"EXPERIMENTAL THERAPEUTIC STUDIES OF *ALLIUM SATIVUM* (LILIACEAE: ALLIACEAE) ON *LEISHMANIA MAJOR* (KINETOPLASTIDA; TRYPANOSOMATIDAE) INFECTION IN BALB/C MICE". //

BY

ATIENO BENTER OBARE

B.Ed. (Sc.)

SCHOOL OF BIOLOGICAL SCIENCES

UNIVERSITY OF NAIROBI

Reg.No.I56/7756/2006

A Thesis Submitted to the University of Nairobi, School of Biological Sciences, in Partial Fulfillment of the Requirements for the Award of Degree of Master of Science in Applied Parasitology.

2009

## DECLARATION

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

_____     _____
Signature                                 Date

**Atieno Benter Obare**

**University of Nairobi**

**Supervisors**

We confirm that the work reported in this thesis was carried out by the candidate under our supervision and has been submitted for examination with our approval

_____     _____
Signature                                 Date

**Prof Horace Ochanda, PhD**

**School of Biological Sciences**

**University of Nairobi**

_____     _____
Signature                                 Date

**Dr. Christopher Anjili, PhD,**

**Centre for Biotechnology Research & Development (CBRD)**

**Kenya Medical Research Institute (KEMRI)**

## DEDICATION

I dedicate this Thesis to my beloved mother, the late Hilda Yon'ge who worked tirelessly hard to ensure that all her children got the best. We remain indebted to you and forever grateful for the virtues of self discipline and hard work you instilled in us.

Many thanks and may God rest your soul in eternal peace.

# ACKNOWLEDGEMENT

# TABLE OF CONTENTS

**CHAPTER ONE**

**1.0. INTRODUCTION AND LITERATURE REVIEW**

## CHAPTER TWO

## 2.0. MATERIALS AND METHODS

## CHAPTER THREE

## 3.0. RESULTS

## CHAPTER FOUR

## 4.0. DISCUSSION, CONCLUSIONS AND RECCOMENDATIONS

# LIST OF TABLES

# LIST OF FIGURES

# ABBREVIATIONS

| Abbreviations | Expanded form |
|---|---|
| μg | microgram |
| % | Percent |
| °C | Degrees Celsius |
| μg | Microgram |
| μl | Micro litre |
| +ve | Positive |
| AIDS | Acquired immune deficiency syndrome |
| ANOVA | Analysis of variance |
| BALB/c | Inbred mice very susceptible to *Leishmania major* |
| CBRD | Centre for Biotechnology Research and Development |
| CL | Cutaneous leishmaniasis |
| CLF | Chloroform fraction |
| IV/IM | Intravenous/Intramuscular |
| CS | Complete Schneider's medium |
| DALYs | Disability adjusted life years |
| DCL | Diffuse Cutaneous leishmaniasis |
| Df | Degrees of freedom |
| DPX | Distyrene Plasticizer Xylene |

| ELISA | Enzyme linked immunosorbent assay |
|---|---|
| FBS | Foetal bovine serum |
| Rk39 | Dipstick test for Visceral Leishmaniasis |
| PCR | Polymerase Chain Reaction |
| HAAT | Highly active antiretroviral therapy |
| HBSS | Hanks Buffered Salt Solution |
| HCL | Hydrochloric acid |
| HIV | Human immunodeficiency virus |
| Hrs | Hours |
| $IC_{50}$ | 50% inhibitory concentration |
| ICIPE | International Centre Of Insect Physiology and Ecology |
| IFS | International Federation for Science |
| IRS | Indoor residual spraying |
| ITN | Insecticide Treated Nets |
| KEMRI | Kenya Medical Research Institute |
| Kg | Kilogramme |
| LEAP | Leishmaniasis east African platform |
| $M\varphi$ | Macrophages |
| MCL | Mucocutaneous leishmaniasis |
| Mg | Milligrams |
| MIC | Minimum inhibitory concentration |

| | |
|---|---|
| ml$^{-1}$ | Per millilitre |
| Mls | Millilitres |
| KJV | King James Version. |
| NLB | Nairobi Leishmania Bank |
| Nm | Nanometer |
| NO | Nitric oxide |
| PBS | Phosphate-buffered solution |
| PKDL | Post kala-azar dermal leishmaniasis |
| RH | Relative humidity |
| RPM | Revolutions per minute |
| RPMI 1640 | Roswell Park Memorial Institute 1640 medium |
| RT | Room temperature |
| Spp | Species |
| -ve | Negative |
| VL | Visceral leishmaniasis |
| WHO | World health organization |
| ZVL | Zoonotic visceral leishmaniasis |

# ABSTRACT

The first line therapy for all types of leishmaniases requires potentially toxic and painful multiple injections of pentavalent antimonies and aromatic diamidines. A major limitation of these drugs is that they are very expensive and cause severe side effects due to their high toxicity, hence not accessible to the majority poor populations. Recently, the emergence of antimony-resistant parasites has compelled the search for new antileishmanial agents. There is therefore an urgent need to develop cheaper, affordable and effective indigenous formulations against *Leishmania* Ross parasites. Several new antileishmanial compounds are under development, but a drug with the capacity to completely cure these infections has not been discovered. Although most active drugs against infectious agents are derived from medicinal plants, scientific evaluation of the medicinal properties of plants remains grossly understudied.

Development of anti- parasite compounds could emerge from screening of natural libraries of plant compounds with recognized anti- parasitic activities. Among these, is *Allium sativum* (L.) (Liliaceae: Alliaceae) which has been described as having immunomodulatory activity and therapeutic properties. This study has investigated the therapeutic effect of extracts from *A. sativum* for activity on *Leishmania major* Yarkimaff and Schokor amastigotes *in vivo* and promastigotes *in vitro*. Serial dilutions of the crude extract at 250μg/ml, 500μg/ml and 1000μg/ml were assayed for their activity against *L. major* in cell free cultures. The analysis program Probit was used to determine 50% inhibitory concentration ( $IC_{50}$.). *A.sativum* crude extract was found to have an $IC_{50}$ of 2.216 mg/ml. *A. sativum* extract was then examined for efficacy in treatment of *L. major*-infected BALB/c mice (*Mus musculus* L.).

The susceptible BALB/*c* mice were subcutaneously inoculated with *L.major* parasites and disease progression monitored for one month. The infected mice were then treated with different formulations of extracts from *A. sativum*.The experimental groups either received topical application of *A.sativum* ointment, oral or intraperitoneal injection with extract formulation for five weeks post infection.

The control groups either were treated with antileishmanial drug Pentostam® as a positive control or olive oil and Phosphate buffered saline (PBS) as negative control.

Parasite load/ numbers were assayed before and after treatment using Leishman Donovan Assay (LDA) and spleen impression smears (LDU) on termination. The disease progression was assessed by measuring footpad lesion sizes weekly with venier caliper for both infected and uninfected footpads of mice.

Mice were significantly protected from *L. major*, displaying decreases in lesion size and parasite burden, particularly those on topical treatment. In contrast, mice that were treated either with olive oil or PBS showed a marked increase in disease progression leading to increased lesion sizes. The ointment from extract of *A.sativum* was found to be more effective than the positive control Pentostam® . Males had larger lesions than females but lesions did not ulcerate in both cases. The marked clearance of *Leishmania* parasites from a susceptible strain of mice suggests that crude extracts of *A. sativum* represent a potentially useful formulation for treatment of cutaneous leishmaniasis.

# CHAPTER ONE

## 1.0 INTRODUCTION AND LITERATURE REVIEW
## 1.1 INTRODUCTION

### 1.1.1   Background information

Leishmaniases are diseases caused by protozoan parasites that belong to the genus *Leishmania* Ross (Reithinger *et al.*,2007),and transmitted by the bite of infected  female Sand flies mainly of the genus *Lutzomyia* Franca, and *Sergentomyia* Franca and Parrot in the New World and *Phlebotomus* Rondani,Franca and Parrot in the Old World (Piscopo *et al.*,2006). It is an important Global public health problem. The World Health Organization (WHO) estimates that 350 million people are at risk worldwide, 12 million people are infected with *Leishmania* parasites and that as many as 2 million new cases occur each year in over 80 Countries. (Desjeux, 2001).

These infections produce a variety of clinical diseases depending on the virulence or tropism of the parasite and differential host immune responses (Magill, 1995).More than 20 different *Leishmania* species can infect humans. Host health status and genetic background can influence the outcome of infection (lipoldova, *et al.*, 2006) and Sundar S, *et al.*, 2007) and HIV co-infection has dramatically increased the incidence of visceral leishmaniasis as reported by Sundar, *et al*, (2007).

The genus *Leishmania* is widely distributed in nature. It has a number of species that are nearly identical morphologically. (Arora, 2006).Differentiation is therefore based on a number of biochemical and epidemiological criteria,
use of monoclonal probes to detect specific antigens, promastigote growth patterns *in vitro* in the presence of antisera,vectors and reservoir hosts. Human infection is caused by about 21 of 30 species that infect mammals. The parasites of the Old world

1

Leishmaniasis are *L.donovani, L. infantum chaggasi; L.tropica, L. major and L.aethiopica.* They are transmitted to humans by the bite of female sand flies of the genus *Phlebotomus.* The New world species include *L.peruviana, L.chagasi, L.mexicana* Biagi complex (*L. mexicana, L. amazonensis,* and *L. venezuelensis*); and *L.brazilensis* complex and are transmitted by sand flies of the genus *Lutzomyia* and *Psychodopygus.* Leishmaniasis is the second-largest parasitic killer in the world (after malaria), responsible for an estimated 60 000 deaths among the half-million infections which occur each year worldwide. These diseases are commonly known as Leichmaniosis, Leishmaniose, and formerly, Orient boils, Baghdad boil, kala a zar, black fever, sand fly disease, Dum-Dum fever or Espundia.Most forms of the disease are transmissible only from animals (Zoonosis), but some can be spread between humans.

The symptoms of leishmaniasis are skin sores which erupt weeks to months after the person affected is bitten by sand flies. Other consequences, which can become manifest anywhere from a few months to years after infection, include fever, damage to the spleen and liver, and anaemia. In the medical field, leishmaniasis is one of the famous causes of a markedly enlarged spleen, (splenomegaly) which may become larger than even the liver.

The major factor that determines the tropism and pathology of *Leishmania* infection is however the species of *Leishmania* (Murray *et al.,* 2005). For example, *L. donovani* Laveran and Mesnil *L. infantum chagasi* Nicolle are closely related members of the *L. donovani* complex that cause visceral leishmaniasis, which is fatal if not treated. *L. major*Yarkimoff and Schokor and *L. tropica* Wright infections usually result in cutaneous lesions that remain localized at the site of the sand fly bite. *L. braziliensis* Vianna causes cutaneous leishmaniasis but can also migrate from the site of initial infection to the nasopharyngeal area resulting in highly destructive mucocutaneous leishmaniasis (Ivens, *et al.,* 2005).

Most forms of the disease are transmissible only from animals (Zoonosis), but some can be spread between humans. Human infection is caused by about 21 of 30 species that infect mammals.

Transmission is also possible by parenteral, sexual and occupational e.g. contaminated needles routes or by blood transfusion. (Otero *et al.,* 2000, Singh, 2006a.).
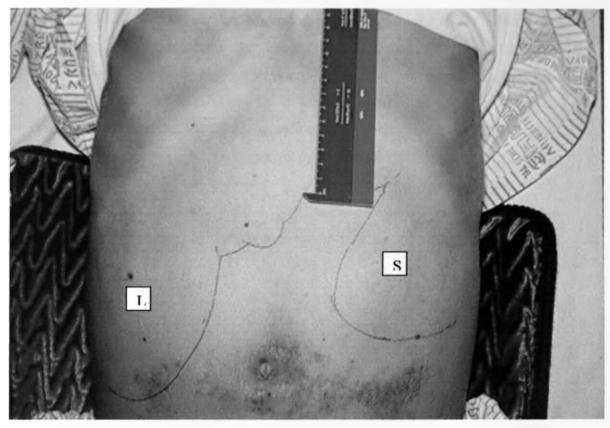
In humans, infection with *Leishmania* parasites causes a wide spectrum of clinical manifestations ranging from localized Cutaneous leishmaniasis (LCL) to mucocutaneous leishmaniasis (MCL), diffuse cutaneous leishmaniasis (DCL) and visceral leishmaniasis (VL) ,(Akilov *et al.,* 2007). The *Leishmania* species are inoculated into the mammalian host as extra cellular promastigotes which invade the host macrophages and replicate extracellularly to amastigotes.

*L. major* is the etiological agent of Old World CL, a disease that is characterized by cutaneous lesions that can be self-resolving with life-long immunity or chronic when accompanied by defective cellular immune responses (Reed and Scott, 2000; Tonui *et al.,* 2004).

CL caused by *L. major* is endemic in the Koibatek District of Rift Valley, Kenya, where it is transmitted by the sand fly *Phlebotomus duboscqi* Neveu-Lemaire (Beach *et al.,* 1984). The reservoirs for *L. major* in this focus are rodents, particularly.Psammomys obesus Cretzschmar,*Tatera robusta* Cretzschmar, *Aethomys kaiseri* Noack, *Taterillus emini* Thomas, and *Arvicanthis nalotica* Geoffrey, which are readily fed on by the sand fly which feeds, breeds and rests in their burrows (Githure *et al.,* 1984)

Control of the Leishmaniases is currently based on chemotherapy to treat infected cases and on vector control to reduce transmission (Davies *et al.,* 2003; Tonui *et al.,* 2004). Chemotherapy remains the mainstay for the control of Leishmaniases as effective vaccines are yet to be developed (Murray *et al.,* 2005).Treatment for Leishmaniases often involves the use of high doses of antimony compounds (known as Pentavalent antimonials), meglumine antimoniate (Glucantime), Sodium stibogluconate ((Pentostam®) and various formulations of Amphotericin B (Gicheru *et al.,* 2001; Croft *et al.,* 2006). These current drugs are highly toxic and patients have to endure painful multiple injections in a hospital bed (Berman, 2003). Moreover they are expensive and there are *Leishmania* strains that are resistant to antimony drugs.

Forms of Leishmaniases include Visceral Leishmaniasis which is also known as kala-a zar or black fever. It is the most severe form of leishmaniasis, and potentially fatal if untreated. The parasite migrates to the internal organs such as liver, spleen (hence 'visceral)' and bone marrow and if left untreated will almost always result in the death of the host. Signs and symptoms include fever, weight loss, anemia and a marked enlargement of the liver and spleen (Hepatosplenomegaly). Refer to Figure1.The etiological agents of VL are primarily *L. donovani* and *L. infantum chagasi* (Schnur *et al.,* 1981). *L. donovani* causes VL in the Mediterranean basin, western Africa and *L.infantum chagasi* in Latin America hence American Visceral leishmaniasis (AVL).

**Source:** http://en.wikipedia.org/wiki/ Visceral leishmaniasis"
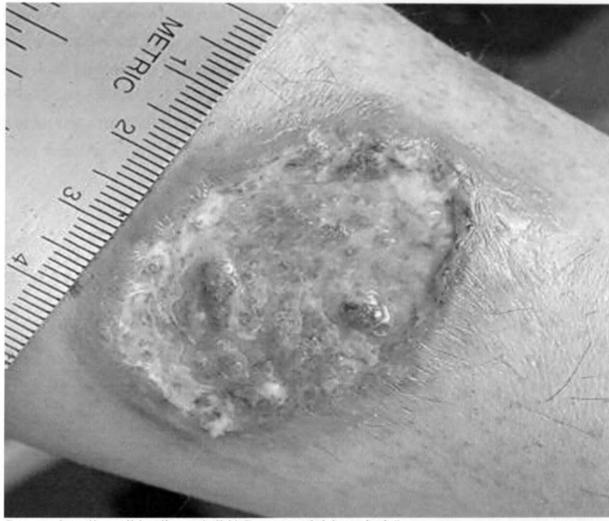
**Figure 1. Hepatosplenomegaly in man suffering from visceral leishmaniasis**


**Key**

**L- Enlarged liver (Hepatomegaly)**

**S- Enlarged spleen (Splenomegaly)**

Cutaneous leishmaniasis is the most common form which causes sores at the site of bite, which heals in a few months to a year, leaving an unpleasant looking scar. This form can progress to any of the other three forms. The etiological agents of CL in the Old World include *L. major, L. tropica* and *L. aethiopica,* whereas *L. braziliensis* and *L. mexicana* complexes are the CL etiological agents in the New World. In addition to CL, *L. aethiopica* in eastern Africa and the *L. mexicana* species complex in the Americas are also the etiological agents of diffuse cutaneous leishmaniasis (DCL). Other causes of CL include *L.amazonensis, L. venezuelensis, L. v. panamensis* Lainson and Show *L.v. guyanensis* Floch *L. v. peruviana* Velez *L. garnhami* Momen *and L. pifanoi* Medina and Romero (Croft and Vanessa, 2002).A typical lesion from *L.major* infection is shown in Figure 2 below.

**Figure 2.Cutaneous leishmaniasis lesion on left forearm coursed by *L.major*.**

Mucocutaneous leishmaniasis is the most feared form of cutaneous leishmaniasis because it produces destructive and disfiguring lesions of the face refer to Figure 3. It commences with skin ulcers which spread causing tissue damage to (particularly) nose and mouth. *L. peruviana* and the complexes of *L. braziliensis* and *L. mexicana* are the known etiological agents of MCL (Ashford and Bates, 1998), but cases caused by *L. aethiopica* have also been rarely described.

**Source:** http://en.wikipedia.org/wiki/ Mucocutaneous leishmaniasis"

**Figure 3.Disfigured human face due to mucocutaneous leishmaniasis**

## 1.1.2 Transmission of Leishmaniases

About 21 *Leishmania* species and subspecies are known to cause disease in humans and 30 Phlebotomine species are confirmed vectors (Desjeux, 1996; Ashford, 1997). All species found within the Americas are considered New World species, while those occurring in the rest of the world are Old World species. These include *P. argentipes* on the Indian sub-continent, *P. duboscqi, P.martini* Parrot and *P.orientalis* Parrot in Africa and the Mediterranean basin, *P. atinensis* and *P. alexandri* Sinton in china. In the new world *Lu. longipalpis* Cunha and Chagas is the only known vector of *L. chagasi* (Murray *et al.*, 2005).

The predominant mode of transmission is the bite of an infected female sand fly as shown in Figure 4 overleaf. Other but uncommon modes of transmission are congenital transmission and through blood transfusion. Rare cases of transmission through needle sharing, pregnancy, sexual intercourse and inoculation of cultures have also been reported (Conjivaram, 2002). The life cycle of Leishmania parasite in both the vertebrate and invertebrate hosts is shown in Figure 4.

**Sandfly Stages**

**Human Stages**

1 Sandfly takes a blood meal
(injects promastigote stage
into the skin)

2 Promastigotes are
phagocytized by
macrophages

8 Divide in midgut and
migrate to proboscis

3 Promastigotes transform
into amastigotes inside
macrophages

7 Amastigotes transform into
promastigote stage in midgut

4 Amastigotes multiply in cells
(including macrophages) of
various tissues

6 Ingestion of
parasitized cell

5 Sandfly takes a blood meal
(ingests macrophages infected
with amastigotes)

= Infective Stage

= Diagnostic Stage

http://www.dpd.cdc.gov/dpdx

**Figure 4 Life cycle of the Leishmania parasite**

The *Leishmania* parasites live in the acidic phagolysosomes of macrophages as round, non-motile amastigotes (3-7 μm in diameter). The macrophages are ingested by the female sand fly during the blood- meal and the amastigotes are released into the midgut of the insect. Almost immediately the amastigotes transform into the motile, elongated (10-20μm), flagellate called the promastigote form. The promastigotes then migrate to the alimentary tract of the fly, where they live extracellularly and multiply by binary fission. Four to five days after feeding, the Promastigotes move forward to the oesophagus and the salivary glands of the insect.

When the sand fly next feeds on a mammalian host, its proboscis pierces the skin and saliva containing anti-coagulant is injected into the wound to prevent the blood from clotting, the *Leishmania* promastigotes are transferred to the host along with the saliva. The presence of these promastigotes in the pharynx causes discomfort forcing the insect to regurgitate them first before sucking blood.

Once in the vertebrate host the promastigotes are taken up by the macrophages where they rapidly revert to the amastigote form. The *Leishmania* parasites are able to resist the microbiocidal action of the acid hydrolases released from the lysozymes and so survive and multiply inside the macrophages, eventually leading to the lyses of the macrophages. The released amastigotes are taken up by other macrophages and the cycle continues. Ultimately all the organs containing macrophages and phagocytes are infected, especially the spleen, liver and bone marrow, Arora, 2006. Amastigotes and promastigotes are shown in Figure 5 and 6 respectively.
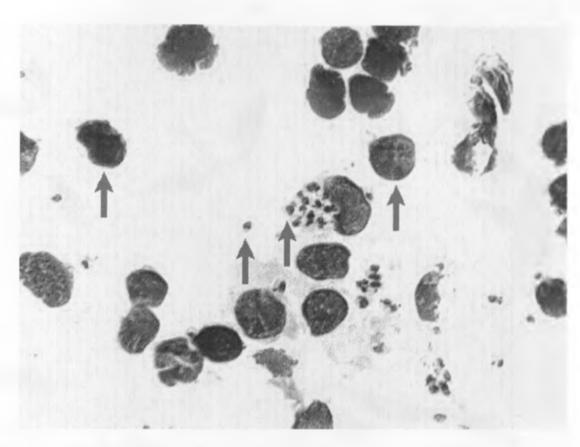
**Figure 5.** **Amastigotes in infected macrophages**

This photograph shows macrophages infected with *Leishmania* amastigotes. The red arrows indicate amastigotes which have been released following macrophage lysis. The green arrows indicate intact macrophages. This is the form in which the parasite appears in vertebrate hosts like man.
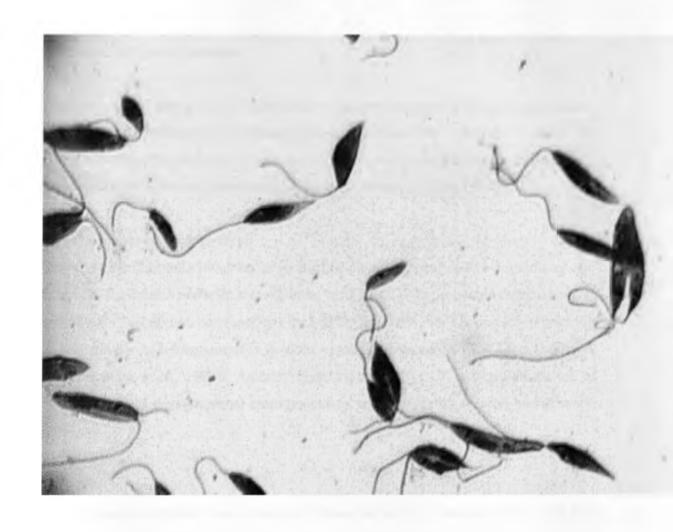
**Figure 6.** *Leishmania* **promastigotes in culture medium**

**Courtesy of Mrs. Tsehay Atlaw, University of East London**

This photograph shows the flagellated *Leishmania* promastigotes in culture. The promastigotes form of the parasites is found only in the sand fly vector or in artificial culture.

### 1.1.3 Control of Leishmaniases

Measures to check the spread of Leishmaniases include treatment of infected individuals, active case detection, and chemotherapy to eradicate the parasite reservoir in anthroponotic transmission cycles and control strategies against the vector and zoonotic reservoir in order to break transmission in zoonotic cycles (Larceda, 1994).

### 1.1.3.1 Animal reservoir control

Reservoir control usually involves the testing and culling of seropositive dogs in zoonotic visceral leishmaniasis endemic areas (Davies *et al.*, 2003). Impregnated collars, a novel method of topical application repellents such as Deltamethrin, have been used in breaking the transmission of leishmaniasis in zoonotic visceral leishmaniasis (ZVL) foci (Mutinga, 1975b; Schreck *et al.*, 1982). Animals' reservoir control for CL is based on the use of poison baits and environmental management to control rodents (Maroli and Khoury, 2004).

### 1.1.3.2 Vector control

The only proven vector of the *Leishmania* parasite is the blood-sucking female sand flies of the genus *Phlebotomus* in the Old World and *Lutzomyia* in the New World (Murray *et al.*, 2005). The insects are 2-3 mm long and are found throughout the tropical and temperate parts of the world. Only 30 or so of the over 500 species of *Phlebotomine* sand flies are known to transmit *Leishmania* parasites in the old world. The *Phlebotomine* sand flies are very susceptible to insecticides and their populations have been observed to drop drastically during control of Anopheles malaria vectors, resulting in interruption of Leishmaniases transmission (Kaul *et al.*, 1994). Indoor residual spraying with insecticide is the most widely used intervention for controlling sand flies that are endophilic and can considerably reduce CL cases (Davies *et al.*, 2000; Reyburn *et al.*, 2000). However, spraying programmes are often unsustainable (Murray *et al.*, 2005). Where sand flies are endophagic and are active when people are asleep, insecticide bed nets impregnated with the synthetic pyrethroids permethrin, deltamethrin and lambda-cyhalothrin provide considerable protection (Bern *et al.*, 2000). The limitations associated with the use of bed

nets include the discomfort generated by smaller mesh nets in warmer climates, the requirement for periodic re-impregnation of the nets and the high cost of long-lasting insecticide-treated bed nets (Murray *et al.*, 2005).

The application of biolarvicides in the field condition is difficult due to diverse breeding habitat of sand fly and their practical application appears to be of limited use in the control of VL (Kishore *et al.*, 2006). Satellite remote sensing for early prediction of disease by identifying the sandflygenic conditions and the use pheromons should be exploited in the control of leishmaniasis (Palit *et al.*, 2002; Kishore *et al.*, 2006).

In regions such as Latin America, Mediterranean basin, central and southwestern Asia where VL is primarily zoonotic, reducing transmission to human beings by targeting the animal reservoir is a feasible strategy (Davies *et al.*, 2003). However, culling infected domestic dogs in Brazil to reduce human VL was not effective because of incomplete coverage; delays between taking blood samples, diagnosis and culling; and the high dog population turnover rate (Courtenay *et al.*, 2002).

In view of the above shortcomings, dipping dogs in insecticide, applying topical insecticide lotions and the use of deltamethrin treated collars are novel strategies that can substantially reduce sand fly bites on dogs and subsequent human infection (Davies *et al.*, 2003). The use of insecticide treated bed nets (ITNs) can offer good protection against transmission of leishmaniasis by endophagic sand fly vector (Davies et al. 2003). In Kenya, most vector species such as the *P. duboscqi, P.guggisbergi* Kirk and Lewis, *P.martini* Parrot, *P.pedifer* Mutinga and Ashford *and P.longipes* Parrot and Martin are exophilic and exophagous, thus negating common strategies such as IRS and ITNs (Clive et al., 2003).The unknown breeding and resting sites of sand flies are other factors that complicate sand fly control strategies. The known life cycle of sand fly is given in Figure 7 below.
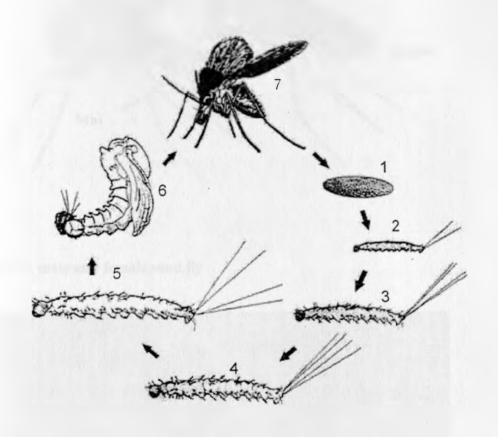
Figure 7a, Life cycle of Sand fly

Key
1. Eggs
2. 1$^{st}$ Instar
3. 2$^{nd}$ Instar
4. 3$^{rd}$ Instar
5. 4$^{th}$ Instar
6. Pupae
7. Adult sand fly
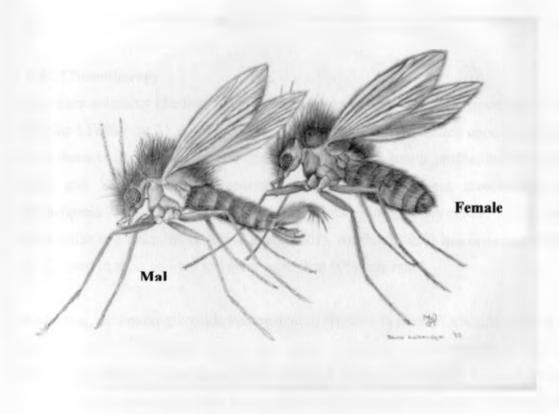
**Figure 7 b: male and female sand fly**
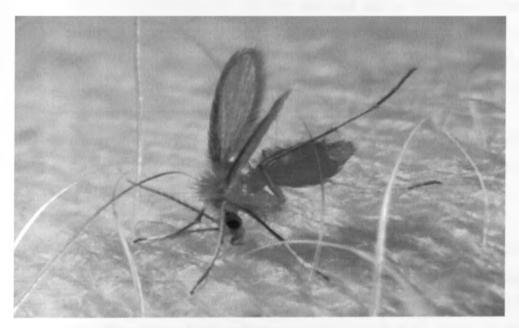


**Figure 7c, Adult gravid female Sand fly**

### 1.1.3.3 Chemotherapy

Pentavalent antimony (Sodium stibogluconate 20mg/kg/d IV/IM), and Aminosidine (16-20mg/kg/d IV/IM for 21 days) are used as primary therapy, depending upon the species of *Leishmania* concerned and resources available to the health professional involved (Croft and Vanessa 2002). Recommended secondary treatment involves use of Amphotericin B (3-4mg/kg IV for 5 days), Pentamidine, Paromomycin, Miltefosine, Sitamaquine and Imiquimod, (Clive *et al.*, 2003). Amphotericin B has been a treatment for VL, used in short courses and gives more than 90% cure rate.

In addition, the amino glycoside Paromomycin, effective in phase II trials, is likely to be approved after a pivotal phase III study is completed. However, it is the alkylphosphocholine Miltefosine (taken orally, 2.5mg/kg; 100mg/day for four weeks), which offers the most hope even among patients with antimony resistance disease (Croft and Coombs, 2003). Another potential oral drug Sitamaquine, an aminoquinoline, lacked a linear correlation between doses and cure rates and had an unsatisfactory safety and efficacy profile (Thakur *et al.*, 1993; Thakur *et al.*, 1999; Clive *et al.*, 2003; Croft and Coombs, 2003). Chemotherapy of VL relies on specific anti-leishmanial drugs and the aggressive management of any concomitant bacterial or parasitic infections, anaemia, hypovolemia and malnutrition (Chappuis *et al.*, 2007).

Successful treatment of VL results in 3% of African and up to 10% of Indian cases developing post-kala-azar dermal leishmaniasis (PKDL), whilst none is known to occur in the New World (Boelaert *et al.*, 2000).

HIV co-infections with *L. infantum chagasi* and occasionally CL have proved difficult to treat, with over 60% failure rate after treatment with most antileishmanial drugs used either alone or in combination .HAART (highly active antiretroviral therapy) has some effect on the relapse rate (Croft and Vanessa, 2002; Clive et al., 2003). A three-week course of antimonial drug is the most common treatment, especially in patients with disfiguring or relapsing cutaneous or mucocutaneous leishmaniasis (Castes *et al.*, 1984; Petersen *et al.*, 1984).Imiquimod, an immunomodulatory has been used to cure CL in Peru while Miltefosine, 133 mg and 150 mg daily cured 100% and 89% of patients

respectively (Conjivaram *et al.*, 2002). Ketoconazole has some potential against *L. mexicana* infection. and recently fluconazole 200 mg/day for six weeks led to healing of cutaneous leishmaniasis (*L. major*) in 79% of patients compared with 34% with placebo (Conjivaram. 2002; Croft and Vanessa, 2002; Clive *et al.*, 2003). Although non-fatal, CL is treated to accelerate cure to reduce scarring, especially in cosmetic sites, and to prevent parasite dissemination (i.e., mucosal leishmaniasis) or relapse. Treatment is commonly given for a persistent duration of over six months for multiple, or large lesions, and for lesions located on joints or on the face (Piscopo and Mallia, 2006).

WHO recommends treating CL with pentavalent antimonial drugs (Sodium stibogluconate or Meglumine antimoniate) at 20 mg/kg per day for 20–28 consecutive days (Reithinger *et al.*, 2007). Barring one exception, this regimen has been shown to be more efficient than a daily dose of 10 mg/kg, 13 mg/kg or 15 mg/kg in treating LCL (Berman, 1997; Oliveira *et al.*, 1997; Croft *et al.*, 2006).

Treatment of MCL with antimonials is unsatisfactory, especially in severe disease (Franke *et al.*, 1990). Amphotericin B and more recently liposomal Amphotericin B have been used successfully in difficult cases (Sampaio *et al.*, 1970; Amato *et al.*, 2000). Steroids may have to be used in patients in whom respiratory compromise is possible.

### 1.1.4 Global distribution of Leishmaniases

Leishmaniases has a long history (Conjivaram, 2006). Designs on pre-Columbian pottery and the existence of thousand-year-old skulls with evidence of leishmaniasis prove that the disease has existed in the America for a long time (Renee, 2005). It has also been present in Africa and India since the mid eighteenth century, at least (Cruz *et al.*, 2002). Geographical distribution of Leishmaniases is restricted to tropical and temperate regions (natural habitat of the sand fly) (Croft and Vanessa, 2002). It is limited by the distribution of the sand fly, its susceptibility to cold climates, its tendency to take blood from humans or animals only and its capacity to support the internal development of specific species of *Leishmania* (Manuel, 2002). Since 1993, regions that are *Leishmania* endemic have expanded significantly

The geographical spread is due to factors such as massive rural-urban migration, agro-industrial projects, man-made projects with environmental impact, like dams, irrigation systems wells and deforestation, which bring non-immune urban dwellers into endemic rural areas (Conjivaram et al., 2002). Aids and other immunosuppressive conditions increase the risk of *Leishmania* infected people developing visceral illness (WHO, 2000).

The epidemiology of the leishmaniases is categorized into two groups namely: zoonotic (animal reservoir hosts are involved in the transmission cycle) and anthroponotic (man serves as the sole reservoir and source of infection for the vector) (Desjeux, 2004). Rodents are usually the reservoirs for both Old and New World CL, whereas dogs are the common reservoirs for Old and New World VL (Fig 7 overleaf). In endemic areas for zoonotic CL, sand fly vectors are usually both exophilic and exophagous, that is, they like to rest and to feed outdoors. In areas of zoonotic visceral leishmaniasis (ZVL) the sand flies are peridomestic, increasing chances of transmission to humans.

In anthroponotic foci such as CL in India and VL in southern Sudan, the sand fly species are both endophilic and endophagous (Davies *et al.,* 2003). Leishmaniasis causes substantial clinical, public health and socioeconomic problems in endemic regions in more than 88 countries in the Indian sub continent, South Western Asia, Southern Europe, Africa, and Central and South America (Desjeux, 2004).

There is a remarkable increase in risk factors for leishmaniases worldwide and the disease burden is increasing (Reithinger e al., 2007). Globally, there are an estimated 350 million people at risk of infection and disease, 14million people are infected and 2 million new cases are reported each year (World Health Organization, Leishmaniasis Control home page: 5 https://www.who.int/gb/ebwha/pdf_files/EB118/B118_4-en.pdf).

The global burden of Leishmaniases has remained stable for some years, causing a morbidity and mortality loss of 2.4 million disability adjusted life-years (DALYs) and approximately 70,000 deaths, a significantly high rank among communicable diseases (Davies *et al.,* 2003; Reithinger *et al.,* 2007).

The global estimate for new cases of VL is 500 000 cases per year out of which 90% of the cases arise in just five countries-Bangladesh, Brazil, India, Nepal and Sudan (Desjeux, 2004). Each year, there are 1.5 million new cases of CL in more than 70 countries worldwide with 90% of the cases reported in Afghanistan, Algeria, Brazil, Islamic Republic of Iran, Peru, Saudi Arabia and Syria (Ghalib and Modabber, 2007). On the other hand, 90% of all cases of MCL cases occur in Bolivia, Brazil and Peru (Desjeux, 2004).

In Africa, Leishmaniases is endemic to countries mostly in the North, Central, East, the Horn of Africa and West Africa (Boakye *et al.*, 2005). Sudan is the most affected country, being one of the five countries that constitute 90% of all global cases of VL (Guerin, *et al.*, 2002). Algeria on the other hand is one of the eight countries that contribute 90% of worldwide cases of CL (Reithinger *et al.*, 2007). The highest incidence of post kala-azar dermal leishmaniasis (PDKL) in the world is also found in Sudan (Ghalib and Modabber, 2007). Between 1984 and 1994, an epidemic in Southern Sudan is thought to have had a mortality rate of 38-57%, killing 100,000 people (Seaman *et al.*, 1996).The world map for Leishmaniases is shown in figure 8.
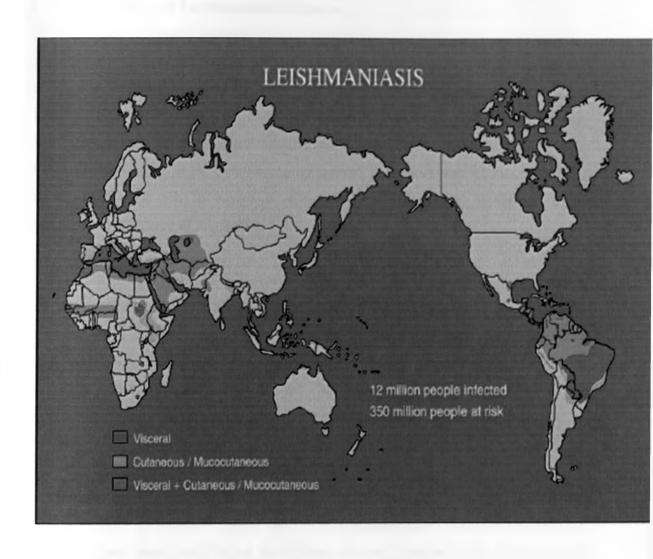
**Figure 8 World map highlighting areas where Leishmaniases are endemic**


**(Adapted from Handman, 2001).**

### 1.1.5 Epidemiology of Leishmaniases in Kenya

In Kenya, both CL and VL are prevalent. Leishmaniasis has been known to be endemic in the country from as far back as early in the 20th century (Fendall, 1961). An outbreak of VL was first reported among King's African Rifles troops encamped north of Lake Turkana in the 1940s (Cole et al., 1942). Since then Turkana. Baringo, Kitui, West Pokot, Machakos, Meru, Keiyo and Marakwet districts have been considered to be endemic for VL with Baringo and West Pokot being considered as endemic foci (Tonui, 2006). The reservoir host for VL in Kenya is still unknown. The disease is thought to be anthroponotic (Githure *et al.*, 1995).

The causative agent of VL in Kenya is *L donovani* transmitted mainly by *P. martini* (Wijers and Kiilu, 1984). In 2001, an outbreak of VL was reported in the previously non-endemic Wajir and Mandera districts of North Eastern Kenya where between May 2000 to August 2001, 904 patients were diagnosed with VL, with patients coming from as far as southern Somalia and southeast Ethiopia (Marlet *et al.*, 2003). In Kenya, CL is caused by *L. major, L. aethiopica* Bray, Ashford and Bray *and L. tropic*a (Mebrahtu *et al.*, 1992).

CL due to *L. major* which is transmitted by *P. duboscqi* is rare in humans, but underreporting is likely. Diffuse cutaneous leishmaniasis (DCL) was first reported in Kenya in 1969 in Bungoma district and the Mount Elgon area (Kungu *et al.*, 1972). *L. aethiopica* has been identified as the etiological agent, tree and rock hyraxes as the animal reservoirs and *P.pedifer* and *P.elgonensis* Ngoka, Madel and Mutinga (Lewis, Mutinga *et, al.,;* Ashford, 1972) to be the vector. (Mutinga, 1975; Sang and Chance, 1993; Ashford, 2000).

Although various aspects of the transmission and control of leishmaniassis have been studied in Kenya, the impact of the disease and particularly VL is still enormous (Tonui, 2006). CL caused by *L .tropica* is endemic in Laikipia district of the Rift Valley Province. In this focus, it is transmitted by *P.guggisbergi* (lawyer *et al.*, 1991). Rodents are suspected reservoirs. The distribution of Leishmaniases in Kenya is shown in Figure 8 below.

Figure 8. Map showing the distribution of the Leishmaniases in Kenya

VL    Visceral Leishmaniasis

CL    Cutaneous Leishmaniasis

DCL   Diffuse Cutaneous Leishmaniasis

25

## 1.2 LITERATURE REVIEW

### 1.2.1 Use of medicinal plants in the treatment of leishmaniases.

With the advent of the human immunodeficiency virus (HIV) pandemic, *Leishmania*/HIV co-infection is emerging as an extremely serious, new disease with important clinical and epidemiological implications in many parts of the world (Alvar *et al.*, 1997; Cruz *et al.*, 2006). Many studies have been conducted to find an effective therapy for Leishmaniases that avoids exposure to potentially toxic drugs including screening of plant extracts and plant derived compounds (Abreu *et al.* 1999, Carvalho and Ferreira, 2001, Rocha *et al.* ,2005). According to WHO,2006, the most pressing research needs for *Leishmania* control are the search for alternative and cheap drugs for oral, parenteral (injections) or topical administration in shorter treatment cycles and identification of mechanisms to facilitate access to existing control measures, including health sector reform in some developing countries.

Development of antileishmanial compounds could emerge from screening of natural libraries of plant compounds with recognized anti- parasitic activities. Among these, *A. Sativum* has been described as having immunomodulatory activity and therapeutic properties. (Pamplona, 2000). Other drugs have been used to treat *L. major* with varying results. Ketoconazole, a fungicidal drug which is an imidazole derivative has been reported to be effective in the treatment of *L. Major* (Berman, 1988), but a high dose of 200-400 mg per kilogram of body weight per day are needed. Several plant products have been tested and found to posses some antileishmanial activity.

Some of these plants contain isoquinoiles such as berberine isolated from *Berberis aristata* D.C (Berberidaceae), benzylisoquinoline alkaloids such as gyrocarpine, isotetradine, berdamine and antioquine that have been isolated from Bolivian plant families Annonaceae, Hernandiaceae and Menispermaceae (Fournet, 1988, 1993). Naphthoquinones such as plumbagin from *Plumbago zeylanica* (Fournet *et al.*, 1992) and 8, 8' biplumbagin isolated from stem and root extracts of *Pera benesis* Rusby (Euphorbiaceae) have been used in folk medicine in Bolivia as a treatment of CL caused

by *L. braziliensis*, when fresh stalks are applied directly on the lesion. lichochalcone, an oxygenated chalcone purified Chinese lichorice plant roots has been shown to inhibit growth of *L. major, L. donovani* and *Plasmodium falciparum* Welch (Chen *et al.*, 1993A). Other constituents from plants that have shown antileishmanial activities are saponin, anthocynadins, alkylamines, sequiterpenes, iridoid glycosides, lignan, diospyrin and aromatic polysulphur compounds (Iwu *et al.*, 1994; Fournet *et al.*, 1993).Many centuries ago, the Greek physicians Hippocrates and Dioscorides recommended *A. sativum* L. (Iwu *et al.*, 1994; Fournet *et al.*, 1993). They recommended *A. sativum* for digestive problems, leprosy, wounds and heart trouble. In the 20th century, Albert Schweitzer used it to treat amoebic dysentery. *A.sativum* has been found to be powerful rubefacient, antitusive, diaphoretic and vermifuge agent.

When intact in the bulb, *A. sativum* does not smell. It contains potent chemicals that are not in contact with each other until a garlic clove is bruised or crushed. When this happens, an enzyme called allinase (a catalyst) comes into contact with alliin (S-allylcysteine sulphoxide) triggering its transformation into allicin (= S-allyl propenthiosulphinate). Allicin is a sulphur ester derivative that produces a variety of diallyl sulphides, one of them being diallyl disulphide that gives garlic its aroma and has curative properties (Pamplona, 2000).

Allyl in novel 3-substituted quinolines has been shown to be effective in the treatment of American Visceral Leishmaniasis (VL) caused by *L. chagasi* (Tempone *et al.*, 2005). The evidence for optimal treatment of cutaneous leishmaniasis is patchy. However, the increasing prevalence of drug-resistant strains and the tendency for patients to relapse after an initially successful regimen of chemotherapy underscore the need for an effective prophylactic vaccine (Croft *et al.*, 2006) and/ or a herbal regimen that is effective, safe and affordable.

Traditional uses of medicinal plants consist of oral administration of crude plant extracts for VL and as topical preparations of the corresponding extracts for the treatment of CL (Croft and Vanessa, 2002). Natural products reported to have antileishmanial activity include quinones, alkaloids (quinolines and isoquinoline analogues, indole analogues and steroidal alkaloids), terpenes (iridoids, monoterpenes, sesquiterpenes, diterpenes, triterpenes and saponins), phenol derivatives (chalcones and flavonoids) and other metabolites such as acetogenins (Manuel and Luis, 2001).Quinone isolated from the bark of *Diospyros montana* (Ebenaceae) is reported to be active against promastigotes of *L. donovani* with a minimum inhibitory concentration (MIC) of 1 µg ml-1, although this activity is not selective against the corresponding amastigotes in macrophages (Manuel and Luis, 2001).

Berberine, a quaternary isoquinolinic alkaloid found in a number of plant families, which includes Annonaceae, Berberidaceae, Menispermaceae, is one of the alkaloids with the highest leishmanicidal activity (Phillipson & Wright, 1991). At a concentration of 10mg ml-1, berberine eliminates effectively *L. major* parasites in peritoneal mice macrophages but shows minimum activity when applied topically on mild cutaneous lesions caused by *L. major* (Manuel and Luis, 2001). Phenol derivative, which include oxygenated chalcone obtained from roots of the Chinese licorice plant (Glycyrrhiza spp., Fabaceae), was shown to inhibit the growth of promastigotes of *L. major*, and *L. donovani in vitro* (Philipson & Wright, 1991; Lunde and Kubo, 2000). Minquartynoic acid, a fatty acid derivative isolated from the cortex of *Minquartia guyanensis* showed a moderated in vitro activity against *L. major* due to its general cytotoxicity (Manuel and Luis, 2001).

### 1.2.2 Use of *Allium sativum* for the treatment of Leishmaniases

The genus *Allium has* over 1250 species, making it one of the largest plant genera in the world. They are perennial bulbous plants that produce chemical compounds (mostly cysteine sulphoxide) that give them a characteristic onion or garlic taste and odor, and many are used as food plants. *Allium* is classified in family *Alliaceae* although some classifications have included it in the lily family (*Liliaceae*). Onion is a term used for

many plants in the genus *Allium*. They are known by the common name "onion" but, used without qualifiers; it usually refers to *Allium cepa L. A. cepa* is also known as the 'garden onion' or 'bulb' or red onion.

*Allium* species occur in temperate climates of the northern hemisphere, except for a few species occurring in Chile, Brazil or tropical Africa .They can vary in height between 5 cm and 150 cm. The flowers form an umbel at the top of a leafless stalk. The bulbs vary in size between species, from very small (around 2–3 mm in diameter) to rather big (8–10 cm). Some species (such as Welsh onion, *A. fistulosum L.*) develop thickened leaf-bases rather than forming bulbs as such.

Members of the genus include many valued vegetables such as onions, shallots, leeks and herbs such as garlic and chives. A strong "oniony" odor is characteristic of the whole genus, but not all members are equally flavorful. The common onion varieties are shown in Figure 9a and 9b. *A scallion*, also commonly known as spring onion, green onion or salad onion, is associated with various members of the genus *Allium* that lack a fully-developed bulb.

*A.sativum* plant (Fig.9b) is hardy and not frost tender. The flowers are hermaphrodite (have both male and female organs)and are pollinated by bees and other insects. The plant prefers light (sandy) and medium (loamy) soils and requires well-drained soil. The plant prefers acid, neutral and basic (alkaline) soils and can grow in very alkaline soil. It cannot grow in the shade. It requires dry or moist soil. *A. Sativum* has a very long folk history of use in a wide range of ailments, particularly ailments such as ringworm, Candidiasis and virginities where it's fungicidal, antiseptic, tonic and parasiticidal properties have proved of benefit.

Other qualities that have been attributed to *A.sativum* include anthelmintic; antiasthma tic; anticholesterolemic; antiseptic; antispasmodic; Cancer; cholagogue; diaphoretic; diuretic; expectorant; febrifuge; stimulant; stings; stomachic; tonic; vasodilator. The plant produces inhibitory effects on gram-negative germs of the typhoid-paratyphoid-enteritis group; indeed it possesses outstanding germicidal properties and can keep amoebic dysentery at bay. It is also said to have anticancer activity. It has also been

shown that garlic aids detoxification of chronic lead poisoning. Daily use of garlic in the diet has been shown to have a beneficial effect on the body, especially the blood system and the heart. For example, demographic studies suggest that garlic is responsible for the low incidence of arteriosclerosis in areas of Italy and Spain where consumption of the bulb is heavy Recent research has also indicated that garlic reduces glucose metabolism in diabetics, slows the development of arteriosclerosis and lowers the risk of further heart attacks in myocardial patients. Externally, the expressed juice is an excellent antiseptic for treating wounds. The fresh bulb is much more effective medicinally than stored bulbs; extended storage greatly reduces the anti-bacterial action

Shallots and ten other onion (*Allium cepa* L.) varieties were evaluated. In general, the most pungent onions delivered many times the benefits of their milder cousins. Shallots have the most phenols, six times the amount found in Vidalia onion, the variety with the lowest phenolic content. Shallots also have the most antioxidant activity, followed by Western Yellow, New York Bold, Northern Red, Mexico, Empire Sweet, Western White, Peruvian Sweet, Texas 1015, Imperial Valley Sweet, and Vidalia. Western Yellow onions have the most flavonoids, eleven times the amount found in Western White, the variety with the lowest flavonoid content.

For all varieties of onions, the more phenols and flavonoids they contain, the more antioxidant and anti-cancer activity they provide. When tested against liver and colon cancer cells, Western Yellow, New York Bold and shallots were most effective in inhibiting their growth. The milder-tasting varieties showed little cancer-fighting ability. The varieties of *Allium* are shown bellow (Figure10).

Figure 9a) *Allium sativum* bulbs (magnification x1)
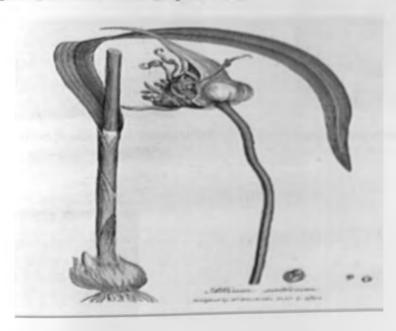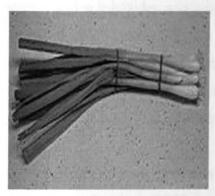


Figure 9b) The *A. sativum* full plant

**Brown and whit onions**



**Yellow onions**



**Spring onions (scallions).(*A. ascalonicum L.*)**



**Leeks *A. ampeloprasum* L.var. porrum**



Red onions     *A.cepa* L.

**Figure 10.Other onion varieties**

### 1.2.2.1 Properties of *A. sativum*

*A.sativum* contains potent chemicals that are not in contact with each other until a garlic clove is bruised or crushed. When this happen an enzyme called allinase comes into contact with alliin triggering its transformation into allicin.

Allicin is a powerful antibiotic and antifungal compound however due to poor bioavailability it is of limited use for oral consumption. Other bioactive compounds in *A.sativum* are ajoene enzymes, diallyl disulphide, vitamins minerals and flavonoids.Diallyl disulphide is responsible for the sharp or hot taste and the strong smell of garlic.

### 1.2.2.2 Onions and eye irritation

As onions are sliced, cells are broken, allowing enzymes called allinase to break down amino acid sulphoxides and generate sulphenic acids. Sulphenic acids are unstable and spontaneously rearrange into a volatile gas called syn-propanethial-S-oxide. The gas diffuses through the air and eventually reaches the eye, where it reacts with the water to form a diluted solution of sulphuric acid. This acid irritates the nerve endings in the eye, making them sting. Tear glands produce tears to dilute and flush out the irritant.

### 1.2.2.3 Studies on antileishmanial activities of *A. sativum*

A study by Gamboa (2007 found that *A.sativum* extracts have an immunomodulatory effect. He showed that control of cutaneous leishmaniasis due to *L. mexicana* is associated with a Th1- type immune response. The study showed that an aqueous extract of dried but not fresh garlic could control an infection by *L. mexicana* in BALB/c mice. Indeed, dried garlic treatment induced a mass reduction in footpad lesions and parasite burden compared with control mice. They also observed a marked increase in IFN-Y production by T cells in response to garlic treatment in agreement with a shift towards a Th1 immune response as reported earlier by Hassan *et al*, (2000).

Taken together these results strongly support the potential of garlic extracts for the immunotherapy of *Leishmania* infection. This Immunomodulatory activity of garlic extract may also be complemented by the direct inhibitory effect of some garlic compounds such as ajoene which can significantly inhibit *Leishmania* growth in vitro (Ledezma *et al.,* 2002).They also found out that only dried garlic extract was effective against *L. mexicana* and that fresh garlic extract had no significant effect pointing out that different extracts have different biological activities as observed before by Kasuga and others (2001).Also different doses of garlic extracts used may have opposite effects. For example, high concentrations of garlic extracts were found to inhibit Concanavalin A, induced Lymphocyte proliferation, while low concentration stimulated it (Colic *et al.,* 2002).In this case drying may have caused the degradation of thermolabile compounds with inhibitory activity. For example, an allicin fraction but not whole garlic extract can decrease IL-10 secretion from LPS- activated blood cells (Keiss *et al.,* 2003).

Alternatively immunomodulatory compounds may have been concentrated increasing the activity of dried extract. In vitro, *A.sativum* extracts reduced macrophages infection through induction of Nitric Oxide (NO) production. *A.sativum* extract may thus act on both T cells and macrophages to stimulate IFN-Y production and NO synthesis for parasite killing. A 10-14 KDa fraction was identified as responsible for the in vitro effect of the whole extract and may lead to the identification of novel immunomodulating drugs and therapeutic alternatives for the treatment of the leishmaniases.

*In vitro,* *A.sativum* extracts reduced macrophages infection through induction of Nitric Oxide (NO) production. *A.sativum* extract may thus act on both T cells and macrophages to stimulate IFN-Y production and NO synthesis for parasite killing. A 10-14 KDa fraction was identified as responsible for the in vitro effect of the whole extract and may lead to the identification of novel immunomodulating drugs and therapeutic alternatives for the treatment of leishmaniases.

A study by Nok *et al.,* (1996) reported that garlic induced death of protozoan with a dose of 5.0mg/ml.The extract of garlic pulp completely suppressed the ability of *Trypanosoma*

*brucei brucei* to cause trypanosomiasis in mice. Allicin. a biologically active compound in garlic cloves has been shown to have antimalarial activity.

Many researchers and laboratory studies demonstrated that garlic had antibiotic like effects. They investigated the ability of *A.sativum* to inhibit antibiotic resistant strains of bacteria. Singh *et al.*, 1984 found that garlic was more effective than many antibodies against a clinical strains of bacteria, Webber *et al.* 1992 also illustrated that garlic had antiviral activity. *A.sativum* has been described as having some immunomodulatory activity (Kasuga *et al.*, 2001) and therapy with fresh aqueous garlic extracts in mice being found to significantly control infection with *L. major* and it also intensified the effect of glucantine (Hassan *et al.*, 2000). This therapeutic effect of *A.sativum* extract was associated with a strong shift in cytokines from Th 2 to Th1 profile. Further more, a single dose of garlic extract in mice was able to induce macrophage recruitment and increase phagocytosis and the killing of *L. major* by macrophages in vivo.

Previous reports showed that garlic extract could stimulate NK cell activity and lectin-induced Lymphocyte proliferation although this depends on the type of extract and its concentration. *A.sativum* also decreases lipopolysaccharides (LPS) - induced proinflamatory cytokines such as TNF- , and IL- 1β from blood cells (Keiss *et al.*, 2003). An investigation of antileishmanial activity of a linalool- rich essential oil from *Croton cajucara* Benth by Maria do Socorro and others in 2002 showed that pretreatment of mouse peritoneal macrophages with 15ng of essential oil per ml reduced by 50%, the interaction between these macrophages and *L. amazonensis* with a concomitant increase by 20% in the level of NO production by the infected macrophages.

Treatment of pre infected macrophages with 15ng of essential oil per ml reduced by 50% the interaction between these cells and the parasites, which led to a 60% increase in the amount of nitric oxide produced by the pre infected macrophages.
 These results provide new prospective on the development of drugs with activities against *Leishmania* as Linalool- rich essential oil is a strikingly potent leishmanicidal plant extract.

Linalool, a terpenic alcohol is the main constituent of the oil and it is more leishmanicidal than the essential oil 50% lethal doses ($LD_{50}$) for promastigotes and amastigotes, 8.3ng/ml and 22ng/ml for the essential oil and 4.3ng/ml and 15.5ng/ml for Linalool respectively. With little or no observed toxicity in uninfected and infected murine macrophages and a potent leishmanicidal action, oil from *C. cajucara* could be a useful source of novel drugs.

Examination of methanolic extracts of four Sudanese plants (*Azadirachta indica* (Neem), *Acacia nilotica, Balanites aegyptica* and *A. sativum* revealed that only three species had a considerable *in vitro* anti- leishmanial activity on *L. major* promastigotes. The plants gave a $LC_{50}$ of 10.2, 4-94 and 89.38ng/ml for *Accacia indica* Juss, *A. sativum* L. and *Accacia nilotica* Willd respectively.

Bioactive fraction from leaves of the plant night jasmine (*Nyctanthes arbortristis* L). has been studied for the treatment of leishmaniasis.Eight weeks infected golden hamsters were treated intra-peritoneally with bioactive fraction at the doses of 100, 150, 200mg/kg body weight and the bioactive fraction plus SAG at the doses of 150+5, 200+5, 200+10 mg/kg body weight respectively for 21days. Controls were infected untreated hamsters. Treated animals received a total of six shots (infections) during three weeks of treatment schedule. Animals were sacrificed after 12 weeks of infection and level of parasite burden in spleen and liver expressed in Leishmania- Donovan Units (LDU).

### 1.2.4 Null hypothesis
Extracts of *Allium sativum* are not effective against experimental cutaneous leishmaniasis.

### 1.2.5 General objective
To investigate the chemotherapeutic effect of methanolic extract of dry *Allium sativum in vivo*.

### 1.2.5.1 Specific objectives

1  To extract the bioactive compounds from *A. sativum* and prepare the candidate drug formulations.

2  To determine the anti-leishmanial activity of extracts from *A.sativum* on *L.major* promastigotes on cell free cultures.

3  To determine parasite load and lesions size in *L major* in infected mice treated with *A. sativum* extracts, Pentostam® and untreated infected mice.

4  To compare disease progression *L major* in infected mice treated with *A. sativum* extracts, Pentostam®and untreated infected mice

## 1.2.6 Problem statement

Infections caused by protozoa of the genus *Leishmania* are major worldwide problems with high endemicity in developing countries (Sharif *et al.,* 2006). The situation has been aggravated by the fact that pentavalent antimonials, the drugs of choice for the treatment of Leishmaniasis, are expensive, exhibit considerable toxicity, variable efficacy and recently, there is the emergence of antimony-resistant strains (Hadighi *et al.*, 2006).

The control of Leishmaniases remains a problem because no vaccines exist and the available chemotherapy still relies on the potentially toxic antimonials which cause serious side effects, require protracted treatments with painful injections under prolonged hospitalization (Olliaro *et al.*, 1993).

The rise in the rates of in vitro antimonials resistance due to intermittent drug exposures, (Fournet *et al.,* 1995), the isolation of antimonials resistant *Leishmania* strains from patients with unresponsive cutaneous leishmaniasis (Alexander *et al.*, 1999), and recently the numerous cases of patients with co infection of *Leishmania* with HIV/AIDS make the search for new agents for the treatment of leishmaniasis very agent.

Plants have always been used as catalysts for our healing. Perhaps 80% of the world populations rely solely upon medicinal plants as the source of remedies in treatment of diseases. The majority of drugs active against infectious agents are in fact derived from natural products.

In order to halt the trend of increased resistance in leishmaniases; it will require a multi-pronged approach that includes the development of new drugs. Phytomedicines usually have multiple effects on the body, their actions often acting beyond the symptomatic treatment of disease (Iwu *et al.,* 1999). Plant based products are cost effective, less toxic, abundant in nature, may lead to oral drugs that are easy to administer and do not require hospitalization. Such plants can also be grown in large numbers (Iwu *et al.,* 1999).

Extensive studies of new drugs with antileishmanial activities including both natural products and synthetic compounds have been undertaken worldwide (Croft *et al.,* 2002), although problems with the side effects of the chemotherapies used at present have not yet been solved. Compounding this problem is the fact that many Countries and regions where the disease is endemic are economically poor. It is estimated that 80% of the 2 million new cases each year affects the world's poorest populations earning less than $2 a day (Clive *et al.,* 2003).

This lack of an effective antileishmanial drug and urgency of the present drug situation has generated a renewed interest in the study of traditional remedies to develop new antileishmanial chemotherapeutic agents with better activity and less toxicity. Leishmaniases are some of the most neglected tropical diseases in terms of the few tools available for control and the lack of clear criteria for methods of control. WHO has focused research on control of leishmaniases and consequently recent strategic research has led to the development of rapid and reliable non invasive diagnostic techniques. Basic research has resulted in the complete mapping of the genome of *L. major* thanks to the *Leishmania* Genome Network. Mapping of the genomes of *L. braziliensis* and *L. infantum chagasi* is underway (WHO, 2006).

38

Recently, oral Miltefosine has become available for the treatment of VL in India. Miltefosine is also effective against antimony-resistant disease. However, the long half-life of Miltefosine (approximately 8 days), may lead to sub-therapeutic levels for a few weeks after a 4-week course, with an attendant risk of inducing resistance. In addition, Miltefosine has a narrow therapeutic window and is contraindicated in pregnancy. Teratogenicity has been clearly demonstrated in animal studies and Miltefosine must, therefore, be used with caution in women of child-bearing age.

Together, these factors may limit the potential use of Miltefosine in public health or VL eradication campaigns.

Thus, an unmet medical need exists for an oral antileishmanial agent or Topical ointment that has efficacy against antimony-resistant disease, is affordable, and can be given safely in a public health setting. The purpose of this study was to investigate the therapeutic potential of *A. sativum* extracts against *L. major* promastigotes in BALB/c mice.

### 1.2.7 Justification of the study

Leishmaniases are on the WHO list of neglected diseases, which are characterized by lack of cheap, effective and safe drugs for chemotherapy (WHO, 2002). Moreover, vector control strategies are hampered by inaccessibility of vector breeding sites and adults due to their exophillic behavior (Mutinga, 1991). In Kenya, most vector species such as the *P.duboscqi* are exophillic and exophagous, thus negating common strategies such as indoor residual spraying (IRS) and insect treated nets (ITNs) (Alexander *et al.,* 1995). The Leishmaniases have an estimated morbidity burden of 2.4 million disability adjusted life years (DALYs) hence causing loss of lives and productivity (Manuel *et al.,* 2001). With no real promising prospects of an effective vaccine against the *Leishmania* parasite in the near future, there is an urgent need to develop highly available, affordable, effective indigenous formulations with low toxicity (WHO, 2002).

The Tropical diseases programmes of the WHO have considered the investigation of medicinal plants for the treatment of leishmaniases as essential and of high priority. Treatment of leishmaniases suffers from problems of drug resistance and severe toxicity

39

and requires prolonged parenteral administration, Davies *et al.*, 2003 and Garnier et *al.*, 2002. Two oral drugs are Sitamaquine and Miltefosine, which is in its clinical trial stage and also an anticancer has been shown to have gastrointestinal toxicity and Teratogenicity, Davies *et al.*, 2003 and therefore cannot be administered to women of childbearing age unless contraception is taken (Croft and Coombs, 2003). The fact that these antimony drugs have been in use for over 50 years despite the underlying problems underscores the need for developing new antiprotozoal compounds with improved pharmacological properties.

*A. sativum* is used all over the world for treatment of many different diseases. More than 3000 publications have provided evidences for the efficacy of this herb in the prevention and treatment of a variety of diseases and for validating its traditional uses. It has been shown that *A. sativum* modulates immune responses according to Ghanzafari *et al*, (2005). Considering that some aromatic poly-sulphur compounds such as the ones from *C.attiensis* (Steruliaceae) (Iwu *et al.*, 1994) and novel quinolines derivatives containing allyl have antileishmanial effects, the known antibiotic and antiseptic properties of allicin and diallyl disulphide in *A. sativum* makes them suitable candidates for testing against CL caused by *L. major* both *in vitro* and *in vivo*.

The findings of this study will be useful in providing new insights on the potential of Plant extracts as therapeutic agents in the control of the leishmaniases.Herbal regimens are relatively cheap, less toxic and takes shorter time to develop and market as opposed to the conventional drugs that take over ten years to develop. The present study sought to test plant extracts from *A. sativum* for its activities against *L. major* promastigotes *in vivo*.

# CHAPTER TWO

## 2.0 MATERIALS AND METHODS

### 2.1 Extraction of bioactive compounds from *Allium sativum*

*A.sativum* bulbs were purchased locally at Kenyatta market Ngumo estate in Nairobi. Voucher Specimens were taken to the Herbarium of National Museums of Kenya in Nairobi for authentification by Taxonomists. Specimen of the bulbs was also deposited at Kenya Medical Research Institute (KEMRI) Center for Traditional Medicine and Drugs Research where the study done. *A.sativum* extract was prepared according Mantis *et al.*Briefly, *A.sativum* bulbs were peeled, chopped into small pieces and air dried until constant weight then ground with an electric blender.

Methanolic extraction was done using absolute methanol as the organic solvent .The powder was soaked overnight and concentrated using rotor everporator.The final dry extracts was used to make the ointment for topical application on the lesions and the injectables as well as oral solution. The homogenate was centrifuged for 20 minutes at 1800g at room temperature and the supernatant filtered through whatman filter paper followed by sterilization through a 0.22µm filter Millipore, Billerica, MA, USA yielding a filtrate weighing 50grams.

### 2.2 Preparation of *A. sativum* ointment

About two hundred milligrams (200mg) of dried garlic extract was dissolved in olive oil until a homogenate paste obtained (Pamplona- Roger, 2000). It is this paste that was topically applied onto the lesions of BALB/c mice infected with *L. major* at 20 µg per milliliter of olive oil as base daily for four weeks post infection.

### 2.3 Cultivation of *Leishmania major* promastigotes.

Promastigote cultures of *L. major* strain **IDUB/KE/83=NLB-144** were used. This parasite was originally isolated from an infected female sand fly *Phlebotomus duboscqi* collected near Marigat, Baringo District, and Rift Valley, Kenya (Beach *et al.*, 1984) .This strain has been maintained by periodic passage in BALB/c mice to maintain its

virulence (Katakura and Kobayashi, 1985,) or maintained in mice by serial subcutaneous passage (Anjili *et al.*, 1994). Parasites were maintained as previously described (Titus *et al.*, 1994). Briefly, *L. major* parasites were cultured in complete Schneider's Drosophila insect medium supplemented with 20% heat -inactivated fetal bovine serum (FBS), glutamine (2mM), penicillin G (100 U/ml), streptomycin (100 μg/ml) and 5-flourocytosine arabinoside (Hendricks and Wright, 1979; Kimber *et al.*, 1981) and filtered in sterile conditions.

Stationary-phase promastigotes were obtained from 5- to 7-day-old cultures. Metacyclic promastigotes for infection were isolated from stationary-phase cultures by negative selection using peanut agglutination (Sacks and Perkins, 1984; Tonui and Titus, 2006) or promastigote concentrations diluted to $1x10^6$cell per milliliter of medium. The mortality rates of promastigotes were determined using Trypan blue stain exclusion principle whereby the promastigotes permeable to the blue dye were the dead ones. The parasites were counted using a Neubauer chamber. The mice were bred and maintained in insect proof rooms at KEMRI animal house Nairobi Kenya.
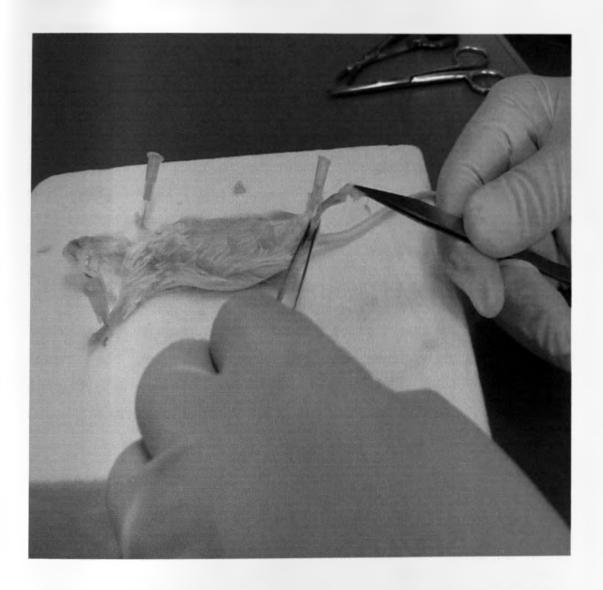
**Figure.11 Harvesting *L. major* promastigotes from an infected foot pad of a BALB/c mouse**.

## 2.4 Infection and treatment of BALB/c mice

### 2.4.1 Infection of mice

Six to eight weeks old BALB/c mice were used in this study. All the mice were challenged with $1x\ 10^6$ metacyclic *L. major* in 50 µL of saline solution in their hind left footpad (Tonui *et al.*, 2004; Al-Wabel *et al.*, 2007) Mice were divided into 3 groups of 20 each. The left hind footpad was inoculated with $1x\ 10^6$ stationary phase culture promastigotes in 40ml phosphate buffered saline (PBS) intradermally. Lesions were left to develop for 4 weeks. Lesion development was monitored by measuring the thickness of the infected footpad with a, vernier caliper weekly for five week post infection. For measurement of lesion size, the infected left footpad was compared to the normal right footpad.

Lesion size was expressed as the difference between the infected and uninfected footpad. Treatment with garlic extract formulations and Pentostam® as positive control started during the fifth week for a period of five weeks. Olive oil was topically applied on lesions of some mice as well as PBS both acting as negative control.

Figure.12 Subcutaneous infection of mice with 1x10$^6$ *L. major* metacyclic promastigotes.

## 2.5 Study design

The study design was a completely randomized block design (CRD) that consisted of six treatments. Sixty female and male BALB/c mice that were used in the experiments were bred and maintained at KEMRI's animal house facility following the laid down institutional guidelines. The mice aged between 6-8 weeks were then randomly assigned into three groups of twenty each. This allowed for four mice from each group to be followed for lesion size throughout the experiment and four subgroups of duplicate mice each to be used for assays to determine parasite burden and the nature of the immune response (Al-Wabel *et al.*, 2007).

Sixty BALB/c mice were randomly assigned into three groups as bellow.

**Table 1.Completly randomized block design used in the study**

| | | |
|---|---|---|
| GROUP A (20 male mice) | Treatment.(12 mice) | Oral administration with solution of extract *A. sativum* daily for four weeks. |
| | Negative Control (4 mice) | Oral administration with PBS daily for four weeks. |
| | Positive control(4mice) | Intraperitoneal injection with known drug Pentostam® daily for 4weeks |
| GROUP B (20 female mice) | Treatment.( 12 mice) | Topical applications with *A. sativum* extract ointment daily for 4 weeks. |
| | Negative Control (4mice) | Topical application with olive oil daily for 4 weeks. |
| | Positive Control (4mice) | Intraperitoneal injection with known drug Pentostam daily for 4weeks. |
| GROUP C (20 male mice) | Treatment.( 12 mice) | Intraperitoneal injection with *A. sativum* extract daily for 4 weeks. |
| | Treatment.(4 mice) | Injection with PBS daily for 4weeks. |
| | Positive Control (4 mice) | Intraperitoneal injection with known drug Pentostam ®daily for 4 weeks. |

In order to compare the drug effects, the measurement of infected and uninfected footpads were taken on a weekly basis and the level of infection determined according to the method of Nolan and Farrell (1987).

To determine whether all parasites died at the site of infection, saline aspirates were taken from the treated groups and cultured for parasite examination.

## 2.6 Limiting dilution assay (LDA) for quantification of parasite numbers.

Parasite burden was evaluated using a limiting-dilution assay. Footpads were skinned and removed from euthanatized mice. Footpads from multiple mice of the same group were ground in a tissue homogenizer in modified Schneider's insect medium (Sigma-Aldrich) containing 10% fetal bovine serum, 10 mM HEPES, 100 U/ml penicillin, 100 µg/ml streptomycin, 50 µg/ml gentamicin (Sigma-Aldrich), 2 mM l-glutamine, 1 mM sodium pyruvate, 0.2 mM l-asparagine, 0.6 mM l-arginine, and 2% sterile-filtered normal human urine. Serial dilutions of the footpad homogenate were then distributed in replicate wells of a 24-well plate, and the plates incubated at 26°C. Plates were sealed and set in the dark at room temperature for 10 to 14 days before evaluation. Plates were scored and the results evaluated using the ELIDA program (PROBIT) for statistical analysis, (Tarswell, 2005).

## 2.7 *In vitro* testing for antileishmanial activity of *A. sativum* extract

Promastigotes ($1 \times 10^6$ cell in 100ml) were loaded into each culture well of negative control group in complete Schneider's media (positive control group in Pentostam®) and experimental groups treated with *A.sativum* extracts. The 24 well plates were wrapped with para film and incubated in a plastic chamber at $28^0$ C for 2-4 days, making observations in the course of the incubation. Serial dilutions were made from the stock solutions of *A. sativum* extracts then introduced into the same wells containing *L major* parasites. The negative control group was incubated in the absence of the extracts. Parasite viability was assessed before and after incubation by staining with trypan blue and examining under a compound microscope. After establishing several inhibitory concentrations, the minimum inhibitory concentration that was able to eliminate 50% of the parasites was determined as the $IC_{50}$.

Figure 13 *L. major* promastigotes (strain NLB 144) cultured in Schneider's medium in a 28 $^0$C incubator.

## 2.8 Quantifying parasite burden using Leishman-Donovan unit (LDU).

All the BALB/c mice that were infected with $1 \times 10^6$ *L. major* stationary phase promastigotes strain (NLB 144) and treated were then euthanized and the spleens collected and weighed.Impresion smears made by making transverse sections on the spleens and stamping on grease free microscope slides. The stamps were dried, methanol fixed and giemsa stained. The slides were then examined under compound microscope for enumerating the number of amastigotes per 1000 host nuclei. The parasite load was expressed as Leishman-Donovan unit (LDU), which is calculated by multiplying the spleen weight in grams by the number of amastigotes per 1000 host nuclei( Roy *et al.*, 1991).The experiments were in accordance with the Committee for the purpose of control and supervision of experiments on animals-approved protocol. Parasites present were counted and the parasite load quantified using the method of Bradley and Kirkley (1977).All BALB/c mice used in this study were sacrificed in accordance with the regulation that has been set by the KEMRI s animal care and use committee. They were injected with 100µl sodium pentabarbitone and disposed by incineration.

## 2.9 Ethical and biosafety considerations

Approval for the study was sought from Kenya Medical Research Institute (KEMRI) s ethical Review Committee, the Board of Postgraduate Studies of University of Nairobi and KEMRI's Animal Care and Use Committee (ACUC). Standard operating procedures (SOPs) available at the *Leishmania* laboratory at the CBRD included immunizing of animals using 57 standard 21 gauge needle, anesthetizing them using 6% sodium Pentabarbitone (Sagatal®) and killing them by using $CO_2$ asphyxiation. Biosafety issues were addressed by sterilizing dead animals through dipping them into 70% ethanol and disposing into appropriate biohazard bags before transfer to the incinerator at KEMRI. Further precautionary measures involved putting on protective gear and carrying out the experiments in a laminar flow hood.

The study was carried out at the Kenya Medical Research Institute's (KEMRI), Centre for Biotechnology Research and Development (CBRD), in Nairobi, Kenya. The Institute has the requisite facilities that ensured that the studies were undertaken successfully

## 2.10 Expected outcome

Successful plant extracts are expected to serve as a source of pharmacologically relevant isolates that are less toxic, readily available and with higher parasite clearance rate than the available chemotherapeutic agents. Once the plant extracts pass through *in vivo*, pre-clinical and clinical trials, it's my hope that Topical, oral and injectable drugs that could be easier to administer, non-toxic and having no resistance problems will be discovered. This will reduce the need for hospitalization. The drug would be cheaper, more affordable and more effective. These immunostimulative plant products should also be recommended for further testing of their role as adjuvants.

## 2.11 Statistical analyses

Statistical analysis was performed using SPSS. The mean and standard errors of the lesions of each treatment group was compared using student's t-test (Paired t-test) and analyzed for statistical significance using the analysis of variance (ANOVA).The mean parasite burden was also compared using analysis of Variance. Probit analysis was used to determine $IC_{50}$ using procedure PROBIT of SAS version 9.1 (SAS Institute, 2002). All pair wise analysis of mortality rates, versus the negative control was done using Two-sample t- test. All analyses were carried out 5% level of significance.

# CHAPTER THREE

## 3.0 RESULTS

### 3.1 Cell free assays

Cell free cultures were done in complete Schneider's insect medium supplemented with fetal bovin saline (FBS), at 25°C. After seven to ten days of incubation, elongated stationary forms of *L. major* promastigotes with active flagella movement were observed. These parasites were incubated further for 72 hours in the presence of the plant extracts for the experimental groups, in complete Schneider's insect medium for the negative control and in Pentostam® for the positive control.

Promastigotes were counted with an improved neubauer chamber after staining with trypan blue dye. The cells that stained blue as shown in figure 14 overleaf were taken to be the dead cells whereas the bright cells as live. The number of dead and live *L. major* promastigotes in experimental, negative and positive control groups was recorded and mortality rates calculated as shown below.

Mortality rate = $\dfrac{\text{No of dead cells}}{\text{Total no. of cells counted.}} \times 100$

Deaths in *L. major* promastigotes cultured in complete Schneider's media were due to growth limiting factors such failure to acclimatize to the *in vitro* environments, accumulation of toxic waste substances, lack of sufficient nutrients supply, depletion in amounts of gases and reduced space.

51
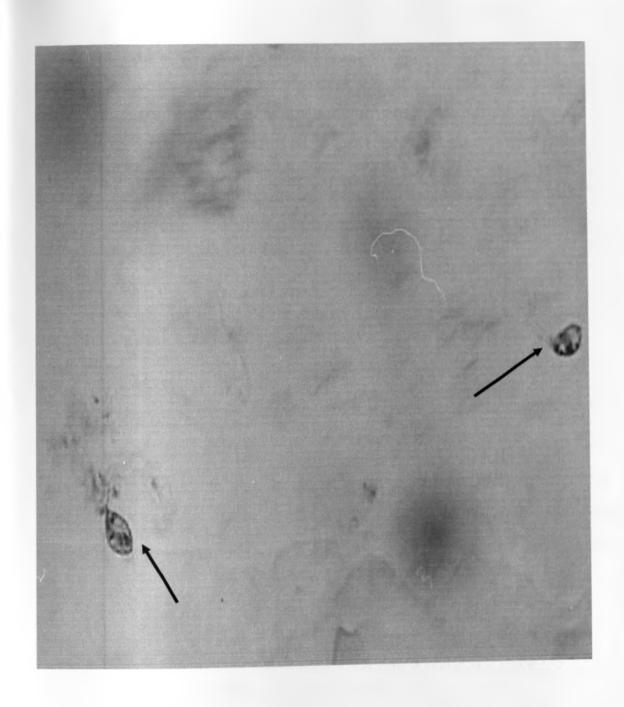
Figure 14 Dead *L. major* promastigotes stained blue (arrowed) after 24 hrs
treatment with *A.sativum* extract at a concentration of 1000 µg/ml (X1000).

### 3.1.1 Mortality rates in *A. sativum* methanolic extracts.

Exposure to *A. sativum* extracts greatly reduced population numbers of *L. major* promastigotes *in vitro*. Methanolic extracts of *A. sativum* caused 88% mortality rate at a concentration of 1000 µg/ml shown in table 2 below. At the same 1000 µg/ml, Pentostam®the current drug of choice caused 74% mortality rate in *L. major* promastigotes *in vitro*.A 5% mortality rate was observed in *L. major* parasites cultivated in complete Schneider's medium. Death in this media was probably due to other causes like overcrowding, lack of food and toxicity.

**Table 2 Mortality rates of *L.major* promastigotes in A.sativum extract at different concentrations.**

| Mortality rate in % | 250µg/ml | 500µg/ml | 1000µg/ml |
|---|---|---|---|
| *A.sativum* extract | 52 | 74 | 88 |
| Pentostam® | 41 | 69 | 74 |
| Schneider's medium | 5 | 5 | 5 |

**Figure 15 Oval shaped slender *L. major* promastigotes forms with short flagella after 24 hrs treatments with *A. sativum* methanolic extract at a concentration of 1000 μg/ml (X1000).**
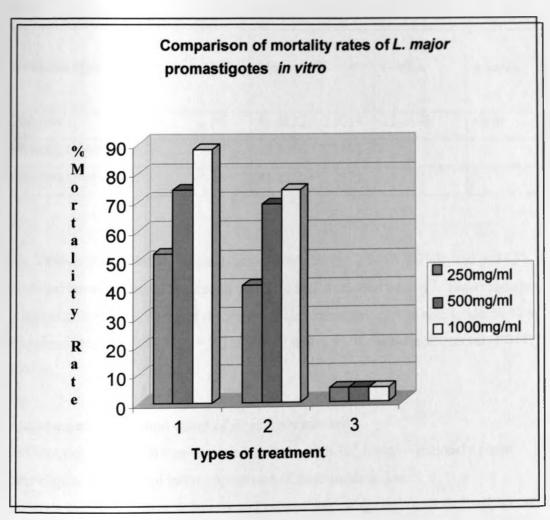
**Figure 16 Effects of *A. sativum* extracts on mortality rates in *L. major* promastigotes after 24 hours of incubation *in vitro*.**

KEY:

1 *A.sativum* extract

2 Pentostam® the antileishmanial drug of choice

3 Phosphate buffered saline (PBS)

Table 3 Statistical values describing leishmanicidal effects in *A.sativum* extracts in relation to Schneider's medium and Pentostam.

| Treatment type | DF | Mean + SE | t-value | p-value |
|---|---|---|---|---|
| *A.sativum* | 36 | 46.5822 + 2.0718 | 12.6193 | <0.05 |
| Schneider's (-ve control) | 36 | 18.6666 + 1.1998 | | |
| Pentostam (+ve control) | 36 | 41.1250 + 3.2589 | | |

Table 3 shows that statistically significant differences (t=12.6193; DF=36 and p<0.05) were found between mortality rates in untreated and *A.sativum* treated *L. major* parasites. It is remarkable that 2.216 mg of *A.sativum* crude extract per ml was able to kill 50 % of the promastigotes in vitro {$IC_{50}$ = 2.216 mg/ml with a 95 % confidence interval (1.0441, 1.2006)}.

### 3.2 *In vivo* antileishmanial effect of *A. sativum* extract

Hind footpads of BALB/c mice were infected with $1 \times 10^6$ *L.major* stationery phase promastigotes and resulted in the appearance of measurable lesions.

Cutaneous ulcers as lesions, tightness and redness were observed in all the BALB/c mice 5weeks post infection. There was no slopping or any other second lesion in other parts of the body except the infected left footpads.Amastigotes were seen in smear preparations from lesions of all the infected BALB/c mice used in the study. At the end of treatment, the lesion sizes of experimental mice were found to have decreased and become softer in groups treated with the extract of *A. sativum* and the reference drug Pentostam®compared to the negative control groups i.e. those that received olive oil and PBS.

**Table 4** Statistical values describing lesion sizes of mice before and after treatment with A.sativum extract formulations and the controls.

| | Mean | N | Std. Deviation | Std. Error Mean |
|---|---|---|---|---|
| VAR00002 | 2.4300 | 10 | 4.830E-02 | 1.528E-02 |
| VAR00012 | 2.4200 | 10 | 4.216E-02 | 1.333E-02 |
| VAR00006 | 2.5200 | 10 | 7.888E-02 | 2.494E-02 |
| VAR00012 | 2.4200 | 10 | 4.216E-02 | 1.333E-02 |
| VAR00010 | 2.5400 | 10 | 8.433E-02 | 2.667E-02 |
| VAR00012 | 2.4200 | 10 | 4.216E-02 | 1.333E-02 |
| VAR00012 | 2.4200 | 10 | 4.216E-02 | 1.333E-02 |
| VAR00014 | 2.5200 | 10 | 7.888E-02 | 2.494E-02 |
| VAR00008 | 3.3200 | 10 | .1033 | 3.266E-02 |
| VAR00012 | 2.4200 | 10 | 4.216E-02 | 1.333E-02 |
| VAR00002 | 2.4300 | 10 | 4.830E-02 | 1.528E-02 |
| VAR00006 | 2.5200 | 10 | 7.888E-02 | 2.494E-02 |
| VAR00002 | 2.4300 | 10 | 4.830E-02 | 1.528E-02 |
| VAR00008 | 3.3200 | 10 | .1033 | 3.266E-02 |
| VAR00006 | 2.5200 | 10 | 7.888E-02 | 2.494E-02 |
| VAR00010 | 2.5400 | 10 | 8.433E-02 | 2.667E-02 |
| VAR00001 | 3.0400 | 10 | .2221 | 7.024E-02 |
| VAR00002 | 2.4300 | 10 | 4.830E-02 | 1.528E-02 |
| VAR00002 | 2.4300 | 10 | 4.830E-02 | 1.528E-02 |
| VAR00014 | 2.5200 | 10 | 7.888E-02 | 2.494E-02 |
| VAR00012 | 2.4200 | 10 | 4.216E-02 | 1.333E-02 |
| VAR00016 | 2.4000 | 10 | 4.714E-02 | 1.491E-02 |
| VAR00002 | 2.4300 | 10 | 4.830E-02 | 1.528E-02 |
| VAR00014 | 2.5200 | 10 | 7.888E-02 | 2.494E-02 |

NB; Key for the variables is on page 60

**Key**

| VAR00002 | LESION SIZE OF MICE ON TOPICAL TREATMENT |
|----------|-------------------------------------------|
| VAR00006 | LESION SIZE OF MICE ON INJECTION TREATMENT |
| VAR00008 | NEGATIVE CONTROL (OLIVE OIL) |
| VAR00010 | LESION SIZE OF MICE ON ORAL TREATMENT |
| VAR00012 | LESION SIZE OF UNINFECTED FOOTPAD BEFORE TREATMENT |
| VAR00014 | POSITIVE CONTROL PENTOSTAM) |
| VAR00001 | INITIAL LESION SIZE OF MICE ON TOPICAL TREATMENT |
| VAR00016 | LESION SIZE OF UNINFECTED FOOTPAD AFTER TREATMENT |

Table 4 above shows the results of statistical analysis of differences between means of lesion sizes of infected mice footpads and those of uninfected footpads. Paired t-test was used to compare the means and test for the hypotheses. The correlations between the treated groups and untreated groups are recorded in table 3 below. The lesion sizes of uninfected mouse footpads before and after treatment was insignificant (t=1.5, r=0.559, p<0.05)

The mean lesion size of BALB/c mice that were topically treated with *A.sativum* extract ointment was compared to test the hypothesis that there is no difference between the means. It was found that there was no significant difference between the means of mice that were treated with extract ointment and those uninfected footpad (t=1.000, r=0.764, df=9,).The means of all other treatment group compared to the mean of untreated was significant (Injection=3.354, r=-0.134, df=9, Oral, t=4.811, r=0.375, df=9and Pentostam t=-3.873, r=0.200, df=9).

Effectiveness of *A.sativum* compared to the antileishmanial drug of choice sodium stibogluconate (Pentostam®) was tested by comparing the mean lesion size of mice on topical treatment using extract ointment with mean of mice on positive control using Pentostam®.There was no significant difference t=-3.250, r=0.117, df=9.There was no significant difference between the mean lesion size of mice on injection formulation and those on oral extract formulation.

However, the lesions of the negative control groups continued to increase and self healing was not observed.

Sixty BALB/c mice of about 8weeks old were used in this experiment. All the mice were injected subcutaneously on the left hind footpads with $1 \times 10^6$ stationery phase *L. major* promastigotes. The footpad sizes were measured before infection and weekly for five weeks post infection. The right hind footpads of each infected mice were measured before infection and weekly for five weeks post infection. Data plotted are the means of weekly footpad lesion sizes in each experimental group.
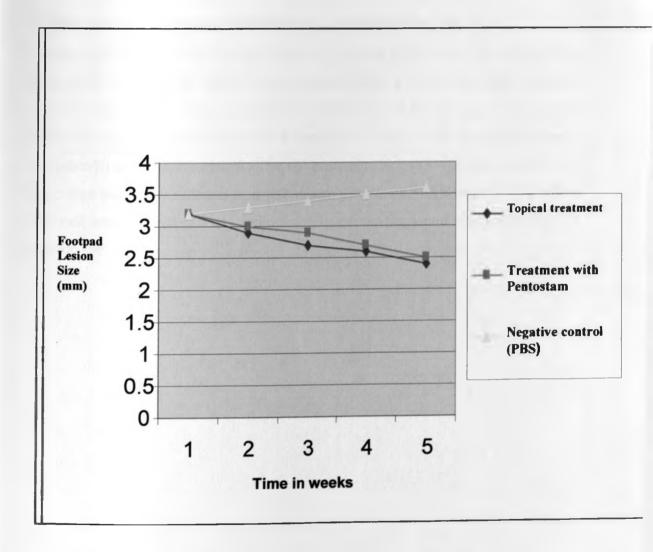


**Figure 18. graph showing the comparison of disease progression between the experimental and control groups of mice infected with *Leishmania major* promastigotes and treated with *Allium sativum* extract and Pentostam®.**

Treatment was done one month after infecting the mice with the *L.major* parasites. Experimental mice were either treated with extract formulations of *A.sativum* or drug of choice Pentostam as a positive control. The negative control groups received topical application with olive oil or injection with PBS.Mice footpad lesion sizes were measured weekly for five weeks post infection. Values plotted are the means of weekly lesion sizes per experimental group.

### 3.3 Effect of *A.sativum* extracts treatment on *L. major* parasite load

*L. major*-infected mice treated with ointment of *A.sativum* and those on Pentostam® had significantly ($P < 0.05$ by ELIDA) lower parasite numbers per footpad than untreated controls (Figure 19). Mice treated with ointment of the extract had parasite numbers/footpad that was progressively lower than those from both the positive control (Pentostam®) and negative control (olive oil) treatment groups ($P < 0.0001$ by ANOVA). Those mice treated with the test drug had a mean parasite number/footpad of 0.4, with a 95% confidence interval of 0 to 1, indicating effective eradication of the parasite from the infection site.

## 3.4 Parasite quantification using Leishman Donovan unit (LDU)

The infected and treated BALB/c mice were sacrificed on the fifth week post infection and their spleens removed, weighed and impression smears done as described by Bradley and Kirkly. Briefly Liver and spleen impression smears were made on clean microscope slides fixed and stained. Parasites found in the spleens of both treated and untreated mice were insignificant. This was expected as *L. major* is a known cutaneous parasite and therefore does not metastasize to visceral organs in higher animal except in rodents.
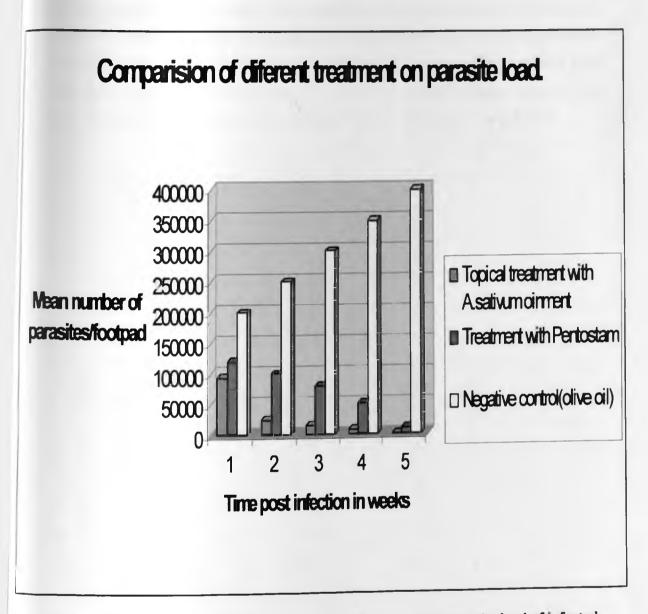
**Figure 19.Effect of *A.sativum* extract ointment treatment on parasite load of infected BALB/c mice.**

Parasite numbers per footpad were determined as described in materials and methods from samples obtained at the indicated time points. Values reported are the means ± 95% confidence limits of parasite numbers based on limiting-dilution analysis (LDA).ELIDA programme was used to determine the parasite loads per mouse footpad.

## 3.4 Parasite quantification using Leishman Donovan unit (LDU)

The infected and treated BALB/c mice were sacrificed on the fifth week post infection and their spleens removed, weighed and impression smears done as described by Bradley and Kirkly.Briefly Liver and spleen impression smears were made on clean microscope slides fixed and stained. Parasites found in the spleens of both treated and untreated mice were insignificant. This was expected as *L. major* is a known cutaneous parasite and therefore does not metastasize to visceral organs in higher animal except in rodents.

# CHAPTER FOUR

## 4.0 DISCUSION, CONCLUSIONS AND RECOMENDATIONS

### 4.1 DISCUSION

This study investigated the chemotherapeutic effect of extracts from *A.sativum* on experimental cutaneous leishmaniasis on BALB/c mice.

All the BALB/c mice used in this study had their left hind footpads measured using a veneer caliper before infection with *L.major* promastigotes and weekly for four weeks post infection. Disease progression was determined by comparing the lesion sizes of infected BALB/c mice before treatment and weekly during treatment with *A.sativum* extract formulations, Pentostam and the untreated mice. *A.sativum* ointment for topical application was the most effective as it was able to reduce footpad lesions back to normal size four weeks after treatment. Extract ointment was found to be more effective than Pentostam which is the current antileishmanial drug. Lesion sizes of mice on olive oil topical application and PBS as negative controls showed marked increase, indicating continued parasite growth.

These *in vivo* studies of effect of *A.sativum* extract against experimental cutaneous leishmaniasis were impressive. *A.sativum* ointment was able to significantly reduce the lesion size of infected mice footpads. The mean lesion size of BALB/c mice after infection and treatment with *A.sativum* ointment was same as the mean of BALB/c mice footpads that were not infected. This shows that the ointment significantly cleared parasites from the footpads of infected mice thus regaining their normal size after treatment. Topical ointment of extract was found to be more effective than the current drug of choice Pentostam in treating cutaneous leishmaniassis. *A.sativum* extract is more effective as an ointment than parenteral or oral formulations. Oral formulation was less effective than the parenteral formulation. A study using fresh garlic extract was shown to be more effective against Leishmaniasis when administered parenteraly.

The cell free assays for leishmanicidal effects were done and the mortality rates of *L.major* promastigotes were indicative of antileishmanial activities of *A.sativum* extracts versus Pentostam against *L. major* promastigotes *in vitro*. The mortality rate of parasites was found to depend on the concentration of the drug used.

*A.sativum* extract had the highest mortality rate at the three different concentrations followed by the conventional antileishmanial drug Pentostam®. At 1000μg/ml the extract had a remarkable mortality rate of 88% compared to Pentostam®, that had 79%. It is clear that *A.sativum* extracts should be prioritized in case of further exploration on antileishmanial properties. Results in this study do not show any significant difference in mortality rates between extract and Pentostam®.

Mortality rates in *L. major* promastigotes treated with 250μg/ml of *A.sativum* was the least 52% compared to those at higher concentrations. At 500μg/ml, leishmanicidal effect rose to 74%, which was higher than that of Pentostam the drug of choice which showed 69%. As expected, leishmanicidal effects in *A.sativum* extracts was dose dependent, and the most effective dose concentration of 1000 μg/ml was used in testing for leishmanicidal properties of *A.sativum in vivo*.

It was found out that methanol extract of *A.sativum* had a higher (67%) leishmanicidal effect than the conventional antileishmanial drug off choice Pentostam® (56%).It is clear that the methanolic extract of dry *A.sativum* should be prioritized in case of further exploration on antileishmanial properties. Mortality rates in *L.major* promastigotes treated with different concentrations of *A.sativum* extract were determined. As expected, leishmanicidal effects of *A. sativum* extract was dose dependent. It is remarkable that 1.2 mg of *A. sativum* crude extract per milliliter was able to kill 50% of promastigotes *in vitro* {$IC_{50}$ 1.2mg/ml with a 95% confidence interval (1.044, 1, 2006)}.Mortality rates in *L.major* promastigotes treated with crude extracts from *A. sativum* (t= 8.292532, DF=36 and $P < 0.05$) were statistically significant.

Deaths in *L. major* promastigotes cultured in complete Schneider's media were due to growth limiting factors such failure to acclimatize to the *in vitro* environments, accumulation of toxic waste substances, lack of sufficient nutrients supply, depletion in amounts of gases and reduced space.

Extra cellular antileishmanial potential in methanolic extract exceeds that of Pentostam® although the latter is also known for killing intracellular *Leishmania* parasites indirectly by activating macrophage mitochondrial mechanisms such as NO production (Philippe *et al.*, 2002). Oval shaped slender *L. major* promastigotes forms with short less vigorous flagella after 24 hours of treatment with *A. sativum* extracts suggests that the extracts interfered with the normal growth and development of parasites polygodials ability to inhibit the mitochondrial ATPase has been demonstrated by Lunde *et al*,( 2000).

The effect of *A.sativum* on parasite load was investigated. The footpads of infected and treated mice were studied using Limiting dilution assays. The footpad lesions of experimental mice on Topical treatment showed insignificant parasite numbers. Absence of parasites in such mice were indicative of the efficacy of *A.sativum* extract as an ointment against cutaneous leishmaniasis.Some *Leishmania* amastigotes were observed in footpads of mice that were treated with *A.sativum* extract for oral and injection.

However the mice that were on negative control i.e. those that got topical application of olive oil and either oral or injection with *A.sativum* extract showed a marked increase of parasite numbers in their footpads. This was also evident as their footpad lesions showed increase in size after treatment contrary to those on experimental and positive control group that had decrease in lesion sizes.

The results of Leishman Donovan unit showed that insignificant *L.major* parasites were able to metastasize to the visceral organs like the spleen. When the impression smears of spleens of mice infected and treated were examined microscopically most slides did not show presence of amastigotes. This was expected as *L.major* is a known cutaneous parasite and not visceral tropic like *L.donovani* and others that cause visceral Leishmaniasis.

65

These results of antileishmanial activity of *A.sativum* are in line with studies done by Nok *et al.*, They examined methanolic extracts of four Sudanese plants (*Azadirachta indica* (Neem), *Acacia nilotica, Balanites aegyptica* and *A. sativum* and revealed that only three species had a considerable *in vitro* anti- leishmanial activity on *L. major* promastigotes. The plants gave a $LC_{50}$ of 10.2, 4-94 and 89.38ng/ml for *Accacia indica* Juss, *A. sativum* L. and *Accacia nilotica* Willd respectively.

These four plant species in Sudan used in traditional medicine were collected. samples of each plant dried, coarsely powdered (except *A.sativum* which was used fresh). 100g of each sample was soaked in 80% methanol over night with continuous shaking at $37^0C$ then filtered and kept at $4^0C$ for *Leishmania* test.

The different statistical procedures using the standard agent Pentostam proved that Neem and *A.sativum* are the more effective agents against *L. major* promastigotes compared to the other agents. They showed 44.04 and 46.7 mean of growth inhibition respectively after 72 hours. They also showed the lowest mean differences with the standard 4.08 and 1.5 respectively. *A.sativum*, the only agent that showed insignificant difference with standard agent Pentostam® ($\rho=0.10$) means that *A.sativum* can be considered to have powerful anti leishmanial activity. *A.sativum* also showed $IC_{50}$ at concentration of 4.94μg/ml while Pentostam® showed $IC_{50}$ 3.07μg/ml. This work had been supported by Atta-Ur-Rahaman *et al,* whose work on Leishmanicidal natural products had led to the identification of ajoene (one of the garlic compounds) as a potent leishmanicidal substance. He reported $IC_{50}$ of *A.sativum* compounds more than 0.39μg/ml.

The ability to survive and multiply within macrophages is a feature of several infectious agents including *Trypanosoma cruzi* and *Leishmania.*In order to sustain a chronic infection ,parasites must subvert macrophage-accessory cell activities and oblate the development of protective immunity as reported by Alexander *et al.*(1999).Nevertheless, the most important mechanism for the killing of *Leishmania* and the control leishmaniasis is the production is the production of nitric oxide NO by macrophages of draining lymph nodes( David *et al.*,1997).New drug therapy regimens have taken advantage of the knowledge obtained from studies on *Leishmania*-macrophage interaction. Concerning mouse peritoneal macrophage infection with *L. major*, when the macrophages were

pretreated with *A. sativum* or pre infected with the parasite and then treated with dried *A. sativum* solution it was showed that *A.sativum* extract reduced macrophage infection through induction of Nitric oxide (NO) production (Gamboa *et al.*, 2007).

*A.sativum* extract may thus act on both T cells and macrophages to stimulate IFN-λ and NO synthesis for parasite killing. It has been described as having some Immunomodulatory activity Kasuga *et al*, 2001 and therapy with a fresh aqueous *A.sativum* extract in mice found to significantly control infection with *Leishmania major* also intensified the effect of Glucantime Ganzafari *et al.*, 2000.This therapeutic effect of garlic extract was associated with a strong shift in cytokines from a Th-2 to Th-1 profile.

Further more; a single dose of *A.sativum* extract in mice was able to induce macrophage recruitment and increase phagocytosis and the killing of *L.major in vivo*. Previous reports showed that *A.sativum* extracts could stimulate NK cell activity Hassan *et al.*, 2003 and lectin-induced lymphocyte proliferation (Morioka *et al.*, 1993).This activity however depends on the type of extract and it concentration. *A.sativum* also decreases lipopolysaccharides LPS-induced proinflamatory cytokines such as TNFα and IL-1β from blood cells. Because of these immunomodulatory properties and the potent effect of *A.sativum* extract against *L. major*, we extended these findings by showing that an aqueous and olive oil based extract of dried *A.sativum* could control an infection by *L. major* promastigotes in BALB/c mice. Indeed dried extract ointment for topical application induced a marked reduction in footpad lesions and parasite burden compared to the control mice. Compared to the dried methanolic extract in PBS for oral and parenteral treatment, the *A.sativum* ointment for topical application was found to be more effective as an antileishmanial agent.

## 4.3 CONCLUSIONS

The results of this study confirmed that the use of medicinal plants in folk medicine contributes significantly to primary health care, and that natural products are potential sources of new and selective agents for the treatment of important tropical diseases caused by protozoans. The killing of *L. major* promastigotes in the plant extract was dependent upon the mode of preparation i.e. type of extract and its concentration.

The present report is based on the investigation of *A. sativum*'s activity in the treatment of BALB/c mice experimentally infected with *L. major* promastigotes. It was observed that infected mice treated with 20 mg/kg/day of *A. sativum* ointment for 4 weeks post infection showed a significant clinical and parasitological response, with reduction in the size of lesions and decreased numbers of parasites.

These promising results pave the way for further testing of this drug as a new alternative in the chemotherapy of leishmaniases. *A.sativum* ointment had the most significant ($p<0.05$) leishmanicidal effect on *L.major* promastigotes *in vitro* therefore they are potential chemotherapeutic agents for leishmaniases. Treatment of BALB/c mice infected with *L. major* with *A. sativum* crude extract resulted in significant decrease in lesion size and parasite burden. BALB/c mice infected with *L. major* represents a model of extreme susceptibility, and the striking and sustained reduction in the number of parasites in treated animals supports the proposal of further testing of this drug in other models of leishmaniasis.

## 4.4 RECOMMENDATIONS

Further laboratory and clinical studies of these plants are required in order to understand their antileishmanial principles in non human primates like vervet monkeys (*Cercopethicus aethiops* L.) that have been shown to be good animal models for studying leishmaniases. Therefore, the most promising extracts should be prioritized for further phytochemical analysis on the isolation and the identification of the active compounds with leishmanicidal activity. Bioassay guided fractionation should be performed on methanolic extract of *A. sativum* to identify the bioactive components.

If an extract continues to exhibit significant parasite clearance rate. no adverse effects and optimum immunostimulative results, then the same should be tested in primates, after which pre-clinical and clinical trials should follow.

# REFERENCE

1. Abok,K.,Cadenas,E and Brunk,U(1988a).An experimental model system for Leishmaniasis;Effects of porhyrin compounds and in drugs responses of *Trypanosoma brucei.Journal of Parasitology.* **81**(6):968-973

2. Alexander, B., Cadena, H., Usma, M.C. and Rojas, C.A. (1995). Laboratory and field evaluations of a repellent soap containing diethyl toluamide (DEET) and permethrin against phlebotomine sand flies (Diptera: Psychodidae) in Valle del Cauca, Colombia. *American Journal of Tropical Medical Hygiene. 52(2):* 169-173.

3. Alexander, J., Satoskar, A.R. and Russel, D.G (1999). Leishmania species: models of intracellular parasitism. *Journal of Cell Science.* **112**: 2993-3002.

4. Alexander, J., Satoskar, A.R. and Russel, D.G (1999). *Leishmania* species: models of intracellular parasitism. *Journal of cell science.* **112**: 2993-3002.

5. Alvar J, Cruz, I., Morales, M. A., Noguer, Rodriguez I.A. and. (2002). *Leishmania* in discarded syringes from intravenous drug users. *Lancet.* **359**: 1124-25.

6. Anjili, C.O., Odongo, S., Ogaja P. Mbati, P.A., and Tonui, W. (2000). *Leishmania major* infections in *Phlebotomus duboscqi* fed on murine models immunized with L. major sub cellular antigens and sand fly gut antigens. *Onderstepoort Journal of Veterenary Resserch.* **67**(1): 57-63.

7. Arfan B. and Simeen R. (2006) Correlation of clinical, histopathological, and microbiological findings in 60 cases of cutaneous leishmaniasis. *Ind. J. of Derm. Ven. And Lep.* **72**:28-32.

8. Arora D.R.(2006) Medical Parasitology.Second edition.

9.  Ashford,R.W (1974).Sand flies(Diptera:Psychodidae) from Ethiopia: Taxonomy and biological notes.*Journal ofMedical Entomology*.11:605-616

10. Ashford, R.W. (1997). The leishmaniasis as model Zoonosis. *AmericanTropical Medical Parasitology*. **91**: *693-701*.

11. Ashford, R.W. and Bates, P. A. (1998). Leishmaniasis in the Old World. Topley & Wilson's, Microbiology and Microbial Infections. Ninth Edition. 5: 215-242.

12. Ashford, R.W., Desjeux, P. and de Raadt, P. (1992). Estimation of population at risk of infection and number of leishmaniasis. *Parasitology Today*. *8: 104-105*.

13. Basimike, M., Mutinga, M.I. (1997). Studies on the vectors of leishmaniasis in Kenya: Phlebotomine sand flies of Sandai location Baringo district. *East African Medical Journal*. **74** (9): 582-5.

14. Beach, R., Kiilu, G., Hendricks, L.D., Oster, C.N. and Leeuwenburg, J. (1984). Cutaneous leishmaniasis: transmission of Leishmania major to man by the bite of naturally infected *Phlebotomus duboscqi*. *Transnational Research Society of Tropical Medical. Hygiene. 78: 747-751*.

15. Bradley D.J and Kirkly J.Regulations of *Leishmania* populations within the host. The variable course of *L.donovani* infections in mice. Clinical and Experimental Immunology 1977; 30:119-129.

16. Beach, R., Young, D.G. and Mutinga, M.J. (1982). *Phlebotomus duboscqi* from Kenya: a new record. *Transnational Research Society of Tropical Medical. Hygiene. 76: 707-708*

17. Berman, J.D. (1997). Human leishmaniasis: clinical, dignostic and chemotherapeutic developments in the last ten years. *National Library of Medicines*. **24** (4): 684-703.

18. Berman J.D 1988.Chemotheraphy of the Leishmaniasis: Bichemical mechanism. Clinical efficacy and future strategies *Rev.infect.Dis.***10**:560-586

19. Berman, J.D. (2005). Infectious diseases in clinical practice. *Clinical. Infectious Diseases.* **13** (2): 84-86.

20. Boelaert, B., Criel, J., Leeuweburg, W., Vandamme, D.. Le ray and Van de S. (2002). Visceral leishmaniasis control: a public health perspective transaction of the Royal Society of Tropical medicine and hygiene. *National Library of Medicines* **94***: 465-471*.

*21.* Boelaert, M., Criel, B., Leeuwenburg, J., Van Damme, W., Le Ray, D. and Van der Stuyft, P. (2000). Visceral leishmaniasis control: a public health perspective. . *Transnational Research Society of Tropical Medical. Hygiene.* **94***: 465-471*.

22. Berman JD, Bhattacharya SK, Jha TK, Sundar S, Thakur CP, Engel J, Sindermann H, Junge K, Karbwang J, Bryceson AD, 2004. Efficacy and tolerability of miltefosine for childhood visceral leishmaniasis in India. *C Clinical. Infectious Diseases.* **38**: 217–221.*[Medline]*

23. Colic M,Savic M.Garlic extracts stimulate proliferation of rat lymphocytes in vitro by increasing Il-2 and IL-4 production.*Immunophamacology and immunotoxicalogy* **2000**;22:163-81

24. Cheng, S. (2003). Evaluation of the roles of four *Candida albicans* genes in virulence by using gene disruption strains. *Immunology.* **71** (10): 6101-6103.

25. Clive, R.D., Paul K., Simon L.C. and Shyam S. (2003). Leishmaniasis: new approaches to disease control. *Biomedical Journal.* **326**: 377-382.

26. Cole, A.C.E., Cosgrove, P.C. and Robinson, G. (1942). A preliminary report of an outbreak of kala-azar in a battalion of King's African Rifles. *Transnational Research Society of Tropical Medical. Hygiene.* **36**: 25-3.

27. Conjivaram, V., Gary, J. N., Mary L. W., Martin, W., Robert W. T. and Russel, S. (2002). Leishmaniasis. e Medicine. www.emedicine.com/ped/topic1292.htm.

28. Croft S.L and Vanessa Y. (2002). Chemotherapy of leishmaniasis. Curr. Pharm.D. 8: 319-342.

29. Chapman WL Jr, Hanson WL, Waits VB, Kinnamon KE, 1979. Antileishmanial activity of selected compounds in dogs experimentally infected with *Leishmania donovani. Rev Institute of Medical Tropical Sao Paulo* **21**: 189–193.[Medline]

30. Chiej. R. Encyclopaedia of Medicinal Plants. MacDonald 1984 ISBN 0-356-10541-5

31. Croft, S.L. and Coombs G.H. (2003). Leishmaniasis: current chemotherapy and recent advances in the search for novel drugs. *Trends in Parasitology* **19** (11): 502-508.

32. Colic M,Vuceviv D,Kilibarda V,Radicevic N,Savic.Modullatory effects of garlic extracts on proliferation of T-lymphocytes *in vitro* stimulated with Concanavalin A.Phytomedicine.2002;9:117-24

33. Castes, M., Agnelli, A. and Rondun, A.J. (1984). Mechanisms associated with immunoregulation in human American cutaneous leishmaniasis. *Clinical Experimental Immunology.* **37**: 295-3

34. Cruz, I., Morales, M. A., Noguer, Rodriguez I.A. and Alvar J. (2002). *Leishmania* in discarded syringes from intravenous drug users. *Lancet.* **359:** 1124-25.

35. Cruz, I., Morales, M. A., Noguer, Rodriguez I.A. and Alvar J. (2002). *Leishmania* in discarded syringes from intravenous drug users. *Lancet.* 359: 1124-25.

36. David, J., Soares, M., Satoskar, A., Brombacher F., Shoemaker, C., Titus, R. and Bozza, M. (1997). Immunomodulatory properties of maxadilan, a peptide derived from *Pesquiera australis. Acta Parasitologia.* **21**: 174.

37. Davies, C.R., Kaye, P., Croft, S.L. and Sundar, S. (2003). Leishmaniasis: New approaches to disease control. *Biomedical Journal.* **326**: 377-38.

38. Desjeux, P. (1996). Leishmaniasis: Public health aspects and control. *Clinical dermatolology* **14**: 417-23.

39. Desjeux, P. (2001). The increase in risk factors of leishmaniasis worldwide. *Trans African Reserch Society of Tropical Medicine.and Hygiene.* **95**: 239-243.

40. Desjeux, P. (2004). Leishmaniasis. TDR. *National. Revision of Microbiology.* **2:** 692-693

41. Dolands illustrated Medical Dictionery.26th Edition.WB Sounders Company Philadelphia 1981 pg.49.

42. Fournet, A.V., Monoz, A.A., Barrios R., Core. A (1992).Effect of natural naphthoquinones in BALB/c mice infected with *L.amazonensis* and *L.venezuelensis.Tropical Medical Parasitology.* **43** (4) 219-222.

43. Fatima, A. Khalid, Nazar.M. Abdalla,Hassam Eldin O.Mohammed,Abdalla M.Toum,Mubark M.A.Magzoub,M.Siddig Ali,2005.*In vitro* Assessment of Anti-Cutaneous Leishmaniasis Activity of some Sudanese Plants.*Acta parasitologia Turcica* **29**(1):3-6,2005.

44. Franke, A., Cuentes, L., Eschew, J.A., Maria, E., Cruz, P.C., Adolfo, A., Carmen, M. and Berman, J.D. (1994). Efficacy of 28 day and 40 day regiments of Sodium Stibogluconate (Pentostam) in the treatment of mucosal leishmaniasis. *American Journal of Tropical Medical Hygiene.* **51** (1): 77-82.

45. Githure, J. I., Schnur, L. F., Blancq, S.M. and Hendricks, L. D. (1986). Characterizations of Kenya *Leishmania* spp. and identification of *Mastomys natalensis*, *Tarterillus emini* and *Aethomys kaiseri* as new hosts of *L. major*. *American Tropical Medical. Parasitology.* **80**: 501-50

46. Gamboa-Leon R.Paraguai de –Souza E.Immunotheraphy against Visceral Leishmaniasis with the Nucleoside hydrolase's-DNA Vaccine of *Leishmania donovani*.Vaccine 2006; 24:4863-73.

47. Githure, J.I., Beach, R.F and Lighter, L.K (1984).The isolation of *L.major* from rodents in Baringo district, Kenya. *Transnational Research Society of Tropical Medical. Hygiene.* **78** (2) 283

48. Gamboa-Leon, I.M.R, Aranda-Gonzallezi, M.Mut-Martini, M.R.Garcia-Miss, E.Dumonteil.In *vivo* and *In vitro* control of *Leishmania mexicana* due to Garlic induced NO Production (2007).*Scandinavian journal of immunology* **66**,508-514

49. Githure, J.I., Muchemi, G.K. Binhazim, A.A., and Reid, G.D.F. (1987). Isolation of Leishmania major from a naturally infected vervet monkey (*Cercopithecus aethiops*) in Kiambu District, Kenya. *Journal of Parasitology. 73*: 1278-12

50. Gharzanfari T.Hassan ZM, Ebtekar M.Ahmdiani A. Naderi G.Azar A. Garlic induces a shift in cytokine pattern in Leishmania major- infected BALB/c mice. *Scandinavian journal of Immunology 2000; 52*:491-5.

51. Gharzanfari T, Hassan ZM. Khamesipour A. Enhancement of peritoneal macrophage activity against *Leishmania major* by Garlic (*Allium sativum*) treatment. *Ethnopharmacolology.2006*; 103:333-7

52. Githure J., Shaefer, K. U., Kurtzhals J. A., Sherwood, J. A. Kager P. A., Muller A. S. (1995). Epidemiology and clinical manifestations of visceral and cutaneous leishmaniasis in Baringo district. Lift valley, Kenya. *Tropical Geoggraphy of Medicines*: 46 (3): 129-33.

53. Herwaldt, B.L. and Berman, J.D. (1992). Recommendations of treating leishmaniases with sodium stibogluconate (Pentostam) and review of pertinent clinical studies. *American Journal of Tropical Medical Hygiene. 46* (3): 296-306.

54. Hassan Z.M,Yaraee R,Zare N, Gharzanfari T,Sarraf Nejad AH.Nazori B.Immunomodulatory effect of R10 Fraction of Garlic extract on Natural killer activity.*Intituite of Immunopharmacology. 2003*;3:1483-9

55. Hassan SM, Thakur CP, Sinha PK, Singh KR. Narain S, 2000. Miltefosine in a case of visceral leishmaniasis: and rising incidence of this disease in India. *Trans Royal Society of Tropical Medical Hygiene. 94*: 696–697.[*Medline*]

56. "http://en.wikipedia.org/wiki/Allium"

57. http://en.wikipedia.org/wiki/Garlic"

58. "http://en.wikipedia.org/wiki/Scallion"

59. Categories: Leaf vegetables | Onions

60. http://en.wikipedia.org/wiki/leishmaniasis"

61. http://en.wikipedia.org/wiki/ Cutaneous leishmaniasis"

62. http://en.wikipedia.org/wiki/ Visceral leishmaniasis"

63. Hendricks, L.D. and Wright (1979).Diagnosis of Cutaneous Leishmaniasis by in vitro cultivation of saline aspirates in Schneider's Drosophila medium *American Journal of Tropical Medical Hygiene.***28**; 962-964.

64. Iwu, M.M., Jackson, J.E.and Schuster, B.G. (1994).Medicinal plants in the fight against Leishmaniasis. *Parasitology Today* **10**:2

65. Iwu, M.W., Duncan, A.R. and Okunji, C.O. (1999). New antimicrobials of plant origin. In: J. Janick (Edition.), Perspectives on new crops and new uses. Alexandria. 57–462.

66. Jha TK, Sundar S, Thakur CP, Bachmann P, Karbwang J, Fisher C, Voss A, Berman J, 1999. Miltefosine, an oral agent, for the treatment of Indian visceral Leishmaniasis. *National English Journal of Medicines.* **341**: 1795–1800.

67. Iwu M.M., Jackson,J.E and Schuster B.G (1994) Medicinal plants in the fight against Leishmaniasis. *Parasitology Today* **10**:2

68. Jha SN, Singh NKP, Jha TK, 1991. Changing response to diamidine compounds in cases of kala-azar unresponsive to antimonial. *Journal of Associations of Physicians in India* **39**: 314–316.

69. Jha TK, Giri YN, Singh TK, Jha S, 1995. Use of amphotericin B in drug-resistant cases of visceral leishmaniasis in north Bihar. *American Journal of Tropical Medical Hygiene.* **52:** 536–538.

70. Johnson, R.N., Ngumbi, P.M., Gachihi, G.S., Mwanyumba, J.P., Mbugua, J., Mosonik, N., Were, J.B., Roberts, C.R. (1993). A new focus of kala-azar due to *Leishmania donovani* sensu lato in Kenya. . *Transnational Research Society of Tropical Medical. Hygiene* . **87** (2): 142-144.

71. Kaul, S.M., Sharma, R.S., Dey, K.P., Rai, R.N. and Verhese, T. (1994). Impact of DDT indoor residual spraying on *Phlebotomous argentipes* in a kala-a zar endemic village in eastern Uttar Pradesh. Bull. WHO **72**: 79-81.

72. Kasuga S,Uda N,Kyo E, Ushijima M,Morihara N.Itakura Y.Pharmacologic activities of aged Garlic extracts in comparison with other garlic  preparations. *Nuture.* **2001**; 131:1080s-4s.

73. Kasuga S Kyo E,Uda N., Itakura Y.Immunomodulatory effects of aged Garlic extract's . **2001**;131:1075s-9s.

74. Keiss HP, Dirsch, Hartung T.Garlic (*Allium sativum* L.) modulates cytokine expression in Lipopolysaccharides-activated human blood therapy inhibiting NF-kappa B Activity. *Journal of naturer* **2003**; 133:2171-5.

75. Katakura, K.and Koboyashi, A (1985).Enhancement of infectivity of *Leishmania donovani* promastigotes by serial mouse passages.*Journal of Parasitologia* **71**(3):393-394

76. Kokwaro J.O. (1993). Medicinal plants of East Africa. Second Edition. East Africa Literature Bureau. Nairobi, Kenya. 1-69.

77. Kungu, A., Mutinga,  M. J., and Ngoka, J.M. (1972). Cutaneous leishmaniasis in Kenya. *East African medical journal..* **48**: 458-465.

78. Kiilu, G., Hendricks, L.D., Oster, C.N. Beach, R., and Leeuwenburg, J. (1984). Cutaneous leishmaniasis: transmission of Leishmania major to man by the bite of naturally infected *Phlebotomus duboscqi. TransnationalResearch society of tropical medical hygiene.* **78**

79. Lacerda, M. M. (1994). The Brazilian leishmaniasis control program. *Memoir Institute Oswaldo Cruz.* **89**: 489-495.

80. Lunde C.S. and Kubo, I. (2000). Effects of Polygodial on the Mitochondrial ATPase of Saccharomyces cerevisiae. *American society of microbiology.* **44** (7): 1943-1953.

81. Manuel J. (2002). Vaccination against Leishmania infections. *Nat. Prod. Rep.* **2**:201-226.

82. Manuel, J.C. and Luis, M.P.  (2001). Plant natural products with leishmanicidal activity. *Nat. Prod. Rep.* **18**: 674- 688.

83. Maroli M. and Khoury C. (2004).  Prevention and control of leishmaniasis vectors: current approaches. *Parassitologia* **46** (1-2): 211-215.

84. Maslov.D.A., Podlipaev, S.A and Lukes, J., (2001). Phylogeny of the Kinetoplastida: Taxonomic problems and insights into Evolution of Parasitism. *J. Mol. Evol.*397-402.

85. Mbati, P.A., Githure, J.I., Kagai, J.M., Kirigi, G., Kibati, F., Wasunna, K., Koech, D.K. (1999). Evaluation of a standardized direct agglutination test (DAT) for the diagnosis of visceral leishmaniasis in Kenya. *Am. Trop. Med. Parasitol* **93** (7): 703-710.

86. Muigai, R., Githure, J.I., Gachihi, G., Were, J.B.O., Leuweenburg, J. and Perkins, P.V. (1987). Cutaneous leishmaniasis caused by Leishmania major in Baringo District, Kenya. *Trans. R. Soc. Trop. Med. Hyg.* **81**: 600-602.

87. Mutinga, M.J. (1975a). Phlebotomus fauna in the cutaneous leishmaniasis focus of Mt. Elgon, Kenya. *East Afr. Med. J.* **52**: 142-151.

88. More B. Bhatt H, Kukreja V, Ainapure S, 2003. Miltefosine: great expectations against visceral leishmaniasis. *J Postgrad Med* **49**: 101–103.[Medline]

89. Mutinga, M.J. (1975b). The animal reservoir of cutaneous leishmaniasis on Mt. Elgon, Kenya. *East Afr. Med. J.* **52**: 142-151.

90. Mutinga, M.J. (1991). A review of investigations on leishmaniasis vectors in Kenya. *Parasitologia* **33**(Supplement): 453-461.

91. Nok AJ, Williams's, Onynekkwe, PC, 1996.*Allium sativum* induced death of African Trypanosomes.*Parasitology research.*, **82**; 634-637.

92. Nolan, T.J and Farrel, J.P. (1987) Experimental infections of the multimammate rat (Mastomys natalensis) with *Leishmania donovani* and *L. major.American journal of tropical medical hygiene.***36** (2):264-269.

93. Ngoka. J.M. and Mutinga, M.J. (1978). Visceral leishmaniasis in Kenya: The onset of an epidemic outbreak in the Machakos district of Kenya. *East African Medical journal.* **55** (7): 328-331.

94. Olobo J.O,Michael M.Gicheru and Anjili C.O (2001).The African Green monkey model for cutaneous and VL.*Trends in Parasitology.***17** (12) 588-592.

95. Pamplona-Roger, G.D. (2000) Garlic heals and efficiently prevents many diseases. Encyclopedia of Medicinal Plants. Education and Health Library. Editorial Safeliz, S.L.Madrid, Spain.pp 230-233.

96. Philippe, H., Denis S., Mireille, C., Isabelle, M., Sylvie, D., 2 Philippe, V., and Jean, L. L. (2002). Nitric Oxide-Mediated Proteasome-Dependent Oligonucleosomal DNA Fragmentation in *Leishmania amazonensis* Amastigotes. *Journal of infectious immunology.* **70**: (7) 3727-3735.

97. Prasad R, Kumar R, Jaiswal BP. Singh UK, 2004. Miltefosine: an oral drug for visceral leishmaniasis. *Indian Journal of peadiatrics.* **71**: 143–144.[Medline]

98. Rosa, M.D., Ricardo, S., Mendonca-Filho, R., Humberto, R.. Bizzo, R., Igor de Almeida R., Rosangela M., Soares, A., Thais Souto-P., Alviano, C. L., and Lopes, A.C.S. (2003). Antileishmanial activity of a Linalool-Rich Essential Oil from *Croton cajucara. Antimicrobiological Agents and Chemicals.* **47**: 1895-1901.

99. Sang, D. K., Okelo, G. B., Chance, M. L. (1993b). Cutaneous leishmaniasis due to *L. aethiopica* on mount Elgon, Kenya. *American journal of tropical medical Parasitology.* **4**: 349-357.

100. Sang, D. K., Okelo, G. B., Chance. M. L. (1993a). Cutaneous leishmaniasis due to *L. aethiopica* on mount Elgon, Kenya. *American journal of tropical medical Parasitology.* **4:** 349-357.

101. Singh DV, Shukla NP, 1984. Activity on multiple resistant bacteria of garlic (*Allium sativum*) extract Fitoterapia5595); 313-315

102. Schnur, L.F., Chance, M.L., Ebert, F., Thomas, S.C. and Peters, W. (1981). The biological and serological taxonomy of visceralizing Leishmania. *Annual Tropical medical parasitology.* . **75**:131-144.

103. Sundar S, Singh VP, Sharma S, Makharia MK, Murray HW, 1997. Response to interferon-ɣ plus pentavalent antimony in Indian visceral leishmaniasis. *Journal of infectious diseases.* **176:** 1117–1119.

104. Sundar S, Jha TK, Thakur CP, Mishra M, Singh VR, Buffels R, 2002. Low-dose liposomal amphotericin B in refractory Indian visceral leishmaniasis: a multicenter study. *American journal of tropical medical Parasitology.* **66**: 143–146.

105. Sundar S, Gupta LB, Makharia MK, Singh MK, Voss A, Rosenkaimer F, Engel J, Murray HW, 1999. Oral treatment of visceral leishmaniasis with miltefosine. *American tropical medical Parasitology.* **93**: 589–597.[ [Medline]

106. Sundar S, Jha TK, Thakur CP, Engel J, Sindermann H, Fischer C, Junge K, Bryceson A, Berman J, 2002. Oral miltefosine for Indian visceral leishmaniasis. *National English journal of medicines.* **347:** 1739–1746.

107. Sundar S, Jha TK, Sindermann H, Junge K, Bachmann P, Berman J, 2003. Oral miltefosine treatment in children with mild to moderate Indian visceral leishmaniasis. *Pediatrics Infectious diseases.* **22**: 434–438.

108. Singh S. and Sivakumar R. (2003). Recent advances in the diagnosis of leishmaniasis.

109. *Journal of postgraduate Medicine.* **49**: (1) 55-60.

110. Schnur, L.F., Chance, M.L., Ebert, F., Thomas, S.C. and Peters, W. (1981). The biological and serological taxonomy of visceralizing *Leishmania. Annual Tropical Medical Parasitology.* **75**:131-144.

111. Tempone A.,da silva,A.C.M.P.,Brant C.A., Martinez F.S.,Borborema,S.E.T.,da Silveira,M.A.B and Andrada J,H.F(2005).Synthesis and antileishmanial activities of novel 3 substituited quinolines.Antimicrobiol Agents Chemotheraphy.,49(30):1076-1080.

112. Thakur CP, Sinha GP, Pandey AK, Kumar N, Kumar P, Hasan SM, Narain S, Roy RK, 1998. Do the diminishing efficacy and increasing toxicity of sodium stibogluconate in the treatment of visceral leishmaniasis in Bihar, India; justify its continued use as a first-line drug? *American journal of tropical medical hygiene.* **92**: 561–569.

113. Thakur CP, Sinha PK, Singh KR, Hassan SM, Narain S, 2000. Miltefosine in a case of visceral leishmaniasis: and rising incidence of this disease in India. *Trans Royal Society of tropical medical hygiene.* **94**: 696–697.[Medline]

114. Thakur, C.P., Sinha, G.P., Sharma, V. and Barat, D. (1993). The treatment of kala-azar during pregnancy. *National medical journal of India.* **6** (6): 263-265.

115. Taswell, C. 1987. Limiting dilution assays for the separation, characterization, and quantization of biologically active particles and their clonal progeny, p. 109-145. In T. G. Pretlow and T. P. Pretlow (ed.), Cell separation: methods and selected applications. Academic Press, Orlando, Fla.

116. Webber ND,Andersen DO, North JA,MurrayBK,lawsonLD,Hubhes BG,1992.In vitro virucidal effects of Allium sativum(garlic)extracts and compounds.*Planta*

*medica*,**58:**417-423.

117. White MR, Chapman WL Jr, Hanson WL, 1989. Chemotherapy with
     experimental visceral leishmaniasis in the opossum. *Journal of Parasitology*.
     **75**: 176–178.[Medline]

118. Wijers, D.J. and Kiilu, G. (1984). Studies on the vector of kala-azar in Kenya,
     VIII. The outbreak in Machakos District: epidemiological features and a
     possible way of control. *American journal of tropical medical Parasitology.*. **78**
     (6): 597-604.

119. World Health Organization (1990). World Health organization Tech. Rep.
     Ser.793: 1-58.

120. WHO, 1990.Control of Leishmaniasis. Report of WHO expert committee,
     Technical series No 793.

121. World Health Organization (2000). The leishmaniasis and Leishmania / HIV co-
     infections, South-western Europe 1990-1998. Geneva: World Health
     Organization .Fact sheet no. 116.

122. White MR, Chapman WL Jr, Hanson WL, 1989. Chemotherapy with
     experimental visceral leishmaniasis in the opossum. *Journal of Parasitology*.
     **75**: 176–178.[Medline] )

123. World Health Organization (2002). Reducing risks and promoting healthy lives.
     The World Health Report 2002. World Health Organization, Geneva
     Switzerland. 192-197.

# APPENDICES

## APPENDIX I: PREPARATION OF SCHNEIDER'S INSECT MEDIUM

**Materials**

Distilled water

Schneider's medium powder

Sodium bicarbonate

Sodium hydroxide

Hydrochloric acid

Calcium chloride

0.45 and 0.22 microns filter units

**Preparation instructions**

1. Measure out 80% of final required volume of water. Water temperature should be 15-20 $^0$C. While gently stirring the water, add the powdered Schneider's medium. Stir until dispersed. Materials will not go in solution completely. Do not heat.

2. Rinse original package with a small amount of water to remove all traces of powder. Add to solution in step 1.

3. To the solution in step 3, add 0.4g Sodium bicarbonate or 5.3 ml of sodium bicarbonate (7.5% w/v) for each litre of final solution for each litre of final volume of media being prepared. Stir until dissolved.

4. To solution in step 5, add 35 ml 1.0 N NaOH solution for each litre of final volume of medium being prepared. Stir for 10 minutes. Solution will be turbid.

5. To solution in step 5, add 35 ml 1.0 N HCL to bring pH to approximately 6.9, solution will clear. Add 0.60 g Calcium chloride dissolved in 50 ml distilled water with rapid stirring for each litre of final volume of medium being prepared

6. While stirring, adjust the pH of the medium to 0.1-0.3 pH of the medium to 0.1-0.3 pH below the desired pH since it may rise during filtration. The use of 1N HCL or 1N NaOH is recommended. Add additional water to bring the solution to final volume.

7. Sterilize immediately by filtration using a membrane with a porosity of 0.45, then 0.22 microns or less. Aseptically dispense medium into sterile containers, label the name, date of preparation and store liquid medium refrigerated at 0-5 $^0$C in the

dark. This Schneider's medium is suitable for the growth of cells derived from *Drosophila melanogaster*.

## APPENDIX II: PREPARATION OF 1 LITRE OF COMPLETE RPMI 1640 MEDIUM

### Materials

- Sterile distilled water
- RPMI 1640 Sachet
- Stirrer
- Measuring cylinder
- Beaker
- Foetal calf serum (FCS), 20%=200ml
- Sodium bicarbonate ($Na(HCO_3)_2$, 35 ml (from stock solution of 7.5% $Na(HCO_3)_2$
- Antibiotics: Penicillin / Streptomycin (5 ml @200 ml of media
- Antimycotic: 5-fluorocytosine, 0.5 g
- Syringe

### 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 colour no longer shows. Top up the volume to 1L. Stir to ensure an even mixture of incomplete RPMI 1640 (RPMI).

2.  Pour out antibiotics and the Antimycotic into another mixing jar. Add Sodium bicarbonate (provides a buffering system for the media).

3.  Add incomplete RPMI till it reaches 1000 ml. The volume of the RPMI will be 1000-[200(FCS) +35 (Sodium bicarbonate) + Antibiotics quantity]. Stir the contents

4.  To these, add FCS, 200 ml. Stir again.

5.    Check the pH. Adjust to 7.0 using 1N HCL if above 7 or 1N NaOH if below. Filter-sterilize the media, label the name, date of preparation and store at 4 $^O$C.

## APPENDIX III: PREPARATION OF PHOSPHATE BUFFERED SALINE (PBS) AND COMPLETE HANKS BALANCED SALT SOLUTION (HBSS)

### A) PHOSPHATE BUFFEREDSALINE (PBS)

**Materials**

- Distilled water
- Phosphate Buffer powder
- NaCl

**Procedure**

1. PBS=PB + 0 85% NaCl. Therefore, weigh 0.1 g of PB powder and dissolve in 100 ml distilled water. Prepare 0.85% saline by dissolving 8.5 g NaCl in 1L of distilled water. [If 0.85 g in 100 ml, then 8.5 g in 1000 mls].

2. Measure 900 ml of saline and add 100 ml of PB solution. Filter-sterilize through 0.2 µm pore filter, label the name, date of preparation and store at 4 $^O$C.

### B) COMPLETE HANKS BALANCED SALT SOLUTION (HBSS)

**Materials**

- Sachet of HBSS
- $Na(HCO_3)_2$
- Distilled water
- pH meter

### Procedure

1. Pour the contents of the sachet into a beaker. Wash the sachet with distilled water and then add into the beaker. Top up the volume to 950 ml. Add 0.4 g Na (HCO3)2. Stir the contents for 30 minutes. Using the Ph meter, check and adjust the Incomplete HBSS to 7.2

2. For complete HBSS with 20% FCS, add 200 ml FCS, 0.05 g 5-fluolocytosine. Adjust the pH to 7.2. Filter-sterilize the media, label the name, date of preparation and store at 4 $^{O}$C.

## APPENDIX IV: HARVESTING AMASTIGOTES FROM A BALB/c MICE FOOT PAD

### Materials

- Infected animals
- Dissecting kit
- Flame
- 70% alcohol
- Sterile PBS
- Petri dishes or sterile tubes

## Procedure

1. Kill mouse by dislocation or by chloroform and pin it on a dissecting board.

2. Sterilize the scissors and tweezers by passing them through the flame, cool them, but observe sterility. Carefully, using scissors, remove any hair close to the lesion. Wash the lesion with sterile PBS, 70 % ethanol and finally sterile PBS.

3. Using sterile scissors and tweezers, remove the foot just above the lesion by cutting at the base of the foot, then chop off the toes before pulling the skin off with the tweezers, and finally cutting the whole footpad at the base.

4. Transfer the lesion to a sterile petri dish on ice containing either PBS of culturing media. Remove the petri dish to the laminar hood and cut the lesion in about 4 pieces of equal size.

5. Wash the lesion in about three changes of sterile PBS before subjecting it to further processing e.g. homogenization or culturing.

6. Remember if you have to hold any portion of the lesion or do any cutting you must pass the scissor or tweezers through the flame, take care not to 'fry the meat' by cooling them.

## APPENDIX V: HARVESTING AND CULTURING MACROPHAGES

### Materials

15ml centrifuge tubes

- Pre-cooled centrifuge
- Chloroform & anesthesia jar
- Ice
- 70%alcohol
- Hanks balanced salt solution
- Haemocytomete

## Procedure

1. Anaesthetize the mouse (one at time) with chloroform in an anesthesia jar with a lid, till it is unconscious. Soak the mouse in 70% ethanol or isopropanol for 5 minutes and let it dry in the hood. Snip the skin and pull it to expose the peritoneum completely.

2. Carefully introduce 5 mls of washing media HBSS into the peritoneal cavity using a 26G or 27G hypodermic needle and massage the abdomen.

3. Withdraw as much as possible of this media using 18G or 19G hypodermic needle and pool the media into a plastic centrifuge tube placed in ice.

4. Centrifuge the pooled media at 2000rpm for 10 minutes at $4^0C$ and suck off the supernatant leaving about 0.5 mls at the bottom.

5. Re suspend in 5mls of same media and make up to 10mls with the same media. Repeat the centrifugation using the washing media, and then repeat using the culture media.

6. Res suspend the cells in 5mls of culture media and do cell counts. Adjust the concentrations of macrophages to $10^5$ live cells per ml.

7. Add 0.5 ml of the suspension into each cell-culturing chamber. Incubate at $37^0c$ 5 % $CO_2$ and 95% humidity for 24 hours. Check that they are growing: - shown by increase in granules and size (if in doubt take a sample of the culture and stain using Trypan blue to show the dead one.

# APPENDIX VI : Probit Analysis Results on inhibitory concentrations in *Allium sativum* extracts

| Probability | Concentrations | 95% Fiducial limits | |
|---|---|---|---|
| 0.01 | -1163.8 | -1416.0 | -965.9 |
| 0.02 | -896.8 | -1112.3 | -727.5 |
| 0.03 | -727.4 | -919.7 | -576.0 |
| 0.04 | -600.0 | -775.0 | -462.0 |
| 0.05 | -496.4 | -657.4 | -369.1 |
| 0.06 | -408.2 | -557.4 | -290.0 |
| 0.07 | -330.8 | -469.8 | -220.6 |
| 0.08 | -261.6 | -391.4 | -158.3 |
| 0.09 | -198.6 | -320.2 | -101.6 |
| 0.10 | -140.6 | -254.7 | -49.3 |
| 0.15 | 99.4 | 15.0 | 168.5 |
| 0.20 | 290.2 | 226.5 | 344.4 |
| 0.25 | 453.9 | 403.6 | 499.7 |
| 0.30 | 600.9 | 556.4 | 645.4 |
| 0.35 | 737.1 | 691.5 | 786.9 |
| 0.40 | 866.3 | 814.8 | 926.0 |
| 0.45 | 991.4 | 931.2 | 1063.6 |
| 0.50 | 1114.4 | 1044.1 | 1200.6 |
| 0.55 | 1237.5 | 1155.9 | 1338.7 |
| 0.60 | 1362.5 | 1269.0 | 1479.6 |
| 0.65 | 1491.8 | 1385.4 | 1625.7 |
| 0.70 | 1628.0 | 1507.7 | 1779.9 |
| 0.75 | 1774.9 | 1639.4 | 1946.7 |
| 0.80 | 1938.6 | 1785.9 | 2132.6 |
| 0.85 | 2129.4 | 1956.5 | 2349.4 |
| 0.90 | 2369.4 | 2170.9 | 2622.5 |

| | | | |
|------|--------|--------|--------|
| 0.91 | 2427.4 | 2222.6 | 2688.5 |
| 0.92 | 2490.4 | 2278.9 | 2760.2 |
| 0.93 | 2559.7 | 2340.7 | 2839.0 |
| 0.94 | 2637.0 | 2409.7 | 2927.1 |
| 0.95 | 2725.2 | 2488.4 | 3027.6 |
| 0.96 | 2828.9 | 2580.8 | 3145.6 |
| 0.97 | 2956.3 | 2694.5 | 3290.7 |
| 0.98 | 3125.6 | 2845.5 | 3483.7 |
| 0.99 | 3392.6 | 3083.4 | 3787.8 |