ISOLATION AND STRUCTURE ELUCIDATION OF LARVICIDAL COMPOUNDS FROM *LAGGERA ALATA* AND *CLAUSENA ANISATA*

MOSES ABEDNEGO OLLENGO

A Thesis Submitted to Graduate School in Partial Fulfillment of the Requirements for the Master of Science Degree in Chemistry of Egerton University

EGERTON UNIVERSITY

APRIL 2011

DECLARATION AND RECOMMENDATION

DECLARATION

I declare that this thesis is my original work and that it has not been previously presented in this or any other university for award of any degree.

MOSES ABEDNEGO OLLENGO

SM11/2025/07

Signature é é é é é é .. Date: é é é é é é é é é é

RECOMMENDATION

This thesis has been presented for examination with our approval as university supervisors:

PROF. J. C. MATASYOH

EGERTON UNIVERSITY

NJORO

Signatureé é é é é é é é é Dateé é é é é é é é é é

DR. J.M. VULULE

KEMRI

KISUMU

Signatureé é é é é é é é é é Dateé é é é é é é é é é

COPY RIGHT

DEDICATION

To my family: Sylvia, Newton, Faith and Mercy.

ACKNOWLEDGEMENT

I am deeply indebted to Prof. J. C Matasyoh and Dr. J.M. Vulule, supervisors of this thesis work, for their great support, guidance and encouragement during the whole period of this work. I thank them for giving me deep insights in the fascinating world of natural products chemistry. Their advice, patience and understanding kept me motivated during my research work.

Profound appreciation to Prof. Martin E. Maier of the Institute of Organic Chemistry, University of Tuebingen, Germany for availing the use of GC-MS and NMR equipment. I am very thankful to Prof. Uzi Ravid of Newe Yaa´r Center in Israel for making available the GCï MS apparatus for this work. I also wish to thank everyone at the Department of Chemistry, Egerton University, especially all the technical staff members for being very cooperative and providing a very good working environment, which kept me strong even at times when I seemed to be overwhelmed by the research work. I am very grateful to Mr. S. M. Kariuki, Chief lab technologist Egerton University, Mrs. R. Chepkorir, very able research lab technician and Mrs. K. Margaret for being supportive.

Thanks to Kenya Agricultural Research Institute, Njoro for availing me, a miller, which I used to grind the samples. Special thanks go to the Kenya Medical Research Institute, for allowing me to use their insectary laboratory to perform larvicidal tests. I thank Mr. R. Amito and his team for his assistance during the bioassays.

Special thanks to the Teachers Service Commission for granting me paid study leave. Moreover, to my classmates Peter R., Nancy N. and the entire MSc. Chemistry 2007 class for their support during the whole period of my study. Utmost gratitude to Sophie Ondiaka of ICIPE- Malaria Programme for her dedication to read through the thesis and insights she gave in this interesting field of malaria research.

Exceptional gratitude goes to my wife, Sylvia and children: Newton, Faith and Mercy for all their love, support, and understanding. Thanks for being such wonderful people in my life. Glory is to GOD.

ABSTRACT

Laggera alata is in the Asteraceae family, and. *Clausena anisata* is in the family of Rutaceae both plants have shown bioactivity against several diseases. Malaria is by far the most important insect transmitted disease. There is no so far vaccine to prevent infection caused by *A. gambiae* mosquito and the malaria parasite is continually developing resistance to the available drugs, so vector control is the best option. Dried, ground and weighed 600g of aerial parts for each plant (*L. alata* and *C. anisata*) were sequentially extracted with hexane, ethyl acetate, chloroform, acetone and methanol. The solvents were removed by rotor evaporation under vacuum to give five extracts for each species. Fresh whole plants of *L. alata* and *C. anisata* were subjected to hydro-distillation in a modified Clevenger-type apparatus for at least four hours according to the British pharmacopoeia. The essential oils obtained was 7g of C. *anisata* oils constituting 4.25% and 5g of *L. alata* oils accounting for 2.78% in a yield of w/w after drying over anhydrous sodium sulphate respectively. The oils were subjected to GC, GC-MS to determine the composition. In *L. alata* oils, the major compounds were: 2, 5-dimethoxy-para -Cymene 24.4%, cis-Chrysanthenol 11.8%. The oils from *C. anisata* gave the following major compound composition: ϐ-Phelandrene (Limonene) 20.1%, Germacrene-D 18.8%, ɔ-Terpinene 13.8%. The bioassays were performed with third instar larvae of *A. gambiae s.s,* carried out in triplicate using 20 larvae for each replicate assay. From larvicidal assay the LC_{50} of the *L. alata* oils was found to be 273.38 mg/l and that of *C. anisata* was 75.96 mg/l. The LC_{99} for *L. alata* was 507.75 mg/l and *C. anisata* was 256.80 mg/l. LC_{50} of *L. alata* hexane fraction was 1161.30 mg/l and the corresponding LC_{99} was 2734.91 mg/l. The *C. anisata* ethyl acetate fraction gave LC_{50} as 2095.46 mg/l and LC_{99} was 4438.75 mg/l. The ethyl acetate fraction of *C. anisata* was subjected to GC-MS analysis to determine total chemical composition. The hexane fraction of *L. alata* was subjected to chromatographic separation leading to isolation and purification of compound 1 and 2. Spectroscopic analysis was done to elucidate the structure of new eudesmane sesquiterpenoids: 3ϐ-angeloyloxy-4ϐ-hydroxy-eudesm-7, 11-en-8-one **(1)** and 3ϐ-angeloyloxy-4ϐ-acetoxy-11-hydroxy eudesm-6-en-8-one (**2).** Application of these extracts to larval habitats may lead to promising results in malaria and mosquito management programmes. The isolated larvicidal compounds can be used as lead compounds for environmentally friendly and biodegradable larvicides.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS AND ACRONYMS

CC	Column Chromatography
CDC	Center for Decease Control
COSY	Correlation Spectroscopy
DDT	Dichlorodiphenyltrichloroethane
DEPT	Distortion less Enhancement by Polarization Transfer
DMSO	Dimethylsulphoxide
ESIMS	Electron Spray Ionization Mass Spectrometry
GC-MS	Gas Chromatography-Mass Spectroscopy
HMBC	Heteronuclear Multiple Bond Correlation
IRS	Indoor residual spray
ITNs	Insecticide treated nets
KEMRI	Kenya Medical Research Institute
LC_{50}	Lethal concentration that is able to kill 50% of the target organisms
LC_{99}	Lethal concentration ï 99%, the dose of an active ingredient, which is expected to cause death in 99% of the test organisms, treated.
NMR	Nuclear Magnetic Resonance
RPM	Revolutions Per Minute
TLC	Thin Layer Chromatography
TMS	Tetra-methylsilane
ID NMR	One Dimensional Proton Nuclear Magnetic Resonance
2D NMR	Two Dimensional Proton Nuclear Magnetic Resonance
^{13}C NMR	Carbon-13 Nuclear Magnetic Resonance
HSQC	Heteronuclear single quantum correlation

Other abbreviations used are standard according to IUPAC

CHAPTER ONE

INTRODUCTION

1.1 Background information

Mosquitoes constitute a major public health problem as vectors of serious human diseases (El Hag *et al.,* 1999). Hubalek and Halouzka (1999) reported that *Culex pipiens* is the vector of West Nile virus that causes encephalitis or meningitis, which is known to affect the brain tissue, finally resulting in permanent neurological damage. Several mosquito species belonging to genera *Anopheles*, *Culex* and *Aedes* are vectors for the pathogens of various diseases like malaria, filariasis, Japanese encephalitis, dengue fever, dengue hemorrhagic fever and yellow fever (Hubalek and Halouzka, 1999). The inefficiency of the organophosphate and carbamate insecticides (Bracco *et al.,* 1999), along with the need for safer methods regarding toxicity to man and the environment has stimulated the search for new means of control. Oil-resin or plant extracts are an alternative with potential for use.

Although some diseases such as yellow fever have been reasonably brought under control by vaccination, no effective vaccine is available for malaria (Matasyoh *et al*., 2008). Several drugs, most of which are also used for treatment of malaria, can be taken preventively. Modern drugs used include mefloquine (*Lariam*), doxycycline (available generically), and the combination of atovaquone and proguanil hydrochloride (*Malarone*). Doxycycline and the atovaquone and proguanil combination are the best tolerated with mefloquine associated with higher rates of neurological and psychiatric symptoms (Jacquerioz and Croft 2009). The choice of which drug to use depends on which drugs the parasites in the area are resistant to, as well as side-effects and other considerations. Use of prophylactic drugs is seldom practical for full-time residents of malaria-endemic areas, and their use is usually restricted to short-term visitors and travelers to malarial regions. This is due to the cost of purchasing the drugs, negative side effects from long-term use, and because some effective anti-malarial drugs are difficult to obtain outside of wealthy nations. Quinine was used historically, however the development of more effective alternatives such as quinacrine, chloroquine, and primaquine in the 20th century reduced its use. Today, quinine is not generally used for prophylaxis. The use of prophylactic drugs where malaria-

bearing mosquitoes are present may encourage the development of partial immunity (Roestenberg *et al*., 2009)

Control of *A. gambiae s.s* is of particular interest because it acts as a vector of malaria. Methods used in order to prevent the spread of disease, or to protect individuals in areas where malaria is endemic, include prophylactic drugs, mosquito eradication and the prevention of mosquito bites. The prevention of malaria may be more cost-effective than treatment of the disease in the long run, but the capital costs required are out of reach of many of the world's poorest people. Therefore, the only efficacious approaches of minimizing the incidence of this disease are to eradicate or control mosquito vectors mainly by application of insecticides to larval habitats. It has been shown that plant- derived natural products used as larvicides have the advantage of being harmless to non-target organisms and no vector resistance observed so far (Wattal *et al*., 1981).

In the recent years, the emphasis to control the mosquito populations has shifted steadily from use of conventional chemicals towards more specific and environmentally friendly materials, which are generally of botanical origin. For this purpose, many phytochemicals extracted from various plants species have been tested for their larvicidal and repellant actions against mosquitoes (Ciccia *et al*., 2000; Ansari and Razdan, 2000). One strategy of the WHO in combating tropical diseases is to destroy their vectors or intermediate hosts. Malaria is a parasitic disease from which more than 300 million people suffer yearly throughout the world. It is one of the main causes of infant and young child mortality (WHO, 1995). As part of continued search of the biodiversity resource available in Kenya for natural products with utilizable bioactivity, two plants of choice were taken for this work. The two have shown good bioactivity from ethenopharmacological point of view. However, no larvicidal activity of these plants has been reported. Compounds with larvicidal activity isolated from *C. anisata* and *L. alata* towards *A. gambiae s.s.* were assayed

1.2 Statement of the problem

The single most important insect transmitted disease globally is malaria. Recent WHO estimates are that there are 300- 500 million cases of clinical malaria per year, with 2.6 million deaths, mainly among African children. Malaria is therefore a major cause of infant mortality and is the only insect borne parasitic disease comparable in impact to the worldôs major killer transmissible diseases: diarrhea, acute respiratory infections, tuberculosis and AIDS. However, there are major problems of parasite drug resistance necessitating policy change to more expensive drugs which the majority of the afflicted cannot afford. Similarly there is a notable vector resistance to insecticide. These has led to interest in the development of malaria vaccines but the only one, which has been extensively field-tested, only gave a limited degree of protection. The main limitation in malarial eradication is resistance of the parasite to anti-malarial drugs, coupled with toxicity of the drugs to human health and their high cost. The situation is much more grave especially for the third world countries which are mostly infested with *A. gambiae*; a notorious carrier of the parasite. In order to reduce incidences of malaria, it is important to look at preventive measures that involve the control of the malaria vector.

1.3 Objectives of the study

1.3.1 Main objective

To isolate, purify and elucidate the structures of larvicidal compounds from *L. alata* and *C. anisata.*

1.3.2 Specific objectives

i. To evaluate larvicidal activity of crude extracts and essential oils from the plants.
ii. To carry out bioassay guided isolation and purification of larvicidal compounds from the plant extracts.
iii. To elucidate the structures of the active compounds using GC-MS and NMR spectroscopy.

iv. To determine the LC_{50} and LC_{99} for the active secondary metabolites.

1.4 Justification

There is no vaccine to prevent infection caused by *A. gambiae* mosquito and the malaria parasite is continually developing resistance to the available drugs, so vector control is the best option (Matasyoh *et al*., 2008). A considerable number of plant derivatives have shown to be effective against mosquitoes (El Hag *et al.,* 1999). Larval control strategies against the vectors of malaria in sub-Saharan Africa should be prioritized for further development, evaluation and implementation as an integral part of controlling malaria. The ideal method of controlling mosquito infestation is the prevention of its breeding through use of larvicides. Many synthetic larvicides have been used in several countries since the 1960ôs (Romi *et al*., 2003). However, resistance to pesticides has guided research to develop new tools to control mosquitoes. In addition, the synthetic insecticides are toxic and adversely affect the environment by polluting soil, water and air. Recent research has focused on natural product alternatives. Plant-based larvicides appear to have no ill effects on non-target populations and are biodegradable, in addition to being available in many parts of the world. The scientific rationalization of the larvicidal activity of the plant extracts can lead to their use in the rural villages ravaged by malaria. The isolated larvicidal compounds can be used as lead compounds for environmentally friendly and biodegradable larvicides. The mosquitoes are also unlikely to develop resistance to these types of larvicides.

CHAPTER TWO

LITERATURE REVIEW

2.1 *Anopheles gambiae*

Mosquitoes undergo complete metamorphosis; they go through four distinct stages of development during a lifetime as shown in its life cycle (Fig. 1) below. The four stages are egg, pupa, larvae, and adult. The full life cycle of a mosquito takes about a month.

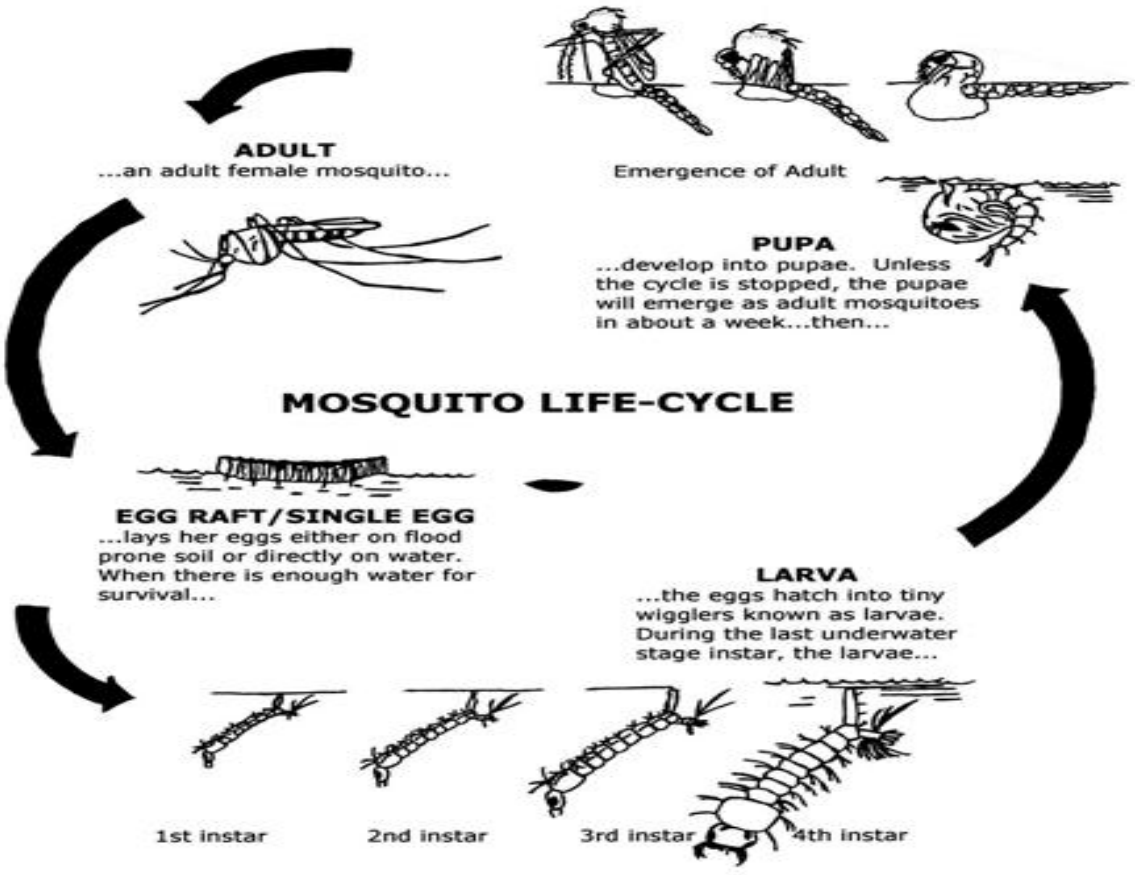


Fig. 1: Life cycle of mosquito (Singh *et al.,* 2006**).**

Anopheles gambiae as in other mosquitoes, only females bite and they use the proteins from a blood meal to produce a batch of eggs (Singh *et al.,* 2006). These are laid in relatively clean

water, such as in marshes, puddles, irrigation water etcetera. Unlike other mosquito larvae, those of anopheles float parallel to the water surface. They develop through four larval instars to a short-lived, motile pupa stage. The whole process from egg to emergence of the pupa takes little more than a week at tropical temperatures.

2.2 Progress in malaria control by targeting the malaria parasite

Malaria is a mosquito-borne infectious disease of humans caused by eukaryotic protists of the genus *Plasmodium*. It is widespread in tropical and subtropical regions, including much of Sub-Saharan Africa, Asia and the Americas. The disease results from the multiplication of malaria parasites within red blood cells, causing symptoms that typically include fever and headache, in severe cases progressing to coma, and death (Kilama, 2009).

Four species of *Plasmodium* can infect and be transmitted by humans. Severe disease is largely caused by *Plasmodium falciparum*. Malaria caused by *Plasmodium vivax*, *Plasmodium ovale* and *Plasmodium malariae* is generally a milder disease that is rarely fatal. A fifth species, *Plasmodium knowlesi*, is a zoonosis that causes malaria in macaques but can also infect humans (Fong *et al.,* 1971, Singh *et al*., 2004)

Malaria transmission can be reduced by preventing mosquito bites by distribution of inexpensive mosquito nets and insect repellents, or by mosquito-control measures such as spraying insecticides inside houses and draining standing water where mosquitoes lay their eggs. Although many are under development, the challenge of producing a widely available vaccine that provides a high level of protection for a sustained period is still to be met (Kilama, 2009) Two drugs are also available to prevent malaria in travellers to malaria-endemic countries (prophylaxis).

A variety of antimalarial medications are available. In the last 5 years, treatment of *P. falciparum* infections in endemic countries has been transformed by the use of combinations of drugs containing an artemisinin derivative. Severe malaria is treated with intravenous or intramuscular quinine or, increasingly, the artemisinin derivative artesunate (Dondorp and Day, 2007) which is

superior to quinine in both children and adults (Dondorp *et al*., 2010) Resistance has developed to several antimalarial drugs, most notably chloroquine (Wellems, 2002)

Each year, there are more than 225 million cases of malaria, (Phillips, 2009) killing around 781,000 people each year according to the latest WHO Report, 2.23% of deaths worldwide. The majority of deaths are of young children in sub-Saharan Africa (Snow *et al.,* 2005) Ninety percent of malaria-related deaths occur in sub-Saharan Africa. Malaria is commonly associated with poverty, and can indeed be a cause of poverty (Phillips, 2009) and a major hindrance to economic development.

2.3 Vector Control

Vector control is an important strategy as a means to control mosquitoes and prevent malaria as well as several other mosquito-borne diseases. Methods in place for the prevention of malaria includes use of insecticide-treated bed nets, indoor residual spraying, outdoor fogging, sterile male technique, source reduction (larval control) and plant extracts as larvicides.

2.3.1 Insecticide-treated bed nets and Lasting Impregnated Nets

Insecticide-treated bed nets (ITNs) are a form of personal protection that has repeatedly been shown to reduce severe disease and mortality due to malaria in endemic regions. In community-wide trials in several African settings, ITNs have been shown to reduce infant mortality by about 20%. Currently, only pyrethroid insecticides are approved for use on ITNs (CDC, 2008). These insecticides have very low mammalian toxicity but are highly toxic to insects and have a rapid knockdown effect, even at very low doses. Pyrethroids have a high residual effect: they do not rapidly break down unless washed or exposed to sunlight.

The need for frequent retreatment was a major barrier to full implementation of ITNs in endemic countries. The additional cost of the insecticide and the lack of understanding of its importance resulted in very low retreatment rates. In most African countries nets had to be retreated at intervals of 6-12 months, more frequently if the nets were washed. nets were retreated by simply

dipping them in a mixture of water and insecticide and allowing them to dry in a shady place (CDC, 2008).

The development of mosquito nets pre-treated with insecticide, Long Lasting Impregnated Nets (LLINs) that last the life span of the net, is a solution to the difficulty of the re-impregnation of conventional nets. Even if they have showed a good efficacy in control conditions, their efficacy in the field, particularly in areas with resistance of *A. gambiae* to pyrethroids, is not well documented (Roch *et al*., 2006). Similarly high mortality rate of vectors can be needed only when the objective of the programme is to achieve community protection through a mass impact of the treated nets on the mosquito population. However, it should be stressed that such protection, equivalent to that obtained with indoor residual spraying, can be achieved only if a very high proportion of the human population (for example > 80%), is effectively protected by nets treated with an insecticide that kills mosquitoes (Pierre, 2008).

2.3.2 Indoor Residual Spraying

Many malaria vectors are endophilic, resting inside houses after taking a blood meal (Sharma e*t al*., 1990). These mosquitoes are particularly susceptible to control through indoor residual spraying (IRS). As its name implies, IRS involves coating the walls and other surfaces of a house with a residual insecticide. For several months, the insecticide will kill mosquitoes and other insects that are exposed to these surfaces. IRS does not directly prevent people from being bitten by mosquitoes. Rather, it usually kills mosquitoes after they have fed, if they come to rest on the sprayed surface. IRS thus prevents transmission of infection to other persons. To be effective, IRS must be applied to a very high proportion of households in an area (usually >70%). IRS with DDT and dieldrin was the primary malaria control method used during the Global Malaria Eradication Campaign (1955-1969). The campaign did not achieve its stated objective but it did eliminate malaria from several areas and sharply reduced the burden of malaria disease in others (Pitasawat *et al*., 2007). As a result of the cost of IRS, the negative publicity due to the failure of the Malaria Eradication Campaign, and environmental concerns about residual insecticides, IRS programs were largely disbanded other than in a few countries with resources to continue them.

One problem with all forms of Indoor Residual Spraying is insecticide resistance via evolution of mosquitos. According to a study published on Mosquito Behavior and Vector Control, mosquito species that are affected by IRS are endophilic species (species that tend to rest and live indoors), and due to the irritation caused by spraying, their evolutionary descendants are trending towards becoming exophilic (species that tend to rest and live out of doors), meaning that they are not as affectedð if affected at allð by the IRS, rendering it somewhat useless as a defense mechanism (Pates and Curtis, 2005).

Recent data re-conýrms the efficacy and effectiveness of IRS in malaria control in countries where it was implemented well. However, there are important considerations that must be taken into account when considering whether to introduce or scale up IRS. In particular, there must be sufficient capacity to deliver the intervention effectively, prevent unauthorized and un-recommended use of public health pesticides, and manage insecticide resistance. Intensiýed research efforts are needed, for example to develop new insecticides, long-acting formulations and improved application technologies (WHO, 2006).

2.3.3 Outdoor fogging

Fogging or area spraying is primarily reserved for emergencies: halting epidemics or rapidly reducing adult mosquito populations when they have become severe pests. Fogging or area sprays must be properly timed to coincide with the time of peak adult activity, because resting mosquitoes are often found in areas that are difficult for the insecticide to reach (for example, under leaves, in small crevices). However, the method is too costly and also eliminates non target organisms

2.3.4 Sterile male technique

Sterile insect technique is emerging as a potential mosquito control method. Progress towards transgenic, or genetically modified, insects suggest that wild mosquito populations could be made malaria-resistant. The first transgenic malaria mosquito (Imperial College, 2000), with the first plasmodium-resistant species was reported in 2002 (Ito *et al*., 2007). Successful replacement

of current populations with a new genetically modified population relies upon a drive mechanism, such as transposable elements to allow for non-Mendelian inheritance of the gene of interest. However, this approach contains many difficulties and success is a distant prospect (Knols *et al.,* 2002). An even more futuristic method of vector control is the idea that lasers could be used to kill flying mosquitoes. Sterile male release has been successfully applied in several small-scale areas. Needless to say, the need for large numbers of mosquitoes for release and driving the genes to fixation makes this approach impractical for most areas. Genetic modification of malaria vectors aims to develop mosquitoes that are refractory to the parasite. This approach is still several years from application in field settings (Knols *et al.,* 2002).

2.3.5 Source Reduction (Larval Control)

Source reduction is the method of choice for mosquito control when the mosquito species targeted are concentrated in a small number of discrete habitats. The larval habitats may be destroyed by filling depressions that collect water, draining swamps and ditching marshy areas to remove standing water. Container-breeding mosquitoes are particularly susceptible to source reduction as people can be educated to remove or cover standing water in cans, cups, and rain barrels around houses. Mosquitoes that breed in irrigation water can be controlled through careful water management. For some mosquito species, habitat elimination is not possible. For these species, chemical insecticides can be applied directly to the larval habitats. Source reduction is an ideal approach to mosquito control (CDC, 2008). Mosquito larvae aggregate in defined areas, and source reduction eliminates mosquitoes before they reach the stage that is responsible for disease transmission.

The malarial control measures should concentrate on stopping the mosquito at larval stage since the larval mosquito breeding sites can be identified and are relatively small in an area. The adults can fly miles and cause problem over a wide area. The water management explains that larvae are vulnerable to removal of water they need to survive (Obomanu *et al.,* 2006). There are areas where you cannot escape standing water like in lakes, swamps and rice growing areas. Biological control uses fish and other predators to feed on the larvae. This larval control method has little impact on the non-target species and does not affect ground water. These materials are extremely

safe for application. Treating the breeding areas does not involve the exposure of the public since materials are applied to water in swamps, marshes and other non-residential areas (Silvagnaname and Kalyanasundaram, 2004).

Anopheles gambiae, one of the primary vectors of malaria in Africa, breeds in numerous small pools of water that form due to rainfall. The larvae develop within a few days, escaping their aquatic environment before it dries out. It is difficult, if not impossible, to predict when and where the breeding sites will form, and to find and treat them before the adults emerge. Therefore, larval mosquito control for the prevention of malaria in Africa has not been attempted on a large scale

2.3.6 Plant extracts as larvicides

Natural product extracts for instance the extract from the leaves of *Blumea balsamifera* are used for the manufacture of borneol, and *Oxalis corymbosa* is used as a source of pyrethrin pesticides (Lin, 1985). This family is a rich source of sesquiterpenoid natural products, especially those with the eudesmane framework. During the last two decades, eudesmane-type sesquiterpenoids and their biological activities from Asteraceae species have been the focus of numerous phytochemical, pharmacological and synthetic studies. Sesquiterpenoids exhibit a wide range of biological activities, and include compounds that are plant growth regulators, insect anti-feedants, anti-fungals, anti-tumour compounds and anti-bacterial (Quan-Xiang *et al*., 2006). Secondary metabolites of plants, many of them produced by the plant for its protection against micro-organisms and predator insects are natural candidates for the discovery of new products to combat mosquitoes.

Several studies have focused on natural products for controlling *Aedes* mosquitoes as insecticides and larvicides, but with varied results (Consoli *et al*., 1988). Repeated use of synthetic insecticides for mosquito control has disrupted natural biological control systems and led to resurgences in mosquito populations. It has also resulted in the development of resistance (WHO, 1992), undesirable effects on non-target organisms and fostered environmental and human health concern (Kumari *et al*., 1998), which initiated a search for alternative control measures.

Controlling the vectors using various methods can interrupt disease transmission. Plants are considered as a rich source of bioactive chemicals (Sharma *et al*., 1990) and they may be an alternative source of mosquito control agents. Natural products of plant origin with insecticidal properties have been tried in the recent past for control of variety of insect pests and vectors.

Due to the problem of pollution and vector resistance, safe plant products are being tested around the world as pest control agents. Appendix 1 provides details of plant products reported for larvicides growth inhibition and repellent activity against mosquito vectors. A survey of literature on larvicidal effects of plant products on mosquitoes indicates that most of the studies included well-known horticultural and commonly grown plants (Obomanu *et al.,* 2006). But, larvicidal activities of wild plants that are found in vast areas on plains as well as on hilly regions is not attempted so far.

Vector control is facing a threat due to emergence of resistance to synthetic insecticides. Insecticides of botanical origin may serve as a suitable alternative bio-control technique in the future (Matasyoh *et al*., 2008). Although several plants show mosquitocidal activity, only a few botanicals have moved from laboratory to field use, because they are poorly characterized, in most cases, active principals are not determined and most of the works are restricted to preliminary screening (Kaushik and Saini, 2008).

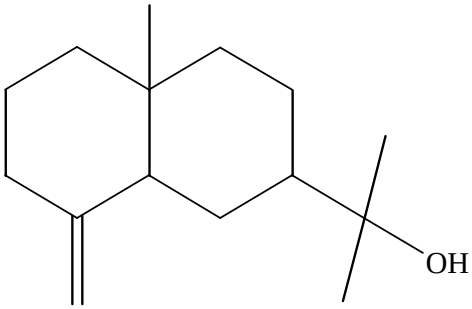
Phytochemicals derived from plant sources acting as larvicides, insect growth regulators, repellent, and ovipositor attractant; have shown different activities observed by many researchers (Kaushik and Saini, 2008). However, insecticides of plant origin have been extensively used on agricultural pests and to a very limited extent, against insect vectors of public health importance. The selective pressure of conventional insecticides is enhancing resistance of mosquito populations at an alarming rate (Matasyoh *et al*., 2007), increasing the demand for new products that are environmentally safe, target-specific and degradable. Co-evolution has equipped plants with a plethora of chemical defenses against insect predators. Aware of this effect, humanity has used plant parts or extracts to control insects since ancient times. Plant derived products have received increased attention from scientists and more than 2000 plant species are already known to have insecticide properties (Sukumar *et al.,* 1991).

2.4 Laggera alata

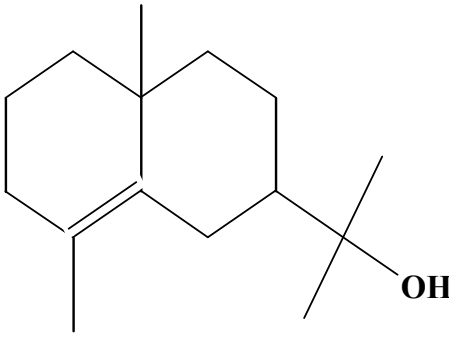
The genus *Laggera* of the Asteraceae family consists of about twenty species found mainly in the tropical Africa and Southeast Asia (Singh *et al.,* 2006). *Laggera alata* (Plate 1) is a robust, much branched glandular pubescent herb, up to 60 -75 cm tall, stem winged, wings herbaceous, entire, rarely somewhat denticulate, and continuous. Leaves oblong, 8 -10 x 0.7-1.5 cm, with decurrent bases and denticulate margins, acute to sub obtuse, densely covered with longer multiseptate hairs. Capitula 1-1.3 cm across, arranged leafy racemes, on short axillary winged branches; phyllaries 4-5-seriate, outer lanceolate 9 x 1 mm, inner longer, up to 10 mm long, green at the apex, glandular pubescent on the outer side. Corolla of female florets 6 mm long, minutely toothed; of bisexual florets 7-8 mm long, 5-lobed. Cypselas dark brown, 1 mm long; pappus setae white, 6-7 mm long (Hyde and Wursten, 2008).

Plate 1*: Laggera alata*

The herb is used as anti-inflammatory agent and for treatment of cancer. The anti-inflammatory activity is due to the inhibition of prostaglandin formation. It is also used as remedy for fever and pneumonia. *L. alata* and *L. pterodonta* are also found in China and both have been employed as traditional herbal medicine for their anti-inflammatory and anti-bacterial activities (Deng, 1963). Both species have undergone phytochemical investigations (Zheng *et al.,* 2003) but no larvicidal studies have been done. Previous investigation of *L. pterodonta* led to the isolation of 20 eudesmane sesquiterpenes and their glucosides (Zhao *et al*., 1997). Phytochemical investigation of the Chinese *L. alata* led to the isolation of four new sesquiterpene glucosides of the eudesmane type and one megastigmane glucoside. Two new eudesmane sesquiterpenes, 7-epi-ɔ-eudesmol and 7- epi- ɓ- eudesmol have been isolated from the Madagascan species (Raharivelomanana *et al*., 1998). The *L. alata* species also grows widely in Kenya. There have been no reports of investigations of larvicidal compounds from this species.



7-epi-ɔ-eudesmol



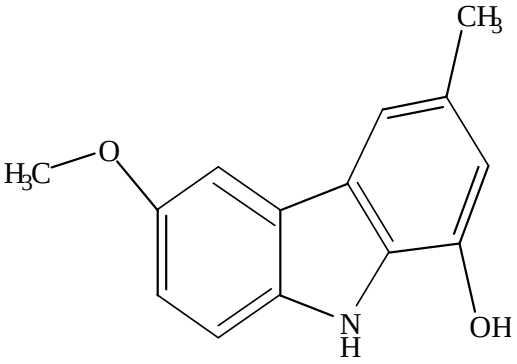
7- epi- ɓ- eudesmol

2.5 *Clausena anisata*

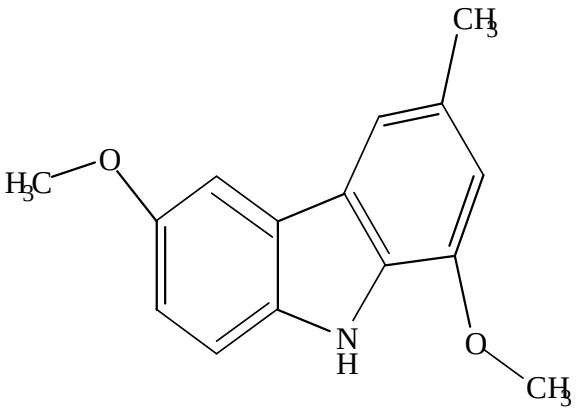
Plate 2: *Clausena anisata*

Clausena anisata (Plate 2) is in the family of *Rutaceae* and grows in the tropics. It is a Shrub or small tree. Leaves are pinnately compound with 10-17 alternate or sub-opposite leaflets and a terminal leaflet. The leaves are densely dotted with glands and have a strong scent when crushed. The scent has been likened to aniseed and opinions vary on its pleasantness. Inflorescence, a branched auxiliary spray; flowers small but attractive, white with orange-yellow stamens (Hyde and Wursten, 2008). It is used as a cure and remedy for epilepsy and convulsions, arthritis, heart ailments, parasitic infections, malaria and diabetes (type 2 *Diabetes mellitus*). There has not been

lot phytochemical investigations of this plant reported but four new carbazole alkaloids were isolated from it as inhibitors of Epsteins-Barr virus early antigen activation induced by 12-O-tetradecanoylphorbol-13-acetate in Raji cells (Ito *et al*., 2000). Chakraborty *et al*. (1995) also isolated two carbazole alkaloids, Clausenol and Clausenine from the alcoholic extract of the stem bark.



Clausenol



Clausenine

CHAPTER THREE

MATERIALS AND METHODS

3.1 Collection and Identification of Plants

Laggera alata is in the well-known medicinal plant family of Asteraceae and grows wildly in the outskirts of Mau forest complex near Molo at an altitude range of 2127 -2137m in Kenya. It is from here that fresh aerial parts of the plant were collected. *Clausena anisata* twigs and leaves were collected from Kakamega equatorial forest in Kenya. The plant grows wildly in the mid altitudes in the range 1500m to 1700m of the tropical rainforest conditions which receive about 2000nm of rainfall a year. The average temperatures remain similar throughout between 15-28^{0}C. A taxonomist identified the plants materials and a voucher specimen deposited at the department of biological sciences of Egerton University.

3.2 Extraction

Both the non-volatile and volatile (essential oils) secondary metabolites were extracted as described in sections 3.2.1, fig. 2 and 3.2.2, fig. 3 respectively.

3.2.1 Non – volatile compounds

The plant materials were dried under shade to constant weight and ground to a fine powder (fig.2). A powder weighing 600 g of the plant powder was extracted sequentially with hexane (3x1.5 L), ethyl acetate (3x1.5 L), chloroform (3x1.5 L), acetone (3x1.5 L) and methanol (3 x 1.5 L) after soaking the sample in each solvent for 24 hours. The extracts were filtered through a Buchner funnel fitted to a vacuum pump with a thin layer of activated charcoal, and then concentrated using a rotary evaporator and the solvent recovered. All crude extracts were partitioned between equal volumes (250 ml each) of distilled water and chloroform to remove sugars. The chloroform fraction was concentrated under reduced pressure. The dry sample was then subjected to column chromatography using hexane (4 x 200 ml), ethyl acetate (4 x 200 ml),

chloroform (4 x 200 ml), acetone (4 x 200 ml), and methanol (4 x 200 ml). The solvents were recovered using rotor evaporator to obtain 17.10 g, 10.60 g, 14.95g, 15.40 g and 14.20 g of dry hexane, chloroform, ethyl acetate, acetone, and methanol soluble fractions of *L. alata* respectively. Same procedure was adopted for 600 g of *C. anisata* powder of which 12.52g, 15.69 g, 17.02 g, 16.45 g and 15.91g of dry hexane, chloroform, ethyl acetate, acetone, and methanol soluble fractions were obtained. The extracts were then subjected to larvicidal assays. The flow chart in Fig. 2 shows the flow diagram for the extraction of non-volatile secondary metabolites.

3.2.2 Essential oils

A weighed amount;180g of fresh whole plants of *L. alata* and 165g of *C. anisata* were subjected to hydro-distillation (fig.3) in a modified Clevenger-type apparatus (Plate 3) for at least four hours according to the British pharmacopoeia. The essential oil obtained was 7g (4.25%) *C. anisata* and 5g (2.78%) *L. alata* in a yield of w/w after drying over anhydrous sodium sulphate respectively. The oil was stored in sealed glass vial (Bijoux bottle) at 4°C.

Plate 3: Modified clevenger-type apparatus

3.2.2.1 GC, GC-MS analysis

Samples of essential oils were diluted in methyl-t-butylether (MTBE) (1:100) and analysed on an Agilent GC-MSD apparatus equipped with an Rtx-5SIL MS (ôRestekô) (30 m x 0.25 mm i.d., 0.25 εm film thickness) fused-silica capillary column. Helium (at 0.8 mL/min) was used as a carrier gas. Samples were injected in the split mode at a ratio of 1:10 ï 1: 100. The injector was kept at 250 ^{o}C and the transfer line at 280 ^{o}C. The column was maintained at 50 ^{o}C for 2 min and then programmed to 260 ^{o}C at 5 ^{o}C/min and held for 10 min at 260 ^{o}C. The MS was operated in the EI mode at 70 eV, in m/z range 42-350. The identification of the compounds was performed by comparing their retention indices and mass spectra with those found in literature (Adams, 1995) then supplemented by Wiley and QuadLib 1607 GC-MS libraries. The relative proportions of the essential oil constituents are expressed as percentages obtained by peak area normalization, all relative response factors being taken as one.

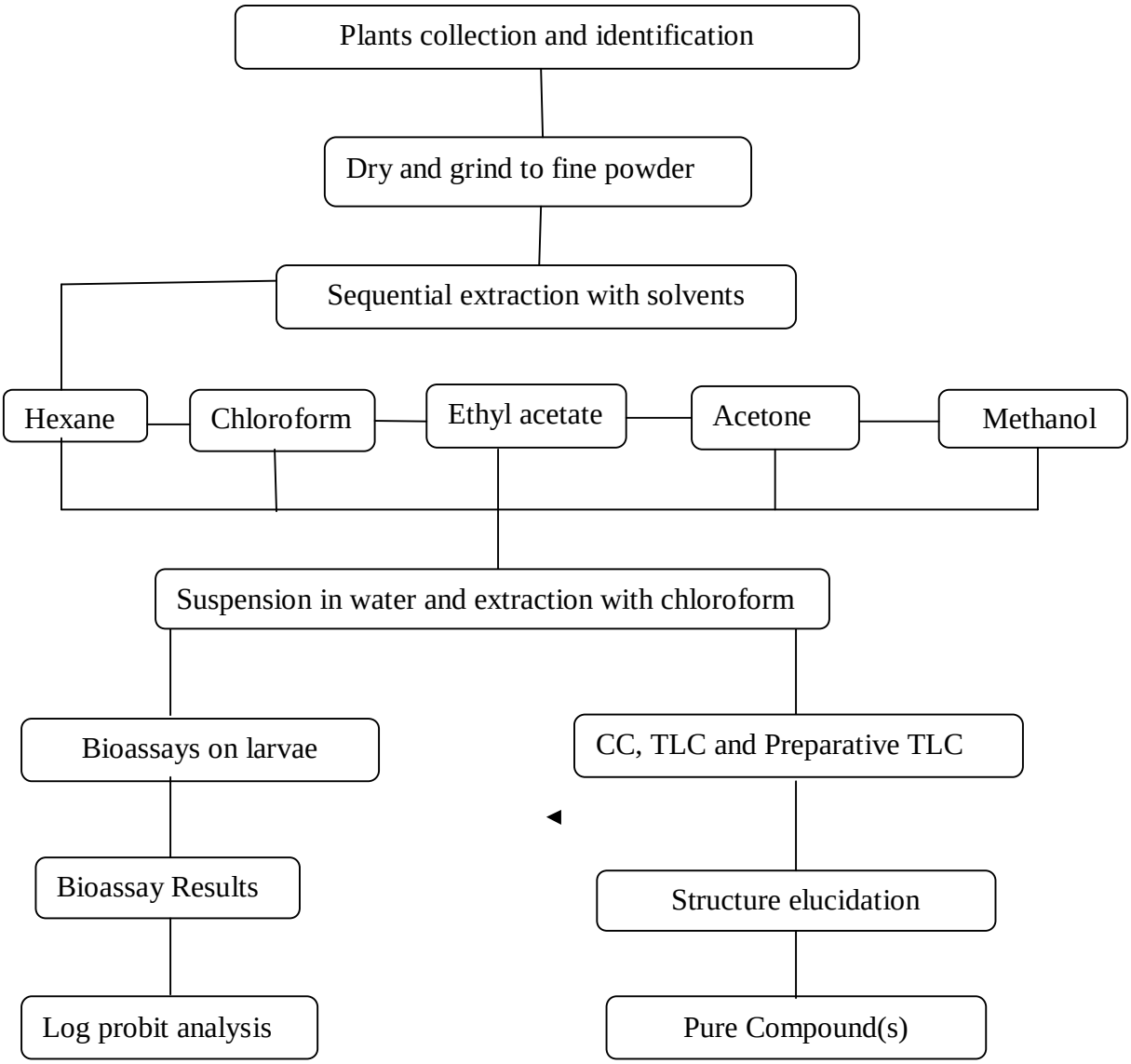


Fig. 2: Extraction of non – volatile compounds (Matasyoh *et al.*, 2008)

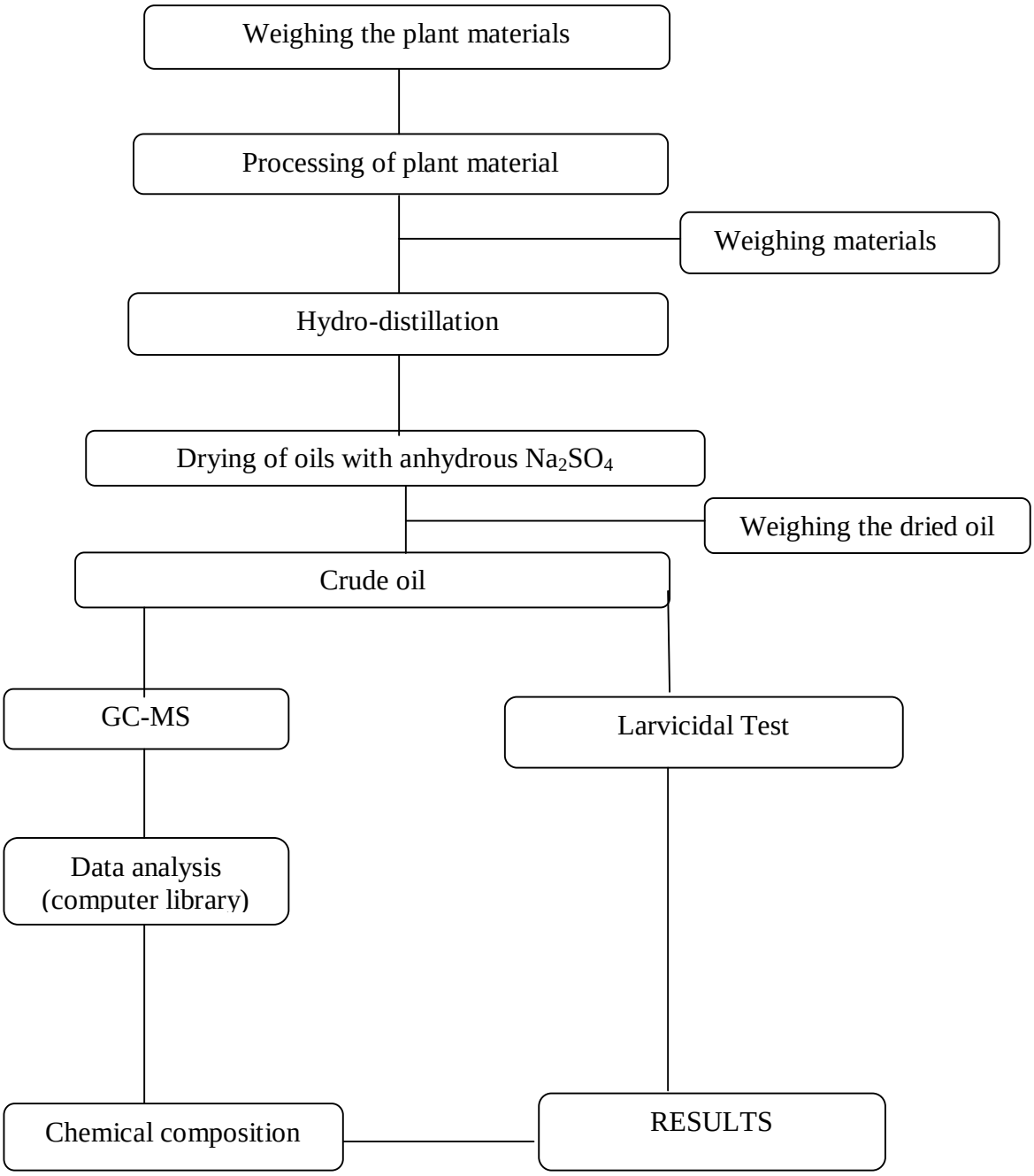


Fig. 3: Extraction of essential oil and Larvicidal activity test (Matasyoh *et al.*, 2007)

3.3 Larvicidal assays

The extracts were solubilized in dimethyl-sulphoxide (DMSO) an analytical reagent obtained from Lobarchemi and diluted to give 2 mg/ml of stock solution with DMSO kept at a concentration of 1%. The bioassay experiments were conducted mainly according to standard WHO procedure (1981) with slight modifications. The bioassays were conducted at the Kenya Medical Research Institute (KEMRI), Centre for Disease Control (CDC), Kisumu, Kenya, where the larvae were reared in plastic and enamel trays in spring river water. The larvae were maintained, and all experiments carried out at 26 ± 3^{o}C and the humidity ranged between 70 to 75%. The bioassays were performed with third in-star larvae of *A. gambiae s.s* and carried out in triplicate using 20 larvae for each replicate assay. The larvae were placed in 50 ml disposable plastic cups containing 15 ml of test solution and fed on tetramin fish feed during all testing. Larvae were considered dead if they were unrousable within a period, even when gently prodded. The dead larvae in the three replicates were combined and expressed as the percentage mortality for each concentration. The negative control was spring river water while the positive control was the pyrethrum-based larvicide, pylarvex.

3.4 Isolation, purification and structure elucidation of larvicidal compounds

To isolate, purify and elucidate the structures of larvicidal compounds from *L. alata* and *C. anisata* the following analytical techniques were employed.

3.4.1 Chromatographic techniques

The crude extracts were analyzed using the TLC to establish suitable solvent system (silica gel, 20×20 cm, 0.20 mm thick, cut into 5×15 cm for use). The main solvents used as the mobile phase were hexane and ethyl acetate. The ratios of the solvent were changed while using the hexane as the main solvent in the following percentages: 0%, 10%, 20% and 30% of ethyl acetate in hexane. The TLC analysis with the above solvent systems showed that hexane and ethyl acetate in a ratio of 7:3 gave the most pronounced separation with distinct spots. Column chromatography was performed using Merck silica gel 60 (70-230 mesh). The hexane and ethyl

acetate extracts of *L. alata* and *C. anisata* were chromatographed on a silica gel column using gradient elution of hexane- ethyl acetate solvent system to give four fractions (Table 3). Column used was of the dimension 50 cm height by 19 mm internal diameter. Silica gel used was about 65 g per column to give 45 cm of gel height.

3.4.2 TLC analysis

The extracts that showed bioactivity were subjected to thin layer chromatographic analysis. This was done on silica gel plates (Merck, 60F254) using the solvent system Hex-EtOAc, 7:3. The solvents were distilled before use. The visualization and identification of spots of the compounds was done using an ultra violet lamp at a wavelength of 254 nm. The retention factor (Rf) values were determined (Table 9).

3.4.3 Isolation of compound 1 and 2

17.10g of hexane fraction was suspended in 250ml of distilled water and extracted with 250ml chloroform using a separating funnel. The chloroform extract was dried using anhydrous sodium sulphate. The solvent was then recovered on vacuum rota evaporator. The dry sample was dissolved in hexane and re-eluted on a column packed with 65g of silica gel. Isolation was carried out using the solvents: hexane, ethyl acetate by increasing polarity (Table 9). A total of 25 fractions (Table 8) were collected. Fractions that showed the same Rf value and the same characteristic color on TLC observed using UV lamp operating at 254nm were combined and subjected to preparative thin layer chromatography on 20x20cm plates. Fractions collected with 100% hexane and hex-EtOAc (9:1) ratios were discarded because their TLC result did not show spots. However fraction with 80% hexane gave very many close spots indicating several compounds with relatively same polarity.

Fraction 7 (Fr-7), (Table 9) was concentrated under reduced pressure (on a rotor evaporator) to yield 2.86gm(1.67%) and was applied on a preparative thin layer chromatography plate and developed in 7:3 ratio of hexane- ethyl acetate as the mobile phase. Two distinct band

separations were observed in this solvent system: a more mobile yellow band and a grey band only visible under UV lamp operating at 254nm. The bands were carefully scraped and extracted using the same solvent system. Concentrating this fraction under reduced pressure yielded 680mg of pure compound 1. Compound 1 is a yellow Gummy substance with Rf value of 0.50 on TLC. Similarly on concentration 275mg of pure white substance compound 2 was obtained. On TLC this compound had Rf value of 0.24. The other combined fractions on preparative TLC showed rather very close and superimposing bands hence no further work was done on them.

3.4.3 Spectroscopic analysis of the compounds

3.4.3.1 GC-MS analysis

Ethyl acetate fractions of *C. anisata* were derivatised using trimethylsilyl ethers in order to increase their volatility to pass through the GC column as well as increasing their stability in gaseous phase. The four fractions were subsequently subjected to GC-MS analysis. The identification of the compounds was performed by comparing their retention indices and mass spectra with those found in literature (Adams, 1995) then supplemented by Wiley and QuadLib 1607 GC-MS libraries. Mass spectra were recorded on Finnigan Triple- Stage-Quadrupol Spectrometer (TSQ-70) with electro spray ionisation (ESI) Method. GC-MS analyses were done using a Hewlett-Packard model 6890 series GC (Table 3).

3.4.3.2 NMR- Spectroscopy

All the spectra were measured on a Bruker Advance 400 spectrometer, which operated at 400 MHz for ^{1}H and 100 MHz for ^{13}C nuclei. ^{1}H and ^{13}C NMR (Appendices 1and 2) spectra were performed in deuterated solvent and chemical shifts were assigned by comparison with the residue proton and carbon resonance of the solvent and tetramethylsilane (TMS) as internal reference ($\delta = 0$). 2D-NMR spectroscopy was used to elucidate the structures and especially establish the connectivities in the molecules. The proton-carbon connectivity (three bonds) was identified using ^{1}H-^{13}C COSY and HMBC (Heteronuclear Multiple Bond Correlation) spectrum (Appendices 6, 7, 8, 9, 10, 11, 12 and 13) in which there was one- dimensional ^{13}C NMR

spectrum along the left and the ^{1}H NMR spectrum along the top. The two-dimensional array of spots forming a ‘square box’ identified the proton-carbon connectivity.

3.5 Statistical analysis

The lethal concentrations were determined using SPSS package version 11.5. The bioassay data was subjected to probit regression analysis according to Finney (1971). Probit analysis of concentration- mortality data was conducted to estimate the LC_{50} and LC_{99} values and associated 95% confidence limits as shown in appendices 2, 3, 4 and 5**.**

CHAPTER FOUR

RESULTS AND DISCUSSION

4.1 The GC-MS Analysis of C. anisata Oils

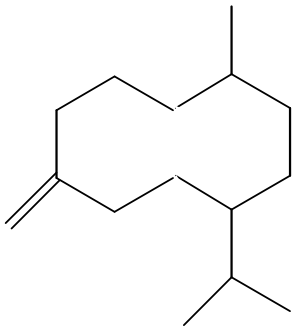
Clausena anisata was oilier and gave on drying 7g of neat oil. Approximately 2g was subjected total chemical analysis. The chemical composition in Table 1 lists twenty-six compounds identified by GC and GC-MS, which constitute 99.1 % of the total oil. The oil was dominated by monoterpenoids, which accounted for 56.7 % of the oil. The monoterpenoids fraction was characterized by a high percentage of ß-Phelandrene (20.1 %), ɔ-Terpinene (13.8%) and Ŭï Phelandrene (4.6%). Considering components with concentrations of about 2 % and above, the other major monoterpenes were (E)-ßï Ocimene (3.7%), Myrcene (3.4%), Ŭ-Pinene (3.4%) and para-Cymene (2.8%). The sesquiterpenoids components were accounting for 42.4% of the total oils constituents. The main constituents were ŭ-Germacrene (18.8%) and ß-Germacrene (6.0%). Other sesquiterpenoids present in appreciable amounts were Bicyclogermacrene (3.7%), Ŭ-Humulene (3.6%), (E)-Caryophyllene (2.5%), and ß-Elemene (2.0%).

Table 1: *Clausena anisata* Oils Chemicals Composition

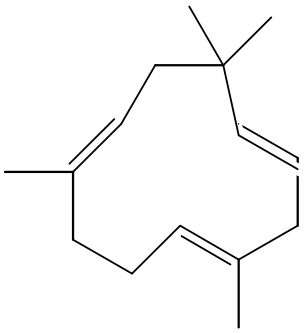
Compounds	R.T.	% of total	RI	ID Method
Monoterpenes				
Ŭ-Thujene	5.4	0.5	925	MS, RI
Ŭ-Pinene	5.6	3.4	932	MS, RI
Sabinene	6.6	0.9	971	MS, RI
ɓ-Pinene	6.7	1.3	976	MS, RI
Myrcene	7.0	3.4	989	MS, RI
Ŭ-Phellandrene	7.5	4.6	1006	MS, RI
Ŭ-Terpinene	7.8	0.6	1017	MS, RI
Cymene<para>	8.0	2.8	1024	MS, RI
ɓ -Phelandrene	8.2	20.1	1032	MS, RI
ɓ -Ocimene(E)	8.6	3.7	1046	MS, RI
ɔ-Terpinene	9.0	13.8	1059	MS, RI
Terpinolene	9.8	1.1	1085	MS, RI
ŭ-Elemene	17.0	0.5	1335	MS, RI
	Total %	**56.7**		
Sesquiterpenes				
Ŭ-Copaene	18.2	0.6	1376	MS, RI
ɓ -Bourbonene	18.4	0.6	1384	MS, RI
ɓ -Elemene	18.5	2.0	1389	MS, RI
Caryophyllene(E)	19.3	2.5	1420	MS, RI
ɔ -Elemene	19.6	0.8	1429	MS, RI
Ŭ-Humulene	20.3	3.6	1456	MS, RI
Germacrene-D	21.0	18.8	1483	MS, RI
Bicyclogermacrene	21.3	3.7	1496	MS, RI
ɓ -Bisabolene	21.6	1.4	1508	MS, RI
ŭ -Cadinene	21.9	0.7	1519	MS, RI
Elemol	22.7	1.1	1549	MS, RI
Germacrene-B	23.0	6.0	1560	MS, RI
Spathulenol	23.4	0.6	1578	MS, RI
	Total %	**42.4**		
Unknown	21.9	0.8	1516	

4.2 Essential Oils of *L. alata* Oils

The oils were dominated by sesquiterpenes which accounted for 50.3% of the oils (Table 2). Considering components with concentrations of about 2 % and above, the sesquiterpenes were 2, 5-dimethoxy-para-Cymene (24.4%) ŭ-Germacrene (8.4%), Ŭ-Humulene (6.2%) whose structures are shown below, (E)-Caryophyllene (2.3%) and ɓ-Bourbonene (2.5%). Monoterpenes accounted for 31.5% of the constituentsô compounds in the oils (Table 2). The main component was cis-Chrysanthenol (11.8%), Chrysanthenone (8.7%), Thymol methyl ether (4.6%), Filifolone (3.5%) and Sabinene (3.6%). A total **of** 15.4**%** were compounds whose identity was not established based on the method employed.



Germacrene-D



Alpha-Humulene

Table 2: *Laggera alata* Oils Chemicals Composition

ID	R.T.	% of total	RI	ID Method
Monoterpenes				
Ŭ-Pinene	5.6	1.1	932	MS, RI
Sabinene	6.6	3.6	971	MS, RI
Filifolone	10.2	3.5	1100	MS
Chrysanthenone	10.8	8.7	1122	MS, RI
Chrysanthenol<cis>	12.1	11.8	1165	MS, RI
Thymol methyl ether	14.0	4.6	1229	MS, RI
	Total	**30.3%**		
Sesquiterpenes				
Ŭ-Copaene	18.2	1.2	1376	MS, RI
ɓ-Bourbonene	18.4	2.5	1384	MS, RI
ɓ-Elemene	18.5	0.8	1389	MS, RI
Cymene<2,5-dimethoxy-para>	19.2	24.4	1414	MS
Caryophyllene(E)	19.3	2.3	1420	MS, RI
ɔ-Elemene	19.6	0.6	1430	MS, RI
Ŭ-Humulene	20.3	6.2	1456	MS, RI
Germacrene-D	21.0	8.4	1482	MS, RI
Muurola-4(14),5-diene<trans>	21.2	0.8	1492	MS, RI
Bicyclogermacrene	21.3	0.8	1496	MS, RI
ŭ-Cadinene	21.9	1.9	1519	MS, RI
Germacrene-D-4-ol	23.4	0.5	1578	MS, RI
Caryophyllene oxide	23.5	0.5	1583	MS, RI
Ŭ-Cadinol	25.3	0.6	1657	MS, RI
	Total	**51.5%**		
Unknown	10.3	1.8	1104	
Unknown	10.4	4.1	1107	
Unknown	12.6	1.5	1181	
Unknown	24.2	0.9	1611	
Unknown	24.7	1.1	1632	
Unknown	25.0	0.8	1644	
Unknown	25.4	1.0	1660	
Unknown	26.0	1.2	1685	
Unknown	27.8	0.5	1767	
Unknown	28.5	0.6	1796	
Unknown	28.6	0.3	1801	
Unknown	31.1	1.1	1964	
Unknown	31.4	0.5		
	Total	**15.4%**		

4.3 Larvicidal Activity of the oils

The *C. anisata* oils showed very high larvicidal activity with lethal concentration that could kill 50% of the target larvae as 75.96 mg/l (44.63- 95.42) and the corresponding LC_{99} of 256.80 mg/l (215.90- 340.84) at 95% confidence interval (appendix 2). Ŭ-Pinene, was found to be inappreciable amounts (3.4%) in *C. anisata* and (1.1%) in *L. alata.* Ŭ-Pinene has been reported to be the cause of the antifungal activity of oil from *Pistacia lentiscus* (ana-cardiaceae) (Matasyoh *et al*., 2007). The observed difference in activity between the oils from the two plants could be due to difference in percentage composition. The LC_{50} of *L. alata* was 273.38 mg/l (239.18-299.55) and LC_{99} of 507.75 mg/l (455.15-603.90), at the same confidence interval (appendix 3). Though major oils compound composition vary greatly in these two plants but ŭ-Germacrene appears in both at significant amount composition, with different percentage composition in both. For instance in *C. anisata* the major compounds are: ɓ-Phelandrene (20.1%), ŭ-Germacrene (18.8%), ɔ-Terpinene (13.8%), ɓ-Germacrene (6.0%) and Ŭ-Phelandrene (4.6%), taking anything above 4.00% as major bound to cause notable activity. Major compounds in *L. alata* oils are 2, 5-dimethoxy-para-Cymene24.4%, cis-Chrysanthenol (11.8%), Chrysanthenone (8.7%), ŭ-Germacrene (8.4%), Ŭ-Humulene (6.2%), Thymol methyl ether (4.6%) and unknown (15.4%). The unknown compounds appear not to have significant influence but we cannot ignore their probable synergistic effect. The unknown compounds therefore need to be carefully isolated, elucidate structures and their larvicidal activity studied. However, compounds likes ɓ-Caryophyllene (2.5%) in *C. anisata* and (2.3%) in *L. alata,* a common sesquiterpene widely distributed in plants, possesses anti-inflammatory and ant-carcinogenic activities (Tellez *et al.*, 1999). Its oxygenated form Caryophyllene oxide is present in a minor quantity of 0.50 % in *L. alata*, (Table 2) it is known to possess antimicrobial properties against a wide range of bacteria and fungi (Guillen *et al*., 1996). The difference in concentrations of the essential oils and the standard larvicide can be explained in terms of the fact that the active components in the oils comprise of only a fraction of the oils used. Therefore, the concentration of the active components could be much lower than the standard larvicide; pylarvex used.

4.4 GC-MS Analysis of *C. anisata* Extracts

4.4.1 Column chromatography

This was done by subjecting 13g of hexane fraction of *C. anisata* to silica gel column chromatography using hexane and ethyl acetate in a ratio of 7:3. Four fractions were obtained: fraction 1 (4.01g), fraction 2 (3.20g), fraction 3 (4.40g) and fraction 4 (0.09g).(Table 3)

Table 3: Column chromatography results crude ethyl acetate extract.

Fraction	Eluting solvent system	Amount in grams, (*C. anisata)* ethyl acetate
1	100% hexane	4.01
2	10% ethyl acetate in hexane	3.20
3	20% ethyl acetate in hexane	4.40
4	30% ethyl acetate in hexane	0.91

The ethyl acetate fraction showed exceptional larvicidal activity compared to other fractions of *C. anisata* (Table 3). This was then considered for further analysis to isolate compounds presenting the observed activity. However, on further application of column chromatography Hex-EtOAc with increasing polarity to 7:3, four fractions were obtained (Table 9). These were then purified using preparative TLC on silica gel but it was discovered that most compounds present were known compounds prompting total compound composition to be done using GC-MS yielding compounds in Tables 5 as major compounds at 90% quality match with Wiley electronic library of organic compounds. 13C NMR, DEPT 135 and the GC-MS data analysis comparison with available known compound proved the presence of ŭ-Germacrene as shown in the figures 4 and 5. Another example is the 99% match of ɓ-Elemene shown in Fig. 6 below. This fraction was dominated by sesquiterpenes.

Table 4: Larvicidal activity screening of the fractions

Sample	Concentration(mg/l)	(%) mortality
Hexane fraction of *L. alata*	4000	100
$CHCl_3$ fraction of *L. alata*	4000	87.50
Acetone fraction of *L. alata*	4000	87.50
Ethyl acetate fraction of *C. anisata*	4000	100
$CHCl_3$ fraction of *C. anisata*	4000	75.00
Hexane: Ethyl acetate 3:7 fraction of *C. anisata*	4000	00
Acetone fraction of *C. anisata*	4000	90.00

Table 5: Major Compounds from Ethyl acetate Fraction of *C. anisata*.

Peak	RT	Area%	Compound	RI	(%) Quality match
1	10.36	1.86	ɓ-.Bourbonene	1384	98
2	10.59	2.48	ɓ-Elemene	1389	99
3	11.30	5.82	*Trans*-(ɓ)-Caryophyllene	1420	98
4	11.69	13.37	gamma.-Elemene	1429	99
5	12.28	8.01	Ŭ-Humulene	1456	98
6	13.01	20.96	ŭ-Germacrene	1483	98
7	3.37	3.37	Cyclohexene	-	94
8	14.09	2.86	ŭ.-Cadinene	1519	98
9	15.04	2.64	ɓ-Germacrene	1590	98
10	42.22	4.39	Squalene	-	93
11	47.11	34.24	Nonacosane	-	98
15	47.14	63.47	Octacosane	-	99
16	53.42	2.72	Neophytadiene	-	95
17	47.05	100.00	Hentriacontane	-	97

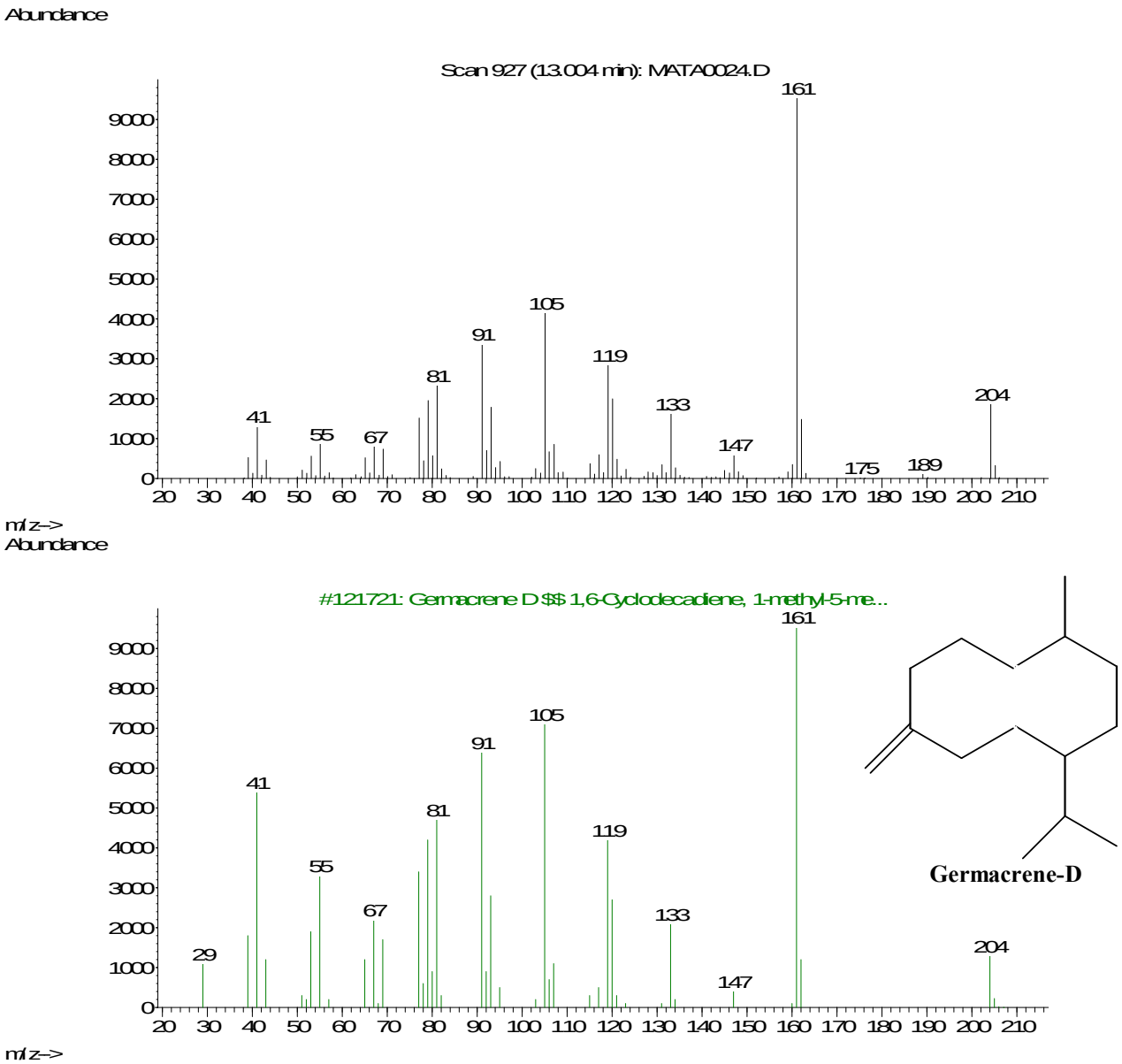


Fig.4: Library MS spectrum match spectrum of δ-Germacrene

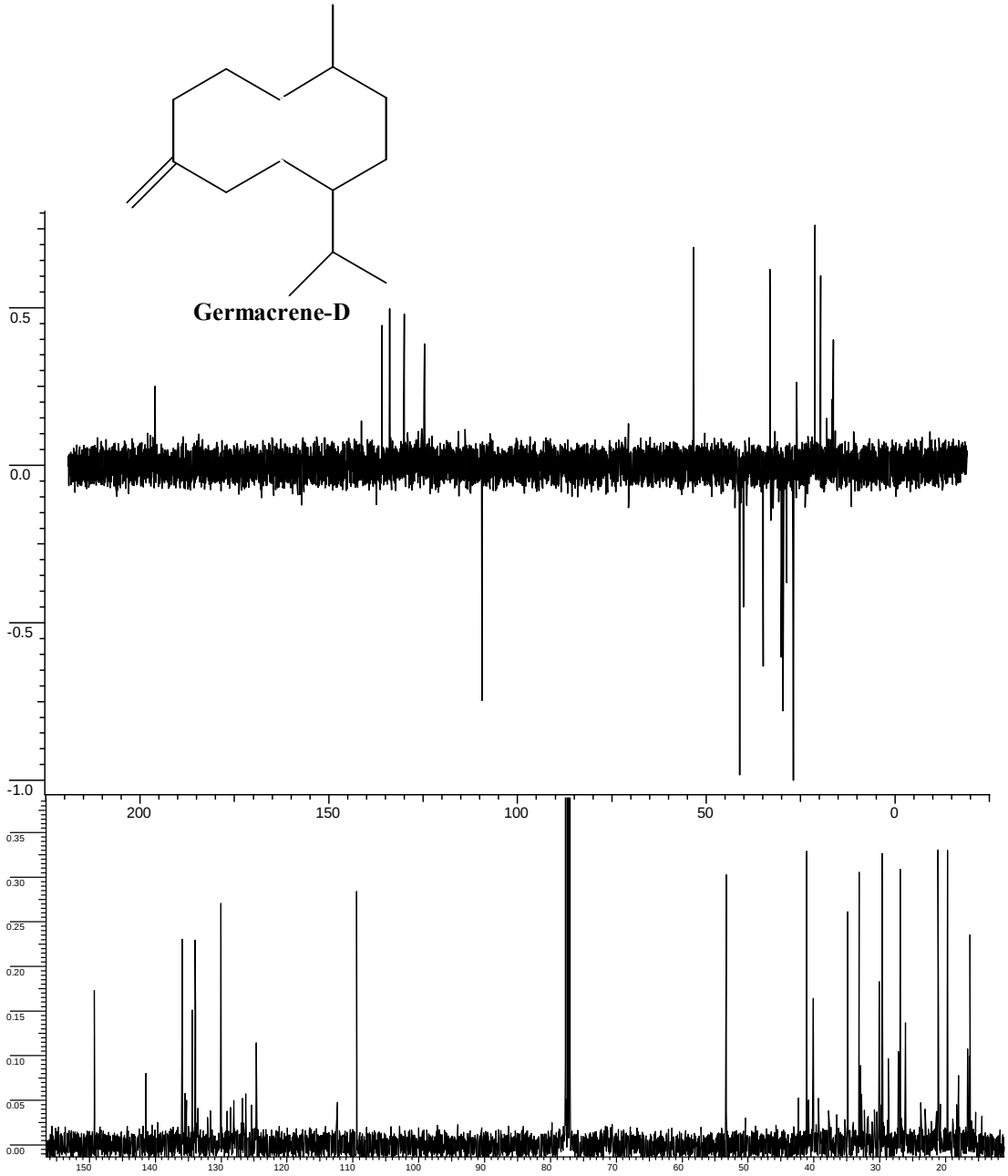


Fig. 5: ^{13}C NMR and DEPT 135 of δ-Germacrene

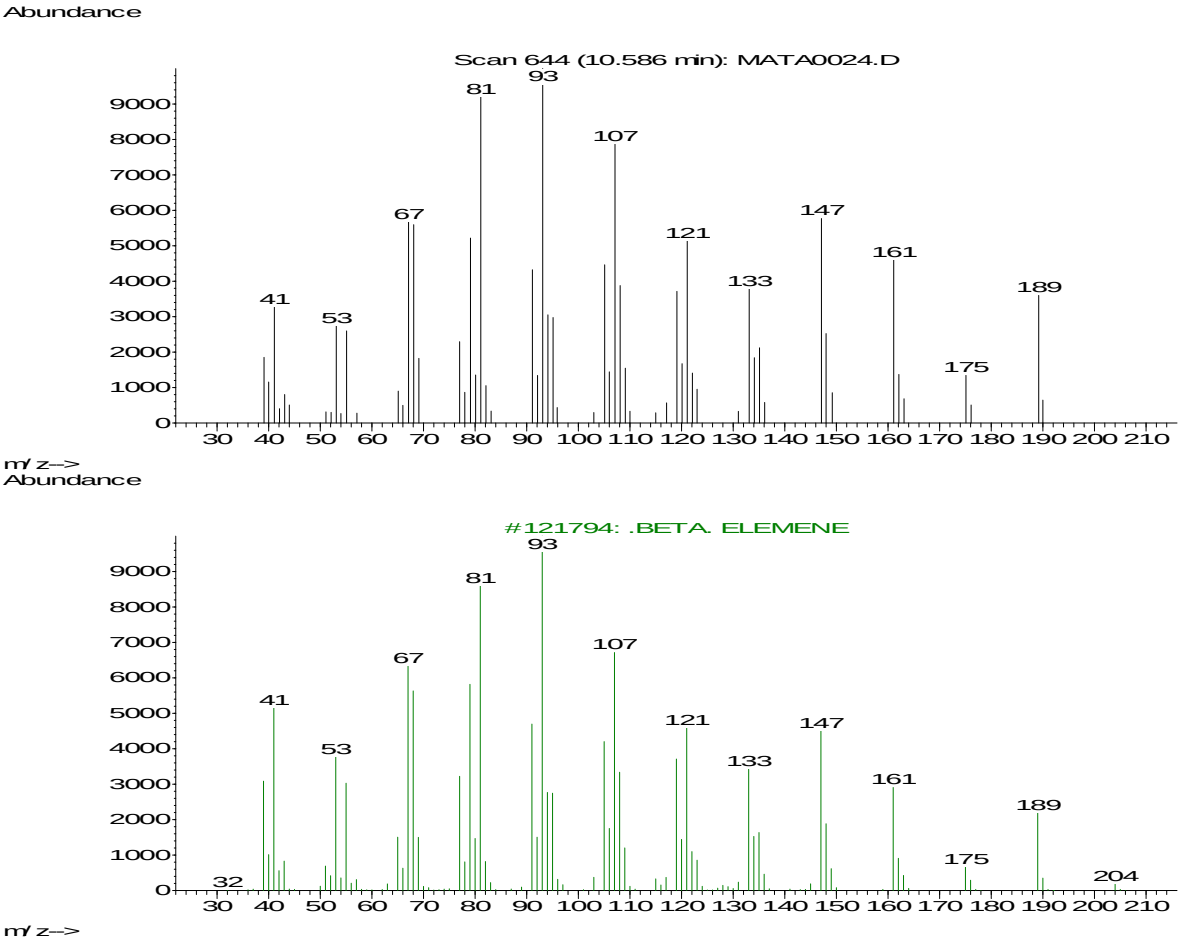
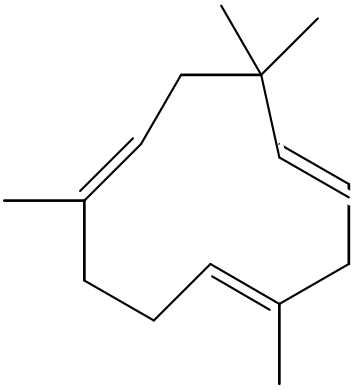
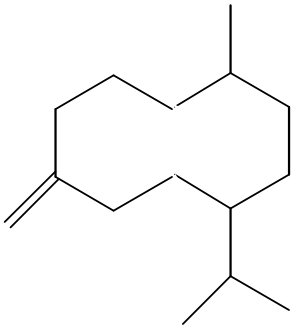


Fig. 6: Library ms spectrum match result of compound β-Elemene

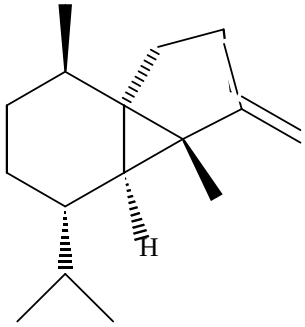
Structures of identified compound in the two plant extracts



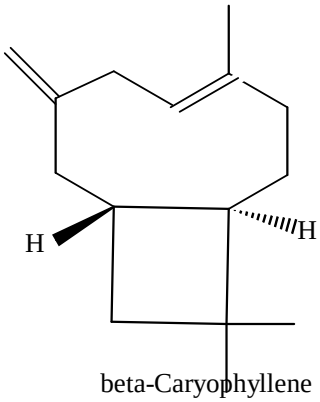
Alpha-Humulene



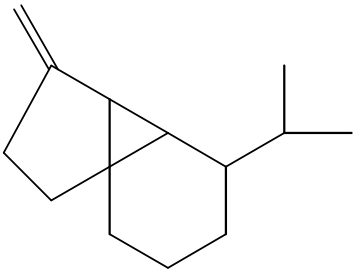
Germacrene-D



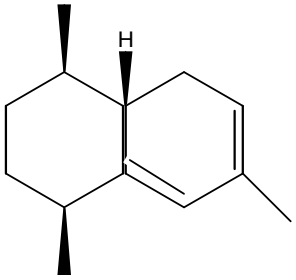
Cubebene



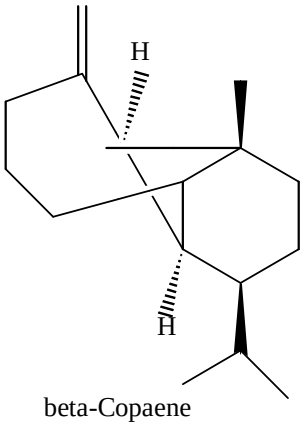
beta-Caryophyllene



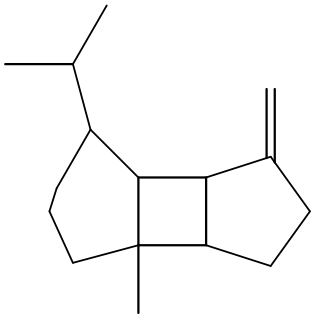
beta-Cubenene



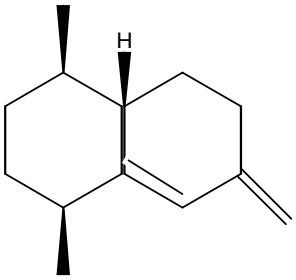
cis- Muurolene-3,5-diene



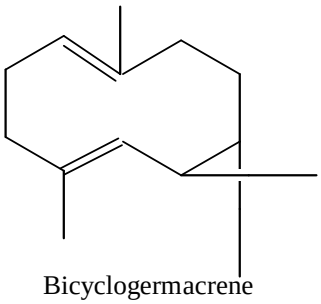
beta-Copaene



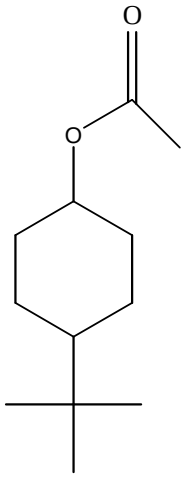
beta-bourbonene



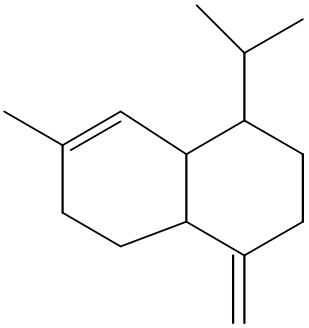
cis- Muurolene-4(14)5-diene



Bicyclogermacrene



4-tert-Butylcyclohexyl acetate



gamma-Muurolene

4.5 Ethyl acetate fraction of *C. anisata* and Hexane fraction of *L. alata*

The hexane fraction *of L. alata* demonstrated reasonable activity (Table 3) against *A. gambiae s.s*. Larvae. This fraction gave an LC_{50} of 1161.30 mg/l and LC_{90} of 2734.91 mg/l. The Ethyl acetate fraction of *C. anisata* gave slightly higher values of LC_{50} (2095.46 mg/l) and LC_{90} (4438.75 mg/l) (Table 4 and 5). The observed differences in the Larvicidal activities of these plants species may be due to the difference in available bioactive secondary metabolites in plants (Kitagawa *et al.,* 1999). The amount of these active metabolites differs in these plants, in as much as most major compounds are similar. These compounds also have been shown to partition exclusively in particular solvents (Kokwaro, 1993). The LC_{50} of 1161.30 mg/l for *L. alata* extract and LC_{50} value of 2095.46 mg/l for *C. anisata* were higher compared with that of 30 mg/l of the standard pylarvex. The big difference in activity of the extracts compared with the reference larvicide could be because the active compounds are only a small percentage of the extracts since; no purification at this stage.

4. 6 Chloroform, Acetone and Methanol fractions

Chloroform, Acetone and Methanol fractions did not show notable activity for *L. alata*. Similarly, hexane, chloroform, acetone and methanol fractions of *C. anisata* did not give 100% mortality at very high concentration of 4000 mg/l (Table 1). The medicinal properties of plant extracts normally depend upon the presence of active compounds (Kokwaro, 1993) possessing specific functional groups that are soluble only in solvents of particular polarity. The active compounds in these extracts of the *L. alata* and *C. anisata* were therefore not soluble appreciably in these solvents.

4.7 Larvicidal activity of *C. anisata* active fraction and oils

Clausena anisata oils showed much better activity compared to the active fraction (Table 4). The log probit analysis gave LC_{50} and LC_{99} as 75.96 mg/l (44.63- 95.42) and 256.80 mg/l

(215.90- 340.84) respectively. While the corresponding LC_{50} as 2095.46 mg/l (1792.75-2315.00) and LC_{99} were 4438.75 mg/l (3936.03-5365.71) for the most active extract.

Table 6: Larvicidal assay for *C. anisata* oils and ethyl acetate extract

Larvicidal assay for *C. anisata* oils		**Larvicidal assay for *C. anisata* ethyl acetate extract**	
Concentration (mg/l)	**(%) mortality**	**Concentration (mg/l)**	**(%) mortality**
250	98.35	4000.00	100
200	98.35	3500.00	93.35
175	88.35	3200.00	86.65
150	75.00	3000.00	75.00
125	73.35	2850.00	73.35
100	71.65	2500.00	60.00
75	50.00	2250.00	55.25
50	33.35	2000.00	49.35

4.8 Larvicidal activity of *L. alata* active fraction and oils

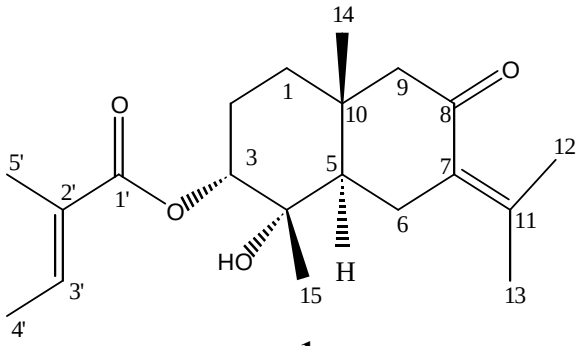
Laggera alata oils showed much better activity compared to the active hexane fraction (Table 6). The log probit analysis gave LC_{50} and LC_{99} as 273.38 mg/l (239.18-299.55) and 507.75 mg/l (455.15-603.90) respectively (appendix 2). LC_{50} of *L. alata* hexane fractions was 1161.30 mg/l (906.99-1356.63) and the corresponding was LC_{99} 2734.91 mg/l (2336.15- 3535.01). The observed big difference in the required concentrations can be explained on the basis of difference in the amounts of the active secondary metabolites in the oils and the non volatile extracts. The oils have higher amounts of the active compounds than the extracts.

Table 7: Larvicidal assay for *L. alata* oils and hexane extract

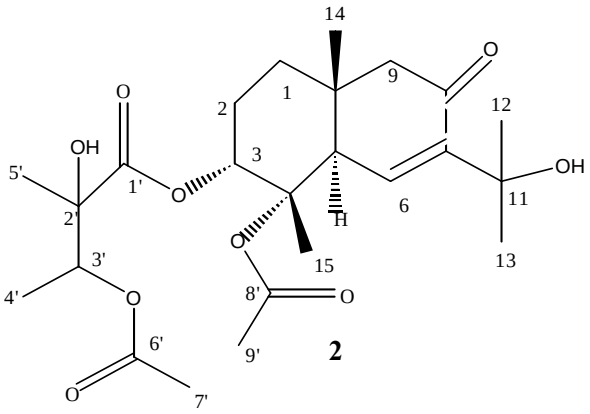
Larvicidal assay for *L. alata* oils		**Larvicidal assay for *L. alata* hexane extract**	
Concentration (mg/l)	**(%) mortality**	**Concentration (mg/l)**	**% Mortality**
500	96.65	4000.00	100
450	93.35	3500.00	100
400	93.65	3000.00	100
350	81.65	2500.00	98.35
300	63.35	2000.00	80.00
250	43.35	1500.00	78.90
200	16.65	1000.00	55.00
		800.00	15.00

4.9 Eudesmane Sesquiterpenoids 1 and 2

Bioassay-guided fractionation of active hexane fraction of *L. alata* led to isolation of 30mg of compounds **1** and 28mg of compound **2.** Elucidation of the structures of the pure compounds was determined using spectroscopic 1D and 2D NMR methods. The NMR data is shown in the Tables 7 and 8. High-resolution mass spectrometry established the molecular formulas of compounds **1** as $C_{20}H_{30}O_4$ and molecular as 357.2034090 the calculated mass was 357.2041791, with double bond equivalence of six. Similarly, the molecular formulae of compound **2** was established as $C_{24}H_{36}O_9$ with corresponding mass of 491.2261970. However, the calculated mass was 491.2257024 with double bond equivalence of seven.



1



2

4.10 Larvicidal activity of *L. alata* compounds 1and 2

The Eudesmane Sesquiterpenoids **1**and **2** were isolated from very active hexane fraction of *L. alata* (Table 7) but themselves did not show appreciable activity. The hexane fraction gave LC_{50} and LC_{99} as 1161.30mg/l (906.99-1356.63) and 2734.91mg/l (2336.15- 3535.01) at 95% confidence interval respectively.

4.10.1 Column chromatography of crude hexane extract of *L. alata*

About 17.10 g of *L. alata* were subjected to the column in similar conditions yielding five fractions; fraction1 (4.21g), fraction 2 (4.24g), fraction 3 (3.96g) and fraction 4 (2.89g) (Table 8).

Table 8: Column chromatography results

Fraction	Eluting solvent system	Amount in grams, *(L. alata)* hexane
1	100% hexane	4.21
2	10% ethyl acetate in hexane	4.24
3	20% ethyl acetate in hexane	3.96
4	30% ethyl acetate in hexane	2.86

4.10.2 Thin layer chromatographic of analysis of crude hexane extracts

The crude hexane of *L. alata* extracts were analyzed using the TLC (silica gel, 20×20 cm, 0.20 mm thick) and was cut into 5×15 cm for use. The main solvents used as the mobile phase were hexane and ethyl acetate. The ratios of the solvent were changed while using the hexane as the main solvent in the following percentages: 0%, 10%, 15%, 20%, 25%, 30% and 50% of ETOAC in HEX. The sample was spotted on the plates and placed into the developing tanks for about 25 fractions (Table 9). Each tank had the solvents in their respective ratios mentioned above. The TLC analysis with the above solvent systems showed that hexane and ethyl acetate in a ratio of 7:3 gave the most pronounced separation with distinct spots at R_f values 0.21, 0.38, 0.53 and 0.74. A repeat TLC of the fractions from the chromatographic column gave single spots at the respective R_f values.

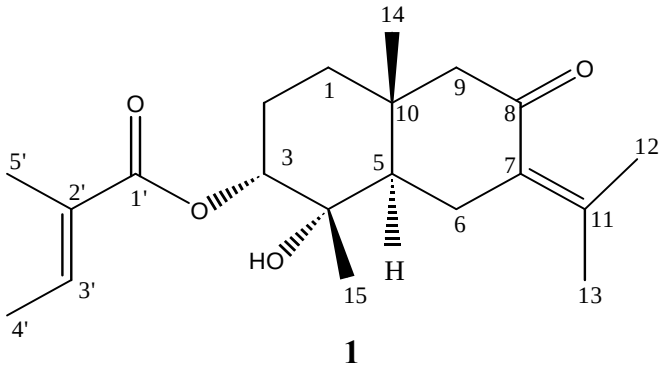
Table 9: TLC results using hexane/ethyl acetate (7:3)

Solvent system	Ratio	Fraction	Volume	Fractions combined	Code
Hex/EtoAc	10:0	1-8	200	1-4 **5-8**	Fr-1 **Fr-2**
Hex/EtoAc	9:1	9-12	200	9-10 **11-12**	Fr-3 **Fr-4**
Hex/EtoAc	8:2	13-19	200	13-16 **17-19**	Fr-5 **Fr-6**
Hex/EtoAc	7:3	20-25	200	20-25	Fr-7

4.11 NMR Structural Elucidation of the Compounds

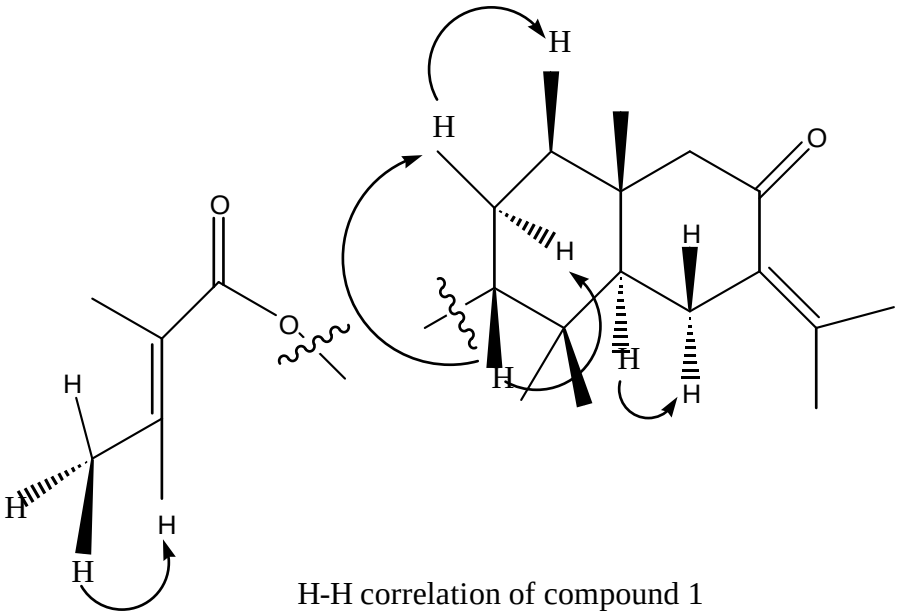
4.11.1 Structure Elucidation of Eudesmane Sesquiterpenoid 1

Both one and two-dimensional NMR were used to determine the structure of the pure compound **1**. ^{13}C-NMR and DEPT (appendices 6 and 11), ^{1}H-^{1}H COSY (appendix 12, Table 10), ^{1}H-^{13}C NMR (HMBC) (appendices 11 and 12, Table 7), HSQC (appendices 9 and 10) techniques achieved the structural elucidation and complete proton and carbon assignments. The comparisons of DEPT spectrum with a broadband decoupled carbon spectrum, made the carbon peaks to be firstly classified into methyl, methylene, methine and quaternary carbon atoms (Table 10). The proton decoupled ^{13}C NMR spectrum (appendix 6, Table 10) of **1** showed well resolved resonance of the 20 carbon atoms. The multiplicity of each carbon atom was determined using DEPT-135 and DEPT 90 experiment, which revealed the presence of six
methyl groups, four methylene groups, three methine groups and seven quaternary carbon (two carbonyl carbon atoms, three vinylic carbon atoms and two saturated carbon atoms, indicating 30 hydrogen atoms attached to carbon atoms. Based on ^{1}H NMR (appendices 6 and 7) and proton decoupled ^{13}C NMR spectrum (appendix 6, Table 10) data of **1** the proposed structure of the compound is shown below and the corresponding fragmentation. The H-H correlation is also shown below.



1

Proposed structure of compound 1



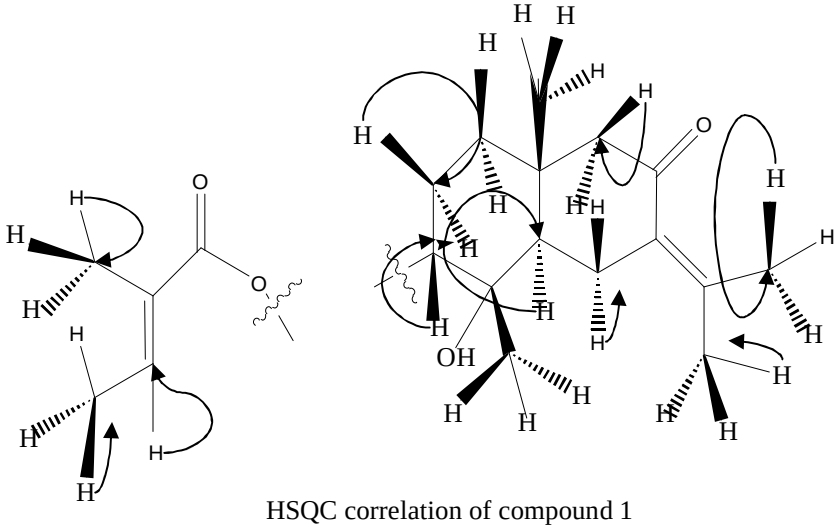
H-H correlation of compound 1

Table 10: NMR Spectral Data of Eudesmane Sesquiterpenoid 1

Carbon no.	^{13}C NMR (ppm)	DEPT	^{1}H NMR (ppm)	H/H COSY	HMBC
1.	38.60	CH_2	1.5	H^1-H^2, H^3-H^2	H-1 ź C-3,C-5, C-9, C-14
2.	25.85	CH_2	1.7, 1.9, 2.2, 3.0	H^2-H^3	H-2 ź C-4, C-10
3.	81.69	CH	4.8	H^3-H^2	H-3 ź C-1, C-5, C-15
4.	74.76	Cq	-	-	-
5.	51.44	CH	1.9	H^5-H^6	H-5 ź C-7, C-9, C-14
6.	25.85	CH_2	1.7, 1.9, 2.2, 3.0	H^6-H^5	H-6 ź C-4, C-8, C-10, C-11
7.	130.64	Cq	-	-	-
8.	202.64	Cq (C=O)	-	-	-
9.	60.21	CH_2	2.2	H^9-H^{14}	H-9 ź C-1, C-5, C-7, C-14
10.	36.76	Cq	-	-	-
11.	145.29	Cq	-	-	-
12.	23.82	CH_3	2.0	-	H-12 ź C-7, C-13
13.	23.13	CH_3	1.8	-	H-13 ź C-7, C-12
14.	19.31	CH_3	1.0	H^{14}-H^9	H-14 ź C-1, C-5, C-9
15.	17.85	CH_3	1.3	-	H-15 ź C-3, C-5
1ô	168.73	Cq (C=O)	-	-	-
2ô	128.21	Cq	-	-	-
3ô	139.08	CH	6.1	$H^{3ć}$-$H^{4ć}$	H-3ô ź C-1ô, C-5ô
4ô	21.02	CH_3	1.9	$H^{4ć}$-$H^{3ć}$	H-4ô ź C-2ô, C-3ô
5ô	16.27	CH_3	2.0	-	H-5ô ź C-1ô,C-2ô, C-3ô
Cq = 7, CH = 3, CH_2 = 4, CH_3 = 6, O = 4					

The above prediction was supported by using its 2D NMR spectral data as follows. ^{1}H-^{1}H correlation spectroscopy (COSY) (Appendix 8, Table 9) showed strong correlation between H-3 (ŭ 4.8) and H-2 (ŭ 1.7) indicating methylene protons at C-2 are in the same environment as hydrogen atom on the oxygen containing carbon which is chiral. There are also coupling between H-3ô (ŭ 6.1) and H-4ô (ŭ 1.9)

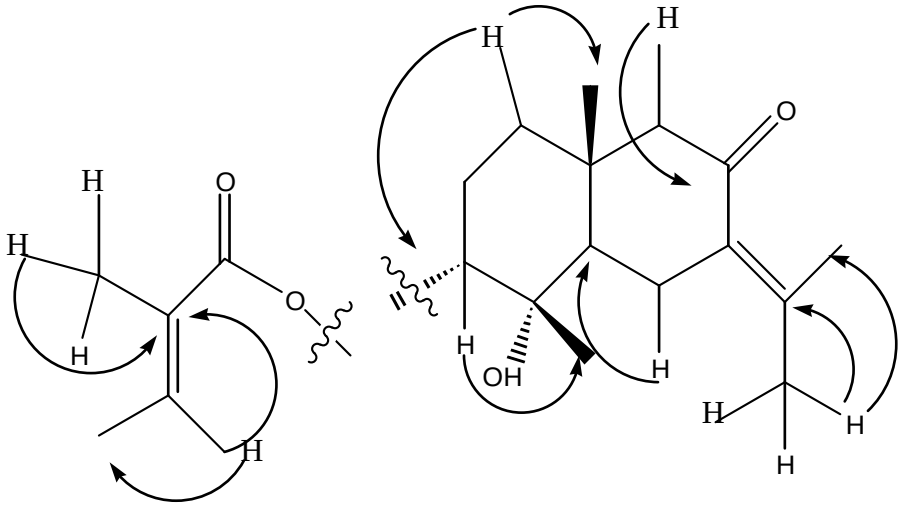
Heteronuclear Single Quantum Correlation (HSQC) experiment correlates the chemical shift of proton with the chemical shift of directly bonded carbon atom. In the HSQC spectral data (appendices 9 and 10), showed three protons at ŭ 1.0 (s) connected with C-14 (ŭ19.31). Three protons at ŭ 1.3 (s) connected with C-15, (ŭ 17.85) three protons at ŭ 1.8 (s) connected with C-13, (ŭ 23.13). Three protons at ŭ 2.0 (s) connected with C-5ô, (ŭ 16.27), six protons at 1.98-2.00 connected with C-4ô, (ŭ 21.02) and C-12, (ŭ 23.82). Two protons at ŭ 1.5 (m) connected with C-1 (ŭ 38.60), two protons at ŭ 2.2 (m) connected with C-2 (ŭ 25.85). One proton at ŭ 4.8 (dd) correlates to C-3, (ŭ 81.69) and C-5, (ŭ 51.44) correlates with a single proton at ŭ 1.9 respectively. Two protons at **δ**.2.2 (d) connected with C-9, (ŭ 60.21). One proton at ŭ 4.8 (dd) attached to C-3, (ŭ 81.69), one proton at ŭ 1.9 (dd) connected with C-5, (ŭ 51.44) and one proton at ŭ 6.1 (q) connected with C-3ô, (ŭ 139.08).



HSQC correlation of compound 1

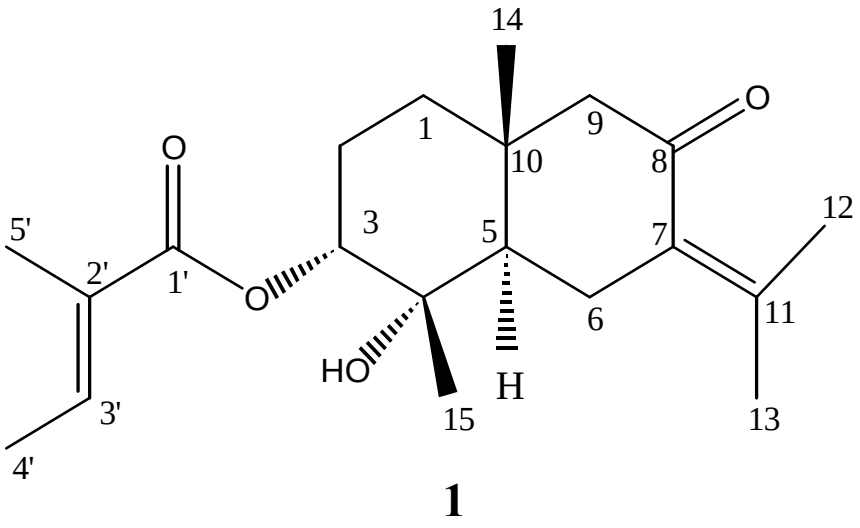
Heteronuclear Multiple Bond Correlation (HMBC) experiment gave information about coupling of hydrogen atoms and carbon atoms that are two or three bonds away. In the HMBC (appendix

10 and 11), the methyl protons at ŭ 1.9 (s) (H-5ô) correlated with ŭ 139.08 (C-3ô); ŭ 128.21 (C-2ô) Methylene proton at ŭ 1.56 (H-1) correlated with ŭ 81.69 (C-3); ŭ 60.21(C-9) and ŭ 19.31 (C-14). The methylene protons at ŭ 2.2 (m) (H-2) correlated with ŭ 74.76 (C-4). Methine proton at ŭ 4.8 (dd H-3) correlated with the ŭ 38.60 (C-1); ŭ 74.76 (C-4) and ŭ168.73 (C-1ô). Another methine proton at ŭ 1.9 (dd H-5) correlated with ŭ 81.69 (C-3); ŭ 74.76 (C-4); ŭ 25.85 (C-6); ŭ 60.21 (C-9); ŭ 36.76 (C-10); ŭ 19.31 (C-14); ŭ 17.85 (C-15). The methylene proton at ŭ 2.68 (dd H-6) correlated with ŭ 51.44 (C-5); ŭ 130.64 (C-7); ŭ 202.64 (C-8); ŭ 36.76 (C-10); ŭ 145.29 (C-11). The methylene protons at ŭ 2.2 (d) H-9 correlated with ŭ 51.44 (C-5); ŭ130.64 (C-7); ŭ 202.64 (C-8); ŭ 36.76 (C-10); ŭ19.31 (C-14). The methyl protons at ŭ 2.0 (s) (H-12) correlated with ŭ 23.13 (C-13) and methyl proton at ŭ 1.8 (s) (H-13) correlated with ŭ 23.82 (C-12). The methyl protons at ŭ 1.0 (s) (H-14) correlated with C-1 ŭ 38.60; (C-5) ŭ 51.44; (C-9) ŭ 60.21 and C-10 ŭ 36.76.



H-C Correlation of compound 1

The methyl protons at ŭ 1.3 (s) (H-15) correlated with C-3 ŭ 81.69; C-4 ŭ 74.76 and C-5 ŭ 51.44. Methine proton at ŭ 6.1 (q) (H-3ô) correlated with ŭ 16.27 C-5ô and ŭ 166.99 C-1ô. The vinylic proton at ŭ 6.1 (q) (H-3ô) correlated with ŭ 16.27 C-5ô; ŭ 168.73 C-1ô and ŭ 21.02 C-4ô. From the literature reported before (Guilhon and Muller, 1996) the configuration of **1** is ß-substituted at C-3 and C-4.The coupling constant of H-5 and H-6 confirm the configuration.



1

3ɓ-angeloyloxy-4ɓ-hydroxy-eudesm-7, 11-en-8-one.

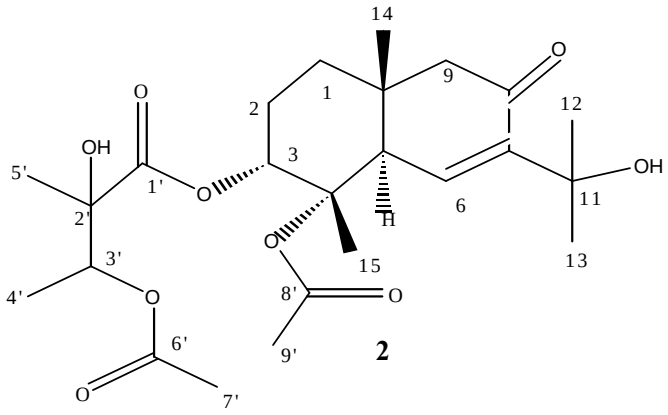
4.11.2 Structure elucidation of Eudesmane Sesquiterpenoid 2

The ^{1}H NMR (Table 10) showed multiplet peak at ŭ 1.23 and 1.47 integrated for two protons indicating the presence of methylene group. Peaks appearing at ŭ 1.91 (^{1}H) and ŭ 2.38 (^{1}H) show diastereotopic protons of methylene groups. A broad peak at ŭ 5.8 (^{1}H) revealed methine proton attached with oxygen substituted tertiary carbon. A complex peak at ŭ 1.91 ï 3.0 integrated for five protons, showed two methylene groups and one methylene proton. A singlet peaks at ŭ 1.33, 1.33, 0.9, 1.55, 1.3 and 1.99 each integrated for three protons indicated methyl protons. A quartet peak at ŭ 5.0 integrated for one proton indicated methine proton. The proton decoupled ^{13}C NMR spectrum (appendix 13, Table 11) of **2** showed 24 carbon atoms. The multiplicity of each carbon atom was determined using DEPT 135 and 90 experiment, which revealed the presence of eight methyl groups, three methylene groups four methine groups and five quaternary carbon atoms indicating 34 hydrogen atoms attached to carbon atoms.

Table 11: NMR Spectral Data of Eudesmane Sesquiterpenoid 2

Carbon no.	^{13}C NMR (ppm)	DEPT	^{1}H NMR (ppm)	H/H COSY	HMBC
1.	32.01	CH_2	1.23, 1.47	H^1-H^2, H^2-H^3	H-1 ź C-3C-5,C-9,C-14
2.	23.40	CH_2	1.91	H^2-H^1, H^2-H^3	H-2 ź C-4,C-10
3.	74.68	CH	5.8 dd 2.55, 2.79 Hz	H^3-H^2	H-3 ź C-1,C-5,C-15
4.	81.87	Cq	-	-	-
5.	48.95	CH	3.0	H^5-H^6	H-5 ź C-7,C-14,C-15
6.	141.23	CH	2.89	H^6-H^5	H-6 ź C-4,C-8,C-10,C-11
7.	145.76	Cq	-	-	-
8.	200.51	C=O	-	-	-
9.	58.02	CH_2	2.38		H-9 ź C-1,C-5,C-7,C-14
10.	39.42	Cq	-	-	-
11.	72.13	Cq	-	-	-
12.	22.54	CH_3	1.33	-	H-12 ź C-7, C-13
13.	22.57	CH_3	1.33	-	H-13 ź C-7, C-12
14.	18.49	CH_3	0.99		H-14 ź C-1, C-5, C-9
15.	19.25	CH_3	1.55	-	H-15 ź C-3,C-5
1â	173.98	C=O	-	-	-
2â	76.24	Cq	-	-	-
3â	74.22	CH	5.0 quartet 6.35 Hz	$H^{3ĉ}$-$H^{4ĉ}$	H-3ô ź C-1â,C-5ô
4â	13.60	CH_3	1.3	$H^{4ĉ}$-$H^{3ĉ}$	H-4ô ź C-2ô
5â	21.47	CH_3	1.99	-	H-5ô ź C-1â,C-3ô
21.	169.70	11-$CH_3\underline{C}O$	-	-	-
22.	170.44	4-$CH_3\underline{C}O$	-	-	-
23.	29.14	11-$\underline{C}H_3CO$	1.45	-	-
24.	29.53	4-$\underline{C}H_3CO$	1.45	-	-
Cq = 9, CH = 4, CH_2 = 3, CH_3 = 8, O = 9					

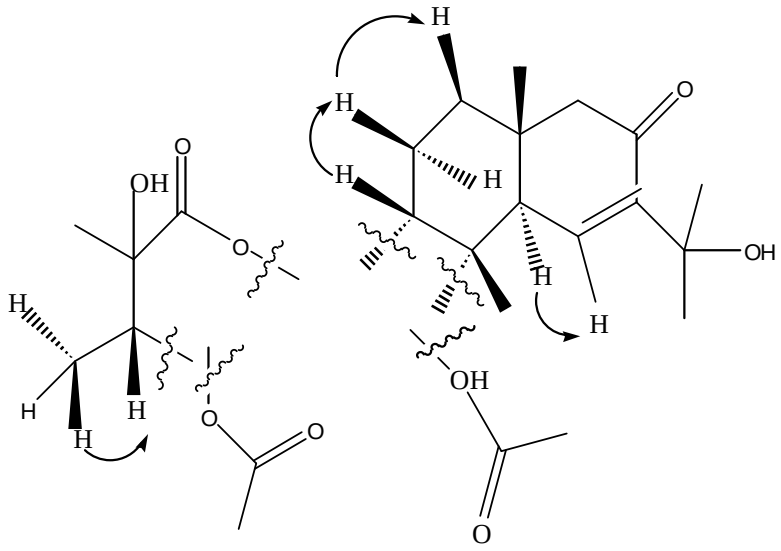
From ^{1}H NMR, Proton Decoupled ^{13}C and DEPT spectra data of **2** (Appendix 15, Table 11). The ^{13}C NMR taken when the machine operating at 400MHz showed two prominent peaks which on analysis came from impurities (Appendix 15). However, on high resolution the machine operating at 600MHz the carbonyl carbon atom (C-10) at 200.51ppm was picked out which was very faint at 400MHz (Appendix 16). The proposed structure of compound **2** is shown below.



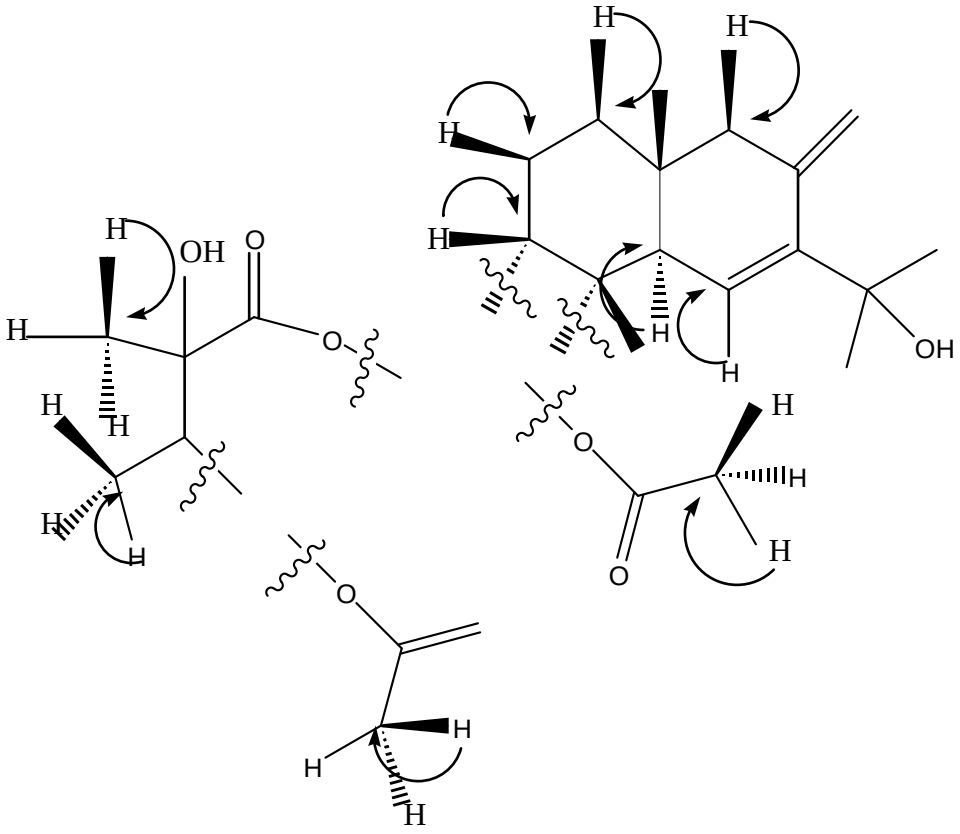
2

3ß-angeloyloxy-4ß-acetoxy-11-hydroxy-eudesm-6-en-8-one

The above prediction was also supported by using its 2D NMR spectral data as follows. ^{1}H-^{1}H correlation spectroscopy (COSY) (Appendix 14, Table 11) showed strong correlation between H-3 ŭ 5.8 and H-2 ŭ 1.91;H-1 ŭ 1.23 and H-2; H-3 and H-1 indicated H-1, H-2 and H-3 exist in the same region. There are also coupling between H-5 (ŭ 2.38) and H-6 (ŭ 0.99). HSQC analysis (Appendix 18) corroborated the assignment of the carbon atom-hydrogen atom connectivities in the molecule a few correlations are shown below on molecule fragments.



H-H Correlation of compound 2

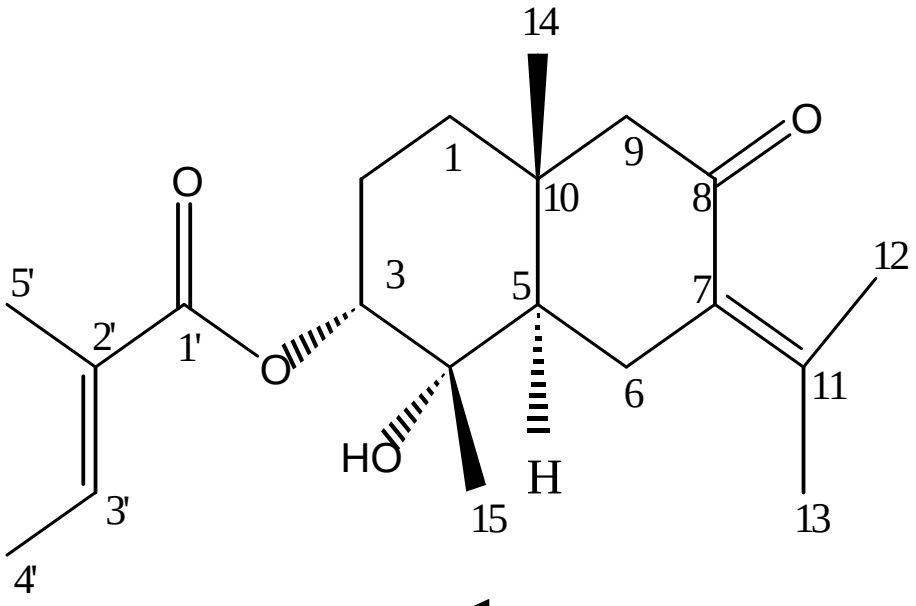


HSQC correlation of compound 2

In the HMBC (Appendix 19,Table 11), the methine proton at ŭ 5.8 (H-3) correlated with ŭ 32.01 C 1 and ŭ 81.87 C-4. Another methine proton at ŭ 3.0 (H-5) correlated with ŭ 81.87 (C-4) ; ŭ 145.76 (C-7); ŭ 58.02 (C-9) and ŭ 39.42 (C-10), methylene proton at ŭ 2.89 (H-6) correlated with ŭ 81.87 (C-4); ŭ 48.95(C-5) ; ŭ 145.76 (C-7); ŭ 39.42 (C-10) and ŭ 72.13 (C-11). A methylene proton at ŭ 2.38 (d) (H-9) correlated with ŭ 145.76 (C-7); ŭ 200.51 (C-8); ŭ 39.42 (C-10);and ŭ 18.49 (C-14). Methyl proton at ŭ 1.33 (s) (H-12) showed correlated with ŭ 22.57 (C-13). The methyl protons at ŭ 0.99 (s) (H- 14) showed correlation with ŭ 32.01 C-1; ŭ 48.95 C-5; ŭ 58.02 (C-9); ŭ 39.42 (C-10). Another methyl protons at ŭ 1.55 (s) (H-15) correlated with ŭ 74.68 (C-3) and ŭ 48.95 (C-5). Methine proton at ŭ 5.0 (q) (H-3ô) displayed correlation with ŭ 21.47 (C-5ô) and ŭ 173.98 (C-1ô). Methyl proton at ŭ 1.3 (d) (H-4ô) correlated with ŭ 76.24 (C-2ô), methyl proton at ŭ 1.99 (s) (H-5ô) correlated with ŭ 173.98 (C-1ô), methyl proton at ŭ 1.45 (s) (H-7ô) correlated with ŭ 74.22 (C-3ô).

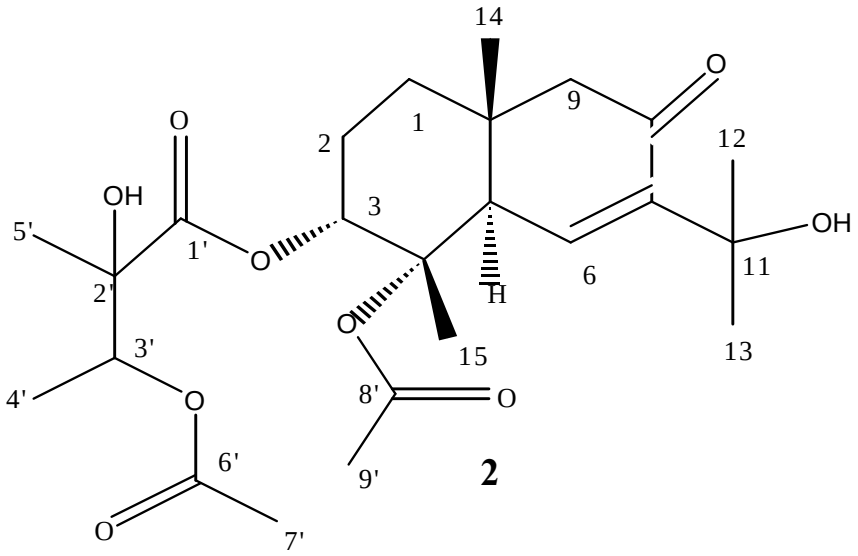
4.12Compounds 1 and 2 similarity and differences

The eudesmane sesquiterpenoids isolated in this work have basically similar skeletal structure except that in compound **2** there is observed hydroxylation at C-13 and C-2ôand acetylation at C-4 and C-3ô. This makes the compound more polar and explains the compoundôs low Rf value compared to compound **1.** Both of them have ɓ- configuration at C-3 and C-4



1

3ɓ-angeloyloxy-4ɓ-hydroxy-eudesm-7, 11-en-8-on



2

3ɓ-angeloyloxy-4ɓ-acetoxy- 11-hydroxy-eudesm-6-en-8-one

CHAPTER FIVE

CONCLUSIONS AND RECOMMENDATIONS

5.1 Conclusions

The two plants under study grow wildly in the rural parts of Kenya where *A. gambiae* is a serious problem. The results show that the crude ethyl acetate extracts of *C. anisata* and hexane extract of *L. alata* could be used in mosquito control instead of synthetic larvicides. As adult mosquitoes transmit diseases, the critical concentrations of the materials which kills 50% (LC_{50}) of the treated larval population from emerging adults are more meaningful (Bhakthratchagan et al., 1993; Moshen et al., 1995). Their corresponding LC_{99} extracts have shown that up to 99% of the larvae population tested can be killed. These indicate with certainty it can help reduce the mosquito population drastically. Considering that a large proportion of the human population living in malaria prone areas suffer from varying degrees of poverty, the discovery of plant extracts that could control the mosquito population is of great value.

The plants *L. alata* contains bioactive Eudesmane sesquiterpenoids, which were isolated and characterized using physical methods of structure elucidation. Mass spectrum coupled to gas chromatography (GC-MS) technique and nuclear magnetic resonance (NMR) spectroscopy and correlation spectroscopic techniques like ^{13}C NMR ^{1}H-^{1}H COSY, HMBC and HSQC proved extremely useful in the identification of these compounds. Two new Eudesmane sesquiterpenoids 3ɓ-angeloyloxy-4ɓ-hydroxy-eudesm-7, 11-en-8-one **(1)** and 3ɓ-angeloyloxy-4ɓ-acetoxy-11-hydroxy-eudesm-6-en-8-one **(2)** isolated and characterized.

Several other known sesquiterpenoids were isolated from the plants and identified. The medicinal use of these plants by the herbalist against various diseases could possibly be attributed to the presence of these compounds. Their active ingredients have no toxicities to humans since the *L. alata* and *C. anisata* plants from which the extracts are obtained have been used as traditional medicine for centuries without any reported illness or side effects resulting from their use (Cheney, 1970).

Hexane fraction of *L. alata* and ethyl acetate fraction of *C. anisata* showed great activity towards *A. gambiae s.s,* larvae. The pure compounds **1** and **2** did not show reasonable activity possibly because of low mass used during the bioassay. The other possibility is that the activity is enhanced by the synergic effect of compounds in the extract. However, the oils of *C. anisata* showed markedly enhanced activity against *A. gambiae s.s,* larvae with an LC_{50} value of 75.96mg/l. The *L. alata* oils gave higher LC_{50} (273.38mg/l) values indicating low activity but on the contrary, the LC_{50} (1161.30mg/l) of its extracts were better than LC_{50} (2095.46 mg/l) of *C. anisata* plant.

The oils formulations require immediate use due to volatility of the constituent compounds, which imply the extracts could prove to be better larvicides than the oils. The plants *L. alata* and *C. anisata* contains bioactive sesquiterpenoids. Application of these extracts to larval habitats may lead to promising results in malaria and mosquito management programmes. The isolated larvicidal compounds can be used as lead compounds for environmentally friendly and biodegradable larvicides.

5.2 Recommendations

In the course of isolation and purification of these compounds, most of the fractions discarded presented a number of compounds with very close retention indices and hence difficult to separate given the bulk of work and elaborate larvicidal assay involved.

- Apart from the ^{13}C NMR, DEPT and ^{1}H NMR employed in this work to elucidate the structure there is need to carry out IR analysis to verify the functional groups in these new Eudesmane compounds **1** and **2**.
- Itôs prudent also to look at their physical properties like density, refractive indices, boiling and melting points to ascertain their purity.
- Isolate more compounds from the plants and assess their larvicidal activity**.**
- Unknown compounds in *L. alata* oils need to be carefully isolated and elucidate their structures.

- Isolate and purify oils compounds from *C. anisata* and *L. alata* and ascertain the bioactivities of each compound.
- The larvicidal assays were basically using laboratory bred larvae *A. gambiae s.s*, field application on *A. gambiae s.l* larvae is necessary.
- Stability of the oils needs to be studied and enhanced to increase their shelf life.

REFERENCE

Adams, R. P, (1995). Identification of Essential Oil Components by Gas Chromatography/Mass Spectroscopy, Allured Publishing Corporation: Illinois, U.S.A. pp. 75-89.

Ansari, M. A. and Razdan, R. K. (2000). (*F. Leguminosae*) oil against mosquitoes. *Biores Technol*. **73**: 207-211.

Bhakthratchagan, R. Rita, C. and Jebansesan. A. J. (1993). Laboratory evaluation of two insect growth regulators against some vector mosquitoes. *Insect Sci*. **6**: 276.

Bracco, J. E., Barata, F. M. and Marinotti, O. (1999). Evaluation of insecticide resistance and biochemical mechanisms in a population of *Culex quinquefasciatus* (Diptera: *Culicidae*) from São Paulo, Brasil. *Mem. Inst. Osvaldo Cruz*. **94**:115-120.

Centers for Disease Control (CDC). (2008) and Prevention, Division of Parasitic Diseases, National Center for Zoonotic, Vector-Borne, and Enteric Diseases (ZVED); Malaria Vector Control. Atlanta, U.S.A. pp. 129-136.

Chakraborty, A., Chowdhury, B. and Bhattacharyya, K. (1995). Clausenol and Clausenine ï two carbazole alkaloids from *Clausena anisata. Phytochem.* **40**: 295-298.

Cheney, R. H. (1970). Aloe drug in human therapy. *Quart. J. Crude Drug Res.* **10**: 1523-1530.

Ciccia, G., Coussio, J. and Mongelli, E. (2000). Insecticidal, repellent activity against *Aedes aegypti* larvae of some medicinal South American plants. *J. Ethnopharmacol. Pharm.***71**: 267-268.

Consoli, R. A., Mendes, N. M., Pereira, J. P., Santos, B. S. And Lamounier, M. A. (1988). Influência de diversos derivados devegetais na sobrevida das larvas *Aedes fluviatilis* (Lutz) (Diptera: Culicidae) em laboratório. *Mem Inst Oswaldo Cruz*. **83**: 87-93.

Deng, S. X. (1963). Defervescence and removing phlegm activities in *Laggera pterodonta*. *J. Yunnan Med.***2**: 28-30.

Dondorp, A. M., Fanello, C. I. and Hendriksen, I. C. (2010). "Artesunate versus quinine in the treatment of severe falciparum malaria in African children (AQUAMAT): an open-label, randomised trial". *Lancet*. **376:** 1647ï 1657.

Dondorp, A. M and Day, N. P. (2007). "The treatment of severe malaria.". *Trans. R. Soc. Trop. Med. Hyg.* **101**: 633ï 634.

El Hag, E. A., Nadi, A. H.and Zaitoon, A. A. (1999). Toxic and growth retarding effects of three plant extracts on *Culex pipiens* larvae (Diptera: *Culicidae*). *Phytother. Res* . **13**: 388.

Finney, D. J. Probit Analysis. 3rd ed. (1971). London: Cambridge University press;. p. 38-39.

Fong, Y. L., Cadigan, F. C. and Coatney, G. R. (1971). "A presumptive case of naturally occurring Plasmodium knowlesi malaria in man in Malaysia". *Trans. R. Soc. Trop. Med. Hyg.* **65**: 839ï 840.

Guilhon, G. M. S. P. and Muller, A. H. (1996), *Photochem.*, **43**: 417-418.

Guillen, M. D., Cabo N. and Burillo, J. J. (1996). *Sci Food Agric*., **70**: 359

Hubalek, Z. and Halouzka, J. (1999). West Nile Fever, A re-emerging mosquito-borne viral disease in Europe. *Emerging Infectious Diseases*. **2**: 519-529.

Hyde, M.A. and Wursten, B. (2008). Flora of Zimbabwe: Species information: *Clausena anisata* and *Laggera alata.* pp. 69-85

Imperial College, London. (2000) "Scientists create first transgenic malaria mosquito". pp.894.

Ito, J., Ghosh, A., Moreira, L. A., Wimmer, E. A. and Jacobs-Lorena, M. (2002). "Transgenic *anopheline mosquitoes* impaired in transmission of a malaria parasite". *Nature.* **417**: 387ï 388

Ito, C., Katsuno, S., Itoigwa, M., Ruangrungsi, N., Mukainaka,T., Okuda, M., Kitagawa, Y., Tokuda, H., Nishino, H. and Furukawa, H. (2000). New carbarzole alkaloids from *Clausena anisata* with anti-tumor promoting activity. *J. Nat. Prod.* **63**: 125- 128.

Jacquerioz, F. A. and Croft, A. M. (2009). "Drugs for preventing malaria in travellers". *Cochrane Database Syst Rev*. **4**: 258-259.

Kaushik. R. and Saini, P. (2008) Larvicidal activity of leaf extract of *Millingtonia hortensis* (Family: *Bignoniaceae)* against *Anopheles stephensi, Culex quinquefasciatus* and *Aedes aegypti J Vector Borne Dis.* **45**, 66ï 69

Kilama, W. and Ntoumi, F. (2009). "Malaria: a research agenda for the eradication era". *Lancet.* **374**: 1480ï 1482.

Kitagawa**,** K., Skowyra, D., Elledge, S.J., Harper, J.W., and Hieter, P. (1999). SGT1 encodes an essential component of the yeast kinetochore assembly pathway and a novel subunit of the SCF ubiquitin ligase complex. *Mol. Cell* **4,** 21ï 33.

Knols, B. G., Njiru, B. N., Mathenge, E. M., Mukabana, W. R., Beier, J. C. and Killeen, G. F. (2002):Malaria sphere ; A green house enclosed simulation of a natural *Anopheles gambiae s.s* (Diptera: Culicidae) ecosystem in western Kenya. *Malaria J.* **5:** 75-76.

Kokwaro , J. O. (1993). Medicinal plants of East Africa. Kampala, Nairobi, and Dar-es-Salaam: East Africa Literature Bureau. pp. 106ï 115.

Kumari, R. Thapar, B. R., Dasgupta, R. K., Kaul, S.M. and Shiv, L. (1998). Susceptibility status of malaria vectors to insecticides in India. *J. Commun. Dis*. **33**: 179ï 85.

Lin, R. and. Chen, Y. L. (1985) Flora Reipublicae Popularis Sinicae. Science Press, Beijing. pp. 1.

Matasyoh, J. C., Wathuta, E. M., Kariuki, S. T., Chepkorir, R. and Kavulani, J. (2008) Aloe plant extracts as alternative larvicides for mosquito control. *Afri. J. Biotechnol*. **7**: 912-915.

Matasyoh, J.C., Kiplimo, J. J., Karubiu, N. M. and Hailstorks, T. P. (2007). Chemical composition and antimicrobial activity of essential oil of *Tarchonanthus camphorates. Food Chem*. **101**: 1183ï 1187

Moshen, Z. H., Jawad, A. M., Al Saadi, M, and Al Naib, B. A. (1995). Anti-oviposition and insecticidal activity of Imperata cylindrical (Graminae). *Med. Vet. Entomol*. **9**: 441.

Obomanu, F.G., Ogbalu, O.K., Gabriel, U.U., Fekarurhobo, G.K. and Adediran B. I., (2006). Larvicidal properties of *Lepidagathis alopecuroides* and *Azadirachta indica* on *Anopheles gambiae* and *Culex quinquefasciatus. Afr. J. Biotechnol*. **5**: 761-765.

Pates, H. and Curtis, C. (2005) "Mosquito behaviour and vector control". *Annual Review on Entomology*, **50**: 53-70.

Phillips, L. and Nicky, N. (2010). "Gorillas in midst of malaria mystery". *Sydney Morning Hearld*. **2:** 56-58

Pierre F. G. (2008) Vector Control, Long Lasting Insecticidal Nets: current status & programmatic issues. WHO Geneva. Pp.465-467

Pitasawat, B., Champakaew, D., Choochote, W., Jitpakdi, A., Chaithong, U., Kanjanapothi R, Tippawangkosol P., Riyong, D., Tuetun, B. and Chaiyasit, D. (2007) Aromatic plant-derived essential oil: An alternative larvicide for mosquito control. *Fitoterapia* **78**, 205-210.

Quan-Xiang W,, Yan-Ping S. and Zhong-Jian J. (2006). Eudesmane sesquiterpenoids from the Asteraceae family. *Nat. Prod.* **10**: 1039-1040

Raharivelomanana, P., Bianchini, J. P., Ramanoelina, A. R. P., Rasoarahona, J. R. E., Faure, R. and Cambon, A. (1998). Eudesmane sesquiterpenes from *Laggera alata*. *Phytochem*. **47**: 1085-1088.

Roch, K. D., Abdoulaye, D., Thierry, B., LéaParé-Toé, Robert, T. G, Jean-Bosco, O., and Ole Skovm (2006) Personal protection of long lasting insecticide-treated nets in areas of *Anopheles gambiae s.s*. resistance to pyrethroids. *Malaria J*, **5**:123-124.

Roestenberg, M., McCall, M. and Hopman, J. (2009). "Protection against a malaria challenge by sporozoite inoculation". *N. Engl. J. Med.* **361**: 468ï 477.

Romi, R., Toma, L., Severini, F., Di Lucas, M. (2003). Susceptibility of Italian populations of *Aedes albopictus* to temphos and other insecticides. *J. Am. Mosquito contr*. **19**: 419-423.

Sharma, R. N., Tera, V.S. and Despande, S.G. (1990). New chemicals, natural products and their permutation and combination to combat insect pest. In: *Impact of environment on animals and aquaculture*. pp 97-100.

Silvagnaname, N. and Kalyanasundaram, M. (2004). Laboratory evaluation of methanolic extract of *Atlantia monophylla (*Family*: Rutaceae*) against immature stages of mosquitoes and non-target organisms. *Mem. Inst. Oswaldo Cruz, Rio de Janeiro*. **99**: 115-118.

Singh, B. and Kim Sung, L. and Matusop, A. (2004). "A large focus of naturally acquired *Plasmodium knowlesi* infections in human beings". *Lancet.* **363**: 1017ï 24.

Singh, R. K., Dhiman, R. C.and Mittal, P. K. (2006). Mosquito larvicidal properties of *Momordica charantia Linn (*Family*: Cucurbitaceae), J. Vect. Borne. Di*s**.* **43:** 88-91.

Snow, R. W., Guerra, C. A., Noor, A. M., Myint, H. Y. and Hay, S. I. (2005). "The global distribution of clinical episodes of *Plasmodium falciparum malaria*". *Nature* **434**: 214ï 7.

Sukumar, K. Perich, M. J. and Boombar, L.R. (1991). Botanical derivatives in mosquito control: A review. *J. Am. Mosq. Contr*. Assoc. **7**: 210ï 237.

Tellez, M. R., Canel, C., Rimando, A.M. and Duke, S.O. (1999). *Phytochem.*, **52**, 1035-1039.

Wattal, B. L., Joshi, G. C. and Das, M. (1981). Role of agricultural insecticides in precipitation vector resistance. *J. Comm. Dis.* **13**: 71-73.

Wellems, T. E., (2002). "Plasmodium chloroquine resistance and the search for a replacement antimalarial drug". *Science* **298**: 124ï 126.

WHO (1981). Instructions for determining susceptibility or resistance of mosquito larvae to insecticides. *WHO/VBC*. **81**: 807-808.

WHO Global Malaria Programme (2006) Indoor residual spraying. WHO. Pp. 1112-1113

WHO Tech Rep Ser (1992). Vector resistance to pesticides: fifteenth report of WHO expert committee on vector biology and control; **818**: 62-63.

WHO Technical report series (1995). Vector control for malaria and other mosquito-borne diseases, No.857. pp 56-57

Zhao, Y., Yue, J. M., He, Y. N., Lin, Z. W. and Sun, H.D. (1997a). Eudesmane derivatives from *Laggera pterodonta*. *J. Nat. Prod.* **60**: 545-549

Zhao, Y., Yue, J.M., He, Y.N., Lin, Z. W. and Sun, H.D. (1997 b). Eudesmane sesquiterpene from *Laggera pterodonta. Phytochem.* **44**: 459-464.

Zheng, Q., Xu, Z., Sun, X., Yao, W., Sun, H., Cheng, C. H. K. and Zhao, Y. (2003). Eudesmane and megastigmane glucosides from *Laggera alata*. *Phytochem*. **63**: 835-939.

APPENDICES

Appendix 1: Plant products reported for bioactivity against mosquito vectors

Plant species (Family)	Plant product	Species tested	Type of activity	References
Artimisia cina (Compositeae)	Aqueous extract	*Culex pipens*	Larvicidal	Yali, M. Z. *et al*. (1996)
Atlantia monophylla (Rutaceae)	Methanol extract	*Cx.quinquefasciatus* *Ae. aegypti, Anapheles spp.*	Larvicidal & Pupicidal	Silvagnaname *et al*.(2004)
Azadirachta indica (Meliaceae)	Aqueous extract	*An.gambiae, Cx.quinquefasciatus*	Larvicidal	Obomanu *et al.,* (2006)
Azadirachta indica (Meliaceae)	Neem leaves extract + *Malathian*	*Culex fatigans*	Larvicidal	Mohammad A. *et al.* (1996)
Azadirachta indica (Meliaceae)	Neem oil-Oil water emulsion on wood scrapping	*Cx.quinquefasciatus*, *An. Stephensi, Ae.aegypti*	Larvicidal, Repellent	Batra *et al.* (1998)
Citrus *spp*.(Rutaceae)	Essential oil	*Cx.pipens, Cx.quinquefasciatus*	Adulticidal, Larvicidal	Al Dakhil and Morsy.(1999)
Cleome droserifolia (Capparidaceae)	Aqueous extract	*Culex pipens*	Larvicidal Larvicidal	Yali, M. Z .*et al.*(1996)
Citrus *spp*.(*Rutaceae*)	Fruit peel oil	*Cx.pipens,Cx.quinquefasciatus*	Adulticidal, Larvicidal	Ezenou *et al.* (2001)
Mentha piperita (Labiatae)	Essential oil	*Cx.quinquefasciatus* , *A*n. Stephensi, Ae.aegypti	Larvicidal, Repellent	Pathak *et al.* (2000)
Mentha piperita (Labiatae)	Fruit peel oil	*Cx.pipens, Cx.quinquefasciatus*	Adulticidal, Larvicidal	Ansari *et al.* (1999)
Momordica charantia	Plant extract	*Cx.quinquefasciatus* *Ae.aegypti,*	Larvicidal	Singh *et al.* (2006)

(Cucurbitaceae)		*Anapheles spp*		
Ocimum sanctum (Labiatae)	Steam distillated essential oil	*Cx.quinquefasciatus , An. Stephensi, Ae.aegypti*	Larvicidal	Pathak *et al.* (2000)
Polyalthia longifolia (Annonaceae)	Leaf extract	*Cx.quinquefasciatus*	Larvicidal Larvicidal, Growth regulator	Murty *et al*. (1997)
Tagetes errecta (Compositeae)	Acetone extract, Steam distillated essential oil	*Cx.quinquefasciatus , An. Stephensi, Ae.aegypti*	Larvicidal,	Pathak *et al.* (2000)
Solanum nigrum Linn. (Solanaceae)	Crude leaf extract	*An. Culicifacies, Cx.quinquefasciatus Ae.aegypti*	Larvicidal	Singh *et al.* (2002)
Solanum nigrum Linn. (Solanaceae)	Ethanolic leaf extract	*Ae. Caspius, Cx pipiens*	Larvicidal, Growth regulator	Ahmed *et al.* (2001)

Appendix 2: P r o b i t a n a l y s i s of *Clausena anisata* oils

Observed and Expected Frequencies

	Number of	Observed	Expected		
concentration	Subjects	Responses	Responses	Residual	Prob
250.00	20.0	19.7	19.748	-.078	.98742
200.00	20.0	19.7	18.894	.776	.94472
175.00	20.0	17.7	17.974	-.304	.89868
150.00	20.0	15.0	16.591	-1.591	.82957
125.00	20.0	14.7	14.719	-.049	.73594
100.00	20.0	14.3	12.429	1.901	.62145
75.00	20.0	10.0	9.902	.098	49509
50.00	20.0	6.7	7.384	-.714	.36922

Confidence Limits for Effective concentration

		95% Confidence Limits	
Probits	concentration	Lower	Upper
.01	-104.88368	-232.39867	-44.25533
.02	-83.69289	-199.32482	-28.50098
.03	-70.24802	-178.36455	-18.48133
.04	-60.13396	-162.61219	-10.92869
.05	-51.90696	-149.81024	-4.77383
.06	-44.90449	-138.92298	. 47413
.07	-38.76469	-129.38485	5.08344
.08	-33.26723	-120.85155	9.21748

.09	-28.26751	-113.09716	12.98353
.10	-23.66526	-105.96507	16.45602
.15	-4.61073	-76.50839	30.90515
.20	10.53322	-53.20639	42.49810
.25	23.52537	-33.32529	52.55375
.30	35.19273	-15.59172	61.70432
.35	46.00427	.70133	70.32343
.40	56.26337	15.99137	78.67258
.45	66.18916	30.56779	86.96735
.50	75.95759	44.62698	95.41676
.55	85.72602	58.29751	104.25480
.60	95.65181	71.65208	113.77144
.65	105.91091	84.72090	124.34181
.70	116.72245	97.53054	136.44434
.75	128.38981	110.19215	150.66686
.80	141.38196	123.03288	167.76288
.85	156.52591	136.75859	188.93212
.90	175.58044	152.83567	216.76085
.91	180.18269	156.58831	223.61279
.92	185.18241	160.62288	231.09866
.93	190.67987	165.01552	239.37336
.94	196.81966	169.87552	248.66079
.95	203.82214	175.36873	259.30281
.96	212.04914	181.76677	271.86158
.97	222.16320	189.56581	287.36754
.98	235.60807	199.84514	308.06812
.99	256.79886	215.89934	340.84212

Appendix 3: P r o b i t a n a l y s i s of *Laggera alata* oils

Observed and Expected Frequencies

	Number of	Observed	Expected		
Concent	Subjects	Responses	Responses	Residual	Probits
500.00	20.0	19.3	19.755	-.425	.98776
450.00	20.0	18.7	19.204	-.534	.96021
400.00	20.0	18.7	17.912	.758	.89559
350.00	20.0	16.3	15.531	.799	.77653
300.00	20.0	12.7	12.084	.586	.60421
250.00	20.0	8.7	8.165	.505	.40826
200.00	20.0	3.3	4.664	-1.334	.23321

Confidence Limits for Effective concentration

		95% Confidence Limits	
Prob	concentration	Lower	Upper
.01	38.99931	-89.31377	107.72384
.02	66.46342	-49.64881	129.02947
.03	83.88852	-24.53130	142.59584
.04	96.99674	-5.66731	152.83222
.05	107.65927	9.65401	161.18182
.06	116.73476	22.67612	168.30737
.07	124.69218	34.07798	174.57108
.08	131.81711	44.27285	180.19360
.09	138.29695	53.53187	185.31988
.10	144.26164	62.04297	190.05046
.15	168.95709	97.13534	209.78222
.20	188.58425	124.80626	225.68377
.25	205.42260	148.32809	239.54324

.30	220.54397	169.21913	252.22177
.35	234.55616	188.31681	264.23129
.40	247.85237	206.13490	275.93089
.45	260.71660	223.01176	287.61275
.50	273.37687	239.18245	299.54798
.55	286.03714	254.82064	312.01572
.60	298.90137	270.07088	325.32420
.65	312.19758	285.08332	339.82945
.70	326.20977	300.05806	355.96191
.75	341.33114	315.30473	374.28481
.80	358.16949	331.33331	395.63753
.85	377.79665	349.04426	421.49906
.90	402.49209	370.29618	455.07125
.91	408.45679	375.30454	463.30458
.92	414.93663	380.70226	472.29215
.93	422.06155	386.59133	482.22049
.94	430.01898	393.11847	493.35890
.95	439.09447	400.50685	506.11819
.96	449.75700	409.12231	521.17365
.97	462.86522	419.63370	539.76263
.98	480.29032	433.49639	564.58382
.99	507.75443	455.15292	603.89788

Appendix 4: P r o b i t a n a l y s i s of Laggera *alata* hexane fraction 1 extract

Observed and Expected Frequencies

	Number of	Observed	Expected		
concent	Subjects	Responses	Responses	Residual	Prob
4000.00	20.0	20.0	20.000	.000	99999
3500.00	20.0	20.0	19.995	.005	.99973
3000.00	20.0	20.0	19.934	.066	.99672
2500.00	20.0	19.7	19.522	.148	.97610
2000.00	20.0	16.0	17.850	-1.850	.89249
1500.00	20.0	15.8	13.834	1.946	.69172
1000.00	20.0	11.0	8.115	2.885	.40576
800.00	20.0	3.0	5.933	-2.933	.29663

Confidence Limits for Effective concentration

		95% Confidence Limits	
Prob	concentration	Lower	Upper
.01	-412.30820	-1428.35716	84.41392
.02	-227.91468	-1144.75072	223.53657
.03	-110.92291	-965.20280	312.19671
.04	-22.91455	-830.38625	379.14263
.05	48.67346	-720.91124	433.78582
.06	109.60610	-627.88382	480.44877
.07	163.03210	-546.44809	521.49416
.08	210.86872	-473.64842	558.36174
.09	254.37421	-407.54583	591.99712

.10	294.42108	-346.79639	623.05664
.15	460.22583	-96.49491	752.86880
.20	592.00219	100.58665	857.88951
.25	705.05458	267.80753	949.84547
.30	806.57911	415.96781	1034.43401
.35	900.65672	550.97945	1115.09883
.40	989.92718	676.41635	1194.31781
.45	1076.29737	794.57284	1274.16825
.50	1161.29820	906.98409	1380.62461
.55	1246.29904	1014.74306	1443.73324
.60	1332.66923	1118.76439	1537.71884
.65	1421.93969	1220.06845	1641.07066
.70	1516.01730	1320.10548	1756.71009
.75	1617.54183	1421.12561	1888.43878
.80	1730.59422	1526.70782	2042.03342
.85	1862.37058	1642.93659	2227.90691
.90	2028.17533	1782.07708	2468.88006
.91	2068.22220	1814.83462	2527.93149
.92	2111.72769	1850.12782	2592.37625
.93	2159.56431	1888.62200	2663.54931
.94	2212.99031	1931.27411	2743.37833
.95	2273.92295	1979.53915	2834.80366
.96	2345.51096	2035.80235	2942.65866
.97	2433.51932	2104.42364	3075.79985
.98	2550.51109	2194.88874	3253.54280
.99	2734.90461	2336.14905	3535.01158

Appendix 5: P r o b i t a n a l y s i s of *Clausena anisata* ethyl acetate fraction 2 extract

Observed and Expected Frequencies

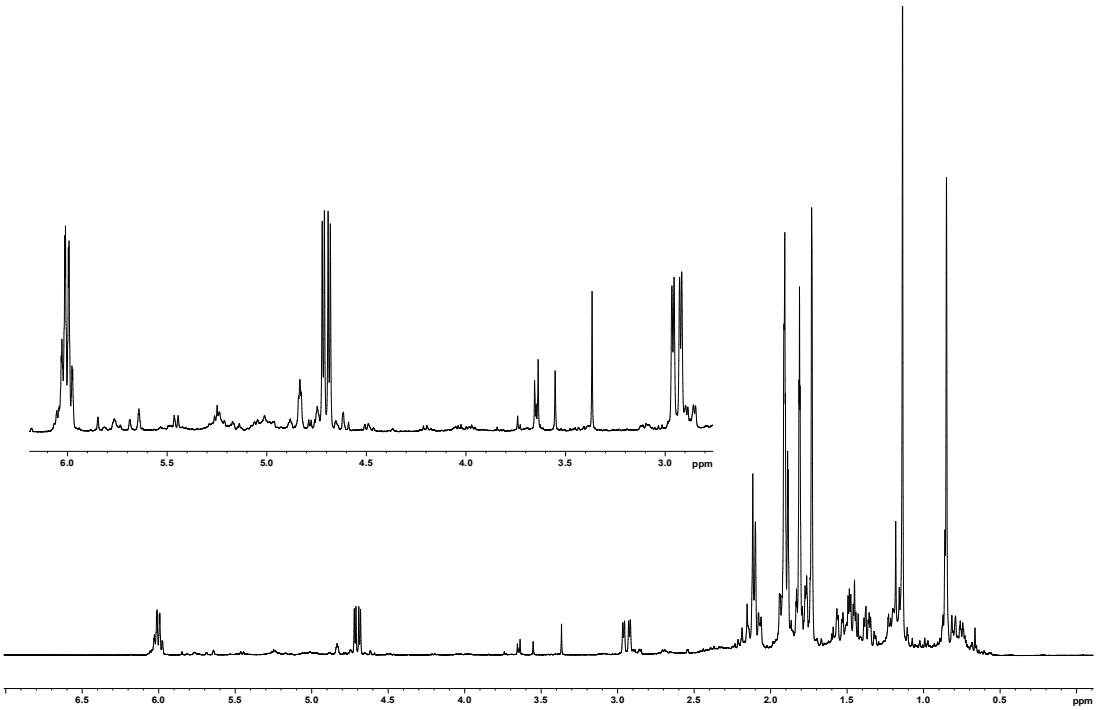
	Number of	Observed	Expected		
Concent	Subjects	Responses	Responses	Residual	Prob
4000.00	20.0	20.0	19.413	.587	.97067
3500.00	20.0	18.7	18.368	.302	.91840
3200.00	20.0	17.3	17.272	.058	.86358
3000.00	20.0	15.0	16.308	-1.308	.81541
2850.00	20.0	14.7	15.462	-.792	.77310
2500.00	20.0	12.0	13.120	-1.120	.65602
2000.00	20.0	11.3	9.245	2.085	.46225
1800.00	20.0	9.0	7.693	1.307	.38464
1500.00	20.0	4.3	5.544	-1.214	.27721

Confidence Limits for Effective concentration

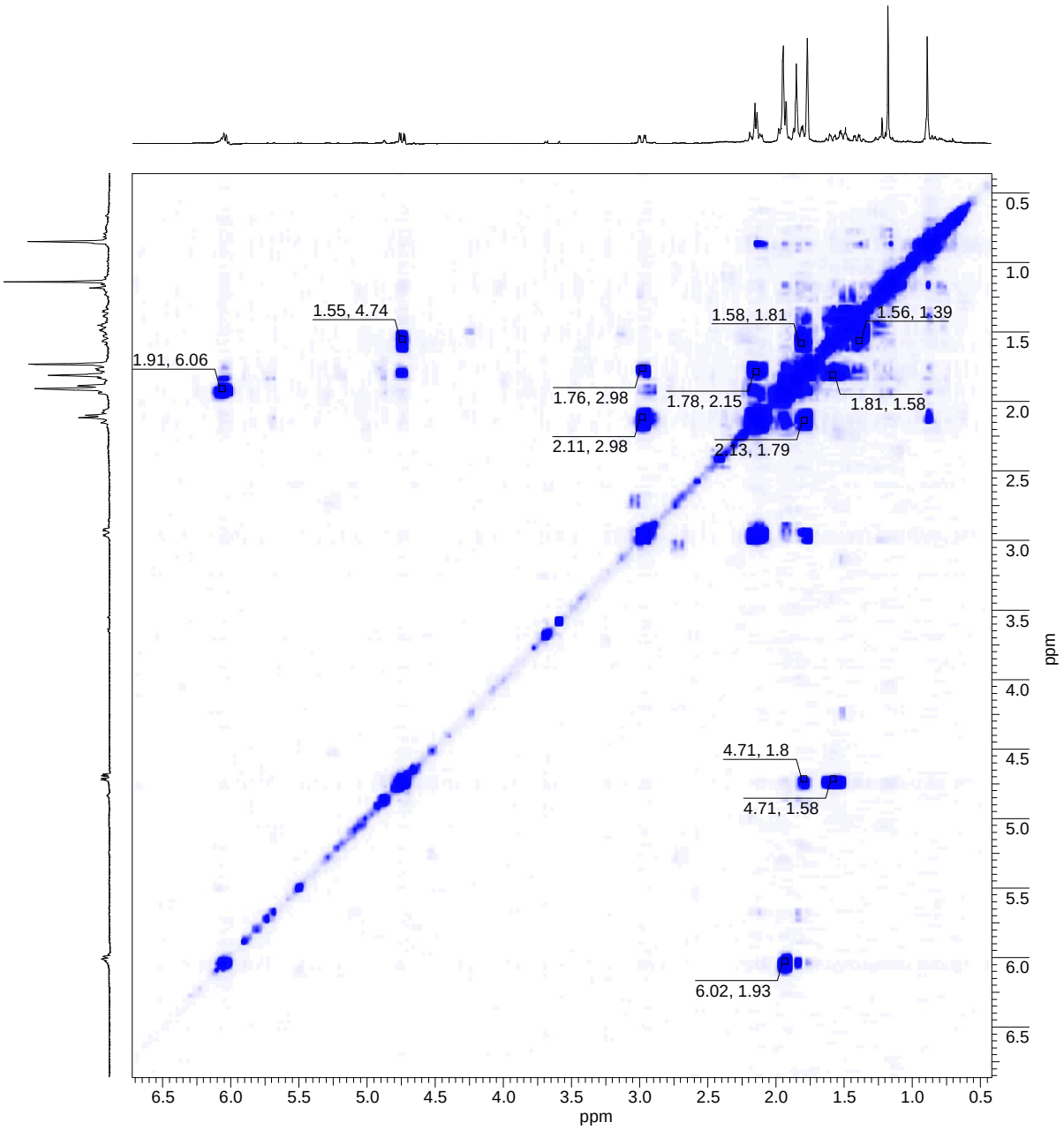
		95% Confidence Limits	
Probits	concentration	Lower	Upper
.01	-247.82254	-1514.36301	428.03933
.02	26.76098	-1117.78159	640.08870
.03	200.97543	-866.50135	774.96520
.04	332.03016	-677.68910	876.64361
.05	438.63308	-524.26700	959.51325
.06	529.36891	-393.81276	1030.18040
.07	608.92648	-279.54312	1092.25488
.08	680.16079	-177.32879	1147.93566

.09	744.94555	-84.46044	1198.66661
.10	804.58003	.94011	1245.44944
.15	1051.48270	353.46392	1440.20017
.20	1247.71312	632.02036	1596.60018
.25	1416.06134	869.34948	1732.42497
.30	1567.24325	1080.66034	1856.21785
.35	1707.33582	1274.34926	1973.05248
.40	1840.26996	1455.55547	2086.50277
.45	1968.88529	1627.61758	2199.52422
.50	2095.46147	1792.75362	2314.95185
.55	2222.03765	1952.42917	2435.83997
.60	2350.65297	2107.65257	2565.70014
.65	2483.58712	2259.38436	2708.62483
.70	2623.67969	2409.18143	2869.35132
.75	2774.86160	2560.02875	3053.60774
.80	2943.20982	2717.29582	3269.49457
.85	3139.44024	2890.43582	3531.31102
.90	3386.34291	3098.38972	3870.63166
.91	3445.97738	3147.49902	3953.70575
.92	3510.76214	3200.47779	4044.32627
.93	3581.99646	3258.34124	4144.35794
.94	3661.55403	3322.54930	4256.49400
.95	3752.28986	3395.32159	4384.84309
.96	3858.89278	3480.29718	4536.15925
.97	3989.94751	3584.12932	4722.81778
.98	4164.16195	3721.29857	4971.80526
.99	4438.74548	3936.02624	5365.70838

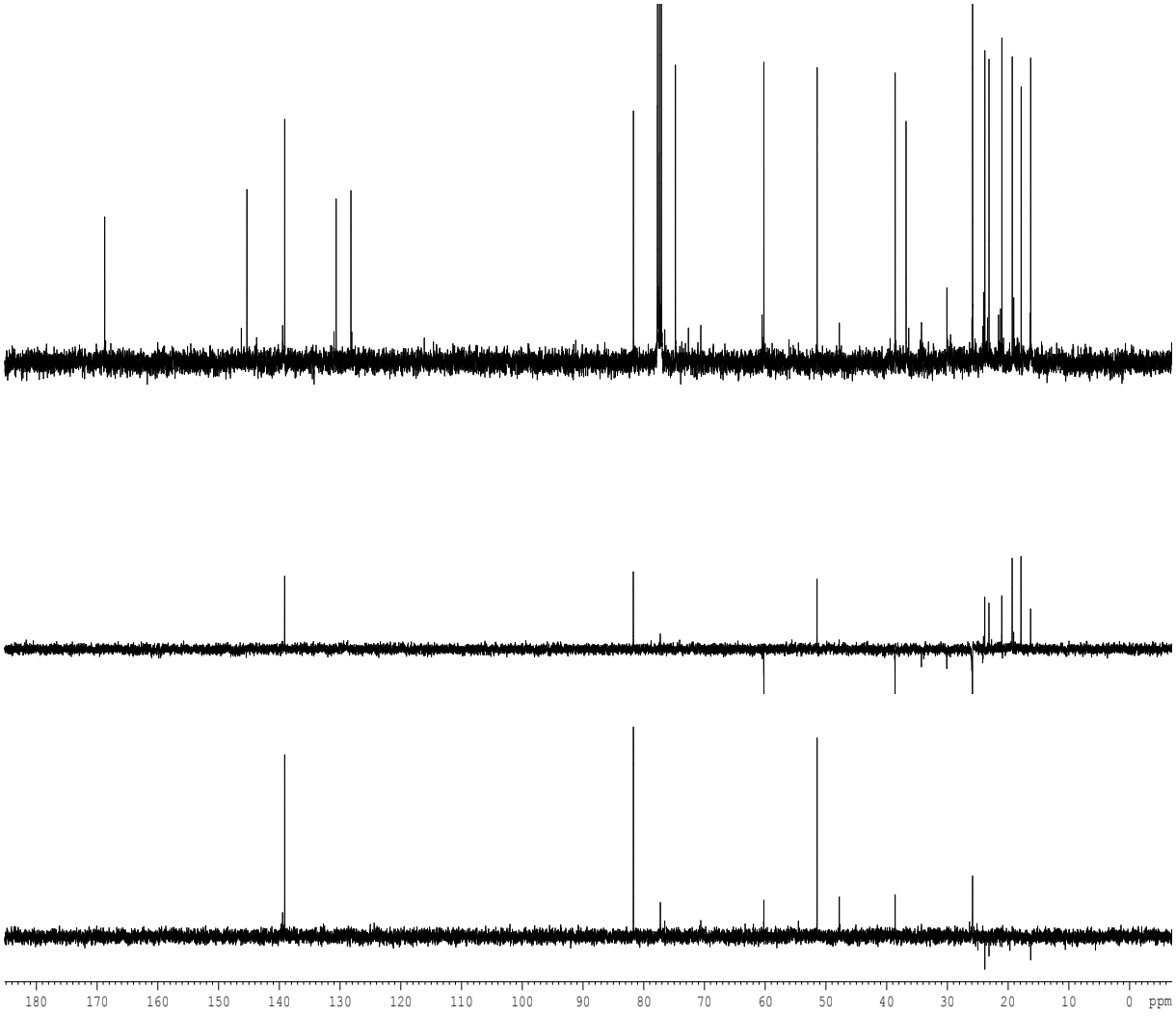
Appendix 6: ^{1}HNMR Analysis of Compound 1



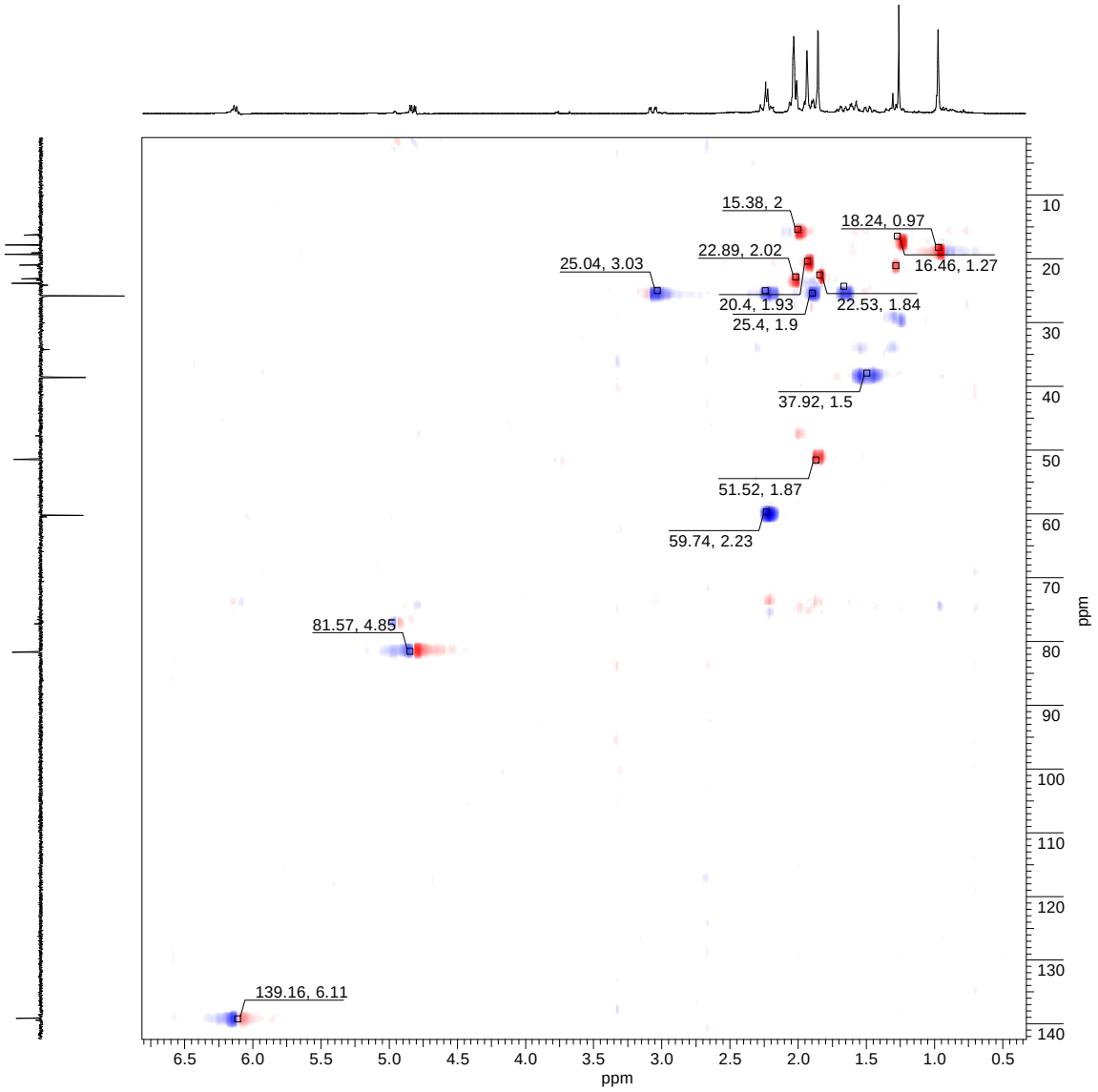
Appendix 7: H/H COSY of Compound 1



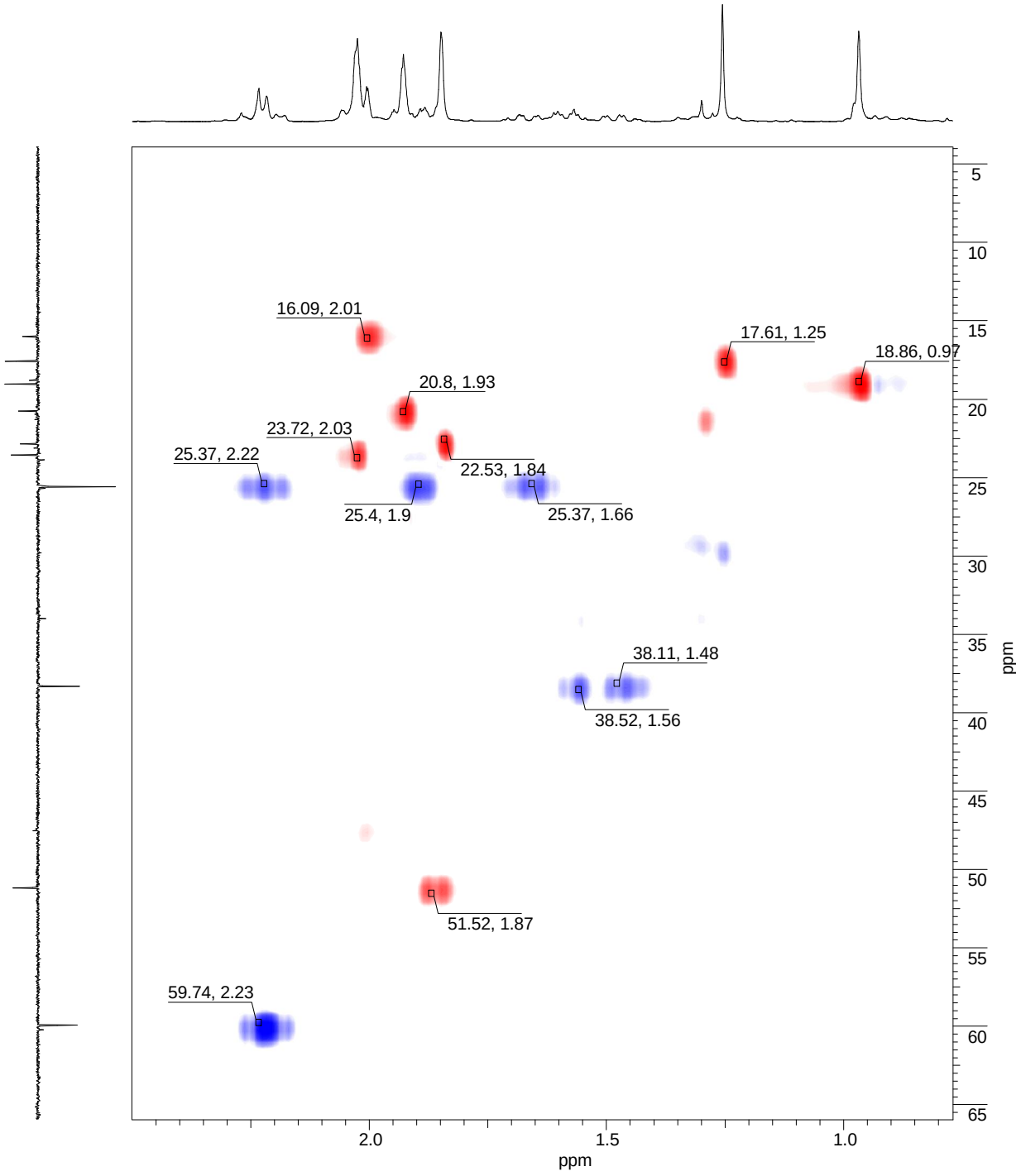
Appendix 8: ^{13}C NMR, DEPT 135 and DEPT 90 spectra of compound 1



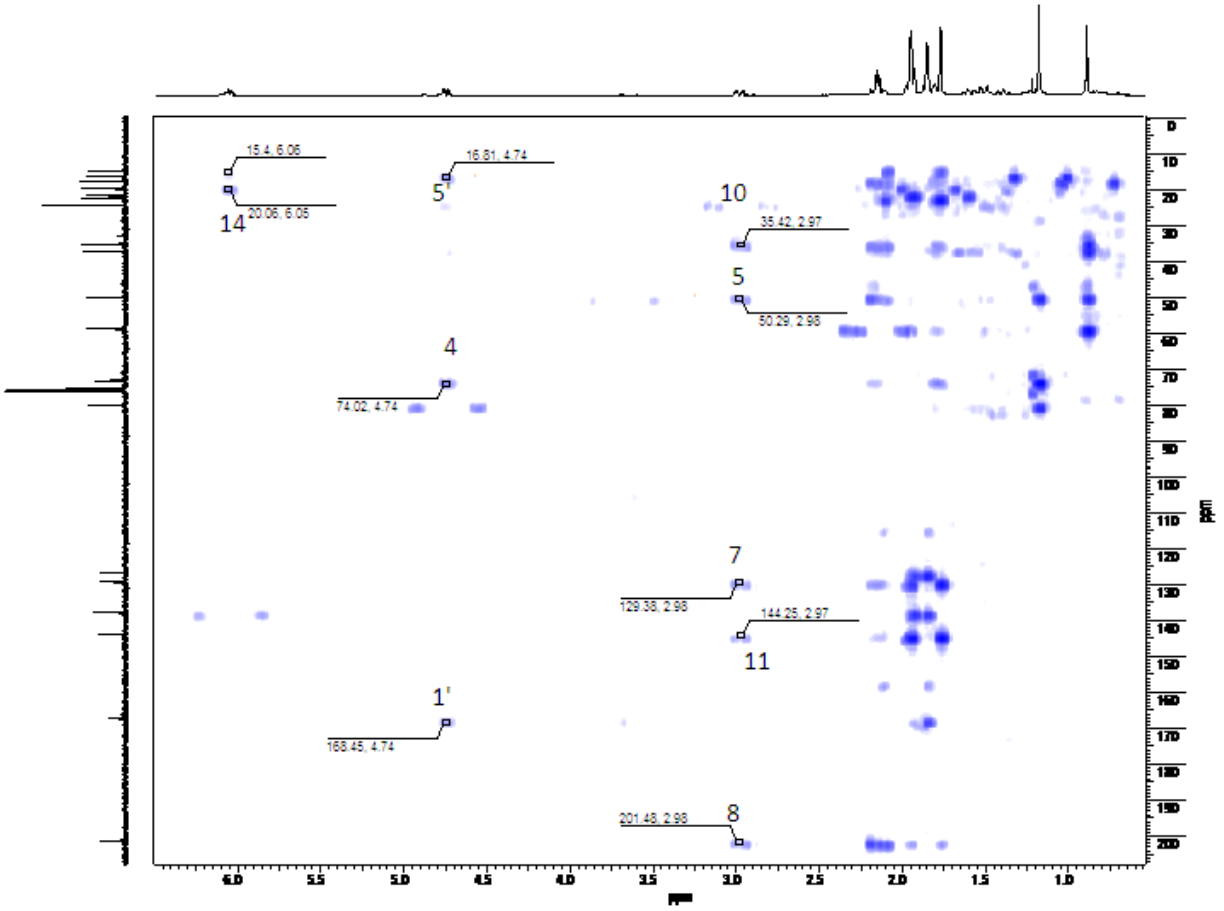
Appendix 9: HSQC spectra of compound 1



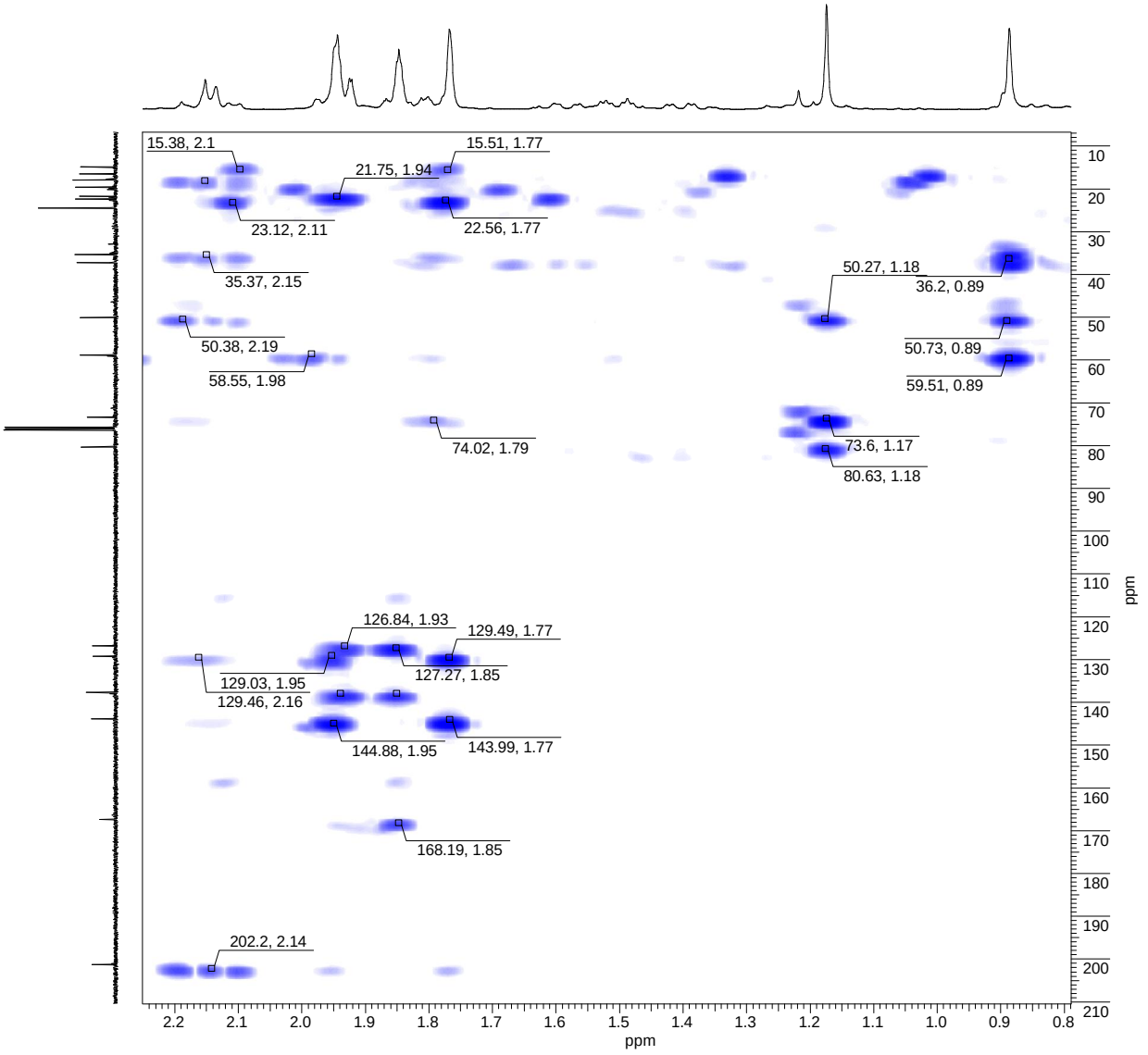
Appendix 10: HSQC Spectra of Compound 1 Cont'd



Appendix 11: HMBC of Compound 1



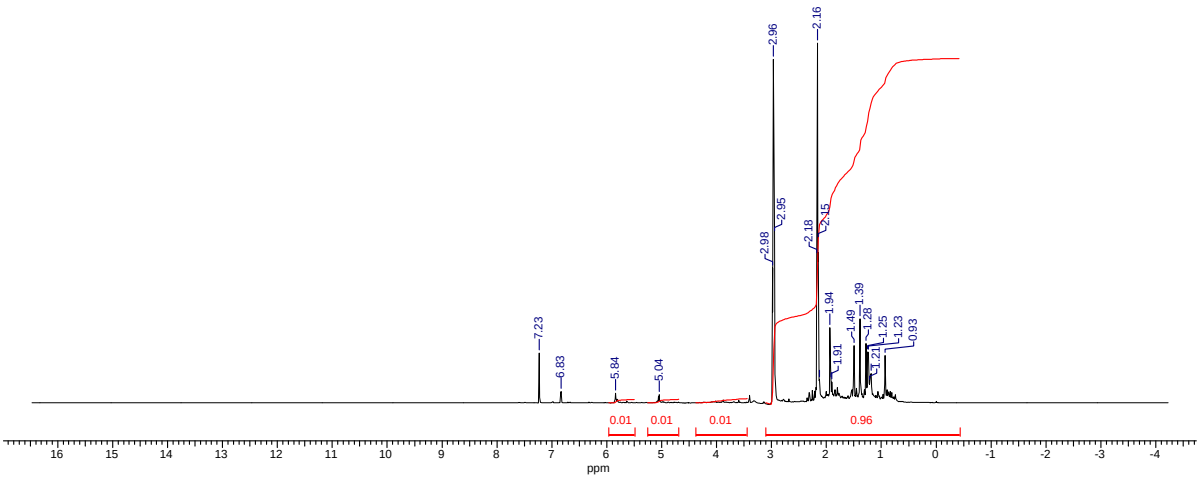
Appendix 12: HMBC of Compound 1 Cont'd



Appendix 13: ^{1}H NMR spectra of compound 2

11 Apr 2011

Acquisition Time (sec)	3.9584	***Comment***	mo6f3ptlc2	***Date***	27 Jun 2009 13:41:44		
File Name	I:\compound 2\20090627-32-majo_004001r			***Frequency (MHz)***	400.16	***Nucleus***	1H
Original Points Count	32768	***Points Count***	32768	***Pulse Sequence***	zg30	***Solvent***	CHLOROFORM-D
Sweep Width (Hz)	8278.15	***Temperature (degree C)***	27.000				



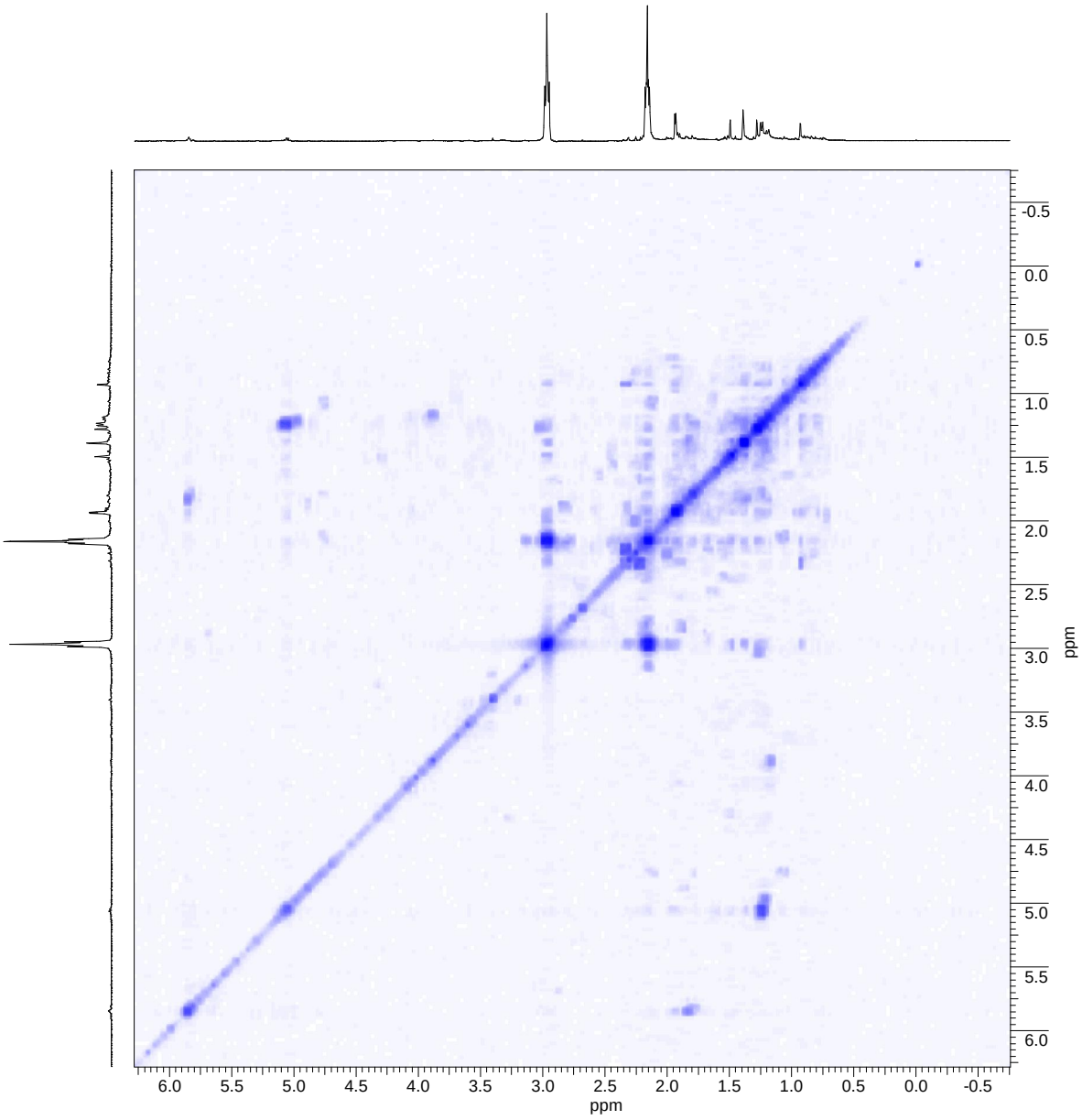
No.	(ppm)	(Hz)	Height
1	0.93	371.9	0.1313
2	1.18	473.7	0.0813
3	1.20	480.3	0.0749
4	1.21	485.3	0.0556
5	1.22	488.6	0.0555
6	1.23	493.7	0.1398
7	1.25	499.7	0.1412
8	1.28	511.8	0.1646
9	1.39	555.6	0.2333

No.	(ppm)	(Hz)	Height
10	1.49	597.5	0.1588
11	1.90	759.9	0.0574
12	1.91	765.5	0.0634
13	1.93	772.1	0.2078
14	1.94	775.1	0.2085
15	2.13	851.6	0.0633
16	2.14	856.4	0.4025
17	2.15	860.5	0.4531
18	2.16	864.0	1.0000

No.	(ppm)	(Hz)	Height
19	2.17	867.3	0.4333
20	2.18	871.3	0.4082
21	2.95	1179.1	0.4691
22	2.96	1186.4	0.9542
23	2.98	1193.7	0.3745
24	5.04	2018.1	0.0222
25	5.84	2337.1	0.0265
26	6.83	2734.8	0.0314
27	7.23	2892.4	0.1383

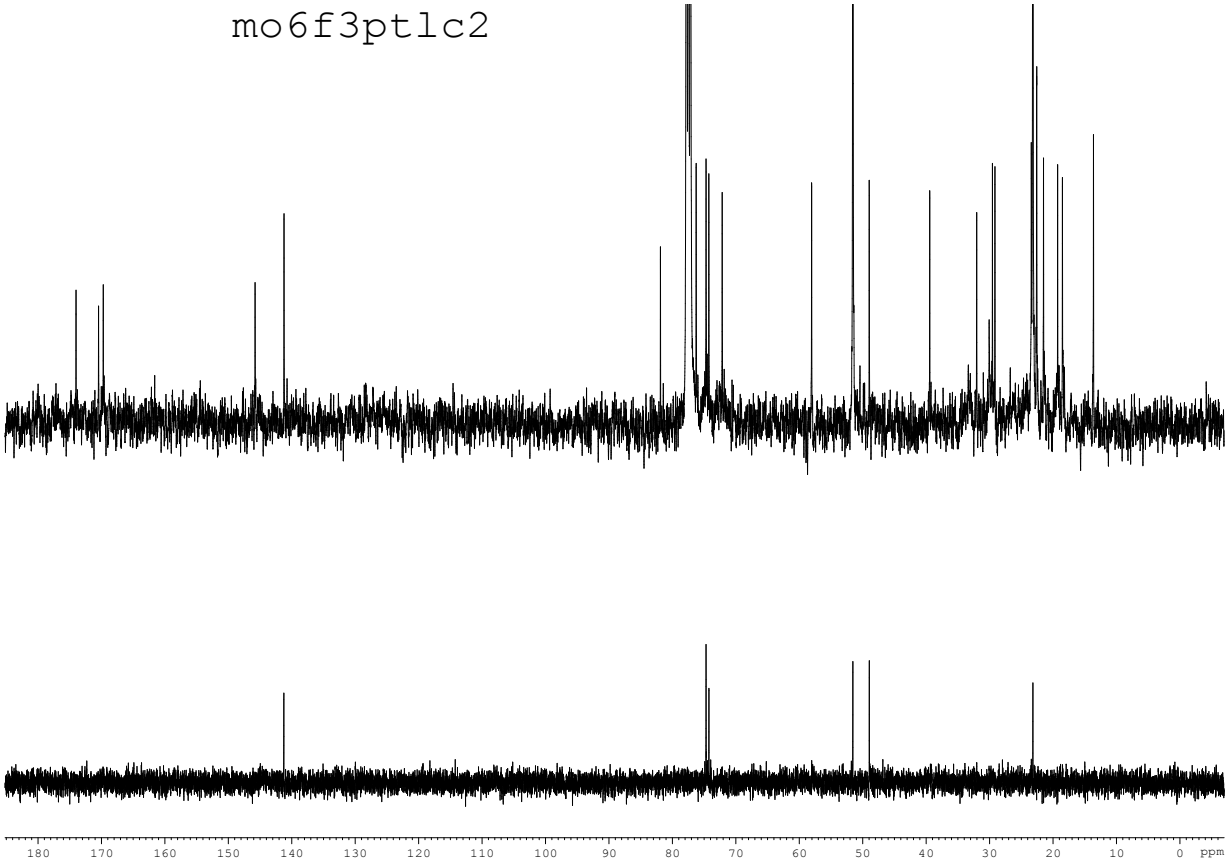
No.	(ppm)	Value	Absolute Value
1	[-0.44 .. 3.10]	0.957	8.06502e+10
2	[3.44 .. 4.38]	0.011	9.60604e+8
3	[4.69 .. 5.25]	0.010	8.01330e+8
4	[5.48 .. 5.96]	0.009	7.71447e+8

Appendix 14: H-H COSY of compound 2



Appendix 15: ^{13}C NMR and DEPT 90 Spectra of compound 2

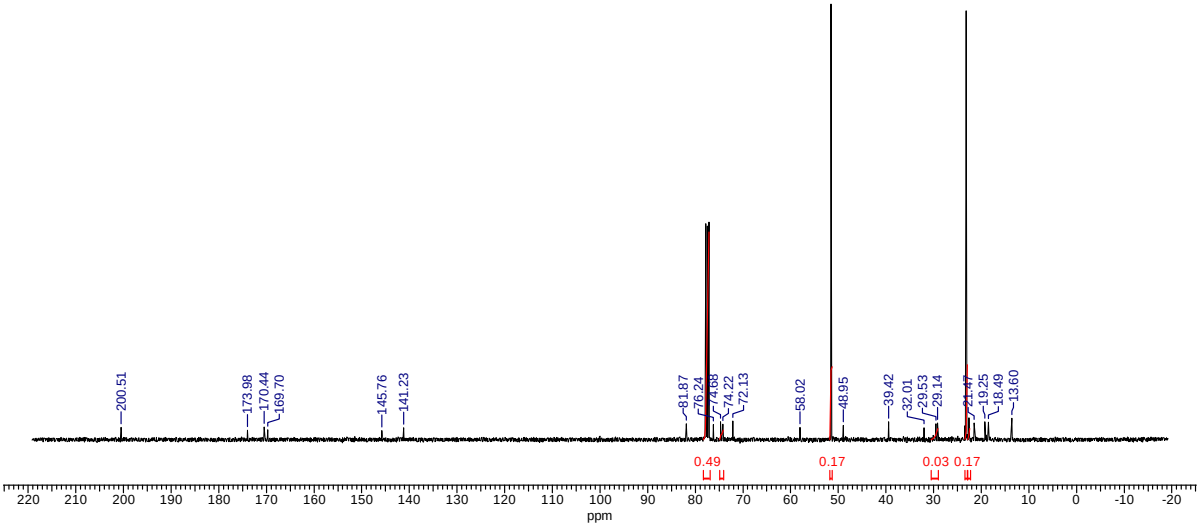
Cq = 9, CH = 4, CH_2 = 3, CH_3 = 8



Appendix 16: ^{13}C NMR spectra of compound on 600MHZ

11 Apr 2011

Acquisition Time (sec)	1.3664	***Comment***	mo6f3ptlc2	***Date***	27 Jun 2009 12:57:00		
File Name	I:\compound 2\20090627-32-majo_002001r			***Frequency (MHz)***	100.62	***Nucleus***	13C
Number of Transients	1024	***Original Points Count***	32768	***Points Count***	32768	***Pulse Sequence***	zgpg30
Solvent	CHLOROFORM-D			***Sweep Width (Hz)***	23980.81	***Temperature (degree C)***	27.000



No.	(ppm)	(Hz)	Height
1	13.60	1368.2	0.0493
2	18.49	1860.7	0.0401
3	19.25	1936.8	0.0406
4	21.47	2160.8	0.0379
5	29.14	2932.2	0.0380
6	29.53	2971.0	0.0361
7	32.01	3220.5	0.0277
8	39.42	3966.3	0.0409
9	48.95	4925.0	0.0329
10	58.02	5838.4	0.0285
11	72.13	7257.5	0.0430

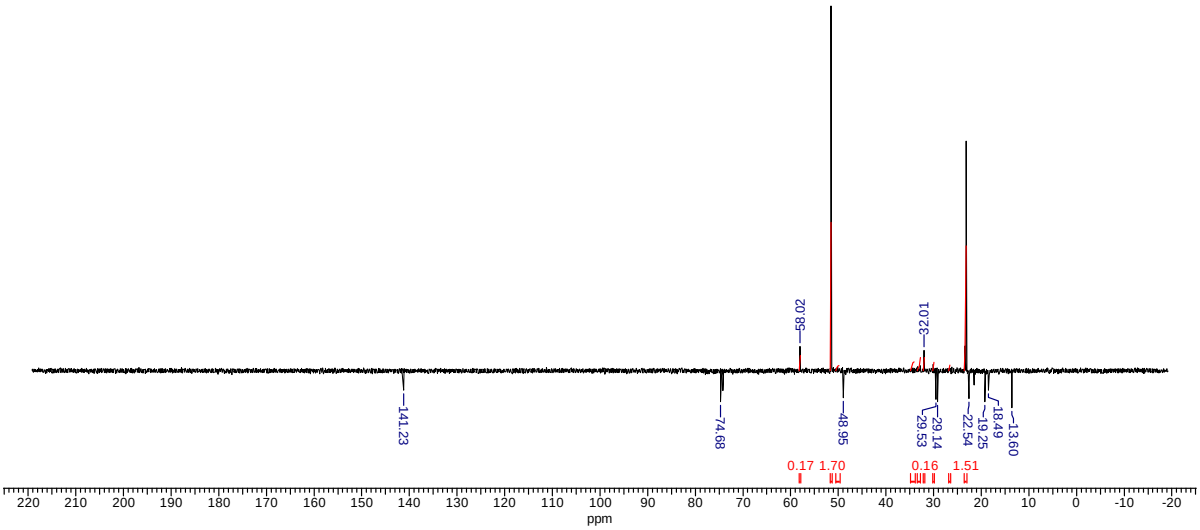
No.	(ppm)	(Hz)	Height
12	74.22	7468.2	0.0356
13	74.68	7514.3	0.0412
14	76.24	7671.0	0.0347
15	81.87	8237.4	0.0362
16	141.23	14210.9	0.0273
17	145.76	14666.8	0.0208
18	169.70	17075.3	0.0226
19	170.44	17150.0	0.0291
20	173.98	17505.7	0.0215
21	200.51	20175.5	0.0284

No.	(ppm)	Value	Absolute Value
1	[22.27 .. 22.86]	0.026	1.50747e+8
2	[22.87 .. 23.42]	0.175	1.00633e+9
3	[29.00 .. 30.53]	0.026	1.51348e+8
4	[51.25 .. 51.74]	0.169	9.74289e+8
5	[74.08 .. 74.88]	0.023	1.34384e+8
6	[76.90 .. 78.32]	0.492	2.83515e+9

Appendix 17: DEPT 135 spectra of compound 2

11 Apr 2011

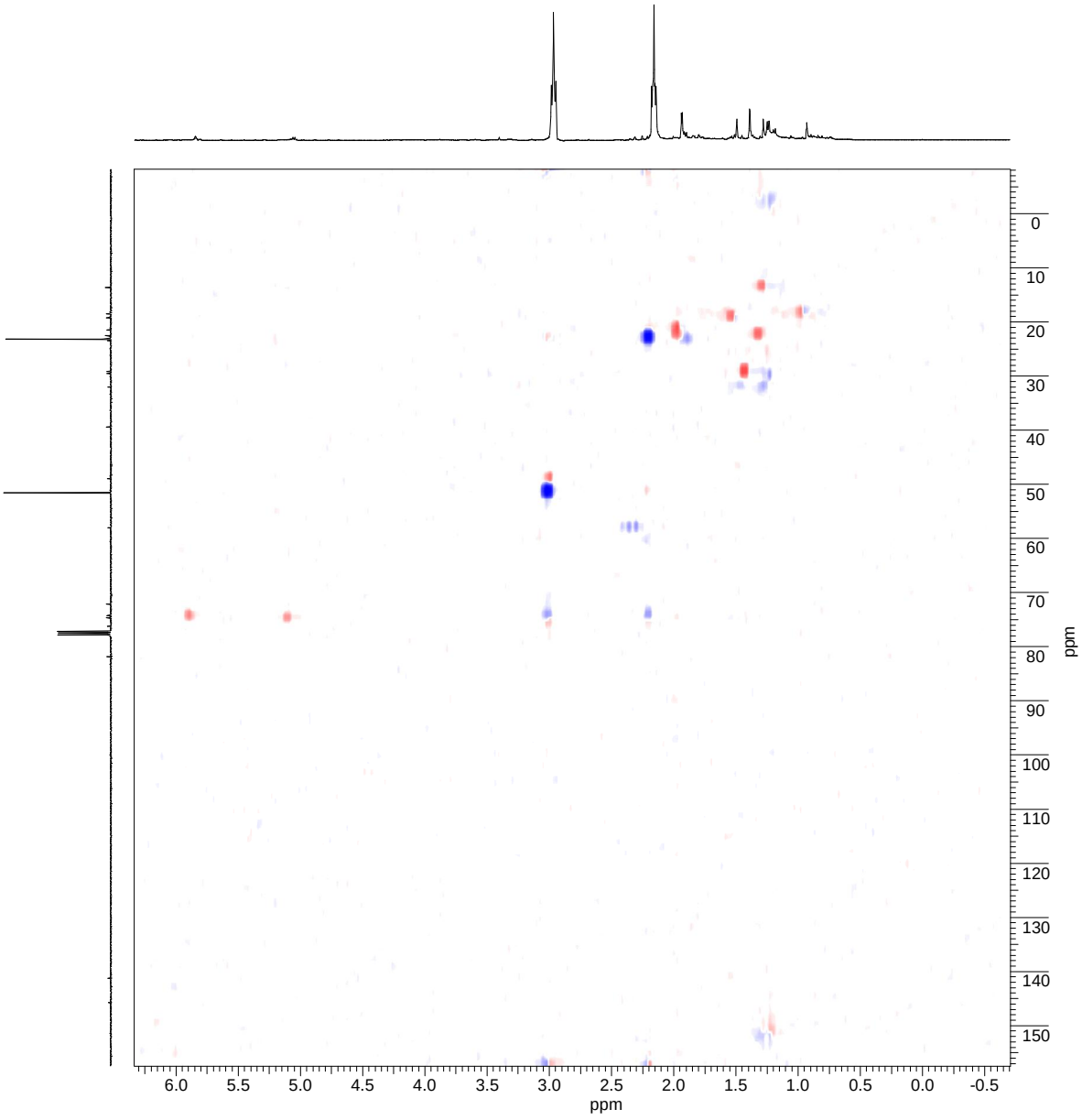
Acquisition Time (sec)	1.3664	***Comment***	mo6f3ptlc2	***Date***	27 Jun 2009 13:38:14		
File Name	I:\compound 2\20090627-32-majo_003001r			***Frequency (MHz)***	100.62	***Nucleus***	13C
Number of Transients	1024	***Original Points Count***	32768	***Points Count***	32768	***Pulse Sequence***	dept135
Solvent	CHLOROFORM-D			***Sweep Width (Hz)***	23980.81	***Temperature (degree C)***	27.000



No.	(ppm)	(Hz)	Height
1	13.60	1368.2	-0.1011
2	18.49	1860.7	-0.0534
3	19.25	1936.8	-0.0848
4	22.54	2268.4	-0.0759
5	29.14	2932.2	-0.0846
6	29.53	2971.0	-0.0781
7	32.01	3220.5	0.0563
8	48.95	4925.0	-0.0745
9	58.02	5838.4	0.0667
10	74.68	7514.3	-0.0855
11	141.23	14210.9	-0.0535

No.	(ppm)	Value	Absolute Value
1	[22.96 .. 23.62]	1.514	1.04737e+9
2	[26.46 .. 26.86]	0.100	6.93312e+7
3	[29.86 .. 30.19]	0.090	6.19503e+7
4	[31.85 .. 32.17]	0.155	1.07399e+8
5	[32.73 .. 33.46]	0.149	1.03240e+8
6	[33.85 .. 34.81]	0.116	8.04290e+7
7	[49.64 .. 50.55]	0.084	5.79716e+7
8	[51.25 .. 51.73]	1.704	1.17909e+9
9	[57.84 .. 58.18]	0.173	1.19630e+8

Appendix18: HSQC spectra of compound 2



Appendix 19: HMBC spectra of compound 2