DETERMINATION OF EFFECTS OF *TARCHONANTHUS CAMPHORATUS, ACALYPHA FRUTICOSA* AND *TAGETES MINUTA* CRUDE EXTRACTS ON THE VECTORIAL CAPACITY OF *PHLEBOTOMUS DUBOSCQI*

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Determination of Effects of *Tarchonanthus camphoratus*, *Acalypha fruticosa* and *Tagetes minuta* Crude Extracts on the Vectorial Capacity of *Phlebotomus duboscqi*

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DECLARATION

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

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DEDICATION

This thesis is dedicated to my father Ogeto, my mother Sabina and my siblings.

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I am indebted to my God who has been so gracious to me. He gave me good health, intellect, wisdom, perseverance and strength; necessary for completion of this work. I will give praise and always glorify your name for you are the corner stone of my life.

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LIST OF ACRONYMS AND ABBREVIATIONS

AIDS — Acquired immunodeficiency Syndrome

ANOVA — Analysis of variance

CBRD — Centre for Biotechnology Research and Development

CL — Cutaneous Leishmaniasis

CCR – Center for Clinical Research

DALYs — Disability Adjusted Life Years

DCL — Diffuse Cutaneous Leishmaniasis

HIV — Human Immunodeficiency Virus

LCL — Localized Cutaneous Leishmaniasis

MCL — Mucocutaneous Leishmaniasis

PKDL — Post Kala-azar Dermal Leishmaniasis

VL — Visceral Leishmaniasis

WHO — World Health Organization

ABSTRACT

The drawbacks of synthetic chemical insecticides have necessitated the search for new therapies against *Phlebotomus duboscqi*. The effects of *Tarchonanthus camphoratus* (Asteraceae), *Acalypha fruticosa* (Fabacea) and *Tagetes minuta* (Asteraceae) crude extracts on the vectorial capacity of *Phlebotomus duboscqi* were investigated. These plants are traditionally used for controlling sand flies and leishmaniasis in endemic areas. This study evaluated the following objectives: effects of *T. camphoratus, A. fruticosa,* and *T. minuta* crude extracts on the fertility of *P. duboscqi*, effects of *T. camphoratus, A. fruticosa, and T. minuta* crude extracts on longevity of *P. duboscqi* and the effects of *T. camphoratus, A. fruticosa, and T. minuta* crude extracts on infectivity of *P. duboscqi* by *Leishmania* parasites. The extracts were prepared from the dried aerial parts of *T. camphoratus, A. fruticosa,* and *T. minuta.* After grinding into a fine powder, the plant material was soaked in methanol and ethyl acetate solvents for 48 hours then filtered and dried using rotary evaporation at 30°C. The extracts obtained were later prepared into appropriate concentration for bioassay. The study was carried out using a completely randomized design. One hundred and sixty *P. duboscqi* adult flies were used; they were randomly divided into two groups; test and control groups. The collected data on bioassays was stored in MS Excel and analyzed using analysis of variance (ANOVA) and Student's *t* test. The extracts were found to reduce the fertility of *P. duboscqi* significantly by 73% (*A. fruticosa*), 53% (*T. minuta*) and 26% (*T. camphoratus*) ($P< 0.05$). The extracts also reduced the survival time of *P. duboscqi* significantly to: 7 days (*A. fruticosa),* 7 days (*T. minuta*) and 9 days (*T. camphoratus*). Development of *L. major* was greatly inhibited by the crude extracts at the nectomonad stage. Percentage inhibition of

L. major development was highest in *A. fruticosa* crude extract (86%) followed by *T. minuta* (79%) and finally *T. camphoratus* (63%). The observation that *A. fruticosa*, *T. minuta* and *T. camphoratus* have effect on infectivity, egg development and longevity of *P. duboscqi* has important implications for vector control because the high level of their activities would potentially reduce the population of sand flies. This study shows that these medicinal plants have effects on the vectorial capacity of sand flies.

CHAPTER ONE

1.0 INTRODUCTION AND LITERATURE REVIEW

1.1 General Introduction

Leishmaniasis is a zoonotic infection caused by protozoa of the genus *Leishmania* **(**Conjivaram and Ruchir, 2007*)*. These parasites enter the immune system cells following the bite of an infected sand fly and spread either to the skin, causing disfiguring lesions, or to internal organs, causing lethal infections (Getti *et al.*, 2009). The infection was named after Leishman who first described it in London, May 1903. The infection is transmitted to humans by infected sand flies of the genus *Phlebotomus* and *Lutzomyia* (Piscopo and Mallia, 2006). Leishmaniasis is endemic in 88 countries in the world with 350 million people being at risk. An estimated 14 million people are infected, and each year about two million new cases occur (WHO, 2007).

The global burden of leishmaniasis has remained stable for some years, causing morbidity and mortality loss of 2.4 million disability adjusted life-years (DALYs) and approximately 70,000 deaths, a significantly high rank among communicable diseases (Ngure *et al.,* 2009). Leishmaniasis is not only associated with poverty but also propagates poverty, because treatment is expensive and either unaffordable or imposes a substantial economic burden, including loss of wages (WHO, 2007). The global prevalence of leishmaniasis has risen in recent times because of an increase in international travel, human alteration of both vector and host habitats, and concomitant factors that increase susceptibility, such as human immunodeficiency virus infection and

malnutrition. Recent international conflicts have also contributed to an increase in and spread of leishmaniasis in previously unaffected countries (Rosypal *et al*., 2003).

Leishmaniasis is a major tropical disease with a wide clinical spectrum of cutaneous, mucocutaneous and visceral involvement whose presentation is often varied and diagnosis can be challenging. The type of disease expressed depends both on the type of *Leishmania* species and on the zymodeme expressed on that species. Clinical manifestation depends on the parasite species and the host's specific immune responses to *Leishmania* antigens (Roberts, 2006).

The two major clinical forms of leishmaniasis are cutaneous leishmaniasis (CL) and visceral leishmaniasis (VL). Cutaneous leishmaniasis manifests as a sore on the skin at the site of the infected sand fly bite and is usually self-healing. These infections normally produce skin ulcers on the exposed parts of the body such as the face, arms and legs (WHO, 2007). In mucocutaneous forms of leishmaniasis, lesions can lead to partial or total destruction of the mucous membranes of the nose, mouth, throat cavities and surrounding tissues. Visceral leishmaniasis ranges from asymptomatic infection to severe life-threatening infection. It is the most severe form of leishmaniasis and is usually fatal within 2 years if left untreated (Conjivaram and Ruchir, 2007). There are an estimated 1–1.5 million cases of CL and half a million new cases of VL annually (Desjeux 2004). Post kala azar dermal leishmaniasis is a complication of visceral leishmaniasis characterized by rashes ranging from papular or nodular, maculopapular, micropapular to macular rashes (Ngure *et al*., 2009).

Currently, a cost effective cure for the disease does not exist. The chemotherapeutic agents most commonly used for treating leishmaniasis, that is, sodium stibogluconate, *N*-methylglucamine antimoniate, pentamidine, and amphotericin B, are not effective when administered orally. Moreover, they often require long periods of treatment and cause serious side effects, including cardiac and renal toxicity (Akendengue *et al.*, 2002). This has prompted the World Health Organization (WHO) to emphasize the need for development of new drugs in the treatment of leishmaniasis (WHO, 2005).

1.2 Global Distribution of Leishmaniasis

Leishmaniasis causes substantial clinical, public health and socioeconomic problems in endemic regions in more than 88 countries in the Indian sub-continent, South Western Asia, Southern Europe, Africa, and Central and South America (Desjeux, 2004). There is a remarkable increase in risk factors for leishmaniasis worldwide and the disease burden is increasing (Reithinger *et al.*, 2007). VL is endemic in the tropical and sub-tropical regions of Africa, Asia, the Mediterranean, Southern Europe, South and Central America. The distribution of VL in these areas however is not uniform; it is patchy and often associated with areas of drought, famine and densely populated villages with little or no sanitation.

The global estimate for new cases of visceral leishmaniasis is 500 000 cases per year out of which 90% of the cases arise in just five countries. These include Bangladesh, Brazil, India, Nepal and Sudan (Desjeux, 2004). Each year, there are 1.5 million new cases of CL in more than 70 countries worldwide with 90% of the cases reported in Afghanistan,

Algeria, Brazil, Islamic Republic of Iran, Peru, Saudi Arabia and Syria (Ghalib and Modabber, 2007). 90% of MCL cases occur in Bolivia, Brazil and Peru (Desjeux, 2004). In India, visceral leishmaniasis is endemic in the districts of Bihar, Uttar Pradesh, Orissa, Tamil, Nadu and Gujarat (fig. 1). Geographical distribution of leishmaniasis is restricted to tropical and temperate regions (Conjivaram and Ruchir, *2007*).

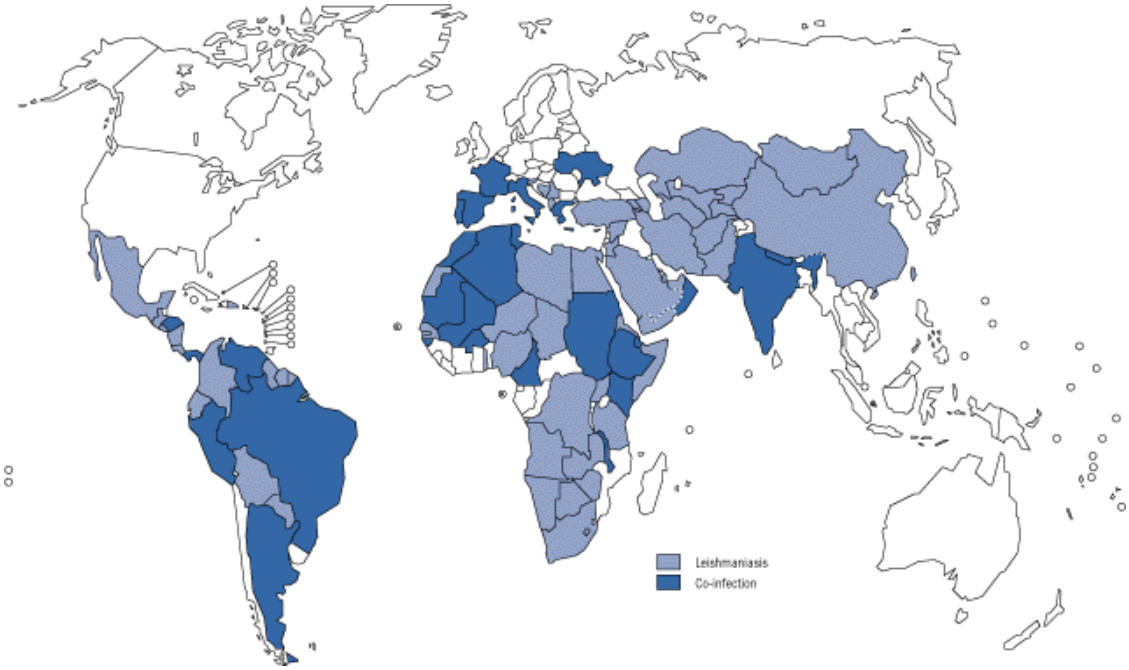

Figure : Global distribution of reported leishmaniasis and *Leishmania*/HIV co-infection, 1990-1998

(Source: http://www.who.int/leishmaniasis/leishmaniasis_maps/en/index.html)

In Africa, VL is a particular problem in Kenya, Sudan, Ethiopia and Eritrea (Wasunna *et al.*, 2005). Sudan is the most affected country, being one of the five countries that constitute 90% of all global cases of VL (Guerin, *et al*., 2002). Sudan also has the highest incidence of Post Kala-azar Dermal Leishmaniasis (PKDL) in the world

(Ghalib and Modabber, 2007). VL has been known in Sudan since 1904 to be endemic along the Blue Nile where it enters Ethiopia and its tributaries (Berman, 2006). Since the first reported case of VL in Sudan, the disease has become wide spread and is endemic in south and eastern parts of the White Nile and Upper Nile states. Other areas affected include the provinces of Kasala, Jonglei and Kapoeta in the south, El Fasher and El Nahud in the west and north of Khartoum (Hashim *et al.,* 1994).

In Ethiopia, there is the presence of a significant number of patients co-infected with HIV and VL (Ngure *et al*., 2009). The etiologic agent associated with the epidemic is *L. donovani* and the sand fly vector is *Phlebotomus orientalis* (Seaman *et al*., 1996). The number of leishmaniasis cases in Ethiopia has increased since 1970 and this appears to correspond to an extensive programme of agricultural development with an annual influx of migrant workers in the Tigray region (Ngure *et al*., 2009). The first case of VL in Ethiopia was documented in 1942 in the southern parts of the country. Since then the disease has spread to become endemic in the Segen, Woito and Gelana river valleys. The highest incidence has been recorded in the Aba Roba area (WHO, 1991). VL cases have also been reported in the villages close to the Segen river valley.

In Somalia, sporadic cases of VL first appeared in 1934, mainly in the Middle Shabelle and Lower Juba areas. Children below the age of 15 years are at the highest risk of infection and males are over three times more susceptible than females (Shiddo *et al*, 1995). In Israel VL is rare and the few cases that have been reported are largely confined

to the run down Arab villages in western Galilee, indicating that the disease is linked to poverty, poor sanitation and sub-standard housing.

In West Africa, leishmaniasis is endemic although it is one of the less recognized or under-reported parasitic infections in this region (Boakye, 2005). Cases of leishmaniasis have been reported in Niger, Mali, Nigeria, Senegal, Cameroon, Burkina Faso, Mauritania, Gambia, and Guinea. There is high prevalence of both HIV and leishmaniasis co-infection in Burkina Faso (Niamba *et al*., 2007). Algeria is among the eight countries that contribute 90% of worldwide cases of CL (Reithinger *et al*., 2007).

1.3 Epidemiology of Leishmaniasis in Kenya

Leishmaniasis has been endemic in Kenya for a long time. The most prevalent forms of leishmaniasis are the cutaneous and visceral forms (Tonui, 2006). In addition, post kala-azar dermal leishmaniasis (PKDL) has been reported (Ngure *et al*., 2009). VL is endemic in the Baringo, Koibatek, Turkana, West Pokot, Kitui, Meru, Keiyo, Marakwet, Mwingi and Machakos districts (Wasunna *et al.*, 2005). Baringo district is the only foci where both VL and CL are known to occur in Kenya. VL was first reported in Kenya among King's African Rifles troops in Lake Turkana, Southwest Ethiopia in the 1940s (Ngure *et al*., 2009). The disease is caused by *L. donovani* and transmitted by *P. martini*, though other vectors including *P. orientalis* have been reported. In 2001, there was an outbreak of VL in Wajir and Mandera districts of North Eastern Kenya with 904 patients diagnosed between May 2000 and August 2001 (Marlet *et al*., 2003).

In Kenya, CL is caused by *L. major, L. aethiopica and L. tropica* (Ngure *et al.*, 2009). Cutaneous leishmaniasis due to *L. major* which is transmitted by *Phlebotomus duboscqi* mainly found in animal burrows where it feeds on rodents that are frequently infected. Diffuse cutaneous leishmaniasis (DCL) was first reported in Kenya in 1969 in Bungoma district and the Mount Elgon area (Kungu *et al.*, 1972). *Leishmania aethiopica* has been identified as the etiological agent, rodents as the animal reservoirs and *P. pedifer* Lewis as the vector of DCL in the Mt. Elgon region (Ashford, 2000). In Kenya, PKDL was first described in 1959 by Manson-Bahr (Ngure *et al.*, 2009). Although various aspects of the transmission and control of leishmaniasis have been studied in Kenya, the impact of the disease and particularly VL is still enormous (Tonui, 2006).

1.4 The biology of *Leishmania*

Leishmania is a protozoon that is capable of infecting animals, humans and rodents. There are at least 20 species of *Leishmania* each causing a disease specific to the species and the host response (Piscopo and Mallia, 2006). *Leishmania* parasites have a digenetic life cycle with an extracellular developmental stage in the female Phlebotomine sand fly and a developmental stage in mammals, which is mostly intracellular (Roberts, 2006). In 1903, Leishman and Donovan separately described the protozoan now called *Leishmania donovani* in splenic tissue from VL patients in India. As a result, the amastigote stage seen in clinical samples is commonly known as Leishman-Donovan (LD) bodies (Singh, 2006).

1.5 Life Cycle of *Leishmania*

Leishmania parasites undergo a series of developmental stages which can be divided into 2 forms: the amastigote form which occurs in humans and the promastigote form which occurs in the sand fly and in artificial culture (Conjivaram and Ruchir, 2007). In the vertebrate host, *Leishmania* parasites survive and multiply intracellularly in mononuclear phagocytes as tiny, ovoid to round, non-motile amastigotes about 3-5 μm in diameter (Singh, 2006). Female sand flies (*Phlebotomus* and *Lutzomyia* species) acquire *Leishmania* parasites when they feed on an infected mammalian host in search of a blood meal.

During feeding, the sand flies take up the amastigote forms from the peripheral circulation. Amastigotes that are taken up by sand flies are not usually found in the peripheral circulation; rather they are present in the skin itself. Parasites present in organs such as liver and spleen are not accessible to sand flies. Amastigotes are intracellular parasites found in phagolysosomes of macrophages and other phagocytes (Handman and Bullen, 2002), and their uptake by the blood feeding sand fly is assisted by the cutting action of the mouthparts. This tissue damage associated with the creation of the wound releases the skin macrophages and/or freed amastigotes into the pool of blood, and enables their subsequent uptake into the abdomen of the sand fly (Fig. 2).

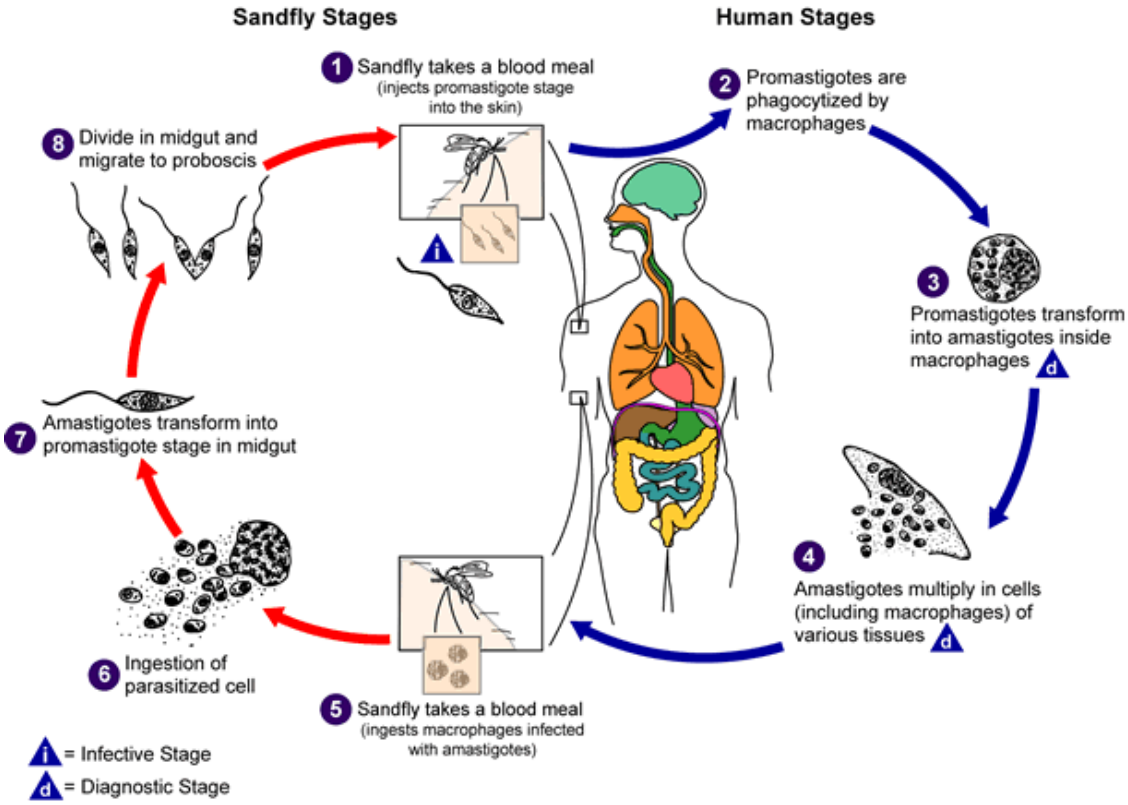

Figure : Life cycle of Leishmania species (Source: http://www.dpdx.cdc.com)

The change in conditions moving from the mammalian host to the sand fly midgut (decrease in temperature, increase in PH) triggers development of the parasite in the vector (Bates and Rogers, 2004; Kamhawi, 2006). The amastigotes transform into motile promastigotes with flagella beating at the anterior end. This first stage in the vector is called a procyclic promastigote which is a weakly motile, replicative form that multiplies in the blood meal. This initial ‘‘blood meal phase’’ is confined by the peritrophic matrix, a chitin and protein mesh secreted by the midgut epithelium that encloses the blood being digested within (Secundino *et al*., 2005).

After a few days, the parasites begin to slow their replication and differentiate into elongate, strongly motile nectomonad promastigotes. These are migratory forms that accumulate at the anterior end of the peritrophic matrix and break out of the blood meal. This escape is facilitated by the action of a parasite secretory chitinase (Dwyer, 2005) and probably by the action of endogenous sand fly chitinase (Ramalho-Ortigao *et al*., 2005). They move towards the anterior midgut, some of them attaching to the microvilli of the midgut epithelium, until they reach the stomodeal valve (cardia) that guards the junction between foregut and midgut.

These nectomonad promastigotes mediate the establishment phase of the infection that marks a true vector including persistence beyond the blood meal and avoidance of expulsion during defecation. Thus the ability to attach is an important property of *Leishmania* promastigotes (Sacks and Kamhawi, 2001). The major parasite surface glycoconjugate lipophosphoglycan (LPG) is responsible for binding to a galectin on the sand fly gut epithelium in certain species such as *Leishmania major* in *Phlebotomus papatasi* (Kamhawi *et al*., 2004), although findings indicate that non-LPG mediated attachment is used by some other *Leishmania* species (Myskova *et al*., 2007). The identity of these alternative receptor–ligand pairs has not been fully described.

Once they reach the stomodeal valve the nectomonad promastigotes transform into haptomonad promastigotes, shorter forms that resume replication (Gossage *et al*., 2003). These are responsible for the secretion of promastigote secretory gel (Rogers *et al*., 2002), which plays a key role in transmission. They later attach themselves to the cuticle-lined

surface of the valve. This form of attachment is mechanistically different to that seen with the midgut epithelium and is mediated by expansion of the flagellar tip into hemi-desmosome-like structures (Wakid and Bates, 2004).

Finally, haptomonads differentiate into metacyclic promastigotes (Rogers *et al*., 2002), which are the mammal-infective stages. These are deposited in the skin of a new mammalian host when the fly takes another blood meal, leading to the transmission of disease.

1.6 Biology of Sand Flies

Sand flies are small blood sucking gnats prevalent along coastal territories and regions with a lot of water, dampness and marshland with high humidity. Only females bite since they need several blood meals before they can lay eggs. They seek blood from birds, amphibians, reptiles and mammals. Eggs will hatch between 7-11 days emerging as larva which feed on dead organic matter present in the breeding site. Larvae molt and undergo development to second, third and fourth instar stages. The fourth instar larvae pupate and adults emerge and immediately look for a good blood meal (fig. 3). Mating occurs at the site of emergence. This life cycle could take as little as a month or more than six months depending on climatic conditions.

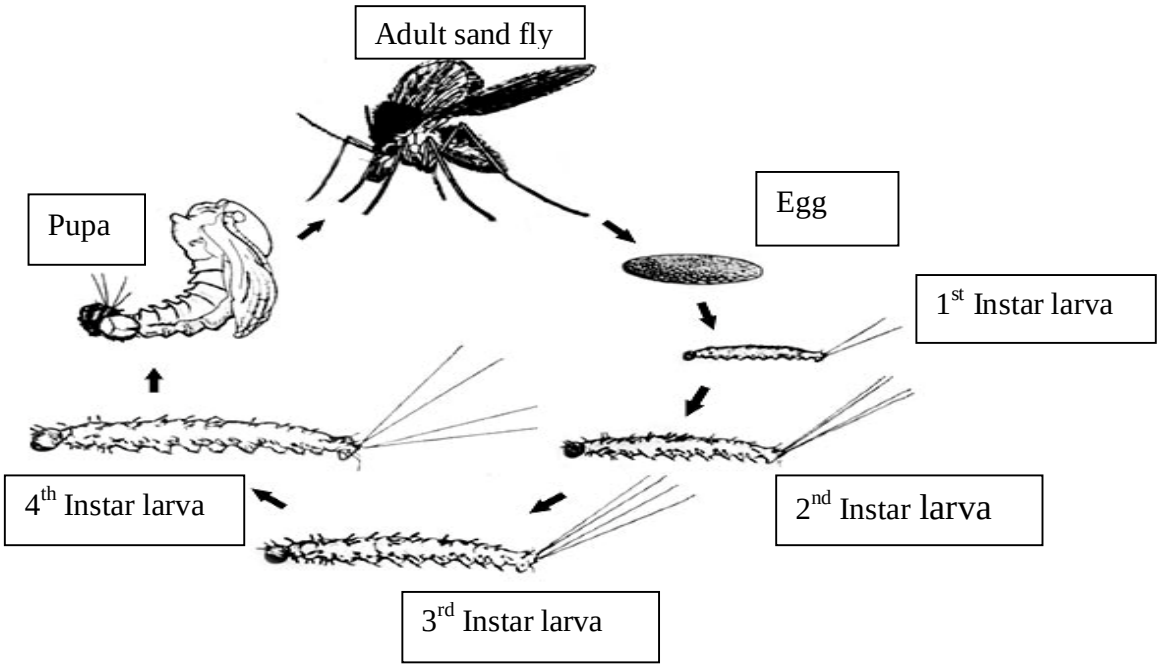

Figure : Life cycle of *Phlebotomus duboscqi*

(Source: http://pcwww.liv.ac.uk/leishmania/life_cycle__habitats.htm)

The sand fly characteristically feeds at dusk, and, being a weak flier, tends to remain close to its breeding area, not too high from the ground. Different species have different feeding and resting patterns. These different characteristics are important in formulating control strategies (Piscopo and Mallia, 2006).

The only proven vector of the *Leishmania* parasite is the blood-sucking female of the genus *Phlebotomus* and *Lutzomyia* (Murray *et al*., 2005). Only 30 or so of the over 500 species of Phlebotomine sand flies are known to transmit *Leishmania* parasites. These include *P. argentipes* on the Indian sub-continent, *P. duboscqi*, *P. martini* and *P. orientalis* in Africa and the Mediterranean basin, *P. chinensis* and *P. alexandri* in china.

In the new world *L. longipalpis* is the only known vector of *L. chagasi* (Murray *et al*., 2005).

1.7 Vectorial Capacity of Sand Flies

The vectorial capacity of a sand fly depends on its ability to develop and harbor parasites in its intestine and the colonization of the foregut by infective promastigotes after a complete digestion of the infective blood meal. The fly should also be able to locate, bite and transmit the parasites to a new reservoir or host. In transmission-competent sand flies, parasites attach to the midgut epithelium and go on to establish a stable infection while in a transmission-refractory vector; unattached parasites are expelled when the sand fly defecates (Dobson *et al*., 2010). Of the 42 species of sand flies known to occur in Kenya to date, *P. pedifer*, *P. martini* and *P. duboscqi* can naturally carry biochemically and serologically confirmed *Leishmania* (*aethiopica, donovani* and *major,* respectively) and *P. rhodhaini, Sergentomyia garnhami, S. squamipleuris, S. africanus, S. kirki, S. ingrami, S. antennatus, S. bedfordi, S. schwetzi, S. affinis, S. graingeri and S. clydei* carry various flagellates of biochemically or serologically unknown character (Kaddu *et al*., 1986).

Current research on naturally infected wild-caught sand flies indicates that *L. aethiopica* promastigotes have close association with structures resembling a peritrophic membrane and invade *P. pedifer* gut cells. Studies on laboratory-reared sand flies shows that *P. martini* is susceptible to *L. donovani* amastigotes and that cultured promastigotes of *L. donovani* can survive in the guts of *S. schwetzi, S. ingrami* and *S. adleri.* Therefore, a key step in *Leishmania* transmission is stage-specific midgut attachment which allows *Leishmania* development to proceed (Dobson *et al*., 2010).

1.8 Pathology of Leishmaniasis

Leishmaniasis infections are zoonotic diseases because the infection is maintained in dogs, wild rodents, and other animals in endemic areas. Three main types of disease patterns occur: visceral, cutaneous and mucocutaneous.

1.8.1 Cutaneous Leishmaniasis

Cutaneous leishmaniasis (CL) is the most common form of leishmaniasis (Desjeux, 2004). Multiple species produce CL in children and adults, primarily *L. major*, *L. tropica*, and *L. aethiopica*, *L. infantuma, L. chagasi*, *L. mexicana*, *L. amazonensis*, *L. braziliensis*, *L. Viannia panamensis ,L.(V) peruviana* and *L.(V) guyanensis* (Murray *et al*., 2005). The disease produces skin lesions mainly on the face, arms and legs (Akilov *et al*., 2007). Cutaneous leishmaniasis starts as a papule at the site of a sand fly bite, which then increases in size, crusts and eventually ulcerates (Piscopo and Mallia, 2006).

Variations of cutaneous leishmaniasis exist. Localized cutaneous leishmaniasis (LCL) is the most common and the least drastic form of cutaneous leishmaniasis. The lesions are caused by *L. major*, *L. tropica*, *L. aethiopica*, and subspecies of *L. mexicana* (Murray *et al*., 2005). The first sign of an infection is typically a small erythema that develops after a variable prepatent period at the site where an infected sand fly has bitten the host. The erythema develops into a papule, then a nodule that progressively ulcerates over a period of 2 weeks to 6 months to become the lesion that is characteristic of localized cutaneous leishmaniasis (Akilov *et al*., 2007). In diffuse cutaneous leishmaniasis, dissemination of

skin lesions rarely occurs over the face and hands and feet, disclosing high parasite numbers owing to poor cell-mediated immune response (Aghaei *et al*., 2004).

Diffuse cutaneous leishmaniasis (DCL) is a chronic, progressive, polyparasitic variant of CL that develops in context of leishmanial-specific anergy and is manifested by disseminated non-ulcerative skin lesions, which can resemble lesions of lepromatous leprosy (Aghaei *et al*., 2004). The lesions of DCL are very similar to those of LCL; except they are spread all over the body. The body's immune system apparently fails to battle the protozoa, which are free to spread throughout the body (Handman, 2001).

1.8.2 Visceral Leishmaniasis (VL)

Visceral leishmaniasis is the most severe form of leishmaniasis and is potentially fatal if left untreated (Garg and Dube, 2006). Visceral leishmaniasis coexists with malaria and other debilitating parasitic infections (Hailu *et al*, 2005). Parasites of the *L. donovani* complex are the typical etiological agents of VL (Mukhopadhyay and Mandal, 2006). *Leishmania donovani* is the principal cause of VL in the Indian sub-continent and East Africa, *L. infantum* in the Mediterranean region and *L. chagasi* in the New World (Murray, 2001; Mukhopadhyay and Mandal, 2006). *Leishmania tropica* has been reported to produce visceral disease in immunocompromised persons while visceralization by *L. amazonensis* has also been reported (Herwaldt, 1999). Migration, lack of control measures and HIV–VL co-infection are the three main factors driving the increased incidence of VL (Chappuis *et al*., 2007). The clinical symptoms of visceral leishmaniasis include splenomegally (causing abdominal distension and pain, which is sometimes

increased by concomitant hepatomegaly), recurring and irregular fever, anemia, pancytopenia, weight loss and weakness. The disease is a silent killer, killing almost all untreated patients (Hailu *et al.,* 2005). A variant of visceral leishmaniasis has been described in the US soldiers characterized by fever, malaise and nausea (Conjivaram and Ruchir, 2007).Visceral leishmaniasis affects children, those weakened by other diseases e.g. HIV and tuberculosis, healthy adults and economically productive social groups. VL symptoms often persist for several weeks to months before patients either seek medical care or die from bacterial co-infections, massive bleeding or severe anemia (Chappuis *et al*., 2007).

1.8.3 Post Kala-azar Dermal Leishmaniasis (PKDL)

Post kala-azar dermal leishmaniasis is a disease that appears after treatment of visceral leishmaniasis (Ghalib and Modabber, 2007), and it requires lengthy and costly treatment (WHO, 2006). PKDL is a dermatropic form of leishmaniasis that develops in some patients who have had visceral leishmaniasis but there are cases without any previous known history of VL (El-Hassan *et al*., 1992). The interval to development of PKDL is variable and PKDL occurs in a small percentage of patients in Africa and India. This is usually due to infection by the *L. donovani sensu stricto* cluster (Piscopo and Mallia, 2006).

The skin lesions are macular, maculopapular or nodular, and usually spread from the peri-oral area to other areas of the body. The symptoms first appear around the mouth; those which do not heal spontaneously become denser and spread over the entire body

(Berman, 1997). PKDL lesions are categorized into 3 types: depigmented macules on the trunk and extremities, erythematous patches on the nose, cheeks, and chin with a butterfly distribution and yellowish pink nodules on the face and replace the earlier lesions (Conjivaram and Ruchir, 2007). PKDL patients may be important sources of infection in VL transmission.

1.8.4 Mucocutaneous Leishmaniasis (MCL)

MCL, also called espundia, produces disfiguring lesions to the face, destroying the mucous membranes of the nose, mouth and throat (WHO, 2007). Mucocutaneous leishmaniasis may occur many years after the initial cutaneous ulcer has healed. After an initial skin lesion, that slowly but spontaneously heals, chronic ulcers appear after months or years on the skin, mouth and nose, with destruction of underlying tissue e.g. nasal cartilage. It is mostly related to *Leishmania* species of the new world such as *L. braziliensis*, *L. panamensis* and *L. guyanensis*. The mucosal lesions have also been reported in old-world leishmaniasis caused by *L. donovani, L. major* and *L. infantum* in immunosuppressed patients (Desjeux, 1996). Ninety percent of all cases of MCL occur in Bolivia, Brazil and Peru.

1.9 Diagnosis of Leishmaniasis

Diagnosis of visceral leishmaniasis is usually based on microscopic detection of amastigotes in smears of tissue of the bone marrow (Piscopo and Mallia, 2006). The parasite can also be detected through direct evidence from peripheral blood, bone marrow, or splenic aspirates. The smears are stained in Leishman, Giemsa, or Wright stains and examined under oil immersion microscope (Conjivaram and Ruchir, 2007).

The clinical signs and epidemiological manifestations of VL and CL cannot be used in diagnosis. They can mimic several other conditions like misdiagnosis for malaria, tropical splenomegally, schistosomiasis, cirrhosis, lymphoma or leukaemia; hence a laboratory diagnosis is necessary to confirm the infection (Singh, 2006).

1.10 Control and Treatment of Leishmaniasis

Currently, no well-defined model for cost-effective control exists (WHO, 2007). Control strategies rely on chemotherapy to alleviate disease and on vector control to reduce transmission (Tonui and Titus, 2006). The drugs currently recommended for the treatment of leishmaniasis include Sodium stibogluconate, meglumine antimoniate and amphotericin B. However these drugs have drawbacks such as serious side effects, long courses of treatment and rampant drug resistance especially of the antimonials (WHO, 2007).

Leishmaniasis experts advocate for vector control especially for areas of anthroponotic transmission (Hailu *et al.*, 2005). In endemic areas, sand fly control is often combined with malaria control to eliminate the vector. This is a cost effective method given the expense of mass spraying with chemicals such as DDT, Malathion, Fenitrothion, Propoxur and Diazinon. Vector control using indoor spraying of insecticides is always determined by the behavior of the species of sand flies present in each area i.e. whether endophilic or exophilic and endophagic or endophagous (WHO, 2007). Measures employed include spraying houses with insecticides where sand flies are endophilic and using treated and untreated bednets where sand flies are endophagic (Piscopo and Mallia, 2006). Personal protection using repellents and nets is an important aspect. In endemic

areas, spraying with dichlorodiphenyltrichloroethane (DDT) and other residual insecticides is effective in sand fly control **(**Conjivaram and Ruchir, 2007*)*. In general, the control of leishmaniasis mainly depends on its epidemiological features. In the zoonotic foci where carriers are involved and dogs are the main vertebrate host, the effective methods include destruction of dogs and elimination of sand flies by environmental and chemical control (WHO, 2005).

1.11 Role of Herbal Plants in Controlling Sand Flies

Studies have confirmed that natural products are potential sources of new and selective agents for the treatment of important tropical diseases caused by protozoans. Plant extracts have also been used as insecticides in controlling Phlebotomine sand flies (Sirak-Wizeman *et al*., 2008). Plant extracts such as pyrethrum, nicotine and rotenone were among the first compounds used to control insects of medical and agricultural importance (Coats, 1994).

Medicinal plants act against insects through different mechanisms. Most of them are either antifeedant, insecticidal, larvicidal or deterrent of oviposition. Morallo-Rejesus, (1986) found out that plants belonging to Asteraceae, Fabacea and Euphorbiaceae families deter oviposition and have repellent activities. Lingathurai *et al*., (2010) reported that *Momordica charantia* leaf extract showed strong feeding deterrent activity and developmental inhibition in *P. xylostella* larvae. Besides this, medicinal plants also have ethnobotanic uses as sedative, insecticidal, antiparasitic, antirheumatic, astringent and emetic (Osorio *et al*., 2007). They are also used as antiprotozoans, anti-diarrheic, anti-

diabetic and abortive properties (Coee and Anderson, 1996). Other plants have antiviral, insecticide, molluscacide and antiparasitic properties (Osorio *et al*., 2007).

Tagetes minuta contains secondary compounds which are effective deterrents of numerous organisms including insect pests through different mechanisms (Jacobson, 1990). *Tagetes minuta* is an erect annual herb growing to a height of 1-2m (Meshkatalsadat *et al*., 2010). The leaves of this herb are slightly glossy green, and are pinnately dissected into 4 to 6 pairs of pinnae (plate 1). *Tagetes minuta* is rich in many secondary compounds, including acyclic, monocyclic and bicyclic monoterpenes, sesquiterpenes, flavonoids, thiophenes, and aromatics (Jacobson, 1990). There is evidence that the secondary compounds in *Tagetes minuta* are effective deterrents of numerous organisms, including: fungi pathenogenic to humans, bacteria, trematodes, nematodes, and numerous insect pests through several different mechanisms (Mohamad *et al*., 2010).

Plate : *Tagetes minuta* plant

A. fruticosa is a medicinal plant used in traditional medicines to cure stomachache, digestive disorders, dyspepsia, colic and diarrhea (Sripathi and Uma, 2010). The leaves of *Acalypha fruticosa* are powdered, soaked in water and the solution applied on animal skin and wound as a repellent or insecticide against ectoparasites and dipteran flies. This plant has many uses including medicinal value, food, fodder and repellent activities against many insects (Bekalo *et al.,* 1996). This plant also has antioxidant and anti-inflammatory activities. This plant belongs to Euphorbiaceae family whose members have insecticidal activities against a wide range of insects. It is an erect clump forming shrub standing 2-3m high. This plant is widely distributed along river banks and flood plains in riparian wood lands in East Africa. This shrub has yellow resinous glands on the lower leaf surface that give off an unpleasant smell when crushed. It has simple, oval and alternate leaves whose edges are round and toothed (plate 2).

Plate : *Acalypha fruticosa* plant

Tarchonanthus camphoratus has derivatives with repellent activities and medicinal uses (Bishay *et al*., 2002). *T. camphoratus* is an evergreen shrub of 9 feet tall, usually much-branched with a narrow crown; young stems densely covered by white felt-like tomentum. Leaves are shortly petiolate and strongly camphor-scented when crushed (plate 3). *T. camphoratus* is wide spread in Africa especially in the stony sites of Rift valley in Kenya, Angola, Ethiopia, Lesotho, Namibia, Somalia, South Africa, Tanzania, Uganda and Zimbabwe (Grant and Thomas, 1998).

Tarchonanthus camphoratus has many uses including high quality fuel wood, hut-building, making of general utensils and hunting weaponry. The essential oil extracted from leaves is the safest and most effective natural product for protection from mosquitoes, midges and many kinds of biting insects (Grant and Thomas, 1998). Several African tribes use this plant as a treatment for bronchitis and chest ailments, for

chilblains, tired legs, sore feet, stomach ailments, asthma, over-anxiety and heartburn (Grant and Thomas, 1998). *Tarchonanthus* essential oil has also been found to have excellent cosmetic and dermatological properties especially as soothing, anti-irritation, decongestant remedy for sensitive skins, dermatitis, sunburns and bedsores (Van Wyk and Van Wyk, 1997).

Plate : *Tarchonanthus camphoratus*

1.12 Statement of the Problem

Leishmaniasis is a major problem threatening 350 million people in 88 countries around the world. According to the World Health Organization, 14 million people are infected with *Leishmania* parasites and there are 2 million new cases each year. In Kenya the infection is endemic in twelve districts. The drugs of choice for the treatment of leishmaniasis are the pentavalent antimonials which include Sodium stibogluconate, meglumine antimoniate and Amphotericin B. These drugs are expensive, toxic, and have variable efficacy with antimony-resistant strains being reported. The long periods of pentavalent antimonial administration requiring 20-30 days causes non-compliance

among patients. In addition, leishmaniasis has a great impact on the socio-economic status of patients in the society. There have been reports on human immunodeficiency virus (HIV) co-infection with leishmaniasis in different parts of the world. This has worsened the infection since leishmaniasis has appeared as a reactivating infection in acquired immunodeficiency syndrome patients (Ngure *et al.*, 2009).

Vector control programs are limited by the high cost of synthetic insecticides, vector tolerance due to rampant use and the high cost of application equipment. Current insecticides are toxic and they cause soil and water contamination. Control of Phlebotomine sand flies is not effective due to the increasing resistance of vectors to insecticides and high costs of insecticides which causes and propagates poverty.

1.13 Justification of the Study

Control strategies for leishmaniasis rely on chemotherapy to alleviate disease and on vector control to reduce transmission. The drugs used for treatment of leishmaniasis require adequate health care infrastructure and the long period of patient hospitalization. In areas where leishmaniasis is endemic, there are no such facilities and if they are, they are very far apart. The painful injections and non-compliance of patients can be overcome by the use of oral administration of the drugs. This will reduce treatment-related socioeconomic difficulties that are present in the areas where the disease is endemic and health facilities are lacking.

Vector control programs are challenged by insecticide resistance and high cost of insecticides hence the need for alternative control methods, including new insecticides.

Use of chemicals poses adverse effects on the environment including non-target effect of insecticides which also kill beneficial organisms. The synthetic insecticides causes soil and water contamination incase of poor handling of the insecticides. Natural alternatives will lower the high cost of treatment, reduce resistance of drugs & insecticides and also reduce environmental pollution.

1.14 Hypotheses

1. Crude extracts from *A. fruticosa, T. minuta* and *T. camphoratus* do not have any effect on the fertility of *P. duboscqi.*
2. Crude extracts from *A. fruticosa, T. minuta* and *T. camphoratus* do not have any effect on longevity of *P. duboscqi.*
3. There is no infectivity of *P. duboscqi* by *Leishmania* parasites after treatment with *A. fruticosa, T. minuta* and *T. camphoratus* crude extracts.

1.15 Objectives

1.15.1 General Objective

To determine the effects of *Tarchonanthus camphoratus, Acalypha fruticosa* and *Tagetes minuta* crude extracts on the vectorial capacity of *Phlebotomus duboscqi.*

1.15.2 Specific Objectives

1. To evaluate the effects of *Tarchonanthus camphoratus, Acalypha fruticosa,* and *Tagetes minuta* crude extracts on the fertility of *Phlebotomus duboscqi.*

2. To establish the effects of *Tarchonanthus camphoratus, Acalypha fruticosa*, and *Tagetes minuta* crude extracts on longevity of *Phlebotomus duboscqi*.
3. To determine the effects of *Tarchonanthus camphoratus, Acalypha fruticosa*, and *Tagetes minuta* crude extracts on infectivity of *P. duboscqi* by *Leishmania* parasites.

CHAPTER TWO

2.0 MATERIALS AND METHODS

2.1 Study Site

The study was carried out at the Kenya Medical Research Institute's Centre for Biotechnology Research and Development (CBRD), Nairobi, Kenya.

2.2 Study Design

The study design used involved a comparative experimental design using *Tarchonanthus camphoratus, Acalypha fruticosa,* and *Tagetes minuta* crude extracts. Their activities were compared against each other for their activities against the adult sand flies. Synergistic effects of the combined extracts were also considered. The extracts were incorporated in filter papers and Karo syrup then used in feeding the flies.

2.3 Collection and Preparation of the Plants

Floral and foliar parts of *Tarchonanthus camphoratus, Acalypha fruticosa,* and *Tagetes minuta* were collected from Marigat division, Baringo district, Rift Valley province, Kenya. Traditionally, these plants have been used for controlling sand flies and leishmaniasis in Baringo district. Botanical identification was carried out with the help of taxonomists from the National Museums of Kenya. All the collected parts of the plants were left to dry completely under a shade for one month and then transported to the laboratory where they were left to dry further under room temperature. The dried specimens were then ground using an electrical mill in readiness for extraction.

The sample extraction procedure was carried out as described by Harborne (1994). Briefly, cold sequential extraction was carried out on plant material with analar grade organic solvents of increasing polarity. The solvents used include methanol and ethyl acetate to prepare two different extracts. Three hundred milliliters of methanol were added to 300g of the shred specimen and flasks placed on a shaker and soaked for 48 hours. The residue was filtered using a Buchner funnel under vacuum until the sample dried. The sample was soaked further with 300 ml of methanol for 24 hours until the filtrate remained clear. The filtrate was then concentrated under vacuum by rotary evaporation at 30 - 35°C. The concentrated extracts were transferred to a sample bottle and dried under vacuum; the weight of the dry extracts was recorded and stored at 4°C until required for bioassay. The process was repeated for ethyl acetate.

2.4 Sand Fly Colony

The sand fly colony used belonged to the Phlebotomine genus specifically *Phlebotomus duboscqi* Neveu Lemaire. This colony is being reared at KEMRI for research purposes. *Phlebotomus duboscqi* flies used were collected from Marigat division, Baringo district in Rift Valley province, Kenya. The colony was established using periodically field captured sand flies and inbreeding. In the insectary, Syrian golden hamsters were used for blood feeding female sand flies for egg development. Blood fed females were aspirated into oviposition vials for egg laying. Temperature was maintained at 25±1°C and a relative humidity of 78-83 %. To determine the sample size, the following formula was used where n is the sample size, Z is the confidence level Z-score, C is the confidence interval and E is a margin of error.

$$n = \frac{1}{4}\left(\frac{z_c}{E}\right)^2$$

2.5 Experimental Animals

BALB/c mice were obtained from KEMRI's animal house facility. They were used for infecting the sand flies with *Leishmania* parasites. BALB/c mice were first infected with *Leishmania major* by injecting them with 100,000 *L. major* metacyclic promastigotes in the hind footpad. Infection establishment was determined by the presence of well developed lesions, 5 weeks after infection. Later, sand flies were infected directly by feeding them on the lesions on the hind footpad of BALB/c mice.

2.6 Effects of *T. camphoratus, A. fruticosa,* and *T. minuta* on Fertility of *P. duboscqi*

Fertility of *P. duboscqi* was assessed according to the methods of Moura *et al*., (2002). Briefly, 2 day-old *Phlebotomus duboscqi* female adults were aspirated into plastic vials partially filled with plaster of Paris and fitted with screen tops. Only the blood fed females were aspirated into the plastic vials where they were fed on sugar solutions laced with crude extracts. The insoluble compounds were dissolved in dimethyl sulfoxide (DMSO, Panreac, Barcelona, Spain) at a concentration of 0.1%, after this had been assayed as non-toxic and without inhibitory effects on parasite growth, as previously demonstrated (Dorin *et al*., 2001). These extracts were then mixed with Karo dark corn Karo syrup (Best Foods, CPC International, Inc., Englewood Cliffs, NJ), in the ratio of 1:1. The plant extracts were then prepared into the following concentrations: 2.5 mg/ml, 5 mg/ml and 10mg/ml which were used in the experiment. Eggs laid in the oviposition vials were counted. Dead flies were dissected and their abdomens observed for any eggs

retained in the abdomen. Three replicates were used in this experiment. Fecundity was compared with sand flies that fed on dark corn syrup that was diluted with distilled water and 0.1% DMSO (dark corn Karo syrup mixed with distilled water and 0.1% DMSO without extracts).

2.7 Effects of *A. fruticosa, T. minuta* and *T. camphoratus* on Longevity of *P. duboscqi*

Longevity was assessed according to the methods of Moura *et al*., (2002). Briefly, two to three day old female and male *P. duboscqi* adults were placed in separate plastic vials partially filled with plaster of Paris and fitted with screen tops. The flies were then fed on dark corn Karo syrup laced with the crude extracts in the ratio of 1:1. The flies were fed *ad libitum* and the number of days they lived was noted. Their longevity was compared with flies that were fed on dark corn syrup diluted with distilled water and 0.1% DMSO. Three replicates were used in this experiment with a total of 10 sand flies per replicate.

2.8 Assessing Development of *Leishmania major*

Two to three day old *P. duboscqi* adults were placed in a Perspex cage and allowed to blood feed on BALB/c mice which were infected with *L. major* parasites. The infected mice were anaesthetized with sodium pentobarbitone (Sagatal®), placed in a smaller cage and only the hind foot with a well developed lesion was exposed to the flies for direct feeding (plate 4). After blood feeding for 30 minutes, ten female *P. duboscqi* flies were carefully aspirated into 300 ml oviposition/rearing jars. Drops of Karo dark corn syrup (Best Foods, CPC International, Inc., Englewood Cliffs, NJ), mixed with the crude extract in the ratio 1:1 were placed on the screen tops of the jars for the sand flies to feed. The

flies were fed with the extracts *ad libitum* and maintained at normal insectary conditions (28±1^{0}C and 80±5% relative humidity, 12:12 L: D photoperiod).

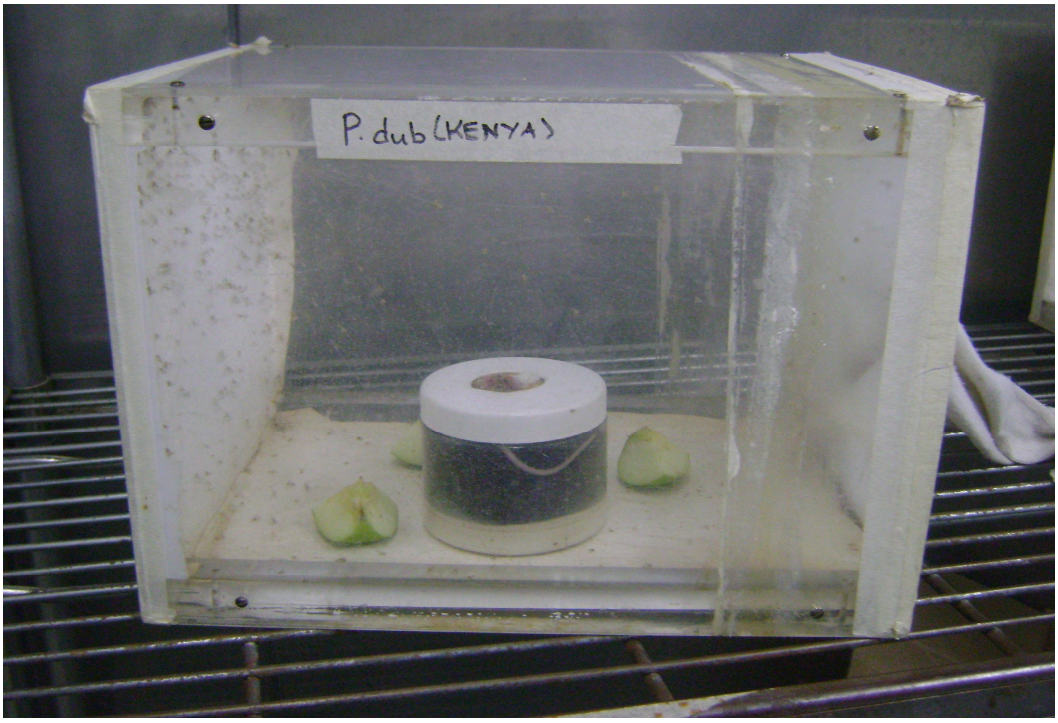

Plate : Experimental set up for infecting *P. duboscqi* with *L. major*

Six days post infection; two sand fly specimens were dissected after every two days in physiologic saline. Their midgut were dissected as described and demonstrated elsewhere (Coleman *et al.,* 2007; Xi *et al.,* 2007) with few modifications whereby only dissecting pins were used instead of fine tipped forceps. Infectivity was determined by looking for presence or absence of promastigotes in the mid guts of the sand flies. Five engorged female sand flies were dissected on days 6, 8, 10, 12 and 14 post-feeding and examined for promastigote forms as previously described. Individual female flies were dissected in a drop of physiologic saline, and their guts examined in wet preparations for parasites. Where parasites were seen, their locations within the guts were noted. In order to

ascertain parasite forms and to enumerate their numbers in the sand flies, slides used in dissections were air-dried, fixed in absolute methanol and stained in Giemsa's stain. The slides were examined under the light microscope (oil immersion) for the presence or absence of procyclics, nectomonads, haptomonads and metacyclic promastigotes. Their morphological features like motility, shape and presence or absence of the flagella were used in differentiating them. The control experiment had flies that fed on dark corn syrup diluted with distilled water and 0.1% DMSO only.

2.9 Determining Parasite Density per Sand Fly

This was carried out according to the methods of Moura *et al.*, (2002) with slight modification. Briefly, the sand flies were allowed to blood feed on the infected BALB/c mice for 30 minutes until their abdomens were full of the blood meal. Then, ten *P. duboscqi* adult flies were placed in 7-dram plastic vials partially filled with plaster of Paris and fitted with screen tops. The flies were then fed on the crude extract mixed with sugar syrup (Karo syrup) in the ratio of 1:1. Six days post feeding, 2 sand flies from each vial were dissected (after every 2 days till day 14) in physiologic saline and examined for parasite development. The gut of each sand fly was carefully drawn out of the abdomen and observed under a light microscope. Infected sand flies were counted and the number recorded. To determine the parasite load, dissected and infected sand flies were air-dried on microscope slides, fixed in absolute methanol and stained in Giemsa's stain. The slides were examined under the light microscope for the presence or absence of parasites and their density graded according to Chulay and Brycesson’s 1983 grading system (Table 1).

Table : Chulay and Brycesson's 1983 parasite grading system

GRADE	AVERAGE PARASITE DENSITY
6+	10-100 Parasites/10 fields
5+	10-100 Parasites/field
4+	1-10 Parasites/field
3+	1-100 Parasites/ 10 fields
2+	1-10 Parasites/100 fields
1+	1-10 Parasites/1000 fields

2.10 Ethical and Biosafety considerations

Before carrying out the study, approval was sought from KEMRI's ethical review committee. The experiment was carried out in compliance with Animal Care and Use Committee regulations. Standard operating procedures were followed all the time. These include injecting of hamsters using a standard 21 gauge needle through IP, anaesthetizing them using 6% sodium pentobarbitone and killing them using carbon dioxide asphyxiation. Dead animals were sterilized by dipping them in 70% ethanol and disposed in biohazard bags before transferring them to the incinerator. Precautionary measures included putting on protective gear and carrying out the experiments in a laminar flow hood where necessary.

2.11 Data Analysis

All the experiments were carried out in triplicate. The mean and standard deviation of three experiments was determined. Statistical analysis of the differences between mean values obtained for the experimental groups was done by analysis of variance (ANOVA) and student's *t* test. The means were separated using LSD. *P* values of < 0.05 were considered significant.

CHAPTER THREE

3.0 RESULTS

3.1 Effects of the Crude Plant Extracts on the Fertility of *P. duboscqi*

The average number of eggs laid by sand flies after exposure to the different crude extracts decreased with increase in extract concentration. At a concentration of 2.5mg/ml the number of eggs laid by sand flies that had fed on *A. fruticosa* crude extract was 8.67±1.20 (mean±SE, n=30, *P*=0.003), *T. minuta* crude extract was 10.20±1.15 (mean±SE, n=30, *P*=0.017) and *T. camphoratus* crude extract was 14.34±1.75 (mean±SE, n=30, *P*=0.06). This difference in the number of eggs laid by flies that had fed on *T. camphoratus* crude extract was not significant when compared to the control group. At a concentration of 5mg/ml, the number of eggs laid was 5.44±2.06 (n=30, *P*=0.016) in *A. fruticosa* extract, 8.50±1.51 (n=30, *P*=0.01) in *T. minuta* crude extract and 12.84±1.24 (n=30, *P*=0.019) in *T. camphoratus* crude extract. Sand flies in the control group had laid 15.00±1.52 eggs (mean±SE).

Feeding the sand flies on 10 mg/ml crude extract concentration, led to further inhibition of egg laying. The sand flies that had fed on *A. fruticosa* crude extract did not lay eggs while those that had fed on *T. minuta* crude extract laid 4.00±2.31 (mean±SE, n=30, *P*=0.014). The sand flies that formed the control group laid 15.00±1.00 eggs (mean±SE). However, the flies that had fed on *T. camphoratus* crude extract laid 10.67±1.76 eggs (mean±SE, n=30, *P*=0.002) (fig. 4). Dissection of the dead flies revealed that their guts were full of undigested blood.

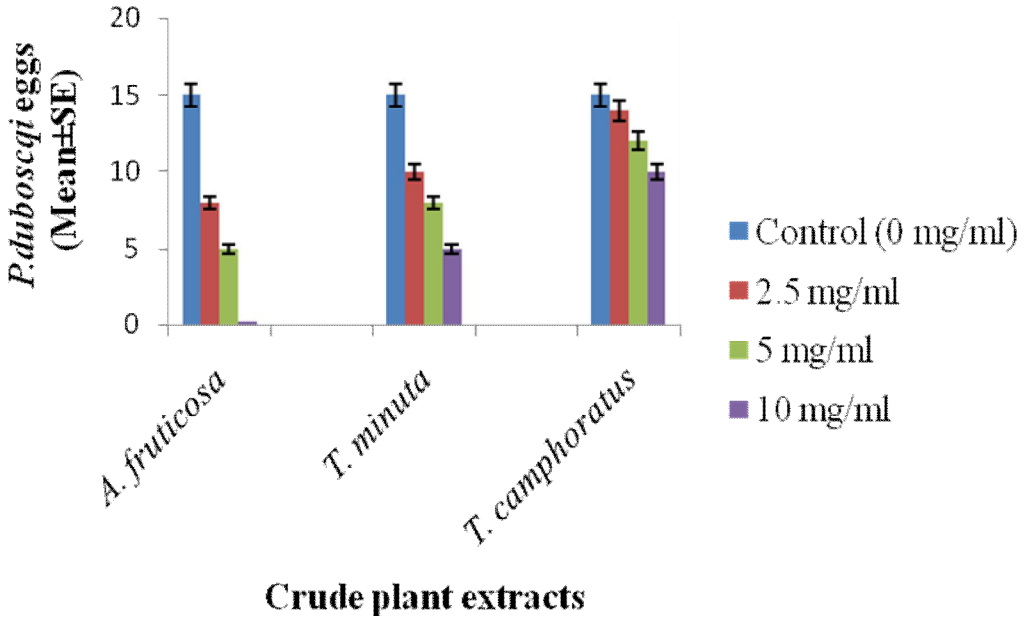

Figure : Mean number of eggs laid by *P. duboscqi* after feeding on methanol crude extracts of *Acalypha fruticosa, Tagetes minuta* and *Tarchonanthus camphoratus*

There was also a significant difference among the sand flies that had fed on ethyl acetate crude extracts. At 2.5 mg/ml, the mean number of eggs laid by the sand flies that had fed on *A. fruticosa* extract was 10.33±2.67 (mean±SE, n=30, *P*=0.019), *T. minuta* crude extract was 13±0.58 (n=30, *P*=0.071) and 14.67±1.33 eggs (n=30, *P*=0.084). The sand flies in the control group had laid 15.48±1.53 (mean±SE).

Feeding the sand flies on the crude extracts at 5 mg/ml concentration inhibited egg laying further. The sand flies that had fed on *A. fruticosa* extract laid 7.68±1.22 eggs (n=30, *P*=0.014), eggs laid after feeding on *T. minuta* extract were 9.33±2.84 (n=30, *P*=0.021)

while those that had fed on *T. camphoratus* laid 11.67±2.33 eggs (n=30, *P*=0.027). At 10 mg/ml concentration, there was a significant inhibition of egg laying in all the three crude extracts. The flies that had fed on *T. camphoratus* had laid the highest number of eggs, 10.67 ± 1.86 (*P*=0.019) followed by those that had fed on *T. minuta* with 8.67 ± 1.33 (*P*=0.011) eggs and finally those flies that had fed on *A. fruticosa*, 5.33 ± 0.33 (*P*=0.001) eggs (mean±SE). Comparing the flies that had fed on methanol extracts with those that had fed on ethyl acetate crude extracts shows that more eggs were laid by the sand flies that had fed on ethyl acetate extracts.

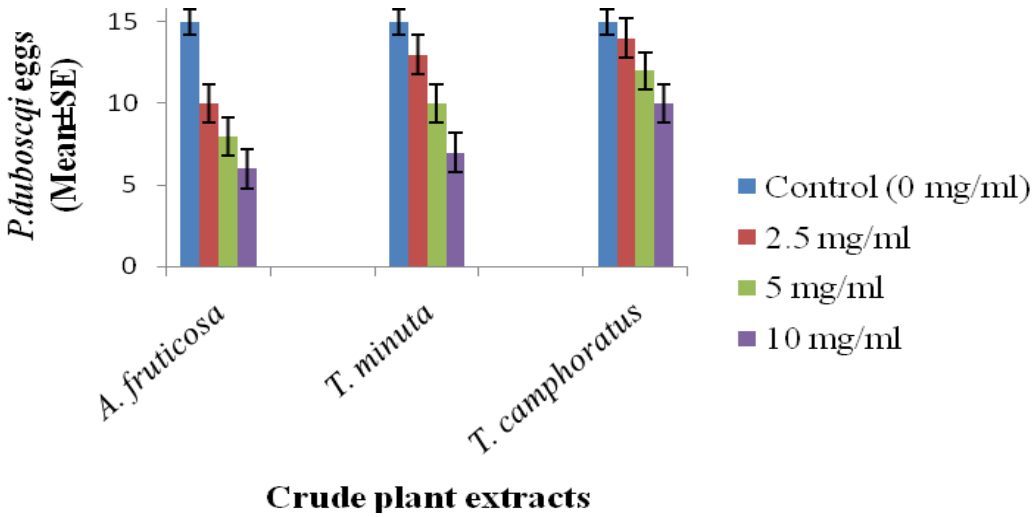

Figure : Mean number of eggs laid by *P. duboscqi* after feeding on ethyl acetate crude extracts of *A. fruticosa, T. minuta* and *T. camphoratus*

3.2 Combined Effects of the Crude Extracts on Fertility of *P. duboscqi*

The number of eggs laid by *P. duboscqi* after feeding on the different combinations of the methanol crude extracts was dependent on the concentration used. At a concentration of 2.5 mg/ml, the sand flies that had fed on *A. fruticosa* + *T. minuta* extract laid 10.70±0.58

eggs (mean±SE, n=30, *P*=0.001), *A. fruticosa + T. camphoratus* extract 11.33±0.67 eggs (mean±SE, n=30, *P*=0.011) *and T. minuta + T. camphoratus* 13.60±1.69 eggs (mean±SE, n=30, *P*=0.081). The sand flies that had formed the control group laid 15.33±1.45 eggs. The difference observed when comparing eggs laid by sand flies that had fed on *T. minuta + T. camphoratus* extract combination with the control group was not significant. Feeding the sand flies on *A. fruticosa + T. minuta* and *A. fruticosa + T. camphoratus* extracts led to a significant decline in egg laying. However, the difference was not significant as compared to the individual crude extracts.

Increasing the crude extract concentration to 5 mg/ml led to further inhibition of egg laying. The sand flies that had fed on *A. fruticosa + T. minuta* extract combination laid 9.00±0.68 eggs (mean±SE, n=30, *P*=0.018), *A. fruticosa + T. camphoratus* 9.93±0.67 eggs (mean±SE, n=30, *P*=0.021) and *T. minuta + T. camphoratus* 11.67±1.63 (mean±SE, n=30, *P*=0.026). The number of eggs laid per sand fly was significant across all the three combinations used as compared to the control. However, the effect of these combined extracts was not significantly different from those of individual extracts.

Highest inhibition was observed when 10 mg/ml concentration was used. The sand flies that had fed on *A. fruticosa + T. minuta* extract laid 5.33±0.57 eggs (mean±SE, n=30, *P*=0.001), *A. fruticosa + T. camphoratus* extract laid 6.73±1.47 (mean±SE, n=30, *P*=0.016) eggs and *T. minuta + T. camphoratus* extract laid 8.35±1.55 eggs (mean±SE, n=30, *P*=0.014). The effects of *A. fruticosa + T. minuta* combination differed

significantly (P<0.001) from the *A. fruticosa* + *T. camphoratus* and *T. minuta* + *T. camphoratus* combinations (fig. 6).

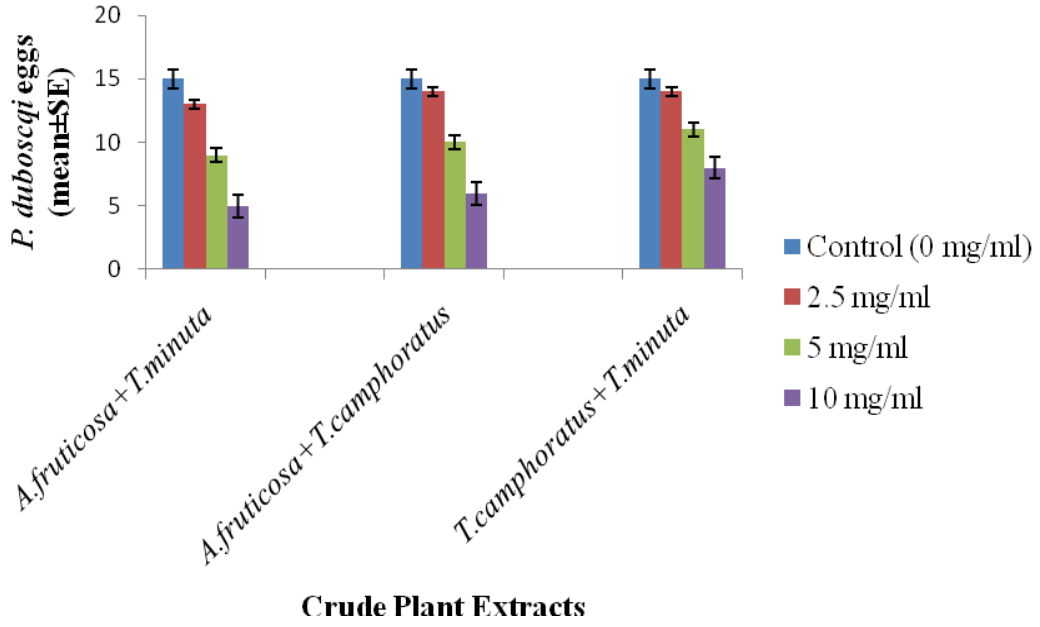

Figure : Mean number of eggs laid by *P. duboscqi* after feeding on the combined methanol extracts

Comparing the sand flies that had fed on methanol and ethyl acetate extracts did not show any significant difference in egg laying. At a concentration of 2.5 mg/ml, the flies that had fed on *A. fruticosa* + *T. minuta* extract laid 11.64±0.16 eggs (mean±SE, n=30, P=0.011). Sand flies that had fed on *A. fruticosa* + *T. camphoratus* extract laid 13.22±1.68 eggs (mean±SE, n=30, P=0.02) while those that had fed on *T. minuta*+ *T. camphoratus* extract laid 14.55±1.45 eggs (mean±SE, n=30). The effects of *T. minuta*+ *T. camphoratus* extract on egg laying was not significant as compared to the control sand flies. At 5 mg/ml, feeding the sand flies on the extracts led to further inhibition of egg laying. Feeding the sand flies on *A. fruticosa* + *T. minuta* extract resulted in 8.43±1.57

eggs (mean±SE, n=30, *P*=0.013) and *A. fruticosa* + *T. camphoratus* resulted in 10.45±1.55 eggs (mean±SE, n=30, *P*=0.021). The sand flies that had fed on *T. minuta* + *T. camphoratus* extract had laid 10.67±1.33 eggs (mean±SE, n=30, *P*=0.024).

Further inhibition was observed when the sand flies were fed on 10 mg/ml extract concentration. The sand flies that had fed on *A. fruticosa* + *T. minuta* extract had laid 4.84±0.66 eggs (mean±SE, n=30, *P*=0.001) while those that had fed on *A. fruticosa*+*T. camphoratus* had laid 7.45±0.65 eggs (mean±SE, n=30, *P*=0.002). The sand flies that fed on *T. minuta* + *T. camphoratus* extract had laid 7.67±1.33 eggs (mean±SE, n=30, *P*=0.011). This difference was significant (fig. 7).

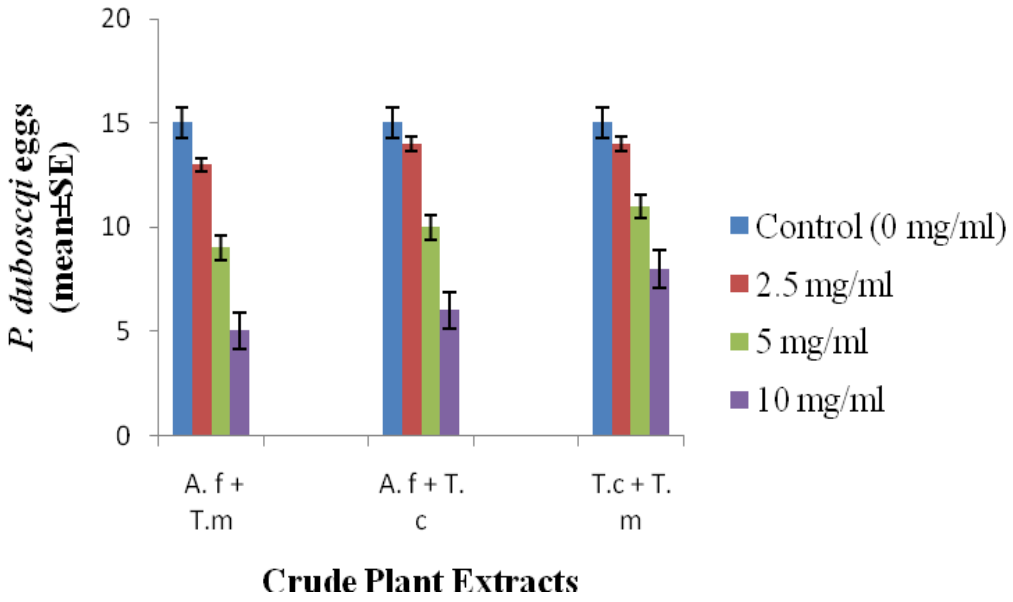

Figure : Mean number of eggs laid by *P. duboscqi* after feeding on the ethyl acetate combined extracts

Key

A. f + T. m = *Acalypha fruticosa + Tagetes minuta*

A. f + T. c = *Acalypha fruticosa + Tarchonanthus camphoratus*

T. c + T. m = *Tarchonanthus camphoratus + Tagetes minuta*

3.3 Effect of the Crude Extracts on Longevity of *P. duboscqi*

After feeding on the crude extracts, the sand flies showed a reduction in the number of days they stayed alive. Survival time decreased with increase in concentration of the plant extract used. At a concentration of 2.5 mg/ml methanol extracts, longevity in days was highest in the sand flies that had fed on *T. camphoratus* extract, 10.67±.33 days (mean±SE, n=30, *P*=0.011). This was followed by the sand flies that had fed on *T. minuta* extract that had lived for 9.44±1.46 days (mean ± SE, n=30 *P*=0.011). However, the sand flies that had fed on *A. fruticosa* extract had the lowest longevity of 8.67±0.33 days.

Further decline in survival time was observed when higher extract concentrations were used. At 5 mg/ml, lowest survival time was observed in sand flies that had fed on *A. fruticosa* extract, 6.00±0.58 days (mean±SE, n=30, *P*=0.001). Sand flies in the control group lived for 12.00±1.00 (mean±SE) under similar conditions. The sand flies that had fed on *T. camphoratus* had the highest longevity of 9.60±2.54 days (mean±SE, n=30, *P*=0.021).

At 10 mg/ml, longevity was further suppressed across all the three extracts used. In the sand flies that had fed on *A. fruticosa* extract lived for 4.40±0.58 days (mean±SE, n=30,

P=0.001). This difference was significant as compared to the sand flies that had formed the control group. Feeding the sand flies on *T. minuta* and *T. camphoratus* extracts resulted in 5.24±0.56 and 7.62±0.28 days of survival (mean±SE) respectively (fig. 8).

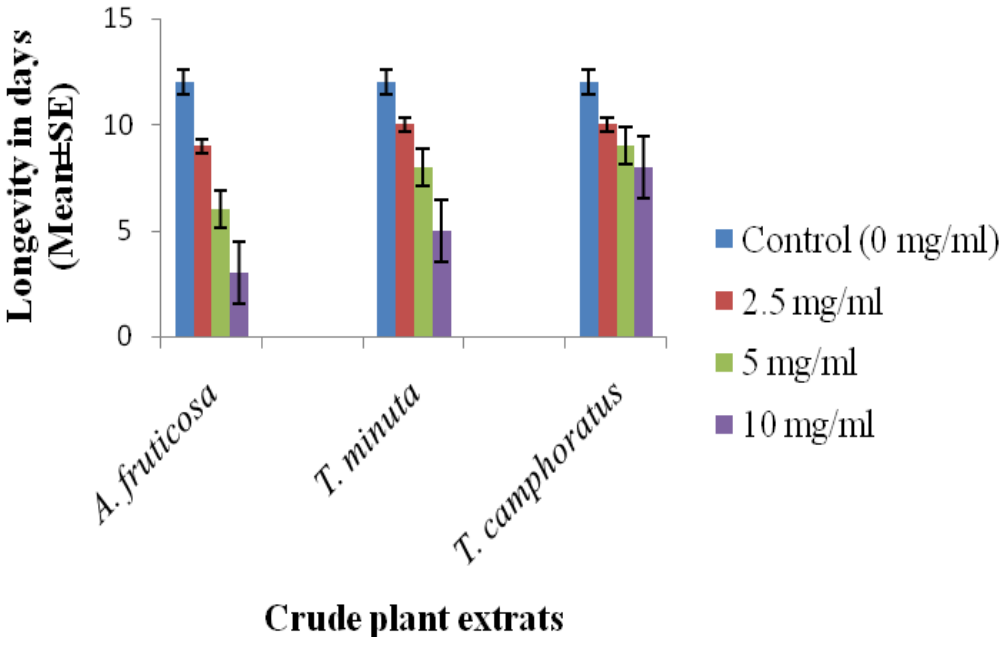

Figure : Longevity of *P. duboscqi* after feeding on the methanol crude extracts of *A. fruticosa, T. minuta* and *T. camphoratus*

In the ethyl acetate crude extract, there was a steady decline in longevity in the sand flies that had fed on the three crude extracts as compared to control group. At 2.5 mg/ml, sand flies that had fed on *A. fruticosa, T. camphoratus* and *T. minuta* crude extracts lived for 11.34±0.26, 10.67±1.33 and 10.44±0.66 days (mean±SE) respectively. At 5 mg/ml concentration, survival time was 7.54±1.46, 9.67±1.33 and 9.00±1.00 (mean±SE) days in the sand flies that had fed on *A. fruticosa, T. camphoratus* and *T. minuta* crude extracts

respectively. Longevity in the control group was 12.48±1.12 (mean±SE) days at the same extract concentration.

At 10 mg/ml, survival time was greatly depressed in all the three extracts. The sand flies that had fed on *A. fruticosa* extract survived for 4.42±1.38 days (mean±SE, n+30, *P*=0.001) while those that had fed on *T. camphoratus* had the highest survival time, 7.67±0.33 (mean±SE, n=30, *P*=0.017). The sand flies that had fed on *T. minuta* crude extract lived for 5.45±1.55 days (mean±SE, n=30, *P*=0.012) (fig. 9). The difference in longevity of the sand flies that had fed on methanol extracts and those that had fed on ethyl acetate extracts was not significant.

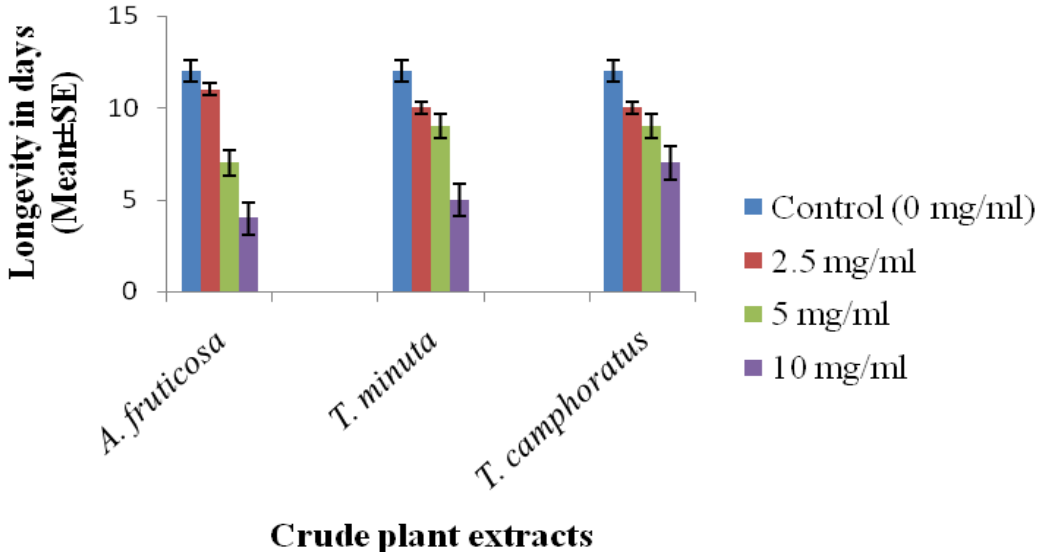

Figure : Longevity of *P. duboscqi* after feeding on *A. fruticosa, T. minuta* and *T. camphoratus* ethyl acetate crude extracts

3.4 Combined Effects of the Crude Extracts on Longevity of *P. duboscqi*

The extracts evoked a significant decline in survival time; however, the difference was not significant as compared to the individual plant extracts. Survival time was dependent on the extract concentration used. Feeding the sand flies on 2.5 mg/ml methanol extracts of *A. fruticosa + T. minuta, A. fruticosa + T. camphoratus* and *T. minuta + T. camphoratus* extract combinations resulted in 11.00±1.00, 11.84±1.46 and 12.34±0.44 days respectively. This difference was not significant as compared to the sand flies that formed the control group.

There was further decline in survival time when 5 mg/ml extract concentration was used. The sand flies that had fed on *A. fruticosa + T. minuta* extract combination had the least survival time of 6.74±1.06 days (mean±SE, n=30, *P*=0.002). This was followed by the sand flies that had fed on *A. fruticosa+T. camphoratus* and *T. minuta + T. camphoratus* extract combinations that lived for 10.67±0.33 (mean±SE, n=30, *P*=0.47) and 9.00±0.58 (n=30, *P*=0.31) days respectively. This difference was not significant as compared to the sand flies that formed the control group (fig. 10).

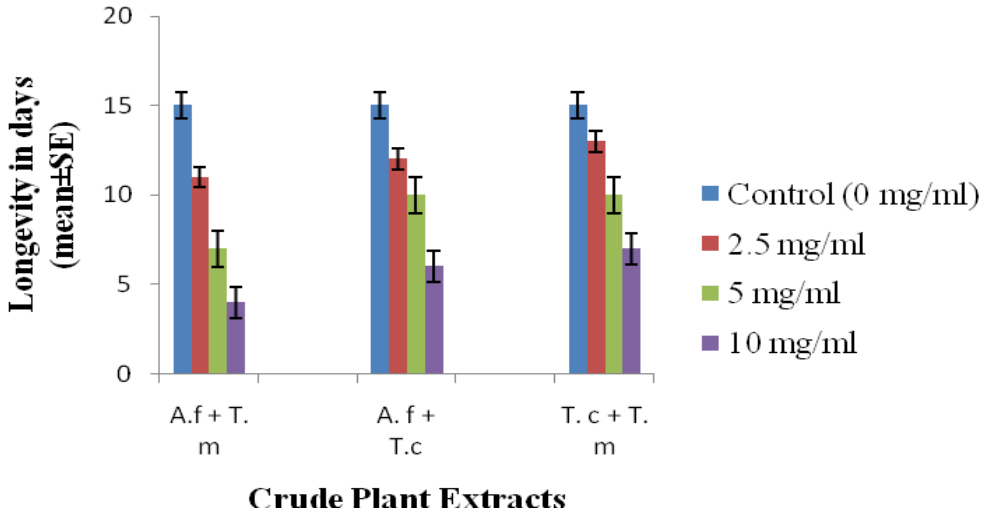

Figure : Longevity of *P. duboscqi* in days after feeding on the combined methanol crude extracts

Key

A. f + T. m= *Acalypha fruticosa* + *Tagetes minuta*

A. f + T. c= *Acalypha fruticosa* + *Tarchonanthus camphoratus*

T. c + T. m= *Tarchonanthus camphoratus* + *Tagetes minuta*

In the ethyl acetate extract combinations, longevity of *P. duboscqi* was dependent on extract concentration used. At 2.5 mg/ml, the sand flies that had fed on the crude extracts of *A. fruticosa* + *T. minuta* combination was 10.10±0.55 (mean±SE, n=30, *P*=0.012), *A. fruticosa* + *T. camphoratus* 11.33±0.67 (mean±SE, n=30, *P*=0.012), *T. minuta* + *T. camphoratus* 12.67±0.88 (mean±SE, n=30, *P*=0.08). This difference was not significant when compared to the control group.

At 5 mg/ml, longevity of the sand flies that had fed on the crude extracts of *A. fruticosa* + *T. minuta* was 8.18±1.52 (mean±SE, n=30, *P*=0.011), *A. fruticosa* + *T. camphoratus* 11.33±0.67 (mean±SE, n=30, *P*=0.012), *T. minuta* + *T. camphoratus* 10.22±0.88 (mean±SE, n=30, *P*=0.012). When 10 mg/ml concentration was used, the sand flies that had fed on the extracts of *A. fruticosa* + *T. minuta* combination was 4.33±0.67 (mean±SE, n=30, *P*=0.001), *A. fruticosa* + *T. camphoratus* 7.33±0.67 (mean±SE, n=30, *P*=0.002), *T. minuta* + *T. camphoratus* 7.67±0.33 (mean±SE, n=30, *P*=0.002) (fig.11). These results showed that both methanol and ethyl acetate crude extracts combinations did not result in any significant difference between the combinations and the individual extracts. Therefore, in the subsequent experiments, combined crude extract bioassays were excluded.

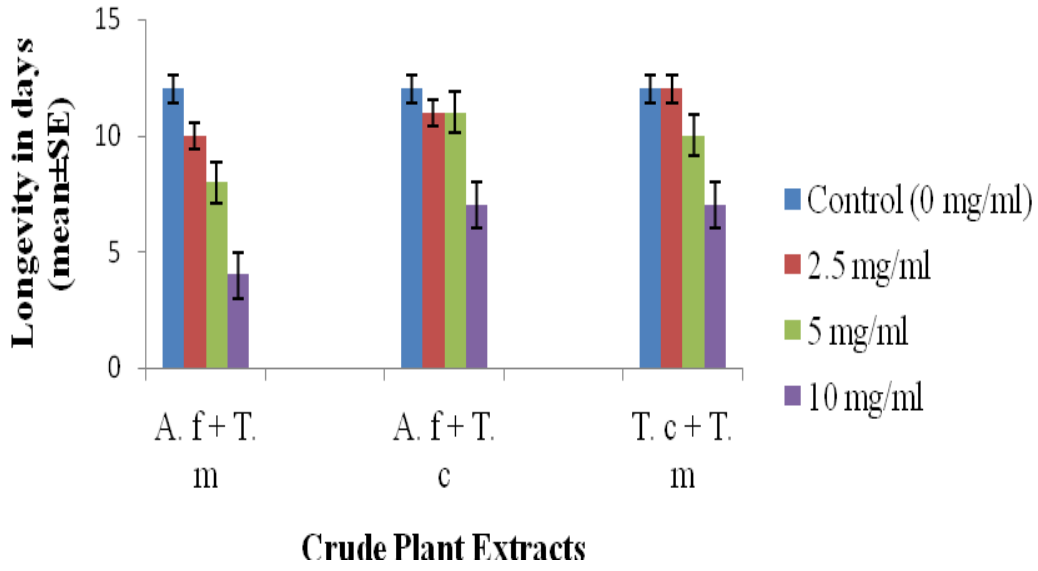

Figure : Longevity of *P. duboscqi* in days after feeding on the combined ethyl acetate crude extracts

Key

A. f + T. m= *Acalypha fruticosa + Tagetes minuta*

A. f + T. c= *Acalypha fruticosa + Tarchonanthus camphoratus*

T. c + T. m= *Tarchonanthus camphoratus + Tagetes minuta*

3.5 Effect of the Crude Extracts on the Developmental Stages of *Leishmania major*

The effect of *Acalypha fruticosa, Tagetes minuta* and *Tarchonanthus camphoratus* extracts on the developmental process of *Leishmania major* was assessed. Development was found to be dependent on the type of extract used. *A. fruticosa* extract was found to be more effective against the parameters assessed, followed by *T. minuta* extract and finally *T. camphoratus* extract as shown in the following sections.

3.5.1 Effect of *A. fruticosa* Methanol Crude Extracts on *L. major* Development

Parasite development from amastigotes to metacyclic stage was dependent on the dose of the crude extract used to feed *P. duboscqi*. At 2.5mg/ml of methanol extract concentration, the number of parasites present was significantly reduced as compared to the control group. At day six post feeding, amastigotes were 19.33±1.33 (mean±SE, n=30, *P*=0.001) in the treatment while in the control group there were 32.00±1.00 amastigotes. The amastigotes were round in form and no division was observed. Further examination showed that there was no evidence of flagella development within the first 6 days.

Transformation of the ingested amastigotes to procyclic promastigotes was first seen 8 days post infection. The number of procyclic promastigotes was 9.33±0.67 (mean±SE,

n=30, *P*=0.014) and were larger than amastigotes although they were short and ovoid in form (plate 5). The rest of the parasites (29%) were amastigotes. Each procyclic parasite had a flagellum that was shorter than the body. They exhibited slight movement within the blood meal. At day 10, 8.00±1.55 (mean±SE, n=30, *P*=0.001) were nectomonads which were longer and slender than the procyclic promastigotes. Their flagella were slightly longer than the body**.** At this time, the control group had numerous (22.00±0.50), long and slender nectomonads which were highly motile. At day 12, haptomonads were 8.67±0.33 (mean±SE, n=30, *P*=0.013). Their flagella were as long as the body size. At day 13, metacyclics were 6.67±2.03 (mean±SE, n=30, *P*=0.001) observed after much searching among the cells of the anterior midgut. They were the shortest of all the parasites, very slender and highly motile. Their flagella were longer than the body. In this concentration, the parasites went through a complete life cycle.

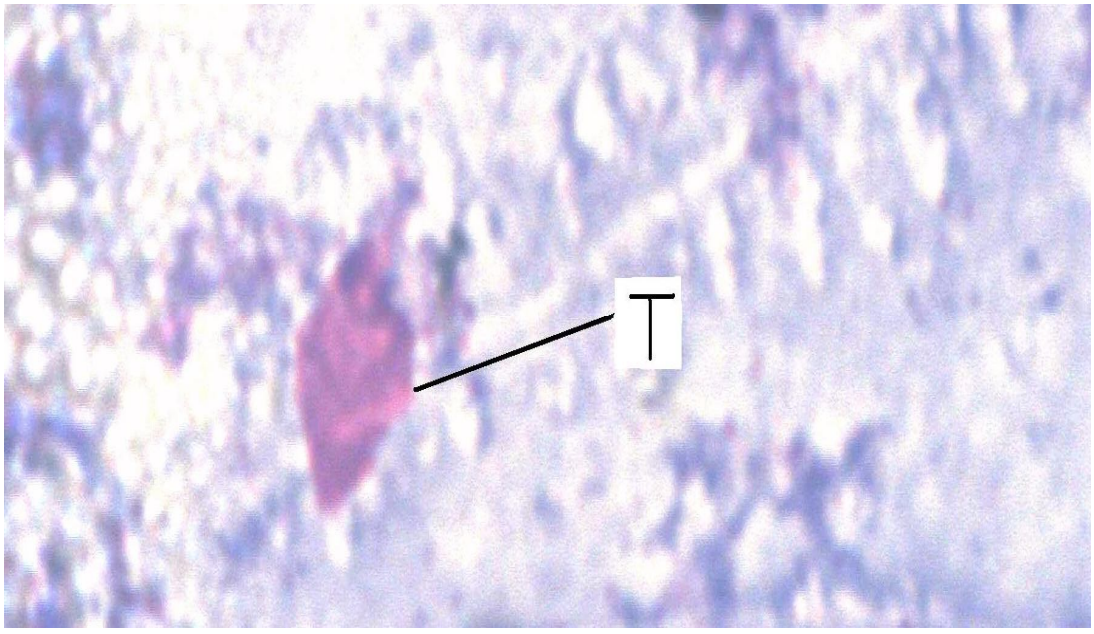

Plate : A procyclic promastigote under *A. fruticosa* extract (Mg=X1000)

T- Represents a fully transformed procyclic promastigote

However, at 5mg/ml and 10mg/ml concentrations, the developmental cycle did not reach completion. At 5mg/ml concentration, development was inhibited at the haptomonad stage while in 10mg/ml; the cycle was inhibited at the nectomonad stage. Amastigotes observed were tiny and spherical in form while procyclic promastigotes were elongate in form without flagella. The inhibition persisted and even increased with time, reaching values of 86% inhibition at 10 days of experimental period. At this time, the number of parasites per fly was greatly diminished when compared with the control. During this time, only the nectomonads were the predominant developmental stage with short flagella. This effect continued and increased slightly over the 14 experimental days. Development was inhibited at the haptomonad stage under 5mg/ml treated group while at 10mg/ml; development was inhibited at the nectomonad stage.

The mean number of the different parasite stages at 5mg/ml extract concentration was: amastigotes 13.67±1.33 (mean±SE, n=30, *P*=0.01), procyclic promastigotes 8.67± 0.83 (mean±SE, n=30, *P*=0.011), nectomonads 6.00±1.55 (mean±SE, n=30, *P*=0.01) and haptomonads 6.67±0.33 (mean±SE, n=30, *P*=0.011). At 10 mg/ml extract concentration, number of parasites was; amastigotes 12.00±1.53 (mean±SE, n=30, *P*=0.001), procyclic promastigotes 7.33±0.67 (mean±SE, n=30, *P*=0.003) and nectomonads 5.00±1.54 (mean±SE, n=30, *P*=0.001). In the control groups, the parasite numbers were; amastigotes 32.00±1.00, procyclic promastigotes 28.00±0.00 nectomonads 22.00±0.00 and haptomonads 32.00±1.00 (fig. 12).

At 10 mg/ml extract concentration, the mean number of the parasites was: amastigotes 11.60±1.30 (mean±SE, n=30, *P*=0.01), procyclic promastigotes 8.27± 0.83 (mean±SE, n=30, *P*=0.011), nectomonads 7.20±1.55 (mean±SE, n=30, *P*=0.01). No haptomonad and metacyclic forms were observed. In the control group, haptomonad and metacyclic forms were 32.00±1.52 and 26.67±1.33 (mean±SE) respectively (fig. 12).

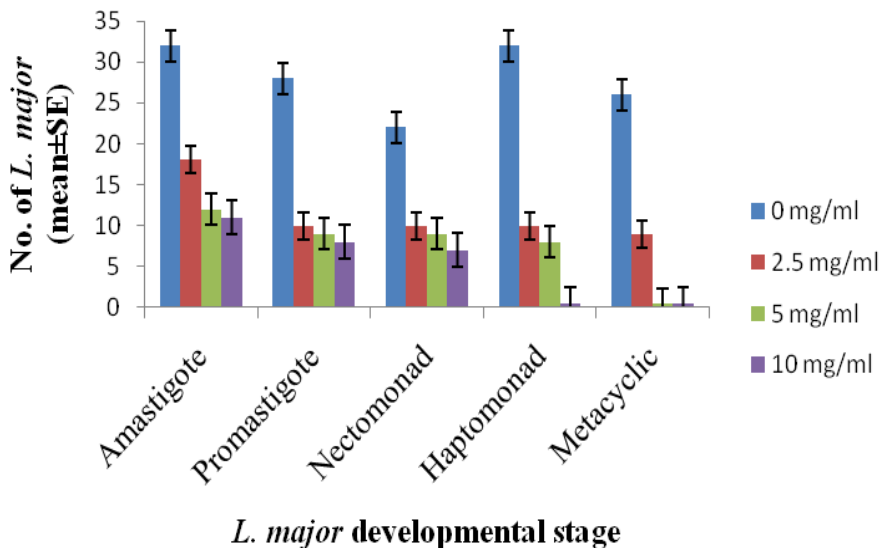

Figure : Average number of *Leishmania* parasites as seen after administering *A. fruticosa* methanol extract

3.5.2 Effect of *A. fruticosa* Ethyl Acetate Crude Extract on *L. major* Development

In 2.5 mg/ml concentration, the parasites showed normal morphology although; there were lower levels of development as compared to the control group. Amastigotes were

21.67±1.33, (mean±SE, n=30, *P*=0.01). This accounted for 95% of the total parasites. 5% were procyclic promastigotes which were short and ellipsoid in form. At days 8, 10, 12 and 14, the numbers of the predominant stages were procyclics 13.33±2.67 (mean±SE, n=30, *P*=0.012), nectomonads 13.00±1.00 (mean±SE, n=30, *P*=0.01), haptomonads 15.33±1.45 (mean±SE, n=30, *P*=0.017), and metacyclics 10.00±1.73 (mean±SE, n=30, *P*=0.001) respectively. However, nectomonads showed the absence of flagella while haptomonads and metacyclics showed normal morphology.

At 5mg/ml concentration, there were mainly amastigotes at 6 days post feeding. However, their number was greatly reduced to 16.67±1.33 (mean±SE, n=30, *P*=0.001) compared to the control group (32.00±1.00). Transformation continued with 12.00±1.55 (mean±SE, n=30, *P*=0.002) of the total parasites being procyclic promastigotes by the end of day 8 of the experimental period, nectomonads were 10.25±1.15 (mean±SE, n=30, *P*=0.011), while haptomonads were 12.00±1.00 (mean±SE, n=30, *P*=0.01). Development was highly inhibited at this stage and by the end of the 14th day; there were no parasites in the sand flies' gut. Sand flies in the control group had mainly metacyclics at day 14.

10mg/ml concentration had the highest inhibition percentage whereby at day 6, there were 16.67±1.33 (mean±SE, n=30, *P*=0.012) amastigotes, 9.00±0.58 (mean±SE, n=30, *P*=0.001) procyclic promastigotes at day 8 and 6.33±0.67 (mean±SE, n=30, *P*=0.001) nectomonads at day 10. The control group had 32.00±1.00 amastigotes, 28.00±0.00 procyclics, 24.00±1.65 nectomonads and 25.00±0.00 metacyclic promastigotes at days 6,

8, 10, 12, and 14 respectively. The difference in number of parasites was significant among the different stages of parasite development (Fig. 13).

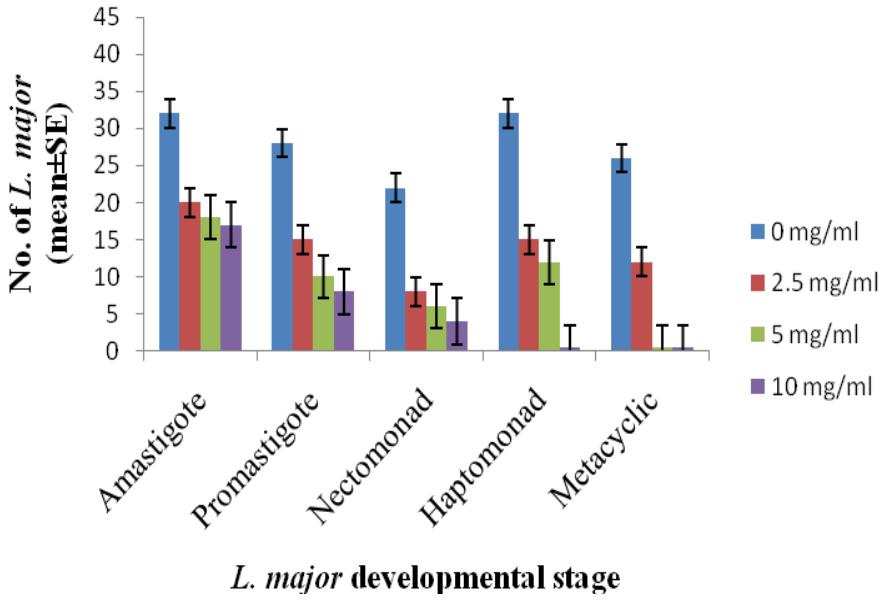

Figure : Average number of *Leishmania* parasites observed after feeding on *A. fruticosa* ethyl acetate crude extract

3.5.3 Effect of *T. minuta* Methanolic Crude Extract on *L. major* Development

At 2.5mg/ml concentration; there was full parasite development though the parasite numbers were significantly low as compared to the control group. At day 8, there were 17.33±2.09 amastigotes (mean±SE, n=30, *P*=0.002), procyclics were the predominant stage with 11.00±3.06 (mean±SE, n=30, *P*=0.001) parasites, nectomonads were 10.67±1.20 (mean±SE, n=30, *P*=0.001) at day 10, 9.33±0.67 (mean±SE, n=30, *P*=0.001) were haptomonads while at day 13; the predominant parasite stage was metacyclics 7.00±1.73 (mean±SE, n=30, *P*=0.001).

At 5mg/ml concentration, there was a reduction in the rate of development of *L. major* parasites. At day 6, all the flies had mainly amastigotes 17.67±1.33 (mean±SE, n=30, *P*=0.013). At this stage the control group showed the longer, broader procyclic promastigotes 17.33±2.85 and fewer nectomonads which are advanced stages as compared to amastigotes in the treatment. However, at day 8, only 7.00±0.58 (mean±SE, n=30, *P*=0.011) were procyclics in the treatment, while the control had mainly nectomonads 22.00±1.78 and a few haptomonads. At days 10 and 12, there were only 6.33±0.88 (mean±SE, n=30, *P*=0.001) nectomonads and 7.33±0.67 (mean±SE, n=30, *P*=0.001) haptomonads respectively. This difference was significant as compared to the control group (*P*<0.05). Parasite development was highly inhibited at the haptomonad stage.

At a concentration of 10mg/ml of *T. minuta* extract, percentage inhibition was highest. At day 8, only 15.33±2.91 (mean±SE, n=30, *P*=0.01) were amastigotes. However, at day 8 parasite densities decreased greatly from 15.33±2.91 amastigotes to 5.67±1.53 (mean±SE, n=30, *P*=0.001) procyclic promastigotes. At this stage, the control group had the longer, broader procyclic promastigotes (16.33±0.88) and mainly nectomonads (5.83±2.33). The transformation from nectomonads to haptomonads was inhibited further with only 4.33±0.67 (mean±SE, n=30, *P*=0.016) of the parasites being haptomonads at day 10. Percentage inhibition was 91% with only 3.33±1.33 (mean±SE, n=30, *P*=0.002) at day12 and this inhibition persisted and even increased with time, reaching values of 100% inhibition at 14 days of *T. minuta* crude extract administration (fig. 14).

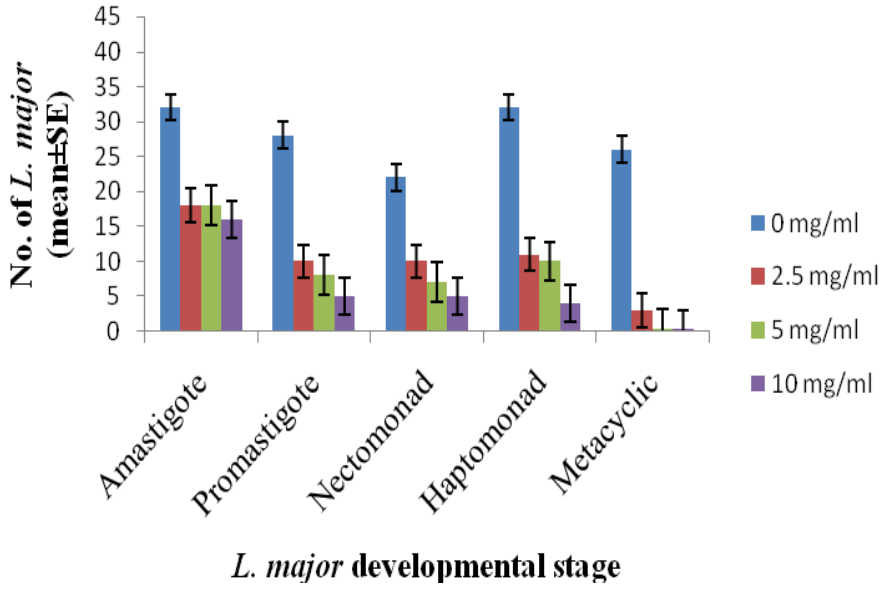

Figure : Mean number of *L. major* parasites observed after exposure to *T. minuta* crude extracts

3.5.4 Effect of *T. minuta* Ethyl Acetate Crude Extract on *L. major* Development

Inhibition of parasite development was dependent on the dosage of the crude extract used. At a concentration of 2.5mg/ml, the parasites went through a full developmental cycle with normal morphology. However, the mean number of parasites at each stage differed significantly from those of the control group. The mean number of amastigotes was 20.67±1.20 (mean±SE, n=30, *P*=0.02), procyclic promastigotes 11.33±2.85 (mean±SE, n=30, *P*=0.001), nectomonads 12.33±1.45 (mean±SE, n=30, *P*=0.017), haptomonads 15.83±2.29 (mean±SE, n=30, *P*=0.003) while that of metacyclic promastigotes was 7.67±1.20 (mean±SE, n=30, *P*=0.001).

At 5mg/ml concentration of *T. minuta*, parasite transformation was inhibited with percentage inhibition reaching 86%. At day 6, there were 18.67±1.33 amastigotes (mean±SE, n=30, *P*=0.001). The control group had 32.00±0.00 amastigotes at day 6. At day 8, there were 10.67±1.20 (mean±SE, n=30, *P*=0.003) procyclic promastigotes, 10.83±2.33 (mean±SE, n=30, *P*=0.001) nectomonads at day 10 and 14.67±2.40 (mean±SE, n=30, *P*=0.019) haptomonads at day 12. At this concentration, development of *L. major* was significantly inhibited at the haptomonads stage.

At 10mg/ml concentration, the mean number of parasites was significantly different from the control group (*P*<0.05). At day 6, the parasite mean number was 17.67±1.76 (mean±SE, n=30, *P*=0.021) amastigotes while at day 8, there were 6.67±0.88 (mean±SE, n=30, *P*=0.01) procyclic promastigotes. At days 10 and 12, parasite numbers were 8.67±0.33 (mean±SE, n=30, *P*=0.001) nectomonads and 6.33±0.33 (mean±SE, n=30, *P*=0.001) haptomonads respectively. The cycle was fully inhibited at the haptomonad stage at day 10 (fig. 15).

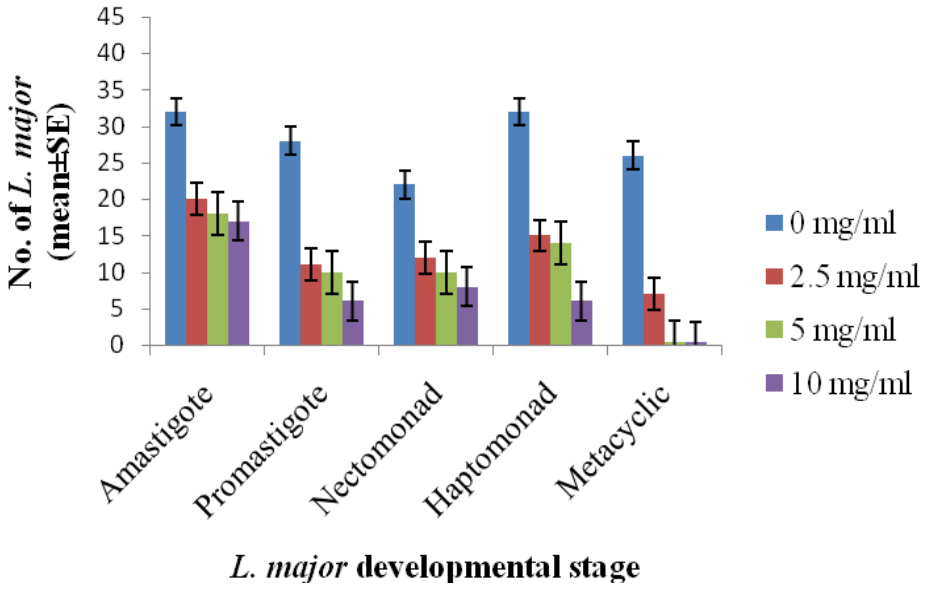

Figure : Mean number of *L. major* parasites observed after feeding on *T. minuta* ethyl acetate crude extracts

3.5.5 Effect of *T. camphoratus* Methanolic Crude Extract on *L. major* Development

Tarchonanthus camphoratus showed the least activity on the transformation of *L. major*. At 2.5mg/ml, a marked fall was noted in the degree of inhibition in the transformation of amastigotes to nectomonads. Transformation in *T. camphoratus* was faster than in *A. fruticosa* and *T. minuta* crude extracts whereby more parasites were observed at each stage. At days 6 and 8, there were 21.67±1.33 (mean±SE, n=30, P=0.031) and 17.33±2.85 (P=0.023) amastigotes and procyclic promastigotes respectively. These numbers were significantly different from the flies which had fed on *A. fruticosa* and *T. minuta* crude extracts (P<0.05). At day 10, 10.00±1.15 (mean±SE, n=30, P=0.01) were nectomonads while at day 12, 15.00±2.52 (mean±SE, n=30, P=0.017) were haptomonads. At day 14,

there was a further transformation with 7.75±2.98 (mean±SE, n=30, *P*=0.001) of the parasites being metacyclics.

At 5mg/ml concentration, 23.06±1.76 (mean±SE, n=30, *P*=0.031) amastigotes were observed at day 6 and 8.00±1.00 (mean±SE, n=30, *P*=0.001) procyclic promastigotes at day 8. The control group had 28.00±0.00 procyclic promastigotes at the same time. The difference observed in number of parasites was significant. Percentage inhibition increased with time and reached 64% at day 10 where 8.67± 1.20 (mean±SE, n=30, *P*=0.001) nectomonads were observed. At day 12, 4.67±2.40 (mean±SE, n=30, *P*=0.001) haptomonads were observed. Development was inhibited at haptomonad stage while at 10mg/ml; development was inhibited at the nectomonad stage (Fig. 16).

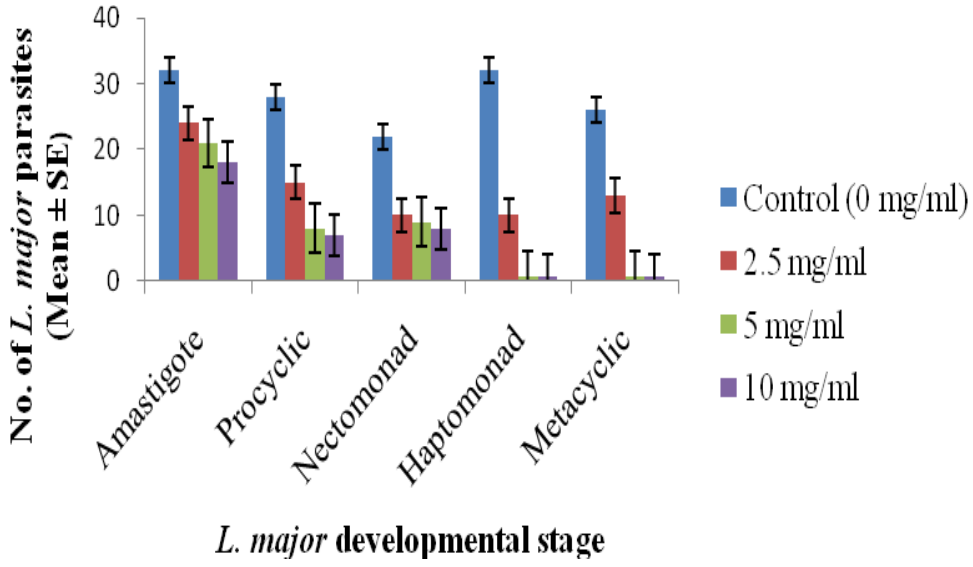

Figure : Mean number of *Leishmania major* parasites observed after exposure to *T. camphoratus* methanol crude extract

3.5.6 Effect of *T. camphoratus* Ethyl Acetate Crude Extract on *L. major* Development

Sand flies that had fed on this crude extract supported full parasite development at a concentration of 2.5mg/ml. However, the mean number of parasites observed was significantly different from those observed in *A. fruticosa* and *T. minuta* crude extracts. At 5mg/ml concentration, a similar trend was observed although the cycle was inhibited at the haptomonad stage with only 12.33±1.45 (mean±SE, n=30, *P*=0.02) parasites. However, at a concentration of 10mg/ml, the cycle was inhibited at the nectomonad stage with 10.00± 1.15 (mean±SE, n=30, *P*=0.011) parasites (fig. 17).

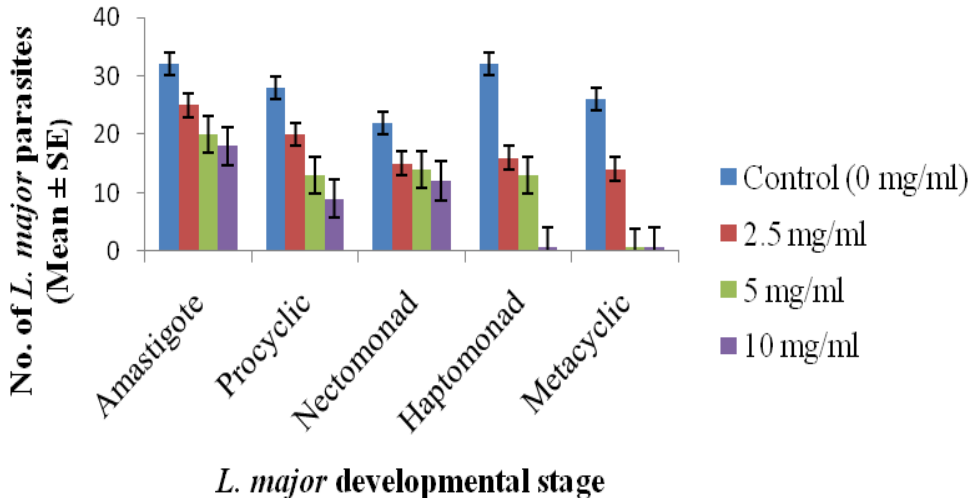

Figure : Mean number of *L. major* Parasites Observed after Exposure to *T. camphoratus* Ethyl Acetate Crude Extract

At a concentration of 10mg/ml, the parasites that fed on *A. fruticosa* crude extract differed from those that had fed in *T. camphoratus* crude extract treatment morphologically. The parasites that were exposed to *A. fruticosa* showed amastigotes which were very tiny and crumpled together while those in *T. camphoratus* were bigger and sparsely distributed.

10% of the parasites were procyclic promastigotes in the dissected flies. The controls mainly had the slender nectomonads and a few amastigotes at the same time. Nectomonads had a slow motility within the midgut of the dissected fly. The spherical amastigotes were significantly more in *A. fruticosa* than in *T. minuta* and *T. camphoratus* crude extracts. Plate 6 shows the amastigotes while plate 7 shows amastigotes transforming to procyclic promastigotes as seen under high power magnification (X1000).

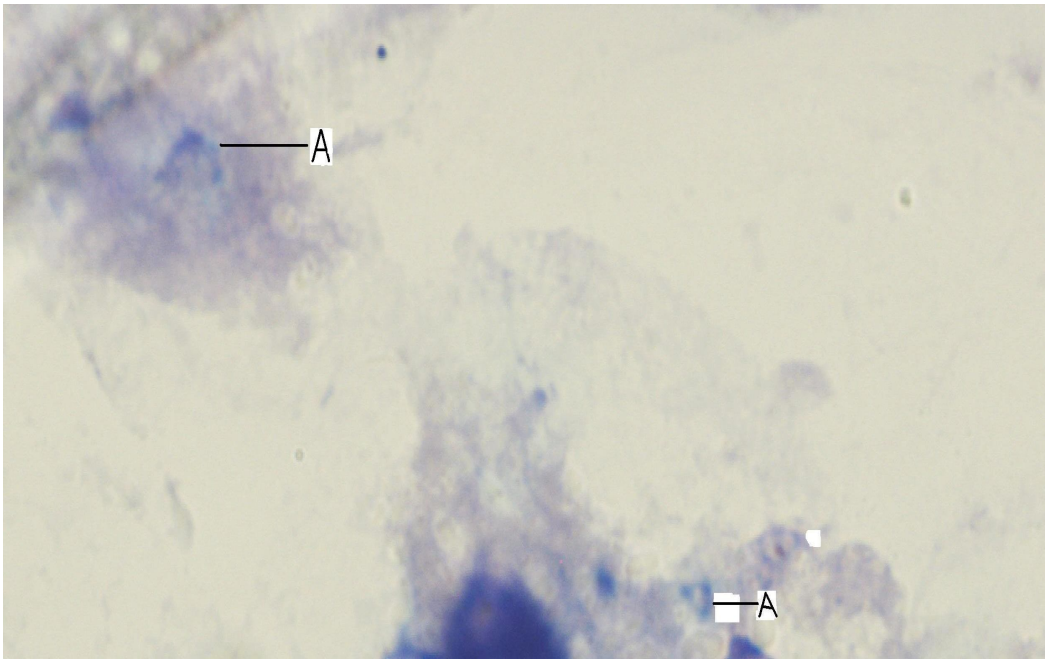

Plate : Spherical amastigotes observed six days post (Mg=X1000)

Key:

A=amastigotes observed after administering *A. fruticosa* crude extract

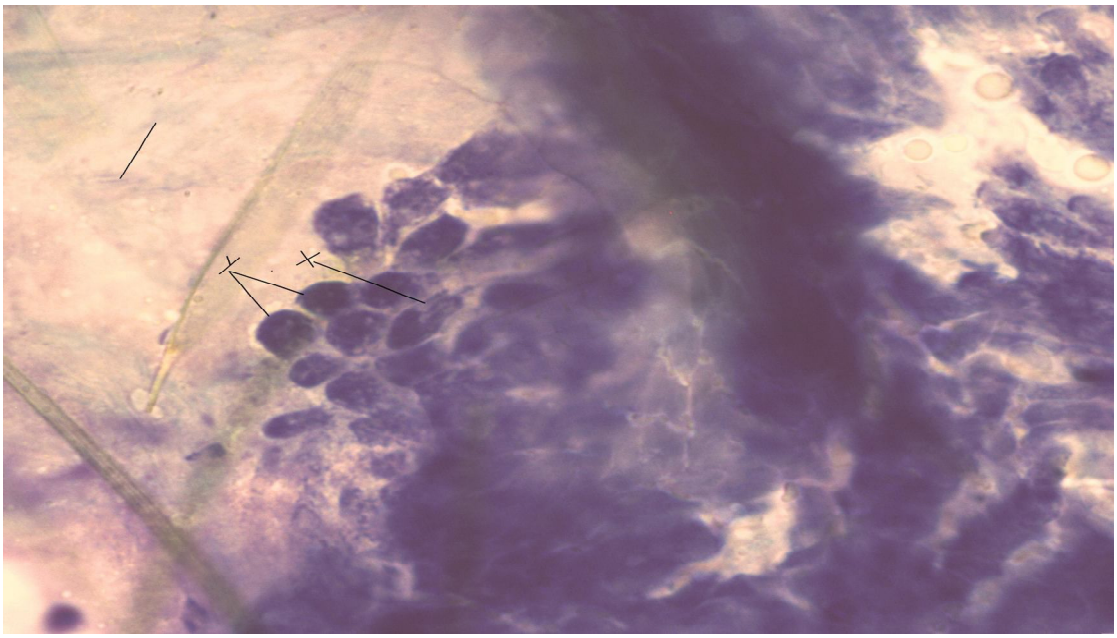

Plate : Amastigotes transforming to procyclic promastigotes (Mg=X1000)

X shows a fully transformed procyclic, Y show amastigote transforming

Nectomonads were seen to be slender, with long flagella freely moving within the gut and generally showed normal morphology. The percentage of nectomonads was significantly lower in the treatments than in the control group (control 71%, *A. fruticosa* 17%, *T. minuta* 14%, *T. camphoratus*15.6%). Haptomonads observed at the 10th day post feeding on the extracts, had a normal morphology, however, their flagella were shorter than the body and they were tightly packed against each other inside the gut (Plate 8). They were present in the anterior midgut area. This stage persisted till day 14 with a few metacyclics being observed in the sand flies that had fed on *A. fruticosa* crude extract. The control sand flies supported full parasite development.

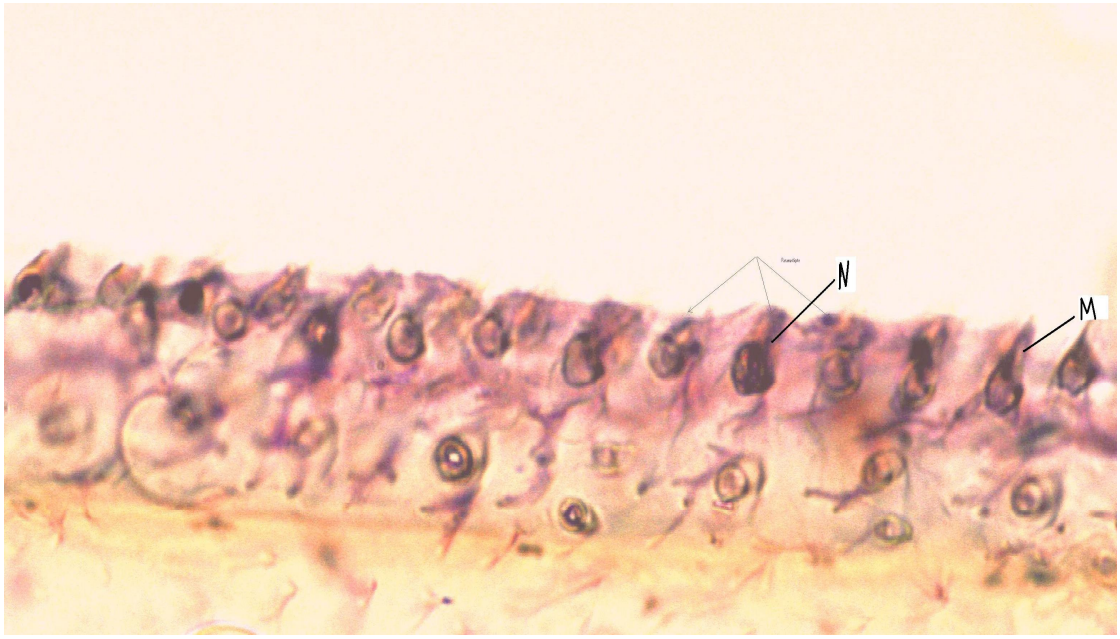

Plate : Haptomonads tightly packed inside the sand fly's gut at 10 days post infection (Mg=X1000)

(Key: N & M=Haptomonads)

3.6 Determining Parasite Density per *P. duboscqi* Fly

Dissection of the sand flies that fed on the extracts showed that the number of parasites per each sand fly was significantly reduced as compared to the control group. Feeding *P. duboscqi* on the crude extracts significantly reduced the number of parasites per fly as compared to the control group. Under 2.5 mg/ml concentration, the flies that had previously fed on *A. fruticosa* crude extracts showed less than 15 amastigotes per 10 fields (grade 3+). Six days post infection, 4 procyclic promastigotes per 1000 fields (grade 1+) were observed. Those that had fed on *T. minuta* crude extract showed 17 amastigotes per 10 fields (grade 3+). However, the sand flies that had fed on *T. camphoratus* showed an average of 4-6 parasites per field (grade 4+).

Between days 6 and 8 post feeding, there were mainly amastigotes and procyclic promastigotes as the commonest parasites forms in the vector. Their numbers differed significantly with 12 amastigotes per 10 fields (grade 3+) in the sand flies that had fed on *A. fruticosa* and *T. minuta*, and 4-6 amastigotes per field (grade 4+) in those that had fed on *T. camphoratus*. Sand flies which had fed on sugar syrup alone (control group) showed an average of 2-5 parasites per field (grade 5+).

Table : Parasite density per fly after different crude extract administration

EXTRACT	SF. NO.	DAY	PARASITE FORM	DENSITY
A. fruticosa	1	6	Amastigotes	3+
			Procyclics	1+
	2	6	Amastigotes	4+
	3	8	Amastigotes	3+
	4	8	Amastigotes	3+
	5	13	Amastigotes	2+
	6	13	Haptomonad	1+
			Nectomonad	2+
	7	8	Nectomonads	3+
			Haptomonads	2+
	8	8	Amastigotes	2+
	9	10	Amastigotes	1+
	10	13	No parasite	-
	11	13	No parasite	-
T. camphoratus	12	6	Amastigotes	4+
			Nectomonads	2+
	13	6	Amastigotes	4+
			Procyclics	1+
	14	13	Amastigotes	2+
	15	8	No parasite	-
	16	6	Amastigotes	3+
	17	8	Nectomonads	1+
			Haptomonads	1+
	18	8	Nectomonads	2+
T. minuta	19	13	Haptomonads	2+
	20	6	Amastigotes	3+
	21	13	Amastigotes	1+
	22	8	Amastigotes	2+
	23	6	Amastigotes	4+
	24	8	Procyclics	3+
			Nectomonads	2+
	25	8	Amastigotes	2+
	26	12	Amastigotes	1+
	27	13	No Parasite	-
Control	28	6	Procyclics	4+
		8	Nectomonads	5+
		10	Nectomonads,	4+
		12	Haptomonads	5+
			Metacyclics	4+

Key: SF- Sand fly number

At day 10 post infection, there were further transformations of the parasites and the commonest parasite form was nectomonad with 3-5 parasites per 100 fields (grade 2+) in the flies that had fed on *A. fruticosa* while those that had fed on *T. minuta* crude extract had 6-7 nectomonads per 100 fields (grade 2+). However, there were 2-5 haptomonad parasites per 100 fields in the flies that had fed on *T. minuta* group. In the flies that had fed on *T. camphoratus,* there were 1-3 nectomonads per 10 fields (grade 3+) and 2-3 haptomonad parasites per 1000 fields (grade 1+).

At day 13, the sand flies that had fed on *A. fruticosa* crude extract had 2-3 metacyclic parasites per 1000 fields (grade 1+) while the control group had an average of 16 metacyclic parasites per 10 fields (grade 3+). Flies that had fed on *T. minuta* crude extract supported averagely 5 metacyclic parasites per 1000 fields (grade 1+). Sand flies that had fed on *T. camphoratus* crude extract showed 8 metacyclic parasites per 1000 fields (grade 1+). It was observed that the flies that had fed on *T. camphoratus* crude extract supported more parasites as compared to those that had fed on *A. fruticosa* and *T. minuta* crude extracts. The experiment was terminated at day 13 once the final infective stage appeared.

Highest inhibition of parasite development was seen in higher concentrations of 5mg/ml and 10mg/ml with the percentage inhibition differing significantly among the three extracts used. At 5mg/ml extract concentration, the sand flies that had fed on *A. fruticosa* showed mainly amastigotes by the end of 8^{th} day of extract administration (grade 3+). Feeding on *T. minuta* extract resulted in 3-5 nectomonad parasites per 100 fields (grade 2+). While in the flies that had fed on *T. camphoratus* extract, there were procyclics and

nectomonads at day 8. Nectomonad load was less than 15 parasites per 10 fields (grade 3+**)** while procyclics were 9 parasites per 100 fields (grade 2+). In the flies that had fed on *A. fruticosa*, development was highly inhibited at the nectomonad stage by the end of the 10^{th} day. At this time, nectomonads were 3-5 parasites per100 fields (grade 2+) while haptomonads were 4-6 parasites per 1000 fields (grade 1+). In the flies that had fed on *T. minuta,* there were mainly nectomonads (grade 2+) while in the group that had fed on *T. camphoratus*, nectomonads dominated with grade 3+.

At day 13, no parasite was found in the sand flies that had fed on *A. fruticosa;* only agglutinated parasites were observed. In the sand flies that had fed on *T. minuta* extract, there were 2-5 nectomonad parasites (grade 1+) that had persisted. In the sand flies that had fed on *T. minuta* extract, there was a mixture of procyclic promastigotes and nectomonads of grade 2+ that had persisted. Parasite development was highly inhibited at the nectomonad stage.

Feeding *P. duboscqi* on 10mg/ml of the crude extracts led to more inhibition of parasite development. In the sand flies that had fed on *A. fruticosa*, parasite development was inhibited at the nectomonad stage at day 10 of extract administration. At this time, there were 3-5 parasites per 1000 fields (grade 1+). In the sand flies that had fed on *T. minuta*, parasite load was 4-6 parasites per 1000 fields (grade 1+). However, in the group that had fed on *T. camphoratus* extract, the nectomonad stage persisted till day 13 (grade 3+) with some procyclic promastigotes.

CHAPTER FOUR

4.0 DISCUSSION

4.1 Effects of *T. camphoratus, A. fruticosa* and *T. minuta* Extracts on Fertility of *P. duboscqi*

Results from this study showed that *Tarchonanthus camphoratus, Acalypha fruticosa* and *Tagetes minuta* crude extracts depressed the mean number of eggs laid per female sand fly depending on the dose of the extract used. From the results, it is evident that *A. fruticosa* and *T. minuta* crude extracts had significant effects on the fertility of *P. duboscqi.* This may be attributed to the fact that these plants have high efficacy on the flies, or the flies fed more on these extracts than on *Tarchonanthus camphoratus* extract. There is evidence that the secondary compounds in *T. minuta* are effective deterrents of numerous organisms, including: fungi pathogenic on humans, bacteria, round worms in general, trematodes, nematodes and numerous insect pests through several different mechanisms (Mohamad *et al*., 2010).

Studies have shown that egg laying period can stretch up to 15 days post feeding (Mauricio *et al.*, 2010) but in this study the number of eggs recorded was observed on the 7^{th} day. This study showed that plant extracts have the ability of inhibiting digestion of the blood meal hence egg formation was inhibited. Investigations have shown that decreased fertility is linked to the quantity of proteins obtained from a blood meal (Volf *et al.*, 2001). Harre *et al.*, (2001) compared fecundity among sand flies fed on various sources of mammalian blood. His study showed that egg development depends on the

blood meal protein. Therefore, the reduced fertility in this study may be due to the inhibition of blood meal digestion.

Acalypha fruticosa was found to be more effective in reducing the fertility of *P. duboscqi* in both methanol and ethyl acetate crude extracts followed by *T. minuta*. *T. camphoratus* had the least effect on fertility and this may be attributed to the strong smell of the crude extract which might have deterred the flies from feeding on the plant extracts. *T. camphoratus* has insect repellent properties when in combination with a natural or synthetic triglyceride which improves its activities. Other studies have shown that *T. camphoratus* has no antimicrobial activity. However, investigation of antimicrobial activity of aqueous, ethanolic and hexane extracts of dried leaves did not demonstrate *in vitro* inhibitory effects against *Staphylococcus aureus*, *Bacillus subtilis*, *Escherichia coli* or *Klebsiella pneumonia (*McGaw *et al.*, 2000*)*. Combined crude extracts had a significant effect on the fertility of *P. duboscqi* when compared to the control although; this effect was not significantly different from the individual crude extracts. This reveals that there are no synergistic effects when the extracts were combined. The extracts were found to reduce the fertility of *P. duboscqi* by 73% (*A. fruticosa*), 53% (*T. minuta*) and 26% (*T. camphoratus*).

The observation that *Acalypha fruticosa*, *Tagetes minuta* and *Tarchonanthus camphoratus* extracts have effect on egg development in *P. duboscqi* has important implications for vector control because the higher level of their activities would potentially reduce the population of sand flies hence reduce leishmaniasis cases. The

approach used here may be useful in the application of these plant extracts against Phlebotomine sand flies which are the vectors for leishmaniasis.

4.2 Effects of *T. camphoratus, A. fruticosa* and *T. minuta* Extracts on Longevity of *P. duboscqi*

Longevity and post oviposition survival time differed significantly after feeding on the crude plant extracts. Sand flies that fed on *A. fruticosa* and *T. minuta* crude extracts lived for a shorter period at a concentration of 10 mg/ml as compared to those that fed on *T. camphoratus*. Higher effects were observed in methanolic extracts than in ethyl acetate extracts. This implies that methanol and ethyl acetate solvents extracted different active compounds from the plants leading to the difference in their effects.

The sugar solution which was mixed with the crude extracts acted as bait for the flies. Various studies have shown that sand flies can feed on aqueous sucrose solutions mixed with noxious plant juices and have their lifespan greatly reduced (Schlein *et al.,* 2001). Normally, vectors feed on plant secretions, juice and nectar hence they end up feeding on insecticidal substances. This greatly reduces their survival time. The sugar that was mixed with the crude extracts might have attracted more females than males to the crude extracts. This might have led to females feeding more on the extracts than males. This probably explains why females lived for a short time as compared to the male sand flies.

It has been shown that plant sugars attract more female sand flies than male sand flies (Muller *et al*., 2011), may be that is why females lived for a shorter period than males

because they fed more on the crude extracts.

It has been shown that female sand flies need a blood meal for egg production, but sugar is their main source of energy and the only food taken by males (Killick-Kendrick, 1999). The sugar feeding behavior of sand flies, therefore, influences survival time and fertility, dispersal, host seeking behavior and ultimately blood feeding and disease transmission (Muller and Schlein, 2004). Female sand flies obtain sugar meals mainly from honeydew excreted by aphids and coccids (Muller *et al*., 2011) and by feeding directly on tissues of plants in the field (Schlein and Jacobson 1994; Schlein and Muller, 1995). In arid areas, there is evidence that availability of suitable sugar sources is a limiting factor for sand fly fitness and survival (Schlein and Jacobson, 2002). A previous study showed that flowering *Tamarix nilotica* is a highly attractive sugar source for sand flies (Muller and Schlein, 2004).

4.3 Effects of *T. camphoratus, A. fruticosa* and *T. minuta* Extracts on Development of *L. major*

The result showed that these crude extracts also have inhibitory effects on the growth of *L. major* parasites. The strength in activity was found to be dependent on species of the plant and the concentration used. Numerous extracts from these plants have been screened for their antibacterial, antifungal, molluscicidal, antiprotozoal or antiviral activities and they have been found to have antimicrobial activities. Current research shows that medicinal plants used in Brazilian traditional medicine had between 49.5 and

99% growth inhibition percentage of *L. amazonensis* amastigotes and promastigotes respectively (Luize *et al*., 2005).

The inhibition of parasite development by *A. fruticosa*, *T. minuta* and *T. camphoratus* crude extracts may be attributed to the fact that these crude extracts have the ability of interrupting the host-parasite interaction. This may have led to the detachment of the parasites from their hosts and eventually their death. This explains the observation that procyclic, and nectomonad promastigotes were observed in the midgut lumen freely moving.

After dissection, most of the parasites were found dead and many were fragmented, and thus, the gut was filled with cell fragments, and intact parasites were scarce. This may be due to the agglutination of the parasites more especially at higher concentrations of *A. fruticosa* and *T. minuta* crude extracts. A similar observation was made by Gonzalez *et al.,* (2005) who determined the activity of alkaloid derivatives in amastigotes and promastigotes in *L. infantum.* Agglutination and death of the parasites may be due to the effect of the crude extracts which disintegrated the cytoplasmic membrane of the parasites leading to direct contact of the parasites with the crude extracts.

The disintegration of the cytoplasmic membrane and failure of its resynthesis may have led to inhibition of the transformation of the parasites from one stage to the next more especially from nectomonads to haptomonads and finally to metacyclic promastigotes. A closely related research revealed that the compounds in plant extracts act fundamentally

at the level of the cytoplasmic membrane of the parasites (Gonzalez *et al*., 2005). This might have inhibited the attachment and tight packing of the parasites to the midgut wall. Agglutination of the parasites led to the high mortality of the parasites which was observed at higher concentrations.

T. camphoratus crude extract was found to have a moderate inhibitory effect on parasite development. This plant is known to be a good deterrent of insects preventing them from feeding on the extract. This may explain why the sand flies that fed on this extract managed to carry the parasites up to the haptomonad stage at higher concentrations.

The crude extracts also had a great effect on the morphology of the parasites. Oval shaped slender *L. major* procyclic promastigote forms with short flagella observed after 8 days of treatment with crude extracts suggest that the extracts interfered with the normal growth and development of the parasites. This observation is in agreement with Githinji *et al*., 2010. In this study, when *W. ugandensis* stem bark extracts were administered against *L. major* promastigotes, their development was delayed and the procyclic promastigotes had a sluggish movement due to their less rigorous flagella. Therefore, these extracts were either quick in causing death to the extra cellular parasites or impaired the developmental process. The multiplication of the parasites was different in each concentration used and type of plant extract. Concentration dependent multiplication in all plant extracts implies that high concentrations inhibited rate of replication in the parasites. Lowest levels of inhibition observed in *T. camphoratus* extract confirm its weak chemotherapeutic potential.

This study also revealed that these crude extracts significantly reduced the mean number of parasites per female sand fly but this was dependent on the plant species used. The most effective concentration in reducing parasite density was 10 mg/ml. *A. fruticosa* induced notable morphological changes in the parasites, which appeared with a rounded and extremely swollen appearance. The parasite numbers were also greatly reduced. Similar alterations were provoked by *T. minuta* crude extract. The parasites appeared very swollen more especially the haptomonads. *A. fruticosa* proved to be the most harmful crude extract to *L. major* promastigotes. This may be attributed to its secondary compounds which have antiprotozoal and antimicrobial activities.

Investigations have shown that *A. fruticosa* is effective against numerous organisms as an antifeedant or insecticide. The inhibition per centage of this plant extracts agrees with the finding that *A. fruticosa* chloroform extract has maximum antifeedant activity of 92.8% at 5% concentration on *Plutella xylostella* (Lingathurai *et al*., 2010). *A. fruticosa* belongs to the family Euphorbiaceae and it has been shown that plants belonging to this family are highly repellent to *P. xylostella* and other lepidopteran pests. This may also extend to most dipterans including Phlebotomine sand flies.

Crude extract administration also reduced the parasite load per fly significantly. Those sand flies that fed on *A. fruticosa* crude extract showed the least number of parasites per fly. This may be due to its active compounds which caused agglutination of the parasites and eventually their death. *T. minuta* and *T. camphoratus* crude extracts also had a

significant effect on parasite load; however, *T. minuta* was more harmful to the parasites than *T. camphoratus* crude extract signifying that *T. minuta* might be having more active compounds than *T. camphoratus.* Parasite development in *T. minuta* was inhibited at the nectomonad stage and the number of these parasites was 2+. This was significant as compared to 2+ (haptomonads) in *T. camphoratus*, a more advanced parasite stage. This shows that *T. minuta* and *T. camphoratus* have active compounds which have antimicrobial activities.

The inhibition bioassays clearly indicated that methanol crude extract of *A. fruticosa* leaves was more effective than ethyl acetate treatments. In all the treatments the percentage inhibition was directly proportional to the concentration of the crude extract used. Feeding deterrent activity of plant extracts and plant products against *P. xylostella* has been reported by many investigators (Ling *et al.*, 2008; Patil and Goud, 2003). Sugar is the main source of energy for the daily activities of sand flies. Considering its importance, there is surprisingly little information on sugar meal specific sources and sand fly attraction to plants, particularly in the field.

Research has shown that toxic sugar baits can be used in controlling various vectors of medical importance. Muller *et al.*, (2010) sprayed the bait solution onto vegetation with the aim of reducing the abundance of mosquitoes in Israel. The bait solution decreased mosquito abundance from approximately 125 to approximately eight per trap and the parity of mosquitoes was reduced from 20% to 3% (Muller *et al.*, 2010). This implies

that the combination of crude extracts and sugar solution reduces the repellency of the crude extracts and attracts more *P. duboscqi* flies to the extract hence reducing their fecundity and inhibiting *L. major* development.

CHAPTER FIVE

5.0 CONCLUSIONS AND RECOMMENDATIONS

5.1 Conclusions

1. *A. fruticosa, T. minuta and T. camphoratus* crude extracts inhibited the fertility of *P. duboscqi*.
2. *A. fruticosa* crude extract was more effective in reducing the survival time of *P. duboscqi* followed by *T. minuta* crude extract and the least in efficacy was *T. camphoratus* crude extract.
3. *A. fruticosa, T. minuta* and *T. camphoratus* crude extracts inhibited the development of *L. major* at the haptomonad stage.

5.2 Recommendations

1. The extracts of *A. fruticosa, T. minuta* and *T. camphoratus* should be processed into insecticides that can be used against sand flies.
2. The extracts of *A. fruticosa, T. minuta* and *T. camphoratus* should be impregnated bed nets and other sand fly monitoring equipment for small scale field trials in leishmaniasis endemic areas.`
3. Field trials should be carried out to test the efficacy of these crude extracts as antileishmanial drugs and transmission blocking vaccines.

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