STUDIES ON THE USE OF REPELLENT PLANTS AND PLANT PRODUCTS AGAINST THE MAIN MALARIA VECTORS IN EASTERN AFRICA

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DECLARATION

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

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DEDICATION

I dedicate this thesis to my parents Debritu Legesse and the late Seyoum Abebe.

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LIST OF ACCRONYMS AND ABBREVIATIONS

BSA	Bovine Serum Albumin
CDC	Communicable Diseases Control
CI	Confidence Interval
DDT	Dichloro-diphenyl-trichloroethane
DEET	Diethyl methylbenzamide
DCM	Dichloromethane
ddH_2O	Double distilled water
dNTP	Deoxynucleotide Triphosphate
ED_{50}	The median effective dose
EDTA	Ethylenediaminetetraacetic acid
FID	Flame Ionization Detector
GC	Gas chromatography
GC-MS	Gas chromatograph with mass spectrometer
GLM	General linear model
ICIPE	International Center of Insect Physiology and Ecology
MS	Mass spectrometer
NIST	National Institute of Science and Technology
PCR	Polymerase Chain Reaction
SAS	Statistical Application System
SPSS	Statisical package for Social sciences
SEM	Standard Error of Mean
Taq	*Thermus aquaticus*
WHO	The World Health Organization

ABSTRACT

Malaria is one of the major public health problems in tropical Africa. The existing vector control tools are not sufficient to reduce the already escalating burden of the disease. The main aims of this study were to search for simple and cost-effective plant-based repellents against the main malaria vectors in Africa, and compare existing and alternative methods of utilizing the plants. The repellency of the following plants suggested by the ethnobotanical survey and the literature was evaluated against *Anopheles gambiae sensu stricto* Giles in experimental huts within a screen-walled greenhouse: *Ocimum americanum* Linnaeus, *Ocimum kilimandscharicum* Guerke, *Ocimum suave* Willd., *Lantana camara* L, *Azadirachta indica* Adrien Jussieu, *Hyptis suaveolens* Poit, *Lippia ukambensis* Spreng and *Corymbia citriodora* Hook. Thermal expulsion, direct burning, and intact potted plants were tested as alternative application methods. When thermally expelled (from modified traditional stoves), only *H. suaveolens* failed to repel mosquitoes, whereas the leaves of *C. citriodora* (74.5% repellency, $P<0.0001$), leaves and seeds of *O. suave* (53.1% repellency, $P<0.0001$) and *O. kilimandscharicum* (52.0% repellency, $P<0.0001$) were the most effective. Leaves of *C. citriodora* also exhibited the highest repellency (51.3% repellency, $P<0.0001$) by direct burning. Intact potted plants of *O. americanum, L. camara,* and *L. ukambensis* repelled on average 39.7, 32.4 and 33.3 % ($P<0.0001$) of the mosquitoes respectively in semi-field experimental system.

The effectiveness of live potted plants and thermal expulsion in repelling *An. gambiae s.l.* and *An. funestus* was also estimated in traditional houses in western Kenya. O*cimum americanum*, *L. camara* and *L. ukambensis* were tested in potted form, and *C. citriodora*, *O. kilimandscharicum* and *O. suave* by thermal expulsion. All plant species showed significant repellency against *An. gambiae s.l.*, with the highest repellency by *C. citriodora* (48.71 %, P<0.0001) followed by an equal level of repellency of *O. kilimandscharicum* and *O. suave* (44.54%, P=0.001) during application of plants by thermal expulsion. All the three also showed residual effects against *An. gambiae s.l.* Similarly, potted plants of *O. americanum* and *L. camara* repelled *An. gambiae s.l.* significantly (37.91%, P=0.004 and 27.22%, P=0.05 respectively). Thermal expulsion of *O. kilimandscharicum* significantly repelled *An. funestus* Giles, although none of the potted plants repelled this species.

Volatile oils extracted by steam distillation of plants (*O. americanum, O. kilimandscharicum, O. suave H. suaveolens* and *L. camara*) were also evaluated against *An. gambiae s.s*. The essential oils from all candidate plants showed complete protection for less than an hour, but this was 4 hours for the standard DEET. Gas chromatographic analysis of the volatiles emitted by thermally expelled *C. citriodora* revealed that the major constituents are citronellal, citronelol and *iso*-pulegol, and that of *O. kilimandscharicum* and *O. suave* are camphor and *trans*-methyl *iso*-eugenol, respectively. The major constituent from potted plants of *O. americanum* is *α*-terpineole. This study showed that modifications of traditional practices represented by thermal expulsion and intact potted plants can reduce domestic exposure to malaria vectors. As such, they may represent a sustainable and readily applicable malaria vector control tool for incorporation into integrated vector management programs.

CHAPTER 1

GENERAL INTRODUCTION AND LITERATURE REVIEW

1.1 GENERAL INTRODUCTION

1.1.1 Global malaria situation

Over two billion people, primarily in tropical countries, are at risk from mosquito-borne diseases, such as malaria, dengue hemorrhagic fever and filariasis (Service, 1993). Malaria continues to be one of the major public health problems in the tropics (WHO, 1997) and is responsible for enormous economic burdens in endemic regions (Gallup and Sachs, 2001). More than 40% of the world's population lives in over 100 countries with endemic malaria including Africa, Latin America and the Caribbean, South East Asia, the Eastern Mediterranean, the western Pacific and parts of Europe (WHO, 1997).

Recently, it has been reported that, at a minimum, between 700,000 and 2.7 million people die annually from malaria, over 75% of them African children (Breman, 2001). Furthermore, the report revealed that between 400 and 900 million acute febrile episodes occur annually in African children under the age of 5 living in malaria-endemic regions, and this number will double by 2020 if effective control interventions are not implemented (Breman, 2001).

1.1.2. Malaria vectors

Although there are about 400 species of *Anopheles* mosquitoes, only 60 of them transmit malaria under natural conditions, and only 30 are of major importance (Bruce-Chwatt, 1985). In Africa the main vectors for malaria are species of the *Anopheles gambiae sensu lato* and *Anopheles funestus* complex Giles (Gillies and De Meillon, 1968). The *An. gambiae s.l.* complex consists of six sibling species. Four are freshwater-breeding species: *An. gambiae sensu stricto* Gilles, *An. arabiensis* Patton, *An. quadriannulatus* Theobold and *An. bwambae* Theobold; and two salt-water species: *An. merus* Theobold and *An. melas* Theobald. The Ethiopian population of *An. quadriannulatus* was recently recognised as being distinct from South African population and is designated as *An. quadriannulatus* species B (Hunt *et al.*, 1998). Among all the sibling species of the *An. gambiae s.l.*, *An. gambiae s.s.* and *An. arabiensis* are primary vectors of malaria in tropical Africa (Garrett-Jones, 1964; White, 1974; Gillies and Coetzee, 1987).

This study was designed to evaluate some plants used traditionally in repelling mosquitoes and to search for cost-effective plant-based repellents against the main African malaria vectors for use in integrated vector management programmes. The candidate plants were identified on the bases of ethnobotanical and existing empirical information (Schreck and Leonhardt, 1991; Jembere *et al.*, 1995; Bekele *et al.*, 1996). The efficacies of candidates were determined as intact potted plants, and by burning and thermal expulsion of plant materials under semi-field and field conditions against the main African malaria vectors. The major compounds emitted by intact potted plants and those produced by thermal expulsion and direct burning were also identified by gas chromatography coupled with mass spectrometry (GC-MS). The research project, therefore, assessed and developed appropriate technologies for feasible use of natural products of plant origin with emphasis on home-grown repellent plants for integrated management of the main malaria vectors in Africa.

1.2 LITERATURE REVIEW

1.2.1 MALARIA CONTROL

The measures for prevention of malaria in individuals and for large-scale control of the disease can be divided into 1) measures designed to eliminate the malaria parasite in the human host, 2) measures designed to destroy adult mosquitoes, 3) measures designed to destroy the larvae of mosquitoes, 4) measures designed to reduce the breeding of mosquitoes by altering the environment and 5) measures designed to prevent mosquitoes from feeding on man (Bruce-Chwatt, 1985). These control measures generally target the vectors and/ or the parasite.

Prompt diagnosis and treatment are among the key basic technical elements of the global malaria control strategy and important for reducing malaria mortality and morbidity (WHO, 1993). Chloroquine was the drug of choice for many years for the treatment of acute attack of malaria for all species of human malaria, *Plasmodium falciparum, P. vivax, P. ovale* and *P. malariae* (Kreier, 1980). However, due to the widespread of chloroquine resistant *falciparum* malaria, sulfadoxine-pyrimethamine is now the first line drug for the treatment of *falciparum* malaria in many countries, but, less effective against other species (WHO, 1995b). Emergence and spread of resistance to both chloroquine and sulfadoxine-pyrimethamine, however, would dictate the use of alternative drugs (such as quinine, mefloquine, halofantrine and artemisinin and its derivatives) that are substantially more expensive and less safe (WHO, 2000).

Vector control is an essential component of malaria control programmes. The vector control options that are currently available are; indoor residual spraying, biological control, larviciding, environmental management, space spraying and personal protection measures including mosquito repellents (WHO, 1995a).

1.2.1.1 Indoor Residual House Spraying

The development of highly effective, residual insecticide, DDT initiated a global eradication programme in 1950s and 1960s, which was initially very successful in many countries such as India, Sri Lanka, and former Soviet Union (Greenwood and Mutabingwa, 2002). Indoor residual house spraying may be considered as an appropriate method for vector control when the following conditions are met: a high percentage of the structures in an operational area have adequate sprayable surfaces, and can be expected to be well sprayed; the majority of the vector population is endophilic, i.e rests indoors; the vector is susceptible to the insecticide in use (WHO, 1995a). Indoor residual spraying can also be prescribed to control the epidemic degrees of malaria, and routine applications by standard techniques will easily control the transmission of infection (Bruce Chwatt, 1985).

In the past, the use of chemical insecticides such as DDT and organophosphate insecticides was the method of choice for mosquito control. Regretfully, the past successes are now eroded because of the appearance of mosquitoes resistant to insecticides (Roberts and Andre, 1994; Chandre *et al.*, 1999; Hargreaves *et al.*, 2000) coupled with long-term detrimental effects of chemical insecticides to non-target organisms and the environment (Attaran and Maharaj, 2000; Zaim and Guillet, 2002).

1.2.1.2 Larviciding and Biological Control

Larval control with either chemicals such as temephos or biological agents such as *Bacillus thuringiensis* var *israelensis* de Berjac and *Bacillus sphaericus* Neide is relevant method of vector control if a high proportion of the breeding sites within mosquito flight range of the community to be protected can be located and are accessible, and the breeding sites are of manageable sizes (WHO, 1995a). It has also a major advantage in the control of mosquitoes before they disperse and transmit diseases (Killeen *et al.*, 2002). Furthermore, anti-larval measures of control are of particular value when employed in conjunction with other means such as environmental management (Bruce-Chwatt, 1985).

1.2.1.3 Environmental management

Environmental management has been defined as 'planning, organisation, implementation and evaluation of deliberate changes of environmental factors, with the view to preventing the propagation of vectors and reducing the man-vector pathogen contact (Bruce-Chwatt, 1985). Environmental management approaches to vector control aim at modifying the environment to deprive the target vector population of its requirements for survival. This reduces human-vector contact and renders the conditions less conducive to disease transmission (WHO, 1995a). Environmental management is a promising approach with which to Roll Back Malaria but has seen little application in Africa for over half a century. However, environmental management programme at cooper mining communities in Zambia showed significant success in the control of malaria (Utzinger *et al.*, 2001). Environmental management integrated with pharmacological, insecticidal and bednet interventions-could substantially increase the chances of rolling back malaria (Utzinger *et al.*, 2001).

1.2.1.4 Insecticide treated bednets and other materials

Bednets are traditionally used to prevent bites of mosquitoes and have long been advocated as a means of personal protection against malaria vectors in Africa (WHO, 1986). However, torn or incorrect usages provide little or no additional protection (Port and Boreham, 1982). For these reasons the application of residual insecticides mainly pyrethroids to bednets was suggested in the late 1970s as a means of reinstating the effectiveness of torn or incorrectly used nets as a human-vector barrier (Curtis *et al.*, 1990). The results of various studies often differ and although some trials with African malaria vectors have demonstrated substantial reductions of vector density, survival and sporozoite rate (Magesa *et al.*, 1991; Robert and Carnevale, 1991), others have found little or no effects on the vector population as a whole (Lindsay *et al.*, 1993; Quinones *et al.*, 1998). In general, it is believed that the feeding and resting habits of the vector and the cultural practices and sleeping habits of the people are important determinants of the efficacy of personal protection measures. It will be most effective when the vectors are endophilic and/ or endophagic; but they are less effective or ineffective when the vectors are exophagic and when people are not protected during the active period of the vectors (WHO, 1995a).

Insecticide-treated bednets protect individuals by either diverting host-seeking vectors to look for a blood meal elsewhere or by killing those that attempt to feed on that person (Killeen *et al.*, 2000). At present, insecticide treated bednets have been widely advocated for the control of malaria in Africa, but give no protection against anopheline mosquitoes in the evening before people go to bed. Exposure to malaria-vectors and to nuisance mosquitoes starts in the early evening (Maxwell *et al.*, 1998). It has been also documented that insecticide treated bednets have been only partially successful in some areas, reducing anaemia in pregnant women, but having no demonstrable effect on incidence of parasitemia (Dolan *et al.*, 1993). One reason for the limited efficacy of insecticide treated bednets may be due to infective bites received outside the nets (Lindsay *et al.*, 1998).

1.2.1.5 Personal protection measures

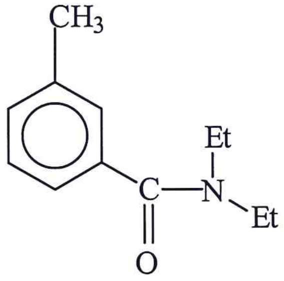
1.2.1.5.1 Insect repellents

Concerns for environmental contamination with residual insecticides coupled with problems of insecticide resistant mosquitoes and the economic restraints imposed by inflation have led to an intense re-examination of new and traditional methods in vector control. One field of research experiencing renewed interest is that of insect repellents as a method of protecting humans against disease vectors (Curtis *et al.*, 1991). Moreover, the cost of synthetic insecticides is too high for most of the less developed nations. The use of insect repellents can be considered as an alternative method where other conventional vector control methods are not feasible (Gupta and Rutledge, 1994). A search for new mosquito repellents may also provide compounds with a mode of action different from classic neurotoxicants, against which many arthropod vectors have become resistant.

Repellents have long been used in protection against biting insects primarily to avoid nuisance. However, through their potential in the reduction of human-vector contact, repellents can also be considered as important tools in the prevention of vector-borne diseases. They are a practical means of protection against nuisance and disease vectors in conjunction with or without other control measures. Insect repellents have a unique role in regions where mosquito vectors bite in the early evening since people often are not sleeping under bednet at this time. Physical barriers such as long clothing are unpopular in the tropics since the evenings are warm. Repellents may therefore provide a valuable supplement to bednet use. Indeed, it may be that in areas where the local vectors feed in the early evening this is the only means of securing a reduction in the level of malaria transmission. Insect repellents can be broadly classified as synthetic repellents and natural or plant derived repellents (Curtis *et al.*, 1991).

1.2.2 SYNTHETIC REPELLENTS

Interest in repellent research was probably brought about with the development of diethyl methylbenzamide (DEET) (Schreck, 1977). The synthesis of DEET (McCabe *et al.*, 1954) proved to be a major breakthrough for repelling a variety of arthropod species, and best studied, insect repellent currently available on the market. The most commonly used insect repellent formulations contain DEET, which is effective against a broad spectrum of insects including mosquitoes, biting flies, chiggers, fleas and ticks (Davis, 1985). The meta isomer of DEET (**I**) is the active ingredient of most commercially available repellents. Until recently, this material has virtually eclipsed other repellents for topical use, and the principal component in use, nearly 50 years after its discovery.



(**I**)

In Zanzibar, DEET provided good protection against *An. gambiae s.s.* in the field despite the low sensitivity of this species in the laboratory (Curtis *et al.*, 1987). Schreck (1985) reported that 100% concentration significantly repel *An. albimanus*, but protected from bites for only 2 hours in test cages containing 1000 to 1500 mosquitoes. Recent studies by Durrheim and Govere (2002) showed the potential of topical application of 15% DEET formulation to contain malaria outbreak in South Africa. In this study, topical application to feet and ankles reduced the overall biting rate of the malaria vector *An. arabiensis* by 69% and recommended it for use in other outbreak-prone settings where infective mosquito bites are sporadic and malaria has unstable endemicity.

In addition to mosquito repellent property, it has also shown larvicidal property against *Aedes albopictus* and *An. albimanus* both under laboratory and field conditions (Xue *et al.*, 2001). Although synthetic repellents containing DEET or other chemicals are effective and widely available, their retail price may be too high for daily use among poor communities in tropical Africa.

1.2.2.1 Mode of action of insect repellents

In spite of the clearly recognized need for cost-effective and safe repellents, there is still lack of sufficient knowledge of their precise mechanisms of action. Deither *et al.* (1960) defined a repellent in terms of the specific behaviour pattern: "A repellent is a chemical which causes an insect to make oriented movements away from its source". Based on this earlier definition, Dogan *et al.* (1999) recently concluded that DEET is not a repellent, but rather an inhibitor of attraction to lactic acid, a component of human sweat that is attractive to mosquitoes (Gibson and Torr, 1999). Repellometer study by Dogan *et al.*

(1999) showed that in the absence of a host, DEET was an attractant and in the presence of a host, it was an inhibitor of attraction. However, DEET continues to be known as insect repellent in all subsequent literature cited, presumably due to alternative definitions proposed after Deither *et al.* (1960), but not cited in Dogan *et al.* (1999). Browne (1977) suggested that the definition be broadened to "a repellent is a chemical that, acting in the vapor phase, prevents an insect from reaching a target to which it would otherwise be attracted". Davis (1985) later refined the definition as: "a chemical that elicits a combination of behavioural responses whose net result is the prevention of biting by an insect". The specific behavioural patterns by which this result is attained are usually not determined.

Two well-known forms of repellents are those that are applied directly to the skin or clothing, and those that are released directly into the air through evaporation or burning. Repellents for skin treatment interfere with the way mosquitoes perceive host stimuli. They are effective in the vapor phase and work only on short-range approach. Based on the work with DEET, Davis (1985) postulated that they interfere with sensory neurons that respond to host cues, and possibly stimulate other behavior receptors simultaneously. A repellent generally turns a mosquito aside before or just before it lands. The mosquito's feeding response depends on automatic responses occurring in a definite sequence, so that anything that interrupts the sequence will prevent landing and feeding.

1.2.2.1.1 Repellents and insect behaviour

Very little is known on how repellents affect an insect's behavioural responses and how they prevent insects from finding and biting their hosts. Earlier studies by Wright and co-workers (Daykin *et al.*, 1965; Rayner and Wright, 1966; Kellogg *et al.*, 1968; Wright, 1968; Kellogg, 1970) investigated repellent mechanisms in mosquitoes and described the host seeking behaviour as follows: "when female mosquitoes encounters an air stream containing host-related stimuli, they do not make apparent response (i.e. do not turn), but on leaving the host air-stream, the mosquitoes do turn, so as to re enter it". No quantitative data were presented for these observations, but the authors stated that if the mosquito encounters a repellent in the host air-stream, it either turns out of the air-stream on entry or fails to turn back towards the air-stream on leaving the repellent-laden host air-stream (Davis, 1985).

Daykin *et al.* (1965) reached a conclusion that female *Ae. aegypti* L. lacks an olfactory sense, and that an insect repellent functions by blocking the pores on the antennal sensilla, thereby preventing the mosquito from detecting host related signals (Wright, 1975). These conclusions are in direct conflict with the results obtained by other investigators (Davis and Rebert, 1972). For example, if a female mosquito were to enter a host air-stream containing a repellent and execute a turn so as to exit from the air-stream as described above, it would have had to perceive the presence of the repellent via an olfactory sense. If the repellent simply blocked the sensillar pores, neither the repellent nor the host odour would have been perceived and the female mosquito would have flown through and out of the host air-stream with no apparent response. It has been also known for some time that host odour components, especially carbon dioxide, activate mosquitoes (Gillies, 1980), and lactic acid and human sweat samples enhance the activation effects of carbon dioxide (Eiras and Jepson, 1991).

1.2.2.1.2 Sensory Physiology of Insects and repellents

Knowledge on the peripheral nervous system of mosquitoes and perspectives on the behaviour of the whole organism may provide the basis for construction of a model of how chemicals repel host-seeking mosquitoes (McIver, 1981). Davis and colleagues conducted neurophysiological experiments on the peripheral sensory system of mosquitoes to examine the responses of single antennal sensory neuron to behaviourally active chemical and physical stimuli. Their studies provided important information regarding the potential mechanisms by which chemical repellents may act (Davis, 1985). It has been generally assumed that all insect repellents act in a common manner and that they have some common property or set of properties to which their mode of action can be attributed. However, Davis (1985) found that some insect repellents appear to affect differently the activity of the five recognized types of antennal chemoreceptor sensilla of *Ae. aegypti* (Davis and Rebert, 1972; Davis, 1977). Table 1.1 summarizes the quantitative responses of the 5 different antennal sensilla types to various repellent compounds.

Table 1.1 Quantitative responses of the antennal olfactory sensilla of female *Aedes aegypti* mosquitoes to insect repellents. Source: (Davis, 1985)

	SENSILLA TYPES				
REPELLENT	Long pointed Sensory hair**	Short pointed hair†	Long blunt hair††	Short blunt hair†	Grooved peg‡
DEET	nt	0	++	+	-/0
612	nt	++	++	++	-/0
Indalone	nt	0	0	+	+
SRI-C6	nt	++	+	++	+
2504-5	nt	0	0	++	0
Citronellal	_	+	0	++	+
Naphtalene	nt	0	0	++	0
Dimethyl phthalate	nt	0	0	nt	nt

** Contains sensory neurons associated with plant nectar sources
† Contains sensory neurons associated with oviposition-site attractant
†† Contains sensory neurons not yet associated with a specific behaviour.
‡ Contains sensory neurons associated with host related attractants
nt = not tested; ++ strong and + moderate nerve cell excitation by substance; _ strong and-moderate nerve cell inhibition; and 0 = nerve cell did not respond to substance.

The repellents tested did not show anything resembling a common response pattern among the different types of sensory neurons. Therefore, it appears that chemical repellents do not have a common mode of action. Thus, Davis and colleagues concluded that repellents do not behave as a single class of compounds with a common mode of action in mosquitoes and identified at least five possible modes of action for repellents: 1) inhibit response to an otherwise attractive signal, 2) switch the sensory message from attraction to repulsion, 3) activate a receptor system that controls a competing behaviour, 4) activate a noxious odour receptor, or 5) activate different receptor types simultaneously causing loss of the specific signal for host finding.

1.2.2.2 Factors influencing the efficacy of insect repellents

Different factors determine how effective any repellent will be; these factors include the target mosquito species, density and concentrations of the active ingredients.

1.2.2.2.1 Sensitivity of mosquito species

In the past, investigators heavily relied on *Ae. aegypti* in tests for mosquito repellents. This species gained popularity early when it was recognized to be easily reared and an avid blood feeder in the laboratory (Schreck, 1977). However, relying on a single species for information on repellents, many compounds may have been consequently, overlooked because they were not effective against *Ae. aegypti*. Laboratory tests with three different synthetic repellents and three mosquito species (Table 1.2) provide an example of the differential effectiveness of the compounds on the various mosquito species.

Table 1.2 Effectiveness of three repellents as skin applications against three species of mosquitoes. Source: Schreck *et al.* (1977)

Repellents	Protection time (minutes) [a]		
	Ae. aegypti	*An. quadriamaculatus*	*An. albimanus*
Diethyl methylbenzamide	426	96	87
Dimethyl phthalate	53	415	42
Ethyl hexanediol	130	380	158

[a] Average of six tests with six subjects and 250 mg of the indicated repellent per forearm.

In general, different species of mosquitoes may react differently to the same repellent (Rutledge *et al.*, 1983). Earlier studies have shown that even strains of the same species differ significantly in their tolerance for the same repellent. The behavioural response of mosquitoes to DEET is variable within and among species as well as the geographic range (Rutledge *et al.*, 1978). *Ae. taeniorhynchus* and *Cx. pipens* were found to be significantly more sensitive to 31 commercial and experimental repellents than were *Ae. aegypti*, the traditional test species for the repellent studies and *An. albimanus*. Patterns of sensitivity to the test compounds were not related to the taxonomic relationships of the species tested. Similarly, Curtis *et al.* (1987) reported that *An. pulcherrimus* Theobold, *An. albimanus* Wied and *An. gambiae s.l.* were less susceptible to DEET than *Ae. aegypti* in laboratory tests. Other investigators such as McGovern *et al.* (1980), Curtis *et al.* (1987) and Frances *et al.* (1993) have also reported similar differences in the overall sensitivity of other species to repellents.

Therefore, selection and screening of appropriate repellents for protection against the bites of mosquitoes should depend greatly upon the species most important from the public health point of view (Gupta and Rutledge, 1994). The response of the target species cannot be efficiently predicted from those of other mosquito species, even if they are co-generic. Behavioural differences to repellents may also occur between the laboratory reared and natural populations of mosquitoes (Schreck, 1985).

On the other hand, climatic differences have not shown differences in protection time at least for some controlled release repellent formulations. Laboratory studies in environmental chambers for the effectiveness of three controlled release repellent formulations of DEET against the laboratory reared mosquitoes (*Ae. aegypti, Ae. taeniorhynches, An. stephensi*, and *An albimanus*) under three climatic regimens provided similar protection for different time periods after application (Gupta and Rutledge, 1991). As expected, significant differences were observed in the time of protection provided by the three repellent formulations.

1.2.2.2.2 Effect of density on efficacy of insect repellents

The effect of DEET was found to be density dependent (Schreck, 1985; Frances *et al.*, 1993). At low densities in laboratory tests, *An. albimanus* was effectively repelled at lower dosages. But at higher densities, the duration of repellency was notably reduced. Indeed, substantial number of laboratory tests suggests that only limited protection against *An. albimanus* can be expected through the use of DEET. Moreover, if there is a direct correlation between the biting rate and population density, then field data can appear to contradict results of laboratory studies with mosquito densities substantially different from those in the field.

1.2.2.2.3 Concentrations of active ingredients

Although, concentrated DEET formulations protect longer than those that are more dilute, little improvement is offered by concentrations of the active ingredient higher than 50 percent (Buescher *et al.*, 1983). Most commercially available formulations now contain 40 percent DEET or less, and the higher concentrations are most appropriate to use under circumstances in which the biting pressures are intense, the risk of arthropod-transmitted disease is great, or environmental conditions promote the rapid loss of repellent from the surface of the skin (Fradin and Day, 2002).

1.2.2.2.4 Stereochemical effects of insect repellents

Field and laboratory studies have showed that racemic, 1-[3-cyclohexen-1-ylcarbonyl]-2-methylpiperidine (coded as AI3-37220 by The U.S. Department of Agriculture in many literatures) is as, or more, effective than the commonly used arthropod repellent, DEET in reducing biting from blood-sucking arthropods (Coleman *et al.*, 1993; Frances *et al.*, 1996; Walker *et al.*, 1996; Frances *et al.*, 1998). This compound contains two asymmetric centers and standard symmetric synthesis yield an equal mixture of four stereoisomers [rectus (R), sinister (S)], SR, SS, and RR (Klun *et al.*, 2001).

Quantitative mosquito bioassay using *Ae. aegypti* showed that (1S, 2'S) and (1R, 2'S) configurations were 2.8 – 3.1 and 1.6 – 1.8 times more effective, respectively than the other two isomers (1S, 2'R and 1R, 2'R) in reducing mosquito bites (Klun *et al.*, 2001). It is therefore, believed that enhanced repellent effect can be realized through the formulation of the most active stereoisomer of the compound (1S, 2'S) to further improve the efficacy against *Ae. aegypti*. This result further indicated that the repellent chemoreceptor system in *Ae. aegypti* accommodates the (2'S) moiety more effectively than (2'R). However, it is not well known that the stereochemical effects seen in this study and the (1S, 2'S) and (1R, 2'S) stereochemical specificity of this compound repellency observed in *Ae. aegypti* generalizes to other blood sucking arthropods or, if in other species, different stereoisomers will be more active. Hypothetically, the receptor binding sites responsible for repellency might not be the same in all blood-sucking arthropods. This information, however, is useful for designing analogous compounds with better or taxon-specific repellent properties. In another recent study (Barasa *et al.*, 2002), however, the four stereoisomers of p-menthane-3,8-diol were equally repellent against *An. gambiae s.s.*

1.2.2.2.5 Influence of infection status of mosquitoes on repellents

Limited studies have been carried out to address the effect of infection in mosquitoes on their behavioural response towards repellents (Robert *et al.*, 1991; Copeland *et al.*, 1995). The response of colonized *An. stephensi* Liston to repellent formulations of DEET and permethrin was independent of infection with either *Plasmodium falciparum* or *P. berghei* (Robert *et al.*, 1991). This species is characterized by low concentrations of salivary apyrase (Ribeiro *et al.*, 1984), and does not show infection-dependent differences in feeding behaviour (Li *et al.*, 1992). A field experiment in western Kenya (Copeland *et al.*, 1995) also showed that repellent formulations are equally effective against *Plasmodium* infected and uninfected populations of *An. funestus*. However, no data are available on the effect of infection in members of the *An. gambiae* complex to the behavioural response of the species towards repellents.

1.2.2.3 Formulations and release technology

Commercial insect repellents are formulated as aerosol and pump sprays, creams, lotions, solutions, gels, sticks, foams, and toweletes and contain DEET at concentrations ranging between 5% and 100% (Fradin, 1998). DEET has been also used in combination with 'thanka' a root paste made from pulp of the wood tree *Limonia acidissima* L. (Rutaceae) traditionally used as a cosmetic by Karen women in Thailand (Lindsay *et al.*, 1998). Bioassays using a laboratory strain of *Ae. aegypti* demonstrated that 'thanka' is itself slightly repellent at high dosage and the mixture with DEET provides protection for over 10 hours. The use of sustained-release technology with topical repellents has provided extended protection against biting mosquitoes in the laboratory (Gupta and Rutledge, 1989).

1.2.2.4. Concerns about the safety of DEET

Unfortunately, there is increasing concern about the safety of DEET-based repellents (Moody, 1989). This compound has been associated with bullous eruptions in the antecubital fossa and contact urticaria and toxic encephalopathy has occurred with excessive or prolonged use (at 75–90% concentrations for 3–4 weeks), particularly in infants and children (Edwards and Johnson, 1987). Furthermore, the ability to penetrate the skin is rapid in both humans and animals, and there have been concerns over its side effects (Miller, 1982; Roland *et al.*, 1985) that, on some occasions, have been resulted in death (Osmitz and Grothaus, 1995). Moreover, it can also damage certain plastics and synthetic materials (Coleman *et al.*, 1994). For these reasons, new compounds are screened continually for repellent actions. The ultimate goal of this process is to identify compounds that are safer and more effective against a broad range of arthropods than DEET.

1.2.2.5 Other modes of usage of insect repellents

1.2.2.5.1 Repellents on Fabric

Many repellents can be applied to clothing for protection against mites, ticks, and biting insects. Those that are or have been widely used in this manner include sulphur, dimethyl phthalate, dibutyl phthalate, benzyl benzoate and DEET (Gupta and Rutledge, 1994).

DEET or other volatile repellents can be absorbed into cotton fabric to form a reservoir that evaporates slowly to give long-term repellency with limited skin contact. Netting jackets impregnated with 0.25 g DEET per gram of netting give several weeks of protection from biting flies (Schreck *et al.*, 1979). Repellents applied to clothing usually retain their effectiveness longer than on skin because they adhere better to cotton and synthetic fibres since there is little loss from abrasion, absorption, or precipitation (Rozendal, 1997).

DEET-impregnated bed nets also provided complete protection up to four months against *Culex quinquefasciatus* and *Ae. aegypti* in Bangkok, Thailand (Gouck and Moussa, 1969). Mosquito nets and other types of fabric can also be treated with pyrethroid insecticides, such as permethrin, that might repel insects at a distance, and when in contact with the treated fabric, and irritate or kill them before they can feed. Pyrethroids are preferable to synthetic repellents as a fabric treatment because they act quickly to repel or kill biting insects, retain activity for several months even after laundering, are relatively safe to use if properly applied, and more effective at low concentrations (Schreck, 1977). When DEET-based repellents are applied in combination with permethrin-treated clothing, protection against bites of nearly 100% can be achieved (Fradin, 1998).

1.2.2.5.2 Mosquito coils and vaporizing mats

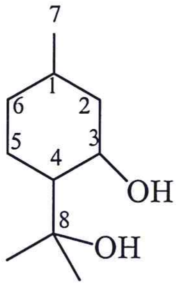
Mosquito coils contain various active ingredients such as pyrethrum, pyrethroids or even DDT, and show a wide range of efficacy, 22 – 87% (Chadwick, 1985; Birley *et al.*, 1987; Coene *et al.*, 1989; Ansari *et al.*, 1990; Mosha *et al.*, 1992), due to variable active ingredients or insecticides and because the effect depends on conditions in which the coil is used. However, some side effects such as smarting of the eyes, headaches and drowsiness have been reported by users (Curtis and Hill, 1988).

Another repellent device marketed in many parts of the world, but less so in Africa, is the vaporizing heater and mats consisting of a small piece of cardboard impregnated with a volatile pyrethroid. The mat is heated electrically causing the pyrethroid to evaporate. Reported protection ranges from 56% to 90.5% (Ansari *et al.*, 1990; Hewitt *et al.*, 1996). Although vaporizing mats are apparently among the most effective space repellents, they are relatively expensive, and their use is restricted by the requirements for electricity.

1.2.3. NATURAL OR PLANT-BASED REPELLENTS

Plant based repellents have the advantage that they can be produced locally, reducing their cost, and may help to boost the local economy. They may also be more culturally acceptable in communities with a tradition of plant use, where synthetic chemicals may be perceived as unhealthy or unpleasant smelling. Plant derived natural products have been traditionally used as repellents for biting insects in many parts of the world. The potential of plant derived repellents for the control of disease vectors has been extensively reviewed by (Curtis *et al.*, 1991). The essential oils of various plants have been shown to have mosquito-repellent properties. These oils, and their mixtures were the bases of many commercial repellents in the past (Granett, 1940).

In the search for effective alternatives to DEET, in Tanzania, *Eucalyptus*-based insect repellent (quwenling) with the principal active ingredient p-methane-3, 8-diol **(II)** showed comparable efficacy and duration of protection to DEET against *An. gambiae* and *An. funestus* (Trigg, 1996). Quwenling, was discovered in China from the waste distillate after the extraction of essential oils of Lemon eucalyptus (*Corymbia citriodora*). It is repellent to mosquitoes, biting midges, and tabanids. In China its use has displaced the use of dimethyl phthalate (Curtis *et al.*, 1991). Though the active ingredients of quwenling is said to be p-methane-3, 8-diol (PMD) it has also *iso*-pulegol, citronellol and citronellal (Collins *et al.*, 1993; Barasa *et al.*, 2002). The repellent product is formulated as a patented mixture of isomers of each component, the repellent effect of which is more persistent than citronella and nearly equal to that of DEET (Trigg and Hill, 1996). However, very recently oil of eucalyptus currently marketed in the USA under two names: "Repel Lemon Eucalyptus Insect Repellent (WPC Brands)" and "Fite Bite Plant-based Insect Repellent (Travel Medicine)" tested against laboratory reared *Ae. aegypti* and showed complete protection for only 2 hours as compared to 6 hours protection time of 23% DEET (Fradin and Day, 2002). The active ingredient of this product is p-menthane-3-8- diol (Fradin, personal communication).



(II)

In laboratory tests in the USA (Barnard, 1999) thyme (*Thymus vulgaris* L.) and clove (*Syzygium aromaticum* L.) oils provided 1 ½ to 3 ½ hours of protection depending on oil concentration against *Ae. aegypti*. Clove oil combined with geranium oil or with thyme oil with equal proportion prevented biting by *An. albimanus* for 1 ¼ to 2 ½ hours. It was found that eugenol, eugenol-acetate, and β-caryophyllene are the major constituents of clove oil that has shown better repellency than DEET against *An. albimanus* (Barnard, 1999). Eugenol is known for its repellent property against *An. gambiae*. However, neither eugenol acetate nor β-caryophyllene is repellent to *Ae. aegypti*, and neither have been tested for repellency against *Anopheles* mosquitoes (Barnard, 1999).

Citronella oil has a lemony scent and was originally extracted from *Cymbopogon nardus* (Fradin, 1998). Ceylon and java types of citronella oil contain citronella, geraniol and citronellol. Java-type is generally considered superior to ceylon oil (Barnard, 1999). Citronella oil is known to be generally less repellent to mosquitoes than dimethyl phthalate or DEET (Barnard, 2000). However, citronella oil, in concentrations ranging from 0.05 to 15% is used alone or in combination with cedar wood, lavender, peppermint, clove, eucalyptus, and garlic in a number of commercial insect repellent products (Fradin, 1998).

1.2.3.1 Traditional use of repellents

The traditional ways of using insect repellents in the community can overcome cost related production problems and formulation. Moreover, their applications are simple and could be used by the community with minimal external help. However, little attention has been given to assess the effectiveness of the traditional ways of using repellent plants for use in integrated vector management program against disease vectors (Curtis *et al.*, 1991).

In many parts of the world, indigenous people have historically relied on locally sourced natural plant-based traditional remedies to repel or prevent biting insects entering their homes. The practice of 'smoking' houses to deter mosquito biting is widespread in rural communities in tropical Africa (Snow *et al.*, 1987; Bockarie *et al.*, 1994; Palsson and Jaenson, 1999; Seyoum *et al.*, 2002a). In China, the burning of certain herbs, such as *Artemisia* and *Calamus* species is practised in remote villages to banish mosquitoes and protect cattle from blood-sucking insects (Hwang *et al.*, 1985). In East Africa, *Ocimum basilicum* has traditionally been used in Luo community to drive away mosquitoes by laying the branches in the house (Kokwaro, 1976). In a Northern Tanzania village (Curtis *et al.*, 1991) more than two thirds of women reported using some kind of natural insect repellent. The most used repellents were one or other of basil-like herbs (*Ocimum* spp. and *Hyptis suaveolens*), while a few women used leaves of neem tree (*Azadirachta indica*). In The Gambia, the woods and resins of aromatic trees are widely sold in the markets under collective term *Churai* to be burned as mosquito repellents (Curtis *et al.*, 1991).

White (1973) demonstrated the repellent effects of the juice of *Ocimum americanum* and *O. suave* on *An. gambiae* in Africa. It was found that biting-rates of host-seeking *An. gambiae* mosquitoes were halved on treated skin using plant juices smeared on skin. This provides experimental rationale for traditional use of such plants in different ways in many African countries against mosquitoes. There have been also interesting information on the traditional use of *O. americanum* in Prachuabkirikhan province in Thailand. The people wear young shoots of this plant behind their ear to repel insects while working in the fields. In this area, haemorrhagic fever transmitted by *Ae. aegypti* is one of the most important public health problems (Chokechaijaroenporn *et al.*, 1994).

In Sierra Leone, Bockarie *et al.* (1994) reported that smoke from household presumably from *Daniella oliveri* Rolfe (Caesalpiniaceae) make rooms unfavourable for resting *An. gambiae* mosquitoes and cause most of them to leave rooms before dawn. However, smoke does not appreciably affect the feeding success of mosquitoes once they have entered a room, as shown by most of the mosquitoes that left the 'smoke' room having taken a blood meal. It is unclear from this study why most mosquitoes left the smoke-filled room soon after feeding.

Recently, Pallsson and Jaenson, (1999a, b) investigated the traditional use of mosquito repellent plants among communities in Guinea Bissau and evaluated their repellency under field conditions. It was found that fresh smouldering of *H. suaveolens* Poit. (Lamiaceae), smoke of the bark of *D. oliveri*, the inflorescence of *Elaeis guineensis* Jacq. (Arecaceae), the seed capsules of *Parkia biglobosa* Jacq. (Mimosaceae), the leaves of *A. indica* A. Juss. (Meliaceae) and *Eucalyptus* sp. (Myrtaceae), fresh *O. canum* Sims (Lamiaceae), and fresh *Senna occidentalis* Link (Caesalppiniaceae) have been traditionally used as mosquito repellents in the communities. Field evaluations showed that all plant species except *S. occidentalis* significantly repelled the malaria vectors in the area.

1.3 JUSTIFICATION OF THE STUDY

Previous studies have concentrated mainly on simple solutions of topical synthetic repellents to prevent the bites of blood sucking arthropods. The use of traditional repellents is not only widespread among the different cultures, but also may be very common within communities. However, little attention has been given to the development of plant-based traditional repellents for integrated management of malaria vectors. The present study aimed at quantifying and understanding the scientific rationale for some of the traditional uses of plants, and evaluating more effective and sustainable methods for integrated management of African malaria vectors in Eastern Africa.

1.4 HYPOTHESES OF THE STUDY

(i) Traditional use of mosquito repellent plants has no impact to reduce domestic exposure of humans to *An. gambiae s.l.*

(ii) Method of use of traditional mosquito repellents cannot be improved to substantially reduce domestic exposure of humans to *An. gambiae s.l.*

(iii) Terpenoids-emitting live, intact potted plants have no impact to protect humans against host-seeking African malaria vectors.

(iv) The effectiveness of essential oils of plant samples collected in day time is not different from plant sampled collected at night against *An. gambiae s.s.*

1.5 OBJECTIVES OF THE STUDY

1. 5.1 General objective

The main objective of this study was to evaluate simple and effective alternative methods of repelling malaria vectors using selected repellent plants that are suitable for use by the rural communities in Eastern Africa.

1.5.2 Specific objectives

(i) To identify mosquito repellent plants based on ethnobotanical and other empirical information, and determine the repellency by thermal expulsion and direct burning against *An. gambiae* s.s in semi-field experimental huts.

(ii) To determine the repellency of intact potted plants against *An. gambiae* *s.s.* in semi-field experimental huts.

(iii) To determine the efficacy of two different application methods of repellent plants, thermal expulsion and intact potted plants in the reduction of African malaria vectors under natural field condition.

(iv) To establish the repellency of essential oils of plant samples taken during different times of the day against *An. gambiae* s.s.

(v) To determine the major components of volatiles from intact potted repellent plants, by thermal expulsion and direct burning of repellent plant materials.

CHAPTER 2

GENERAL MATERIALS AND METHODS

2.1 Mosquito colony and rearing

Semi-field tests (Chapters 3 and 4) on the repellency of nine candidate plants and various combinations were carried out with laboratory-reared *An. gambiae s. s.* maintained at the Mbita Point Research and Training Centre, the International Centre of Insect Physiology and Ecology (00°25'S, 34°13'E). The colony was originally initiated from adults collected in 1996 at Njage village (70 km from Ifakara) southeast Tanzania.

The adults were maintained at ambient temperature and humidity in standard 30cm × 30cm × 30cm mosquito cages made up of mosquito netting and metal frames. The females were routinely fed on human arm for 10 minutes three times a week and 6 % glucose solution (for both females and males) soaked in filter paper wicks. The eggs were collected on egg laying cups with filter paper disks (9 cm diameter) and transferred to plastic pans with fresh lake water (from Lake Victoria). The mosquito larvae were reared under ambient temperature and light conditions in screen house insectaries. The larvae were reared using fresh lake water and were fed daily on Tetramin® (fish food) and periodically transferred to fresh lake water. Upon pupation, insects were transferred to cages for emergence. Throughout the semi-field experiments (Chapters 3 and 4), 5-7 days old female mosquitoes, which had never received a blood meal, were starved for six hours before experimental use.

2.2 Mosquito sampling

2.2.1 Human landing catch

Human landing catch according to WHO (1975) was used for sampling mosquitoes in experimental huts for semi-field evaluation of candidate plants for repellency against *An. gambiae* s.s. (Chapters 3 and 4). The human volunteers sat at the middle of the huts (control and treatment huts) and exposed their legs from knee to ankle to collect host-seeking mosquitoes (laboratory reared and infection free) released at the center of the screen house (Chapters 3 and 4). The volunteers washed their legs and dried before the start of each experiment. This was done to minimize effect in differential attractiveness of the collectors to mosquitoes. The mosquitoes were collected using mouth aspirators and kept in separate labelled paper cups for each individual, experimental huts and time of collection (20.00 – 24.00 hrs). The data was recorded in data sheets designed for this purpose (Appendix 2).

2.2.2 CDC light trap collections

Communicable Disease Control (CDC) miniature light traps (model 512, John W. Hock company, USA) set close to occupied untreated bed nets inside bedrooms were used for sampling mosquitoes under natural field condition to estimate the reduction of mosquito density in the treatment huts (with repellent plants) as compared to the reference control huts without plant treatments (Chapter 5).

2.2.3 Morphological identification and preservation

Anopheline mosquitoes collected from the field tests (Chapter 5) were identified to the species level using morphological characteristics (Gillies and De Meillon, 1968). The mosquitoes were also categorized into four gonotrophic stages (Bruce Chwatt, 1985) and recorded in data sheet. Samples of *Anopheles gambiae s.l.* were preserved in 95% ethanol for further identification to sibling species by Polymerase Chain Reaction (PCR).

2.3 PCR identification of *Anopheles gambiae* complex

Samples of the *An. gambiae* species complex collected from the field trials (Chapter 5) on the repellency of selected candidate plants by thermal expulsion and in intact potted form in Lwanda, western Kenya, were identified to sibling species using PCR following the protocol developed by Scott *et al.* (1993).

2.3.1 DNA Extraction

Whole mosquito samples were homogenized using pestles in 1.5 ml eppendrof tube containing 100 µl of grinding buffer, and incubated in water bath at 65 °C for 30 minutes. In each sample 14 µl of 8 M potassium acetate was added and incubated on ice for 20 minutes. The samples were then centrifuged for 10 minutes at 13000 rpm. The supernatants were then pipetted out and transferred to corresponding 1.5 ml tubes containing 95% ethanol, and incubated at –70°C for 2 – 3 hours or at –20 °C overnight. The samples were span at 13000×g for 20 min, the supernatant decanted and washed with 200 µl of 70 and

95 % ethanol and spinned for 10 and 5 min, respectively. The supernatants were discarded and the DNA samples were air dried and suspended in 100 µl of Tris-EDTA buffer pH 8.0 (Scott *et al.*, 1993).

2.3.2 Polymerase Chain Reaction

Extracted DNA template was suspended into 100 µl of tris-EDTA. The PCR reaction mix was prepared in a total volume of 14 µl as follows: 10 X PCR buffer (1.5 µl), 5mM of dNTP-mix (0.6 µl), 2.5 mM $MgCl_2$ (1.8 µl), Taq ^{TM} (0.075 µl), 12.5ng ml^{-1} universal primer (0.6 µl), 6.25 ng ml^{-1} *An. gambiae* primer (0.6 µl), 18.75 ng ml^{-1} *An. arabiensis* primer (0.6 µl), 12.5 ng ml^{-1} *An. meru* primer (0.6 µl), 25 ng ml^{-1} *An. quadriannulatus* primer (0.6 µl); 1 $mgml^{-1}$ BSA (1.5 µl), plus ddH_20 (5.525 µl).

PCR reaction mix (14 µl) was distributed into PCR tubes. DNA template of 1 µl was added to the corresponding PCR mix and labelled. The samples were loaded into PCR machine with the controls. The PCR products (15 µl of PCR product containing 3 µl of agarose dye) were then loaded into a 2.5 % w/v agarose gel. The PCR products with agarose dye were electrophorosed at 180 v in an electrophoresis unit containing TAE (running buffer), and stained with ethidium bromide for 20 minutes. The lanes were visualized and scored.

2.4 Data analyses

Logistic regression analysis was used to determine the significance of the effect due to plant treatment and other sources of variations using SAS 8.1 for Windows (SAS, 1999) for the semi-field evaluation of repellent plants (Chapter 3 and Chapter 4). The proportion of recaptured mosquitoes recovered in the treatment hut for each candidate plant was analysed by logistic regression. By considering only those mosquitoes that actually chose to bite in a given 30 min period, probability of mosquitoes biting in the treatment hut was modelled as a proportion of the total caught in the treatment and control huts. A forward stepwise selection procedure was used to fit the model, including only parameters reflecting other potential sources of variation, that were found to be significant ($P < 0.05$). The list of parameters from which these were chosen includes arrangement of huts, arrangement of collectors, replicates and time. Standard Errors of the Means (SEM) and 95% confidence intervals (CI) of the fitted parameters were estimated by maximum likelihood analysis. The percentage repellency (R) was calculated using the proportions of the total catch in the control P(C) and treatment huts P(T) (Sharma and Ansari, 1994),

$$R\ (\%) = 100 \times (P(C) - P(T))/P(C) \qquad \mathbf{1}$$

Given that all the mosquitoes recaptured either chose the treatment or control hut:

$$P(C) = 1 - P(T) \qquad \mathbf{2}$$

Substituting into **1**:

$$R = 100 \times (1 - 2P(T))/\ (1 - P(T)) \qquad \mathbf{3}$$

Repellency was estimated using the parameter estimate of the intercept (β_0). By definition (Collett, 1991), β_0 is the natural log of the treatment over the control:

$$\beta_0 = \ln (P(T)/1-P(T)) \qquad \mathbf{4}$$

Which by rearrangement (Collett, 1991), yields:

$$T = \exp (\beta_0)/ (1 + \exp (\beta_0)) \qquad \mathbf{5}$$

Mean repellency and confidence intervals for that estimate were therefore calculated by substituting **5** into **3**, using the estimates for β_0 and its confidence intervals respectively, as derived from logistic regression analysis.

2.5 Gas chromatography analysis of volatile compounds

The chemical profile of the volatiles emitted from intact potted repellent plants, thermally expelled and directly burnt repellent plant materials (Chapter 7) were initially obtained on gas chromatograph (GC). Nitrogen gas was used as carrier gas. Methyl silicone capillary column (Hewlett Packard 50-m long, 0.22-mm id) was used. Two micro liters of samples were injected into injection port of GC. The column temperature program comprised of an initial temperature of 50°C (5 min), a rise to 280° C at 5°C/min and final hold at 280°C for 20 or 40 min.

2.5.1 Gas chromatography-mass spectrometery (GC-MS)

The constituents of volatiles from potted plants of *O. americanum*, and thermal fumigation and direct burning of plant materials (*C. citriodora*, *O. kilimandscharicum* and *O. suave*) were analysed on a Fisons VG-platform II mass spectrometer (MS) coupled to Fisons GC 8060 series II gas chromatograph (GC) equipped with data system linked to Hewlett Packard 5790 gas chromatograph (GC). The GC parameters comprised of HP pana cross linked methyl silicone capillary column with 50ml x 6.2 mm internal diameter (id) and 0.5 μm film thickness. Helium was the carrier gas and the flow rate was 0.84 m/min. The injection port temperature was 270°C and the detector (ion solute) temperature was 180°C. The column temperature programme that was used for the GC analysis was also used for GC-MS. The mass spectrometer parameters comprised of EI ion mode, 70 ev ion energy, with ion source temperature 180°C. The MS library was NIST (National Institute of Science and Technology) published by J. Willey publishers. Control of GC-MS software is Mass Lynx NT.

The identity of major constituents were confirmed by comparison of the retention times and co-injections with reference compounds (as suggested by GC-MS) on a Hewlett Packard 5890A gas chromatograph equipped with splitless injector and flame ionization detector. The standards were purchased from Sigma-Aldrich Co. Ltd., UK.

CHAPTER 3

ETHNOBOTANICAL STUDIES ON TRADITIONAL USE OF MOSQUITO REPELLENT PLANTS IN WESTERN KENYA AND THEIR EVALUATION BY THERMAL EXPULSION AND DIRECT BURNING AGAINST *ANOPHELES GAMBIAE* IN SEMI-FIELD EXPERIMENTAL HUTS[*]

3.1 INTRODUCTION

In many parts of the world, plant-derived natural products have traditionally been used as repellents against insects, primarily to avoid nuisance biting (Curtis *et al.*, 1991). By reducing human-vector contact, repellents can also be considered as important tools in the prevention of vector-borne diseases (Gupta and Rutledge, 1994). Although synthetic repellents have been studied extensively, little effort has been made to investigate and promote the traditional use of plant-derived natural repellents. Moreover, in contrast with synthetic repellents and commercial products that incorporate them, traditional ways of using insect repellents are usually simple, cost-effective and accessible to communities with minimal external input.

The use of traditional repellents is widespread among the different cultures and communities of Africa and beyond (Snow *et al.*, 1987). In spite of the

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widespread traditional use of mosquito repellent plants by rural communities in the developing world only limited studies have been carried out to demonstrate their impact on human contact with disease vectors. Recent studies in Guinea Bissau, West Africa, showed that smoke produced by burning of the shrub *H. suaveolens* and the bark of the tree *D. oliveri* indoors at night significantly repelled endophagic mosquitoes (Palsson and Jaenson, 1999a; Palsson and Jaenson, 1999b). This study revealed that burning the bark of *D. oliveri* and *H. suaveolens* (whole plant, excluding the root) more effectively repelled *Mansonia* than *Anopheles* species. Similar differences in the sensitivity of mosquito species towards synthetic repellents such as diethyl methylbenzamide have also been widely documented (Rutledge *et al.*, 1978; Robert *et al.*, 1991). It is therefore important to evaluate natural repellents against target species involved in disease transmission, under conditions that simulate their routine application by communities, and avoid exposure to potentially infectious mosquito bites.

This study was conducted to identify candidate plants traditionally used by the community to repel mosquitoes on Rusinga Island and Rambira location, Western Kenya, and quantify the repellency of selected plants by periodic thermal expulsion and direct burning against *Anopheles gambiae* Giles in experimental huts in an exposure-free semi-field environment.

3.2 MATERIALS AND METHODS

3.2.1 Study areas

Ethnobotanical studies on the traditional use of mosquito repellent plants were carried out among communities on Rusinga Island (0.24 S and 34.10 E) in Lake Victoria, and Rambira location around Kendu Bay (0.22 S and 34.39 E), Western Kenya between May and July, 2000. Both communities belong to the Luo ethnic group, and the predominant economic activities are fishing and farming with the main crops being maize and millet. The rainy seasons are from March through May and October to November, and the vegetation type is bushy shrub in both locations. Most of the houses are made of mud and wattle walls roofed with corrugated iron sheets.

3.2.2 Interviews

Interviews were undertaken in a total of twenty-seven villages in seven sub-locations of Rusinga Island (n = 195) and Rambira location (n = 197) (Table 3.1). One adult from each one of these 392 households was interviewed on the traditional uses of repellent plants using a pre-tested questionnaire specifically designed for this purpose (Appendix 1). The questionnaire was prepared in English and translated to Dholuo (the native language) during the interview. Male and female respondents from all age groups were included in the survey. The main questions focused on 1) usage and knowledge of insect repellent plants, 2) names of plants used or known, 3) insects against which the plants are used, 4) methods of application, and 5) parts of the plant material used as mosquito repellent.

3.2.3 Mosquito species and candidate plants

Semi-field tests on the repellency of eight candidate plants and various combinations thereof were carried out with laboratory-reared *An. gambiae s. s.* maintained at the Mbita Point Research and Training Centre, ICIPE. Leaves of *Corymbia citriodora* Hook (Myrtaceae) previously referred to as *Eucalyptus citriodora* Hook (Myrtaceae), *Lippia ukambensis* Spreng (Verbenaceae), *Lantana camara* L. (Verbenaceae), and *Azadirachta indica* A. Juss; leaves and seeds of *Ocimum americanum* L.(Labiatae), *Ocimum kilimandscharicum* Guerke (Labiatae), and *Ocimum suave* Willd. (Labiatae); seeds of *L. camara* and leaves and flowers of *H. suaveolens* (Lamiaceae) were tested for their repellency by periodic thermal expulsion or direct burning in experimental huts inside a screen-walled greenhouse (see Figure 3.1). The plant species where selected based on ethnobotanical information and the literature. Furthermore, the combined effect of two different plant combinations, *L. camara* (leaves) combined with *O. kilimandscharicum* (seeds and leaves), *L. ukambensis* (leaves) combined with *O. kilimandscharicum* (seeds and leaves), and seeds and leaves of *O. suave* combined with *O. kilimandscharicum* were tested for possible additive/ synergistic effects.

Table 3.1 Study villages included in the ethnobotanical study on Rusinga Island and Rambira location, western Kenya

Location	Sub location	No.of villages	No. of respondents
	Wanyama	5	53
Rusinga	Kaswanga	6	81
West	Kamasengre (East)	3	34
	Kamasengre (West)	3	27
	4	17	195
Rambira	Kagwa	4	95
	Kamser-seka	2	74
	Karabondi	4	31
	3	10	197
TOTAL	7	27	392

3.2.4 Semi-field tests on the repellency of plants

Two similar experimental huts were constructed inside each of two screen-walled greenhouses (11.5m × 7.1m) lined with a mosquito net barrier along the walls on the inside at the Mbita Point Research and Training Centre, ICIPE, for evaluating the repellency of candidate plants (Figure 3.1). The roofs of the greenhouses were covered with glass and the sides with mesh (density 90%). A layer of reed mats was placed beneath the roof so as to lower temperatures. The huts were rectangular (3.2m × 2.7m) and made of hard board (plywood), internally painted with white paint. The roofs were covered with black cloth inside, and reed mats outside. The huts have a single door each and no windows. Eaves (height 10 cm) all around provide ventilation and serve as the only entry point for mosquitoes during experimentation.

Traditional stoves, locally known as "Jiko" (Plate 3.1), were modified and used for burning and thermal expulsion of the plants in experimental huts. Two hundred and fifty grams of charcoal was used to light the stove and subsequently supplemented with 150 g of charcoal after 2 hrs (half-way through the experiment), to burn or thermally expel the plant materials. Direct burning was carried out by placing plant material directly on the burning charcoal (Plate 3.1 B) whereas it was thermally expelled by placing it on a thin (<0.5 mm) metal plate directly above but not in contact with the charcoal (Plate 3.1 A). Every 30 minutes, 5 g of fresh plant material was placed on the flame of the charcoal (direct burning) or on the upper plate of the stoves (thermal expulsion) in the treatment huts. The control huts were treated only with burning charcoal to which no plant material was added. Each test night, 150 mosquitoes were released from a paper cup at the center of screen house, midway between the 2 experimental huts at 20.00 hours and recaptured by human landing catch in both huts up to mid night.

The two persons involved in the human landing catch were exchanged between huts every 30 minutes in order to offset any personal difference in attractiveness and collection skill. Experiments for each plant species were replicated four times on four different nights by changing the assignments of the two huts (the one served as treatment hut changed into the control at different nights) and by exchanging the person to start in each hut on different nights. The possible residual effect of the smoke of the plant materials was also checked before changing the assignments of the two huts as treatment or control by comparing the two huts on subsequent nights without any charcoal burning or plant use.

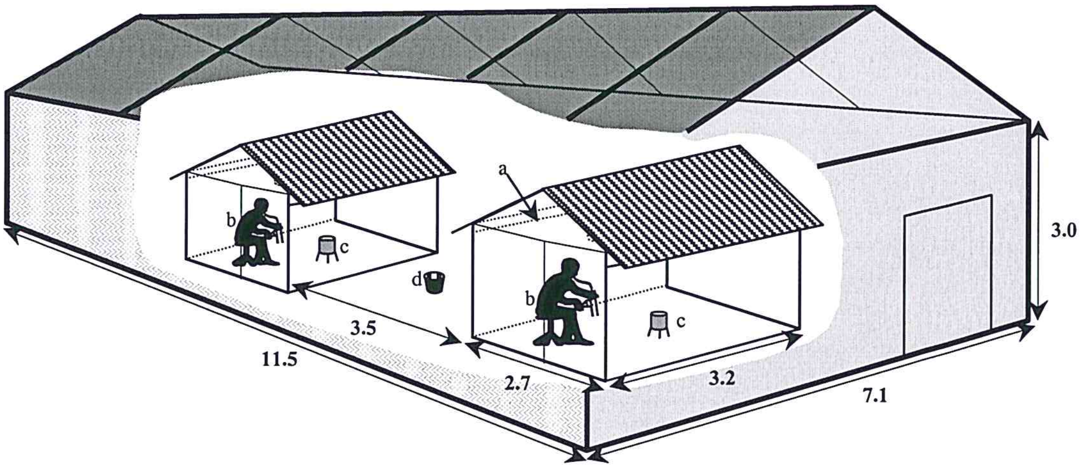

Figure 3.1 Semi-field experimental set up for testing the repellency of plants by thermal expulsion and direct burning. Experimental huts with eaves (a) were constructed inside a screen-walled greenhouse, within which human baits (b) and traditional stoves (c) were placed. Mosquitoes were released from a paper cup (d) placed half way between the huts. Numbers describe dimensions in meters.

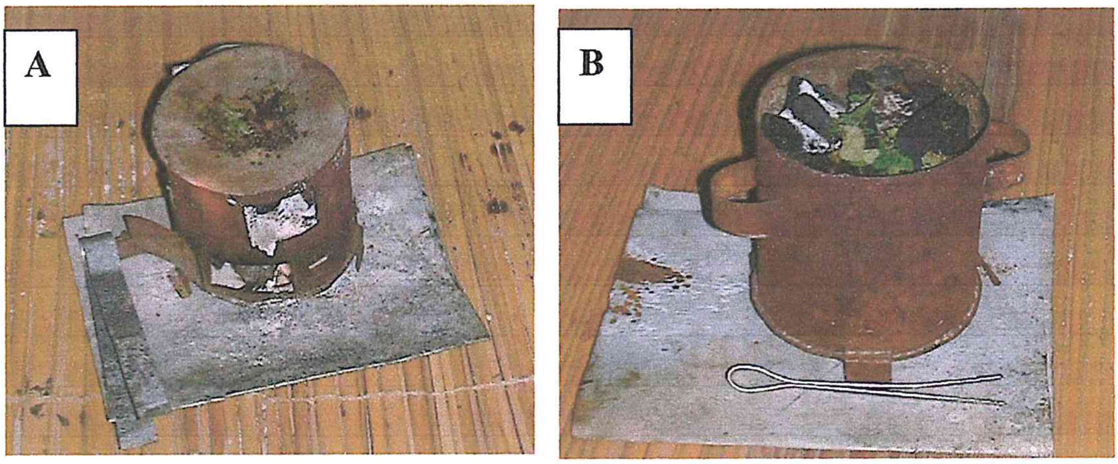

Plate 3.1 Traditional stove ('Jiko') used for thermal expulsion (A) or direct burning (B) of the repellent plant materials. Note the thin metal plate covering the charcoal for thermal expulsion. The stoves are 17 cm in diameter and 15 cm high (excluding legs).

3.2.5 Data analyses

Data analyses were carried out using SAS 8.1 for windows. The proportion of recaptured mosquitoes recovered in the treatment hut for each candidate plant was analysed by logistic regression as described in Chapter 2.

3.3 RESULTS

The ethnobotanical studies (Table 3.2) revealed that the most commonly used and known repellent plant on Rusinga Island is *O. americanum*, locally known as "Mweny", followed by *L. camara*, *T. minuta*, L. and *A. indica*. *Hyptis* *suaveolens* is the most commonly used and known plant around Rambira location followed by *L. camara* and *O. basilicum*. *Ocimum americanum, L. camara* and *O. basilicum* are mainly used by burning plant material once each night. Overall, cutting the branches or whole plants and placing these inside the house or bruising the leaves of the plants and hanging them around the beds are the main methods of application for *H. suaveolens, A. indica* and *T. minuta.* Although traditional use of several species of plants as mosquito repellents is very common in these communities, considerable proportions of the population know about but do not use them.

The parts of plants used appeared to depend on the size of plant species. Whole plants being used for the small herbs whereas, branches, leaves and seeds of plants mainly for shrubs and trees as mosquito repellents (Tables 3.3). The repellency of plants by periodic thermal expulsion and direct burning is shown in Tables 3.4 and 3.5 respectively. All species except *H. suaveolens* showed significant repellency ($P<0.05$) by thermal expulsion and the highest repellency was exhibited by the leaves of *C. citriodora* (74.5%, $P<0.0001$), followed by leaves and seeds of *O. suave* (53.1%, $P<0.0001$) and *O. kilimandscharicum* (52%, $P<0.0001$). Leaves of *C. citriodora* also exhibited the highest repellency (51.3%, $P<0.0001$) by direct burning followed by leaves of *L. ukambensis* (33.4%, $P=0.0004$) and leaves plus seeds of *O. suave* (28%, $P=0.0255$). Although no major synergistic effect was observed the combinations of either *O. kilimandscharicum* with *L. ukambensis* by thermal expulsion or with either

L. camara or *L. ukambensis* by direct burning was as effective as either plant alone (Tables 3.4 and 3.5). Combining *O. kilimandscharicum* with either *L. camara* or *O. suave* by thermal expulsion failed to repel mosquitoes (4.6%, P=0.6091 and 14.9%, P=0.0979, respectively) even though each plant was effective on its own (Table 3.4). The combination of *O. kilimandscharicum* and *O. suave* by direct burning actually resulted in an overall increase (-24.4%, P=0.0212) in exposure to mosquitoes (Table 3.5).

The results obtained from these experimental hut trials showed that thermal expulsion of the plant materials was found to be the better method of application to repel host-seeking *An. gambiae*. When thermally expelled, all plant species except *H. suaveolens* reduced exposure to biting mosquitoes whereas three of them failed to do so when directly burned. The distribution of mosquitoes in test and control huts was also influenced by the arrangement of huts, the person carrying out the human-landing catch, replicate and time (Tables 3.6 and 3.7). Despite substantial variability due to other sources of variation, the cross-over design allowed treatment effects to be detected and were included in the logistic model estimating repellency.

Table 3.2 Traditional use of repellent plants, knowledge about them and methods of application in communities on Rusinga Island (n = 195) and Rambira location (n = 197), Western Kenya

	Rusinga					Rambira				
Plant species	Usage (%)	Knowledge** (%)	Method of use (%)			Usage (%)	Knowledge (%)	Method of use (%)		
			Burning	Natural*	Both			Burning	Natural*	Both
Azadirachta indica	7.7	8.7	33.3	66.7	0	0	3.5	0	100	0
Bidens pilosa L.	1.0	2.0	100	0	0	1.0	2.0	100	0	0
Capsicum frutescens L.	1.0	1.0	100	0	0	0	0	0	0	0
Cassia occidentalis L.	0	0	0	0	0	3.0	4.0	100	0	0
Eucalyptus saligna Sm.	0	0	0	0	0	2.0	8.1	100	0	0
Hyptis suaveolens	1.5	3.0	66.7	33.3	0	25.4	49.2	30.0	57.8	12.2
Lantana camara	7.7	17.9	100	0	0	15.7	30.9	100	0	0
Ocimum americanum	32.8	64.1	68.8	20.3	10.9	0	0	0	0	0
Ocimum basilicum	0	0	0	0	0	12.7	30.4	58.8	31.9	9.3
Psiadia punctulata Dc.	0	3.0	100	0	0	2.0	2.0	100	0	0
Schkuhria pinnata Lam.	5.1	8.6	90.9	9.1	0	5.6	7.1	94.7	5.3	0
Tagetes minuta	4.1	11.3	35.5	54.8	9.7	8.1	11.6	36.0	56.0	8.0
Others (6 species)	1.0	3.5	50.0	50.0	0	7.5	15.5	50.0	50.0	0

* Placing branches or whole plants inside houses

** Refers to percentage of the total who knows but do not use repellent plants

Table 3.3 Parts of the plants used as mosquito repellents among communities in Rusinga & Rambira locations

	Rusinga			**Rambira**		
Plants name	whole plant	leaves	branches	whole plant	leaves	branches
A. indica	0	60.0	40.0	0	0	0
B. pilosa	100	0	0	0	0	0
C. occidentalis	0	0	0	80.0	0	20.0
E. saligna	0	100	0	0	100	0
H. suaveolens	100	0	0	79.6	8.2	12.2
L. camara	0	38.5	61.5	0	45.2	54.8
O. americanum	67.2	7.8	25.8	0	0	0
O. basilicum	0	0	0	88.0	4.0	8.0
P. punctulata	0	0	0	0	33.3	66.7
R. chalepensis	100	0	0	0	0	0
S. pinnata	40.0	0	60.0	100	0	0
T. minuta	75.0	25.0	0	68.8	12.5	18.8

Table 3.4 The repellency of plants against *Anopheles gambiae s.s.* by periodic thermal expulsion in semi-field experimental huts

Plant species	Plant parts	No. recaptured*		Intercept Parameter estimate ($\beta_o \pm$ SEM)[a]	% Repellency (95% CI)	*P*
		Treatment	Control			
1. *Azadirachta indica*	Leaves	144	176	-0.2817 ± 0.1192	24.5 (4.2, 40.6)	0.0181
2. *Corymbia citriodora*	Leaves	110	402	-1.3681 ± 0.1160	74.5 (67.9, 79.8)	<0.0001
3. *Hyptis suaveolens*	Leaves & flowers	265	236	0.1252 ± 0.0911	-13.3 (-36.0, 5.5)	0.1691
4. *Lantana camara*	Leaves	141	245	-0.5525 ± 0.1057	42.4 (28.9, 53.4)	<0.0001
5. *Lantana camara*	Seeds	187	241	-0.3423 ± 0. 1040	29.1 (12.7, 42.4)	0.0010
6. *Lippia ukambensis*	Leaves	117	229	-0.6145 ± 0.1198	45.9 (31.3, 57.4)	<0.0001
7. *Ocimum americanum*	Leaves & Seeds	129	225	-0.5635 ± 0.1116	43.1 (28.8, 54.5)	<0.0001
8. *Ocimum kilimandscharicum*	Leaves & Seeds	115	233	-0.7344 ± 0.1162	52.0 (39.5, 62.0)	<0.0001
9. *Ocimum suave*	Leaves & Seeds	112	204	-0.7566 ± 0.1683	53.1 (34.3, 66.9)	<0.0001
4 + 8		240	251	-0.0468 ± 0.0916	4.6 (-14.6, 20.5)	0.6091
6 + 8		172	352	-0.7951 ± 0.1007	54.8 (44.8, 63.1)	<0.0001
9 + 8		220	261	-0.1613 ± 0.0974	14.9 (-3.4, 30.0)	0.0979

* Total caught during 4 test nights, SEM = Standard Error of the mean, [a] see data analysis section in chapter 2

Table 3.5 The repellency of plants against *Anopheles gambiae s.s.* by periodic direct burning in semi-field experimental huts

Plant species	Plant parts	No. recaptured*		Intercept Parameter estimate ($\beta_o \pm$ SEM)[a]	% Repellency (95% CI)	*P*
		Treatment	Control			
1. *Azadirachta indica*	Leaves	137	151	0.0039 ± 0.1280	0 (-29.7, 22.3)	0.9760
2. *Corymbia citriodora*	Leaves	174	393	-0.7193 ± 0.1115	51.3 (39.1, 61.0)	<0.0001
3. *Hyptis suaveolens*	Leaves & flowers	220	282	-0.2337 ± 0.0932	20.8 (4.6, 34.3)	0.0122
4. *Lantana camara*	Leaves	143	170	-0.1886 ± 0.1154	17.2 (-4.3, 34.3)	0.1023
5. *Lantana camara*	Seeds	214	195	0.1290 ± 0.1018	-13.8 (-39.5, 7.2)	0.2051
6. *Lippia ukambensis*	Leaves	159	240	-0.4067 ± 0.1142	33.4 (16.3, 47.0)	0.0004
7. *Ocimum americanum*	Leaves & seeds	185	228	-0.2348 ± 0.1022	20.9 (3.0, 35.5)	0.0215
8. *Ocimum kilimandscharicum*	Leaves & seeds	145	209	-0.3064 ± 0.1137	26.4 (7.6, 41.4)	0.0070
9. *Ocimum suave*	Leaves & seeds	109	165	-0.3284 ± 0.1470	28.0 (3.4, 46.3)	0.0255
4 + 8		215	267	-0.2262 ± 0.0946	20.2 (3.6, 34.0)	0.0168
6 + 8		220	313	-0.3526 ± 0.0880	29.7 (16.2, 41.1)	<0.0001
9 + 8		272	224	0.2183 ± 0.0947	-24.4 (-50.3, -2.9)	0.0212

* Total caught during 4 test nights, SEM = Standard Error of the Mean, [a] see data analysis section in chapter 2

Table 3.6 Sources of variation affecting host choice, other than plant treatment by thermal expulsion, as determined by logistic regression

Plant species	Plant parts	Hut location		Person		Replicate		Time	
		P	OR[a]	*P*	OR[b]	*P*	OR[c]	*P*	OR[d]
1. *Azadirachta indica*	Leaves	NS	NA	NS	NA	0.0001	2.266	NS	NA
2. *Corymbia citriodora*	Leaves	0.0003	2.282	<0.0001	2.719	NS	NA	NS	NA
3. *Hyptis suaveolens*	Leaves & flowers	NS	NA	NS	NA	0.0191	2.168	NS	NA
4. *Lantana camara*	Leaves	NS	NA	NS	NA	NS	NA	NS	NA
5. *Lantana camara*	Seeds	NS	NA	NS	NA	<0.0001	3.838	NS	NA
6. *Lippia ukambensis*	Leaves	NS	NA	NS	NA	0.0006	1.754	NS	NA
7. *Ocimum americanum*	Leaves & seeds	0.0135	0.576	NS	NA	NS	NA	NS	NA
8. *Ocimum kilimandscharicum*	Leaves & seeds	0.0361	0.614	NS	NA	NS	NA	NS	NA
9. *Ocimum suave*	Leaves & seeds	NS	NA	NS	NA	0.0038	2.351	0.0566	3.587
4 + 8		NS	NA	0.0002	1.980	NS	NA	NS	NA
6 + 8		NS	NA	<0.0001	2.940	0.0009	1.690	NS	NA
9 + 8		NS	NA	0.0006	1.960	<0.0001	4.589	NS	NA

OR = Odds ratio, [a] Hut 1 versus Hut 2, [b] Person 1 versus Person 2, [c] Greatest difference between replicates, [d] Greatest difference between 30 minutes time intervals, NS = Not significant, NA = Not applicable

Table 3.7 Sources of variation affecting host choice, other than plant treatment by direct burning, as determined by logistic regression

Plant species	Plant parts	Hut location		Person		Replicate		Time	
		P	OR[a]	*P*	OR[b]	*P*	OR[c]	*P*	OR[d]
1. *Azadirachta indica*	Leaves	NS	NA	NS	NA	0.0026	3.470	NS	NA
2. *Corymbia citriodora*	Leaves	NS	NA	0.0047	0.575	NS	NA	<0.0001	5.247
3. *Hyptis suaveolens*	Leaves & flowers	NS	NA	NS	NA	<0.0001	3.792	NS	NA
4. *Lantana camara*	Leaves	0.0024	2.015	NS	NA	NS	NA	NS	NA
5. *Lantana camara*	Seeds	<0.0001	2.376	NS	NA	NS	NA	NS	NA
6. *Lippia ukambensis*	Leaves	NS	NA	NS	NA	<0.0001	1.250	0.0058	4.024
7. *Ocimum americanum*	Leaves & seeds	NS	NA	NS	NA	0.0005	3.398	NS	NA
8. *Ocimum kilimandscharicum*	Leaves & seeds	NS	NA	NS	NA	0.0005	3.024	NS	NA
9. *Ocimum suave*	Leaves & seeds	NS	NA	0.0036	2.222	NS	NA	0.0543	3.603
4 + 8		NS	NA	<0.0001	2.741	NS	NA	NS	NA
6 + 8		NS	NA	NS	NA	NS	NA	NS	NA
9 + 8		NS	NA	0.0183	0.637	0.0124	1.947	NS	NA

OR = Odds ratio, [a] Hut 1 versus Hut 2, [b] Person 1 versus Person 2, [c] Greatest difference between replicates, d Greatest difference between 30 minutes time intervals, NS = Not significant, NA= Not applicable

3.4 DISCUSSION

The ethnobotanical studies showed that direct burning of plants is the most common application method for repelling host-seeking mosquitoes in the surveyed communities. This study developed thermal expulsion as an alternative application method and demonstrated that it is generally superior to direct burning except for *H. suaveolens*. This may indicate possible variations in the type or amount of repellent compounds produced by the two application methods. Indeed up to 2- and 4-fold reductions in exposure to host-seeking *An. gambiae* were achieved by direct burning and thermal expulsion respectively. Thus, traditional methods can serve as a good starting point for research to optimise the use of plants as mosquito repellents. Furthermore, the new semi-field system was found to be ideal for screening large numbers of candidate repellents under conditions that compare well with full-field trials and allow year-round experimentation with fixed densities of non-infected mosquitoes.

The type of plant species used as mosquito repellent can vary from place to place depending to a large extent upon the availability of plant species in a given area. In this study it was observed that the most commonly used and known plant on Rusinga Island was *O. americanum*, while this was *H. suaveolens* among communities around Rambira location. With a distance of only 60 km between these localities, this shows that traditional use of plants may vary considerably and necessitates localised surveys prior to introducing such low-input technology on a wider scale. Similar ethnobotanical studies in a locality in Zimbabwe

(Lukwa *et al.*, 1999) showed that *Lippia javanica* most commonly used compared to other seven plant species by all age groups for mosquito control. Apart from direct burning and use of plants in its original form observed in this study some members of the community traditionally use plants by crushing and application on the skin in Zimbabwe (Lukwa *et al.*, 1999).

Bockarie *et al.* (1994) have shown that smoke from household fires, presumably *D. oliveri* wood, reduce endophily but not endophagy of *An. gambiae* in Sierra Leone. However, direct burning of several plant species from Guinea-Bissau reduced biting rates by up to 80% in the field (Palsson and Jaenson, 1999a, b) and citronella candles were found to reduce mosquito biting rates by 42% in the Gambia (Lindsay *et al.*, 1996). Furthermore, studies on the effectiveness of mosquito coils of the Chinese brand 'white crane' in Zaire showed a weak protective effect (22%) against mosquito bites (Coene *et al.*, 1989). It seems that the level of domestic repellency exhibited by some of the plants, evaluated here under controlled semi-field conditions, compare well with those levels reported from full field tests. The application methods described here are also readily practicable by communities with minimum external input. In addition to direct burning, which is already used by most people in study areas (Table 3.2), thermal expulsion is also easy to implement, requiring only a simple modification of traditional stoves. However, in spite of the substantial repellency observed for several plants, it remains unclear to what extent this contributes to disease reduction (if any) or if it is primarily for nuisance control.

The most effective repellent plant found in this study was *C. citriodora* (lemon eucalyptus) by both thermal expulsion and direct burning. It has long been known that oil of lemon eucalyptus plants can repel mosquitoes and that its principal ingredients are citronella, citronellol, geraniol, *iso*-pulegol, delta pinene, and a sesquiterpene (Curtis *et al.*, 1991). Although, the topical repellency of these ingredients is very low (one hour protection or less) against *Aedes aegypti* L. (Curtis *et al.*, 1991), cloth treated with quwenling (the waste distillate after extraction of oil from the lemon eucalyptus), at greater than twice the dosage of DEET, was effective against *Ae. albopictus* Skuse and *An. quadriamaculatus* Say for at least 28 days (Schreck and Leonhardt, 1991). The major ingredient in the waste distillate was reported to be *p*-menthane-3,8-diol (PMD) (Schreck and Leonhardt, 1991). Here it has been shown that *C. citriodora* can also be highly effective when used with domestic stoves. Despite the strong scent produced by fresh *C. citriodora*, fresh cut branches hung underneath the eaves failed to repel *An. gambiae* under semi-field conditions (Chapter 4).

No synergetic effects were observed for the combination of *O. kilimandscharicum* with *L. ukambensis* by thermal expulsion or with either *L. camara* or *L. ukambensis* by direct burning. Furthermore, combining *O. kilimandscharicum* and *O. suave* by direct burning actually appeared to be attractive even though both plants are repellent when used alone. The low-level repellency of some of the combinations of repellent plants could be due to the low dose (2.5 g of each plant) used, which may not be enough to repel host-seeking mosquitoes. The blends of compounds that result from mixing two different repellent plants may elicit a very different response from those produced by the individual plants. Detailed bioassays of different blends are needed to shed light on this question, but it is clear that rigorous evaluation is required before plants, or their constituent compounds, can be combined for use by people in malaria endemic areas.

This is the first time that repellents have been evaluated in a risk-free, semi-field experimental system and the results have proven very satisfactory. Although standard laboratory methods for evaluating repellents have the advantage of being readily standardised, the conditions under which they are performed are somewhat artificial and the results are not always readily translatable into field efficacy. On the other hand, normal village huts vary considerably in size, construction and the number of occupants, making it difficult and often time-consuming to properly evaluate the effect of mosquito repellent plants. Variability of mosquito densities between huts and daily fluctuations in their numbers further compound rigorous evaluation. Specially constructed experimental huts inside screenhouses clearly minimize external factors that can affect the test results and, unlike the field tests, there is no potential risk to the collectors of acquiring malaria as a result of infective bites from mosquitoes. Using this experimental set up other sources of variation are minimised and these are easily quantified using logistic regression analysis. As a result, significant repellency can be detected at levels as low as 21% within four days. Given that this threshold is far lower than that required for practical use amongst affected communities, this approach represents a promising new way to screen large numbers of candidate repellents under conditions that compare well with full-field trials. Nevertheless, field studies are required to evaluate the effectiveness of these plants in community dwellings.

Although this semi-field system has many clear advantages, full field trials are essential to rule out possible artefacts resulting from overlap of the repellent range of the treatment hut with control hut and behavioural variants of mosquitoes where they are not constrained. In this semi-field system, where mosquitoes are released at a mid-way point between control and treatment huts at a distance of 1.75 m, most mosquitoes can easily fly towards the control hut and avoid the treatment hut when the candidate plant has repellent properties. However, under field conditions, where distances between houses can be much greater, the use of the same repellent may not have the same effect without an alternative host nearby and mosquitoes may therefore be less easily diverted.

In conclusion, modification of traditional practices represented by thermal expulsion of plant materials have shown better protection against *An. gambiae s.l.* in semi-field experimental huts as compared to direct burning of the same plant species. Therefore, thermal expulsion is a better alternative for use by the rural communities in areas where the plant species traditionally used by direct burning.

CHAPTER 4

REPELLENCY OF LIVE POTTED PLANTS AGAINST *ANOPHELES GAMBIAE* IN SEMI-FIELD EXPERIMENTAL HUTS[*]

4.1 INTRODUCTION

Vector-borne diseases are among the most important public health problems and obstacles to socio-economic development of developing countries, particularly in the tropics. To date, no method of malaria control has proven effective enough to control the high transmission intensities found in sub-Saharan Africa (Lengeler *et al.*, 1997; Lengeler *et al.*, 1998; Beier *et al.*, 1999; Killeen *et al.*, 2000). Even the most efficacious of these, such as pyrethroid treated bed nets, have proven difficult to implement on a sustainable basis for reasons of availability, acceptability and cost (Binka and Adongo, 1997; Muller *et al.*, 1997; Snow *et al.*, 1999). However, with increasing problems of toxicity to non-target organisms and the resistance of mosquitoes to synthetic insecticides (WHO, 1976) interest in natural or botanical insecticides has been revived. Natural and synthetic insect repellents are well established as means of personal protection against biting arthropods and the various pathogens they may carry (Gupta and Rutledge, 1994; Braverman *et al.*, 2000).

This study examined the potential use of mosquito repellent plants and their modes of application, with an emphasis on materials and methods that

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[*] The subject of this chapter is published as: Seyoum *et al.* (2002b). Repellency of live potted plants against *Anopheles gambiae* from human baits in semi-field experimental huts. *American Journal of Tropical Medicine and Hygiene*, **67**: 191-195. (Abstract of the paper is reproduced in Appendix 4)

can be locally used by communities with minimal external input. Ethnobotanical studies of traditionally used mosquito repellent plants in Western Kenya showed that many of the most commonly used plants are indeed effective against malaria vectors when burned or thermally expelled with domestic charcoal stoves (Chapter 3). Moreover, these surveys also revealed that some of the plants were commonly applied by cutting the branches and placing them inside houses, particularly around beds. It is therefore, hypothesized that live, intact, potted plants may work in a similar way and represent a sustainable and cost-effective way to protect humans against host-seeking malaria vectors. This thesis chapter focuses on the repellency of selected potted plants against the malaria vector *Anopheles gambiae s. s.* Giles by using semi-field experimental system.

4.2 MATERIALS AND METHODS

4.2.1 Mosquito and plant species

Semi-field tests of the repellency of nine individual candidate plants and some combinations thereof were carried out with a laboratory colony of *An. gambiae s.s.* maintained at the Mbita Point Research and Training Center, ICIPE, as previously described (Chapter 3) within a large semi-field greenhouse.

The plant species tested were, *Ocimum americanum*, *Lantana camara*, *Azadirachta indica*, *Tagetes minuta*, *Hyptis suaveolens*, *Ocimum suave*, *Ocimum kilimandscharicum,* *Corymbia citriodora* and *Lippia ukambensis.* The latter four species were included in the test as candidate repellent plants based on empirical information from unpublished work and from the literature (Schreck and Leonhardt, 1991; Jembere *et al.*, 1995; Bekele *et al.*, 1996). The other five species, were selected for experimentation based on ethnobotanical information obtained locally (Chapter 3). Furthermore, the combined use of two repellent plants (*O. americanum* with either *L. camara* or *L. ukambensis*) in house entry and human feeding behaviour by mosquitoes was also evaluated.

4.2.2 Semi-field quantification of the repellency of selected potted plants

The effect of potted plants on human-vector contact of *An. gambiae* was determined under semi-field conditions inside a screen-walled (shade netting 90%) green house (7.1 × 11.5 m) with two experimental huts (Figure 4.1). These were rectangular (3.2 × 2.7 m) and made of hard board (plywood), internally painted with white paint. The roofs were covered with black cloth inside, and reed mats outside. Each night, ten potted test plants (fresh branches of the tree for *C. citriodora*) were suspended under the eaves of one of the huts (5 on each side) while ten pots with *Hyparrhenia rufa* Stapf (Poaceae), a local wild grass, were placed in a similar position on the other hut to serve as a control, one hour before the start of the experiment. Some leaves of the plants were bruised by hand 5 min before the onset of the experiment in order to enhance release of repellent volatiles. Five potted plants of each species were used for testing the combined use of two repellent plants. A cross-over design was used in which the treatment and control were exchanged between the two huts on different nights. Each night 200 (which were 5-7 day old) unfed female *An. gambiae s.s.*, from a colony which was originally initiated from adults collected in 1996 at Njage village, South-East Tanzania, were used in the experiments. They were starved for six hours, released from a paper cup at the centre of the screenhouse at 20.00 hours and recaptured in the two huts by the human landing catch until midnight. Human collectors (both African males, 35 and 20 yrs old) were exchanged between the huts every 30 min in order to minimize bias caused by differential attractiveness and collection skills. Experiments were replicated four times on four different nights by changing the arrangement of treatment and control plants to the huts.

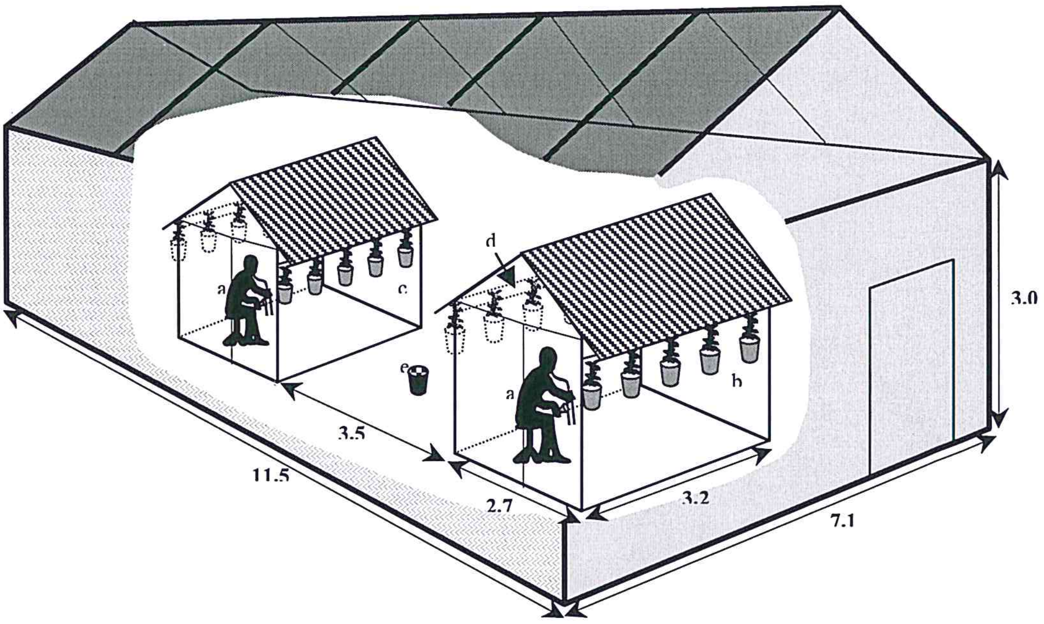

Figure 4.1 Set up for testing the repellency of potted plants. Experimental huts were constructed inside a screen-walled greenhouse, within which human baits sat (a) and the test plants (b) as well as control plants (c) were placed under the eaves (d). Mosquitoes were released from a paper cup (e) placed half way between the huts. Figures describe dimensions in meters.

4.2.3 Data analyses

Logistic regression analysis was used to determine the significance of the effect due to plant treatment and other sources of variations using SAS 8.1 for Windows (SAS 1999) as described in Chapter 2.

4.3 RESULTS

Taking into account the possible effects due to other factors such as the arrangement of huts, collectors, time of collections and replicates, *O. americanum, L. camara* and *L. ukambensis* were found to be relatively effective repellents, reducing domestic exposure by 30 to 40 % ($P<0.0001$). Combining *O. americanum* with either *L. camara* or *L. ukambensis* was similarly effective (31.6 % and 45.2%, respectively, $P<0.0001$ for both treatments) but no obvious synergism was observed (Table 4.1). Neither *O. kilimandscharicum, O. suave, C. citriodora*, *A. indica*, *T. minuta* nor *H. suaveolens* proved to be even mildly repellent to *An. gambiae* even though some of these plants were found to be quite potent when directly burned or thermally expelled (Chapter 3).

The effects of other sources of variation in this experimental setup are shown in Table 4.2. Logistic regression using forward stepwise selection indicated that the arrangement of huts was a significant ($P < 0.05$, OR=0.554 to 0.664) source of variation during the test periods for five candidate plants. Large differences were also observed between the catches of mosquitoes by the two persons ($P<0.0001$, OR=0.157 to 0.494) involved in human landing catches for all but one of the trials but replicate was found to be a significant

($P<0.05$, OR=0.664 and 0.622) source of variation on only two occasions. No significant effect ($P > 0.05$) due to time of collection was observed in any of the trials, indicating that live plants do not appreciably vary in their repellent properties at different times of the night.

Table 4.1 The repellency of potted plants against *Anopheles gambiae s.s.* in semi-field experimental hut trials

Plant species	No. recaptured[a]		Intercept parameter estimate($\beta_o \pm$ SEM)[b]	% Repellency (95% CI)	*P*
	Treatment	Control			
1. *Azadirachta indica*	379	339	0.0479 ± 0.082	-4.9 (-2.3, 10.9)	0.5595
2. *Corymbia citriodora*	319	302	0.0656 ± 0.0824	-6.8 (-25.9, 9.4)	0.4263
3. *Hyptis suaveolens*	179	202	-0.1209 ± 0.1027	11.4 (-8.8, 27.8)	0.2390
4. *Lantana camara*	264	432	-0.3915 ± 0.0863	32.4 (19.7, 43.1)	<0.0001
5. *Lippia ukambensis*	273	408	-0.4055 ± 0.0814	33.3 (21.5, 43.3)	<0.0001
6. *Ocimum americanum*	283	456	-0.5063 ± 0.0779	39.7 (29.6, 48.4)	<0.0001
7. *Ocimum kilimandscharicum*	335	325	-0.0353 ± 0.0826	3.5 (-13.9, 18.2)	0.6693
8. *Ocimum suave*	343	372	-0.1170 ± 0.0793	11.0 (-4.2, 24.1)	0.1403
9. *Tagetes minuta*	388	351	0.0817 ± 0.0772	-8.5 (-26.6, 7.0)	0.2901
4 + 6	290	414	-0.3800 ± 0.0800	31.6 (19.7, 41.7)	<0.0001
5 + 6	221	408	-0.6008 ± 0.0875	45.2 (34.7, 54.0)	<0.0001

SEM = Standard Error of the Mean, [a] Total number caught for 4 test nights, [b] see data analysis section in chapter 2

Table 4.2 Sources of variation affecting host choice, other than plant treatment, as determined by logistic regression analysis

Plant species	Hut Location		Person		Replicate	
	P	OR[a]	*P*	OR[b]	*P*	OR[c]
1. *Azadirachta indica*	NS	NA	<0.0001	0.195	0.0361	1.96
2. *Corymbia citriodora*	0.0128	0.664	<0.0001	0.470	NS	NA
3. *Hyptis suaveolens*	NS	NA	NS	NA	NS	NA
4. *Lantana camara*	NS	NA	<0.0001	0.157	NS	NA
5. *Lippia ukambensis*	NS	NA	<0.0001	0.322	NS	NA
6. *Ocimum americanum*	0.0021	0.622	<0.0001	0.494	NS	NA
7. *Ocimum kilimandscharicum*	NS	NA	<0.0001	0.266	NS	NA
8. *Ocimum suave*	NS	NA	<0.0001	0.282	0.0193	0.586
9. *Tagetes minuta*	0.0001	0.554	<0.0001	0.324	NS	NA
4 + 6	0.0029	0.620	<0.0001	0.341	NS	NA
5 + 6	0.0183	0.663	<0.0001	0.304	NS	NA

OR = Odds ratio, [a] Hut 1 versus Hut 2, [b] Person 1 versus Person 2, [c] Greatest difference between replicates, NS = Not significant, NA = Not applicable

4.4 DISCUSSION

This study is the first to demonstrate that live intact plants can repel mosquitoes to reduce domestic exposure. Although the 'mosquito plant' *Pelargonium citrosum* Voiget (Geraniaceae) is said to repel host-seeking mosquitoes from areas near the plant, it appears ineffective when evaluated under field conditions (Jensen *et al.*, 2000). Intact plants may represent a new and readily applicable vector control tool to be integrated into vector management programs and indeed one of these plants, *L. camara*, is already commonly used for hedges around the huts in many villages around Lake Victoria.

However, field studies are required to fully evaluate the effectiveness of these plants in community dwellings. Although this semi-field system has many clear advantages (Chapter 3), full field trials are essential to rule out possible artifacts resulting from overlap of the repellent range of the treatment hut with the control hut and possible differences in the behaviour of mosquitoes where they are not constrained. In this semi-field set up, where mosquitoes are released at a mid-way point between control and treatment huts at a distance of 1.75 m, mosquitoes can readily choose to fly towards the control hut and avoid the treatment hut when the candidate plant is indeed repellent. As explained in Chapter 3, under real field conditions, where distances between dwellings are usually much greater, the use of the same plant-derived repellent may not have the same effect without an alternative, unprotected host nearby so mosquitoes may be less easily diverted from humans by repellent volatiles.

Of the three plants found to be repellent, *O. americanum* is the most commonly used plant by the community on Rusinga island, western Kenya (Chapter 3). Although there are no previous reports on the repellency of intact plants considered in this study, extracts from the closely related species, *O. canum* Sims (Labiatae) showed strong larvicidal properties against *An. gambiae s.s.* in Tanzania (Lukwa, 1994). Similarly, topical application of *L. camara* flower extract in coconut oil resulted in 94.5% protection against *Aedes albopictus* Skuse and *Ae. aegypti* L. with a mean protection time of 1.9 hours in India (Dua *et al.*, 1996). Essential oil from leaves of *L. ukambensis* also has mosquito repellent properties (M. Omolo *et al.* unpublished data). The essential oils of closely related species *Lippia javanica* also showed mosquito repellent property with 76% repellency for four hours against *An. arabiensis* in South Africa (Govere *et al.*, 2000). The same plant species has been traditionally used as mosquito repellent in Central Africa (Lukwa *et al.*, 1999).

T. minuta, A. indica, O, kilimandscharicum, O. suave, H. suaveolens and *C. citriodora* have not shown significant repellency in this study even though they all have been documented to have repellent or insecticidal properties. Whole plant Soxhlet and steam distillation extracts of *T. minuta* are larvicidal to *Ae. aegypti* and *An. stephensi* Liston (Perich *et al.*, 1994). Neem (*A. indica*) cream (5% neem oil in vanishing cream) was also reported to afford over 90 % protections against both anopheline and culicine mosquitoes in the field (Singh *et al.*, 1996). Kerosene lamps containing neem oil also reduce the biting and indoor resting density of mosquitoes with better protection against *Anopheles* than *Culex* (Sharma and Ansari, 1994). *Ocimum suave* and *O. kilimandscharicum* repel insect pests of stored products (Jembere *et al.*, 1995; Bekele *et al.*, 1996) and cloth treated with quwenling was effective against *Ae. albopictus* and *An. quadriamaculatus* Say (Schreck and Leonhardt, 1991).

The precise effect of potted plants in the reduction of human vector contact is difficult to compare with effects obtained with existing vector control tools, given the inherent differences in the methods of evaluation of their impact. However, the repellent plants observed in this study can be considered as effective (about 40% reduction in human vector contact) and may be used in combination with other vector control tools in integrated vector management programmes. Although such reductions by repellent plants are unlikely to yield significant reductions of disease burden in malaria endemic settings (Lengeler *et al.*, 1997; 1998; Beier *et al.*, 1999), integrated control with a few modestly effective tools can meaningfully reduce malaria transmission (Killeen *et al.*, 2000). Intact repellent plants may therefore contribute to control programs that combine several tools, and is perhaps the most easily implemented method of domestic protection reported to date.

Furthermore, the results obtained from the present study can also help to develop 'push-pull' or stimulo-deterrent diversionary strategies (Miller and Cowles, 1990) whereby certain species of vector mosquitoes (zoophilic anophelines) could be repelled from human hosts and simultaneously attracted to alternative hosts such as cattle. Recent research on agricultural pests has demonstrated that such approaches can be very successful: intercropping with the non-host molasses grass, *Melinis minutiflora* Beauv. (Poaceae), significantly repelled female *Chilo partellus* Swinhoe stemborers from maize crops and simultaneously attracted foraging female *Cotesia sesamiae*

Cameron, a larval parasitoid of this important pest (Khan *et al.*, 1997). There is also some evidence that domestic use of insecticides with excito-repellent properties can lengthen the feeding cycle of anthrophophilic mosquitoes and discourage them from feeding on humans (Garrett-Jones, 1964; Charlwood and Graves, 1987; Bogh *et al.*, 1998; Killeen *et al.*, 2000). Thus, bed nets and insecticides seem to have the potential to enhance or enable zooprophylaxis and similar approaches could be explored for the use of intact repellent plants. Although this approach is unlikely to work for highly anthropophilic species such as *An. gambiae* and *An. funestus*, malaria transmission across much of tropical Africa is dominated by *An. arabiensis* (Gillies and Coetzee, 1987) and appears to be very sensitive to changes in the relative availability of cattle and humans (Killeen *et al.*, 2001).

In conclusion, this study showed that potted plants of *O. americanum, L. camara* and *L. ukambensis* significantly reduce domestic human exposure to the malaria vector *An. gambiae* in semi-field trials. Potted plants may represent a new, sustainable and readily applicable malaria control tool for incorporation into integrated programs, particularly if used to enable or enhance zoo-prophylaxis by diverting the repelled mosquitoes to alternative host such as cattle.

CHAPTER 5

FIELD EFFICACY OF THERMALLY EXPELLED AND LIVE POTTED REPELLENT PLANTS AGAINST AFRICAN MALARIA VECTORS IN WESTERN KENYA*

5.1 INTRODUCTION

Malaria remains an important public health problem, and is endemic in over 100 countries in the world (Remme *et al.,* 2001). The major impact of malaria is in sub-Saharan Africa where at least 90% of deaths from malaria occur (Greenwood & Mutabingwa, 2002). Because of the poor performance of health service delivery systems for malaria control and other diseases, vector control has had limited success in highly endemic countries (Breman, 2001). There is, however, growing interest in the control of mosquitoes both with classical vector control technologies to prevent and control epidemics and personal protection methods such as insecticide treated bednets (Lengeler & Snow 1996; Nevill *et al.,* 1996; Schellenberg *et al.,* 2001; Guyatt *et al.,* 2002; Maxwell *et al.,* 2002) and insect repellents (Curtis *et al.,* 1987; Curtis *et al.,* 1991).

Although insecticide-treated bednets have been widely advocated for protection against mosquitoes and malaria in many parts of the world, people may

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*The subject of this chapter has been accepted for publication as: Seyoum *et al* (2003). Field efficacy of thermally expelled or live potted repellent plants against African malaria vectors in western Kenya. *Tropical Medicine and International Health.* - Abstract of the paper is reproduced in Appendix 5 often contract the disease in the early evening before they retire to the confines of the net. Exposure to malaria vectors and to nuisance mosquitoes starts in the early evening before going to bed (Maxwell *et al.,* 1998). Thus, there is the need to find supplemental protective measure to insecticide treated nets that can easily be adopted in rural communities of Africa.

In an effort to develop low-cost plant-based household protection methods that can be used by communities with minimal external input, several plant species were evaluated with respect to their repellent properties under semi-field experimental huts in western Kenya (Chapters 3 and 4). Thermal expulsion of *Corymbia citriodora*, *Ocimum suave* and *O. kilimandscharicum* repelled between 52 to 74% host-seeking *An. gambiae sensu stricto* in semi-field experimental huts, using a simple modification of typical African traditional stoves (Chapter 3). Furthermore, intact potted plants of *O. americanum, Lantana camara*, and *Lippia ukambensis* also reduced biting by *An. gambiae* in semi-field experimental huts by 30-40% (Chapter 4).

However, field trials are required to demonstrate the impact of promising repellents in community dwellings under fully natural conditions. Furthermore, different mosquito species may display different behavioural responses towards a given vector control tool under natural field conditions where transmission is often mediated by two or more mosquito species. The impact of selected potted repellent plants and thermal expulsion of plant materials was, therefore, evaluated against the malaria vectors in Lwanda village, western Kenya where transmission is mediated by *An. gambiae* Giles, *An. arabiensis* Patton and *An. funestus* Giles, the three major vectors of malaria in Africa.

5.2 MATERIALS AND METHODS

5.2.1 Study area

The studies were carried out in Lwanda village (S 00° 28.621' and E 034° 17.331'), Suba district, western Kenya, about 420 km west of Nairobi in Lake Victoria basin (Figure 5.1). The study village has an altitude of 1169 m above sea level, with the main rainy season from March through May, and a short rainy season from October to November. It is a rural village with many small man-made ponds, swamps, hoof prints and a variety of other mosquito breeding habitats (Minakawa *et al.,* 1999). Mosquito density is high in the main and small rainy seasons but lower in the dry season. Although the bulk of anopheline populations comprises of members of the *An. gambiae s.l.*, *An. funestus* complex can also be found at relatively high densities. No previous vector control programme had been in operation in the village, nor were there any on-going control activities.

Five homesteads were selected for the field tests of the candidate plants by thermal expulsion and four for potted plants. All experimental houses were mud-walled with grass-thatched roofs in which the open eaves and the unscreened windows allowed ready access to mosquitoes. An open eave is one of the architectural features that enable vectors of malaria to enter houses (Lindsay &

Snow, 1988). The sizes of houses ranged from 12.6 to 15.7 m^2. The distances between huts vary from approximately 100 to 300 meters (the distance between houses number 1, 2 and 3 was approximately 150 meters, where as the distance between houses number 3 and 4 was approximately 300 meters. The distance between house number 4 and 5 was about 200 meters. The distance between house number 5 and 1 was about 100 meters).

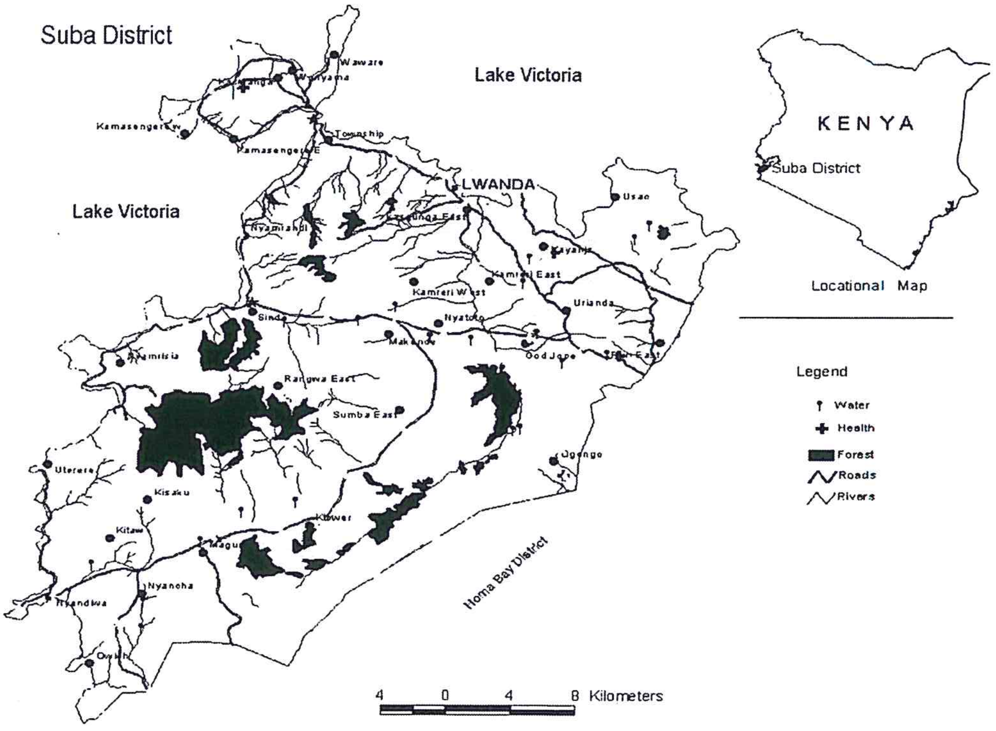


Figure 5.1 Map showing location of the study area (Lwanda village) in relation to Suba District, Kenya (Inset – map of Kenya showing Suba District)

5.2.2 Test plants

Candidate plants were selected for the field trial on the basis of their repellency in semi-field experimental huts trials. These comprised potted plants of *O. americanum*, *L. camara*, and *L. ukambensis* (Seyoum *et al.*, 2002b) and, leaves of *C. citriodora* (lemon eucalyptus); and leaves and seeds of *O. kilimandsharicum* and *O. suave* (Seyoum *et al.*, 2002a) for thermal fumigation. Mosquito coils (Baygon®, product of BAYER East Africa Ltd.) with 0.20% w/w pyrethrins as the active ingredient was also tested to serve as a positive control.

5.2.3 Repellency tests

Ten potted plants of each species were placed under the eaves of houses (one species per house) from 18.30 hrs to 06.30 hrs during each experimental night. The leaves of the plants were bruised by hand at 18.30 and at 21.00 hrs to enhance the release of repellent volatiles. For thermal expulsion, the plant materials were placed on the top of thin metal plates placed directly above the charcoal in a traditional stove or "Jiko" (Chapter 3) and the houses were fumigated by applying fresh plant material every hour from 18.30 - 21.30 hrs local time. Each hour 10 g of preweighed plant materials were placed on the thin metal plate of the traditional stove with burning charcoal for thermal fumigation of individual huts. Three hundred gram of charcoal was used to light the traditional stoves followed by additional 150 g of charcoal every hour.

A 4×4 Latin square design was used for testing potted plants, three represent the repellent plants and one the control, a potted local wild grass, *Hyparrhenia rufa*. Similarly, for the thermal expulsion experiment, a 5 × 5 Latin square design was used, three treatment huts, one with mosquito coil (positive control) and one without any treatment (negative control). The huts were randomly assigned for the candidate plants and the control(s) on the first sampling night. The treatments and the control(s) were then assigned by rotation in consecutive sampling nights in different huts to compensate for potential spatial variations of mosquito density in individual huts selected for experimentation.

CDC light traps set close to occupied untreated bed nets inside bedrooms were used for sampling mosquitoes (Plate 5.1). Each light trap was operated on a 6 V 10 Ah battery and fitted with a 150 mA bulb (6.3 V) and a lid. The traps were positioned at the foot end of the bed near the top of the bednet (Mboera *et al.,* 1998). Traps were operated between 18.30 and 06.30 hrs. For thermal expulsion experiment, the collection bags were replaced at 22.30 hrs to compare effects during fumigation and after. The mosquitoes were identified to species level using morphological characteristics (Gillies & De Meillon, 1968). Samples (5%) of *An. gambiae s.l.* were preserved in 95% ethanol for further identification to sibling level by Polymerase Chain Reaction (Scott *et al.,* 1993). Tests involving thermal fumigation were carried out with minimum intervening periods of three nights to avoid potential residual effects. Tests with potted plants were carried out over consecutive nights. The experiments were replicated 24 nights for potted plants (six blocks) and 30 nights (six blocks) for experiments for thermal expulsion. These blocks were run over different seasons (June 2001 to May 2002). Thus each treatment or control was assigned six times to all experimental huts for both methods.

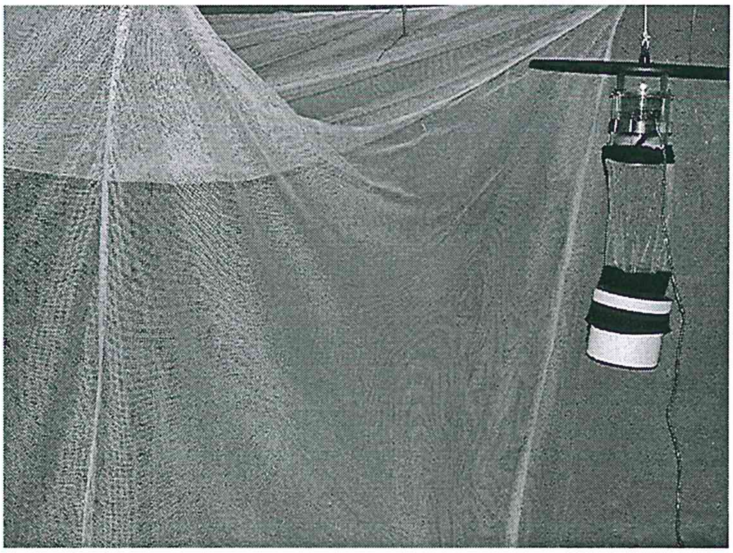


Plate 5.1 CDC light trap set close to occupied bednet for sampling mosquitoes indoor.

5.2.4 Data analyses

A Generalized linear model (GLM) procedure was used to determine the significance of differences of the total catch of mosquitoes in each treatment and control huts using SPSS 10 for windows. The data were analysed separately for the two most common anopheline species, *An. gambiae s.l.* and *An. funestus*, and for the two application methods. For experiments with thermal expulsion, data for the catches during and after application of the treatments were analysed separately. All-night catches were used in the analysis of data from experiments with potted plants.

The effects of repellent plants was analysed, and allowed for differences between experimental units by generalised linear modelling of the relationship between mosquito catches in control (C) and treatment huts (T). The effects of treatment repellency (R) and the effects of different household experimental units (E) were modelled as:

$$T = (1-R)\,E\;C \qquad \mathbf{1}$$

Because mosquito catches are usually highly aggregated and vary over wide ranges (Smith, 1995) they are best expressed in logarithmic form to minimize heterogeneity of variances for generalized linear modelling. To enable the inclusion of nights with zero catches, 1 was added to all counts, assume $T + 1 \approx T$ and $C + 1 \approx C$ and log transform both sides of equation **1**.

$$\mathrm{Log}\,T \;=\; \mathrm{Log}\,(1-R)\,E\,C \;=\; \mathrm{Log}\,(1-R) + \mathrm{Log}\,E + \mathrm{Log}\,C \qquad \mathbf{2}$$

which can be fitted to a generalized linear model of the form:

$$y \;=\; \beta_1 x_1 + \beta_2 x_2 + \beta_3 x_3 \qquad \mathbf{3}$$

where β_1 represents the effects of different repellent plants, β_2 represents the effects of different experimental units (huts and residents), and β_3 represents the equivalence of catches where treatment and experimental unit effects are negligible. Therefore replicate or sampling night was treated as a randomly varying factor determining overall catch size and estimated values for β_1 and β_2 as fixed factors that influence this outcome. Substituting β_1 into the first term of equation **2** and rearranging allows calculation of proportional repellency as follows:

$$R = 1 - 10^{\beta 1} \qquad \mathbf{4}$$

5.3 RESULTS

5.3.1 Trap catches and species composition

An. gambiae s.l. and *An. funestus* were the main species present during the study period, with a higher proportion of the former than the latter. A total of 16,347 anopheline mosquitoes were caught from all the treatment and control huts for both studies, of which 79.5 % were *An. gambiae s. l.* and 20.5 % *An. funestus*. Out of a total of 649 *An. gambiae s.l.* samples screened using PCR, 18.5 % were *An. gambiae s. s.* and 81.5% *An. arabiensis*.

5.3.2 Repellency by thermal expulsion and potted plants

All the three candidate plants for thermal expulsion experiments, *C. citriodora, O. suave* and *O. kilimandscharicum* showed repellency against *An. gambiae s.l.* both during application of plant treatments and as residual effects in post-application periods (Tables 5.1 and 5.2, respectively). The highest repellency against *An. gambiae s.l.* both during and after application of the plant treatments was by *C. citriodora* (48.71%, P<0.0001 during application and 44.02%, P<0.0001 post application periods) followed by *O. kilimandscharicum* (44.54%, P=0.001 during application and 39.61%, P<0.0001 post-application periods) and *O. suave* (44.54%, P=0.001 during application and 36.03%, P=0.001 post-application periods). None of candidate plants except thermal expulsion of *O. kilimandscharicum* showed significant protection against *An. funestus* (P>0.05).

Intact potted plants of *O. americanum* and *L. camara* also showed significant repellency (37.91%, P=0.004 and 27.22%, P=0.05, respectively) against *An. gambiae s.l.* under field conditions (Table 5.3). However, none of the candidate potted plants showed significant protection against *An. funestus* (P>0.05) under natural field conditions.

Table 5.1 The repellency of plants against *Anopheles gambiae s.l.* and *Anopheles funestus* by thermal expulsion during application of plants (18.30 – 22.30 hrs sampling period) in Lwanda, western Kenya

Plant Species	*Anopheles* species	Parameter estimate of treatments ($\beta_1 \pm$ SEM)	Repellency % (95% CI)	*P*
C. citriodora	*An. gambiae s.l.*	-0.290 ± 0.072	48.71 (28.88, 63.02)	<0.0001
	An. funestus	-0.07054 ± 0.071	15.0 (-17.58, 38.48)	0.323
O. kilimandscharicum	*An. gambiae s.l.*	-0.256 ± 0.072	44.54 (23.09, 60.01)	0.001
	An. funestus	-0.205 ± 0.071	37.63 (13.69, 54.92)	0.005
O. suave	*An. gambiae s.l.*	-0.256 ± 0.072	44.54 (23.09, 60.01)	0.001
	An. funestus	-0.07472 ± 0.071	15.81 (-16.45, 39.19)	0.295
Mosquito coil (control)	*An. gambiae s.l.*	-0.349 ± 0.072	55.23 (37.91, 67.72)	<0.0001
	An. funestus	-0.186 ± 0.071	34.84 (9.89, 52.90)	0.010

Table 5.2 The residual repellency of plant volatiles against *Anopheles gambiae s.l.* and *Anopheles funestus* between 22.30 – 06.30 hrs, following a period of thermal expulsion (18.30-22.30 hrs) in Lwanda, western Kenya

Plant Species	*Anopheles* species	Parameter estimate of treatments ($\beta_1 \pm$ SEM)	Repellency % (95% CI)	*P*
C. citriodora	*An. gambiae s.l.*	-0.252 ± 0.057	44.02 (27.56, 56.75)	< 0.0001
	An. funestus	-0.07459 ± 0.067	15.78 (-12.45, 37.91)	0.267
O. kilimandscharicum	*An. gambiae s.l.*	-0.219 ± 0.057	39.61 (21.84, 53.44)	<0.0001
	An. funestus	-0.164 ± 0.067	31.45 (6.96, 49.42)	0.016
O. suave	*An. gambiae s.l.*	-0.194 ± 0.057	36.03 (17.12, 50.57)	0.001
	An. funestus	0.01682 ± 0.067	-3.95 (-40.93, 23.44)	0.802
Mosquito coil (control)	*An. gambiae s.l.*	-0.351 ± 0.057	55.43 (42.19, 65.57)	<0.0001
	An. funestus	-0.232 ± 0.067	41.39 (20.51, 56.75)	0.001

Table 5.3 The repellency of potted plants against *Anopheles gambiae s.l.* and *Anopheles funestus* in Lwanda, western Kenya

Plant Species	*Anopheles* species	Parameter estimate of treatments ($\beta_1 \pm$ SEM)	Repellency % (95% CI)	*P*
O. americanum	*An. gambiae s.l.*	-0.207 ± 0.069	37.91 (14.51, 54.81)	0.004
	An. funestus	0.02651 ± 0.103	-5.92 (-71.00, 33.93)	0.798
L. camara	*An. gambiae s.l.*	-0.138 ± 0.069	27.22 (0.04, 47.16)	0.050
	An. funestus	0.06801 ± 0.103	-16.95 (-87.93, 27.22)	0.513
L. ukambensis	*An. gambiae s.l.*	-0.125 ± 0.069	25.01 (-3.08, 45.55)	0.075
	An. funestus	-0.129 ± 0.103	25.70 (-19.46, 53.87)	0.216

Table 5.4 The distribution of *An. gambiae s.s.* and *An. arabiensis* from samples identified using PCR by methods and durations of applications.

Methods (duration)	Total PCR samples	*An. gambiae s.s.* (%)	*An. arabiensis* (%)
Thermal (18.30-22.30)	206	18 (8.7)	188 (91.3)
Thermal (22.30-6.30)	250	58 (23.2)	192 (76.8)
Potted plants (18.30-6.30)	193	44 (22.8)	149 (77.2)
Total	649	120 (18.5)	529 (81.5)

5.4 DISCUSSION

This study evaluated the level of protection conferred by thermal expulsion of plants and intact potted plants under natural field conditions in western Kenya. The results show that both are effective in repelling *An. gambiae s.l.*, the principal malaria vector in tropical Africa. The methods are simple and highly adaptable under varied local situations in rural communities of Africa where traditional methods such as direct burning of plant materials and bruising the leaves of the plants and hanging them around the bed are already being practised to repel house-entry mosquitoes (Seyoum *et al.*, 2002a).

The repellency of all candidate plants for *An. gambiae s.l.* by thermal expulsion was comparable to that of a mosquito coil that was used as a positive control in this field trial. The level of repellency of mosquito coil in this study is in agreement with previous reports of 24 – 88 % reduction from Tanzania and Papua New-guinea (Hudson & Esozed, 1971; Charlwood & Jolly, 1984). The continuing repellency of residual fumigants from thermal expulsion showed that fumigation in early hours of the night persists during the remaining part of the night. This finding indicates that thermal fumigation of such plants may have potential for integration with untreated bednets (Takken, 2002) and can, under some situations, replace bednets impregnated with pyrethroids for excito-repellent effects.

A similar approach to thermal expulsion of plant materials has been recently developed using kerosene-burning lamp (‘*korobois*’) modified to heat and vaporize a volatile pyrethroid insecticide to repel host-seeking mosquitoes in Tanzania (Pates *et al.,* 2002). In this study, a modified lamp containing a tin with a mixture of an insecticide (0.1% transfluthrin) and vegetable oil heated to 120°C just above the flame gave 50-75% reduction in the biting rate of *Culex quinquefasciatus* Say. However, direct comparisons cannot be made with the results of this study due to differences in mosquito species.

Live potted plants of *O. americanum* were more effective in repelling *An. gambiae s.l.* compared to the other two species. This is in agreement with the results obtained in the semi-field tests (Seyoum *et al.,* 2002b), and suggests that such readily applicable use of local plant products merits further investigation as a means to incrementally roll back malaria. In addition, the result validates the efficacy of semi-field systems (Seyoum *et al.,* 2002a; Seyoum *et al.*, 2002b; Knols *et al.,* 2002) to screen large numbers of plants in a short period of time.

Although, the level of repellency of intact potted plants and thermal expulsion of plant materials in this study is lower than that required to substantially reduce the incidence of malaria in highly endemic areas, it may usefully contribute to integrated programmes. Integrated vector management with a number of modestly effective control tools can significantly lower entomological inoculation rates (Killeen *et al.,* 2000).

None of plant species tested except *O. kilimandscharicum* gave significant ($P > 0.05$) reduction against *An. funestus* by either method of application. *Ocimum kilimandscharicum* significantly repelled *An. funestus* both during and after fumigation periods (37.63% and 31.45%, respectively). Differences in the sensitivity of mosquito species to synthetic repellents such as diethyl-methylbenzamide are also widely documented (Curtis *et al.,* 1987; Walker *et al.,* 1996; Tawatsin *et al.,* 2001). Walker *et al.* (1996) reported that *An. funestus* was significantly less sensitive ($P < 0.001$) than *An. arabiensis* to synthetic topical repellents, such as DEET and a piperidine compound, AI3-37220 in western Kenya. Similarly, it has been reported (Tawatsin *et al.,* 2001) that DEET provided protection for at least eight hours against *Ae. aegypti* L. and *Cx. quinquefasciatus*, but for only six hours against *An. dirus* Peyton & Harrison in

Thailand. It was also found that laboratory tests of six insect repellents (DEET, di-methyl phthalate, ethyl-hexanediol, permethrin, citronella and cedarwood oil) by different methods showed that *An. stephensi* Liston was consistently more susceptible than *An. gambiae* Giles, *An. albimanus* Wiedemann or *An. pulcherrimus* Theobold (Curtis *et al.,* 1987).

Similar studies in rural Papua New Guinea (Paru *et al.,* 1995) also demonstrated the repellent activity of burning wood of various plants outdoors, and or bruised (rubbed) leaves of the plants applied on the legs of humans against anopheline and culicine mosquitoes. It was found that wood smoke and topical applications reduced biting of human volunteers by 79% and 51%, respectively. More recently, Pålsson & Jaenson (1999a, b) showed that direct burning of several plant species from Guinea-Bissau reduced biting rates by up to 80% in the field. Overall, different results obtained in this and other reports can be accounted for by differences in the type of mosquito species, plant species, methods of applications and evaluation procedures.

This study used CDC light traps to evaluate the impact of mosquito repellent plants in the reduction of mosquito density. This is the first time that CDC light traps were used to evaluate the impact of personal protection methods and has proven to be a satisfactory sampling tool. Studies by Mathenge M. (personal communication) in the same village revealed that catches by CDC light traps are proportional to human landing catches of *An. gambiae s.l.* and *An. funestus* in the area and sample equivalent host-seeking cohorts of the vector population. Similarly, studies in Tanzania and Sierra Leone showed that catches of *An. gambiae s.l.* in light traps set beside occupied untreated nets were proportional to the human biting catches, and the age distribution of the mosquitoes caught by the two methods were similar (Lines *et al.,* 1991; Magbity *et al.,* 2002), and recommended the use of light traps as a surrogate for human bait catches in estimating biting rates of *An. gambiae.* Therefore, light traps set beside occupied bed nets present an alternative to human biting catches and avoid the possible ethical problem that arises when mosquito collectors deliberately expose themselves to disease vectors.

In conclusion, thermal expulsion of *C. citriodora*, *O. kilimandscharicum* and *O. suave*, and intact potted plants of *O. americanum* and *L. camara* significantly repelled *An. gambiae* under natural field condition in western Kenya, and can be incorporated into integrated vector management in areas where the plant species are available and where the dominant vector of malaria is *An. gambiae s.l.* Similarly, thermal expulsion of *O. kilimandscharicum* can also be considered in areas where *An. funestus* is important vector of malaria. Both methods of application may offer cost-effective alternatives as additional means of household protection, and a useful complement to bednets, particularly for the early part of the evening before bedtime.

CHAPTER 6

LABORATORY EFFICACY OF ESSENTIAL OILS OF MOSQUITO REPELLENT PLANTS AGAINST *ANOPHELES GAMBIAE*

6.1 INTRODUCTION

Mosquito repellent plants have been reported to be effective, and are widely used traditionally among communities in East and West Africa (Curtis *et al.*, 1991; Palsson and Jaensson, 1999a, b; Seyoum *et al.*, 2002 a). There are different traditional practices, such as fumigating houses by burning whole or part of a plant, and by placing fresh branches of plants around beds to prevent mosquito's bites. Topical applications of synthetic and plant-derived insect repellents are also widely used as a means of personal protection against biting arthropods (Gupta and Rutledge, 1994; Trigg and Hill, 1996).

Personal protection measures, including application of repellents to exposed skin, have long been advocated for minimizing the number of bites from mosquitoes (Gupta and Rutledge, 1994) and are considered important in reducing the risk of contracting insect borne diseases (Curtis, 1992). It can also be the most ideal protection method against mosquitoes when people stay outdoor during early hours of the night, where other vector control tools such as insecticide treated nets and indoor residual house spraying are irrelevant.

The majority of commercial insect repellent preparations contain diethyl methylbenzamide (DEET), first synthesized in 1954 (McCabe *et al.*, 1954). There are, however, some disadvantages associated with DEET usage, which stem from its activity as a solvent of paints, varnishes and certain plastics and synthetic fibers (Trigg and Hill, 1996). Concerns arising from reports of the occasional toxicity of the central nervous system (encephalopathy) to humans, particularly in children have highlighted the need for alternatives to DEET (Moody, 1989). Recently, Qui *et al.,* (1998) reviewed the pharmacokinetics, formulations, and safety of DEET, and concluded that although, DEET exhibits a good margin of safety, but does manifest some adverse effects in human.

To find safer and more acceptable repellents for topical use, the essential oils of a number of plants have been tested and shown to have mosquito repellent properties (Barnard, 2000). One of the advantages of using plant materials, as a repellent is the cost, compared to other conventional vector control methods (Gupta and Rutledge, 1994).

The aim of this study is to evaluate the repellency of essential oils of some of mosquito repellent plants that have shown significant repellency by thermal expulsion and in intact potted form in semi-field and field studies (Chapters 3-5). This chapter focuses on the efficacy of these essential oils of selected repellent plants in dose response studies and the duration of protection as compared to the standard synthetic repellent, DEET, against laboratory colony of *Anopheles gambiae* s.s. This study also evaluated the efficacy of essential oils of plant samples taken during the day and night time.

6.2 MATERIALS AND METHODS

6.2.1 Isolation of essential oils

Essential oils from the leaves of *Ocimum americanum*, *O. kilimandscharicum*, *O. suave,* and *Lantana camara*, flowers and leaves of *Hyptis suaveolens,* and seeds of *O. suave* were extracted by hydrodistillation, using clavenger apparatus and concentrated using rotavapor until the solvent (hexane) completely evaporated at temperature of about 40°C. The plant samples were taken during the day and night for *O. americanum*, *O. kilimandscharicum*, *O. suave* and *L. camara,* but only during the day for *H. suaveolens*.

6.2.2 Bioassay condition

Repellency tests were carried out in a room using 50x50x50 cm cages following the World Health Organization protocol for the dose response studies at different concentrations, and to determine protection time (Curtis *et al.*, 1987; WHO, 1996; Frances *et al.*, 1998). The room was maintained at 28 ± 2°C and relative humidity 70 ± 5% during the tests. On any one day, only one repellent preparation was tested. Laboratory colony of *An. gambiae s.s.* maintained at ICIPE-Mbita Point Research and Training Center, Kenya, was used for the bioassay tests. The details of rearing procedures are given in Chapter 2.

6.2.3 Dose response studies

One hundred nulliparous females (5 - 7 days old, starved for 6 hours) were placed into laboratory test cages. Clean cages of fresh mosquitoes were used each day. Tests were conducted by first exposing the 'control forearm' of a person treated uniformly with 0.7-0.9 ml (depending up on the size of the arm of volunteers) of acetone, to the mosquitoes inside cages followed by the ‘test forearm’ in the same cage. The 'test forearm' was treated with acetone solutions of test samples (0.7-0.9 ml based on surface area of the arm) at a rate of $0.001 ml/cm^2$. Three adult male volunteers and three adult female volunteers, aged 20 – 34 years old were involved in this study. The arms were first cleaned with water and 95% ethanol, and air-dried before application of the treatments and control. Each volunteer used the right arm for treatment and the left arm for control. Separate cages were used for each individual. The repellent was spread evenly over the forearm of a volunteer wrist to elbow. A surgical glove was worn during the tests to prevent biting on the untreated palms and fingers. The exposure time in the cages was 30 seconds, and the number of mosquitoes landed on or probed the arms exposed were counted and recorded for each subject. The high avidity of the laboratory colony of *An. gambiae s.s.* used in this study necessitated 30 second exposure periods instead of the usual 1 minute exposure time (Frances *et al.*, 1998) to obtain adequate landing and biting counts during the test periods for all repellent doses. Mosquitoes were shaken off the arm before they had a chance to imbibe any blood. Exposure of control arms was alternated with treated arms to provide a standard for comparing avidity of biting.

The tests were conducted on series of concentrations (0.08% to 10%) of the repellent and untreated control. The concentrations were presented one after the other (from lowest to highest concentration) to the same caged mosquitoes for a particular repellent. Each test was done at an hour interval. The amount of each repellent applied on the arm was determined from the relative surface area of the arm. The surface area of the arm was calculated using the following formula: $A= (1/3(a+b+c)) \times h$, where A represents the application area, a = the circumference of the arm at the joint between the femur and tibia, b = the circumference of the arm halfway between the joint and the wrist, c = the circumference of the arm at the wrist, h= the length of the forearm between the joint and the wrist.

6.2.4 Evaluation of protection time

Similarly, protection time of the essential oils of all candidate plants was determined at 20% test concentrations. Formulated commercial DEET (20%) was also tested as a standard for comparison of protection time to that of the candidate essential oils. It is a common standard against which the protection time of repellents is compared in many laboratories. The oils and the control applied on the forearms and tested in a similar way described in section 6.2.3. After application of repellents, subjects were instructed not to rub, touch, or wet the treated arms. Tests were undertaken immediately after application of repellents followed by 1, 2, 3, 4, 5, 6 and 7 hours post-application, and the experiment was only stopped when no significant protection was observed by the essential oils of the candidate plants. The subjects were restricted to stay in a room in between test periods in order to minimize the loss of repellent effects of oils on treated arms.

6.2.5 Data analyses

The averages of bites in the treatment and control arms were used in calculating percentage protection. Percentage protection, defined as the average number of bites received by individuals in a treatment arm relative to that of the control was calculated as $(N_c - N_t)/N_c \times 100$ (Sharma and Ansari, 1994), where, N_c denotes the mean number of mosquitoes landing on the control forearm, and N_t denotes that at the treated forearm. This was calculated for each of the concentrations of the crude oil extracts in dose response studies and at each time interval in the tests involving evaluation of protection times.

The median effective dosage (ED_{50}) of the oils was determined following probit plane procedure using computer software for probit analysis after Finney (1971). Significant differences were determined by comparing the 95% confidence intervals of the ED_{50} values.

6.3 RESULTS

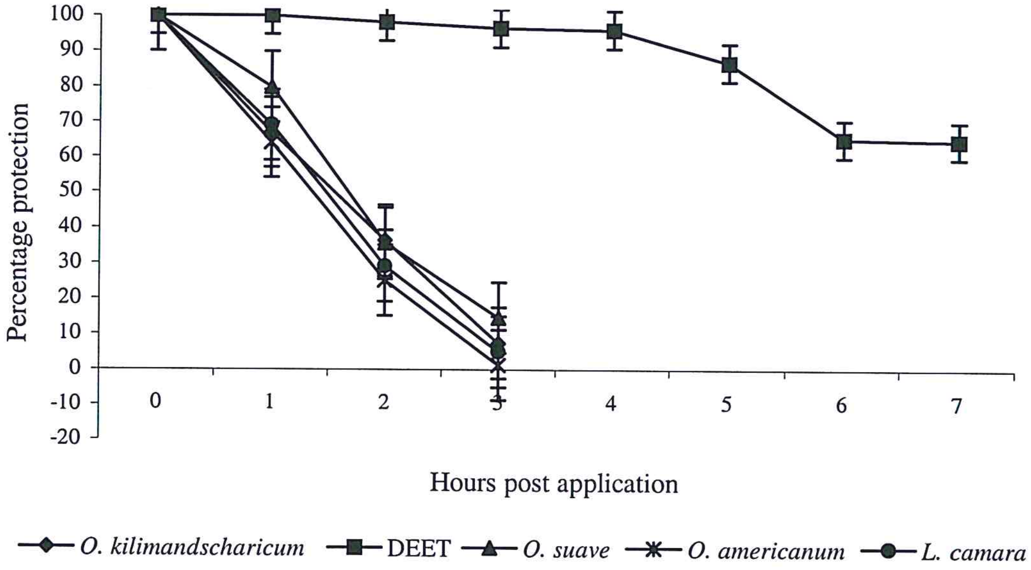
Table 6.1 summarized the efficacy of essential oils from mosquito repellent plants samples taken during the day and night against *An. gambiae s.s* in dose response studies. The essential oils of all plant species showed high level of repellency with the ED_{50} ranging from 0.266 (95% CI=0.173-0.386) for leaves of *O. suave* (night) to 3.155% (95% CI=2.406-3.864) for leaves of *O. kilimandscharicum* (day). The efficacy of essential oils of plant samples taken during the night was not significantly different from those taken during the day ($P > 0.05$). Figure 6.1 shows protection time of the essential oils of candidate plants with reference to the standard DEET. All showed complete protection against *An. gambiae* at 20% test concentration for freshly applied oils soon after the time of application. However, none of the essential oils of test plants provided even 1 hour of complete protection against *An. gambiae*, and after 3 hours biting rates were similar for both the treated and control arms (Figure 6.1). The repellent effect of the standard formulated DEET lasted much longer than that of the unformulated essential oils of the other candidate plants. DEET at 20% test concentration provided 90 – 100% relative protection up to 5 hours, against *An. gambiae* s.s., but declined to 65 % after 6 - 7 hours (Figure 6.1.).

Table 6.1 The ED_{50} values of essential oils of mosquito repellent plants against adults of *Anopheles gambiae s.s.* in laboratory dose response studies

Plant species	Part of plant	Time of collection	ED_{50} (95% CI)	χ^2(df=2)
O. americanum	Leaves	Day	0.568^{a} (0.380, 0.771)	1.588
O. americanum	Leaves	Night	0.626^{a} (0.492, 0.793)	3.431
O. kilimandscharicum	Leaves	Day	3.155^{b} (2.406, 3.864)	0.460
O. kilimandscharicum	Leaves	Night	1.772^{b} (1.123, 2.902)	6.053
O. suave	Leaves	Day	0.554^{a} (0.364, 0.701)	1.579
O. suave	Leaves	Night	0.266^{a} (0.173, 0.386)	0.053
O. suave	Seeds	Day	0.388^{a} (0.246, 0.573)	3.567
H. suaveolens	Flowers & leaves	Day	1.623^{b} (1.136, 2.409)	3.497
L. camara	Leaves	Night	1.062^{b} (0.832, 1.360)	0.638
L. camara	Leaves	Day	1.122^{b} (0.823,1.545)	3.332

ED_{50} values followed by different letters are significantly different from one another at $P < 0.05$.

Figure 6.1 Mean Percentage protection provided by the essential oils of candidate plants and the standard DEET against *Anopheles gambiae* at 20% test concentration



6.4 DISCUSSION

The data in Table 6.1 show that the essential oils from all candidate plants collected during the night are equally effective as the same plant samples collected during the day. This may imply that the amount and type of repellent compounds produced by the plant species during the day when plants under go photosynthesis is not very different from those produced during the night when plants undergo only respiration. The essential oils from both the aerial parts of the plants (leaves and seeds) exhibited similar level of repellency for *O. suave* in dose-response studies. However, the amount of essential oil produced from the seeds was quite small as compared to that from the leaves.

The volatile oils from all test plants were effective at 20% test concentrations against *An. gambiae*. Although the essential oils of all candidate plants were repellent, the duration of complete protection was less than an hour. This suggests that faster loss of repellent effect is due to faster loss by evaporation rather than to a lower effectiveness of the repellent compounds. Appropriate formulations may extend the protection time. In a similar study in Thailand, the protection time of volatile oils from plants (turmeric, citronella grass and hairy basil) was significantly increased by incorporation of 5% vanillin (Tawatsin *et al.*, 2001). The use of controlled-release technology with topical repellents has provided extended protection against biting mosquitoes in the laboratory studies (Gupta and Rutledge, 1989).

The duration of protection exhibited by the essential oils of the candidate plants are comparable to the one reported for citronella oil against *Anopheles gambiae* (Trigg and Hill, 1996). Citronella oil has long been used in commercial preparations of repellents and is popular in India, though generally rated as less effective than repellents with synthetic active ingredients (Curtis *et al.*, 1987).

The repellency of essential oils of *O. americanum* was earlier reported against *Ae. aegypti*, *An. dirus* and *Cx. quinquefasciatus* in Thailand (Chokechaijaroenporn *et al.*, 1994; Tawatsin *et al.*, 2001). But, it has not been previously reported against the African malaria vector, *An. gambiae s.s.* In a mouse experiment by Chokechaijaroenporn *et al.* (1994), the oils of *O. americanum* showed significant protection for only about 15 minutes against *Ae. aegypti*. However, studies on human subjects by Tawatsin *et al.* (2001) reported complete protection for three hours against *Ae. aegypti* and *An. dirus* and for eight hours against *Cx. quinquefasciatus*. This shows wide variation in the sensitivity of different mosquito species to a given repellent. Accordingly, it is important to test the sensitivity of even closely related mosquito species to a given repellent. Indeed, several authors argued that repellent test data of a chemical agent obtained from one species of mosquito cannot be used to predict its effectiveness against another species (Rutledge *et al.*, 1978; Robert *et al.*, 1991).

L. camara flower extract in coconut oil was previously reported against *Ae. albopictus* Skuse and *Ae. aegypti* L. in India (Dua *et al.*, 1996), which gave 94.5% protection with a mean protection time of 1.9 hours. The essential oil of *Ocimum suave*, with eugenol as the active constituent also showed mean protection time of 105 minutes against *Ae. aegypti* (Chogo and Crank, 1981). These studies have shown somewhat better protection time than reported in this study, suggesting a lower sensitivity of *An. gambiae* compared to these species.

In this study, formulated DEET has shown the highest duration of protection as compared to the essential oils of all candidate plants. In a previous study, *An. gambiae* was found to be less sensitive to DEET than *An. stephensis* and *An. freeborni*, but more sensitive than *An. albimanus* (Coleman *et al.*, 1993). The result obtained from this study is similar to that reported for DEET against *An. stephensi* (Coleman *et al.*, 1993). *Anopheles gambiae* and *An. albimanus* also have been reported to be less sensitive to DEET in laboratory experiments than *Ae. aegypti* (Curtis *et al.*, 1987).

In summary, although, the essential oils from all candidate plants have shown repellent properties with high level of efficacy, all have shown reduced repellency after one hour of application. Appropriate criteria for repellent products are at least 80% reduction in biting for 6-8 hours after application (Barnard, 2000). Formulations studies on the oils of the plants need to be carried out to determine if their protection duration can be substantially improved before they can be considered as candidates for routine topical use.

CHAPTER 7

GAS CHROMATOGRAPHIC ANALYSIS OF VOLATILES TRAPPED BY THERMAL EXPULSION, DIRECT BURNING AND POTTED REPPELENT PLANTS

7.1. INTRODUCTION

Plant products play important role in traditional methods of protection against crop pests and disease vectors in Africa (Hassanali *et al.*, 1990). Several plant species have been traditionally used as mosquito repellents in Africa, and few of these plant species were tested for repellency against the main malaria vectors (White, 1973; Palsson, 1999a, b; Seyoum *et al.*, 2002a). However, previous studies have not shown the basis for the repellency of plant species in terms of the chemical constituents emitted from the plants, which could provide the requisite scientific rationale for future use in integrated vector management.

Thermal expulsion, direct burning, live intact potted plants and the essential oils of selected plants have shown repellency against the main African malaria vectors in the semi-field, field and laboratory studies (Chapters 3-6). The repellency of these plant species appear to be associated with the presence of one or more volatile repellent compounds emitted by the different alternative application methods described in the preceding chapters. As part of elucidating the basis of their repellent properties it was important to identify the major constituents emitted from the most efficacious plant species in different methods of application using gas chromatographic techniques. Furthermore, as the same plant species have shown differences in efficacy when thermally expelled and by direct burning (Chapter 3), it was also considered of interest to identify the major constituents emitted by the two alternative application methods.

Gas chromatography (GC) is one of the techniques for separation and analyses of volatile compounds and the most widely used analytical instrument. Mass spectrometry (MS) on the other hand is one of the most information-rich detectors. It provides data for qualitative identification of unknown compounds, as well as their quantification. This study used GC and GC-MS for the identification of the volatile compounds trapped by direct burning and thermal expulsion of selected repellent plants and reports the major constituents. The volatiles from live, intact potted plants were also trapped and analyzed to determine the major constituents of plants.

7.2. MATERIALS AND METHODS

7.2.1. Plant species

The plant species were selected based on their efficacy both in the semi-field and field tests (Chapters 3-5). *Ocimum americanum,* the most efficacious repellent plant in the study, was selected among the plant species tested in its intact potted form. Similarly, *Corymbia citriodora, O. kilimandscharicum* and *O. suave* were selected from plant species evaluated by thermal expulsion and direct burning. All these plant species have shown significant protection against the main Afro-tropical malaria vector, *An. gambiae s.l.* in both semi-field and field studies (Chapters 3 – 5).

7.2.2 Trapping volatiles from intact potted plants

Potted plants were placed inside a glass chamber. The traps, 0.5 g reverse-phase silica gel (C-18 packed in fine mesh bags), were suspended inside the glass chamber within the foliages for three consecutive days and nights. The volatiles were eluted from the traps using 10 ml of dichloromethane (DCM) and collected in glass vials and kept in deep freezer until used for analysis. Two ml of the samples were concentrated by blowing the samples in vials with nitrogen gas for analyses (GC and GC-MS).

7.2.3 Trapping volatiles by thermal expulsion and direct burning

A total of 80 g of plant samples (in four equally divided doses) were placed on the upper part of a thin metal plate of a modified traditional stove with burning charcoal to thermally expel the plant materials (Plate 7.1 A). Similarly, a total of 80 g of plant materials were directly placed on burning charcoal to trap the volatiles by direct burning (Plate 7.1 B). A glass funnel (15-cm diameter) was placed just above the metal plate or burning charcoal. The funnel was connected to the tube that led to the sample collection vial that contained 2 ml of DCM. The volatiles in plume mixtures passed through the funnel to the connecting tube and collected in a sample vial. Nitrogen gas was used to channel the volatiles to the sample vial via the connecting tube. The inside portions of the funnel and the connecting tube were also washed with 8 ml of DCM into the sample vial to make a total volume of 10 ml. The samples were kept in deep freezer until analyses.

Identification of the major constituents of volatiles trapped from selected candidate plants by thermal expulsion, direct burning and potted repellent plants were carried out using GC and GC-MS analyses as described in the general materials and methods sections (Chapter 2).

A

B

Plate 7.1. Devices for trapping volatile compounds by thermal expulsion (A) and direct burning (B)

7.3 RESULTS

The chromatograms for plant volatiles trapped from each candidate repellent plant and methods of application are shown in Figures 7.1 – 7.7. Identification information on the numbered peaks of the major components of the volatiles trapped from intact potted plants, by thermal expulsion and direct burning of repellent plants, is summarized in Table 7.1.

Thermal expulsion of *C. citriodora* produced citronellal, *iso*-pulegol and citronellol as the major constituents, and appeared at the retention time of 28.63, 28.98, and 31.28 minutes, respectively on the GC profile (Figure 7.1); on the other hand, direct burning of the same plant species produced, *cis* and *trans*-citral as the major constituents (Figure 7.2) at the retention time of 31.48 and 32.35 minutes, respectively. Camphor was the major constituent (31.10%) of thermally expelled *O. kilimandscharicum* (Figure 7.3) and appeared at the retention time 28.70 minutes but was not found in sample trapped by direct burning of the same plant species (Figure 7.4). Thermal expulsion of *O. suave* produced *trans*-methyl *iso*-eugenol with the retention time 39.35 minutes as one of the major constituents (Figure 7.5) but was not found by direct burning of the same plant species (Figure 7.6). The major constituent (83.43%) emitted from live potted plants of *O. americanum* was α-terpineol (Figure 7.7). The mass spectra of the individual major constituents are given in Appendix 6.

Table 7.1. Major compounds identified from volatiles trapped by thermal expulsion, direct burning and intact potted repellent plant

Plant species	Trapping method	Peak number	Retention time	Components	Percentage peak area
O. americanum	Potted plant	1	30.30	α-terpineol	83.43
		2	38.38	Caryophyllene	3.81
		3	40.25	Unidentified	10.47
C. citriodora	Thermal expulsion	1	22.3	Phenol	1.43
		2	28.48	Citronellal (cyclic)	3.72
		3	28.63	Citronellal	7.0
		4	28.98	*iso*-pulegol	5.20
		5	29.33	Unidentified	2.34
		6	31.28	Citronellol	5.95
		7	33.90	Unidentified	2.07
		8	35.00	Citronellyl acetate	1.50
		9	48.03	Phytol	1.26
		10	50.68	Palimitic acid	4.24

Table 7.1 continued

	Direct burning	1	28.58	Camphor	3.77
		2	31.48	*cis*-Citral (Neral)	21.40
		3	32.35	*trans*-Citral (Geranial)	40.32
		4	35.30	Unidentified	8.74
O. suave	Thermal expulsion	1	39.35	*trans*-methyl *iso*-eugenol	14.27
		2	86.88	Squalene	43.36
	Direct burning	1	35.45	Eugenol	1.78
		2	39.23	Unidentified	7.46
		3	86.48	Squalene	11.17
O. kilimandscharicum	Thermal expulsion	1	28.70	Camphor	31.10
		2	84.88	Squalene	3.50
	Direct burning	1	86.50	Squalene	68.14

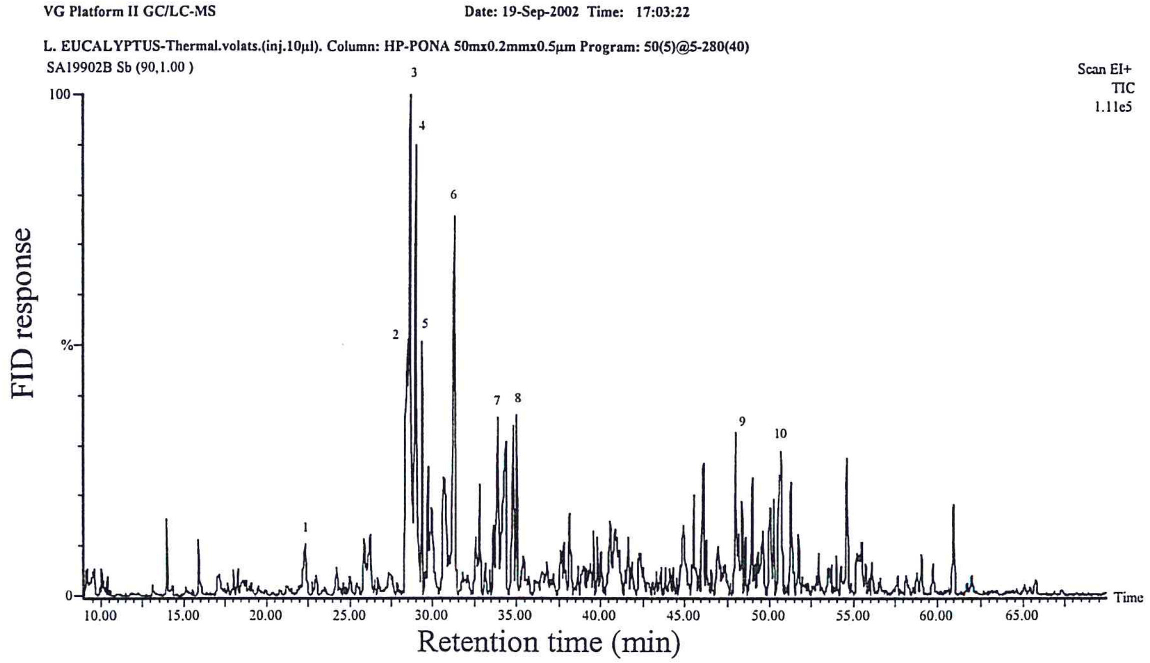


Figure 7.1 Chromatogram of volatiles trapped from *Corymbia citriodora* by thermal expulsion

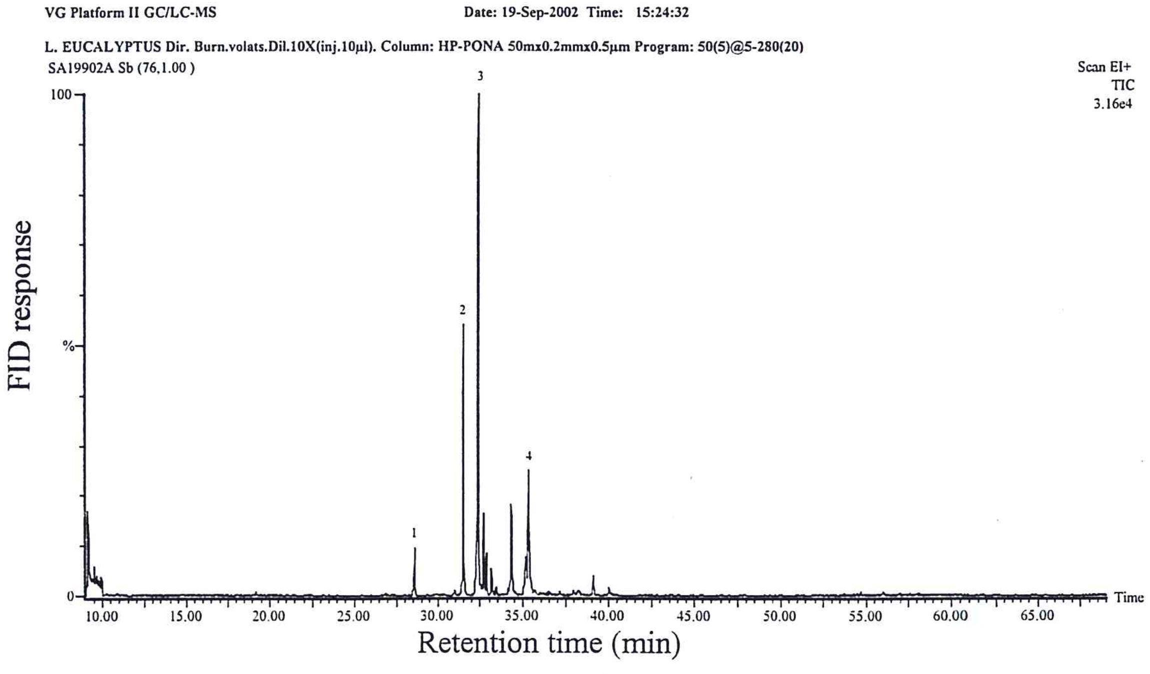


Figure 7.2 Chromatogram of volatiles trapped from *Corymbia citriodora* by direct burning

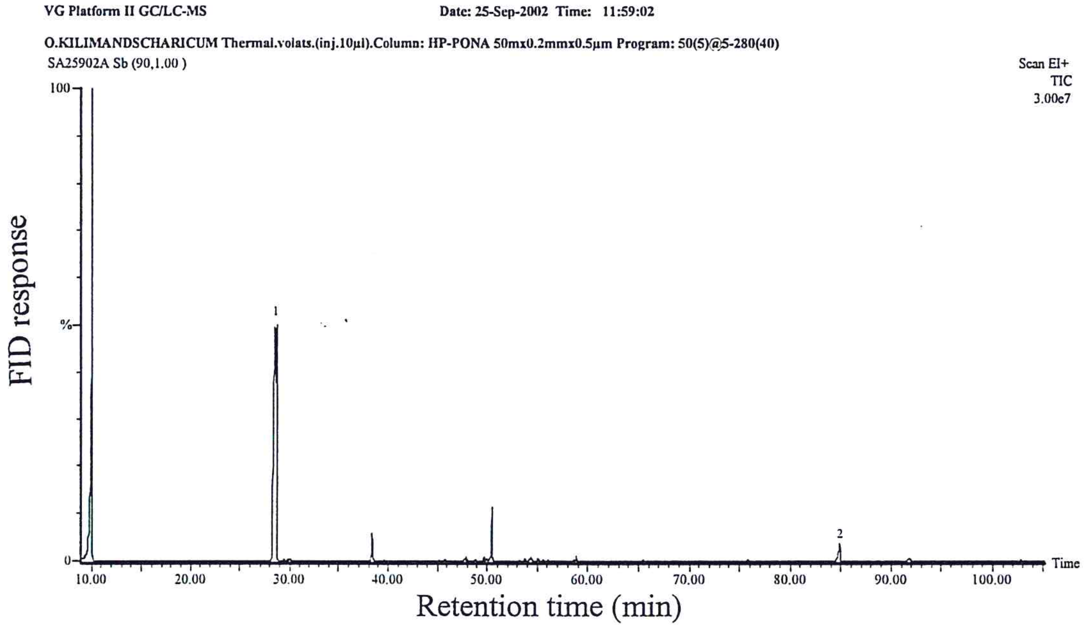


Figure 7.3 Chromatogram of volatiles trapped from *Ocimum kilimandscharicum* by thermal expulsion

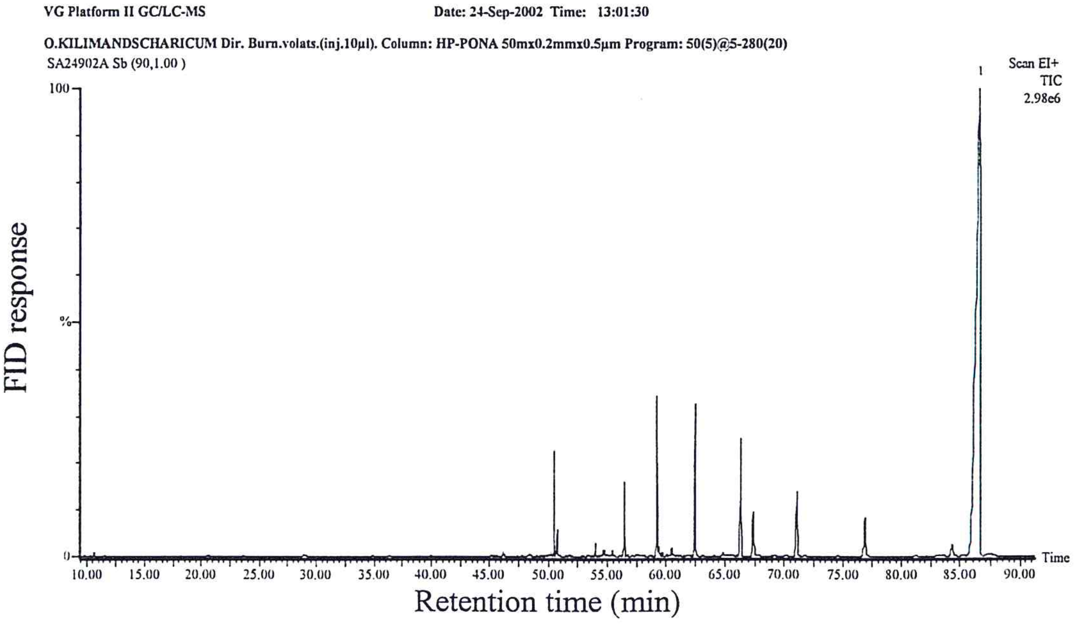


Figure 7.4 Chromatogram of volatiles trapped from *Ocimum kilimandscharicum* by direct burning

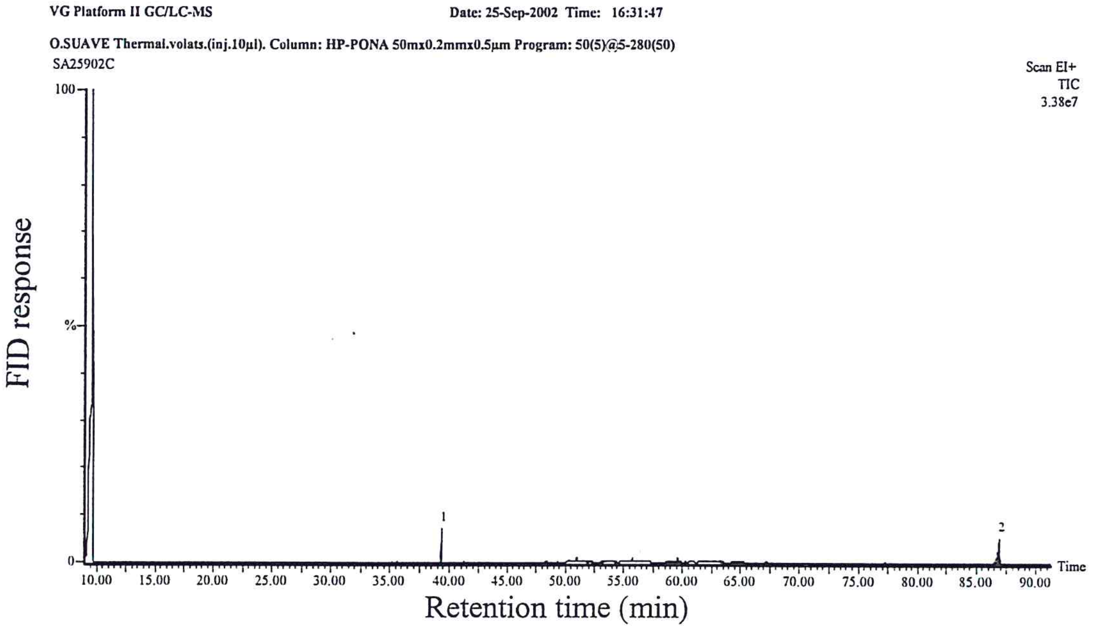


Figure 7.5 Chromatogram of volatiles trapped from *Ocimum suave* by thermal expulsion

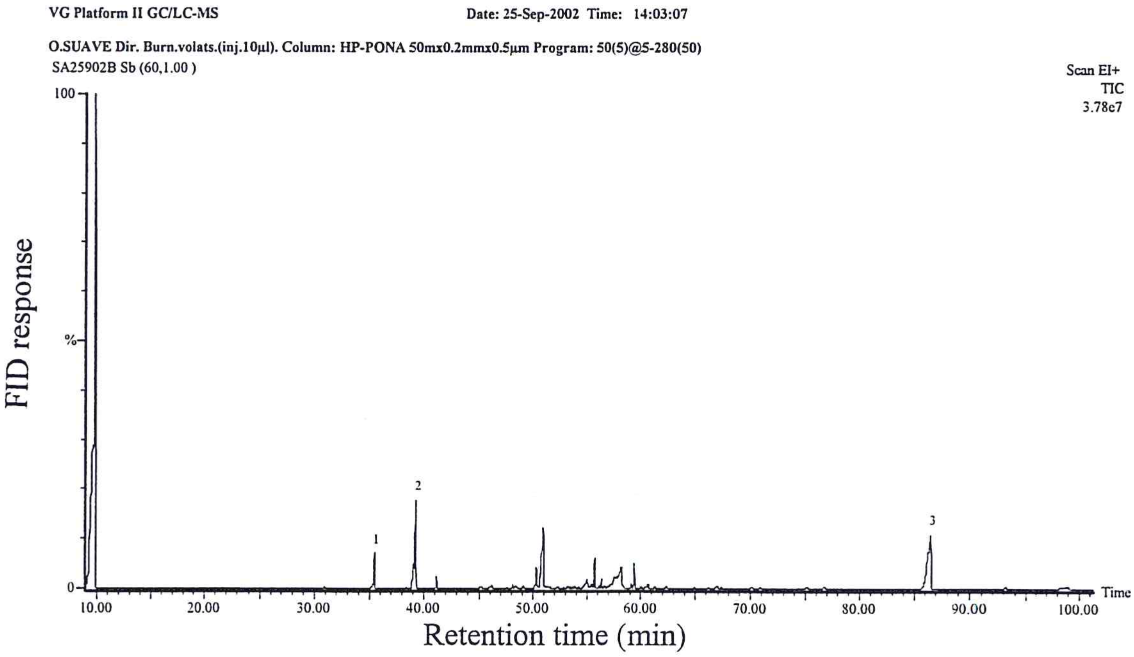


Figure 7.6 Chromatogram of volatiles trapped from *Ocimum suave* by direct burning

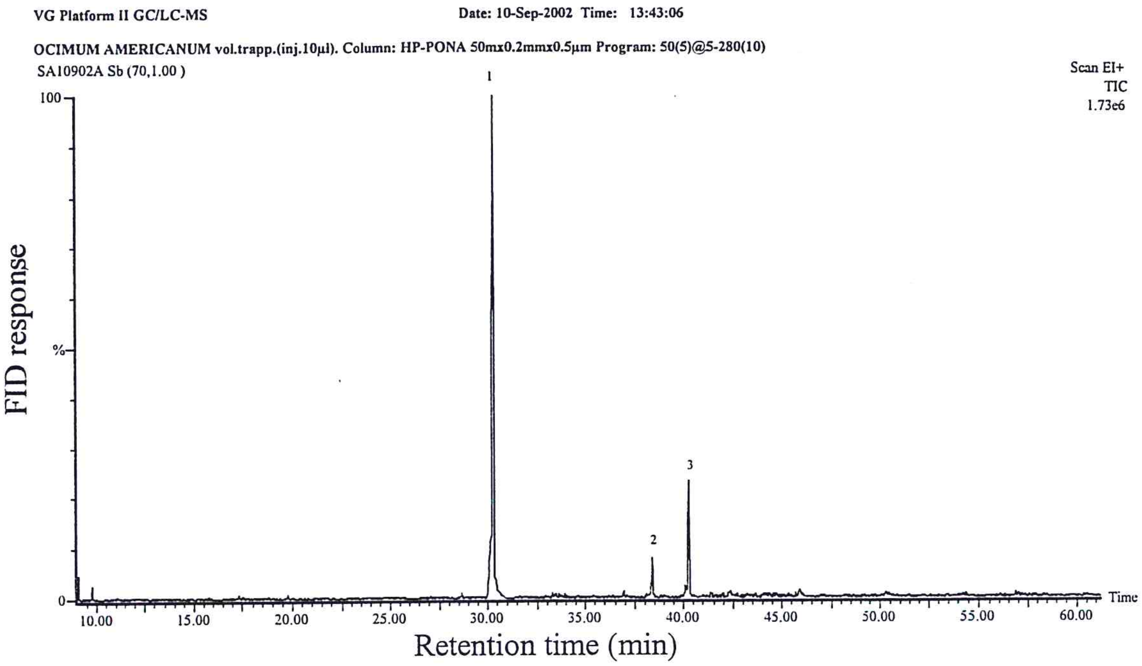


Figure 7.7 Chromatogram of volatiles trapped from intact potted plants of *Ocimum americanum*

7.4 DISCUSSION

Most of the compounds identified from the plant volatiles in this study are already known to have repellent properties to insects and have been previously reported as constituents of the essential oils of the same or different plant species (Chogo and Crank, 1981; Hwang *et al.*, 1985; Chokechaijaroenporn *et al.*, 1994; Thorsell *et al.*, 1998; Barasa *et al.*, 2002). This study showed that citronellal (10.72 %), *iso*-pulegol (5.20 %) and citronellol (5.95 %), are the major constituents of thermally expelled volatiles of *C. citriodora* (Figure 7.1). This is similar to the essential oil composition of *C. citriodora* (Barasa *et al.*, 2002). During thermal expulsion, a lot of moisture is released, which is likely to volatilise the major volatile components of the essential oils. In direct burning rapid dehydration of the plant material may make this difficult and, in any case, higher temperatures are likely to lead to rapid transformation and degradation of the plant constituents.

The main constituent of citronella oil is citronellal (Curtis *et al.*, 1991; Barasa *et al.*, 2002) or citronellol (Thorsell *et al.*, 1998) depending up on the plant variety. Citronella oil, in concentrations ranging from 0.05% to 15%, is used alone or in combination with cedarwood, lavender, peppermint, clove, eucalyptus, and garlic in a number of commercial insect repellent products (Fradin, 1998). However, these compounds have less potential (effective for less than an hour or 1 –2 hours protection time). However, thermal expulsion is a slow release method, and repeated application of the plants (every 30 minutes in the semi-field and every hour in the field tests) makes the plants more effective. In addition, additive or synergistic effects among the different constituents of thermally expelled volatiles may have improved the repellent effect of the plume mixtures.

Citronella oil is a popular repellent in India and is one of the few natural products being used in commercial repellents in Europe and North America (Curtis *et al.*, 1991). Citronella is as effective as dimethyl phthalate (DMP) when freshly applied, but it did not last as long (Curtis *et al.*, 1991). However, frequent reapplication of the repellent can partially compensate for this (Fradin, 1998). It has been available in the form of candle for many years and its effectiveness is largely limited to indoor areas (Gupta and Rutledge, 1994). A synthetic derivative of citronellal has been used as the active ingredient of a commercial repellent (Curtis *et al.*, 1991). Quwenling from the waste distillate after the extraction of the essential oils from *C. citriodora* has better repellency as compared to the essential oils. The principal active component in the residue is p-menthane-3-8-diol (50%) with additional *iso*-pulegol and citronellol (Collins *et al.*, 1993). However, there is no evidence for the presence of p-menthane-3-8 diol in the volatiles trapped either by thermal expulsion or direct burning of *C. citriodora* in this study. Thus, the other constituents such as *iso*-pulegol, citronellol and citronellal that have been found in fairly large quantities (Figure 7.1) in thermally expelled volatiles of *C. citriodora* may have been primarily responsible for the repellency of this plant species in the semi-field and field studies.

The major constituents emitted by thermal expulsion were different from direct burning of *C. citriodora.* Unlike thermal expulsion, the major constituent was citral when the plant volatiles trapped by direct burning. Citronellal, citronellol and citral are all reported from the essential oil of citronella at concentrations 1.9%, 19.5% and 4.7%, with complete protection time for < 1 hr, 1-2 hrs and 2-3 hrs, respectively against laboratory colony of *Ae. aegypti* when applied as a formulation on the hand (Thorsell *et al.*, 1998). However, the higher repellency exhibited by thermal expulsion than direct burning of leaves of *C. citriodora* against *An. gambiae* in the semi-field tests (Chapter 3) may be due to the blend effects of the major and other minor constituents or due to differential sensitivity of mosquito species to one of these compounds.

Dehydrogenation of citronellal from the leaves of *C. citriodora* by direct burning may have produced cis and trans isomers of citral. Camphor has not been so far known from the essential oils of *C. citriodora*, but was found in the volatiles from direct burning of this plant species (Figure 7.2). Cyclization of the cis and trans isomers of citral may have resulted in the formation of camphor in volatiles from directly burnt plant samples and, therefore, could be a byproduct of the two isomers.

Camphor is a major constituent of the essential oil of *O. kilimandscharicum* (Bekele and Hassanali, 2001). Interestingly, volatiles emitted by thermal expulsion of *O. kilimandscharicum* (Figure 7.3) in this study also showed the same compound as major constituent (31 %) but, direct burning of this plant species has not produced camphor (Figure 7.4), probably accounting for the lower repellency in the semi-field study. Direct burning of *O. kilimandscharicum* has only produced long chain hydrocarbons with retention time more than 50 minutes (Figure 7.4) as suggested by mass spectrometry.

Field studies (Chapter 5) showed that only thermal expulsion of this plant species significantly repelled *An. funestus*. Camphor alone or in combination with other constituents may have an effect on this species. Detailed studies of individual constituents or blends of compounds are required to elucidate the basis of the exceptional efficacy of thermally expelled volatiles of this plant species against *An. funestus* in contrast to the other plant species. Camphor from the essential oil of *Artemisia vulgaris* at 1.4 mg/cm^2 also showed 87 –97 % repellency against *Ae. aegypti* mosquitoes (Hwang *et al.*, 1985).

Trans-methyl *iso*-eugenol is the major volatile constituent from thermal expulsion of *O. suave* (Figure 7.5). Interestingly, the major constituent, eugenol

(58%), of *O. suave* essential oil from Kenya (Jembere, 1995) was detected only in trace amount. It was also found in small amount (1.78 %) in the volatiles from direct burning of the same plant species (Figure 7.6), and may account for low repellency of volatiles from direct burning of the plant in semi-field tests. However, methyl eugenol was also reported as the main constituent (56%) of the essential oil of *O. suave* from Kenya (Coomber and Cosgrove, 1946). On the other hand, the essential oil of *O. suave* from two different places in Tanzania was reported to contain 71.5% eugenol (Chogo and Crank, 1981) and 53% phenols (cited in White, 1973) as the major constituents. These variations may suggest the presence of different chemotypes in Africa. In the present study, whether *trans*-methyl *iso*-eugenol detected in thermally expelled volatiles arises from methyleugenol in the plant samples or formed by methylation during thermal treatment remains to be determined. Samples of the plant from different sources have not always given a similar result, and biological evaluation and chemical analyses of diverse collections of this plant species may be required prior to large-scale propagation for household protection in a given area.

The essential oil of *O. suave* from Tanzania with active constituent eugenol, has an excellent mosquito repellent and has moderate antimicrobial activity (Chogo and Crank, 1981). The mean protection time of eugenol was 105 minutes and longer than citronella oil tested at the same time that showed mean protection time of 75 minutes against *Ae. aegypti*. White (1973) also showed that when smeared on the legs, the juice *O. suave* reduced biting by caged *An. gambiae*. The repellency was attributed to the constituent eugenol (Chogo and

Crank, 1981), which is the major components of clove oil, and other essential oils reputed to have repellent properties (Curtis *et al.*, 1991). Methyleugenol is also known as mosquito repellent, and one of the commercial insect repellents (Chokechaijaroenporn *et al.*, 1994).

The results from the semi-field tests (Chapter 3) showed that thermal expulsion of *O. suave* repelled *An. gambiae s.s.* better than direct burning of the same plant species that produced higher quantities of eugenol. The results, therefore, suggests that the *trans-* methyl analogue of *iso*-eugenol could also be important for better activity in the volatiles produced by thermal expulsion, since this has been the major constituent of volatiles emitted by thermal expulsion. However, detailed studies are required on analogues to reveal precise structural requirements for optimal repellency of thermally expelled plant materials against mosquitoes. In general, the type and amount of repellent compounds produced by thermal expulsion and direct burning of plant species are different and could be the basis for the differences on the degree of repellency observed in the semi-field screening of potential candidate repellent plants.

Squalene was commonly found as one of the major constituents of volatiles of *O. kilimandscharicum* and *O. suave* by both thermal expulsion and direct burning. The compound has not been known as mosquito repellent. However, it is popularly sold and widely consumed as health supplement in many developed countries (Zhang *et al.*, 2002).

Intact potted plants of *O. americanum* released α-terpineol as a major constituent (Figure 7.7). α-Terpineol has been found also from the essential oil of

Lippia ukambensis, L. javanica, Plectranthus marruboides, Totradenia riparia and *Tarchonanthus camphorates*, and showed repellency (89 %) against *An. gambiae s.s* when the pure compound was tested at the concentration 10^{-2} gm/l applied on the human arms (Omolo, 2001). It is also a major constituent of brich/ pine tar oil and showed repellency against *Ae. aegypti* (Thorsell *et al.*, 1998). A closely related compound, terpinen-4-ol from the essential oil of *Artemisia vulgari*, has shown high level of repellency against *Ae. aegypti* and was as effective as dimethyl phthalate (Hwang *et al.*, 1985) .

In summary, most of the constituents trapped by thermal expulsion are closely similar to those reported from the essential oils of the same plant species. Thus, thermal expulsion of plant materials is an inexpensive way of applying plant materials to repel house entry mosquitoes by the rural communities in Africa. In conclusion, plant species contain various repellent compounds, which vaporize into air and repel mosquitoes when the plant volatiles are expelled thermally from simple modification of African traditional stove. The rural communities where the plant species are available and malaria is a major public health problem can therefore consider it as cost effective alternative application method for household protection against the main Afro-tropical malaria vectors.

CHAPTER 8

GENERAL DISCUSSION, CONCLUSION AND RECOMMENDATIONS

8.1 GENERAL DISCUSSION

Previous studies on mosquito repellents by and large seem to have concentrated on the extraction and synthesis of repellent compounds for either topical applications on the skin or treatments on fabrics (Curtis *et al.*, 1991). Relatively little has been known about the effectiveness of traditional ways of using insect repellent plants. This study has focused mainly on the development of simple and low-cost plant based alternative household protection methods based on traditional practices of repellent plants against the major Afro-tropical malaria vectors. The methods evaluated in this study show varying degree of efficacy and the more promising ones can easily be adopted by the rural communities in tropical Africa.

Differences in the sensitivity of mosquito species towards a given repellent were observed in this study. Both semi-field (Chapters 3 and 4) and field evaluations (Chapter 5) showed that live potted plants of *O. americanum* and *L. camara*, and thermal expulsion of *C. citriodora*, *O. kilimandscharicum* and *O. suave* significantly reduced domestic exposure by the major African malaria vector, *An. gambiae s.l.* However, only thermal expulsion of *O. kilimandscharicum* significantly reduced *An. funestus* under natural field condition. This is similar to the previous report by Walker *et al.* (1996) who reported lower sensitivity of *An. funestus* as compared to *An. arabiensis* to synthetic repellent, DEET, and differential sensitivity of mosquito species towards a given repellent (Curtis *et al.*, 1987). The major constituent of volatiles from thermally expelled *O. kilimandscharicum* is camphor, which may be primarily responsible for the effectiveness of the plant against *An. funestus*. Therefore, selection of appropriate or effective repellent plants for household protection should depend greatly upon the target species. Of special interest is the finding that all plant species tested by thermal expulsion in the field showed residual effect against *An. gambiae s.l.* Therefore, application of plant materials in the early hours of the evening can equally protect mosquito bites in the remaining duration of the night.

Apart from mosquito repellent property of the plant species reported in this study, some of the plants have other potential benefits to humans in various ways. *Ocimum kilimandscharicum* is traditionally used for the treatment of serious colds and coughs, abdominal pains, measles and mild diarrhea in children in many parts of East Africa (Kokwaro, 1976). It is also used and shown to be effective as grain protectants in East Africa (Jembere *et al.*, 1995). *Ocimum suave* also has a variety of indigenous uses as a body perfume and grain protectant (Hassanali *et al.*, 1990), and has been used for treating coughs, eye and ear complaints, and abdominal pains (Kokwaro, 1976). *Ocimum americanum* is also traditionally used in Ghana to control insect pests of stored grains and legumes (Belmain *et al.*, 2001). The essential oil from the leaves of *O. americanum* growing in the Democratic Republic of Congo was recently reported to have antibacterial activity (Cimanga *et al.*, 2002). Studies on the essential oils of *L.*

camara showed that the oil is rich in sesquiterpenes, which may be suitable for blending for enhancement of the longevity of the perfumery products (Misra and Laatsch, 2000). The essential oil of lemon eucalyptus, with its high content of citronellal, is one of the perfumery oils distilled in commercial scale from the *Eucalyptus* species (Mwangi *et al.*, 1982). In addition to yielding important essential oils from its leaves, lemon eucalyptus is grown for its excellent sawn timber. It is also noteworthy that Australian/ Tasmanian eucalyptus trees have long been reported to be useful in the elimination of mosquito breeding places (Klocke *et al.*, 1987). In the past, Tasmanian blue gum trees were introduced into the coastal and wet regions of southern and western Europe (especially in the Mediterranean regions of Italy and Spain), Africa, the middle east, India, and many other parts of the world with many large breeding places of the malaria mosquitoes. These very large and rapidly growing trees, which have large water requirements, have been found to be extremely useful in draining marshy areas and in reclaiming swampy lands, making them mosquito-and malaria free areas within a few years (Klocke *et al.*, 1987). These multipurpose uses of mosquito repellent plants can help to enhance the willingness of rural communities to cultivate the plants and to adopt potential plant-based mosquito control technologies in the future.

This study also has demonstrated the merit of using green house based semi-field experimental system for screening mosquito repellent plants. The system has some clear advantages such as: 1) external factors can be controlled and easily quantified; 2) it avoids potential risks of infective bites of malaria mosquitoes; and 3) large number of candidate plants can be screened in a short period of time. Furthermore, experiments can be conducted all-year round, with fixed numbers of mosquitoes of known age in a malaria parasite-free environment. In this semi-field system, within a year of nearly continuous experimentation, the repellency of eight plant species and three combinations were evaluated through thermal expulsion or direct burning (Chapter 3) and nine species and two combinations were tested in intact potted form (Chapter 4).

The overall similarity of the major constituents of volatiles from thermally expelled plants and those reported from the essential oils of plants showed that the same repellent compounds may account for the repellency of plants by this technique both in the semi-field and field tests. Thermal expulsion of plants is, therefore, cheap and effective for alternative use by the rural communities against the major African malaria vectors.

This study has examined a limited number of plants for mosquito repellency. Screening of a larger profile of plants selected from ethnobotany or from taxonomic considerations may help to identify more effective plants. The use of such plants in live potted form, or by thermal expulsion of the foliage, can significantly reduce house-entry by malaria vectors. These tools can augment the protection offered by bednets at household level at minimal cost; particularly during the early hours of the evening before bednet occupancy commences.

The potential integration of selected candidate repellent plants and zooprophylaxis in a 'push-pull' model can be further explored in areas where zoophilic mosquitoes such as *An. arabiensis* are important vectors of malaria. However, zooprophylactic effect of cattle against *An. arabiensis* can be variable in different ecological settings and variable results have been reported from different studies (Hadis *et al.*, 1997; Bogh *et al.*, 2001; Habtewold *et al.*, 2001; Minakawa *et al.*, 2002; Seyoum *et al.*, 2002c). It is, therefore, important to first elucidate the impact of domestic cattle keeping on the human vector contact of *An. arabiensis* and malaria transmission in different ecological settings.

8.2 CONCLUSIONS

1. The semi-field experimental system developed in this study is a promising alternative for screening large number of candidate repellent plants in short periods of time.

2. The use of locally available repellent plants and modifications of traditional practices represented by thermal expulsion of plant materials and live, intact potted plants have reduced domestic exposure to African malaria vector mosquitoes. The methods developed are effective and appropriate for households in many developing countries that do not have sufficient incomes to pay for insecticide-based vector control.

3. Thermal expulsion of plants is generally better than the traditional direct burning of plants in reducing biting by *An. gambiae s.s.* for most candidate repellent plants

4. Both semi-field and field studies showed that *C. citriodora* and *O. americanum* are the most effective repellents among plant species tested by thermal

expulsion and in intact potted form, respectively, against *An. gambiae s.l.* However, only thermal expulsion of *O. kilimandscharicum* significantly reduced *An. funestus* in field studies.

5. The essential oils of plant samples collected during the day are equally effective as plant samples collected during the night.

6. The results presented in this thesis demonstrate the scientific rationale and objective basis for future incorporation of thermal expulsion of *C. citriodora, O. kilimandscharicum* and *O. suave*, and intact potted plants of *O. americanum* into integrated vector management programmes in rural communities where malaria is a major public health problem and transmission is mediated by the major malaria vector *An. gambiae s.l.*

8.3 RECOMMENDATIONS

The following recommendations are made from this study:

1. Large-scale application studies should be carried out to integrate plant-based mosquito repellents with other vector control tools such as insecticide treated or untreated bed nets in integrated vector control programmes.
2. The potential of mosquito repellent plants as a 'push' agent and zooprophylaxis as a 'pull' agent can be studied in controlled field

experiments and in appropriate ecological settings to develop a 'push-pull' tactic to further enhance the level of protection conferred by plants either in intact potted form or by thermal expulsion.

3. All plant species evaluated by thermal expulsion have shown residual effects in field trials. Therefore, the possibilities on cumulative effect of repellents by continuous applications over long period of time should be studied for greater efficacy.

4. The development of plant-based mosquito coils should be considered using plant species that have shown significant repellency by thermal expulsion both in the semi-field and field studies.

5. Formulations of essential oils of selected repellent plants can improve the duration of protection, and therefore can be explored for product development.

6. Other plant species could have major constituents similar to those reported in this study and others may similarly have different repellent constituents; and therefore, more plant species need to be screened to discover a large profile of plants that are potentially useful in mosquito control.

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Appendix 1. Questionnaire used for the ethnobotanical study on the traditional use of mosquito repellent plants in western Kenya

Name interviewer: Date of Interview:

Sub location: Village:

Reason for early ending……………………………………………..

1. Are you a resident in the village? ☐ **Yes** ☐ **No (end)**
2. Name of respondent……………………………………………
3. Sex of respondent: ☐ **Man** ☐ **Women**
4. Marital status ☐ **Married** ☐ **Single**
5. Status in homestead: ☐ **Head** ☐ **Son** ☐ **Daughter**
   ☐ **Wife** ☐ **Other**……………………..
6. How old are you? ☐ **<25** ☐ **25–40** ☐ **41-55** ☐ **> 55**
7. Circle the level of education:

   Standard **0** **1** **2** **3** **4** **5** **6** **7** **8**

   Form **I** **II** **III** **IV** **Other**…………………

*8. Occupation: ☐ **Farming** ☐ **Fishing**

☐ **House wife** ☐ **Casual employment**

☐ **Regular employment** ☐ **Trading**

☐ **Civil servant** ☐ **Traditional healer**

☐ **Other……………………**

9. Tribe: ☐ **Luo** ☐ **Other**………………………..

* Tick more than one if appropriate.

Appendix 1 continued

10. Do you use plants to keep away insects? ☐ **Yes** ☐ **No**

11. What are the names of the plants you are using to keep away insects?

a) Which part of these plants are you using?

b) To which insects are you using these plants?

c) How do you use these plants?

d) How frequently do you use or apply these plants? (indicate per day, week, month or year)

Name of plant:..			
Part of plant	**Insect**	**Method of use**	**Frequency**

Appendix 1 continued

Name of plant:..

Part of plant	**Insect**	**Method of use**	**Frequency**

Name of plant:..

Part of plant	**Insect**	**Method of use**	**Frequency**

Appendix 1 continued

12. Do you know any other plant that you don't use that keep insects away?

☐**Yes** ☐**No**

13. What are the names of these plants?

a) Which part of these plants is used?

b) To which insects are these plants being used?

c) How are these plants being used?

d) How frequently are these plants being used? (indicate per day, week, month or year)

Name of plant:………………………..…………………			
Part of plant	**Insect**	**Method of use**	**Frequency**

Appendix 1 continued

If the respondent use plants against mosquitoes.

Name of plant:...

Part of plant	**Insect**	**Method of use**	**Frequency**

Name of plant:...

Part of plant	**Insect**	**Method of use**	**Frequency**

Appendix 1 continued

14. If not mentioned any thing about mosquitoes before,

Do you know the names of any plants that are used to specifically keep away mosquitoes? ☐ **Yes** ☐ **No**

a) What are the names of these plants?

b) Which part of these plants are used?

c) To which insects are these plants being used?

d) How are these plants being used?

e) How frequently are these plants being used? (indicate per day, week, month or year)

Name of plant:...			
Part of plant	**Insect**	**Method of use**	**Frequency**

15. For what reasons you are not using these plants?

Reasons..

....

Appendix 1 continued

16. Do you know if your parents/ grand elders used any plant to keep insects away? **Yes** ☐ **No** ☐

a) what are the names of these plants?

b) Which part of these plants are used?

c) To which insects are these plants being used?

d) How are these plants being used?

e) How frequently are these plants being used? (indicate per day, week, month or year)

Name of plant:………………………..……………………			
Part of plant	**Insect**	**Method of use**	**Frequency**

17. Remarks____

____

____

____

APPENDIX 2. DATA SHEET FOR THE EVALUATION OF PLANTS IN SEMI-FIELD EXPERIMENTAL HUTS

Date of test__________________________ Plant's name_________________________

Method of application__________________ Number of mosquitoes released__________

Period recapture_______________________

Experimental hut A/B__________________ Control hut A/B______________________

Hours	**Numbers recaptured**	
	Person "A"	**Person "B"**
8:00 – 8:30		
8:30 – 9:00		
9:00 – 9:30		
9:30 – 10:00		
10:00 – 10:30		
10:30 – 11:00		
11:00 – 11:30		
11:30 – 12:00		
Sub total / Person		
Total catch (MLC)		
Total inside hut		
Total outside hut		
Total not recaptured		

Remarks____

Appendix 3: **Abstract of paper published**: Traditional use of mosquito repellent plants in Western Kenya and their evaluation in semi-field experimental huts against *Anopheles gambiae*: ethnobotanical studies and application by thermal expulsion and direct burning. *Transactions of the Royal Society of Tropical Medicine and Hygiene. 96*: 225-231.

Ethnobotanical survey in two communities in Western Kenya revealed that the most commonly known repellent plants were *Ocimum americanum* L. (64.1%), *Lantana camara* L. (17.9%), *Tagetes minuta* L. (11.3%) and *Azadirachta indica* A. Juss (8.7%) on Rusinga Island and *Hyptis suaveolens* Poit. (49.2%), *L. camara* (30.9%) and *Ocimum basilicum* L. (30.4%) in Rambira. Direct burning of plants is the most common method of application for *O. americanum* (68.8%), *L. camara* (100%) and *O. basilicum* (58.8%). Placing branches or whole plants inside houses is most common for *H. suaveolens* (33.3 & 57.8% for the respective locations), *A. indica* (66.7 & 100%), and *T. minuta* (54.8 & 56.0%). The repellency of plants suggested by the ethnobotanical survey and other empirical information was evaluated against the malaria vector *Anopheles gambiae sensu stricto* Giles in experimental huts within a screen-walled greenhouse. Thermal expulsion and direct burning were tested as alternative application methods for the selected plants *O. americanum*, *Ocimum kilimandscharicum* Guerke, *Ocimum suave* Willd., *L. camara, A. indica, H. suaveolens*, *Lippia ukambensis* Spreng and *Corymbia citriodora* Hook. When thermally expelled, only *H. suaveolens* failed to repel mosquitoes, whereas the leaves of *C. citriodora* (74.5%, $P < 0.0001$), leaves and seeds of *O. suave* (53.1%, $P < 0.0001$) and *O. kilimandscharicum* (52.0%, $P < 0.0001$) were the most effective. Leaves of *C. citriodora* also exhibited the highest repellency (51.3%, $P < 0.0001$) by direct burning followed by leaves of *L. ukambensis* (33.4%, $P = 0.0004$) and leaves and seeds of *O. suave* (28%, $P = 0.0255$). The combination of *O. kilimandscharicum* with *L. ukambensis* repelled 54.8% of mosquitoes ($P < 0.0001$) by thermal expulsion. No combination of plants increased repellency by either method. The semi-field system described appears a promising alternative to full-field trials for screening large numbers of candidate repellents without risk of malaria exposure.

Appendix 4: **Abstract of paper published**: Repellency of live potted plants against *Anopheles gambiae* from human baits in semi-field experimental huts. *American Journal of Tropical Medicine and Hygiene. 67*: 191-195.

The repellency of potted plants against the malaria vector *Anopheles gambiae sensu stricto* Giles was quantified in experimental huts under semi-field conditions inside a screen-walled greenhouse. *Ocimum americanum* Linnaeus (Labiatae), *Lantana camara* L. (Verbenaceae), and *Lippia ukambensis* Spreng (Verbenaceae) repelled on average 39.7 [CI: 29.6 – 48.4], 32.4 [CI: 19.7 – 43.1], and 33.3 [CI: 21.5 – 43.3] % of the mosquitoes respectively ($P < 0.0001$ for all treatments) as determined by logistic regression, allowing for variations associated with different bait hosts, sampling huts and replicate test nights. In contrast, *Ocimum kilimandscharicum* Guerke (Labiatae), *Ocimum suave* Willd. (Labiatae), *Corymbia citriodora* Hook (Myrtaceae), *Azadirachta indica* A. Juss (Meliaceae), *Tagetes minuta* L. (Asteraceae) and *Hyptis suaveolens* Poit. (Lamiaceae) did not significantly repel mosquitoes. The combination of *O. americanum* with either *L. camara* or *L. ukambensis* repelled 31.6 [CI: 19.7 – 41.7] and 45.2 [CI: 34.7 – 54.0] % of the mosquitoes respectively ($P < 0.0001$ for both treatments). This study is the first to show that live, intact plants can reduce domestic exposure to malaria vector mosquitoes. As such, they may represent a new, sustainable and readily applicable malaria vector control tool for incorporation into integrated vector management programs.

Appendix 5: **Abstract of paper accepted**: Field efficacy of thermally expelled or live potted repellent plants against African malaria vectors in western Kenya. *Tropical Medicine and International Health*

Objective To estimate the effectiveness of live potted plants and thermal expulsion of plant materials in repelling African malaria vectors in traditional houses in western Kenya.

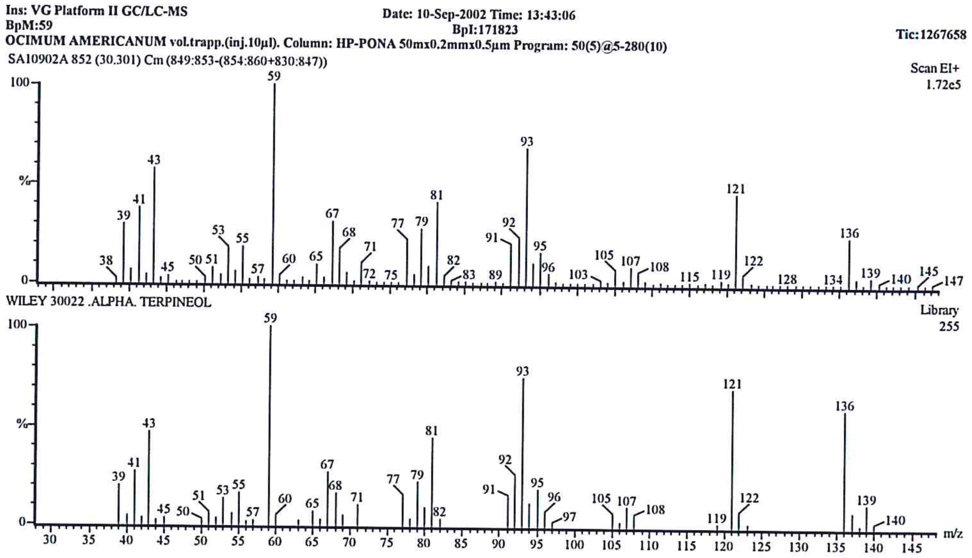
Methods *Ocimum americanum*, *Lantana camara* and *Lippia ukambensis* were tested in live, intact potted form whereas leaves of *Corymbia citriodora*, leaves and seeds of *O. kilimandscharicum* and *O. suave* were tested by thermal expulsion from modified traditional stoves. A latin square design was applied for randomly assigning the treatment and control plants to experimental houses over different nights.

Results All plant species showed significant repellency against *Anopheles gambiae sensu lato* Giles (Diptera: Culicidae) (81.5% *An. arabiensis* Patton and 18.5% *An. gambiae sensu stricto* Giles), the main vectors of malaria in Africa, with the highest repellency by *C. citriodora* (48.71 %, $P < 0.0001$) followed by an equal level of repellency of *O. kilimandscharicum* and *O. suave* (44.54%, $P = 0.001$) during application of plant material by thermal expulsion. All the three plant species also showed residual effect against *An. gambiae s.l.* with 36 to 44% repellency post-application period (22.30 – 06.30 hrs) following a period of thermal expulsion. Similarly, intact potted plants of *O. americanum* and *L. camara* repelled *An. gambiae s.l.* significantly (37.91%, $P = 0.004$; and 27.22%, $P = 0.05$, respectively). Thermal expulsion of leaves and seeds of *O. kilimandscharicum* significantly repelled *An. funestus* Giles, though none of the potted plants repelled this species.

Conclusion Both methods of application may offer cost-effective alternatives as additional means of household protection, and a useful complement to bednets, particularly for the early part of the evening before bedtime.

Appendix 6. Mass spectra of compounds identified from volatiles trapped by thermal expulsion, direct burning and intact potted repellent plant with reference to the WILEY library

ALPHA TERPINEOL (852) - SA10902A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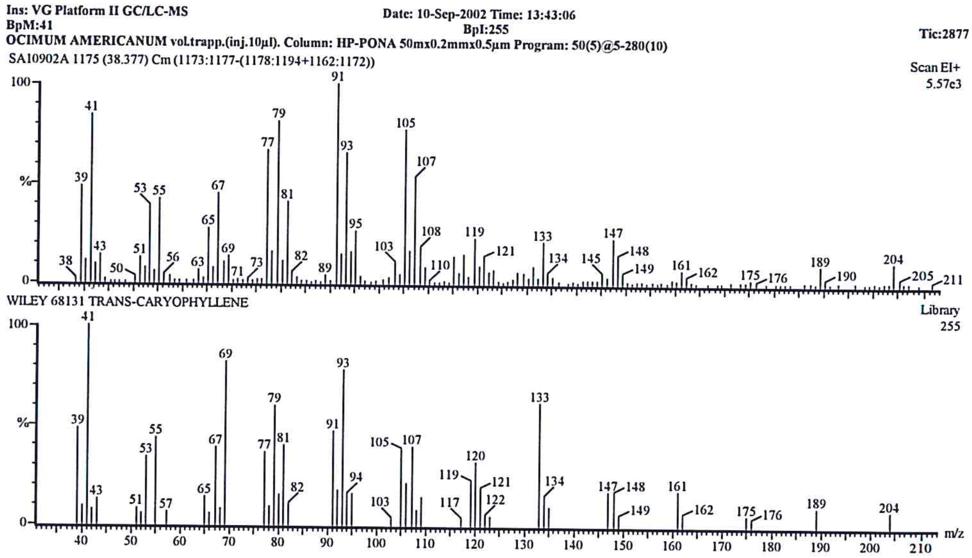
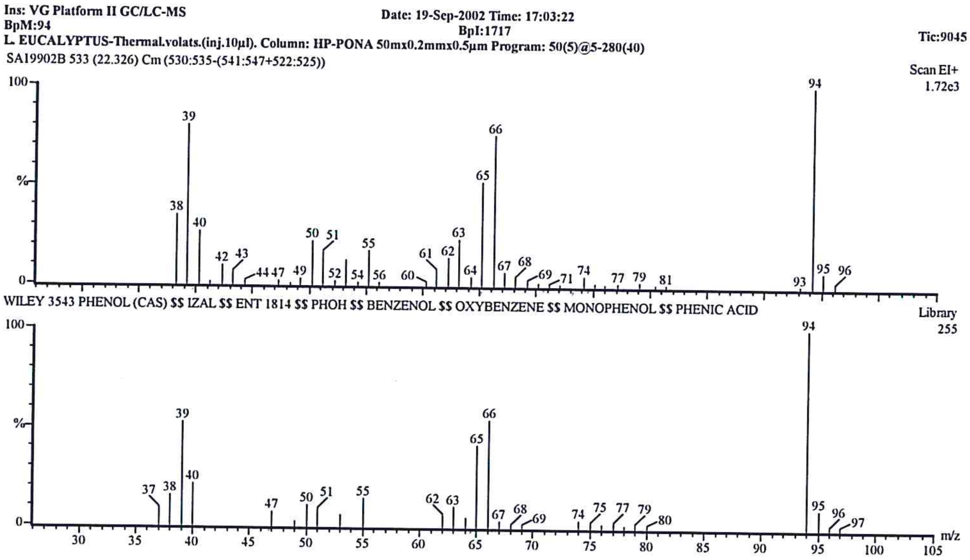
OCIMUM AMERICANUM vol.trapp.(inj.10µl). Column: HP-PONA 50mx0.2mmx0.5µm Program: 50(5)@5-280(10)
SA10902A 852 (30.301) Cm (849:853-(854:860+830:847))
Scan EI+
1.72e5
WILEY 30022 .ALPHA. TERPINEOL
Library
255

CARYOPHYLLENE (1175) - SA10902A

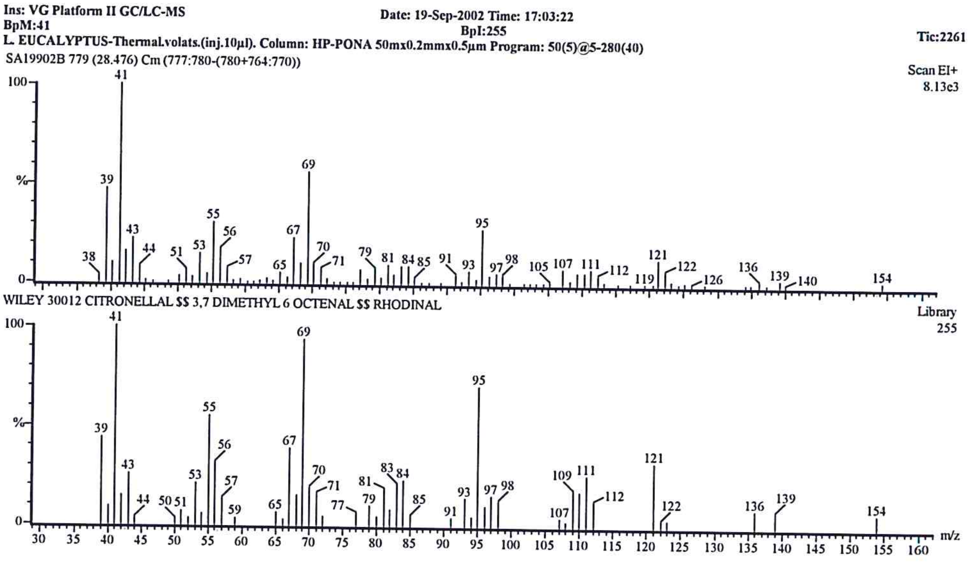

Ins: VG Platform II GC/LC-MS **Date: 10-Sep-2002 Time: 13:43:06**
BpM:41 **BpI:255** **Tic:2877**
OCIMUM AMERICANUM vol.trapp.(inj.10µl). Column: HP-PONA 50mx0.2mmx0.5µm Program: 50(5)@5-280(10)
SA10902A 1175 (38.377) Cm (1173:1177-(1178:1194+1162:1172))
Scan EI+
5.57e3
WILEY 68131 TRANS-CARYOPHYLLENE
Library
255

Appendix 6 continued

PHENOL (533) - SA19902B

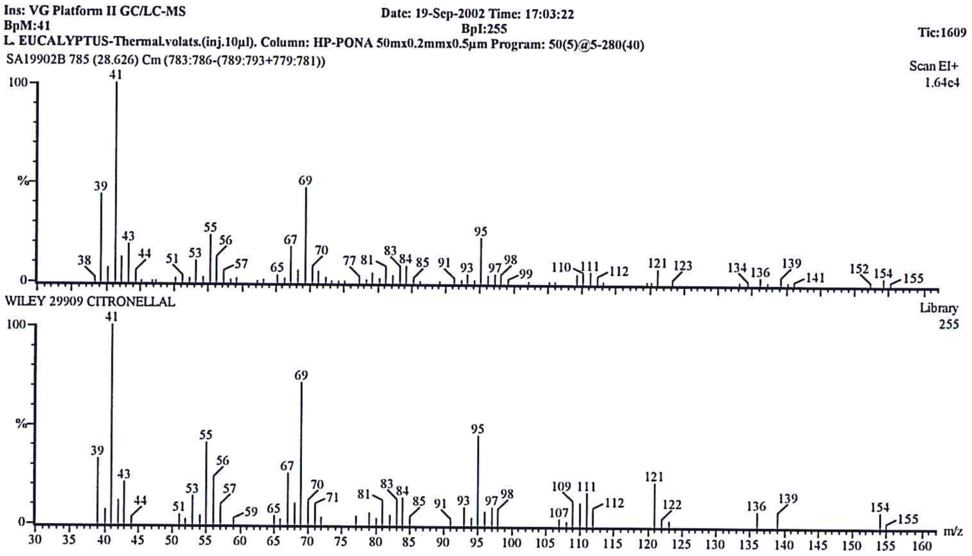


CITRONELLAL (779) - SA19902B

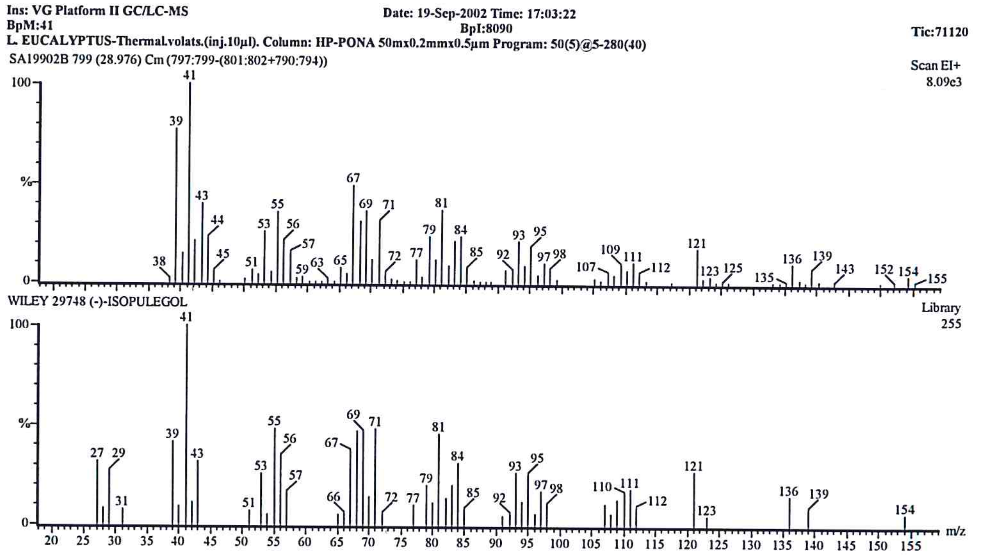


Appendix 6 continued

CITRONELLAL (785) - SA19902B

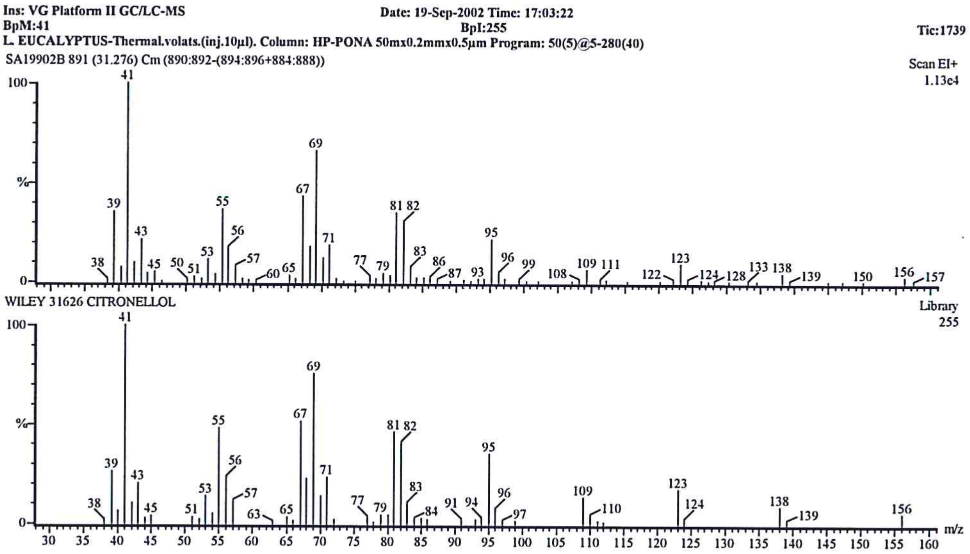


(-)-ISOPULEGOL (799)- SA19902B

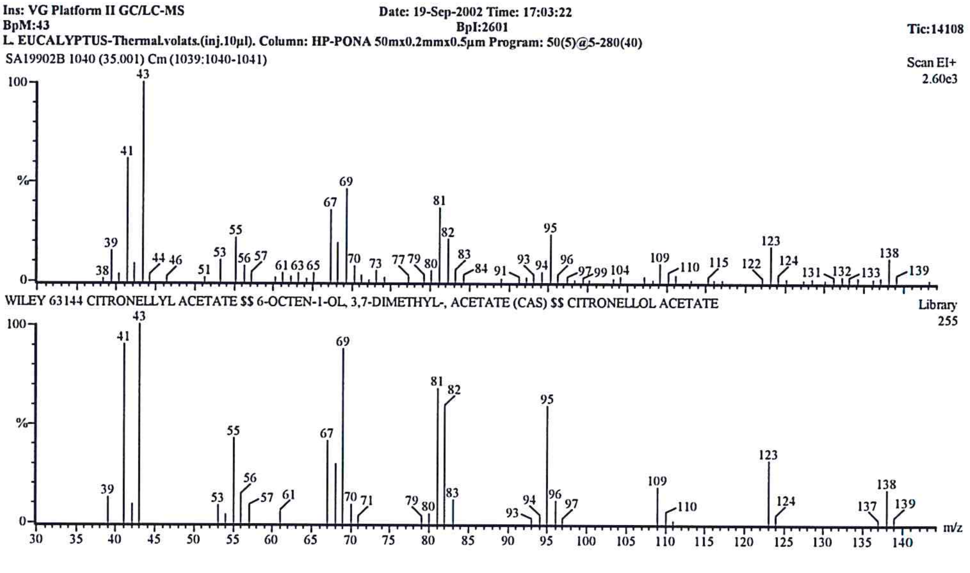


Appendix 6 continued

CITRONELLOL (891) - SA19902B

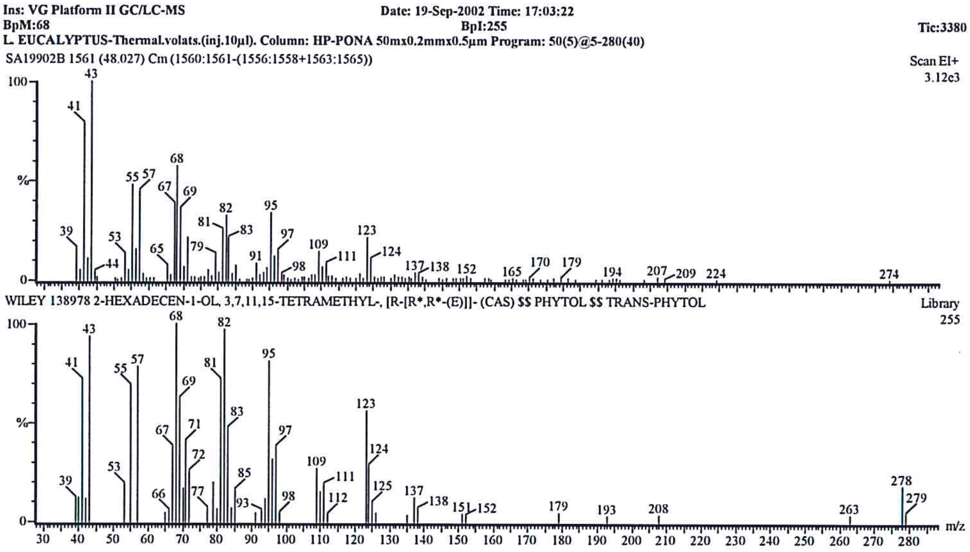


CITRONELLYL ACETATE (1040)- SA19902B

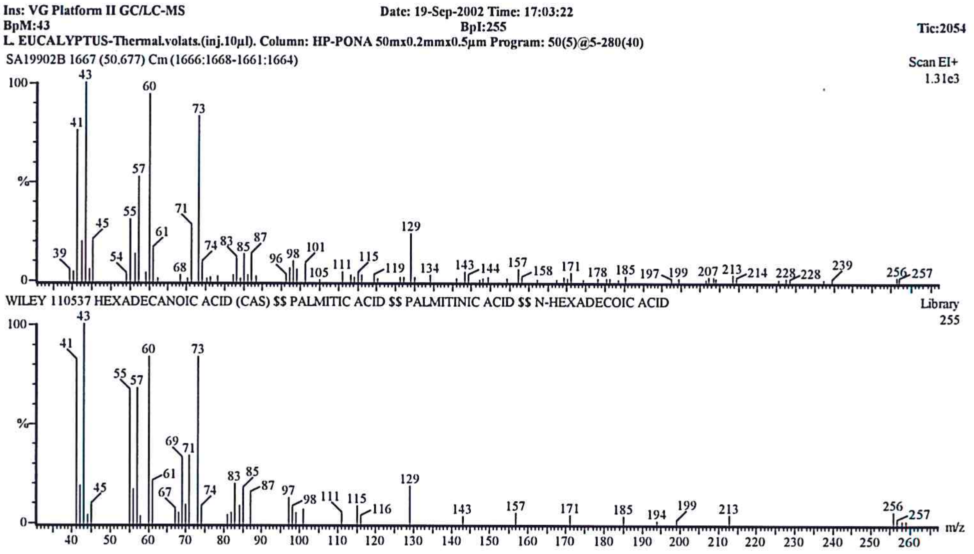


Appendix 6 continued

PHYTOL (1561) - SA19902B

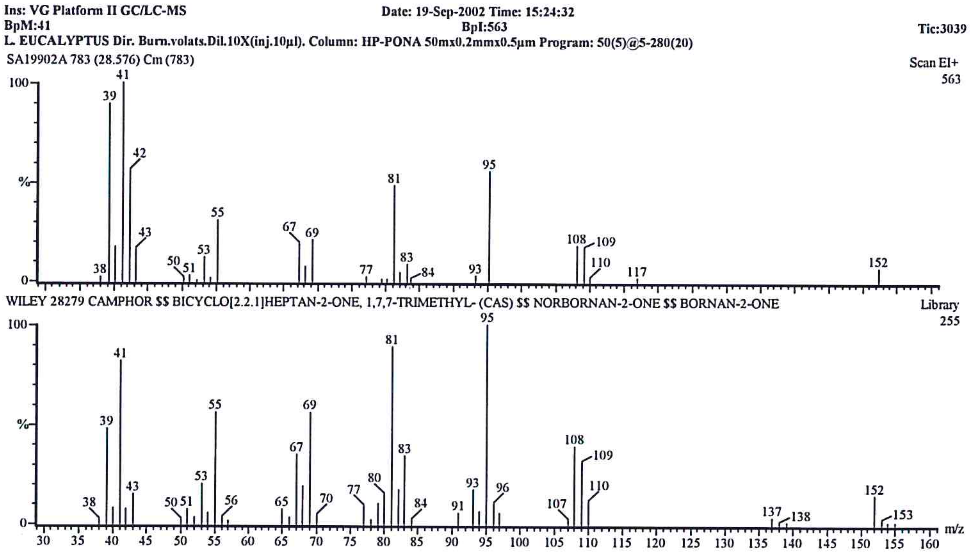


PALMITIC ACID (1667) - SA19902B



Appendix 6 continued

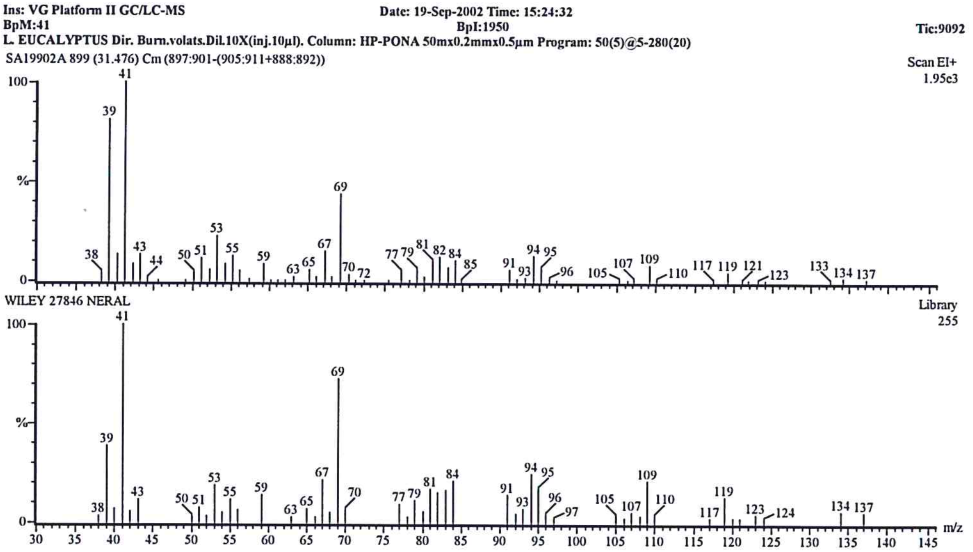
CAMPHOR (783) - SA19902A



Ins: VG Platform II GC/LC-MS **Date: 19-Sep-2002 Time: 15:24:32**
BpM:41 **BpI:563** **Tic:3039**
L. EUCALYPTUS Dir. Burn.volats.Dil.10X(inj.10µl). Column: HP-PONA 50mx0.2mmx0.5µm Program: 50(5)@5-280(20)
SA19902A 783 (28.576) Cm (783)
Scan EI+
563

WILEY 28279 CAMPHOR $$ BICYCLO[2.2.1]HEPTAN-2-ONE, 1,7,7-TRIMETHYL- (CAS) $$ NORBORNAN-2-ONE $$ BORNAN-2-ONE
Library
255

CIS-CITRAL (899) - SA19902A



Ins: VG Platform II GC/LC-MS **Date: 19-Sep-2002 Time: 15:24:32**
BpM:41 **BpI:1950** **Tic:9092**
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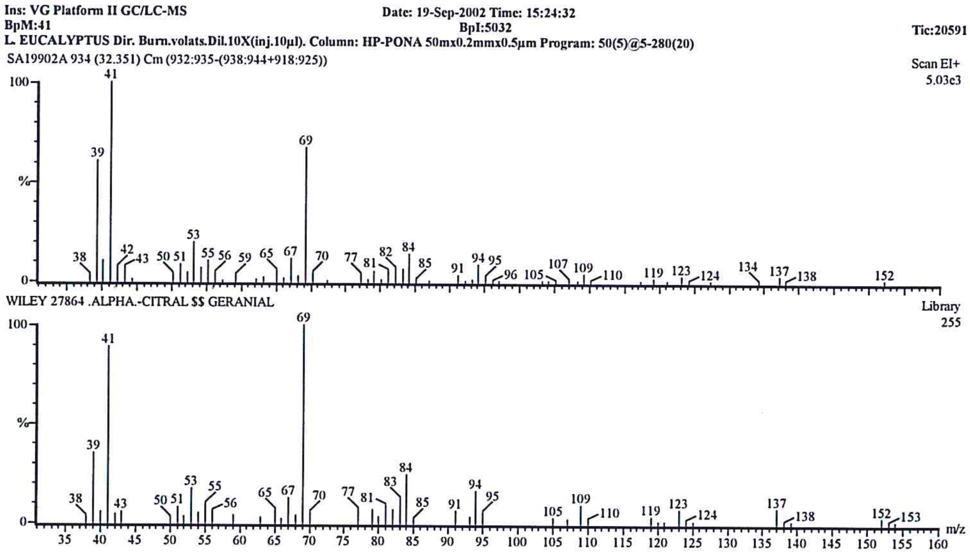
SA19902A 899 (31.476) Cm (897:901-(905:911+888:892))
Scan EI+
1.95e3

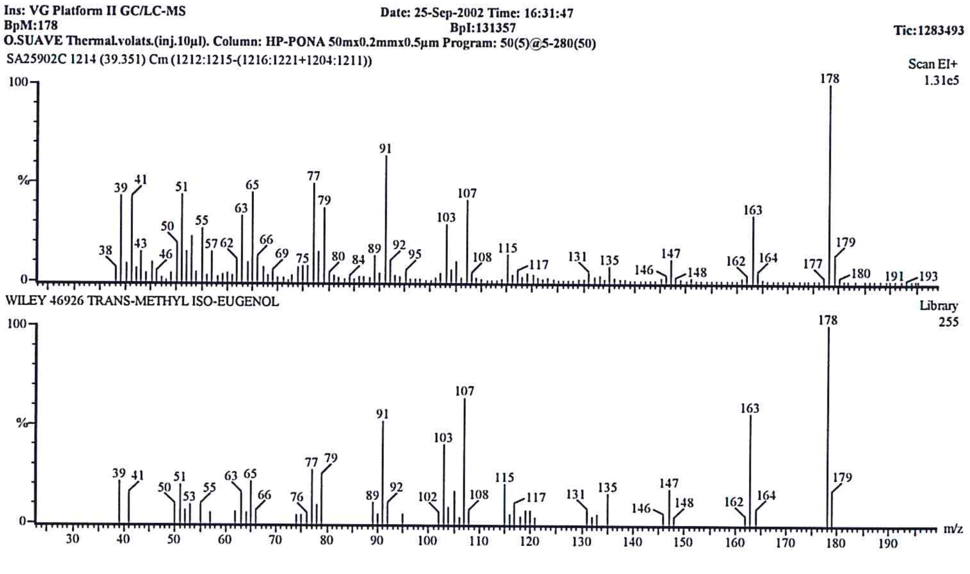
WILEY 27846 NERAL
Library
255

Appendix 6 continued

TRANS-CITRAL (934) - SA19902A

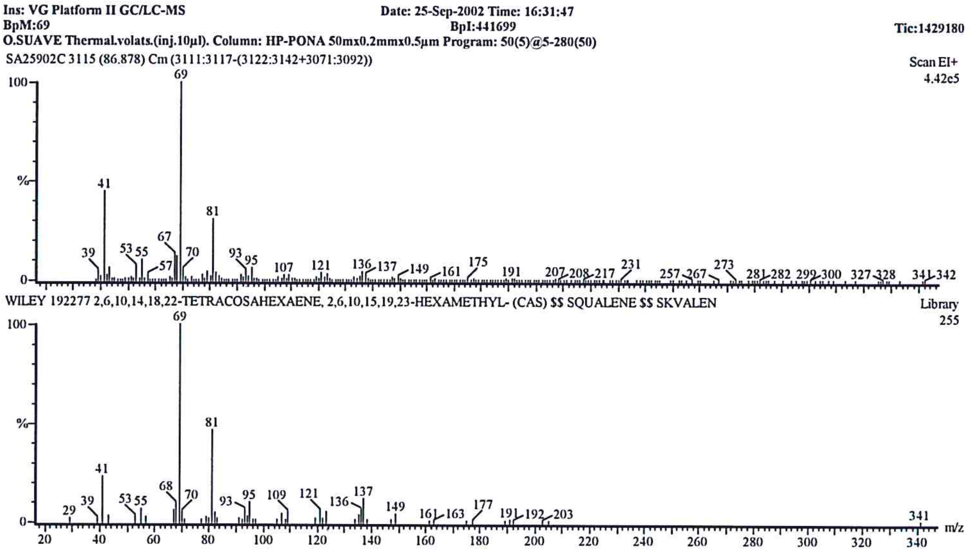


TRANS-METHYL ISO-EUGENOL (1214) - SA25902C



Appendix 6 continued

SQUALENE (3115) - SA25902C



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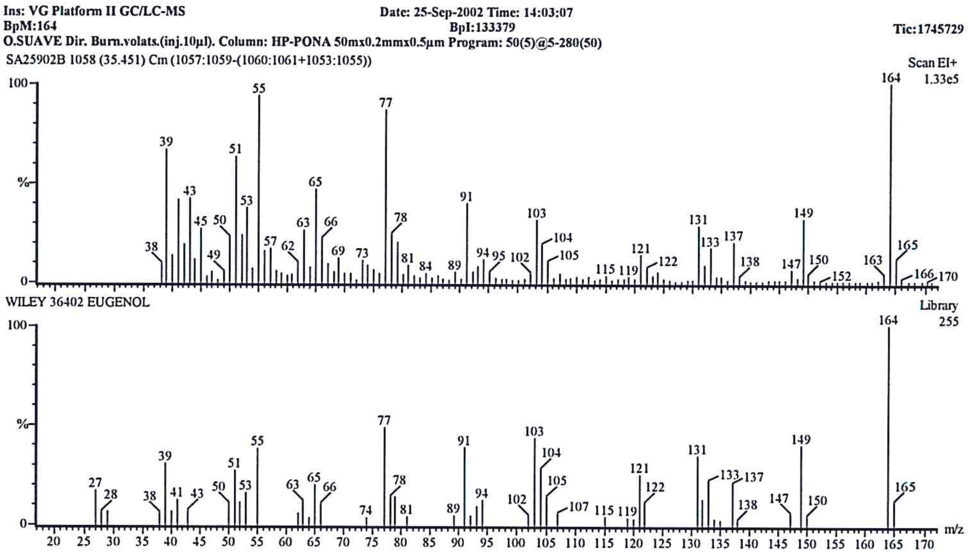
BpM:69 **BpI:441699** **Tic:1429180**
O.SUAVE Thermal.volats.(inj.10µl). Column: HP-PONA 50mx0.2mmx0.5µm Program: 50(5)@5-280(50)
SA25902C 3115 (86.878) Cm (3111:3117-(3122:3142+3071:3092))
Scan EI+
4.42e5

WILEY 192277 2,6,10,14,18,22-TETRACOSAHEXAENE, 2,6,10,15,19,23-HEXAMETHYL- (CAS) $$ SQUALENE $$ SKVALEN
Library
255

EUGENOL (1058) - SA25902B

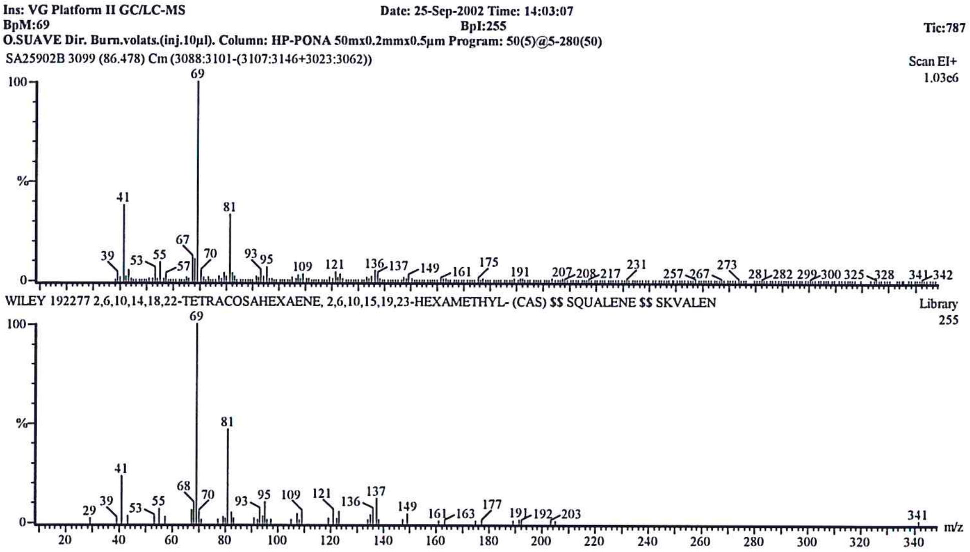


Ins: VG Platform II GC/LC-MS **Date: 25-Sep-2002 Time: 14:03:07**
BpM:164 **BpI:133379** **Tic:1745729**
O.SUAVE Dir. Burn.volats.(inj.10µl). Column: HP-PONA 50mx0.2mmx0.5µm Program: 50(5)@5-280(50)
SA25902B 1058 (35.451) Cm (1057:1059-(1060:1061+1053:1055))
Scan EI+
1.33e5

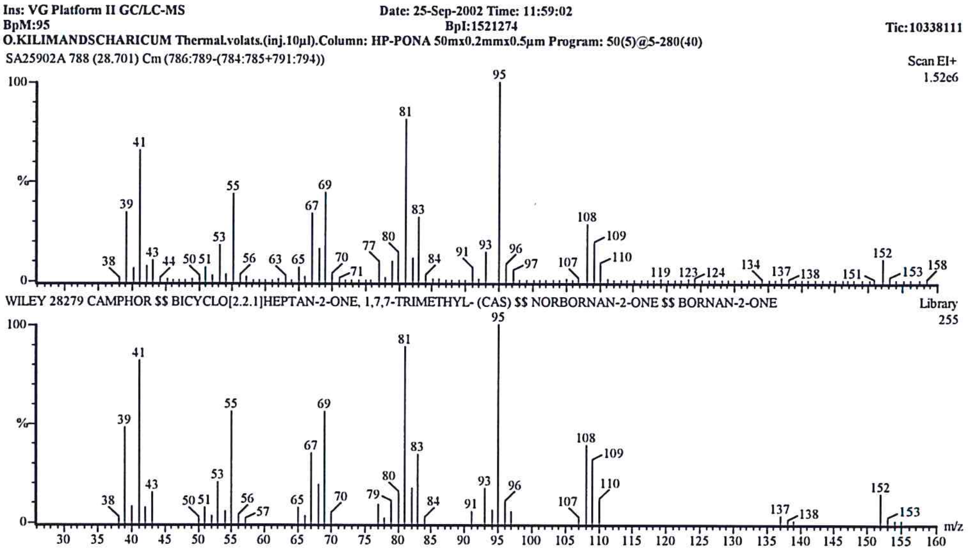
WILEY 36402 EUGENOL
Library
255

Appendix 6 continued

SQUALENE (3099) - SA25902B

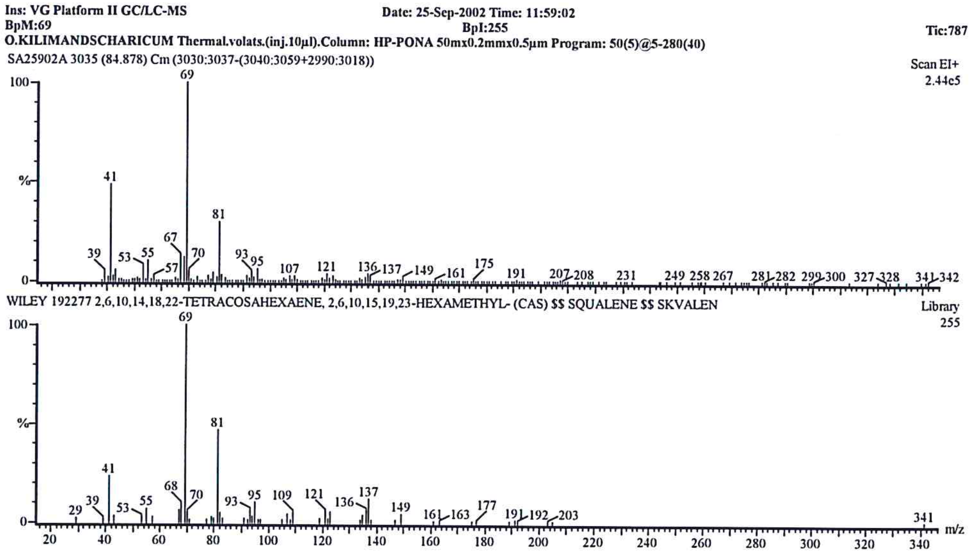


CAMPHOR (788) - SA25902A



Appendix 6 continued

SQUALENE (3035) - SA25902A



Ins: VG Platform II GC/LC-MS **Date: 25-Sep-2002 Time: 11:59:02**
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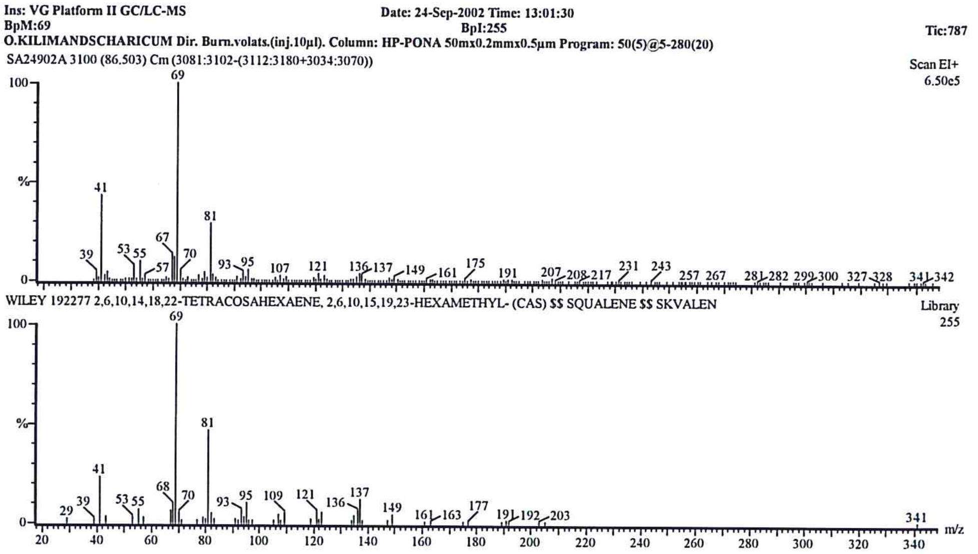
O.KILIMANDSCHARICUM Thermal.volats.(inj.10µl).Column: HP-PONA 50mx0.2mmx0.5µm Program: 50(5)@5-280(40)
SA25902A 3035 (84.878) Cm (3030:3037-(3040:3059+2990:3018))
Scan EI+
2.44e5

WILEY 192277 2,6,10,14,18,22-TETRACOSAHEXAENE, 2,6,10,15,19,23-HEXAMETHYL- (CAS) $$ SQUALENE $$ SKVALEN
Library
255

SQUALENE (3100) - SA24902A



Ins: VG Platform II GC/LC-MS **Date: 24-Sep-2002 Time: 13:01:30**
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O.KILIMANDSCHARICUM Dir. Burn.volats.(inj.10µl). Column: HP-PONA 50mx0.2mmx0.5µm Program: 50(5)@5-280(20)
SA24902A 3100 (86.503) Cm (3081:3102-(3112:3180+3034:3070))
Scan EI+
6.50e5

WILEY 192277 2,6,10,14,18,22-TETRACOSAHEXAENE, 2,6,10,15,19,23-HEXAMETHYL- (CAS) $$ SQUALENE $$ SKVALEN
Library
255

Appendix 7. Plant species with the highest repellency by thermal expulsion (A= *C. citriodora*, B = *O. suave*, C = *O. kilimandscharicum*) and in potted form (D = *O. americanum*).