ANTI-LARVAL AND GROWTH DISRUPTING CONSTITUENTS FROM *MELIA VOLKENSII* (GÜRKE) ON *ANOPHELES GAMBIAE S. S.*

Karenga Samuel Mukiha

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

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DEDICATION

To the glory of God the Father, the Son and the Holy spirit, whose rod and staff comfort me,and to my late parents, Samson and Agnes Karenga, brothers and sisters.

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ABSTRACT

Malaria is a major global health problem. The disease is transmitted through the bite of anopheline mosquitoes infected with *Plasmodium* parasites. Control measures have focused on the parasite and the vector. Hopes for eradication of malaria have diminished due to the emergence of drug-resistant strain of the parasites and insecticide resistant vectors. Given the prevalent situation of malaria in Africa, the search for alternative control measures for the parasite and vector is of significant importance. Recent research has focused on traditional plant remedies for malaria, with few studies on the vector. In West and East Africa, plants of the Meliaceae family are widely used as traditional anti-malarials. Meliaceae plants contain compounds that exhibit anti-insect, anti-protozoa, anti-bacterial and anti-fungal activities. Although the anti-insect activity of *Melia volkensii* is known, the active compound(s) or blend(s) are unknown. Dry, powdered ripe fruits from *Melia volkensii* were extracted sequentially using hexane, chloroform, ethyl acetate and methanol. The crude extracts were assayed for their anti-larval activities against 3rd-instar *Anopheles gambiae s.s* larvae. The methanol extract was the most active (LD_{50} 36.3 ppm, after 24 h). Column and high performance liquid chromatography of the *n*-hexane and ethyl acetate fractions, respectively yielded nine pure compounds; seven known, salannin (**51**), volkensin (**54**), epi-volkensin (**76**), 1-cinnamoyltrichilinin (**56**) melianin B (**64**) stigmasterol (**72**) and ohchinin-3-acetate (**52**); and two new ones: methyl 2-acetyl-2,3-dimethyl-3-{[4''-(6'''-oxotetrahydro-2H-pyran-2'''-yl)-2''-E-butenyloxy]methylene}octanoate (**73**) and 1-benzoyltrichilinin (**74**). While two of these compounds **72** and **73,** were isolated from the hexane extract fraction, the rest were isolated from the ethyl acetate extract fractions. Only Pure methyl 2-acetyl-2,3-dimethyl-3-{[4''-(6'''-oxotetrahydro-2H-pyran-2'''-yl)-2''-E-butenyloxy]methylene}octanoate (**73**) was isolated in adequate amount for bioassay. This compound was bio-assayed against 3rd-instar *An. gambiae s.s* larvae and found to be a potent larvicide(LD_{50} 68.5 ppm, after 24 h). Although the other compounds were not isolated in adequate amounts for bioassay, their activity could be inferred from the activity of the fractions from which they were isolated. The study showed that the anti-larval compounds from *M. volkensii* were toxic at high doses and possessed growth regulation properties such as delayed pupation and larval-pupal intermediates at lower doses against *An. gambiae*.

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LIST OF ABBREVIATIONS

^{13}C NMR	Carbon 13 nuclear magnetic resonance
^{1}H NMR	Proton nuclear magnetic resonance
BCED	Behavioural and Chemical Ecology Department
Bti	*Bacillus thuringiensis* var *israelensis*
Bs	*Bacillus sphaericus*
$CDCl_3$	Deuterated chloroform
DDT	Dichlorodiphenyltrichloroethane
DEET	*N,N*-Diethyl-*m*-toluamide
HPLC	High perfomance liquid chromatography
ICIPE	International Center for Insect Physiology and Ecology
IGR	Insect growth regulator
IR	Infra-red spectroscopy
ITN	Insecticide-Treated Nets
LD	Lethal dose
MIM	Multilateral Initiative on Malaria
MS	Mass spectroscopy
NMR	Nuclear magnetic resonance
TDR	Tropical Diseases Research
TLC	Thin layer chromatography
UV	Ultra-Violet Spectroscopy
WHO	World Health Organization

N/B. other abbreviations are standard according to IUPAC

CHAPTER ONE

INTRODUCTION

1.1 Malaria

Human malaria, an important cause of morbidity and mortality, is the most important parasitic disease of man in the tropics. Globally, 300 million people are infected by malaria and 1.5-3 million die from the disease every year (WHO, 2003). Ninety percent of deaths due to malaria occur in sub-Saharan Africa, mostly among children below age five. In Kenya, malaria kills approximately 34,000 children every year (92 deaths a day) and is responsible for 30% outpatient cases and 19% of hospital admissions (Anonymous, 2003).

1.2 Malaria Parasite and its Life Cycle

The disease is caused by single-celled protozoan parasites, in the genus *Plasmodium*, and is transmitted exclusively by mosquitoes in the genus *Anopheles*. There are four species of human malaria parasites; *Plasmodium vivax, P. malariae, P. ovale and P. falciparum. Plasmodium falciparum* is the most life threatening and common in Africa. It predominates in sub-saharan Africa, Asia, Oceania and the Amazons (Anne, 2002).

An attack of malaria is caused by inoculation of the parasite (*Plasmodium*) into the host by an infected female *Anopheles* mosquito during blood feeding (Fig. 1.1). The parasite undergoes a two phase life cycle: sexual (sporogony) and asexual (schizogony). Asexual phase occurs in man (Fig. 1.2a). Sporozoites inoculated by an infected mosquito develop and multiply in the liver after which they develop into merozoites. The merozoites enter the red blood cells, multiply, cause the erythrocytes to rupture and invade other red blood cells. The incubation period lasts between 6-16 days depending on the parasite species (Rozendaal, 1997).

The sexual phase of the parasite life cycle takes place in female anopheline mosquitoes (Fig. 1.2b). A mosquito sucking blood from an infected person picks up the gametocytes that fuse to form a zygote. The zygote penetrates the mosquito stomach to form an oocyst within which large numbers of slender sporozoites develop. Some of these sporozoites pass to the salivary glands. When an infected mosquito feeds on a new host, it

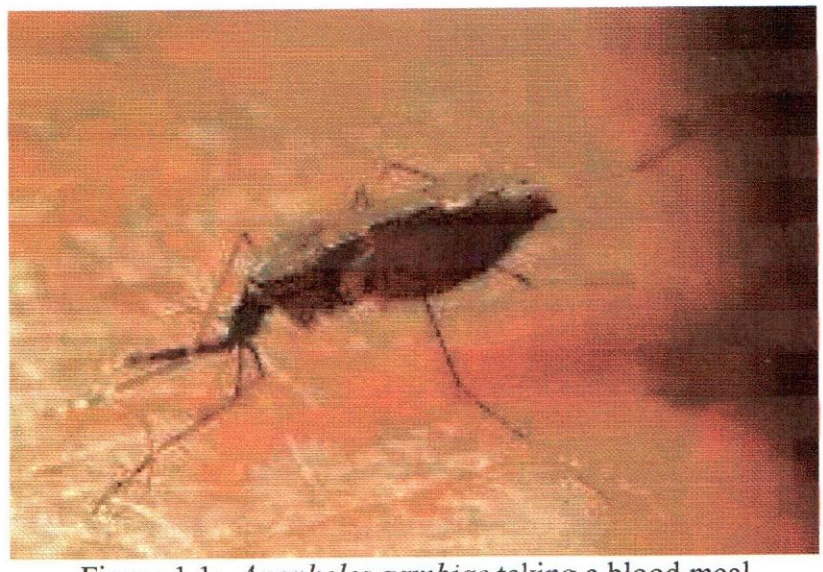


Figure 1.1: *Anopheles gambiae* taking a blood meal

Adapted from(http://www.westchestergov.com/health/WNVmosquito_life_cycle.htm)

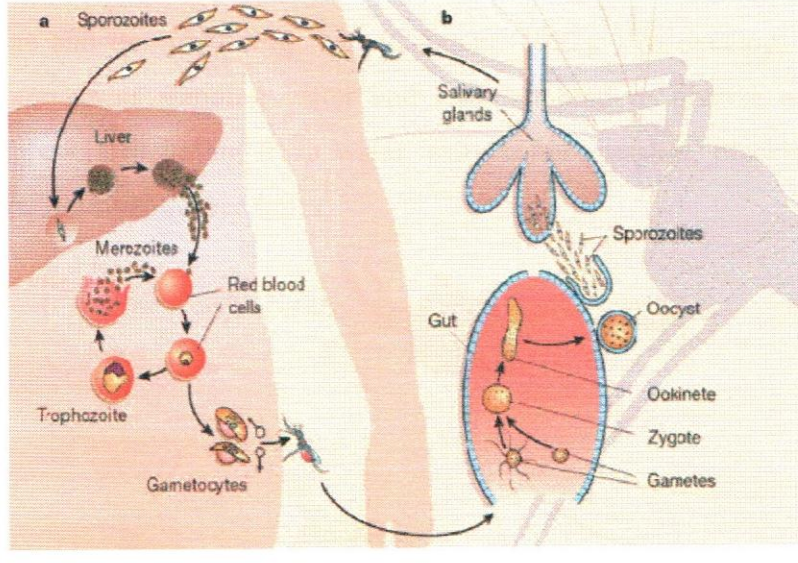


Figure 1.2: Lifecycle of plasmodium; (a) sporogony, (b) schizogony

Adapted from (http://encarta.msn.com/media_461541582/html)

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inoculates the sporozoites from its salivary glands into the host and the asexual life starts again. Incubation period of the parasites in mosquitoes varies with temperature and the species of the parasite. The life cycle is summarized in Figure 1.2.

1.3 The Malaria Vectors

All human malaria vectors belong to the genus *Anopheles* within the family Culicidae. Out of 250 species of *Anopheles* mosquitoes, about 60 are able to transmit malaria and 25 are notorious in many parts of the world (Clement, 1992). In sub-Saharan Africa, the major vectors of malaria are *An. gambiae, An. arabiensis* and *An. funestus*. *Anopheles gambiae* and *An. funestus* are anthropophilic (have a strong attraction to man) while *An. arabiensis* is both anthropophilic and zoophilic. Both female and male mosquitoes feed on nectar and other plant fluids. However, female mosquitoes also feed on blood from vertebrates. One blood meal (Fig. 1.1) is required to produce a batch of 30-150 eggs. The life cycle consists of four stages namely: eggs, larvae, pupae and the adult (Fig. 1.3) (Clement, 1992). To develop, larval mosquitoes require an environment of standing water and have adapted to diverse aquatic habitats to complete their life cycle including fresh water, salty water marshes, blackish water or water found in containers, old tyres or tree holes.

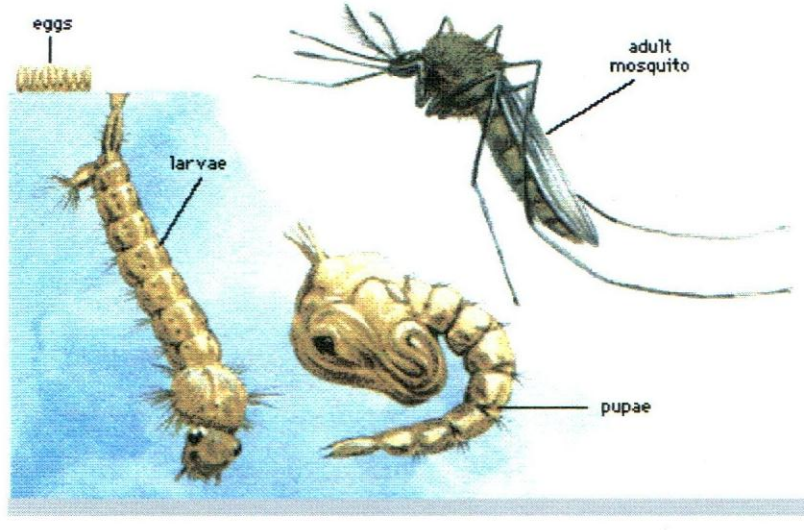


Figure 1.3: Mosquito life cycle

Adapted from (http://www.cdc.gov/malaria/biology/mosquito/)

Factors involved in attracting mosquitoes to human hosts are complex and have not been fully understood. It has been estimated that 300-400 compounds are released from the body as by-products of metabolism and that more than 100 volatile compounds can be detected in human breath. Of these, only a fraction have been isolated and fully characterised. Mosquitoes have chemo-receptors on the antennae that are stimulated by lactic acid (Bock and Cardew, 1996).

1.4 Control Measures for Malaria

Control measures that have been employed for malaria eradication focus on chemotherapy and chemoprophylaxis, development of malaria vaccine and vector control strategies. The latter includes personal protection i.e. use of insecticide treated nets (ITNs), repellents, insecticides and the use of larvicides. However, these have not been without drawbacks. Problems of controlling malaria are aggravated by inadequate health structures and poor socio-economic conditions. The situation has become even more complex over the last few years with the increase in resistance to the several anti-malarial drugs by the parasite (WHO, 2003). Drug-resistance can be considered as a natural response to the selection pressure. However, it is exacerbated by several factors including abuse, underuse or misuse, poor patient compliance, and poor quality of available drugs. The problem of anti-malarial drug resistance is further aggravated by the existence of cross-resistance among drugs belonging to the same chemical group (http://www.who.int/csr/drug). There is no guarantee that the current promising approaches to malaria vaccine development will result in a cost-effective malaria vaccine soon (WHO, 1998a; Mwaniki, 2003).

Another approach to malaria control is by management of the population of the vectors responsible for the transmission (Maillard *et al.*, 1993). The emergence of physiological and behavioural resistance to insecticides, high cost of insecticides and adverse effects of insecticides to the environment have complicated the situation.

These concerns have stimulated increased interest of the World Health Organization (WHO) and other agencies in integrated control measures, particularly those that result in reduced dependence on synthetic insecticides (Lacey and Cynthia, 1990). The degradation of natural products under field conditions takes place faster than in the laboratory because of the effects of ultra violet (UV) light, temperature, pH, and microbial action (Mulla & Tiamin, 1999). In an effort to develop environment-friendly insecticides, considerable attention has focused on

botanicals. One plant family that has shown potential as a viable source of environment-friendly insecticides is Meliaceae. The genus *Azadarachta* and *Melia* have been investigated extensively (Schmutterer and Ascher, 1987). Extracts of *Melia volkensii* exhibit larvicidal, insecticidal, growth inhibiting and anti-feedant effects on insects (Mwangi, 1982; Mwangi and Mukiama, 1988; Mwangi and Rembold, 1988). It may therefore offer an alternative to expensive synthetic insecticides for the control of insect pests and vectors of diseases such as malaria in Africa. Unfortunately, the compounds responsible for the anti-mosquito activity have not been identified. It is also possible that specific mixtures (blends) may be responsible for the activity.

In this study, the identity of the bio-active components of *M. volkensii* extracts or blend(s) therein associated with the observed anti-larval activity were investigated.

1.5 Statement of the Problem

Anti-insect components of *M. volkensii* that may be developed into safe, bio-degradable, cheap, target specific products for tropical disease vectors such as *An. gambiae sensu stricto* remain unidentified.

1.6 Objectives

1. To extract *M. volkensii* fruits using sequential solvent extraction with chloroform, ethyl acetate, methanol and n-hexane.
2. To screen and evaluate the anti-larval activity of the crude *M. volkensii* fruit extracts using *An. gambiae s.s.*
3. To isolate and characterize anti-larval compounds from *M. volkensii* fruits
4. To investigate the effect of *M. volkensii* fruit extracts and isolated compounds on pupation, adult emergence, fitness and egg production of *An. gambiae s.s.*

1.7 Hypotheses

1. The chemical constituents of *M. volkensii* can be extracted.
2. The crude extracts of *M. volkensii* can be bioassayed
3. The anti-laval compounds from *M. volkensi* can be isolated and characterized.
4. The anti-laval compounds from *M. volkensi* can affect the pupation, adult emergence, fitness and egg production of *An. gambiae s.s.*

1.8 Significance of the Study

As a social burden, malaria costs Africa between $3-$12 billion annually (Anne, 2002). Since mosquitoes are important vectors of human malaria, use of larvicides ensures that they are eliminated at an early stage before they can feed on human blood, acquire the malaria parasites and infect humans. The adverse environmental impacts already caused by the use of synthetic larvicides are long lasting, may take long to reverse and need not be enhanced. The vectors have become physiologically-resistant to many of these compounds and there is need to discover new chemicals that are more effective, safe to the environment and non-target organisms.

Plants of the family Meliaceae may offer alternative natural insecticides for insect pest and disease vectors as demonstrated in the neem tree. From the few studies already done on *M. volkensii*, (Mwangi, 1982; 1997; Mwangi and Mukiama, 1988; Rajab *et al.,* 1988 a), the plant is a potential source of anti-insect products. There is need to identify the anti-mosquito compounds and blends in *M. volkensii* to facilitate the development of alternative corresponding products.

CHAPTER TWO

LITERATURE REVIEW

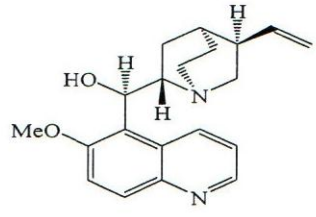
2.1 Efforts to Combat Malaria

These have been based on chemotherapy or chemoprophylaxis, vaccines and vector control as described below.

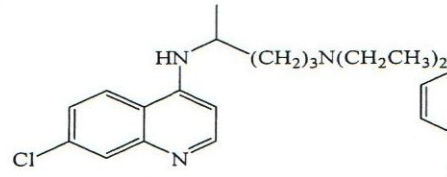
2.2 Chemotherapy and Chemoprophylaxis

Malaria is curable and preventable (WHO, 2000). 4-Aminoquinoline-based drugs such as quinine (**1**), chloroquine (**2**) mefloquine (**3**), mepacrine (**4**), amodiaquine (**5**) and pyronaridine (**6**); the benzoquinones such as atovaquone (**7**); the antifoliates like chloroproguanil (**8**) and pyrimethamine (**9**); Sulphoxide and sulphonamide-based drugs like dapsone (**10**) and sulphadoxine (**11**) have been employed in efforts to treat malaria (Service, 2001). Unfortunately, *P. falciparum* has developed resistance to all monotherapy with current anti-malarial drugs (Andersen *et al.*, 1995). A new class of anti-malarial drugs that are effective against the multiple drug-resistant strains of *P. falciparum*, with virtually no toxicity include artemisinin (**12**) derivatives (Gabor, 2001). Artemisinin (**12**) is a potent anti-malarial drug that was initially isolated from the aerial parts of the plant *Artemisia annua* L (Asteraceae) (Wallart *et al.*, 1999). It is a sesquiterpene lactone with an unusual endoperoxide group essential for its activity. It is available under the trade name Cotexin® (Obong, *et al.*, 2003).

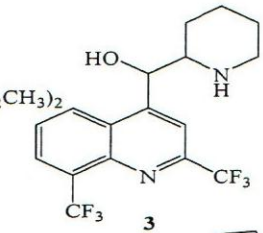
However, artemisinin (**12**) and derivatives (**13-15**) require long treatment courses (White and Olliaro, 1998). When artemisinin (**12**) is used alone, recrudescence can be expected within the first four weeks after treatment because of its short half-life (Seidlein and Bojang, 1998).



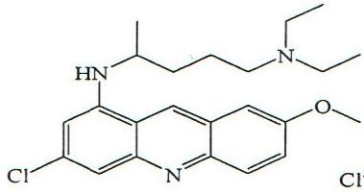
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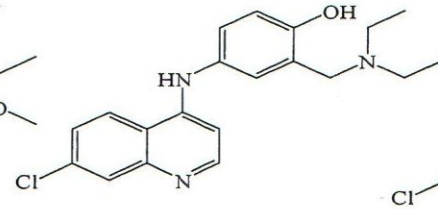
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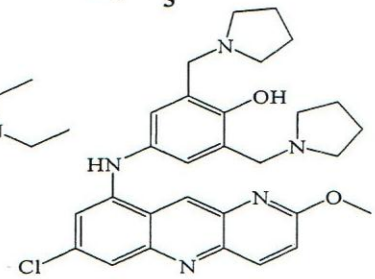
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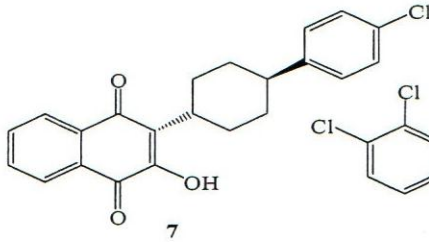
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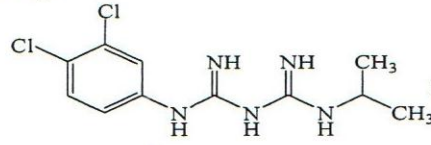
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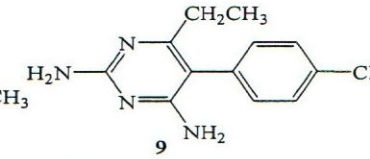
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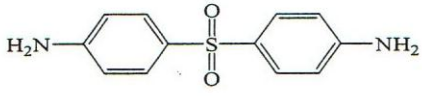
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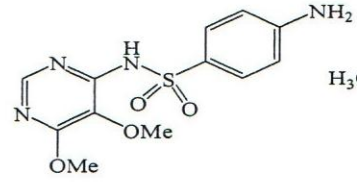
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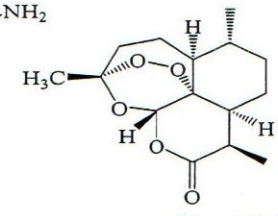
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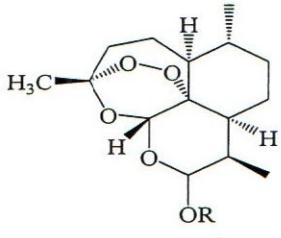


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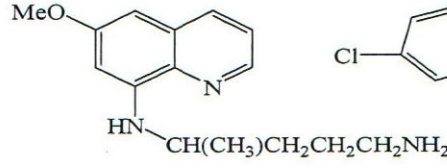


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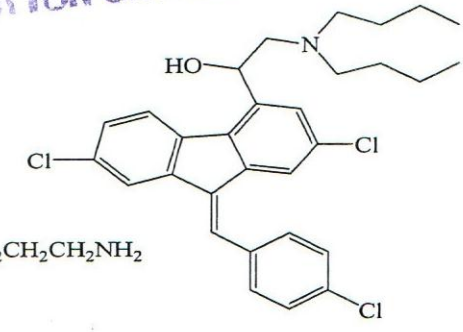
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13 R = $CO(CH_2)_2CO_2H$
14 R = Me
15 R = Et



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Due to widespread parasite resistance to single therapy the use of anti-malarial drug combinations has recently been introduced (Wiesner *et al.*, 2003). The usefulness of

combination chemotherapy depends on the enhancement of efficacy as compared to the component drugs given individually, and in the delay of development of resistance by the pathogen. Ideally, the combined drugs should have similar pharmacokinetics, pharmacodynamics, and display no enhanced adverse effects. Originally it was thought that it is highly unlikely that a parasite will simultaneously develop resistance to two drugs with different modes of action (Service, 2001). However, Fansidar® a combination of sulphadoxine (**11**) and pyrimethamine (**9**) was introduced several years ago but resistance to it has been reported in South America, South East Asia and in East Africa (White, 1992). Other combinations used are Maloprin®-pyrimethamine (**9**) + Dapsone (**10**), Malarone®-Atovaquone (**7**) + chloroproguanil (**8**) (Bertrand *et al.*, 1998). A new drug, Lapdap®, a combination of chloroproguanil (**8**) and dapsone (**10**), has been developed (Lang and Greenwood, 2003). Due to recrudescence and long treatment regime for artemisinin-based anti-malaria drugs, artemisinin combination therapy has been introduced. The most common is Coartem® which is a combination of artemether (**14**) and lumefantrin (**17**) (Wiesner *et al.*, 2003). Other combinations involving artesunate (**13**) and arteether (**15**) and other antimalarials especially 4-aminoquinolines also exist. Examples include artesunate (**13**) + mefloquine (**3**), artesunate (**13**) + pyradodine (**6**), artesunate (**13**) + sulphadoxine and pyrimethamine (SP), arteether (**15**) + mefloquine (**3**) (Wiesner *et al.*, 2003).

The development of resistance to double therapy has facilitated triple therapies. Mefloquine (**3**) has been used in combination with sulphadoxine (**11**) and pyrimethamine (**9**) (MSP) (White and Olliaro, 1996). Amodiaquine (**5**) and SP have also been shown to be effective against resistant malaria parasite strains (Thayer and Houston, 2005). The team working on Lapdap® has also started to develop the triple combination of chloroproguanil (**8**), dapsone (**10**) and artesunate (**13**), (CDA), as a low cost combination therapy for uncomplicated malaria (Annon., 2004). This drug would enhance clinical efficacy and also delay the development of resistance by the malaria parasites. However, it has been observed that combination therapy only delays resistance a fact that emphasizes the need for alternative malaria control strategies (Mcintosch and Greenwood, 1998).

Chemoprophylaxis (or intermittent treatment) is recommended for pregnant women and all persons travelling to malaria regions from non-endemic areas. Chloroquine (**2**) is commonly used for prophylaxis where malaria parasites are sensitive to it. In regions of chloroquine-resistance, mefloquine (**3**) is the drug of choice (Service, 2001). Chloroquine in combination

with chloroproguanil is safe in pregnancy. Primaquine (**16**) has been a drug of choice for radical cure and terminal prophylaxis against malaria for more than 40 years (Baird *et al.,* 1995). Sulphadoxine + pyrimethamine combination is not recommended for prophylaxis due to the rare occurrence of severe and occasionally fatal skin reactions (Service, 2001).

Varied patterns of resistance to therapeutic agents by *P. falciparum* and *P. vivax* together with low tolerance of some drugs by many people restricts options for chemoprophylaxis against malaria (Baird *et al.,* 1995).

2.3 Vaccine Development

There are three types of vaccines currently under development: pre-erythrocytic vaccines, that prevent the sporozoite stage from invading liver cells; asexual blood-stage vaccines, that prevent the merozoite stage from invading red blood cells, and transmission-blocking vaccines that work by inhibiting the sexual development of the parasite within the mosquito. Several candidate vaccines are currently in clinical test like MuStDo (Multi-stage DNA vaccine operation), which is currently on tests in Gambia. A different asexual-blood stage vaccine type is based on cocktail of antigens, for example SPf66 that has been tested in field trials in S. America, Africa and S. E. Asia. It has only been partialy effective to date (WHO, 1998a and b).

Another candidate vaccine developed to control malaria is the liver stage antigen-3 vaccine (LSA-3) (Wakelin, 1996). The vaccine consists of inactivated sporozoites that when injected into test animals protects them from subsequent infections by the parasite. Test animals immunised with LSA-3 produce anti-bodies, which can activate the immune system to eliminate the parasite. Another advantage of LSA-3 is that its shape does not vary as much as other sporozoite proteins from one parasite strain to another. Since molecular configuration is crucial to protein anti-body interaction, the vaccine is effective in all strains of malaria (Hoffman and Doolan, 2000).

Another most advanced pre-erythrocytic vaccine in clinical development today is the RTS, S/ASO2 vaccine developed by Glaxo SmithKline through a long-standing collaboration with the Walter Reed Army Institute Research (WRAIR). The vaccine was first tested in adults in Belgium, Kenya, Gambia and the US. It has since been tested in Mozambique (Alonso *et al.*, 2005). The efficacy against clinical malaria attacks is30 %. Efficacy against primary infection with *Plasmodium falciparum* is 45% and efficacy against severe disease was 58%. However,

researchers recognise that the vaccine will never achieve 100% efficacy. It has been recommended for phase two paediatric trials (WHO, 2004).

The search for an effective anti-malarial vaccine is hampered by several factors including the ability of malaria parasites to evade host immune responses, the difficulty to cultivate malaria parasites in sufficient quantities and the cost of testing requiring expensive and time-consuming animal model systems (Service, 2001).

Although the recent sequencing of *An. gambiae* and *P. falciparum* genes may open up new avenues for rapid vaccine development, for now and foreseeable future, only efficient vector control can hold promise for the future (Maramorosch, 1985).

2.4 Vector Control

Vector control targets both the adult and the larval stages with little emphasis on control of the eggs by the use of ovicides.

2.4.1 Control of Adult Mosquitoes

Control of adult mosquito bites is possible by the use of either insecticides or repellents.

2.4.1.1 Insecticides

Global eradication of malaria seemed possible with the discovery of DDT (**18**) in the 1940s. Despite the initial success there was a complete failure to eradicate malaria in most countries due to resistance developed by anopheline mosquitoes to DDT (Anne, 2002). The natural insecticide pyrethrum had been used successfully against adult *anophelines* in South Africa from 1932-1933. Residual insecticides like, malathion (**19**) and permethrin (**20**) when applied to the interior surfaces of houses and other structures, can be highly effective against adult mosquitoes. However, their application to a large number of homes is time consuming, labour-intensive and expensive. Furthermore, by 1991, resistance to all major classes of insecticides in public health use was reported in more than 150 species of vectors and nuisance pests (WHO, 1996).

There has been increased interest in the use of insecticide treated nets (ITNs) for malaria control. The introduction of ITNs in the communities has remarkably reduced parasite prevalence and malaria cases (Marbiah *et al.*, 1998). Currently, synthetic pyrethroids (SPs) such

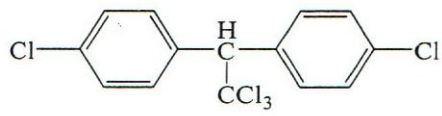
as permethrin (**20**) are the most commonly used anti-mosquito agents on ITNs. Pyrethroids are synthetic chemical insecticides that kill mosquitoes on contact. They are considered to pose slight risks of acute toxicity to humans, but at high doses can affect the nervous system. Pyrethroid resistance has been reported in Africa, from the Ivory Coast to South Africa, and in East Africa, from Kenya to Tanzania (http//www.wellcome.ac.uk/en/). However, even though bed nets are now available in many rural areas, the insecticide is not. Underdosing and untimely re-impregnation may increase the risk of reducing susceptibility of malaria vectors to synthetic pyrethroids.

A new mosquito net, Olyset® manufactured in Tanzania and now being tested there, is expected to go far in helping the continent fight malaria. Olyset®, is made with fibres containing permethrin, which is slowly released over a five year period. However, at $ 5.50 it is relatively expensive and beyond the reach of many poor families (http://www.VOAa).

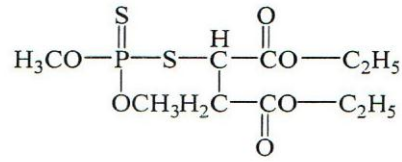
2.4.1.2 Repellents

Repellents suppress or neutralize attraction of animals, plants or materials. They are not acceptable to pests and disturb their sensory receptors or are sensed as alarm signals. Repellents may be mildly poisonous to the target organism (Mafong and Kaplan, 1997). The search for new repellents led to the discovery of *N,N*-diethyl-*m*-toluamide (DEET) (**21**), which is the most potent of the modern synthetic repellents (Kirk and Othmer, 1992). However, questions have been raised about the safety of DEET, its effectiveness against some species of *Anopheles* mosquitoes in addition to attacking paint and some hard plastics (Stinecipher and Shah, 1999). Several DEET analogues and derivatives have since been made and tested. Disadvantages of synthetic repellents include toxicity to other animals, high cost, and environmental pollution.

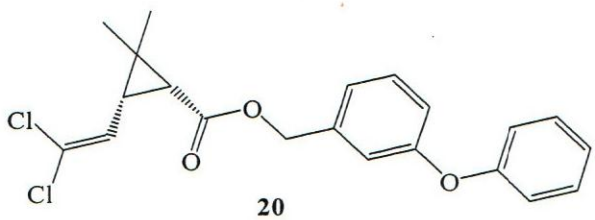
Natural repellents have also been used to control mosquitoes. These include pyrethrin (**22**) (WHO, 1984), essential oils of some plants like lemon grass (citronella) geranium, pine, wintergreen and eucalyptus (Curtis *et al.,* 1991). Citronellal (**23**), geraniol (**24**), camphor (**25**), and *p*-menthane-3, 8-diol (**26**) are examples of natural repellents isolated from plants (Trigg, 1996). Plant derived repellents are generally volatile compounds and as a result do not protect for long when applied on skin or clothing, requiring repetitive treatment.



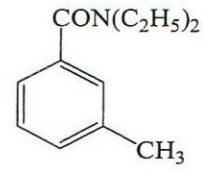
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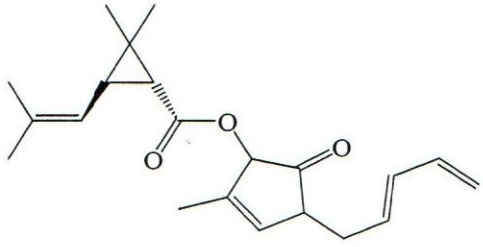
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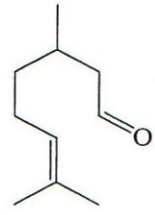
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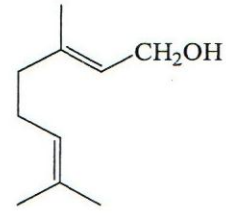
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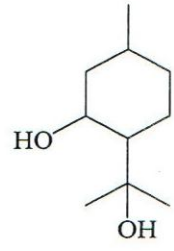
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Although the control of mosquito bites is attractive, the adults rest in varied places and may be found outdoor or indoor only during feeding. This demands that a more vulnerable stage be targeted for efficient vector control.

2.4.2 Larval Control

This strategy has advantage in that the larvae are found only in water bodies and therefore can be eliminated before they mature into adults. Besides, habitats of adult mosquitoes are less likely to be accessible than larval breeding sites (Jamieson *et al.*, 1994). Methods of larval control include environmental management, biological control, chemical larvicides and insect growth regulators (IGRs).

2.4.2.1 Environmental Management

This includes physical transformations of the breeding sites that are somewhat permanent such as drainage, ponds, wells, water storage tanks and dams. Land filling and grading can prevent, eliminate or reduce mosquito breeding habitats. It may also involve activities that are designed to reduce contact with vectors and pathogen transmission such as location of human settlements away from vector breeding sources, use of bed nets and mosquito proofing of houses. Environmental management has advantages in that it tends to be more effective for longer periods of time than conventional chemical insecticides. Although the initial costs are high, it reduces over time since it lasts for long and the environmental impact is often slight (Lacey and Cynthia, 1990).

2.4.2.2 Biological Control

This involves the reduction of an insect pest or vector population by natural enemies (predators or pathogens). It involves the use of bio-larvicides including microbial control agents, toxins produced by fungi among other organisms (WHO, 1996). Attempts have been made to isolate, identify and develop bacterial and fungal agents for vector control. Among these, *Bacillus thurigiensis* var. *israelensis* (*Bti*), *B. sphaericus(Bs)* and *Tolypocladium cylindrosporium* (Deuteromycotina: Hyphomycetes) have been commercialized and used for the control of disease vectors (Nadeau and Boisvert, 1994; WHO, 1996). The insecticidal ingredients of *Bacillus thuringiensis* consist of proteins produced by the bacteria. In particular, compounds known as the δ-endotoxins, which form crystals inside the bacteria, kill the insect (http://www.colostate.edu/depts/dept//pm/cd15.pdf). The primary advantage of *Bti* products is their safety resulting from selectivity and non-toxicity to humans. However, since *Bti* is an insect-stomach poison, for it to be effective, the insect must eat it. *Bti* formulations used for mosquito control are adversely affected by water turbidity.

Several species of nematodes are known as specific parasites to mosquito or black fly larvae. Among these *Romanomermis culicirorax* has been developed for vector control. The nematode exhibits a relatively broad host range among mosquito species. However, it is active only in relatively warm waters and is inhibited by salinity and other environmental factors.

The mosquito fish, *Gambusia affinis,* continues to occupy a unique place in worldwide distribution as a tool for control of mosquitoes. Laboratory experiments also showed that a

cyprinodontid fish, *Aphanius mento*, could be used to control *Culex pipiens*. Though the use of mosquito feeding fish has been successful experimentally large scale application has its set backs. To date, there is no mosquito feeding fish production program adequate for stocking on a large operational scale and fishes may have negative effect on other fish species when introduced in areas outside their normal habitat (Blaustein and Byard, 1993).

Biological control agents require thorough understanding of the population dynamics and ecological impact. Besides, they are slow in action and thus unsuitable for use in emergency situations like epidemics (Jaroslav, 1991).

2.4.2.3 Insect Growth Regulators (IGRs)

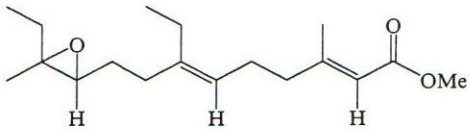
These are chemicals exhibiting insect growth inhibitory properties. Most of the IGRs so far discovered can be classified as juvenile hormone (**27**) mimics or chitin synthesis inhibitors. Methoprene (**28**) is a compound first registered with the Environmental Protection Agency (EPA) in 1975 for its IGR activity. It mimics the action of juvenile hormone (**27**) and prevents the normal maturation of insect larvae. Other examples of IGRs include fenoxycarb (**29**) and diflubenzuron (**30**) that are chitin synthesis inhibitors (Mulla, 1995). These compounds are safe to humans and have mild or no toxicity to most non-target organisms. They are active against immature stages of mosquitoes, flies and other insects. Some IGRs induce sterility, among other reproductive disorders, in the adults (WHO, 1996). It has been observed that IGRs have a great potential for the control of mosquitoes in their developmental sites like pools and wastewater accumulations.

2.4.2.4 Chemical Larvicides

Use of chemical larvicides can reduce overall pesticide usage in a control program. Killing mosquito larvae before they emerge into adults can reduce or eliminate the need for ground or aerial application of pesticides to kill adult mosquitoes (WHO, 2003). Inorganic larvicides that have been used include paris green dust [Cu $(C_2H_3O_2)_2.3Cu(AsO_2)_2$], which is expensive due to high copper content and toxic to the environment due to the presence of arsenic in it (Rozendaal, 1997).

The earliest used synthetic organic larvicides were organochlorines (OCs) like DDT (**18**) and methoxychlor (**31**). Due to vector resistance and toxicity to non-target organisms and

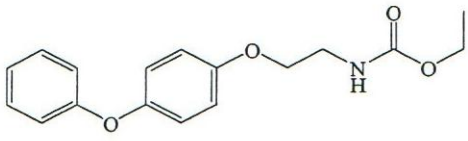
persistence in the environment, the OCs were replaced by the organophosphates (OPs) like malathion (**19**), and fenthion (**32**). The OPs were found to be toxic to mammals.



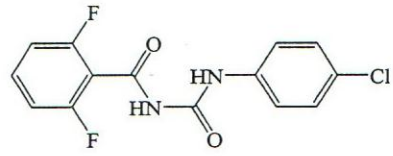
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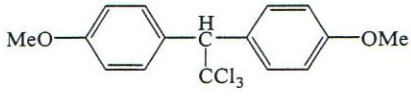
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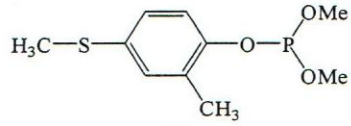
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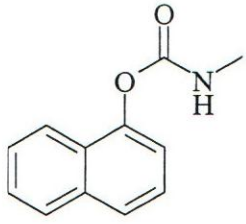


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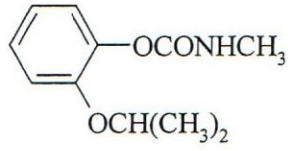


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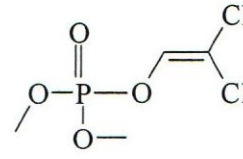
Due to development of resistance to organophosphates, organocarbamates like carbaryl (**33**), propoxur (**34**) and dichlorvos (**35**) have been used. Organic larvicides may also include IGRs (Methoprene), oils and mononuclear films.



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Vector resistance to the existing synthetic insecticides, their high cost and public concern over environmental pollution have necessitated a continued search for alternative vector control methods which would be environmentally safer and specific in their action (Peng *et al.*, 1998). Therefore, the evaluation of the potential of local plants as repellents or natural IGRs is worth

being explored. This will contribute novel approaches in providing cheap and reliable control strategies for mosquitoes in the rural communities.

2.5 Natural Products used in Mosquito Control

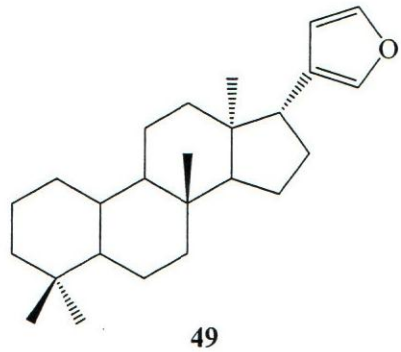
The early Romans used extracts from plants as insecticides, a practice that continues to date. For example, they used *Hellebore* as an insecticide. Furthermore, the use of *Derris* as a mosquito repellent has been common practice among the Chinese (Jacobson, 1989). The use of insecticidal plants is especially prevalent in the developing countries where they grow and are cheaper to use by subsistence farmers than synthetic chemical pesticides. Plants belonging to the families Meliaceae, Pipereceae, Plumbaginaceae, Rutaceae, Asteraceae and Annonaceae have been shown to contain potent anti-insect properties (Sadek, 2003). Over 2000 plant species have been reported to exhibit insecticidal activity (Muthukrishnan *et. al.,* 1999). Plants that exhibit anti-mosquito properties have been reviewed but many of them remain uninvestigated chemically (Aklilu *et al.*, 2002).

There are many naturally occurring compounds reported to have anti-mosquito properties (repellents, larvicides or insecticides). For instance, plumbagin (**36**) extracted from *Plumbago zeylanica* L. (Plumbaginaceae) showed larvicidal activity against *Ae. aegypti* (LC_{50} = 0.60 ± 0.05 μM/ml) (Bilton *et al.,* 1987). Similarly, 5E-ocimenone (**37**) from *Tagetes minuta* (Maradufu *et al.,* 1978), rotenone (**38**) from *Derris elliptica* (Ameen *et al.,* 1983), azadirachtin (**39**) (Schmutterer and Ascher 1987), nimocinolide (**40**) and isonimocinolide (**41**) from *A. indica* (Siddiqui *et al.*, 1986), capillin (**42**) from *Artemisia nilagirica* (Banerji *et al.,* 1990), eupomatenoid-5 (**43**) and eupomatenoid-6 (**44**) from *Piper decurrens,* quassin (**45**) from *Quassia amara* (Evans and Kaleysa, 1991), *n*-hexanedecanoic acid (**46**) from *Feronia limonia* (Rahuman *et al.,* 2000), arborine (**47**) from *Glycosmis pentaphylla* (Muthukrishnan *et al.*, 1999), and goniothalamin (**48**) from *Bryonopsis laciniosa* (Kabir *et al.*, 2003) have demonstrated anti-mosquito activity.

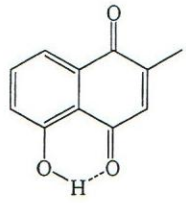
2.5.1 The Meliaceae Family

The Meliaceae family is well represented in eastern Africa with over 40 species in 11 genera. The biological activities of the Meliaceae plant species have been attributed mainly to

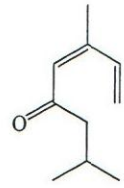
the presence of limonoids. These are modified triterpenes (tetranotriterpenoids) with or derived from a precursor with a 4,4,8-trimethyl-17-furanyl steroid skeleton (**49**).



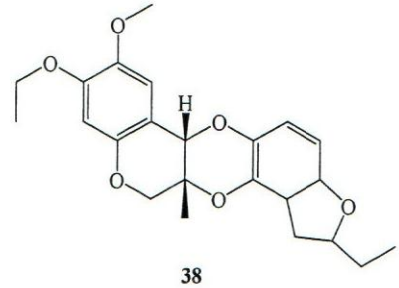
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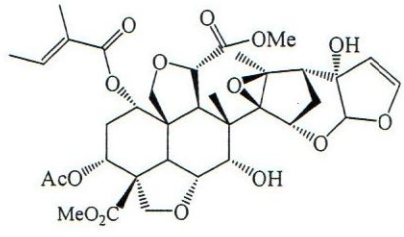
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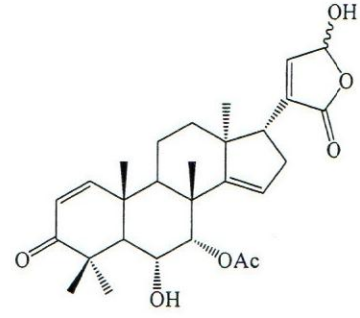
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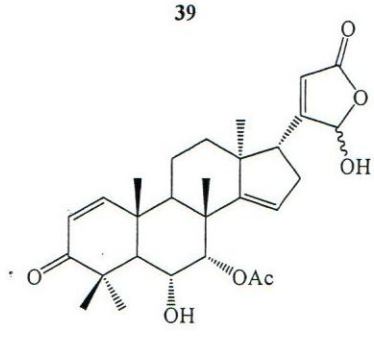
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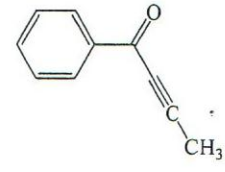
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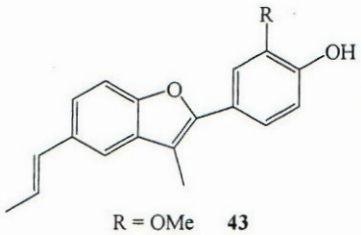
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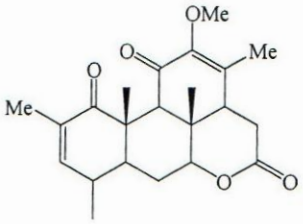
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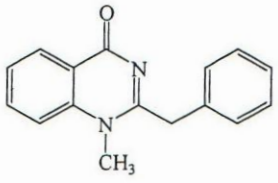
R = OMe **43**
R = H **44**



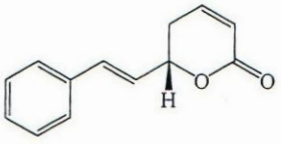
45

$CH_3(CH_2)_{14}COOH$

46

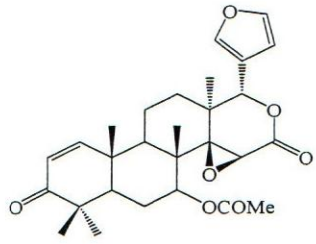


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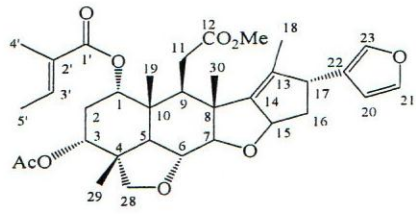


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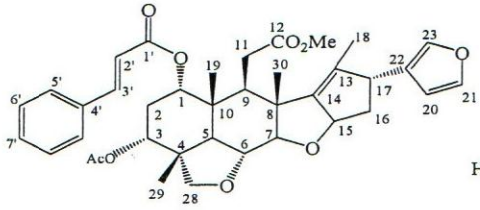
Arrangements of sub-groups and ring structures within this basic building block provide host characteristics that have generated interest in these plant products. These characteristics include insecticidal, insect growth regulation, insect anti-feedant and medicinal effects to animals and humans such as anti-bacterial, anti-viral and anti-fungal properties (Abdelgaleil and Nakatani, 2003). Extracts of several tropical Meliaceae have also been found to have anti-malarial activity *in vitro* against *P. falciparum* (Connolly, 1983). The roots of *Chukrasia tabulans* have been reported to be active against chloroquine-resistant strains of *P. falciparum* (Taylor, 1981), while gedunin (**50**) is the anti-malarial agent of *A. indica* juss and *M. azedarach* (Khalid *et al.*, 1989). Plants of the family Meliaceae were first reported to contain insecticidal properties (Mwangi and Mukiama, 1988). Until recently, much of the work reported on Meliaceae insect growth inhibitory and feeding deterrent properties concentrated mainly on the extracts of the neem tree, *A. indica* juss and the China berry tree, *M. azedarach* Linn, whose main bioactive compounds are azadirachtin (**39**), salannin (**51**), ohchinin-3-acetate (**52**) and meliantriol (**53**) (Schwinger *et al.*, 1984). Besides the use of such compounds as agricultural pest control agents, their use in mosquito larval control is an interesting perspective. This possibility has previously been investigated (Schmutterer and Ascher, 1987).



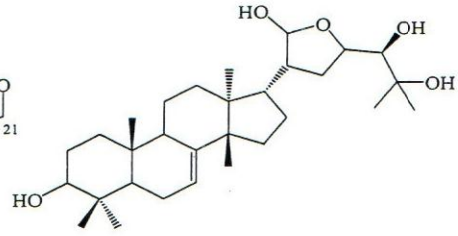
50



51



52



53

Hexane and ethanol extracts of seeds from ten plant species of this family were found to produce significant mortality, reduced larval growth rate and increased time of pupation of *Spodoptera frugiperda Smith* at a concentration of 16 ppm (Breuer and Schmidt, 1995). The same extracts were found to inhibit feeding and induce mortality in the larvae of the striped cucumber beetle, *Acalymma vittatum* F. (Taylor, 1986).

Limonoids appear to be plant chemicals produced in response to insect attack. To date over 300 limonoids have been isolated from Meliaceae. They are abundant and diverse in Meliaceae and characterise the family (Huang *et al.,* 1996). Recently, limonoids have attracted much attention because of the marked insect anti-feedant and insect growth regulating (IGR) activity of azadirachtin (**39**) and related highly oxidized limonoids from the neem tree *(A. indica)*. Azadirachtin (**39**) and extracts containing this compound have been shown to affect over 200 species of insects and mites (Bentley *et al.,* 1992).

2.5.2 The Genus *Melia*

Species in this genus include *Melia azedarach*, *Melia toosendan* and *Melia volkensii*.

2.5.2.1 Melia volkensii

Melia volkensii (Güerke), *Melia*, tree of knowledge (English), *Mkau* (kamba), *Kirumbutu* (Taita), *Mukau* (Kikuyu), *Maramarui* (Samburu), *Boba* (Somalia) (Mwangi and Kabaru, 2002), is a sub-tropical tree found in eastern areas of Africa (Figure 2.1). Natural distribution include

the semi-arid zone of Ethiopia, Somalia, Kenya and Tanzania where it is found at altitudes between 350 and 1700 m, in areas with mean annual rainfall of 300-800 mm. It is a deciduous tree, 6-20 m tall with a diameter of 25 cm. The leaves are bright green, flowers white and fruit ovoid drupe and yellow at maturity. Like neem, *M. volkensii* contains compounds that are toxic to insects and aqueous extracts of the fruits are traditionally used to control fleas and ticks (Mwangi and Rembold, 1988). Tea prepared from the bark is used to alleviate pain and is reported to be poisonous in overdose (Kokwaro, 1993). Previously, the root bark extract of this plant was investigated for potential anti-cancer constituents. Several limonoids were isolated and characterized (Zeng *et al.*, 1995a and b; Rogers *et al.*, 1998). The root-bark extracts of *M. volkensii* were also found to be potent larvicides against *An. gambiae s.s* (Ndung'u, 2002).

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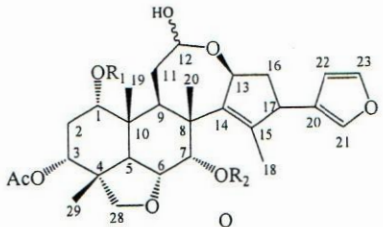
Figure 2.1: Melia volkensii plant with ripe fruits

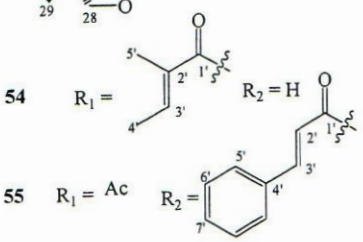
Some limonoids and limonoid-containing extracts of Meliaceae, such as *A. indica* and *M. azedarach* (Lee *et al.*, 1991; Rembold, 1995), *Trichilia harvanensis* (Lopez-Olquin *et al.*, 1997) and *T. pallida* have been implicated as possible disruptants of the endocrine system of insects. Because of their structural similarity to ecdysteroids, limonoids have been specifically considered as potential ecdysteroid agonists or antagonists. Two limonoids from *Turraea*

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obtusiofolia have been shown to antagonise 20-hydroxyecdysone action in a *Drosophila* cell line (Sarker *et al.*, 1997).

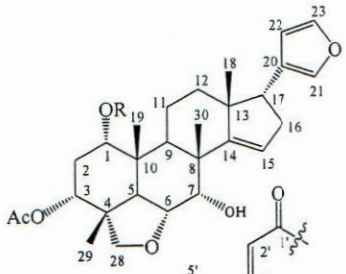
The crude extracts of the seed kernels of *M. volkensii* are reported to have potential anti-feedant activity against the desert locust, *Schistocerca gregaria* (Mwangi, 1982). Volkensin (**54**) and salannin (**51**) are examples of limonoids that have been isolated from fresh *M. volkensii* fruits and which have shown high anti-feedant activity against larvae of the fall armyworm *Spodoptera frugiperda* (Rajab *et al.*, 1988a). Dried kernel extracts of *M. volkensii* fruits have also demonstrated growth-inhibitory activity against larvae of *Ae. aegypti* and *An. arabiensis* mosquitoes (Mwangi and Mukiama, 1988; Mwangi and Rembold, 1988; Al-sharook *et al.*, 1991). Two groups of constituents have been implicated, one toxic and the other growth-inhibitory. A number of limonoids from the seed kernels have been isolated from this plant (Rajab *et al.*, 1988a; 1988b; Rajab and Bentley, 1988). These include volkensin (**54**); salannin (**51**) (Rajab *et al.*, 1988a); nimbolin B (**55**) (Zeng *et al.*, 1995a); 1-cinnamoyltrichilinin (**56**); 1-tigloyltrichilinin (**57**); 1-acetyltrichilinin (**58**) (Rajab *et al.*, 1988b), melianin C (**59**); melianin A (**60**); meliavolkinin (**61**); 1,3-diacetylvilasinin (**62**); meliavolkin (**63**) (Rogers *et al.*, 1998), melianin B (**64**) (Zeng *et al.*, 1995b), 12β-hydroxykulactone (**65**); 6β-hydroxykulactone (**66**); kulonate (**67**) (Cantrell *et al.*, 1999). (E)-volkendousin (**68**); (Z)-volkendousin (**69**); meliavosin (**70**) and 2,19-oxymeliavosin (**71**) are bioactive steroidal compounds previously isolated from *M. volkensii* (Lingling *et al.*, 1998).

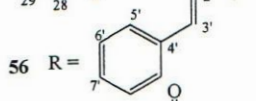




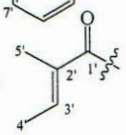
54 R_1 = R_2 = H

55 R_1 = Ac R_2 =

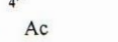




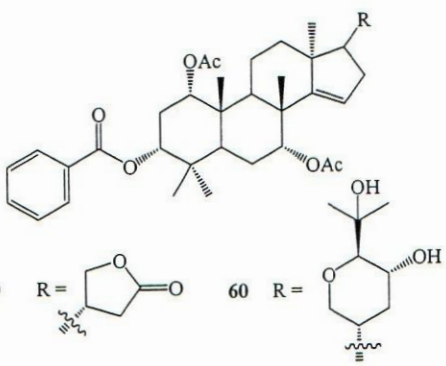
56 R =



57 R =

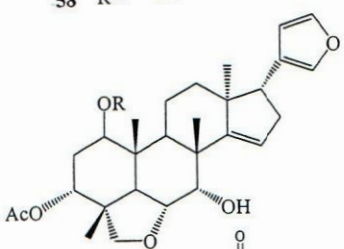


58 R = Ac

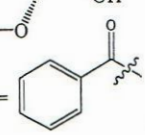


59 R =

60 R =

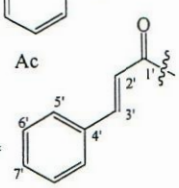


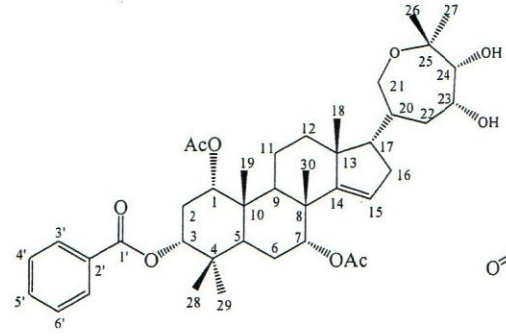
61 R =



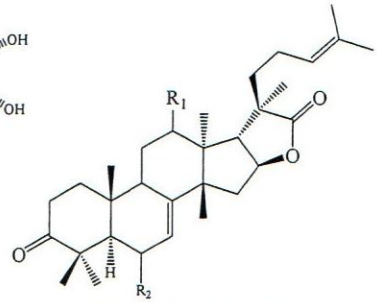
62 R = Ac

63 R =





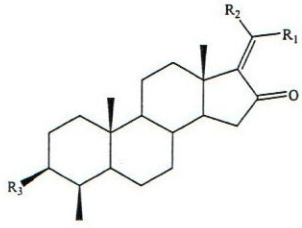
64



65 R_1 = OH R_2 = H

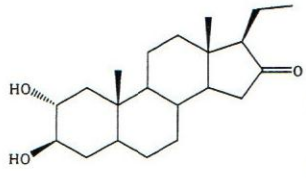
66 R_1 = H R_2 = OH

67 R_1 = H R_2 = H

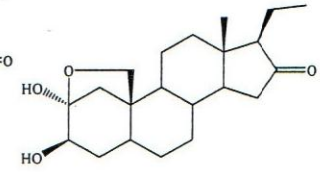


68 R_1 = H R_2 = CH_3 R_3 = OH

69 R_1 = CH_3 R_2 = H R_3 = OH



70



71

Although several pure compounds have been isolated from *M. volkensii* fruit, it is still not clear which of these are responsible for the observed anti-mosquito activities (Mwangi and Kabaru, 2002). The fruits were chosen in this study since literature search has shown that most of the compounds previously isolated are from the fruits. Previous work has also shown that inactive components of a plant may synergize active compounds or the active ones can synergise one another, or a blend of inactive compounds may produce some activity (Jembere and Hassanali, 2001).

CHAPTER THREE

MATERIALS AND METHODS

3.1 Collection and Identification of the Plant Materials

The ripe fruits of *M. volkensii* were collected from Kibwezi, Kenya. The plant was identified by Mr. S. Mathenge, a taxonomist at Botany Department, University of Nairobi, Kenya. A voucher specimen is deposited in the Herbarium of the Botany Department, University of Nairobi, Kenya.

3.2 Extraction of Plant Material

The fruits were dried under shade for 3 months and ground in readiness for the extraction. Sequential solvent extraction method was carried out on the powdered plant material at room temperature using hexane, chloroform, ethyl acetate and methanol, with occasional swirling to obtain different portions of the crude extracts. The powder (2 kg) was soaked twice in each extracting solvent (2.5 litres) in succession for 2-3 days with occasional swirling, filtered and concentrated in *vacuo* to obtain the crude extracts (Scheme 3.1).

3.3 Larvicidal Assays

The bio-assays were carried out using 3rd instar *An. gambiae.s.s.* larvae reared at the ICIPE-Nairobi, mosquito insectary using the standard WHO procedure (WHO, 1996). The temperature and humidity of the rearing and the bio-assay room was maintained at 28°C and 85% respectively. The different solvent extracts were subjected to larvicidal assays as detailed below

A stock solution was prepared by dissolving 1 g of the crude extract in 20 ml of acetone for each of the extracts, to obtain a concentration of 50 mg/ml (50,000 ppm). Serial dilution of stock solution was done to achieve 1000, 500, 250, 125, 62.5, 31.3 and 15.7 ppm concentrations. The prepared solution was divided into four portions of 20 ml each in 250 ml beakers and 20 3rd instar *An. gambiae* larvae introduced into each test beaker, fed with fish food (Tetramin®), mortality monitored after 24, 48, 72, 96 and 120 h and the larvicidal activity calculated. Control experiments were prepared by adding 1 ml acetone to a volumetric flask (10 ml) and topping to the mark with distilled water.

The larvicidal data of crude extracts and fractions, at varying concentrations (1000, 500, 250, 125, 62.5, 31.3 and 15.7 ppm), were subjected to statistical analysis. Statistical Analysis System (SAS) was used to analyze the data. Probit analysis was used to compute the LD_{50} values (Ndung'u, 2002). Adjusted % Mortality was calculated using Abbot's formula (Abbot, 1925) as:

% Mortality (adjusted) = (% AC - % AT)/ % AC

$$= \frac{(\%\ DT - \%\ DC)}{(100 - \%DC)} \times 100$$

where, AC = Alive in control, DT = Dead larvae in test, AT = Alive in treatment and DC = Dead larvae in control

The adjusted mortality was transformed to fit a general linear model (GLM) and Tukey test used for comparison of means. Probit analysis was used to determine LD_{50} values (Finney, 1978). Statistical Analysis System (SAS 2000), version 8.1 was used. The extracts with high larvicidal activity were further fractionated in order to identify the bio-active principles.

3.4 Effect of Solvent Extracts on Pupation and Adult Emergence

The procedure was similar to the larvicidal one described earlier in section 3.3. The mortality was assessed after every 24 hours up to the emergence of the adults or the death of the last pupa. The activity of each fraction or sub-fraction was based on the magnitude of inhibition of the adult mosquito emergence. Any emerging adults were monitored further to see whether they produced F_1 and F_2 generations. The % pupation and % emergence was calculated using the formula:

% Pupation = (A/B) x 100,

where A = total no of pupae, B = total no of larvae

Similarly, the % emergence was calculated using the formula:

% Emergence = (C/A) x 100,

where C = total no of emerged adults.

3.5 Fractionation

The active extracts were separated into several fractions by column chromatography using silica gel (Kiesegel G_{60} silica gel). All fractions were monitored using 0.20mm silica gel

60 ALUGRAM® SIL G/UV_{254} TLC plates (5×10 cm) with fluorescent indicator. The TLC locating agents used included UV lamp (254 and 366nm), iodine or 5% sulphuric acid in methanol. The fractions obtained were assayed as detailed above and those with substantial larvicidal activity noted for further purification.

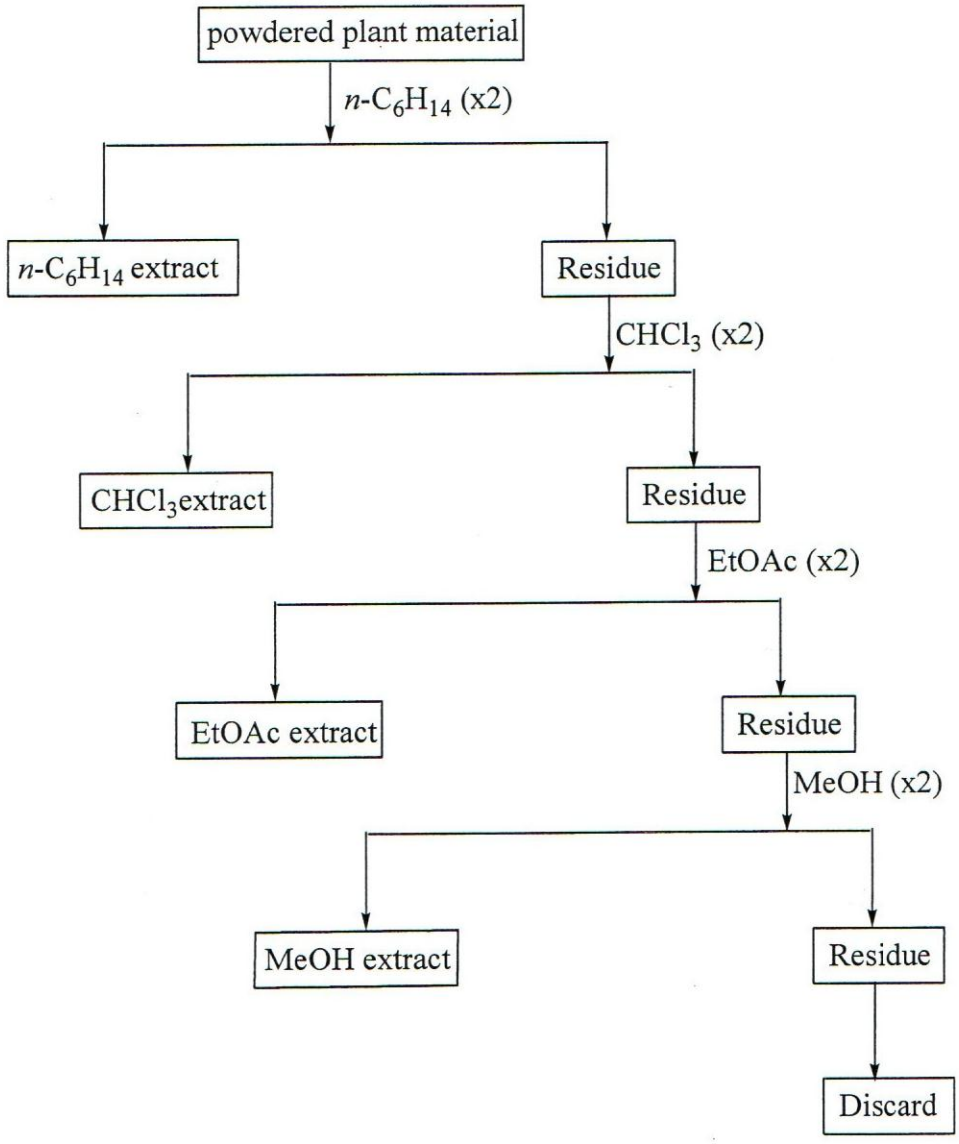
3.6 Isolation, Structure Elucidation and Identification of the Compounds

The fractions and sub-fractions with anti-insect activity, obtained from column chromatography, were purified further through high performance liquid chromatography (HPLC) and re-crystallization. The isolated pure compounds were identified through conventional physical and spectroscopic techniques (IR, MS and NMR).

3.7 Larvicidal Assay of the Isolated Pure Compounds

One of the isolated pure compounds was subjected once again to larvicidal and IGR assays as described earlier in section 3.3 and 3.5.

Scheme 3.1: Extraction procedure



CHAPTER FOUR

RESULTS AND DISCUSSION

4.1 *Melia volkensii* Solvent Extraction Yields

After extracting the dry fruit powder (2 kg), thick brown gummy oils were obtained. The % yields of the various solvent extracts are summarised in Table 4.1.

Table 4.1: Yield% of *M. volkensii* solvent extracts

Extract	Mass (g)	% Yield
n-C_6H_{14}	25	1.25
$CHCl_3$	106	5.30
EtOAc	12	0.60
MeOH	32	1.60

4.2 Effects of *M. volkensii* Crude Extracts on 3rd instar *Anopheles gambiae* Larvae

Melia volkensii solvent extracts assayed against 3rd-instar *An. gambiae* larvae at 1000, 500, 250, 125, 62.5, 31.3 and 15.7 ppm are presented in Table 4.2. For the hexane extract, 100% mortality was achieved within 24 h at high doses (1000 and 500 ppm) while at lower doses (250 and 125 ppm) the same level of mortality was achieved after 48 and 72 h, respectively. At the lower doses (62.5, 31.3 and 15.7 ppm) 100 % mortality was not achieved. Generally, larvicidal activity of the extract increased with extract dosage.

With chloroform extracts, 100% mortality was achieved within 24 h for 1000 and 500 ppm and after 48 and 72 h for the 250 and 125 ppm, respectively. These results are similar to those of the hexane extract. TLC analysis for this extract revealed that it contained compounds with the same R_f values as those in the hexane extract. The two extracts were combined and fractionated together.

For the ethyl acetate extract, a 100% larval mortality was achieved after 24h at 1000 and 500 ppm and after 48 h at 250 and 125 ppm. At lower doses (62.5, 31.3 and 15.7 ppm) the activity was higher than that recorded for hexane and chloroform extracts.

Table 4.2: Cumulative mortality (%) of 3rd instar *An. gambiae* larvae in water treated with *M. volkensii* solvent extracts

Conc (ppm)	Extract	24 h	48 h	72 h	96 h	120 h
1000	*n*-C_6H_{14}	100±00				
	$CHCl_3$	100±00				
	EtOAc	100±00				
	MeOH	100±00				
500	*n*-C_6H_{14}	100±00				
	$CHCl_3$	100±00				
	EtOAc	100±00				
	MeOH	100±00				
250	*n*-C_6H_{14}	86±1.3	100±00			
	$CHCl_3$	95±2.9	100±00			
	EtOAc	95±3.2	100±00			
	MeOH	100±00				
125	*n*-C_6H_{14}	63±3.2	95±2.9	100±00		
	$CHCl_3$	73±1.4	95±2.9	100±00		
	EtOAc	81±2.0	100±00			
	MeOH	98±3.2	100±00			
62.5	*n*-C_6H_{14}	14±2.4	25±2.1	30±2.0	35±2.0	45±2.9
	$CHCl_3$	15±4.6	20±4.8	30±2.1	33±1.4	50±2.4
	EtOAc	55±5.0	90±2.0	93±1.4		
	MeOH	84±3.8	95±2.0	97±1.4	100±00	
31.3	*n*-C_6H_{14}	0±00	6±1.3	16±1.3	29±1.4	
	$CHCl_3$	0±00	8±1.3	17±1.4	28±1.6	
	EtOAc	20±2.1	34±4.3	35±3.5	55±3.5	
	MeOH	70±2.9	76±2.4	83±4.3	89±3.2	93±0.9
15.7	*n*-C_6H_{14}	0±00	0±00	0±00	8±1.1	10±1.4
	$CHCl_3$	0±00	0±00	0±00	10±2.1	
	EtOAc	0±00	6±1.2	16±1.3		
	MeOH	0±00	23±7.2	26±6.3	28±4.4	30±4.0

The methanol extract was the most active with 100% mortality for 1000, 500, 250 ppm, after 24 h and at 125 and 62.5 ppm, after 48 and 96 h, respectively. At 31.3 and 15.7 ppm, 93% and 30% mortality was recorded after 120h, respectively. Methanol extract exhibited delayed

mortality in the treatment at lower doses (≤62.5 ppm). Probit analysis for the larvicidal data for various solvent extracts was carried out (Figure 4.1).

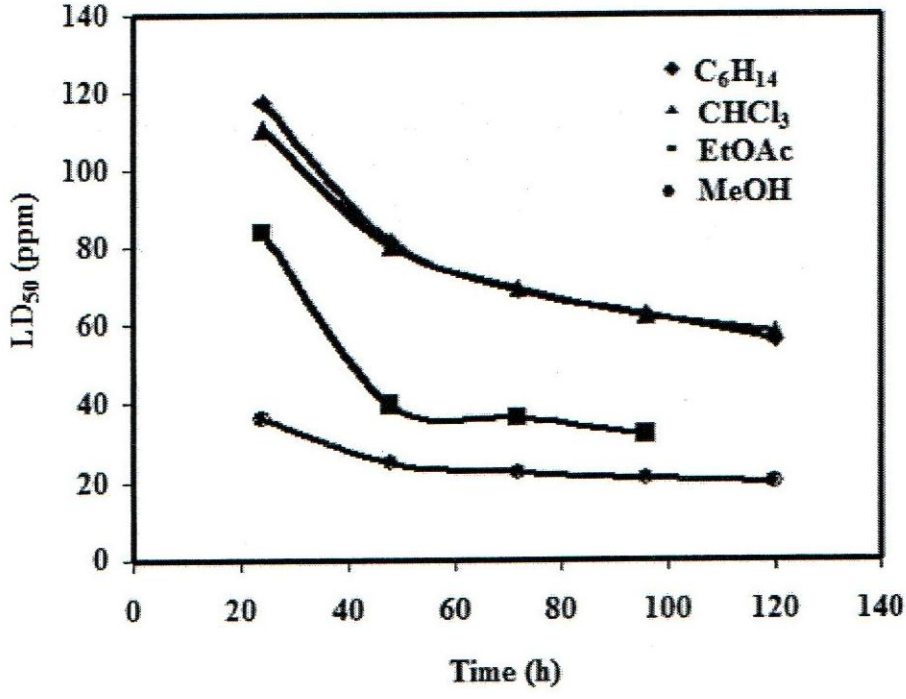


Figure 4.1: Probit analysis (LD_{50}) of larvicidal data for *An. gambiae* treated with *M. volkensii* extracts

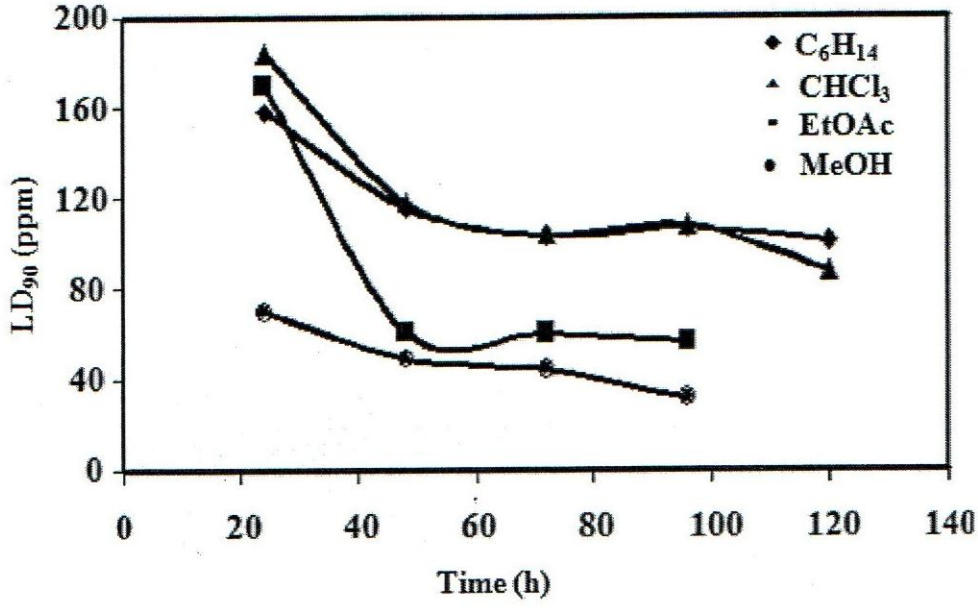


Figure 4.2: Probit analysis (LD_{90}) of larvicidal data for *An. gambiae* treated with *M. volkensii* extracts

The probit analysis reveals that the less polar solvents (hexane and chloroform) have the highest LD_{50} and LD_{90} values as compared to the more polar ones (ethyl acetate and methanol). It is also noted that both the LD_{50} and LD_{90} values decrease over the 24-120 hour range. This implies that whereas a high dose is required to kill the larvae within a short time, it would require almost half that dose to kill the same number of larvae after a longer time. The fruit kernel extracts of *M. volkensii* were previously evaluated for their toxicity against *Ae. aegypti* and LD_{50} values of 215.5, 135.0 and 86.7 ppm for the hexane, ethyl acetate and methanol extracts, respectively reported (Mwangi R.W, 1997). The reported values are higher than those recorded for the hexane, ethyl acetate and methanol extracts (110.8, 84.1 and 36.3 ppm, respectively) in this study. Another study carried out with *An. gambiae* on the root-bark extract of *M. volkensii* (Ndung'u *et al.*, 2004) reported LD_{50} values of 115.5 and 67.0 ppm for the methanol and chloroform extracts respectively. The LD_{50} for the methanol extract observed during this study of 36.3 ppm is lower, suggesting that most active compounds are found in the fruits and not on the root-bark. However, the opposite is true for the chloroform extract.

Some of the larvae survived at lower concentrations of the extracts and pupated. The effect of the extracts on pupation of *An. gambiae* is summarized in Table 4.3.

Table 4.3: Cumulative pupation (%) of 3rd instar *An. gambiae* larvae in water treated with *M. volkensii* solvent extracts

Conc (ppm)	extracts	48 h		72 h		96 h	120 h	144 h
		T	C	T	C	T	T	T
62.5	*n*-C_6H_{14}	0	95	15	100	35	45	55
	$CHCl_3$	0	100	20		35	50	
	EtOAc	0	90	0	100	0	0	
	MeOH	0	95	0	100	0	0	
31.3	*n*-C_6H_{14}	0	100	25	100	45	60	70
	$CHCl_3$	0	100	20	100	50	65	70
	EtOAc	0	100	10	100	20	45	
	MeOH	0	100	0	100	0	0	5
15.7	*n*-C_6H_{14}	30	85	50	100	75	90	
	$CHCl_3$	25	90	50	100	70	90	
	EtOAc	0	95	40	100	65	85	
	MeOH	0	100	25	100	35	50	70

Where T = treatment, C = control

Treatments with *n*-hexane and chloroform extracts (the least polar) recorded the highest % pupation within 48-144 h period. Hexane and chloroform extracts had similar % pupation except for the duration. For instance, there was 55% pupation after 144 h for hexane, whereas there was 50% cumulative pupation for chloroform after 120 h at 62.5. ppm. These results show no significance difference except for duration.

For the ethyl acetate extract, the pupation was delayed to 72 h at the low concentrations unlike in the control experiments where pupation took place within 48h. It achieved 10 and 40% pupation at 31.3 and 15.7 ppm, respecively after 72 h. This was lower than for hexane and chloroform extracts at the same concentrations. Some larvae did not moult into pupae but stayed at the larval-pupal intermediate (Figure 4.3 b) stage and eventually died.

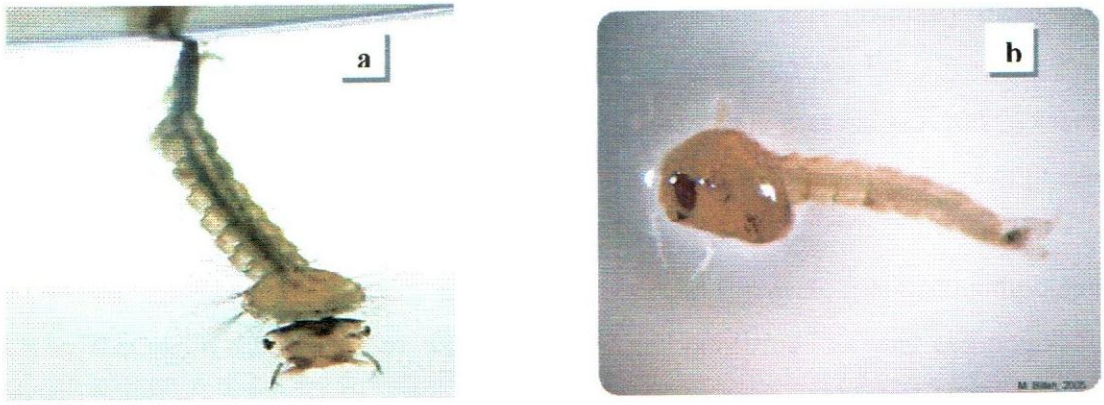


Figure 4.3: (a) normal mosquito larvae, (b) larval-pupal intermediate

The methanol extract, being the most active as a larvicide, recorded pupation only at 31.3 ppm (5%) and 15.7 ppm (70%) after 144 h. However, many dead larval-pupal intermediates (Figure 4.3b) were recorded for treatments with low doses of methanol extract.

Some pupae died before they could emerge into adults while others died as they tried to emerge. However, some of the pupae that did not die emerged into adults. The emergence data is summarized in Table 4.4.

Treatments with hexane and chloroform extracts, the least polar solvents, recorded the highest % emergence over the 72-120 h period. Whereas, the pupae in the control emerged within 24h after pupation, those treated with extracts took up to 48 h before emergence. However, the emerged adults were unable to fly due to deformed wings compared to those from

the control. Consequently, they drowned to death after emergence. This is attributable to the growth regulatory properties of the extracts at lower doses (Table 4.4).

For the ethyl acetate extract, not all the pupated larvae emerged into adults in the treatments. However, the % emergence for treatments with ethyl acetate extract was lower than that for hexane and chloroform. The adults that emerged had deformed or smaller wings than those from the control experiment and could not fly and died within two days of emergence.

The lowest % emergence was recorded for the methanol extract in comparison to the others. Like the ethyl acetate extract, not all the pupated larvae emerged into adults. Most pupae died before emergence while others died while trying to emerge

4.2.1 Bioactivity of n-hexane Extract's Fractions on An. gambiae

The combined hexane and chloroform extract (131g) was fractionated into 16 fractions (F_{1H}-F_{16H}) by column chromatography eluting with 100 % hexane and gradually increasing polarity to 100 % ethyl acetate. Fractions F_{1H}, F_{2H}, F_{3H}, F_{5H}, F_{6H}, F_{11H}, F_{12H}, F_{14H}, F_{15H} and F_{16H} were assayed for larvicidal activity. Fractions F_{1H} and F_{2H} were oils that caused 100 % larval mortality within 24 h for 1000, 500, 250 and 125 ppm due to suffocation of the larvae (Table 4.5).

Thin layer chromatographic analysis revealed that F_{1H} and F_{2H} were identical compounds (R_f 0.72, SiO_2 9:1 EtOAc:*n*-C_6H_{14}). They were combined and confirmed as pure compound **73** (1.82 g) by TLC. Fractions F_{15H} and F_{16H} showed some activity but none achieved 100% activity level even after 96 h. F_{16H} exhibited 49, 44 and 43% larvicidal mortality after 48 h for 1000, 500 and 250 ppm, respectively. F_{15H} only exhibited activity after 72 h for concentration above 125 ppm and above (Table 4.5). Fraction F_{7H} was re-chromatographed and a pure compound **72** (9.0 mg) obtained. It was assayed but found to be inactive. All the other six fractions (F_{3H}-F_{14H}) were assayed but found to be inactive as none achieved 100 % mortality at 1000 ppm, even after 96h. Although F_{15H} and F_{16H} were not very active even at high doses, none achieved 100% pupation (Table 4.6).

Table 4.4: Cumulative emergence (%) of 3rd-instar *An. gambiae* larvae in water treated with *M. volkensii* solvent extracts

Conc(ppm)	extracts	72 h		96 h		120 h
		T	C	T	C	T
62.5	*n*-C_6H_{14}	0	85	9	100	54
	$CHCl_3$	0	90	16	100	60
	EtOAc	0	95	-	100	
	MeOH	0	100	-		
31.3	*n*-C_6H_{14}	0	85	36	100	64
	$CHCl_3$	0	95	40	100	50
	EtOAc	0	100	5		20
	MeOH	0	90	-	100	
15.7	*n*-C_6H_{14}	0	85	56	100	89
	$CHCl_3$	0	95	50	100	90
	EtOAc	0	100	41		71
	MeOH	0	100	-		36

Where T = Treatment C = Control

Table 4.5: Cumulative mortality (%) of 3rd instar *An. gambiae* larvae in water treated with *M. volkensii n*-hexane extract fractions

Fraction	Conc (ppm)	24 h	48 h	72 h	96 h
F_{1H}	1000	100±00			
	500	100±00			
	250	100±00			
	125	100±00			
F_{2H}	1000	100±00			
	500	100±00			
	250	100±00			
	125	100±00			
F_{15H}	1000	0±00	0±00	48±9.7	50±8.7
	500	0±00	0±00	38±3.2	46±2.4
	250	0±00	0±00	26±4.2	31±4.2
	125	0±00	0±00	16±6.6	21±6.6
F_{16H}	1000	0±00	49±1.3	61±5.5	76±4.7
	500	0±00	44±2.4	49±2.4	49±2.4
	250	0±00	43±11.3	48±6.3	48±6.3
	125	0±00	0±00	14±3.2	14±3.2

The % cumulative pupation of the control experiment for both F_{15H} and F_{16H} were higher than those for the treatment. This difference is attributable to the growth disrupting properties of these extract fractions. Fractions F_{15H} and F_{16H} did not achieve 100% emergence rate even after 120h (Table 4.7).

The % cumulative emergence of the control experiment for both F_{15H} and F_{16H} were higher than those for the treatment. This difference is attributable to the growth disrupting properties of these extract fractions.

4.2.2 Effect of Ethyl Acetate Extract's Fractions on An. gambiae Larvae

The ethyl acetate extract (12g) afforded 23 fractions (F_{E1}-F_{E23}) from column chromatography eluting with 100% *n*-hexane and gradually increasing polarity to 100% ethyl acetate. Fractions F_{E6}, F_{E7}, F_{E11}, F_{E12}, F_{E14} and F_{E23} were assayed for anti-larval activity, and the data is summarized in Table 4.8.

F_{E23} was the most active achieving 100% mortality at 1000, 500 ppm after 24 h and at 250, 125 ppm after 48 h. F_{E12} achieved 100 % mortality at 1000, 500, 250 ppm after 48 h, and at 125 ppm after 72 h, and was the second most active fraction. F_{E6} was next with 100 % mortality after 24, 48 and 72 h at 1000, 500 and 250 ppm, respectively. F_{E14} and F_{E11} achieved 100 % mortality at 72 and 96 h at 1000 and 500 ppm, respectively. F_{E7} was the least active fraction with 100 % mortality only at 1000 ppm after 96 h. The larvae that survived in treatments with ethyl acetate extract fraction pupated. The pupation data is summarized in Table 4.9.

Table 4.6: Cumulative pupation (%) of 3rd-instar *An. gambiae* larvae in water treated with *M. volkensii* hexane extract fractions

Fraction	Conc(ppm)	48h		72h		96 h
		T	C	T	C	T
F_{15H}	1000	0	85	15	100	40
	500	10	95	30	100	50
	250	20	100	45		70
	125	30	90	50	100	75
F_{16H}	1000	0	100	10		25
	500	15	95	35	100	50
	250	25	100	50		50
	125	50	95	75	100	75

T = Treatment, C = Control

Table 4.7: Cumulative emergence (%) of 3rd-instar *An. gambiae* larvae treated with *M. volkensii* hexane extract fractions

Fraction	Conc(ppm)	72 h		96 h		120 h	144
		T	C	T	C	T	T
F_{15H}	1000	0	100	10		30	70
	500	27	85	73	100	80	
	250	43	95	79	100	90	
	125	55	100	82		95	
F_{16H}	1000	0	100	20			
	500	15	100	50			
	250	25	100	50			
	125	50	100	85			

T = Treatment, C = Control

Table 4.8: Cumulative mortality (%) of 3rd-instar *An. gambiae* larvae in water treated with ethyl acetate extract fractions

Fraction	Conc (ppm)	24 h	48 h	72 h	96 h	120 h
F_{E6}	1000	100±00				
	500	99±1.3	100±00			
	250	68±3.2	73±3.2	100±00		
	125	0±00	29±4.3	44±5.2		
F_{E7}	1000	66±6.6	76±6.6	88±3.2	100±00	
	500	55±4.6	66±9.4	69±9.4	75±8.4	
	250	0±00	5±1.0	43±6.0	46±5.5	
	125	0±00	0±00	8±1.4	8±1.4	
F_{E11}	1000	54±4.2	76±6.9	100±00		
	500	24±4.3	53±1.4	84±4.3	100±00	
	250	0±00	53±3.3	60±5.4	84±1.3	84±1.3
	125	0±00	0±00	13±3.2	41±4.3	44±3.8
F_{E12}	1000	49±2.4	100±00			
	500	28±3.2	100±00			
	250	15±3.5	100±00			
	125	0±00	78±3.2	100±00		
F_{E14}	1000	0±00	84±4.2	100±00		
	500	0±00	78±3.2	78±3.2	100±00	
	250	0±00	68±3.4	71±4.5	93±2.1	
	125	0±00	29±6.3	30±3.3	30±3.3	
F_{E23}	1000	100±00				
	500	100±00				
	250	96±3.5	100±00			
	125	90±2.4	100±00			

Table 4.9: Cumulative pupation (%) of 3rd-instar *An. gambiae* larvae in water treated with *M. volkensii* ethyl acetate extract fractions

Fraction	Conc (ppm)	48h		72h		96h
		T	C	T	C	T
F_{E6}	1000	0	-	0	-	0
	500	0	-	0	-	0
	250	0	-	0	-	0
	125	0	100	50	-	0
F_{E7}	1000	0	100	0	-	0
	500	5	85	25	100	0
	250	35	85	55	100	0
	125	75	80	90	100	0
F_{E11}	1000	0	-	0	-	0
	500	0	-	0	-	0
	250	0	90	5	100	15
	125	25	95	55	100	0
F_{E14}	1000	0	25	0	90	0
	500	0	5	0	85	0
	250	0	90	0	45	5
	125	30	95	70	10	0

T = Treatment, C = Control

The % cumulative pupation of the control experiments for all the fractions (F_{E6}, F_{E7}, F_{E11} and F_{E14}) was higher than that for the treatments. F_{E7} appeared to be the least active recording % pupation of 35 and 75 at 250 and 125 ppm, respectively after 48 h followed by F_{E14} which recorded a % pupation of 30 after 48 h, at 125 ppm. F_{E11} had a % pupation of 25 at 125 ppm after 48 h, and 55 at the same concentration after 72 h. F_{E6} appeared to be the most active with % cumulative pupation of 50 after 72 h at 125 ppm. Fractions F_{E11} and F_{E14} demonstrated growth disruption properties as pupation was delayed to 96h unlike in the controls. The pupae that survived treatments with ethyl acetate extract fractions emerged into adults. The emergence data is summarized in Table 4.10.

Table 4.10: Cumulative emergence (%) of 3rd instar *An. gambiae* larvae in water treated with *M. volkensii* ethyl acetate extract fractions

Fraction	Conc (ppm)	72 h		96 h		120h
		T	C	T	C	T
F_{E6}	1000	0	-	0	-	0
	500	0	-	0	-	0
	250	0	-	0	-	0
	125	15	95	20	100	50
F_{E7}	1000	0	-	0	-	0
	500	0	90	20	100	40
	250	6	85	63	100	0
	125	50	80	94	100	0
F_{E11}	1000	0	-	0	-	0
	500	0	-	0	-	0
	250	0	85	33	100	0
	125	18	95	45	100	0
F_{E14}	1000	0	-	0	-	0
	500	0	-	0	-	0
	250	0	70	0	95	0
	125	0	90	36	100	64

T = Treatment, C = Control

While the pupae in the control experiments emerged within 96 h, those under treatment from the fractions took up to 120 h demonstrating growth regulation properties. F_{E6} recorded 50% emergence after 120 h at 125 ppm, while F_{E7} recorded 40% emergence after 120 h at 500 ppm and F_{E14} recorded 64% emergence after 120 h at 125 ppm.

Fractions F_{E6}, F_{E12} and F_{E23}, which gave a positive response for limonoids when sprayed with Ehrich's reagent (2% 4-dimethylaminobenzaldehyde in ethanol) and developed in a hydrogen chloride gas chamber (Torto *et al.*, 1996), were further purified and pure limonoids isolated. The structure elucidation of the isolated limonoids is extensively discussed in chapter five.

4.2.3 Fractionatuion of Methanol Extract

Although the methanol extract was found to be the most active, its fractionation and purification was not done. This is because the said fractionation and purification could not be accomplished within the time limits of the program.

4.2.4 Bioassay Data for 73

The pure compound (**73**), obtained from the hexane extract was assayed and the larvicidal data summarized in Table 4.11.

Table 4.11: Mean cumulative mortality (%) of 3rd instar *An. gambiae* larvae reared from water treated with pure 73

Conc (ppm)	24 h	48 h	72 h	96 h	120 h	144 h
1000	100±00					
500	100±00					
250	100±00					
125	100±00					
62.5	33±2.6	100±00				
31.3	13±1.6	44±9.4	65±9.4	75±8.4	95±3.6	
15.7	0±00	40±1.0	57±6.0	60±5.5	80±2.3	
7.9	0±00	15±1.1	30±1.4	40±1.4	50±2.0	
3.9	0±00	11±1.9	15±1.0	35±1.3	49±1.2	
2.0	0±00.3	8±1.4	15±1.3	17±1.1	20±1.5	24±1.6
1.0	0±00	4±1.0	10±1.4	10±1.3	11±1.3	16±1.0

From the data in Table 4.11, the isolated compound was very active and toxic to the larvae achieving 100% mortality for 1000, 500, 250 and 125 ppm after 24 h and at 62.5 ppm after 48 h. High larval mortality was also noted for the lower concentrations. The larval mortality was 95, 80, 50 and 49% after 120 h for 31.3, 15.7, 7.9 and 3.9 ppm, respectively. From probit analysis (Figure 4.4), the compound was found to have a LD_{50} of 68.5 ppm compared to 110.8 ppm observed for the hexane extract. The compound may contribute significantly to the high mortality observed for the hexane extract.

The LD_{50} value for the pure compound (68.5 ppm, after 24 h) is lower than that for both the hexane and chloroform extracts. This implies that this compound is quite active and can

make a very good larvicide. The larvae that did not die pupated and the pupation data is summarised in Table 4.12.

The pure compound has growth disrupting property from the significant pupation observed in the control experiments after 48 h while it was delayed to 72 h for larvae in the treatment. Some larvae pupated after 120 h long after the ones in the control experiments had pupated. Many of the pupae did not emerge into adults while others died as they tried to emerge especially at the higher concentrations (31.3, 15.7, 7.9 and 3.9 ppm) (Table 4.13).

The emergence data for **73** (Table 4.13) shows that whereas the pupae in the controls emerged after 24 h, those in the treatment were delayed to 48 h after pupation. The adults that emerged had difficulty flying due to deformed wings and drowned within 24 h after emergence. The structure elucidation of **73** is discussed in great detail below.

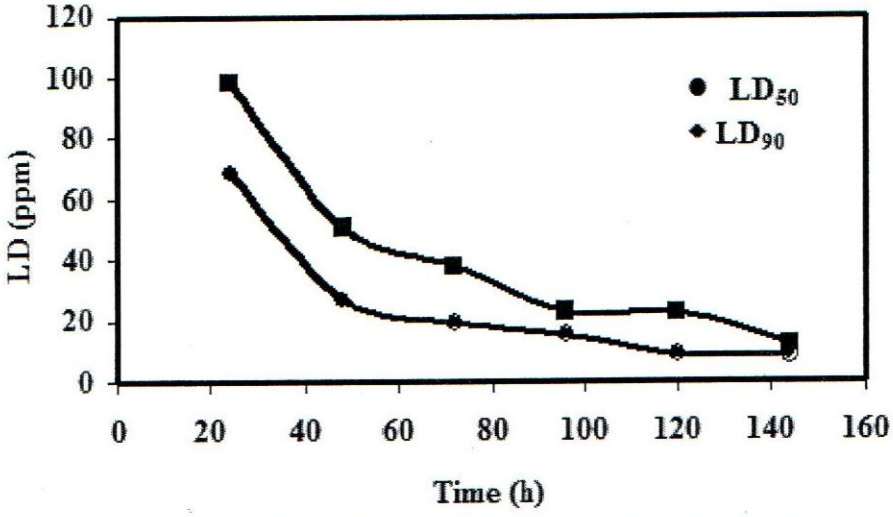


Figure 4.4: Probit analysis data for pure **73**

Table 4.12: Cumulative pupation (%) of 3rd-instar *An. gambiae* larvae in water treated with pure 73

	48 h		72 h		96 h	120 h
Conc (ppm)	T	C	T	C	T	T
31.3	0	90	0	100	5	
15.7	0	90	10	100	15	20
7.9	0	95	20	100	45	50
3.9	0	85	30	100	50	
2.0	0	95	40	100	75	
1.0	0	75	55	100	85	

Where T = Treatment, C = Control

Table 4.13: Cumulative emergence (%) of 3rd-instar *An. gambiae* larvae in water treated with pure 73

	48 h		72 h		96 h	120 h
Conc(ppm)	T	C	T	C	T	T
31.3	0	80	0	100	5	
15.7	0	80	10	100	15	20
7.9	0	85	20	100	45	50
3.9	0	85	30	100	50	
2.0	0	95	40	100	75	
1.0	0	70	55	100	85	

Where T = Treatment, C = Control

4.3 Structure Elucidation

A total of nine compounds 51, 52, 54, 56, 64, 72, 73, 74, and 76 were isolated and purified from *M. volkensii*. Compounds 72 and 73 were isolated from the hexane extract fraction while all the others were isolated from the ethyl acetate extract fraction These compounds were analysed by NMR, IR and MS spectroscopy

4.3.1 Stigmasterol, (72)

Stigmasterol, **72** was the first compound isolated by column chromatography from the haexane extract as white needle like crystals (R_f 0.65, SiO_2 9:1 EtOAc:C_6H_{14}). IR spectrum gave

the following diagnostic peaks: ν_{max} (cm^{-1}) 3700-3400 (OH str), 2921 (C-H str), 1656 (C=C str) and 1256(C-O str). The structure elucidation of **72** was based on a combination of ^{1}H-, ^{13}C-NMR, DEPT, HMQC, HMBC and COSY.

^{1}H NMR (Table 4.14) revealed 4 main signal regions integrating for 48 H. The signal at δ 5.35 (d, 1H) and was assigned to olefinic protons. The signal at δ 3.51 (m, 1H) suggested the presence of an α-hydroxy group typical of sterols hydroxylated at C-3. The rest of the signals were in a complex mass of multiplets between δ 0.68-2.35. On further comparison with literature data, this compound exhibited ^{1}H and ^{13}C NMR signals that are superimposable on those of stigmasterol, (**72**) (Ponce *et al.*, 2000).

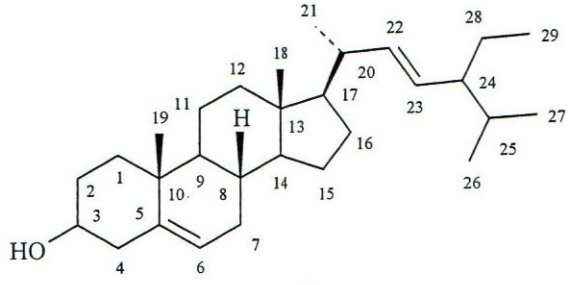
Table 4.14: ^{1}H NMR (200 MHz, $CDCl_3$) data for stigmasterol, (72)

Postion	*δ_{lit} (ppm)	δ_{obs} (ppm)
	1.1-2.28 (m, 38H)	1.1-2.35 (m, 29H)
3	3.51 (m, 1H)	3.51 (m, 1H)
6	5.34 (d, j=4, 1H)	5.35 (d, j=4, 1H)
18	0.70 (s, 3H)	0.70 (s, 3H)
19	1.00 (s, 3H)	0.70 (s, 3H)
22	5.17 (dd, j=15.2, 8.6, 1H)	5.12 (dd, j=15, 9, 1H)
23	5.04 (dd, j=15.2, 8.6, 1H)	5.17 (dd, j=15, 9, 1H)
26	0.85 (d, 3H)	0.86 (d, j=9.3, 3H)
27	0.80 (d, 3H)	0.82 (d, j=4.7, 3H)
29	0.81 (t, 3H)	0.82 (d, j=5, 3H)

* Literature data for stigmasterol (**72**) are taken from Ponce *et al.*, 2000, Forgo and Kover., 2004; multiplicity and coupling constants j in Hz are in parenthesis.

From ^{13}C NMR and DEPT (Table 4.15) 29 signals were observed.

From the above data the structure of stigmasterol (**72**) was confirmed.



72

Table 4.15: ^{13}C-NMR (50.3 MHz, $CDCl_3$) data for stigmasterol, (72)

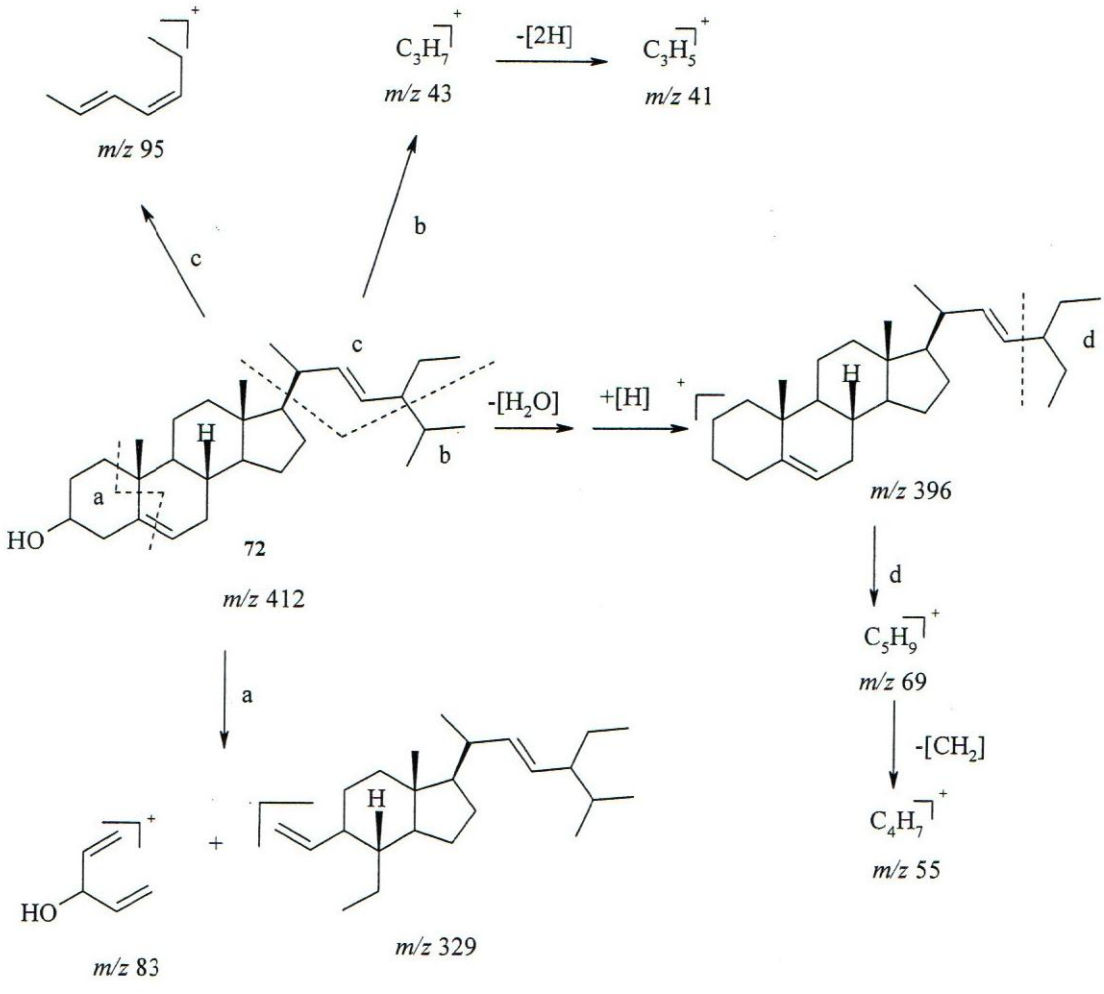
Position	*δ_{lit} ppm,	δ_{obs} (ppm)	DEPT
1	37.5	37.2	CH_2
2	31.7	31.6	CH_2
3	71.8	71.8	CH
4	42.4	42.3	CH_2
5	140.9	140.7	C
6	121.7	121.8	CH
7	31.9	31.9	CH_2
8	31.9	31.9	CH
9	50.3	50.1	CH
10	36.6	36.4	C
11	21.1	21.1	CH_2
12	39.8	39.7	CH_2
13	42.4	42.3	C
14	57.0	56.8	CH
15	24.4	24.3	CH_2
16	28.9	28.9	CH_2
17	56.0	56.0	CH
18	12.2	12.0	CH_3
19	19.4	19.0	CH_3
20	40.5	40.5	CH
21	21.1	21.1	CH_3
22	138.4	138.4	CH
23	129.4	129.2	CH
24	51.3	51.2	CH
25	31.9	31.9	CH
26	19.0	19.0	CH_3
27	21.1	21.1	CH_3
28	25.4	25.4	CH_2
29	12.0	12.3	CH_3

* Literature data for stigmasterol (**72**) are taken from Ponce *et al.*, 2000

The structure was further confirmed by EIMS, which revealed a molecular ion peak at *m/z* 412, a value that is in agreement with the molecular formula $C_{29}H_{48}O$. The IHDs of 6 agrees

with 4 rings and the 2 double bonds in support of the structure above. Other fragment ions observed in EIMS were; *m/z* 396 (29), 329 (16), 95 (68), 83 (70), 69 (78), 55 (88), 43 (100) and 41 (77). The MS can be rationalised by the fragmentation pattern in Scheme 4.1.

Scheme 4.1: Proposed mass fragmentation pattern for stigmasterol, (72)



Stigmasterol is found in many higher plants such as *Zea mays* and wheat germ, where it has a role to play in cell membrane formation (Harbone and Barter, 1993). The spectral data obtained was consistent with what has previously been reported (Rubinstein *et al.*, 1976, Greca *et al.*, 1990, Connolly and Hill, 1991; Ponce *et al.*, 2000; http://www.spectroscopynow.com/spy/basehtml).

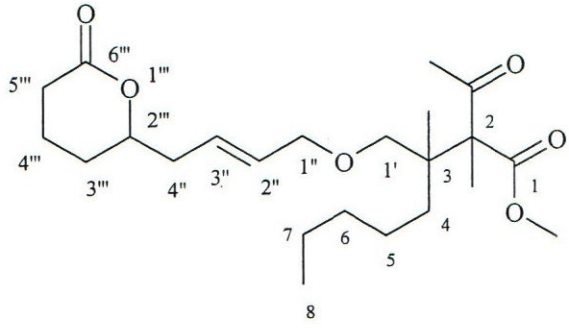
4.3.2 Methyl-2-acetyl-2,3-dimethyl-3-{[4''-(6'''-oxotetrahydro-2H-pyran-2'''-yl)-2''-E-butenyloxy]methylene}octanoate, 73

This compound was isolated by column chromatography from the hexane extract as colourless oil (R_f 0.72, SiO_2, 9:1 EtOAc: C_6H_{14}). The IR spectrum gave the following diagnostic peaks: ν_{max} (cm^{-1}) 2923 (C-H str), 1746 (C=O str esters or saturated lactones), 1653 (C=C str), 1461 (C-H str), 1163 (C-O str). The absence of a peak at ν_{max} 3700-3200 cm^{-1} in the IR spectrum indicates lack of OH group. The structure elucidation of **73** was based on a combination of ^1H, ^{13}C NMR, DEPT, HMQC, HMBC and COSY (appendices I-VI).

From ^1H NMR and HMQC data (Table 4.16), the signals at δ 5.35 (ddd, J=3.9, 7.5, 13.2 Hz, 1H) and δ 5.27 (ddd, J=6.6, 8.1, 13.2 Hz, 1H) were associated with carbons resonating at δ 128.5 and δ 130.0, respectively, and were assigned to 2 olefinic protons. A coupling constant of 13.2 Hz was an indication that the substitution across this double bond was trans. The signal at δ 5.37 (m, 1H) was associated to a methine carbon attached to an oxygen atom (δ 69.3). The signals at δ 4.29 (dd, J=11.4, 17.6 Hz, 1H) and δ 4.18 (dd, J=6, 17.6 Hz, 1H) suggested two non equivalent protons of a methylene group attached to an oxygen atom, which is characteristic of an ester group. From HMQC and DEPT data these two protons were both attached to a CH_2 carbon signal at δ 62.5. From the COSY data (Table 5.3), the signals at δ 4.29 and 4.18 were coupled to the peak at δ 5.35 (ddd, J=3.9, 7.5, 13.2, 1H) and these three protons are therefore next to each other. From HMQC and DEPT data, the protons resonating at δ 3.75 (d, J=14.6 Hz, 2H) and δ 3.70 (d, J=14.6 Hz, 2H) were attached to the carbon signal at δ 58.8 and were assigned to another methylene group attached to an oxygen atom. A peak at δ 3.50 (s, 3H) was associated with the carbon signal at δ 51.1 and was assigned to a methoxyl group. The signal at δ 2.32 (t, J=11.0, 2H) suggested the presence of a methylene group that is deshielded, and probably next to an electron withdrawing group like carbonyl. The peak at δ 2.19 (s, 3H) was associated with the carbon signal at δ 31.3, and was assigned to the methyl of the acetyl group. The signal at δ 2.07 (ddd, J=7.5, 11, 12.5 Hz, 2H) was assigned to the 2 allylic protons pendant to the lactone ring while another signal at δ 2.03 (m, 2H) was assigned to 2 methylene protons in the lactone ring. The protons at δ 2.07 (ddd, J=7.5, 11, 12.5 Hz, 2H) are coupled to the signal at δ 5.35 (ddd, J=3.9, 7.5, 13.2, 1H) from the COSY spectrum. The signals due to the methyl groups at C-2 and C-3 were observed at δ 2.1 (s, 3H) and δ 1.28 (s, 3H) in the ^1H NMR and were associated with

the peaks at δ 21.1 and δ 18.7 respectively, in ^{13}C NMR (HMQC). The peak at δ 0.90 (t, J = 6.7 Hz, 3H) was associated with the carbon signal at δ 14.5 and was assigned to the terminal CH_3 that was coupled to a CH_2 at δ 1.23-1.31 (m).

A combination of ^{13}C and DEPT NMR data helped in the rationalization of the 23 carbon signals as follows: 5 methyl (δ 14.5, 18.7, 21.1, 31.3, 51.1), 10 methylenes (δ 23.1, 27.6, 27.6, 29.5, 29.6, 29.9, 30.2, 34.4, 58.8, 62.5), 3 methines (δ 69.3, 128.5, 130.0) and 5 quartenary carbon atoms (δ 32.3, 34.6, 173.3, 177.1, 207.6). The signals due to two methyl groups attached to quartenary centers were observed at δ 18.7 and δ 21.1. The presence of oxygenated carbons was supported by the signals at δ 58.8, 62.5, 69.3, while the signals due to the 3 carbonyl groups were observed at δ 173.3 (ester), 177.1(lactone) and 207.6 (ketone). From ^{1}H, ^{13}C, DEPT, HMQC, HMBC and COSY NMR data, the molecular formula was proposed as $C_{23}H_{38}O_6$ suggesting 5 IHDs. From the spectroscopic data, the compound has four double bonds (3 × C=O, 1 × C=C) and 1 lactone ring. The structure of this compound was proposed as methyl-2-acetyl-2,3-dimethyl-3-{[4''-(6'''-oxotetrahydro-2H-pyran-2'''-yl)-2''-E-butenyloxy]methylene}octanoate (**73**) based on the spectroscopic data.



73

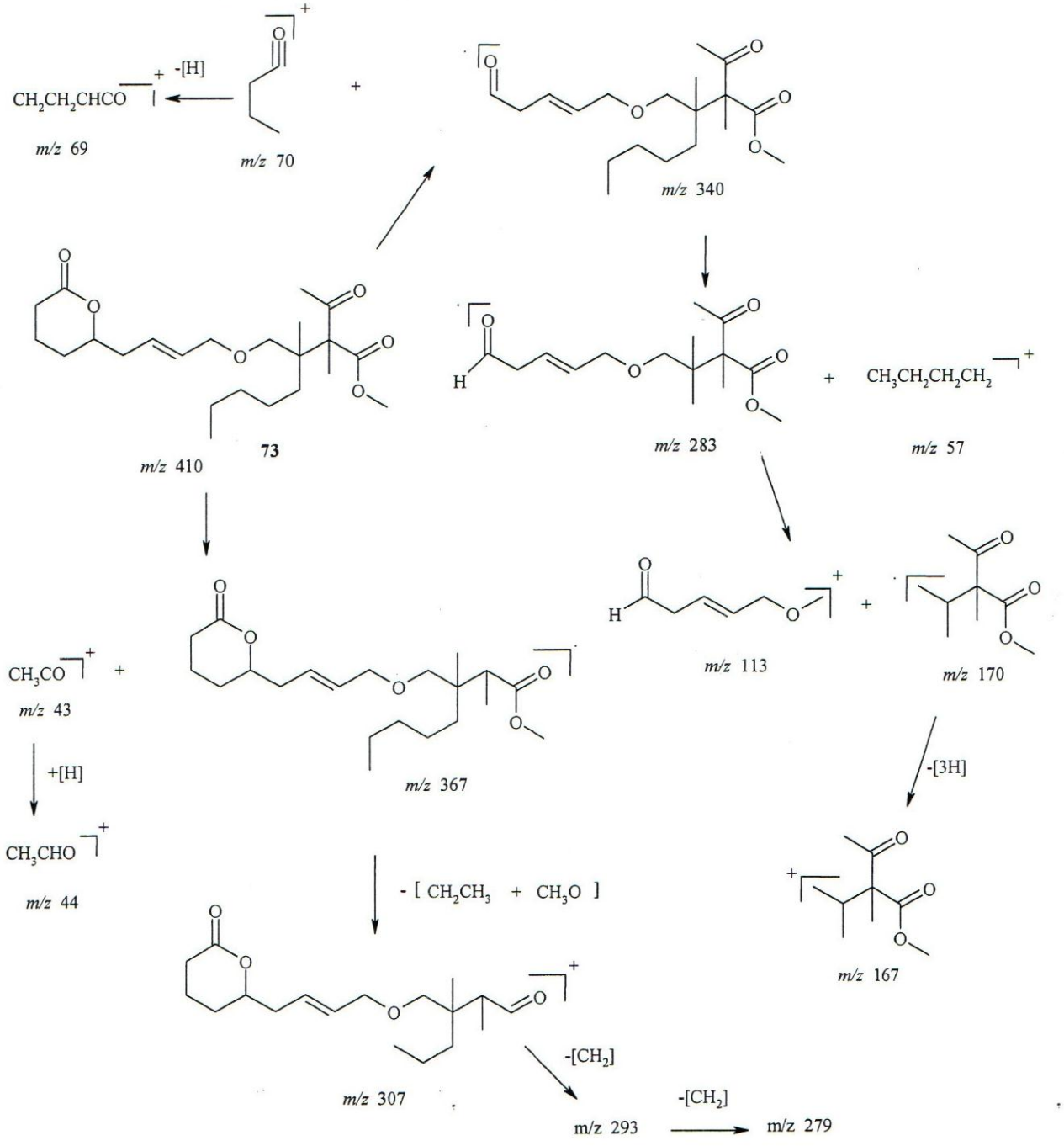
The EIMS analysis revealed the expected molecular ion peak at *m/z* 410 (10) consistent with the molecular formula $C_{23}H_{38}O_6$. Some notable mass fragments are *m/z* 43(61), 44(100), 57(58), 69(21), 70(25), 113(10), 170(5), 167(24), 279(50), 283(30), 293(48), 307(15), 340(7) and can be rationalized by the mass fragmentation pattern presented in Scheme 4.2.

Table 4.16: ^{1}H-NMR (300MHz, $CDCl_3$) and ^{13}C-NMR (75.5 MHz, $CDCl_3$) data for Methyl-2-acetyl-2,3-dimethyl-3-{[4''-(6'''-oxotetrahydro-2H-pyran-2'''-yl)-2''-E-butenyloxy]methylene}octanoate, 73

Position	δ_H	COSY	δ_C	DEPT	HMQC	HMBC
1			173.3*	C		
2			34.6	C		
3			32.3	C		
4	1.23-1.31 (m)		29.9	CH_2	H-4	
5	1.23-1.31 (m)		29.5	CH_2	H-5	
6	1.23-1.31 (m)		30.2	CH_2	H-6	
7	1.23-1.31 (m)		23.1	CH_2	H-7	
8	0.90 (t, J=6.7, 3H)	H-7	14.5	CH_3	H-8	C-7, C-6
OCH_3	3.50 (s, 3H)		51.1	CH_3	OCH_3	CO_2
2-CH_3	2.1 (s, 3H)		21.1	CH_3	2-CH_3	
2-$CH_3\underline{C}O$			207.6	C	OCH_3	
2-$\underline{CH_3}CO$	2.19 (s, 3H)		31.3	CH_3	2-$CH_3\underline{C}O$	2-$CH_3\underline{C}O$, C-2, CO_2
3-CH_3	1.28(s, 3H)		18.7	CH_3	3-CH_3	
1'α	3.75 (d, J=14.6, 2H)		58.8	CH_2	H-1'β	3-CH_3, C-4, C-1''
1'β	3.70 (d, J=14.6, 2H)				H-1'α	
1''α	4.29 (dd, J=11.4, 17.6, 1H)	H-1''BH-2'', H-3'' H-1''A	62.5	CH_2	H-1''	C-1'', C-2'''
1''β	4.18 (dd, J=6, 17.6, 1H)					
2''	5.35 (ddd, J=3.9, 7.5, 13.2, 1H)	H-1''A,B,	128.5	CH	H-2''	C-4'', C-1
3''	5.27 (ddd, J=6.6, 8.1, 13.2, 1H)		130.0	CH	H-2''	C-3''
4''	2.07 (ddd, J=11, 7.5, 12.5, 2H)	H-3'', H-2'''	27.6	CH_2	H-4''	
2'''	5.37 (m, 1H)	H-3''	69.3	CH	H-2'''	C-6'''
3'''	2.03 (m, 2H)		27.6	CH_2	H-3'''	C-3
4'''	1.23-1.31 (m, 2H)		29.6	CH_2	H-4'''	
5'''	2.32 (t; J=11.0, 2H)	H-4'''	34.4	CH_2	H-5'''	C-6''', C-3'''
6'''			177.1*	C		

Multiplicity and coupling constants (J in H_Z) are in parenthesis *interchangeable assignments

Scheme 4.2: Proposed mass fragmentation pattern for Methyl-2-acetyl-2,3-dimethyl-3-{[4''-(6'''-oxotetrahydro-2H-pyran-2'''-yl)-2''-E-butenyloxy]methylene}octanoate, (73)



There is no information documented in the chemical literature that this compound has been isolated from *M. volkensii* or any plant in the Meliaceae family. This compound was assayed and found to be very toxic (Table 4.11) to *An. gambiae* larvae by suffocation. It is also

likely that this compound was responsible for the high toxicity observed for the hexane extract of *M. volkensii*.

4.3.3 Salannin, 51

The limonoid positive ethyl acetate fraction (F_{E6}) eluting with 50:50 EtOAc:*n*-C_6H_{14} was further purified by HPLC on ultrasphere® C-18 reverse phase column (10 mm × 250 mm, 50μM) using 31:69 H_2O:CH_3CN mixture yielding compounds **51** (10 mg) and **52** (4 mg).

Compound 51 identified as was obtained as a white amorphous solid with a melting point of 165.5-167.5 °C. IR spectrum gave the following diagnostic peaks: ν_{max} 3454 cm^{-1} (O-H str), 2955 cm^{-1} (C-H), 1735 cm^{-1} (C=O str of ester), 1710 cm^{-1} (C=O str of a conjugated ester), 1652 cm^{-1}, 1446 cm^{-1} (C=C str), 1264 cm^{-1} (C-O str), 874 cm^{-1}(furan).

The structure elucidation of **51** was based on ^1H NMR, ^{13}C NMR, HMQC and HMBC NMR data (Table 4.17-4.18). The ^1H NMR revealed 22 signals integrating to 44H. The ^1H and ^{13}C-NMR spectra indicated that **51** had 8 methyl groups (including 1 OCH_3), 4 methylenes, 12 methines (including 4 olefinic groups) and 10 quartenary carbons (including 3 ester carbonyls and 4 olefinic groups). From the HMQC data the methyl signals were rationalized as follows; δ 3.22 (s, -CO_2CH_3), 1.93 (s, CH_3CO_2-), 1.93(s, H-5'), 1.80 (dd, J = 0.8, 7.1 Hz, H-4') 1.65 (d, ^{2}J=1.5 Hz, H-18), 1.29 (s, H-30), 1.20 (s, H-29) and δ 0.97 (s, H-19). The signals at δ 6.28 (d, J=1.6 Hz, 1H), 7.25(s, 1H), and 7.32 (d, J=1.6 Hz, 1H) suggested the presence of a β-Substituted furan ring (Cui *et al.*, 2005). The signals at δ 3.57 (d, J=7.5 Hz, 1H) and 3.67 (d, J=7.5 Hz, 1H), were characteristic of a C-28 methylene group forming an ether linkage with C-6, and strongly suggesting that the limonoid belonged to the nimbin class, in which ring C is modified through oxidation. Such limonoids are common in Meliaceae family (Zhou *et al.*, 1997). The ^1C NMR data (Table 4.18) revealed the presence of 3 carbonyl groups at δ 166.8, 170.4, and 172.8 with the ones at δ 170.4, and 172.8 suggesting the presence of acetate groups in those positions while the one at δ 166.8 indicated the presence of a tiglate group.

On further comparison with the spectroscopic data of compounds previously isolated from Meliaceae, the ^1H and ^{13}C NMR data were superimposable on those of salannin (**51**) (Nakatani *et al.*, 1996, Koul *et al.*, 2004). Consequently, the ^1H and ^{13}C spectra of **51**were assigned relative to those of Salannin, (**51**) (Table 4.17-4.18).

Table 4.17: ^{1}H NMR (500 MHz, $CDCl_3$) data for salannin, (51)

Position	δ_{lit} (ppm)	δ_{obs} (ppm)	COSY	HMBC
1	4.79 (t, J=2.9, 1H)	4.77 (t, J=2.8, 1H)	H-2α,β, H-3	
2α	2.20 (m, 1H)	2.18 (t, J=2.8, 1H)	H-1, H-2β, H-3	
2β	2.20 (m, 1H)	2.20 (t, J=2.8, 1H)	H-1, H-2α, H-3	
3	4.96 (t, J=2.9, 1H)	4.95 (t, J=2.8, 1H)	H-2	C-5
5	2.82 (d , J=12.6, 1H)	2.80 (d, J=12.8, 1H)	H-6	C-19, C-29
6	3.99 (dd, J = 3.2, 12.6, 1H)	3.97 (dd, J=3.3, 12.6, 1H)	H-5, H-7	
7	4.18 (d, J=3.3, 1H)	4.16 (d, J = 3.0, 1H)	H-6	
9	2.75 (dd, J=4.0, 8.1, 1H)	2.73 (dd, J=4.1, 8.4, 1H)	H-11α,β	C-5,
11α	2.20 (dd, J=4.4, 14.6, 1H)	2.20 (dd, J=4.4, 14.6, 1H)	H-11β, H-9	
11β	2.31 (dd, J=8.1, 14.6, 1H)	2.30 (dd, J=8.5, 14.9, 1H)	H-11α, H-9	
15α	5.44 (br t, J=6.9, 1H)	5.42 (dd, J=1.7, 6.5, 1H)	H-16α, β	
16α	2.13 (ddd, J = 6.6,8.8,11.9, 1H)	2.13 (ddd, J=6.4, 8.4, 12.1 1H)	H-15α, H-16B, H-17	
16β	2.24 (dd, J=6.6, 11.9, 1H)	2.24 (ddd, J=6.4, 8.4, 12.3, 1H)	H-15α, H-16B, H-17	
17	3.64 (br d, J = 8.8, 1H)	3.62 (dd, J = 8.5, 12.3, 1H)	H-16α, β	C-14 C-15 C-22
18	1.67 (d, J=1.1, 3H)	1.65 (d, ^{2}J=1.5, 3H)		C-8,
19	0.98 (s, 3H)	0.97 (s, 3H)		C-3,
21	7.26 (br s, 1H)	7.25 (br, s, 1H)		C-23
22	6.30 (br s, 1H)	6.28 (d, J=1.6, 1H)	H-23	
23	7.33 (t , J=1.4, 1H)	7.32 (d, J=1.6, 1H)	H-22	C-20, C-21
28α	3.69 (br d, J=7.6, 1H)	3.67 (d, J=7.5, 1H)	H-28β, H-29	
28β	3.58 (d, J=7.4, 1H)	3.57 (d, J=7.5, 1H)	H-28α, H-29	C-6
29	1.21 (s, 3H)	1.20 (s, 3H)		C-1,
30	1.30 (s, 3H)	1.29 (s, 3H)		
$CO_2C\underline{H_3}$	3.24 (s, 3H)	3.22 (s, 3H)		
3-$O_2C\underline{CH_3}$	1.94 (s, 3H)	1.93 (s, 3H)		
3'	6.96 (d J=1.3, 7.0, 1H)	6.94 (dd, J=1.4, 7.1, 1H)		
4'	1.81 (dq, J=1.1,7.0, 3H)	1.80 (dd, J=0.8, 7.1, 3H)		C-1'
5'	1.77 (br s, 3H)	1.93 (s, 3H)		C-1'

* Literature data for Salannin, (51) are taken from Nakatani *et al.*, 1996; multiplicity and coupling constants (J in Hz) are in parenthesis.

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Table 4.18: ^{13}C-NMR (125.8MHz, $CDCl_3$) data for salannin, (51)

Position	δ_{lit} (ppm)	δ_{obs} (ppm)	HMQC	DEPT
1	71.2	71.3	H-1	CH
2	27.5	27.6	H-2	CH_2
3	71.4	71.3	H-3	CH
4	42.7	42.7		C
5	39.9	40.0	H-5	CH
6	73.6	72.6	H-6	CH
7	85.7	85.7	H-7	CH
8	48.9	49.1		C
9	39.2	39.4	H-9	CH
10	40.3	40.6		C
11	29.7	30.7	H-11	CH_2
12	172.9	172.8		C
13	134.7	134.9		C
14	146.5	146.6		C
15	87.9	87.9	H-15	CH
16	41.2	41.4	H-16	CH_2
17	49.5	49.4	H-17	CH
18	12.9	13.1	H-18	CH_3
19	15.5	15.1	H-19	CH_3
20	127.2	127.1		C
21	142.9	138.8	H-21	CH
22	110.8	110.6	H-22	CH
23	138.9	143.0	H-23	CH
28	77.8	77.7	H-28	CH_2
29	19.6	19.6	H-29	CH_3
30	16.9	16.9	H-30	CH_3
12-$CO_2\underline{C}H_3$	51.6	51.5		CH_3
3-$\underline{C}O_2CH_3$	170.1	170.4		C
3-$CO_2\underline{C}H_3$	20.9	20.9		CH_3
1'	166.6	166.8		C
2'	128.2	129.0		C
3'	137.6	137.3	H-3'	CH
4'	14.5	14.4	H-4'	CH_3
5'	12.1	11.9	H-5'	CH_3

* Literature data for salannin are taken from Koul *et al.*, 2004 and Rajab *et al.*, 1988.

The MS analysis revealed the expected molecular ion peak at m/z 596 consistent with $C_{34}H_{44}O_9$. Other major peaks were m/z 565 $[M+H]\text{-}MeOH]^+$, 421 [M+H- TigOH -CH_3OH-$H_2O]^+$, 411[M+H-TigOH-AcOH]$^+$, 83$[C_5H_7O]^+$ (base peak), 55$[C_4H_7]^+$, (Attygalle *et. al.*2007, Schaaf *et al.*, 2000).

Salannin, (**51**) is a C-seco limonoid, which was first isolated from the seed oil of *Melia azadirachta* (Henderson *et al.*, 1968). It was subsequently isolated from the fruits of *M. volkensii* and the insect anti-feedant activity confirmed on *Schistocerca gregaria* (Rajab *et al.*, 1988a).

4.3.4 Ohchinin-3-acetate, 52

Due to the small amount of sample obtained (4 mg), structural elucidation of **52** was limited to ^1H, ^{13}C, HMQC COSY and HMBC NMR spectroscopic analysis (Tables 4.19-4.20). The spectra obtained indicated that **52** was not very pure and the structure elucidation had to be rationalized from complex NMR spectra. On a close look at the ^1H NMR spectral data (Table 4.19), signals similar to those obtained for salannin (**51**) (Table 4.17) were evident, except for a few but subtle differences. Methyl signals were rationalized as follows; δ 3.15 (s, CO_2CH_3), 1.94 (s, CH_3CO_2-), 1.63 (d, ^2J=2.2 Hz, H-18), 1.25 (s, H-29) 1.13 (s, H-30), 1.06 (s, H-19). Unlike in salannin (**51**), the methyl signals at δ 1.93 (s, H-5') and δ 1.80 (dd, J = 0.8, 7.1Hz, H-4') were absent indicating the absence of the tiglate group in **52**. HMQC data (Table 4.20) revealed that the signal at δ 5.53 (s, 1H) is attached to the peak at δ 88.5 in the ^{13}C NMR spectrum and was assigned to H-15 (Ochi *et al.*, 1978).

The ^1H NMR signals at δ 6.17 (d, J=1.7 Hz, 1H), 7.13 (dd, J=9.6, 1.7 Hz, 1H), and 7.25 (d, J=1.7, 1H) suggested the presence of a β-Substituted furan ring (Cui *et al.*, 2005). The signals at δ 3.59 (d, J=7.4) and 3.79 (d, J=7.4), are characteristic of a C-28 methylene group forming an ether linkage with C-6, and strongly suggesting that the limonoid belonged to the nimbin class in which ring C is modified through oxidation. Such limonoids are common in Meliaceae family (Zhou *et al.*, 1997). The ^{13}C NMR data (Table 4.19) revealed the presence of 3 carbonyl groups resonating at δ 165.2, 171.0, and 173.3. The 2 carbonyls at δ 171.0, and 173.3 suggested the presence of acetate groups while the one at δ 165.2 indicated the presence of a cinnamate group. Therefore, **52** differed from salannin (**51**) in that it has a cinnamate group at position 1 instead of a tigliate.

The cinnamate and acetate groups must be attached to C-1 and C-3, respectively, rather than the reverse, because the signal due to the ester methyl group in **52** is observed at an unusually high field (δ 3.15) as in salannin (**51**) (Henderson *et al.*, 1968). On a closer look at the information in the chemical literature, it was discovered that the spectral data for **52** is super-imposable on that of ohchinin-3-acetate (**52**) (Ochi *et al.*, 1978). Consequently the ^{1}H and ^{13}C NMR data were assigned according to the reported information on ohchinin-3-acetate, (**52**) (Ochi *et al.*, 1978).

Table 4.19: ^{1}H NMR (500 MHz, $CDCl_3$) data for Ohchinin-3-acetate, 52.

Position	δ_{lit} (ppm)	δ_{obs} (ppm)	COSY	HMBC
1	4.87 (t, , J = 4.9, 1H)	4.84 (dd, J = 3.0, 4.8, 1H)	H-1, H-2α, B, H-3	C-9
2α	2.31 (m, 1H)	2.26 (dd, , J = 3.0, 4.8, 1H)	H-1, H-3	C-5
2β	2.31 (m, 1H)	2.31 (dd, , J = 3.0, 4.8, 1H)	H-1, H-2α, H-3	C-9, C-29
3	5.01 (t, , J = 3.0, 1H)	4.96 (t, , J = 3.0, 1H)	H-1, H-2β	C-10
5	2.93 (d , , J = 12.0, 1H)	2.89 (d, , J = 12.3, 1H)	H-6	
6	4.05 (dd, , J = 3.0, 12.0, 1H)	4.02 (dd, , J = 2.9, 12.3, 1H)	H-5, H-7	
7	4.25 (d, , J = 3.0, 1H)	4.20 (d, , J = 2.9, 1H)	H-6	
9	2.77 (d, , J = 4.0, 1H)	2.77 (dd, J = 3.8, 12.6, 1H)	H-11α, β	
11α	2.20 (m, 1H)	2.20 (dd, J=5.1, 12.6, 1H)	H-9, H-11β	
11β	2.18 (m, 1H)	2.18 (dd, J=5.1, 12.6, 1H)	H-9, H-11α	
15α	5.53 (m, 1H)	5.53 (dd, J=9.5, 11.4, 1H)	H-16 α, β	
16α	2.13 (m, 1H)	2.13 (dd, J=9.5, 11.4, 1H)	H-15	
16β	2.24 (m, 1H)	2.24 (dd, J=9.9, 11.3, 1H)	H-15, H-17	C-8, C-21
17	3.64 (m, 1H)	3.62 (dd, J=9.9, 11.4, 1H)	H-16α, β	
18	1.55 (d, , J = 2.0, 3H)	1.63 (d, ^{2}J=2.1, 3H)		
19	1.05 (s, 3H)	1.06 (s, 3H)		C-4, C-8, C-30
21	7.16 (m, 1H)	7.13 (dd, J=9.6, 1.7, 1H)		C-21
22	6.23 (m, 1H)	6.17 (d, J=1.4, 1H)	H-23	
23	7.18 (m, 1H)	7.25 (d, J=1.7, 1H)	H-22	
28α	3.63 (d, ,J = 8, 1H)	3.59 (d, J=7.4, 1H)	H-28β	C-10
28β	3.73 (d, , J = 8, 1H)	3.79 (d, J=7.4, 1H)	H-28α	C-2
29	1.24 (s, 3H)	1.25 (s, 3H)		C-2, C-10
30	1.14 (s, 3H)	1.13 (s, 3H)		C-10
CO_2CH_3	3.21 (s, 3H)	3.15 (s, 3H)		
3-OCO(CH_3)	1.95 (s, 3H)	1.94 (s, 3H)		
2'	6.47 (d, , J = 16.0, 1H)	6.45 (d, , J = 16.1, 1H)	H-3'	
3'	7.8 (d, 16.0, 1H)	7.75 (d, , J = 16.1, 1H)	H-2'	
5'	7.37-7.51 (m, 1H)	7.51 (m, 1H)	H-6'	
6'	7.37-7.51 (m, 1H)	7.39 (m, 1H)	H-5'H-7'	
7'	7.37-7.51 (m, 1H)	7.38 (m, 1H)	H-6'	

* Literature data for Ohchinin-3-acetate (**52**) are taken from Ochi *et al.*, 1978; multiplicity and coupling constants (J in Hz) are in parenthesis.

Table 4.20: ^{13}C-NMR (125.8 MHz, $CDCl_3$) data for Ohchinin-3-acetate, 52

Position	δ_{lit} (ppm)	δ_{obs} (ppm)	HMQC	DEPT
1	71.7	71.7**	H-1	CH
2	27.3	27.1	H-2	CH_2
3	72.6	73.2**	H-3	CH
4	42.7	42.4		C
5	39.9	39.9	H-5	CH
6	71.3	72.0	H-6	CH
7	85.8	86.2	H-7	CH
8	49.1	49.0		C
9	39.5	39.9	H-9	CH
10	40.7	40.6		C
11	30.7	30.2	H-11	CH_2
12	172.6	173.0		C
13	134.9	135.4		C
14	146.4	146.4		C
15	88.0	88.5	H-15	CH
16	41.3	41.4	H-16	CH_2
17	49.4	49.5	H-17	CH
18	13.0	13.5	H-18	CH_3
19	16.9	16.7	H-19	CH_3
20	126.9	127.4		C
21	138.6	139.0	H-21	CH
22	110.4	110.9	H-22	CH
23	142.8	143.3	H-23	CH
28	77.6	77.7	H-28	CH_2
29	19.6	19.9	H-29	CH_3
30	15.2	15.6	H-30	CH_3
12-$CO_2\underline{C}H_3$	51.5	52.0		CH_3
3- $O_2C\underline{C}H3$	170.3	171.0		C
3- $O_2C\underline{C}H3$	21.1	21.4		CH_3
1'	165.4	165.2		C
2'	118.7	119.0	H-2'	CH
3'	144.6	145.1	H-3'	CH
4'	134.3	134.8	H-4'	C
5'	127.9	128.4	H-5'	CH
6'	129.0	129.4	H-6'	CH
7'	130.3	130.8	H-7'	CH

* Literature data for Ohchinin-3-acetate (**52**) are taken from Ochi *et al.*, 1978;
** assignments may be interchanged.

Ohchinin-3-acetate, $C_{38}H_{44}O_9$ (**52**) was first isolated from *Melia azedarach* Linn (Ochi *et al.*, 1978). It was subsequently isolated from the fruits of *M. volkensii* (Rajab and Bentley, 1988).

4.3.5 1-cinnamoyltrichilinin, 56

The limonoid positive fraction (F_{E23}) eluting with 55:45 EtOAC-*n*-C_6H_{14} mixture was further purified by HPLC on ultrasphere® C-18 reverse-phase column (10mm × 250mm, 50μM) using 25:75 H_2O:CH_3CN mixture as solvent to yield compound **56** (5.8 mg) as a white crystalline solid. IR spectrum gave the following diagnostic peaks: ν_{max} 3439 (O-H str), 2933 (C-H), 1727 (C=O str of ester), 1708 (C=O str, conjugated ester), 1636, 1447 (C=C str), 1246 (C-O str) and 871 cm^{-1} (furan).

The structure elucidation of **56** was based on ^1H, ^{13}C, HMQC and HMBC NMR data (Table 4.21-4.22). The ^1H NMR spectrum revealed 16 signals integrating to 44 H out of which 43 H could be easily assigned. Signals at δ 7.26 (d, J=1.5 Hz 1H), 7.15 (m, 1H), and 6.18 (d, J = 1.5 Hz, 1H) indicated the presence of a β-Substituted furan ring. The peak at δ 5.71 (dd, J = 1.6, 3.0 Hz, 1H) confirmed the presence of a tri-substituted olefinic proton H-15 (Cui *et al.*, 2005). The presence of an isolated AB-type CH_2 due to the C-28/C-6 oxymethylene bridge was confirmed by the peaks at δ 3.67 (d, J=7.6 Hz, H-28A) and δ 3.61(d, J=7.6 Hz, H-28B) (Nakatani *et al.*, 1999). The signal at δ 1.86 (6H) indicated the presence of 2 methyl groups attached to 2 carbonyl groups suggesting that **56** is a di-acetate. The signal at δ 4.24 (d, J=2.9 Hz) suggested the presence of a free hydroxyl attached to C-7. The peak at δ 4.20 (dd, J=2.9, 12.5 Hz) was assigned to H-6 like in salannin (**51**). Consequently, unlike salannin, **51**, **56** has an intact ring C.

The ^{13}C-NMR (Table 4.22) revealed the presence of 36 carbon atoms 6 of which were assigned to methyl groups. The signals at δ 111.6 (C-22), 124.4 (C-20), 140.2 (H-21) and 142.0 (H-23) confirmed the presence of a β-Substituted furan ring. The two signals at δ 170.1, 170.8 were assigned to the carbonyls in the acetate groups while the one at δ 165.5 was assigned to the carbonyl of a cinnamoyl group (Rajab and Bentley, 1988). Overall, the ^{13}C-NMR revealed that **56** had 6 olefinic bonds, 4 quartenary methyls, 2 acetates and a cinnamoyl group.

The spectroscopic information suggested that **56** belongs to the havanensin group of limonoids within the Vilasinin class. Members of this group have intact rings A, B, C and D. On further comparison with other previously isolated limonoids belonging to this class, the spectral data for **56** was found to be super-impossable on that of 1-cinnamoyltrichilinin (**56**) (Rajab and Bentley, 1988). ^{1}H and ^{13}C-NMR signals were assigned based on, COSY, HMQC, HMBC and comparative chemical shifts reported in the chemical literature (Rajab and Bentley, 1988).

Table 4.21: ^{1}H-NMR (500MHz, $CDCl_3$) data for 1-cinnamoyltrichilinin (56)

Position	*δ_{lit} (ppm)	δ_{obs} (ppm)	COSY	HMBC
1	4.71 (t, J=2.2, 1H)	4.71 (t, J=2.5, 1H)	H-2α, β	C-3', C-5'
2α, β	2.24 (m, 2H)	2.23 (dd J=2.0, 16.0 2H)	H-3	
3	4.96 (t, J=2.7, 1H)	4.95 (t, J=2.6, H)	H-2α, β	C-5
5	2.84 (d, J=12.0, 1H)	2.82 (d, J=12.5, 1H)	H-5	C-19, C-29
6	4.21(dd, J=3.0, 12.0, 1H)	4.20 (dd, J=2.9, 12.5, 1H)	H-7, H-5	
7	4.25 (d, J=3.0, 1H)	4.24 (d, J=2.9, 1H)	H-6	C-10
9	2.92 (m, 1H)	2.91 (dd, J=7.6, 12.5, 1H)	H-11α, β	C-20
11α	1.20 (m, 1H)	1.20 (ddd, J=5.2, 7.3, 12.7, 1H)		C-14
11β	2.18 (m, 1H)	2.16 (ddd, J=5.2, 7.5, 12.7, 1H)	H-9, H-12β	
12α, β	5.07 (dd, J=7.0, 9.0, 1H)	5.05 (dd, J=7.1, 8.9, 1H)	H-11α, β	C-17, C-19
15	5.72 (dd, J=1.5,3.0, 1H)	5.71 (dd, J=1.6, 3.0, 1H)	H-16α, β,	
16α, β	2.50 (m, 2H)	2.45 (ddd, J=3, 11.1, 15.8, 1H) 2.52 (ddd, J=3, 11.1, 16, 1H)	H-16α, β, H-17	C-18, C-20
17	2.99 (m, 1H)	3.00 (dd, J=7.9, 10.7, 1H)	H-16α, β	C-13
18	0.97 (s)	0.96 (s, 3H)		C-10, C-12 C-14
19	1.05 (s, 3H)	1.04 (s, 3H)		C-1, C-12
21	7.26 (m, 1H)	7.15 (m, 1H)		
22	6.19 (m, 1H)	6.18 (d, J=1.5, 1H)	H-21	
23	7.17 (m, 1H)	7.26 (d, J=1.5, 1H)	H-22	
28α	3.68 (d, J=7.6, 1H)	3.67 (d, J=7.6, 1H)		C-5, C-29
28β	3.62 (d, J=7.6, 1H)	3.61 (d, J=7.6, 1H)	H-28β, H-29	
29	1.24 (s, 3H)	1.24 (s, 3H)	H-28α, H-29	C-5, C-28
30	1.14 (s, 3H)	1.14 (s, 3H)		C-7, C-9, C-14
12-$OCO(CH_3)$	1.86 (br. S, 3H)	1.86 (br s, 3H)		
3-$OCO(CH_3)$	1.86 (br s, 3H)	1.86 (br s, 3H)		
2'	6.40 (d, J=15.9, 1H)	6.40 (d, J=16.0, 1H)	H-3'	C-4'
3'	7.76 (d, J=15.9, 1H)	7.74 (d, J=16.1, 1H)	H-2'	C-5'
5'	7.37-7.51 (m, 1H)	7.47 (d, J=1.7, 1H)	H-6'	C-3', C-7'
6'	7.37-7.51 (m, 1H)	7.37 (dd, J=1.6, 2.8, 1H)	H-5', H-7'	C-4', C-5'
7'	7.37-7.51 (m, 1H)	7.45 (dd, J=1.7, 2.8, 1H)	H-6'	C-3', C-5', C-6'

* Literature data for 1-cinnamoyltrichilinin, (**56**) are taken from Rajab and Bentley, 1988; multiplicity and coupling constants (J in Hz) are in parenthesis

The ^{1}H-NMR of **56** fits that of 1-cinnamoyltrichilinin, (**56**) (Rajab and Bentley, 1988).

Table 4.22: ^{13}C NMR (125.8MHz, $CDCl_3$) data for 1-cinnamoyltrichilinin, (56)

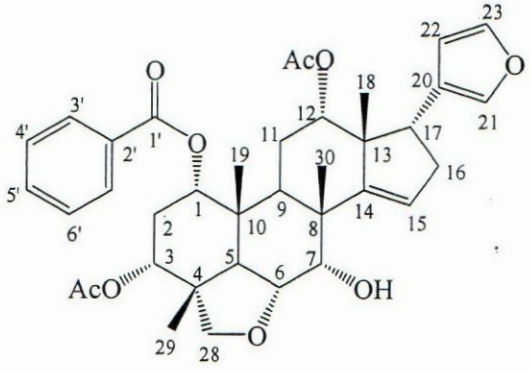
Position	*δ_{lit} (ppm)	δ_{obs} (ppm)	DEPT	HMQC
1	72.6	72.2	CH	H-1
2	27.3	27.3	CH_2	H-2
3	71.7	71.7	CH	H-3
4	42.3	42.3	C	
5	39.6	39.6	CH	H-5
6	74.0	73.9	CH	H-6
7	72.2	72.5	CH	H-7
8	45.3	45.4	C	
9	34.4	34.4	CH	H-9
10	39.4	39.3	C	
11	24.3	24.2	CH_2	H-11
12	77.1	77.0	CH	H-12
13	51.4	51.4	C	
14	157.2	157.2	C	
15	122.7	122.8	CH	H-15
16	36.6	36.6	CH_2	H-16
17	50.4	50.3	CH	H-17
18	26.8	26.8	CH_3	H-18
19	15.2	15.2	CH_3	H-19
20	124.4	124.4	C	
21	140.2	140.2	CH	H-21
22	111.6	111.6	CH	H-22
23	142.0	142.0	CH	H-23
28	77.9	78.0	CH_2	H-28
29	19.6	19.6	CH_3	H-29
30	15.2	15.4	CH_3	H-30
12-$O_2\underline{C}CH_3$	170.1	170.1	C	
3-$O_2\underline{C}CH_3$	170.8	170.8	C	
12- $O_2C\underline{C}H_3$	21.2	21.3	CH_3	
3- $O_2C\underline{C}H_3$	21.0	21.1	CH_3	
1'	165.4	165.5	C	
2'	118.0	117.9	CH	H-2'
3'	145.5	145.5	CH	H-3'
4'	134.1	134.0	C	H-4'
5'	128.0	128.0	CH	H-5'
6'	129.0	129.0	CH	H-6'
7'	130.5	130.6	CH	H-7'

* Literature ^{13}C NMR data for 1-cinnamoyltrichilinin, (**56**) are taken from Rajab and Bentley, 1988.

4.3.6 1-Benzoyltrichilinin, 74

The limonoid positive fraction (F_{E23}) eluting with 55:45 EtOAc-*n*-C_6H_{14} mixture was purified further by HPLC on ultrasphere® C-18(10 mm × 250 mm, 50μM) using 26:74 H_2O:CH_3CN mixture as the solvent to give compound **74** (3.4mg) as a white amorphous solid. The IR spectrum gave the following diagnostic peaks; ν_{max} 3437 (O-H str), 2933 (C-H), 1730 (C=O str of ester), 1717 (C=O str of a conjugated ester), 1653, 1446 (C=C str), 1246 (C-O str), 871 cm^{-1} (furan).

The structure elucidation of **74** was done based on ^1H, ^{13}C, COSY, HMQC and HMBC NMR data (Table 4.23-4.24 and appendices VIII-XII). The spectroscopic data for **74** was found to be similar to that of 1-cinnamoyltrichilinin, (**56**) except for a few subtle differences. The ^1H-NMR spectrum revealed benzoate signals between, δ 8.08 and 7.4 (Nakatani *et al.*, 1999). The presence of a benzoate moiety was further supported by the observation of a base peak at m/z 105 in the MS (Zhou *et al.*, 2004). The ^{13}C NMR data (Table 4.24) revealed the absence of one double bond when compared to that of 1-cinnamoyltrichilinin (**56**). On the basis of the spectroscopic data, **74** was identified as 1-benzoyltrichilinin. 1-benzoyltrichilinin, (**74**) was therefore different from 1-cinnamoyltrichilinin, (**56**) on substitution around C-1. Whereas 1-cinnamoyltrichilinin, (**56**) has a cinnamoyl group on C-1, **74** has a benzoyl group attached to this carbon. The location of the 1-benzoyloxy on C-1 was deduced from the peak at δ 4.83 (dd J=2.9, 2.9 Hz, H-1β) that is weakly coupled to the one at δ 4.95 (t, J=2.9, Hz, H-3β) through long-range interactions (Nakatani *et al.*, 1999).



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The spectral data for **74** is summarised in Tables 4.23-4.24.

Table 4.23: ^{1}H NMR (500MHz, $CDCl_3$) data for 1-benzoyltrichilinin, (74)

Position	δ ppm	COSY	HMBC
1	4.83 (dd, J=2.9, 2.9, 1H)	H-2	C-1'
2α, β	2.28 (dd, J=2.8, 2.8, 2H)	H-3	
3	4.95 (dd, J=2.9, 2.9, 1H)	H-2	
5	2.91 (d, J=12.6, 1H)	H-6	C-8
6	4.22 (dd, J=3.0, 12.5, 1H)	H-5	
7	4.25 (d, J=2.9, 1H)	H-6	
9	2.95 (m, 1H)		C-4
11α	1.15 (ddd, J=5.3,9.2, 12.4, 1H)	H-9	
11β	2.20 (ddd, J=5.3,9.2, 12.4, 1H)	H-12 β	
12β	5.02 (dd, J=7.1 ,9.0, 1H)	H-11α, β	C-30
15	5.71 (dd, J=1.7, 3.0, 1H)	H-16α β	C-13
16α	2.52 (ddd, J=3, 10.1, 16.2, 1H)	H-15, H-17	
16β	2.45 (ddd, J=3, 10.1, 16.2, 1H)		
17	2.97 (dd, J=8.0, 9.9, 1H)		C-8, C-30
18	0.74 (s, 3H)		
19	1.07 (s, 3H)		
21	7.13 (s, 1H)		
22	6.15 (d, J=1.2, 1H)	H-23	
23	7.25 (d, J=1.2, 1H)	H-22	
28α	3.66 (d, J=7.6, 1H	H-28β, H-29	C-7, C-10
28β	3.62 (d, J=7.6, 1H)	H-28α, H-29	
29	1.26 (s, 3H		C-10
30	1.14 (s, 3H		
7-OH	2.50, (m), 1H		
12-CO_2CH_3	1.79 (s, 3H		
3- CO_2CH_3	1.55 (s, 3H		
4'6'	7.42 (dd, J=7.8, 1.1, 1H	H-3', 7'	
3'7'	8.08 (dd, J=8.0, J=1.1, 1H	H-3', 4', 6'	
5'	7.53 (dt, J=8, J=1.1 1H		

Multiplicity, coupling constants (J in Hz) and integrals are in parenthesis

Table 4.24: ^{13}C NMR (125.8MHz, $CDCl_3$) data for 1-benzoyltrichilinin, (74)

Position	δ	DEPT	HMQC
1	71.6	CH	H-1
2	27.8	CH_2	H-2
3	72.6	CH	H-3
4	42.4	C	
5	39.6	CH	H-5
6	72.0	CH	H-6
7	73.9	CH	H-7
8	45.5	C	
9	34.4	CH	H-9
10	39.5	C	
11	23.9	CH_2	H-11
12	77.0	CH	H-12
13	51.3	C	
14	157.4	C	
15	122.8	CH	H-15
16	36.2	CH_2	H-16
17	50.3	CH	H-17
18	26.8	CH_3	H-18
19	15.3	CH_3	H-19
20	124.4	C	
21	140.2	CH	H-21
22	111.6	CH	H-22
23	142.0	CH	H-23
28	78.0	CH_2	H-28
29	19.7	CH_3	H-29
30	15.3	CH_3	H-30
12-CO_2 ($\underline{C}O$)	170.9	C	
3- CO_2 ($\underline{C}O$)	170.3	C	
12- CO_2 ($\underline{C}H_3$)	21.3	CH_3	H-12
3- CO_2 ($\underline{C}H_3$)	20.6	CH_3	H-3
1'	165.2	C	
2'	130.0	C	
3'7'	129.8	CH	H-3', 7'
4'6'	128.4	CH	H-4', 6'
5'	133.4	CH	H-5'

The MS analysis did not reveal a molecular ion peak at m/z 632 consistent with the formula $C_{37}H_{44}O_9$. However, the highest peak at m/z 572 was attributed to the loss of acetic acid

[M-AcOH]$^+$ peak. Other peaks that could be rationalized were 357[M-2AcOH-BzOH-H_2O-CH_3]$^+$, 105[C_7H_5O]$^+$ (base peak), 77[C_6H_5]$^+$, 44[CO_2], 43[CH_3CO]$^+$.

Literature search revealed that there is no previous documented chemical information on this compound from *M. volkensii* or any other plant in Meliaceae. However, other limonoids belonging to the same class have been isolated and characterised from Meliaceae (Rajab and Bentley, 1988; Nakatani *et al.*, 1999).

4.3.7 Melianin B, 64

The limonoid positive fraction (F_{E23}) eluting with 50:50 EtOAc- *n*-C_6H_{14} mixture was purified further by HPLC on ultrasphere® C_{18} reverse-phase column (10mm × 250mm, 50μM) using 35:65 H_2O:CH_3CN as the solvent to yield compound **64** (3.1 mg) as a white amorphous solid. The IR spectrum gave the following diagnostic peaks: ν_{max} 3407 (O-H str), 2930 (C-H), 1728 (C=O str of ester), 1708 (C=O str of a conjugated ester), 1651, 1440 (C=C str), 1268 (C-O str). From the HPLC profile, it appeared that **64** was contaminated with another compound since it was only 91% pure. Consequently, the ^{1}H and ^{13}C NMR spectra were overloaded with too many complex signals.

The presence of a benzoate group could be discerned from the ^{1}H NMR signals at δ 8.07 d (d, J=7.4 Hz), δ 7.55 (t, J=7.4 Hz), and δ 7.42 (dd, J=1.5, 7.8, Hz). The methyls of the two acetate groups resonated at δ 1.63 (1-OAc) and δ 2.01(7-OAc) while an olefinic proton signal was observed at δ 5.33 (H-23).

It was possible to rationalize the other compound in the mixture as salannin whose structure elucidation was discussed in section 4.3.3. Apart from the signals assigned to salannin (**51**), the remaining signals, were similar to those of Melianin B, (**64**), which has been previously isolated from *M. volkensii* (Rogers *et al.*, 1998). The comparative spectral data for melianin B, (**64**) are summarized in Tables 4.25-4.26.

The MS analysis did not give the expected molecular ion peak at m/z 694 consistent with $C_{41}H_{58}O_9$. However, the highest peak at m/z 512 was attributed to simultaneous loss of acetic and benzoic acid [M-AcOH-BzOH]$^+$. Other fragments observed were 417[M-2AcOH-BzOH-$2H_2O$+H], 120[BzOH-2H]$^+$ and 105[C_7H_7O]$^+$. Melianin B (**64**) was previously isolated from the root bark of *M. volkensii* and shows marginal cytotoxicity against some human tumour cell lines (Rogers *et al.*, 1998).

Table 4.25: ^{1}H-NMR (500 MHz, $CDCl_3$) data for Melianin B, (64)

Position	δ_{lit}	δ_{obs}	HMBC
1	4.67 (t, J=3.0, 1H)	4.77 (dd, J=2.9,16, 1H)	
2	2.15 (dd, J=3.0,16.5, 1H)	2.15 (ddd, J=2.9, 15.5, 6.3 1H)	C-5, C-9
	2.27 (td, J=3.0, 16.5, 1H)	2.25 (ddd, J=2.9, 15.5, 6.0 1H)	
3	4.87 (t, J=3.0, 1H)	4.88 (t, J=3.0, 1H)	C-9
5	2.51 (t, J=8.0, 1H)	2.51 (t, J=8.0, 1H)	
6	1.82 (dd, J=3.0, 8, 2H)	1.81 (ddd, J=3.0, 7.8, 6.0, 2H)	
7	5.16 (t, J=3.0, 1H)	5.17 (d, J=3.0, 1H)	
9	2.62 (dd, J=6.0, 12, 1H)	2.64 (dd, J=6.0, 11.3, 1H)	
11α	1.22 (m, 1H)	1.23 (m, 1H)	C-1, C-8 ,C-19
11β	1.48 (m, 1H)	1.40 (m, 1H)	
12α	1.52 (m, 1H)	1.54 (m, 1H)	
12β	1.68 (m, 1H)	1.68 (m, 1H)	
15	5.32 (br d. J=2.5, 1H)	5.33 (dd, J=2.6, 10, 1H)	
16α	1.98 (m, 1H)	1.98 (dd, J=3.0, 9.6, 1H)	C-8
16β	2.25 (m, 1H)	2.25 (dd,J=3.0, 9.6, 1H)	
17	1.85 (m, 1H)	1.85 (m, 1H)	
18	0.99 (s, 3H)	0.99 (s, 3H)	C-11
19	0.98 (s, 3H)	0.98 (s, 3H)	C-4, C-7 ,C-8
20	1.85 (m, 1H)	1.80 (m, 1H)	C-14
21	3.43 (dd, J=3.0, 13, 1H)	3.41(dd, J=2.8, 12.5, 1H)	
	3.56 (dd, J=3.0, 13, 1H)	3.58 (dd, J=2.8, 12.6, 1H)	
22α	1.60 (m, 1H)	1.63 (m, 1H)	
22β	1.92 (m, 1H)	1.89 (m, 1H)	
23	3.79 (ddd, J=3.0, 9.0, 9.0, 1H)	3.79 (ddd, J=2.8, 9.0, 9.0, 1H)	
24	3.40 (d, J=9.0, 1H)	3.40 (d, J=9.1, 1H)	
26	1.12 (s, 3H)	1.11(s, 3H)	C-6, C-7
27	1.27 (s, 3H)	1.30(s, 3H)	
28	0.88 (s, 3H)	0.88 (s, 3H)	
29	0.97 (s, 3H)	0.97 (s, 3H)	C-10
30	1.13 (s, 3H)	1.14 (s, 3H)	
1-CH_3CO_2	1.62 (s, 3H)	1.63 (s, 3H)	
7- CH_3CO_2	2.03 (s, 3H)	2.01 (s, 3H)	
3'7'	8.06 (d, J=8, 2H)	8.07(d, J=7.4, 2H)	C-1', C-5', C-6'
4',6'	7.40 (t, 8, 2H)	7.42 (dd, J=1.5, 7.8, 2H)	
5'	7.54 (tt, 5, 8, 1H)	7.55 (t, J=7.4, 1H)	C-3',

^{1}H NMR (300 MHz, $CDCl_3$) literature data for melianin B (**64**) is taken from Rogers *et al.*, 1998; Multiplicity, coupling constatnts (J in Hz) and integrals are in parenthesis

Table 4.26: ^{13}C NMR (125.8 MHz, $CDCl_3$) data for Melianin B, (64)

Position	δ_{lit}	δ_C	HMQC	DEPT
1	72.6	73.2	H-1	CH
2	25.5	25.5	H-2	CH_2
3	77.1	77.2	H-3	CH
4	36.6	36.1	H-4	C
5	37.4	38.2	H-5	CH
6	22.8	23.6	H-6	CH_2
7	75.6	76.4	H-7	CH
8	42.1	41.3	H-8	C
9	35.4	35.9	H-9	CH
10	40.3	40.3	H-10	C
11	16.1	16.8	H-11	CH_2
12	35.0	35.0	H-12	CH_2
13	46.3	46.5	H-13	C
14	159.4	159.4	H-14	C
15	119.2	119.1	H-15	CH
16	34.3	34.6	H-16	CH_2
17	54.2	54.5	H-17	CH
18	20.1	20.2	H-18	CH_3
19	16.2	16.8	H-19	CH_3
20	36.4	36.2	H-20	CH
21	64.3	65.0	H-21	CH_2
22	37.7	37.3	H-22	CH_2
23	68.2	68.6	H-23	CH
24	80.8	81.4	H-24	CH
25	76.2	76.4	H-25	C
26	22.3	22.3	H-26	CH_3
27	26.4	26.4	H-27	CH_3
28	28.0	28.6	H-28	CH_3
29	21.5	21.3	H-29	CH_3
30	26.8	27.0	H-30	CH_3
1-$O_2\underline{C}CH_3$	169.7	167.5		C
1-$O_2C\underline{C}H_3$	21.0	21.8	1-$O_2C\underline{C}H_3$	CH_3
7-$O_2\underline{C}CH_3$	170.1	170.5		C
7-$O_2C\underline{C}H_3$	21.0	21.8	7-$O_2C\underline{C}H_3$	CH_3
1'	165.2	166.3		C
2'	130.7	130.0	H-2'	C
3'	129.5	130.0	H-3'	CH
4'	128.3	129.1	H-4'	CH
5'	133.0	133.6	H-5'	CH
6'	128.3	129.1	H-6'	CH
7'	129.5	130.0	H-7'	CH

^{13}C NMR (75.5 MHz, $CDCl_3$) literature data for melianin B (**64**) is taken from Rogers *et al.*, 1998

4.3.8 Volkensin, 54 and epi-volkensin, 76

The limonoid positive fraction (F_{E12}) eluted with 50:50 EtOAc: *n*-C_6H_{14} mixture was purified further by HPLC on ultrasphere® C_{18} (10mm × 250mm, 50μM) using 34:66 H_2O:CH_3CN as the solvent to give compounds **54** and **76** (4.1 and 2.1 mg, respectively) as white yellowish solids. The IR spectrum gave the following diagnostic peaks: ν_{max} 3410 (O-H str), 2924 (C-H), 1731 (C=O str of ester), 1708 (C=O str of a conjugated ester), 1647, 1440 (C=C str), 1264 (C-O str), 873 cm^{-1} (furan).

During their separation by preparative HPLC, it was noted that every time an individual peak was collected, it appeared as two peaks on re-injection after a few minutes, (Figure 4.5) suggesting that the two compounds easily inter-converted into one another. The ^1H NMR spectra (Fig. 4.6) of the two compounds were identical suggesting that they were interconverting epimers. From the ^1H and ^{13}C NMR data (Tables 4.27-4.28) the following functional groups were indicated: one hydroxyl, one carbonyl, one tiglate, one acetate, one alkene and a β-substituted furan ring. The characteristic diastereotopic C-28-methylene protons, forming an ether linkage with C-6 as in salannin (**51**), were observed at δ 3.62 (d, J = 7.6 Hz) and δ 3.65 (d, J=7.6 Hz). However, the ^1H NMR signals at δ 4.18 (H-7) and 5.44 (H-15); and the corresponding ^{13}C NMR peaks at δ 85.7 (C-7) and δ 87.9 (C-15) usually observed in salannin (**51**) were absent in **76**. The ^{13}C NMR data (Table 4.28) indicated the presence of a pair of hemi-acetal carbons at δ 91.7 and 98.9 (Ana *et al.*, 2005). Other pairs of signals were observed for C-9, C-11, C-12, C-13 and C-15 in the ^{13}C NMR data (Table 4.28) analysis, indicating an epimeric mixture. After a thorough chemical literature search, it was discovered that the spectra data for **54** was similar to that of Volkensin (**54**) which has been previously isolated as a mixture of epimers from the fruits of *M. volkensii* (Rajab *et al.*, 1988a). Consequently, one of the compounds in the mixture was identified as Volkensin (**54**). It equilibrates with the hydroxy aldehyde (**75**) resulting from the hemi-acetal ring opening (Rajab *et al.*, 1988a). Cyclization of the hydroxyaldehyde (**75**) results in volkensin (**54**) and epi-volkensin (**76**) leading to epimerization (scheme 4.3). The other compound in the mixture was subsequently identified as epi-volkensin (**76**).

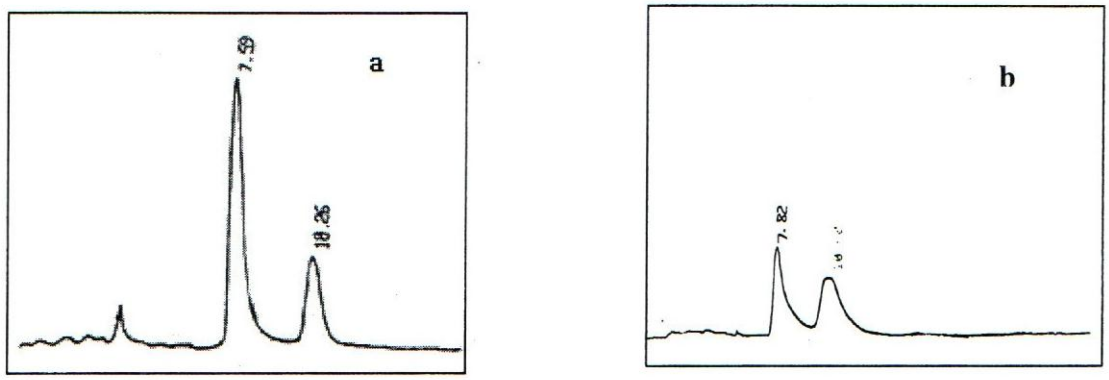


Figure 4.5: HPLC profile for (a) volkensin, (**54**) (b) epi-volkensin, (**76**)

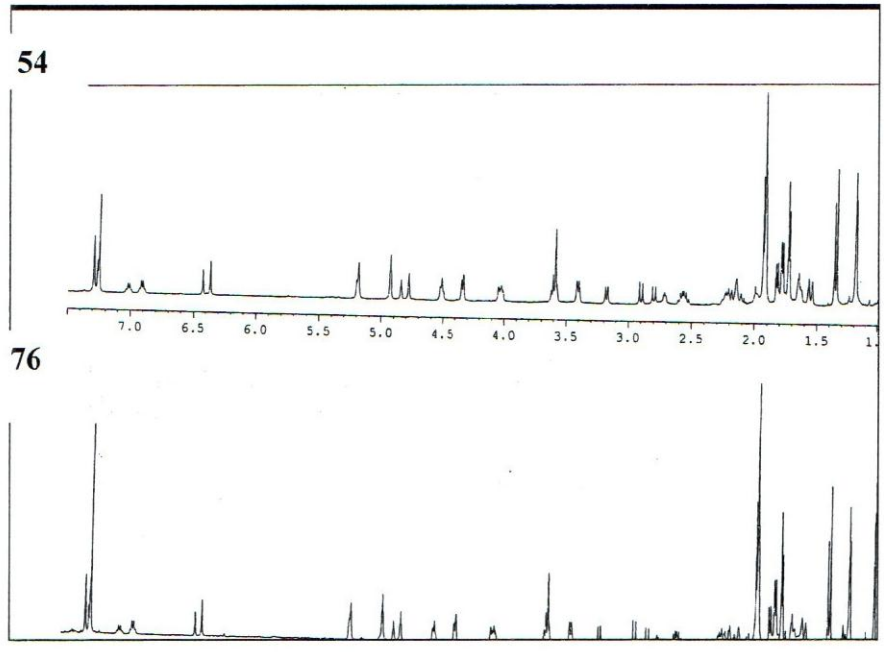
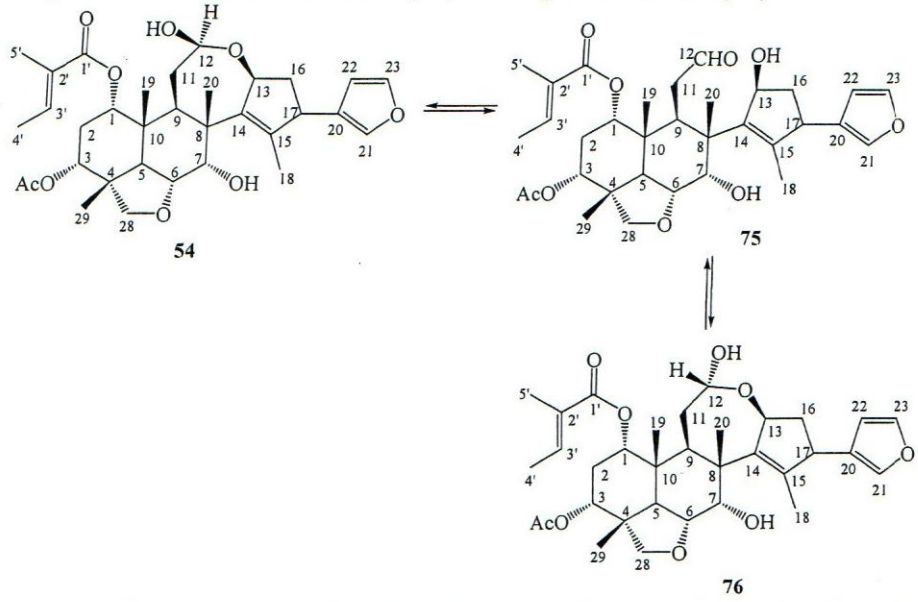


Figure 4.6: ^{1}H NMR data for volkensin, (**54**) and epi-volkensin, (**76**)

Scheme 4.3: Epimerization of volkensin (54) and epi-volkensin (76)



Limonoids of this class, in which the C ring has been oxidatively cleaved, followed by rotation and closure to C-15, have β stereochemistry of the furan at C-17. The obtained spectra are consistent with this D-ring structure type. The spectra data for Volkensin, (**54**), and epi-volkensin, (**76**) are summarized in Tables 4.27-4.28.

MS analysis revealed a peak at m/z 584 consistent with the molecular formula $C_{33}H_{44}O_9$. Other peaks present were m/z 421 $[M+3H\text{-}TigOH\text{-}AcOH]^+$ and 43 $[CH_3CO]^+$ (base peak), 71$[TigOH\text{-}CO\text{-}H]^+$. Volkensin (**54**) is a limonoid belonging to the Nimbin class of limonoids (Mulholland *et al.*, 2000). It was first isolated from the fruits of *M. volkensii* (Rajab *et al.*, 1988a) and assayed as an anti-feedant against 3rd-instar larvae of the fall armyworm (*Spodoptera frugiperda*). The ^1H and ^{13}C NMR spectra were not completely interpreted due to the overlapping signals arising from the epimeric mixture (Rajab *et al.*, 1988a). However, CrO_3 oxidation to the stable C-ring lactone (**77**) assisted in structure elucidation.

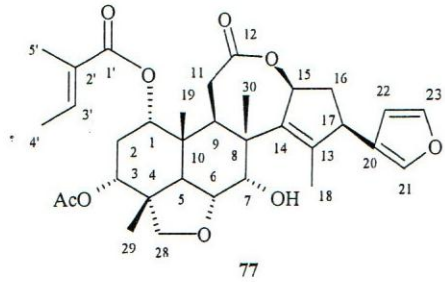


Table 4.27: ^{1}H-NMR (500 MHz, $CDCl_3$) data for Volkensin, (54) and epi-volkensin, (76)

Position	δ_{lit} (ppm)	δ_{obs}	COSY	HMBC
1	4.96 (t, J = 3, 1H)	4.94 (t, J = 3.0, 1H)	H-2α, β	3-CH_3O_2
2α, β	2.25 (t, J = 3.0, 2H)	2.25 (dd, J = 3.1, 6.1, 2H)	H-1, H-3	C-1'
3	4.78 (t, J = 3.0, 1H)	4.84 (dd, J = 3.0, 6.1, 1H)	H-2α, β	C-4, C-6
5	2.80 (d, J = 12.5, 1H)	2.83 (d, J = 12.6, 1H)	H-6	C-8
6	4.05 (dd, J = 3, 12.5, 1H)	4.05 (dd, J = 2.8, 12.7, 1H)	H-5, H-7	
7	4.26 (d, J = 3, 1H)	4.33 (d, J = 2.6, 1H)	H-6	C-10, C-11
9	3.18 (d, J = 11.0, 1H)	3.22 (dd, J = 10.1, 5.0, 1H)	H-11β	C-4, C-6
11α	1.63 (m, 1H)	1.63 (ddd, J = 5.3, 9.3, 10, 1H)	11 β, H-12	C-14
11β	2.26 (m, 1H)	1.80 (ddd, J = 5.3, 9.4, 11, 1H)	11α, H-12	
12-H	5.21 (m, 1H)	5.20 (dd, J = 5.0, 10, 1H)	H11α, β	
13α	5.19 (d, 7, 1H)	5.24 (dd, J = 6.7, 9.4, 1H)	H-16α, β	C-21
16α	2.5-2.7 (m, 1H)	2.5-2.7 (m, 1H)	H-15, H-16β, H-17	C-8, C-13
16β	2.28 (m, 1H)	2.5-2.7 (m, 1H)	H-15, H-16β, H-17	
17	3.35 (d, J = 9.0, 1H)	3.42 (d, J = 9.4, 1H)	H-16α, β	C-10, C-13
18	1.78 (s, 3H)	1.82 (s, 3H)		
19	1.00 (s, 3H)	1.04 (s, 3H)		C-6
21	7.30 (m, 1H)	7.32 (s, 1H)		
22	6.37 (s, 1H)	6.37 (dd, 1.4, 2, 1H)	H-23	
23	7.29 (m, 1H)	7.29 (dd, 1.5, 2, 1H)	H-22	C-21
28α	3.62 (s, 1H)	3.65 (d, J = 7.6, 1H)	H-28β, H-29	C-6, C-10
28β	3.62 (s, 1H)	3.62 (d, J = 7.6, 1H)	H-28α, H-29	
29	1.22 (s, 3H)	1.22 (s, 3H)	H-28α, β	C-6, C10
30	1.35 (s, 3H)	1.34 (s, 3H)		C-6
12-OH	2.39-2.52 (m, 1H)	2.2-2.6 (m, 1H)		
3-CH_3CO_2	1.98 (s, 3H)	1.99 (s, 3H)		
3'	7.00 (dd, J = 1.5, 7, 1H)	6.92 (dd, J = 1.2, J = 6.9, 1H)	H-4'	
4'	1.85 (m, 3H)	1.85 (m, 3H)		C-1', C-5'
5'	1.88 (d, J = 1.0, 3H)	1.90 (d, J = 1.0, 3H)		C-1', C-4'

*^{1}H NMR(300 MHz) literature data for Volkensin, (**54**) and epi-volkensin, (**76**) are taken from Rajab *et al.*, 1988a; Kraus and Bokel, 1981, multiplicity, coupling constants (J in Hz) and integrals are in parenthesis

Table 4.28: ^{13}C NMR (125.8 MHz, $CDCl_3$) data for Volkensin, (54) and epi-volkensin, (76)

Position	*δ_{lit} (ppm)	δ_{obs} (ppm)	DEPT	HMQC
1	71.2	71.3	CH	H-1
2	7.5	27.6	CH_2	H-2α, β
3	72.0	71.5	CH	H-3
4	42.8	42.5	C	
5	39.6	39.2	CH	H-5
6	73.0	73.8	CH	H-6
7	75.0	73.6	CH	H-7
8	45.7	45.6	C	
9	35.8/38.4	36.2/38.8	CH	H-9
10	40.8	40.9	C	
11	32.3/34.6	30/32.4	CH_2	H-11α, β
12	91.6/99.2	91.7/98.9	C	
13	141.0/141.5	140.0/141.0**	C	
14	143.6	144.8**	C	
15	78.0/86.6	78.3/86.8	CH	H-15α
16	37.2	37.2	CH_2	H-16α, β
17	47.4	47.2	CH	H-17
18	15.8	15.7	CH_3	H-18
19	15.8	15.7	CH_3	H-19
20	126.9	126.9	C	
21	139.1	139.2	CH	H-21
22	110.3	110.2	CH	H-22
23	143.0	143.1	CH	H-23
28	78.1	78.5	CH_2	H-28α, β
29	19.6	20.0	CH_3	H-29
30	20.9	21.0	CH_3	H-30
3-$O_2C\underline{C}H_3$	21.2	21.4	CH_3	3-CH_3CO_2
3-$O_2\underline{C}CH_3$	170.1	170.2/171.6	C	
1'	166.2	166.3.0	C	
2'	128.3	128.9	C	
3'	137.9	137.9	CH	H-3'
4'	14.6	14.7	CH_3	H-4'
5'	12.1	12.2	CH_3	H-5'

* Literature data for Volkensin, (**54**) and epi-volkensin, (**76**) are taken from Rajab *et al.*, 1988a; Kraus and Bokel, 1981; **assignments can be interchanged.

CHAPTER FIVE

CONCLUSIONS AND RECOMMENDATIONS

5.1 Conclusions

(a) The four solvent extracts of powdered ripe fruits of *M. volkensii* were all screened for their anti-larval activity against *An. gambiae ss.* The methanol extract was found to be the most potent (LD_{50} 36.3 ppm) followed by the ethyl acetate (LD_{50} 84.1 ppm), *n*-hexane (LD_{50} 110.8 ppm) and chloroform (LD_{50} 117.4 ppm).

(b) The isolation and characterization of the nine compounds: stigmasterol (**72**), , methyl 2-acetyl-2,3-dimethyl-3-{[4''-(6'''-oxotetrahydro-2H-pyran-2'''-yl)-2''-E-butenyloxy]methylene}octanoate (**73**), salannin (**51**),), ohchinin-3-acetate (**52**), 1-cinnamoyltrichilinin (**56**), 1-benzoyltrichilinin (**74**), melianin B (**64**), volkensin (**54**) and epi-volkensin (**76**), was achieved. Only methyl 2-acetyl-2,3-dimethyl-3-{[4''-(6'''-oxotetrahydro-2H-pyran-2'''-yl)-2''-E-butenyloxy]methylene}octanoate (**73**) was isolated in an adequate amount for anti-larval assay.

(c) The effect of *M. volkensii* solvent extracts on adult emergence and fitness was investigated. It was discovered that the extracts that did not kill the larvae and caused delayed pupation and emergence. The adults that emerged drowned and died immediately while others could not fly due to malformed wings and similarly drowned to death. It was also noted that these anti-larval compounds in *M. volkensii* fruit extract had toxicity at high doses and insect growth disrupting properties at lower doses against *An. gambiae* s.s.

(d) Apart from methyl-2-acetyl-2,3-dimethyl-3-{[4''-(6'''-oxotetrahydro-2H-pyran-2'''-yl)-2''-E-butenyloxy]methylene}octanoate (**73**), bioassay of all the isolated pure compounds was not accomplished due to the small amounts of samples obtained. However, the activity of these compounds can be inferred from that of the fractions from which they were isolated. The effect of the extracts and the isolated pure compounds on egg production was also not done since the adults treated with the *melia volkensii* solvent extracts did not survive long enough for such experimentation.

(e) Methyl-2-acetyl-2,3-dimethyl-3-{[4''-(6'''-oxotetrahydro-2H-pyran-2'''-yl)-2''-E-butenyloxy]methylene}octanoate (**73**) was assayed and found to be a potent larvicide (LD_{50} 68.5 and 8.8 ppm, after 24 and 120 h, respectively.

5.2 Recommendations

(a) The methanol extract that was found to be the most active should be fractionated and purified to isolate the active compounds for anti-larval assays.

(b) It was not possible to bioassay the pure compounds except for methyl-2-acetyl-2,3-dimethyl-3-{[4''-(6'''-oxotetrahydro-2H-pyran-2'''-yl)-2''-E-butenyloxy]methylene}octanoate (**73**), due to small yields obtained. The compounds can be synthesized or extracted in larger amounts to avail them in reasonable quantities for anti-larval assays against *An. gambiae s.s.* Blending of the compounds should be done to investigate which compounds synergize others.

(c) The toxicity of methyl-2-acetyl-2,3-dimethyl-3-{[4''-(6'''-oxotetrahydro-2H-pyran-2'''-yl)-2''-E-butenyloxy]methylene}octanoate (**73**) non-target organisms including mammals should be investigated.

(d) The yield of methyl-2-acetyl-2,3-dimethyl-3-{[4''-(6'''-oxotetrahydro-2H-pyran-2'''-yl)-2''-E-butenyloxy]methylene}octanoate (**73**) from extraction was 1.82 g from only 25 g of the extract (7.28%). This means that extraction would be a worthwhile undertaking and formulations that can be commercialized as larvicides against mosquito larvae made. Such a formulation would be environmentally friendly as it is biodegradable.

(e) The kernel of *M. volkensii* fruits contain oils that can be extracted and their efficacy against other arthropods, pests and vectors investigated.

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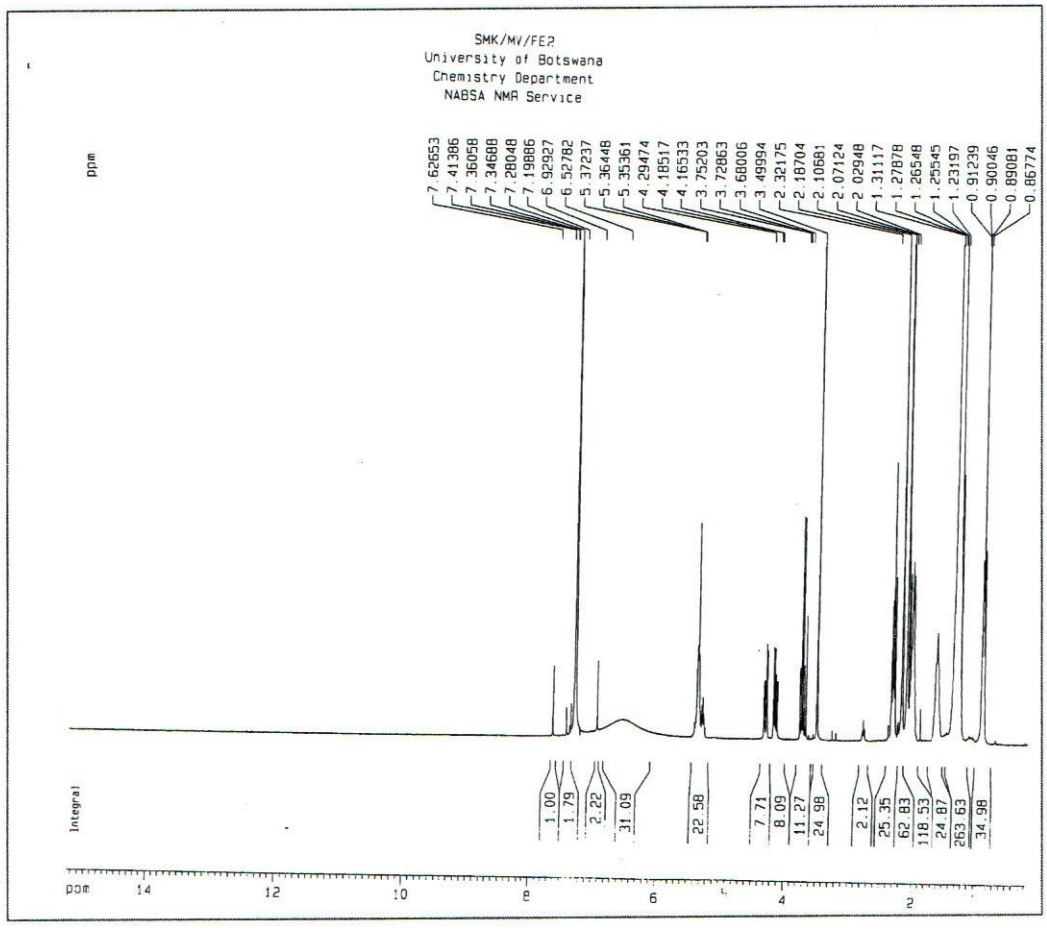
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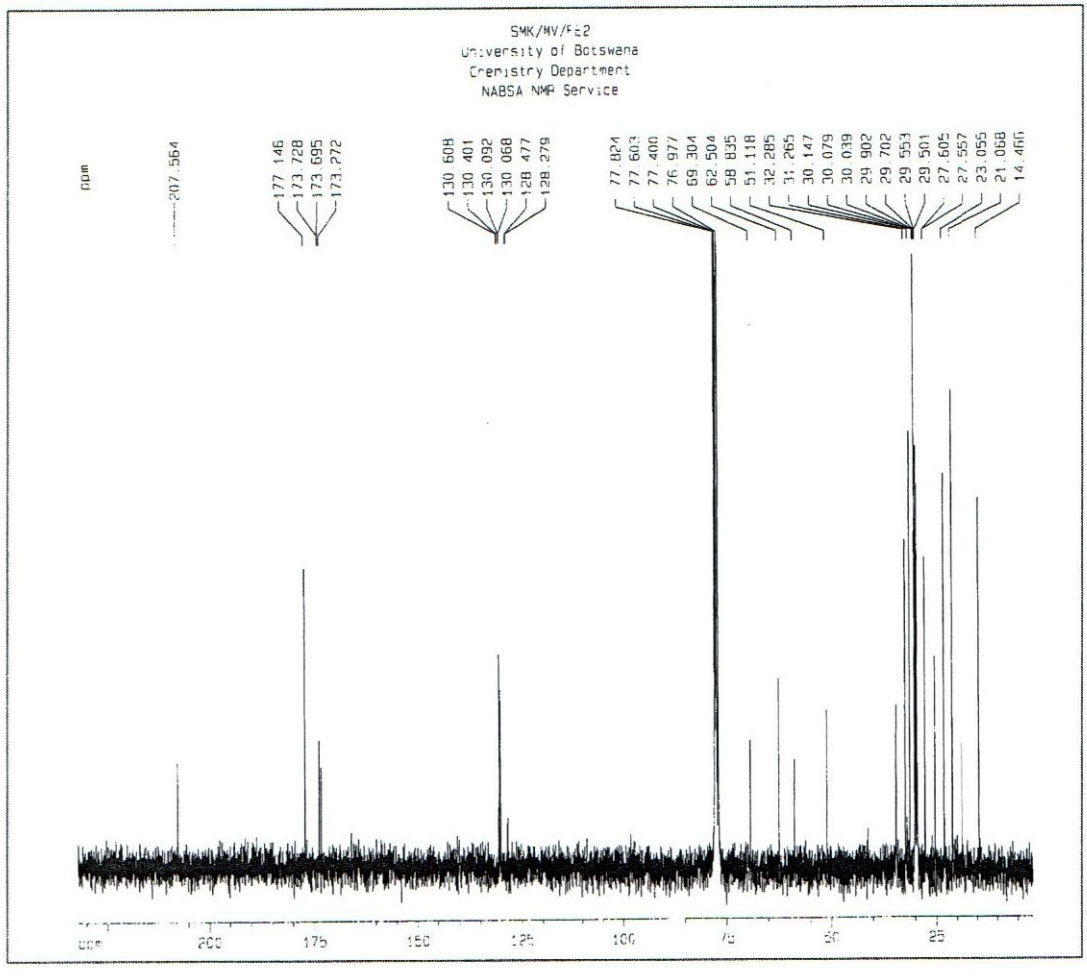
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APPENDICES

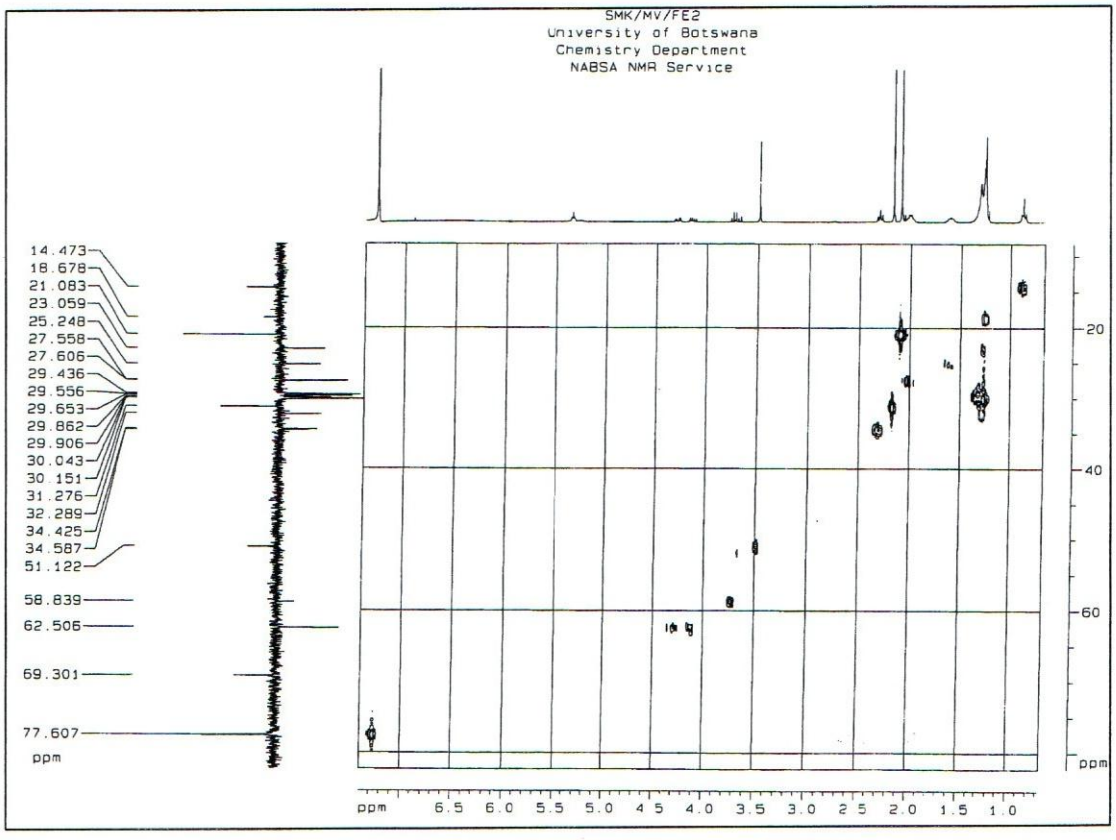
Appendix I: ^{1}H NMR spectrum of (E)-methyl-2-acetyl-2,3-dimethyl-3-((4-(6-oxotetrahydro-2H-pyran-2-yl)but-2-enyloxy)methyl) octanoate, (73)



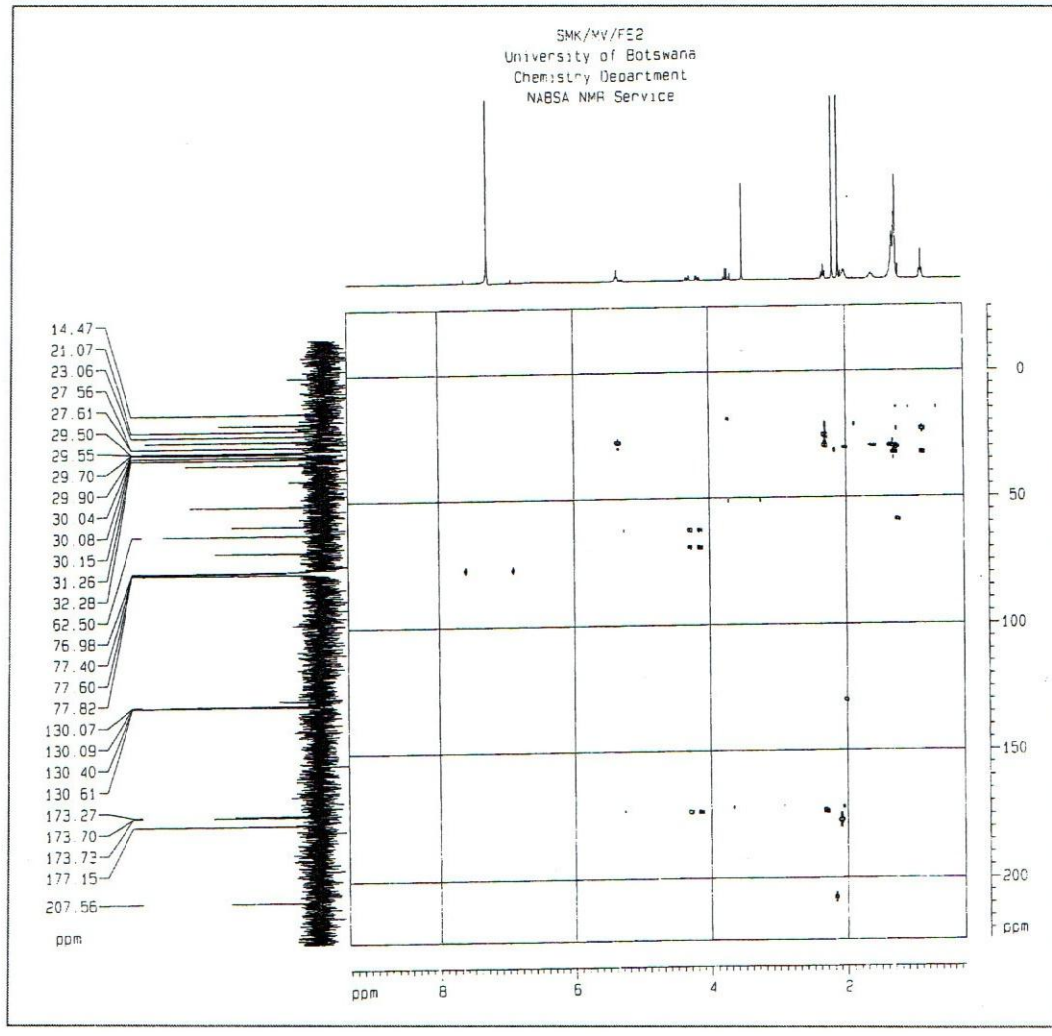
Appendix II: ^{13}C NMR spectrum of (E)-methyl-2-acetyl-2,3-dimethyl-3-((4-(6-oxotetrahydro-2H-pyran-2-yl)but-2-enyloxy)methyl) octanoate, (73)



Appendix III: HMQC spectrum of (E)-methyl-2-acetyl-2,3-dimethyl-3-((4-(6-oxotetrahydro-2H-pyran-2-yl)but-2-enyloxy)methyl) octanoate, (73)

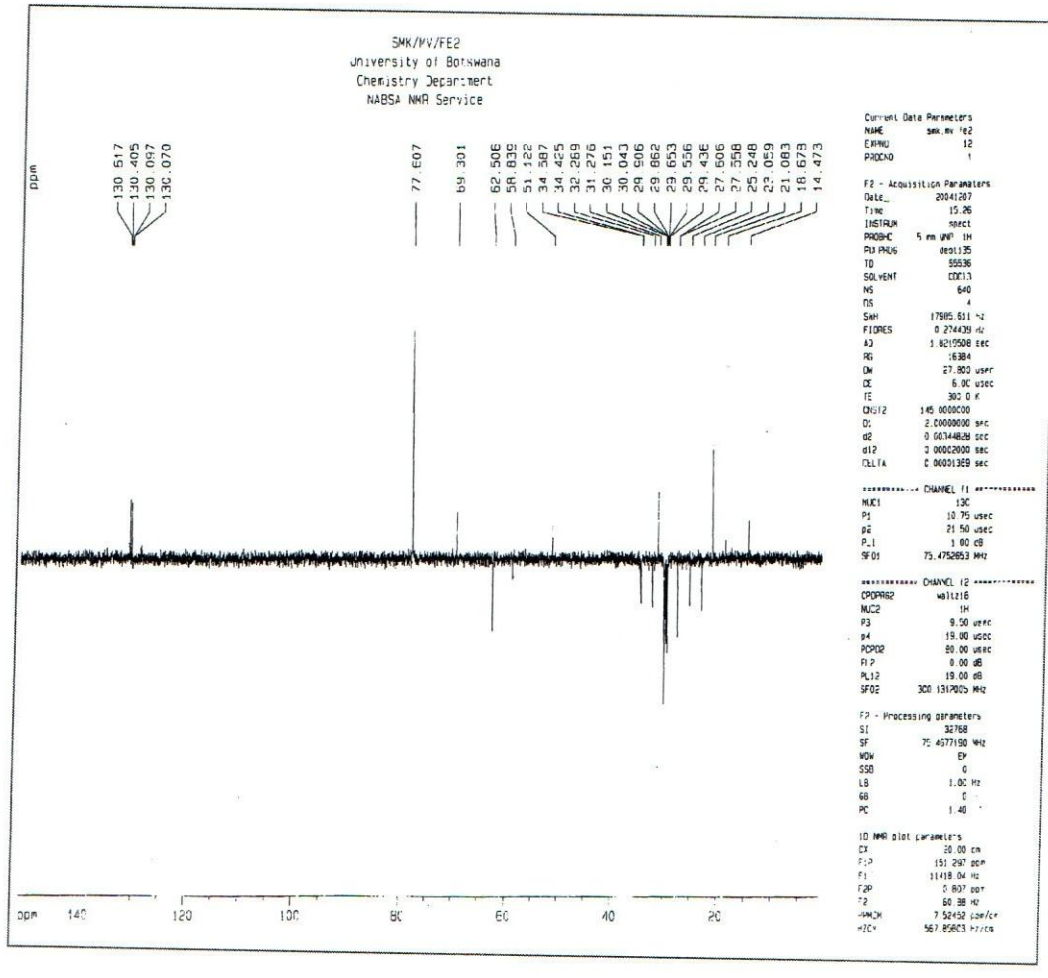


Appendix IV: HMBC spectrum of (E)-methyl-2-acetyl-2,3-dimethyl-3-((4-(6-oxotetrahydro-2H-pyran-2-yl)but-2-enyloxy)methyl) octanoate, (73)

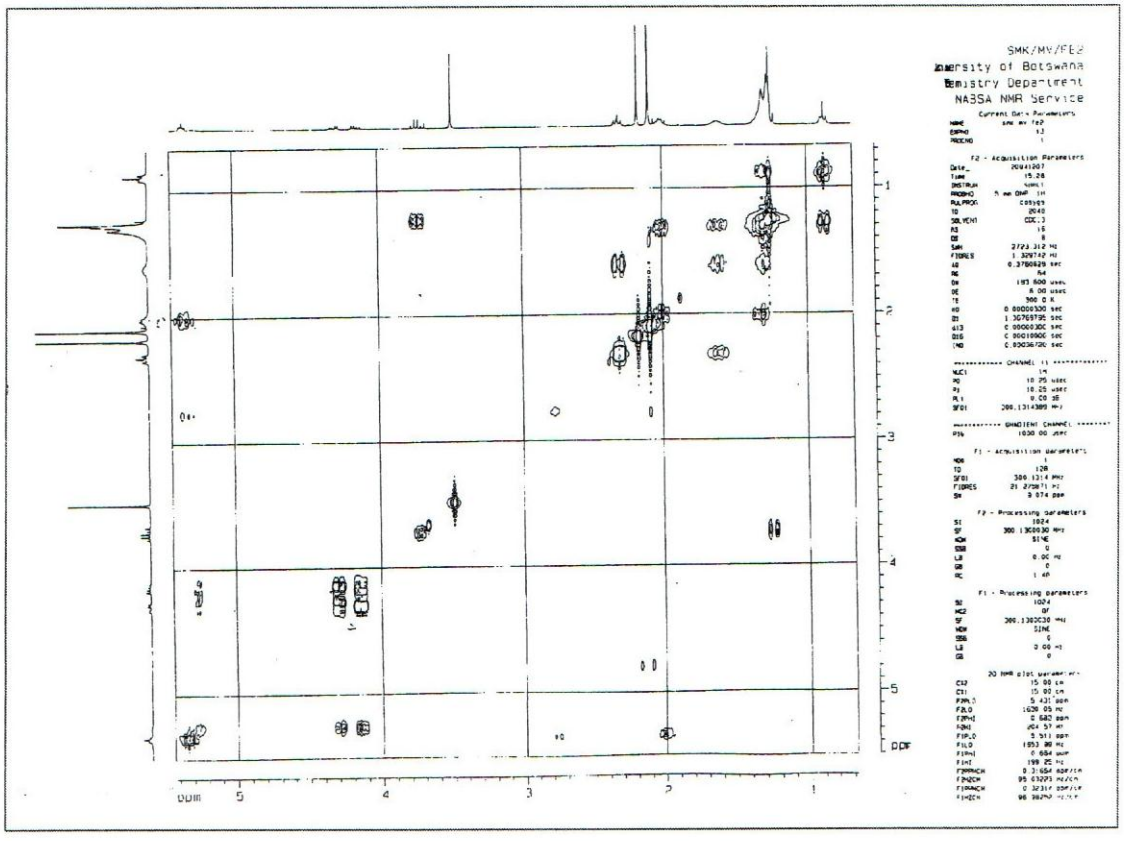


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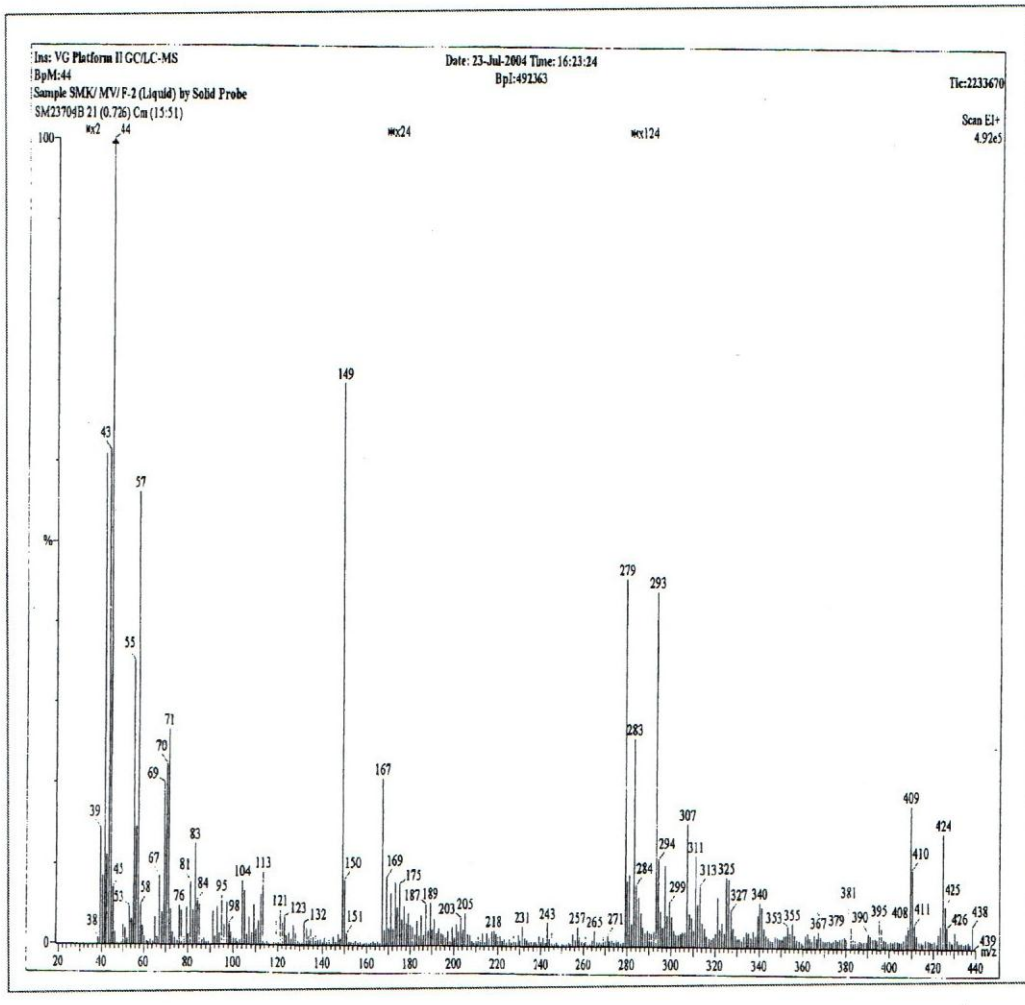
Appendix V: DEPT spectrum of (E)-methyl-2-acetyl-2,3-dimethyl-3-((4-(6-oxotetrahydro-2H-pyran-2-yl)but-2-enyloxy)methyl) octanoate, (73)



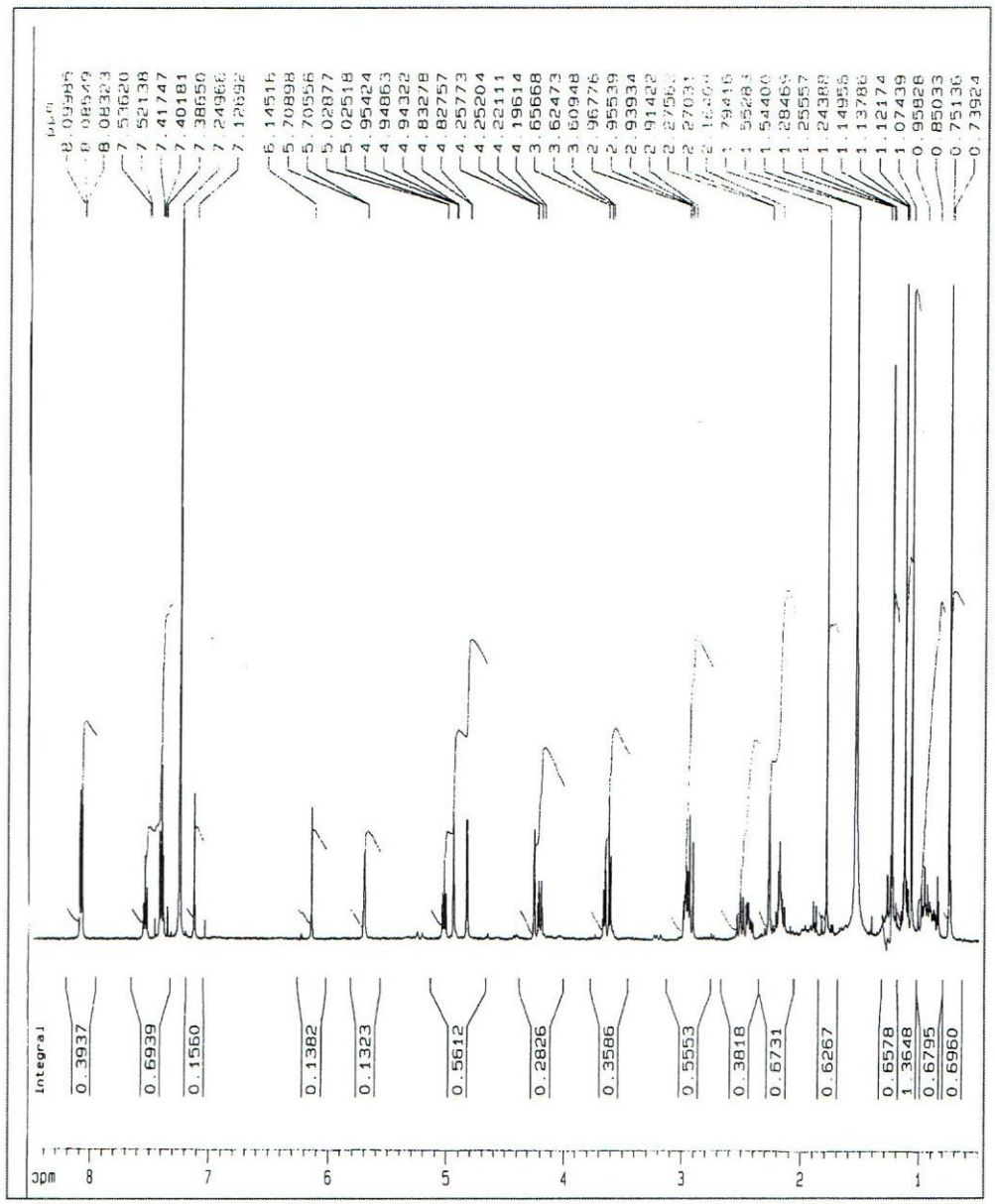
Appendix VI: COSY spectrum of (E)-methyl-2-acetyl-2,3-dimethyl-3-((4-(6-oxotetrahydro-2H-pyran-2-yl)but-2-enyloxy)methyl) octanoate, (73)



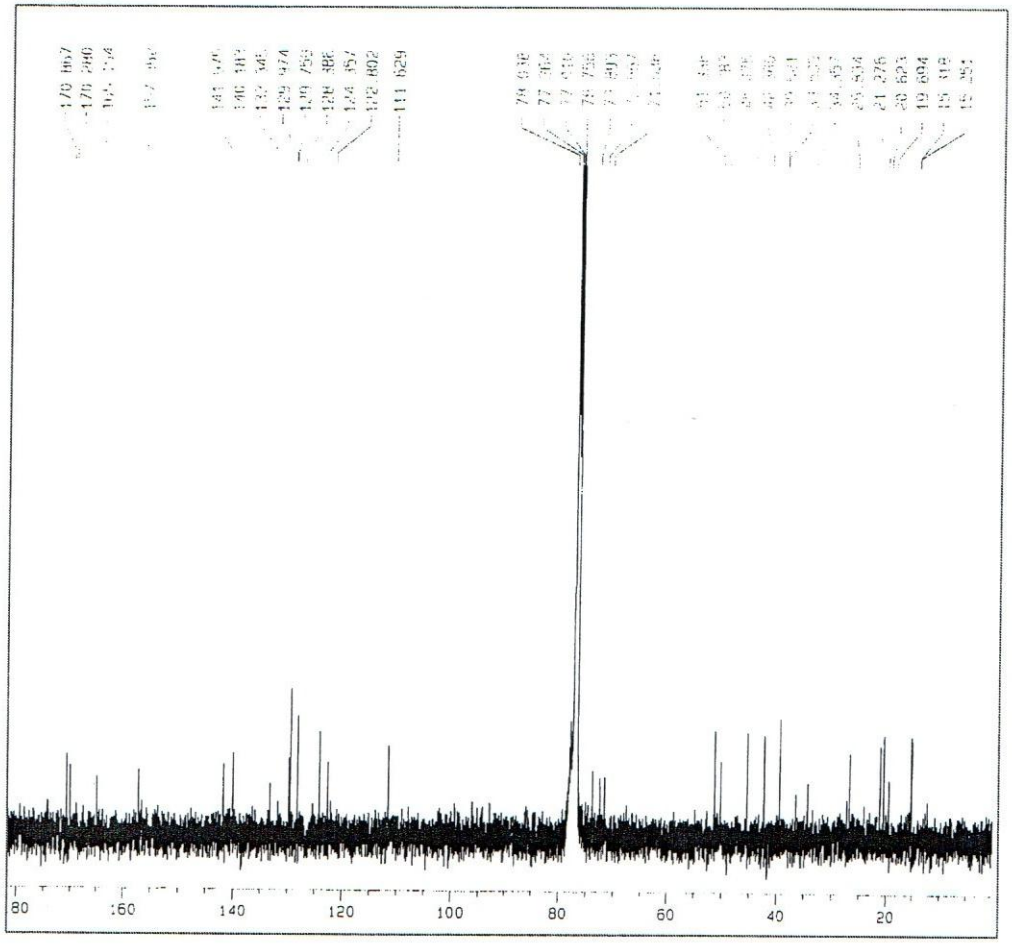
Appendix VII: Mass spectrum of (E)-methyl-2-acetyl-2,3-dimethyl-3-((4-(6-oxotetrahydro-2H-pyran-2-yl)but-2-enyloxy)methyl) octanoate, (73)



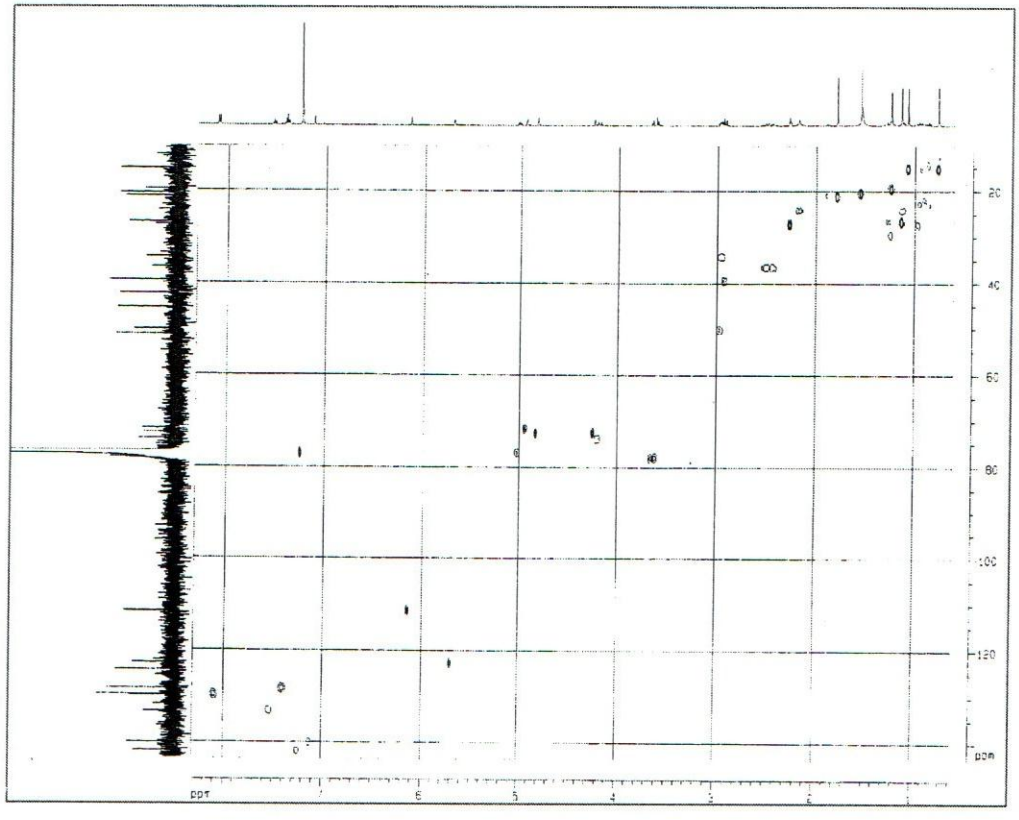
Appendix VIII: ^{1}H NMR spectrum of 1-Benzoylltrichilinin, (74)



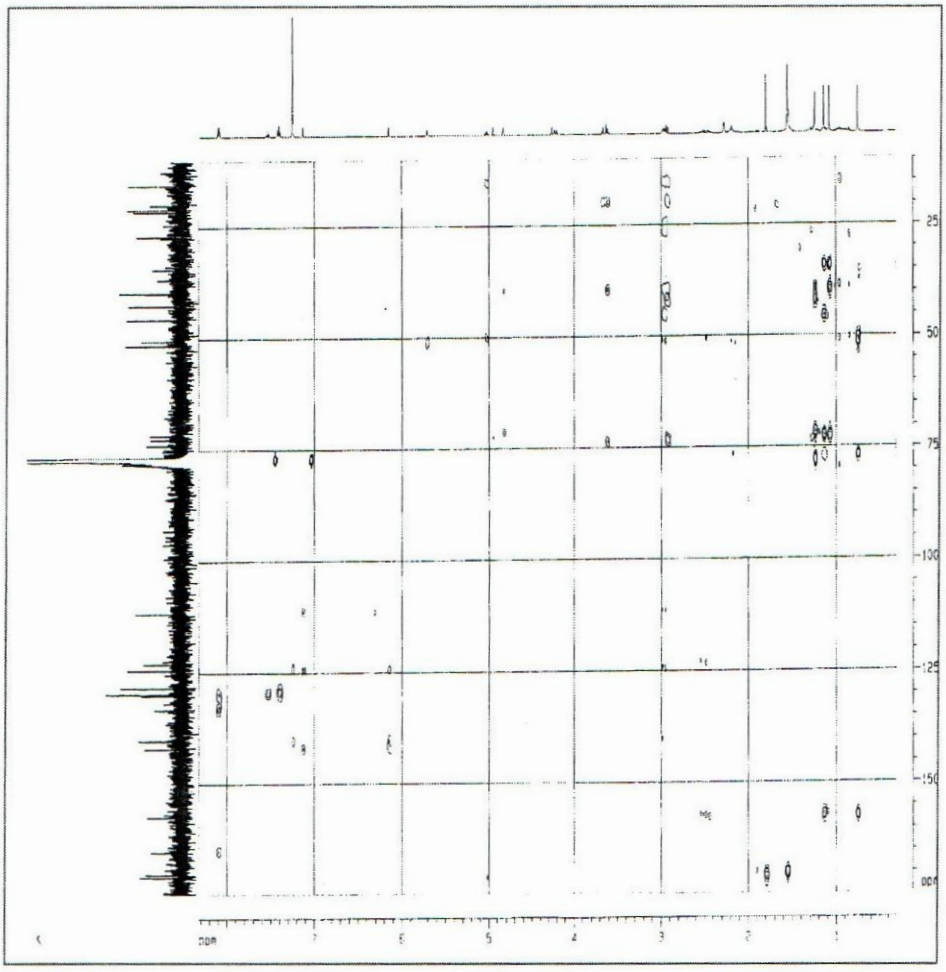
Appendix IX: ^{13}C NMR spectrum of 1-Benzoylltrichilinin, (74)



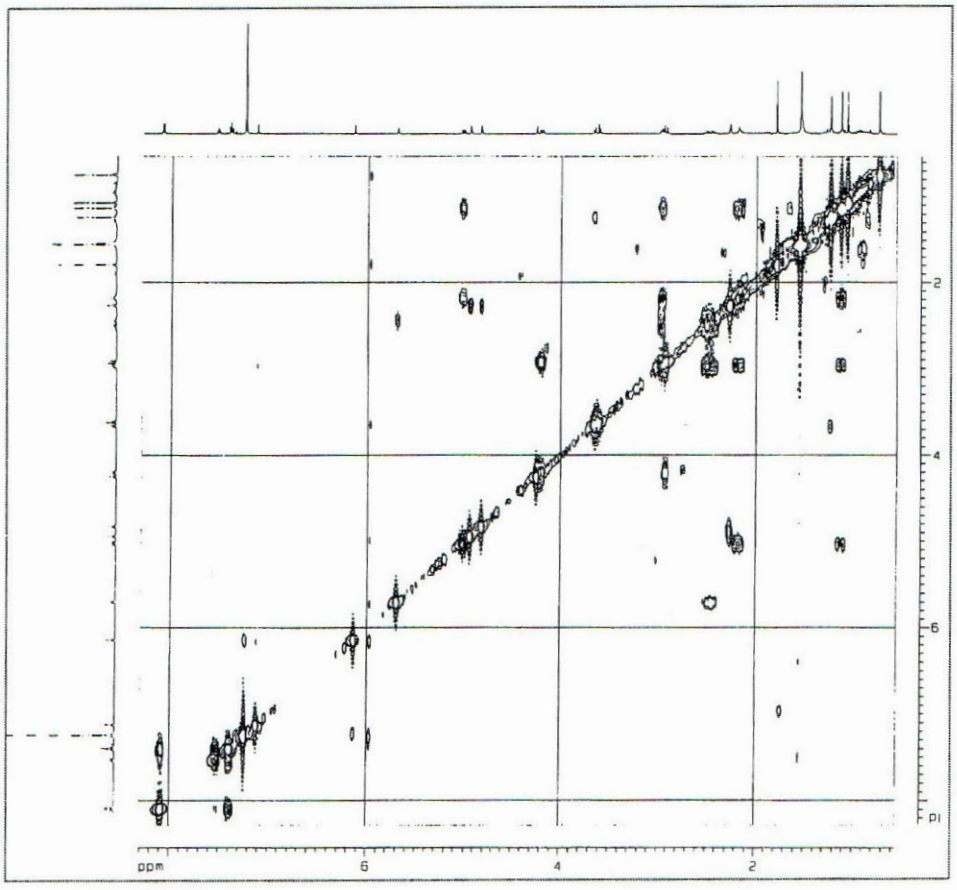
Appendix X: HMQC spectrum of 1-Benzoylltrichilinin, (74)



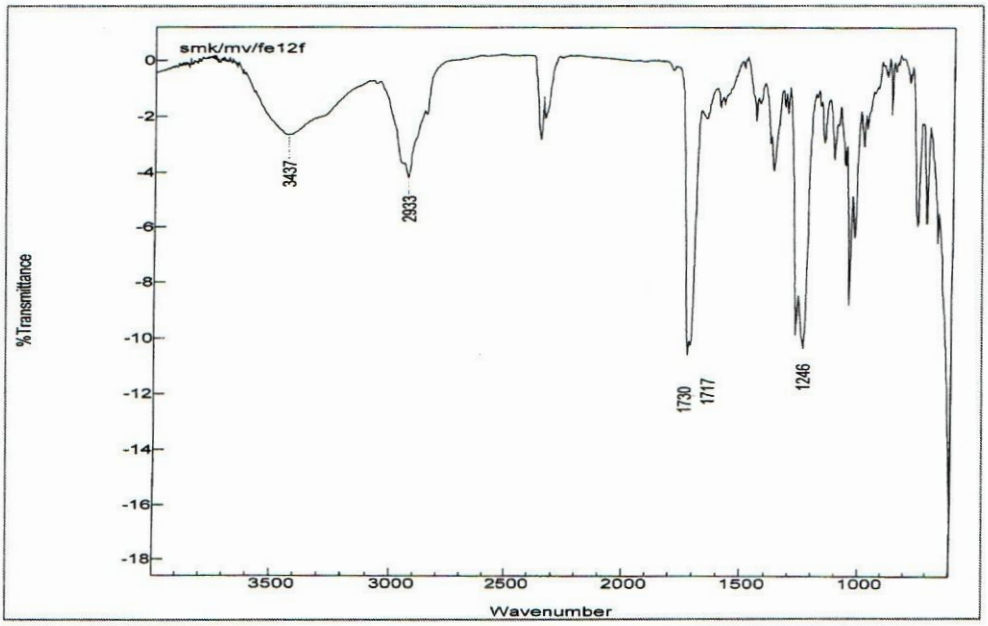
Appendix XI: HMBC spectrum of 1-Benzoylltrichilinin, (74)



Appendix XII: Cosy spectrum of 1-Benzoylltrichilinin, (74)



Appendix XIII: IR spectrum of 1-Benzoylltrichilinin, (74)



Appendix XIV: Mass spectrum of 1-Benzoylltrichilinin, (74)

