LARVICIDAL COMPOUNDS FROM THE PLANT *Zanthoxylum gilletii* AGAINST MALARIA VECTOR *Anopheles gambiae* sensu stricto

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EGERTON UNIVERSITY

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DECLARATION AND RECOMMENDATION

DECLARATION

This thesis is my original work and has not been submitted or presented for examination in any institution.

Signature …… Date ……...

Ombito Omollo Japheth

SM11/3065/11

RECOMMENDATION

We wish to confirm that this thesis has been prepared under our supervision and is presented for examination as per the Egerton University regulations with our approval.

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DEDICATION

This work is dedicated to my guardian Mr. Washington Masinde, siblings, fiancée and friends for their moral, emotional and financial support.

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ABSTRACT

Malaria is a serious health problem in many African countries. The *Anopheles gambiae* mosquito, which is the major vector for this disease, has developed resistance against synthetic pyrethroids, which are the main stay of insecticide treated bed nets. The development of insecticide resistance and side effects associated with synthetic pesticides has triggered intense research efforts towards natural products (for vector control) such as essential oils and the non-volatiles because of their efficacy and safety. In this study, the larvicidal potential of essential oil and secofuroquinoline alkaloids isolated from the leaves of *Zanthoxylum gilletii* was studied against larvae of the malaria vector *An. gambiae* s.s. The essential oil was extracted by hydrodistillation and its chemical compositions determined by GC - MS. The oil was dominated by monoterpenes and sesquiterpenes which accounted for 34.00 % and 38.30 %, respectively. For the larvicidal assay 20 third instar larvae were used for each concentration of the solution. The oil recorded LC_{50} and LC_{90} values of 57.73 and 140.24 ppm after 24 h exposure period, respectively. Methanol extract (LC_{50} = 497.62 ppm), ethyl acetate (LC_{50} = 155.65 ppm) and hexane extract (LC_{50} = 274 ppm) were all active against the third instar larvae of *An. gambiae s.s*. Bioassay-guided column fractionation of the ethyl acetate crude extract (6:4 ethyl acetate/hexane as eluting solvent mixture) afforded four fractions F_1, F_2, F_3 and F_4. All the four fractions were active against the larvae of *An. gambiae* s.s, F_1, (LC_{50} = 705.24 ppm), F_2 (LC_{50} = 542.33 ppm), F_3 (LC_{50} = 146.80 ppm) and F_4 (LC_{50} = 83.59 ppm). Further purification of F_3 and F_4 by Preparative Thin Layer Chromatography (PTLC) afforded mixture **Z.** Purification of mixture **Z** by Preparative High Performance Liquid Chromatography (PHPLC) afforded a pair of isomeric secofuroquinoline alkaloids *Z*-Dimethylrhoifolinate (**43**) and *E*-Dimethylrhoifolinate (**44**). Identification of compound **43** and **44** was done by the use of Mass Spectrometry, 1D and 2D NMR. Mixture **Z** exhibited LC_{50} value of 110.31 ppm and LC_{90} value of 216.31 ppm when tested against third instar larvae of *An. gambiae*. Compound **43** and **44** were isolated for the first time from *Z. gilletii*. The results obtained show that both the essential oil and the Secofuroquinoline alkaloids **43** and **44** isolated from the leaves of *Z. gilletii* have larvicidal effects and potentially can be used in malaria vector control.

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LIST OF ABBREVIATIONS AND ACRONYMNS

COSY	Correlation Spectroscopy
DDT	Dichlorodiphenyltrichloroethane
DMSO	Dimethyl sulphoxide
GC-MS	Gas Chromatography-Mass Spectrometry
HMBC	Heteronuclear Multiple Bond Correlation
HSQC	Heteronuclear Single Quantum Coherence
IRS	Indoor Residual Spraying
ITNs	Insect-treated bed nets
LC_{50}	Lethal concentration to kill 50% of the treated larvae
LC_{90}	Lethal concentration to kill 90% of the treated larvae
LLIN	Long-lasting insecticidal nets
NMR	Nuclear magnetic resonance spectroscopy
PTLC	Preparative Thin Layer Chromatography
SPSS	Statistical package for social sciences
TLC	Thin layer chromatography

CHAPTER ONE
INTRODUCTION

1.1 Background information

Mosquitoes are known vectors of various diseases which are life threatening. Some of these diseases include; malaria which is transmitted by *Anopheles gambiae*, yellow and dengue fever transmitted by *Ae. aegypti* and filarial disease which is transmitted by *Cx. quinquefasciatus* (Cheng *et al*., 2003; Das and Ansari, 2003; Magalhaes *et al*., 2010). According to the latest WHO estimates, there were approximately 219 million cases of malaria globally in 2010 and 660,000 fatal cases: approximately 90% of these fatal cases occurr in Africa (WHO, 2012). In Kenya, 76% of the entire population is at risk and one in thirteen children die before their fifth birthday (DHS, 2009). There are over 150 species of *Plasmodium* of which *Plasmodium falciparum*, which is transmitted by *An. gambiae*, is considered the most pathogenic human malaria species. It has the highest rates of complications and mortality, it accounts for about 80% of all human malarial infections and approximately 90% of the deaths (Keiser family foundation, 2007). Mosquito bites are also known to cause allergic responses including local skin reactions and systemic reactions such as urticaria and angioedema (Peng *et. al*., 2004).

Control of malaria is of serious concern in developing countries due to several factors and options for control. Lack of vital information regarding vector habits, such as: time of biting (evening and night), flight range of the vector (3km), feeding preference of adult female mosquito (humans), larval habitat preference and adult behavior-particularly, preference for biting and resting indoors and development of resistance (Walker, 2002). Vector control, which includes both anti-larval and anti-adult measures, constitutes an important aspect of any mosquito control programs. Either control by biological or chemical means is the basic requirement for planning an effective vector control strategy. Synthetic insecticides are today at the forefront of mosquito-controlling agents. However, overreliance on these chemical insecticides has generated several problems such as insecticide resistance, environmental pollution, safety risks for humans and domestic animals all of which have increased during the last five decades (Wattanachai and Tintanon, 1999; Amer and Mehlhorn, 2006). One of the strategies of the WHO in combating tropical diseases is to destroy their vectors or intermediate hosts. Among the various vector control measures, Larviciding has the greatest control impact on

mosquito populations because the larvae are concentrated, immobile and accessible (Tiwary *et al*., 2007).

Although several vaccines are under development, an effective vaccine for malaria is not yet available (Färnert *et al*., 2009). In recent years, control of mosquito populations has shifted steadily from the use of conventional chemicals towards more specific and environmentally friendly materials of botanical origin (Navneet *et al*., 2011). Crude solvent extracts of plant parts belonging to different families, essential oils or their chromatographic fractions have been shown to have various levels of bioactivity against different developmental stages of malaria vector mosquitoes (ICMR, 2003). Vector control by application of insecticides to larval habitats rather than the application of residual insecticides to houses is more effective (Tiwary et al., 2007). Botanical and microbial insecticides have been increasingly used for mosquito control because of their efficacy and documented non-toxic effects on non-target organisms (Ascher *et al*., 1995). For this purpose, many phytochemicals extracted from various plants species have been tested for their larvicidal and repellent actions against mosquitoes (Ciccia *et al.,* 2000; Ansari and Razdan, 2000). As part of the continued search for the biodiversity resource available in Kenya for natural products with utilizable bioactivity, larvicidal activity towards *An. gambiae* of extracts from *Zanthoxylum gilletii* species growing in Kenya were assayed.

Zanthoxylum gilletii is a tropical rainforest species from the family *Rutaceae*, distributed at an altitude of 1524 m above the sea level. It is known by various names among different communities across the east African region; among the Sukumas of Tanzania, it is known as Mfwakumbi, Munyeye in Luganda and Shikhuma among the Luhya community in Kenya. In Kenya the plant is found in Kakamega forest and is important in traditional medicine. The bark of *Z. gilletii* is used in traditional anti-malarial preparations among the Luhya community (Nyunja *et al.,* 2009). In Ghana and Nigeria, the bark of stem and roots is commonly used as an analgesic, especially to treat burns, rheumatism, headache, stomachache, toothache and pain after childbirth. The leaves are used to treat heart complaints and snake bites, whereas a leaf decoction is taken to treat cough, gonorrhea and schistosomiasis. The genus *Zanthoxylum* has great importance due to its phytochemistry and biological activity, and it is a promising source of various secondary metabolites. Various parts of the plants from the genus *Zanthoxylum* have been reported to exhibit larvicidal activity against a number of mosquito species. Alkaloids, sesquiterpene, lactones, coumarins, triterpenoids and limonoids have been isolated from *Z.*

gilletii, *Z. limonella*, *Z. rhoifolium*, *Z. usambarense*, *Z. syncarpum*, *Z. zanthoxyloides*, *Z.chalybeum* (Patino *et al*., 2012). Assessment of mosquito larvicidal properties of *Z. gilletii* leaf extracts against the medically important malaria vector *An. gambiae s.s* was done, aiming at the development of a new agent for mosquito control based on plant source.

1.2 Statement of the problem

The malaria vector *Anopheles gambiae s.s* has developed resistance against the current synthetic pyrethroid insecticides used for its management; as a result, a huge human population continues to die of malaria despite the presence of these insecticides in the market. Over 500 million people globally are infected with malaria and approximately 1.2 to 2.7 million die per year, of which over 75% of these deaths occur in African children under the age of five years. In addition, some of the synthetic insecticides such as DDT have been documented as having generated several problems including environmental pollution and harmful effects on non-target organisms.

1.3 Objectives

1.3.1 General objective

To determine the larvicidal activity of the crude extract(s) and the pure compounds from *Z. gilletii* leaves against the larvae of *An. gambiae s.s*

1.3.2 Specific objectives

1. To screen the crude extract(s) from *Z. gilletii* leaves against malaria vector *An. gambiae s.s*
2. To perform bioassay-guided chromatographic isolation and purification of the active compounds.
3. To determine the mosquito larvicidal activity of essential oil and characterize its compounds.
4. To elucidate the structures of the isolated larvicidal pure compounds using NMR and MS spectroscopic techniques.

1.4 Hypotheses

1. That the crude extracts from *Z. gilletii* leaves will not exhibit significant larvicidal activity against malaria vector, *An. gambiae s.s*
2. That the isolated and purified compounds will not possess significant mosquito larvicidal activity.
3. That the crude essential oil will not exhibit significant larvicidal activity against *An. gambiae s.s*
4. That the spectroscopic data obtained will not provide the requisite information for the structure elucidation of the bio-active secondary metabolites.

1.5 Justification

The emergence and spread of resistance to a majority of present synthetic insecticides by *An. gambiae s.s* and the environmental pollution coupled with the safety risks for both human and domestic animals posed by these insecticides means that alternative ways of managing *An. gambiae s.s* need to be developed urgently. Botanical and microbial insecticides have been documented as being effective against mosquitoes, having non-toxic effects on non-target organisms as well as being environmentally friendly. Several compounds isolated from both the non-volatiles and the volatiles (essential oil) of various plant parts from the *Zanthoxylum* genus have been documented as possessing either adulticidal or larvicidal activity against the *An. gambiae*. The use of larvicidal active compounds such as those from *Z. gilletii* leaf extracts may constitute an important alternative to these synthetic insecticides for the control of *An. gambiae*.

CHAPTER TWO
LITERATURE REVIEW

2.1 Vector control techniques

Mosquitoes are known vectors of various diseases which are life threatening. Some of these diseases include; malaria which is transmitted by *Anopheles gambiae*, yellow and dengue fever transmitted by *Ae. aegypti* and filarial disease which is transmitted by *Cx. quinquefasciatus* (Cheng *et al*., 2003; Das and Ansari, 2003; Magalhaes *et al*., 2010). Malaria vector *An. gambiae* has developed resistance against the synthetic insecticides (Walker, 2002). Various methods have been employed over time to reduce the mosquito population, some of which include:

2.1.1 Environmental Modification

As an alternative to complete elimination of wetlands, modification projects could involve the creation of channels to improve water flow in areas of standing water, filling small ponds or water-collecting depressions, or changing the banks of water impoundments to reduce mosquito populations. As rivers and streams can create anopheline larval breeding sites, particularly in slow-moving pools with heavy vegetation, regrading streams and even straightening riverbanks may reduce vector populations (Thevasagayam, 1985). Some of these activities require regular maintenance, whereas others represent permanent changes to the landscape (which may require substantial initial effort and expense). An important component to environmental modification addresses problems of man-made vector breeding sites associated with water-holding structures in mini-dams and small-scale irrigation projects.

2.1.2 Environmental Manipulation

Environmental manipulation refers to activities that reduce larval breeding sites of the vector mosquito through temporary changes to the aquatic environment in which larvae develop. Water management activities include changing water levels in reservoirs, flushing streams or canals, providing intermittent irrigation to agricultural fields (particularly rice), flooding or temporarily dewatering man-made or (where feasible) natural wetlands, and changing water salinity. Manipulation of vegetation may also be useful. Planting water-intensive tree species, such as *Eucalyptus robusta*, can reduce standing water in marshy areas (Sharma and Sharma, 1998). Planting shade trees near potential larval habitats may help reduce the abundance of vectors, such as *An. gambiae*, *An. funestus, An. minimus*, and *An. sundaicus* that prefer sunny conditions for larval development (Rafatjah, 1988).

2.1.3 Modification of Human Habitations or Behaviours

Changes in placement and structure of human habitations as well as changes in behaviour may reduce human-vector contact (WHO, 1992; Ault, 1994). Humans have long practiced a simple form of malaria prevention by locating houses away from breeding sites, although settlements must be near enough to a water source to supply domestic needs. Even though many anopheline adults can fly as far as 3 km from their larval habitat, locating settlements 1.5 to 2 km away from major breeding sites may significantly reduce transmission (WHO, 1992). Preferred housing sites should also be on well drained, high ground, upwind (rather than downwind) of probable breeding sites. Raising houses on poles may also reduce transmission, as many vector species tend to fly low (Charlwood *et al*., 1984). Although it has often been suggested that removing vegetation from around houses may control mosquitoes by removing resting sites, one of the few studies evaluating this practice found it had no effect on anophelines (Stephens *et al*., 1995).

2.1.4 Indoor residual spraying (IRS)

Indoor residual spraying **(**IRS) involves the coordinated, timely spraying of the interior walls of homes with insecticides. Twelve insecticides including DDT belonging to four chemical classes are recommended for IRS in vector control of malaria (Najera and Zaim, 2001), which collectively address only three different modes of toxic action (Nauen, 2007). Apart from its toxic action, DDT also has repellent and irritant properties (Roberts *et al*., 2000). Thus indoor spraying with DDT repels some of the mosquitoes from entering a house, and causes others to be irritated upon contact with the chemical and leave without biting and picking up a lethal dose of the toxicant (Kuhlow, 1962); those that are exposed to DDT are killed by the toxic action.

Compared to methods aimed at reducing vector population densities, the strength of IRS lies in its effect on shortening the life span of adult mosquitoes near their human targets, which has a critical impact on malaria transmission (MacDonald, 1957). The bulk of evidence on the impact of IRS on transmission reduction is from observational data obtained during the malaria eradication era and additional small-scale trials in the 1950s and 1960s (Bruce-Chwatt, 1984). In northern Nigeria in the 1970s, IRS substantially reduced malaria transmission (Molineaux and Gramiccia, 1980), though not as much as in western Kenya in the same period. The difference was attributed to the greater indoor resting habit of the local vector in Kenya.

Contemporary data from a range of sentinel sites in Africa indicate that the occurrence of resistance to DDT is widespread, especially in West and Central Africa (ANVR, 2005). The major African vector *Anopheles gambiae* s.s. showed resistance to DDT in the majority of tests. Further, there is recent evidence of resistance in *An. gambiae* s.l. in Ethiopia (PMI, 2008) a country which for many years has been the largest DDT user on the continent. There are signs of DDT resistance in *Anopheles arabiensis* from Uganda, Cameroon, Sudan, Zimbabwe and South Africa. Pyrethroids appear to be the most cost-effective alternatives to DDT in malaria control (Walker, 2000). However, resistance to pyrethroids is already widespread, and the occurrence of cross-resistance between DDT and pyrethroids severely limits the choice of insecticides.

2.1.5 Insecticide-treated bed nets (ITNs)

The main current alternative to IRS is the use of insecticide-treated bed nets (ITN) (RBM, 2008). An insecticide-treated net is a mosquito net that repels and/or kills mosquitoes being exposed to the insecticide on the netting material. Two categories of ITNs are available: conventionally treated nets and long-lasting insecticidal nets (LLIN). Conventionally treated nets require regular retreatment; a follow-up action which has proven difficult to achieve at field level. The latter is a relatively new technology, which retains the efficacy for at least three years, thus removing the need for retreatment (Malima *et al*., 2008).

Pyrethroid insecticides, which are used to treat nets, have an excito-repellent effect that adds a chemical barrier to the physical one, further reducing human-vector contact and increasing the protective efficacy of the mosquito nets. Most commonly, the insecticide kills the malaria vectors that are exposed to the ITN. By reducing the vector population in this way, ITNs, when used by a majority of the target population, provide protection for all people in the community, including those who do not themselves sleep under nets (Binka *et al*., 1998; Hawley *et al*., 2003). Although ITNs have been shown to avert around 50% of malaria cases (Clarke *et al*., 2001), Pyrethroid resistance in malaria vectors has been reported from West, East and southern Africa (ANVR, 2005) involving several resistance mechanisms. World Health Organization currently recommends the purchase of LLIN (WHO, 2007), but scaling up of LLIN to meet the demand is a challenge (RBM, 2008). Furthermore, disposal of LLIN is a potential problem.

2.1.6 Larviciding

Larviciding involves the use of chemical insecticides as larvicides to control mosquito breeding in aquatic habitats such as the edges of swamps, lakes, pools, and riversides. Larviciding has the greatest control impact on mosquito populations because the larvae are concentrated, immobile and accessible (Tiwary *et al*., 2007). Larviciding was the main intervention responsible for the eradication of introduced *An. gambiae* s.l. populations from Northeastern Brazil in the 1930s and in the Nile Valley of Egypt in the 1940s (Shousha, 1948). Larviciding is not necessarily a recommended option for control in situations where this species breeds in numerous types and sizes of water bodies.

World Health Organization recommends four organophosphate insecticides for larviciding (WHO, 2006), but the broad-spectrum effect of these chemicals when applied to aquatic ecosystems are a concern. Larviciding and other larval control methods are promising supplementary interventions to IRS and ITN where they are applicable and feasible, particularly in urban settings. Generally, larviciding for malaria vector control is effective where (and when) breeding sites are easily accessible and manageable (UNEP, 2008).

2.1.7 Repellents and attractants

Chemical repellents could have a useful supplementary role to ITN (Rowland *et al*., 2004). Repellents are available for application to the skin or clothes or as repellent soaps, or as low-cost vaporizers. Some novel compounds have shown to be promising candidates for mosquito repellents (Katritzky *et al*., 2008). Moreover, innovative work is in progress on the biochemical mechanisms underlying host finding and feeding through identification of the key components of human odour that are responsible for differences in attractiveness to malaria vectors.

2.1.8 Predation

The use of predatory fish that feed on mosquito larvae was one of the oldest suggested methods for controlling vector diseases at the larval stages. Prior to the 1970s, mosquito control by means of fresh water *Gambusia affinis* predominated. These native southeastern United States species were widely introduced around the world for mosquito control (Walker, 2002). Other fish species, like those belonging to the family *Cyprinodontidae*, were also copiously used, for at least 100 years, in larval control (Meisch, 1985). As compared to chemical agents, larvivorous fish were shown to be more effective. These fish are harmless to both humans and wildlife,

cheap to produce in most cases, and exhibit minimal risks of mosquito resistance (Yap, 1985). Although promising, the use of larvivorous fish as a means of vector control agent was questioned with time. Introducing new fish species into certain aquatic environments showed great variability at the level of efficacy and exerted many negative impacts on the native fauna where these fish were brought in. The introduction of *Gambusia* in certain habitats, for example, resulted in the elimination of many native fish species from these habitats (Rupp, 1996).

2.1.9 Fungi

Insect pathogenic fungi have only partly been explored for their potential in vector control. A selected fungus has shown promising results for controlling adult *Anopheles* mosquitoes (Scholte *et al*., 2004). When applied to surfaces inside houses where female mosquitoes rest after blood meals, the fungus infects and kills the insects upon contact without being ingested. Studies have predicted that malaria transmission could be substantially reduced by this method (Scholte *et al*., 2005), but several issues related to residual activity, resistance development and effects on non-target organisms remain to be addressed (Kanzok and Jacobs, 2006).

2.1.10 House improvement

House improvement can contribute significantly to malaria transmission control. Plastering of walls and ceiling fills the crevices that serve as the refuge for adult mosquitoes. A study in Sri Lanka showed that the risk of malaria was 2.5 times higher in poorly constructed houses than in houses of good construction (Gunawardena *et al*., 1998). Moreover, screening of houses or sleeping quarters to keep mosquitoes out at night is a protective option for houses with solid walls (Lindsay *et al*., 2002).

2.2 Botanicals as prospective insecticides

Natural products of plant origin with insecticidal properties have been tested in the recent past for control of various insects, pests and vectors. Secondary metabolites of plants, mostly produced by plants for their 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).

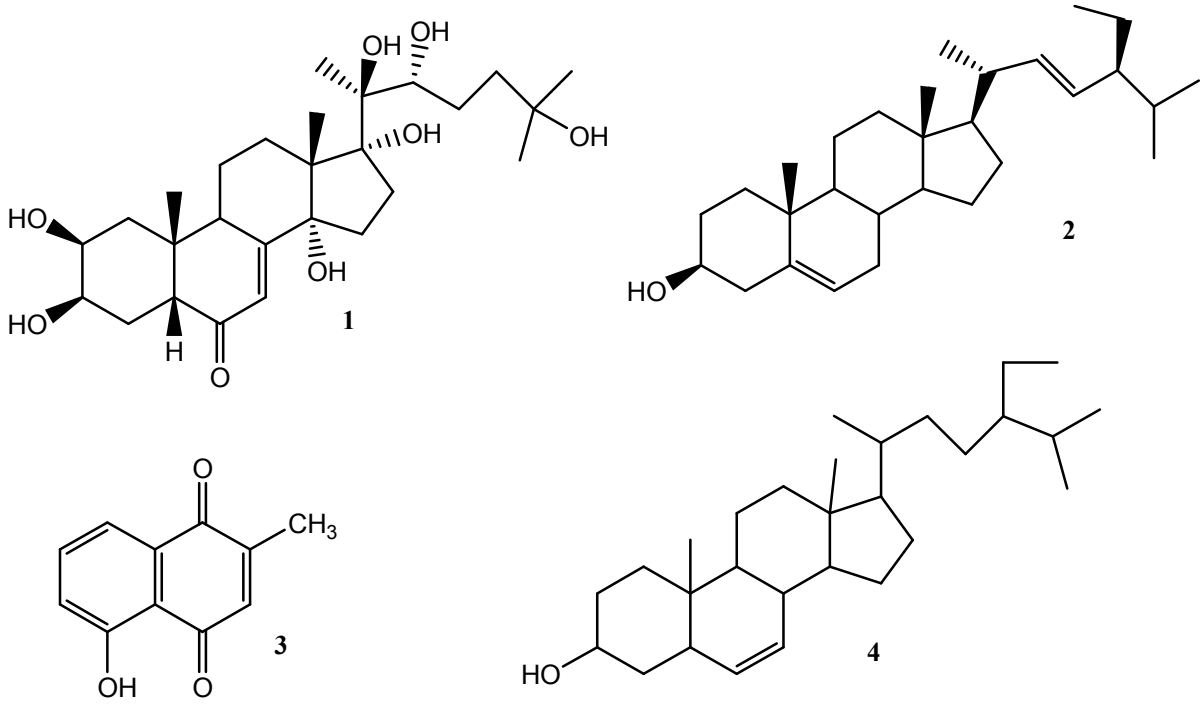
Secondary metabolites of plants (such as steroids, alkaloids, terpenoids, saponins, phenolics, and essential oil) are the active toxic ingredients of plant that are evolved to protect

them from herbivores and are associated with a wide range of biological activities (Chowdhuri *et al*., 2007). These secondary metabolites produce toxic substances with relatively non-specific effects on a wide range of molecular targets. These targets range from proteins, nucleic acids such as DNA and biomembranes (Rattan, 2010). This in turn, affects insect physiology in many different ways and at various receptor sites, the principal of which is abnormality in the nervous system.

2.2.1 Plant products as mosquito larvicides

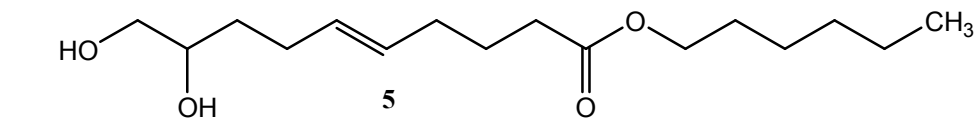
Members of different plant families have been documented to exhibit larvicidal activity against vectors of different diseases such as malaria, yellow and dengue fever, filiriasis and many others. Both their volatiles (essential oil) and the non-volatiles have been shown to be active against the *Anopheles* species. From acetone extract of stem bark of *vitex schiliebenii* (verbenaceae) two compounds phytoecdysteroid 20-hydroxyecdysone (**1**) and stigmasterol (**2**) were isolated and showed activity against *An. gambiae* larvae (Nyamoita *et al*., 2012). These active principles in the acetone extract of stem bark had LC_{50} values of 1.00 and 8.145 ppm against late third and early fourth instar larvae after 72 h exposure period, respectively. Methanolic leaf extract of *Ocimum gratissimum* (Lamiaceae) was found to be active against *An. gambiae* larvae with LC_{50} and LC_{90} values of 73.6 and 1021 mg/mL, respectively (Ofoegbu *et al*., 2013).

Barasa *et al* (2009) isolated two compounds 5-hydroxy-2-methyl-1, 4-naphthoquinone (**3**) and β-sitosterol (**4**) from ethyl acetate root extract of *Plumbago dawei* (Plumbaginaceae) which showed larvicidal activity against *An. gambiae*. Ethyl acetate leaf extract of *Aloe turkanensis* (Aloeaceae) was found to have high larvicidal activity with 100% mortality at a concentration of 0.2 mg/mL. Its LC_{50} value of 0.11 mg/mL against third instar larvae of *An. gambiae* was reported (Matasyoh *et al*., 2008). Apart from the non-volatiles, the volatiles have also been shown to have activity against *An. gambiae*. Essential oil from the leaves of *Plectranthus amboinicus* (Lamiaceae) showed activity against third instar larvae of *An. gambiae* (Kweka *et al*., 2012). The oil showed good larvicidal potential after 48 h of exposure period against *An. gambiae* with LC_{50} and LC_{90} values of 25.51 and 111.17 ppm, respectively.



2.2.2 Rutaceae family as a potential source of mosquito larvicide

Plants from Rutaceae family have been documented to exhibit larvicidal, adulticidal or repellent activities against various species of mosquitoes. Three compounds hexyl-9,10-dihydroxydec-5-enoate (**5**), methylheneicosane ester derivative (**6**) and Phenanthrene carboxylic acid derivative (**7**) from the leaves of *Fagaropsis angolensis* (Rutaceae) showed larvicidal activity against third instar larvae of *An. gambiae* (Mudalungu *et al*., 2013). The compounds **5**, **6** & **7** exhibited LC_{50} values of 245.5 mg/L, 144.4 mg/L and 147.6 mg/L, respectively. Their LC_{90} values were 471.6 mg/L, 259.4 mg/L and 292.1 mg/L respectively against the third instar larvae of *An. gambiae*. Leaf extract of *Feronia limonia* exhibited larvicidal activity against three mosquito species *Cx. quinquefasciatus*, *An. stephensi* and *Ae. aegypti* (Rahuman *et al*., 2000). These leaf extracts had LC_{50} values of 129.24, 79.58 and 57.23 ppm, respectively for the three species. Methanolic leaf extract of *Atlanta monophylla* had LC_{50} values of 0.05 mg/L, Insect growth regulating activity with IE_{50} value of 0.065 mg/L against *An. stephensi* (Sivagnaname and Kalyanasundaram, 2004).



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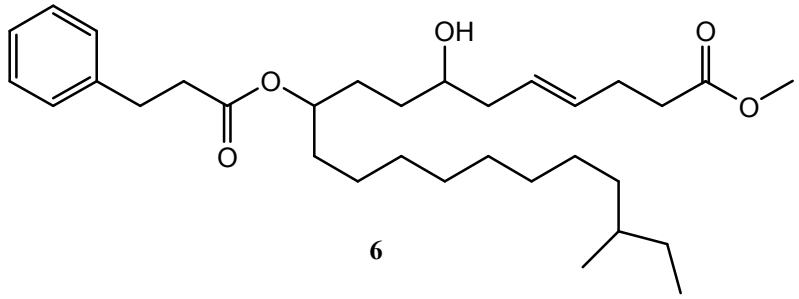

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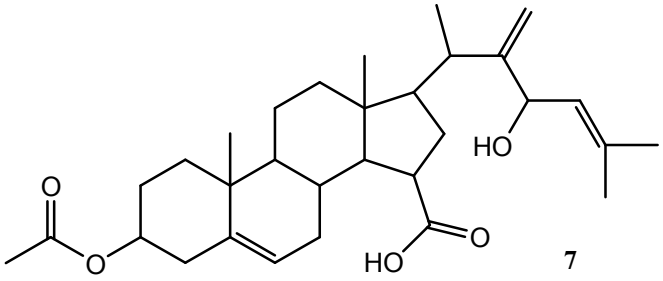

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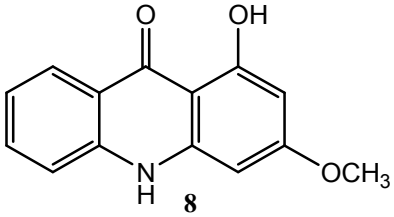
2.2.3 The genus *Zanthoxylum* as a potential source of mosquito larvicides

The genus *Zanthoxylum* is from the family *Rutaceae* and has great importance due to its phytochemistry and biological activity. Various parts of the plants from the genus *Zanthoxylum* have been reported to exhibit larvicidal activity against a number of mosquito species. Several classes of secondary metabolites such as alkaloids, sesquiterpene, lactones, coumarins, triterpenoids and limonoids have been isolated from *Z. gilletii* (Fig.1), *Z. limonella*, *Z. rhoifolium*, *Z. usambarense*, *Z. syncarpum*, *Z. zanthoxyloides*, *Z.chalybeum* (Patino *et al*., 2012). Four alkaloids, chelerythrine, nitidine, 6-acetonyl-N-methyl-dihydrodecarine and 10-O-dimethyl-17-O-dimethylisoarnottianamide from the roots of *Z. lemairei* showed larvicidal activity against third instar of larvae of *An*. *gambiae* (Talontsi *et al*., 2010).

Three acridone alkaloids, 1-hydroxy-3-methoxy-9-acridone (**8**), 1-hydroxy-3,4-dimethoxy-10-methyl-9-acridone (**9**), and 1-hydroxy-3-methoxy-10-methyl-9-acridone (**10**) were isolated from the plant *Zanthoxylum leprieurii* (Rutaceae) and evaluated for mosquito larvicidal activity against the malaria vector *Anopheles gambiae* (Matasyoh *et al*., 2011). Compounds **8** & **9** were the most potent with LC_{50} and LC_{90} values of 39.61, 189.76 and 77.53, 475.41 ppm

respectively. Compound **10** was less potent and achieved only 33% mortality at a concentration of 1000 ppm.



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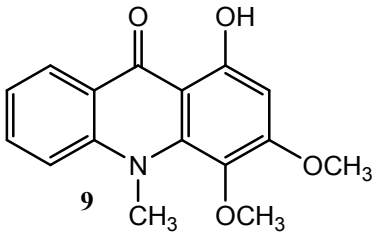

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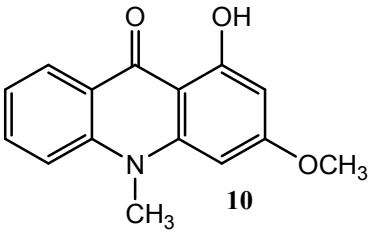

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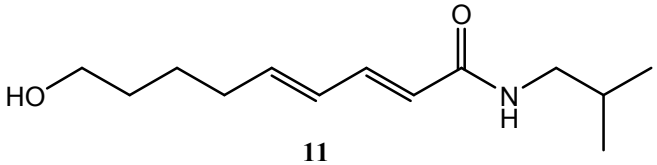
2.2.4 Some secondary metabolites isolated from *Zanthoxylum gilletii*

Most alkaloids isolated from different parts of *Z. gilletii* (Fig. 1) have been tested for antiplasmodial activity. Two alkaloids, N-isobutyldeca-2, 4-dienamide (**11**) and securinine (**12**) were isolated from *Z.gilletii* and were shown to exhibit moderate antiplasmodial activity against the K-1 strain of *P. falciparum* (Magadula and Erasto, 2009). Each of the two alkaloids had IC_{50} value of 5.4µg/mL. Four alkaloids nitidine (**13**), tembetarine, oblongine and magnoflorine were isolated from *Z. gilletii* and were reported to possess antiplasmodial activity against *P. falciparum* (Zirihi, 2006). Nitidine **13** which was the main alkaloid was reported to give the plant its antipaludic properties, by blocking the synthesis of the DNA of the *P. falciparum*. Dihydronitidine (**14**) alkaloid isolated from the bark of *Z. gilletii* showed antiplasmodial activity against *P. falciparum* with IC_{50} value of 0.16µg/mL.



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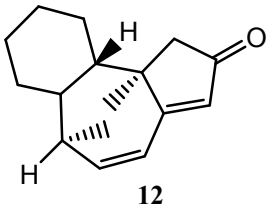

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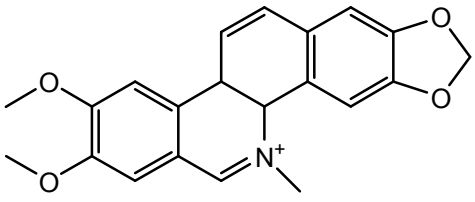

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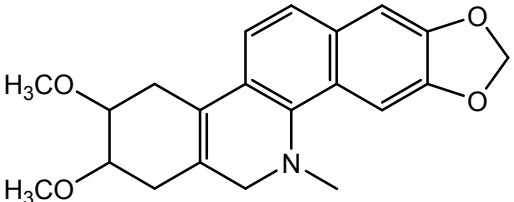

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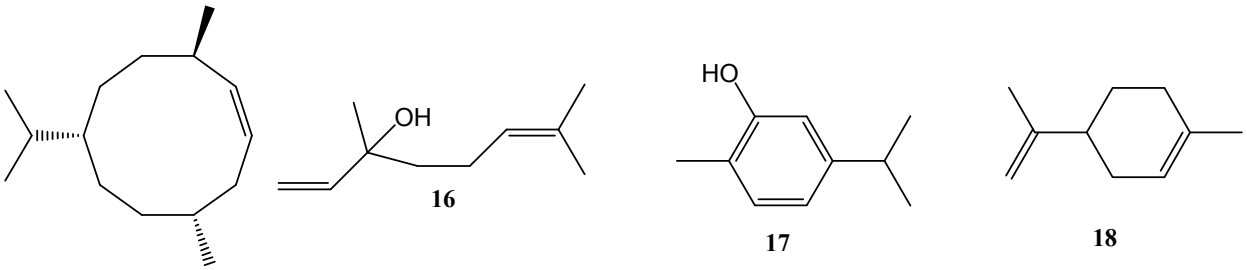
Figure 1: Photograph of *Zanthoxylum gilletii*

2.2.5 Mosquito larvicidal activity of essential oils

Essential oils are simply volatile fractions obtained by either steam distillation of medicinal and aromatic plants (Rabha *et al*., 2012). Most essential oils contain majorly monoterpenes and sesquiterpenes which are responsible for their activity (Mohamed *et al*., 2010). Essential oils have received considerable renewed attention as potent bioactive compounds against various species of mosquitoes. They are potentially suitable for application in larval control management because they constitute a rich source of bioactive compounds that are effective and naturally biodegradable into non-toxic products (Lucia *et al*., 2007; Cheng *et al*., 2008; Cheng *et al*., 2009).

Essential oils from a variety of plant families have been shown to possess mosquito larvicidal properties (Phasomkusolsil and Soonwera, 2010) and have received attention as potentially controlling vectors of mosquito borne diseases (Sutthanont *et al*., 2010). Essential oils from *Cedrus atlantica* (Pinaceae), *Cymbopogon nardus* (Poaceae), *Myrtus caryophyllum* (Myrtaceae), *Myristica fragrans* (Myristicaceae), *Citrus sinensis* (Rutaceae) and *Ocimum sanctum* (Lamiaceae) have been reported to be active against *Ae. aegypti* with LC_{50} values of 947.09, 1374.05, 135.20, 93.62, 85.93 and 92.48 ppm respectively (Tennyson *et al*., 2013).

Germacrene D (**15**), a sesquiterpene isolated from the essential oil of *Blumea martianana* aerial parts showed activity against larvae of *An. anthropophagus* with LC_{50} and LC_{90} values of 44.61 and 96.18 mg/L respectively (Zhu and Tian, 2011). Compound **15** was also active against larvae of *Cx. quinquefasciatus*, *Ae. aegypti* and *An. stephensi* with LC_{50} values of 21.28, 18.76 and 16.95 ppm respectively. Monoterpenoids: linalool (**16**) and carvacrol (**17**) have been documented to demonstrate *Ae. aegypti* larvicidal activity with LC_{50} value of 38.64 and 58.9 ppm respectively (Govindarajan, 2010; Lima *et al*., 2011). From the essential oil of *Clausena excavata*, a monoterpene limonene (**18**), was isolated and showed activity against the larvae of *Ae. aegypti* with LC_{50} and LC_{90} of 19.4 and 34.0 µg/ml respectively (Cheng *et al*., 2008).



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CHAPTER THREE

MATERIALS AND METHODS

3.1 Collection of the plant material

The leaves of *Z. gilletii* were collected from Kakamega forest, a tropical rain forest in Kenya which stretches from 0^0 $10^{'}$- 0^0 $21^{'}$ N and longitude 34^0 $44^{'}$- 34^0 $58^{'}$ E and an altitude of 1524 m above the sea level. The help of a taxonomist identified the leaves.

3.2 Extraction of volatile compounds

3.2.1 Preparation of material and apparatus set-up

Fresh plant leaves of *Z. gilletii* were sorted and cut into small pieces to increase the surface area during the hydrodistilation process. These plant leaves (500g) were then packed into a 2.0 L round bottomed flask together with 500 mL of water and then placed on a heating mantle, which was set at 100^{o}C. Hydro-distillation process was carried out for 6 h in the modified-type Clevenger apparatus. The essential oil obtained was then dried over anhydrous sodium sulphate. The dried oil was then stored in a sealed glass vial at 4^{o}C be used for larvicidal activity and analysis of its chemical composition (Fig. 2).

3.2.2 Larvicidal assay of *Z. gilletii* essential oil

The bioassay experiments were carried out following the standard World Health Organization larval bioassay method (WHO, 2005), with slight modifications. Since oil does not dissolve in water, 90 mg of it was first solubilized in dimethyl sulphoxide (DMSO, analytical reagent, Lobarchemi) and diluted with spring river water to make a stock solution of 1000 ppm (volume = 90 ml). Serial dilutions of the stock solution were done at different concentrations using the formula C_1 $V_1 = C_2$ V_2 which included 500, 250, 200, 150, 125, 100, 62.5, 55, 45, 40, 31.25, 15.6 and 7.8 ppm. The concentration of DMSO was kept below 1% since at this level it does not affect larval mortality. The bioassays were conducted at the Kenya Medical Research Institute (KEMRI), Centre for Global Health Research (CGHR), Kisumu, Kenya, where the *An. gambiae* mosquitoes were reared in plastic and enamel trays in spring river water. All experiments were carried out at a room temperature of 26 ± 3 ^{0}C and the humidity range between 70% and 75%. The bioassays were performed with third instar larvae of *An. gambiae s.s* and carried out in triplicate using 20 larvae for each replicate assay. The replicates were run simultaneously yielding a final total of 60 larvae for each concentration. The larvae were collected by direct pipetting from the enamel trays and transferred to 25 ml disposable plastic

cups containing 10 ml of test solution and fed on tetramin fish feed during all testing. Mortality and survival was established after 24 h of exposure. Larvae were considered dead if they were unarousable within a period of 1 min, even when gently prodded with a micro-pipette. The dead larvae in the three replicates were combined and expressed as the percentage mortality for each concentration. The negative control was 1% DMSO in spring river water while the positive control was the natural pyrethrum based larvicide, pylarvex.

3.3 Essential oil analysis

3.3.1 GC-MS analysis

Samples of essential oils were diluted in methyl-t-butyl ether (MTBE) (1:100) and analyzed 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.

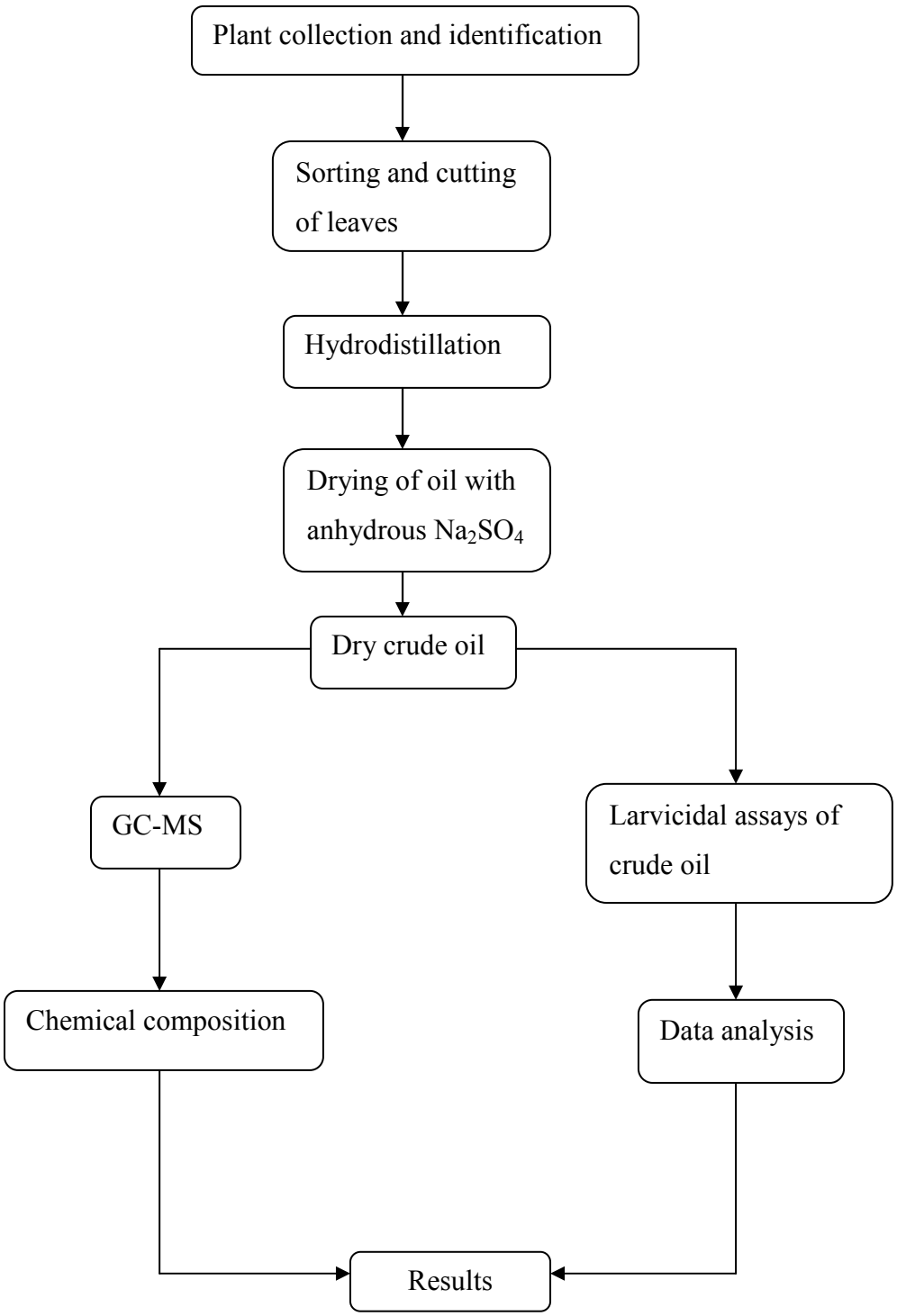


Figure 2: Extraction of essential oil and larvicidal assays

3.4 Extraction of non-volatile compounds

The plant leaves were air dried under shade in order to retain their active compounds for 21 days and later ground into powder using a blending machine (Thomas-Wiley Mill Model 4) at Kenya Agricultural Research Institute (KARI), Njoro. In a Winchester bottle of 2.5 L, 500g of powdered leaves were soaked in methanol for three days. The materials were then extracted exhaustively with 30 L of distilled methanol solvent at room temperature. After several cycles of extraction, the solution was filtered with Whatman filter paper (Whatman® No.1) and then the extraction solvent was removed under vacuum using a rotary evaporator machine (BUCHI-R 205). Part of the methanol crude was kept in a vial for larvicidal assays. The remaining portion of methanol crude extract obtained was then suspended in distilled water to remove the sugars. The crude extract free of sugars was then sequentially extracted with distilled hexane followed by ethyl acetate. Part of the hexane and ethyl acetate extracts were kept in vials for larvicidal assays. Since the hexane extract was less potent in comparison to the ethyl acetate extract, the ethyl acetate extract (56 g) was subjected to column chromatography to isolate the compounds responsible for its activity (Fig. 3).

3.5 Bioactivity guided fractionation

A column of diameter 2 cm and 50 cm height fitted with a tap at the bottom was used for column chromatography. Silica gel (70-230 mesh) was used as the stationary phase for packing the column and ethyl acetate-hexane solvent mixture in the ratio 6:4 (v/v) used as the mobile phase. Since the ethyl acetate extract was more potent in comparison to the hexane one, the ethyl acetate crude extract was then fractionated using 6:4 (v/v) ethyl acetate-hexane as eluting solvent mixture to give 24 fractions. The visualization and identification of the spots of all the compounds on the TLC plate was done using a UV lamp at a wavelength of 254 nm. These 24 fractions were combined according to their TLC patterns to give four major fractions namely F_1, F_2, F_3 and F_4, which were dried over anhydrous sodium sulphate to remove water, filtered and kept in vials. Methanol crude extract, hexane crude extract, ethyl acetate crude extract and the four fractions were all subjected to larvicidal assays at different concentrations. Fractions F_1 and F_2 showed low activity against third instar larvae of *An. gambiae s.s* and were therefore not considered for further purification. Fraction F_3 and F_4 which were more potent against the third instar larvae of *An. gambiae s.s* were considered for purification to isolate the possible pure compounds responsible for their activity against *An. gambiae s.s*.

3.6 Purification of compounds

Fractions F_3 and F_4 which showed high larvicidal activity when subjected to larval toxicity tests were considered for purification by repetitive Preparative Thin Layer Chromatographic techniques followed by Preparative High Performance Liquid Chromatography. The Preparative Thin Layer Chromatography plates were prepared locally in the laboratory before use.

3.6.1 Preparation and Development of PTLC Plates

Transparent glass plates measuring 20 by 20 by 0.3 cm were used for PTLC. A known amount (180 g) of the adsorbent silica gel (Kiesel 60, 70-230 mesh) was weighed and mixed with 40 ml of distilled water. A clean glass rod was used to stir in order to obtain homogeneous slurry. The plates were placed on a flat surface and then the slurry was evenly spread over the clean plates to obtain a thickness of 1.0 mm. The prepared plates were then left to dry overnight. Activation of the plates was achieved by heating them at 140 ^{0}C in an oven for 1 h. The plates were then allowed to cool before use to prevent glass breakage.

A mixture of ethyl acetate-hexane in the ratio of 6:4 (v/v) was prepared and used as the eluting solvent. The mixture was then poured in the development tank and the tank covered with a glass to obtain maximum saturation in the tank. On the dry PTLC plates the sample (F_3 and F_4) was applied as thin stripes on the baseline using a Pasteur pipette and left to dry for 3 minutes. The plates were then carefully placed in the development tank and allowed to develop by capillary action. The compound of interest was obtained by scraping off the adsorbent region which appeared as purple colour when visualized under a UV lamp at a wavelength of 254 nm. The scrapped sample was then eluted with ethyl acetate and filtered using Whatman filter paper (Whatman® No.1) and the solvent of extraction was removed under vacuum using a rotary evaporator machine (BUCHI-R 205).

3.6.2 Preparative High Performance Liquid Chromatography (PHPLC)

The sample from Preparative TLC was taken to PHPLC in order to separate and refine high-purity target compounds from this mixture. The compounds were separated on a reverse phase preparative HPLC using water and methanol as mobile phases. Gradient elution i.e a separation in which the mobile phase composition is changed during the separation process, was

used. The gradient started with 100% water and 0% methanol ending at 0% water and 100% methanol after 40 minutes.

3.7 Larvicidal assay of crude extracts, fractions and pure compounds

The larvicidal assays were carried out following the standard World Health Organization larval bioassay method (WHO, 2005), with slight modifications. For the crude extracts of methanol, hexane and ethyl acetate, as well as the four fractions F_1-F_4, 180 mg of each was separately solubilized in dimethyl sulphoxide (DMSO, analytical reagent, Lobarchemi) and diluted with spring river water to make a stock solution of 2000 ppm. In the case of pure compounds, 90 mg were dissolved in DMSO and diluted with spring river water to make a stock solution of 1000 ppm. Serial dilutions of the stock solutions were done at different concentrations using the formulae $C_1 V_1 = C_2 V_2$. The bioassays were performed with third instar larvae of *An. gambiae s.s* and carried out in triplicate using 20 larvae for each replicate assay. The replicates were run simultaneously yielding a final total of 60 larvae for each concentration. The larvae were collected by directly pipetting from the enamel trays and transferred to 25 ml disposable plastic cups containing 10 ml of test solution and fed on tetramin fish feed during all testing. Mortality and survival was established after 24 h of exposure. Larvae were considered dead if they were unrousable within a period of time, even when gently prodded with a micro-pipette. The dead larvae in the three replicates were combined and expressed as the percentage mortality for each concentration. The negative control was 1% DMSO in spring river water while the positive control was the natural pyrethrum based larvicide, pylarvex.

3.8 Structure elucidation of bioactive pure compounds

The NMR spectra were measured on a Bruker Advance (500 MHz) spectrometer. Structure elucidations of pure non-volatile compounds were carried out by a combination of Nuclear Magnetic Resonance spectroscopy and Mass spectroscopy. ^{1}H NMR was used to determine all the number of protons present in the compound while ^{13}C NMR was used to determine the number of carbon atoms present in the compound. In ^{1}H–^{1}H COSY (Correlation spectroscopy) the off-diagonal elements were used to identify the spin-spin coupling interactions. ^{1}H–^{13}C HMBC (Heteronuclear Multiple Bond Correlation) spectrum was used to identify proton-carbon connectivity of two to three bonds away 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. ^{1}H–

^{13}C HSQC spectrum (Heteronuclear Single Quantum Coherence) was used to distinguish the proton resonances of different groups (methines, methylene and methyl) along with their corresponding carbon resonances.

All the readings were done in deuterated chloroform ($CDCl_3$) solvent, chemical shifts were assigned by comparison with the residue proton, carbon resonance of the solvent, Tetramethylsilane (TMS) was used as an internal standard, and chemical shifts were given as δ (ppm). The spectra were simulated using ACD NMR manager program to obtain the chemical shifts of both proton and carbon.

3.9 Statistical analysis

Probit analysis (Finney, 1971) of concentration mortality data was conducted to estimate the LC_{50} and LC_{90} values and the statistical software package SPSS version 15.0 was used.

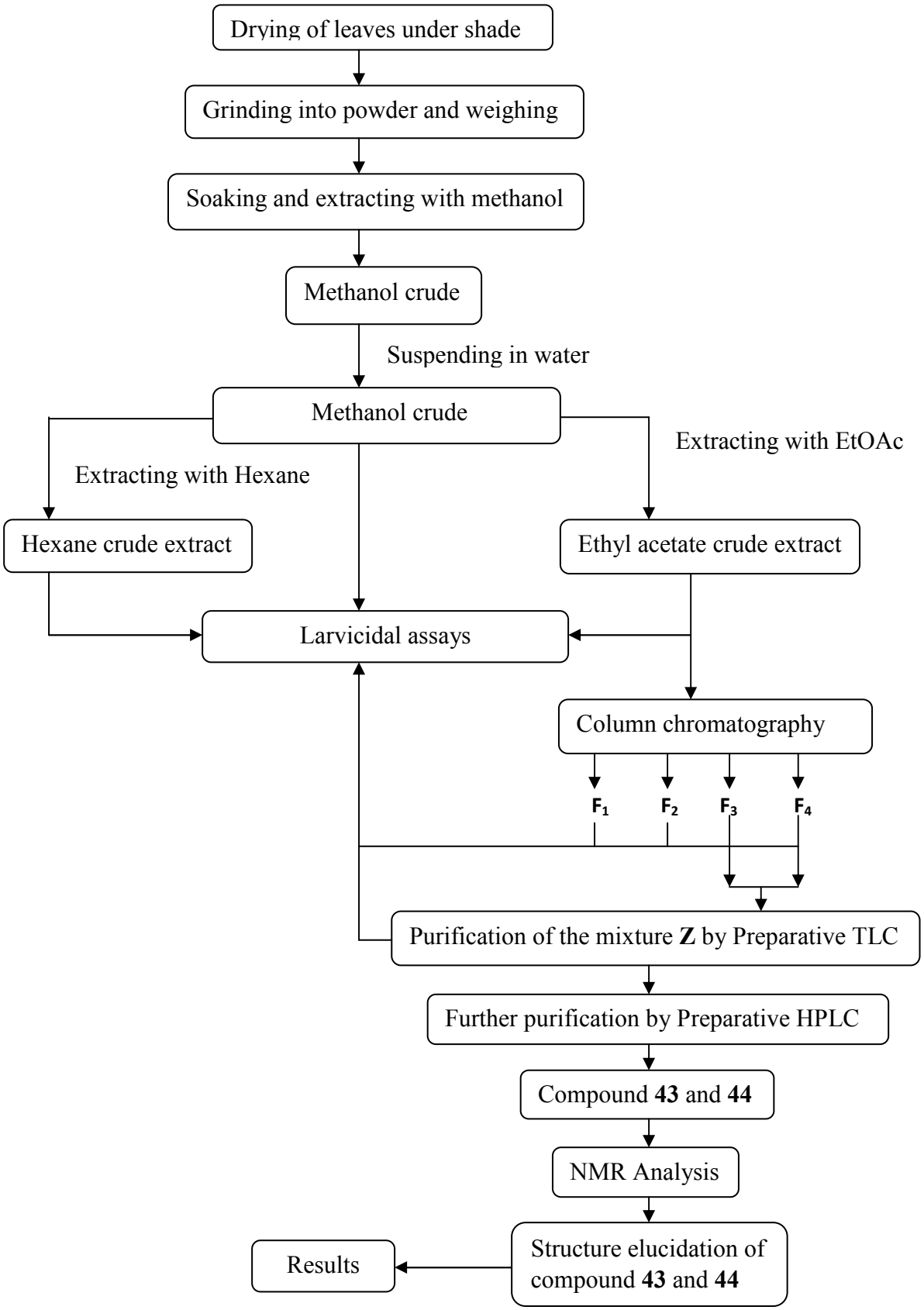


Figure 3: Extraction of non-volatile compounds and larvicidal assays

CHAPTER FOUR

RESULTS AND DISCUSSION

4.1 Chemical composition of the essential oil of *Z. gilletii*

4.1.1 Identified compounds

A total of 47 compounds (Appendix 11) were identified by comparing the peaks on their chromatograms and matching with the mass spectra in the computer databases (Wiley 7N.l, HPCH 1607.L, FLAVORS.L) and in literature (Adams, 2007). This number of compounds only constituted 69.11% of the total possible number of compounds present in the essential oil of *Z. gilletii*. Table 1 shows eighteen compounds each of which had greater than one percent concentration.

The essential oil of *Z. gilletii* was dominated by monoterpenes and sesquiterpenes which accounted for 34.00% and 38.30% respectively. The major monoterpene components included γ-terpinene (10.62%), β-myrcene (5.16%), sabinene (4.89%), β-ocimene (3.12%) and camphene (2.56%). The main sesquiterpene components were *trans*-caryophyllene (9.82%), caryophyllene oxide (4.4%), α-cadinol (2.71%), 1,1,4,8-tetramethyl-4,7,10-cycloundecatriene (2.62%), δ-cadinene (2.52%) and τ-cadinol (2.29%) (Appendix 11).

Table 1: Identified compounds of the essential oil from *Z. gilletii* with > 1.00% concentration

Compound no.	Compound name	Retention times (min)	Concentration %
25	γ-terpinene	6.93	10.62
26	trans-caryophyllene	20.46	9.82
27	β- myrcene	8.54	5.16
28	sabinene	7.96	4.89
29	caryophyllene oxide	24.39	4.40
30	2- undecanone	17.44	3.64
31	β-ocimene	10.19	3.12
32	α-cadinol	25.99	2.71
33	1,1,4,8-tetramethyl-4,7,10-cycloundecatriene	21.26	2.62
34	camphene	7.25	2.56
35	δ- cadinene	22.84	2.52
36	τ- cadinol	25.67	2.29
37	β- cubebene	19.56	1.70
38	phytol	35.02	1.51
39	2-isopropyl-5-methyl-9-methylene-bicyclo[4.4.0]dec-1-ene	22.26	1.43
40	alloocimene	12.37	1.35
41	β- selinene	21.99	1.35
42	bornyl acetate	16.77	1.16

4.2 Larvicidal assay of essential oil of *Z. gilletii*

The dry essential oil was subjected to larvicidal assay against third instar larvae of *An. gambiae* and found to be active (Table 2). From a stock solution of 1000 ppm, other lower concentrations were prepared up to a concentration of 7.8 ppm. The data obtained from the larvicidal assay which includes percent mortality of the larvae at various concentrations of the oil was recorded and presented in Table 2.

Table 2: Larvicidal results of essential oil against 3rd instar of *An. gambiae s.s*

Concentration (ppm)	**% Mortality ± SD**	**LC_{50} (ppm)**	**LC_{90} (ppm)**
7.80	0.00 ± 0.00		
15.60	1.67 ± 2.89		
31.25	3.33 ± 2.89		
40.00	13.33 ± 5.77		
45.00	33.33 ± 5.77		
55.00	46.67 ± 2.89	57.73 (45.40- 73.05)	140.24 (105.73-217.05)
62.50	68.33 ± 5.77		
100.00	76.67 ± 2.89		
125.00	80.00 ± 10.00		
150.00	85.00 ± 8.66		
200.00	88.33 ± 2.89		
250.00	96.67 ± 5.77		
500.00	100.00 ± 0.00		
1000.00	100.00 ± 0.00		
[X]Pylarvex (100 ppm)	100.00 ± 0.00		
[Y]Spring water + DMSO	0.00 ± 0.00		

X-Positive control, *Y*-Negative control.

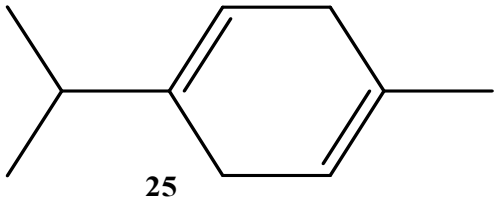
The LC_{50} and LC_{90} values which were reported to be 57.73 and 140.24 ppm after 24 h exposure (Table 2), were calculated by log probit analysis (95% confidence level) using SPSS 15.0 software. The negative control showed no activity against *An. gambiae s.s* after 24 h exposure while the positive control showed 100 % mortality at a concentration of 100 ppm. The oil achieved 100% mortality at a concentration of 500 ppm.

Reports from earlier studies have shown the essential oil of *Fagaropsis angolensis* (Rutaceae) as possessing larvicidal activity against third instar larvae of *An. gambiae* with LC_{50} and LC_{90} values of 83.7 and 324 ppm respectively (Mudalungu *et al*., 2013). Essential oil of *Zanthoxylum armatum* was active against late third instar larvae of *Cx. quinquefasciatus*, *Ae. aegypti* and *An. stephensi* with values of 49, 54 and 58 for LC_{50} and 146, 171 and 185 ppm for LC_{90} respectively (Tiwary *et al*., 2007). Both essential oils from wild and cultivated *R.*

chalepensis (Rutaceae) plants were able to exert a very good toxic activity against *Ae. albopictus* larvae with wild plants having LC_{50} of 35.66 ppm while the cultivated plants had LC_{50} of 33.18 ppm (Conti *et al*., 2013). Essential oil from seeds and fruits of *Zanthoxylum limonella* Alston has been reported to be effective as repellent against three medically important mosquito species, *Ae. aegypti* (L.), *Cx. quinquefasciatus* Say, and *An. dirus* (Trongtokit *et al*., 2005).

Larvicidal toxicity and repellency rates of sweet *Citrus sinensis* L. and *Citrus limon* L. essential oils against *Ae. albopictus* were reported (Giatropoulos *et al*., 2012). In comparison to the essential oil of *F. angolensis* which was tested against *An. gambiae*, essential oil of *Z. gilletii* had better larvicidal activity against third instar larvae of *An. gambiae*. Essential oil from *Z. gilletii* therefore could be classified as a promising mosquito larvicide.

According to the mass spectrum of γ-Terpinene (**25**), it had a retention time of 6.93 minutes and its percentage concentration in the entire oil was found to be 10.62. The peak at *m/z* 136 corresponded to the molecular ion peak of γ-Terpinene which was also present in the GC-MS of the compound in the library database (Fig. 4). The fragmentation peak observed at *m/z* 121 belonged to a fragment $[C_9H_{13}]^+$ after the loss of a methyl radical. The peak at *m/z* 93 belonged to a fragment $[C_7H_9]^+$ after the loss of a fragment of mass 43 (isopropyl radical). Another peak was observed at *m/z* 77 corresponding to the fragment $[C_6H_5]^+$ (figure 4). Following these fragmentation peaks, the compound was identified as γ-terpinene.



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Reports from earlier studies have shown γ-Terpinene isolated from the essential oil of *Clausena excavate* being active against the larvae of *Ae. aegypti* and *Ae. albopictus* with LC_{50} values of 26.8 and 22.8 µg/ml respectively (Cheng *et al*., 2009). Zhu and Tian, (2011) isolated this compound from the aerial parts of *Blumea martiniana* and tested it against *An. anthropophagus* larvae. The compound was found to be active and recorded LC_{50} of 29.21 mg/L and LC_{90} of 63.10 mg/L. In a previous study, γ-Terpinene isolated from the oil fractions of *Cymbopogon nardus* had an excellent effect against third instar larvae of *Cx. quinquefasciatus* after 24 h exposure period, with LC_{50} value of 0.8 mg/L (Ranaweera and Dayananda, 1996). The

larvicidal activity of *Z. gilletii* oil may be attributed partly to the presence of γ-Terpinene in the oil.

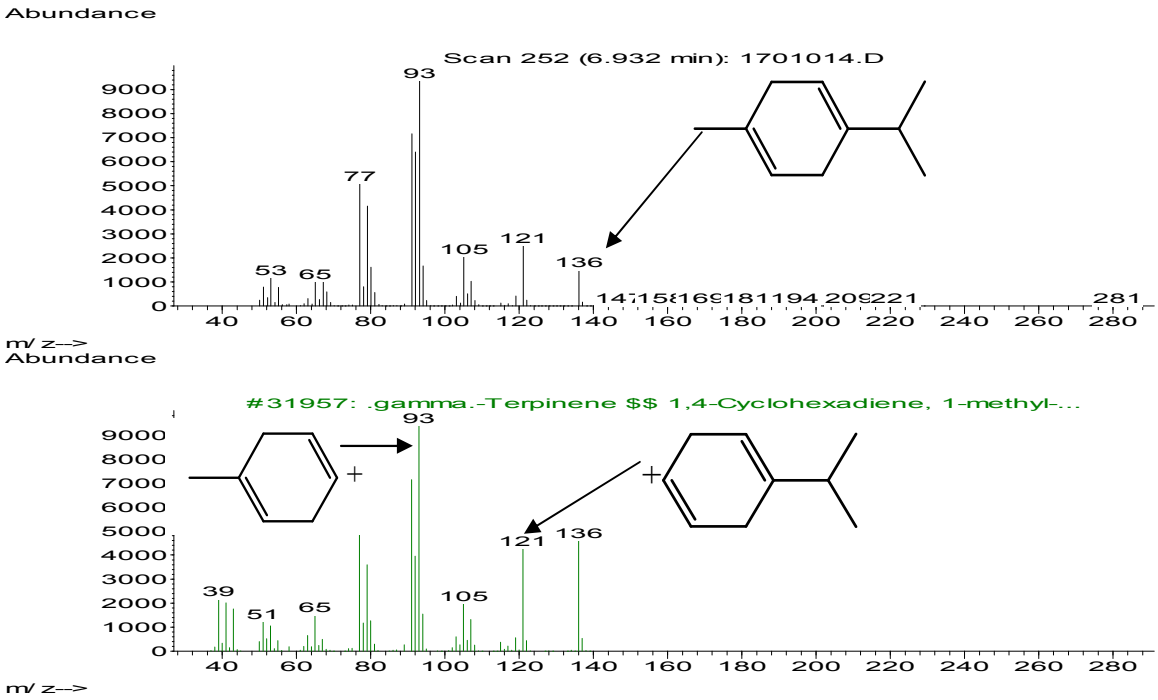
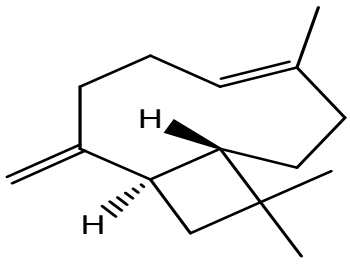


Figure 4: GC-MS spectrum of compound **25** with comparison to database

trans-Caryophyllene (**26**) occurring at retention time 20.46 minutes and comprising of 9.82 % concentration of the total oil with a molecular mass of 204 was identified with the help of the library database. The oil spectra and the spectra of the oil in the library database showed five major peaks (Fig. 5). The peak occurring at *m/z* 161 resulted from the detachment of the isopropyl radical leaving the fragment $[C_{12}H_{17}]^+$ while the peak *m/z* 133 was attributed to the loss of pentyl unit leaving the fragment $[C_{10}H_{13}]^+$. The fragmentation peak at 69 was attributed to the fragment $[C_5H_9]^+$. Earlier reports indicate that *trans*-caryophyllene, isolated from the essential oil of *Croton sonderianus*, had larvicidal activity against *Ae. aegypti* with LC_{50} value of 104 ppm (Morais *et al*., 2006).



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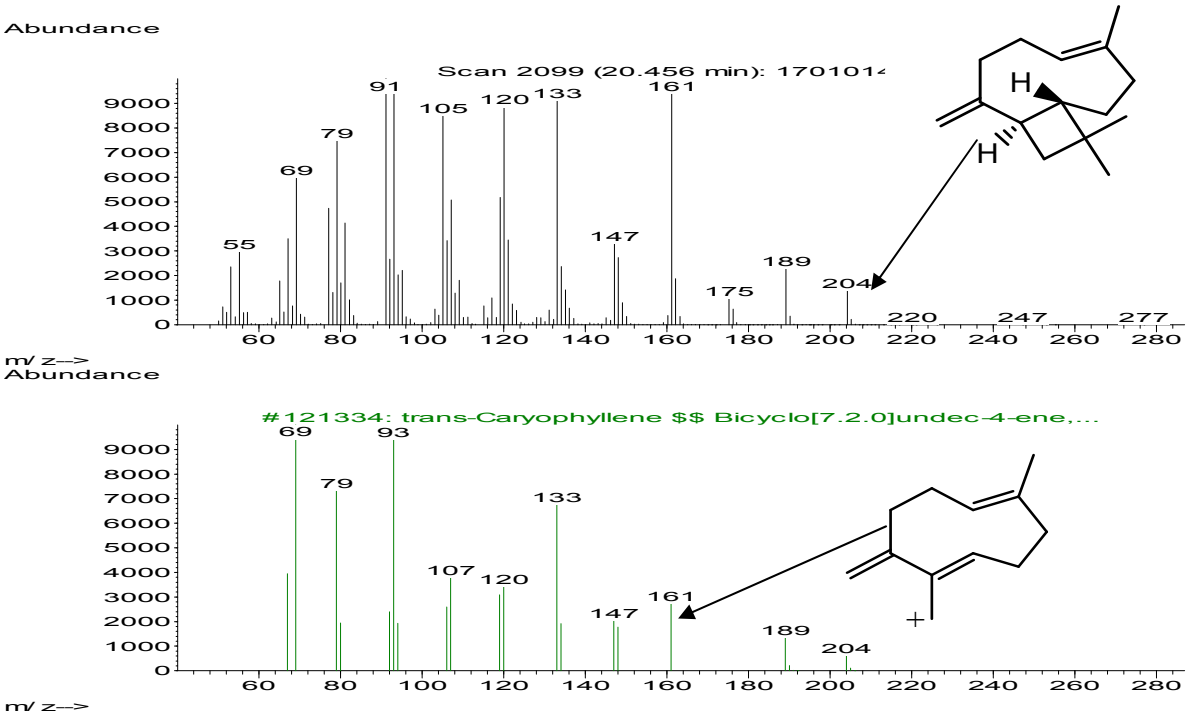
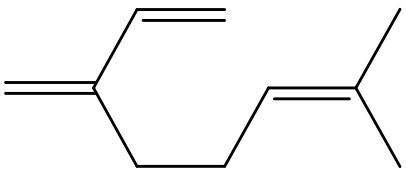


Figure 5: GC-MS spectrum of compound **26** with comparison to database

The peak occurring at retention time 8.54 minutes was assigned to β-myrcene (**27**). The oil spectra and the spectra of the compound in the database showed two major peaks (Figure 6).. The fragmentation peak observed at *m/z* 136 corresponded to the molecular ion peak of the compound (Fig. 6). An intense peak at *m/z* 93 belonged to a fragment $[C_7H_9]^+$ after the loss of isopropenyl radical. Another fragmentation peak observed at *m/z* 69 belonged to a fragment $[C_5H_9]^+$ (Fig. 6). Following all these fragmentation peaks, the acyclic unsubstituted monoterpene was therefore concluded to be β-myrcene.



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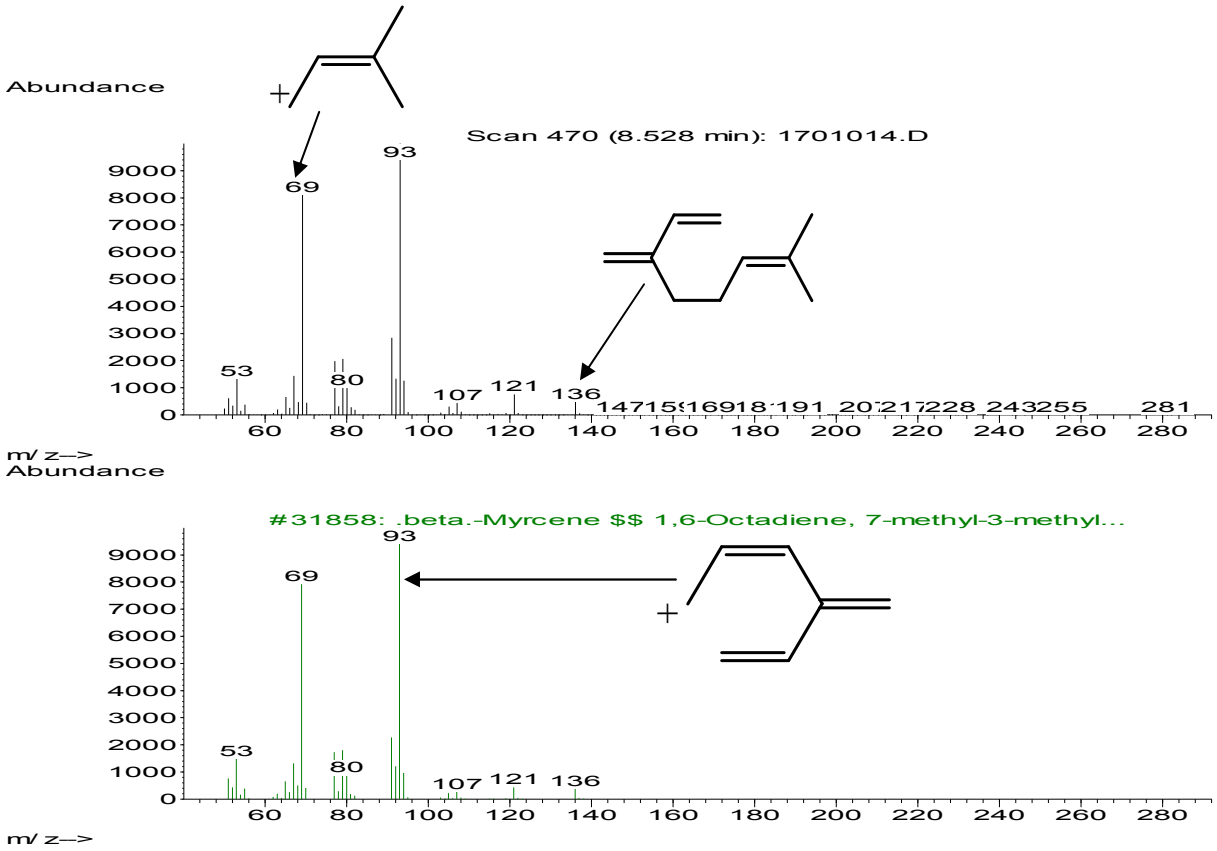


Figure 6: GC-MS spectrum of compound **27** with comparison to database

Basing on literature, β-myrcene has been isolated from the essential oil of *Clausena excavata* leaves. This compound showed larvicidal activity against *Ae. aegypti* and *Ae. albopictus* larvae with LC_{50} values of 27.9 and 23.5 µg/ml, respectively (Cheng *et al*., 2009c).

The mass spectrum of sabinene (**28**) showed its retention time at 7.96 minutes with its concentration in the oil being 4.89 %. The chromatograms obtained from the mass spectra at a retention time of 7.96 minutes showed closely matching peak to the chromatograms obtained from the library databases (Fig. 7). The GC-MS detection identified major fragmentation peak at *m/z* 136 which corresponded to the molecular ion peak of the compound. The peak at *m/z* 121 belonged to a fragment $[C_9H_{13}]^+$ after one methyl radical was detached from it. Another intense peak was observed at *m/z* 93 and was attributed to the loss of an isopropyl radical. Following all these fragmentation peaks, compound **28** was identified to be sabinene.

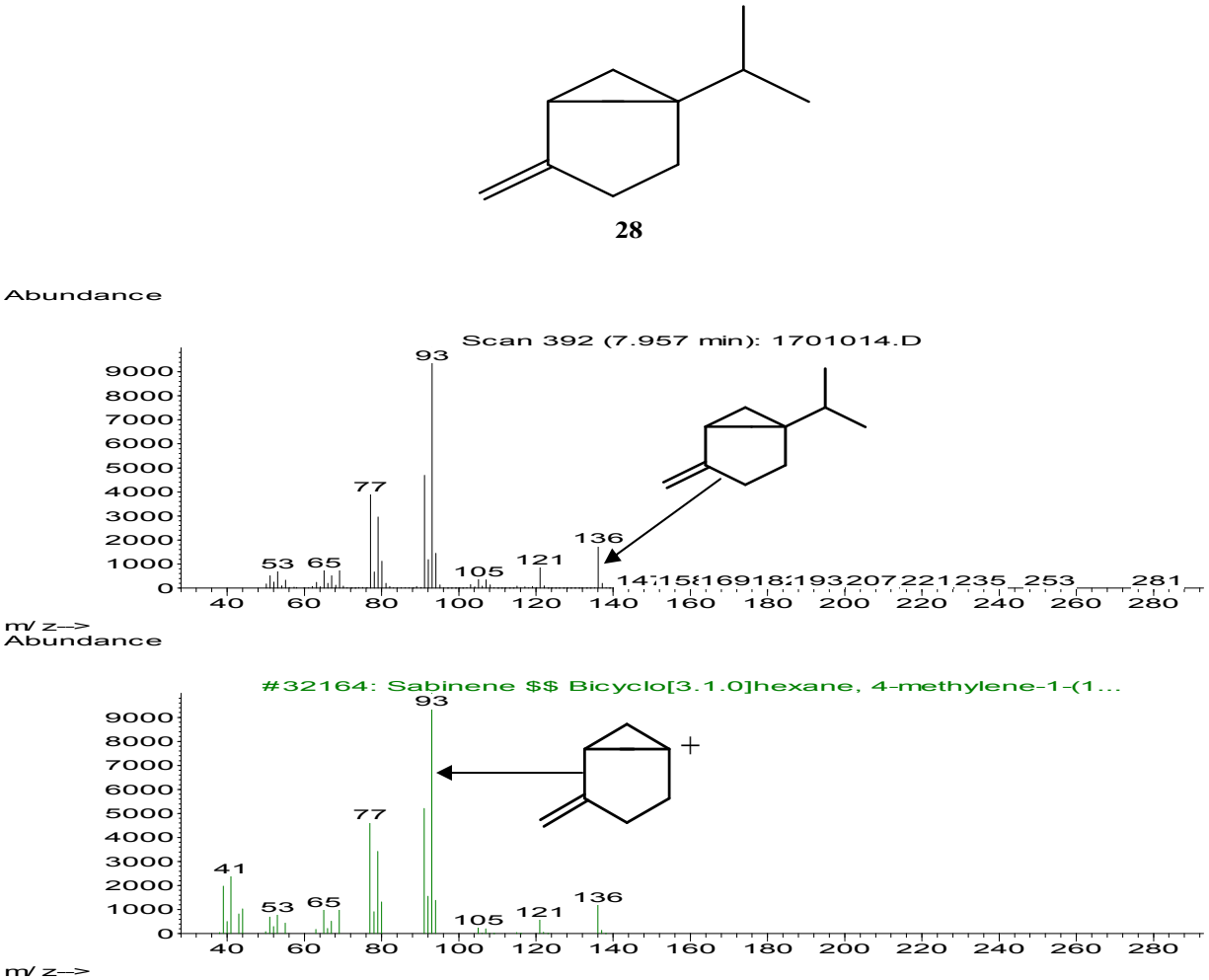


Figure 7: GC-MS spectrum of compound **28** with comparison to database

Sabinene was isolated previously from the essential oil of *Clausena anisata* leaves and evaluated against three mosquito species (Govindarajan, 2010). This compound was found to be active against third instar larvae of *Cx. quinquefasciatus, Ae. aegypti* and *An. stephensi* with LC_{50} of 25.01, 21.20 and 19.67 ppm, respectively. The LC_{90} of this compound was 45.15 ppm against *Cx. quinquefasciatus*, 39.22 ppm against *Ae. aegypti* and 36.45 ppm against *An. stephensi*.

Analysis of GC-MS spectral peaks at retention time 24.39 minutes revealed caryophyllene oxide (**29**) which had a molecular mass of 220 and accounted for 4.4 % concentration of the total oil. The GC-MS chromatograms obtained for these compound showed a close match to the spectra present in the library databases (Fig. 8). Two major peaks at *m/z* 79 and *m/z* 93 were observed from the oil spectra and that of the database library.

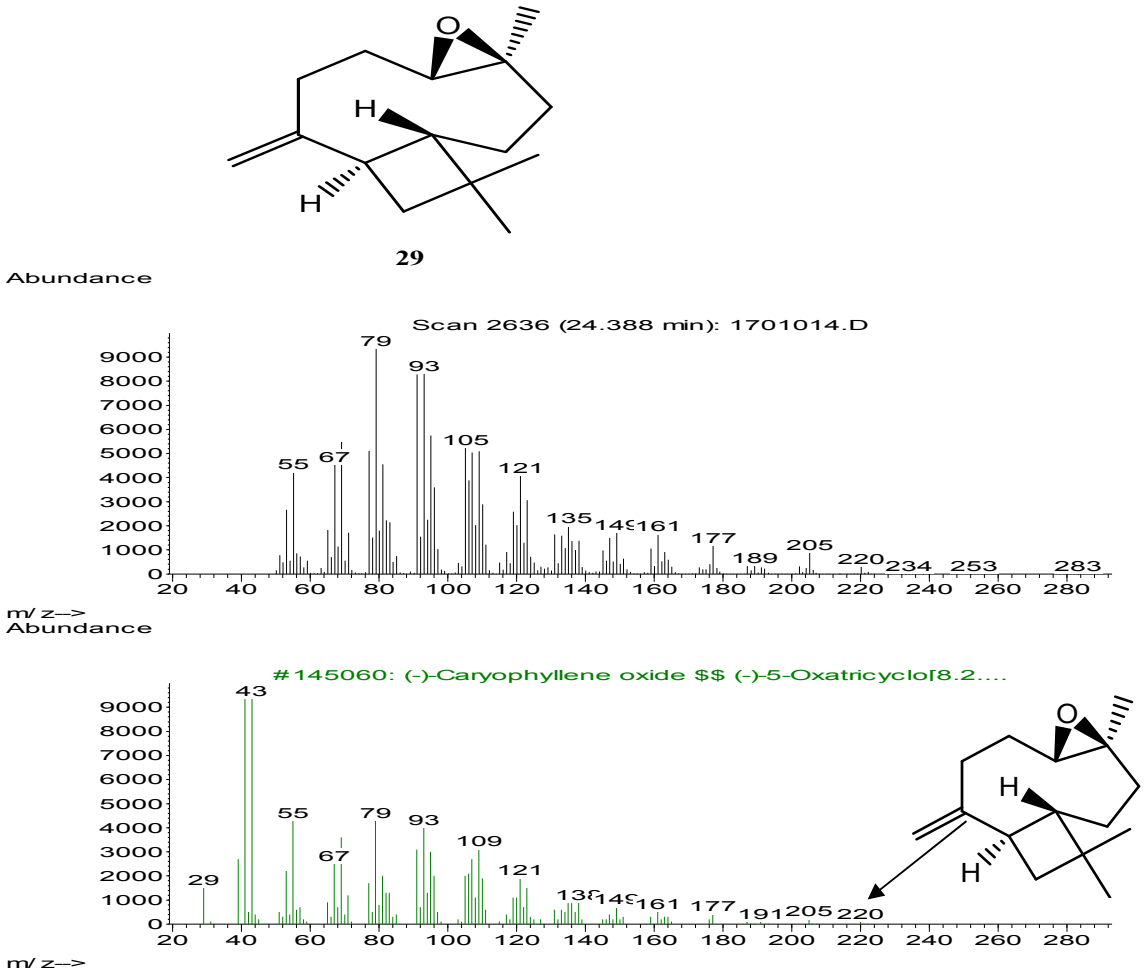
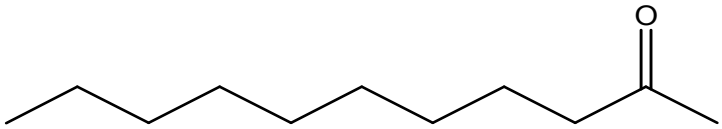


Figure 8: GC-MS spectrum of compound **29** with comparison to database

Previous studies have shown caryophyllene oxide as having larvicidal activities. Zhu and Tian, (2013) isolated this compound from the essential oil of *A. gilvescens*. Upon evaluation against fourth instar larvae of *An. anthropophagus*, caryophyllene oxide exhibited LC_{50} and LC_{90} values of 49.46 and 115.38 mg/L, respectively.

The mass spectrum of 2-undecanone (**30**) showed a peak at retention time 17.44 minutes with its concentration in the oil being 3.64 %. The computer library compound spectra showed two major peaks which were also observed in the spectra of 2-undecanone (Fig. 9). The molecular mass ion peak *m/z* 170 was observed. The peak at *m/z* 71 belonged to a fragment $[C_4H_7O]^+$ after the loss of a heptyl radical. The peak *m/z* 58 could be attributed to the fragment $[C_3H_6O]^+$ after the loss of an octyl radical followed by McLafferty rearrangement. Following all these fragmentations, compound **30** was concluded to be 2-undecanone. This compound has not

been reported to possess any larvicidal activity and therefore the activity of the oil could not be attributed to the presence of this compound in the oil.



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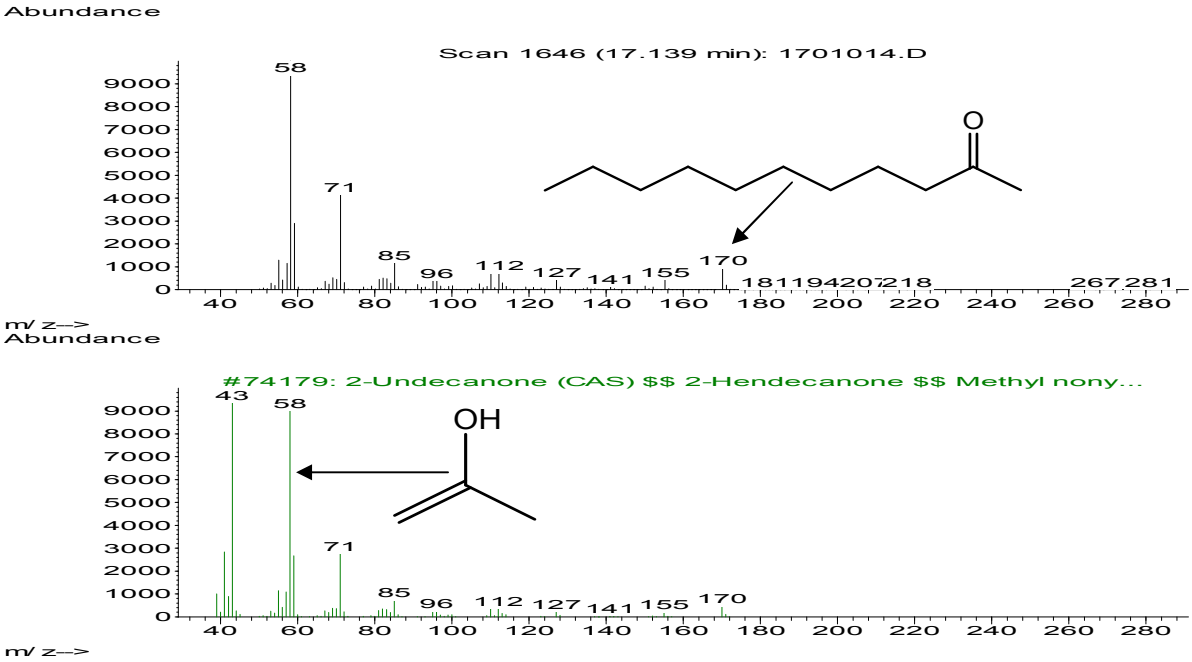
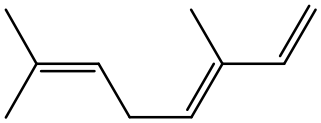


Figure 9: GC-MS spectrum of compound **30** with comparison to database

Compounds **31- 42** had retention times 10.19, 25.99, 21.26, 7.25, 22.84, 25.67, 19.56, 35.02, 22.26, 12.37, 21.99, 16.77 minutes, respectively. Although the activity of these compounds against mosquito larvae has not been reported, their activity may not be ruled out.



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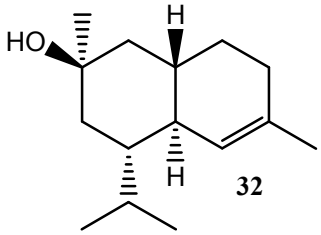

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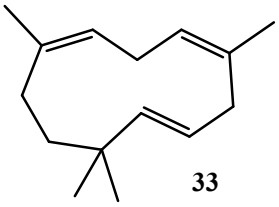

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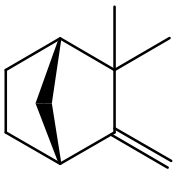

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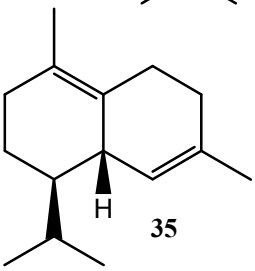

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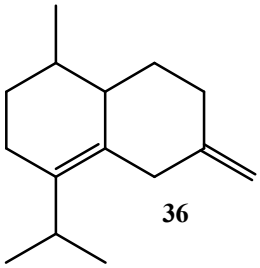

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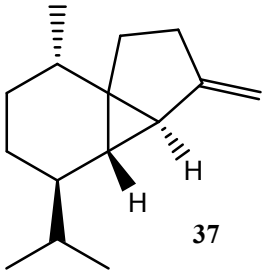

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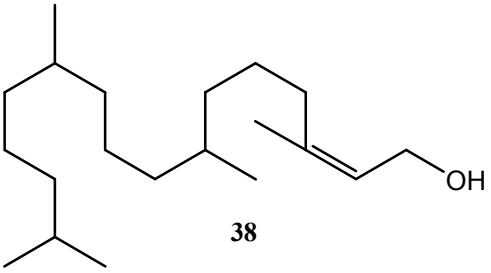

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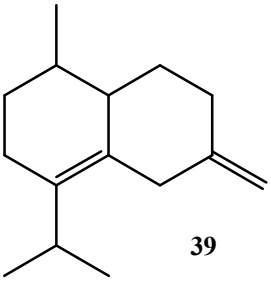

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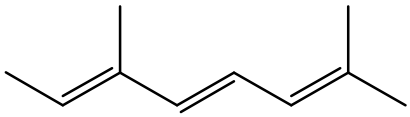

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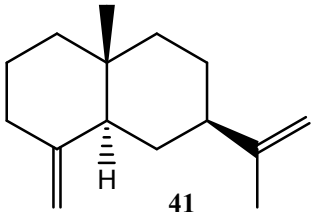

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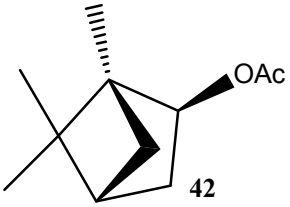

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4.3 Structure elucidation of compounds 43 and 44

Analysis of the NMR spectra of compounds **43** and **44** indicated that they were a pair of *Z/E* isomeric secofuroquinoline alkaloids. Both compounds **43** and **44** had fourteen carbon atoms, thirteen hydrogen atoms, one nitrogen atom and six oxygen atoms leading to a molecular formula of $C_{14}H_{13}NO_6$. The NMR data of compound **43** in comparison with data from literature is shown in Table 3.

Table 3: ^{1}H NMR and ^{13}C NMR spectral data of compound **43**

Position	**δ ^{13}C (ppm)**	**DEPT**	**δ ^{1}H/HSQC (ppm)**	**J (Hz)**	**HMBC**	**COSY**	**δ $^{13}C^{\#}$ (ppm)**
2	145.1	CH	7.71 (d)	2.60	2',3',3	3	145.1
3	105.3	CH	7.02 (d)	2.60	2,2',3'	2	105.3
4	158.7	Q					158.4
5	137.7	CH	7.26 (d)	11.90	4,5',7	6	137.7
6	121.8	CH	6.16 (d)	11.90	7	5	121.7
7	166.1	Q					166.0
8	165.9	Q					166.0
2'	162.7	Q					163.0
3'	109.1	Q					109.0
5'	141.2	Q					-
8'	160.1	Q					-
OMe-4	59.1	CH_3	4.23(s)				59.1
CO_2 Me-7	51.2	CH_3	3.56(s)				50.3
CO_2 Me-8	52.8	CH_3	3.93(s)				51.2

$^{\#}$(Mara *et al*., 1992).

The ^{13}C NMR (appendix 13) and DEPT (appendix 14) spectra of compound **43** confirmed the presence of fourteen carbon atoms, consisting of four methine, five quaternary, two methoxycarbonyl and three methoxylated carbon atoms. The chemical shifts of the four methine carbons were observed to occur at δ 105.3, 121.8, 137.7 and 145.1. The chemical shifts of the five quaternary carbons were observed to occur at δ 109.1, 141.2, 158.7, 160.1 and 162.7, the two methoxycarbonyl carbon atoms had chemical shifts of δ 165.9 and 166.1, while the chemical shifts of the three methoxylated carbon atoms were observed to occur at δ 51.2, 52.8

and 59.1. The ^{1}H NMR spectrum (appendix 12) of compound **43** revealed the presence of four aromatic protons. Four doublets resonating at δ 6.16, 7.02, 7.26 and 7.71 as well as three singlets at δ 3.56, 3.93 and 4.23 for methoxy groups were observed.

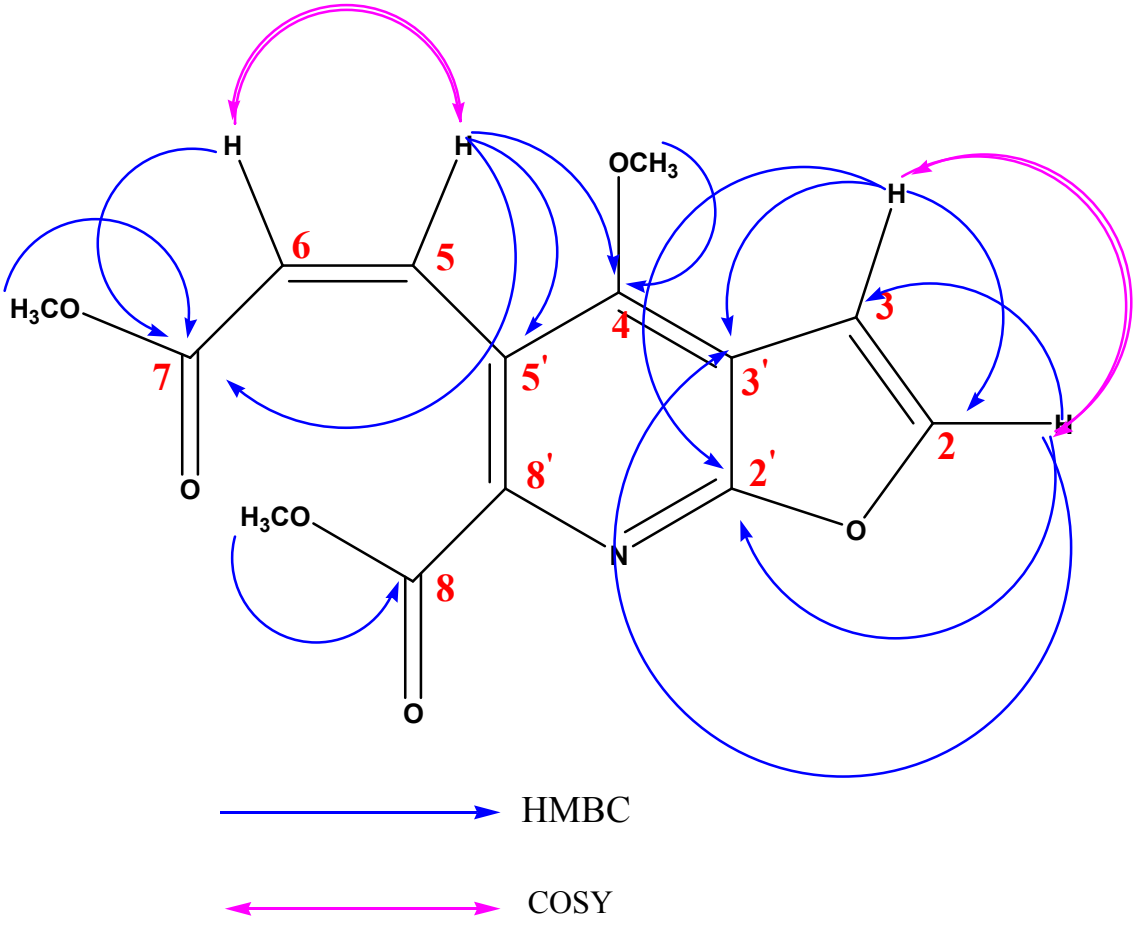
The chemical shifts occurring at δ 166.1 and 165.9 were assigned to methoxycarbonyl carbons (C-7) and (C-8), respectively. Chemical shifts of the quaternary carbons (C-2', C-3', C-4, C-5' and C-8') were observed to occur at δ 162.7, 109.1, 158.7, 141.2 and 160.1, respectively. The four methine carbons (C-2, C-3, C-5 and C-6) were observed to absorb at δ 145.1, 105.3, 137.7 and 121.8, respectively. The methoxy group carbon attached to C-4 appeared at δ 59.1, that attached to C-7 appeared at δ 51.2 while the one attached to C-8 appeared at δ 52.8.

The HSQC spectrum (appendix 15) of compound **43** showed correlations of carbon atoms and the protons directly attached to them. There was a correlation between C-3 and a proton which was observed to resonate at δ 7.02, while a proton resonating at δ 7.71 was observed to correlate with C-2. Carbons C-5 and C-6 were observed to correlate with protons resonating at δ 7.26 and 6.16, respectively. The methoxy carbon absorbing at δ 51.2 was correlating with a proton resonating at δ 3.56, the one absorbing at δ 52.8 was correlating with a proton resonating at δ 3.93 while the methoxy carbon resonating at δ 59.1 was correlating with a proton absorbing at δ 4.23.

From the HMBC spectrum (appendix 16), the proton H-2 resonating at δ 7.71 showed correlation with C-2', C-3' and C-3. Proton H-3 absorbing at δ 7.02 was observed to correlate with C-2, C-2' and C-3'. A proton resonating at δ 7.26 (H-5) of α, β-unsaturated ester showed correlation with C-4, C-5' and C-7 while that resonating at δ 6.16 (H-6) was observed to correlate with C-7. The attachment of methoxy groups to carbons C-4, C-7 and C-8 was substantiated by the correlations of protons resonating at δ 4.23, δ 3.56 and δ 3.93 with C-4, C-7 and C-8, respectively.

The ^{1}H-^{1}H COSY (appendix 17) correlation for compound **43** was also determined. From COSY spectrum, H-2 was correlating with H-3. The coupling constant for peaks absorbing at δ 7.71 (H-2) and δ 7.02 (H-3) was calculated to be 2.60 Hz. Proton H-5 (δ 7.26) and H-6 (δ 6.16) showed COSY correlation as well with a coupling constant of 11.90 Hz. The coupling constant of *cis* isomer is always smaller than that of *trans* isomer and is always in the range of 7-12 Hz. The *Z*-isomer of compound **43** was determined based on the coupling constant of H-5 (δ 7.26) and H-6 (δ 6.16) which was 11.90 Hz. Since this coupling, constant falls within the range 7-12

Hz, compound **43** was therefore confirmed to be a *Z*-isomer based on the double bond at H-5 and H-6. Based on the 1D and 2D NMR information, the proposed structure for compound **43** is shown below.



Z-Dimethylrhoifolinate

Compounds **43** and **44** both had a molecular mass of 291.256 calculated for $C_{14}H_{13}NO_6$. Both compound **43** and **44** had a similar mass spectrum (Fig. 10). The compounds were analyzed at wavelength range of 250-2500 nm and therefore only the M peak was recorded. This corresponded with the retention time at 9.33 minutes. The positive electron impact mass spectrometry (EIMS) for compounds **43** and **44** revealed a peak at *m/z* 292.08 representing the molecular ion $[M + H]^+$ corresponding to molecular formula $C_{14}H_{13}NO_6$. The mass spectrum for compound **43** and **44** is in figure 10.

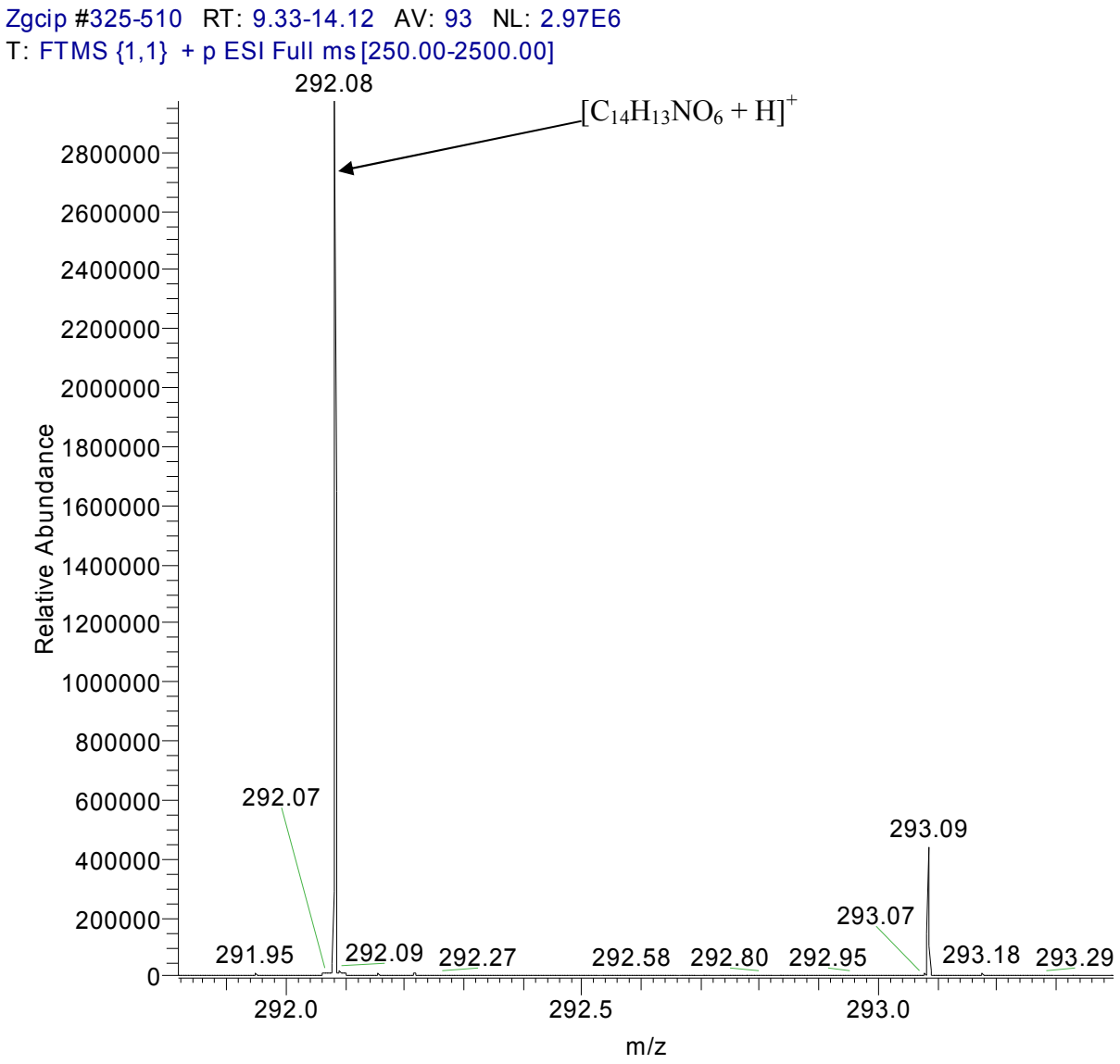


Figure 10: Mass spectrum of compounds **43** and **44**

The NMR data of compound **44** in comparison with data from literature is shown in Table 4.

Table 4: ^{1}H NMR and ^{13}C NMR spectral data of compound **44**

Position	**δ ^{13}C (ppm)**	**DEPT**	**δ ^{1}H/HSQC (ppm)**	**J (Hz)**	**HMBC**	**COSY**	**δ ^{13}C$^{\#}$ (ppm)**
2	145.1	CH	7.71 (d)	2.60	2',3',3	3	145.1
3	105.3	CH	7.05 (d)	2.60	2,2',3'	2	105.3
4	158.7	Q					158.4
5	136.9	CH	7.96 (d)	16.17	4,5',7	6	142.0
6	123.6	CH	6.48 (d)	16.17	7	5	123.0
7	166.1	Q					166.0
8	165.9	Q					166.0
2'	162.7	Q					163.0
3'	109.1	Q					109.0
5'	141.2	Q					-
8'	160.1	Q					-
OMe-4	59.3	CH_3	4.34(s)				59.1
CO_2 Me-7	51.8	CH_3	3.81(s)				49.8
CO_2 Me-8	53.2	CH_3	3.98(s)				50.9

$^{\#}$(Mara *et al*., 1992).

The ^{13}C NMR (appendix 19) and DEPT (appendix 20) spectra of compound **44** confirmed the presence of fourteen carbon atoms, consisting of four methine, five quaternary, two methoxycarbonyl and three methoxylated carbon atoms. The chemical shifts of the four methine carbons were observed to occur at δ 105.3, 123.6, 136.9 and 145.1. The chemical shifts of the five quaternary carbons were observed to occur at δ 109.1, 141.2, 158.7, 160.1 and 162.7. The chemical shifts of the two methoxycarbonyl carbon atoms were observed to occur at δ 165.9 and 166.1, while the chemical shifts of the three methoxylated carbon atoms were observed to occur at δ 51.8, 53.2 and 59.3. The ^{1}H NMR spectrum (appendix 18) of compound **44** revealed the presence of four aromatic protons. Four doublets resonating at δ 6.48, 7.05, 7.96 and 7.71 as well as three singlets at δ 3.81, 3.98 and 4.34 for methoxy groups were observed.

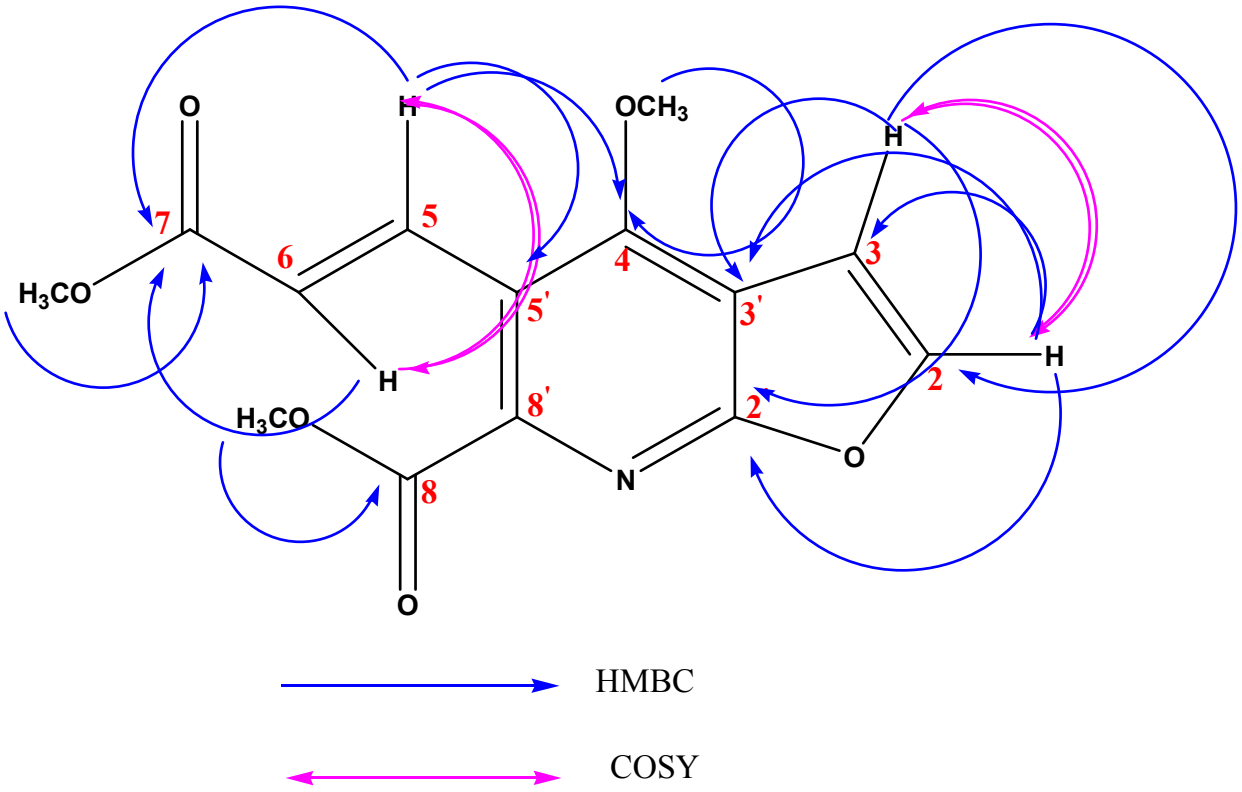
The chemical shifts occurring at δ166.1 and 165.9 were assigned to the methoxycarbonyl carbons (C-7) and (C-8), respectively. Chemical shifts of the quaternary carbons (C-2', C-3', C-4,

C-5' and C-8') were observed to occur at δ 162.7, 109.1, 158.7, 141.2 and 160.1, respectively. The four methine carbons (C-2, C-3, C-5 and C-6) were observed to absorb at δ 145.1, 105.3, 136.9 and 123.6, respectively. The methoxy group carbon attached to C-4 appeared to absorb at δ 59.3, C-7 appeared to absorb at δ 51.8 while that attached to C-8 appeared to absorb at δ 53.2.

The HSQC spectrum (appendix 21) of compound **44** showed correlations of carbon atoms and the protons directly attached to them. There was a correlation between C-3 and a proton, which was observed to resonate at δ 7.05, while a proton resonating at δ 7.71 was observed to correlate with C-2. Carbons C-5 and C-6 were observed to correlate with protons resonating at δ 7.96 and δ 6.48, respectively. The methoxy carbon absorbing at δ 51.8 was correlating with a proton resonating at δ 3.81, that absorbing at δ 53.2 was correlating with a proton resonating at 3.98 while the methoxy carbon resonating at δ 59.3 was correlating with a proton absorbing at δ 4.34.

The HMBC spectrum (appendix 22) showed correlations between carbons and protons. HMBC spectrum gives correlation information between protons and carbon atoms, which are two to three bonds away. From the HMBC spectrum the proton H-2 resonating at δ 7.71 showed correlation with C-2', C-3' and C-3. Proton H-3 absorbing at δ 7.05 was observed to correlate with C-2, C-2' and C-3'. A proton resonating at δ 7.96 (H-5) of α, β-unsaturated ester showed correlation with C-4, C-5' and C-7 while that resonating at δ 6.48 (H-6) was observed to correlate with C-7. The attachment of methoxy groups to carbons C-4, C-7 and C-8 was substantiated by the correlations of protons resonating at δ 4.34, 3.81 and 3.98 with C-4, C-7 and C-8, respectively.

The ^{1}H-^{1}H COSY (appendix 23) correlation for compound **44** was also determined. COSY correlation gives information about protons, which are attached to adjacent carbons. From COSY spectrum, H-2 was correlating with H-3. The coupling constant for peaks absorbing at δ 7.71 (H-2) and δ 7.05 (H-3) was calculated to be 2.60 Hz. H-5 (δ 7.96) and H-6 (δ 6.48) showed COSY correlation as well with a coupling constant of 16.17 Hz. The coupling constant of *trans* isomer is always larger than that of *cis* isomer and is always in the range of 12-18 Hz. The *E*-configuration of compound **44** was determined based on the coupling constant of H-5 (δ 7.96) and H-6 (δ 6.48) which was 16.17. Since this coupling constant falls within the range 12-18 Hz, compound **44** was therefore confirmed to be *E*-isomer. Based on the 1D and 2D NMR information, the proposed structure for compound **44** is shown below.



E-Dimethylrhoifolinate (**44**)

4.4 Larvicidal bioassay of non-volatile crude extracts

4.4.1 Methanol crude extract

The methanol crude extract of *Z. gilletii* was tested against third instar larvae of *An. gambiae s.s*. Serial dilutions were made from the stock solution of 2000 ppm using the formula $C_1\ V_1 = C_2\ V_2$ and all tests were done in triplicates. The results of the test were tabulated as shown in table 5.

Table 5: Larvicidal results of methanol crude extract against 3rd instar of *An. gambiae s.s*

Concentration (ppm)	**% Mortality ± SD**	**LC_{50} (ppm)**	**LC_{90} (ppm)**
31.25	0.00 ± 0.00		
62.50	1.67 ± 2.89		
125.00	1.67 ± 2.89		
250.00	11.67 ± 2.89		
300.00	21.67 ± 7.64		
400.00	31.67 ± 5.77		
500.00	36.67 ± 2.89		
600.00	48.33 ± 5.77	497.62 (314.16 - 718.87)	1014.91 (706.64 – 3706.23)
700.00	66.67 ± 7.64		
800.00	83.33 ± 5.77		
900.00	96.67 ± 2.89		
1000.00	98.33 ± 2.89		
2000.00	100.00 ± 0.00		
^{X}Pylarvex (100 ppm)	100.00 ± 0.00		
^{Y}Spring H_2O + DMSO	0.00 ± 0.00		

X-Positive control, *Y*-Negative control.

From table 5, it can be seen that the crude extract was active against the larvae of *An. gambiae s.s*. At a concentration of 2000 ppm, a mortality of 100 % was recorded while at the lowest concentration used of 31.25 ppm no mortality was recorded. From table 5 it can also be seen that an increase in the concentration of the crude extract led to an increase in the percent mortality. Therefore, it can be concluded that the percent mortality was concentration dependent. The LC_{50} value of 497.62 ppm and LC_{90} value 1014.91 ppm after 24 h exposure period recorded for the extract were calculated using log probit analysis at 95 % confidence level. The negative control showed zero mortality after 24 h of exposure while the positive control recorded a mortality of 100 % at a concentration of 100 ppm.

4.4.2 Ethyl acetate crude extract

Ethyl acetate crude extract was also subjected to larvicidal activity against third instar larvae of *An. gambiae s.s* at various concentrations as per the procedure described in section 3.6. The LC_{50} and LC_{90} values after 24 h exposure period were calculated using log probit analysis (95 % confidence level). The results were tabulated as shown in Table 6.

Table 6: Larvicidal results of ethyl acetate crude extract against 3rd instar of *An. gambiae s.s*

Concentration (ppm)	**% Mortality ± SD**	**LC_{50} (ppm)**	**LC_{90} (ppm)**
31.25	0.00 ± 0.00		
62.50	3.33 ± 2.89		
100.00	10.00 ± 5.00		
125.00	26.67 ± 7.64		
150.00	53.33 ± 7.64	155.65 (138.32 - 173.59)	273.12 (237.45 – 335.36)
200.00	76.67 ± 10.41		
250.00	86.67 ± 10.41		
300.00	91.67 ± 7.64		
400.00	96.67 ± 2.89		
500.00	100.00 ± 0.00		
1000.00	100.00 ± 0.00		
2000.00	100.00 ± 0.00		
^{X}Pylarvex (100 ppm)	100.00 ± 0.00		
^{Y}Spring H_2O + DMSO	0.00 ± 0.00		

X-Positive control, *Y*-Negative control.

The ethyl acetate extract was active against third instar of *An. gambiae s.s* larvae with LC_{50} and LC_{90} values of 155.65 and 273.12 ppm, respectively. The negative control showed no activity and recorded 0.00 % mortality while the positive control recorded 100 % mortality at a concentration of 100 ppm. Following the activity of ethyl acetate crude extract against the third instar larvae of *An. gambiae s.s*, the extract was further subjected to column chromatography in order to isolate the compounds responsible for its activity.

4.5 Larvicidal bioassay of fractions

All the four fractions (F_1, F_2, F_3 and F_4) were subjected to mosquito larvicidal activity against third instar of *An. gambiae s.s* larvae at different concentrations. Serial dilution of the stock solutions was performed as per the procedure in section 3.6. The results were tabulated as shown in Tables 7, 8, 9 and 10.

Table 7: Larvicidal results of F_1 against 3rd instar of *An. gambiae s.s*

Concentration (ppm)	**% Mortality ± SD**	**LC_{50} (ppm)**	**LC_{90} (ppm)**
31.25	0.00 ± 0.00		
62.50	0.00 ± 0.00		
125.00	0.00 ± 0.00		
250.00	0.00 ± 0.00		
300.00	1.67 ± 2.89		
400.00	5.00 ± 5.00		
500.00	20.00 ± 8.66		
600.00	26.67 ± 2.89		
700.00	46.67 ± 7.64	705.24 (646.88 – 772.82)	1133.81 (992.33 – 1407.59)
800.00	58.33 ± 5.77		
900.00	76.67 ± 2.89		
1000.00	90.00 ± 5.00		
2000.00	100.00 ± 0.00		
^{X}Pylarvex (100 ppm)	100.00 ± 0.00		
^{Y}Spring H_2O + DMSO	0.00 ± 0.00		

X-Positive control, *Y*-Negative control.

Table 8: Larvicidal results of F_2 against 3rd instar of *An. gambiae s.s*

Concentration (ppm)	% Mortality ± SD	LC_{50} (ppm)	LC_{90} (ppm)
31.25	0.00 ± 0.00		
62.50	0.00 ± 0.00		
125.00	1.67 ± 2.89		
250.00	3.33 ± 2.89		
300.00	16.67 ± 7.64		
400.00	33.33 ± 5.77		
500.00	35.00 ± 10.00	542.33 (484.88 – 603.42)	1036.48 (889.41 – 1304.55)
600.00	51.67 ± 2.89		
700.00	58.33 ± 7.64		
800.00	80.00 ± 5.00		
900.00	86.67 ± 2.89		
1000.00	96.67 ± 2.89		
2000.00	100.00 ± 0.00		
^{X}Pylarvex (100 ppm)	100.00 ± 0.00		
^{Y}Spring H_2O + DMSO	0.00 ± 0.00		

X-Positive control, *Y*-Negative control.

From Tables 7, 8, 9 and 10, it can be seen that all the four fractions were active against the third instar larvae of *An. gambiae s.s*. Fraction one was the least potent with LC_{50} and LC_{90} values of 705.24 and 1133.81 ppm against the third instar larvae of *An. gambiae s.s*, respectively. Fraction two was more potent against third instar larvae of *An. gambiae s.s* in comparison to fraction one. Fraction two had LC_{50} of 542.33 ppm and LC_{90} of 1036.48 ppm. In comparison to fraction one and two, fraction three was more potent with LC_{50} value of 146.80 ppm and LC_{90} value of 348.34 ppm. Fraction four which was the most potent among the four fractions had LC_{50} and LC_{90} values of 83.59 and 244.61 ppm, respectively. Following the good larvicidal activity of fraction three and four, they were further purified as described in section 3.6 to isolate the compounds responsible for their activity against third instar larvae of *An. gambiae s.s*.

Table 9: Larvicidal results of F_3 against 3rd instar of *An. gambiae s.s*

Concentration (ppm)	**% Mortality ± SD**	**LC_{50} (ppm)**	**LC_{90} (ppm)**
31.25	5.00 ± 5.00		
62.50	15.00 ± 5.00		
100.00	23.33 ± 5.77		
125.00	28.33 ± 7.64		
150.00	48.33 ± 5.77	146.80 (125.52 – 169.56)	348.34 (286.28 – 463.96)
200.00	68.33 ± 7.64		
250.00	73.33 ± 12.58		
300.00	88.33 ± 2.89		
400.00	96.67 ± 2.89		
500.00	100.00 ± 0.00		
1000.00	100.00 ± 0.00		
2000.00	100.00 ± 0.00		
^{X}Pylarvex (100 ppm)	100.00 ± 0.00		
^{Y}Spring H_2O + DMSO	0.00 ± 0.00		

X-Positive control, *Y*-Negative control.

Table 10: Larvicidal results of F_4 against 3rd instar of *An. gambiae s.s*

Concentration (ppm)	**% Mortality ± SD**	**LC_{50} (ppm)**	**LC_{90} (ppm)**
31.25	16.67 ± 5.77		
62.50	35.00 ± 10.00		
100.00	51.67 ± 5.77	83.59 (65.30 – 101.10)	244.61 (196.79 – 334.79)
125.00	66.67 ± 7.64		
150.00	73.33 ± 5.77		
200.00	83.33 ± 5.77		
250.00	88.33 ± 5.77		
300.00	96.67 ± 2.89		
400.00	96.67 ± 2.89		
500.00	100.00 ± 0.00		
1000.00	100.00 ± 0.00		
2000.00	100.00 ± 0.00		
^{X}Pylarvex (100 ppm)	100.00 ± 0.00		
^{Y}Spring H_2O + DMSO	0.00 ± 0.00		

X-Positive control, *Y*-Negative control.

4.6 Larvicidal bioassay of mixture Z

Mixture **Z** that consisted of a pair of *Z/E* isomeric secofuroquinoline alkaloids (**43** and **44**) was subjected to larvicidal assays at various concentrations as seen in section 3.7. Controls, 1% DMSO in spring river water as negative and pyrethrum based larvicide pylarvex, as positive were also performed for comparison. Larval mortality was observed after 24 h exposure period. The LC_{50} and LC_{90} values were calculated using log probit analysis at 95 % confidence level. The results of these tests were tabulated as shown in Table 11.

Table 11: Larvicidal results of mixture Z against 3rd instar of *An. gambiae s.s*

Concentration (ppm)	**% Mortality ± SD**	**LC_{50} (ppm)**	**LC_{90} (ppm)**
3.90	0.00 ± 0.00		
7.80	0.00 ± 0.00		
15.60	0.00 ± 0.00		
20.00	0.00 ± 0.00		
31.25	0.00 ± 0.00		
40.00	1.67 ± 2.89		
50.00	8.33 ± 5.77		
62.50	20.00 ± 0.00		
80.00	21.67 ± 5.77		
90.00	31.67 ± 7.64		
100.00	48.33 ± 5.77	110.31 (97.48 – 128.65)	216.31 (174.83 – 304.95)
125.00	71.67 ± 7.64		
250.00	85.00 ± 5.00		
500.00	98.33 ± 2.89		
1000.00	100.00 ± 0.00		
^{X}Pylarvex (100 ppm)	100.00 ± 0.00		
^{Y}Spring H_2O + DMSO	0.00 ± 0.00		

X-Positive control, *Y*-Negative control.

The positive control showed 100 % mortality at a concentration of 100 ppm while the negative control showed no mortality. Mixture **Z** was active against third instar larvae of *An. gambiae s.s* with LC_{50} and LC_{90} values of 110.31 and 216.31 ppm, respectively (Table 11). At a concentration of 1000 ppm, 100 % mortality was observed while no mortality was observed at a concentration of 31.25 ppm and below. In comparison to fraction four (F_4) and the essential oil which had LC_{50} values of 83.59 and 57.73 ppm, mixture **Z** was less potent. The higher larvicidal activity of fraction four and the essential oil could be attributed to the natural synergism of the various compounds present in both the fraction and the essential oil.

Although this is the first time compounds **43** and **44** have been isolated from *Z. gilletii*, this pair of isomeric secofuroquinoline alkaloid has been isolated before from the fruits, leaves and stems of *Zanthoxylum rhoifolium* (Mara *et al*., 1992). Most furoquinoline alkaloids that have been isolated before have been tested against antiplasmodial activity. From the roots of *Vepris uguenensis*, Cheplogoi *et al* (2008) isolated three furoquinoline alkaloids namely flindersiamine, maculosidine and syringaldehyde. With the exception of flindersiamine (which lacked antimalarial efficacy against all strains, alkaloids maculosidine and syringaldehyde exhibited moderate antimalarial activity against two strains of *P. falciparum* with IC values of 29.2 and 13.0mg/mL (chloroquine-susceptible 3D7 strain) and 40.4 and 21.4mg/mL (chloroquine-resistant FCM29 strain), respectively. Although much has not been reported on the activity of furoquinoline alkaloids against mosquitoe larvae, furoquinolines such as Fagarine and Dictamine isolated from *Spiranthera odoratissima* were reported to exhibit insecticidal activity against leaf-cutting ants (Terezan *et al*., 2010). Apart from the furoquinoline class of alkaloids, other classes of alkaloids have been isolated from various plant species and tested against larvae of *An. gambiae* (Matasyoh *et al*., 2011). Generally alkaloids are known to possess medicinal and in insecticidal properties.

This is the first time a secofuroquinoline alkaloid has been isolated from *Z. gilletii* and tested against larvae of *An. gambiae s.s*. This secofuroquinoline showed a good larvicidal activity against third instar larvae of *An. gambiae s.s* mosquito and potentially can be used in malaria vector control.

CHAPTER FIVE
CONCLUSIONS AND RECOMMENDATIONS

5.1 Conclusions

The ethyl acetate crude extract of *Zanthoxylum gilletii* leaves possess mosquito larvicidal activity against the third instar of *An. gambiaes s.s*. This crude extract exhibited LC_{50} and LC_{90} values of 155.65 and 273.12 ppm, respectively. Bioassay-guided column fractionation of ethyl acetate crude extract afforded four fractions (F_1, F_2, F_3 and F_4). The four fractions were active against the third instar larvae of *An. gambiae s.s*, F_1 (LC_{50} = 705.24 ppm; LC_{90} = 1133.81 ppm), F_2 (LC_{50} = 542.33 ppm; LC_{90} = 1036.48 ppm), F_3 (LC_{50} = 146.80 ppm; LC_{90} = 348.34 ppm) and F_4 (LC_{50} = 83.59 ppm; LC_{90} = 244.61 ppm). Further purification of fractions three and four by Preparative TLC afforded mixture **Z** which consisted of a pair of isomeric secofuroquinoline alkaloids *Z*-Dimethylrhoifolinate **43** and *E*-Dimethylrhoifolinate **44** which were successfully identified by NMR and MS spectroscopic analyses. Mixture **Z** exhibited LC_{50} value of 110.31 ppm and LC_{90} value of 216.31 ppm and was more potent in comparison with fractions one, two and three but less potent than fraction four and the essential oil.

The essential oil from *Z. gilletii* leaves was shown to possess mosquito larvicidal activity against the third instar of *An. gambiae s.s*. The oil exhibited LC_{50} and LC_{90} values of 57.73 and 140.24 ppm, respectively. Of the total 68 components of the essential oil, only 47 (69.11%) were identified indicating presence of new compounds in the oil whose mass spectra could not be found in the GC-MS databases that already exist. The oil was dominated by monoterpenes and sesquiterpenes which accounted for 34.00 % and 38.30 %, respectively.

The results of this study suggest that both the essential oil and the Secofuroquinoline alkaloids *Z*-Dimethylrhoifolinate and *E*-Dimethylrhoifolinate from the plant *Zanthoxylum gilletii*, holds great promise as potential mosquito larvicides. Furthermore these outcomes could be useful in the search for newer more selective, biodegradable and natural larvicidal compounds. These findings also offer an opportunity for developing alternatives to inorganic insecticides which have been documented not only to affect non-target population but have constantly increased mosquito resistance to these insecticides as well.

5.2 Recommendations

1. Individual compounds from the essential oil should be isolated and tested against the third instar larvae of *An. gambiae s.s*.
2. The mode of action of the essential oil and compound **43** and **44** on mosquito larvae should be studied.
3. The effect of the extracts on non-target organism should be investigated with the aim of formulating a botanical larvicide.

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APPENDICES

Appendix 1: Generated LC values for Methanol 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	136.466	10.756	245.536	2.135	1.032	2.390
	.020	158.805	16.415	270.973	2.201	1.215	2.433
	.030	174.839	21.446	288.710	2.243	1.331	2.460
	.040	187.959	26.208	302.985	2.274	1.418	2.481
	.050	199.353	30.839	315.250	2.300	1.489	2.499
	.060	209.595	35.407	326.196	2.321	1.549	2.513
	.070	219.006	39.953	336.210	2.340	1.602	2.527
	.080	227.791	44.505	345.532	2.358	1.648	2.538
	.090	236.087	49.080	354.326	2.373	1.691	2.549
	.100	243.989	53.694	362.705	2.387	1.730	2.560
	.150	279.623	77.649	400.786	2.447	1.890	2.603
	.200	311.620	103.598	435.998	2.494	2.015	2.639
	.250	341.973	131.990	471.085	2.534	2.121	2.673
	.300	371.743	163.100	507.968	2.570	2.212	2.706
	.350	401.637	197.057	548.533	2.604	2.295	2.739
	.400	432.224	233.803	595.042	2.636	2.369	2.775
	.450	464.032	273.030	650.475	2.667	2.436	2.813
	.500	**497.621**	**314.161**	**718.871**	**2.697**	**2.497**	**2.857**
	.550	533.641	356.442	805.704	2.727	2.552	2.906
	.600	572.913	399.192	918.386	2.758	2.601	2.963
	.650	616.543	442.119	1067.264	2.790	2.646	3.028
	.700	666.124	485.556	1267.878	2.824	2.686	3.103
	.750	724.112	530.593	1545.980	2.860	2.725	3.189
	.800	794.643	579.280	1949.306	2.900	2.763	3.290
	.850	885.573	635.327	2579.658	2.947	2.803	3.412
	.900	**1014.908**	**706.639**	**3706.230**	**3.006**	**2.849**	**3.569**
	.910	1048.880	724.197	4049.875	3.021	2.860	3.607
	.920	1087.077	743.472	4461.166	3.036	2.871	3.649
	.930	1130.683	764.936	4963.865	3.053	2.884	3.696
	.940	1181.455	789.281	5595.113	3.072	2.897	3.748
	.950	1242.149	817.572	6416.902	3.094	2.913	3.807
	.960	1317.453	851.599	7542.408	3.120	2.930	3.878
	.970	1416.314	894.711	9206.972	3.151	2.952	3.964
	.980	1559.311	954.428	12014.212	3.193	2.980	4.080
	.990	1814.564	1054.809	18308.722	3.259	3.023	4.263

a . Logarithm base = 10.

Appendix 2: Generated LC values for Hexane 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	43.536	25.286	62.612	1.639	1.403	1.797
	.020	54.012	33.132	75.058	1.732	1.520	1.875
	.030	61.930	39.312	84.246	1.792	1.595	1.926
	.040	68.643	44.697	91.917	1.837	1.650	1.963
	.050	74.636	49.607	98.690	1.873	1.696	1.994
	.060	80.148	54.199	104.864	1.904	1.734	2.021
	.070	85.315	58.566	110.611	1.931	1.768	2.044
	.080	90.223	62.765	116.039	1.955	1.798	2.065
	.090	94.932	66.837	121.220	1.977	1.825	2.084
	.100	99.483	70.810	126.208	1.998	1.850	2.101
	.150	120.767	89.814	149.341	2.082	1.953	2.174
	.200	140.886	108.272	171.063	2.149	2.035	2.233
	.250	160.797	126.851	192.583	2.206	2.103	2.285
	.300	181.066	145.934	214.650	2.258	2.164	2.332
	.350	202.121	165.804	237.875	2.306	2.220	2.376
	.400	224.360	186.703	262.866	2.351	2.271	2.420
	.450	248.203	208.880	290.301	2.395	2.320	2.463
	.500	**274.140**	**232.618**	**321.000**	**2.438**	**2.367**	**2.507**
	.550	302.788	258.281	356.008	2.481	2.412	2.551
	.600	334.966	286.371	396.718	2.525	2.457	2.598
	.650	371.821	317.624	445.083	2.570	2.502	2.648
	.700	415.059	353.161	504.009	2.618	2.548	2.702
	.750	467.376	394.779	578.140	2.670	2.596	2.762
	.800	533.431	445.575	675.622	2.727	2.649	2.830
	.850	622.297	511.514	812.677	2.794	2.709	2.910
	.900	**755.437**	**606.452**	**1028.775**	**2.878**	**2.783**	**3.012**
	.910	791.654	631.638	1089.534	2.899	2.800	3.037
	.920	832.968	660.083	1159.794	2.921	2.820	3.064
	.930	880.889	692.723	1242.500	2.945	2.841	3.094
	.940	937.676	730.952	1342.111	2.972	2.864	3.128
	.950	1006.922	776.967	1465.817	3.003	2.890	3.166
	.960	1094.839	834.529	1626.217	3.039	2.921	3.211
	.970	1213.511	910.875	1848.258	3.084	2.959	3.267
	.980	1391.411	1022.809	2192.065	3.143	3.010	3.341
	.990	1726.219	1226.775	2870.749	3.237	3.089	3.458

a. Logarithm base = 10.

Appendix 3: Generated LC values for Ethyl acetate 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	56.085	38.286	71.153	1.749	1.583	1.852
	.020	63.211	44.788	78.491	1.801	1.651	1.895
	.030	68.195	49.461	83.559	1.834	1.694	1.922
	.040	72.201	53.285	87.601	1.859	1.727	1.943
	.050	75.633	56.605	91.046	1.879	1.753	1.959
	.060	78.682	59.586	94.095	1.896	1.775	1.974
	.070	81.457	62.324	96.862	1.911	1.795	1.986
	.080	84.024	64.875	99.416	1.924	1.812	1.997
	.090	86.429	67.282	101.805	1.937	1.828	2.008
	.100	88.703	69.571	104.063	1.948	1.842	2.017
	.150	98.775	79.838	114.052	1.995	1.902	2.057
	.200	107.589	88.955	122.826	2.032	1.949	2.089
	.250	115.776	97.483	131.049	2.064	1.989	2.117
	.300	123.657	105.704	139.075	2.092	2.024	2.143
	.350	131.438	113.789	147.146	2.119	2.056	2.168
	.400	139.273	121.858	155.459	2.144	2.086	2.192
	.450	147.299	130.005	164.204	2.168	2.114	2.215
	.500	**155.648**	**138.320**	**173.587**	**2.192**	**2.141**	**2.240**
	.550	164.471	146.897	183.841	2.216	2.167	2.264
	.600	173.949	155.853	195.261	2.240	2.193	2.291
	.650	184.319	165.349	208.233	2.266	2.218	2.319
	.700	195.917	175.616	223.302	2.292	2.245	2.349
	.750	209.253	187.015	241.302	2.321	2.272	2.383
	.800	225.175	200.147	263.629	2.353	2.301	2.421
	.850	245.269	216.128	292.941	2.390	2.335	2.467
	.900	**273.118**	**237.451**	**335.357**	**2.436**	**2.376**	**2.526**
	.910	280.306	242.828	346.605	2.448	2.385	2.540
	.920	288.328	248.778	359.296	2.460	2.396	2.555
	.930	297.415	255.456	373.836	2.473	2.407	2.573
	.940	307.903	263.088	390.828	2.488	2.420	2.592
	.950	320.316	272.025	411.222	2.506	2.435	2.614
	.960	335.541	282.858	436.632	2.526	2.452	2.640
	.970	355.254	296.693	470.152	2.551	2.472	2.672
	.980	383.262	316.023	518.915	2.583	2.500	2.715
	.990	431.957	348.849	606.640	2.635	2.543	2.783

a. Logarithm base = 10.

Appendix 4: Generated LC values for Fraction one (F_1)

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	297.873	213.948	361.886	2.474	2.330	2.559
	.020	329.528	245.458	392.483	2.518	2.390	2.594
	.030	351.335	267.742	413.337	2.546	2.428	2.616
	.040	368.685	285.777	429.830	2.567	2.456	2.633
	.050	383.428	301.297	443.791	2.584	2.479	2.647
	.060	396.440	315.134	456.084	2.598	2.498	2.659
	.070	408.212	327.754	467.188	2.611	2.516	2.669
	.080	419.048	339.452	477.404	2.622	2.531	2.679
	.090	429.154	350.424	486.929	2.633	2.545	2.687
	.100	438.671	360.809	495.903	2.642	2.557	2.695
	.150	480.372	406.777	535.385	2.682	2.609	2.729
	.200	516.325	446.760	569.877	2.713	2.650	2.756
	.250	549.306	483.412	602.188	2.740	2.684	2.780
	.300	580.716	518.000	633.841	2.764	2.714	2.802
	.350	611.423	551.232	665.890	2.786	2.741	2.823
	.400	642.060	583.561	699.203	2.808	2.766	2.845
	.450	673.163	615.333	734.580	2.828	2.789	2.866
	.500	**705.244**	**646.884**	**772.818**	**2.848**	**2.811**	**2.888**
	.550	738.853	678.608	814.777	2.869	2.832	2.911
	.600	774.645	711.009	861.475	2.889	2.852	2.935
	.650	813.462	744.752	914.236	2.910	2.872	2.961
	.700	856.475	780.747	974.960	2.933	2.893	2.989
	.750	905.449	820.309	1046.598	2.957	2.914	3.020
	.800	963.287	865.521	1134.160	2.984	2.937	3.055
	.850	1035.382	920.131	1247.196	3.015	2.964	3.096
	.900	**1133.809**	**992.334**	**1407.587**	**3.055**	**2.997**	**3.148**
	.910	1158.953	1010.425	1449.584	3.064	3.005	3.161
	.920	1186.900	1030.387	1496.725	3.074	3.013	3.175
	.930	1218.409	1052.717	1550.439	3.086	3.022	3.190
	.940	1254.588	1078.146	1612.836	3.099	3.033	3.208
	.950	1297.164	1107.800	1687.220	3.113	3.044	3.227
	.960	1349.035	1143.563	1779.194	3.130	3.058	3.250
	.970	1415.656	1188.955	1899.419	3.151	3.075	3.279
	.980	1509.336	1251.857	2072.304	3.179	3.098	3.316
	.990	1669.733	1357.349	2378.124	3.223	3.133	3.376

a. Logarithm base = 10.

Appendix 5: Generated LC values for Fraction two (F_2)

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	167.352	110.887	216.419	2.224	2.045	2.335
	.020	192.072	132.774	242.286	2.283	2.123	2.384
	.030	209.618	148.808	260.351	2.321	2.173	2.416
	.040	223.865	162.105	274.873	2.350	2.210	2.439
	.050	236.166	173.769	287.320	2.373	2.240	2.458
	.060	247.166	184.334	298.392	2.393	2.266	2.475
	.070	257.233	194.107	308.480	2.410	2.288	2.489
	.080	266.593	203.278	317.829	2.426	2.308	2.502
	.090	275.402	211.977	326.603	2.440	2.326	2.514
	.100	283.767	220.298	334.918	2.453	2.343	2.525
	.150	321.192	258.130	372.004	2.507	2.412	2.571
	.200	354.426	292.366	404.957	2.550	2.466	2.607
	.250	385.666	324.873	436.163	2.586	2.512	2.640
	.300	416.059	356.593	466.939	2.619	2.552	2.669
	.350	446.358	388.096	498.225	2.650	2.589	2.697
	.400	477.146	419.773	530.840	2.679	2.623	2.725
	.450	508.954	451.935	565.605	2.707	2.655	2.753
	.500	**542.326**	**484.879**	**603.423**	**2.734**	**2.686**	**2.781**
	.550	577.887	518.951	645.351	2.762	2.715	2.810
	.600	616.411	554.616	692.694	2.790	2.744	2.841
	.650	658.928	592.553	747.170	2.819	2.773	2.873
	.700	706.913	633.785	811.214	2.849	2.802	2.909
	.750	762.624	679.908	888.583	2.882	2.832	2.949
	.800	829.843	733.579	985.663	2.919	2.865	2.994
	.850	915.708	799.735	1114.755	2.962	2.903	3.047
	.900	**1036.478**	**889.408**	**1304.549**	**3.016**	**2.949**	**3.115**
	.910	1067.960	912.266	1355.439	3.029	2.960	3.132
	.920	1103.246	937.666	1413.123	3.043	2.972	3.150
	.930	1143.393	966.303	1479.554	3.058	2.985	3.170
	.940	1189.959	999.197	1557.639	3.076	3.000	3.192
	.950	1245.387	1037.935	1651.973	3.095	3.016	3.218
	.960	1313.816	1085.187	1770.424	3.119	3.036	3.248
	.970	1403.114	1145.993	1928.155	3.147	3.059	3.285
	.980	1531.287	1231.762	2160.445	3.185	3.091	3.335
	.990	1757.485	1379.417	2586.070	3.245	3.140	3.413

a. Logarithm base = 10.

Appendix 6: Generated LC values for Fraction three (F_3)

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	30.585	17.727	43.231	1.486	1.249	1.636
	.020	36.756	22.490	50.302	1.565	1.352	1.702
	.030	41.302	26.147	55.397	1.616	1.417	1.743
	.040	45.089	29.278	59.580	1.654	1.467	1.775
	.050	48.424	32.093	63.226	1.685	1.506	1.801
	.060	51.457	34.697	66.514	1.711	1.540	1.823
	.070	54.271	37.149	69.546	1.735	1.570	1.842
	.080	56.922	39.487	72.386	1.755	1.596	1.860
	.090	59.445	41.737	75.076	1.774	1.621	1.876
	.100	61.866	43.917	77.648	1.791	1.643	1.890
	.150	72.984	54.161	89.367	1.863	1.734	1.951
	.200	83.229	63.874	100.100	1.920	1.805	2.000
	.250	93.157	73.460	110.513	1.969	1.866	2.043
	.300	103.078	83.144	120.996	2.013	1.920	2.083
	.350	113.212	93.076	131.847	2.054	1.969	2.120
	.400	123.748	103.375	143.347	2.093	2.014	2.156
	.450	134.874	114.150	155.798	2.130	2.057	2.193
	.500	**146.799**	**125.517**	**169.555**	**2.167**	**2.099**	**2.229**
	.550	159.780	137.615	185.065	2.204	2.139	2.267
	.600	174.145	150.634	202.910	2.241	2.178	2.307
	.650	190.352	164.850	223.889	2.280	2.217	2.350
	.700	209.066	180.691	249.166	2.320	2.257	2.396
	.750	231.331	198.847	280.571	2.364	2.299	2.448
	.800	258.926	220.502	321.264	2.413	2.343	2.507
	.850	295.272	247.920	377.444	2.470	2.394	2.577
	.900	**348.337**	**286.281**	**463.957**	**2.542**	**2.457**	**2.666**
	.910	362.523	296.271	487.887	2.559	2.472	2.688
	.920	378.590	307.469	515.369	2.578	2.488	2.712
	.930	397.080	320.215	547.472	2.599	2.505	2.738
	.940	418.800	335.012	585.807	2.622	2.525	2.768
	.950	445.025	352.648	632.955	2.648	2.547	2.801
	.960	477.942	374.460	693.397	2.679	2.573	2.841
	.970	521.764	403.001	775.923	2.717	2.605	2.890
	.980	586.301	444.131	901.445	2.768	2.648	2.955
	.990	704.604	517.214	1142.717	2.848	2.714	3.058

a. Logarithm base = 10.

Appendix 7: Generated LC values for Fraction four (F_4)

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	11.901	5.127	19.779	1.076	.710	1.296
	.020	14.955	6.955	23.783	1.175	.842	1.376
	.030	17.287	8.437	26.743	1.238	.926	1.427
	.040	19.278	9.755	29.215	1.285	.989	1.466
	.050	21.066	10.976	31.399	1.324	1.040	1.497
	.060	22.718	12.133	33.389	1.356	1.084	1.524
	.070	24.272	13.247	35.241	1.385	1.122	1.547
	.080	25.754	14.329	36.989	1.411	1.156	1.568
	.090	27.180	15.389	38.658	1.434	1.187	1.587
	.100	28.562	16.432	40.263	1.456	1.216	1.605
	.150	35.074	21.540	47.690	1.545	1.333	1.678
	.200	41.293	26.677	54.629	1.616	1.426	1.737
	.250	47.500	32.009	61.458	1.677	1.505	1.789
	.300	53.865	37.651	68.404	1.731	1.576	1.835
	.350	60.522	43.700	75.649	1.782	1.640	1.879
	.400	67.599	50.253	83.371	1.830	1.701	1.921
	.450	75.232	57.414	91.770	1.876	1.759	1.963
	.500	**83.586**	**65.302**	**101.100**	**1.922**	**1.815**	**2.005**
	.550	92.866	74.060	111.703	1.968	1.870	2.048
	.600	103.353	83.863	124.056	2.014	1.924	2.094
	.650	115.437	94.947	138.866	2.062	1.977	2.143
	.700	129.705	107.654	157.208	2.113	2.032	2.196
	.750	147.087	122.535	180.822	2.168	2.088	2.257
	.800	169.196	140.579	212.758	2.228	2.148	2.328
	.850	199.196	163.764	259.098	2.299	2.214	2.413
	.900	**244.612**	**196.790**	**334.790**	**2.388**	**2.294**	**2.525**
	.910	257.052	205.505	356.539	2.410	2.313	2.552
	.920	271.285	215.333	381.908	2.433	2.333	2.582
	.930	287.846	226.596	412.051	2.459	2.355	2.615
	.940	307.540	239.770	448.726	2.488	2.380	2.652
	.950	331.651	255.613	494.788	2.521	2.408	2.694
	.960	362.403	275.419	555.285	2.559	2.440	2.745
	.970	404.145	301.679	640.319	2.607	2.480	2.806
	.980	467.169	340.188	774.563	2.669	2.532	2.889
	.990	587.045	410.446	1047.218	2.769	2.613	3.020

a. Logarithm base = 10.

Appendix 8: Generated LC values for Essential oil

Confidence Limits

		95% Confidence Limits for con			95% Confidence Limits for log(con)(a)		
	Probability	Estimate	Lower Bound	Upper Bound	Estimate	Lower Bound	Upper Bound
PROBIT	.010	11.525	5.622	17.526	1.062	.750	1.244
	.020	13.919	7.288	20.414	1.144	.863	1.310
	.030	15.691	8.586	22.504	1.196	.934	1.352
	.040	17.170	9.708	24.228	1.235	.987	1.384
	.050	18.476	10.725	25.737	1.267	1.030	1.411
	.060	19.666	11.670	27.102	1.294	1.067	1.433
	.070	20.771	12.564	28.365	1.317	1.099	1.453
	.080	21.814	13.420	29.553	1.339	1.128	1.471
	.090	22.808	14.246	30.681	1.358	1.154	1.487
	.100	23.763	15.048	31.764	1.376	1.177	1.502
	.150	28.159	18.842	36.745	1.450	1.275	1.565
	.200	32.227	22.460	41.382	1.508	1.351	1.617
	.250	36.182	26.038	45.953	1.558	1.416	1.662
	.300	40.146	29.652	50.628	1.604	1.472	1.704
	.350	44.205	33.354	55.540	1.645	1.523	1.745
	.400	48.436	37.184	60.817	1.685	1.570	1.784
	.450	52.915	41.185	66.599	1.724	1.615	1.823
	.500	**57.726**	**45.402**	**73.049**	**1.761**	**1.657**	**1.864**
	.550	62.976	49.893	80.378	1.799	1.698	1.905
	.600	68.799	54.737	88.863	1.838	1.738	1.949
	.650	75.384	60.043	98.894	1.877	1.778	1.995
	.700	83.006	65.976	111.056	1.919	1.819	2.046
	.750	92.099	72.799	126.278	1.964	1.862	2.101
	.800	103.402	80.954	146.194	2.015	1.908	2.165
	.850	118.339	91.285	174.040	2.073	1.960	2.241
	.900	**140.235**	**105.726**	**217.656**	**2.147**	**2.024**	**2.338**
	.910	146.105	109.482	229.866	2.165	2.039	2.361
	.920	152.760	113.690	243.958	2.184	2.056	2.387
	.930	160.429	118.477	260.513	2.205	2.074	2.416
	.940	169.449	124.029	280.406	2.229	2.094	2.448
	.950	180.358	130.639	305.047	2.256	2.116	2.484
	.960	194.075	138.807	336.901	2.288	2.142	2.528
	.970	212.375	149.481	380.833	2.327	2.175	2.581
	.980	239.402	164.840	448.534	2.379	2.217	2.652
	.990	289.151	192.069	581.225	2.461	2.283	2.764

a. Logarithm base = 10.

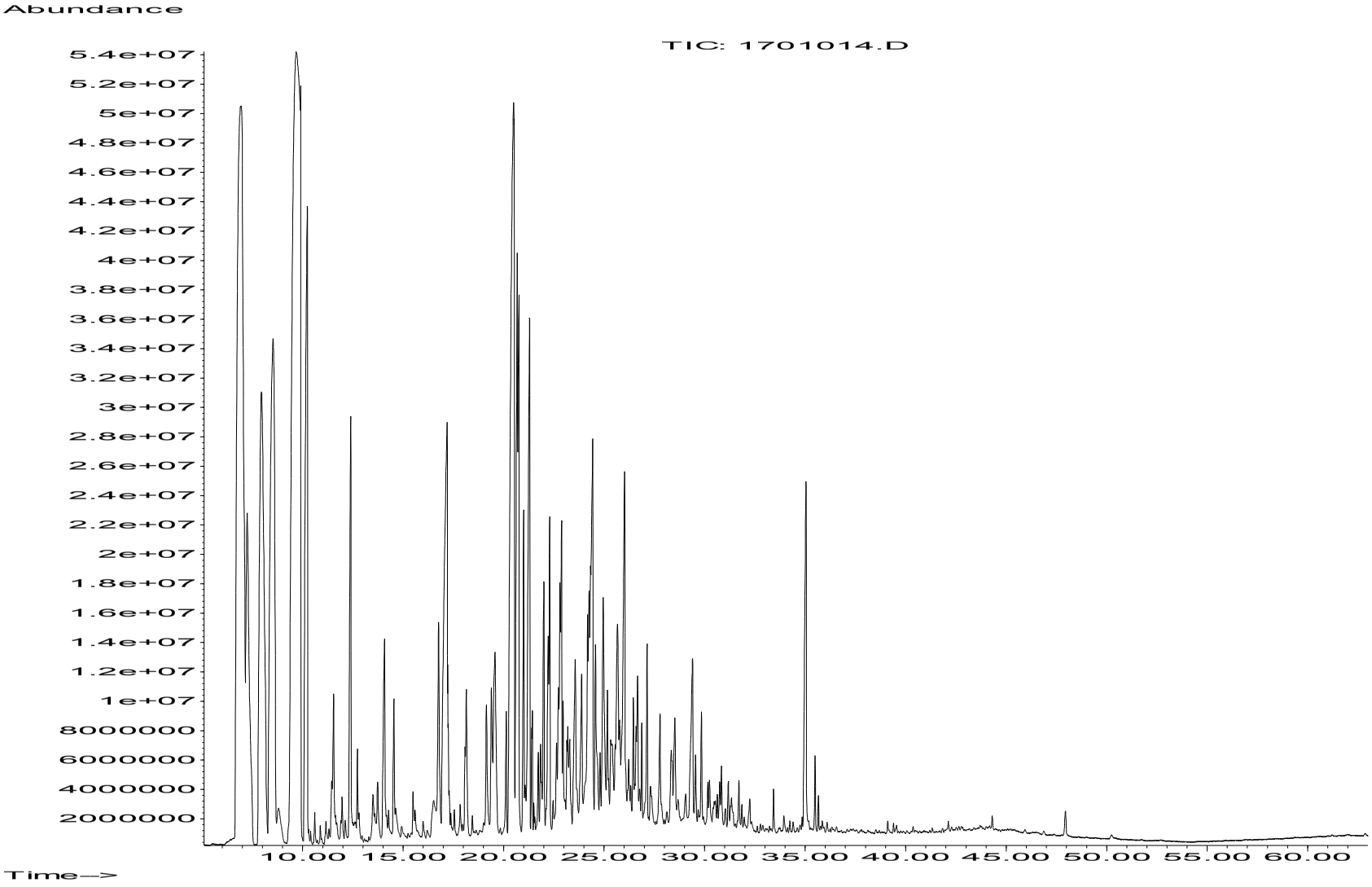
Appendix 9: Generated LC values for mixture **Z**

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	32.492	21.964	41.276	1.512	1.342	1.616
	.020	37.495	26.576	46.410	1.574	1.424	1.667
	.030	41.062	29.974	50.024	1.613	1.477	1.699
	.040	43.968	32.800	52.948	1.643	1.516	1.724
	.050	46.482	35.283	55.471	1.667	1.548	1.744
	.060	48.735	37.534	57.727	1.688	1.574	1.761
	.070	50.800	39.616	59.796	1.706	1.598	1.777
	.080	52.723	41.570	61.723	1.722	1.619	1.790
	.090	54.535	43.421	63.543	1.737	1.638	1.803
	.100	56.258	45.190	65.278	1.750	1.655	1.815
	.150	63.991	53.185	73.153	1.806	1.726	1.864
	.200	70.889	60.315	80.379	1.851	1.780	1.905
	.250	77.395	66.949	87.455	1.889	1.826	1.942
	.300	83.746	73.270	94.670	1.923	1.865	1.976
	.350	90.095	79.392	102.229	1.955	1.900	2.010
	.400	96.564	85.408	110.301	1.985	1.931	2.043
	.450	103.265	91.405	119.049	2.014	1.961	2.076
	.500	**110.313**	**97.482**	**128.648**	**2.043**	**1.989**	**2.109**
	.550	117.843	103.745	139.312	2.071	2.016	2.144
	.600	126.020	110.323	151.324	2.100	2.043	2.180
	.650	135.069	117.381	165.083	2.131	2.070	2.218
	.700	145.309	125.140	181.182	2.162	2.097	2.258
	.750	157.232	133.927	200.564	2.197	2.127	2.302
	.800	171.664	144.276	224.851	2.235	2.159	2.352
	.850	190.167	157.174	257.188	2.279	2.196	2.410
	.900	**216.306**	**174.832**	**304.947**	**2.335**	**2.243**	**2.484**
	.910	223.141	179.357	317.806	2.349	2.254	2.502
	.920	230.810	184.395	332.410	2.363	2.266	2.522
	.930	239.548	190.086	349.268	2.379	2.279	2.543
	.940	249.699	196.634	369.135	2.397	2.294	2.567
	.950	261.801	204.361	393.210	2.418	2.310	2.595
	.960	276.772	213.806	423.552	2.442	2.330	2.627
	.970	296.355	225.987	464.145	2.472	2.354	2.667
	.980	324.548	243.212	524.294	2.511	2.386	2.720
	.990	374.528	272.965	635.543	2.573	2.436	2.803

a. Logarithm base = 10.

Appendix 10: GC-MS spectrum for *Zanthoxylum gilletii* essential oil

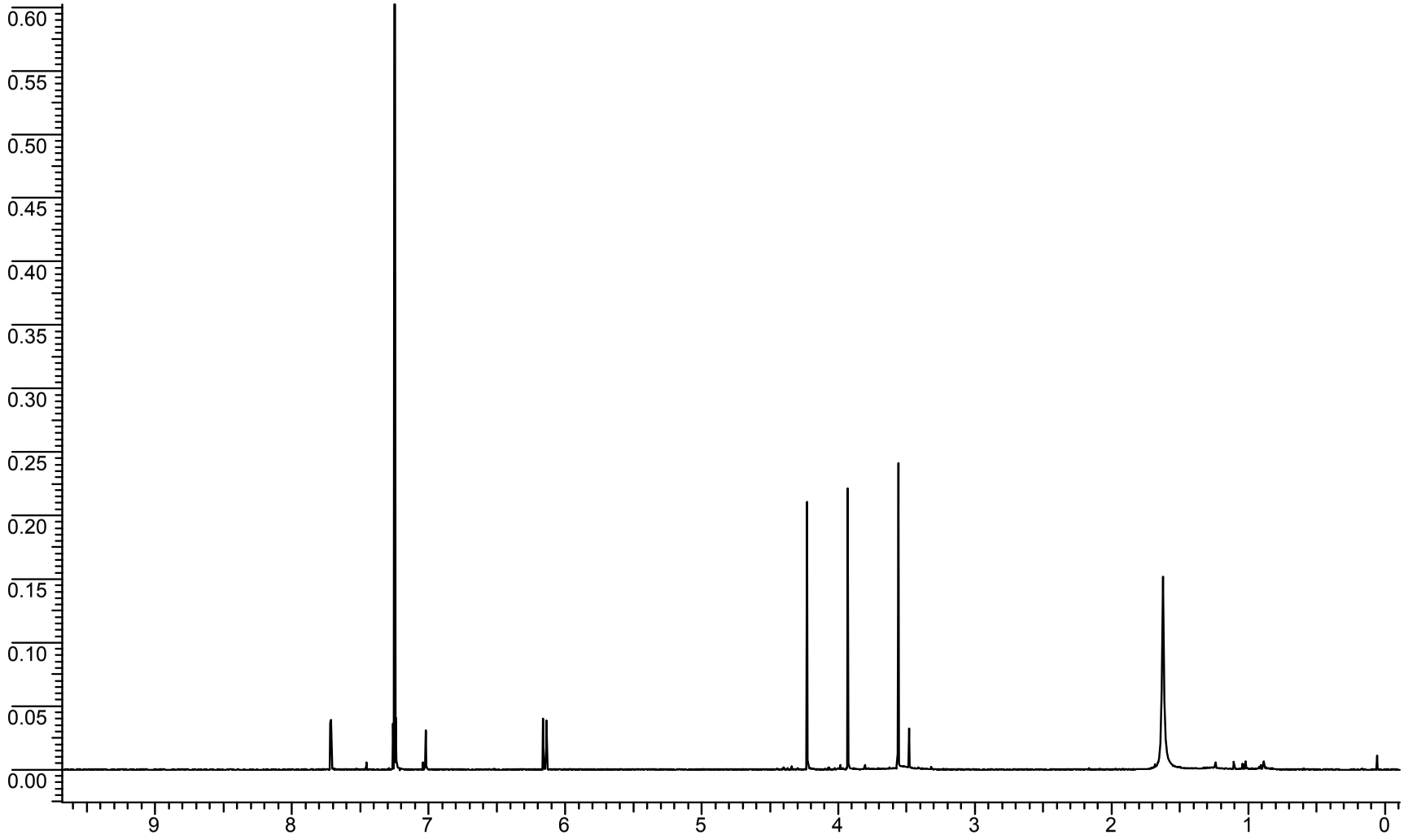


Appendix 11: Identified compounds of the essential oil from *Z. gilletii*

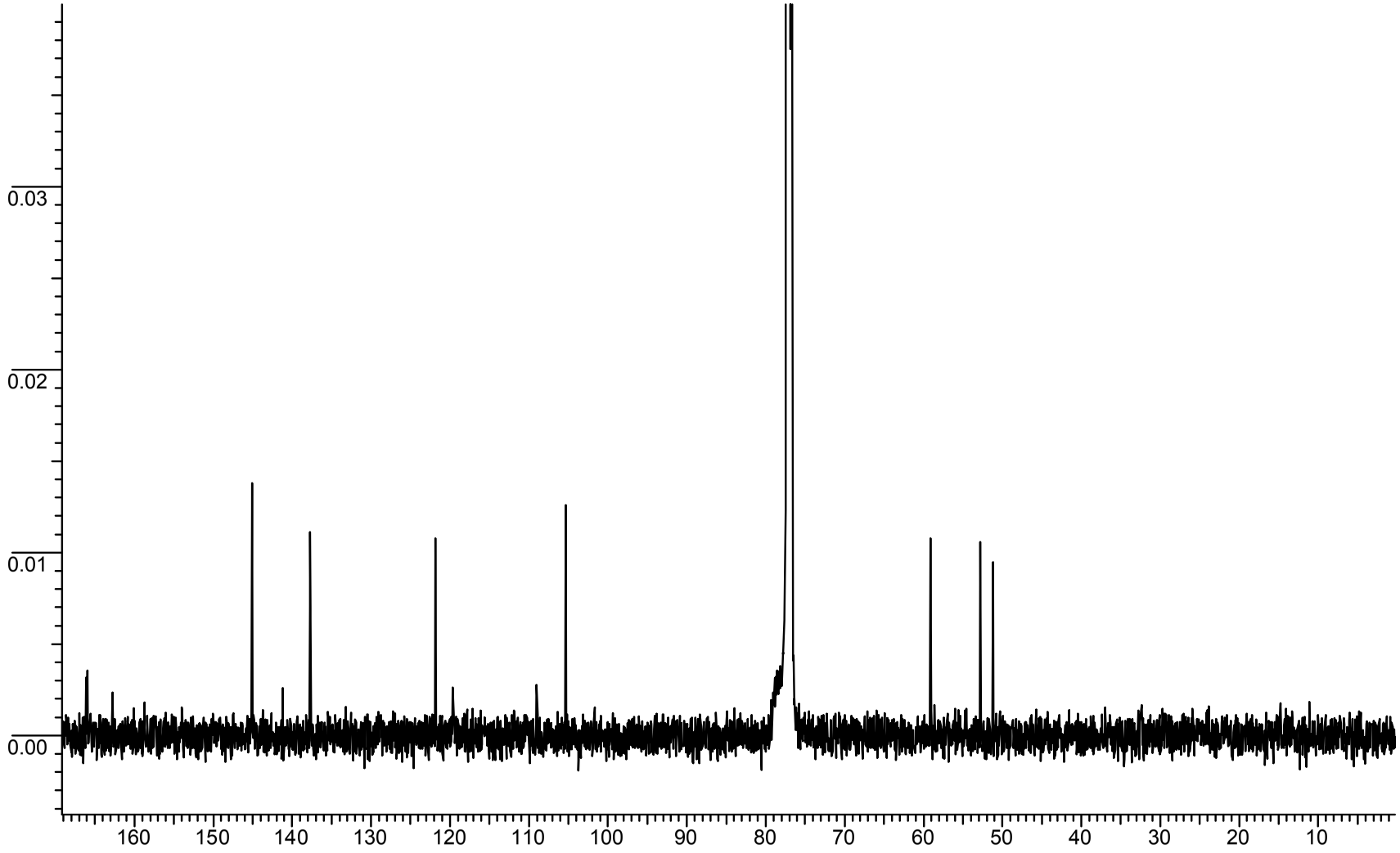
Compound number	Retention time (min)	Compound name	Concentration %
1	6.93	γ-terpinene	10.63
2	7.25	camphene	2.56
3	7.96	sabinene	4.89
4	8.54	β-myrcene	5.16
5	8.81	tricyclene	0.32
6	10.19	β-ocimene	3.12
7	10.61	trans Sabinene hydrate	0.13
8	11.53	3,7-dimethyl-1,6-octadien-3-ol	0.84
9	12.37	alloocimene	1.35
10	12.73	cis-epoxyocimene	0.44
11	13.73	4-terpineol	0.55
12	14.06	cryptone	1.00
13	14.55	n-decanal	0.58
14	14.95	trans-(+)-carveol	0.12
15	15.53	2-methyl-2-phenylpropanal	0.37
16	16.01	(2-methylpropyl)-benzene	0.16
17	16.77	bornyl acetate	1.16
18	17.14	2-undecanone	3.64
19	18.46	α- cubebene	0.23
20	19.15	α-copaene	0.58
21	19.56	β-cubebene	1.70
22	20.46	trans Caryophyllene	9.82
23	20.99	germacrene D	0.87
24	21.26	1,1,4,8-tetramethyl-4,7,10-cycloundecatriene	2.62
25	21.99	β- selinene	1.35
26	22.26	2-isopropyl-5-methyl-9-methylene-bicyclo[4.4.0]dec-1-ene	1.43
27	22.84	δ-cadinene	2.52

28	23.21	1a,2,3,4,4a,5,6,7b-octahydro-1,1,4,7-tetramethyl-1H-cycloprop(e) azulene	0.84
29	23.55	4-ethenyl-cyclohexenemethanol	0.98
30	23.87	2,6,6-trimethyl-5-(3-methyl-2-butyl)-1-cyclohexene-1-methanol	0.77
31	24.39	caryophyllene oxide	4.40
32	25.67	τ-cadinol	2.29
33	25.99	α- cadinol	2.71
34	26.86	juniper camphor	0.31
35	27.13	8-dodecenol	0.30
36	29.05	β-oplopenone	0.30
37	30.22	3-thujopsanone	0.39
38	30.81	octahydro-4a-methyl-7-(1-methylethyl)-2-(1H)-naphthalenone	0.84
39	32.24	palmitic acid	0.24
40	33.43	cis-nerolidol	0.29
41	33.96	5-(decahydro-5,5,8a-trimethyl-2-methylene-1-naphthalenyl)-3-methyl-2-pentenoic acid	0.28
42	35.02	Phytol	1.51
43	38.53	2,6,10-trimethyl-13-(1-methylethenyl)-2,5,9-cyclotetradectrien-1-ol	0.04
44	41.32	n-eicosane	0.04
45	44.30	nonadecane	0.24
46	47.95	nonacosane	0.16
47	50.25	(1S,2S,5R)-(+)-4-isopropyl-7-methyl-oxaspiro(2,5) octane	0.03

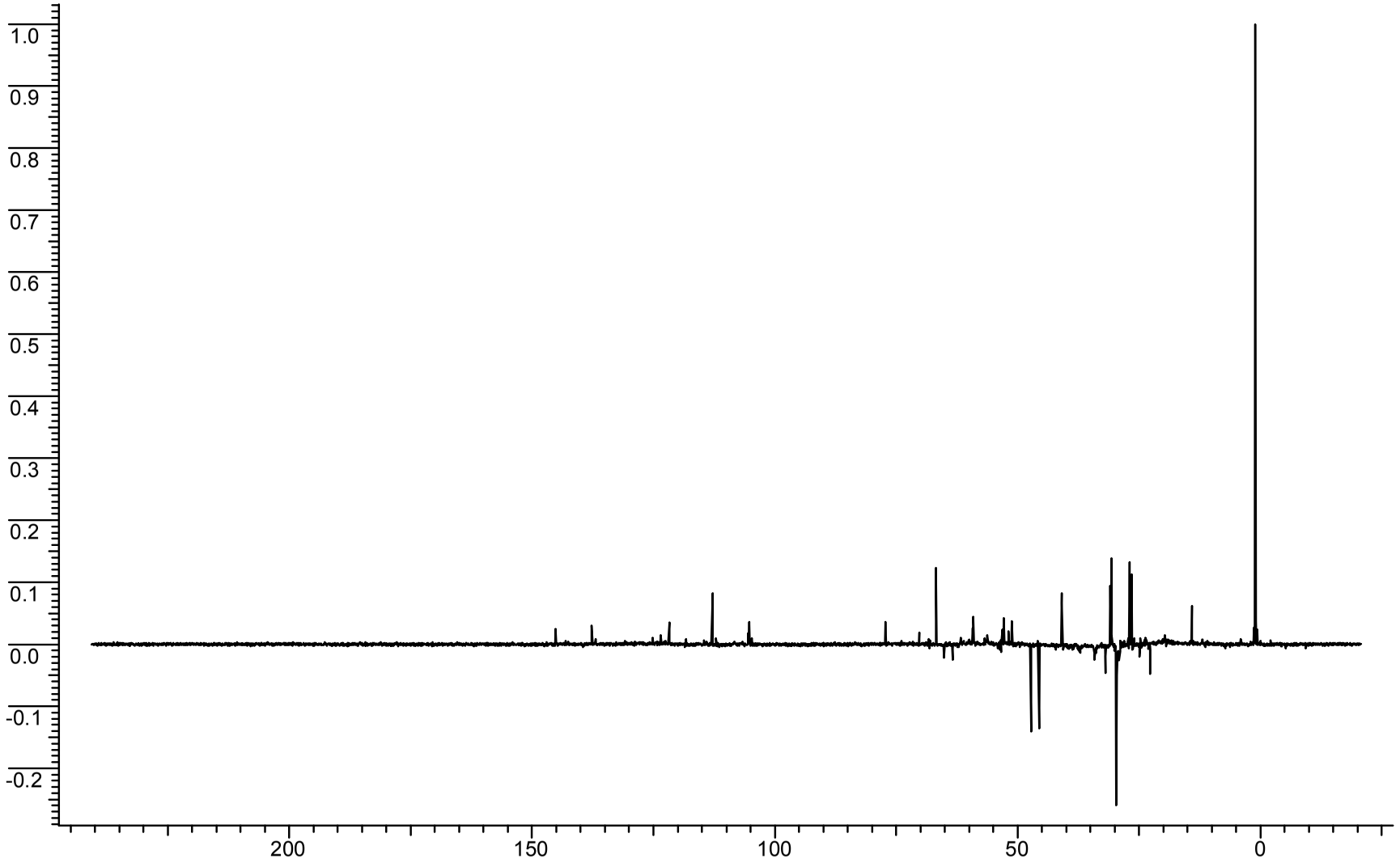
Appendix 12: ^{1}H-NMR spectrum for compound **43**



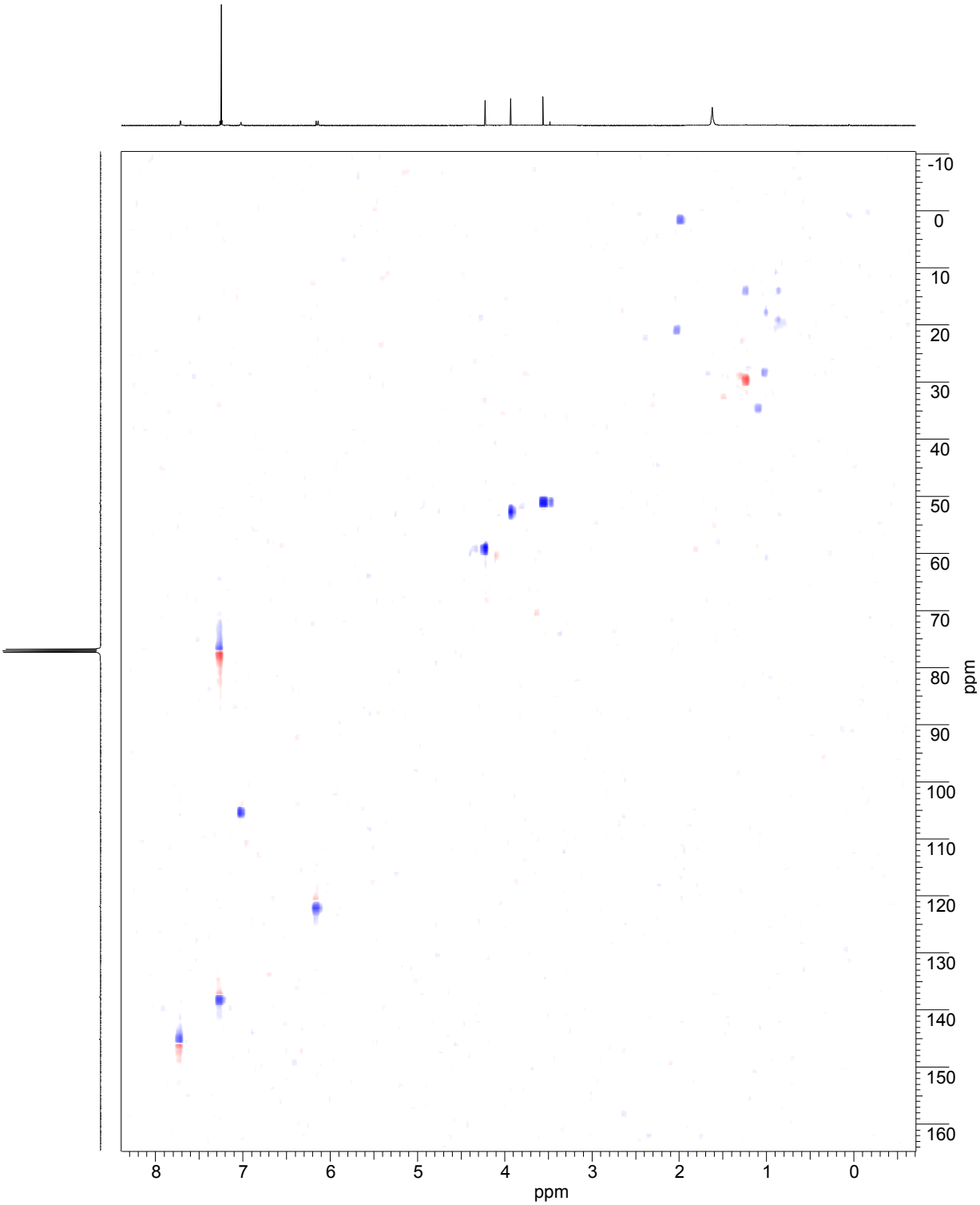
Appendix 13: ^{13}C-NMR spectrum for compound **43**



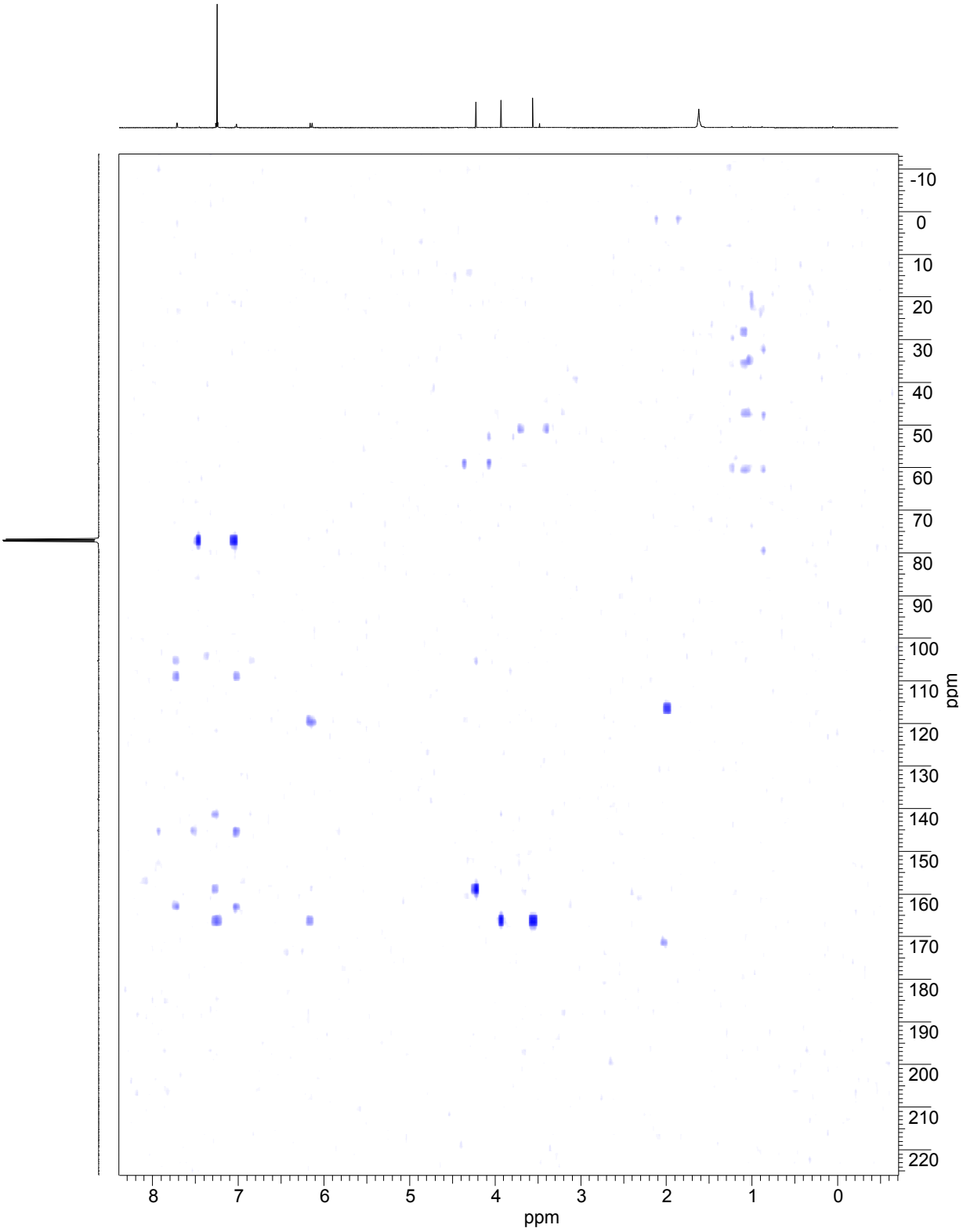
Appendix 14: DEPT NMR spectrum for compound **43**



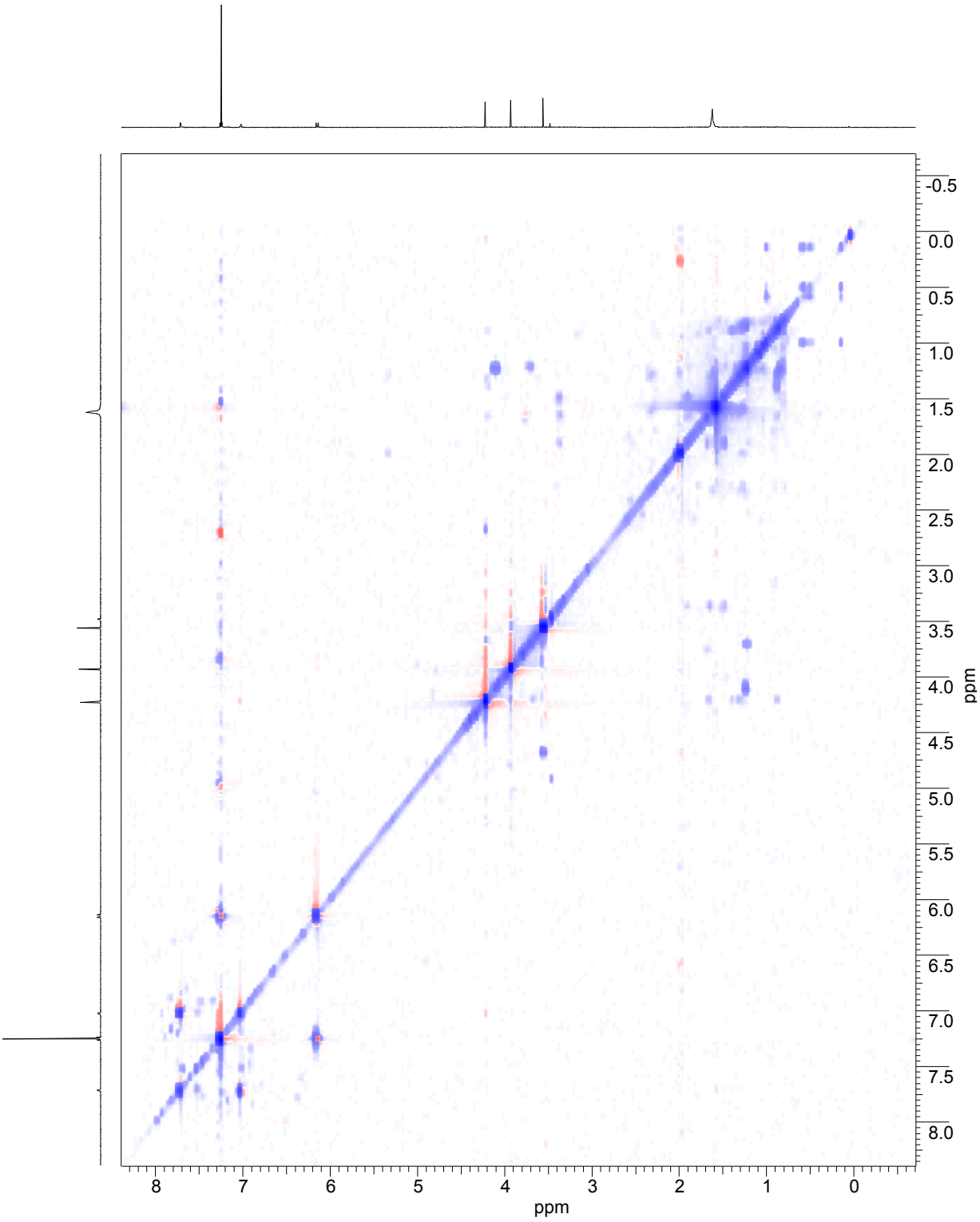
Appendix 15: HSQC NMR spectrum for compound **43**



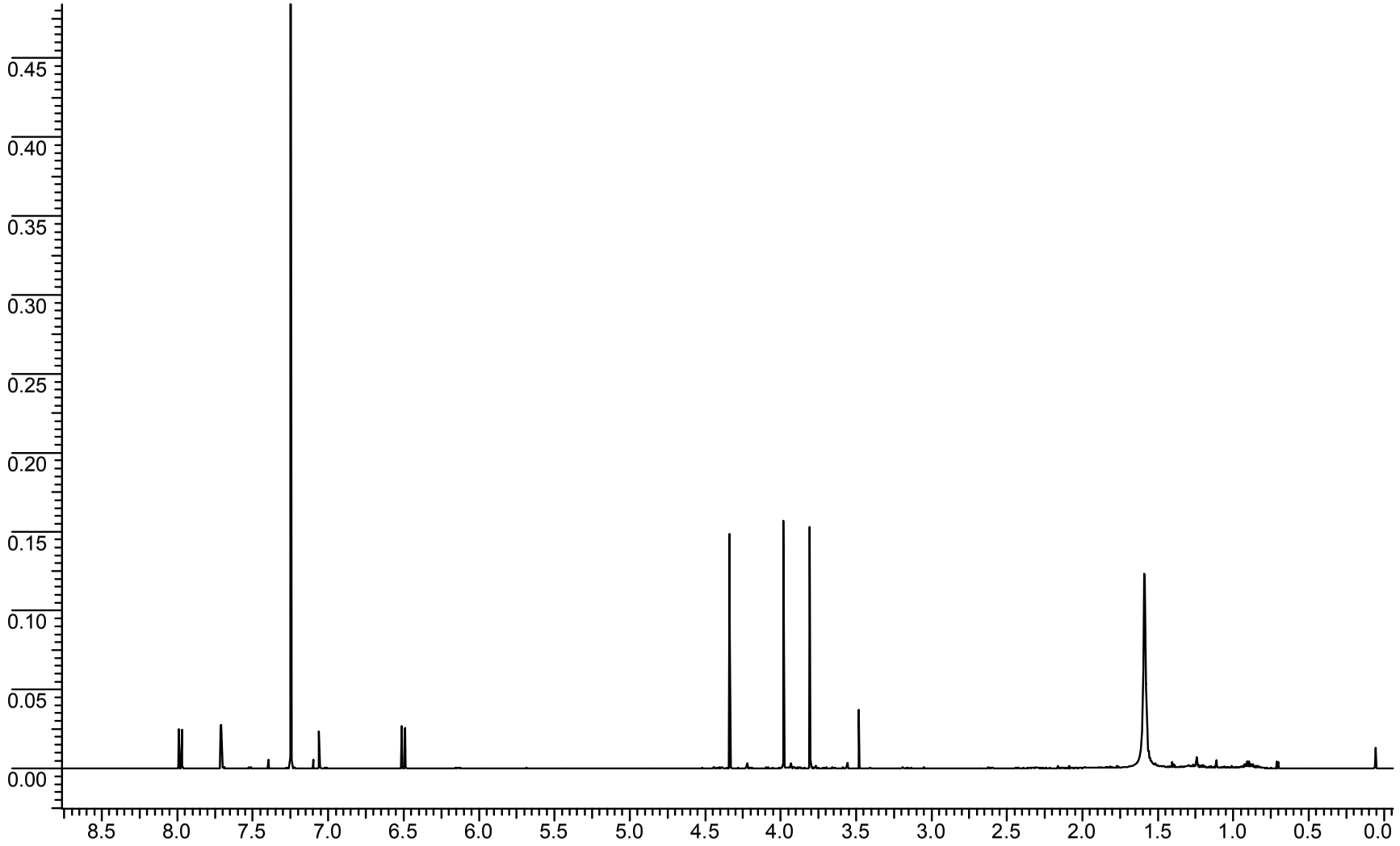
Appendix 16: HMBC NMR spectrum for compound **43**



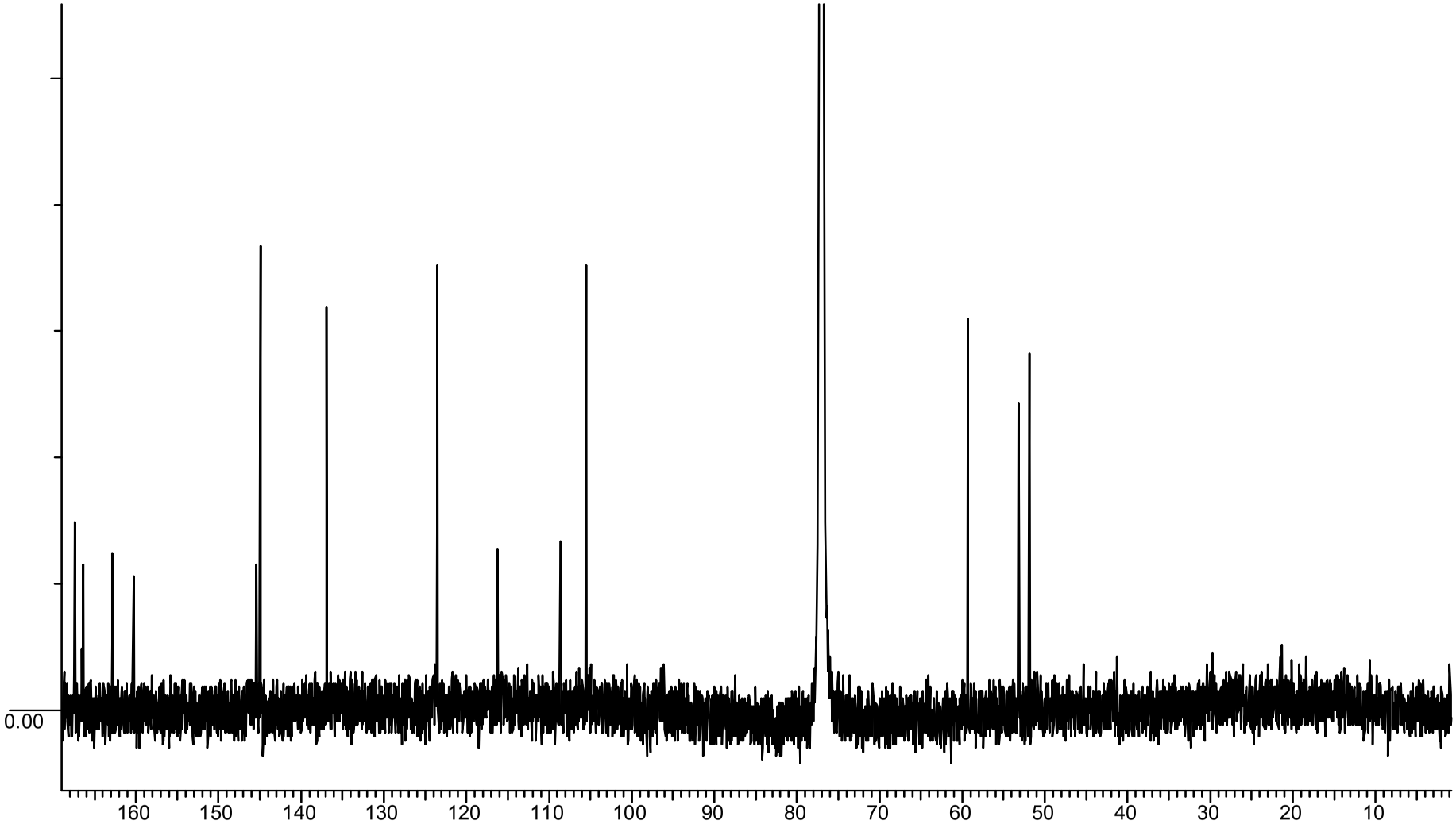
Appendix 17: COSY NMR spectrum for compound **43**



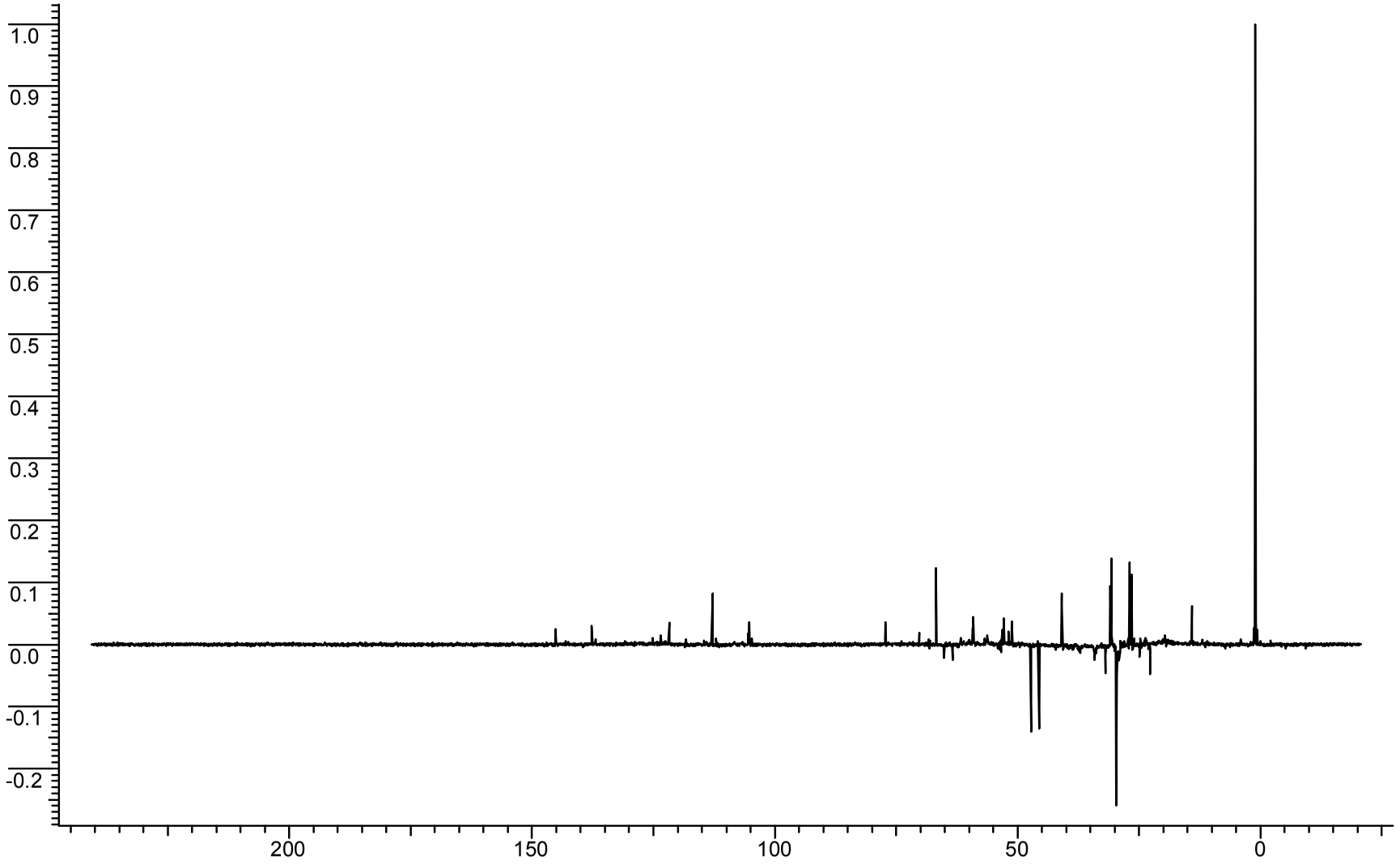
Appendix 18: ^{1}H-NMR Spectrum for compound **44**



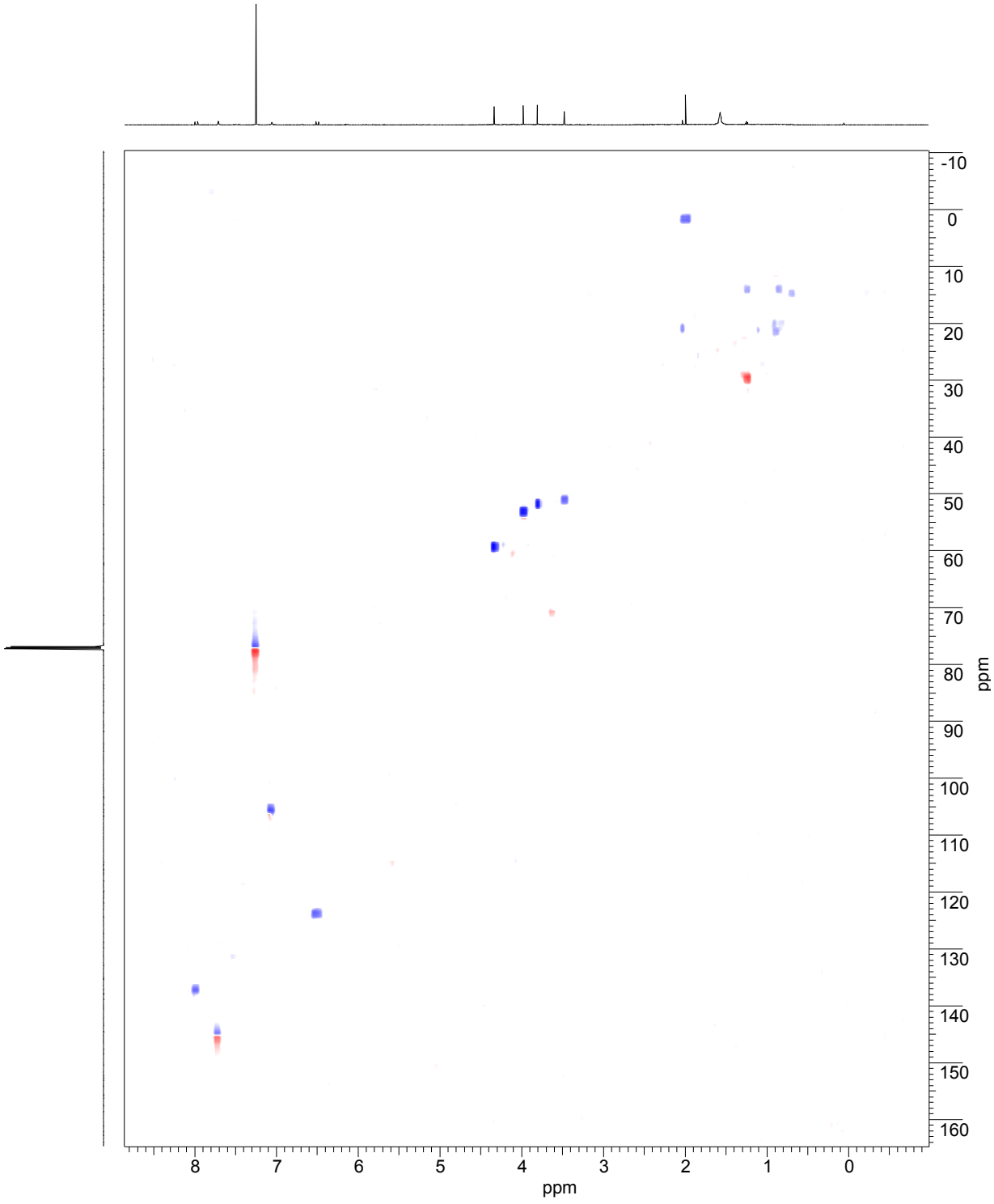
Appendix 19: ^{13}C-NMR Spectrum for compound **44**



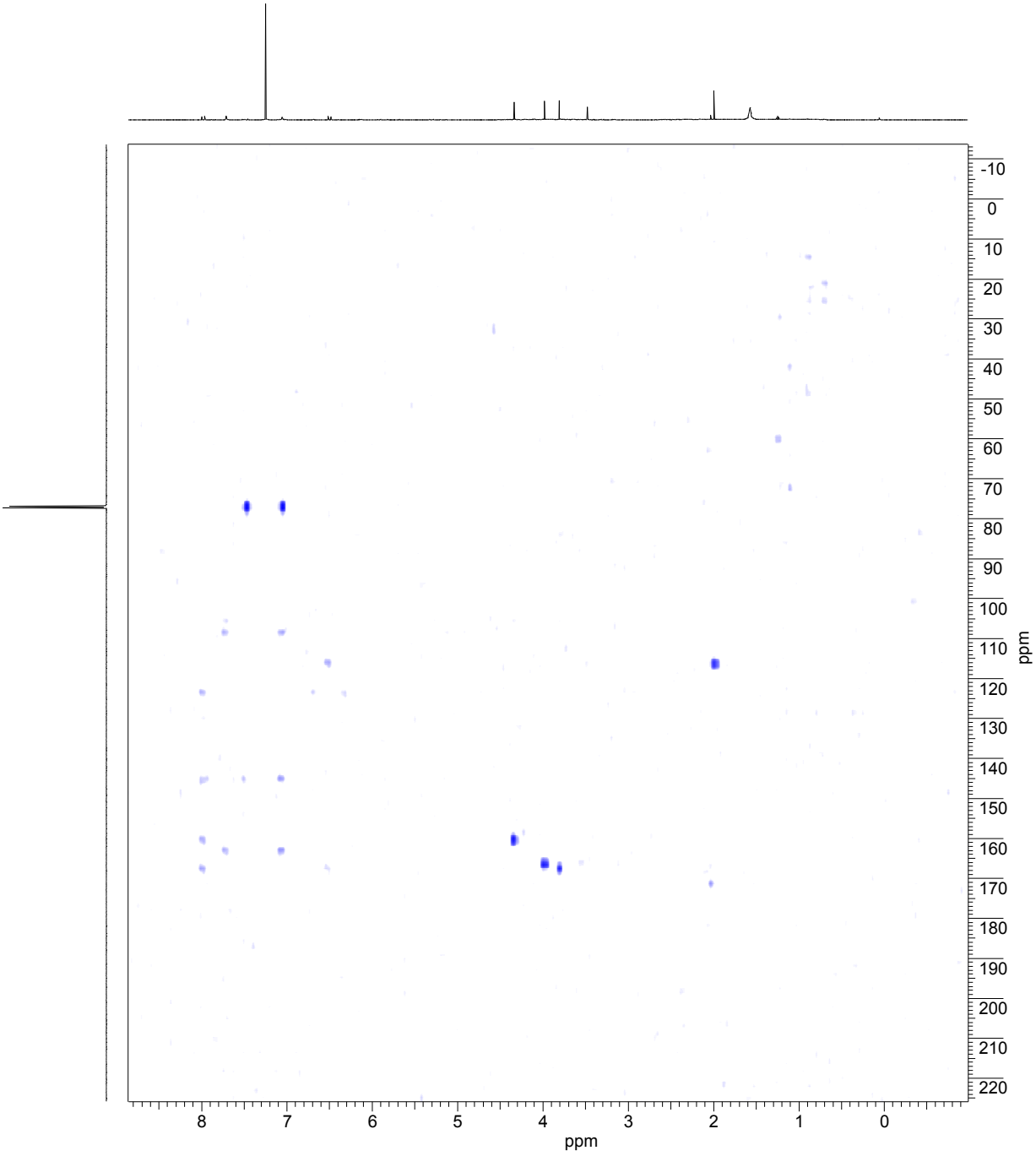
Appendix 20: DEPT NMR spectrum for compound **44**



Appendix 21: HSQC NMR spectrum for compound **44**



Appendix 22: HMBC NMR spectrum for compound **44**



Appendix 23: COSY NMR spectrum for compound **44**