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EVALUATION OF SELECTED MEDICINAL PLANT EXTRACTS FOR INSECTICIDAL PROPERTIES AGAINST PHLEBOTOMINE SAND FLIES (DIPTERA: PSYCHODIDAE)

BY

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Evaluation of selected medicinal

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DECLARATION

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

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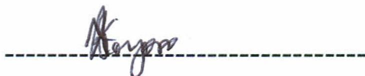

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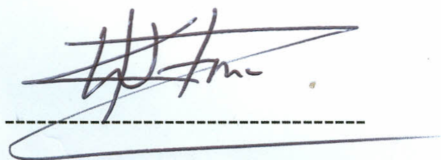

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DEDICATION

Dedicated to my wife Mary and children; Edwin, Pauline, Victoria, Lenny and Martin.

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ABBREVIATIONS AND ACRONYMS

μ	Micro
CBRD	Centre for Biotechnology Research and Development
CL	Cutaneous leishmaniasis
CTMDR	Centre for Traditional Medicine and Drug Research
DCL	Diffuse cutaneous leishmaniasis
DDT	Dichlorodiphenyltrichloroethane
DEET	N, N-diethylmethyltoluamide
DMSO	Dimethylsulphoxide
EC	Emulsifiable concentrate
ELISA	Enzyme linked immunosobent assay
HIV	Human immunodeficiency virus
IRS	Indoor residual spray
ITMs	Insecticide treated materials
ITNs	Insecticide treated nets
kDa	kilo Dalton: Unit of molecular weight
KEMRI	Kenya Medical Research Institute
L.	*Leishmania*
L.d.	*Leishmania donovani*
L: D	Light: dark
LD_{50}	Lethal dose

LLTNs	Long lasting insecticide treated nets
Lu.	*Lutzomyia*
MCL	Mucocutaneous leishmaniasis
mg	Milligram
ml	Millilitres
P.	*Phlebotomus*
PCR	Polymerase chain reaction
PKDL	Post kala azar dermal leishmaniasis
RH	Relative humidity
Serg.	*Sergentomyia*
S.l	*Sensu latu*
Spp	Species
SP15	Secretory protein
TDR	Tropical Diseases Research
TM	Trade mark
V/V	Volume by volume
VL	Visceral leishmaniasis
W/V	Weight by volume
WHO	World Health Organization
®	Registered trade mark

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ABSTRACT

Phlebotomine sand flies are the vectors of *Leishmania* species, the causative agents of leishmaniases that are a global health problem and are usually endemic in developing countries affecting predominantly the poor in society. Complementally control measures through use of biodegradable, safe and species selective natural compounds are urgently needed. Plant based compounds are safe in most biosystems. Some synthetic chemical insecticides are toxic, expensive and discriminate use may cause vector resistance. In this study, the aerial parts of *Tagetes minuta* Linnaeus (Asteraceae), *Acalypha fruticosa* Forssk (Euphorbiaceae) and *Tarchonanthus camphoratus* L. (Compositae) currently being used in endemic areas as repellents were extracted using methanol and ethyl acetate prior to being filtered, dried out by rotary evaporation at 30-35°C then dissolved in Dimethylsulphoxide (DMSO) and prepared into 2.5, 5 and 10mg/ml using distilled water. The different concentrations of the extracts were evaluated against *Phlebotomus duboscqi* while incorporated in filter papers. Other sets of similar concentrations were prepared using 10% sucrose solution and used in feeding technique using cotton wool balls. Pyrethrin incorporated in artificial food was also investigated for insecticidal efficacy using feeding techniques to 10 adult sand flies and 10 larvae and replicated three times. Pyrethrin (0.6 %) was diluted in 10% sucrose solution at 0.08, 0.25 and 0.5 mg/ml and used in the adult feeding bioassays while bioassays on the larvae were conducted using the extracts, powdered plant parts, 0.2% pyrethrin powder and 0.5% EC incorporated in larval food. Mortality was recorded every 24 hours of exposure and the data entered into MS Excel and subjected to computerized probit analysis. The extracts had significant mortality to adult sand flies in both contact and feeding methods but exhibited no larvicidal activity. Extracts of *A. fruticosa* and *T. minuta* had significant mortality ($P<0.05$) to adult sand flies at 48 hours of exposure than those of *T. camphoratus*. The combination of the extracts yielded weaker insecticidal properties than separate extracts. The feeding techniques had higher LD_{50} values in all the bioassays at 48 hours of exposure when compared to the contact bioassays using similar concentrations. In the pyrethrin bioassay there was no significant difference between male and female LD_{50} at 48 hours of exposure. The powdered plant parts were not larvicidal. The two pyrethrin formulations were effective against larvae with a LD_{50} of 0.1mg/ml in the third instar larvae groups yielding mortality of about 100% in only 96 hours of exposure. The plant extracts were found to be effective against adult phlebotomine sand flies while pyrethrin products were found to be efficient larvicides when incorporated in artificial sand fly larvae food and are therefore proposed to be harnessed, made into appropriate formulations and used alongside other control strategies.

CHAPTER ONE

1 GENERAL INTRODUCTION

1.1 Background

Phlebotomine sand flies are the only known vectors of human leishmaniases, a disease complex which clinically manifests itself in four different forms, namely; visceral (VL), cutaneous (CL), diffuse cutaneous (DCL) and muco-cutaneous leishmaniasis (MCL). Visceral leishmaniasis causes hepatospleenomegally, a fatal condition unless treated. Cutaneous leishmaniasis affects the skin forming disseminated papules. Muco-cutaneous leishmaniasis disfigures nasopharyngeal membranes and diffuse cutaneous forms lepromatous papules on the skin. Leishmaniases are prevalent in both tropical and sub-tropical regions of the world (WHO, 2004; Hide *et al,* 2007) (Figure I). They are neglected diseases that have recently generated much interest due to the worldwide increase in their incidences caused probably by global warming, other environmental factors and the formidable HIV/leishmaniases co-infection (Alvar, 1994; Tonui, 2006). In Kenya, leishmaniasis endemic areas include Turkana, Baringo, Machakos, Meru, West Pokot and Elgeyo Marakwet Districts (Mutinga *et al*., 1994).

The causative agents of leishmaniases are a range of morphologically similar parasites of the Genus *Leishmania* supported by a wide range of vectors and reservoirs in both tropical and sub-tropical regions of the world (Ashford, 2000; WHO, 2004; Consingli *et al.,* 2006). In Kenya, *Leishmania* species include *Leishmania donovani, L. major, L. aethiopica* and *L. adleri. L. donovani* is the causative agent for visceral leishmaniasis, commonly known as

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kala azar whereas *L. adleri* affects lizards. *Leishmania major* and *L. aethiopica* causes cutaneous and diffuse cutaneous leishmaniases, respectively.

Current efforts to control leishmaniases are primarily focused on chemotherapy, vector control through use of chemicals, destruction of rodent burrows, termite moulds, use of impregnated dog collars, environmental manipulation and improvement of housing. Personal protections through use of lotions and chemical impregnated bed nets are also major tools in vector control (Tonui, 2006). However, these control strategies have been hampered by various factors including rampant spread of drug resistance, high cost of classical anti-leishmanial drugs, lack of suitable vaccines and resistance to the insecticides by vectors. Also, sand flies are usually dispersed in subterranean habitats such as rock crevices, bases of trees, leaf litter and animal burrows, which are not easily penetrated by conventional insecticides (Ferro *et al.,* 1997). Alternative compounds and especially botanicals are critical sources of ideal control agents because they are safe to most flora and fauna. Control interventions call for a better understanding of the biology and diversity of *Leishmania* parasites, sand flies and the reservoir hosts (Tonui, 2006).

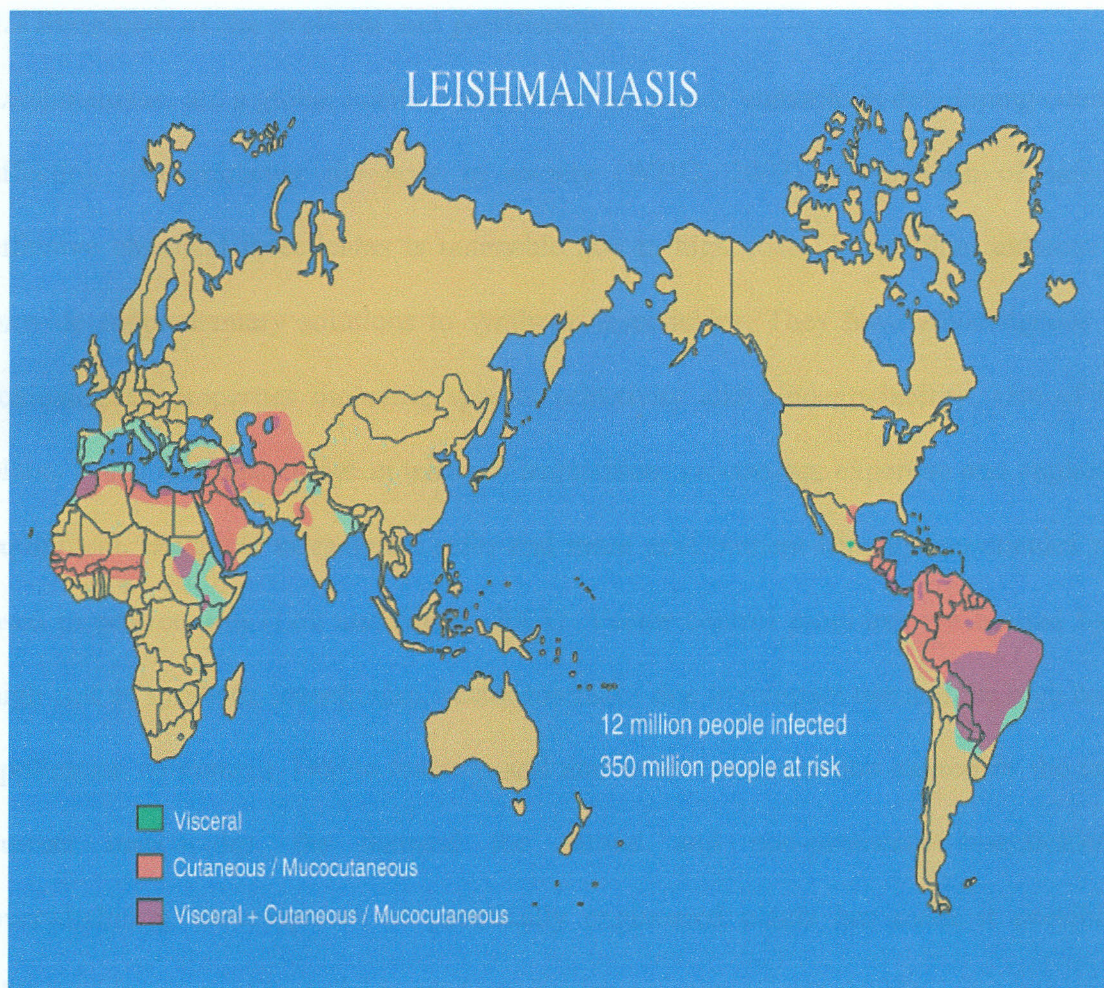

Figure 1. World map highlighting areas where cutaneous, visceral, and muco-cutaneous leishmaniasis is endemic (adopted from Handman Emmanuela, Leishmaniasis: Current state of Vaccine Development, 2001)

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1.2 Statement of the problem and justification

Leishmaniases are a global health problem and are highly endemic in developing countries affecting predominantly the poor in society (WHO, 2007). Acquisition of safe and effective chemical insecticides is untenable due to high costs. Local plant extracts may provide supplementary solutions to synthetic insecticides. They have advantageous eco-toxicological properties including species selectivity and biodegradability. Use of local plants needs minimum input in terms of acquisition, processing, expertise and application equipment. Botanical extracts are safer and more environment-friendly insecticides when used in pest control (Lok and Singh, 2003). 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. Due to toxicity, soil and water contamination caused by the use of current insecticides, the demand for natural and non-persistent insecticides is overwhelming. Development of genetically engineered plants has made it possible to obtain wide arrays of insecticide products in high amounts (Coats, 1994).

Plant-derived products such as azadirachtin from neem (*Azadirachta indica*) have shown potency as insect-growth regulators and are therefore ideal for future use as effective insecticides (Wood, 1990). Selective, safe and environmentally friendly plant extracts may probably be an important addition to the currently available measures used in the control of sand flies resulting in the reduction of leishmanial burden. This study was aimed at evaluation of insecticidal effects of extracts from selected plants obtained from Baringo district, an endemic focus of leishmaniasis and pyrethrin through feeding, and to explore for simple effective methods of their application. The plant extracts were from *Tagetes*

minuta (Asteraceae), *Acalypha fruticosa* (Euphorbiaceous) and *Tarchonanthus camphoratus* (Compositae). These were selected through a wider project on "Search for antileishmanial and insecticidal agents from Kenyan Medicinal plants" initiative. Some communities in *Leishmania*-endemic areas of Kenya purport to use these plants as sand fly repellents (Tonui, personal communication). The emulsifiable concentrates have not been tried in a laboratory setup or in the field to control phlebotomine sand flies and especially when used in sugar baits. The results of this investigation will be useful in development of additional methods in leishmaniases control.

1.3 Research questions

(i) What are the effects of *Acalypha fruticosa, Tagetes minuta* and *Tarchonanthus camphoratus* extracts and pyrethrin against sand fly adults?

(ii) What are the effects of *Acalypha fruticosa, Tagetes minuta* and *Tarchonanthus camphoratus* extracts and pyrethrin against sand fly larvae?

(iii) What are the synergistic effects of combined extracts of *Acalypha fruticosa, Tagetes minuta* and *Tarchonanthus camphoratus* against adult sand flies and larvae?

1.4 Null hypotheses

(i) *Acalypha fruticosa*, *Tagetes minuta* and *Tarchonanthus camphoratus* extracts and pyrethrin do not have any effect against adult sand flies.

(ii) *Acalypha fruticosa*, *Tagetes minuta* and *Tarchonanthus camphoratus* extracts and pyrethrin do not have any effect against sand fly larvae.

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(iii) There are no synergistic effects of combined plant extracts against phlebotomine sand fly adults and larvae.

1.5 Objectives

1.5.1 General objective

To assess the adulticidal and larvicidal activities of extracts from selected plants against phlebotomine sand flies (Diptera: Psychodidae) in the control of leishmaniases.

1.5.2 Specific objectives

(i) To determine adulticidal properties of *Acalypha fruticosa, Tagetes minuta*, *Tarchonanthus camphoratus* extracts and pyrethrin against *Phlebotomus duboscqi* male and female sand flies.

(ii) To determine larvicidal properties of *Acalypha fruticosa, Tagetes minuta*, *Tarchonanthus camphoratus* extracts and pyrethrin against *Phlebotomus duboscqi* larvae.

(iii) To determine the synergistic effects of combined *Acalypha fruticosa, Tagetes minuta* and *Tarchonanthus camphoratus* extracts against male and female sand flies and larvae.

1.6 Significance and anticipated output

Leishmaniases are neglected diseases and necessitates newer, economical and safer control methods. Although sand fly control in Kenya has not been a priority, this study was aimed at searching for insecticidal local plants which could be used in endemic areas of Kenya. These plants may provide cheap, species specific and biodegradable products unlike synthetic chemicals used in vector control programmes.

The chemicals used in mosquito control with consequential sand fly population reduction are expensive to buy since these diseases occur in poor resource areas and these plants are abundant here. Pyrethrin, a natural plant product is abundant in Kenya has routinely been applied to materials such as curtains and as indoor residual spraying. Pyrethrin-sucrose formulations will be made and used in sand fly invested areas. This may elucidate novel and easier methods of application as sugar baits to sugar-feeding pests, sand fly resting and breeding sites. Larval food mixtures will be made into suitable formulations and can be blown into termite hills and rodent burrows. This will be an added contribution to the current integrated vector management strategies and development of safe and effective future generation insecticides.

CHAPTER TWO

2 LITERATURE REVIEW

Phlebotomine sand flies are small haematophagous insects and brownish in daylight but their bodies are densely covered in oily hairs which give the insects a whitish appearance when illuminated. They bite at dawn and dusk and are weak fliers and therefore feed within their breeding sites. Their control is complicated since they breed and rest in obscure locations, difficult to apply conventional insecticides and therefore need novel control strategies.

2.1 Importance and distribution of phlebotomine sand flies

Phlebotomine sand flies transmit many zoonotic pathogens that include arboviruses, *Bartonella* and *Leishmania*, which are significant pathogens of humans. They also constitute a serious but localized biting nuisance (Mehlhorn, 2001). There are over 600 known phlebotomine sand fly species belonging to six genera within the sub-family Phlebotominae. The genera *Phlebotomus, Lutzomyia* and *Sergentomyia* have 70 species that are anthropophagus, of these species 30 have been identified as vectors of *Leishmania* parasites (Killick-Kendrick, 1990). The other three genera *Chitinius, Brumptomyia* and *Warileya* do not have species that are disease vectors. The phlebotomine sand flies are found throughout the world's intertropical and temperate regions (WHO, 1990). Only female sand flies suck blood, and hence transmit diseases. Males feed on plant juices and sugars.

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Worldwide there are two million new leishmaniases cases each year and one tenth of the world's population is at risk. The various forms of the disease are prevalent in 88 countries in tropical and subtropical regions in four continents and the range may expand with global warming (WHO, 2004). Leishmaniasis is considered one of the most important protozoal diseases in the world (WHO, 1990). Leishmaniasis causes serious disabilities, destruction of mucus membranes and if left untreated within two years may lead to fatalities (WHO, 2004). It is well documented that leishmaniasis is a major health problem in some tropical countries.

2.2 Classification of sand flies

Sand flies are members of the phylum Arthropoda and belong to the class Insecta and order Diptera. Class Insecta is the largest and the most evolved group of animals with respect to the number of species which are over 773,000 and individuals (Mehlhorn, 2001). The number of genera depends on the hierarchical classification adopted, but according to the most widely accepted concept six genera in the family Psychodidae do exist. These are *Phlebotomus* with ten subgenera, *Sergentomyia* seven subgenera and *Chitinius* with only one species, in the Old World and *Lutzomyia* with twenty six subgenera, *Brumptomyia* twenty two species and *Warileya* six species, found in the New World. Only sand flies in the genera *Phlebotomus* and *Lutzomyia* are proven vectors of *Leishmania*, though in other genera man-biting flies also occur (Lainson *et al.,* 1986; Lane, 1986; Tonui, 2006). Diverse subgenera within the different genera exist (Lewis *et al.,* 1977)

Like mosquitoes, sand flies are members of the suborder Nematocera. Both members can be recognized by their many segmented antennae, which are usually long. Majority of the nematocerans are small, slender and long-legged. Within the suborder Nematocera many flies are of economic importance, as pests or disease vectors (Mehlhorn, 2001). The nematoceran larvae possess a well-developed head, and most live in water or in moist habitats, this forming an important food item of many freshwater fishes as aquatic larvae (Killick-Kendrick *et al.,* 1994). The anatomy of the head, the structure of the male genitalia, the presence and arrangement of teeth in the cibarium (i.e. mouth cavity) and partly the structure of the female spermathecae are used as characters for the subdivision of genera, subgenera and species of sand flies (Killick- Kendrick, 1978, 1994; Peters, 1992; Pession *et al.,* 1994). Methods such as isoenzyme analysis are used for closely related and morphologically indistinguishable species like *P. duboscqi* and *P. papatasi* (Fryauff *et al*., 1991; Pesson *et al.,* 1994). Polymerase Chain Reaction (PCR) technique may be more useful in sand fly identification and classification for its simplicity and ease of application.

2.3 The life cycle of sand flies

Female sand flies lays eggs in humid rodent burrows, termite moulds, barks of old trees, ruined buildings, cracks in walls, in animals shelters and household rubbish dumps. First instar larvae with a pair of caudal bristles hatch between 7-11 days and feed on dead organic matter present in the breeding site. The larvae molt and undergo development to second, third and fourth instar stages. The second stage develops a second pair of caudal bristles. The fourth instar larvae pupate and adult sand flies emerge after about 5-7 days

(Figure 2). Mating occurs at the site of emergence whereby male and female sand flies pair up for copulation. The sand fly life cycle takes 4-6 Weeks from eggs to adult emergence.

Female sand flies require blood to support growth of eggs though both sexes take "sugar meals" consisting of plant sap or honeydews excreted by aphids and coccids to sustain their daily activities (Schlein and Warburg, 1986; Moore *et al*., 1987; Walbanks *et al*., 1991). The females suck blood from human or other mammalian hosts for egg development. Males feed on sugars and plant juices. Optimum temperature of about 29-30° C and high humidity of 80 to 90 % are essential factors for sand fly development.

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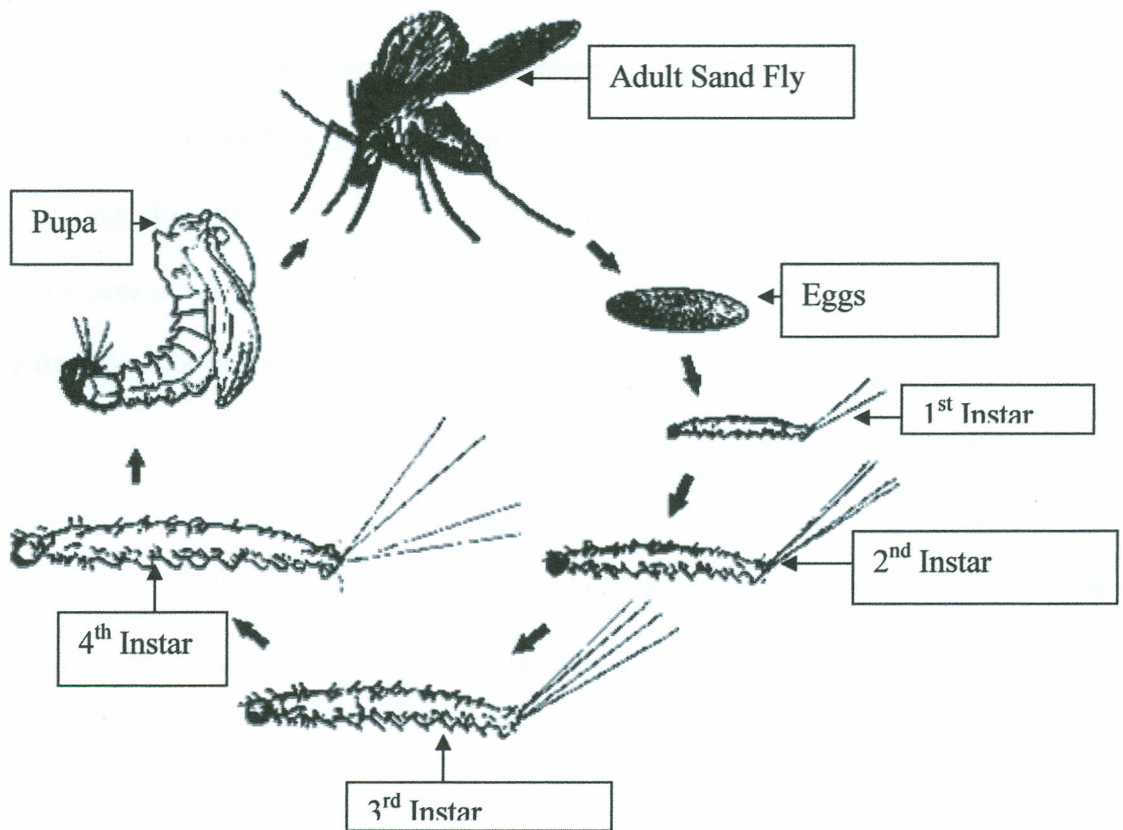

Figure 2; The life cycle of sand flies (Psychodidae).

2.4 Identification of adult sand flies

Sand flies are minute insects which are hump-backed, have black eyes, long antennae and short mouth parts unlike mosquitoes. Most adult sand fly species vary in length, between 1.2-3.7 mm (Perfil'ev, 1968) (Plate 1). They range in colour from white as phlebotomine species of the old world to black as the *Lutzomyia* of the new world (Killick-Kendrick, 1999). Male sand flies have bi-furcated genitalia while females have a rounded abdominal tip. Phlebotomines at rest hold their wings in a raised "V". Their bodies are covered with long hairs which give a "fluffy" appearance, legs are long and slender (Perfil'ev, 1968). In

its search for blood, usually in the evening and at night, the female covers a radius of a few metres around its habitat. They have short hopping flights. When they locate a host, they hop around before settling down to feed and their attacks are very silent unlike those of other biting flies like chrysops, tsetse flies and mosquitoes (Killick-Kendrick,1999). Females have developed mandibulate sucking mouthparts of which their detailed structures vary from one species to another. Males have undeveloped mouthparts. The head bears the feeding apparatus for blood feeding and for nectar uptake. The proboscis opens into the cibarium which acts as the feeding pump and leads into the midgut via the pharynx and the esophageal valve. The midgut is a long sac where digestion and absorption of food takes place. It opens to the pylorus and leads to the hind gut and ends at the rectum.

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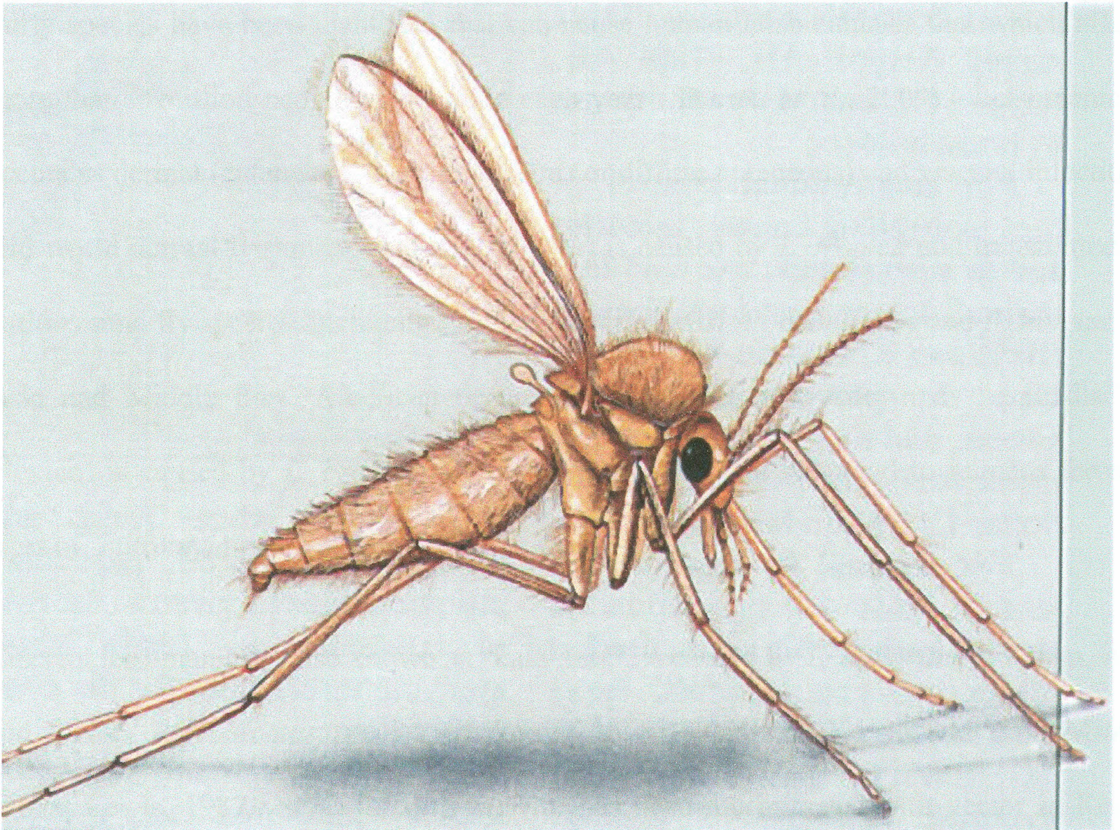

PLATE 1. Adult female sand fly. Golden brown to black, minute dipterans (1.2-3.7 mm) with long slender legs, hairy body and holds wings in a "V" shape when resting and moves with short hopping flights.

(Source:http://www.who.int/leishmaniasis/disease_epidemiology/en/,)

2.5 *Leishmania* parasites and their transmission by phlebotomine sand flies

Leishmania parasites occur in two forms the rounded forms referred to as amastigotes found in vertebrate hosts tissues, and the promastigote found in the vector, the sand fly. They are the causative organisms of leishmaniases and belongs to the genus *Leishmania,* family Trypanosomatidae. They are transmitted by a host of sand fly species. At least

thirty species have been identified that can cause human leishmaniasis that which affects more than 2 million people world wide each year (Sharma *et al.*, 2009). Leishmaniases occurs as dermal (cutaneous, muco-cutaneous or diffuse cutaneous) and visceral infections. Old world dermal leishmaniasis (oriental sore) is caused by *L. tropica* and transmitted by various sand fly species, including *Phlebotomus papatasi, P. caucasicus* and *P. longipes* in Asia and Middle East. American dermal leishmaniasis (also known as "espundia" in Mexico) is caused by *L. braziliensis* and *L. mexicana,* and is spread to humans through bites of *Lutzomyia* species.

Visceral leishmaniasis, also known as "kala-azar", is caused by *L. donovani* in Kenya, Asia and Sudan. *Phlebotomus* species are known to be vectors in Kenya and Iran (Minter, 1963a; Lewis, 1987). *Phlebotomus martini* has been incriminated as a vector in Kenya (Perkins *et al.*, 1988; Tonui *et al.,* 2006). The sand fly *Phlebotomus duboscqi* is widely spread from north Africa to east Africa and transmits *Leishmania major* that causes cutaneous leishmaniasis in the vast Savanna and desert areas of Africa (Ashford and Bettini, 1987). *Phlebotomus pedifer* transmits *Leishmania aethiopica,* the causative agent for diffuse cutaneous leishmaniasis in Kenya and Ethiopia.

2.6 Life cycle *of Leishmania* parasites

The amastigotes of *Leishmania* parasites are ingested together with infected blood meal when the sand fly feeds, develop in the gut and undergo major transformations within 4 to 25 days forming infective promastigotes. The ability of *Leishmania* parasites to attach to the sand fly midgut as the blood meal is sequentially digested is essential for the

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development of transmissible infections (Killick-Kendrick *et al.*, 1975). The infective forms, the promastigotes travel to the mouthparts and are introduced into the wound of the host during feeding. In human or other mammalian hosts, the metacyclic promastigotes are immediately phagocytosed by the macrophages at the bite site and round up to form amastigotes, which multiply by binary fission at the site and are later disseminated into the blood and other organs where they infect other macrophages. The amastigotes are taken up by sand flies during their next blood meal initiating another developmental cycle (Figure 3).

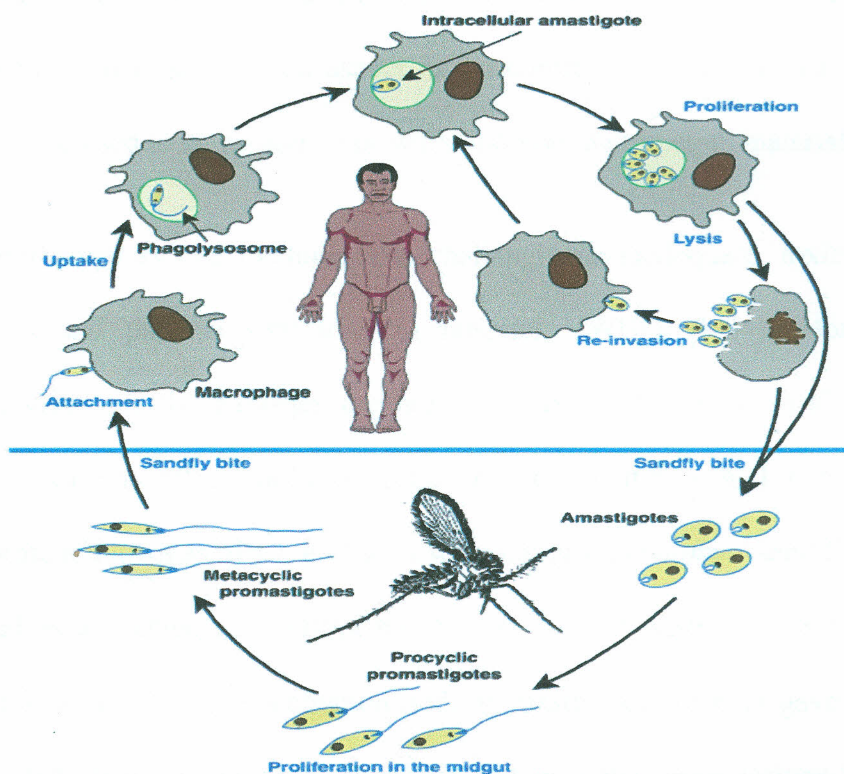

Figure 3; Schematic diagram of the *Leishmania* digenetic lifecycle (Adopted from Handman Emmanuela, Leishmaniasis: Current status of Vaccine Development, 2001).

2.7 Current approaches to leishmaniases control and prevention

The present strategies for leishmaniases control includes reduction of leishmanial infections, prevention of death and management of social and economic losses due to these diseases through several methods which include;

A) Chemotherapy by using drugs including pentostam, amphotericin B and miltefosine

B) Control of reservoir hosts which may include curling infected dogs, destroying rodent burrows and using rodenticides.

C) Vector control by physical destruction of anthills, clearing bushes, personal protection using lotions, screening houses and use of bed nets among others. Public health education is also a vital aspect in the control of leishmaniases. Chemicals could also be used to spray houses or while incorporated in cloth materials.

Although chemotherapy is one method; it is challengingly inadequate, toxic and have resistant patterns and patients stay long in hospitals (WHO, 2004). Moreover the diagnostic techniques for the parasites are non-predictive and complex and have poor biomarkers. The vaccines that could be used are very complex and parasite- stage dependent. A protein known as SP15, a 15 kDa secreted by *Phlebotomus* sand flies, is now being developed as a vaccine to interrupt the transmission of leishmaniasis through the insect (Valenzuela *et al*., 2001). Vaccination with promastigotes however gave promising results when tested. Inoculations with scrapings and exudates from lesions have been tested with appreciable results (Khamesipour *et al*., 2006).

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Sand flies (*Lutzomyia* and *Phlebotomus*) are controlled using chemicals (pyrethroids) for indoor residual spraying (IRS) and spraying of animal shelters. This is never routinely however except when there is an outbreak or when controlling other insects. Use of insecticide treated materials (ITMs) e.g. curtains and bed sheets; and long lasting treated nets (LLTNs) have also had a great impact in sand fly control. Impregnated dog collars and personal protection through application of repellents or lotions laced with insecticides to the skin or fabrics have also been encouraged (Vyokov, 1980; Buescher *et al.,* 1982; Maroli and Majoli, 1991). Destruction of animal burrows, anthills, use of nets of fine mesh and improvement of housing may reduce incidence of man-vector contact. Control strategies however depend on the underlying specific situations (Killick-Kendrick, 1999). Chemicals including organochlorides, carbamates, pyrethroids and organophosphates used to control other pests also reduce sand fly populations considerably (White, 1991). With increasing resistance to insecticides by other insect vectors, and the environmental pollution by synthetic chemicals, there is an urgent demand for natural, safe and non-persistent insecticides from plants.

Insecticide resistance has been reported in only three sand fly species (*Phlebotomus papatasi, P. argentipes* and *Sergentomyia shortii*) against DDT in India (Singh *et al*., 2001). However DDT tolerance has been reported in several countries although this chemical was banned for use in Kenya. Sand flies have been shown to possess detoxification mechanisms that could confer cross resistance between DDT and other compounds (Alexander and Maroli, 2003). Insecticide resistance may arise through selection resulting from leishmaniasis vector control measures or exposure to insecticides

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applied for other purposes, for example, agricultural or domestic pest control, peri-focal spraying against dengue vectors or residual house spraying against vectors of malaria or chagas disease (Alexander and Maroli, 2003).

2.8 Role of herbal plant extracts in the control of sand flies

Crude plant extracts and inorganic larvicides were largely used as natural insecticides before the organic laboratory-synthesized insecticides became available in the 1940's (Balandrin *et al.*, 1985; Raw, 1986; Rozendaal, 1997). Plant extracts such as pyrethrum, nicotine and rotenone were among the first compounds used to control insects of medical and agricultural importance (Grainge and Ahmed, 1988; Coats, 1994). Pyrethrins, a complex of esters extracted from flowers of *Chrysanthemum cinerariefolium* (Compositae), are still used to enhance commercial preparations of household insecticides (Bel *et al.,* 1990). Nicotine extracts from *Nicotiana glauca* and its nicotinoid derivatives are choice molecules for the manufacture of new insecticides. Rotenone and rotenoids, isoflavanoids found in several genera of tropical leguminosae plants such as *Derris* (Papillionaceae), *Antonia* (Loganiaceae) and *Lonchocarpus* (Fabaceae), were shown to have insecticidal properties against *Lutzomyia longipalpis* Lutz and Neiva, the vector for *Leishmania chagasi* in Brazil (Luitgards-Moura *et al.*, 2002). Essential lemon oil was found to be 70% protective against sand fly bites (Rojas *et al.*, 1991). A concentration of two per cent neem oil mixed in coconut or mustard oil provided 100% protection against *P. argentipes* throughout the night in field conditions (Sharma *et al.*, 1993). Pyrethrin esters have been found to be effective repellents against *P. argentipes* the vector of Indian kala azar (Kishore *et al.*, 2006).

2.8.1 *Tagetes minuta*

Tagetes is a genus of about 60 species in the daisy family Asteraceae. *Tagetes minuta* (Plate 2 A) is an annual plant growing 1-2m tall (Meshkatalsadat *et al.,* 2010). It was introduced to Africa from south America and is abundantly available in Kenya (Hillard, 1977). It is rich in many secondary compounds (Rodriquez and Mabry, 1977; Garg and Mehta, 1998; Kadriya *et al.,* 2004; Meshkatalsadat *et al.,* 2010). Secondary compounds in *Tagetes* are effective deterrents of numerous organisms including insect pests through different mechanisms (Usher, 1974; Maradufu *et al*., 1978; Saxena and Koul, 1982; Jacobson, 1990). Dried plants can be hung indoors as an insect repellent (Brown, 1995). *T. minuta* was found to be larvicidal against *Aedes aegypti* larvae at 10 ppm (Green *et al*., 1991). The terpene, ocimenone in *Tagetes* was found to be larvicidal only at higher concentrations than the whole oil. The discovery of insecticide activity of phototoxins present in Asteraceae species has stimulated the interest in this plant family as part of the search for new plant derived insecticides (Rawls, 1986). Although *T. minuta* is perceived to have insecticidal activities, its action against phlebotomine sand flies has not been evaluated.

2.8.2 ***Acalypha fruticosa*** Forssk belongs to the family Euphorbiaceae is an erect clump-forming shrub standing 15 to 25m high often forming a pure stand (Plate 2 B). It is widely distributed along riverbanks and flood plains in riparian woodlands in East Africa. The shrub has yellow resinous glands on the lower leaf surface that give off an unpleasant smell when crushed and has no stinging hairs or white latex. The leaves are simple, ovate and alternate, 1.8 cm long, the tip long or short but usually blunt, edges round toothed,

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grey-green below, the leaf stalk shorter than the blade (strap shaped stipules). The male and female flowers are tiny yellow-green to reddish, in hanging catkin-like spikes, usually male above and female below, or on separate plants, small sepals but an enlarged bract surround the female flowers and fruit, 4 mm x 8 mm, wavy edged and ribbed. Fruits are tiny capsules, 2.3 mm and contain the seeds. The plant has been used extensively for its medicinal value, food, fodder and repellent activities against insects (Bekalo *et al.,* 1996). Stems are also used for weaving granaries and local doors or as withies. Its leaves are powdered, soaked in water and the solution applied on animal skin and wound as a repellent or insecticide against ectoparasites and dipteran flies which would facilitate super infection.

2.8.3 *Tarchonanthus camphoratus* L (Compositae) is a dioecious shrub widespread in tropical Africa especially in the stony sites in the Rift Valley and grows to about 6 metres tall (Plate 2 C). Its derivatives and formulations have repellant activities and medicinal uses (McGaw *et al*., 2000 Bishay *et al.,* 2002). Distilled leaf extracts yielded compounds with insecticidal activities. Wild animals have been seen rubbing against the plant to keep off biting insects (De stepanis, 1924; Van Wyk and Van Wyk, 1997). The plant is known in Swahili as 'leleshua' in Kenya.

2.8.4 *Chrysanthemum cinerariefolium* The white *Chrysanthemum* flower, pyrethrum, is mentioned in early Chinese history and it is believed to have passed into Europe along the silk routes. However, the species of the plant was unknown, and its history starts with the mention of the identified species of *C. cinerariefolium* (Plate 2D) found growing in

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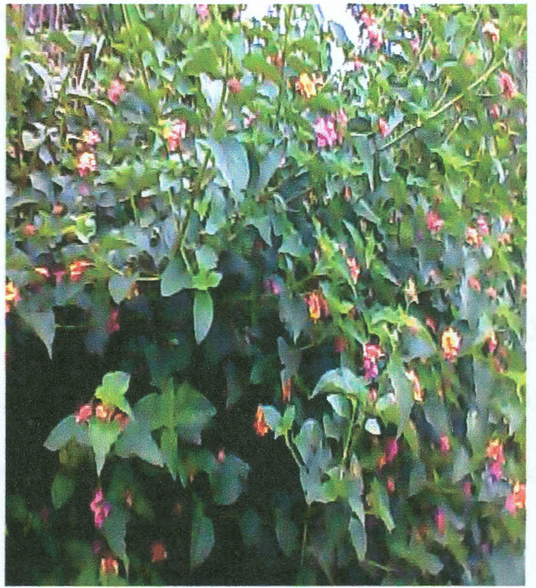
Dalmatia (now part of Croatia) in 1847. The flowers were ground and used for the control of crawling insects. It was known as Dalmatian insect powder. Since then, it has been processed into a myriad of insecticide formulations. Pyrethrum is the oldest insecticide known to man. It is still used to enhance commercial preparations of household insecticides and agricultural pesticides (Bell *et al*., 1990). Pyrethrum has six esters comprising the active ingredients. The relative amounts of each vary depending on the particular plant type, geographical source and time of harvest. Pyrethrins, like all members of the pyrethroid insecticide family, kill insects by disrupting their nervous systems. Although pyrethrin resistance has been reported in about fifteen insect species, resistance ratios between resistant and susceptible hosts are often relatively low (Cox, 2002).

Pyrethroids are synthetic chemical compounds similar to the natural pyrethrin. They are usually broken down by sunlight in one or two days, and do not significantly affect water quality. Currently, WHO, (2007) rates pyrethroid insecticides as important weapons against insect pests of both economic and medical importance. These products show remarkably high toxicity and rapid action against a wide range of insects, but relatively low mammalian toxicity. Pyrethroid insecticides are also easily biodegraded to a harmless product, and hence do not accumulate in biological systems. First generation pyrethroids are less stable than the second generation but are enhanced by the addition of a synergist such as piperonyl butoxide. According to Heal *et al,* (1950), Farnsworth (1966), Kokwaro (1976) and Sukamar *et al*, (1991), more than 2000 other plant species are catalogued as having insecticidal properties.



A B

C D

Plate 2. Plants used in this study- A- *Tagetes minuta*, B- *Acalypha fruticosa*, C- *Tarchonanthus camphoratus*, D- *Chrysanthemum cinerariefolium*. Only the commercially prepared pyrethrin, from the flowers of *C. cinerariefolium* (D) was used in the experiment.

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CHAPTER THREE

3 MATERIALS AND METHODS

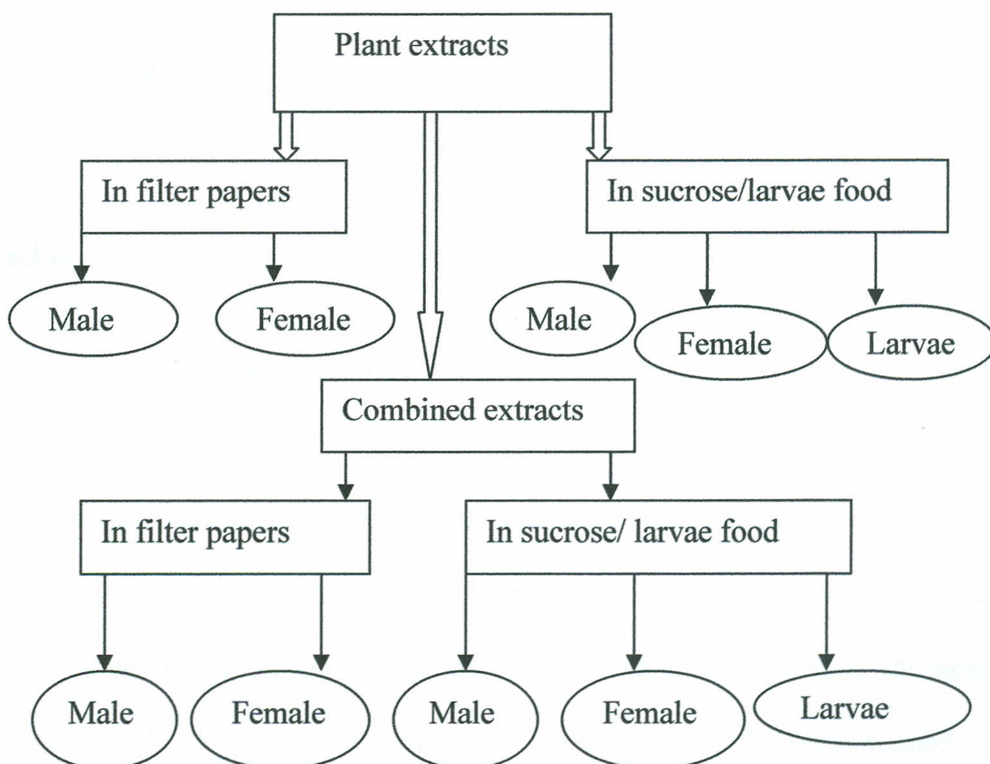
3.1 Study site

This study was conducted in the Leishmaniases Section, at the Centre for Biotechnology Research and Development (CBRD), KEMRI, off Mbagathi road, Nairobi which is mandated to undertake basic and biotechnology-related research on human diseases and training in Kenya.

3.2: Study Design

The study was a comparative experimental design consisting of three plants each with two different extracts and commercial pyrethrin that were set and compared against each other for their activity against male and female sand flies and the four larval instars. Extracts were incorporated in filter papers and used in one set while extracts in sucrose were used in the second set of adulticidal experiments. Extracts were also incorporated in larval food and used in feeding the larvae. Ten specimens were used in each male, female and larval jar and all experiments were done in triplicate (Figure 4).

Figure 4; Experimental set up



Ten specimens were used in each experiment and concentration and the experiments were done in triplicate

3.3 Plant collection and preparation

Floral and foliar parts of *Tarchonanthus camphoratus*, *Acalypha fruticosa*, and *Tagetes minuta* were collected from Baringo district in the Rift Valley Province of Kenya. These plant parts were dried under shade before packaging in paper bags for transportation to the laboratory for further drying. Voucher specimens of the plant parts were taken to the National Museums of Kenya herbarium for identification and future referencing. The plants were left to dry completely under a shade for a month for the extraction. Three pyrethrum products, 0.6%

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pyrethrin EC (Pymos™), 0.5% EC (Pylarvex™) and 0.2 % pyrethrin powder (Moskil®) were acquired from the Pyrethrum Board of Kenya, in Nakuru for bioassays. Pyrethrin (0.6%) is normally used as a mosquito adulticide and 0.5% EC is used as a mosquito larvicide. The dusting powder, 0.2 % pyrethrin is generally used for controlling crawling insects.

3.4 Preparation and extraction of test extracts

The dry plant samples were ground to fine powder using laboratory Waring blender. One hundred grams of each sample was weighed and put in one litre conical flasks. Bioassay-guided extraction of the same samples was done using 300 mls of distilled organic solvents of increasing polarity, starting with N-hexane, dichloromethane, ethyl acetate and methanol. The solvents were added into flasks and placed on a shaker and soaked for 48 h. The samples were soaked further with 300 mls of the solvents for 24 h until the filtrates remained clear. The extracts were then filtered using Whatman filter paper number 1, concentrated and dried under vacuum using rotary evaporator at 30-35° C (Harborne, 1994) (Appendix 1). The concentrates were then transferred to sample bottles and the weight of the dry extracts recorded and stored at -20 °C. The extracts were later assayed for larvicidal and adulticidal properties.

3.5 Preparation of test commercial products

Three commercial products of *Chrysanthemum cinerariefolium*, 0.6% pyrethrin EC (w/v), 0.5% pyrethrin EC (w/v), and 0.2 % pyrethrin (w/w) dusting powder were tested. Concentrations of 0.08, 0.25 and 0.5 mg/ml were prepared from 0.6% concentrate, equivalent to 6mg pyrethrin per ml using 10 % sucrose solution and used to feed sand flies.

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This was arrived at from the preliminary tests after feeding adult sand flies on the mixtures. The concentrate of 0.5% pyrethrin, equivalent to 5mg pyrethrin per ml was reconstituted and solution concentrations of 0.05mg/ml to 1.0mg/ml prepared using distilled water and each mixed with 1g of larval food. The mixtures were left to dry in a shaded place overnight and small amounts introduced into vials containing the four stages of larvae to feed on. Concentrations of 0.05 mg/g to 1.0 mg/g of the active powder were prepared, mixed with one gram of larval food and small amounts fed to the four stages of the sand fly larvae.

3.6 Sand fly colony

A colony of *Phlebotomus duboscqi* Neveu Lemaire (Diptera: Psychodidae) that originated from Marigat, Baringo District, Rift Valley, and which was maintained at KEMRI insectaries was used. The colony of *P. duboscqi* was established using field-captured females and sustained according to the methods of Beach *et al*, 1986. This colony was being restocked by regular re-collection of field isolates from the same locality and in-breeding. The female sand flies were fed on blood using Syrian golden hamsters anaesthetized with sodium pentobarbitone (Sagatal®) for egg development. The hamsters were usually shaved under the belly for convenient feeding. The sand flies were reared at 28 ±1°C, and an average RH of 85-95%, 12:12 (L: D) photoperiod in Perspex insect rearing cages and were fed using slices of apples as a source of energy.

Larvae for the experiments were obtained at intervals from female sand flies which were reared and fed on blood using Syrian golden hamsters anaesthetized with sodium pentobarbitone (Sagatal®). Almost an equivalent number of males were included for

copulation purposes. They were then gently aspirated using a mouth aspirator into rearing jars to lay eggs which were left to hatch. The rearing of the sand flies was done at two weeks intervals to obtain the larval instar required for the bioassays. The larvae were gently placed into rearing vials using a camel hair brush wetted in distilled water. Little amounts of larval food of an equal mixture of rabbit droppings and rabbit chow, ground to fine powder and let to ferment was then sprinkled into the vials (appendix 2). Optimum temperature ($28 \pm 1^{o}C$) and humidity (85-95%) was maintained throughout the entire rearing and experimental period. All the four stages of the larvae were used for the experiments.

3.7 Adult sand fly bioassays

3.7.1 Detection of sucrose in the sand fly guts

In all the adulticidal bioassays, 10 flies were first fed on 20mg/ml of each extract and then tested for an evidence of sugar feeding using cold anthrone prepared (Sigma, St. Louis, MO, USA; Lot 13HO404) before the main experiments were set (Appendix 4). Whole guts were removed and put in micro well plates (Appendix 5). Three to four drops of cold anthrone were added in the wells containing the sand fly guts (Van Handel, 1985). Each gut was ground with a plastic grinder to expose the sugar contents in the gut. The cold anthrone, in the presence of sugar turned from yellow to greenish blue. The intensity of the colour depended on the amount of the sugar present in the guts after one hour of incubation at room temperature. It was noted that a few males and females did not feed on the sugar mixtures. The highest percentages were those of males fed on pyrethrin/sucrose mixture, with a maximum mean of 10% after repeating three times. The plant extract mixtures gave non-feeding mean of 12% for both sexes. This indicated that some sand flies in the experimental

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groups may not have fed on the solutions, either due to repellency effects of the compounds or other unknown factors. For those that fed on sucrose alone, 7.6% gave a negative anthrone test.

3.7.2 Contact method using filter paper technique (Luitgards-Moura *et al*, 2002).

Table 1- Experimental set up for contact method using extracts incorporated in filter papers. Ten sand flies were aspirated for each extract concentration. In the negative control (0), sand flies were aspirated into jars containing filter papers soaked in distilled water and dried.

	Plant*	*A. fruticosa*		*T. minuta*		*T. camphoratus*
		Methanol	ethyl acetate	Methanol	ethyl acetate	Methanol
	Conc (mg/ml)					
Male	0	10	10	10	10	10
	2.5	10	10	10	10	10
	5	10	10	10	10	10
	10	10	10	10	10	10
Female	0	10	10	10	10	10
	2.5	10	10	10	10	10
	5	10	10	10	10	10
	10	10	10	10	10	10

*Plant- extraction was done using N- hexane, Dichloromethane, Ethyl acetate and finally Methanol. The former two had no insecticidal activity. Only the methanolic extract of *T. camphoratus* shown insecticidal activity. Experiments were done in triplicate.

3.7.3 Feeding sand flies with extracts while incorporated in sucrose

Table 2- Experimental set up for *P. duboscqi* feeding method using cotton wool balls. Ten sand flies were aspirated each concentration. In the negative control (0), sand flies were fed on sucrose solutions only. Experiments were done in triplicate.**

Plant*		*A. fruticosa*		*T. minuta*		*T. camphoratus*
Conc (mg/ml)		Methanol	ethyl acetate	Methanol	ethyl acetate	Methanol
Male	0	10	10	10	10	10
	2.5	10	10	10	10	10
	5	10	10	10	10	10
	10	10	10	10	10	10
Female	0	10	10	10	10	10
	2.5	10	10	10	10	10
	5	10	10	10	10	10
	10	10	10	10	10	10

*Plant- extraction was done using N- hexane, Dichloromethane, Ethyl acetate and finally Methanol. The former two had no insecticidal activity. Only the methanolic extract of *T. camphoratus* shown insecticidal activity.

** Feeding method was also used to feed sand flies on Pyrethrin (Conc. of 0, 0.08, 0.25 and 0.5 mg/ml).

3.8 Determination of larvicidal effects of the extracts from plants

This was done according to the methods described by Luitgards-Moura *et al*, (2000). Ten *P. duboscqi* larvae were gently placed into four triplicate vials (Appendix 3B) using a camel hair brush wetted with distilled water. The first triplicate vials contained first instar larvae, the

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second, third and fourth triplicate contained second, third and fourth instar larvae, respectfully. One gram of larval food prepared from a fungal growth obtained from rabbit chow was mixed with 40mg/ml (stock solution) of each extract and let to dry overnight under shade. Small amounts of the prepared dry food-extract mixtures were then sprinkled into the vials each day. The triplicate vials were set for each extract. Larvae that fed on larval food alone without the extracts were used as controls. The larvae were also fed on plain powdered plant parts and without any larvae food mixture. Those that fed on larval food alone formed the control groups. Larvae were monitored daily and mortality recorded for analysis (Plate 3). Mean lethal dosage designated LD_{50}, was determined every 24 hours of exposure.

3.9 Determination of larvicidal effects of pyrethrin

Ten *P. duboscqi* larvae were transferred into triplicate vials for each instar. One gram of larval food was thoroughly mixed with concentrations of 0, 0.2, 0.5 and 1 mg/g of 0.2% pyrethrin powder and sprinkled into the vials. Mortality was then monitored and recorded. In the pylarvex (0.5% pyrethrin EC) bioassay, concentrations of 0, 0.2, 0.5 and 1 mg/ml were prepared and mixed with a gram of larval food each, homogenized and dried overnight under shade. The mixtures were introduced into marked larval vials in small bits as the larvae feed. The larvae that fed on larval food alone formed the control groups. Mortality was recorded every 24 hours of exposure for statistical analysis.

3.10 Data analysis

All experiments were done in triplicate, whereby mortality of between 10% and 90% were considered and data entered into Microsoft excel program. Control groups in the experimental bioassays with more than 20% mortality were repeated.

Where mortality in the control groups fell between 5 and 20%, the observed percentage mortality was corrected using Abbott's formula (Abbott, 1925):

$$\text{Observed \% mortality} = \frac{(\text{Test \% mortality - control \% mortality})}{(100 - \text{control \% mortality})} \times 100$$

Data on the dose-mortality effects of different extracts on both larvae and adults were subjected to computerized Probit analysis (Finney, 1971) (PROBIT model: (PROBIT (p)) = Intercept + BX): for LD_{50} values and Pearson's goodness-of-fit χ^2 for significance. Variation effects of the extracts and those of males and females were subjected to ANOVA (Edwards, 1960). Values of ≤ 0.05 were considered significant.

3.10.1 Ethical approval

This was sought from KEMRI scientific and ethical review committee and Kenyatta University Board of Postgraduate Studies after conforming to the required research protocols.

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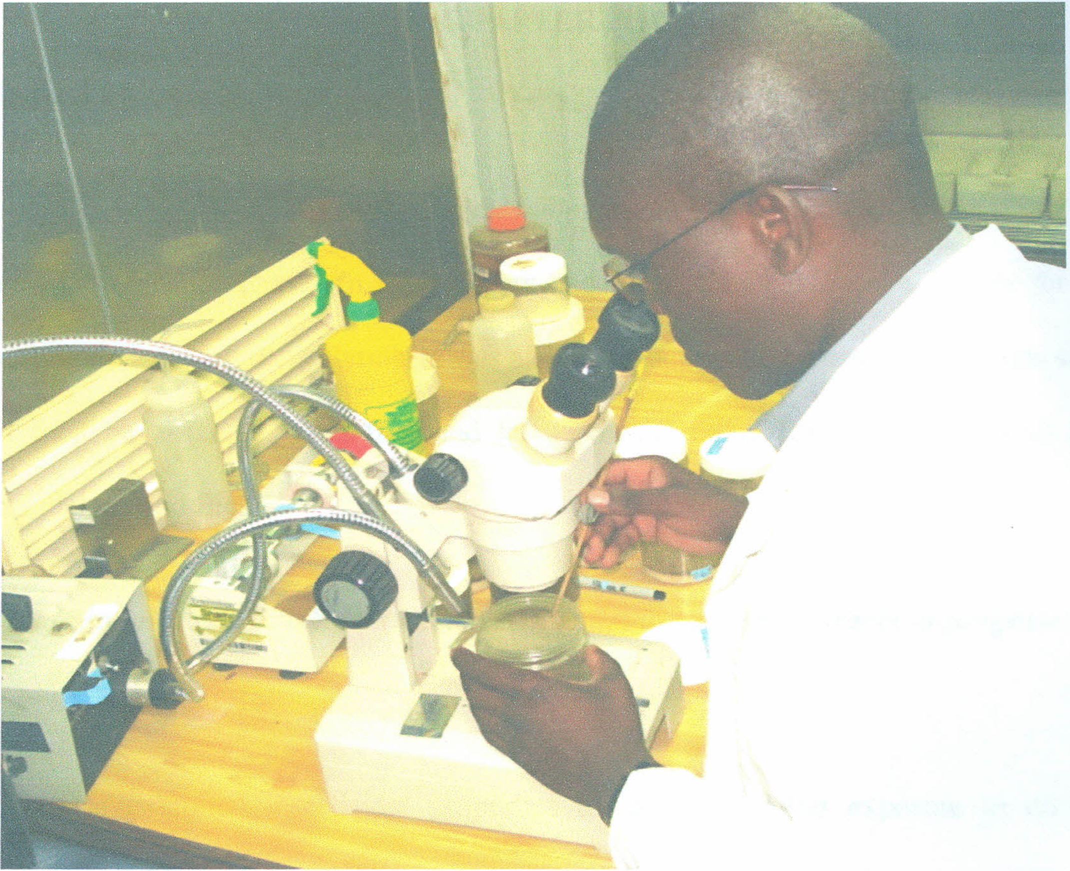

Plate 3. Examination and manipulation of hatched sand fly larvae. A wet camel hair brush was used to transfer the larvae into the vials where their behavior was observed.

CHAPTER FOUR

4 RESULTS

The extracts of *Acalypha fruticosa, Tagetes minuta* and *Tarchonanthus camphoratus* were evaluated while impregnated in filter papers and incorporated in sucrose to test for their efficacy against *P. duboscqi*. Pyrethrin was also evaluated while incorporated in sucrose and larval food against adult sand flies and larvae, respectfully. They were all found to be insecticidal to sand flies.

4.1 Mortality of adult *Phlebotomus duboscqi* exposed to plant extracts impregnated in filter papers.

The extracts exhibited insecticidal properties to sand flies after exposure to different concentrations. Mortality values between male and female sand flies were not significantly different ($F = 1.00$, $P = 0.318$) (ANOVA) in contact bioassays although individual sexes showed significant mortality. The most suitable time factor considered here in detail is the forty-eighth hour where significant mortalities were realized.

4.1.1 Comparison of mortality rates in male and female sand flies exposed to *A. fruticosa* extracts.

There was significant female mortality ($P < 0.05$) in methanol extract at 48 hours amongst the concentrations used. The LD_{50} for the females was 8.95 mg/ml ($\chi^2 = 39.4$) at 48 hours of exposure. Males had no significant mortality in the 48-hour period. The LD_{50} for the males at 48 hours was 3.26 mg/ml ($\chi^2 = 7.3$). Mortality in 5mg/ml and above increased steadily with

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time for both sexes. In the ethyl acetate bioassay, both male and female groups gave significant mortality (P <0.05) values at 48 hours post exposure. The LD_{50} for females at 48 hours was 3.5mg/ml (χ^2= 12.7) and that of males was 2.35mg/ml (χ^2= 18.4). Males in both extract bioassays were affected more than the females with mortality of over 60% in the lowest concentration of 2.5mg/ml at 48 hours of exposure and beyond. There was however significant mortality (P<0.05) difference ($F = 33.7$, $P < 0.05$) between the concentrations used. Males had 100 % mortality in methanol 10 mg/ml assay while the least was obtained from methanol 2.5 mg/ml for the female group which had 20 % mortality (Figure 5).

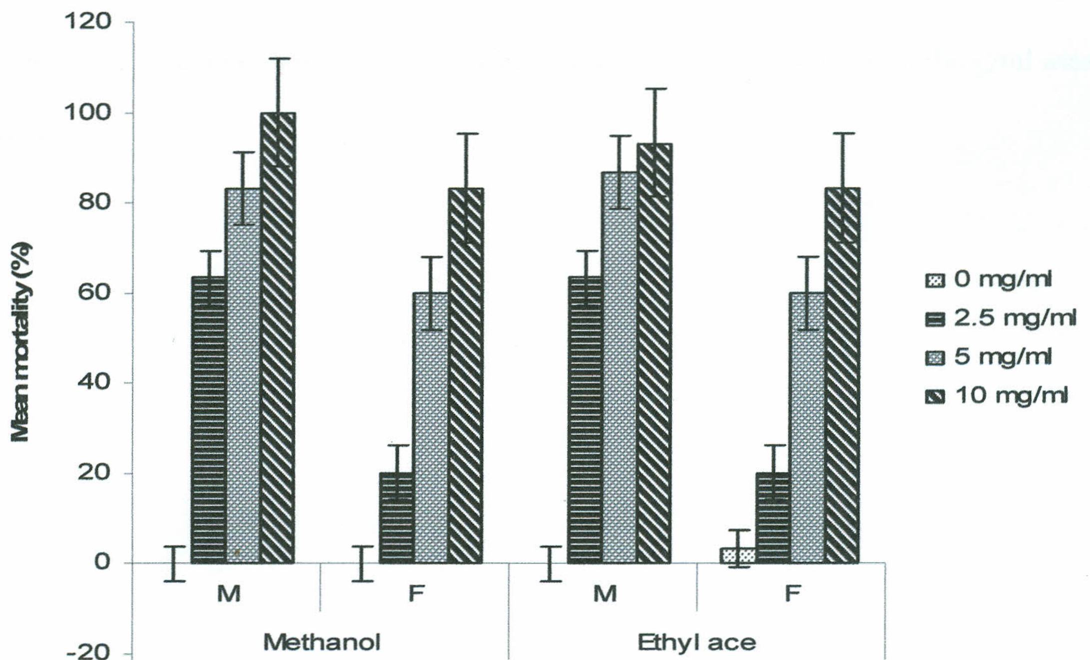

Figure 5; Mortality rates of male and female *P. duboscqi* exposed to *A. fruticosa* extracts incorporated in filter papers at 48 hours of exposure. F= female, M= male

4.1.2 Mortality of *P. duboscqi* males and females when exposed to the extracts of *T. minuta* incorporated in filter papers.

Both males and females had significant mortality ($P < 0.05$) in the methanol extract bioassay at 48 hours post exposure. The LD_{50} for females at 48 hours was 1.6 mg/ml (χ^2= 17.7). The LD_{50} for males at 48 hours was 1.2mg/ml (χ^2= 26.5). Mortality for both sexes was dose dependent and males were affected more than the females in 48 hours and above. In the ethyl acetate extract bioassay, females had significant mortality ($P < 0.05$) while male mortality were not significant ($P = 0.48$). The LD_{50} at 48 hours for this extract for males was 2.44 mg/ ml (χ^2= 4.4) and 3.09 mg/ml for females χ^2 (= 19.1) Mortality for both males and females was 100% for both extracts of *Tagetes minuta* in 10mg/ml assay (Figure 6).

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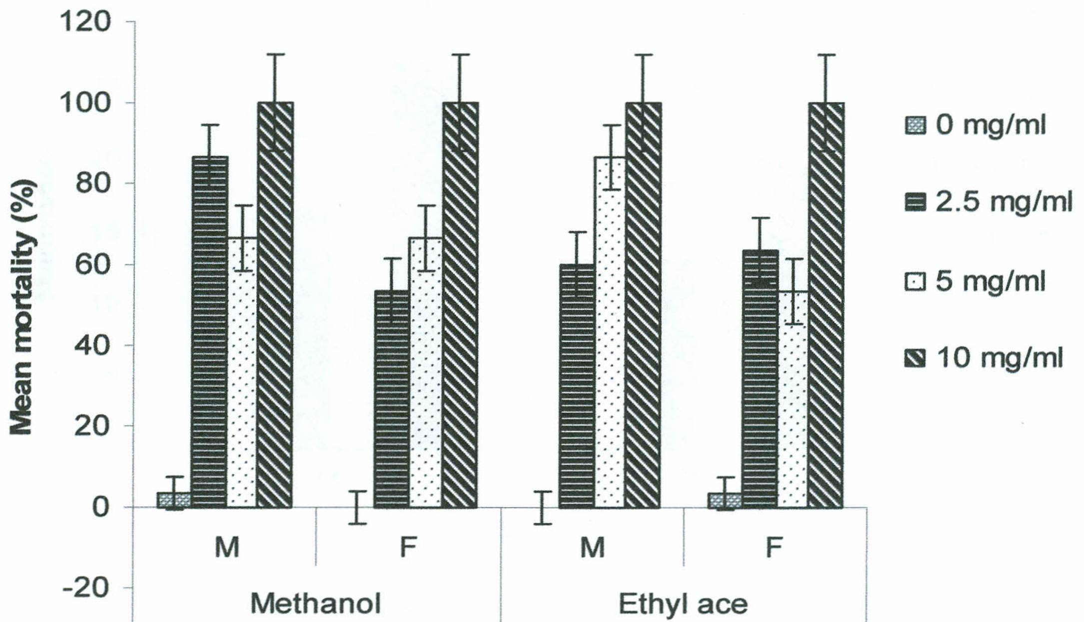

Figure 6; Mortality rates of adult *P. duboscqi* exposed to *T. minuta* extracts incorporated in filter papers at 48 hours of exposure. F= female, M= male

4.1.3 Mortality of *P. duboscqi* due to *Tarchonanthus camphoratus* methanol extract

There was no significant difference in mortality between males and females (P = 0.592) in this experiment. In this bioassay, the LD_{50} for females at 48 hours of exposure to this extract was 49.9 mg/ml (χ^2= 3.09) while males had a LD_{50} of 24.5mg/ml (χ^2= 7.19). These were higher values than those obtained from other extracts therefore showing weaker properties. Maximum mortality was 90 % in the male assay which was in the 10 mg/ml group (Figure 7).

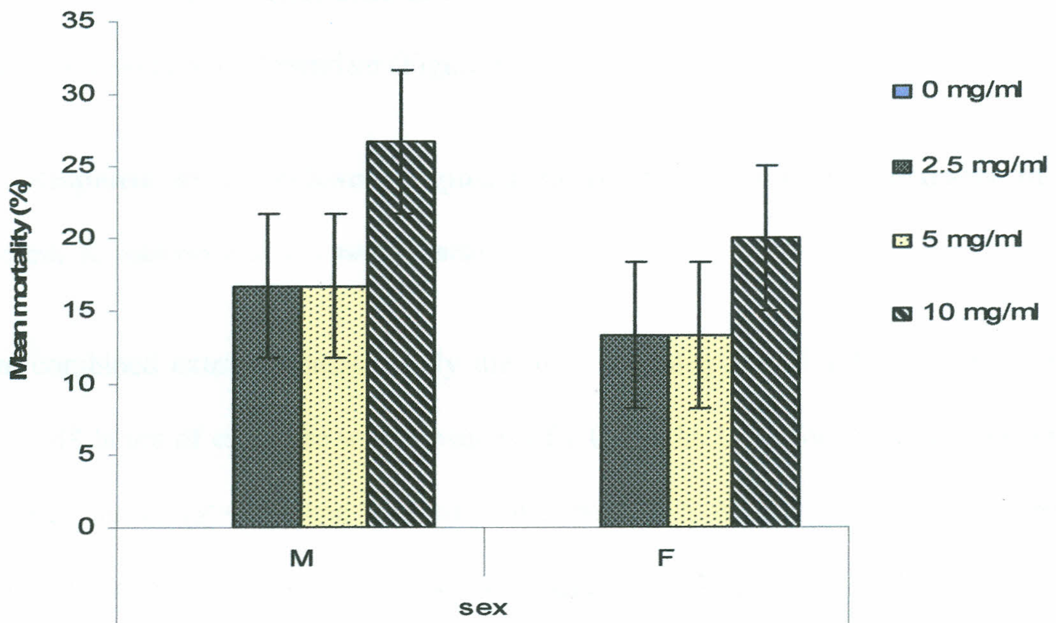

Figure 7; Mortality rates of adult *P. duboscqi* exposed to the methanol extract of *T. camphoratus* incorporated in filter papers at 48 hours of exposure. F= female, M= male

4.1.4 Response of *P. duboscqi* exposed to *A. fruticosa* and *T. minuta* combined extracts.

There was significant mortality ($P < 0.05$) in the methanol bioassays for both males and females values at 48 hours post exposure. The LD_{50} for the female sand flies at 48 hours of exposure was 9.47 mg/ml (χ^2= 23.8) while males had a LD_{50} of 9.93 mg/ml (χ^2= 27.1) in the methanol extract bioassay. Similarly there was significant mortality ($P < 0.05$) for both sexes in the ethyl acetate bioassay at 48 hours of exposure. LD_{50} for females was 4.23 mg/ml (χ^2= 60.7) and a LD_{50} of 4.88 mg/ml χ^2 (= 32.3) for the males at 48 hours of

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exposure. There was no difference in mortality for both sexes and died in almost equal numbers and were dose dependent (Figure 8).

4.1.5 Response of *P. duboscqi* exposed to combined methanol extracts of *A. fruticosa, T. minuta* and *T. camphoratus.*

In the combined extract bioassay, only the male mortality had significant values ($P < 0.05$) at 48 hours of exposure with P values of 0.026. The LD_{50} for females at 48 hours was 18.8 mg/ml (χ^2=3.8) and that of males was 19.5 mg/ml (χ^2=12.6). Maximum mortality for both was 40% at 48 hours of exposure in the 5mg/ml assay (figure 9).

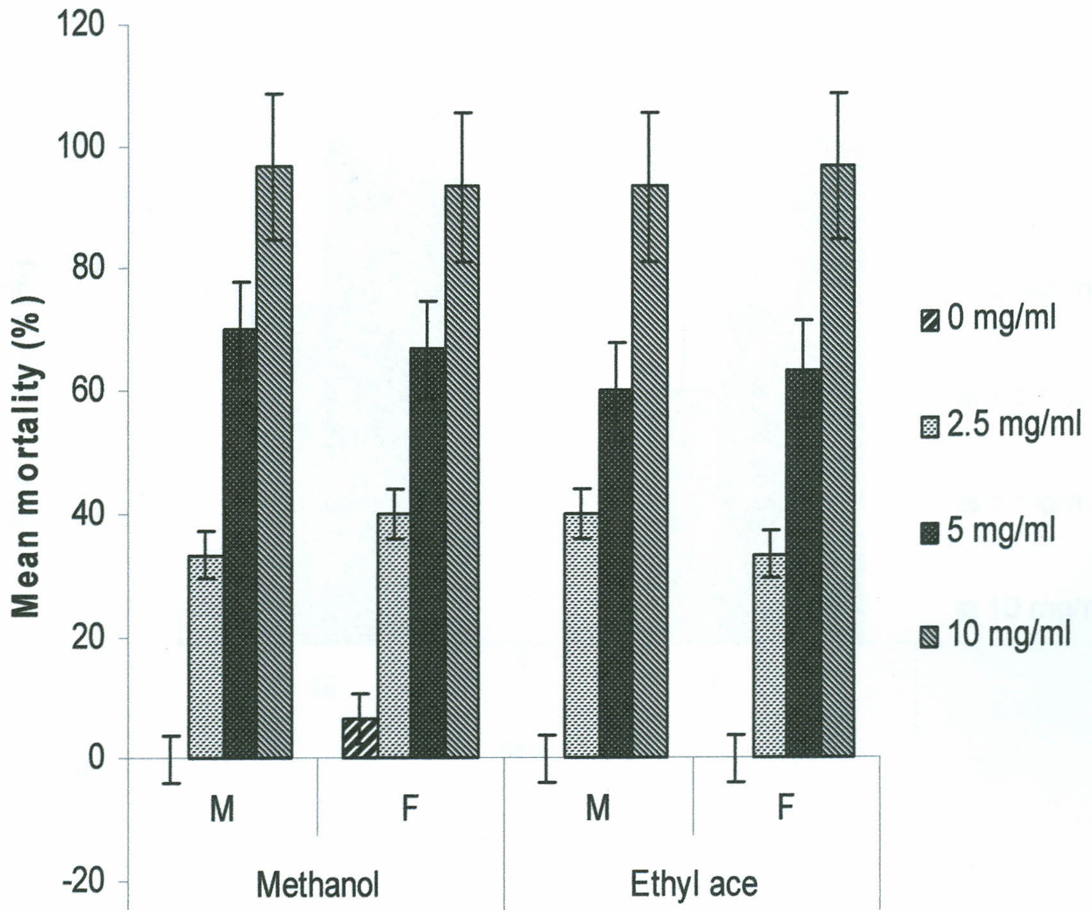

Figure 8, Response of *P. duboscqi* exposed to *A. fruticosa* and *T. minuta* extracts incorporated in filter papers at 48hours of exposure. F= female, M= male

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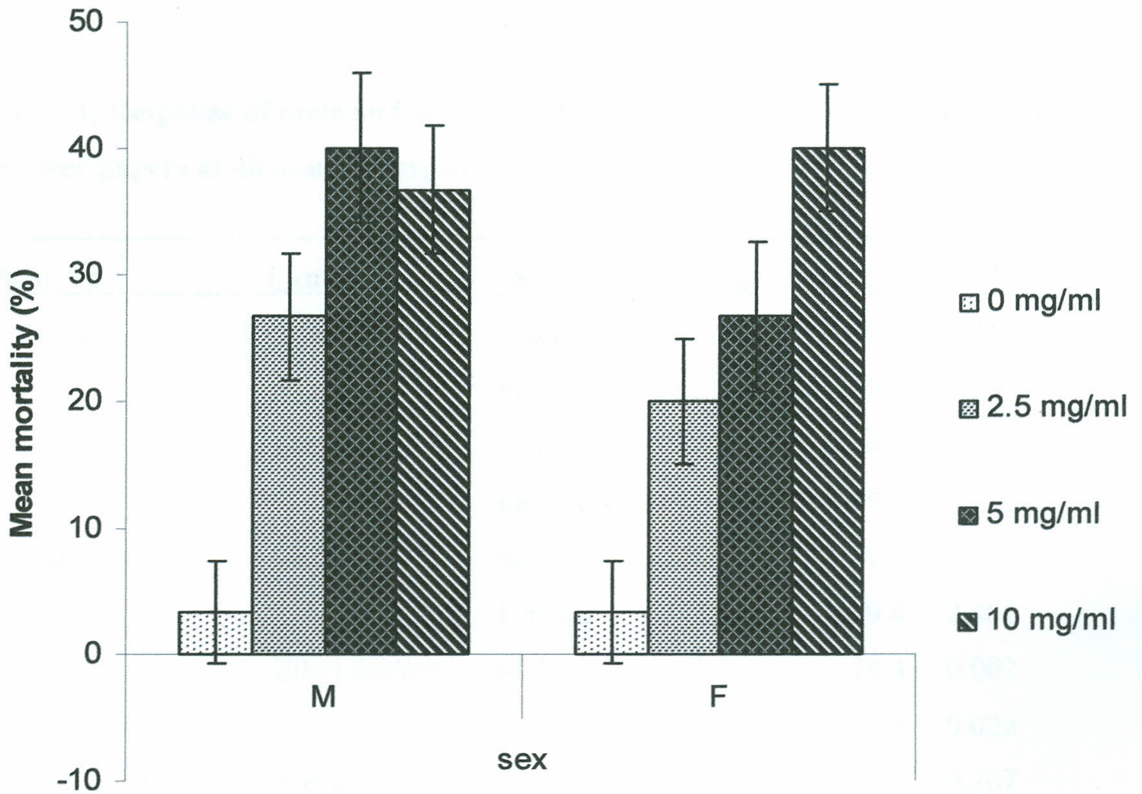

Figure 9; Response of *P. duboscqi* exposed to *A. fruticosa, T. minuta* and *T. camphoratus* combined methanol extracts at 48 hours of exposure. F= female, M= male

4.1.6 Mortality of *P. duboscqi* after exposure to different extracts.

Tagetes minuta extracts were established to be more effective in mortality of sand flies than other extracts (Table 3, Figure 10). Males had a LD_{50} of 1.2 mg/ml while the females had a LD_{50} of 1.6 mg/ml in the methanol extract bioassay. *T. camphoratus* had a LD_{50} of 19.5 and 18.8 mg/ml in male and female assays respectively. However there was no significant difference in mortality between extracts (F = 1.4, P = 0.240) but

there was significant mortality (*P*<0.05) difference between the concentrations used (F = 33.7, P < 0.05 Coefficient of Variation = 75.8 %)

Table 3; Response of male and female sand flies when exposed to different extracts in filter papers at 48 hours of exposure. N= 30

Plant	Extract	Sex	LD_{50}[1]	X^2	*P*
T. minuta	Methanol	male	1.2	26.5	0.001
		Female	1.6	17.7	0.007
	Ethyl acetate	male	2.44	4.4	0.487
		Female	3.99	19.1	0.002
A. fruticosa	methanol	male	3.26	7.3	0.202
		Female	8.95	39.4	0.001
	Ethyl acetate	Male	2.56	18.4	0.002
		Female	3.5	12.7	0.026
T. camphoratus	Methanol	male	24.5	7.19	0.207
		Female	49.9	3.09	0.685
Acalypha/Tagetes	Methanol	male	9.93	27.1	0.001
		Female	9.47	23.8	0.001
	Ethyl acetate	Male	4.88	32.3	0.001
		Female	4.23	60.7	0.001
Acalypha/Tagetes/ Tarchonanthus	methanol	male	19.5	12.6	0.026
		Female	18.8	3.8	0.575

[1]LD_{50}=mg/ml; sex variation F= 1.00, *P*> 0.05; Concentration variation F= 33.7, *P*< 0.05

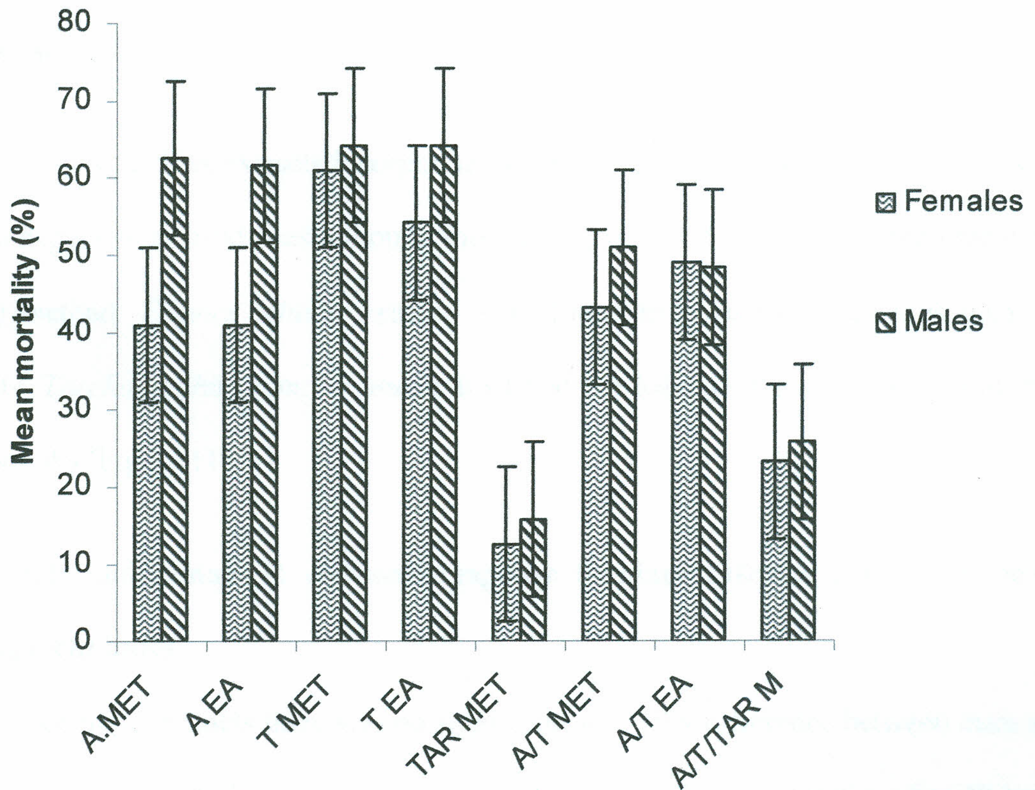

Figure 10; Mean mortality of sand flies exposed to different extracts in filter papers at 48 hours of exposure

Legend

A. MET= *Acalypha* methanol, A. EA= *Acalypha* ethyl acetate

T. MET= *Tagetes* methanol, T. EA= Tagetes ethyl acetate

TAR. MET=*Tarchonanthus* methanol, A/T. MET= *Acalypha/Tagetes* methanol

A/T. EA= *Acalypha/Tagetes* ethyl acetate

A/T/TAR. M= *Acalypha/Tagetes/Tarchonanthus* methanol

4.2 Mortality of adult *Phlebotomus duboscqi* exposed to plant extracts incorporated in sucrose solutions.

Exposure of sand flies extracts incorporated in sucrose solutions revealed that the extracts were insecticidal to sand flies although this technique was slow in action than the contact (filter) method. *Tagetes minuta* extracts were found to be more efficacious than other extracts. *Tarchonanthus camphoratus* extract had the least activity with female mortality of about 6% (Figure 11).

4.2.1 Male and female *P. duboscqi* response to plant extracts determined using feeding techniques

After exposure to extracts there was no significant mortality difference between male and female sand flies in their response ($F = 0.00$, $P = 0.929$). Mean mortality after 48 hours for female flies was 8.875 ± 1.228 and that of male flies was 8.719 ± 1.228. Both female and male sand flies had similar rates of mortality in the same concentration levels of the extracts used. Sand fly mortality significantly differed at various concentrations tested on the adults ($F = 27.2$, $P < 0.05$, Coefficient of Variation = 52.2 %). Highest mortality was noted in concentration 10 mg/ml (mean 15.5 ± 1.149). Concentration of 5 mg/ml had a mean of 10.88 ± 1.149; 2.5 mg/ml had a mean of 7.563 ± 1.149.

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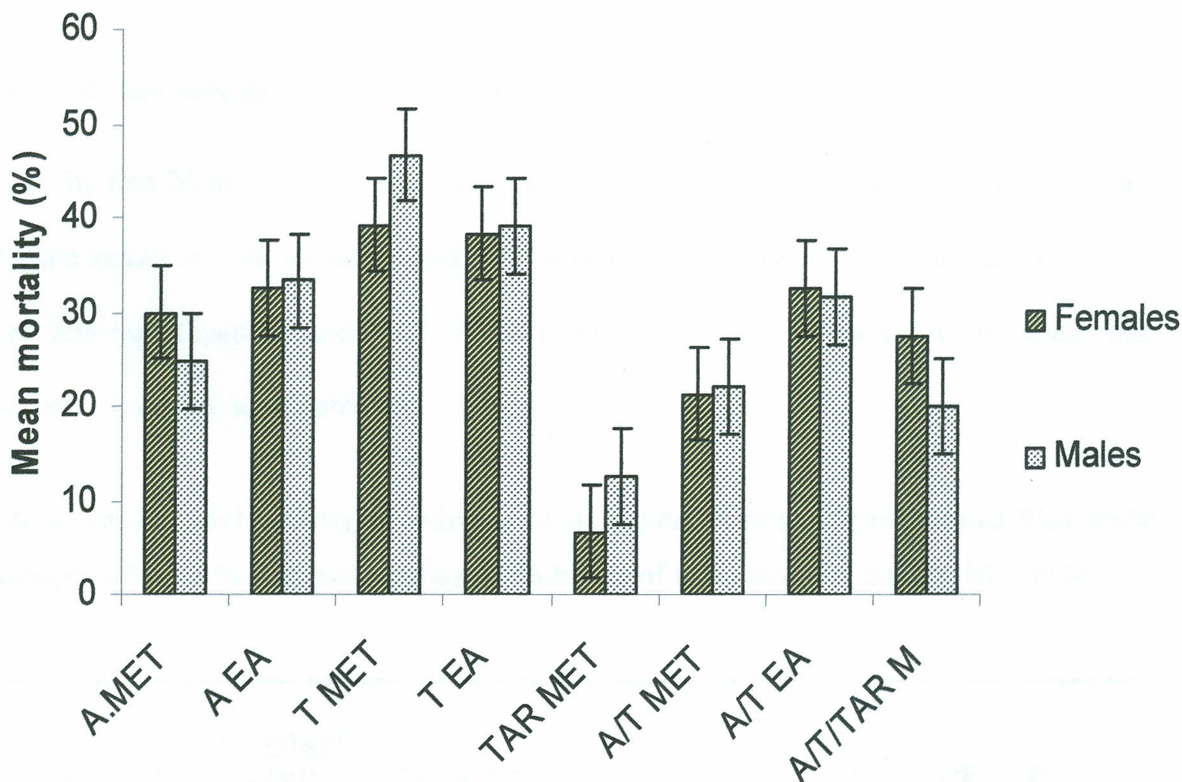

Figure; 11; Mean mortality of sand flies exposed to different extracts in sucrose solutions at 48 hours of exposure

Legend

A. MET= *Acalypha* methanol, A. EA= *Acalypha* ethyl acetate

T. MET= *Tagetes* methanol, T. EA= Tagetes ethyl acetate

TAR. MET=*Tarchonanthus* methanol, A/T. MET= *Acalypha/Tagetes* methanol

A/T. EA= *Acalypha/Tagetes* ethyl acetate,

A/ T/TAR. M= *Acalypha/Tagetes/Tarchonanthus* methanol

4.2.2 To determine the response of *P. duboscqi* to extracts of *A. fruticosa.*

Adult sand flies were fed on various concentrations of the extract in 10% sucrose solution.

During the first 24 hours of exposure, there was no significant mortality ($P > 0.05$) in female and male assays in both methanol and ethyl acetate extracts and in the concentrations used. There was significant mortality ($P < 0.05$) during 48 hours of exposure for males and females for both extracts (Table 4).

Table 4; Insecticidal activity of extracts of *Acalypha fruticosa* against sand flies while incorporated in 10% sucrose solution at 48 hours of exposure. F= female, M= male

Extract	sex	Treatment (mg/ml)	% Mortality	SE	χ^2	LD_{50}[1]	DF	*P*
Methanol	F	0	0.0					
		2.5	20.0					
		5	30.0					
		10	70.0	0.02	22.0	11.2	5	0.001
	M	0	0.0					
		2.5	16.7					
		5	40.0					
		10	43.3	0.014	17.5	15.5	5	0.004
Ethyl acetate	F	0	0.0					
		2.5	40.0					
		5	40.0					
		10	50.0	0.015	20.9	10.6	5	0.001
	M	0	3.3					
		2.5	30.0					
		5	46.7					
		10	53.3	0.04	22.8	12.4	5	0.001

[1]LD_{50} – expressed in mg/ml

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4.2.3 Response of *P. duboscqi* to *T. minuta* extracts incorporated in sucrose solution.

Adult *P. duboscqi* were fed on various concentrations. Both methanol and ethyl acetate extracts gave significant mortality ($P < 0.05$) in the concentrations used for both male and female bioassay. The LD_{50} were 9.9 and 12 mg/ml in the methanol extract for male and female respectively at 48 hours of exposure. Similarly the LD_{50} in the ethyl acetate assay were 10.5 and 10.6 mg/ml for male and female assays respectively. The χ^2 values obtained were significantly high for these extracts (Table 5).

Table 5; Insecticidal activity of the extracts of *T. minuta* against sand flies while incorporated in 10% sucrose solution at 48 hours of exposure. F= female, M= male

Extract	sex	Treatment (mg/ml)	% Mortality	SE	χ^2	LD_{50}[1]	DF	*P*
Methanol	F	0	6.6					
		2.5	30.0					
		5	53.3					
		10	66.7	0.01	13.3	12.0	5	0.021
	M	0	3.3					
		2.5	53.3					
		5	63.3					
		10	66.7	0.01	26.3	9.9	5	0.00
Ethyl acetate	F	0	0.0					
		2.5	46.7					
		5	33.3					
		10	73.3	0.01	31.2	10.64	5	0.00
	M	0	9.9					
		2.5	33.3					
		5	53.3					
		10	60.0	0.01	17.4	10.5	5	0.004

[1]LD_{50} – expressed in mg/ml

4.2.4 Response of *P. duboscqi* to *T. camphoratus* extracts.

There was no significant mortality ($P > 0.05$) in the methanol extract bioassays of this plant extract in 48 hours of exposure. The LD_{50} values were high symbolizing weaker properties than for those other extracts. The highest mortality was realized in the male bioassay which was 20 % (Table 6).

Table 6; Insecticidal activity of methanol extract of *T. camphoratus* against sand flies when incorporated in 10% sucrose solution at 48 and hours of exposure. F= female, M= male

Extract	sex	Treatment (mg/ml)	% Mortality	SE	χ^2	LD_{50}[1]	DF	P
Methanol	F	0	3.3					
		2.5	3.3					
		5	6.7					
		10	13.3	0.02	3.6	34.4	5	0.61
	M	0	3.3					
		2.5	13.3					
		5	13.3					
		10	20.0	0.02	3.85	37.7	5	0.57

[1]LD_{50} – expressed in mg/ml

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4.2.5 Response of *P. duboscqi to A. fruticosa* and *T. minuta* extracts.

Female and male mortality rates in the combined methanol extracts of the two plants had significant ($P < 0.05$) values at 48 hours post treatment. However, these combined extracts had lower mortality values than those of individual extracts (Table 7).

Table 7; Mortality of sand flies when exposed to combined extracts of *T. minuta and A. fruticosa* incorporated in 10% sucrose solution at 48 hours of exposure. F= female, M= male

Extract	sex	Treatment (mg/ml)	% Mortality	SE	χ^2	LD_{50}[1]	DF	*P*
Methanol	F	0	0.0					
		2.5	20.0					
		5	40.0					
		10	25.0	0.02	25.3	9.8	5	0.0
	M	0	3.3					
		2.5	20.0					
		5	40.0					
		10	25.0	0.02	40.1	10.9	5	0.0
Ethyl acetate	F	0	0.0					
		2.5	36.7					
		5	46.7					
		10	46.7	0.02	13.3	12.0	5	0.021
	M	0	6.6					
		2.5	36.7					
		5	33.3					
		10	50.0	0.02	14.9	14.7	5	0.01

[1]LD_{50} – expressed in mg/ml

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4.2.6 Response of *P. duboscqi* to *A. fruticosa, T. minuta* and *T. camphoratus* combined extracts.

There was no significant mortality ($P > 0.05$) obtained from the bioassays on the combination of the methanol extracts of the three plants at 48 hours of exposure. Similarly these combined extracts had weaker insecticidal properties than individual extracts although mortality at 10mg/ml was 46.7 % (Table 8).

Table 8; Insecticidal activity of *A. fruticosa, T. minuta* and *T. camphoratus* combined methanol extracts incorporated in 10% sucrose solution against sand flies at 48 hours of exposure. F= female, M= male

Extract	sex	Treatment (mg/ml)	% Mortality	SE	χ^2	LD_{50}[1]	DF	*P*
Methanol	F	0	6.6					
		2.5	23.3					
		5	33.3					
		10	46.7	0.02	3.21	28.0	5	0.42
	M	0	0.0					
		2.5	20.0					
		5	26.7					
		10	33.3	0.01	5.9	26.4	5	0.32

[1]LD_{50} – expressed in mg/ml

4.3 Effects of pyrethrin (Pymos™) in 10% sucrose solutions on adult *P.duboscqi*

When the sand flies were subjected to pyrethrin in 10% sucrose both male and female flies had significant (P> 0.05) mortality after feeding on the solutions. However, there was no significant mortality difference between male and female assays (F = 0.1, P = 0.812). Males recorded mean of 59.13 % ± 6.396 while female had a mean of 59.97% ± 6.396. The concentration of 0.5 mg/ml had 100 % mortality to both males and females while the lowest concentration had 35 and 53 % for male and females respectively (Figure 12). Females had χ^2 value of 19.8, LD_{50}= 0.1; Males χ^2 as 1.13 and LD_{50} of 0.1 mg/ml. In 48 hours of exposure to pyrethrin incorporated in 10% sucrose solutions, only females had significant mortality (*P* = 0.001) while males had no significant mortality (*P*= 0.95) (Table 9).

Table 9; Insecticidal activity of different concentrations of pyrethrin in 10% sucrose solution when fed to sand flies in 48 hours of exposure. F= female, M= male.

Extract	sex	Treatment (mg/ml)	% Mortality	SE	χ^2	LD_{50}[1]	DF	*P*
Pyrethrin	F	0	0.0					
		0.08	53.3	1.655	19.85	0.10	5	0.001
		0.25	86.6					
		0.5	100.0					
	M	0	3.3					
		0.08	36.6	2.064	1.132	0.112	5	0.951
		0.25	96.6					
		0.5	100.0					

[1]LD_{50} – expressed in mg/ml

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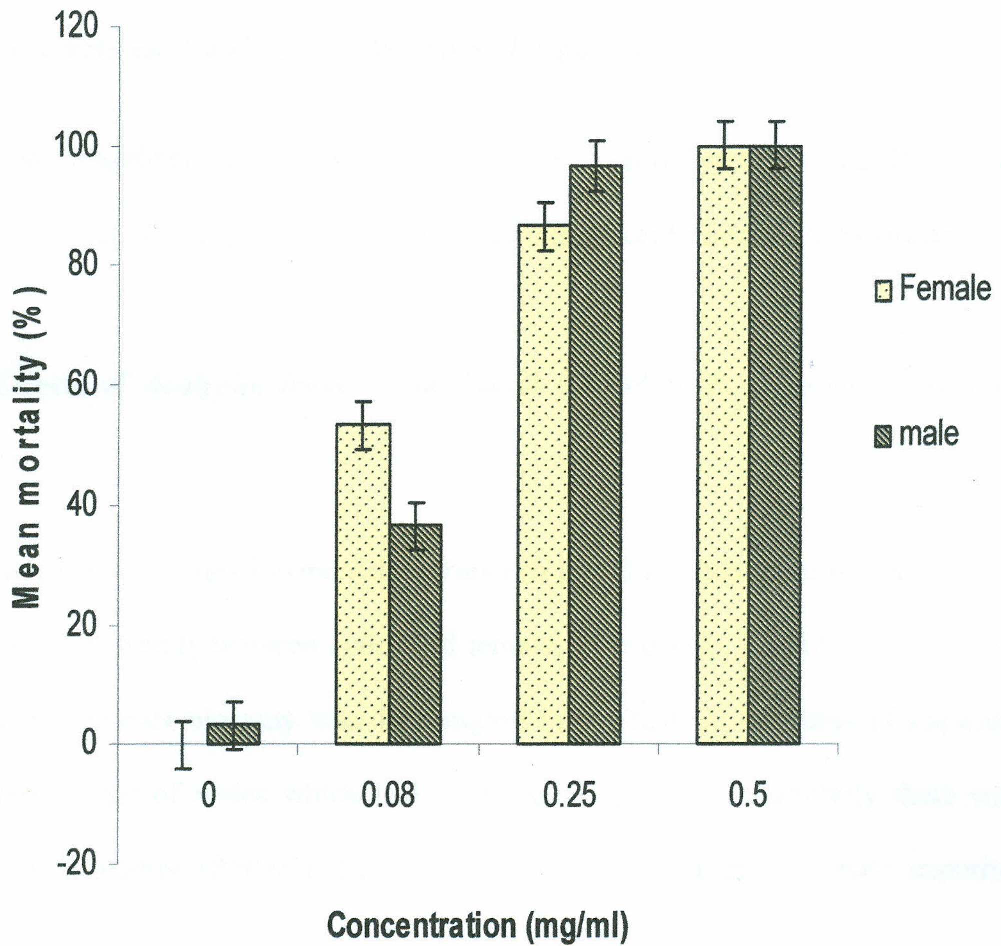

Figure 12: Male and female mortality due to various concentrations of pyrethrin (Pymos™) fed while in 10% sucrose solution at 48 hours of exposure.

4.4 Comparative effects of *A fruticosa, T. minuta* and *T. camphoratus* methanolic extracts on *P. duboscqi* in 48 hours of exposure

This study established that there was no significant mortality difference between male and female assays (P=0.473) after feeding on various concentrations of the extracts.

4.4.1 Comparative effects of *A. fruticosa, T. minuta* and *T. camphoratus* ethyl acetate extracts on *P. duboscqi* in 48 hours of exposure

This study established that there was no significant mortality difference (P = 0.483) in male and female bioassays after feeding on various concentrations of the extracts.

4.4.2 Effects of *Acalypha fruticosa* methanol extract to *P. duboscqi* at 48 hours of exposure

Bioassays using methanol extracted *A. fruticosa* found out that there was no significant difference in mortality between males and females (P = 0.912). The LD_{50} for the females in *Acalypha* extract bioassay was 11.2 mg/ml (χ^2 = 22.0) at 48 hours of exposure as compared to that of males which had 15.5 mg/ml (χ^2= 17.5). Similarly there was no significant mortality (P>0.05) difference was observed in ethyl acetate experiments (Table 10)

4.4.3 Effects of *Tagetes minuta* methanol extract to *P. duboscqi* at 48 hours of exposure

There was no significant difference in mortality between males and females (P = 0.914) bioassays nonetheless, there was significant mortality difference in separate male and female assays. The LD_{50} for male and female assays were 9.90 and 12.0 mg/ml; χ^2- 26.3 and 13.3 respectively. This extract was found to be more efficacious than other extracts and had lower LD50 values than other extracts (Table 10).

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4.4.4 Effects of *Tarchonanthus camphoratus* methanol extract to adult *P. duboscqi*

Similarly there was no significant difference in mortality between males and females ($P = 0.592$) in this experiment. In this bioassay, the LD_{50} for females at 48 hours of exposure to this extract was 49.9 mg/ml (χ^2= 3.09) while males had a LD_{50} of 24.5mg/ml (χ^2= 7.19). These were higher values than those obtained from other extracts therefore showing weaker properties (Table 10).

4.4.5 Effects of *Acalypha* and *Tagetes* combined methanol extracts to adult *P. duboscqi*

When combined methanol extracts of *Acalypha* and *Tagetes* were used in the bioassay, the study established that there was no significant mortality difference ($P = 0.592$) in both sexes. Exposure to these combined extracts yielded LD_{50} for the female sand flies at 48 hours of exposure as 9.47 mg/ml (χ^2= 23.8) while males had a LD_{50} of 9.93 mg/ml (χ^2= 27.1) (Table 10)

Table 10; Response of male and female *P. duboscqi* sand flies exposed to extracts incorporated in 10% sucrose solutions at 48 hours of exposure. N= 30

Plant	Extract	Sex	LD50[1]	X^2	*P*
T. minuta	Methanol	male	9.90	26.3	0.051
		Female	12.0	13.3	0.021
	Ethyl acetate	male	10.5	17.4	0.004
		Female	10.6	31.2	0.001
A. fruticosa	methanol	male	15.5	17.5	0.004
		Female	11.2	22.0	0.004
	Ethyl acetate	Male	12.4	21.4	0.002
		Female	10.6	20.0	0.001
T. camphoratus	Methanol	male	37.7	3.85	0.571
		Female	34.4	3.58	0.612
Acalypha/Tagetes	Methanol	male	10.9	40.1	0.001
		Female	9.76	25.3	0.001
	Ethyl acetate	Male	14.7	14.9	0.011
		Female	12.0	13.3	0.021
Acalypha/Tagetes/ Tarchonanthus	methanol	male	26.4	5.89	0.317
		Female	28.0	3.21	0.420

[1]LD_{50}=mg/ml; sex variation F= 0.00, $P>0.05$; Concentration variation F= 27.2, $P<0.05$

4.5 Response *P. duboscqi* larvae fed to whole plant powder and crude extracts

The plant extracts were tested against all the stages of the sand fly larvae and with the stock solutions of 40mg/ml and no mortality was recorded. There were no abnormal behaviors and the larvae fed normally and developed into pupa. The larvae were also fed on plain powdered plant parts and without any larvae food mixture. The only effect observed was stunting of the instars and time taken from one instar to the other and pupation increased between two and three days. Adult sand flies with the same morphological features and size as those in the main insectary later emerged.

4.6 Response of larvae after feeding on pyrethrin mixed with larval food

4.6.1 Mortality of *P. duboscqi* larvae due to Pylarvex™ in food

Larval mortality did not significantly differ in the various stages of the instars ($F = 0.1$, $P = 0.962$). More mortality were however noted in first instar larvae (mean 20.25 ± 6.664) while second instars had a mean of 15.5 ± 6.664, third instars 19.00 ± 6.664 and fourth instars mean was 19.00 ± 6.664 (Figure 13). Probit analysis showed χ^2 values as 15.1, 18.7, 44.3 and 88.7 for first, second, third and fourth instar larvae respectively. LD_{50} values for the instars were 0.19, 0.29, 0.14 and 0.18mg/ml for first, second, third and fourth instar larvae respectively.

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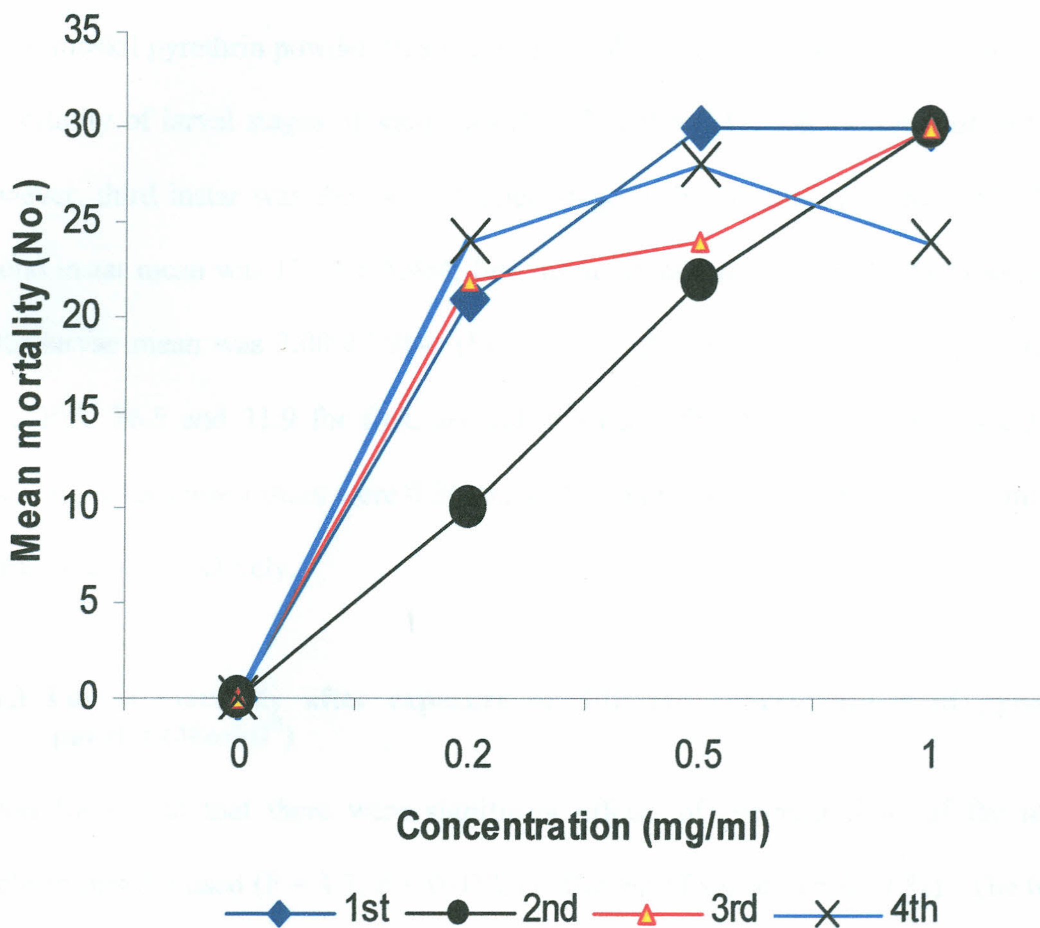

Figure 13: Larval mortality after feeding on pyrethrin (Pylarvex™) incorporated in larval food at 48 hours of exposure

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4.6.2 Mortality of *P. duboscqi* larvae due to Moskil® pyrethrin powder

Using moskil pyrethrin powder, this study established there was no significant difference in mortality of larval stages of sand flies (F = 2.0, P = 0.173) at 48 hours of exposure. However, third instar was the most affected stage of larvae (mean 21.00 ± 5.964), the second instar mean was 19.75 ± 5.964, first instar larvae (mean 17.75 ±5.964) and fourth instar larvae mean was 3.00 ±5.964) (Figure 14). Probit analysis showed χ^2 values as 16.5, 40.3, 26.5 and 31.9 for first, second, third and fourth instar larvae respectively. LD_{50} values for these instars were 0.26, 0.23, 0.1 and 0.1mg/g for first, second, third and fourth instars respectively.

4.6.3 Larval mortality after exposure to different concentrations of pyrethrin powder (Moskil®)

It was found out that there were significant effects of concentrations of the moskil pyrethrin powder used (F = 4.7, P = 0.022, coefficient of variation= 64.4 %). The higher the concentration the more larval mortality was experienced although fourth instar experience no mortality in 0.5mg/g assay. Concentration of 1.0 mg/ml had an overall mean of 24.5 ± 4.951; 0.5 mg/ml had a mean mortality of 19.75 ± 4.951, 0.2mg/ml (mean mortality 17.25 ± 4.951) and control mean mortality of 0.0.

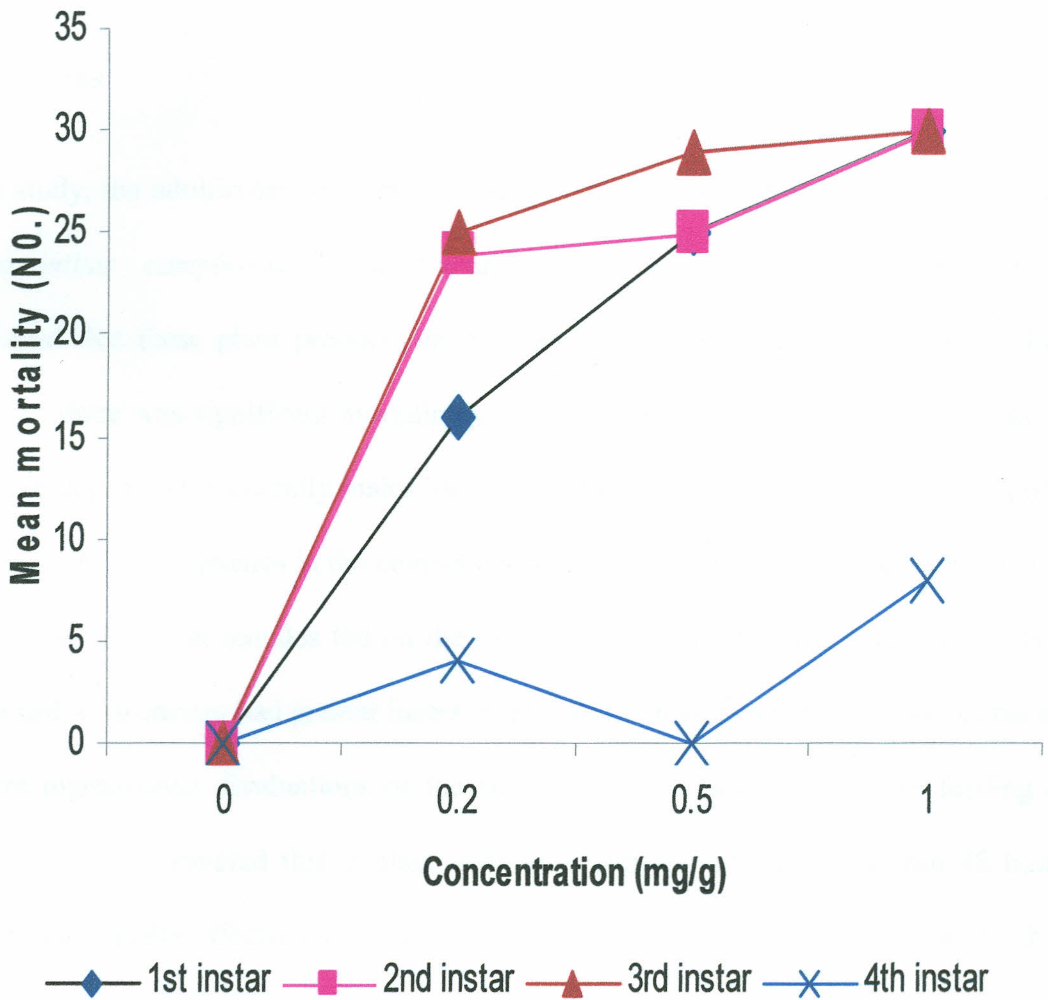

Figure 14: Larval mean mortality after exposure to pyrethrin powder (Moskil®) incorporated in larval food at 48hours of exposure.

CHAPTER FIVE

5 Discussions

In this study, the adulticidal and larvicidal activities of *Tagetes minuta, Acalypha fruticosa, Tarchonanthus camphoratus* extracts and pyrethrin against *Phlebotomus duboscqi* established that these plant products are potential insecticides against sand flies. Results show that there was significant mortality ($P< 0.05$) in both male and female bioassays and were dose dependent. Generally males had lower LD_{50} values portraying quicker response to the extracts than the females in the contact and vice versa in the feeding methods. This could be due to the fact that females fed on the extracts frequently than the males. Extracts of *T. minuta* and *A. fruticosa* had greater insecticidal activity than those of *T. camphoratus* due to different ingredients. Evaluations of the filter techniques with the sucrose feeding of *A. fruticosa* extracts revealed that contact method is superior to feeding within 48 hours of exposure but equally effective. However both techniques gave 90-100% mortality by the 96th hours in 5mg/ml and above. Data on insecticidal evaluation on the above plant to sand flies is not fully understood. Observations on the efficacy of these plant extracts as potential insecticides are scanty. However Bekalo *et al,* (1996) noted that *A. fruticosa* has been used extensively for its medicinal value, food, fodder and repellent activities against insects. Dried leaves can be powdered, soaked in water and the solution applied on animal skin and wound as a repellent or insecticide against ectoparasites and flies (Bekalo *et al.,* 1996). This could explain the reaction of the sand flies to the extracts probably due to insecticidal properties exhibited in the present study.

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Although *T. minuta* is perceived to have insecticidal activities, its action against phlebotomine sand flies is still not fully understood. In this study, its activities against male and female sand flies caused significant mortality ($P<0.05$). Filter methods were more superior than feeding at 48 hours post exposure and with lower LD_{50} values. *Tagetes* species essential oil has been described by other research groups as having insecticidal properties and some of its active derivatives have been identified. Macedo *et al,* (1997) described crude extracts from *T. minuta* aerial parts as effective larvicides to mosquito larvae with LC_{50} and LC_{90} of 1.5mg/l and 1.0mg/l respectively. Seyoun *et al,* (2002) also reported repellent activity of *Tagetes* species against *Anopheles gambiae*, the vector for malaria. Sarin, (2004) successfully evaluated *Tagetes spp* against stored product pests and Cestari *et al,* (2004) evaluated the potential of 100 ppm of *T. minuta* essential oil against head rice *Pediculus humanus capitis* (Phthiraptera: Pediculidae) and achieved a lethal time (LT_{50}) of 16.4 ±1.62 minutes denoting toxicity of the essential oil. According to Perich *et al*, (1995) the *T. minuta* oil essential terpenes were responsible for the toxic effects reported in dipterans and possibly in the present study. Brown, (1995) reported that dried plants can be hung indoors as insect repellents.

The hexane, ethyl acetate and dichloromethane extracts of *T. camphoratus* were not insecticidal although the methanol extract showed some activity but inferior to those of *Tagetes minuta* and *Acalypha fruticosa*. *T. camphoratus*, a semi-deciduous shrub that is 6m in height and is widespread especially in the stony sites in the Rift Valley of Kenya has been shown to have repellant activities and medicinal uses (Bishay *et al.,* 2002). De stefanis, (1924) observed that distilled leaves of the plant yielded compounds with insecticidal

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activities and a spray lotion containing 3% *Tarchonanthus* essential oil was shown to be protective against mosquitoes. observed that wild animals were observed to browse the leaves in south Africa to keep off biting insects (Van Wyk and Van Wyk, 1997).

Although pyrethrum is the oldest insecticide known to man and well recognized for its low mammalian toxicity and non-persistence in the environment, it has only been used routinely to treat materials such as curtains and bed nets, and as indoor residual sprays (Rozendaal, 1997). Pyrethrin resistance has been reported in about fifteen insect species and is caused by repeated exposure to synthetic insecticides including pyrethroids (Cox, 2002). The result of the evaluation of pyrethrin in sucrose against sand flies in this study reveals promising insight into a novel vector control strategy. Under laboratory conditions, *P. duboscqi* males and females readily fed on weak mixture of pyrethrin and sucrose solutions with significant mortality. Despite the very low concentrations used in this study, mortality was higher than those obtained from the plant extract bioassays during 48 hours of exposure depicting greater potency than those of the extracts. The LD_{50} for both males and females were appreciably low. There are still no records available on the use of pyrethrin products in arthropod feeding trials in available literature. The study described here was based on the hypothesis that vectors feed on plant secretions, juice and nectar and may therefore feed on the solutions used. This was confirmed and is in agreement with other previous studies. In a similar study conducted by Leon *et al*, (1997), it was shown that sand flies could feed on aqueous sucrose solution containing a larval toxicant *Bacillus sphaericus* Neide, when sprayed on vegetation cover near burrows and termite hills. Schlein *et al*, (2001) demonstrated that sand flies could feed on noxious plants juice but had their lifespan reduced considerably. Although in the

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past plant extracts have been used in phlebotomine sand fly control, the present observations revealed that none has been evaluated while incorporated in sugars as baits in feeding bioassays. These approaches may be useful for the application of these plant extracts as control agents against phlebotomine sand flies especially those that are exophilic and exophagic, not usually targeted by residual indoor spraying.

In similar studies on other plants, it has been observed that *Derris amazonica* and *Antonia ovata* had insecticidal effects on *Lutzomyia longipalpis* Lutz and Neiva, the vector for *Leishmania chagasi* in Brazil (Luitgards-Moura *et al.*, 2002). In comparison, the plant extracts used in this study are more effective since the working concentrations in that study were as high as 250mg/ml while the highest in this study were 10mg/ml. This could be attributed to the composition of the active products and the sand fly species used. Rojas *et al*, (1991) noted that application of essential lemon oil to human skin was 70% protective against sand fly bites. Bioassays have revealed that 2 % neem oil mixed in coconut or mustard oil offered 100% protection against *P. argentipes* (Sharma *et al.*, 1993).

No abnormal behavior, feeding patterns or mortality of larvae were observed after feeding on the extracts and plain powdered plant parts. Larvae were observed to feed pretty well since there was plenty of frass (waste material) in the experimental vials (Mascari *et al.,* 2007). The only effect observed was stunting of the instars and time taken from one instar to the other increased between two and three days, but gradually pupated and adult flies consequently emerged signifying that they were not larvicidal. Due to limited information on vector competence, seasonal abundance patterns, species association, systematics and evolution

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designing new control strategies may entail rigorous studies to embrace the current trends as noted by Ngumbi *et al*, (1998).

The results obtained in the evaluation of pyrethrum product showed that first instar larvae were more susceptible to Pylarvex™ than Moskil®. There was no mortality of fourth instars in the first 24 hours in the 0.2% pyrethrin bioassays. Fourth instar larvae fed less avidly than second and third instars. The behaviour of fourth instar larvae could be attributed to the fact that they were able to withstand long hours without feeding in preparation to undergo pupation or other factors not tested here. Other instars had significant mortality ($P<0.05$) in both pyrethrin-larvae food preparations at 48 hours of exposure. Pylarvex™ mixtures were more effective than those of moskil® in 24 hours of exposure. This could be attributed to permeability and homogeneity of the solutions in the larvae food as compared to the physical mixing of the powdered formulation and the larvae food. In all the bioassays, all the larval instars succumbed to the preparations before molting to the next stage; hence pupal stage was never realized. The larvae showed evident physiological changes twenty four hours after feeding on the food mixtures. These included gradual shortening, changing from translucent to opaque, looking decrepit and scarcely feeding until they died, depicting the potential of pyrethrin combination with other materials to control sand fly larvae. Use of such material could form an efficient means of controlling sand flies by exploiting their habitat, concomitantly rendering them inhospitable as witnessed by Killick- Kendrick, (1980) and Ghosh *et al*, (1999).

CHAPTER SIX

6 Conclusions and recommendations

6.1 Conclusions

1. Extracts of *Acalypha fruticosa, Tagetes minuta* and *Tarchonanthus camphoratus* [10mg/ml and pyrethrin 0.08 mg/ml] had insecticidal activity against adult *Phlebotomus duboscqi.*

2. *A. fruticosa, T. minuta* and *T. camphoratus* extracts were not effective against *P. duboscqi* larvae.

3. Pyrethrin was found to be a potent larvicide when incorporated in larval food.

4. There were no synergistic effects of *A. fruticosa, T. minuta* and *T. camphoratus* extracts against adult sand flies

6.2 Recommendations

1. Extracts of *Acalypha fruticosa, Tagetes minuta* and *Tarchonanthus camphoratus* should be harnessed and made into suitable insecticides for the control of sand flies

2. Pyrethrum products showed potent insecticidal properties and should be formulated into fine powder, incorporated into dry powdered plant parts or animal waste products as food baits and blown into breeding sites, and also design ideal concentrates for use as formulations for spraying on substrates as sugar baits.

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3 Whereas the synergistic effects of the plant extracts was not shown, further work is necessary in order to investigate possible effects of these extracts on the vector capacity to support parasite proliferation, fecundity and the after-effects on the adults and possible interference in developmental time of immature stages in nature.

4 Further work should be done and evaluated on the plant extract-sucrose combinations as baits in the sand fly natural habitats to design possible simpler application techniques since both males and females feed on sugar, and their food search is limited to local fauna and flora.

5 Studies are needed to establish the repellent effects of these extracts for complementing other personal protection measures since chemical impregnated clothing cannot offer 100% protection against leishmaniasis in the field conditions.

6 Studies should be extended to other medicinal plants in a bid to identify more potent extracts in sand fly control since botanical insecticides remain the only ideal option if aggressively harnessed as has been discussed and advocated in this study.

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APPENDICES

Appendix 1.

The rotary evaporator used in the vacuum evaporation and drying of the plant extracts at 30- 35°C, at the CTMDR laboratory, KEMRI

Appendix 2

Sand fly larval food preparation

1. Weigh and mix equal amounts of rabbit chow and dried feaces.
2. Grind into fine powder and spread the powder in a layer of 3-4 cm deep in a large plastic tray.
3. Thoroughly wet the mixture with tap water and cover it tightly in a plastic paper bag to prevent drying.
4. Keep the mixture at ambient temperature for 4-6 weeks to age, while stirring up 2-3 times per week to aerate and disrupt fungal growth.
5. Several tablespoons of beef liver powder may be added to enrich it.
6. The mixture is allowed to dry, sterilized by autoclaving and kept at room temperature until required for use.

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Appendix 3

A- Experimental jars used in the adult sand fly feeding and filter paper techniques. **B-** Larval vial used in the larvae bioassays. Their bases were filled with plaster of Paris and wet with distilled water to maintain optimum relative humidity of 80-95%.

A

B

Appendix 4.

Anthrone reagent preparation, application and principle

(I) Preparation and application of Anthrone solution

Procedure:

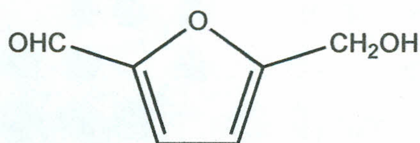
1) Pour carefully, and while cooling, 350 ml. concentrated sulfuric acid into 150 ml. distilled water
2) Mix 150 mg anthrone powder with the diluted sulfuric acid (anthrone reagent). The prepared anthrone reagent should be kept in a refrigerator after use and is usually stable for many weeks.

(II) The sugar test:

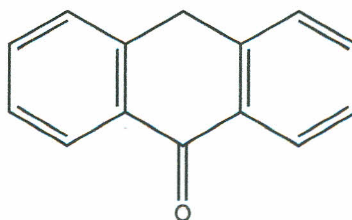
1) Remove and place the sand fly gut in a test tube or ELISA micro wells.
2) Add 0.5 ml anthrone reagent and crush the gut with a glass rod or plastic grinder. Alternatively each sand fly gut may be treated with a drop of chloroform-methanol mixture ratio of 1: 1. This removes the wax to allow easy penetration of anthrone reagent.
3) The specimens are stood for about one hour. The reagent will turn green or blue at room temperature depending on the amount of sugar present. Any specimen that may not have changed its yellow colour after one hour will be considered negative for sugar.

(III) The anthrone Principle

The anthrone test can be used for the qualitative and quantitative estimation of polysaccharides as well as monosaccharides. The test is highly sensitive (Van Handel 1972). It is based on the dehydration of monosaccharides to furfural derivatives, e.g. hydroxymethylfurfural. Furfural derivatives react with anthrone to form a deep blue-green color (Van Handel, 1985).



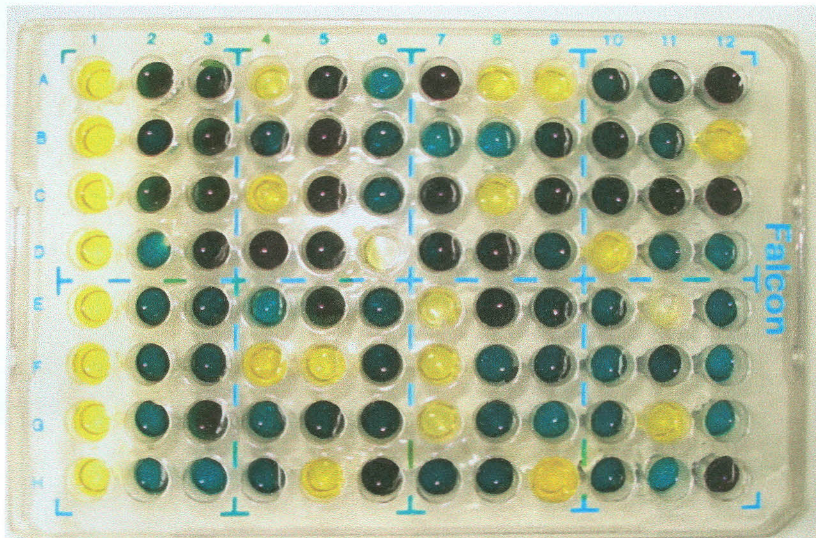
Hydroxymethylfurfural



Anthrone

Appendix 5

Micro well plate as used for testing for evidence of sugar feeding using cold anthrone prepared using Anthrone (Sigma, St. Louis, MO, USA; Lot 13HO404).



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