PURIFICATION AND CHEMICAL CHARACTERISATION OF LARVICIDAL COMPOUND (S) OF EXTRACTS PREPARED FROM SUBMERGED CULTURES OF BASIDIOMYCETES AGAINST A*EDES AEGYPTI*

BY

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A thesis submitted to the Graduate School in partial fulfilment for the requirements of the Degree in Master of Science in Chemistry of Egerton University.

EGERTON UNIVERSITY

SEPTEMBER 2008

DECLARATION AND RECOMMENDATION

DECLARATION

This thesis is my original work and has not been submitted for an award in this or any university to the best of my knowledge.

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DEDICATION

I dedicate this work to:

My husband Victor, whose love and support enabled me to reach this far.

My sons Kevin, Brian, Trevor, Franc and the late Chris who gave me a good working atmosphere.

Dr. J. Ouma Omolo and Dr. P. K. Cheplogoi, who inspired the work.

ACKNOWLEDGEMENTS

Utmost compliments go to my supervisors Dr. J. O. Omolo and Dr. P. K. Cheplogoi for their competent supervision. I am deeply indebted to them for their tireless guidance, valuable advice, encouragement and their useful ideas, which enabled me to accomplish my research objectives. I wish to acknowledge the Department of Chemistry, Egerton University and the entire technical staff for their competent technical assistance and granting me unlimited access to laboratory facilities. To mention a few, Mrs. A. Njue for her tireless guidance as it regards to the study. I am also grateful for the help and constructive critics of Mr. Karubiu (IBRL) and Esther (Dairy) both of Egerton University. My sincere appreciation goes to the technical staff of Pyrethrum Board of Kenya (PBK) Entomology Section for availing mosquito larvae used bioassay in this work.

I thank my colleagues Regina and Tana with whom I exchanged excellent ideas during the period of my research work. I would like to acknowledge Mr. Moses Langat for obtaining the spectroscopic data for this research. Above all, I am greatly indebted to my family whose love and support saw me through my educational endeavours. I wish to thank all who helped me in one way or another and did not get to be mentioned in person, may God bless you.

ABSTRACT

The diseases transmitted by mosquito continue to be rampant and in most case fatal especially in the developing countries. Mosquito is the main vector for malaria. Malaria is a scourge and according to the World Health Organisation (WHO) estimations, over 40% of the world population remain exposed to malaria. In Kenya, about 25,000 children die of malaria each year; although the actual number of deaths is unknown since most of them occur at home. Since prevention is better than cure, there is an urgent and immediate demand by the society to manage the growing population of mosquito. The chemicals used in managing the mosquito are natural and synthetic. The "miracle" of chemical technology five decades ago has not, however, provided a viable solution. In addition to inefficacy, chemical pesticide can cause mutant strains of fauna and flora. They can also produce potent toxic chemicals to the human body. In this regard, there is need to search for natural larvicides, especially from the unexplored fungal genera, as they are known to produce biologically active compounds with great diversity. Natural compounds are known to be more selective and biodegradable.

This study involved evaluation of two basidiomycetes JO4012 and JO4014 as sources of larvicides against mosquito *Aedes aegypti.* These two basidiomycetes were collected as fruiting bodies of mushrooms from within the precincts of Egerton University. They were selected after preliminary screening and isolated as pure cultures on solid media and then cultured in liquid media. Extraction of culture filtrate and mycelia were done using ethyl acetate and acetone solvents. Bioassay of the crude extract of the culture filtrate and mycelia of both species exhibited significant larvicidal activity. Higher concentrations of the crude extract were required to kill larvae within a shorter period of time. In this study, the mycelium crude extract from JO4012 had the highest larvicidal activity. Chromatographic techniques; thin layer chromatography (TLC) and column chromatography (CC) were used to purify the crude extracts. CC yielded fractions that were subjected to bioassay to verify the activity observed in the crude extract. Further purification of the active fractions was done using Sephadex LH20. A glycosidic moiety was proposed as part of the compound responsible for the larvicidal activity of the culture filtrate extract from JO4012. However, based on the NMR experiments, the aglycone moiety signals were too weak to be observed. This study needs to be pursued to address the problem of mosquito.

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LIST OF ABBREVIATIONS

^{13}CNMR	Carbon-13 Nuclear Magnetic Resonance
^{1}H NMR	Proton Nuclear Magnetic Resonance
AMCA	American Mosquito Control Association
AZA	Azadarachtin
Bti	*Bacillus thuringiensis israelensis*
CDCP	Centre for Disease Control and Prevention
COSY	Correlation Spectroscopy
DDT	Dichlrodiphenyltrichloroethane
DEPT	Distortion less Enhancement by Polarisation Transfer
DFID	Department for International Development
DHF	Dengue Hemorrhagic Fever
HMBC	Heteronuclear Multiple-Bond Correlation
HSQC	Heteronuclear Single-Quantum Correlation
IBRL	Integrated Biotechnology Research Laboratory
ICMR	Indian Council of Medical Research
ICP	Insecticidal Crystal Protein
ITNs	Insecticide-Treated Bed Nets
LC	Lethal Concentration
NMR	Nuclear Magnetic Resonance
NOESY	Nuclear Overhauser Effect Spectroscopy
PDA	Potato Dextrose Agar
TLC	Thin Layer Chromatography
U.S. EPA	United States Environmental Protection Agency
WHO	World Health Organisation

CHAPTER ONE

INTRODUCTION

Mosquitoes are insects belonging to the order Diptera. There are over 2500 different species of mosquito throughout the world, and they can be an annoying and a serious health problem to man. They interfere with work and spoil hours of leisure time. Their attacks on farm animals can cause loss of weight and decreased milk production. Over 1 million people die of mosquito-borne diseases annually (AMCA, 2006a).

Several mosquito species belonging to genera *Anopheles*, *Culex*, and *Aedes* are vectors of pathogens for various diseases like malaria, filariasis, Japan encephalitis (JE), dengue fever, dengue hemorrhagic fever (DHF), yellow fever etc. Mosquitoes also cause allergic responses to humans, which include local skin reaction and systemic reactions such as angioedema and urticaria (Peng *et al.*, 1999).

The World Health Organization estimates that 300 to 500 million people are diagnosed with malaria annually, causing 1.1 to 2.7 million deaths. Approximately 1 million of these deaths are among children in sub-Saharan Africa, where 90% of all malaria cases occur (WHO, 2000; UN, 2007). Malaria is the leading cause of mortality and morbidity in Kenya, particularly among pregnant women and children under five years (Rahul, 2002; DFID, 2005). Studies in Kenya suggest that between 20 - 25% of all deaths can be attributed to malaria. Up to twenty eight million Kenyans (70% of the population) are at risk and at any one time 1.5 million pregnant women are susceptible (DFID, 2005). A reduction in under-five mortality is a reliable indicator of the impact of a malaria intervention. In Kenya under-five mortality is currently 115 per 1000 live births up from 112 in 1998 (DFID, 2005).

The female mosquito when biting for blood meal normally transmits diseases. These mosquitoes are found mainly in less developed tropical countries that have ecosystem favourable for high reproduction rates of mosquitoes (Carlson *et al.*, 2004). Mosquito resistance to currently used insecticides, demographic changes and developmental activities have escalated the diseases transmitted by mosquitoes in the affected countries (Sengottayan, 2005). Consequently the most reliable approaches which would eventually eradicate these diseases would require interruption of the

mosquito life cycle by either targeting the larvae or killing the adult (Joseph *et al*., 2003; Prabakar and Jebanesan, 2004).

Chemicals commonly used in controlling mosquito are both synthetic and natural. Some compounds from herbal products such as nicotine **(1)** obtained from tobacco leaves, *Nicotiana tabacum*, anabasine **(2)** and lupinine **(3)**, the alkaloids extracted from Russian weed *Anabasis aphylla* (Campbell *et al.*, 1993), rotenone **(4)** from *Devris eliptica* and pyrethrins **(5)** from *Chrysanthemum cinerariaefolium* flowers (Hartzell and Wilcoxon, 1947), had been used as natural insecticides even before the discovery of synthetic organic insecticides.

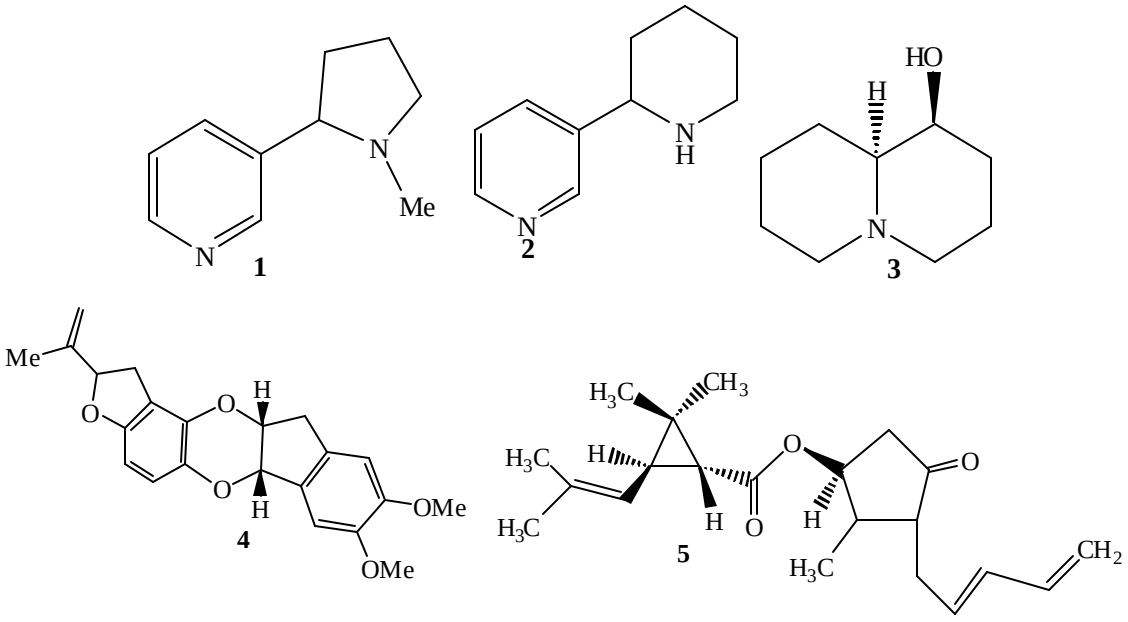

Figure 1 Structure of Nicotine, Anabasine, Lupinine, Rotenone and Pyrethrins

The discovery of Dichlorodiphenytrichloroethane's (DDT) insecticidal properties in 1939 and the subsequent development of organochlorine and organophosphate insecticides suppressed natural product search for insecticides since the answer to insect control was thought to have been found (Essam *et al.*, 2005). The extensive use of synthetic chemicals e.g. DDT **(6)**, resulted in environmental hazards and also in the development of resistance by the mosquito species. This has necessitated the need for search and development of environmentally safe, biodegradable, low cost larvicides to be used as an alternative to the synthetic ones (Thekkevilayil *et al.,* 2003).

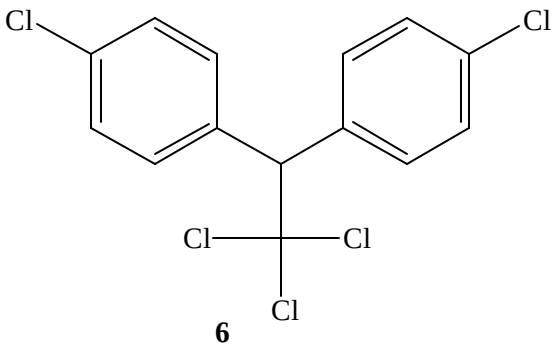

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Figure 2 Structure of DDT

Secondary metabolites of plants, many of them produced for protection against microorganisms and insect predators, are natural candidates for the discovery of new products to combat mosquitoes. Several studies have focused on natural products for controlling mosquitoes as larvicides and insecticides, but very few plant products have shown practical utility for mosquito control (ICMR, 2003). It is noteworthy that other genetic resources like tropical fungi remains under-explored for discovery of insecticides.

1.1 Statement of the problem

Fungi have been known to possess diverse biologically active molecules. A number of chemical constituents have been isolated from them. However, they have not been fully investigated for larvicidal compounds. Therefore, this prompted the study on these basidiomycetes in order to look for and isolate larvicidal compounds.

1.2 Objectives

General objectives

To search for larvicidal compounds from submerged cultures of basidiomycetes

Specific objectives

The specific objectives of this study were:

1) To prepare crude extracts from submerged cultures of two basidiomycetes.
2) To screen the crude extracts for larvicidal activity against *Ae. aegypti.*
3) To carry out larvicidal activity guided purification of the active compound(s) using chromatographic techniques.
4) To characterise the purified compound(s) responsible for the larvicidal activity using spectroscopic methods.

1.3 Significance of the study

Efforts to eradicate mosquitoes by the use of synthetic chemicals have been successful in many countries. Nevertheless, the increase of resistance rates, the concern for the environment and food safety, the unacceptability and restrictions in use of many organophosphates and organochlorines and the rising prices of new synthetic insecticides have hindered their use (Thekkevilayil *et al.,* 2003). This has stimulated the search for cheap, efficient, eco-friendly and selective larvicidal compound(s) from indigenous bio resources.

So far no major discovery has been made involving the new and novel larvicidal compounds from unexplored fungal genetic resources. In this regard, it is worthy to carry out this research, as it will trigger off further investigations to be done on basidiomycetes.

1.4 Hypothesis

From the screening of the crude extracts of the two species of basidiomycetes, they will show significant and reproducible activity against *Ae. aegypti.*

CHAPTER TWO

LITERATURE REVIEW

2.1 Some of the diseases transmitted by mosquitoes

Mosquitoes transmit a number of diseases to humans and animals. Some important ones are as follows:

2.1.1 Yellow fever

The virus that causes this disease is from the family of Flaviviridae. Yellow fever is characterized by high fever and jaundice (CDC, 2004). *Aedes. aegypti*, is the main vector for the arbovirus responsible for yellow fever (or urban yellow fever) in Central and South America and in West Africa (Ciccia *et al.,* 2000. This vector can give forth to offspring in household water making it a transmitter of yellow fever in areas of very low rainfall, or during dry seasons (Carvalho *et al.,* 2003). Yellow fever in its native environment is spread between humans and monkeys. Mosquitoes that feed on carrier monkeys that live in jungle canopies transmit the virus to humans (CDC, 2004).

According to World Health Organisation, 200,000 cases of yellow fever are reported worldwide leading to 30,000 deaths yearly. There is no specific curative treatment for yellow fever, although it has been reasonably brought under control with its vaccine (17D) (Carvalho *et al.,* 2003). A successful way of reducing the mosquito densities to a level where yellow fever epidemics do not occur is by attacking the larval breeding places (Gluber, 1989). Yellow fever is quite rare in Kenya. This makes *Ae. aegypti* a good organism for serotome laboratory screening for larvicides (Ciccia *et al.,* 2000; Carvalho *et al.,* 2003; Ndungu *et al.,* 2003).

2.1.2 Malaria

The principle vectors of malaria are mosquitoes of the species *Anopheles gambiae* complex and *Anopheles funestus.* The distribution of malaria and its vectors follows the rainfall pattern. Malaria has long been one of the greatest causes of serious illness and death in the world. It is reported that malaria kills one child every 40 seconds (AMCA, 2006a). Besides many children experience initial malaria infection during their first two years of life, when they have not yet developed sufficient

immunity, making these early years particularly dangerous (Global Health Reporting, 2005).

The reappearance of malaria is attributed to a number of factors (Carlson, 2004; Wilairatana *et al.,* 2003). This includes: financial constraints, resistance to available drugs and insecticide, poverty in most endemic regions, unavailability of an effective malaria vaccine, war damaged infrastructure, altered metrological conditions, and an incomplete understanding of the biology of the parasite and of the response to parasite infection.

Currently, a number of anti-malarial drugs are available, but their high costs, toxicity, and increasing parasite resistance hinder their use. It is essential therefore to optimise the use of currently available agents and to develop new approaches to malaria control (Wilairatana *et al.,* 2003).

Since development of vaccine is not obvious in the near future, chemotherapy and chemoprophylaxis remain the currently used methods for controlling this disease. However, with the increase in cases of drug resistance and failure, the continued management of malaria will likely require multiple new effective agents. This includes targeting the vectors (ICMR, 2003; CDC, 2006).

2.1.3 Dengue fever

This is a viral infection characterized by pain in muscles and joints, skin rash headache and fever. Dengue hemorrhagic fever (DHF) which is a complex form of this disease may cause severe and fatal bleeding. This disease is one of the most rapidly increasing insect-borne illnesses today (WHO, 2003). Both dengue fever and DHF are caused by various viruses and are transmitted by female mosquito from one person to another. *A. aegypti* and *A. alboliticus* are vectors of DHF, which is endemic to southeast Asia, the Pacific Island area, Africa and America (Carvalho *et al.,* 2003).

The incidence of dengue fever has increased dramatically over the last decade. It has become endemic in more than 100 countries and more than 2.5 billion people are at risk, mainly in Africa, America, western Mediterranean, South and East Asia and Western Pacific (Wandscheer *et al*., 2004). The spread of dengue throughout the world can be directly attributed to uncontrolled demographic increase, poor urban planning, reduced epidemiological surveillance and the proliferation and adaptation of these mosquitoes (Gluber and Kouri, 2002; Guzman and Kouri, 2002; AMCA, 2006a).

There is no vaccine available for dengue fever and the only way of reducing the incidence of these diseases is to combat *A. aegypti.* The use of aerial toxicants for the control of this mosquito is ineffective since they are highly domesticated as many adults rest indoors in hidden places such as closets (Gluber, 1989). Consequently the ideal control method is the systematic treatment of their breeding places by use of larvicides and insecticides.

2.2 Life cycle of *Aedes aegypti*

Ae. aegypti, belongs to the phylum Insecta; order Diptera, family Cullicidae, sub-family Culicinae and genus Aedes. The eggs are white when first deposited and becomes dark within an hour or two. They are laid singly near water. Hatching of the eggs occurs after several days to weeks and is temperature dependent. They hatch quickly when water level rises but can remain dormant for several weeks or months in winter. The eggs can withstand desiccation to a very marked degree, even up to a year's time (Boardman, 2005).

The larvae are quite robust and the breathing siphon is comparatively short, heavy and black. Position of the larvae in the water is almost vertical in comparison to other Culicine species (James, 1967). Larvae molt four times, the last at the time of pupation. All the instars are aquatic and require a minimum of four days to complete the development. Larvae generally feed on small aquatic organisms, algae and particles of plant and animal material with less feeding towards pupation time (Boardman, 2005). Pupa usually emerges from a T-shaped hole at the back of the last larval skin and they do not feed. They are aquatic and mobile using paddle like, oval extensions attached terminally on the abdomen to move up and down in water. Pupae have broadly triangular breathing trumpets. Normally a minimum of few hours is spent in the pupal stage.

Inside the pupal case, the pupa transforms into an adult. The adult uses air pressure to break the pupal case open, and is released from the pupa through a hole or split in the back (dorsum) of the pupal cuticle. The pupal case then floats on the surface of the water. The adult then crawls to a protected area and rests while its external skeleton hardens, spreading its wings out to dry. The wings are fully expanded and hardened after 24 hours and mosquito is then soon able to fly (Boardman, 2005). Once this is complete, it can fly away and live on the land.

2.3 Methods used in controlling mosquito

Methods used in controlling mosquitoes mainly involve the use of chemicals. Nonetheless, the use of chemicals is, at best, a temporary expedient that should be limited to only those situations for which no other alternatives exist. Chemical control can be divided in two major operations. The first, larviciding, is the most efficient and effective. The second, adulticiding, is less efficient and should be used for supplemental or emergency purposes (Williams *et al*., 2002). Most mosquito control programmes targets the larval stage in their breeding sites with larvicides, because adulticides may only reduce the adult population temporarily (El-Hag *et al*., 1999, 2001; Dharmaggada *et al*., 2004).

2.3.1 Control by insecticides

A number of insecticides have been registered for use in mosquito control. The relative value of chemical control varies with the mosquito species and the location conditions. Since each situation differs care must be taken to select the proper insecticide for a particular situation (Williams *et al*., 2002). Some of these factors include: relative toxicity to humans and animals, contamination of food, cost, availability in quantities needed, need for residual action in some situations, chemical stability, flammability, ease of preparation, corrosiveness, offensive odour, staining etc.

The extensive use of synthetic organic insecticides during the last five decades has resulted in environmental hazards and also development of physiological resistance in the major vector species (Hemingway and Ranson, 2000; Brooke *et al.*, 2002; ICMR, 2003). Moreover, the present occurrence of the diseases caused by mosquitoes is due to high number of breeding places resulting in increasing resistance of mosquito to contemporary insecticides (Carvalho *et al.*, 2003).

Insecticides that are sprayed have the advantage of being effective indoors, nevertheless outdoors; their particles disperse rapidly and may not kill many mosquitoes. The major disadvantage of space spraying is that it cannot manage insects for along period of time. On the other hand, larvicides have the advantage of reducing overall insecticides usage by application at localized points. This eliminates the need for ground or aerial pesticide to kill adult mosquito hence broad environmental effect (US EPA, 2002).

Organophosphates are also used as larvicides. Their toxicity is exhibited through inhibitory effects on cholinesterase enzymes in the nervous system. Organophosphates contain a central phosphorous atom with a double bond to sulphur or oxygen, R_1 and R_2 groups that are either ethyl or methyl in structure, and a leaving group, which is specific to individual organophosphate. The general structure of organophosphates is illustrated below **(7)** and a specific example is chlorpyrifos **(8)**.

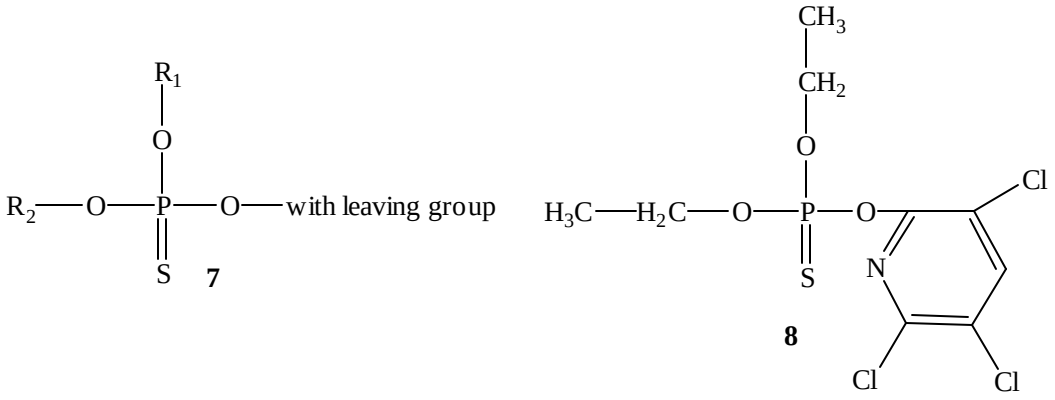

Figure 3 General structures of organophosphate and the structure chlorpyrifos

In the control of mosquito larvae, organophosphates such as temephos and fenthion are used. Additionally, insect growth regulators such as diflubenzuron and methoprene are also applied for larval control (Yang *et al.*, 2002). Methoprene mimics a natural juvenile hormone, and when present in the larval habitat it keeps immature insects from maturing into adults. Unable to metamorphose, the mosquitoes die in the pupal stage. Although effective, their repeated use has disrupted natural biological control systems and has led to outbreaks of new insect species, which has sometimes resulted in the widespread development of resistance, had undesirable effects on non-target organisms and fostered environment and human health concerns (AMCA, 2006b). For instance, temephos presents relatively low risk to birds and terrestrial species; conversely it is more toxic to aquatic invertebrates (US EPA, 2002). To make the matter worse, more than 50 species of *Anopheles* are now showing resistance to chemical pesticides (Moazami, 2005). These problems have highlighted the need for the development of new strategies for selective mosquito larval control.

As a result of prolonged exposure to an insecticide, mosquitoes, like other insects, may develop resistance strains, a capacity to survive contact with an insecticide. Since mosquitoes can have many generations per year, high levels of resistance can arise very quickly. Resistance of mosquitoes to some insecticides has

been documented just within a few years after the insecticides were introduced for example methoprene and temephos. There are over 125 mosquito species with documented resistance to one or more insecticides (CDC, 2006).

2.3.2 Biological control

Larvicides include biological insecticides, such as microbial larvicides *Bacillus thuringiensis israelensis* (*Bti*) and *Bacillus sphaericus (Bs). B. thuringiensis* is a naturally occurring soil bacterium while *Bs* is a naturally occurring bacterium that is found throughout the world. These bacteria are used for the control of mosquito larvae. Mosquito larvae eat the *B. thuringiensis* product that is made up of the dormant spore form of the bacterium and an associated pure toxin. Mosquito larvae also ingest *B. sphaericus* as a toxin. These disrupt the gut in the larvae by binding to receptor cells present in insects causing it to stop eating and die. Contrary this does not occur in mammals (US EPA, 2002).

A compound *Cry4Ba*, an Insecticidal Crystal Protein (ICP) was isolated from *B. thuringiensis.* It is specifically toxic to *Aedes* and *Anopheles* mosquitoes. The mechanism of toxicity of the ICP is not clearly understood (Boonserm *et al.*, 2005). Although *B. thuringiensis* is quite specific in its activity, some types of non-biting midges, which serve as food for fish and wild life, are also susceptible and may be affected. *B. thuringiensis* is only effective to active feeding larvae thus it cannot affect the fourth instar larvae, which does not feed. Furthermore *B. thuringiensis* is liable to degradation by sunlight (Cranshaw, 2006).

On the other hand, the biolarvicidal formulation from *B. sphaericus* has been reported to be less effective against *Anopheles culicifacies* and hardly effective against *A. aegypti* (Mittal, 2003). Besides, recent reports have pointed out that *Culex pipiens* has developed resistance to *B. thuringiensis* and *B. sphaericus* (Dharmaggada *et al.*, 2004).

2.3.3 Insecticide-Treated Bed Nets

Insecticide-treated bed nets (ITNs) are a form of personal protection that has repeatedly been shown to reduce severe disease and mortality due to malaria in endemic regions. In community-wide trials in several African settings, ITNs have been shown to reduce mortality by about 20% (CDC, 2006). Untreated bed nets forms a

physical protective barrier around persons using them. However, mosquitoes can feed on people through the nets or even before getting into the nets. Nets with even a few small holes provide little, if any, protection. The application of a residual insecticide enhances the protective efficacy of bed nets (UN, 2007).

Currently, only pyrethroid insecticides are approved for use on ITNs. These insecticides have very low mammalian toxicity but are highly toxic to insects and have a rapid knockdown effect, even at very low doses (UN, 2007). Pyrethroids have a high residual effect; they do not rapidly break down unless washed or exposed to sunlight. To maintain the efficacy of ITNs, the nets must be retreated at intervals of 6-12 months, more frequently if the nets are washed (CDC, 2006). The need for frequent re-treatments is one of the most difficult barriers to full implementation of ITNs in endemic countries. The additional cost of the insecticide and the lack of understanding of its importance result in very low re-treatment rates in most African countries (DFID, 2005).

2.3.4 Mosquito traps

Mosquito trapping devices are designed to mimic a mammal by emitting a plume of carbon dioxide, heat and moisture which often combined with an additional attractant, i.e. octenol, to create an attractant to the mosquitoes. After drawing the insect to the trap, a vacuum device sucks it to the net or cylinder where they dehydrate or die. Scientific data relative to the effectiveness of the traps is sparse. Additionally some of the mosquito traps are quite expensive (AMCA, 2006b).

2.3.5 Repellents

Until the advent of synthetic compounds, essential oils and their mixtures formed the basis of most commercial repellent formulations (Curtis, 1990). However, due to their high volatilities, they have been abandoned in favour of synthetic repellents, principally DEET (N,N–diethyl-meta-toluamide) **(9)**, which provide long protection against blood feeding insects (Fradin, 1998; Goodyer and Brethrens, 1998). For more than 40 years DEET has been the standard in mosquito repellents (AMCA, 2006c). Nevertheless, rapid skin penetration and bio-distribution of DEET in both human and animals have raised concern on its toxic side effects (Miller, 1982; Roland *et al.*, 1985; Cheng *et al.*, 2004). Persons who have used products containing high concentrations such as 50% or 75% of DEET or exposed to excessive amount of it have

experienced rashes, blisters and skin mucous membrane irritation. In few cases of overdose and misuse, brain effects (encephalopathy) and seizures occur in children (Cheng *et al*., 2004).

2.4 Mosquito larvicides, repellents and insecticides from plants

The use of preparations from plants and their various products in the control of mosquito has been well established (Karam and Bansal, 2003). Numerous plants have been shown to produce pesticidal compounds (Ciccia *et al.*, 2000), as a chemical defence mechanism against predators or infection. Although many plants have been shown to produce insecticidal effects most of the report are based on laboratory observation only. Thus, majority may not be of practical significance (ICMR, 2003).

Azadirachta indica (Meliaceae) was investigated for anti malarial compounds (Nkunya *et al.*, 1995) and was found to have mild activity. Azadarachtin (AZA) **(10)** is an insecticidal compound that was isolated from this plant (Mulla and Su, 1999). It is the most efficient plant substance with moult inhibiting activity. It significantly inhibits the ecdysteroid biosynthesis *in vitro* in ovaries and in abdominal integument of female adult cricket (Muthukrishnan, 1999).

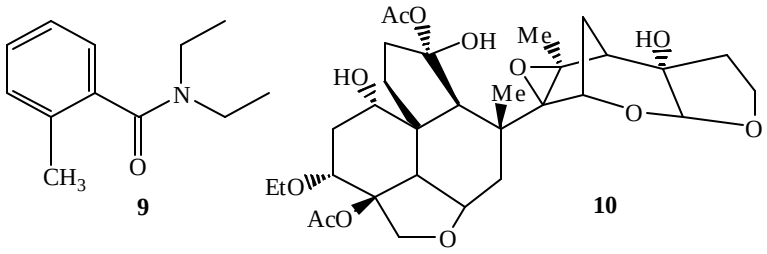

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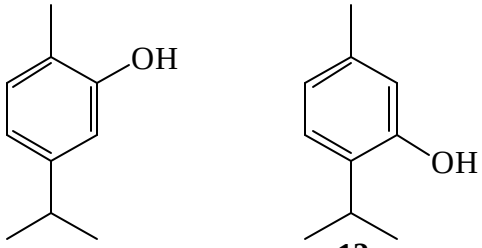
Figure 4 Structures of DEET and Azadarachtin

Though Neem products show a high larvicidal activity, which is attributed to the epoxide ring function, they do not show adulticidal action (ICMR, 2003). It has been suggested that azadarachtin acts as an anti-ecdysteroid and thus kills the larvae by growth inhibition effect (Zebitz, 1984).The plant *Quillaja saponaria* (native to China, Peru and the arid zones of Chile) has been found to have a bark rich in saponins.

These saponins are steroids or triterpene glycosides possessing a wide variety of activities, for example, cancer related anti-inflammatory, antiallergic, antiviral and molluscidal activities (Laccaille- Dubois and Wagner, 1996). It was found that young larva of both *Ae. aegypti* and *Cx. pipiens* were susceptible to saponin application (Pelah

et al., 2003) although the major components responsible for larvicidal activity were not isolated.

A study was carried on essential oil obtained from the shoot of *Lippia sidoides* in which hydrolate (saturated solution of essential oil in water) was produced. Pure hydrolate caused larval mortality of *Ae. aegypti* and it was revealed that its main constituents were carvacrol **(11)** and thymol **(12)**. It was also found that thymol was the active essential oil hydrolate (Carvalho *et al.,* 2003). Hence, the use of plant essential oils in insect control is therefore an alternative pest control for minimizing the noxious effect of some pesticide compounds on the environment (Fatope *et al.,* 1993).



11 **12**

Figure 5 Structures of Carvacrol and Thymol

Larvicidal activity of a stem wood hexane extract of *Cybistax antisyphilitica* has been evaluated against *Ae*. *Aegypti* larvae. A natural quinone identified as 2-hydroxy-3-(3-methyl–2-butenyl)-1,4-napthaquinone **(13)** was isolated. This compound was potent against *Ae. aegypti* larvae. Several activities have been reported for this compound including anticancer, antimicrobial, leishmanicidal, antimalarial, molluscidal and schistosomicidal (Rodrigues *et al*., 2005).

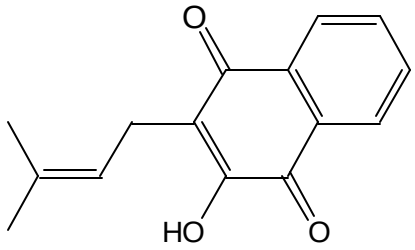


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Figure 6 Structure of 2-hydroxy-3-(3-metyhl – 2- butenyl)-1, 4-napthoquinone

Extract from the tubers of *Neorautanenia mitis* were tested against *An. gambiae* and *Cx. quinquefaciatus*. The extracts exhibited activity against the larvae of these mosquitoes. The active extract yielded coumarin derivative pachyrrhizine **(14)**, the isoflavanoids, neotenone **(15)** and neorautenone **(16)** and the pterocarpans, neoduline

(17), nepseudin **(18)** and 4-methoxyneoduline **(19)** as active constituents. These appeared as attractive candidates for further investigation of their mosquitocidal potency (Joseph *et al*, 2003).

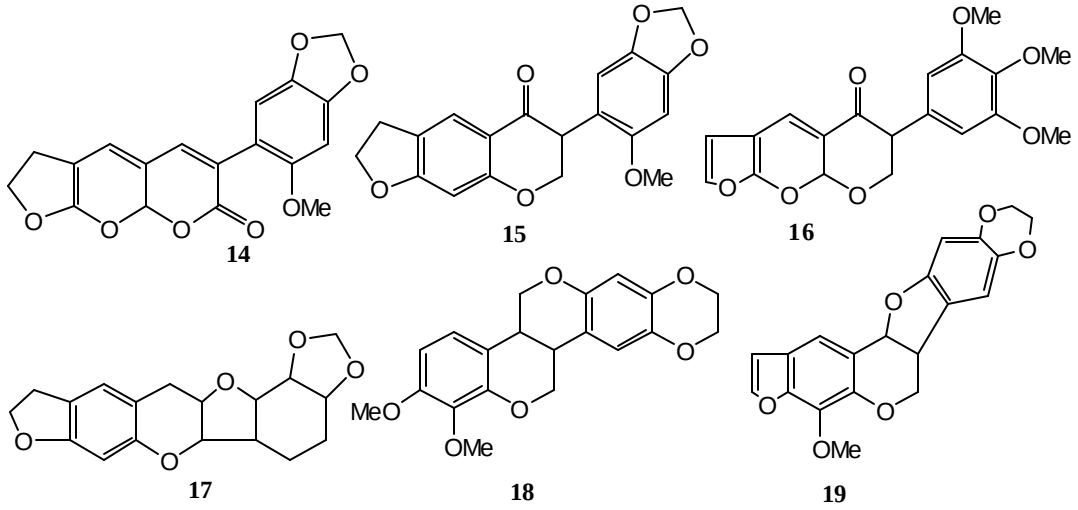


Figure 7 Structures of Pachyrrhizine, Neotenone, Neorautenone, Neoduline, Nepseudin and 4-Methoxyneoduline

The crude methanol extract of the seeds of *Derris trifoliata* showed potent and dose dependent larvicidal activity against the second instar larvae of *Ae. aegypti.* From this extract two unusual rotenoid derivatives, a rotenoloid named 7α-*O*-methyl-12α-hydroxydeguelol **(20)** and a spirohomooxarotenoid named (spiro-13-homo-13-oxaelliptone) **(21)**, were isolated and characterized. Though, they were not screened for larvicidal activity (Yenesew *et al*., 2006).

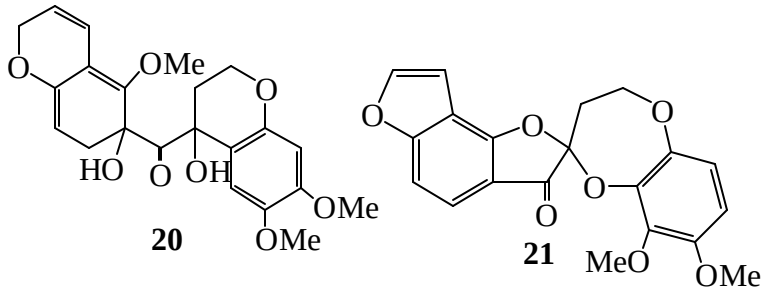


Figure 8 Structures of 7α-O-methyl-12α-hydroxydeguelol and spiro-13-homo-13-oxaelliptone

In addition, a rare natural chromanone (6, 7-dimethoxy-4-chromanone **(22)** and compounds; 7α-O-methyldeguelol **(23)**, 12α-hydroxyelliptone **(24)**, 13-homo-13-oxa-6α,12α- dehydrorotenone **(25)** and 7α-*O*-methydeguelol **(26)** were also isolated from

seeds of *D. trifoliata.* The larvicidal activity of the crude extract is mainly due to rotenone (Yenesew *et al*., 2006).

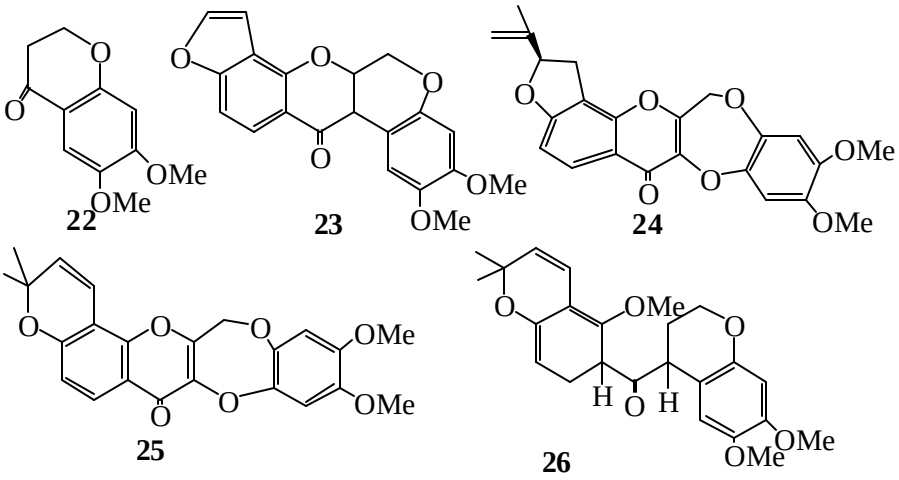


Figure 9 Structures of 6, 7-dimethoxy-4-chromanone, 7a-O-methyldeguelol, 12a-hydroxyelliptone, 13-homo-13-oxa-6a, 12a-dehydrorotenone and 7a-O-methyldeguelol

Some limonoids were isolated from the root back of *Turraea wakefieldii* and were characterized as tecleaninoid derivatives. However, there was a five membered ring, which was discovered and proposed the name neotecleanin. It was suggested that this compound (neotecleanin) could serve as an intermediate in the pathway for synthesis of tecleanin and related compounds.

Among these compounds 1(11β,12α-diacetoxyneoetecleanin) **(27)**, 2(11β,12α–diacetoxy-4β, 15β-epoxyneotecleanin), **(28)** and 3(7α,12α,-diacetoxy-11β -hydroxyneoteclanin) **(29)** showed strong larvicidal activity against *An. gambiae* (Ndungu *et al.*, 2003).

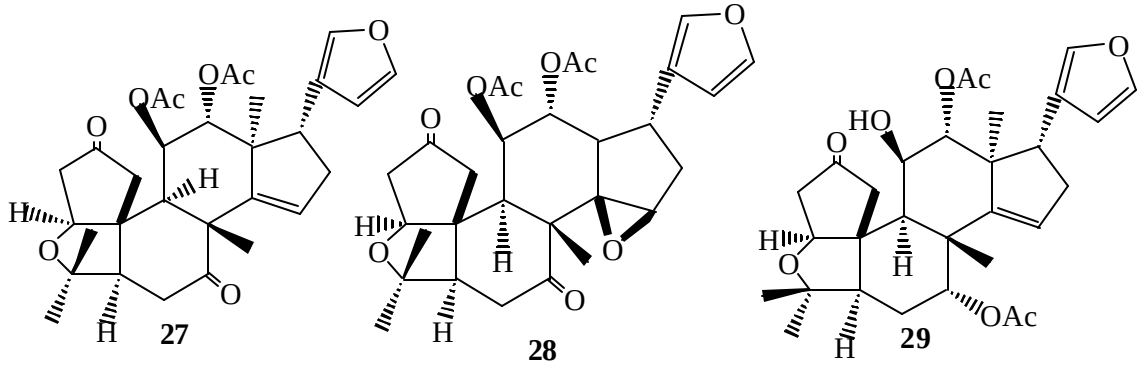


Figure 10 Structures of 1(11b,12a-diacetoxyneoetecleanin), 2(11b,12a–diacetoxy-14b,15b-epoxyneotecleanin) and 3(7a,12a,-diacetoxy-11b-hydroxyneoteclanin

It is an acknowledged fact that tropical plants are of great promise from the point of view of discovering and developing new botanical insecticides (Berenbaum, 1989). Members of the family Meliaceae, Rutaceae, Asteraceae, Labiatae and Caneliaceae seem to possess the most promising botanicals for use at the present (Jacobson, 1989; Muthukrishnan, 1999). For successful application of plant compounds in insect bio-control, it is obligatory to understand the mechanism of their action in the targeted insects as well as the spectrum of the insects affected by them.

The choice of extraction method can affect the efficacy of plant constituent. Different solvents ranging from polar to non-polar have been used to extract compounds that have larvicidal activity. A converse relationship is said to exist between extract effectiveness and solvent polarity, where efficiency increases with decrease in polarity (Mulla and Su, 1999). Some of the solvents that have been used are summarised in the Table 1 below.

Table 1: Some of the solvents used for extraction and their efficacy as larvicides

Plant species	**Plant part**	**Solvent**	**Mosquito**	**mg/ml (LC)***	**Source**
Abuta grandifolia	Fruit	Dichloromethane	*Ae. aegypti*	0.0026	Ciccia *et al.*, 2000
Alnus glutinosa	Old litter	Tannic acid	*Aedes* and *Cx. coquilletidia*	13-30	Rey *et al.*, 2001b
Alnus glutinosa	Old litter	Polyphenols	*Ae. Aegypti* *Ae. albopictus*	0.2-0.4 0.2-0.4	Rey *et al.*, 2001a
Byronopsi slaciniosa	whole	Goniothalamin	*Cx. quiquefasciatus*	0.215	Kabir *et al.*, 2003
Cassia obtusifolia	seed	Methanol	*Ae. Aegypti* *Cx. pipiens pallens*	0.004 (0.051) 0.04 (0.069)	Jang *et al.*, 2002
Cassia tora	seed	Methanol	*Ae. Aegypti* *Cx. pipiens pallens*	0.02 (0.059) 0.02 (0.078)	Jang *et al.*, 2002

* Unless stated, all LC_{50} values for test substance involve a 24h-exposure period.

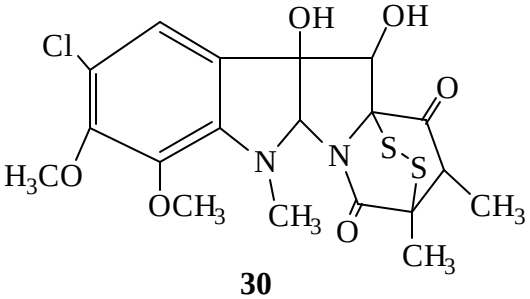
2.5 Some secondary metabolites from fungi

Secondary metabolites are compounds produced by the fungus and are not essential to the basic metabolism of the fungus. The compounds are generally produced following active growth, and many have an unusual chemical structure. Some metabolites are found in a range of related fungi, while others are only found in one or

a few species. The restricted distribution implies lack of general function of secondary metabolites in fungi (Southcott, 1996).

Among the compounds produced by fungi, there are varieties, which cause toxicosis in mammals, especially humans, insects and other herbivores. Fungi absorb nutrients from the environment, which are used for their growth and development. When the energy resource becomes depleted, the production of secondary metabolites increases, including compounds that are toxic.

Medically, the most important toxins formed by fungi include alkaloids, cyclopeptides, coumarins, muscarine, monomethylhydrazine, and the amatoxins (Southcott, 1996). These compounds are active at extremely low concentrations and have a rapid effect. They appear to act directly on the central nervous system, and other primary organs in mammals (Southcott, 1996). Examples of toxins are sporodesmin **(30)** and vomitoxin **(31)**.



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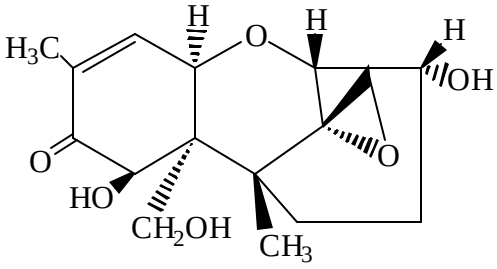

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Figure 11 Structures of sporodesmin and vomitoxin

A study showed that *Tolypocladium* species produces efrapeptins, a group of toxic peptides, in *vivo* but the quantities were too small to account for the insect death. This suggested that these insecticidal compounds work in concert with other pathogenicity determinants. There is inter- and intra-specific variation in efrapeptin production in *vitro* by *Tolypocladium* species. *T. parasiticum* produced only efrapeptin E, in small quantities. Efrapeptins were detectable 48 hours after inoculation and increased with biomass and mortality was dose-related. Efrapeptins also exhibited limited antifungal and antibacterial activity. *Micrococcus luteus* was considered an excellent indicator of efrapeptin presence in culture filtrate extracts because of its extreme sensitivity to these compounds (Bandani *et al*., 2000).

In a screening for nematicidal activities in cultures of basidiomycetes, cultures of *Pleurotus pulmonarius* and *Hericium coralloides* exhibited toxic effects towards the

saprophytic nematode *Caenorhabditis elegans*. Linoleic acid **(32)**, *p*-anisaldehyde **(33)**, *p*-anisyl alcohol **(34)**, 1-(4-methoxyphenyl)-1,2-propanediol **(35)**, and 2-hydroxy-(4'-methoxy)-propiophenone **(36)** were isolated from submerged cultures of *P. pulmonarius*. All these compounds showed nematicidal activities towards *C. elegans*. The most active compound was linoleic acid from cultures of *H. coralloides*, which exhibited both repellant and nematicidal effects; a nematicidal fatty acid mixture was obtained, containing linoleic acid, oleic acid, and palmitic acid as its main components (Stadler *et al.*, 1994).

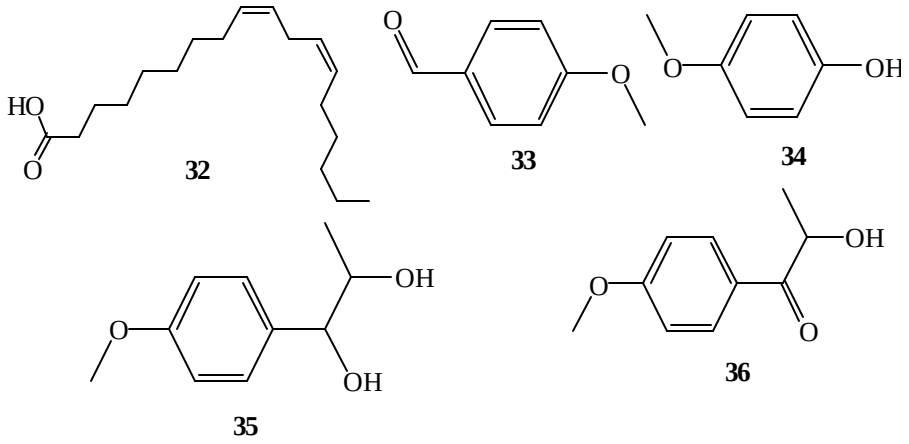


Figure 12 Structures of linoleic acid, *P*-anisaldehyde, *P*-anisyl alcohol, 1-(4-methoxyphenyl)-1,2-propanediol, and 2-hydroxy-(4'-methoxy)-propiophenone.

2.6 Insecticidal fungi

The available literature on entomopathogenic fungi for mosquito control is rather scattered and void of reviews (Scholte *et al.*, 2000). A number of entomopathogenic fungal strains including *Langedium giganteum*, *Metarhizium anisopliae*, *Tolypocladium cylinrosporum* and C*ulicinomyces clavisporus* have been investigated as possible biological control agents against various mosquito species. Preliminary observations suggested that fungus acts as toxin producers rather than invasive pathogens. This is because ungerminated spores were found in the guts of mosquitoes (Deepak and Bharat, 1999).

Laboratory bioassays evaluated the larvicidal activity of the entomopathogenic fungus *Tolypocladium cylindrosporum* against *Ae. triseriatus*. All larval instars were found to be susceptible at temperatures from 18 °C to 25°C. Mortality was proportional to exposure time in assays. *T. cylindrosporum* was also active against *Simulium*

vittatum black fly larvae, but the mortality recorded was much lower than with mosquito larvae (Nadeau and Boisvert, 1994).

A study was carried on a novel entomopathogenic fungus, *Leucothecium emdenii*, which proved effective against several mosquito species under laboratory conditions. The species of mosquito used were second instar larvae of *Ae. aegypti, An. culicifacies, An. subpictus* and *Cx. quinquefasciatus*. These were all found to be susceptible to *L. emdenii.* The compounds accountable for this activity were not elucidated (Deepak and Bharat, 1999). Several entomopathogenic fungi have been screened for larvicidal activity against various species of mosquito. However, the structures of the compounds responsible for this activity have not been elucidated. These extracts have not been used practically. Some of these fungi are listed in the Table 2.

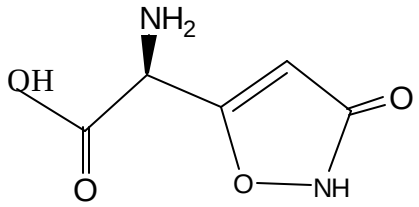
Table 2: Some of the fungal species tested for larvicidal activity.

Fungal species	**Mosquito species**	**Place of test**	**Reference**
Coelomomyces numulartus	*An. squamosus*	Laboratory	Ribeiro and Da Cunha Ramos (2000)
Tricophyton ajelloi	*An. stephensi* *Cx. quinquefasciatus*	Laboratory	Mohanty and Prakash (2000)
Coelomomyces stegomytae var. stegomytae	*A. aegypti*	Laboratory and field	Lucarotti and Shoulkamy (2000)
Paecilomyces lilacinus	*A. aegypti*	Field	Agarwalda *et al*., (1999)
Pythium carolinianum	*A. albopiticus*	Field	Sur *et al*., (2001)
Fusarium semitectum	*A. cantans* *An. stephensi*	Field	Sur *et al*., (1999)

Biologically active natural products have been isolated from Aphyllophorales, many of which are known as polypores. Polypores are a large group of terrestrial fungi of the phylum Basdiomycota (basidiomycete). There are about 25 000 species of

basidiomycetes, of which about 500 are members of the Aphyllophorales, a polyphyletic group that contains the polypores. Many of these fungi are found in North America, Europe, and Asia and broad distributions on all inhabited continents and Africa. The compounds from these fungi also display antiviral, cytotoxic, and/or antineoplastic activities. However, tropical habitats remain largely unexplored.

Insecticidal secondary metabolites have been reported from cultures of basidiomycetes. Insecticidal activity against houseflies was described for ibotenic acid **(37)** isolated from fruiting bodies of *Amanita muscaria*, *A. strobiliformis* and *A. .pantherina*.



37

Figure 13 Structure of ibotenic acid

A cyclopeptide, beauvericin was also isolated from basidiomycetes *Polyporus sulphureous* and from entomopathogenic fungi *Beauveria bassiana* and *Paecilomyces fumosoroseus.* Insecticidal activity was exhibited against mosquito larvae, brine shrimps, houseflies and cockroaches' cardiac cells *in vitro* (Roberts, 1981). It is evident that basidiomycetes provide a rich yet quite untapped source of bioactive compounds.

CHAPTER THREE

METHODOLOGY

3.1 Instruments

pH meter (Consort C830), Magnetic stirrer, Autoclave (Rustri-DK 2820 Gentofte-Denmark), Weighing balance (EK-1200 GD max 1200g d=0.1g), Sterile laminar flow hood (sterilGARD The Baker company Stanford-class II Type A/B3 US PAT NO. 3,8950), water bath, Suction pump, Refrigerator, Analytical balance (PRECISA 310 SWISS QUALITY), Vacuum rotary evaporator (TYPE 349/2), Bruker instrument (500 MHz).

3.2 Media and other materials

Maize flour, potato dextrose agar (PDA), filter paper disks (Rundfilter MN61 12, 50), disposable hand gloves, aluminium foil, cotton wool, Parafilm, marker pens, pencils, ruler and third instar larvae of *Ae. aegypti.*

3.3 The growth of the selected basidiomycetes

From the preliminary screening for larvicidal activities of the crude extracts prepared from six submerged cultures of strains of basidiomycetes, the two (JO4012 and JO4014) were found to have significant mosquito larvicidal activities. The basidiomycetes were collected within the precincts of Egerton University and immediately brought into culture in the Integrated Biotechnology Research Laboratory (IBRL). Pieces of the inner lamella tissues were eccentrically trapped in Petri dish lid and allowed to rest, to allow the spores to be trapped. The trapped spores were incubated on PDA until the mycelium formed. The two species of basidiomycetes were brought into pure cultures, which were preserved, on agar slants and the corresponding herbarium materials kept in Integrated Biotechnology Research Laboratory (IBRL).

3.3.1 Preparation of PDA media

19.5 g of PDA was weighed and suspended in 500 ml distilled water in a 1 L Erlenmeyer flask. The content was swirled to mix and sterilized in autoclave twice for 15 minutes at 121°C. The PDA was left to cool in water bath to 45°C and dispensed

into sterile Petri dishes. The Petri dishes were then left to set for 30 minutes, followed by storage in the refrigerator at 4°C (Finkelstein and Ball).

3.3.2 Sub culturing of the selected basidiomycetes

Laminar flow hood was left to run while the Bunsen burner inside it lit. Petri dishes (from section 3.5.1) together with the agar slants of the selected species of basidiomycetes were placed inside the laminar flow hood. These slants were cut using a blade sterilized in by the flame from the Bunsen burner and placed on to the Petri dishes that had the PDA. The PDA plates were sealed using parafilm and incubated at normal room temperature while monitoring for any contamination. Mycelial colonies were noted and immediately sub cultured onto new plates (plates with PDA) (Finkelstein and Ball).

3.4 Preparation of culture media

The selected basidiomycetes strains produced larvicidal compounds (in the crude extracts tested) when inoculated in maize meal. This was prepared by mixing 10g of maize flour, 20 g of glucose, 0.5 g of potassium chloride (KCl), 0.5 g of sodium nitrate ($NaNO_3$), 1.5 g of potassium dihydrogen phosphate (KH_2PO_4), 0.5 g of magnesium sulphate ($MgSO_4$) and dissolved in distilled water. The solution was then made to 1 litre. This was divided into 250 ml Erlenmeyer flasks and their pH kept at 5.5. The flasks were then corked with cotton wool and the top portion wrapped with aluminium foil paper. These media were sterilized twice by autoclaving at 121°C for 15 minutes. They were left on the bench for about 2-3 hours to cool (Finkelstein and Ball).

3.5 Inoculation of the liquid media

Four agar plugs (1 cm x 1 cm) with mycelium of each basidiomycete (from section 3.5.2) were introduced aseptically into cooled maize meal media. This process was repeated for each of the basidiomycetes. The inoculated media was left to grow as still cultures under normal conditions of 12 hours of light-darkness cycle and occasionally agitated.

From the growing cultures, probe samples were withdrawn under sterile conditions to monitor growth parameters: mycelium accumulation, glucose levels and larvicidal activity. Growth was stopped when there was evidence of no more

accumulation of mycelium and depletion of glucose levels. The maximum larvicidal activity was also used to monitor and stop growth. In case peak larvicidal activity occurred before glucose levels were exhausted, the activity was prioritised (Finkelstein and Ball).

3.6 Preparation of crude extracts

Immediately growth was stopped, mycelium was separated from culture filtrate by filtration. Each of the mycelium was soaked in acetone and stirred continuously using a magnetic stirrer for 4 hours. Mycelium was filtered and the organic filtrate was concentrated under reduced pressure using rotary evaporator at 56°C. The remaining aqueous solution was further extracted thrice using equal volume of ethyl acetate (EtOAc) by the use of separating funnel. The combined ethyl acetate extract was dried with anhydrous sodium sulphate (Na_2SO_4). This was filtered and concentrated to dryness at a temperature of 76°C using rotary evaporator. The dried extract was dissolved in a minimum amount of methanol (MeOH) and stored in screw capped glass vial. The extract was dried under reduced pressure in nitrogen atmosphere to a constant weight. The weight of the extract was determined, recorded and kept at 4°C for further analysis.

The culture filtrate of each culture was passed four times through the Mitsubishi HP 21 resin. The eluted solvent was discarded. To elute the trapped compounds, acetone was passed through the resin followed by methanol. The organic eluents were concentrated separately using a rotary evaporator under reduced pressure at 56°C and 76°C respectively. The remaining aqueous solutions were combined and extracted thrice using equal volume of ethyl acetate (EtOAc). Anhydrous sodium sulphate (Na_2SO_4) was used to dry this extract (EtOAc). This was filtered using filter paper and the filtrate was concentrated to dryness. The remaining organic extract was dissolved in a minimal amount of methanol (MeOH) then transfered and stored in a screwed capped glass vial. The extract was dried under reduced pressure in nitrogen atmosphere to a constant weight. The weight of the extract was determined, recorded and stored at 4°C awaiting further analysis.

3.7 Screening for larvicidal activity

Third and early fourth instar larvae of *Ae. aegypti* were used. They were collected from a colony that was reared in the laboratory at the right conditions from

Pyrethrum Board of Kenya. These were 28±2°C photo periods of 12 hour of light and dark along with a relative humidity of 70±10%. Their food was made from a mixture of blood, liver, sugar and yeast.

Universal bottles were used for the screening in which 10 and 20 larvae were placed in 4 ml of distilled water using a modified pressure controlled Pasteur pipette. Various volumes (50 µll, 100 µl, 150 µl and 200 µl) of the test solutions were added. Each volume was done in triplicate. In each experiment a set of controls i.e. methanol and untreated sets of larvae in distilled water were also set up for comparison. The experiments were carried out at 28±2°C.

Mortality was recorded after 2, 4, 8 and 24 hrs of exposure during which no food was given to the larvae (WHO, 2005). Larvicidal activity was evaluated using standard lethality indicators called LC_{50} (the concentration of the test solution that kills 50% of the larvae) and LC_{90} (the concentration of the test solution that kills 90% of the larvae). Data was evaluated by regression analysis using a computer software mini-programme designed in Microsoft excel 2003. From the regression line, the LC_{50} and LC_{90} values were recorded.

3.8 Scale-up of metabolite production

The two strains of basidiomycetes were cultured in larger amounts. This was done by cultivating them in larger quantity of liquid media (1L). Each strain was inoculated in two 1L flask. Growth was stopped as described in section 3.7. Crude extract for each was prepared and larvicidal activity was conducted as described in sections 3.8 and 3.9 respectively.

3.9 Purification of the active compounds

3.9.1 Thin layer chromatography

This technique was used to determine the purity of the extracted compounds and the best working range of the solvent system. The solvent systems used were methanol, cyclohexane and ethyl acetate in various ratios. The solvent system were freshly prepared and poured into chromatography tank. Filter paper was placed inside the tank before being covered with aluminium foil. This was done to enhance saturation of the tank. The solvent front position was marked 1 cm from the top and the point of application of the spots was also marked 1cm from the bottom. Drawing lines using pencil and ruler across the length of the plate did this. The extract was dissolved in

methanol and the solution spotted on the precoated silica TLC plates using a capillary tube.

The plates were then placed inside the chromatography tank that contained the solvent systems. They were left to run until solvent front was reached. The plates were removed and left to dry before being sprayed using anisaldehyde solution. They were warmed at 110°C for 15 minutes to visualize the separation. The solvent systems that showed good separation on the TLC plates were noted and used in elution in the column chromatography.

3.9.2 Column chromatography

The crude extract was dissolved in minimal amount of methanol and mixed with silica gel before being left to dry. Approximately 50 g of Silica gel was measured and mixed with 100 ml cyclohexane. The mixture was swirled and the trapped air was removed using the suction pump before being slurry packed in a glass column. The height and the diameter of the column were also recorded. The crude extract was then loaded into the silica gel carefully ensuring that air was not trapped. This formed a cylindrical disc above the packed column. The column was then anchored using acid washed sand.

The column was eluted using discrete solvent gradient mobile phase with increasing polarity from cyclohexane to ethyl acetate to methanol. The mobile phase consisted of solvent gradient as 100% cyclohexane, 7:3, 1:1, and 3:7 ratios of cyclohexane- EtOAc and then 100% EtOAc. This was followed by 7:3, 1:1, 3:7 ratios of EtOAc-MeOH and then finally 100% MeOH. For each solvent ratio, a total volume of 200 ml of the mobile phase was prepared and used to elute the column successively starting with 100% cyclohexane. About 200 ml of each column fraction was collected.

Each fraction was concentrated at 70°C using rotary evaporator under reduced pressure. The dried fractions were dissolved in a minimum amount of methanol and stored in screwed capped glass vials.Each fraction was then screened for larvicidal activity and the % mortality was recorded.

Fractions with potent activity were further purified using sephadex LH20 **(Figure 14)**. Sephadex LH20 was slurry-packed in a glass column. Methanol was used as the solvent system. The eluents were collected in labelled test tubes and spotted on a TLC plate to confirm their purity. These were pooled together depending on the appearance of the spots on the TLC. These were then kept in labelled glass vials and

dried under reduced pressure in nitrogen atmosphere to a constant weight. Their weight was determined and recorded. These were then kept in the refrigerator at 4°C, for further experiments**.**

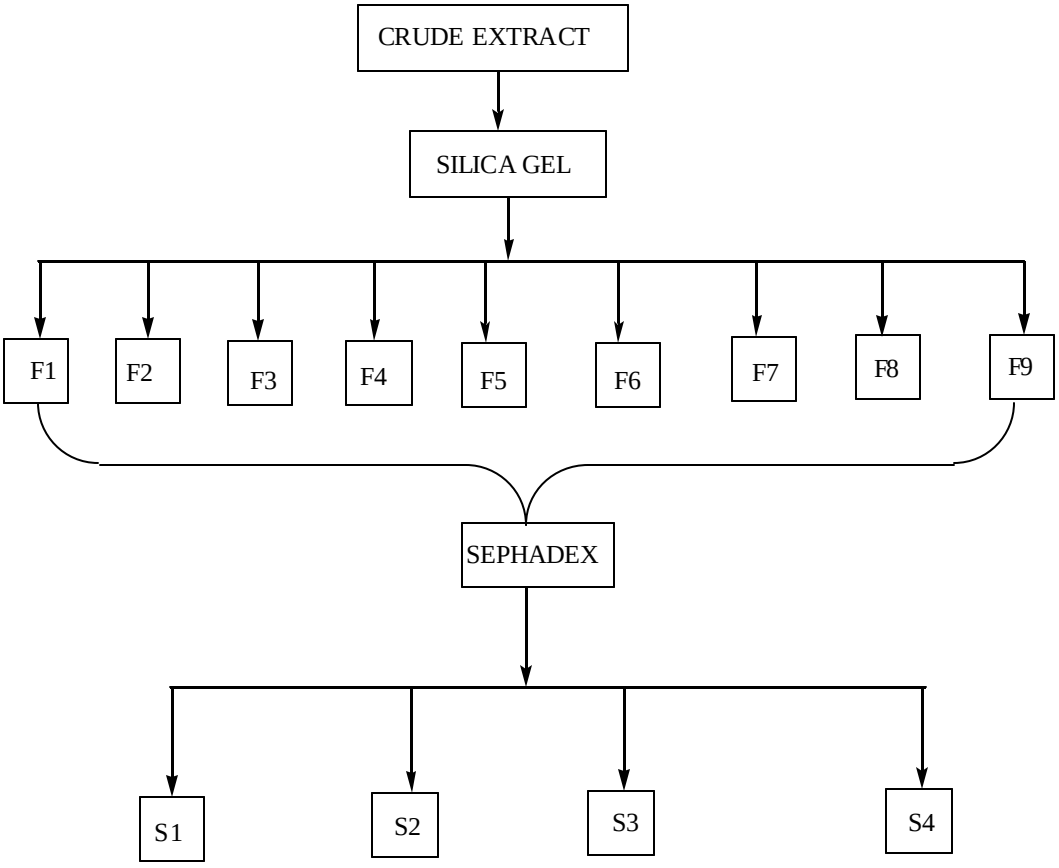


Figure 14 Scheme of separation

3.10 NMR spectral analysis

NMR spectroscopy was carried out on a 500 MHz Bruker instrument. The pure fractions which had reasonable quantity and showed potent larvicidal activity were ran to obtain the spectral data for structure elucidations.^{1}H NMR (500 MHz) and ^{13}C NMR (125 MHz, fully decoupled) were recorded. Samples were prepared in 5 ml of CD_3OD solution onto a 5 mm NMR tubes. The chemical shifts were reported in ppm relative to the d-solvent peaks, 4.87 for CD_3OD. The acquired FID was processed using TOPSIN software and the spectra attached onto a PNG file.

CHAPTER FOUR

RESULTS AND DISCUSSION

4.1 RESULTS

4.1.1 Results of LC_{50} and LC_{90} of the crude extracts

Initial screening for larvicidal activities of both the mycelium and culture filtrate for crude extracts of JO4012 and JO4014 was done. The results obtained for the LC50 (lethal concentration that kills 50% of larvae) and LC90 (lethal concentration that kills 50% of larvae) are shown in Table 3 below.

Table 3: Table showing the LC_{50} and LC_{90} of the crude extracts

	LC_{50} (mg/ml)				**LC_{90} (mg/ml)**			
Time Crude extract	2 hrs	4 hrs	8 hrs	24 hrs	2 hrs	4 hrs	8 hrs	24 hrs
JO4012 Kex	315.0	77.1	32.6	22.5	555.0	128.6	50.6	33.4
JO4012 Mex	12.0	8.9	3.6	2.7	20.0	14.3	5.2	4.7
JO4014 Kex	28.4	22.7	14.1	12.7	42.6	33.4	24.0	23.4
JO4014 Mex	105.0	11.9	1.2	1.1	185.0	16.9	11.4	11.4

Key:

Kex- culture filtrate extract

Mex – mycelium extract

4.1.2 Graphical representations for the LC_{50} and LC_{90} of the crude extracts

The following are graphical representations of LC_{50} and LC_{90} of each crude extract from the basidiomycetes as represented in Table 3.

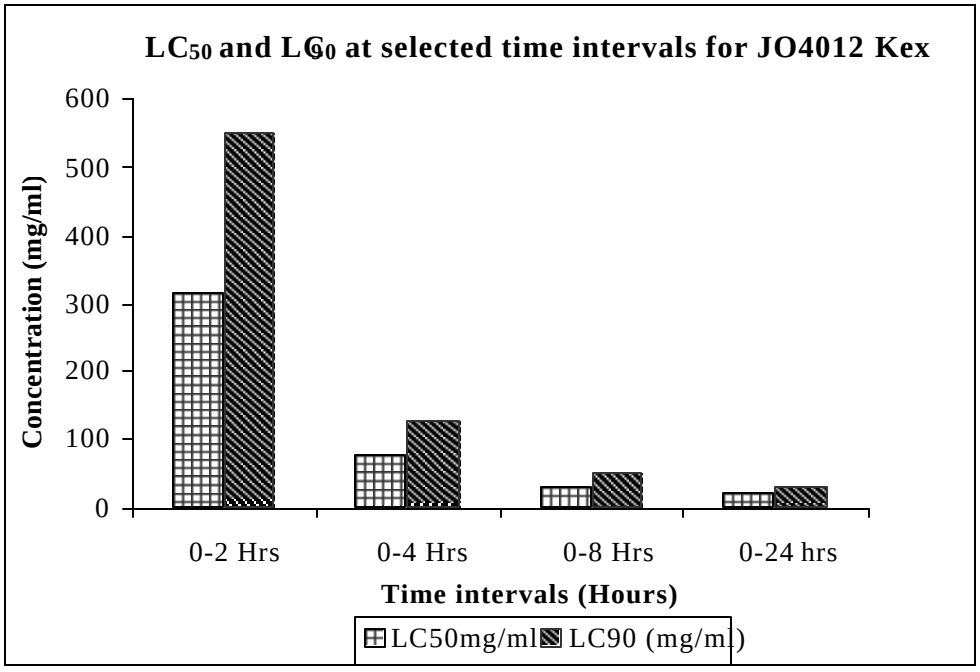


Figure 15 LC_{50} and LC_{90} for JO4012 Kex

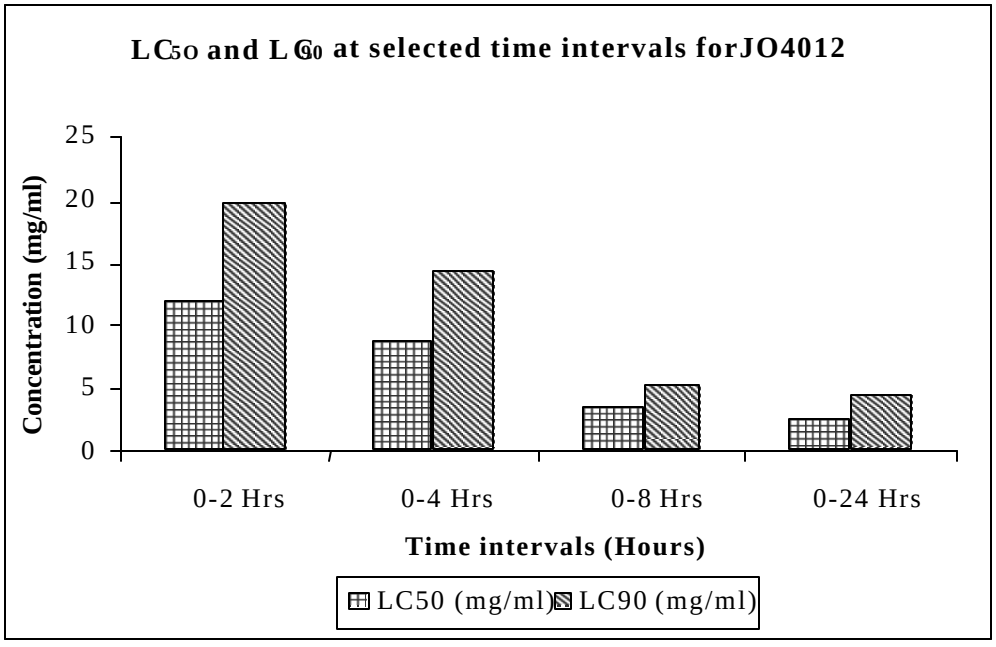


Figure 16 LC_{50} and LC_{90} for JO4012 Mex

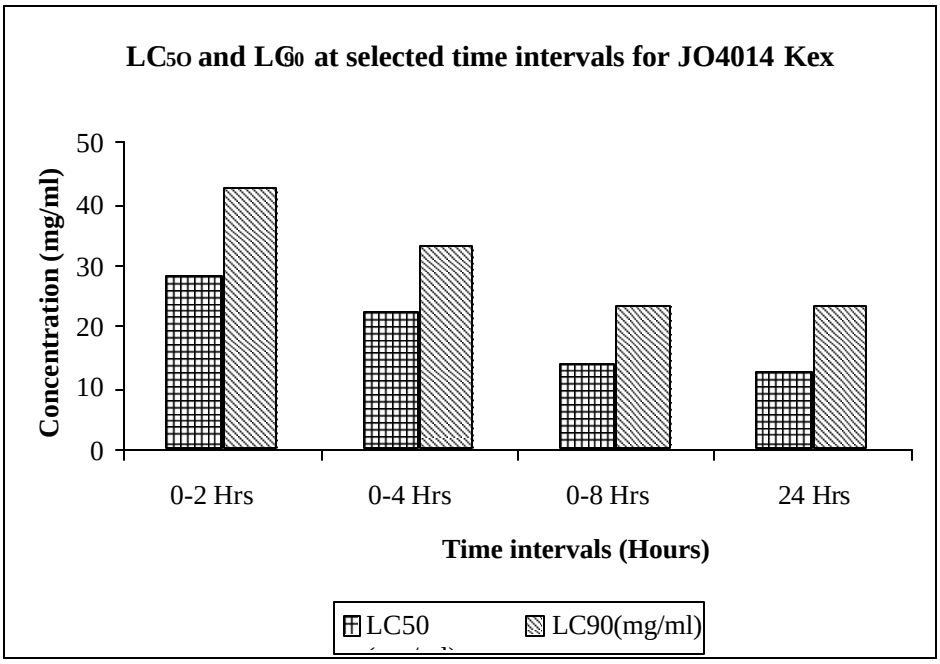


Figure 17 LC_{50} and LC_{90} for JO4014 Kex

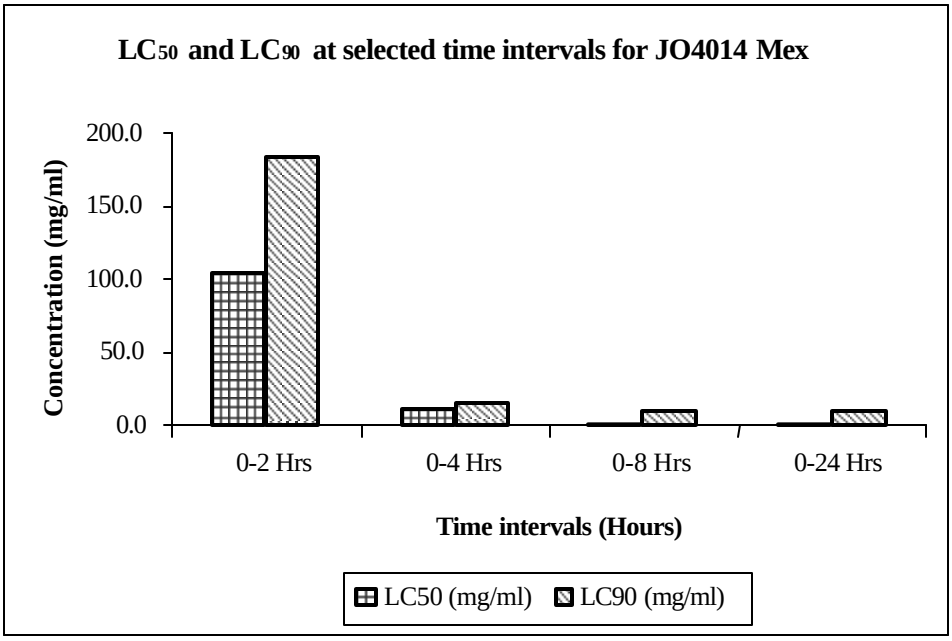


Figure 18 LC_{50} and LC_{90} for JO4014 Mex

4.1.3 Results for the larvicidal activity for Chromatographic fractions

There were 9 Chromatographic fractions for each crude extract. Each fraction was screened for larvicidal activity and the results obtained are represented in Table 4 below.

Table 4: Results for the larvicidal activity for Chromatographic fractions

% mortality after 24 hours				
Fractions tested	JO4012 Kex	JO4012 Mex	JO4014 Kex	JO4014 Mex
FI	100%	100%	40%	10%
F2	100%	100%	100%	90%
F3	100%	100%	0%	100%
F4	100%	100%	0%	100%
F5	0%	0%	100%	90%
F6	100%	0%	100%	100%
F7	60 %	0%	0%	40%
F8	0%	0%	0%	0%
F9	100%	0%	0%	0%
Distilled H2O	0%	0%	0%	0%
Methanol	0%	0%	0%	0%

4.1.4 Fractions from JO4012 and JO4014 that were elucidated from Sephadex LH20

Fractions from the column chromatography that had significant larvicidal activity were further purified using Sephadex LH20. The fractions that resulted from Sephadex LH20 for both basidiomycetes species are listed in Table 5 and 6, below.

Table 5: Fractions from JO4012 that were elucidated from Sephadex LH20

Silica fractions	Sephadex LH20 fractions	Weight	TLC	Appearance	New code
JO4012 Kex F1-F4	S1 S2 S3	3 mg 2 mg 2 mg	Spots absent	Colourless	12KF1-4S1 12KF1-4S2 12KF1-4S3
F6-F7	S1 S2 S3	4 mg 8 mg 3 mg	Spots present	Colourless	12KF6-7S1 12KF6-7S2 12KF6-7S3
F9	S1 S2 S3	4 mg 8 mg 4 mg	Spots present	Colourless	12KF9S1 12KF9S2 12KF9S3
JO4012 Mex F1-F2	S1 S2 S3	1 mg 4 mg 2 mg	Spots absent	Colourless	12MF1-2S1 12MF1-2S2 12MF1-2S3
F3-F4	S1 S2 S3	1 mg 6 mg 7 mg	Spots absent	Colourless	12MF3-4S1 12MF3-4S2 12MF3-4S3

Table 6: Fractions from JO4014 that were elucidated from Sephadex LH20

Silica fractions	Sephadex LH20 fractions	Weight	TLC	Appearance	New code
JO4014 Kex F2	S1 S2 S3	6 mg 1 mg 3 mg	Spots absent	Green-yellow	14KF2S1 14KF2S2 14KF2S3
F5	S1 S2 S3	6 mg 1 mg 3 mg	Spots absent	Pink	14KF5S1 14KF5S2 14KF5S3
F6	S1 S2 S3	4 mg 1 mg 3 mg	Spots present	Green-yellow	14KF6S1 14KF6S2 14KF6S3
JO4014 Mex F2-F4	S1 S2 S3 S4	1 mg 3 mg 4 mg 8 mg	Spots present	colourless	14MF2-4S1 14MF2-4S2 14MF2-4S3 14MF2-4S4
F5	S1 S2 S3 S4	2 mg 6 mg 8 mg 8 mg	Spots absent	colourless	14MF5S1 14MF5S2 14MF5S3 14MF5S4

4.1.5 Sub fractions selected for NMR analysis

Various purified fractions from Sephadex LH20 were selected for NMR analysis. The fractions selected for NMR analysis are listed in Table 7 below.

Table 7: Sub fractions selected for NMR analysis

Sub fractions	TLC	Yield
12KF6-7S2	Dark spots	8 mg
12KF9S3	Dark spots	4 mg
12MF3-4S3	No spots	7 mg
14KF2S1	Green yellow spots	6 mg
14KF5S3	No spots	4 mg
14KF6S1	Pink spots	4 mg
14MF2-4S2	Dark spots	3 mg
14MF2-4S4	Dark spots	8 mg
14MF5S4	No spots	8 mg

4.1.6 Results for NMR analysis for 12KF6-7S2

NMR analysis was done for12KF6-7S2. Compound 12KF6-7S2 had ^{1}H NMR, ^{13}C NMR, DEPT, and HMBC COSY spectra **(Appendix 6-12)**. Its NMR data is shown (Table 8) below.

Table 8: **NMR data for 12KF6-7S2**

(^{1}H NMR 500MHz, ^{13}C NMR 500 MHz CD_3OD, J in Hz)

Position	^{13}C	^{1}H	COSY	HMBC	NOESY
1	98.482 94.038	4.45dd (3.7) 5.09 dd (7.0)	5 3	3 -	3,5
2	78.157	3.38	4,6	4	4,6
3	74.951	3.11	1,5	5	1,5
4	73.078	3.64	2,6	1,3,5	2,6
5	71.932	3.50	1,3,4	2,4,6	1,3
6	62.815	3.74	2,4	5	2,4

4.2 DISCUSSION

4.2.1 Discussion for taxonomic identification of the basidiomycetes

The basidiomycetes JO4012 and JO4014 that were investigated in this study were picked from within the precincts of Egerton University Njoro Campus. They were immediately brought into pure culture using PDA under sterile conditions. The hyphal strands showed very distinct clamp connections under a microscope, a characteristic feature of basidiomycetes. The morphological features at the time of collections indicated that these were typical mushrooms but each had unique features. However, further mycological investigation needs to be done on the herbarium materials preserved in IBRL, Egerton University and molecular markers should be used to unequivocally identify the two basidiomycetes to the corresponding species.

4.2.2 Discussion of the larvicidal activity of the crude extract

In reference to Table 3, in each crude extract the LC_{50} was less than the LC_{90} which is consistent with the expectations. Mortality rate depended on the concentrations of the crude extracts where by high concentrations of the crude extract killed high percentage of the larvae. Both the LC_{50} and the LC_{90} of each crude extract decreased with the increase in time. This meant that a smaller concentration of the crude extract was needed to kill the larvae for a longer period of time. In order for the larvae to be killed within a shorter duration, then higher concentrations of the crude extract were required. The crude extract that had the highest bioactivity against the larvae was JO4012 Mex. JO4012 Kex was the least active as it showed activity at higher concentrations.

4.2.3 Discussion for larvicidal activity of crude extract for JO4012 Kex

In reference to graph (**Figure14)** above, the lethal concentrations decreased with the increase in time, for example at 24 hours the LC_{50} and the LC_{90} were 22.5 mg/ml and 33.4 mg/ml respectively. In the first two hours the lethal concentration was very high in comparison to other time intervals. This meant that more concentrations of these extracts were needed to kill the larvae within a shorter period of time.

4.2.4 Discussion for larvicidal activity of crude extract for JO4012 Mex

In the reference to graph **(Figure 15)** above**,** the lethal concentrations also decrease with the increase in time interval. However these concentrations were not as

high as observed in JO4012 Kex. This implies that less concentrations of crude extract of JO4012 Mex were used to kill the larvae as compared to the crude extract of JO4012 Kex. Hence, for this particular species of basidiomycetes, the crude extract from mycelium is more active than the crude extract from the culture filtrate.

4.2.5 Discussion for larvicidal activity of crude extract for JO4014 Kex

In reference to above graph **(Figure 16)**, the lethal concentrations decreased with the increase in time. In comparison to the crude extract of JO4012 Kex, lower concentrations of this extract were required to kill the larvae. In addition the LC_{50} was less as compared to the LC_{90}. In relation to the potency of the activity of the culture filtrate, JO4014 Kex is more active than JO4012 Kex.

4.2.6 Discussion for larvicidal activity of crude extract for JO4014 Mex

In reference to the above graph **(Figure 17)** the first two hours had higher lethal concentrations in relation to other time intervals. This implied that low concentrations of these extracts were needed to kill the larvae for a longer period of time. The mycelium crude extract of JO4012 Mex is quite potent in comparison to JO4014 Mex. However, for this specific strain of basidiomycete, JO4014, the crude extract from the culture filtrate (JO4014 Kex) is less active than the crude extract from the mycelium (JO4014 Mex). This is evident by the LC_{50} and LC_{90} at 4 hours, 8 hours, and 24 hours for JO4014 Mex being lower than for JO4014 Kex.

4.2.7 Discussion of the larvicidal activity of the chromatographic fractions

Larvicidal activity of the fractions was done to verify the origin of the activity posed by the crude extract and to analyse the compounds responsible for this activity. In reference to Table 4, For JO4012 Kex, each of F1, F2, F3, and F4 had 100% mortality. These were eluted with cyclohexane and ethyl acetate, an indication that these compounds were ranging from non-polar to middle polar. It is speculated that these fractions were interfered with by the presence of fatty acids and steroids but this remains to be proved. F1 to F4 fractions were pooled together as they had similar TLC spots pattern. They were expected to have the same larvicidal compound(s). F6 and F7 had 100% and 60% mortality respectively. These were eluted with solvent mixtures of methanol and cyclohexane, indicating their compound(s) to be ranging from polar to middle polar. They were also pooled together as they showed similar spots pattern on

the TLC. F9, which had 100% mortality, was eluted with pure methanol thus expected to have polar compound(s).

As presented in table 4, FI, F2, F3 and F4 fractions originating from JO4012 Mex had 100% mortality. F1 and F2 were pooled together due to their similar spots pattern on TLC. They were eluted with solvents gradient varying from cyclohexane to ethyl acetate, indicating that these compound(s) were non-polar and middle polar. It is speculated that these fractions are often interfered with by high concentrations of the fatty acids and steroids, which are commonly found in the mycelium. F3 and F4 fractions were also mixed together as they had similar spots pattern on the TLC plate. This compound(s) was expected to range between polar to middle polar. However, they had less concentration of fatty acids and steroids in comparison to F1and F2.

For JO4014 Kex, FI showed 40% mortality as presented in table 4. It had less percentage mortality in comparison to FI of JO4012 Kex, which had 100% mortality. It was eluted with pure cyclohexane thus expected to harbour purely non-polar compounds. It was not purified further as its yield was low compared to other fractions. F2, a green-yellow colour solution had 100% mortality. It was eluted with a mixture of cyclohexane and ethyl acetate. It showed green-yellow spots on the TLC plate. It was expected to have middle-polar compound(s). Both F5 and F6 had 100% mortality. F5, which was a pink solution, was eluted with pure ethyl acetate. It was expected to have middle polar compound(s). F6, which was a pink-yellow solution, was eluted using a mixture of ethyl acetate and methanol. Middle polar to polar compounds was expected in this fraction.

In reference to table 4, F1 from JO4014 Mex had 10% mortality. This yield was low thus it was not purified. The low mortality rate could be due to the presence of lesser active compounds as it had low yields. It was expected to contain non-polar compounds as it was eluted using cyclohexane. F2, F3 and F4 had 90% 100% and 100% mortality, respectively. These were eluted with mixtures of ethyl acetate and cyclohexane. Hence non-polar to middle polar compounds were expected to be in them. F2, F3 and F4 were pooled together as they had similar spots pattern on TLC. F5 and F6 had 90% and 100% mortality respectively. They were eluted with a mixture of cyclohexane and methanol, indicating the presence of non-polar to polar compound(s). These fractions were pooled together due to their equivalent TLC spots. F7 had 40%

mortality but it was not purified as it had a low yield. This low yield could have contributed to its low activity.

In general, for JO4014 Kex and JO4014 Mex, the larvicidal activity of polar fractions was less compared to activity of middle polar fractions. The fractions which showed potent activity were further purified using Sephadex LH20 as illustrated **(figure 14)**. The fractions, which were pooled together, were also purified using Sephadex LH20. Fractions from Sephadex LH20 were newly coded as shown in Table 5 and 6. The fractions that had low activity were not investigated further. Nevertheless, there is a possibility that they could be active if their yields are increased.

4.2.8 Discussion for NMR Spectral Analysis of the Purified Compounds from Sephedex LH20

The purified fractions from Sephadex were selected for NMR analysis. The selection was based on the activity of the fractions from the silica gel column, yield and the purity of the fractions as visualised on the TLC plates. The selected sub fractions are shown in Table 7.

12MF3-4S3 and 14MF5S4 had no visible spots on the TLC. However, they were chosen for NMR experiments because they had high percentage mortality (potent larvicidal activity) and reasonable yield. Furthermore, there could have been presence of the spots on the TLC plates if other visualization techniques which were not available in this study were applied (e.g. UV lamp).

Some fractions had only ^{1}H NMR spectra. This was because some of them had limited yield. In addition the compounds that had reasonable yield had impurities. Purifying them resulted in reducing their yield, thus, further NMR experiments could not be carried out. Fraction 14MF2-4S2 had ^{1}H NMR and ^{13}C NMR spectra **(Appendix 1-2)**. However due to minimal yield other NMR experiments could not be carried out. Fraction 14KF2S1 had ^{1}H NMR, ^{13}C NMR and DEPT spectra **(Appendix 3-5)**. The compound was impure and due to its limited yield, other NMR experiments could not be carried out. Structure elucidation could not be done for these compounds as they lacked enough NMR spectra.

4.2.9 Discussion of the 1H NMR and 13C NMR data for 12KF6-7S2

In the region of sugar skeleton atoms (δ= 60-100) in the ^{13}C NMR, 9 carbon signals were found. Hence, the compound is a monosaccharide consisting of a hexose. The numerous number of carbon signals is attributed to the flipping of the molecule.

The chemical shift of anomeric carbon C-1 and the attached proton is δ 98.482 and δ 4.450 respectively. In ^{1}H NMR, a doublet was observed at δ 5.085 which correspond to δ 94.038 in the anomeric region. This indicates that there was flipping of the molecule which shifted C-1 from δ 98.482 to δ 94.038. In addition, the coupling constant, 7 Hz, proves that the sugar is in β- configuration i.e. H-I is in axial position (Duddeck *et al*, 1998).

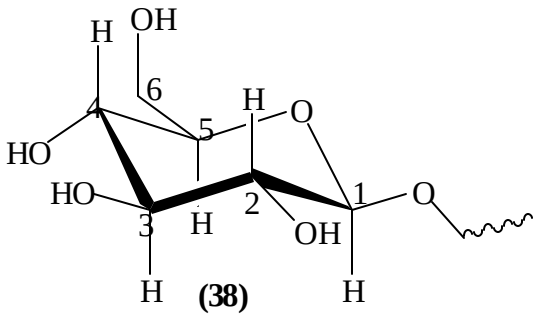
C-6 is differentiated from other carbons due to different numbers of attached hydrogen. This is confirmed by DEPT to be a $-CH_2$ group, thus, it is a $-CH_2OH$ group. HMBC spectrum shows correlations between C-6 and H-5. The 2H-6 methylene group proton resonances showed a NOESY correlation with H-2 and H-4. C-5 resonance occurred at δ 71.932 as it was not attached to the –OH group. In the COSY spectrum, there was coupling between H-5 and H-1, H-3 and H-4 which resulted to a multiplet at δ 3.496.

C-4 resonance occurred at δ 73.078. In the COSY spectra there was coupling between H-4 and H-2 and H-6. This indicated that these hydrogens were on the same side of the ring. NOESY correlation could be seen between H-4 and H-2 and H-6. C-3 resonated at δ 74.951. In the NOESY spectrum there was coupling between H-3 and H-1 and H-5. This confirmed that H-1, H-3 and H-5 were on the same side of the molecule which was in the β- configuration. HMBC correlations were between C-3 and H-5 confirming the position of C-3.

Considering a well known "roofing effect" between H-1and the double doublet signal at δ 5.085 in the 1D 1H NMR spectrum, H-2 can be assigned, hence C-2 (Duddeck *et al,* 1998).

HMBC and NOESY spectra confirmed the proposed structure of this compound, which is known as β–D-glucopyranose **(38)**. It is possible that the compound, which had larvicidal activity, was having glycosidic moiety. The glycosidic part of this compound was β–D-glucopyranose. The aglycone part of this molecule could have been linked to the glycosidic part through C-1. The glycosidic linkage could have been cleaved off due to longer handling period before analysis. The molecule could have been labile to ambient handling conditions leading to degradation. The signals for the aglycone moiety were not detected. Hence, there is need to pursue these in future work on these compounds.

This compound could not have originated from the maize meal media that was used in the growth of these species. The compound present in the maize meal media is α-D-glucopyranose (Duddeck *et al*, 1998).



(38)

Figure 19 Structure of b –D-glucopyranose

CHAPTER FIVE

CONCLUSIONS AND RECOMMENDATIONS

5.1 Conclusions

JO4012 and JO4014 produce larvicidal compounds when inoculated in maize meal media. These larvicidal compounds range from polar to non-polar. This was evident by the solvents used in extraction and elution of the column and the activity of the fractions. Larvicidal activity observed in these basidiomycetes is supported by the fact that some basidiomycete species poses insecticidal activity.

High concentrations of the crude extract are required to kill larvae for a shorter period of time. In addition, high concentrations of crude extract kill high percentage of the larvae. In this study, crude extract from mycelium of JO4012 is the most active. This is evident by its lower concentrations being used in killing the larvae in comparison to other crude extracts.

One of the compounds responsible for the larvicidal activity of the culture filtrate from JO4012 is a glycoside. This is a polar compound and this is supported by the solvents (methanol, acetone and ethylacetate) used in is extraction and elution from the column chromatography (mixtures of methanol and ethyl acetate).

In this study JO4012 and JO4014 was found have significant larvicidal activities. However, mycelium crude extracts from these basidiomycetes had potent bioactivity than their culture filtrate extracts Thus, this study should be pursued, as it will help to address the problem of mosquito.

5.2 Recommendations

The following recommendations were made in relation to this study:

1) Crude extract yield from these basidiomycetes should be increased and various purification techniques should be used in order to get pure compounds for structure elucidation.

2) Other visualisation techniques should be applied to e.g UV lamp to make the TLC spots visible.

3) Other spectral analysis techniques should be done for the compounds in order to verify the structures of all active compounds.

4) The active extracts to be subjected to field study to verify their safety and eliminate the prediction that may be the observed activities were due to non-selective toxicity of the extracts.

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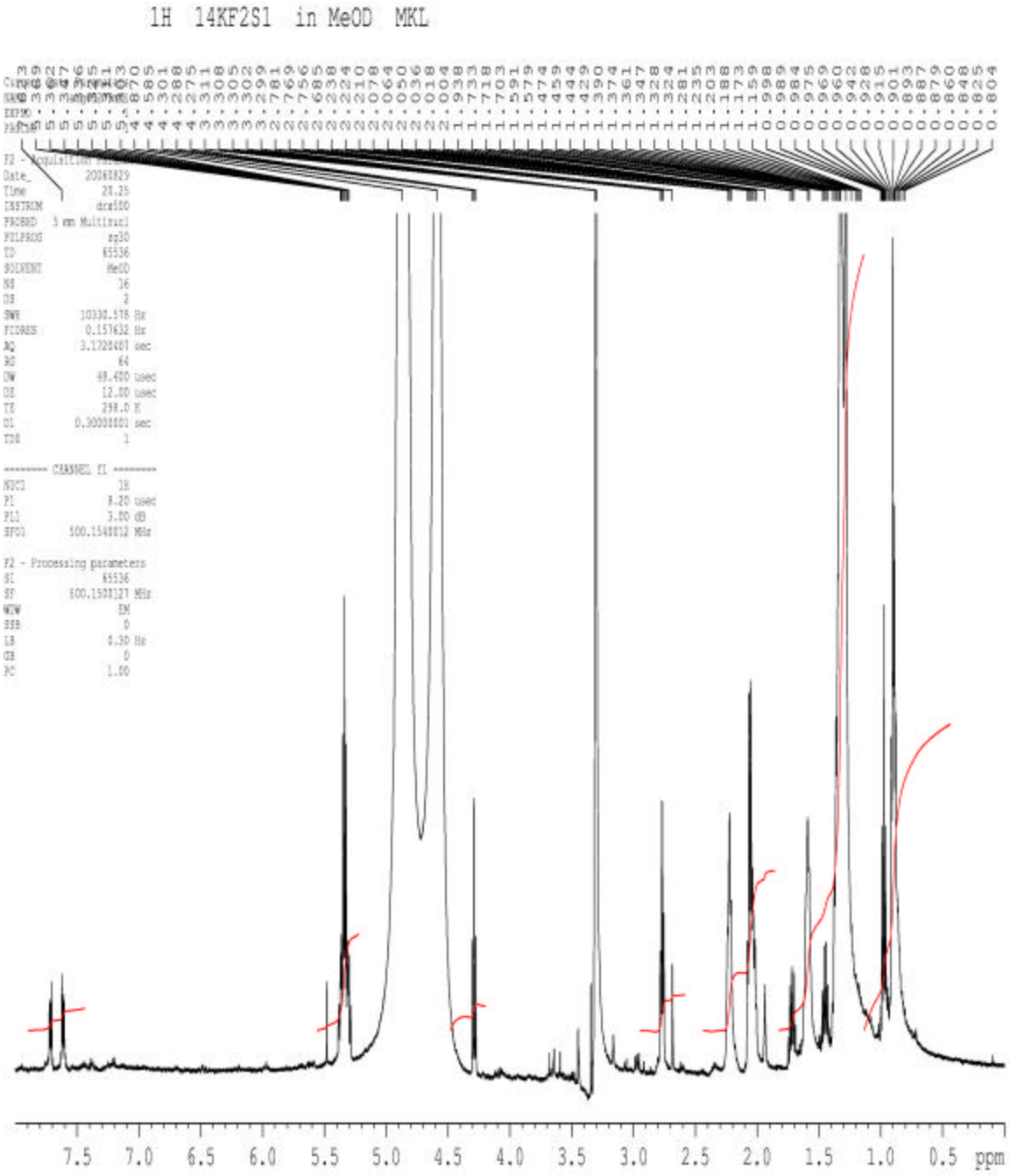
APPENDICES

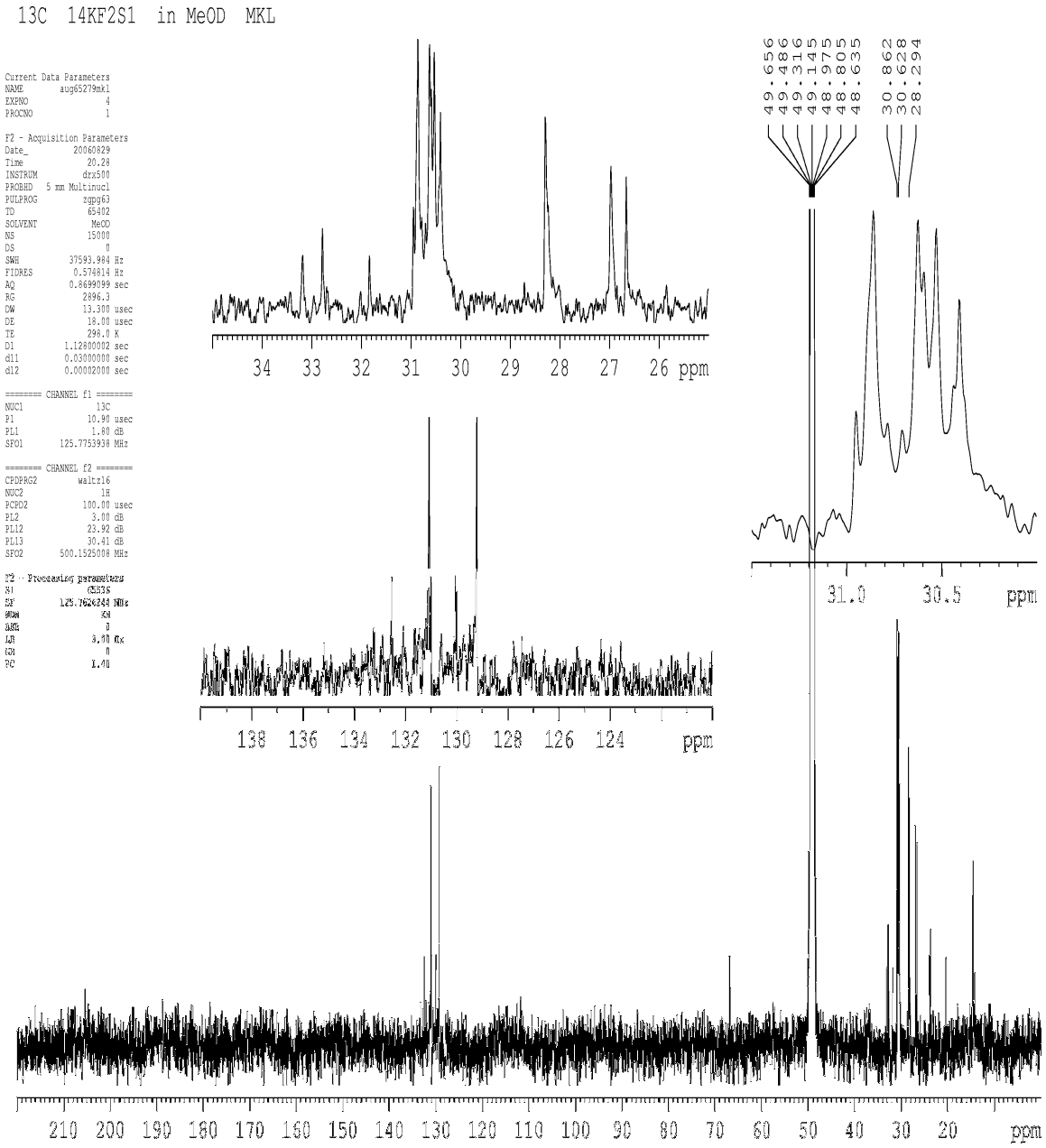
Appendix 1: ^{1}H NMR for 14MF2-4S2

Appendix 2: ^{13}C NMR for 14MF2-4S2

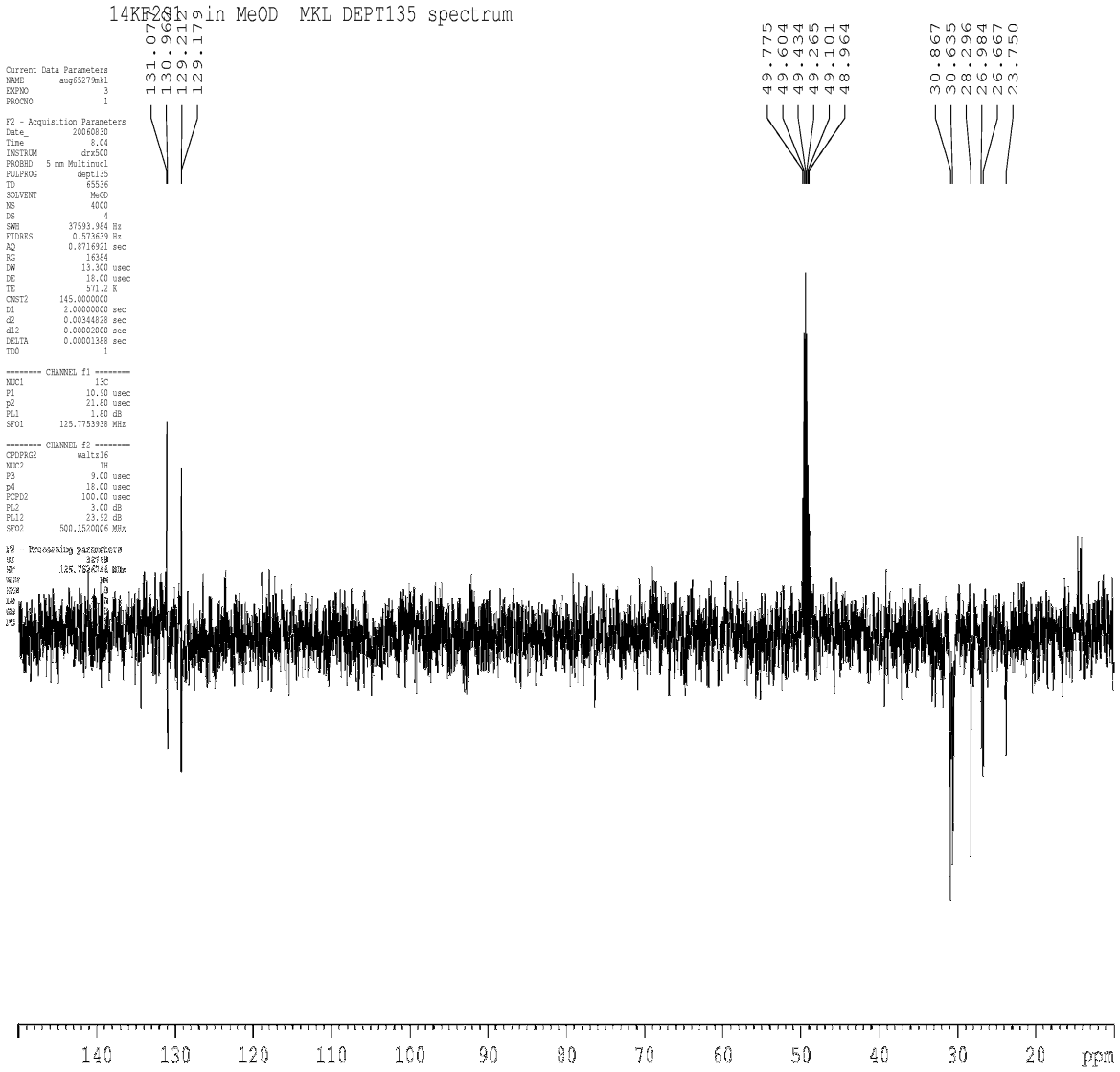
Appendix 3: ^{1}H NMR for 14KF2S1



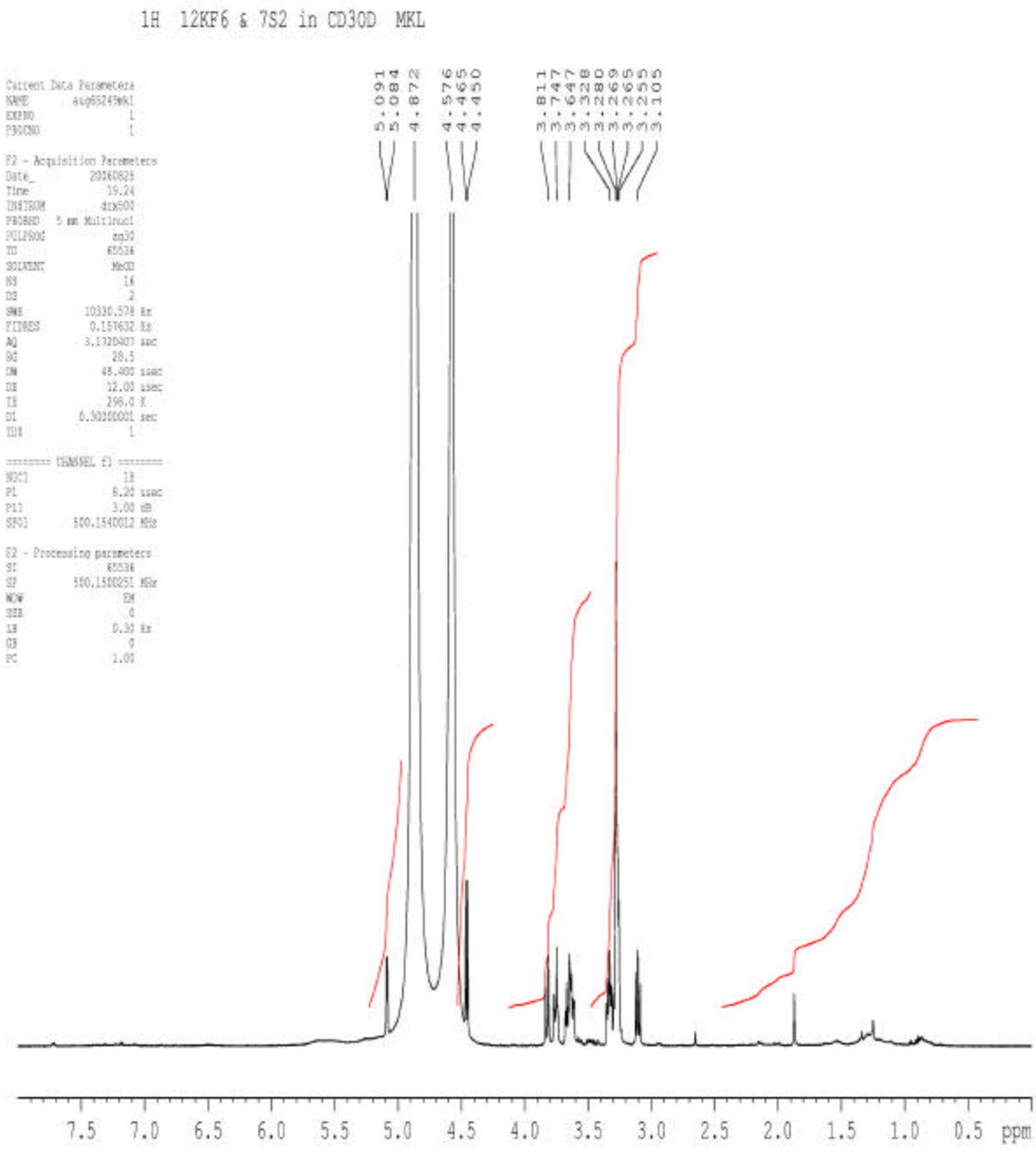
Appendix 4: ^{13}C NMR for 14KF2S1



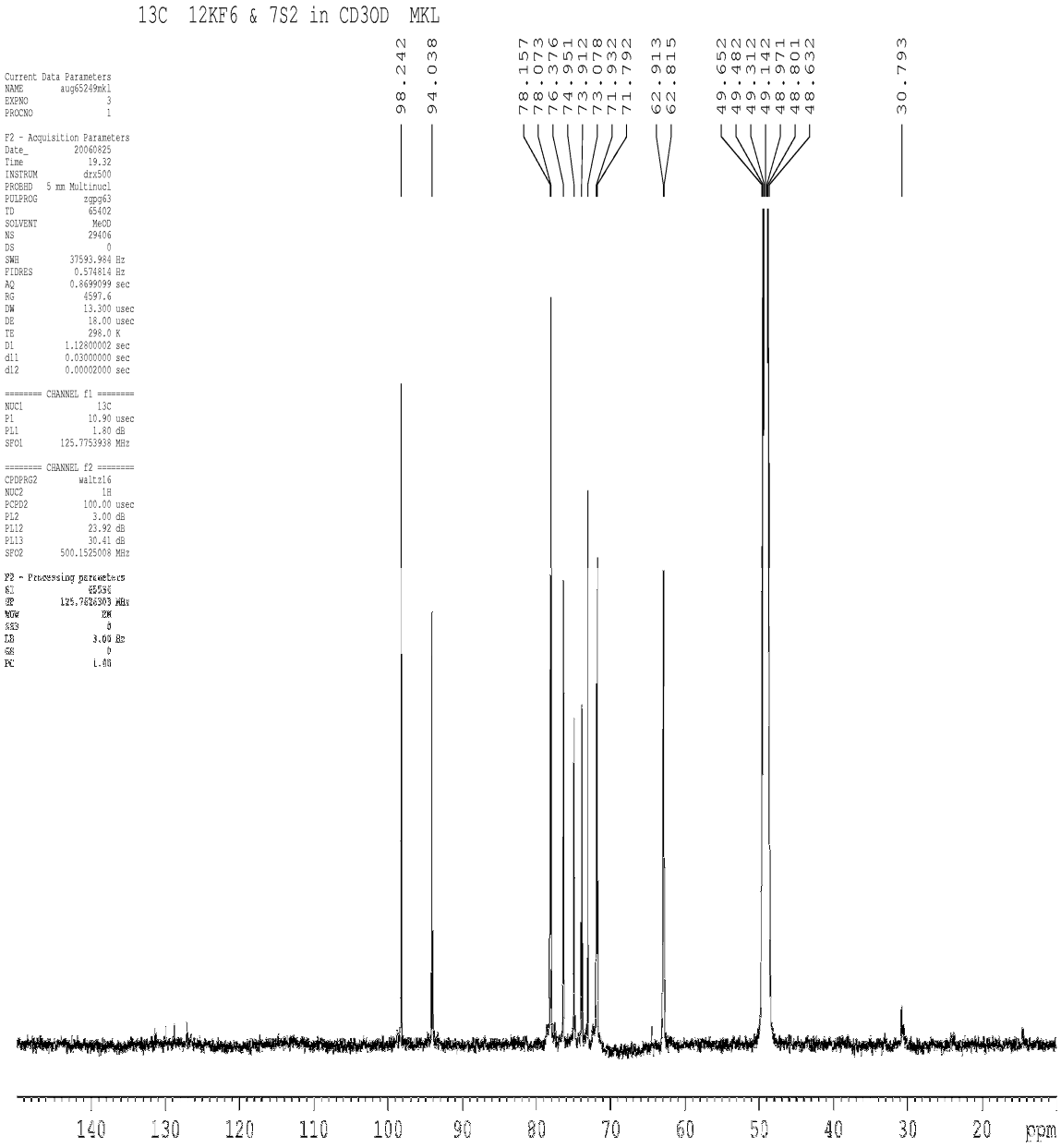
Appendix 5: DEPT Experiment for 14KF2S1



Appendix 6: ^{1}H NMR for 12KF6-7S2 (β-glucopyranose)

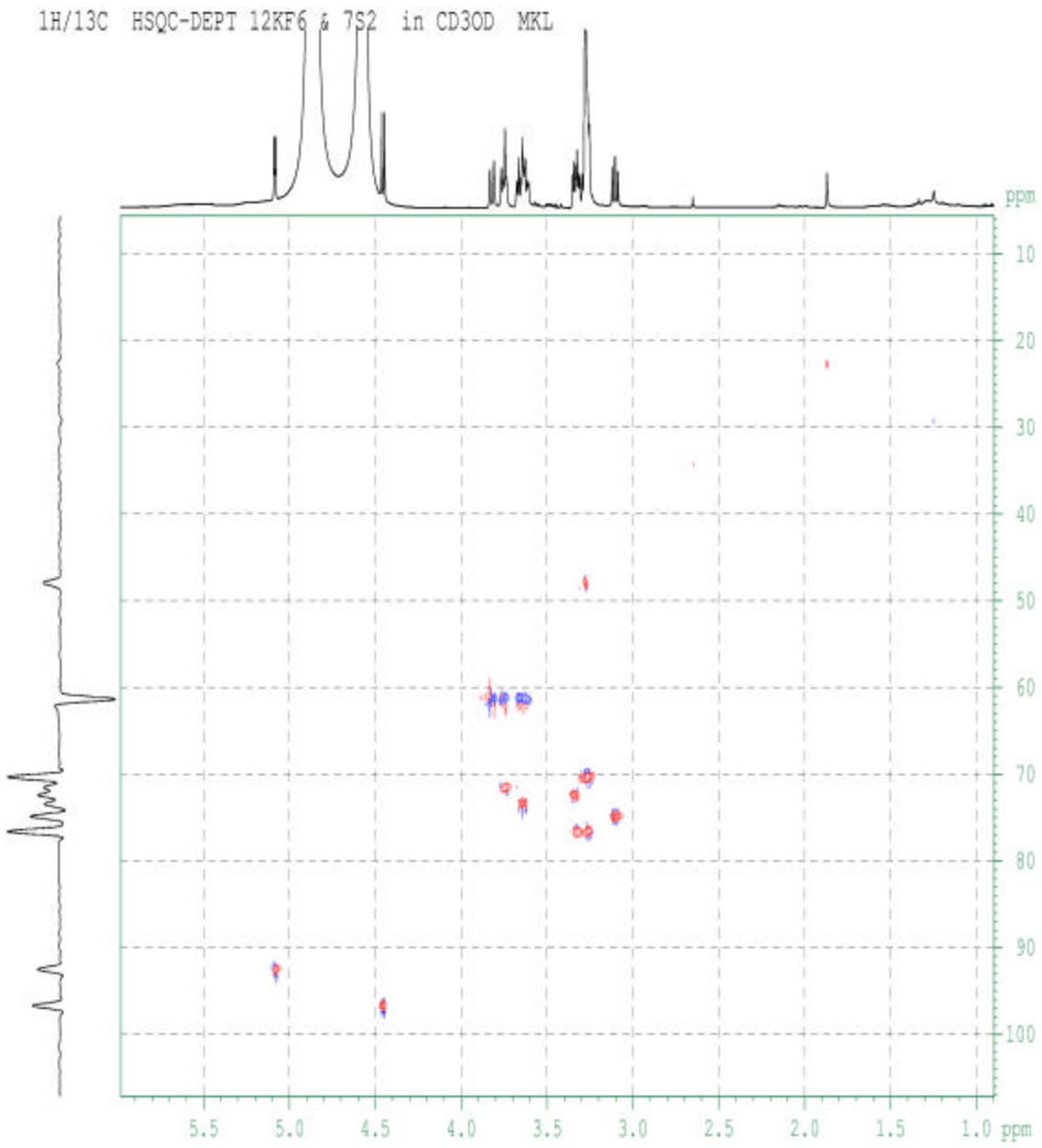


Appendix 7: ^{13}C NMR for 12KF6-7S2 (b-glucopyranose)

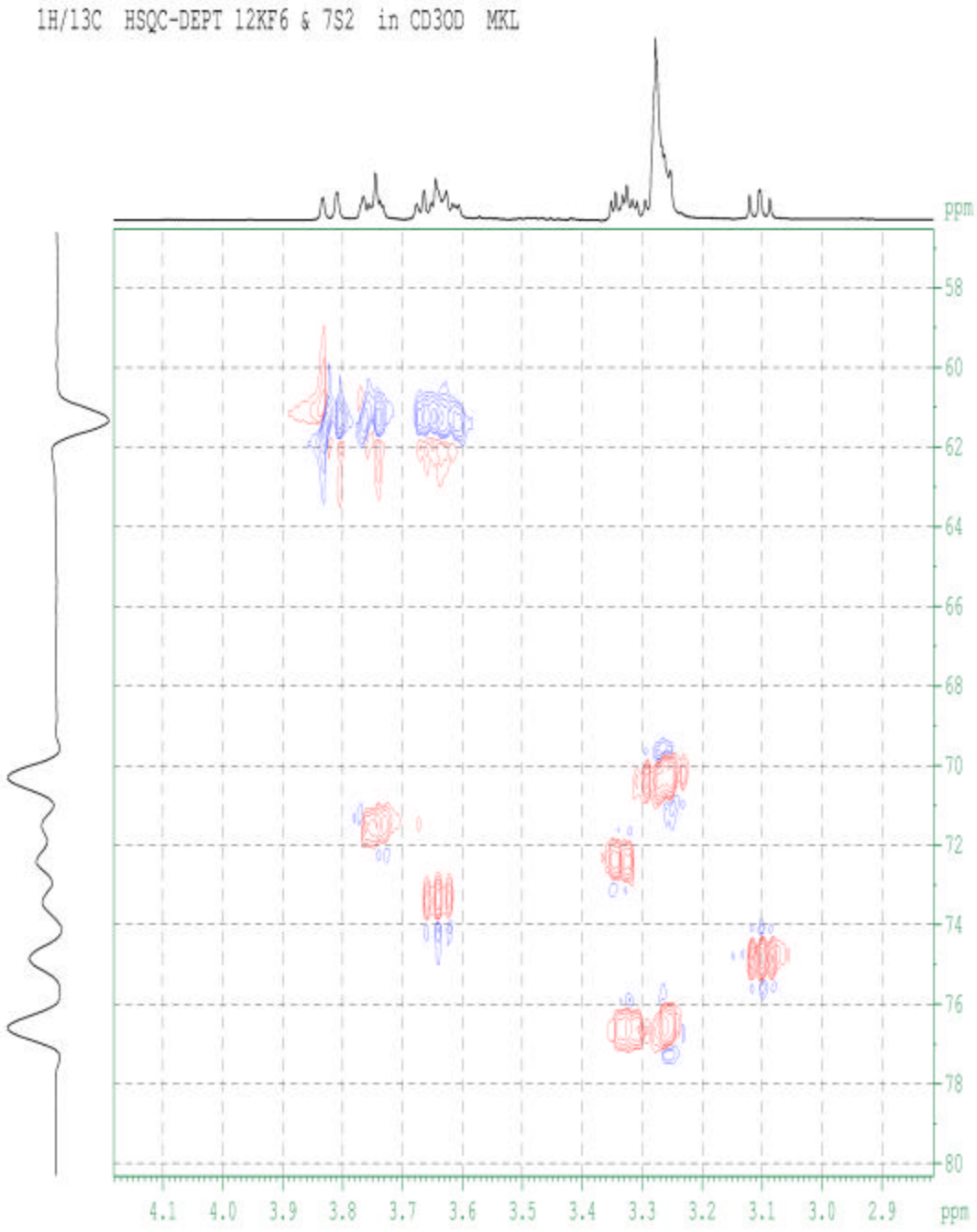


Appendix 8: DEPT Experiment for 12KF6-7S2 (β-glucopyranose)

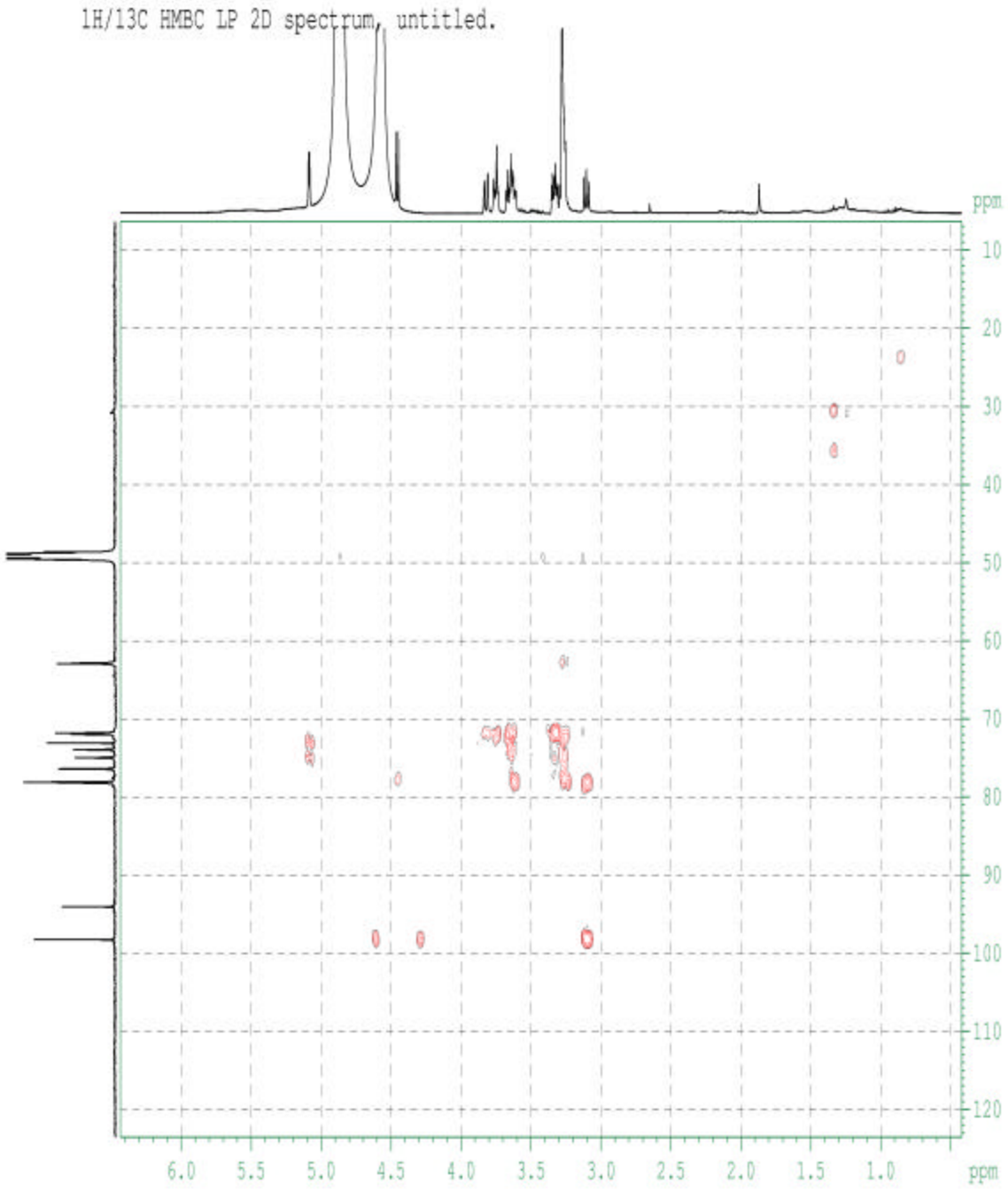
Appendix 9: HSQC Experiment for12KF6-7S2 (b -glucopyranose)



Appendix 10: HSQC Experiment for12KF6-7S2 (ɒ-glucopyranose)



Appendix 11: HMBC Experiment for12KF6-7S2 (b-glucopyranose)



Appendix 12: COSY Experiment for12KF6-7S2 (b -glucopyranose)