PURIFICATION AND STRUCTURAL ELUCIDATION OF MOSQUITO LARVICIDAL COMPOUND(S) FROM CULTURES OF AN ASCOMYCETE SPECIES JO5035 AGAINST *Aedes aegypti*

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

EGERTON UNIVERSITY

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DECLARATION AND RECOMMENDATION

Declaration

I, Judith Muteitsi, hereby declare that this thesis is my original work and has not been submitted wholly or in part for any award in any other institution.

Judith Muteitsi

SM11/1911/07

Signature:……. Date ……

Recommendation

We confirm that this thesis was done under our supervision and has our approval to be presented for examination as per the Egerton University rules and regulations.

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DEDICATION

This thesis is dedicated to my beloved parents, sisters and brothers whose vision and energy, moral, financial and emotional support has positively shaped my destiny.

ACKNOWLEDGEMENT

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ABSTRACT

Mosquito transmitted diseases such as yellow fever, elephantiasis, dengue and malaria are some of the most deadly vector-borne diseases, affecting millions of people mainly in the tropics. In spite of major efforts undertaken for control of the diseases, through drug treatment and vector control, an increase in mosquito borne disease incidences has been witnessed in the last decades. Africa suffers most from mosquito borne diseases since mosquito vectors are distributed almost throughout the whole continent, and the parasite reservoir is big and continuously increasing. The search for new strategies or natural products to control destructive insects and vectors of diseases is desirable. Thus in this study secondary metabolites from higher fungi were investigated for possible mosquito larvicidal activity to control mosquitoes. An ascomycete serialized JO5035 was collected from undisturbed habitat in Mount Kenya forest. The ascomycete JO5035 was sub-cultured onto potato dextrose agar (PDA) to form a well grown culture. The strain was preserved as agar slant and the corresponding herbarium material kept in a fungal culture collection in the Integrated Biotechnology Research Laboratory at Egerton University. On initial screening it was found to produce active compounds in culture against *Aedes aegypti* larvae. It was cultured in sterile submerged nutrient liquid malt media. From the culture two sets of crude extracts were prepared with intracellular secondary metabolites prepared from mycelium (Mex) while extracellular secondary metabolites prepared from the cultured filtrate (Kex). The crude extracts were tested for larvicidal activities against late third instar larvae and early fourth instar larvae of *Ae. aegypti* before activity guided purification of the active compounds was carried out. The crude extracts were then fractionated, guided by mosquito larvicidal activity and which were subjected to NMR experiments (both 1D and 2D). The chemical structures of the compounds were determined using the NMR spectral data. A mosquito larvicidal compound, 2-para-tolylethanol was purified, with LC_{50} of 279.6 ppm and LC_{90} of 624.8 ppm. The compound was produced from cultures of an ascomycete JO5035 and reported to have mosquito larvicidal activity for the first time. During this study it was demonstrated that the ascomycete JO5035 had a potential as a source of mosquito larvicidal compounds despite heavy limitations of necessary research equipment. It is the recommendation of this work that these compounds can be investigated further for product development.

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LIST OF ABBREVIATIONS

COSY	Correlated Spectroscopy
DDT	Dichlorodiphenyltrichloroethane
DHF	Dengue Haemorrhage Fever
HMBC	Heteronuclear Multiple Bond Correlation
HMQC	Heteronuclear Multiple Quantum Coherence
DEPT	Distortionless Enhanced by Polarization Transfer
WHO	World Health Organisation
DEET	N, N-Diethyl-3-methylbenzamide
ITNs	Insecticide Treated Bed nets
IRS	Indoor Residual Spraying
JE	Japan Encephalitis
JO5035	Serial Number of the Fungal Strain Investigated
LC_{50}	Lethal Concentration that Kills 50% of Mosquito Larvae
LC_{90}	Lethal Concentration that Kills 90% of Mosquito Larvae
PDA	Potato Dextrose Agar
DNA	Deoxyribonucleic acid
NMR	Nuclear Magnetic Resonance
H-NMR	Proton NMR Spectroscopy
C-NMR	Carbon NMR Spectroscopy
MIC	Minimum Inhibitory Concentration
TLC	Thin Layer Chromatography
UV-VIS	Ultraviolet – Visible Spectroscopy
MOH	Ministry Of Health
IBRL	Integrated Biotechnological Research Laboratory

CHAPTER ONE
INTRODUCTION

1.1 Background Information

Despite the advances in the techniques used for control of mosquitoes during recent decades, the mosquito continues to pose a serious public health problem. In addition to the persistent irritation the mosquitoes cause human beings and animals simply by virtue of their blood-sucking behaviour and the itching, mosquitoes are also the principal vector of a variety of serious diseases, including malaria, yellow fever, dengue and encephalitis (David and Kubo, 1999; Cheng *et al*., 2004; Hemingway, 2004; Kuo *et al*., 2007). It is estimated that there are about 100 trillion mosquitoes with at least 3,450 different species in the world. They are found from the tropics to the arctic regions. Of all diseases transmitted by the mosquito, malaria is the most deadly and of late it has proved to be the most costly serious problem to manage in countries where it is endemic. Worldwide, approximately 2.7 million human deaths occur each year solely as a result of malaria transmitted by mosquitoes (Nasrin, 2005).

Like other true flies, mosquitoes exhibit 'complete metamorphosis. The juvenile form passes through both the larval and pupal stages. From eggs to the new adult, *Anopheles* takes 7-12 days depending on temperature. Female *Anopheles* needs a blood meal for each batch of eggs, 100 or more produced at two or three day's intervals. They can feed on nectar or fruit juices but without blood cannot produce eggs (Jacobs-Lorena, 2003).

The larvae are anatomically different from the adults, live in different habitats and feed on different type of foods (Clements, 1992). Mosquitoes are a host to a variety of pathogens and parasites including viruses, bacteria, fungi, and nematodes. The blood sucking habits of mosquitoes renders adult mosquitoes prone to acquire parasites or pathogens from one vertebrate host to another, but even so, many aspects of the mosquito's ecology and physiology must be appropriate for it to acquire, harbour, and transmit a particular organism (Haq *et al.*, 2003). Mosquitoes also cause allergic responses to humans that include local skin reaction and systemic reactions such as angioedema and urticaria (Peng *et al.*, 1999).

Malaria is a parasitic disease caused by *Plasmodium* species. The parasite is transmitted by the *Anopheles* mosquitoes that flourish in warm climates (Knell, 1991). Mosquitoes of the genus *Anopheles* are common in most temperate and tropical countries provided that there are suitable breeding sites (Clement, 1992). In Kenya, more than 90% of infections are caused by *P.*

falciparum, which is the most dangerous type of infection and can cause death (WHO, 2003). The cumulative human suffering and economic loss caused by malaria is immense (Snow *et al.*, 1998), and pregnant women suffer severe anaemia and have a high likelihood of delivering infants with low birth weight. All Kenyan households are affected by the financial hardship caused by malaria. It is estimated that 170 million working days are lost each year because of malaria illness, which in turn affects the country's economy, leading to increased poverty (MOH, 2008).

The level of endemicity of malaria in Kenya varies from region to region with a big variation in the risk which is mainly driven by climate and temperature (including the effects of altitude). It is estimated that 77% of Kenya's population lives in areas where the disease is transmitted. Malaria is endemic along the Lake Victoria region and the Coastal area where transmission takes place for more than six months in a year (Martens *et al.*, 1999). In the areas where malaria is seasonal and in epidemic prone areas all age groups are affected. These are situated in the highlands of the Rift Valley, Nyanza and Western regions (MOH, 2008).

Epidemic dengue has become more common since the 1980s and is now second only to malaria as the most important mosquito-borne disease affecting humans. Each year 50 to 100 million cases of dengue fever occur and about 500,000 of them exhibit severe dengue hemorrhagic fever, which has a case fatality rate of about 5% (WHO, 2006). Dengue viruses occur as four antigenically related but distinct serotypes, which cause a broad range of disease, including clinically asymptomatic forms, classic dengue fever (characterised by the sudden onset of fever, headache, retro-orbital pain and myalgia), and the more severe forms such as dengue hemorrhagic fever-dengue shock syndrome. Dengue fever was first described during an epidemic in Philadelphia in 1780, and intermittent pandemics have affected Asia, Africa and the Americas at intervals of 10– 30 years. After World War II, marked changes in human and vector ecology fostered the transmission of multiple dengue serotypes, and the tropical world is currently in the grip of a continuing dengue pandemic (Omena *et al.*, 2007).

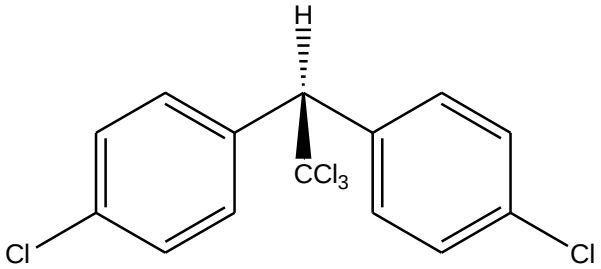
The global distribution of dengue is expanding and is now comparable to that of malaria. It is a mosquito-borne viral disease spread through a bite by *Aedes aegypti* which is the principal vector and there is no vaccine or treatment. *Aedes aegypti* is an invasive species spread inadvertently around the world by human trade and travel and is now distributed widely in tropical and subtropical regions, most notably in urban environments where it has adapted to

breed in artificial containers and refuse (Inoue *et al.*, 2003). This increase in the geographic distribution of the vector is accompanied by the emergence of the viruses and disease in new areas. There is currently no vaccine or specific therapeutic drug available for dengue; therefore, control focuses on the mosquito. Bed nets are largely ineffective against this mosquito that normally bites during the day, making source reduction and space spraying the mainstays of control. However, the mosquito breeds in a wide variety of containers, and finding and treating sufficient numbers of them is extremely challenging or impossible for even the most well-funded and organized programs (Gubler, 1998).

Among the complex set of factors influencing dengue transmission, global warming is particularly important because it can create warmer and wetter conditions that increase the risk of infection. Warmer temperatures boost the speed of development of adult mosquitoes, increasing their numbers. Female mosquitoes bite more frequently in hotter temperatures, and warmer winters enable mosquitoes to survive in areas that were formerly too cold. Higher temperatures also shorten the time it takes for the virus inside the mosquito to develop and become infective. This means that the mosquitoes become dangerous to humans more rapidly (Phillips, 2008).

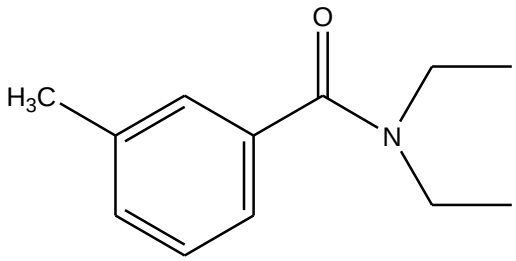
Encephalitis is an inflammation of the brain and the causal virus is primarily transmitted by the mosquito, *Culex tarsalis*. The mosquito transmits the virus to birds, horses, mules and occasionally people. Birds serve as the most important host reservoir for the virus in the disease cycle (Dawn *et al.*, 2001).

Attempts to control mosquito vector over the past seven decades have included the destruction of breeding grounds for mosquitoes, drainage of stagnant water and indoor residual spraying (IRS) which was developed to routinely bring DDT **(1)** into contact with the adult female mosquitoes at the most epidemiologically significant point of contact between man and insect.



p,p'-Dichlorodiphenyltrichloroethane (DDT) (1)

The most common mosquito repellent products available in the market contain DEET (*N*, *N*-Diethyl- 3-methylbenzamide (**2**). It is a broad-spectrum repellent that is effective against mosquitoes and other biting insects. However, its allergic reactions and toxicity to man as well as its ability to act as a good solvent for plastics and other synthetic materials, has led to the search for alternative synthetic and natural repellents (Odalo *et al*., 2005).



N, N-Diethyl-3-methylbenzamide (2)

Long-lasting ITNs were developed in the 1990s using technology to ensure that the insecticide remained on the net for the useful life of the net itself. There has been little innovation in effective treatments since then, despite the seriousness of the health problem. Coils, impregnated mats and aerosols are readily available in the consumer markets, but there is little direct evidence for these impacting on disease transmission and the annual cost of routinely using these interventions is high despite the low cost of the individual coils or sprays (Hemingway, 2009).

The discovery of DDT in 1942 and its first use in Italy in 1944 made the ideal of global eradication of the insect seem possible (Murdock, 2001). Other applications included organophosphates such as temephos and fenthion and insect growth regulators such as diflubenzuron and methoprene generally used for the control of mosquito larvae (Yang *et al*., 2002). Although effective, their repeated use has disrupted natural biological control systems and has led to outbreaks of insect species, which sometimes resulted in the widespread development of resistance, had undesirable effects on non target organisms, and fostered environmental and human health concerns (Sen-Sung *et al*., 2003). Further, certain synthetic pesticides, such as acylamides, phenylureas and phenoxyacetates may break down into more toxic derivatives, while others may persist in nature as recalcitrant environmental pollutants, injurious to humans and wildlife (Munnecke *et al.*, 1982). Thus, there is need to develop non-polluting safe pesticides. An ideal pesticide should have a number of properties, including selective toxicity, and be degradable into smaller harmless molecules such as water, carbon dioxide and ammonia.

Most of the mosquito control programmes target the larval stage in their breeding sites with larvicides, because adulticides may only reduce the adult population temporarily (El Hag *et al.*, 2001). In application of larvicides, mosquito larvae are killed before they disperse to human habitations, and that mosquito larvae, unlike adults (Charlwood and Graves, 1987), cannot change their behaviour to avoid control activities targeted at the larval habitat (Killeen *et al.*, 2002). This can also reduce the overall application of pesticides needed to control mosquito population (Dharmagadda *et al.*, 2005). This in itself is an advantage that can be exploited to search for new and novel larvicides.

Natural products remain the most promising source of novel secondary metabolites. The impact of microbial biodiversity to disease suppression favours the chance of isolating new antibiotics. Identification of microorganisms that produce bioactive compounds is of great interest in the development of new molecules to fight against many pathogens. Fungi produce a wide range of secondary metabolites with high therapeutic value as antibiotics, cytotoxic substances, insecticides, compounds that promote or inhibit growth (Demain, 1999). These metabolites are being exploited in different fields of medicine and industries (Huisman and Gray, 2002). There is evidence that fungi, being simple but multi-cellular eukaryotic systems have similar biochemical mechanisms to those in mammals. Among fungi classes, ascomycetes are reported to be active producers of antimicrobial and larvicidal fungal compounds, which have high therapeutic values (Quang *et al.*, 2002).

Phylum Ascomycota is a taxonomic group comprising 75% of all described genera in Kingdom Fungi. They can be found on all continents and most general display a pan global distribution. Ascomycetes are sac fungi and they produce spores in sac like structures. The Ascomycota harbours representatives of a very wide range of fungi, from food-spoilage organisms (e.g. *Penicillium*, *Aspergillus*), plant pathogens (e.g. *Fusarium*, *Magnaporthe*) to producers of organic compounds, enzymes and pharmaceutical products (*Cordyceps*) (Pedras *et al*., 2002).

1.2 Statement of the Problem

Most of the widely used vector interruption methods against the mosquito are synthetic insecticide- based. These synthetic insecticides not only affect the non-target population but also constantly increase mosquito resistance to the insecticides like DDT, so this strategy becomes

inefficient. In this regard, the development of techniques that would provide more efficient insect control, not have any ill effects on the non-target population, and are easily degradable are always sought necessitating the urgent development of new effective eco-friendly larvicides. Vector management of the mosquito at larval stage has long been neglected. There is need to explore use of secondary metabolites produced by fungi which are often novel molecules with large potential for larvicidal applications for control of mosquitoes. With the advances in biotechnological techniques, this research is geared towards finding compounds from an ascomycete JO5035.

1.3 Objectives

1.3.1 Main Objective

To purify, chemically characterize and elucidate the structures of mosquito larvicidal compounds from the ascomycete serialized JO5035.

1.3.2 Specific Objectives

1. To culture the ascomycete JO5035 in nutrient liquid media.
2. To prepare crude extracts from the mycelium and culture filtrate at the end of cultivation.
3. To test for mosquito larvicidal activities of the crude extracts and purify the responsible compounds using chromatographic techniques.
4. To elucidate structures of pure compounds using spectroscopic techniques.

1.4 Hypotheses

1. That when the ascomycete JO5035 is cultured in sterile nutrient media can produce secondary metabolites.
2. That the secondary metabolites produced by the ascomycete JO5035 can be obtained as crude extracts from mycelium and culture filtrate.
3. That the crude extracts prepared from the mycelium and culture filtrate can show significant mosquito larvicidal activity. And that the compounds responsible for the observed activities can be purified completely using chromatographic techniques.
4. That the chemical structures of the purified compounds can accurately be determined using spectroscopic techniques.

1.5 Justification

Mosquito transmitted diseases including malaria, filiriasis, Japan encephalitis (JE), dengue fever, dengue hemorrhagic (DHF), yellow fever among others are very persistent to human. These diseases lead to adverse effects on human health and well being, death and the economic losses thus the stimulants for the search of new, safe and effective larvicides. Fungal genetic resources have been shown to display great diversity in the production of biologically active molecules especially in the pharmaceutical industry. Little if any research has been conducted on possibility of utilizing the biologically and chemically diverse secondary metabolites from fungi for mosquito control. The mosquito vector may be targeted at its larval stage in order to interrupt its lifecycle. Compounds that are derived from the ubiquitous ascomycete could offer unique solutions to unique problems like malaria bedevilling a relatively poor country like Kenya.

CHAPTER TWO

LITERATURE REVIEW

2.1 Biotechnological Applications of Fungi

Fungi are an extremely diverse group of microorganisms that are exploited for various biotechnological applications. They are used in the production of foods, enzymes, polysaccharides, antibiotics and other pharmaceutical agents, as agents of biological control of pests, insects, fungi and weeds as well as in biomass conversion. Tropical habitats house the greatest diversity of organisms that are abundant and have higher species-diversity than in other geographical regions of the world (Finkeistein and Ball, 1992). Filamentous fungi are among the most prolific producers of secondary metabolites. Some fungal secondary metabolites are also extremely important to our health and nutrition and have tremendous economic impact. In addition to the multiple reaction sequences of fermentations, fungi are extremely useful in carrying out biotransformation processes. These are becoming essential to the fine-chemical industry in the production of single-isomer intermediates. Recombinant DNA technology, which uses yeasts and other fungi as hosts, has markedly increased markets for microbial enzymes. Molecular manipulations have been added to the traditional mutational techniques as a means of increasing titres and yields of microbial processes and in the discovery of new drugs. Moreover, the best is yet to come as genomes of additional species are sequenced at some level (cDNA, complete genomes and expressed sequence tags) and gene/protein arrays become available (Adrio and Demain, 2003).

Filamentous fungi synthesize a wide variety of natural products collectively referred to as secondary metabolites. These are generally low molecular weight molecules that are not required for growth or development of the producing organism under laboratory conditions, but are thought to aid the fungus in competing successfully with other organisms in its natural habitat. Accordingly, many secondary metabolites tend to be compounds that have toxic or inhibitory effects on other organisms (David and Christopher, 1992). Because of these bioactive properties, many fungal secondary metabolites have been adopted by humans for use as pharmaceuticals such as antibiotics, cholesterol-lowering agents, tumour inhibitors, and immunosuppressant's form transplant operations. Other natural products of fungi have negative impacts on society, including phyto- and mycotoxins produced by plant pathogenic fungi and enhancers of virulence in fungal pathogens of humans and other organisms. Because of the impact of these compounds,

fungal secondary metabolites have received considerable attention from the scientific community (Keller and Hohn, 1997).

2.2 Mosquito Larvicidal Compounds from Essential Oils

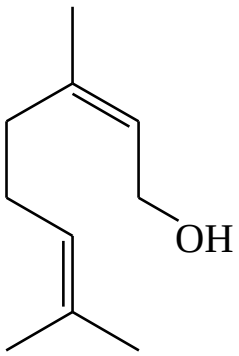
In many parts of the world, plant-derived products have been used to repel or kill mosquitoes and other domestic insect pests. Plant essential oils in general have been recognized as an important natural resource of insecticides (Adebayo *et al*., 1999; Gbolade *et al*., 2000). Their lipophilic nature facilitates them to interfere with basic metabolic, biochemical, physiological and behavioural functions of insects (Nishimura, 2001). They have the potential of being acute ovicidal, fumigant, insect growth regulator and insecticidal against various insect species (Tsao *et al*., 1995) and concurrently being developed as ecologically sensitive pesticides (Isman, 2000). Generally they are safe to humans and other mammals (Tripathi *et al*., 2000; Tripathi *et al*., 2002; Prajapati *et al.,* 2005).

Solvent extracts and essential oils of many plants show varying levels of insect-repellent properties. Indeed, until the advent of synthetic compounds, essential oils and/or their mixtures formed the basis of most commercial repellent formulations (Seyoum *et al*., 2002). However, due to their relative high volatilities, they have been abandoned in favour of synthetic repellents, principally DEET (N, N-diethyl-*m*-toluamide), which provides relatively long protection against blood-feeding *Anopheles gambiae* insects (Seyoum *et al*., 2003).

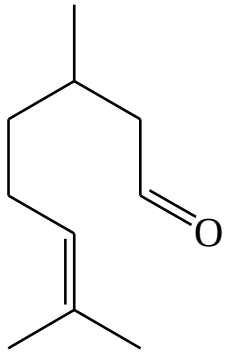
Essential oils of six plants were screened for repellent activities against *Anopheles gambiae*. The oils of *Conyza newii* (Compositeae) and *Plectranthus marrubioides* (Labiateae) were the most repellent followed by *Lippia javanica* (Verbenaceae), *Lippia ukambensis* (Verbenaceae), *Tetradenia riparia*, *Iboza multiflora* (Labiateae) and *Tarchonanthus camphoratus* (Compositeae). Four synthetic blends of the major components of the essential oils were found to exhibit comparable repellent activity to the parent oils. Of the seven more repellent constituents against *An. gambiae*, geraniol **(3)** provided the longest reduction in mosquito biting pressure, with protection lasting 4 hours post-repellent application and (R)-(+)-citronellal **(4)** were previously reported as mosquito repellents (Curtis *et al*., 1991).

Cinnamon oil shows promise as a great-smelling, environmentally friendly pesticide, with the ability to kill mosquito larvae. Eleven compounds in cinnamon leaf oil were tested for their ability to kill emerging larvae of the yellow fever mosquito, *Ae. aegypti*. Four compounds - cinnamaldehyde, cinnamyl acetate, eugenol and anethole - exhibited the strongest activity

against *Ae. aegypti* in 24 hours of testing, using the LC_{50} value. Cinnamaldehyde is the main constituent in cinnamon leaf oil and is used worldwide as a food additive and flavouring agent. A formulation using the compound could be sprayed just like a pesticide, but without the potential for adverse health effects - plus the added bonus of a pleasant smell (Sen-Sung *et al*., 2009).



Geraniol (3)



Citronellal (4)

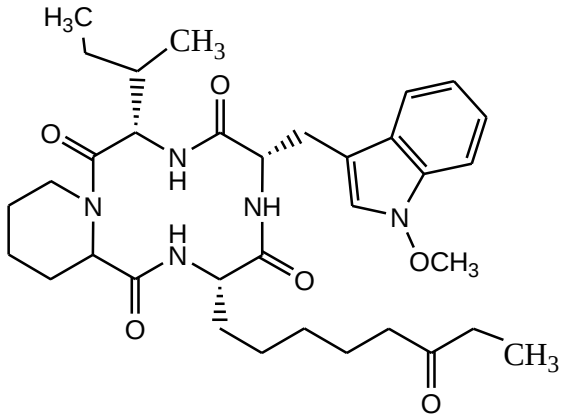
Cinnamon oil shows promise as a great-smelling, environmentally friendly pesticide, with the ability to kill mosquito larvae. Eleven compounds in cinnamon leaf oil were tested for their ability to kill emerging larvae of the yellow fever mosquito, *Ae. aegypti*. Four compounds - cinnamaldehyde, cinnamyl acetate, eugenol and anethole - exhibited the strongest activity against *Ae. aegypti* in 24 hours of testing, using the LC_{50} value. Cinnamaldehyde is the main constituent in cinnamon leaf oil and is used worldwide as a food additive and flavouring agent. A formulation using the compound could be sprayed just like a pesticide, but without the potential for adverse health effects - plus the added bonus of a pleasant smell (Sen-Sung *et al*., 2009).

2.3 Fungal Metabolites used against Mosquitoes

Fungi products are toxic to mosquitoes, yet have low toxicity to non-target organisms. Accordingly, the use of entomophagous fungi and their derived products may be a promising approach for biological control of mosquitoes (Kirschbaum, 1985). Ampicidin **(5)** is a cyclic tetrapeptide [cyclo-(*N*-*O*-Methyl-(L)-Trp-(L)-Ile-(D)-Pip-(L)-2-anuno-8-oxo-decanoyl)] isolated from ascomycete.

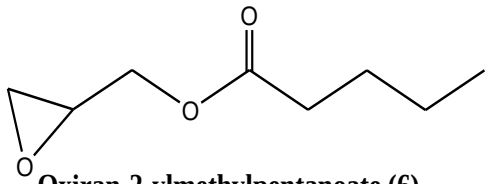
One basidiomycete *Cyptotrama asprata* showed strong larvicidal activity against mosquito *Ae. aegypti*. From submerged cultures of *C. asprata*, a secondary metabolite (oxiran-2-yl)methylpentanoate **(6)** was isolated due to its mosquito larvicidal activity against *Ae. aegypti*. The compound is a new secondary metabolite, whose structure was determined by NMR spectroscopy and comparison with closely related structures from literature (Njogu *et al.*, 2009).

The compound, (oxiran-2-yl)methylpentanoate, showed strong larvicidal activity against the *Ae. aegypti* larvae with LC_{50} of 1.50 ppm and an LC_{90} of 1.90 ppm after 24 hours. These were found to be much lower



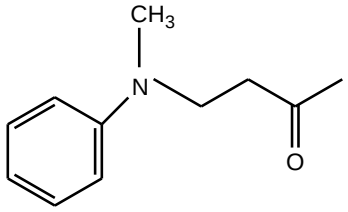
Ampicidin (5)

concentration those reported for some natural products. It shows great potency to kill mosquito larvae and this can be attributed to its relatively high polarity from the epoxide ring and the ester functional group. Presence of the ring makes the compound a more potent insecticide compared to pyrethrins which have got a cyclopropane ring (Njogu *et al.*, 2009).

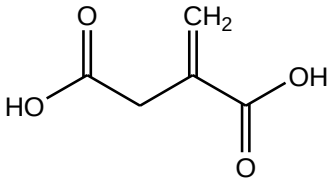


Oxiran-2-ylmethylpentanoate (6)

Two compounds isolated from an ascomycetes fungi *Aspergillus gorakhpurensis, 4* - (*N*-methyl-*N*-phenyl amino) butan-2-one **(7)** and 2-methylenesuccinic acid **(8)** showed moderate antimicrobial and larvicidal activities (Siddhardha *et al.*, 2009).



4-(N-Methyl-N-phenylamino)butan-2-one (7)

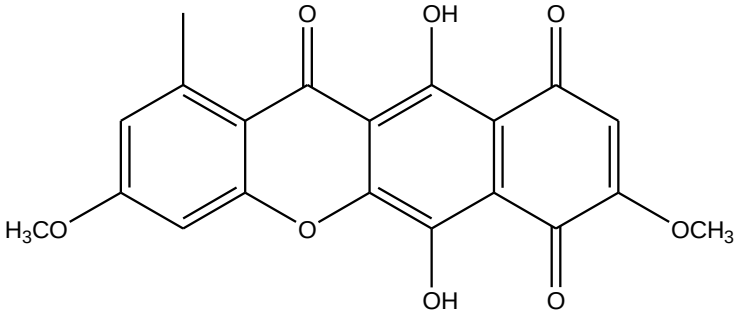


2-methylenesuccinic acid (8)

Conidia of *Beauveria bassiana* are effective in killing mosquito larvae when applied as a conidial dust to the water surface of breeding sites (Clark *et al*., 1968). Conidia are hydrophobic, thus floating on the water surface and contact mosquito larvae that feed below the surface mainly at the tip of the siphon, although (Miranpuri and Khachatourians 1991) reported that the head to be an equally important infection site. *B. bassiana* found to be effective against *C. quinquefasciatus* at all the concentration (10^4 to10^7 spores/ml) than *Paecilomyces fumosoroseus*, and 50% mortality obtained even at lowest spore concentration.

2.4 Metabolites Profiling from Ascomycetes

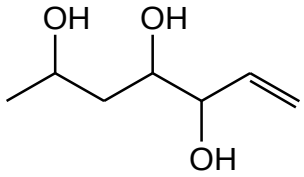
The ascomycete *Gibberella fujikuroi* is well known for the production of gibberellins, but also produces many other secondary metabolites, including the red polyketide pigment bikaverin. Bikaverin **(9)** was first isolated by Kreitman and co-workers, who referred to it as lycopersin but later renamed it bikaverin to avoid confusion with the carotenoid pigment lycopersene (Kreitman and Nord, 1949). It is a mycotoxin with antiprotozoal and antifungal activities (Balan *et al.*, 1970; Cornforth *et al*., 1971; Kjaer *et al*., 1971).



Bikaverin (9)

Fungi of the ascomycete genus Daldinia (Xylariaceae) have been shown to be a good source of bioactive secondary metabolites. The compound hept-6-ene-2, 4, 5-triol **(10)** obtained

from this ascomycete was found to have both antimicrobial and nematicidal activities (Wang and Liu, 2004).



hept-6-ene-2,4,5-triol (10)

2.5 Medicinal Ascomycetes

The medicinal mushroom such as *Cordyceps* species is a valuable source of useful natural products having diverse biological activities (Gu *et al*., 2007). *Cordyceps sinensis* (Figure **1**) is an ascomycetes fungus that grows on the larvae of the Lepidoptera Moth (Koh *et al*., 2003). *Cordyceps* has been used to treat a wide range of conditions, including respiratory, pulmonary diseases, renal, liver, cardiovascular diseases and hyposexuality. It is also regularly used in all types of immune disorders, and as an adjunct in cancer therapy (Zhang *et al*., 2005). *Cordyceps* is thought to be a remedy for weakness and fatigue and is often used as an overall rejuvenator for increased energy while recovering from serious illness. *Cordyceps* is often prescribed for the elderly to ease general aches and pains.

Figure 1: *Cordyceps sinensis*

One of the most exciting benefits of *Cordyceps* is its potential as a source of new anti-cancer drugs. *Cordyceps* is used by a growing number of doctors worldwide as adjunct to chemotherapy, radiation and other conventional and traditional cancer treatments (Wang *et al*., 2005). It has shown remarkable prowess in not only inhibiting the growth of and in some cases even dissolving certain types of tumours (Holliday and Cleaver, 2008), but also as a means by

which the immune system and indeed the body in general may be kept strong and vital as it is being devastated by the effects of chemotherapy and radiation treatment (Nakamura *et al.*, 2003).

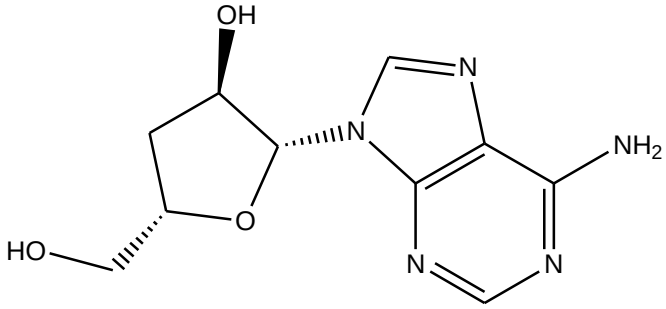
The mechanism by which *Cordyceps* inhibits the growth of various cancer cells might occur by one of several means: by enhancing immunological function and nonspecific immunity; by selectively inhibiting RNA synthesis, thereby affecting the protein synthesis; by restricting the sprouting of blood vessels (angiogenesis); by inducing tumor cell apoptosis; by regulation of signal pathways; anti-oxidation and antifree radical activity; anti-mutation effect; interfering with the replication of tumor-inducing viruses; and by inducing nucleic methylation (Zhou *et al*., 2009). Its secondary effects on immune function helps the body to more efficiently manage its immune resources while undergoing the stresses of the attack by cancer (Shin *et al.*, 2003) allowing it to recognize, eradicate, and prevent abnormalities and disease, both at the local and the systemic level (Koh *et al.*, 2002).

It is also posited that the naturally occurring antiretroviral compounds found in *Cordyceps* 2, 3-dideoxyadenosine could result in increased effectiveness or decreased dosage requirements for patients undergoing concurrent therapy with other antiretroviral drugs. Extracts of *Cordyceps* species are effective against HIV infections. *Cordyceps sinensis* containing formula named Immune Assist 24/7TM has recently been introduced throughout West Africa for use in treating HIV infections and other immune-deficient states (Holliday and Cleaver, 2008) and is quite popular with both the doctors and the patients due to its low toxicity and cost when compared with other antiretroviral drug options (Holliday *et al*., 2010).

Cordyceps militaris (**Figure 2**) an entomopathogenic fungus, is one of the most important medicinal mushrooms, belonging to the class Ascomycetes, has been used popularly as a crude drug and a folk tonic food in East Asia. It possesses many kinds of active components (such as cordycepin, polysaccharides, ergosterol, and mannitol), and due to its several physiological activities, it is used for multiple medicinal purposes (Nag and Wang, 2005).

Figure 2: *Cordyceps militaris*

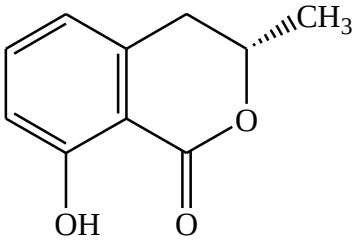
The main active constituent of *C. militaris* fruiting bodies is cordycepin (**11**), which was first extracted from *C. militaris* and then found to be present in *Cordyceps sinensis* and *Cordyceps kyushuensis* (Ling *et al*., 2002). The studies have demonstrated that the active principles of the medicinal mushroom *Cordycpes militaris* are practically beneficial to act as pro-sexual (Yu *et al*., 2007) and, anti-inflammatory, anti-oxidant/anti-aging, anti-tumor/anti-cancer/anti-leukemic, anti-proliferative, anti-metastatic, immunomodulatory, insecticidal, larvicidal, anti-fibrotic, steroidogenic, hypoglacaemic, hypolipidaemic, anti-angiogenetic, anti-diabetic, anti-HIV, anti-malarial, anti-fatigue, neuroprotective, liver-protective, reno- protective as well as pneumo-protective (Shonkor *et al*., 2010).



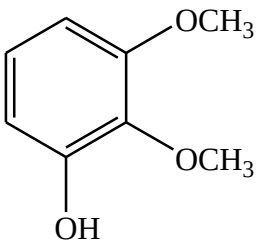
Cordycepin (11)

2.6 Metabolites Extracted from Basidiomycetes

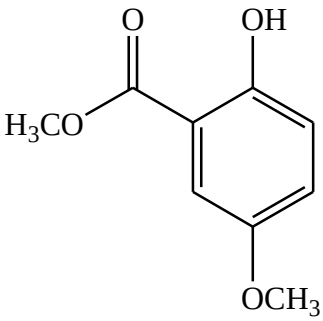
Some larvicidal active compounds were isolated from the basidiomycete serialized JO5182. Three compounds; Methyl-2-hydroxy-4-methoxybenzoate (**12**), 2, 4-dimethoxyphenol (**13**) and (R)-(-)-8-hydroxy-3-methyl-3, 4-dihydro-1H-2-benzopyran-1-one (**14**) were isolated and their structures are as shown below (Kendagor, 2008).



8-Hydroxy-3-methyl-isochoromen-1-one (12)

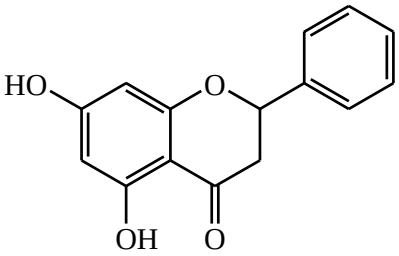


2, 4-dimethoxyphenol (13)

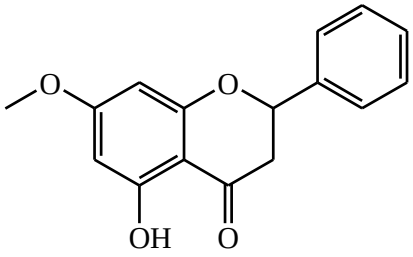


Methyl-2-hydroxy-5-methoxybenzoate (14)

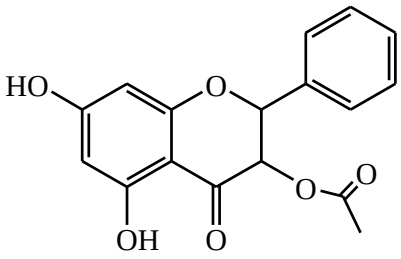
Antibacterial compounds (**15**, **16** and **17**) isolated from basidiomycete JO5191, *Collybia* species had their minimum inhibitory concentration (MIC) found to be in the range of 3 µg/ml–12 µg/ml, agreeing considerably well with those reported from previous literature (Mutambi, 2007).



2,3-dihydro-5,7-dihydroxy-2-phenylchromen-4-one (15)



2,3-dihydro-5-hydroxy-7-methoxy-2-phenylchromen-4-one (16)



3,4-dihydro-5,7-dihydroxy-4-oxo-2-phenyl-2*H*-chromen-3-yl acetate (17)

2.7 Fungi as Parasites: - Entomophathogenic Fungi

The zoospores of entomophathogenic fungus usually invade host larval mosquitoes through the buccal cavity or penetrate the cuticle of the head capsule. Mycelium develops in the hemolymph and eventually the insect is filled with resting spores or sporangia. If sporangia are

present, tubes are forced through the cuticle into the water. The sporangia contents migrate to the tips of the exit tubes. The zoospores formed from this material directly infect other mosquitoes (Galagali *et al.,* 1984*).*

Lagenidium giganteum can probably be regarded as the fungus with the best properties for larval mosquito control, though only for stagnant waters, such as rice fields. *Lagenidium giganteum* is produced and delivered for mosquito control as mycelium (May *et al*., 2006). It is biologically safer for non-target organisms too. *L. giganteum* has been evaluated in a variety of habitats including rice fields, seepage, ditches, and irrigated pastures and fields (Kerwin and Washino, 1986; 1988). Moreover, that fungus has the distinct advantage of being able to recycle in stagnant water, infecting multiple and overlapping generations of mosquitoes. Despite that, it has short life, mass production yields of mycelia stage, and need to keep the mycelium completely hydrate (Kerwin and Petersen, 1997). The limited use is also due to the technical aspects of production and distribution (Vandergheynst *et al*., 2006). *L. giganteum* products have been quite difficult, expensive to store, contaminated easily, and refrigeration was necessary to maintain efficacy (Scholte *et al.,* 2005).

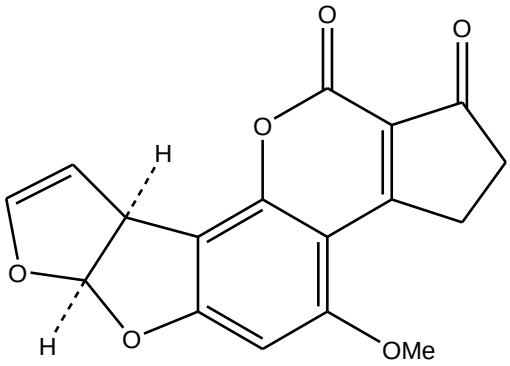
Concerning mosquito-pathogenic fungi, three genera are generally considered important; *Lagenidium*, *Coelomomyces*, and *Culicinomyces* (Roberts 1974; Lacey; Undeen 1986. Each one of these has one or more traits useful for mosquito control, but none of them possesses the full array of properties needed for general applied and cost-effective control (Federici, 1995).

Coelomomyces spp. are very effective in killing many mosquito species, although individual species have narrow host ranges, and have often been reported to cause epizootics, with the major obstacle of dependence on *in vivo* production, making mass-production difficult. *Culicinomyces clavisporus* showed considerable interest initially, but this declined when the high dosages required for effective control and the low persistence of conidia in the environment became apparent (Service, 1983; Lacey; Undeen, 1986).

2.8 Toxic Ascomycete: - Aflatoxin Producers

Aflatoxins are secondary metabolites produced by species of Aspergilli, specifically *Aspergillus flavus* and *Aspergillus parasiticus*. These moulds are ubiquitous in nature and grow on a variety of substrates, thereby producing aflatoxins. Aflatoxins are of great concern due to their biochemical and biological effects on living organisms (Ellis *et al.,* 1991; Cullen and Newberne, 1993). Aflatoxins are potent carcinogenic, mutagenic, and teratogenic metabolites.

Foods and feeds, especially in warm climates, are susceptible to invasion by aflatoxigenic *Aspergillus* species and the subsequent production of aflatoxins during preharvesting, processing, transportation and storage conditions (Eaton *et al.*, 1993 and Shapira *et al.*, 1996). Aflatoxin B1 **(18)** is the most potent natural carcinogen and is usually the major aflatoxin produced by toxigenic strains.



Aflatoxin B1 (18)

CHAPTER THREE

METHODOLOGY

3.1 Chemicals, Reagents & Other Materials Used

The main chemicals and reagents used in this project were; methanol 99.98% purity, (Spain), ethyl acetate (GPR) Belami fine chemicals, cyclohexane 99.99% purity (AR grade, Fisher Scientific, UK (GPR, Kobian Kenya Ltd). silica gel (60-200 mesh), acetone ST for column chromatography) Macherey – Nagel Germany and Mitsubishi HP21-DIAION resin.

3.2 Equipment

The equipment used in this project were: weighing balance (Denver instrument Model – XL-31000), autoclave (Danfoss 59407-3 No. 375), analytical balance (Precision 310M), pH meters (Fishes Acument ® model 620A), magnetic stirrer hotplate (Gallenlamp), vacuum rotary evaporator type 349/2, biological safety cabinet (Steril GARD, Baker Company Sanford – Class II Type A/B3 US PAT NO. 3,895,570) and Suction pump (Arboles model)

3.3 Apparatus & Other Materials

60 Capillary tubes, 20-250 ml and 100 ml conical flasks, 10-100 ml measuring cylinders, Buchner funnel filtration system, 1 packet filter funnel, 20 petri dishes, 2 chromatographic tanks, 2 chromatographic column (glass), 5-250 ml glass beaker, 5-100 ml glass beaker, 20-250 ml rounded round bottomed flask, 2-500 ml separating funnel, inoculating blade (spear), acid washed sand, TLC plate, {Macharey-Nagel, ALUGRAM ® SIL G/UV_{254} 0.25 mm Duren, Germany}, cotton wool, 2 aluminium foil, 1 packet glucose testing strips (Diabur-test ® 5000 Roche).

3.4 Preliminary Preparations

3.4.1 Sterilization of Apparatus & Materials Used

To ensure that cultures were not contaminated, hands and working benches were thoroughly and constantly sterilised using 70% ethanol. The working environment in the hood was maintained in sterile state by use of hot Bunsen flame. The hot flame was also used to sterilise inoculating blade as well opening and closing culture flasks. The liquid and solid media as well as all the apparatus were sterilised using an autoclave at a maintained temperature of 121°C and a steam pressure of 1.5 bars for 15 minutes. Steam sterilisation was used to destroy infected cultures and used old test plates.

3.4.2 Preparation of Liquid Media

Liquid media was prepared by dissolving 10.0 g of molasses, 4.0 g glucose and 4.0 g of yeast extract in 1.0 L of water. The pH of the media was adjusted to 5.5 using 1M hydrochloric acid and 1M sodium hydroxide where necessary. The media was then dispensed using 250 ml Erlenmeyer flasks. These were corked with cotton wool and then wrapped with aluminium foil. Each of the set-ups was autoclaved at 121°C and steam pressure of 1.5 bars for 15 minutes and then left to cool. Once cool, they were placed in the working bench of a sterile laminar flow hood. These were used to prepare the starter cultures. When preparing the molasses liquid media for growth of the already growing cultures, the media was similarly prepared as described above and then dispensed in twenty one Erlenmeyer flasks and sterilized at the same conditions.

3.5 Sub-Culturing of the Ascomycete JO5035

The ascomycete was obtained from the agar slant and sub-cultured onto potato dextrose agar (PDA). From a well grown solid culture, agar plugs were cut and introduced into the sterile media in lamina flow hood. These were allowed to grow as still cultures with regular agitation (at least twice a week) under ambient laboratory conditions of 25^0C . The growth of each of the cultures was monitored closely and evaluated daily to check the biomass build up and presence of any contamination. The growth of the culture was stopped when there was no apparent biomass build up and glucose levels in the medium were depleted.

3.6 Preparation of Crude Extract

3.6.1 Crude Extract from Mycelium

Immediately growth was stopped, mycelium was separated from the culture filtrate using filtration. The mycelium was soaked in acetone and continuously stirred using magnetic stirrer for 5 hours. The mixture was further filtered using the Buchner funnel and the residue discarded. From the acetone extract filtrate, acetone was recovered using rotary evaporator under reduced pressure. Ethyl acetate was then used to obtain the crude extract from the remaining aqueous layer (ratio 1:1 v/v) using a separatory funnel. This was repeated 5 times to optimal extraction of the desired crude extract. The combined ethyl acetate extract was dried using anhydrous sodium sulphate, then filtered and concentrated under reduced pressure using the rotary evaporator. The dried extracts were transferred using 2 ml of methanol and stored in screw capped glass vials. The extracts were kept at 4^oC in a refrigerator waiting for analysis. This was then screened for

larvicidal activity against the test organism (ref section 3.8). The crude extract was then dried using 2 g of silica gel in a 50 ml glass beaker and its weight determined

3.6.2 Crude Extracts from Culture Filtrate

The combined volume of the culture filtrate obtained by filtration in section 3.6.1 was passed three times through a resin (Mitsubishi HP21 DIAION resin) packed in a column. The resin was packed in vertically mounted column of glass (diameter 2.5 cm and height 60 cm) using distilled-deionised water. Once all the culture filtrate passed through the resin, the trapped secondary metabolites were eluted with 1500 ml of acetone followed by 1000 ml of methanol. The eluents were collected and concentrated in a residual aqueous remain, which was extracted five times with ethyl acetate. The combined ethyl acetate extract was dried using anhydrous sodium sulphate and concentrated using rotary evaporator under reduced pressure. The eluents were concentrated under reduced pressure using rotary evaporator to remove acetone and methanol, respectively. The concentrate were transferred into screw capped vials, then dried under nitrogen atmosphere and were kept at 4^{o}C awaiting further analysis.

3.7 Purification of Compounds in the Enriched Fractions

The dried enriched fractions from section 3.6.1 and 3.6.2 were further purified in column with internal diameter 45±0.05 mm, and length of effective column was 7.0 cm for all. The solvent system to develop the column was optimised in the polarity range which was determined for that particular fraction.

The mycelia crude extract (Mex) and culture filtrate (Kex) were separately fractionated and purified using repeated silica chromatography. Column chromatography work was guided by thin layer chromatography (TLC) to determine the solvent system and the level of purity at different stages of the fractionation and purification. Suitable solvent system was determined by spotting the respective crude extracts (that is Mex and Kex) on aluminium coated silica TLC plates, which were developed in various TLC chambers saturated with various solvents in different ratios. At this stage the solvents used were dichloromethane, cyclohexane, ethyl acetate and methanol. The developed plates were visualized using UV lamp and then sprayed with anisaldehyde spraying reagent before drying in the oven to aid further visualization. From the TLC results, the best solvent system that is the one that gave better separation was used in the column chromatography. A vertically mounted glass column was slurry packed with silica gel suspended in the less polar organic solvent. This slurry was constituted by suspending about 60 g

of silica gel in the appropriate solvent system (cyclohexane). It was degassed by use of a suction pump and poured into the column. This was anchored in place by use of acid washed sand. The silica adsorbed sample were introduced slowly into the column with the aid of pipette and the sample was also anchored in place by use of acid washed sand so as to avoid turbulence during elution. Various fractions were collected and every eluent collections were spotted on a TLC plate. TLC analysis was used to determine the purity of the eluents which were later pooled into various fractions based on TLC results and then concentrated under reduced pressure. Repeated column chromatography and TLC were done until fractions were deemed clean. The weight of the purified fractions was determined and then transferred to screw-capped vials and kept in fridge at 4 °C awaiting larvicidal tests and structure elucidation experiments.

3.8 Mosquito Larvicidal Assay

3.8.1 Source of Mosquito Larvae

The mosquito larvae used in this project were obtained from Pyrethrum Board of Kenya, Nakuru where they are reared under standard conditions. Both the crude extracts and the purified samples were subjected to mosquito larvicidal activity test against late 3^{rd} and early 4^{th} in star larvae of *Ae. aegypti*. The late third and early fourth in star larvae were used since the response of freshly hatched larvae is not fully developed and they do not migrate satisfactorily during the first few hours. The larvicidal tests were carried out in the IBRL, Egerton University.

3.8.2 Mosquito Larvicidal Assay

Standard methods for assaying larvicidal activity in scientific research laboratories as recommended by the WHO were followed in all experiments (WHO, 2005). Both the crude extracts and the purified samples were subjected to mosquito larvicidal activity. Bioassays were carried out in duplicate using 10 larvae for each replicate assay. Several fractions of varying concentration (50 ppm, 100 ppm, 200 ppm, 500 ppm and 1000 ppm) were made by dissolving 0.2 mg , 0.4 mg, 0.8 mg, 2.0 mg and 4.0 mg respectively and tested against the larvae of *Ae. aegypti*. Aqueous solutions of methanol were employed as the control experiments. The larvae were placed in test plastic pots containing 4 ml of the test solution. Larvae were considered dead when they were unable to reach the surface of the solution when the test plates were shaken. The number of dead larvae was determined at the start of the experiment and after 2, 4, 8, and 24 hours to monitor the larval mortality. The analysis of larvicidal assay data was carried out using regression analysis. The LC_{50} and LC_{90} values which were the concentration values for killing

50% and 90%, respectively of the mosquito larvae in 24 hours were obtained from the regression analysis.

3.9 Structure Elucidation Nuclear Magnetic Resonance (NMR Spectroscopy)

Structures of purified larvicidal active compounds were elucidated using Nuclear Magnetic Resonance (NMR) spectroscopic technique. NMR spectroscopic experiments were performed on 300 MHz Bruker AVANCE NMR spectrometers at the Division of Chemical Sciences, Faculty of Health and Medical Sciences, University of Surrey, Guildford, UK. The spectra were recorded in deuterated chloroform ($CDCl_3$) and the chemical shifts were recorded in parts per million (ppm) relative to the solvents. The deuterated chloroform was referenced according to the central line at δ 7.260 in the ^{1}H NMR spectrum and at δ 77.23 in the ^{13}C NMR. The purified compounds were dissolved in deuterated chloroform ($CDCl_3$) in a clean vial. The solution was then transferred to an NMR tube and was placed in the probe for analysis. The same sample was used to obtain the spectra (^{1}H, ^{13}C, DEPT, NOESY, HMBC and COSY) data for the compound. Data was acquired from the NMR machine as computer print out. From the spectroscopy experiment, structures were proposed based on the interpretation of the spectra.

CHAPTER FOUR

RESULTS AND DISCUSSION

4.1 Results of Cultivation, Purification and Structure Elucidation

4.1.1 Extraction of the Crude Extracts

The ascomycete JO5035 first growth on Potato Dextrose Agar appeared as white cottony mycelia. Agar plugs of the ascomycete were cut and introduced into the sterile liquid media. The growth of the ascomycete in submerged culture was stopped after 21 days. Immediately growth was stopped, crude extracts were prepared for both intra- and extra-cellular secondary metabolites. On extracting compounds from the culture filtrate and mycelium, 0.00155 mg and 0.00025 mg respectively of crude ethyl acetate extracts were obtained as shown in figure **3** below

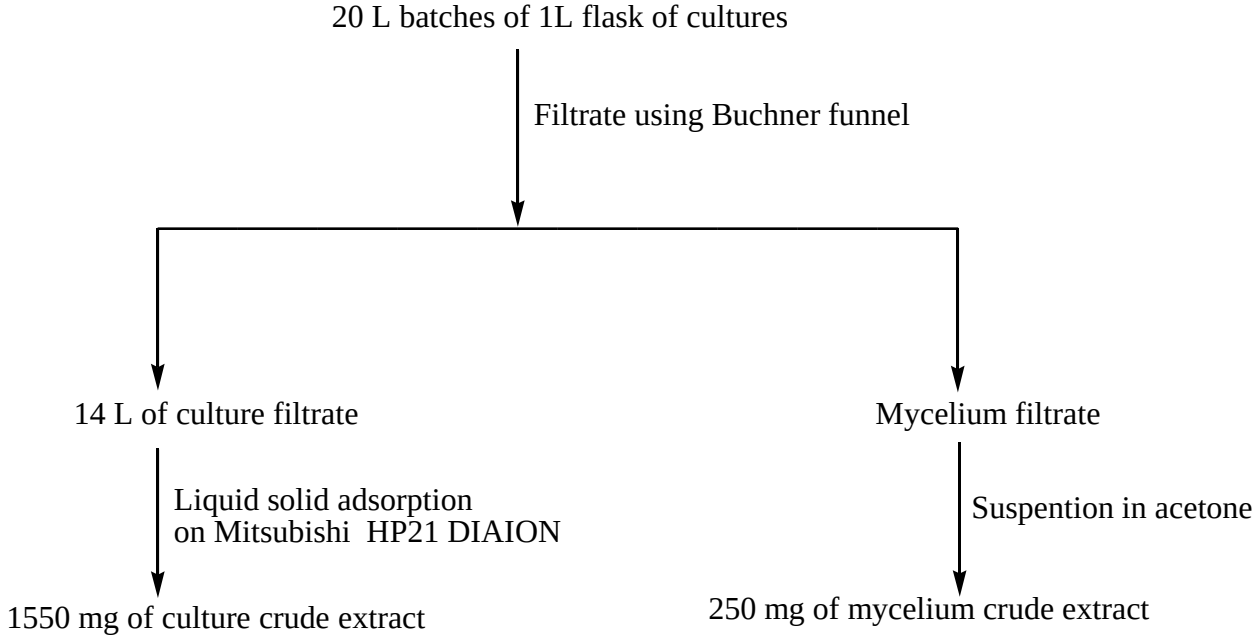


Figure 3: Scheme of Extraction

4.1.2 Larvicidal Tests of the Mycelia and Culture Filtrate Crude Extracts

The mycelial crude extract was prepared in defined concentrations in the range 0-1000ppm. These were then tested for larvicidal activity against *Ae. aegypti*. The crude extracts prepared at different concentrations were tested in set-ups that were evaluated for percent mortality after 2, 4, 8 and 24 hours. All the tests were done in duplicate and the mean value is reported in the table **1**.

Table 1: Larvicidal Test for the Crude Extracts (Mex)

	% mortality of the larvae			
Concentration (ppm)	2 hours	4 hours	8 hours	24 hours
0	0	0	0	0
50	0	0	0	0
100	0	0	0	0
200	0	0	0	20
500	0	60	60	100
1000	100	100	100	100

Table 2: Larvicidal Test for Crude Extracts (Kex)

	Percentage mortality of the larvae			
Concentration (ppm)	2 hours	4 hours	8 hours	24 hours
50	0	0	0	0
100	0	0	0	0
200	0	0	10	30
500	40	80	100	100
1000	100	100	100	100

Table 3: Methanol Control

	Percentage mortality of the larvae			
Concentration (ppm)	2 hours	4 hours	8 hours	24 hours
50	0	0	0	0
100	0	0	0	0
200	0	0	0	0
500	0	0	0	0
1000	0	0	0	0

4.1.3 Purification of the Culture Crude Extract of the Ascomycete JO5035.

The 1.55 g crude extract of the culture filtrate was further purified to identify the compounds responsible for the activity. A total of 5 fractions were collected, each having a volume of approximately 300 ml. These fractions were further purified. Each of the fractions was spotted on TLC plate. The developed TLC plate was allowed to dry and was viewed under UV radiation (λ = 254 nm and 365 nm). The plate was further sprayed with *p*-anisaldehyde solutions and dried for 15 minutes at 110 °C. These two procedures enable the fractions to be pooled together into 36 main fractions based on the pattern and R_f values of the spots on the TLC plate. These intermediate fractions were further subjected to chromatographic separation, which led to elution of 37.2 mg of pure compound JM212 as shown in figure **4** below.

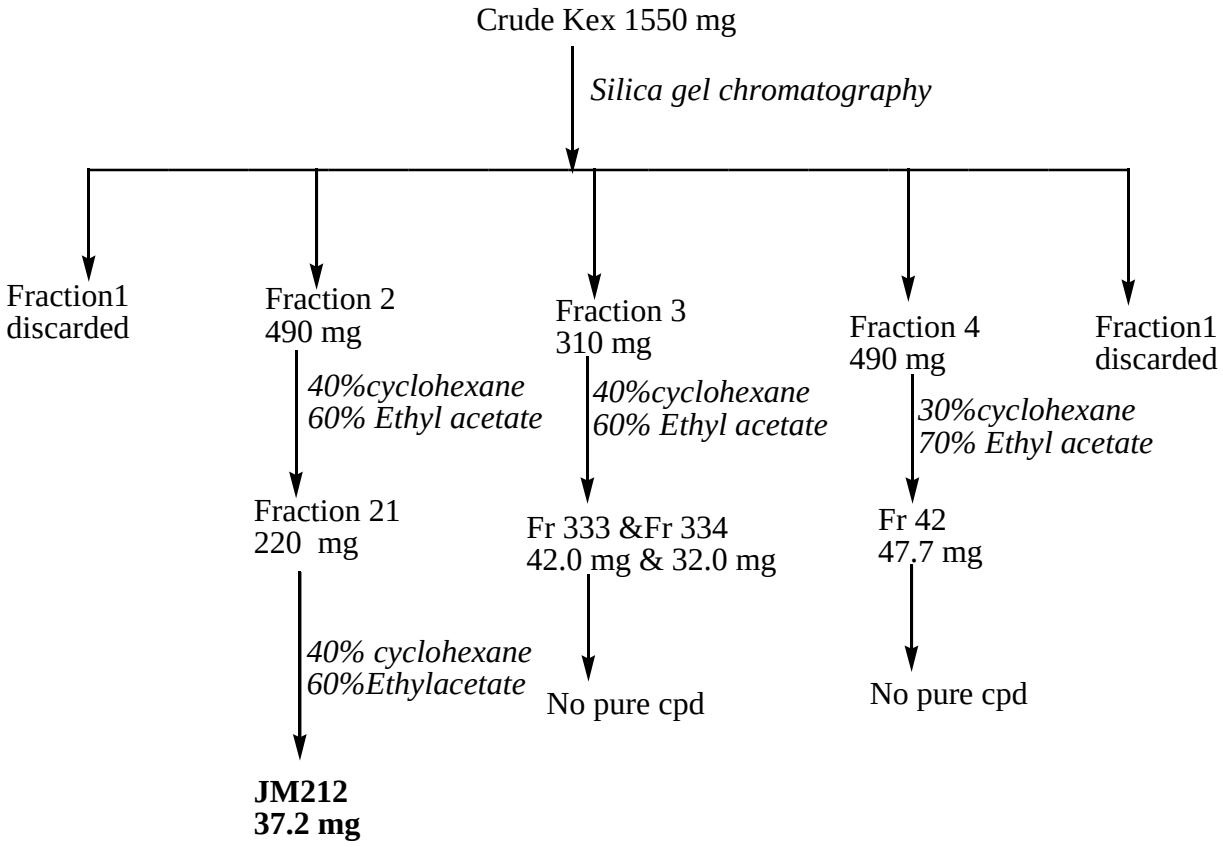


Figure 4: **Purification of the Culture Filtrate**

4.1.4 Purification of the Mycelia Crude Extract of the Ascomycete JO5035.

The 250 mg of the mycelia extract was also subjected to chromatographic purification. Each of the fractions was spotted on TLC plate. The developed TLC plate was allowed to dry and was viewed under UV radiation (λ = 254 nm and 365 nm). The plate was further sprayed with *p*-anisaldehyde solutions and dried for 15 minutes at 110 °C. These two procedures enable the fractions to be pooled together into 10 main fractions based on the pattern and R_f values of the spots on the TLC plate. These fractions were sent for analysis but no pure compound was obtained from them.

4.1.5 Mosquito Larvicidal Activity of the Pure Compound

The purified compound, JM212, was tested for larvicidal activity against *A. aegypti* and the results of the test are summarized in table **4** below;

Table 4: Larvicidal Bioassay for 2-*p*-tolylethanol

	% Mortality of the larvae			
Concentration (ppm)	2 Hours	4 Hours	8 Hours	24 Hours
50	0	0	0	0
100	0	0	0	40
200	0	0	20	80
500	0	10	70	100
1000	20	50	100	100

The results given in table **4** indicate that there was no observable activity up to 8 hours since the start of the experiment. From the results it is the mortality data at 24 hours that was correlated to obtain of the LC_{50} of 279.6 ppm and LC_{90} of 624.8 ppm.

4.1.6 Results of NMR Experiments

The purified compound was subjected to one- and two-dimensional NMR experiments. The ^{1}H-NMR, ^{13}C-NMR and distortionless enhances polarization transfer (DEPT) experiments were carried out and the corresponding spectra are attached in appendices **1-10**.

4.2 Discussion of the Results

4.2.1 Growth of the Ascomycete JO5035

The growth of the ascomycete JO5035 was stopped when there was no apparent growth of the ascomycete and the glucose levels were depleted. Glucose served as the initial carbon source before the ascomycete can immobilise carbon from more complex carbon sources like from molasses. In filamentous ascomycetes, an individual hypha (a multinucleate, multicellular filament with incomplete crosswalls, or septa) grows by hyphal tip extension and branching. Hyphal tip growth is believed to be mediated by delivery of the components for cell wall extension to hyphal tips by a vesicle supply center. As filamentous ascomycetes grow, fusions between hyphae are continuously formed, yielding a network of interconnected hyphae, or mycelium that makes up the fungal individual. Networked hyphae are presumably important in intra-hyphal communication and homeostasis in an individual colony during growth and reproduction. Growth in filamentous fungi thus consists of three balanced processes: hyphal tip extension, branching, and fusion (Louise *et al*, 2000). When growth was stopped the culture was

separated into mycelium and culture filtrate from which crude extracts, Mex and Kex were prepared.

4.2.2 Larvicidal Activity of the Crude Extracts

From the table **1**, it was noted that higher concentration of 1000 ppm of the mycelia crude extract (Mex) was more efficacious from the start of the 2 hours, which had 100% mortality. The 500 ppm was able to kill 60% of the larvae after 4 hours and which remained steady after 8 hours. However, at 24 hours its efficacy was 100%. Concentrations of 200 ppm was able to cause only 20% mortality after 24 hours. For concentrations less that 100 ppm, there was no percent mortality observed at all even after 24 hours. The observed activity at 1000 ppm after 24 hours of 100% mortality was still significant given that it was observed for crude extract.

The culture filtrate crude extract (Kex) table **2** was similarly prepared and tested in duplicate for larvicidal activity against *Ae. aegypti*. Just like the mycelial crude extract above, only 1000 ppm showed larvicidal activity after 24 hours. The 500 ppm of the culture filtrate (Kex) had a higher activity as compared to the same concentration of the mycelium filtrate (Mex). It was also noted that the culture crude extract (Kex) had a relatively higher activity compared to the mycelia crude extract (Mex) because of synergism, but this can only be approximate since these are crude extracts.

4.2.3 Purification of the Culture Filtrate (Kex)

The purification of the 1550 mg of the culture crude extract by use of column chromatography from figure **4** generated five fractions. Fractions one and five were discarded because one was non-polar and contained only fatty acids while five was polar hence hard to be separated. Fractions two, three and four were further purified by column chromatography guided by thin layer chromatography. A pure compound was obtained from fraction **2** as JM212**.**

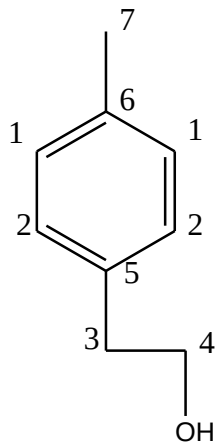
4.2.4 Mosquito Larvicidal Activity of the Pure Compound

Compound JM212 larvicidal tests in table **5** showed clear that sensitivity of *Ae. aegypti* larvae concentration dependent correlated at 24 hours. At other evaluation intervals (after 2, 4, and 8 hours) there was no significant activity observed and correlation to be determined. It was also noted that there were not much activity below concentration of 100ppm. The control had all the larvae active after 24 hours. From the table **5** results, the concentration at 100ppm showed 40% mortality after 24 hours, 200ppm had 80% mortality, 500ppm had 100% mortality and 1000ppm had 100% mortality after 24 hours. Regression analysis was used to determine the

LC_{50} and LC_{90} values. The regression equation is Y= 0.180X + 8.362 and the R^2 = 0.950. From the regression equation the values of LC_{50} and LC_{90} were obtained as 279.6 ppm and 624.8 ppm respectively.

4.2.5 Structure Elucidation of the Pure Compound

The pure compound JM212 was obtained as a yellow liquid weighing 37.2 mg. The chemical structure of the compound was determined based on 1D and 2D NMR experiments. The purified compound whose proposed name is 2-*p*-tolylethanol (**19**) was purified from the enriched fraction 2 using column chromatography. The solvent system used constituted 40% cyclohexane and 60% ethyl acetate. The compound was collected after approximately 400 ml of the eluting solvent were passed. 2-*p*-tolylethanol (**19**) was obtained as yellowish–oily liquid and had pink colouration on the TLC plate after spraying with anisaldehyde. It has a molecular formula $C_9H_{13}O$ as determined by the analysis of ^1H and ^{13}C NMR spectral data. Its structure was studied by use of NMR spectroscopy – both 1D and 2D NMR experiments as attached from appendices **1-10**.



2-*p*-tolylethanol (19)

The NMR spectra (both 1D and 2D) were used in interpretation of structure of 2-*p*-tolylethanol (**19**). From the ^{13}C NMR spectrum (appendix **2**), seven signals are evident from the chemical shifts (δ = 115.7, 130.8, 36.9, 64.0, 155.3, 143.3 and 14.5 ppm). δ = 115.7 and 130.8 ppm represents two carbon atoms each as they are in the same environment. Therefore the total number of carbons present in the molecule is 9. From the ^{13}C NMR spectrum it is evident that there is an aromatic ring carbon due to the high chemical shifts (δ = 115.7(C_1), 130.8(C_2), 155.3(C_5), and 143.3 ppm (C_6)). The signals at δ = 115.7 and 130.8ppm were isochronous, each

representing 2 pairs of carbon atoms in a benzene ring, indicating unequivocally that the ring was having symmetry of sorts. This accounts for six signals namely; δ =115.7, 115.7, 130.8, 130.8 155.3 and 143.3 ppm

The chemical shift at δ = 14.5ppm (appendex **1**) is a typical sp^3 hybridized carbon while that at δ = 39.4 ppm is due to the deshielding effect of the benzene ring. The chemical shift of the carbon at δ = 64.0 ppm is a typical sp^3 hybridized carbon attached to an electronegative atom, the hydroxyl group causing deshielding.

From the DEPT(appendex **6**), four different types of carbon atoms are evident; two quaternary carbons at (δ = 155.3 ppm and 143.3 ppm), four methine carbons in two pairs at (δ = 115.7ppm and 130.8 ppm), two methylenic carbon atom (δ = 36.9 and 64.0 ppm) and one methyl carbon (δ = 14.5 ppm). The carbon signal at δ = 64.0 ppm is typical of aliphatic oxygenated sp^3-hybridised carbon and that at δ = 14.5 ppm is a methyl group.

The HMBC spectrum (appendix **7**)clearly reveals that the aromatic methine carbon at δ = 115.7 ppm is correlating with aromatic protons atoms at δ = 6.79 and 7.09 while that δ =130.8 ppm is correlating with aromatic protons at δ = 6.79, 7.09, 3.83 and 2.80 ppm as summarized in the table **5** below.

Table 5: Summarized Data from 1-D and 2-D NMR Experiments

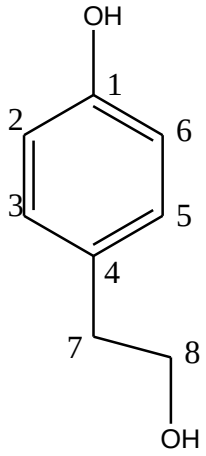
Position	^{13}C	^{1}H	DEPT	HMBC	COSY	NOESY
1	115.7	6.79	CH	H-1/H-2	H-1/H-2	H-2
2	130.8	7.09	CH	H-1/H-2/H-3/H-4	H-1/H-2	H-1
3	36.9	3.83	CH_2	H-2/H-3	H-4	H-4
4	64.0	2.80	CH_2	H-4	H-3	H-3
5	155.3	-	-	H-2/H-1	-	-
6	143.3	-	-	-	-	-
7	14.5	2.38	CH_3	-	-	-

The compound 2-*p*-tolylethanol is prepared in the lab by the reaction of 2-Chloro-1-p-tolyl-ethanone. The reaction needs reagents sodium cyanoborohydride, Zinc iodide and solvent

1, 2 –Dichro-ethane with other condition of heating for 36 hours. This compound is used to produce 4- methylbenzaldehyde.

The observed LC_{50} and LC_{90} values were significant though they were not within the range of those previously isolated larvicidal compounds reported from the literature. Sesquiterpenoids such as (E)–nerolidol and farnesol showed LC_{50} values of 17 ppm and 13 ppm, respectively. Ocimenone, a monoterpenoid isolated from *Tagetes minuta* oil exhibited LC_{50} value of 40 ppm and a triterpene from *Azadiracta indica* showed an LC_{50} value of 21 ppm (Geris *et al.*, 2008). However, the study has revealed the potential of this ascomycete as source of larvicidal compounds. The objectives of the study were achieved to a limited extent due to low yield of the extracts, presence of impurities in the compounds isolated and lack of advanced purification techniques such as HPLC.

Tyrosol (**20**) extracted from a basidiomycete JO5289 showed larvicidal activity. It has an LC_{50} and LC_{90} of 231 ppm and 453 ppm at 24 hours. Tyrosol generally has pharmacological interests and shows an antioxidant activity. It has also been used in atheroscelrosis treatment, protecting low density lipoproteins (LDL) from oxidation which play a role in the initiation and progress of cardiovascular diseases (Chirchir, 2010).



Tyrosol (20)

CHAPTER FIVE

CONCLUSIONS AND RECOMMENDATIONS

5.1 Conclusions

The ascomycete JO5035 was grown in liquid nutrient media composed of 1% malt extract, 0.4% yeast extract and 0.4% glucose. During its growth of 21 days in the media it produced secondary metabolites in both the culture and mycelium extracts. The crude extracts from both mycelium (Mex) and culture filtrate (Kex) were tested for mosquito larvicidal activity against *Ae. aegypti*. Both crude extracts showed larvicidal activity. The culture filtrate crude extract (Kex) showed 100% mortality of the mosquito larvae for a concentration of 500 ppm within 8 hours while the mycelia crude extract (Mex) showed 100% mortality of the mosquito larvae for a concentration of 500 ppm within 24 hours. The results showed that the culture crude extract (Kex) was more active than the mycelia crude extract (Mex).The crude extracts were further purified using column chromatography and thin layer chromatography to determine the responsible larvicidal compounds. All the purified fractions were subjected to both 2-D and 3-D NMR experiments for structure elucidation. Only fraction 212 from the culture filtrate (Kex) had all the requisite experiments for structure elucidation, hence a complete chemical structure was proposed for the compound JM212.

The structure of the pure compound was successfully determined based on NMR spectral data. The compound 2-*p*-tolylethanol (**19**) showed clear larvicidal activity. The compound **19** had a considerable activity with LC_{50} of 279.6 ppm and LC_{90} of 624.8 ppm against the third instar larvae of *Ae. aegypti*.

The larvicidal activity of the crude extract was higher than the pure compound due to synergism. Generally the isolated compound showed moderate larvicidal activity compared with those reported from literature.

The study has revealed the potential of Kenya ascomycete JO5035 as a source of mosquito larvicidal compounds. Full identifications of the components present in the active fractions that might be responsible for the larvicidal activity will be important in realizing full exploitation of the fungus for biotechnological applications.

5.2 Recommendations

This research recommends the following;

1. The ascomycete JO5035 should be subjected to taxonomic identification so as to determine its classification.
2. The ascomycete JO5035 should be cultured in bulk to obtain higher yields of the active compound 2-*p*-tolylethanol (**19**) and also investigate its chemical properties to obtain IR and MS spectral data.
3. Further critical activities like toxicological studies need to be done to validate the potential application of the compound 2-*p*-tolylethanol (**19**) as a commercial larvicidal agent. The mode of action of the active compound as well as possible effects on non target organism should be studied before it can be practically used as natural mosquito control agent.
4. Repeated bioassay coupled with analytical fractionation and more modern equipment is needed to confirm other compounds that might have not been isolated.

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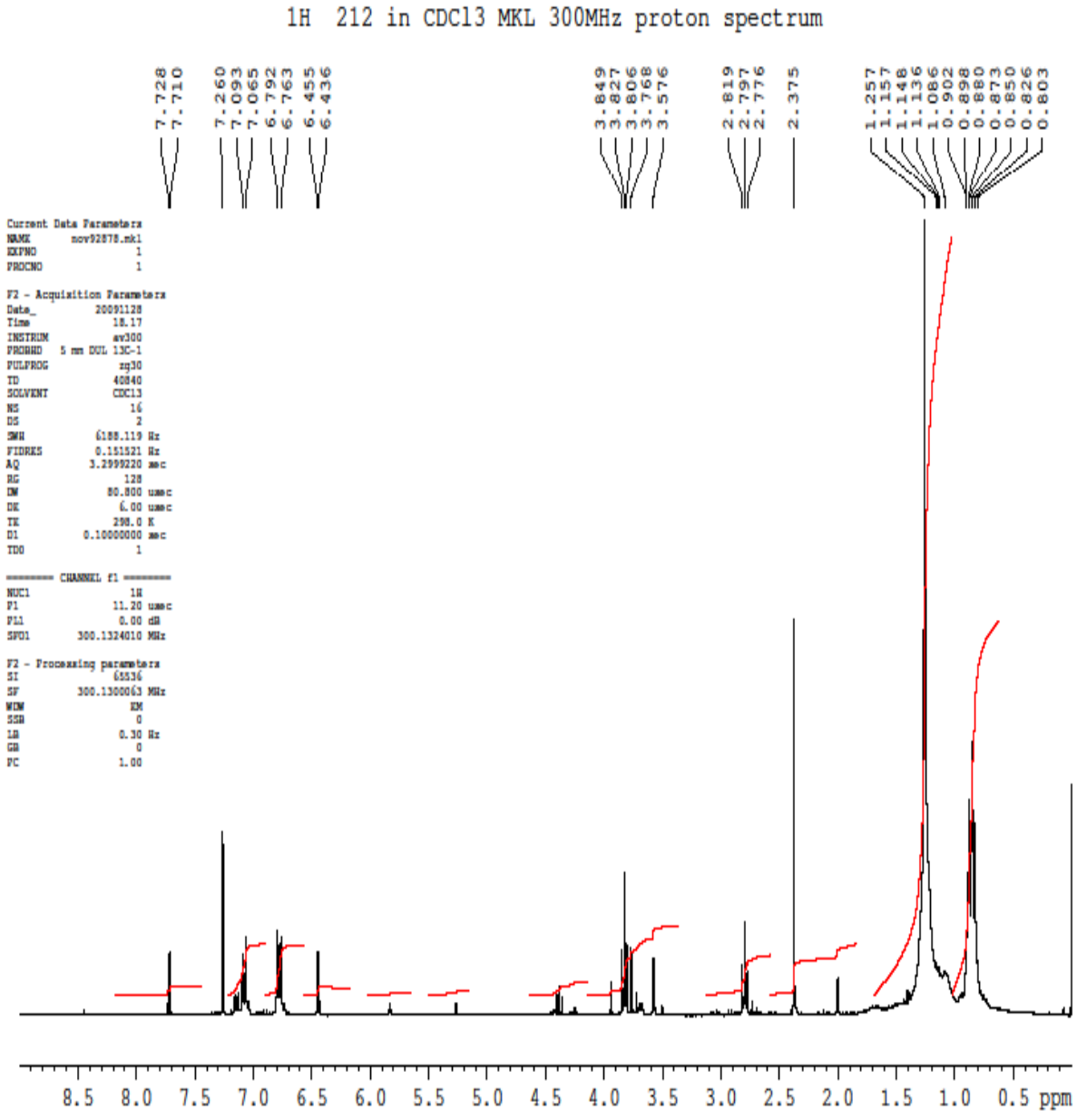
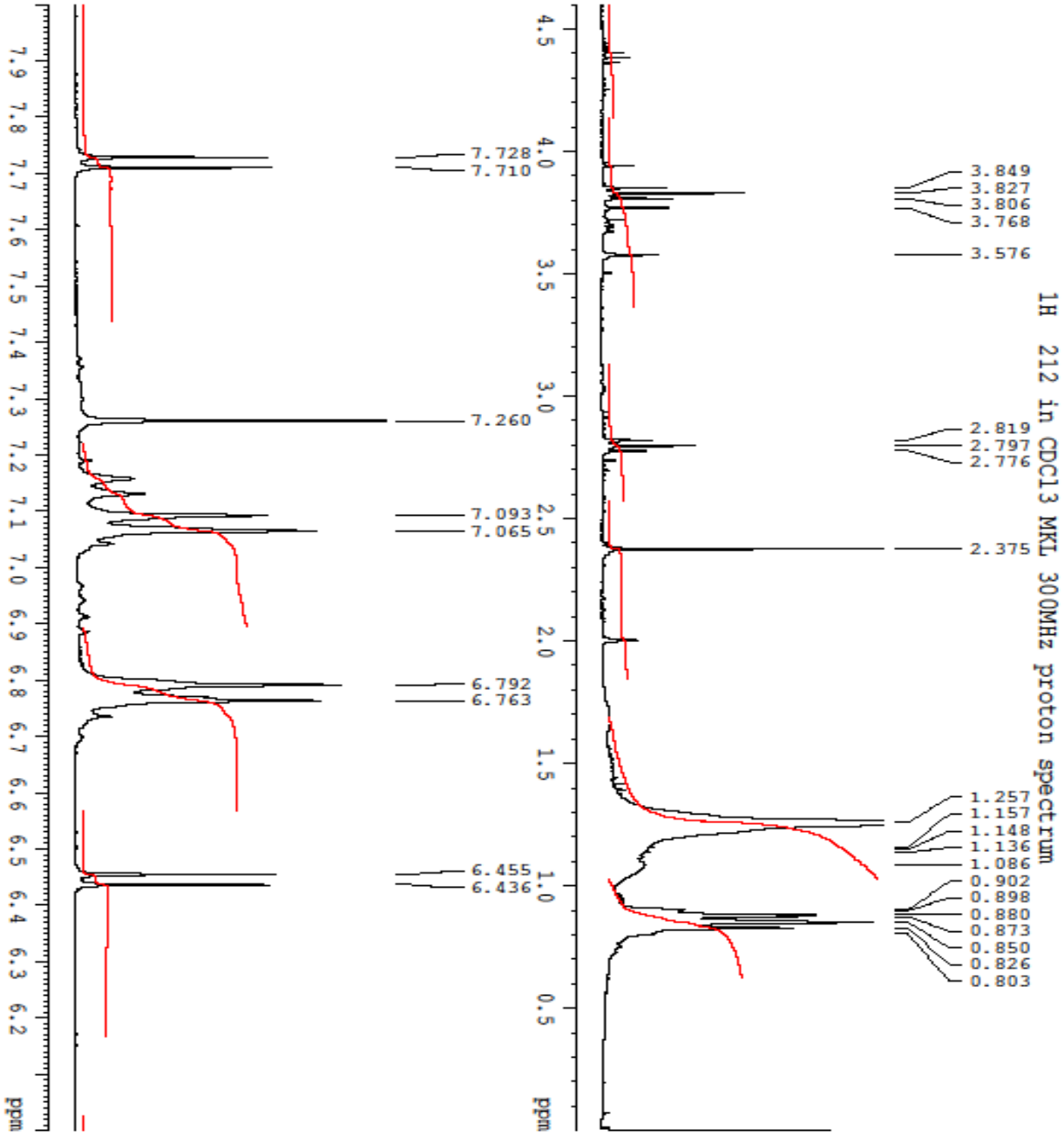
APPENDICES

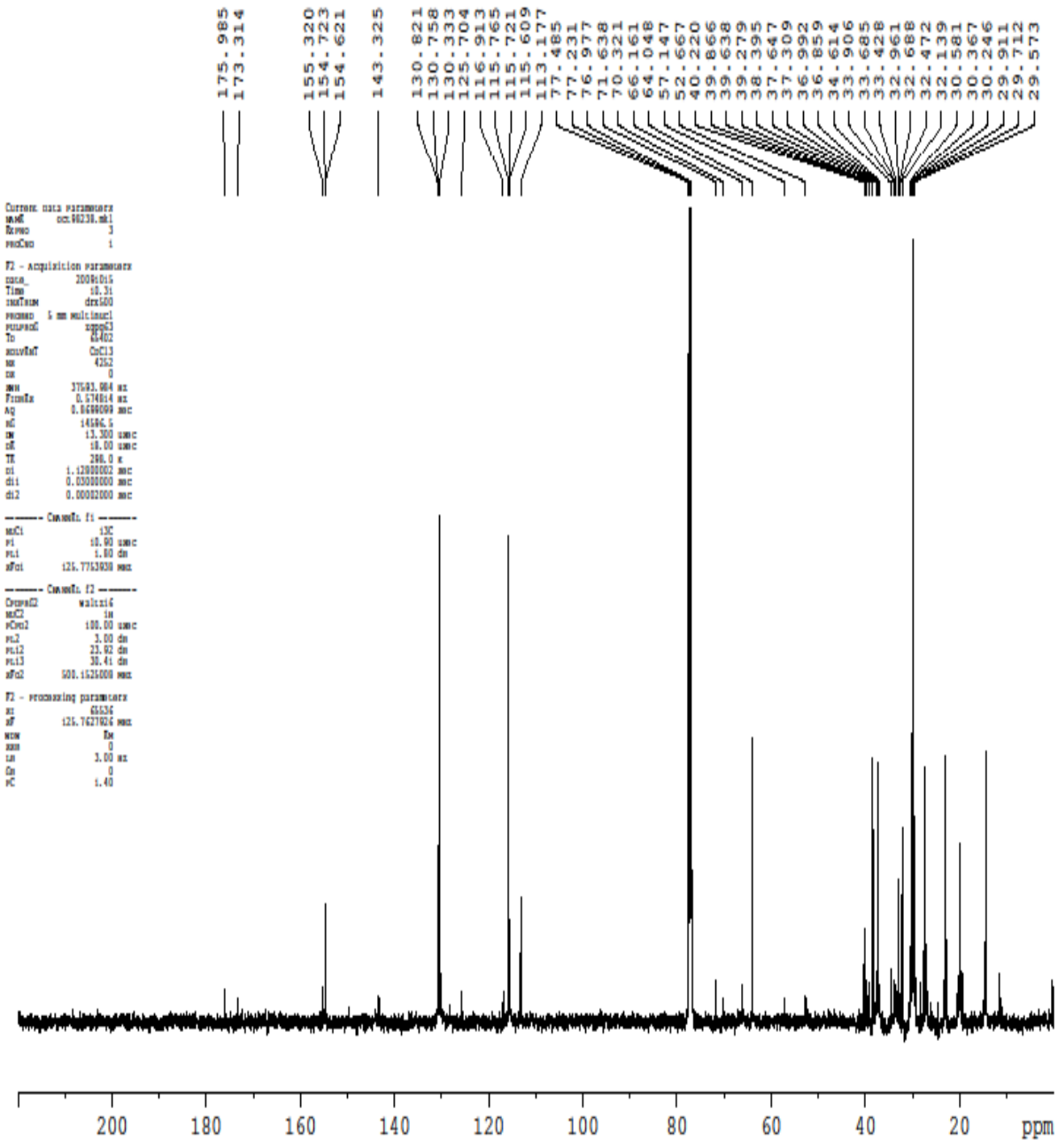
Appendix 1: ^{1}H NMR Spectrum for Compound JM212



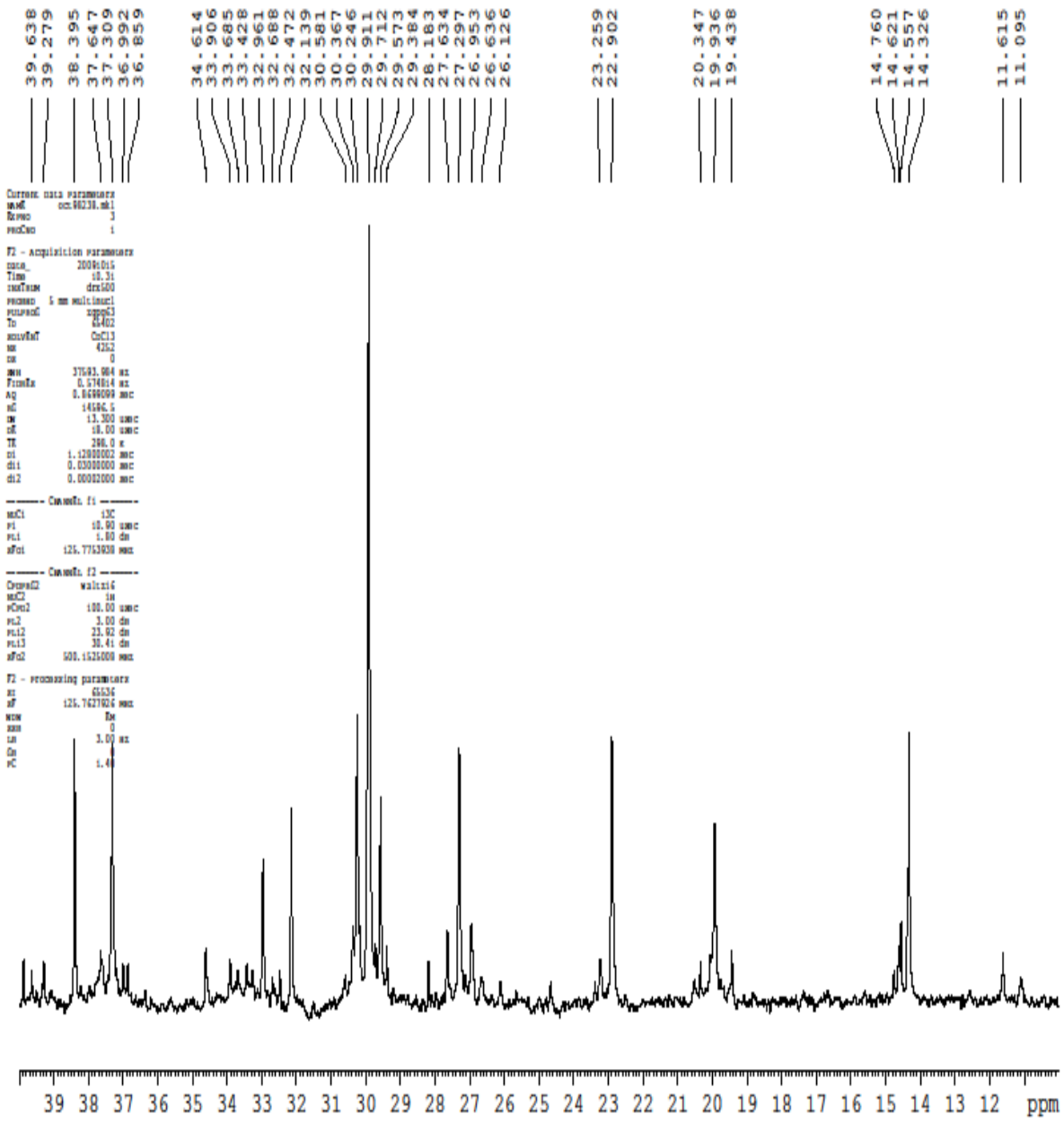
Appendix 2: ^{1}H NMR Spectrum for Compound JM212



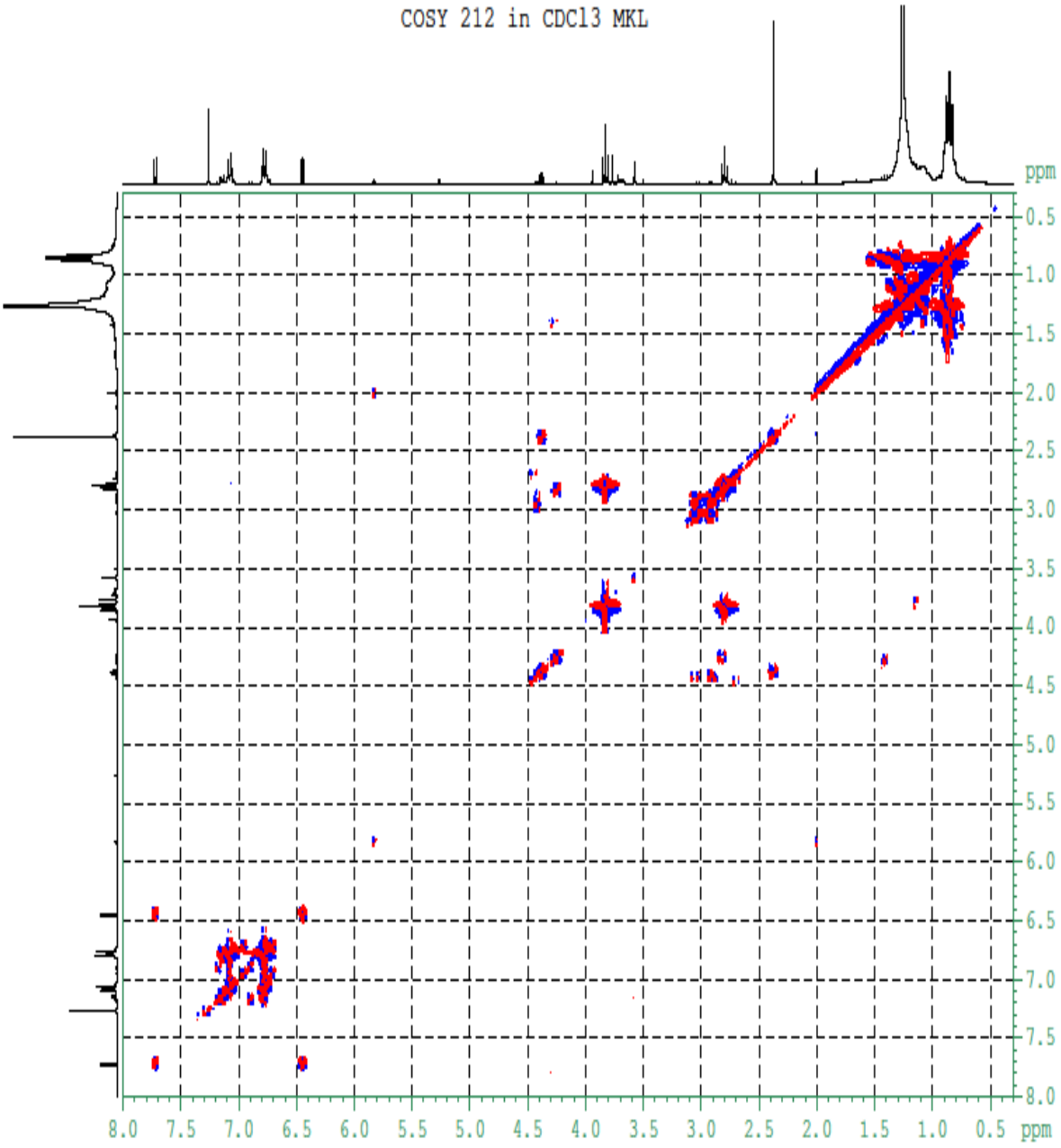
Appendix 3: ^{13}C NMR Spectrum for Compound JM212



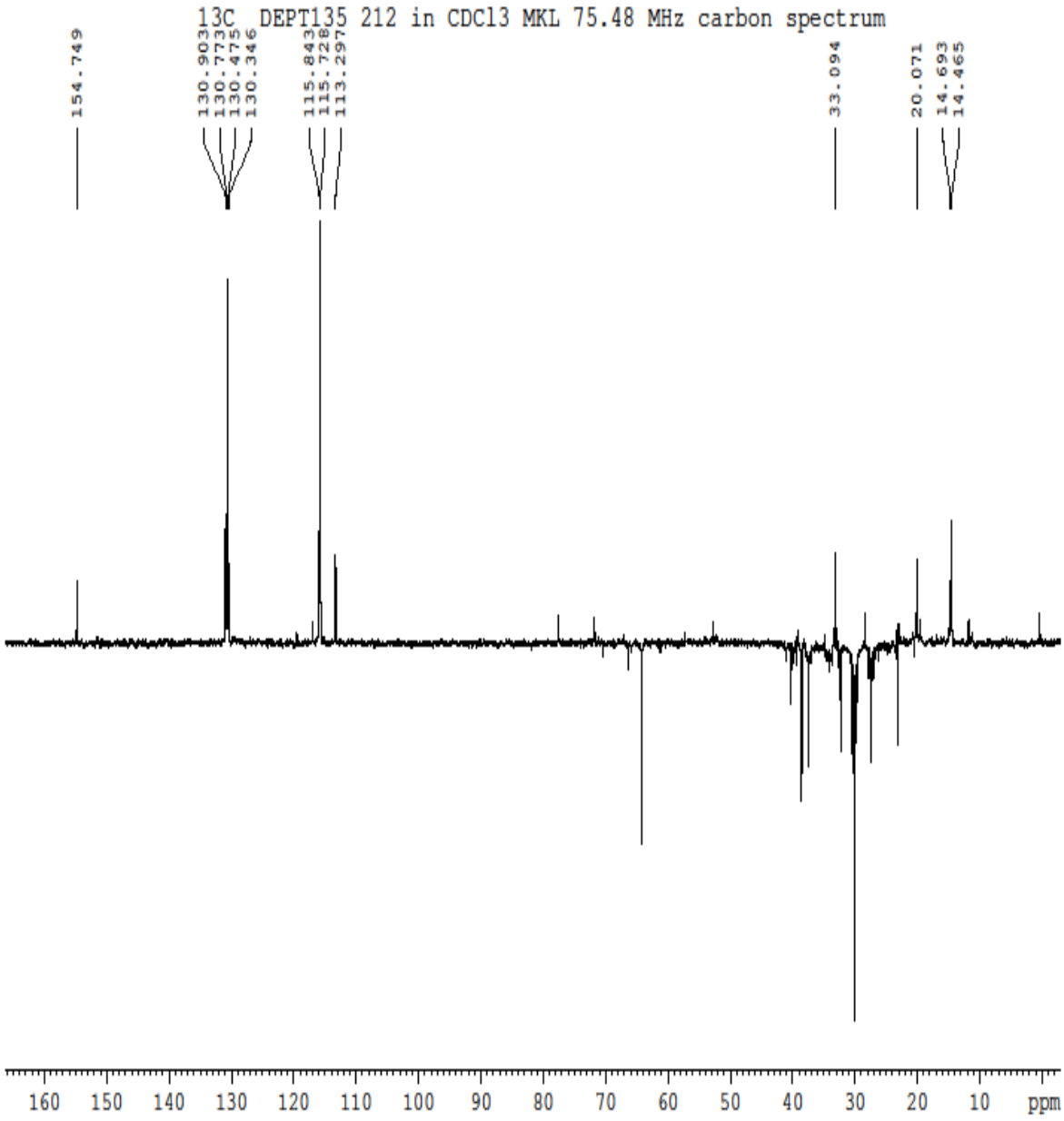
Appendix 4: ^{13}C NMR Spectrum for Compound JM212



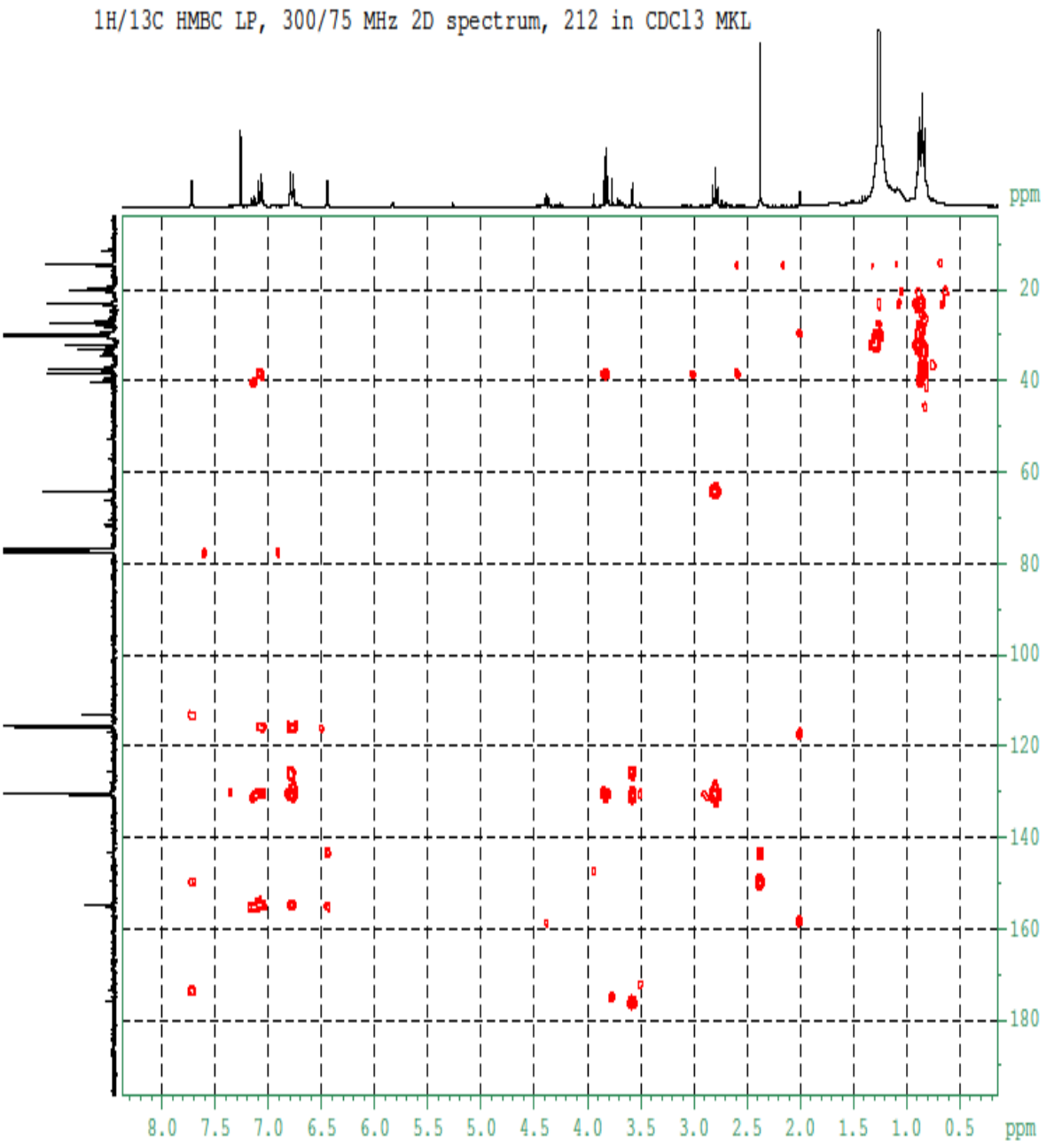
Appendix 5: COSY NMR Spectrum for Compound JM212



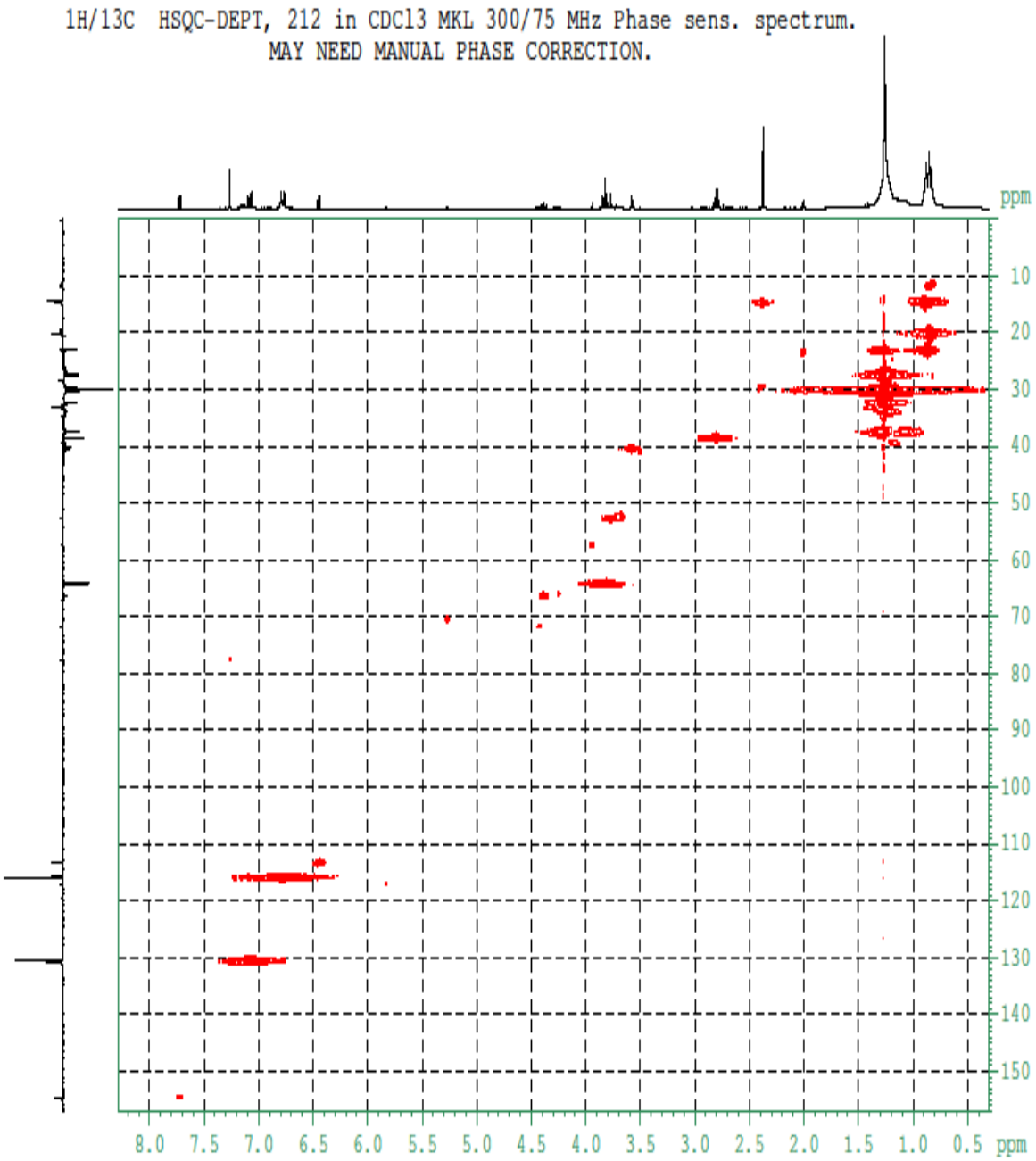
Appendix 6: DEPT NMR Spectrum for Compound JM212



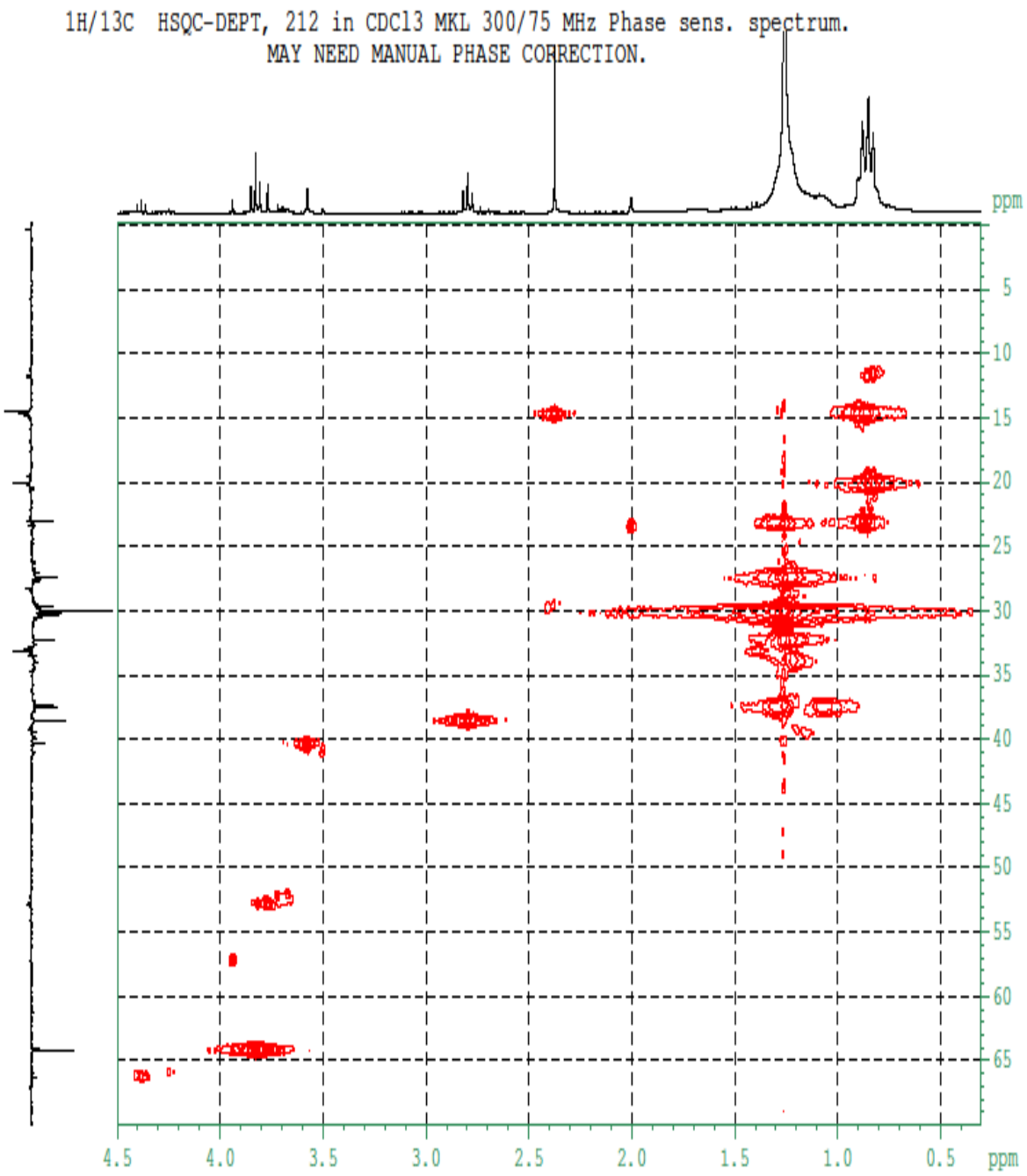
Appendix 7: HMBC NMR Spectrum for Compound JM212



Appendix 8: HSQC-DEPT NMR Spectrum for Compound JM212



Appendix 9: HSQC-DEPT NMR Spectrum for Compound JM212



Appendix 10: $^{1}H/^{1}H$ NMR Spectrum for Compound JM212