ANTI-MOSQUITO TERPENOIDS AND OTHER CONSTITUENTS OF SELECTED TANZANIAN PLANTS

By

Ester Innocent

A Thesis Submitted in Fulfilment of the Requirements for the Degree of Doctor of Philosophy (Chemistry) of the University of Dar es Salaam

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CERTIFICATION

The undersigned certify that they have read and hereby recommend for acceptance by the University of Dar es Salaam, a thesis entitled: '***Anti-mosquito terpenoids and other constituents from selected Tanzanian plants***' in fulfillment of the requirements for the degree of Doctor of Philosophy (Chemistry) of the University of Dar es salaam.

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DECLARATION

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I, **Ester Innocent,** declare that this Thesis is my own original work and that it has not been presented and will not be presented to any other University for a similar or any other degree award.

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DEDICATION

To my beloved husband
CHARLES S.M. KINUNG'HI

our sons
NOEL and FRANK

For their love, support, prayers and patience during the entire period of my studies

ABSTRACT

This Thesis reports on phytochemical, repellency and larvicidal investigations of seven plants species against *Anopheles gambiae s.s.* mosquito. During the studies, structures of seventy six essential oil constituents were identified from *Uvariodendron gorgonis* Verdc., *Clausena anisata* (Willd.) Benth., *Steganataenia araliacea* Hochst., *Suregada zanzibariensis* Verdc. and *Lantana viburnoides* ssp. *viburnoides* var. *kisi* (A. Rich) Verdc. Most of them were tested for mosquito repellent activity. Some essential oils and methyl ketones constituents showed good repellency activity (**Chapter 2**). Extracts from five plant species, namely *Synadenium glaucensen* Verdc., *Kotschya uguenensis* Verdc., *L. viburnoides*, and *S. araliacea* were screened for larvicidal activity. Four extracts, namely dichloromethane extract from the root bark of *L. viburnoides* (LRRD), dichloromethane root bark extract of *S. araliacea* (SARD) and methanol extracts from the root (KURM) and stem barks (KUSM) of *K. uguenensis* showed pronounced larvicidal and insect growth disruption properties (**Chapter 3**). Bioassay guided fractionation of LRRD yielded four series of inseparable mixtures of closely related furanonaphthaquinone derivatives, three triterpenoids, a bis-phenyl peroxide and stigmasterol (**Chapter 4**). Bioassay guided fractionation of SARD yielded four bisbenzocyclooctadiene lactone lignans, two fatty acids and β-sitosterol (**Chapter 5**) while KURM and KUSM yielded unidentified, unstable compounds as active principles, together with a butenyl monoterpene disaccharide (kotside) (**Chapter 6**). Although structures of the unstable compounds could not be established because of instability, they exhibited significant bioactivity, which prompted further bioassay of the extract and powdered plant materials formulations. The formulations (KURP, KUSP, KURME and KUSME) showed good results in reducing mosquito larval and pupal population, with complete mortality occurring in 2-8 days **Chapter 6** and **7**.

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LIST OF ABBREVIATIONS

CC = Column Chromatography EI = Electron Ionization Mass Spectrometry

$CHCl_3$ = Chloroform

CH_2Cl_2 = Dichloromethane

DMSO = Dimethyl Sulfoxide

COSY = Correlation Spectroscopy

^{13}C NMR = Carbon-13 Nuclear Magnetic Resonance

DDT =1,1,1-Trichloro-2,2-*bis*-(*p*-chlorophenyl)ethane

DEET = *N,N*-Diethyl-m-toluamide

DEPT = Distortionless Enhancement by Polarization Transfer

EtOAc = Ethyl Acetate

GC = Gas Chromatography

GC-MS = Gas Chromatography- Mass Spectroscopy

HCH = Hexachlorocyclohexane

HMBC = Proton detected Heteronuclear Multiple Bond Correlation

HMQC = Proton detected Heteronuclear Quantum Coherence

^{1}H NMR = Proton Nuclear Magnetic Resonance

IGR= Insect growth regulators

ITN = Insecticide treated bednet

IR = Infra Red

KURME = Emulsion of the extract from the root bark of *Kotschya uguenensis*

KUSME = Emulsion of the extract from the stem bark of *Kotschya uguenensis*

KURP = Powdered plant materials from the root bark of *Kotschya uguenensis*

KUSP = Powdered plant materials from the stem bark of *Kotschya uguenensis*

LC_{50} = Lethal Concentration to cause 50% response of the test species

m/z = Mass to Charge Ratio

MDA = Mass drug administration

MS = Mass Spectroscopy or Mass spectrum or Mass spectra

MeOH = Methanol

µg/ml =micro-gram per milliliter

Na_2SO_4 –Sodium sulphate

NOESY = Nuclear Overhauser Effect Spectroscopy

ppm = Parts per Million

RBM = Roll back malaria

RC_{50} = Repellency Concentration to cause 50% response of the test species

TLC = Thin Layer Chromatography

TOF MS EI = Time of Flight Electron Ionization Mass spectrometry

UV = Ultra Violet

VLC = Vacuum Liquid Chromatography

WHO = World Health Organization

NOTE: For the list of abbreviations of the extracts which were screened for larvicidal activity see ***Table 3.1*** *on page 66*

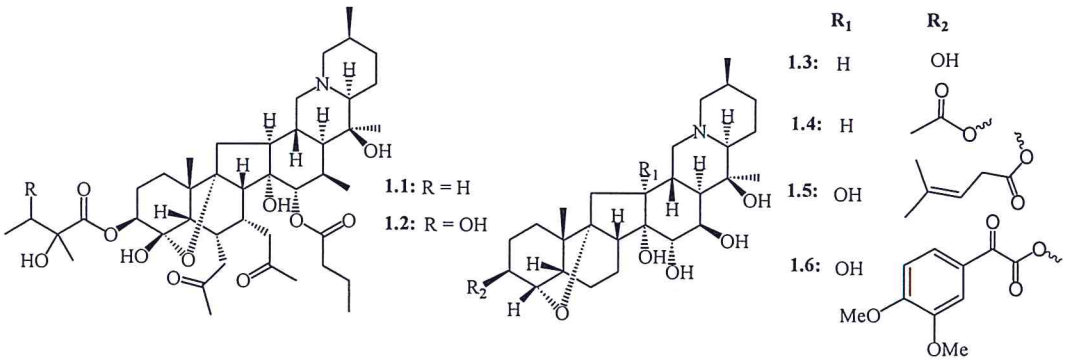
CHAPTER ONE

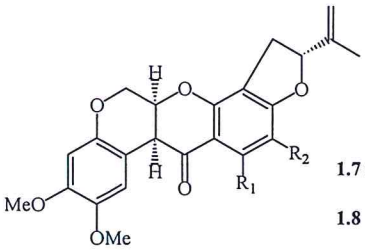
INTRODUCTION AND LITERATURE REVIEW

1.1 INTRODUCTION

1.1.1 Botanical insecticides

From ancient times, different plants have been used for purposes of controlling or managing pests.[1] The Romans used hellebore (*Veratrum album,* Melanthiaceae) as an insecticide while the Chinese used different *Derris* species (Fabaceae) as pesticidal plants. It was not until the development of chromatographic and spectroscopic techniques in the 20th century when it was discovered that *V. album* contains steroidal alkaloids; protoveratrine A (**1.1**), protoveratrine B (**1.2**), germine (**1.3**), zygacine (**1.4**), veratrine (**1.5**) and veratridine (**1.6**). *Derris* species contain rotenoids rotenone (**1.7**), sumatrol (**1.8**), elliptone (**1.9**) and deguelin (**1.10**), all having insecticidal properties.[1-5]

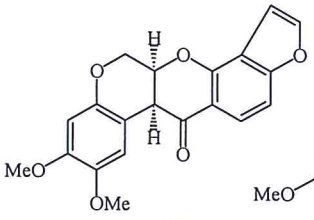




R_1 R_2

1.7: H H

1.8: OH OH



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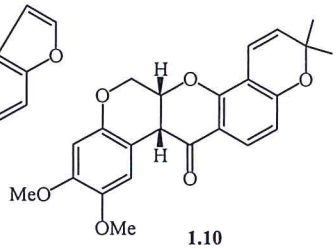


1.10

Ethnobotanical information on the use of plants and other natural resources in insect management is scarcely documented in Africa. However, plant species belonging to several families have been used in Africa and other developing countries for managing insect pests. The most convenient way of obtaining indigenous pest management information is through interviews in the field where the indigenous people depend on traditional methods as the first line solution. Of all known plant families, only about sixty have been reported to have insecticidal properties (**Table 1.1**).[5-8]

Table 1.1: Plant families having insecticidal properties

No.	Plant Family	No.	Plant Family	No.	Plant Family
1	Acanthaceae	21	Ebenaceae	41	Myrsticaceae
2	Agavaceae	22	Ericaceae	42	Myrtaceae
3	Annonaceae	23	Euphorbiaceae	43	Papaveraceae
4	Apocynaceae	24	Flacourtiaceae	44	Piperaceae
5	Araceae	25	Gultiferae	45	Poaceae
6	Aristolochiaceae	26	Helleboraceae	46	Polygonaceae
7	Asclepiadaceae	27	Hippocastanaceae	47	Polypodiaceae
8	Balanitaceae	28	Hypericaceae	48	Ranunculaceae
9	Berberidaceae	29	Illiciaceae	49	Rosaceae
10	Boraginaceae	30	Juglandaceae	50	Rubiaceae
11	Brassicaceae	31	Labiatae (Lamiaceae)	51	Rutaceae
12	Burseraceae	32	Lauraceae	52	Sapindaceae
13	Capparaceae	33	Leguminosae (Fabaceae)	53	Sapotaceae
14	Capparidaceae	34	Liliaceae	54	Simaroubaceae
15	Celastraceae	35	Loganiaceae	55	Solanaceae
16	Chenopodiaceae	36	Lycopodiaceae	56	Stemonaceae
17	Composite (Asteraceae)	37	Magnoliaceae	57	Taxaceae
18	Convolvulaceae	38	Malvaceae	58	Theaceae
19	Cucurbitaceae	39	Meliaceae	59	Umbelliferae (Apiaceae)
20	Dioscoreaceae	40	Menispermaceae	60	Verbenaceae

In recent phytochemical studies of some families such as Annonaceae, Apiaceae, Asteraceae, Cyperaceae, Ebenaceae, Euphorbiaceae, Fabaceae, Lamiaceae, Lauraceae, Meliaceae and Verbenaceae were found to offer great promise as sources of insecticidal compounds.[5-8] The plants under current investigation, *viz. Synadenium glaucenscen* Pax (Euphorbiaceae), *Steganotaenia araliacea* Hochst (Apiaceae), *Lantana viburnoides* subsp. *viburnoides* var. *kisi* (A. Rich) Verdc. (Verbenaceae), *Kotschya uguenensis* Verdc. (Fabaceae), *Clausena anisata* (Willd.) Benth. (Rutaceae), *Uvariodendron gorgonis* Verdc. (Annonaceae) and *Suregada zanzibariensis* Baill. (Euphorbiaceae), belong to some of these families. Although most of them are used traditionally in central Tanzania in traditional medicine and/or as insecticides, there are no insecticidal and phytochemical studies that have been carried out on *S. glaucenscen, S. zanzibariensis, L. viburnoides* and *K. uguenensis*. The leaves of *S. zanzibariensis* have been reported to exhibit antimalarial activity against a chloroquine resistant *Plasmodium falciparum* strain.[9] *Steganotaenia araliacea* exhibit insecticidal activity against *Tribolium castaneum* and *Culex quinquefasciatus.*[10] *Clausena anisata* is traditionally, used to repel mosquitoes and other insects, and is reported to exhibit larvicidal activity against *An. gambiae* mosquitoes.[11-13] In central Tanzania, leaves of *L. viburnoides* are used traditionally for repelling mosquitoes while the aerial parts of *K. uguenensis* are employed in repelling mites. Phytochemical investigations of *U. gorgonis* have been reported and eugenol derivatives were isolated as the main constituents.[14] However, its essential oil composition has not been studied.

Due to their pesticidal properties, the plant species chosen for these investigations were presumed to have constituents responsible for anti-mosquito activities. This study was therefore conceived with the aim of assessing the potency of constituents of the selected plant species as inhibitors of the malaria transmitting mosquitoes at larval and adult stages in order to establish appropriate scientific approaches in using the plant products in minimizing the malaria burden among the communities in central Tanzania and the world at large, in addition to generation of revenue for rural communities in East Africa. Some of the compounds may have unique chemical structures, hence the investigations would contribute to the scientific knowledge about these plant species.

1.1.2 Transmission and control of malaria

1.1.2.1 Malaria transmission

Malaria is a disease of public and economic importance that affects about 40% of the world population mainly in Asia, Latin America, the Middle East, Eastern Europe and Africa. In North America and Europe the disease was eradicated after massive spraying with 1,1,1-trichloro-2,2-*bis*(*p*-chlorophenyl)ethane (DDT).[15] Currently, malaria occurs in about 100 countries, most of them being in Africa South of the Sahara where about 90% of all cases occur.[16]

Malaria, which is transmitted to humans by mosquitoes of the genus *Anopheles,* is a vector born disease caused by a unicellular parasite of the genus *Plasmodium*. According to the World Health Organization (WHO), the disease remains a major killer and ranks first in this regard. It is estimated that, about 300-500 million clinical cases of malaria

are reported worldwide annually.[16] Between 1.5-2.7 million deaths occur annually, the majority of victims being children under 5 years and pregnant women. Thus, in every 40 seconds malaria kills a child.[17]

Plasmodium parasites are specific to vertebrates as hosts and *Anopheles* mosquitoes as vectors. So far, more than 100 species of *Plasmodium* have been described from vertebrates.[15] All the four species that occur in humans, namely *Plasmodium falciparum, P. vivax, P. malariae,* and *P. ovale,* cause human malaria. Although *P. vivax* is prevalent globally, *P. falciparum* is the most deadly species, being responsible for 95% of the malaria deaths worldwide.[15] It often causes severe fever and complications such as anemia, cerebral malaria and even death if diagnosis and appropriate treatment is delayed.[18]

There are about 422 species of *Anopheles* mosquitoes which have been described worldwide. Only 68 species are associated with malaria transmission under natural conditions, and 30 species are the main vectors.[15] The life cycle and life span of the mosquito is largely dependent on the temperature and the type of species. *Anopheles* mosquitoes in tropical, sub tropical and temperate regions, take about 7-14 days to develop from an egg to an adult (**Fig 1.1**).[15,19] The climatic and ecological characteristics of these regions favour survival of the vector and transmission of the disease. The adult *Anopheles* mosquito is an active flying insect, while the eggs, larvae and pupae are aquatic and occur only in water. Within two to three days the eggs hatch into larvae which live in the water for seven to fourteen days, where they develop from first,

second, third and fourth instars. The larvae will only come to the surface of water to breathe and feed on micro-organisms and plants until they develop into pupae. The pupa lives in the water but does not feed. After one to four days the mosquito emerges from the pupal case as a fully developed adult. The adult mosquito then rests on the surface of the water to allow its body to dry and harden before it can fly away. The life-span of a female mosquito may be 3 weeks to several months, during which time she will lay up to 500 eggs in batches of 50-100. She needs a blood meal for ovarian and egg development.[15,19]

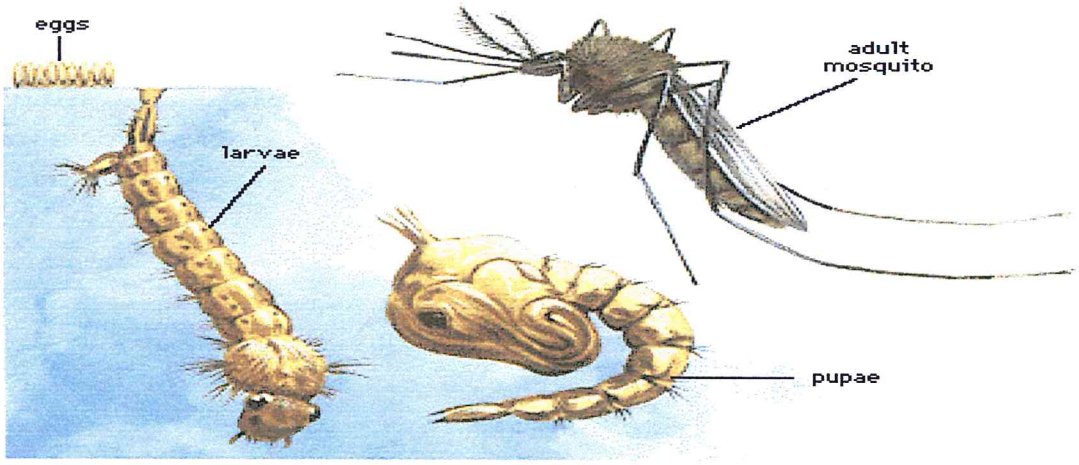


Fig 1.1 Mosquito Life cycle (http://www.westchestergov.com/health/WNVmosquito_life_cycle.htm)

The two important African malaria vectors, namely *Anopheles gambiae* complex and *An. funestus* prefer temperatures of 20-30 °C.[18] *Anopheles gambiae* complex breeds in fresh to brackish water and it is predominant during the rainy season. *Anopheles funestus* breeds in shaded permanent water bodies especially at the edge of slow moving streams, rivers, lakes and in swamps. It is predominant in dry seasons.[18] The

six members of *An. gambiae* complex are morphologically indistinguishable but are known to differ widely in such biological attributes as larval habitat and host preference. The fresh water breeding species and cattle feeder *An. quadriannulatus* Theobald is not a malaria vector.[20,21] Other sibling species include the salt water *An. merus* Donitz and *An. melas* Theobald, *An. bwambae* White, *An. arabiensis* Patton and *An. gambiae s.s* Giles.[20,21] *An. gambiae* s.s and *An. arabiensis* are mostly anthropophilic and endophilic fresh water breeding species although *An. arabiensis* sometimes feed on other animals.[17] *An. gambiae s.s* is the vector associated with stable malaria transmission in Africa because it is strongly anthropophilic and exclusively feeding on humans.[21]

Malaria is transmitted when mosquito feeds on human blood in more than one occasion. This means that, the probability of a mosquito to transmit malaria is related to the product of the two probabilities of an individual vector feeding on humans twice (i.e $p^2=1$).[15] The transmission cycle involves a female *Anopheles* mosquito when sucking blood from an infected person which contains gametocytes of human *Plasmodium* parasite (**Fig 1.2**).[15,16] It transfers the parasite as it sucks blood from a healthy person in the second feeding cycle. *Anopheles gambiae*, which is anthropophilic transmits the parasite even when it is present in low density. This is due to the fact that, the probability of it feeding on humans twice is very high ($p^2=1$).[15]

The transmission cycle is also dependent on the successful sporogonic development of the parasite which is temperature dependant. Cases of malaria do not occur below 16 °C

and above 33 °C and altitudes greater than 2000 m.[22] This is due to the fact that, mosquitoes surviving the parasite incubation period will be few. High rainfall and humidity enhances breeding and transmission.[21,22]

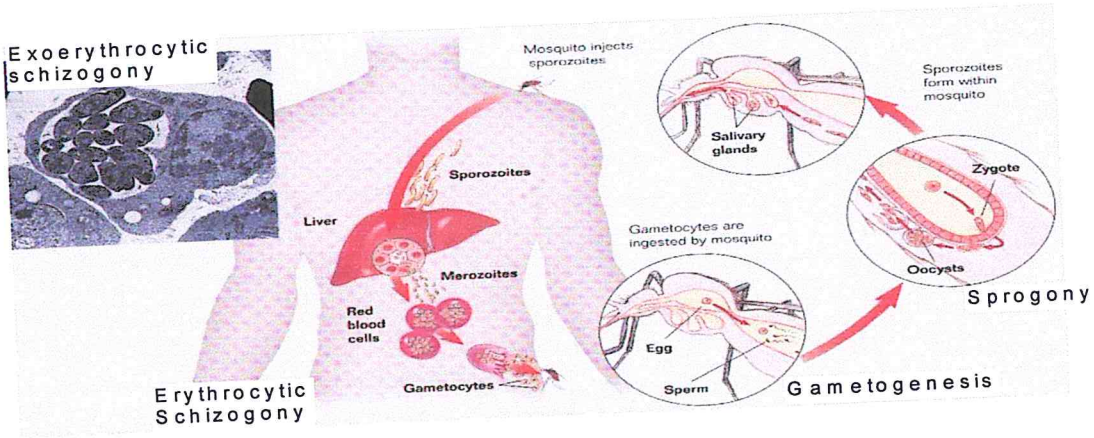


Fig 1.2: Malaria Transmission Cycle (Source: http://www.sirinet.net/~jgjohnso/plasmodium.html)

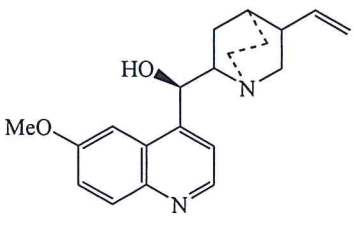
Theoretically malaria control appears simple from the transmission cycle point of view, but in actual practice the approach for the control of malaria is difficult. The cycle involves three organisms of importance, the pathogen, the host vertebrate and the insect vector. From this cycle, malaria can be controlled either directly by eliminating the parasite in the blood stream using anti-malarial drugs or indirectly by eliminating the vector. So far a lot of work has been done to combat the disease, yet no single method has been successful.

1.1.2.2 Control of the malaria parasite

Chemotherapies remove the parasite when already established in the blood stream. The Jesuit missionaries of South America were the first people to learn and use the bark of

Cinchona ledgerian (Rubiaceae) for the treatment of malaria.[23] It was later discovered that the tree contained an anti-malarial agent quinine (**1.11**). Today, quinine (**1.11**) is still a useful drug especially for cerebral malaria although most of its synthetic analogues such as chloroquine and mefloquine have been resisted by *P. falciparum*.[24] Currently, the WHO has recommended the use of Artemisinin Combination Therapy (ACT) as the first line drug [25] Some countries recommend the use of artemisinin (**1.12**) from *Artemisia annua* (Asteraceae) and its derivatives such as artemether and sodium artesunate, but these are expensive drugs and also require long treatment courses. [26,27] In recent endeavors, some synthetic analogues of artemisinine have been prepared and tested including compound OZ 277 (**1.13**) that is currently under clinical trials. [28]

Chemoprophylaxis with chloroquine and fansidar which was used in the past are highly discouraged because of drug resistance.[29] However, chemoprophylaxis is still given to travellers from non-endemic malaria countries, pregnant mothers and children under five years. There is no guarantee that parasites will never develop resistance to the currently available drugs, and therefore a search for new anti-malarial drugs is still needed.



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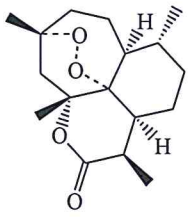

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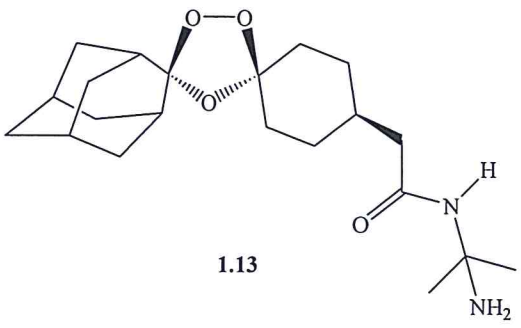


1.13

Vaccination is the best method in disease prevention and control. The three major types of vaccines being developed for malaria are anti-sporozoite vaccines that are designed to prevent infection, anti-asexual blood stage vaccines designed to prevent severe manifestation of the disease and the transmission-blocking vaccines designed to arrest the development of the parasite in the mosquito.[25] So far, attempts to develop efficacious vaccines for worldwide use in humans seem to be difficult. For example, the synthetic vaccine, SPf-66 gave hope when it was tested preliminarily in Morogoro region, in Tanzania, but it did not work in other trials in Gambia.[30] Recent reports on the complete sequence of *P. falciparum* genome and sequencing of the *An. gambiae* genome is expected to open a new era in the control efforts by solving long existing problems of insecticide resistance, antimalarial resistance and identification of potential vaccine antigens.[31]

With these and other reasons, vector control is undoubtly necessary to prevent an epidemic when the conditions leading to a sudden increase in transmission or human exposure have been detected in epidemic-prone and endemic areas. It is often suggested that vector control might be the appropriate for areas where parasite resistance to common anti-malarial drugs poses serious problems in disease treatment due to high selection pressure following massive and incorrect use of anti-malarials.[32]

1.1.2.3 Control of the malaria vector

The three vector control methods, namely environmental management, chemical insecticides and repellents and biological control represent the most important integrated

method in the manipulation of mosquito populations. The wide use of chemical insecticides such as DDT, hexachlorocyclohexane (HCH), dieldrin and lindane is not recommended by the WHO as alternatives to the eradication of mosquitoes since they result in adverse side effects.[16,32] HCH and lindane are not recommended due to resistance developed against them by many insects while dieldrin is toxic to humans.[32] These chemicals which are not environmentally biodegradable have been found to accumulate in the food chains, water bodies and soil, thereby endangering human health and threatening the survival of other non-target organisms.[16,32] However, in recent years, some countries have started using organochlorines for controlling mosquitoes. Furthermore, cross resistance has occurred to most classes of insecticides. For example, *An. gambiae s.s.* has been reported to be resistant to DDT, permethrin and lambdacyhalothrin in West Africa.[33] In Turkey, *An. sacharovi* is resistant to DDT, propoxur, bendiocarb, permethrin, lambdacyhalothrin and cypermethrin.[33] In Indonesia, *Ae. aegypti* is reported as highly resistant to propoxur, bendiocarb and pyrethroids.[33] High levels of multiresistance involving organophosphorus, carbamates and pyrethroids has been reported in *Culex quinquefasciatus* populations in many countries.[33] Resistance is mainly physiological whereby the population of insects can tolerate doses of an insecticide that would be lethal to the majority of the individuals in a normal population of the same species. It develops as a result of the selection pressure exerted by the insecticide.[32] The most important factor in such mechanism is the possession of detoxication enzymes or the modification of the target receptor of the insecticide to prevent it from binding at the site of action. Sometimes behavioural resistance by some

vectors can be developed. In such cases, they avoid contact with an insecticide implying deterrent activity.[32]

1.1.2.3.1 The use of insecticide treated bed-nets (ITNs)

Bednets are currently being used for protection against mosquitoes due to recent discovery of synthetic pyrethroids of relatively low mammalian toxicity but exhibiting significant knockdown effect.[32] Host seeking mosquitoes are attracted by the odour and carbon dioxide emitted by a sleeping person inside the net, thus making the treated net a baited trap. Furthermore, the quick acting insecticide directly in the path of the host seeking mosquito assists the physical barrier of a normal bednet. Pyrethroids are non-carcinogenic, non-teratogenic, non-mutagenic and their volatility is very low. Pyrethroids irritate, repel or kill mosquitoes before they can find a place to bite through the net.[32] That is why insecticide treated nets (ITN's) are reliable and cheaper method to combat malaria.[17] The Abuja declaration of 1999, attended by African heads of state proposed that, at least 60% of the population in Africa should be using ITN's by the year 2010.[17] The aim is to reduce the burden of malaria particularly in pregnant women and children under five years by 50%. ITNs are considered as suitable for personal protection especially when combined with other interventions which are accessible and affordable in preventing malaria infections and sufferings.[17] Though ITN is basically for personal protection, as coverage increases to 100% in the community, the 'mass killing' effect on the vector population may become sufficient to interrupt malaria transmission.

Though the use of ITNs is the method of choice in combating the transmission of malaria, until to date only the pyrethroids have been proved to be safe and effective in treating mosquito nets and curtains.[16,32] The present lack of an alternative class of insecticides for this application is a cause for concern because the emergence of resistance remains a possibility. It was recently reported that, mosquitoes have developed resistance to permethrin, deltamethrin and lambdacyhalothrin, the only pyrethroid insecticides currently used to impregnate bednets and curtains in some areas.[18] ITNs however, give no protection against mosquitoes in the evening before people go to bed.[34]

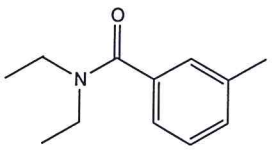
1.1.2.3.2 The use of indoor and outdoor residual spraying of insecticides

Indoor and outdoor residual spraying of organochlorines and organophosphates showed a remarkably fast reduction of mosquito populations to negligible level.[35] However, indoor residual spraying of insecticides cannot be accepted for long time use, it may only be considered as an interim measure in an integrated vector control program. Apart from the adverse effects of organochlorines, it has also been reported that, propoxur which is the main constituent of many organophosphate sprays, produces limited effects on malaria transmission even when combined with mass drug administration (MDA).[35] Currently, pyrethroids are the predominant insecticides for use in residual spraying while organochlorines and organophosphates are less acceptable because of their objectionable odours and effects on non-target organism. In all cases, spraying is

expensive to apply on a large scale for a long time and unaffordable to ordinarily poor people in developing countries .[35]

1.1.2.2.3 The use of mosquito repellents

Personal protection is a common human practice in several certain cultural setting. In rural communities, different practices to deter mosquitoes have been used.[36] These include the use of local materials such as plants or synthetic mosquito repellents. When repellents are applied directly on the skin or released in the air, they turn mosquitoes away before or just after they land, thereby interrupting the landing and feeding behaviour. Mosquito repellents have a unique role in malaria endemic regions, especially in the evening before bedtime. Besides protection against the pain of being bitten, they reduce human vector contact, hence minimize incidences of malaria transmission.[37] *N,N*-diethyl-*m*-toluamide (DEET) (**1.14**), a synthetic insect repellent, has been effective in repelling various insects including mosquitoes.[32]



1.14

In addition to its repellent property, DEET was recently reported to exhibit larvicidal properties against *An. albimanus* and *Ae. albiopictus* under laboratory and field conditions.[32] However, it has been reported to be an irritant when applied to the skin and causing central nervous system (CNC) disturbances when used for a long time.[38,39]

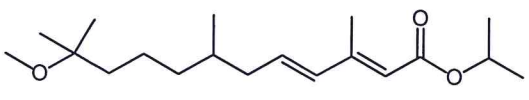
Therefore, in this regard, there is need to search and develop alternative mosquito repellents.

1.1.2.3.4 The use of mosquito larvicides

Large-scale mosquito control with community participation can be achieved by destroying breeding sites, using insect growth regulators (IGRs), chemical larvicides and adult insecticides.[16] Larviciding chemicals include insecticides of biological origin that varying in their mode of action. Insecticides of biological origin are target, specific and environmentally safe. *Bacillus thuringensis* and *B. sphaericus* are examples of bio-larvicides. They produce toxins which are effective in killing mosquitoes and black fly larvae upon ingestion. They are harmless to fish, higher animals and humans at normal dosage but can transmit diseases at high doses.[16,32] However, *B. thuringensis* and *B. sphaericus* have the disadvantage since they are effective only upon ingestion, furthermore, they are heavy and therefore sink into water while Anophelines are surface feeders.

Currently, larviciding practices rely mainly on the use of organophosphate insecticides, despite the increasing level of resistance in some areas. Temephos and fenthion, which have a very low mammalian toxicity are the most widely used mosquito larvicides worldwide. They may be applied to drinking water at dosages not exceeding 1 mg/l (1 ppm) but are toxic to fish.[32] Pyrethroids are not suitable for use as larvicides because of their broad-spectrum activity on non-target arthropods and fish.[32] Insect growth regulators (IGRs) such as methoprene (**1.15**) and diflubenzuron (**1.16**) have been used in

the control of mosquito populations by inhibiting growth at the larval stage. Methropene (**1.15**) which is marketed as Altosid® is designed to disrupt the insect growth cycle at critical development stages while diflubenzuron **(1.16)** is designed to prevent production of substances for the formation of the cuticle, thereby leaving them unprotected. IGRs are effective especially in species which breed in containers, pools, swamps and wells. Most IGRs used are juvenile hormone mimics and chitin synthesis inhibitors. They are environmentally friendly because of their low toxicity to fish and other vertebrates.[16,32] IGRs and larvicides require a method which releases the active ingredients slowly to encounter the target larvae.



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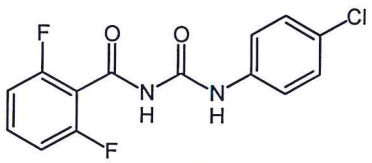


1.16

In any case no single vector control method can be cost effective or even effective in all situations. Therefore, it is logical to assume that a combination of a number of methods will compensate for the deficiencies of each individual method.

There is need to learn much more about malaria before long lasting preventive and curative methods can be assured. The ultimate solution will come through research and development efforts. The International Centre of Insect Physiology and Ecology (ICIPE) has continued to carry out research on the behaviour and chemical ecology of mosquitoes while the Natural Products Research Laboratory at the University of Dar es

Salaam (UDSM) is working on phytochemicals that mediate plant-insect ecological interaction so as to exploit them for monitoring and control of mosquitoes

1.2. RESEARCH OBJECTIVES

The use of plants has been considered primarily as a tradition or a community based way of protection against the malaria vector. This study has been conceived with the aim of assessing the potency of the investigated plant species as inhibitors of the malaria transmitting mosquitoes. The work targets on the control of mosquitoes at larval and adult stages. Therefore, the aim of this study was to identify, isolate, characterize, formulate and test at semi-field, the active compounds exhibiting repellency, larvicidal and insect growth regulation. The scientific information from this investigations would be useful in choosing suitable plant based larvicides and/or repellents that could be easily accessible and affordable in the living environment in rural and peri-urbun populations for managing the malaria vector mosquitoes. Such larvicides and/or repellents would contribute to the development of integrated malaria control programs that already exist.

1.3 LITERATURE REVIEW

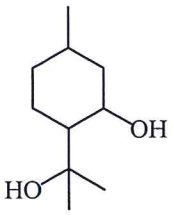
1.3.1. Plants as sources of mosquito repellents

From time immemorial human beings have been using plants for food, treatment of human and animal health disorders, protection of crops and prevention of insects bites. In this regard several plant species have been used to control insects. While few plant

species have been reported for this purpose, many others have neither been documented nor tested for their insecticidal properties. Previous evidence shows that, compounds from plants such as Pyrethrum, Pines, *Ocimum, Eucalyptus* and *Azadrichta indica* repel and/or kill mosquitoes.[37,40-41] Reported plants have been used in different forms such as extracts, smoke from burning materials or in pure natural forms. For example in Nigeria, *Ocimum gratissimum* L. is known as a 'mosquito plant' because it repels mosquitoes and other insects when cultivated near houses, on account of its fragrance.[42] Smoke produced from burning certain aromatic plants such as C*yperus articulatus* L., *Hyptis spicigera* Lam., *Citrus sinensis* L. peel and *Ocimum* species serve to repel mosquitoes.[42,43] In Zimbabwe, a leaf infusion of *Lippia javanica* Spreng and *Ocimum canum* Sims are applied on the skin to control mosquito bites.[44] Herbal smoke produced from burning a mixture of charcoal and plant powder from *Azadirachta indica or Ocimum sanctum* serves as a good repellent and adulticide.[45] In Tanzania, the infusion of *Neorautanenia mitis* is widely used as a mosquitocide and insecticide.[38,46] Most people in the rural communities resort to the use of local materials like plants because they cannot afford the high cost of synthetic repellents and insecticides, some of which are even not easily available.

Repellent properties of many plants are attributed to their volatile compounds. The activities are dependant on the relative amounts of the repellent compounds in the plant and may be species specific. It has been observed that, different strains of mosquitoes may behave differently to the same repellent substance and may be pronounced for different species. For example, the plant based repellent *p*-menthane-3,8-diol (**1.17**), that

was first discovered from waste distillate of essential oils of lemon eucalyptus (*Corymbia citriodora* formerly *Eucalyptus citriodora maculata*, Rutaceae) showed comparable repellency and protection time to DEET against *An. gambiae* and *An. funestus*.[47,48] However, when the oil was tested against *Ae. aegypti,* it exhibited complete protection for only two hours.[47,48] Lemon eucalyptus also contains *iso*-pulegol (**1.18**), citronellal (**1.19**) and citronellol (**1.20**) all of which are mosquito repellents.[47,48]



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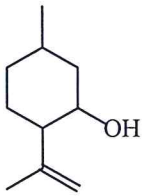

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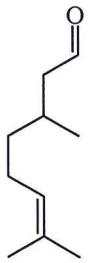

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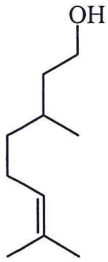
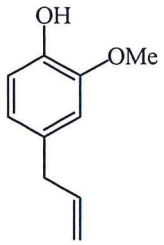


1.20

The oil cloves, *Syzygium aromaticum* L., which is well known as a source of spices and is used also in food storage exhibits good insect repellency. The main constituents of clove oil are eugenol (**1.21**) and β-caryophyllene (**1.22**). These compounds have shown better repellent activity against *An. albimanus* than DEET, but neither repels *Ae. aegypti*.[49] In another study, DEET was more repellent against *An. pulcherrimum*, *An. albimanus* and *An. gambiae s.s.* than to *Ae. aegypti* in laboratory tests.[50] So far, several plant derived insect repellents have been developed and commercialized. Examples of commercial plant derived mosquito repellents include *p*-menthane-3,8-diol (**1.17**), citronellal (**1.19**), eugenol (**1.21**), linalool (**1.23**) and camphor (**1.24**).[38,40-41]



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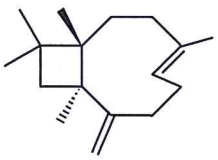

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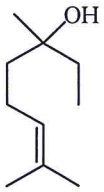

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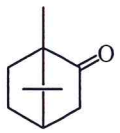
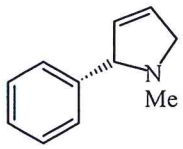


1.24

1.3.2. Plants as sources of larvicides and insecticides

Nicotine (**1.25**) and *nor*-nicotine (**1.26**) from *Nicotiana tabacum* (Solanaceae), and anabasine (**1.27**) from *N. glauca* are effective insecticides and larvicides against a wide range of insects.[5] These compounds kill the larvae and adults of *Culex pipiens* Linn, *C. territans* Walker and *C. quinquefasciatus* Say.[5,51]



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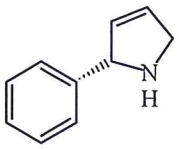

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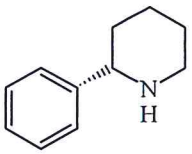
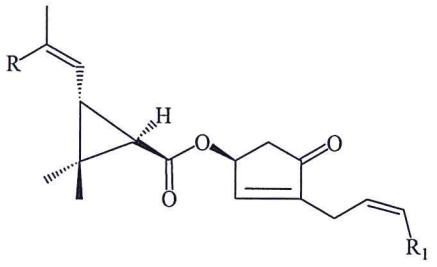


1.27

Pyrethrin is an excito-repellent/insecticidal compound used widely as an alternative to organochlorines, organocarbamates and organophosphates.[52] The active toxicants are six terpenoids extracted from pyrethrum flowers, *Chrysanthemum cinerariafolium* (Asteraceae), namely pyrethrin I (**1.28**), pyrethrin II (**1.29**), cinerin I (**1.30**), cinerin II (**1.31**), jasmolin I (**1.32**) and jasmolin II (**1.33**).[53] Natural pyrethrins are known to act rapidly in killing or immobilizing (knock-down) a wide range of insect species[5] and have low mammalian toxicity under normal circumstances. However, they are highly

unstable in light and are rapidly metabolized thus limiting their potency and application.[54] Due to their rapid metabolism, no resistance has been reported to natural pyrerhrins[5] and only few cases for its analogues.[18]



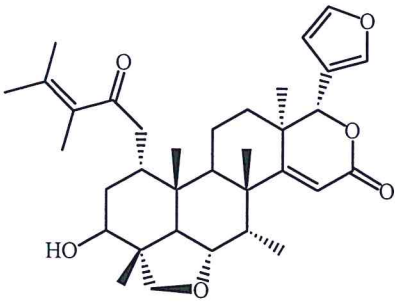
	R	R_1
1.28:	Me	$CH=CH_2$
1.29:	COOMe	$CH=CH_2$
1.30:	Me	Me
1.31:	COOMe	Me
1.32:	Me	Et
1.33:	COOMe	Et

Rotenone (**1.7**) is another potent natural insecticide that is obtained from the roots of *Derris elliptica* Juss and other plant species of the family Fabaceae.[5] *Deris elliptica* originated from India and Indonesia but has been cultivated in the tropics since its roots are a valuable source of rotenone (**1.7**). The compound is a non-systemic contact insecticide and feeding repellent for many vegetable, fruit and cattle pests.[5,55]

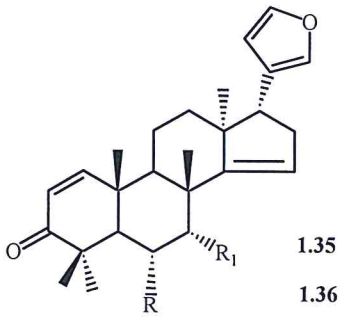
1.3.3 Phytochemical review of other mosquitocidal agents

The contribution of other families in the control of mosquitoes include natural products from the family Meliaceae. The family has been reported to have plant species with insecticidal properties.[56] *Azadirachta indica* A. Juss (Meliaceae) is reported in traditional medicine to have an anti-bacterial, anti-fungal, anti-inflamatory, anti-pyretic, anti-tumour as well as pesticidal properties.[56] Phytochemical investigations of the plant yielded two limonoids, meliacinol (**1.34**) and 6α-*O*-acetyl-7-deacetylnimocinol (**1.35**) from methanolic extracts of the fresh leaves.[56] 6α-*O*-Acetyl-7-deacetylnimocinol (**1.35**)

and nimocinol (**1.36**) which were obtained earlier from the same source, showed toxicity on the fourth instar larvae of *Ae. aegypti* mosquito, with LC_{50} values of 21 and 83 ppm, respectively but meliacinol (**1.34**) had no effect.[57]

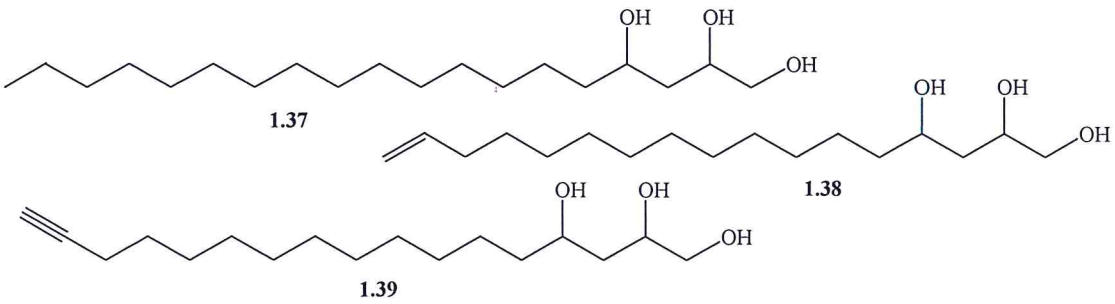


1.34



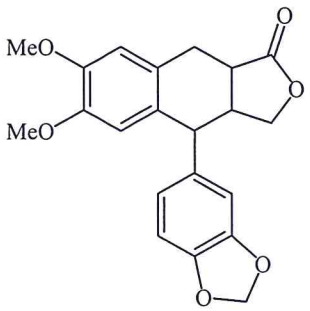
	R	R_1
1.35	:OAc	OH
1.36	: OH	Ac

The family Lauraceae is known to have plant species with insecticidal properties. Thus, phytochemical investigations of unripe avocado fruits (*Persea americana* Miller, Lauraceae) yielded three cytotoxic and insecticidal constituents.[58] These included 1,2,4-trihydroxynonadecane (**1.37**), 1,2,4-trihydroxyheptadec-16-ene (**1.38**) and 1,2,4-trihydroxyheptadec-16-yne (**1.39**). Compound **1.39** was more potent than insecticide rotenone (**1.7**), when tested against the yellow fever mosquito larvae *Ae. aegypti.*[58] The three compounds also had potent activity against six human tumour cell lines with compound **1.39** having selectivity for human prostate adenocarcinoma (PC-3) cells *in vitro*.[58]



1.37 1.38 1.39

The plant species of the family Euphorbiaceae are reported to contain a number of interesting biologically active compounds. *Hura crepitans* (Euphorbiaceae) exhibited 80-100% adulticidal activity[59] while *Croton bonplandianus* (Euphorbiaceae) showed larvicidal activity with LD_{50} values of 0.079 µg/ml against *C. quinquefasciatus*, 0.190 µg/ml against *Ae. aegyptii*, and 0.575 µg/ml against *An. stephensi*.[60] *Jatropha gossypifolia* is known for the treatment of cancer and as an insecticide.[61] Phytochemical investigations the plant yielded the lignan gossypifan (**1.40**) as the main constituent.[61] while a poisonous *Croton hovarum*, yielded two diterpenes 3,12-dioxo-15,16-epoxy-cleroda-13(16),14-dien-9-al (**1.41**) and 3α,4β–dihydroxy-15,16-epoxy-19-*nor*-12-oxo-cleroda-5(10),13(16),14-triene (**1.42**).[62]



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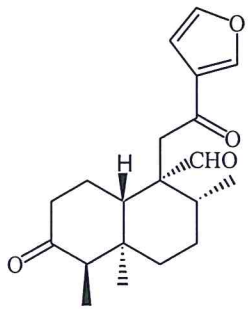

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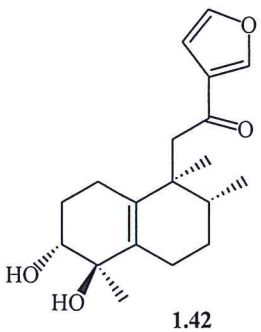
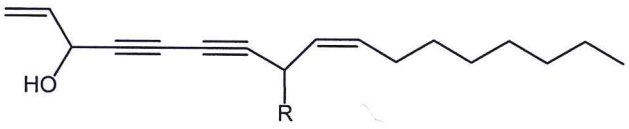


1.42

Cryptotaenia canadensis (Apiaceae) exhibited larvicidal acticvity against *C. pipiens*.[63] Two acetylenic compounds with insecticidal activities, falcarinol (**1.43**) and falcarindiol (**1.44**) were isolated from the roots and fruits of *C. canadensis* (Apiaceae).[63] The compounds were active against *Culex pipiens* larvae, having LC_{50} values of 3.5 and 6.5 ppm, respectively.[63]

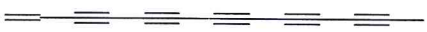


R

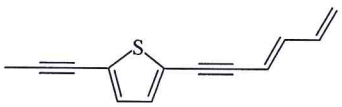
1.43: H

1.44: OH

Three polyacetylene derivatives **1.45-1.47** were isolated from *Rudbeckia hirta* (Asteraceae). The compounds had strong insecticidal activity against mosquito larvae under different light regimes (in dark, visible and visible plus ultraviolet light).[64] Tridecapentaynene **1.45** was highly toxic against *Aedes atropalpus* larvae while thiophine (**1.46**) was highly toxic against *Ae. atropalpus* in the presence of UV/VIS irradiation. Thiarubrine (**1.47**) generated the most lethal activity under both dark and UV/VIS treatments at a concentration of 50 mg/g of diet.[64] Compound **1.47** was previously isolated from *Aspilia mossambicensis* (Asteraceae) and is associated with curative properties when the plant is eaten by chimpanzees.[64]



1.45



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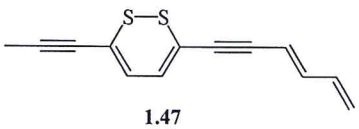
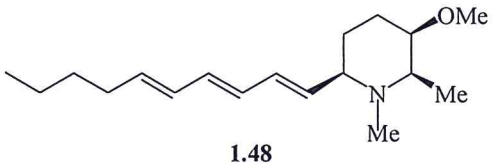


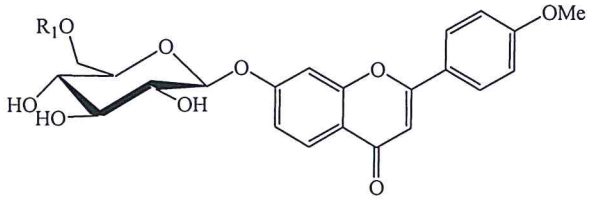
1.47

The insecticidal alkaloid **1.48** was isolated from the stem bark of *Microcos paniculata* (Tiliaceae) and was active against the second instar larvae of *Ae. aegypti* mosquitoes with an LC_{50} of 2.1 ppm.[65]



1.48

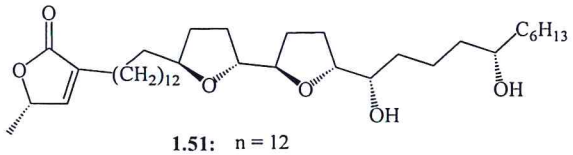
Phytochemical and insecticidal studies of *Latana camara L* (Verbenaceae), was found to be more toxic to *C. pipiens* and *Ae. aegyptii* than to *An. gambiae*.[66] In another investigation, the aerial parts of *Lantana camara* yielded the nematocidal agents lantanoside (**4.49**) and linaroside (**4.50**).[67] The compounds exhibited 85% and 90% mortality, respectively, of the nematode *Meloidogyne incognita* at 1.0% concentration at 24 h post-exposure while at 72 h mortality was 100%,[67]



	R_1
4.49:	Ac
4.50:	H

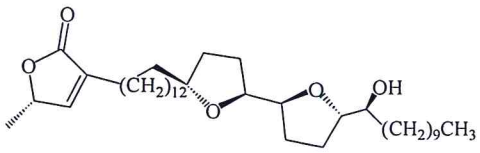
The family Annonaceae is rich in annonaceaous acetogenins such as annonin-1 (**1.51**), neoannonin (**1.52**) and bullatacin (**1.53**) which have been isolated from *Annona squamosa* and *Annona bullata,* respectively.[68] The compounds were shown to be active against the yellow fever mosquitoes, *Ae. aegypti*, whereby 80% of the test insects were killed at 10 ppm.[68] These compounds were also active against cotton aphids (*Aphis*

gossypi) when applied by foliage spraying.[68] They also possess a strong ovicidal and larvicidal activity against the vinegar fly *Drosophila melanogaster*.[68] The insecticidal and other activities of annonaceous acetogenins have been attributed to the ability of these compounds to impair oxidative phosphorylation by interfering the electron transport function of complex I in cell mitochondria.[68]



1.51: n = 12

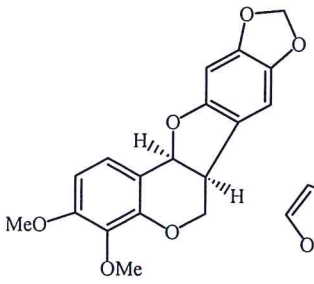
1.52: n = 10



1.53

1.3.4 Insecticidal agents from Tanzanian plants

Tanzania is endowed with a wide range of plant species, of which about 1,200 are endemic and have not yet been investigated for insecticidal activity or for other applications except for a few phytochemical investigations. Recent studies at the Department of Chemistry, University of Dar es Salaam revealed that, extracts and some pure compounds from plants can inhibit the malaria-transmitting vector, *An. gambiae* in different stages of its life cycle. Thus, phytochemical investigations of *Neorautanenia mitis* (Papilionaceae) yielded pterocarpan nepseudin (**1.54**), neoduline (**1.55**), 4-methoxyneoduline (**1.56**) and an isoflavonone neotenone (**1.57**). Compounds **1.54** and **1.55** were found to be active against adult *An. gambiae* mosquitoes having LC_{50} values of 0.005 and 0.003 mg/ml respectively.[46] The mixture of two compounds **1.54** and **1.55** was found to be even more active, thus showing some synergism between them.[46]



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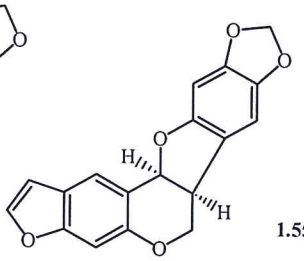
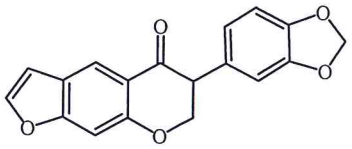


R

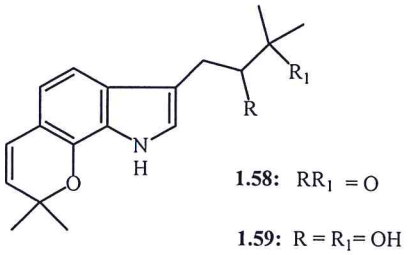
1.55: H

1.56: OMe



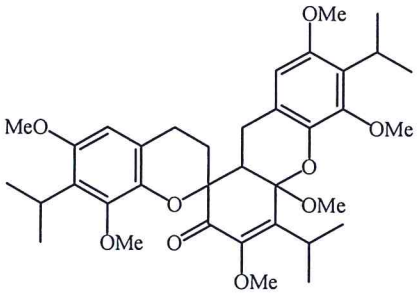
1.57

The pet ether extract of the root bark of *A. lutea* yielded 2',3'-epoxyasteranthine (**1.58**) and 2',3'-dihydroxyasteranthine (**1.59**) which were active against the fourth instar larvae of *An. gambiae* having LC_{50} values of 0.0023 and 0.0775, and 0.0005 and ~ 0.0338 mg/ml after 24 and 48 h. of exposure, respectively.[69] The chloroform extract of the root bark of *U. scheffleri*, gave schefflone (**1.60**) and espintanol (**1.61**). Compound **1.61** was active against *An. gambiae* larvae, having LC_{50} values of 0.9305, 0.0183 and 0.0005 mg/ml after 48, 72 and 96 h., respectively.[69] Espintanol (**1.61**) was found to be the most active against both *Anopheles* mosquito larvae and adults, with LC_{50} values of 0.0164, 0.0049 and 0.002 mg/ml (against larvae) after 24, 48 and 72 h., respectively, while it exhibited a 100% mortality at a dose of 0.012 mg/cm^2 within 24 h. against adult *An. gambiae*.[69]



1.58: $RR_1 = O$

1.59: $R = R_1 = OH$



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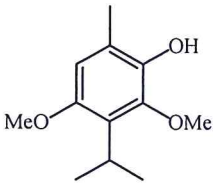
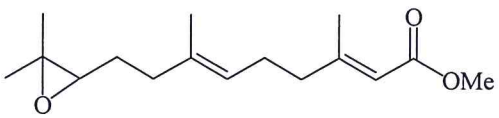


1.61

Juvenile hormone III (**1.62**) was isolated from a monotypic and endemic species *Lettowianthus stellatus* (Annonaceae).[70,71] The compound exhibited mild activity against the K1 and NF54 strain of *P. falciparum*, having IC_{50} value of 29 and 28, respectively.[70] The occurrence in *L. stellatus,* of juvenile hormone III (**1.62**) which is an insect hormone for regulating development and reproduction in insects, is a good example of defense against insects by chemical mimicking. Compound **1.62** was also isolated from *Cyperus iria* and *C. aromaticus* (Cyperaceae).[72]



1.62

The literature review presented in this chapter indicates the great potential of plants as sources of larvicidal, mosquito repellent and mosquitocidal principles and hence the need to investigate plants for such substances is quite appealing, as reported in the forthcoming chapters in this thesis.

CHAPTER TWO

CHEMICAL COMPOSITION OF LEAF ESSENTIAL OILS FROM FIVE PLANT SPECIES AND THEIR MOSQUITO REPELLENCY PROPERTIES

ABSTRACT

This chapter describes the essential oil composition of five plant species and their repellency activity against *Anopheles gambiae s.s.* Gas chromatography (GC) and GC-Mass Spectrometry (GC-MS) analyses of essential oils from leaves of *Uvariodendron gorgonis* Verdc., *Clausena anisata* (Willd.) Benth and *Lantana viburnoides* ssp *viburnoides* var *kisi* (A. Rich.) Verdc., *Steganataenia araliacea* Hochst. and *Suregada zanzibariensis* Verdc. indicated the presence of 76 components. Eugenol (89.82%) and estragol (88.38%) were the major compounds in *U. gorgonis* and *C. anisata* oils, respectively, while *L. viburnoides* ssp *viburnoides* var. *kisi* oil contained piperitenone (25.25%), artemisia ketone (13.96%), limonene (7.80%), linalool (4.15%), *trans*-caryophyllene (4.47%), 1,6,9-tetradecatriene (6.64%) and (+)-verbenone (7.99%) as the major compounds. *Steganotaenia araliacea* contained α-copaene (9.51%), α-armorphene (8.63%), germacrene D (9.33%) and δ-cadinene (9.50%) while *S. zanzibariensis* contained phenylacetaldehyde (14.38%), artemisia ketone (10.13%), (1*S*)-(-)-verbenone (12.08%) and geranyl acetone (9.35%) as the main constituents. The repellency properties (RC_{50} values) of the oils from the five plant species against *Anopheles gambiae* s.s. were 38.3 x 10^{-4}, 56.0 x 10^{-4}, 110 x 10^{-4}, 107 x 10^{-4} and 88.4 x 10^{-4} mg/cm^2 for *U. gorgonis*, *L. viburnoides* ssp *viburnoides* var. *kisi*, *C. anisata*, *S. araliacea* and *S. zanzibariensis* oils, respectively. The prospect of using essential oils from *U. gorgonis*, *L. viburnoides* ssp *viburnoides* var. *kisi* and *S. zanzibariensis* as sources of mosquito repellents is discussed.

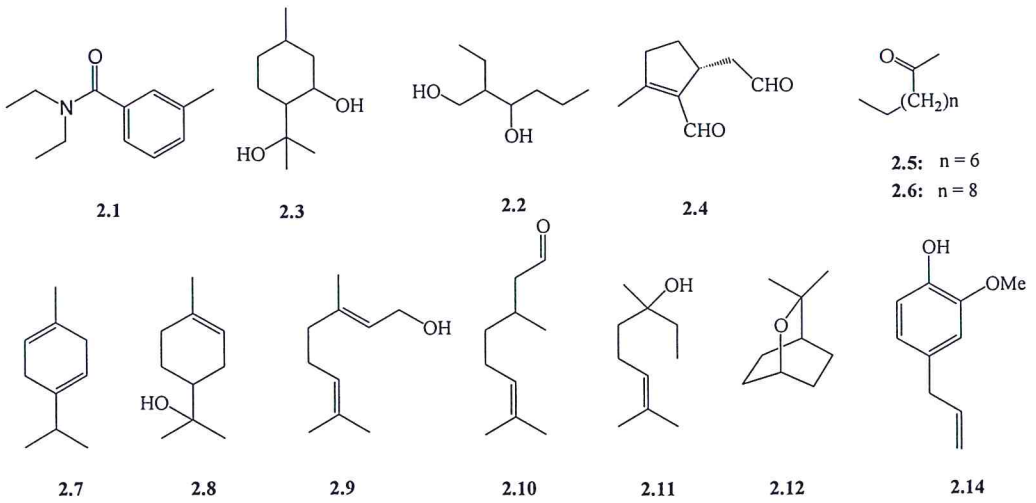
2.1 INTRODUCTION

2.1.1 Synthetic and natural repellents

Many factors play roles in determining the effectiveness of a repellent substance/compound against mosquitoes and other organisms. These factors include among others the frequency and uniformity of application, the species and number of the organisms attempting to bite, and the user's inherent attractiveness to blood-sucking arthropods.[73] Effectiveness of a repellent also depends on volatility of the repellent compound in the formulation. For example, it has been observed that, abrasion by clothing, evaporation and absorption into the skin surface, wash-off by sweat or rain, higher temperatures, or windy environment decrease effectiveness of a repellent.[74] To date, no repellent has been discovered that meets such criteria and thus, there is need to search for a most effective insect repellent, particularly for the control of malaria transmitting mosquitoes.

N,N-Diethyl-*m*-toluamide (DEET) (**2.1**), a synthetic insect repellent, has been shown to be effective against various haematophagus insects including mosquitoes.[37] Its protective efficacy at 40% formulation is about 8 hours. Its effectiveness might be due to low volatility attributed to its high molecular weight compared to components of plant essential oils which are mostly monoterpenes and sesquiterpenes having hydroxyl, aldehyde or epoxide functional groups. Alcohols seem to give good mosquito repellency results than aldehydes and ketones. This might be due to high polarity of alcohols and low volatility compared to aldehydes and ketones. For example, 2-ethyl-1,3-hexanediol

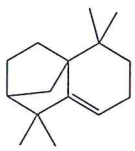
(**2.2**), and *p*-menthane-3,8-diol (**2.3**) have mosquito repellency efficacy comparable to DEET.[51,52] Rotundial (**2.4**) which was isolated from *Vitex rotundifolia* is an example of an aldehyde repellent that is effective against mosquitoes.[41] The compounds 2-undecanone (**2.5**) and 2-tridecanone (**2.6**), which are found in the grandular trichomes of wild tomatoes of the genus *Lycopersicon* (Solanaceae) are repellents towards *C. quinquefasciatus* and *Ae. aegypti* mosquitoes.[75,76] They provided excellent initial repellency, equal to or better than most of the other alternatives to DEET .[75,76] Other common insect repellent compounds from plants include γ-terpinene (**2.7**), α-terpineol (**2.8**), geraniol (**2.9**), citronellal (**2.10**), linalool (**2.11**), camphor (**2.12**), 1,8-cineole (**2.13**) and eugenol (**2.14**).[40,77] They also exhibited insecticidal activity at high concentrations.



2.1.2 Literature review of plant species hereby studied

Essential oil composition and mosquito repellent properties of *Lantana viburnoides* ssp. *viburnoides* var. *kisi* (A. Rich) Verdc. have not been reported in the literature. The aerial

parts of the plant are used locally in Iringa region, Tanzania as mosquito repellents. The essential oil of the related species, *Lantana camara,* contains isolongifolene (**2.15**) (7.79%), δ-cadinene (**2.16**) (6.47%), eudesma-3,11-diene (**2.17**) (7.12%), β-gurjunene (**2.18**) (6.73%), α-selinene (**2.19**) (8.11%), γ-cadinene (**2.20**) (5.59%), δ-selinene (**2.21**) (5.75%), sabinene (**2.22**) (4.51%), α-phellandrene (**2.23**) (3.52%) and α-pinene (**2.24**) (3.02%), and sesquiterpene alcohols as minor constituents.[78] None of the compounds exceeds 10% of the total composition. Monoterpenes account for only 10%, sesquiterpene hydrocarbons 65% and sesquiterpene alcohols 20%.[78]



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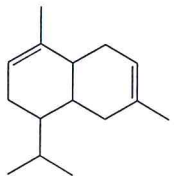

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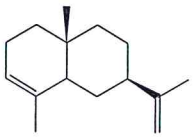

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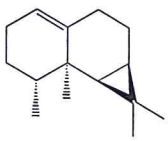

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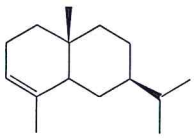

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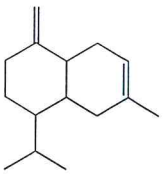

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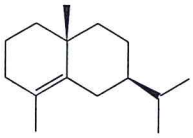

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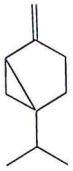

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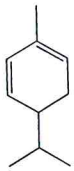

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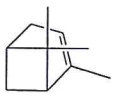
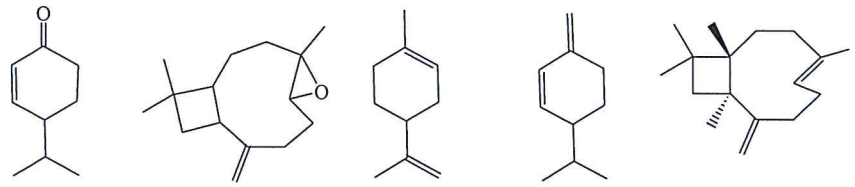


2.24

The essential oil from *Steganatenia araliacea* was analyzed for its chemical composition but no biological activity tests were carried out.[79] The yield of components appeared to vary with seasons and geographical location of collection. For example, cryptone (**2.25**) constituted 16.6% of the essential oil composition of the plant collected from Togo in September but the yield was only 6.7% for the same month from the plant collected from Benin.[79] Caryophyllene oxide (**2.26**) was found in a significant amount (5.70-

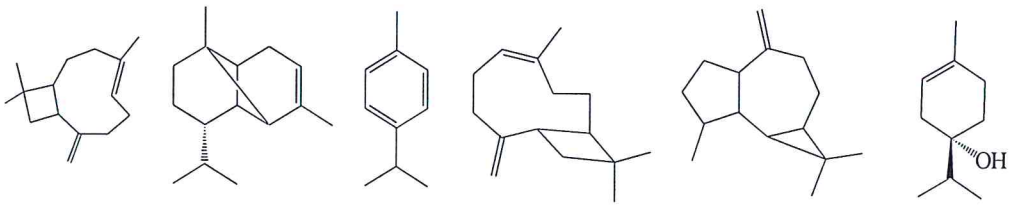
7.02%) in essential oil collected from Togo but the compound was not present in the essential oil of the plant collected from Benin.[79] The major component for the Togo specimen was sabinene (**2.22**) (12.8-24.98%) while that for the plant sample from Benin was limonene (**2.27**) and β-phellandrene (**2.28**) (27.90-35.90%). There was a significant change of the quantities of composition with time. In general, limonene (**2.27**) and β-phellandrene (**2.28**) (11.78-35.90%), sabinene (**2.22**) (9.40-24.98%), β-caryophyllene (**2.29**) (2.00-14.91%), α-pinene (**2.24**) (4.89-11.40%) and cryptone (**2.25**) (3.60-16.60%) were the main constituents.[79] Traditionally, in central Tanzania, *Steganatenia araliacea* plant material is placed near houses to repel snakes.



2.25 **2.26** **2.27** **2.28** **2.29**

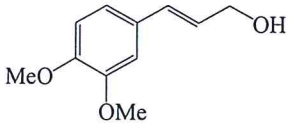
The extract from the stem and root barks of *Clausena anisata* has been shown to exhibit larvicidal activity against *An. gambiae* mosquitoes.[13] Studies of the chemistry and repellent properties of essential oil of *C. anisata* collected from western Kenya revealed promising results against *An. gambiae*,[80] and prompted us to evaluate the plant species from central Tanzania for this property. The essential oil of *C. anisata* collected from western Kenya consisted of limonene (**2.27**) (23.33%) and γ-terpinene (**2.7**) (16.42%) as the main components.[80] Eleven other compounds in the oil were linalool (**2.11**) (2.71%), *trans*-caryophyllene (**2.30**) (2.12%), α-copaene (**2.31**) (0.95%), *p*-cyemene (**2.32**)

(0.93%), isocaryophyllene (**2.33**) (0.92%), (+)-alloaromadendrene (**2.34**) (0.71%), caryopyllene oxide (**2.26**) (0.21%) and terpin-4-ol (**2.35**) (0.26%).[80] The plant is widely used in traditional medicine for repelling ticks and insects, and crop in post-harvest protection of cereals.[12] In central Tanzania the leaves of this plant species are used to repel mosquitoes.



2.30 **2.31** **2.32** **2.33** **2.34** **2.35**

Essential oil composition of *Suregada zanzibariensis* and *Uvariodendron gorgonis* have not been studied. However, phytochemical investigations of less polar components of *U. gorgonis* which grows in Tanzania reported the presence of the phenylpropanoids eugenol (**2.5**), 3,4-dimethoxycinnamyl alcohol (**2.36**), 3,4-dimethoxyphenylprop-2-ene (**2.37**), and dehydrodieugenol (**2.38**) as a trace constituent.[14] The essential oil extracted contained about 90% of eugenol.



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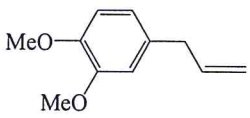

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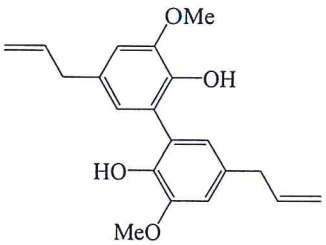


2.38

2.2 RESULTS AND DISCUSSION

2.2.1 Plant materials and extraction of essential oils

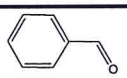
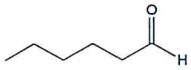
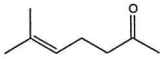
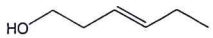
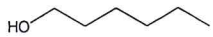
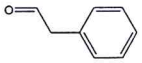
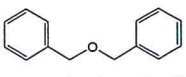
Five plant species, *viz. Uvariodendron gorgonis* Verdc (Annonaceae) (Reference No. LBM 113377), *Clausena anisata* (Willd.) Benth (Rutaceae) (Reference No. FMM 3293), *Lantana viburnoides* ssp. *viburnoides* var. *kisi* (A. Rich) Verdc. (Verbenaceae) (Reference No. FMM 3290), *Steganotaenia araliacea* Hochst. (Apiaceae) (Reference No. FMM 3289) and *Suregada zanzibariens* Verdc. (Euphobiaceae) (Reference No. LBM 10448) were collected from Iringa Region and Pugu Forest Reserve in Tanzania, respectively. Fresh leaves or aerial parts were air-dried and then hydro-distilled over solvent or water to obtain essential oils. The resulting oil was concentrated and dried over anhydrous Na_2SO_4 then stored at -4 °C before further analysis. The yield of essential oil was 0.39% from leaves of *U. gorgonis,* 0.32% from leaves of *C. anisata,* 0.36% from aerial parts of *L. viburnoides* ssp *viburnoides* var *kisi,* 0.08% from leaves of *S. araliacea* and 0.004%. the from leaves of *S. zanzibariensis.*

2.2.2 GC profiles and chemical composition of essential oils

Gas chromatography (GC) and GC-Ms (**Figs 2.1-2.6**) profiles of the essential oil constituents provided clues of the identities of the components (**Table 2.1**) when compared with compounds in the Wiley and NIST MS-library.[81,82] Commercially available compounds were used to positively identify components in the oils through GC-coinjections. GC-MS analysis was used for identification of the remaining

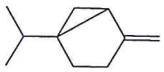
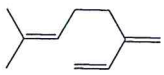
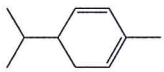
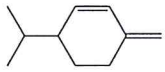
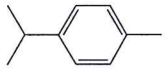
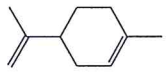
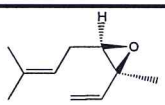
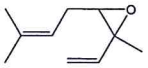
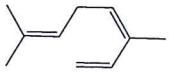
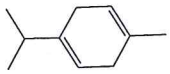
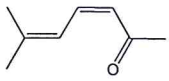
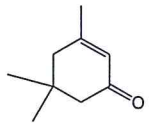
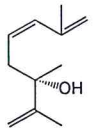
compounds that were not commercially availabe. The relative percentage compositions of the oils were analysed based on the GC-MS detectable peaks with the least component being 0.0001 (0.01%) of the most abundant one in each of the individual essential oil. About 76 compounds were identified from the essential oils of the five plant species and identity of 51 compounds been confirmed by GC-coinjection (**Table 2.1**). The structures of the identified compounds and their respective percentage composition as indicated by the peak areas in the chromatograms (**Figs 2.1-2.6; Tables 2.1**) are presented in the following sub-sections.

Table 2.1 Structures of compounds identified and their percentage composition in each of *U. gorgonis* (*Ug*), *C. anisata* (*Ca*), *L. viburnoides* (*Lv*), *S. araliacea* (*Sa*) and *S. zanzibariensis* (*Sz*) essential oils

Pn[a]		% Area					Pn[a]		% Area				
	Compound	*Ug*	*Ca*	*Lv*	*Sz*	*Sa*		Compound	*Ug*	*Ca*	*Lv*	*Sz*	*Sa*
1	 Pyrrole (**2.39**)	-	-	-	-	0.25	6	 Benzaldehyde (**2.44**)	-	-	-	2.89	-
2	 *N*-Hexanal (**2.40**)	-	-	-		0.31	7	 6-Methyl-5-hepten-2-one* (**2.45**)	-	-	-	2.48	0.15
3	 *trans*-3-Hexenol (**2.41**)	-	-	-	0.58	-	8	 Octanal (**2.46**)	-	-	-	-	1.00
4	 1-Hexanol (**2.42**)	-	-	-	0.83	0.63	9	 Benzene acetaldehyde (**2.47**)	-	-	-	14.38	2.99
5	 Benzyl ether (**2.43**)	-	-	-	-	0.52	10	 α–Pinene (**2.24**)	0.43	0.63	-	-	-

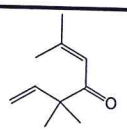
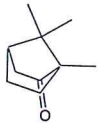
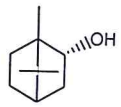
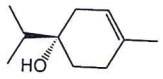
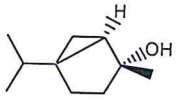
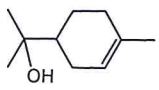
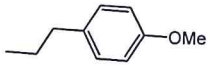
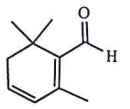
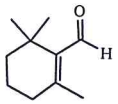
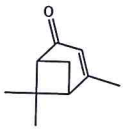
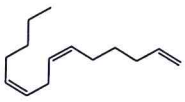
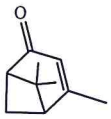
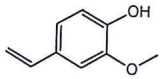
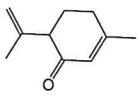
Pn[a] = Peak number; * = except these, all components confirmed by coinjection with authentic sample; # = Isomers not established

Table 2.1 cont.....

Pn[a]	Compound	*Ug*	*Ca*	*Lv*	*Sz*	*Sa*
11	 Camphene (**2.48**)	0.03	-	0.04	-	-
12	 Sabinene (**2.22**)	-	0.42	0.17	-	-
13	 β-Pinene (**2.49**)	0.03	0.30	-	-	-
14	 β-Myrcene (**2.50**)	0.03	0.48	3.66	-	-
15	 α-Phellandrene (**2.23**)	-	0.02	-	-	-
16	 β–Phellandrene (**2.28**)	-	0.04	-	-	-
17	 *p*-Cymene (**2.32**)	-	0.20	0.43	-	-
18	 Limonene (**2.27**)	9.03	0.74	7.80	0.85	0.30
19	 Linalool oxide# (**2.51**)	-	-	-	-	0.42
20	 Linalool oxide# (**2.52**)	-	-	-	0.28	0.27
21	 β–Ocimene (**2.53**)	-	0.08	0.85	-	-
22	 γ̃-Terpinene (**2.7**)	-	4.79	0.46	-	-
23	 (E)-6-Methyl-3,5,heptadien-2-one* (**2.54**)	-	-	-	1.03	-
24	 Linalool (**2.4**)	0.02	0.02	4.15	1.78	-
25	 α-Isophorone* (**2.55**)	-	-	-	0.56	-
26	 Hotrienol* (**2.56**)	-	-	-	-	1.32

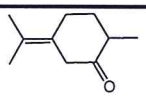
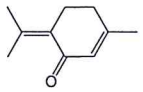
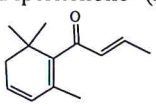
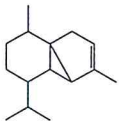
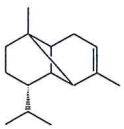
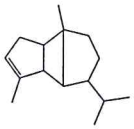
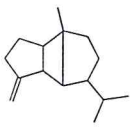
Pn[a] = Peak number; * = except these, all components confirmed by coinjection with authentic sample; # = Isomers not established

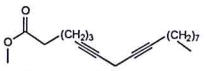
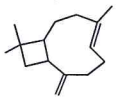
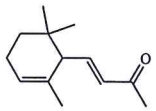
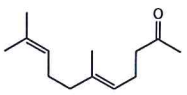
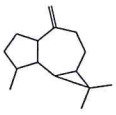
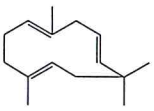
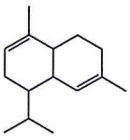
Table 2.1 cont....

Pn[a]	Compound	*Ug*	*Ca*	*Lv*	*Sz*	*Sa*
27	 Artemisia ketone (**2.57**)	-	-	13.96	10.13	2.87
28	 Camphor (**2.11**)	-	-	1.05	0.56	-
29	 Endo-borneol (**2.58**)	0.02				-
30	 Terpinen-4-ol (**2.35**)	-	-	0.83	-	-
31	 *trans*-Sabinene hydrate (**2.59**)	-	-	-	0.41	-
32	 α-Terpineol (**2.8**)	0.22	-	2.50	0.45	-
33	 Estragol (**2.60**)	-	88.38	-	-	-
34	 Safranal* (**2.61**)	-	-	-	0.27	-
35	 β-Cyclocitral* (**2.62**)	-	-	-	0.14	-
36	 (1*S*)-(-)-Verbenone (**2.63**)	-	-	-	12.08	1.47
37	 1,6,9-Tetradecatriene* (**2.64**)	-	-	6.64	2.49	
38	 (+)-Verbenone (**2.65**)	-	-	7.99	-	
39	 4-Vinyl-2-methoxy-phenol* (**2.66**)	-	-	-	1.38	
40	 Isopiperitenone* (**2.67**)	-	-	2.47	-	

Pn[a] = Peak number; * = except these, all components confirmed by coinjection with authentic sample; # = Isomers not established

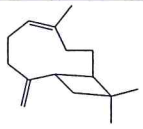
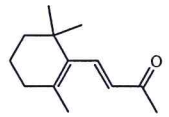
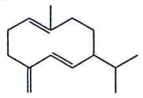
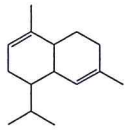
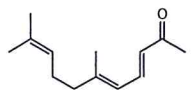
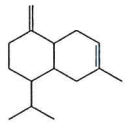
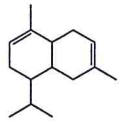
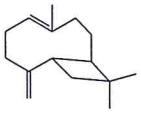
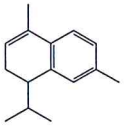
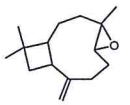
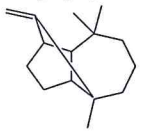
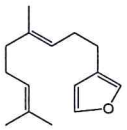
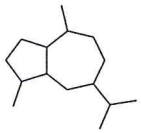
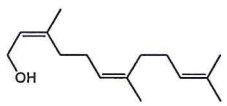
Table 2.1 cont....

Pn[a]	Compound	*Ug*	*Ca*	*Lv*	*Sz*	*Sa*
41	 (+)-Carvone (**2.68**)	-	-	0.21	-	
42	 Piperitenone* (**2.69**)	-	-	25.25	-	-
45	 *Trans*–β-Damascenone* (**2.71**)	-	-	-	2.01	-
50	 α-Cubebene (**2.72**)	-	-	-	-	0.47
51	 α-Copaene (**2.31**)	0.10	-	0.11	0.17	9.51
52	 α-Bourbonene* (**2.73**)	-	-	0.31	-	-
49	 β-Bourbonene* (**2.74**)	-	-	-	0.29	1.05

Pn[a]	Compound	*Ug*	*Ca*	*Lv*	*Sz*	*Sa*
50	 6,9-Octadecadiynoic-methyl ester* (**2.75**)	-	-	-	-	0.76
51	 *Trans*-Caryophyllene (**2.30**)	0.03	0.57	4.47	-	1.47
52	 α-Ionone (**2.76**)	-	-	-	0.93	-
53	 Geranyl acetone (**2.77**)	-	-	-	9.35	-
54	 Alloaromadendrene (**2.34**)	-	-	-	1.31	2.45
55	 α-Humulene (**2.78**)	-	0.85	1.02	0.47	0.61
56	 α-Amorphene* (**2.79**)	-	-	-	-	8.63

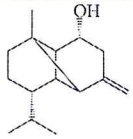
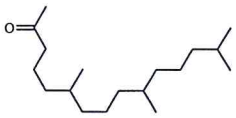
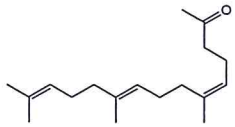
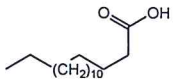
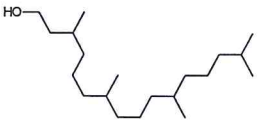
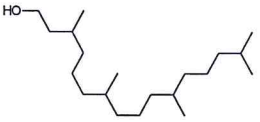
Pn[a] = Peak number; * = except these, all components confirmed by coinjection with authentic sample; # = Isomers not established

Table 2.1 cont....

Pn[a]	Compound	*Ug*	*Ca*	*Lv*	*Sz*	*Sa*
57						
	Isocaryophylene (**2.33**)	-	-	0.50	-	-
58						
	β-Ionone (**2.80**)	-	-	-	0.55	-
59						
	Germacrene-D* (**2.81**)	-	0.67	2.92	1.33	9.33
60						
	α-Muurolene* (**1.82**)	-	-	0.57	-	3.26
61						
	Psedoionone* (**2.83**)	-	-	-	0.95	-
62						
	γ-Cadinene* (**2.20**)	-	-	-	-	3.55
63						
	δ-Cadinene* (**2.16**)	0.02	0.03	0.53	-	9.50
64						
	β-Caryophyllene (**2.29**)	-	-	0.83	-	-
65						
	α-Calacorene*(**2.84**)	-	-	-	-	4.00
66						
	Caryophyllene oxide (**2.26**)	-	-	-	-	1.88
68						
	Junipene* (**2.84**)	-	-	-	-	1.38
71						
	Torreyol* (**2.85**)	-	-	-	-	1.99
72						
	Azunol* (**2.86**)	-	-	-	-	1.57
73						
	Farnesol (**2.87**)	-	-	-	0.79	-

Pn[a] = Peak number; * = except these, all components confirmed by coinjection with authentic sample; # = Isomers not established

Table 2.1 cont....

Pn[a]	Compound	*Ug*	*Ca*	*Lv*	*Sz*	*Sa*
74	 β-Copaen-4-α-ol* (**2.88**)	-	-	0.30	-	1.01
76	 6,10,14-Trimethyl-2-pentadecanone * (**2.89**)	-	-	-	2.26	1.19
77	 Farnesyl acetone (**2.90**)	-	-	-	2.82	0.17
78	 Hexadecanoic acid (**2.91**)	-	-	-	0.88	-
89	 Phytol# (**2.92**)	-	-	-	2.27	0.42
90	 Phytol#(**2.92**)	-	-	-	-	0.76

Pn[a] = Peak number; * = except these, all components confirmed by coinjection with authentic sample; # = Isomers not established

2.2.2.1 Gas chromatography profiles and chemical composition of *Uvariodendron gorgonis* (*Ug*), *Clausina anisata* (*Ca*) and *Lantana viburnoides* ssp. *viburnoides* var. *kisi* (*Lv*) essential oils

Of the total components of the essential oil from *Uvariodendron gorgonis*, 99.78% of the constituents were identified and the major compounds were found to be eugenol (**2.14**) (89.82%) and limonene (**2.27**) (9.03%). Similarly 97.53% of the components of essential oil from *Clausena anisata* were identified with estragol (**2.60**) (88.38%) and γ-terpinene (**2.7**) (4.79%) as major components (**Table 2.1**). Most of the constituents of the essential oils from leaves of *U. gorgonis* and *C. anisata* were monoterpenoids which

constituted 99.63% and 95.41%, respectively of all the constituents of the essential oils. From the essential oil from *L. viburnoides* ssp. *viburnoides* var. *kisi,* 91.08% of the components were identified and sesquiterpenoids accounted for 11.72% while the rest were monoterpenoids. Piperitenone (**2.69**) (25.25%), artemisia ketone (**2.57**) (13.96%), limonene (**2.27**) (7.80%), linalool (**2.11**) (4.15%), *trans*-caryophyllene (**2.30**) (4.47%), 1,6,9-tetradecatriene (**2.64**) (6.64%) and (+)-verbenone (**2.65**) were the major compounds in the essential oil.

Previous studies on the composition of the essential oil from *C. anisata* collected from western Kenya and Nigeria gave different results.[11,80] Thus, while the essential oil composition of the plant collected from western Kenya was found to have limonene (**2.27**) (23.33 %) and γ-terpinene (**2.7**) (16.42 %) as the main components,[80] that from Nigeria contained estragol (**2.60**) as the major compound.[11] In the current investigations the essential oil from *C. anisata* collected from central Tanzania was found to consist of estragol (**2.60**) and γ-terpinene (**2.7**) as the main constituents, this being in agreement with results reported from the Nigerian essential oil.[11] Different results on the composition of the essential oil collected from Kenya, Nigeria and Tanzania may be due to either seasonal or geographical variations. On the other hand, although the essential oil from *C. anisata* collected from central Tanzania showed low repellent effects against *An. gambiae,* the oil from western Kenya showed promising repellency to *An. gambiae* mosquitoes.[80] The variation in the repellent properties of the oils from Kenyan and Tanzanian plant materials may be explained by the difference in the chemical composition.

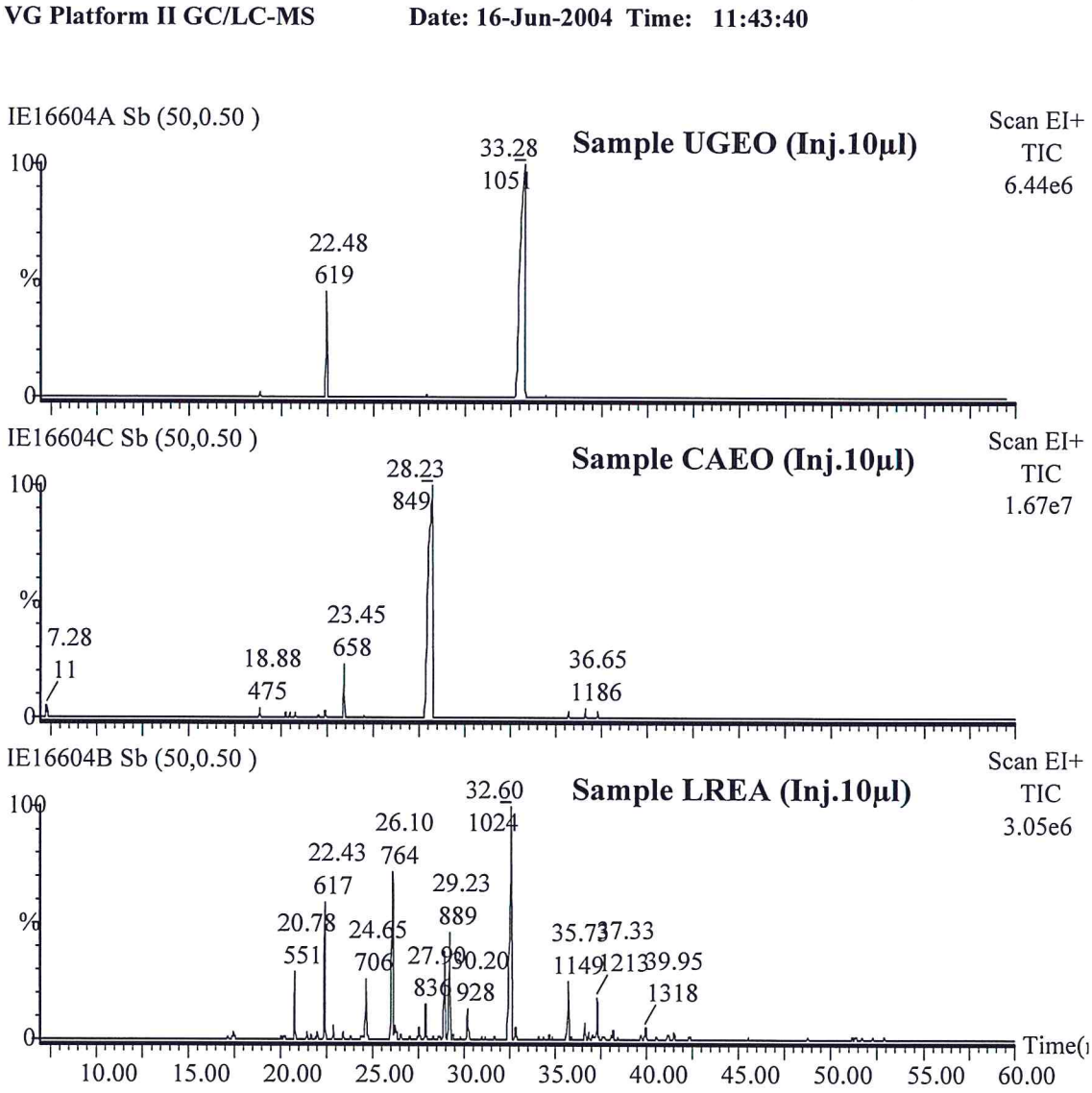


Fig 2.1 **GC-MS profiles of *Uvariodendron gorgonis (Ug), Clausena anisata (Ca)* and *Lantana viburnoides ssp. viburnoides* var. *kisi (Lv)* essential oil**

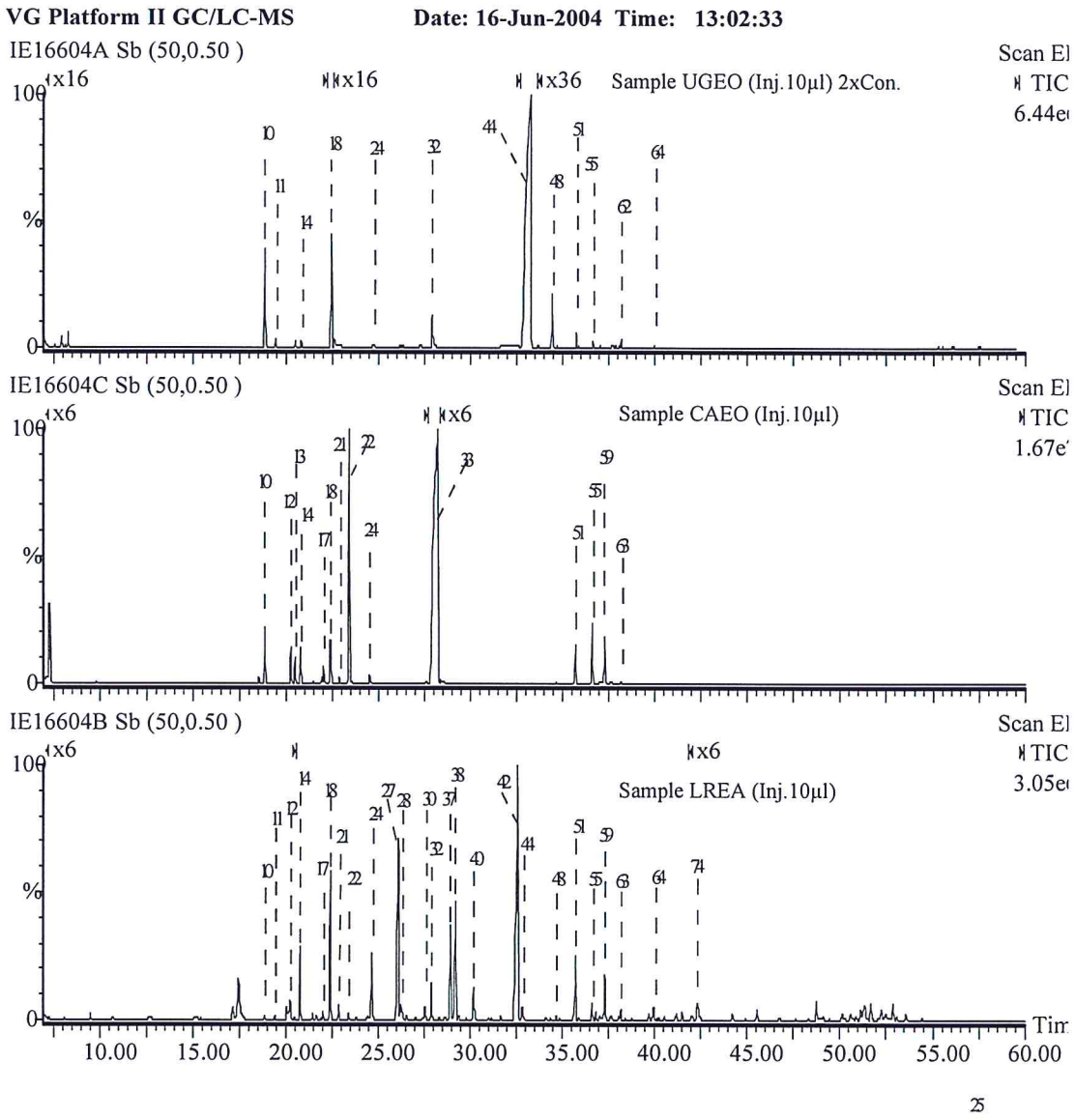


Fig 2.2 **Magnified sections of GC-MS profiles of *U. gorgonis* (*Ug*), *C. anisata* (*Ca*) and *L. viburnoides* ssp. *viburnoides* var. *kisi* (*Lv*) essential oils showing peak labels corresponding to compounds in Table 2.1**

2.2.2.2 Gas chromatography profiles and chemical composition of *Steganotania araliacea* (*Sa*) essential oil

The chemical composition of the essential oil from *Steganotaenia araliacea* was also analysed in these investigations, whereby 85.03% of the components were identified. About 68.07% (**Table 2.1**) were sesquiterpenoids consisting of α-copaene (**2.31**) (9.51%), α-armorphene (**2.79**) (8.63%), germacrene D (**2.81**) (9.33%) and δ-cadinene (**2.16**) (9.50%). Monoterpenoids accounted for 8.76% of the total essential oil constituents while 5.85% were other hydrocarbons.

These results differ from previous reports on the composition of the essential oil from *S. araliacea* collected from Togo and Benin[79] where sabinene (**2.22**) was the major constituent and accounted for 12.8-24.98% of the essential oil from Togo. On the other hand the *S. araliacea* essential oil from Benin contained limonene (**2.27**) and β-phellandrene (**2.28**) as the main constituents, at 27.9-35.9 % of the total essential oil.[79] In general, both the essential oil from Togo and Benin contained limonene (**2.27**) and β-phellandrene (**2.28**) (11.78-35.90%), sabinene (**2.22**) (9.40-24.98%), β-caryophyllene (**2.29**) (2.0-14.91%), α-pinene (**2.24**) (4.89-11.40%) and cryptone (**2.25**) (3.6-16.6%) as the main components.[79]

VG Platform II GC/LC-MS **Date: 18-Jun-2004 Time: 13:11:41**

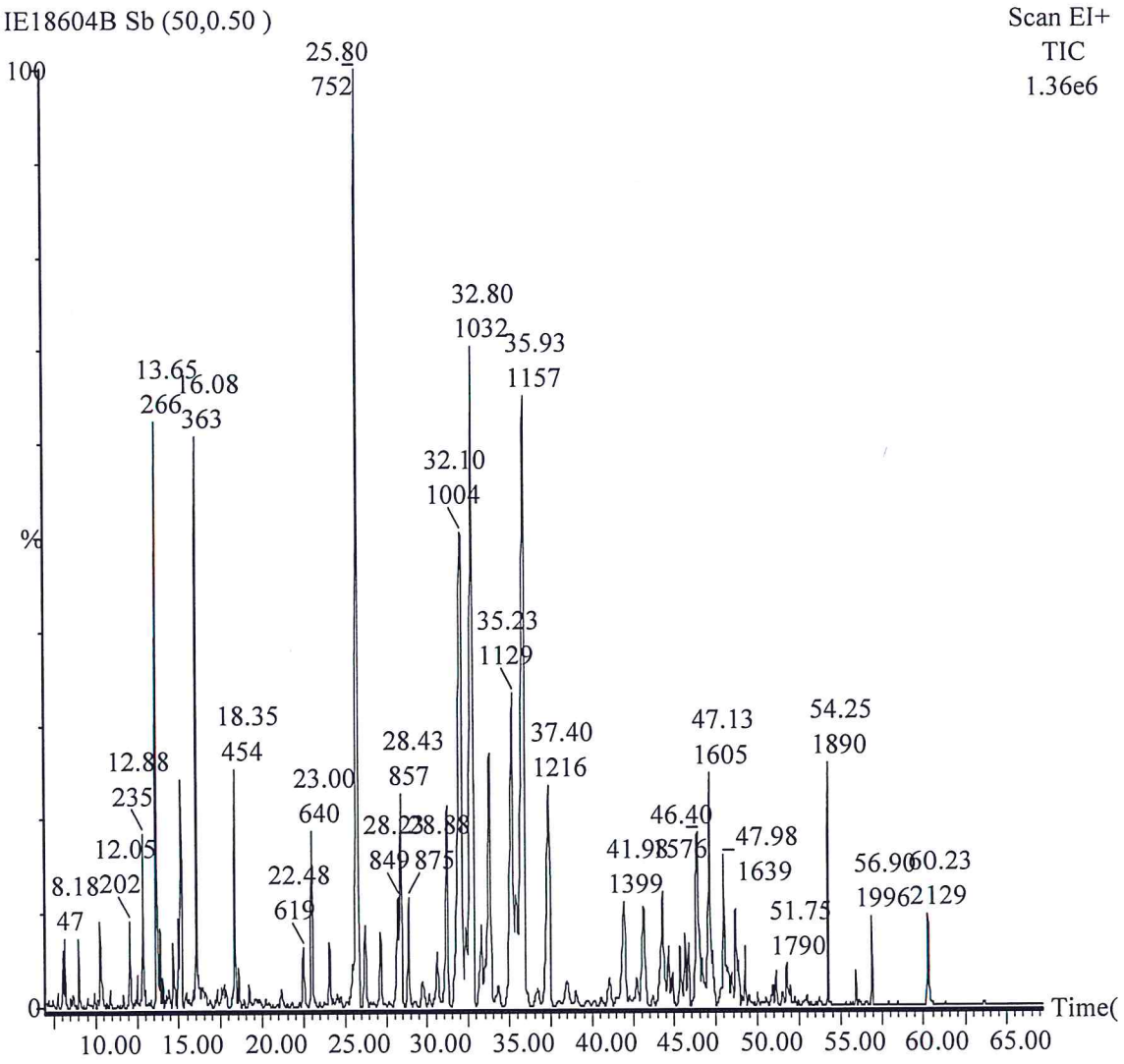


Fig 2.3 **GC-MS profile of *Steganotaenia araliacea* (*Sa*) essential oil**

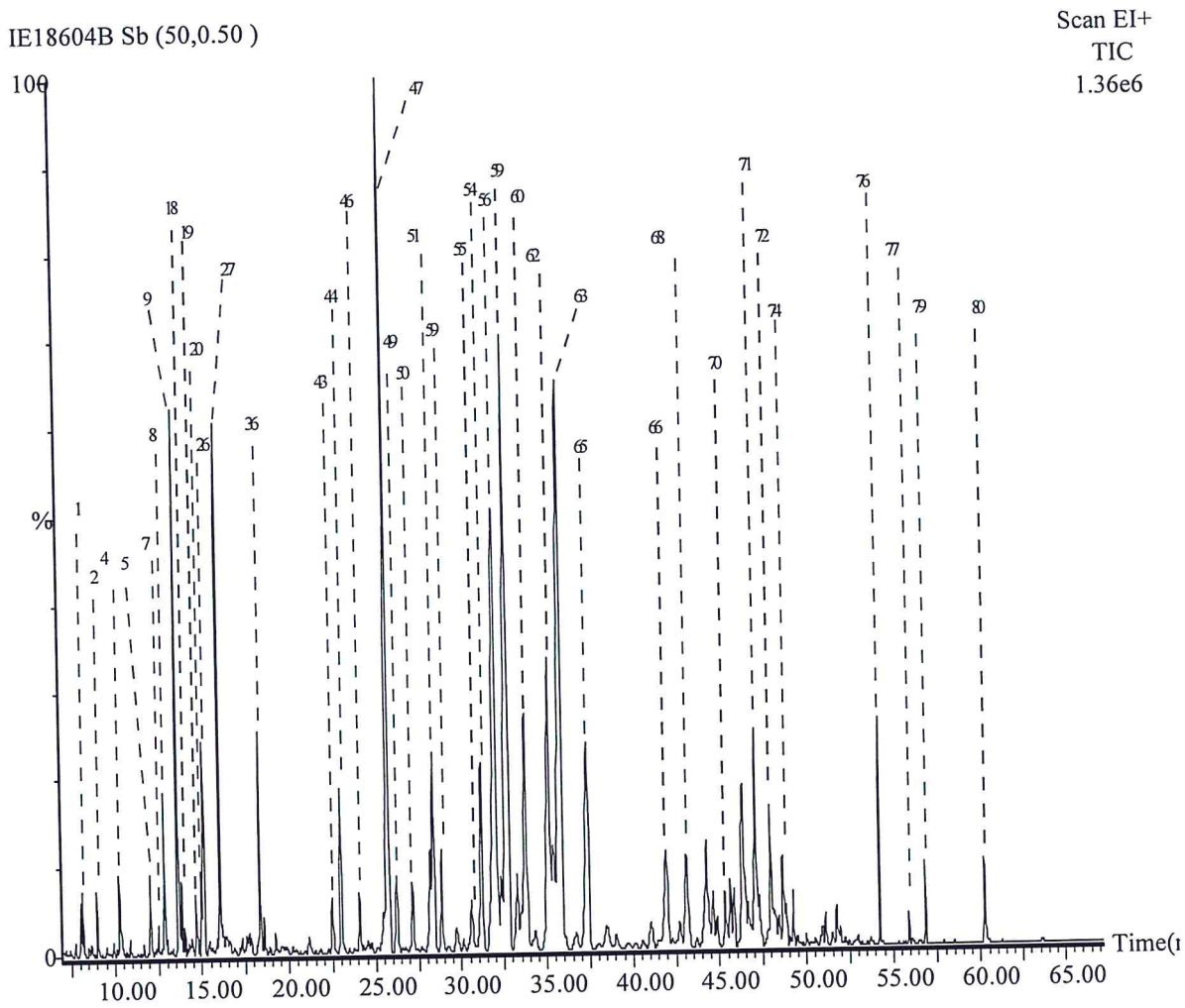


Fig 2.4 **GC-MS profile of *S. araliacea* (*Sa*) essential oil showing peak labels corresponding to compounds in Table 2.1**

2.2.2.3 Gas chromatography profiles and chemical composition of *Suregada zanzibariensis* (*Sz*) essential oil

The composition of the essential oil from *Suregada zanzibariensis* was also analysed in these investigations, whereby 82% of the components were identified, of which phenylacetaldehyde (**2.47**) (14.38%), artemisia ketone (**2.57**) (10.13%), (1*S*)-(-)-verbenone (**2.63**) (12.08%) and geranyl acetone (**2.77**) (9.35%) were the main constituents (**Table 2.1**). Among the identified components, 56% were terpenes with addition of an acetone moiety, suggesting presence of acetone transferase enzyme in the leaves. Hydrocarbons accounted for about 21%, these compounds, being formed during storage and processing of the leaves while monoterpenoids accounted for about 36% and sesquiterpenes were 24.37% of the essential oil constituents (**Table 2.1**).

VG Platform II GC/LC-MS Date: 18-Jun-2004 Time: 11:33:13

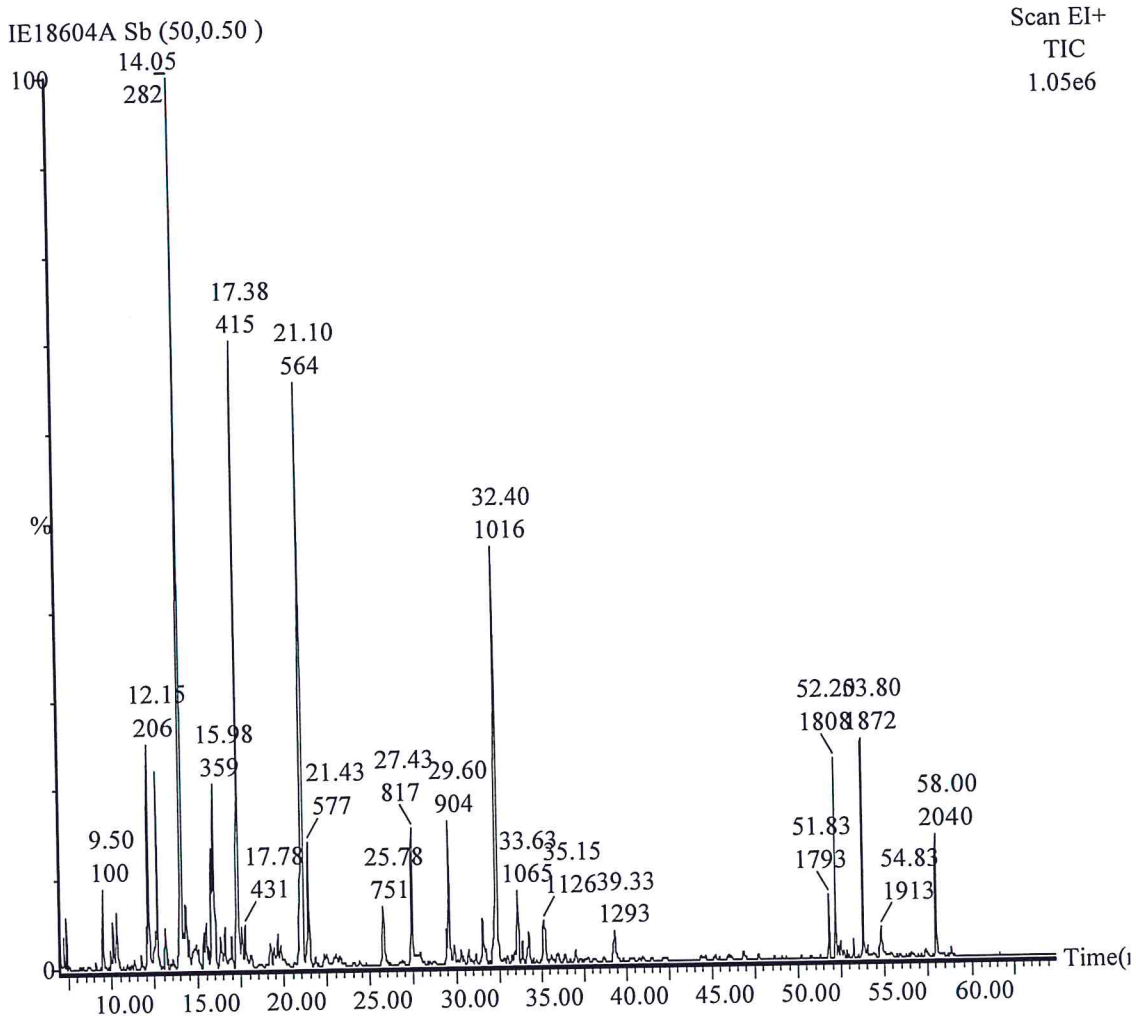


Fig 2.5 GC-MS profile of *Suregada zanzibariensis* (*Sz*) essential oil

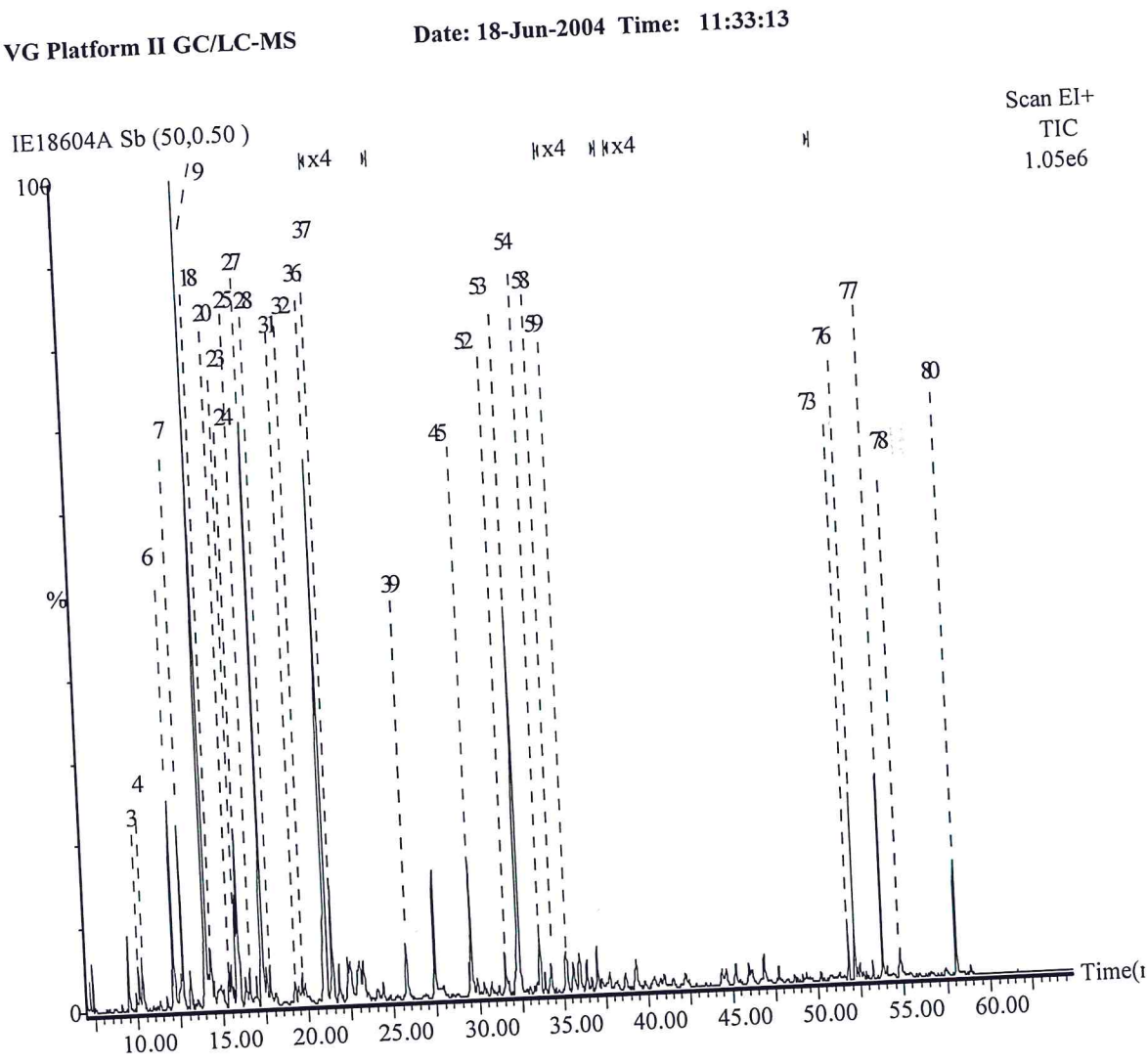


Fig 2.6 **Magnified section of GC-MS profile of *S. zanzibariensis* (*Sz*) essential oil showing peak labels corresponds to compounds identification given in Table 2.1**

2.2.3 Bioassay of essential oils, standards and blends

2.2.3.1 Essential oils

The dose-response repellency of the extracted essential oils were assayed for mosquito repellency at different concentrations by applying 1 ml of sample at each concentration to an average area of 696.6 cm^2 in the fore arms. The repellent effects of the crude oils is summarized in **Table 2.2**. The order of activity is as follows; *U. gorgonis* > *L. viburnoides* > *S. zanzibariensis* > *S. araliacea* > *C. anisata.* There was significant difference in repellency of the tested essential oil samples with *U. gorgonis* (*Ug*) (RC_{50} = 38.3 x 10^{-4} mg/cm^2), *L. viburnoides* (*Lv*) (RC_{50} = 56.0 x 10^{-4} mg/cm^2) and *S. zanzibariensis* (RC_{50} = 88.4 x 10^{-4} mg/cm^2) showing high activity. *Uvariodendron gorgonis* (*Ug*) and *L. viburnoides* (*Lv*) also showed reasonable stable repellency activity in the range of 10^{-2}-10^{-4} g/ml.

Table 2.2 Repellent efficacy of essential oils from five plant species against *An. gambiae s.s*

Essential oil	Conc. (g/ml)				RC_{50} (CI) (mg/cm^2) x 10^{-4}
	10^{-5}	10^{-4}	10^{-3}	10^{-2}	
Ug	29.675±13.91^{aB}	48.9±7.24^{bA}	57.875±10.67^{Aa}	64.15±2.37^{cbA}	38.3 (21.1, 55.6)
Lv	26.47±20.00^{aB}	46.05±8.66^{bAB}	54.58±21.37^{aAB}	62.66±9.59^{cbA}	56.0 (40.5, 73.9)
Ca	13.45±11.55^{aB}	21.91±4.25^{aB}	42.925±14.74^{Aa}	56.925±13.68^{cA}	110.0 (96.0, 126.8)
Sa	12.475±9.07^{aB}	14.475±11.73^{aB}	40.275±27.18^{aAB}	59.325±16.40^{cA}	107.0 (96.0-120.7)
Sz	15.125±5.74^{aB}	23.775±18.57^{aB}	28.925±18.44^{aB}	68.825±10.41^{cbA}	88.4 (79.8, 98.3)

Mean values with the same small letters within the same concentration level and mean values with the same capital letters for a particular treatment are not significantly different at p>0.05 by Student Newman-Keuls (SNK) test; Value in parentheses represent lower and upper confidence limit at p>0.05 by Lackfit inversel; *Ug = Uvariodedron gorgonis; Lv = Lantana viburnoides; Ca = Clausena anisata; Sa = Steganotaenia araliacea; Sz = Suregada zanzibariensis.*

2.2.3.2 Standards and blends of the main constituents in each of the oils

The standards of compounds identified from the five plant essential oils were assayed for repellency, individually, against *An. gambiae* (**Table 2.3**). Subsequently, the major compounds in essential oils were combined in the proportion of their abundance in the mixture to obtain the blends, except for *S. araliacea* (*Sa*) which had an even distribution of the compounds and *L. viburnoides* (*Lv*) whose major component, piperitenone (**2.69**) could not be obtained commercially. The components whose repellency against *Anopheles gambiae* had previously been reported by other workers[80,83-84] were not evaluated singly, but as mixtures in the blends.

There was no significant difference in repellent activity of blend one (B1) (RC_{50} = 41.2 x 10^{-4} mg/cm^2) compared to *Uvariodendron gorgonis* essential oil (RC_{50} = 38.3 x 10^{-4} mg/cm^2). Eugenol (**2.14**) which is the main constituent in *U. gorgonis* is an effective repellent and insecticide for insects in the order Coleoptera,[85,86] Hymenoptera[87] and Diptera.[50] Therefore, the high abundance of this compound in *U. gorgonis* indicates that the leaves of this plant species can be exploited in insect control especially as repellent and grain protectants against post harvest insect pests even at community level without destroying entire plants.

There was a decrease in repellent activity of blend two (B2) (RC_{50} = 196.2 x 10^{-4} mg/cm^2 compared to *C. anisata* essential oil (RC_{50} = 110 x 10^{-4} mg/cm^2). These results showed that, the major compounds in *C. anisata* essential oils make a significant contribution towards mosquito repellency, although the contribution of other repellent

compounds present in small amounts in the essential oils cannot be ignored. Despite the wide use of *C. anisata* in traditional medicine and in pest control, toxicological data of its major compound, estragol (**2.60**) show that it is a naturally occurring genotoxic carcinogen, and hence it is unsuitable for human use.[88] In central Tanzania, *C. anisata* is primarily used as an insect repellent due to the strong scent (of estragol, **2.60**) which is produced by fresh leaves.

High repellency activity of crude oil from the leaves of *L. viburnoides* (RC_{50} = 56.0 x 10^{-4} mg/cm^2) accounts for its traditional use for repelling mosquitoes in some parts of Tanzania. With the exception of 1,6,9-tetradecatriene (**2.64**) and piperitenone (**2.69**), repellent properties of all major compounds in the oils against *An. gambiae s.s.* has been established (**Tables 2.1** and **2.3**).[80,83-84] Previously, piperitenone oxide (**2.93**) was found to be highly toxic and repellent against adults of *An. stephensi*.[89] It may be interesting to establish the repellency property of piperitenone (**2.69**), against *An. gambiae* as it is the major constituent of the essential oil.

Repellent activity of the main constituents in *Suregada zanzibariensis* essential oil, *viz.* artemisia ketone (**2.57**) (RC_{50} = 29.8 x 10^{-4} mg/cm^2), (1*S*)-(-)-verbenone (**2.63**) (RC_{50} = 15.6 x 10^{-4} mg/cm^2) and geranyl acetone (**2.77**) (RC_{50} = 49.0 x 10^{-4} mg/cm^2) was higher except for phenylacetaldehyde (**2.47**) (RC_{50} = 79.5 x 10^{-4} mg/cm^2) whose repellent activity was comparable to the parent oil (RC_{50} = 88.7 x 10^{-4} mg/cm^2). The repellency activity of synthetic blends of the major constituents in the appropriate proportion found in *S. zanzibariensis* essential oil (B_3; RC_{50} = 64.4 x 10^{-4} mg/cm^2) was higher than the

crude essential oil. Similarly, subtraction of phenylacetaldehyde (**2.47**) (B_4) and/or verbenone (**2.63**) (B_6) in the oil resulted in an increased activity (**Table 2.3**). Removal of artemisia ketone (**2.47**) (B_7) gave activity comparable to that of the crude essential oil while presence of geranyl acetone (**2.77**) in the blend caused a significant increase in the activity (**Table 2.3**). Previous studies showed that, the repellent effects of synthetic blends of selected constituents of the oils were either higher or comparable to the corresponding crude oils, thereby indicating additive or synergistic effects of the essential oil constituents.[80,83-84]

Among the compounds tested for repellent activity against *An. gambiae s.s.*, farnesol (**2.87**) (RC_{50} value of 10.7×10^{-4} mg/cm^2) was the most active, followed by methyl ketone terpenes whose RC_{50} values were as follows: β-Ionone (**2.80**) (17.3×10^{-4}), α-ionone (**2.76**) (21.4×10^{-4}), farnesyl acetone (**2.90**) (24.8×10^{-4}) and geranyl acetone (**2.77**) (49.0×10^{-4}) mg/cm^2. The repellent activities compared well with those of other plant based mosquito repellents.[40-41,77] Nonetheless, the repellency activity of compounds found in essential oils as reported in **Table 2.3** was much lower than the repellency shown by DEET. However, despite the weak repellent property of the essential oil constituents, there is still possibility of using the oils from *U. gorgonis, L. viburnoides* and *S. zanzibariensis* in small-scale programs of controlling mosquitoes and other insects.

Table 2.3 **Repellenct efficacy of standard compounds and blends of the major constituents of essential oils of five plant species against *An. gambiae s.s.***

Treatment	RC_{50} (CI) (mg/cm^2) x 10^{-4}
Farnesyl acetone (**2.90**)	24.8 (20.2, 29.4)
Farnesol (**2.87**)	10.7 (8.6, 13.2)
Beta ionone (**2.80**)	17.3 (15.4, 19.7)
α-Humulene (**2.78**)	220.1 (187.9, 264.4)
Geranyl acetone (**2.77**)	49.0 (44.9, 53.5)
Alpha ionone (**2.76**)	21.4 (17.1, 25.9)
Verbenone (**2.63**)	15.6 (21, 88)[17]
Estragol (**2.60**)	208.0 (176.0, 257.1)
Endo-borneol (**2.58**)	286.2 (229.3, 389.2)
Artemisia ketone (**2.57**)	29.8 (18.4, 40.6)
Phenylacetaldehyde (**2.47**)	79.5 (69.8, 90.9)
Alloaromadendrene (**2.33**)	131.3 (119.9, 145.1)
Limonene (**2.27**)	18.0 (26, 03)[17]
γ–Terpinene (**2.7**)	27.4 (2.0, 316.6)[17]
Eugenol (**2.14**)	13.2 (2.0-29.2)[17]
B_1 (**2.14 + 2.27**)	41.2 (37.3, 45.5)
B_2 (**2.60 + 2.7**)	196.2 (161.1, 239.6)
B_3 (**2.47 + 2.57 + 2.63 + 2.77**)	64.4 (59.5, 69.6)
B_4 (**2.57 + 2.63 + 2.77**)	36.8 (32.4, 41.6)
B_5 (**2.47 + 2.63 + 2.77**)	61.6 (56.1,67.5)
B_6 (**2.47 + 2.57 + 2.77**)	31.4 (27.5, 35.5)
B_7 (**2.47 + 2.57 + 2.63**)	87.3 (80.3, 95.3)
DEET (**1.1**)	8.5 (7.8, 9.1)

Value in parentheses represent lower and upper confidence limit at $p>0.05$ by Lackfit inversel; Proportional of weight used in preparation of blends are Eugenol: limonene (9:1); estragol: γ-terpinene (9:0.5); phenylacetaldehyde: verbenone: geranyl acetone: artemisia ketone (14:12:9:10); [17]= data from Omolo *et al.* 2004.

2.4 EXPERIMENTAL

2.4.1 General

The glassware were cleaned by soaking in water overnight and washed with water and soap, rinsed with acetone and then with distilled water and finally left overnight to dry in the oven at 105 °C.

2.4.2 Analysis of essential oils

2.4.2.1 Gas chromatography

Analysis of the essential oils was carried out on a Hewlett Packard 5890A gas chromatograph (GC) equipped with flame ionization detector (FID) and a Hewlett Packard 3396 series II integrator. A cross-linked methyl silicon capillary column (50 m x 0.2 mm id x 0.33 μm film thickness) was used for separation of the essential oil components. Nitrogen was used as the carrier gas at a flow rate of 0.84 ml/min. The injector and detector temperatures were maintained at 250 and 270 °C, respectively. For each sample, the GC oven was operated at different temperature programmes to give good resolution as presented below. Thus, for *U. gorgonis* (*Ug*), *L. viburnoides* (*Lv*) and *C. anisata* (*Ca*) essential oil, analysis, the temperature was maintained at 50 °C for 5 min then increased at 5 °C/min up to 280 °C where it was held for 20 min. For the *S. araliaceae* (*Sa*) essential oil analysis, temperature was raised from 60 °C at 10 °C /min to 160 °C and maintained for 30 min then increased at 8 °C/min to 280 °C where it was maintained for 10 min. For *S. zanzibariensis* (*Sz*) essential oil analysis, temperature was

raised from 40 °C at 10 °C /min to 140 °C where it was maintained for 15 min, increased to 180 °C at 10 °C /min and maintained for 15 min and increased at 10 °C/min to 280 °C where it was maintained for 15 min

2.4.2.2 Gas chromatography-mass spectrometry

Identification of the essential oil components was carried out on Hewlett Packard 5790A series GC coupled to the VG Masslab 12-250 mass spectrometer (MS) with mass range of *m/z* 1-1400. The MS operated in the EI mode at 70 eV with the temperature of the source being held at 180 °C; multiplier voltage at 1350V; scan cycle of 1.5 s (scan duration of 1 s and inter-scan delay of 0.5 s); and scan ranges *m/z* 38–650. The instrument was calibrated using heptacosafluorotributyl amine, $[CF_3(CF_2)_3]_3N$ (Apollo Scientific Ltd., UK). The column and temperature programme used for GC-MS was the same as the one described for GC analysis except for the carrier gas which was helium in this case. The MS machine is equipped with a computerized data system running on MASSLYNX software with Wiley Version 6 and NIST Version 1.0 MS libraries. Where possible, the essential oil components were confirmed by GC co-injection of authentic samples.

2.4.3 Bioassay

2.4.3.1 Mosquitoes

Female adult *An. gambiae* s.s. mosquitoes as used in all experiments were supplied by ICIPE insectary. The mosquito colony used was that previously obtained from Ifakara in Tanzania in 1996 and reared under standard insectary conditions by the ICIPE. The

larvae were reared in a room where the temperature was maintained at 32-36 °C, and fed on TetraMin® food (Tetra GmbH, Germany). The adult mosquitoes were maintained on a 6% glucose solution and females fed on human blood thrice a week. Rearing temperatures and relative humidity in the adult insectary were 26-28 °C and 70-80%, respectively. Female mosquitoes used in the experiments were 5-7 days old, initially fed on glucose (6% solution) and then starved for 18 h, before carrying out the bioassays.

2.4.3.2 Ethical clearance and volunteer safety

As the experiment requires human landing catches, local volunteers were recruited with the informed consent. The discomfort and potential risks of mosquito bites to volunteers was explained. The individuals were all having good knowledge of malaria transmission. Five adult volunteers (3 male and 2 female) were involved in the experiments and do not object to their being revealed for publication. A parasite free environment was ensured through regular screening of the volunteers' peripheral blood for *plasmodium* parasite. Also only one person was allowed to feed the mosquitoes in the insectary for 5 days after the mosquitoes were given the first blood meal. However, sulphadine-pyriproxyfen prophylaxis was provided to all volunteers. A research protocol was submitted to the International centre of insect physiology and ecology, based at Duduvile- Nairobi and the Kenya National Ethical Review Committees, based at the Kenya Medical Research Institute (KEMRI). Ethical clearance was obtained from Kenya National Ethics Board.

2.4.3.3 Repellency assays

The repellency assays were performed in a dark room with red light as the only source of illumination.[33] The room temperature and humidity were controlled at 28 ± 2 °C and 75 ± 5 % respectively to mimic the feeding conditions for female *An. gambiae* s.s. mosquitoes. Cages (50 x 50 x 50 cm) made with aluminium sheet at the bottom, pyrex window screen on sides and top, and a cotton stockinet sleeve for access on the front were used in the dose response assays. Different concentrations of samples (10^{-5} - 10^{-1} g/ml) were prepared by dissolving the samples (1 or 0.1 g) in analytical grade acetone (10 ml) followed by ten-fold dilution to obtain the subsequent concentrations. Blend one (B_1) consisted of 9:1 ratio of eugenol to limonene while blend two (B_2) had a 9:0.5 ratio of estragol to γ-terpinene. Blend three (B_3) contained 1.4:1.0:1.2:0.9 ratio of phenylacetaldehyde, artemisia ketone, verbenone and geranyl acetone. Blend four (B_4) had 1.0:1.2:0.9 ratio of artemisia ketone, verbenone and geranyl acetone. Blend five (B_5) contained 1.4:1.2:0.9 ratio of phenylacetaldehyde, verbenone and geranyl acetone. Blend six (B_6) contained 1.4:1.0:0.9 ratio of phenylacetaldehyde, artemisia ketone and geranyl acetone while Blend seven (B_7) contained 1.4:1.0:1.2 ratio of phenylacetaldehyde, artemisia ketone and verbenone. Acetone acted as a control in all experiments. Fifty test mosquitoes and five adult human volunteers who did not apply any lotion, perfume, oil or perfumed soap on the day of the bioassay were used.

The forearm (average area of 696.6 cm^2) of each volunteer from the elbow to the hand was washed with water, left to dry and then introduced with the hand covered by a glove

in order to be unattractive to mosquitoes. The test sample (1 ml) was spread evenly over the treatment area and presented one after the other to the same caged mosquitoes for a particular sample and person. Sequential exposure to mosquito cages of the arms with zero and then progressively high doses (10^{-5}, 10^{-4}, 10^{-3}, 10^{-2} and 10^{-1} g/ml) of the repellent for 3 min was done. After testing each concentration, the hands were washed using a non-perfumed soap and tap water, and allowed to dry naturally for at least 20 min before dispensing the subsequent concentration. The number of mosquitoes landing on or probing the arm was counted and recorded for each volunteer. Mosquitoes were shaken off the arm before they took a blood meal. The right arm was treated with the samples while the left arm as a control. Exposure of the treated and control arms was alternated to provide a standard for comparing the avidity of biting.

2.4.4 Data analysis

Percentage protective efficacy (PE) was calculated using the formula PE = (C-T/T) x 100%, where C and T are the mean numbers of mosquitoes that landed on the control and test arm, respectively.[90-92] The data were subjected to analysis of variance (ANOVA) and the mean percentage protection compared using Student-Newman-Keuls (SNK) of the SAS package.[93] Probit analysis was done using the Lackfit Inversel procedure of the SAS programme,[93] to compute repellency concentration required for 50% protection (RC_{50})

CHAPTER THREE

EFFECTS OF LONG-TERM EXPOSURE OF *ANOPHELES GAMBIAE* S.S LARVAE TO PLANT EXTRACTS, FRACTIONS AND BLENDS

Abstract

This chapter reports results on effect of long-term exposure of *Anopheles gambiae s.s.* larvae to plant extracts, fractions and blends. The underlying hypothesis was that, the metabolites/compounds associated with the target plants may not be toxic but may be slowly acting as agonistic or antagonistic of insect hormones. The cumulative mean percentage mortality at different concentrations are given. Comparison of means was obtained using Student Newmann Keuls (SNK) and Dunnett's tests while probit was obtained by using Lackfit inversel of the SAS system. The results for the dichloromethane extracts of the rootbark of *Lantana viburnoides* ssp. *viburnoides* var. *kisi* (LRRD) (LC_{50} 126.63, 14.07 and 8.49 ppm after 24, 48 and 72 h, respectively) and rootbark of *Steganotaenia araliacea* (SARD) (LC_{50} of 89.63, 18.08 and 7.68 ppm, in 24, 48 and 72, h respectively) showed good larvicidal activities while methanol extracts of the stem (KUSM) and root (KURM) barks of *Kotschya uguenensis* exhibited insect growth disruption activity and larvae attained complete mortality in 6-8 days at 100 and 50 ppm. The extract from *Synadenium glaucensen* was mildly active. The results reflect effectiveness of each extract and indicate that extracts LRRD, SARD, KUSM and KURM are potential sources of botanical larvicides.

3.1 INTRODUCTION

Chemical agents that can destroy malaria vectors have offered hope for the control of the disease.[15,34,38] Destruction of mosquito breeding sites by draining stagnant water and applying insecticides such as DDT, were very successful in North America, Europe and Cuba[15,34,38] but unfortunately not in Africa, Asia or Latin America. According to the

World Health Organization (WHO), larviciding mosquito breeding sites was one of the first outstanding methods of vector control, but lost its predominance when malaria control was extended to cover entire endemic areas.[32]

Mosquito larvae feed on micro-organisms and organic materials, especially from plants, in ponds. Therefore, it is advantageous to incorporate botanical larvicides as one of the strategies of reducing mosquito populations. However, besides their usefulness, botanical insecticides are expensive to produce and therefore have not been developed to a large extent as compared to synthetic insecticides. Cost factors have hampered efforts to develop new synthetic insecticides, let alone biological ones. It is estimated that, the cost of developing a new pesticide is about US$ 15-30 million.[94] Hence, multi-national corporations are reluctant to pursue investigations in this area because in most cases production costs are too high and sometimes the botanicals might prove to be less effective than synthetic ones.[38,94] There also fear that financial returns will be low since most of the potential clients for such products would belong to poor regions in the developing world whose purchasing power is low.[38,94] Furthermore, the majority of the multi-national coroperations are based in the developed countries and invest in research and development almost exclusively targeting the developed world markets since the products will be readily marketable and profitable. This implies that, development of drugs and pesticides for tropical diseases and pests such as malaria have continued to attract very little attention and investments except for the recently established public-private partnerships.[95] Diseases such as sleeping sickness and chaggas disease are even

more neglected in terms of the development of appropriate drugs and vector control agents.[95]

One of the public sector institutions working with private partnerships is the Special Programme for Research and Training in Tropical Diseases Research (TDR), which was establishment in 1975. The United Nations Development Program (UNDP), the World Bank and the World Health Organization (WHO) among others sponsor TDR. TDR aims at helping co-ordinating support and influence global efforts to combat a portfolio of major diseases of the poor and disadvantaged.[96] TDR has two main goals. The first one being research and development so as to improve existing and to develop new approaches for preventing, diagnonising, treating and controlling neglected infectious diseases.[96] Also to develop new approaches that are applicable, acceptable and affordable to developing countries, which can be readily integrated into the health services, and which focus on the health problems of the poor people. The second goal is training and strengthening the capacity of developing countries to undertake research required for developing and implementing new and improved disease control approaches.

The currently increasing international research efforts are being directed at the development of new and improved tools and methodologies for malaria control and initiatives with links to public-private partnerships abound.[96] The initiatives include the formation of the Malaria Foundation International (MFI) in 1993, Multilateral Initiative for Malaria (MIM) in 1997, Medicines for Malaria Venture (MMV) in 1999 and Roll

Back Malaria (RBM) in 1999, among others.[97] Efforts also are directed to the possibility of involving industry in partnership for the development of new pesticides, through an initiative led by the WHO Pesticide Evaluation Scheme (WHOPES).[33]

However, in the developing world, it is not just a matter of a new pesticide but it must be accessible, easy to use, acceptable, affordable and having low toxicity to non-target organisms including animal. This is because most communities in rural areas are poor and have limited knowledge. Furthermore, since most of the rural population rely on plants as the first line solution to health and pest problems, it is important to understand how, why and what kind of plants are used traditionally for controlling diseases like malaria and other vector borne mosquitoes. Some plants contain pesticidal ingredients, which would act slowly, but effectively. Such plants are good candidates in the Integrated Vector Management (IVM) programmes and hence the need to study and develop them for use in their natural forms or as formulations. These facts inspired the investigations on larvicidal activities and searching for insect growth regulators from some Tanzanian plant species used traditionally for pest and vector control. Results from these investigations are presented in this chapter.

3.2 RESULTS AND DISCUSSION

Tests for larvicidal activity against *An. gambiae s.s.* mosquito larvae were carried out on a total of 20 samples derived from the four plant species *Lantana viburnoides* ssp *viburnoides* var. *kisi* (A. Rich) Verdc. (Verbenaceae), *Steganotaenia araliacea* (Apiaceae), *Kotschya uguenensis* Verdc. (Fabaceae) and *Synadenium glaucenscen* Pax.

(Euphorbiaceae) which were chosen on the basis of their ethnobotanical uses (**Table 3.1**). Since crude extracts from plants are known to be slowly acting and do not usually exhibit knockdown effects, it was therefore important to study residual effect of these extracts as it is hereby reported (**Tables 3.2-3.4**). The cumulative mean percentage mortality at different concentration levels for each extract was recorded for three days. The active extracts were taken as those exhibiting more than 50% mortality at 50 ppm after 72 h of exposure (**Table 3.2-3.4**).

As shown in **Tables 3.2-3.4**, the Euphorbiaceae plant species (*Synadenium glaucenscen*) tested in these investigations was mildly active as a larvicidal agent. The n-hexane and dichloromethane extracts from the leaves, stem bark and root barks of *S. glaucenscen* were more potent than methanol extracts (**Tables 3.2-3.4**), showing that, the larvicidal principle(s) are moderately polar. Extracts from this plant species were not investigated further.

A good pesticide is the one that is effective at low concentrations. Thus, although preliminary bioassay results (**Tables 3.2-3.4**) suggested extracts LRRH, KUAH, LRRD, LRRM and SARD to be good larvicidal agents at 50 ppm after exposing larvae into them for 72 h, extracts KUAH and LRRM were not chosen for further investigations because their activity was highly reduced at low concentrations (**Table 3.5**). Larvicidal activity for extract LRRH (0.21%) was moderate, being effective with time and increased concentration. The extract was not investigated further due to its low yield. High larvicidal effect was observed post-exposure of larvae to dichloromethane extract

of the root barks of *L. viburnoides* ssp. *viburnoides* (LRRD), giving LC_{50} values of 126.63, 14.07 and 8.49 ppm after 24, 48 and 72 h, respectively. Likewise, significant larvicidal activity of dichloromethane extracts of the root barks of *S. araliacea* (SARD) was observed giving LC_{50} values of 89.63, 18.08 and 7.68 ppm, in 24, 48 and 72 h, respectively (**Table 3.5**). Larvae treated with extracts from the stem bark of *L. viburnoides* ssp. *viburnoides* var. *kisi* had low larvicidal activity than the root bark (**Tables 3.2-3.4**). Results for the difference in the bioactivity between the stem bark and root bark of *L. viburnoides* may be a result of diverse distribution of compounds.

Table 3.1 General information of the plants tested for larvicidal activity using *Anopheles gambiae s.s* mosquito larvae

Plant species	Ethnobotanical uses	Part extracted	Solvent used (Extract code as presented in Tables 3.2-3.4)
Synadenium glaucenscen Pax. (Euphorbiaceae)	Poisonous* Poisonous to fish and humans[98,99] Boils[99] Cough[100]	Rootbark	Hexane (SGRH) Methanol (SGRM)
		Stembark	Hexane (SGSH) Dichloromethane (SGSD)
		Leaves	Hexane (SGLH) Dichloromethane (SGLD) Methanol (SGLM)
Lantana viburnoides ssp *viburnoides* var *kisi* (A. Rich.) Verdc. (Verbenaceae)	Leaves as mosquito repellent* Stomach relief and fruits as famine food[6]	Rootbark	Hexane (LRRH) Dichloromethane (LRRD) Methanol (LRRM)
		Stembark	Hexane (LRSH) Dichloromethane (LRSD) Methanol (LRRSM)
Kotschya uguenensis Verdc. (Fabaceae)	Repel mites and milk preservation*	Rootbark	Dichloromethane (KURD) Methanol (KURM)
		Stembark	Methanol (KUSM)
		Aerial parts	Hexane (KUAH) Dichloromethane (KUAD)
Steganataenia araliacea Hochst. (Apiaceae)	Repel snakes* , Treatment of malaria[99,101] Snakebite, sore throat and lung diseases for animals like cattle, goats and calves[6]	Rootbark	Dichloromethane (SARD) Methanol (SARM)

*Ethnobotanical uses in Iringa region, Tanzania

Table 3.2 Mean percentage mortality ± S.E. of larvae in water treated with different plant extracts after 24 h of exposure

Extract code	Conc (ppm)					
	50	100	250	500	750	1000
KUAD	0±0	0±0	86.67±10.93	100.00±0	100.00±0	100.00±0
KUAH	0±0	61.67±6.01	100.00±0	100.00±0	100.00±0	100.00±0
KURD	0±0	0±0	0±0	0±0	-	0±0
KUSM	0±0	0±0	0±0	3.33±1.67	1.67±1.67	5.00±0
LRRD	11.67±4.41	20.00±2.89	40.00±7.64	61.67±6.01	95.00±2.89	100.00±0
LRRH	3.33±1.67	45.00±8.66	53.33±1.67	73.33±6.01	93.33±4.41	100.00±0
LRRM	0±0	3.33±1.67	35.00±16.07	76.67±8.33	91.67±4.41	96.67±1.67
LRSH	26.67±19.22	91.67±8.33	98.33±1.67	100.00±0	100.00±0	100.00±0
SARD	15±5.77	21.67±1.67	25±10.41	28.33±8.82	-	28.33±8.82
SGLD	0±0	3.33±1.67	26.67±6.67	45.00±2.89	100.00±0	98.33±1.67
SGLH	1.67±1.67	48.33±17.40	100.00±0	100.00±0	100.00±0	100.00±0
SGRH	0±0	56.67±13.33	98.33±1.67	100.00±0	100.00±0	100.00±0
SGSD	0±0	0±0	46.67±19.22	33.33±14.81	85.00±2.89	100.00±0
SGSH	0±0	3.33±3.33	75.00±10.00	98.33±1.67	100.00±0	100.00±0
LRSM	0±0	0±0	0±0	1.67±1.67	1.67±1.67	38.33±23.51
SGRM	0±0	1.67±1.67	13.3±6.67	3.33±1.67	6.67±3.33	18.33±8.82
KURM	0±0	0±0	0±0	0±0	1.67±1.67	0±0
SGLM	0±0	0±0	6.67±1.67	8.33±1.67	8.33±1.67	13.33±1.67
LRSD	0±0	1.67±1.67	0±0	1.67±1.67	3.33±1.67	10±2.88
SARM	0±0	0±0	0±0	3.33±3.33	0±0	1.67±1.67

Table 3.3 Mean percentage mortality ± S.E. of larvae in water treated with different plant extracts after 48 h of exposure

Extract code	Conc (ppm)					
	50	100	250	500	750	1000
KUAD	10.00±2.89	16.67±6.01	86.67±10.93			
KUAH	38.33±6.01	81.67±7.26				
KURD	0±0	0±0	0±0	25.00±5.00	-	70.00±2.89
KUSM	0±0	0±0	1.67±1.667	8.33±6.01	5.00±2.89	25.00±10.41
LRRD	88.33±3.33	96.67±3.33	98.33±1.67	100.00±0	100.00±0	
LRRH	35±2.89	70.00±2.88	83.33±1.67	100.00±0	100.00±0	
LRRM	28.33±11.67	51.67±23.33	83.33±3.33	93.33±3.33	100.00±0	100.00±0
LRSH	26.67±19.22	95.00±5.00	100.00±0			
SARD	83.33±7.26	96.67±3.33	98.33±1.67	100.00±0	-	100.00±0
SGLD	0±0	8.33±8.33	76.67±13.33	100.00±0	100.00±0	100.00±0
SGLH	1.67±1.67	53.33±13.64				
SGRH	1.67±1.67	70.00±2.89	100.00±0			
SGSD	1.67±1.67	0±0	61.67±16.91	83.33±7.26	100.00±0	
SGSH	0±0	3.33±3.33	78.33±11.67	100.00±0		
LRSM	0±0	0±0	0±0	3.33±1.67	11.67±4.41	85±8.66
SGRM	0±0	1.67±1.67	25±10.41	56.67±20.88	83.33±3.33	86.67±4.41
KURM	0±0	3.33±1.67	28.33±8.82	83.33±9.28	90±5.77	90±5.77
SGLM	5±5	8.33±10.41	100.00±0	100.00±0	100.00±0	100.00±0
LRSD	0±0	1.67±1.67	6.67±1.67	5±5	33.33±3.33	91.67±4.41
SARM	0±0	0±0	50±20.82	100.00±0	100.00±0	100.00±0

Table 3.4 Mean percentage mortality ± S.E. of larvae in water treated with different plant extracts after 72 h of exposure

Extract code	Conc (ppm)					
	50	100	250	500	750	1000
KUAD	15.00±0	38.33±9.28	98.33±1.67			
KUAH	50±20.82	100				
KURD	0±0	0±0	1.67±1.67	31.67±1.67	-	85.00±2.89
KUSM	3.33±1.67	23.33±1.67	33.33±8.82	41.67±11.67	71.67±3.33	91.67±1.67
LRRD	100.00±0	100.00±0	100.00±0			
LRRH	68.33±3.33	98.33±1.67	100.00±0	100.00±0		
LRRM	56.67±20.88	73.33±14.53	91.67±6.01	100.00±0		
LRSH	26.67±19.22	100.00±0				
SARD	100.00±0	100.00±0	100.00±0			
SGLD	8.33±6.01	11.67±11.67	95.00±2.89			
SGLH	1.67±1.67	60.00±15.00				
SGRH	1.67±1.67	71.67±4.41				
SGSD	1.67±1.67	1.67±1.67	71.67±12.01	96.67±3.33		
SGSH	0±0	8.33±8.33	86.67±10.93	100±0		
LRSM	0±0	0±0	5±2.89	15±5.77	81.67±6.01	100.00±0
SGRM	0±0	3.33±1.67	40±25.17	83.33±9.28	93.33±6.67	100.00±0
KURM	0±0	13.33±7.26	78.33±4.41	85±7.64	98.33±1.67	100.00±0
SGLM	11.67±4.41	53.33±16.91				
LRSD	1.67±1.67	3.33±3.33	8.33±3.33	8.33±3.33	33.33±3.33	91.67±4.41
SARM	1.67±1.67	1.67±1.67	53.33±19.22	100.00±0	100.00±0	100.00±0

Table 3.5 Larvicidal activity (LC_{50}) of the most active extracts against *An. gambiae s. s.*

Extract Code	Yield (%)	LC_{50} (ppm)		
		24h	48h	72h
LRRD	**1.01**	126.63 (97.19-194.11)	14.07 (12.33-15.76)	8.49
LRRH	**0.21**	131.99 (97.97-240.01)	60.35(49.77-79.39)	22.37 (19.78-25.06)
LRRM	**2.83**	-	61.63(57.80-66.79)	47.96 (46.15-49.93)
KUAH	**0.32**	-	68.66 (65.80-71.69)	49.93 (48.12-51.96)
SARD	**1.72**	89.63 (76.55-111.31)	18.08 (16.24-19.92)	7.68 (7.06-8.29)

LC_{50} (CI) values are significant at 95% confindence level by Lackfit inversel; Value in parentheses represent lower and upper confidence limit

As shown in **Table 3.5**, significant larvicidal activities of extract LRRD were observed giving LC_{50} value of 126.6 ppm in 24 h, which increased to 8.49 ppm 72 h post-exposure (**Table 3.5**). Likewise, significant larvicidal activity was observed when larvae were exposed to SARD extract giving LC_{50} value of 89.6 and 7.68 ppm in 24 and 72 h, respectively. Besides their high activity, the yield of extracts LRRD (1.01%) and SARD (1.72%) were good enough for further phytochemical analysis (**Chapters 4** and **5**, respectively).

Vacuum liquid chromatography of extracts LRRD and SARD which was carried out on silica gel, eluting with n-hexane, ethyl acetate and methanol (**Chapters 4** and **5**) yielded six fractions each (i.e. LF1, LF2, LF3, LF4, LF5 and LF6 for LRRD and SF1, SF2, SF3, SF4, SF5 and SF6 for SARD). Percentage yield of each fraction was calculated and used

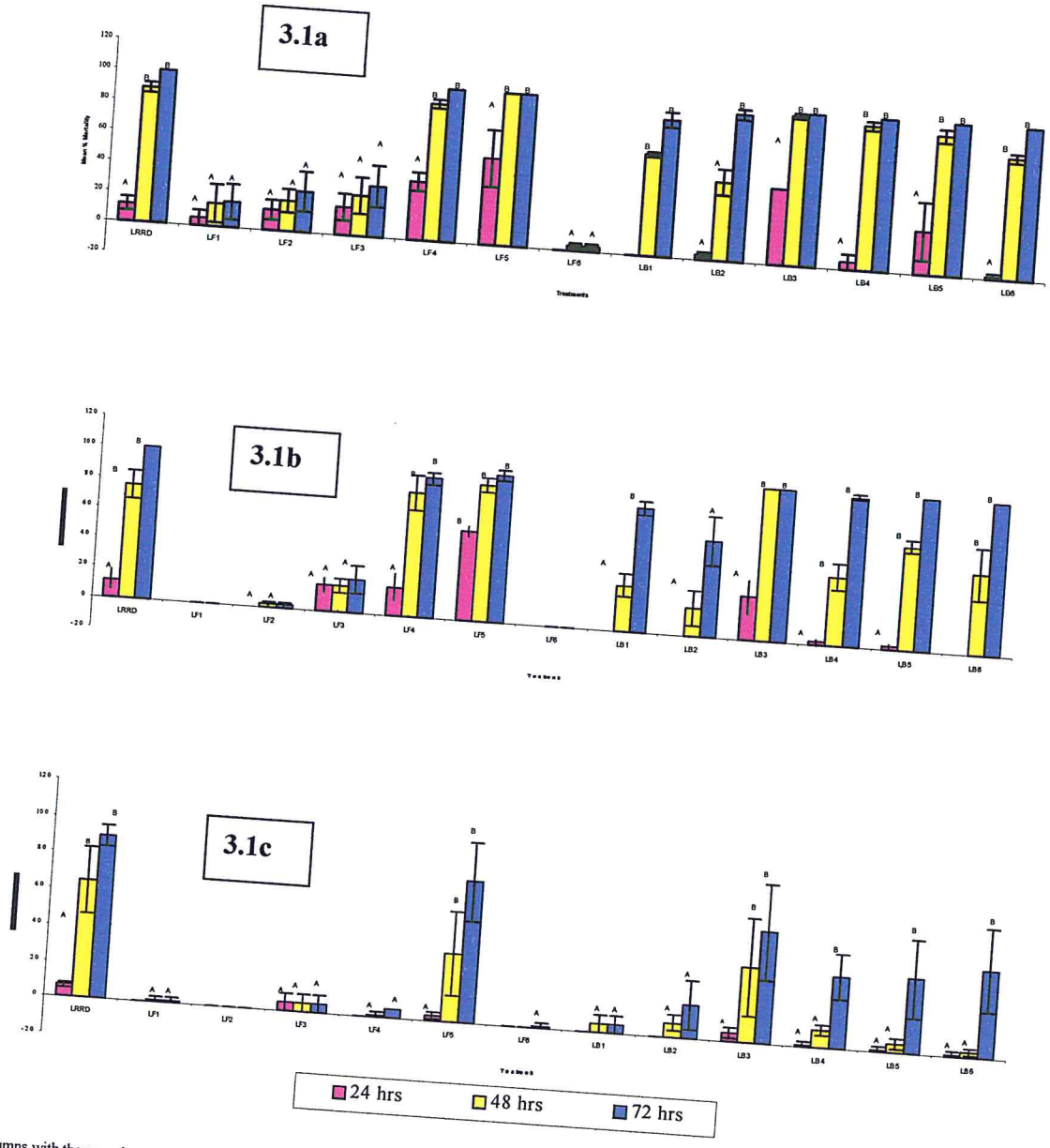
in estimating the amount to be included in the blends for subtraction bioassay. Blend one (LB1) of LRRD was prepared by omitting fraction one (LF1). Similarly, LB2, LB3, LB4, LB5, LB6, SB2, SB3, SB4, SB5 and SB6 were prepared by omitting LF2, LF3, LF4, LF5 and LF6, SF1, SF2, SF3, SF4, SF5 and SF6, respectively. SFI was obtained in trace amount and was not included in the investigations.

Larvicidal assay for fractions and blends from dichloromethane extract of the root bark of *L. viburnoides* ssp *viburnoides* var. *kisi* (LRRD) were also carried out for three days. Larvicidal activity of fractions LF4 and LF5 compared well with the parent extract (LRRD) at 50 and 20 ppm suggesting that, the active compounds were in fractions LF4 and LF5. However, the larvicidal activity of fraction LF4 was reduced at 10 ppm in comparison to LF5, showing that it performed well above this concentration. There were no significant differences between the activity of the extract (LRRD) and blend three (LB3). However, significant differences were observed between the activity of the extract (LRRD) and fraction three (LF3). These observations suggested presence of less active compounds in fraction three (LF3) (**Fig 3.1c; Chapter 4**). Similarly, there was no activity for fraction six (LF6), probably because the fraction was obtained by washing the chromatographic column (residue) with methanol. Low activity was followed by fraction one (LF1), fraction two (LF2), blend two (LB2) and blend one (LB1) (**Fig 3.1a-c**).

There were no significant differences in larval mortalities of the dichloromethane extract of the root barks of *S. araliacea* (SARD) and its fractions and blends after 48 h at 50

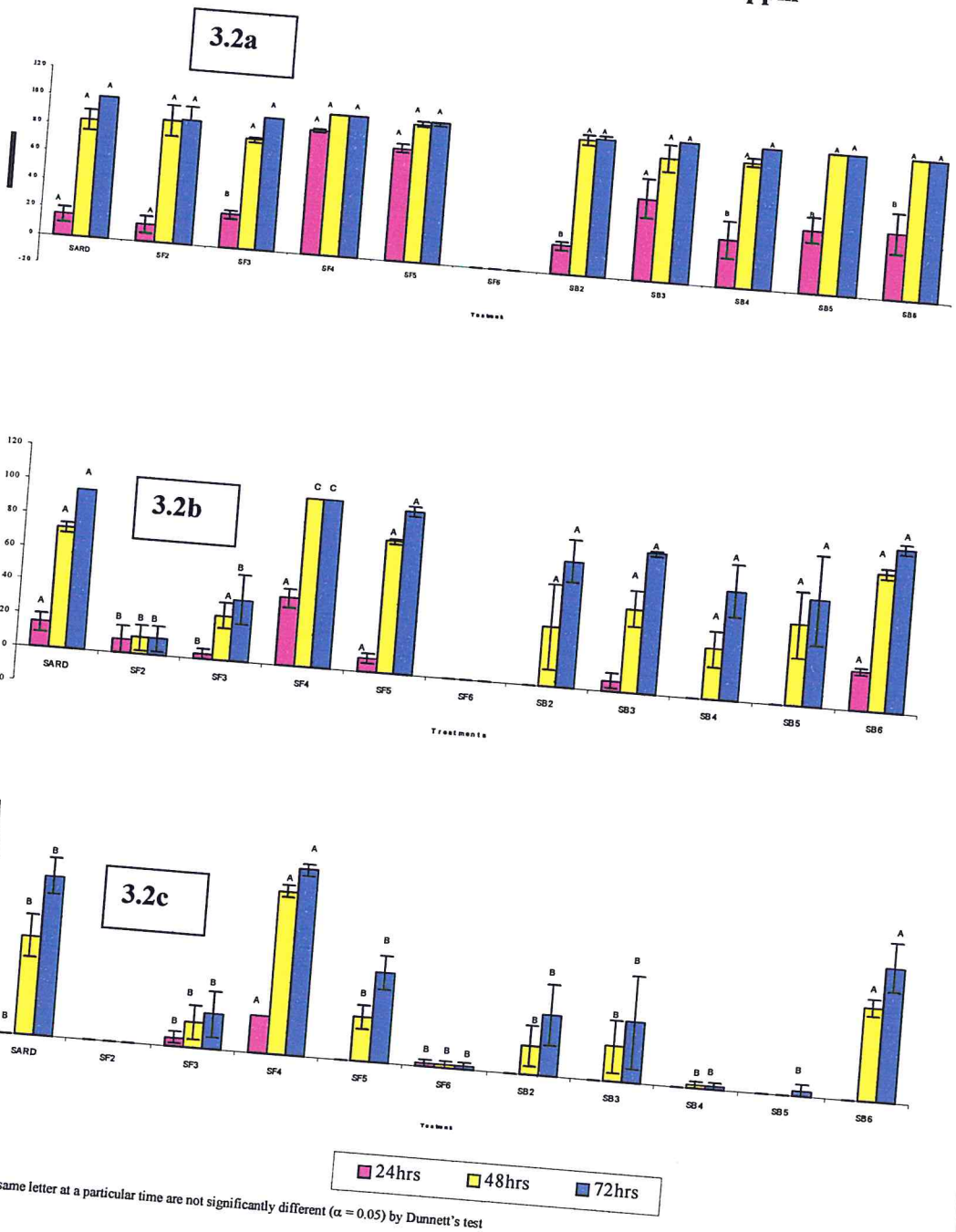
ppm, except for SF6 (**Fig 3.2a**). Blend six (SB6) caused high larvicidal activity at all concentration levels while low activity was shown for fractions two (SF2) and three (SF3), especially at low concentration (**Fig 3.2a-c**). At 20 and 10 ppm, fraction four (SF4) showed higher larvicidal activity than the parent extract (SARD), especially after 48 h post exposure (**Fig 3.2b-c**). Again subtraction of SF4 and SF5 especially at low doses (20-10 ppm) caused significant decrease in larvicidal activity, showing that, the active compounds are in these fractions and may be acting synergistically (**Fig 3.2b-c**). The presence of active compounds in SF4 and SF5 was confirmed by high larvicidal activity of SB6 that was an enriched fraction of extract SARD. SF6 was not active at all tested concentrations, yet, SB6 was highly active confirming the presence of larvicidal compounds in SF4 and SF5. Unlike LF6 which was just a remnant (washout), SF6 was a real subsequent fraction to SF5 and therefore must have had inactive compounds.

Fig 3.1 **Larvicidal efficacy of extract, fractions and blends of dichloromethane extract of the root bark of *Lantana viburnoides* ssp *viburnoides* var. *kisi* (LRRD) extract at a) 50 ppm, b) 20 ppm and c) 10 ppm**



Columns with the same letter at a particular time are not significantly different (p = 95) by Dunnett's test

Fig 3.2 **Larvicidal efficacy of extract, fractions and blends of the dichloromethane extracts of the root bark of *Steganotenia araliacea* (SARD) extract at a) 50 ppm, b) 20 ppm and c) 10 ppm**



Columns with the same letter at a particular time are not significantly different ($\alpha = 0.05$) by Dunnett's test

Methanol extracts of the root (KURM) and stem bark (KUSM) of *K. uguenensis* exhibited larvicidal activity at high concentration levels (250-1000 ppm) (**Tables 3.2-3.4**) and insect growth inhibition at low concentration level (50-100 ppm) ((**Figs. 3.3-3.8**). The activity of extracts KUSM and KURM was slow but effective with time. Larval abnormalities were observed after 24 h of exposure. Larvae elongated by forming tail-like structures, which attained a dark brown colouration and were shaded in 48-72 h post exposure. When viewed under a microscope, the tail like structure was identified to be part of the gut being elongated and extruded through the anal cavity. larva-pupa and pupa-adult intermediates were observed and most of them were short-lived and died before completing the moulting process (**Figs. 3.3-3.8**).

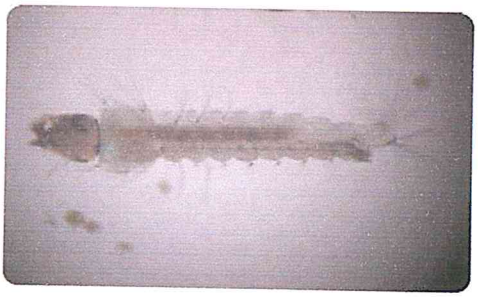


Fig 3.3 A Normal larva abdomen

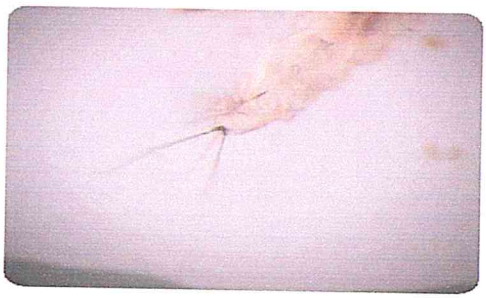


Fig 3.4 Anatomy of a normal larval tail

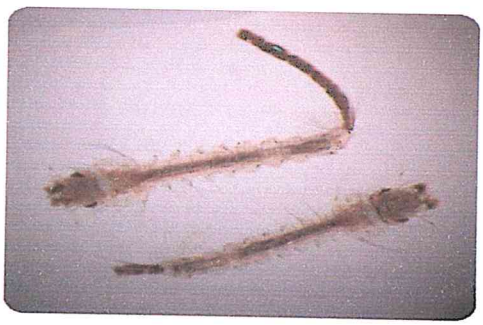


Fig 3.5 Affected larval abdomen due to elongated gut

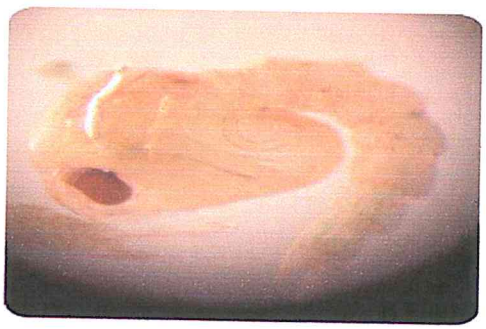


Fig 3.6 Normal pupa

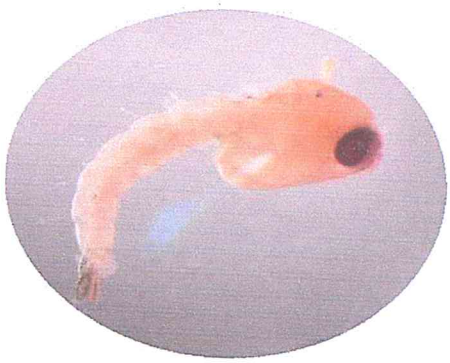


Fig 3.7 Larva-pupa intermediate

Fig 3.8 Pupa-adult intermediate

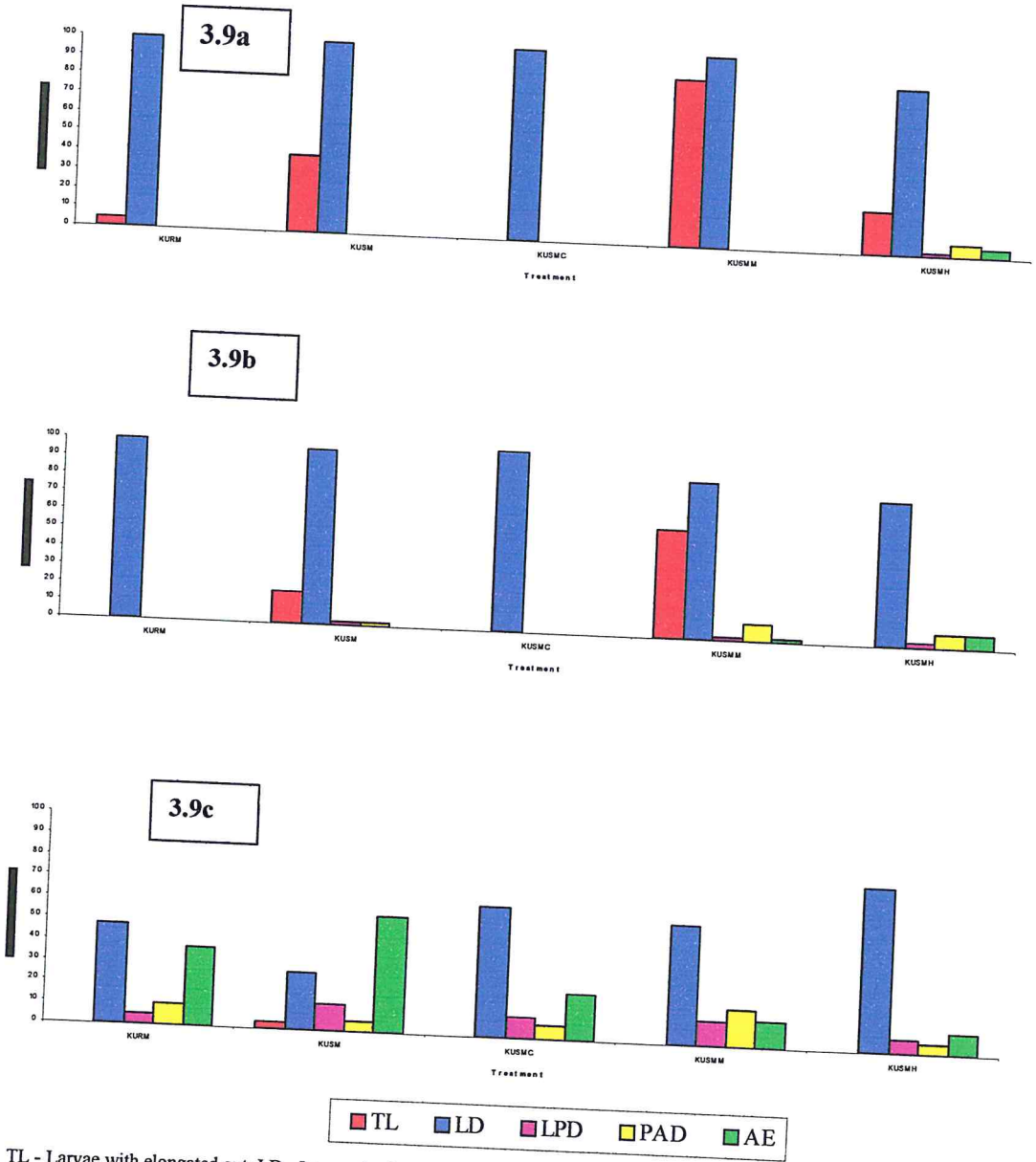
Observations from these investigations show that, exposure time may have a significant effect when botanicals are used as larvicides. The extracts KURM and KUSM that showed good results as candidate larvicides/insect growth regulators were further analyzed for the identification of the responsible chemicals (**Chapter 6**).

Partitioning of the methanol extract of the stem bark (KUSM) of *K. uguenensis* (80 g) between water and dichloromethane yielded a dichloromethane soluble fraction (KUSMC, 3.32%), a methanol soluble fraction (KUSMM, 40.63%) and a water soluble fraction (KUSMH, 56.04%) (**Chapter 6**). HPLC analysis of the methanol extracts of the root bark and stem bark of *K. uguenensis* and the three fractions showed insignificant quantitative differences of the constituents.

Detailed assays of the methanol extract of the stem bark and root bark of *K. uguenensis* and the subsequent fractions were performed at low doses (**Fig 3.9a-c**). At 100 and 50 ppm, larvae with elongated gut amounted to about 40 and 18% for the methanol extract

of the stem bark of *K. uguenensis* while in the methanol soluble fraction they amounted to 88 and 60%, respectively (**Fig 3.9a-b**). This observation showed that, the compounds responsible for larval deformities is present in large amount in these fractions. Although root bark extract KURM, and the stem bark fractions KUSMH and KUSMC gave few deformed larvae, they were active, attaining complete larval mortality in 6-8 days at 100 and 50 ppm. Below 50 ppm adults emerged for all treatments, showing that, the active compounds performed well at concentration above 50 ppm. Larvae that survived from treatments with 10 ppm were reared and monitored through their life cycle until adults were able to lay eggs and eggs hatched to larvae. The morphological features of the emerged adults (first generation, F_1) were as normal as those from the control experiment. This observation suggests that the larval deformities may not affect the reproduction system. The results also revealed that, although the polar extracts of *K. uguenensis* did not exhibit acute effects, most of the mosquitoes died at larval stage or before completing the moulting process. However, a few larvae emerged to adulthood especially at low concentrations below 50 ppm (**Fig. 3.9a-c**).

Fig 3.9 **Larval mortality (%) and deformity (%) on exposure to *Kotschya uguenensis* stem bark (KUSM) and root bark (KURM) extracts and fractions at a) 100 ppm, b) 50 ppm and c) 10 ppm**



TL - Larvae with elongated gut; LD - Larvae death; LPD - Larva-pupa intermediate death; PAD - Pupa-adult intermediate death; AE -; Adult emerged

The relationship between plants and insects is the most predominant ecological interaction on earth due to the large number of species in the two groups.[102] It is also known that, most insects live underground. The tropical and sub-tropical climatic habitat favour proliferation of various plant pathogens and phytophagus insects. Indeed the high insecticidal activities of the extracts from the root bark of *S. araliacea*, *K. uguenensis* and *L. viburnoides* indicate that, roots of tropical and sub-tropical plants must have evolved complex biochemicals, some of which are responsible for defence (survival) against attack by their natural enemies, and prosperity (ecological) in their natural selection pressure. The production of these chemicals (natural products or secondary matabolites) is important because plants cannot move away from hostile situations and must adopt effective survival strategies in the competitive environment. Complex chemical interactions are often involved in deterrence and repulsion of predators or inhibition of the pathogens and competitors. Consequently, the plant accumulates a large number of metabolic by-products. The accumulation of these by-products is particularly of interest since unlike animals which have specialized organs of excretion of unwanted products of biochemical processes such as kidneys, sweat pores, livers etc., plants have none. Therefore, plants would be expected to accumulate such products in leaves, flowers, fruits, seeds, stem and root barks after their transformation into secondary metabolites. Some of these compounds may turn out to be essential for the survival and prosperity of the plants and other organism due to their ability to exhibit different biological activity[103,104] as demonstrated by *K. uguenensis, S. araliacea* and *L.*

viburnoides ssp. *viburnoides* var. *kisi*. These compounds may be metabolized into other useful products or disposed with old leaves, flowers, fruits or seeds.

3.3 EXPERIMENTAL

3.3.1 General

For general procedures, see **chapter two, section 2.4.1**.

3.3.2 Plant material and extraction

Plants were chosen on ethnopharmacological and ethnobotanical information. Plant materials of *Lantana viburnoides* ssp *viburnoides* var. *kisi* (A. Rich) Verdc. were collected in April and October, 2003 from Lugaga village near the Mafinga Army Camp in Mufindi district, Tanzania (voucher specimen; Ref. No. FMM 3290). Plant materials of *Steganotaenia araliacea* were collected in April, 2003 from Ndolezi village near Mafinga in Mufindi District, Tanzania (Ref. No. FMM 3289). Plant materials of *Kotschya uguenensis* Verdc. (Fabaceae) were collected at Ngwazi dam and near Kisolombe farm in Mufindi district along Iringa-Mbeya road 12 km from Iringa town, Tanzania (Ref. No. 3292) while *Synadenium glaucenscen* Pax. (Euphorbiaceae) materials (Ref. No. LBM 10520) were collected from Ifunda in Iringa Region, Tanzania. The plant specimens were authenticated assigned voucher specimen reference numbers and deposited at the Herbarium of the Department of Botany, University of Dar es Salaam, Tanzania. The plant materials were air-dried under shade, pulverised and soaked twice sequentially in n-hexane, dichloromethane and methanol for 72 h with

occasional shaking. Similar solvent extraction were combined and then filtered. The crude filtrates were concentrated *in vacuo* using a rotary evaporator while maintaining the water bath temperature at about 40 °C in order to avoid thermal decomposition of labile compounds. The crude extracts were stored at -4 °C until the time of analysis.

3.3.3 Preparation of samples for bioassay

All solvents were of analytical grade as purchased from the Sigma-Aldrich Chemical Company. Acetone was used as the universal solvent but in some cases ethanol or dimethyl sulphoxide (DMSO) was used for this purpose. A stock solution (50 mg/ml) was prepared by dissolving 1.25 g of each extract in 25 ml of acetone, or ethanol, or 20% DMSO in acetone. The solvent used to dissolve extracts was used as the control. Acetone was used to dissolve the extracts KUAH, LRRH, LRRD, LRRM, SGLD, SGLM, KUAD, SARD, SARM and SGSD while ethanol (99.5%) was used to dissolve the extracts KUSM, KURD, SGRM, LRSM, KURM and LRSD and 20% DMSO in acetone was used to dissolve SGLH, SGSH, LRSH and SGRH.

3.3.4 Bioassay guided fractionation and subtraction bioassay of fractions (blends)

Bioassay-guided fractionation using different chromatographic techniques and subtraction bioassay of fractions (blends) to study the contribution of compounds to the observed larvicidal activities were carried out on the dichloromethane extracts of the root barks of *Lantana viburnoides* ssp. *viburnoides* var. *kisi* (LRRD) and *Steganotenia araliacea* (SARD). Vacuum liquid chromatography (VLC) of the extracts LRRD and SARD was carried out on silica gel (230-400 mesh size) using glass columns (15 cm i.d.

x 25 cm). Each of the extracts LRRD and SARD was fractionated using n-hexane, ethyl acetate and methanol. Extract LRRD yielded six fractions, namely LF1 (0-5% ethyl acetate/n-hexane), LF2 (10-20% ethyl acetate/n-hexane), LF3 (30-40% ethyl acetate/n-hexane), LF4 (50-70% ethyl acetate/n-hexane), LF5 (0-20% methanol/ethyl acetate/) and LF6 (100% methanol). Similarly, extract SARD yielded six fractions SF1 (100% n-hexane), SF2 (2-5% ethyl acetate/n-hexane), SF3 (7.5-10% ethyl acetate/n-hexane), SF4 (20-40% ethyl acetate/n-hexane), SF5 (50-75% ethyl acetate/n-hexane) and SF6 (100-80% ethyl acetate/methanol). The percentage yield of each fraction was calculated and used in estimating the amount to be included in the blends. Subtraction bioassay was carried out by omitting one fraction at a time and its contribution to the extract studied. Thus, blend one (LB1) of extract LRRD was prepared by omitting fraction one (LF1), Blend two (LB2) was prepared by omitting fraction two (LF2), and likewise for LB3, LB4, LB5 and LB6. For extract SARD, blend two (SB2) was prepared by missing fraction two (SF2), and likewise for SB3, SB4, SB5 and SB6. Fraction SFI was available in trace amounts, and was not included in the investigations. Partitioning of the methanol extract of the stem bark (KUSM) of *Kotschya uguenensis* (80 g) between water and dichloromethane yielded the dichloromethane soluble KUSMC), methanol soluble (KUSMM) and water soluble fractions (KUSMH).

3.3.5 Bioassay

3.3.5.1 Mosquito larvae

Twenty late 3rd or early 4th instar of *An. gambiae* s.s larvae were obtained from a colony maintained at ICIPE-Nairobi insectary that was originally obtained from ICIPE-

Mbita Point Research station in 2003. Larvae were allowed to emerge in plastic containers filled with distilled water and transferred to large plastic pans (37 x 31 x 6 cm) in batches of 200-300. Larvae were fed on Tetramin® fish food, and water temperature was maintained at 29±1 °C throughout larval development period.

3.3.5.2 Mosquito larvicidal assay

Larvicidal assay was carried out by exposing 20 late 3rd or early 4th instar larvae to distilled water treated with diffeent concentrations (50, 100, 250, 500, 750 and 1000 ppm) of the plant extracts and 1 mg Tetramin® fish food added to each beaker everyday. A known volume of stock solution was added in beakers to make up 100 ml of treated water solution (water temperature 28±2 °C). Further assay was carried out using lower doses (10, 20 and 50 ppm) for the fractions and blends of the dichloromethane extract of the root bark of *Lantana viburnoides* ssp. *viburnoides* var. *kisi* (LRRD) and *Steganotenia araliacea* (SARD). The test was done in triplicate from different batches of *An. gambiae s.s* larvae. The number of larval death was recorded.

3.3.5.3 Insect growth regulators (IGR's) assay

Detailed bioassay was carried out using lower doses (10, 50 and 100 ppm) for the extracts KURM and KUSM and for their subsequent fractions. Ethanol (99.5%) was used to dissolve samples and as blank in the control experiment. The test was done in triplicate from different reared batches of *An. gambiae s.s.* larvae. Mortality delay was studied by recording the cumulative number of dead larvae, deformities and emerged

adults after every 24 h for fourteen days. During the experiment (water temperature, 28±2 °C) larvae were fed on Tetramin® fish food at 1 mg per beaker per day.

3.3.6 Data analysis

Data was subjected to analysis of variance (ANOVA) and mean percentage mortality was compared using Student Newmann Keuls (SNK) and Dunnetts' tests of the SAS package.[93] Probit analysis was done using the Lackfit Inversel procedure of the SAS Program to compute LC_{50}.[93]

CHAPTER FOUR

LARVICIDAL COMPOUNDS FROM *LANTANA VIBURNOIDES* SSP *VIBURNOIDES* VAR *KISI* (A. RICH.)

Abstract

This chapter reports on bioassay-guided fractionation of the root bark extract of *Lantana viburnoides* ssp *viburnoides* var. *kisi* (A. Rich) Verdc. and isolation of larvicidal compounds and/or those associated with that activity against *Anopheles gambiae s.s.* mosquito. Camaric acid, inseparable mixtures of 6-hydroxy-naphtho[2,3-b]furan-4,9-dione, 7-hydroxy-naphtho[2,3-b]furan-4,9-dione and naphtho[2,3-b]furan-4,9-dione, and 6-methoxy-naphtho[2,3-b]furan-4,9-dione and 7-methoxy-naphtho[2,3-b]furan-4,9-dione were isolated as larvicidal compounds, with LC_{50} values of 8.31, 5.70 and 5.48 ppm, respectively, after 72 h. Other isolated compounds included betulinic acid, a mixture of 6-hydroxy-2-(1-hydroxy-1-methyl-ethyl)-naphtho[2,3-b]furan-4,9-dione, 7-hydroxy-2-(1-hydroxy-1-methyl-ethyl)-naphtho[2,3-b]furan-4,9-dione and 2-(1-hydroxy-1-methyl-ethyl)-naphtho[2,3-b]furan-4,9-dione, a mixture of 6-hydroxy-2-(1-hydroxy-1-methyl-ethyl)-dihydronaphtho[2,3-b]furan-4,9-dione, 7-Hydroxy-2-(1-hydroxy-1-methyl-ethyl)-dihydronaphtho[2,3-b]furan-4,9-dione and 2-(1-hydroxy-1-methyl-ethyl)-dihydronaphtho[2,3-b]furan-4,9-dione, sigmasterol and a hirtheto unreported dimeric peroxide of 4-methoxy-3-methylphenol. Structures of the isolated compounds were established from extensive spectral data analysis.

4.1 INTRODUCTION

Lantana viburnoides ssp *viburnoides* var. *kisi* (A. Rich) Verdc. belong to the family Verbenaceae which comprises 100 genera and 2,600 species that grow as herbs, shrubs or trees. The genus *Lantana* consists of about 150 species occurring in tropical and sub-tropical countries.[8,105,106] *Lantana* species are herbs and shrubs whereby the true number

of species is probably much less because some of them are cultivated and hybridization is apparently widespread.[8,105,106]

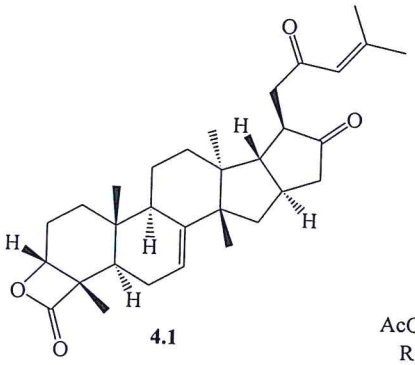
Lantana viburnoides consists of two sub-species and two varieties. The two sub-species are *L. viburnoides* ssp *masaica* Verdc. and *L. viburnoides* ssp *viburnoides* (A. Rich.) Verdc. The sub-species *L. viburnoides* ssp *masaica* Verdc. is a small shrub or woody herb, 0.9-1.5 m tall whose leaves are usually small (1.5-3 cm long and 1-1.6 cm wide). Its leaves are densely pubescent beneath, very closely crenate, inflorescences are small, mostly 0.8-1 cm long.[105] The sub-species *L. viburnoides* ssp *viburnoides* has two varieties which are *L. viburnoides* ssp *viburnoides* var. *viburnoides* and *L. viburnoides* ssp *viburnoides* var. *kisi*. *L. viburnoides* ssp *viburnoides* var. *viburnoides* is a 0.3-2.5 m tall shrub whose leaves are ovate to elliptic-lanceolate up to 12 cm long and 7 cm wide.[1,2] Leaves are less discolourous, not so densely thick and white velvety tomentose beneath, and inflorescences mostly exceeding the leaves. *Lantana viburnoides* ssp *viburnoides* var. *kisi* (A. Rich) Verdc. has leaves that are ovate to elliptic-ovate, mostly 4-4.5 cm long and 2 cm wide.[8,105] Leaves are very discolourous, densely thick and white velvety tomentose beneath. The indumentum totally obscures the surface and they are very rugose above.[105]

Lantana viburnoides ssp *viburnoides* var. *kisi* initially known as *L. rugosa* is indigeneous to Tanzania. The plant materials for these investigations were collected from Iringa and Mbeya regions where the leaves are used as mosquito repellents and sometimes chewed for stomach relief. The fruits are used as famine food by the Zulu in

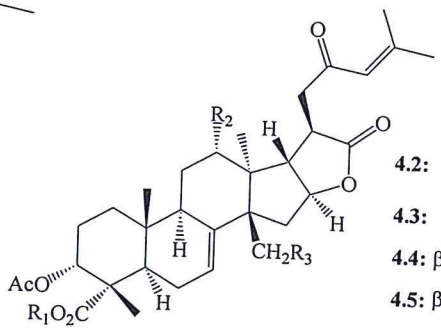
South Africa.[6] The Jaluo of Northern Tanzania regard the plant as poisonous if eaten in large amount but non-poisonous to sheep and goats.[6]

Phytochemical studies of plants of the genus *Lantana* have indicated triterpenoids as the main constituents. The presence of flavanoids, iridoids, phenylpropanoid glycosides, volatile oils, alkaloids, and some hydrocarbons have also been documented.[6,7]

Lantana camara has been reported to consist of 30 taxa, among which 9 have pesticidal properties.[106,107] The plant species has been domesticated as an ornamental plant and exhibits a variety of biological activities. Due to this reason, it has been extensively investigated. The plant is toxic to grazing animals which upon ingestion of the leaves develop hepatotoxicity and photosensitization.[108] A study of a hematological change in sheep after *L. camara* poisoning indicated a significant increase in coagulation and prothrombin time with an associated decrease in blood sedimentation rate, total plasma protein and fibrinogen.[108] In one phytochemical investigation, the leaves of *L. camara* yielded a 5,5-*trans*-fused cyclic lactone-containing euphane tritepenoids **4.1-4.5** that exhibited thrombin inhibitory activity.[108] The mechanism of inhibition of the blood cascade is *via* acylation of the active-site Ser-195 residue of thrombrin.[108] The acylation activity has been found to be generic against other serine protease enzymes.[108]

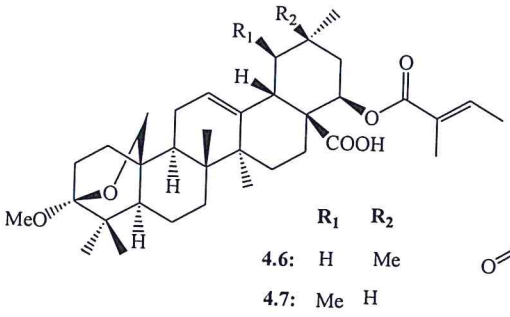


4.1

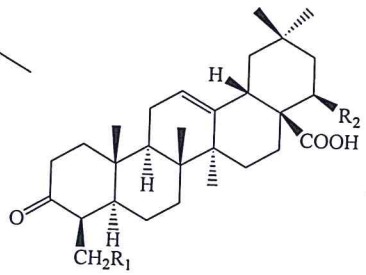


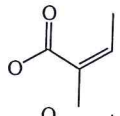
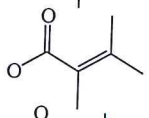
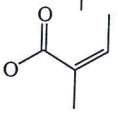
	R_1	R_2	R_3
4.2:	H	H	H
4.3:	H	OH	H
4.4:	β–D-Glucose	OH	OH
4.5:	β–D-Glucose	OH	OAc

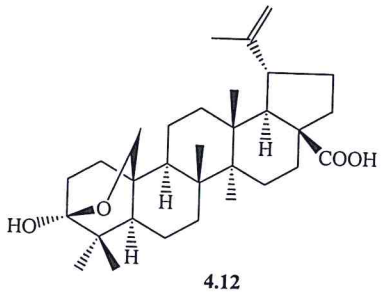
In another pharmacological study, the extracts of the shoots exhibited antibacterial properties. Upon phytochemical analysis, the extracts yielded camarilic acid (**4.6**), camaracinic acid (**4.7**), oleanonic acid (**4.8**), lantadene A (**4.9**), lantadene B (**4.10**), icterogenin (**4.11**), lantabetulinic acid (**4.12**), lantanolic acid (**4.13**), and compounds **4.14** and **4.15**.[109-111]



	R_1	R_2
4.6:	H	Me
4.7:	Me	H



	R_1	R_2
4.8	H	H
4.9:	H	
4.10:	H	
4.11:	OH	



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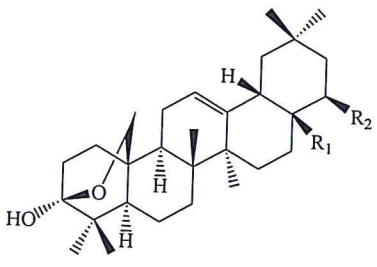
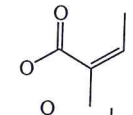
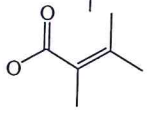
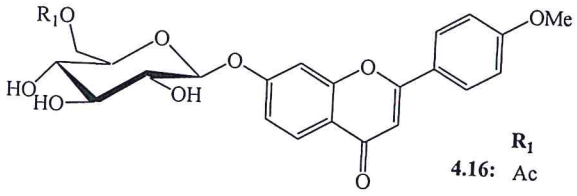
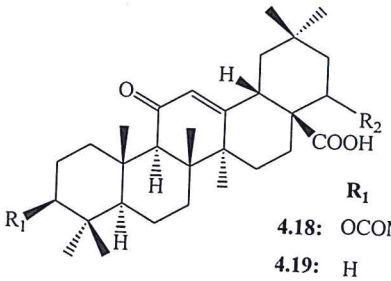


	R_1	R_2
4.13:	COOH	H
4.14:	Me	
4.15:	Me	

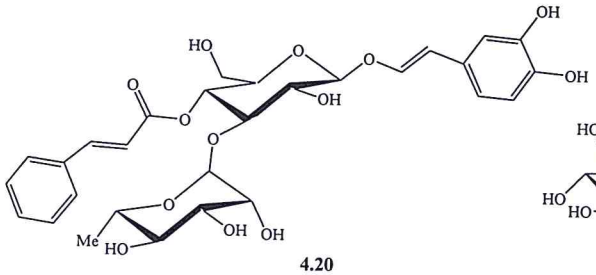
In another investigation, the aerial parts of *Lantana camara* yielded the nematocidal agents lantanoside (**4.16**), linaroside (**4.17**), lantanone (**4.18**) and camarinic acid (**4.19**).[112] Compounds **4.17**-**4.19** exhibited 85, 90 and 100% mortality of the nematode *Meloidogyne incognita* at 1.0% concentration at 24 h post-exposure while at 72 h mortality was 100% for all compounds.[112] This activity being comparable with that of the known nematocidal agent furadan.[112] Camarinic acid (**4.19**) also exhibited antimicrobial and antimutagenic activity against *Staphylococcus aureus* and *Salmonella typhii,* having antimicrobial index (AI) of 0.95 and 0.55, respectively.[114] The antimicrobial activity is comparable to that of tetracycline (AI = 0.80 at 30 μg) while the antimutagenic properties of reducing the number of micronucleated polychromatic erythrocytes (MPCE) induced by mitomycin C was 76.7%.[114] The non-polar components of the leaves yielded a hitherto novel phenylpropanyl glycoside camaraside (**4.20**) and a flavone glycoside lantanaside (**4.21**).[113] Evaluation of allelochemicals of the plant suggested that these compounds have insecticidal, pesticidal and weedicidal activity.[113]



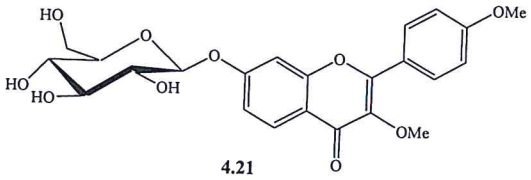
R1
4.16: Ac
4.17: H



	R1	**R2**
4.18:	OCOMe	H
4.19:	H	OCOMe

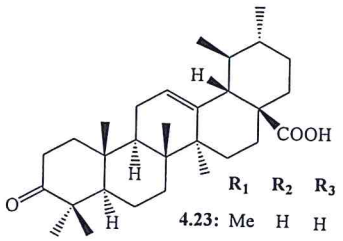


4.20

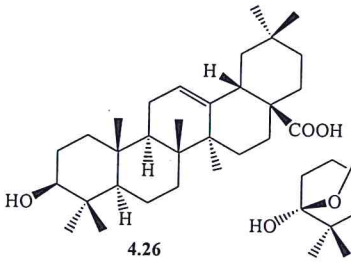


4.21

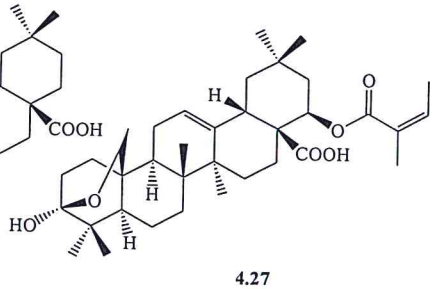
Phytochemical investigations of *L. tiliaeforia* Cham. yielded ursonic acid (**4.23**), oleanonic acid (**4.8**), 24-hydroxy-3-oxours-12-en-28-oic acid (**4.24**) and 24-hydroxy-3-oxoolean-12-en-28-oic acid (**4.25**) and oleanolic acid (**4.26**), [115] It has been indicated that the plant species does not contain either lantadene A (**4.9**) or lantadene B (**4.10**).[115] Oleanolic acid (**4.26**) and camaric acid (**4.27**) have also been isolated from *L. camara* whereby the former compound exhibited antiinflamatory, anticancer, antimicrobial, antifertility and hypoglycaemic activity. Some cosmetic and pharmacological preparations of compound **4.26** have also been patented in Japan.[116]



	R_1	R_2	R_3
4.23:	Me	H	H
4.24:	Me	H	OH
4.25:	H	Me	OH



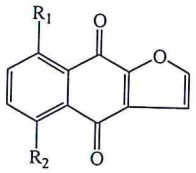
4.26



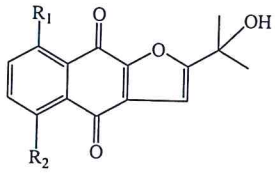
4.27

The hexane extract of the roots of a sample of *L. camara* collected from Sri Lanka, contained diodantunezone (**4.28**), previously isolated from *L. achyrantifolia* Desf. and its regioisomer **4.29**.[106,117-119] The corresponding methyl ether was suggested to occur in *L. achyrantifolia* but was not isolated.[106,117,119] Close scrutiny of the quinones isolated

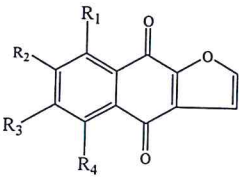
from Sri Lankan plant materials showed that compounds **4.28** and **4.29** occurred as a mixture of isomers and co-occurred with two other isomeric compounds **4.30** and **4.31**.[106,117] Other pairs of inseparable isomers **4.32**, **4.33** and **4.34**, **4.35** were also isolated from this extract. The methanol extract of the roots contained inseparable mixture of **4.36** and **4.37**.[106,117] Previously, diodantunezone (**4.28**) was synthesized and tested for cytotoxicity against KB epidermoid nasopharynx, K562 human leukaemia and P388 lymphocytic leukaemia cell lines and found to be active (IC_{50} 6.76, 9.2 and 7.94 µmol/l, respectively).[106,118] Other compounds isolated from the aerial parts of *L. achyrantifolia* include the flavones penduletin (**4.38**) and chrysosplenetin (**4.39**)[119] while *L. trifolia* yielded the flavonoid umuhengerin (**4.40**), which exhibited *in vitro* antibacterial and antifungal properties at 20 µg/ml against *Staphylococcus aureus, Salmonella typhimurium, Candida tropicalis, Aspergillus niger, A. fumigatus, Trichophyton mentagrophytes* and *Microsporum canis,* and having MIC value of 5 µg/ml against *S. typhimurium*.[120]



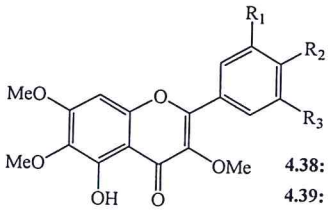
	R_1	R_2
4.28:	H	OH
4.29:	OH	H



	R_1	R_2
4.30:	OH	H
4.31:	H	OH

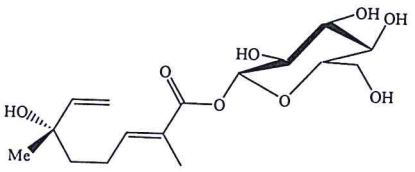


	R_1	R_2	R_3	R_4
4.32:	H	OMe	OH	H
4.33:	H	H	OMe	OH
4.34:	H	OMe	OH	OH
4.35:	OH	OH	OMe	OH
4.36:	H	H	OMe	OH
4.37:	OH	OMe	H	OH

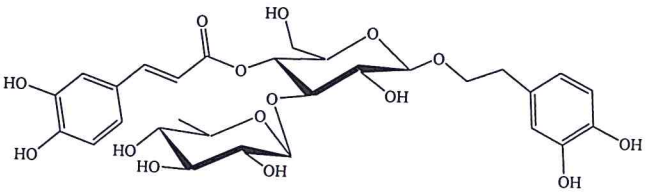


	R_1	R_2	R_3
4.38:	H	OH	OH
4.39:	OH	OH	OH
4.40:	OMe	OMe	OMe

In Brazil, *L. lilacia* Desf. is used as a carminative agent and as a decoction or a syrup for the treatment of bronchitis.[121] Upon phytochemical investigation, a monoterpene glucoside **4.41** and acteoside (verbascoside, **4.42**) were isolated. Compound **4.41** may be assumed to be responsible for the claimed medicinal use.[121]



4.41



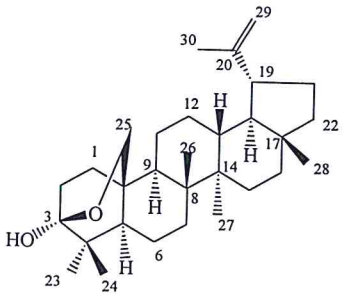
4.42

In the present investigation of the dichloromethane extract of the root bark of *L. virbunoides* ssp *virbunoide* var. *kisi*, the lupane triterpenoids lantalupeol (**4.43**) and betulinic acid (**4.44**), and the oleanolic acid derivative camaric acid (**4.27**) were isolated as well as the hirtheto unreported peroxide dimer of 4-methoxy-3-methylphenol (**4.45**), four inseparable mixtures of furanonaphthaquinones consisting of the isomers 6-hydroxy-naphtho[2,3-b]furan-4,9-dione (**4.46**), 7-hydroxy-naphtho[2,3-b]furan-4,9-dione (**4.47**) and naphtho[2,3-b]furan-4,9-dione (**4.48**); 6-methoxy-naphtho[2,3-b]furan-4,9-dione (**4.32**) and 7-methoxy-naphtho[2,3-b]furan-4,9-dione (**4.33**); and 6-hydroxy-2-(1-hydroxy-1-methyl-ethyl)-naphtho[2,3-b]furan-4,9-dione (**4.49**), 7-hydroxy-2-(1-hydroxy-1-methyl-ethyl)-naphtho[2,3-b]furan-4,9-dione (**4.50**) and 2-(1-hydroxy-1-methyl-ethyl)-naphtho[2,3-b]furan-4,9-dione (**4.51**), a mixture of 6-Hydroxy-2-(1-hydroxy-1-methyl-ethyl)-dihydronaphtho[2,3-b]furan-4,9-dione (**4.52**); 7-ydroxy-2-(1-hydroxy-1-methyl-ethyl)-dihydronaphtho[2,3-b]furan-4,9-dione (**4.53**) and 2-(1-hydroxy-1-methyl-ethyl)-dihydronaphtho[2,3-b]furan-4,9-dione (**4.54**); and stigmasterol

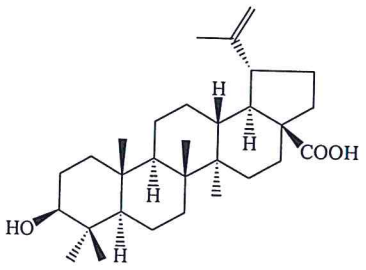
(**4.55**). The isolation and structural determination of the compounds and mosquito larvicidal activity are hereby being reported.

4.2 RESULTS AND DISCUSSION

4.2.1 Lantalupeol (4.43) and betulinic acid (4.44)



4.43



4.44

Silica gel column chromatography of the dichloromethane extract from the root bark of *L. viburnoides* ssp *viburnoides* var. *kisi* (A. Rich) Verdc. yielded several fractions as complex mixtures of compounds (**Chart 4.1**) which were detected only upon spraying with an anisaldehyde followed by heating at about 100 °C. Lantalupeol (**4.43**) was obtained as the major constituent in these mixtures upon repeated chromatography of the combined fractions containing that compound. The related compound betulinic acid (**4.44**) was isolated only in the second workup of another batch of plant material (**Chart 4.2**) and in this case lantalupeol was neither isolated nor detected. Structures **4.43** and **4.44** were established on the basis of interpretation of their NMR and mass spectral data (**Figs 4.1-4.10**) in comparison with those reported in the literature for betulinic acid (**4.44**).[122]

Besides several complex signals typical for pentacyclic triterpenoids, the high field region of the ^{1}H NMR spectrum of each compound (**Figs 4.1** and **4.4**) consisted of signals due to protons corresponding to six quaternary methyl groups, one of which is allylic (δ, 1.69 for lantalupeol and 1.68 for betulinic acid (**Table 4.1**) as expected for structures **4.43** and **4.44**. Furthermore, the ^{1}H and ^{13}C NMR spectra (**Figs 4.1-4.6**) of the two compounds revealed signals at δ_H 4.74 (br *s*) and 4.61 (br *s*), and δ_C 150.43 (Cq) and 109.71 (CH_2) for compound **4.43** and at δ_H 4.73 (br *s*) and 4.61 (br *s*) and δ_C 149.48 (Cq) and 107.63 (CH_2) for compound **4.44 (Table 4.1)**. These signals were ascribed to an exocyclic isopropenyl unit in each of the two compounds, a feature that is pertinent to lupane triterpenoids.[122-125] While the ^{1}H and ^{13}C NMR spectra of compound **4.44** exhibited signals expected for a C-3 carbinol group (δ_H 3.12, *dm*, and δ_C 76.82), such signals were not observed in the corresponding spectra for compound **4.43**. Instead the ^{1}H and ^{13}C NMR spectra of the latter compound consisted of a singlet at δ 4.01 (*s*, 2H), and a carbon resonance at δ 79.0 due to an oxygenated methylene group. Furthermore, the ^{13}C NMR spectrum exhibited an additional signal in the low field region at 113.51 (Cq), which could not be ascribed to an unsaturated carbon atom since it was the only one. Thus, these spectral features suggested the presence of a hemiketal unit in the molecule. It was conceived on biosynthetic considarations that C-3 was the hemiacetal carbon and therefore responsible for ^{13}C NMR signal at δ 113.51 (Cq) forming a 3,25-oxygen bridge since the ^{1}H NMR spectrum lacked an H-25 resonance at *ca.* δ 0.88 as in the spectrum for compound **4.44**.

The MS of betulinic acid consisted of a molecular ion peak at *m/z* 456 corresponding to the molecular formula $C_{30}H_{48}O_3$. Loss of a water molecule from the molecular ion gave a fragment ion at *m/z* 438 followed by cleavage of ring C to give a base peak at *m/z* 189 (**Scheme 4.1**). Other fragment ions (**Scheme 4.1**) were in agreement with the fragmentation process reported for betulinic acid.[122] Lantalupeol (**4.43**) was obtained in a small quantity and hence no sample was available for MS analysis. Furthermore, lantalupeol was analyzed in a low resolution NMR spectrometer (200 MHz), some of the peaks in both the ^1H and ^{13}C NMR spectra were weak or not detected. Again, as expected, assignment of all proton resonances in both compounds **4.43** and **4.44** could not be completed due to the overlapping nature of the signals in these triterpenoids.

Table 4.1 ^1H NMR spectral data for lantalupeol (4.43) and betulinic acid (4.44)

H	4.43		4.44			
	$^c\delta_{Observed}$	Multiplicity	$^a\delta_{Observed}$	Multiplicity	$^b\delta^{122\text{-}125}$	Multiplicity
3	-		3.12	*dm*	3.19	*dm*
19	3.15	*m*	2.37	*m*	2.42	*m*
23	0.82	*s*	0.77	*s*	0.82	*s*
24	1.26	*s*	1.02	*s*	1.03	*s*
25	4.01	*s*	0.88	*s*	0.87	*s*
26	0.93	*s*	0.98	*s*	0.93	*s*
27	0.97	*s*	0.97	*s*	0.95	*s*
28	0.75	*s*	-	-	-	-
29a	4.61	*s*	4.61	*s*	4.60	*s*
29b	4.74	*s*	4.73	*s*	4.74	*s*
30	1.69	*s*	1.68	*s*	1.69	*s*

[a]run in 1:1 v/v MeOD and Me_2CO; [b]run in pyridine-d_5; [c]run in $CDCl_3$

Table 4.2 ^{13}C NMR spectral data for lantalupeol (4.43) and betulinic acid (4.44)

C	4.43	4.44	
	$^{c}\delta_{Observed}$	$^{a}\delta_{Observed}$	$^{b}\delta^{122\text{-}125}$
1	38.29	35.46	38.5
2	27.32	-	28.2
3	113.51	76.82	78.1
4	40.62	39.35	39.4
5	55.26	54.20	55.9
6	19.33	19.52	18.7
7	32.11	33.02	34.7
8	38.83*	37.37	41.0
9	50.44	-	50.9
10	37.14	37.45	37.5
11	-	24.28	21.1
12	-	25.62	26.0
13	-	36.98	39.2
14	-	41.01	42.8
15	29.65	29.16	30.8
16	34.24	35.76	32.8
17	42.38	54.74	56.6
18	49.19	49.34	49.7
19	46.82	47.84	47.7
20	150.43	149.48	151.4
21	30.47	30.72	31.1
22	38.63*	-	37.4
23	27.96	26.16	28.5
24	15.99	13.67	16.2
25	79.00	14.26	16.3
26	16.12	14.21	16.2
27	14.66	12.66	14.8
28	18.25	176.51	179.0
29	109.71	107.63	110.0
30	20.79	17.12	19.4

*interchangable assignment; [a]run in 1:1 v/v MeOD and Me_2CO; [b]run in pyridine-d_5; [b]run in $CDCl_3$

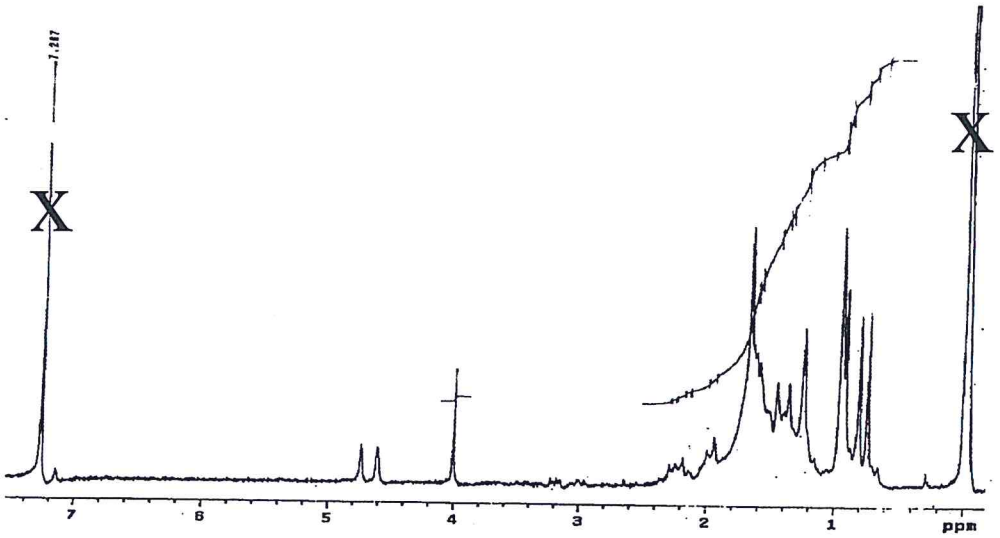


Fig 4.1 ^{1}H NMR spectrum (200 MHz, $CDCl_3$) of lantalupeol (4.43)

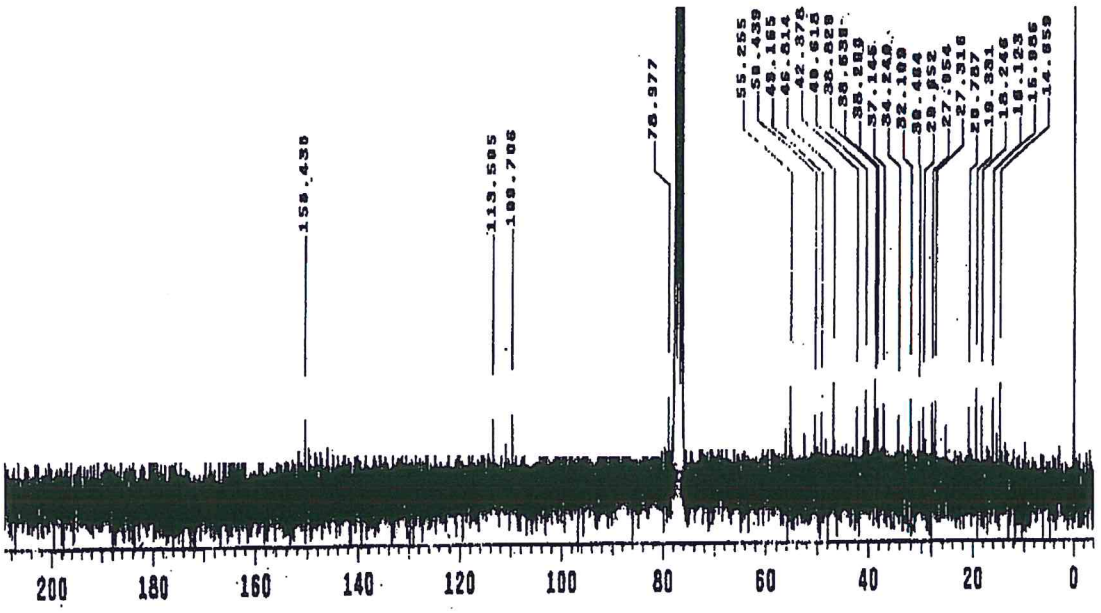


Fig 4.2 ^{13}C NMR spectrum (200 MHz, $CDCl_3$) of lantalupeol (4.43)

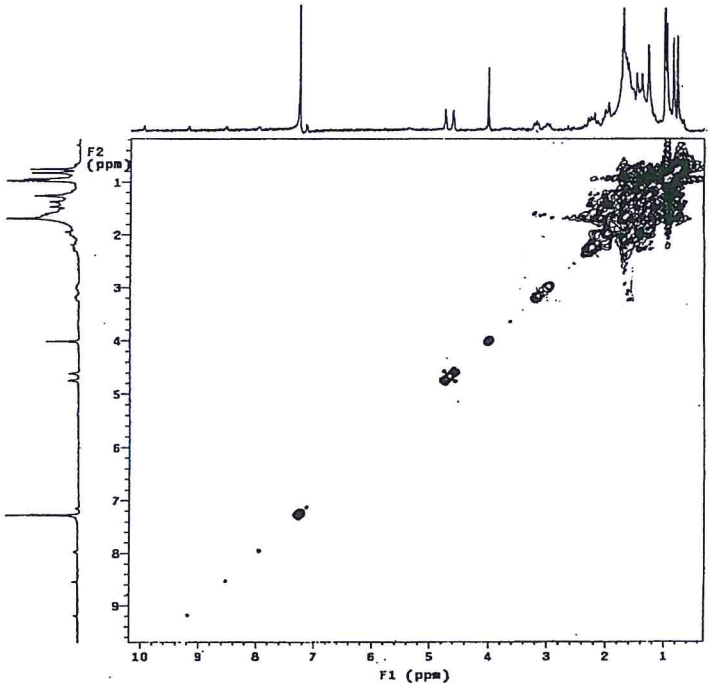


Fig 4.3 H/H COSY Plot (200 MHz, $CDCl_3$) for lantalupeol (4.43)

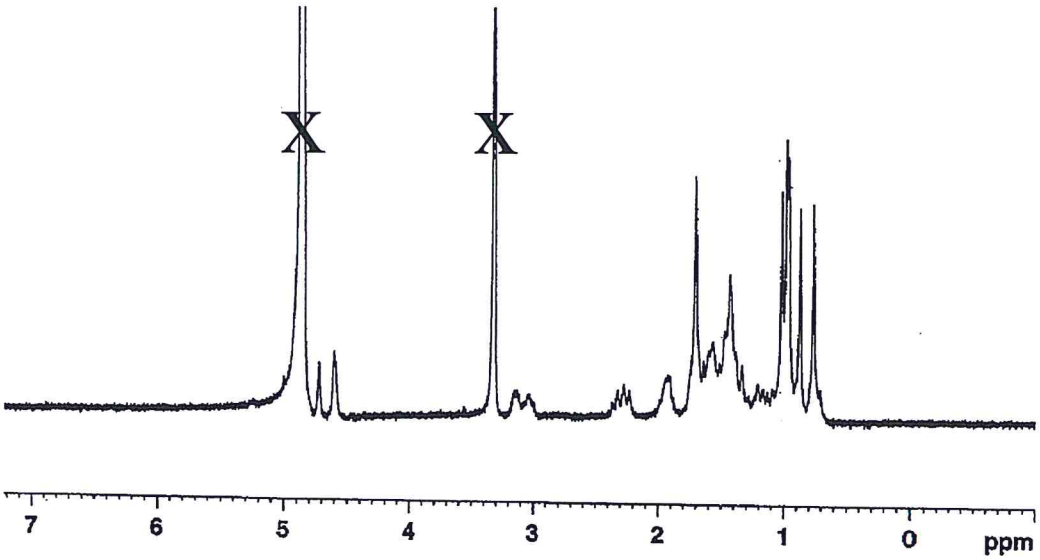


Fig 4.4 ^1H NMR spectrum (300 MHz, MeOD and Me_2CO) of betulinic acid (4.44)

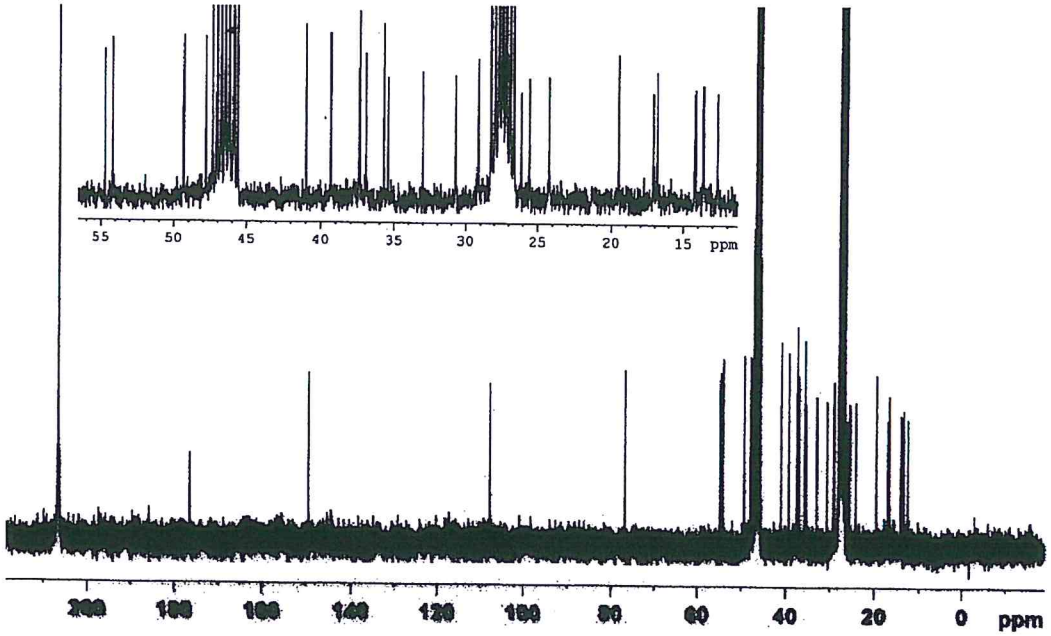


Fig 4.5 ^{13}C NMR spectrum (300 MHz, MeOD) of betulinic acid (4.44)

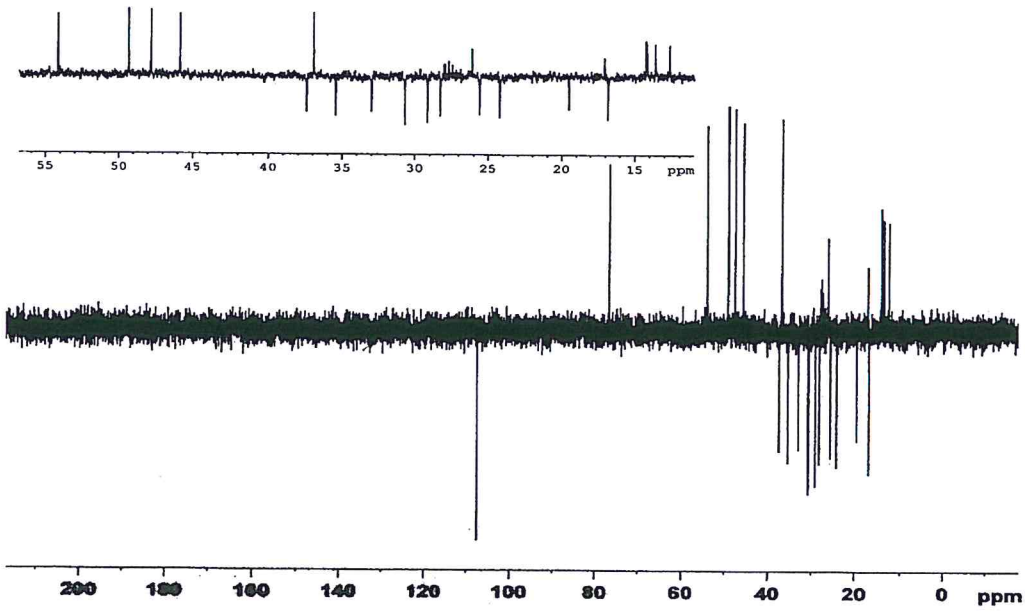


Fig 4.6 DEPT 135 spectrum (300 MHz, MeOD and Me_2CO) for betulinic acid (4.44)

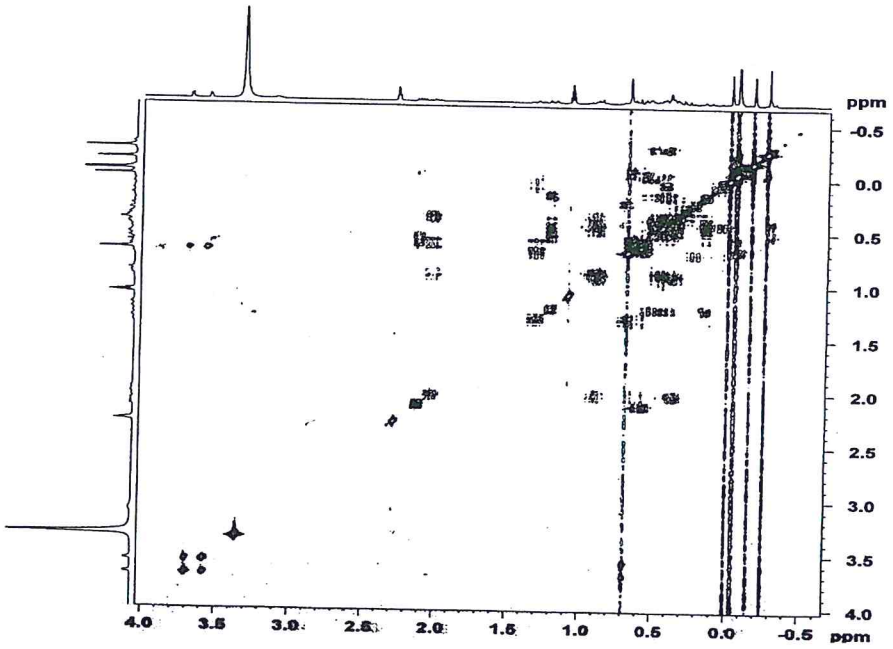


Fig 4.7 **H/H COSY plot (300 MHz, MeOD and Me_2CO) for betulinic acid (4.44)**

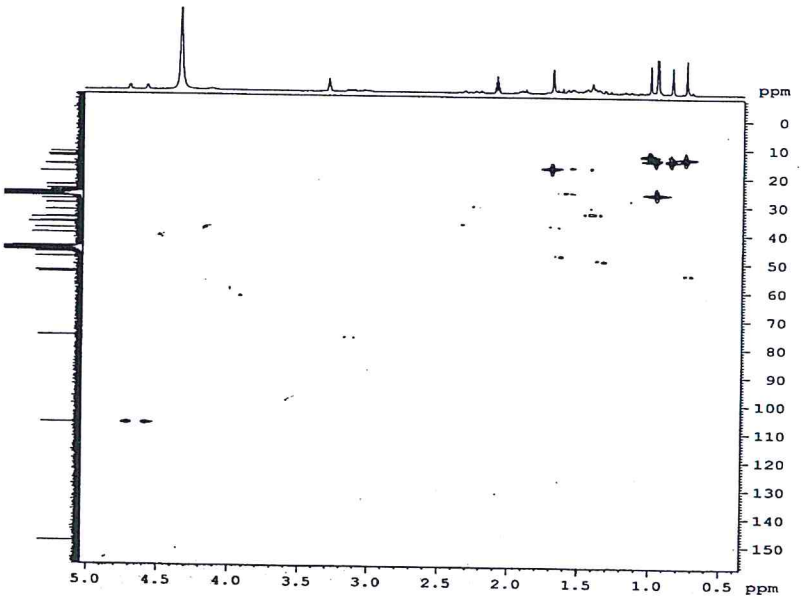


Fig 4.8 **HMQC plot (300 MHz, MeOD and Me_2CO) for betulinic acid (4.44)**

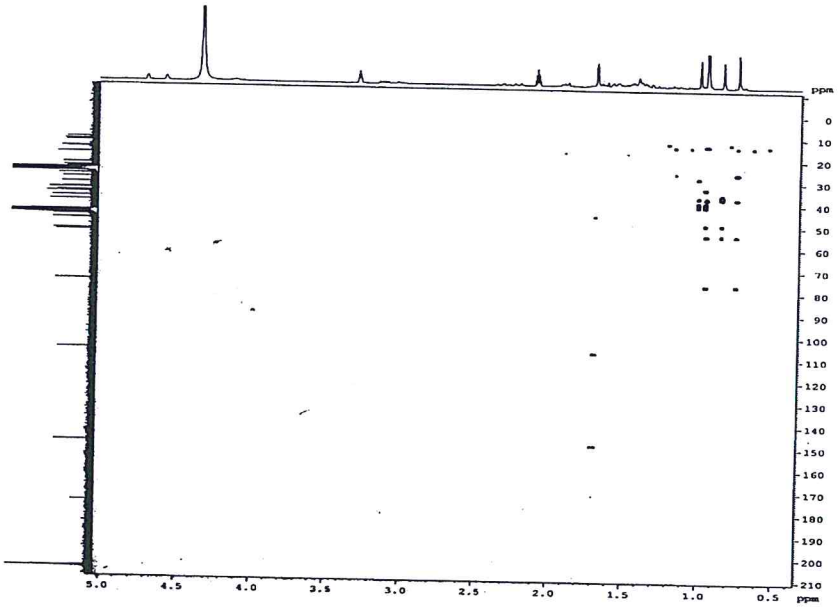


Fig 4.9a HMBC plot (300 MHz, MeOD and Me_2CO) for betulinic acid (4.44)

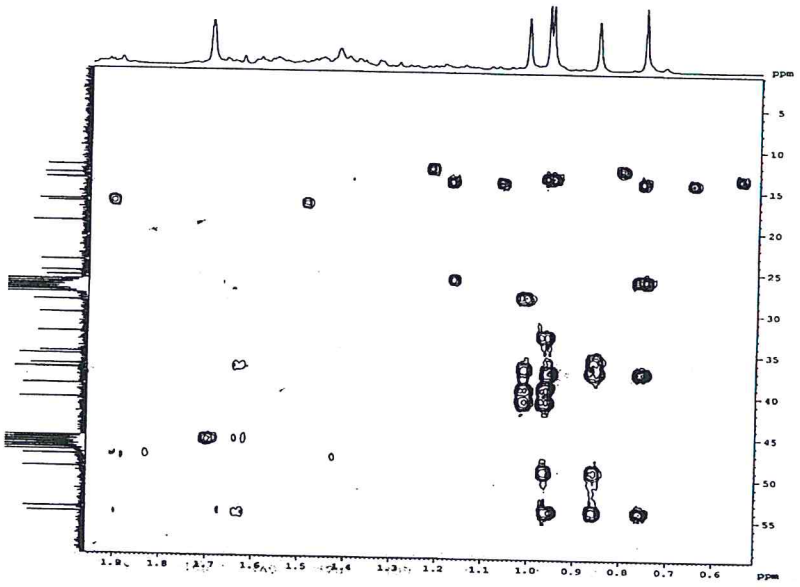


Fig 4.9b Expansion of HMBC plot (300 MHz, MeOD and Me_2CO) for betulinic acid (4.44) between δ 0.5-2.0 and 0-60 ppm

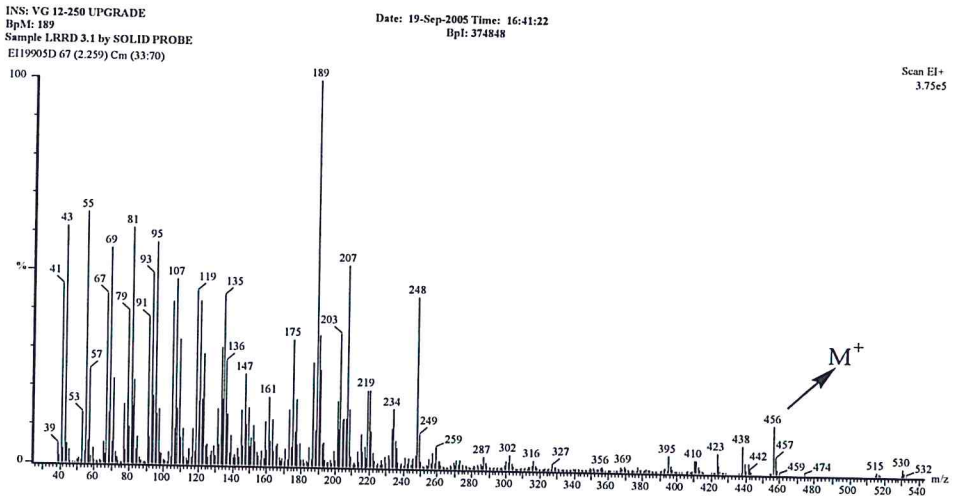
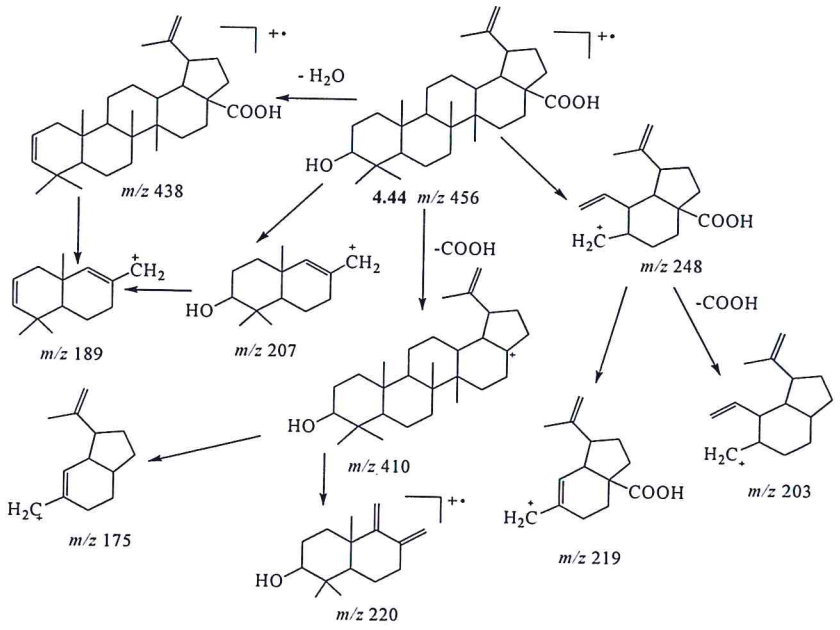


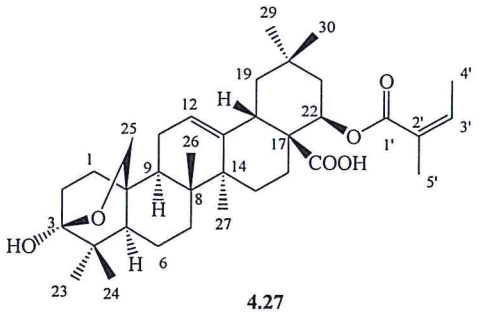
Fig 4.10 EI-Mass spectrum of betulinic acid (4.44)



Scheme 4.1 Proposed mass spectral fragmentation pattern for betulinic acid (4.44)

These investigations have indicated that *L. virburnoides* ssp *virbunoides* var. *kisi* accumulates each of the two lupane triterpenoids lantalupeol (**4.43**) and betulinic acid (**4.44**) in different seasons. Thus, while the less stable hemiacetal lantalupeol (**4.43**) is accumulated during the wet season (around April), betulinic acid (**4.44**) is biosynthesized during the dry season (around October). Such information would be important should the two compounds be found to have viable usefulness in mosquito control, medicinal applications etc, and hence their occurrence pattern would guide the exploitation scheme for the lupanoids.

4.2.2 Camaric acid (4.27)



4.27

Repeated column chromatography of the fourth and fifth VLC fractions of the dichloromethane extract of the root bark of *L. viburnoides* ssp *viburnoides* var. *kisi* yielded 325 mg of a compound which was identified to be an acrylic ester derivative of an oleanane type triterpenoid, having structure **4.27**. The structure **4.27** for the isolated compound was established on the basis of ^{1}H (**Fig 4.11**) and ^{13}C NMR (**Fig 4.12**), IR and mass spectral data as compared with reported literature values for camaric acid.[109] The ^{1}H NMR spectrum (**Fig 4.11**; **Table 4.3**) consisted of a complex signal pattern in the high field region as expected for oleanane triterpenoids. Furthermore, the ^{1}H NMR

spectrum displayed singlets due to protons of six methyl units instead of eight methyl groups expected for underivatized oleanane triterpenoids. This suggested that, two of the oleanane methyl groups were derivatized. In fact, one of the methyl carbon atoms was envisaged to have formed an ether linkage with C-3, since the ^{1}H and ^{13}C NMR spectra did not exhibit signals due to carbinol H-3 and C-3, which is usually the case in oleanane triterpenoids as dictated by biosynthetic considarations. Instead, a quaternary carbon resonance was observed in the ^{13}C NMR spectrum at δ 98.08 that was conceived to be due to a hemiacetal C-3, with the hemiketal oxygen bridge also being attached to C-25 since H-25 resonance at *ca.* δ 0.88 that is observed in oleanolic acid[109,110] was not observed in the ^{1}H NMR spectrum for **4.27**. Instead, two diastereotopic proton signals were observed at δ 4.29 and 3.91 for the oxygenated C-25 in **4.27** (**Table 4.3**).

Biosynthetic considarations led to the assignment of the second derivatized methyl group at C-17, the substituent being a carboxylic unit that accounted for the ^{13}C NMR resonance at δ 176.55 and the enhanced deshielding of H-18 (from δ 2.8 in oleanane to 3.06 in structure **4.27**) at the β-position relative to the carboxylic group.[109]

The ^{1}H and ^{13}C NMR spectra also exhibited signals due to a side chain acrylic acid ester moiety (**Table 4.3**). A three-proton quintent at δ 1.78, a doublet of quartets at δ 1.94 and a one-proton quartet of quartets at δ 6.06 confirmed the presence of 2,3-dimethyl acrylic acid ester with the *Z*-configuration.[109,110]

However, it was difficult to establish unambiguously the position of the acrylonyl unit based on the available spectral evidence. Comparison of the spectral data for the isolated compound with those of camaric acid confirmed the acrylonyl unit to be at C-22.[109,110]

The MS (**Fig 4.18**) exhibited a molecular ion peak at *m/z* 568 corresponding to the molecular formula $C_{35}H_{52}O_6$. The base peak was observed at *m/z* 83 corresponding to the fragment ion $C_5H_7O^+$, while the fragment ion at *m/z* 468 was conceived to result from loss of 100 atomic mass units from the molecular ion, which was indicative of a pentacyclic triterpenoid having an α,β–dimethylacrylic acid ester side chain. The MS fragmentation pattern also indicated the acrylic acid ester moiety to have been substituted in the E ring. These observations, together with the appearance of characteristic fragment ions at *m/z* 246 and 285 in the MS, that were due to a retro-Diels-Alder fragmentation process[126] (**Schemes 4.2**) was indicative of structure **4.27** for the isolated compound.

Table 4.3 ^{1}H NMR spectral data of camaric acid (4.27)

	[a]Observed		[b]Reported[109]	
H	δ	***J* (Hz)**	δ	***J* (Hz)**
11a	2.05	*m*	2.0	*m*
11b	1.8	*m*	1.8	*m*
12	5.36	*br s*	5.36	*br s*
18	3.06	*dd*, $J_{18,19a}$ = 13.68, $J_{12,18}$ = 4.1	3.03	*dd*, $J_{18,19a}$ = 13.8, $J_{12,18}$ = 3.7
21	1.21	*m*	-	-
22	5.06	*t*, $J_{21,22}$ = 3.15	5.00	*t*, $J_{21,22}$ = 3.00
23	0.99	*s*	1.01	*s*
24	0.82	*s*	0.75	*s*
25a	4.29	*dd, ABX*, $J_{25a,25b}$ = 8.5, $J_{9,25a,}$ = 2.5	4.21	*dd,* ABX, $J_{25a,25b}$ = 8.38, $J_{9,25a}$ = 2.28
25b	3.91	*d*, , $J_{25a,25b}$ = 8.5	3.88	*d*, , $J_{25a,25b}$ = 8.38
26	0.93	*s*	0.99	*s*
27	1.21	*s*	0.99	*s*
29	1.04	*s*	0.88	*s*
30	1.04	*s*	1.14	*s*
3'	6.06	*qq*, $J_{3',4'}$ = 7.2, $J_{3',5'}$ = 1.5	5.97	*qq*, $J_{3',4'}$ = 7.24, $J_{3',5'}$ = 1.52
4'	1.96	*dm*, , $J_{3',4'}$ = 7.2, $J_{3',5'}$ = 1.5	1.94	*dq*, $J_{3',4'}$ = 7.24, 1.52
5'	1.83	*dm*, $J_{3',5'}$ = 1.5	1.78	*qn*, $J_{3',5'}$ = 1.52

[a]run in MeOD; [b] run in $CDCl_3$

Table 4.4 ^{13}C NMR spectral data of camaric acid (4.27)

C	$^{a}\delta$ observed	$^{b}\delta^{109}$	C	$^{a}\delta$ observed	$^{b}\delta^{109}$
1	-	34.7	19	45.96	45.9
2	27.79	27.8	20	29.95a	30.1
3	98.08	98.9	21	37.91	37.9
4	38.54	38.4	22	76.67	76.1
5	50.65	50.4	23	26.61	27.3
6	-	19.7	24	16.36	17.3
7	31.35a	31.0	25	67.59	67.7
8	40.18	40.3	26	18.27	18.3
9	-	42.0	27	-	26.2
10	35.03	35.2	28	176.55	178.9
11	23.38	23.8	29	-	33.7
12	122.76	122.8	30	-	25.4
13	143.42	143.1	1'	166.98	166
14	42.24	42.0	2'	128.24	127
15	29.33	29.5	3'	138.02	138
16	-	24.3	4'	15.3	15.6
17	50.85	50.8	5'	23.38	20.5
18	39.79	39.2			

[a]run in MeOD; [b]run in $CDCl_3$

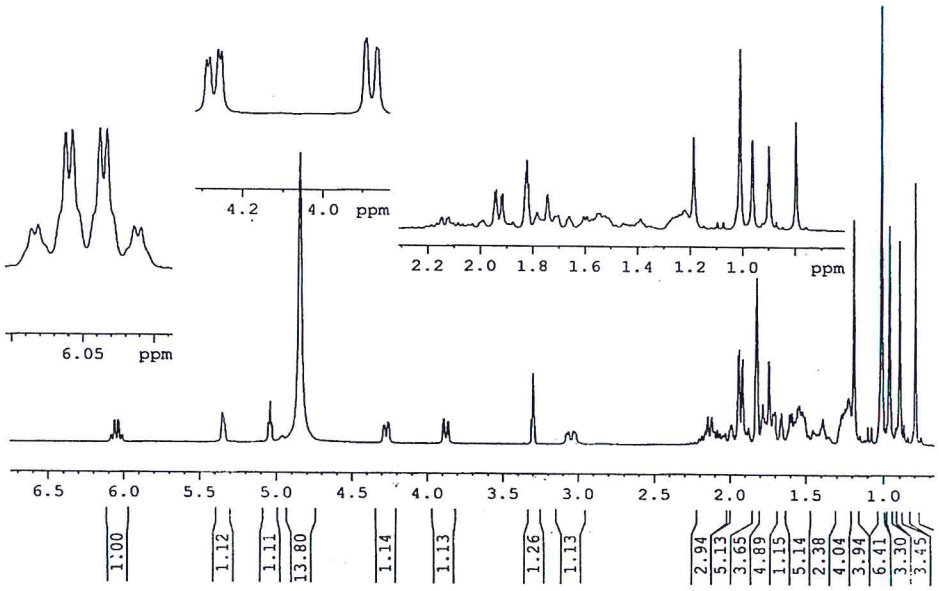


Fig 4.11 ^{1}H NMR spectrum (300 MHz, MeOD) of camaric acid (4.27)

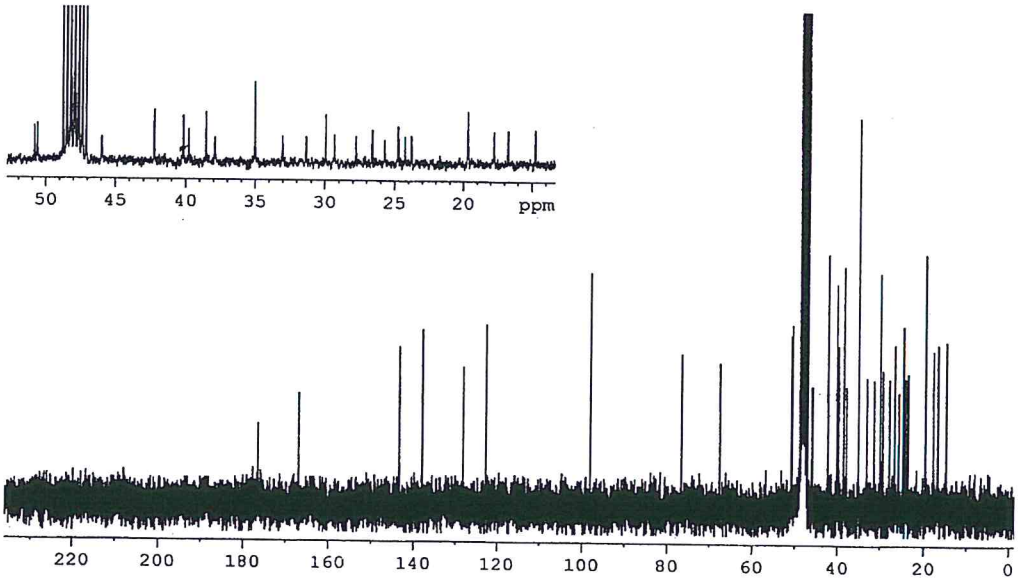


Fig 4.12 ^{13}C NMR spectrum (300 MHz, MeOD) of camaric acid (4.27)

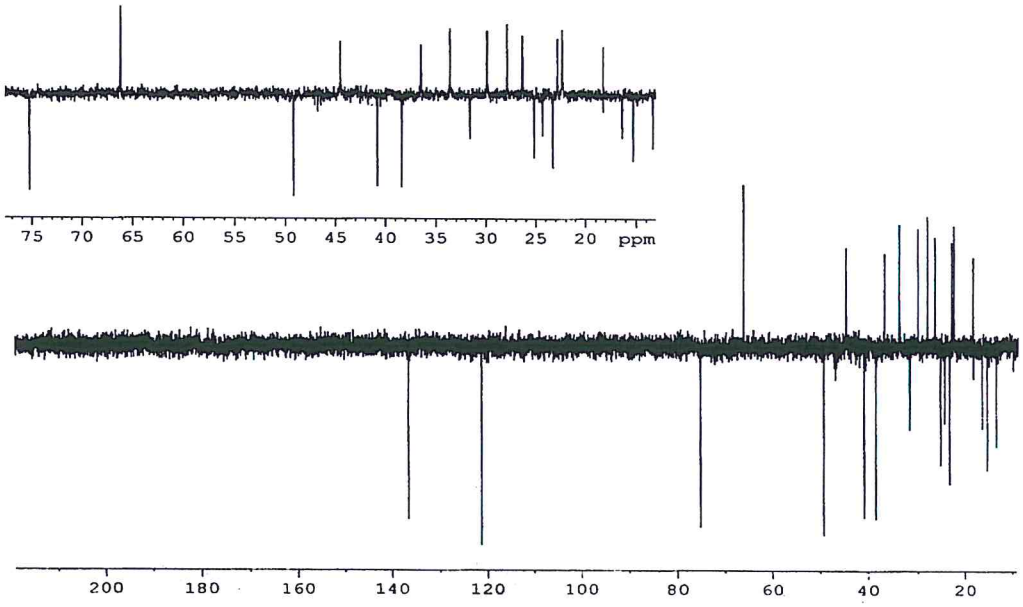


Fig 4.13 **DEPT 135 (Inverse phase) NMR spectrum (300 MHz, MeOD) for camaric acid (4.27)**

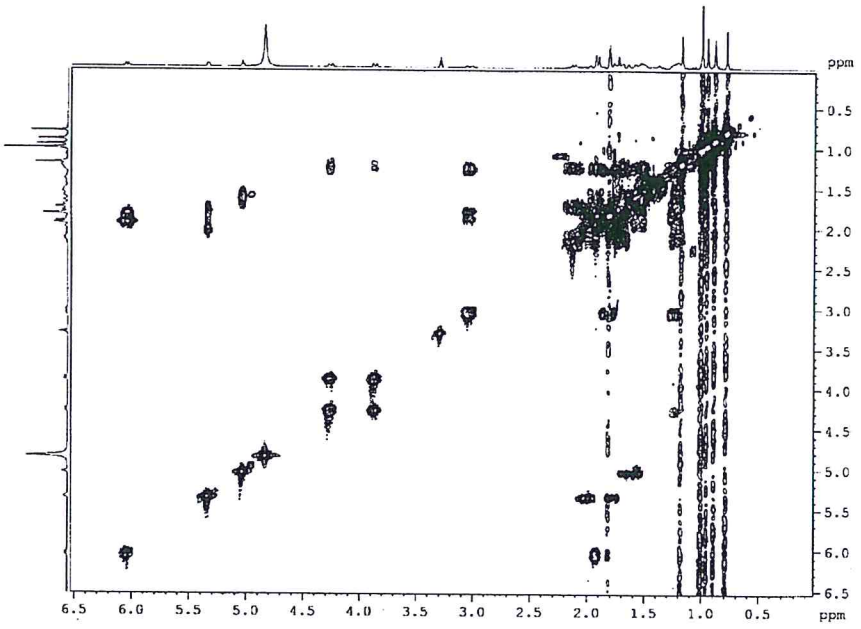


Fig 4.14 **H/H COSY plot (300 MHz, MeOD) for camaric acid (4.27)**

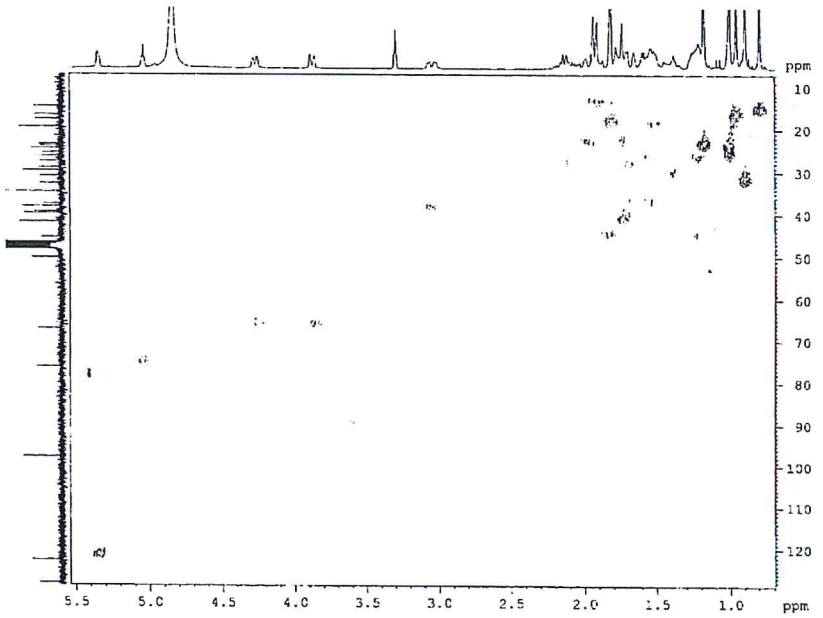


Fig 4.15 HMQC plot (300 MHz, MeOD) for camaric acid (4.27)

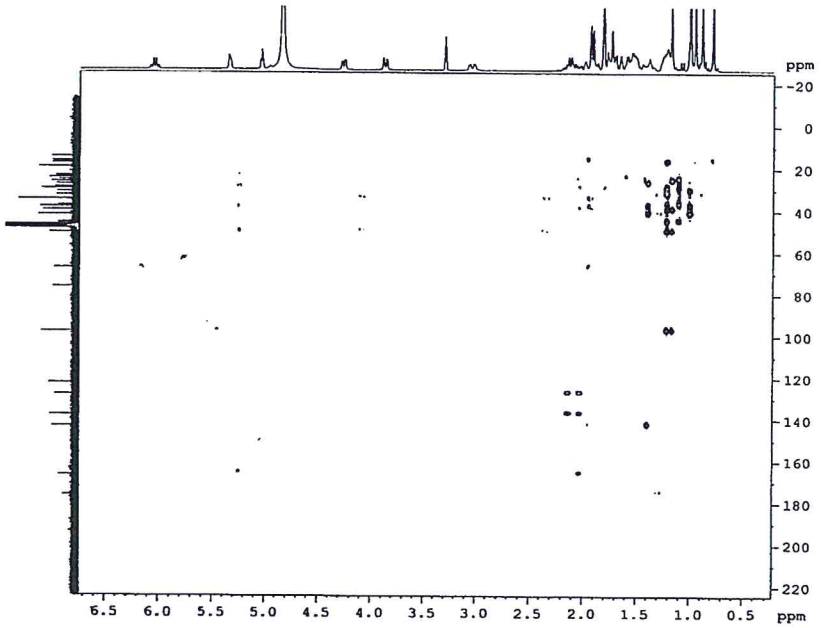


Fig 4.16 HMBC plot (300 MHz, MeOD) for camaric acid (4.27)

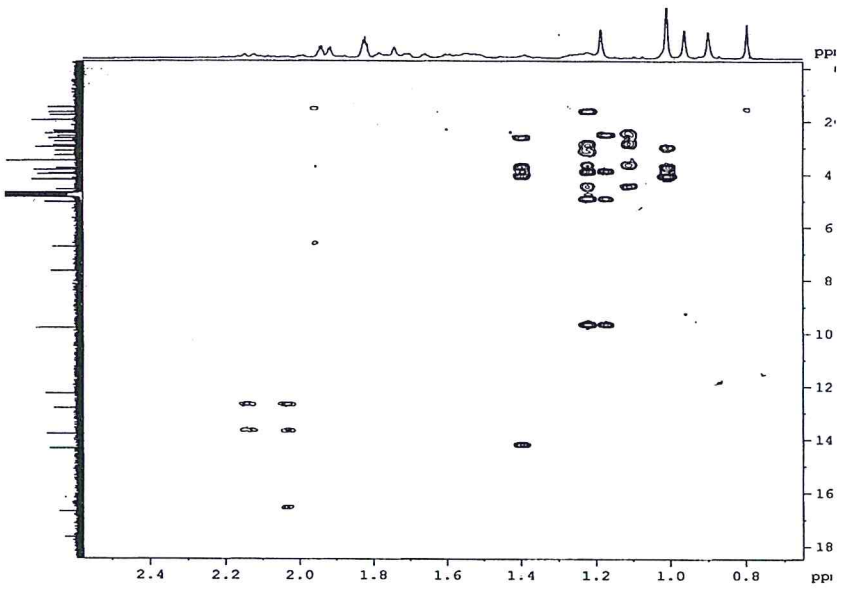


Fig 4.17 **Expansion of HMBC plot (300 MHz, MeOD) for camaric acid (4.27) between δ 0.6-2.6 and 0-180 ppm**

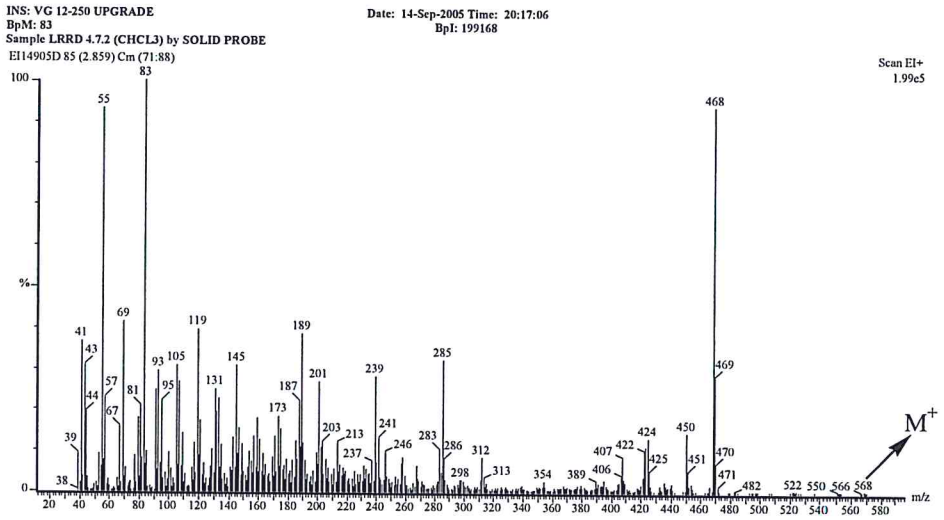
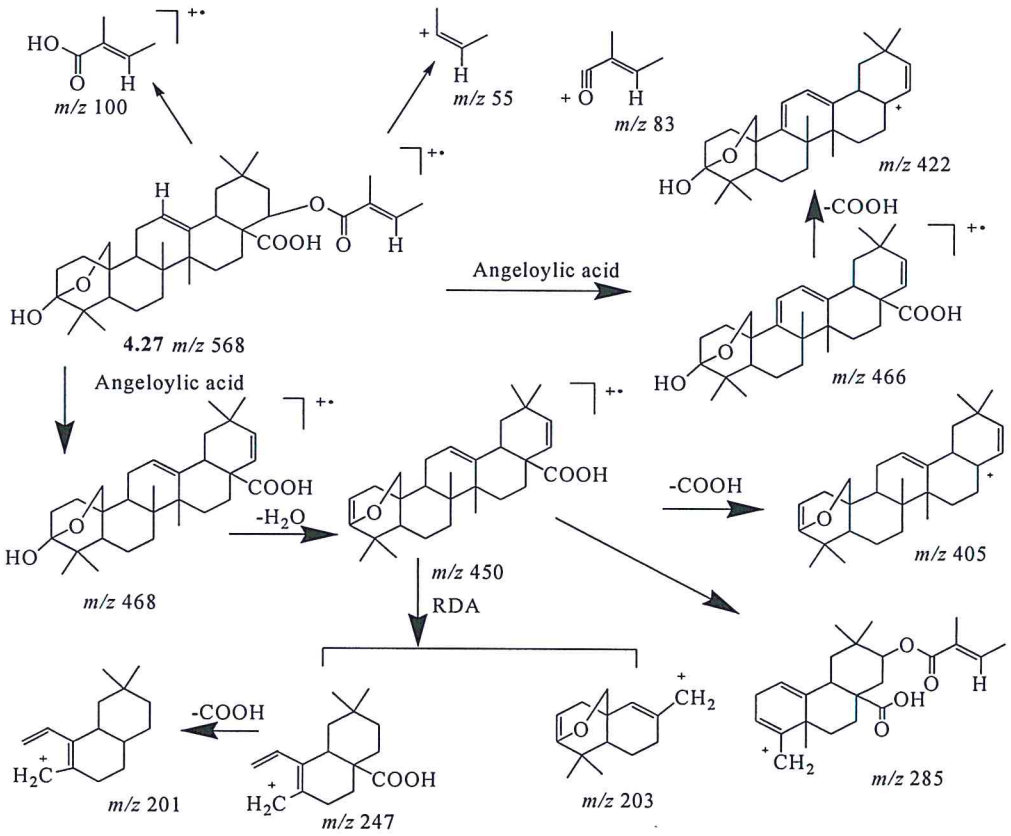
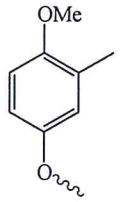


Fig 4.18 **EI-Mass spectrum of camaric acid (4.27)**

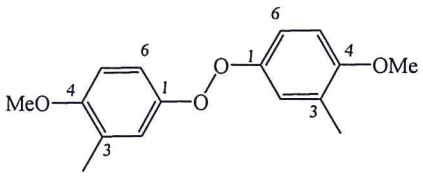


Scheme 4.2 Proposed mass spectral fragmentation pattern for camaric acid (4.27)

4.2.3 Lantaperoxide (*bis*-4-methoxy-3-methyl-phenoxide) (4.45)



4.45a



4.45

Repeated column chromatography of the fourth VLC fraction of the dichloromethane extract of the root bark of *L. viburnoides* ssp *virbunoides* var. *kisi* yielded greenish oil

whose structure **4.45** was established on the basis of ^{1}H and ^{13}C NMR, H/H COSY and mass spectral data. The ^{1}H NMR spectrum (**Fig 4.19**) showed an arylmethoxy proton signal at δ 3.77 (s, 3H) and an arylmethyl proton signal at δ 2.23 (s, 3H), presence of which was also indicated in the ^{13}C NMR (δ_{OMe} 55.59 and δ_{Me} 18.55, respectively). Furthermore, the ^{1}H NMR spectrum exhibited three signals in the aromatic region at δ 6.65 (d, J = 7.8 Hz), 6.55 (dd, J_1 = 7.8, J_2 = 1.8 Hz) and 6.71 (d, J = 1.8 Hz) due to *ortho* and *meta* coupling protons H-2 and H-3, and H-3 and H-5, respectively in partial structure **4.45a**. The ^{13}C NMR spectrum with a DEPT 135 experiment (**Fig 4.20**) showed six aromatic carbon signals, three of them being substituted and these also corresponded to partial structure **4.45a**. That the compound was a asymmetrical dimmer forming a peroxide bridge and the proposed partial structure **4.45a** as the monomeric unit was deduced from the MS, which revealed the molecular ion peak at *m/z* 274 corresponding to the formula $C_{16}H_{18}O_4$ and a fragment ion peak for the proposed partial structure **4.45a** at *m/z* 137 (**Fig 4.21**). Appearance of a methoxy carbon resonance in the ^{13}C NMR spectrum at δ 55.59 showed that the corresponding methoxy group was flanked by only one substituted *ortho* carbon as in the proposed partial structure **4.45a**.[127,128] Literature search indicated that compound **4.45** has not been reported and hence it has been given the trivial name lantaperoxide.

Table 4.5 ^{1}H and ^{13}C NMR spectral data of lantaperoxide (4.45)

H*/C	δ_H	*J (Hz)*	δ_C
1	-		147.38
2	6.65	d, $J_{2,3}$ = 7.8	113.78
3	6.55	dd, $J_{2,3}$ = 7.8, $J_{3,5}$ = 1.8	111.25
4	-		143.59
5	6.71	d, $J_{3,5}$ = 1.8	119.83
6	-		126.98
1-OCH_3	3.77	s,	55.59
6-CH_3	2.23	s	18.55

^{1}H NMR spectrum in MeOD, OH signal not observed

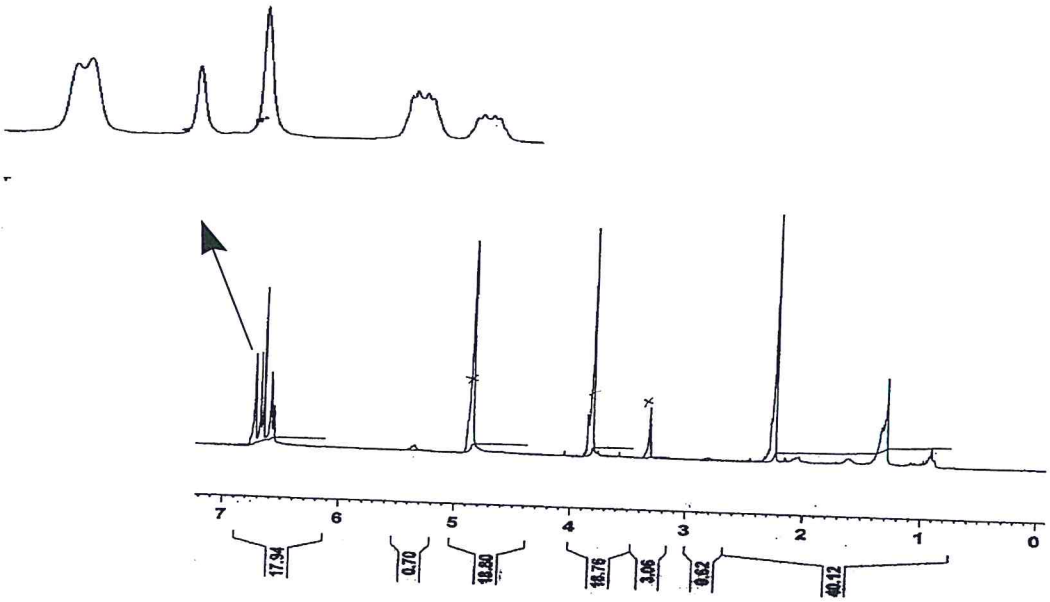


Fig 4.19 ^{1}H NMR spectrum (300 MHz, MeOD) of lantaperoxide (4.45)

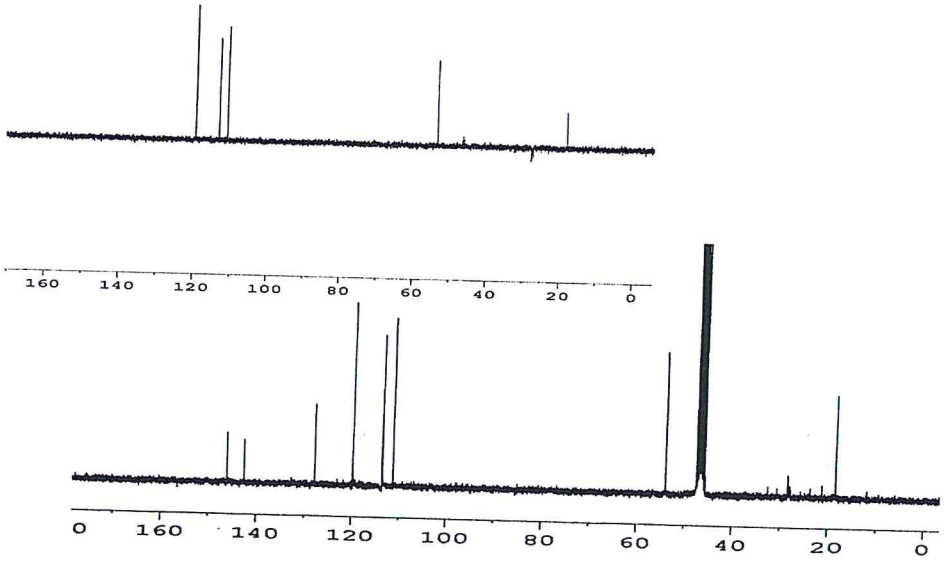


Fig 4.20 ^{13}C NMR spectrum (300 MHz, MeOD) of lantaperoxide (4.45)

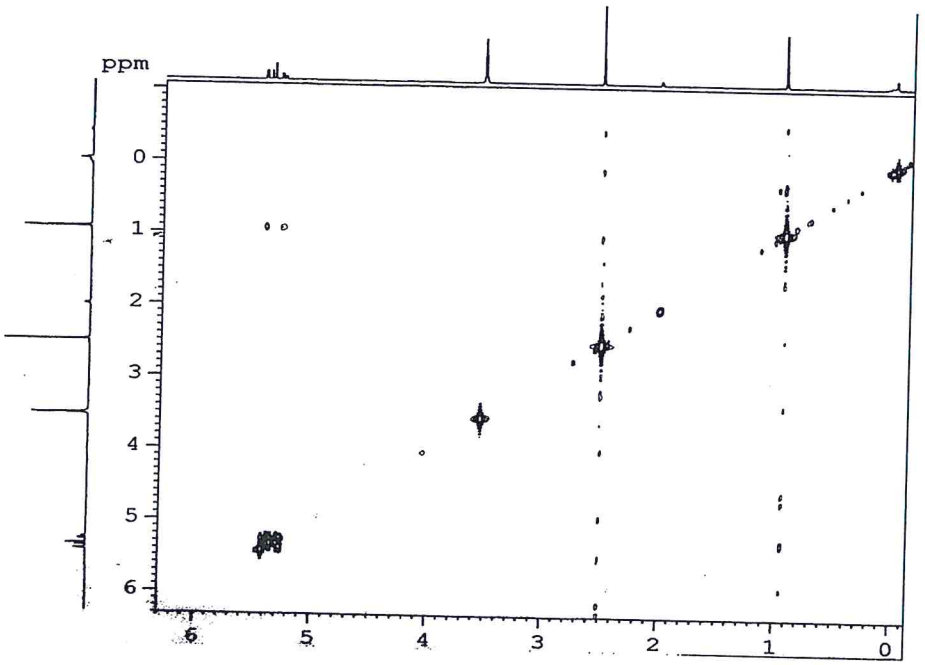


Fig 4.21 H/H COSY plot (300 MHz, MeOD) for lantaperoxide (4.45)

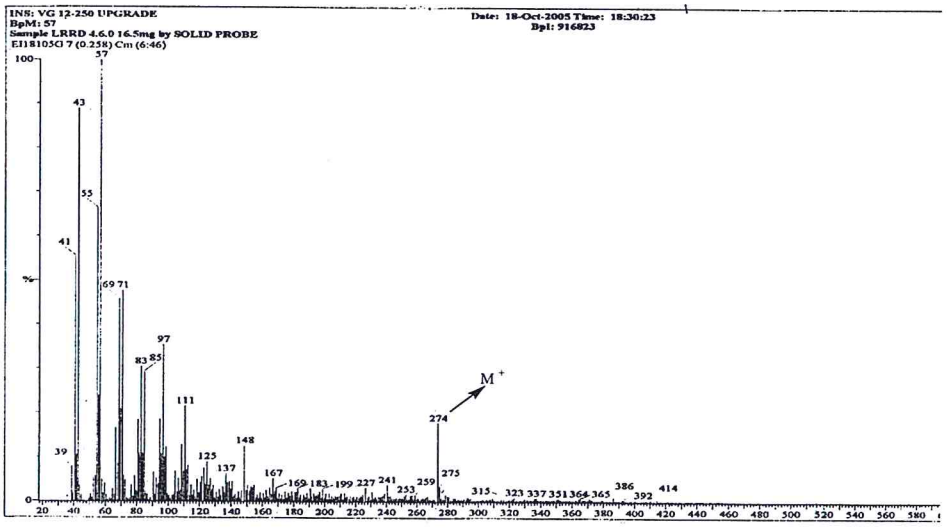
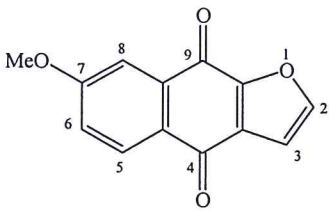
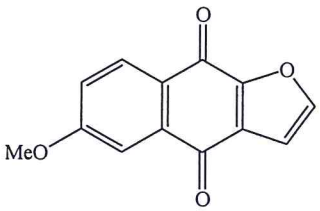


Fig 4.22 EI-Mass spectrum for lantaperoxide (4.45)

4.2.4 7-Methoxy-naphtho[2,3-b]furan-4,9-dione (4.32) and 6-methoxy-naphtho[2,3-b]furan-4,9-dione (4.33)



4.32



4.33

Repeated column chromatography of the second VLC fraction of the dichloromethane extract of the root bark of *L. viburnoides* ssp *viburnoides* var. *kisi* yielded light yellow crystals. The ^{1}H and ^{13}C NMR spectral features (**Figs 4.23-4.26**) indicating the presence of the two regio-isomers **4.32** and **4.33** as the major and minor components, respectively. The UV spectrum of the mixture exhibited absorptions at 214, 255, 299 and 339 nm indicating the conjugation nature of each of the two compounds. The ^{13}C NMR spectrum (**Fig 4.26**) of the mixture showed duplicate signals due to 13 carbon atoms in each

compound, consisting of two pairs of carbonyl, 10 aromatics (5 x Cq and 5 x CH) and one methoxyl carbon.

The skeletal framework and substitution pattern in each of the two compounds was deduced from close examination of the ^{1}H and ^{13}C NMR spectra (**Figs 4.23-4.26**). as further verified from H/H and C/H interactions observed in the COSY, HMQC and HMBC spectra (**Figs 4.27-4.31**). Thus, the ^{1}H NMR spectrum consisted of a superimposed methoxy proton signal for the two compounds (δ 3.96, s) as revealed from the integration ratio; one set of signals due to *ortho* coupling protons appearing at δ 8.15 (d, J = 8 Hz) and δ 7.36 (dd, J_1 = 8, J_2 = 2.5 Hz) and at δ 8.04 (d, J = 8 Hz) and δ 7.35 (dd, J_1 = 8 Hz, J_2 = 2.5 Hz) (**Table 4.6a & 4.6b**). These signals were ascribed to H-5 and H-6 in structure **4.32**, and H-8 and H-7 in structure **4.33**, as further indicated by the corresponding cross peaks in the COSY spectrum of the mixture. Furthermore, the ^{1}H NMR spectrum of the mixture consisted of a pair of signals due to *meta* coupling protons appearing at δ 7.64 and 7.65 (each d, J = 2.5 Hz) which were ascribed to H-8 and H-5 respectively in structures **4.32** and **4.33** hence indicating the different substitution patterns found in ring A of both compounds.

The low field position of all the above signals indicated that the corresponding aromatic protons were part of the conjugated α,β–unsaturated carbonyl system, as in structures **4.32** and **4.33,** with the β-protons (H-5 and H-8) in the two compounds, as expected, being the most deshielded due to anisotropic effects. The isolated protons H-5 and H-8 in the two compounds **4.32** and **4.33**, respectively despite being β to the conjugated

carbonyl system are less deshielded due to their vicinal position relative to the methoxy substituent which would exert anisotropic shielding effects.[117,118,128-131]

The presence of the furanoid ring fused to the quinonoid ring B of each of the two compounds was deduced from signals due to the *ortho* coupling (COSY) of superimposed furanoid protons (intergration ratio) at δ 8.05 and 7.05, respectively, (each d, J = 1.8 Hz) in the ^{1}H NMR spectrum of the mixture. Also, a pairs of superimposed and relatively shielded ^{13}C NMR carbonyl signals at δ 179.33 and 172.89 (**Table 4.6a & 4.6b**, **Fig 4.26**) as previously reported for this type of compounds.[117] Obviously, the shielding effect of the quinonoid carbonyls would be the result of the presence of the fused furanoid unit. It would appear that the extended conjugation of the naphthoquinone unit through fusion of the furanoid moiety onto the former causes shielding of the quinonoid carbonyls due to resonance effects resulting from high electron density at the carbonyl carbon nuclei. The assignment of the rest of the ^{13}C NMR signals was accomplished through comparison of the observed resonances with those reported in the literature for these type of compounds.[117]

The high resolution MS which exhibited the molecular ion peak at at *m/z* 228.0404 that was also the base peak suggested the molecular formula $C_{13}H_8O_4$ for each of the two compounds in the mixture. Extrusion of an oxygen atom or carbonyl group from the molecular ion of each of two compounds would result in the fragment ions as indicated in **Scheme 4.3**.

Table 4.6a ^{1}H and ^{13}C NMR spectral data for 7-methoxy-naphtho[2,3-b]furan-4,9-dione (4.32)

	[a]Observed			[b]Reported[117] ($CDCl_3$)	
H/C	δ_H	*J* (Hz)	δ_C	δ_H	*J* (Hz)
2	8.05	*d*, $J_{2,3}$ = *1.8*	149.72	7.72	*d*,1.9
3	7.05	*d*, $J_{2,3}$ = *1.8*	108.12	6.96	*d*, 1.9
3a			149.21		
4			179.33		
4a			130.67		
5	8.15	*d*, $J_{5,6}$ = *8*	126.44	8.15	*d*, 8.6
6	7.36	*dd*, $J_{5,6}$ = *8*, $J_{6,8}$ = *2.5*	118.90	7.17	*dd*, 8.6, 2.7
7			164.30		
8	7.64	*d*, $J_{6,8}$ =*2.5*	111.04	7.63	*d*, 2.7
8b			128.99		
9			172.89		
9a			152.70		
6-OCH_3					
7-OCH_3	3.98	*s*	55.63	3.96	*s*

[a]run in 1:1 v/v MeOD and Me_2CO; [b]run in $CDCl_3$

Table 4.6b ^{1}H and ^{13}C NMR spectral data for 6-methoxy-naphtho[2,3-b]furan-4,9-dione (4.33)

	[a]Observed			[b]Reported[117] ($CDCl_3$)	
H/C	δ_H	*J* (Hz)	δ_C	δ_H	*J* (Hz)
2	8.08	*d*, $J_{2,3}$ = *1.8*	149.72	7.74	*d*, 1.9
3	7.05	*d*, $J_{2,3}$ = *1.8*	108.32	6.97	*d*, 1.9
3a			149.21		
4			179.33		
4a			129.11		
5	7.65	*d*, $J_{5,7}$ = *2.5*	111.35	7.66	*d*, 2.7
6			164.30		
7	7.35	*dd*, $J_{7,8}$ =*8*, $J_{5,7}$ = *2.5*	118.99	7.18	*dd*, 8.6, 2.7
8	8.04	*d*, $J_{7,8}$ = *8*	126.44	8.12	*d*, 8.6
8b			134.83		
9			172.89		
9a			152.70		
6-OCH_3	3.98	*s*	55.63	3.96	*s*
7-OCH_3					

[a]run in 1:1 v/v MeOD and Me_2CO; [b]run in $CDCl_3$

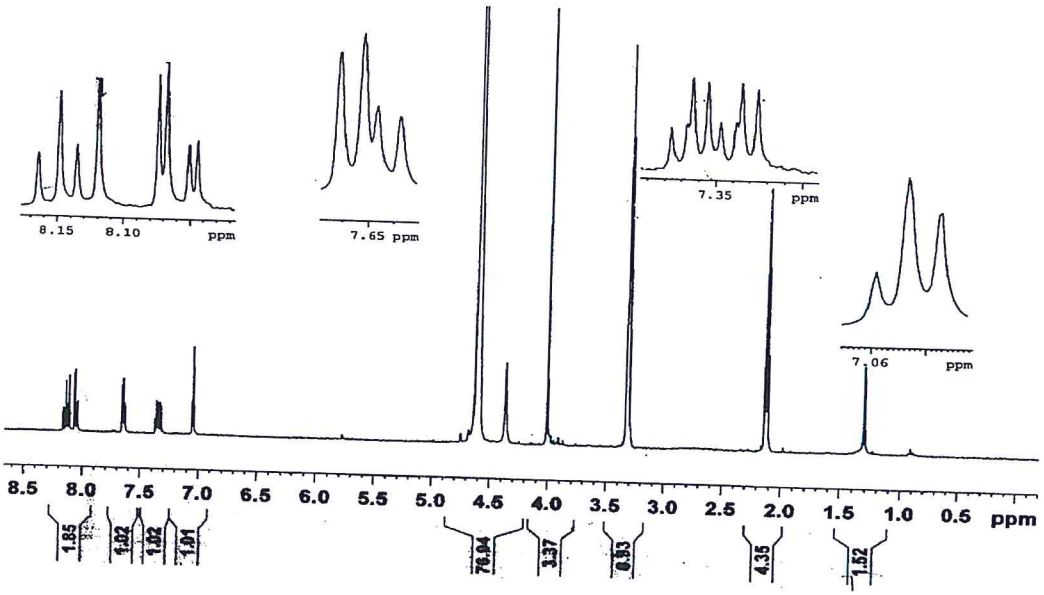


Fig 4.23 ^{1}H NMR (300 MHz, MeOD and Me_2CO) spectrum of a mixture of compounds 4.32 and 4.33

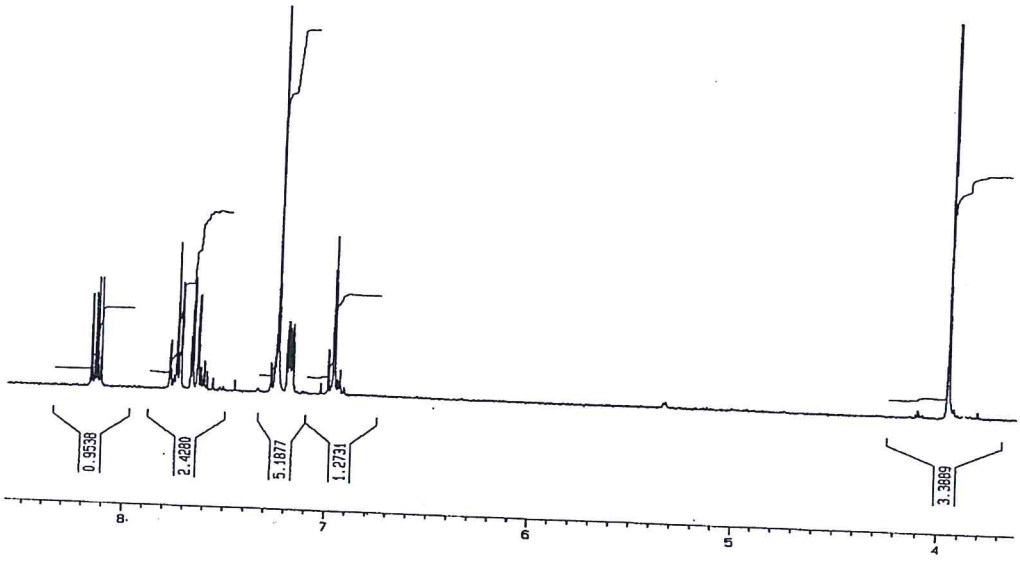


Fig 4.24 ^{1}H NMR (500 MHz, $CDCl_3$) spectrum of a mixture of compounds 4.32 and 4.33

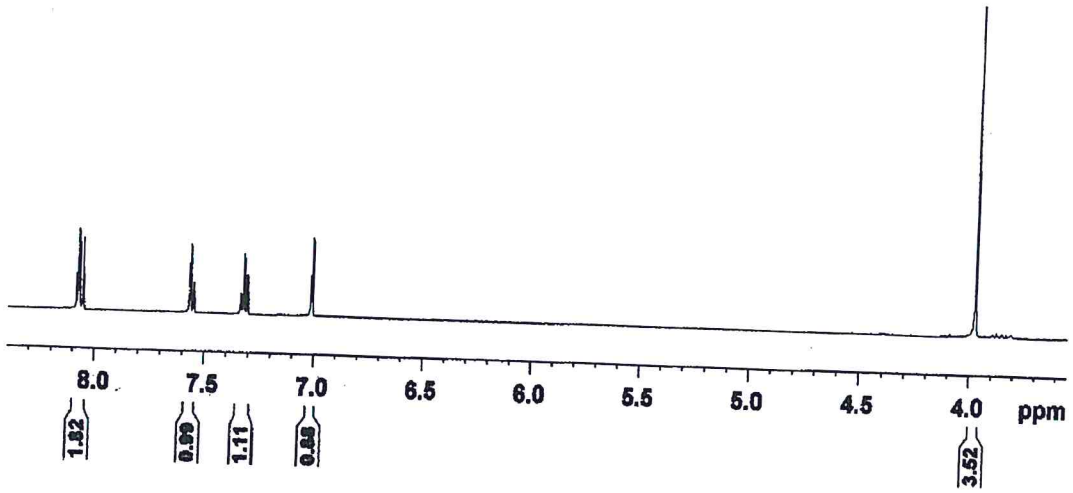


Fig 4.25 ^{1}H NMR (600 MHz, Me_2CO)) spectrum of a mixture of compounds 4.32 and 4.33

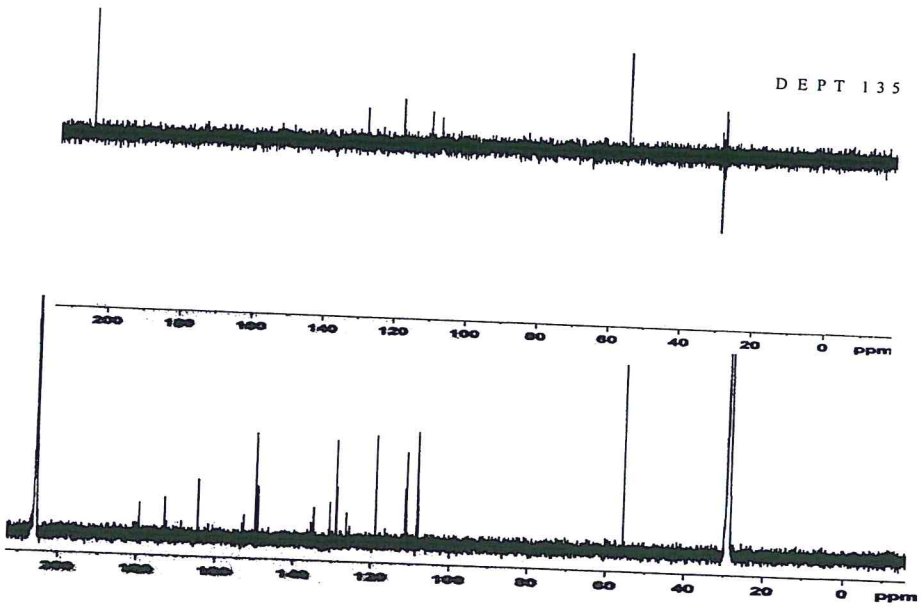


Fig 4.26 ^{13}C NMR (150 MHz, MeOD and Me_2CO) spectrum of a mixture of compounds 4.32 and 4.33

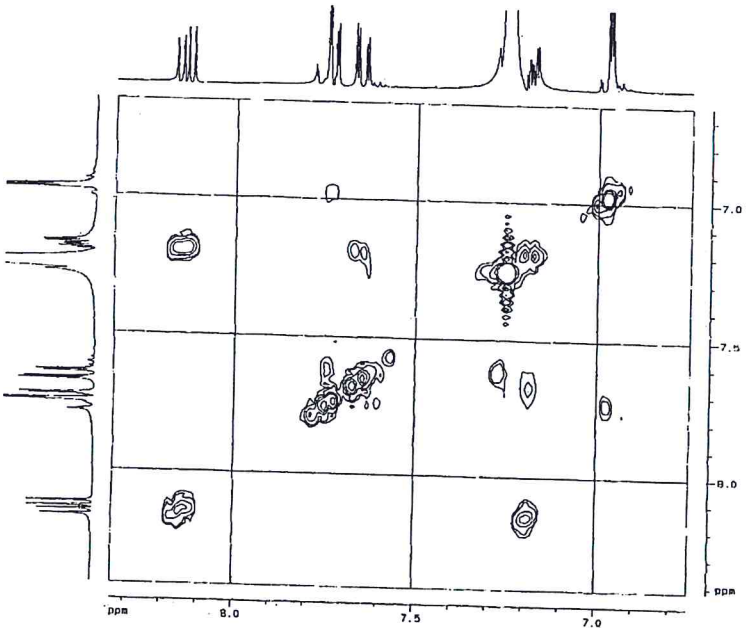


Fig 4.27 **H/H COSY (250 MHz, $CDCl_3$) plot for a mixture of compounds 4.32 and 4.33**

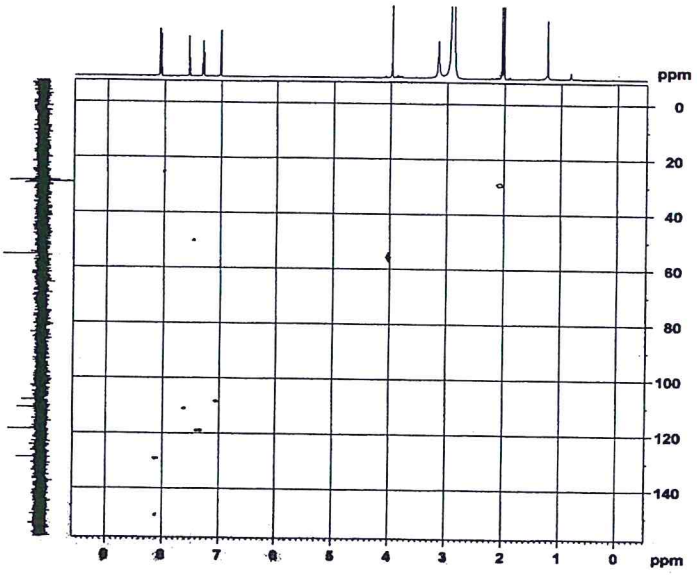


Fig 4.28 **HMQC plot (600 MHz, Me_2CO) for a mixture of compounds 4.32 and 4.33**

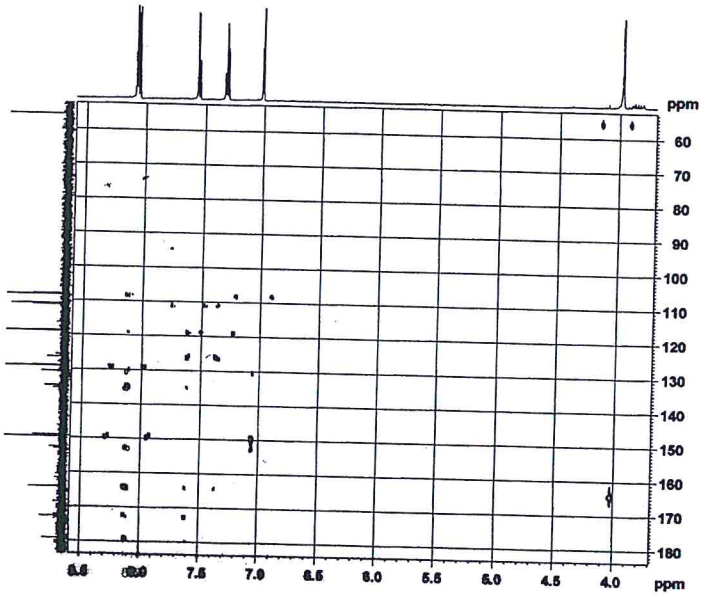


Fig 4.29 **HMBC plot (600 MHz, Me_2CO) for a mixture of compounds 4.32 and 4.33**

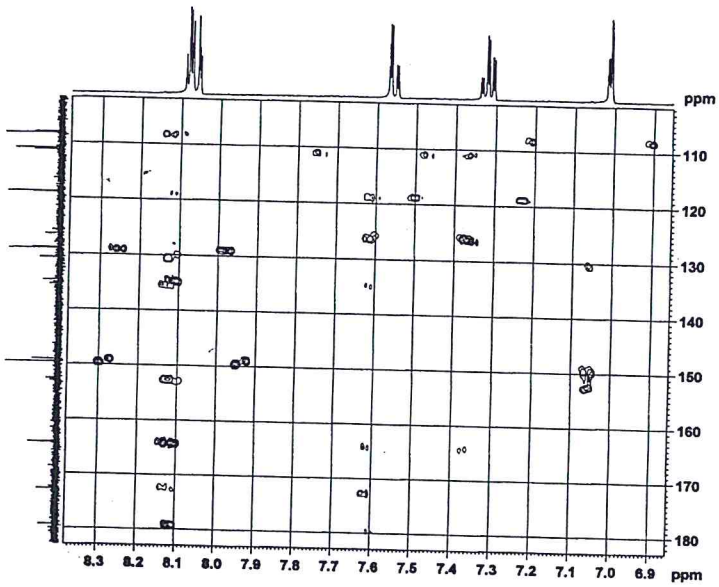


Fig 4.30 **Expansion of HMBC plot (600 MHz, Me_2CO) for a mixture of compounds 4.32 and 4.33 between δ 6.9-8.4 and δ 100-180**

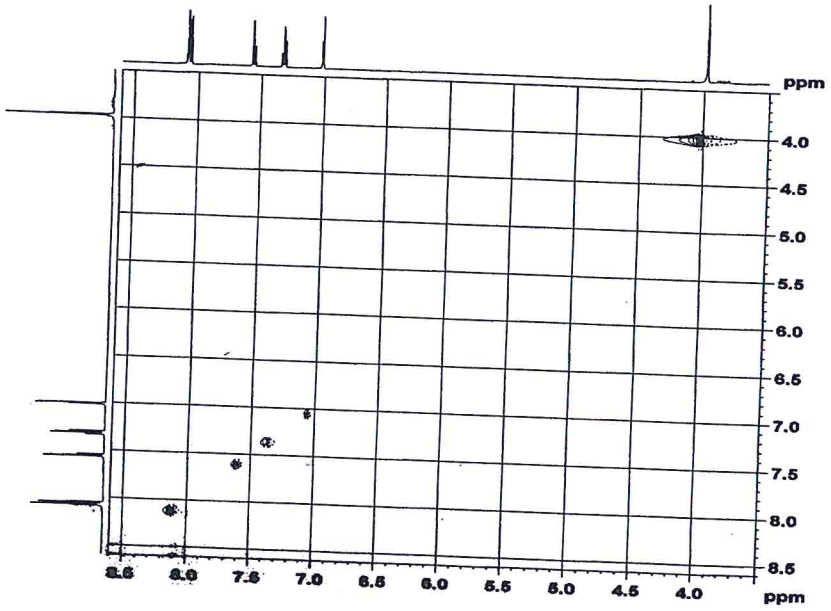


Fig 4.31 **NOESY (600 MHz, Me_2CO) spectrum of a mixture of compounds 4.32 and 4.33**

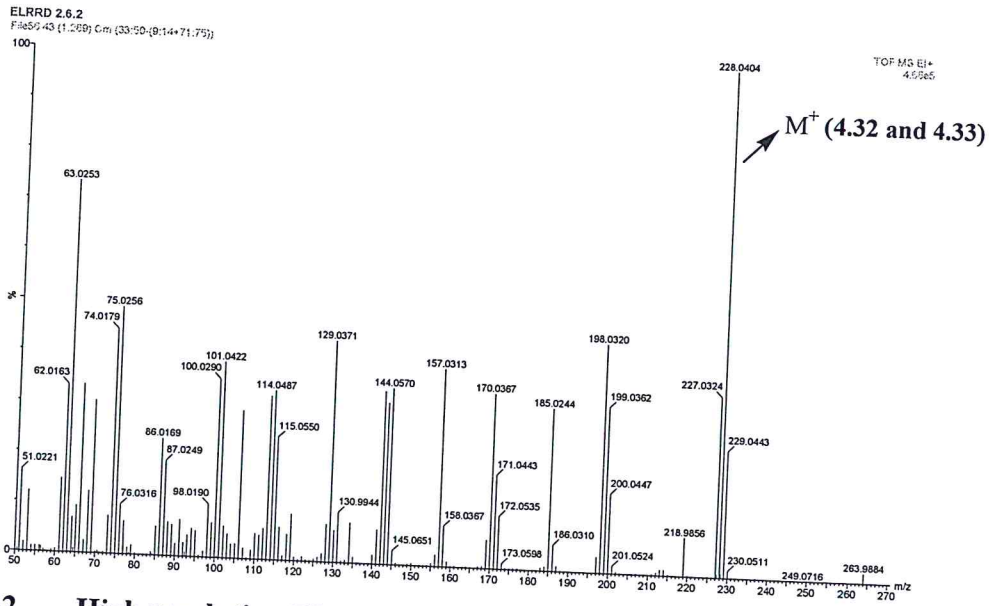
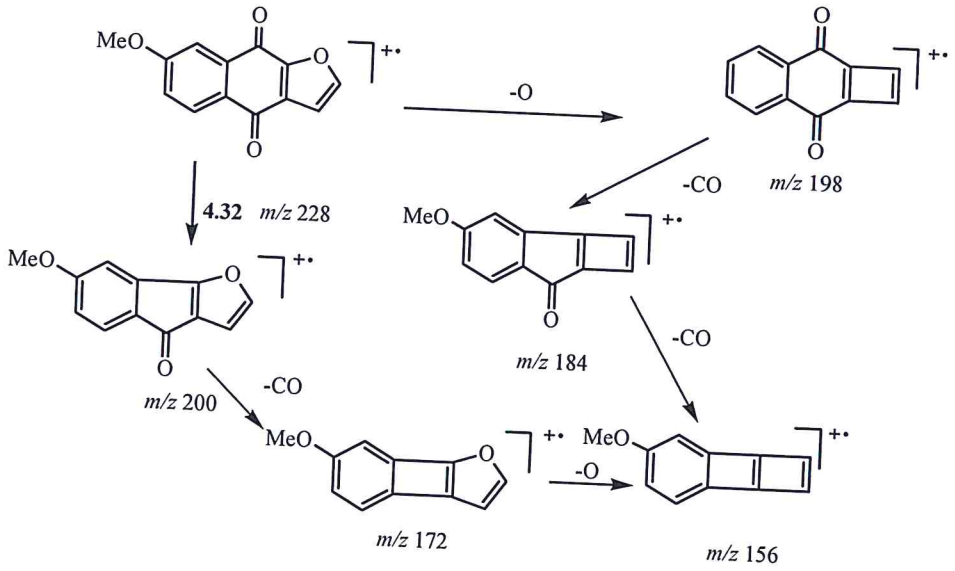
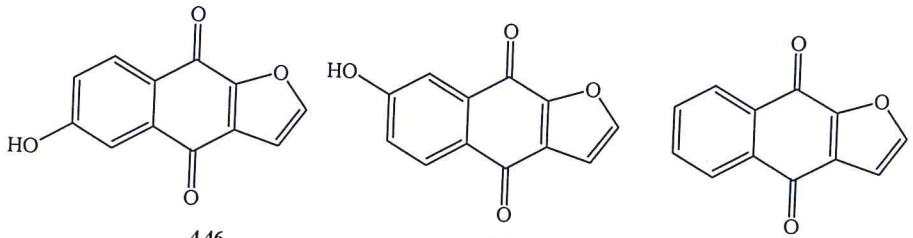


Fig 4.32 **High resolution EI-mass spectrum of a mixture of compounds 4.32 and 4.33**



Scheme 4.3 Proposed mass spectral fragmentation pattern for compound 4.32

4.2.5 6-Hydroxy-naphtho[2,3-b]furan-4,9-dione (4.46), 7-hydroxy-naphtho[2,3-b]furan-4,9-dione (4.47) and naphtho[2,3-b]furan-4,9-dione (4.48)



4.46 4.47 4.48

Repeated column chromatography of the second VLC fraction of the dichloromethane extract of the root bark of *L. viburnoides* ssp *viburnoides* var. *kisi* yielded yellow crystals that consisted of an inseparable mixture of furanonaphthaquinone derivatives **4.46, 4.47** and **4.48**. The UV spectrum of the mixture exhibited absorptions at 201, 245 and 291 nm, which indicated the conjugation nature of the constituents of the inseparable mixture. The IR spectrum displayed bands at 3446, 1674, and 1643 and

1572 cm^{-1}, which were ascribed to hydroxyl, carbonyl and aromatic C-H stretching vibrations, respectively.

The composition of the mixture was deduced upon analysis of the ^{1}H and ^{13}C NMR (**Figs 4.33-4.35; Tables 4.7** and **4.8**), and mass spectra (**Fig 4.40**). Thus, the ^{1}H and ^{13}C NMR spectra were closely related to those reported for the mixture of compounds **4.32** and **4.33**, except for the absence of a methoxyl signals. The high resolution MS exhibited the highest mass peak at *m/z* 214.0251, that was lower than the M^{+} peak for compounds **4.32** and **4.33** (*m/z* 228.0404) by 14 amu. This confirming that the mixture consisted of demethylated compounds **4.46** and **4.47**, having the molecular formula $C_{12}H_6O_4$ that corresponds to the M^{+} peak at *m/z* 214.0251. Besides the molecular ion peak for compounds **4.46** and **4.47**, the MS of the mixture exhibited a peak at 198.0318 which could have resulted from loss of an oxygen atom from the molecular ion of each of two compounds. However, this was not possible considering the structural framework of the two compounds. Therefore, the MS peak at *m/z* 198.0318 was ascribed to a third compound **4.48** in the mixture, being a dehydroxy derivative of the two regio-isomers **4.46** and **4.47**.

Presence of the latter compound in the inseparable mixture was in agreement with the observed ^{1}H and ^{13}C NMR data. Thus, apart from signals due to compounds **4.46** and **4.47**, in the ^{1}H NMR and H/H COSY spectra of the inseparable mixture, an *ortho* coupled set of protons resonating at δ 8.21 and 7.84 as multiplets was observed. The fact that the two protons did not show any long range coupling in the H/H COSY spectrum suggested that, the signals were due to more that one proton and being in the same

chemical environment. This was only possible if ring A was not substituted, as in structure **4.48**, as further confirmed from the H/C interactions as observed in the HMQC plot (**Fig 4.38**). Thus, the signals at δ 8.21 and 7.84 coupled only to C-5 and C-8 and C-6 and C-7 at δ 126.28 and 133.78, respectively, in the ^{13}C NMR spectrum of the mixture.

Table 4.7 ^{1}H NMR spectral data for 6-hydroxy-naphtho[2,3-b]furan-4,9-dione (4.46) 7-hydroxy-naphtho[2,3-b]furan-4,9-dione (4.47) and naphtho[2,3-b]furan-4,9-dione (4.48)

	4.46		4.47		4.48	
H/C	δ_H	*J* (Hz)	δ_H	*J* (Hz)	δ_H	*J* (Hz)
2	8.06	*d*, $J = 1.8$	*8.06*	*d*, $J = 1.8$	8.06	*d*, $J = 1.8$
3	7.07	*d*, $J = 1.8$	*7.07*	*d*, $J = 1.8$	7.07	*d*, $J = 1.8$
5	7.72	*d*, $J_{5,7} = 2.5$	*7.72*	*d*, $J_{5,6} = 7.5$	8.21	*m*
6			*7.29*	*dd*, $J_{5,6} = 7.5$, $J_{6,8} = 2.5$	7.84	*m*
7	7.29	*dd*, $J_{7,8} = 7.5$, $J_{5,7} = 2.5$			7.84	*m*
8	7.72	*d*, $J_{7,8} = 7.5$	*7.72*	*d*, $J_{6,8} = 2.5$	8.21	*m*

[a]run in MeOD

Table 4.8 ^{13}C NMR spectral data for 6-hydroxy-naphtho[2,3-b]furan-4,9-dione (4.46) 7-hydroxy-naphtho[2,3-b]furan-4,9-dione (4.47) and naphtho[2,3-b]furan-4,9-dione (4.48)

H/C	$\delta_{4.46}$	$\delta_{4.47}$	$\delta_{4.48}$
2	150.05	150.05	150.06
3	108.19	108.18	107.91
3a	149.53	149.53	-
4	-	-	-
4a	126.44	133.68	-
5	119.40	136.28	126.28
6	149.52	124.49	133.78
7	124.49	149.52	133.78
8	136.28	119.40	126.28
8b	133.68	126.44	-
9	-	-	-
9a	160.80	160.80	160.80

[a]quaternary carbon signals not observed due to small amount of sample; run in MeOD

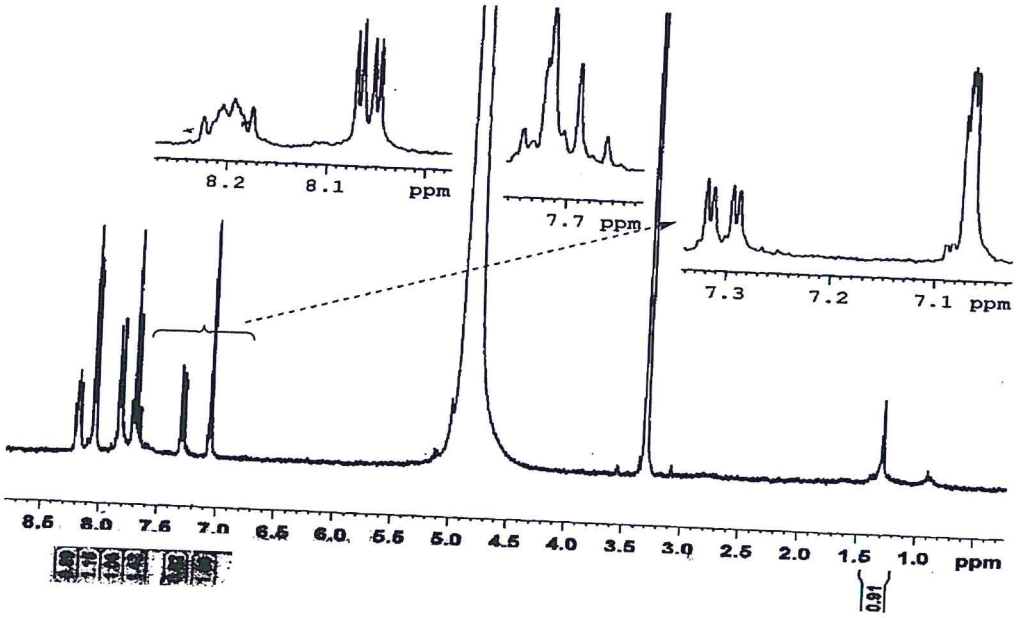


Fig 4.33 **^{1}H NMR (300 MHz, MeOD) spectrum of an inseparable mixture of compounds 4.46, 4.47 and 4.48**

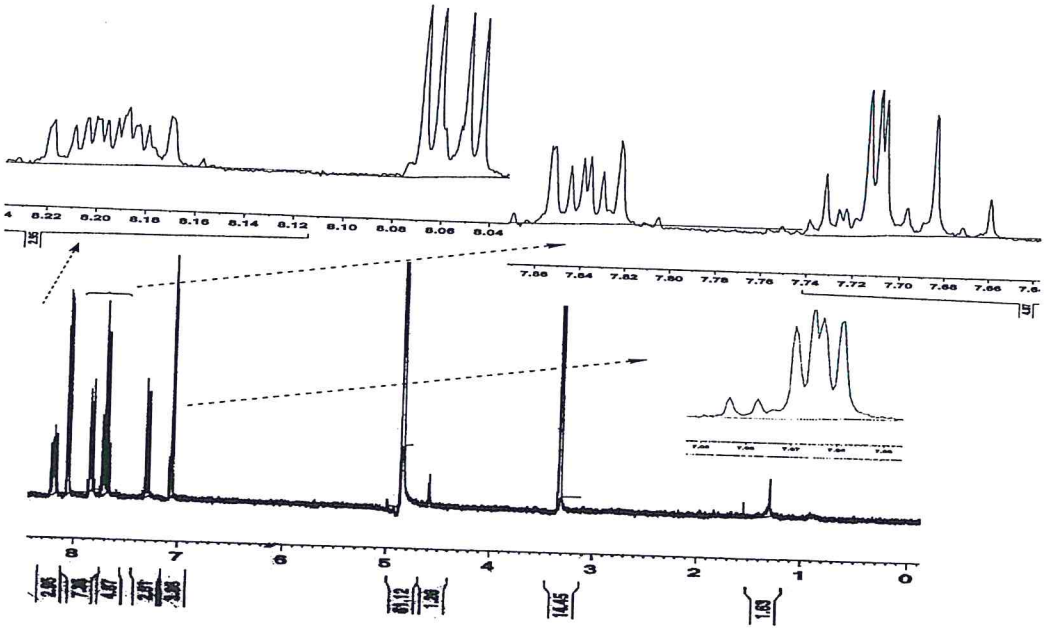


Fig 4.34 **Expansions of ^{1}H NMR (300 MHz, MeOD) spectrum of an inseparable mixture of compounds 4.46, 4.47 and 4.48**

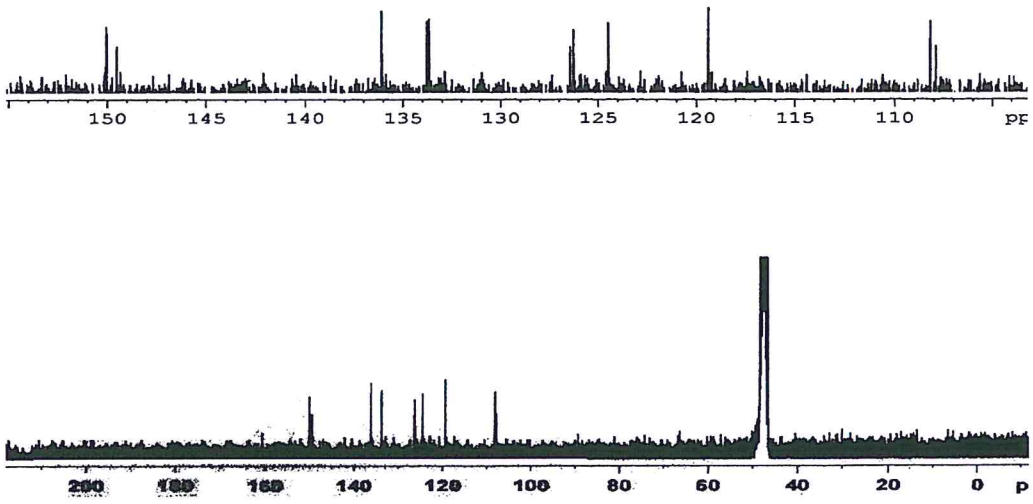


Fig 4.35 ^{13}C NMR (75 MHz, MeOD)) spectrum of an inseparable mixture of compounds 4.46, 4.47 and 4.48

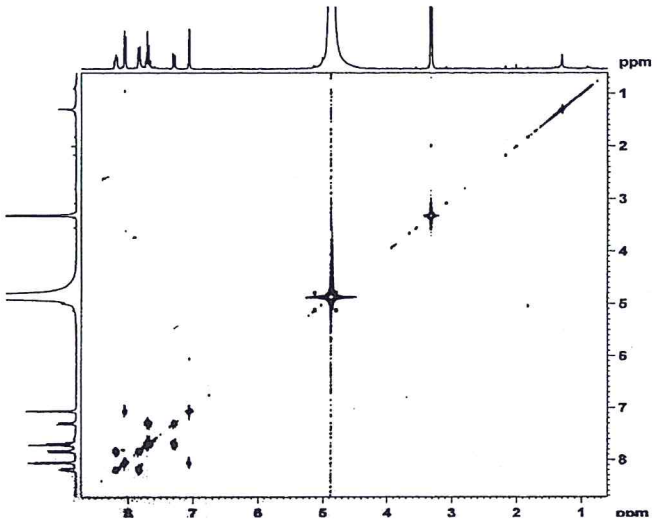


Fig 4.36 H/H COSY (300 MHz, MeOD) plot for an inseparable mixture of compounds 4.46, 4.47 and 4.48

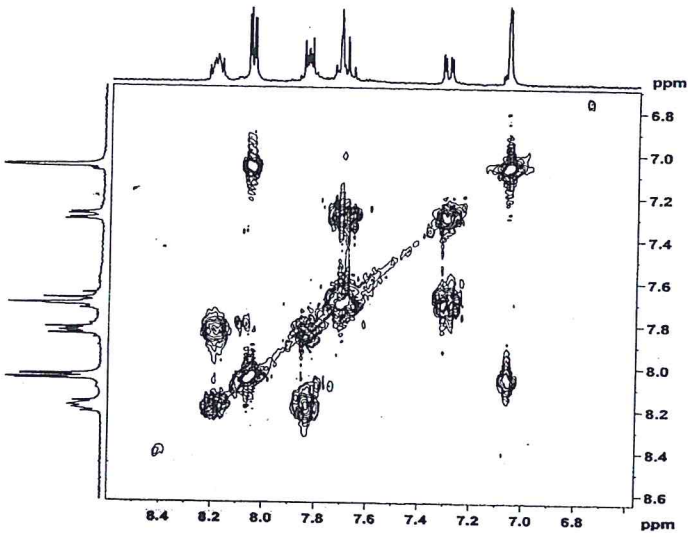


Fig 4.37 **Expansion of H/H COSY (300 MHz, MeOD) plot for an inseparable mixture of compounds 4.46, 4.47 and 4.48 between δ 6-7-8.4**

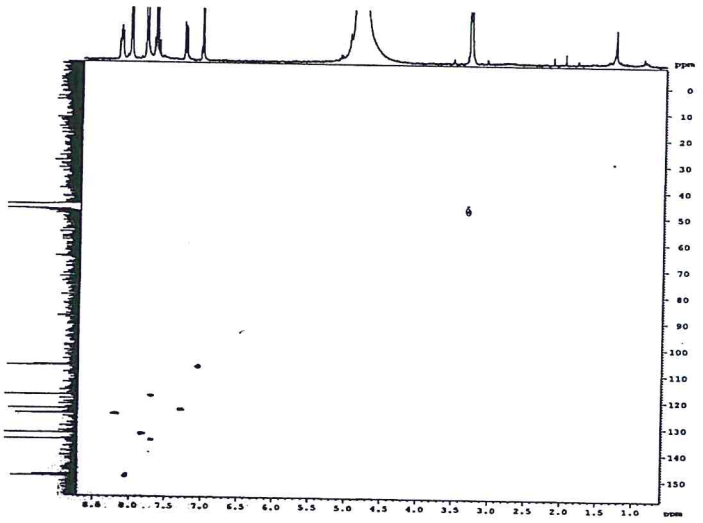


Fig 4.38 **HMQC (300 MHz, MeOD) plot for an inseparable mixture of compounds 4.46, 4.47 and 4.48**

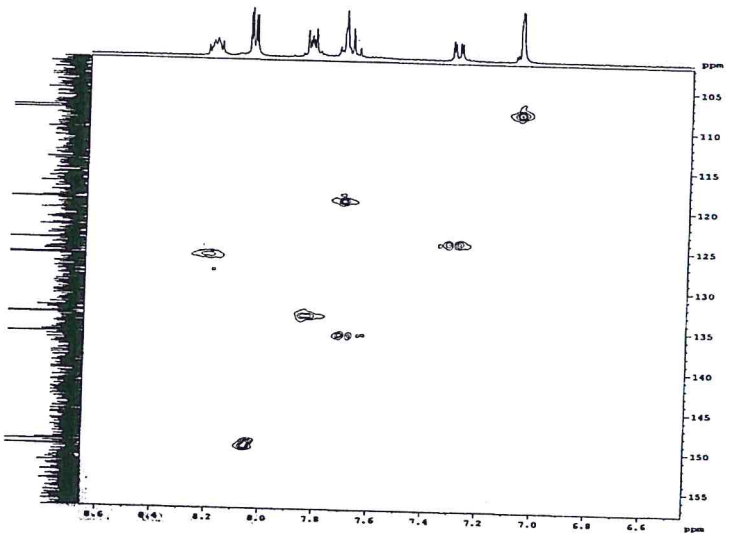


Fig 4.39 **Expansion of HMQC (300 MHz, MeOD) plot for an inseparable mixture of compounds 4.46, 4.47 and 4.48 between δ 6.7-8.6 and δ 100-155**

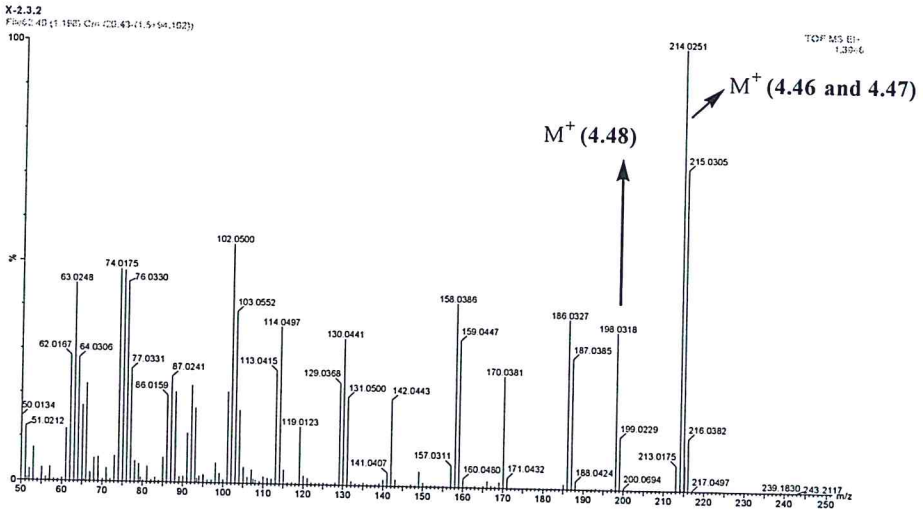
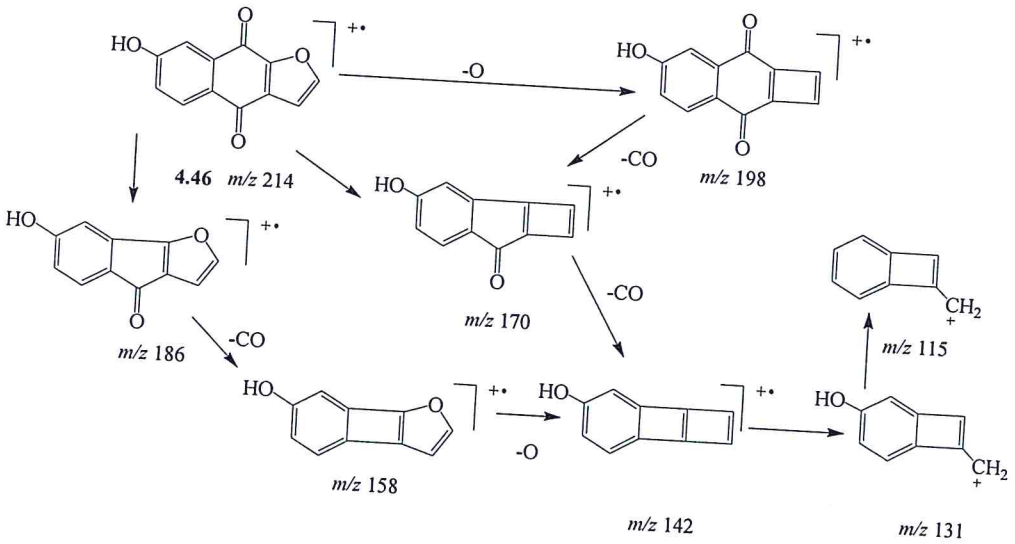
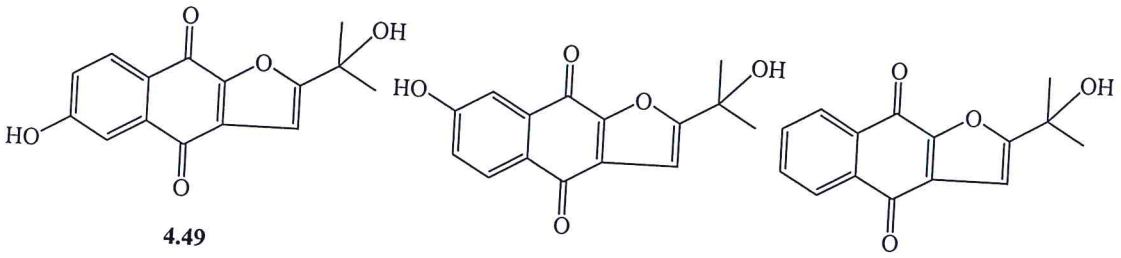


Fig 4.40 **High resolution EI-mass spectrum of an inseparable mixture of compounds 4.46, 4.47 and 4.48**



Scheme 4.4 Proposed mass spectral fragmentation pattern of compound 4.46

4.2.6 6-Hydroxy-2-(1-hydroxy-1-methyl-ethyl)-naphtho[2,3-b]furan-4,9-dione (4.49), 7-hydroxy-2-(1-hydroxy-1-methyl-ethyl)-naphtho[2,3-b]furan-4,9-dione (4.50) and 2-(1-hydroxy-1-methyl-ethyl)-naphtho[2,3-b]furan-4,9-dione (4.51)



4.49 **4.50** **4.51**

Repeated column chromatography of the fourth VLC fraction of the dichloromethane extract of the root bark of *L. viburnoides* ssp *viburnoides* var. *kisi* yielded a yellow gum that consisted of inseparable mixtures of furanonaphthaquinones **4.49**, **4.50** and **4.51**.

The UV spectrum of the inseparable mixture exhibited absorptions at 202, 248 and 298 nm, indicating the aromatic nature of the constituents. The IR spectrum displayed bands at 3393, 1649, 1515 and 1460 cm^{-1}, due to hydroxyl, carbonyl and aromatic functional groups.

The structures were established by ^1H NMR (**Fig 4.41**) to be similar to compounds **4.32** and **4.33**, **4.46-4.48** except that they lacked signals at δ *ca.* 8. Instead they exhibited singlet signals at *ca.* δ 6.85 and 1.65, suggesting that, H-2 of the furanoid system was substituted and in this case, by an isopropane unit, as indicated for compounds **4.49**, **4.50** and **4.51**. The ^1H NMR and H/H COSY spectrum indicated three coupling protons in which two of them were resonating at δ 7.72 (d, H-8, J = 7.8 Hz) and δ 7.70 (d, H-5, J = 2.25 Hz) for **4.49** and δ 7.63 (d, H-5, J = 7.4 Hz) and δ 7.70 (d, H-8, J = 2.25 Hz) for **4.50**, respectively, while the third proton resonated at δ 7.28 (dd, J_1 = 7.8, J_2 = 2.25 Hz) for **4.49** and δ 7.3 (dd, J_1 = 7.4, J_2 = 2.25 Hz) for **4.50** (**Table 4.9** and **Figs 4.41** and **4.42**). Another set of signals for two *ortho* coupled protons resonating as multiplets at δ 7.85 and 8.20 were observed in the low field region of the ^1H NMR spectrum, and were ascribed to H-6 and H-7 and H-5 and H-8 in compound **4.51**.

The high resolution MS (**Fig 4.43**) of the mixture exhibited the molecular ion peak at *m/z* 272.0653 for the two regio-isomers **4.49** and **4.50** corresponding to the formula $C_{15}H_{12}O_5$. Other peaks at *m/z* 258.1477 and 258.0457, were ascribed to a fragment ion formed upon extrusion of the furanoid oxygen from the molecular ions for the two regio-isomers **4.49** and **4.50** (**Scheme 4.5**). The latter MS peak was ascribed to the molecular

ion for the third furanonaphthaquinone constituent **4.51** confirming the formula $C_{15}H_{12}O_4$ in the mixture.

Table 4. 9 **^{1}H and ^{13}C NMR spectral data for 6-hydroxy-2-(1-hydroxy-1-methyl-ethyl)-naphtho[2,3-b]furan-4,9-dione (4.49), 7-hydroxy-2-(1-hydroxy-1-methyl-ethyl)-naphtho[2,3-b]furan-4,9-dione (4.50) and 2-(1-hydroxy-1-methyl-ethyl)-naphtho[2,3-b]furan-4,9-dione (4.51)**

	[a]4.49		[b]4.31[117]	[a]4.50		[b]4.30[117]	[a]4.51	
H/C	δ_H	*J* (Hz)	δ_H	δ_H	*J* (Hz)	δ_H		
2-Me	1.64	*s*	1.68	1.64	*s*	1.68	1.65	*s*
3	6.87	*s*	6.79	6.87	*s*	6.81	6.88	*s*
5	7.70	*d*, $J_{5,7}$ = 2.25	7.59	7.63	*d*, $J_{5,6}$ = 7.4		8.20	*m*
6			7.24	7.30	*dd*, $J_{5,6}$ = 7.4, $J_{6,8}$ = 2.25	7.24	7.83	*m*
7	7.28	*dd*, $J_{7,8}$ = 7.8, $J_{5,7}$ = 2.25	7.69			7.58	7.83	*m*
8	7.72	*d*, $J_{7,8}$ = 7.8		7.70	*d*, $J_{6,8}$ = 2.25	7.69	8.20	*m*

[a]run in MeOH; [b]chemical shift of a related compound, run in $CDCl_3$

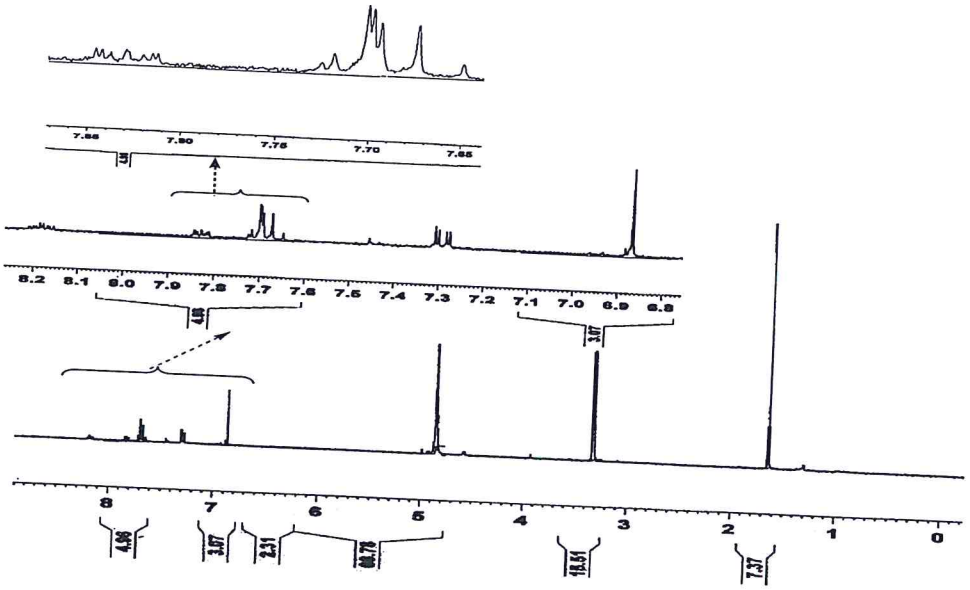


Fig 4.41 ^{1}H NMR spectrum (300 MHz, MeOD) of an inseparable mixture of compounds 4.49, 4.50 and 4.51

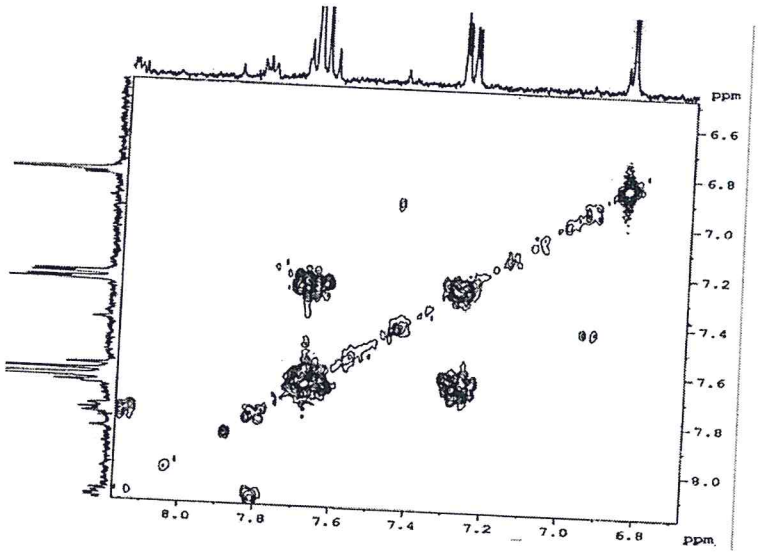


Fig 4.42 H/H COSY (300 MHz, MeOD) plot for an inseparable mixture of compounds 4.49, 4.50 and 4.51

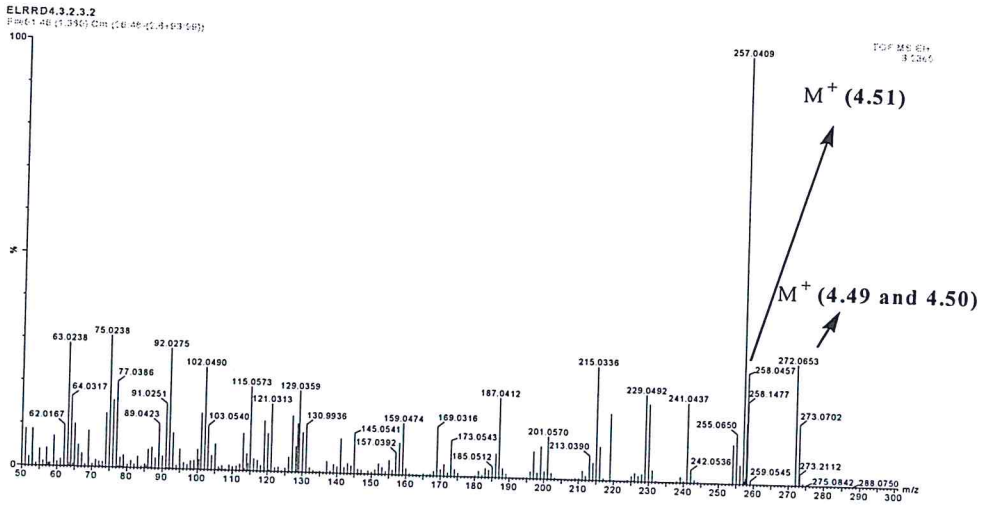
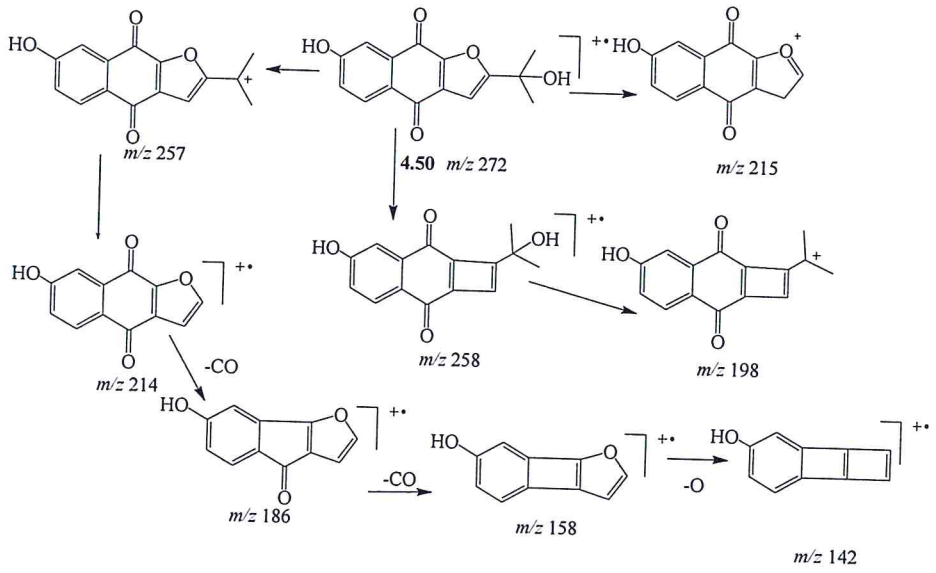
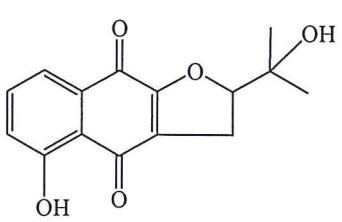


Fig 4.43 **High resolution EI-mass spectrum of an inseparable mixture of compounds 4.49, 4.50 and 4.51**

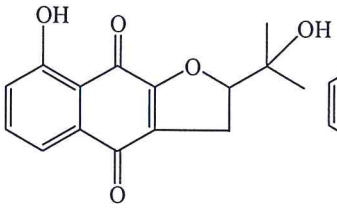


Scheme 4.5 Proposed mass spectral fragmentation pattern for compound 4.50

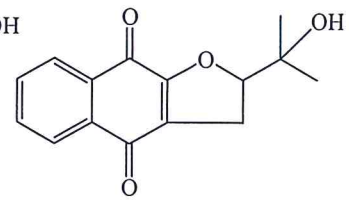
4.2.7 5-Hydroxy-2-(1-hydroxy-1-methyl-ethyl)-2,3-dihydro-naphtho[2,3-b]furan-4,9-dione (4.52), 8-hydroxy-2-(1-hydroxy-1-methyl-ethyl)-2,3-dihydro-naphtho[2,3-b]furan-4,9-dione (4.53) and (1-hydroxy-1-methyl-ethyl)-2,3-dihydro-naphtho[2,3-b]furan-4,9-dione (4.54)



4.52



4.53



4.54

Repeated column chromatography of the fourth VLC fraction of the dichloromethane extract of the root bark of *L. viburnoides* ssp *viburnoides* var. *kisi* yielded a dark yellow gum which consisted of an inseparable mixture of the furanonaphthaquinones **4.52**, **4.53** and **4.54**. The UV spectrum of the mixture exhibited absorptions at 200, 248 and 292 nm and IR bands at 3447, 1644, 1621 and 1512 cm^{-1}, the latter being due to hydroxyl, carbonyl and aromatic functional groups. The spectrum of the mixture resembled that of compounds **4.49**, **4.50** and **4.51** as discussed in **Section 4.2.6** except that, they had undergone dihydrogenation at the furanoid ring at C-2/C-3 bond, as revealed from the ^{1}H and ^{13}C NMR spectra, H/H COSY, HMQC, HMBC and MS (**Figs 4.44-4.50**).

The high field region of the ^{1}H NMR spectrum displayed signals due to six methyl protons attached to a carbinol carbon (δ 1.2, s, 12H and δ 1.4, s, 6H), three methine (δ 4.88, dd, J_1 = 3.10, J_2 = 1.03 Hz, 2H, H-2 for each of the compounds **4.52** and **4.53**; δ 4.92, d, J = 3.12 Hz, 1H, H-2 for compound **4.54**) and three methylene protons (δ 3.13, dd, J_1 = 6, J_2 = 2.3 Hz, 2H, H-3 for each of the compounds **4.52** and **4.53** and δ

3.16, J_1 = 6, J_2 = 1.3 Hz, 2H, H-3 for compound **4.54**). These ^{1}H NMR features suggested the presence of an isopropane unit in each of the structures **4.52**, **4.53** and **4.54**. Furthermore, the ^{1}H NMR and H/H COSY spectrum revealed signals due to three *ortho* coupling protons (δ 7.2, dd, J_1 =8, J_2 = 1.25 Hz; δ 7.52, dd, J_1 =7.5, J_2 = 1.8 Hz and δ 7.62, dd, J_1 = 7.5, J_2 = 7.44 Hz for each of the compounds **4.52** and **4.53**). Another set of signals due to two *ortho* coupling protons appeared at δ 7.75 and 8.0 and were attributed to C-6 and C-7 and C-5 and C-8 in compound **4.54**. Presence of the three compound in the inseparable mixture was in agreement with the observed ^{1}H and ^{13}C NMR data (**Tables 4.10-4.11**; **Figs 4:44-4.45**).

The high resolution MS (**Fig 4.50**) of the mixture exhibited the moleculr ion peak for the two regio-isomeric furanonaphthaquinones **4.52** and **4.53** at *m/z* 274.0868 suggesting the formula $C_{15}H_{14}O_5$ while the molecular ion peak for the third constituent **4.54** of the mixture of furanonaphthaquinones was observed at *m/z* 256.0764 suggesting the molecula formula $C_{15}H_{14}O_4$. The proposed MS fragmentation process for the three furanonaphthaquinones **4.52**, **4.53** and **4.54** are represented by the fragmentation pattern for compound **4.52** shown in **Scheme 4.6**. Thus the three compounds involved cleavage of a 2-hydroxyprenyl unit from the molecular ion, followed by extrusion of the furanoid oxygen and then serial cleavage of carbonyl units to give rearranged fragment ions.

Table 4.10 **^{1}H NMR spectral data for 5-hydroxy-2-(1-hydroxy-1-methyl-ethyl)-2,3-dihydro-naphtho[2,3-b]furan-4,9-dione (4.52), 8-hydroxy-2-(1-hydroxy-1-methyl-ethyl)-2,3-dihydro-naphtho[2,3-b]furan-4,9-dione (4.53) and (1-hydroxy-1-methyl-ethyl)-2,3-dihydro-naphtho[2,3-b]furan-4,9-dione (4.54) in an inseparable mixture**

H/C	4.52		4.53		4.54	
	δ	*J* (Hz)	δ	*J* (Hz)	δ	*J* (Hz)
2	4.88	*dd*, $J_1 = 3.12$, $J_2 = 1.03$	4.88	*dd*, $J_1 = 3.12$, $J_2 = 1.03$	4.92	*dd*, $J_1 = 3.12$, $J_2 = 1.03$
3	3.13	*dd*, $J_1 = 6$, $J_2 = 2.3$	3.15	*dd*, $J_1 = 6$, $J_2 = 1.3$	3.13	*dd*, $J_1 = 6$, $J_2 = 2.3$
5			7.52	*dd*, $J_{6,7} = 8$, $J_{6,8} = 1.25$	8.00	*m*
6	7.52	*dd*, $J_{5,6} = 7.49$, $J_{5,7} = 1.25$	7.62	*dd*, $J_{6,7} = 7.44$, $J_{7,8} = 7.49$	7.75	*m*
7	7.62	*dd*, $J_{6,7} = 7.44$, $J_{5,6} = 7.49$	7.20	*dd*, $J_{6,7} = 7.49$, $J_{6,8} = 1.25$	7.75	*m*
8	7.20	*dd* $J_{6,7} = 8$, $J_{5,7} = 1.25$			8.00	*m*
2Me	1.2	s	1.4	s	1.2	s

run in MeOD

Table 4.11 **^{13}C NMR spectral data for 5-hydroxy-2-(1-hydroxy-1-methyl-ethyl)-2,3-dihydro-naphtho[2,3-b]furan-4,9-dione (4.52), 8-Hydroxy-2-(1-hydroxy-1-methyl-ethyl)-2,3-dihydro-naphtho[2,3-b]furan-4,9-dione (4.53) and (1-hydroxy-1-methyl-ethyl)-2,3-dihydro-naphtho[2,3-b]furan-4,9-dione (4.54)**

C	$\delta_{4.52}$	$\delta_{4.53}$	$\delta_{4.54}$
2	93.60	93.60	93.60
3	29.25	29.25	29.25
3a	160.0	160.0	160.0
4	183.00	183.00	183.00
4a	135.28	127.03	134.72
5	-	119.72	126.78
6	124.87	137.90	134.22
7	137.90	124.87	134.22
8	119.72	-	126.78
8a	127.03	135.28	135.28
9	182.88	182.88	182.88
9a	162.00	162.00	162.00
2-C	72.23	72.23	72.23
2CMe	25.33	25.33	25.33

-quaternary carbon signal not observed due to small amount of sample; run in MeOD

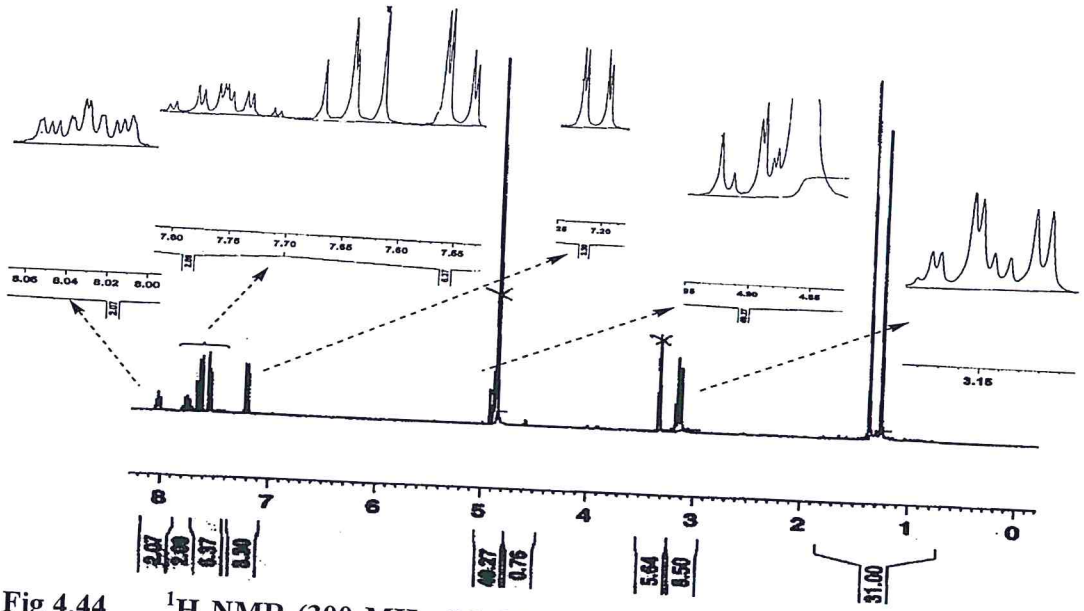


Fig 4.44 ^{1}H NMR (300 MHz, MeOD) spectrum of an inseparable mixture of compounds 4.52, 4.53 and 4.54

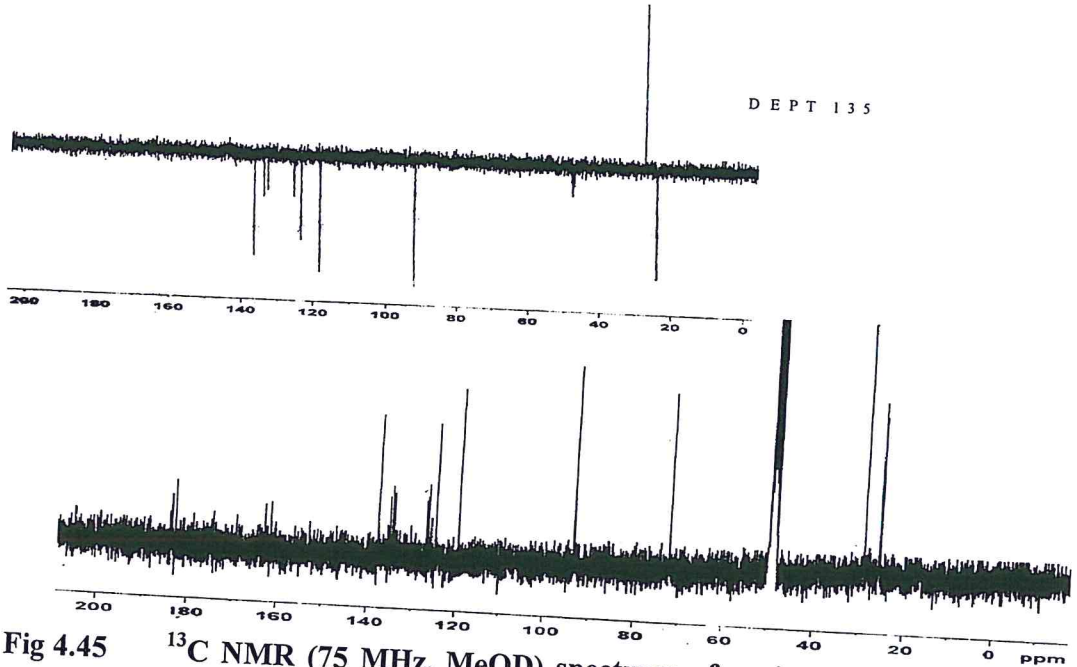


Fig 4.45 ^{13}C NMR (75 MHz, MeOD) spectrum of an inseparable mixture of compounds 4.52, 4.53 and 4.54

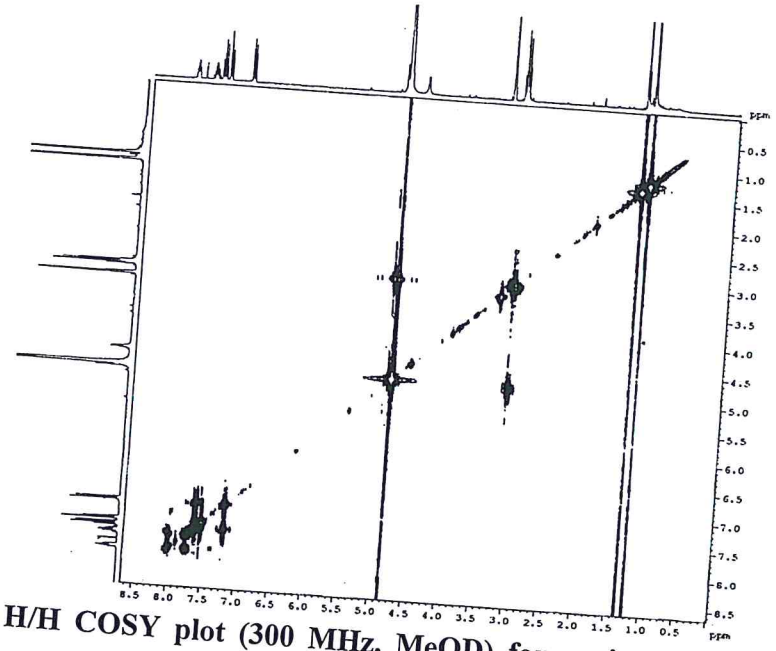


Fig 4.46 **H/H COSY plot (300 MHz, MeOD) for an inseparable mixture of compounds 4.52, 4.53 and 4.54**

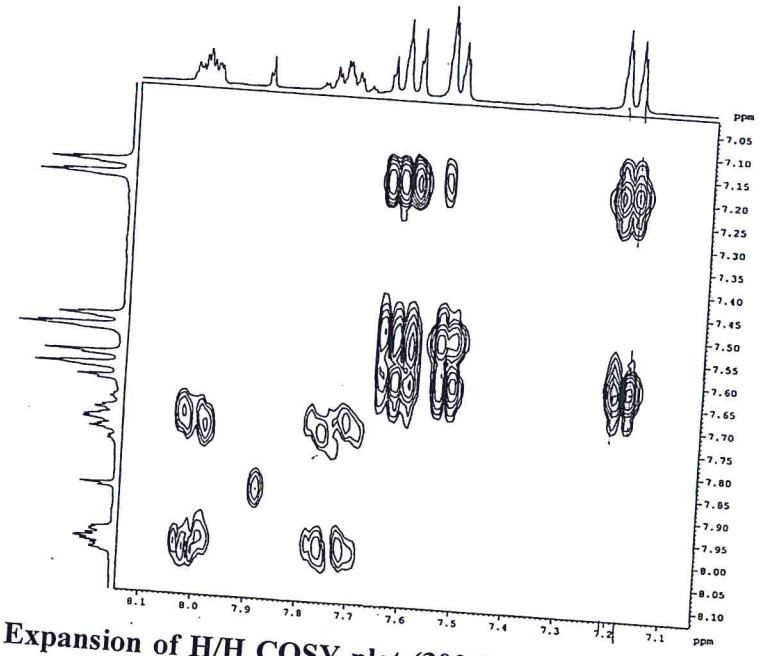


Fig 4.47 **Expansion of H/H COSY plot (300 MHz, MeOD) for an inseparable mixture of compounds 4.52, 4.53 and 4.54 between δ 7.0-8.15**

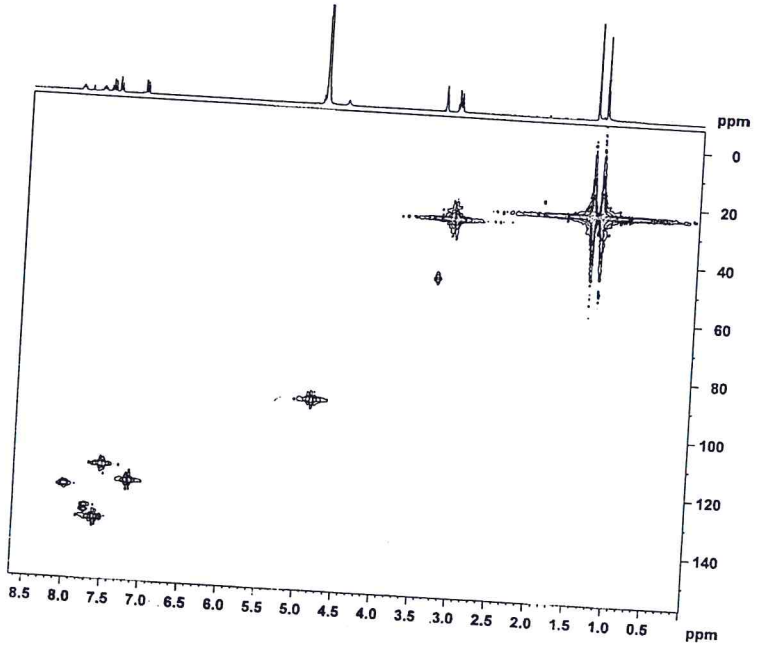


Fig 4.48 **HMQC plot (300 MHz, MeOD) for an inseparable mixture of compounds 4.52, 4.53 and 4.54**

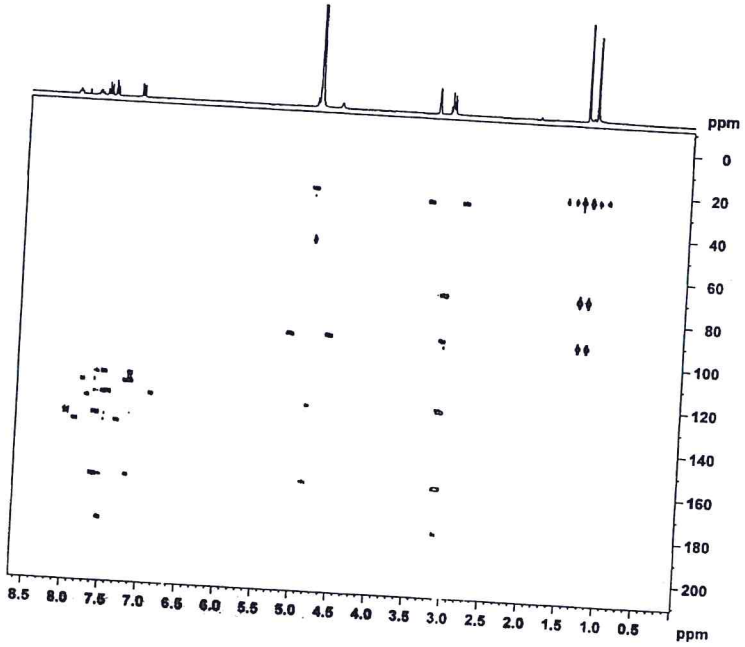
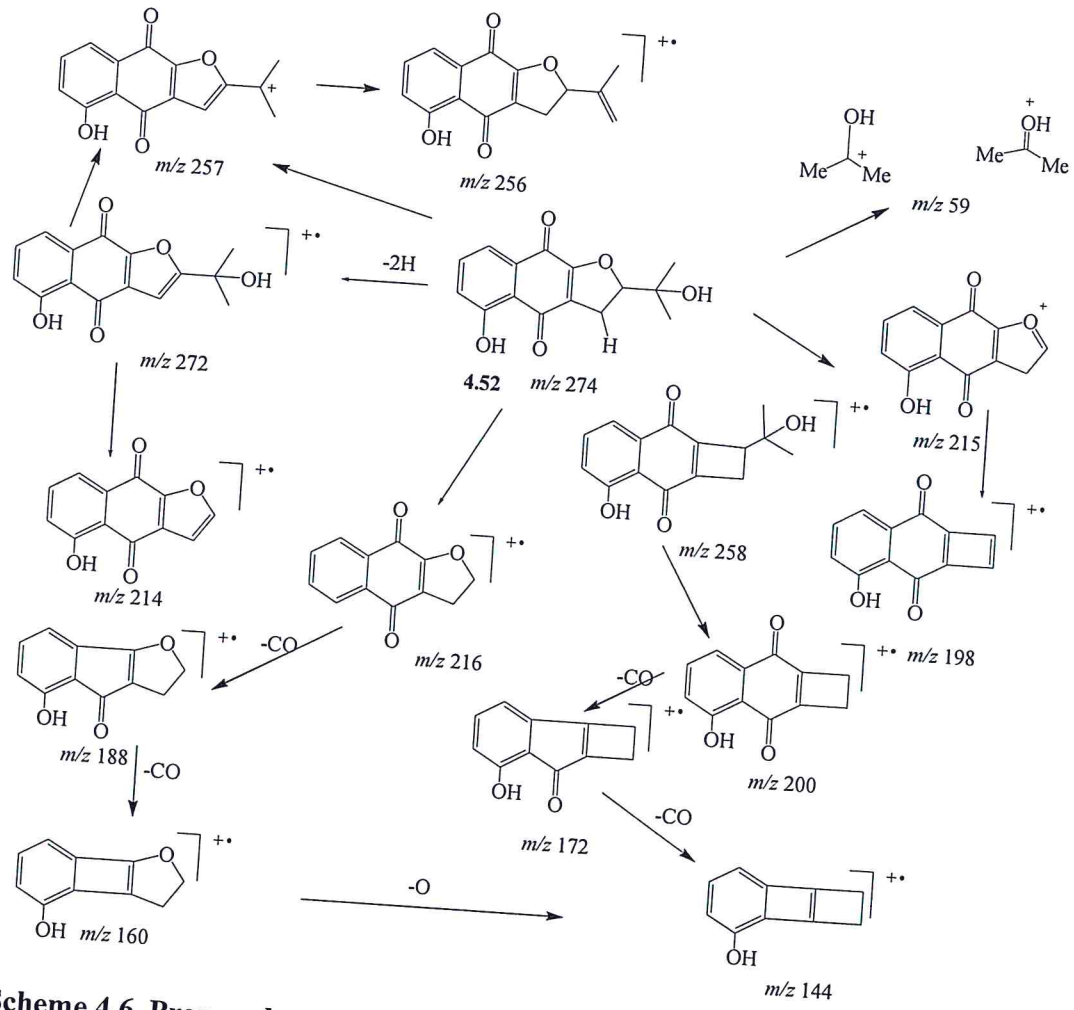


Fig 4.49 **HMBC plot (300 MHz, MeOD) for an inseparable mixture of compounds 4.52, 4.53 and 4.54**



Scheme 4.6 Proposed mass spectral fragmentation pattern for compound 4.52

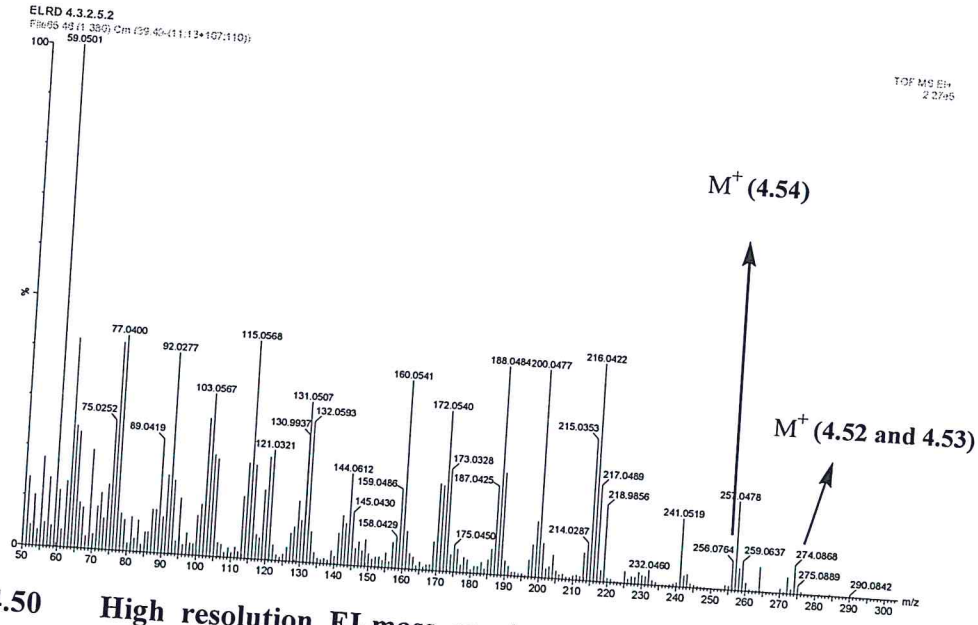
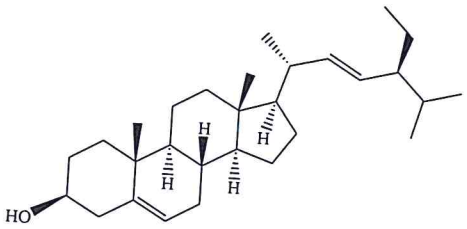


Fig 4.50 **High resolution EI-mass spectrum for the inseparable mixture of compounds 4.52, 4.53 and 4.54**

The isolation of furanonaphthaquinones **4.32**, **4.33** and **4.46-4.54** from *Lantana viburnoides* ssp *vibunoides* var. *kisi* as inseparable mixtures as has been previously reported[106,117-119] further indicates the ability of *Lantana* species to metabolize regio-isomeric furanonaphthaquinones.

4.2.8 Stigmasterol (4.55)



4.55

Stigmasterol (**4.55**), which is a common constituent of many higher plants, was isolated as white crystals from the 5th sub-fraction of the second VLC fraction of the

dichloromethane extract of the root bark of *L. vibunoides*.ssp *vibunoides* var. *kisi*. Its structure was established on the basis of spectral properties.[132]

4.2.9 Lavicidal assay for pure compounds from *Lantana viburnoides* ssp *viburnoides* var. *kisi* (A. Rich) Verdc.

Camaric acid (**4.27**) that was obtained from the 4th and 5th fractions (LF4 and LF5) of the dichloromethane extract of the root bark of *L. viburnoides* ssp *viburnoides* var. *kisi* was found to be very active against the third and fourth instar of *An. gambiae* mosquito larvae. The LC_{50} values for camaric acid were 6.19 and 4.60 ppm after 72 and 96 h exposure, respectively (**Table 4.12**). The mixture of inseparable regioisomeric furanonaphthaquinones **4.49-4.54** that was obtained from LF4 was not active at the concentration range tested. However, furanonaphthaquinones **4.46-4.48** and **4.32-4.33** were found to be more active compared to the mother fraction. The LC_{50} values of the mixtures of the furanonaphthaquinone **4.46-4.48** and **4.32-4.33** was 5.70 and 5.48 ppm respectively, after 48 h (**Table 4.12**). These compounds were obtained in small quantities from the 2nd VLC fraction (LF2) and hence only small samples were available for the bioassays. This could have contributed to the observed low activity of LF2 or the fraction and hence crude extract could also have been obscured by betulinic acid (**4.44**) which was the major constituent of the plant species and had only mild activity (LC_{50} >50 ppm in 24 h; **Chapter 3** and **Scheme 4.4**). Due to the small amounts of the compounds obtained from LF2, synergistic studies with compounds obtained from LF4 could not be undertaken.

Table 4.12 Larvicidal activity (LC_{50}) of isolated compounds against *An. gambiae*

Compound/Mixture	Lethal concentration (LC_{50}) in ppm			
	24 h	48 h	72 h	96 h
4.32 and 4.33	11.52	5.48	-	-
4.44	50.25	10.95	10.43	10.43
4.46, 4.47 and 4.48	7.28	5.70	5.25	5.11
4.27	11.83	8.31	6.19	4.60

- Fiducial confidence limits could not be computed from the concentration range used due to cluster of data.

The betulinic acid (**4.44**) has been isolated from many plant species and shown to exhibit a variety of biological activities, including inhibition of human immunodeficiency virus (HIV), antibacterial, antimalarial, antiinflammatory, anthelmintic and antioxidant properties.[133-141] The antifeedant activity against the larvae of *Achoea janata,* and antibacterial activities were found to be mild.[133,134] The *in vitro* antiplasmodial activity of betulinic acid against chloroquine resistant (K1) and sensitive (T9-96) *Plasmodium falciparum* malaria parasites were close (IC_{50} 19.6 and 25.9 µg/ml, respectively).[135] However, in the *in vivo* assay of betulinic acid in a murine malaria model (*P. berghei*) at 250 mg/kg/day revealed the compound to be toxic and inactive in reducing parasitaemia.[135] Betulinic acid also exhibits antitubercular activity against *Mycobacterium tuberculosis* (MIC 15 µg/ml).[136]

As it is generally acknowledged, betulinic acid has been shown excellent activity in controlling cancer cells as it is selective towards melanoma cells and does not affect normal cells.[137] This feature makes betulinic acid a unique metabolite in comparison to compounds that are currently used in cancer therapy, such as taxol, camptothecin, elipticine, etoposide, vinblastine or vincristine.[138] Recently, it has been demonstrated

that betulinic acid is cytotoxic against other non-melanoma (neuroectodermal and malignant brain tumor) human tumor cell varieties.[137-139] Because of the high anti-HIV activity, selective cytotoxicity against tumor cells and favorable therapeutic index, it is a promising chemotherapeutic agent for the treatment of HIV infection and cancer.[140,141]

Previously, lantadene A (**4.9**) and lantadene B (**4.10**) were shown to have insecticidal and cytotoxcic activity.[136] In these investigations, neither lantadene A nor lantadene B was isolated. However, the related compound, camaric acid (**4.27**) which exhibited high larvicidal activity was obtained in fairly large quantities. These results suggest that, the 3,25-ether linkage which is characteristic of triterpenoids found in the genus *Lantana* may be responsible for the observed larvicidal activity. However, lantalupeol need to be tested for larvicidal activity. Camaric acid has been reported to possess nematicidal activity against *Meloidogyne incognita,* whereby at 0.5% concentration causing 95% mortality.[142] It also exhibit antitubercular properties against *Mycobacterium tuberculosis,* (MIC 36 μg/ml)[136] but showed no significant *in vitro* antiplasmodial against chloroquine resistant (K1) and sensitive (T9-96) *Plasmodium falciparum.*[143]

Previously, a number of furanonaphthoquinones were shown to exhibit antimicrobial activity against Gram-positive bacteria and fungi,[144] inhibitory effects on the Japanese encephalitis virus,[145] antiparasitic activity against *Trypanosoma* parasites[146] and cytotoxicity to a range of tumour cell lines.[106,117] Because of the latter activity diodantunezone (**4.28**) and other related compounds have been snythesized and tested for cytotoxicity against KB epidermoid nasopharynx, K562 human leukaemia and P388 lymphocytic leukaemia cell lines. The compound was found to be active (IC_{50} 6.76, 9.2

and 7.94 mmol/l, respectively) while the corresponding methyl ether (**4.34**) showed higher activity (IC_{50} 1.3, 1.32 and 1.86 mmol/l, respectively) against the three cell line.[106,118] Studies on structure activity relationships revealed the enhancement of activity by an alkyl group at position 2 and a hydroxyl group at positions 5 or 8.[147] In another study, 5-hydroxy-2-(1-hydroxyethyl)naphtho [2,3-*b*]furan-4,9-dione (**4.56**) and 8-hydroxy-2-(1-hydroxyethyl)naphtho [2,3-*b*]furan-4,9-dione (**4.57**) isolated from Bignoniaceae plants *Tecoma ipe* Mart (syn. *Tabebuia impetiginosa*, *Tabebuia cassinoides*, and *Tecoma avellanedae*) and *Kigelia pinnata*, were found to be pontential antimicrobial agents.[144]

However, in the present investigations, furanonaphthaquinone mixtures **4.32-4.33** and **4.46-4.48** that have no substitution in the furanoid ring showed higher larvicidal activity than those substituted at C-2 (**4.49-4.54**). Indeed further research is needed to devise a workable separation of the individual constituents of the mixtures so as to test each individual compound for larvicidal or/and any other biological activities.

4.3 EXPERIMENTAL

4.3.1 General

For general remarks and information about the plant materials, extraction and mosquitoes see **Chapter Three, section 3.3.2** and **3.3.5.1**

4.3.2 Phytochemical work

4.3.2.1 Chromatography

Glass columns wet packed with silica gel were used for gravity column chromatography. Gel chromatography was carried out using Sephadex® LH-20 (Pharmacia). Analytical thin layer chromatography (TLC) was performed on plastic or aluminium sheets precoated with silica gel (60 F_{254}, Merck) and sprayed with an anisaldehyde reagent for visualization. Preparative thin layer chromatography was done either by using normal or reversed phase silica gel (F_{254}, Merck). Vacuum liquid chromatography (VLC) or flash chromatography was carried out using normal phase silica gel whereby the vacuum was generated by a water pump. The compounds were further purified by HPLC linked to a Diode Array detector using a C-4 reversed phase HPLC column (RP C4-SCD100-2585), eluting with a mixture of water and acetonitrile (2:3 v/v). The UV absorption spectrum was scanned and recorded for each sample.

4.3.2.2 Structural elucidation

Structures of compounds isolated in these investigations were determined using various spectroscopic methods, including infrared spectroscopy (FT-IR, Shimadzu 8400, at the Jomo Kenyatta University of Agriculture and Technology, Kenya) and ultra violet-visible (UV-VIS, 168 diode array detector, at the International Center of Insect Physiology and Ecology). High resolution nuclear magnetic resonance (NMR) spectra were done at the Rothamsted Research Institute, in the United Kingdom, the University of Botswana and the university of Nairobi. Both 1D and 2D spectra were recorded on a Bruker Avance DRX 600 MHz, Varian Inova 500 MHz, DPX 300 MHz NMR and 200

MHz spectrometers, operating at 150, 125 and 75 MHz for ^{13}C NMR (δ = 0; TMS internal standard). Low resolution mass spectra were recorded on a VG Masslab 12-250 mass spectrometer with mass range of *m/z* 1-1400 at ICIPE while high resolution MS was recorded on a TOF MS EI machine at the University of Botswana.

4.3.3 Mosquito larvicidal assay

Larvicidal assay was performed by exposing 20 late 3rd or early 4th instar larvae of *An. gambiae* to various concentrations which were prepared by adding a known volume of stock solution in beakers to make up 100 ml of water-sample solution (water temperature 26±2 °C). Thus, 10, 20 and 50 ppm of the dichloromethane extracts of the root barks of *L. viburnoides* ssp *viburnoides* var. *kisi* (LRRD) and 1, 5 and 10 ppm of pure compounds or inseparable mixtures were used in the assay. The tests were done in triplicate from separately reared batches of mosquito larvae. Larval death was recorded after every 24 h. During the experiment, larvae were fed on Tetramin® fish food at 1 mg per beaker per day.

4.3.4 Data analysis

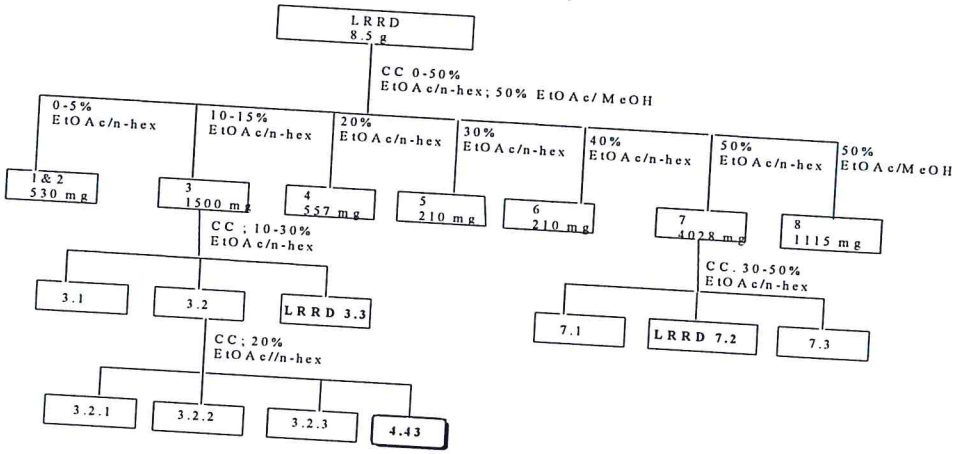
Data were subjected to analysis of variance (ANOVA). Probit analysis to compute lethal doses (LD_{50}) was done using the Lackfit inversel procedure of the SAS program.

4.3.5 Extraction, fractionation and isolation of compounds

About 8.5 g of the crude dichloromethane extract of the root bark of *L. viburnoides* ssp *viburnoides* var. *kisi* (A. Rich) Verdc was obtained from the first materials collected in April, 2003. The crude extract was subjected to column chromatography on silical gel

eluting with a mixture of ethyl acetate and n-hexane (1:4-1:1 v/v gradient) and finally washed out with a mixture of methanol and ethyl acetate (1:1 v/v) (**Scheme 4.1**). Separation was monitored by thin layer chromatography (TLC) and eight fractions were obtained upon combination of fractions having similar composition. The third fraction was further chromatographed on silica gel, eluting with a mixture of ethyl acetate and n-hexane (1:9-3:7 gradient) to obtain three sub-fractions. Lantalupeol (**4.43**) was obtained as white amorphous solid after further chromatography of the second sub-fraction of the third fraction on silica gel, eluting with a mixture of ethyl acetate and n-hexane (1:4 v/v). Other two compounds coded **LRRD 3.3** and **LRRD 7.2** were obtained in small amounts for which the 200 MHz NMR machine could not show clear spectral data. Attempted chromatographic separation of the rest of the fractions was not successful because of the small amount of samples consisting of complex mixtures which could not be worked out further.

Chart 4.1 **Fractionation and isolation of pure compounds from the dichloromethane extract of the root bark of *Lantana viburnoides* ssp *viburnoides* var. *kisi* (1st collection)**



Large scale recollection of the root barks of *L. viburnoides* ssp. *viburnoides* var. *kisi* was done in October, 2003 from which 23 g of crude dichloromethane extract was obtained. Vacuum liquid chromatography of the crude extract (LRRD, 23 g) on silica gel, eluting with n-hexane, ethyl acetate and methanol yielded six fractions (**Scheme 4.2**). Fractions one and six which showed no larvicidal effect were not further analysed.

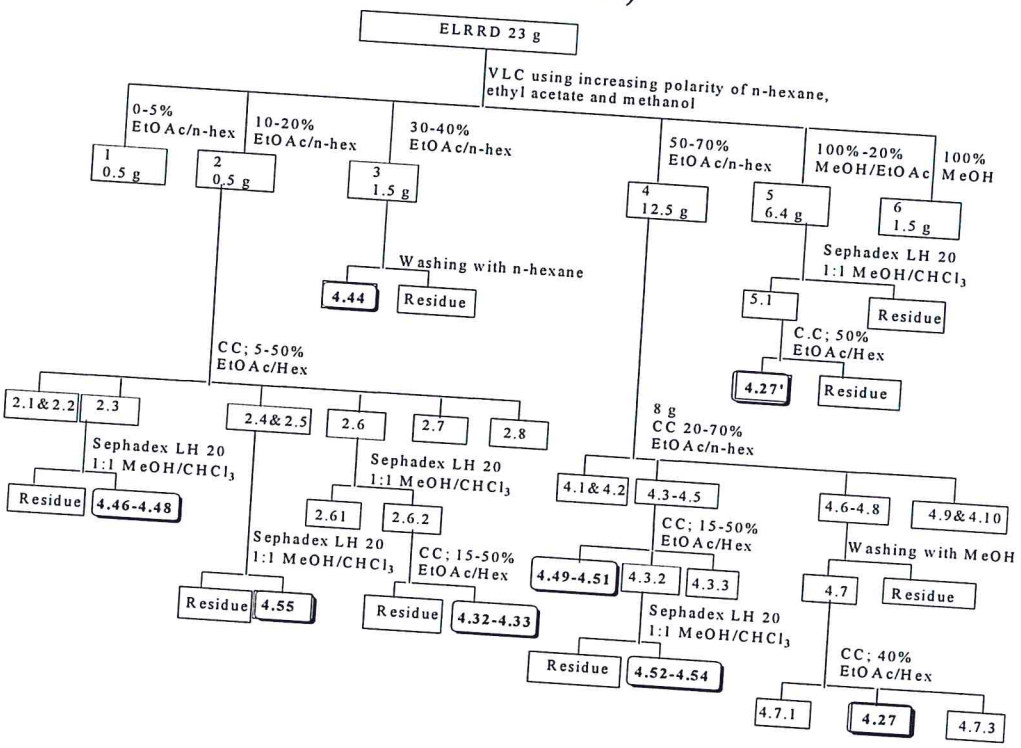
Column chromatography of the second VLC fraction eluting with a mixture of ethyl acetate and n-hexane (1:19-1:1v/v gradient) yielded eight fractions. The 3rd, 5th and 6th fractions were separately subjected to column chromatography on Sephadex® LH-20 eluting with a mixture of methanol and chloroform (1:1 v/v) to yield yellow crystals as an inseparable mixture consisting of the furanonaphthaquinones **4.46, 4.47 and 4.48**, compound **4.55** as white crystals. Another yellow crystals consisting of inseparable mixture of the furanonaphthaquinones **4.32** and **4.33**, was also obtained after further purification on silica gel eluting with a mixture of ethyl acetate and n-hexane (3:17-1:1 v/v gradient). Betulinic acid (**4.44**) was obtained as white amorphous solid after several washing of the 3rd VLC fraction with cold hexane.

Column chromatography of the fourth VLC fraction on silica gel, eluting with a mixture of ethyl acetate and n-hexane (3:7-7:3 v/v gradient), yielded ten fractions which were re-combined to obtain four fractions. Repeated column chromatography of the combined 3rd fraction on silica gel, eluting with a mixture of ethyl acetate and n-hexane (3:7 v/v) yielded an inseparable mixture consisting of furanonaphthaquinones **4.49**, **4.50** and **4.51**, as yellow gum. Further column chromatography of the 3rd fraction on Sephadex® LH-20

eluting with a mixture of methanol and chloroform (1:1 v/v) gave another inseparable mixture consisting of compounds **4.52**, **4.53** and **4.54**.

Repeated column chromatography of the fourth VLC fraction yielded camaric acid (**4.27**), which was obtained after washing with cold methanol and purified by column chromatography on silica gel, eluting with a mixture of ethyl acetate and n-hexane (2:3 v/v). Camaric acid (**4.27**) was also obtained from the 5th VLC fraction upon column chromatography on Sephadex® LH-20, eluting with a mixture of methanol and chloroform (1:1 v/v), and purification by column chromatography on silica gel, eluting with a mixture of ethyl acetate and n-hexane (1:1 v/v gradient).

Chart 4.2 **Fractionation and isolation of pure compounds from the dichloromethane extract of the root bark of *Lantana viburnoides* ssp *viburnoides* var. *kisi* (2nd collection)**



Betulinic acid **(4.44)**. White amorphous (MeOH), (Lit[132] m.p. 316-318 °C); yield, 308 mg; anisaldehyde - black; IR (KBr) ν_{max} cm^{-1}, 3438 br, 3072, 2945, 2872, 1696, 1646, 1558, 1377, 1232, 1187, 1031 and 887; MS, *m/z* (% rel. int.) 456 ($[M]^+$, 12), 438 (7), 410 (5), 248 (45), 219 (20), 220 (20), 207 (50), 189 (100), 175 (32), 161 (20), 147 (25), 135 (45), 119 (47), 107 (48), 95 (57), 69 (55) and 55 (65); ^1H and ^{13}C NMR (see **Tables 4.1** and **4.2**).

Camaric acid **(4.27)**. White amorphous (MeOH), (Lit[109] m.p. 188-189 °C); yield, 325 mg; anisaldehyde - greenish-yellow; IR (KBr) ν_{max} cm^{-1}, 3430 br, 2951, 2877, 1716, 1649, 1556, 1513, 1462, 1376, 1235, 1152, 1042, 938 and 845; MS, *m/z* (% rel. int.) 568 ($[M]^+$, 0.1), 469 (30), 468 (100), 450 (20), 424 (18), 407 (10), 285 (34), 239 (28), 201 (27), 189 (40), 119 (42), 83 (100), 69 (42) and 55 (94); ^1H and ^{13}C NMR (see **Tables 4.3** and **4.4**).

Lantaperoxide **(4.45)**. Creamy oil. yield, 12.4 mg; ninhydrin reagent - red; UV, λ_{max} (2:3 v/v H_2O/MeCN) nm, 215, 278; IR (KBr) ν_{max} cm^{-1}, 3397, 2924, 2855, 1715, 1650, 1607, 1515, 1460, 1372, 1270, 1236, 1152, 1080, 1040 and 970; MS, *m/z* (% rel. int.) 274 ($[M]^+$, 18), 148 (13), 137 (7), 111 (22), 97 (35), 85 (29), 83 (30), 71 (49), 69 (48), 57 (100) and 55 (68); ^1H and ^{13}C NMR (see **Table 4.5**).

Mixture of 7-methoxy-naphtho[2,3-b]furan-4,9-dione **(4.32) and** ***6-methoxy-naphtho[2,3-b]furan-4,9-dione*** **(4.33)**. Yellow crystals (MeOH), yield, 6.4 mg; anisaldehyde- yellow; UV, λ_{max} (2:3 v/v H_2O/MeCN) nm, 214, 255, 299 and 339; HRMS, *m/z* (% rel. int.) 228.0404 ($[M]^+$, 100 for each of the two compounds; calc. for

$C_{13}H_8O_4$: 228.023), 198.0320 (42), 185.0244 (22), 157.0313 (20) and 129.0371 (20); ^1H and ^{13}C NMR (see **Table 4.6a** and **4.6b**).

***Mixture of 6-hydroxy-naphtho[2,3-b]furan-4,9-dione* (4.46), *7-hydroxy-naphtho[2,3-b]furan-4,9-dione* (4.47) and *naphtho[2,3-b]furan-4,9-dione* (4.48)**. Yellow crystals (MeOH); yield, 9 mg; anisaldehyde - yellow; UV, λ_{max} (2:3 v/v H_2O/MeCN) nm, 201, 245 and 291; IR (KBr) ν_{max} cm^{-1}, 3857, 3748, 3446, 3147, 2924, 2855w, 2468, 1674, 1643, 1572, 1453, 1367, 1316, 1264, 1189, 1058, 1025 and 949; HRMS, *m/z* (% rel. int.) 215.0305 ([M^++1], 80; calc. for $C_{12}H_6O_4$: 215.0266), 214.0251 ([M^+], 100 for **4.46**&**4.47**), 198.0318 (([M^+], 40 for **4.48**; calc. for $C_{12}H_6O_3$: 198.0317), 187.0385 (35), 186.0327 (40), 170.0381 (30), 159.0447 (35), 158.0386 (45), 130.0441 (30) and 129.0268 (40); ^1H and ^{13}C NMR (see **Tables 4.7** and **4.8**).

***Mixture of 6-hydroxy-2-(1-hydroxy-1-methyl-ethyl)-naphtho[2,3-b]furan-4,9-dione* (4.49), *7-hydroxy-2-(1-hydroxy-1-methyl-ethyl)-naphtho[2,3-b]furan-4,9-dione* (4.50) and *2-(1-hydroxy-1-methyl-ethyl)-naphtho[2,3-b]furan-4,9-dione* (4.51)**. Yellow gum; yield, 7.5 mg; anisaldehyde - yellow; UV, λ_{max} (2:3 v/v H_2O/MeCN) nm, 200, 248 and 292; IR (KBr) ν_{max} cm^{-1}, 3856, 3744, 3447b, 3019, 2976, 2932, 2328, 1644, 1621, 1512, 1455, 1382, 1241, 1193, 1070, 1012 and 966; HRMS, *m/z* (% rel. int.) 273.0702 ([M^+ +1], 15 for **4.49** and **4.50**), 272.0653 (M^+, 30 for **4.49** and **4.50**; calc. for $C_{15}H_{12}O_5$: 272.0685), 257.0409 (M^+ +1], 100 for **4.51**; calc. for $C_{15}H_{12}O_4$: 256.736), 241.0437 (20), 229.0492 (20), 215.0336 (30) and 187.0412 (25); ^1H and ^{13}C NMR (see **Table 4.9**).

***Mixture of 6-hydroxy-2-(1-hydroxy-1-methyl-ethyl)-2,3-dihydro-naphtho[2,3-b]furan-4,9-dione* (4.52), *7-hydroxy-2-(1-hydroxy-1-methyl-ethyl)-2,3-dihydro-naphtho[2,3-b]furan-4,9-dione* (4.53) and *(1-hydroxy-1-methyl-ethyl)-2,3-dihydro-naphtho[2,3-b]furan-4,9-dione* (4.54).** Yellow gum; yield, 12 mg; anisaldehyde - yellow; UV, λ_{max} (2:3 v/v H_2O/MeCN) nm, 200, 248 and 292; IR (KBr) ν_{max} cm^{-1}, 3856, 3744, 3447, 3019, 2976, 2932, 2328, 1644, 1621, 1512, 1455, 1382, 1241, 1193, 1070, 1012 and 966; HRMS, *m/z* (% rel. int.) 274.0868 ($[M]^+$, 10 for **4.52** and **4.53**; calc. for $C_{15}H_{14}O_5$: 274.0841), 257.0478 (($[M^+-1]$, 45 for **4.54**; calc. for $C_{15}H_{14}O_4$: 258.0892), 241.0519 (15), 216.0422 (50), 200.0477 (45), 188.0484 (45), 160.0541 (40) and 131.0507 (35); ^1H and ^{13}C NMR (see **Table 4.10** and **4.11**).

***Stigmasterol* (4.55).** White crystals (MeOH), m.p. 112-114 °C; yield, 29 mg; Anisaldehyde - blue; IR (KBr) ν_{max} cm^{-1}, 3423 br, 2935, 2858, 1651, 1513, 1461, 1376, 1245, 1104, 1054 and 962; MS, *m/z* (% rel. int.) 414 ($[M]^+$, 90), 396 (50), 329 (30), 303 (29), 255 (35), 213 (30), 159 (35), 145 (40), 105 (48), 81 (60), 55 (58) and 43 (100).

CHAPTER FIVE

LARVICIDAL COMPOUNDS FROM *STEGANOTAENIA ARALIACEA* (APIACEAE)

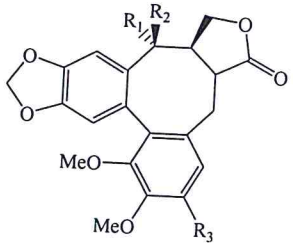
Abstract

This chapter reports results from bioassay-guided fractionation of the dichloromethane extract of the root bark of *Steganataenia araliacea* which led to the isolation of two isomeric lignans, namely steganoate A and isosteganoate A as well as steganacin from mosquito larvicidal fractions. The mixture of the two isomers and steganacin exhibited larvicidal activity with LC_{50} value of 3.92 and 9.10 ppm after 72 h, respectively. Combination of isomers (steganoate A and isosteganoate A) and steganacin in one to one proportion showed slight enhancement of activity (LC_{50} 3.13 ppm after 72 h) indicating that the three compounds may have additive larvicidal effects. Other compounds isolated included the lignan steganangin, β–sitosterol, and saturated and mono-unsaturated fatty acids. The structures of these compounds were established by a combination of various spectroscopic techniques.

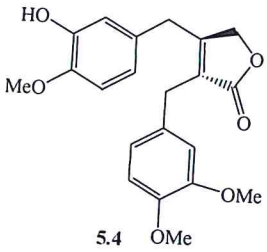
5.1 INTRODUCTION

Steganotaenia araliacea belongs to the family Apiaceae, initially called Umbelliferae, which comprises 300 genera with about 3,000 species.[148] The plants are herbs, shrubs and trees which are mainly distributed in the Mediteranian area.[148] *Steganotaenia araliacea* is a glabrous small tree, rarely a bush, 2-12 metres tall. The tree is straggling and untidy with regularly spreading crown.[148] The plant is cosmopolitan and is used in the treatment of malaria in some parts of Tanzania.[99,101] It is also used as a snakebite antidote, for the treatment of sore throat and lung diseases in animals like cattle, goats and calves.[6] The Shona people of Southern Africa believe that, the plant is capable of inducing abortion in goats.[6] The plant exhibited insecticidal activity against *Tribolium castaneum* and *Culex quinquefasciatus* mosquitoes.[10]

Although the major constituents of *Steganotaenia araliacea* are bibenzocyclooctadiene lactone lignans, other compounds such as coumarins, terpenes, and flavanoids have also been reported in *Steganotaenia* species.[148] So far, phytochemical work has only been reported on the stem bark and leaves but not on roots. The reported compounds include the anticancer bibenzocyclooctadiene lactone lignans such as 10-demethoxystegane (**5.1**), steganones **5.2** and **5.3**, prestegane B (**5.4**), and neoisostegane (**5.5**) from the stem bark. Demethylsteganone (**5.3**) exhibited anti-proliferative activity (2.5 ±0.5 μg/ml) against ovarian cancer cell lines (OVCAR-3).[149]



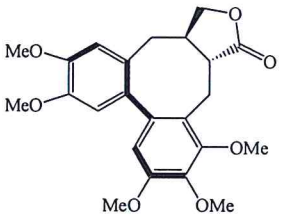
	R_1	R_2	R_3
5.1:	H	H	H
5.2:	$R_1R_2=O$		OMe
5.3:	$R_1R_2=O$		OH



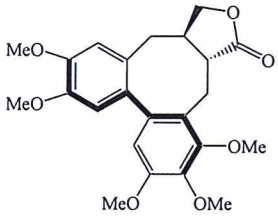
5.4

Other bibenzocyclooctadiene lactone lignans isolated from the stem bark include stegane (**5.6**), isostegane (**5.7**), neostegane (**5.8**), steganolide A (**5.9**), steganolide B (**5.10**), steganolide C (**5.11**), steganacin (**5.12**), steganangin (**5.13**), episteganangin (**5.14**), episteganacin (**5.15**), steganoate A (**5.16**), steganoate B (**5.17**), steganol (**5.18**), episteganol (**5.19**) and araliagine (**5.20**), which are active against eleven human tumour cell lines.[150-155] Steganacin (**5.12**) and steganangin (**5.13**) showed resemblance to colchicines and podophyllotoxin in biological action by binding tubulin, and hence preventing spindle formation and cell division, thereby inhibiting polymerization of tubulin.[152,156] They exhibit *in vivo* antileukaemia activity in the murine P-388 lymphocytic leukaemia cell line.[156] Substitution at C-5 was found to be the significant

factor for high cytotoxic activity. Thus, compounds like neoisostegane (**5.5**), stegane (**5.6**), isostegane (**5.7**) and neostegane (**5.8**), which lack such substitution pattern are less cytotoxic, than those with C-5 substitution with neoisostegane (**5.5**) exhibiting *in vitro* cytotoxic activity at a relatively low level (ED_{50} = 6.6 μg/ml) against the KB cell culture.[153,154]



5.5



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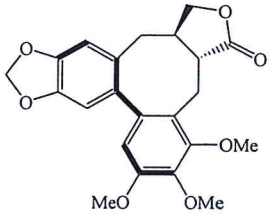
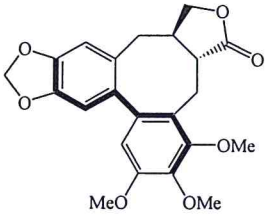
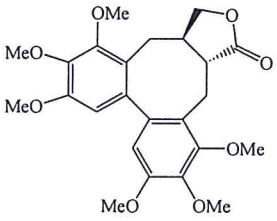


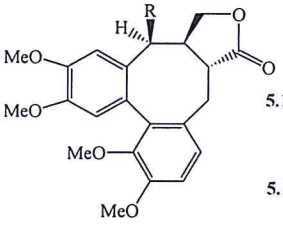
5.7

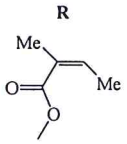


5.8

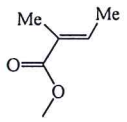


5.9

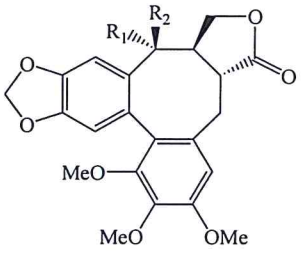


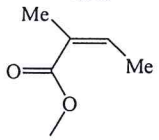
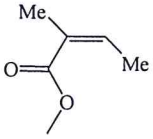


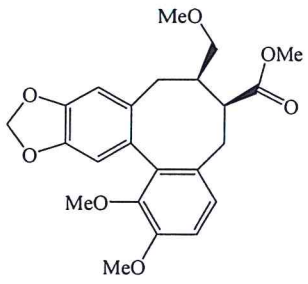
5.10:



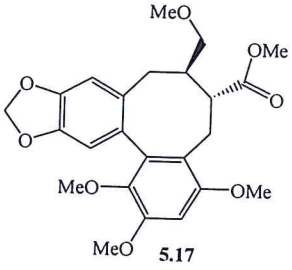
5.11:



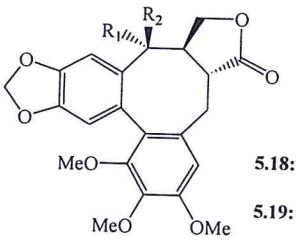
	R_1	R_2
5.12:	H	OAc
5.13:	H	
5.14:		H
5.15:	OAc	H



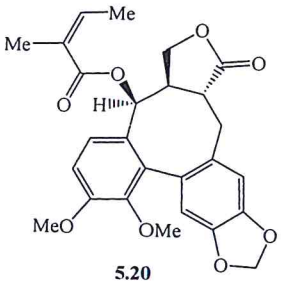
5.16



5.17

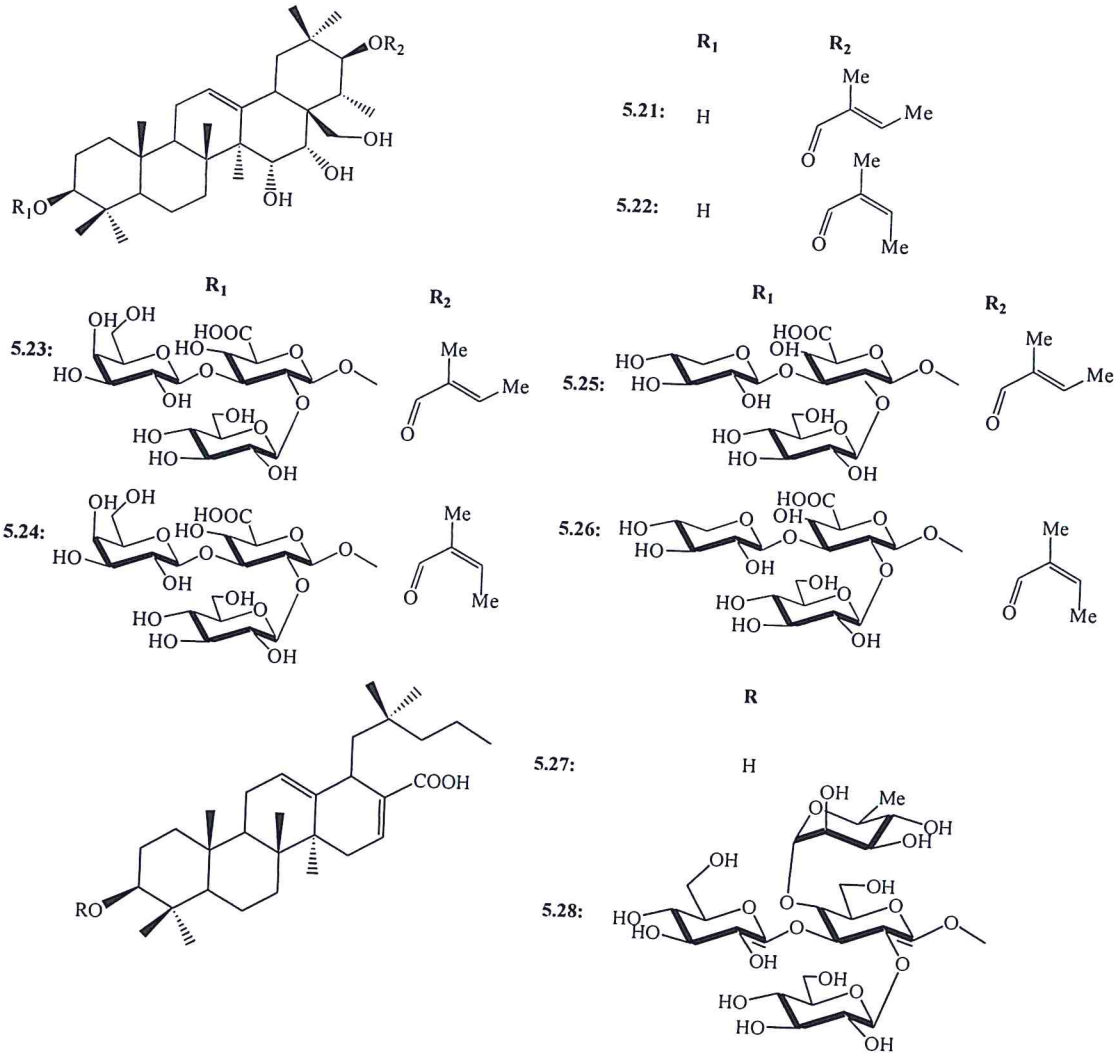


	R_1	R_2
5.18:	OH	H
5.19:	H	OH

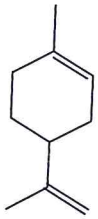


5.20

The leaves of *S. araliacea* are rich in monoterpenes, sesquiterpene and triterpenoids together with the corresponding saponins, as well as s and The most abundant triterpenoids are 21-*O*-tigloyl-barrigenol (**5.21**) and 21-*O*-angeloyl-barrigenol (**5.22**) which were also isolated from *Aesculus glabra*.[157] Other triterpene saponins occurring in *S. araliacea* include 3-*O*-[β-D-galactopyranosyl(1→2)-(β-D-galactopyranosyl(1→3))-β-D-glucuronopyranosyl]-21-*O*-tigloyl-barrigenol (**5.23**), 3-*O*-[β-D-galactopyranosyl(1→2)-(β-D-galactopyranosyl(1→3))-β-D-glucuronopyranosyl]-21-*O*-angeloyl-barrigenol (**5.24**), 3-*O*-[β-D-glucuronopyranosyl(1→2)-(β-D-xylopyranosyl(1→3))-β-D-glucuronopyranosyl]-21-*O*-tigloyl-barrigenol (**5.25**), 3-*O*-β-D-glucuronopyranosyl(1→2)-(β-D-xylopyranosyl(1→3))-β-D-glucuronopyranosyl]-21-*O*-angeloyl-barrigenol (**5.26**), a 17,22-*seco*-olea-12,16-dien-28-oic acid derivative steganogenin (**5.27**) and the corresponding saponin 3-*O*-[β-D-glucuronopyranosyl(1→2)-β-D-glucuro (1→3))-α-L-rhamnopyranosyl(1→4))-β-D-glucuronopyranosyl]-21-*O*-tigloyl-barrigenol (**5.28**).[157]

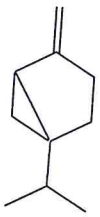


Comparison the essential oil composition of fresh and dried leaves of *S. araliacea* from two difference localities and time of collection showed significant difference in the quantity of essential oil and its composition. In general, the main constituents were limonene (**5.29**) and β-phellandrene (**5.30**) (11.78-35.9%), sabinene (**5.31**) (9.4-24.98%), β-caryophyllene (**5.32**) (2.00-14.91%), α-pinene (**5.33**) (4.89-11.4%) and cryptone (**5.34**) (3.6-16.6%).[79]

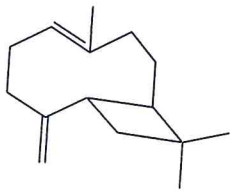


5.29

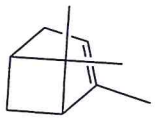
5.30



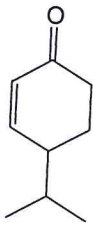
5.31



5.32



5.33

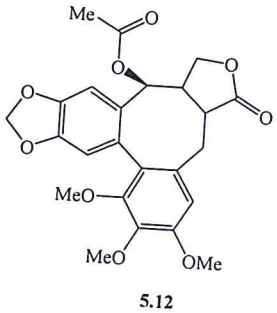


5.34

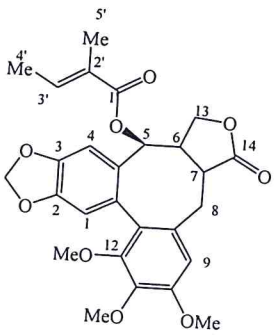
In this study, the roots were collected for further phytochemical investigations and mosquitocidal assays against the malaria vector, *An. gambiae s.s.* Giles. From these investigations, two isomeric lignans, namely steganoate A (**5.16**) and isosteganoate A (**5.35**) as well as steganacin (**5.12**) were isolated from mosquito larvicidal fractions, together with previously isolated steganangin (**5.13**), β–sitosterol (**5.36**), saturated **5.37** and mono-unsaturated fatty acids **5.38**.

5.2 RESULTS AND DISCUSSION

5.2.1 Steganacin (5.12) and steganangin (5.13)



5.12



5.13

Repeated column chromatography of the 4th and 5th VLC fractions of the dichloromethane extract of the root bark of *S. araliacea* (**Chart 5.1**) yielded the

bibenzocyclooctadiene lactone lignans (**5.12**) steganacin and steganangin (**5.13**). The structures and were established on the basis of ^{1}H and ^{13}C NMR spectral properties, including H/H and C/H interactions such as COSY, HMQC and HMBC plots (**Figs 5.1-5.8**) as well as IR and MS properties as compared with reported data.[150-151,153-155]

In the low field region, the ^{1}H NMR spectrum of each of **5.12** and **5.13** exhibited similar patterns, except for the appearance of an additional signal at δ 6.0 in the spectrum of **5.13** due to H-3'. The ^{1}H NMR spectrum of each compound displayed three singlets due to aromatic protons at δ 6.46, 6.56 and 6.99 for compound **5.12,** and δ 6.46, 6.61 and 6.92 for compound **5.13**, which were assigned to H-9, H-1 and H-4, respectively (**Table 5.1a** and **5.1b**). Furthermore, the ^{1}H NMR spectra of **5.12** and **5.13** respectively showed a doublet at δ 5.83 and 6.00 (each J = 9 Hz) and singlet at δ 6.04 and 6.05 which are typical resonances for bibenzocyclooctadiene lignan lactones substituted at C-5 and having dioxymethylene moeities.[150-151,153-155]

The ^{1}H NMR and H/H COSY spectra for **5.13** showed an additional one proton signal at δ 6.00, which also displayed coupling with protons of two methyl groups resonating at δ 1.72 (q, J = 1.5 Hz) and 1.89 (qq, J_1 = 7.2, J_2 = 1.5 Hz), suggesting presence of an angelate moeity in the compound. The ^{13}C NMR spectrum (δ 139.01, 127.21, 20.20 and 15.69) and single bond C/H correlations (HMQC), were in agreement with structure **5.13** (**Table 5.1b** and **5.2; Figs 5.5-5.8**).

Other signals in the ^{13}C NMR and high field region of the ^{1}H NMR spectrum (**Figs 5.2** and **5.5**) of each of the two compounds were similar in pattern. The assignment as well

as stereochemistry of the two compounds were established based on comparison of the spectral data with those reported in the literature.[150-151,153-155] Both compounds **5.12** and **5.13** showed the normal biaryl configuration in combination with a lactone due to the coupling between H-5α and H-6 (δ 5.83, *d*, H-5; δ 2.55, *m*, H-6 for compound **5.12**, and δ 5.83, *d*, H-5; δ 2.56, *m*, H-6 for compound **5.13**), and also between H-7 and H-8β (δ 3.99, m, H-7; δ 2.60, *d*, H-8 for compound **5.12**, and δ 3.92, m, H-7; δ 2.60, *d*, H-8 for compound **5.13**) (**Figs 5.1, 5.4** and **5.6**). The '*iso*' biaryl configuration and *trans*-lactone would not show $H_{5\alpha,6}$ and $H_{7,8\beta}$ coupling.[150-151,153-155] Placement of methoxy groups at C-10, C-11 and C-12 was possible upon considering long range H/C interactions observed in the HMBC plot (**Fig 5.8**).

The MS of steganacin (**5.12**) and steganangin (**5.13**) consisted of molecular ion peaks at *m/z* 456 and 496 which were also base peaks, and corresponding to the molecular formulae $C_{24}H_{24}O_9$ and $C_{27}H_{28}O_9$, respectively. The fragment ion at *m/z* 396 would be formed after cleavage of the ester side chain in each of the two compounds, while the fragment ion at *m/z* 366 would be formed by cleavage of a methoxy group (**Scheme 5.1**). Other ions as shown in **Scheme 5.1** were in agreement with the fragmentation process reported for steganacin (**5.12**) and steganangin (**5.13**).[150-151,153-155]

Table 5.1a ^{1}H NMR spectral data for steganacin (5.12)

H	$\delta_{observed}$	*J* (Hz)	$\delta^{150,153-155}$
1	6.61	*s*	
4	6.92	*s*	
5β	5.83	*d*, $J_{5\beta,6} = 9$	
6	2.55	*t*, $J_{6,7} = 9$	2.52
7	3.99	*m*	
8β	2.60	*t*, $J_{8\alpha,8\beta} = 12.9$	2.59
8α	3.00	*d*, $J_{8\alpha,8\beta} = 12.9$	3.07
9	6.46	*s*	
13β	4.00	*t*, $J_{13\alpha,13\beta} = 9$	
13α	4.30	*dd*, $J_{3\alpha,3\beta} = 9$, $J_{13\alpha,6} = 6$	
2-OCH_2O	6.04	*s*	
10-OCH_3	3.93	*s*	
11-OCH_3	3.88	*s*	
12-OCH_3	3.74	*s*	
2'	1.9	*s*	

Run in $CDCl_3$

Table 5.1b ^{1}H NMR spectral data for steganangin (5.13)

H	$\delta_{observed}$	*J* (Hz)	$\delta^{150,153-155}$	*J* (Hz)
1	6.59	*s*		
4	6.99	*s*		
5β	6.00	*d*, $J_{5\beta,6} = 9.6$		$J_{5\beta,6} = 9.8$
6	2.56	*t*, $J_{6,7} = 9.6$		$J_{5\beta,6} = 9.8$
7	3.92	*obscured*		
8β	2.60	*m*, $J_{8\alpha,8\beta} = 12.9$	2.52	*dd*, $J_{8\alpha,8\beta} = 13.2$, $J_2 = 6.5$
8α	3.10	*d*, $J_{8\alpha,8\beta} = 12.6$	2.57	*dd*, $J_{8\alpha,8\beta} = 13.3$, $J_2 = 10.6$
9	6.46	*s*		
13β	4.00	*t*, $J_{13\alpha,13\beta} = 9$		
13α	4.30	*dd*, $J_{13\alpha,13\beta} = 9$, $J_{13\alpha,6} = 6.5$		
2-OCH_2O	6.05	*s*		
10-OCH_3	3.90	*s*		
11-OCH_3	3.88	*s*		
12-OCH_3	3.72	*s*	3.07	
3'	1.89	*qq*, $J_{3',4'} = 7.2$, $J_{3',5'} = 1.5$	1.98	*qq*,
4'	6.00	*qq*, $J_{3',4'} = 7.2$, $J_{3',5'} = 1.5$		
5'	1.72	*q*, $J_{3',5'} = 1.5$	1.99	*q*, $J_{3',5'} = 1.5$

Run in $CDCl_3$

Table 5.2 ^{13}C NMR spectral data for steganacin (5.12) and steganangin (5.13)

C	5.12		5.13	
	$\delta_{observed}$	$\delta^{150,155}$	$\delta_{observed}$	$\delta^{150,155}$
1	108.0		107.48	
2	141.62		141.04	
3	147.37		146.99	
4	112.78		112.4	
4a	131.41		130.93	
5	77.93	77.2	76.94	77.6
6	42.74	43.4	42.48	42.5
7	44.4	45.0	43.7	44.8
8	30.88	30.5	30.4	30.5
8a	129.6		129.25	
9	152.34		151.85	
10	112.91		112.59	
11	153.8		153.35	
12	148.44		147.97	
12a	127.61		127.11	
12b	133.39		132.82	
13	71.53	71.1	71.15	71.1
14	177.59		177.46	
2-OCH_2O	102.06		101.63	
9-OCH_3	56.45		55.99	
11-OCH_3	61.10		60.68	
12-OCH_3	61.38		60.82	
1'	170.45		167.08	
2'	21.31		127.21	
3'			139.01	
4'-CH_3			20.2	
5'-CH_3			15.69	

Run in $CDCl_3$

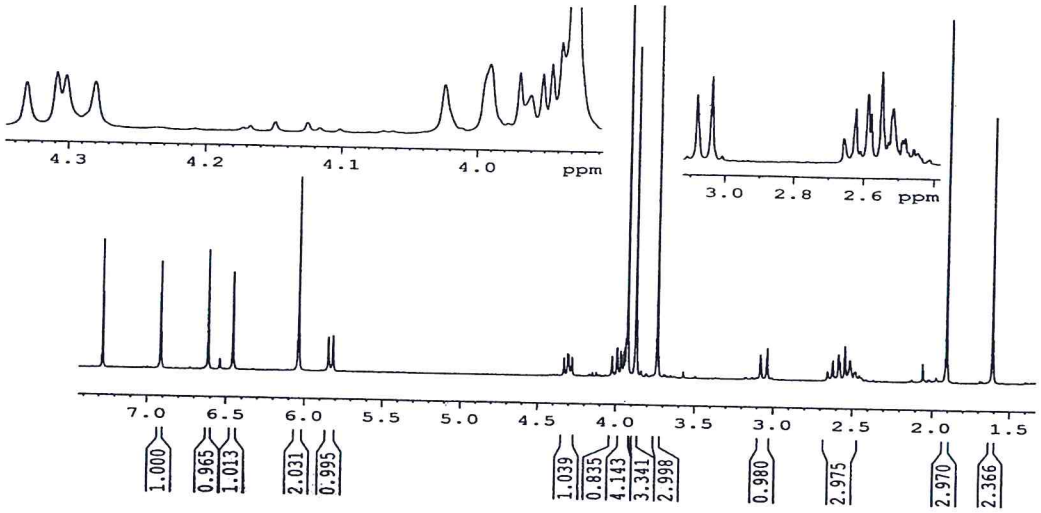


Fig 5.1 ^{1}H NMR spectrum (300 MHz, $CDCl_3$) of steganacin (5.12)

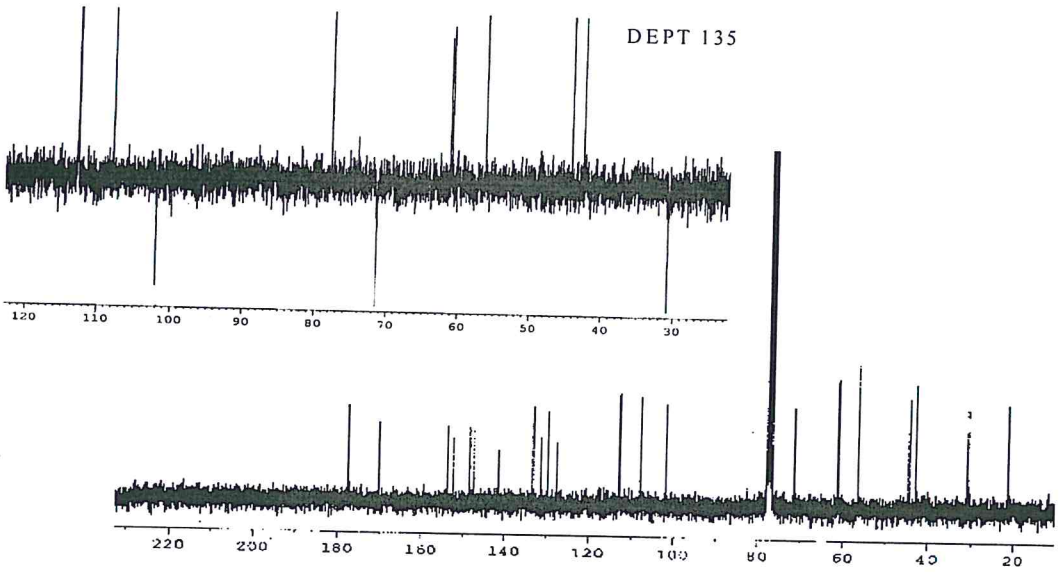


Fig 5.2 ^{13}C NMR spectrum (300 MHz, $CDCl_3$) of steganacin (5.12)

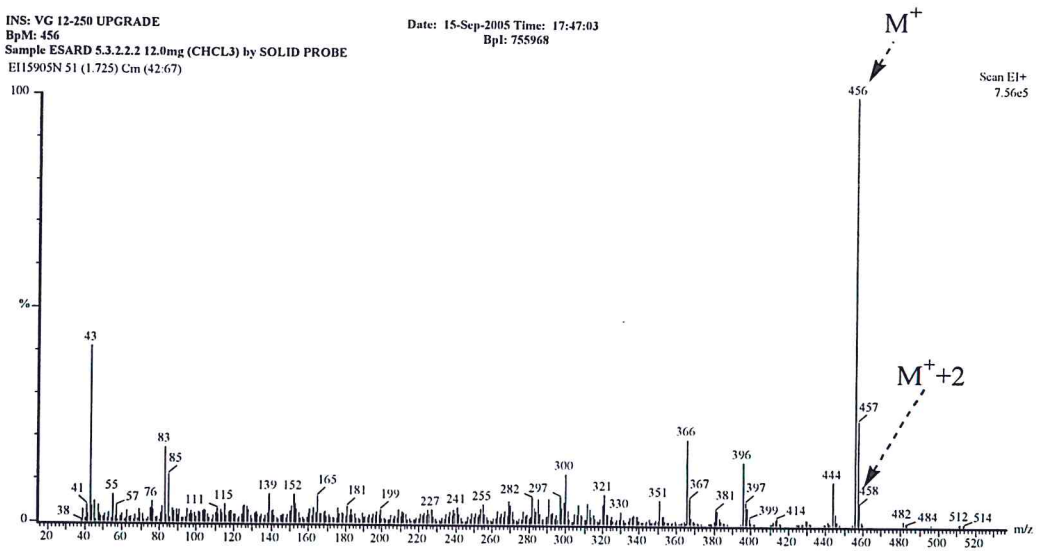


Fig 5.3 EI-Mass spectrum of steganacin (5.12)

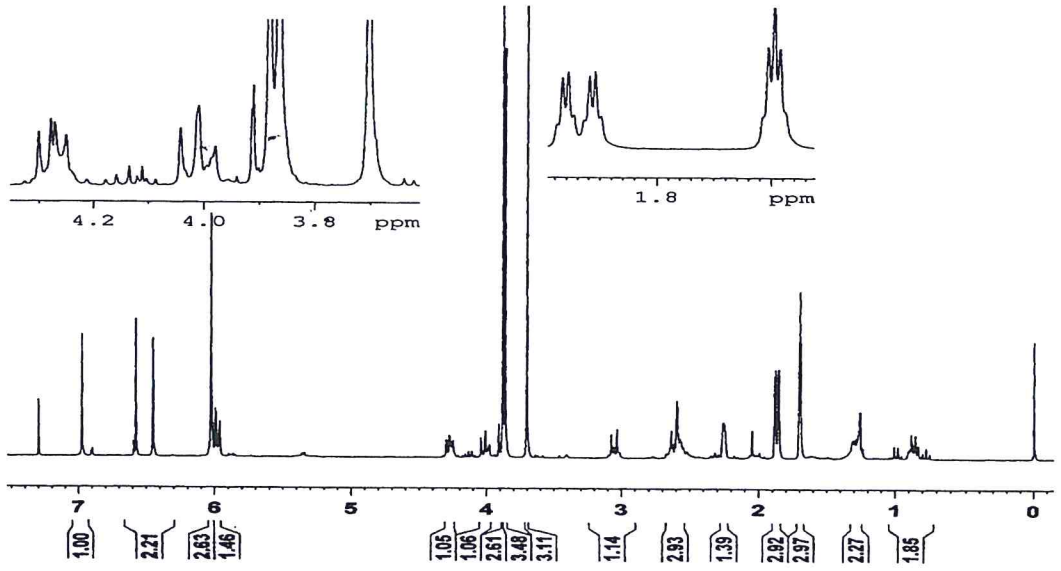


Fig 5.4 ^{1}H NMR spectrum (300 MHz, $CDCl_3$) of steganangin (5.13)

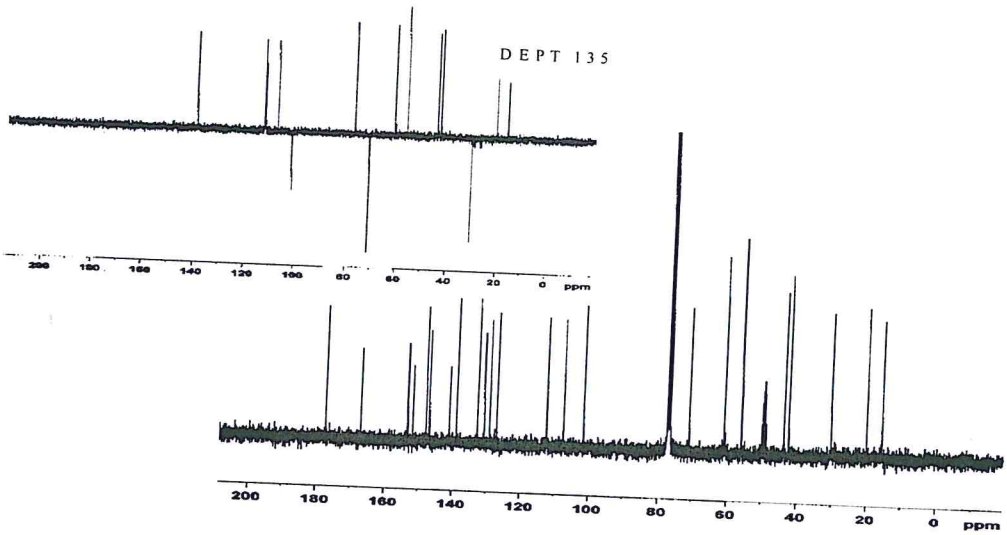


Fig 5.5 ^{13}C NMR spectrum (300 MHz, $CDCl_3$) of steganangin (5.13)

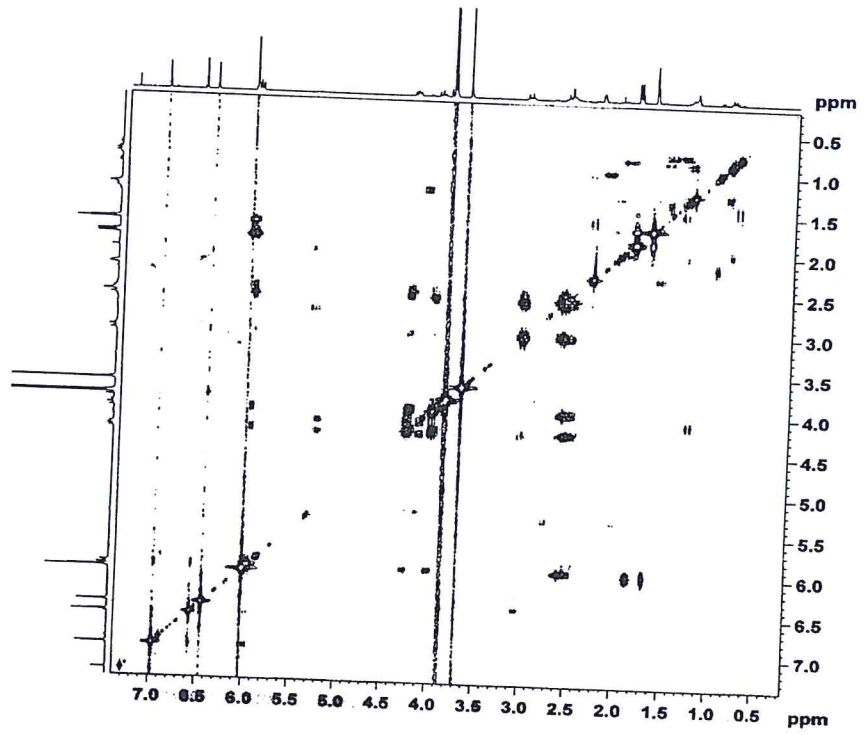


Fig 5.6 H/H COSY Plot (300 MHz, $CDCl_3$) for steganangin (5.13)

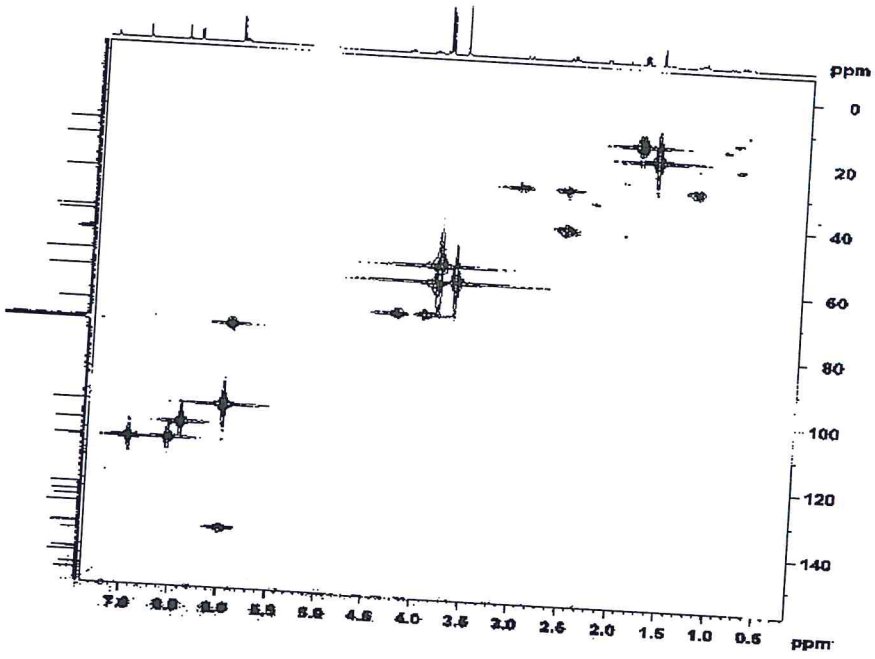


Fig 5.7 HMQC Plot (300 MHz, $CDCl_3$) for steganangin (5.13)

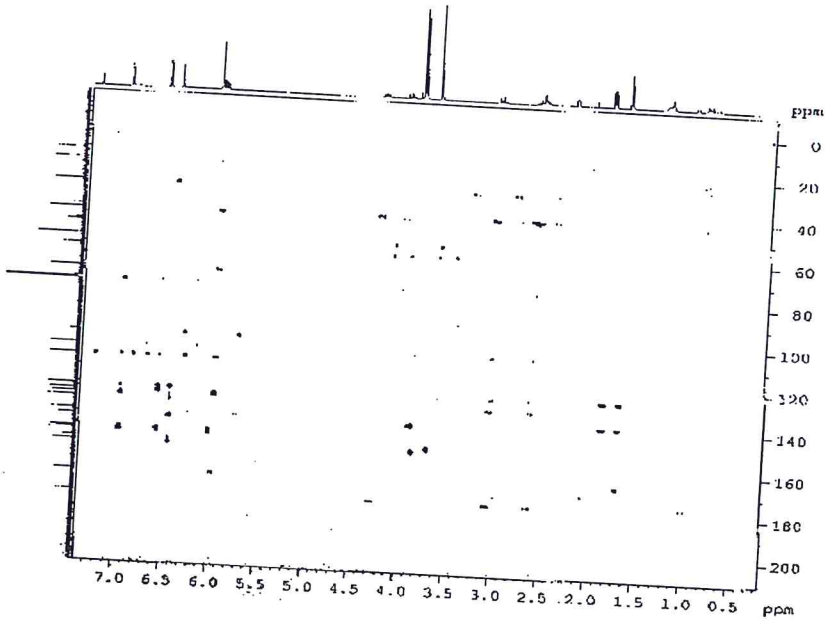


Fig 5.8 HMBC Plot (300 MHz, $CDCl_3$) for steganangin (5.13)

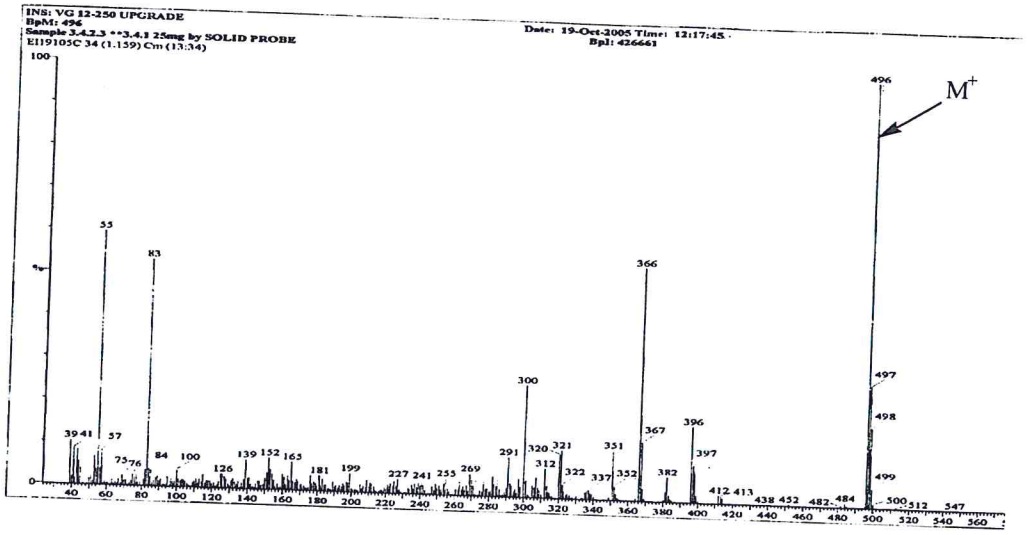
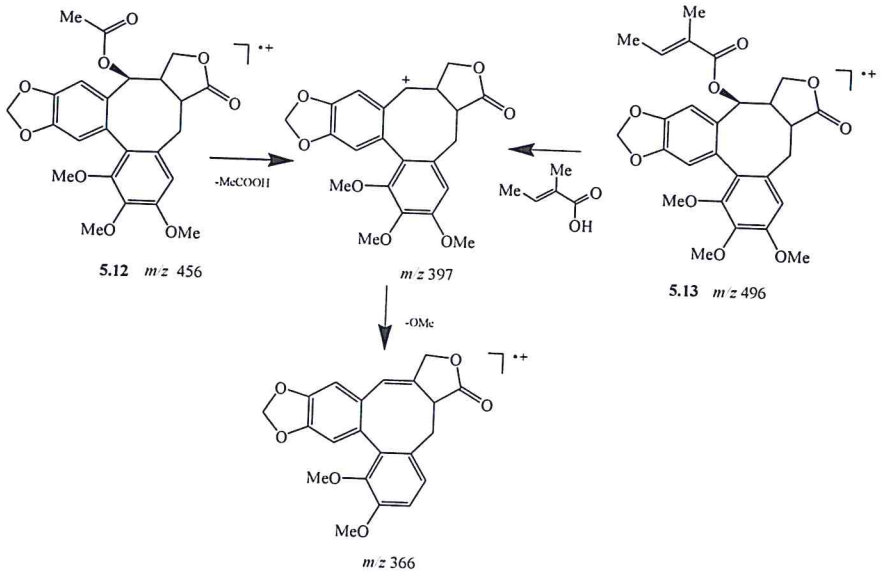
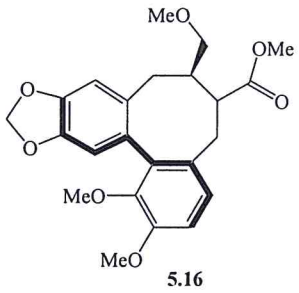


Fig 5.9 **EI-Mass spectrum of steganangin (5.13)**

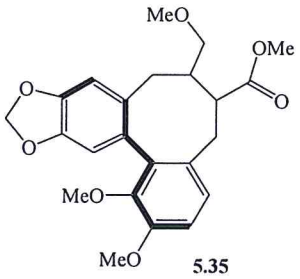


Scheme 5.1 **Proposed mass spectral fragmentation pattern of steganacin (5.12) and steganangin (5.13)**

5.2.2 Steganoate A (5.16) and isosteganoate A (5.35)



5.16



5.35

Repeated column chromatography of the 4th VLC fraction of the dichloromethane extract of the root bark of *S. araliacea* yielded a mixture of the two bibenzocyclooctadiene type lignan isomers steganoate A (**5.16**) and isosteganoate A (**5.35**) in the ratio 2:1 as white oil but then crystallized from methanol to white crystals (**Chart 5.1**). The two compounds could not be purified further even by reverse-phase HPLC chromatography. The structures of the two isomers were established on the basis of ^{1}H and ^{13}C NMR spectra and H/H and C/H correlations, IR and mass spectral properties as compared with those reported in the literature.[150]

Similarities in spectral properties between these compounds and the well characterized lignan, steganoate A indicated the same skeleton type for the two compounds **5.16** and **5.35**.[150] For example, the IR spectrum displayed a carbonyl absorption at 1734 cm^{-1} indicating the presence of an ester group in the molecule. In the low field region, the ^{1}H NMR spectrum (**Fig 5.10** and **5.11**) showed two doublets at δ 6.83 and 6.82 for compound **5.16** and at δ 6.98 and 6.86 for compound **5.35** (each J = 7.2 Hz). Furthermore, the ^{1}H NMR spectrum showed four aromatic proton singlets at δ 6.72 and

6.70 for compound **5.16** and δ 6.79 and 6.65 for compound **5.35**, and two signals (δ 5.97, *dd*, J_1 = 6.3, J_2 = 1.5 Hz for compound **5.16** and 5.99, *d*, J = 1.5 Hz for compound **5.35**) due to two dioxymethylene protons (**Table 5.3** and **Figs 5.10-5.11**).

In the high field region, the ^{1}H NMR spectrum consisted of signals due to eight methoxy groups whose intergration indicated that the signals were due to a mixture of isomers in the ratio 2:1. Both ^{1}H (**Fig 5.10-5.11**) and ^{13}C NMR (**Fig 5.12**) spectra for the two compounds **5.16** and **5.35** showed characteristic peaks which indicated that two methoxy groups were attached to aromatic units (δ_H 3.41, δ_C 60.16, C-9; δ_H 3.86, δ_C 55.80, C-12 for compound **5.16** and δ_H 3.47, δ_C 60.31, C-9; δ_H 3.89, δ_C 55.90, C-12 for compound **5.35**), one methoxy group attached at a carbonyl group (δ_H 3.66, δ_C 51.02, C-15 for compound **5.16** and δ_H 3.71, δ_C 51.17, C-15 for compound **5.35**). Another methoxy group attached to the aliphatic part of the molecule (δ_H 3.35, δ_C 58.48, C-14 for compound **5.16** and δ_H 3.31, δ_C 58.52, C-14 for compound **5.35**) (**Tables 5.3** and **5.4**). These spectral data were further supported by H/C interactions in the HMQC and HMBC plots (**Figs 5.15-5.18**) suggesting the presence of two isomeric bibenzocyclooctadiene lignans lacking substituents at C-5 and having no lactone moiety.[150-151,153-155]

Further assignment of signals in the high field region of the ^{1}H NMR spectrum was difficult due to overlap of protons of the two compounds. However, this was possible with the help of H/H COSY and C/H correlation in the HMQC and HMBC.(**Figs 5.14-5.18**) whereby both compounds **5.16** and **5.35** showed similar coupling partners. In the

H/H COSYspectrum, signals due to H-13α (δ 3.47-3.41 overlapping with methoxy proton signals) showed coupling with H-13β and H-6 (δ 3.01 and 2.51, respectively). Furthermore, H-6 was coupled to protons resonating at δ 3.0 and 2.87 (H-7 and H-5, respectively). The proton H-7 was further coupled to another proton resonating at δ 2.51 and this was concluded to be H-8. These assignments were in agreement with the C/H correlations as shown in the HMQC and HMBC plots (**Figs 5.15-5.18**).

Due to the similarities in the spectra data of the two compounds in the high field region and slight difference in the chemical shift of the aromatic protons resonating in the low field region, it was concluded that the two compounds were bibenzocyclooctadiene lignan isomers and differing in the biaryl configuration in combination with the aliphatic part, and that they were having a methoxyl group attached to C-12 as discussed above.

The MS of the mixture of steganoate A (**5.16**) and isosteganoate A (**5.35**) revealed the molecular ion peak at *m/z* 414 which was also the base peak corresponding to the molecular formula $C_{23}H_{26}O_7$. The fragment ion at *m/z* 382 would be formed after cleavage of a methoxyl group from the molecular ion. Other fragment ion peaks observed in the MS were in agreement with the fragmentation process reported for these type of compounds.[150]

Table 5.3 ^{1}H NMR spectral data for steganoate (5.16) and isosteganoate A (5.35)

H	5.16				5.35	
	$\delta_{observed}$	*J* (Hz)	δ^{150}	*J (Hz)*	$\delta_{observed}$	*J* (Hz)
1	6.70	*s*	6.71		6.65	*s*
4	6.82	*s*	6.80		6.79	*s*
5α & 5β	2.78	*dd,* $J_{5\alpha,5\beta}$ = *10.2,* $J_{5\beta,6}$ = 3	2.77	*m*	2.78	*m*
6	2.54	*m*	2.54	*m*		
7	2.94	*m*	2.97	*d, J* = 9.5	2.94	*m*
8α			2.96	*d,* $J_{8\alpha,8\beta}$ = 10.4		
8β	*2.51*	*dd,* $J_{8\alpha,8\beta}$ = 16.2, $J_{7,8\beta}$ = 4.7	*2.46*	*dd,* $J_{8\alpha,8\beta}$ =10.4, $J_{7,8\beta}$ = 9.5	*2.51*	*m*
9	6.83	*d,* $J_{9,10}$ = 8.7	6.89	*d,* $J_{9,10}$ = 10.2	6.98	*d,* $J_{9,10}$ = 8.7
10	6.82	*d,* $J_{9,10}$ = 8.7	6.83	*d,* $J_{9,10}$ = 10.2	6.86	*d,* $J_{9,10}$ = 8.7
13β	3.01	*t,* $J_{13\alpha,13\beta}$ = 8.7	3.03	*dd,* $J_{13\alpha,13\beta}$ = 9.2, $J_{13\beta,6}$ = 8.7	3.01	*t*
13α	*obs*		3.45	*dd,* $J_{13\alpha,13\beta}$ = 9.2, $J_{13\alpha,6}$ = 7.2		
2-OCH_2O	5.98	*d, J* = 6.13, J_2 = 1.5	6.01	*dd, J = 6.3*	5.94	*d, J* = 1.5
11-OCH_3	3.86	*s*	3.89		3.89	*s*
12-OCH_3	3.41	*s*	3.43		3.47	*s*
13-OCH_3	3.35	*s*	3.37		3.31	*s*
14-OCH_3	3.66	*s*	3.69		3.71	*s*

[Obs]obscured by other peaks; Run in $CDCl_3$

Table 5.4 ^{13}C NMR spectral data for steganoate A (5.16) and isosteganoate A (5.35)

C	5.16		5.35
	$\delta_{observed}$	δ^{150}	$\delta_{observed}$
1	110.23	110.0	109.18
2	145.38	145.2	145.70
3	145.70	145.3	146.38
4	110.67	110.3	110.56
4a	130.91	130.7	130.65
5	29.85	29.3	29.48
6	35.08	34.9	35.15
7	43.33	43.1	42.86
8	31.56	30.8	30.62
8a	129.49	129.2	129.89
9	124.86	124.7	125.77
10	111.24	110.7	111.05
11	151.51	151.3	151.14
12	146.73	146.6	147.08
12a	146.25	130.7	131.07
12b	135.24	135.0	134.92
13	73.98	73.8	74.03
14	174.83	174.7	174.83
2-OCH_2O	100.88	100.8	100.94
11- OCH_3	55.80	55.6	55.90
12-OCH_3	60.16	60.0	60.31
13- OCH_3	58.48	59.1	58.52
14-OCH_3	51.02	50.9	51.17

Run in $CDCl_3$

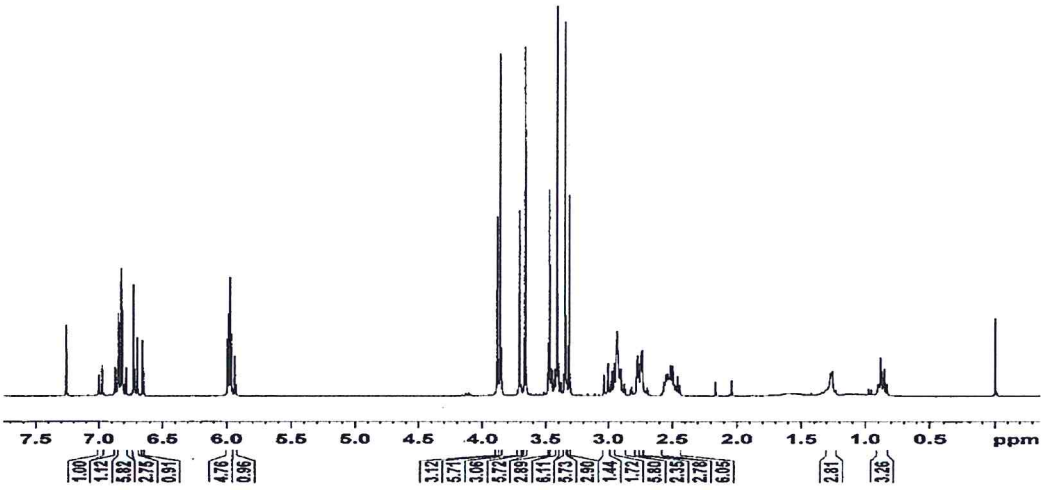


Fig 5.10 **^{1}H NMR spectrum (300 MHz, $CDCl_3$) of the mixture of steganoate A (5.16) and isosteganoate A (5.35)**

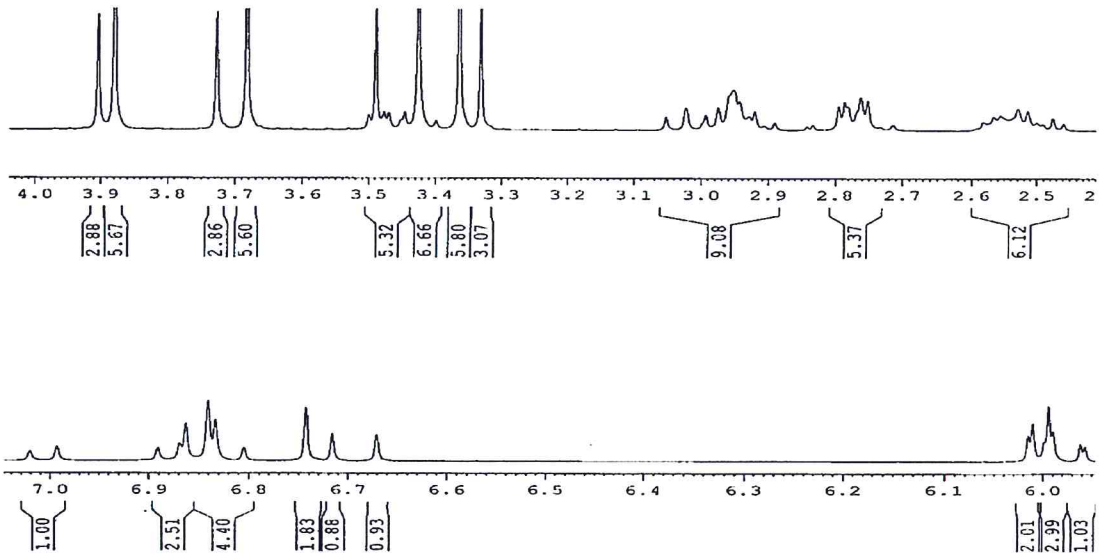


Fig 5.11 **Expansion of the ^{1}H NMR spectrum (300 MHz, $CDCl_3$) of the mixture of steganoate A (5.16) and isosteganoate A (5.35)**

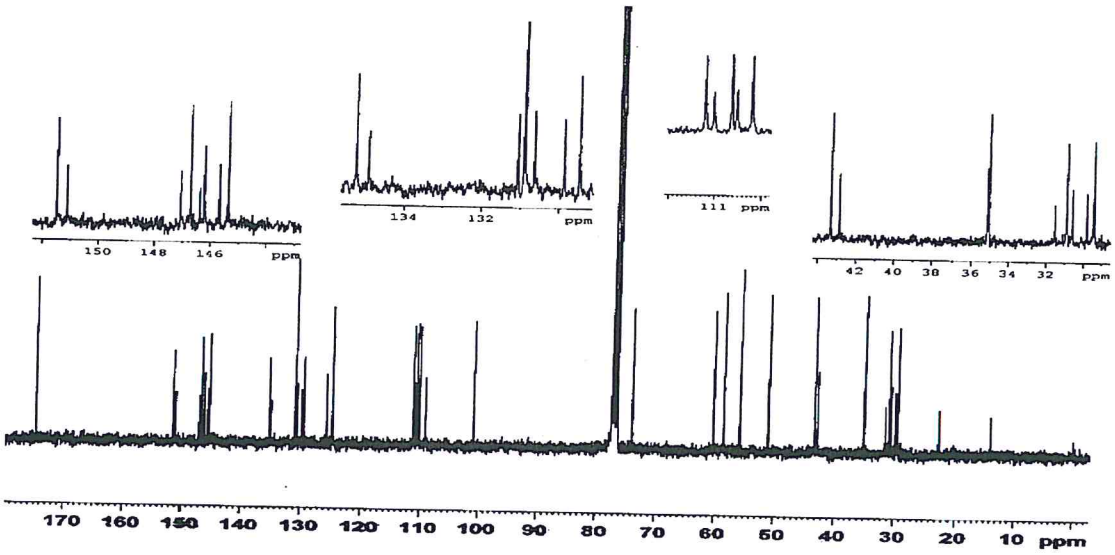


Fig 5.12 ^{13}C NMR spectrum (300 MHz, $CDCl_3$) of the mixture of steganoate A (5.16) and isosteganoate A (5.35)

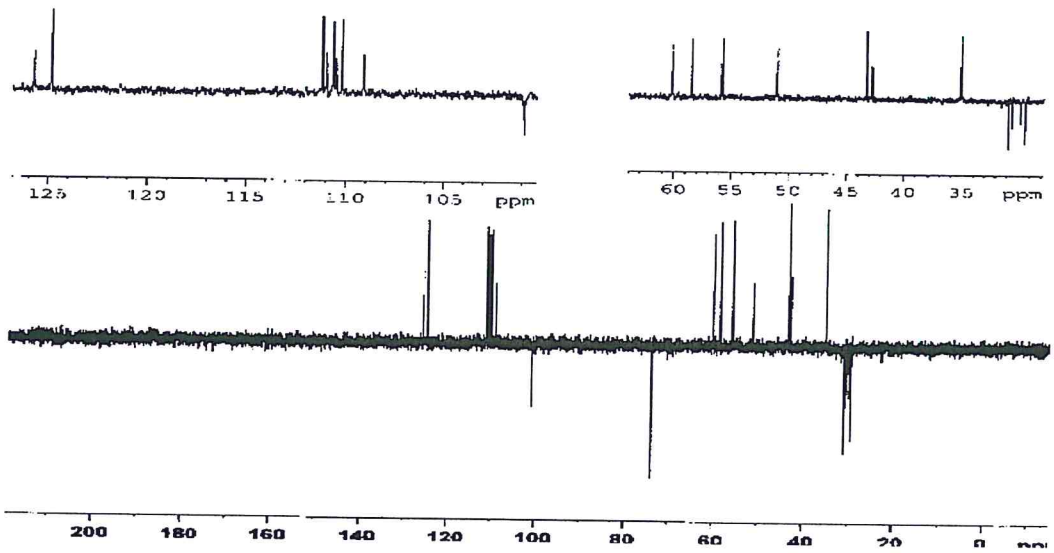


Fig 5.13 DEPT 135 spectrum (300 MHz, $CDCl_3$) of the mixture of steganoate A (5.16) and isosteganoate A (5.35)

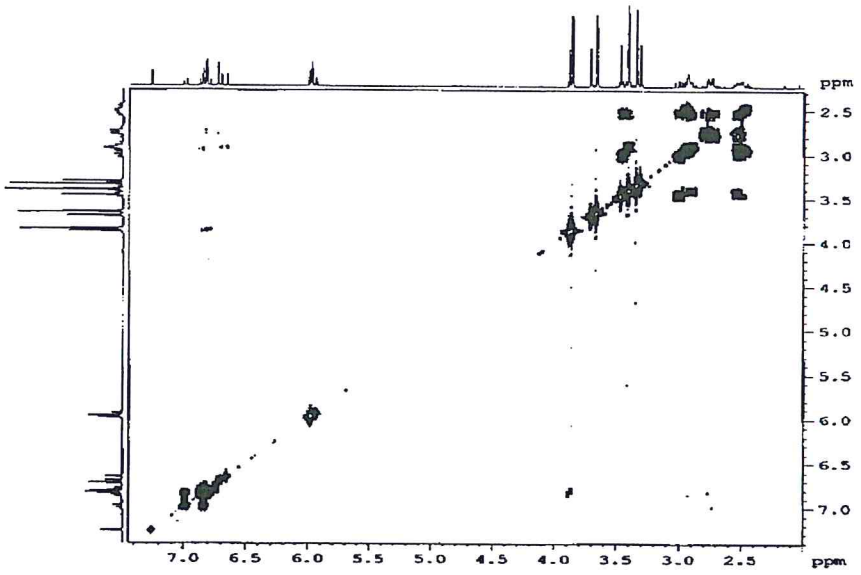


Fig 5.14 **H/H COSY plot (300 MHz, $CDCl_3$) for the mixture of steganoate A (5.16) and isosteganoate A (5.35)**

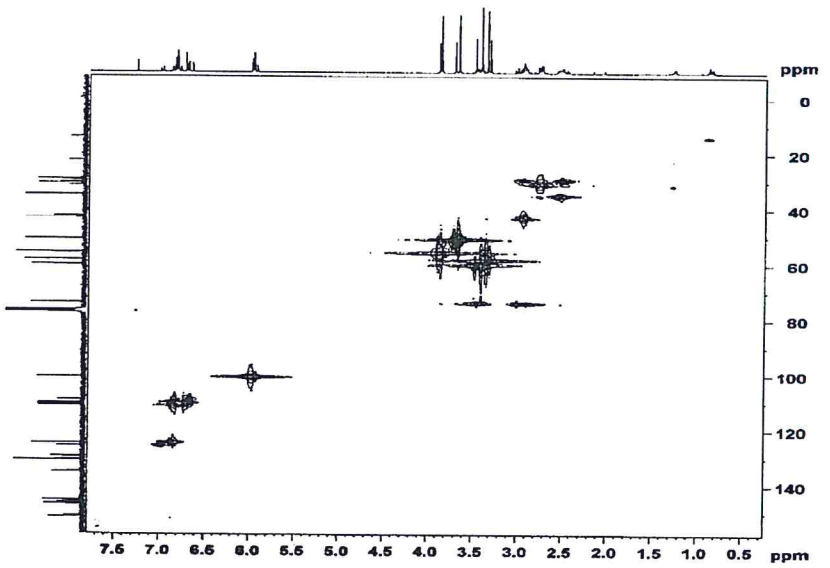


Fig 5.15 **HMQC plot (300 MHz, $CDCl_3$) for the mixture of steganoate A (5.16) and isosteganoate A (5.35)**

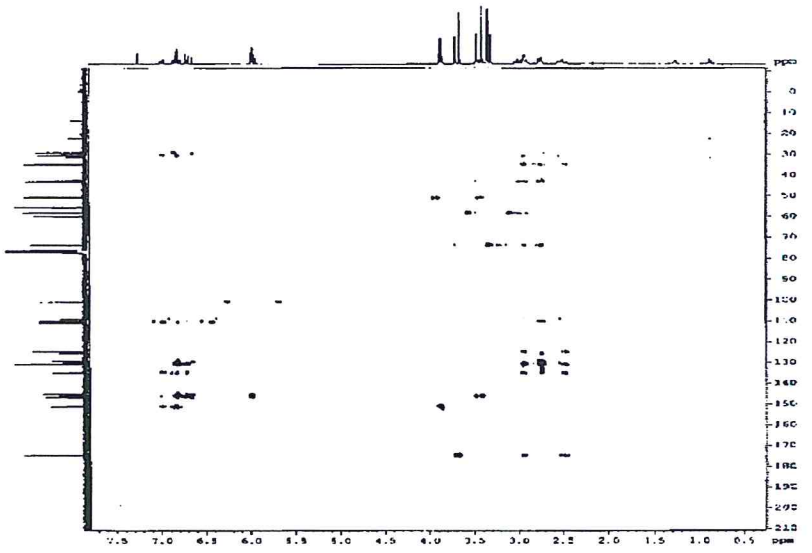


Fig 5.16 **HMBC plot (300 MHz, $CDCl_3$) for the mixture of steganoate A (5.16) and isosteganoate A (5.35)**

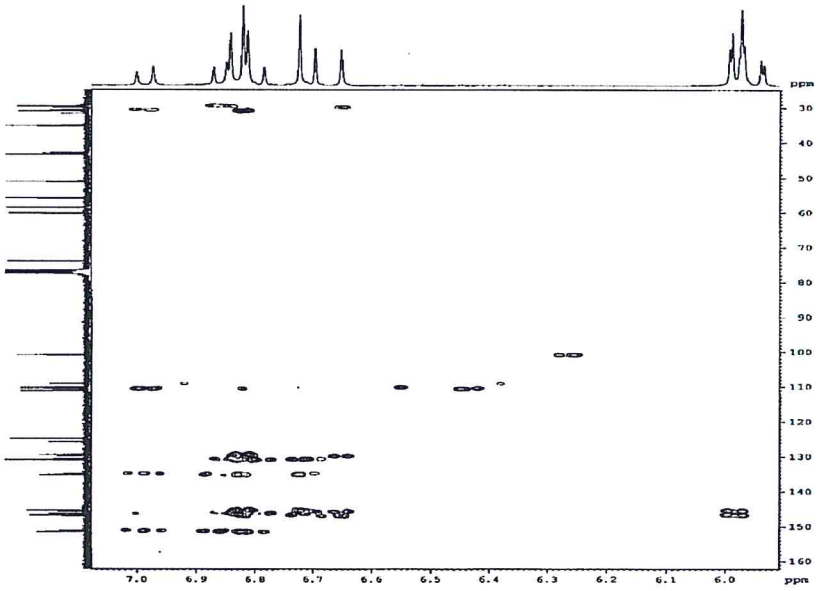


Fig 5.17 **Expansion of HMBC plot (300 MHz, $CDCl_3$) for the mixture of steganoate A (5.16) and isosteganoate A (5.35) between δ 5.9-7.7**

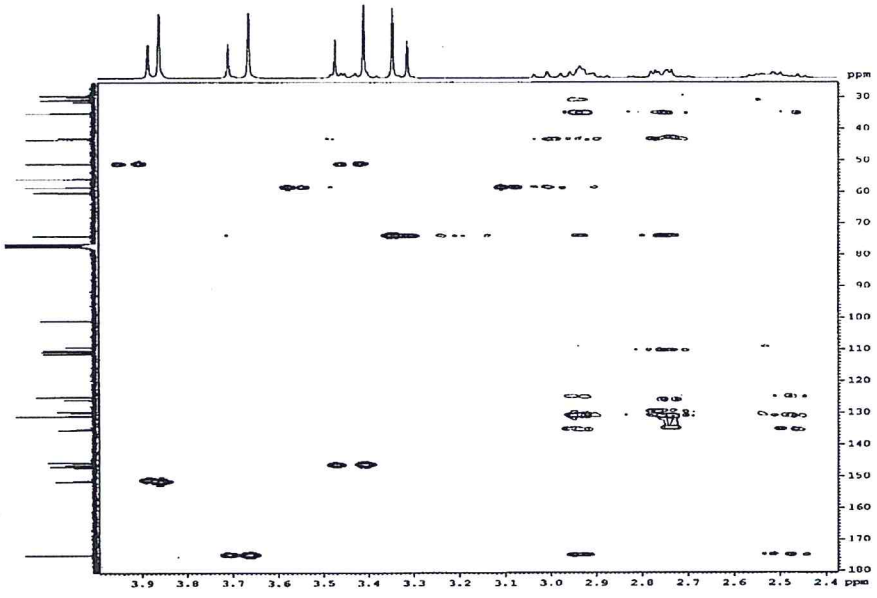


Fig 5.18 **Expansion of HMBC plot (300 MHz, $CDCl_3$) for the mixture of steganoate A (5.16) and isosteganoate A (5.35) between δ 2.4-4.00**

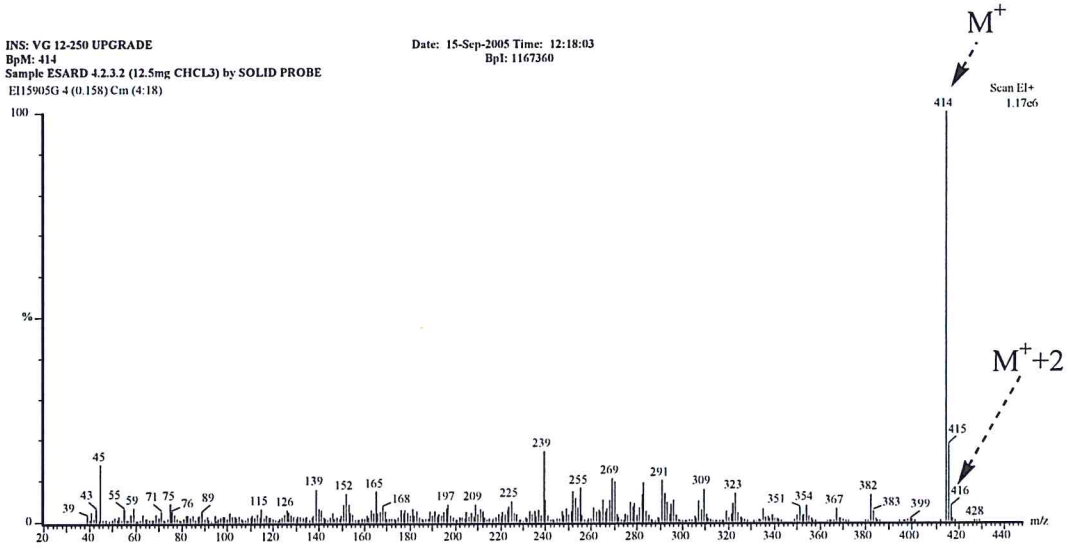
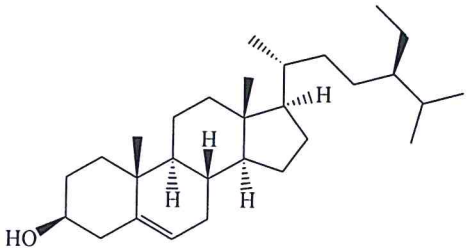


Fig 5.19 **EI-Mass spectrum of the mixture of steganoate A (5.16) and isosteganoate A (5.35)**

5.2.3 Steroids and fatty acids

Repeated column chromatography of each of the 2nd and 4th VLC fractions of the dichloromethane extract of the root bark of *S. araliacea* gave β-sitosterol (**5.36**) and octacosanoic acid (**5.37**) as white crystals, while the 4th VLC fraction yielded 25,26-dihydroxy-20-methyl-hexacos-20-enoic acid (**5.38**) as cream oil. Compounds **5.36** – **5.38** are common constituents of higher plants. Therefore, their structures were established by close analysis of their spectral and physical properties and comparison with literature information.[132]



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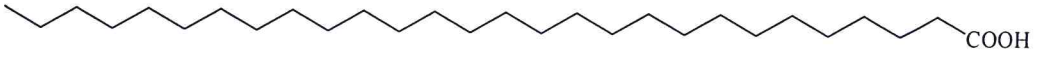

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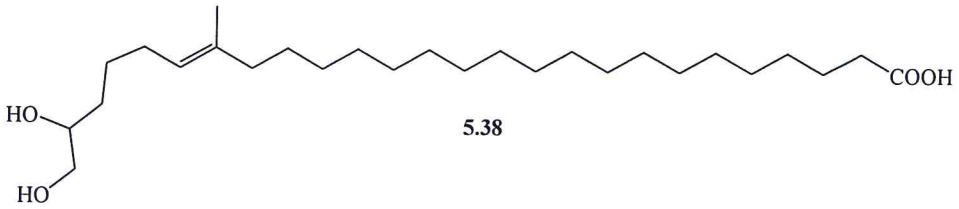


5.38

5.2.4 Larvicidal activities of compounds from *S. araliaceae*

The mixture of steganolignan isomers steganoate A (**5.16**) and isosteganoate A (**5.35**) as well as steganacin (**5.12**) were isolated as larvicidal constituents of the dichloromethane extract of the root bark of *S. araliacea* (SARD). The larvicidal activities of the isolated compounds were similar in trend to that of their mother fractions (**Chapter 3**). The mixture of steganoate A (**5.16**) and isosteganoate A (**5.35**) (LC_{50} 3.92 ppm after 72 h) obtained from the fourth chromatographic fraction (SF4) had higher activity compared to steganacin (**5.12**) (LC_{50} 9.10 ppm after 72 h) which was obtained from fraction five (**Chart 5.1**; **Table 5.5**). Combination of isomers **5.16** and **5.35**, and steganacin (**5.12**) in a one to one proportion showed slight activity enhancement (LC_{50} 3.13 ppm after 72 h) suggesting that, the three compounds act additively. Furthermore, the fact that compounds **5.16** and **5.35** had high activity than compound **5.12** suggest additive effects of the three compounds. Steganangin (**5.13**) and β–sitosterol (**5.36**), and the fatty acids **5.37** and **5.38** were not active at the concentration range tested.

Table 5.3 Larvicidal activity (LC_{50}) of isolated compounds and their blends against *An. gambiae s.s*

Composition of 5.16 and 5.35:5.12 (ratio)	LC_{50} (ppm)			
	24 h	48 h	72 h	96 h
5:0	8.19	5.44	3.92	3.20
1:1	9.18	5.74	3.13	2.47
0:5	-	11.83	9.10	5.28

- Fiducial confidence limits could not be computed from the concentration range used due to cluster of data.

Previously, bibenzocyclooctadiene lactone lignans such as steganacin (**5.12**) and steganangin (**5.13**) have been shown to resemble colchicines and podophyllotoxin in biological action by binding tubulin.[152,156] They prevent spindle formation and cell division (inhibition of polymerization of tubulin) and are potent anti-tumor agents.[152,156] However, substitution at C-5 was found to be a significant factor for high cytotoxic activity. Consequently, compounds like steganoate A (**5.16**) are relatively weakly cytotoxic against KB cell culture. In the present study, it is not clear what contributes to the higher larvicidal activity of mixture of steganoate A (**5.16**) and isosteganoate A (**5.35**) than that of steganacin (**5.12**).

5.4 EXPERIMENTAL

5.4.1 General

For general experimental procedures, information on plant materials, extraction protocol, mosquito larvae and procedures for larvicidal experiments for fractions and pure compound see **Chapters Three and Four**.

5.4.2 Fractionation of bioactive compounds

The crude extract (30 g) of the dichloromethane extract of the root bark of *S. araliacea* obtained through sequential soaking (**Chapter 3**) was subjected to vacuum liquid chromatography (VLC) on silica gel, eluting with a mixture of ethyl acetate and n-hexane by increasing polarity and six fractions were obtained (**Chart 5.1**). The resulting fractions and their blends were subjected to larvicidal assays to identify the active fractions and/or those contributing to the activity (**Chapter 3**). The 4th and 5th VLC

fraction which showed high activity were further fractionated by using different chromagraphic techniques (**Chart 5.1**).

Repeated column chromatography of the 4th VLC fraction eluting with a mixture of n-hexane and ethyl acetate (1:4-3:2 v/v gradient) yielded four sub-fractions. Fractionation of the first sub-fraction on silica gel eluting with a mixture of ethyl acetate and n-hexane (1:4-2:3 v/v gradient) yielded pure **5.38** as colourless oil. Further fractionation of this sub-fraction by column chromatography over Sephadex® LH-20, eluting with a mixture of methanol and chloroform (1:1 v/v) yielded compounds **5.37** and **5.36** as white crystals.

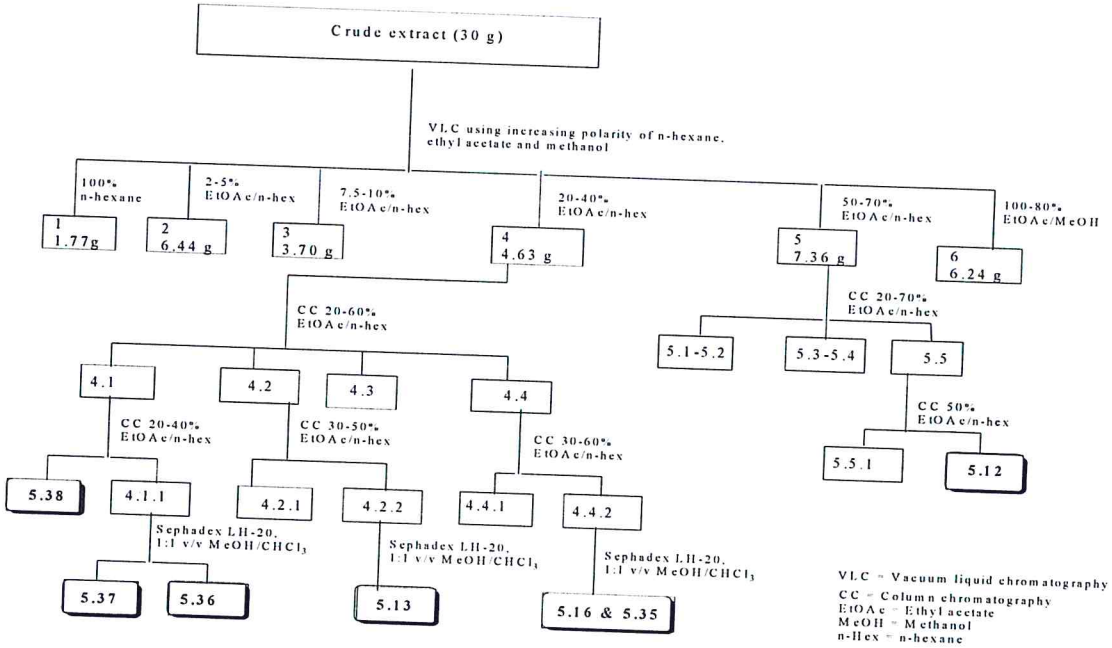
Compound **5.13** was obtained by fractionation of the 2nd sub-fraction on silica gel eluting with a mixture of ethyl acetate and n-hexane (3:7-1:1 v/v gradient) and purified by column chromatography over Sephadex® LH-20 eluting with a mixture of methanol and chloroform (1:1 v/v).

Repeated column chromatography of the 4th sub-fraction on silica gel, eluting with a mixture of ethyl acetate and n-hexane (3:7-3:2 v/v gradient), followed by column chromatography over Sephadex® LH-20, eluting with a mixture of methanol and chloroform (1:1 v/v), afforded a mixture of **5.16** and **5.35** as colourless oil which formed white crystals when kept in the refrigerator (4 °C).

Column chromatography of the 5th VLC fraction on silica gel, eluting with a mixture of ethyl acetate and n-hexane (1:4-7:3 v/v gradient), yielded five sub-fractions which were further re-combined to obtain three sub-fractions. The 3rd sub-fraction was further

separated on silica gel chromatography, eluting with a mixture of ethyl acetate and n-hexane (1:1 v/v isocratic), to give compound **5.12** as colourless oil.

Chart 5.1 Fractionation and isolation of pure compounds from the dichloromethane extract of the root bark of *Steganotaenia araliacea*



***Steganangin* (5.13)**. White crystals (MeOH); m.p. 123-124 °C (Lit.[150] m.p. 138 °C, Lit.[153] m.p. 142 °C, Lit.[150] m.p. 156-158 °C); yield, 101 mg; anisaldehyde - no reaction; IR (KBr) ν_{max} cm^{-1}, 3425, 2938, 1774, 1719, 1651, 1596, 1485, 1458, 1408, 1339, 1306, 1229, 1111, 1068, 1037 and 929; MS, *m/z* (% rel. int.) 496 ($[M]^+$, 100), 396 (20), 366 (55), 300 (30), 83 (55) and 55 (60); ^1H and ^{13}C NMR (**see Tables 5.1** and **5.2**).

***Steganacin* (5.12)**. colourless oil; yield, 114 mg; anisaldehyde - no reaction; IR (KBr) ν_{max} cm^{-1}, 3017, 2939, 2779, 1774, 1734, 1597, 1486, 1410, 1370, 1339, 1231, 1142,

1109, 1030, 941 and 867; MS, *m/z* (% rel. int.) 456 ($[M]^+$, 100), 396 (20), 366 (25), 300 (15) and 83 (18); ^1H and ^{13}C NMR (**see Tables 5.1** and **5.2**).

***Steganoate A* (5.16)** and ***isosteganoate A* (5.35)**. White crystals (MeOH); m.p. 138-139 °C (Lit.[150] m.p. 157-158 °C for steganoate A); yield, 48.5 mg; anisaldehyde- no reaction; UV, λ_{max} (2:3 v/v H_2O/MeCN) nm, 200, 248 and 292; IR (KBr) ν_{max} cm^{-1}, 3857, 3744, 3395, 3015, 2933, 2362, 1734, 1487, 1457, 1426, 1376, 1270, 1223, 1183, 1120, 1038 and 935; MS, *m/z* (% rel. int.) 414 ($[M]^+$, 100), 382 (10), 239 (18) and 139 (10); ^1H and ^{13}C NMR (**see Tables 5.3** and **5.4**).

***β-Sitosterol* (5.36)**. White crystals (MeOH); m.p. 149-150 °C (Lit.[132] m.p. 145-146 °C); yield, 28.4 mg; anisaldehyde - black; IR (KBr) ν_{max} cm^{-1}, 3837, 3751, 3427, 2939, 2866, 2377, 1746, 1653, 1518, 1459, 1375, 1055 and 967; MS, *m/z* (% rel. int.) 412 ($[M]^+$, 70), 351(22), 300 (35), 271 (50), 255 (52), 83 (85), 69 (70) and 55 (100).

***Octacosanoic acid* (5.37)**. White crystals (MeOH); m.p. 62-63 °C; yield, 48.5 mg; anisaldehyde - black; IR (KBr) ν_{max} cm^{-1}, 3858, 3748, 3426, 2918, 2850, 2645w, 1705, 1653, 1554, 1513, 1430, 1298 and 937; MS, *m/z* (% rel. int.) 424 ($[M]^+$, 5), 410 (4), 396 (20), 368 (22), 129 (30), 97 (35), 83 (37), 73 (55), 57 (75) and 43 (100).

***25,26-Dihydroxy-20-methyl-hexacos-20-enoic acid* (5.38)**. Colourless oil; yield, 280 mg; anisaldehyde - black; IR (KBr) ν_{max} cm^{-1}, 3857, 3745, 3426, 3013, 2925, 2856, 1742, 1653, 1512, 1459, 1373, 1220, 1164, 1102 and 985; MS, *m/z* (% rel. int.) 439 ($[M-1]^+$, 20), 339 (25), 313 (60), 262 (100), 239 (70) and 201 (30).

CHAPTER SIX

INSECT GROWTH DISRUPTING COMPOUNDS AND OTHER CONSTITUENTS OF *KOTSCHYA UGUENENSIS* VERDC. (FABACEAE)

Abstract

This chapter reports on chemical investigations of *Kotschya uguenensis* whereby the methanol extracts from the root (KURM) and stem (KUSM) bark exhibited disruption of growth of *Anopheles gambiae* s.s mosquito larvae by extruding intestines even at low doses. The extracts yielded unstable compounds **II** and **IV** as active principles, together with another set of unstable, interconverting compounds **I** and **III** and a butenomonoterpene, kotside. The extracts KURM and KUSM, compounds **II** and **IV** attained 100% mortality at 100 and 50 ppm in 2-8 days. Compound **II** was the most active isolate, causing complete mortality without deformity, followed by **IV** that caused larvae deformities before death. Compound **I** was the least active. The structures of compounds **I**, **II** and **IV** whose spectral properties were similar and complex were not established as they were slowly inter-converting into one another. Therefore, only the structure of the glycoside kotside was established based on interpretation of results from a combination of spectroscopic data.

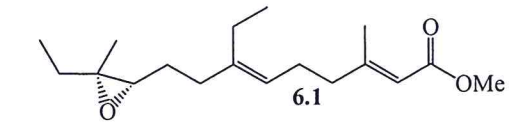
6.1 INTRODUCTION

Insect growth regulators (IGRs) specifically affect the development of insects in the early stages of the latter's life cycle. The effects of IGRs on insects include causing abnormal moulting, formation of twisted wings, loss of mating behaviour, and sometimes death of embryos in eggs.[16,31,158] IGRs are effective insecticides especially for insect species which breed in containers, pools, swamps and wells. The compounds include mimics and inhibitors of two groups of insect hormones, namely the juvenile

and the molting hormones.[159] Also, some few insect growth regulators of botanical origin have been reported. [60-61,161-167]

6.1.1 Insect growth regulators for mosquito control

The juvenile hormones I-III (**6.1-6.3**) and their mimics such as methoprene (**6.4**), hydroprene (**6.5**), kinoprene (**6.6**), fenoxycarb (**6.7**) and pyriproxyfen (**6.8**) interfere with normal metamorphosis processes and prevent immature insects from completing development into normal reproductive adults.[31,158]



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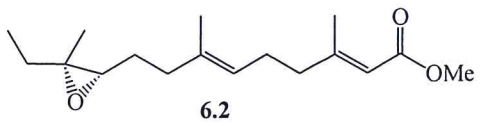

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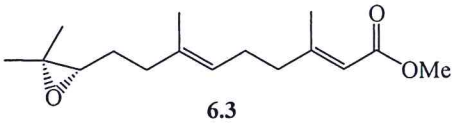

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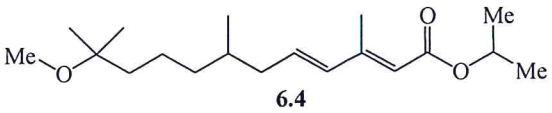

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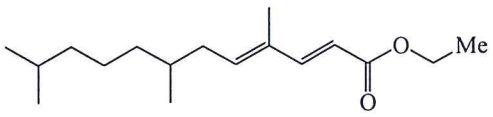

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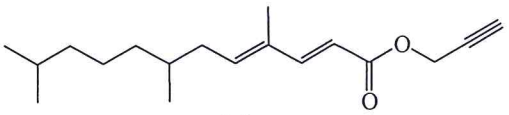

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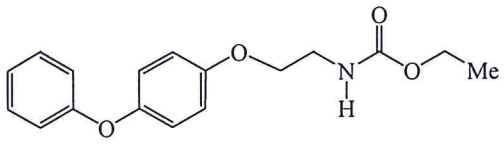

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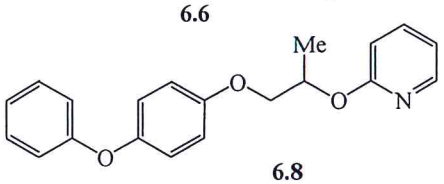

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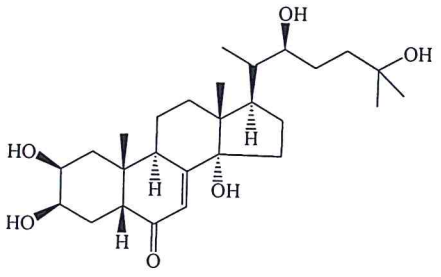
Moulting hormones include α-ecdysone (**6.9**). Other synthetic moulting hormones include the chitin synthesis inhibitors such as diflubenzuron (**6.10**) and teflubenzuron (**6.11**), moulting antagonists (inhibitors) such as diofenolan (**6.12**) and moulting hormone agonists such as chromafenozide (**6.13**), halofenozide (**6.14**), methoxyfenozide

(**6.15**) and tebufenozide (**6.16**).[158] Chitin synthesis inhibitors interfere with the development and molting of immature insects by causing death the next time an insect attempts to molt. Compounds showing promise in the control of the immature growth of mosquitoes include methoprene (**6.4**) and diflubenzuron (**6.10**).[160] Methoprene, marketed as Altosid® is designed to disrupt the insect growth cycle at critical developmental stages while diflubenzuron (**6.10**) is designed to prevent production of substances that create invertebrates' hard shells, hence leaving the insects unprotected.[160] These compounds are environmentally friendly because of low toxicity to fish and other vertebrates. Growth regulators act slowly and kill the insect only when it molts into the next stage of the developmental life cycle.[16] However, they are better alternatives to larvicides and insecticides such as organochlorines and organophosphorus compounds which have adverse environmental effects or cause high levels of resistance to insecticides.[31]



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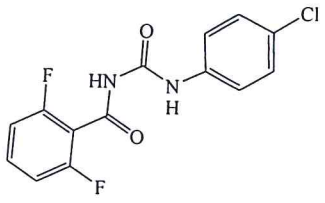

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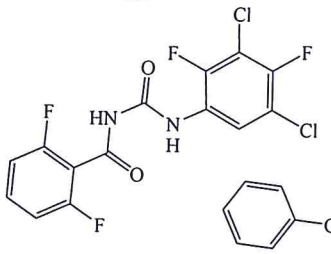

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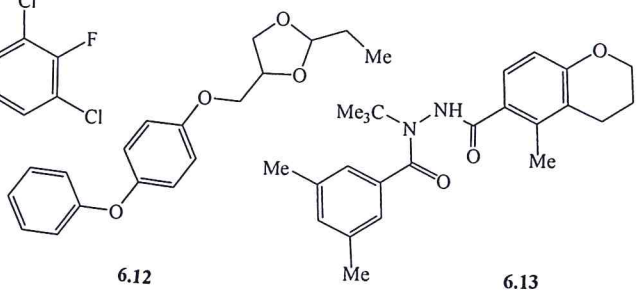

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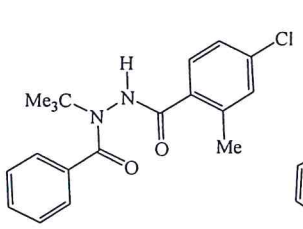

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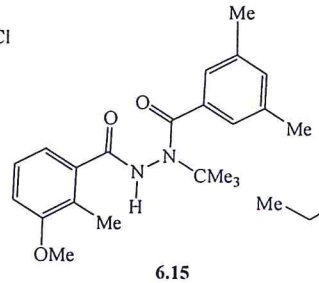

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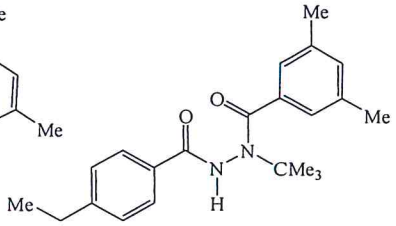


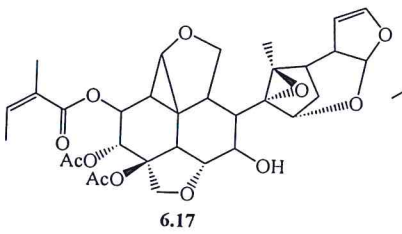
6.16

6.1.2 Limonoid insect growth regulators for mosquito control

Limonoids are modified triterpenoids that are confined to plants in the order Rutales, particularly in the family Meliaceae.[161] Some limonoids are also reported to occur in the families Rutaceae, Cneoraceae and Simaroubaceae. Most of the pesticidal properties of limonoids are related to insect antifeedant and insect growth regulation.[6] So far compounds with the highest activity against insects are the C-*seco* limonoids that are restricted to the family Meliaceae. These compounds also posses antibacterial, antifungal and antiviral activity, hence suggesting their broader role in plant defence.[60,161]

Azadirachta indica A. Juss (Meliaceae) (*Mwarobaini* in Kiswahili) is reported in traditional medicine to have a wide range of activities, such as antibacterial, antifungal, antiinflamatory, antipyretic, antitumour as well as pesticidal properties.[61,162] Extracts from this plant exhibited insecticidal, insect repellent, antifeedant, antioviposition and growth regulating properties.[163] The most active compound is azadirachtin (**6.17**). However, the plant contains several other bioactive *nor*-tetraterpenoids (C-*seco* limonoids).[164,165] Phytochemical investigations of *A. indica* reported some six years ago yielded the two limonoids meliacinol (**6.18**) and 6α-*O*-acetyl-7-deacetylnimocinol (**6.19**)

which were isolated from methanolic extracts of the fresh leaves.[61] The compounds 6α-*O*-acetyl-7-deacetylnimocinol (**6.19**) and nimocinol (**6.20**) which were previously obtained from the same source showed toxicity on the fourth instar larvae of *Ae. aegypti* (LC_{50} 21 and 83 ppm respectively) while meliacinol (**6.18**) had no effect.[61]



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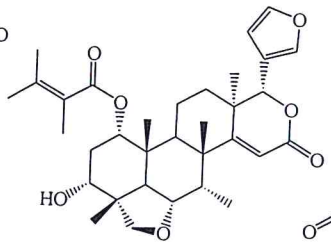

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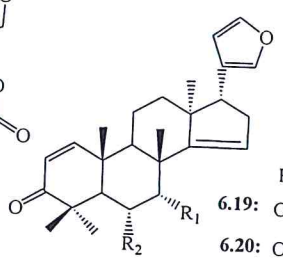
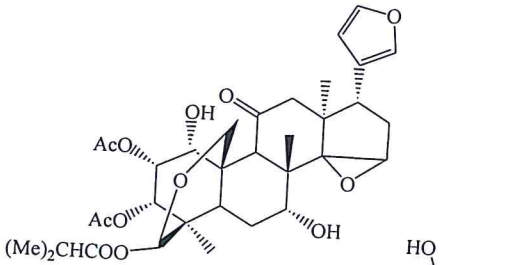


	R_1	R_2
6.19:	OH	OAc
6.20:	OAc	OH

Another plant species, *Melia azedarach,* has been used traditionally for the treatment of a variety of diseases, including being deployed as an antihelminthic agent.[166] It is also reported to be poisonous to humans as well as livestock. The poisonous compounds having been established to be meliatoxin A2 (**6.21**) and meliatoxin B1 (**6.22**) which were isolated from the fruits.[166] Oral admission of meliatoxins to pigs caused poisoning in 2-4 h after dosing and death (LC_{50} = 6.4 mg/kg) occurred within 30 h.[166] Furthermore, meliatoxin A2 (**6.21**) and meliatoxin B1 (**6.22**) exhibited feeding and growth inhibition activity against *Spodoptera litura* at 300 and 400 ppm, respectively.[167] Two other compounds nimocinolide (**6.23**) and isonimocinolide (**6.24**) obtained from the same plant species showed insect growth regulation against *Ae. aegypti* mosquitoes, having LC_{50} values of 0.625 and 0.74 ppm, respectively.[161]



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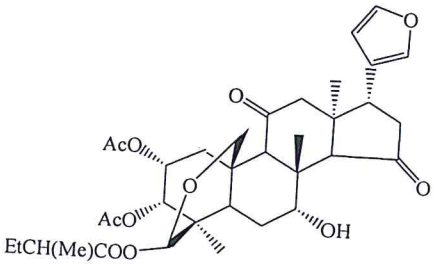

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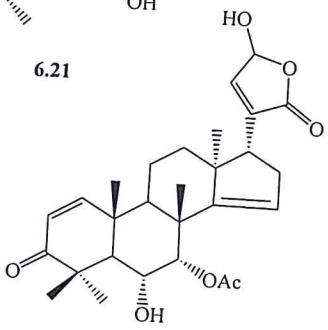

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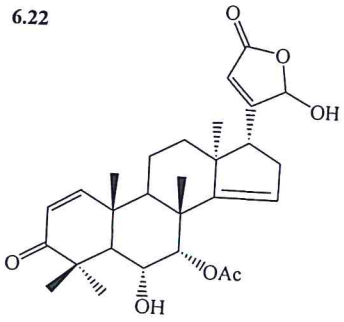


6.24

6.1.3 The family Fabaceae

Kotschya uguenensis Verdc. belongs to the family Fabaceae which was previously known as Leguminosae. The family comprises three sub-families, namely Mimosoideae, Caesalpinioideae and Papilionoideae.[168,169] The sub-family Papilionoideae has about 440 genera and 12,000 species are widely distributed in most parts of the world but with greatest diversity being in tropical and sub-tropical regions.[168,169] The genus *Kotschya* comprises about 30 species which are distributed in tropical continental Africa and Madagascar.[168,169] All species in this genus have not been investigated phytochemically or tested in any biological assay system.

Plant species belonging to the genus *Kotschya* are herbs, shrubs or sub-shrubs and rarely small trees.[168,169] They are normally erect, sometimes spreading plants. The stem is

generally covered with tubercular based, often-glandular hairs. *Kotschya uguenensis* is an erect shrub of about 1-6 m tall, with an unpleasant smell. Its stem is covered with sticky spreading bristly yellow tubercular based hairs.[168,169] The plant species is well distributed in Tanzania where it is found in edges of upland forests and evergreen bush-land, and in swamp forests at an altitude of 1000-2010 m.[168,169] The plant species is also reported to occur in Mozambique, Malawi and Zambia.[168] Indigenous people use the plant species to repel ticks/flies, whereby scientific studies as presented in this chapter indicated the effectiveness of an extract from this plant species as an IGR substance.

6.2 RESULTS AND DISCUSSION

6.2.1 Properties of compounds isolated from the methanol extract of the root bark of *Kotschya uguenensis*

Analysis of the compounds occurring in the methanol extract of the stem bark of *Kotschya uguenensis* (KUSM) and its fractions, as well as the methanol extract of the root bark (KURM) by HPLC showed small qualitative and quantitative differences (**Chapter 3** and **Figs 6.1** and **6.2**). The methanol extract of the root bark (KURM) which exhibited comparable activity with the methanol extract of the stem bark (KUSM) was chosen for further fractionation and the two unstable active compounds **II** and **IV** obtained together with **I**, **III** and **6.25**.

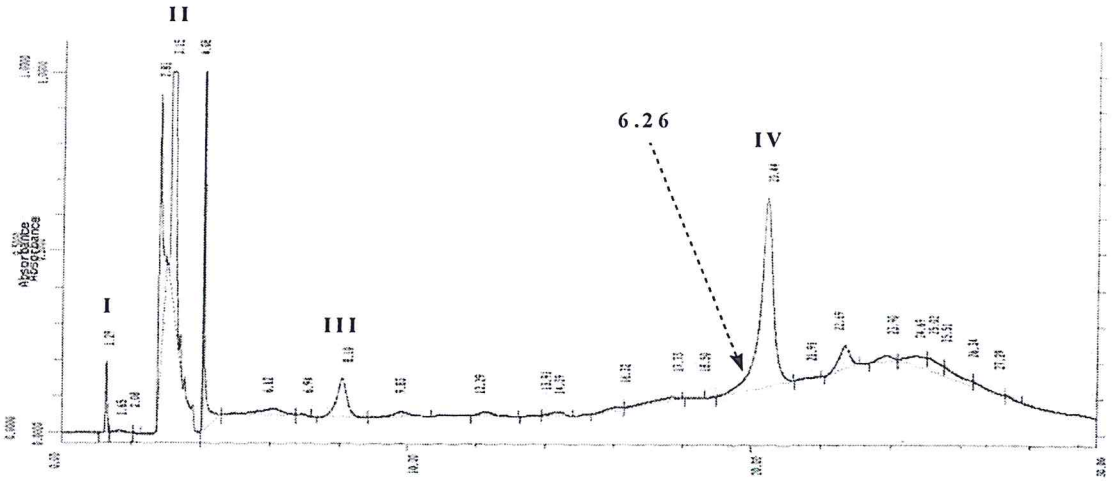


Fig 6.1 **HPLC profile of the methanol extract of the stem bark of *K. uguenensis* (KUSM)**

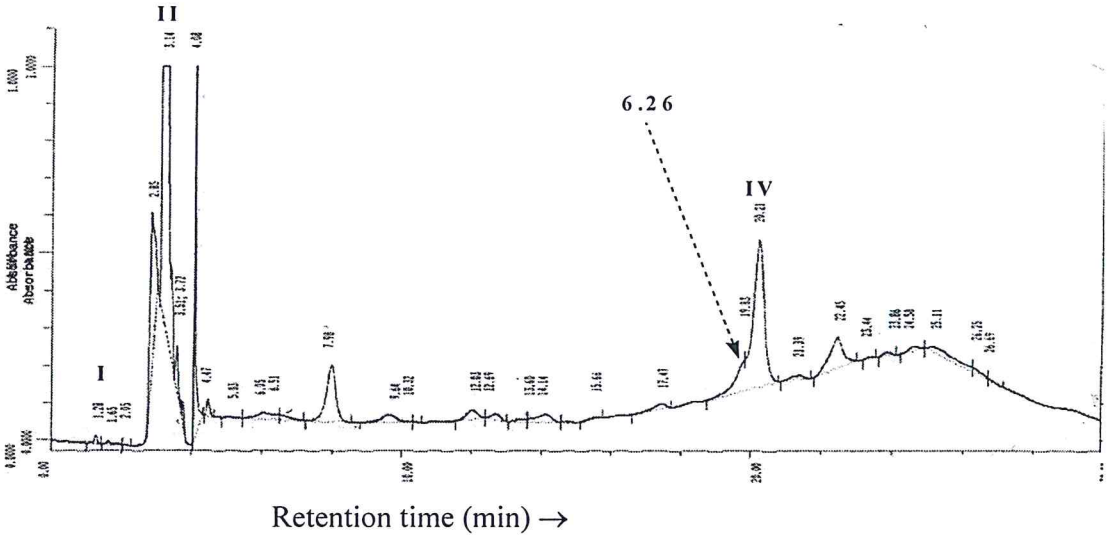
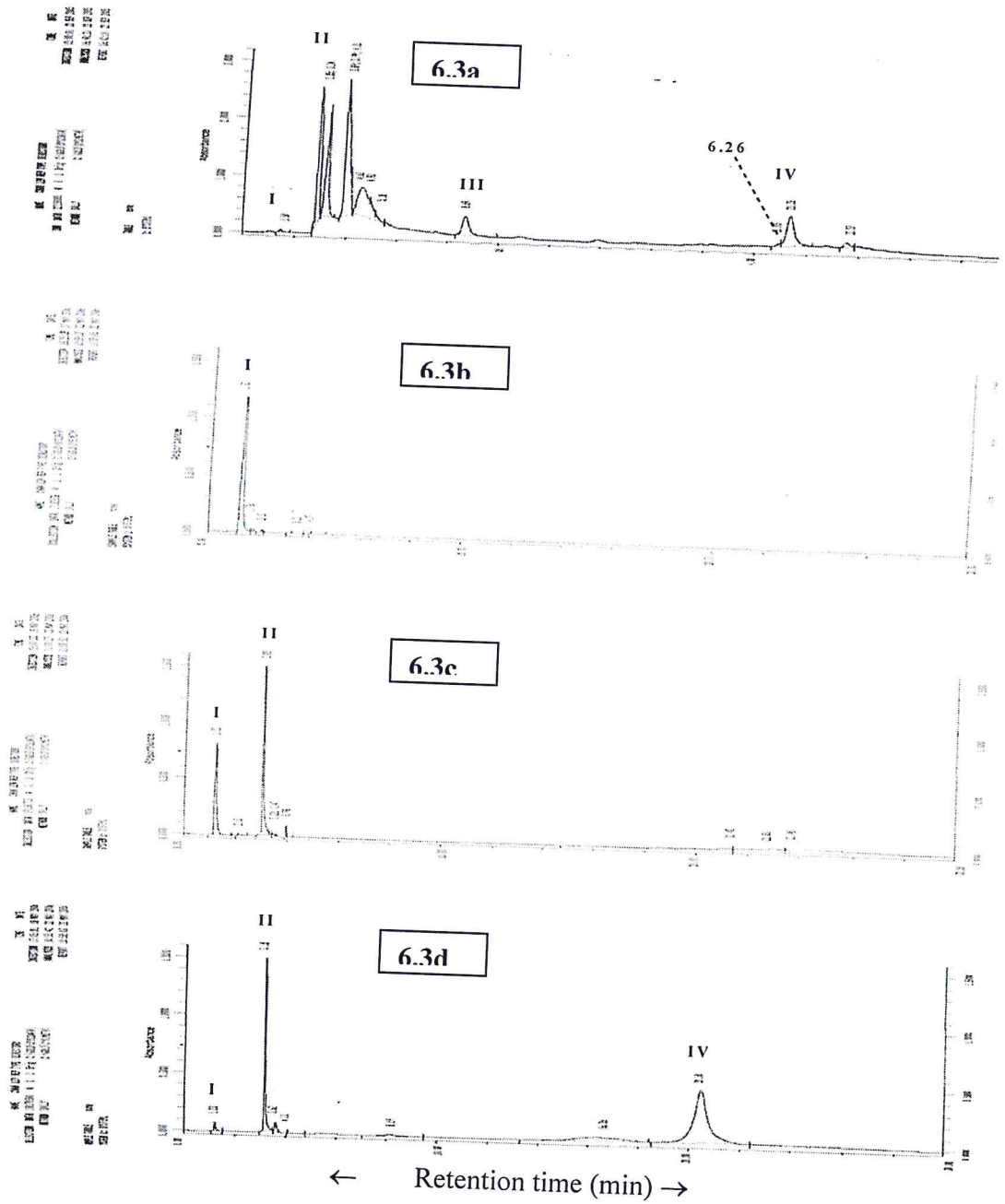


Fig 6.2 **HPLC profile of the methanol extract of the root bark of *K. uguenensis* (KURM)**

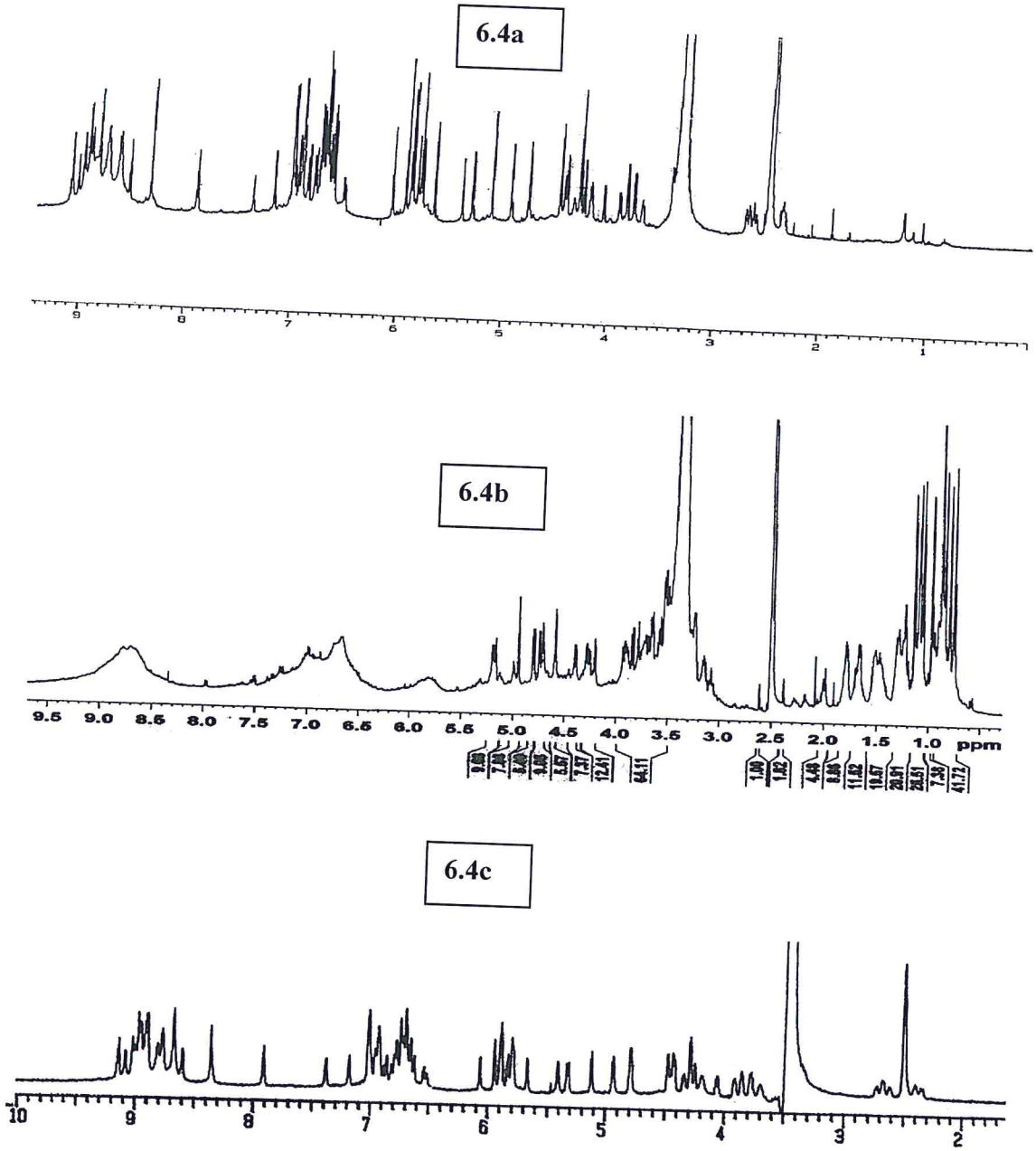
Fractionation of the methanol extract of the root bark (KURM) by reversed phase silica gel (C-8) chromatography, eluting with a mixture of water (100-85 %, solvent A) and methanol (solvent B, gradient), gave fractions which contained only a few compounds that were rather unstable on prolonged exposure to air (**Fig 6.3a-d**). Only compounds **III** and **6.25** were obtained as stable and pure compounds from the 8th and 17th fractions, respectively. The two compounds **III** and **6.25** were obtained as brown gums after isolation by HPLC, eluting with a mixture of water and acetonitrile (17:3 v/v; isocratic conditions). Unfortunately, spectral data for **III** were not available up to the time of submission of this thesis. Structures of the other three compounds **I, II** and **IV** were not identified because of their instability when exposed to air (**Fig 6.3a-d**). Compound **IV** was slowly transforming into **II** (**Fig 6.3d**), which also transformed slowly into **I** (**Fig 6.3c**) that was relatively stable (**Fig 6.3a**). This interconversion was reversible but was more favourable in the direction mentioned above. The NMR analyses of these compounds done using different spectrometers of 500 and 600 MHz, and at different times showed an interesting pattern.

The ^{1}H NMR spectra of compounds **I** and **IV** were closely related. Likewise, spectra for compounds **I** and **II** were very similar (**Figs 6.4 a-c**). This suggested that compounds **I, II** and **IV** might be conformers (rotamer) that kept undergoing conformational interconversion upon exposure to air. The fact that compounds **I, II** and **IV** were unstable and having complex spectra made it impossible to isolate any of them in pure form even by HPLC.



[6.3a]Compounds isolated from KURM extract; [6.3b]Compound I which is relatively stable; [6.3c]Compound II which was converting into I; [5.3d]Compound IV which was converting into II then into I

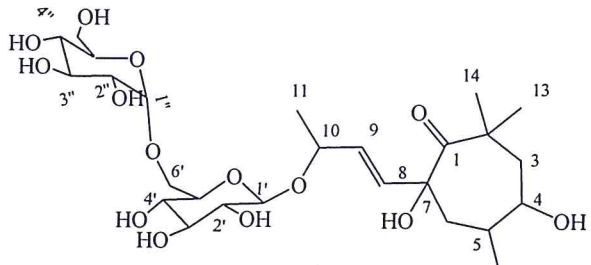
Fig 6.3a-d Chromatograms showing the interconversion of compounds I, II and IV on exposure to air above 25 °C



[6.4a]Compound I which is relatively stable; [6.4b]Compound II which was converting into I; [6.4c]Compound IV which was converting into II then to I

Fig 6.4a-c ^{1}H NMR spectrum of the interconverting compounds I, II and IV isolated from *Kotschya uguenensis*

6.2.2 Kotside (10-*O*-[β-D-glucopyranosyl (1→6)-α-glucopyranosyl-2,5-dihydroxy-2-(3-hydroxy-but-1-enyl)-4,7,7-trimethyl-cycloheptanone) (6.25)



6.25

Column chromatography of the methanol extract of the root bark of *K. uguenensis* on reversed phase silica gel (C-8), eluting with water (85%, solvent A) and acetonitrile (15%, solvent B) in an isocratic condition yielded fraction 17, which upon further HPLC fractionation gave a gum whose structure **6.25** was established on the basis of extensive analysis of ^{1}H and ^{13}C NMR spectroscopic data (**Table 6.1**) and H/H and C/H interactions as observed in the COSY (**Fig 6.8**) and HMQC spectra (**Fig 6.9**).

The structure of the disaccharide sugar residue was deduced upon comparison of the observed spectral data (^{1}H and ^{13}C NMR) with those reported in the literature for the sugar residue[170-173] Homonuclear H/H and single bond C/H interactions as observed in the COSY and HMQC plots respectively, were particularly useful in locating bond connectivities in the aglycone unit. As expected, resonances due to the sugar moiety protons were intensively overlapped between δ 2.90-4.90.

The ^{13}C NMR spectrum consisted of signals due to two sugar residues appearing in the *ca.* 60-110 ppm region, whereby the resonances at δ 102.27 and 110.12 were ascribed to

anomeric carbons, indicating that the two sugar residues were joined at these C-atoms.[170-173] Appearance of prochiral methylene carbon signals at δ 74.12 and 62.45 as revealed by the HMQC plot showed that the two sugar units were both derived from glucose and that, one of the glucose methylene groups in the sugar units was substituted and therefore forming a 1→6-*O*-glycosidic linkage between the two glucosyl moieties.[170-173] Furthermore, the ^{1}H NMR spectrum displayed two signals at δ 4.17 (1H, *d*, J = 7.6 Hz) and 4.9 (1H, *d*, J = 2.7 Hz) due to protons at the two anomeric carbons, this showing the two sugar residues to be D-β-glucose and α-glucose, having been joined through a 1→6-*O*-α-glycosidic linkage, as shown in structure **6.25**.[170-173]

Further analysis of the H/H and C/H interactions as observed in the COSY and HMBC plots (**Figs 6.8-6.9**), and the mass spectral fragmentation pattern showed that, apart from NMR resonances due to the sugar unit, the isolated compound consisted of a hydroxybutenylcycloheptyl monoterpenoid aglycone with a molecular weight of 256 amu since the MS consisted of a fragment ion at *m/z* 256 that would have resulted from α-cleavege of the sugar moiety (**Scheme 6.1**). The ^{1}H NMR spectrum consisted of a doublet of doublets at δ 5.62 (1H, J_1 = 15.6, J_2 = 7.6 Hz) and a doublet at δ 5.41 (1H, J = 15.6 Hz), suggesting a *trans* configuration of vicinally disposed vinylic protons in the aglycone (**Figs 6.5** and **6.6** and **Table 6.1**), which could only be achieved if the olefinic group was exocyclic, as in structure **6.25**. Furthermore, the olefinic proton resonating at δ 5.62 was coupled to a carbinol proton resonating at δ 4.15 (1H, *d*, J = 7.5 Hz) which was confirmed by COSY and HMQC as H-10 in structure **6.25**, also further vicinally

coupling with methyl protons resonating at δ 1.12 (J = 6.3 Hz). Further still the ^{1}H NMR spectrum showed another methine proton signal at δ 1.83 and the proton coupling with methyl protons resonating at δ 0.72 (3H, *d*, J = 7.6 Hz). These latter two signals were assigned to H-5 and H-12 respectively in structure **6.25**, whereby H-5 showed further coupling with diastereotopic methylene protons resonating at δ 1.38 and 1.67 due to H-6. Further analysis of cross peak signals in the H/H COSY spectrum indicated the presence of another carbinol proton signal at δ 3.6, the corresponding coupling with the diastereotopic methylene protons whose signals appeared at δ 1.38 and 1.58. These signals were assigned to H-4 (carbinol) and H-3 (CH_2 group), respectively (**Figs 6.5** and **6.8**.and **Table 6.1**). Other ^{1}H NMR resonances were also assigned from the coupling pattern observed in the COSY spectrum as well as the presence of signals due to H-13 and H-14, and two geminal methyl groups as shown in **Table 6.1** and **Figs 6.8** and **6.10**.

The point of connectivity between the sugar unit and the aglycone was established by considering the chemical environment of the three oxygenated carbons in the aglycone as well as the MS fragmentation pattern for the latter moiety (**Scheme 6.1**). Thus, the ^{1}H and ^{13}C NMR signals due to H-4 and C-4 indicated chemical shifts that were in agreement with those expected for an underivatized carbinol group.[128-131] Furthermore, the MS fragmentation pattern indicated cleavage of the oxygenated side chain unit of the aglycone, yielding a fragment ion at *m/z* 185 due to a hydroxonium heptacyclic monoterpene ion (**Scheme 6.1**), confirming the unsubstituted nature of the C-7 hydroxyl group in structure **6.25**. Based on the above observations it was therefore concluded that the sugar residue was linked to C-10 of the aglycone unit and accounting for the relative

deshielding of H-10 (**Fig 6.8** and **Table 6.1**). The rest of the signals observed in the ^{1}H and ^{13}C NMR spectra, H/H COSY and HMQC plot and MS (**Scheme 6.1**) were in agreement with structure **6.25** for the isolated compound which has been given a trivial name kotside.

Table 6.1 ^{1}H and ^{13}C NMR spectral data (500 MHz, DMSO) for kotside (6.25)

H/C	δ_H	*J* (Hz)	δ_C
1	-	-	198.54
2	-	-	obs
3	1.40	*m*	42. 40
	1.58	*m*	
4	3.73	*m*	74.61
5	1.83	*m*	34.52
6	1.38	*m*	37.50
	1.67	*m*	
7	-	-	79.72
8	5.62	*d*, 15.6	135.67
9	5.41	*dd*, 15.6, 5.6	132.11
10	4.15	*m*	67.42
11	1.12	*d*, 6.3	25.38
12	0.72	*d*, 6.6	17.03
13	0.89	*s*	25.38
14	0.80	*s*	26.35
1'	4.17	*d*, 7.6	102.27
2'	2.89	*m*	73.48
3'	3.11	*m*	76.72
4'	3.73	*m*	77.60[a]
5'	3.64	*m*	76.21[a]
6'	3.64	*d*, 9.5	74.12
	3.83	*d*, 9.5	
1''	4.90	*d*, 2.7	110.12
2''	3.73	*m*	76.80
3''	3.05	*m*	71.16
4''	3.30	*m*	77.76[a]
5''	3.83	*d*, 9.5	70.95
6''	3.41	*d*, 7.1	62.45
	3.64	*d*, 7.1	
9xOH	1-1.35	*s*	

[a]Assignment from HMQC interaction; values may be interchanged; obs = obscured

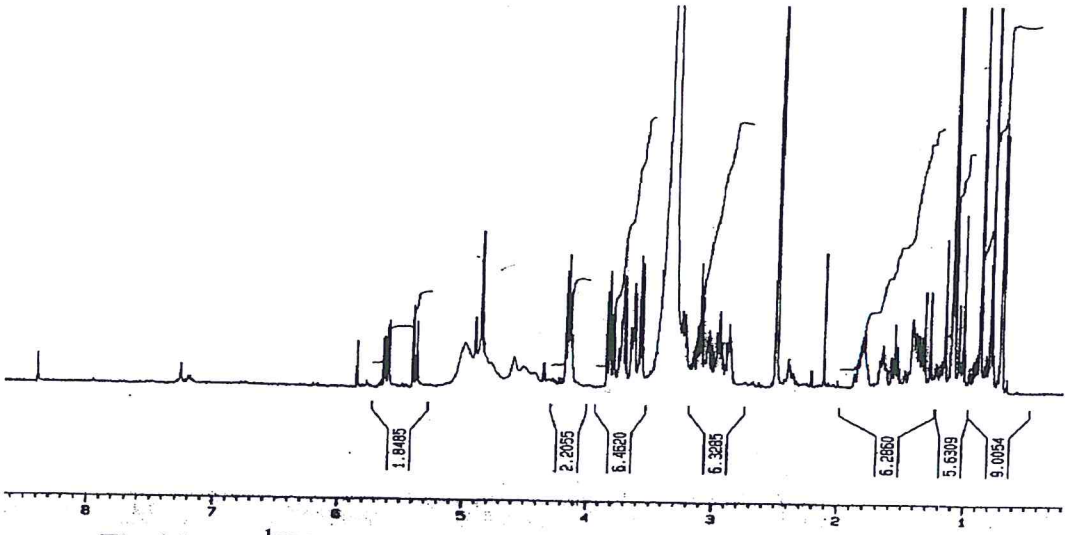


Fig 6.5 ^{1}H NMR spectrum (500 MHz, DMSO) of kotside (6.25)

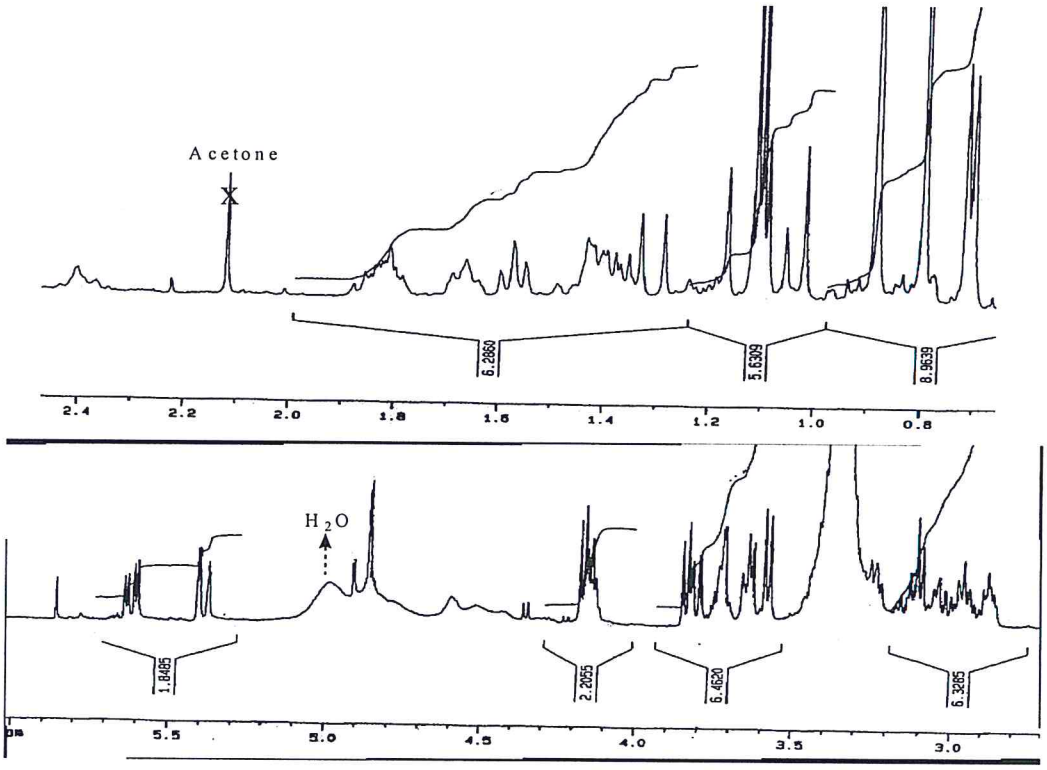


Fig 6.6 Expansion of the ^{1}H NMR spectrum (500 MHz, DMSO) of kotside (6.25)

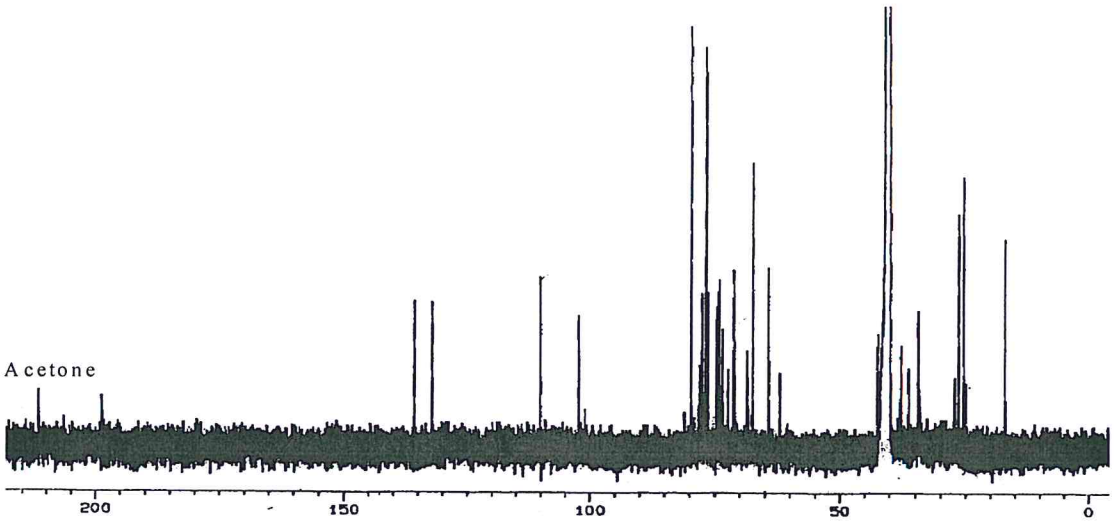


Fig 6.7 ^{13}C NMR spectrum (500 MHz, DMSO) of kotside (6.25)

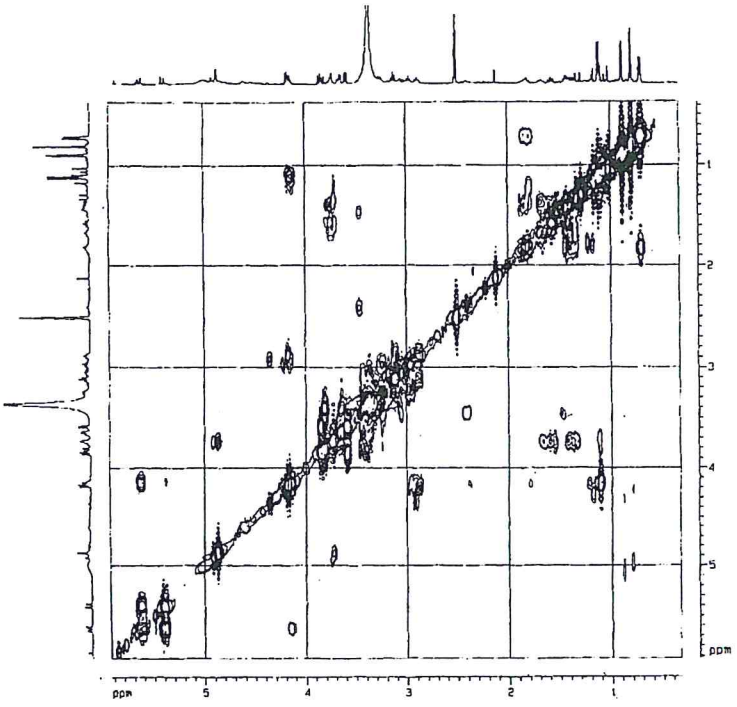


Fig 6.8 H/H COSY Plot (500 MHz, DMSO) for kotside (6.25)

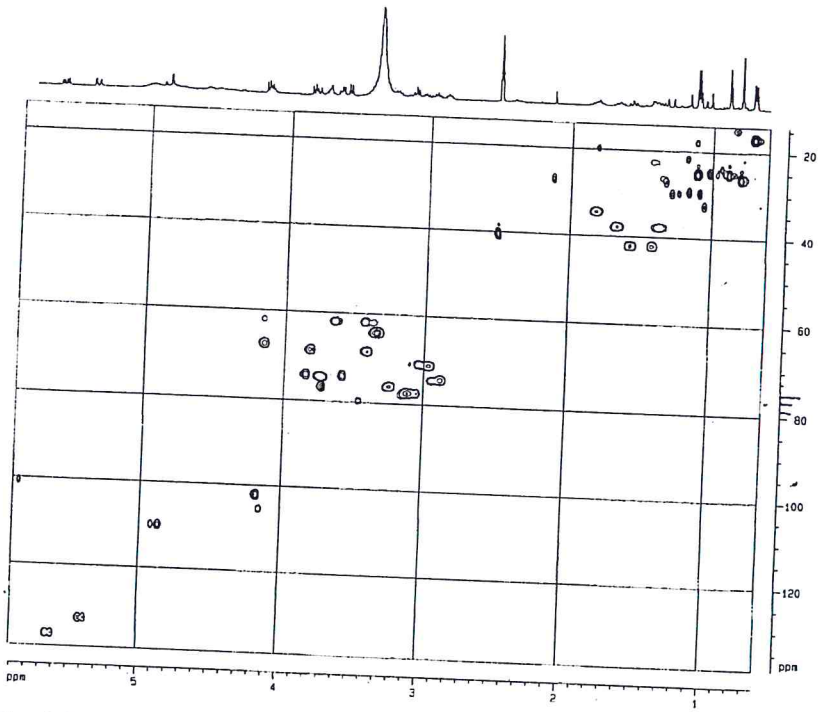


Fig 6.9 **HMQC Plot (500 MHz, DMSO) for kotside (6.25)**

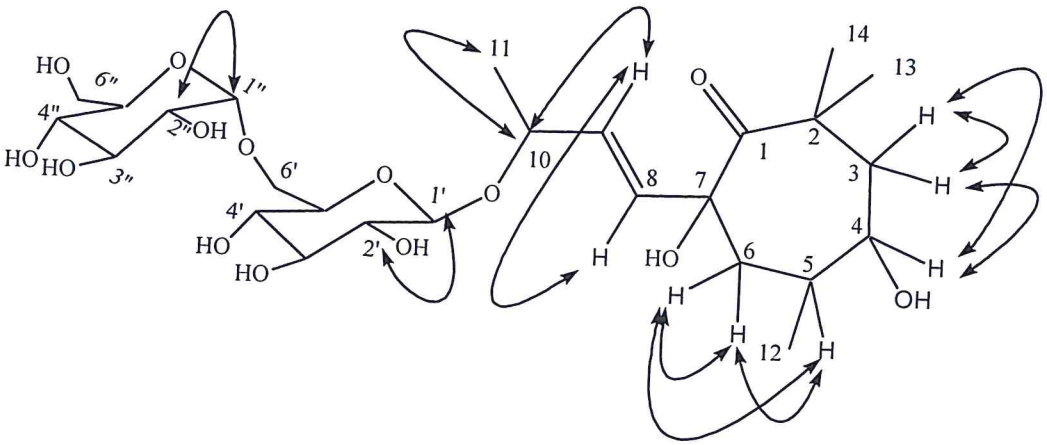


Fig 6.10 **H/H Correlations observed in the COSY spectrum of kotside (6.25)**

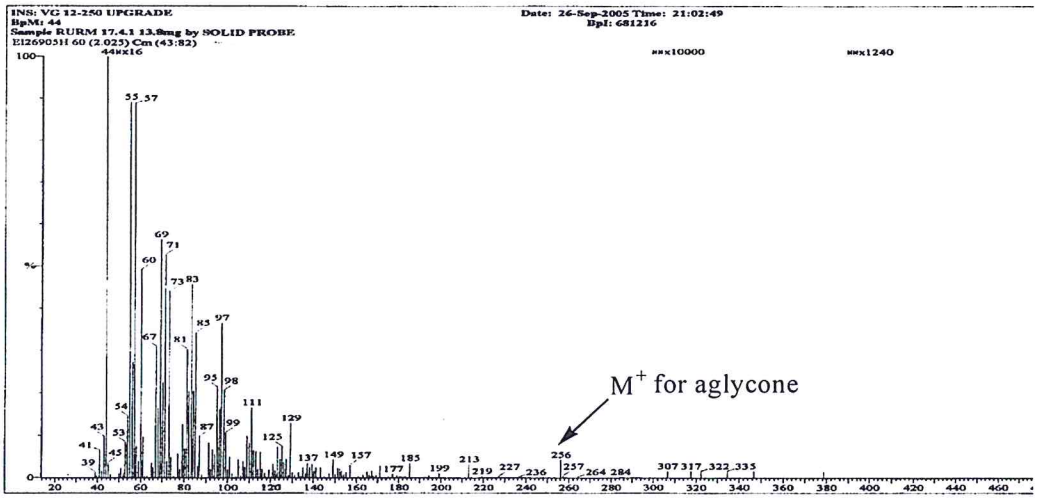
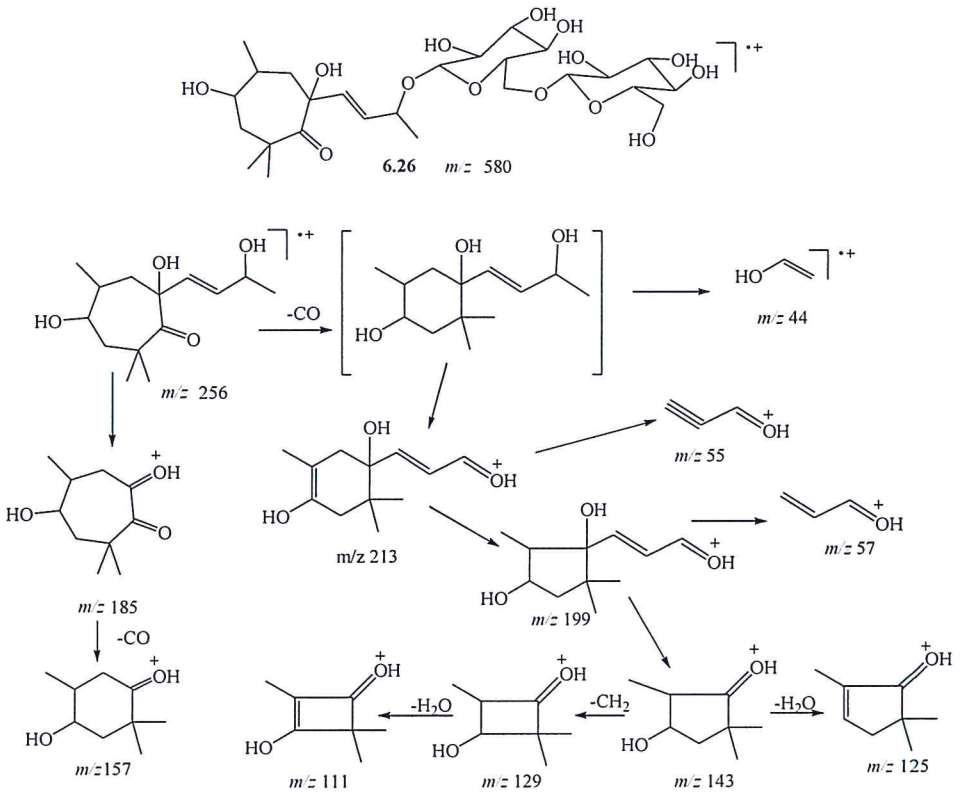


Fig 6.11 Mass spectrum fragmentation pattern of kotside (6.25)

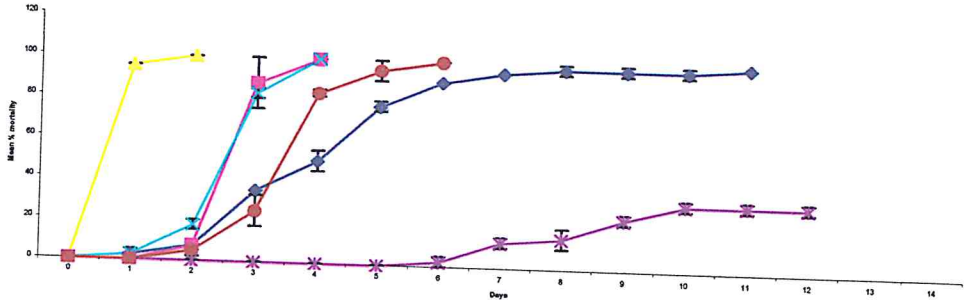


Scheme 6.1 Proposed mass spectral fragmentation pattern for kotside (6.25)

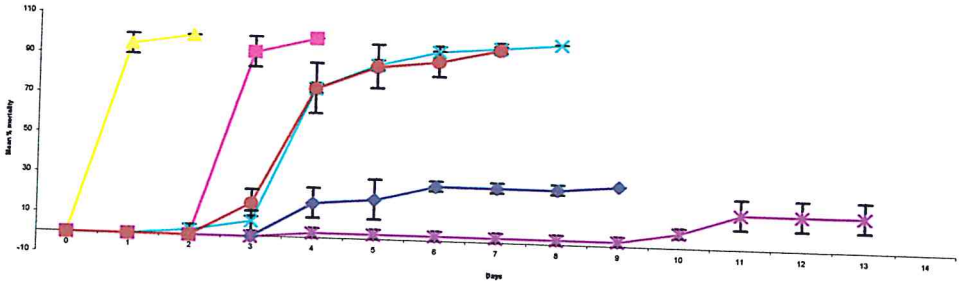
6.2.3 Mosquito larvicidal activity of compounds from *K. uguenensis*

Growth inhibitory assays of the methanol extracts of the root and stem barks of *Kotschya uguenensis* and the isolated compounds were carried out using *Anopheles gambiae* s.s larvae. The larvicidal properties of the three major compounds **I**, **II** and **IV** isolated from *K. uguenensis* were compared with the activity of the parent extracts. Extracts KURM, KUSM and compounds **II** and **IV** exhibited 100% larval mortality at 100 and 50 ppm (**Figs 6.11a-c**). Compound **II** was the most active, causing death of larvae without deformity. This was followed by **IV** and KURM which caused larvae deformation before death. Compound **I** was the least active, causing <35% larval mortality at 50 ppm (**Fig 6.11a**). At 10 ppm, adults emerged when extracts, and compounds **II** and **IV** were tested suggesting that, the active compounds perform well above 50 ppm. Again, larvae that survived at 10 ppm were reared and monitored through their life cycle until the end of F_2 generation. The F_1 adults from all treatments were normal as those from the control experiments.

6.11a



6.11b



6.11c

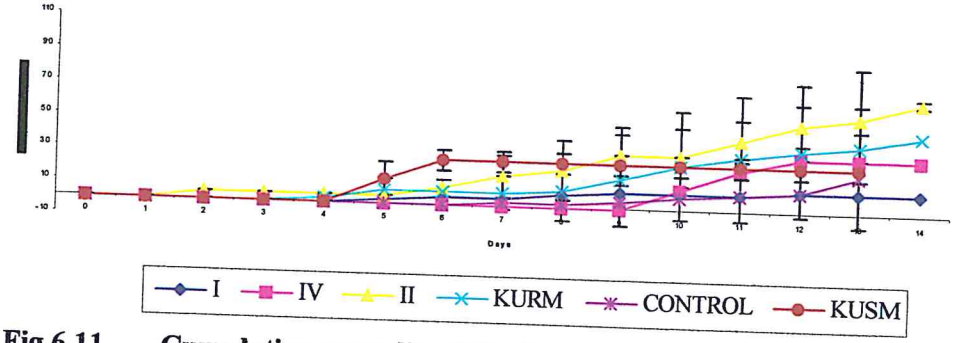


Fig 6.11 **Cumulative mortality (%) of *An. gambiae s.s.* larvae treated with methanol extracts of the root (KURM) and stem (KUSM) barks of *Kotschya uguenensis* and isolated compounds at a) 100 ppm, b) 50 ppm and c) 10 ppm**

6.3 EXPERIMENTAL

6.3.1 General

For general experimental procedures, information for plant materials, extraction and mosquito larvae see **Chapter three**.

6.3.2 Insect growth regulators (IGR's)

Determination of insect growth regulation (IGRs) was performed as described in **Chapter three**. The extracts and compounds showing mortality delay were assessed and recorded after every 24 h until the larvae died, pupated or an adult emerged. During the experiment, larvae were fed on Tetramin® fish food at 1 mg per beaker per day.

6.3.3 Fractionation

The crude extract (80 g) of KUSM was partitioned three times using a mixture of water and chloroform (1:1 v/v). Three layers were obtained upon standing, which were chloroform soluble layer (KUSMC), water soluble layer (KUSMH) and an intermediate gum like layer that was soluble in methanol (KUSMM) (**Chart 6.1**). Analysis by HPLC showed insignificant qualitative and quantitative differences of the compounds in KUSM and its fractions as the constituents of the KURM extracts. Thus, extract KURM was choosen for further fractionation in order to obtain the active compounds. Large scale fractionation of 80 g of extract KURM was carried out on reversed phase silica gel (100 C8-RP, Fluka) eluting with a mixture of water and methanol (gradient: water, followed by 30% methanol in water and finnally washed with methanol). The fractions

obtained were further fractionated by HPLC, eluting with a mixture of water and acetonitrile (17:3 v/v 20 min. under isocratic conditions), and analysed for purity and identity by HPLC (gradient 9:1-17:3 v/v for 10 min. followed by 17:3 v/v, 10 min. and finally 17:3-9:1 for 10 min.). Compounds **I, II, III** and **IV** were obtained as brown gums while **6.25** was a coulorless oil.

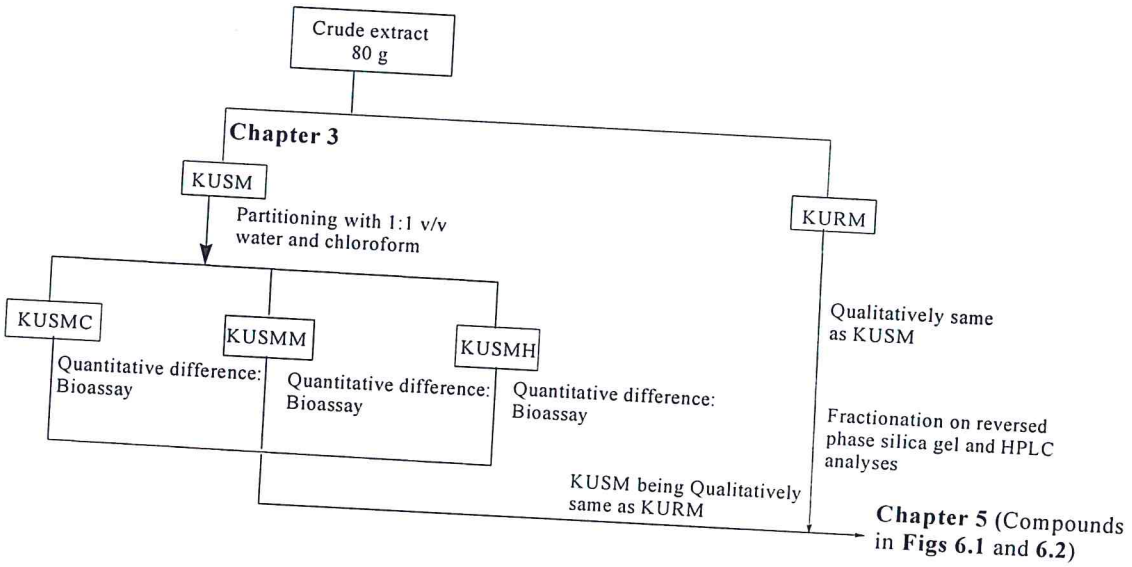


Chart 6.1 Partitioning and fractionation of extracts from *K. uguenensis*

6.3.4 High-performance liquid chromatographic (HPLC) analysis

Analytical HPLC was conducted using a Beckman Gold equipment fitted with a 128 solvent pump module and a reversed phase column (HI-5C8-2610 column, Hichrom Ltd; 250 x 4.6 mm, 5 μm particle size). All samples were dissolved in methanol prior to injection (20 m) into the HPLC. The mobile phase consisted of water (solvent A) and acetonitrile (solvent B), under solvent gradient or isochratic conditions (9:1-17:3 v/v).

The elution was done at a rate of 1 ml/min. and compounds in the eluant detected using an ultra violet (UV) 168 diode array detector set at 380 nm. Compounds eluted from the column were collected and subsequently concentrated on a rotary evaporator. Instrument control and data analysis were performed using the Gold computer system software on an IBM Aptiva PC.

Kotside, 10-O-[β-D-glucopyranosyl (1→6)-α-glucopyranosyl-2,5-dihydroxy-2-(3-hydroxy-but-1-enyl)-4,7,7-trimethyl-cycloheptanone (**6.25**). Brown gum; yield, 12.5 mg; IR (KBr) ν_{max} cm^{-1}, 3855, 3748, 3675, 2927, 2856, 2364, 1613, 1521, 1445, 1372, 1285, 1209, 1113, 1065, 1013 and 977; MS, *m/z* (% rel. int.) 580 ($[M]^+$, <0.5), 256 (8), 227 (1), 213 (5), 185 (5), 157 (4), 97 (38), 69 (55), 57 (90), 55 (90) and 44 (100); ^1H and ^{13}C NMR, see **Table 6.1**.

Compound I. Brown gum; yield, 27.25 mg; IR (KBr) ν_{max} cm^{-1}, 3857, 3747, 2929, 1612, 1521 and 1444.

Compound II. Brown gum; yield, >500 mg; IR (KBr) ν_{max} cm^{-1}, 3857, 3747, 3396, 2932, 2377, 2312, 1613, 1518 and 1446.

Compound III. Brown gum; yield, 25.0 mg; IR (KBr) ν_{max} cm^{-1}, 3857, 3746, 3419, 2933, 2378, 1618 and 1514.

Compound IV. Brown gum; yield, >500 mg; IR (KBr) ν_{max} cm^{-1}, 3857, 3747, 3383, 2936, 1614, 1522, 1448, 1369, 1285, 1210, 1112, 1068, 1011 and 973.

CHAPTER SEVEN

LARVICIDAL EFFICACY OF FOUR FORMULATIONS OF *KOTSCHYA UGUENENSIS* AT LABORATORY AND SEMI-FIELD LEVEL

Abstract

This chapter reports on different methods of formulation of the active ingredients of *Kotschya uguenensis* and their evaluation in semi-field situation. Polar compounds from this plant were found to discrupt growth of *Anopheles gambiae s.s.* larvae leading to death. Therefore, methanol extracts from the root (KURME) and stem barks (KUSME) in the form of emulsions and powdered plant materials from the root (KURP) and stem barks (KUSP) of *K. uguenensis* were investigated for larvicidal effects. Powdered plant materials performed better than emulsions in both cases. Under semi-field conditions, lethal time (at 95 % confidence level) at which 80% (LT_{80}) of the larval and pupal density would be reduced was calculated using probit analysis. The LT_{80} for KURP was 4.37 days while for KUSP it was 4.17 days at 0.1% w/v. At 0.05% w/v, the LT_{80} for KURP was 8.28 days and for KUSP it was 8.27 days. The LT_{80} for KURME at 200 µg/ml, was 5.52 days while that for KUSME was 7.74 days. Efficacy of the formulations was exhausted after 14 days under semi-field conditions. Weekly use of *K. uguenensis* powder formulation for controlling anopheline larvae is recommended due to cost effectiveness and utilization of simple technology.

7.1 INTRODUCTION

Chemicals that are used as pesticides are normally mixed with other substances for the purpose of preservation, stabilization, storage and dispensing. Pesticide formulation is the process of transforming a pesticide chemical into a product that can be applied by practical methods for its effectiveness, safety and economic use. Different formulations

determine the physical states of a pesticide and how they are applied. The compound in the pesticide formulation that kills, destroys, repels, or mitigates any pest, or that functions as regulator, desiccant, or defoliant is refered to as the active ingredient. Substances that are added to the active ingredient such as solvents, stabilizers, emulsifiers, spreaders, stickers, preservatives, surfactants, defoamers which make the product easy and safe apply, are termed as the inert ingredients or excipient. The function of exipients is simply to enhance or prolong the activity of an active ingredient. However, a chemical may be an active ingredient in one formulation and an inert ingredient in another. Sometimes mixed function oxidase inhibitor (anti-oxidizing agent) such as piperonyl butoxide, sodium ascorbate and mixture of liquid paraffin-polyoxyethylene glycerin oleate are added to enhance stability of the formulation. The use of a buffer or acid adjuvant can stabilize or lower the pH and improve the stability of the pesticide being used.[174] The common pesticide formulations for solid chemicals include tablets, capsules, liquid dosage or emulsion concentrates, micro-encapsulated formulations and wettable powders.

Little effort has been made in processing and formulation bioactive components of African plants to products for commercial use. However, two plants, *viz*. Neem tree *(A. indica,* Meliaceae) and Pongam tree (Fabaceae) have been acknowledged in recent years as biopesticides in semi-purified forms.[175] The *Pongamia* extract is a good synergist and hence has been used in formulations of several pest control products.[175-179] On the other hand, the solvent extract and pure oil from *A. indica* has been used to formulate larvicides, insect repellents and growth regulators.[180-181] This investigation aimed at the

formulation larviciding products from *K. uguenensis* and test them against anopheline larvae at semi-field conditions. Previous laboratory studies as reported in **Chapter 3** and **6** showed that compounds from *K. uguenensis* exhibit larvicidal and insect growth disruption to *An. gambiae* s.s mosquitoes. It was therefore anticipated from this study that, some of the formulated products would be prospective candidates in the integrated vector management programs. The methods used in extraction, formulation and dispensing are simple and cheap and hence mass production of the larvicide by the community is practical. However, for commercial purposes, the local community may undertake ex-situ conservation of *K. uguenensis* through cultivation.

7.2 RESULTS

7.2.1 Formulation of bioactive components

Four formulations were prepared from the root and stem barks of *K. uguenensis* for semi-field investigations (**Figs 7.1-7.2**). In the first approach, emulsions were obtained after mixing the extracts in different solvents until homogeneity was observed. The best emulsion was formed by mixing the extracts in methanol and 30% n-hexane/methanol. Attempts to form emulsions using 20% DMSO/ethanol, 30% n-hexane/ethanol, ethanol, 20% ethanol/water or water were not successful.

Insects, including mosquito larvae have an outer covering known as cuticle, that is made up of hydrocarbons.[182] When the two emulsion formulations were tested against mosquito larvae, the formulation containing 30% of n-hexane/methanol caused larvae to

shrink followed by death. The larvae appeared dry probably because most of the cuticle hydrocarbons had dissolved by n-hexane. n-Hexane is toxic to aquatic organisms and can cause impaired fertility hence not suitable for use in this formulation.[183-184]

The formulation containing methanol (solvent), 10% or 5% of tween-80 (emulsifier/surfactant) and the extract (as the active ingredient), gave good results. Methanol has low acute toxicity to humans (LC_{50} of 1140 - 2000 mg/kg in 12 h) and aquatic organisms (LC_{50} for *Culex restuans* mosquito is 20,000 mg/L or 2,000 mg/kg in 18 h).[185-187] Methanol permeates the peritrophic membrane of *An. gambiae*,[188] and the small amount, which may be swallowed by larvae in the process of feeding can be easily excreted from the gut. Due to low toxicity of methanol it would be unlikely to be toxic to terrestrial animals around the environment.[186,187] Tween-80 that is used in pharmacological preparations of most drugs is safe for human consumption.[189] In the laboratory, both 10 and 5% tween-80 emulsion formulations of the methanol extract of the root bark of *K. uguenensis* gave 100% mortality in five days at 50 μg/ml (**Fig 7.3**).

In the second approach, two mesh size powders, 30-60 μm and 100-150 μm (**Fig 7.2**) from the root bark of *K. uguenesis* were subjected to a preliminary test in the laboratory. Each of the two sizes of powdered plant (100 mg) in 100 ml water (~50 μg/ml) gave comparable larvae deformities achieving 100% mortality in five days (**Fig 7.3**). These results formed the basis of determining the quantity of the plant material that would achieve complete mortality.

Fig 7.1 Water treated with emulsions formulations

Fig 7.2 Water treated with powdered plant material formulations

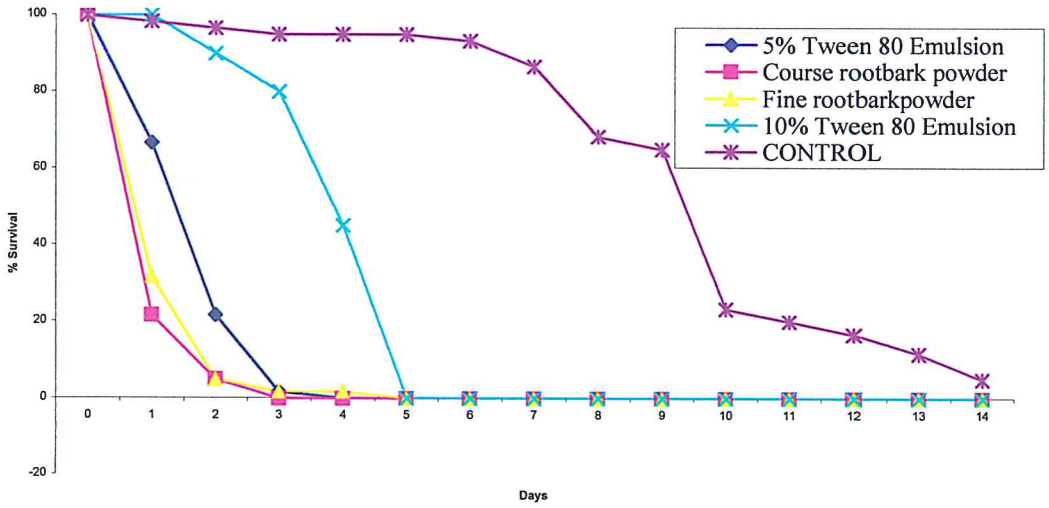


Fig 7.3 Cumulative mean survival (%) of *An. gambiae s.s.* larvae treated with emulsion of the methanol extract and powdered materials of the root bark of *Kotschya uguenensis* at ~50 μg/ml

In this experiment, no pupa was formed upon treatment with either emulsion or powdered plant materials. Therefore, a combination of the two sizes of powdered plant materials (30-150 μm) and 5% tween-80 formulation of the emulsion were selected for semi-field studies.

7.2.2 Dispersion and diffusion rate tests

Diffusion of the emulsion and dispersion of the plant particles in water were very fast (<5 min). The plant particles dispersed and remained suspended up to 6 h before they became heavy and sunk at the bottom of the beaker. However, the active ingredients from plant particles continue to diffuse into the aqueous medium and as was indicated by changes in the colour of water from colourless to brown. The relative amounts of active ingredients dissolved in water were monitored by HPLC analysis hourly for 12 hours.

7.2.3 Laboratory evaluation of emulsion and powdered plant materials

Both emulsions of the methanol extract from the stem (KUSME) and root barks (KURME) of *K. uguenensis* gave 100% larval reduction at 100 and 50 μg/ml in the laboratory (**Table 7.1**). Emulsion KURME was more effective compared to KUSME, causing complete larval mortality in 4 and 10 days, respectively, at 50 μg/ml (**Table 7.1**). There was no significant reduction of larval population for KUSME at 10 μg/ml while KURME gave complete reduction in 10 days (**Table 7.1**).

The powdered plant materials of the stem (KUSP) and root bark (KURP) of *K. uguenensis* gave comparable larval deformities, achieving 100% mortality within five days at 0.1% and 0.05% w/v under laboratory conditions. KURP was not effective at 0.01% w/v while KUSP gave complete larval reduction in 10 days (**Table 7.2**).

7.2.4 Semi-field evaluation of emulsion and powdered plant materials

Activities of emulsions were low under semi-field conditions when compared to the controlled laboratory conditions. However, KURME was still more effective than KUSME. Emulsion KURME attained 94.8% larval and pupal reduction in 10 days while KUSME had 88% reduction and 11.4% of emerged adults for the same period at 200 µg/ml (**Table 7.3**). In general, ponds with high concentrations caused prolonged time of larval development. For example, until day 8, larvae in ponds of the control experiment had all pupated while none had pupated in the ponds treated with 200 µg/ml of KURME. Both emulsions caused no significant larval and pupal reduction at 100 and 50 µg/ml (**Table 7.3**).

Tests in simulated ponds with powdered plant materials of the stem bark (KUSP) gave complete (100%) reduction of larvae at 0.1 % and 0.05 % w/v while reduction due to powdered materials of the root bark (KURP) was 91 and 74 % at 0.1 and 0.05 % w/v, respectively, by day 10. However, both formulations did not give any significant reduction at 0.025 % (**Table 7.4**).

Table 7.1: Reduction of *Anopheles gambiae* larvae and pupae after treatment with emulsion of *Kotschya uguenensis* under laboratory conditions

Days Post treatment	Conc (µg/ml)	Control (L+P)[a]	KURME (L+P)[a]	KURME %R	KUSME (L+P)[a]	KUSME %R
0	100	20.00[b]	20.00[b]		20.00[b]	
1		19.67	6.67	66.10	12	38.98
2		19.33	0	100.00	0.67	96.55
3		19.00			0	100.00
4						
5						
6						
8						
10						
0	50	20.00[b]			20.00[b]	
1		19.33	13.3	31.03	19.67	-1.72
2		19.33	4.33	77.59	11.33	41.37
3		18.67	0.33	98.21	4.67	75.00
4		18.67	0	100	2.67	85.71
5		17.67			1.33	92.45
6		16.33				93.87
8		5.67				88.23
10		2.00			0	100.00
0	10	20.00[b]	20.00[b]		20.00[b]	
1		20.00	16.33	18.33	20.00	0
2		20.00	10.67	46.67	20.00	0
3		19.00	7.00	63.16	20.00	-5.26
4		16.33	4.00	75.51	18.67	-14.28
5		15.67	2.33	85.11	17.67	-12.77
6		13.67	1.33	90.24	16.67	-21.95
8		6.67	0.33	95.00	11.67	-75.00
10		2.33	0	100.00	6.00	-157.14

[a] Mean number of larvae and pupa population; [b] Pre-treatment population; KURME and KUSME: Emulsion of methanol extract of the root and stem barks, respectively; Negative value shows no reduction in treated ponds in respect to the control due to delayed larvae and pupa development; [%R]Percentage reduction based on Mulla's formula

Table 7.2 Reduction of *Anopheles gambiae* larvae and pupae after treatment with powdered plant materials of *Kotschya uguenensis* under laboratory conditions

Days Post treatment	Conc (% w/v)	Control (L+P)[c]	KURP (L+P)[a]	KURP %R	KUSP (L+P)[a]	KUSP %R
0		20.00[b]	20.00[b]		20.00[b]	
1	0.1	19.67	4.33	77.97	12.67	35.59
2		19.67	1.00	94.92	0	100.00
3		18.67	0.00	100.00		
4						
5						
6						
8						
10						
0	0.05	20.00[b]	20.00[b]		20.00[b]	
1		19.67	17.00	13.56	16.67	15.25
2		19.67	6.00	69.49	0.67	96.61
3		18.67	3.00	83.93	0	100.00
4		16.00	0.67	95.83		
5		16.00	0	100.00		
6						
8						
10						
0	0.01	20.00[b]	20.00[b]		20.00[b]	
1		19.67	19.00	3.39	19.00	3.39
2		19.67	19.00	3.39	8.00	59.32
3		18.67	19.00	-1.79	5.67	69.64
4		16.00	18.33	-14.58	5.00	68.75
5		16.00	17.67	-10.41	1.67	89.58
6		16.00	17.33	-8.33	1.00	93.75
8		16.00	14.67	8.33	0.67	95.83
10		16.00	8.33	47.92	0	100.00

[a] Mean number of larvae and pupa population; [b] Pre-treatment population; [c] Mean number of larvae, pupae and emerged adults in the control due to pupation of about 1/3 of the larvae from day three; KURP and KUSP: Powdered plant materials of the root and stem barks, respectively; Negative value shows no reduction in treated ponds in respect to the control due to delayed larvae and pupa development; [%R]Percentage reduction based on Mulla's formula

Table 7.3 Reduction of *Anopheles gambiae* larvae and pupae after treatment with emulsion of *Kotschya uguenensis* at semi field conditions

Days Post treatment	Conc (µg/ml)	Control (L+P)[a]	KURME (L+P)[a]	%R	KUSME (L+P)[a]	%R
0	200	5.30[b]	4.92[b]		5.48[b]	
1		5.10	5.00	-5.88	4.68	10.99
2		5.30	3.36	31.53	3.28	39.96
3		5.20	2.12	55.97	2.2	58.96
4		5.00	2.04	55.94	2.52	51.11
5		5.30	1.80	63.32	2.88	47.29
6		3.8	1.84	47.71	1.88	52.01
8		0.8	1.32	-78.2	0.28	66.05
10		-		94.80		88.00
0	100	4.80[b]	5.05[b]		4.20[b]	
1		4.90	5.88	-13.40	4.88	-13.53
2		4.40	5.00	-7.38	4.64	-20.22
3		5.30	3.56	36.52	4.12	11.38
4		4.30	4.24	6.82	3.84	-1.80
5		4.80	3.08	39.3625	4.20	0.25
6		5.7	4.04	32.36	3.84	23.20
8		0.4	2.76	-552.05	1.56	-344.60
10		-		71.60		42.20
0	50	4.30[b]	5.24[b]		4.80[b]	
1		5.10	6.32	-1.61	5.36	5.83
2		4.20	4.64	9.41	5.08	-8.37
3		4.40	3.92	26.95	5.08	-3.44
4		4.80	4.16	28.93	5.08	5.17
5		4.80	3.84	34.40	5.72	-6.77
6		4.70	4.44	22.54	4.80	8.49
8		0.1	1.44	-1080.8	2.68	-2301.3
10		-		-0.4		-12.6

[a] Mean number of larvae and pupa population; [b] Pre-treatment population; - Ponds free of larvae and pupa due to emergence of adults Negative value shows no reduction in treated ponds in respect to the control due to delayed larvae and pupa development; KURME and KUSME mean Emulsion of methanol extract of the root and stem barks, respectively; [%R]Percentage reduction based on Mulla's formula

Table 7.4 Reduction of *Anopheles gambiae* larvae and pupae after treatment with powdered plant materials of *Kotschya uguenensis* at semi field conditions

Days Post treatment	Conc (% w/v)	Control (L+P)[a]	KURP		KUSP	
			(L+P)[a]	%R	(L+P)[a]	%R
0	0.1	3.27[b]	3.88[b]		3.04[b]	
1		3.93	3.64	22.08	3.00	18.01
2		4.03	2.64	44.89	1.48	60.55
3		4.13	1.68	65.78	1.52	60.47
4		3.50	1.12	73.06	0.84	74.20
5		3.37	0.6	84.99	0.36	88.50
6		1.77	0.28	86.66	0.08	95.13
8		1.20	0.08	94.39	0.08	92.83
10		0.83	0.08	91.88	0	100.00
0	0.05	3.27[b]	3.64[b]		3.44[b]	
1		3.93	4.52	-3.08	3.00	27.54
2		4.03	2.64	41.29	1.28	69.85
3		4.13	2.36	48.78	2.24	48.52
4		3.50	2.24	42.59	1.68	54.40
5		3.37	2.24	40.32	1.28	63.88
6		1.20	1.08	45.16	0.60	67.74
8		0.83	0.64	52.16	0.40	68.33
10		0.83	0.24	74.06	0	100.00
0	0.025	3.27[b]	3.88[b]		3.48[b]	
1		3.93	3.16	32.35	2.8	33.08
2		4.03	3.44	28.19	2.68	37.54
3		4.13	3.48	29.11	2.04	53.61
4		3.50	3.32	20.13	2.44	34.47
5		3.367	2.44	38.97	3.08	14.01
6		1.20	1.08	48.53	2.08	-10.67
8		0.83	1.64	-15.07	1.48	-15.93
10		0.83	0.4	59.42	0.76	13.92

[a] Mean number of larvae and pupa population; [b] Pre-treatment population; ˙ Ponds free of larvae and pupa due to emergence of adults Negative value shows no reduction in treated ponds in respect to the control due to delayed larvae and pupa development; KURP and KUSP: Powdered plant materials of the root and stem barks, respectively; [%R]Percentage reduction based on Mulla's formula

Table 7.5 shows the conditions in the screen house during the experiments and the estimated lethal time to cause a 50 and 80% reduction of larval and pupal densities due to treatment with different concentrations of the four formulations. For the four formulations, LT_{80} were KURP (4.37 days) and KUSP (4.17 days) at 0.1% w/v; KURP (8.28 days) and KUSP (8.27 days) at 0.05% w/v; and KURME (5.52 days) and KUSME (7.74 days) at 200 µg/ml. There was a slight increase in pH from the first day of treatment onwards and it was in the range of 7.50 - 8.70. The pH was slightly high in the control experiments and in ponds having low concentrations while the average temperature was comparable for all experiments (**Table 7.5**).

Table 7.5 Lethal time (LT) for larvae and pupa population due to treatment with high concentrations of emulsion and powdered plant materials of *Kotschya uguenensis* at semi field conditions

Treatment/ Formulation	Conc.	Av. pH	Average Temp (°C)			Lethal time (days)	
			Afternoon	Evening	Water	LT_{50} (CI)	LT_{80} (CI)
CE		8.25±0.22	32.22±1.30	23.75±1.16	23.87±0.40		
KURME	200 µg/ml	7.74±0.06				3.62 (3.35, 3.93)	5.52 (5.06, 6.19)
KUSME	200 µg/ml	7.80±0.07				4.00 (3.48, 4.79)	7.74 (6.43, 10.47)
CP		8.44±0.37	35.00±1.55	24.00±0.87	23.73±0.54		
KURP	0.1%	7.46±0.09				2.45 (2.14 ,2.72)	4.37 (4.01, 4.85)
	0.05%	8.07±0.24				4.77 (4.17, 5.83)	8.28 (6.88, 11.18)
KUSP	0.1%	7.32±0.11				2.31 (1.99, 2.58)	4.17 (3.84, 4.62)
	0.05%	8.29±0.32				2.51 (1.33, 3.28)	8.28 (6.32, 14.62)

CI represent lower and upper confidence limit at p>0.05,value are not significant if coincides; KURME and KUSME: Emulsion of methanol extract of the rootbark and stembark, respectively; KURP and KUSP: Powdered plant materials of the root bark and stembark, respectively; CE and CP: Control for the emulsion and powdered plant materials

After all larvae in the first batch had died or emerged into adulthood, residual studies to check for longevity of efficacy of all the formulations were carried out. This was done

by introducing a new batch of 100 third instar *An. gambiae* larvae on the 14th day from the date of first treatment. The test solutions were not changed in all experimental ponds. For all the four formulations tested none produced any significant reduction even at the highest concentration (0.1% and 200 µg/ml) (**Tables 7.6** and **7.7**).

Table 7.6 Residual effects of treatment of ponds with emulsion of *Kotschya uguenensis* at semi natural conditions

Days Post treatment	Conc (µg/ml)	Control (L+P)[a]	KURME (L+P)[a]	KURME %R	KUSME (L+P)[a]	KUSME %R
0	200	4.50[b]	4.00[b]		4.12[b]	
1		6.50	4.76	17.62	6.00	-0.62
2		5.70	4.44	12.37	5.64	-7.85
3		6.50	3.88	32.85	4.44	25.54
4		5.30	3.96	15.94	4.40	9.51
5		3.80	4.12	-21.97	4.60	-31.95
6		1.70	4.2	-177.94	3.24	-107.74
0	100	5.01[b]	4.56[b]		4.00[b]	
1		6.20	5.44	1.73	4.92	-1.57
2		6.40	4.76	16.70	4.76	4.80
3		6.00	4.08	23.84	3.80	18.93
4		6.60	4.08	30.76	4.04	21.65
5		4.40	2.8	28.73	3.52	-2.40
6		1.80	2.68	-66.75	4.76	-238.49
0	50	4.80[b]	4.20[b]		4.36[b]	
1		6.70	5.12	12.88	5.40	11.34
2		5.30	4.44	4.49	5.04	-4.60
3		5.20	3.36	26.34	4.16	12.00
4		6.60	4.48	22.62	4.52	24.67
5		3.20	3.36	-19.7	3.76	-29.25
6		0.30	0.96	-264.8	5.36	-1865.33

[a] Mean number of larvae and pupa population; [b] Pre-treatment population; KURME and KUSME: Emulsion of methanol extract of the rootbark and stembark, respectively; Negative value shows no reduction in treated ponds in respect to the control due to delayed larvae and pupa development; %R Percentage reduction based on Mulla's formula

Table 7.7 Residual effects due to treatment of powdered plant materials of *Kotschya uguenensis* at semi natural conditions

Days Post treatment	Conc (% w/v)	Control (L+P)[a]	KURP		KUSP	
			(L+P)[a]	%R	(L+P)[a]	%R
0	0.1	3.80[b]	2.96[b]		2.68[b]	
1		4.47	3.68	-5.46	3.56	-13.18
2		7.73	5.96	1.35	7.52	-38.08
3		9.17	9.48	-32.38	9.84	-52.43
4		7.77	9.60	-58.21	5.20	4.93
5		5.67	6.24	-40.95	7.04	-76.41
6		4.43	2.64	23.78	3.12	0.07
0	0.05	3.80[b]	3.20[b]		2.92[b]	
1		4.47	3.88	-3.37	3.20	6.87
2		7.73	5.28	18.75	7.72	-29.78
3		9.17	10.04	-30.34	8.52	-20.83
4		7.77	7.24	-10.93	4.96	16.98
5		5.67	7.04	-47.84	6.12	-40.40
6		4.43	2.84	23.77	2.76	19.07
0	0.025	3.80[b]	3.44[b]		2.32[b]	
1		4.47	4.08	-0.48	4.00	-46.87
2		7.73	6.24	11.24	7.24	-53.54
3		9.17	9.40	-12.80	7.80	-39.55
4		7.77	8.60	-21.80	6.28	-32.61
5		5.67	5.76	-11.81	5.00	-44.71
6		4.43	1.36	66.26	3.36	-24.29

[a] Mean number of larvae and pupa population; [b] Pre-treatment population; KURP and KUSP: Powdered plant materials of the root bark and stem bark, respectively; Negative value shows no reduction in treated ponds in respect to the control due to delayed larvae and pupa development; [%R]Percentage reduction based on Mulla's formula

7.3 DISCUSSION

For the powdered plant materials, the stem bark (KUSP) of *K. uguenensis* had a higher larvicidal activity than the root bark (KURP) under laboratory and semi field conditions. This is because, the amount of extractable active ingredients was higher (about two times) in the stem bark than the root bark (**Chapter 5**). HPLC chromatograms of the bioassay solution of KUSP and KURP at 0.1% w/v, and of KURME and KUSME at 200 μg/ml showed insignificant qualitative and quantitative differences in the pattern and relative amounts of those compounds that had diffused into the aqueous medium. Thus, the least polar compound **IV** was slowly diminishing from the second day post treatment to compound **II** which attained > 60 % of the total composition on the fifth day (120 hours). HPLC analysis on the 14th day showed mainly presence of compound **I**. Residual studies of all the formulations from the 14th day didnot show any significant reduction in mortality. This could be attributed to the predominance of compound **I** that is least active (**Chapter 5**) or rapid biodegradation of natural products from the plant extracts. The emulsion KURME was more effective than KUSME, under laboratory and semi-field conditions. This would be due to the fact that the root bark contains more of toxic compound **II** than the stem bark (**Chapter 5**). The larvicidal activities and behaviour of the three compounds have been described in **Chapter 5**.

It is not yet known what contributed to poor performance of the formulations at semi-field environment. Probably the high afternoon temperatures might have facilitated fast biodegradability of the compounds in the ponds. Whatever the case more precise timing

and frequent applications (preferably 10-14 days interval) are recommended because the compounds seem to affect early developmental stage of larvae. Mortality is an outcome of disruption of endocrine systems, and in most cases cause prolonged larval development stage thereby causing indirect toxicity, followed by death.

Similar observations have been reported when *A. indica* extracts were used for the control of immature stages of mosquitoes. Experiments indicates that much of the effect of the neem components is due to growth regulating effects, as opposed to direct toxicity.[181] For example, the methanolic and ethanolic leaf-extracts (0.2%) on 1st instar *Anopheles stephensi* larvae caused 100 % mortality within 9 days.[182] Aqueous extract of deoiled neem showed 100% mortality and induced prolongation of larval in *C. quinquefasciatus* at 50 ppm.[190] Application of 2.5 % of neem oil at 5 ml/m^2 in water bodies cleared anopheline larvae for two weeks.[191] Similary, 5% neem oil-water emulsion at 50 ml/m^2 in pools and at 100 ml/m^2 in tanks caused a 91.2 % reduction in the larval density of *Cx. quinquefasciatus* after 14 days. Likewise, 10% neem oil-water emulsion in desert coolers at 40-80 ml per cooler results in complete inhibition of *Ae. aegypti* pupal production.[192]

In the present investigations, powdered plant materials performed better than emulsions in all experiments with > 90 % reduction at 0.1 % w/v within 10 days. This suggest that the root and stem barks of *K. uguenensis* can be ground and be used in their natural form for managing larval population in mosquito breeding sites. *K. uguenensis* powder formulation is cost-effective, with minimal external inputs (cost of the grinder and low

technology) needed to use as larvicide by individuals or communities. The plant can be propagated easily *via* both seeds and cuttings.

7.4 EXPERIMENTAL

7.4.1 General

For plant materials and extraction of the root and stem barks of *Kotschya uguenensis* Verdc. (Fabaceae) and Mosquito larvae, see **Chapter three**.

7.4.2 Qualitative and quantitative determination of the active compounds

The amounts of the active compounds in the bioassay solutions of the four formulations were monitored by HPLC and compared for any quantitative differences. During experiments, composition of treated ponds and controls were analyzed daily for 14 days. This was done on an analytical Hewlett-Packard (HP) 1090 liquid chromatograph fitted with a reversed phase column (HI-5C8-2610 column, manufactured by Hichrom Ltd; 250 mm x 4.6 mm, 5 μm particle size). The mobile phase consisted of a mixture of water (solvent A) and acetonitrile (solvent B), with a gradient system (9:1 v/v for 10 min, 9:1 to 17:3 v/v for 10 min, and 17:3 v/v to 9:1 for 10 min). Elution was carried out at a rate of 1 ml/min and pressure of 2.9 mmHg. Constituents of the eluant were monitored using an ultra violet (UV) diode-array detector (HP 1040M) at 380 nm and the relative abundance analyzed using an HP Chemstation software on a PC.

7.4.3 Formulation of larvicidal agents

Formulations were carried out in a fume chamber to avoid contamination by particulates and volatile organic compounds. Different solvents were tested in the preparation of emulsions by mixing the extracts until homogeneity was observed, and methanol was found to be the best solvent. The methanol extract (8 g) of the stem and root bark of *K. uguenensis* were separately dissolved in 40 ml of methanol to make up 200 mg/ml stock solution. About 5 % (400 mg) of tween 80 (polyoxyethylene (20) sorbitan monooleate containing 65 hydroxyl number and 45.55 saponification value) was added as a surfactant and dispersing reagent.

The root and stem bark was pulverized to 30-150 μm. The amount of the plant powder to be added in water was estimated from the yield of the methanol extract of the root bark [which had low yield compared to the stem bark (13.78 %)] as follows:

The methanol extract of the root bark 123.1 g (5.352 %) was obtained after soaking 2300 g of root bark materials. The desired concentrations were 200, 100, 50 and 10 μg/ml and the extract 10,000 μg (0.01g) was needed to prepare 100 ml of a 100 μg/ml solution for laboratory assays. Since 2300 g of plant material yielded 123.1 g of the active extract, then 0.01 g of the extract (active ingredient) can be obtained from 0.18684 g of the plant material. Therefore, 186.84 mg of powdered plant material in 100 ml of water would yield extractable crude material which will make

up ~ 100 µg/ml. The amounts of powdered plant materials to be added to achieve other concentrations were similarly worked out.

7.4.4 Laboratory determination of efficacy of the four formulations

Preliminary assay to compare growth inhibitory effect of *K. uguenensis* formulations were carried out using *Anopheles gambiae* s.s larvae. Thus, 20 late 3rd or young 4th instar larvae were exposed in 100 ml of distilled water treated with various concentrations (10, 50 and 100 µg/ml for emulsions and 0.01, 0.05 and 0.1 % w/v for powdered plant materials). Equal proportion of tween 80 was added to the control set of emulsions while distilled water was used as blank in the control experiment of the powdered plant materials. The test were done in triplicated from separately reared batches of larvae. Living larvae and pupa were recorded every 24 h and reduction of larvae and pupal density calculated using Mulla's formula[193];

$$\% \text{ reduction} = 100 - \frac{C1T2}{C2T1} \times 100$$

Whereby C1 = pre-treatment larval density in controls
C2 = post-treatment larval and pupal density in controls
T1 = pre-treatment larval density in treatments
T2 = post-treatment larval and pupal density in treatments

During the experiment (water temperature 26 ± 2 °C) larvae were fed on Tetramin® fish food at 1 mg per beaker per day. Results from laboratory investigations were used to decide the amount of the emulsions or plant material required to achieve complete mortality in semi-field experiments.

7.4.5 Determination of efficacy of the four formulations in the simulated ponds

The trial was carried out in a screen house (7 x 3.5 x 2 m) built on a pesticide free area at the ICIPE compound. The walls of the screen house were made of netting material from the ground up to 1 m high and the roof was covered with a polyethene sheet (λ = 205 nm) (**Fig 7.6**). A total of 36 circular pools were dug in the ground of the screen house to fit large cylindrical plastic dishes of 50 cm diameter and 20 cm height. The dishes were filled with 3 litres of spring water from Githurai River, Nairobi. Mud obtained in the screen house was smeared inside the dishes to mimic natural aquatic mosquito habitats.

Fig 7.4 Screen house for semi-field studies

Third instar *An. gambiae s.s.* Giles mosquito larvae (100 in number) were introduced in each of the artificial habitats and left for one hour to acclimatize before introducing test samples. Based on the preliminary laboratory evaluation of these formulations, three concentrations of 50, 100 and 200 µg/ml for emulsion and 750, 1500 and 3000 mg of powdered plant materials were selected. The ponds were covered with modified

'Saliternick' mosquito cages (50 x 50 x 50 cm) made from iron rods and covered with a net to prevent the escape of emerging adults. Larval and pupal densities and inhibition of the emerging adults was monitored after every 24 h. Standard dipping technique using an enamel bowl (400 ml) was used. This technique involves immersing a mosquito dipper (enamel bowl, with a long handle) in ponds at 45°.[194] The test organisms would flow in the bowl and then picked by pipettes, counted and returned into the pond. An interval of 2-3 min between each dip was used to allow larvae and pupae to return to the surface. In every pond, larval and pupal densities in five dips were counted and recorded. The pH and temperature of water was also recorded. Larvae were supplemented with Tetramin® fish food. The test was replicated five times from separately reared batches of larvae. Percentage reduction of larval and pupal density was calculated using the Mulla's formula. Residual studies to check for longevity of efficacy of all the formulations was carried out using 100 reared third instar *An. gambiae s.s.* Giles mosquito larvae from the 14th day. The test solutions were not changed in all experimental ponds.

7.4.6 Study design and statistical analysis

Block design was used in semi-field studies, whereby two formulations at three concentration levels and the control were arranged in five replicates per experiment. The mean number of larvae and pupae in five replicates was calculated after determining the average number per five dips. The percentage reduction in larval and pupal density against post treatment days (1, 2, 3, 4 and 5 days) was used to estimate the duration of

effective control (LT_{50} and LT_{80}) as determined by probit analysis.[93] The level of significance of the four formulations at six application rates (200 μg/ml for KURME and KUSME; and 0.1 % and 0.05 % w/v for KURP and KUSP) was calculated at 95 % confidence limits.

CONCLUSIONS AND RECOMMENDATIONS

Investigations reported in this thesis were aimed at screening different parts of the plant species *Uvariodendron gorgonis* Verdc. (Annonaceae), *Clausena anisata* (Willd.) Benth. (Rutaceae), *Suregada zanzibariensis* Verdc. (Euphorbiaceae), *Lantana viburnoides* ssp. *viburnoides* var. *kisi* (A. Rich) Verdc. (Verbenaceae), *Steganataenia araliacea* Hochst (Apiaceae), *Synadenium glaucensen* Verdc. (Euphorbiaceae) and *Kotschya uguenensis* Verdc. (Fabaceae) for repellency and larvicidal activities against the malaria transmitting mosquito, *Anopheles gambiae s.s.* The investigations formed part of the on-going collaborative work between the Department of Chemistry at the University of Dar es Salaam and the International Centre of Insect Physiology and Ecology (ICIPE) in Nairobi, Kenya aimed at bio-prospecting for phytochemicals that could mediate plant-insect ecological interactions so that they may be exploited for monitoring and/or controlling malaria transmitting mosquitoes. Essential oils and extracts that demonstrated some activity were subjected to chromatography in order to isolate and identify structures of the compounds which may have contributed to the repellency and larvicidal activities. The identified compounds could save as chemical markers in the active extracts or plant materials so as to provide a basis for quality control in large-scale applications. In this regard, bioassay-guided fractionation and subtraction bioassays were employed in order to identify the bioactive components. Different pure compounds were tested in the laboratory and finally, quality-controlled small-scale semi-field experiments to evaluate different formulations and/or forms of botanicals of plant species having promising activities were

undertaken. During the studies results of which are reported in this thesis, 100 compounds were characterized and consisting of seventy-six essential oil constituents, eleven closely related furanonaphthaoquinone derivatives, three triterpenoids, four bibenzocyclooctadiene lignans, two fatty acids, one butenomonoterpene disaccharide, one dimeric peroxide and two known steroids.

Gas chromatography (GC) and GC-mass spectrometry (GC-MS) analysis of essential oils from leaves or aerial parts of *U. gorgonis, C. anisata, L. viburnoides, S. araliacea* and *S. zanzibariensis* revealed the presence of 76 compounds. Eugenol (89.82%) and estragol (88.38%) were the major compounds in *U. gorgonis* and *C. anisata* oils, respectively, while *L. viburnoides* ssp *viburnoides* var. *kisi* oil contained piperitenone (25.25%), artemisia ketone (13.96%), limonene (7.80%), linalool (4.15%), *trans*-caryophyllene (4.47%), 1,6,9-tetradecatriene (6.64%) and (+)-verbenone (7.99%) as the major compounds. *Steganotaenia araliacea* contained α-copaene (9.51%), α-armorphene (8.63%), germacrene D (9.33%) and δ-cadinene (9.50%) while *S. zanzibariensis* contained phenylacetaldehyde (14.38%), artemisia ketone (10.13%), (1*S*)-(-)-verbenone (12.08%) and geranyl acetone (9.35%) as the main constituents. The repellent activity (RC_{50} values) of the oils from the five plant species against *An. gambiae* s.s. mosquitoes were 38.3 x 10^{-4}, 56.0 x 10^{-4}, 110 x 10^{-4}, 107 x 10^{-4} and 88.4 x 10^{-4} mg/cm^2 for *U. gorgonis*, *L. viburnoides* ssp *viburnoides* var. *kisi*, *C. anisata, S. araliacea* and *S. zanzibariensis* oils, respectively. Among the compounds tested for repellent activity against *An. gambiae s.s.,* farnesol (RC_{50} 10.7 x 10^{-4} mg/cm^2) was the most active,

followed by methyl ketone terpenes whose RC_{50} values were as follows: β-Ionone (17.3 x 10^{-4}), α-ionone (21.4 x 10^{-4}), farnesyl acetone (24.8 x 10^{-4}) and geranyl acetone (49.0 x 10^{-4}) mg/cm^2. Previously, methyl ketones were reported to be repellent to blood sucking insects. In this study, repellent activity observed are comparable to other plant based mosquito repellents, showing that methyl ketones are potential mosquito repellent(s). Most of these compounds are components in the essential oil from *S. zanzibariensis*.

Despite the weak repellent activity of the essential oils from *U. gorgonis* and *L. viburnoides* compared to than the commercial substance, DEET, there is still possibility of using the oils in small-scale programmes for prevent mosquito and other insects bites since they were obtained in good amount (*L. viburnoides,* 0.36%; and *U. gorgonis,* 0.39%). Furthermore, eugenol (89.82%) that accounted for most of the essential oil from *U. gorgonis* is a known effective repellent and insecticide for many insects in the order Hymenoptera, Isopteran, Diptera and Coleoptera, including post-harvest pests. *U. gorgonis* is therefore a potential source of this useful phytochemical. About 80% of the constituents of *L. viburnoides* essential oils were monoterpenoids. They may be the factor which may have contributed to the repellent property of the plant species and therefore accounting for its traditional use for repelling mosquitoes in some parts of Tanzania.

The major constituent of the essential oil from *C. anisata* collected for these investigations was estragol (>88%), which results are similar to those reported for oils obtained from plant materials collected from Nigeria. However, results for plant materials collected from western Kenya and Zimbabwe are different from these findings. The difference between

this and previous results on *C. anisata* suggested the existence of at least two major chemo-types. In view of risks posed by high estragol-containing botanicals to human health, it is therefore important to carry out extensive surveys of the volatile constituents of the plant growing in different geographical locations if the essential oil is to be used for mosquito repellency.

The presence of sesquiterpenoids in the essential oil from *S. araliaceae* as the main constituents might have contributed to low mosquito repellency activity, since they are less volatile. As far as repellent property is concerned with sense of smell, monoterpenoids surpass sesquiterpenoids in terms of repellency potency.

Four plant species, namely, *L. viburnoides*, *S. araliacea*, *S. glaucensen* and *K. uguenensis* were investigated for larvicidal activity. *Anopheles gambiae* mosquito larvae were exposed to extracts, fractions, blends and pure compounds. Chemical and biological investigations of the dichloromethane extracts from the root bark of *L. viburnoides* (LRRD) (LC_{50} = 126.63, 14.07 and 8.49 ppm after 24, 48 and 72 h, respectively) and root bark of *S. araliacea* (SARD) (LC_{50} = 89.63, 18.08 and 7.68 ppm, after 24, 48 and 72 h, respectively) showed good larvicidal activity while methanol extracts from the stem (KUSM) and root (KURM) bark of *K. uguenensis* induced insect growth disruption resulting in larval midgut elongation. Most of the larvae died in the 6^{th} – 8^{th} day at 100 and 50 ppm. The extracts from *S. glaucensen* were mildly active.

Bioassay-guided fractionation of the root bark of *L. viburnoides* to isolate active compounds and/or those associated with larvicidal activity yielded camaric acid, a mixture

of 6-hydroxy-naphtho[2,3-b]furan-4,9-dione, 7-hydroxy-naphtho[2,3-b]furan-4,9-dione and naphtho[2,3-b]furan-4,9-dione, a mixture of 6-methoxy-naphtho[2,3-b]furan-4,9-dione and 7-methoxy-naphtho[2,3-b]furan-4,9-dione, and betulinic acid that had LC_{50} values of 8.31, 5.70, 5.48 and <10 ppm, respectively in 72 h. Other isolated compounds include a mixture of 6-hydroxy-2-(1-hydroxy-1-methyl-ethyl)-naphtho[2,3-b]furan-4,9-dione, 7-hydroxy-2-(1-hydroxy-1-methyl-ethyl)-naphtho[2,3-b]furan-4,9-dione and 2-(1-hydroxy-1-methyl-ethyl)-naphtho[2,3-b]furan-4,9-dione, a mixture of 6-hydroxy-2-(1-hydroxy-1-methyl-ethyl)-dihydronaphtho[2,3-b]furan-4,9-dione, 7-hydroxy-2-(1-hydroxy-1-methyl-ethyl)-dihydronaphtho[2,3-b]furan-4,9-dione and 2-(1-hydroxy-1-methyl-ethyl)-dihydronaphtho[2,3-b]furan-4,9-dione, sigmasterol and a hitherto unreported dimeric peroxide of a 4-methoxy-3-methylphenol. While betulinic acid was associated with activity reduction when compared to the parent extract, a mixture of 6-hydroxy-naphtho[2,3-b]furan-4,9-dione, 7-hydroxy-naphtho[2,3-b]furan-4,9-dione and naphtho[2,3-b]furan-4,9-dione, and a mixture of 6-methoxy-naphtho[2,3-b]furan-4,9-dione and 7-methoxy-naphtho[2,3-b]furan-4,9-dione showed activity enhancement.

A mixture of 6-hydroxy-2-(1-hydroxy-1-methyl-ethyl)-naphtho[2,3-b]furan-4,9-dione, 7-hydroxy-2-(1-hydroxy-1-methyl-ethyl)-naphtho[2,3-b]furan-4,9-dione and 2-(1-hydroxy-1-methyl-ethyl)-naphtho[2,3-b]furan-4,9-dione and a mixture of 6-hydroxy-2-(1-hydroxy-1-methyl-ethyl)-dihydronaphtho[2,3-b]furan-4,9-dione, 7-hydroxy-2-(1-hydroxy-1-methyl-ethyl)-dihydronaphtho[2,3-b]furan-4,9-dione and 2-(1-hydroxy-1-methyl-ethyl)-dihydronaphtho[2,3-b]furan-4,9-dione were not effective at the concentration range tested. These results show that furanonaphthaquinones having no substitution in the furanoid ring

are prominent larvicidal agents than those having substituted at C-2. Previous, studies on structural activity relationships of furanonaphthaquinones in cancer cell line assays revealed that activity was enhanced by an alkyl group at position 2 and a hydroxyl group at positions 5 or 8.

Lantalupeol and betulinic acid were separately isolated from two different collections, thus indicating that *L. virburnoides* accumulates each of the two lupane triterpenoids in different seasons. Thus, the less stable hemiacetal lantalupeol is metabolized during the wet season (around April) while betulinic acid is biosynthesized during the dry season (around October). Such information would be of importance should lantalupeol or betulinic acid be found to have viable usefulness, and hence their occurrence pattern would guide seasonal exploitation process for these lupanoids.

Previously, betulinic acid was reported to exhibit a variety of biological activities, including inhibition of the human immunodeficiency virus (HIV), antibacterial, antimalarial, antiinflammatory, anthelmintic and antioxidant properties. Betulinic acid also exhibited antitubercular activity against *Mycobacterium tuberculosis,* having an MIC of 15 μg/ml. As it is generally acknowledged, betulinic acid has been showing excellent activity in controlling cancer cells as it is selective towards melanoma cells since it does not affect normal cells. This feature makes betulinic acid unique in comparison to compounds that are currently used in cancer therapy, such as taxol, camptothecin, elipticine, etoposide, vinblastine or vincristine. Because of its selective cytotoxicity

against tumor cells and favorable therapeutic index, it is a very promising new chemotherapeutic agent for the treatment of HIV infection and cancer.

Previously, lantadene A and lantadene B were found to have insecticidal and cytotoxic activity. In the present investigations, neither lantadene A nor lantadene B was isolated but their sibling compound, camaric acid, which had high larvicidal activity was obtained instead. These results suggest that either an acrylonyl unit side chain or a 3,25-ether linkage which is perculiar to the genus *Lantana* is responsible for the observed bioactivities. Camaric acid also possessed nematicidal activity against *Meloidogyne incognita*, antitubercular properties against *Mycobacterium tuberculosis* and antimalarial activity against *Plasmodium falcipurum.*

Bioassay-guided fractionation of the dichloromethane extract of the root bark of *S. araliacea* yielded two isomeric steganolignans, namely steganoate A and isosteganoate A, as well as steganacin, all of which were isolated from the larvicidal fractions. Other isolated compounds included steganagin, β–sitosterol, octacosanoic acid and 25,26-dihydroxy-20-methyl-hexacos-20-enoic acid. The larvicidal activity of a mixture of inseparable steganolignan isomers, steganoate A and isosteganoate A, was quite high (LC_{50} value of 3.92 ppm) compared to steganacin (LC_{50} 9.10 ppm after 72 h). Mixing of the steganolignan isomers (steganoate A and isosteganoate A) and steganacin in one to one proportion showed slight activity enhancement (LC_{50} of the mixture 3.13 ppm after 72 h), in comparison to steganacin. These observations showed that, the three compounds may have additive effects.

Previous chemical and biological investigations of compounds from *S. araliacea* concentrated on the constituent bibenzocyclooctadiene lactone lignans that showed resemblance to colchicines and podophyllotoxin in their biological action by binding tubulin, and preventing spindle formation and cell division. Compounds such as steganacin and steganangin are potent anti-tumor agents, and substitution at C-5 was found to be a significant factor for high cytotoxic activity. Thus, compounds like steganoate A are weakly cytotoxic against KB cell culture. In this study, it is not clear what might have contributed to the high mosquito larvicidal activity of steganoate A and isosteganoate A over steganacin. Therefore, this calls for evaluation of many bibenzocyclooctadiene lignans in order to ascertain the factors responsible for the larvicidal activity.

The comparable activity for pure compounds and extracts from the dichloromethane extract of the root bark of *L. viburnoides* and *S. araliacea* showed that semi purified fractions may be utilized as potential sources of mosquito larvicides. On the other hand, the structures of the isolated active compounds might serve as leads for the development of new insecticides through structural activity relationship (SAR) studies.

Methanol extracts from the root (KURM) and stem bark (KUSM) of *K. uguenensis* exhibited disruption of growth of *An. gambiae* s.s mosquito larvae by extrusion of intestines even at low doses. Phytochemical studies of the extracts yielded **II** and **IV** as active principles, together with **I**, **III** and the butenomonoterpene glycoside kotside. Compounds **I, II** and **IV** were the major constituents of the extracts and were tested for

larvicidal activity. The extracts KURM and KUSM, compounds **II** and **IV** caused 100% mortality at 100 and 50 ppm in 2-8 days. Compound **II** was the most active isolate causing death without deformity, followed by **IV** that caused larval deformity before death while compound **I** was the least active. The structures of compounds **1, II** and **IV** whose NMR spectral properties looked similar and complex were not established because of interconversion between them. Therefore, only the structure of the butenomonoterpene glycoside kotside was established based on interpretation of results from a combination of spectroscopic techniques.

Compound **IV** was slowly transforming into **II**, which also slowly underwent transformation into **I** that was relatively stable. This transformation was reversible but more favourable in the direction mentioned above. NMR (500 and 600 MHz) analysis of these compounds at different time intervals showed interesting spectral patterns. Thus, the ^{1}H NMR spectra of compounds **IV** and **I** at one point appeared to be similar while at another point the spectra for compounds **I** and **II** were similar. This observation suggests that compounds **I, II** and **IV** may be conformers that kept on changing when exposed to air.

Although the structures of the compounds **I, II** and **IV** were not established, such interesting behavioural pattern and promising larvicidal activity prompted further bioassays of these constituents as improved blended manner. Therefore, methanol extracts from the root (KURME) and stem bark (KUSME) in emulsion form and powdered plant materials from the root (KURP) and stem bark (KUSP) of *K. uguenensis* were prepared

and investigated for larvicidal activity. The efficacy of the four formulations against *An. gambiae* under laboratory and semi-field conditions showed that powdered plant materials performed better than emulsions in both cases. Under semi-field conditions, lethal time at which 80% (LT_{80} at 95 % confidence level) of the larval and pupal density would be reduced was 4.37 and 4.17 days for KURP and KUSP, respectively at 0.1% w/v. At 0.05% w/v, the LT_{80} value was 8.28 and 8.27 days for KURP and KUSP, respectively while LT_{80} value for KURME and KUSME at 200 µg/ml was 5.52 and 7.74 days respectively. During the experiments, HPLC profile of the bioassay solution of KUSP and KURP at 0.1% w/v, and of KURME and KUSME at 200 µg/ml were monitored. The least polar compound **IV** slowly diminished from the second day post treatment to **II** which attained > 60 % of the total composition in the fifth day (120 h). The HPLC analysis on the 14th day showed mainly presence of compound **I**.

Residual studies to check for longevity of efficacy of the formulations at semi-field level did not show any significant reduction of larval and pupal population after the 14th day. This may be attributed to the predominance presence of the less active compound **I** or due to fast biodegradation of natural products from this plant. The high afternoon temperature may have caused fast inter-conversion and/or biodegradation of the compounds in the ponds. In whichever case more precise timing and frequent application (preferably 10-14 days interval) is recommended because the compounds seem to affect early developmental stage of larvae. Mortality is an outcome of disruption of endocrine system and in most cases causing prolonged larval developmental stage, thereby causing indirect

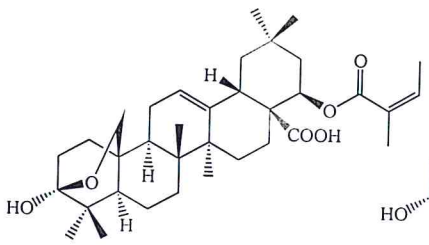
toxicity and death. Similar observations have been reported when *A. indica* components were used in the control of immature stage of mosquitoes. The effect of the neem components is due to growth regulating effects, as opposed to direct toxicity.

Also, it is anticipated from this study that, some of the evaluated products will be prospective candidates in an integrated vector management programme and therefore would require detailed toxicological studies against other invertabrates to establish selectivity of constituents of *K. uguenensis* before field studies. Furthermore, the fact that the three compounds **I, II** and **IV** were not stable and had complex NMR spectra suggested a sophiscated method of fractionation and high resolution NMR analysis, probably one which is directly linked to an HPLC system (LC-NMR), as there is still need to establish the structures of the unstable compounds.

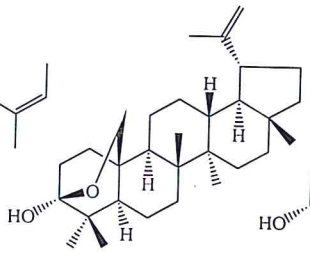
Kotschya uguenensis appeared to be a good source of readily available botanical larvicide that contains active water-soluble compounds. The active compounds do not exhibit acute effects, but slowly caused disruption of larvae and death before completing the moulting process. The methods of extraction, formulation and dispensing as reported in these investigations are simple and of low cost, and hence mass production of the larvicide at community level would be practical. However, for commercial purposes, the communities would be required to conserve *K. uguenensis* through cultivation.

STRUCTURES OF COMPOUNDS ISOLATED IN THESE INVESTIGATIONS

Compounds isolated from *Lantana viburnoides* ssp. *viburnoides* var. *kisi* (A. Rich) Verdc. (Verbenaceae)



4.27



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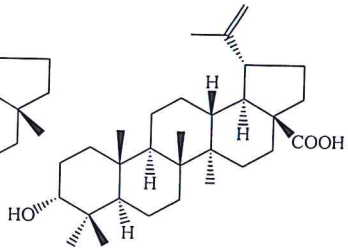

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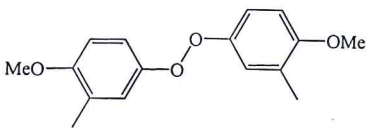

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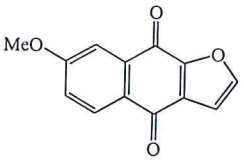

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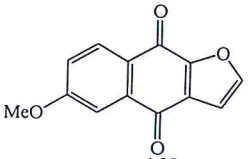

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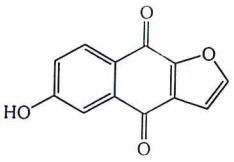

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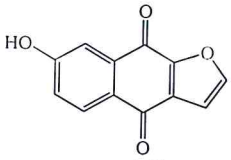

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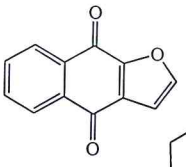

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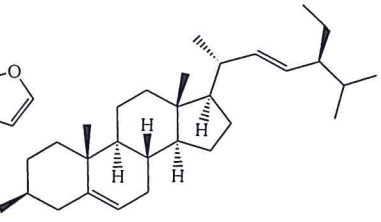

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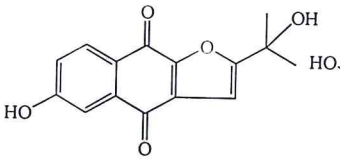

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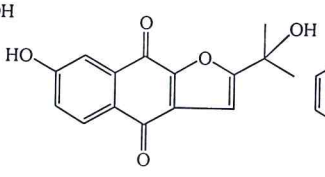

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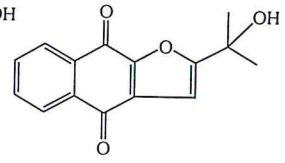

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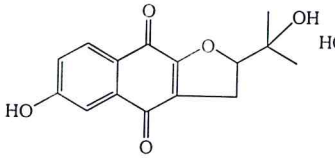

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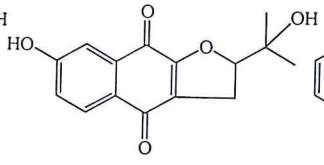

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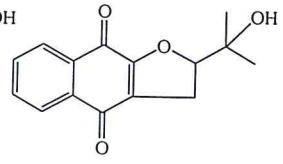
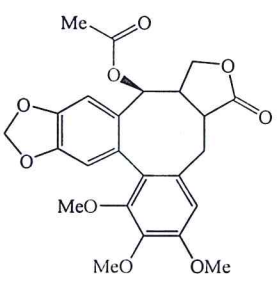


4.54

Compounds isolated from *Steganataenia araliacea* Hochst (Apiaceae)



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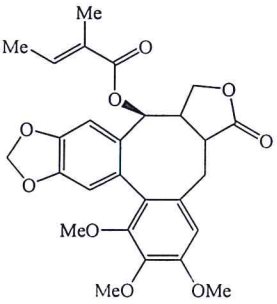
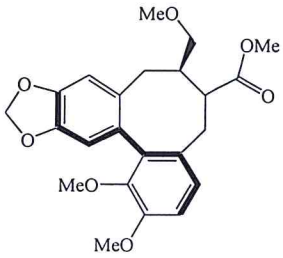
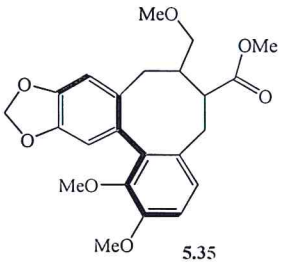


5.13



5.16



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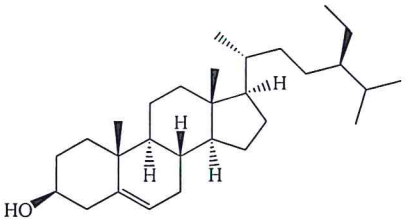
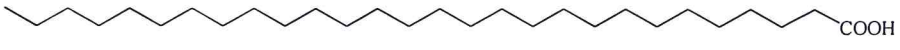


5.36



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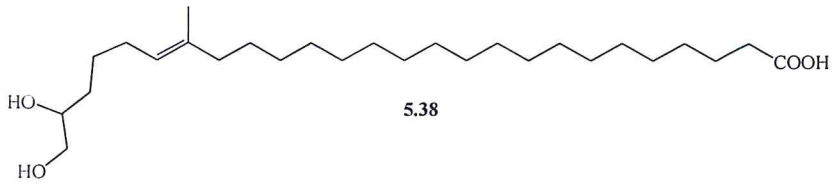
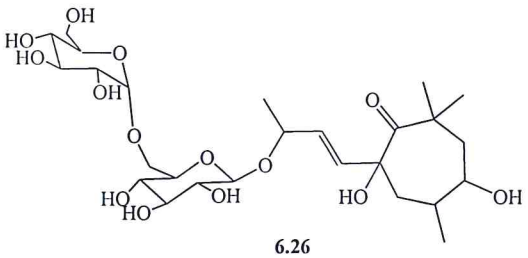


5.38

Compounds isolated from *Kotschya uguenensis* Verdc. (Fabaceae)



6.26

NOTE: Essential oil constituents are not included in this section (See pages 36-41)

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