MOSQUITO LARVICIDAL COMPOUNDS FROM THE PLANT *Fagaropsis angolensis* (Engl. Dale) AGAINST *Anopheles gambiae*

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

EGERTON UNIVERSITY

MAY, 2013

DECLARATION AND RECOMMENDATION

DECLARATION

I, Cynthia Muhavi Mudalungu, declare that this research thesis is my original work and has not been submitted wholly or in part for any award in any institution.

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DEDICATION

I wish to dedicate this work to my parents: Thomas and Hellen Mudalungu, for their financial and moral support throughout my studies.

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ABSTRACT

Anopheles gambiae mosquitoes transmit malaria resulting into about 500 million infections globally every year. Recent studies estimate that more than 50% of the world's population is at risk of malaria infections. Apart from the development of insecticide resistance, the use of synthetic insecticides to control mosquito vectors continues to cause adverse effects to the environment, human health and non-target organisms. Plant derived larvicidal compounds are increasingly being explored as possible alternatives in vector control methods. The plant *Fagaropsis angolensis* has been used in the past as traditional medicine in treating various ailments. This study investigated the potential larvicidal activity of both volatile and non-volatile compounds from *F. angolensis* leaves. The chloroform extract of the non-volatile compounds was prepared and subjected to a series of bioassay guided fractionation (100% and 7:3 Chloroform/ethyl acetate solvent mixture) and purification steps using column chromatography technique. The essential oil from the leaves of *F. angolensis* was obtained by hydro-distillation method and analyzed by GC/GC-MS technique. Three larvicidal compounds namely a phenanthrene carboxylic acid derivative (**32**), hexyl-9,10-dihydroxydec-5-enoate (**33**) and methyl -10-(3-phenylpropanoyloxy)-7-hydroxy-19-methylhenicosa-4, 13, 16-trienoate (**34**) were identified using mass spectrometry, 1D and 2D NMR spectroscopy. The compounds **32**, **33** and **34** exhibited LC_{50} values of 245.5 ppm, 147.6 ppm and 144.4 ppm respectively when tested against the third instar of *An. gambiae* larvae. Their LC_{90} values were 471.6 ppm, 292.1 ppm and 259.4 ppm respectively. The oil showed an LC_{50} of 83.7 ppm and LC_{90} of 324.0 ppm at 95% confidence interval. Only 2.64% of the essential oil components were fully identified, 67.83% partially identified and unknown (29.0%). The oil contained mainly new compounds whose mass spectra could not be found in the GC – MS databases used. The isolated compounds and the oils can be used in the development of natural mosquito larvicides. Results of this study indicate that the three naturally occurring larvicidal compounds and the essential oil of *F. angolensis* leaves could have the potential applicability in the control of the larval stages of the malaria vector - *An. gambiae* mosquitoes.

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LIST OF ABBREVIATIONS AND ACRONYMS

ACD	Advanced Chemistry Development
^{13}C NMR	Carbon–13 Nuclear Magnetic Resonance Spectroscopy
^{1}H NMR	Proton Nuclear Magnetic Resonance Spectroscopy
CDC	Center for Disease Control
$CDCl_3$	Deuterated Chloroform
COSY	Correlation Spectroscopy
DDT	Dichlorodiphenyltrichloroethane
DEPT	Distortionless Enhancement by Polarization Transfer
DMSO	Dimethylsulfoxide
GC/MS	Gas Chromatography/Mass Spectrometry
HMBC	Heteronuclear Multiple Bond Correlation Spectroscopy
HSQC	Heteronuclear Single Quantum Coherence
IBM	International Business Machines
IRS	Indoor residual spray
ITNs	Insecticide treated nets
KEMRI	Kenya Medical Research Institute
LC_{50}	Lethal concentration dosage that kills 50% of the treated larvae
LC_{90}	Lethal concentration dosage that kills 90% of the treated larvae
LLINs	Long-lasting insecticidal nets
MS	Mass Spectrometry
OECD	Organisation for Economic, Co-operation and Development
ppm	Parts per million
SPSS	Statistical Package for Social Science
TLC	Thin Layer Chromatography

CHAPTER ONE

INTRODUCTION

1.1 Background information

Malaria is one of the world's most common and severe tropical diseases. It is caused by mainly *Plasmodium falciparum* protozoa that are transmitted by the female *Anopheles* mosquitoes (Gutie' rreza *et al*., 2008). Among all the anopheline mosquito species, the *Anopheles gambiae* is considered the most virulent. Malaria infects more than 300 million humans each year, killing approximately 1.5 to 3 million people globally each year with about 90% of all infections occurring in Africa, south of the Sahara (Breman *et al.*, 2004; Snow *et al.*, 2005). Malaria epidemics have devastated large populations and continue to pose a serious threat to economic progress in many developing countries (Keiser family foundation, 2007).

Malaria is also one of the leading causes of morbidity and mortality in Kenya with 25 million out of a population of 34 million being at a risk (http://www.stopmalarianow.org). The most vulnerable group to malaria infections are often pregnant women and children under 5 years of age (Steketee *et al.,* 2001). Malaria infections accounts for 30-50% of all outpatient attendance and 20% of all admissions to health facilities. Malaria is also estimated to cause 20% of all deaths in children under the age of five years (MOH, 2006).

Despite considerable efforts to eradicate or control malaria, no effective malaria vaccine is yet available (CDC, 2008; Matasyoh *et al*., 2008). At the same time, the anopheles mosquitoes have developed resistance to many synthetic commercial insecticides (Srisilam and Veersham, 2003). The extensive use of chemical insecticides for control of vector borne diseases has also created problems such as adverse environmental effects, high operational cost and community acceptance (Brown, 1986; Milam *et al.,* 2000; Cheng *et al.,* 2003; Soderlund and Knipple, 2003) which has led to increased negative public perception towards their continued usage.

Vector control is so far the most successful method for reducing incidences of mosquito borne diseases, however, there is emergence of widespread insecticide resistance and the potential environmental issues associated with some synthetic insecticides (ICMR, 2003). Due to the concern over the quality and safety of life and the environment, the

emphasis on controlling mosquito vectors has shifted steadily from the use of conventional chemicals toward alternative insecticides that are target-specific, biodegradable, and environmentally safe, and these are generally botanicals in origin. This has propelled the search for and use of eco-friendly plant based products for the control of insects such as mosquitoes (Navneet *et al*., 2011).

Larval control measures are intended to reduce malaria transmission indirectly by reducing the vector population density near human habitations. As the larvae are exclusively aquatic, their distribution is determined by the locations of suitable water bodies. Immature stages prefer slow-moving or still water in which they can stay close to the surface with their breathing orifices open to the air. Unlike some other mosquito genera, anophelines require relatively clean stagnant water for development (Service and Townson, 2002). One advantage of targeting larvae is that they cannot escape from their breeding sites until the adult stage and therefore the larvae cannot easily avoid control measures (Killeen *et al*., 2002). Larval control as a vector control strategy has in the recent past been explored as an important alternative in curbing the spread of malaria.

Currently, numerous products of botanical origin, especially the secondary metabolites, are increasingly receiving considerable worldwide attention as potentially bioactive agents used in insect vector management (Navneet *et al*., 2011). Numerous varieties of plant products have been reported either as insecticides for killing larvae insect growth regulators, repellents and ovipositor attractants (Venketachalam and Jebasan, 2001; Thomas *et al*., 2004). A lot of phytochemicals extracted from various plant species have been tested for their larvicidal and repellent actions against mosquitoes (Ciccia *et al*., 2000; Ansari *et al*., 2000). Ethnobotanical and laboratory based studies have revealed the existence of insecticidal plants belonging to different families in different parts of the world. Crude solvent extracts of plant parts belonging to different families, essential oils (Harve and Kamath, 2004) or their chromatographic fractions are shown to have various levels of bioactivity against different developmental stages of malaria vector mosquitoes (ICMR, 2003).

Fagaropsis angolensis belongs to the Rutaceae family and to the genus *Fagaropsis*. The Rutaceae plants are herbs, shrubs and trees with glandular punctate, commonly strongly smelling herbage comprising about 150 genera and 1,500 species (Harish *et al*., 2010) that are further characterized by the common occurrence of spines and winged petioles. The leaves are

alternate or opposite, simple or pinnately compound. The tree grows in evergreen rainforests and woodlands, at 1000–2600 metres altitude. In Kenya the plant is found in Kakamega equitorial rain forest and some of its common names are: Muyinja (Swahili), Olmoljoi (Maasai), Shingululutso (Luhya), Mfule (Chagaa), Lisongote (Kurya) and Mulungulungu (Hehe).The stem bark is used in traditional medicine to treat malaria, and the root is chewed as an expectorant in Kenya. In Malawi and Zimbabwe root powder is taken in drinks or gruel to treat male sterility (Lemmens, 2008). This family has been found to contain numerous secondary metabolites such as alkaloids, coumarins and lignans (Sagˇ lam *et al.,* 2000; Joseph, 2005).

The aim of this work was to investigate mosquito larvicidal compound(s) contained in the leaves of *F. angolensis* plant that can help stem the spread of malaria.

1.2 Statement of the problem

The malaria vector - *An. gambiae* has not been entirely eradicated despite the current vector control methods. New malaria infections resulting into about 1.5 to 3 million deaths are still being reported annually. The effect and cost of the disease has had enormous negative impacts on the economic growth of many African countries. Some of the synthetic insecticides that target mosquitoes have been found to cause serious environmental contamination and harm to humans. The *An. gambiae* mosquitoes are also increasingly developing resistance towards the currently used commercial insecticides.

1.3 Objectives

1.3.1 General objective

To evaluate the mosquito larvicidal activity of both the essential oil and the non-volatile compounds from the leaves of *F. angolensis* against the *An. gambiae* mosquito larvae.

1.3.2 Specific objectives

1. To determine the mosquito larvicidal activity of the chloroform crude extract from *F. angolensis* leaves.

2. To carry out bioactivity guided fractionation on the crude extract to obtain the non-volatile larvicidal compounds.
3. To determine the larvicidal activity of the essential oil from *F. angolensis* leaves and identify its components.
4. To elucidate the structure(s) of the isolated mosquito larvicidal compounds from *F. angolensis*.

1.4 Hypotheses

1. That the crude extract from *F. angolensis* leaves will not show any mosquito larvicidal activity.
2. That the bioactivity guided fractionation carried out on the crude extract will not afford non-volatile larvicidal compounds.
3. That the essential oil from *F. angolensis* leaves will not exhibit any larvicidal activity and its components will not be identifiable.
4. That the elucidated pure non-volatile compounds from *F. angolensis* leaves will not display any larvicidal activity.

1.5 Justification

The development of mosquitoes resistant to some of the commonly used synthetic insecticides and the occurrences of environmental insecticide contamination means that alternative strategies for controlling the populations of the malaria vector in a more ecologically friendly way need to be developed. Mosquito vector control at larval stages using plant-derived natural products is being explored as a viable alternative due to their advantage of being easily biodegradable and generally having lower mammalian toxicity. Some mosquito larvicidal compounds and essential oils obtained from certain plant species including the Rutaceae family with varied ethnobotanical uses have been reported in the past and more potent ones are still being explored.

CHAPTER TWO

LITERATURE REVIEW

2.1 Morbidity and mortality of malaria

The World Health Organization currently estimates that each year, malaria causes 300 to 500 million infections and 1.5 to 3 million deaths. This is an alarming rate given that during the six-month Ebola outbreak in the Democratic Republic of the Congo in 1995, about 250 people died while malaria kills over 5,000 Africans every day. The malaria parasite is seen to increase greatly one's susceptibility to other infections via generalized immuno-suppression. For instance, a baby born to a pregnant woman infected with malaria will have a 40 percent greater chance of low birth weight, and congenital malaria may account for as many as half of all childhood deaths in Africa (OECD, 2010). Figure 1 illustrates the habitats of Anopheline mosquito species in the world. The Sub-Saharan Africa harbors the most deadly anophelines species, *Anopheles gambiae,* which is the malaria vector.

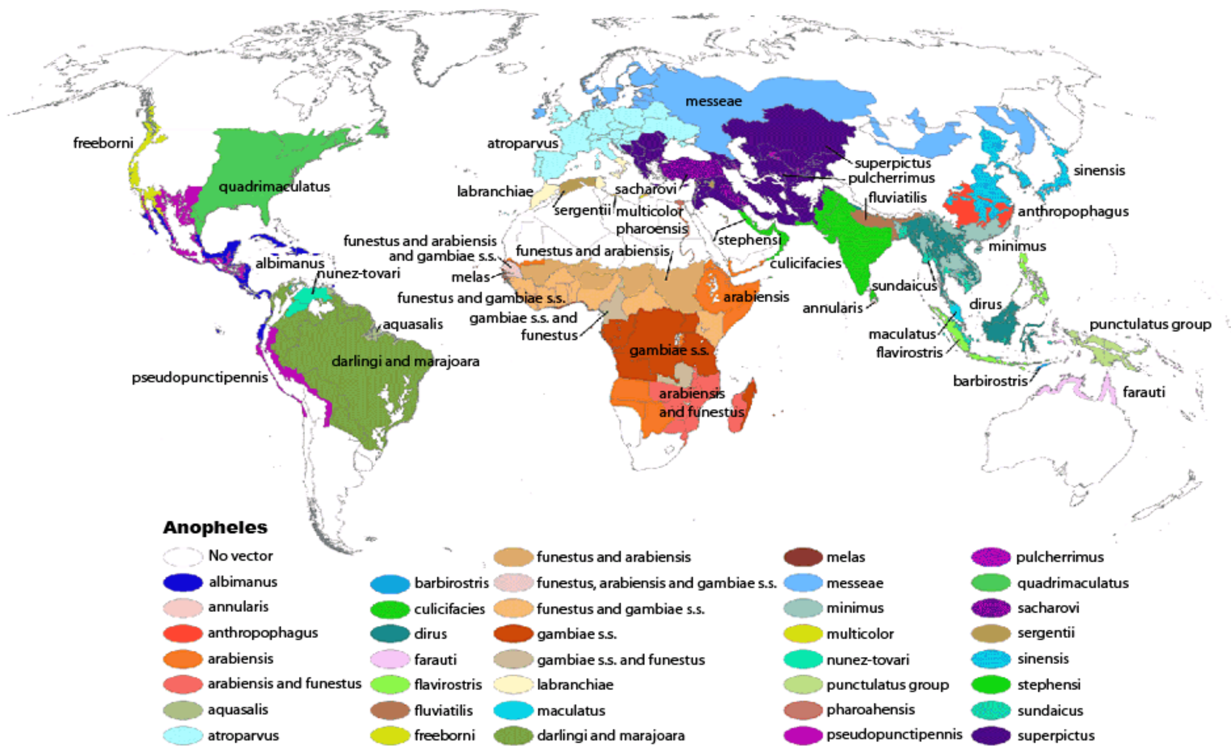


Figure 1: Habitats of anopheline mosquito species world wide

source: (Kiszewksi *et al*., 2004)

Although Africa bears the biggest burden, it is estimated that more than one-third of clinical malaria cases occur in Asia and 3% occur in the Americas. The estimated cost to effectively control malaria in the 82 countries with the highest burden is about US $3.2 billion annually (Keiser family foundation, 2007). Nevertheless, the burden of the disease remains unacceptably high (WHO, 2006).

2.2 Vector and host

The high incidence rates of malaria are affected by the unusual nature of the parasite itself and its vector. Not only is the malaria parasite highly complex, but its vector is a sexually reproducing organism capable of mixing genes during reproduction process. About 50-200 eggs are laid per oviposition on the surface of stagnant water and these eggs develop into adult mosquitoes in a span of about 5-14 days, passing through the stages of larvae and pupae (see figure 2). High humidity and ambient temperature between 20-30ºC provide ideal conditions for breeding of *Anopheles* mosquitoes. Common sites of breeding for *Anopheles* mosquitoes include rainwater pools and puddles, borrow pits, river bed pools, irrigation channels, seepages, rice fields, wells, pond margins, sluggish streams with sandy margins, hoof prints, tyre tracks etc. Water stagnation due to construction of dams, reforestation, shrimp farming and fish ponds have also been identified as possible sites of *Anopheles* breeding.

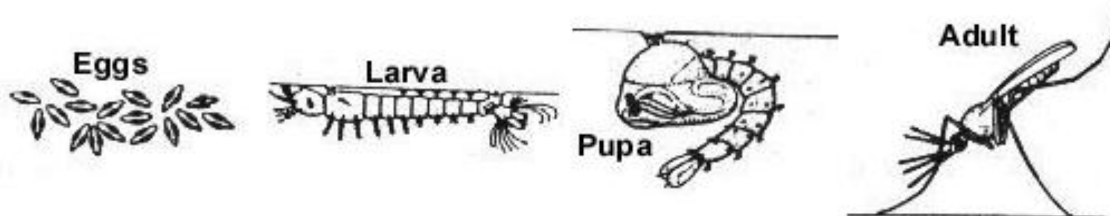


Figure 2: Life cycle of anopheline mosquito

source: (http://upload.wikimedia.org)

The *Anopheles* mosquito serves as the Plasmodium delivery system, or vector and only female mosquitoes can transmit malaria since males don't take blood meals from their

hosts. These mosquitoes are mainly known to bite during night time hours (IDRC, 2008). (See figure 3).

Figure 3: Female anopheline mosquito taking blood meal

Source: Centre for Disease Control

2.3 Vector control strategies

Vector control strategies have been devised to reduce the prevalence of malaria globally. These strategies include various methods aimed at reducing human - vector contact such as insecticide-treated bed nets, use of repellants, indoor residual spraying and finally methods aimed at reducing vector density such as space spraying, sterile male, source reduction through environment management and larviciding. Vector control has been touted as primordial and an essential means for controlling transmission of not only malaria but also several other mosquito-borne diseases such as yellow fever, dengue fever, filariasis and Japanese encephalitis (James, 1992; Vatandoost and Vaziri, 2001; Radhika *et al*., 2011).

2.3.1 Insecticide-treated bed nets

Insecticide-Treated Nets (ITN's) provide protection against adult mosquitoes. Some of the netting material is treated with synthetic pyrethroids insecticide. The insecticides are highly toxic to insects, which repels mosquitoes (CDC, 2008). The most commonly used

pyrethroids are permethrin, deltamethrin and lambda cyhalothrin. Studies in Ghana, Gambia, Kenya and Tanzania found that ITN's reduced child illness by 29% to 63% and childhood mortality by between 17% to 63% depending on net coverage and malaria transmission pressure (Lengeler and Snow, 1996). Pyrethroids are known to have a high residual effect because they do not rapidly break down unless washed or exposed to sunlight.

The need for frequent retreatment of the nets before use was a major barrier to full implementation of ITN's 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 and nets had to be retreated at intervals of 6-12 months or 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 shade (CDC, 2008).

Currently, the advent of Long-lasting insecticidal nets (LLINs) and treatment technologies has opened up prospects for improving ITN interventions by addressing the issue of treatment and re-treatment. Unfortunately, there is evidence of insecticide resistance among major malaria vectors in various parts of Africa. If a mosquito develops resistance to one insecticide, in most cases it is immediately resistant to all other insecticides in the same class. Resistance against pyrethroids is a particular cause for concern as no other insecticide class can be used for ITNs (http://www.malariaconsortium.org).

On the contrary, a lot still needs to be done since 10 million Kenyans who need protection do not have access to bed nets (http://www.stopmalarianow.org/) and its main limitation is that there is potential for transmission by early biting vectors before people retire to sleep.

2.3.2 Use of repellents

Repellants are applied either directly on the skin (as a cream or lotion) or on clothes. The use of repellents is also only a measure of individual protection that can be recommended as a complement to the use of bed nets to be used before retiring under the mosquito net or by people who have to stay outdoors during some parts of the night. The most widely marketed synthetic based insect repellents is N, N-diethyl-3-methylbenzamide (DEET), which has been used worldwide since 1957 (Fradin and Day 2002). In some parts of the world, especially America, Lemongrass oil, a distillate of *Cymbopogon citratus* or *Cymbopogon flexuosus*

leaves, is traditionally used to repel mosquitoes (Sears, 1996) and it is repellent to *An. darling* and other disease vectors (Moore *et al*., 2002). To lower the cost of the repellents and maintain its efficacy, the active ingredients of the repellents are combined with some low-cost ingredients (fixatives) that extend the repellent effect by slowing the evaporation of volatile repellent actives (Rutledge *et al*., 1996). However, in the endemic areas the cost effectiveness of these repellants is still doubtful.

2.3.3 Indoor residual spraying

Since the 1950s, IRS has been used widely in many areas of the world, especially in Asia, Latin America and Southern Africa. IRS with DDT and other insecticides like pyrethroids and organophosphates has been one of the main interventions which led to the elimination of malaria in about half of the world's regions, for example in much of southern Europe, North America, Japan, Central Asia and Latin America (Lengeler and Sharp, 2003).

The vector control arm of malaria prevalence reduction, using indoor residual spraying (IRS) of houses with synthetic insecticides, is challenged by the emergence of insecticide resistant vectors (Jeffery, 1984). Furthermore, the success of indoor residual spraying of houses to control adult anopheline vectors of malaria depend on mosquitoes resting indoors before or after feeding, the presence of walls and surfaces to be sprayed in human shelters, access to the interior of all houses, willingness of people to accept spraying and availability of permanent homesteads (WHO, 2006).

Unlike the highly mobile flying adult vectors that can easily detect and avoid synthetic indoor residual spray chemicals, immature stages of mosquitoes including larvae are confined within relatively small aquatic habitats and cannot readily escape control measures (Killeen *et al*., 2002). Although pyrethrum was the insecticide first used, indoor spraying of insecticides became the most popular method of malaria vector control with the introduction of DDT and other residual insecticides. Its main limitation is that exophilic vectors may exist and may not come into contact with sprayed surfaces. In addition, high cost of synthetic insecticides, environment and food safety concerns, unacceptability and toxicity of many organophosphates and organochlorines on a global scale have stimulated research towards potential botanicals (Severini *et al*., 1993). For instance, in the USA, the use of pyrethroids and organophosphate insecticides (such as chlorpyrifos) has faced restrictions due to the

presence of unacceptably high concentrations in some waterways thus presenting serious sediment contamination issues (Weston *et al.,* 2004; Banks *et al.,* 2005).

2.3.4 Space spraying

This is the application of non-residual insecticides to the outdoor environment in order to immobilize infective mosquitoes and contain their transmissions. It is particularly recommended for urban areas where many people congregate outdoors. Space spraying has been extensively used for controlling epidemics of mosquito-borne diseases such as dengue and some types of encephalitis. It has only occasionally been used in malaria epidemic control and as a complementary measure against exophilic vectors (WHO, 2002).

Its main limitation is the difficulty of applying them at night, when vectors are flying, and the poor penetration of insecticide fogs into the day time resting places of the vectors (e.g., under leaves, in small crevices). Therefore, space spraying must be restricted to an hour or two in the early morning or evening, when the temperature is lowest and thermal currents, which cause excessive dispersion of the insecticide, are at a minimum. According to (WHO, 2003) this method requires specialist equipment and is expensive to implement on a routine basis and the insecticide used has no residual action. As it has a short term effect, frequent re-application is necessary for substantial impact.

2.3.5 Sterile insect technique

This is done by release of spermless male mosquitoes that can be used to prevent the spread of malaria by preventing female mosquitoes from successfully reproducing. Since a widespread release of sterile males could have a major impact on transmission rates of the malaria disease, one needs to make sure that the insects continue to mate as normal and unaware that their sexual mechanisms have been interfered with (Oliva *et al*., 2011). This technique is aimed at not changing the behavior of the female mosquitoes after mating, they should consume blood meals and critically they should not seek out for another sexual partner therefore laying infertile eggs that cannot develop. In the small scale areas, sterile male release has been successfully applied.

Apart from that, there is renewed interest in the scientific community to improve or even replace the SIT through the techniques of molecular biology to make *Anopheles*

mosquitoes incapable of transmitting the *Plasmodium* protozoan parasite (Knols *et al.,* 2002; Klassen, 2009). However, the need for large numbers of mosquitoes for release makes this approach impractical for most areas. Additionally, the immigration of females already inseminated by fertile males outside the release area is a major obstacle to progress using sterile insects.

2.3.6 Source reduction through environmental management

Since the discovery of the role of *Anopheles* mosquitoes in malaria transmission over one hundred years ago, malaria control experts recognized the value of changing mosquito larval habitats to reduce or eliminate malaria transmission. This Environmental Management (EM) was referred to as the concept of modifying vector habitat to discourage larval development or human vector contact. Habitat elimination or modification efforts have included general programs to reduce the abundance of all mosquitoes as well as more targeted projects of "species sanitation" directed at the principal malaria vectors (Bruce-Chwatt, 1985).

Container-breeding mosquitoes are particularly susceptible to source reduction as people can be educated on how 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. But because source reduction is an ideal approach to mosquito control (CDC, 2008), mosquito larvae are concentrated in defined areas; mosquitoes are eliminated before they reach the stage that is responsible for disease transmission. The adults are believed to 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. Larval control methods have been cited as having little impact on the non-target species and do not impact ground water. According to Silvagnaname and Kalyanasundaram, (2004), treating the breeding areas does not involve the exposure of the general public since materials are applied to water in swamps, marshes and other non-residential areas. On the other hand, 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. In addition, these methods have relatively high investment

costs and may be cost-effective only in urban areas or some types of development projects. Nevertheless, they are suitable for the elimination of permanent breeding places.

2.3.7 Larviciding

This involves the killing of the mosquito larvae by application of chemical insecticides and those of biological origin, such as the toxin of *Bacilus thuringiensis israelensis (Bti)* and insect growth regulators to stagnant breeding sites (Lacey, 2007). The mosquito larvae (figure 4) are mainly found in water bodies and feed on the algae and small organisms which live in the water.

Figure 4: Mosquito larva

Source: Centre for Disease Control

Some botanical products appear to be promising larvicides. When community mosquito control is needed to reduce mosquito-borne diseases, use of larvicidal applications to the breeding source of mosquitoes is recommended. Larvicides are more effective and less toxic compared to adult mosquito sprays, and their applications are unlikely to result in human exposure. Also, larviciding exerts a stronger selection pressure on vector populations than indoor residual spraying and the use of insecticide-treated mosquito nets, as it acts on both sexes (WHO, 2002).

2.4 Prospect of mosquito larvicides from botanical sources

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. According to Kroken (2009)

secondary metabolites are low-molecular-weight unique products that are generated naturally in response to environmental, abiotic and biotic stimuli, with diverse chemical structures. Their intricate molecular frameworks often appear to chemists to be quite idiosyncratic. Natural products of plant origin with insecticidal properties have been tried in the recent past for control of a variety of insects, pests and vectors. Many developing countries of the world are endowed with vast resources of natural products. Secondary metabolites of plants, mostly 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. The phytochemicals derived from plant sources have revealed larvicides, insect growth regulators, repellent, and ovipositor attractants (Kaushik and Saini, 2008). The plant *Azadirachta indica* is one of the most commonly studied for the control of mosquitoes and it is known as neem in India (ICMR, 2003). For example, it has gained wide acceptance in some countries as an antifeedant (Isman, 1997) and also neem products show a high larvicidal activity (ICMR, 2003). In addition, many essential oils from plant origin such as citronella, calamus, thymus, and eucalyptus are reportedly promising mosquito larvicides (Chowdhury *et al*., 2008).

The use of herbal products is one of the best alternatives for mosquito control. The search for herbal preparations that do not produce any adverse effects in the non-target organisms and are easily biodegradable remains a top research issue for scientists associated with alternative vector control (Rahuman *et al*., 2008). Many plant species are known to possess biological activity that is frequently assigned to the secondary metabolites. Among these, essential oils and their constituents have received considerable attention in the search for new bio-pesticides.

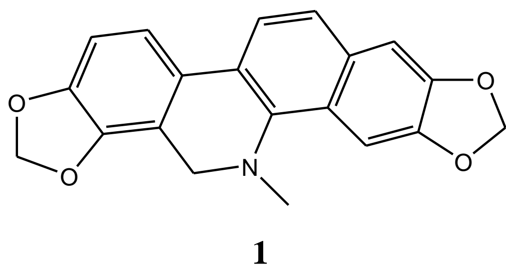
2.5 Some beneficial biological values of *Fagaropsis angolensis*

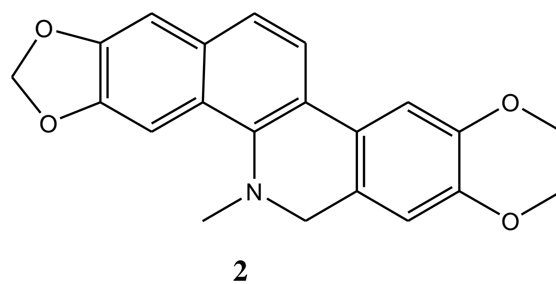
The Rutaceae family has been found to contain many secondary metabolites such as alkaloids, coumarins and lignans with a large spectrum of biological activities that is, antiprotozoal activity against *Leishmania* parasites, peripheral stimulants of parasympathetic nervous system, trypanocidal activity (Mafezoli *et al.*, 2000), anti-ulcer (Li *et al.*, 2005), apoptosis inductor (Roy *et al.*, 2005; Cortez *et. al.*, 2006), giardicidal (Amaral *et al.*, 2006), acetylcholinesterase inhibition (Barbosa-Filho *et al.*, 2006) and antiplasmodial (Dolabela *et al.*, 2008). Terpenoids are other common compounds identified in this family (Mafezoli *et al.*,

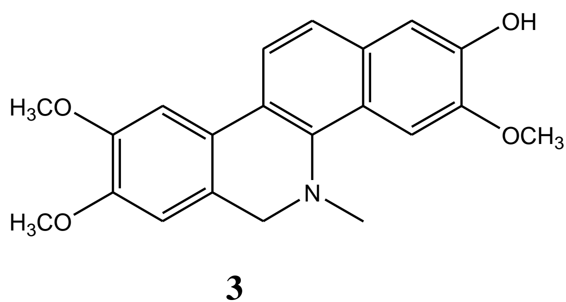
2000). Little is known, however, about the pharmacological potential of Rutaceae species. The Rutaceae is also widely known by their ethnobotanical uses in many countries in the world (Lorenzi and Matos, 2002; Moshi *et al.*, 2005). Chemical investigation of some compounds of the Rutaceae family have been shown to possess mosquito larvicidal activity, the alkaloids isolated from *Zanthoxylum lemairei* roots has been documented to show larval mortality (Matasyoh *et al.,* 2010).

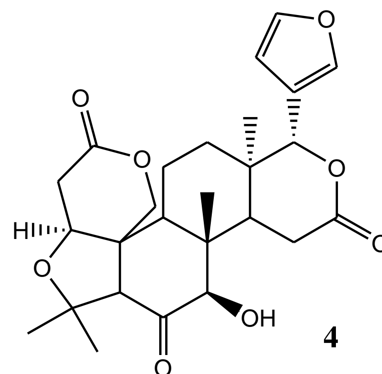
Figure 5: Image of *Fagaropsis angolensis* species

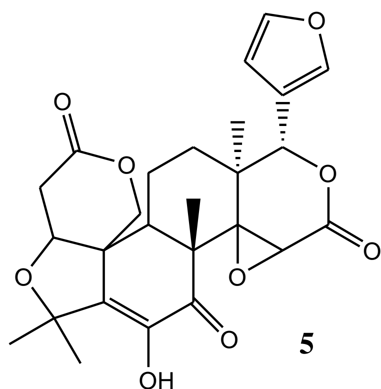
Several alkaloids and limonoids have been isolated from the stem bark of *F. angolensis*, these include: dihydrosanguinarine **(1)**, dihydronitidine **(2)** and the anti-malarial benzophenanthridine alkaloid nitidine **(3)**. Nakagawa *et al*., (2006) reported that limonoids and flavonoids are among the active chemical components of *F. angolensis*. The limonoids that have been identified are rutaevin **(4)** and limonin diosphenol **(5)**. The ethanol extract of *F. angolensis* has exhibited the strongest antimicrobial effect, inhibiting growth of *S. aureus* and *C. albicans* with Minimum Inhibitory Concentrations of 64 and 32 µg/mL respectively. Some of the compounds are illustrated below.

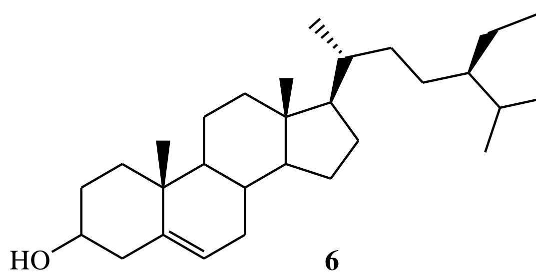


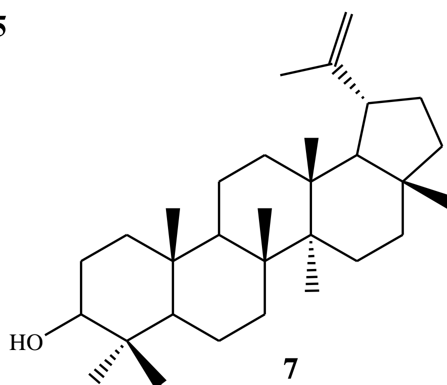










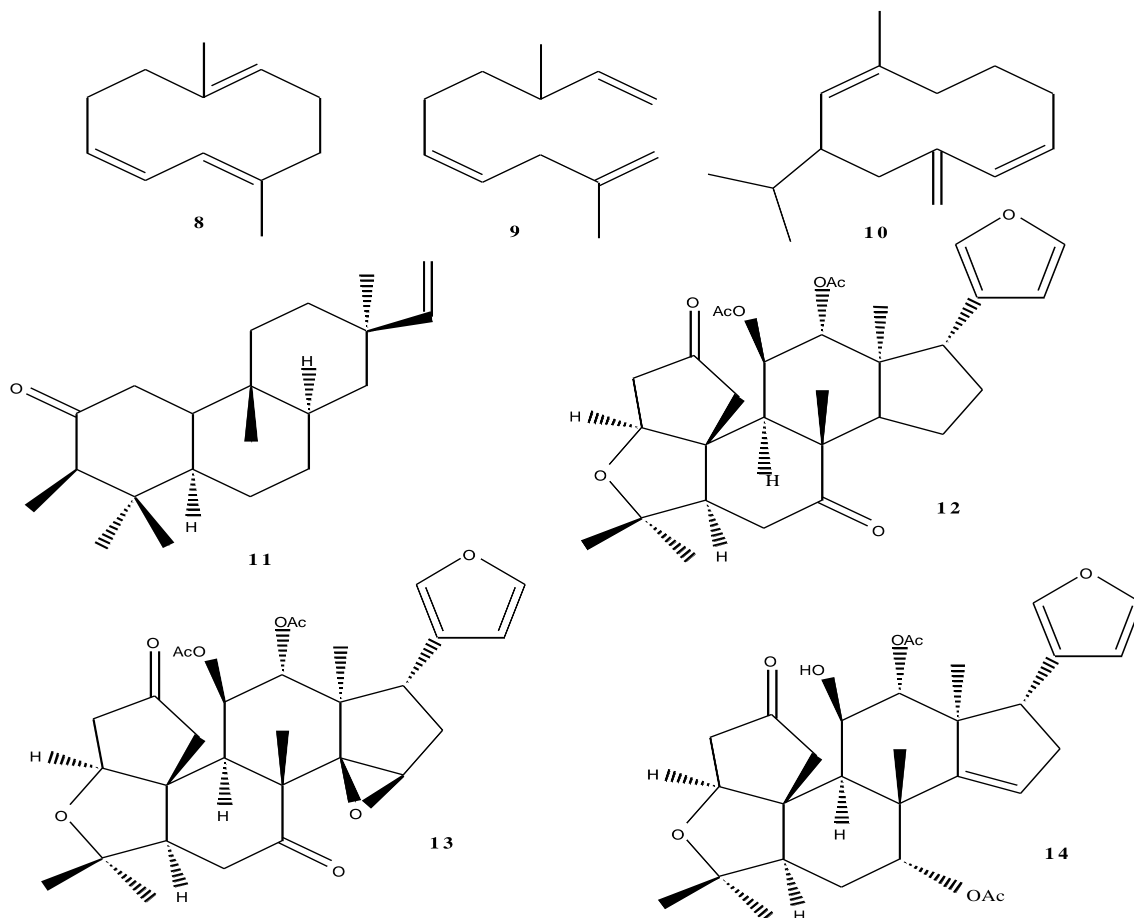


In addition, sterols such as β-sitosterol (**6**) appear ubiquitous in nature and the triterpene lupeol (**7**) appears restricted to the *Zanthoxylum* species in the Rutaceae family. The two compounds are reported to be usually associated with stigmasterol, campesterol and β-

amyrin. They have been isolated from the various morphological parts of all the *Zanthoxylum* species investigated (Adesina, 2005).

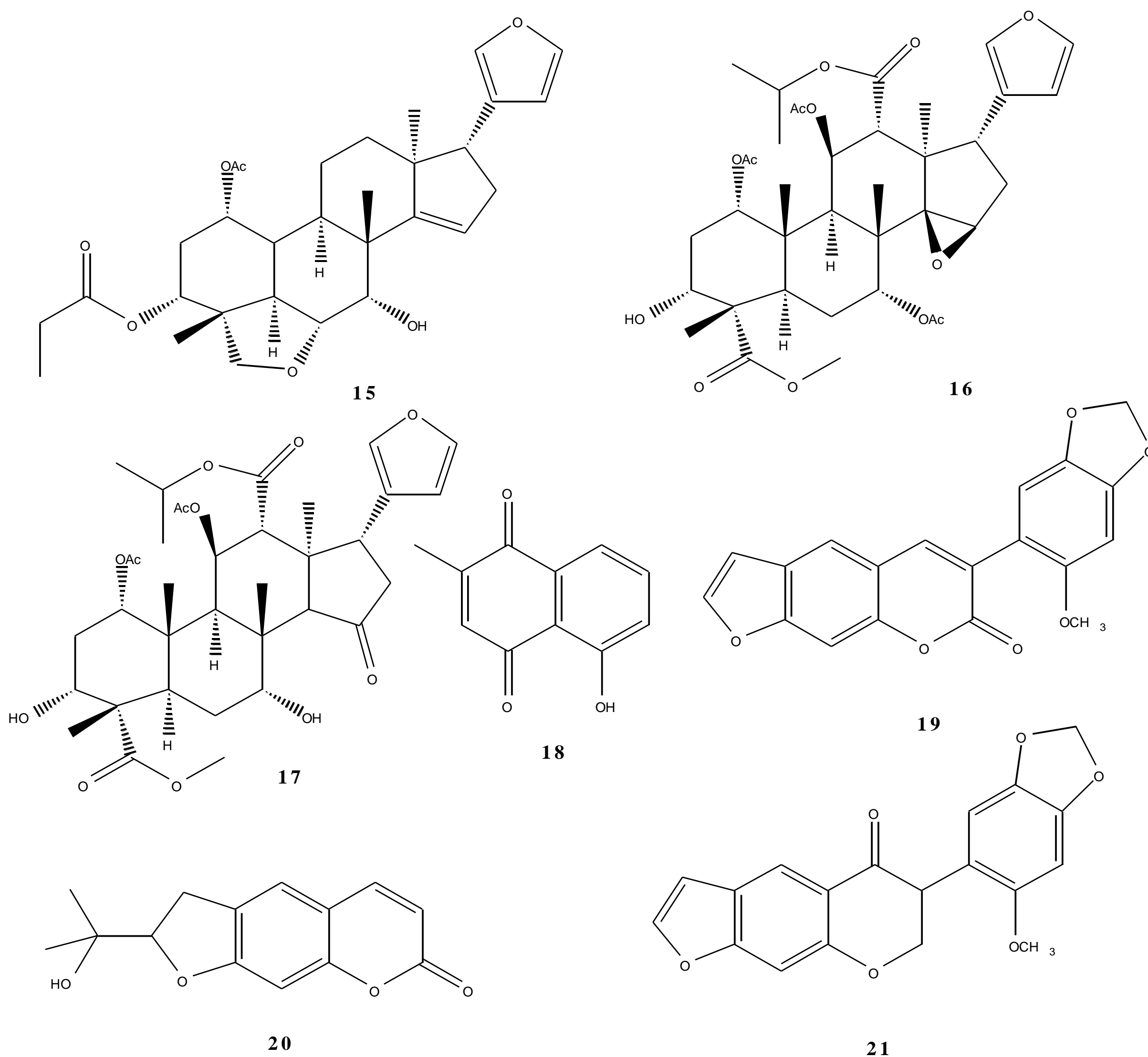
2.6 Some natural larvicidal compounds active against *An. gambiae*

The pregeijerene **(8)**, geijerene **(9)**, and germacrene D **(10)** are sesquiterpenes isolated from the leaves of *Chloroxylon swietenia* which possess activity against *An. gambiae* with LD_{50} values of 1800, 3000, 4200 ppm respectively (Kiran and Devi, 2007). Hugorosenone **(11)** isolated from the *Hugonia castaneifolia* also has been shown to display larvicidal activity against mosquito larvae of *An. gambiae* with LC_{50} values of 302.8 ppm at 24 hr (Baraza *et al.*, 2008). The three limonoids (**12-14)**, isolated from the root bark of *Turraea wakefieldii* also exhibit activity against late third or early fourth-instar larvae of *An. gambiae*.



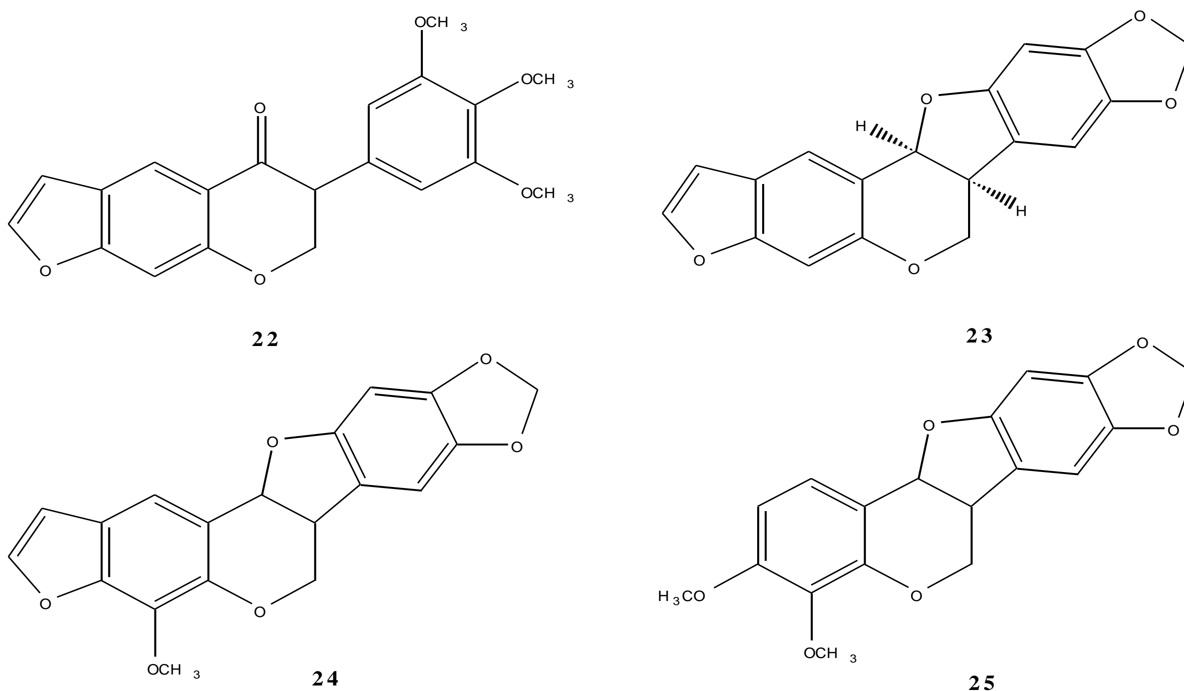
Several other classes of secondary metabolites have also been found to show good larvicidal activities; the tetranortriterpenoids **(15-17)** isolated from *Turraea wakefieldii* and *T.*

floribunda exhibit toxicity against *An. gambiae* larvae at LD_{50} values of 7.1, 4.0, and 3.6 ppm respectively, the naphthoquinone, plumbagin **(18)** isolated from *Plumbago zeylanica* (Kishore *et al*., 2010) and other plant species exhibit larvicidal activity against *An. gambiae* with LC_{50} value of 1.9 ppm.



Coumarin, pachyrrhizine **(19)**, isolated from *Neorautanenia mities* exhibits activity against *An. gambiae* adults with an LC_{50} value of 7.0 ppm. The marmesin **(20)** isolated from *Aegle marmelos* also exhibits toxicity against *An. gambiae* adults with LC_{50} and LC_{90} values of 0.082 and 0.152 ppm, respectively (Joseph *et al*., 2004). The isoflavonoids neotenone (**21**) and neorautanone (**22**) isolated from *Neorautanenia mities* displays activity against adult *An.*

gambiae mosquitoes with LD_{50} values of 8.0 and 9.0 ppm, respectively (Puyvelde *et al.*, 1987).



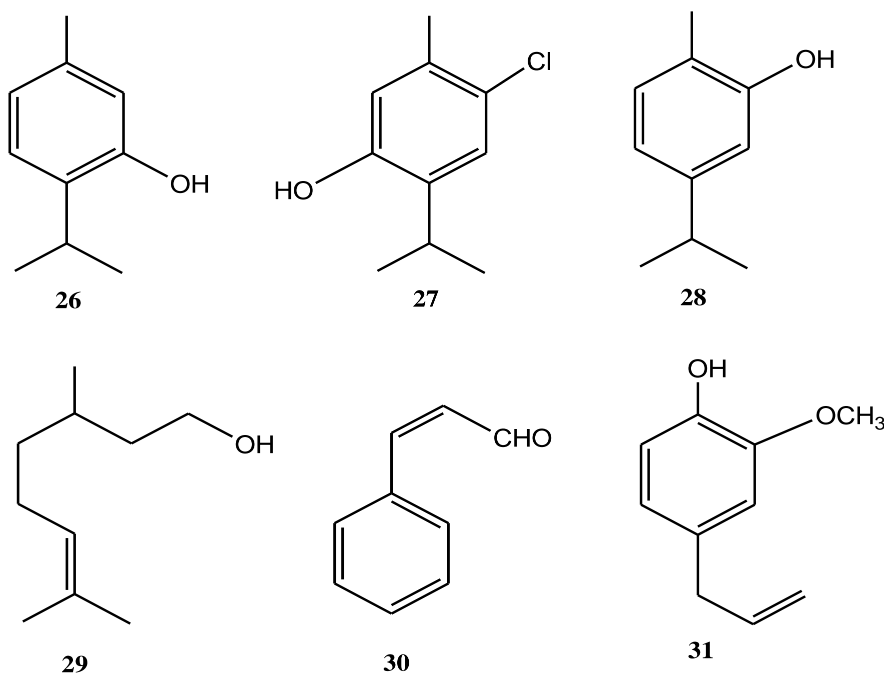
The pterocarpans, neoduline **(23)**, 4-methoxyneoduline **(24)**, and nepseudin **(25)** isolated from tubers of *Neorautanenia mities* exhibit mosquitocidal activity against *An. gambiae* and *Cx. quinquefaciatus* larvae with LD_{50} values 5.0, 11.0 and 3.0 ppm, respectively (Joseph *et al.*, 2004).

2.7 Essential oils and their mosquito larvicidal activity

Essential oils are volatile fractions obtained by steam or water distillation of medicinal and aromatic plants. They are characterized by a strong odour fragrance and are used in perfumes, cosmetics and as food additives (Ebrahimi *et al.*, 2011). Furthermore, they are formed by varied and complex volatile mixtures of chemical compounds, with predominance of terpene associated to aldehyde, alcohols and ketone which were deposited in various structure of the plant (Linares e*t al.*, 2005; Mohamed *et al.*, 2010). In nature, many essential oils play an important role in protecting plants against pathogens and also against herbivores by reducing their appetite for such plants. They also may attract some insects to favour the

dispersion of pollens and seeds, or act as repellent for other undesirable insects (Bakkali *et al.,* 2008).

Some essential oils extracted from different plant families have been shown to be effective in the control of mosquito larvae (Phasomkusolsil and Soonwera, 2010). Essential oils with larvicidal activity against third-instar of *A.aegypti* have been extracted from plants of the Myrtaceae (Cheng *et al*., 2009), Piperaceae (Morais *et al*., 2007), Poaceae (Furtado *et al.*, 2005), Lamiaceae (Cavalcanti *et al.,* 2004; Furtado *et al.*, 2005), Rutaceae (Cavalcanti *et al.,* 2004; Furtado *et al.*, 2005; Kiran *et al.,* 2006; Pitasawat *et al.,* 2007), Verbenaceae (Cavalcanti *et al.,* 2004; Furtado *et al.*, 2005), Apiaceae and Zingiberaceae (Pitasawat *et al.,* 2007).



Many essential oils from plant origin such as citronella, calamus, thymus, and eucalyptus have previously been reported as promising mosquito larvicides (James, 1992; Hemingway, 2004; Wandscheer *et al*., 2004; Shaalan *et al.,* 2005; Ruhaman *et al.,* 2008). Among the monoterpenoid, essential oil components such as thymol (**26**), cholorothymol (**27**), carvacrol (**28**), β-citronellol (**29**), cinnamaldehyde (**30**) and eugenol (**31**) isolated from a number of plant species are reported to possess mosquitocidal activity against forth instar larvae of *Culex pipiens* with LC_{50} values of 37.95, 14.77, 44.38, 89.75, 58.97 and 86.22 ppm, respectively (Navneet *et al.,* 2011).

CHAPTER THREE
MATERIALS AND METHODS

3.1 Chemicals and working techniques

All the reagents were obtained from the commercial suppliers and were used without further purification unless stated. The solvents were distilled prior to use except for those that were analytical reagent grades. Chemicals that were air or water sensitive were stored under inert conditions.

3.2 Collection of the plant and its identification

The leaves of *F. angolensis* were collected from Kakamega Forest, a tropical rain forest in Kenya. The plant grows wildly between latitudes of 00°10’N and 00°21’N and longitudes of 34°47’E and 34°58’E at about 1600 m above sea level. The tropical rainforest conditions receive approximately 2000 mm of rainfall per annum. It was identified by a taxonomist at the department of biological sciences of Egerton University, where a voucher specimen was deposited.

3.3 Extraction of the non-volatile compounds

The collected plant leaves were air dried in the shade for 7 days and ground using a blending machine (Thomas-Wiley Laboratory Mill Model 4) at Kenya Agricultural Research Institute, Njoro. The powdered materials of *F. angolensis* (2.0 kg) were exhaustively extracted with 10 litres of methanol (MeOH) solvent at room temperature for 48 hours. The filtrate was concentrated to dryness under reduced pressure using rotavapor machine (BUCHI – R 205). The green crude extract obtained was suspended in distilled water to remove the available sugars and extracted with chloroform. Chlorophyll matter which gave the crude extract the deep green colouration was removed using 50 g of activated charcoal followed by five filtration steps. The filtrate was concentrated to dryness under reduced pressure using the rotor vapor. This afforded 70 g of brown chloroform crude extract.

3.4 Bioactivity guided fractionation

A glass tube with a diameter 2 cm and a height of 50 cm fitted with a tap at the bottom was used for column chromatography. It was packed using silica gel (70-230 mesh) as the stationary phase and 100% chloroform as the mobile phase. The chloroform crude extract (65 g) was fractionated using 100% chloroform solvent. This led to 36 fractions that were combined according to their TLC patterns to give three major fractions namely C_1, C_2 and C_3. Fractions C_2 and C_3 had high larvicidal activity when subjected to bioactivity tests (see appendices 3 and 4) while fraction C_1 (23.1 g) showed a very low larvicidal activity and therefore left out for further work (see appendix 2).

Subsequent fractionation of C_2 fraction (16.6 g), with chloroform – ethyl acetate solvent mixture in the ratio of 7:3 (v/v) led to five fractions C_2F_1 to C_2F_5. Fraction C_2F_5 had a deep yellow colouration and was only visible at 365 nm on the multiband UV-254/365 nm lamp (UV GL-58) when developed on pre-coated silica gel 60 F_{254} aluminium TLC plates (5 x 8 cm x 0.25 mm) with fluorescence indicator. Also, further fractionation of C_3 fraction (9.7 g) using the same solvent mixture afforded three sub-fractions namely C_3F_1, C_3F_2 and C_3F_3.

According to the bioassays, fraction C_2F_5 (2.4 g) was the most active and was therefore further fractionated using chloroform – ethyl acetate solvent mixture in the ratio of 7:3 (v/v) to give other three fractions namely C_2F_5A, C_2F_5B and C_2F_5C (see figure 6). On comparison of the low yields obtained from C_2F_5A and C_2F_5C, they could not be considered for any promising further work. Despite the relatively good larvicidal activity obtained from fraction C_2F_1, it appeared to be oily and impure when spotted and developed on the analytical TLC plate. From this, it was not considered for further purification due to its low amounts. In addition, fractions C_2F_3, C_2F_4 and C_3F_1 were left out on regard to their low amounts obtained from each. The fractions C_2F_2, C_2F_5B, C_3F_2 and C_3F_3 were purified using preparative TLC as explained in section 3.5. Their retardation factors (RF) were calculated as the ratio of the distance moved by the analyte to the distance moved by the solvent. The obtained RF values for these compounds C_2F_5B (**32**), C_3F_2 (**33**), C_3F_3 (**34**) and C_2F_2 (**35**) on the TLC under UV light were 0.51, 0.40, 0.19 and 0.83 respectively.

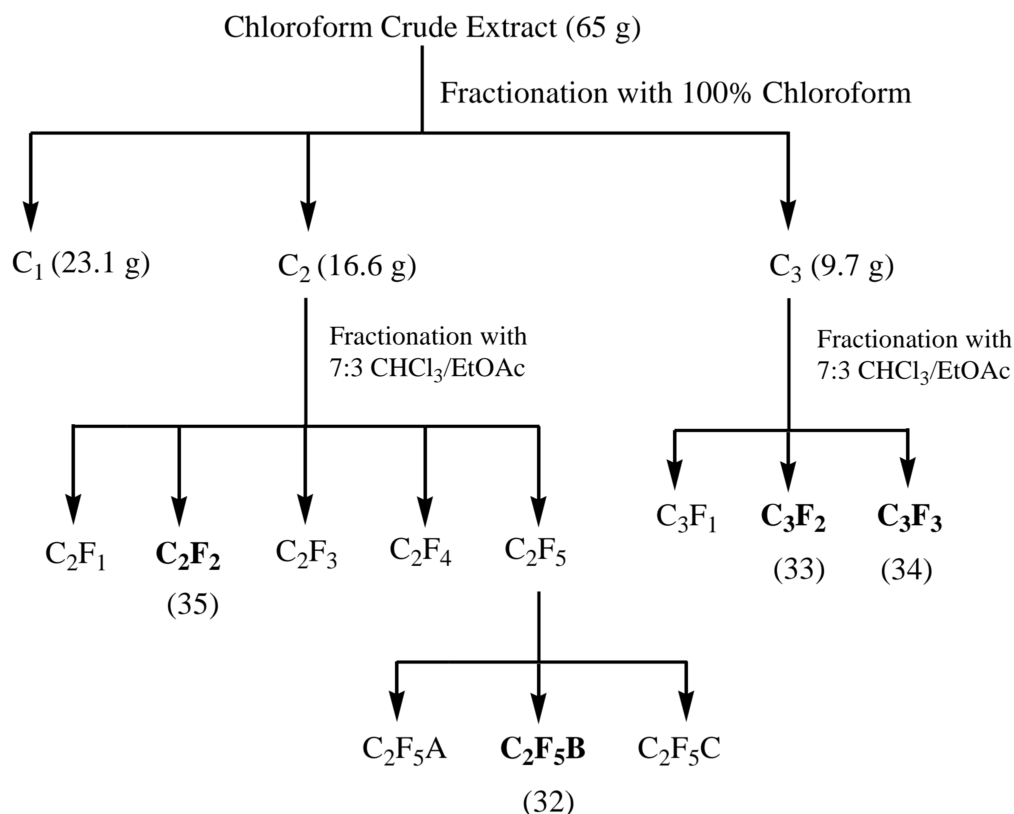


Figure 6: Summary of the bioassay–guided fractionation of the non-volatiles

3.5 Purification of the compounds

All the fractions of interest were purified using the Preparative Thin Layer Chromatography techniques with slight modifications. The PTLC plates were prepared locally in the laboratory prior to use.

3.5.1 Preparation of the preparative TLC plates

For preparative TLC (PTLC), 20 x 20 cm x 0.25 mm glass plates were used. The plates were prepared by mixing the adsorbent silica gel with a small amount of calcium sulphate as the inert binder and water. The powdered silica gel (kiesel 60, 70-230 mesh) was weighed (180 g) and mixed with 45 g of calcium sulphate for binding the slurry on the glass. The slurry was made by the addition of 400 ml of distilled water to the mixture and by the use of a magnetic stirrer to obtain a uniform mixture. The cleaned glasses were placed on a flat surface prior to the application of the slurry to allow uniform coating to all the edges and for good separation during development. After the application of the thick slurry, the resultant

plates were allowed to stay overnight to dry and then activated by heating in the oven for one hour at 140° C. They were allowed to cool before use so as to avoid breakage during the development process.

3.5.2 Development of the PTLC plates

The prepared PTLC plates were placed on clean sheets of plain papers, using a pencil and a ruler two straight lines were drawn 1 cm and 1.5 cm away from the edge of the glass (parallel to each other) which was used as the baseline for the application of the sample. The sample was applied as long streaks using a Pasteur pipette and allowed to dry for 5 minutes. The solvent mixture for separation 7:3 (v/v) chloroform to ethyl acetate was prepared, poured into the development tank and closed with a cover glass for saturation to be obtained in the tank. The plates were developed inside the tank and solvent allowed to move up the plate by capillary action as on normal TLC plates. The plates were removed when the solvent front reached no higher than the top of the plate. The samples were recovered by scraping the adsorbent region from the plate according to their separation distances (Retardation factors). The scraped samples were then eluted with ethyl acetate solvent, filtered under gravity with whatman no. 1 filter papers and finally the solvent recovered under reduced pressure evaporation using BUCHI rotavapor R-205. The obtained sample was put into small sample collection bottles and left open to allow evaporation of the remained solvent.

3.6 Larvicidal assays of the crude extract, fractions and target compounds

The stock solutions for the crude extract and the fractions were prepared according to their yields obtained for screening purposes. However, exactly 0.06 g of each of the four targeted compounds (**32, 33, 34** and **35**) were weighed and dissolved in dimethylsulfoxide (analytical grade, Lobarchemi) and diluted using spring water to 60 ml making a concentration of 1000 ppm as stock solutions. Serial dilutions ranging from 750 ppm, 500 ppm, 250 ppm, 125 ppm upto 3.9 ppm were prepared from the each of the four stock solutions (using the formula $C_1V_1= C_2V_2$) in readiness for the larvicidal tests. The concentration of DMSO was kept below 1% since a higher one leads to the larval mortality. The bioassay experiments were conducted according to standard WHO procedure (2005) with slight modifications. All the bioassays were conducted at the Kenya Medical Research Institute,

Centre for Global Health Research (CGHR), Kisumu, Kenya, where the insects were reared in plastic and enamel trays in spring river water. The experimental temperatures were maintained and carried out at 26±3°C at humidity between 70% and 75%. The bioassays were performed on third instar larvae of *An. gambiae* and carried out in triplicate using 20 larvae for each replicate assay. The replicates were run simultaneously yielding a total of 60 larvae for each dosage. The larvae were placed in 50 ml disposable plastic cups containing 15 ml of test solution and fed on tetramin fish feed (TetraMin®) during all testing. Mortality and survival was established after 24 hours of exposure. Larvae were considered dead if they were unrousable even when gently prodded. The number of the dead larvae in the three replicates was expressed as the percentage mortality for each concentration. The negative and positive controls used were 1% DMSO in spring river water and the pyrethrum based larvicides: pylarvex respectively.

3.7 Extraction of volatile compounds (essential oil)

3.7.1 Preparation and set-up of the Clevenger apparatus

Fresh plant leaves of *F. angolensis* were sorted out and cut into small pieces that were meant to increase the surface area during the heating process. A 2.0 litre round bottomed flask was packed with the leaf pieces (including water) and placed on a heating mantle. Hydro-distillation process was carried out for at least four hours according to the British pharmacopoeia (Papachristos and Stamopoulos, 2004). The essential oils obtained were dried over anhydrous sodium sulphate to ascertain the yield as w/w. The oil was then stored in sealed glass vials at 4°C until chemical composition analysis and larvicidal activity.

3.7.2 Larvicidal assays of the essential oils

The essential oil was weighed (0.1 g) and solubilized into a 100 ml volumetric flask with less than 1% Dimethylsulfoxide to make a stock solution of 1000 ppm. Prior to the setting up of the experiments, the third instar *An. gambiae* larvae were sorted out and counted into small disposable plastic cups. Serial dilutions of the stock solution were done at different concentrations such as 1000 ppm, 650 ppm, 500 ppm, 400 ppm, 250 ppm, 125 ppm, 62.5

ppm, 31.3 ppm, 15.6 ppm, 7.8 ppm and 3.9 ppm. Time was recorded immediately the solutions of different concentrations were added into the disposable cups that contained the mosquito larvae. All the larvae in triplicates were fed on tetramin fish feed (TetraMin®) during the testing. Each test solution comprised of twenty *An. gambiae* mosquito larvae. Mortality and survival rate was established after 24 hours of exposure. Larvae were considered dead if they were unrousable within a period of time, even when gently prodded. The negative control was 1% DMSO in spring river water while the positive control was the pyrethrum based larvicides: pylarvex.

3.8 Determination of the chemical components and spectroscopic analysis

3.8.1 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 ('Restek') (30 m x 0.25 mm, 0.25 μm 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 – 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 electron impact ionization (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, 2007) and supplemented by Wiley 7N.l, HPCH 1607.L and FLAVORS.L 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.

3.8.2 Mass spectrometry

The mass spectra were recorded on Finnigan Triple Stage Quadrupol Spectrometer (TSQ-70) with electrospray ionization (ESI) Method. The Thermo Xcalibur Qual computer software was used in analysis of the mass chromatograms.

3.8.3 Nuclear Magnetic Resonance (NMR) Spectroscopy

The NMR spectra (^{1}H, ^{13}C, DEPT, COSY, HSQC and HMBC) were recorded on the Bruker Advance 500 MHz NMR spectrometer at the Technical University of Berlin, Germany. All the readings were done in Deuterated chloroform solvent and chemical shifts were assigned by comparison with the residue proton and carbon resonance of the solvent, tetramethylsilane (TMS) was used as an internal standard and chemical shifts were given as δ (ppm). The structures were simulated using ACD NMR manager program to obtain the chemical shifts of both proton and carbon.

3.8.4 Two dimensional NMR Spectroscopy

In the ^{1}H – ^{1}H COSY (Corrrelation spectroscopy) the off- diagonal elements were used to identify the spin – spin coupling interactions. The proton - carbon connectivity of three bonds away was identified using ^{1}H–^{13}C HMBC (Heteronuclear Multiple Bond Correlation) spectrum in which there was one – dimensional ^{13}C NMR spectrum along the y – axis on the left and the ^{1}H NMR spectrum along the x – axis at the top. The proton resonances of different groups (methines, methylene and methyl) were distinguished along with their corresponding carbon resonances by use of the ^{1}H–^{13}C HSQC spectrum (Heteronuclear Single Quantum Coherence).

3.8.5 Statistical analysis

The larval mortality data were subjected to probit regression analysis according to Finney (1971). Probit analysis of the concentration-dependent mortality data was conducted to estimate the LC_{50} and LC_{90} values associated at 95% confidence limits with the statistical package IBM SPSS 19 software.

3.8.6 Flow chart diagram

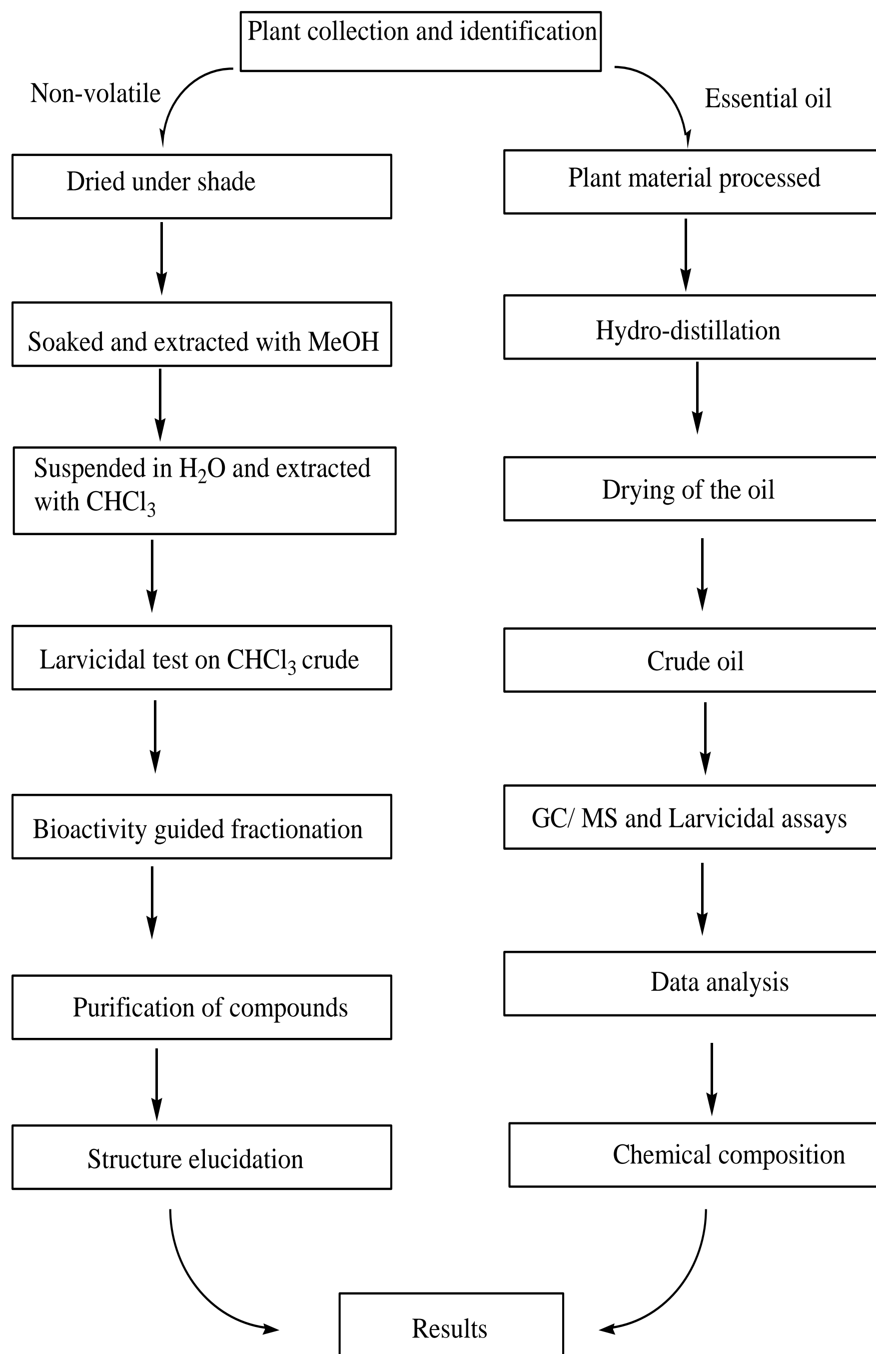


Figure 7: A summary chart of the methodology

CHAPTER FOUR
RESULTS AND DISCUSION

4.1 Larvicidal assay of the chloroform crude extract of *F. angolensis*

The chloroform crude extract obtained from the leaves of *F. angolensis* was subjected to mosquito larvicidal activity tests against the third instar larvae of *An. gambiae* in three replicates. The concentration dilutions were obtained from the stock solution as described in section 3.6. Data obtained from the larvicidal test is shown in table 1 which gives the percentage mortality of the larvae at various concentrations of the crude extract. The LC_{50} and LC_{90} values after 24 hours of exposure were calculated using log probit regression analysis (95% confidence level) with IBM SPSS 19 software. The negative control used was 1% of DMSO which showed no activity against the larvae after 24 hours and the positive control was Pylarvex that gave 100% mortality at 100 ppm.

Table 1: Larvicidal results for the chloroform crude extract of *F. angolensis* (Appendix 1)

Concentration (ppm)	% Mortality ± SD	LC_{50} (ppm)	LC_{90} (ppm)
31.25	3.3 ± 2.9		
62.5	10.0 ± 5.0		
125	10.0 ± 5.0		
250	30.0 ± 13.2		
500	83.3 ± 7.6	264.8 (205.9 - 340.0)	766.9 (565.1 - 1194.5)
1000	96.7 ± 2.9		
1500	100.0 ± 0.0		
2000	100.0 ± 0.0		
Pylarvex (100 ppm)[a]	100.0 ± 0.0		
Spring water + DMSO[b]	0.0 ± 0.0		

[a]Positive control, [b]Negative control.

Based on table 1, it is evident that the chloroform crude extract is active against the *An. gambiae* mosquito larvae. There is a positive correlation between the percent mortality values and the crude extract concentrations. Therefore it is true that the percentage mortality values depend on the concentration of the crude extract. One hundred percent larval mortality was achieved at a concentration of 1500 ppm. The lowest concentration used from serial dilution of the stock solution was 31.25 ppm which showed a 3.3% larval mortality. According to the log probit analysis done (see table 1), the crude extract had an LC_{50} value of

264.8 ppm and an LC_{90} value of 766.9 ppm at 95% confidence limit. Owing to the positive results from the crude extract larvicidal assay, it is evident that there are compound(s) in the crude extract responsible for the actual larvicidal activity against the *An. gambiae* larvae. This was the basis for further fractionation of the crude extract.

4.2 Larvicidal assays of the major fractions from the crude extract

The initial stage bioactivity guided fractionation of the crude extract led to three major fractions namely C_1, C_2 and C_3. The major fractions were subjected to larvicidal tests against the third instar larvae of *An. gambiae* in three replicates and the data obtained was reported in tables 2, 3 and 4. The LC_{50} and LC_{90} values for each of the three major fractions were also analyzed using log probit regression analysis (appendices 2-4) and reported in their respective tables (2, 3 and 4).

Table 2: Larvicidal assay results for the C_1 major fraction (Appendix 2)

Concentration (ppm)	% Mortality ± SD	LC_{50} (ppm)	LC_{90} (ppm)
337.5	8.3 ± 2.9		
675.0	76.7 ± 10.4		
1250.0	75.0 ± 5.0		
2500.0	95.0 ± 5.0	633.2 (261.8 - 1032.0)	603.6 (993.4 - 9659.7)
5000.0	100.0 ± 0.0		
Pylarvex (100 ppm)[a]	100.0 ± 0.0		
Spring water + DMSO[b]	0.0 ± 0.0		

[a]Positive control, [b]Negative control.

Table 3: Larvicidal assay results for the C_2 major fraction (Appendix 3)

Concentration (ppm)	% Mortality ± SD	LC_{50} (ppm)	LC_{90} (ppm)
62.5	30 ± 17.3		
125	31.7 ± 25.7		
250	96.7 ± 2.9		
500	100.0 ± 0.0	113.1 (47.6 - 210.6)	259.7 (157.4 – 3852.2)
1000	100.0 ± 0.0		
1200	100.0 ± 0.0		
Pylarvex (100 ppm)[a]	100.0 ± 0.0		
Spring water + DMSO[b]	0.0 ± 0.0		

[a]Positive control, [b]Negative control.

Table 4: Larvicidal assay results for the C_3 major fraction (Appendix 4)

Concentration (ppm)	**% Mortality ± SD**	**LC_{50} (ppm)**	**LC_{90} (ppm)**
18.8	0.0 ± 0.0		
37.5	0.0 ± 0.0		
75.0	0.0 ± 0.0		
150.0	45.0 ± 15.0	159.1 (135.5 - 178.3)	231.5 (202.4 - 310.6)
200.0	73.3 ± 2.9		
300.0	100.0 ± 0.0		
Pylarvex (100 ppm)[a]	100.0 ± 0.0		
Spring water + DMSO[b]	0.0 ± 0.0		

[a]Positive control, [b]Negative control.

All the three major fractions were active to different extents against the *An. gambiae* larvae. According to tables 2, 3 and 4, it can be seen that the percent mortalities for C_1, C_2 and C_3 are all concentration dependent. Fraction C_1 had a 100% larval mortality at 5000 ppm while fractions C_2 and C_3 had 100% larval mortality at 500 ppm and 300 ppm respectively. Fractions C_2 and C_3 also showed comparable activities that was markedly different from that of C_1 fraction and were far more potent. The LC_{50} values for fractions C_1, C_2 and C_3 were 633.2, 113.1 and 159.1 ppm respectively and the LC_{90} values were 603.6 ppm, 259.7 ppm and 231.5 ppm respectively. Therefore, the C_2 and C_3 fractions were further fractionated and larvicidal tests carried out on their sub-fractions as shown in sections 4.3 and 4.4. The negative control which was 1% DMSO showed no activity against the larvae after 24 hours.

From the observed mortality of the crude extract and the major fractions, the current findings compare well with the aforementioned studies of extracts from Rutaceae family. For instance, the Rutaceae families had earlier on been shown to induce insecticidal effects against mosquitoes (Tiwari *et al.,* 2007). Also, *Murraya koenigii* (Rutaceae) had been previously reported to possess mosquitocidal properties through effects of hormone regulation with subsequent disruption of instar development of *An. stephensi*, *Cx. quinquefasciatus* and *A. aegypti* (Arivoli and Tennyson, 2011).

4.3 Larvicidal assays of fractions from the C_2 major fraction

Fraction C_2 was subjected to further bioactivity guided fractionation which led to five fractions namely C_2F_1, C_2F_2, C_2F_3, C_2F_4 and C_2F_5. All the fractions from C_2 except C_2F_3 which had very low yields were subjected to larvicidal assays against *An. gambiae* larvae in

three replicates. The mortality data obtained was tabulated in tables 5, 6, 7 and 8. The LC_{50} and LC_{90} values for each of the four fractions were also analyzed using probit analysis and reported in their respective tables (as shown in tables 5, 6, 7 and 8).

Table 5: Larvicidal assay results for the C_2F_1 fraction (Appendix 5)

Concentration (ppm)	**% Mortality ± SD**	**LC_{50} (ppm)**	**LC_{90} (ppm)**
12.5	0.0 ± 0.0		
25.0	1.7 ± 2.9		
50.0	13.3 ± 5.8		
100.0	53.3 ± 20.2		
125.0	100.0 ± 0.0	78.6 (65.9 – 91.9)	133.9 (111.6 – 183.0)
250.0	100.0 ± 0.0		
500.0	100.0 ± 0.0		
1000.0	100.0 ± 0.0		
1500.0	100.0 ± 0.0		
2000.0	100.0 ± 0.0		
Pylarvex (100 ppm)[a]	100.0 ± 0.0		
Spring water + DMSO[b]	0.0 ± 0.0		

[a]Positive control, [b]Negative control.

Table 6: Larvicidal assay results for the C_2F_2 fraction (Appendix 6)

Concentration (ppm)	**% Mortality ± SD**	**LC_{50} (ppm)**	**LC_{90} (ppm)**
27	0.0 ± 0.0		
53	0.0 ± 0.0		
106	1.7 ± 2.9		
213	80.0 ± 13.2	171.8 (143.7 - 202.7)	252.5 (212.0 - 363.0)
425	100.0 ± 0.0		
850	100.0 ± 0.0		
1700	100.0 ± 0.0		
2500	100.0 ± 0.0		
3400	100.0 ± 0.0		
Pylarvex (100 ppm)[a]	100.0 ± 0.0		
Spring water + DMSO[b]	0.0 ± 0.0		

[a]Positive control, [b]Negative control.

Table 7: Larvicidal assay results for the C_2F_4 fraction (Appendix 7)

Concentration (ppm)	**% Mortality ± SD**	**LC_{50} (ppm)**	**LC_{90} (ppm)**
168.3	16.7 ± 2.9		
337.5	25.0 ± 15.0		
675.0	100.0 ± 0.0		
1250.0	100.0 ± 0.0	343.8 (189.6 - 668.3)	643.9 (416.0 -6275.3)
2000.0	100.0 ± 0.0		
2500.0	100.0 ± 0.0		
Pylarvex (100 ppm)[a]	100.0 ± 0.0		
Spring water + DMSO[b]	0.0 ± 0.0		

[a]Positive control, [b]Negative control.

Table 8: Larvicidal assay results for the C_2F_5 fraction (Appendix 8)

Concentration (ppm)	**% Mortality ± SD**	**LC_{50} (ppm)**	**LC_{90} (ppm)**
10	0.0 ± 0.0		
20	5.0 ± 5.0		
40	50.0 ± 10.0		
50	100.0 ± 0.0		
80	96.7 ± 5.8	35.3 (29.9 - 40.3)	54.6 (47.3 - 69.2)
100	100.0 ± 0.0		
200	100.0 ± 0.0		
400	100.0 ± 0.0		
600	100.0 ± 0.0		
800	100.0 ± 0.0		
Pylarvex (100 ppm)[a]	100.0 ± 0.0		
Spring water + DMSO[b]	0.0 ± 0.0		

[a]Positive control, [b]Negative control.

According to the larvicidal assay results analyzed and reported in tables 5, 6, 7 and 8, fraction C_2F_1 had 100% larval mortality at 125.0 ppm while C_2F_2 had 100% mortality at 425.0 ppm. Also, fractions C_2F_4 and C_2F_5 had 100% mortality at 675.0 ppm and 50.0 ppm respectively. The percent mortalities for all the four fractions were concentration dependent. The LC_{50} values for fractions C_2F_1, C_2F_2, C_2F_4 and C_2F_5 were 78.6 ppm, 171.8 ppm, 343.8 ppm and 35.3 ppm respectively. The LC_{90} values were 133.9 ppm, 252.5 ppm, 643.5 ppm and 54.6 ppm respectively. Comparing the LC values of all the four fractions, it can be seen that fraction C_2F_5 was the most potent followed by C_2F_1 and thirdly C_2F_2. Fraction C_2F_4 showed the least potent larvicidal activity. The negative control showed no activity against the larvae after 24 hours.

4.4 Bio-assays of the isolated pure compounds from *F. angolensis*

From the bioassay guided fractionation and purification procedures illustrated under section 3.4 in figure 6, four compounds were isolated and numbered (**32**), (**33**), (**34**) and (**35**). Each of the four compounds was subjected to larvicidal tests against the third instar larvae of *An. gambiae*. Three replicates were set per concentration during the experiments and 1% DMSO which showed no activity was used as the negative control. Their activity results were reported in the tables 9-12.

Table 9: Larvicidal activity of compound **32** (Appendix 10)

Concentration (ppm)	% Mortality ± SD	LC_{50} (ppm)	LC_{90} (ppm)
3.9	0.0±0.0		
7.8	0.0±0.0		
15.6	0.0±0.0		
31.3	0.0±0.0		
62.5	0.0±0.0	245.5 (199.9-297.3)	471.6 (379.0-662.5)
125.0	11.7±7.6		
250.0	46.7±34.0		
500.0	96.7±5.8		
750.0	96.7±5.8		
1000.0	100.0±0.0		
Pylarvex (100 ppm)[a]	100.0 ± 0.0		
Spring water + DMSO[b]	0.0 ± 0.0		

[a]Positive control, [b]Negative control.

Table 10: Larvicidal activity of compound **33** (Appendix 11)

Concentration (ppm)	% Mortality ± SD	LC_{50} (ppm)	LC_{90} (ppm)
3.9	0.0±0.0		
7.8	1.7±2.9		
15.6	0.0±0.0		
31.3	3.3±2.9		
62.5	5.0±5.0	147.6 (97.0-233.5)	292.1 (195.1-870.9)
125.0	21.7±7.6		
250.0	91.7±10.4		
500.0	100.0±0.0		
750.0	100.0±0.0		
Pylarvex (100 ppm)[a]	100.0 ± 0.0		
Spring water + DMSO[b]	0.0 ± 0.0		

[a]Positive control, [b]Negative control.

Table 11: Larvicidal activity of compound **34** (Appendix 12)

Concentration (ppm)	% Mortality ± SD	LC_{50} (ppm)	LC_{90} (ppm)
3.9	0.0±0.0		
7.8	0.0±0.0		
15.6	0.0±0.0		
31.3	0.0±0.0		
62.5	6.7±5.8	144.4 (118.9-176.4)	259.4 (206.4-389.2)
125.0	30.0±8.7		
250.0	91.7±2.9		
500.0	100.0±0.0		
750.0	100.0±0.0		
Pylarvex (100 ppm)[a]	100.0 ± 0.0		
Spring water + DMSO[b]	0.0 ± 0.0		

[a]Positive control, [b]Negative control.

Table 12: Larvicidal activity of C_2F_2 purified as compound **35** (Appendix 13)

Concentration (ppm)	% Mortality ± SD	LC_{50} (ppm)	LC_{90} (ppm)
3.9	0.0±0.0		
7.8	0.0±0.0		
15.6	0.0±0.0		
31.3	0.0±0.0		
62.5	0.0±0.0	423.0 (290.0-682.2)	957.0 (616.4-3593.5)
125.0	11.7±7.6		
250.0	6.7±2.9		
500.0	46.7±27.5		
750.0	96.7±5.8		
Pylarvex (100 ppm)[a]	100.0 ± 0.0		
Spring water + DMSO[b]	0.0 ± 0.0		

[a]Positive control, [b]Negative control.

The four isolated compounds from *F. angolensis* leaves showed strong larvicidal activities after 24 hours of exposure at a concentration of 750 ppm; however, the highest larval mortality was found in compound (**34**) against the third instar larvae of *An. gambiae* (LC_{50} = 144.4 ppm; LC_{90} = 259.4 ppm) and the least potent of the compounds was (**35**) (LC_{50} = 423.0 ppm; LC_{90} = 957.0 ppm). The lethal concentrations were calculated at 95% confidence level. From tables 8 and 9, it is clearly indicated that the C_2F_5 fraction was more active than the purified compound **32** (C_2F_5B). Synergistic effects could have been a factor in explaining their difference in activities. Also, the loss of activity could be associated to the dissociation of the compound when left in liquid organic solvent for a long period of time.

4.5 Structure elucidation of the isolated pure larvicidal compounds

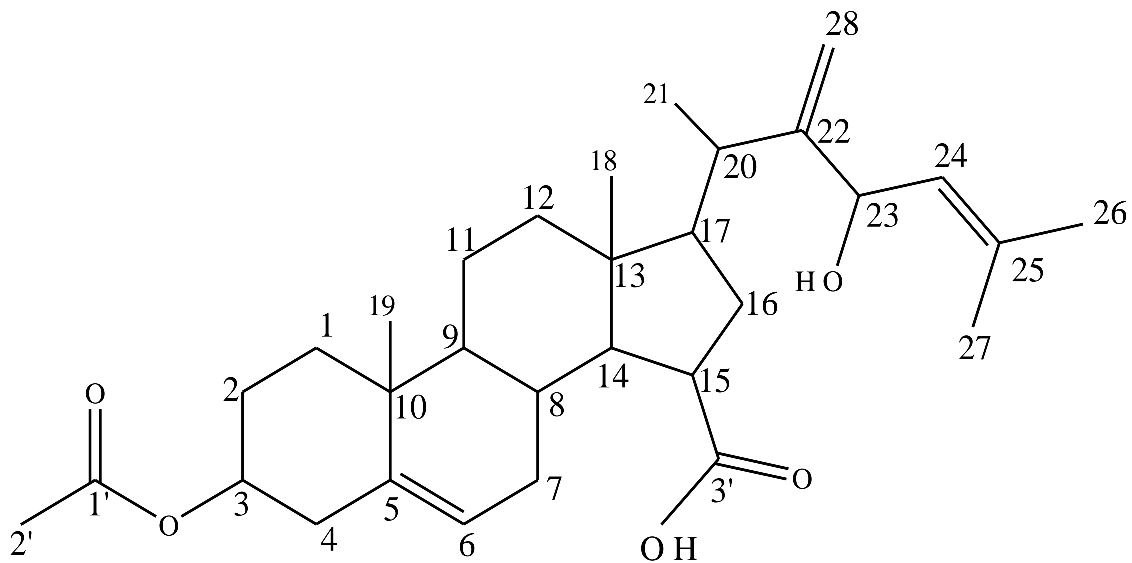
4.5.1 Structure elucidation of the larvicidal compound 32

The compound (**32**) was obtained as a yellow oily substance (66.8 mg). Its ^{13}C NMR spectrum (125 MHz, $CDCl_3$) in appendix 23 indicated thirty one carbon signals (ignoring the solvent signal at δ 77.2). The data from both the 1D and 2D NMR spectra were as summarized in table 13.

The ^{1}H NMR spectrum (500 MHz, $CDCl_3$) showed the characteristic pattern of three olefinic proton resonances at δ_H 4.99 (H-28), δ_H 5.05 (H-24, H-28) and δ_H 5.27 (H-6) and the deshielded methine proton resonance at δ_H 3.49 (H-3) owing to a hydroxylated substituent group at this position. On close examination of the methyl group region in the ^{1}H NMR spectrum and in comparison with the literature (Langlois, 2000; Akhtar *et al.,* 2010), the protons of the methyl groups in positions 18, 19, 21, 26 and 27 were assigned to δ_H 0.77,1.52 (H-18), δ_H 0.77, 1.96 (H-19), δ_H 1.06,1.96 (H-21), δ_H 1.96 (H-26) and δ_H 0.77, 1.54 (H-27). However, the ^{1}H NMR coupling constants (*Jz*) could not be derived from this spectrum due to the poor resolution of the peaks and overlapping effect of the peak signals in the methylene group region. The methylene proton signals appeared from δ_H 1.06 to δ_H 5.05. The exomethylene (C-28) protons resonated at δ_H 4.99 and δ_H 5.

The ^{13}C NMR spectrum revealed the presence of four characteristic olefinic resonances at δ_C 121.69 (C-6), δ_C 125.03 (C-24), δ_C 135.20 (C-25) and δ_C 140.76 (C-5). Two oxygenated methine carbons resonances occurred at δ_C 71.81(C-3), δ_C 75.01(C-23) and the two carbonyl groups present appeared at δ_C 170.40 and 177.83 (C-1' and C- 3') respectively. The exo-methylene carbon C-28 resonated at δ_C 110.45 while the quarternary carbon C-22 resonated at δ_C 148.68. The remaining carbon resonances are as illustrated in table 13.

The ^{1}H-^{1}H COSY correlated the olefinic hydrogen H-6 at δ_H 5.27, 5.05 with H-7 at δ_H 1.96, 1.42 and H-4 at δ_H 2.24. It also correlated with H-4 at δ_H 2.24. Other ^{1}H-^{1}H COSY correlations suggesting the parent sterol structure are those between H-2 (δ_H 1.78,1.19) and H-3 at δ_H 3.49 and to H-4 δ_H 2.24.



Phenanthrene carboxylic acid derivative (**32**)

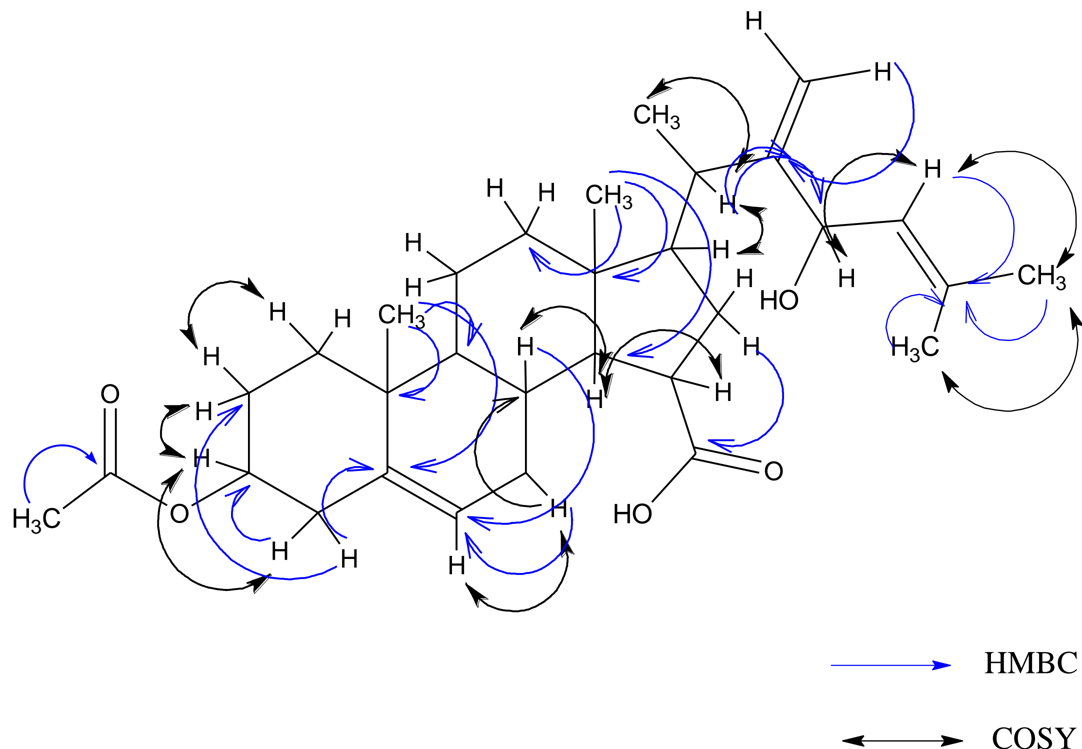
Table 13: Summary of 1D and 2D NMR data values for compound **32**

CARBON	^{13}C (δ)	^{1}H (δ)	DEPT	COSY	HMBC	^{13}C (δ) Literature*	^{13}C (δ) Literature[#]
1	37.27	1.06,0.80	CH_2	2	2, 10	37.33	-
2	29.67	1.19,1.96	CH_2	1, 3	1, 3, 4	31.63	-
3	71.78	3.49	CH	2, 4	2, 4, 5, 1’	71.73	-
4	42.21	2.24	CH_2	3	2, 3, 5, 6	42.00	-
5	140.76	-	Cq	-	-	140.71	-
6	121.69	5.27	CH	7	4, 7, 8	121.16	-
7	31.90	1.96, 1.42	CH_2	6	6, 8	31.96	-
8	32.78	2.24	CH	9	6, 7	31.81	-
9	51.46	0.80,1.52	CH	8	7, 10, 11	51.13	-
10	36.50	-	Cq	-	-	36.43	-
11	22.67	1.60,0.80	CH_2	12	9,10, 12	21.09	-
12	39.36	1.96	CH_2	11	13	39.79	-
13	42.31	-	Cq	-	-	42.37	-
14	56.76	3.30	CH	12, 15	8, 15, 3’	56.75	-
15	45.82	0.80	CH	16	14, 17	-	-
16	24.77	1.96	CH_2	17	3’	-	-
17	56.05	0.99	CH	16	12, 16, 20	56.05	-
18	14.10	0.77,1.52	CH_3	14	12, 13, 14	11.84	-
19	19.74	0.77,1.96	CH_3	2, 9	2, 5, 9,10	19.46	-
20	36.14	1.19,0.77	CH	17, 21	22, 23, 28	36.07	-
21	23.43	1.06,1.96	CH_3	20	17, 20, 22	-	-
22	148.68	-	Cq	-	-	-	-
23	75.01	4.08	CH	24	24, 25,28	-	-
24	125.03	5.05	CH	23	25, 27	-	123.6
25	135.20	-	Cq	-	-	-	132.2
26	25.68	1.96	CH_3	27	24. 25, 27	-	25.7
27	16.00	0.77, 1.54	CH_3	26	24, 25, 26	-	17.6
28	110.45	4.95,5.05	CH_2	20, 23	22, 23	-	-
1’	170.40	-	Cq	-	-	-	-
2’	20.99	1.96	CH_3	-	1’	-	-
3’	177.83	-	Cq	-	-	-	-

* (Akhtar *et al.,* 2010) [#] (Gwala, 2011)

The partial sterol parent substructure was determined effectively by HMBC couplings assigning two of the five methyl groups to C-18 and C-19 respectively. The two methyl carbons of these functionalities resonated at δ_C 14.10 and δ_C 19.72. The long range heteronuclear ^{1}H-^{13}C coupling (HMBC) showed ‘solenoid’-like correlations between C-5 at

δ_C 140.76 and the methyl hydrogens H-19 at δ_H 0.77; between C-14 at δ_C 56.76 and the H-18 at δ_H 0.77 and between C-3 at δ_C 71.78 and H-2 at δ_H 1.96, 1.19. The sterol substructure was connected to side chains by the coupling between C-17 δ_C 56.05 and H-20 at δ_H 0.77. The strong correlations between C-24 at δ_C 124.41 and the protons of C-26 and C-27 at δ_H 1.96 and δ_H 0.77, 1.54 respectively. Other ^1H-^1H and ^1H -^{13}C correlations in the molecule are illustrated below as obtained from their respective spectra shown in appendices 24 and 25**.**



The positive electron impact mass spectrometry (EIMS) of compound (**32**) showed a molecular ion peak at *m/z* 498 corresponding to the molecular formula $C_{31}H_{46}O_5$ which indicated nine indices of hydrogen deficiency. This corresponded with the retention time at 10.39 minutes. The Positive and Negative electron impact mass spectrometry fragmentation pattern in figure10 (a,b,c) is also characteristic of steroids as it showed peaks at *m/z* 521[M+Na]$^+$, *m/z* 483[M-CH_3]$^+$, *m/z* 497[M-H]$^+$ and *m/z* 387[M-$C_7H_{11}O$]$^+$.

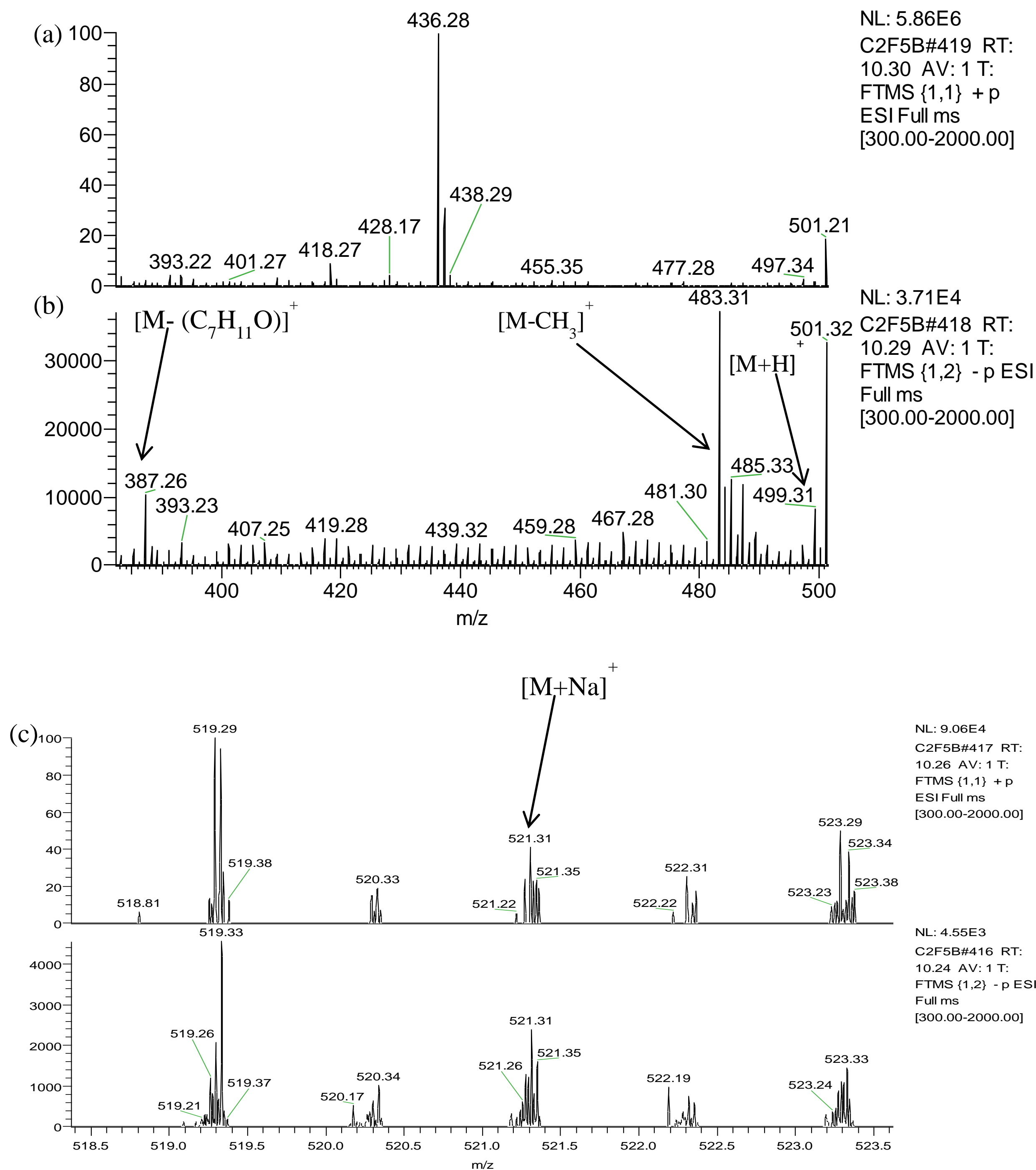


Figure 8: MS fragmentation (a, b and c) patterns of compound **32**

The EIMS and ^{1}H NMR spectral data of compound (**32**) compares well with some of the literature values of similar sterol compounds isolated by Langlois, (2000), Dzeha *et al*., (2003), Kongduang *et al*., (2008), Akhtar *et al.,* (2010) and Gwala, (2011). This further confirmed that compound (**32**) was a phenanthrene carboxylic acid derivative. Previous

phytochemical investigations on the aerial parts of *Zanthoxylum setulosum* (Rutaceae) have yielded the sterol 22-oxo-24-methylcholest-5-en-3β-ol, the triterpene lupeol and other components as cerotic acid (Angulo and Cuca, 2002).

Apart from that, reports of various authors have shown larvicidal activity of *Cestrum* (Rutaceae) plant species in which a steroidal bioactive compound responsible for the mosquitocidal activity was found (Ghosh and Chandra, 2006; Ghosh *et al*., 2008). Also, Rahuman *et al*., (2008) reported that β-sitosterol isolated from *Abutilon indicum* possessed strong larvicidal activity against *Ae. aegypti*, *An. stephensi* and *Cx. quinquefiasciatus* mosquito larvae. Thus related literature and these current findings conclude that β-sitosterol derivatives possess mosquito larvicidal activity.

4.5.2 Structure elucidation of the larvicidal compound 33

The yield obtained from the yellow compound (**33**) was 95.6 mg. In the ^{1}H NMR spectrum of this compound, the resonance caused by the terminal methyl group occurred at δ 0.79. The signal at δ_H 4.07 and δ_H 2.25 are due to the methylene protons of $–CH_2CO$-O- moieties. Rest of the methylene protons resonated at δ_H 1.18 and δ_H 1.55 (methylene protons β - to the ester group). There was presence of a pair of olefinic protons occurring at δ_H 5.27 and δ_H 5.40. The ^{13}C NMR spectrum showed a peak at δ_C 179.09 which corresponds to the carbon of the ester group. Peaks at δ_C 33.94 and 31.92 correspond to the methylene carbons at α- and β- to the ester and alkene groups respectively. The signals observed at δ_C 29.67-22.69 correspond to the remaining methylene carbons. The peak at δ_C 14.11 corresponds to the terminal methyl carbon (see table 14). The present olefinic carbon atoms resonate at δ_C 128.84 and δ_C 130.92 while the signals at δ_C 70.30 and δ_C 65.13 were attributed to carbinolic carbons in positions 9 and 10, allowing the proposal of the molecular formula $C_{16}H_{30}O_4$ for compound (**33**) with two unsaturation indices. The data values obtained from the various spectra for this proposed compound corresponds with the ones reported in literature (Mehta *et al*., 2006; Pateh *et al.,* 2009).

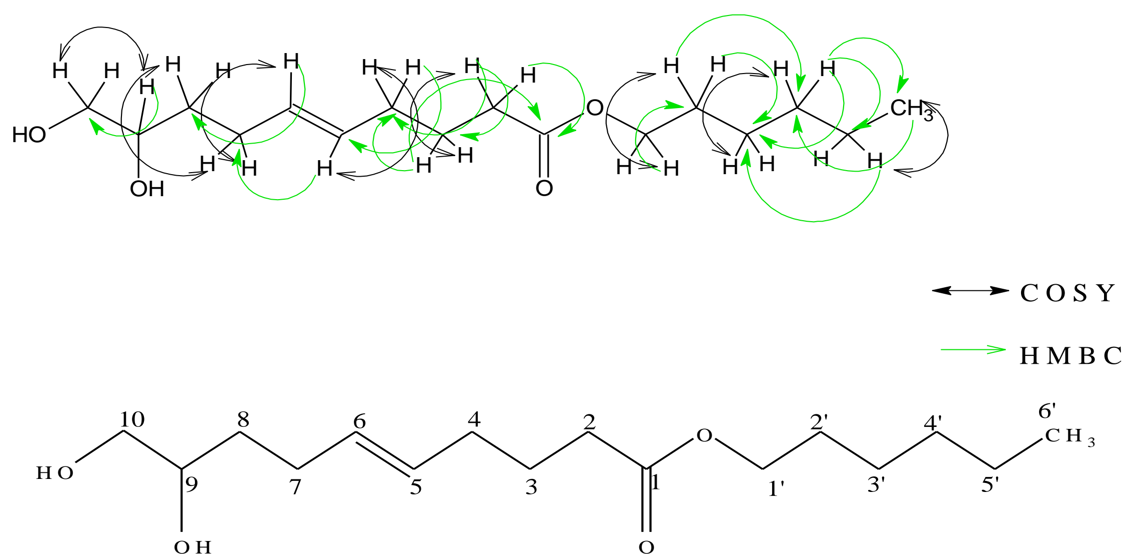
The COSY NMR spectrum established homonuclear correlations between δ_H 1.18 and proton resonances at δ_H 0.79, δ_H 1.55, δ_H 1.96 and δ_H 4.07 ppm showing the proximity of groups bonded together as shown in table 14. Nevertheless, proton multiplicities could not be determined due to the high magnitude of the methylene carbons showing overlapping signals.

Table 14: Summary of 1D and 2D NMR data values for compound **33**

CARBON	^{13}C (δ)	^{1}H (δ)	DEPT	COSY	HMBC	^{13}C (δ) Literature*
1	179.09	-	Cq	-	-	180.0
2	33.94	2.25	CH_2	3	1, 3, 4	34.5
3	24.71	1.55	CH_2	2, 4	1, 2, 4	25.1
4	29.25	1.18	CH_2	2, 3	5	29.2
5	130.92	5.40	CH	4	7	-
6	128.84	5.27	CH	7	8	-
7	29.36	1.18,0.79	CH_2	6, 8	8, 9	29.3
8	31.92	2.25,1.96	CH_2	7	6, 7	32.0
9	70.30	3.47	CH	10	10	-
10	65.13	4.23	CH_2	8, 9	9	-
1’	63.33	4.07	CH_2	2’	2’	-
2’	29.07	1.18,1.55	CH_2	1’,4’	3’, 4’	28.7
3’	29.44	1.18	CH_2	2’, 4’	4’	29.4
4’	29.69	1.18,1.96	CH_2	5’, 6’	3’, 5’, 6’	29.7
5’	22.69	1.55	CH_2	4’	3’	22.7
6’	14.11	0.79	CH_3	4’ 5’	4’, 5’	14.2

*(Mehta *et al*., 2006).

The corresponding COSY and HMBC correlations of compound (**33**) are illustrated below.



Hexyl-9, 10-dihydroxydec-5-enoate (**33**)

A close watch on the electron impact mass spectrometry of this compound could not help in suggesting the position of the molecular ion peak and its fragmentation patterns. This

was due to the fact that both the positive and negative experimental mass ranges used were higher than the actual mass of the compound. Therefore, the proposed compound (**33**) was elucidated using the 1D and 2D NMR spectroscopic techniques only and comparison with literature values.

4.5.3 Structure elucidation of the larvicidal compound 34

Compound (**34**) was also obtained as a yellow substance with 120 mg as the yield. At retention time of 12.52 minutes the Electron Impact MS chromatogram in figure 9 exhibited a weak molecular ion peak at *m/z* 512, which established a molecular formula of $C_{32}H_{48}O_5$, implying nine degrees of unsaturation. Other fragmentation peaks observed were *m/z* 535 $[M+Na]^+$ and *m/z* 551 $[M+K]^+$. Negative electron impact mass spectrometry gave the $[M-H]^+$ at *m/z* 511, *m/z* 483 $[M-CH_2CH_3]^+$, *m/z* 499 $[M-OH]^+$, *m/z* 441$[M-C_5H_{11}]^+$ and there was an evident $[M-C_6H_{10}O_2]^+$ peak displayed at *m/z* 397.

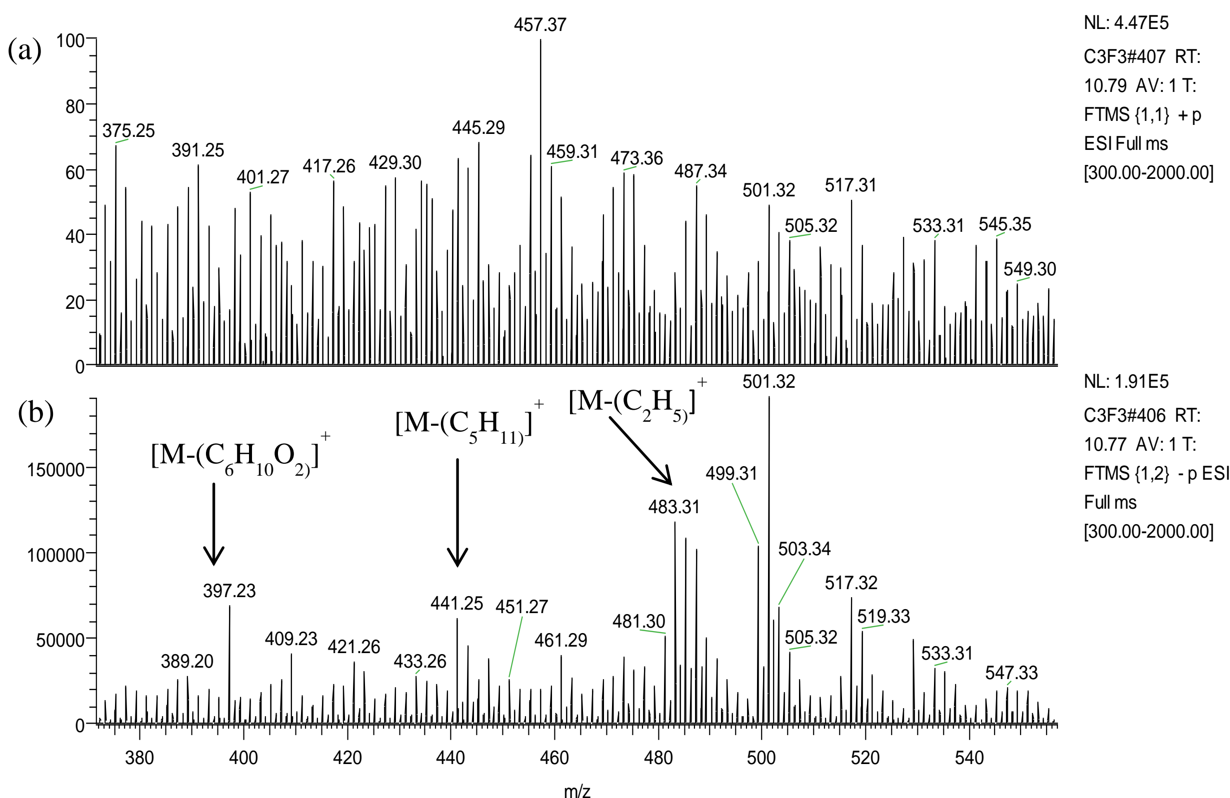


Figure 9: MS fragmentation patterns of compound **34**

The ^{13}C NMR spectra was indicative of a thirty two carbon molecule having three methyls, twelve sp^3 methylenes, three sp^3 methines including two oxymethines, eleven sp^2 methines, three sp^2 quaternary carbons as confirmed the ^{13}C DEPT spectrum(see table 15).

Table 15: Summary of 1D and 2D NMR data for compound **34**

CARBON	^{13}C (δ)	^{1}H (δ)	DEPT	COSY	HMBC	^{13}C (δ) Literature*
1	174.42	-	Cq	-	-	174.0
2	34.09	2.23	CH_2	3	1, 3	33.9
3	27.17	1.96,1.54	CH_2	2, 4	1, 2, 4	26.7
4	130.22	5.27	CH	3	3, 6	129.5
5	123.04	5.27	CH	6	6	-
6	36.67	0.99,1.18	CH_2	5	7	-
7	71.73	3.41	CH	5, 8	8	-
8	31.90	2.23	CH_2	9	7	-
9	29.67	1.18,1.54	CH_2	8	8, 11	-
10	77.27	3.41	CH	9, 11	8	-
11	29.33	1.18	CH_2	10,12	9, 12	-
12	29.12	1.96	CH_2	11, 13	11, 14	29.2
13	131.91	5.27	CH	12	15	130.0
14	128.03	5.27	CH	15	15	128.3
15	24.77	1.54,2.7	CH_2	14, 16	16, 17	25.5
16	127.10	5.24	CH	15	15, 17	128.1
17	129.99	5.27	CH	18	15,19	129.5
18	37.26	1.96	CH_2	17,19	19, 20	-
19	32.76	1.18	CH	18	17	-
20	29.23	1.18,1.54	CH_2	19, 21	19, 21, 22	-
21	14.09	0.79	CH_3	22	19, 20, 22	13.7
22	19.72	0.79,1.96	CH_3	20, 21	20, 21	22.7
OCH_3	51.42	3.6 (s)	-	-	1	51.4
1’	140.06	-	Cq	-	-	-
2’	129.71	7.45	CH	3’	3’	-
3’	128.23	7.65	CH	2’	2’	-
4’	127.71	7.65	CH	2’, 6’	2’, 6’	-
5’	128.23	7.65	CH	6’	6’	-
6’	129.71	7.45	CH	5’	5’	-
7’	39.34	1.96,1.06	CH_2	8’	1’, 6’,9’	-
8’	37.41	1.18,0.99	CH_2	7’	1’,9’	-
9’	170.55	-	Cq	-	-	-

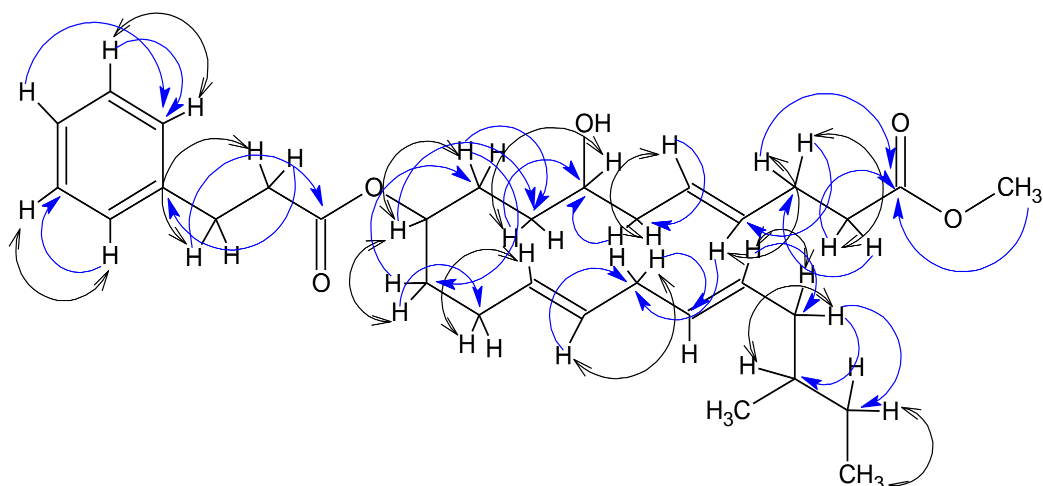
*(Viron *et al*., 2000)

Present in the ^{1}H NMR spectrum were two overlapping signals at δ_H 3.41 and 3.6, their deshielded chemical shifts confirming the presence of two hydroxyl groups at C-7, C-10 and O-CH_3 protons. The phenyl ring protons resonated at δ_H 7.45 (H-2', H-6') and δ_H 7.65 (H-3', 4', 5'). The olefinic protons showed signals at δ_H 5.24 (H-16) and δ_H 5.27 (H-4, 5, 13, 14). The rest of the protons are as shown in column three of table 15.

The ^{13}C NMR spectrum (appendix 32) shows the characteristic functional groupings; the signals at δ_C 174.42(C-1) and δ_C 170.55(C-9') are characteristic of carbonyl carbon groups and this is further confirmed by the absence of the signals in the DEPT NMR spectrum in appendix 33. The eleven signals ranging from δ_C 123.03 to δ_C 131.91 are characteristic of C=C bond. The phenyl ring is characterized by δ_C 140.06 (C-1'), δ_C 129.71 (C-2', C-6'), δ_C 128.23 (C-3', C-5') and δ_C 127.71 (C-4'). The two hydroxylated carbon atoms were observed at δ_C 71.73 and 77.27 and positioned at (C-7) and (C-10) respectively. Similarly, the signal at δ_C 51.42 was characteristic of a methoxy group (OCH_3). The present methyl groups showed signals at δ_C 14.09 (C-21) and δ_C 19.72 (C-22).

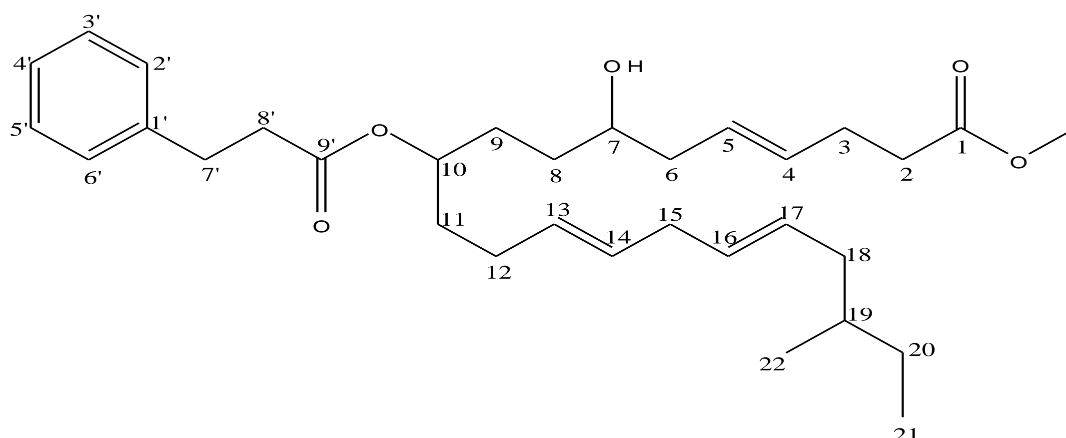
Moreover, its ^{1}H-^{1}H COSY spectrum (appendix 35) revealed correlations of protons 0.79 with 1.18 and 1.54. The other correlations observed were between 5.27 with 1.96 and 2.7. The proton 0.99 couples with 1.18 while 7.65 couples with 7.45. The HMBC spectrum (appendix 36) showed long range couplings between OCH_3 protons at δ_H 3.6 and the carbonyl carbon at position 1 with δ_C 174.42. Apart from that, the methyl groups at positions 21(δ_H 0.79) and 22 (δ_H 0.79, 1.96) indicated correlations with δ_C 32.76(C-19), δ_C 29.23(C-20), δ_C 19.72(C-22) and δ_C 29.23 (C-20), δ_C 14.09(C-21) respectively. The rest of the correlations are as indicated in column six of table 15.

By studying the above ^{1}H, ^{13}C NMR, COSY, HSQC, HMBC and comparison with published spectral data (Viron *et al*., 2000), it was concluded that compound (**34)** was an aliphatic acid methyl ester (specifically Methyl-10-(3-phenylpropanoylxyl)-7-hydroxy-19-methylhenico-4, 13, 16-trienoate). The homonuclear correlations and the long range couplings in the molecule as obtained from the respective spectra are illustrated below.



COSY

HMBC



Methyl-10-(3-phenylpropanoylxyl)-7-hydroxy-19-methylhenico-4, 13, 16-trienoate (**34**)

Since compounds (**33**) and (**34**) fall in the same class of organic compounds, the two are assumed to have similar chemical properties of aliphatic acid esters. On the other hand, account for the activity and toxicity of compound (**34**) is explained by Ghayal *et al*., (2010) in that phenols are generally known to be important source of potent insecticides, fungicides, bacteriocides and herbicides. Also, the fatty acid constituents, linoleic acid and oleic acid isolated from *Dirca palustris* are reported to exhibit mosquitocidal activity against fourth instar *Ae. aegypti* larvae with LD_{50} values of 100 µg/mL at 24 hours, each (Ramsewak *et al*., 2001). In addition, Amin *et al.,* 2012, reported that palmitic acid isolated from a medicinal plant *Acanthus montans* was evaluated for activity against female adults of *A. aegypti*

mosquito and showed 90% mortality at 1.25µg/ml of concentration. Therefore, the above observed activities of these compounds could be attributed to the ester functional group present in the compounds.

Apart from the mentioned compounds (**32, 33** and **34**), the NMR experiments were also conducted on compound (**35**) whose larvicidal activity is reported in section (4.4). The obtained spectra were aberrant in nature and therefore could not facilitate required information for its structure elucidation. Compound (**35**) was obtained with a yield of 138.0 mg.

4.6 Larvicidal assay of the *F. angolensis* essential oil

The yellow essential oil yield of *F. angolensis* leaves was 0.037% (w/w) and the density of concentrated essential oil was determined to be 0.88 g/ml. It was evaluated for larvicidal activity against the third instar mosquito larvae (*An. gambiae)* and found to be active. The data obtained from the larvicidal test was reported in table 16 which includes the percentage mortality of the larvae at various concentrations of the oil prepared from the stock solution. At a dosage of 500 ppm, leaf essential oil induced 100% larval mortality towards *An. gambiae* larvae within 24 hours. When the dosage was decreased to 250 ppm, the larval mortalities of *An. gambiae* larvae against leaf essential oil were 76.7% and at a concentration of 3.9 ppm no larval mortalities were observed.

Table 16: Larvicidal assay results for the *F. angolensis* essential oil

Concentration (ppm)	% Mortality ± SD	LC_{50} (ppm)	LC_{90} (ppm)
3.9	0.0±0.0		
7.8	3.3±5.8		
15.6	3.3±5.8		
31.3	13.3±10.4		
62.5	55.0±17.3	83.7 (63.4 -108.8)	324.0 (236.2 - 497.7)
125.0	55.0±18.0		
250.0	76.7±2.9		
400.0	90.0±13.2		
500.0	100.0±0.0		
650.0	100.0±0.0		
1000.0	100.0±0.0		
Pylarvex (100 ppm)[a]	100.0 ± 0.0		
Spring water + DMSO[b]	0.0 ± 0.0		

[a]Positive control, [b]Negative control.

The LC_{50} and LC_{90} values after 24 hours were also calculated by log probit analysis (95% confidence level) and reported as 83.7 ppm and 324.0 ppm respectively (see table 16). It was noted that some of the fractions from the non-volatiles of *F. angolensis* showed more potent results than its oil. The negative control also showed no activity against the larvae after 24 hours of exposure.

In the earlier reported studies, the larvicidal activity of essential oils from Brazilian plants *Citrus limonia* Osbeck and *Citrus sinensis* Osbeck (Rutaceae) against third instar *A. aegypti* exhibited LC_{50} values of 519 ppm and 538 ppm respectively (Cavalcanti *et al*., 2004). The comparison of these LC_{50} values with the current study indicates that leaf essential oil of *F. angolensis* possessed higher larval toxicities. Additionally, Tiwari *et al.,* (2007) found out that the essential oils obtained from seeds of *Zanthoxylum armatum* (Rutaceae) displayed promising larvicidal activity when tested against *An. stephensi*, *Cx quinquefasciatus* and *A. aegypti* larvae. In general, the bioactivity of the *F. angolensis* oil was comparable with many essential oils reported recently as mosquito larvicides (Cavalcanti *et al*., 2004). Hence the Rutaceae essential oils could be classified to possess toxic effects against mosquito larvae.

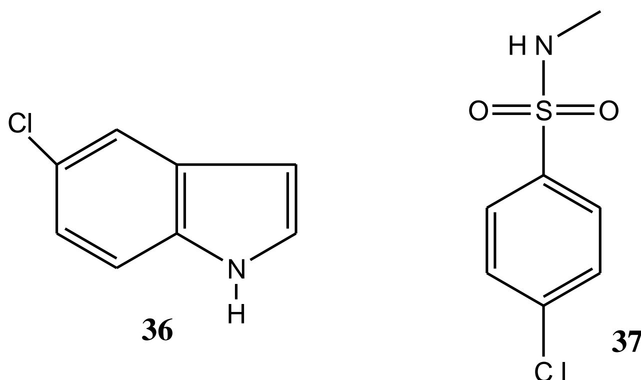
4.7 Chemical composition of the essential oil

4.7.1 Identified compounds

Compounds were identified by comparing the retention times of the peaks on chromatograms and matching with the mass spectra in the computer library databases (Wiley 7N.l, HPCH 1607.L, FLAVORS.L) and in literature (Adams, 2007).

Table 17: GC-MS identified compounds from *F. angolensis* essential oil

Compound No.	Retention time (minutes)	Compound name	Concentration %
36	13.42	5-Chloroindole	0.53
37	23.67	N-methyl-p-chlorobenzenesulfonamide	0.60
38	28.97	Hexadecane	0.32
39	32.10	11-cyclopentylheneicosane	0.06
40	33.54	1-Butyl-2-ethyloctahydro-4,7-epoxy-1H-inden-5-ol	0.21
41	43.08	3-Methylheneicosane	0.14
42	18.70	1,1-Dicyano-2-methyl-4-(p-cyanophenyl) propene	0.46
43	28.97	Hahnfett	0.32



Only eight compounds constituting of 2.64% were positively identified as shown in table 17. According to the mass spectrum of compound (**36**) in figure 10, it had a retention time of 13.42 minutes with 0.53 percentage concentration of the total oil. There was a close match of the electron impact mass spectrum obtained from the oil to that of the libraries in the

database. The most intense peak at *m/z* 151 corresponded to the molecular ion peak of the compound. In addition, a small peak which was adjacent to the molecular ion peak indicated the M+1 peak as it is applicable in mass spectrometry. The compound was therefore concluded to be 5-Chloroindole.

From literature, indoles as a class of organic compounds have been reported to have possessed insecticidal activity (Becher *et al*., 2007). For instance, hapalindoles isolated from cyanobacteria were reported to show larvicidal activity against *Chironomous riparius* in their highest concentration of 37 µM and 26 µM. Also, (Maharani *et al*., 2008) evaluated the insecticidal activity of bufadienolides against the third instar larvae of silkworm and noted that bufadienolides having an orthoacetate, α-pyrole moiety and presence of oxygenated substituent enhanced the insecticidal activity. In addition, compound (**36**) has an α-pyrole group and therefore the mosquito larvicidal activity of the *F. angolensis* essential oil could be attributed to this functional group in the molecule.

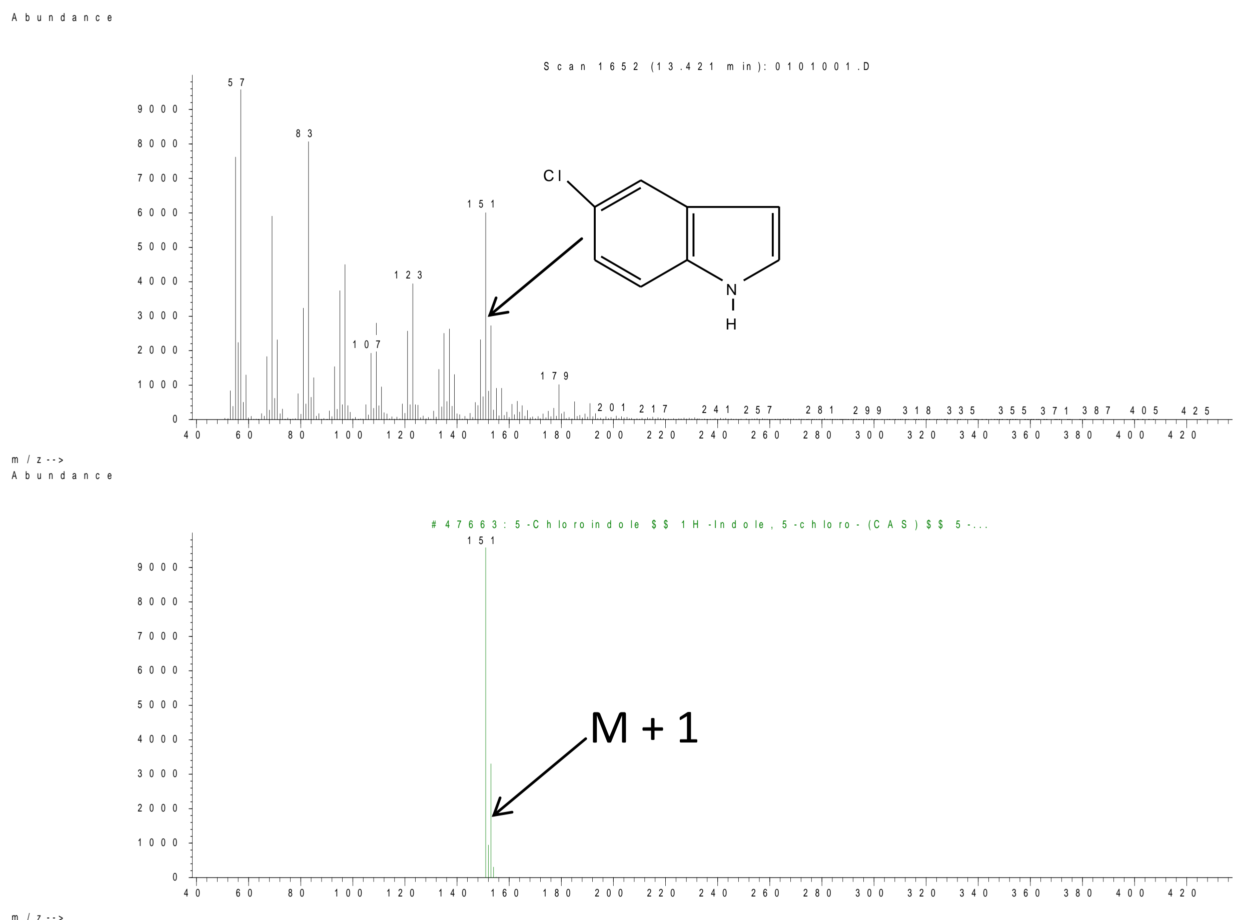


Figure 10: GC-MS Spectrum for compound **36** with comparison to the database

Analysis of GC-MS spectral peaks (appendix 18) at a retention time of 23.67 minutes revealed the amide (**37**) with its concentration in the oil being 0.60 %. The chromatograms obtained from the mass spectra at this retention time showed closely matching peaks to the chromatograms obtained from the library databases (see figure 11). The detection by GC-MS was able to show the major fragmentation peaks at *m/z* 205 which corresponded to the molecular ion peak of the compound. Other fragmentation patterns (figure 11) were observed at *m/z* 175 (M-(NH+CH_3)), *m/z* 141(M-(NH+CH_3+Cl)) and *m/z* 111(M-(NH+CH_3+O_2+Cl). From the above fragmentation peaks, the compound was evident to be N-methyl-p-chlorobenzenesulfonamide.

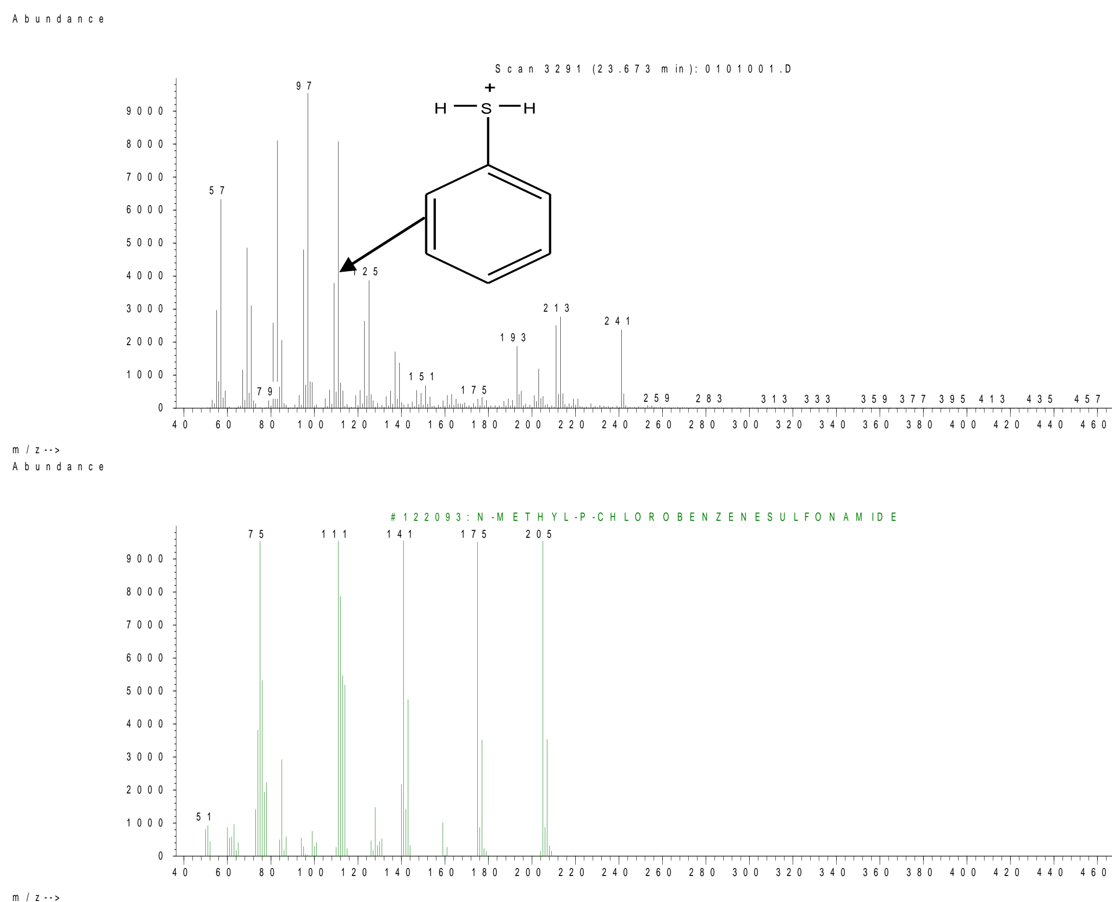
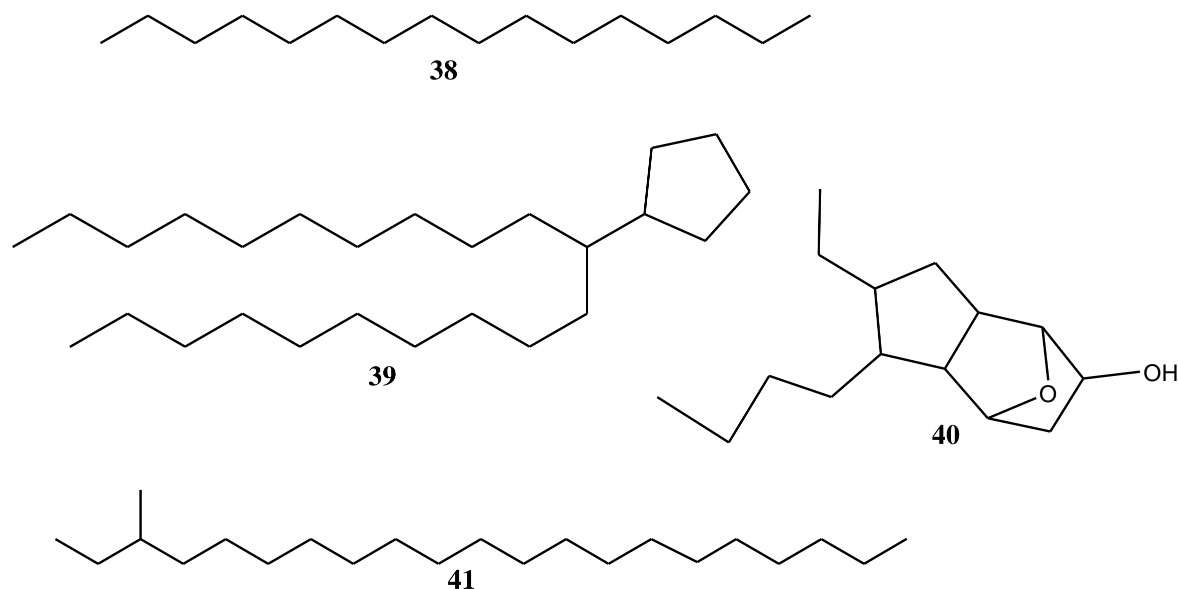


Figure 11: GC-MS Spectrum for compound **37** with comparison to the database

Basing on literature, Batista-pereira *et al*., (2006) reported that amides exert strong toxicity against armyworm insects. The most active amide synthesized as N-[3-(3', 4'-methylenedioxyphenyl)-2-(E)-propenoyl]piperidine was evaluated to possess an LD_{50} of 1.07 ppm against *Spodoptera frugiperda* larvae. Also, the genus *Zanthoxylum* which belongs to the family Rutaceae is characterized chemically by the frequent accumulation of olefinic alkamides (unsaturated aliphatic acid amides) and there is biogenetic capacity derived from the condensation of fatty acids such as linolenic and linoleic acids with isobutyl amines (Mester, 1983). Biologically, the isobutyl amides have been shown to have strong insecticidal properties (Chaaib, 2004; Adesina, 2005). In account, the compound could have contributed to the documented activity of the essential oil.



The saturated aliphatic hydrocarbons hexadecane (**38**), 11-cyclopentylheneicosane (**39**) and 3-methylheneicosane (**41**) were recorded at 28.97, 32.10 and 43.08 minutes as their retention times from the GC-MS spectra respectively. Their percentage concentrations were also recorded as 0.32, 0.06 and 0.14 respectively. The GC-MS chromatograms obtained for these compounds indicated a close match to the spectra present in the library databases (see appendices 14, 15 and 16). Fragmentation patterns for some ions in the spectrum are also shown. From previous literature, the alkane octacosane isolated from *Moschosma polystachyum* has been reported to have shown significant larvicidal activity against *Culex*

quinquefasciatus mosquito with LC_{50} value of 7.2±1.7 ppm (Rajkumar, 2004). Consequently, reports by Seenivasagan *et al*., (2009), explains that n-heneicosane identified and characterized from the larval cuticle of *A. aegypti* have the principle role as an attractant to the gravid female mosquitoes to oviposit in treated substrates among other chemical components.

The continuous analysis of *F. angolensis* essential oil also led to identification of the following compounds 1-butyl-2-ethyloctahydro-4,7-epoxy-1H-inden-5-ol (**40**), 1,1-dicyano-2-methyl-4-(*p*-cyanophenyl) propene (**42**) and hahnfett (**43**) with their retention times recorded as 33.54, 18.70 and 28.97 minutes respectively. Their concentrations were recorded as 0.21, 0.46 and 0.32 percent respectively and they have not been reported previously to possess insecticidal activity.

4.7.2 Partially identified compounds

Analysis of the *F. angolensis* oil revealed that a higher percentage of the essential oil contained partially and unknown compounds some of which had mass spectra patterns related to those of known compounds. These compounds include: Cis-1- ethinyl-2-methyl-1-cyclohexanol, 2-Butyldecahydronaphthalene, 1-(4-Chlorophenyl)-5-(2-diethylaminoethenyl)-1H-tetrazole and N-isobutyl-2-quinolone. A total of forty seven compounds (67.83%) were classified to be in this category (see appendix17) while 29.0% were classified as unknowns. Thus the activity of the oil against the mosquito larvae may be attributed to the additive or synergistic effect of many or some of the constituents in the oil. Such an effect has been previously observed with some essential oils where their activity was due to the combination of the major constituents, none of which was found to exhibit significant activity, individually (Papachristos *et al*., 2004; Omolo *et al*., 2005).

CHAPTER FIVE
CONCLUSIONS AND RECOMMENDATIONS

5.1 Conclusions

The chloroform crude extract obtained from *F. angolensis* leaves was found to possess larvicidal activity against the malaria vector *An. gambiae* larvae. From its larvicidal assay results, the crude extract had LC_{50} value of 264.8 ppm and LC_{90} value of 766.9 ppm. This clearly indicated that there were secondary metabolites in the crude extract that contributed to this activity.

The essential oil, which was the volatile component obtained from the leaves of *F. angolensis* was also found to have larvicidal activity against the third instar *An. gambiae* larvae. It exhibited LC_{50} value of 83.7 ppm and an LC_{90} value of 324.0 ppm. From the observed activity, it was also evident that the oil had chemical components which were responsible for the activity. Only 2.64% of the total oil concentration was positively identified from the GC-MS spectra. This implied that the oil contained mainly new compounds whose mass spectra could not be found in the GC – MS databases used.

Bioassay-guided fractionation of the non-volatile component *of F. angolensis* leaf extract (section 3.4) afforded four compounds of which three were successfully identified by spectroscopic analyses including MS, NMR and by comparison with published data. The identified compounds were classified as a phenanthrene carboxylic acid derivative (**32**) and aliphatic acid esters (**33)** and **(34**). Furthermore, the potency of the compounds was observed more in compound (**34**) (LC_{50} = 144.4 ppm; LC_{90} = 259.4 ppm) which had the least retardation factor (RF) values and having a phenylpropanoyloxy substituent group. The least potent was compound (**35**) with LC_{50} value of 423.0 ppm and LC_{90} value of 957.0 ppm.

Results from this study indicate that the three naturally occurring larvicidal compounds and the essential oil of *F. angolensis* leaves could have potential applicability in the control of the malaria vector - *An. gambiae* mosquitoes. This study underscores the fact that bioactive constituents of plants hold potential to be employed as useful agents in controlling mosquito vectors and hence contributing to stem the spread of malaria. Such findings also offer an opportunity for developing newer more selective, biodegradable and natural larvicidal compounds or lead compounds for the development of more potent

mosquito larvicides as alternatives to rather expensive and environmentally hazardous inorganic insecticides.

5.2 Recommendations

In relation to this study, the following recommendations were made:

i. That toxicity tests towards non-target organisms and field evaluation tests to be carried out for the three non-volatile compounds and the essential oils.
ii. That further structure determination experiments should be done on the unidentified components of the essential oil and non-volatile extracts.
iii. That mode of action of each larvicidal non-volatile compound and the essential oil be investigated.

REFERENCES

Adams, R. P. (2007). *Identification of Essential Oil Components by Gas Chromatography/Mass Spectrometry*, 4th edn. Allured publishing corporation: Carol Stream. Illinois. USA.

Adesina, S. K. (2005). The Nigerian *Zanthoxylum*: Chemical and Biological Values. *African Journal of Traditional, Complementary and Alternative medicines*. **2**: 282-301.

Akhtar, P., Ali, M., Sharma, P. M., Farooqi, H. and Khan, N. H. (2010). Phytochemical investigation of fruits of *Corylus colurna* Linn. *Journal of phytology*. **2**: 89-100.

Amaral, F. M. M., Ribeiro, M. N. S., Barbosa-Filho, J. M., Reis, A. S., Nascimento, R. F. and Macedo, R. O. (2006). Plants and chemical constituents with giardicidal activity. *Brazilian Journal of Pharmacognosy.* **16**: 696-720.

Amin, E., Radwan, M. M., El-Hawary, S. S., Fathy, M. M., Mohammed, R., Becne, J. J. and Khan. I. (2012). Potent Insecticidal Secondary Metabolites from the Medicinal Plant *Acanthus montanus. Records of Natural Products*. **6**: 301-305.

Angulo, O. A. A, and Cuca, S. L. E. (2002). Nuevo esterol y otros constituyentes de *Zanthoxylum setulosum*, Rutaceae. *Revista Colombiana de Química*. **31**: 87 - 93.

Ansari, M. A., Razdan, R. K., Tandan, M. and Vasudevan, P. (2000). Larvicidal and repellent actions of Dalbergia sisoo Roxb.(*F. Leguminosae*) oil against mosquitoes. *Bioresource Technology*. **73**: 207-211.

Arivoli, S. and Tennyson, S. (2011). Studies on the mosquitocidal activity of *Murraya koenigii* (L.) Spreng (Rutaceae) leaf extracts against *Aedes aegypti*, *Anopheles stephensi* and *Culex quinquefasciatus* (Diptera: Culicidae). *Asian Journal of Experimental Biological Science.* **2**: 721–730.

Bakkali, F., Averbeck, S., Averbeck, D. and Idaomar, M. (2008). Biological effects of essential oils – A review. *Food Chemistry Toxicology*. **46**: 446–475.

Banks, K. E., Hunter, D. H. and Wachal, D. J. (2005). Chlorpyrifos in surface waters before and after a federally mandated ban. *Environment International*. **31**: 351- 356.

Baraza, L. D., Joseph, C. C., Munissi, J. J. E., Nkunya, M. H. H., Arnold, N., Porzel, A. and Wessjohann, L. (2008). Antifungal rosane diterpenes and other constituents of *Hugonia castaneifolia*. *Phytochemistry.* **69**: 200-205.

Barbosa-Filho, J. M., Medeiros, K. C. P., Diniz, M. F. F. M., Batista, L. M., Athayde-Filho, P. F., Silva, M. S., Da-Cunha, E. V. L., Almeida, J. R. G. S. and Quintans-Júnior, L. J. (2006). Natural products inhibitors of the enzyme acetylcholinesterase. *Revista Brasileira de Farmacognosia*. **16**: 258-285.

Batista-pereira, L. G., Castral, T. C., Da Silva, T. M., Amaral, B. R., Fernandes, J. B., Vieira, P. C., Fatima, M. and Da Silva, G. F. (2006). Insecticidal activity of synthetic amides on *Spodoptera frugiperda*. *Naturforsch*. **61**: 196-202.

Becher, P. G., Keller S., Jung, G., Su¨ssmuth, R. D. and Ju¨ ttner, F. (2007). Insecticidal activity of 12-epi-hapalindole J isonitrile. *Phytochemistry*. **68**: 2493–2497.

Breman, J. G., Alilio, M. S. and Mills, A. (2004). Conquering the Intolerable Burden of Malaria: What's New, What's Needed: A Summary. *American Journal of Tropical Medicine and Hygiene.* **71**: 1–15.

Brown, A. W. A. (1986). Insecticide resistance in mosquitoes: a pragmatic review. *Journal of the American Mosquito Control Association.* **2**: 123-140.

Bruce-Chwatt, L. J. (1985). Essential Malariology, 2nd ed. John Wiley & Sons, Inc., New York. pp. 45-49.

Cavalcanti, E. S. B., Morais, S. M., Lima, M. A. A. and Santana, E. W. P. (2004). Larvicidal activity of essential oils from Brazilian plants against *Aedes aegypti* Larvae. *Memorias Instituto Oswaldo Cruz.* **99**: 541–544.

CDC - Centers for Disease Control and Prevention and Division of Parasitic Diseases (ZVED). "Vector Control and CDC Malaria." (2008). Available at: http://www.cdc.gov/malaria/control_prevention/vector_control.htm (accessed on 28th May, 2011 at 9.23 am).

Chaaib, K. F. (2004). Investigation Phytochimique d'une Brosse à Dents Africaine *Zanthoxylum zanthoxyloides* (Lam.) Zepernick et Timler (Syn. *Fagara zanthoxyloides* L.) (Rutaceae). Thèse de doctorat. pp. 11-44.

Cheng. S. S., Chang, H. T., Chang, S. T., Tsai, K. H. and Chen, W. J. (2003). Bioactivity of selected plant essential oils against the yellow fever mosquito *Aedes aegypti* larvae. *Bioresource Technology.* **89:** 99-102.

Cheng, S. S., Huang, C. G., Chen, Y. J., Yu, J. J., Chen, W. J. and Chang, S. T. (2009). Chemical compositions and larvicidal activities of leaf essential oils from two *Eucalyptus* species. *Bioresource Technology.* **100**: 452–456.

Chowdhury, N. Ghosh, A. and Chandra, G. (2008)**.** Mosquito larvicidal and antimicrobial activity of protein of *Solanum villosum* leaves. *BMC Complementary and Alternative Medicine*. **8**: 10-11.

Ciccia, G., Coussio, J. and Mongelli, E. (2000). Insecticidal, repellent activity against *Aedes aegypti* larvae of some medicinal South American plants. *Journal of Ethnopharmacology and Pharmaceuticals.* **71**: 267-269.

Cortez, L. E. R., Cortez, D. A. G., Ferreira, A. G., Vieira, P. C., Silva, M. F. G. F. and Fernandes, J. B. (2006). Constituintesquímicos de *Almeideacoerulea* (Nees& Mart.) A. St.-Hil. Rutaceae. *Revista Brasileira de Farmacognosia*. **16**: 164-169.

Dolabela, M. F., Oliveira, S. G., Nascimento, J. M., Peres, J. M., Wagner, H., Póvoa, M. M. and Oliveira, A. B. (2008). *In vitro* antiplasmodial activity of extract and constituents from *Esenbeckia febrifuba*, a plant traditionally used to treat malaria in the Brazilian Amazon. *Phytomedicine.* **15**: 367-372.

Dzeha, T., Jaspars, M. and Tabudravu, J. (2003). Clionasterol, a Triterpenoid from the Kenyan Marine Green Macroalga Halimeda macroloba. *Western Indian Ocean Journal of Marine Science*. **2**: 157–161.

Ebrahimi, A., Moaveni, P., Dashtbozorg, A. T. and Farahani, H. A. (2011). Effects of temperature and varieties on essential oil content and quantity features of chamomile. *Journal of Agricultural Extension and Rural Development.* **3**: 19-22.

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

Fradin, M. S. and Day, J. F. (2002). Comparative efficacy of insect repellents against mosquito bites. *The New England Journal of Medicine*. **347**: 13–18.

Furtado, R. F., Lima, M. G. A., Andrade-Neto, M., Bezerra, J. N. S. and Silva, M. G. V. (2005). Atividade larvicida de óleosessenciais contra *Aedes aegypti* L. (Diptera: Culicidae). *Neotropical Entomology*. **34**: 843–847.

Ghayal, N., Padhye, A. and Dhuma, K. (2010). Larvicidal activity of invasive weeds *cassia uniflora* and *Synedrella nodiflora. International Journal of Pharma and Bio Sciences.* **1:** 1-10.

Ghosh, A. and Chandra, G. (2006). Biocontrol efficacy of *Cestrum diurnum* L. (Solanaceae: Solanales) against the larval forms of *Anopheles stephensi*. *Natural Products Research*: **20**: 371-79.

Ghosh, A., Chowdhury, N. and Chandra, G. (2008). Laboratory evaluation of phytosteroid compound of mature leaves of Day Jasmine (Solanaceae: Solanales) against larvae of *Culex quinquefasciatus* (Diptera: Culicidae) and nontarget organisms. *Parasitology Research*: **103**: 271-77.

Gutie' rreza, L. A., Nelson, N., Luz, M. J., Carlos, M. S. L., Jan, E. C. and Margarita, M. C. (2008). Natural infectivity of anopheles species from the pacific and atlantic regions of colombia. *Acta Tropica.* **107**: 99-105.

Gwala, E. P. (2011). The anti-platelet aggregation activity of *Rapanea melanophloeos*-A zulu medicinal plant. Msc Thesis University of Zululand. pp. 54-56.

Harish, K. H., Prashanth, K. J. and Shruthi, S. D. (2010). Pharmacognostic and phytochemical studies on the leaves of *murraya koenigii* (L) spreng. *Pharmacophore* . **1**: 231-238.

Harve, G. and Kamath, V. (2004). Larvicidal activity of plant extracts used alone and in combination with known synthetic larvicidal agents against *Aedes aegypt*. *Indian Journal of Experimental Biology.* **42**: 1216-1219.

Hemingway, J. (2004). Taking aim at mosquitoes. *Nature*. **430**: 936 - 937.

http://www.stopmalarianow.org/malaria-in-kenya.html**.** Towards a Malaria-free Kenya. (accessed on June 9th, 2012 at 11.23 am).

IDRC – International Development Research Cenre. (2008). Case study Mexico (malaria), fighting malaria without DDT, Better management of environments a key factor to disease control.

ICMR - Indian Council of Medical Research. (2003). Prospects of using herbal products in the control of mosquito vectors. *ICMR Bulletin.* **33:** 377 - 491.

Isman, M. B. (1997). Neem and other Botanical insecticides: Barriers to commercialization. *Phytoparasitica*. **25**: 339–344.

James, A. A. (1992). Mosquito molecular genetics: the hands that feed bite back. *Journal of Science.* **257**: 37–38.

Jeffery, G. M. (1984). The role of chemotherapy in malaria control through primary health care: constraints and future prospects. *Bulletin of the World Health Organization*. **62**: 49-53.

Joseph, C. C., Ndoile, M. M., Malima, R. C. and Nkunya, M. H. H. (2004). Larvicidal and mosquitocidal extracts, a coumarin, isoflavonoids and pterocarpans from *Neorautanenia mitis*. *Transactions of the Royal Society Tropical Medicine and Hygiene*. **98**: 451-455.

Joseph, P. M. (2005). Quinoline, quinazoline and acridone alkaloids. *Natural Products Report.* **22**: 627-645.

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

Keiser family foundation. (2007). http://www.globalhealthreporting.org. (accessed on July 19th, 2011 at 10.05 pm).

Killeen, G. F., Fillinger, U. and Knols, G. J. (2002). Advantages of larval control for African malaria vectors: low mobility and behavioral responsiveness of immature mosquito stages allow high effective coverage. *Malaria Journal.* **1:** 1–7.

Kishore, N., Mishra, B. B., Tiwari, V. K. and Tripathi, V. (2010). Difuranonaphthoquinones from *Plumbago zeylanica* roots. *Phytochemistry Letters Journal.* **3**: 62-64.

Kiszewski, A., Mellinger, A., Spielman, A., Malaney, P., Sachs, S. E. and Sachs, J. (2004). A global index representing the stability of malaria transmission. American Journal of Tropical Medicine and Hygiene. **70**: 486-498. In Global Distribution (Robinson Projection) of Dominant or Potentially Important Malaria Vectors. Retrieved April, 2013. http://www.cdc.gov/Malaria/about/biology/mosquitoes/map.html.

Kiran, S. R., Bhavani, K., Devi, P. S., Rao, B. R. R. and Reddy, K. J. (2006). Composition and larvicidal activity of leaves and stem essential oils of *Chloroxylon swietenia* DC against *Aedes aegypti* and *Anopheles stephensi. Bioresource Technology*. **97**: 2481–2484.

Kiran, S. R. and Devi, P. S. (2007). Evaluation of mosquitocidal activity of essential oil and sesquiterpenes from leaves of *Chloroxylon swietenia* DC. *Parasitology Research.* **101**: 413-415.

Klassen, W. (2009). Development of the Sterile Insect Technique for African malaria vectors. *Malaria Journal*. **8:** 1-10.

Knols, B. G., Njiru, B. N., Mathenge, E. M., Mukabana, W. R., Beier, J. C. and Killeen, G. F. (2002). Malaria sphere; A greenhouse enclosed simulation of a natural *Anopheles gambiae* (Diptera: Culicidae) ecosystem in western Kenya. *Malaria Journal*. **1**: 19 - 20.

Kongduang, D., Wungsintaweekul, J. and De-Eknamkul, W. (2008). Biosynthesis of β-sitosterol and stigmasterol proceeds exclusively via the mevalonate pathway in cell suspension cultures of *Croton stellatopilosus*. *Tetrahedron Letters*. **49**: 4067-4072.

Kroken, S. (2009). Evolution of Secondary Metabolism in Microbes. *Annual Review of Microbiology*. **44**: 395 - 427.

Lacey, L. A. (2007). Bacillus thuringiensis serovariety israelensis and Bacillus sphaericus for mosquito control. *Journal of the American Mosquito Control Assocation*. **23**: 133 - 163.

Langlois, A. (2000). The chemical investigation of four medicinal plants, University of KwaZulu-Natal, South Africa. MSc Thesis. pp. 49 – 50.

Lemmens, R. H. M. J. (2008). *Fagaropsis angolensis* (Engl.) Dale. Available at http://database.prota.org/PROTAhtml/Fagaropsis%20angolensis_En.html. (accessed January 25th, 2012 at 4.20 pm).

Lengeler, C. and Snow, R. W. (1996). From efficacy to effectiveness: insecticide-treated bednets in Africa. *Bulletin of the World Health Organization*. **74**: 325-332.

Lengeler, C. and Sharp, B. (2003). Indoor residual spraying and insecticide treated nets. In: Reducing malaria's burden: evidence of effectiveness for decision makers. Washington: Global Health Council. pp. 17–24.

Li, Y., Xu, C., Zhang, Q., Liu, J. Y. and Tan, R. X. (2005). *In vitro* anti-*Helicobacter pylori* action of 30 Chinese herbal medicines used to treat ulcer diseases. *Journal of Ethnopharmacology*. **98**: 329-333.

Linares, S., Gonzalez, N., Gómez, E., Usubillaga, A. and Darghan, E. (2005). Effect of the fertilization, plant density and time of cutting on yield and quality of the essential oil of *Cymbopogon citrates* Stapf. *Revist de la Facultad de AgronĆa LUZ*. **22**: 247-260.

Lorenzi, H. and Matos, F. J. A. (2002). Medicinal Plants in Brazil**:** Exotic and Native. Nova Odessa: *Instituto Plantarum.* **176**: 137-142.

Mafezoli, J., Vieira, P. C., Fernandes, J. B., Silva, M. F. G. F. and Albuquerque, S. (2000). *In vitro* activity of Rutaceae species against the trypomastigote form of *Trypanosoma cruzi. Journal of Ethnopharmacology.* **73**: 335-340.

Maharani, R., Fajriah, S., Hardiawan, R. and Supratman. U. (2008). Insecticidal bufadienolides from the leaves of *Kalanchoe daigremontiana* (Crassulaceae). *Proceeding of The International Seminar on Chemistry Report*. pp. 236-239.

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

Matasyoh, J. C., Talontsi, F. M., Ngoumfo, R. M. and Chepkorir, R. (2010). Mosquito larvicidal activity of alkaloids from *Zanthoxylum lemairei* against the malaria vector *Anopheles gambiae*. *Pesticide Biochemistry and Physiology journal.* **99**: 82–85.

Mehta, B. K., Gupta, M. and Verma, M. (2006). Steroid and aliphatic esters from the seeds of *Nigella sativa*. *Indian Journal of chemistry*. **45**: 1567-1571.

Mester, I. (1983). Structural Diversity and Distribution of Alkaloids in the Rutales, in, "Chemistry and Chemical Taxonomy of the Rutales: (P. G. Waterman and M. F. Grundon Eds.) Academic Press, London. pp. 31-96.

Milam, C. D., Farris, J. L. and Wilhide, J. D. (2000). Evaluating mosquito control pesticides for effect on target and non-target organisms. *Archives of Environmental Contamination and Toxicology*. **39**: 324-328.

MOH - Ministry of Health. (2006). Malaria in Kenya at a glance. Available at http://www.kemri.org/index.php/help-desk/search/diseases-a-conditions/29-malaria/113-kenya-malaria-fact-sheet (accessed on June 13th, 2012 at 9.11 am).

Mohamed, A. A., El-Emary, G. A. and Ali, H. F. (2010). Influence of some citrus essential oils on cell viability, glutathione-S-transferase and lipid peroxidation in *Ehrlich ascites* carcinoma cells. *Journal of American Science*. **6**: 820-826.

Morais, S. M., Facundo, V. A., Bertini, L. M., Cavalcanti, E. S. B., Anjos-Junior, J. F., Ferreira, S. A., Brito, E. S. and Souza-Neto, M. A. (2007). Chemical composition and

larvicidal activity of essential oils from *Piper* species. *Biochemical System Ecology*. **35**: 670–675.

Moshi, M. J., Kagashe, G. A. B. and Mbwambo, Z. H. (2005). Plants used to treat epilepsy by Tanzanian traditional healers. *Journal of Ethnopharmacology.* **97**: 327-336.

Moore, S. J., Lenglet, A. and Hill, N. (2002). Field evaluation of three plant-based insect repellents against malaria vectors in Vaca Diez province, the Bolivian Amazon. *Journal of American Mosquito control Association*. **18**: 107-110.

Nakagawa, H., Takaishi, Y., Tanaka, N., Tsuchiya, K., Shibata, H. and Higuti, T. (2006). Chemical constituents from the peels of *Citrus sudachi*. *Journal of Natural Products.* **69**: 1177-1179.

Navneet, K., Bhuwan, B., Mishra, V., Tiwari, K. and Tripathi, V. (2011). A review on natural products with mosquitosidal potentials. *Mosquitosidal natural products.* **2011**: 335-365.

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*. *African Journal of Biotechnology*. **5**: 761-765.

OECD - Organisation for Economic Co-operation and Development. (2010). International maternal newborn and Child Health. The African Partnership Program. *Phytoparasitica*. **25:** 339-340.

Oliva, C. F., Benedict, M. Q., Lempérière, G. and Gilles, J. (2011). Laboratory selection for an accelerated mosquito sexual development rate. *Malaria Journal.* **10**: 135-136.

Omolo, M. O., Okinyo, D., Ndiege, I. O., Lwande, W. and Hassanali, A. (2005). Fumigant toxicity of the essential oils of some African plants against *Anopheles gambiae sensu stricto*. *Phytomedicine.* **12***:* 241–246.

Papachristos, D. P. and Stamopoulos, D. C. (2004). Fumigant toxicity of three essential oils on the eggs of *Acanthoscelides obtectus* Say (Coleoptera: Bruchidae). *Journal of Stored Products Research.* **40**: 517-525.

Papachristos, D. P., Karamanoli, K. I., Stamopoulos, D. C. and Menkissoglu-Spiroudi, U. (2004).The relationship between the chemical composition of three essential oils and

their insecticidal activity against *Acanthoscelides obtectus* Say. *Pest Management Science*. **60***:* 514–520.

Pateh, U. U., Haruna , A. K., Garba, M., Iliya, I., Sule, I. M., Abubakar, M. S. and Ambi A. A. (2009). Isolation of stigmasterol, β-sitosterol and 2-hydroxyhexadecanoic acid methyl ester from the rhizomes of *Stylochiton lancifolius* pyer and Kotchy (Araceae). *Nigerian Journal of Pharmaceutical Sciences*. **8**: 19 – 25.

Phasomkusolsil, S. and Soonwera, M. (2010). Potential larvicidal and pupacidal activities of herbal essential oils against *Culex quinquefasciatus* Say and *Anopheles minimus* (Theobald). *Southeast Asian Journal of Tropical Medicine and Public Health*. **41**: 1342-1351.

Pitasawat, B., Champakaew, D., Choochote, W., Jitpakdi, A., Chaithong, U., Kanjanapothi, D., Rattanachanpichai, E., 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.

Puyvelde, V. L., Dekimpe, N., Mudaharanwa, J. P., Gasiga, A., Schamp, N., Declerq, J. P. and Meerssche, V. M. (1987). Flavonoids and isoflavonoids from *Tephrosia fulvinervis* and *Tephrosia pentaphylla. Journal of Natural Products*. **50**: 349-351.

Radhika, D., Ramathilaga, A. and Prabu, C. S. (2011). Evaluation of larvicidal activity of soil microbial isolates (Bacillus and Acinetobactor *Sp*.) against *Aedes aegypti* (Diptera: Culicidae) - the vector of Chikungunya and Dengue. *Proceedings of the International Academy of Ecology and Environmental Sciences*. **1**: 169-178.

Rahuman, A. A., Gopalakrishnan, G., Venkatesan, P. and Geetha, K. (2008). Mosquito larvicidal activity of oleic and linoleic acids isolated from *Citrullus colocynthis* (Linn.) Schrad. *Parasitology Research*. **102**: 981-983.

Rajkumar, S. and Jebanesan, A. (2004). Mosquitocidal activities of octacosane from *Moschosma polystachyum* Linn (Lamiaceae). *Journal of Ethnopharmacology*. **90**: 87-89.

Ramsewak, R. S., Nair, M. G., Murugesan, S., Mattson, W. J. and Zasada, J. (2001). Isolation and characterization of aromatase inhibitors from Brassaiopsis. *Journal of Agricultural Food Chemistry.* **49**. 5852-5856.

Roy, M. K., Thalang, V. N., Trakoontivakorn, G. and Nakahara, K. (2005). Mahanine, a carbazole alkaloid from *Micromelum minutum*, inhibits cell growth and induces apoptosis in U937 cells through a mitochondrial dependent pathway. *Brazilian Journal of Pharmacognosy.* **20**: 502-505.

Rutledge, L. C., Gupta. R. K., Mehr, Z. A., Buescher, M. D. and Reifenrath. W. G. (1996). Evaluation of controlled-release mosquito repellent formulations. *Journal of American Mosquito control Association*. **12:** 39-44.

Sagˇ lam, H., Gözler, T., Gözler, B. (2000). A new prenylated arylnaphthalene lignan from *Haplo - phyllum myrtifolium*. *Fitoterapia.* **74**: 564-569.

Sears, R. (1996). An ethnobotanical survey of insect repellents in Brazil**.** *TRI News*. **15:** 8-10.

Seenivasagan, T., Sharma, K. R., Sekhar, K., Ganesan, K., Prakash, S. and Vijayaraghavan R. (2009). Electroantennogram, flight orientation, and oviposition responses of *Aedes aegypti* to the oviposition pheromone *n* –heneicosane. *Parasitology Research.* **104**: 827-833.

Service, M. W. and Townson, H. (2002). The Anopheles vector Essential Malariology, 4thedn (ed. by D. A. Warrell and H. M. Gilles). pp. 85 – 106. Arnold Publishers, U.K.

Severini, C., Rom, R., Marinucci, M. and Rajmond, M. (1993). Larvicidal effects of various Euro-Asiatic plants against *Culex quinquefasciatus* larvae. *American Journal of mosquito control*. **9**: 164–165.

Shaalan, E., Canyon, D., Faried, M. W., Abdel-Wahab, H. and Mansour, A. (2005). A review of botanical phytochemicals with mosquitocidal potential. *Environment International*. **31**: 1149-1166.

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, (eds. G.K. Manna and B.B. Jain). 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. *Memorias Instituto Oswaldo Cruz, Rio de Janeiro*. **99**: 115-118.

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**: 214–217.

Soderlund, D. M. and Knipple, D. C. (2003). The molecular biology of knockdown resistance to pyrethroid insecticides. *Journal of Insect Biochemistry and Molecular Biology.* **33:** 563–577.

Srisilam, K., and Veersham, C. (2003). Antimalarials of plant origin. In Nishan, I. and Khanu, A. (Eds) Role of Biotechnology in medicinal and aromatic plants. *Journal of Ethnopharmacology.* **7**: 17-47.

Steketee, R. W., Nahlen, B. L., Parise, M. E. and Menendez, C. (2001). The Burden of Malaria in Pregnancy in Malaria-Endemic Areas. *American Journal of Tropical Medicine and Hygiene.* **64**: 28–35.

Thomas, T. G., Rao, S. and Lal, S. (2004). Mosquito larvicidal properties of an indigenous plant, *Ipomoea cairica* Linn. *Japan Journal of Infectious Diseases.* **57**: 176-177.

Tiwari, M., Naik, S. N., Tewary, D. K., Mittal, P. K. and Yadav, S. (2007). Chemical composition and larvicidal activities of the essential oil of *Zanthoxylum armatum* DC (Rutaceae) against three mosquito vectors. *Journal of Vector Borne Disease.* **44**: 198–204.

Vatandoost, H. and Vaziri, M. (2001). Larvicidal activity of neem extract (*Azadirachta indica*) against mosquito larvae in Iran. *Pestology*. **25**: 69-72.

Venketachalam, M. R. and Jebasan, A. (2001). Larvicidal activity of *Hydrocotyl javanica* Thunb (Apiaceae) extract against *Cx. quinquefasciatus*. *Journal of Experimental Zoology India.* **4**: 99-101.

Viron, C., Saunois, A., Andre, P., Perly, B. and Lafosse, M. (2000). Isolation and identification of unsaturated fatty acid methyl esters from marine micro-algae. *Analytica Chimica Acta.* **409**: 257-266.

Wandscheer, C. B., Duque, J. E., da Silva, M. A. N., Fukuyama, Y., Wohlke, J. L., Adelmann, J. and Fontana, J. D. (2004). Larvicidal action of ethanolic extracts from fruit endocarps of *Melia azedarach* and *Azadirachta indica* against the dengue mosquito *Aedes aegypti. Toxicology.* **44**: 829–835.

Weston, D. P. You, J. and Lydy, M. J. (2004). Distribution and Toxicity of Sediment-Associated Pesticides in Agriculture-Dominated Water Bodies of California's Central Valley *Environmental Science and Technology.* **38**: 2752 – 2759.

Wikimedia /wikipedia/commons/a/ad/Anopheles-range-map.png (accessed on July 10th, 2011 at 3.40 pm).

World Health Organization (WHO). (2002). Decision making criteria and procedures for judicious use of insecticides. WHO, Geneva; *document.* WHO/CDS/WHOPES/2002.5 Rev.1. pp.11-16.

World Health Organization (WHO). (2003). Communicable Disease Control, Prevention and Eradication. WHO, Geneva; *document.* WHO/CDS/WHOPES/2003.5 pp. 8-20.

World Health Organization (WHO) (2005). Guidelines for laboratory and field testing of mosquito larvicides. Communicable disease control, prevention and eradication, WHO pesticide evaluation scheme. WHO, Geneva; WHO/CDS/WHOPES/GCDPP/1.3.

World Health Organization (WHO). (2006). Malaria vector control and personal protection. 936th technical report series.

www.malariaconsortium.org/media-downloads/19 (accessed on October 29th, 2012 at 7.00 pm).

APPENDICES

APPENDIX 1: Generated LC values for chloroform crude extract

Confidence Limits

		95% Confidence Limits for concentration			95% Confidence Limits for log(concentration)[a]		
	Probability	Estimate	Lower Bound	Upper Bound	Estimate	Lower Bound	Upper Bound
PROBIT	.010	38.433	19.083	59.952	1.585	1.281	1.778
	.020	48.187	25.610	72.345	1.683	1.408	1.859
	.030	55.622	30.839	81.571	1.745	1.489	1.912
	.040	61.963	35.448	89.325	1.792	1.550	1.951
	.050	67.649	39.685	96.209	1.830	1.599	1.983
	.060	72.899	43.675	102.517	1.863	1.640	2.011
	.070	77.836	47.489	108.415	1.891	1.677	2.035
	.080	82.540	51.175	114.009	1.917	1.709	2.057
	.090	87.064	54.764	119.371	1.940	1.738	2.077
	.100	91.447	58.278	124.553	1.961	1.766	2.095
	.150	112.071	75.226	148.848	2.049	1.876	2.173
	.200	131.731	91.850	172.047	2.120	1.963	2.236
	.250	151.325	108.690	195.386	2.180	2.036	2.291
	.300	171.392	126.068	219.656	2.234	2.101	2.342
	.350	192.355	144.233	245.526	2.284	2.159	2.390
	.400	214.614	163.414	273.670	2.332	2.213	2.437
	.450	238.597	183.858	304.858	2.378	2.264	2.484
	.500	**264.816**	**205.859**	**340.031**	**2.423**	**2.314**	**2.532**
	.550	293.916	229.796	380.410	2.468	2.361	2.580
	.600	326.762	256.188	427.660	2.514	2.409	2.631
	.650	364.574	285.774	484.161	2.562	2.456	2.685
	.700	409.166	319.662	553.522	2.612	2.505	2.743
	.750	463.426	359.613	641.592	2.666	2.556	2.807
	.800	532.355	408.653	758.745	2.726	2.611	2.880
	.850	625.745	472.622	925.874	2.796	2.675	2.967
	.900	**766.866**	**565.122**	**1194.464**	**2.885**	**2.752**	**3.077**
	.910	805.475	589.720	1270.978	2.906	2.771	3.104
	.920	849.624	617.525	1359.954	2.929	2.791	3.134
	.930	900.967	649.461	1465.339	2.955	2.813	3.166
	.940	961.985	686.902	1593.142	2.983	2.837	3.202
	.950	1036.636	732.020	1753.105	3.016	2.865	3.244
	.960	1131.776	788.531	1962.416	3.054	2.897	3.293
	.970	1260.786	863.596	2255.389	3.101	2.936	3.353
	.980	1455.330	973.867	2715.571	3.163	2.988	3.434
	.990	1824.675	1175.388	3643.637	3.261	3.070	3.562

a. Logarithm base = 10.

APPENDIX 2: Generated LC values for C_1 fraction

Confidence Limits

		95% Confidence Limits for concentration			95% Confidence Limits for log(concentration)[b]		
	Probability	Estimate	Lower Bound	Upper Bound	Estimate	Lower Bound	Upper Bound
PROBIT[a]	.010	117.233	1.504	275.273	2.069	.177	2.440
	.020	142.851	2.835	312.010	2.155	.453	2.494
	.030	161.936	4.236	338.173	2.209	.627	2.529
	.040	177.955	5.724	359.532	2.250	.758	2.556
	.050	192.148	7.310	378.088	2.284	.864	2.578
	.060	205.116	8.997	394.797	2.312	.954	2.596
	.070	217.204	10.791	410.201	2.337	1.033	2.613
	.080	228.631	12.694	424.637	2.359	1.104	2.628
	.090	239.545	14.710	438.334	2.379	1.168	2.642
	.100	250.051	16.844	451.455	2.398	1.226	2.655
	.150	298.683	29.410	511.822	2.475	1.468	2.709
	.200	343.995	45.562	568.485	2.537	1.659	2.755
	.250	388.309	65.968	625.451	2.589	1.819	2.796
	.300	432.949	91.422	685.595	2.636	1.961	2.836
	.350	478.883	122.821	751.822	2.680	2.089	2.876
	.400	526.968	161.119	827.772	2.722	2.207	2.918
	.450	578.082	207.216	918.587	2.762	2.316	2.963
	.500	**633.224**	**261.752**	**1032.019**	**2.802**	**2.418**	**3.014**
	.550	693.625	324.843	1180.158	2.841	2.512	3.072
	.600	760.904	395.859	1382.155	2.881	2.598	3.141
	.650	837.307	473.551	1668.792	2.923	2.675	3.222
	.700	926.142	556.737	2091.168	2.967	2.746	3.320
	.750	1032.611	645.414	2740.252	3.014	2.810	3.438
	.800	1165.634	741.952	3797.185	3.067	2.870	3.579
	.850	1342.467	852.787	5684.553	3.128	2.931	3.755
	.900	**1603.559**	**993.433**	**9659.651**	**3.205**	**2.997**	**3.985**
	.910	1673.889	1028.105	11007.574	3.224	3.012	4.042
	.920	1753.793	1066.225	12696.834	3.244	3.028	4.104
	.930	1846.061	1108.766	14868.466	3.266	3.045	4.172
	.940	1954.860	1157.159	17753.036	3.291	3.063	4.249
	.950	2086.794	1213.624	21755.414	3.319	3.084	4.338
	.960	2253.219	1281.901	27659.307	3.353	3.108	4.442
	.970	2476.116	1369.044	37212.833	3.394	3.136	4.571
	.980	2806.921	1491.032	55319.514	3.448	3.173	4.743
	.990	3420.304	1699.628	103710.571	3.534	3.230	5.016

a. A heterogeneity factor is used.

b. Logarithm base = 10.

APPENDIX 3: Generated LC values for C_2 fraction

Confidence Limits

	Probability	95% Confidence Limits for concentration			95% Confidence Limits for log(concentration)[b]		
		Estimate	Lower Bound	Upper Bound	Estimate	Lower Bound	Upper Bound
PROBIT[a]	.010	24.998	.106	54.538	1.398	-.975	1.737
	.020	29.835	.227	60.895	1.475	-.644	1.785
	.030	33.378	.368	65.397	1.523	-.434	1.816
	.040	36.318	.529	69.064	1.560	-.277	1.839
	.050	38.899	.710	72.248	1.590	-.149	1.859
	.060	41.240	.911	75.117	1.615	-.040	1.876
	.070	43.409	1.134	77.765	1.638	.054	1.891
	.080	45.447	1.378	80.252	1.658	.139	1.904
	.090	47.383	1.645	82.616	1.676	.216	1.917
	.100	49.239	1.935	84.888	1.692	.287	1.929
	.150	57.727	3.775	95.460	1.761	.577	1.980
	.200	65.504	6.368	105.649	1.816	.804	2.024
	.250	73.007	9.886	116.272	1.863	.995	2.065
	.300	80.474	14.523	128.033	1.906	1.162	2.107
	.350	88.073	20.481	141.774	1.945	1.311	2.152
	.400	95.947	27.928	158.702	1.982	1.446	2.201
	.450	104.234	36.937	180.666	2.018	1.567	2.257
	.500	**113.088**	**47.401**	**210.592**	**2.053**	**1.676**	**2.323**
	.550	122.695	58.997	253.102	2.089	1.771	2.403
	.600	133.292	71.256	315.506	2.125	1.853	2.499
	.650	145.209	83.770	409.657	2.162	1.923	2.612
	.700	158.921	96.373	556.055	2.201	1.984	2.745
	.750	175.176	109.229	793.650	2.243	2.038	2.900
	.800	195.239	122.841	1205.678	2.291	2.089	3.081
	.850	221.544	138.199	2000.800	2.345	2.141	3.301
	.900	**259.734**	**157.410**	**3853.213**	**2.415**	**2.197**	**3.586**
	.910	269.905	162.103	4523.362	2.431	2.210	3.655
	.920	281.407	167.244	5387.863	2.449	2.223	3.731
	.930	294.619	172.957	6535.118	2.469	2.238	3.815
	.940	310.111	179.426	8113.951	2.492	2.254	3.909
	.950	328.775	186.934	10394.444	2.517	2.272	4.017
	.960	352.144	195.954	13919.635	2.547	2.292	4.144
	.970	383.161	207.379	19957.099	2.583	2.317	4.300
	.980	428.663	223.212	32274.424	2.632	2.349	4.509
	.990	511.597	249.885	69060.741	2.709	2.398	4.839

a. A heterogeneity factor is used.

b. Logarithm base = 10.

APPENDIX 4: Generated LC values for C_3 fraction

Confidence Limits

	Probability	95% Confidence Limits for concentration			95% Confidence Limits for log(concentration)[a]		
		Estimate	Lower Bound	Upper Bound	Estimate	Lower Bound	Upper Bound
PROBIT	.010	80.568	40.790	104.530	1.906	1.611	2.019
	.020	87.257	47.243	110.600	1.941	1.674	2.044
	.030	91.786	51.845	114.661	1.963	1.715	2.059
	.040	95.348	55.591	117.831	1.979	1.745	2.071
	.050	98.346	58.830	120.488	1.993	1.770	2.081
	.060	100.973	61.729	122.808	2.004	1.790	2.089
	.070	103.333	64.384	124.890	2.014	1.809	2.097
	.080	105.494	66.853	126.792	2.023	1.825	2.103
	.090	107.498	69.176	128.556	2.031	1.840	2.109
	.100	109.376	71.381	130.209	2.039	1.854	2.115
	.150	117.508	81.223	137.385	2.070	1.910	2.138
	.200	124.401	89.902	143.534	2.095	1.954	2.157
	.250	130.635	97.970	149.200	2.116	1.991	2.174
	.300	136.500	105.697	154.672	2.135	2.024	2.189
	.350	142.169	113.239	160.151	2.153	2.054	2.205
	.400	147.766	120.688	165.810	2.170	2.082	2.220
	.450	153.391	128.100	171.824	2.186	2.108	2.235
	.500	**159.135**	**135.505**	**178.396**	**2.202**	**2.132**	**2.251**
	.550	165.095	142.910	185.771	2.218	2.155	2.269
	.600	171.380	150.322	194.257	2.234	2.177	2.288
	.650	178.127	157.761	204.245	2.251	2.198	2.310
	.700	185.525	165.292	216.246	2.268	2.218	2.335
	.750	193.853	173.067	230.994	2.287	2.238	2.364
	.800	203.568	181.377	249.673	2.309	2.259	2.397
	.850	215.509	190.764	274.510	2.333	2.280	2.439
	.900	**231.532**	**202.393**	**310.640**	**2.365**	**2.306**	**2.492**
	.910	235.578	205.201	320.221	2.372	2.312	2.505
	.920	240.053	208.259	331.024	2.380	2.319	2.520
	.930	245.072	211.634	343.388	2.389	2.326	2.536
	.940	250.801	215.425	357.816	2.399	2.333	2.554
	.950	257.499	219.780	375.098	2.411	2.342	2.574
	.960	265.597	224.951	396.576	2.424	2.352	2.598
	.970	275.903	231.398	424.814	2.441	2.364	2.628
	.980	290.224	240.147	465.698	2.463	2.380	2.668
	.990	314.320	254.411	538.697	2.497	2.406	2.731

a. Logarithm base = 10.

APPENDIX 5: Generated LC values for C_2F_1 fraction

Confidence Limits

	Probability	95% Confidence Limits for concentration			95% Confidence Limits for log(concentration)[a]		
		Estimate	Lower Bound	Upper Bound	Estimate	Lower Bound	Upper Bound
PROBIT	.010	29.928	16.511	40.437	1.476	1.218	1.607
	.020	33.514	19.595	44.121	1.525	1.292	1.645
	.030	36.009	21.835	46.649	1.556	1.339	1.669
	.040	38.007	23.681	48.659	1.580	1.374	1.687
	.050	39.715	25.292	50.367	1.599	1.403	1.702
	.060	41.228	26.745	51.877	1.615	1.427	1.715
	.070	42.602	28.084	53.245	1.629	1.448	1.726
	.080	43.872	29.336	54.507	1.642	1.467	1.736
	.090	45.059	30.519	55.687	1.654	1.485	1.746
	.100	46.180	31.648	56.802	1.664	1.500	1.754
	.150	51.127	36.737	61.733	1.709	1.565	1.791
	.200	55.434	41.285	66.075	1.744	1.616	1.820
	.250	59.417	45.554	70.163	1.774	1.659	1.846
	.300	63.237	49.676	74.180	1.801	1.696	1.870
	.350	66.995	53.727	78.251	1.826	1.730	1.893
	.400	70.769	57.762	82.486	1.850	1.762	1.916
	.450	74.621	61.818	86.991	1.873	1.791	1.939
	.500	**78.617**	**65.931**	**91.883**	**1.896**	**1.819**	**1.963**
	.550	82.828	70.138	97.299	1.918	1.846	1.988
	.600	87.337	74.483	103.412	1.941	1.872	2.015
	.650	92.256	79.029	110.452	1.965	1.898	2.043
	.700	97.739	83.871	118.744	1.990	1.924	2.075
	.750	104.023	89.156	128.786	2.017	1.950	2.110
	.800	111.497	95.132	141.415	2.047	1.978	2.150
	.850	120.890	102.264	158.237	2.082	2.010	2.199
	.900	**133.839**	**111.577**	**182.966**	**2.127**	**2.048**	**2.262**
	.910	137.170	113.895	189.591	2.137	2.057	2.278
	.920	140.882	116.446	197.094	2.149	2.066	2.295
	.930	145.079	119.295	205.728	2.162	2.077	2.313
	.940	149.915	122.532	215.865	2.176	2.088	2.334
	.950	155.628	126.300	228.094	2.192	2.101	2.358
	.960	162.618	130.837	243.423	2.211	2.117	2.386
	.970	171.644	136.586	263.786	2.235	2.135	2.421
	.980	184.422	144.543	293.675	2.266	2.160	2.468
	.990	206.520	157.880	348.150	2.315	2.198	2.542

a. Logarithm base = 10.

APPENDIX 6: Generated LC values for C_2F_2 fraction

Confidence Limits

	Probability	95% Confidence Limits for concentration			95% Confidence Limits for log(concentration)[a]		
		Estimate	Lower Bound	Upper Bound	Estimate	Lower Bound	Upper Bound
PROBIT	.010	85.391	45.060	110.863	1.931	1.654	2.045
	.020	92.683	52.158	117.753	1.967	1.717	2.071
	.030	97.630	57.203	122.405	1.990	1.757	2.088
	.040	101.525	61.299	126.064	2.007	1.787	2.101
	.050	104.807	64.830	129.151	2.020	1.812	2.111
	.060	107.684	67.983	131.862	2.032	1.832	2.120
	.070	110.272	70.862	134.308	2.042	1.850	2.128
	.080	112.641	73.533	136.556	2.052	1.866	2.135
	.090	114.841	76.040	138.651	2.060	1.881	2.142
	.100	116.903	78.413	140.624	2.068	1.894	2.148
	.150	125.843	88.926	149.312	2.100	1.949	2.174
	.200	133.434	98.069	156.932	2.125	1.992	2.196
	.250	140.310	106.443	164.110	2.147	2.027	2.215
	.300	146.786	114.336	171.186	2.167	2.058	2.233
	.350	153.054	121.910	178.396	2.185	2.086	2.251
	.400	159.249	129.268	185.939	2.202	2.111	2.269
	.450	165.481	136.480	194.008	2.219	2.135	2.288
	.500	**171.852**	**143.602**	**202.809**	**2.235**	**2.157**	**2.307**
	.550	178.469	150.691	212.579	2.252	2.178	2.328
	.600	185.453	157.814	223.608	2.268	2.198	2.349
	.650	192.959	165.063	236.276	2.285	2.218	2.373
	.700	201.198	172.570	251.119	2.304	2.237	2.400
	.750	210.485	180.536	268.955	2.323	2.257	2.430
	.800	221.331	189.287	291.160	2.345	2.277	2.464
	.850	234.682	199.411	320.348	2.370	2.300	2.506
	.900	**252.629**	**212.179**	**362.530**	**2.402**	**2.327**	**2.559**
	.910	257.166	215.286	373.694	2.410	2.333	2.573
	.920	262.187	218.677	386.276	2.419	2.340	2.587
	.930	267.821	222.427	400.673	2.428	2.347	2.603
	.940	274.257	226.645	417.472	2.438	2.355	2.621
	.950	281.786	231.499	437.596	2.450	2.365	2.641
	.960	290.895	237.268	462.610	2.464	2.375	2.665
	.970	302.500	244.470	495.508	2.481	2.388	2.695
	.980	318.645	254.254	543.168	2.503	2.405	2.735
	.990	345.859	270.219	628.357	2.539	2.432	2.798

a. Logarithm base = 10.

APPENDIX 7**:** Generated LC values for C_2F_4 fraction

Confidence Limits

	Probability	95% Confidence Limits for concentration			95% Confidence Limits for log(concentration)[b]		
		Estimate	Lower Bound	Upper Bound	Estimate	Lower Bound	Upper Bound
PROBIT[a]	.010	110.039	2.664	195.934	2.042	.426	2.292
	.020	125.754	4.644	213.901	2.100	.667	2.330
	.030	136.869	6.596	226.533	2.136	.819	2.355
	.040	145.873	8.580	236.791	2.164	.933	2.374
	.050	153.632	10.616	245.692	2.186	1.026	2.390
	.060	160.561	12.716	253.715	2.206	1.104	2.404
	.070	166.893	14.888	261.132	2.222	1.173	2.417
	.080	172.774	17.135	268.112	2.237	1.234	2.428
	.090	178.302	19.461	274.769	2.251	1.289	2.439
	.100	183.547	21.870	281.185	2.264	1.340	2.449
	.150	206.956	35.222	311.411	2.316	1.547	2.493
	.200	227.671	50.904	341.299	2.357	1.707	2.533
	.250	247.089	69.038	373.387	2.393	1.839	2.572
	.300	265.933	89.622	409.938	2.425	1.952	2.613
	.350	284.676	112.473	453.606	2.454	2.051	2.657
	.400	303.682	137.184	507.849	2.482	2.137	2.706
	.450	323.276	163.134	577.309	2.510	2.213	2.761
	.500	**343.795**	**189.604**	**668.261**	**2.536**	**2.278**	**2.825**
	.550	365.615	215.966	789.307	2.563	2.334	2.897
	.600	389.206	241.869	952.722	2.590	2.384	2.979
	.650	415.190	267.312	1177.166	2.618	2.427	3.071
	.700	444.452	292.645	1493.181	2.648	2.466	3.174
	.750	478.349	318.550	1955.060	2.680	2.503	3.291
	.800	519.146	346.136	2669.616	2.715	2.539	3.426
	.850	571.111	377.306	3879.183	2.757	2.577	3.589
	.900	**643.948**	**416.021**	**6275.306**	**2.809**	**2.619**	**3.798**
	.910	662.891	425.396	7057.611	2.821	2.629	3.849
	.920	684.101	435.619	8022.081	2.835	2.639	3.904
	.930	708.208	446.922	9239.957	2.850	2.650	3.966
	.940	736.136	459.641	10825.855	2.867	2.662	4.034
	.950	769.335	474.297	12977.439	2.886	2.676	4.113
	.960	810.258	491.753	16069.448	2.909	2.692	4.206
	.970	863.563	513.620	20917.164	2.936	2.711	4.321
	.980	939.890	543.493	29735.810	2.973	2.735	4.473
	.990	1074.117	592.743	51891.863	3.031	2.773	4.715

a. A heterogeneity factor is used.

b. Logarithm base = 10.

APPENDIX 8: Generated LC values for C_2F_5 fraction

Confidence Limits

	Probability	95% Confidence Limits for concentration			95% Confidence Limits for log(concentration)[a]		
		Estimate	Lower Bound	Upper Bound	Estimate	Lower Bound	Upper Bound
PROBIT	.010	15.961	9.382	20.818	1.203	.972	1.318
	.020	17.517	10.811	22.354	1.243	1.034	1.349
	.030	18.581	11.824	23.395	1.269	1.073	1.369
	.040	19.424	12.647	24.214	1.288	1.102	1.384
	.050	20.138	13.356	24.904	1.304	1.126	1.396
	.060	20.767	13.989	25.510	1.317	1.146	1.407
	.070	21.334	14.568	26.056	1.329	1.163	1.416
	.080	21.855	15.105	26.557	1.340	1.179	1.424
	.090	22.340	15.610	27.023	1.349	1.193	1.432
	.100	22.795	16.088	27.461	1.358	1.207	1.439
	.150	24.783	18.215	29.374	1.394	1.260	1.468
	.200	26.486	20.080	31.026	1.423	1.303	1.492
	.250	28.040	21.808	32.553	1.448	1.339	1.513
	.300	29.513	23.459	34.026	1.470	1.370	1.532
	.350	30.947	25.071	35.492	1.491	1.399	1.550
	.400	32.372	26.669	36.990	1.510	1.426	1.568
	.450	33.813	28.272	38.554	1.529	1.451	1.586
	.500	**35.294**	**29.894**	**40.222**	**1.548**	**1.476**	**1.604**
	.550	36.840	31.553	42.040	1.566	1.499	1.624
	.600	38.480	33.263	44.062	1.585	1.522	1.644
	.650	40.251	35.046	46.361	1.605	1.545	1.666
	.700	42.207	36.934	49.042	1.625	1.567	1.691
	.750	44.424	38.973	52.258	1.648	1.591	1.718
	.800	47.030	41.246	56.265	1.672	1.615	1.750
	.850	50.262	43.909	61.542	1.701	1.643	1.789
	.900	**54.645**	**47.307**	**69.178**	**1.738**	**1.675**	**1.840**
	.910	55.760	48.140	71.199	1.746	1.683	1.852
	.920	56.997	49.052	73.476	1.756	1.691	1.866
	.930	58.388	50.063	76.081	1.766	1.700	1.881
	.940	59.983	51.206	79.119	1.778	1.709	1.898
	.950	61.855	52.525	82.755	1.791	1.720	1.918
	.960	64.128	54.101	87.269	1.807	1.733	1.941
	.970	67.039	56.080	93.198	1.826	1.749	1.969
	.980	71.113	58.789	101.767	1.852	1.769	2.008
	.990	78.043	63.257	117.027	1.892	1.801	2.068

a. Logarithm base = 10.

APPENDIX 9**:** Generated LC values for *F. angolensis* essential oil

Confidence Limits

	Probability	95% Confidence Limits for concentration			95% Confidence Limits for log(concentration)[a]		
		Estimate	Lower Bound	Upper Bound	Estimate	Lower Bound	Upper Bound
PROBIT	.010	7.212	3.271	12.242	.858	.515	1.088
	.020	9.615	4.692	15.600	.983	.671	1.193
	.030	11.538	5.896	18.207	1.062	.771	1.260
	.040	13.235	6.997	20.460	1.122	.845	1.311
	.050	14.798	8.040	22.504	1.170	.905	1.352
	.060	16.273	9.047	24.411	1.211	.957	1.388
	.070	17.687	10.032	26.221	1.248	1.001	1.419
	.080	19.056	11.002	27.960	1.280	1.041	1.447
	.090	20.393	11.963	29.647	1.309	1.078	1.472
	.100	21.707	12.919	31.295	1.337	1.111	1.495
	.150	28.111	17.731	39.226	1.449	1.249	1.594
	.200	34.522	22.741	47.068	1.538	1.357	1.673
	.250	41.176	28.083	55.173	1.615	1.448	1.742
	.300	48.238	33.856	63.795	1.683	1.530	1.805
	.350	55.858	40.157	73.171	1.747	1.604	1.864
	.400	64.199	47.090	83.562	1.808	1.673	1.922
	.450	73.453	54.779	95.290	1.866	1.739	1.979
	.500	**83.862**	**63.377**	**108.769**	**1.924**	**1.802**	**2.037**
	.550	95.746	73.087	124.559	1.981	1.864	2.095
	.600	109.547	84.186	143.449	2.040	1.925	2.157
	.650	125.906	97.075	166.599	2.100	1.987	2.222
	.700	145.796	112.362	195.808	2.164	2.051	2.292
	.750	170.800	131.033	234.059	2.232	2.117	2.369
	.800	203.721	154.816	286.764	2.309	2.190	2.458
	.850	250.185	187.128	365.123	2.398	2.272	2.562
	.900	**323.986**	**236.163**	**497.687**	**2.511**	**2.373**	**2.697**
	.910	344.860	249.625	536.762	2.538	2.397	2.730
	.920	369.062	265.045	582.864	2.567	2.423	2.766
	.930	397.640	283.011	638.340	2.599	2.452	2.805
	.940	432.180	304.412	706.815	2.636	2.483	2.849
	.950	475.250	330.665	794.250	2.677	2.519	2.900
	.960	531.366	364.235	911.344	2.725	2.561	2.960
	.970	609.515	409.947	1079.905	2.785	2.613	3.033
	.980	731.474	479.271	1354.451	2.864	2.681	3.132
	.990	975.105	612.048	1938.922	2.989	2.787	3.288

a. Logarithm base = 10.

APPENDIX 10: Generated LC values for compound **32**

Confidence Limits

	Probability	95% Confidence Limits for concentration			95% Confidence Limits for log(concentration)[a]		
		Estimate	Lower Bound	Upper Bound	Estimate	Lower Bound	Upper Bound
PROBIT	.010	75.064	41.341	105.165	1.875	1.616	2.022
	.020	86.245	50.259	117.527	1.936	1.701	2.070
	.030	94.188	56.857	126.184	1.974	1.755	2.101
	.040	100.642	62.365	133.162	2.003	1.795	2.124
	.050	106.216	67.218	139.158	2.026	1.827	2.144
	.060	111.203	71.630	144.503	2.046	1.855	2.160
	.070	115.768	75.723	149.386	2.064	1.879	2.174
	.080	120.014	79.573	153.921	2.079	1.901	2.187
	.090	124.011	83.233	158.187	2.093	1.920	2.199
	.100	127.808	86.740	162.239	2.107	1.938	2.210
	.150	144.805	102.745	180.423	2.161	2.012	2.256
	.200	159.911	117.290	196.745	2.204	2.069	2.294
	.250	174.121	131.138	212.344	2.241	2.118	2.327
	.300	187.955	144.679	227.846	2.274	2.160	2.358
	.350	201.754	158.161	243.699	2.305	2.199	2.387
	.400	215.783	171.765	260.289	2.334	2.235	2.415
	.450	230.284	185.645	278.003	2.362	2.269	2.444
	.500	**245.506**	**199.956**	**297.271**	**2.390**	**2.301**	**2.473**
	.550	261.734	214.870	318.614	2.418	2.332	2.503
	.600	279.323	230.604	342.701	2.446	2.363	2.535
	.650	298.746	247.452	370.448	2.475	2.393	2.569
	.700	320.678	265.841	403.183	2.506	2.425	2.606
	.750	346.156	286.432	442.978	2.539	2.457	2.646
	.800	376.916	310.335	493.372	2.576	2.492	2.693
	.850	416.237	339.628	561.201	2.619	2.531	2.749
	.900	**471.591**	**378.982**	**662.494**	**2.674**	**2.579**	**2.821**
	.910	486.030	388.951	689.942	2.687	2.590	2.839
	.920	502.216	400.000	721.190	2.701	2.602	2.858
	.930	520.637	412.423	757.347	2.717	2.615	2.879
	.940	542.010	426.651	800.069	2.734	2.630	2.903
	.950	567.460	443.355	851.983	2.754	2.647	2.930
	.960	598.890	463.658	917.607	2.777	2.666	2.963
	.970	639.926	489.682	1005.693	2.806	2.690	3.002
	.980	698.860	526.215	1136.740	2.844	2.721	3.056
	.990	802.961	588.710	1380.445	2.905	2.770	3.140

a. Logarithm base = 10.

APPENDIX 11**:** Generated LC values for compound **33**

Confidence Limits

	Probability	95% Confidence Limits for concentration			95% Confidence Limits for log(concentration)[b]		
		Estimate	Lower Bound	Upper Bound	Estimate	Lower Bound	Upper Bound
PROBIT[a]	.010	42.734	8.073	72.270	1.631	.907	1.859
	.020	49.413	11.170	80.188	1.694	1.048	1.904
	.030	54.182	13.706	85.784	1.734	1.137	1.933
	.040	58.071	15.970	90.336	1.764	1.203	1.956
	.050	61.439	18.072	94.285	1.788	1.257	1.974
	.060	64.460	20.066	97.840	1.809	1.302	1.991
	.070	67.230	21.983	101.120	1.828	1.342	2.005
	.080	69.811	23.844	104.196	1.844	1.377	2.018
	.090	72.244	25.662	107.120	1.859	1.409	2.030
	.100	74.559	27.447	109.925	1.873	1.438	2.041
	.150	84.960	36.089	122.923	1.929	1.557	2.090
	.200	94.251	44.546	135.285	1.974	1.649	2.131
	.250	103.028	53.009	147.863	2.013	1.724	2.170
	.300	111.605	61.556	161.230	2.048	1.789	2.207
	.350	120.188	70.220	175.894	2.080	1.846	2.245
	.400	128.942	79.007	192.390	2.110	1.898	2.284
	.450	138.019	87.922	211.329	2.140	1.944	2.325
	.500	**147.575**	**96.977**	**233.460**	**2.169**	**1.987**	**2.368**
	.550	157.792	106.209	259.745	2.198	2.026	2.415
	.600	168.899	115.690	291.489	2.228	2.063	2.465
	.650	181.202	125.545	330.564	2.258	2.099	2.519
	.700	195.138	135.977	379.823	2.290	2.133	2.580
	.750	211.381	147.307	443.949	2.325	2.168	2.647
	.800	231.066	160.073	531.359	2.364	2.204	2.725
	.850	256.336	175.267	659.267	2.409	2.244	2.819
	.900	**292.093**	**195.083**	**870.878**	**2.466**	**2.290**	**2.940**
	.910	301.453	200.014	932.290	2.479	2.301	2.970
	.920	311.961	205.445	1004.257	2.494	2.313	3.002
	.930	323.938	211.509	1090.215	2.510	2.325	3.038
	.940	337.859	218.404	1195.431	2.529	2.339	3.078
	.950	354.469	226.436	1328.507	2.550	2.355	3.123
	.960	375.028	236.116	1504.759	2.574	2.373	3.177
	.970	401.945	248.404	1755.085	2.604	2.395	3.244
	.980	440.740	265.452	2155.747	2.644	2.424	3.334
	.990	509.628	294.151	2986.734	2.707	2.469	3.475

a. A heterogeneity factor is used.

b. Logarithm base = 10.

APPENDIX 12**:** Generated LC values for compound **34**

Confidence Limits

	Probability	95% Confidence Limits for concentration			95% Confidence Limits for log(concentration)[a]		
		Estimate	Lower Bound	Upper Bound	Estimate	Lower Bound	Upper Bound
PROBIT	.010	49.858	26.771	68.481	1.698	1.428	1.836
	.020	56.473	32.326	75.460	1.752	1.510	1.878
	.030	61.119	36.410	80.306	1.786	1.561	1.905
	.040	64.864	39.802	84.192	1.812	1.600	1.925
	.050	68.079	42.779	87.518	1.833	1.631	1.942
	.060	70.941	45.476	90.477	1.851	1.658	1.957
	.070	73.549	47.969	93.173	1.867	1.681	1.969
	.080	75.965	50.308	95.675	1.881	1.702	1.981
	.090	78.231	52.525	98.025	1.893	1.720	1.991
	.100	80.377	54.642	100.256	1.905	1.738	2.001
	.150	89.907	64.230	110.265	1.954	1.808	2.042
	.200	98.280	72.825	119.273	1.992	1.862	2.077
	.250	106.082	80.890	127.931	2.026	1.908	2.107
	.300	113.615	88.655	136.604	2.055	1.948	2.135
	.350	121.072	96.255	145.556	2.083	1.983	2.163
	.400	128.600	103.784	155.014	2.109	2.016	2.190
	.450	136.329	111.320	165.208	2.135	2.047	2.218
	.500	**144.389**	**118.937**	**176.387**	**2.160**	**2.075**	**2.246**
	.550	152.925	126.722	188.848	2.184	2.103	2.276
	.600	162.116	134.782	202.971	2.210	2.130	2.307
	.650	172.196	143.265	219.269	2.236	2.156	2.341
	.700	183.498	152.380	238.492	2.264	2.183	2.377
	.750	196.529	162.444	261.815	2.293	2.211	2.418
	.800	212.131	173.977	291.244	2.327	2.240	2.464
	.850	231.887	187.935	330.663	2.365	2.274	2.519
	.900	**259.379**	**206.439**	**389.168**	**2.414**	**2.315**	**2.590**
	.910	266.493	211.087	404.957	2.426	2.324	2.607
	.920	274.444	216.220	422.901	2.438	2.335	2.626
	.930	283.460	221.971	443.627	2.452	2.346	2.647
	.940	293.881	228.531	468.068	2.468	2.359	2.670
	.950	306.234	236.197	497.701	2.486	2.373	2.697
	.960	321.412	245.466	535.067	2.507	2.390	2.728
	.970	341.106	257.272	585.074	2.533	2.410	2.767
	.980	369.167	273.711	659.191	2.567	2.437	2.819
	.990	418.154	301.495	796.277	2.621	2.479	2.901

a. Logarithm base = 10.

APPENDIX 13**:** Generated LC values for compound **35**

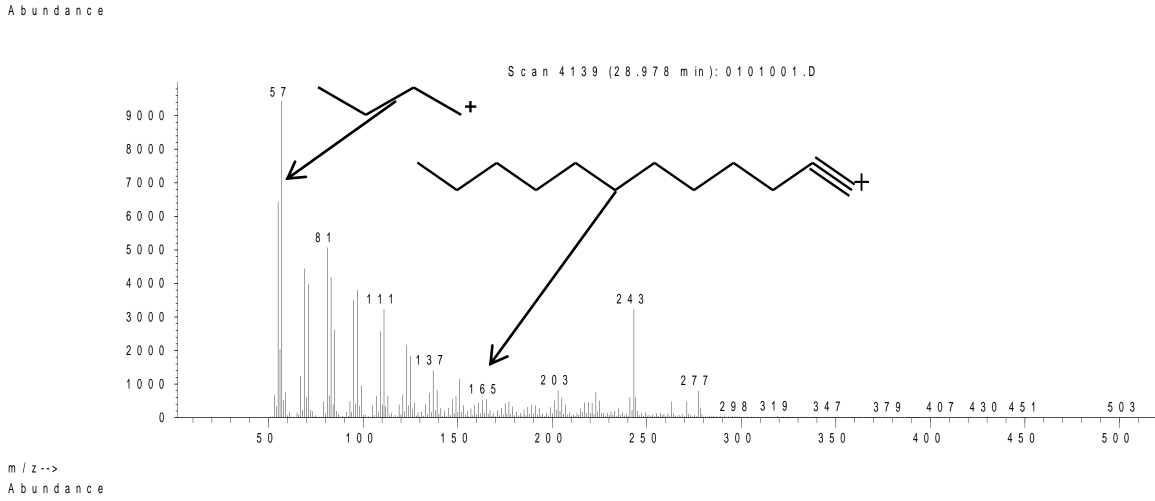
Confidence Limits

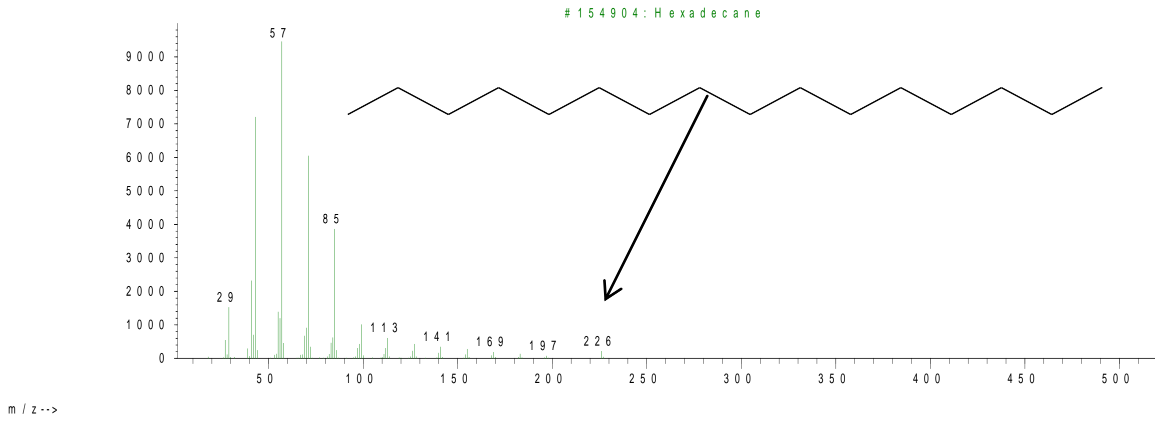
	Probability	95% Confidence Limits for concentration			95% Confidence Limits for log(concentration)[b]		
		Estimate	Lower Bound	Upper Bound	Estimate	Lower Bound	Upper Bound
PROBIT[a]	.010	96.078	14.470	170.371	1.983	1.160	2.231
	.020	114.302	21.444	192.186	2.058	1.331	2.284
	.030	127.617	27.483	207.749	2.106	1.439	2.318
	.040	138.647	33.092	220.490	2.142	1.520	2.343
	.050	148.318	38.461	231.597	2.171	1.585	2.365
	.060	157.080	43.685	241.639	2.196	1.640	2.383
	.070	165.186	48.819	250.935	2.218	1.689	2.400
	.080	172.799	53.901	259.688	2.238	1.732	2.414
	.090	180.027	58.954	268.034	2.255	1.771	2.428
	.100	186.948	63.995	276.070	2.272	1.806	2.441
	.150	218.547	89.399	313.688	2.340	1.951	2.496
	.200	247.428	115.607	350.205	2.393	2.063	2.544
	.250	275.229	142.863	388.336	2.440	2.155	2.589
	.300	302.847	171.134	430.189	2.481	2.233	2.634
	.350	330.908	200.231	477.881	2.520	2.302	2.679
	.400	359.935	229.888	533.793	2.556	2.362	2.727
	.450	390.441	259.854	600.742	2.592	2.415	2.779
	.500	**422.986**	**289.996**	**682.175**	**2.626**	**2.462**	**2.834**
	.550	458.243	320.375	782.528	2.661	2.506	2.894
	.600	497.080	351.282	907.882	2.696	2.546	2.958
	.650	540.684	383.265	1067.149	2.733	2.583	3.028
	.700	590.782	417.165	1274.301	2.771	2.620	3.105
	.750	650.065	454.260	1552.899	2.813	2.657	3.191
	.800	723.106	496.604	1946.513	2.859	2.696	3.289
	.850	818.666	547.922	2546.995	2.913	2.739	3.406
	.900	**957.042**	**616.441**	**3593.498**	**2.981**	**2.790**	**3.556**
	.910	993.831	633.769	3907.909	2.997	2.802	3.592
	.920	1035.402	652.971	4281.863	3.015	2.815	3.632
	.930	1083.121	674.563	4735.848	3.035	2.829	3.675
	.940	1139.020	699.298	5301.709	3.057	2.845	3.724
	.950	1206.304	728.349	6032.274	3.081	2.862	3.780
	.960	1290.448	763.690	7023.306	3.111	2.883	3.847
	.970	1401.978	809.041	8472.249	3.147	2.908	3.928
	.980	1565.302	872.821	10880.051	3.195	2.941	4.037
	.990	1862.199	982.256	16161.816	3.270	2.992	4.208

a. A heterogeneity factor is used.

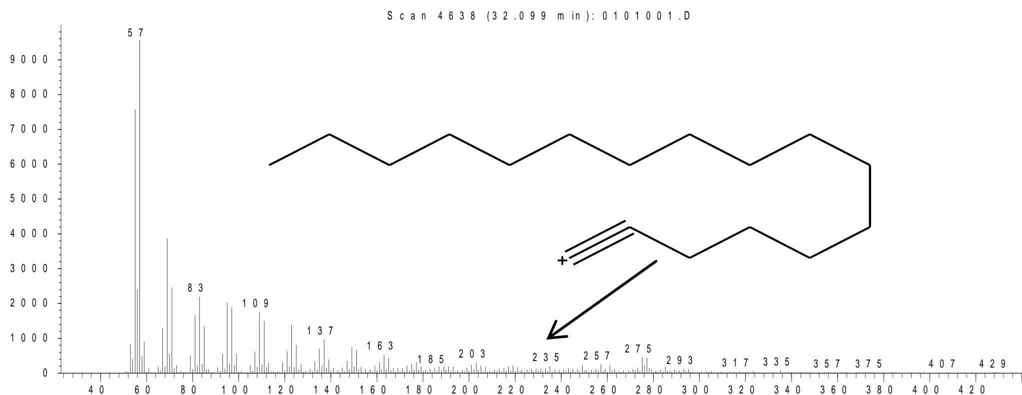
b. Logarithm base = 10.

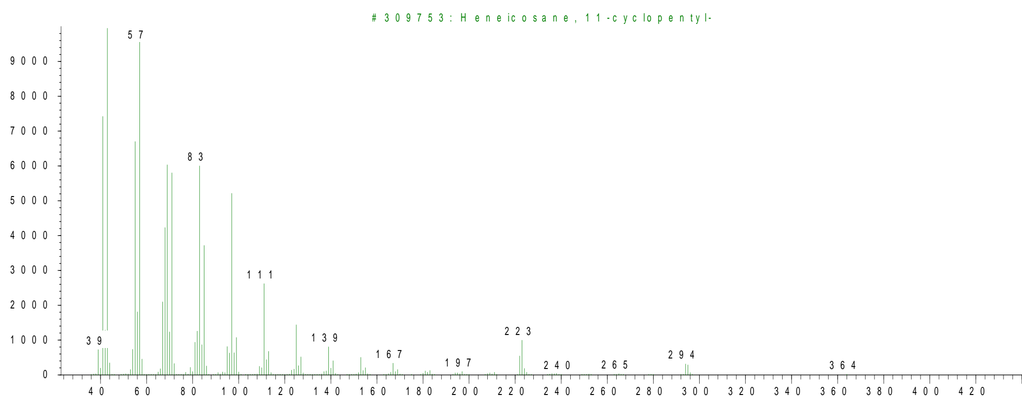
APPENDIX 14: GC-MS spectrum of compound **38** in comparison with database



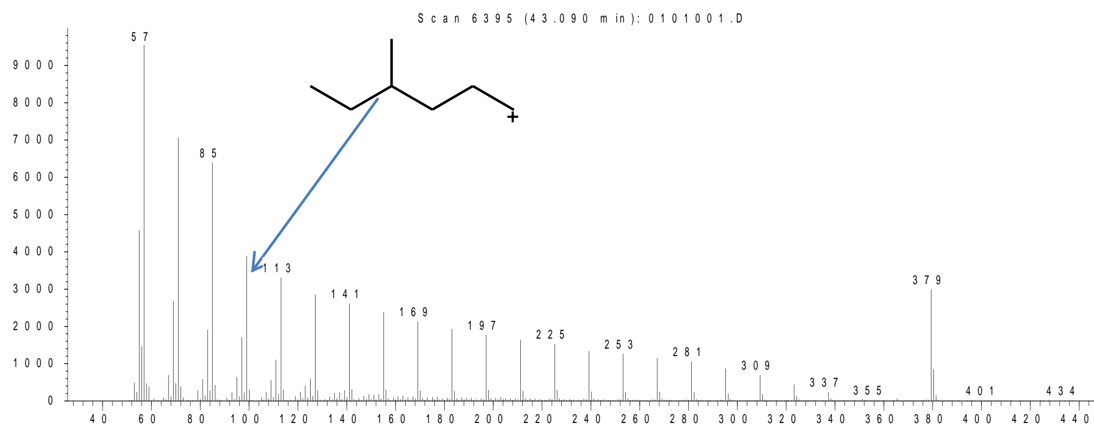


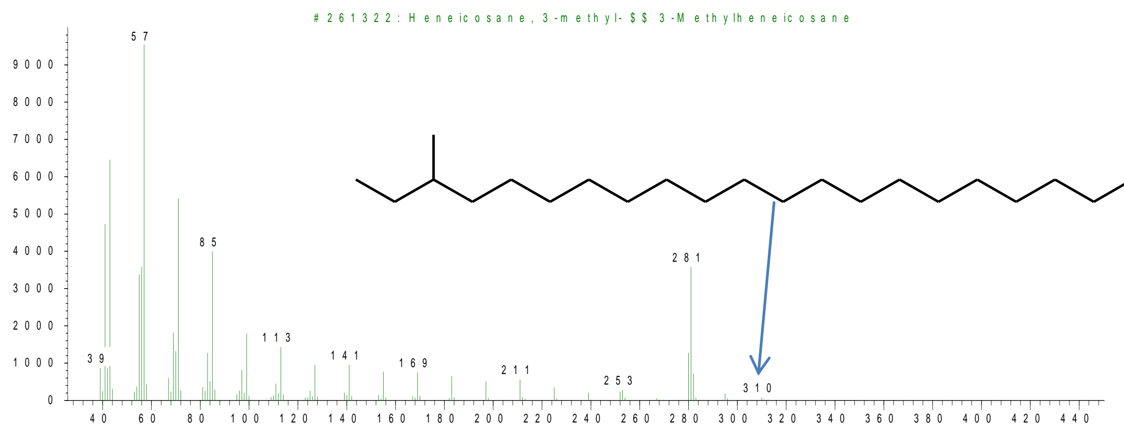
APPENDIX 15: GC-MS spectrum of compound **39** in comparison with database





APPENDIX 16: GC-MS spectrum of compound **41** in comparison with database





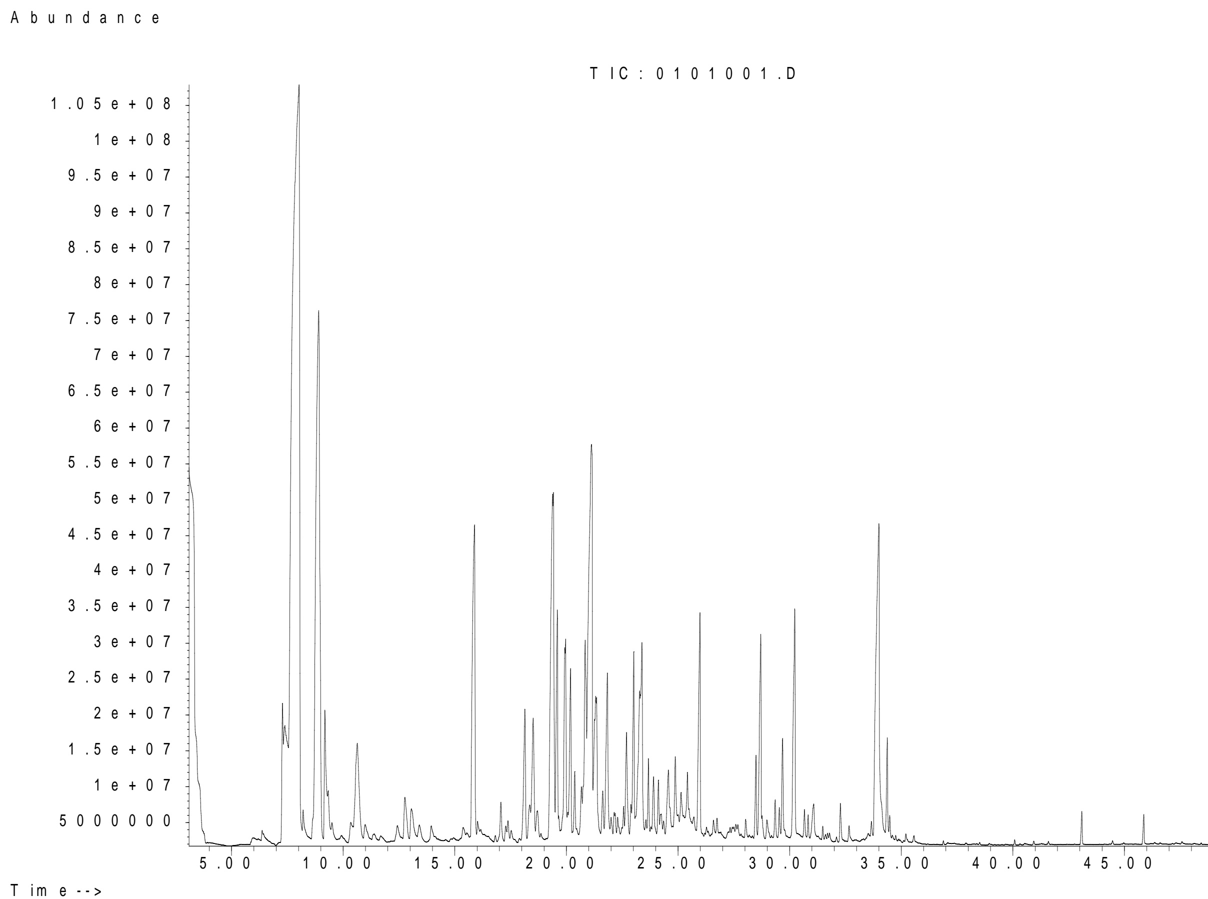
APPENDIX 17: Partially identified compounds by GC-MS from *F. angolensis* oil

No.	Retention Times	Compound	Concentration %
1	5.98	5-Acetoxymethyl-2,6,10-trimethyl-2,9-undecadien-6-ol	0.22
2	7.29	1-Isopropenyl-4-methylcyclohexane methanol	2.61
3	**8.00**	**Cis-1-Ethinyl-2-methyl-1-cyclohexanol**	**21.81**
4	8.22	3-Methylene-1,7,7-trimethylbicyclo [2.2.1]hept-2-ylestercyclopentane carboxylic acid	0.92
5	**8.89**	**2-Butyldecahydronaphthalene**	**8.50**
6	9.19	Endo-3-oxo-8-oxabicyclo[3.2.1]oct-6-ene-2-ethanal	1.40
7	10.99	(E)-3,4,7,11,15-pentamethyl-2,6,10,14-hexadecatetren-1-ol	0.34
8	12.77	Cis,trans-3-ethylbicyclo[4.4.0] decane	0.55
9	13.95	4-Nitro-O-cresol	0.43
10	14.95	5-Nitro-2,11-dioxo-cycloundecane-1-carboxylatemethyl	0.19
11	17.38	1a,2,3,4,4a,5,6,7b-Octahydro-1,1,4,7-tetramethyl-1H-cycloprop(e)azulene	0.29
12	17.90	4-(5,5-Dimethyl-1-oxaspiro[2,5]oct-4-yl)-3-buten-2-one	0.05
13	18.14	2,3,4,4a,4b,5,6,7,8,8a-decahydro-9(1H)-phenanthrone	1.06
14	18.14	2-(Hydroxyphenylmethyl)-1-hexen-3-one	1.06
15	**19.39**	**N-isobutyl-2-quinolone**	**4.59**
16	20.18	1-Hydroxymethyl-5,8,9-endo-10-exo-tetramethyltricyclo [6.3.0.0(5,11)]undecane	1.22
17	20.38	2-(Hydroxyphenylmethyl)-1-hexen-3-one	0.55
18	21.33	(1R,Cis)-3-Aminomethyl-1,2,2-	1.85

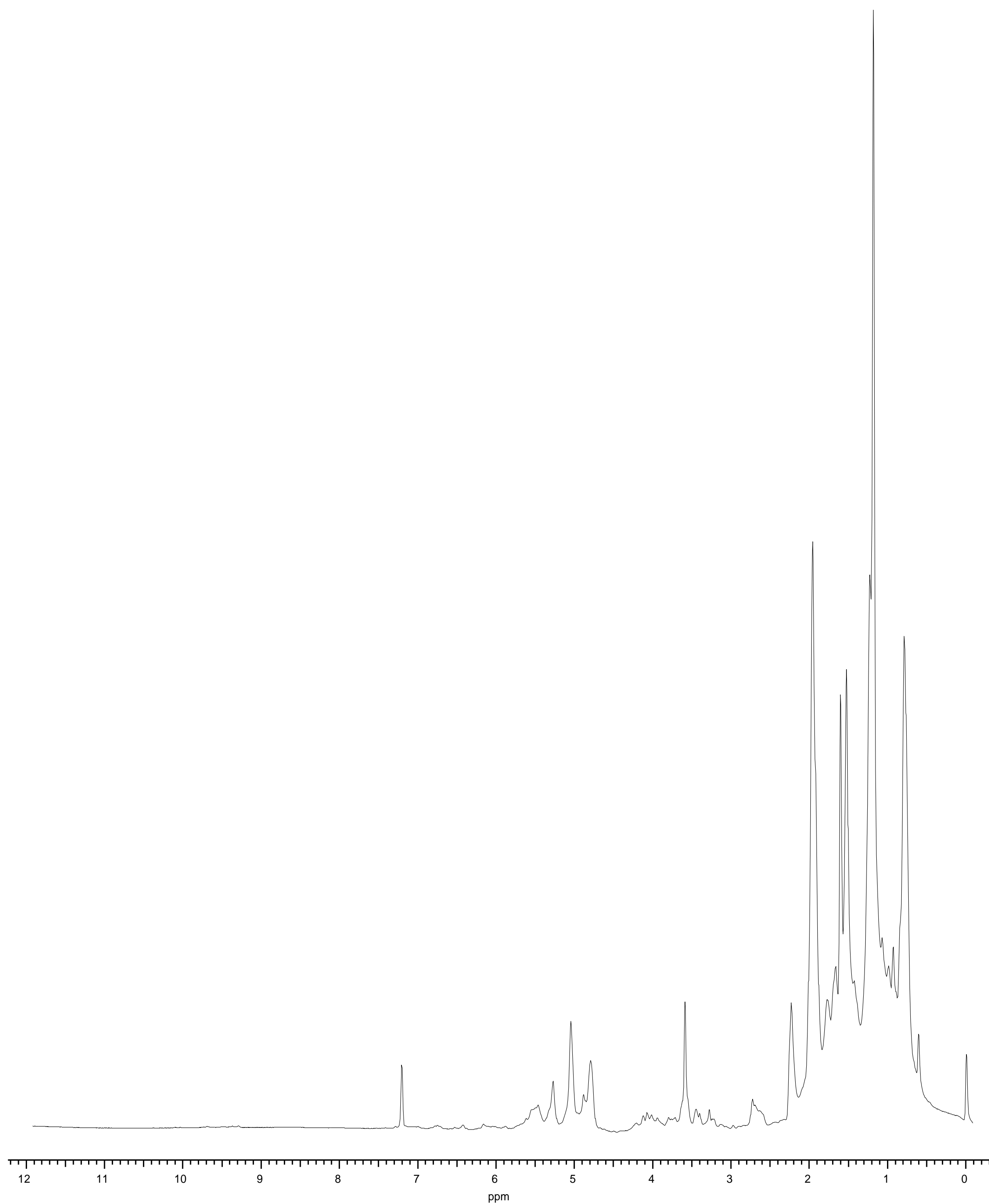
		trimethylcyclopentylmethanol	
19	21.83	Chlorophenamine	1.41
20	22.16	1,2-Dibromo-3-deuteriopropane	0.53
21	22.69	(Z)-3-Methyl-4-nonen-1-ol	1.13
22	23.01	2,2,4-Trimethyl-3-(3,8,12,16-tetramethyl-heptadeca-3,7,11,15-trietraenyl)cyclohexanol	1.32
23	23.37	Cis,trans-2,3-Dimethylthiochroman-4-carbonitrile	2.87
24	24.88	Octahydro-3,3,6-trimethyl-2-(1-methylethylidene)-1H-inden-1-one	0.91
25	25.14	2,3,5,6-tetrahydro-6-phenylimidazo[2,1-b]thiazole	0.64
26	25.42	5-Chloro-6-methyl-3-(4-methylphenyl)-2H-1,4-oxazin-2-one	0.90
27	25.97	2-Cyclododecylidene ethanol	1.78
28	27.68	Hydroxyneoisolongifolane	0.40
29	28.97	(4-Octyldodecyl)-cyclopentane	0.32
30	29.54	(E)-11,13-Dimethyl-12-tetradecen-1-ol acetate	0.21
31	29.68	(E,Z)-2-Acetyl-5-[beta.,(4’-methyl-5’-thiazolyl)vinyl]thiophene	0.75
32	30.67	Tetracosane	0.25
33	31.07	7-phenyl-2-azafluoren-9-one	0.56
34	32.10	6-Nitrocyclohexadecane-1,3-dione	0.06
35	32.11	(2,4,4,4,16,16-D6)-3.aplha.,17.betadihydroxy-5beta androstane	0.06
36	32.28	(+)-(9-.beta.H)-labda-8(17),13(E)-diene-5-ol	0.28
37	33.53	2-Dodecen-1-yl(-)succinic anhydride	0.21
38	33.54	Dihydrophytol	0.21
39	**33.98**	**1-(4-Chlorophenyl)-5-(2-diethylaminoethenyl)-1H-tetrazole**	**4.64**
40	34.76	2-Dodecen-1-yl(-)succinic anhydride	0.12

41	35.21	(Tetrahydroxycyclopentadienone) tricarbonyliron(0)	0.21
42	35.57	11-cyclopentylheneicosane	0.10
43	36.89	3-Methylheptadecane	0.01
44	41.59	Methyl-9-octadecenoate	0.02
45	43.08	Hentriacontane	0.14
46	44.47	9,10-Dideuterooctadecanoic acid	0.02
47	45.86	Heptacosane	0.13

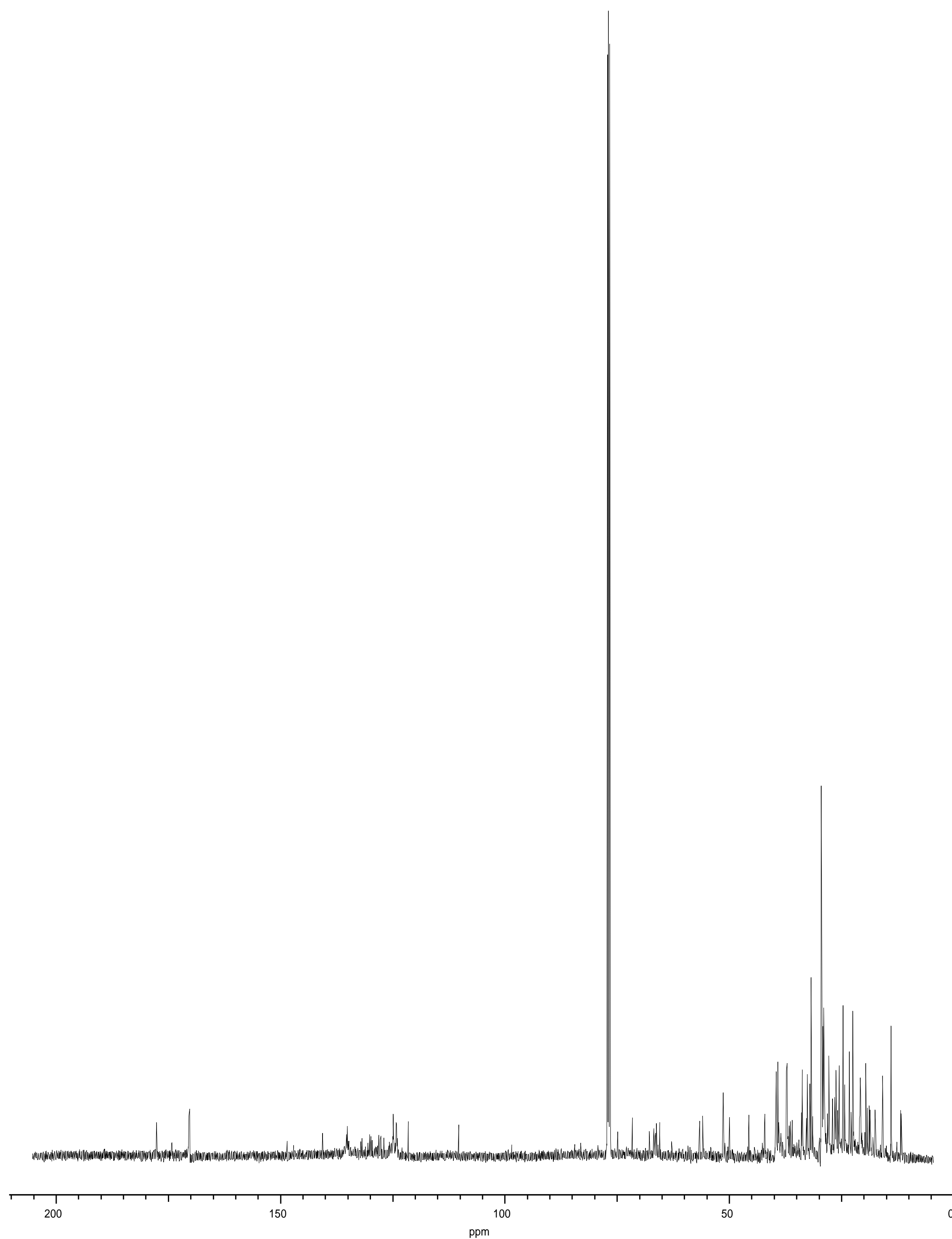
APPENDIX 18: GC-MS Spectra of *F. angolensis* essential oil



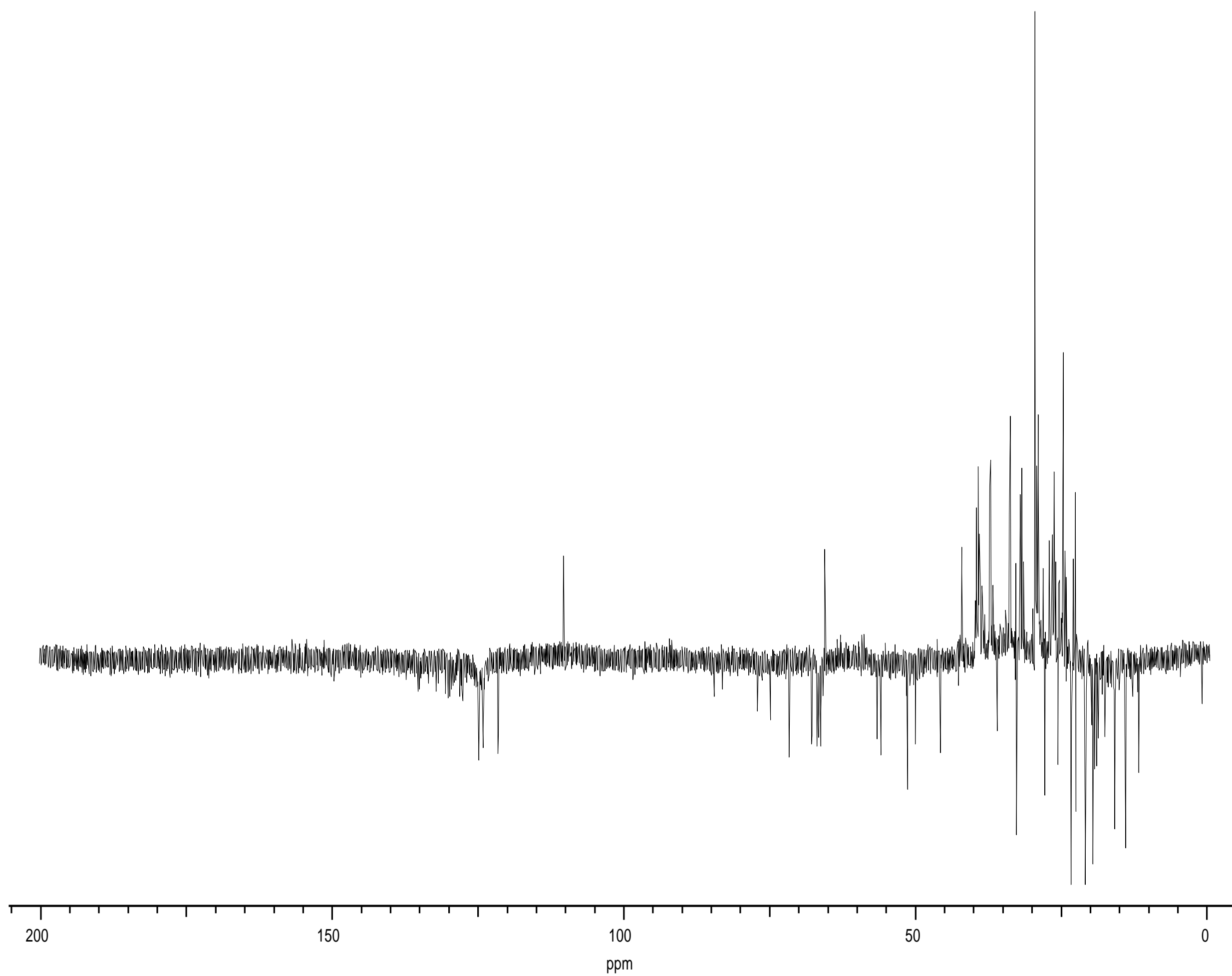
APPENDIX 19: ^{1}H NMR spectrum of compound **32**



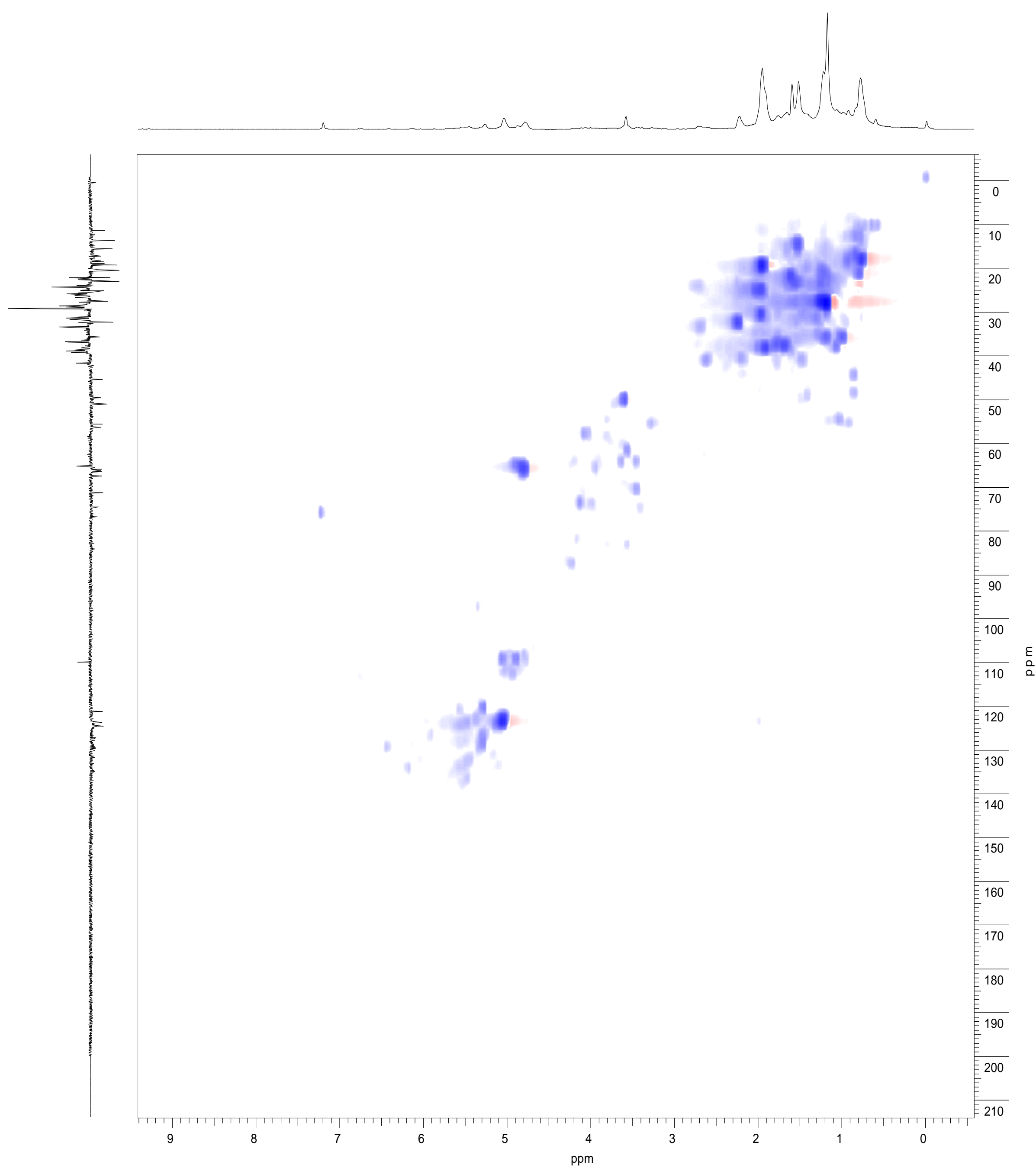
APPENDIX 20: ^{13}C NMR spectrum of compound **32**



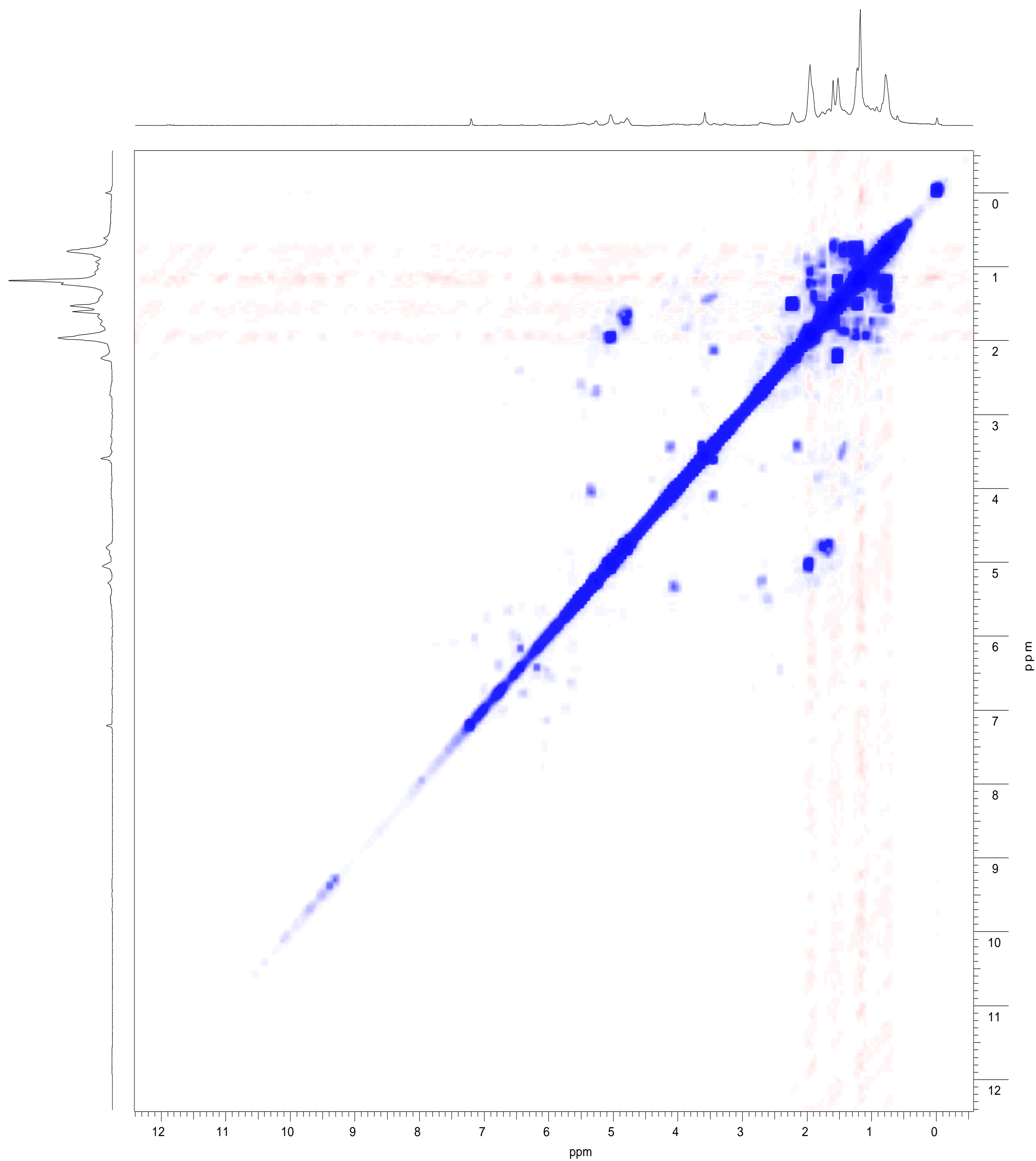
APPENDIX 21: DEPT NMR spectrum of compound **32**



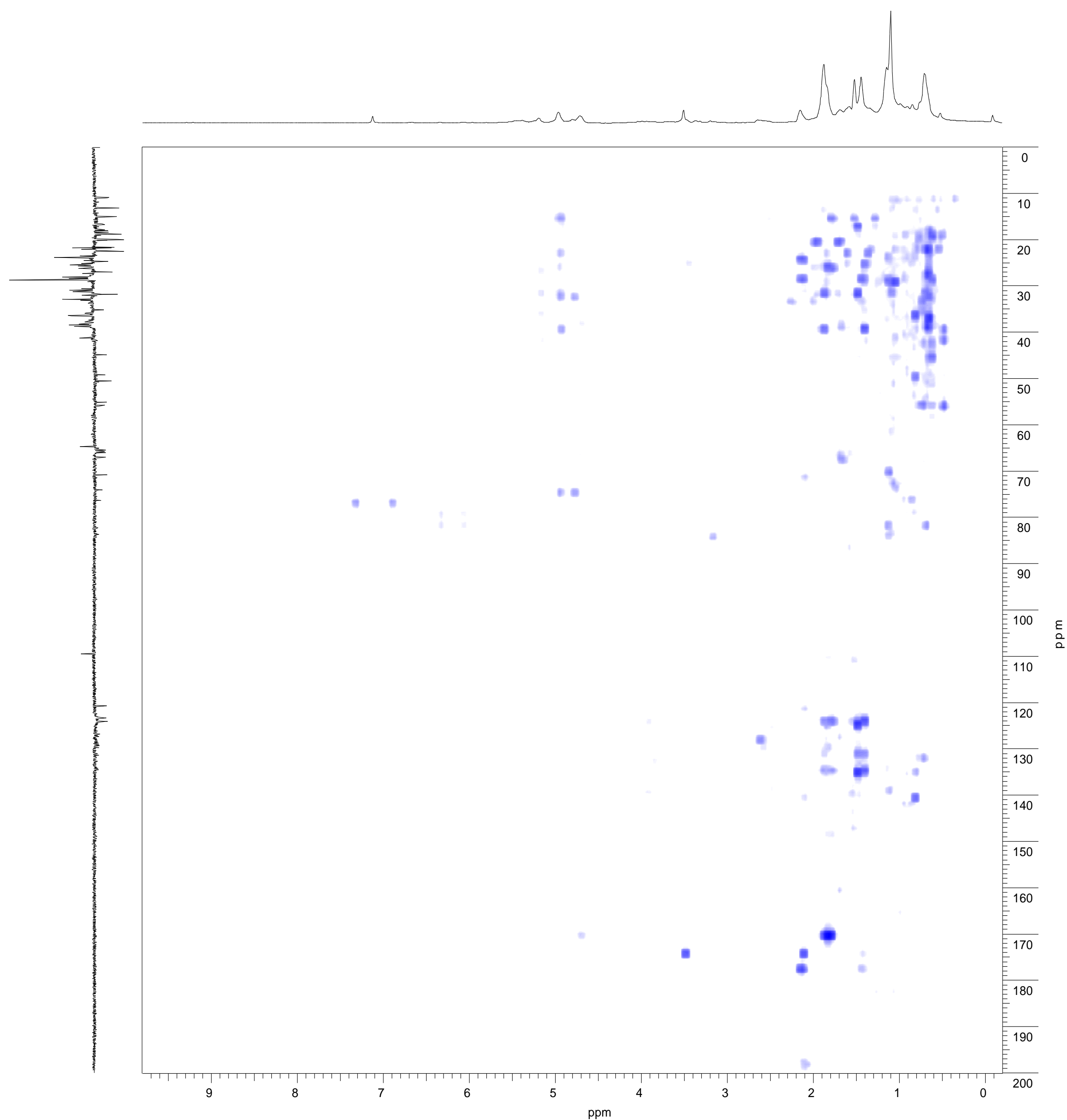
APPENDIX 22: HSQC NMR spectrum of compound **32**



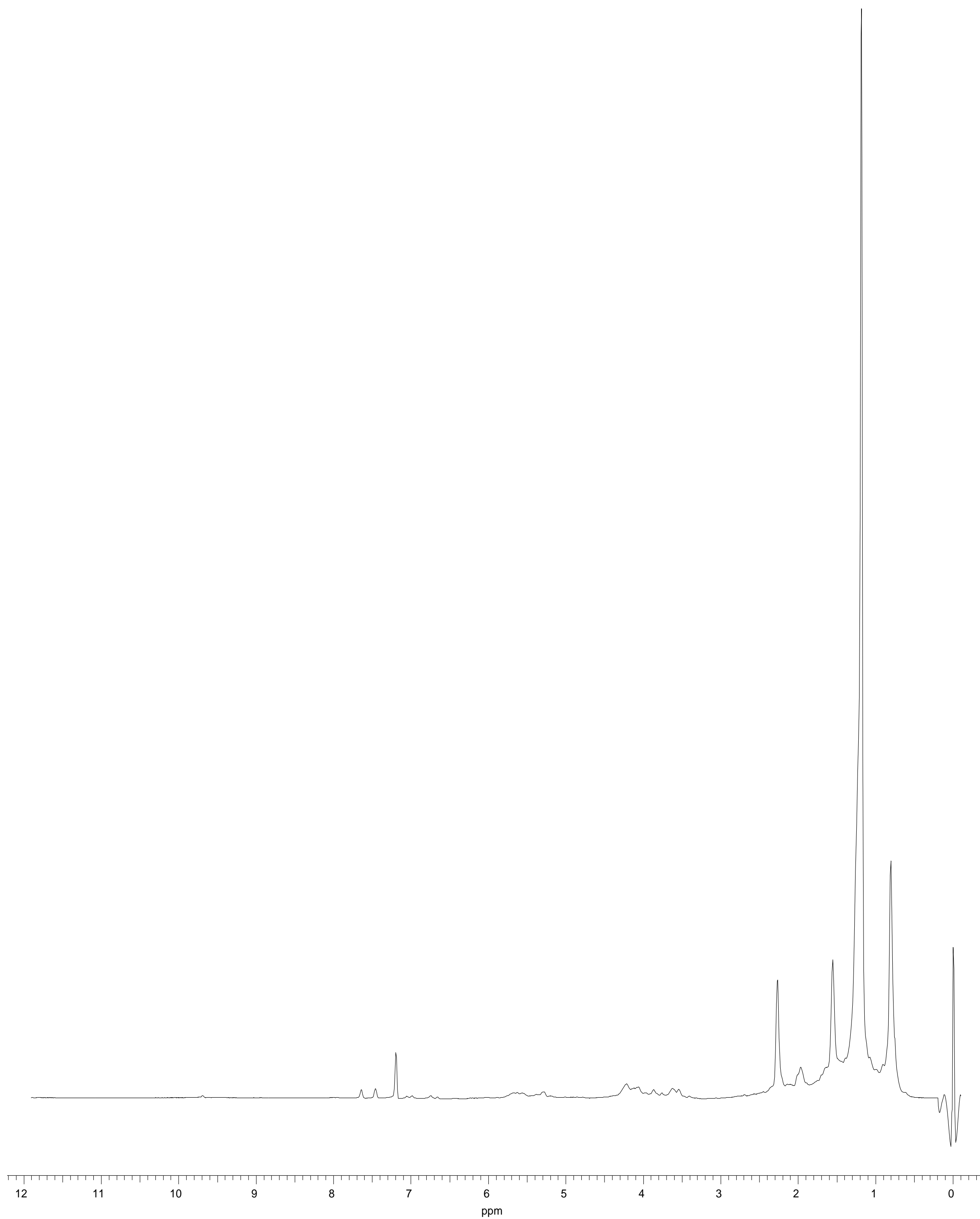
APPENDIX 23: $^1H/^1H$ COSY NMR spectrum of compound 32



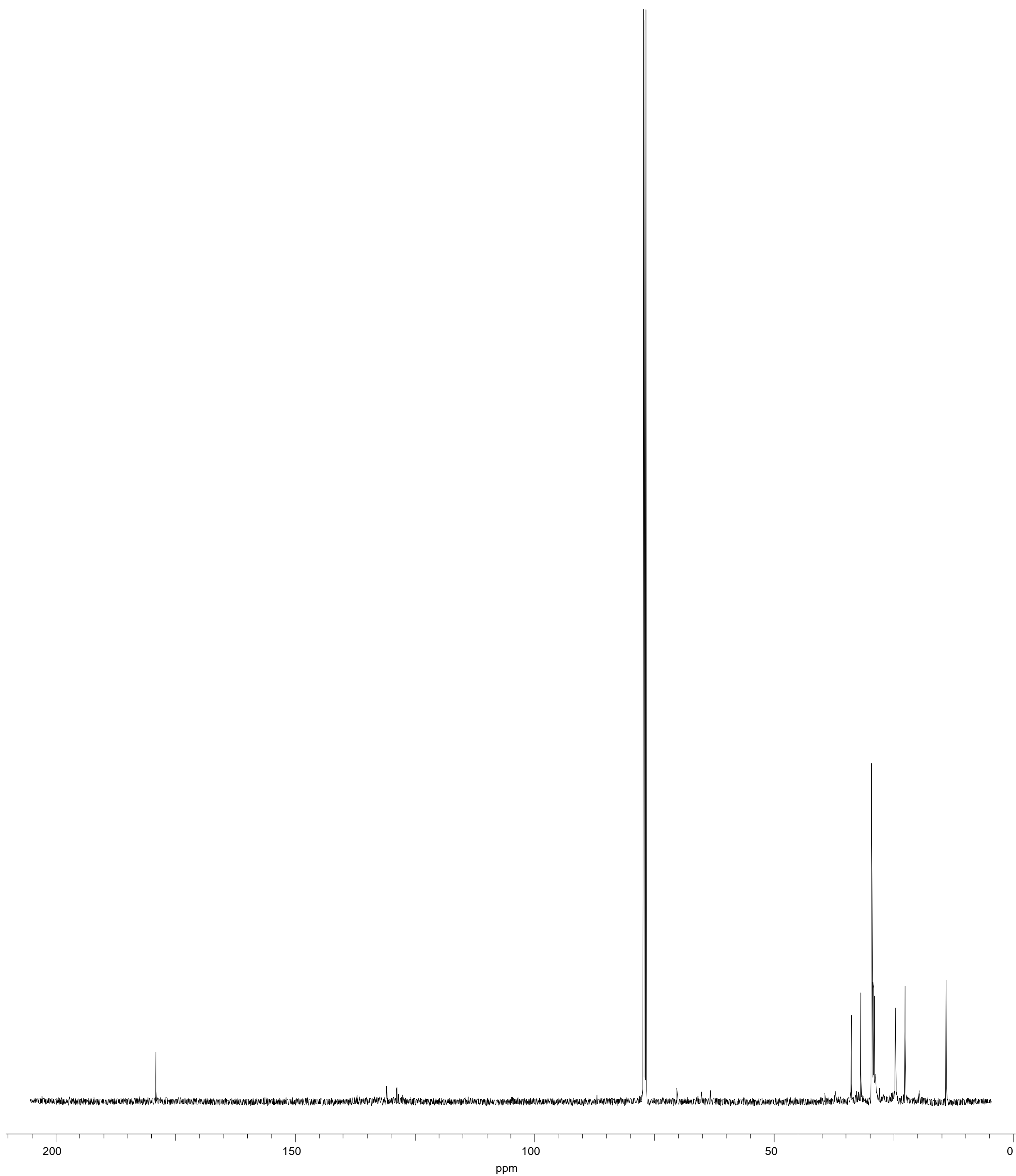
APPENDIX 24: ^{1}H- ^{13}C HMBC NMR spectrum of compound **32**



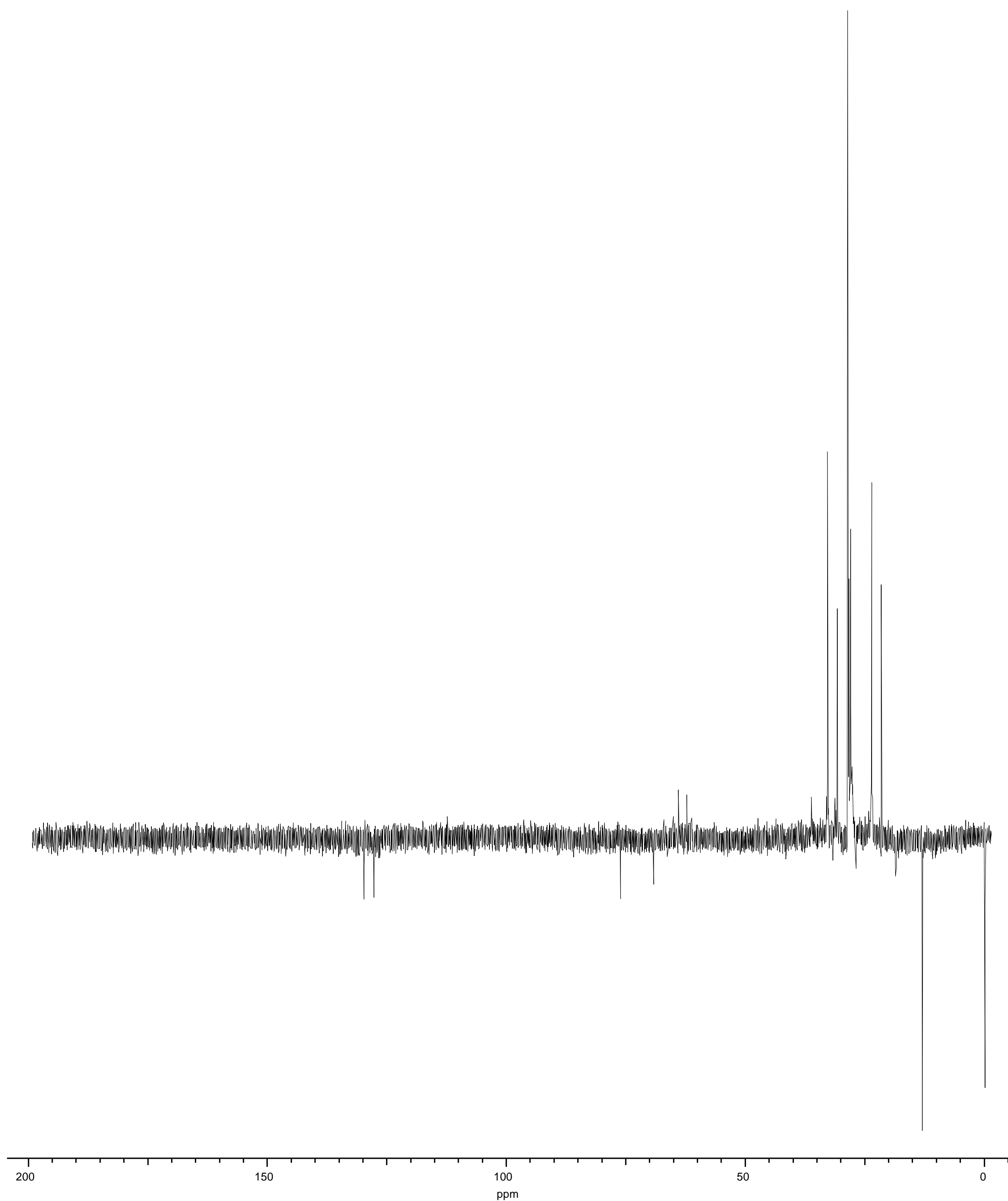
APPENDIX 25: ^{1}H NMR spectrum of compound **33**



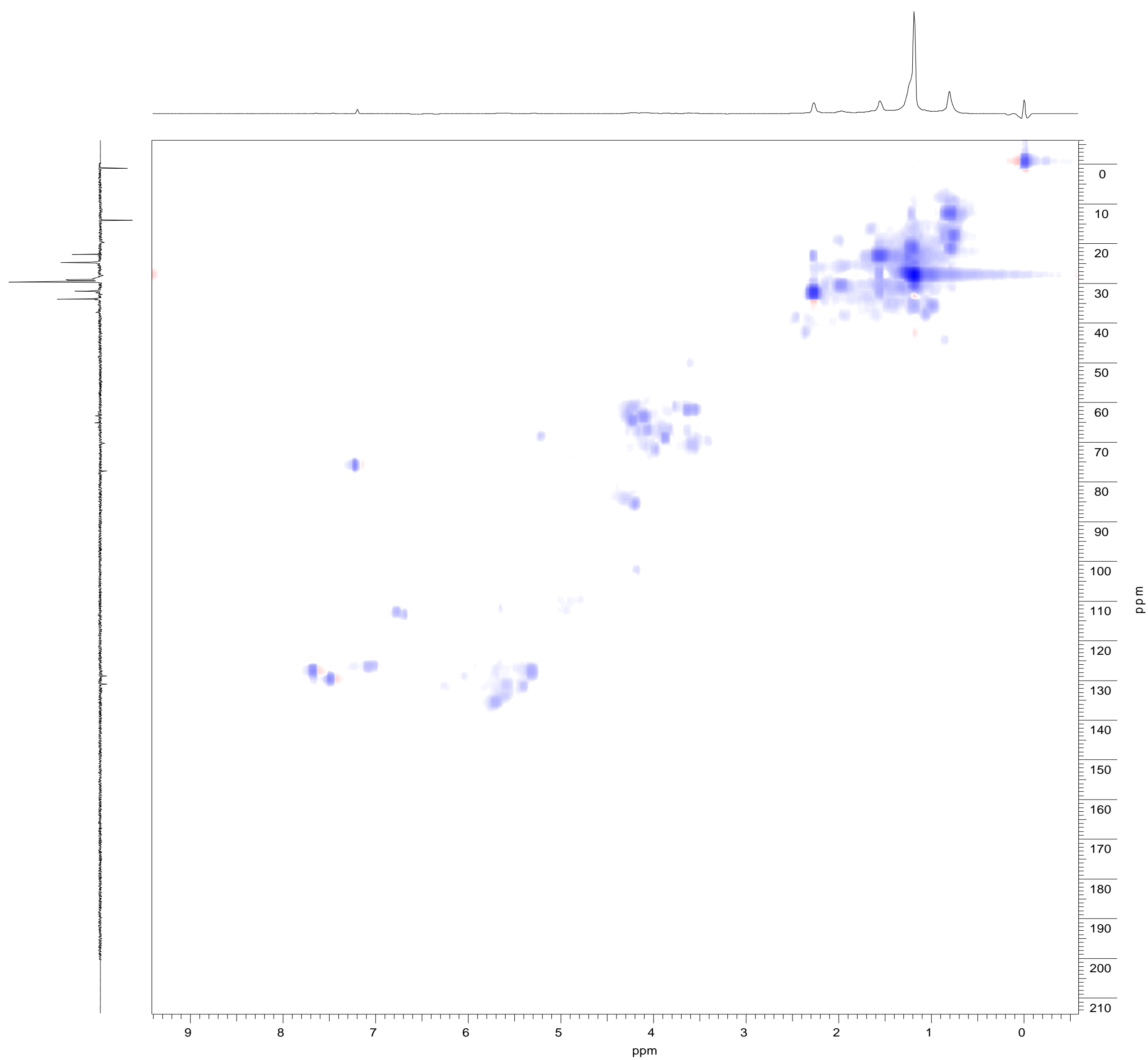
APPENDIX 26: ^{13}C NMR spectrum of compound **33**



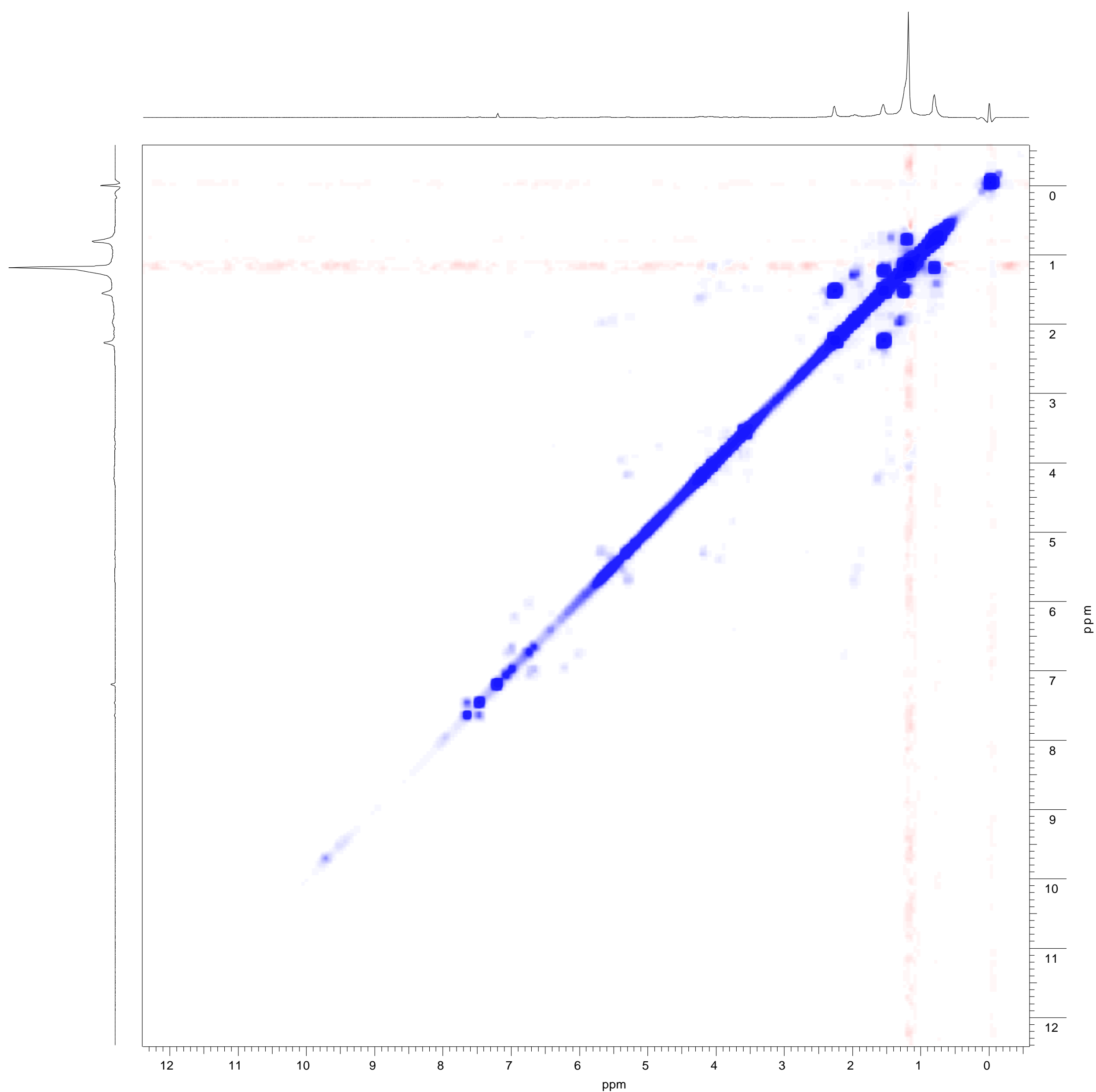
APPENDIX 27: DEPT NMR spectrum of compound **33**



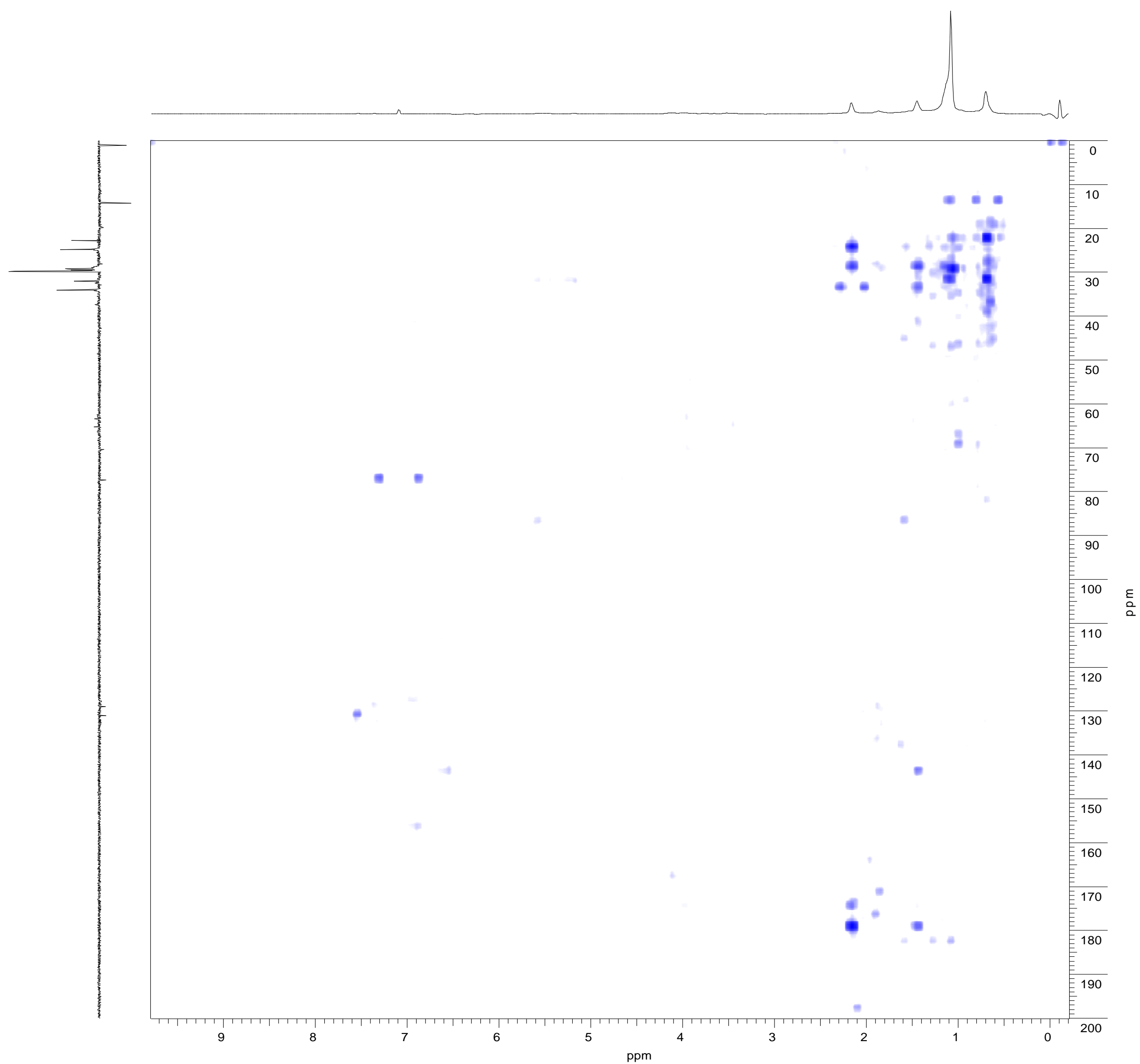
APPENDIX 28: ^{1}H- ^{13}C HSQC NMR spectrum of compound **33**



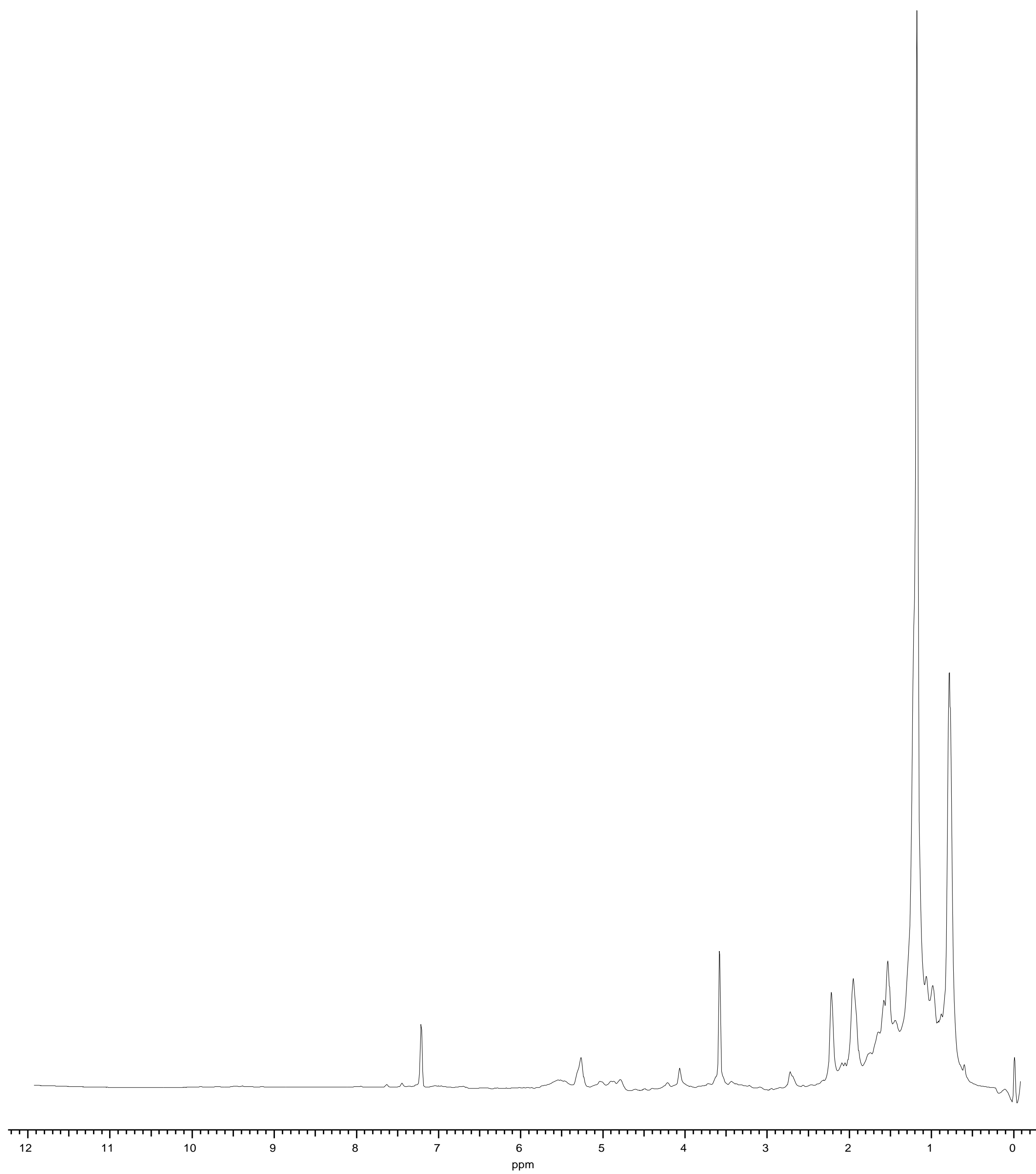
APPENDIX 29: $^1H/^1H$ COSY NMR spectrum of compound **33**



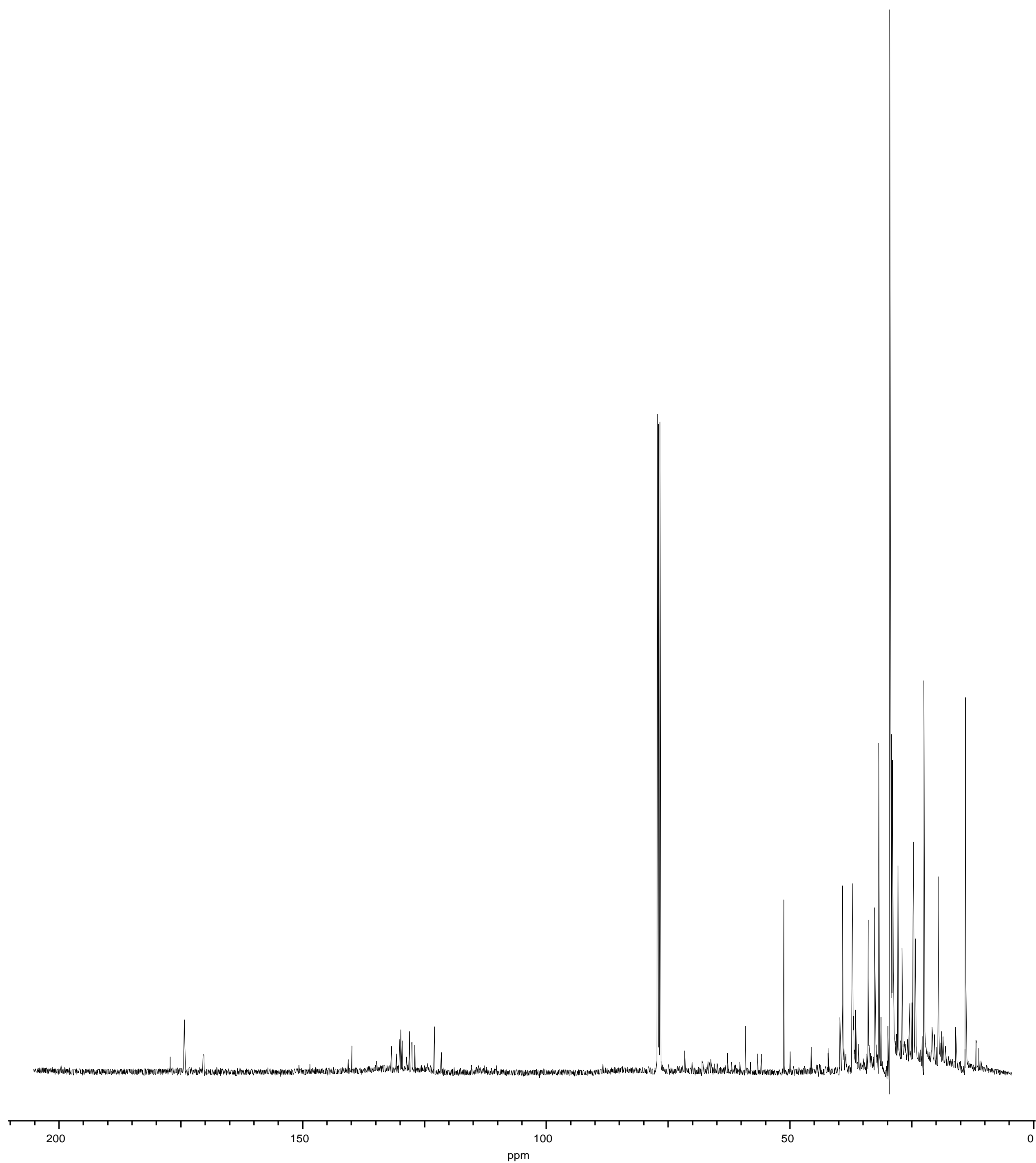
APPENDIX 30: ^{1}H- ^{13}C HMBC NMR spectrum of compound **33**



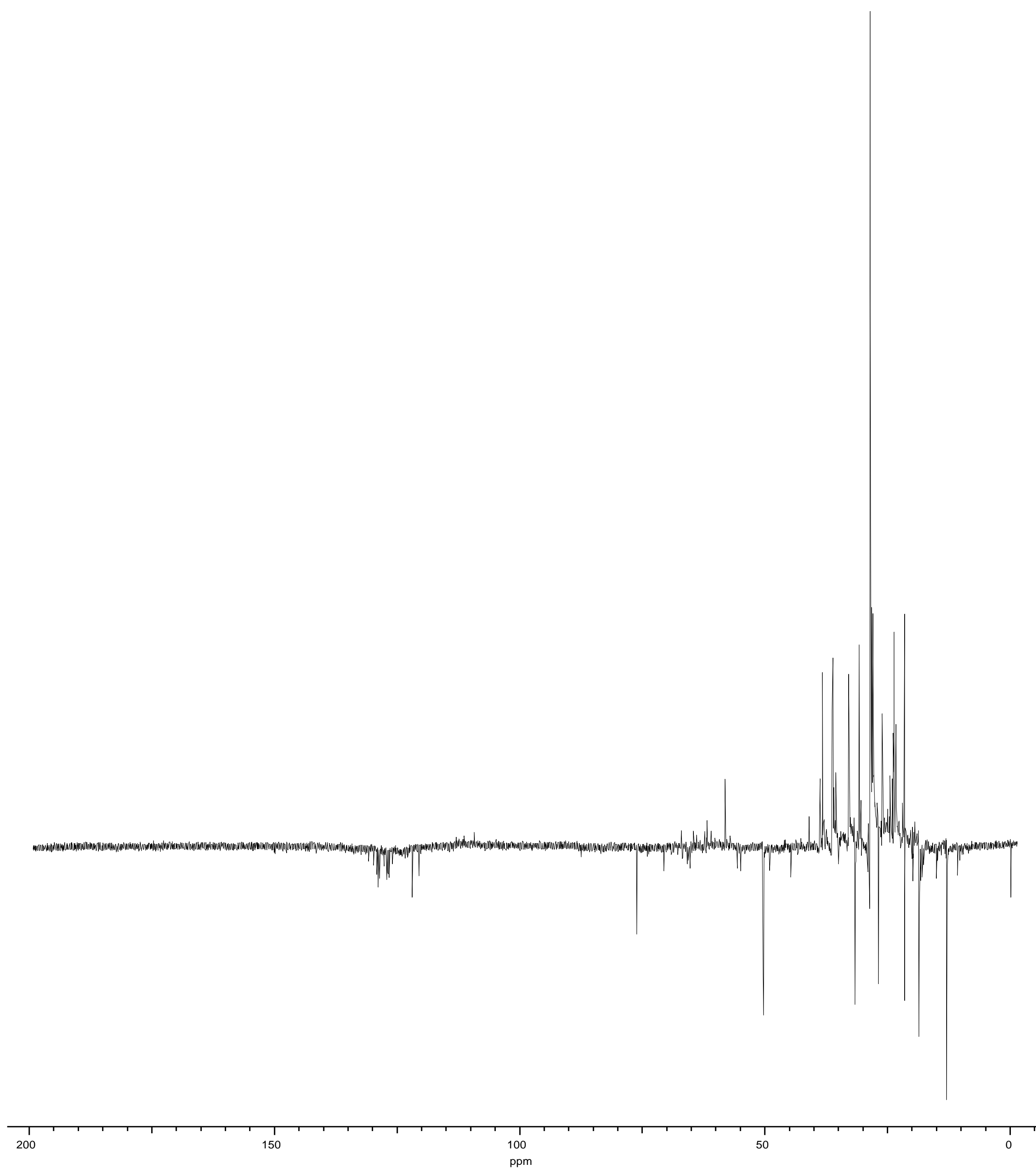
APPENDIX 31: ^{1}H NMR spectrum of compound **34**



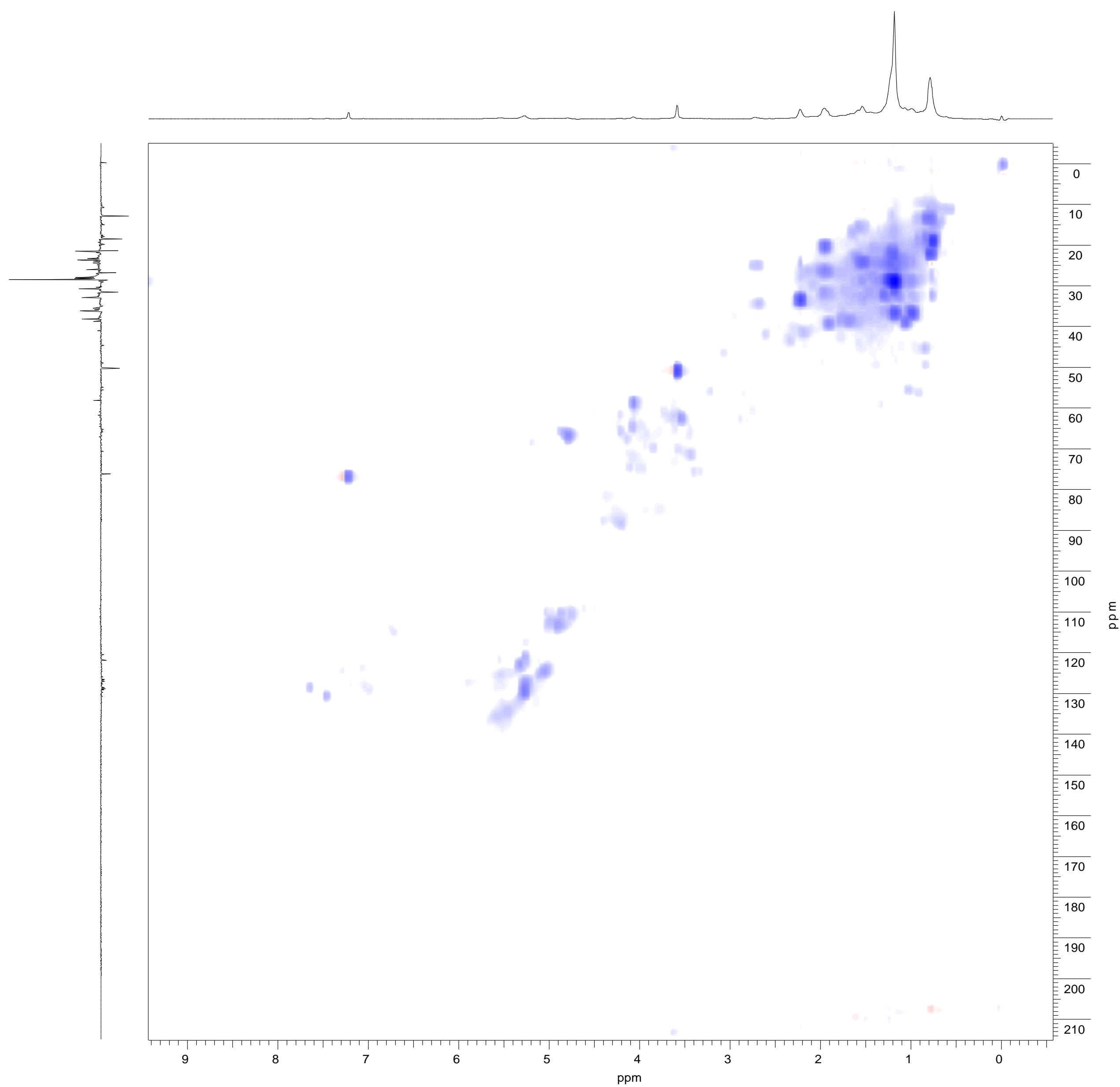
APPENDIX 32: ^{13}C NMR spectrum of compound **34**



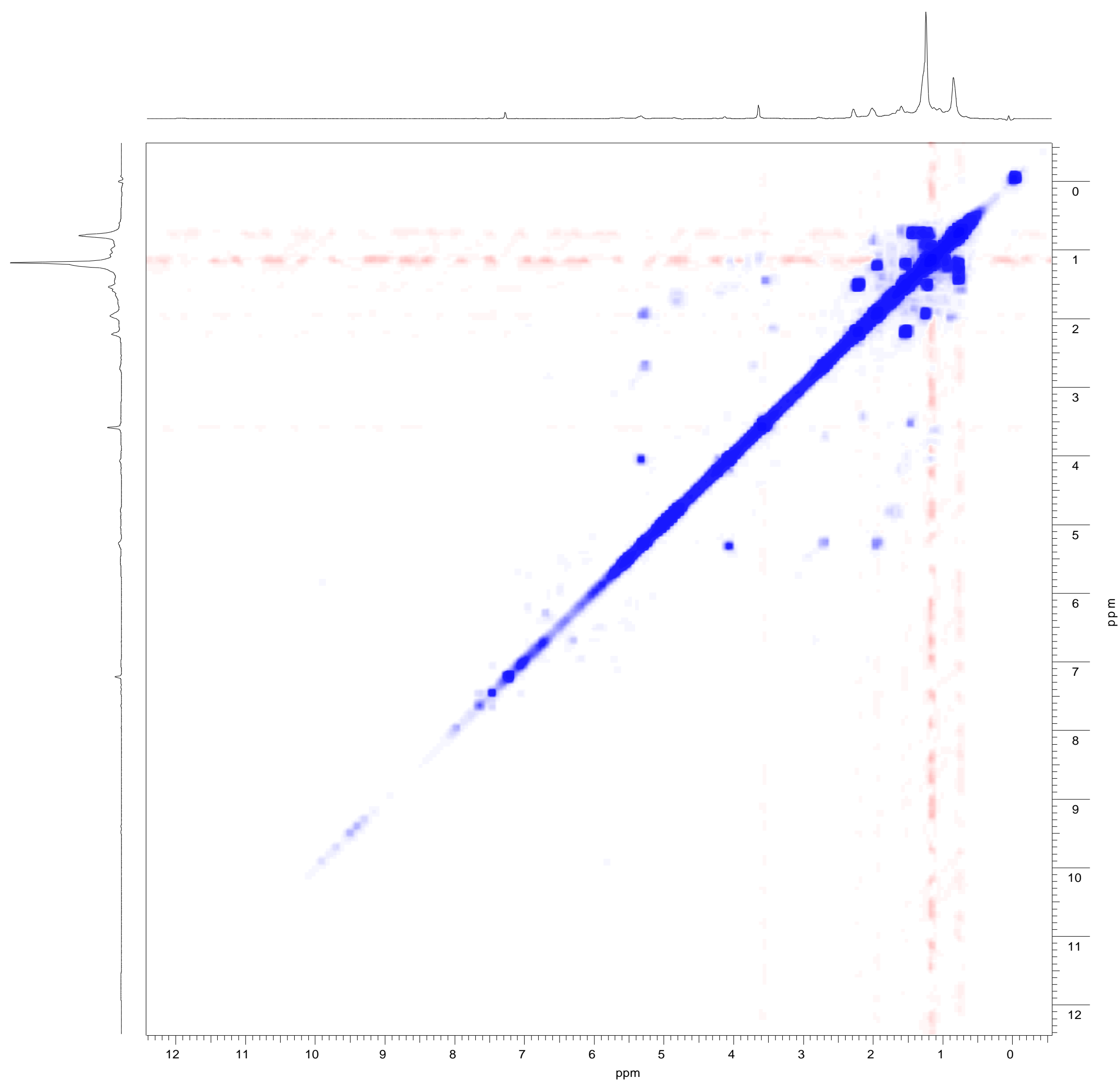
APPENDIX 33: DEPT NMR spectrum of compound **34**



APPENDIX 34: ^{1}H- ^{13}C HSQC NMR spectrum of compound **34**



APPENDIX 35: $^{1}H/^{1}H$COSY NMR spectrum of compound **34**



APPENDIX 36: ^{1}H- ^{13}C HMBC NMR spectrum of compound **34**