REPELLENCY EFFICACY OF ESSENTIAL OILS OF SELECTED PLANTS GROWING IN WESTERN KENYA AND THEIR EAG-ACTIVE CONSTITUENT BLENDS AGAINST *Anopheles gambiae*

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DECLARATION

I hereby declare that this thesis is my original work and has not been presented for the award of any degree in another university.

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DEDICATION

This work is dedicated to my wife **Ruth Mutanda Amayeye**, my son **Ridge Omondi Otieno** my father **Alfred Ywaya Onyango** and mother **Rose Amolo Ywaya** for their prayers, encouragement and moral support they gave me during my course work and research period.

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ABBREVIATIONS AND ACRONYMS

ACT	Artemisinin-based combination therapy
AIDS	Acquired immune deficiency disease
CDC	Center for disease control and prevention
DDT	1, 1, 1-Trichloro-2,2-bis (*p*-chlorophenyl)ethane
DEET	Dethyl-m-toluamide
DNA	Deoxyribonucleic acid
EAD	Electroantennographic detection
EAG	Electro-antennogram
EPA	Environmental protection agency
FCB	Five - component blend
FID	Flame ionization detector
GC	Gas chromatography
GM	Genetically modified
HIV	Human immunodeficiency virus
HP	Hewlett Packered
ICIPE	International Center of Insect Physiology and Ecology
IRS	Indoor residual spraying
ITNs	Insecticide treated mosquito net
IVM	Integrated vector management
MS	Mass spectrometer/spectrometry
RD	Repellent dose
TCB	Three-component blend
WHO	World Health Organization

ABSTRACT

Control of disease vectors by use of synthetic chemical pesticides has been associated with a series of problems, including resistance development, environmental pollution, and safety risks for humans and domestic animals. Plant-based products have been used for generations in traditional practices to control different arthropods. These have been used in space or personal protection either to repel the arthropods from distance or to deter them from blood-feeding on contact. In the present study, the repellence of essential oils of three plants (*Ocimum gratissimum* L., *Vitex keniensis* Turill and *Hyptis suaveolens* (L.) Poit used in Siaya County in western Kenya were screened against the malaria vector *Anopheles gambiae* sensu stricto. The essential oils were extracted by steam distillation using Clevenger apparatus. The efficacy of protection provided by oil from each plant and some blends of electrophysiologically active components was tested using WHO-approved topical application bioassay. All the oils were repellent, with that of *O. gratissimum* showing the highest repellence (RD_{50} = 2.77 × 10^{-5} mg cm^{-2}, 95% CI), followed by *V. keniensis* (RD_{50} = 5.68 × 10^{-5} mg cm^{-2} , 95% CI), and then *H. suaveolens* (RD_{50} = 6.25× 10^{-5} mg cm^{-2}, 95% CI). The three essential oils were analyzed by gas-chromatography (GC) and gas-chromatography-linked Mass Spectrometry (GC-MS). The constituents of the essential oils were identified by direct comparison of their mass spectra with Wiley NBS and NIST databases. The major constituents of *O. gratissimum* were (*Z)*-ocimene (29.73%), eugenol (21.76%), (R)-(+)-germacrene D (9.65%), *β*-caryophyllene (5.86%), and *β*-linalool (4.13%). The constituents that dominated *H. suaveolens* essential oil were (*E*)-caryophyllene (21.27), *γ*-elemene (9.75%), (*E*)-*α*-bergamotene (5.07%), (Z)-*α*-cis bisabolene epoxide (4.54%), and spathulenol (4.35%). That of *V. keniensis* was dominated by *α*-cadinol (16.1%), *δ*-cadinene (12.67%), *β*-cubebene (10.88%), tau-muurolol (9.79%), and patchulane (4.60%). Gas chromatography-linked elecroantennographic analyses (GC-EAG), followed by co-injections with authentic standards on the GC were deployed to identify the constituents in each oil that were perceived by the antennae of *An. gambiae*. The active components in *O. gratissimum* essential oils were *α*-pinene, *β*-pinene, hexyl acetate, (*Z*)-ocimene, (*Z*)-4,8-dimethyl-1,3,7-nonatriene,(*E,E*)-2, 4-decadienal, eugenol, and (*R*)-(+)-germacrene D. *α*-Pinene, *p*-cymene, (*E*)-caryophyllene, spathulenol and *α*-cadinol in *V. keniensis* essential oil were found to be electrophysiologically active, and in essential oil of *H. suaveolens*, *β*-pinene, *p*-cumenol, (*E*)-caryophyllene, *α*-copaene, *α*-gurjonene, bicyclogermacrene were active. Subtractive bioassays of blends of selected electrophysiologically active components of *O. gratissimum* essential oil were then carried out to determine their roles in conferring repellence to *An. gambiae.* Absence of hexyl acetate, *α*-pinene and (*E,E*)-decadienal resulted in the drop in repellency activity against the *An. gambiae.* The repellency activity reported herein explains and verifies their efficacy in ethno-practices. In addition, the improved repellency of selected blend of constituents warrants further investigation so as to formulate a cheap, affordable and environmental friendly repellent against *An. gambiae.*

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CHAPTER ONE

INTRODUCTION

1.1 Background

Mosquitoes belong to the family *Culicidae* that consist of two major sub-families the *Anopheline* and *Culcinae.* A few species are harmless or even useful to humanity. However, most are a nuisance because they suck blood from vertebrates, including humans. The females of many species are blood sucking pests and dangerous vectors of malaria, filariasis, yellow fever and dengue fever (Molavi, 2003; WHO, 2010b). All human malaria vectors belong to the genus *Anopheles* and some 60 species of *Anopheles* are found in the tropical and sub-tropical regions (Manson and Bell, 1987). The three efficient vectors of malaria are *An. gambiae, An. arabiensis* and *An. funestus* (Temu *et al*., 1998; Palson, 1999). In sub-Saharan Africa, two important *Anopheles* species are *An. gambiae* and *An. funestus* that prefer humans to other animals. *An. gambiae* is found everywhere and breed in quiet water bodies exposed to sunlight in comparison with *An. funestus* which is confined to grassy edges of slow moving streams (Wigglesworth, 1976).

Key interventions to control malaria include: prompt and effective treatment with artemisinin-based combination therapies; use of insecticidal nets by people at risk; and indoor residual spraying with insecticide to control the vector mosquitoes (WHO, 2006; Kokwaro, 2009; Enayati and Hemingway, 2010 WHO, 2010; CDC, 2010; Raghavendra *et al*., 2011). In 2010, an estimated 219 million cases of malaria occurred worldwide and 660,000 people died, 91% of deaths occurred in the African region (WHO, 2010a). This

is equivalent to roughly 2000 deaths every day (Nadjm and Behrens, 2012). Malaria mortality rates have fallen by more than 25% globally since 2000 and by 33% in the WHO African region. Most deaths occur among children living in Africa where a child dies every minute from malaria (Greenwood *et al*., 2005; WHO, 2010). However malaria outbreaks are being reported in some locations of Africa that had been previously thought to be at elevations too high for malaria transmission such as the highlands of Kenya. A 2012 study estimated the number of documented and undocumented deaths in 2010 was 1.24 million (Olver, 2012; Murray *et al*., 2012).

Malaria is one of the leading causes of morbidity and mortality in Kenya and it kills an estimated 34,000 children under the age of five who have not developed immunity to malaria every year. About 3.5 million children are at risk of infection and developing severe malaria. Seventy seven percent of Kenya population lives in areas where the disease is transmitted. Pregnant women, whose immunity has been decreased by pregnancy, also face high risks. There are about 1.1 million pregnancies per year in malaria endemic areas. Patients with HIV/AIDS, non-immune travelers in high transmission areas are at considerably higher risk of contracting malaria and suffering from, or dying of it. Economically, it is estimated that 170 million working days in Kenya are lost each year because of malaria illness (KENYA, 2011).

1.2 Malaria Prevalence

A new global map of malaria incidence suggest that 50% more people are suffering from malaria than previously thought (Olver, 2012; Murray *et al*., 2012). Malaria prevalence

depends mainly on climatic factors such as temperature, humidity, and rainfall. Temperature is particularly critical. For example, at temperatures below 20^{o}C, *Plasmodium falciparum* which causes severe malaria cannot complete its growth cycle in the *Anopheles* mosquito and thus cannot be transmitted (CDC, 2012; Layne, 2006; Prothero, 1999). Malaria is transmitted in tropical and subtropical areas where *Anopheles* mosquitoes can survive and multiply and malaria parasites can complete their growth cycle in mosquitoes.

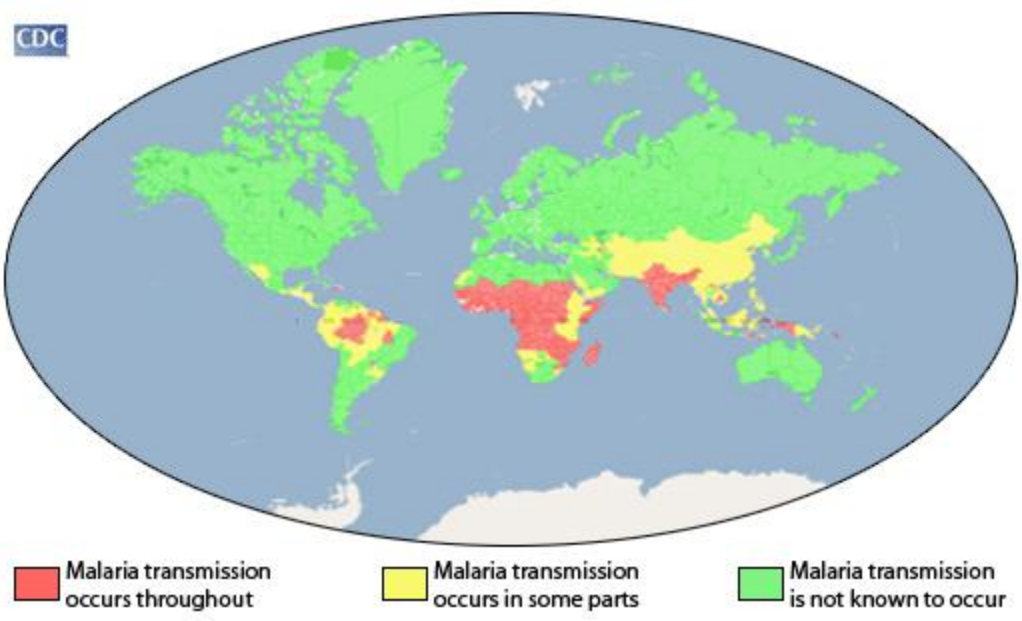


Figure 1.1: Malaria risk areas (CDC, 2012)

Generally in warmer regions closer to the equator, transmission is more intense and malaria is transmitted year-round. The highest transmission is found in Africa South of Sahara and in parts of Oceania such as Papua New Guinea (Prothero, 1999; Layne, 2006; CDC, 2012).

In many malaria-endemic countries, malaria epidemics may be contributed to climatic changes such as global warming, El-Nino weather phenomenon and new development projects such as agro forestry, irrigation, mining, quarrying, logging, and road and dam construction among others (Palson, 1999). The conditions provide good breeding grounds for mosquitoes and enabling substantial increase in their densities. Epidemics also are attributed to the disintegration of national health services, armed conflicts, mass movement of refugees, vector resistance to insecticides and emergence of multi-drug resistance strains of the parasite. Malaria attack may depend on parasite virulence, host's immunity, genetics, nutritional status and other infections and innate individual difference among people in attracting mosquitoes (Lindsay *et al*., 1993; De Jong and Knolls, 1995; Knolls *et al*., 1995).

In cooler regions, transmission will be less intense and more seasonal where; *P. vivax* might be more prevalent because it is more tolerant of ambient temperature. In many temperate areas, such as western Europe and the United States, economic development and public health measures have succeeded in eliminating malaria (CDC, 2012).

1.3 Malaria Parasite

Earlier theories were that malaria was caused by bad air ("mala aria" in Italian), formerly called ague or marsh fever due to its association with swamps and marshlands (Reiter, 2000). In 1889, Alphonse Laveran working in Algeria discovered a protozoan parasite, an agent causing malaria (Joy *et al*., 2003). He was awarded the Nobel Prize in 1907 in

recognition of his discovery of the malaria parasite and for his overall work on protozoa as causes of disease (Laveran, 1880). In 1897 Ronald Ross demonstrated that *Anopheles* mosquito was the vector for malaria (Ross, 1897). Ross's discoveries about malaria were immediately followed by a series of important works (Simmons, 1979).

Malaria is caused by parasitic protozoan belonging to the *Plasmodium* genus. Out of about 300 known species of which only five species, *P. falciparum, P. vivax, P. malariae and P. ovale* and *P. knowlesi* are known to cause malaria in humans (Mueller *et al*., 2007; Collins, 2012).

Plasmodium vivax is found mostly in Asia, Latin America and in some part of Africa. Because of population densities especially in Asia, it is probably the most prevalent human malaria parasite. *Plasmodium vivax* as well as *P. ovale* has dormant liver stages ("hypnozoites" that can activate and invade the blood ("relapse") several months or years after the infecting mosquito bite (Nadjm and Behrens, 2012).

Plasmodium ovale is found mostly in Africa (especially west Africa) and the islands of the Western Pacific. It is biologically and morphologically very similar to *P. vivax*. However, differently from *P. viva*x, it can infect individuals who are negative for the Duffy blood group, which is the case for many residents of sub-Saharan Africa. This explains prevalence of *P. ovale* (rather than *P. vivax*) in most of Africa (Mendis *et al*., 2001).

Plasmodium malariae is found worldwide and is the only human malaria parasite species that causes fevers that recur at approximately three-day intervals, therefore occurring every fourth day (a quartan fever), unlike three other species with a tertian (two day) cycle. If untreated, *P. malariae* causes a long-lasting, chronic infection that in some cases can last a lifetime. In some chronically infected patients *P. malariae* can cause serious complications such as nephrotic syndrome (Mueller *et al*., 2007; Collins, 2012).

Plasmodium knowlesi is found throughout Southeast Asia as a natural pathogen of long-tailed and pig-tailed macaques. It has recently been shown to be a significant cause of zoonotic malaria in that region, particularly in Malaysia. *Plasmodium knowlesi* has a 24-hour replication cycle and so can rapidly progress from an uncomplicated to a severe infection; fatal cases have been reported (CDC, 2010; Collins, 2012). *Plasmodium falciparum* infections are the most virulent, resulting in severe anemia, cerebral malaria and occasional deaths if not treated (Idro *et al*., 2005; Beare *et al*., 2011).

1.4 Malaria Disease

The malaria parasite requires specific human and mosquito tissue to complete its life cycle. Grassi and Felleti (1900) described the developmental cycles of *P. falciparum* and *P. vivax* which are the most common. A female *Anopheles* mosquito carrying malaria-causing parasites feeds on a human and injects the parasites in the form of sporozoites into the blood stream. The sporozoites travel to the liver and invade liver cells. Over 5-16 days the sporozoites grow, divide, and produce tens of thousands of haploid forms called merozoite, per cell (Bledsoe, 2005). Some malaria parasite species remain dormant for

extended periods in the liver, causing relapses weeks or months later (Richter *et al*., 2010). The merozoite exits the liver cells and re-enter the blood stream, beginning a cycle of invasion of red blood cells, asexual replication, and release of newly formed merozoite from the red blood cells repeatedly over 1-3 days.

This multiplication can result in thousands of parasite-infected cells in the host blood stream, leading to illness and complications of malaria that can last for months if not treated. Some of the merozoite-infected blood cells leave the cycle of asexual multiplication. Instead of replicating, the merozoite in this form develops sexual forms of the parasite, called male and female gametocytes, which circulate in the bloodstream (Talman *et al*., 2004).

When a mosquito bites an infected human, it ingests the gametocytes. In the mosquito gut, the infected human blood cells burst, releasing the gametocytes, which develop further into mature sex cells called gametes. Male and female gametes fuse to form diploid zygotes, which develop into actively moving ookinetes that burrow into the mosquito midgut wall and form oocysts. Growth and division of each oocyst produces thousands of active haploid forms called sporozoites. After 8-15 days the oocyst bursts releasing sporozoites into the body cavity of a mosquito, from which they travel to and invade the mosquito salivary glands. The cycle of human infection re-starts when the mosquito takes a blood meal, injecting the sporozoites from its salivary glands into the human blood stream (Talman *et al*., 2004).

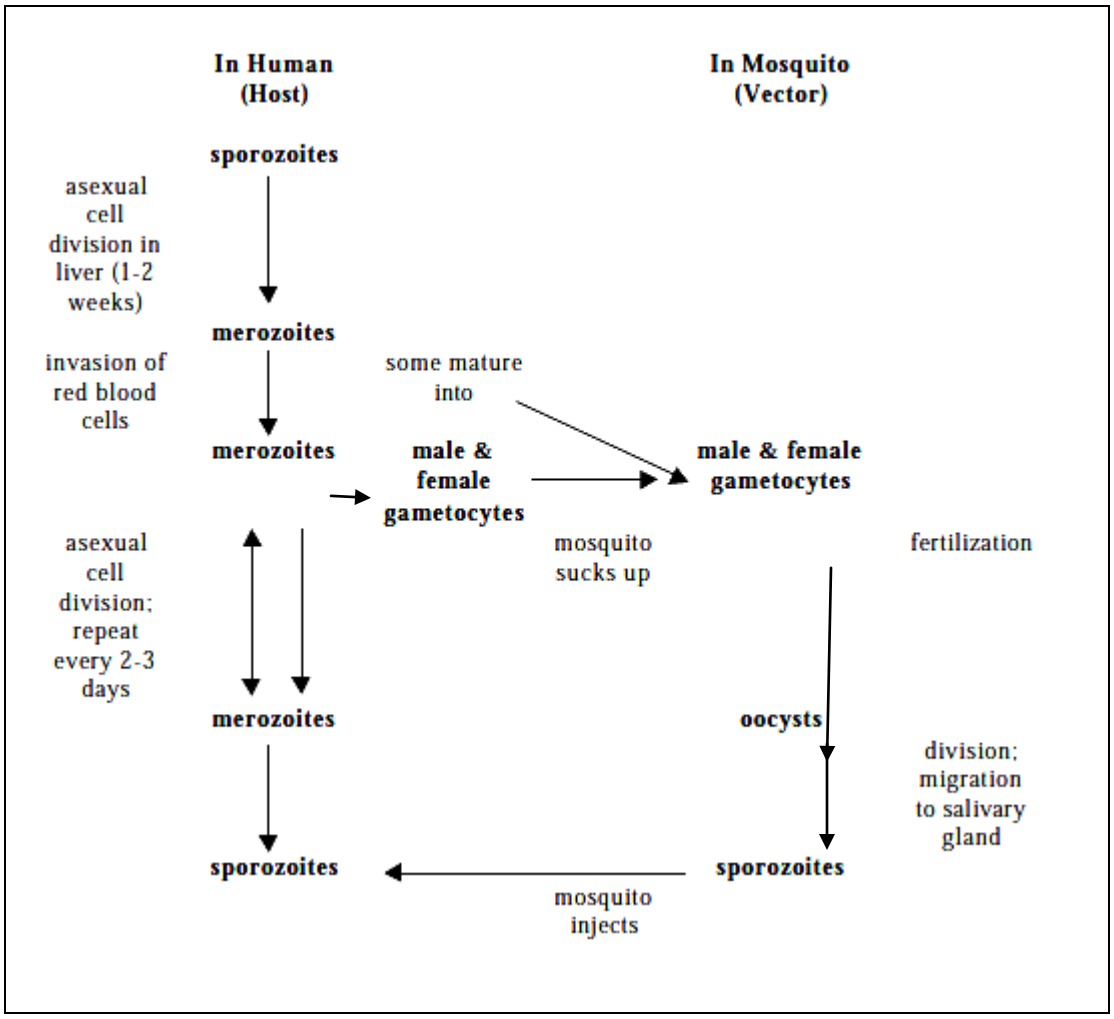


Figure 1.2: Life cycle of malarial parasite *Plasmodium* (Ahmed *et al*., 2009)

Infection with malaria parasite may result in a wide variety of symptoms, ranging from absent or very mild symptoms to severe disease and even death (WHO, 2010; Mandell *et al*., 2010; Nadjm and Behrens, 2012). In general malaria is curable disease if diagnosed and treated promptly and correctly. All the clinical symptoms associated with malaria are caused by the asexual erythrocyte or blood stage parasites. When the parasite develops in erythrocyte, numerous known and unknown waste substances such as hemozoin pigment

and other toxic factors accumulate in the infected red blood cell. These are dumped into the blood stream when the infected cells lyse and release invasive merozoites. *Plasmodium falciparum*-infected erythrocytes adhere to the vascular endothelium of venular blood vessel walls and do not freely circulate in the blood. When this sequestration of the infected erythrocytes occurs in the vessel of the brain it is believed to be a factor causing the severe disease syndrome known as cerebral malaria which is associated with high mortality (Chen *et al*., 2000; Grau and Craig, 2012).

If not treated, malaria can quickly become life threatening by disrupting the blood supply to vital organs. In many parts of the world, the parasites have developed resistance to a number of malaria medicines (Dondorp *et al*., 2010; Phyo *et al*., 2012; WHO, 2012).

1.5 Malaria Transmission

Malaria is transmitted among humans exclusively through the bites of female mosquitoes of the genus *Anopheles* (Talman *et al*., 2004). Female mosquitoes take blood meals to carry out egg production. Of the approximately 430 *Anopheles* species, only 30-40 transmit malaria in nature. All of the important vector species bite at night. *Anopheles* mosquitoes breed in water and each species has its own breeding preference; for example some prefer shallow collections of water, such as puddles, rice fields and hoof prints. Transmission is more intense in places where the mosquito lifespan is longer (so that the parasite has time to complete its development inside the mosquito) and where it prefers to bite humans rather than other animals. For example, the long lifespan and strong human-

biting (anthropophilic) habit of the African vector species is the main reason why more 90% of the world's malaria deaths are in Africa (Charlwood *et al*., 1997).

Transmission also depends on climatic conditions that may affect the number and survival of mosquitoes, such as rainfall patterns, temperature and humidity. In many places, transmission is seasonal, with the peak during and just after the rainy season. Malaria epidemics can occur when climate and other conditions suddenly favour transmission in areas where people have little or no immunity to malaria. They occur when people with low immunity move into areas with intense malaria transmission, for instance to find work or as refugees (CDC, 2010).

Human immunity is another important factor, especially among adults in areas of moderate or intense transmission conditions. Partial immunity is developed over years of exposure, and while it provides complete protection, it does reduce the risk that malaria infection will cause severe disease. For this reason, most malaria deaths in Africa occur in young children, whereas in areas with less transmission and low immunity, all age groups are at risk (WHO, 2012).

1.6 Vectors of Malaria

Like all mosquitoes, *Anophelines* go through four stages in their life cycle: egg, larva, pupa, and adult. The first three stages are aquatic and last 5-14 days, depending on the species and the ambient temperature (Wigglesworth, 1933). The adult stage is when the

female *Anopheles* mosquito acts as malaria vector. The adult females can live up to a month (or more in captivity) but most probably do not live more than 1-2 weeks in nature. Adult females lay 50-200 eggs per oviposition in water (Garret-Jones *et al*., 1980). Eggs are laid singly directly on water and are unique in having floats on either side. Eggs are not resistant to drying and hatch within 2-3 days, although hatching may take up to 2-3 weeks in colder climates (Kosova, 2003).

Mosquito larvae have a well-developed head with mouth brushes used for feeding, large thorax, and segmented abdomen. In contrast to other mosquitoes, *Anopheles* larvae lack a respiratory siphon and for this reason position themselves so that their body is parallel to the surface of water. Larvae breathe through spiracles located on the 8^{th} abdominal segment and therefore must come to the surface frequently. Larvae develop through four stages, or instars, after which they metamorphose into pupae (Spielman and D'Antonio, 2001).

The pupa is a comma-shaped when viewed from the side. The head and thorax are merged into a cephalothorax with the abdomen curving around underneath. As with the larvae, pupae must come to the surface frequently to breathe through respiratory trumpets on the cephalothorax. After a few days as a pupa, the dorsal surface of the cephalothorax splits and the adult mosquito emerges. The duration from egg to adult varies considerably among species and is strongly influenced by ambient temperature. Mosquitoes can develop from egg to adult in as little as 5 days but usually take 10-14 days in tropical conditions (Huang *et al*., 2006).

Adult *Anophelines* have slender bodies with head, thorax and abdomen. The head contains the eyes and a pair of long, many segmented antennae. The antenna is important for detecting hosts odor as well as odors of breeding sites where female lays eggs. The head also has an elongate, forward-projecting proboscis used for feeding, and two sensory palps. Three pairs of legs and a pair of wings are attached to the thorax. This segmented abdomen is specialized for digestion and egg development expands considerably when a female takes a blood meal (Kaufmann and Briegel, 2004; Harzsch and Hafner, 2006).

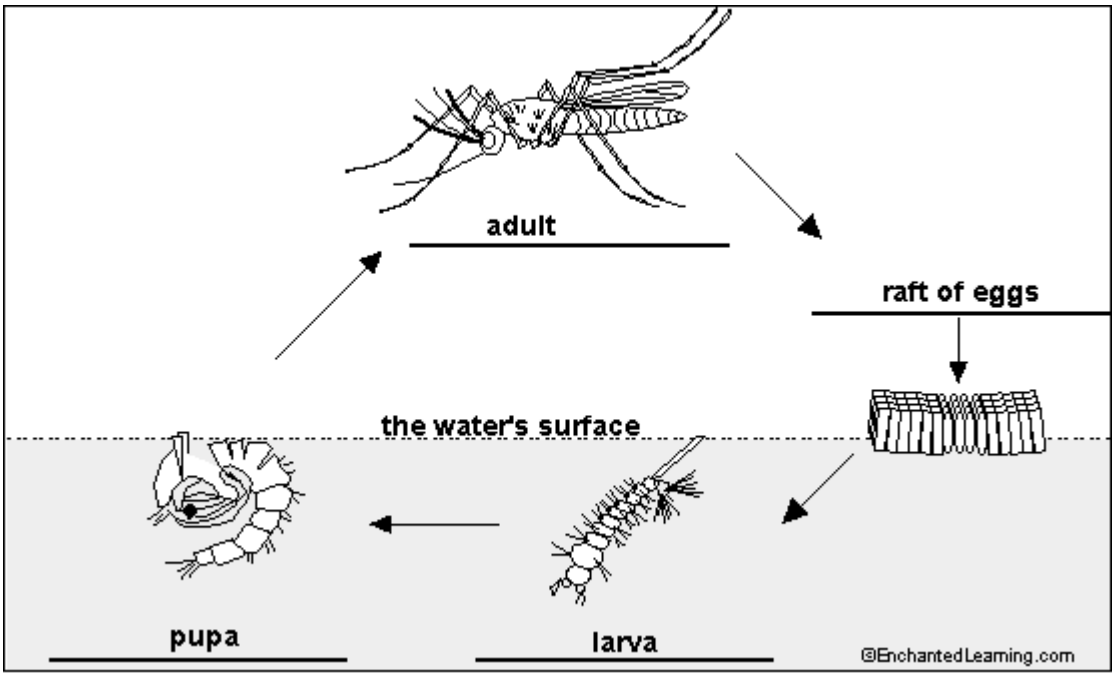


Figure 3: Life cycle of *Anopheline* mosquito (Charlwood *et al*., 1997)

Anopheles mosquitoes can be distinguished from other mosquitoes by the palps, which are as long as the proboscis, and by the presence of discrete blocks of black and white scales on the wings. Adult *Anopheles* can also be identified by their typical resting

position: males and females rest with their abdomens sticking up in the air rather than parallel to the surface on which they are resting (Besansky *et al*., 1994; Fanello *et al*., 2002; Pennetier *et al*., 2009). Adult mosquitoes usually mate within a few days after emerging from the pupal stage. In most species, the males form large swarm usually around dusk, and the females fly into the swarms to mate. Males live for about a week, feeding on nectar and other sources of sugar. Females will also feed on sugar sources for energy but usually require a blood meal for the development of eggs. After obtaining a full blood meal, the female will rest for a few days while the blood is digested and eggs are developed (Talman *et al*., 2004). This process depends on temperature but usually takes 2-3 days in tropical conditions. Once the eggs are fully developed, the female lays them and resumes host seeking. The cycle repeats itself until the female dies. The females' chances of survival depend on temperature and humidity, but also their ability to successfully obtain a blood meal while avoiding host defenses (Arrow *et al*., 2004).

One important behavioral factor is the degree to which an *Anopheles* species prefers to feed on humans (anthropophyly) or animals such as cattle (zoophyly). Anthrophilic *Anopheles* is more likely to transmit malaria parasites from one person to another. The primary malaria vectors in Africa, *An. gambiae* and *An. funestus*, are strongly anthropophilic and consequently, are two of the most efficient malaria vectors in the world (Temu *et al*., 1998).

1.7 Malaria Control

The goal of most malaria control programs and malaria activities is to reduce the number of malaria related cases and death. Various strategies have been adopted towards malaria control. These include medication, mosquito eradication and prevention of bite.

1.7.1 Chemotherapy

Malaria is curable if the right drugs are used for the correct period of time. Treatment should be initiated immediately and taking into consideration the type of infecting *Plasmodium* species, the severity of symptoms and geographical area where the infection was acquired. The first effective treatment for malaria was the bark of the cinchona, which contains quinine (Pelletier and Caventou, 1820; Kyle and Shampe, 1974; Kaufman and Ruveda, 2005). Quinine became the predominant malarial medication before other medications began to be developed. Chloroquine replaced quinine as the treatment of *falciparum* malaria until resistance occurred (Brasseur *et al*., 1992; Wongsrichanalai *et al*., 1992; Edoh *et al*., 1997; Achan *et al*., 2011). The most effective strategy to treat uncomplicated malaria infection is by use of artemisinins in combination with other antimalarials called artemisinin-combination therapy (ACT). This is done to reduce risk of resistance against artemisinin (Hsu, 2006; Kokwaro, 2009; WHO, 2010). However its failure increased on both sides of Thai-Cambodia borders due mainly to local emergence of resistance to artemisinin derivatives (WHO, 2009; Dondorp *et al*., 2010; Phyo *et al*.,

2012). This has been attributed to extraction of one element from *Artemisia annua* plant for cure instead of the entire plant structure (Tunoi, 2013).

The most used treatment for severe malaria was quinine, but artesunate has been shown to be superior to quinine in both children (Dondorp *et al*., 2010a) and adults (Dondorp and Day, 2007; Achan *et al*., 2011). Several drugs, most of which are used for treatment of malaria, can be taken to prevent contracting the disease during travel to endemic areas (Jacquerioz and Croft, 2009; Turschner and Efferth, 2009). These drugs are expensive, have negative adverse effects and are difficult to obtain in poor countries (Freedman, 2008).

1.7.2 Vaccine Development

The first promising studies demonstrating the potential for a malaria vaccine were performed in 1967 by immunizing mice with live, radiation-attenuated sporozoites, which provided significant protection to the mice upon subsequent injection with normal, viable sporozoites (Van den Berg, 2009) Since the 1970s, there has been a considerable effort to develop similar vaccination strategies within humans. It was determined that an individual can be protected from a *P. falciparum* infection if they receive over 1,000 bites from infected yet irradiated mosquitoes (Hill, 2011).

A completely effective vaccine is not yet available for malaria, although several vaccines are under development (Geels *et al*., 2011). SPf66 was tested extensively in endemic areas in the 1990s, but clinical trials showed it to be insufficiently effective (Graves and

Gelband, 2006). Other vaccine candidates, targeting the blood-stage of the parasite's life cycle, have also been insufficient on their own (Graves and Gelband 2006a; Bray, 1976). Several potential vaccines targeting the pre-erythrocytic stage are being developed, with RTS, S showing the most promising results so far (Hill, 2011).

1.7.3 Genetic Modification

Advances in genetic engineering technologies has made it possible to introduce foreign DNA into the mosquito genome and either decrease the lifespan of the mosquito, or make it more resistance to the malaria parasite (Raghavendra *et al*., 2011). Introducing a modified *Wolbachia* strain in *An. gambiae* was enough to shorten the insect's lifespan, halt the development of *Plasmodium* pathogen and activate the parts of mosquito's immune system that affect whether an infective parasite will live or die within the host. However, problems have hindered its effective deployment (Kambris *et al*., 2009; Raghavendra *et al*., 2011).

In sterile insect technique, large numbers of sterile males' mosquitoes are reared and released. Mating with the wild females reduces the wild population in the subsequent generation; repeated releases eventually eradicate the target population. However, survival of the genetically modified (GM) mosquito in the wild still poses a great challenge to the scientists. Even if the GM mosquito survives in the wild, mass production may pose a major challenge (Batler, 1999).

1.7.4 Vector Control

Integrated vector management (IVM) is an approach that makes use of a variety of control methods each in its most appropriate settings to eliminate or greatly reduce malaria transmission. The vector control strategies include indoor spraying of insecticides, personal protection measures, larval control and environmental control (Yeye, 2001).

1.7.4.1 Attractants

Researchers have known for years that lactic acid present on human skin and carbon dioxide produced by human exhalation is shown to be major attractants to mosquitoes (Acree *et al*., 1968; Elissa *et al*., 2004; Devlin, 2010).

Special traps have been developed to attract and catch large numbers of mosquitoes and removing them from a fairly wide radius around the trap (Govella, 2009). The device emits a plume of carbon dioxide together with other mosquito attractants such as sugary scents, lactic acid, octenol, warmth, water vapour and sounds (Okumu *et al*., 2010). By mimicking a mammal's scent and outputs, the trap draws female mosquitoes toward it, where they are typically sucked into a net or holder by an electric fan where they are collected.

1.7.4.2 Indoor Residual Spraying

Indoor residual spraying (IRS) involve applying residual insecticides pesticides recommended by WHO on the interior walls of houses and structures in malaria affected

area (WHO, 2006). The insecticide remains on the treated surface upon which the mosquitoes will rest before or after taking a blood meal (Research Triangle Institute, 2007). The residual effect of the insecticide is sufficient to kill mosquitoes for a period ranging from 3 to 12 months depending on the insecticide, the surface on which it is applied and the local conditions (Enayati and Hemingway, 2010).

The first pesticide used for IRS was 1,1,1-trichloro-2,2-bis (*p*-chlorophenyl)ethane (DDT) (CDC, 2010). Although it was initially used exclusively to combat malaria, its use quickly spread to agriculture. In time, pest control, rather than disease control, came to dominate DDT use, and this large-scale agricultural use led to the evolution of resistant mosquitoes in many regions leading to its ban on agricultural applications of DDT in many countries (Van den Berg, 2009).

Twelve insecticides used in IRS operations, including DDT as well as alternative insecticides such as the pyrethroids, permethrin and deltamethrin (WHO, 2006; Brown, 1986; Kirk and Orthmer, 1981; Metacalf *et al*., 1962) and small amounts of DDT (Van den Berg, 2009). However, because of its legacy, many developed countries previously discouraged DDT use even in small quantities (Rosenberg, 2004). One problem with all forms of IRS is insecticide resistance via evolution (Pates and Curtis, 2005).

1.7.4.3 Larviciding

Larviciding is ecologically safe because it controls the population of adult mosquitoes even before they become flying, biting, disease spreading adults (CCMCD, 2007). It

involves physical changes to the mosquito larval breeding habitat and treating the breeding sites directly with chemical or biological agents that kill larvae. Biological control include introduction of parasites, pathogens and predators to target mosquitoes (Kathleen, 2002). Control of larvae can be accomplished through use of contact poisons, growth regulators, surface films, stomach poisons (including bacterial agents), and biological agents such as fungi, nematodes, copepods, and fish (Wigglesworth, 1976; Lacey and Lacey, 1990; Meek and Hayes, 1993; Kenneth, 1995; Das and Amalraj, 1997; CDC, 2001; Marten and Reid, 2007; Walker and Lynch, 2007; Research Triangle Institute, 2007).

Mosquitoes breed in standing water, source reduction can be done by emptying water from containers around the home, clearing clogged gutters and repairing leaks around faucets, regularly changing water in bird baths and by filling or draining puddles, swampy areas, and tree stumps, pools, tires, buckets. Eliminating such mosquito breeding areas can be an extremely effective and permanent way to reduce mosquito populations without resorting to insecticides (Surtees, 1970; Sharma *et al*., 1986; Rafatjah, 1988; Lacey and Lacey, 1990; Research Triangle Institute, 2007). However, this may not be possible in parts of the developing world where water cannot be readily replaced due to irregular water supply.

1.7.4.4 Personal Protection Measures

Personal protection involves then use of insecticide treated mosquito nets (ITNs) and repellents. Most malaria-carrying mosquitoes bite at night. ITNs are estimated to be twice

as effective as untreated nets and offer greater than 70% protection compared with no net (Raghavendra *et al*., 2011). These nets are dip treated using a synthetic pyrethroid insecticide such as deltamethrin or permethrin which double the protection over a non-treated net by killing and repelling mosquitoes. The distribution of mosquito nets impregnated with insecticides (permethrin or deltamethrin) has shown to be an extremely effective method of malaria prevention, and it is also one of the most cost effective methods of prevention (Christopher, 1939; Maxwell *et al*., 2002). Although ITNs are proven to be very effective against malaria, only about 13% of households in sub-Saharan countries own them (Miller *et al*., 2007).

An insect repellent is a substance applied to skin, clothing, or other surfaces which discourage insect (and arthropods in general) from landing or climbing on that surface (Peterson and Coats, 2001). Insect repellents works by masking human scent or by emitting scent which insects naturally avoid. There are different types of repellents namely synthetic repellents, natural repellents and ultrasonic mosquito repellent (Collins *et al*., 1993; Mishra *et al*., 1995; Ibrahim and Zaridah, 1998; Fradin and Day, 2002; Cilek *et al*., 2004; Jeong-Kyu *et al*., 2005; Trongtokit *et al*, 2005; Caroll and Loye, 2006). Synthetic repellents containing N,N-diethyl-m-toluamide (DEET) is widely used. It has been regarded as safe, but toxic effect has been recorded (Carl and Leonhardt, 1991). Several other compounds have been evaluated for repellent activity, but none have had the commercial success of DEET. Repellents of natural origin include citronella, eucalyptus, lemon grass, marigolds, neem, peppermint, pyrethrum, *Lantana camara*, garlic among others (Eisner, 1964; White 1973; Chogo and Crank, 1981; Sharma *et al*.,

1993; Palson, 1999). These plants have been smoldered to produce smoke carrying chemical compounds that would repel mosquitoes away from the house. Lately, use is being made of essential oils extracted from these plants. More information on exploitation of essential oils from these plants by man to control mosquitoes is discussed in the next chapter.

1.8 Statement of the Problem

Malaria continues to be the biggest contributor to disease burden in terms of death and suffering (WHO, 1997; WHO, 2013). Based on documented cases, the WHO estimates that there were 216 million cases of malaria in 2010 resulting in 655,000 deaths worldwide. The vast majority cases (~89%) were in the Africa region, followed by the eastern Mediterranean (6%) and south east Asia Region at 5% (WHO, 2010). The actual number of deaths may be significantly higher, as precise statistics are unavailable in many rural areas, and many cases are undocumented (Breman, 2001). An estimate, based on a systematic analysis of all available mortality data combined with empirical methods for estimating causes of death, places the number of deaths in 2010 at 1.24 million (Olver 2012; Murray *et al*., 2012). In Kenya, 16,000 people die annually from the disease and more than 25 million are at risk (Ratemo, 2010). At present there is no drug that offers full protection against malaria and some of these drugs used to treat the disease have some extreme unpleasant side effects on a small percentage of people. In Kenya the parasite has now become 80% resistance to quinine (WHO, 1987). This resistance is said to be one of the highest rates in the world. The absence of adequate health services makes the synthetic malarial drugs to be inaccessible to many. In many parts of the world, plant-

derived natural products have been used as repellent against malaria vector, but little effort has been made to investigate and promote the traditional use of plant-derived natural repellent (Curtis *et al*., 1991; Mishra *et al*., 1995; Fradin and Day, 2002).

1.9 Justification of the Study

Control of malaria effectively can be achieved by controlling the vector itself, yet most *Anopheles* mosquitoes have become resistance to DDT, the most effective general purpose insecticide available in the market. The available synthetic insecticides are not friendly to the environment as they accumulate in the food chains with serious environmental health repercussions. Important malaria vectors such as *An. pulcherrimus, An. albimanus, An. arabiensis, An. gambiae* and *An. funestus* are less susceptible to DEET, the most potent ingredient of many commercially available repellents. Moreover, DEET attacks paint, varnish and some hard plastics (WHO, 1996). Alternative repellents must therefore be found urgently. It is clear then that the vector strategies of the future must include potent, more selective and biodegradable insecticides / repellents discovered from natural sources such as higher plants and animals. Plants commonly used as repellents in western Kenya include, *Ocimum americanum* L., *Ocimum basilicum* L., *L. camara* L., *Tagetes minuta* L., *Azadirachta indica* A. Juss, *Hyptis suaveolens* (Seyoum *et al*., 2002), *Lippia ukambensis*, *Corymbia citriodora* (Seyoum *et al*., 2003) by either placing branches / whole plants inside houses or direct burning of the plants. Other plants used include *O. gratissimum, V. keniensis*.

1.10 Research Questions

i. Do plants such as *O. gratissimum, V. keniensis* and *H. suaveolens* used in western Kenya have mosquito repellent properties?

ii. What is the relative repellence of the volatile oils of these plants?

iii. What volatile constituents of the plant are primarily responsible for repellency?

1.11 Hypotheses

i. Essential oils from *O. gratissimum, V. keniensis* and *H. suaveolens* from Western Kenya are repellent to mosquitoes.

ii. The repellence of essential oil of a given plant depends upon its composition.

1.12 Objectives

1.12.1 General Objective

To determine the repellency, composition and contribution of constituents of essential oils of *O. gratissimum, V. keniensis* and *H. suaveolens* from western Kenya against *An. gambiae*.

1.12. 2 Specific Objectives

i. To carry out repellency tests of essential oils derived from the aerial parts of *O. gratissimum, V. keniensis* and *H. suaveolens* against *An. gambiae.*

ii. To determine chemical composition of essential oil of aerial parts of candidate plants by GC-MS technique.

iii. To identify the constituents primarily responsible for repellency by GC-EAD and subtractive bioassays of different blends against *An. gambiae*.

1.13 Scope and Limitation

The research was able to standardize light, temperature, humidity, air quality and repellent dose. Not all electrophysiologically active constituents of the three essential oils were available; therefore, it was not possible to determine their relative contribution to the overall repellency of the plant; thus, the basis of the repellence of the essential oils could not be elucidated.

1.14 Significance of the Study

The study provides useful baseline information on the repellent properties of the essential oils of three African plants and constituents responsible for repellence against one of the principal Afro-tropical malaria vectors. The information can be used to formulate cheap repellent if more work is done.

CHAPTER TWO

LITERATURE REVIEW

2.1 Vector Resistance to Insecticides

Insecticide resistance has been a problem in all insect groups that serve as vectors of emerging diseases. As of 1992, the list of insecticide-resistant vector species included 56 *Anophelines*, 39 *Culcinae* mosquitoes, body lice, bedbugs, triatomides, eight species of fleas and nine species of ticks (WHO, 1996). This number has been increasingly steadily. Resistance has developed to every chemical class of insecticides including microbial drugs and insect growth regulators. Pyrethroid resistance is emerging despite early optimism that because of its rapid toxicologic action this newest large class of insecticides would not produce resistance (Brogdon and McAllister, 1998).

Insecticide resistance is already rampant in 64 malaria-ridden countries and may result in as many as 26 million more cases of malaria a year (Coetzee *et al*., 2006). Mosquitoes in sub-Saharan African countries are becoming resistant to pyrethroid insecticides, which are used extensively for household spraying and treating bed nets as well as to DDT, which is still used in many parts of the world to control mosquitoes (Lines and Nassor, 1991; Hunt *et al*., 2005; Anto *et al*., 2009; Christian *et al*., 2011). WHO is recommending rotating classes of pesticides used to spray inside homes and developing a new non-pyrethroid insecticide to treat bed nets (Graeme, 2005). A reliance on chemical programs to combat a pest problem is not a sustainable approach because the predicable consequence of repeated pesticide use results in resistance (Miller, 2004).

Insects are known for their ability to develop resistance to insecticides. In a population of insects there may be a few individuals that carry the genes for resistance. These genes arise from mutations and are rare. Upon exposure to insecticides, insects that do not carry the resistance genes die, thus allowing the individuals with the resistance genes to survive and reproduce, creating more resistant insects. With every generation the number of resistant insects increases (Ferro, 1993; Alyokhin *et al*., 2008).

2.2 Repellents

Historically, repellents have included smoke, plants hung in dwellings or rubbed on the skin as the fresh plants or its brews, oils, pitches, tars and various earths applied to the body. Before a more edified approach to insect olfaction and behavior was developed, it was wrongly assumed that if a substance was repugnant to humans it would likewise discourages arthropods from landing or climbing on a surface where the substance is applied. Among many repellents, only DEET has survived and is used worldwide against biting flies and mosquitoes. Most of the others have lost their registrations and are no longer available (Williamson, 2002; CDC, 2007).

Repellents are chemical substances that protect animals, plants or materials such as fabrics, grain, timber from insect attack by rendering them unattractive, unpalatable or offensive (Metcalf *et al*., 1962; Fradin and Day, 2002). The most common mosquito-repellent formulations available on the market contain a synthetic chemical called DEET. It was developed and patented by the U.S army in 1946 for use by military personnel in insect-infested areas. DEET was recognized as one of the few products effective against

mosquitoes and biting flies. It was registered for use by the general public in the U.S in 1957 (EPA, 1980). The efficacy of DEET in providing long-lasting protection against a wide variety of mosquito species has been documented in several studies that have shown excellent repellency against mosquitoes (Schreck and McGovern, 1989; Fradin and Day, 2002; Roberts and Reigart 2004). Several other compounds have been evaluated for activity but none have had the commercial success of DEET.

Although DEET is an effective repellent against mosquitoes, there are concerns associated with its use. It is irritating to mucous membranes, and its concentrated formulations dissolve plastic. Some human toxicity effects have been reported after applications of DEET, varying from mild to severe (Briassoulis and Hatzis, 2001; Bell *et al*., 2002). Because of these undesirable side effects, research on repellents derived from plant extracts is needed to find alternatives that would be safer but still effective.

The repellent properties of plants to mosquitoes and other insects' pest were well known before the use of synthetic chemicals. Traditionally, people used natural compounds to protect themselves against insect bites. Some plant species contain insecticidal and/or insect-repellent substances. A review by Sukumar (1991) highlighted the potential of plants for use in mosquito control, either as repellents, larvicides, or insecticides. Extracts of several plants-neem (*Azadirachta indica*), basil (*Ocimum basilicum*), and lemon eucalyptus (*Corymbia citriodora*) have been studied as possible mosquito repellents and have demonstrated good efficacy against some mosquito species (Sharma *et al*., 1993; Trigg and Hill, 1996; Ansari *et al*., 2000).

2.2.1 Repellent Mode of Action

DEET was historically believed to work by blocking insect olfactory receptors for carbon dioxide, *L*-lactic acid, 1-octen-3-ol, a volatile substance that is contained in human sweat and breath (Acree *et al*., 1968; Snow, 1970; Davis and Sokolove, 1976; McIver, 1981; Dogan *et al*., 1999). DEET does not appear to affect the insect's ability to smell carbon dioxide, as had been suspected earlier (Anna, 2008; Mathias *et al*., 2008).

However, more evidence shows that DEET serves as a true repellent in that mosquitoes intensely dislike the smell of the chemical repellent (Zainulabeuddin and Walter, 2008). A type of olfactory receptor neuron in special antennal sensilla of mosquitoes that is activated by DEET as well as other known insect repellents such as eucalyptol, linalool, and thujone has been identified. Moreover, in a behavioral test DEET had a strong repellent activity in the absence of body odor attractants such as 1-octen-3-ol, lactic acid, or carbon dioxide. Female and male mosquitoes showed the same response (Fox and Wiessler, 2008; Syed and Leal, 2008).

A recent structural study has revealed that DEET binds to *An. gambiae* odorant binding protein 1 (AgamOBP1) with high shape complementarity, suggesting that AgamOBP1 is a molecular target of DEET and perhaps other repellents (Tsitsanou *et al*., 2011).

2.2.2 Chemical Properties of Repellents

The only parameter that has direct correlation with repellent activity is the vapour pressure or boiling point (Skinner and Johnson 1980) which is a rather obvious property, in that if a chemical is going to repel mosquitoes, it will most likely act in the vapour phase. In addition, it should not evaporate too fast lest it loses its ability to protect. Other properties, such as partition coefficients, melting points (except that liquids work better than solids), molecular weights (except as they relate to boiling points), infrared absorption, have been shown to have no correlation with repellency.

Compounds that have been reported to have high repellency and most effective are amides, imides, alcohols, and phenols (Skinner and Johnson, 1980). Interestingly, one of the molecular structures indicated as having a repellent activity was R^1-CHBr-COOR. If a certain set of substitutions is made (an-OH for –Br, a $–CH_3$ for R^1, and an ethyl for R), the result is ethyl lactate ($CH_3CH(OH)$-$COOC_2H_5$), a weak oviposition attractant for *An. triseriatus* (Bently *et al*., 1979; Davis, 1985).

2.2.3 Sensory Physiology of Insect

The general assumption is that all insects repelling substances act in a common manner and that they have common property or set of properties to which their mode of action can be attributed. However it was found that some chemical substances classed as insect repellents appear to affect differently the neural activity of the five recognized types of antennal chemoreceptor sensilla of *Aedes aegypti* (Davis and Rebert, 1972; Davis and

Sokolove, 1976; Davis, 1977). Thus, concluding that chemical repellents do not behave as a single class of compounds having a common mode of action.

2.3 Chemistry of Essential Oils

The essential oils are defined as any volatile oil(s) that have strong aromatic components and that give distinct odor, flavour and scent to a plant. They are the by-products of plant metabolism and are commonly referred to as volatile plant secondary metabolite (Koul *et al*., 2008). Traditionally, all natural compounds built up from isoprene subunits and for the most part originating from plants is denoted as terpenes or isoprenoids. Terpenes are extracted by steam distillation and are used to create fine perfumes, to refine the flavors and the aroma of food drinks and produce medicines of plant origin (Adam, 2008; Glenn, 1993).

Many plants produce volatile terpenes in order to attract specific insect for pollination or otherwise to expel certain animals using these plants as food. Less volatile but strongly bitter tasting or toxic terpenes also protect some plants from being eaten by animals (antifeedant) and fromm heat and cold. Terpenes also play an important role as signal compounds and growth regulators (phyto-hormones) of plants. In nature, terpenes occur predominantly as hydrocarbons, alcohols and their glycosides, ethers, aldehydes, ketones, carboxylic acids and esters (Breitmaier, 2006).

The chemicals present in essential oils are synthesized during normal development of the herbs and may be classified as hydrocarbons built from multiple of 5-carbon hemi-

terpenoid units, oxygenated derivatives of these hydrocarbons, and compounds containing sulphur or nitrogen (FRIDGE, 2004). Chemically, the essential oils are composed of terpenoids and aromatic polypropanoids synthesized via the mevalonic acid pathway for terpenes and the shikimic acid pathway for aromatic polypropanoids (Simon, 190).

Plants have been estimated to collectively synthesize more than 30,000 different terpenoids which constitute the largest family of natural products exceeding in number the alkaloids and phenylpropanoids combined (Simon, 1990). They are synthesized from the condensation in a head to tail fashion of 5-carbon isoprene (or hemiterpene) units. Isoprene itself does not undergo the building process but rather the activated forms, isopentenyl diphosphate and dimethylallyl diphosphate. Major terpenoid classes include mono-, sesqui- and di-terpenes, which are mostly secondary metabolites as well as tri- and tetraterpenes, which are generally primary metabolites (Broun and Somerville, 2001). However, the vast majority are secondary metabolites, including the volatile constituents of essential oils. Monoterpenes are the primary constituents of many essential oils.

It was thought that the synthesis of terpenoids in higher plants was by mevalonate pathway only. However, during the last ten years it has become clear that plants also use a non mevalonate pathway which takes place in plastids of plants (Rohmer and Rohmer, 1999; Eisenreich *et al*., 2004; William, 2007). In both pathways, isopentenyl diphosphate is isomerized to dimethylallyl diphosphate by the enzyme isopentenyl pyrophosphate isomerase. Plants use the mevalonate-dependent pathway to synthesize sesquiterpenes

and triterpenes whereas other major terpenoids are derived from the non-mevalonate pathway, 1-deoxy-D-xylulose-5-phosphate synthase (DXPS) pathway. Because discovery of the plastidial route in plants is relatively recent, little is known of the mechanisms that limit flux through the DXPS pathway (Broun and Somerville, 2001).

2.4 Potential Insecticidal Plants

About 1200 plants have been listed in literature as being potential insecticidal value (Roark, 1947). Most remain uninvestigated either chemically or biologically. Many more plants used traditionally for preventing mosquito bites and hence malaria control and remain uninvestigated and the bioactive compounds therein are therefore unknown. In this research, three plants were investigated. The approach used to choose the plants was on the basis of enthnobotanical information (Kokwaro, 1993), supplemented by inspection of the plants growing naturally in the field for emission of specific odor and avoidance by insects.

2.4.1 *Vitex keniensis* Turill

Vitex keniensis (also called Meru Oak) is a species of plant in the Verbenaceae family (Kigomo, 1985; ICRAF, 1992). It is known as ‘kalembe’ in Luo, ‘muhuru’ in Kikuyu and ‘muuru’ in Meru languages. It is indigenous to Tanzania, Kenya and has been introduced in Uganda. It is a deciduous tree 12-30 meters tall, up to 1.8 (maximum 3) meters in diameter (Joker and Mngulwi, 2000).

It is threatened by habitat loss. Due to its use as a source of wood that is both durable and has an attractive grain it has been severely over-exploited and is now very rare. It is common in moist evergreen forest and on rocky hills, prefers deep sandy-loam soils. It is a fairly fast-growing tree. It is used to produce hard and durable timber, very pale and similar to teak. The wood is also suitable for firewood. It is a popularly ornamental tree and is sometimes planted as a windbreak. The fallen leaves produce a useful mulch of litter improving the soil. The fruits are edible but in most areas only eaten in times of food shortage (Sang and Mung'a, 1978; Albrecht, 1993). There is scarcity in literature on phytochemical analyses and pharmacological uses of this plant.

2.4.2 *Ocimum gratissimum* L

Ocimum gratissimum is an herbaceous perennial plant, which is woody at its base. Locally it is known as 'bwar' in Luo language. It prefers wet and fertile conditions, but can tolerate drought after flowering. It is a weed that is frequently encountered along roadsides but also in pasture (Swarbrick, 1997; Wagner *et al*., 1999; Orwa *et al*., 2009).

Ocimum gratissimum has been reported to contain alkaloids, flavonoids, tannins, phenols and saponins (Akubu, 1984; Gill, 1988; Edeoga *et al*., 2006). Leaves and flowering tops of the plant yield an essential oil on steam distillation. *O. gratissimum* from Kenya has been reported to be dominated by eugenol (which accounted for 68%) followed by methyl eugenol (13.21%). Other components include cis-ocimene (7.47), germacrene D (4.25%), trans-caryophyllene (1.69) and β-pinene (1.10%) (Matasyoh *et al.*, 2007). Thymol, citral, geraniol and linalool have been extracted from essential oils in its leaves

and stem (Sulistiarini, 1999). In south-eastern Nigeria, it is cultivated around houses to repel mosquitoes (Sofowara, 1982; Oparaocha *et al.,* 2010). The plant has been reported for its numerous medical uses in Kenya and Saharan African communities (Matasyoh *et al.,* 2007). Studies on *O. gratissimum* proved the plant extract can be source of medication for people living with Human Immunodeficieciency Virus (HIV) and Acquired Immune Deficiency Diseases, AIDS (Elujoba, 2000). Its oil is used to flavor foods, dental and oral products, in fragrances and aromatherapy and in traditional rituals and medicines.

The essential oil is also important insect repellent. The whole plant and the essential oil have many applications in traditional medicine, especially in Africa and India. Preparations from the whole plants are used to treating sunstroke, headache and influenza. The seeds have laxative properties and are prescribed against gonorrhea. The essential oil is applied against fever, inflammations of the throat, ears or eyes, stomach pain, diarrhea and skin diseases (Correa, 1931; Onajobi, 1986; Ilori *et al*., 1996). The essential oil is being tested for antimicrobial activity. In Indonesia (Sumatra) a tea is made from the leaves, while in Thailand the leaves are applied as a flavoring. In Indonesia the eugenol-type *O. gratissimum* is used in ceremonial washing of corpses (Orwa *et al*., 2009). No studies have been done on repellency of its essential oils against *An. gambiae.*

2.4.3 *Hyptis suaveolens* (L.) Poit

Hyptis suaveolens locally known as ‘nya ywe thiang’ in Luo language belongs to lamiaceae family and genus *Hyptis* Jacq. The genus consists of over 300 species that occur in tropical American (Harley and Reynolds, 1992). The aggressive annual weedy species, *H. suaveolens* is distributed in the tropic and subtropics, and it is not commonly found over 500 meters above sea level (Wulff, 1987). The plant is normally restricted to places where soils have been profoundly disturbed (Wulff, 1973).

Hyptis suaveolens has been reported to contain alkaloid, phenols, flavonoids and tannin but not saponins (Edeoga *et al*, 2006). The presence of these bases in the plant account for its usefulness as a medicinal plant. Several species from this genus have been reputed to posses’ medicinal properties and are frequently reported in the treatment of gastrointestinal infections, cramps and pain as well as in the treatment of skin infections (Correa, 1931; Asekun *et al*., 1999). Some work dealing with the composition, repellent and insecticidal activities of its essential oil against four stored-grain coleopteran pests (Tripathi and Upadhyay, 2009), composition and antifungal (Pandey *et al*., 1982; Singh *et al*., 1992), antibacterial (Iwu *et al*., 1990) and anticonvulsant (Akah and Nwambie, 1993) activities of *H. suaveolens* leaf oil have been previously reported.

It is reported that aerial part oils of *H. suaveolens* exhibited several constituents characterized by the occurrence of *β*-caryophyllene 41% Malaysia (Din *et al*., 1988); 22.15% Nigeria (Iwu *et al*., 1990; Asekun and Ekundayo, 2000), 1,8-cineole and sabinene 38.71% and 19.91% USA (Ahmed *et al*., 1994); 35.3% and 15.05% India

(Mallavarapu *et al*., 1993); 35.9% and 12.0% Aruba (Fun and Baerheim, 1990), 1,8-cineole and *β*-pinene 37% and 18% Brazillian Amazonian (Gottlieb *et al*., 1981), and 1,8-cineole 30.88% Brazilian Northeast (Luz *et al*., 1984) as major components. However, by the time of this study, no work had been reported on repellent activity of the oil from the leaf of the plant against *An. gambiae.*

CHAPTER THREE

MATERIAL AND METHODS

3.1 Plant Collection, Identification and Preparation

Collection of plants was based on ethno-botanical information. The aerial parts of *V. keniensis, O. gratissimum* and *H. suaveolens* were collected from different areas in western Kenya. The plant samples were collected randomly from five different locations in an area. The samples were collected at their different stages of growth. The collected plants were identified by a plant taxonomist Mr. Lucas Karimi Kaime from Kenyatta University, Department of Pharmacy and Complementary/Alternative Medicine and sample specimen deposited at the university herbarium. The samples (leaves, flowers or whole aerial parts) were air-dried under a shade for seven days before extraction.

3.2 Chemicals and Solvents

The solvent (acetone) used was of pure analytical HPLC grade. All the essential oils standards that were used for the GC co-injections and bio-assays were purchased from Aldrich Chemicals Company in Britain.

3.3 Extraction

The essential oils from the plants samples (leaves, flowers or aerial parts) were extracted by steam distillation using modified Clevenger apparatus. Five hundred grams of each of the plant material were put into a 2 liter round-bottom flask and 500 ml of water added. The flask was then fitted with the Clevenger apparatus and a double pocket condenser.

The plant materials were steam distilled for 4 hours. The essential oil was collected on water layer in the Clevenger apparatus. It was separated, dried with anhydrous sodium sulphate and stored in amber-colored vials at 0 ^{o}C until use.

3.4 Mosquito Repellency Assay

The essential oils were tested for their repellency activity against the female *An. gambiae* [ex-Ifakara (Tanzania) strain] that were reared under standard conditions at ICIPE Duduville mosquito insectary. The repellence assay was done as per WHO (1996) protocols on laboratory and field evaluation of insecticides and repellents. The bio-assays were carried out in a dark room with red light as the only source of illumination. The room temperature and humidity were artificially set using a heater and a humidifier to mimic the host feeding conditions for the female *An. gambiae* (temperature 27-35 ^{o}C and relative humidity greater than 65%).

All repellency tests were done using 5-7 days old female *An. gambiae* that had been starved overnight but previously fed on 6% glucose solution. Six human volunteers who did not show mild or no allergic reaction to mosquito bites or candidate oils were used in these assays. The subjects were given written informed consent before participating. They were not allowed to use lotion, perfumes, oils and perfumed soaps on the day of the bio-assay. The volunteers were provided with a standardized log sheet to ensure reliability of the data collected.

3.4.1 Mosquito Repellency Bio-assay of Essential Oils and Data Analysis

One gram of the neat oil from each plant sample was dissolved in 10 mls of acetone to give 10^{-1}g/ml (10%) solution. By serial dilution, 1% (10^{-2}g/ml), 0.1% (10^{-3}g/ml), 0.01% (10^{-4}g/ml), and 0.001% (10^{-5}g/ml) solutions in acetone were prepared. Each of these solutions was screened against *An. gambiae* using six different human volunteers. The test solution was applied on the right forearm of a volunteer from the wrist to the elbow. The rest of the hand was covered with glove to make it unattractive to the mosquitoes. Acetone (0.5ml) was applied on the left forearm to act as control. The control arm was the first to be introduced into the cage and was left for 3 minutes. The number of mosquitoes that landed on that arm was recorded. The treated arm was then introduced into the cage for the same period of time and the numbers of mosquitoes landing on the arm were recorded. The screening was done sequentially starting from the lowest dose (0.001%) and ended with the highest one (10%).

Each concentration was screened with fresh batch of 50 female *An. gambiae* mosquitoes in each cage, measuring 50×50×50 cm. After the bioassay of each concentration, the arms were washed with bar soap, rinsed well with tap water and then allowed to dry for 15-20 minutes, before application of the next dose of the sample. For each dosage the volume of repellent solution applied on the test arm depended on the surface area of the volunteer arms which was estimated using a paper. The amount of repellent was applied evenly to 500 cm^2 of the forearm skin between the wrist and the elbow on a volunteer. The % protective efficacy (P.E) was calculated as described by (WHO, 1996) as follows:

$$PE = 100\times (PCM - PTM)/ PCM$$

Where (PCM) Percent control mean and (PTM) percent test mean of mosquitoes landing on the control and treated arms respectively (Mehr *et al*., 1985). The repellency for the treated and the control was compared using the one-way analysis of variance (ANOVA) (Miller and Miller, 1984). The means were ranked using Student-Newman-Keuls (SNK) test (SAS Institute, 2000). The RD_{50} and RD_{75} values for the essential oils was calculated using Probit analysis (Busvine, 1971; Finney, 1971).

3.5 Chemical Identification

Characterization, identification and determination of the components of the essential oil from the repellent plants were done by gas chromatography (GC) and gas chromatography linked to mass spectrometry (GC-MS). Bioactive components of the oils were determined by gas chromatography linked to elecroantennogram (GC-EAG), and GC co-injection of the essential oils with authentic samples.

3.5.1 Gas Chromatography (GC)

Gas chromatographic separation was performed on a 6890N gas chromatography (GC) (Agilent Technologies) equipped with split-splitless injector (230 ^{o}C) and flame ionization detector (FID). Hydrogen was used as the carrier gas. The GC was equipped with a HP-1 capillary column (10m x 0.53mm i.d., 2.65 μm film thickness). The oven temperature was initially set at 30 ^{o}C for 0.5 min, followed by gradient increase to 150 ^{o}C at 5 ^{o}C/min for 0.1 min, and finally increased to 250 (10 ^{o}C/min) for 45 minutes. The components of essential oils were initially obtained with an enhance integrator (HP Chemstation).

3.5.2 Gas Chromatography-linked Mass Spectrometry (GC-MS)

GC-MS analyses were performed using a fused silica capillary column (50m x 0.32mm i.d., film thickness 0.52 μm, DB-1, J and W Scientific) attached to a cool on column injector, which was directly coupled to HP 5972 mass spectrometric detector (MSD). Ionization was by electron impact (70 eV, source temperature 250 ^{o}C). Helium was used as the carrier gas. The oven temperature was maintained at 30 ^{o}C for 5 minutes, and programmed at 5 ^{o}C/min to 250 ^{o}C. The calculation of retention indexes were made through co-injection with an n-alkenes' series (Van Den Dool and Kratz, 1963). Identification of the oil constituents were made based on the retention indexes (Adams, 1995) and by comparison of mass spectra with computer search using NIST21 and NIST107 libraries (NIST, 2005). The concentration of individual constituents was calculated from the GC peak areas and they are arranged in order of GC (HP-1) elution.

3.5.3 Gas chromatography-linked Electrophysiological Analyses

Gas chromatography-linked elecroantennographic analyses (GC-EAG) was used to identify volatile compounds within a sample that elicited an electrophysiological response from antenna of female *An. gambiae* (Wadhams, 1990). Elecroantennogram (EAG) recording from 5-7 day old female *Anopheles* mosquitoes was made using Ag-AgCl glass electrodes filled with ringer solution (Maddrell, 1969). The insects were anaesthetized by chilling and the head was excised and inserted in the tip of indifferent electrode. The signals was passed through a high impendence amplifier (UN-06, Syntech, The Netherlands) and analyzed by using a customized software package (Syntech, The Netherlands).

The essential oil was separated by injecting 1µl of the oil on an AI 93 GC equipped with a cold on column injector and an FID. The column used was a 50 m x 0.32 mm i.d. HP-1 column (non polar). Oven temperature was maintained at 40 ^{o}C for 2 minutes and then programmed at 5 ^{o}C/min to 100 ^{o}C and finally at 10 ^{o}C/min to 250 ^{o}C. The carrier gas was hydrogen (15psi). The outputs from the EAG amplifier and the FID were monitored simultaneously and with the software package (UN-06; Syntech, the Netherlands). A standard stimulation was done at the beginning and at the end of each recording to correct for loss of sensitivity of the preparation. This experiment was repeated six times. Identification of components of essential oils which gave rise to peaks associated with EAG-activities was confirmed by peak enhancement on GC co-injections of the crude essential oils with authentic samples (Pickett, 1990).

3.6 Repellency Tests of Synthetic Blends of Electrophysiological Active Constituents

Synthetic blends of electrophysiological active constituents were mixed in approximate relative amounts in which they occur in the oils of the three plants. The selected EAD active compounds in *O. gratissimum* essential oil included *α*–pinene, *β*-pinene, hexyl acetate, (*E*,*E*)-decadienal and eugenol which consisted of first five-component blend (FCB). One compound was subtracted at a time to form subsequent blends. *β*-Pinene, *p*-cuminol and (*E*)-caryophyllene formed the first three-component blend (TCB) of *H. suaveolens* essential oil. Succeeding blends were obtained as explained above. The selected EAD active compounds in *V. keniensis* essential oil that formed the first three-

component blend (TCB) included *α*-pinene, *p*-cymene and (*E*)-caryophyllene, subsequent blend were obtained by subtracting each component from the blend in turn.

Selected blends of electrophysiologically active components were used to find out the effect of subtracting one component at a time from a blend so as to check the dose response of the blend against *An. gambiae* (Owaga *et al*., 1988). These were tested in concentration range 10^{-5} – 10^{-1} g/ml as detailed in section 3.4.1 above and the repellency data was subjected to probit analysis.

CHAPTER FOUR

RESULTS AND DISCUSSION

4.1 Yields of Essential Oils

The amounts of essential oil obtained from dried aerial parts (500 g) of *H. suaveolens, V. keniensis* and *O. gratissimum,* differed greatly. Their percent yields are shown in Table 4.1.

Table 4.1: Percentage yields of essential oils obtained by hydrodistillation

Plant	Percent of essential oil by dry weight
O. gratissimum	0.12±0.05
V. keniensis	0.05±0.05
H. suaveolens	0.08±0.05

Ocimum gratissimum produced the highest percentage of the essential oil of 0.12±0.05% w/w while *H. suaveolens* and *V. keniensis* produced yields of 0.08±0.05 % w/w and 0.05±0.05 % w/w, respectively. The essential oils collected were less dense than water. *H. suaveolens* oil was red while those of *O. gratissimum* and *V. keniensis* were yellow and pale yellow, respectively. The oils were insoluble in water but soluble in ethanol, ether and dichloromethane (El Deeb *et al*., 2004).

4.2 Evaluation of Repellency of the Essential Oils of *O. gratissimum, V. keniensis* and *H. suaveolens* against *An. gambiae*

Results of the repellence assays of the essential oils of *O. gratissimum, V. keniensis and H. suaveolens* against *An. gambiae* s. s. are summarised in Table 4.2 and appendix 7. The results show that *O. gratissimum* oil is significantly more repellent than oils of *V. keniensis* and *H. suaveolens* and comparable to that of DEET.

Table 4.2: Mean percent repellencies of *O. gratissimum, V. keniensis, H. suaveolens* oils and DEET and their respective RD_{50} and RD_{75} (95% CI) values against *An. gambiae*

Dose (g/ml)	*O. gratissimum* % PE±SE	*V. keniensis* % PE±SE	*H. suaveolens* % PE±SE	DEET % PE±SE
10^{-5}	47.66±10.84[a]	36.53±14.83[a]	42.59±5.52[a]	51.11±13.32[a]
10^{-4}	64.94±10.01[a]	57.78±9.35[a]	58.06±12.06[a]	86.22±4.51[b]
10^{-3}	85.47±6.48[b]	58.38±8.58[a]	67.39±6.23[a]	94.29±3.69[b]
10^{-2}	100.00±0.00[c]	73.34±5.92[b]	68.79±11.23[a]	100.00±0.00[c]
10^{-1}	100.00±0.00[c]	91.67±5.69[c]	94.38±2.69[b]	100.00±0.00[c]
RD_{50} ($\times 10^{-5}$ mg cm^{-2})	2.77(1.22-3.25)[A]	5.68(4.12-6.72)[B]	6.27(4.48-7.28)[B]	1.25(0.82-2.13)[A]
RD_{75} ($\times 10^{-5}$ mg cm^{-2})	10.5(10.5-63.0)[A]	851(158-26100)[B]	597(124-10600)[B]	7.45(3.37-16.2)[A]

Mean values followed by same small letters within the same column are not significantly different $p < 0.05$, while RD_{50} and RD_{75} (95% CI) values in the same row followed by the same capital letter(s) are not significantly different $P < 0.001$.

Previously, Oparaocha *et al.* (2010) evaluated the fumigant toxicity of methanol extract of a chemotype of *O. gratissimum* in Nigeria and found it to reduce the biting rate of different species of mosquitoes. However, the active constituents responsible for this effect were not characterised. Similarly, in a thermal fumigation experiment, *H. suaveolens* was found to be an effective source of repellent blend against *An. gambiae* (Seyoum *et al.*, 2002). In another study, controlled-release of essential oil of *H. suaveolens* from a formulation with wood flour and starch was found to repel mosquitoes in households (Vineet *et al*., 2011). No previous reports on the mosquito repellence of *V. keniensis* essential oil have been reported.

4.3 Composition of the Essential Oils of *O. gratissimum*, *V. keniensis* and *H. suaveolens* and their EAG-active Constituents

Generally, a total of 53 constituents were identified in the essential oils of the three plants. The constituents characterized from essential oils were mainly sesquiterpene (58.49%) monoterpenes (33.96%), and others (7.55%).

Table 4.3: The chemical composition of essential oil of *O. gratissimum*, EAG- active constituents and their respective percentage antennal responses

A total of 24 constituents were identified in the essential oil of *O. gratissimum* (Table 4.3). The oil was dominated by monoterpenes (58.3%) and sesquiterpenes (37.5%).

Compound	% Peak area	EAG activity (% response frequency)
Monoterpenoid		
α-Thujene	0.24	
α-Pinene	0.51	Active (50.0)
Sabinene	0.45	
β-Pinene	3.66	Active (33.3)
α-Terpinene	0.68	
β-Cymene	0.66	
(*Z*)-Ocimene	29.73	Active (83.3)
(*E*)-Ocimene	2.44	
β-Linalool	4.13	
Terpinolene-4-ol	1.27	
Eugenol	21.76	Active (83.3)
Sesquiterpenoids		
α-Copaene	1.93	
β-Cubebene	1.80	
β-Caryophyllene	5.86	
α-Humulene	0.23	
Germacrene D	9.65	Active (50.0)
Elemecin	1.94	
δ-Cadinene	0.14	
β-Caryophyllene oxide	3.14	
Asarone	1.49	
Others		

1-Octen-3-ol	0.46	
Hexyl acetate	1.21	Active (50.0)
(*Z*)-4, 8-Dimethyl-1,3,7-nonatriene	0.76	Active (50.0)
(*E, E*)-Decadienal	0.86	Active (50.0)

Table 4.4 gives the 31 constituents identified in *V. keniensis* essential oil. Sesquiterpenes, 70.97%, and monoterpenes, 22.58% dominated the oil.

Table 4.4: The chemical composition of essential oils of *V. keniensis*, EAG-active constituents and their respective antennal responses

Compound	% Peak area	EAG-activity (% response frequency)
Monoterpenoid		
α-Pinene	0.49	Active (33.3)
β-Myrcene	0.53	
α-Phallandrene	1.57	
p-Cymene	1.40	Active (33.3)
β-Thujene	0.40	
(*E*)-Ocimene	0.96	
β-Linalool	1.25	
Sesquiterpenoids		
α-Cubebene	0.30	
α-Copaene	1.03	
β-Cubebene	10.88	
β-Elemene	1.01	
(*Z, Z*)-α-Farnesene	0.32	
(*E*)-Caryophyllene	2.71	Active (50.0)
Trans-*α*-Bergamotene	0.21	
E-*β*-Farnesene	1.00	
α-Humulene	1.88	
α-Curcumene	1.14	
α-Selinene	1.15	
α-Muurolene	3.75	
γ-Elemene	1.83	
δ-Cadinene	12.67	
α-Gurjunene	2.03	
Germacrene D-4-ol	2.39	
Spathulenol	0.90	Active (33.3)
Patchulane	4.60	
γ-Eudesmol	0.64	

α-Bisabolol	0.95	
Tau-Muurolol	9.79	
α-Cadinol	16.01	Active (83.3)
Others		
1-Octen-3-ol	0.51	
Citronellol acetate	0.32	

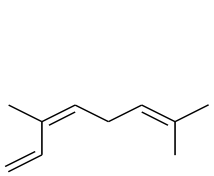
A total of 22 constituents were identified in essential oil of *H. suaveolens.* Table 4.5 shows that the oil was dominated with sesquiterpenes, 65%, and monoterpenes, 30%.

Table 4.5: The chemical composition of essential oils of *H. suaveolens*, EAG-active constituents and their respective percentage antennal responses

\

Compound	% Peak area	EAG-activity (% response frequency)
Monoterpenes		
Sabinene	4.13	
β-Pinene	0.63	Active (66.7)
Limonene	1.02	
γ-Terpinene	0.56	
α-Terpinolene	1.60	
Sesquiterpenes		
α-Copaene	0.45	Active (33.3)
α-Gurjonene	3.72	Active (33.3)
(*E*)-Caryophyllene	21.27	Active (66.7)
(*E*)-*α*-Bergamotene	5.07	
Bicyclogermacrene	2.19	Active (50.0)
α-Selinene	2.01	
γ-Elemene	9.75	
Spathulenol	4.35	
β-Caryophyllene oxide	3.70	
Ledol	1.16	
Globulol	0.88	
(*Z*)-*α*-Trans-bisabolene epoxide	1.29	
(*Z*)-*α*-Cis bisabolene	4.54	
(*Z*)-*α*-Trans-bergamotol	0.66	
Other		
1-Octen-3-ol	0.83	
p-Cuminol	1.80	Active (66.7)

The GC-MS chromatograms of *O. gratissimum*, *V. keniensis* and *H. suaveolens* are shown in the appendices 1, 3, and 5 respectively. The peaks labeled correspond to the major constituents in each chromatogram as listed in appendices 2, 4 and 6. The constituents above 3.14% in *O. gratissimum* essential oil were (*Z*)-ocimene (**1**), eugenol (**2**), (R)-(+)-germacrene D (**3**), *β*-caryophyllene (**4**), *β*-linalool (**5**), and *β*–pinene (**6**) among others as indicated in Table 4.3.



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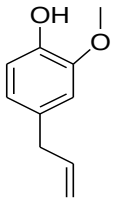

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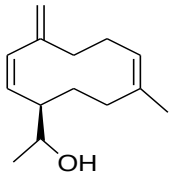

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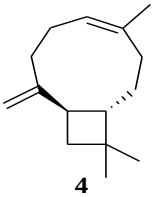

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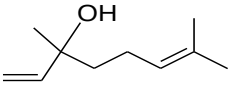

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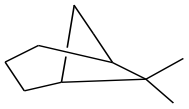
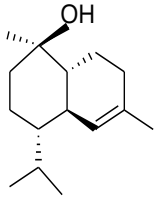


6

Vitex keniensis essential oil constituents above 3.75% were *α*-cadinol (**7**), *δ*-cadinene (**8**), *β*-cubebene (**9**), tau-muurolol (**10**), and patchulane (**11**) as indicated in Table 4.4.



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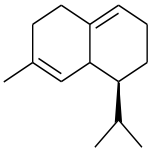

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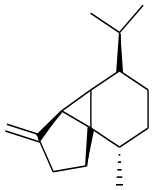

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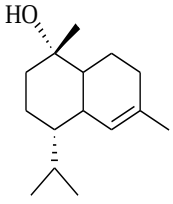

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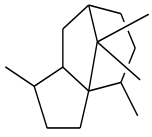
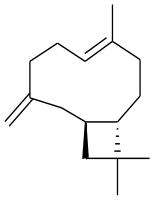


11

The constituents of essential oil of *H. suaveolens* above 4.13% were (*E*)-caryophyllene (**12**), *γ*-Elemene (**13**), (*E*)-*α*-bergamotene (**14**), (*Z*)-α-cis bisabolene epoxide (**15**), and spathulenol (**16**) among other compounds as given in Table 4.5.



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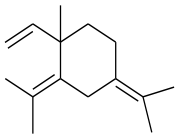

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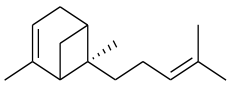

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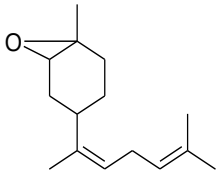

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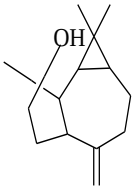

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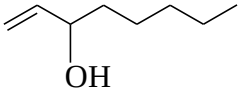
The essential oil constituents namely 1-octen-3-ol **(17)**, *β*-pinene **(6)** and *α*-humulene **(18)** were present in all the essential oils but in small amounts.



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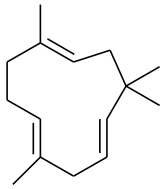

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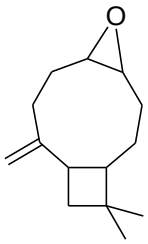

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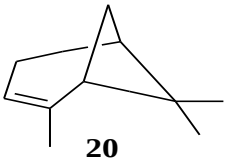
The high concentration of eugenol as one of the main constituent of essential oil of *O. gratissimum* was in agreement with previous studies (Matasyoh *et al.*, 2007; Dubey *et al*., 2000; Vasconcelos-Silva *et al*., 1999). However, eugenol **(2)** as a constituent of essential oil in *O. gratissimum* obtained from Portugal Island was not reported in a previous study (Martins *et al*., 1999). This is the first time that chemical constituents of the essential oil of *V. keniensis* are characterized.

The chemical constituents of essential oils of *H. suaveolens* varied significantly as reported in literature. For example, *α*-bergamotene **(14)** was reported as a major constituent in *H. suaveolens* from Nigeria (Asekun and Ekundayo, 2000) but was found

at low concentration of 5.07 % in this sample. This may be due to chemotypic or climatic differences. Caryophyllene oxide **(19)**, which was at low concentration of 3.70% in this sample, was detected as the main component in *H. suaveolens* oil from Cuba (Pino *et al*., 2003). The high concentration of *(E)*-caryophyllene **(12)** as main constituent of essential oil of *H. suaveolens* was in agreement with studies by Azevedo *et al*., (2002) that indicated a higher content of (*E*)-caryophyllene **(12)** as a constituent of the essential oil in *H. suaveolens* from Brazilian Cerrado.

4.4 Electrophysiologically Active Constituents of the Essential Oils

Gas chromatography-electroantennography (GC-EAG) data showed that the mosquito responded to many but not all of the compounds present in the essential oils as shown in Appendices 11, 12, and 13. Interestingly, some minor compounds still unidentified, elicited strong response from the mosquitoes. In *O. gratissimum* essential oil, online EAG responses were found repeatedly at ten different retention times; eight of the components were identified by high resolution GC-MS analyses. The EAD active compounds included α–pinene **(20),** β-pinene **(6)**, hexyl acetate **(21)**, (*E,E*)-2, 4-decadienal **(22)**, eugenol **(2)**, (*Z*)-ocimene **(1)**, (*Z*)-4, 8-dimethyl-1, 3, 7-nonatriene **(23)** and (*R*)-(+)-germacrene D **(3)**. (*Z*)-ocimene **(1)** and eugenol **(2)** gave the highest percentages of antennae responses as indicated in Table 4.3 and appendix 11.



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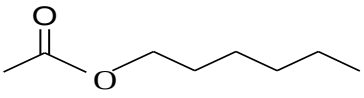

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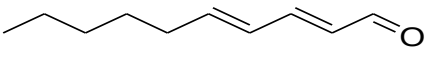

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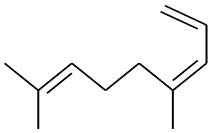

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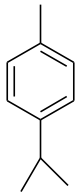
Six responses were found repeatedly at six different retention times in *V. keniensis* essential oil and five components were identified as shown in Table 4.4 and appendix 12. These included *α*–pinene **(20)**, *p*-cymene **(24),** (*E*)-caryophyllene **(12)**, spathulenol **(16)** and α-cadinol **(7)** with the latter giving the highest percentage of antennal response.



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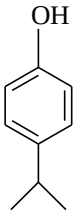

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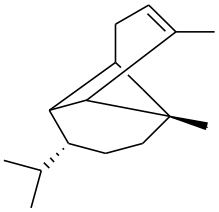

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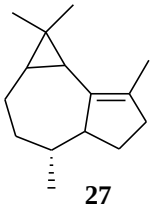

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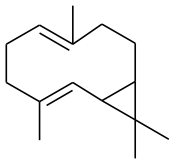

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In *H. suaveolens* essential oil, EAG responses were found repeatedly at ten different retention times and the six identified components were *β*–pinene **(6)**, *p*-cumenol **(25)**, (*E)*-caryophyllene **(12)**, *α*-copaene **(26)**, *α*-gurjonene **(27)**, bicyclogermacrene **(28)** with the first three components giving the highest percentages of antennae responses. The data is given in Table 4.5 and appendix 13.

4.5 Evaluation of Repellency of the Blends and Determination of their Dose Response

4.5.1 Evaluation of Repellency of *O. gratissimum* Essential Oil and its EAG- active Selected Blends and Determination of their Dose Response

The mean percent repellencies and repellency dose-response, RD_{50} and RD_{75} (the dose that will repel 50% and 75% of *An. gambiae* mosquitoes) values for subtractive assay of the selected electrophysiologically active components in *O. gratissimum* essential are given in Table 4.6. RD_{50} indicated that repellency activity of five-component full blend mixture was lower than the essential oil of *O. gratissimum* suggesting that other component(s) of the blend may actually inhibit the repellency of active compounds in the parent oil; alternatively, other than these five compounds, there are other EAG-active components may also be contributing to the repellent activity of the oil of this plant. RD_{50} values show that repellency dosage of 5-component blend (FCB) was significantly increased by subtraction of hexyl acetate (**21)**, (*E*,*E*)-decadienal **(22)** and *α*-pinene **(20)**. Their removal made the resultant respective blends less repellent as evidenced in Table 4.6. Subtraction of *β*–pinene **(6)**, eugenol (**2**) from their respective blend had no significant effect on repellent effects of resultant blends.

The subtractive assays provided information into the relative contribution of EAD-active components to the overall repellency. Absence of hexyl acetate **(21)**, (*E*,*E*)-decadienal **(22)** and α-pinene **(20)** reduced the activity of resulting four-component blend showing these components contribute most to the activity of *O. gratissimum* essential oil.

FCB minus (*E*,*E*)-2, 4-decadienal **(22)** had a negative repellency at the lowest concentration of 10^{-5} g/ml. It is worth noting that this blend maintained the lowest repellency at different doses as compared to other blends as shown in Table 4.6. However the blend had a repellency of 100% at the highest dose concentration of 0.1g/ml. Repellents may interact with and inhibit the response of a sensory neuron to a normally attractive chemical signal. For example it was found that DEET inhibits the response of lactic acid (Davis and Sokolove, 1976). The degree of interference of repellent with the cell's response to lactic acid alone is dependent on the intensity of the repellent. A chemical that is an attractant at low stimulus intensities may at higher intensities, alter the response of insect from attraction to repulsion.

Table 4.6: Mean percent repellencies, RD_{50} and RD_{75} values of *O. gratissimum* oil and its selected blends

Dose (g/ml)	*O. gratissimum*	5-component blend (FCB)	FCB minus α-pinene	FCB minus β –pinene	FCB minus Hexyl acetate	FCB minus (*E, E*)-2,4-decadienal	FCB minus eugenol
10^{-5}	47.66±10.84[a]	30.89±12.01[a]	26.40±29.02[a]	22.75±8.15[a]	4.04±9.84[a]	-16.88±7.67[a]	21.07±10.77[a]
10^{-4}	64.94±10.01[b]	43.05±6.48[b]	61.26±6.06[b]	49.95±7.30[b]	26.75±8.36[b]	25.29±4.94[b]	51.64±7.2[b]
10^{-3}	85.47±6.48[c]	48.66±5.27[b]	63.61±12.29[b]	66.05±7.90[c]	39.79±5.42[b]	37.99±5.66[c]	55.29±3.64[b]
10^{-2}	100.00±0.00[d]	71.93±9.16[c]	93.06±3.17[c]	89.90±4.60[d]	82.32±6.71[c]	55.49±7.45[d]	76.09±3.64[c]
10^{-1}	100.00±0.00[d]	100.00±0.00[d]	100.00±0.00[d]	100.00±0.00[e]	100.00±0.00[d]	100.00±0.00[e]	100.00±0.00[d]
RD_{50}($\times 10^{-5}$ mg cm^{-2})	2.77 (1.22-3.25)[A]	19.3 (4.02-36.0)[B]	682 (425-533)[E]	13.3 (3.89-26.6)[B]	71.9 (36.9-87)[C]	168 (96.1-362)[D]	16.4 (6.17-35.8)[B]
RD_{75} ($\times 10^{-5}$ mg cm^{-2})	10.5 (10.5-63)	571 (172-4080	36.8 (4.39-246)	122 (60.1-2700)	5160 (243-14600)	318 (678-5500)	24.4 (142-937)

Mean values followed by same small letters within the same column are not significantly different, $p < 0.05$, while RD_{50} values in the same row followed by the same capital letter(s) are not significantly different $P < 0.001$. Values in parentheses represent lower and upper confidence limits at 95%.

.

For example, it has been reported that at low levels, DEET will attract female *A. aegypti* (Kost *et al*., 1971). Repellency is observed only when the amount of DEET in the vapour phase is increased. Similar paradoxical patterns have been observed in the oviposition attraction response of *An. triseriatus*. At normal low levels (3 ppm), *p*-cresol attracts these mosquitoes to an oviposition site but at higher levels it repels them (Bently *et al*., 1979). Moreover, lactic acid alone, which is normally a host attractant at high intensity levels, will repel female *A. aegypti* (Muller, 1968). Absence of eugenol (**2**) and *β*-pinene **(6)** had no effect on overall repellency of resulting blends.

4.5.2 Evaluation of Repellency of *V. keniensis* Essential oil and its EAG-Active Selected Blends and Determination of their Dose Response

Table 4.7 gives the mean percent repellencies and RD_{50} values obtained from repellency data of blends of selected EAG active components of *V. keniensis*. The three component blend (TCB) of (*E*)-caryophyllene **(12)**, *α*-pinene **(20)** and *p*-cymene **(24)** was less effective than *V. keniensis* essential oil against *An. gambiae*. This demonstrated that other component(s) of the blend may actually inhibit the repellency of the active compounds in the blend or, other than these three compounds, there might be some other(s) that could be contributing to the repellent activity of the oil of this plant.

Subtraction of *α*-pinene **(20)** reduced the activity of resulting two-component blend showing this component contributes most to the activity of *V. keniensis* essential oil. Subtraction of each of the other two constituents namely, (*E*)-caryophyllene **(12)** and *p*-cymene **(24)** resulted in a significance decrease in the dosage of the resulting blend. Their removals made the resultant respective blends more repellent. This is in agreement with a

study on mosquito larvicidal activity of dichloromethane extract of the root bark of *Lantana viburnoides sp*. against *An. gambiae* which showed that subtraction of some fractions resulted in enhanced activity (Innocent *et al.,* 2008).

p-Cymene **(24)** and (*E*)-caryophyllene **(12)** were identified as the least active components of the blend. However, (*E*)-caryophyllene (**12**) has been reported to contribute towards the overall repellency of *Nepata cataria* oil against *An. gambiae* (Birkett *et al*., 2011), but studies elsewhere on repellency assays of individual constituents have shown that (*E*)-caryophyllene **(12)** and *p*-cymene **(24)** did not show any repellent activity against *An. gambiae* (Omolo *et al.,* 2004). A binary blend of *p*-cymene (**24**) and (*E*)-caryophyllene (**12**) (Table 4.7) was moderately active (44.74±6.76%) against *An. gambiae*. This is in agreement with previous report (Bekele and Hassanali, 2001), illustrating the lethal activity of quarternary mixture of methyl chavicol, ethyl isovalerate, α-humulene, and 1, 8-cineole against *R. dominica,* while none of the single components was individually toxic to *R. dominica.* Interestingly presence of α-pinene in the binary blend with *p*-cymene (**11**) and (*E*)-caryophyllene (**12**) respectively (Table7) demonstrated repellency comparable to DEET at RD_{50}.

Table 4.7: Mean percent repellencies, RD_{50} and RD_{75} (95% CI) of *V. keniensis* oil and its selected EAD-active blends

Dose (g/ml)	*V. keniensis*	3-component blend (TCB)	TCB minus *α*-pinene	TCB minus *p*-cymene	TCB minus (*E*)-caryophyllene	DEET % PE±SE
10^{-5}	36.53±14.83[a]	22.72±16.33[a]	40.19±8.38[a]	55.80±7.76[a]	52.07±7.81[a]	51.11±13.32[a]
10^{-4}	57.78±9.35[a]	24.05±11.63[a]	44.74±6.76[a]	56.29±5.22[a]	59.25±5.61[a]	86.22±4.51[b]
10^{-3}	58.38±8.58[a]	44.55±12.34[a]	56.89±10.60[a]	72.99±2.31[b]	61.79±3.58[a]	94.29±3.69[c]
10^{-2}	73.34±5.92[b]	64.13±4.45[b]	68.43±8.76[a]	79.54±8.77[b]	67.00±3.34[a]	100.00±0.00[d]
10^{-1}	91.67±5.69[c]					100.00±0.00[d]
RD_{50} (×10-^{5} mg cm-^{2}	5.68 (4.12-6.72)[B]	66 .6 (20-225)[C]	251 (230-1000)[D]	0.838 (0.437-3.2)[A]	0.412 (0.23-1.09)[A]	1.25(0.82-2.13)[A]
RD_{75} (×10-^{5} mg cm-^{2}	851 (158-26100)	65000	15000	450 (70.4-470)	112000	7.45(3.37-16.2)[A]

Mean values followed by same small letters within the same column are not significantly different, $p < 0.05$, while RD_{50} and RD_{75} values in the same row followed by the same capital letter(s) are not significantly different while, $P < 0.001$.

4.5.3 Evaluation of Repellency of *H. suaveolens* Essential oil and its EAD-active Selected Blends and Determination of Dose Response

Table 4.8 gives the mean percent repellencies and RD_{50} values obtained from repellency data of blends of selected EAG active constituents of *H. suaveolens* essential oils against *An. gambiae*. The results showed that the activity of *H. suaveolens* essential oil was less than mixture of the three components of *β*-pinene (**6**), *p*-cuminol (**25**) and (*E*)-caryophyllene (**12**). This suggested that other components of the essential oil may actually inhibit the repellence of active compounds. Subsequent subtraction of each constituent decreased the repellency action of the resulting blend indicating that the three constituents enhanced the repellent activity of one another.

The dose-response results showed that *α*-pinene (**20**), *p*-cuminol (**25**), hexyl acetate (**21**) and (*E*, *E*)-decadienal (**22**) increased repellency of their respective blends against *An. gambiae*. Of special interest is the contrasting ways in which (*E*)-caryophyllene **(12)** and *β*–pinene **(6)** contributed to repellency of resultant blends against mosquitoes. *p*-Cymene **(24)** was found to reduce the repellency of its respective blends. Eugenol (**2**) had no significant effect on the activity of its blends. Given the structural diversity of the compounds, there is no conclusive generalization that can be made on structural requirements for potency in repellent activity against *An. gambiae.*

Table 4.8: Mean percent repellencies and RD_{50} and RD_{75} (95% CI) of *H. suaveolens* oil and its selected blends

Dose (g/ml)	*H. suaveolens*	3-component blend (TCB)	TCB minus *β*-Pinene	TCB minus *p*-cuminol	TCB minus (*E*)-caryophyllene	DEET % PE±SE
10^{-5}	42.59±5.52[a]	45.47±5.33[a]	35.23±8.61[a]	30.25±6.41[a]	30.70±11.76[a]	51.11±13.32[a]
10^{-4}	58.06±12.06[a]	63.42±4.17[b]	58.17±5.54[b]	47.20±7.51[b]	67.20±3.70[b]	86.22±4.51[b]
10^{-3}	67.39±6.23[a]	68.80±4.89[b]	60.30±5.79[b]	61.77±5.83[c]	72.63±4.21[b]	94.29±3.69[c]
10^{-2}	68.79±11.23[a]	86.66±6.42[c]	100.00±0.00[c]	81.45±5.03[d]	90.55±1.56[c]	100.00±0.00[d]
10^{-1}	94.38±2.69[b]	100.00±0.00[d]	100.00±0.00[c]	100.00±0.00[e]	100.00±0.00[d]	100.00±0.00[d]
RD_{50}(×10-^{5} mg cm-^{2})	6.27(4.48-7.28)[C]	3.95 (2.94-4.10)[B]	9.42 (5.51-16.14)[C]	20.6 (18.63-42.3)[D]	7.27 (5.46-15.1)[C]	1.25(0.82-2.13)[A]
RD_{75}(×10-^{5} mg cm-^{2})	597 (124-10600)	103 (44.3-277)	96.3 (45.4-248)	317 (15-832)	88.8 (44.2-202)	7.45(3.37-16.2)

Mean values followed by same small letters within the same column are not significantly different, $p < 0.05$, while RD_{50} values in the same row followed by the same capital letter are not significantly different, $P < 0.001$.

CHAPTER FIVE

5.0 CONCLUSIONS AND RECOMMENDATIONS

5.1 Conclusions

This study demonstrates that essential oils from aerial parts of *O. gratissimum, V. keniensis* and *H. suaveolens* are repellent to *An. gambiae.* A number of compounds from their essential oils elicited consistent electrophysiological responses in the antennae of *An. gambiae*. These compounds were identified using GC-EAD and GC-MS analyses and constituted several classes of compounds including monoterpenes (**1**, **6**, **20**, and **24)**, sesquiterpenes (**3**, **27**, and **28)**, bicyclic sesquiterpene (**12)**, tricyclic sesquiterpene (**26)**, sesquiterpenoid alcohol, (**7, 16)**, an ester, (**21)**, an aldehyde (**22)**, olefin (**23)** and phenylpropanoids (**2**, **25)**. The GC-EAD analysis can be used to identify minor components of an essential oil that are also responsible for repellency. All full blends (FB) of essential oils and their respective blends produced a repellence of 100% at maximum dose concentration of 10^{-1} g/ml. This implied that a constituent that reduces the activity of a blend at low concentration may increase its activity at higher concentration.

Some constituents which reduced the repellent activities of their respective blends (*p*-cymene and (*E*)-caryophyllene) can become moderately active in binary mixture. This blend effect suggest that synergism of some repellent constituents produces mixture that are active than linear summation of their individual activities. Repulsion may well be enhanced through combining different repellent plants that can be locally sustainable part in mosquito control efforts. From the study, repellent activity of an essential oil cannot be assigned to a particular compound.

Malaria mosquitoes bite mainly during night, dusk and dawn. Consequently the essential oils could be used as alternative, relatively safe, natural insect repellent to protect people from mosquito bites. Since the extraction technique is simple, the bases that can be used to prepare the lotions are affordable and plants are abundant and the local people could be taught how to prepare the lotions. The formulation could equally supplement the protection afforded by window and nets for those who because of cost and/or odour of permethrin do not like to sleep under the insecticide-treated bed nets or those who stay outside their houses due to social and religious responsibilities.

5.2 Recommendations

The following are recommendations from this research:

i. Extend the study to include all the compounds in the essential oils of the plants that elicited consistent electrophysiological responses with the antennae of *An. gambiae*, and carry out subtractive bioassays to determine the relative contribution of each component to the overall repellency of EAG-active blend. The optimally active blend can then be evaluated and developed for personal and space protection.

ii. No studies have previously been reported on the biological activities and chemical constituents of *V. keniensis*. A more detailed investigation on the medicinal, pesticidal and phytochemistry of *V. keniensis* is warranted.

iii. To evaluate fumigant toxicity of essential oils of *O. gratissimum*, *V. keniensis* and *H. suaveolens* against *An. gambiae*.

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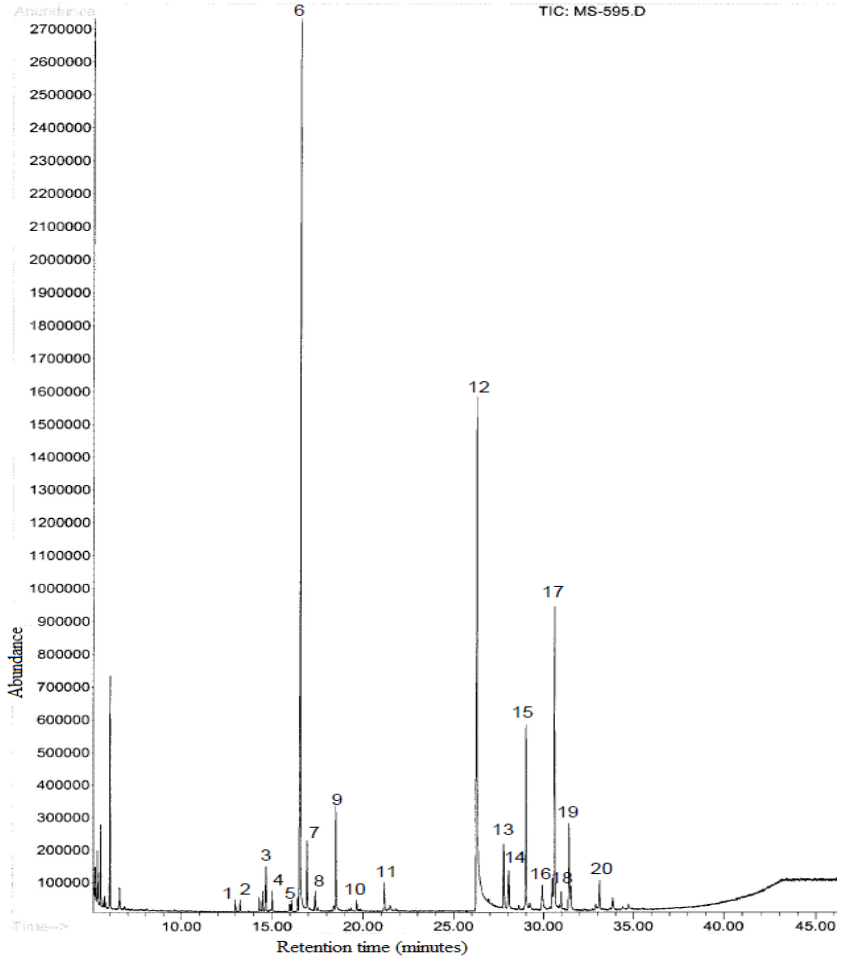
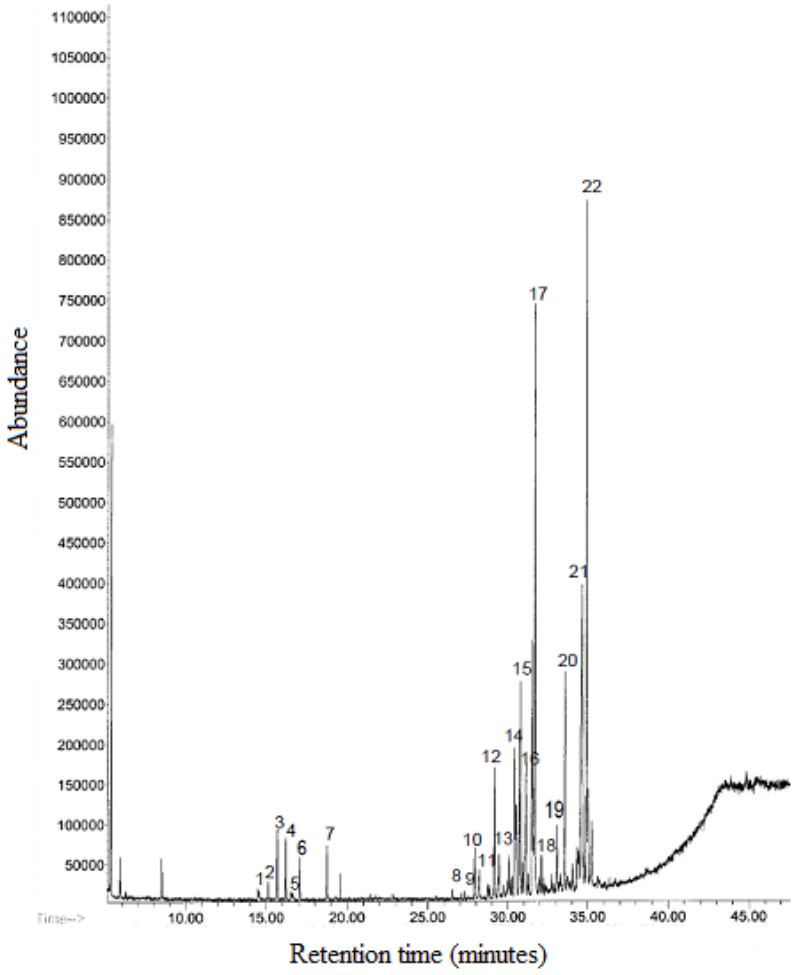
APPENDICES



Appendix 1: The GC-MS chromatogram of O. *gratissimum* essential oil. The labeled peaks correspond to the major constituents as listed in Appendix 2

Appendix 2 Major constituents in *O. gratissimum* essential oil and their relative proportion

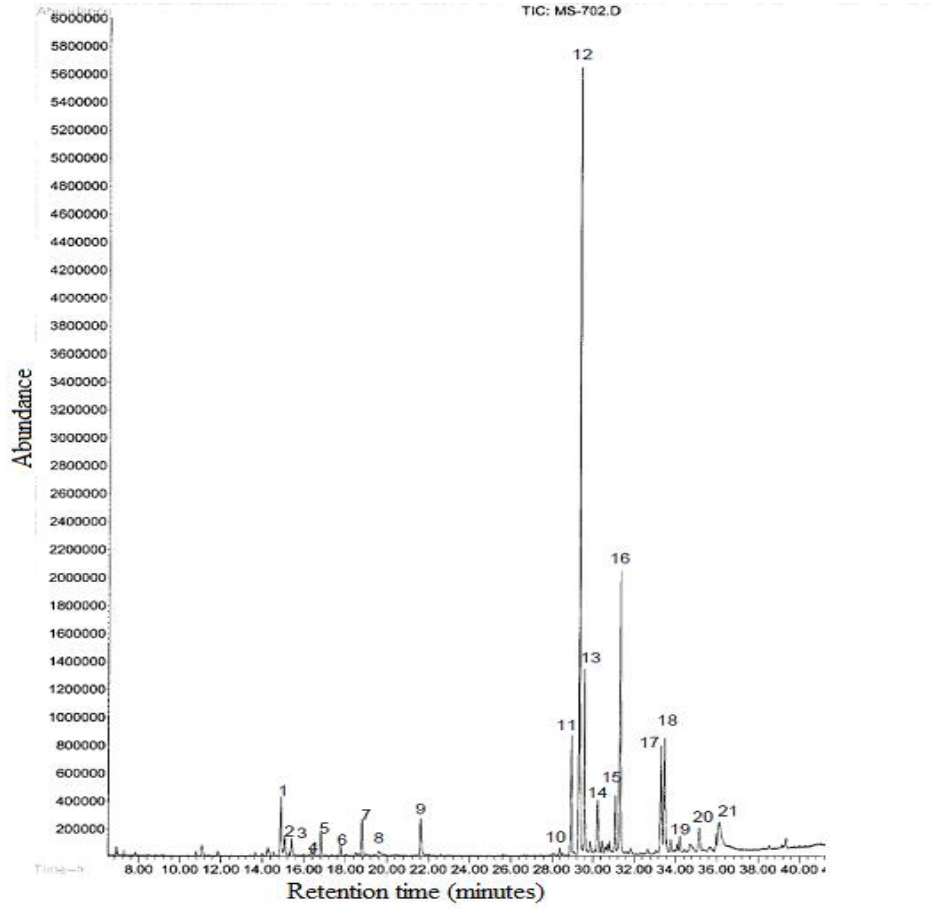
Peak	Kovat Index (KI)	Compound	% Peak Area
1	930	*α*-Thujene	0.2417
2	939	*α*-Pinene	0.5123
3	973	*β*-Pinene	3.6620
4	981	*β*-myrcene	1.2088
5	1002	*α*-Terpinene	0.6814
6	1024	(*Z*)-Ocimene	29.7345
7	1040	(*E*)-Ocimene	2.4431
8	1057	*γ*-Terpinene	0.7623
9	1098	*β*-Linalool	4.1342
10	1136	*α*-Terpinolene	0.8650
11	1182	Terpinen-4-ol	1.2667
12	1350	Eugenol	21.7606
13	1375	*α*-Copaene	1.9333
14	1399	*β*-Cubebene	1.7974
15	1450	*β*-Caryophyllene	5.8579
16	1489	(*Z*,*E*)-*α*-Farnesene	0.2348
17	1493	Germacrene D	9.6517
18	1508	Bicyclogermacrene	0.1258
19	1527	Elemicin	1.9363
20	1593	Caryophyllene oxide	3.1413



Appendix 3: The chromatogram of essential oil of *V. keniensis*. The peaks labeled correspond to the major constituents as listed in Appendix 4

Appendix 4: Major compounds present in *V. keniensis* essential oil and their relative Proportions

Peak	Kovat Index ((KI)	Compound	% Peak Area
1	969	*β*-Pinene	0.4864
2	981	*β*-Myrcene	0.5338
3	991	*α*-Phellandrene	1.5710
4	1007	*p*-cymene	1.4028
5	1025	(*Z*)-Ocimene	0.4032
6	1040	(*E*)-Ocimene	0.9618
7	1098	*β*-Linalool	1.2508
8	1354	Citronellol acetate	0.3166
9	1375	*α*-Cubebene	0.2991
10	1389	*α*-Copaene	1.0300
11	1399	*β*-Elemene	1.0092
12	1450	(*E*)-caryophyllene	2.7102
13	1455	(*E*)-β-Farnesene	1.0006
14	1465	*α*-Curcumene	1.1407
15	1476	*β*-Cubebene	10.8769
16	1498	*γ*-Muurolene	7.1415
17	1530	*δ*-cadinene	12.6734
18	1537	*α*-Gurjunene	2.0306
19	1586	Germacrene D-4-ol	2.3928
20	1642	*γ*-Eudesmol	0.6431
21	1648	Tau-muurolol	9.7906
22	1661	*α*-Cardinol	16.0073

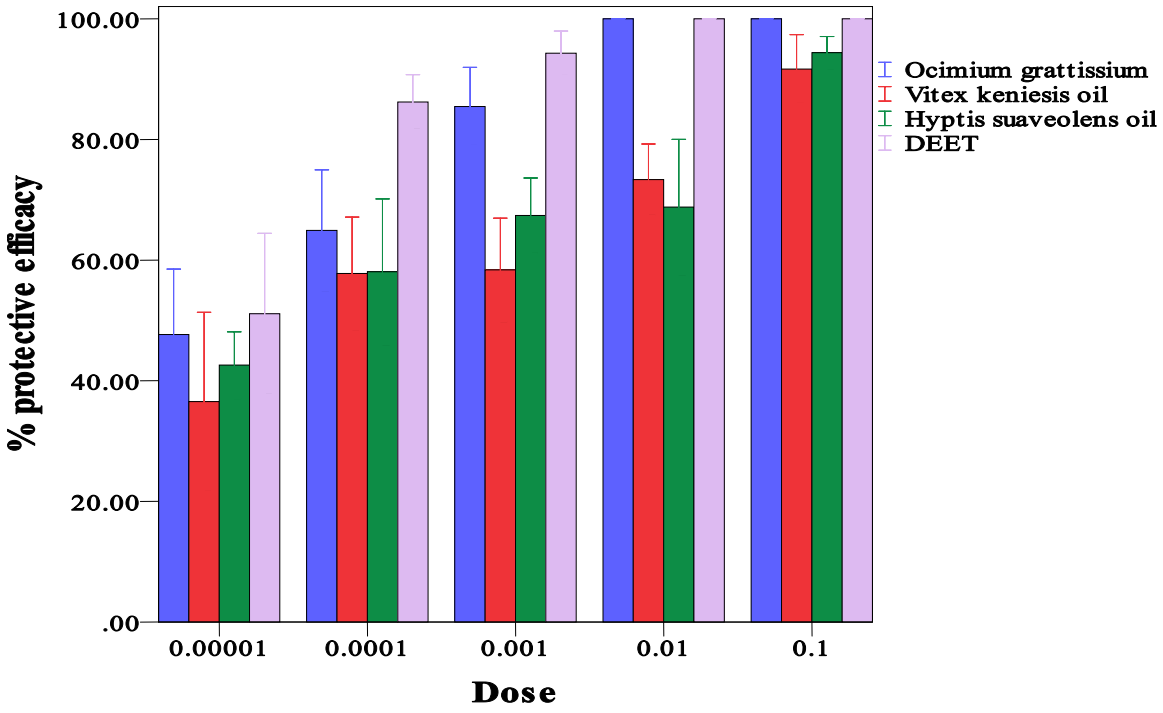


Appendix 5: The GC-MS chromatogram of essential oil of *H. suaveolens*. The peaks labeled correspond to the major constituents as listed in Appendix 6

Appendix 6: Major compounds present in *H. suaveolens* and their relative proportions

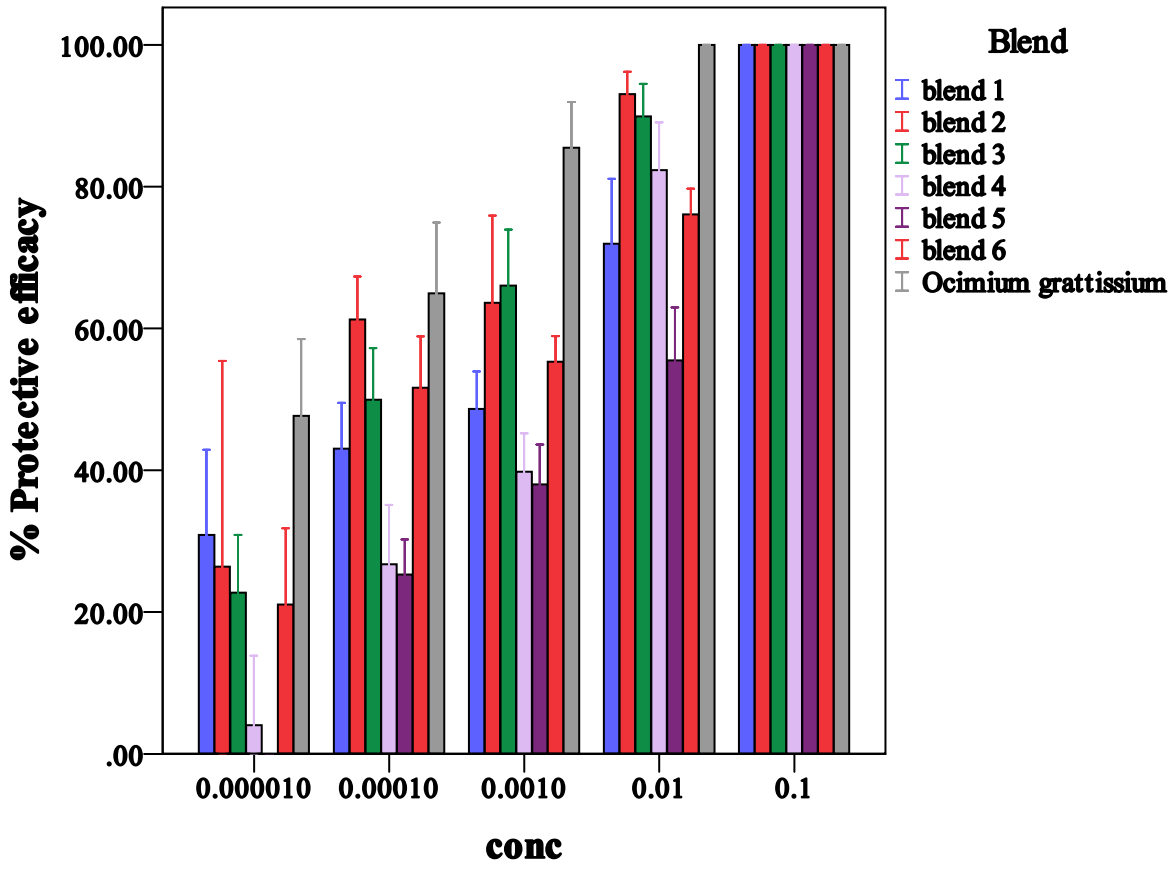
Peak	Kovat Index (KI)	Compound	% Peak Area
1	969	Sabinene	4.1267
2	972	*β*-pinene	0.6335
3	979	3-Octanol	0.4879
4	1005	*p*-Cymene	1.0169
5	1019	Limonene	1.0169
6	1056	*γ*-Terpinene	0.5563
7	1087	Terpinolene	1.6029
8	1098	Linalool	0.5465
9	1181	Terpinen-4-ol	1.8015
10	1413	*β*-Elemene	0.7988
11	1435	Isocaryophyllene	3.7230
12	1450	(*E*)-Caryophyllene	21.2713
13	1459	Trans-*α*-Bergamotene	5.07224
14	1482	*α*-Humulene	2.1946
15	1514	*β*-Selinene	2.0052
16	1523	*γ*-Elemene	9.7535
17	1586	Spathulenol	4.3472
18	1592	*β*-Caryophyllene oxide	3.6949
19	1623	Globulol	0.8787
20	1662	(*Z*)-*α*-cis Bisabolene epoxide	4.5389
21	1705	(*Z*)-*α*-Bergamotol	0.6630

.



Appendix 7: Mean (±SE) percentage protective efficacy provided by *O. gratissimum, V. keniensis, H. suaveolens* oils and N, N-diethyltoluamide (DEET) against *An. gambiae*

Dose in grams per milliliter (g/ml)

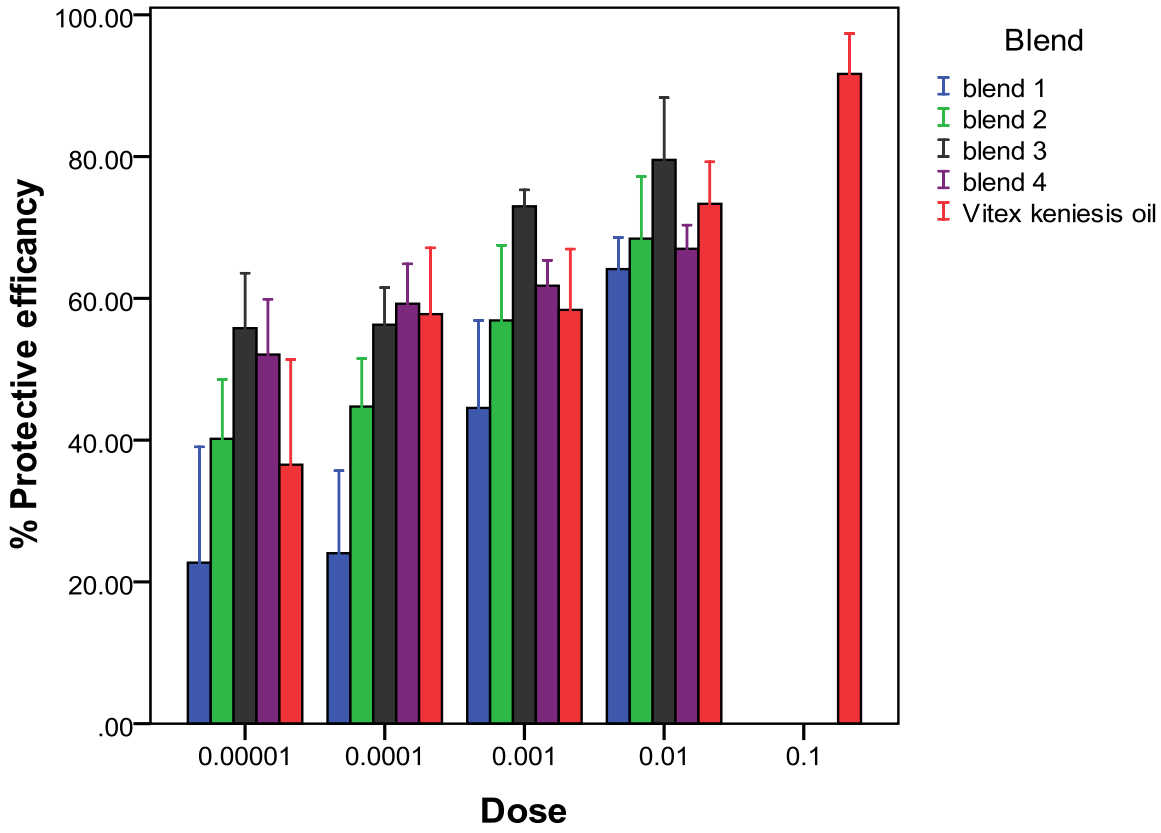


Appendix 8: Mean (±SE) percentage protective efficacy of *O. gratissimum* oil and selected blends against *An. gambiae* mosquitoes

Key

Blend 1: α-pinene, β-pinene, hexyl acetate, (E, E)-decadienal and eugenol
Blend 2: β-pinene, hexyl acetate, (E, E)-decadienal and eugenol
Blend 3: α-pinene, hexyl acetate, (E, E)-decadienal and eugenol
Blend 4: α-pinene, β-pinene, (E, E)-decadienal and eugenol
Blend 5: α-pinene, β-pinene, hexyl acetate and eugenol
Blend 6: α-pinene, β-pinene, hexyl acetate and (E, E)-decadienal,

Conc is concentration which is grams per milliliter (g/ml)

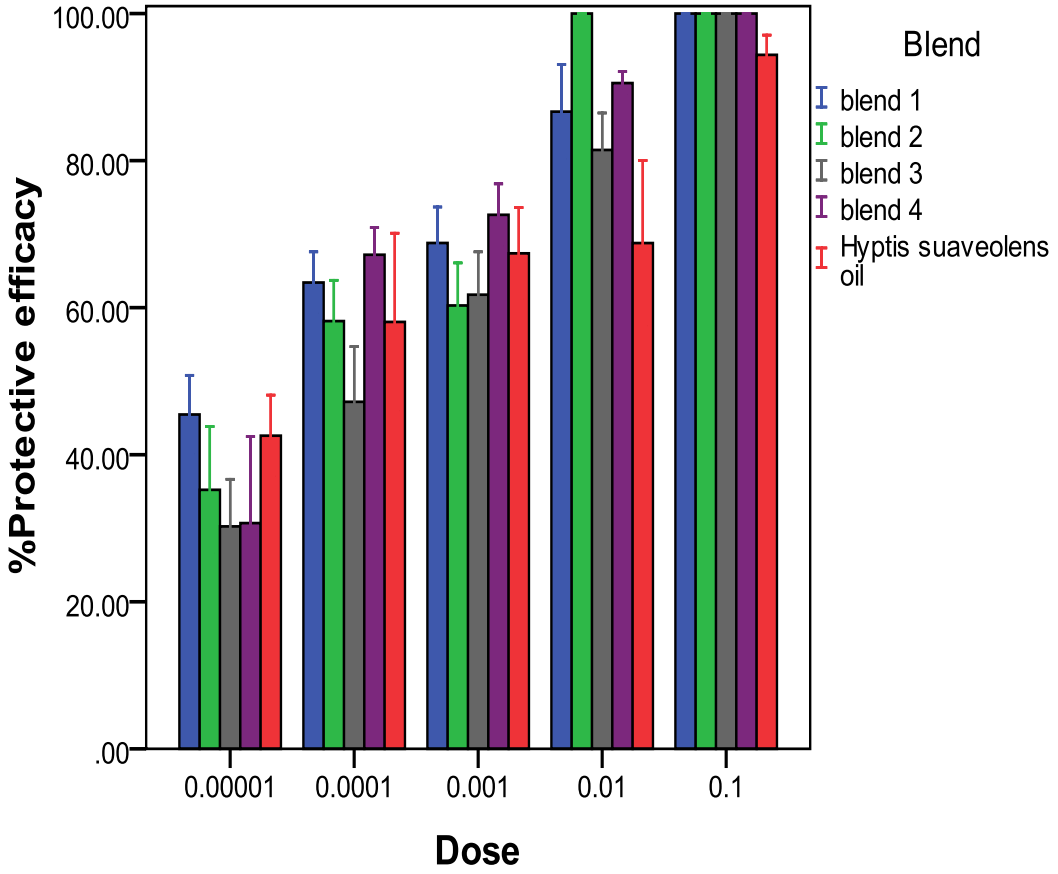


Appendix 9: Mean (±SE) percentage protective efficacy provided by *V. keniensis* oil and selected blends against *An. gambiae* mosquitoes

Key

Blend 1: *α*-pinene, *p*-cymene and (*E*)-caryophyllene
Blend 2: *p*-cymene and (*E*)-caryophyllene
Blend 3: *α*-pinene and (*E*)-caryophyllene
Blend 4: *α*-pinene, *p*-cymene

Dose is in grams per milliliter (g/ml)

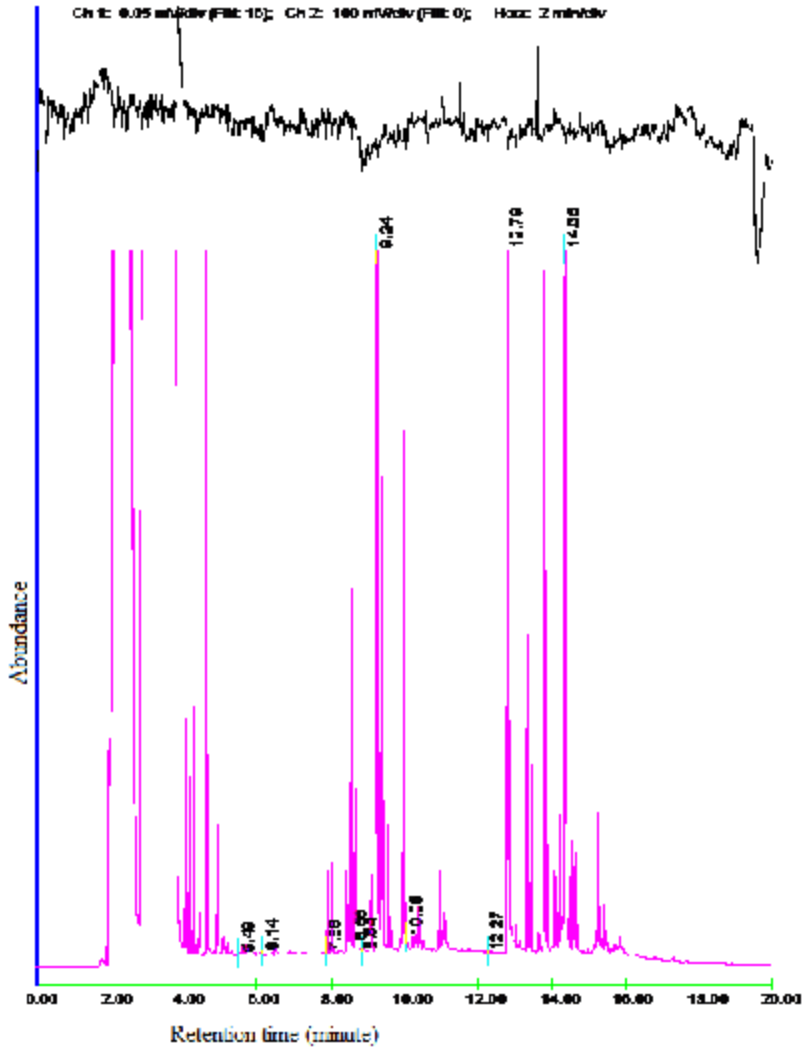


Appendix 10: Mean (±SE) percentage protective efficacy provided by *H. suaveolens* oil and selected blends against *An. gambiae* mosquitoes

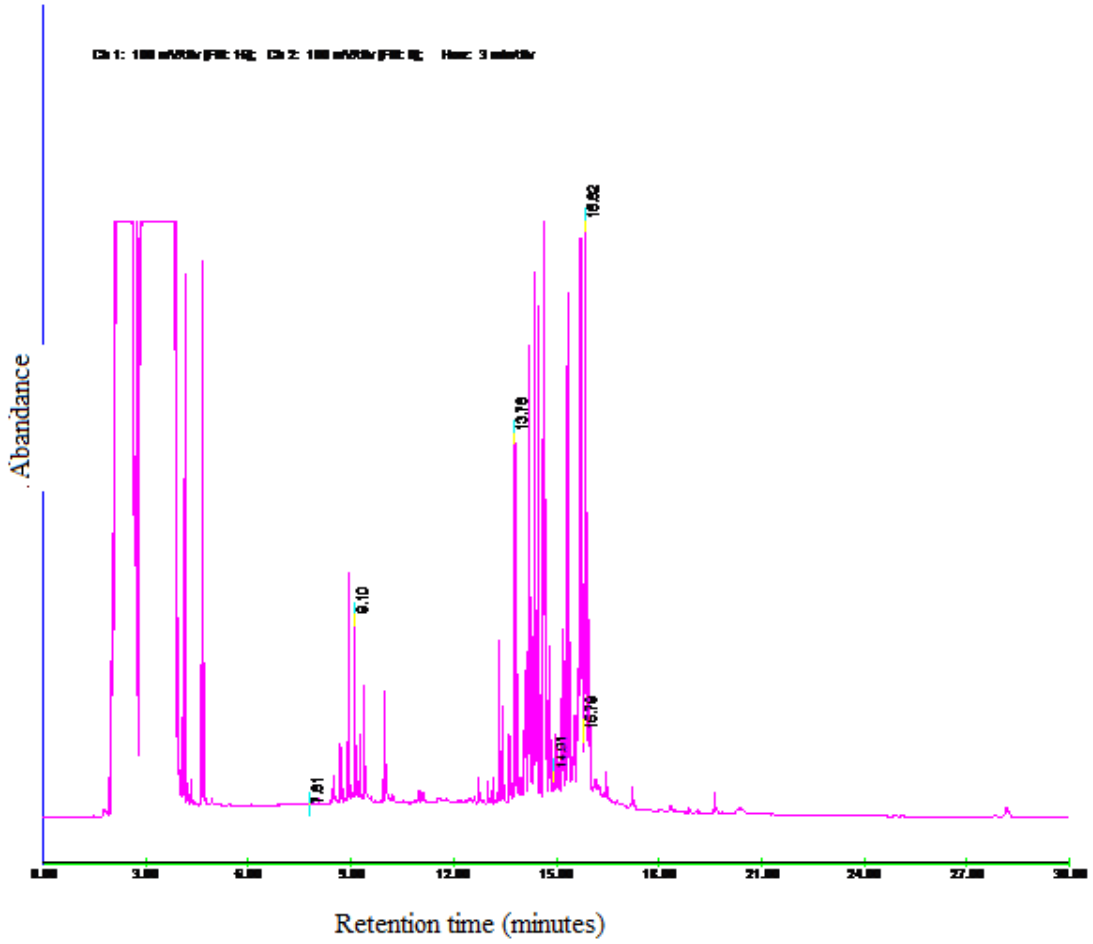
Key

Blend 1: *β*-pinene, *p*-cuminol and (*E*)-caryophyllene
Blend 2: *p*-cuminol and (*E*)-caryophyllene
Blend 3: *β*-pinene and (*E*)-caryophyllene
Blend 4: *β*-pinene and *p*-cuminol

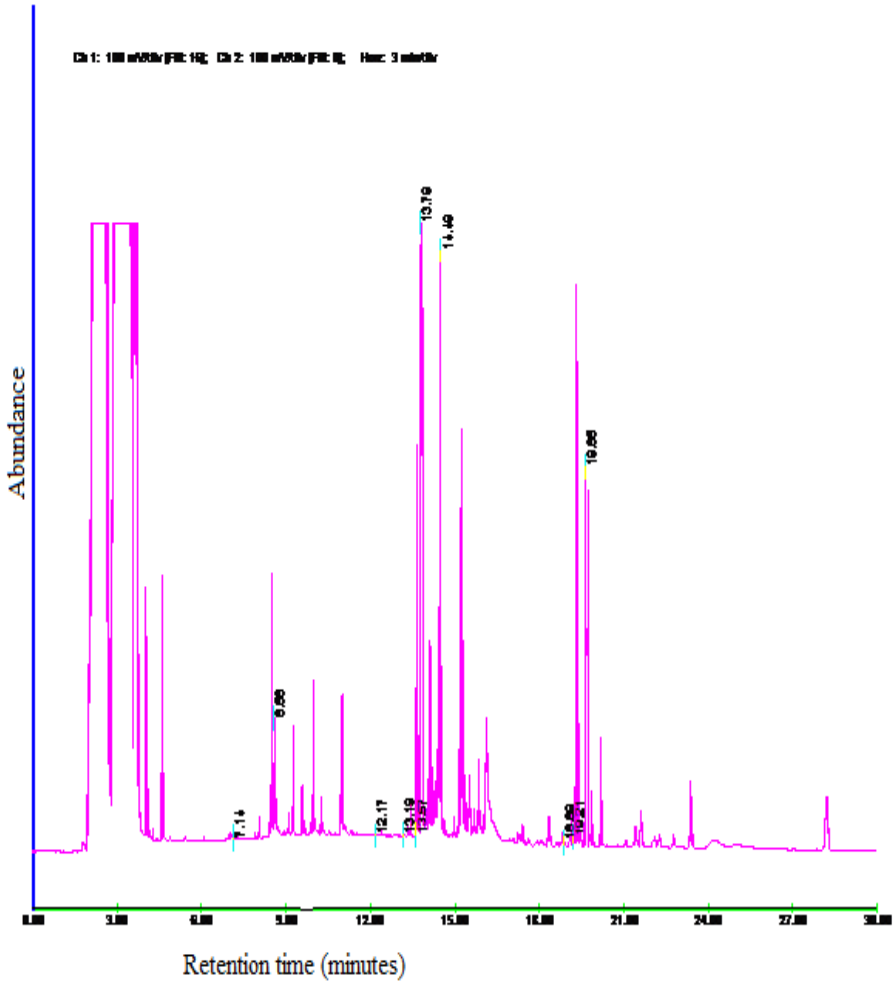
Dose is in grams per milliliter (g/ml)



Appendix 11: Peaks of GC Chromatogram of *O. gratissimum* essential oil (indicated by retention time) that elicited electrophysiological response from antennae of female *An. gambiae*



Appendix 12: Peaks of GC Chromatogram of *V. keniensis* essential oil (indicated by retention time) that elicited electrophysiological response from antennae of female *An. gambiae*



Appendix 13: Peaks of GC Chromatogram of *H. suaveolens* essential oil (indicated by retention time) that elicited electrophysiological response from antennae of female *An. gambiae*