CHEMICAL CHARACTERIZATION AND THE EFFICACY OF THE ESSENTIAL OILS OF *TAGETES MINUTA* L. AND *CYMBOPOGON CITRATUS* STAPF. AGAINST *PHLEBOTOMUS DUBOSCQI* NEVEU-LEMAIRE

BY

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DECLARATION

This thesis is my original work and has not been presented for a degree in any other University. No part of this thesis may be reproduced without the prior written permission of the author and/or University of Eldoret.

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DEDICATION

This dissertation is dedicated to my late mum Susana Jemosop Sutter who passed away in 2008 after a brave fight with cancer and to my loving spouse Lydia and our children Jerry and Ian.

ABSTRACT

Phlebotomine sandflies transmit leishmaniases, a group of diseases which, the World Health Organization (WHO) estimates that over 2.3 million new cases occur each year and that, at least 12 million people are presently infected worldwide. In Kenya, Turkana, Baringo, Kitui, Machakos, Meru, West Pokot and Elgeyo Marakwet Counties are endemic for the disease with serious debilitating effects and which is spreading fast to new areas. The sandfly *Phlebotomus duboscqi* is a major vector for cutaneous leishmaniasis. The current management strategy for leishmaniasis is mainly chemotherapy of cases and use of insecticides in vector control. However, usage of highly persistent and toxic synthetic insecticides has led to development of resistance in vector populations and environmental pollution. Thus, the harmful side effects of these chemicals on both animals and humans have progressively limited their usage and have led to increased interest in new natural products that are environmentally safe, affordable and effective in leishmaniases control. This study sought to evaluate the chemical composition and the insecticidal activity of the essential oils from *Cymbopogon citratus* Stapf and *Tagetes minuta* L. on the eggs, larval and adult stages of *Phlebotomus duboscqi*. Leaves of *C. citratus* were collected from the equatorial rainforest in Kakamega while the aerial and foliar parts of *T. minuta* were collected from the leishmaniasis endemic focus of Marigat, Baringo County. The essential oils from the two botanicals were extracted by hydro-distillation and then analysed by Gas Chromatography–Mass Spectrometry (GC-MS) to identify the constituent compounds. Each essential oil (EO) extract was tested at graded concentrations of 0.125; 0.250; 0.500; 0.750 and 1mg/ml along with a negative control Tween 80 and a positive control of dimethyl-3-methylbenzamide (DEET) for their ovicidal, larvicidal, adulticidal and repellent activity against *P. duboscqi*. Chemical analysis revealed the presence of a wide range of compounds, including terpenes. The extract from *C. citratus* at 1mg/ml demonstrated significant inhibitory activity of 100% on the hatching of sandfly eggs while. *T. minuta* oil achieved 84.44% inhibition at the same concentration. Both the essential oils of *T. minuta* and *C. citratus* were highly potent against adult sand flies with *C. citratus* and *T. minuta* causing mortality levels of 100.00 and 82.22 % on female sandflies and 100.00 and 88.89 % on male sandflies, respectively at 72 hours post treatment. *C. citratus* was significantly more potent ($P < 0.05$) than *T. minuta* on killing of male and female sandflies. Repellency increased with increasing doses of the essential oils, demonstrated by biting rates which decreased with increasing concentrations of the oils. Further, the oil of *C. citratus* was more potent than that of *T. minuta* with regard to protection time and biting deterrence. The effective doses at 50% (ED_{50}) and at 90% (ED_{90}) for the oil of *C. citratus* were 0.04 and 0.79 mg/ml, respectively while those of *T. minuta* were 0.10 and 12.58mg/ml. In addition, the percentage repellency of *C. citratus* and *T. minuta* against the sandflies was 100% and 88.89%, respectively after 180 minutes exposure. The oils showed concentration dependent larval mortality. However, *C. citratus* EO achieved higher repellency, ovicidal, larvicidal and adulticidal rates than *T. minuta* at all tested concentrations. In conclusion, these findings, together with previous studies by other researchers, lend strong credence to the consideration of *C. citratus* Eos as potentially valuable agents for the control of phlebotomine sandflies at the egg, larval and adult stages. The study further recommends for more investigations to identify the specific active chemical constituents of the extracts of *T. minuta* and *C. citratus* that are responsible for the larvicidal, adulticidal, ovicidal and repellent activities as well as their specific mechanisms of action.

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LIST OF ABBREVIATIONS

CL	-	Cutaneous leishmaniasis
DDT	-	Dichlorodiphenyltrichloroethane
DEPA	-	N-diethyl phenyl acetamide
GC-MS	-	Gas Chromatography–Mass Spectrometry
IGR	-	Insect Growth Regulator
KEMRI	-	Kenya Medical Research Institute
MCL	-	Mucocutaneous leishmaniasis
RH	-	Relative Humidity
USSR	-	Union of the Soviet Socialist Republics
VL	-	Visceral leishmaniasis
WHO	-	World Health Organization

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CHAPTER ONE

INTRODUCTION

1.1 Background information

Phlebotomine sandflies transmit leishmaniases, a group of diseases that puts at risk of disease 350 million people in 88 countries (Desjeux, 2004). In some countries, sandflies also carry and transmit other zoonoses such as bartonellosis (Birtles, 2001), phleboviruses (Ready, 2013; Xu *et. al*. 2007a), certain flaviviruses, orbiviruses and vesiculoviruses (Comer and Tesh, 1991; Ashford, 2001), that cause health problems for humans and domestic animals.

The World Health Organization (WHO, 2015) estimates that over 2.3 million new leishmaniases cases occur each year and that at least 12 million people are presently infected worldwide. Sandflies, which are vectors for several species of *Leishmania*, comprise more than 40 species of *Phlebotomus* in the Old World and 30 *Lutzomyia* species in the Americas (Alexander and Maroli, 2003).

The three major clinical forms of leishmaniases are cutaneous leishmaniasis (CL), mucocutaneous leishmaniasis (MCL) and visceral leishmaniasis (VL), also known as kala-azar (Desjeux, 2004; Murray *et al*., 2005). Visceral leishmaniasis is a life-threatening systemic infection while cutaneous leishmaniasis (CL) is a serious disease characterized by cutaneous lesions that can be self-healing with life-long immunity or chronic when accompanied by defective cellular immune responses (Reithinger *et al*., 2007; Modabber, 2010). On a global scale, there are an estimated 1.0 to 1.5 million cases of CL annually (Desjeux 2004; Reithinger *et al*., 2007). In Kenya, CL has been reported in Baringo (Muigai *et al*., 1987), Kitui (Mutinga *et al*., 1994), Kiambu

(Binhazim *et al*., 1987), and the Rift Valley districts of Laikipia, Samburu, Isiolo, Nakuru and Nyandarua (Sang and Chance, 1993). In Kenya, CL has been shown to be caused by three species of *Leishmania* parasites, *L. major*, *L. Tropica* and *L. aethiopica* (Reviewed in Tonui, 2006). *Leishmania major* is the most prevalent form in many areas including the leishmaniasis endemic focus of Baringo county (Tonui, 2006). The vector for *L. major* has been shown to be *P. duboscqi* (Mutinga *et al*., 1994) while *P. guggisbergi* has since been shown to be the vector for *L. tropica* in Kenya (Lawyer *et al*., 1991; Tonui, 2006).

The WHO (2010) Expert Committee recommended integrated surveillance and control, which has advantages for controlling leishmaniasis, a disease often neglected compared with malaria and Chagas' disease in the same regions. Control of leishmaniases is currently based on chemotherapy to treat infected cases and on vector control to reduce transmission (Tonui, 2006). There are currently no vaccines for leishmaniasis (Murray *et al*., 2005; Modabber, 2010). The drugs available for leishmaniasis treatment are toxic, expensive and effective dosages are administred for long periods of time (Croft and Yardley, 2002; Croft *et al*., 2006), so vector control is crucial in minimizing and/or preventing bites from potentially infectious sand flies.

Vector control using insecticides has been recommended by the World Health Organization (WHO, 2010). Depending on the application techniques, timing and the target species, sandflies are known to be highly susceptible to insecticides (Alexander *et al*., 1995a; Alexander and Maroli, 2003; Wilamowski and Pener, 2003; Orshan *et al.*, 2006). In certain areas, effective control has been achieved as a side-effect of malaria control programmes (Kishore *et. al*., 2006). Residual formulations of DDT

have been used expressly to control sandflies (Hertig and Fisher, 1945; Hertig and Fairchild, 1948; Hertig, 1949), and has demonstrated insecticidal activity against sand flies in Sudan (Hassan *et al*. 2012). The synthetic pyrethroid deltamethrin has been used against sandflies in Bolivia (Le Pont *et al.*, 1989) and Brazil (Bermudez *et al*., 1991; Marcondes and Nascimento, 1993; Courtenay *et al*., 2007). In other countries where sandfly vectors are endophilic, control of leishmaniasis has traditionally been based on residual insecticide spraying in houses, with significant effectiveness (Alexander *et al.*, 1995a; Vieira and Coelho, 1998; Alten *et al*., 2003). Other studies have tested the efficacy of insecticide impregnated textiles, such as curtains, bed nets or bed covers, with varying degrees of success (Alexander *et al.*, 1995a, 1995b; Basimike and Mutinga, 1995; Kroeger *et al*., 2002; Courtenay *et al*., 2007). Environmental modification, involving the total eradication of rodents, destruction of burrow systems and spraying of herbicides to kill their food plants, has been demonstrably effective in controlling CL caused by *L. major* in foci in the Asian republics of the former USSR and in Tunisia (Vioukov, 1987; WHO, 1990).

However, acquired resistance and environmental pollution due to the repeated application of persistent synthetic insecticides have led to increased interest in new natural chemicals (Viegas-Junior, 2003). Other disadvantages include high toxicity and harmful side effects for both animals and humans, and their potential for environmental pollution which have progressively limited their usage (Reviewed in Rogan and Chen, 2005). In this context, screening of natural products for their effectiveness has received the attention of researchers around the world. Since many diseases transmitted by insects including malaria, dengue fever, yellow fever, leishmaniasis and Chaga's disease are endemic in developing countries, the search for

insecticides and repellents of botanical origin in these countries has been driven by the need to find new products that are effective, but also safer and cheaper than current synthetic products (De Paula *et al*., 2004).

In recent decades, research on the interactions between plants and insects has revealed the potential use of plant metabolites or allelochemicals for the control of arthropods that vector diseases (Pavela, 2004). It is known that some chemical constituents of essential oils from various plants have insecticidal properties (Spitzer, 2004). In some studies, essential oils obtained from commercial sources have been used for the control of sand flies (Maciel *et al*., 2010). Specific compounds isolated from plant extracts or essential oils have also been tested for fumigation purposes (Rajendran and Sriranjini, 2008).

The lemon grass, *Cymbopogon citratus* belongs to the family Poaceae is a genus of about 55 species of grasses, native to warm temperate and tropical regions of the Old World and Oceania (Akhila, 2010). In Kenya, lemon grass is native to the Kakamega tropical rainforest (Matasyoh *et al*., 2011). It is a tall perennial grass. Common names include lemon grass, barbed wire grass, silky heads, citronella grass or fever grass amongst others. Lemon grass is commonly used by man in preparing teas, soups, and curries. It is also suitable for poultry, fish, beef, and seafood. It is often used as a tea in African countries such as Togo and the Democratic Republic of Congo and in Latin American countries such as Mexico (Adeniran and Fabiyi, 2012).

Lemongrass essential oil is obtained from the aerial parts of the plant. The plant has been widely recognized for its enthnobotanical and medicinal usefulness (Shah *et al*.,

2011**)**. The insecticidal (Arias *et al*., 1992; Aziz and Abbas, 2010; Kabera *et al*., 2011; Phasomkusolsil and Soonwera, 2011; Pushpanathan *et al*. 2006; Hindumathy, 2011), antifungal (Matasyoh *et al*., 2011), antimicrobial (Syed *et al*., 1995; Akin-Osanaiye *et al*., 2007), and the therapeutic properties (Shah *et al*., 2011) of its oil and extracts are known. Trado-medicinal preparations of the oil have been used both internally for alleviating colds and fever symptoms (Comerford, 1996) and externally to treat skin eruptions, wounds and bruises (Spring, 1989). Plant essential oils in general have been recognized as an important natural source of pesticides–insecticides (Raguraman and Singh, 1997; Gbolade, 2001), larvicides (Adebayo *et al*., 1999; Cavalcanti *et al*., 2004), and repellents (Thorsell *et al*., 1998; Oyedele *et al*., 2002).

Tagetes minuta L., also known as Mexican marigold, is an annual, strongly aromatic herb. The stem is erect, on average of 1m tall, branched and furrowed. Some plants may reach a height of 2m. Leaves are opposite (sometimes alternate on smaller branches). The leaves are up to 5-15cm long, divided into one terminal and several (3-7) lateral leaflets (Wang and Chen, 2006). The plant has been shown to have both larvicidal as well as adulticidal activity against mosquitoes (Green *et al.,* 1991; Perich *et al*., 1995; Macedo *et al*., 1997; Pathak *et al*., 2000). Active components have been isolated from different parts of this plant.

Green *et al*. (1991), reported mosquito larvicidal activity in the extract of *Tagetes minuta* flowers. Perich *et al*. (1995) compared biocidal activities of the whole-plant extracts of three *Tagetes species* and showed that *T. minuta* had the greatest biocidal effect on the larvae and adults of *Aedes aegypti* (L.) and *Anopheles Stephensi* (L). Bioassays of simultaneous steam distillated extracts of *T. minuta* flowers showed

larval mortality at LC90 of 4 and 8 ppm and against the adult at 0.4 and 0.45% against *Aedes aegypti* and *Anopheles stephensi*, respectively (Perich *et al*., 1995). In the study, the extract from *T. minuta* was found to be most active among 83 plant species belonging to the compositae family, with a LC50 of 1mg/l against *Aedes fluviatilis* (Macêdo *et. al*., 1997). Active components of *T. minuta* have also been identified as thiophene derivatives, a class of compounds present in many plants of family Asteraceae (Perich *et al*. 1995). Insecticidal activity of *Tagetes* species against *Anopheles gambiae*, the vector for malaria has also been demonstrated (Seyoun *et al*., 2002). Previously, Ireri *et al*., (2010) demonstrated that the methanol and ethyl acetate crude extracts of *T. minuta* derived from the aerial parts had significant mortality against both males and females *P. duboscqi*, Neveu Lemaire (Diptera: Psychodidae) while Mong'are *et al*. (2012) found that the same crude extracts reduced the fecundity of *P. duboscqi* by 53%.

In light of the above, the present study sought to evaluate the insecticidal effects of lemon grass oil and Mexican marigold oil against sand flies *P. duboscqi*. Specifically the study sought to achieve this by evaluating the essential oils' ovicidal, larvicidal, adulticidal and repellent activities against the flies with a view to recommending them as natural products with long lasting insecticidal activity. These would be safe to humans and animals when applied in their environments or when used as a repellent cream on the skin, thus, providing alternatives to synthetic chemical insecticides and repellents.

1.2 Statement of the problem

The phlebotomine sandflies are of medical and veterinary concern (Pessoa *et al*., 2007; Xu *et al*., 2007a). In particular, they are vectors of *Leishmania* parasites, the causative agents of the leishmaniases, one of the world's most neglected diseases, affecting largely the poorest of the poor, mainly in developing countries. Globally, about 350 million people are considered at risk of contracting leishmaniasis, and some 2 million new cases occur yearly (Desjeux, 2004). In fact, among the protozoan diseases, leishmaniasis is second to malaria (Lawyer and Perkins, 2004). Phlebotomine sand flies are the vectors for several species of *Leishmania*. More than 40 species of *Phlebotomus* species in the Old World and 30 *Lutzomyia* species in the Americas have been implicated to be vectors (Alexander and Maroli, 2003).

The lack of an effective vaccine, the prohibitive cost of treatment and the side effects and difficulties associated with chemoprophylaxis have served to emphasize the importance of vector control for disease prevention. However, control of sandflies remains a difficult problem throughout the world. Although the use of insecticides remains the most effective method of sand fly control, high cost and resistance to synthetic insecticides has proved to be major challenges (Reviewed in Kishore *et al*., 2006). In Kenya, in as early as 1994, the use of bed nets, permethrin impregnated wall cloth, repellents and other personal protective measures had proved to be unreliable (Mutinga *et al*., 1994). In addition, high toxicity and harmful side effects to both animals and humans have progressively limited the use of synthetic insecticides. With these shortcomings, there is need to identify novel natural insecticides against the Phlebotomine flies that are not harmful to man and animals and have no adverse effects on the environment.

1.3 Justification of the study

In the leishmaniasis endemic areas of Baringo in Kenya, resident communities traditionally hang dried herbs of *Tagetes minuta* along the inside of the walls of their huts to repel mosquitoes and other biting insects, including the sandflies, *P. duboscqi.* So far, no work has been done to validate this practice and elucidate the chemical components in this plant that play a role in repelling sand flies and whether its' essential oil has any adverse effects on the various developmental stages of the flies. On the other hand, the lemon grass, *Cymbopogon citratus* essential oil is known to have high antifungal activity (Matasyoh *et al*., 2011), high larvicidal, ovicidal and repellent activities against the filarial transmitting mosquito *Culex quinquefasciatus* **(**Pushpanathan *et al*., 2006; Phasomkusolsil and Soonwera, 2011), *Aedes aegypti* and *Anopheles dirus* (Phasomkusolsil and Soonwera, 2011). The essential oil has also been demonstrated to have very high insecticidal activity against the maize weevil, *Sitophilus zeamais* (Kabera *et al*., 2011) and the cow pea beetle (Aziz and Abbas, 2010). The essential oil has also demonstrated activity against house dust mites (Hanifah *et al*., 2011). However, no studies have been conducted to determine the insecticidal activity of *C. citratus* and *T. minuta* essential oil(s) against *Phlebotomus duboscqi* sandflies. This study therefore sought to investigate the insecticidal activity of the essential oils of *T. minuta* and *C. citratus* against *P. duboscqi* sandflies, by evaluating their ovicidal and larvicidal effects on the developmental stages and adulticidal and their repellent effects on adult flies.

1.4 Overall Objective

To investigate the chemical components and biological activities of *Cymbopogon citratus* and *Tagetes minuta* essential oils against *Phlebotomus duboscqi* sand flies.

1.5 Specific Objectives

1. To identify constituent compounds in extracted essential oils of *T. minuta* and *C. citratus* using Gas Chromatography – Mass Spectrometry (GC –MS) analytical techniques.
2. To determine the ovicidal effects of the essential oils of *T. minuta* and *C. citratus* on the sand fly, *P. duboscqi* eggs' viability by measuring the hatching success of the eggs following treatment;
3. To determine the larvicidal and adulticidal effects of the essential oils of *T. minuta* and *C. citratus* against *P. duboscqi* sandfly by quantifying their mortalities after treatment;
4. To evaluate the effects of the essential oils of *T. minuta* and *C. citratus* on the fecundity of females of the sand fly, *P. duboscqi* by assessing their oviposition success after treatment.
5. To determine the repellent effects and protection time of the essential oils of *T. minuta* and *C. citratus* against adults of the sand fly, *P. duboscqi* by estimating their biting rates and protection periods;

1.6 Research Questions

1. What are the bioactive compounds that are present in the extracted essential oils of *T. minuta* and *C. citratus* that could have insecticidal, ovicidal and/ or repellent activities to the various life stages of the sand fly, *P. duboscqi*?
2. Do the essential oils of *T. minuta* and *C. citratus* have ovicidal activity on the eggs of the sand fly, *P. duboscqi*?

3. Do the essential oils of *T. minuta* and *C. citratus* have larvicidal and adulticidal activities on the sand fly, *P. duboscqi*?
4. Do the essential oils of *T. minuta* and *C. citratus* adversely affect the fecundity of the female *P. duboscqi* sand flies?
5. Do the essential oils of *T. minuta* and *C. citratus* have repellent activity against adult sand fly, *P. duboscqi*?

CHAPTER TWO

LITERATURE REVIEW

2.1 Leishmaniasis

Leishmaniasis is an anthropozoonosis of great public health concern affecting more than 80 countries around the world. The disease, transmitted by phlebotomine sandflies currently puts at risk of disease 350 million people in 88 countries (Desjeux, 2004). The World Health Organization (WHO) estimates that over 2.3 million new leishmaniasis cases occur each year and that at least 12 million people are presently infected worldwide (WHO, 2015). Infection of humans with the *Leishmania* parasite causes a spectrum of clinical manifestations ranging from healing cutaneous leishmaniasis (CL) to mucocutaneous leishmaniasis (MCL) and visceral leishmaniasis (VL) (WHO, 2010). There are an estimated 1.0 to1.5 million cases of CL and half a million new cases of VL annually (Reithinger *et al*., 2007).

In Kenya, leishmaniasis has been known to be endemic in parts of the country from as far back as early in the 20th century (Fendall, 1961). Cutaneous leishmaniasis has been reported in Baringo County (Muigai *et al*., 1987), Kitui (Mutinga *et al*., 1994), Kiambu (Binhazim *et al*., 1987), and the Rift Valley districts of Laikipia, Samburu, Isiolo, Nakuru and Nyandarua (Sang and Chance, 1993). The disease (CL) has been shown to be caused by three species of *Leishmania* parasites; *L. major, L. Tropica* and *L. aethiopica* (Reviewed by Tonui, 2006).

2.2 Phlebotomine Sandflies

The phlebotominae are of medical and veterinary concern as they vector leishmaniasis, bartonellosis and some arboviruses (Pessoa *et al*., 2007), such as

fleboviruses (Xu *et al*., 2007b), flaviviruses, orbiviruses (Traore-Lamizana *et al*., 2001) and vesiculoviruses (Comer and Tesh 1991). The proven vector species for leishmaniasis belong to two genera: *Phlebotomus* in the Old World and *Lutzomyia* in the New World (Killick-Kendrick, 1990; Alexander and Young 1992; Morrison *et al*., 1993). The leishmaniasis vectors are represented by more than 40 species in the Old World and 30 species in the Americas (Alexander and Maroli 2003). In Kenya, *Leishmania major* is transmitted by *P. duboscqi* (Beach *et al*., 1984) while *P. guggisbergi* is the vector for *L. tropica* in Kenya (Lawyer *et al*., 1991; Tonui, 2006).

2.3 Taxonomy of Phlebotomine Sand Flies

2.3.1 Family Psychodidae

Phlebotomine sand flies belong to the family Psychodidae, which is among the most primitive families of Diptera (Young and Duncan 1994). The family Psychodidae is characterized by their wing venation (the presence of numerous parallel veins running to wing margin), and the presence of dense hairs on the wings and thorax (Triplehorn and Johnson, 2005).

2.3.2 Subfamily phlebotominae

Phlebotomine sand flies are differentiated from other subfamilies within Psychodidae by the presence of biting mouthparts that are longer than the head, five-segmented palps, nearly cylindrical antennae, a five-branched radial vein on the wing, and the absence of an eye-bridge (Triplehorn and Johnson, 2005). Some general attributes that can often be used to distinguish sand flies from other small flies include their size (1.5 to 2.5 mm in length), characteristic hopping flight, and the “V” position in which they hold their wings while resting.

2.3.3 Phlebotomine sand fly genera

There are three New World genera within subfamily Phlebotominae: *Brumptomyia* França and Parrot, *Warileya* Hertig, and *Lutzomyia* França (Young and Duncan, 1994). Sand flies in the genus *Brumptomyia* have not been reported -as feeding on humans, and are distinguished from sand flies in other genera by differences in the morphology of male external genitalia (Young and Duncan 1994). Sand flies in the genus *Warileya* are reported to be anthropophilic, but they have not been implicated in the transmission of any human pathogens (Young and Duncan,1994). Sand flies in the genus *Lutzomyia* feed on mammals and are the only medically important genus of sand flies in the New World. *Lutzomyia* is distinguished from *Brumptomyia* by the number of rows of teeth on the cibarium (*Lutzomyia* has 1 row of transverse teeth, *Brumptomyia* has 4 horizontal rows of teeth), and differ from *Warileya* by the presence of episternal (*Lutzomyia* has episternal setae, and *Warileya* does not).

There are two Old World genera within the subfamily Phlebotominae: *Sergentomyia* França and *Phlebotomus* Rondani and Berté (Lewis, 1982). Sand flies in the genus *Sergentomyia* feed primarily on lizards, and may be the vectors of the agents of saurian leishmaniasis. Sand flies of the genus *Phlebotomus* feed on mammals, and represent all of the medically important sand flies in the Old World. Sand flies of the genus *Phlebotomus* can often be distinguished from those within *Sergentomyia* by the cibarium; *Phlebotomus* does not have a row of teeth and usually does not have a patch of pigment (Lewis, 1982).

2.4 Sand fly Biology, Ecology, and Sampling Methods

2.4.1 Immature stages of sand flies

The eggs of phlebotomine sand flies are dark brown or black and elliptical in shape. The eggs have ridges in species-specific patterns that potentially could be used for identification. The number of eggs laid by a single female at one time varies greatly by species and by factors such as species of bloodmeal host or ambient temperature, but typically is between 40 to 70 eggs (Young and Duncan, 1994). Eggs are laid singly on moist surfaces or substrates, and the presence of conspecific eggs can serve as an oviposition attractant and stimulant (Elnaiem and Ward,1991, Kalyanasundaram *et al*., 1994). The hatching of eggs usually occurs within 10 days after oviposition, but hatching of some eggs in a batch is sometimes delayed for as long as 30 days (Young and Duncan,1994).

Sand fly larvae have four instars. Sand fly larvae are covered in setae along the length of their bodies, and have four caudal setae by the time they reach 4^{th} instar. Sand fly larvae feed on organic matter near the site of oviposition. The larval stage of phlebotomine sand flies is completed in approximately 18 days, but typically lasts longer and can be dependent on temperature (Young and Duncan,1994). Before pupation, sand fly larvae cease feeding and some species may travel a short distance upward to a drier location. Pupae sometimes attach to rocks or other fixed objects (Young and Duncan,1994).

The sand fly larval habitats have been identified for only a handful of species. In the Old World, immature stages of *P. argentipes*, *P. martini*, *P. papatasi*, *P. celiae*, *P. ariasi*, *P. perfiliewi*, and *P. langeroni* have been recovered from soil taken from inside

of structures housing humans or domesticated animals (Dhiman *et al.,* 1983; Bettini *et al*., 1986; Killick- Kendrick, 1987; Mutinga *et al.,* 1989; Doha *et al.,* 1990). Larvae of *P. martini*, *P. papatasi*, and *P. duboscqi* have been consistently recovered from soil taken from inside of rodent burrows (Perfil'ev 1968; Artemiev *et al.,* 1972; Dedet *et al.,* 1982; Mutinga *et al.,* 1986; Doha *et al*. 1990; Morsy *et al.,* 1993). Larvae of the sand flies *P. martini* and *P. celiae* have been recovered from termite mounds in East Africa (Mutinga *et al.*, 1989).

In the New World, structures housing livestock have been shown to be a larval habitat for *L. longipalpis* and *L. intermedia* (Forattini, 1954; Deane and Deane, 1957). Larvae of other species, including many of medical importance (including, *L. trapidoi, L. umbratalis, L. anduzei*, and *L. whitmani*), have been found among soil and leaf litter on the forest floor (Rutledge and Ellenwood, 1975).

For many of the species listed above, very few immature specimens have been recovered (Feliciangeli, 2004), and thus little can be stated about the importance of their larval habitats. However, for some species, enough evidence has been compiled to make more definitive conclusions about their larval habitat. The larvae of *P. duboscqi* have been recovered consistently from inside of rodent burrows; this is considered to be the principal larval habitat for this species (Mutinga *et al*., 1989).

The first sand fly larva (*P. mascittii*) recovered in nature was found by direct examination of a soil sample taken from a cellar in Rome (Grassi, 1907). Direct examination of soil to find sand fly larvae was the method used throughout the early 20th century and is still the preferred method of some more recent researchers

(Dhiman *et al.*, 1983). A method of extracting immature sand fly larvae from soil samples though differential flotation in salt or sugar solutions also has been used, but there is no improvement in the rate of success and it is no less labor intensive (McCombie-Young *et al.*, 1926). This method has been modified by combining differential flotation with passing the soil samples through a series of nested sieves, but the modified method still was no simpler or productive than flotation or direct examination (Hanson, 1961). The larvae of *P. papatasi* also have been extracted from soil samples through dessication with some success in Iran (Seyedi- Rashti and Nadim, 1975). This method was validated in the laboratory by extracting larvae from soil samples that had been spiked with larvae from a laboratory colony (Killick-Kendrick, 1987). Breeding sites also have been identified by isolating soil samples and recovering adult sand flies as they emerge either through the incubation of soil samples in the laboratory, or by placing emergence traps over suspected breeding sites in the field (Mutinga and Kamau, 1986; Bettini *et. al.*, 1986).

2.4.2 Adult sand flies

Male adult sand flies typically emerge before females from the same egg batch, and they become sexually mature within 1 day (Young and Duncan, 1994). Male sand flies can find potential mates through the use of pheromones, or by locating vertebrate hosts or resting sites to which female sand flies also may be attracted. Both specific pheromones and wing-beat rhythms have been identified for mate location for the sand fly *L. longipalpis* (Phillips *et al.*, 1986; Ward and Morton,1991).

Adult male and female sand flies obtain energy by ingesting sugars. Sugar meals can be obtained from a variety of sources, including the sap of plants and honeydew from

aphids (Schlein and Warburg 1986; Killick-Kendrick, 1987; Cameron *et al.*, 1995). In arid areas where sand flies are found, the available sources of sugar can be limited to a handful of plant species (Schlein and Yuval, 1987). Female sand flies also are required to feed on the blood of vertebrate hosts for the production of eggs. Females of most species take bloodmeals only once per gonotrophic cycle, though females of some species, such as *L. shannoni*, will feed multiple times throughout the gonotrophic cycle (Young and Duncan, 1994). Because of their characteristic short, hopping flight, sand flies are often perceived as weak fliers unable to travel long distances. For many species this holds true: the longest recorded dispersal distance for a *P. papatasi* sand fly was 280 m. Sand flies in forested areas of the New World also do not have long flight ranges; in one study in Panama in which 20,000 sand flies were marked with fluorescent powder and released, the majority of re-captured sand flies were collected within about 50 m of the release site; four sand flies were recaptured 200 m away (Chaniotis *et al.*, 1974). However, *P. ariasi* sand flies have been shown to fly as far as 2 km in southern France (Killick-Kendrick *et al.*, 1984).

2.5 The sand fly leishmaniases vector

The only known vector of the leishmaniases is the small dipteran fly known commonly as a "sand fly." The subfamily Phlebotominae is comprised of the bloodsucking sand fly vectors of leishmaniasis and other diseases, including bartonellosis (Carrion's disease), phlebotomus fever (sand fly fever) and vesicular stomatitis (Tesh, 1988). Like all true flies (Order: Diptera), sand flies undergo complete metamorphosis and exhibit four complete life stages: egg, larva, pupa and the adult. Sand fly eggs are laid in a suitable habitat by the female adults. They are initially white or light gray in color but often turn dark brown or black within a few

hours of oviposition, depending on the species. They are banana-shaped and nearly microscopic in size (0.3 to 0.5mm in length). Time-to-hatch is highly temperature dependent but averages 6 to17 days. The eggs are usually laid in a mass of high organic content, like animal excreta and soil, providing the newly emerged larvae with shelter, moisture and nutrition (Claborn, 2009).

Larvae are caterpillar-shaped with head capsules and small leaf-like antennae. Distinctive caudal setae can help identify the larvae as sand flies, but larvae are rarely used in taxonomy because very few are ever collected in nature (Lawyer and Perkins, 2004). There are four larval instars ranging in size from 0.55 mm long in the 1st to about 3.2 mm long in the 4th. The 1st instar larvae usually have two long caudal setae, but the 2nd instar larvae have 4 caudal setae upon molting. The larvae move very little distance from the oviposition site.

Pupae resemble a small butterfly chrysalis except that the 4th stage larval exuvium (cast-off exoskeleton) is attached at one end. The exuvium acts as glue which is attached to a solid substrate and holds the pupa upright (Lawyer and Perkins, 2004).
Adult sand flies are about one-third the length of a small mosquito, usually less than 3.5 mm in length. They are covered with dense hairs and hold their wings in a characteristic "V" shape over their backs when at rest. The wing veins are parallel to each other and have numerous small "hairs." The eyes are large and dark. The antennae are long and filiform, with 16 segments. Mouthparts are short, dagger-shaped and oriented downward. The thorax is distinctively humped, pushing the head below the upper surface of the thorax. The legs are very long and delicate. Both female and male sand fly adults obtain carbohydrate nutrition from plant juices;

however, most females also require at least one blood meal in order to complete development of egg batches. Some are autogenous (Harwood and James, 1977). Acquisition of disease agents is therefore incidental to blood meals.

Sand flies are very susceptible to dehydration, so most are nocturnal. They seek shelter in animal burrows, tree buttresses or holes, caves, rocks and other protected habitats, including human habitations. Generally weak flyers, they usually fly close to the ground in short hops (Goddard, 1996). Their range is typically very short (about 300 m), but some have been known to travel up to 2300 m in desert environments.

The short flight range usually restricts the adult to the general vicinity around the larval development site. These sites are usually organically rich, moist soils. In the New World, the flies are often found near tree buttresses and caves (Feliciangeli, 2004).

2.6 Control of leishmaniasis

Control of leishmaniasis is currently based on chemotherapy to alleviate the disease and on vector control to reduce transmission (Kishore *et al*., 2006; Monzote, 2009). Pentavalent antimonies have remained the drugs of choice for the treatment of visceral and cutaneous leishmaniasis, except in Bihar India, where visceral leishmaniasis is highest, because of resistance to the drugs (Guerin *et al*., 2002; Croft *et al*., 2006). The traditional second line treatment consists of pentamidine and amphotericin B whereas sitamaquine is still under development (Guerin *et al*., 2002; Monzote, 2009). The high cost of these drugs, long courses of parenteral administration coupled with the need for hospitalization and high toxicity (Croft *et al*. 2006), lack of an effective vaccine (Handman, 2001; Khamesipour *et al*., 2006;

Modabber, 2010) has necessitated the search for better alternatives in the control of leishmaniasis. Vector control is therefore crucial to prevent bites from potentially infectious sand flies.

2.7 Vector control in curbing leishmaniasis

Depending on application techniques, timing and target species, sandflies are known to be highly susceptible to insecticides (Alexander *et al*., 1995a; Alexander and Maroli, 2003; Wilamowski and Pener, 2003; Orshan *et al*., 2006) and in certain areas effective control has been achieved as a side-effect of malaria control programmes (Vioukov, 1987). Residual formulations of DDT have also been used expressly to control sandflies (Hertig and Fisher, 1945; Hertig and Fairchild, 1948; Hertig, 1949). The synthetic pyrethroid deltamethrin has been used against sandflies in Bolivia (Le Pont *et al*., 1989) and Brazil (Bermudez *et al*., 1991; Marcondes and Nascimento, 1993; Courtenay *et al*., 2007). In other countries where sandfly vectors are endophilic, control of leishmaniasis has traditionally been based on residual insecticide spraying in houses, with significant effectiveness (Alexander *et al*., 1995a; Vieira and Coelho, 1998; Alten *et al*., 2003). Other studies have tested the efficacy of insecticide impregnated textiles, such as curtains, bed nets or bed covers, with varying degrees of success (Alexander *et al*., 1995a, 1995b; Basimike and Mutinga, 1995; Elnaiem *et al*., 1999; Kroeger *et al*., 2002; Courtenay *et al*., 2007). Environmental modification, involving the total eradication of rodents, destruction of burrow systems and spraying of herbicides to kill their food plants, has been demonstrably effective in controlling CL caused by *L. major* in foci in the Asian republics of the former USSR and in Tunisia (Vioukov, 1987; WHO, 1990). However, acquired resistance and environmental pollution due to the repeated application of persistent synthetic

insecticides, high toxicity and harmful side effects for both animals and humans have progressively limited their usage and have led to increased interest in new natural chemicals that are environmentally safe and effective in leishmaniases control.

2.7.1 Historical perspective of the control of sandflies

Initial attempts to control sandflies using modern insecticides were carried out using DDT (dichlorodiphenyltrichloroethane) in the Rimac Valley which was Peruvian focus of bartonellosis, in January 1944. Field trials were subsequently carried out in Palestine, Italy and Greece to combat outbreaks of sandfly fever and leishmaniasis. In India, Ghosh (1950) carried out the first field evaluations of DDT and BHC (benzene hexachloride) against sandflies. In comparison with DDT, BHC was much less effective and sandflies reappeared within 1 month. Both BHC and DDT were employed extensively in the Soviet Union for sandfly control (Perfilev, 1966). Insecticidal control of visceral leishmaniasis (VL) in the People's Republic of China dates back to 1958 and has been based on residual spraying of houses and caves containing livestock with DDT, the carbamate 3,5-Methylcarbamate (MC), BHC and more recently deltamethrin, as well as aerial spraying with BHC.

Brazil accounts for 90% of leishmaniasis cases in the New World (WHO, 2015). In this South American country, attempts to control sandflies with chemical insecticides dates back to 1954 when evaluated spraying of houses with DDT in a focus of CL in Rio de Janeiro State. After 5 years of periodic spraying there was a decrease both in sandfly population levels and in incidence of disease. The success was subsequently replicated in the Brazilian states of Ceara and Minas Gerais against *Lu. longipalpis* until 1964 (Oliveira Filho, 1994).

Reduction of the number of leishmaniasis cases has been also postulated as a collateral benefit of the malaria intervention programmes in Bangladesh (Elias *et al*., 1989), Syria (Tayeh *et al*., 1997), with a modest reduction also observed in response to BHC spraying in Azerbaijan (Nadzharov, 1955). In addition, Tesh and Papaevangelou (1977) reported that antimalaria activities in Greece produced a reduction in the number of sandfly fever cases. However, no reduction in morbidity from zoonotic visceral leishmaniasis (ZVL) was recorded as a result of antimalaria measures in Portugal or Greece, (Lane, 1991) and incidental suppression of leishmaniasis could only be expected in areas where the transmission cycle is largely anthroponotic (Saf'janova, 1971).

In Kenya, Wijers and Kiilu (1984) credited pesticide use on cotton by Kenyan farmers and its storage in human dwellings with the suppression of man-biting by both *P. martini* and the malaria vector *An. gambiae*. Urban populations of *P. papatasi* in Saudi Arabia were reduced by ground and aerial application of diazinon against synanthropic flies such as *Musca* (Büttiker, 1980).

2.7.2 Residual spraying of houses and animal shelters

Sand fly control programmes often rely on spraying potential sand fly resting sites with residual insecticides (Alexander and Maroli, 2003). While regular spraying can offer some protection to human beings from infection (Davies *et al*., 2000), such strategies are often challenging hence difficult to sustain effectively, particularly in rural communities, where there may be large numbers of potential resting sites that must be revisited and resprayed on a near regular basis. Spraying also requires

training to be conducted effectively, in order to ensure that indiscriminate killing is avoided and that the correct concentration of insecticide is applied to kill sand flies, while minimizing exposure to sub-lethal amounts which might promote the onset of resistance (Alexander *et al*., 2009). A cluster randomized controlled trial evaluating the impact of indoor residual spraying (IRS) on vector density was conducted in India, Nepal and Bangladesh in 2006-2007 (Joshi *et al*. 2009). IRS of houses and cattle sheds, supervised by the researchers, reduced the indoor *P. argentipes* density by 72.4 per cent in intervention clusters compared to control. This effect was consistent in all sites (Joshi *et al*. 2003) and confirmed in Nepal where additional entomological surveys were conducted (Das *et al*., 2010). Benzerroug *et al*. (1992) compared human infections with *L. infantum* in Algeria before and after a house-spraying campaign based on DDT and found that annual incidence dropped from 426 cases per 100 000 inhabitants to only 17.9 one year later.

In other studies, Davies *et al*. (2000) evaluated the effect of spraying interior walls and ceilings of houses in the Peruvian Andes with lambdacyhalothrin on transmission of *Le. peruviana* resulting in a significant reduction in the proportion of susceptible householders acquiring leishmaniasis. Marcondes and Nascimento (1993) evaluated three different concentrations of an emulsifiable concentrate of deltamethrin applied to walls for the control of *Lu. longipalpis* in Brazil. Although the number of sandflies aspirated from the walls of treated houses was significantly lower than that from untreated dwellings, this effect disappeared within only 2 months and some insects were collected as early as 14 days after spraying. Alexander *et al*. (1995b) found that residual spraying of walls with deltamethrin in a Colombian village surrounded by forest had no perceptible effect on the number of sandflies entering houses, although

the insecticidal activity of the treated surfaces was undiminished during the study period.

2.7.3 Use of insecticide-treated mosquito nets (ITNs)

Insecticide-treated mosquito nets (ITNs) have been evaluated against phlebotomine sandflies and leishmaniasis in several countries, including Italy (Maroli and Lane, 1989), Burkina Faso (Majori *et al*., 1989), Syria (Tayeh *et al*., 1997; Desjeux, 2000), Sudan (Elnaiem *et al*., 1999a, 1999b), Kenya (Mutinga *et al*., 1992, 1993; Basimike and Mutinga, 1995; Robert and Perich, 1995), India (Ostyn *et al*., 2008), Bangladesh (Chowdhury *et al*., 2011; Mondal *et al*., 2013), Colombia (Alexander *et al*., 1995b) and Venezuela (Feliciangeli *et al*., 1995; Kroeger *et al*., 2002). The use of ITNs may represent the most sustainable method of reducing intradomiciliary transmission of *Leishmania* in communities surrounded by forest, where the diurnal resting sites of vectors are unknown or inaccessible (Alexander and Maroli, 2003). There have been arguments that the small size of sandflies means that the mesh used on bed nets would have to be extremely fine to confer protection against their bites, and that this would increase cost and limit their acceptability to potential users. Das *et al*. (2010) in crossover trials conducted in a rural village in Morang district (South-eastern Nepal) demonstrated that reducing the size of the holes in treated nets (625 holes/inch^2) increased the barrier effect of Long lasting insecticide treated nets (LN) by 77% (95% confidence interval (CI): 56% to88%) compared with treated nets with larger holes (156 holes/inch^2). Treating nets with alpha-cypermethrin reduced the number of *P. argentipes* captured inside the nets by 77% (95% CI: 27% to 93%) compared with untreated nets.

Previously, Maroli and Lane (1989) found out that permethrin-impregnated nets of 1cm^2 mesh placed over windows significantly reduced the numbers of *P. perfiliewi* entering houses in Italy while Majori *et al*. (1989) obtained a similar result using permethrin-impregnated curtains of slightly smaller mesh (5 mm^2) in Burkina Faso. Feliciangeli *et al*. (1995), however, found that sandflies (especially *Lu. ovallesi*) were able to pass through 6 mm mesh treated with deltamethrin at 15 mg/m^2 and bite volunteers. The insects could also rest on the impregnated surfaces for periods of up to two minutes with no significant mortality observed. When mesh size was reduced to 4 mm and concentration raised to 60 mg/m^2 all sandflies were killed within 30 min after 10 min of exposure (Feliciangeli *et al*.,1995).

Impregnated curtains hung across doors and windows may reduce sandfly access before the occupants retire for the night but since the insects are preadapted to resting in very confined spaces during the day, even small gaps in the walls or roof of a dwelling would allow them to enter. Elnaiem *et al*. (1999a) carried out laboratory and field evaluations of curtains impregnated with permethrin on *P. papatasi* in Sudan and found that exposure to mesh treated with 0.5 mg active ingredient per cubic meter (a.i./m^2) for 3 min killed all the insects within24 hoursours. Both the human biting rate and the resting density of this species (although not its nocturnal activity) were also significantly reduced. Basimike and Mutinga (1995) evaluated screens made of polyester netting and impregnated with 0.5% permethrin, hung beside beds and each occupying an area equivalent to half the surface area of a one-roomed house. The screens were treated at 6-month intervals and percentage reduction of *P. martinii* numbers inside houses increased to a maximum recorded value of 88.8% after eight treatments.

Mutinga *et al*. (1992, 1993) hung cotton wall cloths impregnated with 0.5 g/m^2 permethrin inside houses in Kenya and found that these retained their insecticidal effect against *P. martini* and *P. duboscqi* for 6 months. The numbers of these species collected inside houses were reduced by 76 and 85%, respectively. Unlike bednets and curtains, the purpose of these cloths was solely to kill sandflies that would normally rest on the inside walls rather than to restrict access to houses or sleeping individuals.

2.7.4 Chemical repellents

In areas where *Leishmania* transmission is extra-domiciliary and leishmaniases are an occupational hazard, use of insect repellents or protective clothing may be the only preventative measures available. Mustafa and Ahmad (2015) evaluated the use of commercial lavender lotion as a repellent against sand fly bites in Gadarif state in Sudan. The field evaluation showed protection up to mean time for 7 h and 40 min against sand flies. Britch *et al*. (2011) specifically, examined the potential for ultra-low volume (ULV) pesticide applications to control Old World sand fly vectors in equitorial Kenya. Sampling of wild populations before and after treatments suggested local population suppression from ULV treatments, as well as a possible repellent effect in nearby untreated areas (Britch *et al*., 2011).

The commercially available insect repellent DEPA (N,N-diethylphenyl acetamide) was compared with neem oil for protection against the bites of *P. papatasi* by Srinivasan and Kalyanasundaram (2001). Neem oil was significantly more effective

than DEPA when applied to mice in the laboratory at concentrations of 1% and 2% but the repellencies of the two compounds were similar at 5%.

Jia *et al*. (1990) studied the efficacy of five repellents against *P. alexandri* in the laboratory and field in China. The most effective compound tested was a mosquito-repellent perfume (MRP) which at 0.25 µl/cm^2 conferred protection for almost 8 h. The least effective was dibutyl phthal (DBP) which repelled sandflies for only 1 h.

Alexander *et al*. (1995a) in Colombia evaluated a relatively inexpensive soap formulation containing DEET and permethrin and found that it retained 67% of its activity (in terms of numbers of sandflies biting volunteers) up to 8 h after application. However the main drawback of this formulation was that repellency was lost if the soap was rinsed off the skin. Perfil'ev (1966) summarized experiences in the Soviet Union with older formulations of repellent soap and long-lasting repellent-treated netting that remained active for the whole season.

Insecticides or repellents applied to clothing rather than skin offer an alternative approach to personal protection against sandfly bites. However clothing impregnated with permethrin did not completely protect volunteers against sandfly bites in Panama (Schreck *et al*., 1982), probably because of the low vapour pressure of the insecticide and the fact that insects landing on the treated surface would be deflected to the exposed skin of the face and hands. Dees *et al*. (1987) found out that *P. papatasi* exhibited probing behaviour during direct contact with permethrin-treated uniforms and readily attacked skin previously covered by treated fabric. Fryauff *et al*. (1996) investigated the effects of laundering and exposure time on the insecticidal activity to

P. papatasi of military uniform fabric impregnated with 0.125 mg/cm^2 permethrin. The insecticide remaining after three washes was toxic to sandflies exposed for as little as one minute, killing 15% of insects within24 hours. Nevertheless significant reductions in the knockdown effectiveness of treated fabric were associated with repeated laundering,24 hours mortality falling from 91% (0 washes) to 63% in sandflies exposed to treated surfaces for 10 min. Based on the disappointing results of these carefully controlled studies, use of impregnated clothing to protect non-military personnel from sandfly bites may be construed as impractical.

2.7.5 Other alternatives for sandfly control

Although sandfly larvae are susceptible in the laboratory to the bacterium *Bacillus thuringiensis* var. *israelensis* (Bti) (De Barjac *et al*., 1981), the difficulty of finding immatures under natural conditions precludes targeting breeding sites as a viable control measure. However, Yuval and Warburg (1989) suggested that microbial agents such as Bti could be used against adult insects by incorporating them in sugar baits sprayed onto substrates in open, dry habitats. Concentrations of 4.4×10^{-2}, 10^{-3} and 10^{-4} mg/ml of Bti killed 100% of adult *P. papatasi*, *P. argentipes* and *Lu. longipalpis* in the laboratory.

Majori and Maroli (1983) studied the larvicidal effect of Bti serotype H-14 against *P. perniciosus*. After 6 days of exposure, they observed 100% mortality among larvae fed on the Bti-treated diet.

Robert *et al*. (1998) evaluated the mosquito larvicidal bacterium *B. sphaericus* against sandflies in the laboratory and found out that aqueous suspensions inhibited hatching

of eggs of *P. dubosqci* and *Sergentomyia schwetzi* by 95% at low concentrations (0.5 and 0.11 mg/cm^2, respectively). In a previous study (Robert *et al*., 1997) they established that sugar solutions containing this bacterium, sprayed onto vegetation and taken up by sandflies, caused significant mortality of larvae at their breeding sites in animal burrows in Kenya. The same study indicated that adult sandfly populations were also significantly reduced by spraying the bacterial solution at burrow entrances, the effect persisting for up to 10 weeks after treatment.

Kassem *et al*. (2001) carried out laboratory evaluations of two avermectins on the sandflies *P. papatasi* and *P. langeroni*, by presenting the compounds in blood (ivermectin) or sugar meals (abamectin). Low concentrations of either avermectin killed sandflies of both species and sublethal doses of ivermectin in blood resulted in reduced survival and fecundity of adult females. The avermectins are environmentally safe compounds and could therefore be used as systemic insecticides, administered to animals used as blood meal sources by sandflies or to the plants from which they obtain sugars.

In Colombia, the entomopathogenic fungus *Beauveria bassiana* is employed to control infestations of the coffee berry borer (*Hypothenema hampei*) in coffee plantations where sandflies transmit *Leishmania* to man. Although Warburg (1991) selected for a strain of the fungus which killed sandflies (*P. papaptasi* and *Lu. longipalpis*) in the laboratory, Reithinger *et al*. (1997) found that live insects were not infected by commercial preparations of *B. bassiana*. Simultaneous application of mixtures of strains pathogenic to *H. hampei* and *Lutzomyia* spp. might, however,

represent a viable alternative in coffee-growing areas where both borer infestions and leishmaniasis occur.

Following the discovery that certain plants (*Capparis spinosa, Ricinus communis, Solanum luteum*) used as sources of sugar by sandflies were toxic to *L. major* (Schlein and Jacobson, 1994; Jacobson and Schlein, 1999; Schlein *et al*. (2001) found that certain exotic species were also able to kill the insects themselves. Planting these (*Bougainvillea glabra, Ricinus communis, Solanum jasminoides*) in barrier zones might therefore provide a low-cost, sustainable alternative to insecticide use in the control of sandflies and leishmaniasis. Luitgards-Moura *et al*. (2002) evaluated the insecticidal effects of two plant extracts used by Amazonian Indians to kill fish. Both were highly toxic to *Lu. longipalpis*, dried leaf extracts dissolved in water of *Antonia ovata* (Loganiaceae) and *Derris amazonica* (Papilionaceae) killing 80% and 100% of females, respectively. These plants could therefore represent a readily available alternative to commercial insecticides for sandfly control in the ZVL focus of Roraima, Brazil.

In recent years the discovery of pheromones produced by male sandflies has led to the suggestion that synthetic copies of these compounds could be used to attract females to insecticide-treated surfaces and potentiate conventional control measures (Lane, 1991). Although attractiveness is not dependent on the presence of a warm-blooded host (Morton and Ward, 1990) the range of the *Lu. longipalpis* pheromone is restricted to a few metres, considerably less than that exerted by CO_2 or host odour (Alexander, 2000) and not comparable to that of semiochemicals currently used for the control of several lepidopteran pests. Sandfly pheromones might be better used as

tropical treatments on dogs or livestock to disrupt mating, at least in the case of *Lu. longipalpis*.

Quesada and Montoya-Lerma (1994) evaluated the insect growth inhibitor chlorfluazuron against second and third-instar larvae of *Lu. longipalpis* and observed a number of lethal and sublethal effects. Larvae ceased to feed and underwent premature moults, cuticular rupturing or imperfect shedding of exuviae. Female adults that had ingested the insecticide in the larval diet were less likely to take a bloodmeal and the wings, abdomens and genitalia of treated males were significantly smaller than those in control groups.

In some situations, integrated vector control can be employed against the sandfly vector species, involving a combination of different methods. One leishmaniasis control programme in Uzbekistan used spraying with hexachlorocyclohexane (HCH) and DDT, elimination of gerbils by poisoned baits, destruction of gerbil burrows/sandfly resting sites, personal protection methods and vaccine prophylaxis (Faysulin, 1980).

2.7.6 Resistance of sandflies to insecticides based on laboratory bioassays

The initial cases of resistance of sand flies to insecticides were reported from Bihar, India, a global focus of visceral leishmaniasis, (Kaul *et al*., 1978; Joshi *et al*., 1979). Since then, reports of resistance to insecticides by sandflies of different genera and species have continued to emerge (Pener and Wilamovsky, 1987; Bansal and Singh, 1996; Mukhopadhyay *et al*., 1996); Amalraj *et al*., 1999). In Sudan, Hassan *et al*. (2012) provided evidence for Malathion and propoxur resistance in the *P. papatasi*

sand fly population of Surogia village in Khartoum state, which probably resulted from anti-malarial control activities carried out in the area during the past 50 years.

Alexander *et al.* (2009) demonstrated the susceptibility to chemical insecticides of two geographically isolated Brazilian populations of *Lu. longipalpis* the visceral leishmaniasis. Biochemical analyses have demonstrated that Montes Claros sand flies had significantly lower insecticide detoxification enzyme activity than Lapinha sand flies. In Italy, Maroli *et al*. (2002a) studied the susceptibility of newly established laboratory colonies of *P. perniciosus* and *P. papatasi* to some insecticides. A laboratory colony of *P. papatasi*, unexposed to insecticides for 10 years, was used as a reference strain. This study showed that the two Italian populations of *P. perniciosus* and *P. papatasi* from Campania region and Rome were susceptible to the insecticides tested.

Lavagnino and Ansaldi (1991) tested the susceptibilities of wild-caught *P. perniciosus* and *P. perfiliewi* from Sicily to DDT, malathion and permethrin. Higher concentrations of DDT (4% vs. 1%) and exposure times to permethrin (30 min vs. 10 min) were required to kill the former species. Mortalities of both species were low when exposed to 0.5% malathion for up to 1 hour, values of only 55% and 65% mortalities were recorded for *P. perniciosus* and *P. perfiliewi* respectively.

El-Sayed *et al*. (1989) showed that insecticide-susceptible sandflies are able to metabolize this insecticide to Dichlorodiphenyldichl (DDE) using the mixed function oxidase and glutathione-S-transferase (GST) mechanisms. The level of GST activity

in *P. papatasi* was lower than that seen in susceptible adults of the mosquito *Culex quinquefasciatus* when expressed in terms of activity per mg soluble protein.

2.8 Insecticidal activities of plant natural products against sand flies

The need for more efficient and safer methods to control insects has stimulated the search for new insecticides in plants (Nogueira and Palmério, 2001). Plants have been important sources of active compounds against insects, such as pyrethroids (pyrethrin and allethrin) and rotenoids. The neem plant (*Azadirachta indica*) oil is repellent to *Phlebotomus papatasi* Scopoli and it has been shown to be more effective when used for three days at concentrations of 1% and 2% (Srinivasan and Kalyanasundaram 2001). A recent study by Ireri *et al*. (2010) demonstrated that the extracts of *Acalypha fruticosa* and *Tagetes minuta* had significantly higher mortality rates on *P. duboscqi* than those of *Tarchonanthus camphoratus.* Death rates of 80% and 100%, respectively, 72h after exposure were recorded. The repellent and anti-feedant effect of the garlic oil (*Allium sativum*) has also been evaluated on *P. papatasi* females. In this study, 1%, the oil had a repellent effect of 97% and an anti-feedant effect of 100% (Valerio and Maroli, 2005). Many other studies have been carried out to evaluate the biological activity of plant components against many pathogens and arthropods (reviewed by Shaalan *et. al*., 2005).

2.9 Essential oils

2.9.1 Background overview of essential oils

Essential oils are composed of lipophilic and highly volatile secondary plant metabolites, reaching a mass below a molecular weight of 300, which can be physically separated from other plant components or membranous tissue (Protzen 1993; Grassmann and Elstner, 2003; Schmidt 2010; Sell, 2010). As defined by the International Organization for Standardization (ISO), the term “essential oil” is

reserved for a “product obtained from vegetable raw material, either by distillation with water or steam, or from the epicarp of citrus fruits by a mechanical process, or by dry distillation” (ISO 9235, 1997), that is, by physical means only.

Essential oils and their derivatives are considered to be an alternative means of controlling many harmful insects (Pillmor *et al*., 1993; Tripathi *et al*., 2009). Essential oils are volatile, natural, complex compounds characterized by a strong odor and are formed by aromatic plants as secondary metabolites (Bakkali *et al*., 2008; Tripathi *et al*., 2009). They are lipophilic in nature and interfere with basic metabolic, biochemical, physiological and behavioral functions of insects (Brattsten, 1983). In nature, essential oils protect plants from herbivores and microorganisms through their antimicrobial or insecticidal properties. Nearly 3,000 essential oils are known from nearly 17,500 aromatic plant species out of which about 300 are commercially important for pharmaceuticals, pesticide or flavor industries (Franzios *et al*., 1997; Chang and Cheng, 2002; Bakkali *et al*., 2008; Tripathi *et al*., 2009). Thus, they are generally recognized as safe by the US Food and Drug Administration (Trongtokit *et al*., 2005).

The majority of essential oils originate in the plant families: Myrtaceae, Lauraceae, Rutaceae, Lamiaceae, Asteraceae, Apiaceae, Cupressaceae, Poaceae, Zingiberaceae and Piperaceae (Regnault-roger, 1997; Tripathi *et al*., 2009). Essential oils exist in wide variety of structures with diverse functions and are often classified into four main groups: terpenes, benzene derivatives, hydrocarbons and other miscellaneous compounds (Ngoh *et al.*, 1998; Tripathi *et al.*, 2009). Their composition may vary considerably between aromatic plant species and varieties, and within the same

variety from different geographic areas (Zygadlo, 2003). Composition, quality, and quantity of essential oils is known to depend on several factors including extraction methods, source, plant growth, climate, plant structure and the vegetative stage of source plant (Masotti *et al*., 2003; Angioni *et al.*, 2006; Tripathi *et al*., 2009). Little is known about the true mode of action of essential oils on insects. The rapid onset of toxic signs suggests a neurotoxic mode of action involving competitive inhibition of acetylcholinesterase (Coats *et al*., 1991; Re *et al*., 2000; Kostyukovsky *et al*., 2000) competitive activation of octopaminergic receptors (Kostyukovsky *et al*., 2000; Enan, 2005; Price and Berry, 2006) or interference with gamma-aminobutyric acid (GABA)-gated chloride channels (Priestley *et al*., 2003). Linalool a constituent of several essential oils has been demonstrated to act on the nervous system, affecting ion transport and the release of acetylcholine esterase (Re *et al*., 2000) whereas eugenol has been shown to mimic octopamine in *Periplaneta americana* and *Drosophila melanogaster*. It has been observed that toxicity of eugenol increases in octopamine deficient mutant *D. melanogaster* (Kostyukovsky *et al*., 2000; Re *et al*., 2000; Enan, 2005; Price and Berry, 2006; Priestley *et al*., 2003). David *et al*. (2000) found that tannic acid from decaying leaves of *Alnus glutinosa, Populus nigra,* and *Quercus robur* primarily affect the midget epithelium and secondarily affect gastric caeca and the malpighian tubules in mosquito larvae (David *et al*., 2000; Priestley *et al*. 2003). Toxic, repellent, ovicidal or growth retardant activity of large number of essential oils or their constituents have been demonstrated on large number of haematophagous insects including mosquitoes, fleas, lice, filth flies, ticks and mites (Barnard, 1999; Gbolade, 2001; Cheng *et al*., 2003; Pavela, 2007; Panella *et al*., 2005; Amer and Mehlhorn, 2006; George *et al*. 2009; Mann *et al*., 2010**)**. However, in insect vectors the bioactivity of essential oils have been evaluated primarily against

mosquitoes and to a lesser extent on other insect vectors, perhaps because of their greater significance in pathogen transmission

2.9.2 Ovicidal activity of essential oils

Besides toxic and repellent properties, essential oils have been shown to have a pronounced effect on the developmental period, growth, adult emergence, fecundity, fertility and egg hatching of insects (Shallan *et al*., 2005; Elango *et al*., 2010). Hexane extract of *Andrographis lineat*, *A. paniculata* and *Tagetes erecta* showed 100% ovicidal activity against *An. subpictus* (Elango *et al*., 2010). Essential oils from *Juniperus macropoda* and *Pimpinella anisum*, *Zingiber officinale* and *Rosmarinus officinalis* showed strong ovicidal properties against *Ae. aegypti*, *An, stephensi*, and *Cx. quinquefasciatus* (Toloza *et al*., 2008). Essential oils of *Piper guineense* and *Xylopia aethiopica* deterred oviposition by gravid female *Ae. aegypti* for up to 48 hours (Tawatsin *et al*., 2006). Essential oils from Aglalia, Alpinia, Curcuma, Eleutherococcus, Hedychium, Houttuynia, Litsea, Manglietia, Melaleuca, Murraya, Myristica, Ocimum, Piper, Schefflera, Vitrex, and Zingiber plants exhibited 16.6 to 94.7% oviposition deterrence against *Ae. Aegypti* (Tawatsin *et al*., 2006). Rosemary oil, pulegone, thymol, and eugenol showed up to 100% oviposition deterrent activity against *Ae. Aegypti* (Fradin, 1998). One of the linalool and 10.0 µL of pine oil completely inhibited oviposition by house flies (Maganga *et al*., 1996). While piperitenone oxide isolated from *Mentha spicata* var. viridis completely inhibited *An. stephensi* oviposition at 75.0 g $m1^{-1}$ dosage.

2.9.3 Repellent activity of essential oils

By definition, repellents are substances that act locally or at a distance, deterring an arthropod from flying to, landing on or biting human or animal skin (or a surface in general) (Blackwell *et al*., 2003; Choochote *et al*., 2007). Usually, insect repellents work by providing a vapor barrier deterring the arthropod from coming into contact with the surface (Brown and Hebert, 1997). Among them, essential oils, complex mixtures of volatile compounds isolated from a large number of plants, have been found to have these properties against various haematophagous arthropods, some of them being the basis of commercial repellent formulations (Curtis *et al*., 1989).

2.9.4 Repellent activity of essential oils on mosquitoes

Essential oils of large number of plants have been found to have repellent properties against various haematophagous arthropods (Adorjan and Buchbauer, 2010; Nerio *et al*., 2010). The oils, which have been reported as potential sources of insect repellents, include citronella, cedar, verbena, pennyroyal, geranium, lavender, pine, cajeput, cinnamon, rosemary, basil, thyme, allspice, garlic and peppermint, among others. Lemongrass *Cymbopogon* spp., has been reported to produce the most used natural repellents in the world (Cheng *et al*., 2003; Nerio *et al*., 2010). Essential oils from *C. martinii martinii* provided 100% repellency for 12 hours against Anopheles mosquitoes in field tests (Ansari and Razdan,1994). *Cymbopogon winterianus* oil, mixed with 5% vanillin, gave 100% protection for 6 hours against *Ae. aegypti*, *Cx. quinquefasciatus* and *Anopheles dirus* (Tawatsin *et al*., 2001). Essential oils obtained from Eucalyptus produced high repellency against the mosquitoes, *Ae. albopictus* and *Mansonia* spp. (Hadis *et al*. 2003; Yang and Ma, 2005). Piperitenone oxide isolated from *Mentha spicata* viridis was found to be highly repellent against *An. stephensi*

(Tripathi *et al*. 2004). Essential oils from *C. excavatus* gave 100% repellency for 2 hours, against *An. arabiensis* (Govere *et al*., 2000). Essential oils of *Melaleuca ericifolia* effectively repelled *Ae. vigilax* and *Verrallina carmenti* mosquitoes (Greive *et al*. 2010). Essential oils from clove, *Syzygium aromaticum* provided 2 hours of complete repellency against *Ae. aegypti*, *Cx. quinquefasciatus* and *An. dirus* (Trongtokit *et al*. 2005). Essential oils from catmint, *Nepeta cataria* at 15% active ingredient conferred complete protection for 7.5 hours from *Ae. intrudens* under field conditions (Spero *et al*., 2008). Recently several new essential oil-based chemistries have been commercialized as mosquito repellants. Examples of such chemistries are use of citronella oil alone or in combination with cedar wood or lavender, peppermint, clove, Eucalyptus and garlic in a number of commercial insect repellent products (Fradin, 1998).

Commercial natural chemical based repellents such as Swamp Buddy Bug Chaser®, All SportTM, Neem Aura®, GONE®, Sun Swat™, Bite Blocker™, Cutter®, 3m™, Ultrathon™, Green Ban™ have been developed against several mosquito species (Qualls and Xue, 2009). However, the field efficacy of these commercial products has been reported to be highly variable depending upon the insect species, product formulation and methods of evaluation.

There have been numerous reports concerning the biological properties of many kinds of essential oils; however, most of the results were obtained from artificial (*in vitro*) testing methods using cloth, filter paper, animal membrane or olfactometry, with few from *in vivo* evaluations utilizing animal or human subjects (Barnard, 1999; Rutledge *et. al*. 1964). Qualls and Xue reported that commercial products Geraniol®, All

Sport™ and Swamp Buddy Bug Chaser™ provided 4, 1.5 and 1 hr protection against *Psorophora ferox*, *Ae. atlanticus*, and *Ae. mitchellae* bites (Qualls and Xue, 2009). However, their protective effects dissipated relatively rapidly (Adorjan and Buchbauer, 2010; Rutledge *et al*., 1964). Commercial candles with 5% geraniol, linalool and citronella caused an 82, 65 and 35% respectively reduction in female mosquitoes trap catches and a 70, 49 and 15% reduction in sand fly trap catches up to a distance of 1.0 m (Muller *et al*., 2008a). The candles also produced comparable repellency when evaluated under high mosquito and sand fly populations (Muller *et al*., 2008a). However, a continuous release diffuser containing these essential oils provided better degree of personal protection than the candles (Muller *et al*., 2008a).

2.9.5 Repellent activity of essential oils on other haematophagous arthropods

Essential oils obtained from Eucalyptus produced high repellency against the human head louse, *Pediculus humanus* capitis (Toloza *et al*., 2008). Essential oils from *Eucalyptus cinerea*, *E. viminalis* and *E. saligna* showed knockdown time 50 (KT50) values of 12.0, 14.9 and 17.4 min, respectively against permethrin-resistant human head lice (Toloza *et al*., 2006). A lemon eucalyptus extract from *E. maculata* citriodon showed good repellent activity against mosquitoes, midges, ticks and stable flies (Curtis, 1989; Trigg *et al*., 1996). Essential oils from *Pogostemon cablin* provided protection up to 3.7 hours against *Stomoxys calcitrans*. Very strong repellency was also produced by *Eugenia caryophyllata*, *Levisticum officinale* (3.2 to 3. 5 hours) and *Thymus vulgaris* (2.1 hours) against this species (Hieu *et al*., 2010). Essential oils of *Melaleuca ericifolia* effectively repelled bush fly *Musca vetustissima* and the biting midges *Culicoides ornatus* and *C. immaculatus* (Carroll *et al*., 2010). Thavara *et al*. (2007) while examining repellency in cock roaches reported that

essential oils from *Citrus hystrix* led to 100% repellency against *Periplaneta americana* and *Blattella germanica* and 88% against *Neostylopyga rhombifolia* under laboratory conditions (Spero *et al*. 2008). *Citrus hystrix* formulated as 20% active ingredient in ethanol, exhibited 86% reduction in *P. americana* and *N. rhombifoha* populations under field conditions. Essential oils from catmint *Nepeta cataria* at 15% active ingredient conferred complete protection for 7.5 hours from *Simulium decorum* under field conditions (Thavara *et al*., 2007).

Eucalyptol from eucalyptus oil showed fumigant activity against first-instar nymphs of the bloodsucking bug *Rhodnius prolixus*, a vector of Chaga's disease, yielding KT50 value of 216 minutes as compared to 30 minutes for dichlorvos (Sfara *et al*., 2009). Essential oils have been shown to have repellency for numerous non-insect arthropods including ticks and chiggers. Those from *Amyris balsamifera* and *Madura pomifera* effectively repelled the blacklegged tick, *Ixodes scapularis*, and the lone star tick, *Amblyomma americanum* up to 4 hours (Carroll *et al*., 2010). Essential oils from *Syzygium aromaticum* exhibited 100% repellency against host seeking chiggers, *Leptotombidium imphalum* (a vector of scrub typhus), at a 5% concentration (Eamsobhana *et al.,* 2009). Essential oils from citronella cloves and lily of the valley repelled *Ix. ricinus* to the same magnitude as DEET (Thorsell *et al*., 2006). Whereas, essential oils of *Melaleuca alternifoha*, *Zingiber cassamunar* and *Eu. globules* exhibited repellency against *L. imphalum* at concentrations ranging from 40 to 100% (Eamsobhana *et al*., 2009). Thyme oil at 0.14 mg oil cm^{-3} effectively repelled the poultry mite, *Dermanyssus gallinae* up to 13 days (George *et al*., 2009). Essential oils obtained from Eucalyptus also produced high repellency against the Ixodes tick (Jaenson *et al*., 2006).

2.10 Toxic activity of essential oils

2.10.1 Toxicity on mosquitoes

Toxic activity of essential oils on mosquitoes has been reviewed by Sukumar *et al.* (1991) and Shallan *et al.* (2005). A survey of literature on insecticidal properties of essential oils from the year 2004 onwards indicates that essential oils from about 90 plant genera belonging to 38 plant families were reported to have toxic properties against mosquito larvae. Although the majority of essential oils are less toxic than synthetic insecticides, LC50 values as low as 0.004 mg L^{-1} of pipercide from *Piper nigrum* against *Cx. pipiens pallens* larvae have been reported (Park *et al*., 2002). Komalamisra *et al*. (2005) evaluated 84 Thai plant species against *Ae. aegypti Cx. quinquefasciatus*, *An. dims* and *Mansonia uniformis* larvae, of which *Rhinacanthus nasutus* extract exhibited the strongest larvicidal activity with LC50 values ranging between 3.9 and 11.5 mg L^{-1} (Komalamisra *et al*., 2005). Pulegone, thymol, and eugenol extracted from rosemary oil showed high larvicidal activity against multiple larval stages of *Ae. aegypti* (Waliwitiya *et al*., 2009). The LC_{50} values for these compounds ranged from 10.3 to 40.8 mg L^{-1} and 2.3 to 3.2 mg L^{-1} against third and first instar larvae, respectively. Piperitenone oxide isolated from *Mentha spicata viridis* showed high larvicidal and adulticidal activities against *An. stephensi* (Tripathi *et al*., 2004).

Essential oils from *Citrus hystrix*, *C. reticulata*, *Zingiber zerumbet*, *Kaempferia galanga*, and *Syzygium aromaticum* showed toxicity to permethrin resistant *Ae. Aegypti* (Sutthanont *et al*., 2010). Pellitorine, a chemical isolated from *Asarum heterotropoides* roots showed comparable toxicity to laboratory susceptible and

fenthion, chlorpyrifos, fenitrothion, deltamethrin, chlorfenapyr, and acypermethrin resistant *Cx. pipiens pallens*, *Ae. aegypti*, and *Oc togoi* mosquitoes (Perumalsamy *et al*., 2010).

A majority of studies have concentrated on the evaluation of essential oils as larvicides; however, essential oils from *Aristolochia indica*, *Cassia angustifolia*, *Diospyros melanoxylon*, *Dolichos biflorus*, *Gymnema sylvestre*, *Justicia procumbens*, *Mimosa pudica*, *Zingiber zerumbet* have previously exhibited good activity against *Cx. Gelidus* and *Cx. quinquefasciatus* adults (Kamaraj *et al*., 2010). The essential oil of *Curcum zedoaria* generated an LC50 ranging from 5.44 to 8.52 mg^{-1} of body weight against *Ae. aegypti* adults. These toxicity doses are comparable to many synthetic insecticides including permethrin and imidacloprid (Chaiyasit *et al*., 2006). Kaufman *et al*. (2011) reported that geranyl aetone, citronellol, beta damascene and rosalva were highly toxic to *Ae. aegypti*, *An quadrimaculatus* and *Ae. albopictus* adults in laboratory and field evaluations with stability up to 8 days under laboratory conditions (Kaufman *et al*., 2011). Terpenoid compounds from clove, coriander, thyme, parsley and anis oils provided high larvicidal activity against *Ochleotatus caspius* with LC50 values ranging from of 7.5 mg L^{-1} to 156 mg L^{-1} (Knio *et al*., 2008). Anthraquinone compound, Emoien isolated from *Cassia nigricans* exhibited LC50 values as low as 2.4 mg L^{-1} against *Anopheles gambiaea* larvae (Georges *et al*., 2008). Similarly piperolein-A and piperine compounds extracted from *Piper nigrum* exhibited LC50 values as low as 1.46 and 1.53 mg L^{-1}, respectively, against *Ae. aegypti* (Simas *et al*., 2007).

2.10.2. Toxic activity of the phytochemicals on other haematophagous arthropods

Essential oil from *Eucalyptus globulus* showed higher toxicity (0.125 mg cm^{-2}) against *Pediculus humanus capitis* than the commercially used pediculides deltaphenothrin or pyrethrum (0.25 mg cm^{-2}) (Khater *et al*., 2009). Essential oils from *Cinnamomum camphora*, *Allium cepa*, *Matricaria piperita*, *M. chamomilla* killed 100% of adult buffalo lice, *Haematopinus tuberculatus* within two minutes under laboratory conditions. Choi *et al.* (2010) reported that essential oils from *Eugenia caryophyllata*, and *E. globulus* provided high mortality to d-phenothrin and pyrethrum resistant (resistance ratios up to 754) *Pediculus humanus capitis* (Choi *et al*., 2010). Palacios *et al*. (2009) reported that essential oils from *Citratus aurantium* (LC50 value 4.8 mg dm^{-3}) and *C. sinensis* (LC50 value 3.9 mg dm^{-3}) were highly toxic to *Musca domestica* (Palacios *et al*., 2009).

Although essential oils or their constituents possess good efficacy and are environmentally friendly, the majority of the essential oils are less effective than synthetic insecticides. Thus essential oil products might be better used in combination with synthetic insecticides rather than stand-alone products. Furthermore, essential oils may be used in rotation with synthetic insecticides for vector control strategies, especially in light of documented insecticide resistance of several active ingredients against haematophagous insects.

2.10.3 Growth regulating activity of essential oils

Essential oils have been reported to have a pronounced effect on the developmental period, growth, and adult emergence (Shallan *et al*., 2005; George *et al*., 2009).

Exposure of insect vectors to active botanical derivatives can affect result in an extension of the duration of development (Shallan *et al*., 2005). It is estimated that over one thousand plant species contain bioactive substances that act as Insect Growth Regulators (IGRs) (Varma and Dubey, 1998). Examples of such IGR's include ajugarins isolated from *Ajuga remora* (Marcard *et al*., 1986). Crushed aqueous extract of *Opuntia tuna, Callistemon lanceolatus, Clerodendron inerme* and *Lantana camara* severely affected *Cx. quinquefasciatus* molting and metamorphosis by interfering in production of larval to-larval, larval-to-pupal, pupal-to-adult intermediates, and supernumerary molts besides causing ecdysial failure and mortality (Neraliya and Srivastava, 1996).

2.10.4 Multiple activities of essential oils

Several essential oils or their constituents have been reported to possess multiple activities (insecticidal, repellent, ovicidal and growth inhibition properties) (Koul *et al*., 2008). Neem oil has been shown to act as a larvicide, oviposition inhibitor and growth regulator, against *Cx quinquefasciatus*, *An. culicifacies*, *An. stephensi*, and *Ae. aegypti* (Koul *et al*., 2008; Datta *et al*., 2010). Rosemary oil, pulegone, thymol and eugenol showed both larvicidal and ovicidal activity against *Ae. aegypti* (Waliwitiya *et al*., 2009). Essential oils from *Azadirachta indica, Z. zerumbet, Dolichos biflorus* and *Al. pudica* showed larvicide, adulticide and repellent activities against *Cx. gelidus* and *Cx. quinquefasciatus* (Kamaraj *et al*., 2010). Flower extract and essential oils of *Tagetes minuta* showed adulticide, larvicide and repellent activity against *Ae. aegypti, An. stephensi* and *Cx. quinquefasciatus* (Datta *et al*., 2010). Pine oil and linalool isolated from pine oil completely suppressed *Al. domestica* feeding and oviposition up to24 hours (Maganga *et al*., 1996). Prajapati *et al*. (2005) evaluated 10 essential oils

extracted from medicinal plants for larvicidal, adulticidal, ovicidal, oviposition-deterrent and repellent activities against *An. stephensi, Ae. aegypti* and *Cx. quinquefasciatus* (Prajapati *et al*., 2005).

Essential oils of *Juniperus macropoda* and *Pimpinella anisum* showed both larvicidal and ovicidal activity against *An. stephensi* and *Ae. aegypti*. Essential oils from *Cinnamomum camphora, Allium cepa, Matricaria piperita, M. chamomilla* killed 100% of adult buffalo lice, *Haematopinus tuberculatus* within two minutes under laboratory conditions. These oils also showed ovicidal properties against this species and protected buffaloes from *M. domestica, S. calcitrans Haematobia irritans* and *Hippobosca equine* flies for up to 6 days (Khater *et al*., 2009). Piperitenone oxide isolated from *Mentha spicata viridis* showed larvicidal, ovicidal, developmental toxicity, and repellent properties against larval and adult *An. stephensi* (Tripathi *et al*., 2004). Butler patented 77 plant and animal based compounds that showed attractants or repellent of which 29 showed insecticidal activities against *Ae. aegypti*, *Ae. albopictus, An. quadrimaculatus, M. domestica*, or *S. calcitrans* (Butler, 2006). Constituents of essential oils such as *Beta damascene*, citronellol, geranyl acetone, and rosalva showed insecticidal activity against several insect species including *Ae. aegypti, Ae. albopictus, An. quadrimaculatus, M. domestica*, and *S. calcitrans* and *Lutzomyia shannoni* (Mann *et al*., 2010, Kaufman *et al*., 2011). Celangulin isolated from *Celasutrus angulatus* showed gastrointestinal toxicity, antifeedant activity, contact toxicity, and inhibition of growth and development in *Ae. albopictus* (Xu *et al*., 2010).

2.10.5 Synergistic activity of phytochemicals

The effect of a phytochemical on the inhibition of insect vector growth and reproductive capacity is governed by insect species, plant species, plant parts, concentration and type of solvents used in an extraction. Most studies on the synergistic, antagonistic and additive toxic effects of binary mixtures involving phytochemicals have been conducted on agricultural pests rather than pests of medical importance (Shallan *et al*., 2005). The mosquitocidal activity of binary mixes has been reviewed by Shaalan *et al*. (2005). *Zanthaxylum piperitum* + 5% vanillin provided better protection against *Ae. gardnerii*, *An. barbirostris*, *Armigeres subalbatus*, *Cx. tritaeniorhynchus*, *Cx. gelidus*, the *Cx. vishnui* group, and *Mansonia uniformis* than *Z. piperitum* or vanillin alone or 25% DEET (Kamsuk *et al*., 2007). The addition of piperonyl butoxide significantly (3-250-fold) increased larvicidal activity of pulegone, thymol, eugenol, trans-anithole, and citronellal and rosemary oil against *Ae. Aegypti* (Waliwitiya *et al*., 2009).

Citronella oil, in concentrations ranging from 0.05% to 15% increased efficiency of cedarwood, lavender, peppermint, clove, garlic and eucalyptus oil against *Ae. aegypti* (Fradin, 1998). Similarly, the essential oils from *Blumea lacera* synergized pyrethrum activity (Kamsuk *et al*., 2007). An increase in the protection time was produced when essential oils from *Eugenia caryophyllata*, *Levisticum officinale* and *Thymus vulgaris* (3.2 to 3.5 h) were mixed with *Calophyllum inophyllum* oil producing protection times comparable to the most widely used synthetic repellent DEET (Hieu *et al*., 2010). The addition of 5% of vanillin in *Eu. globules* oil improved the protection time against *Ae. albopictus* (Ma *et al*., 1999). Whereas, addition of vanillin to *Zanthoxylum*

piperitum essential oils had a repellant effect with DEET-tolerant *Armigeres subalbatus* mosquitoes (Kamsuk *et al*., 2007).

Shallan *et al.* (2005) has argued that a less active natural pesticide could possess exceptional synergistic qualities in combination with other synthetic or natural insecticidal agents. Furthermore, joint-action of natural pesticides and synthetic pesticides might enhance control activities and minimize the development of insecticide resistance (Shallan *et al*., 2005).

2.10.6. Structure-Activity Relationships

Terpenes and other low molecular weight aromatic compounds such as alcohols, ketones, aldehydes, and carboxylic acid constitute the primary ingredients of essential oils, which determine the biological properties of the essential oils (Bakkali *et al*. 2008; Tripathi *et al*., 2009).

2.11 Essential oil chemistry

The volatile components of essential oils can be classified into four main groups: terpenes, benzene derivatives, hydrocarbons and other miscellaneous compounds (Haagen-Smit, 1948; Ngoh *et al*., 1998). Monoterpenoids are the most representative molecules constituting 90% of the essential oils and allow a great variety of structures with diverse functions. They are ten carbon hydrocarbon or their related compounds such as acyclic alcohols (e.g. linalool, geraniol, and citronellol), cyclic alcohols (e.g. menthol, isopulegol, terpeniol), bicyclic alcohols (e.g. borneol, verbenol), phenols (e.g. thymol, carvacrol), ketones (carvone, menthone, thujone), aldehydes (citronellal, citral), acids (e.g. chrysanthemic acid) and oxides (cineole).

The main group is composed of terpenes and terpenoids and the other of aromatic and aliphatic constituents all characterized by low molecular weight terpenes mainly the monoterpenes (C10) and sesquiterpenes (C15), but hemiterpenes (C5), diterpenes (C20), triterpenes (C30) and tetraterpenes (C40) also exist. Aromatic compounds occur less frequently than the terpenes and are derived from phenylpropane e.g. Aldehyde: cinnamaldehyde; Alcohol: cinnamic alcohol; Phenols: chavicol, eugenol; Methoxy derivatives: anethole, elemicine, estragole, methyl eugenols; Methylene dioxy compounds: apiole, myristicine, safrole. Essential oil extraction composition of oil varies to a large extent depending on the isolation method used. The chemical profile of the essential oil products differs not only in the number of molecules but also in the stereochemical types of molecules extracted. Steam distillation is the procedure most frequently used to isolate essential oils by Clevenger-type apparatus (Clevenger, 1928). However, distillation may influence the composition of the oil isolated, because isomerization, saponification and other reaction may occur under distillation conditions (Tripathi *et al*., 2009). The other methods of isolation of essential oils are solvent extraction, simultaneous distillation extraction, supercritical carbon dioxide and microwave ovens. The extraction product can vary in quality, quantity and in composition according to climate, soil composition, plant organ, age and vegetative cycle stage (Masotti *et al*., 2003; Angioni *et al*., 2006).

2.12 Insecticidal activities of *Tagetes minuta* and *Cymbopogon citratus*

Lemongrass oil is the essential oil obtained from the aerial part of *Cymbopogon citratus*. The plant has been widely recognized for its enthnobotanical and medicinal usefulness (Shah *et al*., 2011). The insecticidal (Arias *et al*., 1992; Aziz and Abbas,

2010; Kabera *et al*., 2011; Phasomkusolsil and Soonwera, 2011; Pushpanathan *et al*., 2006; Hindumathy, 2011), antifungal (Matasyoh *et al*., 2011), antimicrobial (Syed *et al*., 1995; Akin-Osanaiye *et al*., 2007), and the therapeutic properties (Shah *et al*., 2011) of its oil and extracts have been reported. Trado-medicinal preparations of the oil have been used both internally for alleviating colds and fever symptoms (Comerford, 1996) and externally to treat skin eruptions, wound and bruises (Spring, 1989). Plant essential oils in general have been recognized as an important natural source of pesticides–insecticides (Raguraman and Singh, 1997; Gbolade 2001), larvicides (Adebayo *et al*., 1999; Cavalcanti *et al*., 2004), and repellents (Thorsell *et al*., 1998). In this light, the present study attempts to ascertain the insecticidal activities of lemongrass oil against sand flies *Phlebotomus duboscqi* by evaluating its ovicidal, larvicidal and repellent activity affordable against the flies with a view to providing a natural product with long lasting insecticidal activity, safe for human life, human and domestic animal skin with no side effect and no feedback of environmental ill effect, as an alternative to synthetic chemical repellents.

Tagetes minuta commonly known as marigold has been shown to have both larvicidal as well as adulticidal activity against mosquitoes (Green *et al.*, 1991; Perich *et al*., 1994; Macedo *et al*., 1997; Pathak *et al*., 2000). Active components have been isolated from different parts of this plant. Green *et al*. (1991), reported mosquito larvicidal activity in the extract of *Tagetes minuta* flowers. Perich *et al*., (1994) compared biocidal activities of the whole-plant extracts of three *Tagetes species* and showed that *T. minuta* had the greatest biocidal effect on the larvae and adults of *Aedes aegypti* (L.) and *Anopheles Stephensi* (L). Bioassays of simultaneous steam distillated extracts of *T. minuta* flowers showed larval mortality at LC90 of 4 and 8

ppm and against the adult at 0.4 and 0.45% against *Aedes aegypti* and *Anopheles stephensi*, respectively (Perich *et al*., 1994). The extract from *T. minuta* was found to be most active among 83 plant species belonging to the compositae family, with a LC50 of 1 mg/l against *Aedes fluviatilis*. Active components of *T. minuta* have also been identified as thiophene derivatives, a class of compounds present in many plants of family Asteraceae (Macedo *et al*., 1994). Insecticidal activity of *Tagetes* species against *Anopheles gambiae*, the vector for malaria has been demonstrated (Seyoun *et al*., 2002). Previously, Ireri *et al*. (2010) demonstrated that the methanol and ethyl acetate crude extracts of *T. minuta* derived from the aerial parts had significant mortality against both males and females *P. duboscqi*, Neveu Lemaire (Diptera: Psychodidae) while Mong'are *et al*. (2012) found that the same crude extracts reduced the fecundity of *P. duboscqi* by 53%.

2.13 Description of *Tagetes minuta* and *Cymbopogon citratus*

Cymbopogon is a genus of about 55 species, which are indigenous in tropical and semi-tropical areas of Asia and are cultivated in South and Central America, Africa and other tropical countries. Common names include lemon grass, lemongrass, and barbed wire grass among others. These are tall, tufted perennial C_4 grasses with numerous stiff stems arising from a short, rhizomatous rootstock, (Weiss, 1997; Kumar *et al*., 2000) as with citrus flavor, and can be dried and powdered or used fresh. This tropical grass grows in dense clumps that can grow to 6 ft (1.8 m) in height and about 4 ft (1.2 m) in width, with a short rhizome (Fig. 2.1). In Kenya, it is native to Kakamega tropical rainforest. On the other hand, *Tagetes minuta* L., also known as Mexican marigold, is an annual, strongly aromatic herb. Stem is erect, on average of 1 m tall, branched and furrowed. Some plants may reach a height of 2 m. Leaves are

opposite (sometimes alternate on smaller branches). They are up to 5-15 cm long, divided into one terminal and several (3-7) lateral leaflets. Leaflets are elliptic, serrated up to 2-8 cm long. Flowers heads are up to 2 mm wide and 10 mm long. They are suited on short stalks, in erect dense corymbs at ends of branches (Fig. 2.2). Flowers are creamy yellow and appear in late summer. The plant blooms from September to December. Fruits are achenes, spindle shaped, flattened, 5-8 mm long and 0.6 mm wide, black, covered with short hairs and on apex of achenes are four pointed scales, one longer than the others (Hulina, 2008).

Fig. 2.1. *Cymbopogon citratus* Stapf plant aerial parts growing in Kakamega forest (Source: Author, 2013)

Fig. 2.2. Aerial and foliar parts of *Tagetes minuta L*. (Source: Author, 2013)

2.14 Chemical composition of essential oils of *T. minuta* and *C. citratus*

The chemical composition of the essential oil of *Cymbopogon citratus* has been shown to vary according to the geographical origin. On the overall, hydrocarbon terpenes, alcohols, ketones, esters and mainly aldehydes have constantly been registered (Abegaz *et al*., 1983; Matasyoh *et al*., 2011). The constituents of the Kenyan *C. citratus* as analysed GC-MS analysis have been shown to be dominated by monoterpene hydrocarbons which accounted for 94.25% of the oil (Aziz and Abbas, 2010; Matasyoh *et al*., 2011). The monoterpene fraction has been demonstrated to be characterized by a high percentage of geranial (39.53%), neral (33.31%), myrecene (11.41%) and geraniol (3.05%) (Fig. 2.3). Only 0.78% of the components identified were found to be sesquiterpenes (Aziz and Abbas, 2010; Matasyoh *et al*., 2011).

These components have been reported to have high antifungal activity (Matasyoh *et al*., 2011) and insecticidal activity (Aziz and Abbas, 2010).

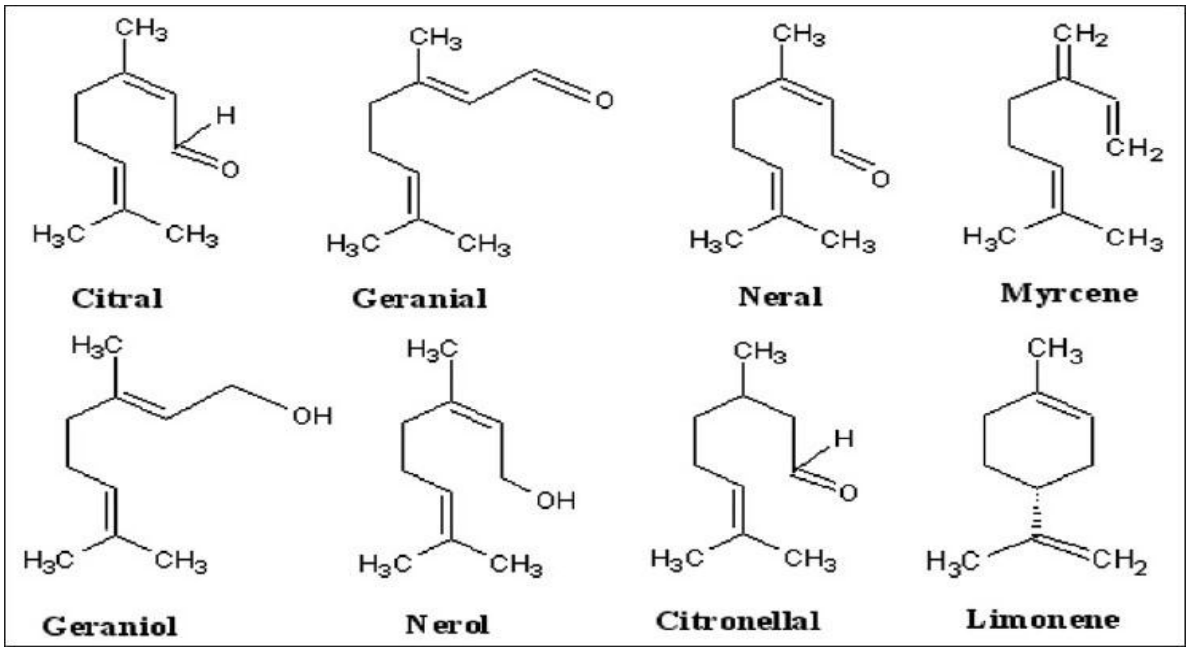


Fig. 2.3. Chemical structure of the major constituents of lemongrass essential oil (Shah *et. al.*, 2011)

On the other hand, a GC-MS analysis of the distillate of the aerial parts of *T. minuta* has revealed that the oil is rich in terpenes (Nchu *et al*., 2012). The major constituents of *T. minuta* essential oil have been demonstrated to be cis-ocimene (28.50%), beta-ocimene (16.83%) and 3-methyl-2-(2-methyl-2-butenyl)-furan (11.94%) (Nchu *et al*., 2012).

CHAPTER THREE

MATERIALS AND METHODS

3.1 Study site

The study was conducted at the Centre for Biotechnology Research and Development (CBRD) of the Kenya Medical Research Institute (KEMRI), Nairobi in the Leishmaniasis laboratory and at the behavioural and chemical ecology laboratory at the International Centre of Insect Physiology and Ecology (ICIPE), Nairobi. The institutes have the requisite facilities that enabled the studies to be undertaken, including a viable sandfly colony that is maintained at KEMRI.

3.2 Collection of plant materials

Fresh leaves of the lemon grass, *Cymbopogon citratus* were collected from the equatorial rainforest in Kakamega, Kenya. The leaves were screened and dry and/or damaged ones were discarded. The remaining undamaged leaves were packed and transported and used for the extraction of essential oils while they were still fresh. Floral and foliar parts of *T. minuta* plant were collected from Marigat sub-county of Baringo County, Kenya. The leaves of *C. citratus* and floral and foliar parts of *T. minuta* were packed in a cooler box and transported to the Behavioural and Chemical Ecology Department laboratory at ICIPE, Nairobi for the extraction of the essential oils. The plants' identities were confirmed by a taxonomist and Voucher specimens were deposited at KEMRI's Center for Biotechnology Research and Development (CBRD) for future reference.

3.3 Study design

The study was a comparative experimental design consisting of two plants with five different oil concentrations that were set and compared against each other and a negative and positive control for their activity against sand fly eggs, larvae, and adults and for the repellent activity against adult sandflies. All experiments were done in triplicate. Five treatments consisting of different concentrations (1.0, 0.75, 0.5, 0.25 and 0.125mg/ml) of the two essential oils *C. citratus* and *T. minuta* were performed along with a negative control consisting of Tween 80 (3%), which was also used for serial dilutions of the essential oils, and a positive control, cypermethrin (0.196 mg/ml). In the ovicidal assay, 30 eggs of the *P. duboscqi* vector were used in each experiment, totalling 450 eggs. On the other hand, in the larvicidal assay, thirty *P. duboscqi* larvae were used. In addition, in the adulticidal assay, thirty adult *P. duboscqi* specimens (15 males and 15 females) were utilized. In the repellency tests, 100 sandflies were used and each triplicate series contained 300 flies per each concentration of the essential oil.

3.4 Extraction of essential oils of *Tagetes minuta* and *Cymbopogon citratus*

Extraction of the essential oil of the lemon grass *C. citratus* was done as described by Adeniran and Fabiyi, 2012. The fresh leaves were again screened and undamaged ones were then immersed into a 500 ml round bottom flask and subjected to steam distillation. The mixture of steam and the volatile oil(s) generated was passed through a condenser and collected in a flask. A separating funnel was used to separate the oil(s) from water. After the extraction, the products were purified using rotary evaporator at fixed temperature of 50°C. After rotovap, the samples were left under fume hood for one hour to make sure all the ethanol left in the crude oil(s) was

completely vaporized to the hood environment. The recovered oil(s) was dried using anhydrous sodium sulphate and kept in a refrigerator at 4 ^{o} C for subsequent use (Adeniran and Fabiyi, 2012).

For the extraction of the essential oil(s) from *T. minuta*, fresh plant material was sliced and hydro-distilled by using a Clevenger-type apparatus (Clevenger, 1928), with slight modifications (Evans, 1989). Heat was provided by a heating-mantle equipped with a thermostat and the temperature maintained at 90 °C. The plant material was immersed into a 500 ml round-bottomed flask and hydro-distilled for 2 hours. The distillate was collected as the essential oil(s) band above the water (Nchu *et al*., 2012).

3.5 Gas chromatography and mass spectrometry analysis of essential oil(s) of *Tagetes minuta* and *Cymbopogon citratus*

The analysis of the essential oils was carried out in the Behavioural and Chemical Ecology Department laboratory at ICIPE, Nairobi. Samples of the essential oil(s) of each of the two plants were diluted in high purity dichlomethane (99.9%, Sigma®, Aldrich) analyzed on a coupled GC-MS using a Hewlett Parckard® (HP) 7890 Series A gas chromatograph (Agilent technologies®, Wilmington, DE, USA) coupled to a 5975 C Series mass spectrometer fitted with an 7683 B Series autosampler (Agilent technologies®, Wilmington, DE, USA) and a triple axis detector (Mburu, 2009). The GC is equipped with a non-polar capillary column (HP5 MS 5% with phenylmethyl silicone) that was 30 m (length × 0.25 µm (i.d.) and 0.25 µm (film thickness) for the separation of the chromatographic peaks. The GC is also coupled to a HP monitor

(L1710) for displaying of the chromatographic data which were acquired and studied using the 3365 MSD ChemStation® software (G1701Ea E.20.00.493).

Samples were injected in the split mode at a ratio of 1:10 to 1: 100. The injector was kept at 250°C and the transfer line at 280°C. The column was maintained at 50°C for 2 min and then programmed to 260°C at 5°C/min and held for 10 min at 260°C. The mass spectrometry was operated in the EI mode at 70 eV, in m/z range 42 to 350. Identification of the compounds was performed by comparing their retention indices and mass spectra with those found in literature (Adams, 1995) and supplemented by Wiley and QuadLib 1607 GC-MS libraries. The relative proportions of the essential oil(s) constituents were expressed as percentages obtained by peak area normalization, all relative response factors being taken as one (Matasyoh *et al.,* 2011).

3.6 Sand fly colony maintenance

Sandflies were obtained from a colony of *P. duboscqi* Neveu Lemaire that originated from Marigat District, Baringo County, Rift Valley, and were maintained at the Centre for Biotechnology Research and Development (CBRD) insectaries in Kenya Medical Research Institute, Nairobi. The colony of *P. duboscqi* was established using field-captured females that were held in cages and maintained according to the methods of Beach *et. al*. (1986), with some slight modifications. Briefly, female sandflies were fed on blood using Syrian golden hamsters that had been anaesthetized with sodium pentobarbitone (Sagatal®). The hamsters' under bellies were usually shaved using an electric shaver for easy access for feeding by sandfly. The sandflies were reared at 28 ± 1°C, and an average relative humidity (RH) of 85-95% and 12:12

h (light: dark) photoperiod in Perspex® insect rearing cages. Sandflies were fed *ad libitum* on slices of apple.

3.7 Assessing sandfly egg viability after treatment with the essential oils

In the *in vitro* tests on *P. duboscqi* eggs, aqueous extract solutions of *C. citratus* and *T. minuta* plant oil(s) were used at varying concentrations. Serial dilutions of the essential oils were done using Tween 80 which was also used as the negative control. DEET was used as the positive control. Tests were performed at 27 ^{0}C and 80% RH using 30 eggs placed in a vial with 1ml of each oil(s) concentration solution. The egg hatching was observed daily for 15 days and larval mortality was counted 25 days post-treatment, with the aid of stereomicroscope. Oil concentrations of 0.125; 0.250; 0.500; 0.750 and 1mg/ml of *C. citratus* and *T. minuta* essential oil(s) with 3 replicates per concentration were used. Each vial contained 30 eggs of *P. duboscqi*, totaling 550 eggs for the treatments and control experiments. The parameter that was under study here was hatching inhibition which was calculated as shown below;

$$\text{Per cent inhibition of egg hatching} = \frac{\text{Total number of eggs - Number of eggs hatched}}{\text{Total number of eggs}} \times 100$$

3.8 Assessing sandfly larval mortality due to the essential oils

Larvicidal activity was determined as previously described (Luitgards-Moura *et al*., 2002) and similar to the one generally applied for the bioassays of *Spodoptera* larvae (Ikbal *et al*., 2007). *Phlebotomus duboscqi* larvae at various stages of development were selected, counted and sprayed at varying concentrations of 0.125; 0.250; 0.500; 0.750 and 1mg/ml of *C. citratus* and *T. minuta* essential oils. Tween 80 and DEET

were used as negative and positive controls respectively. During the experimental set-up, thirty *P. duboscqi* larvae of each instar were gently placed into four triplicate series of vials using a camel hair brush wetted in distilled water to avoid stress and damage. The experiment was arranged such that first triplicate series contained 1st instar larvae, the second triplicate 2nd instar larvae, the third triplicate contained the 3rd instar larvae and the fourth triplicate contained the 4^{th} instar larvae in each vial. Mortality of larvae was recorded after24 hoursrs of treatment. While recording the percentage mortalities for each concentration, the moribund and dead larvae in five replicates were combined. It has been described that dead larvae are those that cannot be induced to move when they are probed with a needle in the siphon or the cervical region; moribund larvae are those incapable of rising to the surface (Luitgards-Moura *et al*., 2002, Ikbal *et al*., 2007).

The percentage mortality was calculated using the formula;

$$\text{Percent mortality} = \frac{\text{Number of dead larvae}}{\text{Number of larvae introduced}} \times 100$$

The corrections for mortality was done using Abbot's (1925) formula;

$$\text{Corrected percentage mortality} = \frac{\text{1- n in T after treatment}}{\text{1- n in C after treatment}} \times 100$$

Where n = number of larvae, T = treated, C = control.

3.9 Insecticidal effects of the essential oils of *Cymbopogon citratus* and *Tagetes minuta* on the adult sandfly, *Phlebotomus duboscqi*

3.9.1 Bioassays on sand flies with essential oils

Each oil concentration (1.0 ml), and the controls, DEET and Tween 80 were applied to the inner surface and bottom of each pot using a pipette. Thirty adult *P. duboscqi*

flies (15 males and 15 females) were released inside of the pots after the application of the oils at concentrations of 0.125; 0.250; 0.500; 0.750 and 1mg/ml of *C. citratus* and *T. minuta* essential oils. In these experiments, the parameters observed were insect mortality after 24, 48 and 72 hours, mortality rate differences between female and male insects and the number of eggs obtained from females subjected to the oils; The percentage mortality was calculated by using the formula below;

$$\text{Percent mortality} = \frac{\text{Number of dead adults}}{\text{Number of adults introduced}} \times 100$$

The corrections for mortality when necessary were done using Abbot's (1925) formula

$$\text{Corrected percentage mortality} = \frac{\text{\% Kill in treated - \% kill in control}}{\text{100 - \% Kill in control}} \times 100$$

3.10 Preparation of the oil extracts for repellent tests

Test samples of the essential oils of *T. minuta* and *C. citratus* essential oils were prepared by reconstituting measured amounts the essential oils in olive oil to have a series of concentrations of 0.125; 0.250; 0.500; 0.750 and 1mg/ml. Separate experiments using different cages were done in triplicates and hamsters were treated with the above preparations of the essential oils To prevent any cross-over effects between treatments with the different concentrations, each test with a given dose of each oil was applied to one hamster per cage. The olive oil and a standard repellent, N,N-diethyl-3-methylbenzamide (DEET) were used as negative and positive controls, respectively.

3.11 Assessing repellent activity of the essential oils

3.11.1 Assessing repellent effects of essential oils on adult sandflies

Sandflies, *Phlebotomus duboscqi* were obtained from a colony was maintained at the CBRD insectary. The basic design of this experiment was a modification of the World Health Organization Pesticide Evaluation Scheme (WHOPES) (2005). Experiments were carried out in the laboratory within tunnels constructed from glass cages with plaster of Paris on their bases. Two such cages, each measuring 25 cm (width) x 25 cm (height) x 40 cm (length), were joined on their open ends with an adhesive tape to form a tunnel measuring 25 x 25 x 80 cm. Before joining the two cages with tapping material, a removable cardboard frame of 1 cm thick that had holes (of 20 mm diameter) drilled though it was fitted in between the cages.

The repellency tests were conducted as previously described by Kasili *et al*. (2010), with some modifications. In the` shorter section of the tunnel, a restrained hamster, anesthetized with sodium pentobarbitone (Sagatal®), and acting as a bait (host) was placed. Separate experiments using different cages were conducted in which hamsters were treated by smearing their legs, tail, and mouth parts with 0.1ml of the various serial concentrations of 0.125; 0.250; 0.500; 0.750 and 1mg/ml of *T. minuta* and *C. citratus* extracts.

On one side of the flight tunnel were 100 sandflies that were held in Perspex cage while on the other, a hamster that had been smeared with the oil preparation on the legs, tail, and mouth parts was placed. Sand flies that were pre-starved for 4 h or more prior to testing were be used for the experiment. Different concentrations of the oils were tested in different cages and each was replicated three times. Each test cage

contained 100 flies, thus for a given dose, 300 flies were used. In addition, for each dose, only one hamster was used. The bioassay set up was such that flies flew freely in in the tunnel but had to make contact with the removable cardboard and pass through the holes to reach the bait (hamster) (Figs. 3.1 to3.2).

Following the release of flies in the tunnel, five minute landing counts were done at intervals of 30 minute, between 08:00hr and 11:00 hrs. Mean percent repellency for each concentration was calculated based on the data of the three replicates at the given times of observation. Percent repellency for the test oils and DEET was calculated using the formula:

$$\text{Repellency (\%)} = (N - R) / N \times 100$$

Where, N = number of flies landing on the negative control side; R = number of flies landing on side treated with test oil or DEET. Thus, efficacy of the candidate repellent could be assessed relative to DEET.

During tests, the bioassay room was maintained at 27°C and 80–95% RH. To obtain an acceptable estimate of effective dose (ED), ED_{50} and ED_{90}, the treated areas on the hamster were swabbed with Isopropanol pads.

3.11.2 Estimation of Protection time

To determine protection time, a modified screened two-cage arena (Barnard, 2005) was used. For the tests, 100 nulliparous, 5-7 day old sandflies, *P. duboscqi* were released into the holding cage. The sandflies were free to fly in the tunnel, make contact with the piece of removable perforated cardboard partition, pass through the holes and locate the restrained hamster in the adjacent cage. As in the other bioassays,

the hamsters were treated by smearing their legs, tail, and mouth parts with 0.1ml of concentrations of 0.125; 0.250; 0.500; 0.750 and 1mg/ml of *T. minuta* and *C. citratus* extracts. Following the release of flies into the tunnel, their biting ability was monitored between 08:00 hours to 11:00 hrs at intervals of 30 minutes. Observations were done for three minutes within each half of the cage and the total number of sandflies biting on the treated and control areas recorded. If no observations were made for the first 3 minutes of every half an hour exposure, the experiment was discontinued until the next half hour. The test was continued until at least two bites occurred and were followed by a confirmatory bite (second bite) in the subsequent exposure period. The time between application of the test oil and the second successive bite was recorded as the protection time.

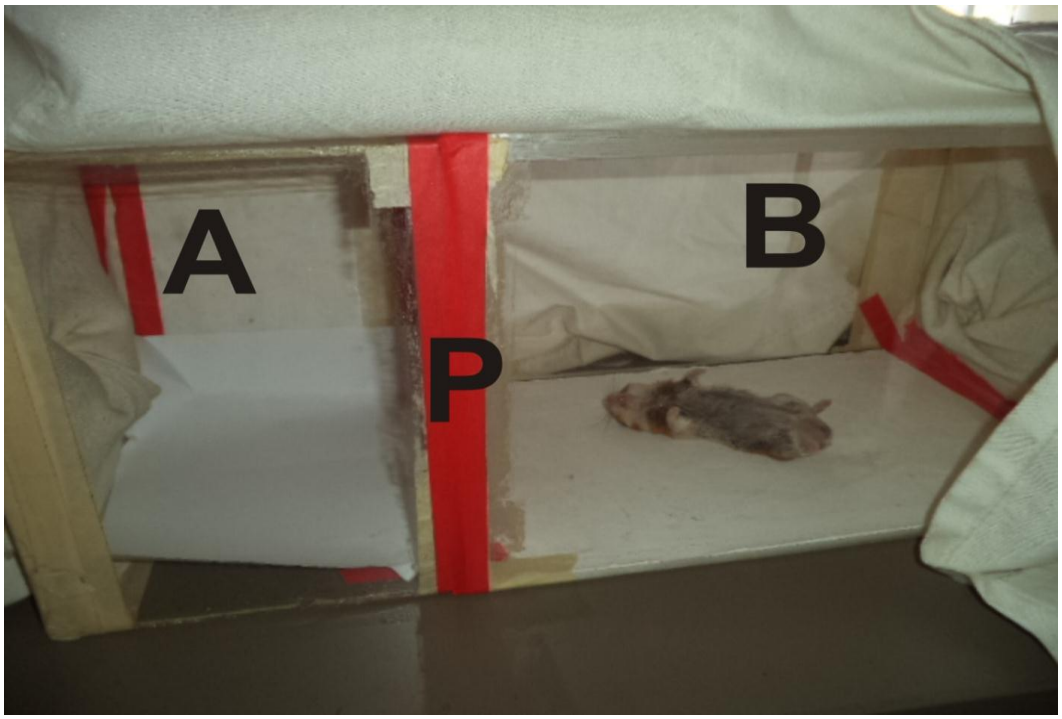


Plate 3.1 Perspex cage showing a restrained hamster on the floor of the second test chamber (B) of the wind tunnel. (Source: Author, 2014) Test insects were held in chamber (A) and moved through the perforated partition (P).

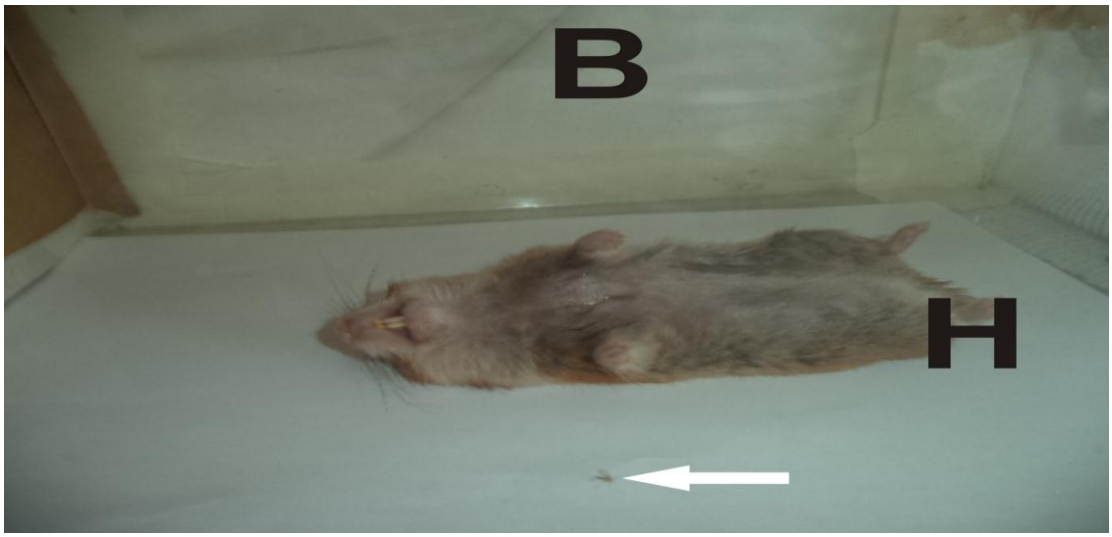


Plate 3.2. Restrained hamster (H) with one sand fly (tip of arrow) that had crossed into the second chamber (B). (Source: Author, 2014)

3.12 Ethical considerations

Approval for the study was granted by the Kenya Medical Research Institute's ethical review committee (IREC) and the Board of Postgraduate Studies of the University of Eldoret. The experiments were done in compliance with KEMRI's Animal Care and Use Committee (ACUC) recommendations and in conformity with Good Laboratory Practices (GLP).

3.13 Data Management and Statistical analysis

All data was recorded in laboratory notebook and transferred to excel spreadsheet protected by a password. Analyses were done using SPSS version 20.0. The dose mortality data was analysed by log-probit method of Finney (1971) and lethal concentrations for 50 and 90% mortality determined. The method allows calculation of LD_{50}, LD_{90} (Time involving respectively the knockdown of 50% and 90% of tested sandflies) and their confidence intervals. For the repellency tests, comparisons between and among the various groups subjected to different essential oils was determined by T-tests and ANOVA respectively.

CHAPTER FOUR

RESULTS

4.1 Chemical composition of *Cymbopogon citratus*

The volatile Lemon grass essential oil obtained from hydro distillation had the light yellow color, a lemony scent, and an extraction yield of 0.6% (v/w) when distilled from the fresh aerial parts of the plant, as was done in the present study. Thirty compounds that constituted 98.28% of the total oil's constituents were identified. The constituents identified by GC-MS analysis, their retention times (RT) and area percentages (%) are summarized in Table 4.1. The oil was dominated by monoterpene hydrocarbons. This monoterpene fraction was characterized by a high percentage of Geranial (20.45%), Myrcene (14.24%), Neral (11.57%), and Verbenene (9.26%) among others.

Table 4.1: Chemical composition of *C. citratus* essential oil identified by GC-MS

Serial no.	Compound	RT	Area (%)
1	1-methyl-1,3-Cyclohexadiene	5.57	3.76
2	3-methylene-Cyclohexene	5.95	0.14
3	Myrcene	11.15	14.24
4	1,3,8-Menthatriene	11.39	7.20
5	alpha-Terpinene	11.55	0.19
6	Verbenene	11.75	9.26
7	(Z)-beta-ocimene	11.93	1.28
8	(E)-beta-ocimene	12.11	1.26
9	gamma-Terpinene	12.31	0.11
10	para-Cymenene	12.89	6.42
11	Terpinolene	13.30	3.66
12	allo-Ocimene	13.47	0.61
13	2,6-dimethyl-1,3,5,7-octatetraene	13.63	1.54
14	2,3,5-Trimethyl-2,3,5-hexanetricarbonitrile	13.77	1.55
15	trans-Chrysanthemal	13.88	0.60
16	(Z)-Isocitral	14.08	1.84
17	Trans-p-Mentha-2 8-dienol	14.37	6.65
18	5-isopropyl-2-methyl-cyclopent-1-enecarbaldehyde	14.95	1.61
19	Citronellyl formate	15.13	0.42
20	Neral	15.33	11.57
21	delta-3-Carene	15.54	0.69
22	Geranial	15.85	20.45
23	2-Undecanone	16.01	1.04
24	thuj-3-en-10-al	16.36	0.14
25	Piperitenone	16.74	0.20
26	2-methyl-3-phenyl-propanal	17.53	0.16
27	3,5-Heptadienal, 2-ethylidene-6-methyl-	17.71	0.39
28	Z-Caryophyllene	17.80	0.17
29	(Z)-alpha-Bergamotene	17.93	0.11
30	2-Tridecanone	18.60	1.02
Total			**98.28**

4.2 Chemical composition of *Tagetes minuta*

The GC-MS analysis of the distillate of the aerial parts of *T. minuta* revealed that the extracted oil is rich in terpenes. A total of 29 compounds were identified representing 98.95 % of the total oil composition, and these are presented in Table 4.2. The major components of the essential oil were Dihydro-Tagetone (21.15%), (E)-Tagetone (16.21%), (Z)-Tagetone (14.99%), (Z)-beta-Ocimene (9.84%), Limonene (7.40%), allo-Ocimene (6.69%) and (Z)-Ocimenone (4.12%). Oxygenated monoterpenes were the most abundant chemical class of compounds in the essential oil.

Table 4.2: Chemical composition of *T. minuta* essential oil identified by GC-MS

Serial no.	Compound	RT	Area %
1	Ethyl 2-methylbutanoate	7.94	0.31
2	Pentanoic acid, ethyl ester	8.03	0.16
3	1-butanol 2-methyl- acetate	8.66	0.42
4	alpha-Thujene	9.69	0.65
5	alpha-Pinene	9.82	0.43
6	Camphene	10.14	0.51
7	Sabinene	10.67	1.17
8	Myrcene	11.03	0.62
9	alpha-Phellandrene	11.28	1.06
10	alpha-Terpinene	11.53	0.58
11	Limonene	11.79	7.40
12	(Z)-beta- Ocimene	11.97	9.84
13	Dihydro-Tagetone	12.33	21.15
14	2-Cyclohexen-1-one, 5-methyl-2-(1-methylethyl)-	12.89	2.33
15	allo-Ocimene	13.52	6.69
16	(E)-Tagetone	13.88	16.21

17	(Z)-Tagetone	14.01	14.99
18	Borneol	14.21	0.53
19	2-propenal,2-methyl-3-phenyl-	14.91	1.30
20	(Z)-Ocimenone	15.13	4.12
21	Car-3-en-2-one	15.24	2.81
22	Thymol	15.96	0.53
23	Piperitenone	16.72	1.79
24	(E)-Caryophyllene	17.80	0.62
25	Aromadendrene	18.04	0.55
26	alpha-Humulene	18.24	0.72
27	Germacrene D	18.58	0.38
28	Bicyclogermacrene	18.76	0.73
29	delta-Cadinene	19.05	0.35
Total			**98.95**

4.3 The effects of the essential oils of *Cymbopogon citratus* and *Tagetes minuta* against *Phlebotomus duboscqi* egg hatching

Results on ovicidal activities of the two plant essential oils against *P. duboscqi* egg hatching at different doses 15 days post exposure is presented in Table 4.3.

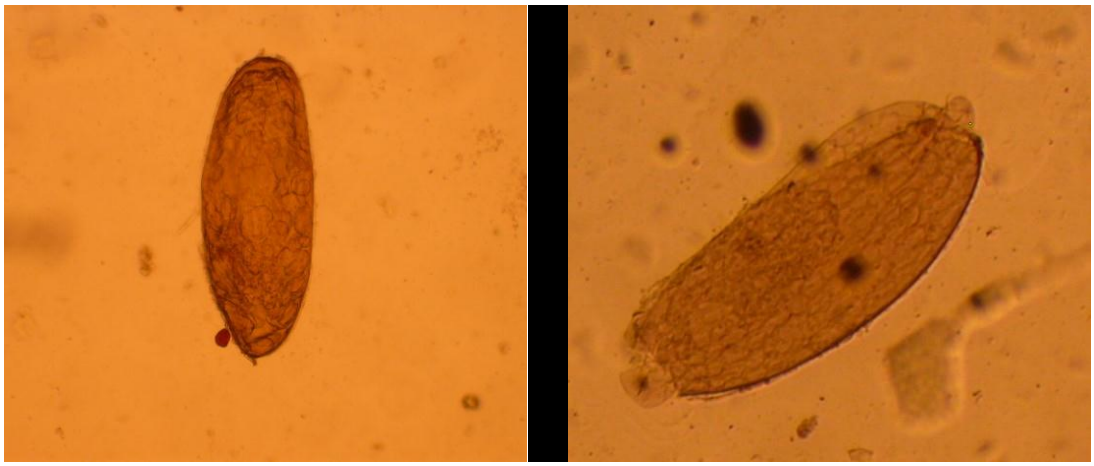
Table 4.3: The effect of *T. minuta* and *C. citratus* essential oils (EOs) on hatching of the eggs of *Phlebotomus duboscqi*

Inhibition of egg hatching (%) mean at 15 days post treatment		
Treatment	*T. minuta (%)*	*C. citratus (%)*
Conc. mg/ml		
0.125	54.44	87.78
0.25	61.11	91.11

0.50	66.67	94.44
0.75	76.67	97.78
1.00	84.44	100.00
Controls		
Tween 80	13.34	15.56
DEET 0.196 mg/ml	100.00	100.00
LD 50 (mg/ml)	0.25	0.077
LD 90 (mg/ml)	1.07	0.36
P value	0.00	0.00
χ^2	1.87	1.59

From the results, it is evident that the highest inhibitory activity was observed with lemon grass essential oil at 1mg/ml. This concentration produced 100% inhibitory activity on the hatching of eggs, and way comparable to that of the standard insecticide, DEET (positive control). *T. minuta* oil was less potent achieving 84.44% inhibition at 1mg/ml concentration. On the overall, all the concentrations of the EOs showed inhibitory effects on the eggs of *P. duboscqi* after 15 days of exposure. There was a direct and significant correlation (r=0.99) between the essential oil concentration and the inhibition of egg hatching (F = 12.098, df = 4, *P*=0.00038). The quantity of *C. citratus* essential oil needed to inhibit the hatching of 50% eggs was 0.077 mg/mL while the quantity required to inhibit hatching of 90% eggs was 0.36 mg/ml. On the other hand, the LD50 and LD90 values for *T. minuta* were relatively higher than those of *C. citratus* being 0.25 mg/ml and 1.07 mg/ml respectively. There was a highly significant difference in the inhibition of egg hatching between *T. minuta* and *C. citratus* (*P*= 0.001), with *C. citratus* achieving higher inhibition levels than *T. minuta*.

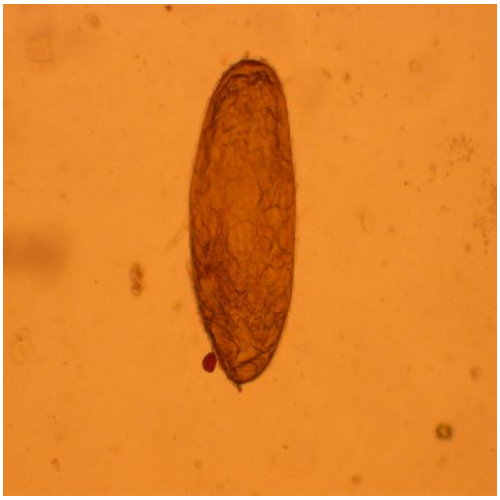
When 1 mg/ml of *C. citratus* was used on eggs in a vial, microscopic examination showed that there was loss of chorionic sculpturing with eggs puffing up and acquiring a more ovoid shape 4.1(b) as opposed to the usual elliptical shape 4.1(a). There was also loss of most of the exochorion constituents. Normal untreated and a treated eggs are shown on Plates 4.1 (a) and (b).

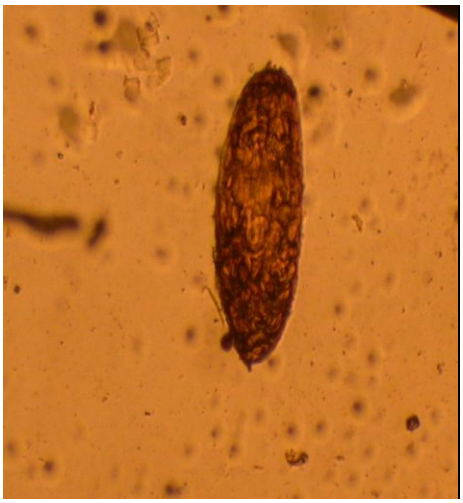


(a) **(b)**

Plate 4.1: A normal *P. duboscqi* egg (a) X300 magnification and (b) an egg treated with 1mg/ml of *C. citratus* (x 300 magnification).

A microscopic examination of *P. duboscqi* eggs exposed to *T. minuta* oil at 1 mg/ml showed that the eggs had darkened coloration. In addition, the eggs had shrunk. Normal untreated and a treated egg is shown on Plates 4.2 (a) and (b).





(a) **(b)**

Plate 4.2: A normal *P. duboscqi* egg (a) X 300 magnification and (b) an egg treated with 1mg/ml of *T. minuta* (x 300 magnification). (Source: Author, 2013)

Hatching period (days) of eggs mean ± S.E. upto 15 days post treatment		
Treatment	*T. minuta*	*C. citratus*
Conc. mg/ml		

0.125	12.33±0.57	12.87±0.57
0.25	12.87±1.15	13.33±1.15
0.50	13.67±0.57	14.33±0.57
0.75	14.33±0.57	14.87±0.57
1.00	15.00±0.57	0.00±0.00
Controls		
Tween 80	8.57±0.83	8.50±1.15
DEET(0.196) mg/ml	0.00±0.00	0.00±0.00

Table 4.4: The effect of *T. minuta* and *C. citratus* Eos on hatching period of the eggs of *Phlebotomus duboscqi*

On the overall, there was a delay in hatching period of the eggs exposed to the essential oils when compared to the controls ($P<0.05$). The hatching period of eggs subjected to *T. minuta* varied from 12 days for 0.125mg/ml to 15 days for 1mg/ml (Table 4.4). On the other hand, the hatching period for *P. duboscqi* eggs exposed to *C. citratus* essential oil varied from 13 days to 15 days with the later achieving a complete deterrent activity on egg hatching at 1mg/ml.

4.4 Larvicidal effects of the essential oils of *Cymbopogon citratus* and *Tagetes minuta* on immature stages of *Phlebotomus duboscqi*

The percent mortality values, LD_{50} and LD_{90} values of the 1st to the 4th instar larvae of *P. duboscqi* treated with different concentrations (ranging from 0.125 mg/ml to 1mg/ml) of the two essential oils extracted from *C. citratus* and *T. minuta* after24 hoursrs treatment are presented in Tables 4.5 and 4.6.

Table 4.5: Larvicidal activity of different concentrations of *C. citratus* against the 1st, 2nd, 3rd and 4th instars of *P.duboscqi* after 24h exposure compared to DEET (0.196mg/ml) and Tween 80

	Mortality (%)			
Concentration mg/ml	1st instar	2nd instar	3rd instar	4th instar
0.125	31.11	24.44	18.89	16.67
0.25	44.44	36.67	30.00	23.33
0.50	71.11	54.44	42.22	38.89
0.75	85.56	74.44	67.78	57.78
1.00	98.00	81.11	78.89	76.67
Controls				
DEET 0.196mg/ml	100.00	100.00	100.00	100.00
Tween 80	2.30	3.00	3.33	3.66
LD50	0.33	0.49	0.57	0.64
LD90	0.82	1.11	1.18	1.27
P value	0.24	0.53	0.82	0.97
χ^2	5.46	3.20	1.54	0.54

Table 4.6: Larvicidal activity of different concentrations of *T. minuta* extract against the 1st, 2nd, 3rd and 4th instars of *P.duboscqi* after24 hoursours exposure compared to positive and negative controls

	Mortality (%) upto24 hoursours post treatment			
Concentration mg/ml	1st instar	2nd instar	3rd instar	4th instar
0.125	27.78	15.56	14.44	8.89
0.25	36.67	21.11	18.89	16.67
0.5	46.67	31.11	31.11	27.78
0.75	68.89	64.44	55.56	51.11
1.00	78.89	75.56	63.33	65.56
Controls				
DEET 0.196	100.00	100.00	100.00	100.00
Tween 80	7.77	6.67	10	6.67
LD50	0.63	0.65	0.75	0.77
LD90	1.38	1.25	1.45	1.44
P value	0.87	0.47	0.68	0.9
χ^2	1.23	3.57	2.21	1.05

The two oils showed increasing concentration dependent larval mortality. *C. citratus* essential oil was the most effective larvicide. It killed 31.11, 44.44, 71.11, 85.56 and 98.00% of the 1st instar larvae in24 hoursrs at 0.125, 0.25, 0.5, 0.75 and 1.0mg/ml concentrations respectively. On the other hand, *T. minuta* killed 27.78, 36.67, 46.67, 68.89 and 78.89% of the 1st instar larvae at the same concentrations during the same time period. More deaths were observed among the 1st instar larvae than in any of the

other instars. The lowest mortality rates amongst the larvae exposed to *C. citratus* for 24h was observed amongst the 4th instar larvae. In this group, *C. citratus* killed 8.89, 16.67, 23.33, 38.89, 57.78 and 76.67 in 24 hours at 0.125, 0.25, 0.5, 0.75 and 1.0mg/ml concentrations respectively. There was no significant mortality difference between the larvicidal activity of the two essential oils of *C. citratus* and *T. minuta* in each of the four instar stages ($P > 0.05$).

4.5 Effects of essential oils of *Cymbopogon citratus* and *Tagetes minuta* on mortality and oviposition in adult sandflies, *Phlebotomus duboscqi*

Insecticidal effects of the essential oils of *C. citratus* and *T. minuta* on adults of the sandfly, *P. duboscqi* 24, 48 and 72 hours post treatment are shown in Tables 4.7 – 4.9. Also, the number of eggs laid by female flies during the same period are included. Among the two oils, that of *C. citratus* was significantly ($P < 0.05$) more potent and caused higher mortality than that of *T. minuta* on both male and female sand flies. The results show that, after24 hours, treatment with the oil of *C. citratus* at a concentration of 1 mg/ml, a mortality of 91.11 and 88.89 % against female and male sandflies, respectively was obtained. However, the essential oil of *T. minuta* at the same concentration, recorded a relatively lower mortality of 71.11% and 66.67 % against female and male sand flies respectively was obtained. The results of this study demonstrate that the effects of the oils were dose-dependent and increased with the concentration of the oil. The low concentrations tested inflicted low levels of mortality. This is clearly evident for all the concentrations tested with the lowest one (0.125 mg/ml) of *C. citratus* and *T. minuta* oils causing 51.11 and 28.89% mortality, respectively in female flies. Further, the mortality levels recorded also increased with time. Thus, the highest mortality levels were observed at 72 hours post treatment for

all the concentrations tested. At 72 hours post treatment, the essential oils of *C. citratus* and *T. minuta* at a concentration of 1 mg/ml recorded a mortality of 100.00 and 82.22 % respectively, on female sandflies (Table 4.8). At the same concentration, *C. citratus* and *T. minuta* oils caused mortalities of 100.00 and 88.89% respectively, in male sandflies (Table 4.9). There was no statistical difference in mortality rates between males and females when subjected to the two oils *C. citratus* and *T. minuta* at24 hours, 48 h and 72 h ($P > 0.05$). However, there was a significant difference between the mortality rates of *C. citratus* and *T. minuta* ($P < 0.05$) observed for both male and females after24 hours (P=0.00014), 48 h (P=0.0000238) and 72 h (0.00084). The LD_{50} values for *C. citratus* and *T. minuta* oils were 0.07mg/ml and 0.2 mg/ml respectively.

Table 4.7: Cumulative mortality (mean percentage ± S.D.) of essential oils of *C. citratus* and *T. minuta* in the first24 hours on adults of *Phlebotomus duboscqi*

		Mortality (%) at 24 hours post treatment					
		C. citratus			***T. minuta***		
	Concentration (mg/ml)	**% males**	**% females**	**No. of eggs laid**	**% males**	**% females**	**No. of eggs laid**
	0.125	44.44±0.58	51.11±0.58	11.33±5.86	26.67±1.00	28.89±0.58	22.67±7.51
	0.25	62.22±1.53	64.44±1.15	7.67±4.51	35.56±0.58	37.77±0.58	17.33±3.51
	0.50	68.89±2.08	75.56±1.15	6.33±3.79	42.22±0.58	42.22±1.53	12.67±5.13
	0.75	86.67±1.00	82.22±0.58	4.67±2.52	51.11±1.53	53.33±1.73	9.33±3.51
	1.00	88.89±1.15	91.11±0.58	2.33±1.53	71.11±0.58	66.67±1.00	6.67±1.52
DEET	0.196	100±0.00					
Tween 80	53.4	2.22±0.58					

Table 4.8: Cumulative mortality (mean percentage ± S.D.) of essential oils of *C. citratus* and *T. minuta* in the first 48 h on adults of *Phlebotomus duboscqi*

		Mortality (%) at 48 hours post treatment					
		C. citratus			*T. minuta*		
	Concentration (mg/ml)	**% males**	**% females**	**No. of eggs laid**	**% males**	**% females**	**No. of eggs laid**
	0.125	75.56±0.58	66.67±1.00	19.67±9.07	46.67±1.00	40.00±0.00	39.67±3.51
	0.25	80.00±0.58	75.56±0.58	12.67±4.51	44.44±1.53	42.22±1.15	33.67±13.43
	0.50	84.44±0.58	84.44±0.58	9.33±1.53	51.11±0.58	53.33±1.00	25.67±1.79
	0.75	95.56±0.58	88.89±0.58	6.67±1.155	55.56±1.52	57.78±1.15	18.33±2.89
	1.00	97.78±0.58	100.00±0.00	3.33±0.58	75.56±0.58	73.33±1.00	12.33±1.53
DEET	0.196	100.00±0.00					
Tween 80	53.4	4.44±0.58					

Table 4.9: Cumulative mortality (mean percentage ± S.D.) of essential oils of *C. citratus* and *T. minuta* in the first 72 h on adults of *Phlebotomus duboscqi*

		Mortality (%) at 72 hours post treatment					
		C. citrates			*T. minuta*		
	Concentration (mg/ml)	**% males**	**% females**	**No. of eggs laid**	**% males**	**% females**	**No. of eggs laid**
	0.125	84.44±0.58	68.89±0.58	29.00±7.94	57.78±0.58	44.44±0.58	51.00±5.57
	0.25	88.89±0.58	77.78±0.58	21.33±4.93	64.44±1.52	46.67±1.00	36.67±19.60
	0.50	93.33±1.00	95.56±0.58	11.00±2.66	73.33±1.00	62.22±0.58	22.00±10.58
	0.75	97.78±0.58	97.78±0.58	7.67±1.53	77.78±0.58	75.56±0.58	23.67±4.51
	1.00	100.00±0.00	100.00±0.00	3.33±0.58	88.89±0.57	82.22±0.58	18.67±8.62
DEET	0.196	100±0.00					
Tween 80	53.4	6.66±0.00					

With regard to the number of eggs that were laid by female sandflies that were treated with the essential oils, those treated with the oil of *C. citratus* oil were significantly lower than those laid by sand flies that were treated with that of *T. minuta* oil (P< 0.05; P= 0.00084). In comparison with the controls, flies subjected to Tween 80 which was a negative control laid significantly higher (P> 0.05) number of eggs than those treated with the essential oils of *C. citratus* and *T. minuta*. The number of eggs laid by female sand flies exposed to 0.125 mg/ml of *C. citratus* essential oil after24 hoursours exposure was 11.33±5.86 compared to 22.67±7.51 laid by those exposed to *T. minuta* during the same period. The number of the eggs oviposited reduced with an increase in concentration of the essential oils so that female sand flies exposed to *C. citratus* essential oil at 1mg/ml and at24 hoursours exposure oviposited 2.33±1.53 while those exposed to *T. minuta* oviposited 6.67±1.52 eggs during the same time period.

4.6 Repellent effects of essential oils of *Cymbopogon citratus* and *Tagetes minuta* on the sandfly, *Phlebotomus duboscqi*

In the dose-response study for determining effective dose, the results on ED_{50} and ED_{90} values are shown in Table 4.10. The ED_{50} and ED_{90} values of essential oil of lemon grass, *C. citratus* were determined to be 0.04 and 0.79 mg/ml respectively while those for the oil of *T. minuta* were 0.1 and 12.58 mg/ml, respectively. In addition, the percentage repellency of the two essential oils for against *P. duboscqi* is presented in Table 4.11. The essential oil of *C. citratus* at three concentrations (1, 0.75 and 0.5mg/ml) provided the highest repellency with 100%, 87.67 and 89.13 respectively at 180min. On the other hand, the repellency of *T. minuta* essential oil at similar concentrations of 1, 0.75 and 0.5mg/ml was relatively lower than that of *C.*

citratus at 88.89%, 79.56 and 52.2 respectively at 180 min. The most potent oil was that of *C. citratus* at 1mg/ml that elicited an average repellency of 99.8% (range, 99.8-100%; ED_{50} 0.04) and a mean biting rate of 0.8 at various concentrations (Table 4.12). In general, the percentage repellency of the two essential oils increased when the concentration of these essential oils increased, in contrast, biting rates decreased when the concentration increased. The results showed significant differences in both the percentage of repellency and the number of sand flies biting ($P<0.05$).

Data on the protection time conferred by different concentrations of the two essential oils are shown on table 4.13. The data shows that 1mg/ml and 0.75 of essential oil of *C. citrates provided* 100% protection for up to 3 hours. On the other hand, 1mg/ml of the oil of *T. minuta* conferred lesser protection as compared to that of *C. citratus* for up to 150 minutes.

Table 4.10: Effectiveness of *C. citratus* and *T. minuta* essential oils against *Phlebotomus duboscqi* tested on hamsters as repellents.

Repellents	No. flies	ED50 (mg/ml)	95%C.L* (mg/ml)	ED90 (mg/ml)	95%C.L* (mg/ml)
C. Citratus	100	0.039	0.039±0.0082	0.79	0.79±0.0082
T. minuta	100	0.10	0.1±0.0367	12.58	12.58±0.0367
Controls					
DEET 0.196mg/ml	100	0.0009	0.0009±0.0001	0. 0015	0. 0015±0.497

*Mean dosages are significantly different ($P<0.05$) from each other if 95% confidence limits (C.L) do not overlap.

ED50: Effective dose that causes 50% of prohibiting of bites; ED90: Effective dose that causes 90% of prohibiting of bites

Table 4.11: Repellent activities (%) of Lemon grass and *T. minuta* essential oils in five concentrations ranging from 0.125 to 1 mg/ml against *P. duboscqi*, Neveu Lemaire

		30 min	60 min	90 min	120 min	150 min	180min
			Repellencies				
	Oil conc.		(%)				
Essential oil	mg/ml						
	0.125	96.875±0.20	84.62±2.08	53.40±2.14	52.05±0.38	43.91±0.19	51.3±1.71
	0.25	96.875±0.19	98.21±0.33	98.81±0.19	90.66±0.51	83.57±1.90	59.13±0.51
C. citratus	0.50	97.81±0.30	97.69±0.20	98.09±0.20	92.94±0.20	93.70±1.00	89.13±1.20
	0.75	99.38±0.20	97.44±0.19	98.34±0.19	99.0±0.19	100.0±0.00	87.67±0.67
	1.00	100.00±0.00	100.00±0.00	98.72±0.20	99.77±0.20	100.00±0.00	100.0±0.00
	0.125	83.72±1.00	58.46±2.14	48.94±2.83	44.31±3.42	28.69±2.36	21.49±2.27
Tagetes minuta	0.25	90.23±0.80	64.10±2.20	70.21±2.08	60.98±4.55	51.3±3.01	46.81±5.81
	0.50	96.88±0.33	91.53±0.33	86.53±0.67	74.51±3.84	58.22±3.34	52.22±7.78
	0.75	98.14±0.40	100.00±0.00	97.87±0.19	88.45±0.84	64.35±2.34	75.96±1.76
	1.00	100.0±0.00	100.00±0.00	98.81±0.19	98.68±0.00	91.30±0.60	88.89±1.57
Controls							
DEET	0.196	100.0±0.00	100.0±0.00	100.0±0.00	100.0±0.00	100.0±0.00	95±1.70

Table 4.12: Biting rates of *P. duboscqi* sandflies when tested against five ranging from 0.125mg/ml to 1.0 mg/ml of *C. citratus* and *T. minuta* essential oils DEET and Tween 80

		30 min	60 min	90 min	120 min	150 min	180min
	Oil conc.		Mean number of biting sandflies				
Essential oil	mg/ml						
	0.125	0.6±0.20	6.0±2.08	21.90±2.14	21.1±0.38	25.8±0.19	22.4±1.71
	0.25	1.0±0.19	0.67±0.33	0.56±0.19	4.11±0.51	7.56±1.90	18.8±0.51
C. citratus	0.5	0.7±0.30	0.9±0.20	0.9±0.20	0.7±0.30	2.9±1.00	5.0±1.20
	0.75	0.20±0.20	0.89±0.19	0.78±0.19	0.00±0.00	0.0±0.00	0.00±0.00
	1	0.00±0.00	0.00±0.00	0.60±0.20	0.1±0.01	0.00±0.00	0.00±0.00
	0.125	0.22±0.19	16.2±2.14	23.8±2.83	28.4±3.42	32.8±2.36	36.9±2.27
Tagetes							
minuta	0.25	0.78±0.19	14.22±2.22	14.0±2.08	19.89±4.55	22.44±3.01	25.0±5.81
	0.5	0.8±0.20	0.2±0.20	6.3±0.67	13.1±3.84	19.22±3.34	22.44±7.78
	0.75	0.78±0.40	0.00±0.00	0.78±0.19	0.33±0.33	16.4±2.34	11.3±1.76
	1	0.00±0.00	0.00±0.00	0.56±0.19	0.67±0.00	0.11±0.20	5.22±1.57
Controls							
DEET	0.196mg/ml	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.33±0.13
Tween 80		43.00±6.30	39.00±2.50	47.00±7.1	51.00±5.5	46±1.00	47.00±7.50

KEY:
DEET - n, n-diethyl-3-methylbenzamide
Tween 80 - Polysorbate 80

Table 4.13: Protection time of Lemon grass and *T. minuta* essential oils in five concentrationsranging from 0.125 to 1.0mg/ml against *P. duboscqi*, Neveu Lemaire

Essential oils			Controls	
Oil conc.	**Protection time (min)**		Protection time (min)	
mg/ml	Lemon grass	*T. minuta*	DEET	Tween 80
0.125	60	30	>180	<30
0.25	120	30		
0.5	120	60		
0.75	180	120		
1	180	150		

CHAPTER FIVE

DISCUSSION

5.1 Chemical composition of *C. citratus* and *T. minuta*

There are many reports on the chemical composition of the oils from the plants belonging to the species *C. citratus* (Chisowa *et al*., 1998; Kasali *et al*., 2001; Menut *et al*., 2000; Olivero-Verbel *et al*., 2010; Sacchetti *et al*., 2005; Sidibé *et al*., 2001; Boukhatem *et al*., 2014; Vázquez-Briones *et al*., 2015). Most of these reports indicate that neral and geranial are the main characteristic constituents of *C. citratus* (Ekpenyong *et al*., 2014). The results of the GC-MS analysis of *C. citratus* obtained for this study concur with previous studies by Matasyoh *et al.* 2011, which demonstrated that the oil was dominated by monoterpene hydrocarbons which accounted for 94.25% of the oil. In the study, the monoterpene fraction was characterized by a high percentage of geranial (39.53%), neral (33.31%), myrecene (11.41%) and geraniol (3.05%). Only 0.78% of the components identified were sesquiterpenes (Matasyoh *et al*. 2011). In other studies, Farhang *et al*. (2013) identified α- citral (39.16 %), Z- citral (30.95 %), limonene (5.83 %), caryophyllene (3.44 %) and ceranyl acetate (3.1 %) as the main components in *C. citratus* essential oil. Gupta *et al*. (2011) found that the main components of *C. citratus* essential oil were dominated by citral (77.8%), limonene and traces of eucalyptol (4.0%), geraniol (2.7%), 6 methyl-5-hepten-2-one (2.4%) and geranyl acetate (1.1%). The differences in the composition of the essential oil might have been derived both from harvest time and local, climatic and seasonal factors, or it may be hypothesized that these samples belong to a different chemotype (Rahimi-Nasrabadi *et al.*, 2013).

The qualitative and quantitative analyses of the essential oil extract obtained from *T. minuta* in this study showed that there are six major components in the extract. The major components of the essential oil were Dihydro-Tagetone (21.15%), (E)-Tagetone (16.21%), (Z)-Tagetone (14.99%), (Z)-beta-Ocimene (9.84%), Limonene (7.40%), and allo-Ocimene (6.69%) which represented more than 70% of the essential oil The results of this study are consistent with those found by Moghaddam *et al.* (2004) and Garcia *et al.* (2012). The *T. minuta* essential oil used in this study was rich in terpenes, as determined by GC–MS analyses.

5.2 The effects of the essential oils of *Cymbopogon citratus* and *Tagetes minuta* against *Phlebotomus duboscqi* egg hatching

Results from this study show that both *T. minuta* and *C. citratus* essential oils demonstrated significant ovicidal activity against *P. duboscqi* sandflies in a dose dependent manner, with the percent hatchability being inversely proportional to the concentration of extracts. In addition, the results indicated that *C. citratus* essential oil demonstrated a higher inhibition potency than *T. minuta* essential oil (P= 0.0015). In fact, at 1mg/ml, *C. citratus* essential oil inhibited egg hatching completely, only comparable to the standard insecticide DEET used in this study. Mong'are *et al*. (2012) demonstrated that crude extracts of *Tarchonanthus camphoratus (Asteraceae), Acalypha fruticosa (fabacea) and Tagetes minuta* (*asteraceae*) reduced significantly the fecundity of *P. duboscqi* by 73% (*A. fruticosa*), 53% (*T. minuta*) and 26% (*T. camphoratus*) in a dose dependent manner. The *Mexican marigold* plant has been credited to have a number of properties including antimicrobial, insecticidal and nematicidal activity (Natarajan *et. al*., 2006; Piccaglia *et al*., 1997; Hulst *et al*., 1989; Romagnoli *et. al*., 1994). In addition, 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; Priyanka *et al.* 2013).

Many essential oils are composed of a variety of terpenoid compounds (De Paula *et al*., 2004). The *T. minuta* essential oil used in this study was also rich in terpenes, as determined by GC–MS analyses. The great majority of the literature on the effects of terpenoids on insects has reported growth inhibition, impaired maturation, reduced reproductive capacity, appetite suppression and death of predator insects by starvation or direct toxicity (Viegas-Ju´nior, 2003). The monoterpene limonene has demonstrated insecticidal activity by penetrating the cuticle of the insect (contact effect), by respiration (fumigant effect) and through the digestive system (ingestion effect) (Prates *et al*., 1998). The essential oils of the tested plants could have affected the eggs probably through contact direct toxicity effects.

T. minuta oil reportedly has aphicidal properties (Tomova *et al*., 2005). The terpenes in *T. minuta* oil are responsible for the toxic effects observed in mosquitoes (Seyoun *et al*., 2002), and the insecticidal activity of *T. minuta* oil has also been observed against stored product pests (Sarin, 2004). The results obtained in this study indicate that the *T. minuta* essential oil prepared in this study includes beta-ocimene, a tick repellent. *Hyalomma rufipes* adults display a significant dose-repellent response to *T. minuta* essential oil (Nchu *et al*., 2012). The compound tagetone may be related to a delayed molting effect in the engorged nymphs of *H. rufipes* (Nchu *et al*., 2012).

In Western Kenya, households' grow and tender *C. citratus* plants around their houses and cattle pens with a view of repelling mosquitoes and other biting insects (Personal information). Lemon grass has been demonstrated to have both repellant and toxic effects against arthropods. A methanol-leaf extract of lemongrass showed various degree of repellency and larvicidal effects against a malaria vector, *Anopheles arabiensis* (Moore *et al*., 2007). In addition, Karunamoorthi and Ilango (2010a) and Karunamoorthi *et al*. (2010b) also demonstrated that lemon grass can provide protection against bites of *Anopheles darlingi* and *Mansonia* spp. while Morsy *et al.* (1998) found out that solvent extracts of lemon grass have larvicidal activity against third instar larvae of *Chrysomyia albiceps*. Lemon grass extract has also been found to reduce a cattle tick, *Boophilus microplus*, infestation on naturally infested Holstein cows (Heimerdinger *et al.* 2006). Pushpanathan *et al.* (2006) reported that distilled oils extracted from lemongrass had larvicidal and ovicidal activity against the mosquito *Culex quinquefasciatus*. Jarongsak *et al.* (2009) reported that the essential oil of lemon grass at the rate of 75 µg/cm^3 has the highest inhibitory effect against dust mites, resulting in 97.3± 4.7 mortality hence proving that lemon grass has the potential to be a chemical control agent of dust mites. The high efficiency of *C. citratus* against the eggs could be attributed to the presence of a large proportion of citral (Geranial 20.45 and Neral (11.57%), in its chemical composition. The biological activity of these compounds was previously demonstrated in the *in vivo* evaluation of lemongrass essential oil against *Plasmodium berghei* (Tchoumbougnang *et al*., 2005) and in the evaluation of *in vitro* antifungal activity of essential oils of citrus on the mycelial growth of *Phaeoramularia angolensis* (Jazet *et al*. 2002).

Other studies have tested different concentrations of the essential oil of lemon grass for their ovipositional inhibition, antifeedant activity and insecticidal properties against the lesser cotton leafworm, *Spodoptera exigua* (Sharaby, 1988). The results obtained showed that 2% concentration of oil emulsion inhibited egg laying and 0.5% concentration more than reduced by 70% the number of eggs deposited on oviposition sites. Older egg masses were more affected by treatment than the newly laid eggs. A concentration of 2% inhibited hatchability when sprayed on egg masses before hatching. Lemon grass oil effectively controlled *S. exigua* as an ovicidal and larvicidal agent (Sharaby, 1988).

On egg hatching period, the results from the current study showed that the mean numbers of eggs that hatched were lower in the treated groups than in the negative control. In addition, there was an inverse relationship between essential oil concentrations and ovicidal activity. As the concentration of essential oil increased from 0.125mg/ml and up to 1.0 mg/ml, the hatching rate decreased. *C. citratus* oil at 1.0 mg/ml produced 100% deterrent activity on egg hatching. The hatching period for *T. minuta* essential oil ranged from 12 days at 0.125 mg/ml to 15 days at 1.0mg/ml. Previous studies by Srinivasan and Panicker (1987) on the hatching period of the phlebotomid sandfly, *P. papatasi* in the laboratory demonstrated that the incubation period of eggs of a single cohort ranged from 7 to 9 days (mean 7.81 ±0.61 days). This study produced similar results for the controls at 8.57±0.83 days. The mode of action of essential oil constituents has not been known yet, although, it may be due to the inhibition of various biosynthetic processes in the egg (Prates *et al*., 1998). Lemon grass oil is also comprised of various groups of chemical compounds such as eugenol, citronellal, geraniol, citral, trans-geraniol and limonene. These compounds have

properties to antibacterial, antifungal and insecticide (Simic *et al*., 2008; Kang *et al*., 2009; Maia and Moore, 2011; Setiawati *et al*., 2011; Shapiro, 2012; Hassain *et al*., 2012; Thein *et al*., 2013).

5.3 Larvicidal effects of *C. citratus* and *T. minuta* on the immature stages of *P. duboscqi*

The results of the present study demonstrate that the essential oils of *T. minuta* and *C. citratus* exhibit larvicidal effects against *P. duboscqi* larvae in an increasing dose dependent manner. In fact, a concentration of 1mg/ml of *C. citratus* essential oil exhibited a mortality of 98% against the 1st instar larvae with an LD_{50} value of 0.33mg/ml and LD_{90} of 0.82 mg/ml. The findings of this study corroborate previous studies which have demonstrated that *C. citratus* essential oils are effective against other arthropod larvae. Karunamoorthi and Ilango (2010) demonstrated that the LC_{50} and LC_{90} values of *Cymbopogon citratus* against *Anopheles arabiensis* Patton, a potent malaria vector were 74.02 and 158.20 ppm, respectively. In their data, a chi-square value of 2.760 was found to be significant at a probability level of 0.05 (Karunamoorthi and Ilango, 2010). In another experiment, three plant essential oils namely *Ocimum gratissimum*, *Cymbopogon citratus* and *Ageratum conyzoides* were tested against the 4^{th} instar larva of *Aedes aegypti* under laboratory conditions. It was found that after24 hoursours of exposure, *A. conyzoides, C. citratus* and *O. gratissiumum* gave 100% larval mortality at 120, 200 and 300 ppm respectively (Sosan *et al*., 2001). The results thus revealed that different formulations of plant essential oils can be effectively used for mosquito control programme strategies (Sosan *et al*., 2001). Further, Cavalcanti *et al.* (2004) demonstrated that the essential oils of *O. americanum* and *O. gratissimun* were as potent as *L. sidoides* and *C. citratus* in the larvicidal activity against *A. aegypti* and caused 100% mortality at a

concentration of 100 ppm. The results of the current study are however not concurrent with previous studies by Soonwera and Sinthusiri (2014), which demonstrated that although lemongrass oils exhibited excellent pupicidal and adulticidal activities against the house fly *Musca domestica* L. pupae and adults, the oil was non toxic to housefly larvae (Soonwera and Sinthusiri, 2014) . The different species of the insects may explain this difference.

Secondary compounds in *Tagetes* have been shown to be effective in the control of numerous organisms including insect pests through different mechanisms (Usher, 1974; Maradufu *et al*. 1978; Saxena and Koul 1982; Jacobson, 1990). Crude extracts from *T. minuta* aerial parts have been found effective against mosquito larvae with LC50 and LC90 of 1.5 and 1 mg/l, respectively (Macedo *et al*., 1997). In addition, *T. minuta* was found to have larvicidal effect against *Aedes aegypti* larvae at 10 ppm (Green *et al*., 1991). The terpene and ocimenone in *Tagetes* were found as larvicidal only at higher concentrations than the whole oil.

The discovery of insecticide activity of phototoxins present in *Asteraceae* species stimulated the interest in this plant family as part of the search for new plant derived insecticides (Rawls, 1986). This study has demonstrated that *T. minuta* has larvicidal activity and thus validates its traditional use. The plant has been used extensively for its medicinal value, food, fodder and repellent activities against insects (Bekalo *et al*., 1996).

Previous studies have also demonstrated the susceptibility of sand fly larvae to plant natural products. In one such experiment, dry, powdered, and otherwise unprocessed

fruit and leaves of the broad-spectrum insecticidal plants *A. indica* and *Melia azedarach* L. were tested in a no-choice feeding experiment against *Lu. longipalpis* first instars which were allowed to develop over a period of 30 days (Andrade-Coelho *et al.,* 2009). All the extracts obtained had significant larvicidal effects as compared to the untreated controls fed on normal diet. *A. indica* fruit extracts totally prevented third instars from moulting, thus resulting in no fourth instars. Feeding *M. azedarach* fruit extracts totally prevented fourth instars of *Lu. longipalpis* from moulting (100% mortality) while feeding leaf extracts of *M. azedarach* on the larvae totally prevented moulting of second instars (100% mortality) (Andrade-Coelho *et al.,* 2009). However, Anjili *et al*. (2014), demonstrated that *Melia sericea* water extracts do not penetrate the egg chorion of *P. duboscqi* and hence did not inhibit egg hatching (Anjili *et al*., 2014). It is also possible to attribute at least partially the origin of the efficiency of *C. citratus* against the larvae of *An. funestus* s.s. to citral; because citral toxic effect in the evaluation of the larvicidal activity of the essential oil of *C. citratus* on larvae of *Anopheles gambiae* was demonstrated (Tchoumbougnang *et al*., 2009). Previously, Luitgards-Moura *et al.* (2000) attributed the larvicidal and insecticidal activities of *C. citratus* against *Ae. aegypti* to citral. Several compounds acting in synergy can also be the source of the toxic effectiveness of an essential oil (Nuto, 1995).

5.4 Effects of essential oils of lemon grass, *Cymbopogon citratus* and the Mexican marigold, *Tagetes minuta* on mortality and oviposition in adult sandflies, *Phlebotomus duboscqi*

The bioassay results of this study demonstrate that both *T. minuta* and *C. citratus* are highly potent against *P. duboscqi* sandflies. Between the two oils tested, that of *C. citratus* was significantly more potent ($P < 0.05$) and caused higher mortality than

that of *T. minuta* against both male and female sandflies. The results further demonstrated that after 24 hours, treatment with the oil of *C. citratus* at a concentration of 1 mg/ml it caused mortality of 91.11 and 88.89 % against female and male sandflies, respectively while *T. minuta* oil at the same concentration, recorded a relatively lower mortality of 71.11% and 66.67 % in female and male sand flies, respectively. The results of this study demonstrate that, the effects of the oils were dose-dependent and increased with the concentration of the oil. The low concentrations tested inflicted low levels of mortality. The highest mortality levels were observed at 72 hours post treatment for all the concentrations tested. In fact, at 72 hours post treatment, the essential oils of *C. citratus* and *T. minuta* at a concentration of 1 mg/ml recorded a mortality of 100.00 and 82.22 % respectively, on female sandflies. At the same concentration, *C. citratus* and *T. minuta* oils caused mortalities of 100.00 and 88.89% respectively, in male sandflies.

The findings of this study concur with previous studies which demonstrated that *C. citratus* and *T. minuta* essential oils are effective against arthropods. Hanifah *et al*. (2011) were able to demonstrate that the mortalities from lemongrass extract were higher than those of neem extracts for both topical and contact activities against the house dust mites *Dermatophagoides farinae* (*D. farinae*) and *Dermatophagoides pteronyssinus* (*D. pteronyssinus*). At 50 % concentration, both the 24 hours topical and contact exposures to lemon grass resulted in more than 91% mortalities for both species of mites. At the same concentration and exposure time, neem resulted in topical mortalities of 40.3% and 15.7% against *D. pteronyssinus* and *D. farinae* respectively whilecontact mortalities were 8.0% and 8.9% against the 2 mites, respectively (Hanifah *et al*. (2011).

Previous studies have demonstrated various biocidal activities of plant natural oils and products against sandfly adults. *Lutzomyia longipalpis* Lutz and Neiva adults were killed by water extracts of the leaves of *Antonia ovata* Pohl (LD_{50} at 233mg/mL) and water extracts of the roots of *Derris amazonica* Killip (LD_{50} at 212mg/ mL) (Luitgards-Moura *et al*. 2002). Also, *Eucalyptus* spp. essential oils exhibit toxic effects on contact with *Lu. longipalpis* adults. Adulticidal effects were observed for lemon ironbark (*E. staigeriana* F. Muell) essential oil whose major components were limonene, Z-citral, α –citral (EC50 at 0.59mg/ml), and lemon eucalyptus (*E. citriodora* Hook) with the major chemical constituent being β-citronellal (ED50= 5.04 mg/ml). Finally, *E. globulus* Labill with essential oil major component being 1,8-cineole had an adulticide effect at an effective concentration of 7.78mg/ml. The superior toxicity of lemon ironbark is evident from these and other data and is presumably due to the activity of the major components of its essential oil, which were not individually evaluated for biological activity (Luitgards-Moura *et al.* 2002).

With regard to the observed reduction in the number of eggs oviposited by the treated female flies, in addition to there being possible adverse physiological effects on female sand flies, the mortality of the flies before ovipositing may have been a major factor.

5.5 Repellent effects of essential oils of *Cymbopogon citratus* and *Tagetes minuta* on the sandfly, *Phlebotomus duboscqi*

The essential oils of the two plants, *C. citratus* and *T. minuta* have not been previously tested against the sandfly *P. duboscqi*. However, most of the previous studies on repellency by essential oil of the lemon grass have been carried out on

mosquitoes. Other plant-derived compounds that have been shown to reduce mosquito and/or sandfly trap catches include geraniol, linalool, and citronella (Muller *et al*., 2008b). The results of the present study demonstrate high repellent effects of these essential oils on the adults of the sandfly, *P. duboscqi*. Grasses of *Cymbopogon* spp. have been traditionally used for repelling mosquitoes in jungle regions such as the Bolivian Amazon (Moore *et al*., 2007). Plants of this genus produce the most used natural repellents in the world (Trongtokit *et al*., 2005). A wide range of extracts and essential oils isolated from these plants have been tested against a broad range of species of arthropods. In particular, formulations of the oil of *C. citratus* in paraffin oil have been successfully utilized (Oyedele *et al*., 2002). However, the essential oil of *Cymbopogon nardus* oil that was evaluated against *Cydia pomonella* (Lepidoptera: Tortricidae) was inactive (Landolt *et al*., 1999). Further, oils of *C. nardus* and *Cymbopogon flexuosus* were ineffective on the cigarette beetle, *Lasioderma serricorne* (Coleoptera: Anobiidae) (Oyedele *et al*., 2002).

In one particular study, in which the ED_{50} was closest to what was obtained for the present study (ED= 0.039 mg/ml), Phasomkusolsil and Soonwera (2011) demonstrated that the essential oils of various species of plants including *C. citratus, Cymbopogon nardus, Syzygium aromaticum* and *Ocimum basilicum* exhibited high repellency against *Ae. aegypti* with the ED_{50} at less than 0.045 mg/cm^2 of the substrate. In the same study, oils of *C. citratus, C. nardus* and *S. aromaticum* showed repellency against *An. dirus* with ED_{50} at less than 0.07 mg/cm^2. On the other hand, the essential oils of *C. citratus, C. nardus, S. aromaticum, O. basilicum* and *Cananga odorata* gave strong effective dose (ED_{50}) values of less than 0.003 mg/cm^2 of substrate when tested against *Culex quinquefasciatus* (Phasomkusolsil and Soonwera ,

2011). Similar findings have been documented by Soonwera and Sinthusiri (Soonwera and Sinthusiri 2014) who obtained 87.9% effective repellency of the essential oil of *C. citratus* among other oils tested on the house fly, *Musca domestica*.

Dried plants of *T. minuta* are usually indoors to repel a broad range of insect species (Perich *et al*., 1995, Seyoun *et al*., 2002). Repellent activity of *Tagetes* species have been reported against *Anopheles gambiae*, the vector of malaria (Seyoun *et al*., 2002). *Tagetes* species have also showed insecticidal activity against stored product pests (Cestari *et al*., 2004). The efficacy of 100 ppm of *T. minuta* essential oil against head lice *Pediculus humanus capitis* (Phthiraptera: Pediculidae) was evaluated and it was found to be toxic to the insects (Cestari *et al*., 2004). The toxic effect of the oil of *T. minuta* to dipterans was attributed to the presence of terpenes (Perich *et al*., 1995). In addition, the GC-MS results obtained in this study indicated that the *T. minuta* essential oil prepared in that study included beta--ocimene, which has been shown to be a tick repellent (Lwande *et al*., 1999). The soft tick, *Hyalomma rufipes* adults showed a significant dose-repellent response to the essential oil *T. minuta* (Nchu *et al*., 2012).

With regard to protection time of *C. citratus* essential oil, a similar study (Phasomkusolsil and Soonwera, 2011) using the method of inserting a human arm treated with 0.21 mg/cm^2 of essential oil into a cage with mosquitoes found that the oil gave the longest protection periods against three mosquito species; 72 min for *Ae. aegypti*, 132 min for *An. dirus* and 84 min for *Cx. quinquefasciatus*. However, the essential oils of *C. nardus* and *Syzygium aromaticum*, exhibited moderate repellency against *Ae. aegypti, An. dirus* and *Cx. quinquefasciatus.*

In a study carried out in Ethiopia, *Phlebotomus bergeroti* Parrot adults that are vectors of visceral leishmaniasis were repelled by neem (*Azadirachta indica*) and chinaberry (*Melea azedarach*) oils at 2 and 5% formulations in coconut oil. These oil formulations provided protection of up to 98.3% protection for up to 9hours at the higher concentration, under laboratory conditions. In tests against field populations of *Phlebotomus orientalis* Parrot and *P. bergeroti*, 2 and 5% neem oil in coconut oil mixtures and DEET, the essential oil and DEET were not effective. Other essential oils that have been tested include garlic clove (*Allium sativum*) oil for which 1% preparation elicited a repellency of 97.0% against mature female sandflies, *Phlebotomus papatasi* Scopoli (Valerio and Maroli, 2005).

Over the years, researchers have demonstrated that effectiveness of repellents over several hours can be improved by synergizing the repellent with a base or fixative materials such as vanillin, salicylic acid and mustard and coconut oils, among others (Stuart *et al*., 2000; Tawatsin *et al*., 2001; Das *et al*., 2003). However, the effectiveness of the repellents depends on multiple factors including the type of repellents (active ingredients), formulation, mode of application, environmental factors (temperature, humidity, and wind), the attractiveness of individual people to insects, loss due to removal by perspiration and abrasion, the sensitivity of the insects to repellents, and the biting density (Rozendaal, 1997; Barnard, 2000; Hossain *et al*., 2011; Govindarajan *et al*., 2011; Singha *et al*., 2011; Wabo *et al*., 2011; Ahmad *et al*., 2012).

CHAPTER SIX

CONCLUSIONS AND RECOMMENDATIONS

6.1 Conclusions

1. The results of the GC-MS analysis of *C. citratus* oil obtained for this study concurred with previous findings which demonstrated that *C. citratus* oil is dominated by monoterpene hydrocarbons including Geranial and Myrcene while *T. minuta* is composed of Dihydro-Tagetone, (E)-Tagetone, (Z)-Tagetone, (Z)-beta-Ocimene and Limonene. The efficacy of these essential oils is possibly based on their chemical compositions in which major and/or minor compounds could have been responsible for insecticidal activities on *P duboscqi*

2. These findings clearly indicated that both *C. citratus* and *T. minuta* essential oils exhibited significantly high ovicidal activities against *P. duboscqi*. However, *C. citratus* essential oil inhibited egg hatching of *P. duboscqi* more than *T. minuta* essential oil. The terpenes that were shown to be present in the essential oils of both *C. citratus* and *T. minuta* were probably responsible for the toxic effects on the eggs leading to inhibition of egg hatching. These findings, among many others by other researchers lend strong credence to the consideration of both *C. citratus* and *T. minuta* as a potent source of natural ovicidal products with activities against the sandfly *P. duboscqi*.

3. The two essential oils of *T. minuta* and *C. citratus* exhibited significant larvicidal and adulticidal activities. However, the essential oil of *C. citratus* recorded higher larval and adult mortalities compared to that of *T. minuta*. Thus, the results obtained suggest that the essential oils are promising as larvicides and adulticides

against *P. duboscqi* larvae and adults and they should be used due to their safety advantage over synthetic chemical insecticides.

4. The essential oils of *T. minuta* and *C. citratus* have a potent effect on the fecundity of *P. duboscqi* and significantly reduced oviposition of female sand flies. However, the the potency of *C. citratus* essential oil was higher than that of *T. minuta* essential oil.

5. The two essential oils were found to be candidate natural repellents that can be used against *P. duboscqi* due to their high efficacy at very low doses, hence, the envisaged safety in their use over synthetic chemical repellents. However, *C. citratus* exhibited higher repellency, longer protection times and lower biting rates as compared to *T. minuta*.

6.2 Recommendations

1. This study recommends more investigations to be undertaken to identify the specific active chemical constituents of the compounds of *T. minuta and C. citratus* that are responsible for the larvicidal, adulticidal, ovicidal and repellent activities as well as their specific mechanisms of action.

2. There is need to carry out bioassays with individual and combinations of the identified compounds to elucidate the candidate biologically active components by testing them in bioassays individually and in blends.

3. This study recommends *C. Citratus* as the priority plant for the sourcing of novel insecticide products against sandflies. The application should target both the immature and mature stages of the vector.

4. Finally, there is need to undertake field studies to determine the efficacy of the essential oils in the field. Owing to their safety record on humans, there is also need to undertake clinical studies to test for the repellent and protection potential of the essential oils.

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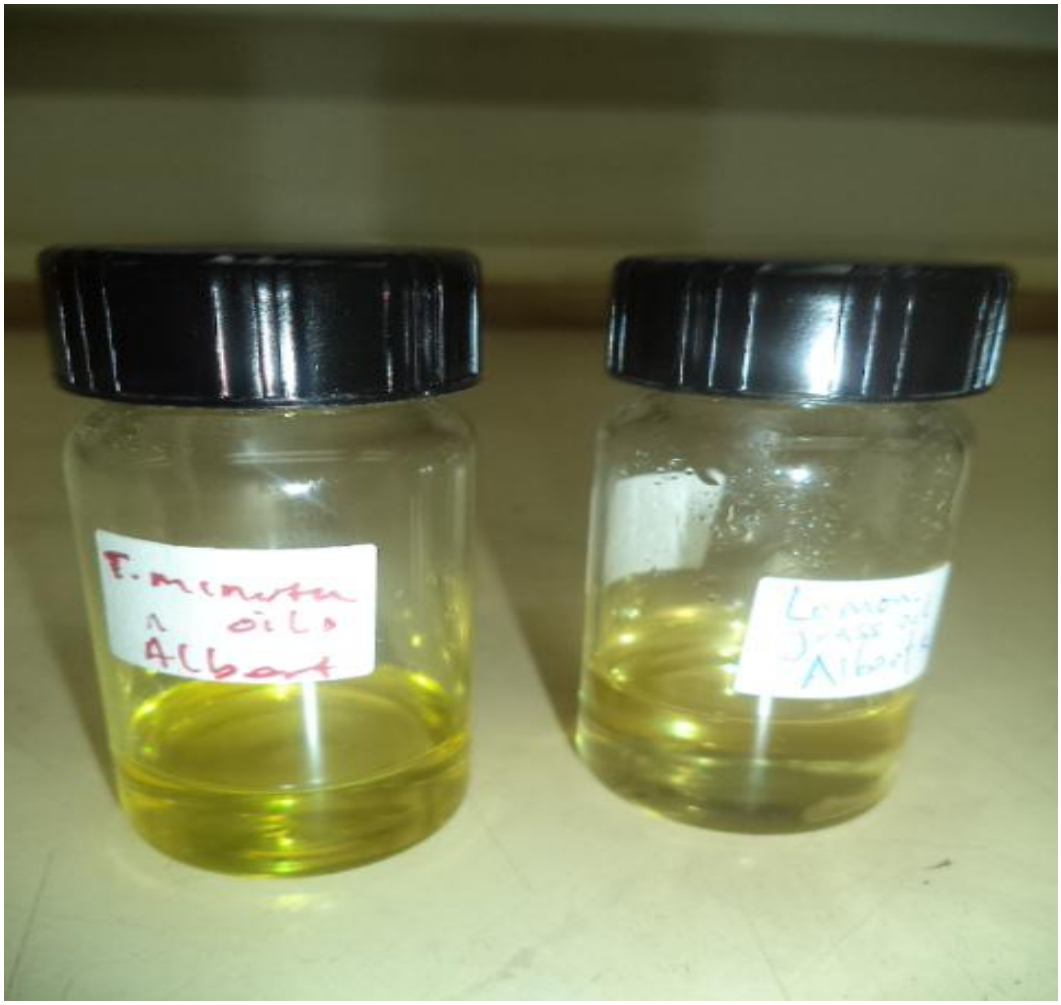
APPENDICES

APPENDIX I: Collection of Lemon grass from Kakamega forest

APPENDIX II: Hydrodistillation of the essential oils at ICIPE

APPENDIX III: *T. minuta* essential oil (left) and C. citratus essential oil (right)



APPENDIX IV: GC-MS acquisition of essential oils