Bioevaluation of Insecticidal and Repellent Plants from Central Region of Kenya and Chemical Identification of Bioactive Derivatives

By

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A thesis submitted in partial fulfillment for the award of the degree of Master of Science of Kenyatta University

April 2004

Dedication

This thesis is dedicated to beloved members of my family especially my brothers Ken and Isaac for their material support, encouragement and assistance at will.

Declaration

This is my original work and I do confirm that it has not been presented for a degree award in any other institution:

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Signed.....Date 25/03/2004

This work is submitted for the university examination with our approval as supervisors

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Acknowledgement

The author wishes to greatly acknowledge Prof. Isaiah O. Ndiege of the Department of Chemistry, Kenyatta University for his tireless support making the research success. Prof. Ahmed Hassanali and Dr. Wilber Lwande both of ICIPE are also acknowledged for their assistance during research and thesis writing.

Special thanks to Wanyama Kaye for running GC-MS spectra, Edward Nyandat for provision of solvents and chemicals, Patrick Mbogo for carrying out bio-assay, Jeremiah Ojude and Milkah Mwangi for the supply of mosquitoes.

I wish to appreciate the contribution from members of the Department of Chemistry, Kenyatta University.

My fellow M.Sc. Students Josiah Odalo, Fedelis Samita, Malele Cathrine, Jackson Wachira and Rono Martin are greatly acknowledged for the moral support they accorded me throughout the research.

Finally not forgetting my long-term friends Charles Wasike, Michael Wafula, Sululu Geoffrey, Wensilas Tabu and Steven Mumello for encouragement particularly when things seemed not to be working

This research was supported by WHO/TDR/MIM research grants No. 99056 and A10638

MAY THE GRACE OF OUR HEAVENLY FATHER BE WITH ALL THAT MADE MY WORK POSSIBLE.

List of abbreviations

AA	*Artemisia afra*
CA	*Clausena anisata*
CG	*Cineraria grandifolia*
CQ	Chloroquine
DCM	Dichloromethane
DEET	*N, N*-Diethyl-toluamide
DDT	Dichloro diphenyl trichloroethane
DMP	Dimethyl phthlate
FAO	Food and Agriculture Organization
HE	Highlands Elgon
HC	Highlands Cherangani
HT	Highlands Tinderet
HM	Highlands Mau
HA	Highlands Aberdares
ICIPE	International Centre for Insect Physiology and Ecology
ITN	Insecticide-treated bet nets
IGR	Insect growth regulator
KIT	Kitale
KAJ	Kajiado
LD	Lethal dose
NA	*Nepeta azurea*
PE	Protective efficacy
PEM	*Pseudocarum eminii*
RD	Repellent dose
SMO	*Senecio moorei*
SP	Sulfadoxine-pyrimethamine
SPS	*Satureja pseudomensis*
SE	Standard error
WHO	World Health Organization
nd	not done
t	trace

List of figures, plates, schemes and tables

Abstract

Despite considerable control efforts, malaria still remains the most prevalent and devastating disease in the tropics. With more than 40% of the world population at risk, malaria undermines the welfare of several South American, Asian and African states, endangering the survival of children (killing one child every 30 seconds) and straining scarce resources. It is estimated that US $ 2 billion is spent on malaria control and treatment programmes in Africa annually. The problem is becoming increasingly difficult to manage because of the continuous intensification and spread of resistance to anti-malarial drugs by the parasites. This poses a serious threat in increased severity of disease and health. Vector resistance to insecticides is a recurring theme and a major problem in malaria control programmes. The safety and efficacy of *N*, *N*-diethyl-*m*-toluamide (DEET), the most potent of the modern synthetic repellent, is questionable. Other problems associated with repellents include vector resistance, avoidance and frequent repetitive application. The recent discovery of *trans-cis*-nepetalactone and *p*-mentane-3,8-diol that are more effective and environmentally friendly than DEET justifies bioprospecting from plants. In our search for new repellents we have continued with bioprospecting activities of the Kenyan flora.

The essential oils from leaves of *Artemisia afra*, *Senecio moorei*, *Cineraria grandifolia* (Asteraceae), *Nepeta azurea*, *Satureja pseudomensis* (Labitae), *Clausena anisata* (Rutaceae) and *Pseudocarum eminii* (Umbelliferae) from Mt. Kenya region were evaluated for their repellency and insecticidal activity against adult female *An. gambiae* mosquitoes. The essential oil of *Nepeta azurea* was the most effective repellent (RD_{50} $6.5x10^{-7}$ mg/cm^2). The essential oils from other species studied also showed significant repellency effect. The order of repellency was: *P. eminii* > *S. moorei* > *C. anisata* > *S. pseudomensis* > *C. grandifolia* > *A. afra* with RD_{50} = $4.39x10^{-4}$, $1.27x10^{-3}$, $7.86x10^{-3}$, $1.15x10^{-2}$, $2.2x10^{-2}$, $3.07x10^{-2}$ mg/cm^2, respectively. The essential oil from *A. afra* was insecticidal at 1% w/v (LD_{50} = $2.26x10^{-2}$ mg/cm^2). The constituent compounds were identified through GC, GC-MS and GC co-injection and bio-assayed for repellent and insecticidal properties.

CHAPTER 1
INTRODUCTION

1.1 Malaria

Globally, malaria is found in at least 100 countries (Plate 1) where over 40% of the world's population lives (WHO, 1997). Malaria is the most important insect-transmitted human disease (WHO, 2000a). However, progress in the control of the disease has been slow especially in Africa where approximately 90% of the infection occurs. It is estimated that nearly 40% of the world's population live in areas with malaria risk (WHO, 1997). The global incidence of malaria is estimated at 300-500 million clinical cases per year and > 1 million deaths worldwide (Anon, 2001a). Vector resistance to insecticide is a recurring theme and a major problem in malaria control programmes (Curtis *et al.*, 1993). Globaly chloroquine resistance and drug failure force governments to adopt more expensive drugs for first line treatments (WHO, 2000b). Advances in molecular biology have led to the development of new vaccines, but large-scale application of this technique is not envisaged soon (Collins and Paskewitz, 1995). Bed nets impregnated with insecticides are facing considerable scrutiny as a tool for prevention of host-vector contact (Amador *et al.*, 1992). Recent large-scale trials with pyrethroid-impregnated bed nets in Africa have demonstrated their impact on child mortality and morbidity (Lengeler *et al.*, 1996), but it remains to be seen whether such effects can be sustained or obtained in regions with intense perennial transmissions.

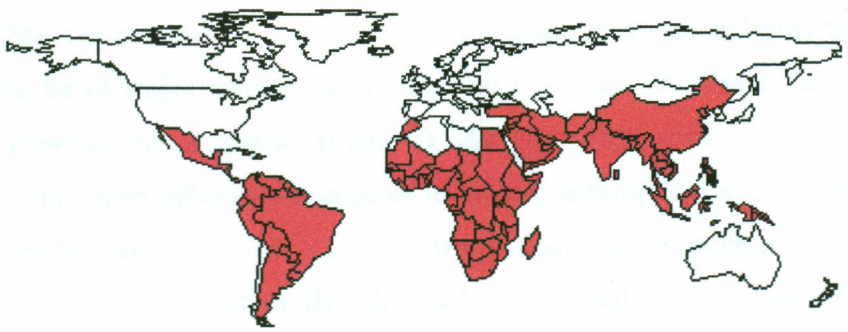


Plate 1. Global distribution of malaria (WHO, 1997)

The current malaria situation is critical as development of alternative strategies is slow and existing methods are rapidly losing efficacy. These have recently called for worldwide-integrated efforts to prevent further deterioration of the global malaria situation. One such effort is the exploitation of

the plant kingdom. The use of plants as natural repellents/insecticides in traditional/cultural systems has been documented for many areas (Curtis *et al.*, 1990). However, most of the products from these plants have not been carefully analyzed. This research focused on identification and characterization of insecticidal or repellent compounds found in plants from central region of Kenya.

1.2 The parasite, transmission and symptoms

Malaria, the human disease is caused by the infection with one of the four morphologically distinct species of malaria parasite in the genus *Plasmodium*: *P. vivax, P. ovale, P. malariae* and *P. falciparum*, with the later being the most virulent species and predominates in sub-Saharan Africa, eastern Asia, Oceania and Latin America (WHO, 1997). The illness is transmitted through the bite of infected mosquito (Fairley, 1947). The parasite enters the blood stream and travels to liver where it multiplies. From the liver, it re-enters the blood stream and invades the red blood cells. It ruptures the cells causing malaria symptoms such as fever, shivering, pain in the joints, headache, repeated vomiting, generalized convulsions and coma (Manson-Bahr and Bell, 1987).

1.3 Vectors of human malaria

Mosquitoes of the genus *Anopheles* are the exclusive vectors of human malaria. Worldwide, there are about 70 species of *Anopheles*, which are known vectors of malaria (Rao, 1984), and these numbers will surely increase as more sibling species of complexes are identified within the genus. Most of the malaria cases are found in tropical Africa and only three malaria vectors are considered to be of major importance (Hunt and Cootzee, 1995) in these regions. These include *Anopheles gambiae sensu stricto* (Plate 2), *An. arabiensis* and *An. funestus*. Of these, *An. gambiae s.s* is the most efficient because of its highly anthropophilic character (Garret-Jones *et al.*, 1980). *An. arabiensis* is known to vary from being anthropophilic to largely zoophilic depending on geographical location (Brack *et al.*, 1994) and is less susceptible to infection with *Plasmodium* parasites than *An. gambiae s.s* (Brack *et al.*, 1994). But due to the high densities in which it may occur, it can be a very important vector in particular locations (Charlwood and Jones, 1980). The combination of both anthropophilicity and endophily put both *An. gambiae s.s* and *An. funestus* in a special place among malaria vectors (Gillies and Cootzee, 1987). Other vectors of importance in tropical Africa include *An. bwambae*, *An. melas*, *An. merus*, *An. moucheti* and *An. nili* among others (Gillies and Cootzee, 1987).

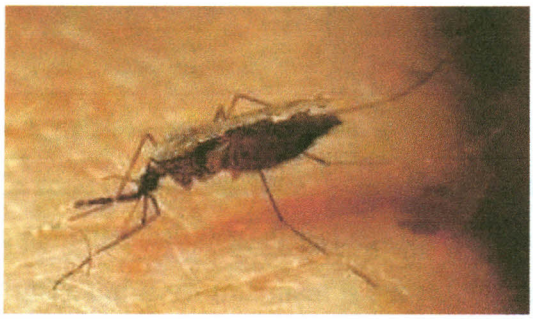


Plate 2. Photograph of *Anopheles gambiae* mosquito (http://www.emblheidelberg de/ixternalinfo/kafatos/ Image2.htm)

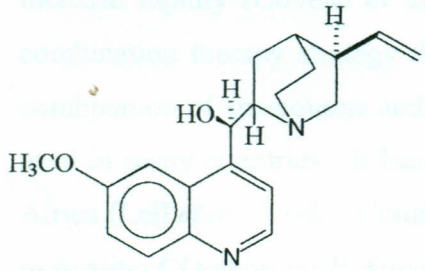
1.4 Control methods

1.4.1 Vaccination

Vaccine development seems the final solution to the desperate malaria situation as the *Plasmodium* parasite continue (s) to defy the latest drugs. Currently, three types of vaccine are envisaged: The first against sporozoites (Franke *et al.*, 1999); the second against asexual erythrocytic stages; and the third against gametes or related sexual stages (WHO, 1998). The search for an effective vaccine has intensified culminating in clinical trials in USA, Gambia, Tanzania and Colombia. Currently, vaccines under research and development include spf66 ((Patarroyo *et al.*, 1992). Spf66 is a widely tested vaccine that has given mixed results in field trials in S. America, Africa and S.E Asia (Anon, 2001b). Recently, a vaccine that has been tested on humans with promising results is RTS,S/AS02 which showed protective efficacy of 47% in Gambia. Further tests are continuing in Mozambique (Bojang *et al.*, 2001). Despite the concerted efforts, vaccine development remains misty as the parasite continue to show a surprising immunologic variability and vaccines strategies that once seemed straightforward have frustratingly been ineffective in recent years. Proposals on the exploitation of the mosquito genome for development of effective vaccines have been floated (Sharakhov *et al.*, 2002). The recent cloning of *An. gambiae s.s* (Zdobnov *et al.*, 2002) and *P. falciparum* (Sumathy *et al.*, 2002) genome has opened new avenues that may yield useful vaccines in the near future.

1.4.2 Chemotherapy

In spite of drug resistance, malaria is a curable disease and an evitable burden. However, due to the emergence of multi-drug resistant strains of the parasite in many parts of the world (Trigg *et al.*, 1997), universal treatment is becoming increasingly difficult. According to WHO (1998), new drugs are badly needed because of the declining efficacy of important drugs such as quinine (**1**), chloroquine (CQ) (**2**), atovaquone (**3**), proguanil (**4**), pyrimethamine (**5**), sulfadoxine (**6**) mefloquine, halofantrine, malaron and artemisinin derivatives. Chloroquine, a drug of choice for many years, has suffered a serious setback due to high levels of parasite resistance (WHO, 1999). Recently, the Kenyan government gazetted prohibition of the sale and use of chloroquine tablets unless prescribed (Anon, 2001a). Artemisinin (**7**), derived from a Chinese herbal remedy, represents one of the most remarkable success stories of anti-malarial compounds from plants (Klayman, 1993). The two most widely used products are the synthetic derivatives, artesunate (**8**) and artemether (**9**). Unfortunately, artemisinin derivatives require long treatment courses and when used alone, recrudescence may occur (WHO, 1998). Given the high rate of treatment failures with artemisinin derivatives, it is now being combined with mefloquine for the treatment of *falciparum* malaria (Looaresuwan, 1992)



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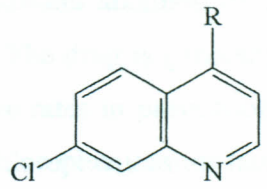
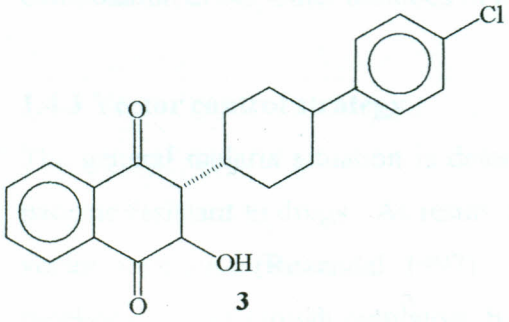


2 R=HNCH(CH_3)(CH_2)$_3$N(C_2H_5)$_2$



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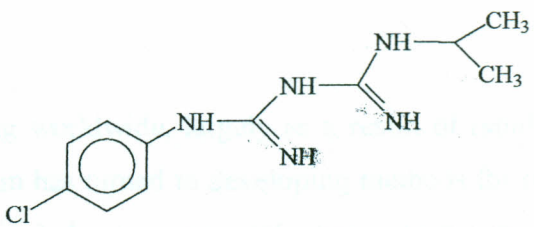

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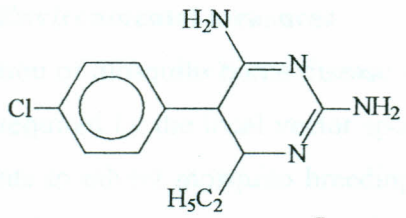

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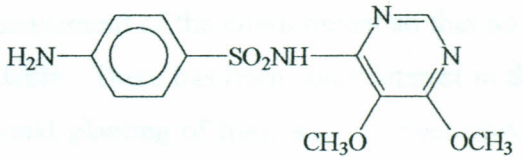

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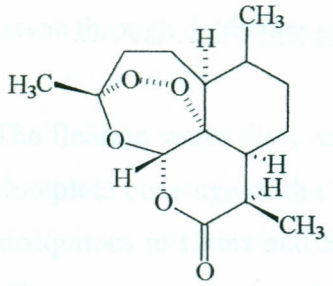

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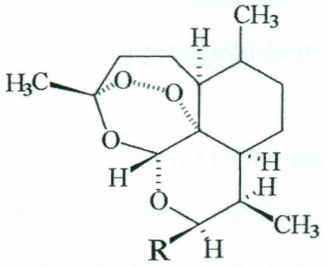


8 R=$CH_2COCH_2CH_2COOH$
9 R=OCH_3

Although SP combinations, like Fansidar®, Orodar® among others, are now available as first line therapy in sub-Saharan Africa, resistance and treatment failure are already prevalent and expected to increase rapidly (Govene *et al.*, 1999). Malarone® is a new anti-malarial drug that represents combination therapy strategy that has received maximum attention by scientists recently. It is a combination of atavaquone and proguanil chloride. The drug is generally accepted and extensively used in many countries. It has registered low failure rates in prevention of *Falciparum* malaria in Africa (Lell *et al.*, 1998). Combination of CQ and chloropheniramine has been found to be effective in treating CQ-resistant *P. falciparum* malaria (Sowunmi and Oduola, 1997). In one of the research studies conducted in Mumbai (India), the combination of CQ and SP was found to be safer and more effective than to CQ alone (Gogtay *et al.*, 2000). Due to resistance development against combination drugs, other methods of malaria control need serious attention.

1.4.3 Vector control strategy

The general malaria situation is deteriorating worldwide, largely as a result of rapid selection for parasite resistant to drugs. As result, attention has turned to developing methods for controlling the vector population (Rozendal, 1997). These include environmental management, use of a variety of repellents, insect growth regulators, biological and chemical insecticides, genetic manipulations and diversion of mosquitoes to other animal hosts.

1.4.3.1 Environmental measures

Eradication of mosquito borne disease calls for management of the environment so that no water of quality required by the local vector species is available. There has been some interest in the use of live plants to inhibit mosquito breeding. Commercial planting of trees such as *Eucalyptus*, which absorb and transpire large amount of water has been attempted in India Sharma (1987). Hackett *et al.* (1938), provides a review of plants that can be used non-specifically against *Anopheles* mosquito larvae through deliberate pollution of water with decaying vegetable matter.

The floating water fern, *Azolla fulcoides*, was tested by Lu (1996) on *Anopheles* and *Aedes* larvae. Complete coverage of the water surface by this plant had an inhibitory effect on the oviposition of mosquitoes in China and Sri Lanka. The fern is widely used in paddy fields and thus confining its effect on mosquito breeding to the rice field breeders. *Azolla fulcoides* is readily cultivated for fertilizer and animal feed, so its effect on mosquito control is an extra benefit. However, proper timing of plant cultivation is of prime importance for complete coverage of water surface.

Stream-breeding mosquitoes have been controlled through "flushing." It is well known that larvae which migrate to the margins of the stream become stranded as the flow subsides and die (MacDonald, 1939). However, this technique requires more research.

Shading has also been used to control mosquito larvae. This method was successfully used in Assam and northern Bengal against *An. minimus* (Rasmay, 1930). *An. minimus* disappeared when suitable bushes were planted along the streams so that they are densely shaded. Unfortunately, the cause of this disappearance is not fully understood.

Protection of breeding places which involves covering all open water surfaces, removal of non-essential water containers from around houses such as jars, storage pots and tins is a well known technique of larvae control (Rajagopalan *et al.*, 1991). However, due to high costs and intensive labour involved, this approach has been rendered impractical in wider scale.

Another environmental control strategy involves activities that are to reduce contact with vectors and therefore pathogen transmission. Such activities include location of human settlements away from vector sources, mosquito proofing of houses, use of betnets, zooprophylaxis and personal protection

measures (Lawrence and Cynthia, 1990). These procedures were effective in the past but have once been discarded due to their high cost and labour intensive nature.

1.4.3.2 Biological control

These involve use of natural enemies of mosquito, pathogens and biological toxins. They include larvivorous fish such as *Gumbussia affinis*, that successfully reduced malaria incidence in Italy and Greece, unstable transmission regions (Wickramasinghe and Costa, 1986). However, environmental problems resulting from competition of *G. affinis* with indigenous fish have led to investigations of many other fish species as control agents. Another fish, *Poecilia (Lebistes) reticulata*, has an advantage of surviving in organically polluted water. Recent trials in Namanjala sub-location, Kitale have shown that *Tilapia* species can be employed as mosquito larvae control agent (Anon, 2001b).

The nematode *Romanomenus culicirax*, used against mosquito larvae, is not effective on all species. Moreover, it cannot develop in polluted habitats. Difficulties with survival and reproduction in fresh water have necessited repeated nematode application (Mogi *et al.*, 1984).

The fungus, *Tolypocladium clindarasporum,* has also been tested against *Aedes eagypti*. However, field trials have not been undertaken hence stability of the fungus under normal environmental conditions has not been established (Nadeeu and Boisvert, 1994).

Some bacteria, particularly *Bacillus thuringiensis*, produce toxins that are lethal to mosquitoes (Lacey and Undeen, 1986; Lacey *et al.*, 1984). Others like *B. sphaericus* is highly active against larvae of *Culex* and *Anopheles* mosquitoes but is virtually non-toxic to *Aedes aegypti*, an important vector of dengue viruses (Margaret *et al.*, 2000). An enormous body of literature in which these toxins are described as biological control agents has accumulated. Unfortunately, *Bacillus* toxins are still expensive. They have no residual activity and require frequent re-application or are only suitable for environments where a one-time control measure produces a valuable outcome (Craven, 1968).

Recently, scientists have created genetically modified (GM) mosquitoes that cannot spread malaria (Collins and Paskewitz, 1995). When a GM mosquito mates with a normal one in the wild, a GM offspring will result. However, no offspring will result if it mates with a natural female mosquito.

Survival of GM mosquito in the wild is anticipated to be problematic. Though GM mosquitoes may survive in the wild, mass rearing will be a major challenge (Battler, 1999).

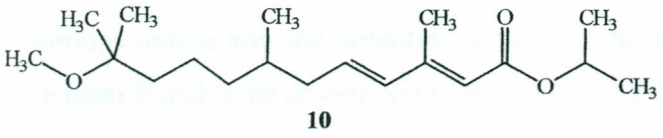
Though biological control methods may seem attractive, they require a good understanding of the population dynamics of the vector compared to other alternative vector control measures. In fact, they are slow-acting and unsuitable in emergencies such as disease epidemics, where insecticides and repellents are more appropriate.

1.4.3.3 Zooprophylaxis

This control method involves the diversion or deflection of potentially infective blood-seeking mosquito from human to animals (Cragg, 1923). In Africa, a good example of a target vector for such an approach to malaria control is *An. arabiensis*, which takes a considerable proportion of its blood-meals from cows when they are present (Petrarca *et al.*, 1991). The effect of this control method on malaria incidence has not been proven and further research is necessary. Push-pull control strategy has been proposed where repellents are used to push a way mosquitoes from humans while cattle are provided as alternative hosts (Hassanali, Personal Communication).

1.4.3.4 Insect growth regulators (IGR)

These specifically affect growth and the development of insects in the early stages of their life cycle. The compounds include mimics and inhibitors of two groups of insect hormones, the juvenile hormone (JH) (Bowers *et al.*, 1976) and the moulting hormone (ecdysone) (Jacobson, 1975). Methropene (**10**) marketed as Altosid$^{(R)}$ is designed to disrupt insect growth cycle at critical development stages. It has shown special promise in the control of mosquito (Schaeser and Wilder, 1977). However, resistance to it is well documented (Brown and Brown, 1974). Other notable examples include hydropene (**11**), neonetin (**12**), diflubenzuron (**13**) and fenoxycarb (**14**) (Kirk and Othmer, 1978, Jose and Mulla, 1986). Though IGRs are known to have little or no toxicity to other non-target organisms, some are responsible for induced sterility and reproductive anomalies in the adult stage of non-target organisms (WHO, 1996a).



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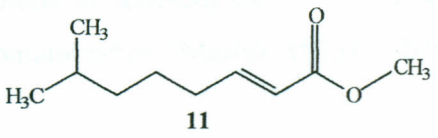

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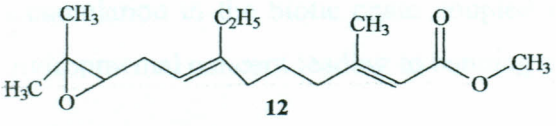

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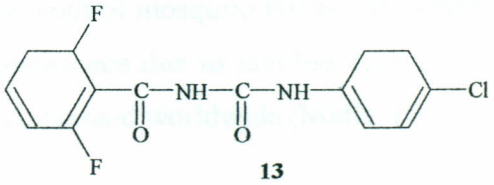

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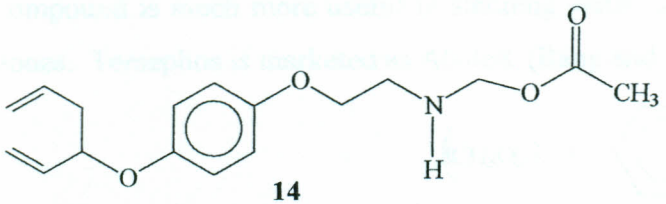

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1.4.3.5 Larvicides

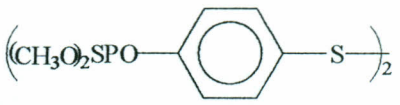
Insecticides are classified by their chemical nature and source as: inorganic, synthetic organic and natural organic compounds. In general, inorganic insecticides are effective only as stomach poisons and synthetic insecticides are employed largely as contact poisons. The natural organic insecticides may act as contact poisons or stomach poisons. Vector control by larviciding has been implemented in some circumcsitances especially when the use of residual adultcides was not effective or too expensive

Most of the inorganic larvicides so far used include fluorine compounds such as fluorosilicic acid [H_2SiF_6], flouraluminic acid [H_3AlF_6], sodium fluorides [NaF] and sodium fluorosilicate [Na_3SiF_6] among others. Arsenic compounds used include calcium arsenate [$Ca_3(AsO_4)_2$], lead arsenate [$PbHAsO_4$], zinc arsenate [$Zn_3(AsO_4)_2$], copper arsenate [$Cu(CuOH)AsO_4$], Paris green {$Cu(C_2H_3O_2)_2.3Cu(AsO_2)_2$} and magnesium arsenate [$Mg_3(AsO_4)_2MgOH_2O$] among others. For instance, larval control with Paris green combined with permethrin house spraying was extremely successfully used in eliminating *An. gambiae s.s* in Brazil (Wernsdorfer and McGregor, 1988).

Inorganic larvicides have been replaced with conventional synthetic insecticides due to the highly poisonous nature and the potential dangers of environmental contamination (Martin, 1973). They have been found to be phytotoxic to some aquatic plants.

DDT, a chlorinated hydrocarborn has been used to control mosquito larvae. However, owing to its accumulation in the biotic chain coupled with persistence due to non-biodegradability has caused environmental concern leading to banning of this compound worldwide (Mulla, 1963).

Temephos (**15**) is an organophosphate pesticide used to control mosquito larvae on large scale. This compound is much more useful in standing water, shallow ponds, swamps, marshes and inter-tidal zones. Temephos is marketed as Abate® (Bang and Pant, 1972).



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Other synthetic larvicides that have been used for larval population management include carbamates such as propoxur and bendiocarb. However, carbamates are highly toxic to non-target organisms (Kirk and Othmer, 1978).

Synthetic pyrethroids such as decamethrin and the chloro-analogue have been found to be 50-100 times more effective than organophosphates against resistant stains of *Ae. nigromaculis* larvae (Mulla *et al.*, 1980).

Control of mosquito larvae has also been achieved through the use of botanical derivatives. Use of botanical alkaloids such as nicotine, *anabasine, methylanabasine* and lupinene extracts from Russian weed, *Anabasis aphylla* against larva of *Culex pipiens* Linn, *C. territans* Walker and *C. quinquefesciatus* is well documented (Campbell and Sullivan, 1933). Pyrethrins derived from flowers of *Chrysanthemum* have also played a key role in larval control. No resistance has been reported for the natural pyrethroids. However, the costs of extraction and the quick biodegradability have limited widespread use.

Despite, the availability of effective larvicides, their application in breeding habitats has always raised environmental concerns. This has spurred further research into adulticides and repellents which target the mature stages of the insect.

CHAPTER 2

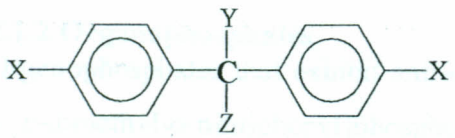
ADULTICIDES AND REPELLENTS

2.1 Synthetic organic insecticides

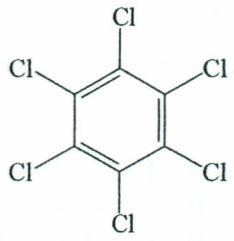
Most of the synthetic organic insecticides fall within five major classes namely, organochlorines, organophosphates, organocarbamates, pyrethroids and natural organic compounds (Dorow, 1993). Currently, organophosphates, organocarbamates and pyrethroids are widely used in public health and agriculture. The concurrent use of these pesticides for medical vector and agricultural pest control is thought to have accelerated resistance development in various insects (Brown, 1986; WHO/UNEP, 1990).

2.1.1 Organochlorines

The most important member of this group is 1,1,1-Trichloro-2,2-*Bis*(*p*-chlorophenyl)Ethane (DDT)(**16**) which has widely been used for mosquito control, agricultural pests and other insect vectors. By 1964 malaria was eradicated from parts of India through the use of DDT house spraying (Sharma and Mehrotra, 1986). However, owing to its ineffectiveness as a result of the emergence of resistant insects and non-degradability (Splindler, 1983), DDT has been phased out. Recently, due to resistance developed to pyrethroids in Natal, DDT was used to control *An. funestus* (Coetzee *et al.*, 2000). The discovery of insecticidal properties of DDT opened up ways for the development analogues such as 1,1-dichlro-2,2-*bis*(*p*-chlrophenyl)ethylene (DDE)(**17**), 1,1-dichloro-2,2-*bis*(*p*-chlorophenyl)ethane (DDD)(**18**), 1,1,1-triclro-2,2-*bis*(*p*-methylphenyl)ethane(methoxychlor)(**19**), prolan (**20**) and bulan (**21**) (Metcalf and Fukuto, 1968). Another member of this group, benzene hexachloride or 1,2,3,4,5,6-hexachlorobenzene (**22**) has also been used.



	X	Y	Z
16	Cl	H	CCl_3
17	Cl	-	$=CCl_2$
18	Cl	H	$CHCl_2$
19	OCH_3	H	CCl_2
20	Cl	$(CHNO_2)C_2H_5$	H
21	$(CHNO_2)CH_3$	H	H



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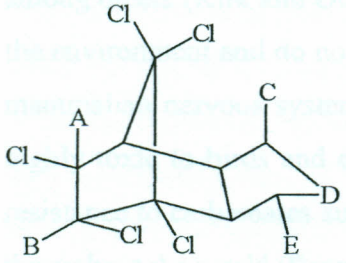
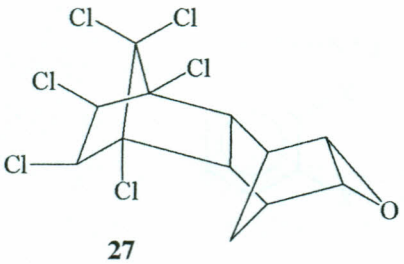
Cyclodiene compounds such as aldrin (**23**), telodrin (**24**), chlordane (**25**), heptachlor (**26**), dieldrin (**27**) and endosulfan (**28**) among others have also been used. The problem of insecticide resistance and negative effects on non-target organisms including man and environment (Rodriguez *et al.*, 2001; Crosa *et al.*, 2001) have discouraged their use and accelerated the search for alternative methods of insect control.



	A	B	C	D	E
23	H	H	H	CH_2	$CH{=}CH_2$
24	-	-	Cl	O	Cl
25	H	H	-	CHCl	Cl
26	H	H	-	-	Cl



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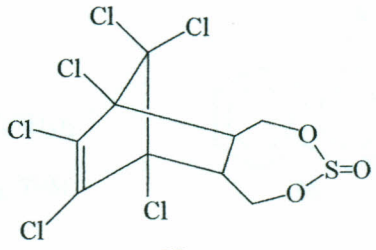

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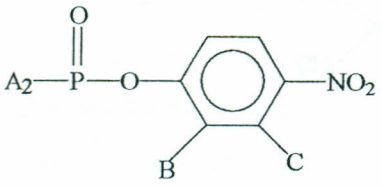
2.1.2 Organophosphates

Organophosphates that exhibit anti-chlolinesterase activity such as parathion (**29**), methylparathion (*o*,*o*-dimethyl-*o*-nitrophenyl)phosphorathioate (**30**), chlordane (*o*,*o*-dimethyl-*o*-3-chloro-4-nitrophenyl)phosphothioate (**31**), fenitrothion (*o*,*o*-dimethyl-*o*-4-nitrophenyl)phosphothioate (**32**) and dichlorvos (**33**) among others have been used in insect-control programmes (Eto, 1974). Wanton use of these compounds, as insecticides in malaria eradication programmes and agricultural pest control may tend to enhance development of resistance in vector populations, thereby negating

their role as a residual indoor adulticides (Lines, 1988). Their use in agriculture exerts selection pressure on the insect vector population leading to the development of resistance (FAO, 1979).

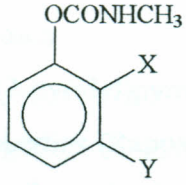


	A	B	C
29	OC_2H_5	H	H
30	OCH_3	H	H
31	OCH_3	H	Cl
32	OCH_3	H	CH_3

33

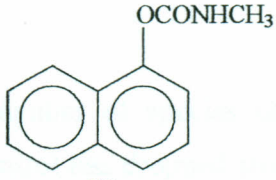
2.1.3 Organocarbamates

Compounds of insecticidal activity in this class include Propoxur (2-isopropoxyphenyl methylcarbamate) (**34**), which is reported to be effective against a wide range of mosquitoes though its use has been limited by the high cost (Johnsen and Hanstbager, 1966), baygon (2-isopropoxyphenyl-*N*-methylcarbamate) (**35**) and carbaryl (1-naphthyl-*N*-methylcarbamate) (**36**) among others (Kirk and Othmer, 1978). Unlike organochlorines, these compounds are short-lived in the environment and do not bio-accumulate (Brown, 1986). However, some are known to affect the mammalian nervous system causing acute and chronic toxicity (Matsumura, 1975). They are also highly toxic to birds and other economically important insects such as bees. Moreover, mosquito resistance to carbamates such as Propoxur or Bendiocarb have been reported in 17 mosquito species throughout the world (Brown, 1986).



34 X=H, Y=$OCH(CH_3)_2$

35 X=$OCH(CH_3)_2$, Y=H



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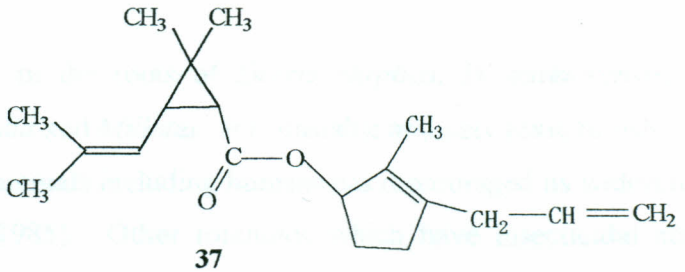
2.1.4 Synthetic pyrethroids

High costs involved in the isolation of natural pyrethrins made them expensive and uneconomical. This led to the development of synthetic derivatives modeled on the natural compounds. Synthetic pyrethroids that have been used in malaria control programmes include allethrin (**37**), permethrin (**38**), cypermethrin (**39**), fenvalerate, cyfluthrin (**40**), resmethrin, cyhalothrin (**41**), decamethrin (**42**), biopermethrin, phenothrin, and bioresmethrin (Kirk and Othmer, 1978). Although the pyrethroids have lower mammalian toxicity, cases of mosquito resistance to synthetic pyrethroids have been reported (Paveling *et al.*, 1994).



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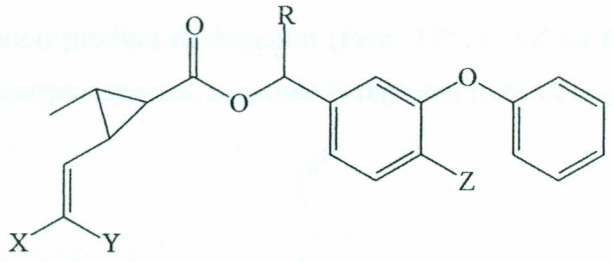


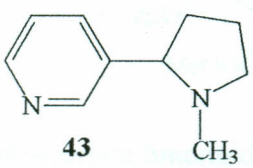
	R	X	Y	Z
38	H	Cl	Cl	H
39	N_3	Cl	Cl	H
40	N_3	Cl	Cl	F
41	H	Cl	CF_3	H
42	H	Br	Br	H

2.1.5 Insecticides of plant origin

Botanical insecticides are plant natural products that are secondary metabolites. These may include alkaloids, terpenoids, polyketides and phenolics. The pool of plants with insecticidal substances is enormous (Jacobson, 1975). Today over 1000 plant species are known to posses some insecticidal activity (Jacobson, 1989). In the middle of 17th century, nicotine, pyrethrum and rotenone were recognized as insect control agents (Crossby, 1966).

2.1.5.1 Nicotinoids

Nicotine, *L*-2-(3-pyridyl)pyrrolidine **(43)**, isolated from a number of species of *Nicotiana* has insecticidal properties (Rappaport, 1992). Its use in insect-control has dropped steadily because of the high cost of production, unpleasant odour, high mammalian toxicity and environmental instability (Schmetz, 1971). Anabasine, 2-(3-pyridyl)piperidine **(44)**, and nornicotine, *L*-2-(3-pyridyl)pyrrolidine **(45)**, are the only other alkaloids of insecticidal importance found in these plants (Kirk and Othmer, 1978).



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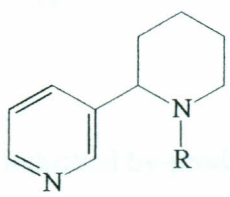
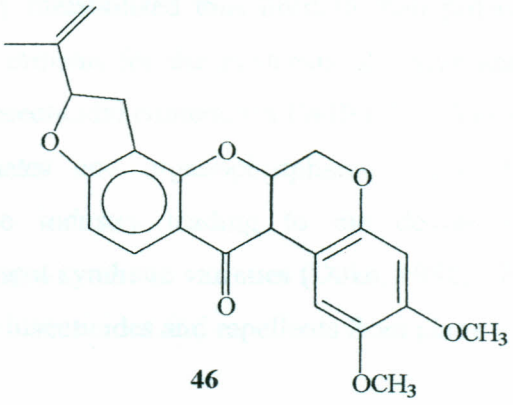


44 R=H

45 R=CH_3

2.1.5.2 Rotenoids

Rotenone (**46**) occurs in the roots of *Derris elliptica*, *D. malaccensis*, and several species of *Lonchocarpus*, *Tephrosia* and *Milletia*. It is unstable and very toxic to fish (Kirk and Othmer, 1978). Toxicity to fish and mammals including humans has discouraged its widespread use as an insecticide (Manuel and James, 1985). Other rotenoids which have insecticidal activity include deguelin, elliptone, malaccol, sumatrol and toxicarol. Tephrosin does not occur naturally in *Derris* resins but is an oxidation product of deguelin (Fear, 1955). Of all the active principles isolated from *Derris* and *Lonchocarpus* species, rotenone is reported to be of major insecticidal importance.



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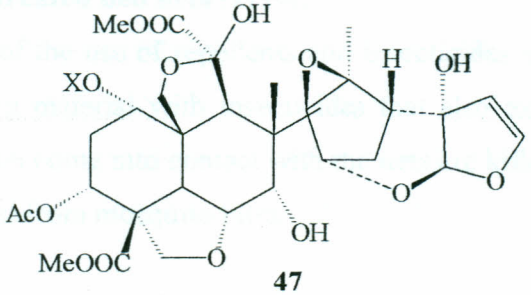
2.1.5.4 Limonoids

Azadirachta indica is one of the plant species with a wide range of activities (Mulla and Su, 1999). Extracts from this tree are insecticidal, repellent, anti-feedant, anti-oviposition and growth regulating (Schmutterrer, 1995). The active principle has been shown to be azidirachtin (**47**) (Rappaport, 1992). It contains several other bioactive nortetraterpenoids (limonoids) (Biswas *et al.*, 2002). It is registred in USA and Europe as an insecticide for horticultural pests (Randhawa, 1997).



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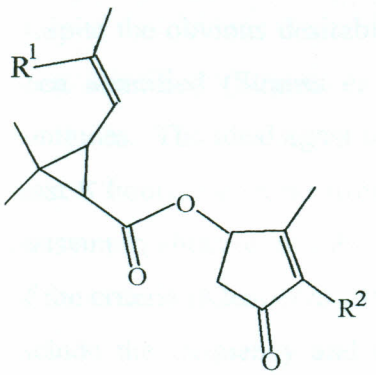
Other anti-feedant limonoids are available as industrial by-products, including limonin, nomilin, and obacunone from the citrus industry and gedunin from the timber industry (Siddiqui *et al.*, 2002)

2.1.5.5 Pyrethrins

The most economically important of the natural insecticidal plant-derived compounds are pyrethrins from the flowers of *Chrysanthemum cinerariafolium* (Casida, 1983; Casida and Quistad, 1995). The active toxicants are six terpenoids that include pyrethrin I (**48**) and II (**49**), jasmolin I (**50**) and II (**51**), cinerin I (**52**) and II (**53**) (Matsui and Yamamoto, 1971). Natural pyrethrins are known to act rapidly by killing or immobilizing (knock-down) a wide range of insect species. They are harmless to mammals under all normal circumstances (Kirk and Othmer, 1978). Due to their rapid metabolism, no resistance has been reported to natural pyrerhrins. However, they are highly unstable in light and are rapidly metabolized thus limiting their potency and application (Casida, 1983). These limitations gave impetus for the synthesis of active analogues (Yamamoto, 1970). Pyrethrins are excito-repellent/insecticidal compounds (WHO, 1984) used widely as an alternative to organochlorines, organocarbamates and organophosphates. The discovery of pyrethrin has revolutionalised the insecticide industry leading to the development of cheap, affordable environmentally friendly and potent synthetic varieties (Duke, 1990). For these reasons, the search for new safer and more effective insecticides and repellents from plants is justified.



	R^1	R^2
48	CH_3	$CH_2CH{=}CHCH{=}CH_2$
49	$COOCH_3$	$CH_2CH{=}CHCH{=}CH_2$
50	CH_3	$CH_2CH{=}CHCH_3$
51	$COOCH_3$	$CH_2CH{=}CHCH_3$
52	CH_3	$CH_2CH{=}CHCH_2CH_3$
53	$COOCH_3$	$CH_2CH{=}CHCH_2CH_3$

2.1.5.6 Insecticide-treated bed nets (ITNs)

It is a combination of the use of repellents and insecticides as malaria control strategy. This is the treatment of clothing material with insecticides that also exhibit repellency. The mosquitoes are repelled but those that come into contact with the nets are killed. Hosts sleeping under these nets are presumably protected from mosquito bites.

Currently, synthetic pyrethroids are the most commonly used anti-mosquito agents on insecticide treated bednets (ITNs) (Lindsay *et al.*, 1992; Sexton *et al.*, 1990). The rapid worldwide expansion in ITN use is a direct result of a change in the perception of the functions of bet nets from that of personal to community protection strategy. Field study of ITNs in Gambia demonstrated a reduction

in child morbidity (Alonso *et al.*, 1991). ITNs also provide protection against bedbugs (Charlwood and Dagoro, 1989), which may be an important factor in gaining community acceptance for bet net use (Curtis, 1994). Bednets programmes are not without setbacks. Washing significantly reduces the efficacy of ITNs (Miller *et al.*, 1994). Resistance to permethrin has been documented for *An. gambiae s.s* in Kenya (Vulule *et al.*, 1994). Other problems include unaffordability by many people in malaria endemic regions and non-compliance. The mosquitoes repelled by ITNs may end up feeding on unprotected people.

2.2 Mosquito repellents

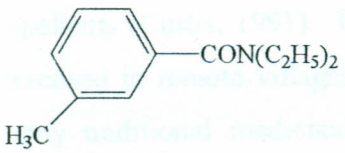
Repellents are defined as chemicals that cause organisms to make oriented movements away from the source due their offensive smell (Dethier *et al.*, 1960). They can give supplementary protection against biting insects indoor or outdoor. Their use can complement ITN or other control strategies. They reduce biting intensity (Gupta and Rutledge, 1994) and can be very useful in short-term strategy to reduce contact with disease vectors.

Despite the obvious desirability of finding an effective oral mosquito repellent, no such agent has been identified (Strauss *et al.*, 1968). Thus, the search for the ideal topical insect repellent continues. The ideal agent would repel multiple species of biting arthropods, remain effective for at least 8 hours, cause no irritation to the skin or mucous membranes, has no systemic toxicity, be resistant to abrasion or rub-off, be greaseless and odourless. No available insect repellent meets all of the criteria (Khan *et al.*, 1969). Many factors play a role in how effective any repellent is. These include the frequency and uniformity of application, the number and species of the organisms attempting to bite, the user's inherent attractiveness to blood-sucking arthropods, temperature, humidity, rain and the overall activity level of the potential host (Schreck, 1995). Abrasion from clothing, evaporation and absorption from the skin surface, wash-off from sweat or rain, higher temperatures, or a windy environment all decrease repellent efficacy (Khan *et al.*, 1976). They can be broadly classified into two groups as natural or synthetic repellents

2.2.1 Synthetic repellents

Among the earliest repellents to be synthesized in the laboratory were indalene and ethylhexanediol (King *et al.*, 1960). The search for the most potent of synthetic repellent led to the discovery of *N*, *N*-diethyl-*m*-toluamide (DEET) (**54**) in 1950s (McCabe *et al.*, 1954). It was shown to be more

effective than dimethyl phthalate (DMP) (**55**) (Kirk and Othmer, 1978). The compounds used as repellents include *n*-propyl-*N,N*-diethyl succinamate, *n*-butyl-6, 6-diethyl-5, 6-dihydro-1, 4-pyrone-2-carboxylate, *o*-chloro-*N,N*-diethylbenzamide among others (Kirk and Othmer, 1978). The disadvantages exhibited by these synthetic repellents include toxicity to other animals especially man, development of resistance (avoidance) by insects, high costs and environmental pollution (Stinecipher *et al.*, 1997). DEET, a well known synthetic commercial repellent is not only reported to be ineffective against *Anopheles* mosquitoes but is also an irritant and some people react badly to it (Carl and Leonhardt, 1991).



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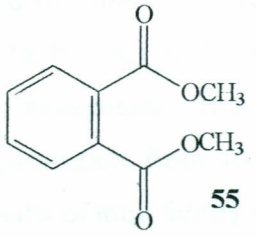

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Other examples of synthetic repellents include IR 3535 marketed in Europe and USA. IR 3535 had a repellency of ≥92% against *An. gambiae s.s* and *An. funestus* for 6 hours in Liberia (Thavara *et al.*, 2001). KBR 3022 available in many countries around the world is known to repel mosquitoes, black flies, stable flies, *Gasterophilus* spp. and ticks (Yap *et al.*, 1998). Toxicological studies have shown that KBR 3022 to be safe for human use (Yap *et al.*, 1998). The efficacy of A13-37220 is shown to be equivalent to DEET against *An. quadrimaculatus*. However, it performs better than DEET against *An. taeniorhychus* (Walker *et al.*, 1996).

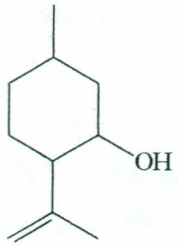
In malaria endemic regions, permethrin treated clothings plus DEET on exposed skin yielded good protective efficacy against mosquito bites (King *et al.*, 1960). Several other synthetic pyrethrins have been used as mosquito repellents (Yap *et al.*, 1990).

2.2.2 Natural repellents

Various substance (s) and methods have been used since ancient times to repel blood-sucking insects. Smoke from open fire repels insects. The repellent effect of smoke may be increased by burning certain materials such as aromatic wood containg resins. The use of plants as natural repellents has been documented for many region (Curtis *et al.*, 1990).

In India leaves of *Vitex negundo* (Nochi) are burnt to repel mosquitoes from houses (Curtis *et al.*, 1991). The practice of smearing the bodies with turmeric and mustard oil before bathing without soap resulted in lower spleen indices among women in India (Philip *et al.*, 1945). Men who avoided this practice were observed to be frequently bitten by *An. fluvicitilis* than were women sleeping nearby. Citronella products are used in India and are effective against *Anopheles* mosquitoes but their protective effects do not last long. In former USSR, burning of air-dried thyme sticks in open air gave 85% protection against mosquito bites for 60 to 70 minutes (Curtis, 1991). In the Gambia, the wood and resin of aromatic trees collectively referred to as *Churai* are burned to repel mosquitoes, although studies showed that users of this method were not protected for malaria (Snow *et al.*, 1987). In East and West Africa, herbs of mint family (*Lamiaceae*) are used as mosquito repellents (Curtis, 1991). Burning of certain herbs such as *Artemisia* and *Calamus* species is still practised in remote villages of China to repel mosquitoes. Herbs of basil family (*Labiatae)* have many traditional medicinal uses including protection from mosquito bites in Africa and Asia (Dalziel, 1937). In Tanzania, the juice from herbs of mint family is applied to the legs for protection against mosquitoes (Curtis *et al.*, 1990). White (1973) showed that juices of *Ocimum* species when smeared on legs reduced biting by caged *An. gambiae s.s.* The repellency of *Ocimum sanctum* leaves was similarly demonstrated (Rathore, 1978). Plants such as *Ocimum* spp, *Ajuga remota* and *Nepeta cataria* (Labitae), *Azadirachta indica* (Meliaceae) and *Lantana* spp (Verbanaceae) among others were smouldered to produce smoke carrying chemical compounds that would repel mosquitoes away from the house. This reduced mosquito numbers indoor and rates of inoculation (Palsson, 1999). However, most of these products have not been carefully analyzed (Schreck, 1995).

The essential oils of several plants have been shown to have mosquito repellent properties. These oils and a mixture of them were the basis of most commercial repellents and many different varieties were produced and tested (Grannet, 1940). These observations have been made in the oils of cassia, camphor, citronella, lemongrass, clove, thyme, geranium, bergamot, baylauvel, pine wintengreen, penyroyal, *Eucalyptus* among others (Knippling, 1945). Some examples of natural repellents that have been isolated from a wide range of plants include isopulegol (**56**), geraniol (**57**), camphor (**58**), pinene (**59**), citronellol (**60**), citronellal (**61**) and *p*-menthane-3,8-diol (**62**). They are usually thought to be part of the plant chemical defense system against herbivorous insects or animals (Steineggar and Hansel, 1972; Dethier, 1947). Coincidentally, they also have repellent properties against disease vectors.



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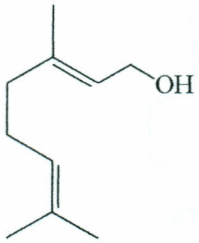

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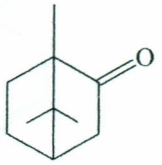

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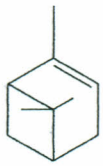

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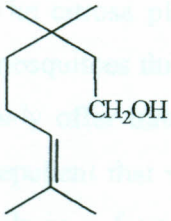

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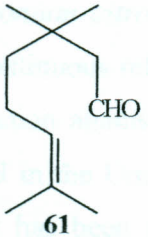

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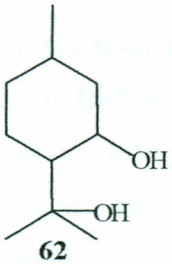
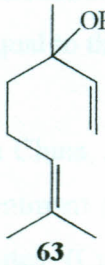


62

Chromatographic analysis of essential oil of *Artemisia vulgaries* yielded several compounds such as linalool **(63)**, camphor, terpene-4-ol **(64)** and borneol **(65)**, which are active as repellents against *Ae. aegypti* (Hwang *et al.*, 1985).



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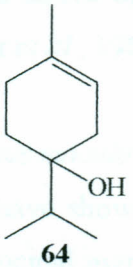

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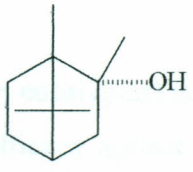
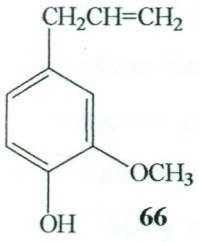


65

The repellency of *Ocimum* species has been attributed to eugenol **(66)**, the major component of clove and other essential oils reputed to have repellent properties (Chogo and Crank, 1981).

Trans-cis-nepetalactone **(67)**, a recently discovered natural mosquito repellent from *Nepeta cataria*, is more effective than DEET (Kumar and Dutta, 1987; Anon, 2001c).



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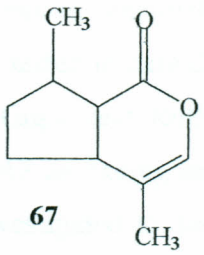


67

The citrosa plant (*Pelargonium citrosum* 'Van Leenii') has been marketed as being able to repel mosquitoes through the continuous release of citronella oil. Unfortunately, when tested, these plants only offer transient protection against bites (Matsuda *et al.*, 1996). Bite Blocker®, a plant-based repellent that was released in the United States in 1997 combines soybean, geranium, and coconut oils in a formulation that has been available in Europe for several years (Pollack *et al.*, 2002). Studies conducted in Canada, showed that this product gave more than 97% protection against *Aedes* mosquitoes under field conditions, even 3.5 hours after application. Several citronella-based products are sold in Europe, USA and India as mosquito repellents. A synthetic derivative of citronellal has been used as the active ingredient of a commercial repellent with efficacy almost equal to that of DEET (Buescher *et al.*, 1982; Rutledge *et al.*, 1985).

In China, an extract of *Eucalyptus maculata citriodora* (lemon eucalyptus) is widely used as topical ointment and tests on humans have shown a high level of efficacy against *Aedes* mosquitoes and mites (Curtis, 1990a). The principal ingredients of *Eucalyptus citriodora* essential oil consists of citronellal, citronellol, geraniol, isopulegol, pinene and sesquiterpenes. However, laboratory assays of these ingredients for repellency to *Ae. aegypti* were rather disappointing (Zhuang *et al.*, 1974). The effective ingredient was finally found in the steam distillate aqueous residue and identified as *p*-menthane-3, 8-diol (Curtis, 1991).

Another derivative designated 9525 isolated from leaves and stems of plants *Clausena kwangsiensis*. Its effective ingredient was identified as *p*-menthane-3, 8-diol (Curtis, 1991).

Pyrethrins (Matsui and Yamamoto, 1971) and *p*-menthane-3, 8-diol (Trigg, 1996) are natural plant-derived repellent that have been found to be environmentally friendly and harmless to non-target organisms.

Thousands of plants have been tested as potential sources of insect repellents (Jacobson 1990). None of the plant-derived chemicals tested to date demonstrate the broad efficacy and long duration of protection exhibited by DEET (Gupta and Rutledge, 1994). A few show repellent activities. Over 1000 plants were listed in 1947 as having excellent repellents or insecticidal value (Roark, 1947). Many of these remain uninvestigated to date, many others may not have been discovered. This project investigated some plants with potential mosquito repellent/insecticidal properties and identified the active ingredients therein. During our bioprospecting activities 7 plants were chosen for insecticidal and repellency studies against *An. gambiae s.s.*

2.3 Plants and families

Research by earlier workers has shown that some plants possess medicinal values as well as mosquito repellent or larvicidal properties (Sukumar *et al.*, 1991). A total of 21 plants within 7 different plant families (Anacardiaceae, Asteraceae, Guttifarae, Labiatae, Rutaceae, Umbelliferae and Verbenaceae) were collected. Surprisingly, only 4 plant families Astereceae, Labiatae, Rutaceae and Umbelliferae contained plants with good mosquito repellent and insecticidal activities.

2.3.1 The family Astereceae

Repellent plants from this family are well documented. Reported cases include *Chrysanthemum cinerariafolium* (Kirk and Othmer, 1978), *Artemisia vulgaris* (Hwang *et al.*, 1985), *A. cana* (Sherif and Hall, 1985), *Hemizonia fitchii* (Klocke *et al.*, 1985) and *Matricaria chamomilla* (Thorsell, 1988) *Conyza newii* and *Tarchonanthus camhoratus* (Omollo, 2002) among others. Bioprospecting work within this family revealed three plants with good activity against mosquitoes: *Artemisia afra*, *Senecio moorei* and *Cineraria grandifolia*.

2.3.1.1 The genus *Artemisia*

Essential oils from plants belonging to the genus *Artemisia* have been widely investigated. Many of the plants contain various monoterpenoids, which vapourize into air and repel blood-sucking insects when the plant is slowly burnt (Hwang *et al.*, 1985). *A. vulgaris* a member of this genus contains mainly monoterpenes such as isoborneol, α- and β-thujone, artemisia ketone, artemisia alcohol, linalool, camphor, terpene-4-ol, 3-nonanone and isobornyl acetate. Beside, artemisia ketone and artemisia alcohol the rest are known to posses mosquito repelling property (Hwang *et al.*, 1985). Out of these compounds, terpene-4-ol was shown to be the most active and effective repellent

against *Aedes aegypti* and as good as dimethyl phthalate (DMP). *A. annua*, whose main essential oil constituents are artemisia ketone, camphor, 1,8-cineole, isoborneol, methylchavicol, artemisia alcohol, artemisia acetate, pinene and camphene (Fabien *et al.*, 2000), is highly regarded for its anti-malarial activity (Klayman, 1985). The essential oil has anti-bacterial activity (Khan *et al.*, 1991). Recent studies also indicate that some qinghaosu (*A. annua*) derivatives have other uses, including anti-parasitic activity against *Schistosoma japonicum* and *Toxoplasma gondii* (Li and Wu, 1998). Research into essential oil of *A. annua* will no doubt continue to produce results of the atmost importance including mosquito repellent activity, which has not been demonstrated yet. Oils from other plants from this genus that have been investigated include *A. californica* (Halligan, 1975) and *A. doughlasian* which was reported to contain irregular monoterpenes, artemisia ketone and artemisia alcohol (Score *et al.*, 1984)

2.3.1.1.1 *Artemisia afra*

Artemisia afra from the family Asteraceae is described as an erect loose shrub to 2 m, with grey-green aromatic foliage. Locally the plant is known by various names such as Sesimwa (Marakwet), Ushemeli (Sukuma) and Shamba (Fiji) (Agnew, 1974). The herb is frequently used in folk medicine for various pharmacological properties (Kokwaro, 1993). A fermentation of the heated herb is given to children with soar throat. The plant is also used for indigestion. Roots are boiled and decotion drunk 2-3 times a day for internal worms. The leaves are chewed and juice swallowed as emetic (Kokwaro, 1993). *A. afra* was collected from Marania Forest, Mt. Kenya via Timau on the way to the Kenya School of Adventure.

The anti-plasmodial activities against chloroquine-resistant *Plasmodium falciparum* and mosquito larvicidal activity of the extracts of *A. afra* has been reported (Kraft *et al.*, 2003) and (Guantai, 1990) respectively. The essential oil composition of *A. afra* from S. Africa, Botswana and Zimbabwe has been reported (Graver *et al.*, 1990; Chagonda *et al.*, 1999). However, no repellent or adulticidal activity had been undertaken. In our preliminary studies, *A. afra* oil exhibited both repellent and insecticidal activity against *An. gambiae s.s* mosquitoes. These findings and the fact that no repellent or insecticidal activity had been reported in the literature for this plant prompted us to embark upon chemical and biological investigations in search of repellent or insecticidal compounds therein.

2.3.1.2 The genus *Cineraria*

The genus name *Cineraria* means grey or ash-coloured from the Latin *cinerarius* and refers to the leaf colour of the first known species. *Cineraria* belong to the Asteraceae (daisy), a huge family of some 1,535 genera and 25,000 species spread all over the world except Antarctica, and is placed in the tribe Senecioneae. This is a genus of herbs and sub-shrubs that consists of approximately 50 species and occur throughout Africa. Only two species are known to occur outside Africa, one with a distribution that extends into Yemen and another one that occurs in Madagascar. The centre of diversification is southern Africa where at least fourty (80%) of the currently described species can be found (Jackson, 1990).

2.3.1.2.1 *Cineraria grandifolia*

This plant in general is described as almost hairless herb, sometimes supported, leaves clasping at base, with long stalks, almost triangular, toothed heads in terminal corymbs, florets yellow, rayed spreading 7-13. It is a common species of roadsides, forest edges and cliffs from 1890-3660 m. The plant is often distributed in HE, HC, HT, KIT below 2135 m, KAJ and HM. The specimen for the study was collected from Meterological Station, Mt. Kenya along Naru Moru route (altitude 10034 ft, East 12.823 min and South 10.214 min). No literature data is available regarding mosquito repellency or insecticidal activities of the plants within this genus. The mosquito repellent activity of this plant is being reported for the first time. Similarly, the essential oil from this plant has been confirmed to possess no insecticidal activity against *An. gambiae s.s* mosquitoes even at the highest concentration tested and we are thus reporting this finding for the first time.

2.3.1.3 The genus *Senecio*

Senecio is the largest genus in the tribe Senecioneae (Asteraceae) of which more than 1,500 species have been reported. This genus is rich in pyrrolizidine alkaloids and sesquiterpenes, in particular eremophilanolide derivatives (Rizk, 1990). For instance, Gonzalez *et al.* (1995) discovered that an ethanolic extract of *Senecio palmensis* had strong anti-feedant activity against Colorado potato beetle (CPB) larvae. Two compounds have been isolated from *S. palmensis*, one from the chemical class of bisabolenes and the other a silphinene sesquiterpene (Gonzalez *et al.*, 1995). Both of these chemicals may alter the host selection process through adult behavioural avoidance because adults are highly mobile and are the primary finders of host plants (Hough, 1990). Bisabolenes serve as effective anti-feedants by causing feeding inhibition.

2.3.1.3.1 *Senecio moorei*

This plant was collected from Chania Falls (altitude of 9878 ft, East 27.204 min and South 43.031 min) in Aberdare National park. It is an erect shrub or woody with variable cobwebby hairs and few or no glands, leaves to 30 mm wide oblanceolate, minutely toothed at the gradually narrowed base, head bell shaped about 15 mm across, 8 mm long in large corymbs. The plant is common in high altitude grasslands especially forest clearing above 2600 m. It is distributed in HE, HT, HA, KIT, and RV. Preliminary investigation of the plant indicated that the essential oil is a mosquito repellent though not insecticidal. Literature search indicated that no mosquito repellency or insecticidal work against *An. gambiae s.s* had been undertaken on the plant. However, mosquito larvicidal activity within the genus has been investigated. The insecticidal activity of essential oils from Bolivian plant species of genus *Senecio* has been previously reported against *Ae. aegypti* larvae with monoterpenes (*E*)-anethol and (*E*)-nerolidol being the active principles of the most toxic essential oils (Chantraine *et al.*, 1998). Therefore, this is the first report on the repellent activity of the leaf oil of *Senecio moorei.*

2.3.2 Family Labiatae

Labiatae family consists of about 3,000 species of plants spread in the warm and temperate regions all over the world. They are mainly grasses and shrubs, very fragrant and rich in medicinal properties and of great worth in natural medicine and pharmacopoeia (Agnew, 1974). Labiatae include about 200 genera. The most important are *Ocimum*, *Nepeta* and *Satureja* among others. From the literature survey, few plants from this family have been reported to exhibit mosquito repellent properties. *Rosemarinus officinalis* has mosquito repellent activity (Thorsell, 1988). Herbs of Basil family (*Labiatae*) are used as mosquito repellent in East and West Africa (Kokwaro, 1993). Others are *Ocimum suave* (Chogo and Crank, 1981), *Nepeta cataria* (Kumar and Dutta, 1987) among others. From the bioprospecting work in this family, two plants *Nepeta azurea* and *Satureja pseudomensis* showed good mosquito repellency activity.

2.3.2.1 The genus *Nepeta*

Mosquito repellent activity has been reported in this genus recently. Essential oil from *Nepeta cataria* (catnip), a perennial herb belonging to the mint family repels mosquitoes (Kumar and Dutta,

1987). Nepetalactone, the major component of the essential oil of catnip that gives the plant its characteristic odour, is ten times more effective in repelling mosquitoes than DEET (Anon, 2001c)

2.3.2.1.1 *Nepeta azurea*

The plant was collected from Marania Forest, Mt. Kenya (altitude 10172 ft, 3° 0.786 min, North and 37° 25.991 min, East) on the way to Kenya School of Adventure. It is an erect heavy perennial herb sometimes branched, leaf lanceolate, heart-shaped at base, densely felt-hairy on both sides. This is a common herb in bushed grasslands at high altitude (1800-3600 m). *N. azurea* is distributed in HE, HC, HM, KSM, NRB and HA (Agnew, 1974). A review of the literature revealed no reports on previous repellent or insecicidal activity of this plant. However, our analysis unearthed mosquito repellent properties from this plant.

2.3.2.2 The genus *Satureja*

The genus *Satureja*, in the family Labiatae has about 30 species distributed in tropical Africa, Europe and North America. Essential oils obtained from the leaves and flowers of *Satureja* species have varied industrial applications as flavouring materials, medicine and perfumes among others (Teklu *et al.*, 1998).

2.3.2.2.1 *Satureja pseudomensis*

This is a herb with weak branching, ascending stems from a woody base, leaves ovate to orbicular, flowers in dense axillary fascicles purple. It is common in forest clearings and edges, mostly upper limits of mountain rain forest and within the heath zone. It is distributed in regions such as HE, HC, HT and KSM. *S. pseudomensis* was collected on the way to Chania Falls, Aberdare Mountains. The plant had low yield of essential oil that was a good mosquito repellent. No previous work on repellency activity of this plant has been reported. The species within the genus *Satureja*, have been widely investigated for repellency of their essential oils to stored-product insect pests. For instance, essential oil of *S. thymbra* repelled *Sitophilus oryzae* adults in food preference tests (Sarac and Tune, 1995).

3.3.3 The family Rutaceae

This family is mainly found in the tropics with many species occurring in South Africa, Australia and South-East Asia. Economically, the most important genus is *Citrus* (orange, grapefruit, lemon, tangerine and lime among others).

A review of the literature reveals several repellent plants from this family. The plants, *Zieria smithii*, *Clausena kwangsiensis* and *Clausena anisata* have been shown to repel mosquitoes (Jacobson, 1958; Curtis, 1991; Okunade and Olaifa, 1987). Only one plant *C. anisata* from this family was collected and bioprospecting work indicated good repellent activity.

3.3.3.1 The genus *Clausena*

Clausena contains about 50 species native to Africa and the Malaysian region with one species *C. anisata*, occurring throughout Africa. Stems of *Clausena* species are used in Africa for evacuant, headache and liniment among others.

3.3.3.1.1 *Clausena anisata*

Okunade and Olaifa (1987) reported repellent activity of this plant against *Culex pipiens* mosquitoes but not against *An. gambiae s.s*, the principal malaria vector in Africa. Among the local communities this plant is referred as Matathi (Kikuyu), Kisimbari (Luhya), Munyapala (Digo) and Msongambwa (Luguru) (Agnew, 1974). Traditionally, the plant has several uses; pounded roots are highly recommended for headache, malaria, influenza and indigestion. Twigs are used as tooth-brushes and are believed to cure toothache. A root decoction is effective for syphilis treatment and pounded roots are put in soup and given to women to cleanse the uterus (Kokwaro, 1993). This plant was collected from Kabati Forest, Mt. Kenya.

Earlier bioprospecting by Okinyo (2002) revealed larvicidal activity of the bark of this plant against *An. gambiae s.s* mosquitoes. However, adulticidal activities were not tested. Given the larvicidal activity of this plant, it was interesting to investigate the adulticidal properties of the essential oil from the leaves. The oil of this plant was found to have a promising repellent activity and no mosquitocidal activity against *An. gambiae s.s*. This is the fist report on mosquito repellent activity of the leaf oil of *C. anisata* againt *An. gambiae s.s*.

2.3.4 The family Umbelliferae

The Umbelliferae family comprises of 300 genera and 3,000 species, which are distributed in most parts of the world, although they are more commonly found in temperate regions. The Umbelliferae was the first family to be recognized and systematically studied by botanists of the 16th century during initial plant classification (Heywood, 1997). Only a few genera of umbellifers are grown as ornamental garden plants including *Eryngium*, *Astrantia*, *Myrrhis*, *Aciphylla*, *Bupleurum* and *Heracleum*, but many members of this family have wide ranging and varying practical uses (Burtt, 1991). For instance, the most well-documented poisonous species, hemlock (*Conium maculatum)*, was probably responsible for the death of Socrates. Culpeper, the herbalist, said that this plant could put a stop to lustful thoughts. He was most probably right, as it has the potential to put a stop to all thoughts! It contains the drug coniine, which although powerfully toxic, now has some medicinal applications (Cannon, 1978).

Some other members of this family were commonly used in medieval times to cure ailments ranging from memory loss to eczema and gout (Schreiber, 1967). A review of the literature on this family indicates that some plants posses both repellent and larvicidal activity against mosquitoes. According to Supavarn *et al.* (1974), root bark extract of *Conium maculatum* was adulticidal to *Ae. aegypti*. Root extract of *Peucedanum ostruthum* was larvicidal to *Culex quinguefascaitus* (Jacobson, 1958) among others.

2.3.4.1 The genus *Pseudocarum*

Pseudocarum species together with eight Malagasy species (Apiaceae), previously misplaced in the African genus *Heteromorpha*, have remarkably unspecialised fruits and do not fit within Drude's classification system for the family, as they combine vittae characteristic of the Saniculoideae and Apioideae. They have oil ducts in the fruit ribs in addition to the normal ones between the ribs. Moreover, *Pseudocarum* species have dentate-serrate leaf segments, and some also have dentate involucral bracts, reminiscent of the Saniculoideae (Van Wyk *et al.*, 1999).

2.3.4.1.1 *Pseudocarum eminii*

The plant was collected at an altitude of 7969 ft, 22° 0.945 min South and 48° 0.046 min East from Meterological Station, Mt. Kenya via Naru moru route. The plant is physically described as almost hairless climber with twining leaf stalks, leaves 3-9 ovate, lanceolate, pointed, toothed leaflets,

bracts and bracleoles oblong, fruit 2 x 3 mm umbel rays elongating and spreading in fruit threat-like. The plant is very common in Bamboo zones of the mountains especially where Bamboo has flowered and dried (1860-3350 m). We could not find any previous reports of repellent or mosquitocidal activity of this plant. Moreover, the essential oils compositions of plant species from this genus have not been well investigated. The plant is repellent as revealed by our bioprospecting work.

2.4 Hypothesis

We hypothesized that there are valuable, unexplored botanical sources with good repellents or insecticidal activity, which could be isolated, identified and developed for practical use or into commercial products for mosquito and therefore malaria control.

2.5 Objectives

The general objective of this research was to investigate the repellent or adulticidal activity of bioactive components of plant extracts from Central region of Kenya against *An. gambiae s.s* mosquitoes. The specific objectives were to extract and screen potential repellent or insecticidal plants used by the local population against mosquitoes and isolate and purify principal ingredients of repellent plant extracts. We also intended to carry out structural elucidation of pure isolates by chromatographic and spectroscopic methods. The bio-assay of pure isolates for their repellent or insecticidal activity against *An. gambiae s.s* mosquitoes was also anticipated.

CHAPTER 3

SCREENING FOR INSECTICIDAL AND REPELLENT ACTIVITY

3.0 Introduction

Various strategies are often employed in choosing plants that may contain new biological agents. One such approach that is no longer practical involves random screening (Soejarto, 1993). This involves collection of all available species from a particular locality regardless of any previous knowledge. In ethnobotanical approach, plants are collected for validation based on the oral or written information on the traditional or cultural medicinal uses of the plant. Alternatively, plants from pre-determined family or genera of interest are sought from diverse location according to chemotaxonomic and phytochemical considerations, where a particular compound or class of compounds is chosen for investigation (Waterman, 1993).

In bioprospecting for anti-mosquito plants from Central region of Kenya, 21 plants were collected. The collection was based on chemotaxonomic approach, where plants of pre-determined family or genera considered to be of potential insecticidal or repellency values were sought from this region. The plants were steam-distilled for essential oils and the residue extracted with chloroform and water respectively. The extracts were subjected to anti-mosquito assays. During the bio-assays, the following tests were performed:

1. Repellency assay of essential oils from 16 plants, chloroform and water extracts from 9 plants.
2. Fumigation assay of essential oils from 16 plants and chloroform extracts from 21 plants.
3. Tarsal contact mosquitocidal assay of the solvent extracts from 21 plants.

In performing the repellency tests, WHO (1996b) protocols were followed. Essential oils, chloroform and water extracts from 21 plants studied were subjected to preliminary assays against female *An. gambiae s.s* mosquitoes using three concentrations. The repellency was assessed as percentage protective efficacy (% PE).

3.1 Repellency assay of water extracts

Water extracts from 9 plants were tested. Extracts from 7 plants were examined in detail (Table 1). Apart from *S. moorei*, none of the extracts tested exhibited a promising repellent activity (>90 %).

The repellent activity of water extracts was established as *S. moorei* > *N. azurea* > *C. anasita* > *S. pseudomensis* > *P. eminii* > *A. afra* > *C. grandifolia.*

Table 1. Detailed mean % protective efficacy of water extracts

Plant material	% Protective efficacy (± SE)	Plant material	% Protective efficacy (± SE)
A. afra	54.03 ± 3.21^{vw}	*S. pseudomensis*	65.39 ± 8.67^{tuv}
C. grandifolia	51.23 ± 3.12^{w}	*C. anisata*	67.85 ± 9.86^{tu}
S. moorei	91.89 ± 8.45^{s}	*P. eminii*	58.35 ± 3.23^{uv}
N. azurea	75.01 ± 8.45^{st}		

Means with same letters show no significant difference in efficacy between the concentrations tested.

3.2 Repellency assay of solvent extracts

Solvent extracts of *A. afra*, *V. urticifolia* and *B. fusca* in acetone were the most effective with protective efficacy > 90 at 0.05 g/ml concentration (Table 2). However, the extracts were not evaluated in detail.

Table 2. Preliminary mean % protective efficacy of solvent extracts from repellent plant species

	% Protective efficacy ± SE		
% Conc (g/ml)	5.0×10^{-2}	5.0×10^{-4}	5.0×10^{-6}
A. afra	92.34 ± 6.05^{abc}	50.62 ± 14.69^{fghij}	49.37 ± 7.97^{ghij}
B. fusca	94.80 ± 6.65^{ab}	67.16 ± 12.13^{efg}	42.34 ± 7.78^{ijklm}
C. grandifolia	65.72 ± 17.47^{defgh}	59.70 ± 11.46^{efghi}	39.77 ± 10.05^{jklmn}
S. moorei	84.19 ± 6.04^{bcd}	63.48 ± 13.0^{efgh}	29.36 ± 7.97^{lmno}
S. nadensis	55.27 ± 19.36^{efghi}	23.42 ± 10.1^{nop}	24.28 ± 12.72^{mno}
V. urticifolia	97.85 ± 2.78^{a}	87.50 ± 0.0^{abc}	59.96 ± 16.21^{efghi}
R. natalensis	85.72 ± 21.46^{abcdef}	63.38 ± 13.83^{efghi}	8.82 ± 11.32^{p}
S. roseflorus	66.52 ± 14.87^{cde}	22.06 ± 10.61^{op}	32.60 ± 8.71^{klmno}
P. edulis	74.36 ± 7.7^{de}	41.36 ± 7.45^{jklm}	40.54 ± 11.31^{ijklm}
C. anisata	78.18 ± 9.97^{cde}	68.75 ± 9.23^{ef}	44.31 ± 8.14^{hijkl}

Means with same letters show no significant difference in efficancy between the concentrations tested

3.3 Repellency assays of essential oils

Table 3 shows preliminary repellency assay data for essential oil from 16 plant species.

Table 3. Preliminary mean % protective efficacy of essential from repellent plant species

	% Protective efficacy (± SE)			
% Conc (g/ml)	10^{-1}	10^{-2}	10^{-3}	10^{-4}
A. afra	100.00±8.39^{ab}	nd	66.63±6.71^{ghi}	57.13±7.43^{hij}
B. fulsca	100.0±22.42^{ab}	nd	77.22±4.64^{ef}	38.61±5.96^{klmno}
C. grandifolia	100.00±9.98^{ab}	nd	63.00±9.23^{ghi}	29.82±8.41^{nopq}
G. tormentosa	nd	53.35±12.7^{hij}	47.29±11.67^{ijkl}	36.46±6.26^{mnop}
S. moorei	95.88±1.47^{ab}	nd	44.60±14.06^{klm}	28.73±3.07^{pq}
S. keniodendrom	92.77±6.38^{ab}	nd	69.52±11.39^{hg}	63.45±17.23^{ghi}
S. schweifurthii	85.07±6.67^{cde}	nd	42.24±9.47^{klmno}	48.82±13.47^{jkl}
S. nandensis	73.98±12.8^{efg}	nd	44.88±4.29^{klm}	26.25±7.85^{pq}
V. urticifolia	91.56±12.86^{ab}	nd	40.22±9.63^{klmn}	37.33±5.88^{jkmnopq}
H. revolutum	90.90±14.69^{abcde}	nd	49.93±14.04^{ijkl}	36.91±7.17^{mnop}
N. azurea	nd	86.96±10.26^{cde}	73.68±15.37^{efg}	41.13±3.72^{klmn}
P. edulis	93.30±9.84^{ab}	nd	68.08±8.65^{hg}	51.97±6.04^{jk}
S. pseudomensis	nd	68.16±6.71^{g}	40.63±5.92^{klmnop}	37.31±3.14^{klmnop}
C. anasita	nd	87.63±1.48^{cd}	72.13±4.82^{g}	58.3±3.07^{i}
P. eminii	90.43±8.24^{b}	nd	48.93±10.61^{jkl}	43.55±6.47^{klmo}
S. biflora	nd	74.13±^{f}	54.45±5.92^{j}	50.16±7.75^{jk}

Means with same letters show no significant difference in efficancy between the concentrations tested.

From the preliminary assay, the repellent activity of >90% was found in the essential oil of 9 plant species (*Artemisia afra*, *Vernonia urticifolia*, *Senecio keniodendrum*, *Cineraria grandifolia*, *Hypericum revolutum*, *Borthrocline fulsca*, *Pseudocarum eminii*, *Senecio moorei* and *Plectanthus edulis*) at the highest concentrations. Although *V. urtifolia* and *B. fusca* were promising during preliminary assay, they were not evaluated in detail because of the low yield of the essential oils. Consequently, the essential oils from 7 plant species were tested in detail (Table 4).

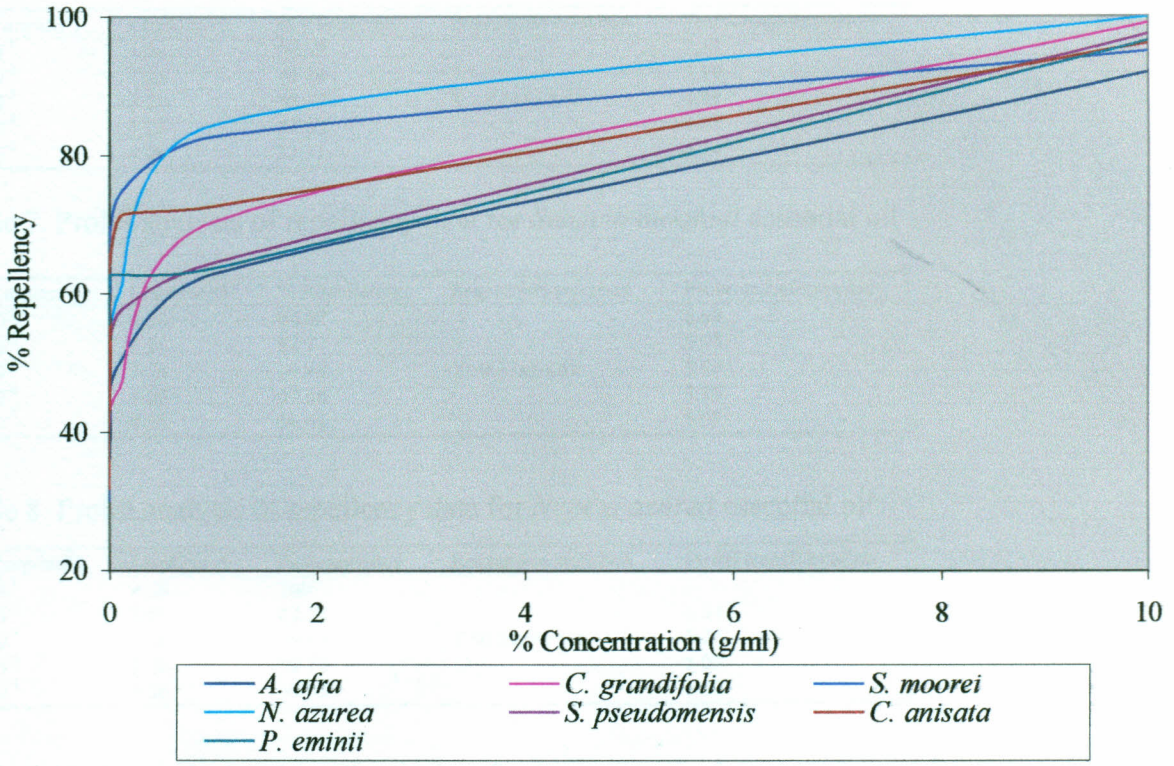
Table 4. Detailed mean % protective efficacy of essential oils from repellent plant species

	% Protective efficacy (± SE)				
% Conc (g/ml)	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}
A. afra	91.97±10.61^{abcd}	63.83±6.67^{ghi}	44.30±4.29^{jklmno}	40.27±12.8^{klmn}	37.04±7.85^{lmnop}
C. grandifolia	99.12±0.0^{ab}	70.34±15.37^{g}	46.37±3.72^{klm}	43.83±14.69^{jkmno}	33.31±14.04^{noph}
S. moorei	94.97±9.84^{ab}	82.70±3.05^{de}	74.44±1.48^{f}	59.66±4.82^{ij}	56.41±3.08^{h}
N. azurea	100±21.18^{a}	84.33±3.63^{cde}	60.13±2.2^{hi}	52.76±9.54^{ikl}	50.03±6.75^{ijkl}
S. pseudomensis	97.5±3.18^{a}	66.45±11.45^{fh}	57.38±6.76^{ij}	46.67±2.64^{kl}	43.46±8.73^{klmno}
C. anasita	96.18±2.23^{ab}	72.63±3.05^{efg}	71.13±3.11^{g}	48.94±9.41^{fjkl}	38.00±8.04^{klmnop}
P. eminii	96.52±2.49^{ab}	63.67±14.28^{fghij}	62.62±15.30^{fhij}	55.88±5.12^{ij}	46.30±3.35^{kl}

Means with same letters show no significant difference in efficancy between the concentrations tested

The % protective efficacies of 91.97, 99.12, 94.97, 100, 97.5, 96.18, 95.7 and 96.52 was achieved at 0.1 g/ml for *A. afra*, *C. grandifolia*, *S. moorei*, *N. azurea*, *S. pseudomensis*, *C. anisata* and *P. eminii*, essential oils respectively. The repellency assay data was subjected to regression analysis and the best fitting curves established (Fig. 1) (SAS® Institude, 2002).

Figure1. Mean % protective efficacy of essential oils from repellent plant species



The regression is based on the equation % PE = exp (log a+ blog x) where x = concentration, log a = intercept, b = slope. For *N. azurea* log a = 82.60 and b = 13.09, *C. grandifolia* log a = 74.41 and b = 15.18, *S. moorei* log a = 83.65 and b = 10.02, *A. afra* log a = 68.82 and b =13.34, *S. pseudomensis* log a = 74.48 and b = 12.89, *C. anisata* log a = 79.38 and b = 14.01, *P. eminii* log a = 72.58 and b = 10.82.

The probit plane model (Busvine, 1971) that relates the response of the mosquito test population to the log of the repellent applied is very important in the testing and evaluation of repellents (Rutledge *et al.*, 1985). In order to study the relative potency of plant derived essential oils, we employed the probit analysis (Tables 5-11).

3.3.1 Probit transformation of repellency data

Table 5. Probit analysis of repellency data for *Artemisia afra* essential oil

Dose (mg/cm^2)	Logdose+5	% Repellency	Regression equation	Probit transformation
$1.8x10^{-1}$	4.26	91.97		6.64
$1.8x10^{-2}$	3.26	63.83		5.41
$1.8x10^{-3}$	2.26	44.3	Y=0.41x+3.98	4.85
$1.8x10^{-4}$	1.26	40.27		4.75
$1.8x10^{-5}$	0.26	37.04		4.67

Table 6. Probit analysis of repellency data for *Cineraria grandifolia* essential oil

Dose (mg/cm^2)	Logdose+5	% Repellency	Regression equation	Probit transformation
$1.8x10^{-1}$	4.26	99.12		7.33
$1.8x10^{-2}$	3.26	70.34		5.39
$1.8x10^{-3}$	2.26	46.37	Y =0.61x+3.57	4.87
$1.8x10^{-4}$	1.26	43.83		4.85
$1.8x10^{-5}$	0.26	33.31		4.64

Table 7. Probit analysis of repellency data for *Senecio mooreei* essential oil

Dose (mg/cm^2)	Logdose+5	% Repellency	Regression equation	Probit transformation
$1.8x10^{-1}$	4.26	94.97		6.64
$1.8x10^{-2}$	3.26	82.7		5.95
$1.8x10^{-3}$	2.26	74.44	Y=0.37x+4.62	5.64
$1.8x10^{-4}$	1.26	59.66		5.15
$1.8x10^{-5}$	0.26	56.41		5.08

Table 8. Probit analysis of repellency data for *Nepeta azurea* essential oil

Dose (mg/cm^2)	Logdose+5	% Repellency	Regression equation	Probit transformation
$1.8x10^{-1}$	4.26	100		-
$1.8x10^{-2}$	3.26	84.33		6.04
$1.8x10^{-3}$	2.26	60.13	Y=0.91x+6.99	5.23
$1.8x10^{-4}$	1.26	52.76		5.05
$1.8x10^{-5}$	0.26	50.03		5.00

Table 9. Probit analysis of repellency data for *Satureja pseudomensis* essential oil

Dose (mg/cm^2)	Logdose+5	% Repellency	Regression equation	Probit transformation
$1.8x10^{-1}$	4.26	97.5		6.88
$1.8x10^{-2}$	3.26	66.45		5.36
$1.8x10^{-3}$	2.26	57.38	Y =0.49x+3.1	5.28
$1.8x10^{-4}$	1.26	46.67		4.82
$1.8x10^{-5}$	0.26	43.46		4.80

Table 10. Probit analysis of repellency data for *Clausena anisata* essential oil

Dose (mg/cm^2)	Logdose+5	% Repellency	Regression equation	Probit transformation
$1.8x10^{-1}$	4.26	96.18		6.48
$1.8x10^{-2}$	3.26	72.63		5.61
$1.8x10^{-3}$	2.26	71.13	Y =0.48x+4.09	5.55
$1.8x10^{-4}$	1.26	48.94		4.85
$1.8x10^{-5}$	0.26	38		4.64

Table 11. Probit analysis of repellency data for *Pseudocarum eminii* essential oil

Dose (mg/cm^2)	Logdose+5	% Repellency	Regression equation	Probit transformation
$1.8x10^{-1}$	4.26	96.52		6.75
$1.8x10^{-2}$	3.26	63.67		5.36
$1.8x10^{-3}$	2.26	62.62	Y =0.41x+4.73	5.33
$1.8x10^{-4}$	1.26	55.88		4.87
$1.8x10^{-5}$	0.26	46.3		4.85

The regression equations (Zar, 1984) were used to calculate the RD values (Table 12). The order *N. azurea* > *P. eminii S. moorei* > *C. anisata* > *S. pseudomensis* > *C. grandifolia* > *A. afra* was established based on the RD_{50} values.

Table 12. RD values of the essential oils from repellent plant species

	RD (mg/cm^2)			
Plant materials	RD_{25}	RD_{50}	RD_{75}	RD_{90}
N. azurea	$1.19x10^{-7}$	$6.5x10^{-7}$	$3.54x10^{-6}$	$1.65x10^{-5}$
P. eminii	$1.12x10^{-5}$	$4.39x10^{-4}$	$1.73x10^{-2}$	$4.9x10^{-1}$
C. anisata	$3.09x10^{-4}$	$7.86x10^{-3}$	$1.96x10^{-1}$	3.65
S. moorei	$2.19x10^{-5}$	$1.27x10^{-3}$	$7.37x10^{-2}$	2.97
S. pseudomensis	4.94^{-4}	$1.15x10^{-2}$	$2.68x10^{-1}$	4.71
C. grandifolia	$1.76x10^{-3}$	$2.2x10^{-2}$	$2.78x10^{-1}$	2.77
A. afra	$7.14x10^{-4}$	$3.07x10^{-2}$	1.32	4.50

3.2 Mosquitocidal assays by fumigation

From preliminary assay, only one plant species (*Artemisia afra*) showed reasonable insecticidal activity. Essential oil of this plant was subjected to detailed assay (Table 13).

Table 13. Mean % mortality of the essential oil from *Artemisia afra* leaves

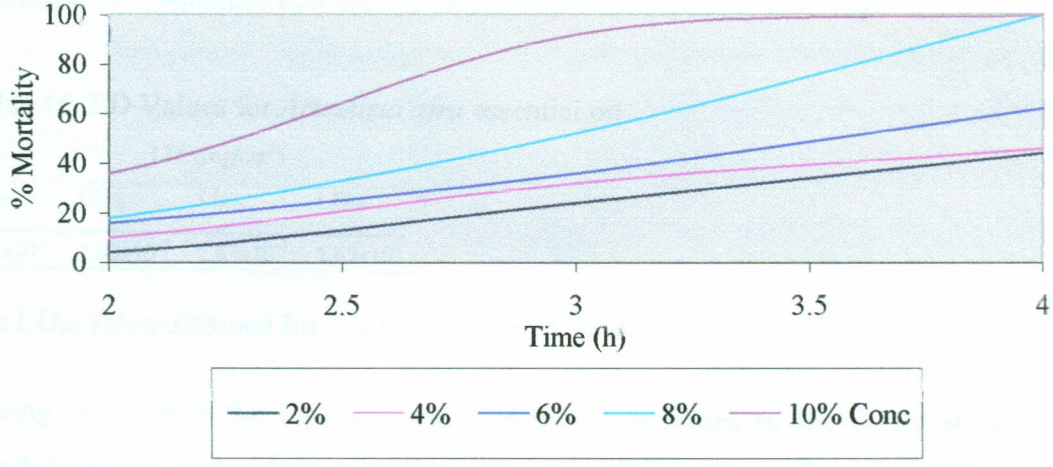
	% Mortality					
% Conc/time (h)	1	2	3	4	5	6
10	20	36	92	100		
8	2	18	52	100		
6	2	16	36	62	86	100
4	0	10	32	46	74	88
2	0	4	24	44	64	86

The complete insecticidal (T_{i100}) and knock down times (T_{KD100}) ranged from 4-6 hours and 15 minutes to 2 hours, respectively. *A. afra* essential oil attained 100% mortality at 8 and 10%

concentration after 4 hours. Mortality of 86 % was achieved at 2% concentration after 6 hours (Table 13).

The insecticidal assay data for *A. afra* was subjected to regression analysis and the best fitting curves established (Fig. 2). From the regression curves 100% insecticidal activity could be achieved after 3.5 and 4 hours at 10 and 8% concentrations, respectively. There was no significant difference in mosquitocidal activity of *A. afra* essential oil between 8 and 10% concentration after 4 hours. Similarly, no difference in activity was found between 2 and 4% concentration after 4 hours. Given that 100% mortality was achieved after 4 hours for both 8 and 10% concentration the optimum concentration for insecticidal activity oil ought to be 8%.

Figure 2. Regression of mean % mortality of essential oil from *Artemisia afra* leaves



The regression values for each concentration based on the equation % PE = exp (log a + blog x) where x = concentration, log a = intercept, b = slope are log a = 12.35 and b = 143.91 (10%), log a = -9.38 and b = 151.84 (8%), log a = -3.52 and b = 94.27 (6%), log a = -4.34 and b = 76.36 (4%), log a = -6.11 and b = 69.89 (2%).

The insecticidal assay data for *A. afra* was subjected to probit transformation and the regression equation obtained (Table 14).

Table 14. Probit analysis of mosquitocidal data for *Artemisia afra* essential oil

Dose (g/ml)	Log dose +4	% Mortality	Regression equation	Probit transformation
10^{-2}	3.00	50.00		5.00
$8x10^{-2}$	2.90	50.00		5.00
$6x10^{-2}$	2.78	31.00	Y =1.19X+0.16	4.50
$4x10^{-2}$	2.68	23.00		4.20
$2x10^{-2}$	2.30	22.00		4.23

The regression equation was used to calculate the LD values (Table 15).

Table 15. LD Values for *Artemisia afra* essential oil

LD. (mg/cm^2)			
LD_{25}	LD_{50}	LD_{75}	LD_{90}
$9.32x10^{-4}$	$2.66x10^{-2}$	$7.63x10^{-1}$	$1.62x10^{1}$

The LD_{50} value obtained for *A. afra* is $2.66x10^{-2}$ mg/cm^2.

Having determined the repellent and insecticidal activities of the essential oils from the seven selected anti-mosquito plants, the stage was set for chemical investigations of their constituents.

CHAPTER 4

CHEMICAL COMPOSITION OF ESSENTIAL OILS

The compositions of the essential oils extracted via hydro-distillation with a modified Clavenger apparatus were determined by gas chromatography/mass spectroscopy. Preliminary identification of essential oil constituents was based upon comparison of retention time and or mass spectrum with that of a standard. The final identity of the component was achieved by GC co-injection of essential oil with standards resulting in the enhancement of the peak area in the chromatogram. In general, the chemistry of essential oils is complex. They consist of several hundreds of compounds, terpenoids, aromatics and fats, in different ratios. The terpenoids can be subdivided into three groups: Hydrocarbons (monoterpenes, sesquiterpenes and diterpenes), oxygenated compounds (esters, aldehydes, ketones, alcohols, phenols and oxides) and miscellaneous (acids, lactones, nitrogen and sulphur compounds) (Williams, 1996).

The volatile components identified and confirmed through co-injection are listed in Table 1 according to the above classification, together with their relative percentages. The peak numbers in figures 3-9 represent the elution order of identified peaks. Analysis using this method resulted in chromatograms that contained > 130 discernible peaks on magnified GC profile. Out of these, 54 compounds were identified constituting 17.71% (*C. anisata, C. grandifolia*), 15.63% (*A. afra*), 14.58% (*P. eminii*), 12.5% (*S. moorei*), 11.45% (*S. pseudomensis*) and 10.42% (*N. azurea*) of the essential oil constituents. In each case, the essential oils consisted of a complex mixture of different substances with terpenoids as the major constituents. Several hydrocarbons were identified. Others were found in trace amount < 0.1% as presented by t in Table 1.

Table 16. Chemical composition of essential oils from repellent plant species

Compound	Peak area (%)						
	AA	CG	SMO	NA	SPS	CA	PEM
a) Hydrocarbons							
Monoterpenes							
α-Pinene	t		22				16.82
β-Pinene	t		t			0.49	9.82
Sabinene						3.89	0.82
(Z)-Ocimene	t					0.58	2.22
(E)-Ocimene	t	t					
(Z)-β- Ocimene		1.58	2.67	1.9	3.48		
(E)- β-Ocimene		1.41	6.28			1.3	
p-Cymene			t				1.86
β-Myrcene		t	19.54			4.61	2.06
(+)-2-Carene							t
(+)-3-Carene							38.96
α-Terpenene						2.51	
γ- Terpenene						16.42	
α-Terpenolene						2.51	
Limonene					6.66	23.33	
Terpenolene	0.5	4.02	10.42	t	0.18	0.39	
α-Phellandrene		t	t			0.64	
Camphene	t						
Sesquiterpenes							
α-Copaene	t			2.09	t	0.95	
α-Cubebene					0.83		
β-Cubebene	t			4.85			
Caryophyllene		0.14					
(Z)-Caryophyllene		0.75		0.83	0.51	2.12	0.26
Isocaryophyllene		1.06				0.92	
Farnesene			0.73	0.63			
Aromadendrene							0.12
Alloaromadendrene	t			1.14		0.71	0.12
α-Gurjunene		1.66					
-(-)-Isoledene		t					
(b) Oxygenated compounds							
Esters							
Linaly acetate							0.97
Bornyl acetate	t						
Aldehydes							
Isopropylbenzaldehyde							0.59
Hexanal			0.93				
Ketones							
Artemisia ketone	18.92	0.29	t				
α-Thujone	15.44						
β-Thujone	1.78						
Methyl decanoate		1.85	2.03				
Carvone					0.34		
Alcohols							
Linalool			2.71	1.2	0.45	t	0.68
1-Nonanol		2.4			0.26		
Terpene-4-ol	0.43					t	
α-Fenchyl alcohol		t				0.29	
Isopinocampheol	0.87						
Phenols							
Carvacrol				0.10			
Isoeugenol					0.72		
Oxides							
(Z)-(E)-linalool oxide				t			
1, 8-Cineole	0.18						
Caryophyllene oxide		t	0.21				t
Lactones							
Nepetalactone				14.67			
Unsaturated aliphatic and aromatic hydrocarbons							
1-Phenyl-1-pentyne		6.62					t
1-Undecene		25.11					
Napthalene				1.49			
1, 3-Butadienylbenzene		32.29					
1-Butynylbenzene		3.51					
Heteroaromatics							
7-Methylquinoline		5.33					

4.5 *Artemisia afra*

A total of 16 compounds were identified and 7 confirmed by GC analysis (Fig.3). Artemisia ketone (18.92%), α-thujone (15.44%), β-thujone (1.78%), 1,8-cineole (5.17%), isopinocampheol (0.86%), terpenolene (0.49%) and terpene-4-ol (0.43%) were identified as the major chemical components of this oil (Table 17). α-Pinene, β-pinene, (*E*)- and (*Z*)-ocimene, camphene, bornyl acetate and α-copaene, α- cubebene, (+) alloaromadendrene are monoterpenes and sesquiterpenes, respectively, that were present in trace amounts (<0.1%).

Figure 3. GC profile of the essential oil from *Artemisia afra* leaves

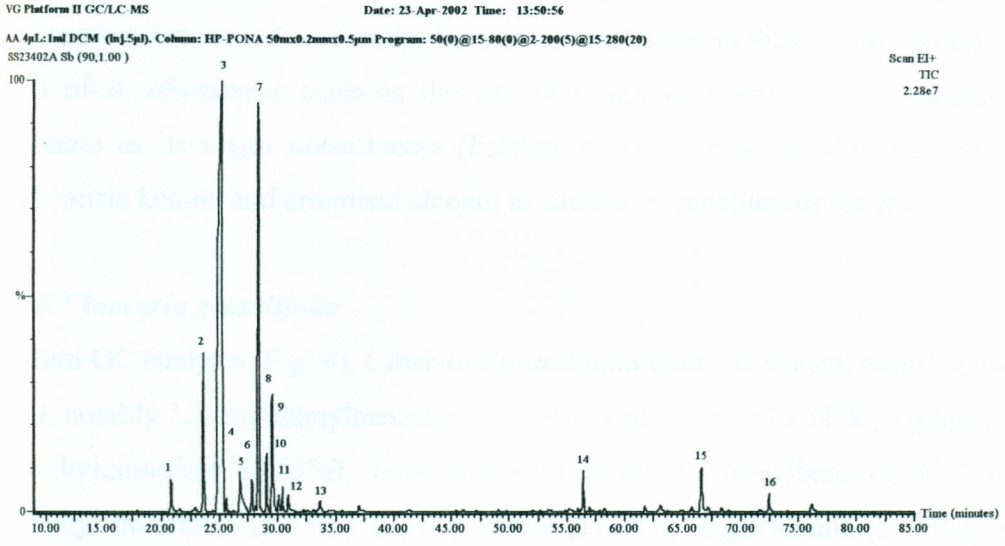


Table 17. Chemical composition of the essential oil from *Artemisia afra* leaves

Peak No	R_t (min)	Compound	Relative %	Co	Peak No	R_t (min)	Compound	Relative %	Co
1	20.85	Yamogi alcohol	1.28		9	29.58	Unidentified	4.98	
2	23.65	1, 8- Cineole	5.17	√	10	30.10	Terpenolene	0.49	√
3	25.33	Artemisia ketone	18.92	√	11	30.40	Accecarbromal	0.75	
4	25.53	4-Ally-1, 6-heptadiene-4-ol	0.36		12	30.90	Isopinocampheol	0.86	√
5	26.75	Artemisia alcohol	3.28		13	33.65	Terpene-4-ol	0.43	√
6	27.80	Isoamylisovalerate	1.19		14	56.43	Germacrene D	1.52	
7	28.43	α-Thujone	15.44	√	15	66.63	β-Eudesmol	2.33	
8	29.05	β-Thujone	1.78	√	16	72.48	Valencene	0.40	

R_t-retention time, Co-coinjection

From the previous work, it has been reported that α-thujone, β-thujone, 1,8-cineole and camphor are the major constituents of the leaf oil of *A. afra* grown in South Africa (Graven *et al.*, 1990). Analysis of the leaf oil of *A. afra* grown in Botwana revealed Yamogi alcohol, 1, 8-cineole, prenal, ethyl-2-hexen-1-ol and *trans*-verbenol as the major compounds (Graven *et al.*, 1990) demonstrating considerable variation with that from South Africa. The major components of leaf oil of *A. afra*

from Zimbabwe were identified as artemisia ketone, 1,8-cineole, α-copaene, camphor and santolina alcohol (Chagonda *et al.*, 1999). Although there is a slight variation in composition, our finding are closer to those of South African and Zimbabwean varieties than Botswana as we have found artemisia ketone, α-thujone, β-thujone and 1, 8-cineole as being the major compounds among others.

Numerous reports on the leaf oils of other *Artemisia* species have been published. For instance, essential oils of *A. annua* (Klayman *et al.*, 1984) and A. *vulgaris* Hwang *et al.*, 1985), comprise many constituents with the major compounds including α-pinene, camphene, ß-pinene, myrcene, 1, 8-cineole, artemisia ketone, linalool, camphor, borneol and ß-caryophyllene among others. Artemisia ketone is found in the highest concentration in these plant species. However, the essential oil of *A. abyssinica* contains the irregular monoterpenes Yamogi alcohol and artemsia alcohol acetate as its major constituents (Esteban *et al.*, 1986). *A. douglasiana* was found to contain artemisia ketone and artemisia alcohol as the major constituents (Score *et al.*, 1984).

4.6 *Cineraria grandifolia*

From GC analysis (Fig. 4), *Cineraria grandifolia* contains several major constituents of the essential oil, notably 1,3-butadienylbenzene (33.29%), 1-undecene (25.11%), 1-phenyl-1-pentyne (6.62%), 7-methylquinoline (5.53%), terpenolene (4.02%), 1-butynylbenzene (3.51%), 1-nonanol (2.4%), methyl decanoate (1.85%), (*Z*)-β-ocimene (1.58%), α-gurjunene (1.66%), (*E*)-β-ocimene (1.41%), isocaryophyllene (1.06%) and (*Z*)-caryophyllene (0.74%), as confirmed by co-injection (Table 18). Other mono- and sesquiterpenes present in trace amounts (< 0.1%) include α-fenchyl acohol, (-)-isoledene, α-phellandrene, caryophyllene oxide, myrcene and (*E*)-ocimene.

Figure 4. GC profile of the essential oil of *Cineraria grandifolia* leaves

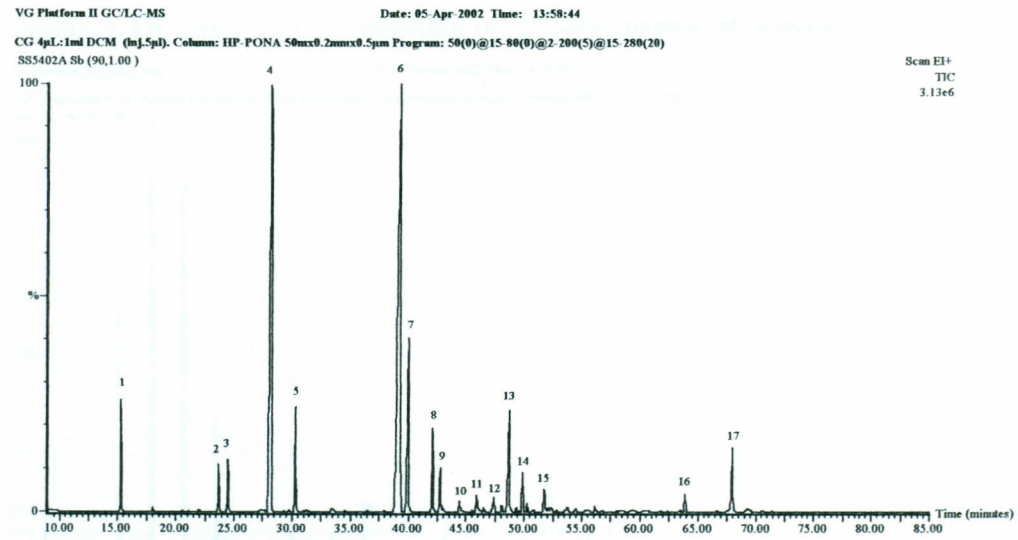


Table 18 Chemical composition of the essential oil from *Cineraria grandifolia* leaves

Peak No	R_t (min)	Compound	Relative %	Co	Peak No	R_t (min)	Compound	Relative %	Co
1	15.30	1-Nonanol	2.4	√	10	44.45	Unidentified	0.54	
2	23.70	(E)-β-Ocimene	1.41	√	11	45.90	Valleral	0.67	
3	24.53	(Z)-β-Ocimene	1.58	√	12	47.41	Caryophyllene	0.74	√
4	28.20	1-Undecene	25.11	√	13	48.73	7-Methylquinoline	5.53	√
5	30.30	Terpenolene	4.02	√	14	49.90	α -Gurjunene	1.66	√
6	39.35	1, 3-Butadienylbenzene	33.29	√	15	51.75	Isocaryophyllene	1.06	√
7	40.03	1-Phenylpentyne	6.62	√	16	63.93	(Z)-Caryophyllene	0.74	√
8	42.13	1-Butynylbenzene	3.51	√	17	68.00	5,9-Tetradecadiyne	3.31	
9	42.78	Methyl decanoate	1.85	√					

R_t-retention time, Co-coinjection

There is no previous work on the oil composition of this plant. A literature survey was conducted to determine the chemical composition of the essential oils of other *Cineraria* species for comparison. However, the survey indicated that the genus has not been investigated for essential oil composition is concerned.

4.2 *Senecio moorei*

The compositon of the essential oil of *S. moorei* as determined by GC (Fig. 5) is summarized in table 19. α-Pinene (22%), myrcene (19.54%), terpenolene (10.42%), 1-nonanol (7.63%), (*E*)-β-ocimene (6.28%), (*Z*)-β-ocimene (2.67%), methyl decanoate (1.31%), hexanal (0.93%) and farnesene (0.73%) were confirmed to be the main chemical constituents by co-injection. Other components of the leaf oil of *S. moorei* are β-pinene, *p*-cymene, artemisia ketone and α-phellandrene which were present in trace amounts (< 0.1%.)

Figure 5. GC profile of the essential oil of *Senecio moorei* leaves

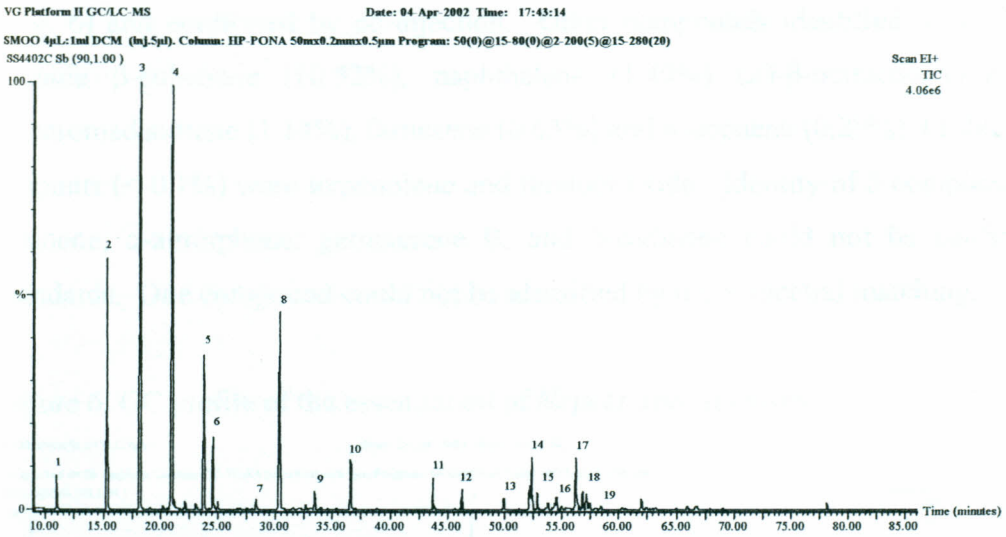


Table19. Chemical composition of the essential oil from *Senecio moorei* leaves

Peak No	R_t (min)	Compound	Relative %	Co	Peak No	R_t (min)	Compound	Relative %	Co
1	10.93	Hexanal	0.93	√	11	43.78	Methyl decanoate	1.30	√
2	15.38	1-Nonanol	7.63	√	12	46.28	Bicyclogermagrene	0.80	
3	18.25	α–Pinene	22.0	√	13	49.95	6, 9, 12-octadedetrienoic acid	0.45	
4	21.05	Myrcene	19.54	√	14	52.38	(Z)-Chrysanthemal	1.77	
5	23.78	(Z)-Ocimene	2.67	√	15	52.85	GermacreneD	0.54	
6	24.58	(E)-α-Ocimene	6.28	√	16	54.53	α-Humulene	0.42	
7	28.30	Octenyl acetate	0.34		17	56.23	Zingiiberene	2.94	
8	30.40	Terpinolene	10.42	√	18	56.80	Farnesene	0.73	√
9	33.43	Unidentified	0.78		19	58.43	α-Cadinene	0.12	
10	36.65	Methyl nonanoate	2.03						

R_t-retention time, Co-coinjection

The composition of the essential oil of *Senecio moorei* is being reported for the first time. Literature survey was done to determine the variations and similarities in oil composition within the genus (*Senecio*). The essential oil of *Senecio graveolens* was found to contain isovaleraldehyde, α-pinene, α-phellandrene, γ-terpenene, *p*-cymene, sabinene, α-terpenene, terpenolene, terpene-4-ol, piperitenone and eudesmol as the major compounds (Perez *et al.*, 1999). Dehydrofukinone was identified as main compound in the essential oil of *S. desfontainei* Druce leaves (El-Shazly, 1999). Similarly, the essential oil of *S. mikanoides* contains α-pinene and myrcene as the main compounds (El-Bahrawy, 2000). The main compounds, α-pinene and myrcene in *S. mikanoides* are same as *S. moorei*. The survey demonstrates significant variations and similarities within the genus. The various plant species may contain compounds in common but in varied percentage composition.

4.1 *Nepeta azurea*

The main constituent of N*epeta azurea* essential oil is nepetalactone (14.67%) as determined by GC (Fig. 6) and confirmed by co-injection. Other compounds identified in the leaf oil of *N. azurea* include β-cubebene (10.52%), naphthalene (1.49%) (*Z*)-β-ocimene (1.9%), linalool (1.2%), alloaromadendrene (1.14%), farnesene (0.63%) and α-copaene (0.29%), (Table 20). Present in trace amounts (< 0.1%) were terpenolene and linalool oxide. Identity of 5 compounds *epi*-nepetalactone, elemene, α-amorphene, germacrene B, and δ-cadinene could not be confirmed due to lack of standards. One compound could not be identified by mass spectral matching.

Figure 6. GC profile of the essential oil of *Nepeta azurea* leaves

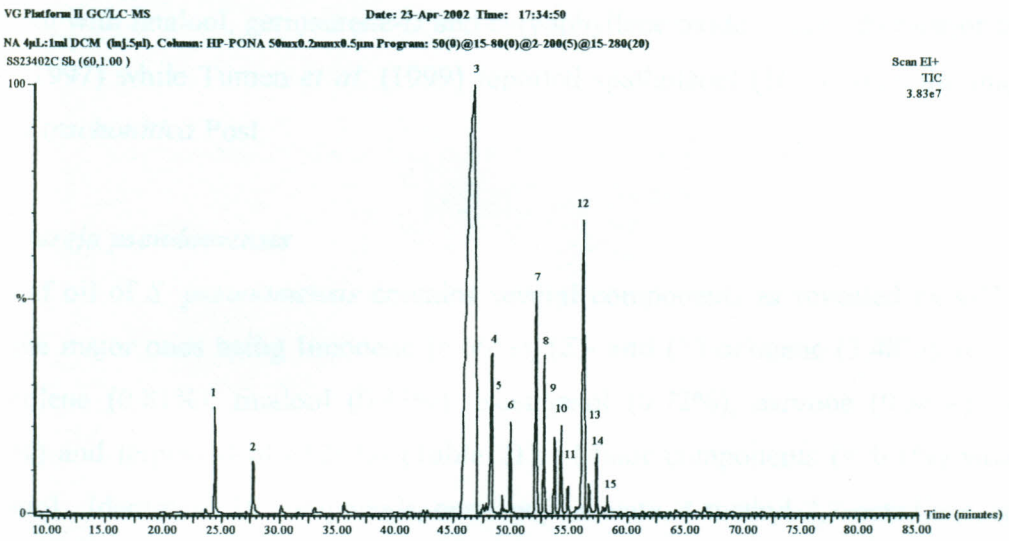


Table 20. Chemical composition of the essential oil from *Nepeta azurea* leaves

Peak No	R_t (min)	Compound	Relative %	Co	Peak No	R_t (min)	Compound	Relative %	Co
1	24.43	(*Z*)-β-Ocimene	1.90	√	9	53.70	Naphthalene	1.49	√
2	27.70	Linalool	1.20	√	10	54.28	α-Amorphene	1.76	
3	46.93	Nepetalactone	14.67	√	11	54.85	Germacrene B	0.54	
4	48.30	*Epi*-nepetalactone	5.15		12	56.25	β-Cubebene	10.52	√
5	49.18	α-Copaene	0.29	√	13	56.68	Farnesene	0.63	√
6	49.90	Elemene	2.09		14	57.33	Alloaromadendrene	1.14	√
7	52.13	Unidentified	4.58		15	58.28	δ-Cadinene	0.33	
8	52.80	Germacrene D	3.64						

R_t-retention time, Co-coinjection

There is no previous report on the chemical constituents of the essential oil of *Nepeta azurea*. However, several reports on the essential oil content of other species within this genus were located. The essential oil from *Nepeta persica* was found to contain 4aα,7α,7aα-nepetalactone, 2,3,4,5-

tetramethyl-1,4-hexadiene, 3α,4β-dihydronepetalactone, α-copaene, caryophyllene oxide and farnesene as the major components (Davis, 1990). *N. cataria* L., commonly known as catnip, is the most extensively studied species and has been shown to contain nepetalactone in the highest concentration. Regier *et al.* (1967) reported the composition of catnip oil as nepetalactone (major component, 26.5%), camphor, caryophyllene, α-humulene, *epi*-nepetalactone and dihydronepetalactone. This reflects some qualitative similarities in the essential oil composition obtained in different species but within the same genus. Of particular interest is that most of these species contain nepetalactone as one of the major components which is consistent with the present finding. The essential oil composition of *Nepeta spicata* Benth is an illustrative example of variation in compostion within this genus. Caryophyllene (31.33%) was the most abundant constituent in this oil along with linalool, germacrene-D and caryophyllene oxide as the other major compounds (Bisht *et al.*, 1997) while Tumen *et al.* (1999) reported spathulenol (16.11%) as the major compound in *Nepeta trachonitica* Post.

4.4 *Satureja pseudomensis*

The leaf oil of *S. pseudomensis* contains several components as revealed by GC analysis (Fig. 7) with the major ones being limonene (6.66%), (*Z*)- and (*E*)-ocimene (3.48%), α-cubebene (0.83%), terpenolene (0.81%), linalool (0.45%), isoeugenol (0.72%), carvone (0.34%), (*Z*)-caryophyllene (0.54%) and terpene-4-ol (0.26%) (Table 21). Minor components (< 0.1%) were α-copaene and carvacrol. Identity of 10 compounds, oct-1-enyl acetate, 4-methyl-2,7-octadiene, spiro[2.4]heptane-4-one, diisoamylene, 2-propyl-2-heptanal, β-boubonene, germagrene D, γ-cadinene, α-amorphene and α-muurolene could not be established due to absence of standards.

Figure 7. GC profile of the essential oil of *Satureja pseudomensis* leaves

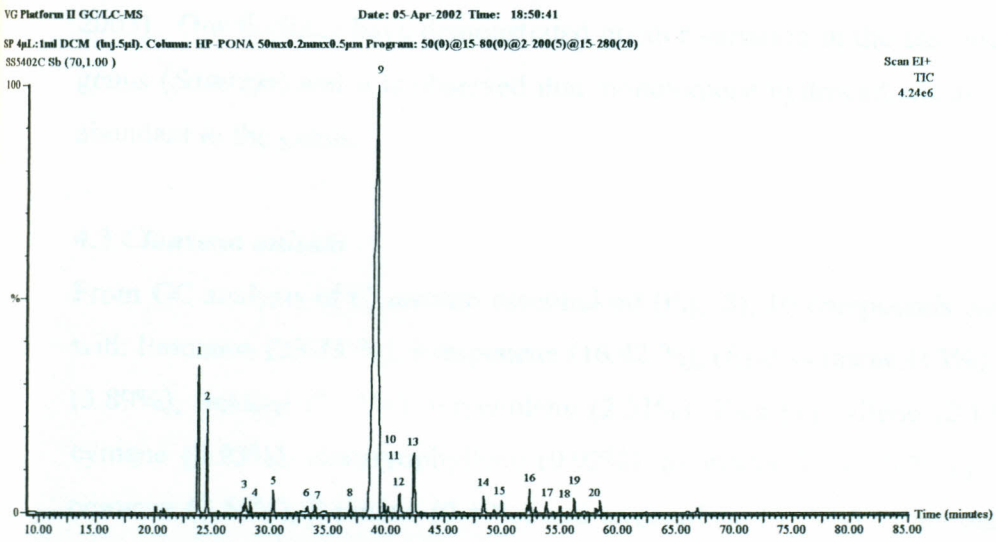


Table 21. Chemical composition of the essential oil from *Satureja pseudomensis* leaves

Peak No	R_t (min)	Compound	Relative %	Co	Peak No	R_t (min)	Compound	Relative %	Co
1	28.83	Limonene	6.66	√	11	40.13	2-Propyl-2-heptanal	0.23	
2	24.58	(Z)-β-Ocimene	3.48	√	12	41.13	Disophenol	1.08	
3	27.85	Linalool	0.45	√	13	42.38	Isoeugenol	2.83	√
4	28.28	Oct-1-enyl acetate	0.33		14	48.35	β-Boubonene	0.51	
5	30.25	Terpenolene	0.81	√	15	49.93	(Z)-Caryophyllene	0.83	√
6	33.18	4-Methyl-2, 7-octadiene	0.32		16	52.33	α-Cubebene	0.44	√
7	33.85	Terpene-4-ol	0.27	√	17	53.75	Germagrene D	0.41	
8	36.90	Spiro[2.4]heptane-4-one	0.54		18	55.58	γ-Cadinene	0.12	
9	39.33	Diisoamylene	23.58		19	56.15	α -Amorphene	0.58	
10	39.78	Carvone	0.34	√	20	58.00	α-Muurolene	0.15	

R_t-retention time, Co-coinjection

Muhayimana *et al.* (1998) reported the major compounds in the essential oil of *S. pseudomensis* as limonene, menthone, pulegone and piperitone oxide demonstrating slight variation with our finding, although the major compound at 39.33 minutes was not confirmed by GC co-injection in our case.

Several reports exist about essential oil composition of members of this genus (Grace and Khattab, 1998) with great variability. For instance, *S. punctata* contains geranial, neral and α-bisabolol as the major components (Teklu *et al.*, 1998). Analysis of the essential oils from *S. coerulea*, *S. icarica S. pilosa* and *S. boissieri* leaves collected from four different localities in Turkey showed that carvacrol was the main component in the leaf oils of these three species. Other major components were identified as *p*-cymene, myrcene, pinene and borneol besides other monoterpenes. In contrast, *S. coerulea* contained mainly sesquiterpenes such as (*Z*)-caryophyllene, germacrene D and farnesene among others (Saener *et al.*, 1995). The main compounds in the essential oil from four populations

of *S. montana* L. from southern France were carvacrol, *p*-cymene and γ-terpenene (Chizzola *et al.*, 2003). Our findings have demonstrated greater variation in the essential oil composition within the genus (*Satureja*) and it is observed that monoterpene hydrocarbons and sesquiterpenes are the most abundant in the genus.

4.3 *Clausena anisata*

From GC analysis of *C. anisata* essential oil (Fig. 8), 16 compounds were identified by co-injection with limonene (23.33 %), γ-terpenene (16.42 %), (*E*)-β-ocimene (13%), myrcene (4.61%), sabinene (3.89%), linalool (2.71%), terpenolene (2.51%), *Z*-caryophyllene (2.12%), α-copaene (0.95%), *p*-cymene (0.93%), isocaryophyllene (0.92%), aromadendrene (0.71%), α-phellandrene (0.64%), β-ocimene (0.58%), β-pinene (0.49%) and terpenolene (0.39%) as the major components (Table 22). Terpene-4-ol and caryophyllene oxide were present in trace amounts. Identity of 9 compounds *p*-mentha-6,8-dien-2-ol, β-boubonene, γ-elemene, γ-gadinene, humulene oxide, germagrene D, bicyclogermagrene, δ-cadinene and 4-β-17-acetoxy-kuran-18-al could not be confirmed due to absence of standards.

Figure 8. GC profile of the essential oil of *Clausena anisata* leaves

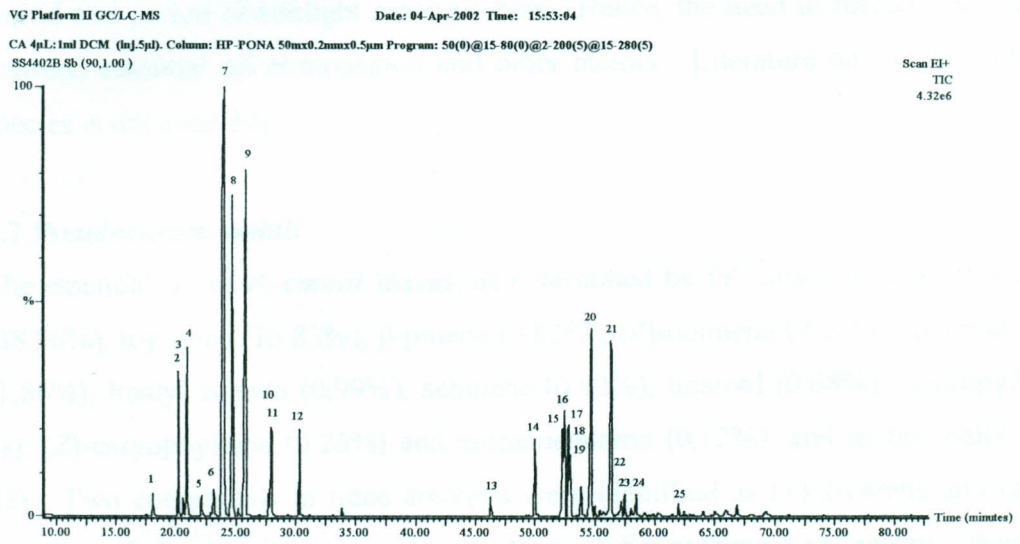


Table 22. Chemical composition of essential oil of *Clausena anisata* leaves

Peak No	R_t (min)	Compound	Relative %	Co	Peak No	R_t (min)	Compound	Relative %	Co
1	18.08	β-Ocimene	0.58	√	14	49.98	β-Boubonene	2.53	
2	20.20	Sabinene	3.89	√	15	52.30	α-Copaene	0.95	√
3	20.60	β-Pinene	0.49	√	16	52.45	(*Z*)-Caryophyllene	2.12	√
4	20.93	Myrcene	4.61	√	17	52.80	γ-Elemene	1.70	
5	22.10	α-Phellandrene	0.64	√	18	52.95	γ-Gadinene	0.89	
6	23.15	*p*-Cymene	0.93	√	19	53.85	Isocaryophyllene	0.92	√
7	24.03	Limonene	23.33	√	20	54.70	Humulene oxide	6.78	
8	24.73	(E)-β-Ocimene	13.0	√	21	56.35	Germagrene D	8.05	
9	25.83	γ-Terpenene	16.42	√	22	57.20	Bicyclogermagrene	0.69	
10	27.73	Terpenolene	0.39	√	23	57.48	Aromadendrene	0.71	√
11	27.95	Linalool	2.71	√	24	58.48	δ-Cadinene	0.86	
12	30.33	α-Terpenolene	2.51	√	25	61.98	4-β-17-acetoxy-kuran-18-al	0.48	
13	36.30	*p*-Mentha-6, 8-dien-2-ol	0.26						

R_t-retention time, Co-coinjection

Previously, the essential oil of *Clausena anisata* leaves was reported to contain phenylpropanoids and methylchavicol (estragole) as the major compounds (Ekundayo *et al.*, 1968). The essential oil of *C. anisata* from Ghana, Togo and different regions of Benin were reported to contain (*Z*)-anethole (Ghana), limonene and phellandrene (Togo), estragole, (*Z*)-caryophyllene, limonene and α-phellandrene (Benin) as the major compounds (Moudachirou *et al.*, 1997). These are considerably different from our results which revealed limonene and γ-terpenene as the major compounds even though they are almost in agreement with those from Togo. This gave rise to suggestion that the essential oil composition may be effected by environmental conditions such as soil, climate, harvest period and period of sunlight among others. Hence, the need to further investigate the correlation between essential oil composition and other factors. Literature on the leaf oil of other *Clausena* species is not available.

4.7 *Pseudocarum eminii*

The essential oil of *P. eminii* leaves, as determined by GC analysis (Fig. 9), contains (+)-3-carene (38.96%), α-pinene (16.82%), β-pinene (9.82%), (*Z*)-ocimene (2.22%), myrcene (2.06%), *p*-cymene (1.86%), linalyl acetate (0.99%), sabinene (0.82%), linalool (0.68%), isopropylbenzaldehyde (0.59 %), (*Z*)-caryophyllene (0.26%) and aromadendrene (0.12%), and as the major constituents (Table 23). Two compounds in trace amounts were identified as (+)-2-carene and caryophyllene oxide. Identity of 8 other components cryptone, 1-*p*-menthene-8-yl acetate, β-elemene, β-elemene, germagrene D, veridiflorol, valerenal and patchulane could not be established due to absence of standards. Two compounds could not be established by mass spectral matching.

Figure 9. GC profile of the essential oil of *Pseudocarum eminii* leaves

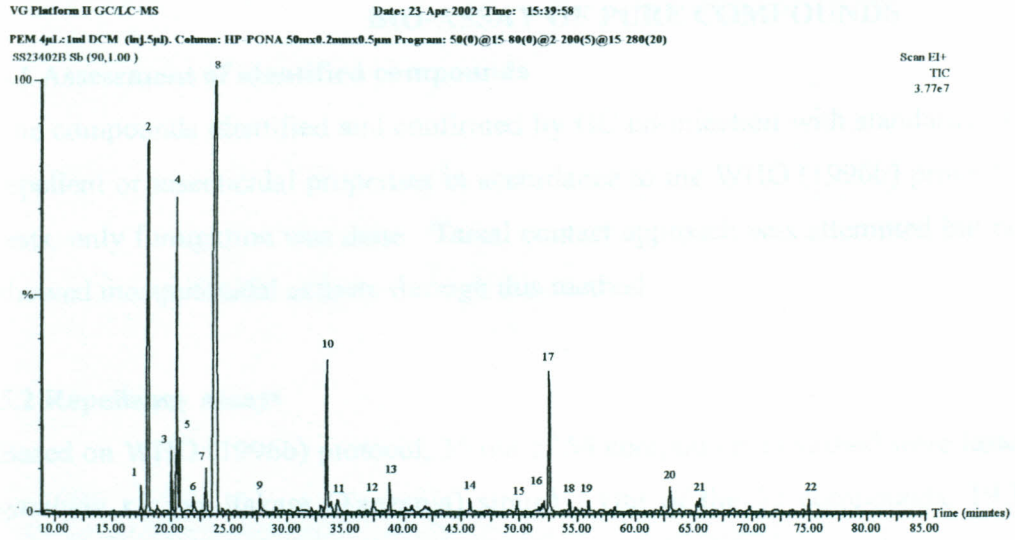


Table 23. Chemical composition of the essential oil from *Pseudocarum eminii* leaves

Peak No	R_t (min)	Compound	Relative %	Co	Peak No	R_t (min)	Compound	Relative %	Co
1	17.43	Sabinene	0.82	√	12	37.40	Isopropylbenzaldehyde	0.59	√
2	18.10	α-pinene	16.82	√	13	38.83	Linaly acetate	0.99	√
3	20.08	(Z)-Ocimene	2.22	√	14	45.73	1-*p*-Menthene-8-yl acetate	0.48	
4	20.58	β-Pinene	9.82	√	15	49.78	β-Elemene	0.45	
5	20.78	Myrcene	2.06	√	16	52.13	(Z)-Caryophyllene	0.26	√
6	21.95	Unidentified	0.64		17	52.58	γ-Elemene	5.87	
7	23.05	*p*-Cymene	1.86	√	18	54.33	(+)-Aromadendrene	0.12	√
8	23.93	(+)-3-Carene	38.96	√	19	55.98	Germagrene D	0.46	
9	27.70	Linalool	0.68	√	20	63.00	Veridiflorol	1.22	
10	33.48	Cryptone	5.74		21	65.63	Valerenal	0.38	
11	34.43	Unidentified	0.89		22	74.95	Patchulane	0.33	

R_t-retention time, Co-coinjection

No previous report on the composition of the leaf oil of *Pseudocarum eminii* is available. This is the first report on the essential oil content of this plant. Literature survey of other species from this genus indicated that essential composition has not been investigated.

Having identified the compounds in the essential oils from the seven anti-mosquito plants, the stage was set for the bio-assay of the identified compounds singly or as blends.

CHAPTER 5

BIO-ASSAY OF PURE COMPOUNDS

5.1 Assessment of identified compounds

The compounds identified and confirmed by GC co-injection with standards were assessed for their repellent or insecticidal properties in accordance to the WHO (1996b) protocol. For mosquitocidal tests, only fumigation was done. Tarsal contact approach was attempted but none of the oils tested showed mosquitocidal activity through this method.

5.2 Repellency assays

Based on WHO (1996b) protocol, 35 out of 54 compounds identified were tested against female *An. gambiae s.s* {ex-Ifakara (Tanzania) strain}. Out of the 35 compounds, 19 had previously been assayed by Omollo (2002) (Table 24). The remaining 16 compounds were assayed and the results are summarized in table 25.

Table 24. Previous repellency assay and RD_{50} values of the essential oil standards

	Mean % protective efficacy				
Concentration (g/ml)	10^{-1}	10^{-2}	10^{-3}	10^{-4}	$RD_{50} \times 10^{-5}$ mg/cm^2
Camphene	65.42	59.60	42.06	39.24	22
Limonene	23.24	45.01	49.18	60.00	10
α-Pinene	51.06	43.38	31.24	20.38	594
β-Pinene	57.69	50.00	46.15	40.78	56
Aromadendrene	68.05	57.14	39.01	36.92	45
Isocaryophyllene	-18.86	-22.25	-5.50	13.04	-
Terpen-4-ol	85.13	42.20	34.01	24.50	48
Isopropylbenzaldehyde	41.75	34.06	22.25	0	329
Thujone	58.26	44.70	30.65	25.65	25
1, 8-Cineole	78.01	50.62	43.87	36.56	21
Linalool	71.42	47.50	37.87	32.00	53
Terpenolene	74.28	47.18	36.16	26.16	55
Linalyl acetate	80.83	52.35	37.28	22.36	4
Caryophyllene oxide	100	50.58	41.47	33.13	14
p-Cymene	-15.38	16.05	36.23	43.33	1
α-Fenchyl alcohol	75.77	45.12	43.21	33.34	35
Carvone	94.34	45.76	43.89	27.91	21
α-terpenene	78.28	51.36	35.26	28.28	40
γ-Terpenene	76.28	44.80	36.05	28.23	74

Table 25. Repellency assay and RD_{50} values of the essential oil standards

	% Protective efficacy					
Concentration (g/ml)	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	RD_{50} ($mgcm^{-2}$)
Artemisia ketone	77.89 ± 3.19^{f}	59.15 ± 3.96^{i}	47.92 ± 5.48^{jkl}	36.96 ± 4.10^{lmno}		$1.93x10^{-1}$
Nepetalactone	nd	78.85 ± 2.57^{ef}	63.29 ± 5.19^{ghi}	62.02 ± 2.51^{hi}	39.63 ± 5.85^{klm}	$1.2x10^{-3}$
(+)-3-carene	66.42 ± 3.82^{gh}	57.00 ± 5.72^{ij}	51.18 ± 3.73^{ij}	46.82 ± 5.11^{kl}		$4x10^{-2}$
(+)-2-carene	56.33 ± 2.09^{ij}	45.7 ± 1.18^{kl}	45.7 ± 1.18^{kl}	23.8 ± 1.72^{q}		$7.4x10^{-1}$
Linalool oxide	70.09 ± 3.07^{g}	62.55 ± 6.08^{ghi}	58.58 ± 2.23^{i}	34.65 ± 2.53^{nop}		$7.3x10^{-2}$
Bornyl acetate	72.82 ± 2.84^{fg}	65.15 ± 2.92^{gh}	63.16 ± 5.00^{ghi}	49.64 ± 3.53^{jkl}		$5.6x10^{-3}$
Hexanal	61.9 ± 3.54^{hi}	49.9 ± 2.19^{ijk}	32.5 ± 2.39^{opq}	19.5 ± 3.33^{r}		$1.06x10^{-1}$
α--Gurjunene	68.7 ± 0.90^{g}	55.4 ± 1.84^{j}	46.9 ± 0.75^{jklm}	33.4 ± 1.63^{op}		$1.95x10^{-1}$
1-Nonanol	79.11 ± 2.52^{e}	72.47 ± 5.19^{g}	63.80 ± 2.51^{ghi}	63.34 ± 5.24^{ghi}		$4.12x10^{-4}$
1-Undecene	75.32 ± 2.41^{f}	69.76 ± 4.63^{g}	66.55 ± 3.31^{g}	52.58 ± 5.30^{j}		$2.6x10^{-3}$
Methyldecanoate	57.64 ± 3.67^{i}	54.32 ± 2.14^{j}	43.48 ± 1.63^{klmo}	38.56 ± 4.46^{nop}		$2.52x10^{-1}$
1-Pheny-1-pentyne	38.05 ± 4.61^{klmno}	40.83 ± 4.61^{klmn}	46.17 ± 4.46^{kl}	50.35 ± 5.94^{jk}		$8.25x10^{-1}$
1,3-Butadienylbenzene	50.94 ± 5.63^{jk}	40.38 ± 5.19^{klmn}	32.79 ± 6.22^{nop}	32.59 ± 4.71^{nop}		$3.92x10^{-1}$
7-Methylquinoline	80.30 ± 2.44^{e}	80.10 ± 3.10^{e}	55.67 ± 4.20^{ij}	49.39 ± 4.21^{jkl}		$1.9x10^{-2}$
Myrcene	75.1 ± 3.01^{f}	66.1 ± 4.24^{g}	56.3 ± 4.82^{ij}	34.1 ± 5.17^{nop}		$7.5x10^{-2}$
Napthalene	55.88 ± 6.45^{ij}	49.98 ± 3.68^{jk}	43.87 ± 4.67^{klm}	42.11 ± 1.67^{klm}		$1.1x10^{-1}$

Means followed by same letters are not significantly different

Blends of major compounds were made in the ratio that they occur in the essential oil. These were assayed for repellency against *An. gambiae s.s* (Table 26).

Table 26. Repellency assay and RD_{50} values of blends of essential oil standards

		% Protective efficacy				
Conc (g/ml)		10^{-1}	10^{-2}	10^{-3}	10^{-4}	RD_{50} ($mgcm^{-2}$)
Plant	Blend					
A. afra	A_1	48.67 ± 5.01^{jkl}	45.87 ± 6.73^{klm}	35.43 ± 7.11^{mnop}	30.61 ± 5.24^{opq}	7.84×10^{-1}
	A_2	65.87 ± 5.54^{gh}	61.33 ± 2.47^{hi}	56.08 ± 3.89^{ij}	55.32 ± 3.41^{j}	10^{-2}
	A_3	82.59 ± 3.57^{cde}	77.14 ± 3.74^{fm}	72.66^{f}	47.14^{jkl}	10^{-4}
C. grandifolia	B_1	78.63 ± 6.89^{ef}	69.49 ± 2.83^{g}	61.78 ± 3.54^{hi}	56.40 ± 3.18^{i}	2×10^{-3}
	B_2	51.24 ± 5.52^{jk}	50.04 ± 4.90^{jk}	45.58 ± 6.82^{klm}	36.92 ± 5.62^{mnop}	1.1×10^{-1}
	B_3	66.38 ± 5.34^{gh}	52.70 ± 5.33^{j}	41.75 ± 4.53^{klmn}	28.58 ± 3.74^{pq}	8.1×10^{1}
	B_4	61.38 ± 2.23^{hi}	52.94 ± 5.55^{j}	44.63 ± 4.87^{klm}	41.20 ± 5.24^{klmn}	10^{-1}
	B_5	60.06 ± 2.21^{hi}	46.20 ± 1.85^{kl}	44.87 ± 3.36^{klm}	41.20 ± 5.44^{klmn}	1.2×10^{-2}
S. moorei	C_1	80.56 ± 3.19^{de}	66.73 ± 3.32^{g}	64.73 ± 2.78^{ghi}	48.48 ± 3.18^{jk}	3×10^{-2}
	C_2	74.04 ± 4.49^{f}	68.44 ± 4.88^{hg}	68.26 ± 2.12^{gh}	28.39 ± 3.3^{q}	6×10^{-2}
	C_3	71.11 ± 3.93^{g}	59.58 ± 3.61^{hi}	43.91 ± 4.86^{klm}	27.78 ± 6.93^{pq}	2.2×10^{-2}
	C_4	69.75 ± 4.63^{g}	66.65 ± 3.64^{gh}	49.24 ± 6.67^{jkl}	28.03 ± 6.17^{pq}	1.9×10^{-2}
	C_5	40.96 ± 5.43^{klmn}	33.88 ± 5.85^{nopl}	32.82 ± 4.46^{nop}	28.33 ± 5.34^{pq}	9.1×10^{-1}
N. azurea	D_1	68.99 ± 2.74^{g}	60.12 ± 5.26^{hi}	41.90 ± 1.67^{klmno}	41.05 ± 3.84^{klmn}	2.2×10^{-2}
	D_2	53.31 ± 4.23^{j}	50.96 ± 4.90^{jk}	41.80 ± 5.41^{klmno}	40.15 ± 6.86^{klmno}	1.93×10^{-1}
	D_3	39.33 ± 5.00^{klmno}	38.04 ± 6.22^{klmnop}	29.61 ± 5.35^{opq}	21.28 ± 8.87^{q}	1.54×10^{-1}
	D_4	47.35 ± 2.23^{jkl}	44.42 ± 1.86^{kl}	41.21 ± 9.24^{klmno}	38.42 ± 5.67^{klmno}	1.2×10^{-1}
S. pseudomensis	E_1	78.18 ± 4.71^{ef}	67.42 ± 3.73^{g}	58.37 ± 5.79^{hij}	46.85 ± 6.37^{jklm}	2×10^{-2}
	E_2	81.22 ± 4.64^{de}	66.89 ± 3.32^{g}	66.03 ± 2.78^{g}	48.78 ± 2.93^{jk}	1.3×10^{-3}
	E_3	66.42 ± 3.82^{gh}	57.00 ± 3.73^{i}	51.48 ± 5.72^{jk}	46.82 ± 5.12^{kl}	1.43×10^{-1}
C. anisata	F_1	77.69 ± 0.77^{ef}	71.03 ± 2.75^{g}	51.31 ± 4.77^{jk}	49.08 ± 3.78^{jk}	1.66×10^{-2}
	F_2	58.39 ± 2.27^{i}	55.89 ± 2.48^{ij}	54.39 ± 4.16^{j}	47.54 ± 5.16^{jkl}	1.2×10^{-1}
	F_3	87.45 ± 3.89^{cd}	70.13 ± 3.05^{g}	63.66 ± 5.17^{ghi}	46.55 ± 3.61^{kl}	1.1×10^{-3}
P. eminii	G_1	75.59 ± 3.88^{f}	70.65 ± 2.68^{g}	60.52 ± 3.51^{hi}	47.84 ± 3.27^{jkl}	1.87×10^{-1}
	G_2	59.24 ± 2.19^{i}	57.21 ± 3.69^{hi}	54.48 ± 5.44^{j}	41.17 ± 4.37^{klmn}	3.7×10^{-4}
	G_3	89.76 ± 3.68^{cd}	78.89 ± 3.16^{bc}	72.41 ± 2.76^{g}	69.69 ± 3.15^{g}	3.6×10^{-4}
	G_4	54.37 ± 3.0^{j}	52.60 ± 4.68^{ijk}	47.84 ± 3.27^{jkl}	46.81 ± 4.48^{kl}	2.4×10^{-2}

Means followed by same letters are not significantly different

5.2.1 *Artemisia afra*

For *Artemisia afra*, artemisia ketone, α- and β-thujone, terpene-4-ol, bornyl acetate, terpenolene, 1, 8-cineole, α- and β-pinene, and aromadendrene were assayed with % protective efficacy (Table 24-25). Terpene-4-ol, artemisia ketone, bornyl acetate and 1,8-cineole had % protective efficacy between 70-80% at 0.1 g/ml. The RD_{50} were 4.8×10^{-4}, 1.93×10^{-1}, 5.6×10^{-3} and 2.1×10^{-4} mg/cm^2, respectively, compared to 3.07×10^{-2} mg/cm^2 for the whole oil.

Repellency of the essential oil of *A. afra* might be due to the synergistic interaction of the major constituents (artemisia ketone, α- and β-thujone and 1,8-cineole). This was confirmed not to be the

case by bio-assay of the blend A_1 (artemisia ketone:α-thujone:β-thujone:1,8-cineole 10.63:8.67:1:2.90), which gave a PE of 48.67% compared to 94.71% for the whole essential oil at 0.1 g/ml. Artemisia ketone is the most abundant component in the essential oil from the leaves and its contribution towards the overall activity is not significant as shown by the bio-assay of the blend A_2 (α-thujone:β-thujone:1,8-cineole 8.67:1:2.90) which gave a PE of 65.87% as compared to 48.67%. However, the compound that appears to be the main contributor to the repellent property is terpene-4-ol as evident in the bio-assay of blend A_3 (artemisia ketone:α-thujone:β-thujone:terpene-4-ol:1,8-cineole 44:35.91:4.14:1:12.02) which gave a PE of 82.59% at 0.1 g/ml. Terpene-4-ol had previously been shown to be the most effective mosquito repellent compound from *Artemisia vulgaris* oil (Hwang *et al.*, 1985) and therefore was included in the blend. It seems that the same compound is responsible for the repellency of *A. afra*. The synergistic action of the other minor compounds in the essential oil cannot be ignored

5.2.2 *Cineraria grandifolia*

The compounds that were assayed from the essential oil of *C. grandifolia* included α-fenchyl alcohol, α-gurjunene, 1-nonanol, 1-undecene, caryophyllene oxide, 1-phenyl-1-pentyne, 1,3-butadienylbenzene, myrcene, isocaryophyllene, 7-methylquinoline and terpenolene with protective efficacies of 75.77, 68.7, 79.11, 75.32, 100, 38.05, 50.94, 75.1, -18.76, 80.3 and 74.28%, respectively, at 0.1 g/ml (Table 24-25). The RD_{50} values were $3.5x10^{-4}$, $1.19x10^{-1}$, $4.12x10^{-4}$, $2.6x10^{-3}$, $1.4x10^{-4}$, $8.25x10^{-1}$, $3.92x10^{-2}$, $7.5x10^{-2}$, -, $1.9x10^{-2}$ and $5.5x10^{-4}$ mg/cm^2, respectively, compared to $2.2x10^{-2}$ mg/cm^2 for the whole essential oil.

Protection against mosquito bites by the essential oil could be due to the action of the major compounds (1-undecen:1-nonanol:1,3-butadienylbenzene:7-methylquinoline:terpenolene: 10.46:1:13.87:2.30:1.68) in a blend, B_1. The PE for the blend was 78.53% at 0.1 g/ml as compared to 99.12% for the whole essential oil at the same concentration. This demonstrated that the contribution from the other minor compounds could not be ruled out. These include caryophyllene oxide, myrcene, α-phellandrene, α-fenchyl alcohol and β-gurjunene which exhibit promising activity individually. A blend B_2, (1-nonanol:1,3-butadienylbenzene:7-methylquinoline:terpenolene 1:13.87:2.30:1.68), gave a PE of 51.24% compared to 99.12% for the whole essential oil mixture and 78.53% for B_1, confirming 1-undecene as major contributor to the repellent activity of the plant oil. The assay of other blends B_3 (1-undecene:1,3-butadienylbenzene:7-methylquinoline:terpenolene

6.25:8.28:1.33:1), B_4 (1-undecene:1-nonanol:1,3-butadienylbenzene:7-methylquinoline 10.46:1:13.87:2.27) and B_5 (1-undecene:1-nonanol:7-methylquinoline:terpenolene 10.46:1:2.27:1.68) gave a PE of 66.37, 61.38 and 60.06% at 0.1 g/ml confirming the significant contribution of 1-nonanol, terpenolene and 1,3-butadienylbenzene, respectively, towards the repellency activity of the oil. The result suggests that all the major components are required in a blend for the repellent activity. However, the repellent activity of the minor compounds cannot be ignored.

5.2.3 *Senecio moorei*

For *Senecio moorei*, 7 out of 14 compounds identified from the essential oil from the leaves were tested for repellency (Table 24-25). Four (4) major components of this oil, α-pinene, 1-nonanol, myrcene, hexanal and terpenolene exhibited a protective efficacies of 51.06, 79.11, 75.10, 61.9 and 74.28 %, respectively, at 0.1 g/ml. The RD_{50} values were $5.94x10^{-4}$, $4.12x10^{-4}$, $7.5x10^{-2}$, $1.06x10^{-1}$ and $5.5x10^{-4}$ mg/cm^2, respectively, compared to $1.27x10^{-3}$ mg/cm^2 for the whole oil.

Repellency of this oil against *An. gambiae s.s* mosquitoes could be due to the major components, α-pinene, 1-nonanol, myrcene, hexanal, and terpenolene. This was confirmed by the assay of the blend C_1 (α-pinene:1-nonanol:myrcene:hexanal:terpenolene 23.66:8.20:21.02:1:11.01), which gave a PE of 80.56% at 0.1 g/ml compared to 93.55% of the leaf oil at the same concentration. The observation suggested the possibility of synergistic contribution of other compounds which are present in smaller amounts but exhibit reasonable activity individually.

The PE of the blends C_2 (α-pinene: 1-nonanol: myrcene: terpenolene 2.88:1:2.56:1.34), C_3 (α-pinene: 1-nonanol:myrcene:hexanal 23.66:8.2:21.01:1) and C_4 (α-pinene:myrcene:hexanal:terpenolene 23.66:21.01:1:11.01) were 71.11, 69.75 and 40.96% at 0.1 g/ml, respectively. The PE of the blend C_4 indicated 1-nonanol as the major contributor towards the activity of the oil while C_2 and C_3 demonstrated the less significant role played by hexanal and terpenolene respectively. As previously demonstrated for *A. afra*, the contribution of artemisia ketone towards the repellent activity of the essential oil of *S. moorei* leaves was insignificant as evident in the assay of a blend C_5 (hexanal:1nonanol:myrcene:artemisia ketone:terpenolene $3.1x10^1$:$2.54x10^3$:$6.5x10^3$:1:$3.41x10^2$) which gave a PE of 74.04%, which is slightly less than 80.56% of the blend C_1. Similarly, the slight reduction in activity of C_2 compared C_1 suggests the insignificant role played by α-pinene.

5.2.4 *Nepeta azurea*

From *Nepeta azurea*, 4 out of 9 compounds identified in the essential oil from leaves were assayed against female *An. gambiae s.s* mosquitoes. Nepetalactone, the major constituent of the oil had a PE of 78.85% at 0.01 g/ml compared to 84.33% for the whole oil mixture with the two means showing no significant difference (Table 4 and 25). The RD_{50} value was $1.2x10^{-3}$ mg/cm^2. Other compounds assayed from this essential oil include linalool oxide, linalool and terpenolene (Table 24-25). The RD_{50} values were found to be $7.3x10^{-2}$, $5.3x10^{-4}$ and $5.5x10^{-4}$ mg/cm^2, respectively.

The repellent properties exhibited by the oil is probably due nepetalactone which is a known mosquito repellent (Anon, 2001c). The PE of nepetalactone indicates that there must be synergestic contribution from other compounds in the essential oil. The compounds most likely to be responsible for enhancing the PE of the oil are naphthalene, alloaromadendrene, β-cubebene, and (*Z*)-β-ocimene. Contribution from linalool oxide, linalool and terpenolene cannot be ignored which are known mosquito repellents (USDA, 1965).

The assay of the blends D_1 (naphthalene:linalool oxide:linalool:terpenolene 74.5:1.5:60:1), D_2 (linalool oxide:linalool 1:6), D_3 (linalool oxide:linalool:terpenolene 1.5:60:1) and D_4 (linalool oxide:terpenolene 1.5:1) gave a PE of 68.99, 53.31, 39.33 and 47.35% at 0.1 g/ml compared to 100% for the whole oil mixture at the same concentration. The big difference between the PE of the blends and whole essential oil mixture suggested that the major component (nepetalactone) is the main contributor towards the repellent activity of the oil. Bioassay of blends with nepetalactone could not be done due to insufficient amounts of sample.

5.2.5 *Satureja pseudomensis*

For *Satureja pseudomensis*, a total of 5 out of 8 compounds identified from the essential oil were assayed, limonene, terpenolene, terpene-4-ol, carvone and carvacrol (Table 24-25). The repellent property exhibited by the leaf oil of *S. pseudomensis* is suspected to be resulting from the major compounds like limonene, diisoamylene, (*Z*)-β-ocimene and isoeugenol. Contributions from other minor compounds (carvacro, linalool, terpenolene, terpene-4-ol, carvone) cannot be ignored since they exhibit repellency individually. The confirmation of the compound responsible for repellency of the leaf oil of this plant was not possible due to unidentified major compound.

However, the blends E_1 (limonene:linalool:isoeugenol 14.8:1:6.29), E_2 (limonene:linalool 1.48:1) and E_3 (limonene:linalool:isoeugenol:carvone 19.59:1.32:8.32:1) were assayed and gave a PE of 78.18, 81.22 and 66.42%, respectively, at 0.1 g/ml compared to 97.50% of the whole essential oil mixture at the same concentration. The observation suggested the likehood of the major component (unidentified) as a major contributor towards the activity of the oil. The test of these major compounds in a blend is necessary to establish where the repellent activity of the oil is emanating from. Thus, identification of the unknown compound in *S. pseudomensis* and bioassays with individual compounds identified from essential oil mixture will be necessary for the complete identification of the active ingredients.

5.2.6 *Clausena anisata*

A total of 10 out of 17 compounds identified as components of the essential oil *C. anisata* leaves were assayed (Table 24-25). Limonene, one of the major chemical component of this oil showed interesting protective properties. The compound seemed to lose efficacy with increasing concentration. For instance, at 0.1 g/ml the protective efficacy was 23.24% compared to 60% at the lowest concentration. These properties were also exhibited by *p*-cymene which behaved as an attractant at higher concentration (Omollo, 2002).

The protection offered by this oil could be due to limonene, linalool, (*E*)-β-Ocimene, myrcene, sabinene and γ-terpinene. A blend F_1 (limonene:myrcene:linalool:terpenolene:γ-terpenene 9.29:1.84:1.08:1:6.54) was assayed giving a PE of 77.69% compared to 96.18% for the whole mixture of the essential oil. The results demonstraded the role of these compounds in the repellent properties of the essential oil. There might also be some contribution from caryophyllene oxide, aromadendrene, (*Z*)-caryophyllene and terpene-4-ol. γ-Terpenene, the second most abundant component in the oil seem to contribute significantly towards the overall repellent activity exhibited as confirmed by the bio-assay of a blend F_2 (limonene:linalool:terpenolene:myrcene 9.29:1.08:1:1.84) with a PE of 58.39%. The PE of blends F_3 (myrcene:terpenolene:linalool:γ-terpenene 1.847:1:1.08:6.54) and F_4 (myrcene:*p*-cymene:linalool:γ-terpenene 4.96:1:2.91:17.66) at 87.45% and 52.24%, respectively, compared to 98.18% for the essential oil mixture at 0.1 g /ml concentration, clearly indicated that limonene and *p*-cymene acted antagonistically while linalool contributed significantly towards the overall repellent activity of the oil. Most of these compounds

(linalool, aromadendrene, terpenolene and γ-terpenene) are known mosquito repellents (Omollo, 2002).

5.2.7 *Pseudocarum eminii.*

For *Pseudocarum eminii*, 9 out of 10 compounds identified and confirmed as components of the essential oil were evaluated for their repellent activity (Table 24-25). Caryophyllene oxide, present in trace in this oil exhibited the highest protective efficacy of 100% (0.1 g/ml). The lowest protection was observed with *p*-cymene having a protective efficacy of -15.38% at the same concentration (0.1 g/ml) (Omollo, 2002). The RD_{50} values were $1.4x10^{-4}$ and $1x10^{-4}$ mg/cm^2 for caryophyllene oxide and *p*-cymene, respectively.

The repellent activity of the leaf oil of *P. eminii* might probably be due to the major components as a blend. A blend G_1 (α-pinene:myrcene:(+)-3-carene:β-pinene 8.17:1:18.91:4.77) had a PE of 75.59% at 0.1 g/ml which was significantly lower than that of the plant oil (96.52%) at same concentration. This suggested that there must be contributions from other minor compounds such as caryophyllene oxide and (+)-2-carene that are present in the essential oil at low concentrations but show high activity individually. The most abundant compound, (+)-3-carene was not the most active compound as confirmed by the PE of the blend G_2 (α-pinene:myrcene:β-pinene 8.17:1:4.77) at 59.24% compared to 75.59% for blend G_1. The contribution of the minor component (caryophyllene oxide), previously demonstrated as an effective mosquito repellent with a PE of 100% at 0.1 g/ml (Omollo, 2002) was confirmed by the assay of the blend G_3 (α-pinene:myrcene:(+)-3-carene caryophyllene oxide:β-pinene $8.41x10^2$:$1.0x10^2$:$1.95x10^3$:1:$4.91x10^2$) with an increased PE of 89.76% compared to 96.52 and 75.59% for the whole essential oil mixture and G_1, respectively, at the same concentration.

Similarly, the assay of the blend G_4 (α-pinene:myrcene:(+)-3-carene:(+)-2-carene:β-pinene $4.2x10^2$: $5.15x10^2$:$9.74x10^2$:1:$2.46x10^2$) gave a PE of 54.37% at 0.1 g/ml which is less than 75.59% for G_1. This observation indicated that of the two minor components present in the oil, caryophyllene oxide contributes more to the repellency than (+)-2-carene.

5.4 Probit analysis

The 8 most effective plant-derived repellent compounds were chosen and the RD_{50} values (Table 25) of each compound estimated by subjecting the repellency data to probit (Busvine, 1971) and regression analysis (Zar, 1984). The compounds analysed included artemisia ketone, nepetalactone, bornyl acetate, linalool oxide, 1-undecene, 1-nonanol, 7-methylquinoline and myrcene (Table 25) with regression equations (Table 27-34).

Table 27. Probit analysis of repellency data for Artemisia ketone

% Conc.	Log +4	% Repellency	Regression equation	Probit transformation
1.0	4.0	59.15		5.23
0.1	3.0	47.92	Y= 0.28x + 4.08	4.87
0.01	2.0	36.96		4.67

Table 28. Probit analysis of repellency data for nepetalactone

% Conc.	Log +4	% Repellency	Regression equation	Probit transformation
1.0	4.0	78.85		5.81
0.1	3.0	59.1563.29	Y= 0.25x + 4.73	5.33
0.01	2.0	47.9262.02		5.31

Table 29. Probit analysis of repellency data for 1-undecene

% Conc.	Log +4	% Repellency	Regression equation	Probit transformation
1.0	4.0	69.76		5.52
0.1	3.0	66.55	Y= 0.22x +4.69	5.44
0.01	2.0	52.58		5.08

Table 30. Probit analysis of repellency data for bornyl acetate

% Conc.	Log +4	% Repellency	Regression equation	Probit transformation
1.0	4.0	65.15		5.39
0.1	3.0	63.16	Y= 0.2x + 4.65	5.33
0.01	2.0	49.64		5.00

Table 31. Probit analysis of repellency data for myrcene

% Conc.	Log +4	% Repellency	Regression equation	Probit transformation
1.0	4.0	66.1		5.41
0.1	3.0	56.3	Y= 0.41x +3.82	5.15
0.01	2.0	34.1		4.59

Table 32. Probit analysis of repellency data for 1-nonanol

% Conc.	Log +4	% Repellency	Regression equation	Probit transformation
1.0	4.0	72.47		5.58
0.1	3.0	63.80	Y= 0.13x +5.03	5.36
0.01	2.0	63.34		5.33

Table 33. Probit analysis of repellency data for 7-methylquinoline

% Conc.	Log +4	% Repellency	Regression equation	Probit transformation
1.0	4.0	80.10		5.95
0.1	3.0	55.67	Y= 0.49x +3.88	5.15
0.01	2.0	49.39		4.97

Table 34. Probit analysis of repellency data for linalool oxide

% Conc.	Log +4	% Repellency	Regression equation	Probit transformation
1.0	4.0	62.55		5.33
0.1	3.0	58.58	Y= 0.36x 3.97	5.23
0.01	2.0	34.65		4.61

The probit analysis revealed the order of activity as 1-nonanol > nepetalactone > 1-undecene > bornyl acetate > 7-methylquinoline > linalool oxide > myrcene > artemisia ketone, based on RD_{50} values. The order confirmed the insignificant role played by artemisia ketone towards the repellent activity of the essential oil of *A. afra*

Nepetalactone, is a known mosquito repellent from *Nepeta cataria* (catnip) oil (Anon, 2001c). Similarly, bornyl acetate, myrcene and linalool oxide are also known mosquito repellents (Omollo, 2002). Although artemisia ketone had been reported in other plants such as *Artemisia vulgaris* (Hwang *et al.*, 1985) its repellent activity against mosquitoes was not investigated. Therefore, this is the first report on the repellent activity of artemisia ketone from *Artemisia afra* oil. Similarly, 1-nonanol, 1, 3-butadienylbenzene and 1-undecene had previously been isolated in other *Senecio* species and are being reported for the first time as mosquito repellents (Gamal and Wink, 2000). 7-Methylquinoline identified from the essential oil of *Cineraria grandifolia* gave PE of 80.30% at 0.1 g/ml. This is the first time such a compound is not only being reported as mosquito repellent but also as a component of essential oils.

5.3 Durational repellency assays of formulated compounds

Out of the 8 most effective repellent compounds, 1-nonanol, myrcene, bornyl acetate, 7-methyquinoline and 1-undecene were formulated in various carrier media and bio-assayed against female *An. gambiae s.s* to determine the longevity of protection. The base materials (aqueous cream, petroleum jelly and emulsificant base) were investigated by mixing them with the compounds of interest. Formation of homogeneous mixture qualified the carrier media for use in formulation. The essence of such formulation with base material is to reduce the evaporation rate of the repellent,

thus extending the duration of efficacy (Dremova, 1971). However, extended-duration formulation is disadvantageous in that they may feel sticky when applied to the skin. The formulated compounds were assayed and PE calculated as earlier described (Table 35).

Table 35. Durational mean % PE of formulated compounds

Compound /Time (h)	0	2	4	6	8
1-Nonanol[1]	78.89±9.76^{ef}	65.21±4.67^{gh}	63.35±5.6^{ghi}	58.21±3.45^{i}	45.23±4.14^{kl}
1-Nonanol[2]	68.37±6.47^{gh}	60.65±2.45^{ih}	56.96±5.4^{h}	56.60±3.37^{h}	53.43±8.03^{j}
Myrcene[1]	64.34±5.89^{gh}	58.46±7.21^{hij}	50.46±8.85^{jk}	25.12±6.37^{pq}	
Myrcene[2]	57.39±11.47^{hij}	51.91±4.67^{jk}	44.72±5.18^{klm}	42.50±6.34^{kjmn}	
7-Methylquinoline[1]	81.09±9.34^{de}	64.29±4.75^{ghi}	60.0±9.91^{hi}	52.27±9.27^{j}	
7-Methylquinoline[2]	74.30±6.74^{f}	64.98±2.52^{gh}	54.48±3.91^{j}	49.14±6.67^{jkl}	
Bornyl acetate[1]	61.9±7.88^{hi}	58.30±10.72^{i}	52.17±3.88^{j}	51.93±7.48^{jk}	
Bornyl acetate[2]	71.25±9.6^{g}	56.85±8.74^{i}	51.90±3.59^{jk}	45.95±2.01^{klm}	
1-Undecene[1]	77.92±7.41^{ef}	76.05±4.45^{f}	60.06±2.21^{hi}	42.09±7.58^{klm}	

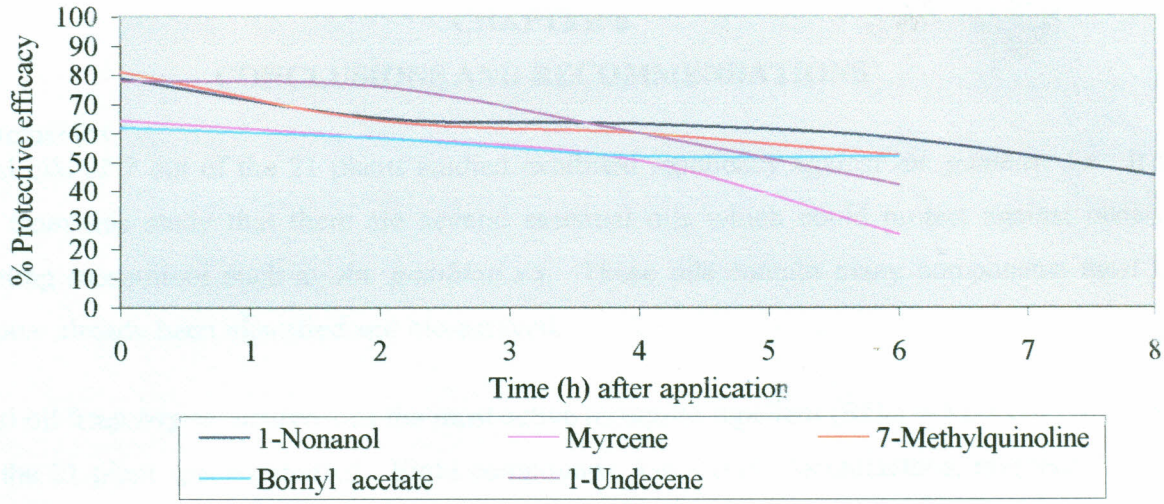
Superscripts 1 and 2 represent petroleum jelly and emulsficant base respectively

1-Undecene gave a PE of 75.32% at 0.1 g/ml when tested singly and was confirmed through the assay of the blend to contribute significantly towards the overall repellent activity of the leaf oil of *C. grandifolia*. Based on this observation, the compound was chosen for formulation to determine its longevity of protection by controlling its rate of release. The compound was formulated in petroleum jelly after other carrier media failed to provide homogeneous mixtures with it. It was observed that 1-undecene had a PE of 77.92, 76.05, 60.06 and 42.19% after 0, 2, 4 and 6 hours respectively. Previously, DEET gave 100% protection after 6 hours which then dropped to 95% after 8 hours (Omollo, 2002). Although 1-undecene does not compare favourably with DEET, a PE of 42.19% after 6 hours is good enough to warrant further formulation tests of this compound with other synersts compounds to improve its PE and longevity of protection.

The emulsificant base containing 10% of 1-nonanol provided high level of protection against mosquito bites. It provided 53.43% protection against mosquito bites after 8 hours compared to petroleum jelly (45.23%). The PE of the formulated compounds (Table 36) demonstrated emulsificant base as better formulation material for 1-nonanol and myrcene while petroleum jelly was best for 7-methylquinoline, 1-undecene and bornyl acetate. However, none of the formulated compounds compared favourably with DEET. Further tests need to be carried out to determine the duration of protection provided by other bases.

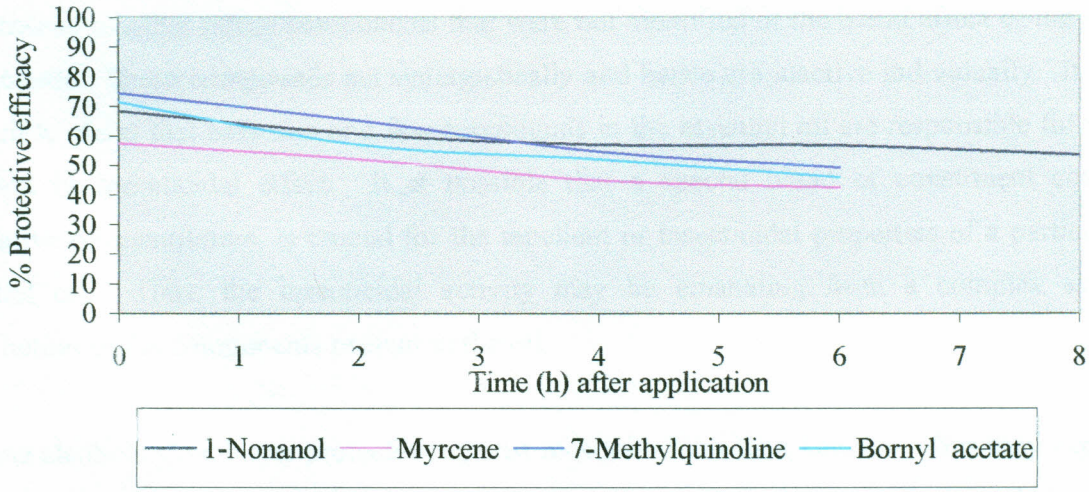
Regression analysis and the best fitting decay curves (SAS® Institude, 2002) were established for the two bases (Fig. 10-11).

Figure 10. Mean durational % repellency curves of compounds formulated in petroleum jelly



The regression equations were established as y = 77.04 – 3.17x, y = 68.44 - 6.28x, y = 78.03 – 4.54x, y = 82.55 – 6.17x and y= 61.48 - 1.80x for 1-nonanol, myrcene, 7-methylquinoline, 1-undecene and bornyl acetate, respectively, in petroleum jelly base.

Figure 11. Mean durational % repellency curves of compounds formulated in emulsificant base



Similarly, for emulsificant base, the regression equations for the best fitting decay curves were established as y = 65.98 – 1.69x, y = 56.91 – 2.59x, y = 73.62 – 4.29x and y = 68.62 – 4.04x for 1-nonanol, myrcene, 7-methylquinoline and bornyl acetate, respectively.

CHAPTER 6

CONCLUSIONS AND RECOMMENDATIONS

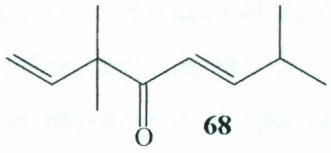
6.1 Conclusions

Essential oils of 7 out of the 21 plants studied exhibited repellency against *An. gambiae s.s.* It is evident from this study that there are several essential oils which could protect against malaria transmitting mosquitoes such as *An. gambiae s.s.* These oils contain many components most of which have already been identified and bio-assayed.

Essential oil from *Nepeta azurea* was the most active mosquito repellent ($RD_{50} = 5.69 \times 10^{-5}$ mg/cm^2) among the 21 plant species studied. Eight compounds, 1-nonanol, nepetalactone, myrcene, bornyl acetate, 7-methylquinoline, 1-undecene, linalool oxide and artemisia ketone were the most active plant derived repellent compounds based on their RD_{50} values. The least effective plant repellent among the 7 selected plant species was *A. afra* ($RD_{50} = 3.07 \times 10^{-2}$ mg/cm^2).

Artemisia afra was confirmed to be the only insecticidal plant ($LD_{50} = 2.66 \times 10^{-2}$ mg/cm^2). The insecticidal activity of *A. afra* oil could not be assigned to any individual component assayed, as none was found to be active singly. This led to the suggestion that the activity could either be due to the presence of other minor components that were not identified or the blend effect of the identified constituents. These compounds act synergistically and hence are inactive individually. It might be difficult to claim that only one or a few compounds in the essential oil are responsible for mosquito repellent or insecticidal effect. It is possible that a special blend of constituent compounds, qualitative or quantitative, is crucial for the repellent or insecticidal properties of a particular plant essential oil. Thus, the insecticidal activity may be emanating from a complex synergestic contribution of the components present in the oil.

Terpene alcohols gave better protection against mosquito bites than ketones. This may explain why *A. afra* oil with artemisia ketone **(68)** as the major component and terpene-4-ol is a better repellent at 0.1 g/ml than *P. eminii* oil with terpenoid hydrocarbons. This agrees with the earlier observations on repellency of essential oils of other plants (Omollo, 2002).



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Variation in the composition of the essential oils of various plant species within the same genus were observed. *Satureja* species from Turkey (Saener *et al.*, 1995) contain compounds which are considerably different from what we obtained from *S. pseudomensis* The observation supports the idea that the essential oil content or composition is a function of other factors such as climate, soil, time of harvest, locality, method of processing and storage methods among others.

The repellent activity of any plant species is not necessarily a function of the most abundant compound(s) present in the essential oil. This was confirmed by artemisia ketone in *A. afra* oil. Although artemisia ketone was the most abundant compound, its contribution towards the overall repellent activity of *A. afra* essential oil was not commensurate with the concentration in the plant.

The relationship between individual repellent activities of a given compound to that of the mixture of compounds does not exhibit direct proportionality. Thus, a compound that exhibits good repellent property individually may not necessarily contribute significantly towards the overall repellent activity of the plant oil. We thus hypothesize that it would be dangerous for one to infer the contribution towards the overall repellency of each compound from the individual activity.

The amount and composition of the essential oil change according to seasonal patterns. A greater amount of oil is extractable in rainy season than dry spells (Halligan *et al.*, 1975). *Nepeta azurea* and *Artemisia afra* harvested during rainy season yielded more oil than those harvested during dry season. The essential oil composition of *A. carlifornica* was found to vary with change in seasons. For instance, α-thujone was found to replace isothujone whereas camphor became more abundant in summer than fal (Halligan *et al.*, 1975). However, the composition of the essential oil *A. afra* did not vary with this seasonal pattern as reported for *A. carlifornica*. The oil composition of *A. afra* examined during dry and rainy season was the same.

α-Pinene (22 %) and terpenolene (10.4 %), the main component of *S. moorei* are known mosquito repellent with protection time of $\leq$ 1 hour (USDA, 1965). Although both *S. moorei* and *A. afra*

essential oils contain artemisia ketone in concentrations of 0.29 and 18.92%, respectively, the former is more active. The observation further confirms the insignificant role of artemisia ketone as mosquito repellent. This explains the importance of special mixture of compounds critical for repellency. The combination of α-pinene, terpenolene and artemisia ketone could account for higher repellent effect of *S. moorei* than *A. afra* oil at 0.1 g/ml.

In conclusion, it has been demonstrated that there are potential repellent plant-derived essential oils. Subsequently, we showed that some compounds in the essential oils also exhibit repellent activity individually and may be useful for protection against mosquito bites. Similarly, we demonstrated the efficacy of the blends of the major compounds in essential oil mixtures as mosquito repellents.

6.2 Recommendations

More bio-prospecting work is needed in both areas covered and not covered by this project as there is no doubt that there could still be more unknown mosquito repellent or insecticidal plants in Kenya

Detailed repellency assay of essential oils of *Bothriocline fusca* and *Vernonia urticifolia* should be followed up as this may lead to novel compound(s) with good repellent or insecticidal activities. This may be possible if more plant materials were secured to avail enough essential oil that may facilitate identification and bio-assay work.

The unidentified major component in the essential oil of *Satureja pseudomensis* should be followed up through isolation and identification by spectroscopic methods, synthesis and confirmation by GC co-injection and assayed to establish major repellent components in the oil.

The identified repellent compounds should be subjected to toxicological assessment and field evaluation to ascertain their safety, stability and applicability for protection against mosquito bites.

Formulation of the repellent compounds and essential oils may be necessary to enhance their duration of protection against mosquitoes such as *An. gambiae s.s*, *An. funestus*, *An. arabiensis*, *An. nili*, *An. melas* and *An. merus* among others.

The active compounds should not only be tested against other anopheline species but also against Culecine mosquitoes (*Culex* and *Aedes* spp) and other blood-sucking arthropods like ticks, lice, bedbugs, tse tse flies and sand flies among others.

Repellent activity of solvent extracts should also be investigated as this might result in the discovery of new active compounds against mosquitoes.

CHAPTER 7
EXPERIMENTAL

7.1 Glassware

All the glassware used were cleaned by soaking in freshly prepared chromic acid overnight, washed with soapy water and rinsed with acetone and finally with distilled water. The glassware were oven dried at 120 °C overnight.

7.2 Reagents and solvents

All solvents used were analytical grade from Sigma-Aldrich Chemical Co. and Fluka Chemica Co. All reagents and chemicals were pure analytical grade, from Sigma Aldrich Chemical Co.

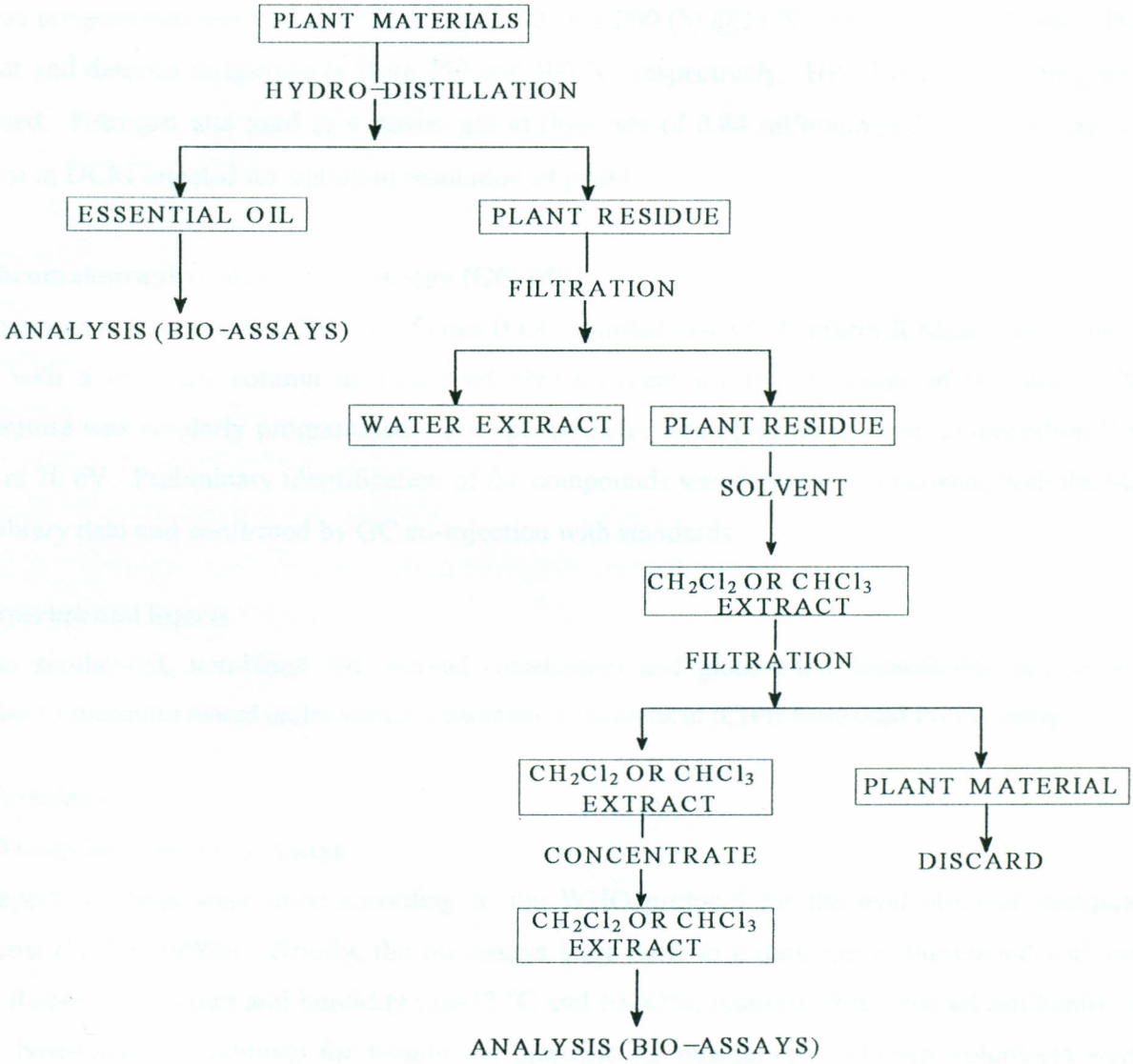
7.3 Collection of plant materials

Leaves were collected from twenty-one plants: *Artemisia afra* (Snmm/1), *Nepeta azurea* (Snmm/2), *Gutenbergia tormentosa* (Snmm/3), *Hypericum revolutum* (Snmm/4), *Rhus natalensis* (Snmm/5), *Salvia marjamie* (Snmm/6), *Satureja biflora* (Snmm/7), *Cineraria grandifolia* (Snmm/17), *Pseudocarum eminii* (Snmm/18), *Senecio roseiflorus* (Snmm/19), *Conyza schimperi* (Snmm/20), *Clausena anisata* (Snmm/21) were collected from Mt. Kenya while *Plectanthus edulis* (Snmm/8), *Senecio schweinfurthii* (Snmm/9), *Senecio keniodendron* (Snmm/10), *Senecio moorei* (Snmm/11), *Bothriocline fusca* (Snmm/12), *Satureja pseudomensis* (Snmm/13), *Solanecio nadensis* (Snmm/14), *Vernonia urticifolia* (Snmm/15) and *Cleodendrum johnstonii* (Snmm/16), from Aberdares National Park. The plants were identified and voucher specimens deposited at the Herbarium, in Botany Department at the University of Nairobi (with reference numbers indicated in brackets against each plant species).

7.4 Handling of plant materials

The collected plants were dried under shade for 7 days. The leaves were hydro-distilled for 12 hours using a Clavenger-type apparatus until no more oil could be collected. The essential oil, collected over water, was dried with anhydrous sodium sulphate and kept in a refregirator at 4 °C for bio-assay and further analysis. The residual material was extracted using dichloromethane or chloroform to avail non-medium polar constituents and concentrated in a rotary evaporator. These were also preserved in a refregirator for bio-assay and further analysis. The final extraction was done with

water and the aqueous extract freeze dried to afford the polar components of the plants. The extraction procedure is summarized in scheme 1.



Scheme 1. The extraction procedure

7.5 Identification and characterization

The chemical composition of the essential oils from the repellent or insecticidal plants were confirmed by GC, GC-MS and GC co-injection with standards supplied by Sigma-Aldrich Company.

7.6 Gas chromatography (GC)

GC analysis was carried out on a Hewlett Packard (HP) 5890 gas chromatograph fitted with cross-linked methylsilicon capillary column, 50 m x 0.2 mm (id) x 0.33 µm (film thickness) and FID. The GC was programmed from 50-280 °C, 50 @15-80 @ 2-200 (5) @15 °C/min-280 °C (20) min). The injector and detector temperatures were 250 and 300 °C, respectively. HP 3393 Series II integrator was used. Nitrogen was used as a carrier gas at flow rate of 0.84 ml/min and 2 µl of essential oil solution in DCM injected for optimum resolution of peaks

7.9 Chromatography- mass spectroscopy (GC-MS)

Analysis was carried out on a HP 8060 Series II GC coupled to a VG Platform II Mass Spectrometer fitted with a capillary column as described above except for the thickness of 0.5 µm. The temperature was similarly programmed. The spectrometer was operated in electron ionization (EI) mode at 70 eV. Preliminary identification of the compounds was done by comparisons with the MS with library data and confirmed by GC co-injection with standards.

7.8 Experimental insects

Five to six-day-old, non-blood fed, starved (repellency) and glucose-fed (insecticidal) female *An. gambiae s.s* mosquito reared under standard insectary conditions at ICIPE were used for bio-assay.

7.9 Bio-assays

7.9.1 Mosquito repellency assays

The repellency tests were done according to the WHO protocol for the evaluation of mosquito repellents (WHO, 1996b). Briefly, the bio-assays were done in a dark room illuminated with red light. Room temperature and humidity (26-32 °C and 65-85%, respectively) were set artificially to mimic host-feeding conditions for female *An. gambiae s.s* mosquitoes. Human volunteers were selected from those who showed no allergic reactions to mosquito bites. The volunteers were not allowed to use perfumes, oils, other chemicals and soap on the day of the bio-assay. The test material (1 g) was dissolved in 10 ml of HPLC grade acetone. This made up the stock solution with a concentration of 0.1 g/ml. Subsequent serial dilutions were done to enable dispensing range of 10-0.001%. The test solutions were applied on the arm between the hand and the elbow. The palms and the fingers were protected by plastic gloves to make them unattractive to mosquitoes. The other arm was similarly treated with acetone to act as control. In performing repellency tests, emphasis was placed on % protection in relation to the applied dose. Preliminary screening was performed to

ascertain the activity. The active oils, standards or mixtures were exposed to detailed bio-assay. The test insects were 5-6 day old, non blood-fed adult female *An. gambiae s.s.*

7.9.1.1 Preliminary bio-assay

Six human volunteers, three male and three female were used. For each test sample, three (3) different concentrations of the essential oils (10, 0.1 and 0.001%) or solvent extracts (5, 0.05, 0.005%) in acetone were tested against twenty-five (25) female *An. gambiae s.s* mosquitoes in a cage. A total of 18 cages each measuring 50 x 50 x 50 cm were required for the three concentrations. The test hand was treated with 1 ml of the plant extract in acetone from the elbow to the wrist while the control hand was treated with 1 ml of acetone alone. The rest of the hand was covered with gloves to make it unattractive to the mosquitoes. After evaporation of the solvent, the mosquitoes in the cage were provoked by hitting the sides of the cages before introducing the control hand. Before the bio-assay of the next concentration, the hands were washed with bar soap, rinsed with water so as to enable the re-establishment of the body odour. A new set of insects was used for each test. The arms were swapped regularly to eliminate any bias. The degree of repellency was expressed in terms of % protective efficacy calculated according to the formula:

% Repellency = (Nc-Nt)/Nc x100%, where Nc and Nt = % control mean and % test mean of mosquitoes landing and probing at control and treated arms, respectively (Weaving and Sylvester, 1967).

The results of preliminary repellency assay are summarized in tables 29-37 where MA, WK, JO, JT, BM and CT are the initials for the names of volunteers while C and T represent control and treated arms, respectively.

Table 36. Preliminary repellency assay data for *Artemisia afra* essential oil

% Conc.	Assay	JO	MA	WK	JO	JT	BM	TOTAL	MEAN	% P.E± SE
0.001	C	14	8	16	11	15	13	77	12.83	57.13± 9.78^{hij}
	T	8	4	5	2	8	6	33	5.5	
0.1	C	10	8	6	7	6	11	48	8	66.63± 10.37^{ghi}
	T	3	4	1	3	2	3	16	2.67	
10	C	6	13	7	13	8	6	53	8.83	100.00± 8.39^{ab}
	T	0	0	0	0	0	0	0	0	

Means with same letters are not significantly different from each other

Table 37. Preliminary repellency assay data for *Bothriocline fusca* essential oil

Conc (%)	Assay	CT	SW	JT	BM	JO	MA	Total	Mean	%PE±SE
0.001	C	7	6	6	11	6	8	44	7.33	38.61±5.69^{klmno}
	T	2	1	9	3	4	8	27	4.5	
0.1	C	10	4	7	10	8	5	44	7.33	77.22±4.64^{ef}
	T	1	1	5	1	1	1	10	1.67	
1.0	C	8	5	4	10	12	8	47	7.83	100±22.42^{ab}
	T	0	0	0	0	0	0	0	0	

Means with same letters are not signficantly different from each other

Table 38. Preliminary repellency assay data for *Cineraria grandifolia* essential oil

% Conc.	Assay	JO	MA	WK	JO	JT	BM	TOTAL	MEAN	% P.E± SE
0.001	C	10	6	4	6	4	7	37	6.17	29.82± 8.14^{nop}
	T	4	4	2	5	5	6	26	4.33	
0.1	C	14	7	7	11	6	9	54	9	63.00± 9.23^{ghi}
	T	4	2	5	3	4	2	20	3.33	
10	C	9	10	7	7	6	10	49	8.17	100.00± 9.98^{ab}
	T	0	0	0	0	0	0	0	0	

Means with same letters are not signficantly different from each other

Table 39. Preliminary repellency assay data for *Senecio moorei* essential oil

%Conc.	Assay	MA	WK	JO	JT	BM	CT	TOTAL	MEAN	%PE± SE
0.001	C	18	4	14	16	12	16	80	13.33	28.73± 7.07^{pq}
	T	12	5	9	10	8	13	57	9.5	
0.1	C	17	12	11	6	8	11	65	10.83	44.60± 6.81^{klm}
	T	6	3	10	4	8	5	36	6	
10	C	10	7	8	9	7	7	48	8	95.88± 1.54^{ab}
	T	0	1	0	0	0	1	2	0.33	

Means with same letters are not signficantly different from each other

Table 40. Preliminary repellency assay data for *Vernonia urticifolia* essential oil

Conc (%)	Assay	CT	SW	JT	BM	JO	MA	Total	Mean	%PE±SE
0.001	C	17	20	18	11	7	4	77	12.83	40.22±5.88^{klmn}
	T	8	10	10	5	5	8	46	7.67	
0.1	C	16	11	9	10	15	6	67	11.17	37.33±18.83^{jklmnopg}
	T	15	7	6	6	3	5	42	7	
1.0	C	16	6	15	10	5	7	59	9.83	91.56±12.86^{ab}
	T	0	0	2	1	1	1	5	0.83	

Means with same letters are not significantly different from each other

Table 41. Preliminary repellency assay data for *Nepeta azurea* essential oil

%Conc.	Assay	MA	WK	JO	JT	BM	CT	TOTAL	MEAN	%PE± SE
0.01	C	15	7	9	19	11	7	68	11.33	41.13± 5.30^{klmn}
	T	3	8	9	10	4	6	40	6.67	
0.1	C	16	14	6	8	9	4	57	9.5	73.68± 21.18^{efg}
	T	0	6	4	1	2	2	15	2.5	
1.0	C	10	10	9	13	12	15	69	11.5	86.96± 7.16^{cde}
	T	1	0	3	4	1	0	9	1.5	

Means with same letters are not significantly different from each other

Table 42. Preliminary repellency assay data for *Satureja pseudomensis* essential oil

% Conc.	Assay	MA	WK	JO	JT	BM	CT	TOTAL	MEAN	% P.E± SE
0.01	C	13	7	11	19	16	17	83	13.83	37.31 ± 9.63^{klmnop}
	T	8	9	4	7	15	9	52	8.67	
0.1	C	21	14	19	16	12	14	96	16	40.63 ± 5.65^{klmnop}
	T	6	8	12	8	17	6	57	9.5	
1.0	C	18	17	15	19	10	12	91	15.17	68.16 ± 2.26^{g}
	T	5	6	3	6	7	2	29	4.83	

Means with same letters are not significantly different from each other

Table 43. Preliminary repellency assay data for *Clausena anisata* essential oil

%Conc.	Assay	MA	WK	JO	JT	BM	CT	TOTAL	MEAN	%PE± SE
0.01	C	7	9	12	11	9	12	60	10	58.3 ± 3.07^{l}
	T	3	5	5	4	4	4	25	4.17	
0.1	C	8	7	8	20	20	16	79	13.17	72.13 ± 4.81^{g}
	T	3	3	2	8	5	1	22	3.67	
1.0	C	4	3	6	23	22	23	81	13.5	87.63 ± 1.47^{cd}
	T	0	0	0	4	3	3	10	1.67	

Means with same letters are not significantly different from each other

Table 44. Preliminary repellency assay data for *Pseudocarum eminii* essential oil

% Conc.	Assay	MA	WK	JO	JT	BM	CT	TOTAL	MEAN	% PE± SE
0.001	C	5	6	7	7	9	12	46	7.67	43.55 ± 13.46^{klmo}
	T	4	5	4	3	5	5	26	4.33	
0.1	C	12	5	5	8	7	8	45	7.5	48.93 ± 8.97^{jkl}
	T	7	4	4	3	3	2	23	3.83	
10	C	9	12	8	4	3	6	42	7	90.43 ± 3.99^{b}
	T	2	2	0	0	0	0	4	0.67	

Means with same letters are not significantly different from each other

7.9.1.2 Detailed bio-assay

Plant derived samples with the highest repellent activity were subjected to detailed assay. The number of experimental insects was increased to 50 per cage. The range of concentrations used was increased to $10\text{-}10^{-3}$ % and $5\text{-}5\text{x}10^{-2}$ % for essential oils and solvent extracts, respectively, and the assays replicated 6. Protective efficacy (%) was calculated as described above. Each experiment was done on two different days and the data subjected to probit analysis (Busvine, 1971) to enable the calculation of the RD_{50} values for the samples. The results are summarized in tables 46-52. Means with same letters are not significantly different from each other.

Table 45. Detailed repellency assay data for *Artemisia afra* essential oil

%Conc.	Assay	MA	WK	JO	JT	BM	CT	TOTAL	Mean	%P.E± SE
0.001	C	13	8	15	19	18	8	81	13.5	37.04 ± 7.85^{lmnop}
	T	8	5	9	13	12	4	51	8.5	
0.01	C	13	12	5	6	13	13	62	10.33	40.27 ± 4.29^{klmn}
	T	6	5	6	4	9	7	37	6.17	
0.1	C	14	5	8	19	12	12	70	11.67	44.30 ± 12.8^{jklmno}
	T	8	4	3	9	9	6	39	6.5	
1.0	C	23	8	7	19	19	18	94	15.67	63.82 ± 6.47^{ghi}
	T	5	2	1	7	9	10	34	5.67	
10	C	29	25	13	10	25	10	112	18.67	91.97 ± 10.61^{abcd}
	T	2	5	2	0	0	0	9	1.5	

Means with same letters are not significantly different from each other

Table46. Detailed repellency assay data for *Cineraria grandifolia* essential oil

%Conc.	Assay	MA	WK	JO	JT	BM	CT	TOTAL	Mean	%PE± SE
0.001	C	10	17	13	33	15	17	105	17.5	33.31 ± 7.17^{nop}
	T	9	3	12	20	10	13	67	11.67	
0.01	C	19	25	13	12	16	20	105	17.5	43.83 ± 14.4^{jklmno}
	T	11	12	12	5	10	9	59	9.83	
0.1	C	17	34	15	12	16	16	110	18.33	46.37 ± 14.69^{klm}
	T	7	12	10	5	13	12	59	9.83	
1.0	C	13	26	12	13	14	13	91	15.17	70.34 ± 3.72^{g}
	T	6	8	2	4	3	4	27	4.5	
10	C	13	22	24	18	25	14	116	19.33	99.12 ± 15^{ab}
	T	0	1	0	0	0	0	1	0.17	

Means with same letters are not significantly different from each other

Table 47. Detailed repellency assay data for *Senecio moorei* essential oil

%Conc.	Assay	MA	WK	JO	JT	BM	CT	TOTAL	Mean	%PE± SE
0.001	C	41	39	35	36	25	35	211	35.17	56.41 ± 8.04^{h}
	T	17	19	9	13	18	16	92	15.33	
0.01	C	42	39	43	23	15	19	181	30.17	59.66 ± 9.42^{ij}
	T	15	11	9	13	8	17	73	12.17	
0.1	C	33	31	35	45	40	39	223	37.17	74.44 ± 3.11^{f}
	T	12	7	9	13	10	6	57	9.50	
1.0	C	40	30	34	40	38	32	214	35.67	82.70 ± 3.05^{de}
	T	6	8	5	3	10	5	37	6.17	
10	C	34	19	33	42	20	31	179	29.83	94.97 ± 2.23^{ab}
	T	1	2	3	0	0	3	9	1.5	

Means with same letters are not significantly different from each other

Table 48. Detailed repellency assay data for *Nepeta azurea* essential oil

%Conc.	Assay	MA	WK	JO	JT	BM	CT	TOTAL	Mean	% PE± SE
0.001	C	12	27	16	11	8	32	106	17.67	50.03 ± 6.75^{ijkl}
	T	13	8	11	8	3	10	53	8.83	
0.01	C	20	15	17	27	26	22	127	21.17	52.76 ± 9.54^{ikl}
	T	9	5	11	6	15	14	60	10	
0.1	C	22	30	26	20	11	29	138	23	60.13 ± 2.2^{hi}
	T	11	7	8	12	7	10	55	9.17	
1.0	C	33	35	38	28	24	40	198	33	84.33 ± 3.6^{cde}
	T	2	15	2	7	3	2	31	5.17	
10	C	40	32	37	46	47	44	246	41	100 ± 3.16^{a}
	T	0	0	0	0	0	0	0	0	

Means with same letters are not significantly different from each other

Table 49. Detailed repellency assay data for *Satureja pseudomensis* essential oil

%Conc.	Assay	MA	WK	JO	JT	BM	CT	TOTAL	Mean	%PE± SE
0.001	C	20	27	27	25	42	27	168	28	43.46± 8.73^{klmno}
	T	14	16	16	20	18	11	95	15.83	
0.01	C	27	35	35	25	36	22	180	30	46.67± 2.64^{kl}
	T	14	11	11	19	22	19	96	16	
0.1	C	28	35	35	30	25	26	179	29.33	57.38± 6.76^{ij}
	T	13	16	16	11	12	7	75	12.5	
1.0	C	29	25	25	27	22	21	149	24.83	66.45± 11.45^{fh}
	T	15	9	9	9	4	7	53	8.33	
10	C	18	18	16	20	23	25	120	20	97.5± 3.18^{ab}
	T	0	0	0	3	0	0	3	0.5	

Means with same letters are not significantly different from each other

Table 50. Detailed repellency assay data for *Clausena anisata* essential oil

%Conc.	Assay	MA	WK	JO	JT	BM	CT	TOTAL	Mean	%P.E± SE
0.001	C	26	17	27	14	24	21	129	21.5	38± 8.04^{klmnop}
	T	8	6	14	17	27	8	80	13.33	
0.01	C	17	23	20	29	15	37	141	23.5	48.94± 9.41^{jkl}
	T	6	12	24	14	9	7	72	12	
0.1	C	35	35	24	25	33	35	187	31.17	71.13± 3.11^{g}
	T	7	10	6	8	11	12	54	9.00	
1.0	C	39	30	47	30	32	34	212	35.33	72.63± 3.05^{efg}
	T	12	7	13	15	5	6	58	9.67	
10	C	35	42	41	29	32	30	209	34.83	96.18± 2.23^{ab}
	T	2	1	2	1	1	1	8	1.33	

Means with same letters are not significantly different from each other

Table 51. Detailed repellency assay data for *Pseudocarum eminii* essential oil

%Conc.	Assay	MA	WK	JO	JT	BM	CT	TOTAL	Mean	%PE± SE
0.001	C	17	15	22	10	17	14	95	15.83	46.30± 3.35^{kl}
	T	6	8	8	7	14	8	51	8.50	
0.01	C	26	16	15	14	15	16	102	17	55.88± 5.12^{ij}
	T	12	5	9	7	2	10	45	7.5	
0.1	C	17	30	13	7	9	7	83	13.83	62.62± 15.30^{fghij}
	T	6	14	3	3	2	3	31	5.17	
1.0	C	18	15	20	11	14	10	88	14.67	63.67± 14.28^{fghi}
	T	6	6	6	8	3	3	32	5.33	
10	C	20	15	16	17	36	39	143	23.83	96.52± 2.49^{ab}
	T	2	0	0	3	0	0	5	0.83	

Means with same letters are not significantly different from each other

7.9.1.3 Mosquito repellency assay of pure compounds

The test solutions of the standards were assayed for their repellency as described above. The highest concentration was either 10% or 1% depending on the quantity of the standard available. The concentrations used were 10, 1, 0.1, 0.01 and 0.001%. The repellency assay results of the 8 most active standards of the identified compounds are summarized in table 25.

7.9.1.4 Repellency assays of the blends of the identified compounds

The main constituents identified from the essential oils from leaves of the most active plants were taken in the ratio in which they occur in the oils and subjected to repellency tests as detailed above. Results are summarized in table 26.

7.9.1.5 Durational repellency assays of formulated compounds

Compounds with good repellent activity were formulated in various carrier media (base material) and tested against *An. gambiae s.s* mosquitoes after 0, 2, 4, 6 and 8 hours to determine the longevity of protection. The protective efficacy was determined as described earlier. Only the carrier media that gave homogeneous mixtures with the test compounds were used. This was attained by allowing the mixture of carrier media and compounds to stand. Separation of the mixture into distinct layers disqualified the base for formulation use. The assay results were subjected to ANOVA and Student t-test (SAS® Institude, 2002) for evaluation of significant difference (Table 36). Regression analysis was also done (Fig. 10-11).

7.9.2 Mosquitocidal assay

Two approaches were followed for mosquitocidal assay of the essential oils and solvent extracts.

7.9.2.1 Fumigant insecticidal assay

7.9.2.1.1 Preliminary assay

Twenty five (25) female *An. gambiae s.s* in a cage measuring 20 x 20 x 35.5 cm were fumigated with 10% solution of plant extract on a small whatman filter paper (diameter 7 cm) placed in a petri-dish (diameter 8 cm). Glucose solution (6%) was provided in the cage to serve as food for the insects. Control experiment was similarly set up minus the plant extracts. The dead mosquitoes were recorded at one-hour interval for six hours. Percentage (%) mortalities were calculated according to the expression: (N/R) x 100%, where N = number of dead mosquito in a test cage less number of dead mosquito in a control cage and R is the total number of mosquitoes introduced in the test cage. Out of twenty-one plants tested for insecticidal activity, only one plant *Artemisia afra* was found to have insecticidal activity necessitating the examination in detail.

7.9.2.1.2 Detailed insecticidal assay

The essential oil from *Artemisia afra* leaves was tested in detail. The experiment was replicated 8 times with the number of the test insects being increased to 50. The oils were tested at 2, 4, 6, 8 and 10 % concentration as summarized in table 13. Percentage mortalities were recorded as described above. The data obtained were subjected to probit analysis (Table 14) and the regression equation established to determine the LD values for the essential oil (Table 15).

7.9.2.2 Tarsal contact insecticidal assay

7.9.2.2.1 Preliminary screening

One small and one big petri-dish (8 and 10 cm diameters, respectively) were used, with the bigger one having a small hole at the centre (0.5 cm). A small filter paper (7 cm diameter) placed in the small dish was treated with 1 ml of 1 mg/ml solution of chloroform extracts in acetone. This was allowed to dry for 24 hours before the small dish was covered by the large one. *An. gambiae s.s* mosquitoes (10) were introduced into the bio-assay chamber through the small opening. The opening was covered with cotton wool and mosquitoes kept in the chamber for the next 1 hour before being introduced into another cage in which glucose solution (6 %) was provided as food. Dead mosquitoes were recorded hourly for the next 6 hours. The control experiment was similarly set up with 1 ml of acetone. From the preliminary work, none of the 21 plants tested by tarsal contact were found to be active and hence no detailed assay was done.

7.9.2.3 Fumigant mosquitocidal assay of pure compounds

Out of the 14 compounds identified in the essential oil from *A. afra* leaves, 8 that were evaluated for mosquitocidal activity (WHO, 1996b) by fumigation included artemisia ketone, α-pinene, β-pinene, 1, 8-cineole, bornyl acetate, terpenolene, terpene-4-ol, α- and β-thujone. None of these compounds showed activity even at the highest concentration considered (0.1 g/ml).

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