-leishmanial activity by inhibition of uptake of radiolabelled ^{3}H-thymidine.

5.3 Infection rates and multiplication indices of *Leishmania major*-infected macrophages treated with *Acacia mellifera.*

Mouse peritoneal macrophages have been shown to be generally comparable with human monocyte-derived macrophages (Berman and Lee, 1984). The use of macrophages is in the bid to use an appropriate model in laboratory conditions. These are the cells often parasitized by *Leishmania* during an infection. Other *in vitro* host cell models that have been used include human monocytes, Chinese hamster ovary, canine peritoneal macrophages (scarcely), and continuous cell lines such as J774G8 (Chang, 1990; Madeira *et al.*, 1999). Evaluation of anti-amastigote activity using intracellular amastigotes in macrophages complements anti-promastigote assays. For the macrophage assays, much lower concentrations of the extracts as well as for Pentostam® were used (lower than 1mg/ml), as opposed to the cell-free assays (higher than 1mg/ml). These results are consistent with earlier reports that this stage was found to be intrinsically more sensitive to antileishmanials than promastigotes (Croft and Yardley, 2002). Amastigotes are reported to differ from promastigotes in morphology, biochemistry, and even at the molecular level, (Croft and Yardley, 2002). These differences are indicated to be the

explanation for the difference in drug sensitivity between the two stages. The amastigotes stage is clinically significant for drug testing, as it is the one found within an infected person. In this study, introducing amastigotes into macrophages mimics the multiplication stage within the infected host. Other researchers prefer introduction of promastigotes into macrophages (Madeira *et al.*, 1999). This method imitates the steps that occur after injection of a promastigote by a vector into a new mammalian host.

The concentrations that had inhibitory activity on the amastigotes encountered toxicity as a challenge. Evaluation for possible cytotoxicity of the fractions was carried out using non-parasitized macrophages. This was to establish whether the activity observed was selective or it was a result of general toxicity of an extract. The fractions of dichloromethane extract affected macrophages at a concentration of 1000µg/ml. Usually macrophages that have not been affected by any toxic substance develop elongations (they have spindle shapes) as they grow. When affected, some round up, others shrivel and others even become lysed. The results suggest that the fractions were not selective at the concentration of 1mg/ml. At the therapeutic level, however, the extract has no or limited toxicity to the host. Earlier work shows that aqueous extracts of *A. mellifera* had significant anti-HSV and was without any cell cytotoxicity (Tolo *et al.*, 2004).

Previous work done by Mutai *et al.* (2004) indicated presence of triterpenoids in the stem bark of *Acacia mellifera*. Triterpenes are reported to have leishmanicidal activity but these metabolites are often also toxic to macrophages (Chan-Bacab and Peña Rodriguez, 2001). Purification of extracts may lead to pure compounds with very high activity; lower

IC_{50}s are usually expected. The fractions that were tested in this study are expected to be a combination of several pure compounds. This is because of the several spots that were observed from TLC analysis. Further fractionation of the fraction may yield invaluable metabolites that would have even higher leishmanicidal activity. In other situations, the pure compounds may not be as effective as the plant extract. Certain impurities in the extracts may render them more active and less toxic (Frieder, 1996). High toxicity has kept many plant metabolites with antileishmanial activity from clinical testing due to their lack of selectivity (Chan-Bacab and Péna Rodriguez, 2001). Chemical modification of moderately active compounds can yield invaluable potential antileishmanials (Grimm, 1999).

Testing of fractions of dichloromethane extract revealed that fraction VII had much higher inhibitory activity than other fractions and Pentostam® ($q=4.98$; $q<0.05$. The activities of fractions VIII and IX had no statistical difference between them suggesting a similar active ingredient(s) in the fractions ($q=0.697$; $q>0.05$). Fraction VI had the least effect on the parasites as it had the highest infection rates and the highest percentage of amastigotes surviving. This result suggests that either the constituents within have no activity against *Leishmania major* amastigotes or there could be at least a few components with activity but the interaction with other constituents antagonize them. These results suggest that the major activity is due to fraction VII and further phytochemical studies should be done on it to identify the active chemicals.

From this study, infection rates of macrophages treated with *Acacia mellifera* extracts did not differ between the methanol and dichloromethane extracts. Infection rates have been used in previous studies to compare the activity of different compounds against amastigotes and also to determine the differences in susceptibility of different *Leishmania* species on given treatments (Madeira *et al.*, 1999). The methanol extract is mainly expected to contain polar compounds whereas the dichloromethane to contain non-polar compounds (Pavia *et al.*, 1976) These support the TLC results, which showed the 100% dichloromethane extract had related profiles with dichloromethane in 50% methanol. There is a likelihood of some non-polar compound of high molecular weight to be eluted with polar compounds as high molecular weight compounds travel slowly.

According to the classification used by Atindehou *et al.* (2004), IC_{50} lower than or equal to 8µg/ml was considered good activity. This classification is however realized more when dealing with pure compounds of plant products. Fraction VII of the dichloromethane extract had the lowest IC_{50} of 54.19µg/ml. This result indicates that the activity of this extract is moderate. Crude methanol extracts of plant species from Sudan had considerable *in vitro* antileishmanial activity. The plants *Azadiracta indica, Maytenus senegalensis* and *Eucalyptus globulus* gave IC_{50}s of 11.5, 55 and 78µg/ml (El-Tahir *et al.*, 1998). In a different classification used by Rukunga *et al.* (2004), high activity was reported for extracts that had IC_{50}s below 50mg/ml. With this classification, the extracts of *Acacia mellifera* therefore can be reported to have good activity as the activities reported were all below the mark given (50mg/ml).

5.4 Immunostimulatory effects of extracts and fractions of *Acacia mellifera*.

Results from the Griess test for nitric oxide showed that the methanol extract of *A. mellifera* had increasing levels of nitric oxide produced than other extracts or fractions. These results suggest that methanol extracts could be having some immunostimulatory effect that enabled the cultures to have lower infection rates than for dichloromethane extract. None of the fractions of dichloromethane had significant amounts of nitric oxide produced. These results suggest that the fractions of dichloromethane did not have immunostimulatory activities at the concentrations that were used. Their activity is therefore likely to be through a different mechanism for killing parasites other than immunostimulation.

The role of the immune system cannot be underestimated. Studies of murine visceral leishmaniasis infection have established that an intact T-cell population is required for antimonials to have a curative antileishmanial effect (Croft and Yardley, 2002). Protective immunity depends on the induction of T-cells producing Th1 cytokines, primarily, interferon gamma, and tumor necrosis factor alpha (TNFα). These cytokines activate the macrophage to kill through the nitric oxide mediated mechanism (Jones *et al.*, 1998). Nitric oxide is a cytotoxic molecule that may induce cell death through various mechanisms some of which are not clearly understood. It may complex with some molecular targets inducing apoptosis (Holzmuller *et al.*, 2002). The nitric oxide molecule is also thought to inactivate enzymes and impair essential cellular functions including DNA synthesis. A trend observed from the results for Pentostam® suggest that the nitric oxide level would have increased with raising the concentration. Immunostimulatory

activity has been found in certain plants with antileishmanial activity such as extracts of *Nyctanthes arbortristis* (Chan-Bacab and Pena-Rodriguez, 2001). Previous studies by Muturi (1998) on the role of nitric oxide in non-specific immunity to *Leishmania donovani* infections demonstrated that a transient rise in serum nitrate levels correlated with improvements in clinical parameters. Higher levels of nitric oxide were demonstrated following treatment of patients with Amphotericin B.

Previous work suggests that there are some extracts that do not demonstrate any immunostimulatory effects. Studies by Kim *et al.* (1999) indicated that certain flavonoids inhibit nitric oxide production in lipopolysaccharide activated RAW 264.7 cells. Activation with lipopolysaccharide triggers expression on nitric oxide synthase and nitric oxide production (Panaro *et al.,* 2001). They indicate that this may be a result of reduction of inducible nitric oxide synthase (iNOS) enzyme expression. These flavonoids included apigenin, wogonin, luteolin, tectorigen, and quercetin (Kim *et al.*, 1999). Different studies by Mittra *et al.* (2000) indicate that luteolin and quercetin inhibit cell cycle progression leading to apoptosis. The conflicting results on the role of these plant-derived flavonoids may therefore need to be clarified.

5.5 Conclusions

5.5.1. The dichloromethane in methanol stem bark extracts of *Acacia mellifera* had inhibitory activity against *Leishmania major* promastigotes *in vitro*.

5.5.2. The stem bark extracts of *A. mellifera* were shown to have no effect on the transformation of amastigotes to the promastigote forms.

5.5.3. Infection rates as well as multiplication indices show that there is inhibition of *Leishmania major* amastigotes in macrophages by three fractions of the dichloromethane extract of *Acacia mellifera*. The *in vitro* culture system for macrophage assays demonstrated the role of macrophage activation as low concentrations of the drug (µg/ml) were acting on parasites as compared to the higher concentrations (mg/ml) required for assays in cell free media. The infection rates of macrophages treated with active fractions were close to the infection rates of macrophages treated with Pentostam®, the present drug of choice. The concentrations of extracts and fractions used, i.e., between 250 and 31.25g/ml were safe on macrophages.

5.5.4. The fractions of dichloromethane extract act through a different mechanism of action on *Leishmania major* amastigotes and not through immunostimulation.

5.6 Recommendations

5.6.1. The dichloromethane in methanol stem bark extract should be evaluated further to establish whether there is synergism resulting from the combination and how this can be explored further for production of anti-leishmanial drugs.

5.6.2. The activity of dichloromethane extract on *Leishmania major* amastigotes in cell free culture should be exploited further in order to establish possible interactions that led to the proliferation and not inhibition of parasite numbers.

5.6.3. Further fractionation of methanol extract and fraction VII and IX should be done to identify the pure compounds responsible for the activity reported. This would lead to the identification of the lead compounds that can be evaluated further in drug development for leishmaniasis.

5.6.4 The potential for stimulation of immunostimulatory activity by the stem bark methanol extract can be explored further to develop compounds that will enhance the activity of the drugs in use or those yet to be developed. *In vivo* studies should follow to investigate the activity of *Acacia mellifera* against *Leishmania major*. This would allow the macrophages to interact better with T-cells and activation of cytokines would be more explicit. This in turn would eliminate any bias with the *in vitro* culture system.

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APPENDIX 1. PREPARATION OF MEDIA AND REAGENTS.

I. RPMI 1640

Incomplete RPMI.

Materials:

a) Sterile distilled water

b) RPMI sachet

c) Stirrer

d) Measuring cylinder

e) Beaker

Procedure

1. Cut the RPMI sachet and pour the contents into a clean beaker. Dissolve the remaining RPMI by adding distilled water into the sachet, and then add this into the beaker. Wash the RPMI sachet until the pink color no longer shows.
2. Top up the volume to 1L.
3. Stir to ensure an even mixture.

Complete RPMI.

Materials for 1L Complete RPMI.

a) Fetal calf serum (FCS), 20% = 200ml

b) Sodium bicarbonate, 35 ml (from stock solution of 7.5%)

c) Antibiotics: Penicillin/ Streptomycin

d) Antimycotic: 5-fluorocytosine, 0.5g.

e) Filters (0.45 and 0.22 μm pore)

f) Measuring cylinder

Procedure

1. Pour out antibiotics and the antimycotic into the mixing jar.
2. To these, add FCS, 200 ml.
3. Add sodium bicarbonate (provides a buffering system for the media).
4. Add incomplete RPMI till it reaches 1000ml.

   Note: The volume of the iRPMI will be 1000ml – [200ml(FCS) + 35ml sodium bicarbonate + Pen-Strep].
5. Stir the contents.
6. Check the pH. Adjust to 7.0 using 1N HCl if above 7 or 1N NaOH if below.
7. Filter-sterilize the media, label the name and date of preparation, then store at $4^{o}C$.

II. HANKS BALANCED SALT SOLUTION (HBSS).

Materials

a) Sachet of HBSS

b) Distilled water

c) Sodium bicarbonate

d) pH meter

Procedure

1. Pour the contents of the sachet into a beaker. Wash the sachet with distilled water, and then add this into the beaker.
2. Top up the volume to 950ml.
3. Add 0.4g sodium bicarbonate.
4. Stir the contents for 30 minutes.
5. Using the pH meter, check and adjust to 7.2.

III. PHOSPHATE BUFFERED SALINE (PBS).

PBS = PB + Saline (0.85% NaCl).

N/B: - For every part of PB, 9 parts of saline are added: -

1ml PB + 9ml Saline (for 10ml PBS)

10ml PB + 90ml Saline

100ml PB + 900ml Saline

Materials

a) Distilled water

b) Sodium chloride (NaCl)

c) Phosphate buffered (PB) powder.

Procedure to prepare 1L PBS.

1. Weigh 0.1g of PB powder and dissolve in 100ml of distilled water.
2. Prepare 0.85% saline by dissolving 8.5g NaCl, in 1L of distilled water.

   [if 0.85% = 0.85g in 100ml, then 8.5 in 1000]
3. Measure 900ml of saline and add 100ml of PB solution.
4. Autoclave to sterilize or filter-sterilize through 0.2µm pore filter.

IV. SCHNEIDER'S INSECT MEDIUM, SIGMA S-9895.

Basic media is prepared as per instructions that come with the medium. Steps to prepare complete media supplemented with 20% serum, for maintaining parasites (1L) are as follows: -

1. Take 200mls of fetal bovine serum (FBS),
2. Add Penstrep solution (25mls), or Gentamycin (1ml),
3. Add 0.5g of 5-fluorocytosine (antifungal),
4. Check pH of the media and adjust to 6.45,
5. Filter-sterilize by passing through 0.45µm pore unit, then a 0.2µm pore filter unit,
6. Store at $4^{o}C$.

V. HEAT-INACTIVATED FETAL CALF SERUM (FCS).

Inactivate FCS by boiling in a water bath at $56^{o}C$ for half an hour.

VI. STARCH (2%)

1. Dissolve 300mg of potato starch in 15 mls of distilled water.
2. Heat the mixture in a waterbath till all bubbles leave the solution.
3. Leave it to cool.
4. Load 2mls into a syringe and inject this into a clean BALB/c mouse intraperitoneally.

Appendix 2. ***Acacia mellifera*** **(Vahl) Benth, subspecies** ***mellifera.***

Appendix 3. A map of the study area, (inset of the map of Kenya), showing Machakos District.