EFFICACY OF NEW PYRETHRUM SYNERGISTS FROM *OCIMUM KILIMANDSCHARICUM* GUERKE AND *TAGETES MINUTA L.* AGAINST MALARIA VECTOR THE *ANOPHELES GAMBIAE*

BY

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A THESIS SUBMITTED TO THE SCHOOL OF SCIENCE IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE AWARD OF THE DEGREE OF MASTER OF SCIENCE IN ORGANIC CHEMISTRY, UNIVERSITY OF ELDORET, KENYA.

DECLARATION

Declaration by the student

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

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DEDICATION

This thesis is dedicated to my parents Gilbert and Eunice Ondigo for the goodwill and wishes to do my studies up to Msc. Level. It is also dedicated to my husband Fred Obimbo, my son Allan, daughters Liza and Gloria for their support, prayers and understanding which enabled me fulfil my academic endeavours.

ABSTRACT

Mosquitoes are vectors that carry disease-causing viruses and parasites from person to person. Some of these diseases such as dengue, malaria, rift valley fever, yellow fever among others can be life threatening. Vector control is by far one of the most successful methods for reducing the incidences of such diseases. However, the emergence of widespread insecticide resistance and the potential environmental concern associated with some synthetic insecticides has indicated that additional approaches to control the proliferation of mosquito population are a priority research area. Concern on quality and safety of life in managing mosquitoes, has shifted steadily from the use of conventional chemicals towards alternative botanical insecticides that are target-specific, biodegradable and environmentally safe. Pyrethrins are natural plant compounds used in commercial vector control. They are usually formulated with synergists to improve quality, increase efficacy, mitigate resistance and make them cost effective. It was thus, necessary to explore new natural synergists to sustain the use of pyrethrins in vector control. In this study, essential oils from *Ocimum kilimandscharicum* leaves and *Tagetes minuta* flowers were extracted by steam distillation. Crude extracts were used with pyrethrins to conduct bioassays for larvicidal and adulticidal activity against 4th instar larvae and 4 day old adult *Anopheles gambiae*. For the bioassay, four concentrations (10ppm, 20ppm, 30ppm and 40ppm) each with 3 replicates, with a final total number of 80 larvae contained in each concentration. For each concentration of synergized mixture, twenty 4th instar larvae were used. Mortalities were recorded after 1, 3, 6, 9, 12 and 24 hour's exposure, during which no food was offered to the larvae. Bioassay data was evaluated by regression and probit analysis and used to determine the lethal doses (LC_{50} and LC_{90}) for the synergist mixtures. The results showed that LC_{50} and LC_{90} values for *O. kilimandscharicum* with pyrethrins were 0.00167 and 0.0076mg/ml while for *T. minuta* with pyrethrins it was 0.00361and 0.01644mg/ml respectively. *Ocimum* was a better synergist than *T. minuta* against larvae and adult stage. The components of the essential oils of *O. kilimandscharicum* was separated and identified by GC-MS. GC-MS resulted to nineteen compounds. The findings of this research could enable investigation of the active compound against *An. gambiae* and further exploration for large scale production of the synergists for commercial application.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF APPENDICES

LIST OF ABBREVIATIONS

µL	Micro litre
AMREF	Africa Medical Research Foundation
An.	Anopheles
CDC	Centre for Disease Control
CPS	Circumsporozoite protein
DDT	1,1-bis(ρ-chlorophtnyl)-2,2,2-trichloroethane
DMSO	Dimethyl sulfoxide
ESD	Estimated Safe Dose
eV	Electron volt
Exp.	Exposed
FOS	Factor of synergism
GoK	Government of Kenya
IPT	Intermittent preventive treatment
IR	Infra red
ITN	Insecticide treated nets
KBr	Potassium bromide
KD	Knockdown
KEMRI	Kenya Medical Research Institute
LD_{50}	Lethal Concentration of 50% Mortality
M/z	Mass to charge ratio
MFO	Mixed Function Oxidases
MoH	Ministry of Health

Mort	Mortality
MPND	Ministry of Planning and National Development
Mw	Molecular weight
NESC	National Economics and Social Council
NMR	Nuclear Magnetic Resonance
PE	Plant Extract
Py	Pyrethrins
R.P	Relative potency
s.s	*Sensu strict*
Temp	Temperature
Rt	Retention time
WHO	World Health Organization
WWII	World War II

ACKNOWLEDGEMENT

I acknowledge with deep gratitude the expert supervision, encouragement and guidance I received throughout the study from my supervisors; Prof. P.K Ndalut of University of Eldoret and Dr. Kefa S. Sum of Pyrethrum Board of Kenya.

My special gratitude goes to the technicians of chemistry department especially Mr. Odero, and Mr. Ongechi for helping with the extraction of plant oil and all technical staff of CDC/KEMRI Kisumu especially Mr. Amito who helped in the preliminary bioassays.

I am deeply indebted to Dr. Vulule the Director of CDC/KEMRI Kisumu for allowing me to carry out larvicidal assay in their laboratories and management of the Government Chemist for allowing me to carry out a GC-MS analysis in their laboratories.

Special thanks to my husband Mr. Obimbo for being a strong pillar in financial support for the project and my welfare.

I cannot conclude without thanking the good Lord for the strength in spirit and good health I have enjoyed throughout my studies.

CHAPTER ONE

INTRODUCTION

1.1Background of the study

Mosquito serves as crucial vector for a number of arboviruses (arthropod-borne viruses) and parasites that are maintained in nature through biological transmission between susceptible vertebrate hosts by blood feeding. These arthropods are responsible for inflammation/encephalitis, dengue, malaria, rift valley fever, yellow fever among others (Rodhain and Rosen, 2001). The World Health Organization (WHO) estimates that each year 300-500 million cases of malaria occur and more than 1 million people die of malaria. In addition, some 2500 million people (two fifth of the world's population) are now at risk from dengue (Tolle, 2009).

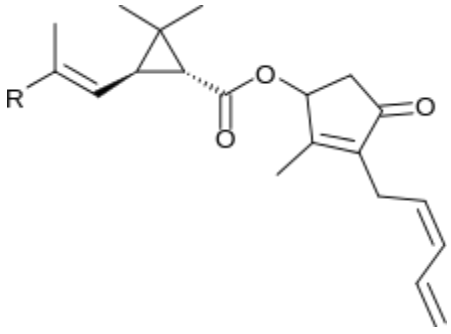
Vector control is by far the most successful method for reducing incidences of mosquito borne diseases (Murray *et al.,* 2012). Pesticides are substances or mixture of substances used to prevent, destroy, repel, attract, sterilize, or mitigate pests (Khater, 2012). The World Health Organization (WHO) estimates that 200,000 people are killed worldwide, every year, as a direct result of pesticide poisoning (Salako *et al.,* 2011). Moreover, the use of synthetic chemicals which are generally persistent in nature has been restricted because of their carcinogenicity, teratogenicity, high and acute residual toxicity, ability to create hormonal imbalance, spermatoxicity, and result in toxic residues in food (Dubey *et al.,* 2011; Khater, 2012). Further, repetitive use of synthetic pesticides has resulted in residue hazards, upsetting the balance of nature through disruption of natural enemies, pollinators and other wild life. Extensive groundwater contamination, evolution of resistance and resurgence of treated populations, outbreaks of secondary pests, that is

those normally kept under control by their natural enemies has also been observed (Dubey *et al.,* 2011). The emergence of widespread insecticide resistance and the potential environmental issues associated with some synthetic insecticides has indicated that an additional approach to control the proliferation of mosquito population is an urgent research priority (Hemingway and Ranson, 2000).

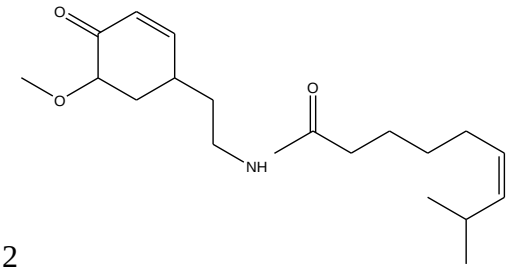
The use of natural products is one of the best alternatives for mosquito control (Murray *et al.*, 2012).The search for herbal preparations that do not produce adverse effects on the non-target organisms and are biodegradable remains a top research issue for scientists associated with alternative vector control (Chowdhury *et al*., 2008). Many plant species are known to possess biological activity that is frequently assigned to the secondary metabolites. Among these, essential oils and their constituents have received considerable attention in the search for new biopesticides. Many of them have been found to possess an array of properties, including insecticidal activity, repellency, feeding deterrence, reproduction retardation and insect growth regulation against various mosquito species (Rice and Coats, 2009).

The plant kingdom is recognized as the most efficient producer of chemical compounds, synthesizing many products that are used to defend plants against different pests (Isman and Akhtar, 2007). Botanicals have been in nature for millions of years without any adversative effects on the ecosystem. The repellency of plant material has been exploited for thousands of years by man by hanging bruised plants in houses, a practice that is still in wide use throughout the developing countries (Moore *et al,* 2006). Currently, numerous products of botanical origin, especially the secondary metabolites, have received considerable renewed attention as potentially bioactive agents for use in insect

vector management. However, there is a little other than anecdotal, traditional or cultural evidence on this topic (Grodner, 1997). Moreover, plants have also been used for centuries in the form of crude fumigants where plants were burnt to drive away nuisance mosquitoes and later as oil formulations applied to the skin or clothes which was first recorded in writings by ancient Greek, Roman and Indian scholars (Maia and Moore, 2011). Botanical pesticides are easily decomposed by a variety of microbes common in most soils and, as a result, they reduce environmental contamination. They also maintain the biological diversity of predators that are often killed by broad-spectrum synthetic pesticides (Grange and Ahmed, 1988). As a form of allelopathy, some pesiticidal plants serve as control agents for pests and diseases (Ogunnika, 2007). The Greek natural philosopher Pliny the Elder (1'st century AD) recorded all the known pest control methods in ''Natural History''. The use of powdered *Chrysanthemum* as an insecticide comes from Chinese record. The other natural products like pyrethrum (**1**), derris (**2**), quassia (**3**), nicotine (**4**), hellebore (**5**), anabasine (**6**), azadirachtin (**7**), *d*-limonene (**8**), camphor (**9**) and turpentine (**10**) were –protective (Pretty, 2006).



1 (a) Pyrethrin **I**; R= CH_3 **1** (b) Pyrethrin **II;** R = CO_2CH_3



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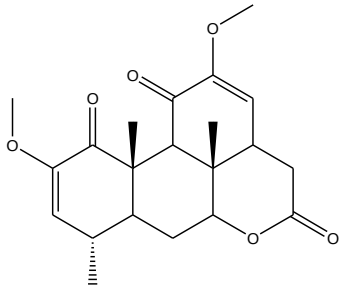

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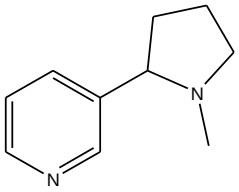

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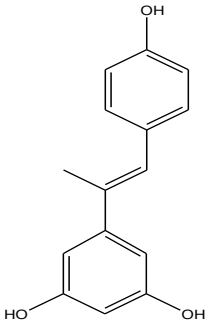

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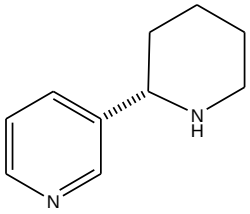

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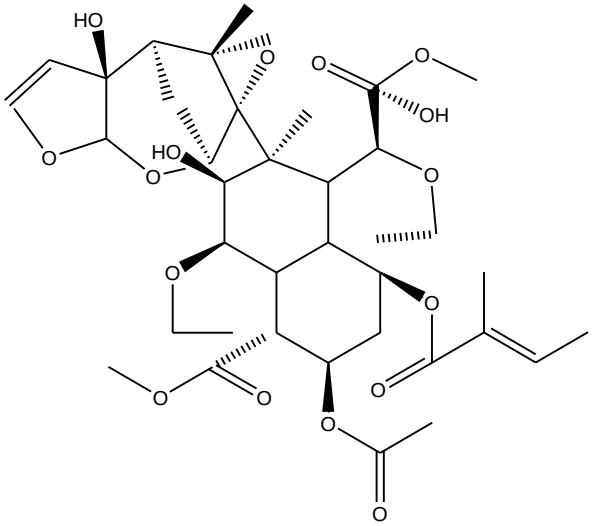

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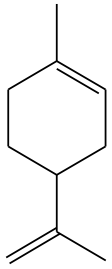

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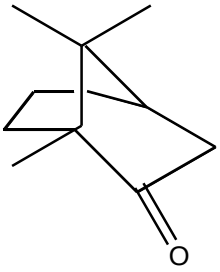

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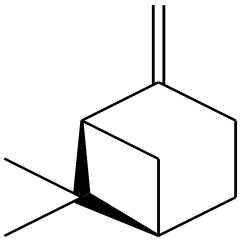

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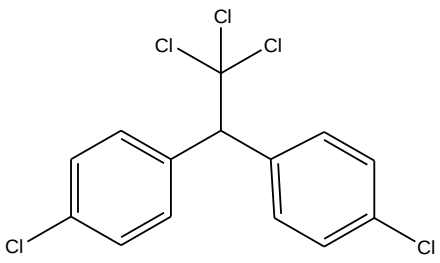
The use of synergists among some phytochemical insecticides is widely used in developed countries (Wood, 2003). The discovery of DDT's (**11**) and the subsequent development of organochlorines, organophosphates and pyrethroids suppressed natural product research as the problem of insect control was thought to have been solved (Turusov *et al.*, 2002). However, high cost of synthetic pyrethroids, environment and food safety concerns, toxicity of many organophosphates and organochlorines, coupled with increased insecticide resistance on a global scale argued for stimulated research towards potential botanicals (Shaalan *et al.*, 2005).

In light of the scanty data on insecticidal efficacy of the herbal extracts especially in the tropical regions where there are large forested land; the aim of this study was to understand the antimosquicidal efficacy of combination application using two plant species and determine possible synergistic relationships with pyrethrum and properties.



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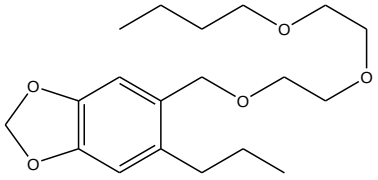
1.2 Problem Statement

Modern synthetic chemicals could provide immediate results for the control of insects or mosquitoes; on the contrary they bring irreversible environmental hazard, severe side effects and serious toxicity to human being and beneficial organisms. Another side effect is the development of pest resistance on applied agents and non-target negative environmental impact. Use of synergists should be advantageous in that, it would increase the potency of the more expensive toxicant while still retaining an effective level of insecticidal activity. Piperonyl butoxide (PBO) (**12**), *N-octyl bicycloheptane dicarboximide* (**13**) as a synergist used in pyrethrum formulations is very costly, toxic and its continuous supply is not guaranteed. This research aimed at identifying a possible replacement of PBO from naturally occurring sources of essential oils. A synergist which is cheap, readily available and environmentally friendly is needed and many botanicals have the potential.



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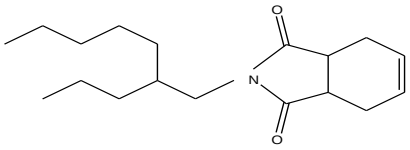

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1.3 Objectives of the study

1.3.1 Main objective

The study was to investigate the synergism of pyrethrins with essential oils from *Ocimum kilimandscharicum* and *Tagetes minuta* against *An. gambiae.*

1.3.2 Specific objectives

1. To determine the activity of pyrethrins and the crude extracts on 4th instar larvae of *An. gambiae.*
2. To compare the efficacy of pyrethrins combined with crude extracts (synergists) against conventional pyrethrum synergist; Piperonyl butoxide (PBO)
3. To determine the average volume per weight (v/w) yields, physical properties and chemical components of the synergistically active compounds.
4. Characterize the more potent using Gas chromatography-mass spectrometry (GC-MS)

1.4 Justification

The pyrethrum industry should benefit greatly from the possibility of using inexpensive additives for lowering the required concentration of the expensive pyrethrins in formulations thus, allowing wide use at favorable cost. While PBO (**12**) is an excellent general purpose synergist, its ratio to pyrethrins or pyrethroids often 5:1 or 10:1 in consumer products is high given that the cost of PBO (**12**) is in fact higher than the cost of production of pyrethrins. The future supply of PBO (**12**) is also in jeopardy. Deforestation in Brazilian rainforest has led to an environmental and political controversy. While Brazil currently supplies a significant proportion of the global safrole, the continued availability largely depended on development of a country's sound environmental policy which is unknown at this time. The rationalization of compounds

with synergistic properties in plants will add value to higher plants and make them more acceptable to the pyrethrum industry.

Moreover, new plant-based insecticidal-products obtained through research and development could diversify and increase efficiency in disease control. This will also improve and possibly expand Medicare in the country, increase economic stability to farmers and increase employment opportunities and provide industries with alternative sources of raw materials. As yet, these benefits continue to elude the country if serious thought of alternative biocides is neglected**.**

CHAPTER TWO

LITERATURE REVIEW

2.1 Synergists and Synergism

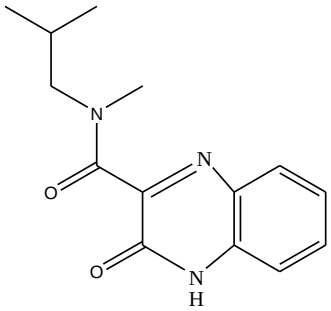
The history of insecticidal synergists originated with attempts to enhance the potency of pyrethrins (B-Benard and Philogene, 1993). An observation made in 1938 showed that *N-isobutylundecylenamide* (**14**) enhanced the insecticidal activity. This discovery initiated the use of insecticide synergists in search for better compounds. The discovery of *methylenedioxyphenyl* synergist started with realization that, the synergistic activity of sesame oil was due to the *sesamin (***15)** and *sesamolin* (**16)** components.

Synthesis and testing of related compounds led to sulfoxide, propylisome, tropital and piperonyl buoxide (**12**). *Propynyl phosphonates* and certain amides such as MGK264 *(N-Octyl bicycloheptene dicarboximide)* (**13**) were also effective. The shifting of compound for effectiveness, economics and toxicology led only to two major synergists for practical use, piperonyl butoxide and MGK264 (Casida and Quistad,1995).

Safrole (**17**) a main component of sassafras oil obtained from *Sassafras albidum*a plant of Lauraceae family is known for its synergist activity. Safrole (**17**) is the precursor for piperonyl butoxide (**12**), a synergist commonly used with pyrethrums (Dewick, 2002). The role of synergists is to increase the potency of the pyrethrum and speeds its reaction time by preventing detoxification within the insects (PESKEM, 1995).

A synergist's mode of action depends on the type of insecticides they are combined with. Piperonyl Butoxide (**12**) is the most commonly used synergist for pyrethrum (**1**) and pyrethroids and has a unique mode of action**.** Insects have in-built, complex systems that always attack an insecticide once it enters the insect body. Mixed Function Oxidases (MFO's) is one of the defense mechanisms. MFO's work by binding with the insecticide active site thereby renders it ineffective. When PBO is present in a compound, it binds with the MFO's, thus making the insecticide available to do its job. MGK 264 also has similar synergistic effects, therefore often used with natural pyrethrum (Hamilton, 1995).

It is in this view that further exploration on botanical synergists was justified. To find new synergists which would replace PBO and also supplement the cost of the same compound. This research tries to understand the antimosquicidal efficacy of combination therapy using two plant species; *Ocimum kilimandscharicum* and *Tagetes minuta* and determine possible synergistic relations



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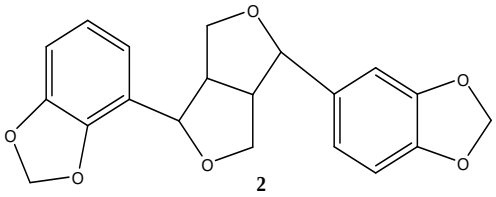

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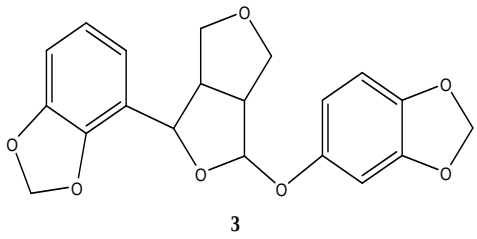

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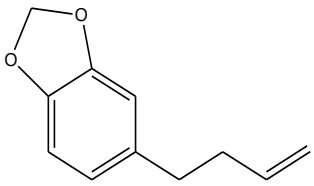


16 **17**

Figure 2.1: Structures of insecticidal synergists, 13,12,13,14, 15, 16 and 17

2.1.1 Synergists in some Botanicals and Factor of synergism

Screening of certain botanicals as pyrethrum synergists revealed that some of the extracts like obtained from the plants listed below exhibited synergism. A convenient way of expressing the increase in toxicity of synergised pyrethrins is by the use of factor of synergism,(FOS) which is usually expressed as;

$$FOS = \frac{Dose\ of\ pyrethrum\ required\ to\ kill\ \ 50\%\ \ of\ \ the\ \ insect\ (L_{50})}{Dose\ of\ the\ synergized\ mixture\ required\ to\ kill\ 50\%\ of\ insect (LD_{50})}$$

Table 2.1: Factors of synergism of different fractions obtained from various plants

S.No.	Name of the plant	Family	Fraction tested	Factor of synergism
1	*Allium sativum*	Liliaceae	P.E extract of bulb	0.87
2	*Azadirachta indica*	Meliaceae	Seed oil	0.80
3	*Boswellia Sevata*	Burseraceae	Oleoresin	0.26
			Gum `	0.23
4	*Calotropis procera*	Asclepiadaoeae	P.E extract of leaves	0.83
			MeOH extract of defatted Leaves	0.80
5	*Cirrulus colocynthis*	Cucurbitaceae	P.E extract of seeds	0.70
			MeOH extract of defatted leaves	0.61
6	*Commiphora mukul*	Burseraceae	P.E extract of resin	1.43
			Ether extract of resin	0.95
7	*Lantana camara*	Verbenaceae	Seed oil	0.54
8	*Mentha aruensis*	Labiatae	Steam volatile oil from leaves	0.94
9	*Moringa oleifera*	Moringaceae	P.E. acetone extract of bark	0.45
10	*Quisqualis indica*	Combretaceae	P.E extracts of leaves	l.l l
			MeOH extract of defatted seeds	0.92

(Pyrethrum post, 1973)

The higher the factor of synergism the better the synergist. However the factor of synergism varies with the properties of synergist in the mixture. It is therefore not possible to say that there is an optimum ratio of pyrethrum to the synergist since the effect varies with insect and the method of application (Scott *et al.,* 2004).

2.2 Synergistic phenomena of essential oils

A synergistic phenomenon among metabolites of essential oils may result in a higher bioactivity as minor constituents found in low percentages and may act as synergists, enhancing the effectiveness of the major constituents through a variety of mechanisms (Berenbaum, 1985); consequently, reducing the dose of polluting substances and the risk of developing resistance. The repellent activity of the mixture of essential oils from *Artemisia princeps* and *Cinnamomum camphora* against the adult weevils, *Sitophillus oryzae* and *Bruchus rufimanu*s was significantly higher than that elicited by individual oils (Liu *et al.,* 2006). Thyme, anise, and saffron oils have synergistic activity (Youssef, 1997).

Mixtures of different monoterpenes produced a synergistic effect on mortality, and a proprietary monoterpene mixture was developed containing 0.9% active ingredient for use against foliar feeding pests (Hummelbrunner and Isman, 2001). Essential oils act as a wood preservative solution by mixing eucalyptus essential oils with pyrethroids and borax (Urabe, 1992). Damage to cotton by the bollworm, *Helicopverpa armigera* (Hubner), can be minimized by mixtures of conventional insecticides at one half the recommended rates by combining extracts of three local plants (*Azadirachta indica, Khya senegalensis*, and *Hyptis suaveolens*) that provided greater efficacy than conventional products alone at their recommended rate. Yet, none of the plant extracts alone provided adequate crop protection (Sinzogan *et al.,* 2006). Note that negative synergism can occur between the essential oil or their components and the other ingredients present in the total formulation.

2.3 Background of the malaria problem

Malaria has been known since antiquity with recognizable descriptions of the disease was recorded in various Egyptian papyri (Nerlich *et al.*, 2008). Malaria significantly impeded progress on construction of the Panama canal early in the 20th century, adversely affected human settlement of a highly malarias area of southern Europe prior to vector control (Sallares *et al.*, 2004), and caused more casualties among allied soldiers in the south Pacific during the Second World War (WWII) than bullets. Some historians believe that the fall of the Roman Empire was partly due to the devastating effects of malaria (Bush *et al.*, 2001).

Malaria is the world's greatest parasitic killer disease and remains endemic in 102 countries with more than half of the world's population living in the tropics at risk as shown in Figure 2.2. Even the most efficacious of these control methods such as pyrethroid-treated bed nets, have proven difficult to implement on a sustainable basis for reasons of availability, acceptability, and cost (Curtis, *et al., 2000)*

Malaria is the most significant public health problem in the world today with a huge economic price for communities in areas where the disease is endemic; in terms of the cost of treatment, school absenteeism, loss of days of work, and disables many individuals greatly reducing their capacity to work (Bush *et al.*, 2005).

Malaria is usually limited to tropics and sub-tropics where it is usually more stable, difficult to control, and far harder to eradicate although severe outbreaks may occur in temperate zones where it is unstable and relatively easy to control or eradicate (Brooks *et al.*, 2004). The burden of malaria has been increasing due to development of resistance

against anti-malaria drugs and insecticides, complex social structures, and rapid environmental changes that have intensified in the last decade.

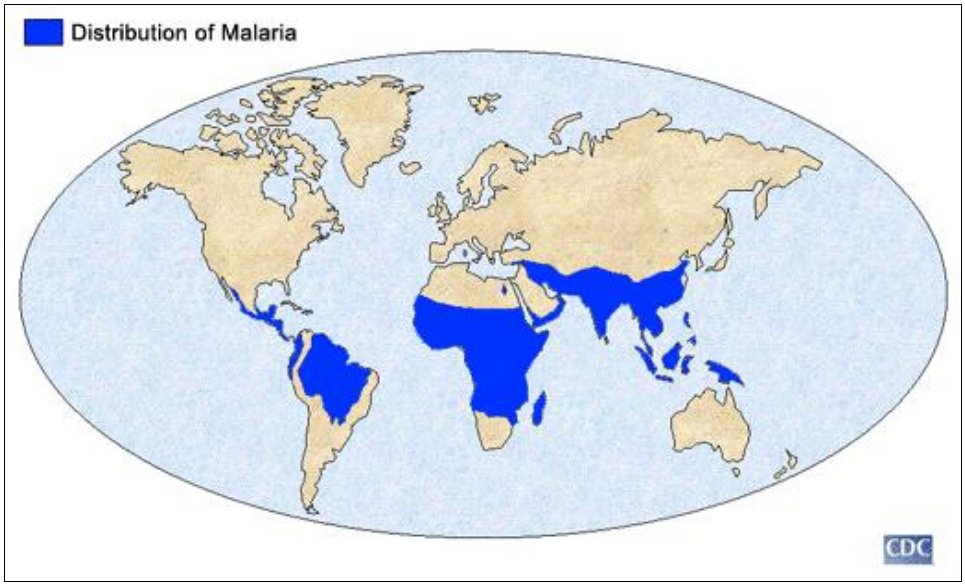


Figure 2.2: Geographic distribution of malaria in the world (adopted from CDC, 2008)

The MoH (2001) described Kenya as a prime victim of the malaria parasite by nature due to existence of a conducive climate for multiplication of the mosquito vector, which is widespread, numerous and intrusive. Besides, the demographics of Kenya expose her population to both endemic and epidemic transmission conditions. The trends in malaria burden and transmission patterns were described by MoH (2001) as varying across the country and four malaria ecological zones were identified, namely:

i. Perennial high transmission near Lake Victoria and the South Coast,

ii. High transmission with seasonal fluctuations in areas adjacent to the areas with perennial transmission,

iii. Stable transmission with seasonal peaks in most of the semi-arid and western highland regions, and

iv. Low transmission risk in the arid and mountain regions.

2.4 Malaria control strategies

Malaria control depends on elimination of mosquito breeding places, personal protection against mosquito bites, and treatment of active cases (Brooks *et al.*, 2013).

2.4.1 Prevention of malaria through vector control

Infection is prevented when malaria-carrying *Anopheles* mosquitoes are prevented from biting humans through vector control which aims to reduce contacts between mosquitoes and humans (CDC, 2008). Vector control measures range from the ancient and simple destruction of larval breeding sites; which can be through environmental control measures such as drainage of stagnant water pools, clearing bushes around homesteads, and proper disposal of empty containers that can hold water. Biological control methods such as use of pathogens and predators are also used to control larvae. Larvae can also be controlled by application of chemicals that suffocate the larvae, such as synthetic pyrethroids and natural pyrethrins.

Adult mosquito control is by judicious use of insecticides, which aims at eradicating the vector but this exercise is too expensive especially to people living in endemic areas majority of who are poor. As a result, strategies geared towards preventing mosquito bites such as sleeping under mosquito nets impregnated with insecticide (ITN) are

advocated for as the most effective and cost-effective prevention method (CDC, 2006). Besides the cost factor, synthetic chemicals have proved not to be the absolute solution to insect problems; they agreeably have a high knockout effect on pests but concern about the long term consequences of using the synthetic insecticides has arisen for several ecological reasons.

2.4.2 Prevention of malaria through case management

This involves administration of drugs to prevent disease by eliminating the parasites that are in the blood, which are the forms that cause disease. Pregnant women are the vulnerable group most frequently targeted. They may receive, for example, "intermittent preventive treatment" (IPT) with anti-malarial drugs given most often at antenatal consultations during the second and third trimesters of pregnancy. Patients with malaria should be treated promptly and correctly because malaria is often a debilitating disease that, when caused by *P. falciparum*, can be fatal (CDC, 2006).

Treatment of the sick limits chances of mosquito infection during a blood meal. This is because treatment eliminates an essential component of the cycle (the parasite) and thus interrupts the transmission cycle. The World Health Organization recommends that anyone suspected of having malaria should receive diagnosis and treatment with an effective drug within 24 hours of the onset of symptoms.

2.4.3 Development of the malaria Vaccine

Currently there is no effective vaccine to prevent *Plasmodium falciparum* malaria, although this one disease causes more than 2 million deaths annually. An effective vaccine would probably have to be directed against a single stage of the parasite's life

cycle because host immunity is stage specific, thus the vaccine being developed targets the circumsporozoite protein (CSP), which plays an important role in the sporozoite's recognition and invasion of the human host's hepatocytes (Fang *et al.*, 2002).

2.5 Use of traditional plant insecticides in malaria control

2.5.1 Pyrethrum

Pyrethrum (**1**) is the most widely and heavily used botanical insecticide worldwide and it is widely used in household aerosols for fast knockdown of pests (Olkwoski *et al.*, 1995). Flowers of *Tanacetum cinerariaefolium* (Asteraceae) are ground to a powder and then extracted with hexane or a similar non-polar solvent; removal of the solvent yields an orange-colored liquid that contains the active compounds (Casida and Quistad, 1995). The majority (> 75%) of the world's supply of pyrethrum was produced in Kenya and Tanzania, but its production began in Tasmania (Australia) in 1996 and it currently produces almost one-half of the world supply (Waceke *et al*., 2002).

Technical grade pyrethrum, the resin used in formulating commercial insecticides, typically contains from 20% to 50% pyrethrins (Casida and Quistad, 1995). Pyrethrins are common active ingredients in the insecticide products, but when used in a formulation without synergists, most of the knocked down insects recover. A synergist such as piperonyl butoxide (PBO) (**12**), derived from sassafras or *N-octyl bicycloheptane dicarboximide (***13***)*, increase insect mortality and extends the shelf life of the product. However as a synergist used in pyrethrum formulations PBO is very costly, toxic and its continuous supply is not guaranteed. This research aimed at identifying a possible

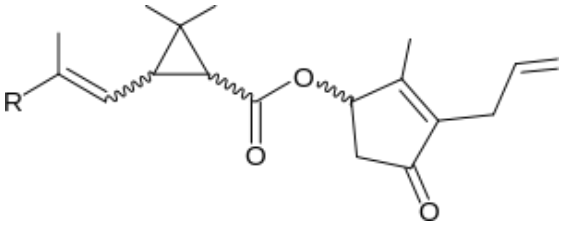
replacement of PBO from naturally occurring sources (essential oils) from flowers and leaves of plants as they are cheap and environmentally friendly.

2.5.1.1 Safety

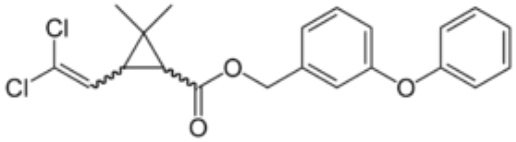
Pure pyrethrins are moderately toxic to mammals (rat oral acute LD_{50} values range from 350 to 500 mg kg^{-1}), but technical grade pyrethrum is considerably less toxic (almost 1500 mg kg^{-1}) (Casida and Quistad, 1995). The half-lives of pyrethrins on field-grown tomato and bell pepper fruits were 2 hours or less (Antonious, 2004). Natural pyrethrum has seldom been used for agricultural purposes because of its cost and instability in sunlight and this has necessitated the need for synergism.

2.5.1.2 Pyrethroids

Pyrethroids (**18a** and **b**) are synthetic chemicals designed to imitate natural pyrethrum, but some are much more toxic and long lasting (Singh and Srivasava, 1999). Sunlight does not break them down easily and they remain on leaf surfaces for weeks, killing any bypassing insect. Even though pyrethroids can be useful insecticides, they induce some deleterious effects as they irritate the human skin; some of them are extremely toxic to natural enemies, honey bees, and fish and are more harmful to the environment than pyrethrum (Dubey, 2011).



18 (a) Allethrin



18 (b) Parmethrin

2.5.1.3 Mode of action of insecticides

Pyrethrum and synthetic pyrethroids (**18**) are axonic poisons that affect the electrical impulse transmission along axons. They affect both the peripheral and central nervous system of the insect. Pyrethrum initially stimulates nerve cells to produce repetitive discharges, leading eventually to paralysis. Such effects are caused by their action on voltage gated sodium channels in synapses (Ware and Whitacre, 2004). There are two types of pyrethroids, type I have a negative temperature coefficient, similar to that of DDT (**11**); whereas Type II have a positive temperature coefficient, showing increased kill with increase in ambient temperature. Pyrethroids dominated world insecticide use from the 1980s to date, representing an example of synthetic pesticide chemistry based on botanical model. As modern pyrethroids bear little structural resemblance to the natural pyrethrins, their molecular mechanism of action differs as well (Ware and Whitacre, 2004; Dubey, 2011; Khater ,2011).

2.6 Essential oils

Essential oils are complex mixtures of volatile organic compounds produced as secondary metabolites in plants. Steam distillation of aromatic plants yields essential oils, long used as fragrances and flavoring in the perfume and food industries, respectively.

Several plant families, for example, Myrtaceae, Lauraceae, Rutaceae, Lamiaceae, Asteraceae, Apiaceae, Cupressaceae, Poaceae, Zingiberaceae, and Piperaceae, have been examined for anti- insect activities (Anupam *et al.*, 2012)

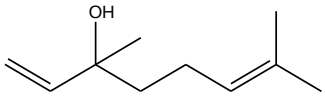
2.6.1 Biological activities of essential oils

Since the middle-ages, essential oils have been widely used for bactericidal, virucidal, fungicidal, parasiticidal, insecticidal, medicinal, and cosmetic applications, especially in the pharmaceutical and sanitary industry (Bakkali *et al.*, 2008). Aromatic plants produce many compounds that are insect repellent or act to alter insect feeding behavior, growth and development, ecdysis and behavior during mating and oviposition (Chang and Cheng 2002).

2.6.2 Chemistry of essential oils

Monoterpenoids (90% of the essential oils) have a great variety of structures with diverse functions. They are ten carbon hydrocarbon compounds or related compounds such as acyclic alcohols (e.g. linalool (**19**)), geraniol (**20**), citronellol (**21**)), cyclic alcohols (e.g menthol (**22**), isopulego (**23**), terpeniol (**24**)), bicyclic alcohols (e.g. borneol (**25**), verbenol (**26**)), phenols (e.g. thymol (**27**), carvacrol (**28**)), ketones (corvine (**29**), menthone (**30**), thujone (**31**)), aldehydes (citronellal (**32**), citral (**33**)), acids (e.g. chrysanthemic acid (**34**) and oxides (cineole (**35**)). The main group is composed of terpenes and terpenoids and the other of aromatic and aliphatic constituents all characterized by low molecular weight terpenes mainly the monoterpenes (C10) and

sesquiterpenes (C15), but hemiterpenes (C5), diterpenes (C20), triterpenes (C30) and tetraterpenes (C40) also exist (Tripathi *et al.*,2009).



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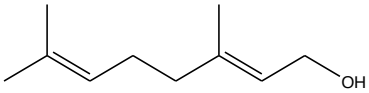

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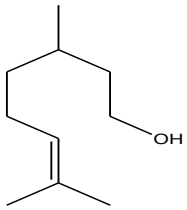

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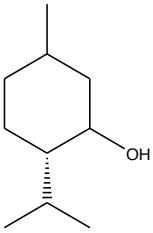

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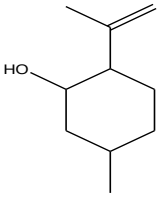

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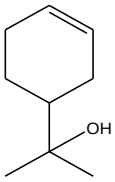

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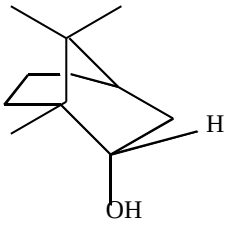

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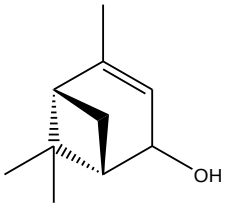

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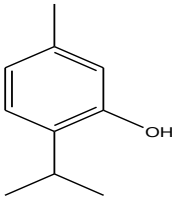

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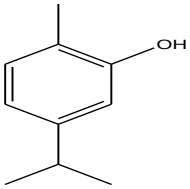

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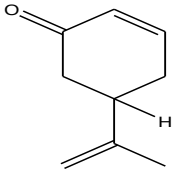

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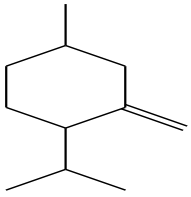

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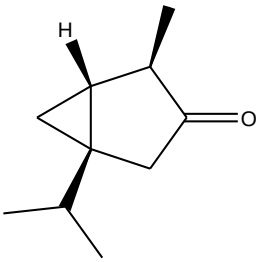

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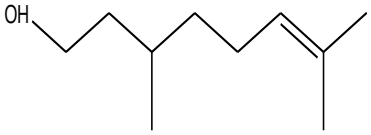

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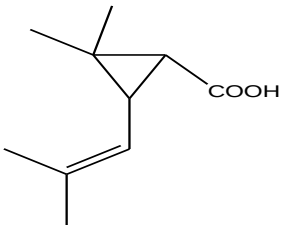

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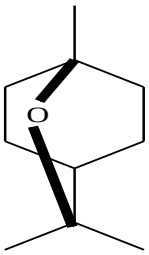

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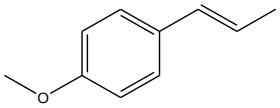
Aromatic compounds occur less frequently than the terpenes and are derived from phenylpropane eg aldehyde: cinnamaldehyde; alcohol: cinnamic alcohol; phenols: chavicol, eugenol methoxy derivatives: anethole (**36)**, elemicine **(37**), estragole (**38**), methyl eugenols (**39**) methylene dioxy compounds: apiole (**40**), myristicine (**41**), safrole (**17**) (Isman, 2006; Tripathi *et al*., 2009).



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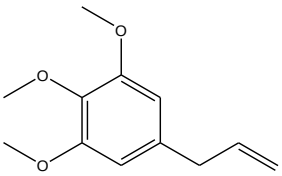

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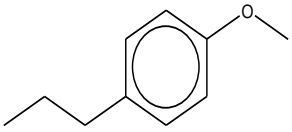

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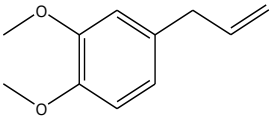

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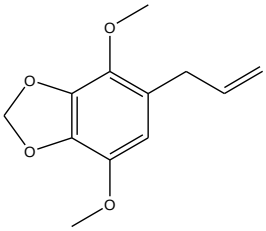

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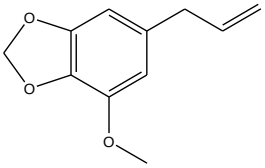

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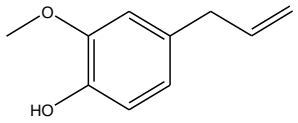
The composition of these oils can vary dramatically, even within species according to the part of the plant from which the oil is extracted (leaf tissue, fruits, stem, etc.), the phonological state of the plant, the season, the climate, the soil type, and other factors, for example, rosemary oil collected from plants in two areas of Italy were demonstrated to vary widely in the concentrations of two major constituents, 1,8-cineole (**35**) (7% to 55%) and α-pinene (**10**) (11% to 30%) (Flamini *et al.*, 2002). Such variation is common and has also been described for the oils derived from *Ocimum basilicum* (Pascual-Villalobos and Ballesta-Acosta, 2003*)* and *Myrtus communis* (Flamini *et al.*, 2004).

2.6.3 Mode of action of essential oils

Essential oils interfere with basic metabolic, biochemical, physiological, and behavioral functions of insects. Insects inhale, ingest or absorb essential oils. The rapid action against some pests is indicative of a neurotoxin mode of action, and there is evidence for interference with the neuromodulator octopamine (Enan, 2005) or GABA-gated chloride channels (Priestley *et al.,* 2003; Khater, 2011). Some essential oils have larvicidal effects and the capacity to delay development and suppress emergence of adults of insects of medical and veterinary importance (Mazyad *et al.,* 1999; Khater, 2003; Shalaby and Khater, 2005; Khater and Shalaby, 2008; Koul *et al.,* 2008; Zhu *et al.,* 2008; Khater and Khater *et al.,* 2009, 2011; Khater, 2011; Kumar *et al.*,2011). Thyme oil and monoterpenoids including thymol (**27**), anethole (**36**), eugenol (**42**) and citronellal (**22**) combinations have been patented for pesticidal activity against cockroaches and the green peachaphid (Bessette & Beigler, 2005 & 2008; Ninkov, 2007). Similarly, citronellal (**32**),

citronellol (**21**), citronellyl (**43**) or a mixture of these has been patented as pest treatment composition against the human louse (Ping, 2007).



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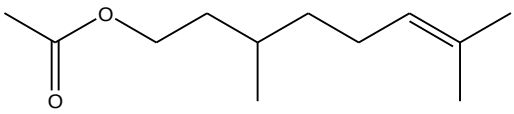

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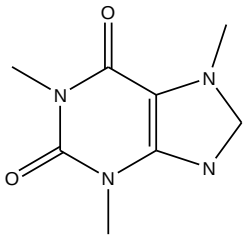
Some essential oils express bioactivity against stored product pests, such as oils of basil, citrus peel, eucalyptus, various mint species, lavender, and rosemary, but not all essential oils are active against all the insect pests (Don-Pedro, 1996; Papachristos & Stamopoulos, 2002). Nutmeg oil has been determined to significantly impact both the maize, *Sitophilus zeamais* and the red-flour beetle, *Tribolium castaneum* and demonstrates both concentration dependent repellent and fumigant properties (Huang *et al.*, 1997). The exact mode of action of these oils as fumigant is unknown, but the oils mainly act in the vapour phase via respiratory system.

2.6.4 Essential oils as chemosterilants

Some of the essential oils and their components are **c**hemosterilants which induce sterility in insect pests. Alkaloids isolated from *Annona squamosa* have shown larvicidal growth regulating and chemosterilant effects against *Anopheles stephensi* at concentrations of 50 to 200 ppm (Saxena *et al.,* 1993). A compound 1, 3, 7-trimethylxanthine (**44**), was isolated from seed extract of *Coffee Arabica* and it proved effective as a chemosterilant

for *Callosobruchus chinensis*, causing nearly 100% sterility at a concentration of 1.5% (Rizvi *et al.*,2009).



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At similar concentration, the compound had no phytotoxic effect on the crop plant *Vigna mungo*. Possible use of the compound for control of stored-grain pests is suggested (Rizvi *et al.*, 1980). The compound β-asarone extracted from rhizomes of *Acorus calamus*, possesses antigonadial activity causing the complete inhibition of ovarian development of different insects (Varma and Dubey, 1999). It is important to note that chemosterilants are important in integrated pest management programs to break the life cycle of pests and to reduce the occurrence of pest resistance.

2.6.5 Improving the efficacy of essential oils

The activity of an essential oil is affected by the salinity and pH. A low pH and salinity (5% NaCl) can potentiate the activity of the product (Lachowicz *et al.*, 1998). The effectiveness of a mosquito repellent in a topical application that contains oil of lemongrass, *Cymbopogon citratus*, was tested on different formulations. Efficacy decreased in the order hydrophilic base > emulsion base > oleaginous base (Oyedele, *et al.*, 2002).

Oil of Indian prickly ash, *Zanthoxylum limonella*, was successfully microencapsulated in glutaraldehyde crosslinked gelatin (a polymer), in order to improve mosquito repellent properties (Maji *et al.,* 2007). Although natural active ingredients are some times more expensive than synthetic products, the essential oil of the Japanese pepper tree, *Zanthoxylum piperitum* is only one example for which there is a potential to be used on the development of combined repellents, especially in situations when DEET is ineffective and impractical (Kamsuk *et al.,* 2007). Citronellal (**32**) and geraniol (**20**) candles are widely sold as outdoor repellents as continuous evaporation of volatile prolongs their repellency.

However, field studies against mixed populations of nuisance mosquitoes show reductions in biting around 50%, although they do not provide significant protection against mosquito bites (Jensen *et al.,* 2000; Müller *et al.,* 2008). In order to increase the repellency of essential oils, some fixative materials such as liquid paraffin, vanillin, salicyluric acid, mustard, and coconut oils have been used. Formulations based on creams, polymer mixtures, or microcapsules for controlled release, resulted in an increase of repellency duration. Essential oils can also be incorporated with polymers into sheets. Attractant adhesive films with essential oils were prepared to control insects (Nerio *et al.,* 2010; Khater, 2011). Furthermore, improving formulations of plant oils increase their longevity through development of nanoemulsions (Nuchuchua *et al.,* 2009), in addition to studying spatial repellency (Bernier *et al.,* 2005) and excitorepellency (Noosidum *et al.,* 2008)

2.6.6 African blue basil (*Ocimum kilimandscharicum)*

2.6.6.1 Local names

This plant is also known as *okita* among the Luo community. (Kokwaro,1976).

Figure 2.3: Aerial parts or *Ocimum kilimandshcaricum*

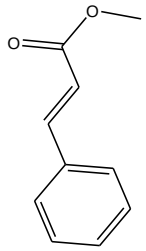
http://www.herbgarden.co.za/mountainherb/herbinfo.php?id=284, 9 10am

2.6.6.2 Distribution of *Ocimum kilimandscharicum*

The genus *Ocimum*, (Lamiaceae formerly Labiatae), collectively called basil has long been recognized as a diverse and rich source of essential oils. *Ocimum* contains between 50 to 150 species of herbs and shrubs from the tropical regions of Asia, Africa, and

Central and South America. Plants have square stems, fragrant opposite leaves, and whorled flowers on spiked inflorescences.

The essential oils of basil extracted via steam distillation from the leaves and flavoring tops are used to flavor foods, dental and oral products, in fragrances, and in traditional rituals and medicines. Extracted essential oils have also been shown to contain biologically-active constituents that are insecticidal, nematicidal, fungistatic or which have antimicrobial properties. These properties can frequently be attributed to predominant essential oil constituents, such as methyl chavicol (Estragole (**38**) , eugenol (**41**), linalool (1**9**), camphor (**9**), and methyl cinnamate (**45**) (Simon *et al.,* 1990)



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Ocimum kilimandscharicum is one of the species of the genus *Ocimum* plant that is native in East Africa. It is an evergreen aromatic perennial shrub belonging to the *Lamiaceae* family. It thrives as a natural rounded woody shrub that can reach 2 m high in warm temperate regions of the tropics and can be propagated both by seeds and vegetative. The plant has pubescent quadrangular branchlets with simple leaves that are opposite and

oblong, narrow at the base and deeply serrated, and contain aromatic oils, which is the essence of the plant (Lwande, 2009).

2.6.6.3 Biocidal activities of *Ocimum kilimandscharicum*

Camphor (**9**) is a major component of essential oil of *O. kilimandscharicum*. The biological activity of camphor against the beetles, *Sitophilus granarius, S. zeamais, Tribolium castaneum* and *Prostephanus truncatus*, has been evaluated by Obeng-Ofori *et al.* (1998) and found camphor to be effective when used in contact toxicity, grain treatment and repellency assays. According to their experimental findings, camphor applied either topically, impregnated on filter papers or whole wheat and maize grains was highly toxic to all the four species. Another study done by Kweka *et al*. (2008) also reported that EOs from *O. kilimandscharicum* and *O. suave* have remarkable knockdown effects (30-50%) when used as repellent against *Anopheles arabiensis, An.gambiae* and *Culex quinquefasciatus.* (Seyoum *et al.,* 2006).

Phytochemicals obtained from plants with proven mosquito control potential can be used as an alternative to synthetic insecticides or along with other insecticides under the integrated vector control. System plant products can be used, either as insecticides for killing larvae or adult mosquitoes or as repellents for protection against mosquito bites, depending on the type of activity they possess. A large number of plant extracts have been reported to have mosquicidal or repellent activity against mosquito vectors (Sukumar *et al.*, 1991), but very few have shown practical utility for mosquito control.

Plant products can be obtained either from the whole plant or from a specific part by extraction with different types of solvents such as aqueous, methanol, chloroform, hexane, etc., depending on the polarity of the phytochemicals. Studies carried out so far have shown that some phytochemicals act as general toxicant (insecticide/ larvicide) both against adult as well as larval stages of mosquitoes, while others interfere with growth and development (growth inhibitors) or with reproduction chemosterilant) or produce olfactory stimuli thus acting as repellent or attractant. (Noosidum *et al.,* 2008).

They have been shown to have insecticidal, nematicidal, fungistatic or and antimicrobial properties (Casals *et al.*, 2003) These properties can frequently be attributed to predominant essential oil constituents, such as methyl chavicol (**38**), eugenol (**42**) linalool(**9**), camphor, (**9**) and methyl cinnamate (**45**).

2.6.7 Marigold (*Tagetes minuta*)

2.6.7.1 Local names

The plant is also known as *Abuba or Anyach* among the Luos, *Igaswende or Inavuzaga* among the Luyha and *Chemusora* among Kalenjin communities (Kokwaro, 1976).

Figure2.4: *Tagetes minuta* flowers

http://www.flickr.com/people/32005048@N06 9.30am

2.6.7.2 Distribution of *T.minuta*

Tagetes minuta, also known as Southern Cone Marigold, Stinking Roger or black mint, (Tyagi, *et al.,* 1994) is a tall upright marigold plant from the genus with small flowers, native to the southern half of South America.

2.6.7.3 Biocidal activities of *Tagetes minuta*

Tagetes species belonging to a family *Asteraceae* commonly known as marigold has shown both larvicidal as well as adulticidal activity against mosquitoes (Pathak *et al.,* 2000). (Green *et al.,* 1999) reported mosquito larvicidal activity in the extract of *Tagetes minuta* flowers. (Perich *et al.,* 1994) compared biocidial activities of the whole- plant extract of the three *Tagetes* species and showed that *T. minuta* had the greatest biocidal effect on the larvae and adult of *Ae. aegypti* (L) and *An. stephensi* (L).

Bioassays of simultaneous steam distilled extract of *T. minuta* flowers showed larval mortality at LC_{90} of 4 and 8 ppm and against the adult at 0.4 and 0.45% against *Ae. Aegypti* and *An. stephensi*, respectively, (Perich *et al.,*1994). The extract of *T. minuta* was found to be most active among 83 plants species belonging to the compositae family with LC_{50} of 1mg /L against *Ae. fluviatilis*. Active component of *T.minuta* has also been identified as thiophene derivatives, a class of compounds present in many plants in the family of asteraceae.

Mosquito larvicidal activity in the extract of *T. minuta* flowers. (Perich *et al.,* 1994) compared biocidial activities of the whole- plant extract of the three *Tagetes* species and showed that *T.minuta* had the greatest biocidal effect on the larvae and adult of *Ae. aegypti* (L) and *An. stephensi* (L).

2.7 Summary on plants with synergism potential

ln view of the compounds employed for synergism it was evident that plants described above held the potential in their chemical constitution. An extensive relation between compounds that bear a **methylenedioxy phenyl** group, a basic framework of chemical structure within which

Likelihood of the compound with synergistic activity is predicted and plants that do produce this compounds aid in restricting these compounds to some plant families such as Lauraceae, Rutaceae. Umbelliferae etc. Special considerations on the choices of plants for this study had been made, bearing in mind the commercialization aspect of the Pyrethrum Board of Kenya's objectives.

CHAPTER THREE

MATERIALS AND METHODS

3.1 Field study sites

A part from pyrethrum that was obtained from Pyrethrum Board of Kenya, the rest of the plant species were obtained from Kipkaren subcounty in Nandi County, situated in the western part of Rift Valley Province. The County borders Kakamega to the North-West, Uasin Gishu district to the North-East, Kericho district to the South-East, Kisumu district to the South-East, and Vihiga district to the West. It lies within latitudes 0º and 0º34' North and longitudes 34º44' and 35º25' East and covers an area of 2873km^2 with a total population of 631,357 (GOK**,** 2009c).Nandi has a cool and moderately wet climate, average annual rainfall is between 1200mm to 2000mm (GOK**,** 2009c**).**

Long rains occur in early March and continue up to end of June, while short rains occur from mid September to end of November. Dry spell is normally experienced between December and March, but there is no month when the district records virtually no rainfall. Highest average rainfall is in Kobujoi and Tindinyo areas to the southwest, and lowest in eastern and northeastern part. This distribution is determined by◦ topography and influence of the southwesterly winds from Lake Victoria. Most parts have mean temperatures between 18-22º C during the rainy season, higher temps average 23º C during drier months of December and January. Coolest temps during cold spell of July and August as low as 12º C. Highest average 26º C recorded only to the southwest area in the neighborhood of Nyando escarpment lying 1300m above sea level **(**GOK, 2009c**).**

3.2 Collection of Plant specimens

The plant specimens were obtained from Kipkaren in Nandi County, which is situated in the western part of Rift Valley Province. It borders Kakamega to the north-west, Uasin Gishu County to the north-east, Kericho County to the south-east, Kisumu County to the south-East, and Vihiga County to the west.

Specimen pyrethrins (oils) samples were obtained from pyrethrum Board of Kenya factory, located in Nakuru. Fresh aerial parts of full-bloom *T. minuta* (flowers) and *O.kilimandscharicum* (leaves) were collected and packed in black polythene bags to prevent further photosynthesis and acquisition of artifact (chemicals acquired from surrounding specially from sunlight). Also to prevent volatilization and leaf damage, so as to reach the laboratory undistorted for identification. Collected plant materials were taken to the University of Eldoret herbarium for identification.

3.3 Preparation of extracts

Fresh leaves of the *O. kilimandscharicum* and *T. minuta* flowers were weighed separately, one kilogram (1000g) each, and were ground using a pestle and a mortar**.** The ground leaves and flowers were placed in round bottomed flask. A liter of water was added to each and the mixtures steam distilled for a period of four hours continuously using Clevenger apparatus in Appendix I.

The solvent was removed by decantation. The resulting crude PE was dried using anhydrous sodium sulphate, re-weighed and then kept at -20ºC until testing for their

synergistic property against *Anopheles* mosquito. After extractions of each single species, the plant species extracts were combined with pyrethrins in the ratio of 1:1 in different proportions of (0.005, 0.001, 0.002, 0.003) ml ready for larvicidal and adulticidal test (synergistic tests).

3.4 Experimental Sites

The experiments were conducted in the laboratories of CDC/KEMRI in Kisumu. KEMRI/CDC Field Research Station is located in Kisian area near Kisumu City on the KEMRI Centre for Global Health Research (CGHR) campus. The Field Research Station is located in an area of western Kenya where *P. falciparum* malaria and HIV are major public health problems.

3.4.1. Collection and rearing of *Anopheles gambiae s.s.*

For this study the test mosquito species chosen was *Anopheles gambiae*. It is the principal mosquito vector of human malaria in tropical Africa, and in Western Kenya and together with *An. funestus*, the most abundant in the latter area (Ndiath *et al*., 2014). Mosquito eggs of *A. gambiae* s.s was collected from CDC/KEMRI Kisumu and hatched in spring water in trays at room temperature in CDC/KEMRI laboratories.

To synchronize and promote hatching, larval food was added to the water 24 h before introducing the eggs as stipulated the WHO (2005a) guidelines. 1000 mosquito larvae were fed on diet with quarter (1/4) finely ground brewer's yeast tablet per day per trough, to avoid strong bacterial growth (which kills the larvae), and the water was

replaced with fresh spring (chlorine free) water after every two days to prevent turbidity and scum that would cause contamination WHO (2005a). Replacement of larvae was done picking out the larvae using a dropper and then transferring them to a clean container with clean water and food, and the amount of food was increased with increasing size of the larvae. On pupation, pupae was put in a separate tray and placed in a cage covered by a mosquito netting material so that when adults emerge, they would not escape and cause a health hazard (WHO, 2005a).

Adult mosquitoes were managed in mosquito netting cage using the methods used by Ghosh *et al.*, (2012). A 10% sugar solution in a Petri dish was placed in the cage as food for the adults, especially the male mosquitoes which naturally feed on nectar. A rabbit in a smaller wire meshed cage was placed in the mosquito netting cage to provide mammalian blood meal for adult female mosquitoes. The rabbit was sheared on the bark to make it easy for the female mosquitoes to feed.

The larvae were fed on brewer's yeast in form of dry powder on a daily basis. Water was added daily to replenish loss due to evaporation. The rearing water was changed every other day to avoid any contamination from waste, bacterial and algal growth stimulated by excess yeast (larval food). Eight days later pupae formed from 4^{th} instar larvae, was picked from the pans using a dropper, and placed in 200 ml paper cups (diameter 7 cm, height 4 cm) each containing 150 ml of distilled water. They were then dispensed in screened breeding cages (30 cm x 30 cm x 30 cm) with fine netting material where adults emerged 24–48 hours later, forming the mother colony. At least 500 adults were reared in each cage at a temperature range of 24-34^{0}C and a relative humidity of 70-80%, while

being continuously fed on 10% ordinary sugar solution provided on wet cotton wool in plastic jars.

During 24–48 hours of post emergence period female adults were separated from males, mating having taken place immediately after emergence (Clements, 1992). These females formed the experimental mother colony. On day 5 post–emergence the females were deprived of sugar feeding for 12 hours, then blood fed on a rabbit. Three blood meals were given to the female mosquitoes. Three (3) days after 1st blood feeding of oviposition substrate (filter paper on wet cotton wool in a Petri dish) were provided in each (mother colony) cage.

3.5 Test for Synergism

3.5.1 Larvicidal bioassay

Methods for testing larvicidal action of the crude extracts were slightly modified from those of World Health Organization (WHO, 1996). A stock solution was prepared by dissolving a known amount (20ml) of the crude extract in 5ml solvent (DMSO) and stored in a refrigerator at 15ºC. Twenty healthy, late 3rd-4th instar larvae was introduced into each testing cup (sterilized plastic drinking cup of 150 ml capacity), which contained 100 ml of distilled water. A measured volume of stock solution (i.e essential oil solutions from the two plants mixed with 0.2% pyrethrins in the ratio of 1:1) was added to obtain the desired concentrations. Experiments was carried out with a series of four concentrations,(10ppm,20ppm 30ppm and 40ppm) each with 3 replicates, with a final total number of 80 larvae for each concentration.

Each batch of replicates contained distilled water (negative control) pyrethrin and pyrethrum-formulated (with PBO, positive control) at the same concentrations as the test solution as treatment standard made of plant extracts in each case. As very few larvae succumbed within an hour of exposure to the test solutions, mortality was recorded after 1, 3, 6, 9, 12 and 24 hours exposure, during which no food was offered to the larvae. The mortalities of mosquito larvae were recorded when moribund larvae were incapable of rising to the surface or of showing the characteristic diving reaction when the water was disturbed or showed discoloration, unnatural position or rigor. Control mortality was accounted for by the formula of Abbott (1925).

3.5.2 Bioassay on adult mosquitoes

The WHO cones were used to evaluate the synergistic activity of the compounds. Twenty adult *An. gambiae* 3-4 days old, were introduced to the test chamber whose surfaces (filter paper) had been smeared with pretreated solutions at defined concentrations (the test compounds and 0.2% dry weight of pyrethrins were mixed in the ratio of 1:1).These concentrations were used until 100% mortality was achieved. The knocked down mosquitoes were removed, provided with food, water, and kept in a cage while the percentage kill was determined after 24 hours. Solutions of pyperonyl butoxide and pyrethrins with same concentration as test solutions were used as standards in each case.

3.6 Separation and identification of the plants components using GC-MS

The composition of the essential oils was determined using an Agilent 7890A Gas Chromatography – Mass Spectrometry instrument from Government chemist- Nairobi. Helium at 25cm/sec (0.73ml/min) was used as a carrier gas, and hydrogen was used for the flame. The GC conditions used were as follows: capillary column; fused silica (polydimethylsiloxane, 0.25 μm film thickness); temperature program: 70 °C for 8 min, 75 – 230 °C for 3 min, 230 – 240 °C for 5 min, 270 °C 5 min; carrier gas, Helium at 5 bar, linear velocity of 25cm/sec (0.73ml/min) ; injection port splitless at 250 °C; injection volume, 0.1 μL. The MS conditions were as follows: ionization EI at 70 eV; m/z range, 30-300 °C; scan rate 1 sec^{-1}; ionization chamber at 180 °C; and transfer line at 280°C. The identification of the essential oil constituents was based on a comparison of their retention times, and these constituents were further identified and authenticated using their MS library search (NIST and WILEY), and by comparison with MS literature data (Adams, 2007).

3.7 Factor of synergism determination

The relative toxicity of the pyrethrins synergized with the various plant extract was expressed using factor of synergism (FOS) as follows the values were obtained from probit graphs.

$$FOS = \frac{\textit{Dose of pyrethrum required to kill 50\% of the insect } (LD_{50})}{\textit{Dose of the synergized mixture required to kill 50\% of insect } (LD_{50})}$$

The interpretation being that, the higher the FOS the higher the potency of the formulation.

3.8 Determination of physical properties of the oils

The colour and odour of the plants obtained were observed while their densities and boiling points were also determined. To determine the boiling point, the synergistic compound was introduced using a pipette into a 5-mm glass tubing sealed at one end, and attached to a thermometer using a rubber band as described by Pavia *et al* (1982). A short piece of capillary tubing with one end sealed was doped, with the open end facing down, into the oil in the 5-mm glass tubing then the whole unit was placed in liquid paraffin contained in a hard glass beaker (Pavia *et al.*, 1982).

The liquid paraffin was heated until a rapid and continuous stream of bubbles emerged from the inverted capillary tube as described by Pavia *et al,* 1982. The boiling point was recorded as the temperature corresponding to the time the oil entered the inverted capillary tube as stipulated by Pavia *et al,* 1982. The densities of the compound were determined using 5 ml density bottles. The density bottles were first weighed while empty using an analytical weighing balance (0.001g) then filled with the plant extract separately and weighed. Mass of the plant extract was obtained by calculating the difference between mass of the plant extract that filled density bottles and empty bottle weight.

3.9 Data analysis

The bioassay data on mortality were corrected for control group deaths using Abbott's formula (1925) and subjected to regression and probit tests (Finney, 1952). Values of the median lethal doses were determined as fitted from probit and regression graphs. The

model of efficacy ranges for the chemicals was fitted using GENSTAT (GenStat Release 4.24DE). Model fit was based on residual likelihood ratio chi-square statistic.

CHAPTER FOUR

RESULTS AND DISCUSSION

4.1 Results

4.1.1 Physical properties

Colour, smell, solubility and boiling point of essential oil of *O. kilimandscharicum* were pale yellow, cinnamon scent (intense scent) slightly soluble in water and boiling point of 253^{o}C respectively. Percentage yield or volume per weight (v/w) yield of the essential oil and density was also determined as show in the Table 4.1

Table 4. 1: Oil Percentage yield for *O. kilimandscharicum*

Weight of round bottom flask I	96.2705g
Weight of round bottom flask I + plant specimen	1096.2705g
Weight of plant specimen	1000g
Weight of vial	53. 2657g
Weight of vial + oil	57.2657g

Weight of plant specimen = 1000.00g (1kg)

Percentage yield of oil = 4g / 1000g x 100%

= 0.4%

The density of the oil was determined as shown;

Weight of empty density bottle		= 25.000g
Weight of empty density bottle + Oil		= 25.928g
Weight of oil	(25.928— 0.928)	= 0.928g

(Density of equal volume of water is $1g/cm^3$)

Density of oil is therefore = $0.928g/cm^3$

The above results showed that the density of essential oil of *O. kilimandscharicum* was 0.928 g/cm3 at 25^0C and volume per weight (v/w) yields of 0.4%. Another study by (Hossain, *et al.*, 2011) also gave similar results.

Tagetes oil had a wild, sweet, fruity almost citrus-like smell, yellow to reddish-amber in color. Had medium viscosity that forms gel-like substance if exposed to the air for a long. The oil was slightly soluble in water, and had a boiling point of 163°C. Density and volume per weight (v/w) yields were also determined as shown in the Table 4.2

Table 4. 2: Oil Percentage yield for *T. minuta*

Weight of round bottom flask I	96.2705g
Weight of round bottom flask I + plant specimen	1096.2705g
Weight of plant specimen	1000g
Weight of vial	53. 2657g
Weight of vial + oil	53.g

Weight of plant specimen = 1000.00g (1kg)

Percentage yield of oil = 1.8 / 1000g x 100%

= 0.18 %

Density of the *Tagates* oil was also determined as shown;

Weight of empty density bottle = 25g

Weight of empty density bottle + Oil = 25.801g

Weight of oil (25.810— 25.0) = 0.810g

(Density of equal volume of water is 1g/cm^3)

Density of oil is therefore = 0.810 g/cm^3.

Results showed that *Tagetes* had a density of 0.810 g/cm^3 and volume per weight (v/w) yields 0.18 %. This values were close to 0.868 g/ cm^3 according to another study by (Merck, *et al.*, 1996).

4.1.2 Larvicidal bioassay

Results presented in Table 4.3 shows that larval mortality varied greatly as varied plants crude extracts were used.

Table 4.3: Effect of combined plant crude extracts with pyrethrins in ratio 1:1 at conc. of 0.01mg/ml on larvae compared to pyrethrins alone

Tests solutions	Time(hrs)	Number Exposed	Number Dead			Mean	% Mort
			Rep.1	Rep.2	Rep.3		
Pyrethrin (Control)	1	20	15	16	14	15	75
	3	20	18	19	18	17.3	91.6
	6	20	20	20	20	20	100
	9-24	20	20	20	20	20	100
Pyrethrins and *Ocimum*	1	20	17	18	17	17.3	86.7
	3	20	20	20	20	20	100
	6	20	20	20	20	20	100
	9-24	20	20	20	20	20	100
Pyrethrin and *T. minuta*	1	20	9	10	8	9	45
	3	20	17	17	16	16.7	83.5
	6	20	20	20	20	20	100
	9-24	20	20	20	20	20	100

Results on comparative effect of different concentrations of the synergistic compounds on mortality of mosquitoes were presented in table 4.4. The performance was further depicted in Figure 4. 1. Bioassay data was evaluated by regression.

Table 4.4: Different concentrations of the synergistically active compounds compared with pyrethrins during a period of 1hr till 24hours

Tests	**Concentration**	**Number Exposed**	**Number Dead**			**Mean**	**% Mort**
			Rep.1	Rep.2	Rep.3		
Dist.Water(N-control)	0	20	0	0	0	0	0
Pyrethrins	0.03	20	20	20	20	20	100
Pyrethrins	0.02	20	14	15	13	14	70
Pyrethrins	0.01	20	11	10	12	11	55
Pyrethrins	0.005	20	5	6	7	6	30
Pyrethrins with A	0.03	20	20	20	20	20	100
Pyrethrins with A	0.02	20	19	18	17	18	90
Pyrethrins with A	0.01	20	15	16	16	15.6	78
Pyrethrins with A	0.005	20	11	10	12	11	55
pyrethrin withB	0.03	20	20	20	20	20	100
pyrethrin with B	0.02	20	14	13	13	13.3	67
pyrethrin with B	0.01	20	10	11	11	10.6	53
pyrethrin with B	0.005	20	6	7	6	6.3	32

KEY: A----*O. kilimandscharicum* B------*T.minuta*

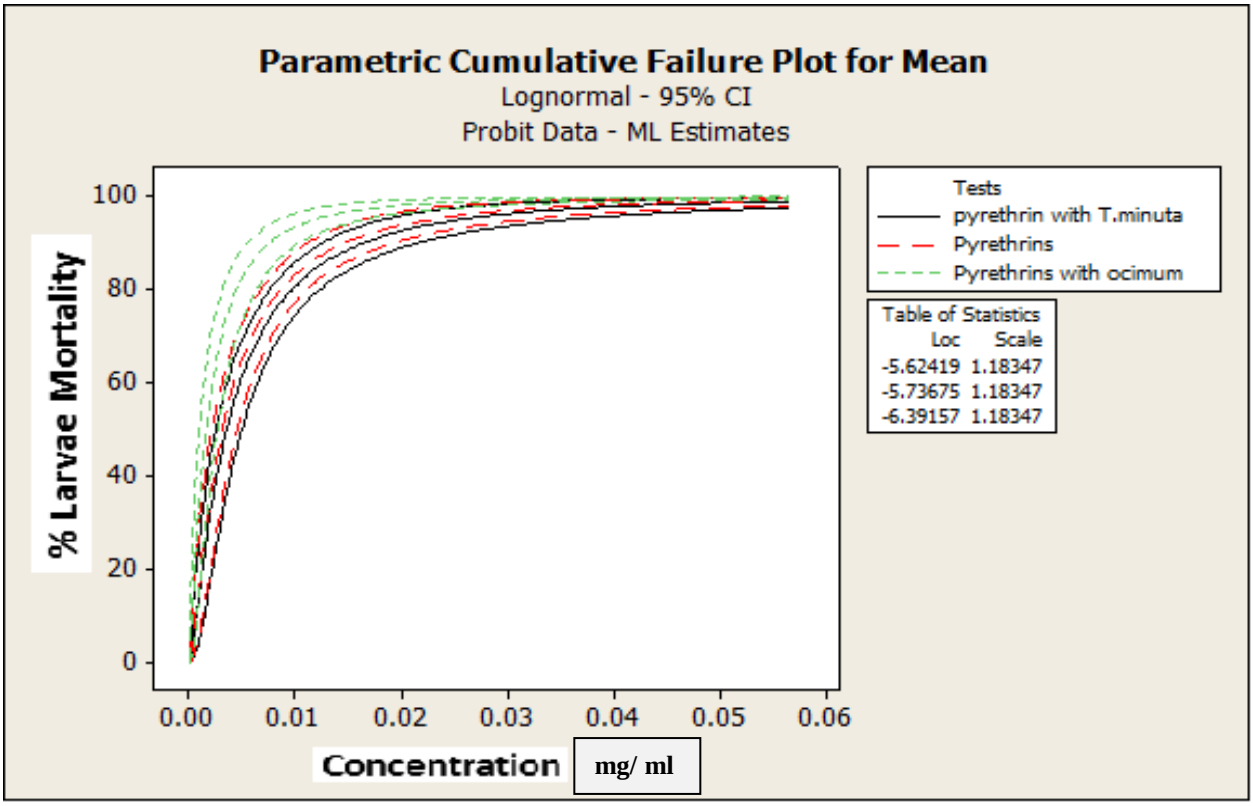


Figure 4.1: % larvae mortality against concentration of synergists

4.1.3 Bioassay on adult mosquito

The results on comparative effects of the two synergists on the knockdown and mortality rates on 3-4 days old adult mosquitoes were shown in Table 4. 3.

Table 4.5: Knockdown and. Mortality rates for different concentrations of the synergized mixtures/solutions on Adult An. *gambiae* in the first 3minutes till 24hours

Test mixture	Conc. mg/ml	No. Exp	KD Rep1	KD Rep2	KD Rep3	Mean	%	Mort Rep1	Mort Rexp2	Mort Rep3	Mean	%
Py	0.1	20	14	15	15	14.7	73	20	20	20	20	100
	0.01	20	10	9	12	10.3	51	20	20	20	20	100
	0.001	20	6	5	8	6.3	32	20	20	20	20	100
Py/A	0.1	20	14	15	14	14.3	71	20	20	20	20	100
	0.01	20	14	12	14	13.3	66	20	20	20	20	100
	0.001	20	10	11	9	10	50	20	20	20	20	100
Py/B	0.1	20	8	7	8	7.8	38	10	12	11	11	55
	0.01	20	5	6	6	5.7	28	12	13	11	12.3	61
	0.001	20	4	4	4	4	20	9	8	11	9.3	48
C	0	20	0	0	0	0	0	0	0	0	0	0
	0.1	20	20	20	20	20	20	20	20	20	20	100
Py/D	0.01	20	20	19	20	20	20	20	20	20	20	100
	0.001	20	18	20	20	19.3	96	20	20	2 0	20	100

KEY: Py---Pyrethtrum

Py/A----Py/*ocimum*

Py/B------Py/*T.minuta*

C----Distilled water (negative control)

Py/D------Py/PBO (positive)

Figure 4.2: Comparative knockdown (KD) and kill of mosquitoes by pyrethrins, pyrethrins plus *Ocimum* and pyrethrins plus *T. minut*a at different Concentrations after 3minutes exposure and 24hours.

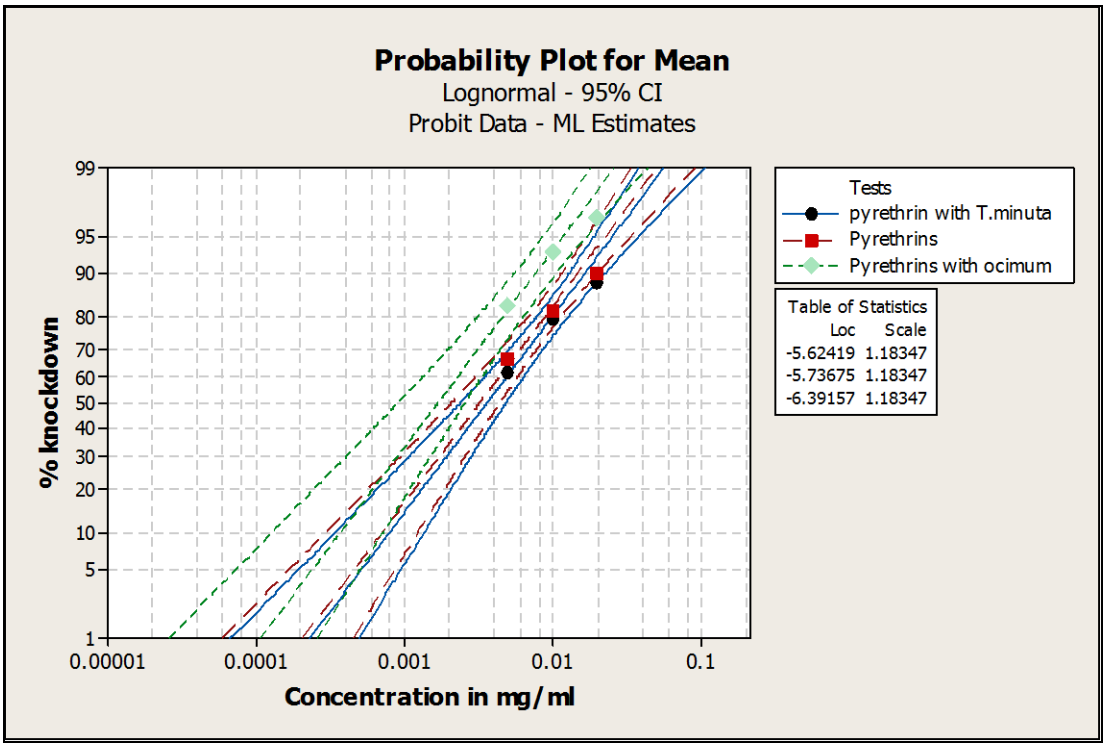


Figure 4.2: probit graph of KD on adult mosquitoes

4.1.4 Factor of synergism

Table 4.6 shows relative potency for the two synergists based on the FOS and lethal concentrations. The Table was generated using the formula given in article 3.8 and probit graphs in Figure 4.1 and 4.2, table of percentile given in Appendix II and tables of percentiles shown in appendix III.

Table 4.6: Relative Potency of the synergists based on the FOS and Lethal concentrations

Solutions	**Ratio Py/Syn**	**Larvae**LC_{50}	**Relative Potency (FOS)**	**Adult** KD_{50} **Potency**	**Relative (FOS)**
Pyrethrins	---	0.00376	1	0.002701	1
Py*/ocimum*	1.1	0.00167	2.2	0.001441	1.874
Py*/T.minuta*	1.1	0.00361	1.04	0.003020	0.894

4.1.5 GC- MS Analysis

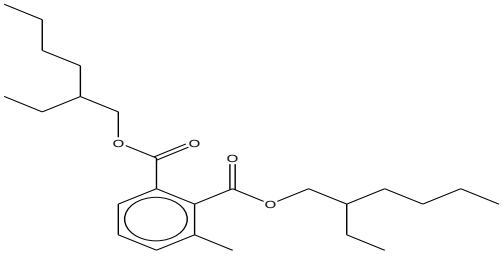
4.1.5.1 Chemical composition of E.O of *kilimandscharicum*

The chemical composition and retention times of the essential oil of leaves of *O. kilimandscharicum* are presented in Table 4.7.

Table 4. 7: Chemical composition of essential oil as found in *Ocimum kilimandscharicum*

Essential oils composition	**RT in min**
Bis (2-ethylhexyl) phthalate (**46**)	23.492
Monooleoylglycerol trimethylsilyl ester (**47**)	23.147
2-propenal, 3-(3,4-dimetho xyphenyl)- (**48)**	16.518
α –Pathchoulene (**49**)	14.461
Germacrene D-4-ol (**50**)	14.365
α Terpineol (**51**)	14.149
ɣ-Muurolene **(52**)	13.473
(-)-Germacrene D **(53**)	13.039
Methyl Eugenol (**42**)	12.504
Eugenol (**39**)	12.604
α-Copaene (**54**)	11.805
o-Menth-8- ene,4-isopropylidene-1-vinyl- (**55**)	11.253
Linalool (**19**)	11.055
Estragol (**38**)	9.265
Terpinenol -4-ol (**56**)	8.985
ß-Ocimene (**57**)	7.393
ß-pinene (**9**)	7.171
Cyclohexene, 1-methyl-4-(1-methylidene)- (**58**)	6.874
Cadinene δ- (**59**)	6.678

Figure 4.3: **Molecular structures of oil components of *O. kilimandscharicum***



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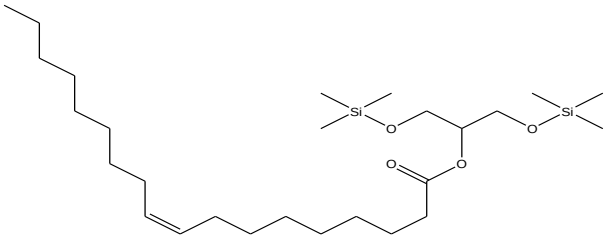

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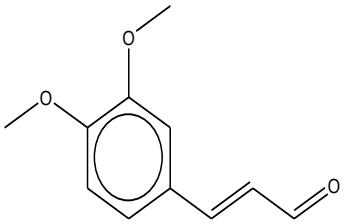

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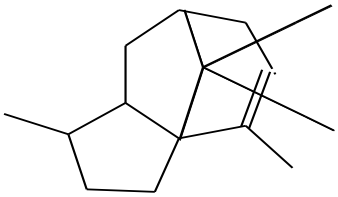

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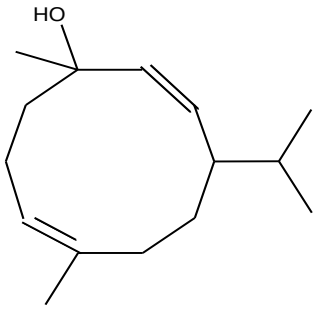

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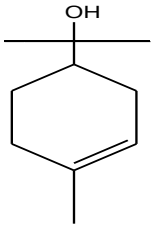

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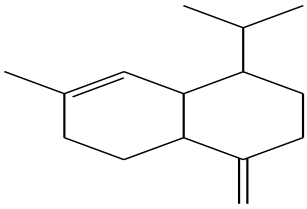

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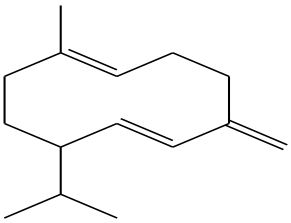

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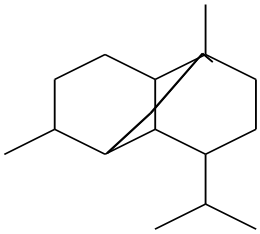

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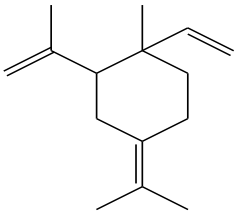

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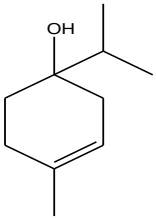

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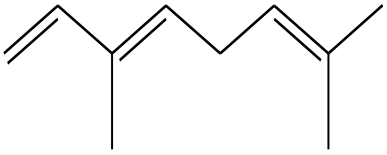

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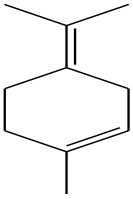

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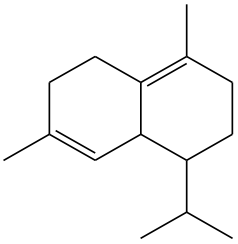

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4.2. Discussion

4.2.1 Larvicidal bioassay

The essential oils from the two plant species showed different activities with *O. kilimandscharicum* being more potent than the *T. minuta* for all treatment periods and concentrations as was shown in Table 4.3.

For example, the highest dose caused 100% mortality after 3-hour incubation with oils of *O. kilimandscharicum* while at the same dose 100% mortality was realized after 6 hours with oils of *T. minuta*. Essential oils from both extracts had larvicidal activity until 6 hours of incubation.

Results on comparative effect of different concentrations of the synergistic compounds on mortality of mosquitoes were presented in table 4.4. The table showed that combination of pyrethrins with *Ocimum* was much more effective on mosquito kill than combination with *T .minuta,* thus comparison by an alternative method was desirable.

The performance was further depicted in Figure 4. 1. Bioassay data was evaluated by regression and probit analysis and used to determine the lethal doses (LC_{50} and LC_{90}) for the synergist's mixtures. The results showed that LC_{50} and LC_{90} values for *O. kilimandscharicum* were 0.00167 and 0.0076 mg/ml while for *T. minuta* it was 0.00361and 0.01644mg/ml respectively as shown in Appendix II.

4.2.2 Bioassay on adult mosquito

The results on comparative effects of the two synergists on the knockdown and mortality rates on 3-4 days old adult mosquitoes were shown in Table 4. 3. These results showed that *T. minuta* was less effective as a pyrethrum synergist for all the concentrations tested. The mortality for mosquitoes after 24hours was also low. A comparison of pyrethrins plus *ocimum* at a specific concentration in the ratio 1:1with pyrethrins plus *T. minuta* at 1:5 showed that adding a large quantity of the less effective synergist does not compensate for its low activity.

Values of the median lethal dose for KD were read from the probit graphs as presented in Figures 4.2. The KD_{50} and KD_{90} values were determined to be 0.00144 and 0.00815mg/ml respectively for the *O. kilimandscharicum* and 0.00302 and 0.01708mg/ ml respectively for the *T. minuta*.

4.2.3 Factor of synergism

Relative potency for the two synergists based on the FOS and lethal concentrations, showed that the synergist *O. kilimandscharicum* had relative potency (R.P) of 2.2 and 1.874 against *T. minuta* which had 1.04 and 0.894 for larvae mortalities and adult knockdown respectively. Meaning that the higher the R.P, the more toxic (better) the synergist.

Similar studies have shown that steam distillation of leaves of *Mentha aruensis* (Labiatae) had a Factor of synergism of 0.94. Other plant families like Burseraceae, P.E

extract of resin of *Commiphora mukul* had FOS of 1.43 and Ether extract of resin had 0.95 (Pyrethrum post, 1973).

4.2.4 GC MS Analysis

The constituents of *O. kilimandscharicum* were listed in order of their elution on the column according to their retention time. The separation and identification of the compounds in *O. Kilimandscharicum* resulted into nineteen compounds. Linalool (1**9**), Methyl Eugenol (**42**) , Eugenol (**39**), Terpinenol -4-ol (**51**), Estragol (**38**) , ß-Ocimene (**57**), ß-pinene (**9**), ɣ - Muurolene (**52**) and alfa-Copaene (**54**) were among the compounds found in the essential oil of *O. kilimandscharicum.* A comparison of a similar study by Joshi (2013) showed that the above compound were most common constituents of *O. kilimandscharicum*.

Another different study by Isman (2006) and Tripathi *et al*., (2009), also indicated that some compounds found in the essential oil, included anethole (**26)**, elemicine **(27**), estragole (**28**), methyl eugenols (**29**) methylene dioxy compounds: apiole (**30**), myristicine (**31**), safrole (**6**).

A similar study by Bhasin (2012) showed that the essential oil obtained by hydro-distillation of the leaves of *Ocimum kilimandscharicum* Guerke, was analyzed by gas chromatography coupled with mass spectrometry (GC/MS). Forty-one constituents were identified. Chemical composition of the essential oil of *O. kilimandscharicum* were

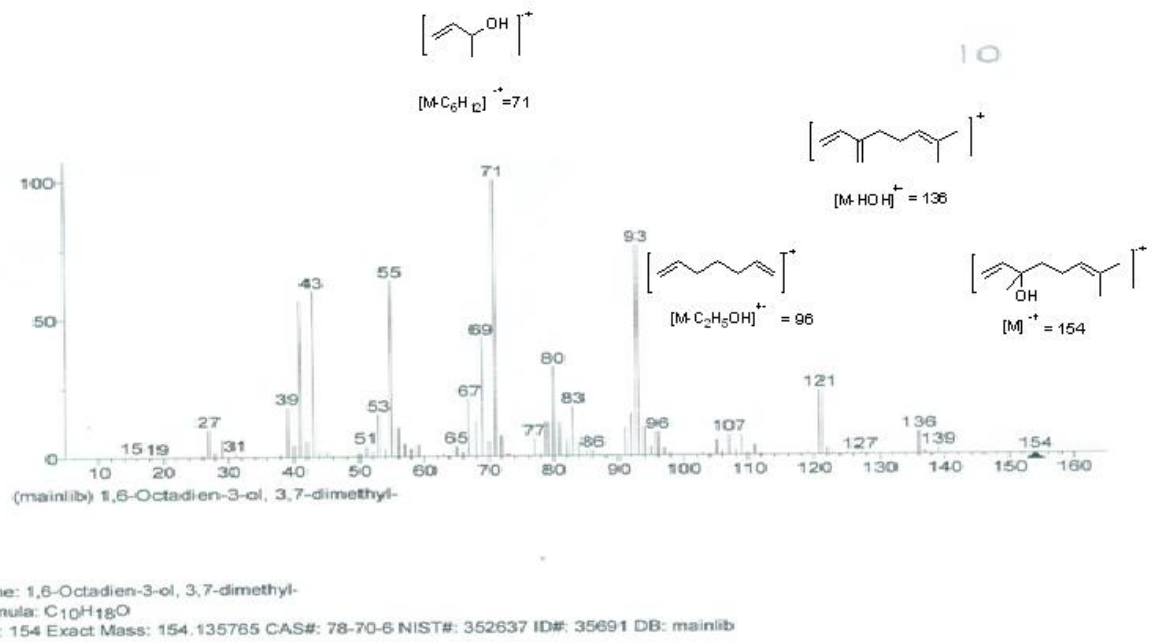
reported from India, where the major constituents were linalool (41.94-58.85%), camphor (17.0-15.82%) and 1,8-cineole (10.18-6.38%) among others.

4.2.5 Fragmentation Patterns

Fragmentation patterns show mass and elemental composition showing the molecular boundaries into which the structural fragments indicated in the spectrum must be fitted. The fragmentation patterns shown below resulted from parent molecules being impacted by electrons to obtain their EIMS. A few examples of the oil constituents suspected to possess synergistic properties were discussed briefly below.

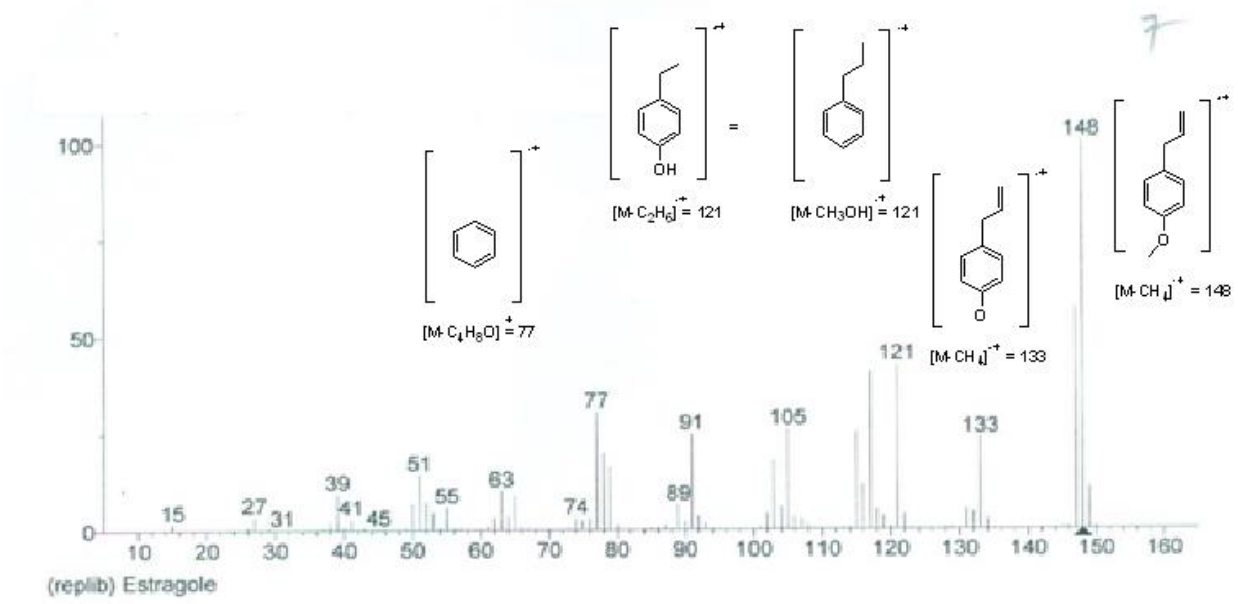
Some of the *Ocimum* oil constituents, the ones likely to have *methyldioxyphenyl,* a group which possess synergistic properties were (**19**), (**38**), (**39**) and(**42**) as observed in their spectra in the Appendices IX, X, XI and XII respectively.

Fragmentation patterns for Compound (19)



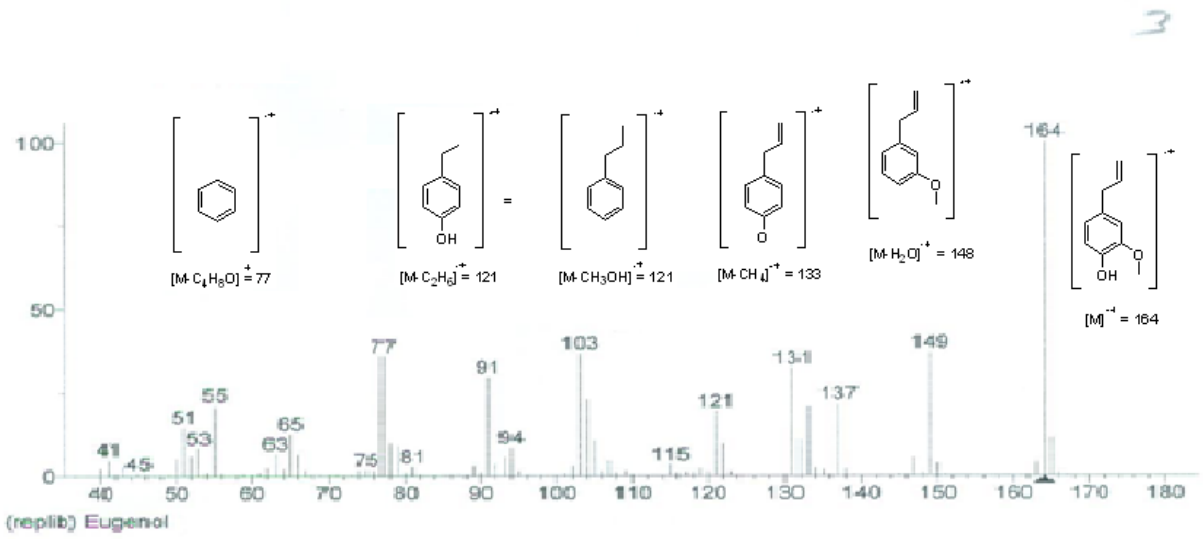
Electron spray ionization mass spectrum of compound (**19)** revealed the following fragmentation patterns. The molecular ion is observed at *m/*z 154. The peak at *m/z* 136 corresponding to $[M-18]^{\cdot+}$ resulting from the loss of water. The *m/z* 96 corresponding to the fragment $[M-58]^{\cdot+}$ due to the loss of ethanol and hydrogen from the molecule. The most abundant fragment ion arising from the molecule was observed *m/z* 71 and this corresponds to $[M-83]^{\cdot+}$ due to the loss of hexane from the molecule during fragmentation.

Fragmentation pattern for Compound (38)



For compounds (**38**) electron spray ionization mass spectrum revealed a molecular ion peak at *m/z* 148 as the base peak. The peak observed at *m/z* 133 corresponds to a fragment ion [M-15] which occurs following the loss of methane molecule. The peak at *m/z* 121 is the second most abundant peak. It corresponds to a fragment ion [M-25] which may be due to the loss of methanol from the molecular ion accompanied hydrogenation on the side chain double bond leading to the formation of relatively more stable n-propylbenzene fragment ion. It is also possible that this fragment occurs due to the formation a relatively stable para-ethylphenol fragment ion and loss of ethane from the molecule. The peak at *m/z* 77 is due to the loss of C_4H_8O side chain by the molecule. It corresponds to benzene [M-61] molecular ion fragment of compound .

Fragmentation patterns for Compound (39)



For compound (**39)** electron spray ionization mass spectrum revealed a molecular ion peak at *m/z* 164 as the base peak. The peak at *m/z* 136 corresponding to $[M-18]^{\cdot+}$ resulting from the loss of water leading to para-3-propenylanisole molecular ion peak. The peak at *m/z* 131, a fragment ion [M-33], corresponds to the loss of methanol from the molecule during fragmentation. The peak at *m/z* 121, a fragment ion [M-43], occurs due to the formation a relatively stable para-ethylphenol fragment ion while that at *m/z* 77 may be due to the loss of $C_4H_9O_2$ side chain by the molecule. It corresponds to benzene [M-97] molecular ion fragment of compound (**39**).

Fragmentation pattern for Compound (41)

This compound is derivative of compound (39).The peak 178 corresponds to the molecular ion M^+ base peak which provides the information in the mass spectrum.

The m/z peak at 163 was due to the loss of methyl, [M-15] and corresponds to a molecular ion fragment **[**$C_{10}H_{11}O_2]^{+\bullet}$

The m/z 147 loss of methoxy, [M-31] and corresponds to molecular ion fragment $[C_{10}H_{11}O]^{+\bullet}$

Furthermore the m/z 115 for example is due to loss of dimethoxy, [M-66] groups from methyl eugenol, and corresponds molecular ion fragment $[C_9H_7]^{+\bullet}$

The m/z 107 is due to loss of [M-58] group and corresponds molecular ion fragment $[C_7H_5O]^{+\bullet}$

The m/z 91 is due to loss of dimethyldimethoxy, [M-87 and corresponds to $[C_7H_7]^{+\bullet}$

The m/z 77 is due to loss of dimethoxyethyl and corresponds molecular ion fragment $[C_6H_5]^{+\bullet}$

The m/z 39 was due to loss of dimethoxyphenyl and corresponds molecular ion fragment $[C_3H_3]^{+\bullet}$

A comparison of the observed EIMS for these oil constituents as shown in appendices mentioned and the Reference from Literature data by Adams (2001) showed a very close correlations.

CHAPTER FIVE

CONCLUSIONS, RECOMMENDATIONS AND SUGGESTIONS FOR FURTHER STUDY

5.1 Conclusions

A comparison of activities of pyrethrins and the two crude extracts on 4th instar larvae of *A.gambiae, O. kilimandscharicum* was the most potent followed by pyrethrins then *T. minuta* for all treatment periods. An increase in concentration of the synergists caused a Significant ($P<0.05$) increase in mortalities both larvae and the adult mosquitoes.

A comparison of the efficacy of pyrethrins combined with crude extracts (synergists) Against. Conventional pyrethrum synergist PBO, showed that PBO was still the most potent (although non- biodegradable).

Characterization of the more potent synergist, *O. kilimandscharicum* resulted to nineteen constituents (compounds) with majority being monoterpenes (C10) and mainly phenylpropane.

5.2 Recommendations

1. The findings of this research would enable further exploration for large scale production of botanicals synergists for commercial application.
2. Focus should be on the development of synergists that would give the most economical control of each intended stage of species, since synergist was observed to be more effective on adult stage than the larval stage.
3. There was a need to find out the most economical way for oil extraction since distillation was rather an expensive and a long process.

4. An attempt should be made into Screening of floral biodiversity in search of pure plant extracts having mosquito larvicidal potentiality in search for better botanical synergists.

5.3 Suggestions for Further Study

1. There was a need to use another separation technique like Flame Ionization Detector (FID) along with GC/FID to identify the components of the essential oil.
2. An attempt should be made to determine which oil component was the most effective against the mosquitos' larvae and the adult stage.

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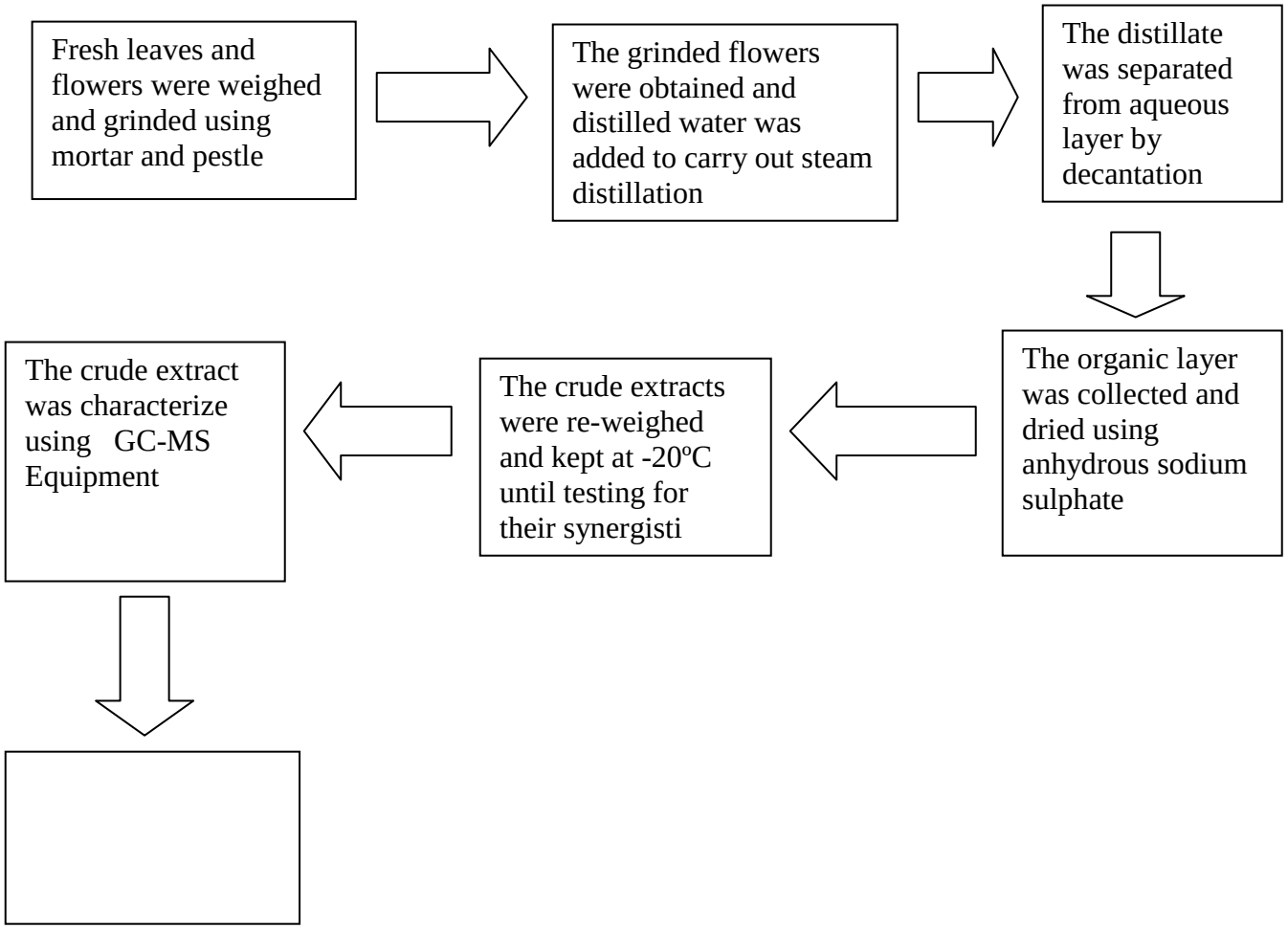
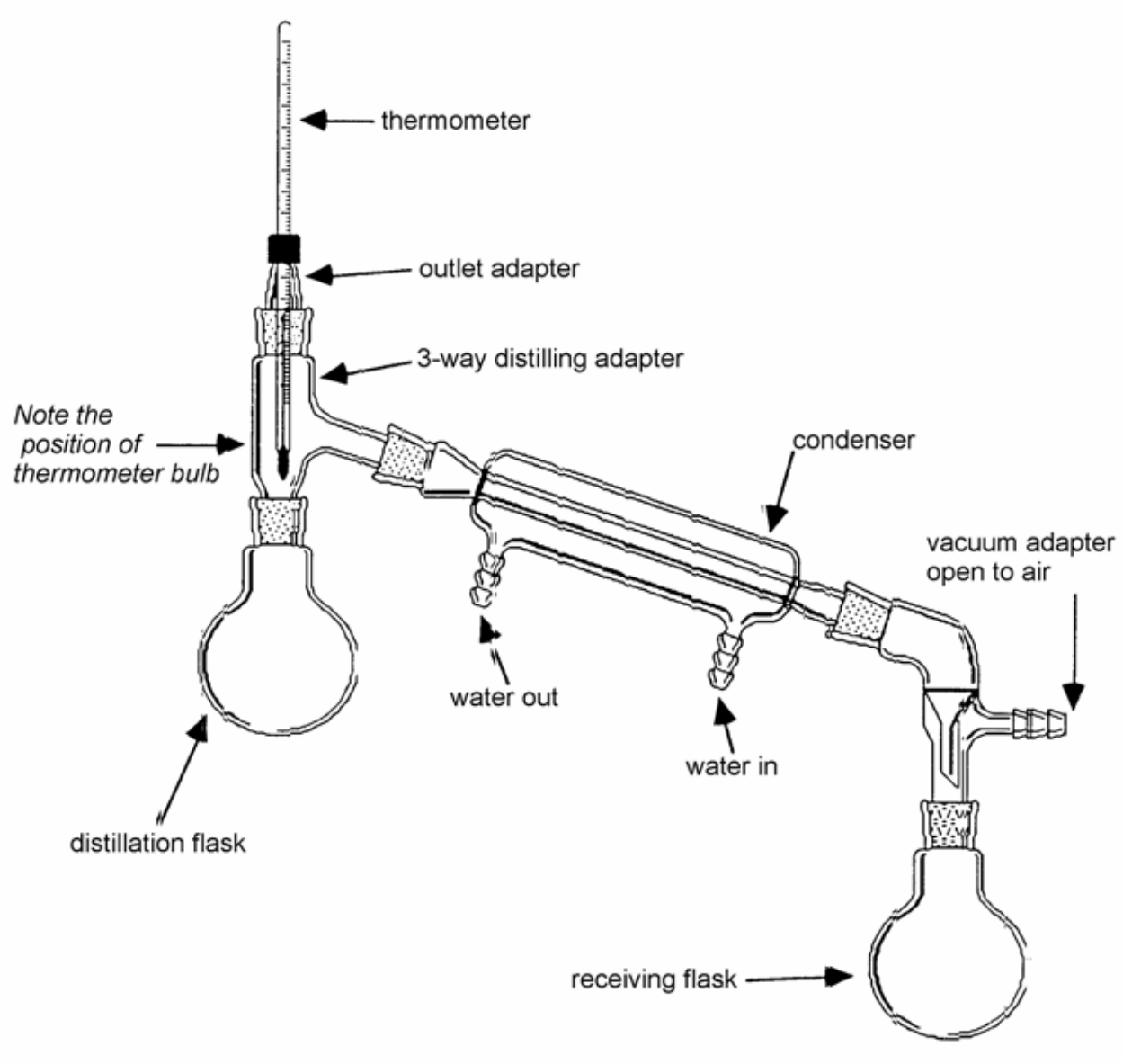
APPENDICES

Appendix I: Methodology




thermometer
outlet adapter
3-way distilling adapter
Note the position of thermometer bulb
condenser
vacuum adapter open to air
water out
water in
distillation flask
receiving flask

Appendix II: Weibull Distribution: Probability Plot for Mean

Probit Analysis: Mean, Trials versus Conc., Tests

Distribution: Weibull

Response Information

Variable	Value	Count
Mean	Success	625
	Failure	95
Trials	Total	720

Factor Information

Factor	Levels	Values
Tests	3	pyrethrin with *T.minuta* , Pyrethrins, Pyrethrins with *ocimum*

Estimation Method: Maximum Likelihood

Regression Table

Variable	Coef	Standard Error	Z	P
Constant	3.65365	0.388575	9.40	0.000
Conc.	0.692840	0.0852835	8.12	0.000
Tests				
Pyrethrins	0.0758004	0.125103	0.61	0.545
Pyrethrins with *ocimum*	0.512742	0.136892	3.75	0.000
Natural Response	0			

Test for equal slopes: Chi-Square = 0.888661 DF = 2 P-Value = 0.641

Log-Likelihood = -234.149

Multiple degree of freedom test

Term	Chi-Square	DF	P
Tests	15.5315	2	0.000

Goodness-of-Fit Tests

Method	Chi-Square	DF	P
Pearson	8.7036	8	0.368
Deviance	11.5848	8	0.171

i) Tests = pyrethrin with T.minuta

Tolerance Distribution

Parameter Estimates: Standard 95.0% Normal CI

Parameter	Estimate	Error	Lower	Upper
Shape	0.692840	0.0852835	0.544323	0.881880
Scale	0.0051259	0.0008371	0.0037219	0.0070596

Table of Percentiles: Standard 95.0% Fiducial CI

Percent	Percentile	Error	Lower	Upper
1	0.0000067	0.0000062	0.0000006	0.0000291
2	0.0000184	0.0000148	0.0000023	0.0000658
3	0.0000332	0.0000244	0.0000050	0.0001063
4	0.0000507	0.0000346	0.0000087	0.0001497
5	0.0000705	0.0000453	0.0000135	0.0001955
6	0.0000924	0.0000564	0.0000192	0.0002435
7	0.0001163	0.0000678	0.0000260	0.0002934
8	0.0001421	0.0000794	0.0000339	0.0003452
9	0.0001697	0.0000912	0.0000427	0.0003989
10	0.0001991	0.0001032	0.0000527	0.0004542
20	0.0005883	0.0002297	0.0002174	0.0010987
30	0.0011576	0.0003622	0.0005246	0.0019169
40	0.0019441	0.0004974	0.0010250	0.0029501
50	**0.0030202**	**0.0006357**	**0.0017997**	**0.0042817**
60	0.0045183	0.0007839	0.0029841	0.0060747
70	0.0067009	0.0009705	0.0048191	0.0086864
80	0.0101876	0.0013059	0.0077909	0.0130795
90	**0.0170833**	**0.0023018**	**0.0133005**	**0.0229645**
91	0.0182229	0.0025078	0.0141571	0.0247461
92	0.0195233	0.0027552	0.0151187	0.0268249
93	0.0210303	0.0030570	0.0162136	0.0292921
94	0.0228122	0.0034333	0.0174838	0.0322852
95	0.0249762	0.0039159	0.0189948	0.0360238
96	0.0277052	0.0045605	0.0208568	0.0408893
97	0.0313487	0.0054764	0.0232786	0.0476267
98	0.0367119	0.0069233	0.0267329	0.0579983
99	0.0464578	0.0097994	0.0327476	0.0780593

ii) Tests = Pyrethrins

Tolerance Distribution

Parameter Estimates: Standard 95.0% Normal CI

Parameter	Estimate	Error	Lower	Upper
Shape	0.692840	0.0852835	0.544323	0.881880
Scale	0.0045947	0.0007874	0.0032839	0.0064289

Table of Percentiles: Standard 95.0% Fiducial CI

Percent	Percentile	Error	Lower	Upper
1	0.0000060	0.0000056	0.0000005	0.0000266
2	0.0000165	0.0000134	0.0000020	0.0000600
3	0.0000298	0.0000222	0.0000044	0.0000969
4	0.0000454	0.0000315	0.0000076	0.0001365
5	0.0000632	0.0000413	0.0000118	0.0001782
6	0.0000828	0.0000514	0.0000168	0.0002220
7	0.0001042	0.0000619	0.0000227	0.0002675
8	0.0001274	0.0000725	0.0000295	0.0003148
9	0.0001521	0.0000834	0.0000373	0.0003636
10	0.0001785	0.0000944	0.0000460	0.0004141
20	0.0005273	0.0002115	0.0001896	0.0010014
30	0.0010376	0.0003354	0.0004577	0.0017464
40	0.0017426	0.0004633	0.0008949	0.0026860
50	**0.0027072**	**0.0005955**	**0.0015731**	**0.0038941**
60	0.0040501	0.0007372	0.0026135	0.0055138
70	0.0060064	0.0009104	0.0042361	0.0078554
80	0.0091319	0.0012023	0.0068961	0.0117465
90	**0.0153129**	**0.0020375**	**0.0119036**	**0.0203978**
91	0.0163344	0.0022109	0.0126861	0.0219528
92	0.0175000	0.0024196	0.0135647	0.0237671

93	0.0188509	0.0026750	0.0145655	0.0259203
94	0.0204481	0.0029944	0.0157265	0.0285327
95	0.0223879	0.0034054	0.0171074	0.0317962
96	0.0248340	0.0039564	0.0188087	0.0360441
97	0.0280999	0.0047420	0.0210204	0.0419277
98	0.0329073	0.0059881	0.0241734	0.0509869
99	0.0416433	0.0084755	0.0296596	0.0685131

iii) Tests = Pyrethrins with ocimum

Tolerance Distribution

Parameter Estimates: **Standard 95.0% Normal CI**

Parameter	Estimate	Error	Lower	Upper
Shape	0.692840	0.0852835	0.544323	0.881880
Scale	0.0024455	0.0005455	0.0015794	0.0037866

Table of Percentiles: Standard 95.0% Fiducial CI

Percent	Percentile	Error	Lower	Upper
1	0.0000032	0.0000032	0.0000002	0.0000154
2	0.0000088	0.0000076	0.0000009	0.0000349
3	0.0000158	0.0000127	0.0000020	0.0000564
4	0.0000242	0.0000181	0.0000035	0.0000794
5	0.0000336	0.0000239	0.0000054	0.0001037
6	0.0000441	0.0000298	0.0000077	0.0001292
7	0.0000555	0.0000360	0.0000105	0.0001557
8	0.0000678	0.0000424	0.0000136	0.0001832
9	0.0000810	0.0000489	0.0000172	0.0002117
10	0.0000950	0.0000556	0.0000212	0.0002411
20	0.0002806	0.0001284	0.0000874	0.0005835
30	0.0005523	0.0002097	0.0002110	0.0010177

40	0.0009275	0.0002986	0.0004130	0.0015640
50	**0.0014409**	**0.0003963**	**0.0007274**	**0.0022628**
60	0.0021556	0.0005063	0.0012139	0.0031897
70	0.0031969	0.0006385	0.0019866	0.0045006
80	0.0048604	0.0008251	0.0033031	0.0065895
90	**0.0081502**	**0.0012223**	**0.0059657**	**0.0109359**
91	0.0086939	0.0012973	0.0063993	0.0116934
92	0.0093143	0.0013869	0.0068900	0.0125725
93	0.0100333	0.0014958	0.0074530	0.0136109
94	0.0108834	0.0016318	0.0081105	0.0148655
95	0.0119158	0.0018071	0.0088970	0.0164275
96	0.0132178	0.0020433	0.0098703	0.0184551
97	0.0149560	0.0023833	0.0111399	0.0212577
98	0.0175148	0.0029297	0.0129531	0.0255669
99	0.0221644	0.0040408	0.0161084	0.0338954

Appendix III: Distribution: Lognormal

Parametric Cumulative Failure Plot for Mean

Probit Analysis: Mean, Trials versus Conc., Tests

Distribution: Lognormal

Response Information

Variable	Value	Count
Mean	Success	625
	Failure	95
Trials	Total	720

Factor Information

Factor	Levels	Values
Tests	3	pyrethrin with T.minuta, Pyrethrins, Pyrethrins with ocimum

Estimation Method: Maximum Likelihood

Regression Table

Variable	Coef	Standard Error	Z	P
Constant	4.75230	0.494859	9.60	0.000
Conc.	0.844976	0.104526	8.08	0.000
Tests				
Pyrethrins	0.0951134	0.147940	0.64	0.520
Pyrethrins with ocimum	0.648419	0.171571	3.78	0.000
Natural Response	0			

Test for equal slopes: Chi-Square = 0.109106 DF = 2 P-Value = 0.947

Log-Likelihood = -234.663

Multiple degree of freedom test

Term	Chi-Square	DF	P
Tests	15.3588	2	0.000

Goodness-of-Fit Tests

Method	Chi-Square	DF	P
Pearson	8.7852	8	0.361
Deviance	12.6129	8	0.126

Tests = pyrethrin with *T.minuta*

Tolerance Distribution

Parameter Estimates

		Standard	95.0% Normal CI	
Parameter	Estimate	Error	Lower	Upper
Location	-5.62419	0.173042	-5.96334	-5.28503
Scale	1.18347	0.146398	0.928666	1.50818

Table of Percentiles

		Standard	95.0% Fiducial CI	
Percent	Percentile	Error	Lower	Upper
1	0.0002300	0.0001102	0.0000672	0.0004928
2	0.0003176	0.0001400	0.0001026	0.0006407
3	0.0003897	0.0001623	0.0001342	0.0007571
4	0.0004546	0.0001811	0.0001642	0.0008584
5	0.0005153	0.0001976	0.0001934	0.0009509
6	0.0005732	0.0002127	0.0002223	0.0010375
7	0.0006294	0.0002267	0.0002512	0.0011201
8	0.0006843	0.0002398	0.0002802	0.0011996
9	0.0007385	0.0002523	0.0003094	0.0012769
10	0.0007921	0.0002642	0.0003390	0.0013525
20	0.0013331	0.0003661	0.0006668	0.0020788
30	0.0019405	0.0004539	0.0010822	0.0028443
40	0.0026744	0.0005378	0.0016303	0.0037328
50	0.0036095	0.0006246	0.0023785	0.0048383
60	0.0048715	0.0007259	0.0034416	0.0063231
70	0.0067139	0.0008746	0.0050364	0.0085428
80	0.0097727	0.0011906	0.0076419	0.0125019
90	0.0164486	0.0022691	0.0128367	0.0225002
91	0.0176424	0.0025092	0.0137035	0.0244607
92	0.0190377	0.0028044	0.0146981	0.0268092

93	0.0206996	0.0031746	0.0158595	0.0296818
94	0.0227278	0.0036513	0.0172468	0.0332913
95	0.0252846	0.0042868	0.0189551	0.0379922
96	0.0286583	0.0051775	0.0211503	0.0444312
97	0.0334289	0.0065237	0.0241600	0.0539516
98	0.0410220	0.0088407	0.0287697	0.0699936
99	0.0566403	0.0141291	0.0377438	0.105893

Tests = Pyrethrins

Tolerance Distribution

Parameter Estimates

		Standard	95.0% Normal CI	
Parameter	Estimate	Error	Lower	Upper
Location	-5.73675	0.183607	-6.09662	-5.37689
Scale	1.18347	0.146398	0.928666	1.50818

Table of Percentiles

		Standard	95.0% Fiducial CI	
Percent	Percentile	Error	Lower	Upper
1	0.0002055	0.0001009	0.0000583	0.0004486
2	0.0002838	0.0001284	0.0000890	0.0005832
3	0.0003482	0.0001491	0.0001164	0.0006891
4	0.0004062	0.0001665	0.0001424	0.0007814
5	0.0004604	0.0001819	0.0001678	0.0008656
6	0.0005122	0.0001960	0.0001928	0.0009444
7	0.0005624	0.0002091	0.0002179	0.0010196
8	0.0006115	0.0002214	0.0002430	0.0010920
9	0.0006599	0.0002331	0.0002684	0.0011623
10	0.0007077	0.0002443	0.0002941	0.0012312
20	0.0011912	0.0003408	0.0005785	0.0018920
30	0.0017339	0.0004253	0.0009392	0.0025879
40	0.0023897	0.0005070	0.0014158	0.0033943
50	0.0032252	0.0005922	0.0020676	0.0043950
60	0.0043529	0.0006905	0.0029977	0.0057325
70	0.0059991	0.0008282	0.0044036	0.0077152
80	0.0087323	0.0011016	0.0067312	0.0112077
90	0.0146975	0.0020097	0.0114417	0.0199336
91	0.0157642	0.0022129	0.0122306	0.0216415
92	0.0170110	0.0024633	0.0131360	0.0236872

93	0.0184960	0.0027784	0.0141933	0.0261897
94	0.0203082	0.0031852	0.0154560	0.0293344
95	0.0225928	0.0037294	0.0170103	0.0334305
96	0.0256074	0.0044943	0.0190068	0.0390419
97	0.0298701	0.0056542	0.0217427	0.0473394
98	0.0366548	0.0076572	0.0259307	0.0613217
99	0.0506104	0.0122461	0.0340791	0.0926099

Tests = Pyrethrins with *ocimum*

Tolerance Distribution

Parameter Estimates

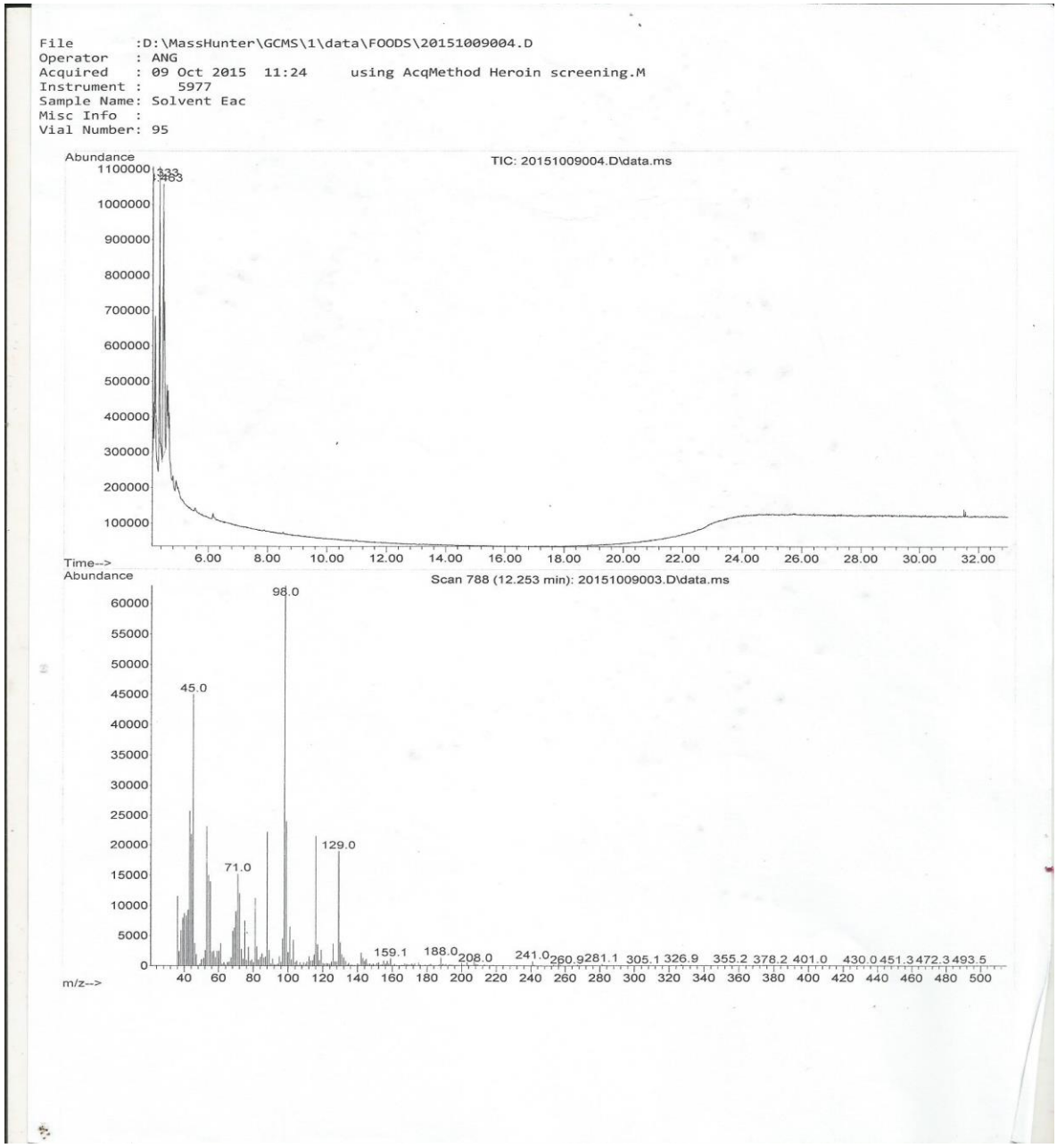
		Standard	95.0% Normal CI	
Parameter	Estimate	Error	Lower	Upper
Location	-6.39157	0.254156	-6.88971	-5.89343
Scale	1.18347	0.146398	0.928666	1.50818

Table of Percentiles

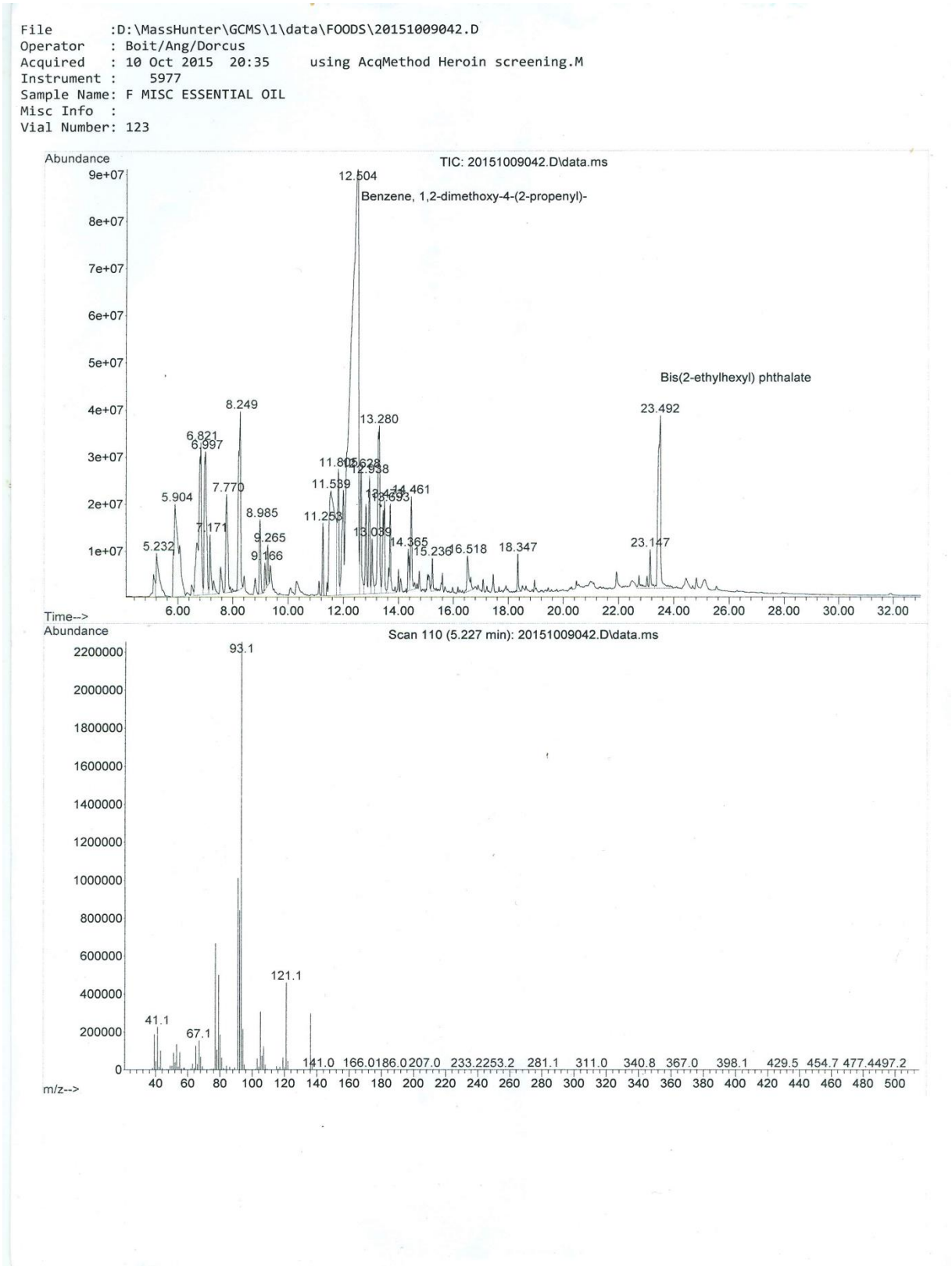
		Standard	95.0% Fiducial CI	
Percent	Percentile	Error	Lower	Upper
1	0.0001068	0.0000599	0.0000254	0.0002610
2	0.0001474	0.0000771	0.0000387	0.0003396
3	0.0001809	0.0000902	0.0000506	0.0004014
4	0.0002110	0.0001014	0.0000619	0.0004554
5	0.0002392	0.0001115	0.0000729	0.0005046
6	0.0002661	0.0001208	0.0000838	0.0005507
7	0.0002922	0.0001294	0.0000946	0.0005946
8	0.0003177	0.0001377	0.0001056	0.0006370
9	0.0003428	0.0001456	0.0001166	0.0006781
10	0.0003677	0.0001532	0.0001277	0.0007184
20	0.0006189	0.0002216	0.0002509	0.0011051
30	0.0009008	0.0002858	0.0004073	0.0015117
40	0.0012415	0.0003524	0.0006144	0.0019813
50	0.0016756	0.0004259	0.0008994	0.0025597
60	0.0022615	0.0005126	0.0013106	0.0033216
70	0.0031168	0.0006257	0.0019469	0.0044209
80	0.0045368	0.0008030	0.0030516	0.0062629
90	0.0076359	0.0012317	0.0054872	0.0105299
91	0.0081901	0.0013190	0.0059144	0.0113376
92	0.0088378	0.0014256	0.0064091	0.0122993

93	0.0096093	0.0015588	0.0069914	0.0134694
94	0.0105509	0.0017304	0.0076918	0.0149329
95	0.0117378	0.0019603	0.0085591	0.0168314
96	0.0133040	0.0022850	0.0096785	0.0194235
97	0.0155186	0.0027819	0.0112173	0.0232458
98	0.0190435	0.0036514	0.0135767	0.0296709
99	0.0262939	0.0056798	0.0181671	0.0440105

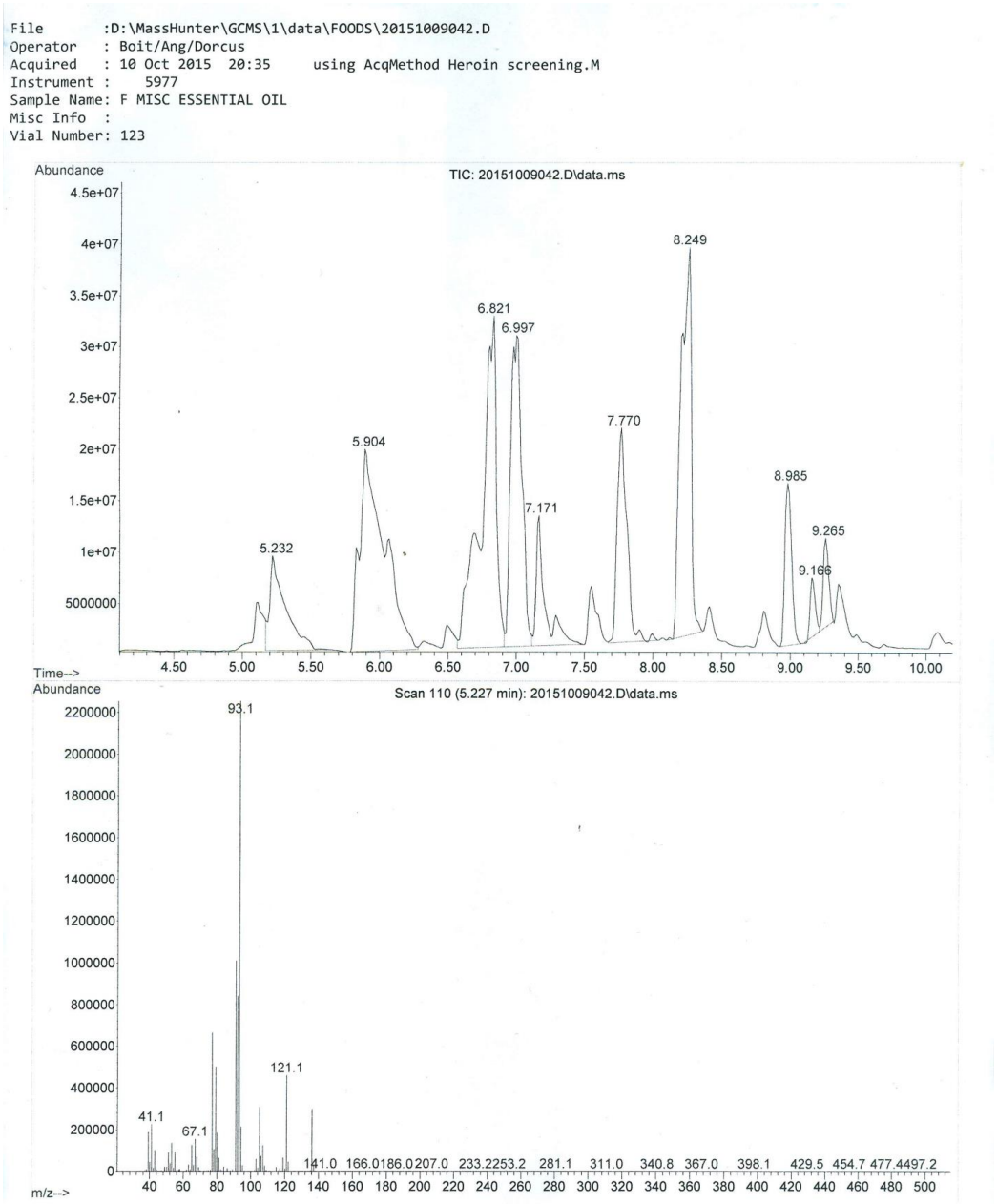
Appendix IV: GC Spectrum for Blank



Appendix V: GC MS Spectrum of the *Ocimum* oil

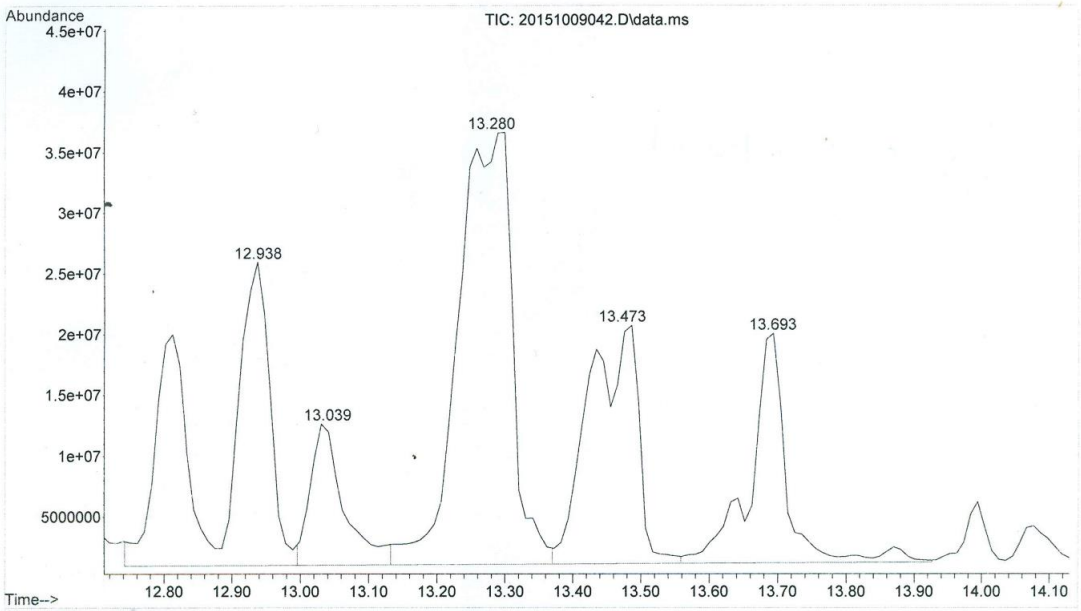


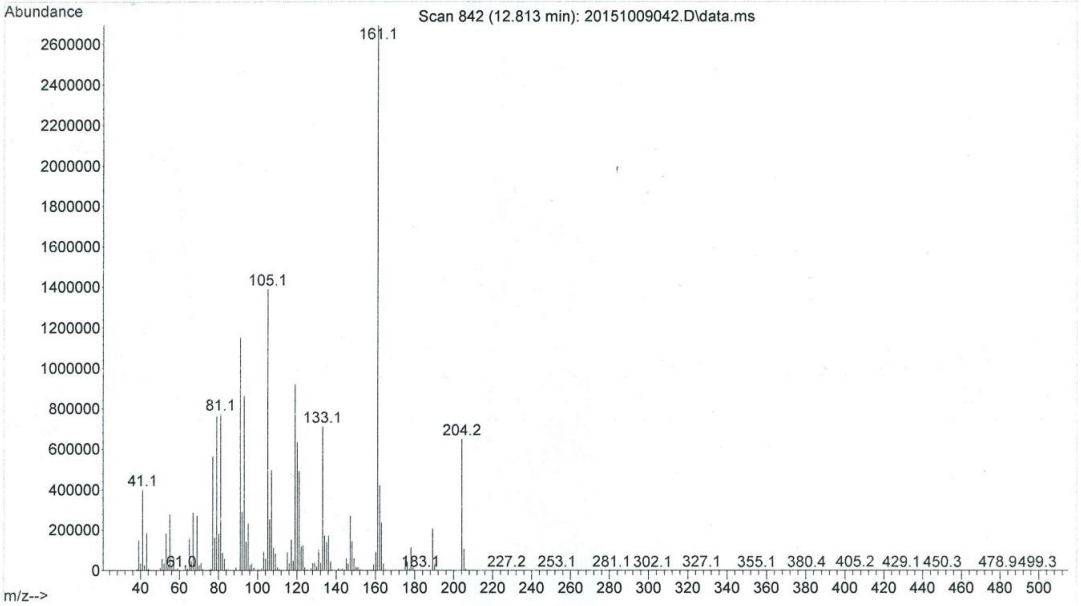
Appendix VI: Expanded GC MS Spectrum of the *Ocimum* oil



Appendix VII: Expanded GC MS Spectrum of the *Ocimum* oil

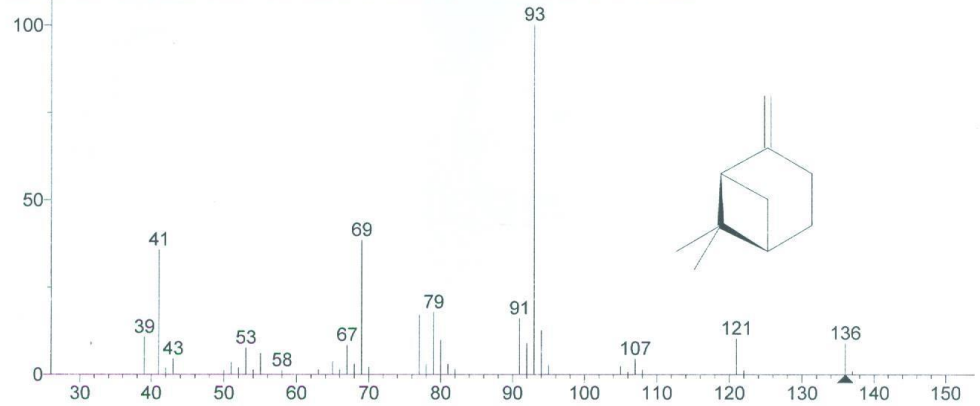
```
File       :D:\MassHunter\GCMS\1\data\FOODS\20151009042.D
Operator   : Boit/Ang/Dorcus
Acquired   : 10 Oct 2015  20:35     using AcqMethod Heroin screening.M
Instrument :    5977
Sample Name: F MISC ESSENTIAL OIL
Misc Info  :
Vial Number: 123
```



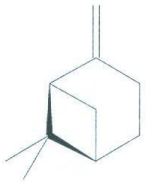


Appendix VIII: MS Spectrum for (9)

Hit 1 : 2(10)-Pinene
C10H16; MF: 946; RMF: 958; Prob 30.9%; CAS: 127-91-3; Lib: w10n11; ID: 35339.



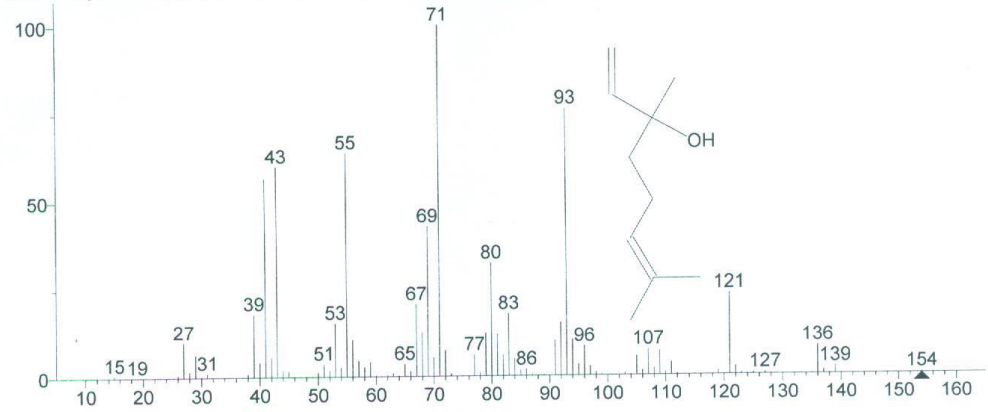
(w10n11) 2(10)-Pinene



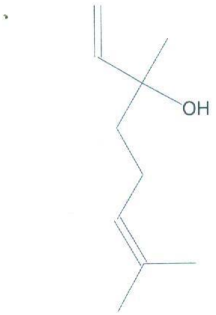
Name: 2(10)-Pinene
Formula: $C_{10}H_{16}$
MW: 136 Exact Mass: 136.1252 CAS#: 127-91-3 ID#: 35339 DB: w10n11
Other DBs: None
Comment: SpectrumID: 1136926; Source: PG-1982-917-0; QI: 976; NBS#: 149871
Synonyms:
1.Bicyclo[3.1.1]heptane, 6,6-dimethyl-2-methylene-
2.(+)-β-PINEN
3.(-)-β-PINEN
4.(-)-PIN-2(10)-ENE
5.(1)-6,6-DIMETHYL-2-METHYLENEBICYCLO(3.1.1)HEPTANE
6.(1S)-(-)-β-PINENE
7.(1S,5S)-6,6-DIMETHYL-2-METHYLENEBICYCLO[3.1.1]HEPTANE
8.β-l-Pinene
9.β-Pinene
10.β-pinene 2-β-pinenenopinen nopinene terebenthenepseudopinen pseudopinene 2(10)-pineneCA: 6,6-dimethyl-2

Appendix IX: MS Spectrum of compound 19

Hit 1 : 1,6-Octadien-3-ol, 3,7-dimethyl-
C10H18O; MF: 954; RMF: 955; Prob 65.0%; CAS: 78-70-6; Lib: mainlib; ID: 35691.



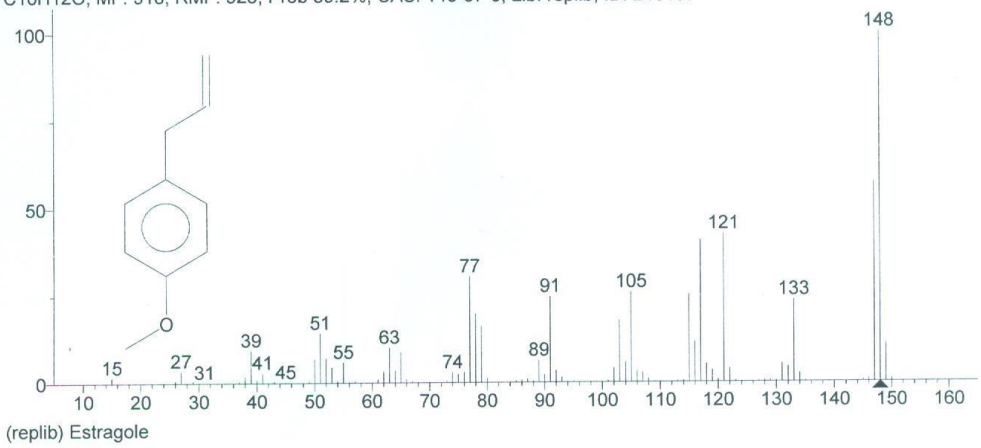
(mainlib) 1,6-Octadien-3-ol, 3,7-dimethyl-



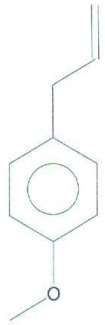
Name: 1,6-Octadien-3-ol, 3,7-dimethyl-
Formula: $C_{10}H_{18}O$
MW: 154 Exact Mass: 154.135765 CAS#: 78-70-6 NIST#: 352637 ID#: 35691 DB: mainlib
Other DBs: Fine, TSCA, RTECS, NIH, EINECS, IRDB
Contributor: NIST Mass Spectrometry Data Center
Related CAS#: 11024-20-7; 22564-99-4
Synonyms:
1.β-Linalool
2.Linalol
3.Linalool
4.Linalyl alcohol
5.2,6-Dimethyl-2,7-octadien-6-ol
6.allo-Ocimenol
7.2,6-Dimethyl-2,7-octadiene-6-ol
8.2,6-Dimethylocta-2,7-dien-6-ol

Appendix X: MS Spectrum of compound 38

Hit 1 : Estragole
C10H12O; MF: 918; RMF: 928; Prob 39.2%; CAS: 140-67-0; Lib: replib; ID: 21619.

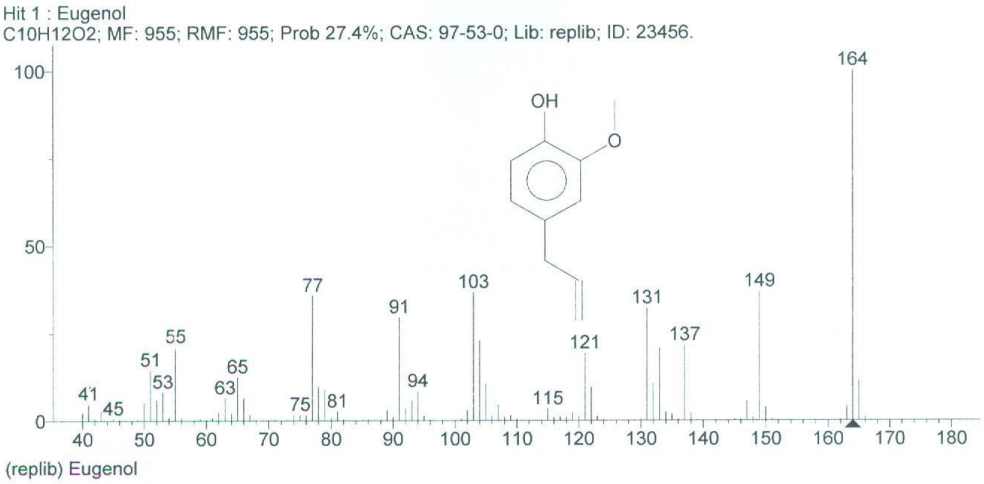


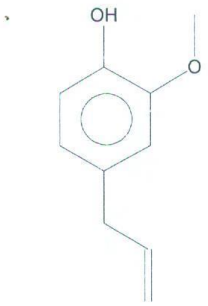
(replib) Estragole



Name: Estragole
Formula: $C_{10}H_{12}O$
MW: 148 Exact Mass: 148.088815 CAS#: 140-67-0 NIST#: 290838 ID#: 21619 DB: replib
Other DBs: Fine, TSCA, RTECS, EPA, HODOC, NIH, EINECS, IRDB
Contributor: NIST Mass Spectrometry Data Center, 1998.
Related CAS#: 1407-27-8; 77525-18-9
Synonyms:
1.Tarragon
2.Anisole, p-allyl-
3.Chavicol, O-methyl-
4.p-Allylanisole
5.p-Methoxyallylbenzene
6.Chavicol methyl ether
7.Esdragol
8.Esdragole
9.Esdragon

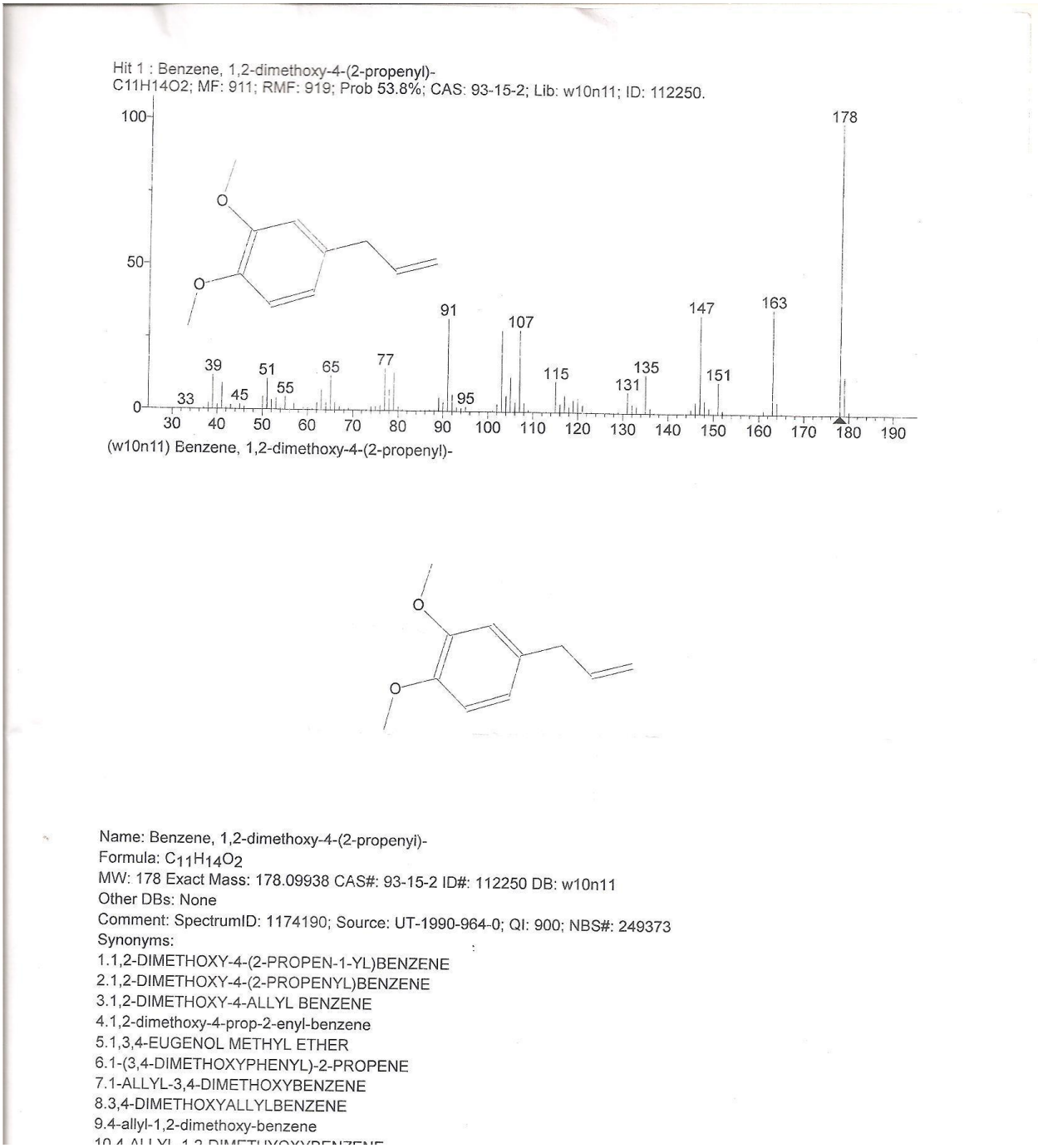
Appendix XI: MS Spectrum of Compound (39)



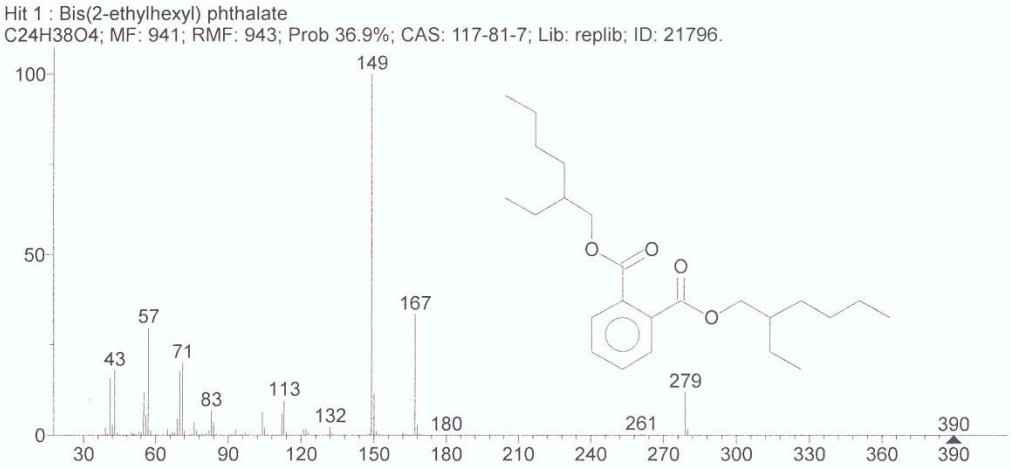


Name: Eugenol
Formula: $C_{10}H_{12}O_2$
MW: 164 Exact Mass: 164.08373 CAS#: 97-53-0 NIST#: 357118 ID#: 23456 DB: replib
Other DBs: Fine, TSCA, RTECS, EPA, USP, HODOC, NIH, EINECS, IRDB
Contributor: Drug Lab
Synonyms:
1.Phenol, 2-methoxy-4-(2-propenyl)-
2.Phenol, 4-allyl-2-methoxy-
3.p-Allylguaiacol
4.p-Eugenol
5.Caryophyllic acid
6.Engenol
7.Eugenic acid
8.2-Methoxy-1-hydroxy-4-allylbenzene
9.2-Methoxy-4-allylphenol

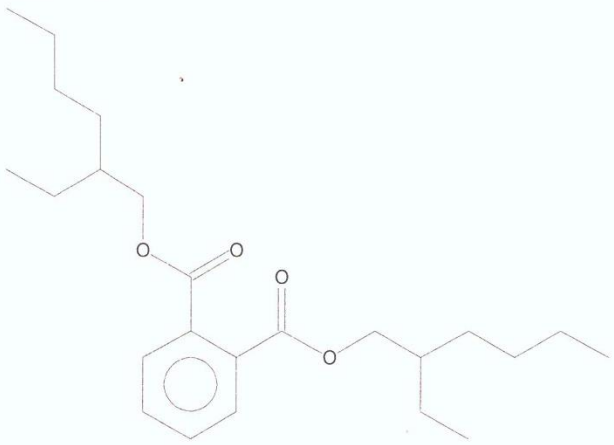
Appendix XII: MS Spectrum of Compound (42)



Appendix XIII: MS Spectrum of Compound (46)

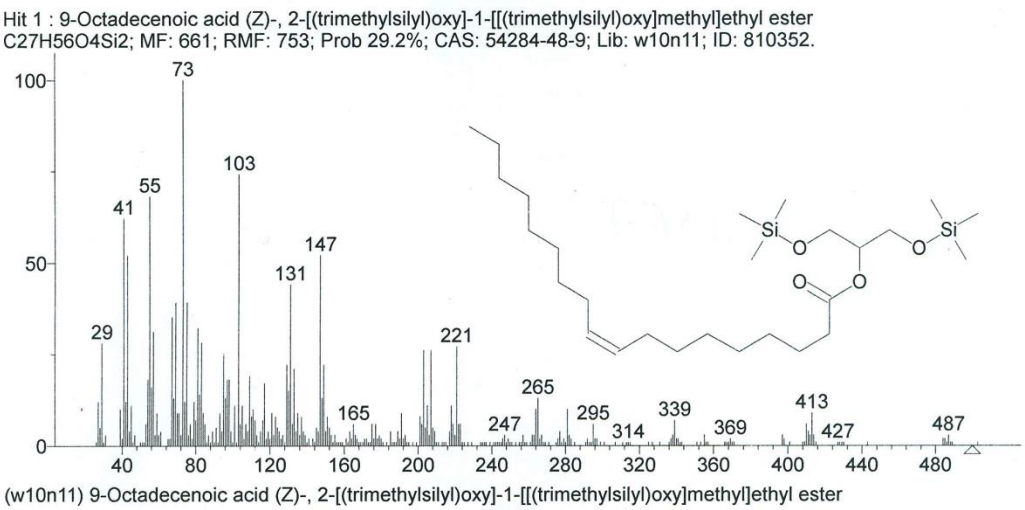


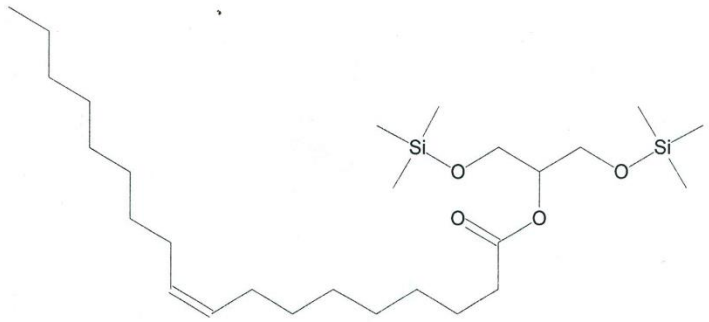
(replib) Bis(2-ethylhexyl) phthalate



Name: Bis(2-ethylhexyl) phthalate
Formula: $C_{24}H_{38}O_4$
MW: 390 Exact Mass: 390.27701 CAS#: 117-81-7 NIST#: 368499 ID#: 21796 DB: replib
Other DBs: Fine, TSCA, RTECS, EPA, HODOC, NIH, EINECS, IRDB
Contributor: Adam Howard, James Little, Eastman Chemical Co.
Related CAS#: 8033-53-2; 137718-37-7; 205180-59-2; 275818-89-8; 40120-69-2; 50885-87-5; 607374-50-5; 109630 -52-6
Synonyms:
1.Phthalic acid, bis(2-ethylhexyl) ester
2.Bis(2-ethylhexyl) 1,2-benzenedicarboxylate
3.Bisoflex 81
4.Compound 889
5.Di(ethylhexyl) phthalate
6.Di(2-ethylhexyl) phthalate
7.DEHP

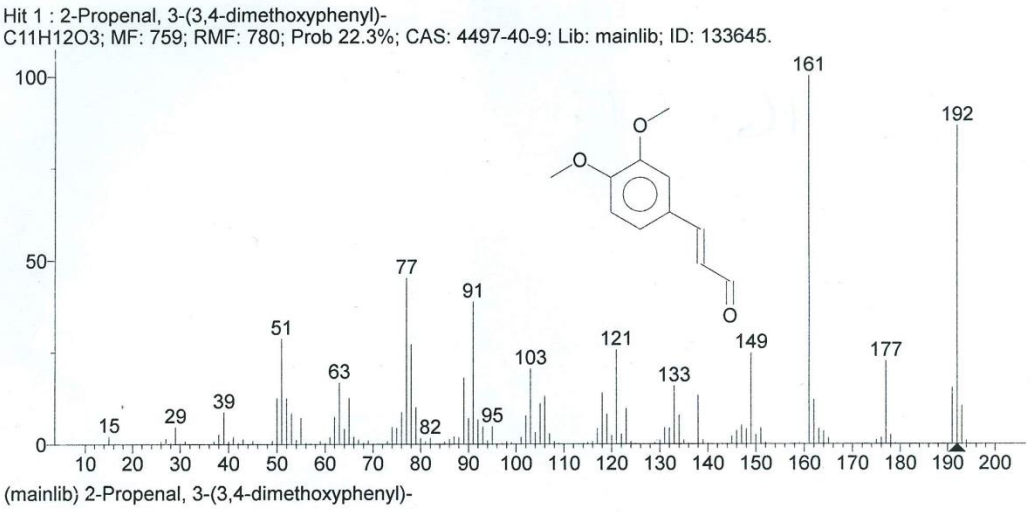
Appendix XIV: MS Spectrum of Compound (47)

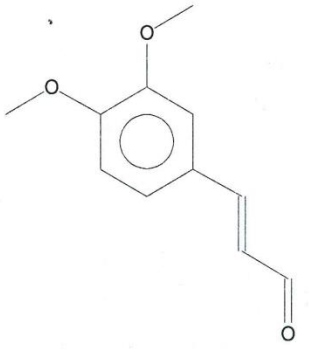




Name: 9-Octadecenoic acid (Z)-, 2-[(trimethylsilyl)oxy]-1-[[(trimethylsilyl)oxy]methyl]ethyl ester
Formula: $C_{27}H_{56}O_4Si_2$
MW: 500 Exact Mass: 500.371714 CAS#: 54284-48-9 ID#: 810352 DB: w10n11
Other DBs: None
Comment: SpectrumID: 62962; Source: AA-0-2243-1; QI: 900
Synonyms:
1.2-Monooleoylglycerol trimethylsilyl ether
2.2-[(trimethylsilyl)oxy]-1-{[(trimethylsilyl)oxy]methyl}ethyl (9Z)-9-octadecenoate
3.TMS ETHER OF 2-MONOOLEGLYCEROL
4.TRIMETHYLSILYL DERIVATIVE OF 2-MONOOLEIN

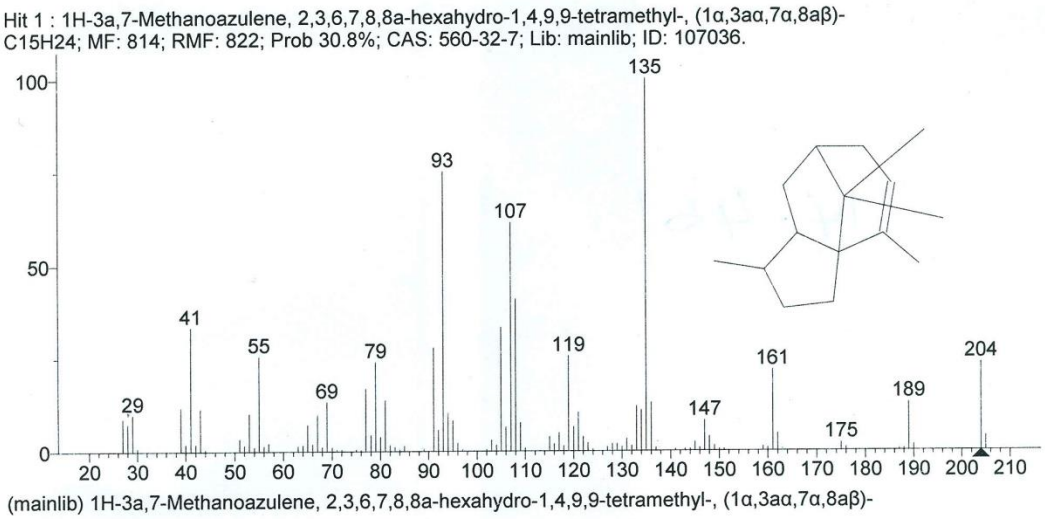
Appendix XV: MS Spectrum Compound (48)

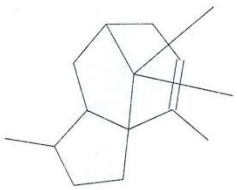




Name: 2-Propenal, 3-(3,4-dimethoxyphenyl)-
Formula: $C_{11}H_{12}O_3$
MW: 192 Exact Mass: 192.078644 CAS#: 4497-40-9 NIST#: 352422 ID#: 133645 DB: mainlib
Other DBs: HODOC
Contributor: NIST Mass Spectrometry Data Center
Synonyms:
1.Cinnamaldehyde, 3,4-dimethoxy-
2.Coniferaldehyde methyl ether
3.Methylconiferylaldehyde
4.3,4-Dimethoxycinnamaldehyde
5.(2E)-3-(3,4-Dimethoxyphenyl)-2-propenal #

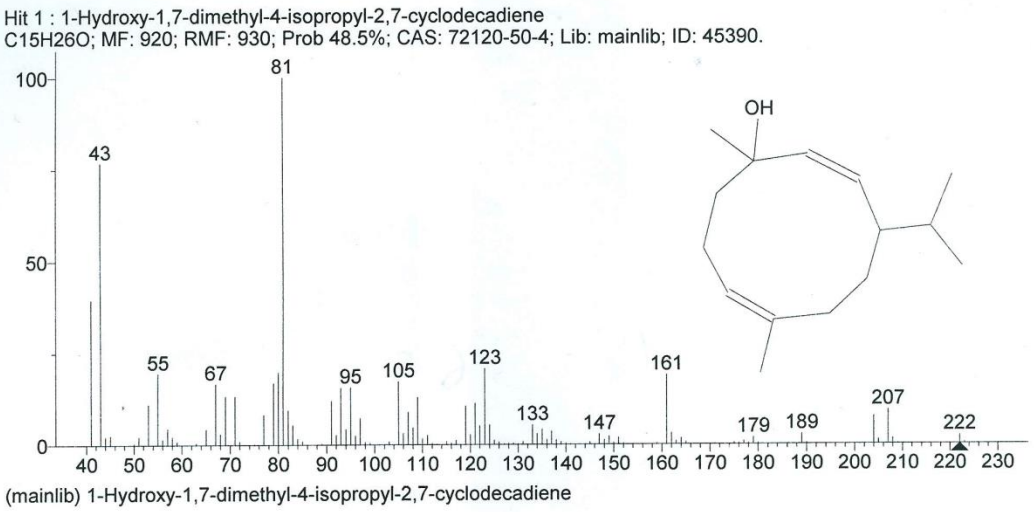
Appendix XVI: MS Spectrum of Compound (49)

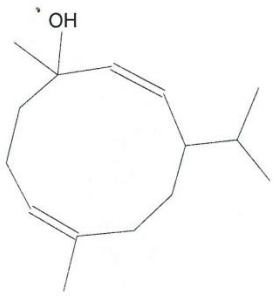




Name: 1H-3a,7-Methanoazulene, 2,3,6,7,8,8a-hexahydro-1,4,9,9-tetramethyl-, (1α,3aα,7α,8aβ)-
Formula: $C_{15}H_{24}$
MW: 204 Exact Mass: 204.1878 CAS#: 560-32-7 NIST#: 22532 ID#: 107036 DB: mainlib
Other DBs: None
Synonyms:
1.1H-3a,7-Methanoazulene, 2,3,6,7,8,8aα-hexahydro-1β,4,9,9-tetramethyl-
2.α-Patchoulene

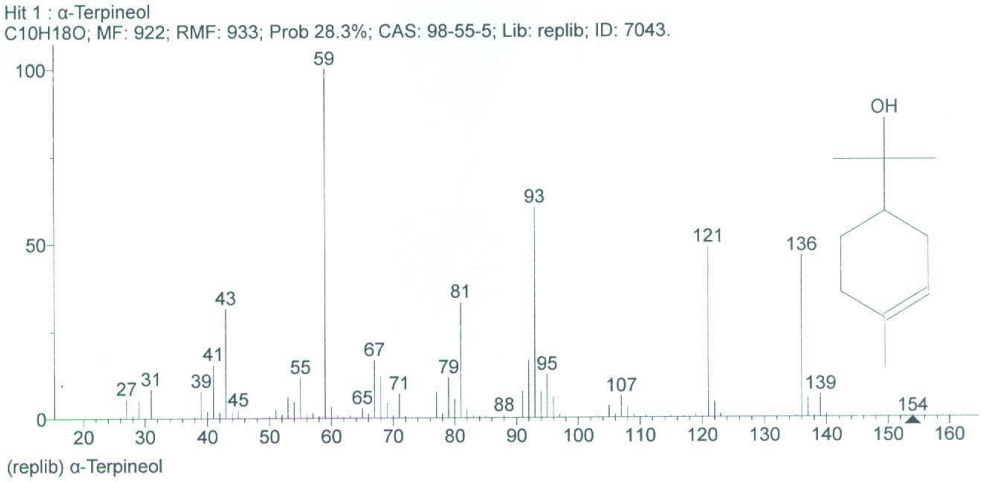
Appendix XVII: MS Spectrum of Compound (50)

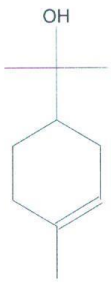




Name: 1-Hydroxy-1,7-dimethyl-4-isopropyl-2,7-cyclodecadiene
Formula: $C_{15}H_{26}O$
MW: 222 Exact Mass: 222.198365 CAS#: 72120-50-4 NIST#: 141062 ID#: 45390 DB: mainlib
Other DBs: None
Contributor: Mark Whitten, Florida Museum of Natural History, U. of Florida
Synonyms:
1.Germacrene D-4-ol
2.4-Isopropyl-1,7-dimethyl-2,7-cyclodecadien-1-ol #
3.Germacren D-4-ol

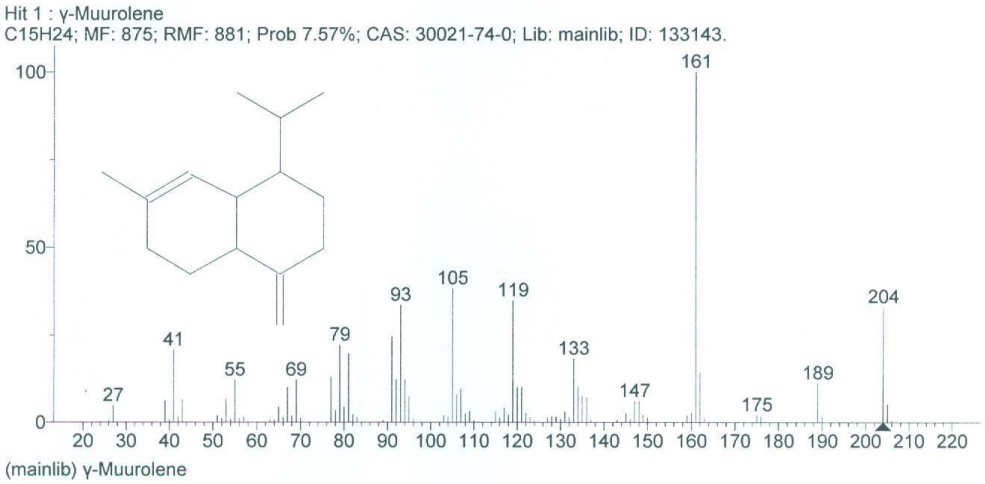
Appendix XVIII: MS Spectrum of Compound (51)

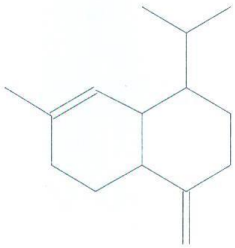




Name: α-Terpineol
Formula: $C_{10}H_{18}O$
MW: 154 Exact Mass: 154.135765 CAS#: 98-55-5 NIST#: 186483 ID#: 7043 DB: replib
Other DBs: Fine, TSCA, RTECS, EPA, NIH, EINECS, IRDB
Contributor: Chemical Concepts
Related CAS#: 2438-12-2; 22347-88-2
Synonyms:
1.3-Cyclohexene-1-methanol, α,α4-trimethyl-
2.p-Menth-1-en-8-ol
3.Terpineol schlechthin
4.Terpineol, α
5.α-Terpinol
6.α,α,4-Trimethyl-3-Cyclohexene-1-methanol
7.2-(4-Methyl-3-cyclohexen-1-yl)-2-propanol #
8.alpha-Terpineol
9.2-(4-methylcyclohex-3-enyl)propan-2-ol

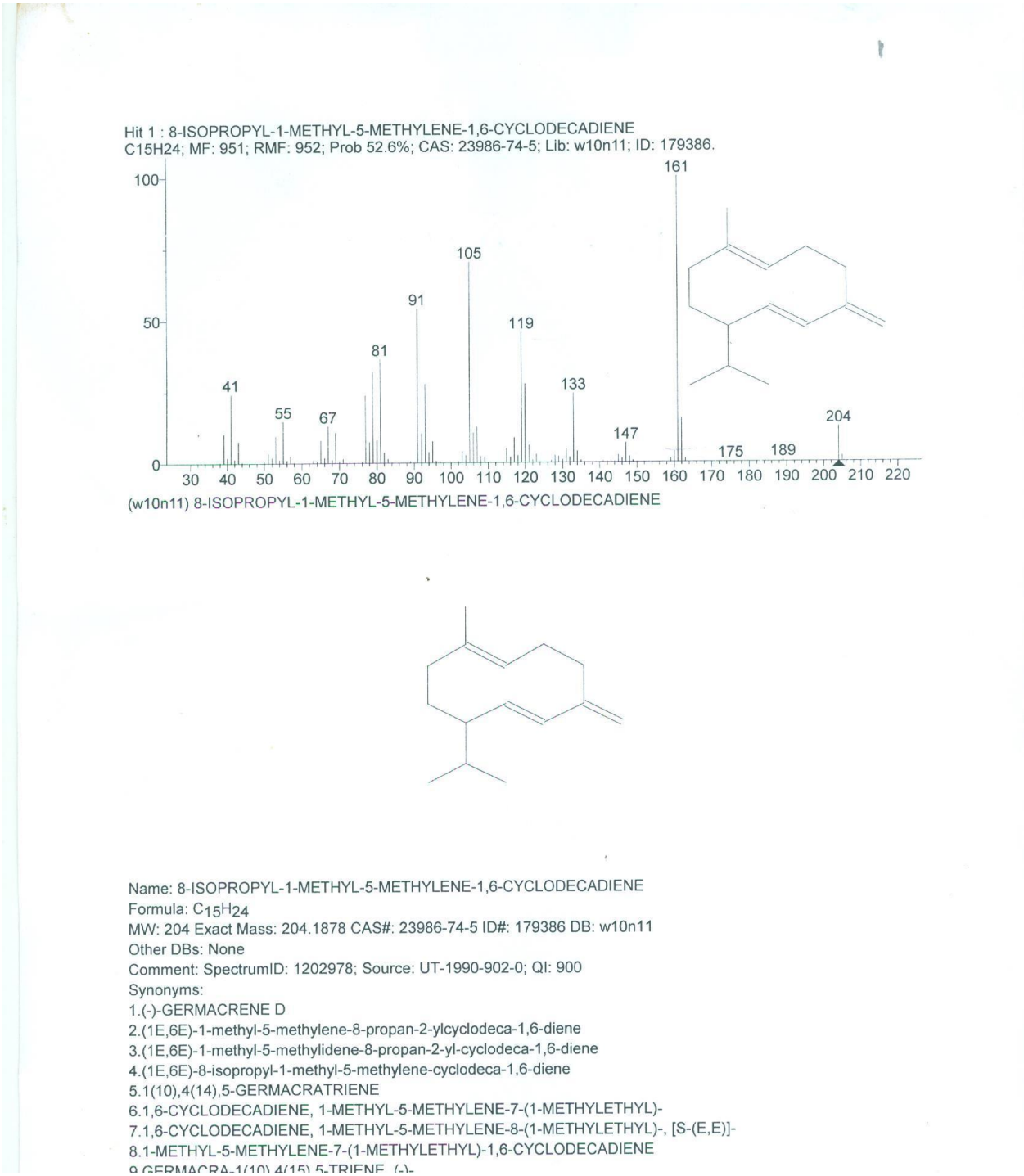
Appendix XIX: MS Spectrum of Compound (52)





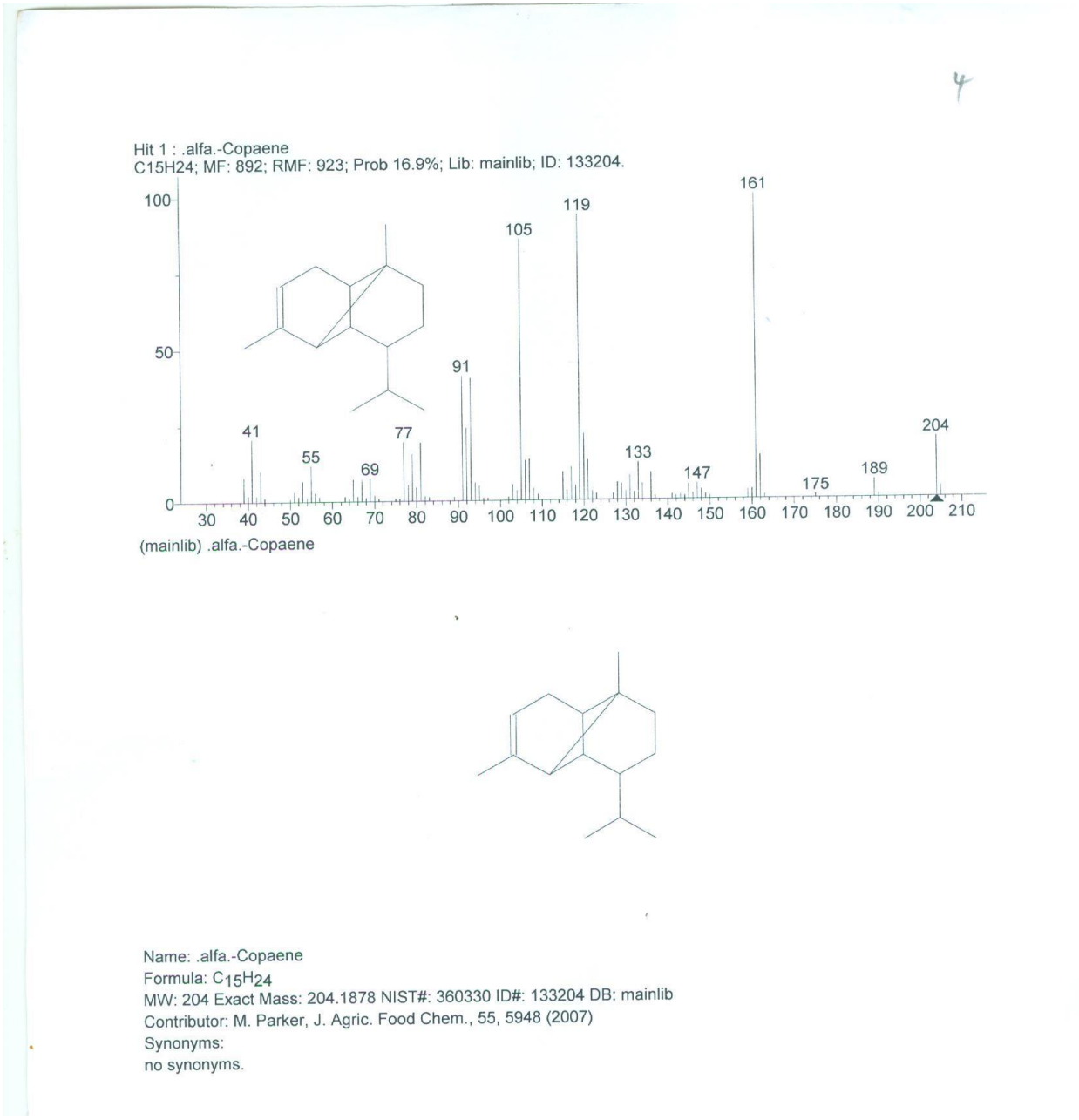
Name: γ-Muurolene
Formula: $C_{15}H_{24}$
MW: 204 Exact Mass: 204.1878 CAS#: 30021-74-0 NIST#: 9234 ID#: 133143 DB: mainlib
Other DBs: None
Synonyms:
1.Naphthalene, 1,2,3,4,4a,5,6,8a-octahydro-7-methyl-4-methylene-1-(1-methylethyl)-, (1α,4aα,8aα)-
2.1-Isopropyl-7-methyl-4-methylene-1,2,3,4,4a,5,6,8a-octahydronaphthalene-, (1α,4aα,8aα)-
3.[1α,4aα,8aα]-Naphthalene,1,2,3,4,4a,5,6,8a-octahydro-7-methyl-4-methylene-1-[1-methylethyl]
4.(±)-γ-Muurolene

Appendix XX: MS Spectrum of Compound (53)



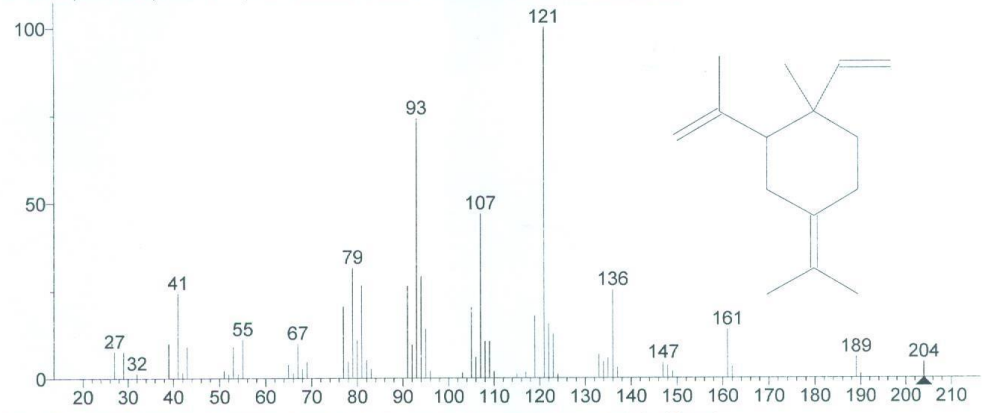
Name: 8-ISOPROPYL-1-METHYL-5-METHYLENE-1,6-CYCLODECADIENE
Formula: $C_{15}H_{24}$
MW: 204 Exact Mass: 204.1878 CAS#: 23986-74-5 ID#: 179386 DB: w10n11
Other DBs: None
Comment: SpectrumID: 1202978; Source: UT-1990-902-0; QI: 900
Synonyms:
1.(-)-GERMACRENE D
2.(1E,6E)-1-methyl-5-methylene-8-propan-2-ylcyclodeca-1,6-diene
3.(1E,6E)-1-methyl-5-methylidene-8-propan-2-yl-cyclodeca-1,6-diene
4.(1E,6E)-8-isopropyl-1-methyl-5-methylene-cyclodeca-1,6-diene
5.1(10),4(14),5-GERMACRATRIENE
6.1,6-CYCLODECADIENE, 1-METHYL-5-METHYLENE-7-(1-METHYLETHYL)-
7.1,6-CYCLODECADIENE, 1-METHYL-5-METHYLENE-8-(1-METHYLETHYL)-, [S-(E,E)]-
8.1-METHYL-5-METHYLENE-7-(1-METHYLETHYL)-1,6-CYCLODECADIENE
9.GERMACRA-1(10),4(15),5-TRIENE, (-)-

Appendix XXI: MS Spectrum of Compound (54)

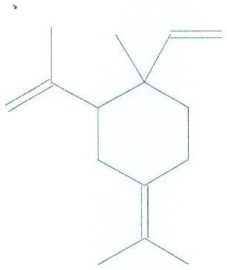


Appendix XXII: MS Spectrum of Compound (55)

Hit 1 : Cyclohexane, 1-ethenyl-1-methyl-2-(1-methylethenyl)-4-(1-methylethylidene)-
C15H24; MF: 928; RMF: 952; Prob 45.3%; CAS: 3242-08-8; Lib: mainlib; ID: 91871.

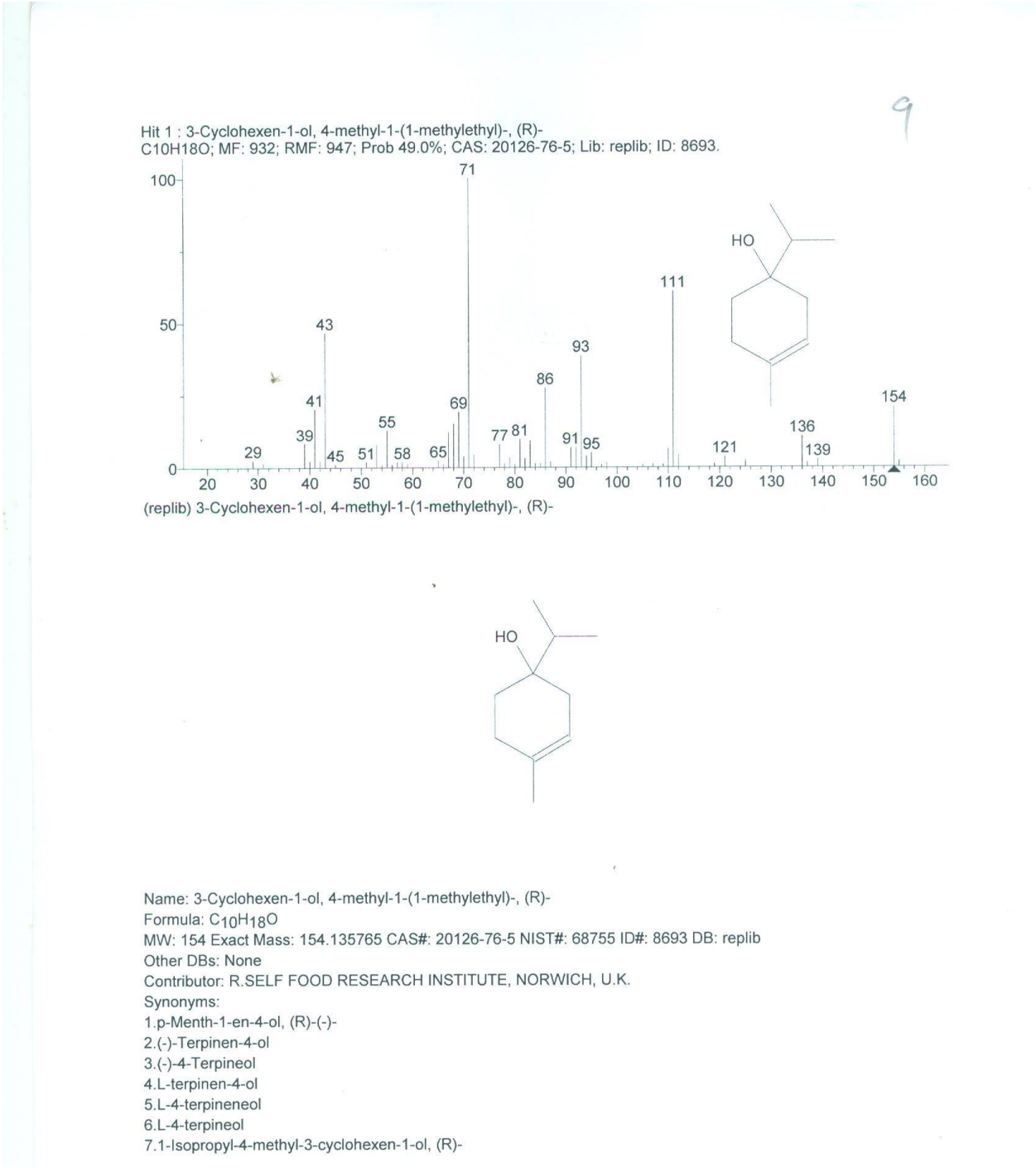


(mainlib) Cyclohexane, 1-ethenyl-1-methyl-2-(1-methylethenyl)-4-(1-methylethylidene)-

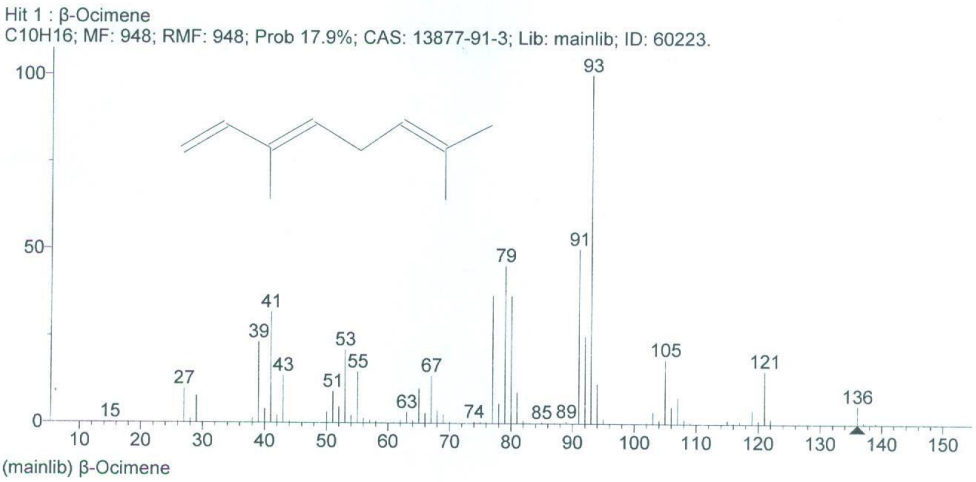


Name: Cyclohexane, 1-ethenyl-1-methyl-2-(1-methylethenyl)-4-(1-methylethylidene)-
Formula: $C_{15}H_{24}$
MW: 204 Exact Mass: 204.1878 CAS#: 3242-08-8 NIST#: 70223 ID#: 91871 DB: mainlib
Other DBs: EINECS
Contributor: N.W. DAVIES, UNIVERSITY OF TASMANIA, TASMANIA, AUSTRALIA
Synonyms:
1.o-Menth-8-ene, 4-isopropylidene-1-vinyl-
2.Elixene
3.2-Isopropenyl-1-methyl-4-(1-methylethylidene)-1-vinylcyclohexane #
4.1-Methyl-4-(1-methylethylidene)-2-(1-methylvinyl)-1-vinylcyclohexane

Appendix XXIII: MS Spectrum of Compound (56)



Appendix XXIV: MS Spectrum of Compound (57)

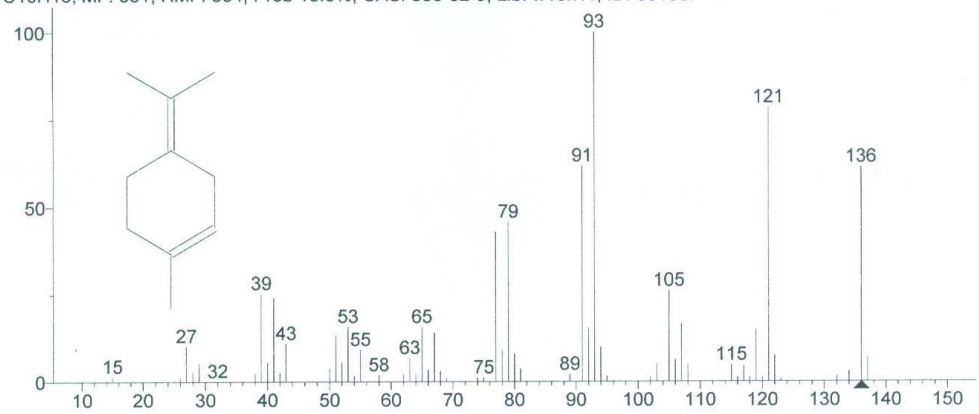


Name: β-Ocimene
Formula: $C_{10}H_{16}$
MW: 136 Exact Mass: 136.1252 CAS#: 13877-91-3 NIST#: 342054 ID#: 60223 DB: mainlib
Other DBs: TSCA, HODOC, EINECS
Contributor: NIST Mass Spectrometry Data Center
Related CAS#: 1856-63-9; 11009-78-2; 11022-64-3
Synonyms:
1.1,3,6-Octatriene, 3,7-dimethyl-
2.Ocimene
3.3,7-Dimethyl-1,3,6-octatriene
4.beta-Ocimene

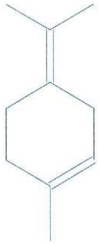
Appendix XXV: MS Spectrum of Compound (58)

12

Hit 1 : Cyclohexene, 1-methyl-4-(1-methylethylidene)-
C10H16; MF: 851; RMF: 864; Prob 18.0%; CAS: 586-62-9; Lib: w10n11; ID: 35136.

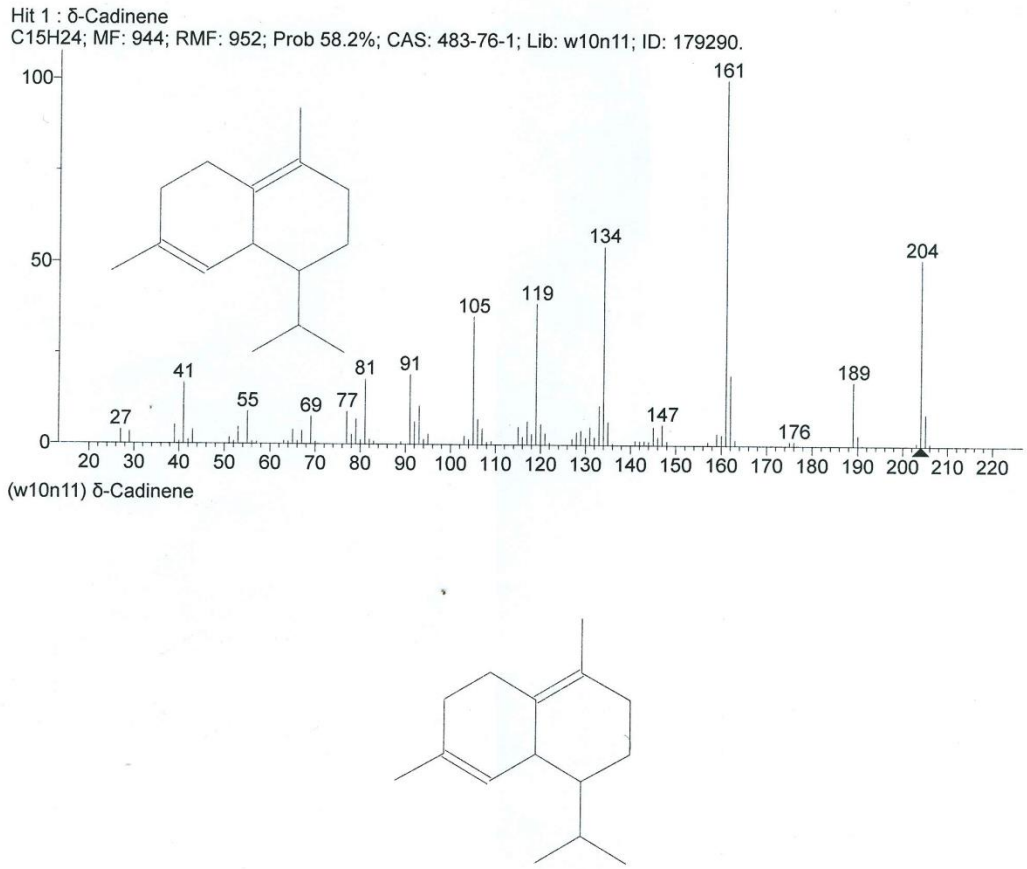


(w10n11) Cyclohexene, 1-methyl-4-(1-methylethylidene)-



Name: Cyclohexene, 1-methyl-4-(1-methylethylidene)-
Formula: $C_{10}H_{16}$
MW: 136 Exact Mass: 136.1252 CAS#: 586-62-9 NIST#: 114838 ID#: 35136 DB: w10n11
Other DBs: None
Comment: SpectrumID: 2060500; Source: NS-6-500-0; QI: 852
Synonyms:
1.p-Mentha-1,4(8)-diene
2.Terpinolene
3.Terpinolen
4.UN 2541
5.α- Terpinolen
6.1-Methyl-4-(1-methylethylidene)-1-cyclohexene #
7.α-Terpinolene
8.4-Isopropylidene-1-methyl-cyclohexene
9.p-Mentha-1,4(8)-diene

Appendix XXVI: MS Spectrum Compound (59)



Name: δ-Cadinene
Formula: $C_{15}H_{24}$
MW: 204 Exact Mass: 204.1878 CAS#: 483-76-1 ID#: 179290 DB: w10n11
Other DBs: None
Comment: SpectrumID: 1202691; Source: A-1-507-1; QI: 1000; NBS#: 69486
Synonyms:
1.Cadina-1(10),4-diene
2.Naphthalene, 1,2,3,5,6,8a-hexahydro-4,7-dimethyl-1-(1-methylethyl)-, (1S-cis)-
3.1-ISOPROPYL-4,7-DIMETHYL-1,2,3,5,6,8A-HEXAHYDRONAPHTHALENE
4.(+)-δ-CADINENE
5.(+)-delta-cadinene
6.δ-CADINENE (ARMOISE-MAROC)
7.δ-CADINENE, (+)-
8.1,2,3,5,6,8a-hexahydro-4,7-dimethyl-1-(1-methylethyl)-naphthalene
9.1,2,3,5,6,8a-hexahydro-4,7-dimethyl-1-(1-methylethyl)naphthalene(1S,8aR)
10.1-ISOPROPYL-4,7-DIMETHYL-1,2,3,5,6,7-HEXAHYDRONAPHTHALENE