EFFECTS OF *RICINUS COMMUNIS* LINNEAUS (EUPHORBIACEAE) EXTRACTS ON *LEISHMANIA MAJOR* PROMASTIGOTES AND ON INFECTED BALB/c MICE

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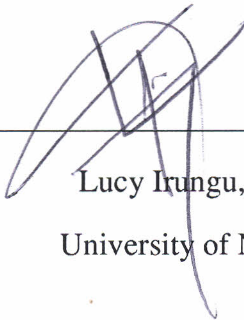
1998

DECLARATION.

I hereby declare that this thesis has not been submitted for a degree in any other university and the contents are my original work

Bernard Achero Okech, BSc.

This thesis has been submitted for examination with my approval as an internal university supervisor.



9th Nov 1998

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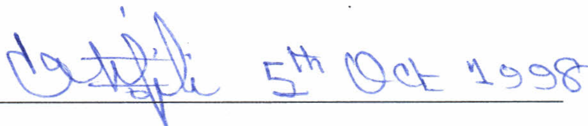

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DEDICATION

I dedicate this thesis to my father who has been instrumental in my pursuing the MSc. degree course and for ably providing financial support to enable me complete my studies. My eldest brother for always being my mentor and my mother for constantly praying for my victory. To all members of my family who have been supportive and encouraged me through this work.

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ABSTRACT

The effect of castor oil plant seed oil fractionally extracted with diethyl-ether and the aqueous extracts of its leaves was tested *in vitro* on *Leishmania major* (Yorkimov and Schokor, 1913) promastigotes and *in vivo* on *Leishmania major* lesions in inbred BALB/c mice. Four experimental groups of 35 female mice each were used namely seed oil group (oil topically applied); Aqueous leaf extract group (extract administered intraperitoneally); Pentostam® (sodium stibogluconate) group (drug administered intraperitoneally) and a control group (untreated infected mice). Before infection, footpad measurements were taken by measuring the left hind footpad (LHFD) and the contralateral right hind footpad (RHFD). All the mice were infected intradermally with *L. major* stationary primary phase metacyclic promastigotes at a dosage of 1 x 10^6 per 10μl on the left hind footpad of every mouse and lesion sizes measured weekly at 7 day intervals. Treatment was commenced 30 days post-infection. Within this time lesion sizes were measured using vanier calipers every 7 days. The experimental groups were treated for six weeks and observed for another 2 weeks before being sacrificed. Impression smears and cultures of spleen were made to determine the parasite load and visceralization while right hind footpad, (RHFD), was left as a contralateral control. Results of the study showed that there was antileishmanial effect due to the *R. communis* oil that was significantly better than the effect of the leaf extracts (Tukey test, $q=4.059$, $P<0.05$). However, the effect compared poorly to the standard treatment drug. Notwithstanding, it was found that the *R. communis* aqueous leaf extracts was much better at preventing metastasis, visceralization, and a much lower *Leishmania donovani* Unit (LDU) than the seed oil possibly due to the that the

leaf infusion was administered intraperitoneally. Surprisingly enough the seed oil appeared to have visible effects at reducing lesion size that was significantly better than the leaf extract. However sodium stibogluconate still proved better. It is recommeded that *R. communis* seed oil be explored furthur as a potential canditate for use in combination therapy with other antileishmanial drugs used for the treatment of cutaneous leishmaniases.

CHAPTER ONE:

INTRODUCTION

AND

LITERATURE REVIEW

1.0 INTRODUCTION

Cutaneous leishmaniases are major causes of morbidity in several developing countries. In Kenya, the causative agents of these diseases in humans are *Leishmania major* (Kung'u *et al.,* 1972; Muigai *et al.*,1987) *L. tropica*, (Mebrahtu *et al.*, 1987, 1988; Lawyer *et al.,* 1991) and *L. aethiopica* (Mutinga, 1975). Most primary lesions of cutaneous leishmaniasis caused by *L. major* heal spontaneously and recovery is thought to confer lifelong immunity (WHO, 1990). Treatment is desirable when there are lesions on the exposed parts of the body especially the face. Another important reason for the need to treat is that it is one of the most important methods of controlling the disease in the population at risk (Marinkelle, 1980).

Unlike *L. major*, infection with *L. aethiopica* and *L. tropica* pose a special problem since they involve large areas of the body surface and tend to have a protracted course. Both infections are in most cases refractory to the therapies that are available for other leishmaniases (Bryceson, 1987; Heughchong, 1986). The pentavalent antimonial compounds, sodium stibogluconate (Pentostam®) and meglumine antimonate (Glucantime®) are the drugs of choice for treatment of all forms of leishmaniases (Neal and Mathews, 1982; Selim *et al.*, 1990). In cases of treatment failure, second line drugs such as pentamidine and amphotericin B are used (Sampaio *et al.*, 1971). The toxic nature of antimonial compounds and the existence of treatment failures and relapses clearly point out the need for alternative antileishmanial agents.

Haematophagous dipteran insects such as mosquitoes (Diptera: culicidae) and sandflies (Diptera: Psychodidae) that are also vectors of human diseases are

known to be phytophagous (Kaddu *et al.*, 1992a, 1992b; Schlein and Jacobson, 1994). Under natural conditions, mosquitoes and phlebotomine sandflies derive their sugar meals from plants and honeydew of aphids (Killick-Kendrick and Killick-Kendrick, 1987). Sandflies have been shown to probe leaves of plants and some of the preferred species include *Bidens pilosa* (Compositae), *Lycopersicum esculentum* (Solanaceae), *Solanum luteum* (Solanaceae), *Capparis spinosa* (Capparridiceae) and *Ricinus communis* (Euphorbiaceae) (Schlein and Jacobson, 1994; Kaddu *et al.*, 1992a, 1992b). Some of these plant sugars have been shown to inhibit *L. major* development in the sandfly gut (Schlein and Jacobson, 1994). Of these plants, *R. communis*, *C. spinosa* and *S. luteum* were shown by Schlein and Jacobson, (1994) to cause over 50% mortality of *Leishmania* in the sandfly and deformation of parasites in 88%, 55% and 46% of the infections respectively. The mechanisms of killing and inhibition of parasite development by these plants are unknown. It is for this reason that the study on the possibility of using extracts from this plant (*R. communis)* in, *in vitro* on *L. major* promastigotes and *in vivo* on the treatment of *L. major* infections in BALB/c mice was investigated

1.1 LITERATURE REVIEW

Leishmaniases are caused by protozoan parasites belonging to the phylum: Sarcomastigophora (Peters and Killick-kendrick, 1987) Order: Kinetoplastida (Peters and Killick-kendrick, 1987; Vickerman, 1976) Genus: *Leishmania* (Ross, 1903). The life cycle is digenetic (Heteroxenous) with promastigotes in the alimentary tract of the phlebotomine insect host, which is limited to blood sucking phlebotomine sandflies (Diptera: Psychodidae) and rounded amastigotes living and dividing in the macrophage and dendritic cells of the

vertebrate host (Moll *et al.*, 1995) and a variety of mammals (Peters and Killick-Kendrick, 1987).

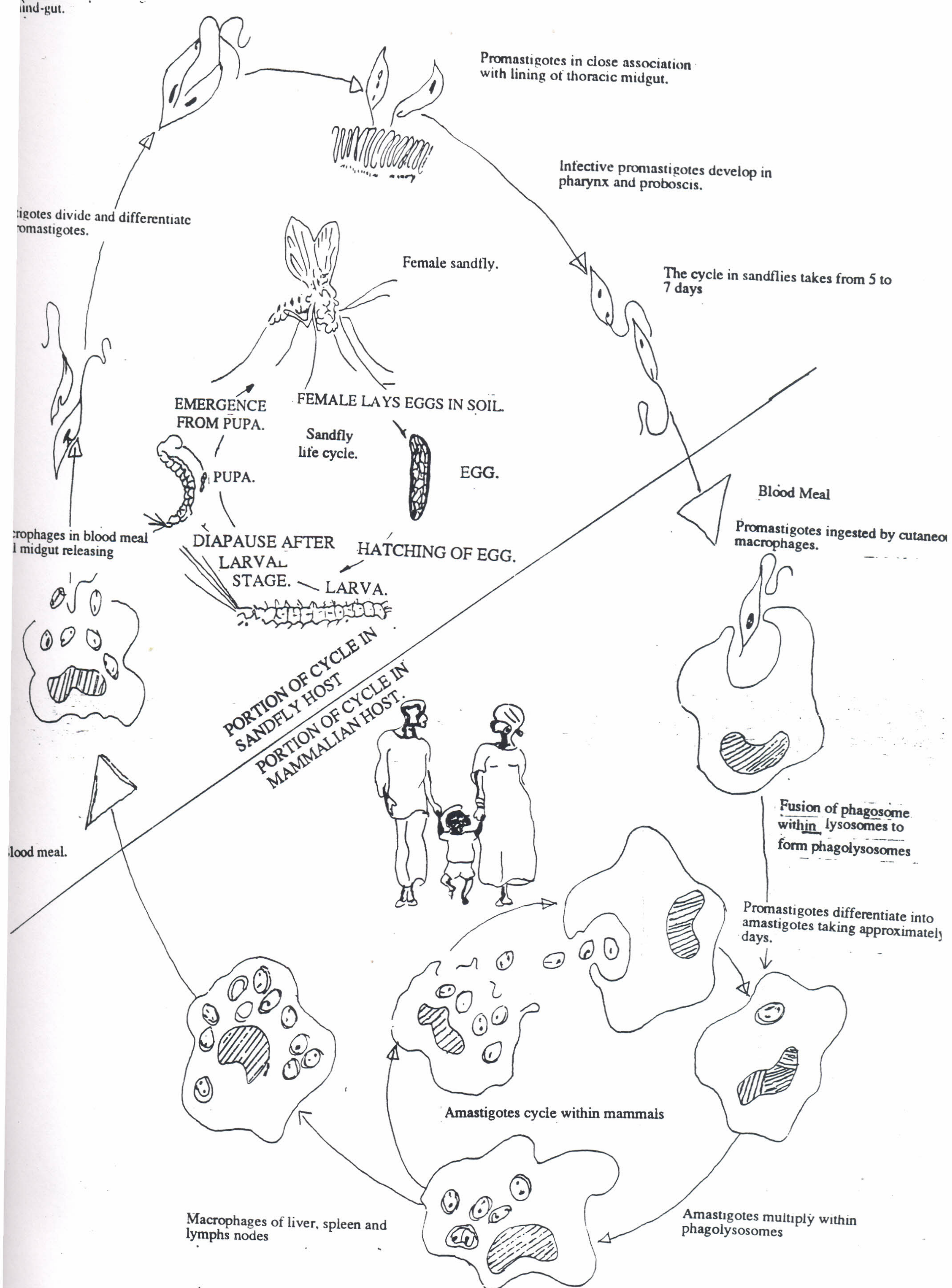

Figure 1. Developmental cycle of the *Leishmania* parasite.

(Adapted from World Health Organization Bulletin, 1990)

1.1.1 The aetiology, distribution and current status of cutaneous leishmaniasis.

The leishmaniases occur worldwide with an estimated 12 million people infected with diferent species of the parasite and an estimated 400,000 new infections yearly (Ashford *et al*, 1992) in Europe, Africa, India and the Americas where these diseases are endemic. As a result of geographical isolation, speciation has occurred and we therefore find several species of *Leishmania* in these different foci. In the Old World, both viscersal and cutaneous leishmaniasis are usually sympatric. Visceral leishmaniasis is nearly exclusively caused by members of *Leishmania donovani* complex. *L. donovani sensu stricto* is mainly confined to the Eastern parts of Indian sub-continent. *L. donovani sensu lato* is more widely distributed throughout the Old World and is thought to be an anthroponosis in Kenya with a human to human transmission through the sandfly. *L. infantum* is also widely distributed in the Old World extending from Central Asia, China, Africa, Mid-East and in countries of the Mediteranean region like Itay and Portugal. It is also a zoonosis with a variety of caniid reservoirs.

Cutaneous leishmaniasis in the Old World is caused by *L. aethiopica, L. tropica, L. major* and *L. donovani* that is typically a visceral form but relapses as post kala-azar dermal leishmaniasis (PKDL) in 2% 0f cases treated with sodium stibogluconate. *L. aethiopica* is confined to the East African region and was isolated in the Ethiopian highlands by Ashford (1977) and in Mt. Elgon, Kenya by Kungu *et al.* (1972) and Mutinga in (1975). It is a zoonotic disease that is self-healing which, if does not heal, may become a diffuse nodular cutaneous leishmaniasis. *L. tropica* is known to be an anthroponosis (WHO,1979) within the mediterranean littoral region and was isolated in

Kenya by Lawyer *et al* (1991) in Muruku, Laikipia District. In Kenya however, studies carried out by Sang and Ashford (1993) suggest that there may be a sylvatic reservoir host. *L. tropica* infections cause dry ulcers that take 6 - 9 months to heal and may result in recidivan. *L. major* is a zoonotic infection which causes wet ulcers and is known to occur in areas as India, Afganistan, Iran, Mongolia, Libya, Senegal in West Africa to Southern Ethiopia and Northern Kenya. The lesions due to *L. major* may spread in the body and become multiple but self healing.

The pathological sequelae of cutaneous leishmaniasis, usually caused by dermatropic species of *Leishmania* involves an early phase with papular lesion and macrophage infiltration; lytic phase with superficial ulceration of the skin; late chronic phase with cell granulomas in the dermis, dermal fibrosis and lesion healing with scarring (Ridley and Ridley, 1983). Uncommon sequelae include recidiva CL with lesions that relapse chronically and increase in cell granulomas in the dermis. The diffuse CL sequelae involves the persistence of early phase lesions with an increase in body skin involvement. Muco-cutaneous complications involves ulceration and secondary bacterial infection that leads to necrosis of the cartilage, collagen and bone (Ridley and Ridley, 1983).

In the New World (Americas) leishmaniasis is caused by parasites of the *L. mexicana* complex and *L. brasiliensis* complex that cause New World cutaneous, NWCL, and mucocutaneous leishmaniases, MCL, and *L. donovani chagasi* that is responsible for sporadic cases of human and canine visceral leishmaniases (Lainson *et al.*, 1969). These parasites extend from Northern Mexico, Texas-USA, through Belize, Dominican Republic, Trinidad, Peru and Brazil (for *L. mexicana*) and through Belize, Honduras, Costa Rica, Panama and Brazil (for *L. braziliensis*). *Leishmania chagasi* is confined only to some

isolated pockets of Brazil (Lainson, 1969, 1983).

CLASSICAL ZCL

MIDDLE EAST ZCL

L. major

SAHELIAN ZCL

L. gerbilli (rodents only)

INDIAN ZCL

L. (L.) aethiopica

Key:

	AREAS WITH ENDEMIC LEISHMANIASIS
INDIAN ZCL	Indian zoonotic cutaneous leishmaniasis.
SAHELIAN ZCL	Sahelian zoonotic cutaneous leishmaniasis.
MIDDLE EAST ZCL	Middle East zoonotic cutaneous leishmaniasis.
CLASSICAL ZCL	Classical zoonotic leishmaniasis.

Figure 2 Distribution of cutaneous leishmaniases in the Old World

(Adapted from Peters and Killick-Kendrick, 1987).

Key:

AREAS WITH ENDEMIC LEISHMANIASIS

Figure 3. Distribution of cutaneous leishmaniasis in Kenya.

(Adapted from Mutinga, 1985)

ETHIOPIA

TURKANA

RIFT VALLEY

EASTERN

NORTH EASTERN

NDA

WEST POKOT FOCUS

BARINGO FOCUS

SOMALIA REPUBLIC

WESTERN

Abadares

CENTRAL

KITUI FOCUS

NYANZA

NAIROBI

MACHAKOS FOCUS

COAST

INDIAN OCEAN

Key:

AREAS WITH ENDEMIC LEISHMANIASIS

Figure 4 Distribution of visceral leishmaniases in Kenya.

(Adapted from Mutinga, 1985).

1.1.2 The treatment of leishmaniasis

The continued search for chemotherapeutic agents against leishmaniasis has been brought about by the fact that there has been a resurgence in the incidences of the leishmaniases throughout the world owing to factors ranging from poor economic conditions, unstable political systems and social upheaval and even to the continued spread of the Aquired Immune Deficiency Syndrome, (AIDS) epidemic (Scalgia, 1989). Leishmaniases have been identified as opportunistic infections in AIDS patients (Scalgia *et al*, 1989; Molina *et al,* 1992) and its continued increase in endemic developing countries due to the latter has added to the urgency of the need to develop and find new chemotherapuetic agents that can help in controlling cases of human leishmaniasis. The World Health Organisation (WHO) approved drugs have undesirable effects and the long treatment regimens and toxicity of these drugs has made them unsuitable and thus spurring more pharmacological investigations into new safer and cheaper drugs.

The drugs of choice in the fight against leishmaniases are the pentavalent antimonials that include sodium stibogluconate (Pentostam®) which is administered intramascularly or intravenously at a dosage of 20mg per kilogram of body weight for a minimum of 20 days for cutaneous leishmaniasis or 28 days for visceral leishmaniasis. However, these dose regimens have been found to be ineffective in treating mucosal leishmaniasis (Franke *et al.* 1994) and there are toxicity and other adverse effects such as anthralgias and myalgia (muscoskeletal complaints), liver malfunctions, abdominal discomforts, nausea and diarrhoea, (Saenz *et al.* 1991). However, sodium stibogluconate has been observed to accelerate healing in *Leishmania major* and *L. tropica* cutaneous leishmaniasis (Oster, 1991; Haidaris and Bonventure, 1983) but the side

effects still persist.

Meglumine antimonate (Glucantime®), a pentavalent antimonial, is also administered systemically or locally at dosages of 10 - 29 mg per kilogram of body weight in every 24 hours for 20 - 30 days. This drug too has been implicated for its side effects and the long duration of administration which makes it undesirable. The pentavalent antimonials are also very costly drugs that cannot be easily afforded by many in the third world countries.

The second line drugs of choice are the aromatic diamidine and amphotericin B. Aromatic diamidine include Pentamidine isothionate (lomidine), stillbamidine isothionate which are primarily indicated in the treatment of trypanosomiasis. These drugs are however only used in cases unresponsive to antimonials and they need careful management to avoid serious side effects that have been encountered with their usage. Pentamidine was only until recently the drug of choice in the treatment of diffuse cutaneous leishmaniasis (DCL) and mucocutaneous leishmaniasis (MCL) caused by *L. aethiopica*. Amphotericin B treatment for leishmaniasis is still of prime importance because of its mode of action. It is a colloidal suspension, intravenously infused at 2 mg per kilogram body weight on alternating days, however it is associated with adverse side effects thus limiting its importance as a first line drug in leishmaniasis treatment (Olliaro and Bryceson, 1993). Amphotericin B which is primarily an antibiotic has been incorporated into liposomes made of phosphatidyl choline, cholesterol and diesteroyl phosphatidyglycerol and these are being tried as leishmaniases drugs. Allopurinol, a drug used in hyperuriceamia treatment has been observed to stop the growth of *Trypanosoma cruzi* and *Leishmania* parasites *in vitro*. When used in combination therapy with sodium stibogluconate, it augments the antileishmanial effects (Kager *et al*, 1981). Other antibiotic drugs that are

indicated in the chemotherapy of the leishmaniases are paromomycin (El-safi *et al.*, 1990) and rifampicin (Selim *et al.*, 1990). Antibiotic treatment for cases of simple cutaneous leishmaniases (CL) has been tried and found successful but is limited only to a narrow range of antibiotic drugs and only when used in combination therapy. In a study conducted in Kenya on the effect of combined therapy and single treatment with paramomycin, Chunge *et al.*, (1990) found better results in cases of combined therapy although single therapy with paromomycin was cheaper and safer, despite spleen aspirates revealing a 21% failure rate. Other studies on antibiotics was done by El-safi *et al.* (1990) who used paramomycin in ointment form and found it unsatisfactory in the treatment of cutaneous leishmaniasis. Rifampicin, an antibiotic drug, has also been tried and used in treating cutaneous leishmaniasis but very conflicting views arise from results of experiments conducted using it. Selim *et al.*, (1990) found rifampicin a safe and effective drug for CL both in early *L. major* and in late ulcerative *L. tropica*. Other antibiotic drugs that have been used include ketoconazale, a fungicidal drug, which is an imidazole derivative, that has been reported to be effective in treatment of leishmaniasis due to *L. major* (Berman and Lee, 1983; Abdel Al, *et al*, 1988). A high percentage of patients treated with this drug were cured at a dosage of 200 - 400 mg per kilogram per day with no reports of major side effects. This drug has also been used to treat visceral leishmaniasis (Wali *et al.* 1990). Itraconazole, a steroid synthetase inhibitor has been used to treat cutaneous leishmaniasis caused by by *L. aethiopica* (Akuffo *et al.*, 1990) and it has been found to be effective in achieving complete (radical) cure. However, no major inhibitory effect was observed in itraconazole treated group that showed a significant difference from a placebo control group(Akuffo *et al.*, 1990).

Monomycin® and Nystatin® antibiotics have also been reported as effective

in anti-leishmaniasis therapy but they are regarded as of secondary and historical importance. Some workers have recommended the use of metronidazole as an effective drug in treating cases of cutaneous leishmaniasis (Pedersen and Sawicky, 1975) but other studies did not confirm this (Griffith, 1976). Metronidazole (flagyl®) for treatment of amoebiasis and trichomoniasis has been used to treat leishmaniasis and clinical cure has been reported (Bassiouny, 1983). It is thought that the selective toxicity of the drug against the parasite is due to reduction of the nitro group on the drug inside the parasite.

Nifurtimox, a drug of choice in chaaga's disease (America trypanosomiasis) when used against certain leishmaniasis at dosage between 10 mg per kg per day for three days to 20 mg per kg per day for 10 days clinical healing of between 40% - 60% has been reported (Guerra *et al,.,* 1981, Marsden *et al.,* 1979). However, side effects were common and severe with higher doses that include anorexia, weight loss, insomnia and personality changes. Eight aminoquinolines are found to be exceptionally effective in treating the hamster model of visceral leishmaniasis (Kimmamonk *et al.* (1979) but no clinical trials have been reported.

Levamisole the antihelminthic drug has also been tried against *Leishmania.* This drug has a property of potentiating T-cells. Cell mediated Immunity (CMI) has been observed tó play a role in subclinical leishmanial infections early in life (Bulter, 1982) but infections increase later with declining CMI levels. Levamisole treatment potentiated the T-cells to assist in combating the parasite, however this still needs to be further investigated.

Furazolidine has also been reported to have a high antileishmanial activity against amastigotes in human macrophages (Berman & Lee, 1983). Phenothiazines which are psychoactive drugs used in treating psychiatric

disorders have antitrypanosomiasis and antileishmanial activity. Treatment of DCL due to *L. aethiopica* with topical chlorpromazine® improves inflammatory responses that increase levels of immune cells and parasite smears were negative after one month (Henriksen and Lenden, 1983). Notwithstanding scars and skin discloration is not altered and therefore a more favourable choice for treatment.

Apart from the medical prophylactic methods of treating leishmaniasis, there are other non medical methods of treating leishmaniasis. Plastic surgery has been used to treat disfiguring scars especially of leishmaniasis recidivans caused by *L. aethiopica* and *L. tropica.* Surgical treatment is quick and simple with few side effects eventhough the cost is high.. In simple cases, it requires one or two visits to the clinic (Heugchong, 1986). This processs usually involves surgical currettage under local anesthetics (Currie, 1983).

Similarly, *Leishmania* parasites being thermosensitive protozoa, have been treated using heat and cold therapy. Heat treatment usually involves a water bath with circulating water through pads wrapped around the lesion and maintained at temperatures between 39° C to 41° C for at least 20 hours for several days. However, different strains/species of *Leishmania* vary in sensitivity to elevated temperatures (Neva *et al.*, 1984). Cold treatment (cryotherapy) has advantage over heat treatment because there tends to be less inflammation. Cryotherapy is used with carbon dioxide cryomachine resulting in good cosmetic results as the skin's collagenous frame work is left intact. Cryotherapy is also simple, rapid and effective and can be used as an alternative to antimonial therapy but may not be used alone in cases of mucosal leishmaniasis due to *Leishmania braziliensis* that get systemic and it is suggested that cryosurgery be used as an alternative (Jalliffe and Bryceson, 1983).

1.1.3 Traditional medicinal drugs in the fight against leishmaniasis.

Traditional medicine has been a major treatment form for several diseases in countries all over the world. In Africa such practices were passed verbally over successive generations and today many still attach great value to traditional medicinal system.

It is estimated that 75% of the African population use traditional medicine (Gbeassor *et al.*, 1989) to treat various diseases such as viral (Sysdiskis *et al*, 1991), protozoal (Philipson and Wright, 1991), and bacterial (Pacheco *et al* 1993). The present scientists in the field of pharmacognosy recognise the fact that before reaching a decision on which medicinal drug to investigate you have to look at a number of factors which presumably are:

(i) ethnobotanical information gained from traditional medical healers/ practitioners.

(ii) the need to develop new drugs and compounds that can combat diseases that do not have a cure and are proving resistant. And the hope is that novel chemotypes may be found in medicinalal plants.

(iii) the adoption of a rational way and enviromentally friendly option of obtaining chemical types from plants that are naturally obtained and biodegradable.

The above factors therefore have led researchers to study or investigate the effects of a number of plant extracts on various parasitic diseases. Some of this work has concentrated on leishmaniasis which is now on the resurgence due to several factors varying from lack of a satisfactory treatment, occurence of relapses, high cost of available drugs, with the overall effect that patients are unable to acquire them and major adverse effects of the available drugs

(Haidarius and Bonventure, 1983). This has prompted an overwhelming interest in the search for new antileishmanial compounds especially from natural plant products. Alot of studies are being conducted all over the world to identify novel chemotypes from plants that may help in the fight against leishmaniasis. Iwu *et al.* (1994) reviewed some medicinal plants that have been identified to have antileishmanial activity. Most of these medicinal plants have been in use in traditional societies (in endemic zones of leishmaniasis) for the treatment of the leishmaniasis. Under more scrutiny and advanced bioanalyses, some of the plants have been found to have active compounds with diverse chemical structures, a factor that makes cross resistance virtually impossible.

The most important and acclaimed naturally occuring antileishmanial group of compounds is the isoquinolines. Berberine, a quatenary isoquinoline alkaloid widely distributed in nature was reported by Vennerstrom *et al.* (1990) to have significant *in vitro* and *in vivo* activity against various species of leishmania. The compound was isolated from *Enantia chlorantha.* Berberine activity against *L. major* is thought to be due to the quatenery nitrogen atom that occurs in the alkaloids and this was demonstrated to be active against *L. major* (El-on and Messer, 1986).

Indole alkaloids have also been shown through laboratory studies to possess significant antileishmanial activities. The extract of *Peganamum hamala* (Apocynaceae) and seed extracts of *Picralima nitida* (Apocynaceae) which posses indole alkaloids have been used in folk medicine in North Africa and West Africa respectively for treatment of dermatosis including cutaneous leishmaniasis (Iwu *et al.*, 1994).

Benzylisoquinoline alkaloids are widely distributed in nature and have been isolated from many plants used in the treatment of many parasitic diseases.

Plant families possesing this chemical include Annonaceae, Berberidaceae, Hemandiaceae, and Menispermaceae that possess the chemical compounds gyrocapine, dapliandrine, and obaberine (Iwu et al. 1994).

Licochalcone A isolated from Chinese licorice is an oxygenated chalcon that has been found to inhibit *in vitro* growth of *Plasmodium falciparum*, the human malaria parasite and offers protection against *Plasmodium yoelii* in mice (Chen *et al.,* 1994) and also inhibits the *in vitro* growth of both *L. major* and *L. donovani* promastigotes and amastigotes (Chen *et al.,* 1993).

Several members of Bignoniaceae have quinones, a group of chemical compounds that possess antileishmanial activity . The synthetic analogues of these quinones such as quinol and quinone acetate have been found to be more active against *L. amazonensis* promastigotes *invitro* (Iwu *et al.*, 1994). The *Polyathia macropoda* (Annonaceae) plant bark contains a terpene (labdane diterpene) which has been shown to be an active antileishmanial agent. Another isolate from *Picrorhiza kurroa*, an iridoid glycoside, has immunostimulating activity owing to the presence of pricroliv, a chemical isolate. This extract was shown to induce a high degree of immune protection in golden hamsters challenged with *L. donovani* promastigotes (Puri *et al.*,1992).

In this research the effect of the castor oil plant (*R. communis*) extracts on the course of leishmaniasis due to *L. major* was investigated both *in vivo* and *in vitro.* This was based on evidence that plant sap from the leaves of this species causes mortality of upto 50% and 88% deformations of *L. major* parasites in infected sandflies that probe its leaves (Schlein and Jacobson, 1994). The leaves of this plant have also been used to forment sores, boils and swellings (Watt and Breyer-Brandwijk, 1962).

The leaf infusion of this plant has been used by the Zulu as a remedy for stomachache when administered orally and the paste of *R. communis* root on toothache when applied on the gums (Watt and Breyer-Brandwijk, 1962). In Southern Rhodesia (now Zimbabwe) the bark was used as a dressing for wounds and sores. Pounded leaves also applied over Guinea worm (*Dracunculus medinensis*) sores is known to extract the worm from the wound (The Wealth of India, vol. IX). The various uses of this plant in traditional medicine is outlined by Watt and Breyer-Brandwijk (1962).

The tree is known to grow wildly even though sometimes it may be cultivated for its seeds. The *R. communis* plant exhibits considerable morphological variations (Smith, 1987) and it is thought to have originated from N.E. tropical Africa (Radcliffe-Smith, 1984) but is now found growing wild and in cultivation in all warm regions. It grows well in tropical summer rainfall areas but may also grow well in wet tropics to the sub-tropical dry regions.

1.2 JUSTIFICATION OF THE STUDY.

Pentavalent antimonials, the drugs of choice in treatment of the leishmaniases are toxic drugs and their use in treatment is due to the inavailability of other equally effective chemotherapeutic agents. Despite their undesirable properties, these drugs are very expensive and in most cases are not available in rural hospitals. The leishmaniases have also emerged as serious opportunistic infections in HIV/AIDS in developed and developing countries (Scaglia *et al.* 1989; Molina *et al.* 1992). *Leishmania*/HIV co-infection impose difficulties in terms of diagnosis and treatement, and can lead to epidemiological changes which modify the traditional patterns of zoonotic visceral and cutaneous leishmaniasis.

The leishmania pathogen is intracellular and they attack the phagocytic cells of the immune system. There is therefore need to identify safer, cheaper and readily available antileishmanial compounds for use in treating *Leishmania* infections. The extract that was tested for anti-*L. major* activity was derived from *R.communis*.. Another important reason for conducting research on the castor oil plant (*R. communis*) is that it is a very common plant and grows wildly or can be easily cultivated under the tropical climatical conditions. This plant if grown on a large scale in areas endemic for leishmaniasis, firstly, will be an easy source of income through the sale of the castor oil seeds and, secondly, the oil from the seeds is of medicinal value used in treating stomachache and as a skin lotion (Watt and Breyer-Brandiwijk, 1962). Sandflies are known to probe the *R. communis* green plants for sap and it has been shown that juices from this plant cause 50% mortality and upto to 80% deformation of *Leishmania* parasites in infected sandflies. This phenomenon may effectively help in reducing disease transmission in an endemic area.

1.3 OBJECTIVES

1.3.1 General objectives

1. To determine whether *R. communis* leaf extracts have quantifiable effects against *L. major in vitro* and *in vivo* in BALB/c mice.

2. To determine whether seed oil from *R. communis* when applied topically has any effect on the course of *L. major* in BALB/c mice.

1.3.2 Specific objectives.

1. To determine effect of aqueous leaf extract on live *leishmania* promastigotes.
2. To compare the effects of treating *L. major* infected mice with *Ricinus communis* aqueous leaf extract, seed oil extract and with Pentostam® (sodium stiboglucomate).
3. To determine the *Ricinus communis* leaf extract optimal dosage for the intraperitoneal treatment of BALB/c mice.

CHAPTER TWO:

MATERIALS

AND

METHODS

2.0 Materials and Methods.

Most of the materials used to execute this project were obtained from the *Leishmania* Research laboratory of the Biomedical Sciences Research Centre, Kenya Medical Research Institute. *Ricinus communis* (castor oil) plant materials were picked from a living plant on site(plate 1) in Kenyatta Market Masandukuni (Latitude: 1^{O} 18' 52"S; longitude: 36° 48' 12"E) about 5 miles from Nairobi city centre. The already harvested seeds were donated by Dr. Chris Anjili of the Biomedical Sciences Research Centre , Kenya Medical Research Centre.

Leishmania major (Strain IDUB/KE/83=NLB-144) routinely maintained in BALB/c mice by serial passage was used in this study. This parasite was initially isolated from a wild caught *Phlebotomus duboscqi* sandfly that was trapped near the town of Marigat, in Baringo District of the Rift Valley Province (Beach *et. al.*, 1985).

One hundred and fourty female weanling inbred BALB/c mice obtained from the KEMRI animal house were used. These were divided into 4 groups of 35 mice each and then seperately labelled with picric acid (BDH Chemicals, Poole, England) for easy identification and to avoid mix-up. Another group of 30 female inbred BALB/c were divided into 6 groups of 5 animals each and were used to determine the optimal *R. communis* leaf infusion dosage in a dosage-response study and toxicity studies.

Plate 1: Photograph showing the *Ricinus communis* plant on site.

2.1 Extraction of plant materials.

(a) Aqueous leaf extract (infusion)

One kilogram of fresh wet leaves picked from the *R. communis* plant was macerated in two litres of distilled water using an electric blender and later straining the juices using a clean cloth from the minced leaves into a clean beaker. The plant sap obtained was filter-sterilized through 0.8µm, 0.45µm, 0.2µm Nalgene filters before being lyophilized in a freeze drier (Chemical Instruments Ltd, UK) at a temperature of -35°C for 72 hours and at a pressure of - 10 atmospheres (Hg) which were directly read from the freeze drier.

(b) *Ricinus communis* seed oil

The dried *R. communis* seeds were crushed using a grinding machine and the pasty material collected in a round bottomed flask. The oily paste was fractionally distilled with diethyl ether in a fractionating chamber and the oil collected in a flat bottomed flask.

2.2 Cultivation of *Leishmania major* parasites.

2.2.1 Media preparation.

Growth media for the *Leishmania* parasites was prepared in line with specifications of Childs *et. al.* (1978). Briefly, 24.08 grammes of bottled sample of Schneider's insect *Drosophila* medium formula, Batch number S-9895 (SIGMA Chemical Co., UK) which comes inclusive of Glutamine, Cytosine and Tyrosine was dissolved with a magnetic stirrer in one litre of distilled water. Sodium bicarbonate (0.4g) and calcium chloride (0.6) respectively were added to the disolution mixture. Four grammes of yeastolate

was dissolved separately and added to the above mixture. The pH was then adjusted 6.45 by using 1N sodium hydroxide and 1N hydrochloric acid. The anti-fungal, 5-flourocytosine (Kimber *et al.*,1981) and antibiotics Penicillin-streptomycin and Gentamycin (Hendricks and Wright, 1979) were added to the mixture to avoid the risk of having the the cultures contaminated with bacteria and/or fungi. Finally, 20% volume/volume of heat inactivated Foetal Bovine Serum (FBS) was added to the mixture to serve as extra nutrient supplement. The final mixture was then filter-sterilized stepwise through 0.8μm, 0.45μm, 0.2μm Nalgene membrane filters (Nalge Company, Rochester, New York, USA) before being stored for usage at 4°C.

2.2.2 Cultivation of *Leishmania major* parasites from infected footpad.

L. major infected BALB/c mice with swollen puss-free lesions were selected and used for parasite isolation. Subcuteneous aspirates were taken by inoculating 0.2 ml of sterile normal saline reconstituted with antibiotics into the edge of the footpad with the aid of gauge 26 inch needle. Without withdrawing the needle it was rotated 3-4 times whilst still in the skin to cut small pieces of tissue from the edge of the needle wound. As much liquid as possible was aspirated from the footpad ensuring that the aspirate contained no more than a trace of blood. This aspirate was then used to inoculate cultures. Culture flasks (corning®) containing Novy Nicolle Mc Neal, (NNN) mediun overlaid with Schneider's *Drosophilla* medium were used and the cultures were left to grow for 4-7days upto the stationary phase. Parasites were then counted using a similar method to Dacie and Lewis, (1966). Briefly, parasites were centrifuged at 2500 rpm to give a pellet. This pellet was resuspended in a small volume of phosphate buffered saline and vortexed to homogenize the

mixture which was then put in hot water to immobilize the parasites. By using a capillary tube, the improved Neaubeur counting chamber was charged before the parasites were counted usinga hand tally counter.

2.3 *IN -VITRO* ASSAYS.

2.3.1 Flagellar motility

An *in vitro* assay was conducted to determine the effects of the infusion on flagellar motility which was determined by counting the number of active flagellar in at least 500 promastigotes per field, growth inhibition and cell deformation.. A 96 well microtiter plate was used in this experiment. Lyophilized *R. communis* aqueous extract stock (1 gm in 10 ml) was serially diluted to 1:100, 1:200, 1:400, 1:800, and 1:1600 and five replicates were set up in the microtiter plate. A seed oil extract and a control containing Schneider's *Drosophila* medium in 20% FBS was also set up in five replicates. The *L. major* parasites were washed as previously described at 2500 rpm for 15 minutes at 4°C and then resuspended in 450 µl of PBS/ Schneider's *Drosophila* medium with 20% FBS. The parasites were counted and adjusted to a concentration of 1 x10^7 per 10 µl. The parasites were introduced into the wells labelled A-G under sterile conditions.

WELLS	CONTENTS.
A	*R. communis* dilution of 1:100
B	*R. communis* dilution of 1:200
C	*R. communis* dilution of 1:400
D	*R. communis* dilution of 1:800
E	Phosphate Buffered Solution, PBS
F	Schneider's *Drosophila* medium +20%
G	*R. communis* oil extract 1:1 (vol/vol)

Table 1: Extracts dilutions of aqueous *Ricinus communis* in the microtiter well plate.

2.3.2 Growth inhibition

Leishmania major parasites were adjusted to 1 x 10^6 parasites and before being introduced into $25cm^3$ culture flasks (Corning®) containing 6 ml of culture media with the extract dilutions. Culture flasks A-F were set up as follows.

(a) One tenth of a gram of lyophilized *R. communis* aqueous extract was dissolved in 10 ml of Schneider's Insect Medium supplemented with 20% volume to volume FBS to give a solution A.

(b) 1 ml of A was added to 10 ml of Schneider's /20%Foetal Bovine Seerum to give us a stock solution that contained 1000µg/ml of the Schneider's Drosophila Medium. Dilution of 500 µg/ml (solution A) was made in sterile hood conditions by adding half of the volume the stock solution to an equal volume of fresh SCH/20%FBS. The 250 µg/ml (B) dilution was obtained by adding equal volumes of 500µg/ml and fresh SCH/20%FBS. The same was done to obtain dilution 125 µg/ml (C), and 62.5 µg/ml (D). E which was fresh SCH/20%FBS served as a control. The cultures were then innoculated with parasites and counting was done after every 2 days to monitor growth.

2.3.3 Cell deformation.

Parasites obtained from the growth inhibition assay cultures were used to determine any deformation caused by the extract. Slide smears were made at the end of the growth inhibition study. They were then air dried and then fixed in methanol before staining with Giemsa at a strength of 1:10 for 30 minutes. These were then observed under x400 and x1000 magnification to see any

abnormal cell morphology.

2.4 *IN- VIVO* EXPERIMENTS.

2.4.1 Toxicity experiments.

Many plant infusions have chemical compounds that are toxic and that can lead to toxicity induced deaths. *R. communis* plant is known to contain some amounts of a proteinous toxin, toxalbumin an alkaloid compound and a naturally occcuring nitrogenous compound ricinine. The leaves are known to contain small amounts of the toxalbimin. Eventhough pure ricin is known to be poisonous and can be found in the leaves, the feeding of cattle on leaves of this plant in india is common and there has reports of deaths due to the laeves but tdue to the seeds. Therefore before injecting the animals intraperitoneally with the aqueous leaf extract, an experiment was conducted to determine the optimum dosage that could be used to treat *Leishmania major* infected BALB/c mice for a period of one month without causing any adverse side effects or causing death. Nine experimental groups of 5 weanling BALB/c mice each were injected with 0.1 ml of *R. communis* extract at concentrations of 500mg/ml, 400mg/ml, 300mg/ml, 200mg/ml, 100mg/ml, 50mg/ml, 30mg/ml, 20mg/ml and a control (PBS). Every week for 12 weeks the weights of the animals were taken and at the end of the treatment period the animals were sacrificed and weights of the spleen were noted down. The mice were observed keenly to monitor their behaviour.

2.4.2 Infection of mice

All female BALB/c weanling mice were marked with picric acid separately into 4 groups of 35 animals each. Hind footpads of all mice were then

measured using a direct reading vanier caliper and the mean footpad difference of all groups determined and are included in the appendices. These animals were inoculated with 1 x 10^6 culture derived *L. major* metacyclic promastigotes on the left hind footpad reconstituted in 10μl of PBS. The parasite was left to grow and establish in the mice for 4 weeks.

2.4.3 Experimental chemotherapy of *L. major* infected BALB/c mice.

Toxicity experiments established the optimal extract dosage suitable for treatment of BALB/c mice. In these experiments the antileishmanial activity of *R. communis* infusion, *R. communis* seed oil, and Pentostam® were compared with an untreated control. Treatment was commenced thirty days post infection . The first group was treated with 100 mg per kg of body weight of Pentostam®, the second group treated with the optimum established dose of 150 mg per kg body weight of the soluble aqueous leaf extract, the third group was treated with *R. communis* seed oil applied topically on the lesion using a paint brush and the fourth group was left as an infected but untreated control. Treatment was done daily for the oinment group while 5 times a week for the pentostam® and the *R. communis* leaf extract group. On a weekly basis, on the same day of the week, lesion measurements were taken for all the groups. After 4 weeks (30 days of treatment), a further 2 weeks extension period for treatment was added before sacrificing the animals. The spleen weights were recorded for each group and impression smears and cultures made to determine the degree of visceralization. Cultures of the uninfected right hind footpad (RHFD) were made to determine whether metastasis occurred. Parasite presence in the spleeen was quantified from the slide impression smears using the Leishmania donovani Unit (LDU) method of Bradley and Kirkley (1977).

Briefly , the number of amastigotes counted was multiplied by the weight of the organ in milligrams and by the constant 2 $\times 10^5$, which represents the difference in counts from organ impressions and dilutions of organ homogenates (Stauber, *et al.*, 1958).

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CHAPTER THREE:

RESULTS

3.0 RESULTS.

It was noted that one kilogram of fresh *Ricinus communis* leaves yields 32.8 grams of lyophilized powder after freeze drying. Solubilized powder of the extract were serially diluted as described and used to determine the direct activity on *L. major* promastigotes in culture. *L. major* parasites that hde been aspirated from a mouse infected footpad that typically grows to stationary phase in 5-7 days were used in the assay.

3.1. IN- VITRO RESULTS.

3.1.1 Motility experiments.

Flagellar activity (motility) were microscopically examined by observing 10 fields which averagely had 500 promastigotes per field. Obsevations were strictly done after every one hour until the eighteen hour when observations were stopped. For each of the field of views, flagellar motility and parasite movement was noted and this was done for each of the extract dilutions for all the replicates. Results indicated that flagellar motility and parasite movement decreased with increasing extract concentration until the 18 hour time lap where all parasites in the microtiter well plates appeared dead except for the control well, F, which showed parasite movement and flagellar motility that was normal. Flagellar motility and parasite movement also decreased with increase in the time of exposure to the extract concentration.

3.1.2 Growth inhibition.

The extent of growth was monitored on a two day basis until day 8 by quantifying the number of promastigote in culture by the method nearly similiar to that of Dacie and Lewis (1966) described before except that the parasites were imobilized with warm water. Briefly, parasites were centrifuged at 2500 rpm to give a pellet. This pellet was resuspended in a small volume of phosphate buffered saline and vortexed to homogenize the mixture which was then put in hot water to immobilize the parasites. By using a capillary tube, the improved Neaubeur counting chamber was charged before the parasites were counted usinga hand tally counter. The results of the assay showed that there was no signifcant differences (ANOVA, $F=0.004$, $P>0.999$) in parasite numbers at the end of the incubation period across the extract dilutions (Figure 5).

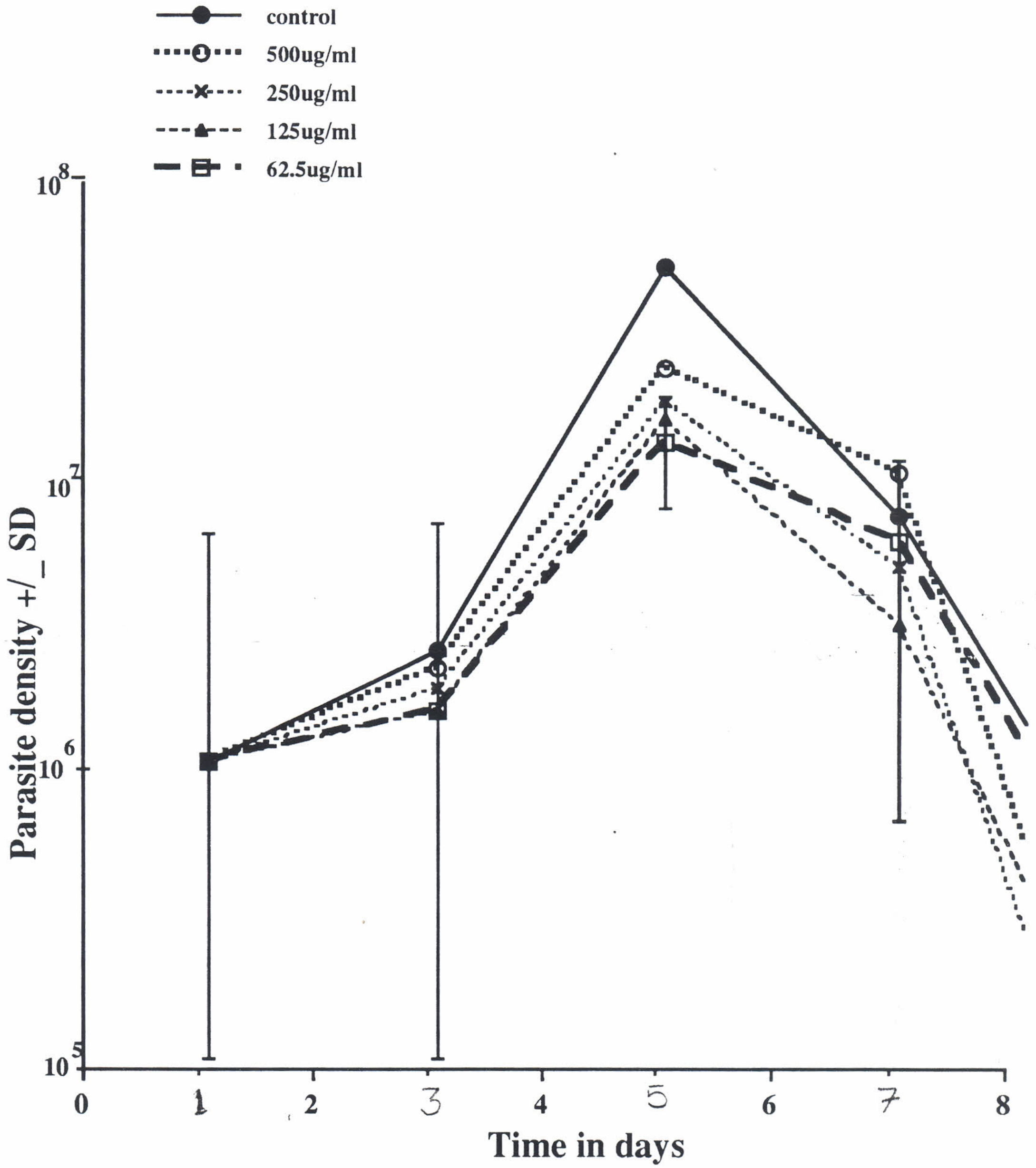


Figure 5: The course of L. major promastigotes growth when incubated with different concentrations of R. communis extracts in cell free culture media for eight days.

3.1.3 Cell deformation.

The effect of *R. communis* extract on cell morphology was determined by light microscopy. They were observed under x1000 and photomicrographs taken using a BH-2 photomicroscope (Olympus, Tokyo, Japan). Promastigote cell morphology was found to be abnormal (plate 2b) In addition, the slides showed that most parasites appeared like paramastigote stage and some parasites had withdrawn cytoplasm, away from the cell membrane. This was largely evident in the aqueous extract set-up which had paramastigotes and cytoplasm shrinkage. Clearly, the level of cell deformation and cytoplasm withdrawal was independent of the concentration of the extract.

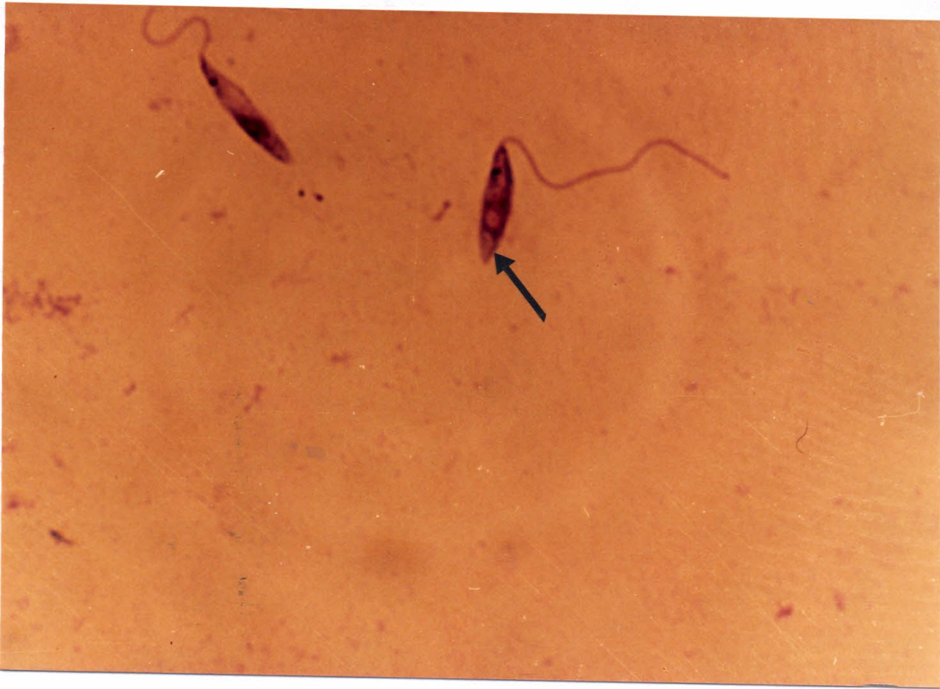


Plate 2a: Photomicrograph of normal (control) *Leishmania* promastigotes.

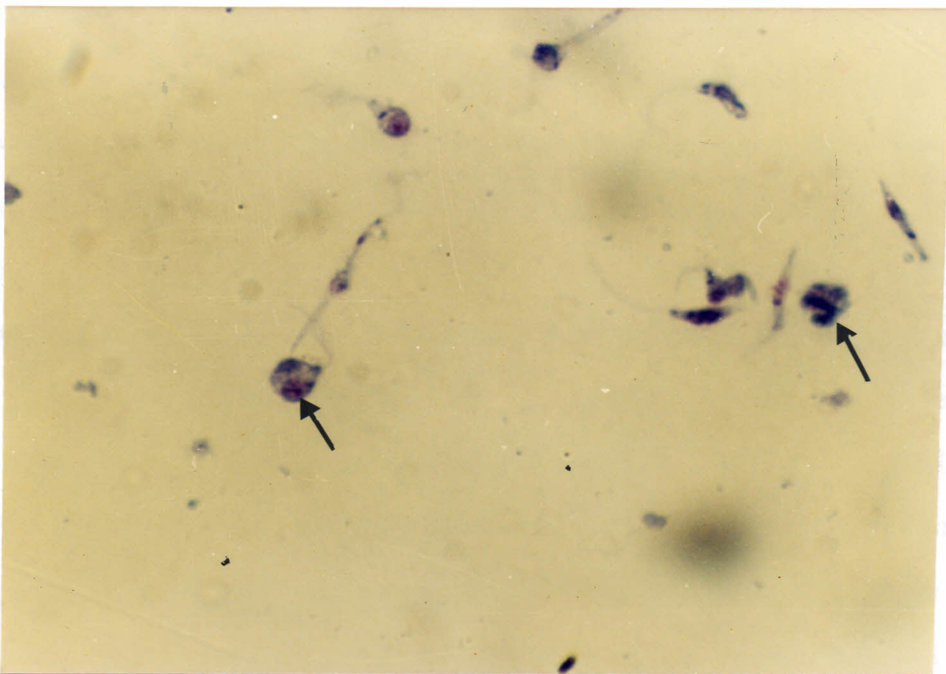


Plate 2b: Photomicrograph of deformed (experimental) *Leishmania* promastigotes

3.2 *IN- VIVO* RESULTS.

3.2.1 Toxicity results.

Toxicity tests were carried out to determine the optimal *R. communis* aqueous leaf extract dosage. Results were recorded on visual examination of the mice following intraperitoneal inoculation of the extract.

After intraperitoneal inoculation of mice with 2500 mg/kg bodyweight, mice develpoed severe muscular spasms indicating some form of shock. However no attempt was made to relieve them of this shock These animals later appeared droopy with ruffled fur . After about 30 minutes, their eyes begun protruding and at exactly 5 minutes later they all died. Mice inoculated with 2000mg/kg bodyweight displayed similar behaviour after 40 minutes but only 2 out of 5 mice died. The others died after about 4 hours. Similar behaviour was displayed by mice inoculated with 1500mg/kg bodyweight. After about 40 minutes , 1 animal was dead. The other animals were severely affected and on the second day of inoculation with the same dosage, all the four animals died.

After the treatment period, the animals were sacrificed and weights of the spleen noted as shown in Appendix 2. Results indicate that there was significant difference in the weekly average weights of the animals (ANOVA, F=142.14, P<0.0001) except for the 100 mg/kg bodyweight and the 150mg/kg bodyweight treatment groups. These two extract dilutions were the best dosages as the average weights of the in-bred BALB/c before treatment did not differ significantly with the weights after the treatment period (Tukey test, q=0.6053, P>0.05). Between the two dosages therefore, the higher dosage that did not affect the mice (ie 150 mg/kg bodyweight) was chosen. The 250

mg/kg bodyweight was considered inappropriate because the mice were affected adversely (prophylactic shock, staggering walk). Other observations made were that in the 250mg /kg bodyweight group, some animals developed tumourous growths on the tail, neck region (see plate 4) and below the nape .

TABLE 2: Weights in grams of BALB/c mice follwed through four weeks of administration of different concentrations of *R. communis* in the dose response study.

Leaf infusion dose	**250 mg/kg**	**150 mg/kg**	**100 mg/kg**	**control, PBS**
week 1	24.0	20.0	19.8	23.2
week 2	24.4	20.8	20.4	23.2
week 3	24.0	20.0	20	22.4
week 4	24.0	20.0	20.2	22.4
Average weight	**24.1**	**20.2**	**20.1**	**22.8**

When the mice were dissected to detect any changes in the viscera particularly the spleen and the liver, no significant differences was observed between the spleen weights (ANOVA, $F= 0.591$, $P> 0.05$) and there was also no significant differences between the liver weights (ANOVA, $F= 1.455$, $P> 0.05$) and hence it can be concluded that the *R. communis* aqueous leaf extract does not affect major changes in viscera of mice with special references to liver and spleen. It is important to note that the liver and the spleen are the organs of importance especially when determining the parasite load and in cases of hepatosplenomegally inferences can be made easily between infected and uninfected animals.

3.2.2 Infected footpads and lesion progression

The mean footpad sizes before infection of the mice did not differ significantly within groups (Students t- test, $t = 2.035$, $P > 0.05$) and betwen groups (Multiple t- test, $t = 2.353$, $P > 0.05$). Footpads of mice were measured once weekly and after the four week period the footpad sizes differed significantly among mice treatment groups (Pentostam®, seed oil, leaf infusion and control) (ANOVA, $F = 12.553$, $P < 0.05$) , however there was no significant difference among the mice treatment group standard deviations (Bartlett's test, $B = 4.714$, $P > 0.05$) as illustrated in Table 3.

TABLE 3. MEAN FOOTPAD LESION MEASUREMENTS IN MM + \- S. E OF MICE PER GROUP POST INFECTION AND POST TREATMENT IN EXPERIMENTAL AND CONTROL ANIMALS FOLLOWED THROUGH WEEK 1 POST-INFECTION TO WEEK 10 POST-INFECTION.

TIME IN WEEKS	MEAN FOOTPAD LESION SIZE SE PER GROUP				
	Pentostam	Seed oil	leaf infusion	Control	statistical difference
1	0.118± 0.058	0.021± 0.39	0.084± 0.04	0.096± 0.05	NS
2	0.184 ±0.032	0.213±0.033	0.199± 0.036	0.197±0.029	NS
3	0.726±0.071	0.559±0.058	0.739±0.044	0.706±0.041	NS
4	1.290±0.081	1.431±0.077	0.903±0.061	0.976±0.061	NS
5	1.249±0.068	1.556±0.093	1.140±0.074	1.285±-0.07	NS
6	1.632±0.082	1.831±0.101	1.363±0.101	2.099±0.079	NS
7	1.867±0.096	2.215±0.127	1.635±0.058	2.272±0.116	NS
8	1.662±0.085	2.407±0.111	1.966±0.111	2.715±0.161	NS
9	1.991±0.094	2.887±0.205	3.547±0.145	3.227±0.185	NS
10	1.597±0.117	2.684±0.175	3.382±0.141	3.252±0.206	NS

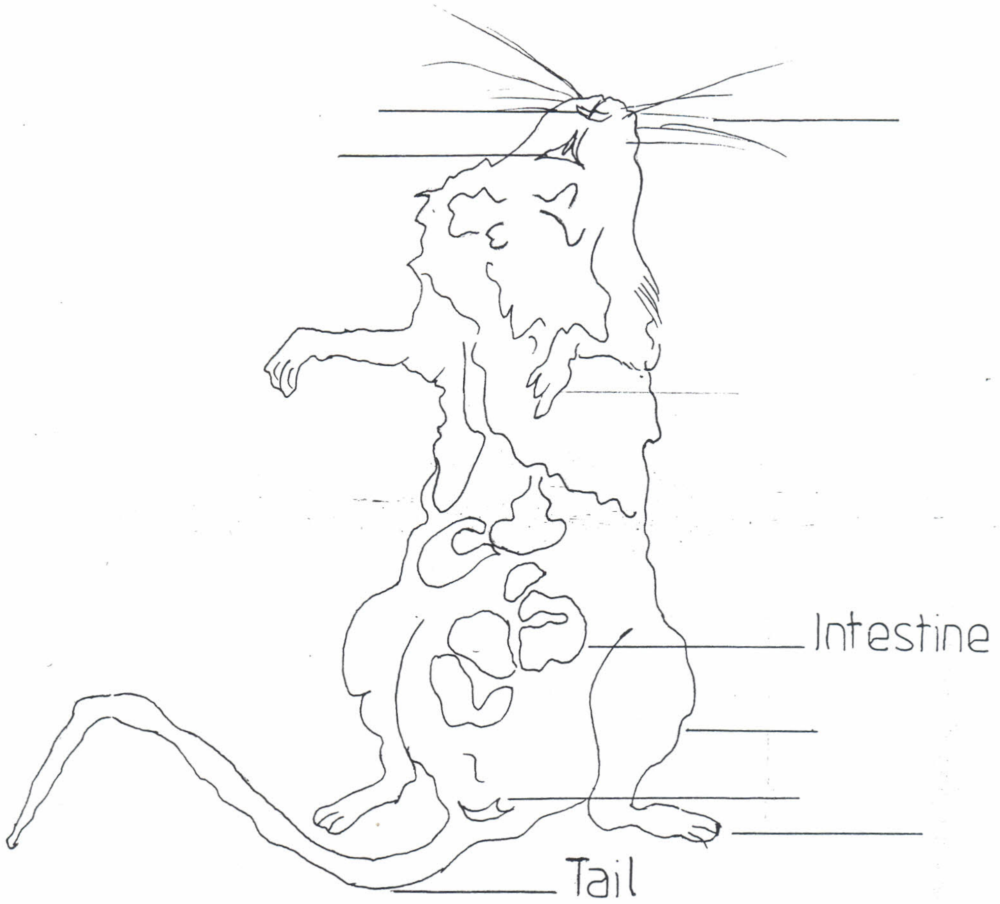

Intestine
Tail

Plate 3: A pathological specimen showing a BALB/c mouse with a huge tumour (T) following treatment with *R. communis* leaf extract (250 mg/kg bodyweight) at weeks 4 post-treatment.

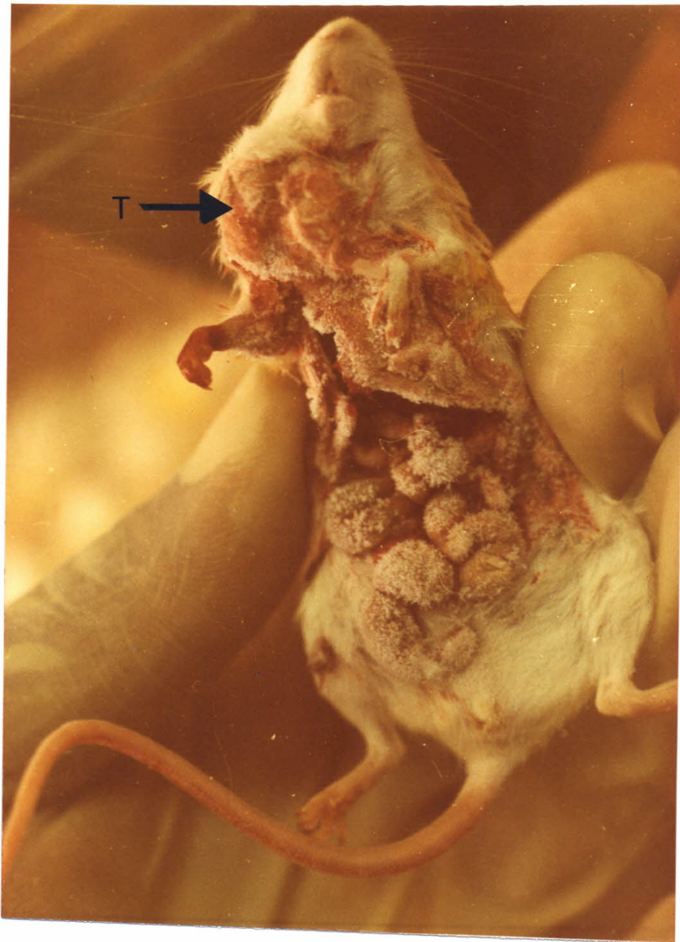
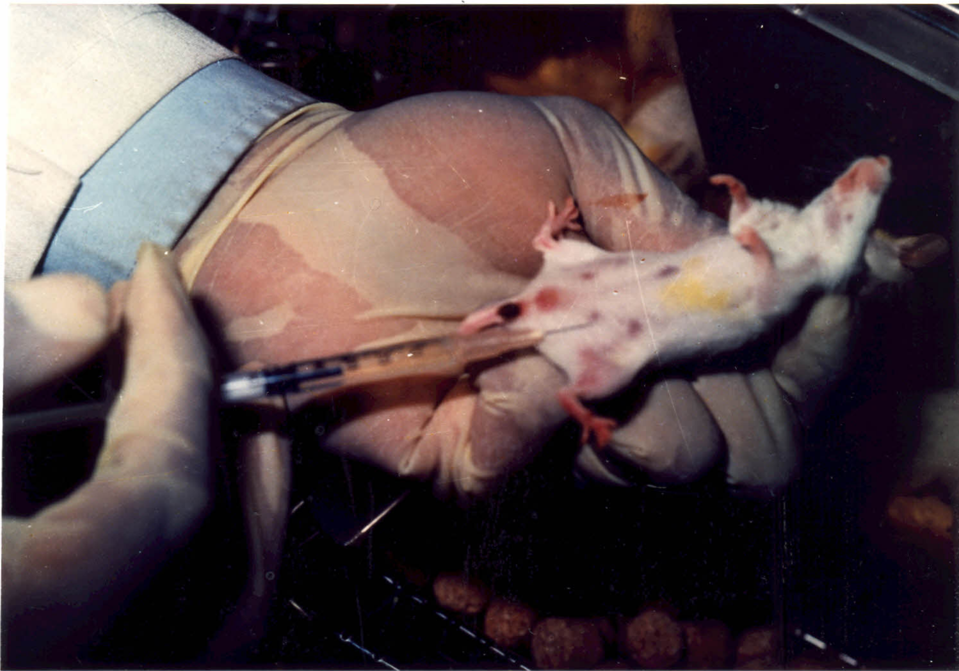


Plate 4: Administration of *R. communis* aqueous extract into BALB/c mouse peritoneal cavity.



3.2.3 Experimental chemotherapy of *Leishmania major* infected BALB/c mice.

Following treatment of the four groups of *L. major* infected BALB/c mice with *R. communis* leaf infusion, topically with the oil and with Pentostam®, a comparison of footpad lesion sizes showed that footpad lesions of the 4 groups of mice after treatment described above between the groups differed significantly (ANOVA, $F = 16.994$, $P < 0.0001$). A Tukey multiple comparison test performed on the groups' footpad lesion size showed that there was no significant differences between control and leaf infusion ($q = 1.973$, $P > 0.05$) and control and seed oil ($q = 2.027$, $p > 0.05$). However there was significant differences between control and Pentostam® ($q = 7.502$, $P < 0.05$), leaf infusion and seed oil ($q = 4.059$, $P < 0.05$), leaf infusion and Pentostam® ($q = 9.061$, $P < 0.001$) and seed oil and Pentostam® ($q = 5.571$, $P < 0.001$). From the statistics done it shows that treatment affected lesion development and based on these facts we can pinpoint the most suitable treatment.

The range in mean footpad differences was 1.2363 for the control vs Pentostam® comparison which indicates that activity of the Pentostam® still remains comparatively better than the other extracts (Figure 6).

Plate 5: *Leishmania major* lesions on footpads of BALB/c mouse 3 weeks post infection

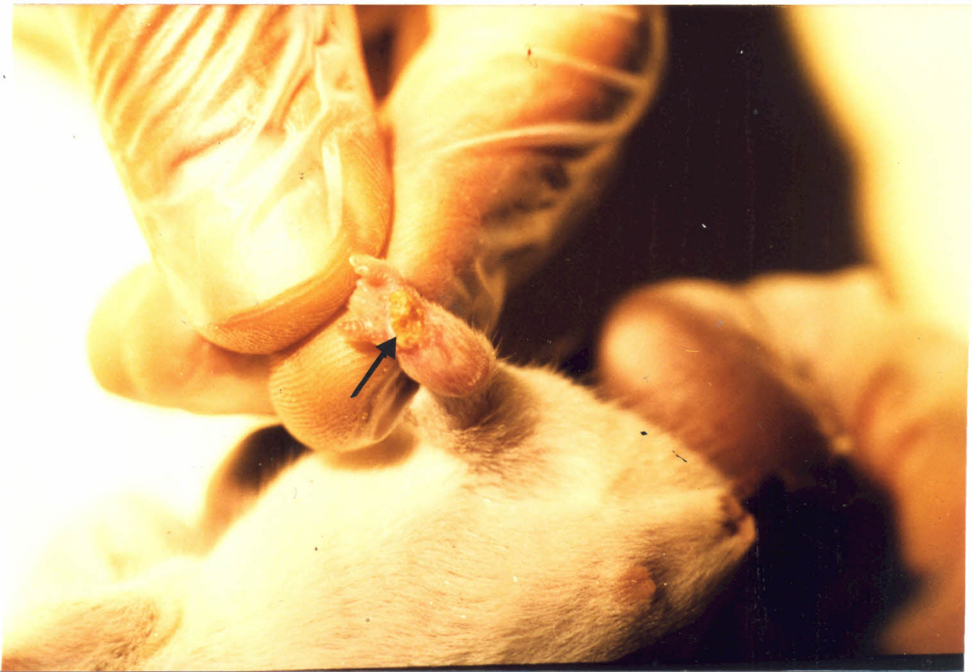


Plate 6: Extreme case of *Leishmania major* infection on BALB/c mouse footpad.

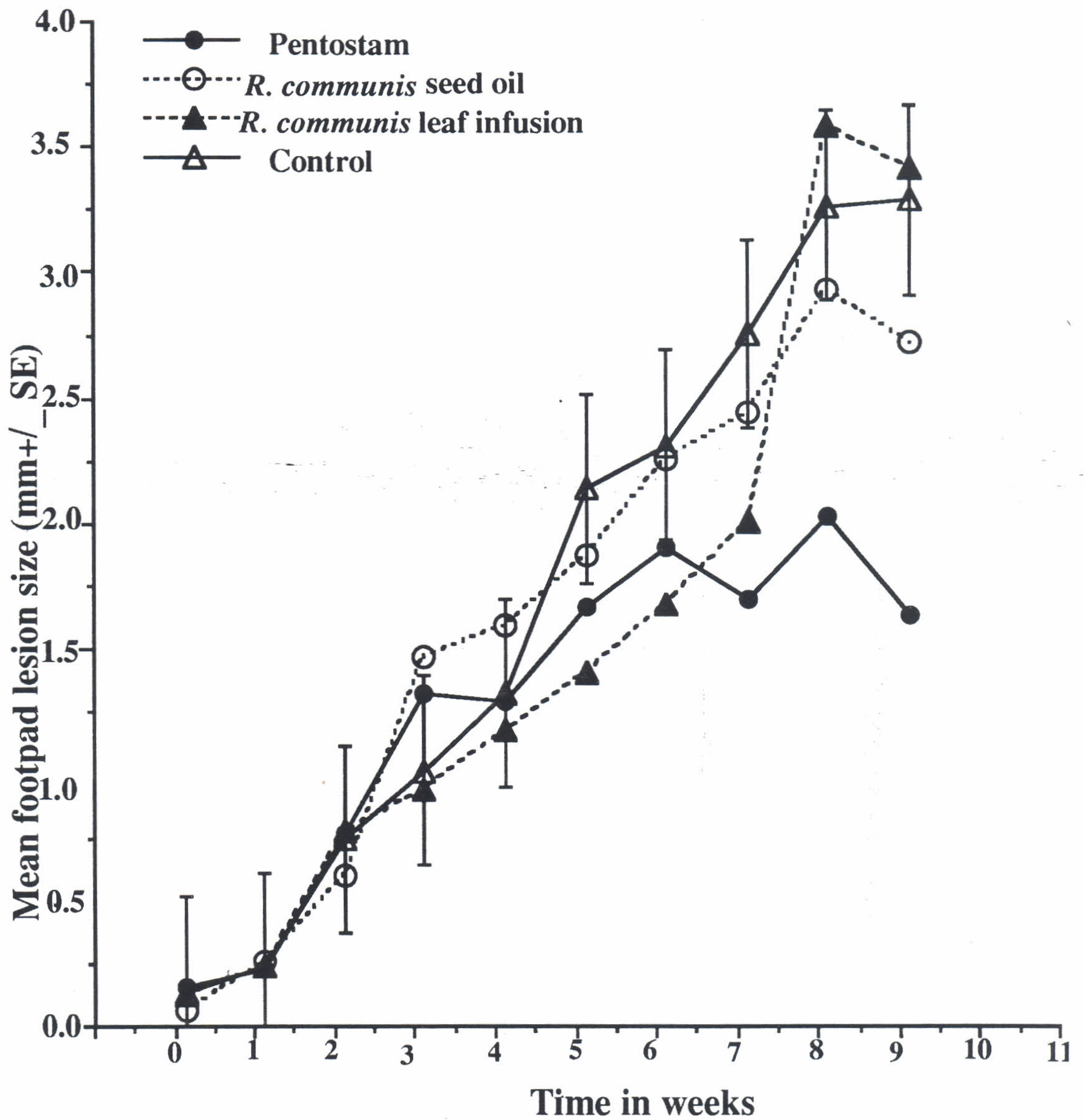


Figure 6: Footpad lesion sizes progression in mice post-infection and treatment with the Ricinus communis extracts, Pentostam and untreated control.

(a) Spleen weights.

At the time of sacrificing the animals at 14 weeks post infection , weights measurements of the spleen indicated that there was significant differences in the spleen weights between the treatment groups (1,2,3,4) (ANOVA, $F = 20.53$, $P < 0.0001$). However on further comparison using the Tukey- Kramer Multiple Comparison test, it was shown that a significant difference existed between all the other groups except for the leaf infusion treatment group and the seed oil treatment groups. (see Table 4 below)

TABLE 4: Tukey- Kramer Multiple Comparison Test of spleen weights between mice treatment groups.

Comparison	mean difference	q	P - value
control vs leaf infusion	- 102.20	4.415	P<0.05*
control vs seed oil	-137.24	5.929	P<0.001***
Control vs Pentostam	110.32	4.494	P<0.05*
leaf infusion vs seed oil	-35.04	1.514	P>0.05 ns
leaf infusion vs pentostam	212.52	8.657	P<0.01***
seed oil vs pentostam	247.56	10.084	P<0.001***

key.

* ....significant.

**.........very significant.

***........extremely significant.

ns.........not significant

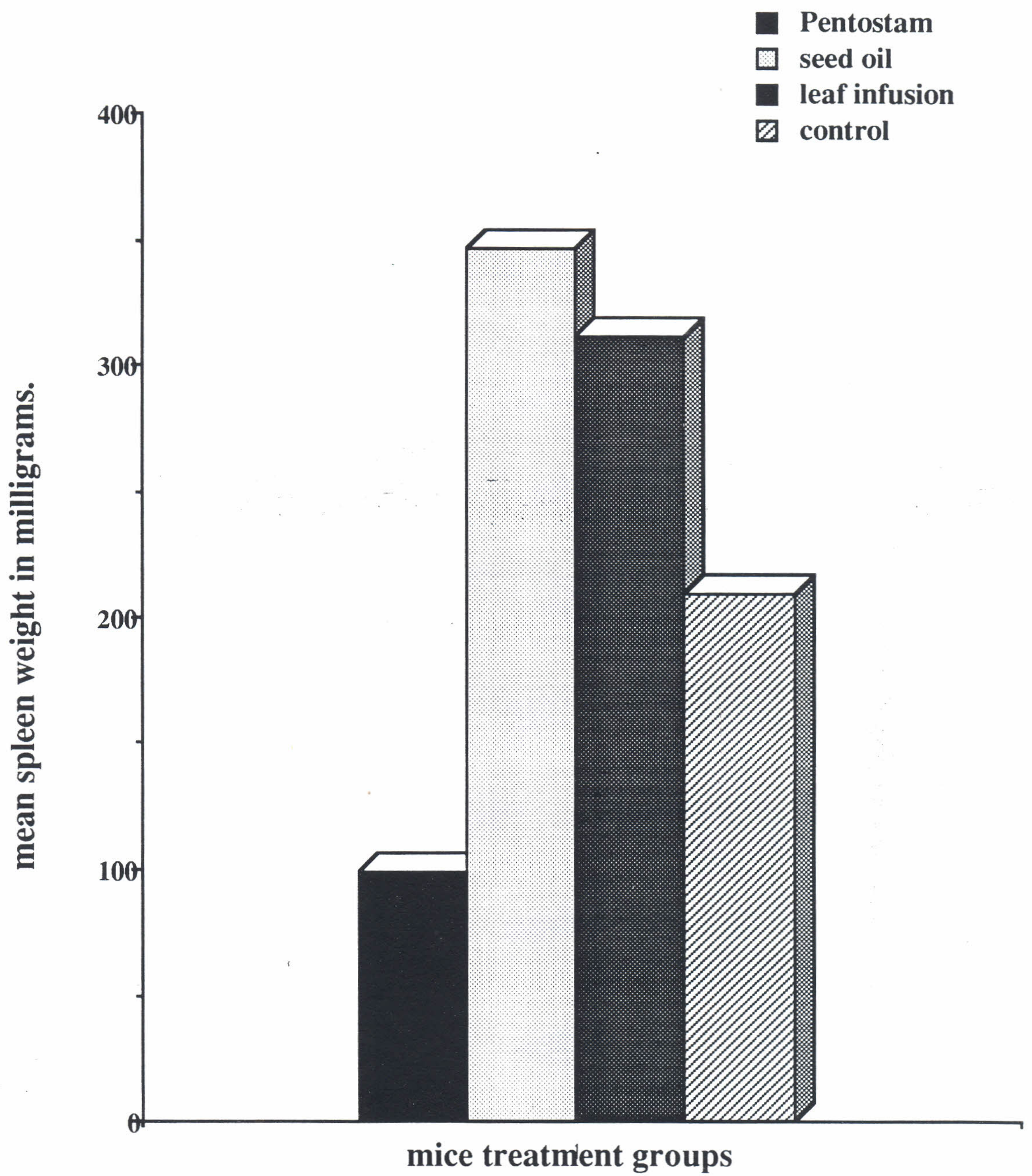


Figure 7: Mean spleen weights at 6 weeks post treatment

Post mortem results also indicated that spleen sizes were very enlarged in the seed oil treatment group (average weight = 346.76 mg) and the leaf infusion treatment group (average weight = 311.72 mg). Spleen size in the control group (average weight = 209.52 mg) only showed slight enlargement and the pentostam group (average weight = 99.20 mg) showed about normal spleen size when compared with the other two groups namely seed oil and leaf infusion.

(b) Culture results.

Cultures of spleen for detection of visceralization and cultures of right hind footpad (RHFP) to detect metastasis were observed everyday for up to14 days. At least 55% (11/20) of the seed oil spleen cultures turned positive after 4 days while 28% (7/25) of the footpad cultures turned positive after the same period. 12% (3/25) of the leaf infusion group spleen cultures turned positive and 8% (2/25) of the right hand footpad cultures of the same group turned positive. Cultures that turned positive after day 7 remained positive with no other culture becoming positive until day 14, the last day. By day 14, 10% (2/20) of both the spleen cultures and footpad cultures tured positive in the pentostam group, 72% (18/25) of spleen cultures and 36% (9/25) of the RHFP cultures turned positive in the seed oil group; 32% (8/25) of spleen cultures and 16% (4/25) of the RHFP cultures turned positive in the leaf infusion group. Seventy percent of (14/20) of spleen cultures and 55% of RHFP cultures turned positive in the control group after 14 days of incubation (Table 5).

TABLE 5: SUMMARY OF ORGAN CULTURES OF MICE TREATMENT GROUPS INOCULATED AT NECROPSY AFTER 14 DAYS.

Results of 14-day old organ cultures at 6 weeks post-treatment with Pentostam, Seed oil and Leaf infusion..

Treatment	RHFP	SPLN
Pentostam®	2/20 (**10%**)	2/20 (**10%**)
Seed oil	9/25 (**36%**)	18/25 (**72%**)
Leaf infusion	4/25 (**16%**)	5/25 (**20%**)
Control	11/20 (**55%**)	8/20 (**40%**)

KEY:

RHFD- Right Hind fotpad.

SPLN - spleen.

(c) **Leishmania donovani Unit counts.**

The *Leishmania Donovani* Unit was calculated and it was found that the control had the highest LDU value followed by the seed oil, leaf infusion and and the least was Pentostam® treated group which had the least. Statistical analysis showed that there was signficant differences between the LDU of the treatment groups (ANOVA, $F = 6.204$, $P < 0.05$). A multiple comparison test (Student Newmann-Keuls statistic) performed on the data indicated that there was significant differences between pentostam group and the control ($q = 5.566$, $p<0.01$), leaf infusion group and the control ($q = 4.787$, $P< 0.01$), and seed oil group and control ($q = 3,021$, $P < 0.05$). No significant differences was noticed in the LDU values on comparison of the leaf infusion and seed oil group ($q = 0.999$, $P > 0.05$)

4.0 DISCUSSION

4.1 *IN VITRO* EXPERIMENTS

4.1.1 Motility experiments.

Flagellar motilities that were observed microscopically at magnifications of x40 (ocular) and x10 (eye piece) indicated that the flagellar motilities decreaesed with increasing extract concentration. This is very indicative of the fact that there is a rapid action by the *R. communis* extract components on the parasite at levels detectable by flagellar motility and for this matter, the longer the time of exposure to the extract dilutions the more evident was the effect. It is not exactly clear why motility of the flagellum is affected but it can be deduced that threr may be some factors from the extract that bind along the flagellum and thus interfering with its motility. Therefore, the aqueous leaf extract effect on promastigote flagellum was seen as slowing its motility.

4.1.2 Growth Inhibition

L. major parasites typically reaches metacyclic form in 5-7 days (Hendricks & Wright, 1979). During the growth inhibition assay, approximately 10^6 parasites were introduced into the experimental culture flasks (Corning®, USA). In all the flasks there was a steady increase in parasite numbers. Control flask (without extract) had highest average parasite density by day 5 followed by 500µg/ml, 250µg/ml, 125µg/ml and least was 62.5µl/mg. This was rather unusal because it was expected that the higher the concentrations, the slower the growth. However, in the last three days of observation, the least dilution 62.5µg/ml and a control had parasite numbers that were almost the same. Similarly it was seen

that the 500, 250 and 125 were within range of each other especially after day 8. When statistical analysis was performed on the day eight final parasite numbers of each dilution, no significant differences were observed (ANOVA, $F=0.0004$ $P>0.999$). These results which are showing us that minimal in-vitro growth inhibition is taking place may be surprising or unexpected but it is consistent with results of Philpson and Wright (1990) who observed that extracts of *R. communis* especially proteinous toxins from the seeds had minimal effect *in vitro* on *Leishmania infantum.* It may also be that there could be some compounds in the aqueous extracts that favour growth of parasite while others are detrimental (acting antagonistically) in the effect on *L. major* promastigotes. However, this is open for further discussion.

4.13 Cell deformations

Deformations of *L. major* caused by extract for *R. communis* has been observed in *P. papatasi* (Schlein and Jacobson, 1994) and in extreme cases these parasites were found dead. Morphological deformations that were observed in our assay showed some parasites having rounded distorted shape (plate 2b) and in some we observed withdrawn cytoplasm from membrane which could indicate that there was systematic evolution of the cytoplasmic contents leading to the death and empty shells observed by Schlein and Jacobson, 1994. It is also probable that there could be shared constituents in the *R. communis* extract that is also present in the parasite cytoplasm, that could create higher osmotic potential leading to movement by it from cytoplasm content into growth medium. This is still a postulation.

4.2 *IN -VIVO* EXPERIMENTS

4.2.1 Toxicity experiments

The optimal *R. communis* dosage injectable intraperitoneally was determined after dilution ranging from 2,500, 2,000, 1,500, 1,000, 500, 250, 150, 50mg/kg bodyweight and control were tested in female BALB/c mice. Optimal dosage was found to be between 250-150 mg/kg bodyweight with 250 showing more toxicity judging from the nature of the shock induced and tumours that developed in the animals (see plate 3). The weights between these two treatment groups did not differ significantly (Tukey-test, $q=0.6053$, $P>0.05$) from a PBS placebo control.

Hence, to keep drug induced distress symptoms at a minimum, the lower optimal dosage (150 mg/kg bodyweight) was chosen . It should be noted that the toxic toxalbumin ricin and ricinine that occurs in this plant is predominantly in the seed. Other plant tissues also are poisonous but to lower levels to an extent that leaves have been used as fodder for cattle in India (Watt and Breyer-Brandijiwik, 1962; The Wealth of India, vol IX). The toxins in the leaves is very low to cause any fatalities unless taken in with seeds that have stuck on them or in unusually high amounts. It is apparent that the experimental mice withstood the dosage administered as toxin levels were neglibible.

4.2.2 Infected footpads and lesions progression.

A typical BALB/c mouse footpad measures between 2mm to 3mm on vertical cross-sectional measurement. Upon intradermal innoculation with *L. major* promastigotes, the footpad will begin to increase in size owing to the establishment of the parasites in the tissue and the disease sequelae. After about two weeks and six weeks when visible evidence of swelling is noticed, there is a

lot of macrophage infiltration in this site and lesion will take the form of a papule (see plate 5). In my study treament was commenced at this stage. From this papular lesion an ulcer will form and this will be observed as a wound (Ridely and Ridely, 1983). A late chronic phase often presents with cell granulomas and fibrosis in the dermis presenting in ten to twelve weeks. It is also at this time that the parasite metastasises and visceralizes (Hill, 1988). In severe and extreme cases, footpads are often cut off (see plate 6) and the mice are left limbless. In the experiments I conducted all these categories were observed. Just before commencement of treatment all footpad sizes did not differ significantly (Multiple t-test, $t = 2.353$, $P > 0.05$) but owing to the different treatment regimens, lesion sizes started differing (ANOVA, $F = 12.533$, $P < 0.05$) upto the last week of treatment.

4.2.3 Experimental infection of *L. major* infected BALB/c mice.

The study demonstrated that the aqueous extracts of the leaves of *R. communis* has a positive effect in lowering the parasite load in the viscera and is also effective in preventing metastasis. The seed oil however is poor in the latter and former but good at preventing lesion size icrease.

In-vivo L. major infected BALB/c mice treated topically with a crude extract of *R. communis* seed oil, initially at weeks 1 and 2 post treatment had a steady lesion progression until the 4th week of treatment when the lesion size started decreasing showing a positive effect. On the other hand, crude aqueous extract of the *R. communis* leaf showed an initial good effect in slowing lesion growth, eventhough lesion size of this treatment group at commencement of treatment was smaller. However there was no significant differences between these lesion sizes for all the groups other than the seed oil group. This apparent slow lesion growth was to be observed only for the first 3 weeks post treatment. At the 4th

week of treatment, the lesion size in this group increased dramatically to be the largest .

The BALB/c mice group treated with the pentostam® displayed the best results. Initially showing a steady increase in lesion size for the first two weeks, the lesion progression levelled off and started decreasing at weeks 3 and 4. By the last week of treatment (week 4) the lesion size had reduced significantly.

The manner in which the *L. major* infection expresses itself overtly on BALB/c mouse footpads under the different treatment regims is quite different from how these work from the inside picture. Splenomegally which occurs when the parasite visceralizes at 4 weeks post infection (Hill, 1988) was evident in all the mice treatment groups. However the degree of splenomegally differed judging from the variable spleen sizes and weights which were however not significantly different. Pentostam® treated mice had no apparent splenomegally as the sizes and weights was consistent with those of naive animals. However the optimum pentostam® dosage that was used to treat the mice that had been established by Mbati *et al*, 1995 having proved effective by the end of the 30 day prescribed treatment period was found to affect the animals negatively and resulted in the mice showing symptoms that typified adverse effects reported in pentostam therapy (Herwaldt and Berman, 1992). A possible explanation is that Mbati, 1994 used *L. donovani* infected BALB/c mice which is not a good *L. donovani* model unlike the hamster (*Mėsocricetus auretus*).

Splenomegally in this study was not only caused by the *Leishmania* infection but other evidence points at the possible role of the crude extracts of the *R. communis* (both seed oil and leaf extracts). During the toxicity study, results at necropsy showed that crude aqueous leaf extracts of *R. communis* caused some slight splenomegally when compared to those of control mice. Speen sizes compared in the order of control, seed oil, aqueous leaf extracts and pentostam®. However

the spleen of aqueous leaf extracts treatment group (3.2×10^4) yielded parasites after a longer period.

5.0 CONCLUSION

At the time of termination of the study, all the objectives set out had been achieved and it was therefore a succesful study. The major achievement was the demonstration that *R. communis* crude seed oil and aqueous leaf extracts had some quantifiable antileishmanial effects both *invivo* and *in-vitro*. *In-vitro* crude aqueous extracts inhibited *L. major* growth in cell free culture media. *In-vivo*, crude aqueous extracts prevented metastasis but not lesion progression wheras crude seed oil prevented lesion progression but not metastasis.

FUTURE CONSIDERATIONS.

In-view of these results, future work on the *R. communis* plant is considered and will include

i) The combined use of the crude seed oil and aqueous leaf extracts, aqueous leaf extracts and paramomycin, an antibiotic that is applied topically.

ii) Another future consideration is the isolation, characterization and testing both *in-vitro* and *in-vivo* of the active constituents in the crude seed oil and aqueous leaf extract.

iii) The testing of seed oil based paromomycin ointment in the topical treatment of cutaeous leishmaniasis due to *L. major*.

iv) Histological analysis of the tissues that heal upon application of seed oil is also an area that will be studied in future. It is important to establish the inflamatory sequences involving macrophages, neutrophils, eosinophils, lymphocytes and mast cells that predisposes to healing.

The major success of the *Ricinus communis* is that it works locally.

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Appendix 1: SPLEEN AND RIGHT HIND FOOTPAD CULTURE RESULTS AFTER 14-DAY PERIOD.

Pentostam® drug		*R. communis* oil		Aqueous leaf ext.		Control group	
SPLN	RHFD	SPLN	RHFD	SPLN	RHFD	SPLN	RHFP
- ve*	- ve*	+ ve*	+ ve*	+ ve *	+ ve*	+ ve*	+ ve*
- ve*	- ve*	- ve*	+ ve*	- ve*	- ve*	- ve *	+ ve*
- ve*	+ ve*	- ve *	- ve *	- ve*	- ve*	- ve*	+ ve*
- ve*	- ve*	+ ve*	+ ve*	+ ve*	- ve*	- ve*	+ ve*
- ve*	- ve*	+ ve *	+ ve*	- ve*	- ve*	+ ve*	- ve*
+ ve*	- ve*	+ ve*	- ve*	- ve*	- ve*	- ve*	- ve*
- ve*	- ve*	+ ve *	+ ve*	+ ve*	- ve*	+ ve*	- ve*
- ve*	- ve*	- ve *	- ve*	- ve*	- ve*	+ ve*	+ ve*
- ve*	- ve*	+ ve*	- ve*	- ve*	- ve*	+ ve*	- ve*
- ve*	- ve *	- ve*	- ve*	conta	- ve*	+ ve*	+ ve*
- ve	- ve	+ ve*	+ ve*	- ve*	- ve*	+ ve	- ve
- ve	- ve	+ ve*	+ ve *	- ve*	- ve*	+ ve	- ve
- ve	- ve	+ ve*	- ve*	- ve*	conta*	+ ve	+ ve
- ve	- ve	+ ve*	- ve*	- ve*	- ve*	+ ve	+ ve
- ve	- ve	+ ve*	- ve*	- ve*	- ve*	- ve	- ve
- ve	- ve	+ ve	+ ve	- ve*	+ ve*	+ ve	+ ve
+ ve	+ ve	+ ve	- ve	- ve	conta	+ ve	- ve
- ve	- ve	+ ve	conta	- ve	- ve	+ ve	+ ve
- ve	- ve	+ ve	- ve	+ ve	- ve	+ ve	+ ve
- ve	- ve	+ ve	- ve	+ ve	- ve	- ve	- ve
		+ ve	conta	- ve	- ve		
		- ve	- ve	+ ve	- ve		
		- ve	- ve	+ ve	+ ve		
		+ ve	conta	+ ve	+ ve		
		- ve	+ ve	conta	- ve		
2/20 **10 % +ve**	2/20 **10 % +ve**	18/20 **72 % +ve**	9/25 **36 % +ve**	8/25 **32 % +ve**	4/ 25 **16 % +ve**	14/ 20 **70 % +ve**	11/ 20 **55 % +ve**

* indicate that the cultures were examined on day 4 ater innoculation. SPLN: Spleen.

All the other cultures were examined between day 7 and day 14 (the last day). RFHP: Right Hind Footpad.

Appendix 2

TABLE SHOWING THE SPLEEN WEIGHTS IN MILLIGRAMS AND SPLEEN SIZES OF MICE GROUPS.

	PENTOSTAM ®	SEED OIL	AQ. LEAF EXT.	CONTROL
1	100, N	288, E	263 N	136, E
2	71, N	348, E	252, N	163, E
3	120, N	363 E	340, E	177, E
4	126, S.E	458, SE	152, N	167, E
5	91, N	383, E	177, SE	195, E
6	95, N	247, E	180, SE	180, E
7	99, N	231, N	123, N	126, SE
8	103, N	243, E	308, E	184, SE
9	74, N	294, E	326, E	145, SE
10	150 E	376, E	172, E	114, SE
11	59, N	471, GE	488, GE	164, SE
12	126, N	486, GE	511, GE	153, SE
13	148, E	288, E	654, GE	178, E
14	126, N	229, E	203, E	363, GE
15	132, N	377, E	220, E	210, N
16	62, N	225, E	415, E	240, N
17	16, N	211, E	235, N	647, E
18	18, SE	415, GE	379, E	165, SE
19	109, N	312, E	340, E	220, SE
20	159, N	612, E	315, N	108, N
21		387, E	314, N	176, SE
22		230, E	350, SE	401, SE
23		774, E	183, N	195, N
24		194, N	340, SE	220, N
25		227, SE	553, E	211, E

KEY: E Enlaged; S. E. Slightly enlarged; G. E. Greatly enlarged; N Normal

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Appendix 3

LEISHMANIA DONOVANI UNIT ACROSS MICE TREATMENT

GROUPS AFTER SACRIFICING

MICE NO.	PENTOSTAM	SEED OIL	LEAF EXT.	CONTROL
1	O	278,400	106,200	216,000
2	0	97,200	0	163,200
3	0	383,000	136,000	871,200
4	0	226,200	0	257,600
5	0	0	52,600	100,800
6	0	0	0	233,800
7	0	46,200	0	78,000
8	0	0	0	106,200
9	0	117,600	0	145,000
10	0	97,200	24,600	228,000
11		0		110,400
12		72,600		

$$\textbf{LDU} = \frac{\text{amastigotes counted}}{\text{1000 Nucleated cells}} \times \text{weight of organ (mg)} \times 2 \times 10^5$$

Mice number and spleen weights that were used corresponds to the those on apendix 2