EFFECTS OF *Acacia polyacantha* CRUDE BARK EXTRACTS ADMINISTRATION IN MICE INFECTED WITH *Leishmania donovani*.

BY

CHRISTINE TOIYOI SALBEI

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENT FOR THE AWARD OF THE DEGREE OF MASTER OF SCIENCE IN ZOOLOGY (PARASITOLOGY) OF THE UNIVERSITY OF ELDORET, KENYA

MAY, 2023

DECLARATION

Declaration by the candidate

This thesis is my own original work and has not been submitted for any other academic award in any other institution; and shall not be reproduced in part or full in any format without prior written permission from the author and /or University of Eldoret

Salbei T. Christine

_____ _____

SC/PGB/023/08 **Date**

Declaration by the Supervisors

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

_____ _____

Dr. Judith Makwali, PhD **Date**
Department of Biological Sciences,
School of Science, University of Eldoret,
P.O Box 1125-30100
Eldoret, Kenya.

_____ _____

Prof M. M Ngeiywa, PhD **Date**
Department of Biological Sciences,
School of Science, University of Eldoret
P.O Box 1125-30100
Eldoret, Kenya.

_____ _____

Christopher O. Anjili, PhD **Date**
Centre for Biotechnology Research and Development,
Kenya Medical Research Institute,
P.O Box 54840,
Nairobi, Kenya.

DEDICATION

The study is dedicated to; my husband, Charles Chemaringo, and children, Debra Jepchirchir and Hosea Kiptarus. I cannot thank you enough for your support. May God bless you.

ABSTRACT

Visceral leishmaniasis (kala-azar) is the severest form of leishmaniasis and is ranked the second deadliest parasitic disease in the world after malaria. Drugs of choice for the treatment of kala-azar are Pentavalent antimonials and Amphotericin B (AmB), which are toxic, and costly and the causative parasites are becoming resistant to them. Cheaper and safer alternative medicines are needed. *Acacia polyacantha* has shown medicinal properties. This study investigated the *in vitro* and *in vivo* efficacy of methanolic and aqueous crude bark extracts of *Acacia polyacantha* against *Leishmania donovani*. Experimental mice were infected with *L. donovani* and treated with plant extract, conventional drugs, and PBS. Their spleen weights and parasite loads were measured and equated between the groups. Data analysis was done using T-test and ANOVA to establish any statistically significant differences in the tested parameters. P values of < 0.05 were considered significant. It was found that the crude bark extract of *A. polyacantha*, is potent against *L. donovani* with MIC of 1.47mg/ml. Spleen weight means of BALB/c mice were significantly reduced by aqueous crude bark extract of *A. polyacantha* by 44% in SAB mice while the methanolic crude bark extract reduced spleen parasite loads by 63% in BALB/c mice, when the extracts were given intra-peritoneally. The methanolic bark extract of *A. polyacantha* was more effective, reducing spleen parasite loads by 63% compared to a reduction of 32% using the aqueous crude bark extract in BALB/c mice when both extracts were given by intra-peritoneal route. The study found that the intra- peritoneal route was more effective than the oral route in reducing spleen parasite loads (IP, LDU = 5.99) and (Oral, LDU = 15.34) in SAB mice, the difference was statistically significant ($p < 0.05$). The study concluded that the methanolic crude bark extract of *A. polyacantha*, administered intra-peritoneally was potent in lowering spleen parasite loads. The study recommends that the methanolic *Acacia polyacantha* bark extract should be administered intra-peritoneally to reduce spleen parasite loads in primates before humans. Further studies should be done on root and leaf extracts of *A. polyacantha* to establish if they have better action against *L. donovani*.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF APPENDICES

LIST OF ABBREVIATIONS /ACRONYMS

ANOVA	Analysis of Variance
AVL	Anthroponotic Visceral Leishmaniasis
BALB/c	An albino, laboratory-bred strain of the house mouse
CBRD	Centre for Biotechnology Research and Development
$CD4^+$T CELLS	Subset of T lymphocytes which signal to provide signal that help activationof CD8+T cells and antibody production by B lymphocytes.
$CD8^+$ T CELLS	Subset of T lymphocytes which are cytotoxic and can eliminate virus-infected
CL	Cutaneous Leishmaniasis
DMSO	Dimethyl sulfoxide
ELISA	Enzyme Linked Immunosorbent Assay
IP	Intraperitoneal
IV	Intravenous
KEMRI	Kenya Medical Research Institute
LDU	Leishman Donovan Units
MEM	Minimum Essential Medium
MIC	Minimum Inhibitory Concentration
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide
NTD	Neglected Tropical Disease
PBS	Phosphate Buffered Saline
PICC	Peripherally Inserted Central Catheter
PKDL	Post-Kala-azar Dermal Leishmaniasis
Rk39	Serodiagnostic rapid Elisa strip kit
RPMI	Roswell Park Memorial Institute
SAB	A mice cross between BALB/c and SWISS albino mice susceptible to *Leishmania donovani* and *Leishmania major* parasites
SSG	Sodium Stibogluconate
SIM	Schneider's Insect Medium

VL	Visceral Leishmaniasis
WHO	World Health Organization
χ^2 **test**	Chi-square test. A statistical test for the null hypothesis
ZVL	Zoonotic Visceral Leishmaniasis

ACKNOWLEDGEMENTS

This study has been accomplished with the assistance of several people. I would like to appreciate everyone who contributed to the success of this work. I acknowledge the late Mr Jacob Wendot who introduced to me the plant that was used in this study. The support and mentorship of Mr. Luka Chemwolo is recognized. His positive and hopeful attitude assisted in the completion of the thesis.

Distinct thank you goes to Mr. Johnstone Ingonga of KEMRI headquarters in Nairobi for the practical support towards the research. He acquainted me with laboratory procedures. My deepest gratitude goes to Ms. Jane Chepsergon for advice on statistical data analysis.

I hold in high esteem my supervisors; Prof. Moses Ngeiywa, Dr. Christopher Anjili (posthumously), Dr. Judith Makwali, and all staff of the Department of Biological Sciences University of Eldoret for the backing given to me during the entire time of the study. Their support cannot be matched.

CHAPTER ONE

INTRODUCTION

1.1 Background information

Leishmaniasis has different clinical manifestations, including visceral (VL or kala-azar), cutaneous (CL), mucocutaneous (MCL), diffuse cutaneous (DCL) and post kala-azar dermal leishmaniasis (PKDL) (Srivastava et al., 2016). Visceral leishmaniasis (VL), is the severest type of leishmaniasis caused by Leishmania parasites. The name kala-azar means “black fever” in Hindi. It was first used in India because patients with the disease developed hyperpigmentation (Craig, 2020).It is mainly transmitted by female phlebotomine sand flies . Other rare methods of transmission include ; during fetal period or at the time of birth, (Mescouto-Borges et al., 2013), through blood transfusion, (Mansueto et al., 2014), by direct person-to-person contact, (Franca et al., 2018), or as a result of laboratory accident, (Safar, 2017) . Kala-azar was first discovered in west Bengal where infection occurs throughout the year although the worst infections were first experienced in North and East Africa. Other affected regions are throughout the Arab world, Sudan and Southern Europe (Waguma & Jallow, 2011).The disease’s geographical range is wide but is not continuous (Chowdhury & Haque, 2019). The disease is perpetuated by drought, famine and high population density in Africa and therefore infection centers mostly in Sudan, Kenya and Somalia where these factors are frequent (Chowdhury & Haque, 2019). In Kenya kala-azar is endemic in hot semi-arid lowlands of the Rift valley, Eastern and North Eastern counties. Even though the disease victims may be under reported in Kenya, about 4000 people are estimated to be infected with the disease every year. The counties mostly affected are; West Pokot, Turkana,

Samburu, Baringo, Isiolo and Wajir (DNDI, 2011).Visceral leishmaniasis in India and East Africa is classically caused by *L. donovani*. This parasite accounts for sporadic cases of visceral leishmaniasis in Ethiopia, Somalia, Kenya and adjacent countries and major epidemics amid refugees in the Sudan (Waleed et al., 2016). Potential reservoirs include ; Rats, gerbils, ground squirrels and small carnivores (Kassahun et al., 2015).

The incubation period of kala-azar is typically weeks to several months, but it may be as short as ten days or as long as several years (MOH, 2017). Usually the onset of kala azar is gradual but can be sudden with high fever like malaria or another acute febrile infection (Craig, 2020). Majority of kala-azar infections are subclinical hence self-resolving ; a subset develop mild symptoms, (Craig, 2020). Only a minority of patients advance to severe kala-azar which is characterized by fever, weight loss, hepatomegaly, severe splenomegaly (Figure 1.1), neutropenia and hypergammaglobinemia (Craig,2020). The definite diagnosis for kala azar is visualization of the amastigotes in splenic aspirate or bone marrow aspirate (Asfaram, 2018).This procedure is done only by skilled personnel and in addition is frequently unavailable in areas of the world where visceral leishmaniasis is endemic (Asfaram, 2018).The commonly used laboratory screening method where leishmaniasis is endemic is serological testing (Leveque, 2020), unfortunately this is not dependable.

Meglumine antimoniate (glucatime), sodium stibogluconate (pentostam), amphotericin B, and miltefosine, are some of the clinically approved medications that have been discovered to treat this endemic condition .On the other hand, excessive use of these chemotherapeutic sources has been linked to antagonistic consequences (Ghobakhloo *et al.*, 2016).Other chemical-based pharmacological medications, such as pentamidine, and

paromomycin, have been used in the treatment of leishmaniasis for numerous years. None of these clinically approved medications could be considered as the ultimate source of treatment , due to the time-consuming method and high toxicity paired with significant adverse effects .Furthermore, parasites are not completely eliminated from all afflicted individuals by the most commonly used medications. Researchers have resorted to looking for natural ways without these undesired effects to treat leishmaniasis (De Menezes *et a*l., 2015).To date there is no licensed vaccine against any form of leishmaniasis (Rezvan & Moafi, 2015; Mutiso *et al*., 2013) coupled with high cost, and toxicity of the current antileishmanial chemotherapeutics which necessitates more research for alternative natural, safe and affordable drug compounds such as plant-based extracts. Another major challenge is the development of drug resistance by the parasite to antileishmanial drugs which necessitates the urgent need for a natural compound to provide affordable, readily available herbal medicines as well as creating a source of income to the local communities where these medicinal plants grow and counter the drawbacks of the current antileishmanial chemotherapeutics (Lage *et al*., 2013).

Acacia polyacantha, has the meaning "many thorns" in Latin (Howard, 1988). It is a flowering tree that can grow up to 25 metres tall and is also known as white thorn (Ross, 1979). The tree has been introduced to the Caribbean although it is native to Africa, India, the Indian Ocean and Asia, (Ross, 1979). Its bark and roots perhaps have medicinal uses. *A. polyacantha's* root extract is applied to wash the skin of children who are agitated at night and useful for snakebites but there is no published use(s) of the bark extract.

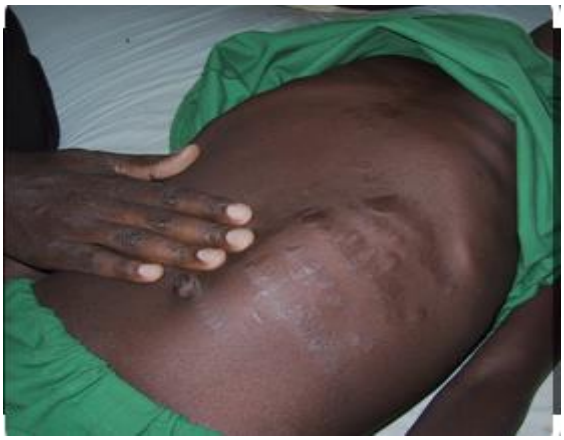


Figure 1.1: **An adult suffering from visceral leishmaniasis with severe splenomegaly (Source: WHO, 2016).**

1.2 Statement of the Problem

Approximately 200,000 to 400,000 VL cases occur each year globally (Alvar *et al*., 2012). Environmental changes such as atmospheric temperature and humidity, ecological conditions affecting the vector, parasite, and its reservoir, and population movements caused by migration and tourism coupled with socioeconomic conditions such as poverty and malnutrition are all risk factors that directly interfere with the world's distribution of leishmaniasis (Dawit *et al*., 2013; Strazulla *et al*., 2013). Visceral leishmaniasis in Kenya is endemic with an estimated 4,000 cases occurring annually, and 5 million people are at risk of infection (MOPS, 2012). Kala-azar is a grave and usually fatal neglected tropical disease (NTD), (Hotez, 2015), second after malaria as the leading cause of death from parasitic infections (Naghavi *et al*., 2013). In addition to the high level of global morbidity is a huge economic impact as well as social stigmatization and other psychological consequences (Litt *et al.*, 2012).

1.3 Justification

The leishmaniases continue to be one of the major tropical diseases affecting people in the Sub-Saharan Africa, Middle East and South America, but the drugs being used in the treatment are very expensive, toxic and require specialized administration in patients (Ghorbani & Farhoudi, 2017). Drug resistance is also becoming a major concern (Ponte-Sucre *et al*., 2017). It is therefore important to have a cheap, non-toxic alternative treatment for visceral leishmaniasis. According to reports from the World Health Organization, (2020) 65% of the world's population have integrated the use of medicinal plants as treatment in the primary modality of healthcare, and in Africa, 80% of individuals incorporate use of traditional herbal medicine (Tilburt & Kaptchuk, 2008). *Acacia polyacantha* in ethnobotanical studies has been found to have medicinal properties, (Ross,1979). In this study, *A. polyacantha* aqueous and methanolic extracts were tried for their *in vitro* and *in vivo* anti-leishmanial activities. The *in vivo* treatment was done using oral and intra-peritoneal routes.

1.4 Objectives

1.4.1 General objective

To investigate the effect of crude bark extracts of *Acacia polyacantha* in the treatment of SAB, BALB/c and Swiss Albino mice infected with *Leishmania donovani*

1.4.2 Specific objectives

1. To determine the effect of aqueous and methanolic crude bark extracts of *A. polyacantha*

   against *in vitro* promastigotes of *Leishmania donovani*

2. To determine the safety levels of *Acacia polyacantha* bark extract against Vero cells
3. To determine the effect of aqueous and methanolic crude bark extracts of *A. polyacantha* on spleen weights of *L. donovani* infected mice.
4. To determine the effect of aqueous and methanolic crude bark extracts of *A. polyacantha* on spleen parasite loads of SAB, BALB/c and Swiss Albino mice infected with *Leishmania donovani*
5. To determine the differences in efficacy of administering the aqueous and methanolic bark extracts of *A. polyacantha* through oral and intra-peritoneal routes in the treatment of SAB, BALB/c and Swiss Albino mice infected with *Leishmania donovani*

1.5 Research questions

1. Are the crude bark extracts of *A. polyacantha* potent against *Leishmania donovani* ?

2. Is the *Acacia polyacantha* bark extract safe to Vero cell?

3. Do crude aqueous and methanolic crude bark extracts of *A. polyacantha* suppress spleen weights of *Leishmani donovani* infected SAB, BALB/c and Swiss Albino mice ?

4. Do aqueous and methanolic crude bark extracts of *A. polyacantha* reduce spleen parasite loads of SAB, BALB/c and Swiss Albino mice infected with *L. donovani*. ?

5. Is there a difference in efficacy when aqueous and methanolic crude bark extracts of *A. Polyacantha* are administered orally and intra-peritoneally in treating, SAB, BALB/c and Swiss Albino mice infected with *L. donovani*.

1.6 Significance of the study

The new World Health Organization (WHO), Neglected Tropical Diseases (NTD) road map 2021-2030 (WHO, 2020) aims to eliminate visceral leishmaniasis as a public health problem, defined as a reduction to <1% in case fatality rate due to primary disease. Critical actions that must be undertaken to achieve these targets include early detection and confirmation of diagnosis to enable early treatment, adequate supply of drugs and diagnostics and the need for more effective diagnostics and treatment especially in Africa. Pentavalent antimonials have been licensed in all East African countries as the standard first line medicines for visceral leishmaniasis (WHO, 2010: Burkit, 2009). Unfortunately, increasing resistance to antimony treatment has been documented in more than half of visceral leishmaniasis patients in some areas in East Africa (Burkit, 2009). Regarding terms of treatment cost, the year 2013 research of the economic cost of visceral leishmaniasis in Sudan, (the only one of its kind in so far for East Africa) showed significantly high costs for both health care providers and affected households (Meheus *et al*., 2013). Pentavalent antimonial which is used as first-line treatment, in addition to the long treatment course of 30 days required for effective treatment, was also found to be mostly associated with this high economic burden (Meheus *et al*., 2013). This study therefore found it needful to identify alternative affordable non-toxic drugs which can be prepared locally for the treatment of leishmaniasis. In this study, the efficacy of crude aqueous and methanolic bark extracts of *A polyacantha* in the treatment of SAB, BALB/C and Swiss Albino mice infected with *L. donovani* was tried.

CHAPTER TWO

LITERATURE REVIEW

2.1 Visceral leishmaniasis

Leishmaniasis is a parasitic disease caused by protozoa of the *Leishmania* genus (Craig, 2020). The parasites usually migrate to the internal organs such as liver, spleen (hence 'visceral ') and bone marrow, and, will almost always result in the death of the host, if not treated (WHO, 2015). Certain species of sandfly of the genus *phlebotomus* (Old world) or *Lutzomyia* (New world), carry out natural transmission of the *Leishmania*. Kala azar is classically caused by the *Leishmania donovani* complex, which includes three species; *Leishmania donovani*, and *Leishmania (chagasi) infantum. Leishmania donovani* complex is responsible for visceral leishmaniasis in children in the Mediterranean basin (Sundar & Rai, 2002)

2.2 The transmission cycles of the *Leishmania* species

Two known transmission cycles of *Leishmania*¸ are the zoonotic cycle (Figure 2.1) that involves a reservoir host on which the sand fly feeds on for onward transmission to man, and an anthroponotic cycle (Figure 2.2) where the infection is perpetuated by a sand fly-man-sand fly transmission pattern. *Leishmania major* cutaneous leishmaniasis with rodents as reservoir hosts (Alemayehu & Alemayehu, 2017), and the sand fly *Phlebotomus duboscqi* Neveu-Lemaireas the vector with *Leishmania aethiopica* whose reservoirs are hyraxes and the giant rat, with *P. pedifer* as the vector, are examples of zoonotic cycle (Anjili *et al*., 2011). For visceral leishmaniasis caused by *L. donovani* no reservoirs have been incriminated in Kenya such that the disease has been considered

anthroponotic in Kenya (MOH, 2017) with *P. martini* as the vector (Waleed *et al*., 2016). The two life cycles are shown in Figures 2.1 and 2.2

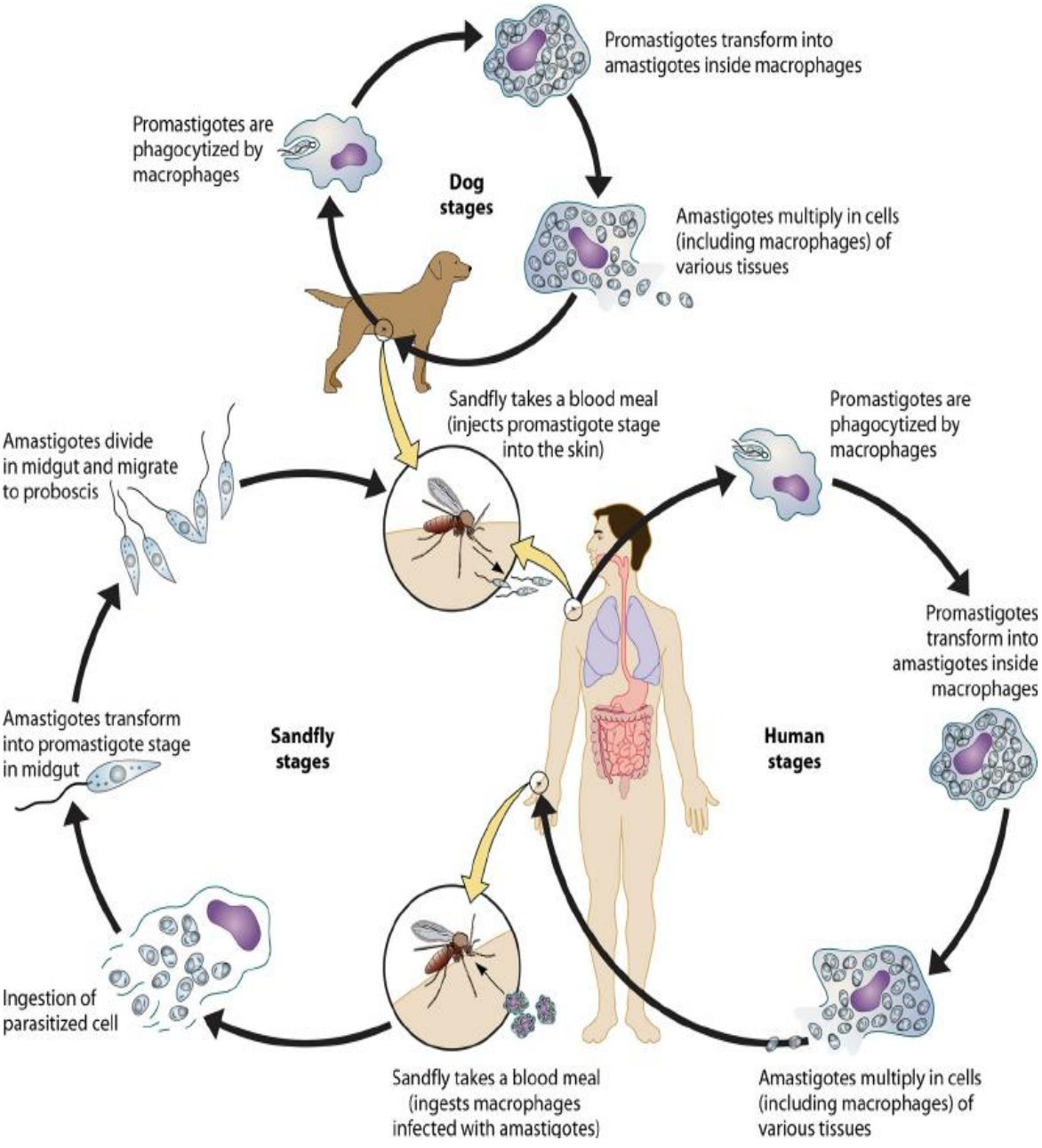


Figure 2.1: The life cycle of zoonotic leishmaniases with dog as reservoir host (Source: CDC, 2013)

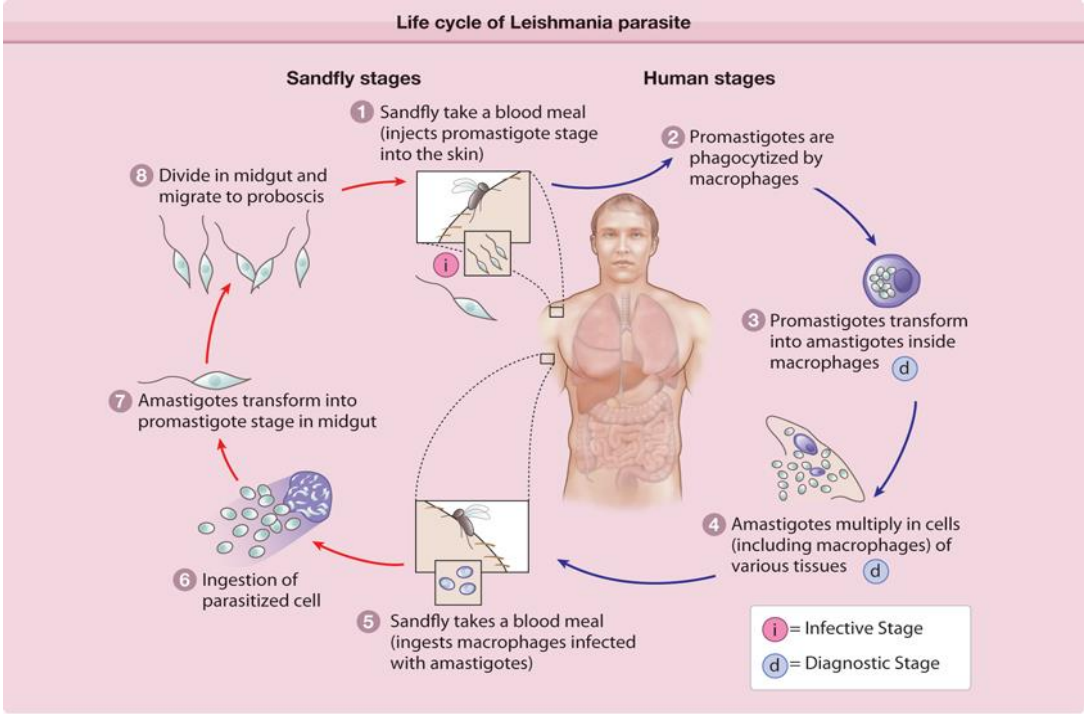


Figure 2.2: Lifecycle of anthroponotic *Leishmania* with no reservoir host (Source: CDC, 2013).

2.3 Development of *Leishmania* parasites in the sand fly and the mammalian host

The adult female sand fly, usually feeds at night on preferred animal host, and is a haematophagous pool feeder (Colacicco-Mayhugh *et al*., 2011). *Leishmania* parasites are ingested along with the prey's blood, when a sand fly bites an infected individual (Dostalova & Volf, 2012). The protozoan is in the its amastigote form, which is smaller, round, non-motile, and only 3–7 micrometers in diameter. The amastigotes quickly transforms into the promastigote form which is elongated and motile once they are inside the stomach of the sandfly. The promastigote is triple the size of the amastigote, spindled-shaped and has a single flagellum that allows mobility (Motuma *et al*., 2016). Promastigotes live extracellularly in the alimentary canal, where they reproduce

asexually before migrating to the proximal end of the gut where they become poised for transmission by regurgitation. During a bite by the fly, the promastigotes are released from the proboscis and introduced locally at the bite site (Sack, 2000).

As soon as the promastigotes are inside the human host, they enter the macrophages host cells. On entering the macrophage cells they transform back into the smaller amastigote form. Amastigotes replicate inside the phagolysosome which is the most hostile part of the macrophage cell, whose normal defensive response they are able to evade (Lodge & Descoteaux, 2008**).** After multiplying severally, they break down their host cell by sheer pressure of mass and possibly by activating exocytosis as is recently speculated (Burleigh *et al*., 2000). The daughter cells of the protozoans then migrate to fresh host cells or through the bloodstream to find new hosts. In this way the infection is progressive, spreading particularly to the spleen and liver in the case of visceral disease and in skin for cutaneous forms (Hartley *et al.*, 2014).

2.4 Visceral leishmaniases in Kenya

The Eastern African countries of Sudan, South Sudan, Ethiopia, Kenya, Uganda and Somali form one of the areas hardest hit by visceral leishmaniasis. In these areas it is mainly caused by *Leishmania donovani* (Anjili *et al*, 2011). In East Africa the visceral leishmaniasis transmission cycle caused by *L. donovani* is generally considered to be anthroponotic (AVL) (Zijstra & el-Hassan, 2001). Humans with active infection from visceral leishmaniasis or post -kala-azar dermal leishmaniasis comprise the AVL reservoir. Some reports suggest that a second zoonotic visceral leishmaniasis (ZVL), in which dogs or rodents are the major reservoir, is also present in some areas (Dereure *et*

al., 2003; Kassahun *et al*., 2015). Major visceral leishmaniasis disease foci can be found along the border between Kenya and Uganda, looking south toward the Rift Valley (Mueller *et al*., 2014; Kolaczinki *et al*., 2008). In Turkana, West Pokot and Baringo counties of Kenya, visceral leishmaniasis has also been reported as an epidemic disease (Mueller *et al*., 2014; Kolaczinki *et al*, 2008) .Other areas where visceral leishmaniasis epidemics in Kenya have been reported are Rift Valley and eastern regions specially Mandera and Marsabit counties (Boussery *et al*., 2001; Schaefer *et al*., 1994). The incidence of the visceral leishmaniasis in Kenya as of the year 2015 stood at 4,000 cases per year (WHO, 2015). The distribution of the visceral leishmaniases in Kenya is as shown in Figure 2.3.

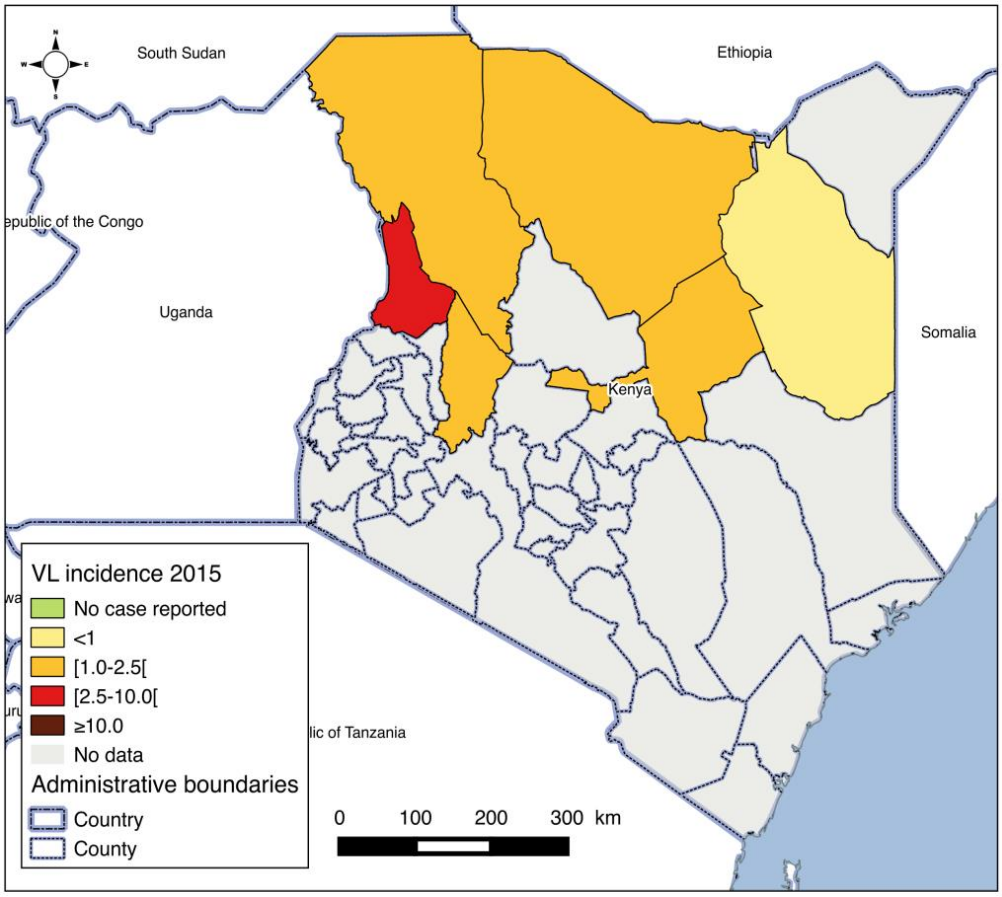


Figure 2.3: Map showing visceral leishmaniasis endemic areas in Kenya (Source: WHO, 2015).

2.5 Immune responses to *L.donovani* in humans

Response to infection by *L. donovani* in human hosts varies a great deal, not only by the strength but also by the type of the patient's immune reaction. People with a history of infection by *Leishmania* that cause visceral leishmaniasis show a range of immune responses ranging from protective to non-protective. Those with a strong type 1 CD4+ response to *Leishmania*, have acquired protective immunity (positive skin test) without ever having visceral leishmaniasis. The hallmarks of protective immunity are antigen

specific interferon-gamma and proliferation with the ability to kill intracellular *Leishmania* (Carvalho *et al*., 1985). Patients with visceral leishmaniasis lack these responses to *Leishmania* and other antigens. Increased interleukin-10 secretion is characteristic of visceral leishmaniasis (McCall *et al.,* 2013). CD8+ T regulatory cells are acute patient peripheral blood mononuclear cells that decrease interferon–gamma secretion and proliferation responses to *Leishmania* and other antigens. They also increase interleukin-10 secretion when added to autologous peripheral blood mononuclear cells harvested after successful treatment (Ghalib *et al.*, 1993). In addition the CD8+ T regulatory cells trigger the immune response characteristic of visceral leishmaniasis. $CD8^+$ T regulatory cells are also associated with post kala azar dermal leishmaniasis (Ganguly *et al.,* 2010a). Neutrophils and not macrophages have been shown to have a protective role against *L. amazonensis* in BALB/c mice in early infections (Sousa *et al.,* 2014).

2.5.1 Reactive oxygen species and the killing of intracellular Leishmania amastigotes

Reactive oxygen species (ROS) are defined as chemically reactive chemical species containing oxygen. Examples include superoxide (O_2-), nitric oxide (NO), hydrogen peroxide (H_2O_2), hydroxyl radical (OH), singlet oxygen ($^10_2$) and peroxides (any class of chemical compounds in which two oxygen atoms are linked together by a singlet covalent bond) (Hayyan *et al*., 2016). The role of nitric oxide (NO) and reactive oxygen species in the killing of *Leishmania braziliensis* by monocytes has been observed in patients with cutaneous leishmaniasis (Horta *et al*., 2012; Carneiro *et al*., 2016). When alone NO is not sufficient to control infection and may contribute to tissue damage. ROS

have been reported not to be very important in *L. amazonensis* killing, but in regulating the inflammatory response by controlling neutrophil numbers in lesions (Roma *et al*., 2016). However, H_2O_2 has been reported to induce apoptosis-like (programmed cell death) in *L. donovani* promastigotes (Das *et al*., 2001; Das *et al.*, 1988).

Studies have shown that ROS are generated following initiation of L-arginine-dependent killing mechanism by *L. major* parasites in interferon gamma-stimulated macrophages by induction of tumor necrosis factor- alpha (Green *et al.*, 1990b). Activated macrophages are then able to kill intracellular amastigotes by the L-arginine-dependent killing mechanism (Green *et al*., 1990).

2.6 Visceral leishmaniasis diagnosis

2.6.1 Parasite detection

The visualization of the amastigote form (Figures 2.4 and 2.5) of the parasite by microscopic examination of aspirates from lymph nodes, bone marrow or spleen is the standard confirmatory test for visceral leishmaniasis. Even though specificity is high, the sensitivity of microscopy varies being higher for spleen (93-99%) than for bone marrow (53-86%) or lymph nodes (53-65%) aspirates (Babiker *et al.*, 2007). Morphologies of the *Leishmania* parasites are shown in Figures 2.4 and 2.5.

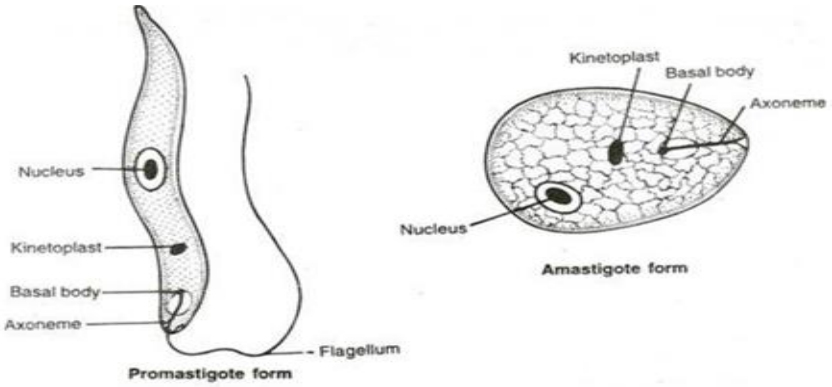
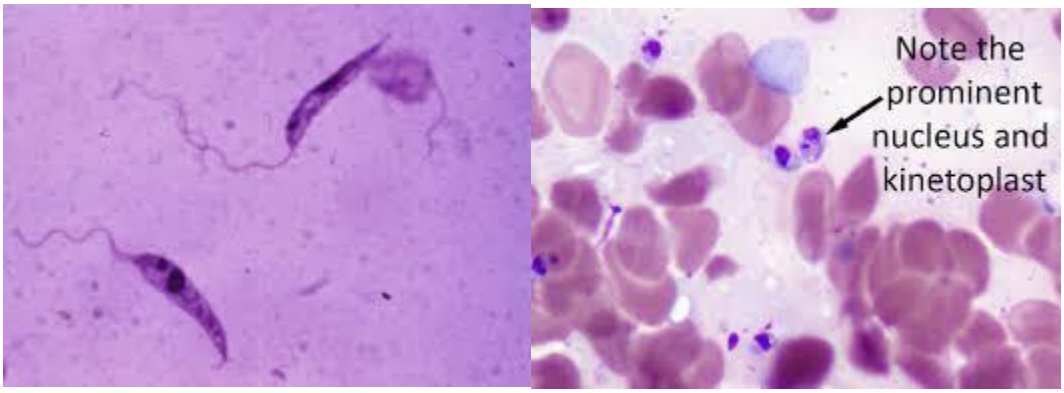


Figure 2.4: **Morphological forms of *Leishmania* parasite. (Source: parasite wonders.blogspot.com, 2018).**



A B

Figure 2.5: **Giemsa stained *Leishmania* promastigotes (A) and amastigotes (B). (Source: Parasite wonders.blogspot.com, 2018)**

2.6.2 Antibody- detection test

The different tests that detect specific anti-leishmanial antibodies have been developed but all have two major limitations. Firstly, although serum antibody levels decrease after successful treatment (Braz, 2002), they remain detectable for several years after cure.

Secondly, a significant proportion of health individuals living in areas endemic for visceral leishmaniasis with no significant history for this disease are positive for anti-leishmanial antibodies owing to asymptomatic infections. The direct agglutination test (DAT) and the rk39-based immunochromatographic test (ICT) are two serological tests that have been specifically developed for field use and have been sufficiently validated (Schallig *et al*., 2002).

Rk39 is a 39-amino acid repeat which is part of a kinesin-related protein in *L. (chagasi) infantum* and is conserved with the *L. donovani* complex (Burns, 1993).The recombinant antigen, rk39 titer correlates directly with the disease activity, indicating its potential use in predicting response to chemotherapy (Kumar *et al*., 2001). On the other hand, Rk39 ICTs are easy to perform, rapid (10-20 minutes), cheap and give reproducible results. Napier's formal gel or aldehyde test and the Chopra antimony test are nonspecific methods for detecting nonspecific immunoglobulins. The two tests depend upon raised globulin levels and can be positive in many other conditions. They lack specificity and have varied sensitivities rendering them highly unreliable (Bray, 1985).

2.7 Visceral leishmaniasis treatment

Sodium Stibogluconate and Meglumine antimoniate which are pentavalent antimonials, have been the first-line treatment for visceral leishmaniasis for more than seventy years (Veeken *et al.,* 2000). Antimonials are toxic drugs with frequent, sometimes life threatening, side effects (Collins *et al.,* 2004). Antimonials have been replaced by conventional amphotericin B (Amb) as the first –line treatment for visceral leishmaniasis in some areas of the Bihar state of India where treatment failure rates for antimonials

reached more than 60 % (Sundar *et al*., 2000a). Liposomal amphotericin B (AmB) is used as first-line treatment in Europe and the United States and is considered by many experts as the best existing drug against visceral leishmaniasis (Bern *et al.*, 2006). Miltefosine on the other hand is the first effective oral drug for visceral leishmaniasis. Data on miltefosine use in East Africa are restricted to only one study that was conducted in northern Ethiopia, in which it was found to be safe and effective as Sodium Stibogluconate in HIV negative patients and safer, but less effective, in HIV co-infected patients (Ritmeijer, 2006). The suggested way forward to increase treatment efficacy, prevent the development of drug resistance, reduce treatment duration and perhaps decrease treatment cost is combination therapy (Bryceson, 2001). A combination of pentavalent antimonals and paromomycin, miltefosine and liposomal amphotericin B (AmB) with meglumine antimonate and miltefosine, are medications used to treat visceral leishmaniasis (de Rossell *et al*., 1992; Thakur & Narayan, 2004; Sundar *et al.*, 2000b)

2.7.1 Complications associated with treatment of visceral leishmaniasis

Some patients develop post-kala-azar dermal leishmaniasis (PKDL) following treatment for VL which is characterized by development of nodular, macular and papular skin lesions (Figure 2.6) (Ganguly *et al*., 2010a and 2010b). Several studies have documented the development of PKDL after treatment with kala-azar and has been reported to be found in two geographical zones, South Asia (India, Nepal, Bangladesh) and East Africa, mainly Sudan and Kenya (Ganguly *et al.*, 2010b; Desjeux &Ramesh, 2011; Rashid *et al.*, 1986). Treatment with Pentostam (sodium stibogluconate) (Rashid *et al.*, 1986), miltefosine and paromycin (Pandey *et al*., 2012, 2018; Goyal *et al*., 2018;

Koley *et al*., 2013) and amphotericin B (AmB) (Kumar *et al*., 2009; Das *et al*., 2013) can result in PKDL development. Parasites can be demonstrated by microscopy in lymph node or bone marrow aspirates in 16% of PKDL patients and, with the aid of the polymerase chain reaction (PCR), in lymph nodes aspirates of 81% of patients, possibly indicating persistent visceral infection (Zijlstra & el-Hassan, 2001). Post-kala-azar-dermal leishmaniasis is difficult to treat and to explain and has long been considered a curious phenomenon , the possible mechanisms behind its pathogenesis still being the subject of much discussion, although the link between administration of sodium stibogluconate and subsequent development of PKDL has been reported (Croft, 2008), and it occurs between 2 months and several years in India and within 6 months in Sudan, (Zijlstra *et al.,* 2003). The treatment of VL with Sodium Stibogluconate (SSG), which has been the mainstay of VL therapy for over 80 years and development of resistance to the drug has reduced the frequency of cure to as low as 30%–40% in some endemic areas (Sundar *et al*., 2000).

Incidences of resistance to SSG led to the introduction of amphotericin B (AmB), as a first-line treatment for VL, in hyper-endemic areas of the Indian state of Bihar (Zijlstra *et al.,* 2003). Ismail *et al*., (1999) in a study done in Sudan, reported that PKDL develops in half of the patients treated with Sodium Stibogluconate and that some patients' lesions heal spontaneously, but in others symptoms are severe and persist for years, while El Hassan *et al*., (2013) found that it is rare in VL caused by *L. infantum chagasi* and it has been reported that persistent PKDL is difficult to treat. Other evidence indicate that inadequate immune response to the parasite and possible genetic predisposition to post-

kala-azar dermal leishmaniasis is rare and generally occurs after treatment of VL. It also shows geographical variation in its clinical manifestation and may be associated with many factors namely; genetics of the patient and or parasite, treatment, nutrition and or co-infection. PKDL may mimic leprosy and that must be recognized, especially in countries where these diseases are endemic (Zijlstra, 2016)

Large number of people travelling leading to increased globalization should also be seriously considered (Trinidade *et al*., 2015). In light of the evidence that PKDL is immunologically mediated, but the immunological changes involved and their role in the pathogenesis of the disease are not known with certainty, together with the high incidences of the diseases, PKDL been considered to be the only reservoir for kala-azar in India (El Hassan *et al*., 2013). Post kala-azar dermal leishmaniasis is increasingly being recognized in Sudan as a complication of visceral leishmaniasis , occurring in 60% of patients, during or after its treatment. The development of PKDL appears to be restricted to parasites of the *Leishmania donovani*. No specific zymodeme has been found to be associated with it. In Sudan, PKDL occurs within 0-6 months after treatment for VL, unlike PKDL in India (Zijlstra & el-Hassan, 2001). Post-kala-azar dermal leishmaniasis patients are considered as reservoirs of *Leishmania donovani* in India where 10% of patients treated for kala-azar develop PKDL (Zijlstra & el-Hassan, 2001; Ramesh *et al.,* 2015) due to persistence of parasites. Apart from treatment with antimonials it has been shown that PKDL can develop following treatment with miltefosine and amphotericin B (AmB) (Kumar *et al.,* 2009). and also a combination of miltefosine and paromycin (Goyal *et al.,* 2018).

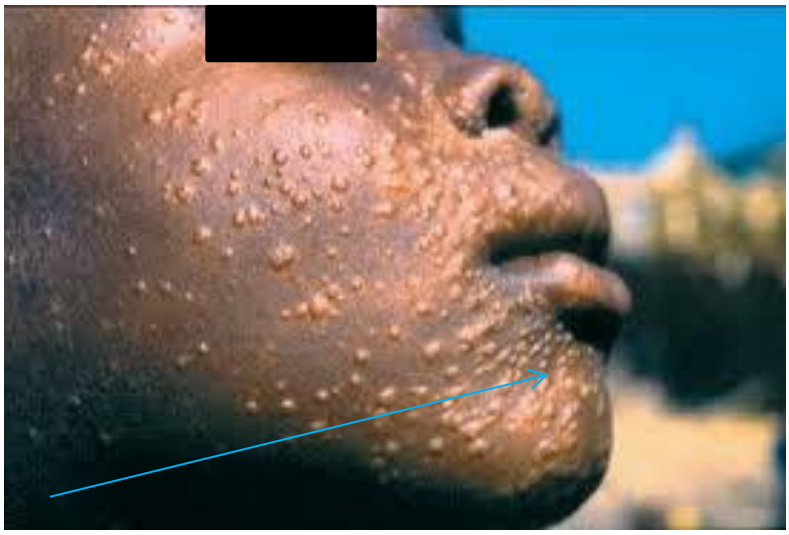


Figure 2.6: **A case of post-kala-azar- dermal leishmaniasis with arrow showing the maculo-papular nodules (Source: Sakib *et al.*, 2018).**

2.7.2: Chemical structures of drugs associated with PKDL

The chemical and molecular formulae of drugs that have been associated with development of PKDL are shown from Figure 2.7 to Figure 2.11.

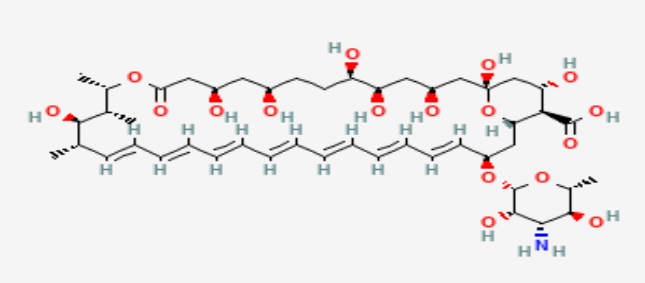


Figure 2.7: **Amphotericin B (Fungizone) $C_{47}H_{73}NO_{17}$ (Source: PubChem, 2018).**

Amphotericin B is an antifungal medication used for leishmaniasis and severe fungal infections. The fungal infections it is used to treat include blastomycosis, aspergillosis,

candidiasis, apodid cryptococcosis and coccidiomycosis (WHO, 2010**).** For some infections it is given with flucytosine **(**WHO,2009**).** According to The American Society of Health System Pharmacist (2015), it is typically administered by intravenous route. Common undesired effects include a reaction with headache, fever and chills immediately after the medication is given, as well as kidney complications. Hypersensitivity symptoms like anaphylaxis may occur. Other serious adverse effects include myocarditis and hypokalemia (WHO, 2010). It appears to be rather safe in pregnancy. There is a lipid formulation that is relatively safe. (The American Society 0f Health System Pharmacist, 2015) It is in the polyene classification of medications and works in partially by interfering with the cell membrane of the fungus.

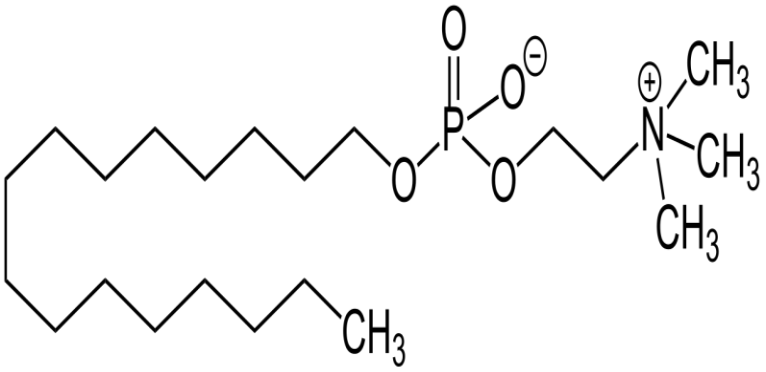


Figure 2.8: Miltefosine ($C_{21}H_{46}NO_4P$) (Source: PubChem, 2018).

Miltefosine , is given orally and is effective against both cutaneous and visceral leishmaniasis (Dorlo *et al.,* 2012). Side effects are generally slight, but still can cause birth defects if taken within 3 months of getting pregnant (Barrett & Croft, 2012). It is not effective against *L. major* or *L. braziliensis* (Minodier & Parola, 2007).

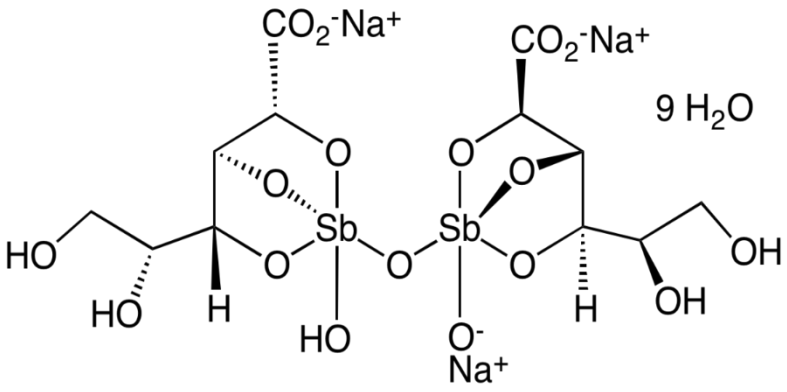


Figure 2.9: **Pentostam (sodium stibogluconate) $C_{12}H_{38}Na_3O_{26}Sb_2$. (Source: Pubmed, 2018)**

Sodium Stibogluconate is very toxic to veins. One of the everyday problems is that after a few injections it can become exceedingly difficult to find a vein in which to inject the next dose. The fixing of a PICC (Peripherally inserted central catheter) does not prevent the problem and can instead worsen it; the whole vein along the course of the PICC line can become inflamed and thrombose (formation of a blood clot inside a blood vessel, obstructing the flow of blood). A PICC is a thin, stretchy tube that is put in into a vein in the upper arm and guided (threaded) into a large vein above the right side of the heart called the superior vena cava **(**International Drug Price Indicator Guide, 2016**)**. It is used for giving intravenous fluids and blood transfusions. Large doses of sodium Stibogluconate are often administered as dilute solutions **(**International Drug Price Indicator Guide, 2016**)**.

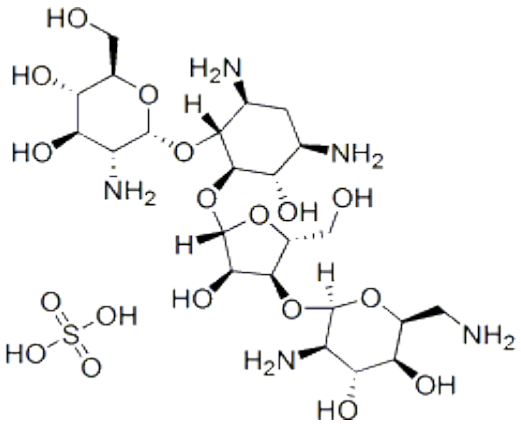


Figure 2.10: **Paromomycin, $C_{23}H_{45}N_5O_{14}$ (Source: PubChem, 2018).**

Paromomycin is an antimicrobial used to treat a number of parasitic infections including leishmaniasis, amebiasis, tapeworm and giardiasis infections. It is a standard treatment for giardiasis or amoebiasis in pregnancy **(**The American Society of Health Systems Pharmacists, 2016; Sundar *et al*., 2007). Ordinarily, it is a second line treatment option. It is given by mouth, topically, or intramuscularly. Common side effects when taken orally include , abdominal pain loss of appetite, diarrhea and vomiting **(**WebMD, 2016). When applied to the skin side effects include blisters, itchiness and redness. When used by injection there may be hearing loss ,fever or liver problems. It is safe when used during breastfeeding. Paromomycin is in the aminoglycoside class of medications and kills microbes by stopping the creation of bacterial proteins (The American Society of Health System Pharmacists, 2015).

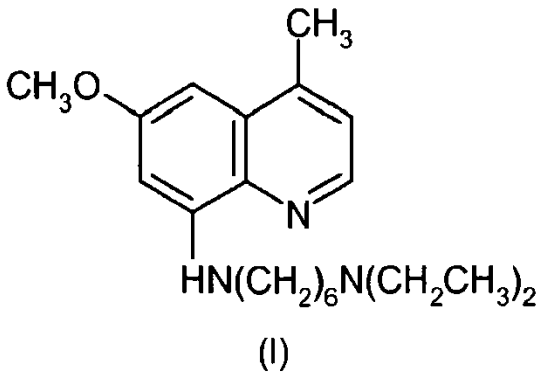


Figure 2.11: Sitamaquine hydrochloride, C21H33N3O (Source: ChemSpider, 2018)

Sitamaquine hydrochloride is an 8-aminoquinoline that is taken by mouth for the treatment of the leishmaniases. It is the second oral drug in development for leishmaniasis treatment (Jha *et a*l.,2005). Experimental studies in Kenya and Brazil demonstrated satisfactory efficacy against different species of *Leishmania* (Sherwood *et al*.,1994 ; Dietze *et al.,* 2001). It interferes with succinate dehydrogenase causing oxidative stress in *L. donovani* promastigotes (Carvalho *et al.,* 2011)

2.7.3 Cure of the leishmaniases and parasite persistence

Mandell and Beverley (2017), reported that protective immune responses through concomitant immunity are maintained by parasites that persist in the host despite being low in numbers. These persistent parasites could be the cause of recurrent cutaneous leishmaniasis (Aebischer, 1994). This is because the leishmaniases have been reported to be opportunistic infections of immunosuppressed individuals such that recrudescence of persistent parasites may be responsible for recurrent disease in a number of individuals who exhibit the disease relapse (Aebischer, 1994). In another study, when de Rossell *et*

al., (1992), treated *L. mexicana* susceptible BALB/c and C57BL/6 partially susceptible mice with Meglumine antimonate, it was observed that the *L. mexicana* lesions disappeared, but when the mice were immunosuppressed using cyclophosphamide and hydrocortisone the lesions reappeared leading to the conclusion that the leishmaniasis are never cured.

2.8 Rodent models for studying leishmaniases infection

Three rodent models have been developed to study infection with leishmaniases

2.8.1 The hamster model:

The Syrian golden hamster (*Mesocricetus auratus*) is highly susceptible to infection with viscerali zing *Leishmania* species (*L. donovani*, *L. infantum chagasi*) and is well-thought-out as the best experimental model to study visceral leishmaniasis (VL) because it reproduces the clinico-pathological features typical of human disease **(**Gifawesen & Farrell, 1989; Pearson *et al*., 1990**)**.The wide use of hamsters yet is limited due to the scarcity of reagents such as cell markers antibodies, and cytokines of well-defined specificity available to study the role of the immune response in disease pathology (Melby *et al*., 1998**)**. In 1978, Chang and Dwyer, provided reckonable evidence indicating an avid ingestion of *L. donovani* amastigotes by hamster macrophages and maintained the early findings that lysosome-phagosome fusion occurs. The photograph of the Syrian golden hamster is as shown in Figure 2.12

Figure 2.12: The Syrian golden hamster (Source; Hellen *et al*., 2012).

2.8.2 The mouse model:

The laboratory mouse owes much of its acceptance as a model organism in biomedical research to the presence of a large assortment of inbred strains that represent an immortal population of genetic clones resulting from repeated brother sister mating **(**Szatkiewicz *et al*., 2008). Owing to the fact that mice from each strain are genetically identical it is possible to collect and combine biological data over time and space leading to a depth of phenotype characterization rarely realized in other mammalian systems. In addition, the existence of a definite set of genetic variances amid inbred strains gives room for scientists to explore the effect of genetic diversity on almost any phenotype of interest. Another advantage of the murine model is the ease of keeping, breeding and reproducing them (Guénet & Bonhomme, 2003).

In the course of the past 40 years, murine models of the human disease cutaneous leishmaniasis have been extensively employed to elucidate the cell types, cytokines, signal transduction cascades and antileishmanial effector mechanisms. All these features

are collectively necessary for the control of parasites, as well as for the clinical resolution of disease, and vaccine development and resistance to a secondary infection **(**Bogdan & Rollinghoff, 1998; Moura *et al*., 2005**)**. Experimental infection of mice with *L. major* promastigotes has permitted understanding of the immunologic mechanisms governing resistance (C57BL/6 strain) and vulnerability to infection (BALB/c strain) (Aguilar-Torrentera & Carlier, 2001)

2.8.3 SAB hybrid mouse:

The new SAB mice hybrid model which is a product of the resistant and susceptible mice provides a suitable model for study of immune responses and disease development in various *Leishmania* species.Moreover, the SAB mice with inherited qualities from resistant and susceptible strains makes available a platform for genetic mapping whose purpose is to identify genes responsible for resistance or susceptibility to *Leishmania* infections. The role of individual genes can be assessed in an attempt to understand the complex genetic interactions (Kiige *et al.*, 2014). The ultimate goal being to recognize therapeutic targets that may lead to medication and vaccine development. The model as such is useful by generating information on a new mouse model that can be used to study disease progression in co-infections as well as determining the role of inbred SAB mice in studies of the pathology of visceral and cutaneous leishmaniases. The SAB mice, so far, is the only mouse model that is easy to rear and use in immunological and parasitological studies using *L. donovani* and *L. major* (kiige *et a*l., 2014). The photograph of laboratory SAB mouse is as shown in Figure 2.13

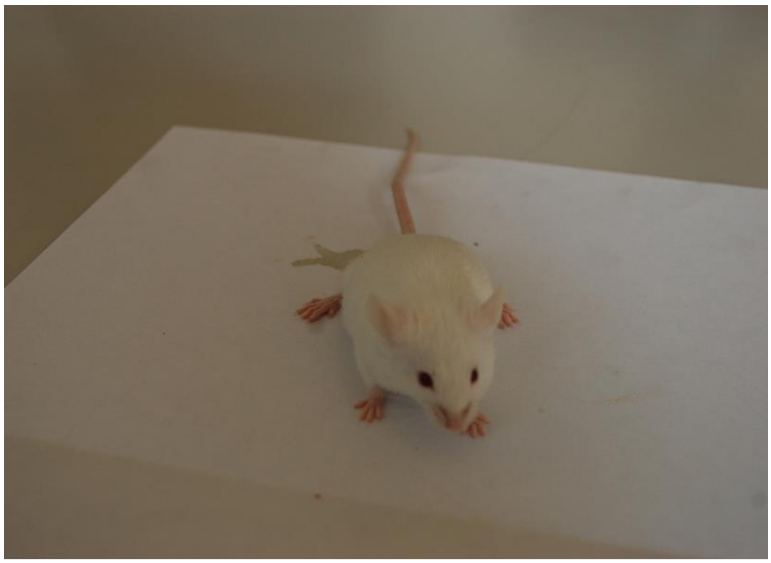


Figure 2.13: **Laboratory SAB mouse (Source: KEMRI animal house Nairobi, 2016)**

2.8.4 BALB/c mouse:

BALB/c is an albino, laboratory-bred strain of the house mouse. BALB/c mice are now widely distributed and among the top 2-3 most widely used inbred strains in animal experimentation (Jackson Laboratory, 2007). BALB/c mice and Syrian hamsters are the most widely used experimental models (Nieto *et al*., 2011). Mice of the BALB/c strain are frequently used, due to their high susceptibility to Leishmania infection. Most of the studies in visceral leishmaniasis use the intravenous or the intraperitoneal routes to inoculate the parasites (Rolão & Campino, 2004). BALB/c mouse is used in biomedical research and particularly in immunology and infectious disease research (Charles River, 2014). In BALB/c mice, the immune response to *L. infantum* and *L. donovani* infection can vary markedly between different organs (liver and spleen) within the same animal. In the liver, the infection can resolve with subsequent immunity to re-infection, whereas in the spleen, *Leishmania* parasites can persist (Engwerda & Kaye ,2000). The BALB/c

mice have a great potential for reproducing the process of genotype 4 Hepatitis E Virus infection (Li *et al.,* 2020).

2.9 Use of natural products to elicit anti-leishmanial activity

Plant-derived products have shown promise in the pursuit for better therapeutics against leishmaniasis (Dupouy-Camet, 2004). As a result of this quest, the leaf exudate of *Aloe vera* **(**Asparagales**:** Asphodelaceae**),** (Dutta *et al.*, 2007b) and triterpenoid saponin of *Careya arborea* (Ericales: Lecythidaceae) (Mandal, 2006) have been reported to be leishmanicidal. Even though water-insoluble, saponins have been shown to have anti-leishmanial effects (Delmas *et al*., 2000: Maes *et al*., 2004). Anti-leishmanial actions of quite a lot of herbal extracts have been investigated in experimental mice and have shown promising results.

The anti-leishmanial action of an ethanolic extract of the leaves of *Artemisia indica* Wild. (Asterales: Asteraceae) was investigated in exponential-phase promastigotes from six strains responsible for mucocutaneous, cutaneous or visceral leishmaniasis. The IC_{50} values for the extract ranged from 0.21 to 0.58 mg ml^{-1} (Ganguly *et al.,* 2006). Leishmanicidal activity has also been demonstrated in methanol extracts of *Aloe nyeriensis***,** *Albizia coriara* (Fabales: Mimosoidae) and *Acacia tortilis* Hayne (Fabales: Fabaceae) which have shown reasonable leishmanicidal activity (Kigondu *et al*., 2009). As a consequence, the current study was undertaken to establish the leishmanicidal efficacy of *Acacia polyacantha* against *L. donovani* and to demonstrate the high safety index of the extracts.

2.9:1 Acacia polyacantha (Fabales: Fabaceae)

Acacia polyacantha is a straight cylindrical, deciduous, erect tree of about 10-15 metres in height found in Tropical Africa, occurring from Gambia to Ethiopia and southwards to Kenya and Zimbabwe (Orwa *et al*., 2018). The gum of this tree is used in the manufacture of candy (Howard, 1988), while the root extracts are beneficial for treating snakebites and are also applied to wash the skin of children who are agitated at night time (Venter & Venter ,1996). The root extracts and perhaps the bark extracts are used in the treatment of dysentery, venereal diseases, and gastrointestinal disorders (Van der Maesen *et al*., 1996). For commercial purposes, bark of *A. polyacantha* is also valuable for tanning (Howard, 1988); notwithstanding the tree's primary use is for wood (Polhiu, 1990). Other important properties of *A. polyacantha include* treatment of rabies, haemorrhoids and typhoid (Mulofwa & Tengnas, 1994). Following a phytochemical study by Ashu *et al.,* (2020), chemical structures and compounds isolated from barks of *Acacia polyacantha* were Lupeol $C30H_{50}O$, dihydroxypropyltetracosanoate $C_{27}H_{54}O_4$ and methyl gallate $C8H8O_5$. In the light of these observations *Acacia polyacantha* becomes a good plant candidate for testing against the leishmaniases. The photograph of *A. polyacantha* is as shown in Figure 2.14.

Figure 2.14: *Acacia polyacantha* in its natural environment (Source: Marigat in Baringo county, 2020)

CHAPTER THREE

MATERIALS AND METHODS

3.1 Study Site

The study was carried out in the Kenya Medical Research Institute (KEMRI), Nairobi , Centre for Biotechnology Research and Development (CBRD) in leishmaniasis laboratory. The facilities necessary for this study were availed in this laboratory. The plant material was collected from Baringo County and transported to KEMRI Nairobi where it was shade dried at room temperature. The dry bark was then ground into a finer powder using a grinder.

3.2 Research Design

Experimental research design was used.

3.3 Sample size Determination

A total of one hundred and eighty mice were used in this study, sixty SAB mice, sixty Swiss Albino and sixty BALB/c mice.

Resource Equation method was used to determine the sample size. According to this method a value “E” is measured, which is the degree of freedom of analysis of variance (ANOVA). The value E = Total number of animals − Total number of groups .The value of E should lie between 10 and 20. If E is less than 10 then adding more animals will increase the chance of getting more significant result, but if it is more than 20 then adding more animals will not increase the chance of getting significant results. Though, this method is based on ANOVA, it is applicable to all animal experiments. Any sample size, which keeps E between 10 and 20 should be considered as adequate.

This research aimed to see the effect of *Acacia. Polyacantha* on *L .donovani* parasites. Five groups (three groups acting as both negative and positive controls and two test groups of aqueous and methanolic solvents of the plant extract) with 12 rats in each group. In this study E was;

$E = (12\times 5) - 5$

$E = 60 - 5 = 55.$

3.4 Experimental Mice

The mice were obtained and maintained at KEMRI animal house where controlled breeding was done through cross breeding between BALB/c and Swiss albino mice to obtain hybrids. In 2008, the cross breeds were named SAB mice by the principal researcher, CBRD, Dr. Christopher Anjili. The F_6 generation was used in this study. The photographs of BALB/c and Swiss Albino mice are as shown in Figures 3.1 and 3.2 respectively.

Figure 3.1: Laboratory BALB/c mouse (Source:Jackson laboratory, 2007).

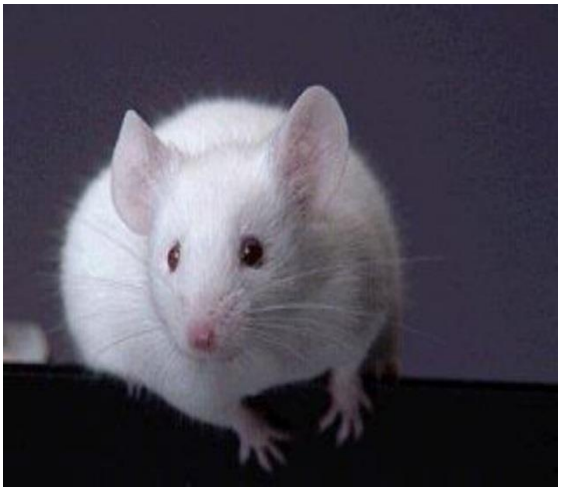


Figure 3.2: Swiss Albino mouse (Source: Charles Rivers, 2014).

3.5 Cultivation of *Leishmania* parasites

Metacyclic promastigote of *L.donovani* strain NLB 065 were used. Parasites were maintained as described by Titus *et al*., (1994). *Leishmania donovani* parasites were cultured in Schneider's insect medium supplemented with 20% Fetal bovine serum, glutamine (2µM), penicillin (1000/ml) and streptomycin (100ug/ml). Stationary phase promastigotes were obtained from day five to seven old cultures. Metacyclics were isolated from stationary phase cultures by negative selection using peanut agglutination (Socks & Perkin, 1984; Tonui & Titus, 2006).

3.6 Extraction of the crude extract from the bark of *Acacia Polyacantha*

The ground material (100g) was soaked in 500ml absolute methanol for 24 hours for the methanolic extraction. The extract was filtered, dried with Na_2SO_4 and the solvent

removed under vacuum in a rotary evaporator at 30-35^{0}C. For aqueous extraction, 100 g of ground material in 600 ml of water was placed in a water bath and maintained at 60^{0}C for 2 hours. The filtrate was freeze-dried, weighed and stored at -20^{0}C until required for use. Reconstitution was done using methanol and water respectively according to the method of Kigondu *et al.,* (2009).

3.7 Experimental procedures

3.7.1 Preparation of the Test Drugs

Stock solutions of the crude extracts were made in culture media for antileishmanial assays at 5mg/ml and re-sterilized by filtering through 0.22µm filter flasks in a laminar flow hood. If some of the extract was found not to dissolve easily in water or media, they were first dissolved in 1% dimethyl sulfoxide (DMSO) to avoid solvent carry-over (Dorin *et al.,* 2001).

All prepared drugs were stored at 4ºC and retrieved only during use.

3.7.2 Bioassays

3.7.2.1 Minimum Inhibitory Concentration (MIC) Anti-Promastigote Assay

Leishmania Donovani promastigotes (1 x 10^6 parasites/ml) were grown and incubated in Schneider's Insect Medium (SIM) culture media. Drugs were weighed and reconstituted in SIM 20% at 5mg/ml for all the extracts and filtered using filter paper for sterilization. Serial concentrations ranging from 5 to 0.025g/ml of the plant extracts and positive control (sodium stibogluconate and amphotericin B) with negative control (normal saline) were added to the wells on a 48 well plates. Parasites were added in equal numbers to each well and incubated for 48 hours. MIC was detected by looking at the motility and

viability in the wells as compared to the negative controls SIM 20%. The lowest concentration of the samples that prevented growth was considered as the MIC.

3.7.2.2: Anti-Amastigote Assay

This was carried out as described by Delorenzi *et al.,* (2001). Briefly, peritoneal macrophages were obtained from BALB/c mice. The mice were injected with 2% starch solution to stimulate macrophage proliferation. After 24 hours, they were anaesthetized using 100µl pentobarbital sodium (Sagatal®). The body surface was disinfected with 70% ethanol. The torso skin was torn dorsoventrally to expose the peritoneum. Using a sterile syringe and needle, 10ml of sterile cold phosphate-buffered saline (PBS) was injected into the peritoneum. After shaking the mouse, peritoneal macrophages were harvested by withdrawing the PBS. The contents were transferred into a sterile 50ml centrifuge tube. The suspension was centrifugally washed at 2000rpm for 10 minutes and the pellet resuspended in complete RPMI 1640 medium.

Macrophages were adsorbed in 24-well plates and allowed to adhere for 4 hours at 37ºC in 5% CO2. Non-adherent cells were washed with cold PBS and the cultures incubated overnight in RPMI. Adherent macrophages were infected with promastigotes a parasite/macrophage ratio of 6:1 and further incubated at 37ºC in 5% CO2 for 4 hours. Free promastigotes were removed by extensive washing with PBS and the cultures incubated in RPMI for 24 hours. Treatment of infected macrophages with the extract dissolved in RPMI was done once. Pentostam® and amphotericin B was used as positive control drug for comparison of parasite inhibition and PBS was used as the negative control. The cells were incubated for 48hrs. The cells were then washed with PBS, dried

fixed in methanol and stained with Geimsa. The number of amastigotes was determined by counting at least 100 macrophages in duplicate cultures, and the results expressed as infection rate (IR) and multiplication index (MI), (Berman & Lee, 1984). The infection rate was used in calculations of the Association Index (AI). The association indices were determined by multiplying the percentages of infected macrophages by the number of parasites per infected cell. Association indices were interpreted as the number of parasites that actually infected the macrophages

3.7.2.3 Nitric oxide Production Assay

Nitric oxide release in macrophage cultures was measured using the Griess reaction for nitrites ,(Hollzmuller *et al.,*2002). Briefly, 100µl of the supernatants was collected 48 hours after introducing the test drug into the culture medium. The assay was done in triplicate wells in 96-well microtiter plates. To this, 60µl of Griess Reagent A (1% sulphanilmide in 1.2M HCl) was added followed by 60µl of Griess Reagent B (0.3% N-(1-naphthyl) ethylenediamine). The absorbance was measured at 540nm in an enzyme-linked immunosorbent assay (ELISA) reader. Nitrite (NO_2^-) was calculated from a standard curve constructed using sodium nitrite in RPMI.

3.7.3 Cytotoxicity Studies

VERO cells were cultured and maintained in Minimum Essential Medium (MEM) supplemented with 10% FBS. The cells were cultured at 37ºC in 5% CO_2 for 24 hours by trypsinization, pooled in 50ml vial and in 100µl cells suspension (1 x 106 cells/ml) put into 2 wells of rows A-H in a 96-well microtiter plate for one sample to attach. The medium was aspirated off and 150µl of the highest concentration (1000µg/ml) of each of

the test samples at 562nm was added into the same row and serially diluted. The experimental plates with the cells were incubated further at 37ºC for 48 hours. The controls used were cells with no extract, medium alone. 10µl MTT reagent was added into each well and the cells incubated for 2-4 hours until a purple precipitate was visible under a microscope. The medium together with MTT were aspirated after which 100µl of DMSO was added and plates shaken for 5 minutes.

The absorbance was measured for each well at 562nm using a micro-titer plate reader (Wang *et al.,* 2006). The results were expressed as the concentration at which the extract inhibited 50% growth of the cells (IC_{50}).

3.8 Experimental animals and infection with parasites

SAB, BALB/c and Swiss albino mice which were used in this study were bred at the KEMRI animal house. They were a total of one hundred and eighty mice of mixed sexes and aged between 8 to 10 weeks. They were drawn in equal proportions of sixty mice each from SAB, BALB/c and Swiss Albino mice models. The mice were divided into eighteen groups of ten mice each. The mice were fed with commercial mice pencil every day and given drinking water *ad libitum*. All the mice were inoculated with $1x10^6$ stationary phase culture *L. donovani* promastigotes intraperitoneally (IP). After four weeks, one mouse from each group was sacrificed. Spleen impression smears were made, fixed with methanol, stained with Giemsa and examined for parasites. Parasite loads were calculated using the method of Bradley and Kirkley (1977). Briefly, the slides were examined under a compound microscope for enumerating the number of amastigotes per 1000 host nuclei. The relative and total number of parasites in the spleen, named

Leishman-Donovan Units (total LDU) were calculated according to the formula by Bradley and Kirkley, (1977), as follows:

LDU = No. of parasites |1000 host nuclei

Total LDU= LDU x organ weight x 2 x 10^5

3.9 Treatment of infected animals with *A. polyacantha* extracts and the controls

For treatment the sixty (60) mice each from SAB, BALB/c or Swiss Albino were divided into five (5) groups each consisting of twelve (12) mice. The first groups of mice were treated orally with a dose of two hundred microlitres with aqueous and methanolic extracts of *A. polyacantha*, using a cannula. The second groups were treated with one hundred microlitres of the aqueous and methanolic extract through IP injection. The third groups were treated with Pentostam through IP, the fourth groups treated with Amphotericin B through IP and the fifth groups were treated with phosphate buffered saline (PBS) orally. Treatment was done for one month. Dosages for the conventional drugs were done as per manufacturer's instructions . All mice were then sacrificed for parasite load determination.

3.10 Determination of efficacy of water and metabolic extracts administered orally and intraperitoneally

At necropsy, all study group mice were sacrificed by inoculation of 100 µl Pentobarbitone sodium IP. Body and spleen weights were recorded. Spleen portions were inoculated into Schneider's medium. The cultures were incubated and observed on a daily basis for six (6) days for emergence of promastigotes. Impression smears of these

organs were made and parasites quantitated using the method of Bradley and Kirkley (1977). Mean parasite counts (LDU) per test and experimental groups were calculated. Mean body and spleen weights were determined and compared between the groups.

3.11 Statistical Analysis

Parasite burden data were expressed as the mean per 1000 per cell nuclei of spleen cells. Statistical analysis of the differences between mean values obtained from the test extracts compared to the controls was done by student's t-test. Results were expressed as mean standard deviation (SD) of three independent experiments. ANOVA was used to determine the differences between the various test extracts. A probability value of $p \leq 0.05$ was considered to be statistically significant. The custom data analysis software program, Chemosen, available at KEMRI was used to determine IC_{50} concentrations

3.12 Ethical Consideration

Approval to carry out the present study was obtained from the Scientific Steering Committee (SSC) and Animal Care and Use Committee (ACUC) of Kenya Medical Research Institute (KEMRI). The stipulated guidelines were strictly adhered to during the research. The approval letter is found in appendix III.

CHAPTER FOUR

RESULTS

4.1 Minimum Inhibitory Concentration of extract and positive control

Promastigotes of *Leishmania donovani* were incubated *in vitro* with various concentrations of pentostam, amphotericin B (AmB) and *A. polyacantha* aqueous and methanolic extracts.The lowest concentrations of the samples that prevented growth were considered as the MIC. The MIC of the extracts and positive control are shown in Figure 4.1

Pentostam was the most active against *L. donovani* promastigotes MIC (0.03mg /ml) followed by amphotericin B MIC (0.08mg /ml) and methanolic extract of *A. polyacantha* MIC (1.47 mg/ml) while the water extract was the least active with MIC (2.35 mg /ml).The methanolic extract of *A. polyacantha* is more active against *Leishmania donovani* promastigotes than the water extract.

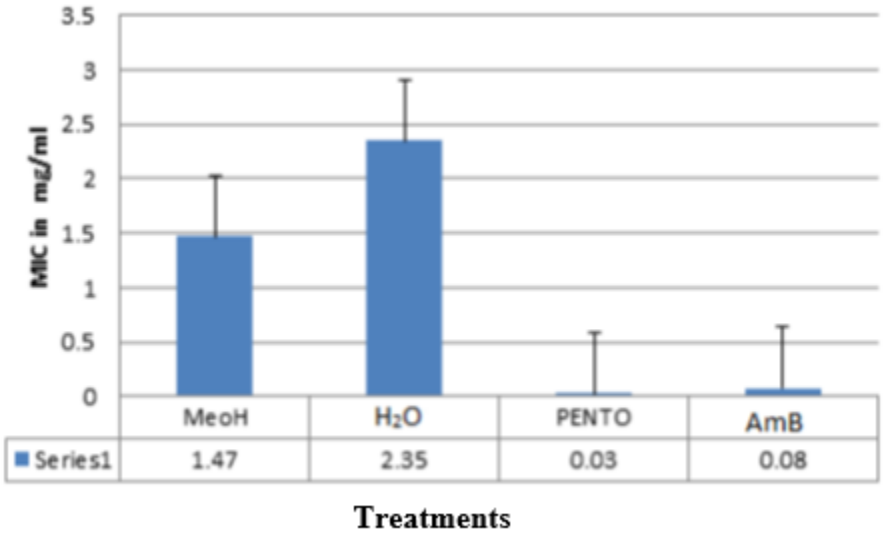


Figure 4.1: MIC for crude bark extract and positive control
Key: MeoH-----Methanolic *A. polyacantha* bark extract PENTO-----pentostam
H_2O----Aqueous *A. polyacantha* bark extract AmB-----Amphoteric

4.2 Inhibition of Vero cells growth

The cells growth inhibition effects of various concentrations of methanolic and aqueous crude bark extracts of *A. polyacantha* was carried out for Vero cells. Several varying concentrations of the extracts were tested (0.01 µg/ml, 0.02 µg/ml, 0.06 µg/ml, 0.19 µg/ml, 0.56 µg/ml, 1.67 µg/ml and 5 µg/ml). The cell growth inhibition potentials of different concentrations of the methanolic and aqueous bark extract of *A.polyacantha* with their IC_{50} values are depicted in Figure 4.2. The IC_{50} values detected for Vero cells against methanolic and aqueous crude bark extracts of *A. polyacantha* were 1.090 µg/ml and 1.127 µg/ml respectively. Vero cell growth inhibition was increased when the concentrations of crude bark extracts of *A.polyacantha* were raised. Maximum inhibition of Vero cells growth occurred concurrently at concentrations of 0.06 µg/ml for both

methanolic and aqueous extracts of *A. polyacantha*. Increasing the concentrations of crude bark extracts above 1.67ug/ml would eventually be lethal for the Vero cells.

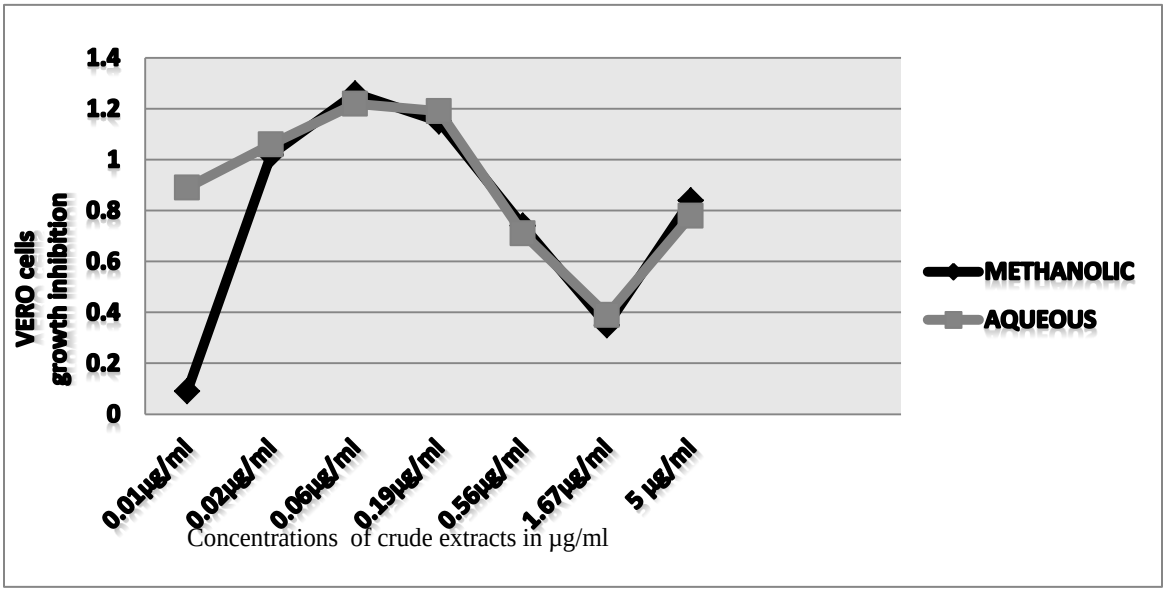


Figure 4.2: Vero cells growth inhibition of various concentrations of methanolic and aqueous A. polyacantha crude bark extracts.

4.3 The mean spleen weights of experimental mice

The oral aqueous crude bark extract of *A. polyacantha* reduced mean spleen weights (Spleen weight mean = 0.16) more than oral methanolic crude bark extract (Spleen weight mean = 0.19) in BAIB/c mice. The difference was significant. Treatment with Pentostam resulted in the lowest mean spleen weights of SAB mice (spleen weight mean = 0.12), Swiss Albino mice (Spleen weight mean = 0.07) and BALB/c mice (Spleen weight mean = 0.14). The mean spleen weights of SAB, Swiss Albino and BALB/c mice infected with *L. donovani* and treated with crude extracts from the bark of *A. polyacantha* and controls are as shown in Table 4.1

Table 4.1: Spleen weight means of SAB, Swiss Albino and BALB/c mice infected with L donovani and treated with extracts from the bark of A. polyacantha and controls.

Route		SAB mice	Swiss Albino	BALB/c
	Extract/control	**Mean±SE ()**	**Mean±SE ()**	**Mean±SE ()**
Oral	Aqueous	0.14±0.035a	0.07±0.000a	0.16±0.007ab
	Methanolic	0.15±0.029a	0.09±0.028a	0.19±0.014b
	PBS	0.15±0.006a	0.11±0.000a	0.15±0.000ab
IP	Aqueous	0.16±0.021a	0.15±0.001b	0.15±0.001ab
	Methanolic	0.16±0.007a	0.15±0.001b	0.16±0.000ab
	PBS	0.16±0.014a	0.15±0.002b	0.16±0.001ab
	PENTO	0.12±0.035a	0.07±0.000a	0.14±0.000a
	AmB	0.14±0.027a	0.14±0.001b	0.15±0.001ab

Means followed by different letters within a column are significantly different at $p < 0.05$.

4.4 The spleen parasite load

After four weeks of treatment with extract, positive control and negative control, PBS, the spleen LDU of SAB, Swiss Albino and BALB/c mice infected with *L. donovani* are as shown in Table 4.2.

There was significant difference in reduction of spleen parasite load ($p < 0.05$) while treating with both methanolic and aqueous bark extracts of *A. polyacantha* in SAB mice using the oral and intra-peritoneal routes. For methanolic crude bark extract (IP, LDU = 7.725, 34% protection) and (Oral, LDU = 12.54 , 42% protection). The performance of aqueous crude bark extract on spleen parasite load of SAB mice was (IP, LDU = 6.59, 44% protection) and (Oral , LDU = 13.66, 29% protection). In BALB/c mice there was

significance difference in reduction of spleen parasite burden when treating with methanolic and aqueous crude bark extra cts using the intra-peritoneal route but not with the oral route. For methanolic crude bark extract (IP, LDU=10.561, 63% protection) and aqueous crude bark extract (IP, LDU = 19.138, 32% protection). No significant difference in reduction of spleen parasite load was observed in Swiss Albino mice after treatment with crude bark extracts. BALB/c mice recorded the highest spleen LDU compared to other experimental mouse models, the highest being (LDU= 28.187) after treatment with negative control (PBS).This difference was significant ($p < 0.05$). Amphotericin B (Amb) was the most potent against spleen parasite loads resulting in LDU (0.641) and 95% protection in SAB mice while in BALB/c, LDU was 0.331 with 99% protection . On the other hand, treatment with Pentostam resulted in a protection of 76% and LDU (3.281) in SAB mice and protection of 79%, LDU (6.021) in BALB/c mice.

Table 4.2: The spleen LDU of SAB, Swiss Albino and BALB/c mice infected with L. donovani and treated with crude extract from the bark of A. polyacantha and controls.

Route		**SAB mice**	**Swiss Albino**	**BALB/c**
	Extract/control	**Mean± SE (10^6)**	**Mean± SE (10^6)**	**Mean± SE (10^6)**
Oral	Aqueous	13.66±7.063d	5.838±0.772c	15.899±8.817c
	Methanolic	12.54±6.595d	4.842±3.482c	17.362±0.342c
	PBS	21.536±2.896e	9.922±3.311c	16.651±1.762c
IP	Aqueous	6.590±2.567c	9.271±2.423c	19.138±0.138c
	Methanolic	7.725±1.566c	7.527±3.456c	10.561±1.231c
	PBS	11.711±1.670d	9.264±2.329c	28.167±7.429d
	PENTO	3.281±1.980b	0.042±0.001a	6.021±1.372b
	AmB	0.641±0.281a	0.252±0.003b	0.331±0.002a

Means followed by different letters within a column are significantly different at $p < 0.05$.

4.5 Effect of route of administration on mean spleen weights and LDU

Following treatment of mice through oral and intraperitoneal routes using the crude bark extracts and positive and negative control PBS, mean spleen weights and LDU were determined and the results are shown in Table 4.3

There was a significant difference in reduction of spleen parasite loads when intra-peritoneal route was used to administer the bark extracts of *A. polyacantha* in SAB mice using IP route, LDU = 5.99 compared to oral route, LDU = 15.34 and in BALB/c mice IP, LDU = 13.89 compared to oral IP, LDU = 16.63. There were no significant differences in parasite spleen loads of Swiss Albino mice whether treatment was given orally or intra-peritoneally (IP, LDU = 6.26, Oral, LDU = 5.27). There was no significant

difference in mean spleen weights of all the experimental mice regardless of route administration of extract and controls.

Table 4.3: Effect of route of administration (oral or intra-peritoneal) of crude extracts from the bark of A. polyacantha on mean spleen weights and LDU in SAB, Swiss Albino and BALB/c mice infected with L. donovani

Mice strain	Route of administration	Mean spleen weight	LDU (10^6)
SAB mice	Oral	0.14±0.024b	15.34±6.52c
	IP	0.14±0.027b	5.99±2.76a
Swiss Albino	Oral	0.09±0.022b	6.26. ±2.76a
	IP	0.13±0.034b	5.27±2.73a
BALB/c	Oral	0.17±0.019b	16.63±4.47c
	IP	0.15±0.011b	13.89±4.62b

Means followed by different letters within a column are significantly different at $p < 0.05$.

CHAPTER FIVE

DISCUSSION

5.1 Minimum Inhibitory Concentration of extract and positive control

The methanolic crude bark extract of *A. polyacantha* is potent against promastigotes of *Leishmania donovani* with MIC of 1.47mg/ml .This proves that this plant extract has the potential to treat kala azar. Other plant extracts which have been tried for kala azar in other studies include; *Azadirachta indica* leaf extract which is an effective leishmanicidal compound having immunomodulatory activity seen in *Leishmania donovani* infection (Dayakar *et al.,* 2015) and *Emblica officinalis* extract which is very effective for treating visceral Leishmaniasis (Kaur *et al*., 2013). *A. polyacantha* crude bark extract was found to be more potent against *L.donovani* than the root extract of *Valeriana wallichii* D (Orya, 2015), a plant tested for kala azar treatment in a similar previous study whose methanolic extract had MIC of 5mg/ml against *L.donovani* promastigotes.

Many plant metabolites have been shown to be effective in treating a wide range of diseases ranging from bacterial, fungal and parasitic infections (Ali shah *et al*., 2016). *A.polyacantha* was shown to have antistaphylococcal activity in a study by Ashu *et al* (2020)

5.2 Safety of *Acacia polyacantha* bark extract on Vero cells

There was no sign of toxicity, and deaths of rats administered with aqueous and methanolic extracts of *A.polyacntha* in the 4-week treatment period. The low toxicity exhibited by *Acacia polyacantha* against vero cells, is a clear indication that it is a safe compound and can be used in the treatment of clinical leishmaniasis without the side effects associated with current antileishmanial drugs. Furthermore, current drugs used for treatment of leishmaniasis are associated with high toxicity rates leading to bodily harm. Toxicity of drugs also makes patients less compliant with dosage leading to development of pathogen resistance and disease relapse (Oryan, 2015).The observation from this study about safety levels of *Acacia polyacantha* echoes those of previous studies that the major merits of herbal medicine include their low cost, low incidence of serious adverse effects and good efficacy (Garcia *et al.,* 2010; Wink, 2012; Sarkar *et al.,* 2013). *Acacia polyacantha* has IC_{50} value of 1.090 µg/ml and compares well with other plant extracts in a previous study whose IC_{50} values determined, *in vitro* were in a range of 0.88 µg/mL (polar fraction of dichloromethane extract of *Boswellia serrata*) to 98 µg/mL (petroleum ether extract of *Murraya koenigii*) (Hassan *et al.,*2022). In this regard *Acacia polyacantha* may be a promising source for the development of new drugs against leishmaniasis.

In addition, plant extracts have proved more effective against Leishmania as compared to current chemotherapeutics in use due to less toxicity (Abdulla *et al*., 2011)

5.3 The mean spleen weights of experimental mice

Progressive splenomegaly is observed in experimental murine VL (de Melo *et al.*, 2020).The current study observed that the crude bark extract of *A. polyacantha* was effective in reducing mean spleen weights in BALB/c mice. This finding is consistent with another study in which BALB/c mice infected with *L.donovani* and treated with ethanolic *Bergenia ligulata* (Wall.) Engl. root extract showed no splenomegaly with normal spleen tissues after treatment (Kaur & Kaur, 2013). In yet another study which agrees with the current trial, there was significant decrease in weight of spleen observed in BALB/c mice at 15 days post-treatment with respect to the infected control mice following treatment with *Coccinia grandis* (L.) Voigt leaf extract (Pramanik *et al.*, 2019).

This fact that *A. polyacantha* crude bark extract reduced spleen weights makes it a good medicine candidate for treating splenomegaly (increase in spleen weight) which is caused by presence of *Leishmania donovani* parasites in the spleen during an infection (de Melo *et al*., 2020; Ishizuka *et al*., 2020).

5.4 Effect of plant extract solvent on spleen parasite load

The concentration of secondary metabolites is affected by the plant species, geographical location and the solvents used for extraction (Demain & Fang, 2000**).**

It was evident from this study that the methanolic crude bark extract of *A. polyacantha* was more effective in reducing spleen parasite loads than the aqueous bark extract when administered intra-peritoneally in BALB/c mice. The methanolic bark extract reduced the spleen parasite loads by 63% compared to a reduction of 34% when using the aqueous extract. The difference was significant ($p < 0.05$). The variation was attributed to the

difference in extractable bioactive compounds by the extraction solvents. This finding demonstrated that methanol yielded high levels of bioactive compounds than water from crude bark extract of *A. polyacantha.* These findings were in agreement with other previous studies with *Limnophila aromatica* (Do, *et al*., 2014) and *Phoenix dactylifera* L. (Kchaou *et al.,* 2013), where methanol extracted more bioactive compounds from the plant extracts than water as an extraction solvent. In yet another study methanol was observed to yield higher bioactive compounds from a plant, *Cedrus deodara* compared to chloroform and ethyl acetate (Narayan *et al*., 2017). Indeed, other studies working *Teucrium orientale* have confirmed the fact that methanol gives the higher extraction yield amongst all solvents (Tawaha *et al.*, 2007). On the contrary to this study, water as an extracting solvent had higher inhibition than methanol as it absorbed more bioactive compounds in the root extract of *Psidium guajava L* (Kidaha *et al* ., 2013)

5.5 Spleen parasite load

Inoculation of experimental mice with *Leishmania donovani* amastigotes induces progressive visceral leishmaniasis (VL) with increasing splenic parasite load (Mukherjee *et al.*, 2003). In the present study showed that *A. polyacantha* crude bark extracts were effective in reducing the spleen parasite loads in experimental mice. Treatment with the intra-peritoneal methanolic crude bark extract of *A. polyacantha* resulted in LDU of 7.725 (34% protection) in SAB mice and in BALB/c mice LDU of 10.561 (63% protection). The intra-peritoneal administration of aqueous crude bark extract of *A. polyacantha* reduced the spleen parasite loads in SAB to LDU of 6.59 (44% protection) and LDU of 19.138 (32% protection) in BALB/c mice. This antileishmanial efficacy of *A. Polyacantha* was

less than that of conventional drugs Pentostam (LDU = 3.281, 76% protection) in SAB mice and (LDU = 6.0217, 79% protection) in BALB/c mice and amphotericin B with LDU = 0.641 (95% protection) in SAB mice and LDU = 0.331(99% protection) in BALB/c mice. Various previous studies have been done by other researchers showing plant extracts with significant antileishmanial activity like *A .polyacantha* including Chouhan *et al.,* (2016), where *in vivo antileishmanial* efficacy of a plant *Piper nigrum* bioactive fractions was assessed in *L. donovani* infected BALB/c mice which showed that *Piper nigrum* hexane seeds (200mg/kg bwt) caused reduction in spleen parasite load (LDU=17.52, 86.66% protection) while *Piper nigrum* ethanolic fractions (200mg/kg bwt) reduced parasite burden in spleen (LDU=34.02, 85.55 protection). In a parallel *in vitro* study by Bolivar *et al.,* (2011), with *Galium mexicanum* , a traditional medicinal plant used in Mexico it was demonstrated that its n- hexane fraction inhibited the growth of *L.donovani* parasites at a concentration of 333µg/ml for a period of 72 hours. Dey *et al.,* (2015), in an *in vitro* study demonstrated that the semi-purified hexane extract of the leaves of a plant *Croton caudates* (JDHex) inhibited proliferation of *L.donovani* promastigotes (IC50=10µg/ml) and intracellular amastigotes (IC50=2.5µg). JDHex was also found efficient in reducing parasite burden in spleen.

5.6 Route of administration

The route of administration for potential therapies is an important consideration in drug discovery Intraperitoneal injection (IP) is particularly useful in discovery laboratories for small animal studies, where it is often preferred over IV due to ease of administration (LiDi & Kerns, 2016). An ideal drug for treatment of leishmaniasis should provide several requirements for patients including being effective in one or few doses, low cost,

not be teratogenic, no side effects, no need for hospitalization, and no induction of resistance (Alviano *et al*., 2012).

The current study found that the most effective route to administer *A. polyacantha* crude bark extract was the intra-peritoneal route in which the spleen parasite load reduced to LDU=5.99 compared to oral route with LDU=15.34. This difference was statistically significant ($p < 0.05$). The observation made in this study about the intraperitoneal route being more effective in administering the *Acacia polyacantha* extract will be associated with reduced side effects during its use as per earlier literature which indicates that the intraperitoneal route of administration is particularly advantageous because medication levels in the IP solution can be maintained one to two orders of magnitude above toxic levels in the plasma. Another advantage for giving *A. polyacantha* intraperitoneally is that when medication is given intraperitoneally, transfer to the systemic circulation is relatively slow, and peak concentrations in the plasma will be a fraction of what they would be if the same dose were administered IV .This provides for maximal pharmacologic activity locally while sparing the remainder of the body from the toxic side effects (Flessner, 2003).

A study by Proulx *et al* (2011) which found that liposomal camptothecin significantly reduced spleen parasite loads by 55% when it was given through the intraperitoneal route is in agreement with the current study. In another study by Marango *et al.*,2017, there was significant ($p < 0.05$) control of parasite burden when *Tephrosia vogelii* extracts were administered intraperitoneally than those treated orally.

CHAPTER SIX

CONCLUSIONS AND RECOMMENDATIONS

6.1 Conclusions

1.*A. Polyacantha* methanolic crude bark extract is potent against *Leishmania donovani* promastigotes

2. Crude bark extract of *A. polyacantha* reduced significantly spleen weights of BALB/c mice infected with *L. donovani* hence making it a good medicinal candidate for treating splenomegaly in kala azar.

3. The methanolic crude bark extract of *A. polyacantha* is more potent against *Leishmania donovani* when compared to the aqueous crude bark extract.

4. The crude bark extract of *A. polyacantha* is more effective when given through intra-peritoneal route than oral route in reducing spleen parasite loads.

5. The crude bark extract of *Acacia polyacantha* is safe in mammalian cells.

6.2 Recommendations

The study recommends that the methanolic .*A. polyacantha* bark extract should be administered through intra-peritoneal route to achieve maximum reduction of spleen parasite loads.

Further studies should be done to establish if the leaf and root extracts have better medicinal value against the leishmaniases.

REFERENCES

Abdullah, I., Farooq, A., Shazia, R., Poonam, S., Omar J.,& Satyajit, D. (2011). Composition, antioxidant and chemotherapeutic properties of the essential oils from two Origanum species growing in Pakistan. *Brazil Journal of Pharmacology* 21:943–952.

Aebischer,T. (1994). Recurrent cutaneous leishmaniasis: a role for persistent parasites?*Parasitolology Today, 10:25-28*

Aguilar-Torrentera F., & Carlier Y. (2001). Immunological factors governing resistance and susceptibility of mice to *Leishmania major* infection. *Rev Latinoam Microbiol*, 43:135–142.

Alemayehu, B., & Alemayehu, M. (2017). Leishmaniasis: A Review on Parasite, Vector and Reservoir Host.*Health Science Journal,* 11(4): 519

Ali,S., Shoab,M., Shafi,U., Asad,U.(2016)Scientific investigation of crude alkaloids from medicinal plants for management of diseases, BMC complementary alternative medicine 16,178

Alvar, J., Vélez, I. D., Bern, C., Herrero, M.,Desjeux, P., & Cano, J. *et al*. (2012). Leishmaniasis worldwide and global estimates of its incidence. *PLoS ONE*, 7(5): e35671.

Alviano, D.S., Barreto, A.L., Dias Fde, A., Rodrigues Ide, A., & Rosa Mdo, S. *et al* (2012). Conventional therapy and promising plant-derived compounds against trypanosomatid parasites. *Front. Microbiology,* 3:283. doi: 10.3389/fmicb.2012.00283

Anjili,O.C. , Ngumbi, M.P., Kaburi,C.J.,& Irungu,W.L.(2011). The phlebotomine sandfly fauna (Diptera: Psychodidae) of Kenya . *Journal of Vector borne diseases,* 48(4): 183-9

Asfaram , S.,Teshnizi ,H.S., Fakhar. M., Salad, E., & Banimostafani.S. (2018). Is urine a reliable clinical sample for the diagnosis of human visceral leishmaniasis? A systematic review and meta-analysis.*Parasitolology International,* 67(5): 575 583

Ashu, F.A., Na-Iya, J., Wamba, B.E.N., Kamga, J., Nayim, P.&Ngameni, B *et al.* (2020). Antistaphylococcal Activity of Extracts, Fractions, and Compounds of *Acacia polyacantha* Wild (Fabaceae). *Evidence Based Complementary and Alternative Medicine*, 2:1-10.

Babiker, Z.O., Davidson,R.,Mazinda,C.,Kipngetich, S, & Ritmeijer, K. (2007). Utility of lymph node aspiration in the diagnosis of visceral leishmaniasis in Sudan. *American Journal of Tropical Medicine and Hygiene,* **76**, 689- 693

Barrett, M.P.,& Croft, S.L.(2012). Management of trypanosomiasis and leishmaniasis. *British Medical Bulletin,104:175–196*

Berman, J.D., & Lee, L.S. (1984). Activity of antileishmanial agents against amastigotes in human monocyte-derived macrophages and in mouse peritoneal macrophages. *Journal of Parasitology, 70*: 220-225.

Bern, C., Adler-Moore, J., Berenguer, J.,Boelaert, M., Figueras, C., & Boer, M.D. *et al* (2006). Liposomal amphotericin B for the treatment of visceral leishmaniasis, *Clinical Infectious Diseases*, 43:917-924

Bogdan, C., & Röllinghoff, M. (1998). The immune response to *Leishmania*: mechanisms of parasite control and evasion. *International Journal of Parasitology*, 28:121–134.

Bolivar, P., Cruz–Paredes, C., Hernández, L. R., Juárez, Z. N., Sánchez–Arreola, E.,& Av–Gay, Y.et al.(2011). Antimicrobial, anti–inflammatory, antiparasitic, and cytotoxic activities of Galium mexicanum. *Journal of Ethnopharmacology, 137(1)*;141–147. doi: 10.1016/j.jep.2011.04.069

Boussery, G., Boelaert, M., vanPeteghem, J., Ejikon, P & Henckaerts, K. (2001). Visceral leishmaniasis(kala-azar) outbreak in somali refugees and Kenyan shepherds, Kenya. *Emerging Infectious Diseases.* 7(3 Suppl):603-4

Bradley, D. J., & Kirkey, J. (1977). Regulations of *Leishmania* population within the host. The variable course of *Leishmania donovani* infection in mice. *Clinical & Experimental Immunology*, **30**:119

Bray, R.S. (1985).*Immunodiagnosis of leishmaniasis*, p .177-181. Ink.p. Chang and R. S. Bray (Ed) Leishmaniasis. Elsevier Science publisher, Amsterdam, the Netherlands

Braz, R.F. (2002). The sensitivity and specificity of *Leishmania chagasi* recombinant k39 antigen in diagnosis of American visceral leishmaniasis and in differentiating active from subclinical infection. *American Journal of Tropical Medicine and Hygiene*, **67**: 344- 348

Bryceson,A. (2001). A policy for leishmaniasis with respect to the prevention and control of drug resistance. *Tropica lmedicine and International Health*, **6**: 928-934

Burleigh, B.,Kima, P.E., &Andrews, N.W. (2000). Surface-targeted lysosomal membrane glycoprotein-1 (Lamp-1) enhances lysosome exocytosis and cell invasion by *Trypanosoma cruzi.Cellular Microbiology*, 2(6): 477-86. PMID: 11207602

Burkit, T. (2009). East African countries struggle with visceral leishmaniasis. *Lancet*,374(9687):371-2

Burns, J.M. (1993). Molecular characterization of a kinesin – related antigen of *leishmania chagasi* that detects specific antibody in African and American visceral leishmaniasis. *Proceedings of the National Academy of Sciences of the United States of America* , 90: 775-779

Carneiro, P.P., Conceição, J., Macedo, M., Magalhães, V., Carvalho, E.M., & Bacellar, O. (2016). The Role of Nitric Oxide and Reactive Oxygen Species in the Killing of *Leishmania braziliensis* by Monocytes from Patients with Cutaneous Leishmaniasis. *PLoS ONE,* 11(2): e0148084.

Carvalho,E.,Badaro,R., Reeds,S., Jones,T., Johnson (1985) "Absence of Gamma Interferon and interleukin 2 production During Activevisceral leishmaniasis". *The Journal of Clinical Invesstigation*, 76 **(6**): 2066- 2069

Carvalho,L., JuanR.L.O., Carmen, L.M.,& Francisco,G. (2011). The 8-Aminoquinoline Analogue Sitamaquine Causes Oxidative Stress in Leishmania donovani Promastigotes by targeting Succinate Dehydrogenase. *Antimicrobial Agents and Chemotherapy*, 55(9): 4204-4210

CDC (2013) Parasites - Leishmaniasis. Centers for Disease Control and Prevention.

Chang, K.P., & Dwyer, D.M. (1978). *Leishmania donovani*. Hamster macrophage interactions *in vitro*: cell entry, intracellular survival, and multiplication of amastigotes. *Journal of Experimental Medicine*, 147: 515–530. ChemSpider, 2018

Chouhan, G., Islamuddin,M., Want, M.Y., Ozbak, H.Y., Hemeg, H, A., & Sahal,D. (2016). Corrigendum:Leishmanicidal Activity of *Piper nigrum* Bioactive Fractions is Interceded via Apoptosis *In Vitro* and Substantiated by Th1 Immunostimulatory Potential. *In V*ivo.*Front Microbiology*, 7: 944

Chowdhury, K.J.I.,&Haque, F.M. (2019). Visceral Leishmaniasis Treatment and Its Challenges. South *Asian Journal of Parasitology*, 3(4): 1-16

Colacicco-Mayhugh, G.M., Grieco, P.J., Putnam, L.J., Burkett, A.D., & Coleman, R.E. (2011). Impact of phlebotomine sand flies on United States military operations at Tallil Air Base, Iraq :5. Impact of weather on sand fly activity. *Journal of Medical Entomology.* 48: 488-545.

Collins, S., Davidson, R., Ritmeijer, K., Keus, K., Melaku, Y., & Kipngetich, S *et al*. (2004). Conflict and kala-azar: determinants of adverse outcomes of kala azar among patients in southern Sudan.*Clinical Infectious Diseases,* **38:** 612- 619.

Craig, G. S. (2020). Leishmaniasis

Croft, S.L. (2008). PKDL-a drug related phenomenon? *Indian Journal of Medical Research*, **128**: 100-111.

Das, V., Pandey, K., Singh, D., Forwood, C., Lal. C. S., & Das, P. (2013). Development of post-kala-azar dermal leishmaniasis in AmBisome treated visceral leishmaniasis: A possible challenge to elimination program in India. *Journal of Postgraduate Medicine*, 59: 226-8

Das M.,Mukherjee, S.B., & Shaha,C. (1988).Hydrogen peroxide induces apoptosis-like death in *Leishmania donovani* promastigotes. *BiosciReports*, 2:131-137.

Das M.,Mukherjee, S.B., & Shaha,C. (2001).Hydrogen peroxide induces apoptosis-like death in *Leishmania donovani* promastigotes. *Journal of Cell Science*, 114: 2461-2469

Dayakar A., Chandrasekaran S., Veronica J., Sundar S.,& Maurya R.(2020). In vitro and in vivo evaluation of anti-leishmanial and immunomodulatory activity of Neem leaf extract in *Leishmania donovani* infection. *Experimental Parasitol.ogy* 153:45–54. doi: 10.1016/j.exppara.2015.02.011.

Dawit, G., Girma, Z.,& Simenew, K. (2013). A review on biology, epidemiology and public health significance of leishmaniasis. *Journal of Bacteriology & Parasitology*, article 166doi: 10.4172/2155-9597.1000166

Delorenzi, J.C., Attias , M.,Gattas, C.R., Andrade, M., Rezende, C., & dacunha pinto, A., *et al*.(2001).Antileishmania activity of an indole alkaloid from peschieraaustralis Antimicrobial Agents and chemotherapy,45(5):1349-1354

Demain, A.L., and Fang, A.(2000).The natural functions of secondary metabolites Advance Biochemical Engineering Biotechnology, 69 , pp. 1-39

De Menezes, J.P.B., Guedes, C.E.S., Petersen, ALd.O. A., Fraga, D.B.M., & Veras, P.S.T. (2015). Advances in development of new treatment for leishmaniasis. *BioMedical Research International*, 2015: 1-11

De Melo, C.V.B., Hermida, M.D., Mesquita, B.R., Fontes, J.L.M., Koning, J.J., & Solcà, M.D.S *et al* (2020).Phenotypical Characterization of Spleen Remodeling in Murine Experimental Visceral Leishmaniasis. *Front Immunology*, 11: 653.

De Rossell, R.H., de Duran, R.J., Rossell, O. & Rodriguez, A.M. (1992). Is leishmaniasis ever cured? *Transactions of the Royal Society of Tropical and Hygiene*, 86: 251-253

Delmas, F., Giogio, C, D., Elias, R., Gasquet, M., Azas, N.,& Mshildadze, V *et al.* (2000). Antileishmanial activity of three saponins isolated from ivy, alpha-hedrin, beta-hedrin and hederacolchiside. *Planta Medica*, 66: 343-347.

Dereure, J., El-Safi, S.H., Bucheton, B., Boni, M., Kheir, M.M., Davoust, Bet al. (2003). Visceral leishmaniasis in eastern Sudan: parasite identification in humans and dogs; host-parasite relationships. *Microbes' infections*,5(12):1103-8.

Desjeux, P., &Ramesh, V. (2011). Post-kala-azar Dermal Leishmaniasis: Facing the Challenge of Eliminating Kala-Azar from South Asia

Dey, S., Mukherjee, D., Chakraborty, S., Swapan, N., Maiti, S., Singh, S&Pal, C. (2015). Protective effect of *Croton caudatus* Geisel leaf extract against experimental visceral leishmaniasis induces proinflammatory cytokines *in vitro* and *in vivo*.*Experimental Parasitology*,151-152:84–95. doi: 10.1016/j.exppara.2015.01.012

Dietze, R., Carvalho, S.F., Valli, L.C., Berman J., Brewer, T.,&Milhous, Wet al. (2001). Phase 2 trial of WR6026, an orally administered 8-aminoquinoline, in the treatment of visceral leishmaniasis caused by *Leishmaniachagasi*. *AmericanJournalof Tropical Medicine and Hygiene*, (65):685–689

Do, Q.D., Angkawijaya, A,E., & Tran-Nguyen,P.L. (2014). "Effect of extraction solvent on total phenol content, total flavonoid content, and antioxidant activity of *Limnophila aromatica*," *Journal of Food and Drug Analysis*, 22(3) : 296–302

Dorin, D., Le Roch,K., Sallicandro, P., Parzy, D., Poullet.P.,&Doerg, C. (2001). Pfnek-1, a NIMA-related kinase from the human malaria parasite *plasmodium falciparum*. *EuropeanJournal of Biochemistry*,268 (9):2600-2608

Dorlo, T.P., Huitema, A.D., Beijnen, J.H., & de Vries, P.J. (2012) Optimal dosing of miltefosine in children and adults with visceral leishmaniasis. *Antimicrobial Agents andChemotherapy*,56:3864–72

Dostalova, A.,&Volf, P. (2012). *Leishmania* development in sand flies: parasite-vector interactions overview. *Parasites and Vectors* 5:276l

Drugs for Neglected Disease Initiative. (2011). New treatment for kala- azar the deadliest parasitic disease after malaria.

Dupouy-Camet, J. (2004). New drugs for the treatment of human parasitic protozoa. *Parassitologia*, 46, 81–84

Dutta, A., Bandyopadhyay, S., Mandal, C., & Chatterjee, M. (2007b). Aloe vera leaf exudate induces a caspase-independent cell death in *Leishmania donovani* promastigotes. *Journal of MedicalMicrobiology*, 56, 629–631

El Hassan, A.M., Khalil, E.A.G., Elamin, W.E., Ahmed, M.E., & Musa, A.M. (2013). Case Report: Misdiagnosis and Mistreatment of Post-Kala-Azar Dermal Leishmaniasis. *Case Reports in Medicine*, Volume 2013, Article ID 351579, 6 pages.

Engwerda, C.R., & Kaye, P.M (2000). Organ-specific immune responses associated with infectious disease. Immunology Today, 21(2) ,73-78. 10.1016/S0167-5699(99)01549-2.

Flessner.F.M.(2003). Encyclopedia of Physical Science and Technology (Third Edition),

França, O.A., Pompilio, A.M., Cury Pontes, J.R.E., Oliveira, P.M., Pereira, R.O.L., &Lima, B.R.et al. (2018)**.** Leishmania infection in blood donors: A new challenge in leishmaniasis transmission?*PLoS One,*13(6)

Ganguly, S., Mukhopadhyay, D., & Das, N. (2010a). "Enhanced lesinal Fox p 3 expression and anergic lymphocytes indicates a role for regulatory T cells in Indian post–kala-azar leishmaniasis."*Journal of Investigative Dermatology,*130 **(4**): 1013- 22

Ganguly, S., Bandyopadhay, S., Bera, A. and Chaterjee, M. (2006). Antipromastigote activity of an ethanolic extract of leaves of *Artemisia indica*. *Indian Journal of Pharmacology*, 38(1): 64-65.

Ganguly, S., Das, N.K., Barbhuiya, J.N., & Chatterjee, M. (2010b). Post-kala-azar dermal leishmaniasis – An overview. *International Journal of Dermatology,*49:921–31.

Ghalib, H., Piuvezam, M., &Skeiky, y. (1993). "Interleukin 10 production correlates with pathology in human *leishmania donovani* infections ". *Journal of ClinicalInvestigation*, 92 **(1)** : 324_ 329

Garcia, M., Monzote, L., Scull, R., & Herrera, P. (2012). Activity of Cuban plants extracts against *Leishmania amazonensis*. *ISRN. Pharmacology*, 2012:104540

Ghobakhloo, N., Motazedian, M.H., & Fardaei M. (2016). Expression analysis of multiple genes may involve in antimony resistance among *Leishmania major* clinical isolates from Fars Province, Central Iran. *Iranian Journal of Parasitology* ,11(2):168-176

Ghorbani, M & Farhoudi, R. (2017). Leishmaniasis in humans: drug or vaccine therapy? *Drugs, Design, Development and Therapy* 12: 25-40

Gifawesen, C., & Farrell, J.P. (1989). Comparison of T-cell responses in self-limiting versus progressive visceral Leishmania donovani infections in golden hamsters. *Infection and Immunity,* 57: 3091

Goyal,V., Mahajan, R., Pandey, K.,Singh, SN., Singh, RS., &Strub-Wourgaft, N., et al. (2018) Field safety and effectiveness of new visceral leishmaniasis treatment regimens within public health facilities in Bihar, India. *PLoS Neglected Tropical Diseases 12(10)*

Green, S.J., Meltzer, M.S., Hibbs, J.B., Jr., & Nacy, C.A. (1990b). Activated macrophages destroy intracellular *Leishmania major* amastigotes by an L-arginine-dependent killing mechanism. *Journal of Immunology,* **144**: 278-283.

Green, S.J., Crawford, R.M., Kockmeyer, J.T., Meltzer, M.S., & Nacy, C.A. (1990). *Leishmania major* amastigotes initiate L-arginine-dependent killing mechanisms in IFN-gamma-stimulated macrophages by tumor necrosis factor-alpha. *Journal of Immunology*,**145:** 4290-4297.

Guénet, J.L., & Bonhomme. F. (2003). Wild mice: an ever-increasing contribution to a popular mammalian model. *Trends Genet*,19:24–31

Hartley, M., Stefan, D., Catherine, R., Stephen, M.B., & Nicolas, F. (2014). Theimmunological, environmental, andphylogrnetiv perpetrators of metastatic leishmaniasis.*Trends in Parasitology,* 30(8):412-422.

Hassan, A. A., Khalid, H. E., Abdalla, A. H., Mukhtar, M. M., Osman, W. J., & Efferth, T. (2022). Antileishmanial Activities of Medicinal Herbs and Phytochemicals In Vitro and In Vivo: An Update for the Years 2015 to 2021. *Molecules (Basel, Switzerland), 27*(21),

Hayyan. M., Hashim. M.A., & Alnashef. I.M. (2016). Superoxideion: generation and chemical implications.*Chemical Reviews,* 116:3029-3085

Helen ,V., Daugherity ., Erin K., Bhupinder, S., Maurer, , & Kirk J. (2012). "The Experimental Use of Syrian Hamsters". In Suckow, Mark A.; Stevens, Karla A.; Wilson, Ronald P. (eds.). *The laboratory rabbit, guinea pig, hamster, and other rodents* (1st. ed.). Amsterdam: Elsevier Academic Press. pp. 875–898. ISBN 978-0123809209.

Holzmuller, P., Sereno, D., Cavaleyra, M., Mangot, L., Daulouede, S.,&Vincendeau, P et al. (2002). Nitric oxide-mediated proteasome-dependent oligonucleosomal DNA fragmentation in *leishmania amazonesis* amastigotes. *Infection and immunity*,70 (7):3727-3735

Horta, M.F., Mendes, B.P., Roma, E.H., Noronho, F.S.M., Macêdo, J.P., &Oliveira, L.S et al. (2012). Reactive oxygen species and nitric oxide in cutaneous leishmaniasis. *Journal of Parasitological Research*, Article 10203818, 11 pages.

Hotez, P.J. (2015). Combating the next lethal epidemic. *Science*,348 (6232) : 296-7

Howard, R. A. (1988). Dicotyledoneae part 1. F1 lessor Antilles Arnold Arboretum In: T.K. Jha, E. Noiri (Eds.), Kala -Azar in South Asia, 111-124, 2011
Inbred Mice | Charles River, 2014

International Drug Price Indicator Guide (2016). Sodium stibogluconate

International Drug Price Indicator Guide. Retrieved 6 December 2016.

Ishizuka,K., Fujii,W., Azuma,N.,Mizobuchii,H., Morimoto., & Sanioba et al. (2020) Pathological roles of MRP14 in anemia and splenomegaly during experimental visceral leishmaniasis. *PLOS Neglected Tropical Disease*

Ismail, A., El Hassan, A.M., Kemp, K., Gasim, S., Kadaru, A.E.,& Moller,T.et al.(1999).Immunopathology of post kala-azar dermal leishmaniasis (PKDL): T-cell phenotypes and cytokine profile. *The Journal of Pathology*, **4**:615-22.
Jackson Laboratory (2007) . *Inbred Strains of Mice*

Jha, T.K., Sundar, S., Thakur, C.P., Felton, J.M., Sabin, A.J., & Horton, J. (2005). A phase II dose-ranging study of sitamaquine for the treatment of visceral leishmaniasis in India. *American Journal of Tropical Medicine and Hygiene*, (73):1005–1011.

Kaur S., Kaur G., Sachdeva H., & Kaur J. (2013). In vivo evaluation of the antileishmanial activity of two immunomodulatory plants, *Emblica officinalis* and *Azadirachta indica* in Balb/c mice. *IJAHM.* 3:1066–1079.

Kaur R, & Kaur S.(2017). Evaluation of *in vitro* and *in vivo* antileishmanial potential of bergenin rich *Bergenia ligulata* (Wall.) Engl. root extract against visceral leishmaniasis in inbred BALB/c mice through immunomodulation. *Journal of Traditional Complement Medicine*, 8(1):251-260. doi: 10.1016/j.jtcme.2017.06.006. PMID: 29322016; PMCID: PMC5756018.

Kassahun ,A.,Sádlová ,J.,Dvorak,V. , Kostalova,T .,Rohousova,I.,Frynta,D et al. **(**2015**).** Detection of *Leishmania donovani* and *L. tropica* in Ethiopian wild rodents .*Acta Tropica*,145:39-44.

Kchaou, W., Abbès, F., Blecker, C., Attia, H & Besbes, S. (2013).“Effects of extraction solvents on phenolic contents and antioxidant activities of Tunisian date varieties (*Phoenix dactylifera* L.),” *Industrial Crops and Products,* 45:262–269

Kidaha, M.L., Alakonya, A.E., & Nyende, A.B. (2013). Bioactivity determination of methanol and water extracts for roots and leaves of Kenyan *Psidium guajava L* landraces against pathogenic bacteria. *Springerplus*, 2:670

Kigondu, E.V.M., Rukunga, G.M., Keriko, J.M., Tonui, W.K., Gathitwa, J.W., & Kirira, P.G *et al*., (2009). Anti-parasitic activity and cytotoxicity of selected medicinal plants from Kenya. *Journal of Ethnopharmacology*, 123(3): 504-509.

Kiige, S.G., Mutiso, J.M., Laban, L.T., Khayeka-Wandabwa, C., & Anjili, C.O. et al.(2014). F_1 cross-breed between susceptible BALB/c and resistant Swiss mice infected with *Leishmania major* exhibit an intermediate ohenotype for lesion sizes and Type 1 cytokines but show low level of total IgG antibodies. *Experimental Immunology*, **79**: 283-291.

Kolaczinski, J.H., Reithinger, R., Worku.D.T., Ochieng, A., Kasimiro, J., & Kabatereine, N et al.(2008). Risk factors of visceral leishmaniasis in East Africa: a case-control study in pokot territory of Kenya and Uganda.*International journal of epidemiology*,37(2):344-52.

Koley, S., Mandal, R.K., Choudhary, S., & Bandyopadhyay, A. (2013). Post-kala-azar dermal leishmaniasis developing in miltefosine-treated visceral leishmaniasis. *Indian Journal Dermatolology,* 58:241.

Kumar, R., Pai, k., Pathak, k., &Sundar, S. (2001). Enzyme – linked immunosorbent assay for recombinant k39 antigen in diagnosis and prognosis of Indian visceral leishmaniasis,*Clinical and DiagnosticLaboratory Immunology*,**8**: 1220 – 1224

Kumar, D., Varadharan, R., Verma, S., & Salotra, P. (2009). Post-kala-azar dermal leishmaniasis (PKDL) developing after treatment of visceral leishmaniasis with amphotericin B and miltefosine. *Pathogens and Global Health*, **103**(8):727-730

Lage, P.S., de Andrade, P.H., Lopes Ade, S., Chavez Fumagalli, M.A., Valadares, D.G., & Duarte, M .C et al.(2013). Strychnos pseudoquina and its purified compounds present an effective in vitro antileishmanial activity. *Evidence Based Complementary and Alternative Medicine,* 2013:304354.

Leveque,F.M.,Lachaud, L., Battery, E., Marty, P., & Pomares,C. (2020). Place of Serology in the Diagnosis of Zoonotic Leishmaniases with a Focus on Visceral Leishmaniasis Due to *Leishmania infantum. Infection Microbiol*ogy

LiDi., & Kerns, H.E (2016). Drug-Like Properties (Second Edition)

Li ,Y ., Long, F ., Yang, C ., Hao, X ., Wu, J ., & Situ, J et al. (2020). BALB/c Mouse Is a Potential Animal Model System for Studying Acute and Chronic Genotype 4 Hepatitis E Virus Infection. *Frontiers Microbiology,* 11:1156. doi: 10.3389/fmicb.2020.01156

Litt, E., Baker, M.C., & Molyneux, D. (2012). Neglected tropical disease and mental health: a perspective on cormorbidity. *Trends parasitology*,28 (5): 195-201

Lodge, R. & Descoteaux, A. (2008). Leishmania invasion and phagosome biogenesis. Subcellular Biochemistry 47 pg 174-181

Maes, L., Germonprez, N., Quirijnen, L., Van Puyvelde, L., Cos, O., & Vanden Berghe, D. (2004). Comparative activities of the triterpene saponin Maesabalide III and liposomal Amphotericin B (AmBisome) against Leishmania*donovani* in hamsters. *Antimicrobial Agents and Chemotherapy,* 48: 2056-2060.

Mandal, D. (2006). A triterpenoid saponin possessing antileishmanial activity from the leaves of *Careya arborea*. *Phytochemistry*, 67(2): 183-190.

Mandell,M.A.,&Beverley,S.M.(2017). Continual renewal and replication of persistent *Leishmania major* parasites in concomitantly immune hosts.

Marango, S.N., Khayeka-Wandabwa, C., Makwali, J.A., Jumba, N.B., & Choge,K.J.,et al., (2017). Experimental therapeutic assays of *Tephrosia vogelii* against *Leishmania major* infection in murine model: in vitro and in vivo. *BMC Res Notes* **10**, 698 Marigat in Baringo,2020

Mansueto, P., Seidita, A., Vitale.G.,& Cascio, A. (2014). *Travel Medicine and Infectious Disease*.12(6):617-627

McCall, L.I., Zhang, W.W.& Matlashewski, G. (2013). Determinants for the development of Visceral Leishmaniasis Disease.*PLoS Pathogens*, 9(1): e1003053

Meheus, F., Abuzaid, A.A., Baltussen, R., Younis, B.M., Balasegeram, M., &Khalil, E.A. G et al. (2013). The economic burden of visceral leishmaniasis in Sudan:an assessment of provider and household costs. *American Journal of Tropical Medicine and Hygiene*,89(6):1146-53.

Melby, P. C., Tryon,V.V., Chandrasekar, B., & Freeman, G.L. (1998). Cloning of Syrian hamster (Mesocricetus auratus) cytokine cDNAs and analysis of cytokine mRNA expression in experimental visceral leishmaniasis. *Infection and Immunity*, 66: 2135

Mescouto-Borges, M.R.M., Maués, E., Costa, D.L, Pranchevicius, M.C., & Romero, G.A.S. (2013). Congenitally transmitted visceral leishmaniasis: report of two Brazilian human cases .*The Brazilian Journal of Infectious Diseases,17(2):263-266*

Minodier, P., & Parola, P. (2007). Cutaneous leishmaniasis treatment. Travel *Medicine andInfectious Disease*,5(3):150-158

Ministry of Public Health and Sanitation (2012). *Integrated Disease Surveillance and Response inKenya: TechnicalGuides.*

MOH. (2017). Prevention, diagnosis and treatment of visceral leishmaniasis (kala-azar) in kenya national guidelines for health workers

Motuma, K., AberaE., Wondu, B., & Negash, A. (2016). Visceral Leishmaniasis in Ethiopia.*Review. European Journal of Biological Sciences* 8(2):70-78

Moura, T.R., Novais, F.O., Oliveira, F., Clarêncio, J., Noronha, A., & Barral A., et al. (2005). Toward a novel experimental model of infection to study American cutaneous leishmaniasis caused by *Leishmania braziliensis*. *Infection and Immunity*,73:5827–5834.

Mulofwa, J.S., & Tengnas, B. (1994). Agroforestry: Nation Institutes of Health (NIH) consensus

Mueller, Y.K., Kolaczinski, J.K., Koech, T., Lokwang, P., Riongoita, M &Velila, E., et al. (2014). Clinical epidemiology, diagnosis and treatment of visceral leishmaniasis in the pokot endemic area of Uganda and Kenya.*AmericanJournal of Tropical Medicine Hygiene*,90(1):33-9

Mukherjee, P., Ghosh, A. K., & Ghose, A. C. (2003). Infection pattern and immune response in the spleen and liver of BALB/c mice intracardially infected with *Leishmania donovani* amastigotes. *Immunology letters*, *86*(2), 131–138. https://doi.org/10.1016/s0165

Mutiso, J. M., Macharia, J. C., Kiio, M. N., Ichagichu, M., Rikoi, H. & Gicheru, M. M. (2013). Development of Leishmania vaccines: predicting the future from past and present experience. *Journal of Biomedical Research,* 27: 85-102.

Naghavi, M., Wang, H., Lozano, R., Davis, ALiang, X.,&Zhou, M. et al. (2013). Global, regional and national age-sex specific all-cause and cause-specific mortality for 240 causes of death ,1990-2013: a systematic analysis for the Global Burden of Disease Study .*Lancet* ,385 (9963) :117-71

Narayan, S., Thakur, C.P., Bahadur, S., Thakur ,M., Pandey, S.N., & Thakur, A.K.et al.(2017). Mitra DK, Mukherjee PK. *Cedrus deodara*: *In vitro* antileishmanial efficacy & immumomodulatory activity. *Indian Journal of Medical Research,* 146(6):780-787. doi: 10.4103/ijmr.IJMR_959_16. PMID: 29664038; PMCID: PMC5926351.

Nieto, A., Domínguez-Bernal, G., Orden, J.A., Madrid-Elena, N ., & Javier, C (2011).Mechanisms of resistance and susceptibility to experimental visceral leishmaniosis: BALB/c mouse versus syrian hamster model. *Veterinary Research*, **42**, 39 (2011). https://doi.org/10.1186/1297-9716-42-39

Orya A.(2015). Plant-derived compounds in treatment of leishmaniasis. *Iranian Journal of Veterinary Research*, 16(1):1-19.

Orwa, C., Mutua, A., Kindt, R., Jamnadass, R., & Anthony, S. (2018). Agroforestree data base: a tree reference and selection guide version 4.0

Pandey, K., Das, V.N.R., Singh, D., Das, S., & Lala, C.S. (2012). Post-kala-azar-dermal leishmaniasis in a patient treated with injectable paromomycin for visceral leishmaniasis in India. *Journal of Clinical Microbiology*, **50**:1478-1479.

Pandey, K., Goyal, V., Das, V.N.R., Verma, N., Rijal, S., & Das, P., et al. (2018). PKDL Development after Combination Treatment with Miltefosine and Paromomycin in a Case of Visceral Leishmanisis.First Ever Case Report *Journal of Medical Microbiology and Immunology Research,2(1).*

Parasite wonders.blogspot.com, (2018)

Pearson, R. D. G., Cox, T., Evans, D. L., Smith, D., Weidel, J.&Castracane. (1990). Wasting and macrophage production of tumor necrosis factor/cachectin and interleukin 1 in experimental visceral leishmaniasis, *American Journal Tropical Medicine and Hygiene,*43: 640

Pramanik, A., Paik, D., Pramanik,K.P., & Chakraborti,T.(2019). Serine protease inhibitors rich *Coccinia grandis* (L.) Voigt leaf extract induces protective immune responses in murine visceral leishmaniasis. *Biomedicine & Pharmacotherapy* , Volume 111,Pages 224-235

Polhiu, R.M. (1990*). Legumineuses*.In Flore des Mascareignes, vol 80

Ponte-Sucre,A.,Gamarro,F., Dujardin,J., Michael P. Barrett, P.M.,& López-Vélez,R.et al . (2017). Drugresistance and treatment failure in leishmaniasis: A 21st century challenge. *Plos Neglected Tropical Diseases*, 11(12) PubChem, 2018

Proulx, M. E., Désormeaux, A., Marquis, J. F., Olivier, M., & Bergeron, M. G. (2001). Treatment of visceral leishmaniasis with sterically stabilized liposomes containing camptothecin. *Antimicrobial agents and chemotherapy*, *45*(9): 2623–2627.

Ramesh,V., Kaushal, H., Mishra, A.K.,Singh, R.,&Poonam Salotra, P. (2015). Clinico-epidemiological analysis of Post kala-azar dermal leishmaniasis (PKDL) cases in India over last two decades: a hospital based retrospective study.*BMC Public Health*, 15: 1092. Doi:10.1186/s12889-015-2424-8.

Rashid J.R., Chunge C.N., Oster C.N.,Wasunna, K.M.,Muigai,R.,& Gachihi.G.S. (1986). PKDL occurring long after cure of VL in Kenya. *East African Medical*, 63: 365-367.

Rezvan, H., & Moafi, M. (2015). An overview on Leishmania vaccines: A narrative review article. *Veterinary Research Forum.* ;6(1):1-7

Ritmeijer, K. (2006). A comparison of miltefosine and sodium stibogluconate for treatment of visceral leishmaniasis in an Ethiopian population with high prevalence of Hiv infection. *Clinical Infectious Diseases*,43: 357- 364

Rolão, N., Melo, C., & Campino, L. (2004). Influence of the inoculation route in BALB/c mice infected by Leishmania infantum. *Acta tropica*, *90*(1), 123–126. https://doi.org/10.1016/j.actatropica.2003.09.010

Roma, E.H., Macedo, J.P., Goes, G.R., Goncalves, J.L., deCastro, W., &Cisalpino, D. et al. (2016). Impact of reactive oxygen species (ROS) on the control of parasite loads and inflammation in *Leishmania amazonensis* infection. *Parasites Vectors*, 9:193

Ross, J.H. (1979). A conspectus of African Acacia.*Memoirs of the Botanical survey of SouthAfrica*, 44: 1-150

Sack,D.L.(2000).Leishmaniasis-sandfly interactions controlling species-specific vector competences.*Cellular Microbiology* 3 (4):189-196

Safar, E.H. (2017). Laboratory-acquired blood-borne parasites from accidental exposures. *Journal of the Arab Society forMedical Research*,12(1):1-5

Sakib, B., Croft, L.S., & Boelaert., M. (2018). Leishmaniasis. The *Lancet,* 392 (10151):951-970 Sitamaquine. (2018). National Center for Biotechnology Information. *PubChem Compound Database; CID=42548*

Schaefer, K.U., Kurtzhals, J.A., Sherwood, J.A., Githure, J.I., Kager, P.A., & Muller, A.S. (1994). Epidemiology and clinical manifestations of visceral and cutaneous leishmaniasis in Baringo District, RiftValley, Kenya.A literature review. *Tropical and Geographical Medicine*,46(3):29-33

Schallig, H. D., Canto-Cavalheiro, M., & da Silva, E. S. (2002). Evaluation of the direct agglutination test and the rK39 dipstick test for the sero-diagnosis of visceral leishmaniasis. *Memorias do Instituto Oswaldo Cruz*, *97*(7): 1015–1018.

Sarkar, J., Pal, S., Bhattacharya, S., & Biswas, M.(2013). In vitro antileishmanial activity of *Pleumeria pudica* leaf extracts on *Leishmania donovani* promastigotes. *American-Eurasian Journal of Scientific Research,* 8:68–71.

Srivastava, S., Shankar, P., Mishra, J., & Singh, S.(2016). Possibilities and challenges for developing a successful vaccine for leishmaniasis. *Parasites Vectors* **9**, 277

Sherwood, J.A., Gachihi, G.S., Muigai, R.K., Skillman, D.R., Mugo, M., & Rashid, J.R., et al. (1994). Phase 2 efficacy trial of an oral 8-aminoquinoline (WR6026) for treatment of visceral leishmaniasis. *Clinical Infectious Diseases*, (19):1034–1039.

Socks, D. L. and Perkins. (1984). Identification of an infective stage of *Leishmania* promastigotes *Science*, 233**:** 1417- 1419

Sousa, L, M.A., Carneiro, M.B.H., Resende, M.E., Martins, L.S., Dos santos, L.M., &Vas, G., et al. (2014). Neutrophils have a protective role during early stages of *Leishmania amazonensis* infection in BALB/c mice. *Parasite immunology*,36(1):13-31

Strazzulla, A., Cocuzza, S., Pinzone, M. R., Postorino, M.C., Cosentino, S., & Serra, A., et al. (2013). Mucosal leishmaniasis: an underestimated presentation of a neglected disease. *BioMed Research International*,2013:7. doi: 10.1155/2013/805108.805108

Sundar , S . , More , D. K . , Singh M.K . , Singh V.P., Sharma ,S., & Makharia , A., et al. (2000). " Failure of pentavalent antimonial in visceral leishmaniasis in India: report from the centre of the Indian epidemic . *Clinical Infectious Disease*, 31 **(4) :** 1104-07

Sundar, S., Makharia, A., More, D.K., Agrawal, G., Voss, A., & Fischer, C. et al.(2000a). Short-course of oral miltefosine for treatment of visceral leishmaniasis. *Clinical Infectious Diseases*, 31:1110–13.

Sundar, S., More, D. K., & Singh, M.K. (2000b). "Failure of pentavalent antimonial in visceral leishmaniasis in India: report from the centre of the Indian epidemic. *Clinical Infectious Diseases*, 31 **(4):** 1104-07

Sundar, S. & Rai, M. (2002). Laboratory diagnosis of visceral leishmaniasis. *Clinical and Diagnostics Laboratory Immunology*, 9 (5): 951-958.

Sundar, S., Jha, T.K., Thakur, C.P., Sinha, P.K& Bhattacharya, S.K. (2007). "Injectableparomomycin for visceral leishmaniasis in India". *New England Journal of Medicine,* 356 (25): 2571–81. doi:10.1056/NEJMoa066536. PMID 17582067.

Szatkiewicz, J.P., Beane, G.L., Ding, Y., Hutchins, L., de Villena, P. F., & Churchill, G.A. (2008). An imputed genotype resource for the laboratory mouse. *Mamm Genome*,19:199–208.

Tawaha,K., Alali, F.Q., Gharaibeh, M., T. Mohammad, T., & M. El-Elimat,M.(2007) Antioxidant activity and total phenolic content of selected Jordanian plant species *Food Chem*, pp. 1372-1378

Tilburt, J.& Kaptchuk, T.J. (2008). Herbal medicine research and global health: an ethical analysis. *Bulletin of the World Health Organization* ,86 (8):594-599

Thakur, C.P., & Narayan, S. (2004) A comparative evaluation of amphotericin B and sodium antimony gluconate, as first-line drugs in the treatment of Indian visceral leishmaniasis. *Annals of Tropical Mdicine&Parasitology*,92(2):129-138

The American Society of Health System Pharmacists, *2015. The American Society of Health-System Pharmacists. Archived from the original on 17th November 2016. Retrieved 3rd December 2016.*

The American Society of Health-System Pharmacists. *Archived from the original on 17th November 2016. Retrieved 3rd December 2016.*

The American Society of Health-System Pharmacists. *Archived from the original on 2015-01-01. Retrieved Jan 1, 2015. (2)*

Titus, R.G., Theodes., C.M., Shanker, A., & Hall, l.R. (1994). Interactions between *Leishmania major* and macrophages. *Immunology series*, 60 :439- 459

Tonui, W. K., & Titus, R.G. (2006). *Leishmania major* solubleexo – antigens (LmSEAgs) protect neonatal BALB c mice from a subsequent challenge with *Leishmania major* and stimulate cytokine production by leishmania naive Human peripheral blood mononuclear cells. *Journal of parasitology*,9**2**: 971- 976

Trindade, M. A. B., Lana, L. C. S., Lucia, M.A.B., Valdir, S.A., Bernard, N.., & Miriam N. S. (2015). Post kala- azar leishmaniasis and leprosy: case report and literature review, *BMC Infectious Diseases,15:543*

Van der Maesen, L.J.G., Vander Burgt, X.M., & Van Medenbach de Rooy, J.M. (1996). The Biodiversity of African Plants.Berlin, Germany: Springer Science Business Media

Veeken, H., Ritmeijer., K. Seaman, J.,& Davidson, R. A. (2000). A randomized comparison of branded sodium stibogluconate and generic sodium stibogluconate for the treatment of visceral leishmaniasis under field conditions in Sudan. *TropicalmedicineandInternational Health* ,5: 312- 317

Venter, F., & Venter, J.A., (1996). *Making the most of IndigineousTrees.* Pretoria, South Africa: Briza publications.

Waguma, J., & Jallow, M. (2011). *Kala -azar in Kenya.*Medicins Sans Frontieres, pg 4

Waleed, A., Jeniffer, R.H., &Peter, J.H. (2016). A review of visceral leishmaniasis during the conflict in South Sudan and the consequences for East African countries *Parasites and Vectors*,9: 460

Wang, X, Ge, J.M, Wang, K, Qian, J., & Zou, Y. (2006). Evaluation of Emodininduced cytotoxicity. *Assay Drug Development and Technology*. 4:203-207

WebMD. Archived from the original on 2016-11-10. Retrieved 2016-11-10.

Wink, M.(2012). Medicinal plants: a source of anti-parasitic secondary metabolites. *Molecules.*17:12771–12791.

WHO (2015).? *Leishmaniasis: Situation and Trends*. World Health Organization

WHO \ Visceral leishmaniasis.www.who.int? Retrieved 2015-10-05

WHO Jagdish, p. (2015). operational guidelines on kala-azar (visceral leishmaniasis) elimination in india

WHO (2010). Control of the leishmaniasis. International. *WHO Technical Report Series,*9492010:22-26, Geneva: Report of the WHO Expert Committee on the Control of Leishmaniases

World Health Organization. 2009. p. 145. ISBN 9789241547659. Archived (PDF) from the original on 13 December 2016. Retrieved 8 December 2016. (3)

*World Health Organization. March (2010). pp. 55, 88, 186. ISBN 9789241209496. Archived (PDF) from the original on 2017-03-15.*9(1)

World Health Organization. (2020). *Ending the Neglect to Attain the Sustainable Development Goals;* A *Road Map for Neglected Tropical Diseases,2021-2030*

World Health Organization.C2016). Visceral leishmaniasis in East Africa.

Zijlstra, E.E. (2016). The immunology of post-kala-azar dermal leishmaniasis (PKDL). *Parasites Vectors*, 9 : 464

Zijlstra, E.E., Musa, A.M., Khalil, E.A.G., El Hassan, I.M., & El Hassan, A.M. (2003). Post-kala-azar dermal leishmaniasis. *Lancet Infectious Diseases*, 3: 87-98.

Zijlstra, E. E., & el-Hassan, A.M. (2001). Leishmaniasis in Sudan. Post kala-azar dermal leishmaniasis. *Transactions of the Royal Society of Tropical Medicine and Hygiene*,95(suppl 1): S59-76.

Zijlstra, E.E., & el-Hassan, M.M. (2001). Leishmaniasis in Sudan. Visceral leishmaniasis. *Transactions of the Royal Society of Tropical Medicine and Hygiene*,95(suppl 1): S27-28.

APPENDICES

Appendix I: Table for cytotoxicity potentials

The cytotoxicity potentials of different concentrations of the methanolic and aqueous bark extract of *A.polyacantha* with IC_{50} value.

Extracts concentrations	0.01µg /ml	0.02µg/ ml	0.06µg/ ml	0.19µg/ ml	0.56µg /ml	1.67µg /ml	5µg/ ml	IC50µg/ ml
Methanolic / growth inhibition	0.09	1.02	1.26	1.15	0.74	0.35	0.84	1.090
Aqueous / growth inhibition	0.89	1.06	1.22	1.19	0.71	0.39	0.78	1.127

Appendix II: Letter of ethical approval

KENYAMEDICALRESEARCHINSTITUTE

Centre for Virus Research, P.O.Box 54628 - 00200 NAIROBI - Kenya

Tel: (254) (020) 2722541 , 254 02 2713349, 0722-205901, 0733-400003 Fax (254) (020) 2726115

Email: cvr@kemri.org

KEMRI/ACUC/ 03.03.15 24" March, 2015

Salbei Christine
P. O. Box 520-30100
Eldoret, Kenya.

Salbei,

RE: Animal use approval for SSC 2930 – "Efficacy of crude bark extract of *Acacia polyacantha* against SAB, BALB/c and Swiss Albino mice infected with *Leishmania Donovani* ''protocol

The KEMRI ACUC committee acknowledges the submission of the above-mentioned protocol.
The committee grants you the approval to use laboratory mice in your study but recommends that you proceed after obtaining all the other necessary approvals that may be required.

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

The committee wishes you all the best in your work sincerely,

Dr. Konongoi
Limbaso Chairperson
KEMR1 ACUC

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

Appendix III: Tables for P values

A. Effect of route (Oral and IP) on the Spleen weight, LDU and splenomegaly development of SAB mice infected with *L. donovani*

Source	Variable	df	T-value	P-value
Route of administration	Mean spleen weight	1	0.187	0.668
	LDU	1	4.22	0.048**

B. Effect of main factors and their interaction on LDU

Source of variation	F- Value	P-value
Strain	4.888	0.025*
Administration method	0.184	0.675
Slovent	5.620	0.007*
Strain* Administration method	2.648	0.106
Strain* Solvent	0.494	0.841
Administration Method Solvent	0.237	0.792
Strain* Administration Method Solvent	0.979	0.450

Appendix IV: Similarity Report

University of Eldoret

Certificate of Plagiarism Check for Synopsis

Author Name	Salbei T. Christine
Course of Study	Type here...
Name of Guide	Type here...
Department	Type here...
Acceptable Maximum Limit	Type here...
Submitted By	titustoo@uoeld.ac.ke
Paper Title	EFFECTS OF Acacia polyacantha CRUDE BARK EXTRACTS ADMINISTRATION IN MICE INFECTED WITH Leishmania donovani.
Similarity	14%
Paper ID	949513
Submission Date	2023-09-04 13:51:34

Signature of Student

University of Eldoret
21
UNIVERSITY LIBRARIAN
P. O. Box 1125, ELDORET-30100

Signature of Guide

Head of the Department

* This report has been generated by DrillBit Anti-Plagiarism Software