CHARACTERISATION OF MOSQUITO LARVICIDAL COMPOUNDS FROM CULTURES OF A BASIDIOMYCETE *Coriolus hirsutus*

OUMA RUSSELL BEN OMONDI

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

EGERTON UNIVERSITY

APRIL, 2012

DECLARATION AND RECOMMENDATIONS

DECLARATION

I 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 of learning to the best of my knowledge.

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RECOMMENDATION

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

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Dr. J. O. Omolo

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Dr. P. K. Cheplogoi

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DEDICATION

This thesis is dedicated to all my family members especially my uncle, Willis Otieno, whose vision and energy, moral, financial and emotional support has positively shaped my destiny.

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ABSTRACT

Mosquitoes transmit serious human diseases, resulting into millions of deaths globally every year. The use of synthetic insecticides to control vector mosquitoes has caused physiological resistance and adverse environmental effects in addition to high operational costs. Mosquito larvicidal compounds from certain plant and fungal species have been reported. Basidiomycetes fungi are known to synthesize a wide range of bioactive secondary metabolites. The objective of this work was to investigate the mosquito larvicidal compound(s) from the fungus *Coriolus hirsutus* belonging to the basidiomycete class of fungi. Crude extracts prepared from fermented liquid nutrient media cultures of *Coriolus hirsutus* were found to exhibit activity against the larvae of *Aedes aegypti* species of mosquito. The active crude extracts were further subjected to bioactivity guided fractionation that led to the purification of two larvicidal compounds. Chemical investigation using NMR spectroscopy and MS experiments identified the two compounds, 17–(5–hydroxy–1,5-dimethyl-hex-3-enyl)-4,4,8,10,13-pentamethyl-4,5,6,7,8,9,10,11,12,13,14,15,16,17-tetradecahydro-3*H*-cyclopenta[α]phenanthren-3-ol and hexanoic acid. 17–(5–hydroxy–1,5-dimethyl-hex-3-enyl)-4,4,8,10,13-pentamethyl-4,5,6,7,8,9,10,11,12,13,14,15,16,17-tetradecahydro-3*H*-cyclopenta[α]phenanthren-3-ol had an LC_{50} and LC_{90} of 147.0 ppm and 285.3 ppm at 95%CI while hexanoic acid had an LC_{50} and LC_{90} of 237.8 ppm and 393.9 ppm at 95%CI. The two larvicidal compounds obtained from *Coriolus hirsutus* may constitute important alternatives to the synthetic insecticides under the integrated vector control strategies.

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LIST OF ABREVIATIONS AND ACRONYMS

^{13}C	Carbon 13 isotope
^{1}H	Hydrogen 1 isotope
BHC	Benzenehexachloride
CC	Column Chromatography
DDD	Dichloro Diphenyl Dichloroethane
DDT	Dichloro Diphenyl Trichloroethane
DEPT	Distortionless Enhancement by Proton Transfer
DHF	Dengue Hemorrhagic Fever
DMSO	Dimethyl sulfoxide
HIV	Human Immunodeficiency Virus
HMBC	Heteronuclear Multiple Bond Correlation
IR	Infra Red
ITNs	Insecticide treated nets
JE	Japan encephalitis
LC_{50}	Lethal concentration that kills 50% of mosquito larvae
LC_{90}	Lethal concentration that kills 90% of mosquito larvae
MS	Mass Spectrometry
MSRA	Methicillin Resistant Staphylococcus aureus
NMR	Nuclear Magnetic Resonance Spectroscopy
NOESY	Nuclear Overhauser Effect Spectroscopy
OR	Optical Rotation
TLC	Thin layer chromatography
UV/Vis	Ultra Violet -Visible
VLBW	Very Low Birth Weight
WHO	World Health Organization

CHAPTER ONE
INTRODUCTION

1.1 Background Information

Mosquito-borne diseases, such as dengue haemorrhagic fever, encephalitis, malaria and filariasis remain a major source of illness and deaths worldwide, particularly in tropical and subtropical climates (Becker and Ascher, 2003). Malaria is one of the most significant health issues that we face today, with 250 million cases, and over 800,000 deaths annually. This impact is multiplied by the fact that 85% of the cases are in children under five years old, and the prevalence is high amongst expectant mothers (WHO, 2007). Protection against insects' bites is achieved by avoiding infested habitats, wearing protective clothing and applying insect repellent (Curtis and Mnzava, 2000). Commercially available repellents can be divided into three categories; synthetic chemicals, botanicals and alternatives such as the combination of synthetic and natural compounds. The most widely marketed synthetic based insecticide is N,N-diethyl-3-methyl benzamide (DEET), used since 1957 but has been found to be unfit for human during use, oily feel and high skin penetration, and it can dissolve plastics and synthetic rubber (Qiu and Jun, 1998).

The world prime choice to curb mosquitoes continues to be based on selective application of residual synthetic insecticides. The public health benefit delivered by these, both in tropical resource-poor settings as well as in temperate zones, cannot be overemphasized for they save thousands of lives each year (WHO, 2004). However, encouraged by a strong industrial lobby, new and more environmental friendly compounds replace older, more harmful ones. Nevertheless, beyond gains in economic and public health terms, the stark reality of environmental impact and ever developing insect resistance remains an issue of grave concern (Scholte *et al.,* 2004). There is a need for new larvicides since the protozoan parasite is constantly becoming resistant to drugs. Larvicides are applied to bodies of water and often only that area where the larvae grow and mature.

The needs for new innovative compounds that can be used to control mosquito require the availability of the large number of compounds. Chemo-diversity in nature offers valuable source for example secondary metabolites, previously regarded as waste products are now recognized for their importunate activity against pests and diseases. Nature has developed an enormous diversity during several years of evolution. It is estimated that there are at least 250,000 different plant species, up to 30 million species of insects, over 1.5 million species of

fungi and similar number of algae and prokaryotes still remain in existence (*en.wikipedia.org/wiki/Fungus)*. All of these species coexist in ecosystems and interact with each other in several ways in which chemistry plays a major role for example in defence, pollination and symbiosis. Considering the number of organisms and the almost infinite number of interactions it is not surprising that an enormous wide variety of secondary metabolites has evolved within organisms. In Kenya there is a rich biodiversity that is underutilised especially for addressing problems of disease such as yellow fever and its vector. The use of *Aedes aegypti* as the test model is recommended due to its survival to harsh conditions than *Anopheles gambia* (Briegel, 1990). This is as a result of the high caloric content which is composed of lipids, proteins and carbohydrates. Lipids are exponentially related to body size thereby considered higher in males while proteins and carbohydrates are linearly related to body size in both sexes (Benoit and Denlinger, 2007). Research also indicates that *An. gambiae* is more susceptible to insecticides than *Aedes* mosquitoes (Badolo *et al*., 2004). The larval stage of *Ae. aegypti* is more stable for experimental purposes (Eveline *et al.*, 2004).

1.2 Statement of the Problem

Mosquitoes transmit diseases that continue to pose a threat to humans. Various methods have been employed to curb mosquitoes but without much success. Limitation of these insecticides include re-emerging resistance, high costs and other chemical limitations for instance stability to heat, smell, and solubility. The recent progress in genetically engineered mosquitoes and Sterile Insect Technique as a way of curbing the anopheline mosquitoes has not gained much either in eliminating mosquitoes. Larval control has been an option of mosquito control but this is grossly underdeveloped and underused approach, particularly in tropical Africa where it may be of much greater utility than generally appreciated. Although tropical fungi are known to possess diverse biological active molecules, they have not been sufficiently investigated for larvicidal compounds. *Coriolus hirsutus,* a basidiomycete (white rot fungus) has been investigated for lignin degradation enzymes but no research on larvicidal activity has been reported. This research therefore focuses on the search for larvicidal compounds from basidiomycetes in the quest for new larvicides to curb diseases spread by mosquitoes.

1.3 Objectives

1.3.1 Main Objectives

To characterise mosquito larvicidal compounds from cultures of a Kenyan basidiomycete *Coriulus hirsutus* (JO5185).

1.3.2 Specific Objectives

1. To prepare crude extracts from the submerged cultures of *Coriolus hirsutus* (JO5185).
2. To test crude extracts from *Coriolus hirsutus* for mosquito larvicidal activity against *Aedes aegypti*.
3. To carry out bioactivity guided fractionation in order to purify the larvicidal compounds from active crude extracts.
4. To elucidate the structure of purified compounds using spectroscopic techniques and other physical methods.

1.4 Justification

Since the diseases spread by mosquitoes are constantly becoming resistant to insecticides, vector control therefore is an option for eliminating these mosquitoes. Identification of novel effective mosquitocidal compounds is essential to combat increasing resistance rate, concern for the environment and food safety. The unacceptability of many organophosphates, N,N-diethyl-3-methylbenzamide (DEET), organochlorines and high cost of synthetic pyrethroids justifies the search for compounds from unique unexplored sources like fungi.

CHAPTER TWO
LITERATURE REVIEW

2.1 Some of the Diseases Transmitted by Mosquitoes

2.1.1 Yellow Fever

Yellow fever is caused by the arbovirus from the family Flaviviridae. The disease is characterized by high fever and jaundice (CDC, 2004). *Ae. aegypti*, is the main vector for the arbovirus responsible for yellow fever in central and south America and in west Africa (Ciccia *et al.,* 2000). The ability of this vector to breed in household water makes 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 (WHO, 2003). There is no specific curative treatment for yellow fever, although it has been reasonably brought under control with its vaccine (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). This makes *Ae. aegypti* a good organism for serotome laboratory screening for larvicides (Ciccia *et al.,* 2000; Carvalho *et al.,* 2003; Ndung'u *et al.,* 2003).

2.1.2 Dengue Fever

Dengue fever 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 (WHO, 2008). Both dengue fever and DHF are caused by various viruses and are transmitted by female mosquito from one person to another. *Ae. aegypti* and *Ae. alboliticus* are vectors of DHF, which is endemic to southeast Asia, the Pacific Island area, Africa and America (Carvallo *et al.,* 2003).

The incidence of dengue fever has increased dramatically in late 20th century. 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, the proliferation and adaptation of these mosquitoes.

There is no vaccine available for dengue fever and the only way of reducing the incidence of these diseases is to combat Ae. *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.

2.1.3 Malaria

Malaria is a major disease in developing countries, causing 1-2 million deaths every year, a majority of cases being young children in Africa (WHO, 2004). This burdensome disease is caused by protozoa parasites *Plasmodium*. The species *Plasmodium falciparum* is by itself responsible for the majority of deaths. The parasite is transmitted by the female Anopheles mosquito and transits through the liver and the blood of the mammalian host. The blood stages of the parasites are those that cause the symptoms. Apart from quinoline-based antimalarials, antifolates, and antibiotics, the endoperoxide artemisinin and its synthetic derivatives are widely used to combat the disease (Malenga *et al*., 2005). Unfortunately, there has been a spread of resistant strains of the parasite to the current therapeutics in numerous countries (Bloland *et al*., 2001; Edwards and Biagini, 2006). The drug resistance, which is also an economic burden to these countries, has become one of the greatest challenges in malaria control (Phillips and Phillips-Howard, 1996). Use of traditional medicine as complementary or alternative solutions in malaria control programmes as advocated by Bertrand is also not conclusive (Graz *et al*., 2011). There is, thus, a need for new therapies, which development can be accelerated by the recent publication of *Plasmodium* genome (Greenwood *et al*., 2005; Yeh and Altman, 2006).

2.2 Modern Control Methods for Mosquitoes and Diseases Caused by Mosquitoes

There are some novel strategies to fight these diseases by targeting the mosquito vectors. However, each approach has its limitations;

Larviciding: - Application of insecticides target immature mosquitoes (the larvae or the pupae). These control agents are applied to bodies of water harbouring the larvae, the immobile phase of the mosquito. When mosquitoes are at their larval stage they are less immobile as compared to adult mosquitoes. When dealing with larval control less acreage is

treated as compared to adulticiding and therefore a better control with decreased risk to the public. This has a better public perception with less environmental factors to affect treatment. However, its limitation accessing all the breeding sites but if this is achieved then the process is more economical.

In-door spraying: - This involves spraying small amounts of *p,p*–dichlorodiphenyltrichloroethane (DDT), to the walls and cervices of the houses. The disadvantage is that DDT is non-biodegradable and ends up in the food web. It accumulates in the fatty tissues leading to growths which can be cancerous and thereby fatal. These observations have led to numerous health and environment concerns.

Biological methods: - Biological control is a method which uses biotic agents that are toxic, antagonostic or lethal to mosquitoes. Available literature cites several bacteria that, according to laboratory and field trials have been used to control the vector at breeding sites, e.g. use of *Bacillus sphaericus* against anophelines. There is also the use of larvivorous fish e.g. *Gambusia affinis* and *Poecillia reticulata*. For instance the fish (*G. affinis*) is reported to consume more than hundred larvae in one day. This is the most ideal environmental approach but the disadvantage is that not all places that the larvae inhabit are appropriate to fish.

Application of repellents: - Commercially available repellents can be divided into three categories: synthetic chemicals, botanicals and alternatives such as the combination of synthetic and natural compounds. The most widely marketed synthetic based insect repellents is N,N-Diethyl-3-methylbenzamide (DEET), which has been used worldwide since 1957. It is still effective and available in commercial formulations for example solutions, lotions, gels, aerosol sprays, creams, sticks or impregnated towelettes (Robbins and Cherniack, 1986). This compound, however, has an unpleasant smell, oily feel, and high skin penetration, and it can dissolve plastics and synthetic rubber (Qiu and Jun, 1998).

Draining stagnant water: - This involves draining stagnant waters and clearing shrubs/grasses near living places. This presents a mammoth task of adherence and supervision in large regions with poor land drainage during rainy seasons.

Larvasonic: - Killing larvae with sound; the larvasonic is an acoustic larvicides system designed to kill larvae using sound. Sound energy is transmitted into water at the resonant frequency of the mosquito larvae air bladders and instantly ruptures the internal tissues causing death.

a) **Use of insecticide treated bed and window nets (ITNs):** - ITNs have proven effective in reducing transmission, especially among children and pregnant women. However, multiple insecticide resistance among the main mosquito vectors *An. gambiae* and

An. funestus, and various complications in the introduction, distribution and proper use of ITNs indicate these tools alone will be insufficient to reduce malaria transmission to a level that could eventually lead to eradication of the disease.

Genetically engineered mosquito vector: - Another form of anti-vector control is gene therapy to engineer unsuitable vector mosquitoes and introduce them into the ecosystem in the hope that they may displace the receptive anopheles species and deprive *Plasmodium* of its vector transmission (Frizzi and Gwadz, 1975). This sounds like a good idea, but the cost to developing countries would probably be very high as suitable genetically engineered mosquitoes may not be easy to obtain.

Sterile Insect Technique (SIT) for African malaria vectors: - This call for a great need for a convenient assemblage of existing information on the suppression and/or eradication of *Anopheles* populations using the release of sterilized mosquitoes. There is renewed interest in the scientific community to improve or even replace the SIT through the techniques of molecular biology to make *Anopheles* incapable of transmitting the *Plasmodium* protozoan parasite (Klassen, 2009). This technique faces a lot of challenges including cost.

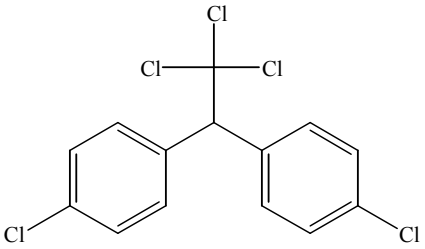
Treatment of malaria: Various approaches of treating malaria have been adopted, A "reverse pharmacology" approach for developing an anti-malarial phytomedicine (Willcox *et al*., 2011.) First trials were done in Mali resulting in a new standardized herbal anti-malarial after six years of research. This has been effective though has not fully eliminated the disease. It is suggested that rigorous evaluation of traditional medicines involving controlled clinical trials in parallel with agronomical development for more reproducible levels of active compounds could improve the availability of drugs at an acceptable cost and a source of income in malaria endemic countries (Ginsburg and Deharo, 2011).

2.3 *p,p*-Dichlorodiphenyltrichloroethane (DDT) as a Synthetic Insecticide

DDT (**1)** the first of the chlorinated organic insecticides was originally prepared in 1873, but it was not until 1939 that its effectiveness as an insecticide was discovered. The use of DDT assumed world proportions after World War II, primarily because of its' effectiveness against the mosquito that spreads malaria and lice that carry typhus (Russell, 1999). The World Health Organization estimates that during the periods of its use, approximately 25 million lives were saved. DDT seemed to be the ideal insecticide; it is cheap and of relatively low toxicity to mammals (oral LD_{50} is 300 to 500 mg/kg). However, problems related to the extensive use of DDT began to appear in the late 1940s. Many species

of insects developed resistance to it; it was also discovered to have a high toxicity towards fish and was associated with extinction of certain bird species (http://www.eco-usa.net/toxics/chemicals/ddt.shtml).

The chemical stability of DDT and its fat solubility compounded the problem. Animals do not metabolize DDT very rapidly; instead, it is deposited and stored in the fatty tissues. The biological half-life of DDT is about eight years; that it takes about eight years for an animal to metabolize half of the amounts it assimilates (http://earthtrends.wri.org/updates/node/317).

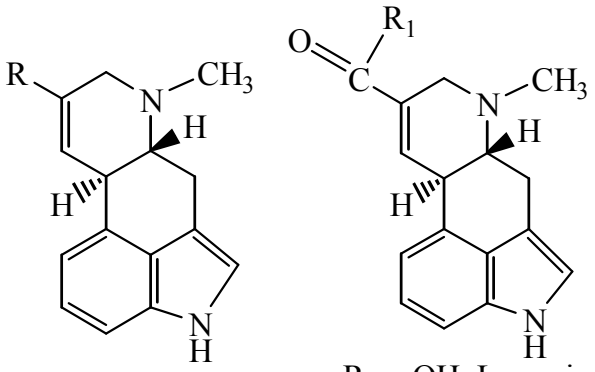


p,p -Dichlorodiphenyltrichloroethane (DDT) (**1**)

2.4 Fungal Therapeutic Metabolites

It is estimated that 50% of the annual 5 million metric tons of cultivated mushrooms might contain functional or medicinal properties, which may be used as a source of biologically and physiologically active substances (Cheung' *et al*., 2004). Fungi are most accomplished chemists in the universe. The compounds they synthesize range from simple ubiquitous molecules such as gluconic acid and citric acid, often referred to as primary metabolites, to complex unusual molecules such as aflatoxin and ergot alkaloids, which are called secondary metabolites.

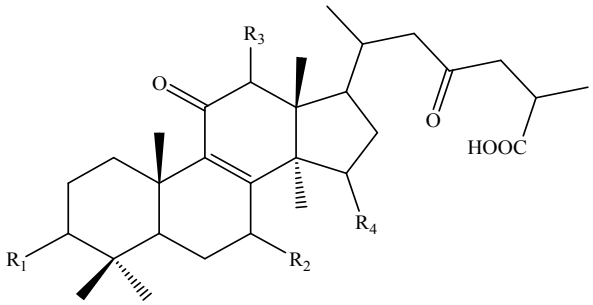
Ergot alkaloids (**2** and **3**), produced by *Claviceps purpurea* from tryptophan, have many pharmacological activities, which include vasoconstriction, induction of uterine contractions, stimulation of dopamine production, and inhibition of prolactin synthesis (Berde and Sturmer, 1978). Ergot alkaloid derivatives have found use in the treatments of migraine, control of uterine motor activity, therapy of Parkinson's disease and of senile dementia and disorders caused by hyperprolactinemia (Sameli, 1978).



Clavine alkaloids (**2**) R_1 = OH; Lysergic acid
R_1 = NH_2; Lysergic acid amide (**3**)

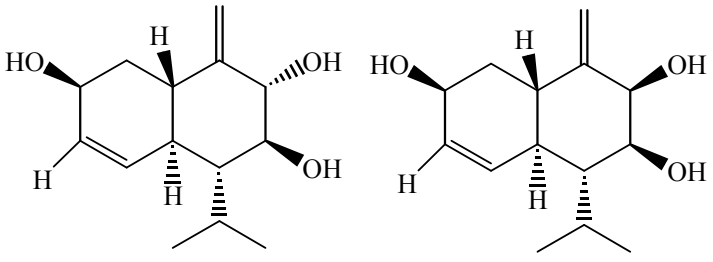
2.4.1 Basidiomycete *Ganoderma lucidum*

Ganoderma, a white rot fungus, is a prolific producer of novel mycochemicals. The fungus of white rot are not only involved in the fundamental process of lignocellulose degradation in nature but is also considered to have healing properties to the body and mind. The preparation has been found to contain bioactive ingredients such as triterpenes and polysaccharides that are beneficial for the prevention and treatment of a variety of ailments. Remarkably, these include ailments such as hypertension, diabetes, hepatitis cancers and AIDS (Huie and Di, 2004). *Ganoderma lucidum* has been found to contain predominantly triterpenes and polysaccharides (Ziegenbein *et al*., 2006). Lingzhi as *G. lucidum* is commonly called in China has been used for treatment of migraine, hypertension, arthritis, asthma, anorexia, gastritis, haemorrhoids and diabetes. Additionally, the presence of neuroactive compounds in the extract of Lingzhi that mediated the neuronal differentiation and neuroprotection of rat PC12 cells has also been demonstrated (Huie and Di, 2004). Unique constituents of Lingzhi isolated and associated with activity are the ganoderic acid B and C (**4**).



Ganoderic acid C2: R_1 = R_2 = *B* - OH, R_3=H, R_4 =OH
Ganoderic acid B:R_1 = R_2= *B* - OH, R_3 = H, R_4 = O (**4**)

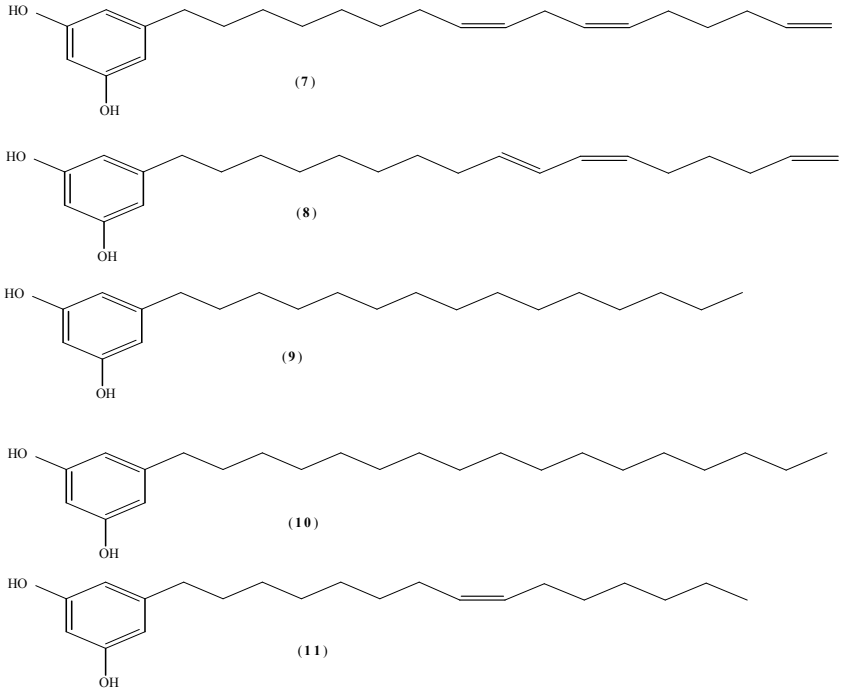
Reports (Li *et al*., 2005), have indicated that *G. lucidum* may have potential immuno-modulating effect in patients with advanced colorectal cancer. A research into polysaccharides includes: water soluble polysaccharides extracted from *G. lucidum* were effective in preventing DNA from strand breakage indicating the anti-tumour and immunomodulating activities of Lingzhi are strongly related to its anti-oxidative property. The anti-oxidant property of *Ganoderma* polysaccharide peptide decreased the oxidation of low density lipoprotein and exhibited anti-oxidant effect by scavenging reactive oxygen species in mice. A proteoglycan having a carbohydrate/protein ratio of 11.5 to 1 isolated from *G. lucidum*, was found to stimulate the proliferation of mouse spleen lymphocytes, resulting in a 3- to 4-fold increase in the (a) percentage of B cells, (b) secretion of immunoglobulin, (c) production of interleukin 2 and (d) expression of protein kinase. It was also found that the polysaccharide extracts from the mycelium of *G. lucidum* exhibited anti-tumour effects against fibrosarcoma in male and female mice and inhibited the metastasis of a tumour to the lung. The bioactive polysaccharides could stimulate blood mononuclear cells to increase cytokines, tumour necrosis factor, interferon and interleukin production, and the lifespan of tumour-implanted mice was found to increase significantly due to the administration of *Ganoderma lucidum* protein. Two protein-bound-polysaccharides, a neutral protein bound polysaccharide (NPBP) and an acidic protein bound polysaccharide (APBP), were isolated from water soluble substances of *G. lucidum*. APBP was found to be more potent than NPBP as an antiviral agent against herpes simplex viruses (HSV). The antiviral activity of APBP appeared to be related to its binding with HSV-specific glycoprotein at the cell membrane (Huie and Di, 2004). *G. lucidum* polysaccharides (Gl-PS) have shown a variety of immune modulating effects. Results confirmed that Gl-PS was a promising biological response modifier and immune potentiator (Zhu and Lin, 2006). Other bioactive compounds that have been isolated from *G. lucidum* include Ganomastenol A and B (**5** and **6**).



Ganomastenol A(**5**) Ganomastenol B(**6**)

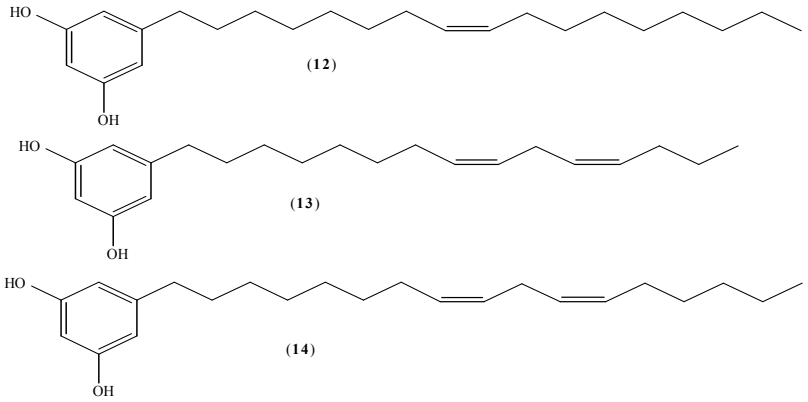
2.4.2 5-Alkylresorcinols from *Merulius incarnatus*

Basidiomycetes are considered by many authors as a major source of compounds having antimicrobial, antiviral, antifungal, anticancer, cardiovascular, anti-inflammatory, antioxidant, nematocidal activities (Zjawiony *et al.,* 2004). Alkylresorcinols have been found to have multiple biological activities, including antimicrobial (Jin and Zjawiony, 2006) and antitumor properties. Compounds (**7 – 14**) isolated from *Merulius incarnatus* were tested against the most common opportunistic infection pathogens such as *Candida albicans, C. glabrata, C. krusei, Cryptococcus neoformans*, methacillin-resistant *Staphylococcus aureus*, *Mycobacterium intracellulare*, and *Aspergillus fumigatus*.



Compounds **9** and **10**, containing saturated side chains, were inactive. The other compounds showed moderate to strong inhibition of MRSA. Compound **7** was the most active, with an IC_{50} of 2.5 µg/mL. Compounds **8**, **11**, **12**, **13**, and **14** were moderately active, with IC_{50} values of 15, 9.5, 8.0, 5.0, and 6.5 µg/mL, respectively, as compared to ciprofloxacin (IC_{50} = 0.1 µg/mL). Compound **12** also showed moderate activity against *Mycobacterium intracellulare*, with an IC_{50} of 10 µg/mL (ciprofloxacin, IC_{50} 0.4 µg/mL), and compound **13** showed weak activity against *Cryptococcus neoformans*, with an IC_{50} of 20µg/mL (amphotericin B, IC_{50} = 0.75 µg/mL). Compound **7** was also active in the anti-leishmanial assay, with an IC_{50} value of 3.6 µg/mL and IC_{90} value of 7.0 µg/mL, as compared

to penatmidine, with an IC_{50} of 0.6 µg/mL and IC_{90} of 1.9 µg/mL, and was not cytotoxic in the Vero cell test at concentrations up to 25 µg/mL (Jin and Zjawiony, 2006).

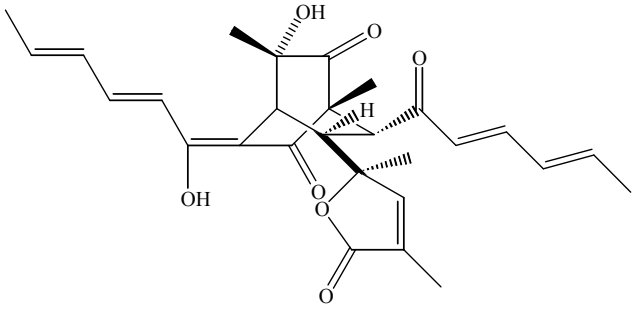


The research indicated the several structure-activity relationships: (7) an increase in the number of double bonds in the side chain (3-5, 5-7, 4-6, 6-8, 8-1) increases anti-MRSA activity, (**8**) the length of the side chain (34, 5-6, 7-8) has little effect on the activity, (**9**) the change of *cis*-*cis* methylene interrupted double bonds in the side chain to conjugated *trans*-*cis*-configuration (1-2) greatly decreases the activity (Jin and Zjawiony, 2006).

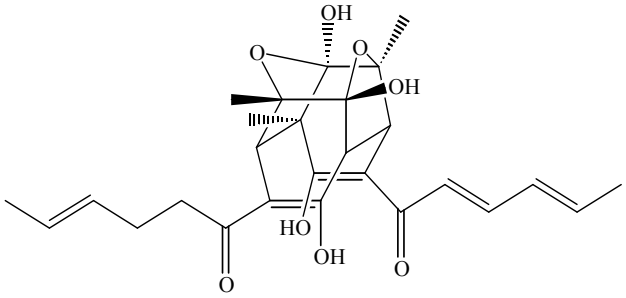
2.4.3 Fungal Secondary Metabolites with Potential Anticancer Activity

Purified bioactive compounds derived from medicinal mushrooms may be an important group of new anticancer agents (Balde *et al.,* 2010). Indeed, many patented anticancer compounds have already been identified in terrestrial fungi, such as polypores, which belong to the class basidiomycete. Apart from small cytotoxic compounds, high molecular weight polysaccharides isolated from the polypore cell walls also display direct anticancer effects and prevention of metastases through immunomodulatory activity (Zjawiony, 2004). Low-molecular-weight secondary metabolites from fungi that display potential anticancer activity target a large set of processes including apoptosis, angiogenesis, metastasis, cell cycle regulation, and signal transduction cascades (Zaidman *et al*., 2005). Some recent potential anticancer fungal metabolites isolated include bislongiquinolide (**15**) and dihydrotricodimerol (**16**) (Balde *et al.,* 2010).

The above compounds belong to the bisorbicillinoid group of compounds that includes all dimeric sorbicillin-derived natural products such as bisvertinoquinol, the bisvertinols, bisvertinolone, trichodimerol, trichodermolide, sorbiquinol, bislongiquinolide, the trichotetronines, bisorbicillinol, demethyltrichodimerol, bisorbicillinolide, and bisorbibetanone, which were all isolated from a new fungal source (Evidente *et al.,* 2009).



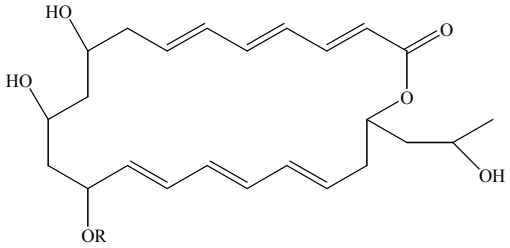
(**15**)



(**16**)

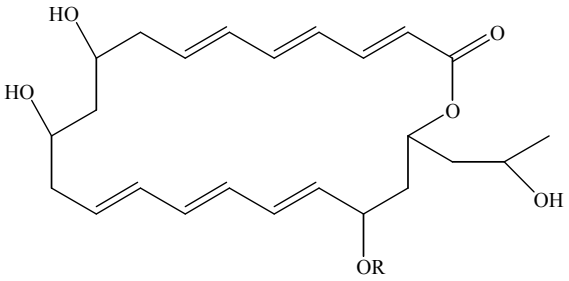
Data describing anticancer activity of (**15**) have already been reported by Liu *et al*., (2005). Dihydrotrichodimerol (**16**) has been reported to activate peroxisome proliferator-activated receptor-Y (PPAR-Y), (Lee *et al*., 2005) which exerts a major role in cancer cell biology (Ondrey, 2009). Dihydrotrichodimerol (**16**) has also been reported to suppress the production of tumour necrosis factor-R (TNF-R) and nitric oxide in LPS-stimulated RAW264.7 cells, (Lee *et al*., 2005).

In a study conducted in 2005, four novel 22 – membered macrolides designated wortmannilactones A – D (**17 - 20**) from a soil filamentous fungus *Talaromyces wortmannii* (Dong *et al.,* 2006*)*



(**17**) R = H

(**18**) R = CH_3



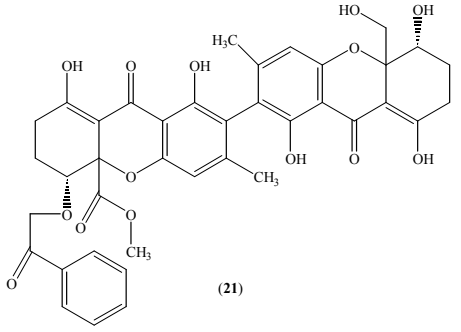
(**19**) R = H

(**20**) R = CH_3

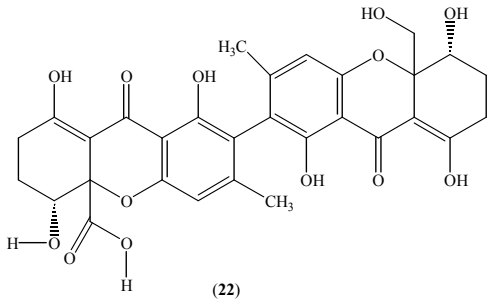
Macrolactin A (**19**) has been reported to show selective antibacterial activity and cytotoxicity against B16 – F10 murine melanoma cancer cells (IC_{50} = 3.5 µg/ml) and antiviral activities against *Herpes simplex* type I and type II (IC_{50} = 5.0 and 8.3 µg/ml respectively) and HIV (Gustafson *et al*., 1989; Choi *et al.,* 2003) as well as squalene synthase inhibitory activity (Choi and Lee, 2003).

2.4.4 Fungal Secondary Metabolites with Anthelmintic Activity

Xanthol isolated from a non-sporulating fungal species exhibited insecticidal and antihelminthic activities against larvae of *Lucila sericata*, *Aedes aegypti* and *Haemonchus contortus* with LD_{90} of 33, 8 and 50 µg/ml respectively (Ondekya *et al.,* 2006).



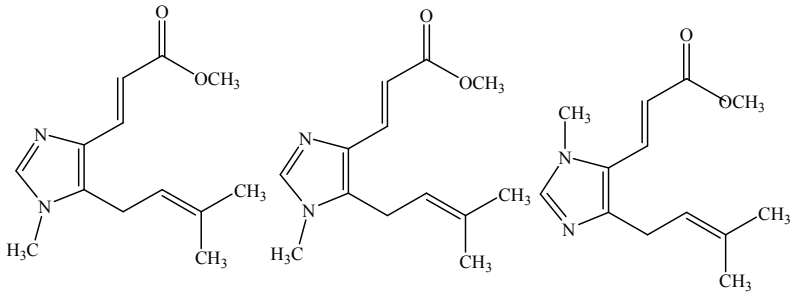
(**21**)



(**22**)

The dimeric compound, xanthol (**21)** belongs to a growing list of bis-xanthones that exhibit various biological activities. The hydrolytic product (**22**) was not active at the highest levels (50 – 500 µg/ml) tested.

New insecticidal antibiotics designated hydroxyfungerins A (**23**) and B (**24**) have been isolated from the culture broth of a fungal strain *Metarhizium* sp. FKI-1079 together with a known compound, fungerin (**25**) (Uchida *et al.,* 2005).

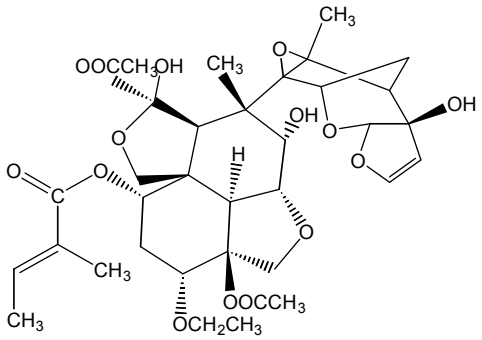


(**23**) (**24**) (**25**)

Fungerin (Fukuda *et al*., 2004) was originally isolated as an antifungal antibiotic from a *Fusarium* species. These compounds from *Metarhizium* species have reported antifungal active against *Penicillium chrysogenum*, *Colletorichum langenarium*, *Altenaria mali* and *Pyricularia oryzae*. In terms of insecticidal activity, fungerin was the most potent with MIC value of 0.39 µg/ml (1.7 µM), hydrofungerin A (**23**) and hydrofungerin B (**24**) exhibited moderate inhibition with MICs of 6.25 µg/ml (25 µM)

2.5 Mosquito Larvicides and Insecticides from Natural Sources

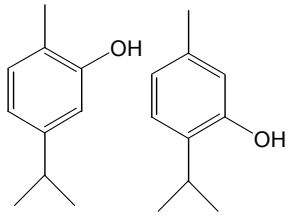
Most natural larvicidal and insecticidal compounds have been reported from plants. Numerous plants have been shown to produce pesticidal compounds, as a chemical defence mechanism against predators or infection. One of the most commonly studied plants for control of mosquitoes is *Azadirachta indica* (Meliaceae) commonly known as neem in India, (ICMR, 2003). Azadirachtin (AZA) (**26**) is an insecticidal compound, isolated from it. Though neem products show a high larvicidal activity, which is attributed to the epoxide ring function, they do not show adulticidal action (ICMR, 2003).



Azadirachtin (**26**)

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 such as cancer related anti-inflammatory, antiallergic, antiviral and molluscidal activities. It was found that young larvae of both *Ae. aegypti* and *C. piperins* were susceptible to saponin application (Pelah *et al.,* 2003) although the major components responsible for the larvicidal activity were not isolated.

Essential oils were the first preservatives used by man, originally in their natural state within the plant tissues and then in oils obtained by water distillation. These oils being composed of isoprenoid compounds; mainly mono- and sesquiterpenes are carriers of smell found in the aromatic plants. Essential oils have received much attention as potentially useful bioactive compounds against insects. A study was carried on essential oils obtained from the shoot of *Lippia sidoides* in which co-distilled water called hydrolate was produced. Pure hydrolate caused larval mortality of *Ae. aegypti* and it was revealed that its main constituents were carvacrol (**28**) and thymol (**27**). It was also found that thymol (**28**) was the active essential oil hydrolate (Carvalho *et al.,* 2003).



Thymol (**27**) Carvacrol (**28**)

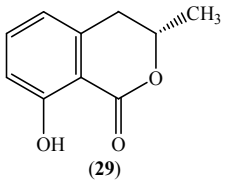
2.6 Biologically Active Material from Microbial Metabolites for Larval Control

Bacillus spp. based larvicides are increasingly replacing, with numerous advantages, chemical insecticides in programmes for controlling black fly and mosquito populations (Regis *et al*., 2000). The use of microbial biopesticides such as *Bacillus thuringiensis* and *Bacillus sphaericus* entomopathogenic microorganisms to control insects is not new. *B.*

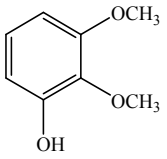
thuringiensis and *B. sphaericus* are widely used to control insect larvae, and entomopathogenic fungi are used to control agricultural pests. What is new is their use against adult mosquitoes. The fungus *Beauveria bassiana* (Blanford and Gilles, 2005) has shown the ability to kill anopheline mosquitoes. The fungi were applied by spraying containers of mosquitoes with an oil formulation of infectious spores. Upon contact with a mosquito, the fungal spores (conidia) begin development and invade the mosquito, after which the fungus multiplies and kills its host within two weeks. Blanford and Gilles (2005) also demonstrated that exposure to the fungus not only caused higher mortality rates in malaria-infected mosquitoes but also reduced the proportion of surviving mosquitoes carrying sporozoites in their salivary glands. Moreover, fungus infected mosquitoes were less likely to take subsequent blood meals than were uninfected mosquitoes.

2.7 Some of the Recent Works on the Basidiomycete Collected from Kenya

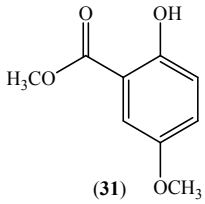
The Kenyan basidiomycetes have been studied and have proved to be rich in bioactive metabolites. Some mosquito larvicidal compounds were isolated from undescribed basidiomycete serialized JO5182. Three compounds; methyl-2-hydroxy-4-methoxybenzoate (**29**), 2,4-dimethoxyphenol (**30**) and (R)-(-)-8-hydroxy-3-methyl-3,4-dihydro-1H-2-benzopyran-1-one (**31**) were isolated and their structures are as shown below (Kendagor, 2008).



(**29**)

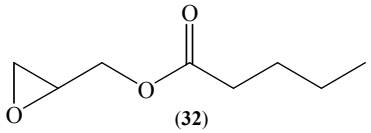


(**30**)



(**31**)

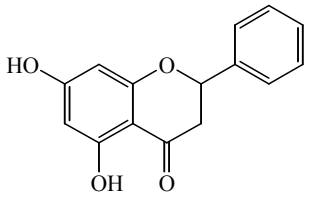
The basidiomycete *Cyptotrama asprata* was also studied and a novel compound identified as (oxiran-2-yl) methyl pentanoate (**32**) was isolated from this fungus. The LC_{50} and LC_{90} after 24 hours of this compound against larvae of *Ae. aegypti* in their late third and early fourth instars were found to be 1.50 ppm and 1.90 ppm. This was quite a high activity as compared to the most compounds isolated from other fungi (Njogu, 2009).



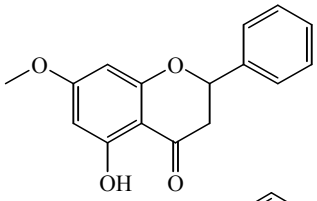
(**32**)

Some antibacterial compounds (**33 – 36)** were isolated from basidiomycete *Collybia* species (JO5191) and their minimum inhibitory concentrations (MIC) were found to be in the

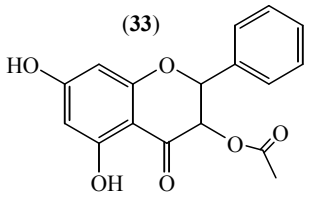
range of 3–12 μg/ml, agreeing considerably well with those reported from previous literature (Mutambi, 2007):



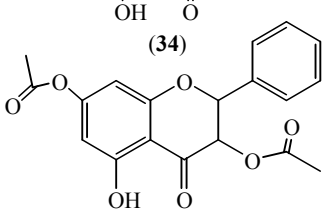
(**33**)



(**34**)



(**35**)



(**36**)

2.8 *Coriolus hirsutus* basidiomycete

Coriolus hirsutus is also found naturally in Europe and North America and it grows on wood. It is inedible, white to cream, yellow or sometimes grey with an odd smell and the normal size usually less than five centimetres.

C. hirsutus is known to possess laccase which has been shown to significantly increase adsorption coefficient and to facilitate irreversible adsorption of atrazine to soil. Laccase introduction aids in atrazine binding to soil. In the presence of laccase, the binding occurs by oxidative binding mechanism. Thus, interaction between atrazine and soil in the presence of laccase results in irreversible binding of the herbicide (Davidchik *et al*., 2008). Whereas only the so-called "free," or unbound atrazine displays toxicity, laccase presence in soil contributes to atrazine detoxication in the environment. Now laccase-producing basidiomycetes and laccases themselves are widely used in biotechnological utilization of lignin containing wastes and in recultivation of media contaminated by polychlorbiphenyls (PCB), polynuclear aromatic hydrocarbons (PAH), synthetic dyes, and herbicides.

CHAPTER THREE
MATERIALS AND METHODS

3.1 The Basidiomycete *Coriolus hirsutus* (JO5185)

The basidiomycete *C. hirsutus* (figure 1) was collected from Mt. Kenya forest in July 2005, the fungus was serialised JO5185 and preserved in Integrated Biotechnology Research Laboratory (IBRL) at Egerton University as a herbarium material and pure culture. The morphological and discernable microscopic features confirmed that the fungus had clamp connections – the unique characteristic distinction of the basidiomycetes.

Figure 1: ***Coriolus hirsutus***

3.2 Test insects (*Aedes aegyti* larvae)

Larvae were collected from Pyrethrum Board of Kenya when they are one day old and brought to laboratory for testing before they pupated. In order to establish LC_{50} and LC_{90} values (the concentrations of extracts in ppm required to kill 50% and 90% of larvae, respectively, within 48 hours), multiple 10-fold dilutions of the extract stock solution were prepared to provide a working concentration range. Three replicate assays were carried out for every sample concentration, each with 10 larvae. Larvae were observed at the start of the assay, 2 hours interval and after 24 and 48 hours, and considered dead when they do not respond to stimulus or when they do not rise to the surface of the solution (WHO, 1981).

Negative controls accompanied each assay and involved treating larvae with water or with 1% DMSO in water. The LC_{50} and LC_{90} were calculated only for the most active extracts. The dead larvae counted after every 2 hours, were used to calculate the percentage mortality reported from the average for the three replicates taken.

3.3.1 Sterilization of apparatus and materials used

To ensure that the cultures of *C. hirsutus* were not contaminated, hands and working benches were thoroughly and constantly sterilised using 70% ethanol. The working environment was maintained sterile by use of bunsen flame that was also used to sterilise the spear blade as well opening and closing corked sterile conical flasks. The liquids and apparatus were sterilised using an autoclave. This involved steam that generated high temperatures and pressure. Sterilisation of materials were done at temperature of 121^{o}C and pressure 1.5 bars. The autoclave (Danfoss 59407-3 No. 375) was also used to destroy infected cultures and used test plates.

3.3.2 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 litre of water. The pH of the media was determined and adjusted to 5.5 using pH meters modelled Fishes Acument ® model 620A. These were corked with cotton wool plugs and then wrapped with an aluminium foil. They were then autoclaved at a regular temperature of 121°C and pressure 1.5 bars for 15 minutes. The media was sterilised two times after which the sterile conical flasks were left to cool at room temperature and then placed in the working bench of a sterile laminar flow hood.

3.3.3 Inoculation and culturing of the basidiomycete *C. hirsustus* (JO5185)

This was done in an integrated biological safety cabinet lamina flow hood under extremely sterile conditions. Preserved mycelia material on an agar slant was transferred on to potato dextrose agar in test plate. Three week old solid cultures were used to inoculate liquid cultures grown in replicate 500 ml Erlenmeyer flasks containing 250 ml of liquid culture. After two weeks, each of these cultures was used to inoculate each of the 2 litres Erlenmeyer flasks containing 1 litre of the liquid media. Cultures were grown for 21 days with regular shaking (at least twice a day) at ambient conditions. The cultures were then harvested by vacuum filtration.

3.4 Preparation of crude extracts

The mycelium collected as the residue after filtration was soaked in acetone with constant agitation using magnetic and stirring rod for 4 hours. This was then decanted and the residue discarded while the filtrate was dried using anhydrous sodium sulphate before concentration using a Rotary vacuum Evaporator type 349/2 to recover the solvent. The concentrated fraction was transferred to screw capped vial and kept in the fridge at 4°C.

3.4.1 Determination of the dry weight of the crude sample

The crude extract was dissolved in minimal amount of methanol. In a tared universal bottle, 2.0 g of silica gel was weighed (using Weighing balance – Denver instrument Model – XL-31000) and to it small amounts of methanol extract solution was slowly added using pasteurised pipettes. The solvent was then evaporated using a heating module. Weighing was done and the difference found to be the mass of the crude extract.

3.4.2 Column chromatography

A glass chromatographic column 45 cm in height and 1.5 cm diameter was mounted vertically using clamps. Approximately 50.0 g of silica gel was suspended in cyclohexane, and then swirled vigorously to obtain homogenous slurry. The slurry was degassed using a water pump to remove any gas trapped in between. The column was slurry packed with the silica gel suspension, ensuring that no air was trapped in between. The dry silica gel adsorbed sample was then loaded onto the column uniformly above the slurry. The set up was anchored in place with acid washed sand. The column was developed with discrete solvent gradient system with increasing polarity from cyclohexane to ethyl acetate and finally methanol as shown in table 1.

Table 1: Solvent system used to elute the column

Solvent mixtures	% Volumes of solvents (ml)		
	cyclohexane	ethyl acetate	methanol
1	70	30	0
2	50	50	0
3	30	70	0
4	0	100	0
5	0	0	100

These eluents were collected and pooled into fractions based on the elution time and the analysis on the TLC plate. The pooled fractions were concentrated by removing the organic solvents under reduced pressure using rotary evaporator. Each was separately washed out using 100% methanol from the round bottomed flask and then transferred to a screw-capped vial and labelled before being kept under refrigeration. Each of these fractions was re-dissolved in 2 ml of methanol and from each several fractions of varying concentrations made they were tested against the larvae according to the procedure outlined in section 3.2.

3.5 Preparation of stock solutions and test concentrations

The concentration of stock solution was 20 ml of 1%, obtained by weighing 200 mg of the technical material and adding 20 ml of solvent to it. It was kept in screw-cap vial; with aluminium foil lining on the lid. This was thoroughly shaken to dissolve the material in the solvent. The stock solution was then serially diluted ten-fold in methanol (2 ml of solution in 18 ml of solvent). Test solutions were obtained by adding 0.1-1.0 ml (100-1000 μl) of appropriate dilution to 100 ml or 200 ml of chlorine-free distilled water starting with the lowest concentration. These small volumes were transferred to test cups by means of micropipettes with disposable tips. The addition of small volumes of solution to 10ml, 200ml or greater volumes of water did not cause noticeable variability in final concentrations. Distilled water was used in preparation of the 1% stock solution and the subsequent serial dilutions according to the content of the active ingredient. This assisted in determination of the lethal concentrations, LC_{50} and LC_{90}.

3.6 Mosquito larvae bio-assay

Initially the mosquito larvae were exposed to wide range of test concentrations and a control to find out the activity range of the materials under test. After determining the mortality of the larvae in the wide range of concentrations, a narrower range of 4-5 concentrations yielding between 10% and 95% mortality in 24 hours or 48 hours was used to determine the LC_{50} and LC_{90} values. Batches of 25 third and fourth instars larvae were transferred to small disposable vessels each containing 10-20 ml of water avoiding transfer of small and unhealthy larvae. The depth of the water in the cups was maintained between 5 cm and 10 cm.

The appropriate volume of dilution was added to 100 ml or 200 ml water to obtain the desired target dosage, starting with the lowest concentration. Four replicates were set for each concentration and an equal number of controls set up simultaneously with tap water to which

1 ml of methanol was added. Each test was done three times on different days. The test containers were held at room temperatures (25-28°C) and preferably photoperiod of 12 hours followed by 12 hours dark (12L: 12D).

After 24 hour of exposure, larval mortality was recorded. For slow acting larvicidal fractions, 48 hour reading was adopted. Moribund larvae were counted and added to dead larvae for calculating percentage mortality. The results were recorded in a form where the LC_{50}, and LC_{90} values and slope and heterogeneity analysis noted. The form contained three separate tests of six concentrations, each of four replicates. The following precautions were taken;

i. Larvae that pupated during the test period negated the test.
ii. If more than 10% of the control larvae were noted to have pupated in the course of the experiment, the test was discarded and repeated.
iii. If mortality in the control was between 5% and 20% then the mortalities of treated groups were corrected according to Abbott's formulae:

$Mortality\ (\%) = \frac{(X - Y)}{X} x100$ where X = percentage survival in the untreated control and Y = percentage survival in the treated sample.

3.7 Data analysis

Data from all the replicates were pooled for analysis. LC_{50} and LC_{90} values were calculated from Log dosage-Probit mortality regression line using SPSS 11.5 software. Standard deviations and confidence intervals of means of LC_{50} and LC_{90} were also recorded.

3.8 Purification of the compounds

Purification was carried out with column chromatography packed with silica gel as the stationary phase. The mobile phases were constituted as discrete gradients of increasing solvent polarity from non-polar to polar solvents using cyclohexane-ethyl acetate-methanol mixtures.

3.9 Structure elucidation

This was done by spectroscopic techniques, this included;

3.9.1 NMR spectroscopy

The purified compound was dissolved in deuterated chloroform $CDCl_3$. Both proton and carbon NMR experiments were performed. For all pure compounds, 1- and 2-D

heteronuclear-correlated NMR spectroscopy was performed. The structures were determined by the interpretation of the spectra (see appendices section). One dimension (^{1}H-NMR and ^{13}C-NMR) and two dimensional (COSY, HMBC, HSQC and NOESY) experiments were performed. All experiments were carried out in Bruker AV drx500 MHz spectrometer. All the spectra were recorded at room temperature and the chemical shifts recorded/given in parts per million (ppm) relative to the TMS peak.

3.9.2 Mass spectrometry

Structures were confirmed by use of mass spectrometry. The relative atomic masses of the compounds were determined. The fragmentation patterns in the mass spectrum were studied and interpreted to determine molecular formulae.

CHAPTER FOUR

RESULTS AND DISCUSSION

4. 1 Results

4.1.1 Cultivation of *Coriolus hirsutus* JO5185

To determine the best period of cultivation an experiment was conducted which concurred with the documented guidelines on cultivation of basidiomycete for the sake of targeting the secondary metabolites. The results were recorded in table 2.

Table 2: Percentage larval mortality in 200 µl of Mex

No of days cultured	Time			
	2 hours	4 hours	8 hours	24 hours
5	0	0	0	40
10	0	0	0	70
15	10	10	10	70
20	20	20	50	90
25	0	0	20	50
30	0	0	0	0

There was notable activity in the mycelia extract above and also in the culture filtrate as shown in table 3 below.

Table 3: Percentage larval mortality in 200 µl of Kex

No of days cultured	Time			
	2 hours	4 hours	8 hours	24 hours
5	0	0	0	30
10	0	0	0	40
15	0	0	0	40
20	20	20	30	100
25	0	0	10	50
30	0	0	0	0

4.1.2 Preparation of crude extracts

Coriolus hirsutus was cultured under laboratory ambient conditions and the growth was stopped after 21 days when the glucose levels were depleted. After preparation of the crude extracts consisting of the culture filtrate and mycelium extracts was done, the extraction was done using distilled solvents followed by concentration, drying and finally weighing. The crude extract from the culture filtrate (Kex) was 750 mg while the mycelium extract (Mex) was 860 mg (see figure 2).

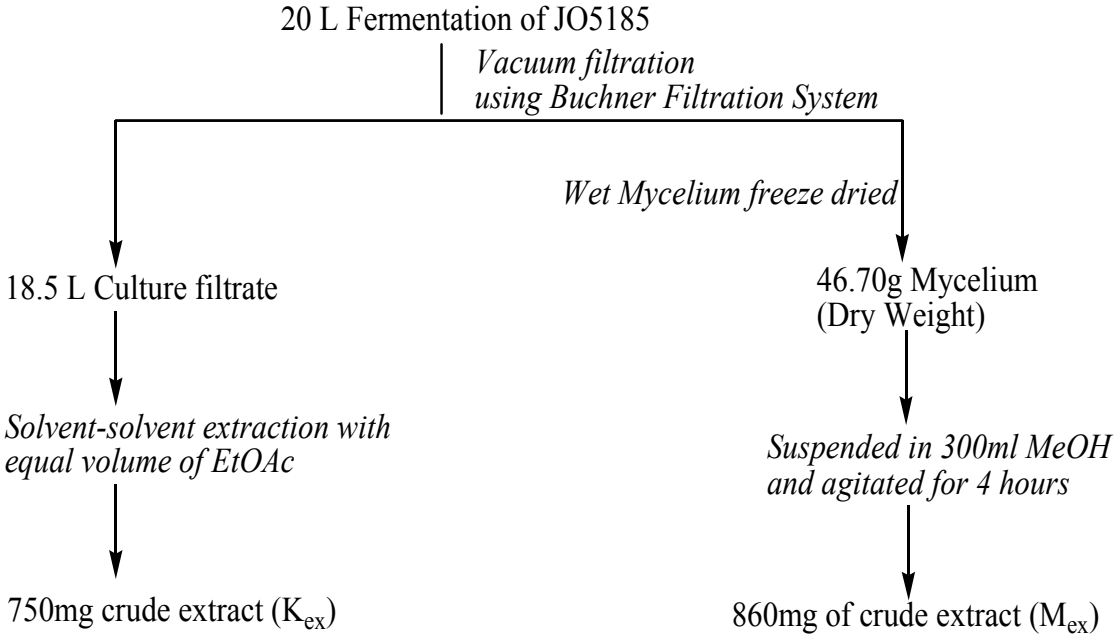


Figure 2: Scheme of extraction of Kex and Mex

4.1.3 Larvicidal activity test for crude extracts

Both the culture filtrate and the mycelium extracts were tested for larvicidal activity as described in section 3.6. The concentration of the extracts ranged between 200-1000 ppm in which larval mortality was scored after 2, 4, 8 and 24 hours. The results are summarized in tables 4 and 5. Distilled water and methanol were used as standards both showed negative results.

Table 4: Percentage larval mortality and lethal concentrations of Kex

Concentration (ppm)	Time			
	2 hours	4 hours	8 hours	24 hours
400	0	0	0	10
600	0	20	40	80
800	10	40	60	100
1000	50	100	100	100
LC_{50} (ppm)	-	872.5	799.2	624.6
LC_{90} (ppm)	-	1512.4	1449.8	1299.8

From table 4, it was noted that the activity was as low as at 400 ppm concentration within the range 0 – 10% mortality for the whole period of mortality (see table 4). High mortality was also observed for concentration of 800 ppm after 24 hours and for concentration of 1000 ppm after 4 hours.

Table 5: Percentage larval mortality and lethal concentrations of Mex

Concentration (ppm)	Time			
	2 hours	4 hours	8 hours	24 hours
200	0	0	0	10
400	0	0	40	60
600	10	40	40	100
800	50	100	100	100
1000	100	100	100	100
LC_{50} (ppm)	872.5	723.3	644.7	479.9
LC_{90} (ppm)	1512.4	1384.8	1317.1	1155.3

According to tables 4, Mex was considered low at a concentration low (10%) after 24 hours. The activity noted to be significantly high (100%) at a concentration of 600 ppm after 24 hours, 800 ppm after 4 hours and 1000 ppm after 2 hours. From the above Mex was found to have a higher activity than Kex comparing tables 4 and 5.

4.1.4 *Coriolus hirsutus* (JO5185) Mex purification

The 0.86 g of the mycelia extract was subjected to larvicidal guided purification using a column of 7.0 cm length and a diameter of 4.5 cm. Five compounds were sent for analysis but only one (RMF2S1) gave spectra that could be used to predict its structure (see figure 3).

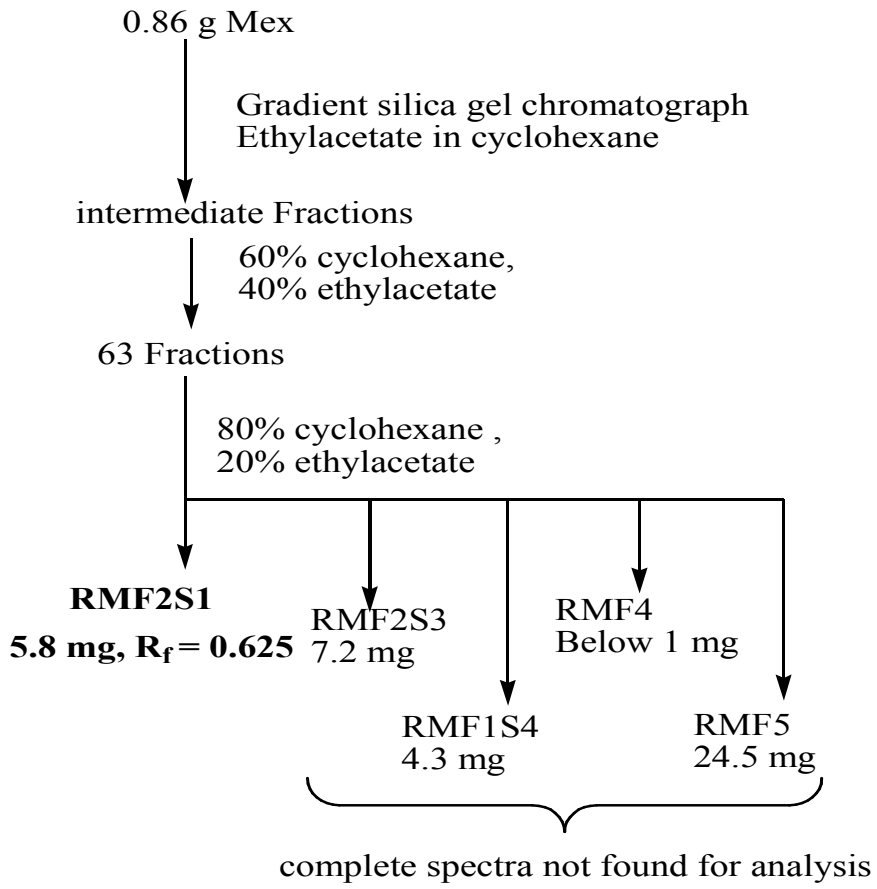


Figure 3: Scheme for purification of mycelium extract (Kex)

4.1.5 *Coriolus hirsutus* mycelium extract purification

The 750 mg of the crude extract (Kex) was subjected to chromatography with gradient elution with increasing polarity of ethyl acetate in cyclohexane. This led to the elution of 14.7 mg of RKF9 at R_f of 0.3 as shown in figure 4.

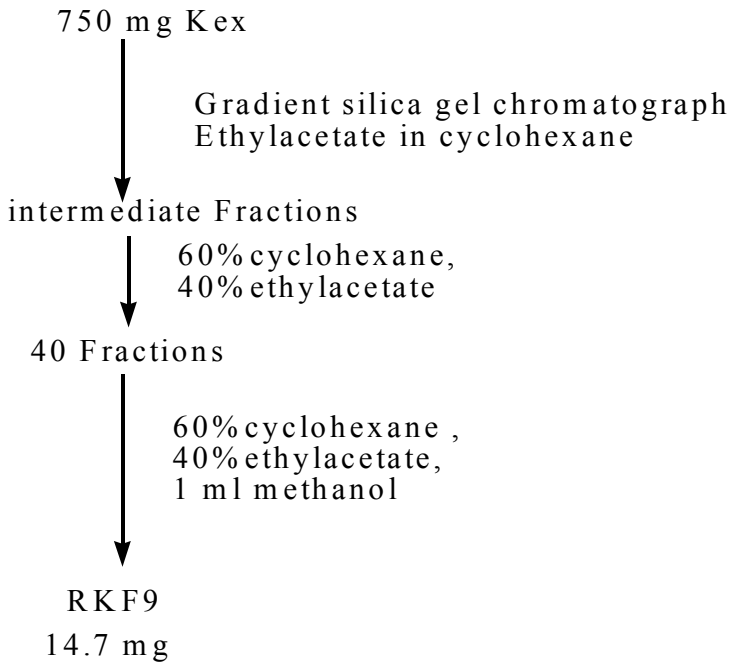


Figure 4: Scheme for purification of Culture filtrate extract (Mex)

4.1.6 NMR and MS experiments

Spectroscopic experiments were run on the samples to determine their chemical structures (see appendices section). One dimensional experiments; ^{1}H – NMR, ^{13}C – NMR, were combined with two dimensional experiments; COSY, HSQC NOESY and HMBC spectra were used in conjunction with MS spectra to propose the structures. The proposed chemical structure was further authenticated by comparison of the spectral data with those available in the literature.

4.2 Discussion

4.2.1 Cultivation period for *Coriolus hirsutus*, (JO5185)

The basidiomycete was collected from Mt. Kenya forest in July 2005, the fungus was serialised JO5185 and preserved in Integrated Biotechnology Research Laboratory (IBRL) at Egerton University as a herbarium material and pure culture. The morphological and discernable microscopic features confirmed that the fungus had clamp connections – the unique characteristic distinction of the basidiomycetes. *Coriolus hirsutus* was cultivated in nutrient liquid media and was found to produce mosquito larvicidal compounds at 50-100 ppm by using the crude extracts.

A plot of percentage mortality versus time was made in order to determine the optimum cultivation periods of basidiomycete for the production of the larvicidal compounds (see figure 5).

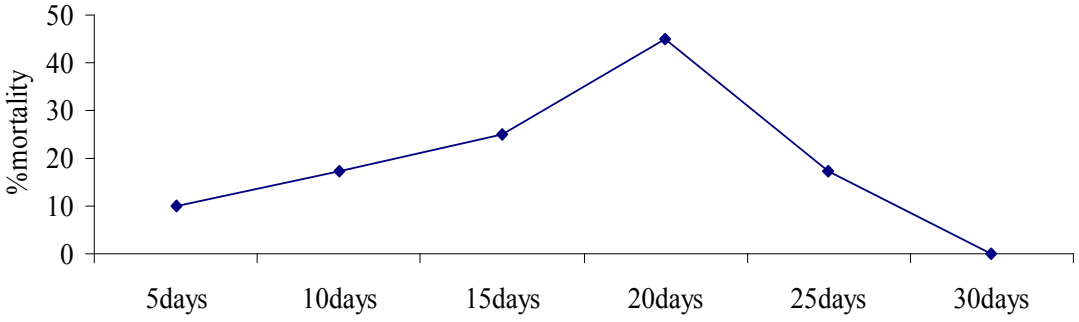


Figure 5: Determination of the optimum cultivation period for Mex harvesting

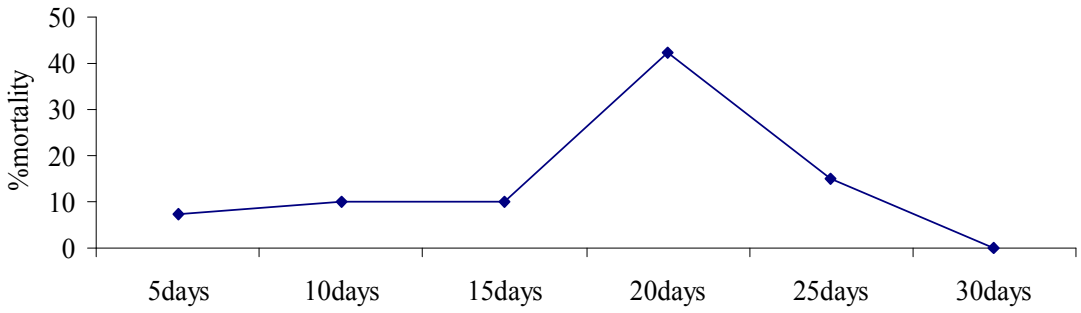


Figure 6: Determination of the optimum cultivation period for Kex harvesting

It can be observed from figures 5 and 6 that the highest larval mortalities were recorded at day 21 in both the Mex and Kex experiments. It can therefore be concluded that the highest concentrations of secondary metabolites with larvicidal activity are produced at day 21.

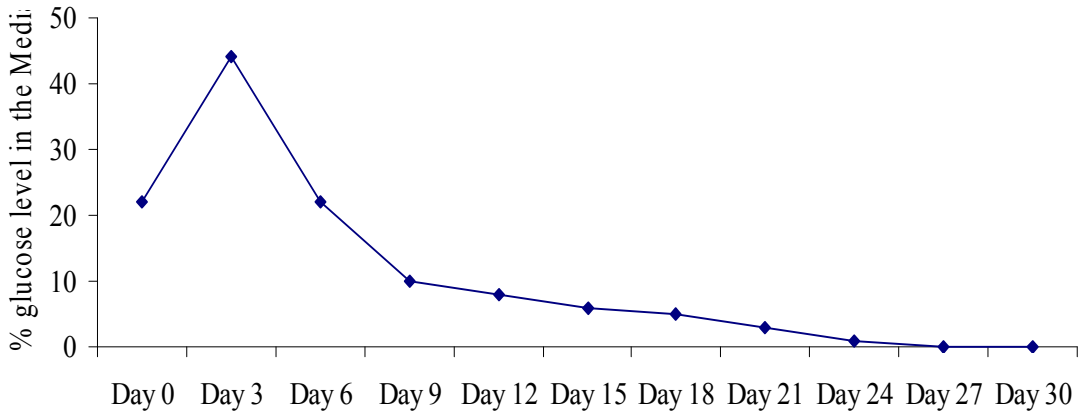


Figure 7: Graph of percentage glucose level in the media over time

The percentage glucose level during the fermentation process was also monitored over time (see figure 7). A rise in the glucose level was noted between day 1 and day 3. This was followed by gradual decrease in the glucose levels between day 7 and day 27 where the entire glucose source was exhausted. The sudden increase of glucose level on the third day would be attributed to the initial fermentation process of molasses in the presence of yeast. As the fungi grow so is the diminishing level of glucose. The liquid media constituted glucose, malt and yeast extract. Glucose served as the initial source of energy before the cultivated mycelia could be able to digest the complex cellulose in molasses. Fungi utilize glucose as the sole source of carbon and energy. Because glucose plays a vital role in the energy and material economies of organisms, its metabolism is the large regulatory phenomenon in fungal metabolism. Glucose is metabolised by glycolysis and a strong fermentation takes place even under aerobic conditions.

The basidiomycete *C. hirsutus* took 27 days to grow in liquid malt media at room temperature in the IBRL. After 21 days the basidiomycete was harvested by vacuum filtration. Immediately after harvesting, crude extracts were prepared (Mex and Kex). These were used in the preliminary bioassay tests.

4.2.2 Bioassay tests

From the table 2, it was noted that higher concentration of 1000 ppm of the mycelia extract (Mex) was more efficacious from the start of the 2 hours, which had 100% mortality. The 400 ppm was able to kill 40% of the larvae after 8 hours and which increased to 60% after 24 hours. However, at 24 hours its efficacy was 100%. Concentrations of 200 ppm was able to cause only 10% 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 1000ppm after 24 hours of 100% mortality was still significant given that it was observed for crude extract. Hence the crude extract was further investigated for the compounds responsible for activity using activity guided separation and purification.

The Culture filtrate crude extract (Kex) table 3 was similarly prepared and tested in duplicate for larvicidal activity against *Ae. aegypti*. Just like the mycelia crude extract above, only 1000 ppm showed 100% larvicidal activity after 4 hours. The 400 ppm of the culture filtrate (Kex) had a lower activity as compared to the same concentration of the mycelium filtrate (Mex). It was also noted that the Mex (LC_{50} = 479.6 ppm) had a relatively higher activity compared to the Kex (LC_{50} = 624.6 ppm), but this can only be approximate since these are crude extracts.

4.2.3 Purification of Kex and Mex

During the purification gradient elution was employed with most compounds being eluted about a concentration of 60% cyclohexane and 40% ethyl acetate confirming that the active compounds were of middle polarity. The following compounds were later subjected to structure elucidation though only two had their structures analysed.

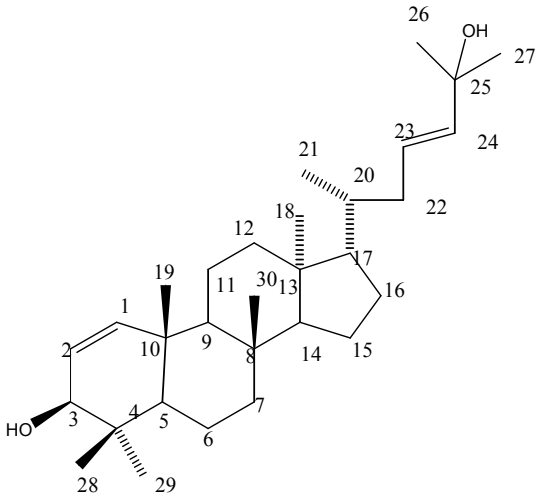
Table 6: Compounds purified and sent for analysis

S/No	Label	Weight of the compound (mg)
1	RKF9	14.7
2	RKF4SI	6.8
3	RMF5	24.5
4	RMF3S4	1.2
5	RMF3S1	4.6
6	RMF2S3	7.2
7	RMF1S4	4.3
8	RMF2S1	5.8

4.2.4 Structure elucidation of RMF2S1

The pure compound RMF2S1 was obtained as a brown amorphous powder with a melting point 660.66 K and boiling point 756 K The molecular formulae of compound **37** was assigned based on the molecular ion peak at m/z 441.73 $[M]^+$ in the Electro Spray Ionisation (ESI) mass spectrum (appendix 14). Also evident is the $[M+C_2H_5^+]$ at m/z = 413.2655.

The ^{1}H (appendix 2) and ^{13}C NMR (appendix 3), $^{1}H - {}^{1}H$ COSY (appendix 4), DEPT (appendix 5), HMBC (Appendix 6) and HSQC (appendix 7) spectra were all instrumental in the constitution of the skeleton structure of the compound. Other structural information was identified and confirmed on the basis of the MS (appendix 13) and NOESY (appendix XVI and XVII) spectra. Both ^{13}C NMR and ^{1}H-NMR indicated the presence of double bond at C1 and C2 spinning at $\delta = 6.49$ and 6.51 respectively for 1H – NMR and both $\delta = 133.6$ and 133.4 for ^{13}C – NMR. The same was also noted for the double bond at position 23 and 24 (see the table and appendices). The DEPT NMR also indicate presence of seven methylene groups as indicated in the table confirming cyclic nature of the compounds. From HSQC spectra, hydrogen atoms attached to carbon atoms can be noted. There is a correlation between protons at $\delta = 6.35$ and 6.38 both at C1and C2 (135.40 and 135.60) respectively indicating a presence of double bond. The molecular ion peak as studied from the mass spectrum is at 442.73 indicating that the molecular mass of the compound is 442.73 and the molecular formulae suggested as $C_{30}H_{50}O_2$ with a molecular mass of 442.73 with the proposed name being 17–(5–Hydroxy–,5-dimethyl-hex-3-enyl)-4,4,8,10,13-pentamethyl-4,5,6,7,8,9,10,11,12,13,14,15,16,17-tetradecahydro-3*H*-cyclopenta[α]phenanthren-3-ol.

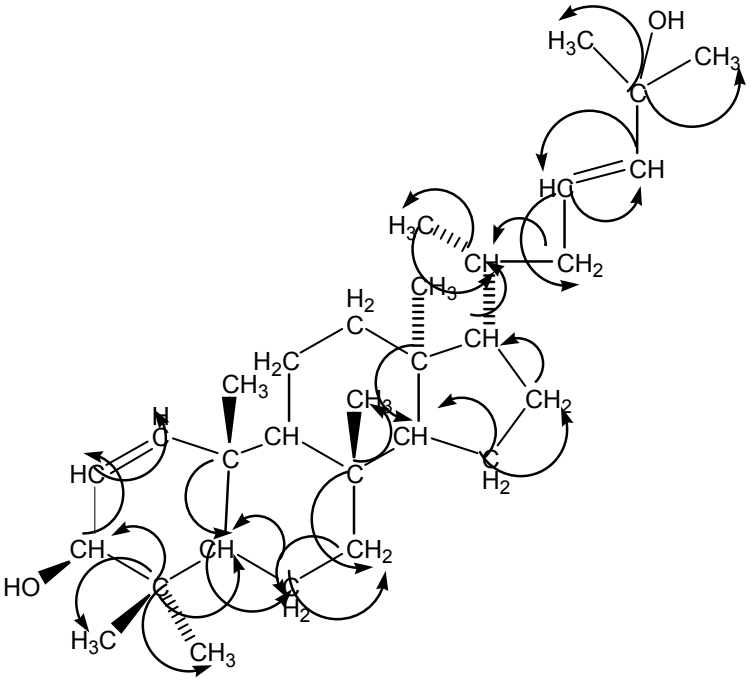


17–(5 –Hydroxy –1,5-dimethyl-hex-3-enyl)-4,4,8,10,13-pentamethyl-4,5,6,7,8,9,10,11,12,13,14,15,16,17-tetradecahydro-3*H*-cyclopenta[α]phenanthren-3-ol. (**37**)

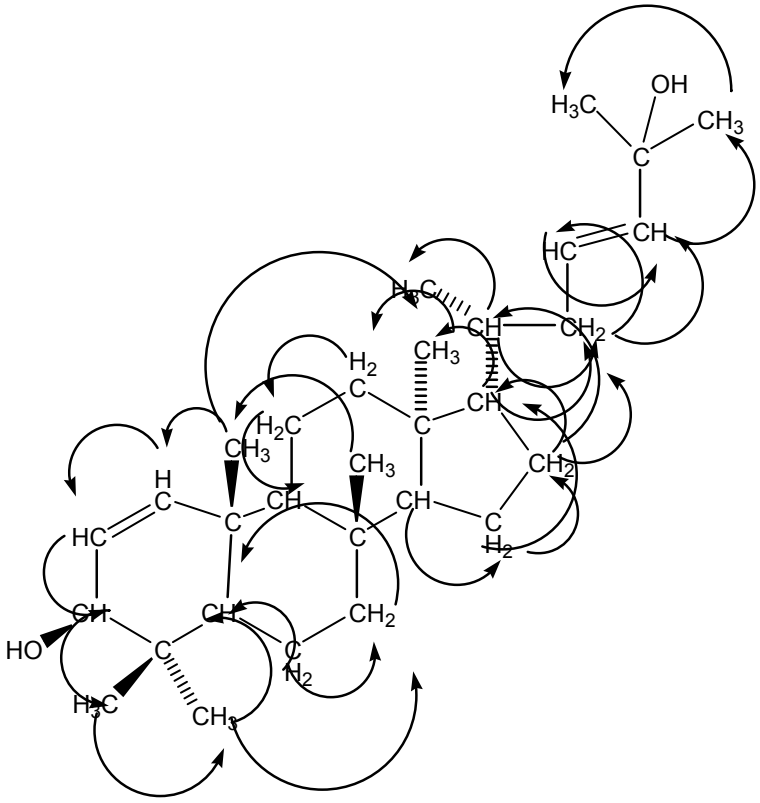
Table 7: NMR spectroscopic data for RMF2S1

Position	^{1}H multiplicity (*J* Hz)	^{13}C	DEPT	NOESY
1	6.493	135.6	CH	H2
2	6.510	135.4	CH	H1/H3
3	3.67	66.7	CH	OH/H2
4	-	56.4	-	-
5	1.60	51.9	CH	H6
6	1.25	51.3	CH_2	H5/H7
7	1.21	44.8	CH_2	H6
8	-	39.9	-	-
9	1.47	37.1	-	H11
10	-	34.9	-	-
11	1.25	33.3	CH_2	H9/H12
12	1.21	30.3	CH_2	H11
13	-	29.9	-	-
14	1.46	28.9	CH	H15
15	1.47	23.6	CH_2	H14/H16
16	1.47	21.1	CH_2	H17/H15
17	1.47	20.9	CH	H16/H20
18	1.16	20.2	CH_3	-
19	1.26	19.9	CH_3	-
20	1.28	18.4	CH	H21/H22
21	1.06	17.8	CH_3	H20
22	1.92		CH_2	H20/H23
23	6.232	132.5	CH	H22/H24
24	6.249	130.7	CH	H23
25	-	71.7	-	-
26	1.36	30.1	CH_3	-
27	1.36	30.1	CH_3	-
28	1.11	19.2	CH_3	-
29	1.11	19.2	CH_3	-
30	1.16	21.3	CH_3	-

400MHz (^{1}H), 100MHz (^{13}C) in $CDCl_3$; δ values are given in ppm and the *J* values (in parentheses) in Hz. All data were assigned based on the observed 2D NMR spectral correlations.

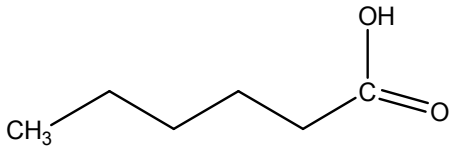


Observed ^{1}H – ^{13}C long range couplings (H-C) for compound (37)



Observed ^{1}H – ^{1}H and long range coupling compound (37)

4.2.5 The structure elucidation of compound RMK9



Hexanoic acid (**38**)

The hexanoic acid **(38)** was purified from the enriched fraction 9 was purified using column chromatography. The solvent system used constituted 60% cyclohexane and 40% ethyl acetate. The compound was collected after approximately 90 ml of the eluting solvent were passed. Hexanoic acid **(38)** was obtained as yellowish–oily liquid and had a molecular formula $C_6H_{12}O_2$ as determined by the analysis of ^1H and ^{13}C NMR spectral data (table 7). Its yield was 14.7 mg of the enriched culture filtrate. On the TLC plate, hexanoic acid gave an R_f of 0.475. Its structure was studied by use of NMR spectroscopy – both 1D and 2D NMR experiments.

The $^1H/^{13}C$ – HMBC NMR spectrum (appendix 11) and $^1H/^{13}C$ HSQC spectrum (appendix 12) revealed presence of one carbonyl group (δ = 179.35 ppm) which was highly deshielded indicating the presence of carboxyl group. The ^{13}C–NMR also indicated that the compound contained six carbons

From ^{13}C–NMR spectrum (appendix 11), there is a carbon having a chemical shift at 14 ppm; this is a characteristic region for sp^3-hybridised carbon atoms which is highly shielded. In the proposed chemical structure this carbon is labelled number 6 in the structure. From $^1H/^{13}C$ HSQC spectrum (appendix 12) this carbon (δ = 14 ppm) is clearly seen to be a methyl carbon and from $^1H/^{13}C$ HSQC-DEPT spectrum (appendix 12) this carbon has a proton with a chemical shift at 1.4 ppm - a characteristic of a terminal carbon, H^B couples with protons at 14.3 ppm.

From COSY $^1H/^1H$ (appendix 10) spectrum, there is coupling of protons at C2 with carbons at C3, C4 and C5 also protons at C3, C4 and C5 also couples with protons at C6 an indication of a straight chain carbon. ^1H - NMR spectrum also gives two triplet peaks one at the shielded region and the other at the deshielded region indicating that one of the carbons with the triplet peaks is close to the carboxyl carbon while the other is far away. This, therefore, depicts a compound which is a straight chain of six carbons with a terminal carboxyl group hence the hexanoic acid (**38).**

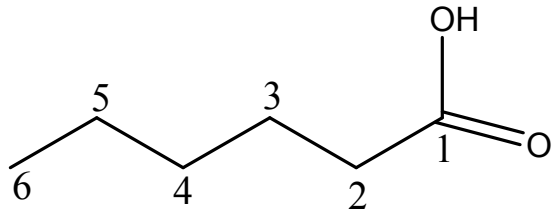
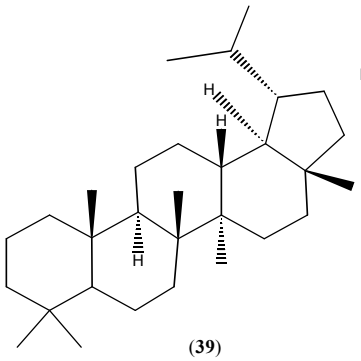


Table 8: NMR spectroscopic data for RMK9

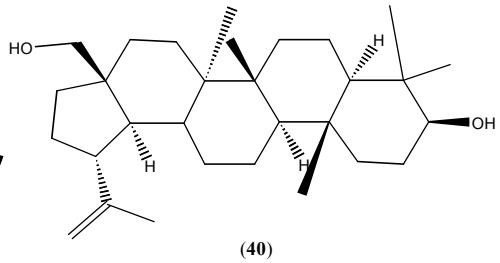
Position	^{1}H multiplicity (*J* Hz)	^{13}C	^{1}H – ^{1}H COSY	DEPT
1		180	-	-
2	2.35	34	H3/H5	CH_2
3	1.64	32	H2/H4/H5	CH_2
4	1.29	24.9	H3/H5/H6	CH_2
5	1.25	20.1	H3/H4/H5	CH_2
6	0.861	14.3	H5/H4	CH_3

4.2.6 Lupane derivatives

17–(5–hydroxy–1,5-dimethyl-hex-3-enyl)-4,4,8,10,13-pentamethyl-4,5,6,7,8,9,10,11, 12,13,14,15,16,17-tetradecahydro-3*H*-cyclopenta[α]phenanthren-3-ol is a triterpenoid of lupane derivatives. The biological activity of natural and semisynthetic lupane triterpenoid has been cited (Tolstikova, *et al.,* 2009). It has been found to have anticancer activity (Laszczyk, 2009). Betulinic acid (**40)** has proven to be the most effective antitumour among more than fifty lupines (Tolstikova, *et al.,* 2009). Ceanothane and lupane (**39**) type triterpenes has shown antiplasmodial and antimycobacteria; activities from *Ziziphus cambodiana* (Suksamrarn, *et al*., 2006). Notable similarity of betulinic acid and the proposed compound RMF2S1 is the same molecular weight and the orientation of hydroxyl group at C3 and C28.



(**39**)



(**40**)

CHAPTER FIVE
CONCLUSION AND RECOMMENDATIONS

5.1 Conclusions

Basidiomycetes fungi, *coriolus hirsitus* can produce compounds that possess larvicidal activities. This was also evident in this research project. The compounds were derived at by using solvent-solvent extraction and solid-liquid adsorption techniques and purified by column chromatography. Structure determination using a variety of spectroscopic techniques showed that the compound isolated during the research were of lupane derivatives.

In the preliminary experiment to determine the period of cultivation, 21 days was found to the optimum period of cultivation for the production of the larvicidal secondary metabolites. During this period, *Coriolus hirsutus* is assumed to have produced enough secondary metabolites which were tapped in both culture filtrate and mycelium extracts. These crude extracts (culture filtrate and the mycelium extracts) were both tested for larvicidal activity against *Aedes aegypti*, which showed positive results. The culture filtrate showed an 80% and 100% mortality against larvae of *Aedes aegypti* using 600 ppm and 800 ppm concentration respectively after 24 hours. Mycelium extract showed relatively high activity against *Aedes aegypti* giving a mortality of 40% and 100% for the 600 ppm and 800ppm respectively after only 8 hours. The results indicated that the culture filtrate and mycelium extracts had a mild activity with LC_{50} of 624.6 ppm and LC_{90} of 1299.8 ppm against the third instar larvae of *A. aegypti* for culture filtrate and LC_{50} of 479.9 ppm and LC_{90} of 1155.3 ppm against the third instar larvae of *A. aegypti* for the mycelium extract.

On purification of both the culture filtrate and the mycelium extract and later subjecting the pure compounds to spectroscopic analysis, with pure compounds labelled RKF9 and RMF2S1 had their structures proposed. Based on 1D and 2D NMR coupled with MS data, RMF2S1 was proposed to be 17–(5–hydroxy–1,5-dimethyl-hex-3-enyl)-4,4,8,10,13-pentamethyl-4,5,6,7,8,9,10,11,12,13,14,15,16,17-tetradecahydro-3*H*-cyclopenta [α]phenanthren-3-ol (**37**) and RKF9 was proposed to be hexanoic acid (**38).**

The pentacyclic structure and the β hydroxy group at C3 accounts for the activity of the suggested compound 17 – (5 –Hydroxy –1,5-dimethyl-hex-3-enyl)-4,4,8,10,13-pentamethyl-4,5,6,7,8,9,10,11,12,13,14,15,16,17-tetradecahydro-3*H*-cyclopenta[α]

phenanthren-3-ol (LC_{50} and LC_{90} of 147.0ppm and 285.3ppm respectively). Hexanoic acid which is supposedly an artefact during the cultivation also showed a very mild activity of (LC_{50} and LC_{90} of 237.8 ppm and 393.9 ppm respectively). Physical properties of 17 – (5 – Hydroxy–1,5-dimethyl-hex-3-enyl)-4,4,8,10,13-pentamethyl-4,5,6,7,8,9,10,11,12,13,14,15, 16,17-tetradecahydro-3*H*-cyclopenta[α]phenanthren-3-ol. The larvicidal activity result produced also indicated that 17 – (5 –hydroxy –1,5-dimethyl-hex-3-enyl)-4,4,8,10,13-pentamethyl-4,5,6,7,8,9,10,11,12,13,14,15,16,17-tetradecahydro-3*H*-cyclopenta[α] phenanthren-3-ol structure and its derivatives can be useful as templates for development of novel larvicidal agents and test in other areas like antitumour, anticancer etc.

5.2 Recommendations

The following recommendations were made in relation to this study:

1. That further purification of the fractions whose structures could not easily be determined by the spectroscopic techniques.
2. That field experiments be conducted for the two compounds so as to assess their effectiveness in elimination mosquito larvae.
3. That the actual mode of action of the compounds be determined.
4. That the toxicity studies be done on the two compounds to assess whether they are toxicity towards non-target organisms.

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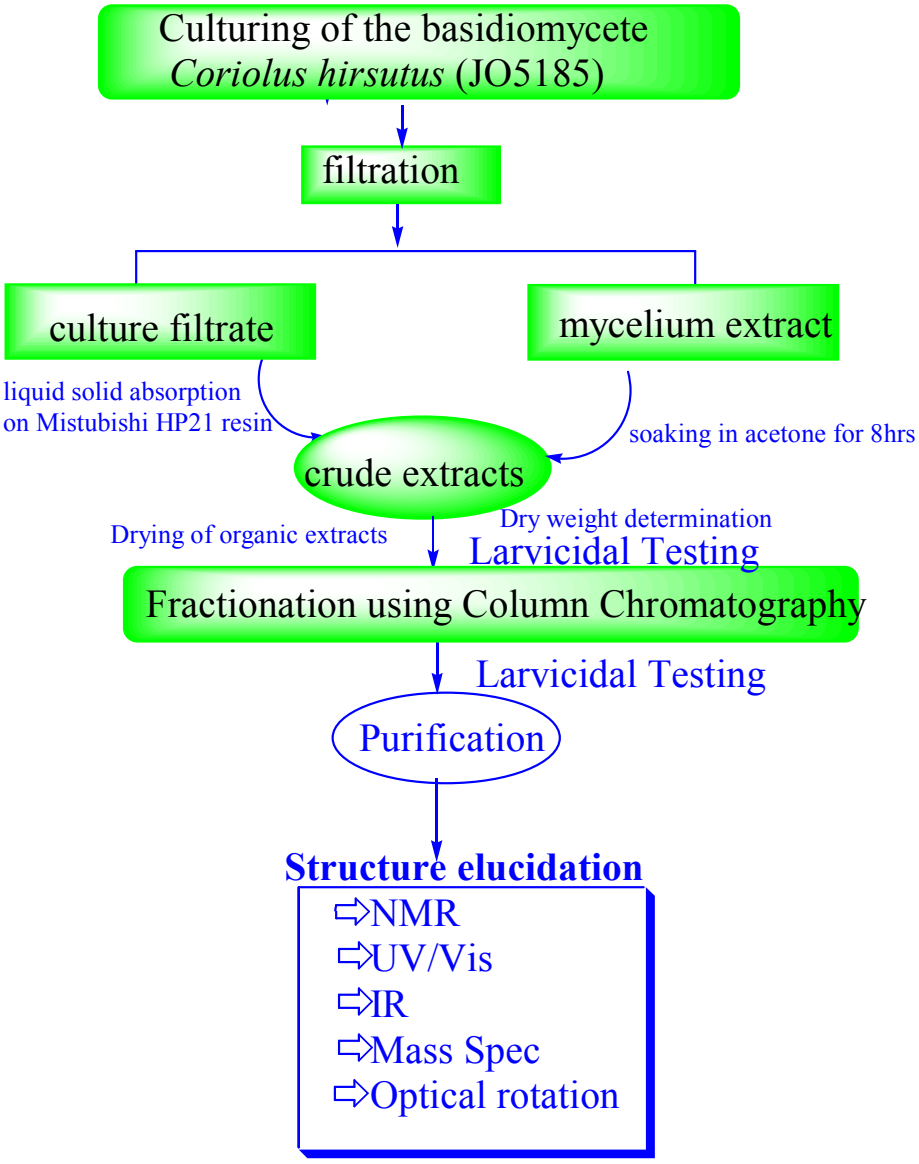
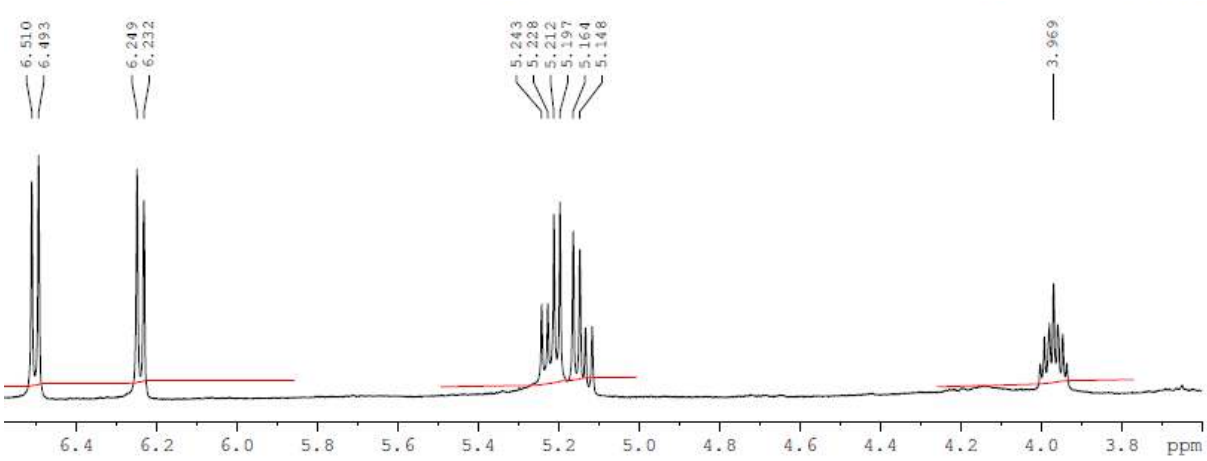
APPENDICES

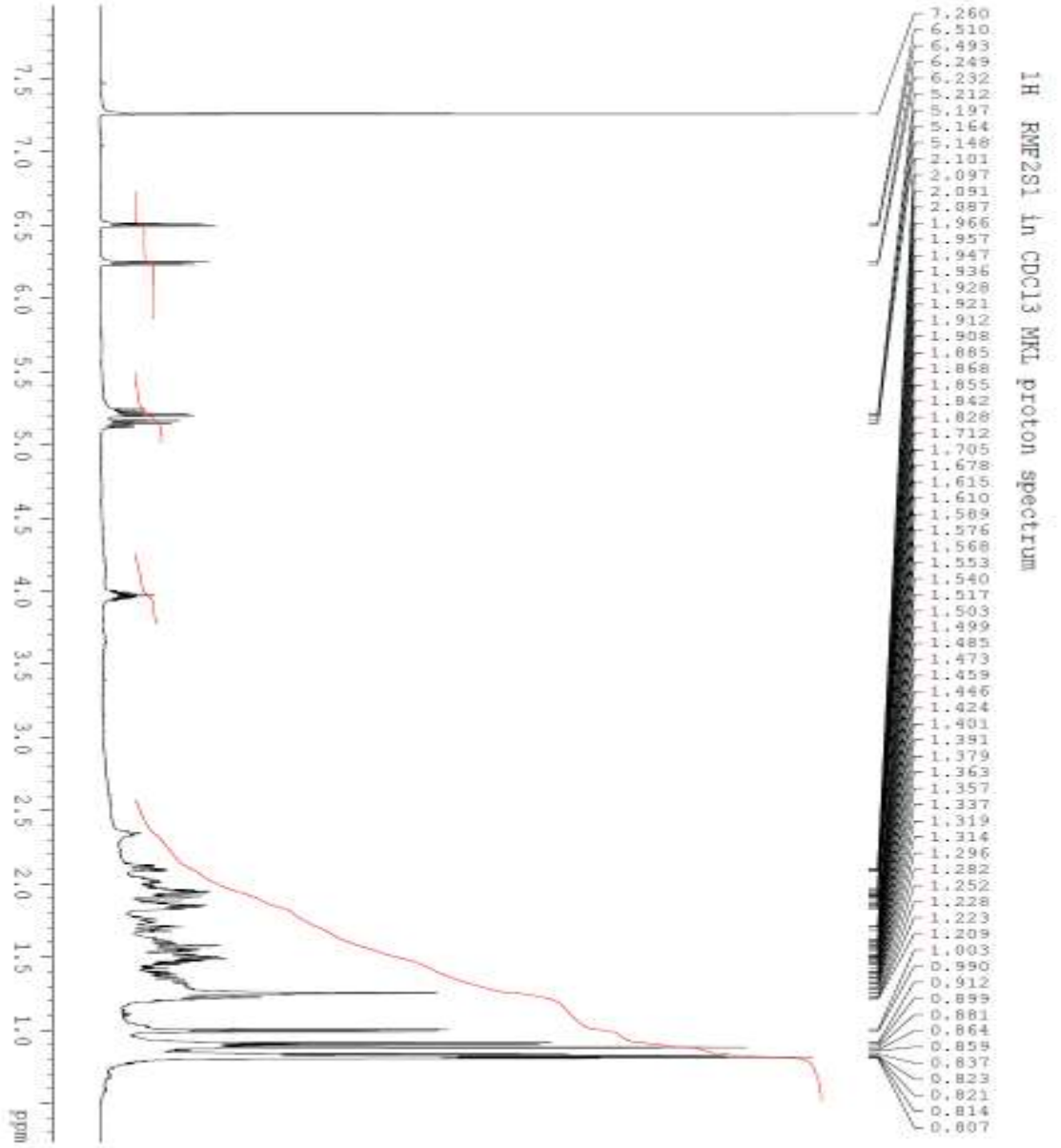
APPENDIX 1: Schematic representation of the methodology

The methodology was based on a schematic diagram given below;

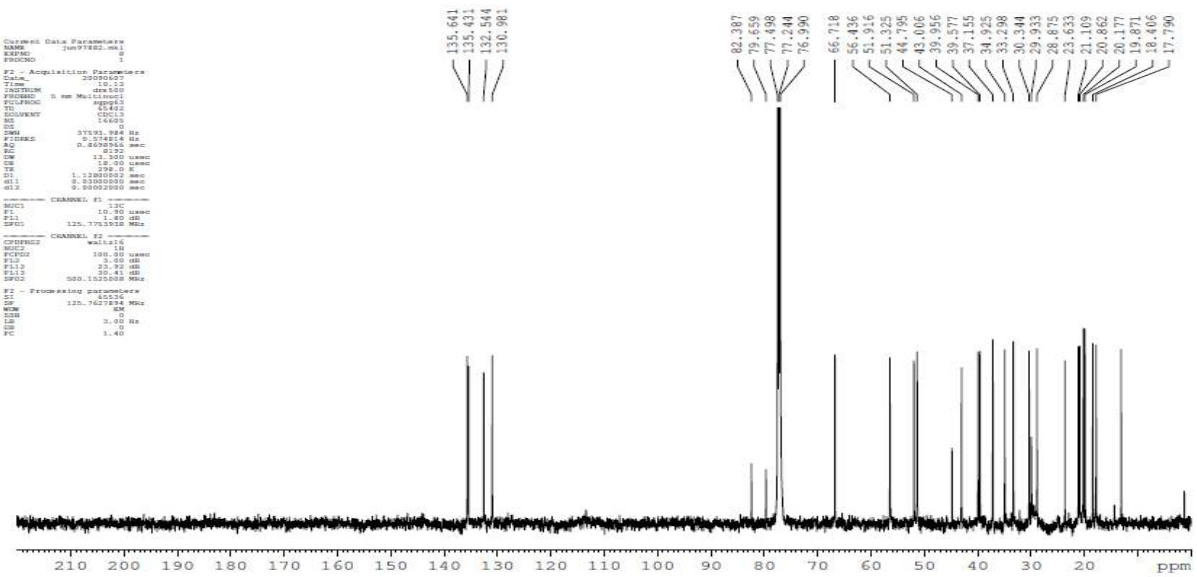


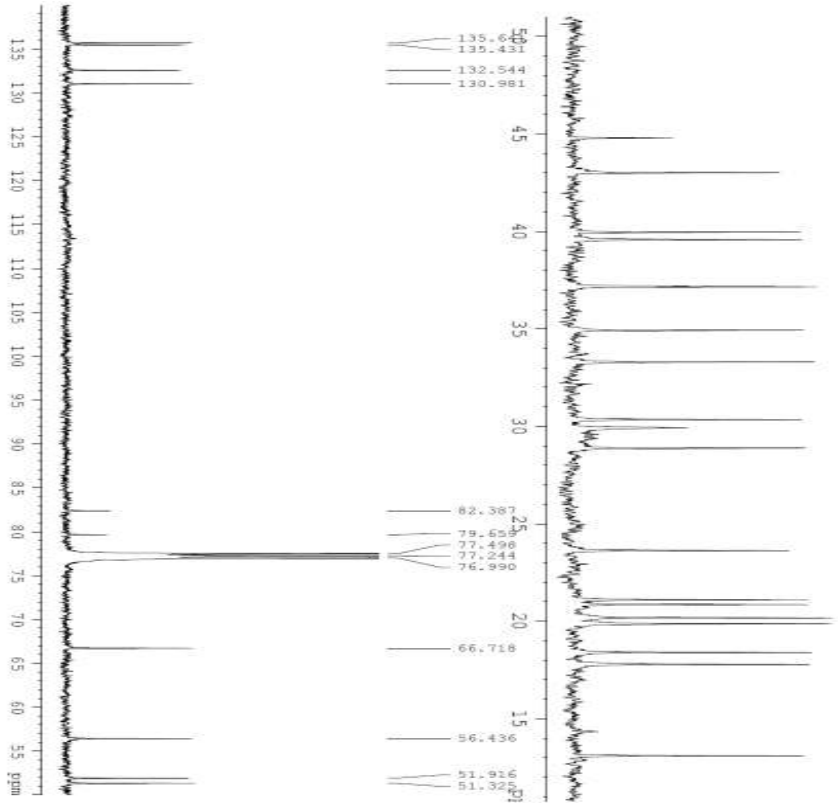
APPENDIX 2: ^{1}H – NMR for RMF2S1



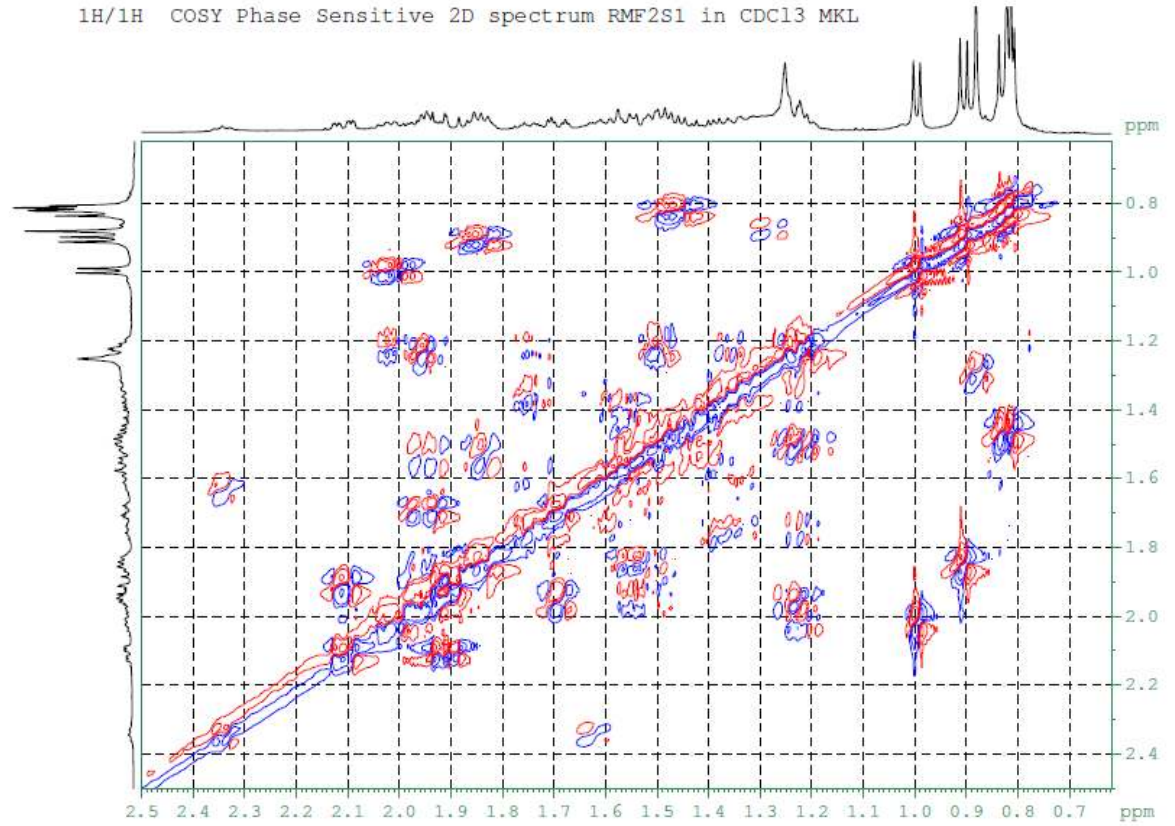


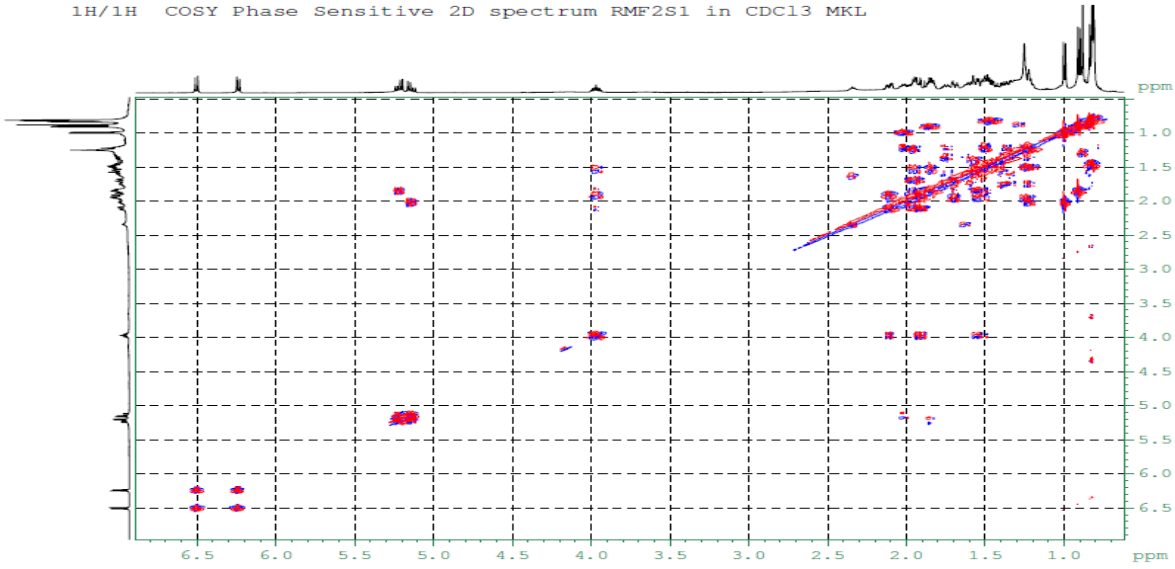
APPENDIX 3: ^{13}C – NMR for RMF2S1



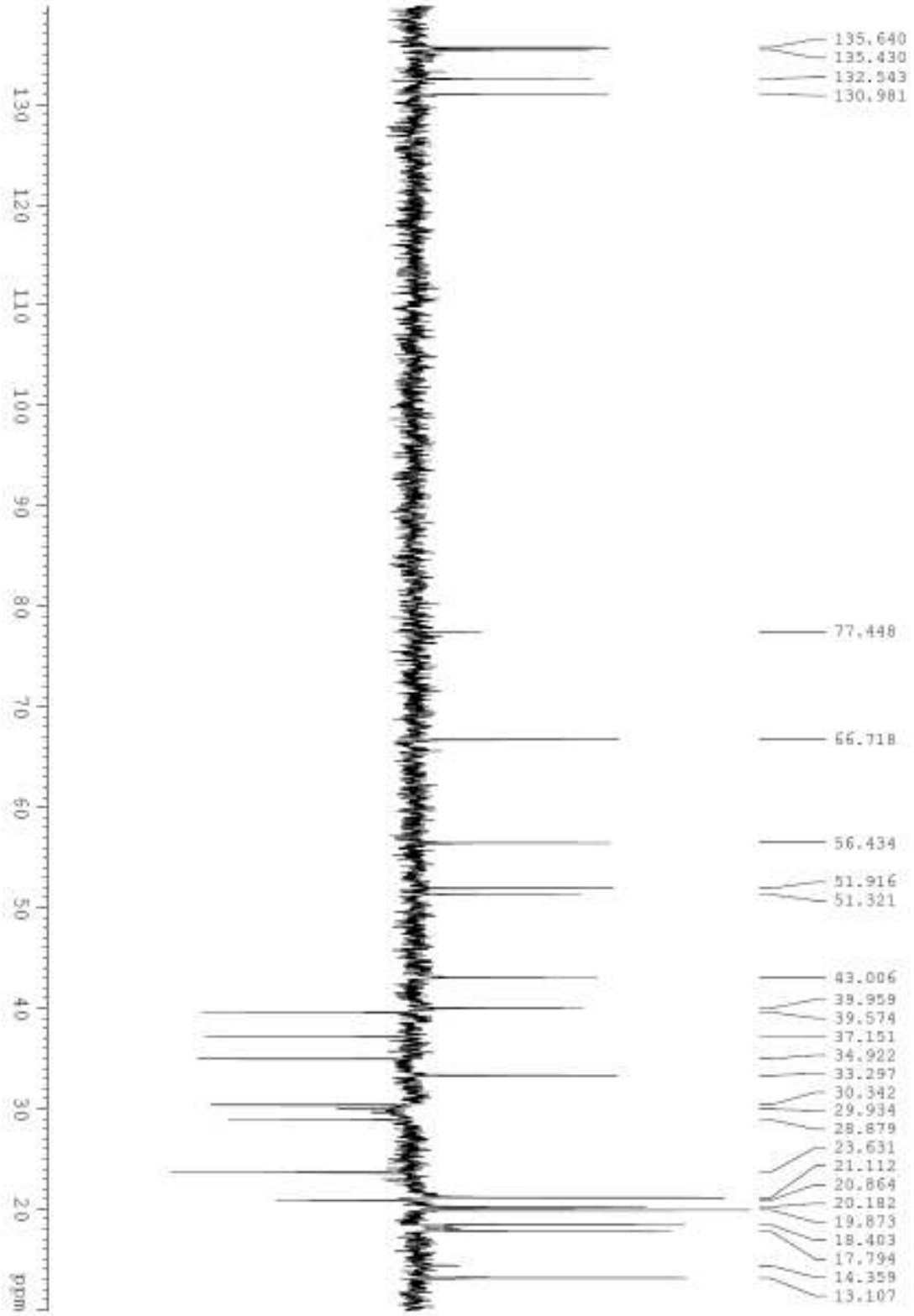


APPENDIX 4: ^{1}H /^{1}H– COSY for RMF2S1

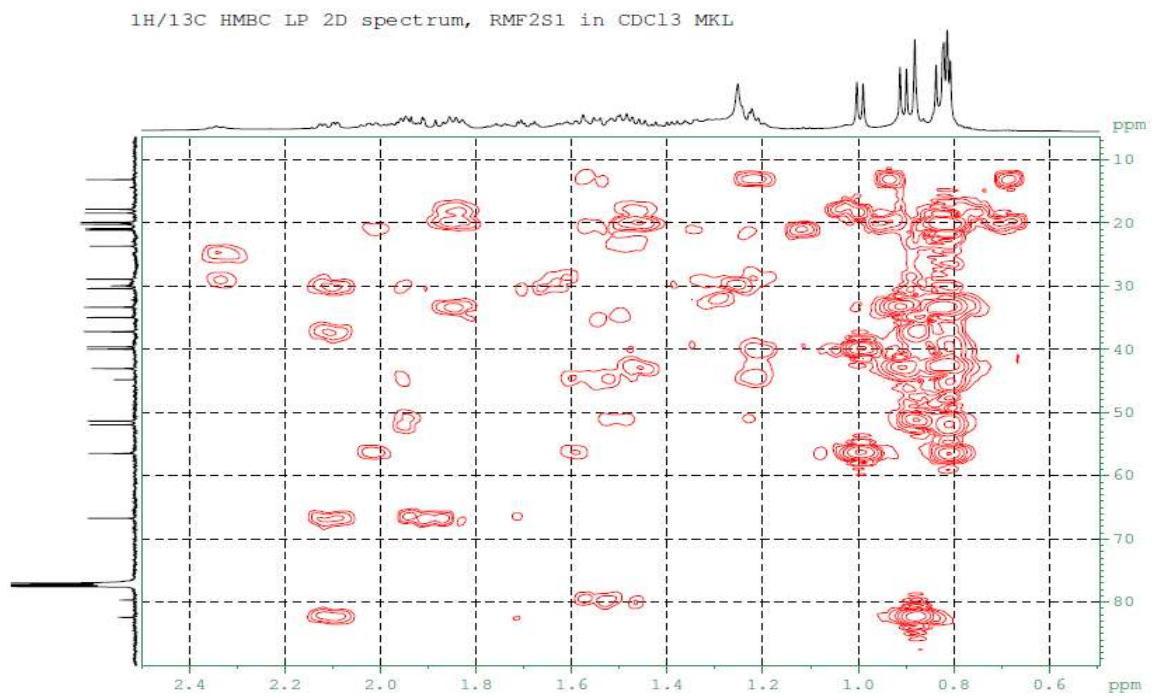


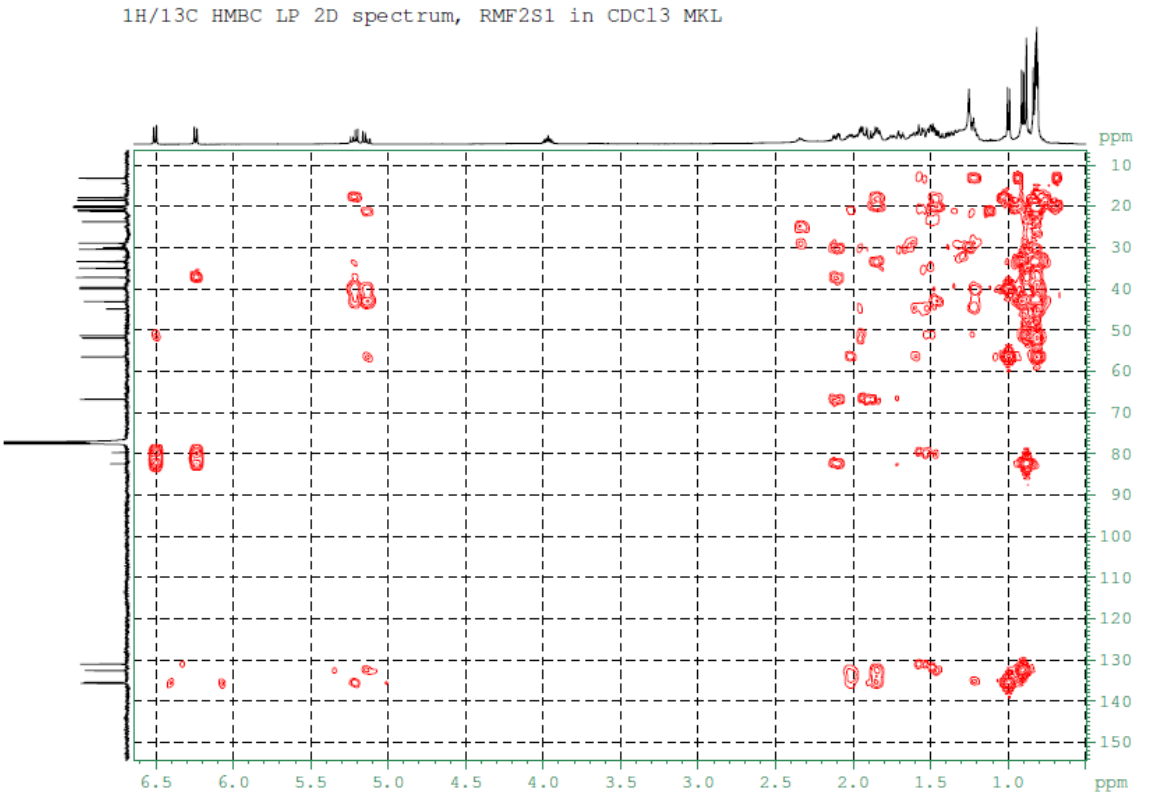


APPENDIX 5: DEPT for RMF2S1

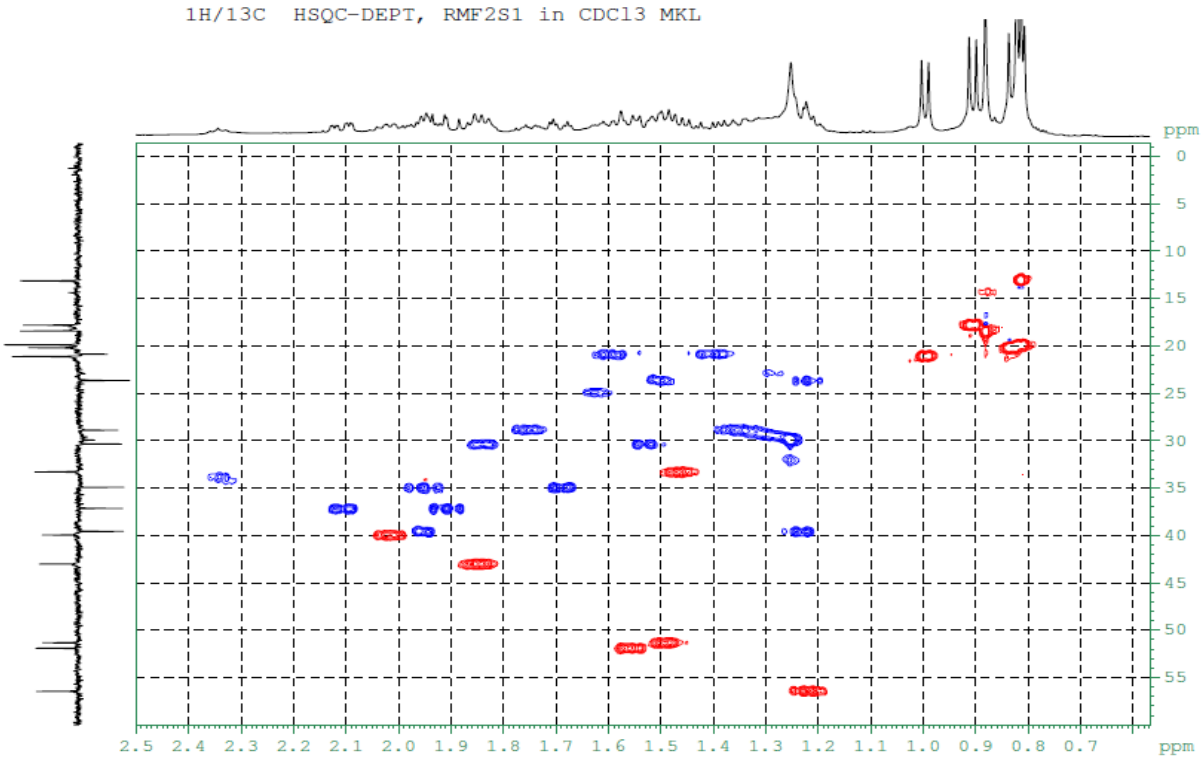


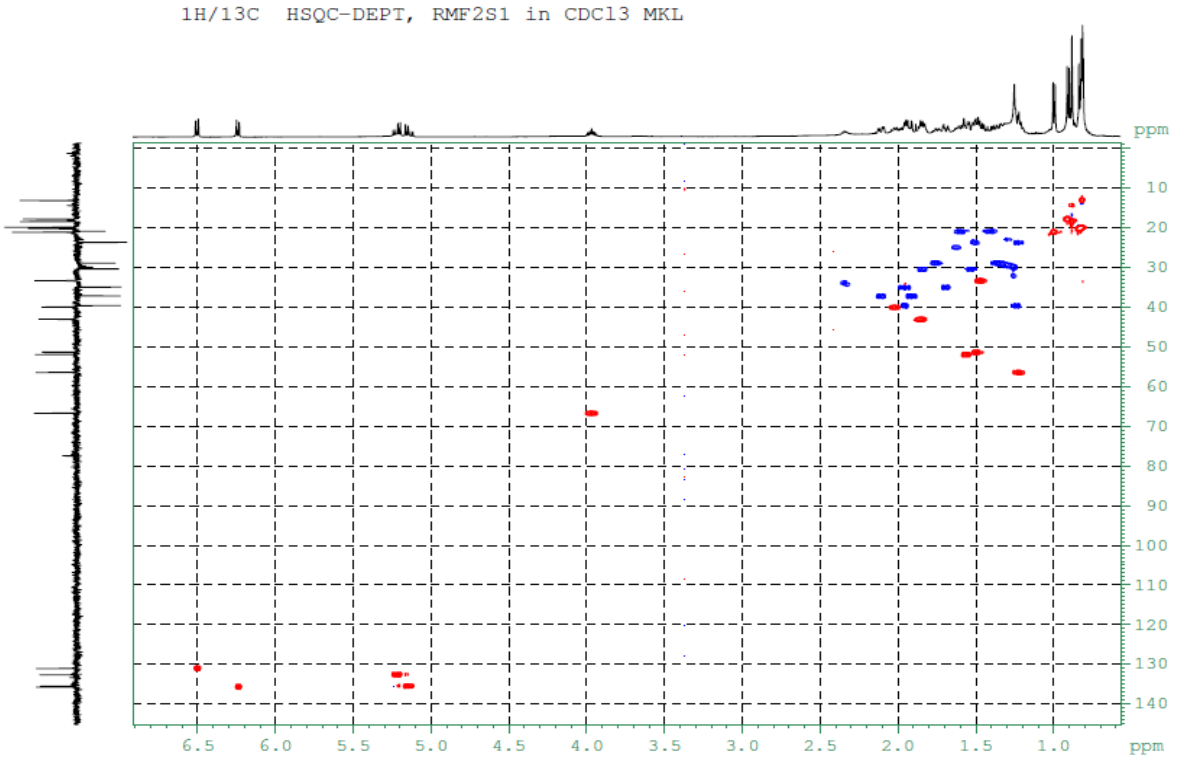
APPENDIX 6: ^{1}H /^{13}C– HMBC for RMF2S1



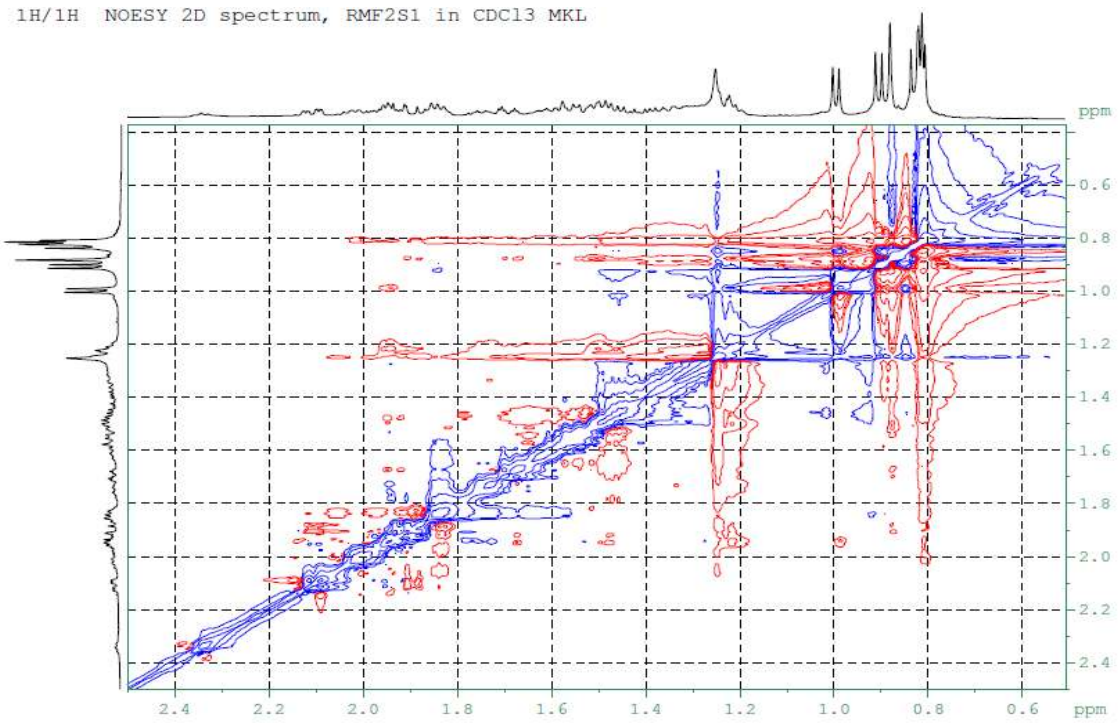


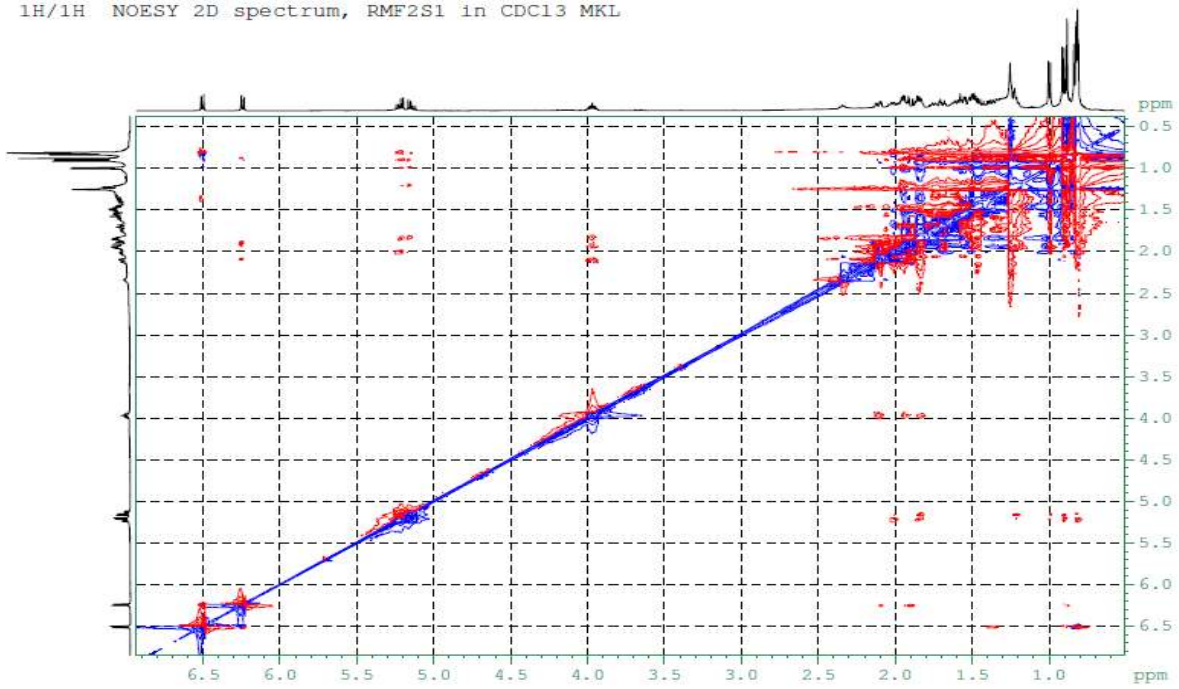
APPENDIX 7: $^{1}H/^{13}C$ HSQC for RMF2S1



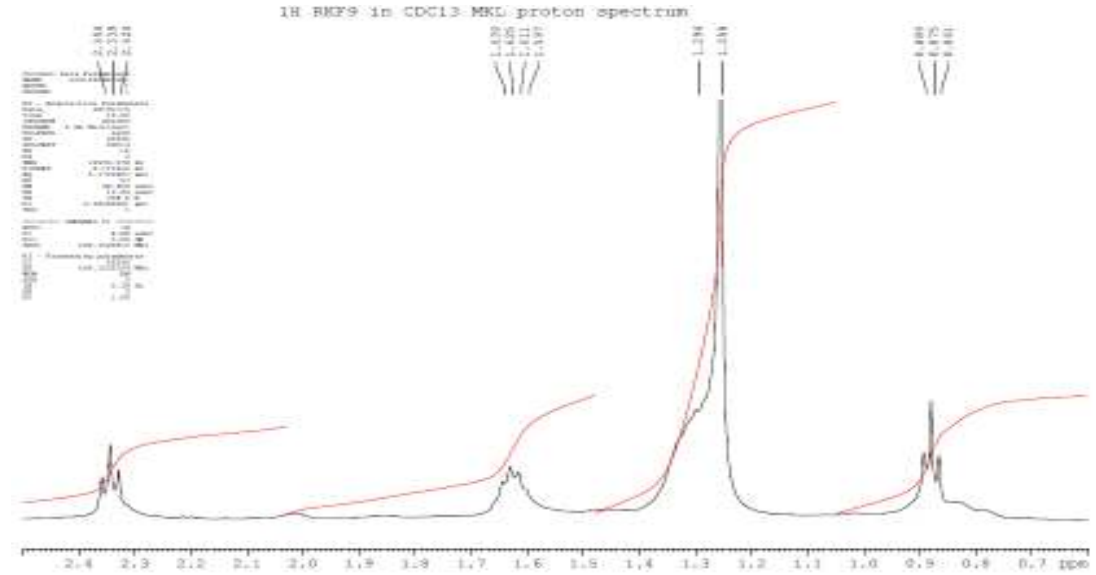


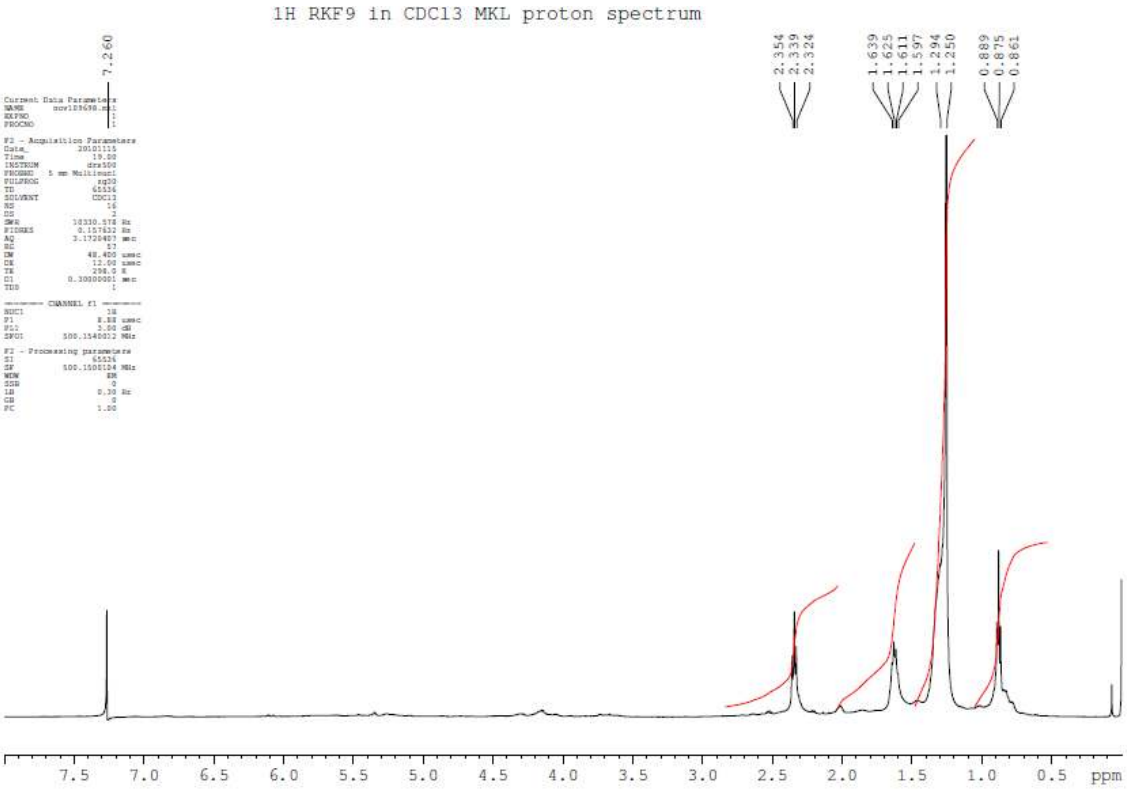
APPENDIX 8: $^{1}H/^{1}H$ NOESY for RMF2S1



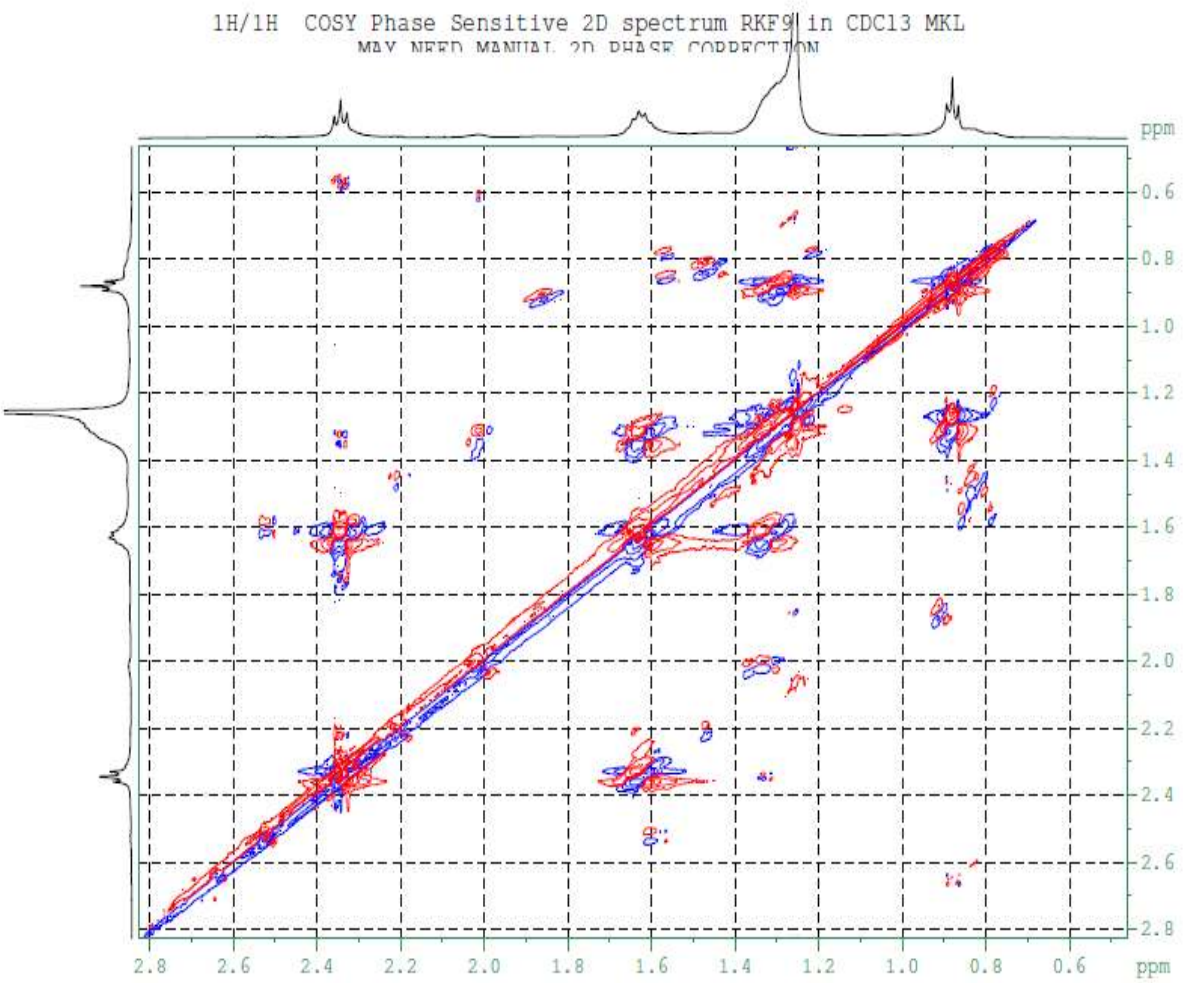


APPENDIX 9: 1H – NMR for RKF9

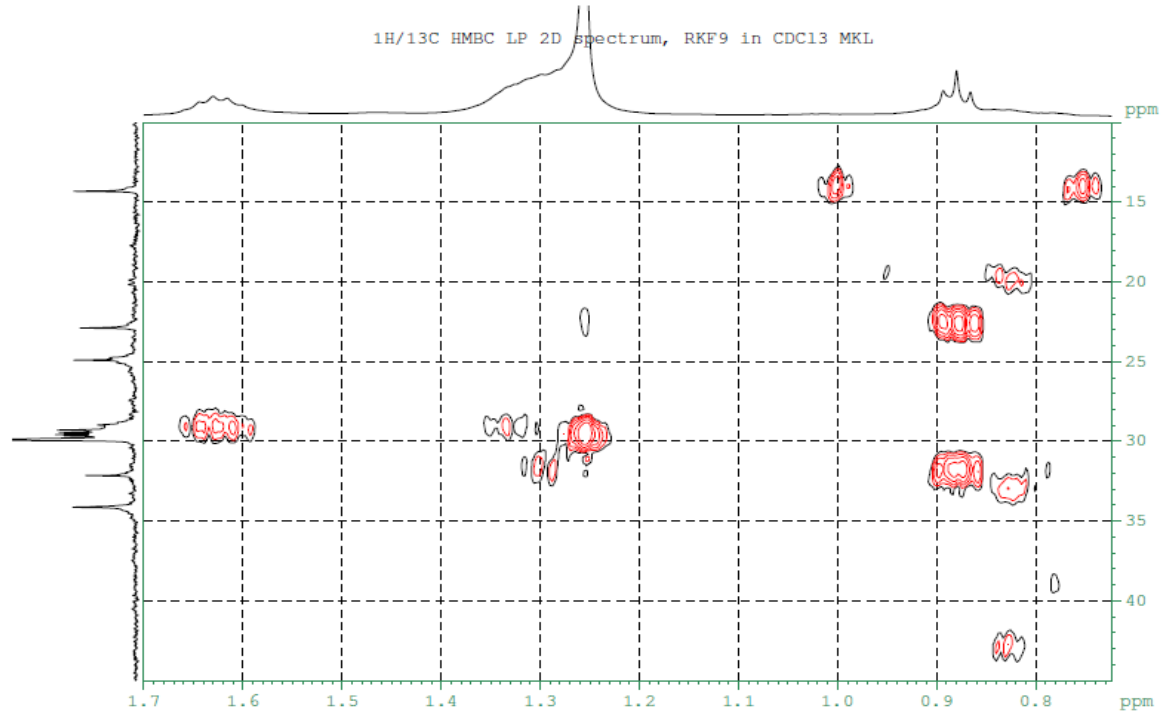


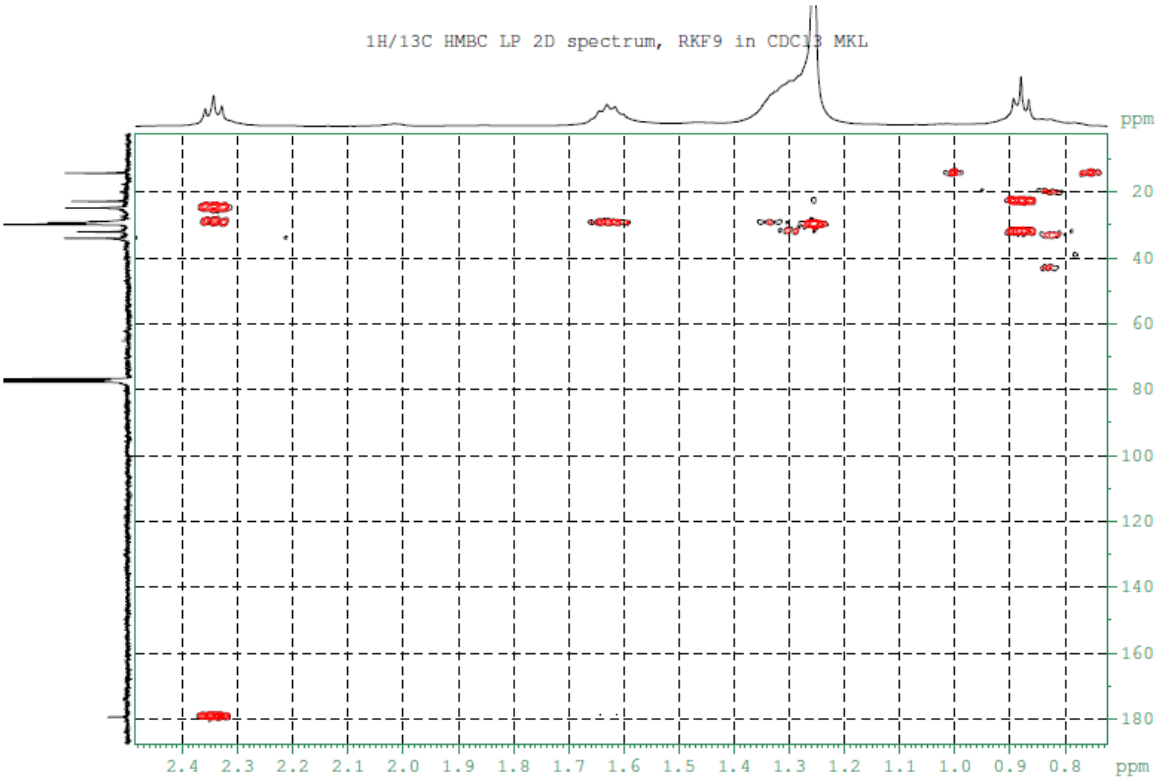


APPENDIX 10: $^{1}H/^{1}H$ – COSY for RKF9

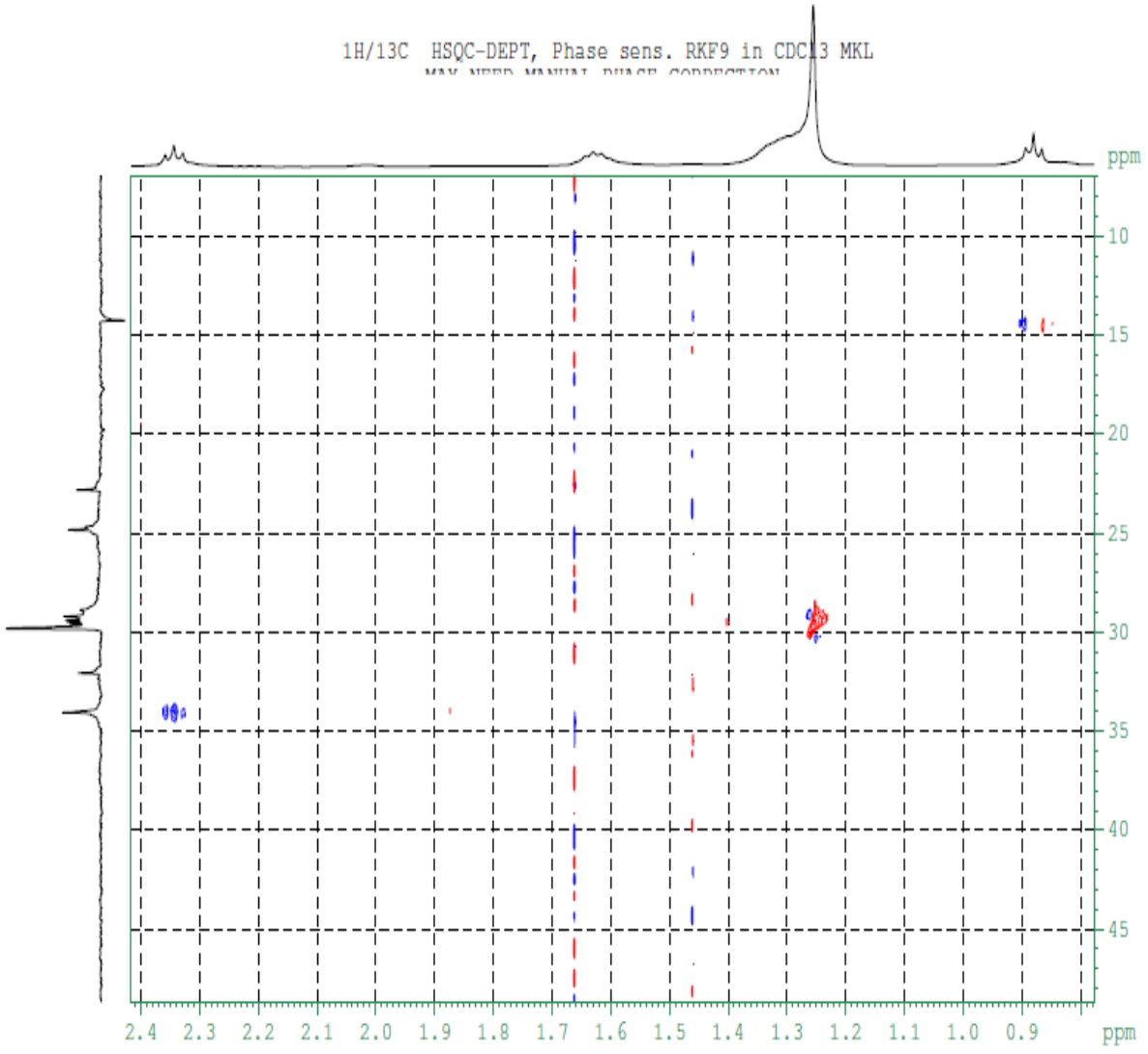


APPENDIX 11: $^{1}H/^{13}C$ – HMBC for RKF9





APPENDIX 12: $^{1}H/^{13}C$ – HSQC for RKF9



APPENDIX 13: MS for RMF2S1