THERAPEUTIC POTENTIAL AND TOXICITY LEVELS OF *SOLANUM ACULEASTRUM* DUNAL. PLANT EXTRACTS AGAINST *LEISHMANIA MAJOR* INFECTION IN BALB/C MICE

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DECLARATION

I declare that this thesis is my original work and that to the best of my knowledge it has not been submitted for a degree or other awards in any other university.

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DEDICATION

With love and appreciation,

*I dedicate this thesis to my dad and mum**,***

Mr. Yuvenalis Laban Chuma

and

Mrs. Dorcah Mokeira Laban

For having loved me first into life, constantly nurturing, challenging and calling me forth to realize my potential to this great achievement.

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ABBREVIATIONS AND ACRONYMS

ACUC	Animal Care and Use Committee
AIDS	Acquired Immunodeficiency Syndrome
AI	Association Index
ANOVA	Analysis of Variance
CBRD	Centre for Biotechnology Research and Development
CI	Confidence Interval for mean
CCR/KEMRI	Center for Clinical Research in Kenya Medical Research Institute
CL	Cutaneous Leishmaniasis
DCL	Diffuse Cutaneous Leishmaniasis
DDRP	Drug Discovery Research Program
DMSO	Dimethyl Sulfoxide
DNDi	Drugs for Neglected Diseases Initiative
ELISA	Enzyme Linked Immunosorbent Assay
FCS	Fetal Calf Serum
FBS	Fetal Bovine Serum
HIV	Human Immunodeficiency Virus
HPLC	High Pressure Liquid Chromatography
IDRI	Infectious Diseases Research Institute
IM	Intramuscular Injection
IPR	Institute of Primate Research
IP	Intraperitoneal
IV	Intravenous Infusion
IR	Infection Rate
KEMRI	Kenya Medical Research Institute

LCL	Localized Cutaneous Leishmaniasis
LDU	*Leishmania Donovani* Unit
M	Mean
MCL	Mucocutaneous Leishmaniasis
MEM	Minimum Essential Medium
MI	Multiplication Index
MIC	Minimum Inhibitory Concentration
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium Bromide
NLB	Nairobi Leishmania Bank
NTD	Neglected Tropical Disease
PBS	Phosphate Buffered Saline
RPMI	Roswell Park Memorial Institute
SIM-F	Schneider's Drosophila culture medium
SSG	Sodium Stibogluconate
TDR/WHO	Tropical Diseases Program of the World Health Organization
VL	Visceral Leishmaniasis
WHO	World Health Organization

ABSTRACT

Solanum acueastrum Dunal. plant (Solanaceae family), growing in most parts of tropical Africa down to South Africa, has been shown to have some chemotherapeutic value. The fruit and leaf of *S. aculeastrum* is used for treating various diseases like jigger wounds, swelling joints in fingers, bronchitis, enlarged spleen, rheumatism, gangrene, gonorrhea, and other inflammatory-related ailments. Scientific studies conducted in S. Africa have also validated that *S. aculeastrum* fruit extracts can be used for the treatment of breast cancer. The current study evaluated *S. acueastrum* leaf and fruit water and methanol extracts for possible antileishmanial activity in BALB/c mice and toxicity levels in vero cells (lineage of cells isolated from kidney epithelial cells of an African Green monkey). Air dried *S. aculeastrum* fruit and leaf were extracted in methanol and water and stored at 4^0C in air tight bottles. In *in vitro* bioassays, *L. major* parasites were exposed to varying concentrations of *S. aculeastrum* fruit and leaf extracts and the inhibitory concentration (IC_{50}) on the promastigotes, percentage infection rate of macrophages by amastigotes and the toxicological effect on vero cells determined. In *in vivo* bioassays, BALB/c mice were infected subcutaneously with 1×10^6 promastigotes and kept for 4 weeks to allow for disease establishment. Infected mice were treated with fruit and leaf methanol and water extracts, amphotericin B (AmB) drug, and sterile phosphate buffered saline (PBS) intraperitoneally at 100 μg/ml for 4 weeks. The mice were sacrificed by ether euthanasia and splenic impression smears made on slides, fixed in methanol, stained with Giemsa and parasites quantified. Fruit methanol extracts were most effective in inhibiting the growth of promastigotes with lowest IC_{50}78.62 μg/ml. Fruit water showed the best activity in inhibiting infection of macrophages by amastigotes. Fruit methanol were most toxic to health vero cells at Ld_{50} = 8.06 mg/ml while AmB antileishmanial control drug showed the highest toxicity of 1.25 mg/ml in killing 50% of vero cells. Analysis of variance computation indicated a statistically significant difference in lesion sizes between experimental and control mice groups (P = 0.0001). Based on splenic impression smears, it was evident that either water or methanol extracts significantly protected the mice against splenic parasites as compared to the control (P = 0.0001). Findings from this study demonstrate that *S. aculeastrum* extracts have potential anti-leishmanial activities and that the medicinal use of the plant also poses considerable health risks exhibited by its toxicity against dividing vero cells.

CHAPTER 1: INTRODUCTION

1.1 Background of the study

Leishmaniasis is a parasitic infection caused by obligate intracellular protozoan of the genus *Leishmania*. Natural transmission of *Leishmania* parasite is carried out by sand flies of the genus *Phlebotomus* (Old world) or *Lutzomyia* (New World). Leishmaniasis is considered endemic in 16 developed countries, and 72 developing countries in 5 continents: Africa, Asia, Europe, South and Central America. A total of 350 million people are at risk of infection, with 15 million clinical cases and 2 million annual infections (WHO, 2007a). There are three clinical forms of leishmaniases: cutaneous leishmaniasis (CL), mucocutaneous leishmaniasis (ML) and visceral leishmaniasis (VL). Cutaneous leishmaniasis is caused by infection with *L. tropica* and *L. braziliensis*. Cutaneous leishmaniasis caused by *L. tropica* is common along the shores of Mediterranean, throughout Middle East, Africa and parts of India. Cutaneous leishmaniasis caused by *L. braziliensis* is mainly confined to C. America and S. America. In Kenya, CL is endemic in Rift Valley, Eastern, Central and Western Provinces (Ngure *et al*., 2009).

In Kenya, in collaboration with other stake holders, the Institute of Primate Research has been conducting research in leishmaniasis since the late eighties. Their first publication on leishmaniases dates way back in 1987, when natural infection of *L. major* was reported in the African Green monkey also known as the vervet monkey (*Chlorocebus aethiops*) (Binhazim *et al.,* 1987). Over the ensuing years, this non human primate model has become well characterized and has been used extensively in *Leishmania* vaccine development studies (Gicheru *et al.,* 2001). The Vervet monkey has proved an invaluable model for vaccine development studies and adjuvant testing. In the IPR research facility, both murine model and non human primate model are currently in use complimented by an *in vitro* maintenance

system for establishing the *Leishmania* parasite lifecycle stages in the hope of understanding the parasite. In further studies on *Leishmania* drug development, a diminazine based drug "Trypan@" was evaluated for its potential as anti-leishmanial agent against *L. major* in BALB/c mice with encouraging results (Macharia *et al*., 2004). Currently, research at IPR is based on different formulation of diminazine based drugs with the hope of providing alternative drugs for treatment of leishmaniases and malaria.

Since 1980, the Kenya Medical Research Institute (KEMRI) has spearheaded research on leishmaniases in Kenya focusing on various aspects including characterization of *Leishmania* species, biology, and ecology of sand fly vectors, development of biological strategies for sandfly control, identification of animal reservoirs, diagnosis, new treatment strategies, new chemotherapeutic agents, and vaccine-related studies. Kenya Medical Research Institute, a founding partner of the Drugs for Neglected Disease Initiative (DNDi), whose overall aim is to address lack of new or improved drugs for neglected diseases (which include leishmaniases, malaria, trypanosomiasis and chagas disease) has made major contributions in leishmaniases research and control in Kenya and the eastern Africa region (Tonui, 2006).

Recent advances in herbal medicine have listed plants and natural derived products that have shown some level of antileishmanial activity. In the family Solanaceae, *Saracha punctata* has been shown to completely inhibit the growth of promastigote forms of *L. braziliensis*, *L. donovani* and *L. amazonensis* (Manuel and Luis, 2001). Several researchers have also reported the effect of both the crude extracts and isolated compounds of *S. aculeastrum* in various biological systems, as antitumor, antimicrobial, antihelminthic, antioxidant as well as molluscicidal agents (Nfi *et al*., 1999; Wanyonyi *et al*., 2003b; Koduru *et al*., 2006c). However, there has been no information which is available on the effects of *S. aculeastrum* on protozoan parasites. There was a need to look for other alternative drugs to supplement the

current leishmaniases chemotherapy. The aim of the study was to investigate the effect of *S. aculeastrum* plant as an anti-leishmanial agent in BALB/c mice as experimental model.

1.2 Statement of the problem

Infections caused by the genus *Leishmania* are major worldwide problems with high endemicity in developing countries (WHO, 2009). The situation has been aggravated by the fact that pentavalent antimonials, the drugs of choice for the treatment of leishmaniases are expensive, exhibit considerable toxicity, variable efficacy and recently, there is the emergence of antimony-resistant *Leishmania* strains (WHO, 2010). The control of leishmaniases remains a problem because no vaccines exist and the available chemotherapy still relies on the potentially toxic antimonials which cause serious side effects and require protracted treatments with painful injections under prolonged hospitalization (WHO, 2009; WHO, 2010).

In the family *Solanaceae,* a Bolivian plant species, *Saracha punctata* has been indicated to completely inhibit the growth of promastigote forms of *L. braziliensis, L. donovani* and *L. amazonensis* (Manuel and Luis, 2001). Different sub species of *S. aculeastrum* plants are found in many parts of Kenya including areas where *L. major* infection is common but there are no reports on the use of the plant as a medicinal herb against leishmaniasis. Since no new drug formulations are currently supplied to substitute the old drugs, if proved effective, *S. aculeastrum* can fill up this gap.

1.3 Justification of the study

Leishmaniases are among the health problems that have not received much attention in the developing countries simply because they are not as widespread as many other tropical diseases like malaria and HIV/AIDS. Leishmaniases are also considered neglected diseases, particularly in terms of new drug development pegged on its poor financial returns. Cutaneous leishmaniasis which forms the bulk of the infection has a self healing nature and as a result,

people do not seek medical attention despite the associated chronic suffering. Treatment of leishmaniases is hindered by misdiagnosis, high drug costs, drug resistance, severe drug toxicity and the requirement for prolonged parenteral administration (Davies *et al.,* 2003). Two oral drugs used are sitamaquine and miltefosine, which are also anticancers have been shown to have gastrointestinal toxicity and teratogenicity, and therefore cannot be administered to women of childbearing age unless contraception is taken (Jha *et al*., 2005; Croft *et al.*, 2005; Croft *et al*., 2006b).

It is essential that the new treatment options become truly accessible and available in endemic areas. It is anticipated that extracts from *S. aculeastrum* may solve this problem of the drug availability, and early treatment for those who wait for the self healing. The findings of this study are useful in providing new insights on the potential of plant extracts as chemotherapeutic agents in the control of the leishmaniases. The availability and accessibility of this drug will be made available in the health care system and to the policy makers.

1.4 Research questions

i. What is the effect *of S. aculeastrum* leaf and fruit methanol and water extracts on *Leishmania* major promastigotes and amastigotes?

ii. What is the toxicological effect *of S. aculeastrum* leaf and fruit methanol and water extracts on vero cells?

iii. What is the effect of *S. aculeastrum* leaf and fruit methanol and water extracts on cutaneous lesion sizes in BALB/c mice infected with *L. major?*

iv. What is the effect of *S. aculeastrum* leaf and fruit methanol and water extracts on splenic parasites in *L. major* infected BALB/c mice following intraperitoneal treatment?

1.5 Hypotheses

i. *Solanum aculeastrum* leaf and fruit methanol and water extracts have no effect on *L. major* promastigotes and amastigotes.

ii. There is no toxicological effect of *S. aculeastrum* leaf and fruit methanol and water extracts on vero cells.

iii. *Solanum aculeastrum* leaf and fruit methanol and water extracts have no effect on cutaneous lesion size of BALB/c mice infected with *L. major*.

iv. *Solanum aculeastrum* leaf and fruit methanol and water extracts have no effect on splenic parasites in *L. major* infected BALB/c mice following intraperitoneal treatment.

1.6 Objectives

1.6.1 General objective

To examine the therapeutic potential and toxicity levels of *S. aculeastrum* plant extracts against *L. major* infection in BALB/c mice.

1.6.2 Specific objectives

i. To determine the efficacy of the leaf and fruit methanol and water extracts on amastigotes and promastigotes.

ii. To determine the toxicological effect of *S. aculeastrum* leaf and fruit methanol and water extracts on vero cells.

iii. To determine the efficacy of *S. aculeastrum* leaf and fruit methanol and water extracts in the treatment of cutaneous leishmaniasis in BALB/c mice.

iv. To determine the splenic parasites in *L. major* infected BALB/c mice following intraperitoneal treatment with *S. aculeastrum* leaf and fruit methanol and water extracts.

CHAPTER 2: LITERATURE REVIEW

2.1 Leishmaniases

Leishmaniases is a general term used for vector-borne parasitic diseases which are transmitted by sandflies and caused by obligate intracellular protozoa of the genus *Leishmania*. Human infection is caused by about 21 of 30 species that infect mammals. These include the *L. donovani* complex with 3 species (*L. donovani*, *L. infantum*, and *L. chagasi*); the *L. mexicana* complex with 3 main species (*L. mexicana*, *L. amazonensis*, and *L. venezuelensis*); *L. tropica*; *L. major*; *L. aethiopica*; and the subgenus *Viannia* with 4 main species (*L. (V.) braziliensis*, *L. (V.) guyanensis*, *L. (V.) panamensis*, and *L. (V.) peruviana*). The different species are morphologically indistinguishable, but they can be differentiated by isoenzyme analysis, molecular methods, or monoclonal antibodies (WHO/TDR, 2004b; Vandana, 2009).

2.2 Parasite morphology

2.2.1 Promastigotes stage

Promastigotes stage of *Leishmania* exists in the sandfly. They are flagellated and spindle-like in shape and measure 15-20 µm in length and 1.5-3.5µm in width. The amastigotes transform to promastigotes in posterior midgut of sandfly within hours of bite while the promastigotes transform to amastigotes inside macrophages. In this form, nucleus is situated at the center and kinetoplast transversely towards the anterior end. Promastigotes also exhibit a single and delicate flagellum 15-28 µm in length. These promastigotes are morphologically similar to those grown in culture (Bari, 2012).

2.2.2 Amastigote stage

The amastigote form exits in the macrophages of reticuloendothelial system of vertebrates such as spleen, liver, bone-marrow and lymph node. They are ovoid and non-flagellated form

of *Leishmania* which are 3-8 µm in length. The centrally located round/oval nucleus and adjacent but smaller round/rod shaped kinetoplast are distinguishable. The flagellum is not functional in amastigotes and does not extend beyond the cell body; however, there is 'Flagellar pocket' which serves as a site of endocytosis and exocytosis. The cytoplasm contains mitochondria, neutral red vacuoles and basophilic, and volutin granules containing RNA. The organism is surrounded by a double membrane below which is a row of 130-200 hollow fibrils (Bari, 2012).

2.3 Mode of transmission

All species are transmitted by small blood-sucking sandflies, notably *Phlebotomus* species in the Old World (Middle-East and Africa) and *Lutzomyia* species in the New World (Central and South America). Infections are confined to tropical and sub tropical areas (Convit *et al.*, 2005). Only the females feed on blood. Amastigotes ingested during feeding transform in the midgut or hindgut of insect vectors into promastigotes which multiply by binary fission. The parasites migrate forward to the foregut and proboscis where some become swept away by saliva into the bite site when the fly feeds and are transmitted via bite to the tissues of vertebrate hosts (Bari and Rahman, 2008). The main reservoir hosts for *Leishmania* are domestic animals (for example dogs, cats and horses), peridomestic animals (for example mice and rats) and wild animals (for example rodents, hyraxes, sloths, bats, oppsumes, kangaroos, wolves and foxes) (Bates, 2007; WHO, 2008).

2.4 Pathogenesis and life cycle of leishmaniasis

Leishmaniases are transmitted as *Leishmania* infected female sandflies take blood from healthy people (Figure 2.1). Two transmission ways are possible: zoonotic which happens when the parasite is transferred from infected non-human reservoir host to healthy individuals (accidental hosts) or anthroponotic where humans are the sole reservoir hosts (Annonymous, 2006). Congenital, parenteral and sexual transmissions have also been suspected (Gilles, 1999;

Sundar *et al*., 2007). Once the sandfly has been infected, the amastigote migrates to the alimentary canal of the insect where it attaches to local epidermal cells. The parasites mature and differentiate into motile promastigotes and move to the pharynx and/or the proboscis of the sandfly (Paredes *et al*., 2003). Upon a subsequent blood meal, the infective promastigotes are injected into the blood stream of the victim. Promastigotes that reach the puncture wound are phagocytized by macrophages and other types of mononuclear phagocytic cells and reside in the parasitophorous vacuole (Hazra, 2001).

Progmastigotes transform back in these cells into the tissue stage of the parasite (amastigotes), which replicate by simple division and are released back into the blood stream, proceeding to infect other mononuclear phagocytic cells and macrophages within the skin, viscera and blood tissues; thus disseminating the disease locally or systemically based on distinct *Leishmania* species involved. Parasite, host, and other factors dictate whether the infection becomes symptomatic and whether cutaneous or visceral leishmaniasis results. The parasites are equipped to evade the digestive enzymes present in the vacuole and they also have a membrane bound molecule, lipophosphoglycan that ensures intracellular survival of the parasite (Sacks and Perkins, 1985).

The incubation period within a human host can range from 10 days up to 2 years. In visceral leishmaniasis the parasite is able to multiply without suppression from the host's immune system leading to complications affecting the spleen, liver, intestinal mucosa, bone marrow and lymph nodes. Hematopoiesis is depressed leading to pancytopenia, prothrombin becomes depleted (leading to bleeding complications when combined with thrombocytopenia), and decreased albumin predisposes the patient to edema. The host's immune system becomes severely compromised as infection spreads and, if left untreated, most patients will die within

2-3 years often from secondary infections such as tuberculosis, pneumonia or dysentery (Mehlhorn, 2004).

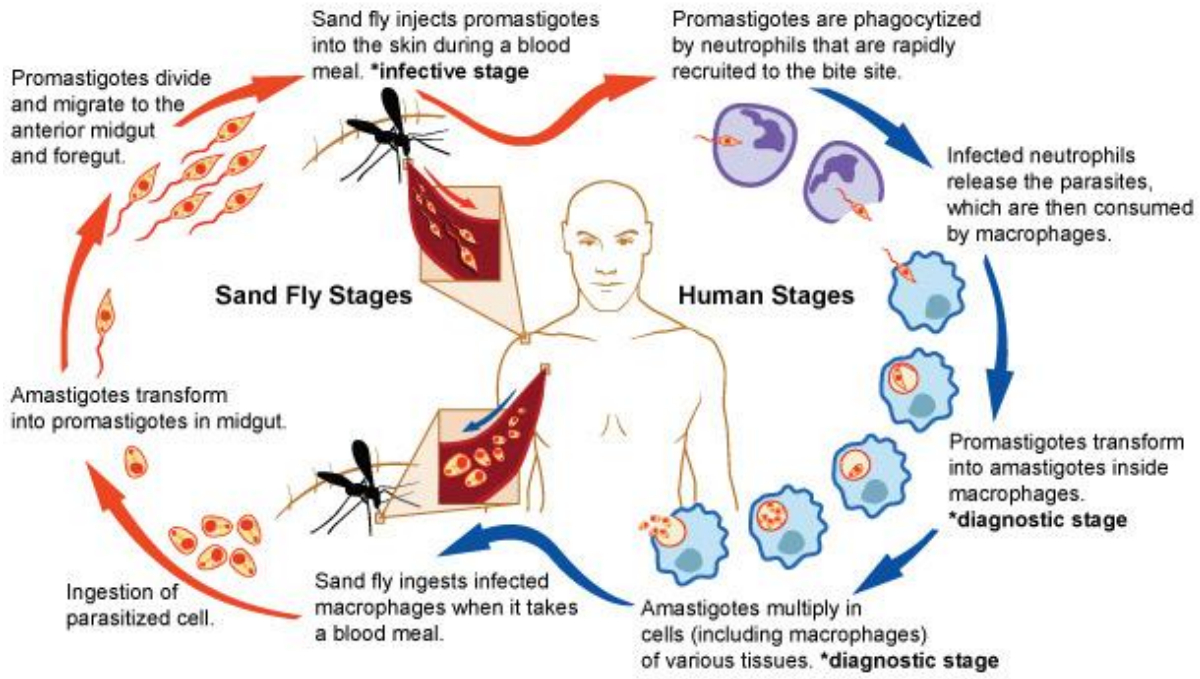

Figure 2.1: Life cycle showing the sand fly and human stages of leishmaniasis. (Retrieved from http://www.niaid.nih.gov/SiteCollectionImages/topics/leishmaniasis/leishDiagram1.jpg. Accessed on 22/05/2014).

2.5 Leishmaniases disease clinical syndromes

Leishmaniases form a range of complex diseases. Depending on strain (s) of the parasite involved in pathogenesis and the immune response established by the host, it can cause clinical symptoms which can range from mild self-limiting cutaneous lesion to fatal visceral diseases. The diseases occur in three forms namely: Visceral leishmaniasis (VL), mucocutaneous leishmaniasis (MCL) and cutaneous leishmanisis (CL) (Bari, 2012).

2.5.1 Cutaneous leishmaniasis

Cutaneous leishmaniasis has many local synonyms such as Tropical sore, Oriental sore, Aleppo sore or Baghdad sore. Cutaneous leishmaniasis is marked by the appearance of itchy

sores, lesions (ulcers) and swelling of the lymph nodes on arms, nose, legs or face and lower limbs (Vandana, 2009). Over time, the sores develop a red raised border and a depression in the middle, and on healing, the sores leave a scar. Although this form is self healing, it can create a serious disability and permanent depression (Hazra, 2001). A severe, non-healing and relapsing disease called recidivans can also occur due to *L. tropica* (Sundar *et al*., 2007). Different *Leishmania* species cause Old World versus New World (American) cutaneous leishmaniasis. In the Old World (the Eastern Hemisphere), the etiologic agents include *Leishmania tropica*, *L. major*, and *L. aethiopica*. The main species in the New World (the Western Hemisphere) are either in the *L. mexicana* species complex (*L. mexicana*, *L. amazonensis*, and *L. venezuelensis*) or the subgenus *Viannia* (*L. [V.] braziliensis*, *L. [V.] guyanensis*, *L. [V.] panamensis*, and *L. [V.] peruviana*). The *Viannia* subgenus is also referred to as the *L. (V.) braziliensis* species complex **(**Bari and Rahman, 2008; Vandana, 2009; Craig, 2011).

2.5.1.1 Localized cutaneous leishmaniasis (LCL)

Localized cutaneous leishmaniasis (LCL) in the old world is caused by *L. major*, *L. tropica*, *L. infantum*, and *L. aethiopica*, primarily by the former two. With minor differences, the clinical lesions produced by all these species are similar (Bari, 2012). Solitary or multiple subcutaneous nodules (smooth, soft, mobile and 0.5-2.0 cm in size) may occur proximal to the skin lesions and usually along the axis between the skin lesions and the regional lymph nodes (Convit *et al.*, 2005). Healing usually takes place in 2-6 months in *L. major* infection and 8-12 months in *L. tropica*. The healing is almost always with a scar that is typically atrophic, hyperpigmented, and irregular. Localized cutaneous leishmaniasis can be differentiated as acute (< 6 months duration), chronic (> 6 months duration) and recidivans. Acute LCL can be further divided into three varieties: nodular-including papules and plaques, nodulo-ulcerative,

and ulcerative. These forms may appear singly or as an overlap picture (Bari and Rahman, 2008).

2.5.1.2 Diffuse (Anergic) cutaneous leishmaniasis

Diffuse cutaneous leishmaniasis (DCL) is a polar form of cutaneous leishmaniasis characterized by disseminated nodules, an abundance of parasites throughout the course of the disease, the absence of parasite-specific cell-mediated immune response, and a poor response to antimonial treatment (WHO, 2009). Diffuse cutaneous leishmaniasis may be caused by *L. amazonensis*, *L. mexicana* and *L. pifanoi* in the new world and by *L. aethiopica* in the Old World, but the disease caused by *L. amazonensis* in Central and South America is more common (Desjeux, 2004). Diffuse cutaneous leishmaniasis related to *L. major* has also been reported. The disease usually begins with an initial primary lesion, which disseminates to involve other areas of the skin. The lesions are often scattered over limbs, buttocks, and face. These progress slowly and become chronic; the improvement following treatment is gradual and relapse is a rule. There is no systemic involvement. The histology shows macrophages loaded with amastigotes (Bari and Rahman, 2008; Craig, 2011; Bari, 2012).

2.5.2 Mucocutaneous leishmaniasis (MCL)

Mucocutaneous leishmaniasis (Espundia) produces extensive disfiguring destruction of mucosa and cartilage of the mouth, nose, ear and pharynx leading to a severe mutilation of the face. The disease may get worse if secondary bacterial or fungal infection occurs (Davies *et al*., 2003; Sundar *et al*., 2007). Mucocutaneous leishmaniasis occurs due to *L. aethiopica* in the Old World and *L. brasiliensis* complex in the New World. Cases due to *L. donovani*, *L. major* and *L. infantum* have also been reported (Paredes *et al*., 2003).

2.5.3 Visceral leishmaniasis (VL)

Visceral leishmaniasis (Kala-azar) is a systemic disease typically caused by *L. donovani* and *L. infantum* (in Europe, Middle East, India, and Africa) and *L. chagasi* (in Central and South America) (Bari, 2012). In visceral leishmaniasis, the parasites are able to invade the spleen and liver, which causes a prolonged splenomegaly. The abdominal swelling of these organs is the most prominent clinical feature of this form of disease. The disease is characterized by irregular fever, weight loss, enlargement of spleen and liver, anemia, leukopenia, skin pigmentation and weakness associated with parasite invasion of spleen, liver, bone-marrow, lungs, oral mucosa, larynx, oesophagus, stomach, small intestine, skin and sex cells (Gilles, 1999; Craig, 2011). The disease may be asymptomatic and self-resolving but usually becomes chronic and is usually fatal if left untreated (Chan-Bacab and Pena-Rodriguez, 2001). Rare VL cases due to *L. tropica* and *L. mexicana* have also been reported (Chappuis *et al.*, 2007).

Some visceral leishmaniasis patients may develop dermal manifestation (depigmented macule, papules, nodules, or infiltrative plaques) called post kal-azar dermal leishmaniasis (PKDL) during or shortly after treatment of VL caused by *L. donovani*. It may also develop on individuals who do not have previous history of VL or its treatment. Post kal-azar dermal leishmaniasis (PKDL) is common in India, Bangladesh and China, less frequent in Africa (regularly in Sudan and Kenya) and rare in the New World (Gilles, 1999). The greatest danger with visceral leishmaniasis is its impact on the immune system. After prolonged infection, the host's immune system deteriorates because these parasites directly attack the reticuloendothelial system. As such, people with visceral leishmaniasis become increasingly susceptible to other infections (Craig, 2011; Mishra *et al*., 2011).

2.6 Epidemiology of leishmaniasis

2.6.1 Global situation

Leishmaniasis is considered endemic in 88 tropical and subtropical countries in Central America, South America, Southern Europe, Asia (excluding South-East Asia), Middle East, and Africa (East and North Africa, with some cases elsewhere) (WHO, 2008). A total of 350 million people are at risk, 82% of which are from developing countries, there are 2 million new cases of leishmaniases annually while 14 million are directly affected by the disease (Ashford, 2000; WHO, 2007b).

There are an estimated 500,000 new cases of visceral leishmaniasis and more than 50,000 deaths from the disease each year. The majority of cases occur in Bangladesh, India, Nepal, Sudan, Ethiopia and Brazil (Chappuis *et al.*, 2007). Cutaneous leishmaniasis is the most common form whose new occurrence is estimated to be 1500,000 each year (Shepherd *et al.*, 2008; Craig, 2011) Cutaneous leishmaniasis caused by *L. tropica* is common along the shores of Mediterranean, throughout Middle East, Africa and parts of India while cutaneous leishmaniasis caused by *L. braziliensis* is mainly confined to C. America and S. America (Tonui, 2006; WHO, 2010) (Table 2.1, 2.2, 2.3). The risk factors for the disease are socioeconomic factors (primitive housing, low hygienic situation and houses near vector breading sites), population movements (for agricultural developments, military activity etc.) and environmental changes. Emergence of HIV/AIDS has also increased reactivity of asymptomatic or previously healed leishmaniasis cases. Currently, *Leishmania* - HIV co-infections have been reported from at least 35 countries (WHO, 2007b).

Table 2.1. Human pathogenic species of cutaneous leishmaniasis in the world, their clinical manifestations and geographical distribution (Bates, 2007; Bari and Rahman, 2008; Mishra *et al*., 2011)

***Leishmania* species**	**Vertebr-ate hosts**	**Clinical pathology**	**Insect vector**	**Geographical distribution**
L. aethiopica	Humans, hyraxes	diffuse/dry cutaneous	*Phlebotomus longipes*	East Africa (Ethiopia, Kenya, Namibia) , Yemen
L. killicki		LCL	*Phlebotomus pedifer*	North Africa: Tunisia, Algeria, Lybia
L. tropica minor	humans, dogs, rodents	dry cutaneous	*Phlebotomus Sergenti, papatasi*	China, C. and N. Africa, C., W, SE Asia, Iran, Iraq, India, S. France, Italy, Indo-Pak subcontinent, Mediterranean regions
L. tropica major	humans, dogs, rodents	rural form of CL. wet cutaneous, oriental sore	*Phlebotomus papatasi, dubosqi, salehi*	Western, Central Asia, India Middle East, Indo-Pak subcontinent, Mediterranean, NW China, Sahel of Africa, C., E., N., and W. Africa
L. peruviana	humans, dogs	uta, cutaneous	*Lutzomyia peruensis, verrucarum*	Peru, Argentina
L. mexicana mexicana	humans, rodents	chicleros ulcer, LCL, DCL	*Lutzomyia olmeca olmeca*	Mexico, USA, the Amazon basin and Venezuela, C., N., and S. America
L. mexicana amazonens-is	humans, rodents	diffuse, cutaneous	*L. flaviscutella-ta*	Dominican Republic, C. and S. America
L. mexicana pifanoi	humans, rodents	Chronic cutaneous, MCL	*Lutzomyia flaviscutella-ta*	Venezuela, S. America
L.m. garnhami	humans, rodents, opossum	Cutaneous	Lutzomyia *townsendi*	South America,Venezuela
L.m. venzuelensis	humans, rodents	Cutaneous	Lutzomyia *olmeca bicor*	Northern South America, Venezuela

L. braziliensis (*L. viannia*) (i). *Leishmania brasiliensis brasiliensis*	humans, rodents, sloths, kinkajo-us	espundia, MLC, LCL	Lutzomyia *wellcomei, complexus, carrerai*	Mexico, Brazil, East Andes, Guyana, Venezuela, Panama, Columbia and Peru, C. and S. America
(ii). *Leishmania brasiliensis guyanensis*			*Lutzomyia umbratilis*	S. America Guyana, French Guyana, Surinam, Brazil
(iii). *L. brasiliensis panamensis*		LCL, Mucosal	*Lutzomyia trapidoi*	Costa Rica, Panama, Colombia, Northern S. America and Southern C. America, Ecuador

Table 2.2. Human pathogenic species of visceral leishmaniasis in the world, their clinical manifestations and geographical distribution (Bates, 2007; Bari and Rahman, 2008; Mishra *et al*., 2011)

Leishmania species	**Vertebrate hosts**	**Clinical pathology**	**Insect vector**	**Geographical distribution**
L. donovani donovani	humans, dogs, foxes	LCL, kala azar, dum-dum fever,	*Phlebotomus Argentipes, orientalis, martini*	C. and SE Asia, China, Mediterranean, E. Africa, Bangladesh, S. America, Indian subcontinent
L. donovani infantum	humans, dogs	Infantile (babies or very young children), visceral, LCL	*Phlebotomus ariasi, perniciosus*	Mediterranean basin, Europe, N. Africa, South, Central and West Asia
L. donovani chagasi	humans, foxes, cats, dogs	New World visceral, sometimes cutaneous		S. America, Argentina, Brazil, Texas, Caribbean, Venezuela and Colombia, isolated cases throughout S. and C. America

2.6.2 Distribution and vectors of leishmaniases in Kenya

The occurrence of VL is rare compared to CL, and the two diseases do not tend to overlap geographically. Baringo district is the only foci reported where both VL and CL are known to occur in Kenya (Tonui, 2006). Visceral leishmaniasis is found predominantly in the arid,

low-lying areas of Turkana, Baringo, West Pokot, Kitui, Meru, Machakos, Tana River, Mandera and Wajir. Visceral leishmaniasis in Kenya is caused by *L. donovani* (Jamjoom *et al*., 2004). The main sand fly vector is *P. martini*, which breeds in termite hills, animal burrows, tree holes and house walls (Mutinga *et al*., 1994). Between 2000 and 2010, Médecins Sans Frontières diagnosed and treated 4,831 patients with visceral leishmaniasis (VL) in the Pokot region straddling the border between Uganda and Kenya. Males between 5 and 14 years of age were the most affected group (Mueller *et al*., 2014). At Amudat Hospital, there were 4,428 admissions for VL investigation between 2000 and 2006, including 2,461 primary VL, 56 relapses, 4 PKDL, and 1,907 cases ultimately viewed as non-VL cases. In Kacheliba, 2,301 cases of leishmaniasis were diagnosed between 2006 and 2010, including 2,144 primary VL, 81 relapses, and 75 PKDL (Mueller *et al*., 2014).

Cutaneous leishmaniasis occurs over a wider range of environmental conditions, from semi-arid lowlands to high plateaus. The aetiological agents for CL include *L. major* which has been reported in Baringo and Kitui; *L. tropica* in Laikipia, Samburu, Isiolo, Nakuru and Nyandarua Districts while *L. aethiopica* has been reported in Mt. Elgon areas. *Phlebotomous duboscqi* and *P. guggisbergi* have been shown to be the vectors of *L. major* and *L. tropica* respectively, while *P. pediffer*, *P. longipes* and *P. elgonensis* have been implicated as vectors of *L. aethiopica* (Tonui, 2006; Ngure *et al*., 2009) (Table 2.3).

Table 2.3. Cutaneous and visceral leishmaniases epidemiology in Kenya (Basimike and Mutinga, 1997; Tonui, 2006)

Leishmaniases type	**Province**	**Current foci (district)**	**Parasite**	**Confirmed vector**
Cutaneous	Rift Valley	Baringo Laikipia Samburu Nakuru Naivasha Gilgil	*L.major* *L. tropica*	*P. duboscqi* *P. guggisbergi* *P. papatasi*
	Eastern	Kitui Isiolo	*L.major* *L. tropica*	*P. duboscqi* *P. guggisbergi*
	Central	Nyandarua	*L. tropica*	*P. duboscqi* *P. guggisbergi*
	Western	Bungoma	*L. aethiopica*	*P. pediffer* *P. longipes* *P. elgonensis*
Visceral	Rift Valley	Turkana Baringo West Pokot	*L. donovani*	*P. martini*
	Eastern	Kitui Meru Machakos	*L. donovani*	*P. martini*
	North Eastern	Mandera Wajir	*L. donovani*	*P. martini (confirmed),* *P. celiae (suspected),* *P. vansomeranae (suspected)*

2.7 Chemotherapy of leishmaniases

Chemotherapy of leishmaniasis is aimed at minimizing morbidity and mortality associated with the disease. It is primarily based on toxic antimony compounds but when this agents lack efficacy, other second-line drugs are used. Several other drugs are also in trial. The most common therapies for leishmaniasis are pentavalent antimonials: meglumine antimoniate (Glucantime), pentamidine (aromatic diamidine) and sodium stibogluconate (SbV) (Pentostam). Others that have been used recently include: liposomal amphotericin B, (AmBisome), paromomycin (Humatin) and miltefosine (Miltex) (Blum *et al.,* 2004).

Cutaneous leishmaniasis which forms the bulk of the infection has a self healing nature. Nevertheless, the disease is associated with great human suffering and loss of life where it occurs. Even though lesions may heal eventually in absence of treatment, the process is often long and produces significant scarring, thereby justifying the use of chemotherapy for example topical application of paromomycin (Blum *et al.*, 2004). The goal of treating cutaneous leishmaniasis is to eradicate amastigotes as well as reducing the size of the lesions so that healing will take place with minimal scarring (Dumonteil *et al.*, 2001).

2.8 Current chemotherapy for leishmaniases

2.8.1 Drugs approved for treatment of leishmaniases

2.8.1.1 Pentavalent antimonials

Pentavalent antimonials are the first anti-leishmanial agents introduced in 1945. Two of these drugs, Pentostam® and Glucantime® contain antimony-sugar polymeric compounds of sodium stibogluconate and meglumine antimonite as main constituents respectively. They are first-line drugs used for VL and CL cases except those caused by *L. aethopica* or MCL due to *L. braziliensis* and DCL due to *L. amazonensis* (Chan, 1998; Laurence *et al*., 2006).

Antimonials are thought to inhibit trypanothione reductase, a key enzyme that mediates redox balance in trypanosomes. Both drugs are parenterally administered at 20 mg/kg/day for 20-28 days. They are not orally active (Donald, 2003; Singh, 2006). Intralesional administration was also found to be effective (Paris *et al.*, 1993). Long term parenteral administration, variable efficacy against VL, CL and MCL cases and adverse effects such as pain at the site of injection, stiff joints, gastrointestinal problems, cardiotoxicity, hepatic and renal insufficiency are some of the factors that limit their wide - spread use (Croft *et al*., 2006a and b). Resistance has also become an increasing clinical problem in India and Bangladesh. Resistance

mechanisms include decreased activation, increased trypanothione levels and increased efflux of the drug (Croft and Coombs, 2003; Croft *et al*., 2006a).

2.8.1.2 Pentamidine

Pentamidine is a cationic aromatic diamine discovered in 1937 as a hypoglycemic compound. It is also toxic to large number of protozoa and some fungi. Pentamidine isethionate (Pentacarinat®) has been used as a second-line drug for treatment of VL, LCL and DCL since 1952 (Gilles, 1999; Croft and Coombs, 2003; Miguel, 2007). Pentamidine interferes with ribosomal aggregation and biosynthesis of DNA, protein and many enzymes by reacting with negatively charged biomolecules such as membrane phospholipids, enzymes, RNA, and DNA (Laurence *et al.,* 2006). The drug is administered intravenously at doses 2-4 mg/kg daily or every other day for 12-15 days. Higher doses (>4 mg/kg/day) can lead to hypotension, tachycardia, stomach upset, anaemia, severe headaches, skin eruptions, abnormal liver function, hypoglycemia, and renal dysfunction. Resistance was reported in India due to increased drug efflux (Jiang, 2002; Donald, 2003; Croft *et al*., 2006a and b).

2.8.1.3 Amphotericin B

Amphotericin B is a polyene macrolide antibiotic extensively used as antifungal. It is also in use for treatment of leishmaniasis as second-line drug since 1960s. Amphotericin B deoxycholate (Fungizone®) is used for treatment of VL, CL and MCL unresponsive to other drugs. AmBisome® was also approved in 1997 for treatment of VL. Other novel amphotericin B formulations are also being considered (Maes *et al*., 2004b). *Leishmania* has membrane sterol similar to fungi and the drug then forms complexes preferentially to these sterols leading to porous parasite membrane permeable to entry and loss of ions. The drug is administered intravenously at a dose of 0.5-1.0 mg/kg daily or every other day for 8 weeks (as Fungizone®) or 3 mg/kg/day for 21 days (as AmBisome®) (Donald, 2003; Croft *et al*., 2006b). Major adverse reaction to amphotericin B is nephrotoxicity. Acute side effects such as fever, chills,

muscle spasms, vomiting, headache, hypotension, and anaphylaxis occur less in lipid complex preparations. Resistance to amphotericin B is emerging probably associated with changes in *Leishmania* membrane sterols (Croft and Coombs, 2003; Croft *et al*., 2006a and b).

2.8.1.4 Paromomycin

Paromomycin (Aminosidine®, Humatin®) is an aminoglycoside used in oral treatment of amoebiasis, cryptosporidiosis and giardiasis and topically for trichomoniasis. Its anti-leishmanial activity was identified in 1960s. The drug was approved in India in 2006 for treatment of VL (WHO, 2007a). Topical paromomycin formulations were also found effective for CL cases (Croft and Coombs, 2003; Croft *et al*., 2006a and b). Paromomycin inhibit protein synthesis binding to polysomes, and causing misreading and premature termination of translation. It is administered parenterally at a dose of 16 - 20 mg/kg daily for 21 days or 12 - 20 mg/kg/d combined with antimonials for 20 days. Toxic effects such as nephrotoxicity and ototoxicity are associated with exceeding the recommended doses and concomitant use of other agents with similar toxicities. Resistance cases were repoted on *L. aethiopica* isolates and *L.donovani* promastigotes associated with decreased drug uptake (Croft *et al*., 2006a; Sundar *et al*., 2007).

2.8.1.5 Miltefosine

Miltefosine (Impavido®) is an alkylphosphocholine analog originally developed as an anticancer agent. Its anti-leishmanial activity was first discovered in mid-1980s. The drug is registered in India, Colombia, Germany, Nepal and Bangladesh for treatment of VL. It is being considered for CL cases, immunocompromized patients and canine leishmaniasis. The mechanism of action of miltefosine is not fully understood. However, experimental results suggest drug's effect on mammalian protein C - kinase, phosphatidylcholine biosynthesis, lipid metabolism, cell signaling and calcium homeostasis (Smith *et al.*, 2000). Miltefosine is given at a daily oral dose of 50-200 mg/Kg for 28 days (Donald, 2003). Major adverse

reactions include gastroinstinal distress, increased hepatic transaminases and serum creatinine levels, teratogenicity and blood hemolysis (parentral) (Croft and Coombs, 2003). Resistance has also been reported related to decreased drug uptake and efflux mechanisms (Croft *et al.*, 2005).

2.8.2 Drugs in clinical trial for treatment of leishmaniases

2.8.2.1 Azoles

The imidazoles and triazoles are well known oral antifungal agents that are well tolerated (Laurence *et al*., 2006). They also have antileishmanial activity against certain species as they inhibit demethylase, a key enzyme in the sterol biosynthesis pathway, thereby interfering with *Leishmanial* cell membrane biosynthesis. Among them, Fluconazole have been used against *L. major* in Old World and Ketoconazole in the New World against *L. panamensis* and *L. mexicana*. Itrakonazole have been used in Old and New World, but a low efficacy has been demonstrated. Posaconazole has shown activity against experimental *L. amazonensis* infection, but has not been evaluated yet in clinical trials (Antony and Lukasz, 2005; Piscopo and Mallia, 2006).

2.8.2.2 Allopurinol

The antileishmanial activity of the purine analogue allopurinol was identified over 30 years ago. Because it had oral bioavailability and it was widely used for other clinical indications, the drug was investigated in clinical trials for CL and VL. However, the results were disappointing. Allopurinol is used as a substrate by various enzymes of the purine salvage pathway of trypanosomatids, and it is selectively incorporated into nucleic acid in the parasite. In recent years, allopurinol was considered as part of a maintenance therapy for canine leishmaniasis (Piscopo and Mallia, 2006).

2.8.2.3 Sitamaquine

Sitamaquine (Lepidine®) is an orally active 8-aminoquinoline analogue (8-aminoquinoline (8-[6-(diethylamino) hexyl] amino)-6-methoxy-4-methylquinoline) known as WR 6026. This new primaquine was originally developed by Walter Reed Army Institute of Research (United States) for malaria. Animal studies showed very encouraging results against VL; although in clinical trials it did not show high efficacy after treatment for 28 days (Jha *et al*., 2005; Croft *et al*., 2006b).

2.8.2.4 Imiquimod

Imiquimod (Aldara®) is an imidazoquinoline derivative which is an ingredient of the topical cream used for treatment of actinic keratosis, superficial basal cell carcinoma and genital warts since 1997. It stimulates a local immune response at the site of application by inducing production of cytokines such as interferon-α, tumor necrosis factor-α and interleukins (IL-1β, IL- 6 and IL-8). In combination with antimonials, the drug showed 90% cure rate on CL cases refractory to antimonials alone (Antony and Lukasz, 2005; Firooz *et al.,* 2006). Iminoquimod, an imidazoquinoline, is the ingredient of Aldara (TM) cream used for the treatment of genital warts. This drug has been shown to induce nitric oxide production in macrophages and it was effective *in vitro* against *L. donovani* (Bilu and Sauder, 2003).

2.8.2.5 Combined Therapy

After increasing unresponsiveness to most of the monotherapeutic regimens, combination therapy has found new scope in the treatment of leishmaniasis. The combination of antileishmanial drugs could reduce the potential toxic side effects and prevent drug resistance (Jha *et al*., 2005). Several works have shown that some drugs increase their antileishmanial effect in conjunction. Paromomycin have been used extensively in Sudan in combination with sodium stibogluconate for the treatment of VL in a period of 17 days. The superiority of this combination has been demonstrated in several studies (Firooz *et al.,* 2006; Mishra *et al*.,

2011). Combined chemotherapy against VL in Kenya was evaluated using oral allopurinol (21 mg/Kg, three times a day for 30 days) with endovenous pentostam (20mg/Kg once a day). The therapy was efficient, but relapses were found in the first month after treatment. This clinical evidence demonstrated the superiority of the combination therapy and can be a hope to develop new formulations (Jha *et al.*, 2005; Firooz *et al.*, 2006).

2.9 Challenges posed by the current therapy

Many times the leishmaniasis infection is misdiagnosed and even where diagnoses are made, treatment is expensive and associated with numerous side effects (Bates, 2007). The standard treatments, outlined above, make effective treatment delivery exceedingly difficult. First, current therapy is toxic and treatment is difficult to administer. The standard antimonials must be administered by intravenous, intramuscular, or by intralesion injections depending on disease progression; more systemic disease calls for IV or IM while localized disease may be treated with intralesion injections. Because antimony is rapidly excreted from the body, treatment with these agents must be repeated daily, especially for individuals suffering from visceral disease. Therefore, these therapy have proven unsatisfactory, a poor clinical agent and a barrier to treatment (Mebrahtu *et al.*, 1987). All these challenges suggest that there is a need and urge to search for alternative natural plant products therapy for the treatment of leishmaniasis (Berman and Lee, 1984).

2.10 Why leishmaniases are difficult to treat

Although it's not known whether antimonials work directly by acting against the parasite in the amastigote stage or indirectly by activating macrophages or other components of the immune system, one recent study done on cutaneous leishmaniasis suggests that antimonials work by inducing high levels of certain cytokines to positively affect macrophage population. However, *Leishmania* parasites are also trying their best to negatively affect the function of macrophages (Ashford, 2000). Metacyclic promastigotes, the infective stage transmitted by the

sand fly, manipulates the complement system to get silently taken up by macrophages (no oxidative burst takes place as a result of crosslinking). While in the macrophages, the parasites down-regulate the expression of MHC class II molecules on the surface of the macrophage; thus, hiding from the immune system and being free to replicate (Sacks and Perkins, 1985). *Leishmania* parasites weaken components of the immune system, making immuno-competent communication, and attempts to counter the immuno-boostering effects of the standard treatments difficult (Convit *et al.*, 2005).

2.11 Herbal anti-leishmanial agents

In recent years, there has been growing interest in alternative therapies and use of natural products, especially those derived from plants. The use of plant based medicine for healing and prophylaxis is as ancient and universal as medicine itself. Recently, the Tropical Diseases Program of the World Health Organization, TDR/WHO with the Drug Discovery Research Program, DDRP has considered a priority, the pharmacological investigation of plants (Monzote, 2009). The revival of interest in the use and importance of African medicinal plants by the WHO and many developing countries has led to intensified efforts on the documentation of ethnomedical data of medicinal plants, since most traditional healers keep no records and their information is passed on, mainly verbally, from generation to generation (Kokwaro, 2009; Mishra *et al*., 2011). Research has been geared towards finding scientific evidence for the claims as to the therapeutic efficacy of African herbs by traditional healers. Most of the published and unpublished written ethnomedicine data with valuable and complementary information are scattered in many documents, some of which are not easily available (Mourice *et al*., 1999; Zhai, 1999).

Extensive studies of activity of natural products against *Leishmania* have reported some of the plant natural products which have been discovered and proved to exhibit leishmanicidal activity. They include: *Morinda lucida* (Rubiaceae), *Stephania dinklagei* (Menispermaceae),

Saracha punctata (Solanaceae), and *Holarrhena curtisii* (Apocynaceae), (Manuel and Luis, 2001; Monzote, 2009). However, the efficacy, dosage, safety and active principles of most herbal preparations have not been well established (Kokwaro, 2009).

2.12 Investigated herbal plants in Kenya against leishmaniasis

In Kenya, about 80% of the population relies on traditional herbal medicine for treatment of various ailments (Kokwaro, 2009). The Kenyan flora is also estimated to contain between 4,000 and 6,000 species of higher plants of which about 12% are endemic. Therefore, scientific evaluation of these plants may provide modern medicine with lead compounds for the development of new drugs (Kokwaro, 2009).

Recent research undertaken in KEMRI has led to discovery of plant-based products with antileishmanial activities. For instance, studies have shown that leaf, root and stem extracts from *Ajuga remota* and *Trichilla emetica* have antileishmanial activity when tested against *L. donovani in vitro* and *in vivo* (Tonui, 2006). Some of the active compounds isolated from plants include iridoid glucosides, limonoids and terpenoids. Kenya Medical Research Institute (KEMRI) has also initiated studies aimed at testing extracts/compounds from medicinal plants used widely by the local people for treating splenomegaly related ailments in leishmaniasis endemic areas of Kenya. A long term goal of this research is to develop oral drugs for leishmaniases. These drugs have an advantage of reducing treatment-related socioeconomic difficulties (inadequate health care infrastructure and the long period of patient hospitalization) prevalent in the endemic areas (Tonui, 2006).

2.12.1 *Warburgia ugandensis* (Canellaceae)

Warburgia ugandensis Sprague, the East African greenheart (family Canellaceae) is one of the most highly utilized medicinal plants in tropical and subtropical Africa. It is rated as the second highest priority medicinal plant species in Kenya (Beentje, 1994). The dried bark of

the tree is commonly chewed and the juice swallowed as a remedy for stomach ache, constipation, toothache, venereal diseases, cough, fever, muscle pains, weak joints and general body pains. The leaf decoction baths are used as a cure for skin diseases while the bark, roots or leaves can be boiled in water and drunk to treat malaria, although this causes violent vomiting (Tonui, 2006; Ngure *et al*., 2009). *Warburgia ugandensis* which is known as "soket" in Tugen tribe is used by traditional healers to treat visceral leishmaniasis (Tonui, KEMRI, Nairobi, personal communication). The stem barks are taken orally in boiled water or soup. Previous studies on *W. ugandensis* have shown good antibacterial, antifungal as well as antiviral activity and trypanocidal effects (Madikane *et al*., 2007).

2.12.2 *Allium sativum*

Allium sativum, commonly known as garlic is a species of the onion family (Alliaceae). A bulb of garlic, the most commonly used part of the plant is divided into numerous fleshy sections called cloves, which are used as seeds for consumption and for medicinal purposes. *Allium sativum* has been found to be a powerful rubefacient, antitusive, diaphoretic and vermifuge agent (Wabwoba *et al*., 2010). When garlic extracts are injected intraperiotneally into infected BALB/c mice, the engulfment and destruction of amastigotes by the peritoneal macrophages is enhanced (Ghazanfari *et al*., 2006). Garlic extracts stimulate the production of interferon gamma (IFN-γ) and nitric oxide (NO) both of which enhance the killing of *Leishmania* parasites in experimental BALB/c mice (Gamboa-Leon *et al*., 2007). The main chemical constituent of garlic is the amino acid alliin, an alklyl derivative of cystein alkyl sulfoxide (Lutomski, 1987). Alliin becomes converted into various sulphur containing compounds most of which have a medicinal importance. Garlic extracts are commonly used in Europe and Asia for medicinal benefits in healing wounds (Lutomski, 1987).

2.12.3 *Aloe secundiflora*

Plants of the genus *Aloe* within the Family Xanthorrhoeaceae are known to have a variety of activities against insects and human and veterinary parasitic diseases (Mandal *et al.*, 2006). Some of the species that have been studied include *A. vera, A. turkanensis, A. ngongensis, A. fibrosa* and *A. secundiflora.* The efficacy of *Aloe vera* leaf exudates against promastigotes of *L. braziliensis* Vianna, *L. Mexicana* Biagi, *L. tropica*, *L. major* and *L. infantum* have been reported to show leishmanicidal activity (Dutta *et al*., 2007). The leaf exudates of *Aloe secundiflora* have been reported to be leishmanicidal. *Aloe secundiflora* contains several major groups of chemical compounds namely tannins, saponins, alkaloids, cardiac glycosides, flavonoids and terpenidoes (Omwenga *et al*., 2009).

2.13 Overview of the Solanaceae family

Solanaceae comprise a very important group of medicinal plants having multifarious uses. These plants belong to the family Solanaceae and genus *Solanum*. Solanaceae is commonly called the 'Brinjal family' (Denton and Nwangburuka, 2011). The Solanaceae family comprises of about 3000 to 4000 species of flowering plants, placed within about 90 genera, a cosmopolitan distribution (NHM, 2008). The family is also informally known as the nightshade or potato family. The family includes the *Datura stramonium* or Jimson weed, eggplant, mandrake, deadly nightshade or belladonna, *Capsicum* (paprika, chili pepper), *Solanum tuberosum* (potato), *Nicotiana tabacum* (tobacco), *Lycopersicon esculentum (*tomato), and petunia (Torkpo *et al*., 2006).

All of the Solanaceae family are toxic in some way, their toxins range from mild to very high (Grubben and Denton, 2004). Many of the plants are edible, while others are poisonous. Some have both edible and toxic parts, which are destroyed on cooking at high temperatures. For example Jimson Weed and mandrake are highly toxic to humans, yet when used correctly can have substantial medical benefits (Denton and Nwangburuka, 2011). Small amounts of Jimson

Weed or mandrake can reverse cholinergic poisoning, halt allergic reaction and can be used to relieve motion sickness. However, in large doses they can easily kill humans (Torkpo *et al*., 2006). Humans have also learned to use the toxicity of Chilli peppers to make pepper spray which is used as deterrent to both humans and animals. *Capsicum* is also used in medical creams and ointments (NHM, 2008).

2.13.1 Alkaloids

The Solanaceae are known for possessing a diverse range of alkaloids. As far as humans are concerned, these alkaloids can be desirable, toxic, or both, though they presumably evolved because they reduce the tendency of animals to eat the plants (Prakash and Jain 2011). One of the most important groups of these compounds is called the tropane alkaloids. Tropane alkaloids are also found in the *Datura*, *Mandragora*, and *Brugmansia* genera, as well as in *Cestum nocturnum*, *Withania somnifera*, *Lycopersicon esculentum*, *Solanum tuberosum* and many others in the Solanaceae family (Torkpo *et al*., 2006).

Chemically, the molecules of these compounds have a characteristic bicyclic structure and include atropine, scopolamine, and hyoscyamine. Pharmacologically, they are the most powerful known anticholinergics in existence, meaning they inhibit the neurological signals transmitted by the endogenous neurotransmitter, acetylcholine. Symptoms of overdose may include mouth dryness, dilated pupils, ataxia, urinary retention, hallucinations, convulsions, coma, and death. Despite the extreme toxicity of the tropanes, they are important drugs when administered in appropriate (and extremely small) dosages (Zubair *et al*., 2011).

One of the most famous alkaloids from the Solanaceae family is nicotine. Like the tropanes, its pharmacology acts on cholinergic neurons, but with the opposite effect (it is an agonist as opposed to an antagonist). Nicotine occurs naturally in the *Nicotiana* or tobacco genus. Capsaicin is structurally unrelated to nicotine or the tropanes and is found in the genus

Capsicum, which includes chili peppers such as tabasco peppers and habaneros (Torkpo *et al*., 2006). The compound is not appreciably toxic to animals. However, it stimulates specific pain receptors in most mammals, those which sense heat, in the oral mucosa as well as many other epithelial tissues. This causes a sensation of burning not unlike an actual heat or chemical burn. It is used in high concentration as a deterrent in pepper sprays, and sought after for many culinary dishes for its "spiciness" (Denton and Nwangburuka, 2011).

Members of Solanaceae provide a variety of culinary, medicinal, and ornamental values. In terms of culinary value, the most important species of this family for the global diet is the potato or *Solanum tuberosum*, whose carbohydrate-rich tubers have been a staple food in many times and places, and which is one of the most grown crops today (Torkpo *et al*., 2006). In many genera, the fruits are the desirable item, for example, tomatoes, tomatillos, eggplants, uchuva, and peppers, such as chili pepper.

Medicinally, as well as in terms of poisoning and psychotropic effects, members of Solanaceae have been prized for their alkaloid content and used throughout history. Important drug plants include deadly nightshade or belladonna *(Atropa belladonna)*, jimson weed *(Datura stramonium)*, henbane *(Hyoscyamus niger)*, and tobacco *(Nicotiana tabacum)* (Torkpo *et al*., 2006). Mandrake, the common name for members of the plant genus *Mandragora*, contains deliriant hallucinogenic tropane alkaloids such as hyoscyamine and the roots sometimes contain bifurcations causing them to resemble human figures, leading to this plant being used in magic rituals and neopagan religions such as Wicca (NHM, 2008).

As ornamental plants, the genera *Petunia*, *Schizanthus* (butterfly flower), *Salpiglossis* (painted or velvet tongue), and *Browallia* (Bush violet, Jamaican forget-me-not) are well-known (Grubben *et al*., 2004). While very popular, some people experience sensitivity or allergy-like symptoms in response to nightshade plants (Denton and Nwangburuka, 2011).

2.14 Medicinal *Solanums*

2.14.1 *Solanum anguivi* Lam. (Poison berry)

Solanum anguivi Lam. is found throughout the tropics, in plains and at low elevations. It is much branched, very prickly under shrub and 0.3-1.5 m in height (Samodien *et al*., 2003). Its roots are useful in vitiated conditions of *vata* and *kapha*, odontalgia, dyspepsia, flatulence, colic, verminosis, diarrhoea, pruritus, leprosy, skin diseases, strangury, cough, asthma, bronchitis, amenorrhoea, dysmenorrhoea, fever, cardiac disorders and vomiting (Kent, 2000). Roots are bitter, acrid, astringent, thermogenic, anodyne, digestive, carminative, anthelmintic, stomachic, constipating, resolvent, demulcent, depurative, diuretic, expectorant, aphrodisiac, emmenagogue, febrifuge and cardiotonic (Graves, 1996).

2.14.2 *Solanum dulcamara* Linn. (Bittersweet, Bitter night shade)

Solanum dulcamara Linn. is found in tropical situations in India and Sikkim (Kent, 2000). The plant is rich in alkaloidal glycosides like solamarine, tomatidenol, solasodine and soladulcine. The berry and twig are alterative, antisyphilitic, diaphoretic, resolvent, narcotic, diuretic, antirheumatic and used in liver disorders and psoriasis (Gibson, 2000).

2.14.3 *Solanum melongena* Linn. (Brinjal, Egg plant)

Solanum melongena Linn. is mainly cultivated as a vegetable throughout the tropics and subtropics (Samodien *et al*., 2003). It is an erect or suffrutescent, herbaceous, armed or unarmed perennial shrub. Leaves are simple, large, entire and lobed. Flowers are blue, in clusters of 2-5. Fruits are large, white, yellow or dark purple berries of different shapes capped with thick persistent calyx. Seeds are many, yellow or cream and discoid (Graves, 1996). The roots, leaves and unripe fruits are useful in cholera, bronchitis, asthma, odontalgia and fever treatments. The roots are laxative, analgesic and cardiotonic. Leaves are sialagogue, narcotic and antiherpetic (Graves, 1996).

2.14.4 Solanum nigrum Linn. (Black night shade)

Solanum nigrum Linn. is seen wild throughout India (Jainu and Devi, 2005). The whole plant is useful in vitiated conditions of *tridosha*, rheumatalgia, swellings, cough, asthma, bronchitis, wounds, ulcers, flatulence, dyspepsia, strangury, hepatomegaly, otalgia, hiccough, opthalmopathy, vomiting, cardiopathy, leprosy, skin diseases, fever, splenomegaly, haemarrhoids, nephropathy, dropsy and general debility (Ncube *et al*., 2008). The plant is bitter, acrid, emollient, antiseptic, antiinflammatory, expectorant, anodyne, vulnerary, digestive, laxative, diuretic, cardiotonic, depurative, diaphoretic, febrifuge, rejuvenating, sedative, alterant and tonic (Prakash and Jain, 2011).

2.14.5 *Solanum surattense* Burm. (Yellow-berried nightshade)

Solanum surattense Burm. is found throughout India and Pakistan in dry situations as weed on roadsides and wastelands (Graves, 1996). The whole plant is useful in vitiated conditions of *vata* and *kapha*, helminthiasis, dental caries, inflammations, flatulence, constipation, dyspepsia, anorexia, leprosy, skin diseases, hypertension, fever, cough, asthma, bronchitis, hiccough, lumbago, haemorrhoids and epilepsy. Fruits contain solasonine, solamargine and solasodine (Priyadarsini *et al*., 2010).

2.14.6 *Solanum viarum* Dunal.

Solanum viarum Dunal. is widely distributed in Khasi, Jaintia and Naga hills of Assam and Manipur up to 2000 m and in Sikkim, West Bengal, Orissa and in the Niligiris. *Solanum viarum* plant and berries contain solasonine (which on hydrolysis yields solasodine), solamargine, khasianine, nantigenin, solasodine, diosgenin and saponin-solakhasianin. *Solanum viarum* plant is spasmolytic and CNS active. The berry is a source of solasodine used in the synthesis of corticosteroidal hormones (Nayak and Patil, 2001).

2.15 *Solanum aculeastrum* Dunal

Solanum aculeastrum (Solanaceae) commonly known as *Omotobo* by the Abagusii community of Kenya is also known as soda apple or goat bitter apple or poison apple. The species name *aculeastrum* refers to the thorns that adorn most parts of the shrub (Appendix V). It is a shrub or small tree native to tropical Africa down to South Africa, in a wide range of soil, terrain and climatic conditions (Aboyade *et al.,* 2009; Aboyade *et al.,* 2010). *Solanum aculeastrum* occurs naturally in grassland, woodland and in forest margins, and also in disturbed places. It has been recorded from gentle to steep slopes and on all aspects, on various soil types e.g. sandy soils, reddish brown clay-loam and brown sandy loam (Watt and Breyer-Brandwijk, 1962).

It is a highly-branched perennial plant that grows and reaches 1–5 m high, usually heavily armed with large, sharp, brown, straight to recurved, broad-based and laterally compressed prickles up to 15 mm long. The leaves are ovate, up to 15 cm long and 13 cm diameter, with lobed margins and a downy underside. The petals are white to pale violet surrounding the ovary, the flower also has a bitter, sour smell (Appendix II). With maturity, the fruits are about 4 to 5 cm in diameter, egg-shaped, and yellow. The surface of the fruit is bumpy - almost thorny, reminiscent of the branches (Appendix IV and V). This plant is a close relative of the tomato. The berries are lemon shaped becoming greenish-yellow when ripe (Watt and Breyer-Brandwijk, 1962; Wanyonyi *et al*., 2003a).

Among the plants that African farmers may be happy to have, growing in their fence rows is *Solanum aculeastrum* Dunal. Sharp and heavy thorns on durable woody branches discourage trespassers, man and beast alike. It is also rarely consumed by goat or cattle, in part because of those sharp thorns. Therefore, in rural areas the goat-apple is used as a hedge and living barrier for containing livestock. It is sometimes used as a windbreak. But mostly, mammalian

herbivores avoid it because the foliage is laced with very bitter and toxic alkaloids. The Soda apple traditionally is often used as a soap replacement, as it is high in saponin. The fruits, both mature and immature, contain the poisonous alkaloid solanine (Hutchings *et al.*, 1996).

2.15.1 Chemistry of *Solanum aculeastrum* plant

The main constituents of *Solanum aculeastrum* which have been implicated in the pharmacological activities of this plant are steroidal alkaloids. These include β-solamarine, solamargine, solasonine, solasodine, solaculine, tomatine and tomatidine (Wanyonyi *et al*., 2002; Koduru *et al*., 2007a). Steroidal alkaloids have been the target of pharmacological investigations because of their structural similarities with anabolic steroids, steroidal hormones and corticosteroids (Gurib-Fakim, 2006). The leaves and berries of *S. aculeastrum* have also been found to contain mainly straight- chain aliphatic hydrocarbons (Koduru *et al*., 2006a). These authors reported the isolation of volatile compounds such as alkanes (64.5%), phthalic acid (14.9%) and fatty acid esters (7.7%) from the berries, while the leaves possessed 17.5% alkanes, 17% aldehydes, 15.2% aromatic hydrocarbons, 11.8% phthalic acid, 2.3% methyl salicylate and 6.5% terpenoids (Koduru *et al*., 2006a).

2.15.1.1 Steroid alkaloids

Steroidal alkaloids are most common in the Solanaceae, Apocynaceae, and Liliaceae. The *S. aculeastrum* fruits, both mature and immature, contain the poisonous alkaloid solanine (Ncube *et al*., 2008). Steroid alkaloids have a fairly complex nitrogen containing nucleus. The *Solanum* type example is solanidine. Solanum-type alkaloids are found in plants in the form of glycosides of alkaloids. Alkaloids are toxic, they are where poisons are found, humans and all classes of livestock are susceptible to poisoning by solunum- type glycoalkaloids (Zubair *et al*., 2011). These alkaloids are not destroyed by cooking or drying at high temperatures (Drewes and van- Staden, 1995).

Many plants in the Solanaceae accumulate steroidal alkaloids based on a C_{27} cholestane skeleton, e.g. solasodine and tomatidine. These are essentially nitrogen analogues of steroidal saponins (Gurib-Fakim, 2006). In contrast to the oxygen analogues, these compounds all have the same stereochemistry at C-25 (methyl always equatorial), but C-22 isomers do exist, as solasodine and tomatidine exemplify. They are usually present as glycosides which have surface activity and haemolytic properties as do the saponins, but these compounds are also toxic if ingested (Koduru *et al*., 2006a). Solasonine from *Solanum* species and tomatine from tomato (*Lycopersicon esculente*) are typical examples of such glycosides. As with the sapogenins, this group of steroidal alkaloids is derived from cholesterol, with appropriate side-chain modifications during the sequence (Wanyonyi *et al*., 2002; Koduru *et al*., 2007a).

2.15.1.2 Solanidine

Solanidine is a poisonous steroidal alkaloid chemical compound that occurs in plants of the Solanaceae family, such as potato and *Solanum americanum*. Human ingestion of solanidine also occurs via the consumption of the glycoalkaloids, α-solanine and α-chaconine, present in potatoes (Nikolic *et al*., 2003). The sugar portion of these glycoalkaloids hydrolyses in the body, leaving the solanidine portion. Solanidine occurs in the blood serum of normal healthy people who eat potato, and serum solanidine levels fall markedly once potato consumption ceases (Drewes and van- Staden, 1995). Solanidine from food is also stored in the human body for prolonged periods of time, and it's been suggested by researchers that it could be released during times of metabolic stress with the potential for deleterious consequences (Agnew and Agnew, 1994). Solanidine is responsible for neuromuscular syndromes via cholinesterase inhibition. Solanidine is a very important precursor for the synthesis of hormones and some pharmacologically active compounds (Nikolic *et al*., 2003).

2.15.1.3 Saponin

Saponins are a class of compounds found in many plant species including the solanaceae family. The soda apple is often used as a soap replacement, as it is high in saponin (Birch *et al.,* 2001).These compounds are high molecular weight glycosylated plant secondary metabolites, which are soap like foam produced when shaken in aqueous solution. Structurally they are composed of one or more hydrophilic glycosides moietes combined with a lipophillic triterpine derivative (Agnew and Agnew, 1994; Price *et al*., 1987). Saponin has been shown to have leishmanicidal effects against *L. major* promastigotes (Mori *et al*., 2007).

2.15.2 Reports on *Solanum aculeastrum* chemotherapy

The fruits of *S. aculeastrum* are used fresh, dried, boiled, or charred (ashed) for the treatment of jigger wounds, toothaches, gonorrhea, bronchitis, rheumatism and in ringworm in cattle and horses (Watt and Breyer-Brandwijk, 1962; Agnew and Agnew, 1994; Wanyonyi *et al*., 2003a). A decoction of the root bark is used in Kenya for sexually transmitted bacterial diseases including gonorrhea while the juice from the berries are used for treatment of jigger infestations as well as acne (Kokwaro, 2009). Ethnobotanical survey revealed that the berries are used in the treatment of breast cancer (Koduru *et al*., 2007a and b).

Methanol and aqueous extracts of the berries have been shown to have moderate antimicrobial activity against *Staphylococcus aureus, Escherichia coli, pseudomonas aureginosa* and *Bacillus subtilis* bacteria (Wanyonyi *et al*., 2003b). The aqueous and methanol extracts of *S. aculeastrum* berries have also been shown to completely inhibit the growth of various fungi for example *Aspergillus flavus, Aspergillus niger, Fusarium oxysporum, Penicillium notatum* (Koduru *et al*., 2006d). The aqueous extracts of the berries have been shown to have more potency against *Biomphalaria pfeifferri* snails than the root bark. Alkaloid-rich fractions obtained from the crude methanolic berry extracts showed 100% mortality in all the fractions at concentrations ranging from 20 to 40 ppm (Wanyonyi *et al*., 2003b). Studies of the

anthelminthic property of *S. aculeastrum* roots and leaves along with other plants used for ethnoveterinary medicine in Cameroon showed that they possess 34.4% anthelminthic activity compared with *Khaya anthoteca* (55.8%) and *Vernonia amygdalina* (52.4%) (Nfi *et al*., 1999).

The aqueous extracts of *S. aculeastrum* berries have been shown to possess higher antioxidant activity on free radicals than the methanol extracts. The higher antioxidant activity of the aqueous extract was attributed to the substantial amount of polar constituents present in the *S. aculeastrum* plant (Koduru *et al*., 2006b). The antiproliferative activity of the aqueous, methanol and acetone extracts of the leaves and berries of *S. aculeastrum* against HeLa, MCF-7 and HT-29 human cancer cell lines has shown that the extracts of the leaves possess no anticancer property while the methanol and aqueous extracts of the berries possess the highest antiproliferative activity (Koduru *et al*., 2006e). The effect of combined compounds of solasodine and tomatidine, steroidal alkaloids isolated from the berries of *S. aculeastrum* has also indicated pronounced activity against human cancer cells rather than the individual compounds (Koduru *et al*., 2007a).

Preliminary pharmacological studies on the extracts of *S. aculeastrum* berries and leaves revealed that the plant parts possess antimicrobial, antitumor and antioxidant activities. The steroidal alkaloids in the berries have also been reported to be active against schistomiasis and cancer (Drews and Van Staden, 1995; Wanyonyi *et al*., 2003b; Koduru *et al*., 2006 d and e; Koduru *et al*., 2007 a and b). However, the effect of the plant on *Leishmania* parasites has not been documented. In view of its use in the local treatment of some painful inflammatory conditions, the present study sought to establish the chemotherapeutic activity of *S. aculeastrum* fresh methanolic and water extracts *in vitro* and *in vivo* on *L. major* infection.

CHAPTER 3: MATERIALS AND METHODS

3.1 Study site

This study was done in the *Leishmania* laboratory at the Centre for Biotechnology Research and Development of the Kenya Medical Research Institute (KEMRI), Nairobi County, Kenya.

3.2 Plant material

Fresh ripe berries and leaves of *S. aculeastrum* Dunal plant naturally occurring in the wild and in homestead hedges of Nyakwerema village of Nyamira County, Kenya, were picked in the months of June-July in the year 2012. They were collected, when the fruits are most mature and ripe between 6 am and 9 am time of the day. This is traditionally believed to be the stage when the fruits are most effective on ailments. The plant was positively identified at the Chiromo Campus herbarium, University of Nairobi. A voucher specimen LLT 2012/001 was prepared and submitted to the herbarium of the same university for preservation as reference material.

3.3 Preparation of plant material and extraction

Dried berry and leaf extracts were prepared according to the method described by Koduru *et al*. (2006a). Briefly, 500 g fresh ripe berries were chopped into small pieces. The chopped fruits and leaves were air dried under shade in a dark room, constantly monitored and weighed till they attained constant weight. The dried parts of the plant were ground using an electric blender and soaked in 1000 ml of fresh absolute methanol on a mechanical shaker with intermittent shaking for 48 hours. The solvents were filtered with a Buchner funnel and Watman No 1 filter paper and the filtrates concentrated to dryness under reduced pressure at 40^0C using a rotary evaporator (Harborne, 1994) to the required doses for further investigation. The final dry extract was stored at 4^0C in air tight bottles to be used for bioassay investigations.

For water extraction, 500 ml of cold water was added to 100 g of ground berry powder and 900 ml cold water was added to 50 g of ground leaves. The two mixtures were filtered into conical flasks and placed on a water bath for one hour and 30 minutes. The filtrates were separately coated in acetone and dry ice and fixed on freeze drier machine for 48 hours. The final dry extract was stored at 4^0C in air tight bottles to be used for further investigations (Koduru *et al.,* 2006a).

3.4 Preparation of the test extracts

Preparation of drug extractions was done as described by Dorin *et al.* (2001). Briefly, stock solutions of the fractions was made in culture media for antileishmanial assay and re-sterilized by passing through 0.22 μm micro-filters under sterile conditions in a laminar flow hood. If the extracts were insoluble in water or culture media, they were first dissolved in 1% dimethyl sulfoxide, DMSO to avoid solvent carry over. The final concentration did not exceed 1%. This is because DMSO is very toxic thus can affect cells. All the prepared drugs were stored at 4°C and retrieved only during use.

3.5 Culture of *Leishmania major* parasites

Metacyclic promastigotes of *L. major* (Strain IDUB/KE/94=NLB-144) used were obtained from an infected mouse at KEMRI by cutting tissues from an infected footpad and the parasites were maintained as previously described by Titus *et al*. (2010). Briefly, parasites were cultured in RPMI 1640 and liquid phase Schneider's Drosophila medium supplemented with 25% foetal bovine serum (FBS), penicillin G (100 U/ml), and streptomycin (100 μg/ml) at 25 ^{0}C. The tubes were checked frequently for any changes in cultivated parasites (Habibi *et al.,* 2008). The promastigotes were washed and purified 3 times in phosphate buffered saline (PBS) by centrifugation at 1500 rpm for 15 minutes. Stationary-phase metacyclic promastigotes of *L. major* ($1x10^6$ cells/ml) was isolated from 5 to 7 day-old cultures. These parasites were used for *in vitro* and *in vivo* experiments.

3.6 Determination of *in vitro* anti-leishmanial activity of *Solanum aculeastrum* extracts

In these bioassays, different concentrations of leaf and fruit extracts were tested against *L. major* parasites to determine the minimum inhibition concentration (MIC) and viability of the promastigotes and amastigotes after exposure to the plant extracts.

3.6.1 Evaluation of minimum inhibitory concentration (MIC)

The MIC was determined and evaluated as described by Wabwoba *et al.* (2010). Briefly, *L. major* promastigotes at 10^6 parasites/ml density were seeded into each well and maintained in SIM culture. Test plant extracts (fruit water, leaf water and fruit methanol) were introduced into each well in duplicate and serially diluted from 3000 µg/ml - 500 µg/ml while AmB reference drug was serially diluted from 500 µg/ml to 62.5 µg/ml. Cell growth was evaluated daily by assessment of visibility and turbidity in order to evaluate MIC. After establishing several inhibitory concentrations, the lowest concentration of the test samples which prevented the growth of any number of *Leishmania* parasites *in vitro* was considered as the MIC.

3.6.2 Anti-promastigote assay

Anti-promastigote assay was carried out as described by Ngure *et al*. (2009). Briefly, *L. major* promastigotes were cultured in RPMI 1640 media overlaid with 2 ml of Schneider's *Drosophila* insect medium supplemented with 20% foetal bovine serum, 100 mg/ml streptomycin and 100 U/ml penicillin-G , and 5-fluorocytosine arabinoside (Kimber *et al*., 1981). Promastigotes were incubated in 24-well plates. After five days of cultivation, aliquots of parasites were transferred and seeded in 96-well micro-titer plate at a density of 10^6 parasites/ml. The parasites were incubated at 27°C for 24 h and 200 µl of the highest concentration (2000 µg/ml) of each of the test samples was added in the first well in duplicate and serial dilution carried out to 62.5 µg/ml.

The experimental plates were incubated further at 27°C for 48 h. The controls used were promastigotes with no drugs and medium alone (no drugs/extract and no cells). A positive control with antimony 20 mg ml^{-1} was used in same plate. Blank wells contained MTT and Schneider's Insect Medium (SIM). Ten microlitres of MTT reagent (final concentration of 0.5 mg/ml) was added into each well and the cells incubated for 2 - 4 hours (Mosmann, 1983). The medium together with MTT was aspirated off from the wells, 100 µl of DMSO (0.2%) added and the plates shaken for 5 min. Absorbance was measured for each well at 562 nm using a micro-titer plate reader. The IC_{50} values were computed by entering the optical density readings in a preset MS excel template. Percentage promastigote viability was calculated using the formula of Mosmann (1983) at each concentration:

$$\text{Viability (\%)} = \frac{\text{(Average absorbance in duplicate drug wells – average blank wells)} \times 100}{\text{Average absorbance of control wells}}$$

3.6.3 Anti-amastigote assay

The anti-amastigote assay was carried out as described by Delorenzi *et al.* (2001). Briefly, peritoneal macrophages were obtained from 4 BALB/c mice. The body surface was disinfected with 70% ethanol, the skin torn dorso-ventrally to expose the peritoneum and a sterile syringe used to inject 10 ml of phosphate buffered saline (PBS) into the peritoneum. After 24 hours mouse peritoneal macrophages were harvested by withdrawing the fluid into sterile centrifuge tubes. The cell suspension was centrifuged at 2000 rpm for 10 minutes and the pellet re-suspended in 5 ml of complete RPMI 1640 medium. Macrophages were adsorbed in 24-well plates and allowed to adhere for 4 hours at 37^0C in 5% CO_2. Non-adherent cells were washed with phosphate buffered saline (PBS), and the macrophages incubated overnight in RPMI 1640 media. Adherent macrophages were incubated with *L. major* promastigotes at a parasite/macrophage ratio of 6:1 at 37^0C in 5% CO_2 for 4 hrs and free promastigotes removed

by extensive washing with PBS. The cultures were further incubated in RPMI 1640 medium for 24 h.

Treatment of the infected macrophages with the extracts and drugs was done once which were serially diluted from 1000 µg/ml to 125 µg/ml. Amphtericin B standard drugs were used as positive control for parasite growth inhibition. The medium and drug were replenished daily for 3 days. After 5 days the monolayers were washed with PBS at 37^0C, fixed in methanol and stained with 10% Giemsa solution. The number of amastigotes was determined by counting at least 100 infected and uninfected macrophages in duplicate cultures, and results expressed as infection rate (IR) and multiplication index (MI) (Berman and Lee, 1984).

% IR = No. of infected macrophages per 100 macrophages

3.7 Evaluation of the toxicological effects of *S. aculeastrum* extracts

Vero cells were cultured and maintained in Minimum Essential Medium MEM, supplemented with 10% FBS as described by Ngure *et al*. (2009). Briefly, vero cells were cultured at 37^0C in 5% CO_2, harvested by trypsinization, pooled in a 50 ml vial and 100 µl cell suspensions ($1x10^5$ cell/ml) put into duplicate wells of rows A-H in a 96-well micro-titer plate for one sample. The cells were incubated at 37^0C in 5% CO_2 for 24 h to attach, the medium aspirated off and 150 µl of the highest concentration (2000 µg/ml) of each of the test samples were put in wells and serially diluted to 62.5 µg/ml. The experimental plates were incubated further at 37^0C for 48 hrs. A positive control with antimony 20 mg ml^{-1} (amphtericin B) were used in same plate. The negative controls used were cells with no drugs, and medium alone (no drugs and no cells), while blank wells containing MTT and Schneider's Insect Medium (SIM). Then 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium Bromide (MTT) reagent (10 µg/ml) was added into each well and the cells incubated for 2 - 4 hrs until a purple precipitate was observed under a microscope. The medium together with MTT were aspirated off from the

wells, after which 100 µl of DMSO was added and the plates shaken for 5 minutes. Absorbance was measured for each well at 562 nm using a micro-titer plate reader. Cell viability (%) was calculated at each concentration as described by Huq *et al*. (2004) using the formula:

Cell viability % = (Average absorbance in duplicate drug wells – average blank wells) x 100
Average absorbance in control wells

3.8 Determination of *in vivo* anti-leishmanial activity of *S. aculeastrum* extracts

3.8.1 Experimental animals

Sixty male BALB/c mice, 6-9 weeks of age and weighing 20-30 g were obtained from the animal house facility of KEMRI. The 60 BALB/c mice were required for this experiment because they were enough for all the test drug groups and would have given sufficient and reliable data of the parameters tested. The age and weith of the BALB/c mice was preferred because of susceptibility and ability to withstand any toxicity they are subjected to. The animals were housed in clean cages placed in a well ventilated house for the whole period of the study. The mice were also allowed free access to food and water *ad libitum*. The study using mice was done following approval from the ethical committee on Animal Care and Use of KEMRI.

3.8.2 Infection and treatment of BALB/c mice

The procedure was carried out as described by Wabwoba *et al*. (2010). Briefly, the left hind footpads of BALB/c mice were inoculated with 1 x 10^6 stationary phase culture of *L. major* promastigotes in 40 µl phosphate buffered saline (PBS) intradermally. Lesion development was monitored by measuring the thickness of the infected footpad using a Vernier caliper weekly. The mice were randomized into 6 cages each comprising of 10 mice. Group I was treated with fruit water extract, Group II was treated with leaf water extract, Group III was

treated with fruit methanol extract, group IV was treated with methanol leaf extract, Group V received PBS, Group VI received amphotericin B. Treatment with the extract, PBS and the standard drugs started four weeks after infection, and groups of mice were treated for four weeks by intraperitoneal injections of 100 μg/ml. To compare the drug effects, the lesion size were measured and expressed as the difference in thickness between the infected and the uninfected contralateral footpad.

3.8.3 Quantification of *L. major* parasites in the spleen of BALB/c mice

At the end of the experiment (after 28 days) all mice were sacrificed by euthanizing them using ether. At necropsy, the spleens of all the treated animals were weighed and impression smears made as described by Chulay and Bryceson (1983). Briefly, the impression smears were fixed in methanol and stained with Giemsa. The slides were examined under a compound microscope and an oil immersion lens to enumerate the number of amastigotes per 1000 host nuclei. At least, 100 microscopic fields were examined before an imprint was reported negative (Titus *et al*., 2010). The relative and total numbers of parasites in the spleen, named Leishman-Donovan Units (LDU) and the total Leishman-Donovan Units respectively were calculated according to the formula by Bradley and Kirkley (1977).

LDU = No. of parasites/1000 host nuclei

3.14 Statistical analysis

Data were analysed using GraphPad InStat software programme utilizing one way analysis of variance (ANOVA) and Tukey-Kramer test statistic as *Post hoc* where applicable. Descriptive statistics and transformation of data were used where appropriate. A *P* value of less than or equal to 0.05 was considered significant. Data were organized into line graphs, tables or bar graphs.

CHAPTER 4: RESULTS

4.1 Activity of *Solanum aculeastrum* extracts and amphotericin B on *Leishmania major* promastigotes

Following incubation of promastigotes with various drug concentrations, a downward decrease in parasite numbers with increase in drug concentration was observed. Fruit water compound demonstrated a complete killing of promastigotes at the highest concentration of 3000 µg/ml while 1×10^6 promastigotes similar to baseline numbers was observed at 2500 µg/ml concentration. It was noted that fruit water compound did not reduce the number of promastigotes to below 1×10^6 in any of the drug concentrations ranging between 2000 µg/ml and 62.5 µg/ml in which a three fold increase was observed between 1000 µg/ml and 62.5 µg/ml concentrations where promastigotes increased from the starting number of 1×10^6 to between 6×10^6 - 2.0×10^7 parasites. Leaf water compound did not completely kill promastigotes in any of the concentrations as there were still 2×10^6 parasites observed at 3000 µg/ml, 5×10^6 at 2500 µg/ml and 7×10^6 at 2000 µg/ml. A two times increase of promastigotes was observed from concentrations ranging between 1500 µg/ml and 62.5 µg/ml where promastigotes increased from the starting number of 1×10^6 to between 1.0×10^7 - 2.8×10^7 parasites.

Fruit methanol compound demonstrated a complete killing of promastigotes at 3000 µg/ml and 2500 µg/ml concentrations while 1×10^6 promastigotes were observed at 2000 µg/ml concentration. A two times increase of promastigotes was observed from 1500 µg/ml to 62.5 µg/ml concentrations where promastigotes increased from the starting number of 1×10^6 to between 2×10^6 - 1.4×10^7 parasites. Leaf methanol compound indicated 1×10^6 promastigotes at 3000 µg/ml while an increase in number of promstigotes was observed in drug concentrations ranging between 2500 µg/ml and 62.5 µg/ml where promastigotes increased from the starting number of 1×10^6 to between 3×10^6 - 2.3×10^7 parasites.

Amongst the test compounds, fruit methanol was most effective in inhibiting the growth of promastigotes. Leaf water and leaf methanol were least effective in the control of promastigotes with leaf methanol showing more inhibitory strength than leaf water (Figure 4.1). Promastigotes increase in the control wells (without drug) ranged from 5.4 x 10^7 to 6.8 x 10^7 indicating at least a five times increase. However, the reference drug, AmB remained the most effective, killing all the promastigotes at all concentrations from 3000 µg/ml to 125 µg/ml. The last concentration 62.5 µg/ml of AmB did not kill promastigotes as there were 2 x 10^6 in wells incubated with this drug compound.

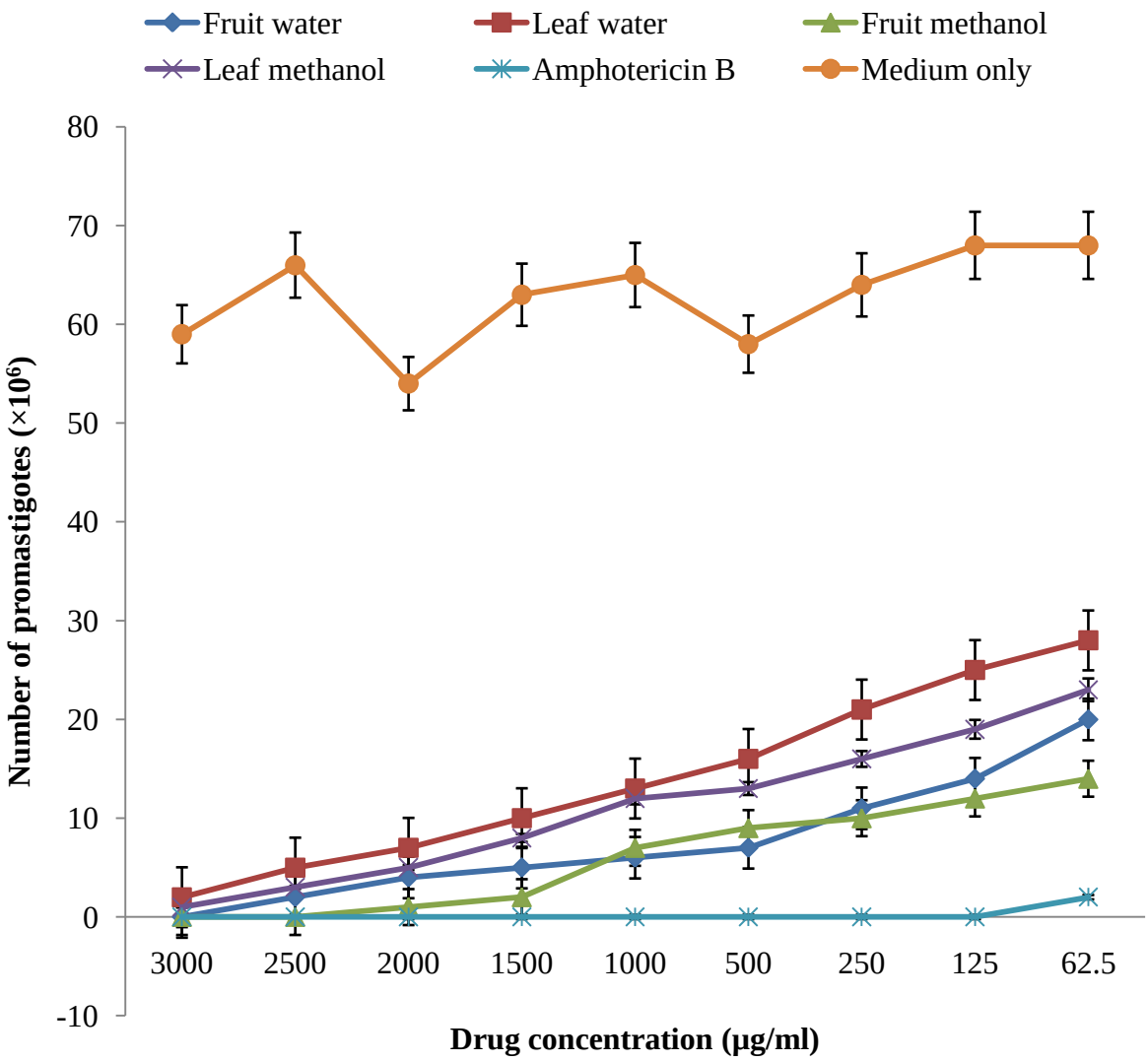

Figure 4.1: The activity of *S. aculeastrum* extracts and amphotericin B on *L. major* promastigotes

Drug concentration inhibiting parasite growth by 50% (IC_{50}) indicated fruit methanol compound with an IC_{50} of 78.62 µg/ml as more effective than other test compounds in inhibiting promastigotes growth. Fruit water, leaf water and leaf methanol were least effective in the control of promastigotes growth in an *in vitro* system indicating IC_{50} of 94.56 µg/ml, 486.79 µg/ml and 450.98 µg/ml respectively. However, the positive control drug, AmB, remained more effective (IC_{50} = 4.38 µg/ml) than fruit methanol in inhibiting parasite growth as it showed 17 fold strength than the fruit methanol drug (Table 4.1).

Table 4.1. The inhibitory concentration (IC_{50}) values of *Solanum aculeastrum* extracts and amphotericin B on *Leishmania major* promastigotes

Treatment	*L. major* promastigotes IC_{50} (mean±SD µg/ml)
Fruit water extracts	94.56±16.95
Leaf water extracts	486.79±125.46
Fruit methanol extracts	78.62±22.27
Leaf methanol extracts	450.98±99.65
Amphotericin B	4.38±2.8

4.2 Activity of *Solanum aculeastrum* extracts and amphotericin B on *Leishmania major* amastigotes

Drug activity which was recorded as percentage macrophage infection rate by *L. major* amastigotes cells following 72 hour incubation with the various drugs used at concentrations ranging from 1000 µg/ml to 125µg/ml indicated that none of all the test compounds inhibited all amastigotes completely at any given concentration. Fruit water demonstrated infection rate of 40% at 1000 µg/ml, 42% at 500 µg/ml, 47% at 250 µg/ml and 48.25% at 125 µg/ml while leaf water demonstrated infection rate of 80% at 1000 µg/ml, 82% at 500 µg/ml, 89% at 250 µg/ml and 85% at 125 µg/ml. Fruit methanol demonstrated infection rate of 48% at 1000 µg/ml, 54% at 500 µg/ml, 52% at 250 µg/ml and 50.75% at 125 µg/ml while leaf methanol demonstrated infection rate of 84% at 1000 µg/ml, 86% at 500 µg/ml, 92% at 250 µg/ml and 88.25% at 125 µg/ml. The control media demonstrated an average of 98% infection rate in all wells.

Fruit water and fruit methanol demonstrated the best activity towards amastigotes with fruit water showing better efficacy (Figure 4.2). The leaf water and leaf methanol extracts treated macrophages were as twice infected by amastigotes as compared to their fruit compounds. However, AmB displayed the best activity against amastigotes with infection rates of 0.6% at 1000 µg/ml, 0.4% at 500 µg/ml, 0.9% at 250 µg/ml and 0.1% at 125 µg/ml. The test drug concentrations demonstrated that infection of the macrophages with *L. major* amastigotes was dependent on the concentration of the drug treatments whereby decreased infectivity was observed with increased concentration, as indicated by reduced mean amastigote percentages at 1000 µg/ml in comparison to 125 µg/ml in all treatments.

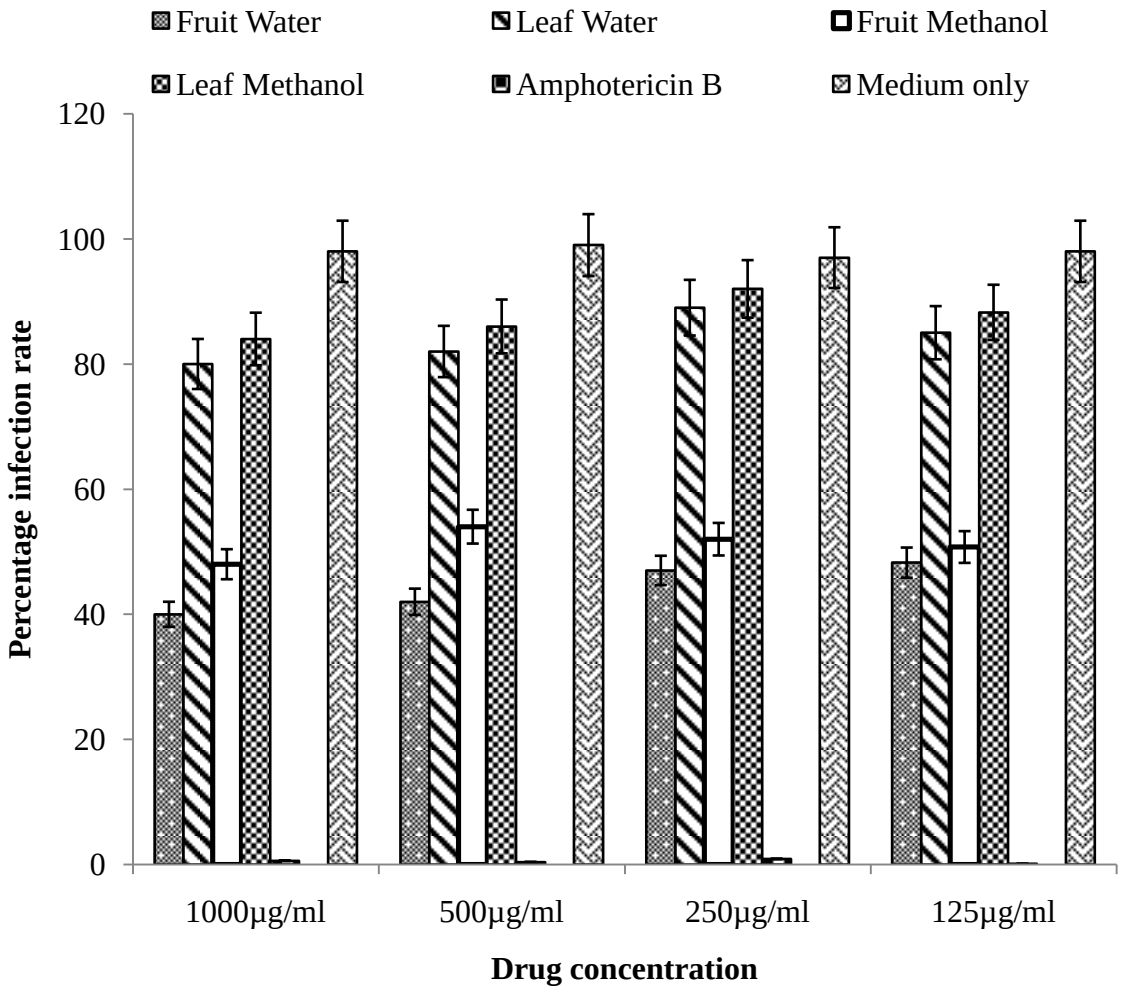

Figure 4.2: *In vitro* activities of *S. aculeastrum* extracts and amphotericin B against *L. major* amastigotes. Data represent percentage infection rates.

4.3 Toxicological effect of *Solanum aculeastrum* and amphotericin B on vero cells

Drug toxicity evaluation indicated that the fruit water test compound killed 85.96% at 20 mg/ml, 47.98% at 10 mg/ml, 23.99% at 5 mg/ml, 11.99% at 2.5 mg/ml, 5.99% at 1.25 mg/ml and 2.99% at 0.625 mg/ml of health vero cells. Leaf water compound killed 71.99% at 20 mg/ml, 35.99% at 10 mg/ml, 17.99% at 5 mg/ml, 8.99% at 2.5 mg/ml, 4.49% at 1.25 mg/ml and 2.24% at 0.625 mg/ml of health vero cells. Fruit methanol compound killed 92.84% at 20 mg/ml, 72.03% at 10 mg/ml, 31.02% at 5 mg/ml, 15.51% at 2.5 mg/ml, 7.75% at 1.25 mg/ml and 3.88% at 0.625 mg/ml while leaf methanol compound killed 80.88% at 20 mg/ml, 45% at 10 mg/ml, 22.5% at 5 mg/ml, 11.25% at 2.5 mg/ml, 5.62% at 1.25 mg/ml and 2.81% at 0.625 mg/ml of vero cells following incubations. The reference drug, AmB depicted a killing of 96.6% at 20 mg/ml, 81.4% at 10 mg/ml, 71.2% at 5 mg/ml, 64.8% at 2.5 mg/ml, 50% at 1.25 mg/ml and 25% at 0.625 mg/ml of health vero cells (Figure 4.3). It was noted that, at the maximum concentration of 20 mg/ml, all test compounds were more lethal to vero cells than at the lowest concentration of 0.625 mg/ml. Amongst the test compounds, fruit methanol appeared the most toxic drug with a killing rate of 92.84% at the highest concentration tested while leaf water displayed lowest cytotoxicity with a killing rate of 71.99% at the highest concentration. However, it was notable that AmB was the most toxic compound with a killing rate of 96.6% at the highest concentration (Figure 4.3).

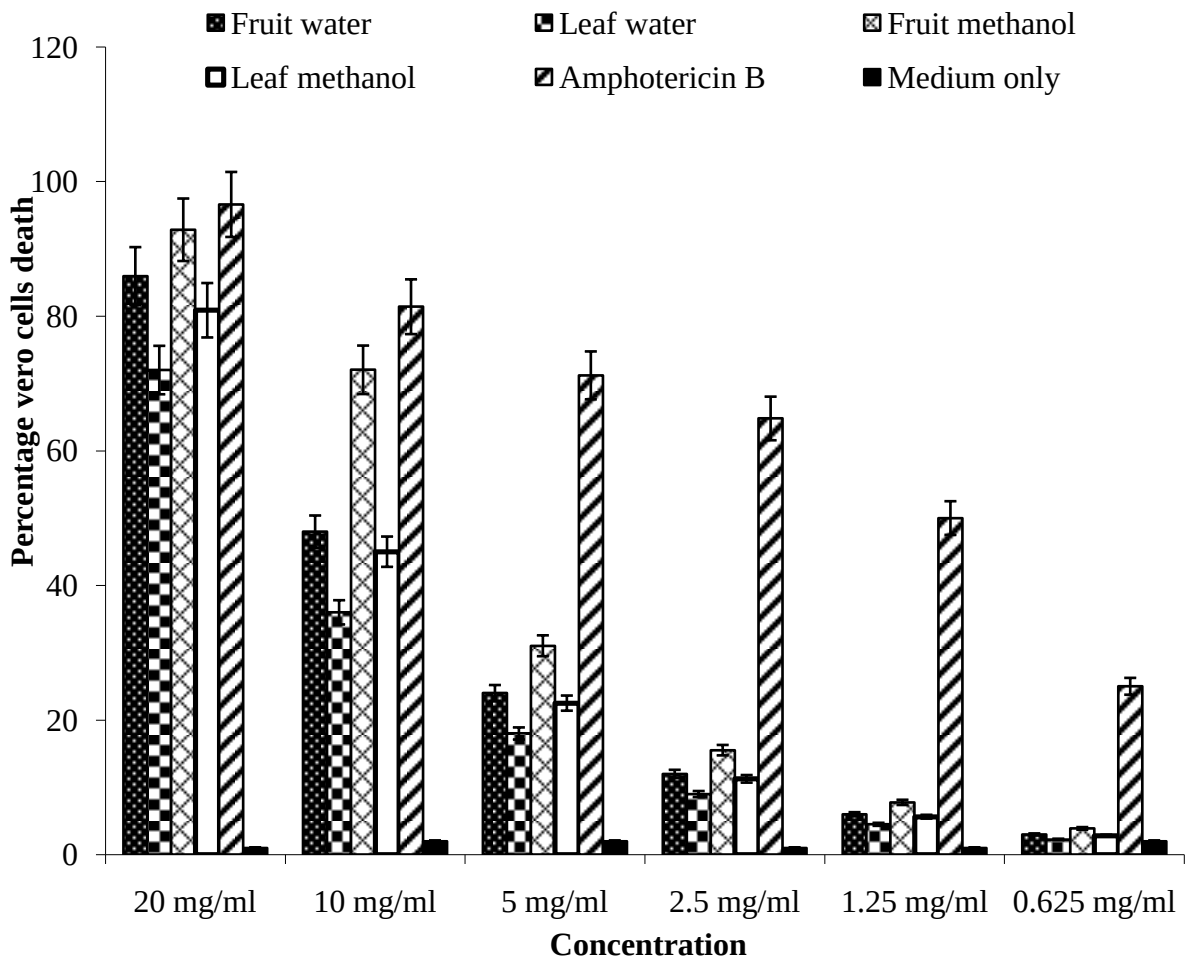

Figure 4.3: The percentage vero cells death following treatment with *S. aculeastrum* extracts and amphotericin B

The lethal dose 50 (Ld_{50}) killing half the number of vero cells indicated that leaf water at Ld_{50} = 13.89 mg/ml and leaf methanol at Ld_{50} = 11.11 mg/ml were less toxic to health vero cells in an *in vitro* test system while fruit water and fruit methanol compounds were more toxic to health vero cells in an *in vitro* system at Ld_{50} = 10.42 mg/ml and Ld_{50} = 8.06 mg/ml respectively. However, AmB (Ld_{50} = 1.25 mg/ml) was 8 times and 6 times more toxic to health vero cells in an *in vitro* system than fruit water and fruit methanol respectively, which were the most toxic test drug compounds (Table 4.2).

Table 4.2. The lethal doseLd_{50} values of *Solanum aculeastrum* extracts and amphotericin B on vero cells

Treatment	Vero cells Ld_{50} (mean±SD mg/ml)
Fruit water extracts	10.42±2.51
Leaf water extracts	13.89±0.15
Fruit methanol extracts	8.06±3.15
Leaf methanol extracts	11.11±0.62
Amphotericin B	1.25±5.00

4.4 Effects of intraperitoneal administration of *Solanum aculeastrum* and amphotericin B on cutaneous lesion sizes (mm)

Intraperitoneal treatment with drug compounds which was used to evaluate drug efficacy *in vivo* by measuring lesion sizes (mm) indicated that fruit water, leaf water, fruit methanol, leaf methanol and AmB recorded lesion sizes of 0.9260±0.07987 mm, 0.8620±0.101 mm, 1.0560±0.22898 mm, 0.65±0.041 mm and 0.8500±0.1118 mm respectively while the control group demonstrated a two fold increase in lesion sizes of 1.76±0.062 mm (Figure 4.4). Analysis of variance computation indicated statistically significant difference in lesion sizes between experimental and control (PBS) mice groups ($F = 11.118$; $P = 0.0001$). Tukey-Kramer multiple comparisons test indicated that lesion sizes were comparable ($P > 0.05$) in all mice groups following treatment with test drug compounds. Results also indicated that there was no significant difference in lesion sizes in mice groups treated with any of the test drug as compared to treatment with AmB ($P > 0.05$). However, treatment of mice with any of the drug compound was significantly protective against cutaneous disease as compared to placebo (PBS) ($P > 0.001$).

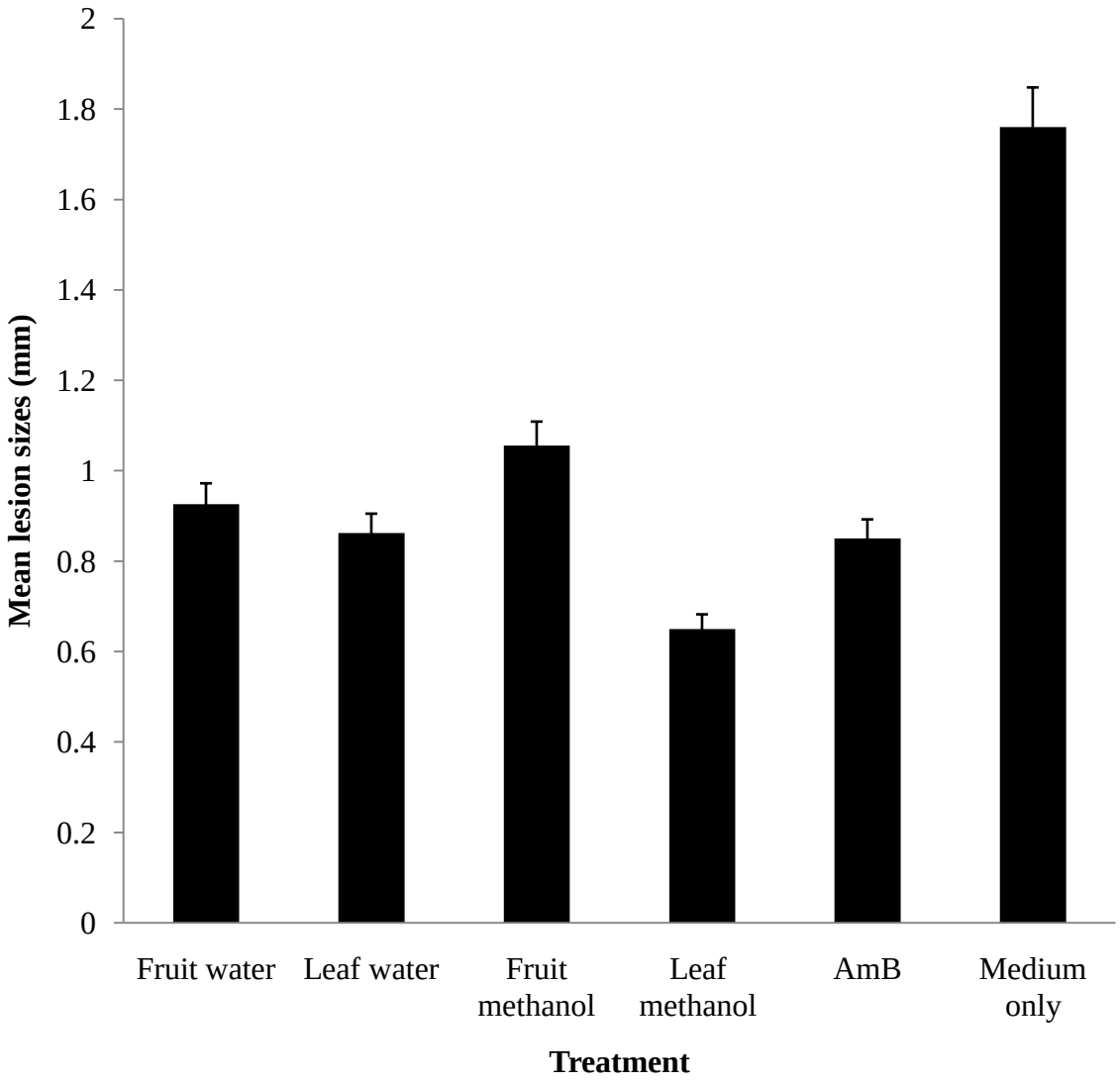

Figure 4.4: The effect of intraperitoneal treatment of *L. major* infected mice with *S. aculeastrum* and amphotericin B on the progression of footpad lesions. Data represent mean lesion sizes ± S.D. (n = 5), four weeks post infection.

4.5 Effects of intraperitoneal administration of *Solanum aculeastrum* extracts and amphotericin B on parasite burden in BALB/c mice

Splenic impression smears showed amastigotes both within infected cells and outside of cells following bursting of some infected macrophages and release of the intracellular amastigotes outside cells. Amastigotes in splenic tissues from treated mice indicated parasitic burden of 9.15 ± 2.634 amastigote per 1000 splenic macrophages in the fruit water treated groups and 19.8 ± 3.718 amastigotes per 1000 cell nuclei in the leaf water treated mice group while a parasitic burden of 11.675 ± 2.211, 13 ± 3.916 and 3 ± 0.909 amastigotes/1000 splenic cell

nuclei was observed in the mice groups treated with leaf methanol, fruit methanol and AmB respectively. The mean parasite numbers in the control (PBS) mice group was 29.25 ± 2.2172 per 1000 splenic cell nuiclei (Figure 4.5). Analysis with ANOVA indicated a highly significant difference in parasitic numbers between the experimental and the control groups ($F = 42.793$; $P = 0.0001$). Although treatment of mice with fruit water indicated significantly more protective capacity as compared to treatment with leaf water ($P < 0.001$), mice group treated with fruit water showed comparable parasitic numbers as compared to mice treated with either leaf methanol or fruit methanol ($P > 0.05$). Parasite numbers were also comparable in mice groups treated with leaf methanol or fruit methanol ($P > 0.05$). However, treatment of mice with fruit methanol was also significantly more protective than treatment with leaf water ($P < 0.05$). In addition, mice treated with leaf methanol were significantly more protected against cutaneous leishmaniasis than mice treated with leaf water compound ($P < 0.01$). Remarkably, treatment of mice with fruit water showed comparable disease parasite outcome as compared to treatment with the positive control drug, AmB ($P > 0.05$). However, treatment of mice with AmB was significantly more protective against disease than treatment with leaf methanol, leaf water, or fruit methanol ($P < 0.001$). Treatment with any of the drug compound recorded significant protection level when compared with the untreated control mice group ($P < 0.01$).

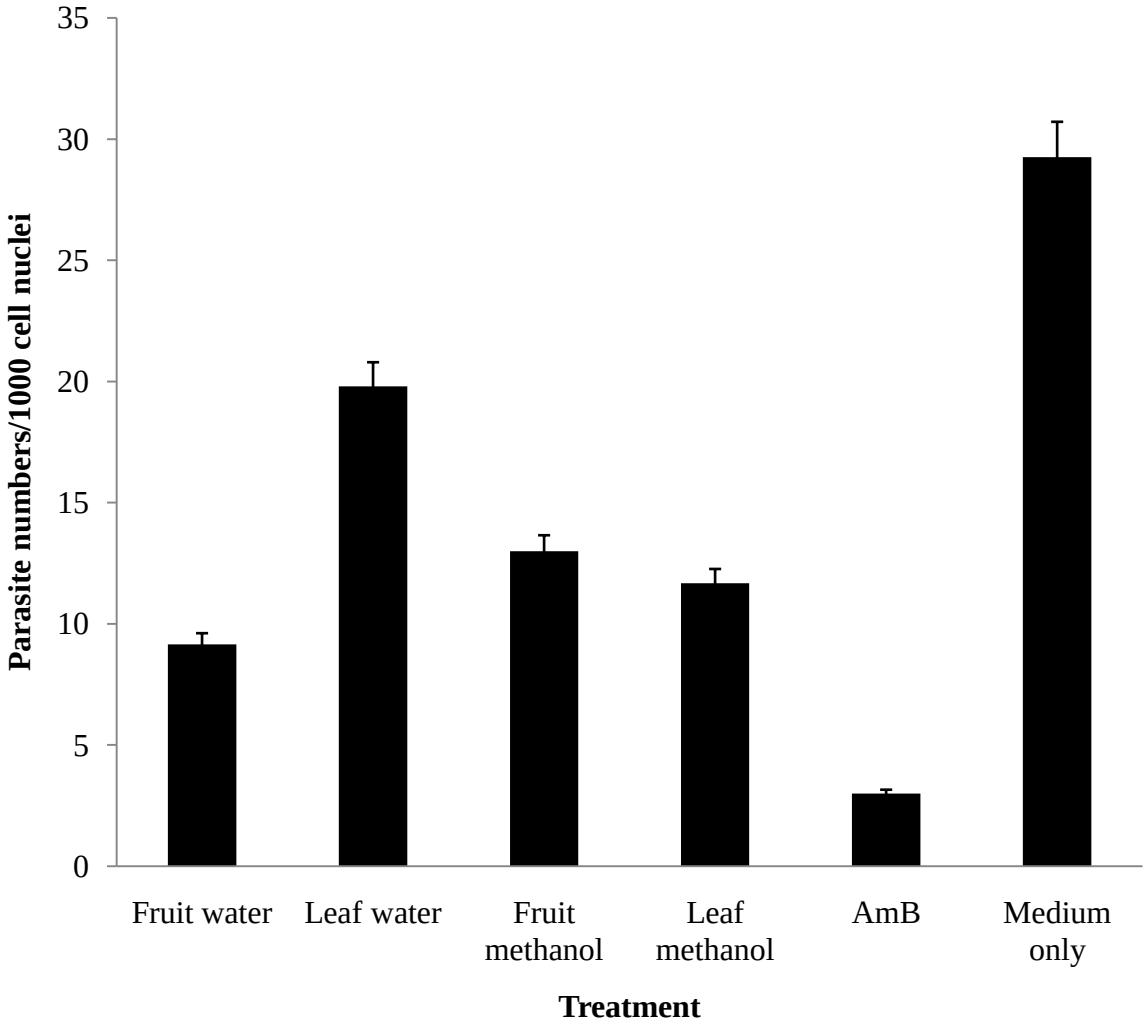

Figure 4.5: Parasite burden in spleens of *L. major*-infected BALB/c 7 days after the end of 4 weeks treatment. Data presented as mean ± S.E.M. of number of amastigotes per 1000 host nuclei.

CHAPTER 5: DISCUSSION

5.0 Overview

The history of herbal medicine is rather old and dates back to the time when the early man became conscious of his environment. Since then, medicinal plants have been used virtually in all cultures as a source of medicine (Lanfranco, 1999). Biologically active natural products developed from these plants are being used either as commercial drugs or as lead structures for the development of modified derivatives possessing enhanced activity (Mourice *et al*., 1999).

In Kenya, about 80 % of the population relies on traditional herbal medicine for treatment of various ailments. The Kenyan flora is also estimated to contain between 4,000 and 6,000 species of higher plants of which about 12 % are endemic. Therefore, scientific evaluation of these plants may provide modern medicine with lead compounds for the development of new drugs (Kokwaro, 2009).

The emergence of antimonial-resistant *Leishmania* strains is on the rise and natural products like *S. aculeastrum* extracts and other plant products that have been tested and found to possess antileishmanial activities may provide alternative treatment against leishmaniasis (Monzote, 2009). In the family *Solanaceae,* a Bolivian plant species *Saracha punctata* has been indicated to completely inhibit the growth of promastigote forms of *L. braziliensis, L. donovani* and *L. amazonensis* (Manuel and Luis, 2001). In the present study, these previous findings are extended to determine the safety and efficacy of *S. aculeastrum* water and methanolic compounds in *in vitro* and *in vivo* test systems against *Leishmania major* infection.

5.1 Activity of *Solanum aculeastrum* against *Leishmania major* promastigotes

In this assay, the lower IC_{50} associated with fruit methanol is an indication of its effectiveness against *L. major* promastigotes in comparison to fruit water, leaf water and leaf methanol compounds. However, with the AmB reference drug indicating a 17 fold strength in killing

promastigotes by half the original number as compared to the methanol compound, the *in vitro* parasite killing strength of the fruit methanol drug may be considered too low if selection of the most effecacious drug compound is to be based on this assay alone. However, the fruit methanol being one and half times and at least six times more effective than fruit water and other test compounds in killing parasites proves to be a potentially efficacious drug compound against leishmaniasis.

5.2 Activity of *Solanum aculeastrum* against *Leishmania major* amastigotes

The phenomenon of substantial amount of polar constituents present in the *S. aculeastrum* plant could explain the higher anti-leishmanial activity of fruit water and fruit methanol compounds in comparison to other test compounds. The *S. aculeastrum* fruits, both mature and immature, contain steroidal alkaloids which have been the target of pharmacological investigations because of their structural similarities with anabolic steroids, steroidal hormones and corticosteroids (Gurib-Fakim, 2006; Ncube *et al*., 2008). In the present study it was interesting to associate fruit water with the highest leishmaniacidal effect on mastigotes than all other test compounds. Furthermore, the aqueous extracts of *S. aculeastrum* berries have been shown to possess higher antioxidant activity on free radicals than the methanol extracts. The higher antioxidant activity of the aqueous extract was attributed to the substantial amount of polar constituents present in the *S. aculeastrum* plant (Koduru *et al*., 2006b). However, with the AmB reference drug indicating a 44 fold strength in inhibiting macrophage infection by amastigotes as compared to the fruit water compound, the activity of fruit water towards amastigotes *in vitro* may be considered too low if selection of the most effecacious drug compound is to be based on this assay alone. However, the fruit water being one times and atleast two times more effective than fruit methanol and other test compounds in reducing macrophage infection by amastigotes proves to be a potentially efficacious drug compound against leishmaniasis.

5.3 Toxicological effect of *Solanum aculeastrum* on vero cells

The current drugs for leishmaniases including AmB are highly toxic (Monzote, 2009). The extremely high toxicity levels associated with the AmB reference drug confirms the concerns, fears and limitations of use of current chemotherapy against leishmaniasis and the need to search for safer and cheaper drugs that can invalidate the use of current toxic compounds. The effect of *S. aculeastrum* extracts on health cells in an *in vitro* test system implies that in addition to efficacy, safety considerations should govern possible therapeutic uses of these plant extracts. A key question for the possible use of the *S. aculeastrum* extracts evaluated in this study against *L. major* treatment should take into consideration the ratio of the effective therapeutic to toxic dose. However, it is worth mentioning that solasodine and tomatidine, compounds found in *Solanum aculeastrum*, have been found to possess significant cytotoxic activity against various cancer cell lines (Cham, 1994; Lee *et al.*, 2004). Although compounds found in *S. aculeastrum* are potent inhibitors of vero cells, it is still imperative to ascertain that any potent inhibitor of *L. major* cells must possess low IC_{50} values when tested against the parasitic actively dividing cells and a high LD_{50} values against confluent cells (cells in a cell culture dish or a flask).

5.4 Effects of *Solanum aculeastrum* on BALB/c cutaneous lesion sizes (mm)

In present study, it is in this assay that the administration of leaf methanol compound demonstrated best activity on reduced mean lesion sizes in comparison to other compounds with one fold strength in comparison to the reference drug, AmB. This is the very first time leaf methanol appeared to have contributed more than the fruit compounds in significantly reducing disease progression in the BALB/c mice. This is a probable indication that the therapeutic effect of *S. aculeastrum* leaf can also be considered when targeting cutaneous leishmaniasis. Besides steroidal alkaloids, *S. aculeastrum* leaves have also been reported to possess alkanes, aldehydes, aromatic hydrocarbons, phthalic acid, methyl salicylate and

terpenoids (Wanyonyi *et al*., 2003a; Koduru *et al*., 2006a), which could be contributing to the medicinal property of this part of the plant.

5.5 Effects of *Solanum aculeastrum* on parasite burden in BALB/c mice

The significant protection against progression of cutaneous leishmaniasis in mice treated with fruit water as opposed to the other drug compounds was an indication of the efficacy and advantages of the fruit water compound against the disease. However, with the AmB reference drug indicating a 3 fold strength in controlling parasite burdens as compared to the fruit water compound, the activity of fruit water towards parasite burdens *in vivo* may be considered too low if selection of the most effecacious drug compound is to be based on this assay alone. However, the fruit water compound being one times and two times more effective than leaf methanol, fruit methanol and leaf water test compounds in reducing parasite burdens is an indication of a potentially efficacious drug compound against leishmaniasis.

This observation may be partly due to different modes of action of fruit water drugs which may effectively reduce parasite resistance. This observation of the fruit water compound being effective in reducing parasite burden in BALB/c mice is consistent with the fruit water activity on amastigotes with general agreement that fruit water compounds were still most effective against amastigotes. Furthermore it has already been indicated before that fruit water compound had higher anticancer property than fruit methanol compound which was attributed to the substantial amount of polar constituents present in the *S. aculeastrum* plant (Koduru *et al*., 2006b). The higher leishmaniacidal activity of the fruit water compound in comparison to the fruit methanol compound could also be attributed to the substantial amount of polar constituents present in the *S. aculeastrum* plant.

A recent study on the antiproliferative activity of the aqueous, methanol and acetone extracts of the leaves and berries of *S. aculeastrum* against HeLa, MCF-7 and HT-29 human cancer

cell lines has shown that the extracts of the leaves possess no anticancer property while the methanol and aqueous extracts of the berries possess the highest antiproliferative activity (Koduru *et al*., 2006e). The effect of combined compounds of solasodine and tomatidine, steroidal alkaloids isolated from the berries of *S. aculeastrum* has also indicated pronounced activity against human cancer cells rather than the individual compounds (Koduru *et al*., 2007a). It therefore follows that a combination of *S. aculeastrum* leaves and fruits may be very effective for treatment of cutaneous leishmaniasis depending on the formulation used if the more effective compound can be used at a higher concentration than the less effective drug to formulate a combination with a desired efficacy.

CHAPTER 6: CONCLUSIONS AND RECOMMENDATIONS

6.1 Conclusions

i. Generally, the water extracts demonstrated better activity to *L. major* in comparison to the methanol extracts. Perhaps the higher antiparasitic activity of the aqueous extract could be attributed to the substantial amount of polar constituents present in the plant.

ii. Fruit methanol compound was most effective in inhibiting the growth of promastigotes in culture and was associated with less IC_{50}. Leaf water and leaf methanol were least effective in the control of promastigotes with leaf methanol showing more inhibitory strength than leaf water.

iii. None of all the test compounds inhibited all amastigotes in culture completely, however, fruit water and fruit methanol demonstrated the best activity towards amastigotes with fruit water showing better efficacy.

iv. Fruit water and fruit methanol compounds were more toxic at Ld_{50} = 10.42 mg/ml and Ld_{50} = 8.06 mg/ml respectively. However, AmB showed a higher toxicity strength of 1.25 mg/ml in killing 50% of vero cells.

v. Leaf methanol drug appeared to have contributed more than the other compounds in significantly reducing disease progression in the BALB/c mice by reducing lesion sizes.

vi. Fruit water compounds significantly protected mice against cutaneous leishmaniasis disease by reducing parasitic burden in splenic macrophages. However, amphotericin B was more protective than fruit water against disease.

6.2 Recommendations

i. Both the methanolic and aqueous extracts of *S. aculeastrum* leaf and berry should be prioritized in case of further exploration on antileishmanial properties. BALB/c mice infected with *L. major* represents a model of extreme susceptibility, and the striking and sustained reduction in the number of parasites in treated animals supports the proposal for further laboratory testing and clinical use of this drug in other models of leishmaniasis for example using non-human primates such as vervet monkeys (*Chlorocebus aethiops*).

ii. The study recommends the development of *S. aculeastrum* fruit extracts for topical application against cutaneous leishmnaisis. This cream can directly and easily be administered by patients.

iii. *Solanum aculeastrum* is very easy to grow and grows easily in several types of soils, terrains, disturbed and undisturbed margins and even in green houses. People in endemic areas should be encouraged to grow and learn how to use this plant.

iv. Further research is required to develop oral formulations and a combination therapy between *S. aculeastrum* leaf and berry for treatment of human leishmaniasis patients.

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APPENDICES

APPENDIX I

Immature seeds of *S.aculeastrum*

APPENDIX II

Early flowering stage of *S. aculeastrum*

APPENDIX III

Early fruit formation in *S.aculeastrum*

APPENDIX IV

Immature fruit of *S. aculeastrum*

APPENDIX V

Mature fruit of *S.aculeastrum*

Appendices I-V photos were taken in a farming area of Nyakwerema village, Nyamira County, Kenya - Africa July 2012.