PURIFICATION, BIOACTIVITY AND STRUCTURE ELUCIDATION OF MOSQUITO LARVICIDAL COMPOUNDS FROM MYCELIUM OF BASIDIOMYCETE JO5444

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DEDICATION

To my mum for her dedication, selflessness and struggle to see me through my formal education, my brother for the support and prayers and to the entire Erasto family for their moral support and encouragement.

DECLARATION AND RECOMMENDATION

DECLARATION

I, Njogu, E. M., hereby declare that this study and 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 study and thesis write up was done under our supervision and has our approval to be presented for examination as per the Egerton University regulations.

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ABSTRACT

As malaria burden persists in most parts of the developing world, the concept of implementation of new strategies to control the disease continue to gain support. In Kenya, mosquito population has increased to unprecedented levels as the insects are now a common sight in places they were previously not known to habit. Mosquitoes species belonging to genera *Anopheles*, *Culex*, and *Aedes* are the principal vectors of etiologic agents of various diseases like malaria, filariasis, Japan encephalitis (JE), dengue fever, dengue hemorrhagic fever (DHF) and yellow fever to mention just but a few. It is known that elimination of the vector is one of the most effective ways of controlling disease transmission. This takes the approach of using adulticides and larvicides. Whereas the former is well established, it has limitations like the challenges of targeting mobile adult mosquitoes and resistance of mosquitoes to insecticides. Larval control has long been an option for disease control but it has been greatly under developed and underutilized approach especially in tropical Africa where it may be of much utility than is generally appreciated. Killing mosquito larvae before they emerge as adults can reduce or eliminate the need for ground or aerial application of pesticides to kill mosquitoes. During the search for new, effective and environmentally benign natural larvicidal compounds, larvicidal activity of nine enriched fractions and one pure compound from mycelium of basidiomycetes serialized JO5444 were investigated. Bioactivity-guided fractionation and purification afforded one pure novel compound identified as (oxiran-2-yl)methylpentanoate by its spectral data. The LC_{50} and LC_{90} at 24 hours of the pure compound against larvae of *Ae aegypti* in their late third and early fourth instars were studied and found to be 1.50ppm and 1.90ppm respectively. The complete structure of the purified compound was obtained from both 1D and 2D NMR experiments, and analysis of the spectra obtained allowed complete ^{1}H and ^{13}C NMR assignments. The ^{1}H NMR and ^{13}C NMR data for the purified compound have been provided. Fungal secondary metabolites are therefore hereby reported and proposed as a complimentary tool for control of mosquitoes and hence reduce transmission of diseases that they vector.

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LIST OF SYMBOLS ABREVIATIONS AND ACRONYMS

Bti- Bacillus thiringiensis var *israeliensis*

BHC – Benzene hexachloride

CC – Column chromatography

DDD - Dichlorodiphenyldichrloroethane

DDT – Dichlorodiphenyltrichloroethane

DEPT – Distortion less Enhancement by Proton Transfer

DFDT - 1,1,1-trichloro-2,2-bis(p-fluorophenyl)ethane

DHF - Dengue hemorrhagic fever

HMBC – Heteronuclear Multiple Bond Correlation

IR - Infra red

ITNs – Insecticide treated net

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 Spectroscopy

PDA – Potato Dextrose Agar

^{13}C – Carbon 13 isotope

^{1}H – Hydrogen 1 isotope

NMR – Nuclear Magnetic Spectroscopy

NOESY – Nuclear Overhauser Effect Spectroscopy

TLC – Thin layer chromatography

UV/Vis – Ultra violet -visible

VLBW - Very low birth weight

WHO - World Health Organization

CHAPTER ONE

INTRODUCTION

1.1 Background

Mosquitoes are a particular problem because they are vectors of human diseases. Mosquitoes are found all over the world except in places that are permanently frozen. Three quarters of all the mosquito species live in the tropics and the subtropics, where the warm moist climate is favourable for rapid development and adult survival, and the diversity of habitats has permitted diversity of many species (Clements, 1992).

Several mosquito species belonging to genera *Anopheles*, *Culex*, and *Aedes* are vectors of etiologic agents of various diseases like malaria, filariasis, Japan encephalitis (JE), dengue fever, dengue hemorrhagic fever (DHF), yellow fever among others (ICMR, 2003). The diseases not only cause high levels of morbidity and mortality, but they also inflict great economic losses and social disruption on countries with high disease endemicity, such as the less-developed countries of Africa, Asia, and Latin America (Peng *et al.*, 1999). Chemical pesticides such as dichlorodiphenyltrichloroethane (DDT), gammaxane, malathion, and chlordane applied with the aim of eliminating mosquitoes have given rise to other serious problems. Not only has mosquito resistance against these chemicals been reported, but the pesticides themselves present threats to both human health and the ecosystem (Phillips, 2001; World Health Organization, 1995). In these circumstances, the possibility of utilizing insecticides from natural sources as alternatives to synthetic chemicals and compliments to other environmentally benign natural insecticides should be examined in depth and indeed all unexplored sources should be sought with more vigour (Scientific Working Group, 2002).

The world's prime choice to curb nuisance biting by mosquito or their transmission of parasitic or arboviral diseases continue to be the selective application of residual synthetic insecticides. The public health benefits delivered by these both in tropical resources-poor settings, as well as in temperate zones cannot be overemphasized; they save thousands of people each year. Powered by strong industrial lobby, new and more environmentally friendly compounds replace older more harmful and less effective ones. However, beyond these gains in economic and public health terms, the stark reality of environmental impact and ever developing resistance remains an issue of grave concern (Chandre *et al.*, 1999; Hemingway and Ranson 2000; Brooke *et al.*, 2002). Besides, some of these diseases transmitted by mosquitoes like malaria are still a scourge.

In spite of the wide-scale withdrawal of DDT as a result of the effect of environmental accumulation, this compound was so effective and so safe (in terms of direct human toxicity) that, originally, the total elimination of mosquitoes was envisaged. The subsequent evolution of DDT resistance has proved naivety but it is still in use in vector control in some African countries, ironically after the subsequent failure of newer compounds, such as pyrethroids, also owing to resistance. Pharmacological studies have since proved DDT to be similar to 'type I' pyrethroids and have shown that resistance to both classes of compounds can be conferred by molecular changes in their common target, the voltage gated sodium channel (Fanello, 1999; Enayati *et al.,* 2003). The search for effective alternative pesticides is not new; previously proposed alternatives for mosquito control have included large scale manipulation of insect populations via the 'sterile insect technique' (SIT), a technique that involves the release of large numbers of sterile males. The use of genetically modified mosquitoes to produce sterile males for SIT has been proposed (Alphey, 2002). Transgenic mosquitoes themselves have been proposed to alter a wide variety of aspects of mosquito's biology refractoriness (i.e. the inability of mosquito to transmit *Plasmodium* owing to change in competency) through the malaria transmitting population, to reduce the mosquito lifespan , to cause the mosquito death upon infection or to alter mosquito blood-feeding habits (Jacobs-Lorena, 2003). These more novel techniques must also be considered in the light of conventional approaches, such as environmental manipulation to reduce mosquito breeding sources and biological control using bacteria or viruses and entomopathogenic fungi. Application of genetically modified mosquitoes and sterile insect technique are not feasible approaches for control of mosquitoes and disease transmission in Sub Saharan Africa.

Given the success of conventional pesticides, and the real concerns about resistance, the simplest and perhaps still the most effective technique is to find alternative chemical compounds so that the life of useful insecticides can be prolonged. Unfortunately, the use of compounds in vector control has always played second fiddle to the use of insecticides in agriculture. In fact such use has always been blamed for subsequent resistance problems in vector control. Thus agrochemical companies developing new products tend to recoup the cost of development in agriculture first and then release products that are useful for vector control. Moreover, little current work is being done to develop new insecticides that are active on mosquitoes, in spite of the wealth of insecticide targets revealed by the ongoing sequencing of several mosquito genomes and recent development of a 'detox' microarray of metabolic genes from *A. gambiae* (David *et al.*, 2005).

1.2 Malaria as a major disease transmitted by mosquitoes and control strategies

Of all diseases transmitted by the mosquito, malaria is the most deadly and of late it has proved to be the most costly malady to manage in countries where it is endemic. There are at least 300 million acute cases annually, resulting in more than 1 million deaths; 90% of them in sub-Saharan Africa. Malaria is the number one killer of children under the age of five years in the African continent, where mosquito vector populations are spread almost throughout the entire continent and where the parasite reservoir is big and is continuously increasing. The disease has, however, been overshadowed by daily reports focused on HIV/AIDS plight but it still remains a titanic problem. WHO estimates that more than 2.4 billion people are at risk, a situation aggravated by poverty, population displacement in times of war and short sighted use of technology in industry and agriculture (Nasrin, 2005). Where malaria prospers most, human societies have prospered least. The global distribution of per-capita gross domestic product shows a striking correlation between malaria and poverty, and malaria-endemic countries also have lower rates of economic growth. There are multiple channels by which malaria impedes development, including effects on fertility, population growth, saving and investment, worker productivity, absenteeism (from duty and school for pupils and students), premature mortality and medical costs (Sachs and Pia, 2002).

The current focus of malaria control programs in Africa is rightly on the management of the sick through early treatment with effective anti-malarial drugs and domestic protection against adult mosquitoes with insecticides (Gerry *et al.*, 2002). However, this cannot be a final strategy. The two first line drugs, chloroquine and sulfadoxine/pyrimethamine (fansidar) are no longer effective in many parts of East Africa, where chloroquine resistance is rampant. Combinations of new drugs has helped to slow the emergence and spread of resistant parasites, but control strategies based on early treatment mean never ending struggle to develop and deploy new drugs before the *Plasmodium* malaria parasite become resistant to existing drugs. Thus, the alternative and the long term strategy must incorporate among others interruption of the transmission of the parasite, though this might be extremely difficult in parts of Africa where people receive as many as 1000 bites yearly by infected mosquitoes (Utzinger *et al.*, 2002).

Indoor insecticide residual spraying and insecticide treated mosquito nets, which are now being vigorously promoted in many parts of Africa reduces bites from infected mosquitoes by 90% particularly in children. However, their effectiveness is already under threat as a result of pyrethroids resistance. Household spraying with residual insecticides

(mostly with organochlorine compounds such as DDT and dieldrin) is highly effective in some parts of Africa, but it is logistically demanding, costly and may have adverse environmental effects. Besides, indoor spraying with organochlorines has caused several behavioural changes in several malaria vector species (for instance changes in biting behaviour, resting behaviour and avoidance of insecticide-treated rooms), mostly due to the selective pressure placed on the target vector population (Willem, 2002). Multiple insecticide resistances coupled with various complications in the introduction, distribution and proper use of ITNs indicate that these tools alone will be insufficient to reduce malaria transmission to a level that could eventually lead to eradication of the disease (George, 2005). There are many ways of reducing malaria transmission, but none can provide a complete block in transmission, particularly in the highly endemic areas of Africa and new approaches are desperately needed. Parasite control appears to be even more problematic; an effective antigen for vaccine production has remained elusive for many years; drug resistant is increasing and new more drugs are unaffordable. On top of this, increasing poverty, bad hygienic conditions and devastating civil conflicts in many endemic countries worsen the situation (George, 2005). Combined deployment of three strategies that each have a potential to reduce malaria transmission by 90% that is, vaccination, drug treatment and vector control should be sufficient to reduce transmission even in highly endemic areas. Among these, vector control has received little attention from bodies and organisations concerned in management of the disease and especially larval control. Malaria control by killing adult mosquitoes is generally favoured because moderately reducing their longevity can radically suppress community-level transmission. By comparison, controlling larvae has a less dramatic effect at any given level of coverage and is often more difficult to implement. Nevertheless, the historically most effective campaign against African vectors is the eradication of accidentally introduced *Anopheles gambiae* in Brazil in the 1930s and early 1940s (Scientific Working Group, 2002). This outstanding success was achieved through an integrated programme but relied overwhelmingly upon larval control. This experience was soon repeated in Egypt and another larval control programme successfully suppressed malaria for over 20 years around Zambian copper mine. These affordable approaches were abandoned and neglected after the advent of DDT and global malaria control policy shifted toward domestic adulticide methods (Gerry *et al.*, 2002). Larval control methods should now be re-prioritized for research, development, and implemented as an additional way to Roll Back Malaria.

One option of eradicating malaria transmitting mosquitoes is the use of chemical insecticides. For the past five decades, humans have been wholly dependent upon synthetic and organic insecticides. Though successful in other fields like agriculture, the miracle of chemical technology has not, however, provided solution when it comes to eradicating malaria and other diseases transmitted by mosquitoes. In addition to their inefficacy, chemical pesticides can cause mutant strains of fauna and flora. Chemical pesticides also pollute water resources and introduce a potent cocktail of toxic chemicals into the human body. To make matters worse, the more than 50 species of *Anopheles* are now showing resistance to chemical pesticides. Initially, this resistance was limited to DDT, Malathion and dieldrin but has been reported in other choice insecticides like pyrethroids (Gerry *et al.*, 2002). Coupled with concerns over environment and the mounting cost of chemical insecticides, the growing resistance of mosquitoes to chemical pesticides makes these less than attractive options for controlling malaria.

1.3 Fungi and biotechnological applications.

Over the past 60 years fungi have proven to be a valuable source for discovery of novel natural products, many of them potential candidates for agrochemical and biomedical development. Fungi are an extremely diverse group of heterotrophic organisms that are exploited by human for various biotechnology applications; they are used in the production of foods, beverages, organic acids, enzymes, polysaccharides and antibiotics and other pharmaceuticals; as agents of biological control of insect pests, pathogenic fungi and weeds; and in biomass conversion. Fungi can be broadly classified as either filamentous or non-filamentous. Filamentous fungi account for virtually all members of the fungal kingdom. Filamentous fungi produce a vast array of secondary metabolites and some species have highly efficient protein secretion mechanisms that can be exploited to express homologous or heterogenous gene products (David and Christopher, 1992). Indeed, the true uniqueness of filamentous fungi in biotechnology lies in their versatility and breadth of utility; they can generate wide industrial products including primary metabolites such as organic acids, secondary metabolites such as β-lactam antibiotics, non-antibiotic drugs and enzymes for use in food production. Whole organisms such as mushrooms can be used as food while others can be used as insecticides and herbicides for biological control. As the name implies filamentous fungi grow as branched filaments termed hyphae which are collectively called mycelia (David and Christopher, 1992).

Ever since the discovery of penicillin, fungi have been recognized as sources of novel compounds important in human health. In addition to providing new pharmacologically active compounds, tropical fungi may act as sources of new antifungal agents, insecticidal compounds (both larvicidal and adulticidal) and enzymes for industrial use including bioconversions. The full array of uses to which fungi can be put is now immense due to the advancement in research technology.

Natural products have been isolated and used as insecticides; nematicidal, pesticides, repellents as well as larvicides. A lot of bio-prospecting and screening has been carried out to get natural larvicides and insecticides but mostly from plants, in addition to repellents. Though plants are good sources of larvicides and insecticides they are limited in that they take long to grow and also the space to grow enough plants to produce enough extracts is lacking in the current situations. Besides, their growth and growth rate is much dependent on climate and weather patterns.

Filamentous fungi are among the most prolific producers of secondary metabolites. Some secondary metabolites are effectors of differentiation; it is therefore not surprising that organisms that engage extensively in morphological differentiation would also be heavily involved in chemical differentiation. Secondary metabolites, also, called idiolites or special metabolites usually contain bizarre chemical structures and unusual chemical linkages such as β-lactam rings, cyclic peptides made of normal and modified amino acids, unsaturated bonds of polyacetylenes and polyenes, and large macrolide rings. Although they are not essential for the producing organism's growth in pure culture they have survival functions in nature. Secondary metabolites are produced by some species of a genus and as a mixture of members of a chemical family because of the low specificity of some enzymes involved in secondary metabolism. Representative compounds include mycotoxins, antibiotics, pigments, pheromones and insecticides. An important characteristic of secondary metabolism is suppression by high specific growth rates of the producing cultures. In addition to growth rate control, individual biosynthetic pathways are affected by regulatory mechanisms such as induction, nutrient repression, synthetase decay, and end-product regulation (David and Christopher, 1992).

However, besides their potential, fungi have been under utilized in search for potential larvicides. This project was designed to isolate, purify and determine the chemical structures of compounds from a basidiomycete with larvicidal and/or adult emergence inhibition properties, which could be developed to larvicides. From an initial screening for novel larvicidal compounds from submerged cultures of selected classes of fungi, one

basidiomycete serialized JO5444 was found to produce active larvicidal compounds against the larvae of *Ae aegypti* in their third and fourth instars. The basidiomycete was collected as fruiting body (appendix 1) growing in an undisturbed habitat in Londiani forest in Rift Valley in Kenya.

1.4 Statement of the problem

Integrated vector control is effective part of any successful vector control program. Mosquito larval control may work best and be the most cost effective in sites where larval habitats occur seasonally, and /or are relatively few, are well defined and accessible and where human population density is high to justify repeated treatment of all breeding sites. These environments are frequently found in sub Saharan Africa and thus mosquito larval control could be effective tool for control of mosquito transmitted diseases in this continent. However, tools and especially insecticides targeting larval stages of mosquito are unavailable and research towards getting effective eco friendly larvicides are lagging far behind and have received little attention from parties concerned with vector control.

1.5 Objectives

1.5.1 Main objective

The main objective of this study was to purify and chemically characterize mosquito larvicidal compounds from the fungus serialized JO5444.

1.5.2 Specific objectives

- To culture the basidiomycete JO5444 in liquid media.
- To separate the mycelium and liquid culture by filtration, extract the compounds in the mycelium and perform larvicidal test for the crude extract.
- To fractionate and purify the compounds in the crude extract using chromatographic techniques.
- To test the several compounds from each fraction in the chromatographic column against *Ae. aegypti* larvae and determine their larvicidal and adult emergence inhibition potential.
- To study and elucidate the chemical structure of pure compounds using spectroscopic techniques.

1.6 Justification of the study

The adverse effects on human health and well being, death and the economic losses initiated by mosquitoes are importance stimulants for the search of new, safe and effective larvicides. Besides, in the light of rapid spread of parasite strains resistant to drugs and the potential of mosquitoes to develop resistance to pyrethroid insecticides, there is need to re-evaluate the role of anti larval tools for disease control to bolster the existing ones. The pool of natural products is an important source for such compounds. Higher fungi have an extensive secondary metabolism at their disposal and obviously some benefit from the credibility to produce metabolites that could kill larvae (i.e. act as larvicides) and insects. Surprisingly little is known about the production of larvicidal metabolites by fungi.

CHAPTER TWO

LITERATURE REVIEW

2.1 Malaria problem

Thoughtless man-made irrigation schemes, dams and the general built environment have provided new habitats for *Anopheles* breeding sites, and resulted in 'man-made' malaria. The extensions of urban areas lead to epidemics in the peripheries of the growing cities. A mass migration of non-immune populations into endemic areas for political reasons further complicates matters. Environmentally disturbed habitats are responsible for malaria transmission during the dry season; accounting for production of 87.5% of the malaria vectors. During the dry season, man-made habitats (such as functional brick making pits and quarries) provide a developmental habitat in which *Anopheles* larvae escape the high degree of predation found in natural environment. With substantial socioeconomic motivation for activities that disturb the natural ecosystems like brick making and quarrying activities, traditional source reduction (eliminating standing water) is unsuitable, and larvicides should be employed (John *et al.*, 2004).

Africa, where the majority of malarial disease is manifest was not even included in the global control mechanism, as it had insufficient infrastructure to support the policy (WHO, 1980). Despite the setbacks, up until 1969, when the global eradication policy was finally abandoned, some European countries such as Hungary, Bulgaria, Romania, Yugoslavia, Spain, Poland, Italy, Netherlands, and Portugal had managed to completely eradicate their endemic malaria by interrupting transmission. The suppression of malaria and its eradication from vast areas was through an extensive vector control where source reduction played the key role. However, the development of dichlorodiphenyltrichloroethane (DDT) may have misled the malaria control community into the ideas that malaria could be controlled and even eradicated by targeting adult mosquitoes with synthetic insecticides (Najera, 2001). Such was the enthusiasms for indoor residual spraying that alternatives were abandoned, neglected and even marginalized (Walker, 2002).

From the early 1970's the malaria situation has slowly and progressively deteriorated, and reduced control measures between 1972 and 1976 due to financial constraints led to a massive 2-3 fold increases in cases globally. Spraying never truly eradicated the mosquitoes anywhere. Since 1976, several new pockets of malaria

transmission have evolved, and a WHO 1980 report recommended that countries that had become non-malarious should maintain at least one malaria vigilance unit.

At present, at least 3 million people are affected by malaria globally, and there are between 1-1.5 million malaria deaths per year (WHO, 1980). Malaria is generally endemic in the tropics, with extensions into the subtropics (WHO, 1980). Distribution varies greatly from country to country, and within the countries themselves, as the flight range of the vector from a suitable habitat is fortunately limited to a maximum of 2 miles.

Ninety per cent of malaria cases are in Africa, with the remainder clustered in nine countries: India, Brazil, Afghanistan, Sri-Lanka, Thailand, Indonesia, Vietnam, Cambodia and China. In Africa, malaria accounts for up to a third of all hospital admissions, and up to a quarter of all deaths of children under the age of five. In Africa it is estimated that an individual receives 40-120 infective mosquito bites per year, compared to only two per year in India (WHO, 1980). Bearing the figures for Africa in mind, it seems incredible that in 1969, global eradication was envisaged from a policy that effectively ignored Africa other than for a couple of pilot schemes.

2.2 Limitations of integrated malaria control

Despite initial success in trying to curb malaria, there was a complete failure to eradicate malaria in many countries due to a number of factors. Although technical difficulties such as vector and parasite resistance to insecticides and drugs respectively have played a part, the main failure to reduce the disease is probably due to social and political factors preventing efficient application of control measures. The malaria control operation was criticized for being too much like a military operation, and the lack of explanations offered to the local populations meant that the control measures received little support or even downright opposition (Bruce-Chwatt, 1980).

Although intra-domiciliary insecticides remain powerful and indispensable tools for malaria control (Curtis and Mnzava, 2000), even these superb interventions often fall short of the efficacy levels required to eradicate malaria in tropical settings. Today malaria remains robustly endemic across most of the tropics especially sub-Saharan Africa where human populations, ideal climatic conditions and exceedingly efficient vectors mediate transmission intensities up to 100 fold higher than required to maintain it (Snow *et al.*, 1999a). In addition the rapid unplanned urbanization observed in many parts of Africa is changing the context of human population and natural system interaction. Human migration, population growth, socio-economic status and behaviours and the modernized

environment can potentially affect the dynamics of a natural system. It is clear that mosquitoes are colonizing new ecological niches in response to appropriate urbanization and increased man made environmental changes. Research needs to keep pace with the rapid evolution of vector to be able to manage the disease (Scientific Working Group, 2002).

Most malaria control interventions are based on antimalarial drugs to treat the sick and use of insecticides to keep the population of mosquitoes in check. Effectiveness of both of these tools is now being compromised (Scientific Working Group, 2002). Insecticide treated mosquito materials are vital component of the Roll Back Malaria Global strategy for vector control, but the number of effective insecticides available is limited, with heavy reliance on pyrethroids. Pyrethroids are already facing problem of resistance and their stability in the environment is a major concern. In the African situation, the availability of environmentally benign insecticides could allow incorporation of larval control into integrated malaria and vector management programmes.

Although indoor spraying and bed nets can reduce exposure by at least an order of magnitude, they often have limited clinical impact when faced with intense transmission by the highly efficient African vector (Lengeler, 1998). High level of absolute coverage, in terms of human population with bed nets are often difficult to achieve in practice (Lines, 1996; Lengeler and Snow, 1996; Snow *et al.*, 1999b), with cost and delivery mechanism being critical determinants of success (Guyatt *et al.*, 2002). Furthermore, the ability of vectors to avoid many domestic insecticide interventions can compromise their effective coverage of the target vector population (Killeen *et al.*, 2002a).

The availability of healthcare services for diagnosis and treatment is crucial to minimizing the mortality risk associated with exposure to malaria infected mosquitoes, but can do little to limit the incidence of clinical malaria in areas of high transmission because it deals only with malaria infection after they occur and has minimal impact in infectiousness of human reservoir (Carter *et al.*, 2000; Killeen *et al.*, 2000). Large scale prophylaxis has played significant role in some notably successful programmes (Romi *et al.*, 2002), but may also have limited impacts and even quite dangerous consequences such as the emergence of drug resistance (Krogstad, 1996; Molyneux, *et al.*, 1999) or even intensification of transmission (Hogh *et al.*, 1998; Puta and Manyando , 1997). Even intensive infection control with active detection, drug treatment and follow up cannot eliminate endemic malaria from most parts of sub-Saharan Africa unless rapid re-infection can be prevented by effective vector control (Beir *et al.*, 1999; Marsh and Snow, 1999). In

addition, it is unlikely that drugs alone will eliminate the disease, and vector control efforts need to be maintained (Scientific Working Group, 2002).

Given that the most commonly advocated malaria control tools for protecting man against adult mosquitoes have clear limitation it is worth considering other technologies not as alternative but as possible additions to integrated programmes (Schiff, 2002; Carter *et al.*, 2000). Currently the development of sophisticated approaches to malaria control, such as transmission blocking vaccines and genetically modified mosquitoes are being vigorously advocated and pursued (Spielman *et al*, 2001; Ito *et al.*, 2002; Richie and Saul, 2002). These approaches are unlikely to bear fruit for several years and their likelihood for success has been questioned (Boete and Koella, 2002; Saul, 1993). In this context it may be useful to reconsider simple control tools that enable the most historically successful attempts to control malaria transmission by *Anopheles gambiae* (Shousha, 1948; Soper and Wilson, 1943) before such approaches were abandoned in favour of modern synthetic adulticides.

Larval control has long been an option for malaria control (Kitron and Spielman, 1989; Rozendaal, 1997), but this is grossly under developed and underused approach, particularly in tropical Africa where it may be of much greater utility than generally appreciated. Given the extensive, diverse, and sometimes obscure nature of breeding sites chosen by Afro-tropical vectors, this targeting represents a formidable challenge but one that has proven feasible in the past (Utzinger *et al.*, 2002). Clearly if ambitions for malaria control in Africa are to go beyond attenuating transmission and minimizing the effects of its consequences, truly integrated control involving all tools at our disposal will be necessary (Killeen *et al.*, 2002b).

2.3 Malaria in Kenya

One of the biggest challenges facing the National Malaria Strategy is the emergence of parasite resistance to the commonly used and relatively cheap drugs (Division of malaria control, Kenya, MoH, 2007). Kenya's ecology provides ideal conditions for the malaria-carrying mosquitoes, especially in the coastal and lake regions. Climatic conditions are conducive to outbreaks of epidemic intensity in other areas such as intensively farmed highlands and semi-arid North Eastern parts of the country where such outbreaks are increasingly frequent.

The level of endemicity of malaria in Kenya varies from region to region and there is a big diversity in risk of malaria infection largely driven by geographical differences in

altitude, rainfall, humidity, climate and temperature (including the effects of altitude). These factors influence transmission patterns, as they determine vector densities and intensity of biting. This notwithstanding, there have been reports of malaria cases in regions where the disease was unheard off in the past. Over 25 million Kenyans are at risk of contracting malaria. Malaria contributes a significant economic burden with an estimated 170 million working days lost annually to malaria illness. In addition, each Kenyan family spends at least 20US$ annually for treating malaria. Malaria reduces the Kenyan GPD by 1% annually (Division of malaria control, Kenya, MoH, 2007)

2.4 Fungal metabolites with known biological activity

Over the past years since the discovery of penicillin, fungi have proven to be a valuable resource for discovery of novel natural products, many of them potential targets for agrochemical and biomedical development among other industrial applications. A large number of bioactive and structurally diverse fungal metabolites have been isolated and characterized over the years and some of these have been used for the development of valuable pharmaceuticals and pesticides. Most fungal metabolites have been reported from fermentations, but also compounds formed in fruiting bodies have attracted attention for several reasons (David and Christopher, 1992).

For decades, biological activity of fungal metabolites has been carried with enormous success. Majority have wide applications in the pharmaceutical industry, agriculture and in food and beverage production industries among others. Secondary metabolites investigated for other biological activity other than insecticidal properties include hispidin and hispolon isolated from *Inonotus hispidus* a parasitic fungus living on a variety of deciduous trees. These showed immunomodulatory and antiviral activity.

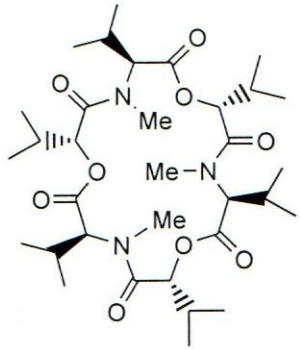
Ganoderma lucidum (Fr.) Krast (Polyporaceae), is a basidiomycete used in traditional Chinese medicine s usually for strengthening the bodies resistance to diseases. In east-Asian countries, *G. lucidum* is also used as health food, especially the submerged cultured mycelia and the cultured broth. The immune-regulation activities of polysaccharides include immune-stimulation and immune-suppression. A galactose rich extracellular polysaccharide (GLP-2), which is composed of galactose, mannose, glucose, arabinose and rhamnose in the molar ratios 103:17:12:10:3 was isolated from the submerged culture broth of a basidiomycete *G. lucidum* and its bioactivity tests showed that GLP-2 enhanced the T- and B- lymphocyte proliferation and exhibited lymphocyte activity,

both *in vitro* and *in vivo* (Yan-Qun Li *et al*, 2007). Immuno-suppressants are used to treat viral hepatitis in medical practices.

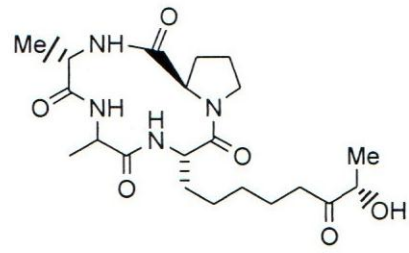
In addition, production of bioactive compounds by macrofungi has been studied and documented and this shows that fungi do indeed produce secondary metabolites. Organic extracts (ethanol, methanol and chloroform) of *Agaricus blazei,* an edible mushroom were found to have anticlastogenic activity in cultured cells by a test for chromosomal aberrations (Luiz *et al.*, 2003). Aqueous extracts of *Ag. blazei* showed an antimutagenic effect against the chemical mutagens cyclophosphamide and methylmethane sulfonate in the micronucleus assay performed *in vivo* and *in vitro* (Delmanto *et al.*, 2001; Menoli *et al.*, 2001). The inhibition of tumour cell growth in rats has also been demonstrated (Takusaburo and Yoshiaki, 1998) and antitumor substances have been isolated from these extracts.

With regard to toxicity, cytotoxic steroid derivatives of cerevisterol and ergosterol have been isolated from extracts of the basidiocarp of *Ag. blazei* obtained with acetone (Kawagishi *et al.*, 1988). Inhibitory effect of a cerevisterol has also been demonstrated on replicative DNA polymerases (Mizushina *et al.* 1998). Other derivatives of ergosterol, such as 4-hydroxy-17-methyl-incisterol, also showed an inhibitory effect on DNA polymerase, which was also found to be dose-dependent (Togashi *et al.*, 1998). *Ag. blazei* also contains a substance with antimicrobial activity, 13-hydroxy-cis-9, trans-11-octadienoic acid, obtained from the hexane extract of its basidiocarp (Osaki *et al.*, 1994).

HC-toxins, (a group of fungal compounds which contain an unusual amino acid 2-amino-8-oxo-9,10-epoxydecanoic acid), were originally isolated from *Cochliobolus carbonum* (Walton *et al.*, 1982; Tanis *et al.*, 1986). The HC-toxins are host selective toxins and act as inhibitors of histone deacetylase, a key nuclear enzyme involved in transcriptional control. The anti-protozoal activity of range of cyclic tetrapeptides related to HC-toxins has been reported (Darkin-Rattray *et al.*, 1996). The metabolite enniatin B **(1)** and a cyclic tetrapeptide, JM47 **(2)** have been isolated from brown rice culture of marine algae *Codium fragile* (Zhong *et al.*, 2002). These two have been tested and have shown remarkable antibiotic activity.

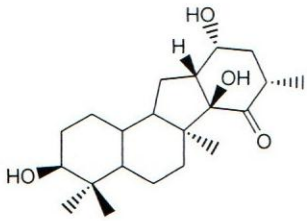


Enniatin **(1)**

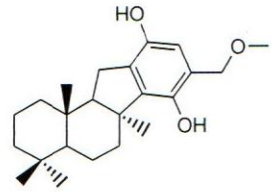


JM47 **(2)**

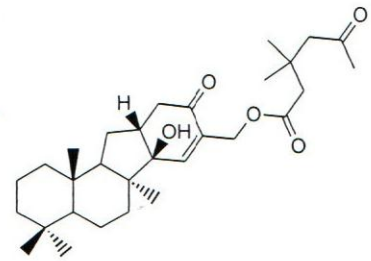
Four terpenoid metabolites named dasyscyphins A **(3)**, B **(4)**, C **(5)** and niveoulone **(6)** were isolated from fermentations of *Dasyscyphus niveus* strain and their cytotoxic properties and antibiotic activities investigated. Dasyscyphin A showed no significant biological activity whereas dasyscyphin B and C and nivuelone showed cytotoxic activities against human cells and modest or weak antibiotic activities (Viktor *et al.*, 2006). Another biologically active triterpenoid, favolon B **(7)** has been isolated from the fermentation broths of *Mycena* species (a Chilean basidiomycete) and showed antifungal activity against *Botrytis cinerea*, *Mucor miehei*, *Paecilomyces variotii* and *Penicillum notatum* (Pedro *et al.*, 2005).



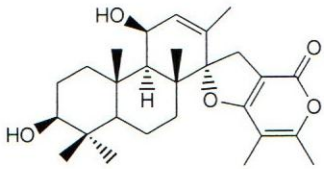
Dasyscyphin A **(3)**



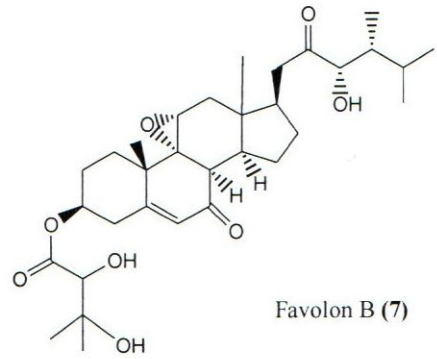
Dasyscyphin B **(4)**



Dasyscyphin C **(5)**



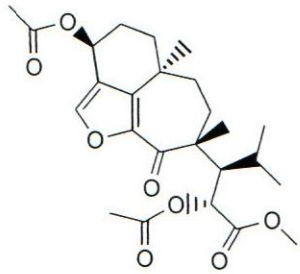
Nivuelone **(6)**



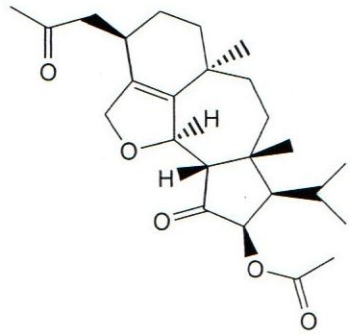
Favolon B **(7)**

Diterpenoids named heptemerones A~G **(8-14)**, were isolated from the broth submerged cultures of *Coprinus heptemerus*, a basidiomycete which had not previously

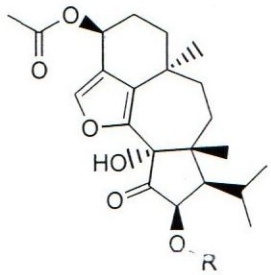
been known to produce secondary metabolites and were investigated for biological activities. Among the biological activities, inhibition of fungal growth was most potent while cytotoxic activities were moderate and only heptemerone D was phytotoxic (Melanie *et al.*, 2005).



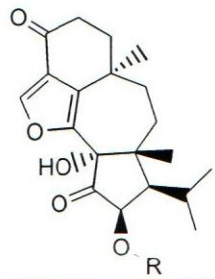
Hepteromones A **(8)**



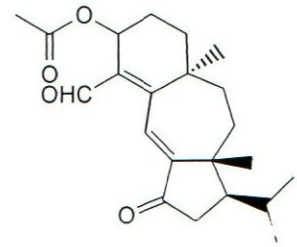
Hepteromones B **(9)**



Hepteromone C: R = CH_3 **(10)**
Heptemerone D; R = CH_3CO **(11)**

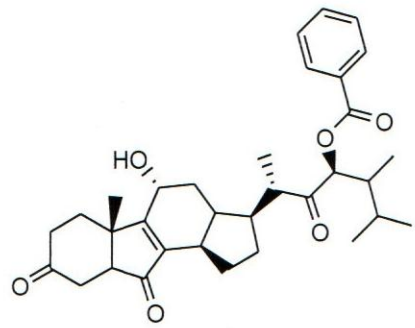


Hepteromone H; R = H **(12)**
Heptemerone E; R = CH_3CO **(13)**



Hepteromone G **(14)**

Laschiatron **(15)**, an antifungal antibiotic compound that exhibits broad *in vitro* activity against several human pathogens has been isolated from fermentations of *Favolaschia* species (Anke *et al.*, 2004).



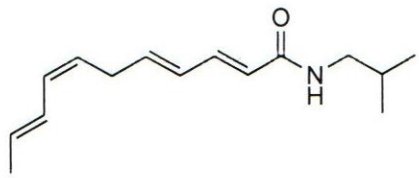
Laschiatrion **(15)**

Phomol a novel antibiotic has been isolated from fermentations of *Phomopsis* species in the course of screening of endophytic fungi from the medicinal plant *Erythrina crista-galli.* The compound exhibits antifungal, antibacterial and weak cytotoxic activity.

Anti-inflammatory activity has also been done for the compound on different reporter gene assays (Weber *et al.*, 2004).

2.5 Mosquito larvicidal compounds from plants

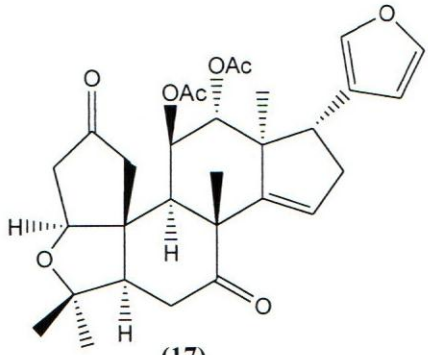
Plants products may be applied against malaria vectors in two principal ways: as sources of repellents to reduce human-vector contact, and in control of mosquito larvae and /or adults. The use of plants as repellents is widespread in different communities in Africa and the performance of many has been evaluated experimentally (Curtis and Mnzava, 2000; Seyoum and Abate, 1997; Snow *et al.*, 1999b). No related use of plant products targeted for vector control has been documented although individual extracts/components of a number of plants have been shown to have potent toxic or growth inhibitory activities against larvae of different species of mosquitoes. Several mosquito larvicides have been isolated form *Spilanthes* species such as *Spillanthes ocelacea* (Jansen) *S. alba,* (Jansen) *S. americana S. acmella* (Jansen) *S. ocymysolia* and *S. mauritiana* (Rich. ex Perse). N-isobutyl-2E, 4E, 8E, 10Z-dodeca-2, 4, 8, 10-tetraenamide **(16)** was isolated from *S. mauritiana* and gave 100% mortality against third and fourth instar larvae of *Ae aegypti* at concentration of 10^{-5} mg/ml (Jondiko, 1986).



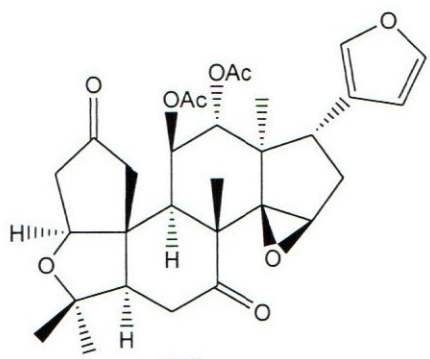
N-isobutyl-2E, 4E, 8E, 10Z-2, 4, 8, 10-tetraenamide **(16)**

Plants in the family Meliaceae are characterized by the presence of limonoids (tetranortriterpenoids) that exhibit a number of anti-insect effects. A number of these plants are used for traditional medicine in Eastern Africa, including *Turraea floribunda* (Hochstetter), a small tree or shrub that is used as an emetic and purgative. Ring A-*seco* mosquito larvicidal limonoids from the root bark of *Turraea wakefieldii* have been reported, (Ndung'u *et al*, 2003). Five limonoids were purified from the root back extracts and were characterized as tecleanin-type limonoids for which the name neotecleanin was proposed to all, i.e. 11β, 12α- diacetoxyneotecleanin **(17)**, 11β, 12α- diacetoxy-14β 15β-epoxyneotecleanin **(18),** 7α, 12α -diacetoxy-14β, 15β-epoxy-11β -hydroxy-neotecleanin **(19)**, 7α, 12α -diacetoxy-11β -hydroxyneotecleanin **(20)** and 11β, 12-diacetoxy-1-deoxo-14β, 15β -epoxy-3β -hydroxy-2-oxo-neotecleanin **(21)**. In mosquito larvicidal assays,

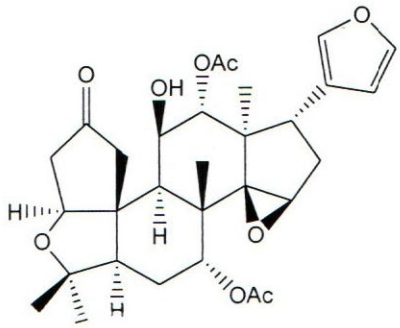
compounds **17**, **18** and **19** showed dose dependent larvicidal activity against larvae of *A. gambiae*.



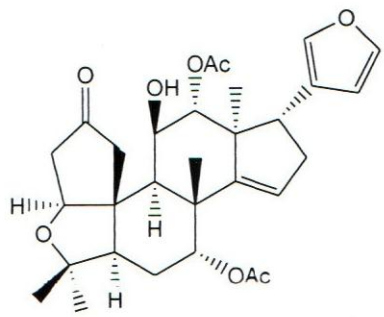
(17)



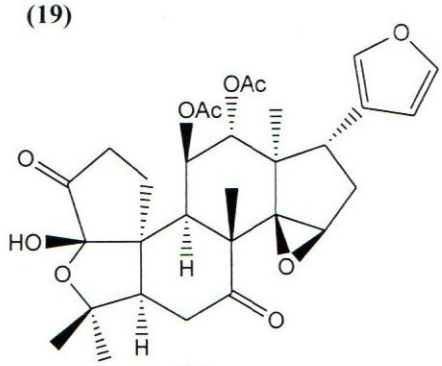
(18)



(19)

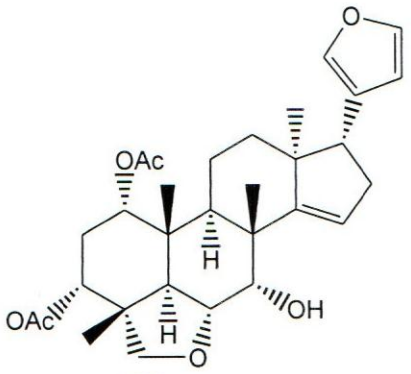


(20)

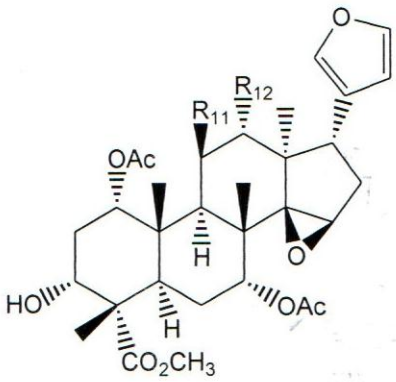


(21)

In addition, a valisininoid **(22)**, a limonoid was isolated from root bark extracts of *T. wakefieldii* while the root bark of *T. floribunda* afforded three limonoids; havanensinoid **(23)**, havanensinoid **(24)** and havanensinoid **(25)**. All these limonoids were found to exhibit larvicidal activity against third-instars larvae of *A. gambiae*. The structures of these compounds are given below (Ndung'u *et al.*, 2004).

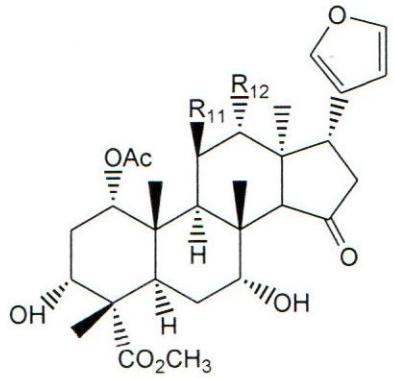


(22)



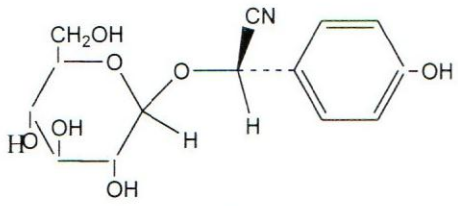
R_{11} = OAc; R_{12} = $OCOCH(CH_2)_3$ **(23)**

R_{11} = OAc; R_{12} = $OCOCH(CH_3)CH_2CH_3$ **(24)**



R_{11} = OAc; R_{12} = $OCOH(CH_2)_3$ **(25)**

Grain sorghum (Moench) extracts derived from seedlings varieties widely cultivated in USA and Egypt for human food and animal fodder have shown larvicidal activity against *Culex pipiens* larvae under laboratory conditions. The activity is greatly attributed to presence of organic cyanogen dhurrin **(26)**, [S]-P-hydroxymandelonitrile β-D-glucopyranoside in grain sorghum. This cyanogenic glycoside is present in high concentrations in immature plant (Nartey, 1981) and is the most likely toxic constituent in grain sorghum capable of larvicidal effects (Branson *et al.*, 1969). Structure of dhurrin is given below (Jackson *et al.*, 1990).

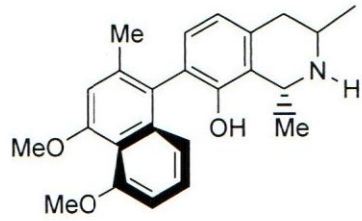


Dhurrin **(26)**

Essential oil from *Ocimum sauntum, O. basilicum* (Watson ex Dallwitz) and *Eucalyptus globules* (White ex Styles) have been studied individually and in combination, which have shown larvicidal activity. Also, essential oils of the plant *Ipomoea cairica* (Johnson) have been extracted and shown to produce mosquito larvicidal activity against larvae of *A stephensi, Ae Aegyptii, Cx. quinquefasciatus* and *Cx. tritaeniorhynchus*. Recently, botanical and microbial insecticides have been increasingly used for mosquito control because of their efficacy and documented non-toxic effects on non-target organisms (Gunasekaran *et al.*, 2004; Senthil, 2006).

Recent studies have also stimulated the investigation of insecticidal properties of chemicals derived from plant material and concluded that they are environmentally safe, degradable, and target specific. Indeed, many medicinally important plant extracts were studied for their efficacy to kill larvae of different species of mosquito (Markouk *et al.*, 2000; Tare *et al.*, 2004).

The larvicidal activity of dioncophylline A **(27)**, a naphthylisoquinoline alkaloid derived from the tropical vine *Triphyophyllum peltatum* (Dioncophyllaceae), was investigated against the malaria vector *A. stephensi.* Inhibition assays showed that the younger larval stages were very sensitive towards this natural product, with LC_{50} values below 1 mg/l. Pronounced effects were observed within 4 hours of exposure. Aging larvae, however, were less sensitive, while pupae were totally insensitive to the action of dioncophylline A (Franqois, 1996).



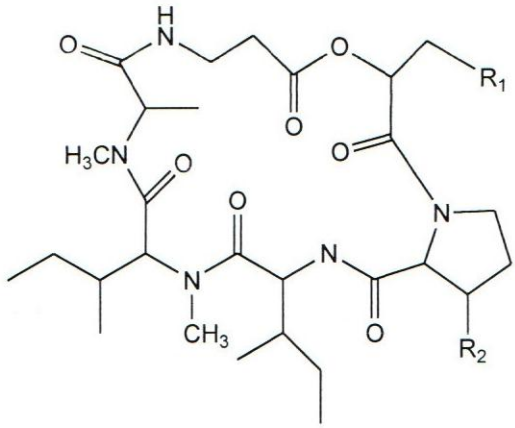
Dioncophylline A **(27)**

Extracts and compounds from tubers of *Neorautanenia mitis* (Verdeourt) have been evaluated against the malaria- and filariasis-transmitting mosquitoes, *A. gambiae* and *Cx. quinquefaciatus*, respectively. The extracts exhibited activity against larvae of *A. gambiae* and *Cx. quinquefaciatus* mosquitoes. The active extracts yielded the coumarin derivative pachyrrhizine, the isoflavonoids neotenone and neorautanone, and the pterocarpans neoduline, nepseudin and 4-methoxyneoduline as the active constituents. The activity of the

crude extracts was at about the same magnitude as that of the constituent natural products, the latter's efficacy being almost at the same level for all the isolated compounds (Joseph *et al.*, 2004). Alcohols from methanol to eicosinol were found to have ability to kill larvae of species *Cx. tarsalis*, the principal vector of the Western equine and St. Louis encephalitis virus common throughout the Western US (David and Christopher, 1992).

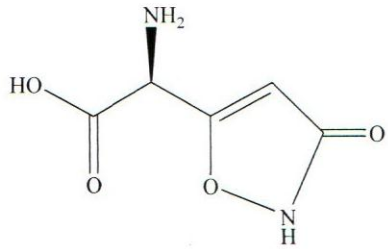
2.6 Fungal metabolites with insecticidal properties

Bio-assay guided fractionation of the organic extracts from liquid cultures of *Aschersonia* spp by silica gel packed column chromatography and reversed phase HPLC afforded two cyclic dipeptides i.e. destruxins A4 **(28)** and destruxin **(29)**. Another compound previously identified as homodestruxin B **(30)** was also identified as a product of this fungus. Destruxins A4 and A5 are the first biologically active molecules from fungus of genus *Aschersonia*. The three compounds showed insecticidal activity against *Drosophilla melanogastus* (Stuart and Gibson, 1996). The general structure of the three compounds is given below. Notable, however, is the scarcity of literature of fungal metabolites used both for mosquito control as well as other insect pests since the field has received limited attention by researchers, scientists and scholars (Stuart and Gibson, 1996).



1 R_1 : $CH{=}CH_2$, R_2 = H**(28)**
2 R_1 : $CH{=}CH_2$, R_2 = CH_3 **(29)**
3 R_1 : $CHMe_2$, R_2 = H **(30)**

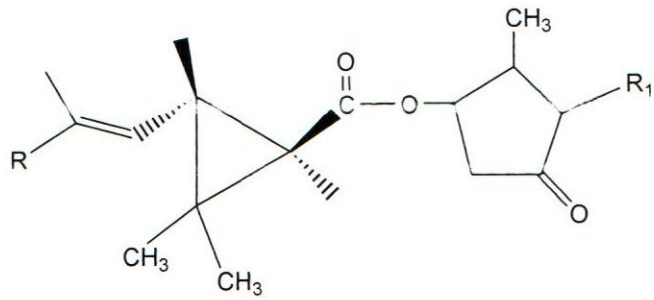
Insecticidal activity against houseflies was described for ibotenic acid **(31)** isolated from fruiting bodies of *Amanita muscaria*, *A. strobiliformis* and *A. pantherina*. A cyclopeptide, beauvericin was also isolated from basidiomycetes *Polyporus sulphureous* (Deol *et al.*, 1978) and from entomopathogenic 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.



Ibotenic acid **(31)**

2.7 Natural and synthetic insecticides used to control mosquitoes

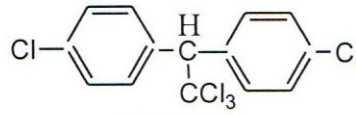
Alkaloids such as nicotine, anabisine, nornicotine and pyrethrum have been widely used as insecticides. Pyrethrum is found in flower heads of certain plants in *Chrysanthemum* genus family Compositae. The active compounds in pyrethrum are pyrethrins I **(32)** and II **(33)** and cinerins I **(34)** and II **(35)**. The insecticidal properties of the pyrethrum are due to five esters i.e. pyrethrins I and II, cinerins I and II and Jasmolin II **(36)** that are present mostly in the achene's of the flowers, ranging from 0.7 to 3.0%.



Where

	R	R_1
Pyrethrin I	$-CH_3$	$-CH_2CH{=}CHCH{=}CH_2$ **(32)**
Pyrethrin II	$-COOCH_3$	$-CH_2CH{=}CHCHCH_3$ **(33)**
Cinerin I	CH_3	$-CH_2CH{=}CHCH_3$ **(34)**
Cinerin II	$COOCH_3$	$CH_2CH{=}CHCH_3$ **(35)**
Jasmolin II	$-COOCH_3$	$-CH_2CH{=}CHCH_2CH_3$ **(36)**

DDT **(37)** is one of the best and most controversial synthetic organochlorinated insecticide ever produced, though its use and application is less at present. DDT in presence of strong alkalis, is decomposed to DDE (2, 2-bis (p-chlorophenyl)-1, 1-dichloroethylene) which is oxidized to pp-dichlorobenzophenone.

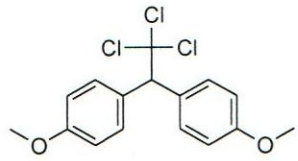


DDT **(37)**

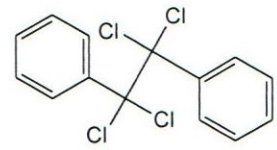
Thermal decomposition point of DDT is 125°C. The tropical and subtropical climate may be sufficiently conducive to decompose technical DDT rapidly causing complete loss of activity in short time (Ramulu, 1985). Although long exposure to ordinary temperature zones may not cause appreciable changes, UV light and in few cases sunlight may cause decomposition of DDT (Ramulu, 1985).

A number of compounds closely related with DDT and have insecticidal activity have been synthesised. These include methoxychlor **(38)**, dichlorodiphenyldichloroethane DDD **(39)**, benzene hexachloride (BHC) **(40),** 1,1,1-trichloro-2, 2-bis (p-fluorophenyl) ethane (DFDT) **(41),** chlordane **(42)** and perthane **(43).** Others include dilan, neotran, sulphenone, and heptachlor.

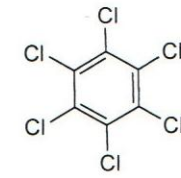
Methoxychlor **(38)** is prepared by reacting anilose and chloral in presence of sulphuric acid or glacial acetic acid. Methoxychlor has limited use due to its high cost and low effectiveness towards some insects. DDD differs from DDT only by the absence of one chlorine atom in the ethane moiety. It has low toxicity on insects thus needs to be applied in high doses. Benzene hexachloride accumulates in animal tissues. It is inconsistent in activity due to presence of isomers of different toxicity and also has received limited application due to its shorter residual action owing to its high volatility. Chlordane has slow action against insects and has been replaced by the more effective heptachlor and other related compounds while perthane has low toxicity to higher animals and is selective but has a low activity.



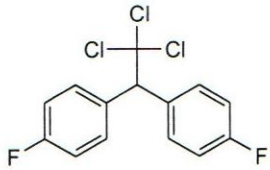
Methoxyclor **(38)**



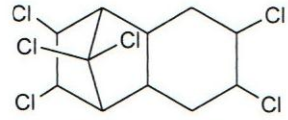
Dichlorodiphenyldichloroethane **(39)**



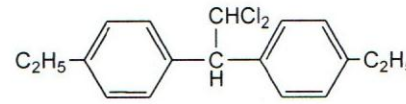
Benzene hexachloride **(40)**



1, 1, 1-trichloro-2, 2-bis
(p-fluorophenyl) ethane **(41)**



Chlordane **(42)**



Perthane **(43)**

Of the large number of DDT analogs, only few like DDD, methoxychlor and DFDT have been developed commercially. Insecticides containing DDT affect growth of certain crops like cucurbits by producing chlorosis, stunting and consequent reduction in yield. In most applications, DDT does not readily break down in soil and under certain conditions causes serious damage to plant life, if it becomes sufficiently deposited in the soil. In addition to these insecticides, we have pyrethroids, organophosphates and organocarbamates (Ramulu, 1985).

CHAPTER THREE

METHODOLOGY

3.1 Reagents and chemicals

The main chemicals and reagents that were used in this project are; Methanol 99.98% purity (Reag. Ph. Eu.) Panreac Quimica of Spain, Ethyl acetate (GPR) Belami fine chemicals, cyclohexane 99.99% purity (ARG) Fisher Scientific, UK, Acetone (GPR) Kobian Kenya Limited, Silica gel (MN 60 Kieselgel 0.063 – 0.2 mm/70 -230 mesh AST for column chromatography) Machery – Nagel Germany and Mitsubishi HP21-DIAION resin

3.2 Equipments

The equipments used in this project were: Weighing balance – Denver instrument Model – XL-31000, Autoclave – Danfoss 59407-3 No. 375, Analytical Balance – Precision 310M Swiss quality, pH meters – Fishes Acument ® model 620A, Magnetic stirrer hotplate – Gallenlamp, Vacuum rotary Evaporator type 349/2, Biological safety cabinet (SterilGARD The Baker Company Sanford – Class II Type A/B3 US PAT NO. 3,895,570) and Suction pump

3.3 Apparatus and other materials

Capillary tubes, 250ml and 100ml conical flask, 100ml measuring cylinders, Buchner funnel filtration system, Filter funnel, Petri dishes, chromatographic tanks, Chromatographic column (glass), 250 ml glass beaker, 100ml glass beaker, 250ml rounded round bottomed flask, 500ml separating funnel, inoculating blade (spear), Acid washed sand, TLC plate, {Macharey-Nagel, ALUGRAM ® SIL G/UV_{254} 0.25 mm Duren, Germany}, cotton wool, Aluminium foil, Glucose testing strips (Diabur-test ® 5000 (Roche).

3.4 Materials used for testing

Disposable tips, 20 µl and 200µl Eppendorf micropipette, syringes, three droppers with rubber suction bulbs, data recording forms, disposable cups (glass bowls) or beakers of 120ml holding 100ml and 250ml holding 200ml and *Ae. aegypti* larvae in their late third and early fourth instars.

3.5 Preliminary preparations

3.5.1 Sterilisation of apparatus and materials used

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

3.5.2 Preparation of liquid media

Liquid media was prepared by dissolving 10.0 g of molasses, 4.0 g glucose and 4.0g of yeast extract in 1.0L of water. The pH of the media was adjusted to 5.5 using 1M hydrochloric acid (and 1M sodium hydroxide where necessary). The media was then dispensed 250ml Erlenmeyer flasks. These were corked with cotton wool plugs/corks and then wrapped with an aluminium foil. They were autoclaved at 121°C and steam pressure of15 Pascal's, for 15 minutes then left to cool. Once cool, they were placed in the working bench of a sterile laminar flow hood. These were used to prepare the starter cultures.

When preparing the molasses liquid media for growth of the already growing cultures, the media prepared similarly as above and then dispensed in 2 l Erlenmeyer flasks and sterilized at the same conditions as above.

3.5.3 Inoculation and culturing of the basidiomycete JO5444

The stock culture agar slant of the basidiomycete used in this project was preserved at the Integrated Biotechnological Research Laboratory, Egerton University. Preserved mycelia material on an agar slant was resuscitated in solid potato dextrose agar in sterile plate (appendix 2). On resuscitation, starter cultures were prepared by inoculating agar plugs in liquid media on 250ml scale replicates. This was done under extremely sterile conditions in the laminar flow hood. After 14 days, each of the starter cultures was transferred in 1 litre scales in 2 litre Erlenmeyer flasks. The bulk cultures (appendix 3) were grown as still cultures for 21 days with regular agitation at room temperature and pressure. In total, sixty 250ml starter cultures were used to inoculate a total of sixty 1 litre scales in 2 litre Erlenmeyer flasks.

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3.6 Preparation of crude extracts from cultures of basidiomycete JO5444

Immediately after the growth was stopped, mycelium was separated from culture filtrate by filtration. The culture broth was not used in this study and was therefore discarded. The mycelium was immediately macerated and soaked in acetone and left for 24 hours with periodic agitation using magnetic stirrer. 250ml Erlenmeyer flasks were used and approximately 150ml of acetone were added to a reasonable quantity of the macerated mycelium. The flasks were corked to reduce evaporation of the solvent. The acetone extract was filtered using the Buchner funnel, filter paper, side arm flask and vacuum pump. The acetone extract concentrated to an aqueous concentrate at reduced pressure using a rotary evaporator and vacuum pump. The concentrate was extracted thrice with equal volume of ethyl acetate in a separating funnel. The total volume of ethyl acetate extract was combined and dried with anhydrous sodium sulphate. This was then filtered and the filtrate collected in clean round bottomed flask before being concentrated under reduced pressure using a rotary evaporator. The final crude extract obtained, was weighed in a screw capped vial and kept in the refrigerator at 4°C. this was then screened for larvicidal activity against the test organism (ref section 3.9). The crude extract was then dried using 4g of silica gel in a 50ml glass beaker and its weight determined.

3.7 Column chromatography

The A glass chromatographic column was mounted vertically. Approximately 80.0g of silica gel were suspended in cyclohexane, and then swirled vigorously to obtain homogenous slurry that was degassed using a vacuum. The column was then slurry packed with the silica gel suspension, ensuring that no air was trapped in between. The dry silica gel adsorbed sample was loaded as a uniform disc 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 cyclohexane/ethyl acetate mixture to ethyl acetate/methanol mixture and finally methanol.

The eluents were collected in 10ml quantities in test-tubes and each spotted on silica coated aluminium TLC plate. The TLC were developed in mid polar solvent system, visualised by the aid of UV lamp (at both long and short wavelength radiation) *p*-anisaldehyde (prepared by mixing 39ml of methanol with 2ml of sulphuric acid and 1ml of anisaldehyde). The eluents were then pooled into nine major fractions depending on their Rf values and colorations as observed under UV radiations and upon spraying with the chemical reagent (*p*-anisaldehyde). Fractions were concentrated by removing the organic

solvents under reduced pressure using rotary evaporator. Each of the fractions was transferred out using 100% pure methanol from the round bottomed flask into a pre-weighed screw capped glass vial before being kept under refrigeration at 4°C. These fractions were then tested individually for their larvicidal potency against the test organism (ref section 3.9).

3.8 Determination of solvent system and purification of compounds in the fractions

Each of the enriched fractions (obtained in section 3.7) were spotted on different pieces of silica coated aluminium TLC plate and then developed in solvents of different polarities. For each, the solvent system was determined by developing a spotted TLC first with a non polar solvent and developed TLC plates were observed under both short and long wavelength UV radiation and then sprayed with *p*-anisaldehyde then heated in the oven at 110°C for 10 minutes and then visualised. Where separation was not satisfactory, a new TLC plate was prepared and developed with a solvent system 5% more polar than the previous one. This was repeated until good separation was observed for individual enriched fractions. The solvent system that gave the best separation of the compounds in the respective fraction was noted and used for purifying the fraction further. Once the solvent for purification was determined, the fractions were dried each on 1g silica gel and its weight determined.

3.8.1 Purification of compounds in the enriched fractions

The dried enriched fractions from section were further purified in column with internal diameter 10.4±0.05mm, and length of effective column was 23.0cm for all. The solvent system to develop the column was optimised in the polarity range which was determined for that particular fraction (refer to section 3.8).

a) Fraction 1

This was not purified.

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b) Fraction 2

The enriched fraction was packed and eluted initially with a 50ml 100% cyclohexane, then 150ml of solvent system consisting 95% cyclohexane and 5% ethyl acetate and finally by 20ml of solvent system consisting 90% cyclohexane and 10% ethyl acetate. A total of 20 collections (approximately 10ml in volume) from the column were made which were then spotted on TLC plate and developed with a solvent system consisting 95% cyclohexane and 5% ethyl acetate. The developed TLC plate was observed

under long and short UV radiations and then sprayed with *p*-anisaldehyde then heated in the oven at 110°C for 10 minutes and then visualised.

c) Fraction 3

The sample was packed in silica gel column and eluted with 200ml of solvent system consisting 80% cyclohexane and 80% ethyl acetate followed by 20ml of solvent system consisting 70% cyclohexane and 30% ethyl acetate. A total of 20 collections approximately 10 ml each in volume were made. These collections were spotted on TLC plate and developed in solvent system consisting 80% cyclohexane and 20% ethyl acetate and visualised as above with the aid of UV lamp and *p*-anisaldehyde.

d) Fraction 4

Was packed and eluted with 400ml of solvent system consisting 60% cyclohexane and 40% ethyl acetate and 40 collections approximately 10ml each in volume each were made. The collections were all spotted on TLC plate and developed using solvent system similar to the one used in eluting the column. The developed TLC plate was observed under long and short UV radiations and then sprayed with *p*-anisaldehyde then heated in the oven at 110°C for 10 minutes and then visualised. From this fraction one pure compound was obtained whose structure elucidation was carried out and larvicidal activity determined.

e) Fraction 5

This was purified with 150ml of a solvent system consisting 50% cyclohexane and 50% ethyl acetate. The collections made were spotted on TLC plate and developed with a solvent system similar to one used to elute the column.

f) Fraction 6

The dried fraction was packed and eluted with 100ml of a solvent system consisting 60% ethyl acetate and 40% cyclohexane. A total of 14 collections were made which were approximately 7ml each in volume. These were spotted on a TLC plate and developed in solvent system similar to the one used in eluting the column. Similarly like the previous ones the developed TLC plate was observed under long and short UV radiations, sprayed with *p*-anisaldehyde then heated in the oven at 110°C for 10 minutes and then visualised

g) Fraction 7

This fraction was packed on column length 23.0cm and eluted with 100ml of ethyl acetate followed by 60ml of solvent system containing 50% ethyl acetate and 50% methanol. This gave 30 collections approximately 5ml each in volume which were spotted on TLC and developed using 100% ethyl acetate. The developed TLC plate was observed under long and short UV radiation and then sprayed with *p*-anisaldehyde.

h) Fraction 8 and 9

These did not show any larvicidal activity at initial stage and were thus not purified further.

3.9 Mosquito larvae bio-activity

The ethyl acetate crude extract was screened for mosquito larvicidal activity against the test organism that is larvae of *Ae aegypti* in their late third and early fourth instars. Approximately 50µl of the crude extract were used. These were dissolved in 20ml of water where batches of 25 third and fourth instars larvae were transferred. The test was done in triplicate and a control was set up for each with 20ml of water and 1 ml of ethyl acetate to which batches of 25 third and fourth instars larvae were transferred.

For the enriched fractions, initially the *Ae. aegypti* larvae were exposed to the 2.5ppm of the enriched fractions and then 1.25ppm. To prepare 2.50ppm test solution, 50µl of enriched fraction were added to 20ml of water in universal glass bottle to which batches of 25 third and fourth instars larvae were transferred The test was performed in triplicate and for each concentration a control was set up simultaneously with tap water to which 1 ml of methanol was added. For the 1.25 test solution, 25µl of enriched fraction were added to 20ml of water to which batches of 25 third and fourth instars larvae were also transferred. This was done for all the enriched fractions individually and tests were performed in different containers. The test containers were held at room temperatures and a photoperiod of 12hrs followed by 12hrs dark. The larvae mortality was recorded after 2, 4, 8 and 24hrs of exposure.

For the pure compound, 2.5mg were dissolved in 25ml of methanol to make the stock solution. From the stock solution, the appropriate volume of dilution was drawn and added to water to obtain the desired target dosage in 20ml water, starting with the lowest concentration. Solutions containing 1.00ppm, 1.25ppm, 1.5ppm, 1.75ppm and 2.00ppm of the pure compound in 20ml. To prepare 1.00ppm test solution, 0.2ml were drawn from the stock solution and topped to make 20ml of the test solution, for 1.25ppm test solution 0.25ml of stock solution were used, whereas for the 1.5ppm test solution 0.3ml of stock solution were used, for 1.75ppm 0.35ml of stock solution were used and finally 0.4ml of stock solution to make 2.00ppm test solution. To each of these different concentrations, batches of 25 third and fourth instars larvae were transferred avoiding transfer of small and unhealthy larvae. 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 and photoperiod of 12 h light followed by 12 h dark (12L: 12D). Larvae mortality was recorded after 24 h exposure. Larvae which pupated during the test period were treated as having negated the test. To get the percentage mortality, Abbott's formula was used;

$$Mortality(\%) = \frac{X - Y}{X} x100$$

Where X = percentage survival in the untreated control and,

Y= percentage survival in the treated sample.

3.10 Data analysis

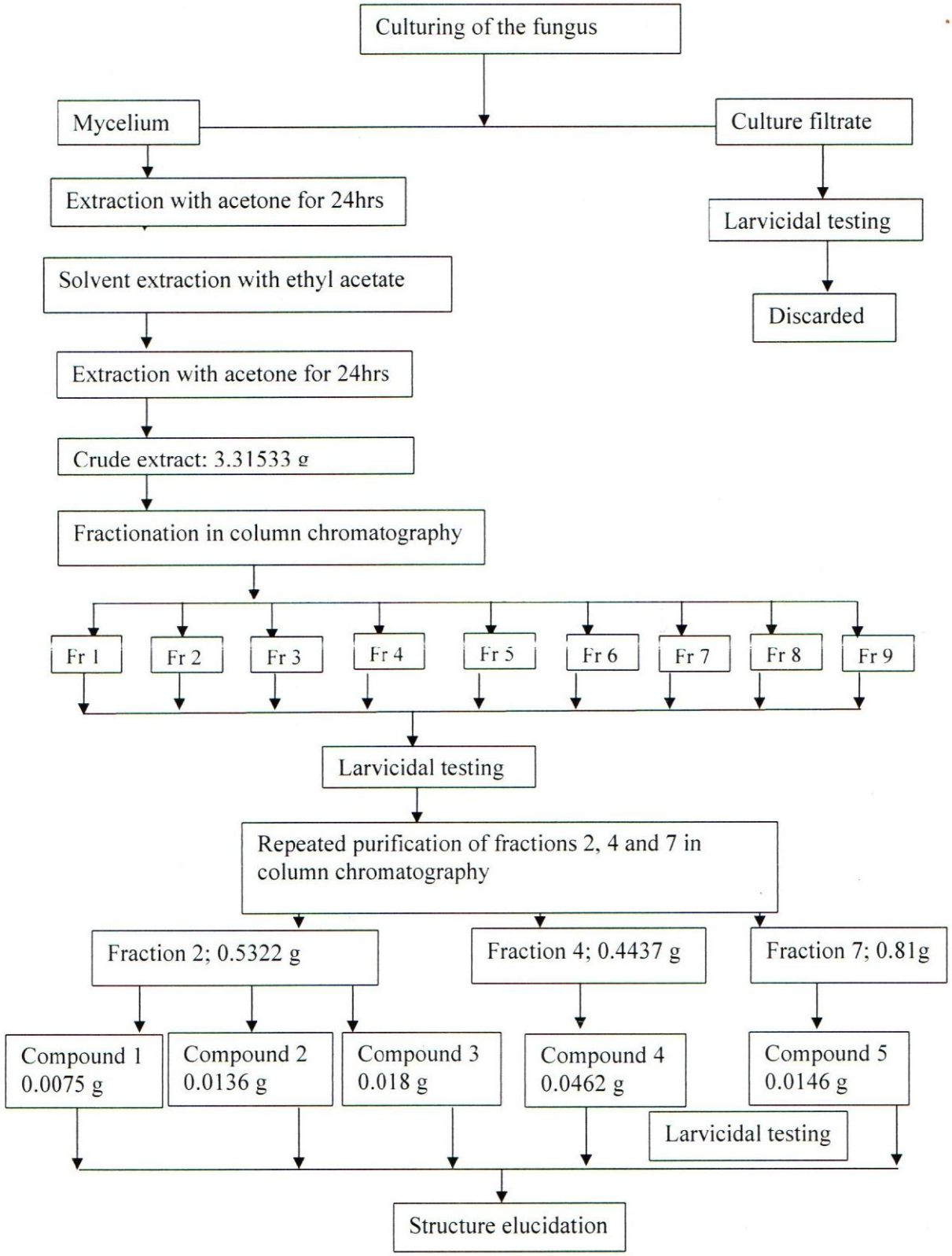
The data obtained from the mosquito larvicidal bioactivity was analysed by linear regression. The LC_{50} and LC_{90} for the purified compound were obtained. The fit (R^2) for the data was also calculated.

3.11 Structure elucidation

The data of the chemical structure of the purified compound from enriched fraction 4 refered to as oxiran-2-yl)methylpentanote and labelled compound **(44).** in consistent with other structures given in this document (refer to section 4.2c) was obtained from an NMR spectrometer. The purified compound was dissolved in deuterated chloroform $CDCl_3$). 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 300 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. t

SCHEME OF EXTRACTION

Bio-activity guided fractionation of the mycelium extract from the basidiomycete JO5444



CHAPTER FOUR

RESULTS AND DISCUSSION

4.1 Results

After twenty one days, growth of the basidiomycete was stopped. On extracting compounds from the mycelium, 3.3153g of crude ethyl acetate extract was obtained. This was tested against larvae of *Ae. aegypti* and showed 100% mortality after 30 minutes of the experiment. The crude extract was purified further in column chromatography. Table 1 below shows the respective yields of enriched fractions purified obtained by column chromatography from the ethyl acetate crude extract of the mycelium of the basidiomycete JO5444.

Table 1: Yields of the enriched fractions

Fraction	Yield (mg)
Fr1	101.3
Fr2	532.2
Fr3	647.0
Fr4	443.7
Fr5	120.0
Fr6	110.1
Fr7	810.0
Fr8	211.0
Fr9	340.0

These enriched fractions were tested against larvae of *Ae. aegypti.* Table 2 next page shows the bioactivity results of the enriched fractions against *Ae. aegypti* larvae at a concentration of 1.25ppm and 2.5ppm

Table 2: Results for the mosquito larvae bioactivity of the enriched fraction

Fraction	Mortality (%)							
	1.25ppm				2.5ppm			
	2hrs	4hrs	8hrs	24hrs	2hrs	4hrs	8hrs	24hrs
Fr1	100	-	-	-	100	-	-	-
Fr2	100	-	-	-	100	-	-	-
Fr3	100	-	-	-	100	-	-	-
Fr4	0	60	100	-	0	100	-	-
Fr5	0	0	0	80	100	-	-	-
Fr6	0	0	0	0	100	-	-	-
Fr7	0	0	0	100	0	0	80	100
Fr8	0	0	0	60	0	0	100	-
Fr9	0	0	0	0	0	0	0	0

The results in table 2 show the percent mortality of larvae upon exposure to a solution containing 1.25ppm and 2.5ppm of the enriched.

Table 3 below shows percent mortality of larvae upon exposure to different concentrations of (oxiran-2-yl)methylpentanoate **(44)** for a period of 24 hours.

Table 3: Results for the mosquito larvae bio-assay of the (oxiran-2-yl)methylpentanoate

Concentration (ppm)	Mortality after 24 hrs				
	Day1	Day 2	Day 3	Day 4	Average
1.00	4	4	3	4	4
1.25	20	20	20	20	20
1.50	44	44	44	44	44
1.75	80	80	80	80	80
2.00	100	100	100	100	100
Control	0	0	0	0	0

The average mortality was plotted against concentration in parts per million (ppm).

4.2 Discussion

a) Extraction and purification

The basidiomycete was resuscitated successfully on potato dextrose agar. A total of seventy five litres of the liquid media were used to grow the basidiomycete for 21 days. After twenty one days, growth of the basidiomycete was stopped and the mycelium separated from the culture broth. On processing the mycelium, a crude ethyl acetate sample weighing 3.3153g was obtained. A 2.5ppm concentration of the mycelia crude extract showed larvicidal activity within 30 minutes of the experiment. The crude extract was fractionated using column and thin layer chromatography. Nine enriched fractions were obtained whose masses are given in table 1(refer to section 4.1).

The nine enriched fractions were tested against the larvae of *Ae. aegypti* and the larvicidal results obtained were tabulated (refer to section 4.1 in Table 2). After spotting and developing the nine enriched fractions on TLC plate, visualization of the developed chromatogram under UV radiation and on spraying with *p*-anisaldehyde showed that Fr1 showed presence fatty acids and steroids due to the polarity discerned from the high Rf values and highly coloured compounds on visualisation. This fraction (Fr1) was thus not purified further.

Upon purification of Fr2 (532.2mg) on column chromatography gave one pure compound weighing 7.5mg after passing 50 ml of 100% cyclohexane, another pure compound after passing 100 ml of a solvent system consisting 95% cyclohexane and 5% ethyl acetate and finally another pure compound after passing 20ml of solvent system consisting 90% cyclohexane and 10% ethyl acetate. The purity of these compounds were discerned from visualisation of a developed TLC chromatogram under UV radiation and after spraying with *p*-anisaldehyde. However, these compounds were in low yields and not much was done on them. Only ^{1}H NMR studies were performed on these compounds due to their low yields. No larvicidal activity was performed on these compounds from enriched Fr2. this also applies to enriched fraction 7 (810mg) which upon purification on column chromatography using 100 ml of ethyl acetate followed by 60 ml of solvent system containing 50% ethyl acetate and 50% methanol gave only one pure compound whose yield was 14.6mg.

Enriched Fr4 gave one pure compound labelled compound 44 and referred to as (oxiran-2-yl)methylpentanoate after passing approximately 200ml of solvent system consisting 60% cyclohexane and 40% ethyl acetate.

Upon purification of Fr3, Fr5 and Fr6 no pure compounds were obtained. Enriched Fr8 and Fr9 did not show any larvicidal activity and were thus not purified further.

b) Bioactivity results

Bioactivity results show differences in sensitivity of larvae to the enriched fractions. The most polar enriched fraction showed no larvicidal potency. Fraction 1, 2 and 3 showed the highest potency against the test organism-causing 100% at concentration of 1.25ppm within the first two hours of the experiment. At the 1.25ppm, enriched Fr4 showed 100% mortality after 8 hours while Fr7 showed the 100% mortality after 24 hours. Fr5 and Fr8 did not achieve the 100% mortality against the test organism. Fr5 showed 80% mortality after 24 hours while Fr8 showed 60% mortality after 24 hours. At the concentration of 1.25ppm, Fr6 and Fr9 did not show any larvicidal activity for the whole experiment period.

At higher concentration of 2.5ppm (double the concentration above), Fr1, Fr2, Fr3, Fr5 and Fr6 showed 100% mortality against the test organism within the first 2 hours of the experiment. The enriched Fr7 and Fr8 showed 100% mortality after 24 and 8 hours respectively while Fr9 did not show any larvicidal activity during the test period. It therefore follows that the enriched fractions 1, 2, and 3 produced rapid efficacy against *Ae. aegypti* larvae. Compounds in Fr 4, Fr7 and Fr8 were long in action .whereas compounds in Fr5 and Fr6 showed dose dependent activity since 100% mortality was realized at higher concentration.

The purified (oxiran-2-yl)methylpentanoate **(44)** (refer section 4.2c) showed high potency against the *Ae aegypti* larvae with LC_{50} of 1.50ppm and an LC_{90} of 1.90ppm. These concentrations of the purified are lower than those reported for some other natural compounds, for example limonoids, vilasininoid isolated from *Turraea wakefieldii,* and havanensinoids (Ndung'u *et al.,* 2004). Figure 1 next page shows a graph of concentration versus percent mortality caused by (oxiran-2-yl)methylpentanoate **(44)** within a period of 24hrs.

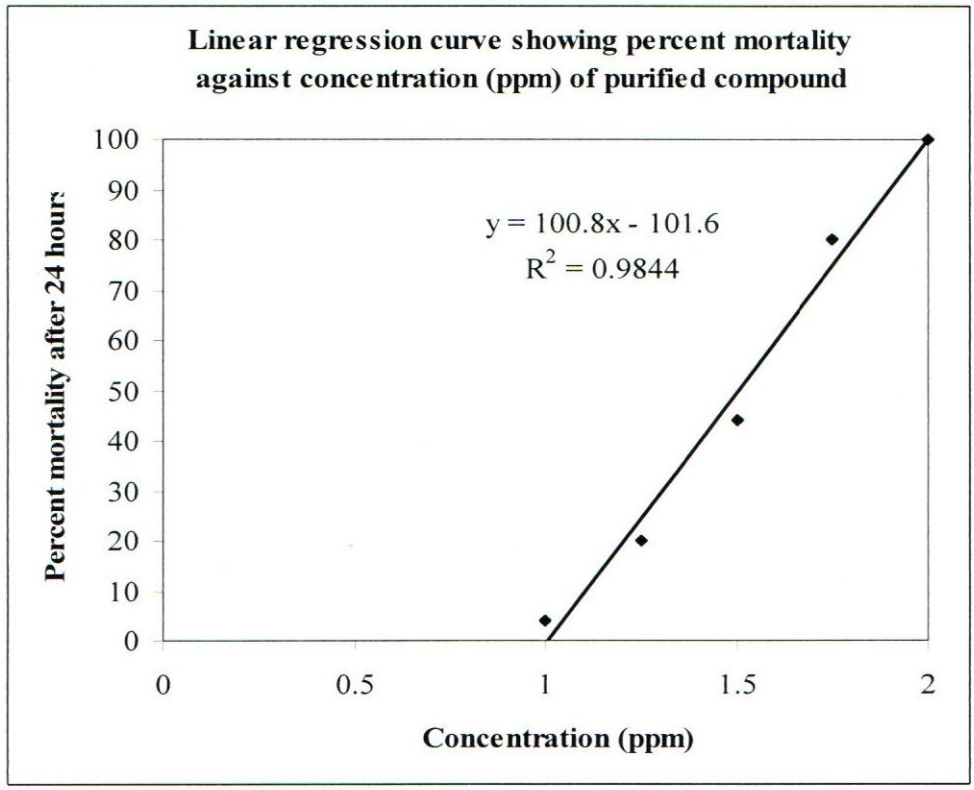


Figure 1: Graph of percent mortality against concentration of (oxiran-2-yl)methylpentanoate **(44)**

c) Structure elucidation of (oxiran-2-yl)methylpentanoate (44)

The (oxiran-2-yl)methylpentanoate **(44)** was purified from the enriched fraction 4 was purified using column chromatography. The solvent system used constituted 60% cyclohexane and 40% ethyl acetate. The compound was collected after approximately 60 ml of the eluting solvent were passed. (oxiran-2-yl)methylpentanoate **(44)** was obtained as yellowish–oily liquid and had a molecular formula $C_8H_{13}O_3$ as determined by the analysis of ^1H and ^{13}C NMR spectral data (table 4). Its yield was 46.2 mg (10.41%) of the enriched Fr4. On TLC plate, (oxiran-2-yl)methylpentanoate **(44)** gave an R_f of 0.54. Its structure was studied by use of NMR spectroscopy – both 1D and 1D NMR experiments.

The ^{13}C NMR spectrum (appendix 5) and DEPT spectrum (appendix 7) revealed presence of one ester carbonyl group (δ = 174.56ppm), two oxygenated methylene carbons (δ = 65.34ppm and 63.58ppm) and one oxygenated methine carbon (δ = 70.46ppm). In addition, three methylene carbons and one methyl carbons were identified. These observations have indicated presence of cyclic functional group (glycidyl) and existence of an alkyl was suggested according to signals in the NMR spectra.

From ^{13}C–NMR spectrum (appendix 5), there is a carbon having a chemical shift at 70.46 ppm; this is a characteristic region for sp^3-hybridised carbon atoms attached to oxygen atom. In the proposed chemical structure this carbon is labeled number 2 i.e. C_2 in figure 17. From DEPT spectrum (appendix 7) this carbon (at chemical shift=70.46 ppm) is clearly seen to be a methine carbon and from $^1H/^{13}C$ HSQC-DEPT spectrum (appendix 10) this carbon has a proton with a chemical shift at 3.9ppm - a characteristic region for protons attached to a carbon which is in turn attached to an electronegative group like oxygen. (For convenience this proton is labeled B i.e. H^B). From COSY $^1H/^1H$-NMR spectrum (appendix 6), H^B couples with protons at 4.1ppm.

These protons (at δ = 4.1 ppm), from $^1H/^{13}C$ HSQC-DEPT spectrum (appendix 10) were assigned to the carbon at 65.34ppm which is a methylene group based on $^1H/^{13}C$ HSQC-DEPT experiment. These protons for convenience have been labeled C i.e. H^c while the carbon is labeled C_3. From the COSY $^1H/^1H$-NMR spectrum (appendix 6), Proton H^B is also coupled to two other protons; at 3.6ppm and the other at 3.7ppm. On the hand, from the $^1H/^{13}C$ HSQC-DEPT (appendix 10) spectrum, the protons at 3.6ppm and 3.7ppm are attached to same carbon at 63.58ppm which also happens to be methylene but having non equivalent protons. Two non equivalent protons on same carbon can only be found in cyclic structures. Theses protons have been labeled H^{A1} and H^{A2} while the carbon is labeled C_1 in the proposed chemical structure. Furthermore, the protons (H^{A1} and H^{A2}) do not couple to any other proton except for H^B.

From COSY $^1H/^1H$ (appendix 6) spectrum, the protons at 4.1ppm (H^C) do not couple with any other proton except H^B which therefore means that C_3 is attached to only one carbon atom that is C_2. The chemical shift of C_3 and its protons is in a region characteristic to carbon atoms attached to an oxygen atom.

From $^1H/^{13}C$ HMBC spectrum (appendix 9) protons at 4.1ppm (H^C) sitting on C_3, are correlating with a C_2 (70.46ppm), C_1 (65.34ppm) and another carbon at 174.56ppm. From ^{13}C-NMR, this carbon falls in the characteristic region for ester carbonyl groups. This peak is also conspicuously absent in the DEPT spectrum (appendix 7) and is also absent in $^1H/^{13}C$ HSQC-DEPT spectrum (appendix 10) which confirms that it is quaternary carbon (specifically carbonyl carbon) atom. This carbonyl carbon has been labeled C_4 in the proposed structure.

From $^1H/^{13}C$ HMBC spectrum (appendix 9), C_4 interacts with two other protons: 2.3ppm and 1.6ppm. The protons at 2.3ppm are attached to methylene carbon at 34.34ppm while those at 1.6ppm are sitting on a methylene carbon at 25.09ppm as depicted by $^1H/^{13}C$

HSQC-DEPT spectrum (appendix 10). The carbon at 34.34ppm has been labeled C_5 and the one at 25.09ppm C_6 while their protons have been given the superscripts "D" and "E" respectively. The COSY $^1H/^1H$ spectrum (appendix 4) shows the couplings between H^D and H^E clearly to further confirm this assertion.

From COSY $^1H/^1H$ spectrum (appendix 6), H^E protons are shown to couple with protons at 1.3 ppm which are in turn attached to a methylene carbon at 22.90ppm. The protons have been assigned the superscript "F" while the carbon has been given subscript 7.

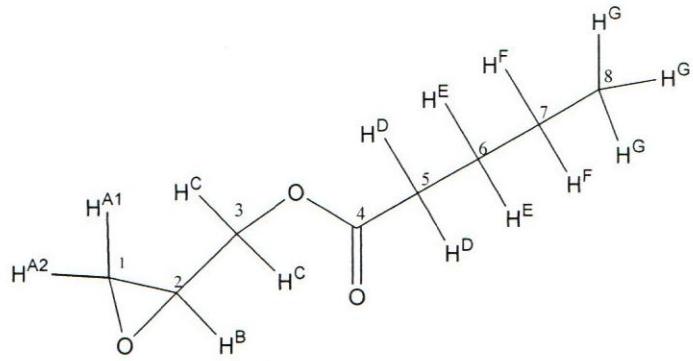
The protons H^F, are coupling with other protons at 0.84ppm which are in turn seen to be attached to methyl carbon at 14.31ppm as shown $^1H/^{13}C$ HSQC-DEPT spectrum (appendix 10). The methyl carbon in this structure has been labeled C_8 and its protons H^G.

The couplings between the various protons in space as depicted by the $^1H/^1H$ NOESY spectrum (appendix 8) are given in table 4. Table 4 below shows the ^{13}C, ^1H, COSY, HMBC and NOESY spectral data for (oxiran-2-yl)methylpentanoate **(44).**

Table 4: The ^1H and ^{13}C NMR data for (oxiran-2-yl)methylpentanoate (44) (δ in ppm)

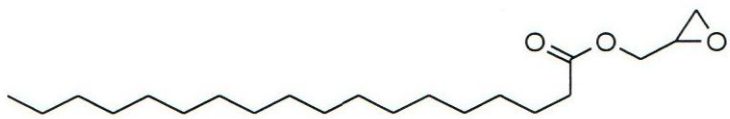
Entry	**^{13}C**	**^1H**	**COSY**	**HMBC (C→ H)**	**NOESY**
1	63.58	3.6 - 3.7br	2	1, 2, 4	1, 2: 1, 2
2	70.46	3.9m	1, 3	1, 3	1, 2
3	65.34	4.1m	2	2, 3	2, 3
4	174.56	-	-	5, 6	
5	34.34	2.3m	6	4, 6, 7	8, 6
6	25.09	1.6m	5, 7	4, 5, 7, 8	5, 7
7	22.90	1.3m	6, 8,	5, 6, 8	5, 6
8	14.31	0.84m	7	6, 7	5, 2

From this analysis, the proposed chemical structure consistent with this data for the purified (oxiran-2-yl)methylpentanoate **(44)** is given below.



Name proposed: (oxiran-2-yl)methyl pentanoate **(44)**

Compounds with an epoxide/glycidyl and ester functional groups' have been reported both from synthetic and natural sources. However, the purified (Oxiran-2-yl)methylpentanoate **(44)** has not been reported from both natural and synthetic sources as evidenced by search in the electronic databases for chemical compounds. Comparison of ^{13}C NMR data of (oxiran-2-yl)methylpentanoate **(44)** with those of (R)-glycidyl octadecanoate **(45)** isolated from basidiomycete *Cortinarius umidicola* (Lin *et al.*, 2003) indicates that the two compounds have similar structures except for the length of the alkyl chain. In addition (oxiran-2-yl)methylpentanoate **(44)**, has ester functional group a property found in many compounds isolated from basidiomycetes e.g. pulvinic acids isolated from mycelia cultures of *Suillus bovines* (Helmut *et al.*, 2008) which has an ester carbonyl atom with chemical shift 172.7ppm, lovastitin isolated from *Pleurotus ostreatus* (Julio *et al.*, 2003) has two ester carbonyl signals at δ = 170.9 and δ = 176.9); cyclopinol, cyclocalopin A and acetylcyclocalopin A isolated from *Boletus calopuis* (Dong-Ze *et al.*, 2008) also have ester carbonyl functional groups. Purpuracolide isolated from *Gomphus purpuraceus* (Meng *et al.*, 2008) has an ester carbonyl group at δ = 175.0ppm.



Glycidyl octadecanoate **(45)**

Table 5 Below shows the comparison of chemical shifts of the respective carbon's and hydrogen's in (oxiran-2-yl)methylpentanoate **(44)** and glycidyl octadecanoate **(45)**.

Table 5: The 1H and 13C NMR data for (oxiran-2-yl)methylpentanoate (**44**) and glycidyl octadecanoate (45) (δ in ppm)

	(oxiran-2-yl)methylpentanoate 44		**Glycidyl octadecanoate 45**	
Entry	**^{13}C**	**^{1}H**	**^{13}C**	**^{1}H**
1	63.58	3.6 - 3.7br	66.8	4.68
2	70.46	3.9m	71.0	4.45
3	65.34	4.1m	64.3	4.12
4	174.56	-	173.8	-
5	34.34	2.3m	34.4	2.35
6	25.09	1.6m	25.3	1.63
7	22.90	1.3m	23.0	1.25
8	14.31	0.84m	14.3	0.85

Finally comparison (oxiran-2-yl)methylpentanoate **(44)** and pyrethrins **(32 – 36)** show marked chemical difference between the two compounds. The three membered ring in pyethrin lacks is a highly substituted cyclopropyl whereas in (oxiran-2-yl)methylpentanoate **(44)** the three membered ring is oxiranyl. In addition, the carbonyl carbon in pyrethrin is attached directly to the three membered ring whereas in (oxiran-2-yl)methylpentanoate, the carbonyl carbon is attached to the three membered ring through an oxygen and a methylene. Pyrethrins has cyclopentanone attached to the carbonyl carbon through an oxygen; the cyclopentanone is lacking in (oxiran-2-yl)methylpentanoate.

CHAPTER FIVE

CONCLUSION AND RECOMMENDATIONS

5.1 Conclusion

The fungus was grown in liquid molasses media for twenty one days and crude extract obtained from the mycelia showed high larvicidal activity. The larvicidal activity of the crude mycelia extract from the fungus JO5444 is indeed as a result of chemical compound. (oxiran-2-yl)methylpentanoate **(44)** purified from this fungus shows great potency to kill mosquito larvae and this can be attributed to its relatively high polarity from the oxygenated three membered ring and the ester functional group. Presence of the oxygenated three membered ring makes the compound more potent insecticide compared to pyrethrins which has got a cyclopropane. The chemical structure of the purified compound was arrived after analysis and interpretation of the spectra obtained from the various NMR experiments performed. The compound obtained in this study is novel and has not been purified from natural sources nor has it been synthesized. Moreover, the enriched fractions from column chromatography showed dose dependent knock down effects on the test organisms. The activity of other fractions against the test organism can be attributed to chemical compounds secreted by the fungus; unfortunately these compounds could not be purified due to among many factors their low yields and the limitations of the apparatus and techniques applied in this study.

5.2 Recommendations for future research

From this study the following has been recommended:

- Systematic identification and classification of the basidiomycete by 18s RNA technology of the fungus should be carried out to find out its true identity and rank.
- The basidiomycete JO5444 should be cultured in bulk to obtain higher yields of active compound and determine its thermal and photo stability and also perform other chemical investigation to obtain IR and MS spectral data.
- The structure activity relationships of the purified larvicidal compound should be carried out to determine its mode of action.
- The purified compound should be synthesized in the laboratory to confirm its chemical structure.

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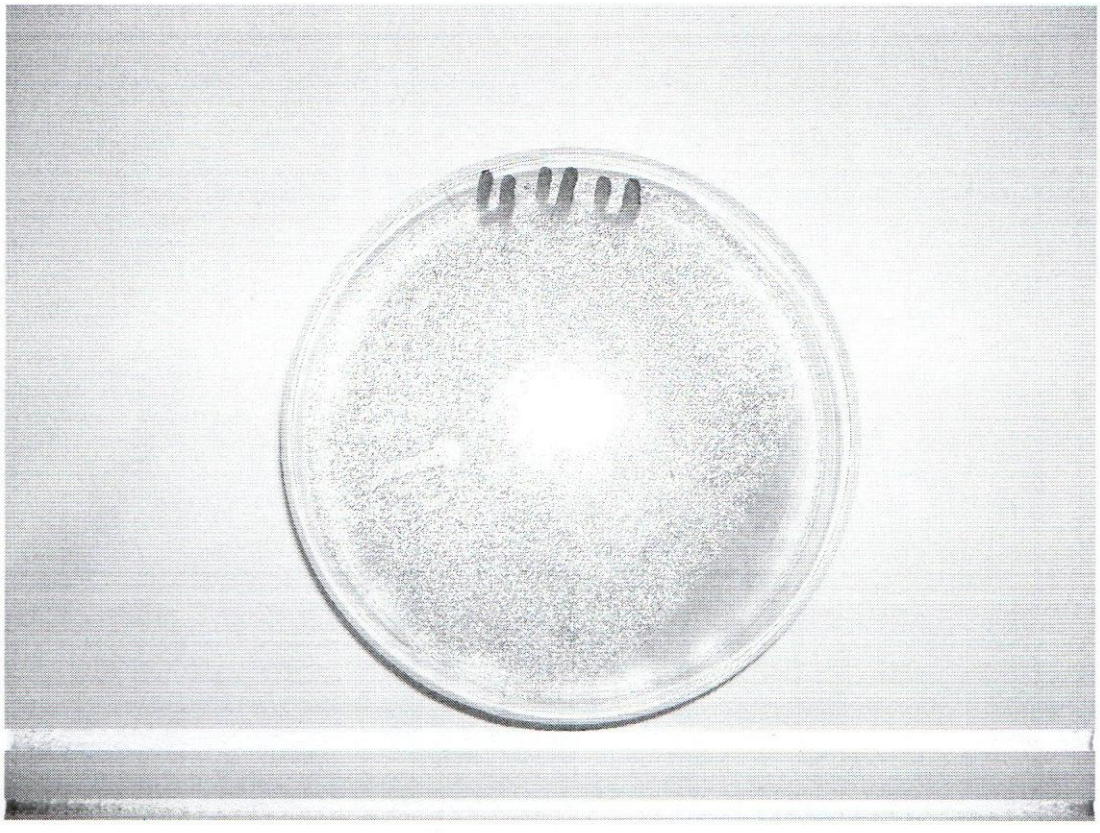
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APPENDICES

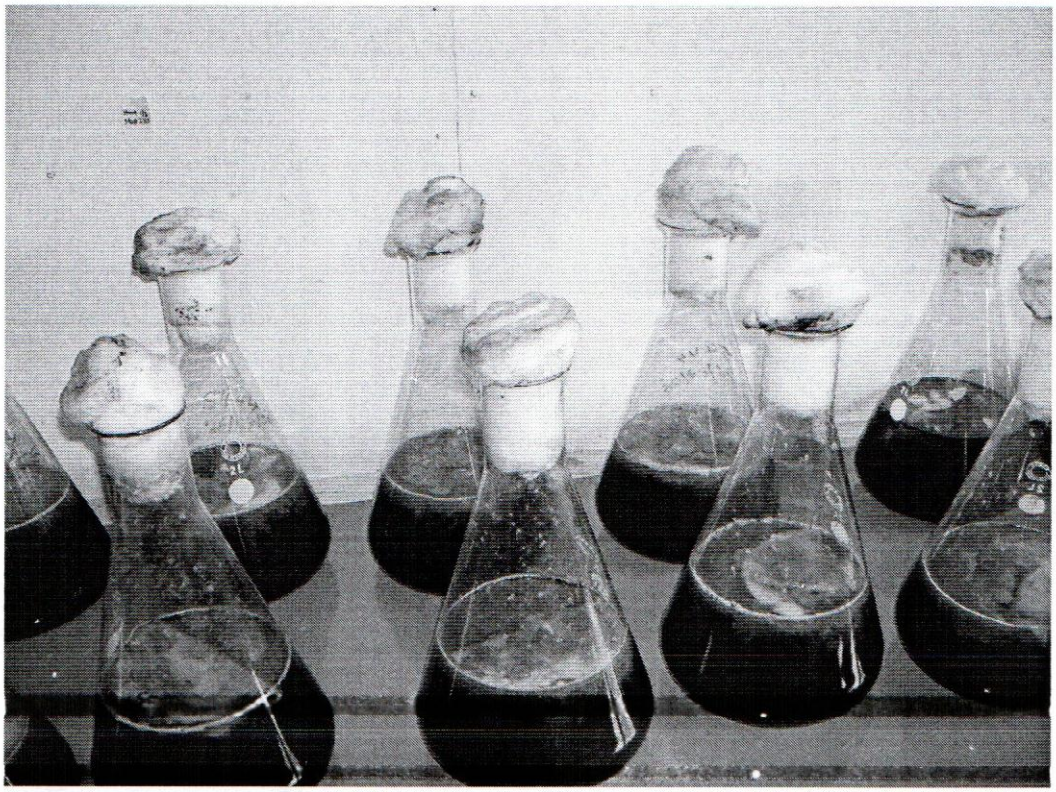
Appendix 1: Picture of the fresh fruiting body of the basidiomycete JO5444

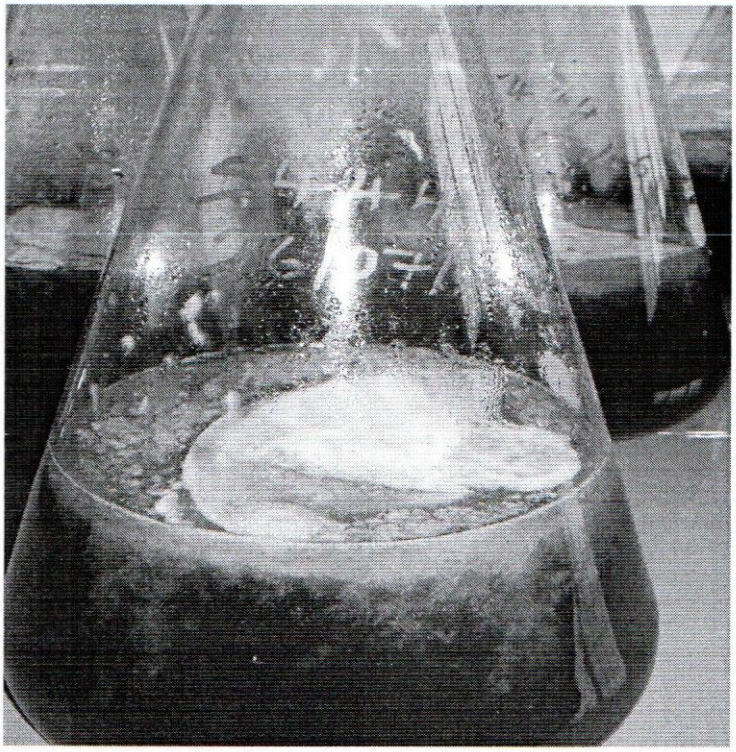


Appendix 2: Picture of the basidiomycete JO5444 growing on PDA

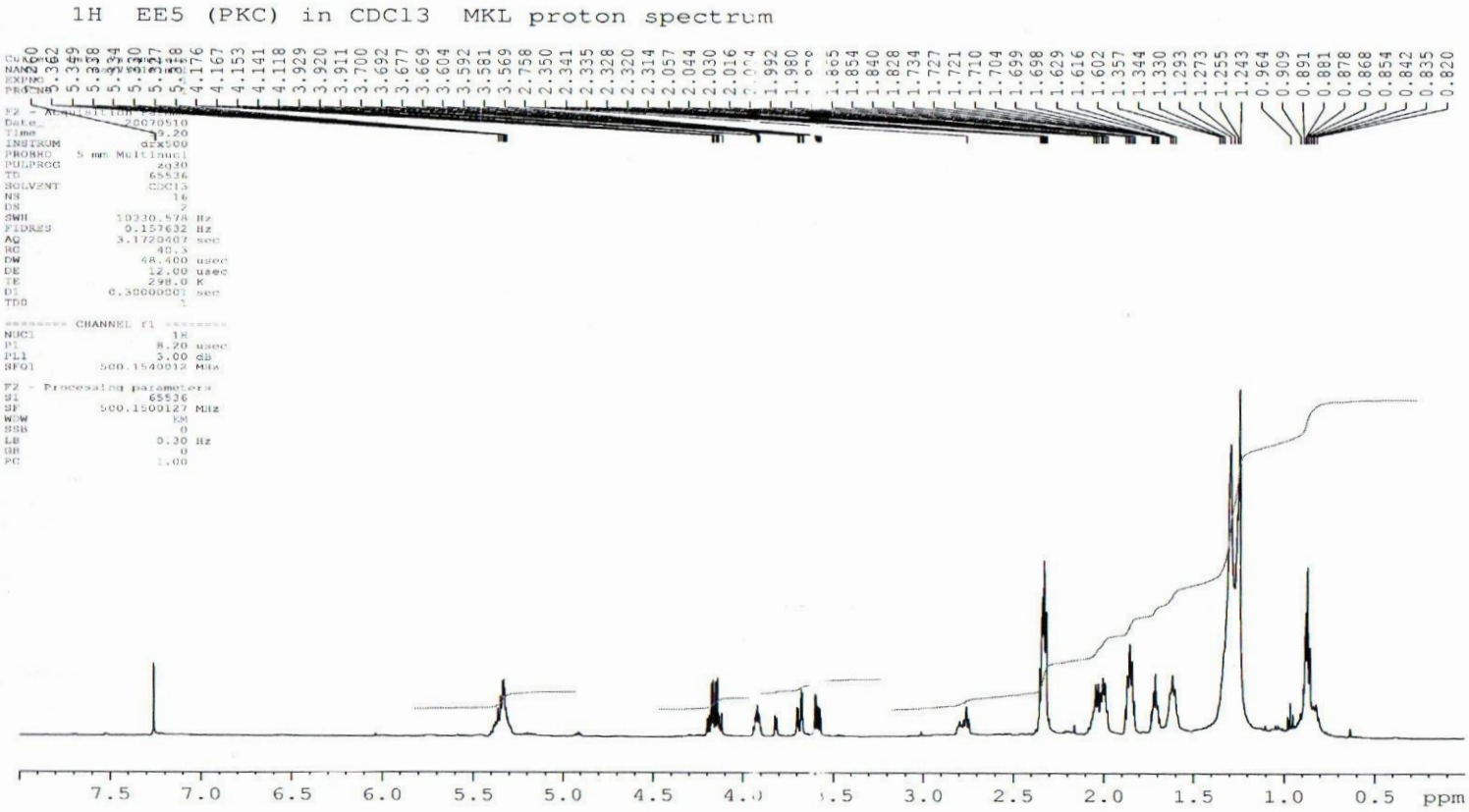


Appendix 3: Picture of the basidiomycete JO5444 growing in liquid culture

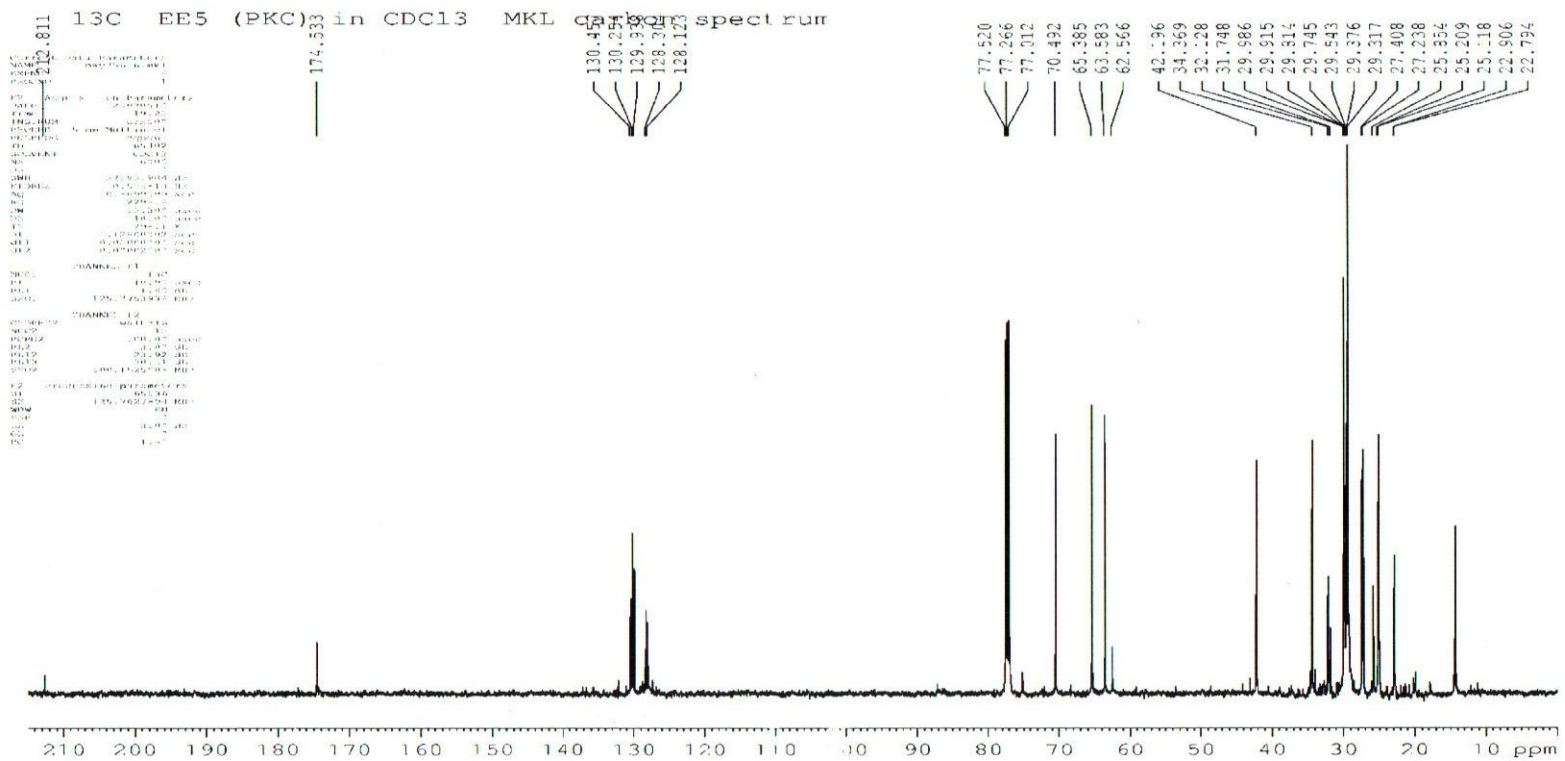




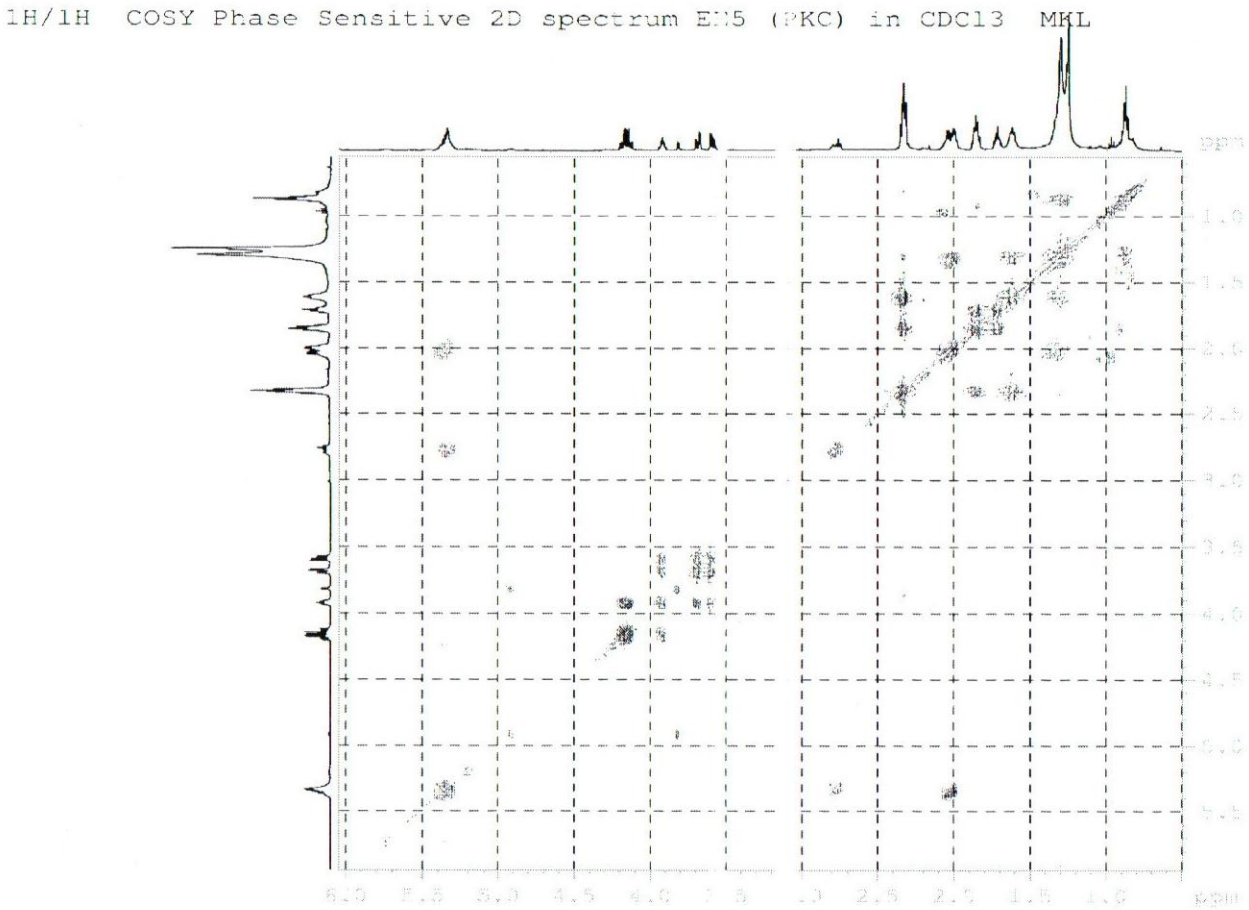
Appendix 4: ^{1}H NMR spectrum for compound 44



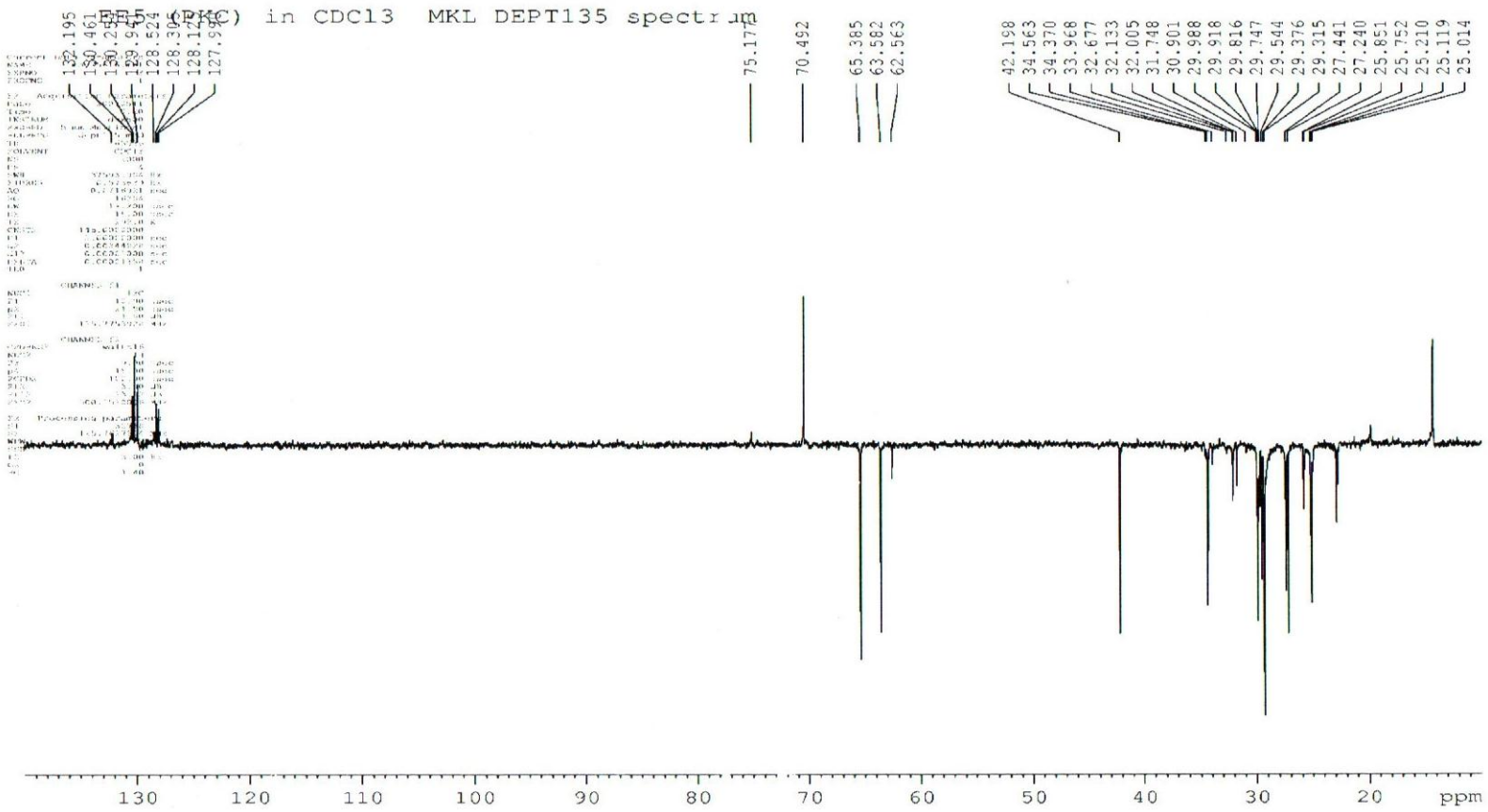
Appendix 5: ^{13}C NMR spectrum for compound 44



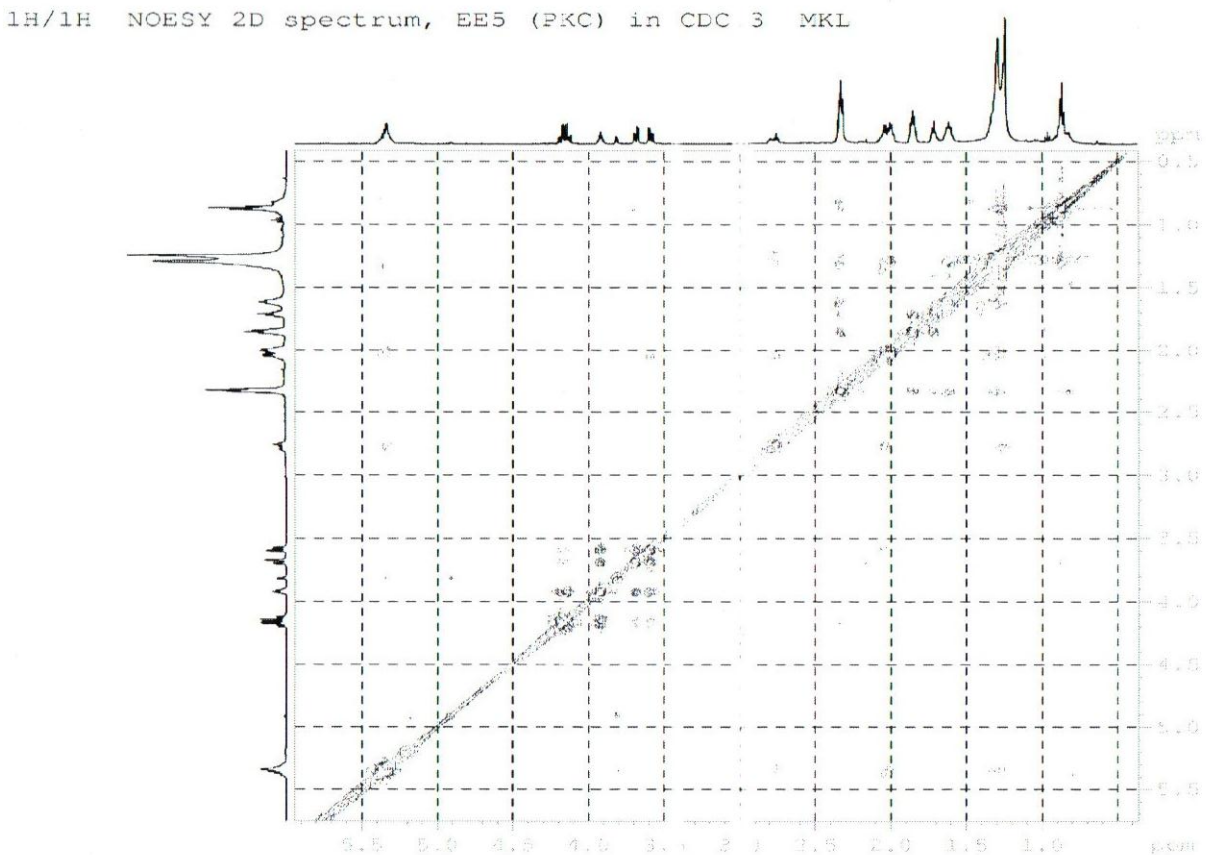
Appendix 6: COSY NMR spectrum for the compound 44



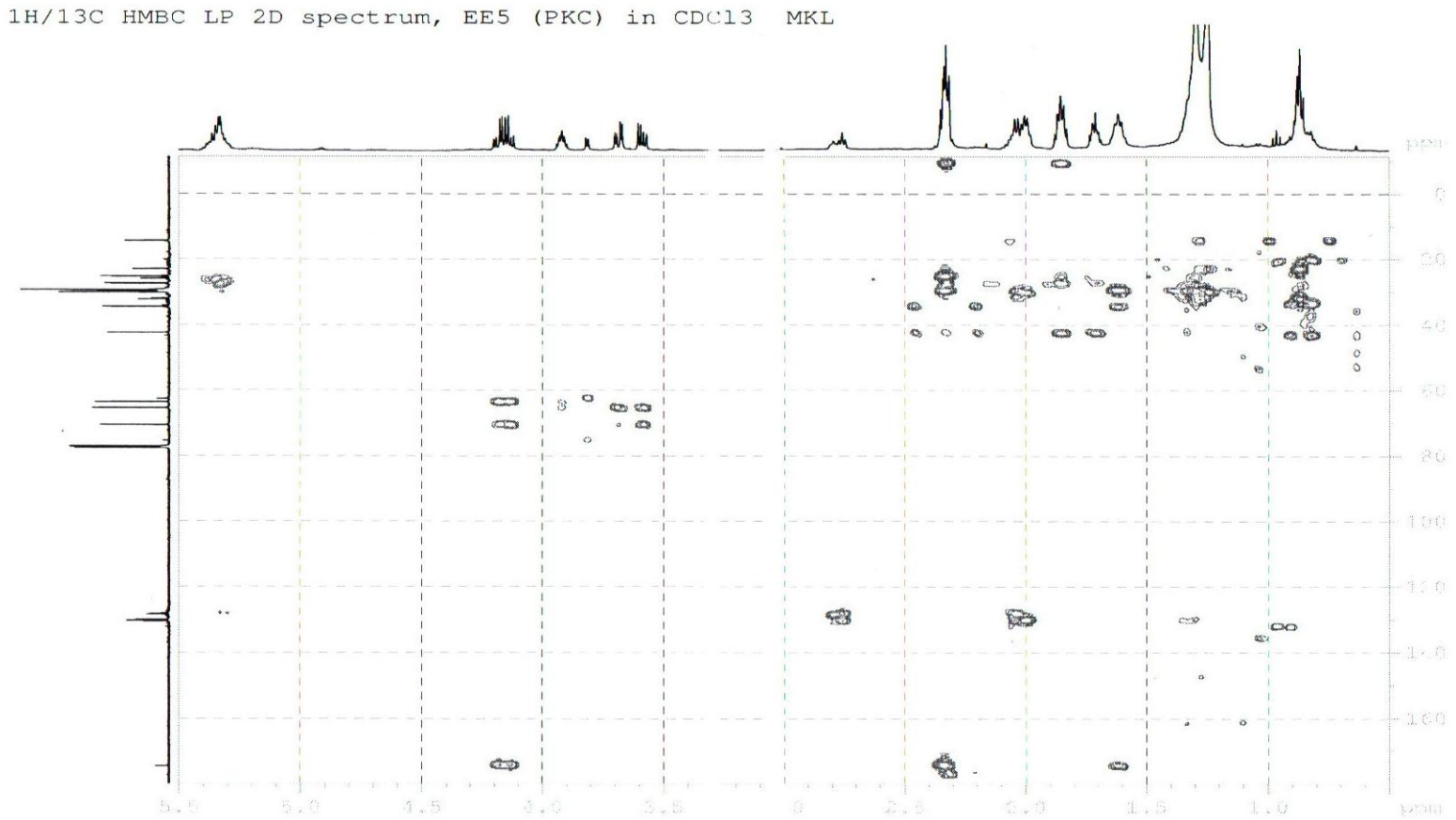
Appendix 7: DEPT spectrum for compound 44



Appendix 8: NOESY spectrum for compound 44



Appendix 9: HMBC spectrum for compound 44



Appendix 10: HSQC-DEPT spectrum for compound 44

