REPELLENCE OF ESSENTIAL OIL OF *Nigella sativa* L. SEEDS AGAINST *Anopheles gambiae* AND IDENTIFICATION OF THE ACTIVE BLEND

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DECLARATION

Declaration by Candidate

This thesis is my original work and has not been presented for award of degree in any other University or any other award.

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DEDICATION

This work is dedicated to my late father Dishon Ndirangu, who encouraged me to pursue graduate studies. My mother Shelmith Wangu, my sisters Charity and Judy, my brother Muriithi and mostly to my dear wife Miriam, and our children Vicky and Frankie who understood my absence.

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ABBREVIATIONS AND ACRONYMS

ACTs	Artemisinin-Based Combination Therapies
AL	Artemether-Lumefantrine
ANOVA	Analysis of Variance
ASAQ	Artesunste-Amodiaquine
ASMQ	Artemisinin-Mefloquine
ASSP	Artesunate-Sulfadoxine-Pyrimethamine
DDT	Trichloro-2,2-bis-(p-chlorophenyl)ethane
DEET	N,N-dimethyl-*m*-toluamide
DHA-PQ	Dihydroartemisinin-Piperaquine
GC	Gas Chromatography
GC-EAD	Gas Chromatography-linked Electro Antennographic Detection
GC-MS	Gas Chromatography/ Mass Spectrometry
ICIPE	International Centre of Insect Physiology and Ecology
ITN	Insect treated bed-net
NIST	Institute of Standards Technology libraries
Rd_{50}	Dose response at 50% confidence level
Rd_{75}	Dose response at 75% confidence level
+SE	Standard error
SNK	Student Newman Keuls
SP	Sulphadoxine-Pyrimethamine
WHO	World Health Organization

ABSTRACT

Anopheles gambiae mosquitoes are vectors of malaria because of their ability to transmit *Plasmodium falciparum* parasites. The major impact of malaria is in sub-Saharan Africa where at least 90% of the deaths from malaria occur. In Kenya, malaria accounts for 30% of all outpatients and 19% of all admissions to health facilities. Malaria can also affect the quality of labour negatively and also can lead to low productivity through absenteeism. *Anopheles gambiae* is the vector associated with stable malaria transmission in Africa because it is strongly anthropophilic, feeding exclusively on humans. One of the greatest challenges facing malaria control is the spread and intensification of parasite resistance to treatment. *P. falciparum* has become resistant to almost all malaria drugs including artemisinin and its derivatives. This means that there is need to come up with effective methods to control mosquito populations as well as diversifying methods of malaria treatment. There is no single mosquito control method which is effective in all situations. Today, the most effective insect repellent is DEET but it has been associated with medical complications when used for a long time. In many parts of the world plant-derived natural products have been used to repel mosquitoes and other insects. The objective of this study was to evaluate the repellence of the essential oil on *Nigella sativa* L. seeds using *An. gambiae* and identify the active constituents and blend. *Nigella sativa* L. seeds were ground and hydro-distilled. Then bioassays of essential oil was conducted on human subjects against newly emerged female *An. gambiae* using DEET as the positive control. It was noted that the repellence (98.81±1.19 and 100.00±0.00 at concentration of 0.01g/ml and 0.1g/ml respectively) of the essential oil against *An. gambiae* was comparable to that of DEET (100.00±0.00 and 100.00±0.00 at concentration of 0.01g/ml and 0.1g/ml respectively) at higher doses; however, it showed lower repellence (36.97±1.81 and 50.41±2.87 against 51.11±13.32 and 86.22±4.51 of DEET at concentration of 0.0001g/ml and 0.0001g/ml respectively) at lower doses. GC-MS and GC-EAD (Gas Chromatography-linked Electro Antennography) analyses of the essential oil led to the identification of eight bioactive constituents namely α-thujene (**19**), longifolene (**38**), 1, 2, 3, 4, 5-pentamethylcyclopentane (**18**), α-pinene (**20**), β-pinene (**22**), tetradecane (**24**), *p*-cymene (**11**), and α-longipinine (**37**). Subtractive bioassays to characterize the constituents that contributed most to the repellence of the oil was then carried out. The most repellent blend was found to contain (+)-β-pinene (**41**), (-)-β-pinene (**42**), (+)-α-pinene (**39**), (-)-α-pinene (**40**), α-longipinene (**37**), tetradecane (**24**) and 1,2,3,4,5 pentamethylcyclopentane (**18**) (RD_{75} = 3.763), though less repellent than DEET (RD_{75}=1.630). Bioassay of pure (+)-α-pinene (**39**) and (-)-α-pinene (**40**) showed that (+)-α-pinene (**39**) was a better repellent than (-)-α-pinene (**40**). More studies need to be undertaken on the essential oil of *N. sativa* seeds to determine the optical stereo-chemistry of the α-pinene (**20**) and β-pinene (**22**) and also establish whether α-thujene (**19**) and longifolene (**38**) contribute to repellency or not against *An. gambiae*. These results form the basis of downstream development of the appropriate blends for personal protection against *An. gambiae*.

CHAPTER ONE

INTRODUCTION

1.1 Background information

Malaria remains an important public health concern in the world. Malaria accounted for estimated 225 million people in 2009, resulting in 781,000 million deaths globally (WHO, 2010a). Each year there are about 500 million people who are afflicted and about 2.7 million people die from malaria (Olliaro and Cattani, 1996; WHO, 2002; WHO, 2005). Approximately 85% of global malaria cases and about 90% of the deaths occur in Africa, the majority being children under five years and pregnant women (Olliaro and Cattani, 1996; WHO, 2002; WHO, 2005). Among children with clinical attacks of malaria, several thousands were estimated to have experienced neurological damage and up to 250,000 have developmental problems (Greenwood *et al.,* 2007). Between 1.5 and 2.7 million deaths occur annually, thus in every 40 seconds malaria kills a child (Roll back malaria, 2002).

Recent estimates have placed the economic losses due to malaria in sub-Saharan Africa to over US dollars 12 billion annually (WHO, 2010b). The disease imposes high and regressive cost burden on households that have a sick family member, with poor households spending a higher proportion of their income on health care than the better off households (Goodman *et al.,* 2000; Russel, 2004). Figure **1.1** shows world distribution of malaria in the world. In Kenya, malaria accounts for 30% of all outpatients and 19% of all admissions to health facilities (Chepkwony *et al.,* 2007). It affects 20 million Kenyans annually and is a debilitating disease that kills 26,000 children per year (Chepkwony *et al.,* 2007). In Kenya, malaria is the leading cause of workdays lost due to the illness. It was estimated that 170 million working days in 2003 were lost annually due to malaria (Republic of Kenya, 2003), the figure may have gone up.

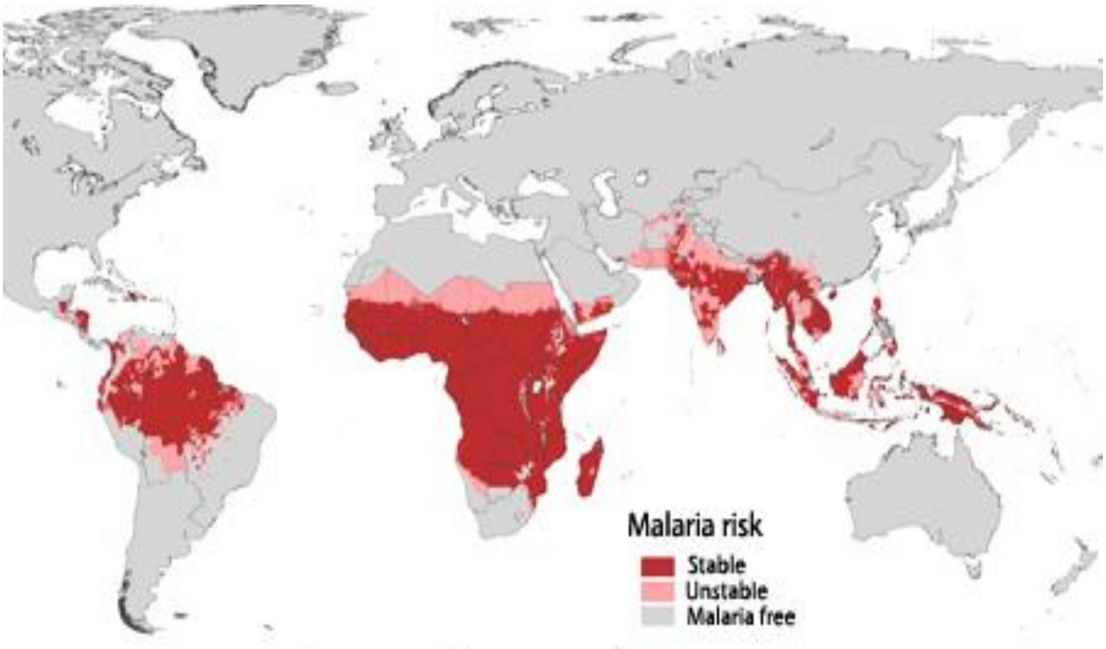


Figure 1.1: Malaria map (http://news.sciencemag.org/2008/new-map-malaria)

Malaria is transmitted in human by mosquitoes of the genus anopheles. *Anopheles gambiae* has been called "the most dangerous animal in the world" as it is such an excellent vector for *Plasmodium falciparum* (Roberts and Schimidt, 2000). *An. gambiae* is the vector associated with stable malaria transmission in Africa because it is strongly anthropophilic and exclusively feeding on humans (Paskewitz *et al*., 1993). Malaria is transmitted when mosquitoes feed on human blood in more than one occasion on different hosts. In parts of rural Africa, a person may suffer 50 to 100 bites per night with one to five of these mosquitoes carrying sporozoites (Robert and Schimidt, 2000). Malaria epidemics has been linked to climatic changes like the El-nino weather phenomena, global warming and interference of man with the environment (Lindsay and Birley, 1996; Jerten *et al*., 1996; Bouma *et al*., 1996,1997; Mouchet *et al*., 1998), drug and insecticide resistance (WHO, 1999). One of the greatest challenges facing malaria control is the spread and intensification of parasite resistance to antimalarial drugs. The availability of limited number of effective drugs has led to increasing difficulties in the development of antimalarial drugs policies and adequate disease management (Baird, 2005).

Essential oils of many plants species have been found to have toxic and repellent effects against different insects (Curtis *et al*., 1991; Regnualt-Roger, 1997). Malaria transmission can be reduced by minimizing mosquito bites by use of insect repellents. Although insecticides treated nets protect against mosquito bites in many parts of the world, people may contact disease in the early evening before they retire to the confines of the net, since exposure to malaria vector and nuisance mosquitoes starts in the early evening (Maxwell *et al*., 1998).

Thus there is need to find supplemental protective measures for personal and/or space protection against this disease vector.

Nigella sativa L. seeds have been used since ancient times mainly as moth balls in wool and silk cloths to protect them from insect attacks (Peter, 2004). In a preliminary study, essential oil of *N. sativa* seeds was found to be repellent to mosquitoes (Bulugahapitiga and Arachchige, 2007). However, the essential oil was not characterized and repellency was based in cage experiments, not according to WHO protocol (WHO,1996a) that allows measurement of protection from feeding mosquitoes on human subjects. This sought to use WHO protocol to measure repellence and identify the constituents of the essential oil using GC-MS and the constituents primarily responsible for repellence be identified using GC-EAD and subtractive assays.

1.2 Problem statement and justification

Insecticides have been used in adult vector control for various diseases, e.g. malaria and sleeping sickness. For example, pyrethrum extracts have been used extensively in mosquito control, but high costs of isolation of pyrethrum coupled with reported resistance to synthetic pyrethrins calls for use of other more effective insecticides (WHO 1995; Mitchell, 1996). Synthetic insecticides such as Baygone, (DDT) [1,1,1-trichloro-2,2-bis(*p*-chlorophenyl)ethane], methoxyclor and synthetic pyrethroids such as allethrin and cyclethrin have been used as effective insecticide. Cross resistance has occurred in some classes of insecticides, e.g. *An. gambiae* has been reported to be resistant to DDT, permethrin and lambdacyhalothrin in West Africa (WHO, 1996b).

The use of insecticide-treated nets (ITNs) reduces malaria incidences and several synthetic pyrethroids are recommended with permethrin topping the list. However, fear of increased resistance to permethrin have been expressed and improper use of ITNs, non-compliance and the unaffordable re-treatment regime has reduced their impact on malaria transmission (Fradin and Day, 2002).

Synthetic repellents such as dimethylphthalate and 2-ethyl-1,3-hexanediol have not provided a great impact in controlling the rate of mosquito bites and transmission of malaria parasites since most of them are highly volatile and thus provides only short-lived protection against the vector (Frances and Cooper, 2002). The use of some mosquito repellents such as *N,N*-diethyltoluamide (DEET) and picaridin (trade name Bayrepel) can reduce malaria as well as the discomfort of insect bites (Costantini *et* *al.,* 2004). DEET is currently the most used synthetic repellent (Frances and Cooper, 2002). However, concerns have been raised about its safety (McGready *et al.,* 2001) and also it is not available in Kenya. The discovery of *p*-menthane-3,8-diol as an effective mosquito repellent from *Eucalyptus citriodora* Hook (Trigg, 1996) has increased the choice of topical repellents.

Mosquitoes control in Kenya relies on insecticide treated bed-nets and insecticides (ITNs), e.g. pyrethrin. Natural pyrethrins from the flowers of *Chrysanthemum cinerariaefolium* L. are powerful insecticides with low mammalian toxicity. Even though, the use of natural pyrethrins depend on the rapid knock down effect, lack of prolonged residual action and cost have restricted their application (Kumar, 1984). Natural mosquito repellents include pyrethrin (an excite-repellent/insecticide) (WHO, 1984), essential oils from some plants like lemon grass, thyme, geranium, bergamot, baylaurel, pine, wintergreen, pennyroyal, and eucalyptus (Curtis *et al.,* 1991). Examples of other natural repellents that have been isolated from a wide range of plants include, geraniol, citronella, linalool, camphor α-pinene, *p*-menthane-3,8-diol, 1,8-cineole, eugenol (Dethier, 1947), *p*-menthane-3,8-diol (Trigg, 1996), perilly alcohol, perillaldehyde, *cis*-verbenol and caryophylene oxide (Omolo *et al.,* 2004). Mosquitoes usually start biting people well before they retire to bed.

Sometimes mosquitoes can bite a person while still in the confines of the net if exposed part of the body comes into contact with the net. Thus personal protection is important to avoid bites from this disease vectors. In addition almost all malaria drugs have become resistant to malaria parasites and thus control of vector population is very important. There is no single vector control method that is effective in all situations, there is need to combine different mosquito control methods so that they can compensate for the deficiencies of each individual method.

1.3 Hypothesis

Essential oil of *N. sativa* contains constituents repellent to *An. gambiae*, and which can be identified by GC-EAD, GC-MS and repellence bioassays.

1.4 Objectives

1.4.1 General objective

To evaluate the repellency of the essential oil of *N. sativa* seeds using *An. gambiae* and identify the active constituents and blend.

1.4.2 Specific objectives

i. To hydro-distil essential oil from *N. sativa* seeds and carry out bioassays at different doses against *An. gambiae*.

ii. To carry out GC-MS (gas chromatography-linked mass spectrometry) analysis of essential oil and identify the major constituents.

iii. To carry out GC-EAD (Gas Chromatography-linked Electro-antennographic Detection) analysis of essential oil of *N. sativa* to identify candidate active compounds associated with repellence against *An. gambiae*.

iv. To carry out subtractive bioassays of candidate active compounds with *An. gambiae* to identify the active repellent blend.

1.5 Scope of the study

Nigella sativa seeds were obtained from the Kenyan market. Bioassay of the essential oil and synthetic blends was carried on the mosquitoes reared in ICIPE. The bioassays of different blends were only carried out with commercially available compounds.

CHAPTER TWO

LITERATURE REVIEW

2.1 Malaria pandemic

Malaria is one of the devastating diseases in the world. In 2007, 2.37 billion people globally lived in areas at risk of *Plasmodium falciparum* transmission; 1 billion lived under unstable malaria risk (Brinkmann and Brinkmann, 1991; Guerra *et al.,* 2008). In 2010, there were 216 million episodes of malaria worldwide and 655,000 malaria deaths, with the vast majority of the episodes and deaths in Africa, and 86% of malaria deaths being children under five years of age (WHO, 2011). Malaria infection in pregnant women may cause low birth weight which is a major risk factor for death in the first month of life (Steketee, 2001). Also repeated malaria infections make young children more susceptible to other common childhood illnesses, such as diarrhea and respiratory infections, and thus contribute indirectly to mortality (Molineaux, 1997).

An estimated 2% of children who recover from malaria infections affecting the brain suffer from learning impairments and disabilities due to brain damage, including epilepsy and spasticity (Murphy and Breman, 2001). Young children who have not yet acquired clinical immunity, and pregnant women, whose immunity to malaria is temporary impaired, are at higher risk of developing clinical diseases (Snow *et al.,* 1998a). The lack of exposure to malaria infection early in life delays the acquisition of immunity, and both children and adults remain at high risk of malaria disease when exposed (Snow *et al.*, 1998b). Perceptions of malaria differ; malaria is perceived as a life-threatening illness in a highland transmission district and as a mild everyday illness in a district with intense transmission (Geissler *et al.*, 2002). Misdiagnosis of malaria is a serious problem everywhere but in areas of low malaria endemicity presumptive treatment of all fevers as malaria can result in over 75% of cases being misdiagnosed as malaria (Amexo *et al.*, 2004). The effect of malaria misdiagnoses on the vulnerable will result in more ill health due to delayed diagnosis and repeat visits, overburdened health services, more severe malaria, loss of faith in health services, increase in real and perceived malaria resistance, chronic disease secondary to untreated infection, increased cost to patient and to health facilities and consistent misdiagnosis will encourage detrimental health seeking behavior (Amexo *et al.,* 2004).

The majority of malaria deaths occur in young children in sub-Saharan Africa, where one in every five childhood deaths is due to malaria (WHO, 2008). Aside from young children, pregnant women are also heavily affected (WHO, 2004). Malaria affects the quality of labour; it can lead to absenteeism, and even though an acute malaria attack might not prevent people from working, it can reduce the quality of productivity and output (Goodman *et al.,* 2000). School going children living in areas of low transmission report more malaria than those in higher transmission settings (Clarke *et* al., 2004). It is estimated that students loose between 3-12 days per year due to malaria (Leighton and Foster, 1994); Brooker *et al*., 2000) and the disease can dramatically affect school attendance (Some, 1994).

2.2 Treatment of malaria

Malaria is usually treated and controlled through chemotherapy. Quinolone containing antimalarial components are the most effective drugs for malaria chemotherapy

(Farooq and Mahajan, 2004). This group of compounds has evolved from the structural modification of quinine and includes 4-aminoquinoline and compounds such as and meloquine of which former is more effective, cheap, safe and commonly available drug (Farooq and Mahajan, 2004). The hydrofolate reductase inhibitors include proguanil, chroloproguanil, pyrimethamine and trimethoprime and sulfa drugs like dapsone, sulfalene, sulfamethoxazole and sulfadoxine. These drugs are used in combinations (Farooq and Mahajan, 2004).

Artemisinin, and its derivatives appear to be the best alternative for treatment of severe malaria. Artemisinin, unusual endoperoxide sesquiterpene lactone was isolated as the active principle from a Chinese herbal remedy *Artemisia annua* L., (Asteraceae) (Klayman, 1985). The three derivatives (artsunate, arteether, arteether) are actually more active than artemisinin itself and seem to have effect on protein synthesis of the malaria parasite (Farooq and Mahajan, 2004). They have shown very rapid parasite clearance in comparison to quinine compounds (Farooq and Mahajan, 2004). In 2001, WHO recommended the use of artemisinin-based combination therapies (ACTs) in countries where *P. falciparum* malaria had developed resistance to the convectional antimalarials, citing high cure rates and the potential to reduce the rate of drug resistance (WHO, 2001).

The use of ACT's results to an increased barrier to resistance by using drugs with different mechanisms of action, forcing the parasite to develop multiple simultaneous mutations in order to become resistant (Lin *et al.*, 2010). Artemisinin and mefloquine combination is being used in some Southeast Asian countries, for the treatment of complicated malaria, where the multidrug resistant strains of *P. falciparum* are prevalent (White *et al.,* 1999). Artemether-lumefantrine (AL) became available in 2004, and has shown to be as effective as and better tolerated than artesunate-mefloquine (Nosten and white, 2007). It is the most widely used ACT in Africa, with more than 30 countries having adopted it as first-line therapy as of 2009 (WHO, 2009; WHO, 2010c). Other ATCs include artesunste-amodiaquine (ASAQ), artesunate-sulfadoxine-pyrimethamine (ASSP) and dihydroartemisinin-piperaquine (DHA-PQ) (Nosten and white, 2007).

One of the greatest challenges facing malaria control is the spread and intensification of parasite resistance to antimalarial drugs. Surveys have shown rates of treatment failure higher than 50% for chloroquine in most affected regions, as well as poor efficacy of sulphadoxine-pyrimethamine in sub-Saharan Africa and Southeast Asia (Baird, 2005). Drug resistance by malaria parasites has been defined as the ability of a parasite to survive or multiply despite the administration and absorption of a drug when given in doses equal to or higher than those normally recommended and within the limits of tolerance of the subject (WHO, 1965). This definition can be applied to antimalarial drugs used as schizontocides, gametocytocides or sporontocides (Farooq and Mahajan, 2004). In India chloroquine resistance in *P. falciparum* was first reported in 1973 (Sehga *et al.,* 1973) and subsequently reported from several parts of the country (Dev *et al.,* 2003; Valecha *et al.,* 2009). By 1989 chloroquine resistance was spread in sub-Saharan Africa (Peters, 1987). The severity of resistance in the west and central Africa was less than in east Africa, but even in west Africa, its intensity varies from an advanced stage with severe effects on morbidity and mortality in focal areas in Senegal (Trape, 2001) to a moderate degree in Ghana (Landgraf, 1994), Cameroon (Ringwald *et al.,* 2000) and at low level in Mali (Djimde, 2001).

Since early 1960s, the increasing chloroquine resistance has led to a significant increase in mortality (Winsstaneley, 2001). The sulphadoxine-pyrimethamine (SP) combination was used as a drug of choice to treat chloroquine resistant malaria (Farooq and Mahajan, 2004). SP resistance has also been reported from large parts of Southeast Asia, southern China and amazon basin (Spencer, 1985; Aramburu *et al.,* 1999; Vasconcelos *et al.,* 2000). In Africa, SP resistance was detected in late 1980s which has since spread more in the east than in the west (Farooq and Mahajan, 2004). In Delhi SP resistance was first reported in 1987 (Choudhury *et al.,* 1987).

Quinine resistance was reported first from South America nearly a century ago (Farooq and Mahajan, 2004) and in Thai-Cambodian border in the mid-1960s (Pickard and Wernsdorfer, 2002). The clinical resistance to quinine therapy has been noticed sporadically in Southeast Asia and western Oceania (Farooq and Mahajan, 2004). Quinine resistance is less frequent in Africa (Jelnek *et al.,* 2001) and South America (Zalis *et al.,* 1998). The widespread use of quinine in Thailand in the early 1980s could be the reason for the development of significant resistance (Shanks, 1994). In the last two decades the drug has been used with combination with tetracycline or doxycycline to enhance its effectiveness (Farooq and Mahajan, 2004).

Mefroquine resistance was first observed in late 1980s near the Thai-Cambodian border (Shanks, 1994; Wongsrichanalai *et al.,* 2001). It is frequent in some parts of South East Asia and has been reported in the Amazon region of South America and sporadically in Africa (Muckenhaupt, 1995). The rapid action of artemisinins and their fast elimination from the plasma are theoretical built-in barriers to the development of drug resistance (Lin and Juliano, 2010). However, failure rates of AL of about 15% have been reported in Uganda and in western Cambodia (Denis, 2006; Kamya, 2007). Reports greater than 20% failure of Artemisinin-Mefloquine (ASMQ) therapy from the two sides of the Thai-Cambodian border first surfaced in the early 2000s (Wongsrichanalai and Meshnick, 2008).

The drug resistance in the parasites can be determined either by *in vivo* or *in vitro* drug susceptibility tests (WHO, 1965). *In vivo* tests are based on the observation of parasite response in the patient to a fixed dose of a drug within limits of tolerability (Wernsdorfer and Payne, 1991), one of the key characteristics of the *in vivo* tests is the interplay between host and parasite (Farooq and Mahajan, 2004). Decreased therapeutic efficacy of a drug can be marked by immune clearance of parasite in patients with a high degree of acquired immunity (White, 1997).

In vitro tests avoid many of the confounding factors, which influence the *in vivo* test, by removing parasites from the host and placing them in a controlled experimental environment (Farooq and Mahajan, 2004). These tests more accurately reflect the intrinsic antimalarial drug resistance (WHO, 2001). Multiple tests can be performed on isolates and response to general drugs can be assessed simultaneously (WHO, 2001). The *in vitro* assays not only yield quantitative results, but also determine the phenotype of the parasite independently of the immune and physio-pathological status of the host (Farooq and Mahajan, 2004). New antimalarials from diverse chemical groups and targeting different parasite stages are urgently needed that would allow implementation without concern for preexisting resistance (Lin and Juliano, 2010). With this trend it is important to come up with effective methods of controlling mosquito populations to reduce incidences of malaria. In addition it is better to control the vector other dealing with the *P. falciparum* which causes a life threatening disease.

2.3 Malaria parasite

Protozoan *Plasmodium* is the cause of malaria disease (Laveran, 1880). There more than one hundred species of *Plasmodia* that have been identified but only four are capable of infecting humans namely; *P. malariae, P. vivax, P. ovale* and *P. falciparum* (Ridley, 1997). *P. falciparum* is predominant in Africa, eastern Asia, Oceania and the Amazons (WHO, 1997; Ridley, 1997). *Plasmodium ovale* occurs in Central, West Africa and in West Pacific regions (Powells, 1989).

Anopheles spp are the only mosquitoes that are capable of transmitting human malaria (Grassi, 1890). The *Plasmodium* exists in two phases, sexual phase in female anopheline mosquito and the asexual phase in humans in the Figure **2.1**. When feeding on an infected human blood mosquitoes take in gametocytes which would later fuse and form a zygote (Rosenberg *et al.,* 1990; Beier *et al.,* 1991; Ponnudurai *et al.,* 1991). The zygotes penetrate the stomach of the mosquito to form an oocyst (Garnham, 1966; Shahabuddin and Kaslow 1994). Large numbers of sporozoites develop from oocyst (Pringle, 1965; Rosenberg and Rungsiwongse, 1991). Sporozoites pass through the body cavity of the mosquito and some enter salivary glands. Sporozoites are injected into a new host when an infected mosquito takes in blood, after which they disappear within one hour in the liver of the host (Fairley, 1947; Bruce-Chwatt, 1985). *P. falciparum* and *P. vivax* cannot complete their developmental cycle below temperature of 18 ^{o}C and 16 ^{o}C respectively (Wernsdorfer and MacGregor, 1988).

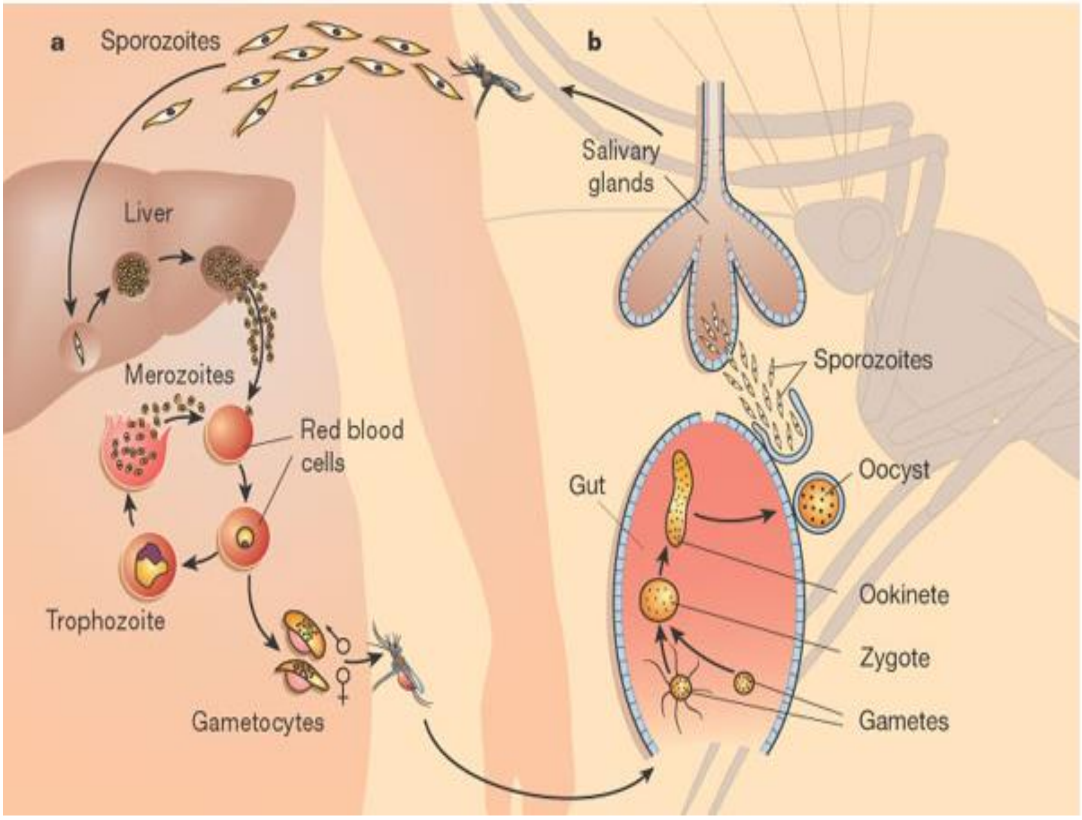


Figure 2.1: Life cycle of P. faciparum (http://www.nature.com/nature/journal)

2.4 Mosquitoes

There are over 3000 species of mosquitoes including the vector of malaria, yellow fever, dengue fever, filariasis and most of the arthroponds borne carborl types of encephahitis (Dawes, 1973). Mosquitoes belong to the order of two winged flies known as diptera, which include the houseflies and others. Mosquitoes belong to the family Culicidae. Culicidae are divided into three sub-families; Anophalinae, Taxorhynchitinae and Culicinae. Anopheline including well known genus Anopheles, are responsible for the transmission of malaria (Gillet, 1971).

Anopheles gambiae is medium sized anopheline with irregular speckled legs pale spot is 3rd dark area of wing vein 1 and lower branch vein 5 mostly pale. It feeds on man and animals by night mostly indoors. It rests inside and outside houses, it transmits malaria and filaria. It breeds on open exposed ground pools of all sizes; brick pits, foot prints, tyres etc. (Gillet, 1972). Very occasionally found in man- made containers such as wheel barrows, motor pans and at times of heavy infestation they have even been found in domestic ant-traps within houses (Gillet, 1972). Mosquitoes usually lay eggs on water or near water. Eggs develop quickly and hatch in 24 to 48 hours depending on temperature. All mosquito larvae are aquatic and most of them are filter feeders but some are predaceous. The larva stage may last for as short as three days to more than a month depending on temperature and species (Marquardt *et al*., 1985). The pupa stage usually lasts only 2 to 4 days; it swims freely but does not feed.Figure **2.2** shows the life cycle of mosquito.

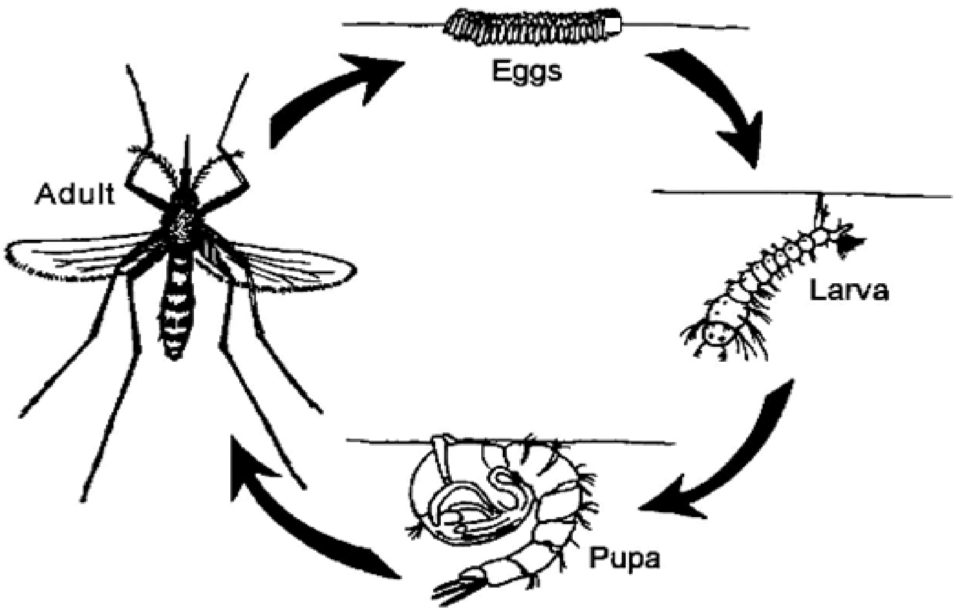


Figure 2.2: Life cycle of the mosquito (hppt://www.dph.state.ct.us)

Both male and female mosquitoes feed on plant juices and nectar but only females take blood. Females require blood for maturation of the eggs (Marquardt *et al.,* 1985).

Mosquitoes locate their host, through use of long-range volatile compounds. According to Reisen *et al.* (2000) chemicals that act as long-range attractants are volatile compounds. Carbon dioxide is one of the mosquito attractant and is dose dependent in its action (Reeves, 1953). There exist a variety of chemicals that are mosquito attractants but none is more important in long-distance orientation than carbon dioxide (Van den Hurk *et al.,* 1997; Mboera and Takken, 1997; Reisen *et al.,* 2000). In absence of all other host odours, mosquitoes are still activated towards carbon dioxide (Healy and Copland, 1995). The attraction range varies with the dose and removal of carbon dioxide from human breath decreases attraction to the host (Constantini *et al.,* 1998). Carbon dioxide is generally considered as a kairomone of mosquitoes (Reisen *et al.,* 2000). Other kiromones are lactic acid, 1-octen-3-ol, acetone and fatty acids (Takken and Knols, 1999). Other studies have also clarified the role of ammonia and phenols in host seeking behaviour (Davis and Bowen, 1994). Human foot also produces odours that are attractive to *An. gambiae.*

Differential attractiveness of *An. gambiae* to the human foot odours is due to the variation in the chemical composition of the volatiles emanating from the feet (Omolo *et al.,* 2013). Skin bacteria play an important role in the host seeking behavior of *An. gambiae* and different combination of bacterial populations produce different compositions of volatile blends (Schulz and Dickschart, 2007; Verhulst *et al.,* 2009; Smallegange *et al.,* 2011). The differences in the attraction of the human foot odours to *An. gambiae* can therefore be attributed to variation in the type and population structure of skin microflora and fauna, which determine the quality and quantity of the volatile odour compositions (Omolo, 2013). This observation explains why some people are more attractive to *An. gambiae* than others. Compound that are present in the foot odour of more attractive person but absent in the least attractive individual, or present in larger relative amounts in the more attractive person, are candidate kairamonal constituents from the human foot odour for *An. gambiae* (Omolo, 2013). Use of host attractants together with mosquito repellents may be helpful in controlling mosquito populations through 'push and pull mechanism'. Repellents can be used as barrier in mosquito prone area, thus pushing mosquitoes away, while attractants can be used to lure mosquitoes into a trap, thus pull them towards into the center of the trap. Tsetse flies population has been controlled using the same method (Gikonyo *et al.*, 2002).

2.5 Control methods of mosquitoes

Control methods of malaria vectors can be classified into three categories: (i) environmental (habitat) management; (ii) use of chemical insecticides and repellents; and (iii) biological control.

2.5.1 Habitat Management

Habitat management includes removal and manipulation of breeding sites. Removal of breeding sites includes drainage of stagnant water which includes land leveling and filling, tree planting designing improved irrigation structures such as drip irrigation among others. Manipulation of breeding sites include temporary removal or disturbance of larval habitat formation, such as intermittent irrigation, stream flushing, manipulating salinity, vegetation removal, expanded polythene beads

(Mouchet *et al*, 1998; Patz *et al*.,2000). However some of the developmental activities have interfered with the environment, leading to global warming and climatic changes that may be responsible for most malaria epidemics (Lindsay and Birley, 1996; Bouma *et al*., 1997; Mouchet *et al*., 1998; Githeko *et al.,* 2000; Patz *et al*., 2000). These malaria vectors have adapted in such ways that enable their larvae to survive in temporary water collections such holes in trunks, animal hoof prints old disused tins and tyres which demands integration of other methods of manipulation of mosquito populations.

2.5.2 Insecticides

Adult mosquito population can be controlled by use of insecticides (Kirk and Othmer, 1992). Commonly used insecticides include chlorinated hydrocarbons, carbamates, organophosphates, and synthetic pyrethroids. Indoor and outdoor residual spraying of organochlorides and organophosphates showed a remarkably fast reduction of mosquito populations to a negligible level (WHO, 2003). In spite of organochlorides having adverse effects, it has been reported that propoxur which is the main constituent of organochlorines, produces limited effects on malaria transmission even when combined with mass drug administration (Fontaine *et al.,* 1979). In all cases, spraying is expensive to apply on a large scale for a long time and unaffordable to ordinarily poor people in developing countries (Fontaine *et al.*, 1979).

Insecticides such as DDT (trichloro-2,2-*bis*-(*p*-chlorophenyl) ethane), HCH (hexachlorocyclohexane), dieldrine and lindane have been used but wide use of these chemicals is not recommended by WHO as alternatives to eradicate mosquitoes since they result in adverse side effects (WHO, 1996a; WHO,2003). HCH and lindane are not recommended due to resistance developed against by insects while dieldrin is toxic to humans (WHO, 2003). These chemicals which are not environmentally biodegradable have been found to accumulate in the food chains, water bodies and soil, thereby endangering human health and threatening the survival of non-target organisms (WHO, 1996b; WHO, 2003). Furthermore cross resistance has occurred in some classes of insecticides, e.g. *An. gambiae* has been reported to be resistant to DDT, permethrin and lambdacyhalothrin in West Africa (WHO, 1996b). Others that are sprayed in the air are pyrenthrins and kill mosquitoes that fly into the spray.

2.5.3 Biological control

Use of predation can used to control mosquito populations e.g. fish known are Gumbusi, Toxonylynchites larvae, Nermatodes e.g. *Romanomermy petersoni* and birds that eat a large number of insects are provided with houses to use in nesting (Marquardt *et al.*, 1985). Disease agent can also be used e.g. micropora such as nosema algerae and bacteria such as *Bacillus thuringiensis* and *B. sphaericus* (Marquardt *et al.,* 1985). Methophone is a juvenile hormone mimic and dimilin is an inhibitor of chitin formation, both are placed in water and cause malformation of larvae (Marquardt *et al.*, 1985). Nematodes have also been used to suppress mosquito populations; they usually enter in the insect through the breathing holes, mouth, and anus or penetrate through the insect’s cuticle (Lacey, 1998). While inside the body of the insect they release special bacteria, which produce toxins that kill the host after a few days (Skovmand *et al*., 2000). Dragonfly nymphs can be used to eliminate the larvae of *Aedes aegypti* (Corbat, 1986) while *Azollo flucoides*, a floating water fern can be used eliminate *Anopheles* and *Aedes* larvae (Lu, 1996).

Since mosquitoes have adapted ways that enable their larvae in temporary water habitats such as tree trunks, hoof prints, old tyres and tins some biological methods of mosquito control becomes difficult to employ. Larvivorus fish cannot survive in temporary water habitats. Other biological control agents are not easy to culture such as *B. thuringiensis* and *B. sphaericuus* and cost of culturing the agents may be expensive resulting to people venturing in other alternative methods.

2.5.4 Use of mosquitoes larvicides

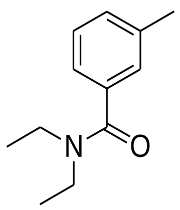
Chemicals and biological larvicides have been used to control mosquito larvae. Petroleum oil sprays have been used on water in USA to control mosquito larvae (Wigglesworth, 1996). Paris green $\{Cu(C_2H_3O)_2 3Cu(AsO_2)_2\}$ and metarsenite $\{Cu(AsO_2)_2\}$ have been used as larvicides (Metcalf and Flint, 1962). Chlorinated synthetic organic compounds like lindane, chlordane and DDT have also been used as larvicides (Kirk and Othmer, 1992). However these chemicals are non-biodegradable and might accumulate in food chains thus causing a health threat. Some plant alkaloids also kill mosquito larvae (Katinka, 1999). Nicotine and methylanabasine which were extracted from *Anabasis aphylla*, were used to eliminate the larvae of *Culex pipiens, Cx. Territans Cx.* and *quinquefasciatus* (Wigglesworth, 1996). Both chemical and biological larvicides are effective on killing of mosquito larvae but are toxic to non-target aquatic organisms (WHO, 1996a).

2.5.5 Insect repellents

Avoiding being bitten by mosquitoes may be one of the best methods of controlling malaria. Repellents are chemical substances that protect animals, plants, or fabrics, grain and timber from insect attack by rendering them unattractive, unpalatable or offensive (Metcalf and Frint, 1962).

2.5.5.1 Personal protection

Repellent or barriers e.g. DEET (N, N-diethyl-*m*-toluamide) (**1**) and ethylhexanediol (**2**) repel insects including mosquitoes and may be placed on the skin or impregnated into clothing (Marquardt *et al*., 1985). These repellents that are applied directly on the skin or released on the air, turn mosquitoes away before or just after they land, and therefore interrupting the landing and feeding behavior. Mosquito repellents usually offer protection especially in the evening before bed time and hence minimize incidences of malaria transmission and pain of being bitten (Xue *et al.,* 2001). DEET which is a synthetic insect repellent has been effective in repelling various insects including mosquitoes. However DEET has been reported to be an irritant when applied to the skin and causing central nervous system disturbances when used for a long time (Lewis, 1996; Nkunya, 2002). In this regard, alternative mosquito repellents need to be searched and developed.



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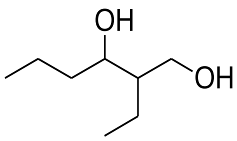

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2.5.5.2 Space protection

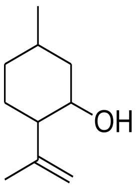
Bed-nets are currently being used for protection against mosquitoes due to recent discovery of synthetic pyrethroids of relatively low mammalian toxicity but exhibiting significant knockdown effect (WHO, 2003). Pyrethroids irritate or kill mosquitoes before they can find a place bite through the net (WHO, 2003). Insect treated nets

(ITN) assist a physical barrier of a bed-net against a host seeking mosquito. ITN's are reliable and a cheap method to combat malaria (Roll back malaria, 2002). Until to date only pyrethroids are proved to be safe and effective in treating mosquito nets and curtains (WHO, 1996b; WHO, 2003). Due to mosquito resistance against pyrethroids which remains a possibility, present lack of alternative possess a great risk against ITN's effectiveness. ITN's however, give no protection against mosquitoes in the evening before people retire to bed. Mosquito coils that are burnt at night produce smoke that repel mosquitoes. However the smoke produced may cause irritation to people with breathing related diseases and conditions, e.g. people suffering from asthma.

2.5.6 Use of plants as mosquito repellents

Human beings have used plants in various ways such as, food, treatment of both human and animal health disorders, protection of crops and prevention of insect bites. Some species plants have been reported as sources of insect repellents but majority have not been tested for their insecticidal properties. Pyrethrum, Pines, *Ocimum, Eucalyptus* and *Azadrichta indica* have been reported repel and/ or kill mosquitoes (Grayson, 2000; Xue *et al.,* 2001). Plants have been burnt to produce smoke, used as extracts or in their pure natural form. *Ocimum gratissimum* is known as 'mosquito' plant in Nigeria because it repels mosquitoes and other insects when cultivated near houses, on account of its fragrance (Dalziel, 1937). *Cyperus articulates, Hyptis spicigera, Citrus sinensis* peel and *Ocimum* spcecies when burnt produce smoke that repel mosquitoes (Dalziel, 1937; Selvaraj-Pandian *et al.,* 1995). A leaf infusion of *Lippia javanica* Spreng and *Ocimum canum* Sims are applied on the skin to control mosquito bites in Zimbabwe (Lukwa *et al.,* 1994). A burning mixture of charcoal and plant powder from *azadirachta* or *Ocimum santum,* produces smoke which serves as a good repellent and an aldulticide (Adebayo, 2001).

Infusion of *Neorautanenia mitis* Rich is widely used as a mosquitocide and insecticide in Tanzania (Joseph *et al*., 2004). Repellent properties of plants are usually attributed to their essential oil. The degree repellency of a certain plant will be dependent on the relative amounts of repellent compounds in the plant. Lemon eucalyptus contains *iso*-pulegol (**3**), citronella (**4)** and citronellol (**5)** which are mosquito repellents (Collin *el al.,* 1993; Fradin and Day, 2002).



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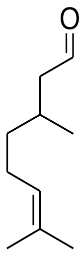

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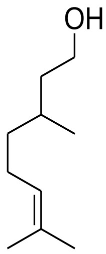

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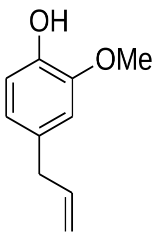
The oil cloves, *Syzygium aromaticum* Merrill and Perry, which is known as source of spices, contains euginol (**6**) and β-caryophyllene (**7**) which are better repellents of *Anopheles albimanus* than DEET but they do not repel *Ae. aegypti* (Bernard, 1999). Several plants derived insect repellents have been developed and commercialized which include *p*-menthane-3,8-diol (**8**), citronellal (**4**), eugenol (**6**) linalool (**9**) and camphor (**10**) (Nkunya, 2002; Grayson, 2000)



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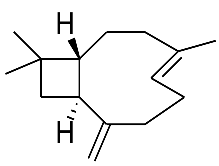

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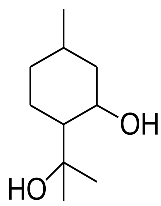

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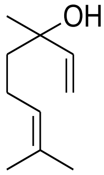

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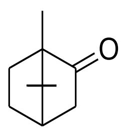

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2.6 *Nigella sativa* L.

Nigella sativa L. commonly known as black cumin, belongs to the family Umbelliferae (Farooqi *et al.*, 2005). It has many regional names which includes Kaloonji, Kalajira, Nallajee, Lakaira, Karunjiragam, Karejrage and also referred to as small fennel among others (Farooqi *et al.*, 2005*)*. *N. sativa* is a small herb growing to a height of 45 cm. The leaves are pinnatisect and 2.5 to 5.0 cm long cut into linear-lanceolate segments (Farooqi *et al.*, 2005). The flowers are pale blue 2.0 to 2.5 cm across, without an invlucre, borne on along peduncles , the seeds are trigonous, black regulose and tubercular (Farooqi *et al*., 2005). The plant matures in 135 to 145 days (Farooqi *et al*., 2005). Figure **2.3** and **2.4** show *N. sativa* plants and *N. sativa* seeds respectively.

Figure 2.3: *Nigella sativa* plant (www.lifestrong.com)

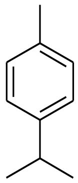
The dry roasted seeds flavor curries, vegetables and pulses. The black seeds taste like oregano and have bitterness to them like mustard-seeds. It can be used as a "pepper" in recipes with pod fruit, vegetables, salads and poultry (Sharma *et al.*, 2009).

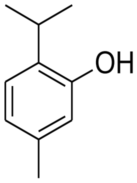
Figure 2.4: *Nigella sativa* seeds (www.phcogrev.com)

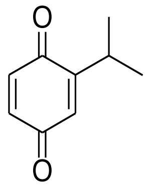
Black cumin is cultivated in Egypt, Syria, Greece, Arabian countries and India (Farooqi *et al.*, 2005*)*. The raw seeds have a little aroma, but their flavor is released when rubbed lightly (Schlosser, 2007). The seeds contain mainly volatile oil (0.5-1.6%) and fatty oil (31%) a bitter principle nigellin, tannins, resins, proteins reducing sugars, saponins, arabic acid and other alcohol-soluble organic acids (Farooqi *et al.*, 2005). Free amino acids like cystine, lysine, aspartic acid, glutamic acid, alanine, tryptophan, valine and leucine are present in dormant seeds (Farooqi *et al.*, 2005).

The main components of *N. sativa* oil from Morocco were reported to be *p*- cymene (33.8%) (**11**) and thymol (26.8%) (**12**), with only small amounts of thymoquinone (3.8%) (**13**) (Moretti *et al*., 2004). Another study by Gurdip *et al.* (2005) identified 38 components of essential oil *N. sativa* seeds representing 84.65% of the total amount, the major component was *p*-cymene (36.2%) followed by thymoquinone (11.27%), *α*-thujene (10.03%), longifolene (6.32%) and carvacrol (2.12%). The main constituents of oil isolated from the plant grown in Poland were similar to plants cultivated elsewhere: *p*-cymene (60%), *γ*-terpinene (12.9%), *α*-thujene (7.2%), carvacrol (3.0%), *α*-pinene (2.0%) and *β*-pinene (2.1%) (Wajs *et al.*, 2008).

Black seed oil was shown to be an effective adjuvant for treatment of patients with allergic diseases (Kalus, *et. al.*, 2003). Also *N. sativa* extract has been used in the treatment of acute tonsillophayngitis (Dirjomuljono *et al.*, 2008). *N. sativa* has been identified as a potent antioxidant acting as a free radical scavenger (Khalife and Lupidi, 2007). Thymoquinone, the active constituent of *N. sativa* seeds, is a pharmacologically active quinone, which possess several properties including analgesic and antiflammatory actions (Abdel- Fattah *et. al.*, 2000). *N. sativa* can significantly prevent hepatotoxity (Turkdogan *et. al.*, 2003) and might have protective effect against hephnatoxicity induced by either disease or chemicals (Hosseinzadeh *et al.*, 2007). Also *N. sativa* was shown to have anti-epileptic effects in children with refractory seizures (Ackhondian *et al.*, 2007)







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A study by Chaubey shows that the essential oils of the *Trachyspermum ammi*, Sprague *Anethum graveolens* Clarke and *N. sativa* killed the larvae and adults of the *Tribolium Castaneum* by vapor action (Chaubey, 2007). The presence of biological active compound such as α-thujene, 2 (1H)-naphthalenone, α-pinene, α-phellandrene, limonene, thymquinone myristicin in *N. sativa* volatile oil contributed its antimicrobial activity of volatile oil (Saptha *et al.*, 2009). Another study by Asma *et al.* (2012) reported *N. sativa* seeds have a considerable antioxidant activity *in vivo* and *in vitro*. Essential oil and extract from *N. sativa* seeds provide antioxidant activity equivalent to or higher than those of synthetic antioxidants and could be better natural antioxidants (Gurdip *et al.*, 2005).

Previous studies on antimicrobial activity of *N. sativa* volatile oil reported the efficacy of the oil to be far better than the standard, tetracycline (Saptha *et al.*, 2009). In addition, the extract of black seed as well as its oil have been reported to possess antibacterial activity; however Gram-positive bacteria are more susceptible to the action of the essential oil, whereas Gram-negative organisms are more sensitive to the extracts of black seeds (Adams, 2007). Burits and Bucar (2000) also reported that *N. sativa* essential oils notably reduced the concentration of DPPH free radical, with an efficacy (IC_{50} of 460 mg/ml) lower than that of reference compound, carvacrol with an IC_{50} of 28.8 mg/ml. No repellence studies of *N. sativa* essential oils against mosquitoes have been reported. Previous studies have focused largely on anti-microbial activities of crude products and specific constituents of *N. sativa* seeds.

CHAPTER THREE

MATERIALS AND METHODS

3.1 Experimental mosquitoes

The *An. gambiae* mosquitoes were obtained from colonies reared in the insectaries at ICIPE (International Centre of Insect Physiology and Ecology) Nairobi, Kenya that are reared according to the WHO protocol (WHO, 1996). The mosquitoes were initially obtained from the field in Mbita. Mosquito eggs were hatched by simultaneously flooding the moist filter paper platforms. Rearing was carried out in the insectary maintained at 27-28^{0}C and approximately 80% humidity on a 12h/12h light and darkness cycle and maintained at optimal larval concentrations to avoid possible effects of competition. Mosquito larvae were fed on ground baby fish food while adults were offered a fresh 10% (w/v) sucrose solution meal daily and on hamsters as a source of blood meals when required to produce eggs.

3.2 Extraction of essential oil by hydro-distillation

The dry *N. sativa* seeds were obtained from the Kenyan local market imported from India. One hundred and twenty five grams of *N. sativa* seeds were weighed and crushed using a grinder, then transferred into a clean flask and 500 ml of distilled water was added. Then 10 g of sodium chloride was added to reduce the foaming during boiling. Then the apparatus for hydro-distillation were set up (Figure **3.1**) and heating was done and regulated at 100^o C. Hydro-distillation was done for 3 hours. The essential oil was diluted with pure acetone to make a concentration of 10% and stored in amber-colored vial at -4^o C for analysis and bio-assays. Uncrushed seeds were also hydro-distilled for comparison.

Figure 3.1: Extraction of *N. sativa* seeds essential oil using hydro-distillation apparatus

3.3 Characterization of the chemical composition of the essential oil

The characterization of chemical composition of the essential oil of *N. sativa* were done using GC and GC-MS techniques (Thollet *et al.*, 2006). The analysis was carried out on capillary GC, Hewlett Packard (HP) 5890 series II, equipped with FID coupled to HP 3393A series II integrator. The carrier gas was nitrogen at flow rate of 0.7ml min^{-1}. The temperature was maintained at 50 ^oC for 5 minutes then increased at 5 ^oC per minute up to 280 ^oC where it was held for 10 minutes. Identification of essential oil components was carried out on GC-MS HP 8060 series II GC coupled with a VG Platform II mass Spectrometer. The MS was operated in the EI mode at 70 eV and an emission of 200 µA, with the temperature of the source held at 180 ^oC multiplier voltage at 300 V. The MS had a scan cycle of 1.5 s (scan cycle of 1 s and inter-delay of 0.5 s) and scan ranges *m/z* 38-650. The carrier gas was helium and the temperature programme involved an initial temperature of 50 ^oC for 5 min then increased to 90 ^oC

at 5 ^{o}C min^{-1}, to 200 ^{o}C at 2 ^{o}C min^{-1}, and finally to 280 ^{o}C at 20 ^{o}C min^{-1} and this temperature maintained for 20 min. The constituents of essential oil were identified by analysis of their mass spectra and direct comparison with the Institute of Standards Technology libraries 98.1 (NIST) and Wiley Registry of Mass Spectral Data, 8th edition database of library of mass spectra, on the GC-MS equipment.

3.4 Identification of active compounds from the essential oil using GC-EAD

Antennae of 5-7 days old female *An. gambiae* mosquitoes were used for the coupled gas chromatography-electroantennographic detector (GC-EAD) analysis. A glass micro-pipette containing Beadle-Ephrussi saline (Ephrussi and Beadle, 1936) was inserted through the head of the insect. The fine tip of the micro-pipette was pushed through the head between the thorax and the head of the insect. The other end of the micro-pipette was sheathed over a silver wire, the recording electrode, which was connected to the input of a universal AC/DC UN-05 amplifier (Syntech, The Netherlands). To complete the circuit, the distal end of the antenna was nipped off with a scalpel and the open end inserted into similar glass micro-pipette containing the saline and was sheathed over a silver wire electrode that was grounded.

GC-EAD tests were performed on HP 5890 series II gas chromatograph equipped with a flame ionization detector (FID) and HP Ultra 1 (cross-linked methyl silicon gum) capillary column (50 m × 0.2 mm × 0.33 µm) using nitrogen at a flow rate of 0.8 ml/min as the carrier gas. The oven temperature was maintained at 60 ^{0}C for 5 min after injection of sample and then programmed at 5 ^{0}C /min to 280 ^{0}C, where it was held for 15 min.

The effluent from the capillary column was split in a ratio of 1:1 into two 50 cm long de-activated silica columns, one connected to the FID and the other connected to a stainless steel tube (5mm i.d.) that was focused onto the antennal preparation. A make-up gas (40 ml/min) was added just before the split point to accelerate the effluent through deactivated columns. The deactivated transfer line carrying the effluent over the antennal preparation was maintained at 150 ^{0}C by a THC-3 temperature control unit (Syntech, The Netherlands). Aliquots (8-10 µl) of *N. sativa* seeds essential at concentration of 0.1% by mass were analyzed by the GC-EAD and GC signals were monitored synchronously using a programme on a GC/EAD interface card (Syntech, The Netherlands) installed in a PC (Harvard Professional computer, American Megatreands Inc.). Subtraction bioassays were undertaken to identify repellent constituents/ blends using *An. gambiae* mosquitoes. Subtraction bioassays by preparing a blend 1 of all EAG acting compounds and then other 7 blends comprising of blend 1 minus each of the compound at a time.

3.5 Synthetic standards

Synthetic standards were purchased from Sigma Aldrich Company and they included: (+)-β-pinene, (-)-β-pinene, (+)-α-pinene, (-)-α-pinene, p-cymene, α-longipinene, tetradecane and 1,2,3,4,5-pentamethylcyclopentane. These compounds were GC-EAD active.

3.6 Blends and individual constituents tested

The blends were prepared for bio-assays according to the relative percentage abundance in the essential oil as determined by the GC. Blend 1 constituted of commercially available compounds that were GC-EAD active namely (+)-β-pinene,

(-)-β-pinene, (+)-α-pinene, (-)-α-pinene, p-cymene, α-longipinene, tetradecane, and 1,2,3,4,5-pentamethylcyclopentane. Blend 1 minus p-cymene (blend 2); Blend 1 Minus (-)-β-pinene (blend 3); Blend 1 Minus (+)-β-pinene (blend 4); Blend 1 Minus (+)-α-pinene (blend 5); Blend 1 Minus (-)-α-pinene (blend 6); Blend 1 Minus α-longipinene, (blend 7); Blend 1 Minus tetradecane (blend 8); Blend 1 Minus 1,2,3,4,5-pentamethylcyclopentane (blend 9). The following constituents were also tested individually: (+)-β-pinene, (-)-β-pinene, (+)-α-pinene and (-)-α-pinene in order to compare their repellency.

3.7 Mosquito repellency assay procedure

Mosquito repellency assays were carried out according to WHO protocol (1996a). The ethical clearance was obtained from Ethical Review Committee of Kenyatta University (Appendix II).The essential oil and blends prepared from standards were assayed for their repellency activity against *An. gambiae* that were reared at ICIPE under standard conditions. For comparison, repellency of DEET (a positive control) was also undertaken at different doses. Repellency assays were done with 5-7 days old females of *An. gambiae* that had been starved for 18 hours but previously fed on 6% glucose solution (Omolo *et al.*, 2004). The screening for bioassay activities of essential oil and blends was carried out at concentrations of 10^{-5}, 10^{-4}, 10^{-3}, 10^{-2} and 10^{-1}.

The assays were performed on 6 human adults (($18yrs \leq age \leq 50yrs$) selected from the Parasitology Laboratory in ICIPE, each of whom signed a standard Ethical Clearance Consent Form (Appendix III). The selection was based on previous involvement of the staff from this laboratory, who also had adequate knowledge of good laboratory practices. The participants did not have a previous history of allergy to insect bites and were not suffering from any illness or not under any medication during assay period. There were no pregnant or lactating mothers. Each assay used different set of *An. gambiae* mosquitoes that was killed after the test. The participants had no contact with lotions, perfumes oil or perfumed soaps on the day of the experiment. In view of the very short exposure time to the mosquitoes in each assay, the probability of mosquito bites is considered very low. However, an insect bite cream was provided to the participants in case of any minor bites and associated irritations. A total of 18 cages each measuring 50×50×50 centimeters were used (Omolo *et al.,* 2004). Test solutions (1ml) were dispensed on one of the forearms of a volunteer from wrist to the elbow covering an area of 500 cm^2. Rest of the hand was covered with a glove. Acetone was dispensed on the other forearm to serve as control. The control and treated arms was interchanged regularly to eliminate bias (Omolo *et al.,* 2004). The control arm was first introduced into the cage immediately after releasing the 50 experimental mosquitoes and kept there for 3 minutes (Omolo *et al.,* 2004). The number of insects that landed on control arm during the test was recorded. The treated arm was then introduced into the cage for the same period of time and the number of landing insects recorded. The different concentration of each test sample was tested starting with lowest concentrations.

3.8 Data analyses

Percentage protective efficacy (PE) was calculated using the formula PE = (C-T/C) ×100% where C and T are the mean numbers of mosquitoes that landed on the control and the test arm respectively (Sharm and Ansari, 1994; Matsuda *et al*., 1996; Yap *et* *al*., 1998). The data obtained was subjected to ANOVA for completely randomized design. The means were ranked using Student-Newman-Keuls (SNK) at the 5% significance level. Dose-response relationship was determined using probit analysis and repellent doses at RD_{50} and RD_{75} values obtained from regression model (Finney, 1971; Busvine, 1971).

Probit [P(dose1]=$ß_0$ +x $ß_1$+ î

Where $ß_0$ =coefficient of the model representing y-intercept,

$ß_1$ = coefficient of the model representing dose 1,

dose 1 =$\log_{10}$ (dose),

î =error term in the data set of the predictor (regressor) variable (x) and

p =repellency probability

CHAPTER FOUR

RESULTS AND DISCUSSION

4.1 Percentage yield of essential oils from the seeds

Hydro-distillation of essential oils from the seeds of *N. sativa* (black cumin) gave 0.68g / 125 g. (0.54%) which agrees with yields reported in literature to be in the range of 0.2 -1.3 % (Burits and Bucar, 2000; Hajhashemi *et al.,* 2003; Michelitsch *et al*., 2004). Uncrushed seeds were also hydro-distilled but no essential oil was collected meaning that the seed husk protected the loss of essential oil. The extracted essential oils had a dark yellow colour and a characteristic odor. The oils did not crystallize at -4°C and was soluble in hexane and acetone.

4.2 Repellency activity of essential oil of *N. sativa* seeds

The repellency of essential oil of *N. sativa* seeds at 5 doses against *An. gambiae* was determined and the mean percentage repellency activity are given in table **4.1** and figure **4.1**

Table 4.1: The mean (±SE) percentage repellency of essential oil at different doses

Conc(g/ml)	0.00001	0.0001	0.001	0.01	0.1
SAMPLE	Mean±SE	Mean±SE	Mean±SE	Mean±SE	Mean±SE
Essential oil	36.97±1.81^{D,a}	50.41±2.87^{C,a}	84.17±0.78^{B,a}	98.81±1.19^{A,a}	100.00±0.00^{A,a}
DEET	51.11±13.32^{C,a}	86.22±4.51^{B,b}	94.29±3.69^{AB,b}	100.00±0.00^{A,a}	100.00±0.00^{A,a}

Means (±SE) with different (i) capital letters in a row, and (ii) small letters within a column are significantly different at $\alpha = 0.05$ (Student-Newman-Keuls test)

From the data obtained, it is clear that the repellence of the essential oil against the *An. gambiae* at higher doses was comparable to that of DEET; however, it showed lower repellence at lower doses. Figure **4.1** shows the repellence of DEET and essential oil against *An. Gambiae.*

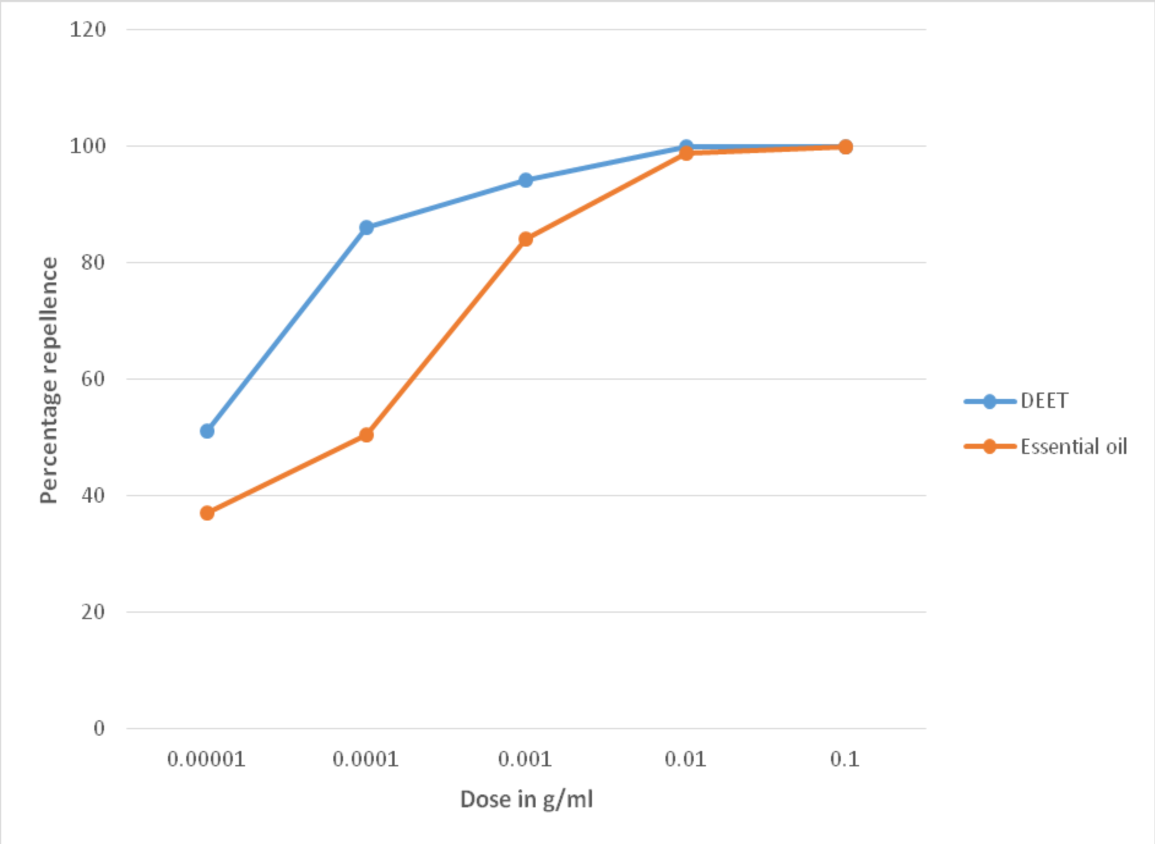


Figure 4.1: Mean percentage repellence of DEET and essential oil of *N. sativa* seeds in various dose against *An. gambiae*

4.2 Gas chromatography and gas chromatography / mass spectrometry analyses of essential oil of *N. sativa* seeds

The GC and GC-MS analysis of the *N. sativa* seeds essential oil gave 26 compounds that were identified by comparing their fragmentation patterns using Institute of

Standards Technology libraries 98.1 (NIST) and Wiley Registry of Mass Spectral Data, 8th edition. The chemical composition of the major compounds and their relative abundances is given in table **4.2**. From the chromatogram the compound whose retention time was 13.05 minutes could not be identified using the NIST data base and Wiley Registry of Mass Spectral Data used. The unidentified compound has been reported in other studies constituting of 4.7% to 11% of the essential oil (Burits and Bucar, 2000). The GC-MS chromatogram of essential oil from *N. sativa* seeds is given figure **4.2.**

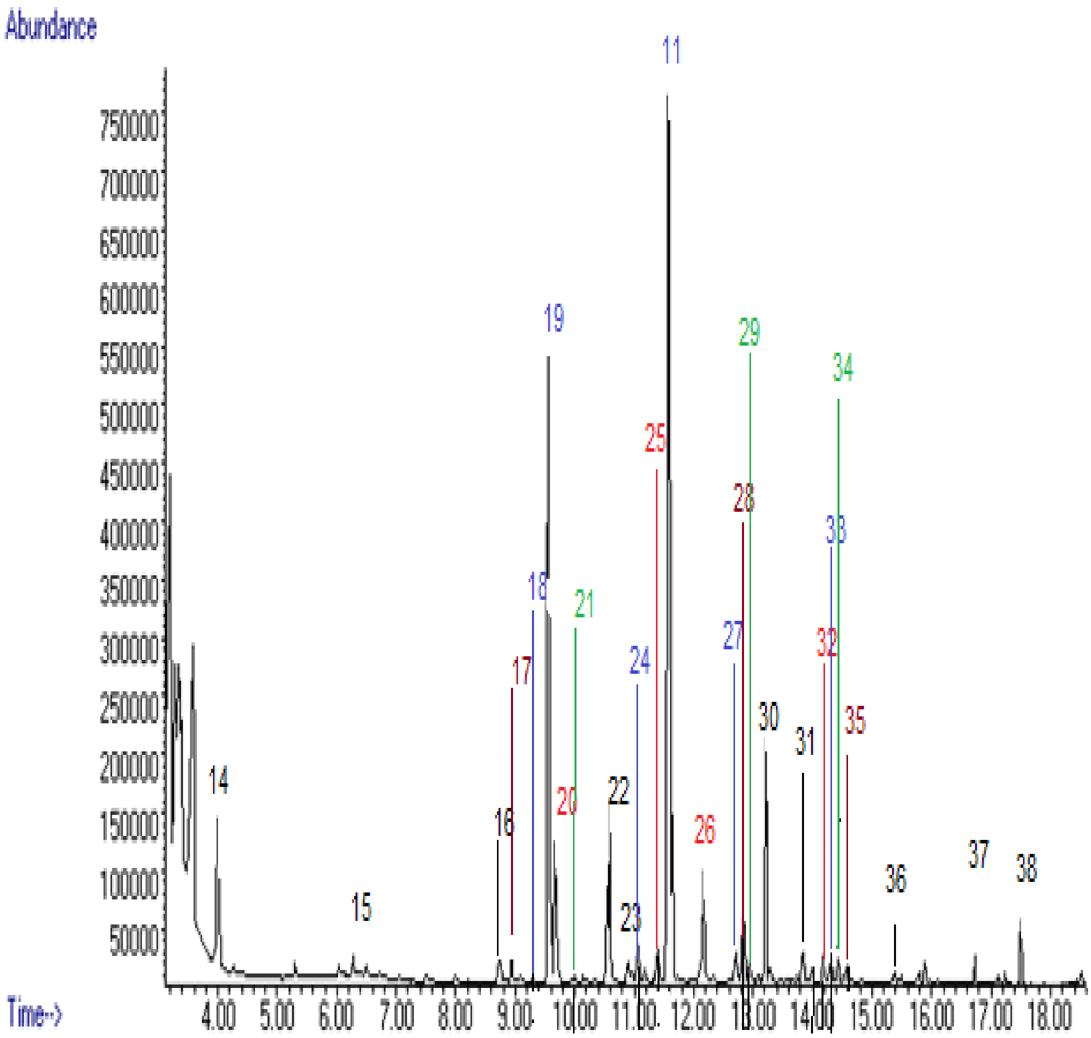


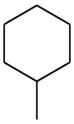
Figure 4.2: GC-MS Chromatogram of essential oil from *N. sativa* seeds

p-Cymene is the major compound with percentage abundance of 34.67% which agrees with previous work that reported 33.8% (Moretti *et al*., 2004). The major component of the essential oil of *N. sativa* seeds were monoterpenes (53.7%) while sesquiterpenes constitute a very small fraction which also agrees with the literature data (Moretti *et al*., 2004).

Table **4.2**: Major compounds in *N. sativa* seeds essential oil identified by GC-MS

NO	**COMPOUND**	**Retention Time in minutes**	**Relative abundance**	**Classification**
14	Methylcyclohexane	4.03	3.11	Non-terpenoid hydrocarbon
15	Octane	6.07	0.02	Non-terpenoid hydrocarbon
16	Styrene	8.57	0.05	Non-terpenoid hydrocarbon
17	Nonane	8.75	0.06	Non-terpenoid hydrocarbon
18	1,2,3,4,5-Penta-methylcyclopentane	9.13	0.08	Non-terpenoid hydrocarbon
19	α-Thujene	9.38	11.55	Monoterpenoid hydrocarbon
20	α-Pinene	9.49	2.82	Monoterpenoid hydrocarbon
21	Propylbenzene	9.99	0.06	Non-terpenoid hydrocarbon
22	β-Pinene	10.41	4.66	Monoterpenoid hydrocarbon
23	1-Decene	10.75	0.07	Non terpenoid hydrocarbon
24	Tetradecane	10.88	0.07	Non terpenoid hydrocarbon
25	δ-Carene	11.22	0.09	Monoterpenoid hydrocarbon
11	p-Cymene	11.42	34.67	Monoterpenoid hydrocarbon
26	γ-Terpinene	12.00	0.26	Non terpenoid hydrocarbon
27	Z-3-Undecene	12.16	0.15	Non terpenoid hydrocarbon
28	E-4- Undecene	12.79	0.09	Non terpenoid hydrocarbon
29	E-5- Undecene	12.94	0.06	Non terpenoid hydrocarbon
30	*Trans*-4-methoxythujane	13.05	5.81	Monoterpenoid ether
31	Pentylbenzene	13.68	0.14	Non terpenoid hydrocarbon
32	Terpinen-4-ol	14.11	0.13	Monoterpenoid alcohol
33	Dodecene	14.15	0.11	Non terpenoid hydrocarbon
34	Dodecane	14.29	0.14	Non terpenoid hydrocarbon
35	β-Cyclocitral	14.42	0.21	Monoterpenoid ketone
36	Hexylbenzene	15.25	0.43	Non terpenoid hydrocarbon
37	α-Longipinene	16.57	0.75	Sesquiterpenoid hydrocarbon
38	Longifolene	17.33	2.55	Sesquiterpenoid hydrocarbon

The following are the structures of the compound identified.



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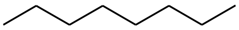

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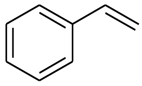

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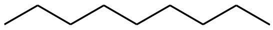

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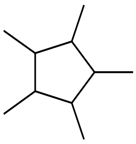

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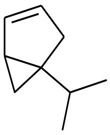

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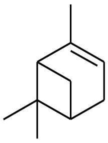

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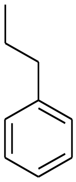

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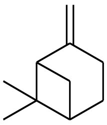

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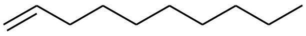

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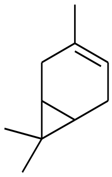

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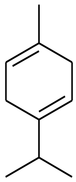

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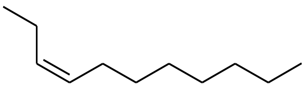

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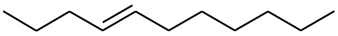

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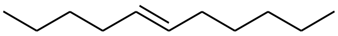

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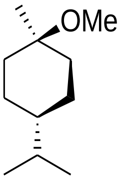

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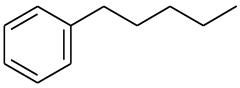

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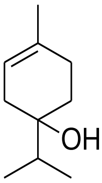

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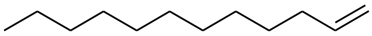

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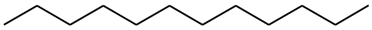

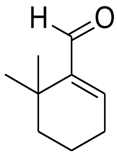

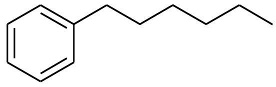

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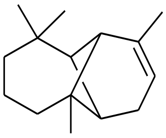
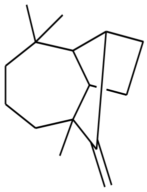
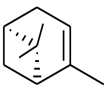


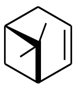


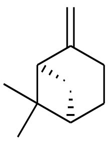
37 **38**

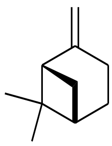
4.3 Gas chromatography/electro-antennographic detection using *An. gambiae*

The gas chromatographic-electroantennographic (GC-EAD) analyses of the essential oil was carried out with *An. gambiae* and eight compounds were found to be EAG-active. These compounds were, 1,2,3,4,5 - pentamethylcyclopentane (**18**), α-thujene (**19**), α-pinene (**20**), β-pinene (**22**), tetradecane (**24**), p-cymene (**11**), α-longipinene (**37**) and longifolene (**38**). The GC-EAD chromatograms is given in figure **4.3,** figure **4.4** and figure **4.5.** Compounds **18** and **24** were non-terpenoids, **19**, **20**, **22**, and **11** were monoterpenes while **37** and **38** were Sesquiterpenoid. Since the GC-MS could not identify the optical isomers of β-pinene and α-pinene isomers both isomers of each compound were bought, (+)-α-pinene (**39**) , (-)-α-pinene (**40**), (+)-β-pinene (**41**) and (-)-β-pinene (**42**).









39 **40** **41** **42**

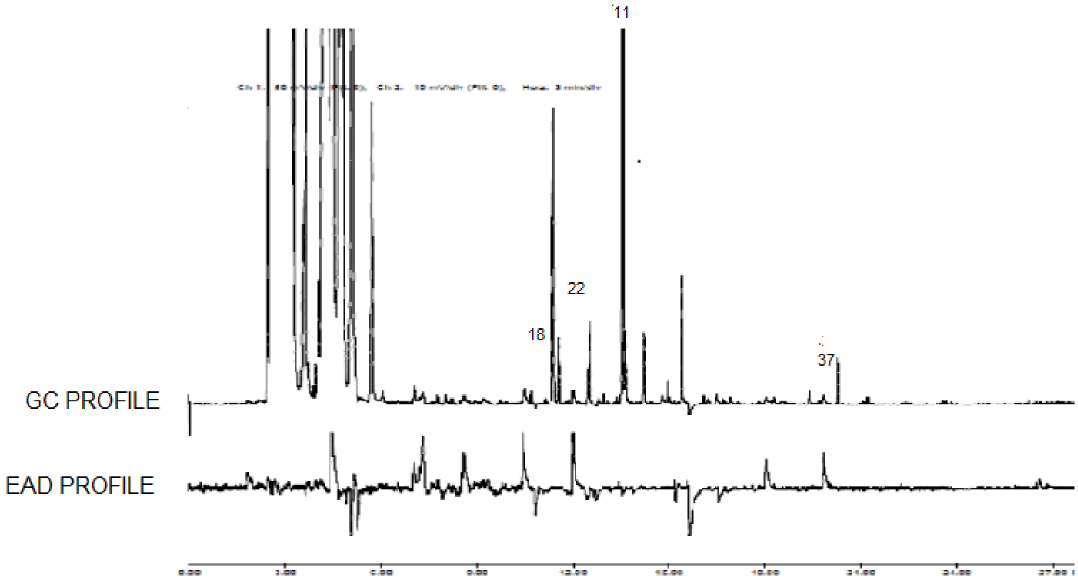


Figure 4.3: GC-EAD chromatograph of the essential oil using *An. gambiae* mosquitoes

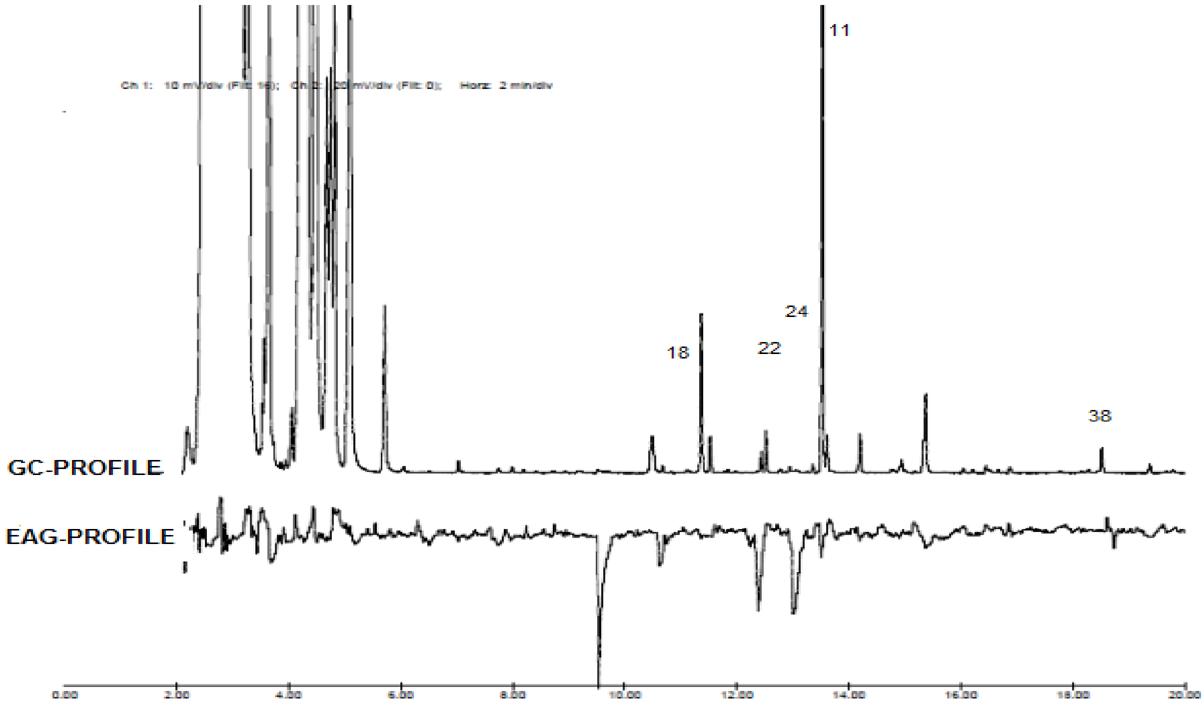


Figure 4.4: GC-EAD chromatograph of the essential oil using *An. gambiae* mosquitoes

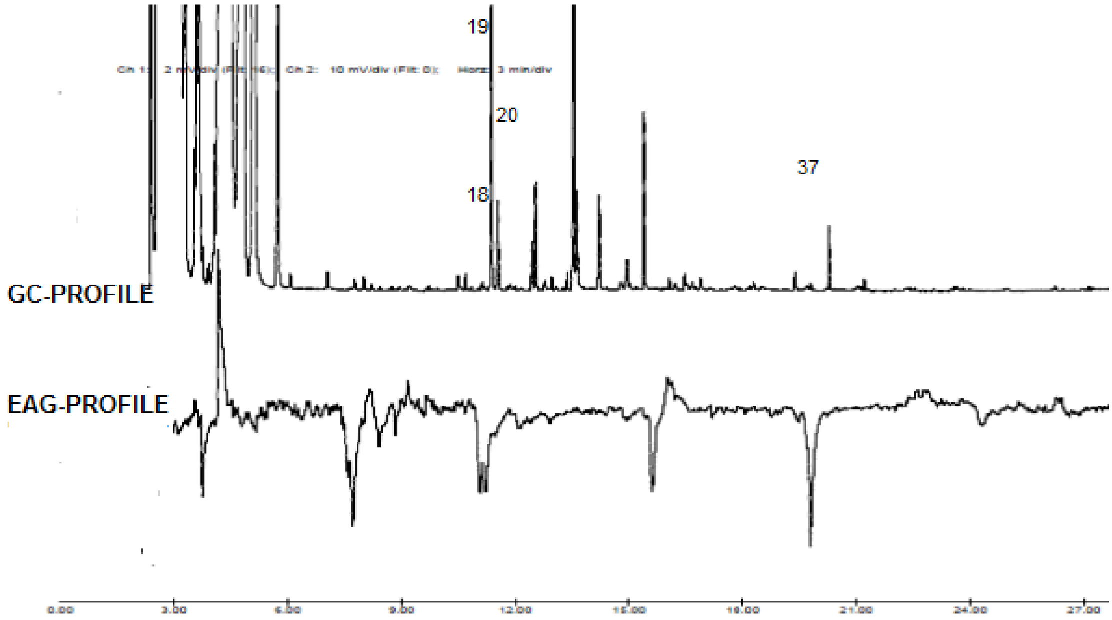


Figure 4.5: GC-EAD chromatograph of the essential oil using *An. gambiae* mosquitoes

4.4 Repellency activity of EAG-active compounds and blends against *An. gambiae* mosquitoes

The mean percent repellency (+SE) of blends and some selected standards obtained at different doses and RD_{50} and RD_{75} values are given in table **4.3**, table **5.1** and table **5.2** in the Appendix 1. It was noted that the repellence (98.81±1.19 and 100.00±0.00 at concentration of 0.01g/ml and 0.1g/ml respectively) of the essential oil against *An. gambiae* was comparable to that of DEET (100.00±0.00 and 100.00±0.00 at concentration of 0.01g/ml and 0.1g/ml respectively) at higher doses; however, it showed lower repellence (36.97±1.81 and 50.41±2.87 against 51.11±13.32 and 86.22±4.51 of DEET at concentration of 0.0001g/ml and 0.0001g/ml respectively) at lower doses. Since the essential oil contained different compound the concentration of the active constituents at low concentrations could have been too low to be detected by the mosquitoes. Blend 1 containing six GC-EAD active compounds was significantly lower than that of essential oil; implying that the

Table 4.3: **Mean percentage repellency (±SE) of blends and standards at different concentrations**

	Dose in g/mL						
Sample	**10^{-5}**	**10^{-4}**	**10^{-3}**	**10^{-2}**	**10^{-1}**	**RD_{50}**	**RD_{75}**
DEET	51.11±13.32^{C,a}	86.22±4.51^{B,a}	94.29±3.69^{AB,a}	100.00±0.00^{A,a}	100±0.00^{A,a}	1.36	1.59
Essential oil	36.97±1.81^{D,b}	50.41±2.87^{C,b}	84.17±0.78^{B,b}	98.81±1.19^{A,a}	100.00±0.00^{A,a}	1.93	2.24
Blend 1	29.21±1.02^{E,d}	40.49±1.01^{D,c}	46.71±2.63^{C,d}	62.36±1.43^{B,de}	86.03±1.32^{A,b}	4.30	5.80
Blend 2	32.32±2.25^{E,bc}	41.96±0.95^{D,c}	60.04±3.61^{C,c}	80.53±1.13^{B,b}	100.00±0.00^{A,a}	2.84	3.50
Blend 3	7.96±2.03^{E,e}	25.80±0.72^{D,e}	39.82±0.85^{C,de}	51.67±2.49^{B,g}	63.96±1.04^{A,d}	5.930	7.48
Blend 4	28.36±0.84^{D,d}	37.78±1.20^{C,cd}	47.13±1.33^{B,d}	55.82±2.29^{A,fg}	58.21±2.18^{A,d}	8.84	14.44
Blend 5	19.21±1.38^{D,bcde}	31.77±1.81^{C,d}	39.63±6.66^{C,de}	51.09±1.56^{B,g}	64.24±2.15^{A,d}	7.20	10.11
Blend 6	11.51±3.95^{E,de}	30.54±2.03^{D,de}	55.46±2.16^{C,c}	74.04±1.73^{B,c}	100.00±0.00^{A,a}	3.29	3.81
Blend 7	20.18±0.75^{E,bcde}	33.02±1.22^{D,de}	39.21±0.63^{C,de}	58.11±1.72^{B,ef}	78.89±1.92^{A,c}	5.12	6.70
Blend 8	20.80±6.63^{B,bcde}	31.51±2.38^{B,de}	45.20±2.08^{A,d}	49.78±2.04^{A,g}	58.01±3.83^{A,d}	8.42	12.49
Blend 9	21.67±1.14^{C,bcde}	23.16±1.57^{C,f}	27.12±1.16^{C,f}	43.20±3.12^{B,h}	52.48±1.80^{A,e}	15.37	24.59
(+)-β-pinene	16.51±0.39^{E,cde}	23.73±1.03^{D,f}	39.79±1.33^{C,de}	50.79±1.03^{B,g}	78.96±2.19^{A,c}	5.40	6.90
(-)-β-pinene	9.44±1.19^{E,e}	23.40±1.33^{D,fg}	42.01±1.98^{C,d}	58.72±1.21^{B,ef}	73.74±1.21^{A,c}	4.95	6.06
(+)-α-pinene	14.76±0.58^{E,cde}	29.39±0.53^{D,ef}	35.94±1.20^{C,de}	64.70±1.15^{B,d}	99.38±0.62^{A,a}	3.86	4.60
(-)-α-pinene	3.74±1.44^{E,e}	20.25±0.91^{D,g}	30.94±0.94^{C,ef}	39.41±0.72^{B,h}	59.12±0.58^{A,d}	6.92	8.59

Means (±SE) with different (i) capital letters in a row, and (ii) small letters within a column are significantly different at $\alpha = 0.05$ (Student-Newman-Keuls test) other two EAG-active constituents, longifolene and α-thujene which were not available for testing, could be contributing significantly to the repellence of essential oil. Subtraction of (+)-β-pinene (**41**), (-)-β-pinene (**42**), (+)-α-pinene (**39**), tetradecane (**24**), or 1,2,3,4,5-pentamethylcyclopentane (**18**) led to significant drop in repellent activities of the resulting blends. This shows that these compounds contribute significantly to the repellent activity of the essential oil. On the other hand, subtraction of (-)-α-pinene (**40**), *p*-cymene (**11**) or α-longipinene (**37**) led to significant rise in the repellence of the resulting blends; thus, these compounds contribute negatively to the repellent activity of the essential oil. The difference in the activities of the two optical isomers of α-pinene was confirmed by comparing the individual repellence of each isomer. The repellence of (+)-α-pinene (**39**) was higher than that of (-)-α-pinene (**40**) (Figure **4.6)**. Interestingly, in another study only positive enantiomers of pinene were found to have microbial activities against *C. albicans*, *C. neoformans* and *R. oryzae* (Silva *et al*., 2012).

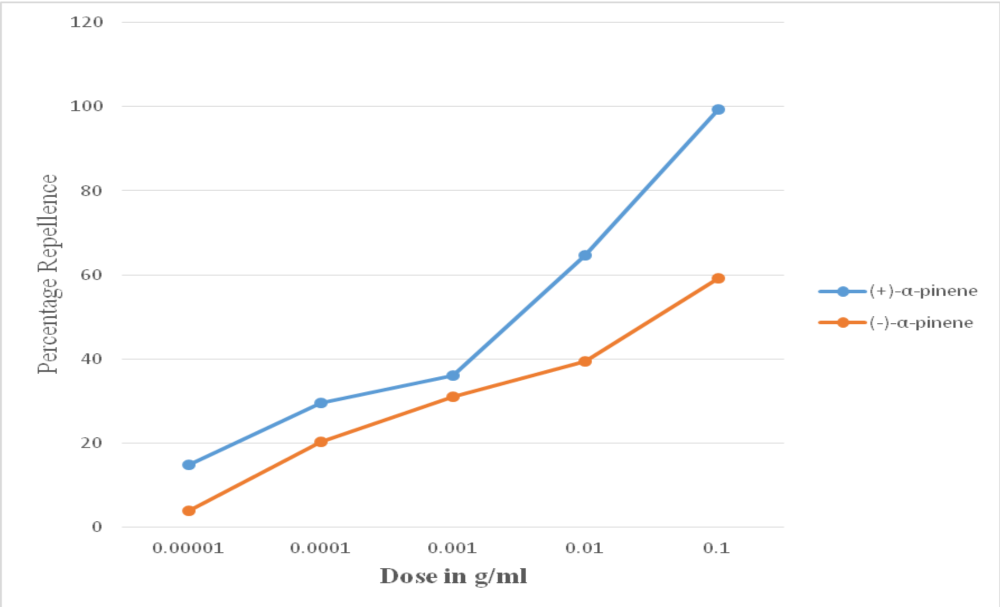


Figure 4.6: Mean percentage repellence of (+)-α-pinene and (-)-α-pinene at different doses

The two optical isomers of β-pinene or isomeric mixture of the two did not show any significant difference in repellence activity (Figure **4.7)**. From RD_{75} and figure **4.8,** it was clear that blend 2 was the most repellent with Rd_{75} of 3.5 among all the blends but was lower to that of DEET and essential oil at lower doses but the same at the highest concentration. Bend 2 did not contain *p*-cymene and which could have acted as an attractant and thus absence of *p*-cymene could contributed to high repellence of blend 2. Blend 2 had lower repellence than that of essential oil and can be attributed to absence of longifolene (**37**) and α-thujene (**19**).

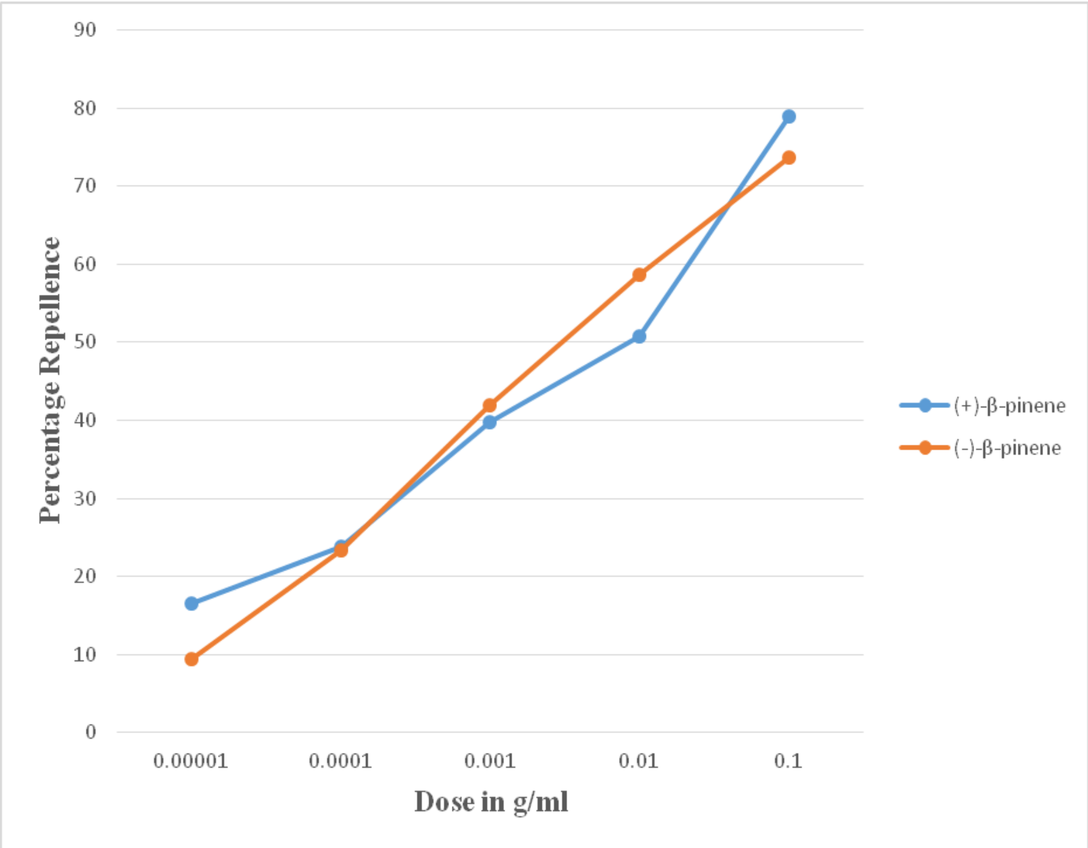


Figure 4.7: Mean percentage repellence of (+)-β-pinene and (-)-β-pinene at different doses

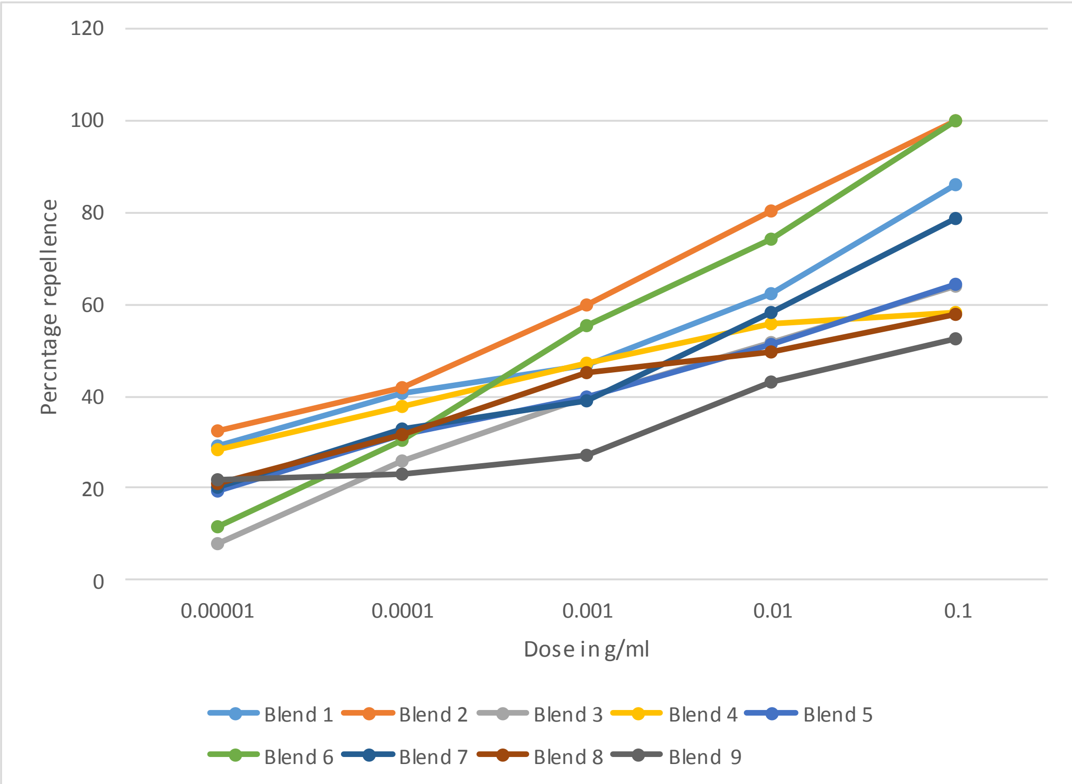


Figure 4.8: Mean percentage repellence of the blends at various doses

Previous studies have focused largely on anti-microbial activities of crude products and specific constituents of *N. sativa* seeds. The essential oil of *N. sativa* was reported to be efficacious against gram positive than gram negative bacteria, and better than the standard, tetracycline (Saptha *et al.*, 2009). In another study, solvent extract of black seed as well as its steam-volatile oil have been reported to possess antibacterial activities. The steam-volatile oil was found to be more effective against Gram-positive bacteria; however, the solvent extract was more effective against Gram-negative bacteria (Adams, 2007). Burits and Bucar (2000) found radical scavenging effect of *N. sativa* essential oil in an experiment with DPPH free radicals; however, it was less efficacious (IC_{50} of 460 mg/ml) than the reference compound, carvacrol (IC_{50} of 28.8 mg/ml).

One constituent (**30)** of the essential oil of *N. sativa* seeds with abundance of 5.81% could not be identified in the present study when compared with NIST data. Its mass spectrum compared closely with that reported by Wajs *et al.* (2008) who identified it as *trans*-4-methoxythujane by comparison of the ^{1}H-NMR, ^{13}C-NMR and MS spectra of the isolated compound with those of a synthetic product. However, in the present study, the compound was not found to be EAG-active. Interestingly EAG-active compound, longifolene was reported to show activity against Gram-positive bacteria (Masaki *et al.*, 1992)

CHAPTER FIVE

CONCLUSIONS AND RECOMMENDATIONS

5.1 Conclusions

Twenty six compounds, constituting 68.147% of the volatile oil, were identified. The oil consisted of five monoterpenoid hydrocarbons (53.79%), one monoterpenoid ketone (0.21%), sixteen nonterpenoid hydrocarbons (5.25%), one monoterpenoid alcohols (0.13%), two sesquiterpenoid hydrocarbons (3.3.0%) and one Monoterpenoid ester. The repellence to the *An. gambiae* of the essential oil was comparable to that of DEET at higher doses. Longifolene and α-thujene could have contributed to the repellence of the essential oil and their absence in the blend containing all other EAG-active compound could have led to lower repellency against *An. gambiae* than the essential oil. Repellence activity of pure (+)-α-pinene was higher than that of pure (-)-α-pinene (**40**). (+)-β-pinene (**41**), (-)-β-pinene (**42**), (+)-α-pinene (**39**), tetradecane (**24**), and 1,2,3,4,5-pentamethylcyclopentane (**18**) were the pure compounds that were found to contribute positively to repellency towards *An. gambiae*. Unlike α-pinenes, pure (+)-β-pinene (**41**) and (-)-β-pinene (**42**) did not show significant difference. *p*-Cymene (**11**) contributed negatively to repellency of the essential oil of *N. sativa* seeds towards *An. gambiae.*

From these results it is evident that essential oil of *N. sativa* seeds contains a blend of compounds that repel *An. gambiae* mosquitoes. It is probable that an insect is less likely to develop resistance to an insect-active blend of several different active compounds than a single compound (Isman, 2006). The results from the present study lays down some groundwork for downstream development of appropriate blends for personal and space protection against *An. gambiae.*

5.2 Recommendations

(i) Two of the GC-EAD active compounds (α-thujene (**19**) and longifolene (**38**)) were not commercially available, and thus should be synthesized and their repellency against *An. gambiae* determined.

(ii) The *N. sativa* seeds were bought from the local market and it would be interesting to see if seeds obtained from plants growing in different regions contain essential oils with different profiles of the constituents, which may confer different levels of activity.

(iii) The optical stereo-chemistry of the α-pinene and β-pinene could not be established thus isolation and determination of the stereochemistry of the natural constituents would clarify the actual optical isomers present.

(iv) More studies should be carried out to determine whether *N. sativa* essential oil is repellent to other nuisance or disease vectors.

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APPENDICES

Appendix I: Rd_{50} and Rd_{75} for DEET, essential oil, pure compounds and blends

Table 5.1: Rd_{50} for blends, essential oil and DEE

Sample	Rd $_{50}$	lower	upper
DEET	1.361	1.322	1.399
Essential oil	1.928	1.775	2.082
Blend 1	4.307	3.947	4.667
Blend 2	2.840	2.627	3.053
Blend 3	5.930	5.555	6.304
Blend 4	8.835	7.839	9.830
Blend 5	7.195	6.615	7.776
Blend 6	3.289	3.120	3.458
Blend 7	5.118	4.760	5.477
Blend 8	8.422	7.662	9.181
Blend 9	15.374	13.865	16.883
(+)-β-pinene	5.404	5.062	5.747
(-)-β-pinene	4.952	4.666	5.239
(+)-α-pinene	3.863	3.654	4.071
(-)-α-pinene	6.915	6.479	7.351

Table 5.2: Rd_{75} for blends, essential oil and DEET

Sample	Rd $_{75}$	lower	upper
DEET	1.585	1.540	1.630
Essential oil	2.236	2.058	2.414
Blend 1	5.796	5.312	6.281
Blend 2	3.500	3.238	3.763
Blend 3	7.482	7.009	7.954
Blend 4	14.444	12.817	16.072
Blend 5	10.109	9.294	10.925
Blend 6	3.809	3.613	4.004
Blend 7	6.702	6.233	7.172
Blend 8	12.488	11.363	13.614
Blend 9	24.587	22.173	27.001
(+)-β-pinene	6.896	6.459	7.334
(-)-β-pinene	6.063	5.713	6.414
(+)-α-pinene	4.600	4.351	4.848
(-)-α-pinene	8.586	8.045	9.128

Appendix II: Certificate of Ethical Clearance

KENYATTA UNIVERSITY
ETHICS REVIEW COMMITTEE

Fax: 8711242/8711575
Email: director-crd@ku.ac.ke
Website: www.ku.ac.ke

P. O. Box 43844
Nairobi, 00100
Tel: 8710901/12

Our Ref: KU/R/COMM/51/16

Date: June 21st, 2011

Mr. Ephantus G. Ndirangu,
Department of Chemistry,
Kenyatta University.

Dear Mr. Ndirangu,

APPLICATION NUMBER PKU006/I05 OF 2011 - 'REPELLENT AND FUMIGANT TOXICITY OF ESSENTIAL OIL OF NIGELLA SATIVA SEEDS AGAINST MOSQUITOES AND SAND-FLIES AND IDENTIFICATION OF THE ACTIVE BLENDS

1. IDENTIFICATION OF PROTOCOL

The application before the committee is with a research topic 'REPELLENT AND FUMIGANT TOXICITY OF ESSENTIAL OIL OF NIGELLA SATIVA SEEDS AGAINST MOSQUITOES AND SAND-FLIES AND IDENTIFICATION OF THE ACTIVE BLENDS' dated 14th June 2011.

2. APPLICANT

Mr. Ephantus G. Ndirangu,
Department of Chemistry,
Kenyatta University.

3. SITE

KENYA

4. DECISION REACHED.

The committee has considered the research protocol in accordance with the Kenyatta University Research Policy (section 7.2.1.3) and the Kenyatta University Ethics Review Committee Guidelines, and is of the view that against the following elements of review,

i. Scientific design and conduct of study,

ii. Recruitment of research participant,

iii. Care and protection of research participants,

iv. Protection of research participant's confidentiality,

v. Informed consent process.

APPROVED that the research proceeds for a period of ONE year.

The approval is for the period 21st June 2011 to 21st June 2012.

5. ADVICE

i. Progress reports are submitted to the KU-ERC every six months and a full report is submitted at the end of the study,

ii. Serious and unexpected adverse events related to the conduct of the study are reported to this board immediately they occur,

iii. Notify the Kenyatta University Ethics Committee of any amendments to the protocol.

Please sign in the space provided below and return to KU-ERC a copy of the letter.

NICHOLAS K. GIKONYO, PhD.
CHAIRMAN ETHICS REVIEW COMMITTEE

I NDIRANGU GITHUI EPHANTUS accept the advice given and will fulfill the conditions therein.

Signature.. Dated this day of 21ST JUNE 2011.

cc. Vice-Chancellor
Director: Institute for Research Science and Technology

AT/

Appendix III: Certificate of Consent

This research involves repellency and fumigant toxicity of essential oils of black seeds against mosquitoes and sandflies. Essential oil in hexane will be dispensed on one of the forearms of the participant. Then forearms of the participant from the wrist to the elbow will be exposed for three minutes to mosquitoes and sandflies, the number of the insects that land of the arm will be counted and then shaken off before imbibing any blood. The insects that will be used will not have any disease causing agent. An insect bite cream will be provided to the participants in case of any minor bites and associated irritations. The results are expected to form the basis of downstream development of the appropriate blends for personal and space protection against the vectors.

I have read the foregoing information, or it has been read to me. I have had the opportunity to ask questions about it, and any questions that I have asked have been answered to my satisfaction. I consent voluntarily to participate as a participant in this research and understand that I have the right to withdraw from the research at any time without in any way affecting my medical care.

Print name of participant: ________________________

Signature of participant: ________________________

Date: ____________________________

Day / Month / Year