MOSQUITOCIDAL AND ANTIMICROBIAL *NOR*-HALIMANOIDS, *ENT*-CLERODANOIDS AND OTHER METABOLITES FROM SOME TANZANIAN *TESSMANNIA* AND ANNONACEAE SPECIES

By

Charles Kihampa

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

University of Dar es Salaam

January, 2008

CERTIFICATION

The undersigned certify that they have read and hereby recommend for acceptance by the University of Dar es Salaam a thesis entitled: ***Mosquitocidal and Antimicrobial nor-halimanoids, ent-clerodanoids and Other Metabolites from Some Tanzanian Tessmannia and Annonaceae species,*** in fulfilment of the requirements for the degree of Doctor of Philosophy (Chemistry) of the University of Dar es Salaam.

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DECLARATION

AND

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

Signature.....

"If I have seen further it is by standing on the shoulders of giants."

Isaac Newton

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DEDICATION

This Thesis is dedicated to my beloved father

Domminick S. Kihampa and my mom Florence Kihampa,

And

to my beloved wife,

Harieth,

and The Boys (our sons),

SAMUEL and SAMSON

ABSTRACT

This Thesis reports results from chemical investigations and larvicidal, mosquitocidal, mosquito repellent and antimicrobial activity of 16 Tanzanian plant species of the families Leguminosae, Annonaceae, Eurphorbiaceae and Meliaceae, and constituents of some of the plant species. Three *Tessmannia* species *T. densiflora, T. martiniana* var *pauloi* and *T. martiniana* var *martiniana* that are reported to occur only in Tanzania, yielded series of *nor*-halimanoid and *ent*-clerodanoid diterpenes, some exhibiting mosquitocidal and antimicrobial activities, while others such as the *nor*-halimanoids tessmannic acid and its methyl, 2-methylpropyl and 1-methylbutyl esters and the *ent*-clerodanoids *cis*-kolavenolic acid, *ent*-(18-hydroxycarbonyl)-cleroda-3,13(*E*)-dien-15-oate, (*9-epi*)-*ent*-(18-hydroxycarbonyl)-cleroda-3,13(*E*)-dien-15-oate as well as the isocoumarin chlotessmin, are novel natural products, and so are *O*-(3-hydroxy-4-hydroxycarbonyl-5-pentylphenyl)-3-chloro-4-methoxy-5-pentyl-2-oxybenzoic acid and 5-pentyl-3-methoxy-*N*-butylaniline. Several other mosquitocidal and antimicrobial compounds were obtained from the Annonaceae species *Uvaria lungonyana*, *Uvariodendron pycnophyllum*, *Polyalthia tanganyikensis* and *Annona squamosa*, among them the aromadendrane sesquiterpenoid tanganyikenol and the kaurane diterpenoid 17-acetoxy-*ent*-kauran-19-al obtained from *P. tanganyikensis* and *A. squamosa* respectively, are being reported for the first time. Structural determination of all the isolated compounds was achieved based on spectroscopic data. These results further demonstrate the abundance of bioactive clerodane and halimane diterpenes in the Caesalpiniaceae family, as previously reported elsewhere.

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LIST OF ABBREVIATIONS

LC_{50} = Lethal Concentration at 50 % Mortality

RC_{50} = Repellency Concentration at 50 % Mortality

HMBC = Proton detected Heteronuclear Multiple Bond Correlation

HMQC = Proton detected Heteronuclear Quantum Coherence

COSY = Correlation Spectroscopy

DEPT = Distortionless Enhancement by Polarization Transfer

APT = Attached Proton Test Correlation Spectroscopy

NOESY = Nuclear Overhouser Enhancement Spectroscopy

MS = Mass Spectroscopy (mass spectrum, or mass spectra)

EIMS = Electron Impact Mass Spectroscopy

^{1}H NMR = Proton Nuclear Magnetic Resonance

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

VLC = Vacuum Liquid Chromatography

PTLC = Preparative Thin Layer Chromatography

UV = Ultra Violet

DMSO = Dimethyl sulfoxide

EtOAc = Ethyl Acetate

CC = Column Chromatography

MeOH = Methanol

$CHCl_3$ = Chloroform

CH_2Cl_2 = Dichloromethane

m/z = Mass to Charge Ratio

ICIPE = International Centre of Insect Physiology and Ecology

NIMR = National Institute for Medical Research

MIRT = Minority Research Training Program, USA

CHAPTER ONE

GENERAL INTRODUCTION AND LITERATURE SURVEY

1.1 Introduction

Malaria is a febrile illness caused by a protozoan parasite of the genus *Plasmodium*. It is spread through the bite of the female *Anopheles* mosquito in which the parasite has undergone part of its life cycle.[1-4] The clinical features of malaria vary from mild to severe, according to the species of the parasite present, the patient's state of immunity, the intensity of the infection and the presence of accompanying conditions such as malnutrition, anaemia and other diseases.[5] There are four types of *Plasmodium* parasites that are known to infect humans, which are *P. falciparum, P. vivax, P. ovale* and *P. malariae*. *P. falciparum* is the most dangerous parasite as it causes much more serious and progressive illness than the other parasites, often leading to a coma and death within a few days. The other three *Plasmodium* species may cause severe illness, but are rarely fatal.[5]

The majority of malaria victims are children under five and pregnant women. The disease kills many children before the age of five and leaves several others disabled. Pregnant women are four times more likely to fall ill, and twice as likely to die from malaria as non-pregnant women. Infants born to mothers with malaria are more likely to have a low birth weight – the single greatest risk factor for death during the first few months of life.[6] As several studies have shown, malaria kills very quickly. In half of those children who die from malaria, death occurs within 48 hours after the first symptoms of disease. Immediate access to effective treatment is a key factor in

reducing malarial morbidity and mortality.[6] Poor people are at greater risk of complications and death because their access to effective treatment is limited. Over a quarter of a very poor family's income can be absorbed by the cost of malaria treatment. This does not include the cost of prevention or the indirect cost of not being able to work.[6] Other high-risk groups include non-immune travellers, refugees, displaced persons, or labour forces entering into endemic areas.[7]

1.2 Global Situation of Malaria

Increasing concern about the worsening malaria situation led the WHO Executive Board and the World Health Assembly in 1980 to resolve affirmatively that malaria must be a global priority, that is essential for achievement of health for all and the objectives of child survival programs.[8] Globally, malaria is by far the most important insect transmitted disease. It is endemic in more than 100 countries and territories worldwide.[9] The current estimates put more than 40% of the world's population - about two billion people–to be living in areas with a high malaria risk, and the great majority of these are found in sub-Saharan Africa.[9] Worldwide, there are about 300-500 million cases of clinical malaria per year, with 1.5–2.6 million deaths annually, 5% of the deaths occurring among African children below the age of 5.[9,10] Countries in tropic Africa bear the brunt of malaria, accounting for more than 90 per cent of the 300-500 million annual cases of malaria (Fig. 1.1).[9,10]

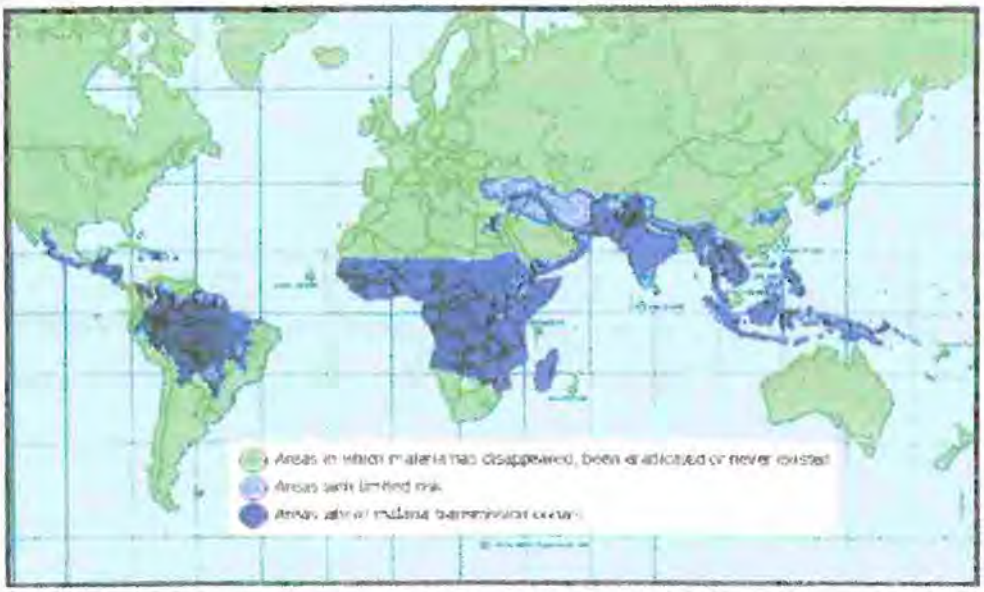


Fig. 1.1. Epidemiological Assessment of the Global Status of Malaria Prevalence (http://www.niaid.nih.gov/publications/malaria/assess.htm, 2007)

Malaria not only cuts lives short but has a huge socio - economic impact, especially in Africa where malaria thrives, people suffer and economies are drained. Patients are often bedridden and incapable of carrying out normal daily activities, therefore suffering considerable loss of income and placing a heavy burden on their families, the health system and the society as a whole. It hits hardest during the rainy season when sowing and harvesting take place. At a country level, it impacts on trade, tourism and foreign direct investment.[6] There is a remarkable correlation between malaria and poverty: Average GDP in malarious countries is five times lower than in non-malarious countries. Malaria keeps poor people poor, thus has social consequences and is a heavy burden on economic development. Malaria slows economic growth in African countries by 1.3 % per year.[11] As a result of the compounded effect over 35 years, the GDP level for African countries is now as much as 32 % lower than it would have been in the absence of malaria.[11] Malaria is

estimated to cost Africa directly and indirectly more than US$12 billion annually,[9,10] and a single bout of malaria is estimated to cost a sum equivalent to over 10 working days in Africa.[13,14] The cost of treatment is between US$ 0.08 and US$ 5.30 according to the type of drugs prescribed as determined by local drug resistance in East Africa (Table 1.1).[6,15]

Table 1.1 Estimated Annual Supplementary Cost of Optimal Treatment at Country Level[15]

Country	Population (Million)	Malaria Cases per year (Million)	% Resistance to Chloroquine	% Resistance to SP *	Supplementary Cost of Optimal Treatment* (in US$ per year)
Burundi	6.5	2	50-90	13-63	1,617,000
Kenya	30	8.2	66-87	27-40	6,135,000
Rwanda	7.2	1.2	40	16-45	945,000
Tanzania	32.8	8.6	28–72	15-34	6,426,000
Uganda	21.1	5.3	10-80	11-60	4,007,000

According to public health experts, presently there are more people suffering from malaria than ever before. Malaria is undergoing an expansion into areas where it was previously absent, or under control.[12,13] There are several factors to be considered which have contributed to this wide occurrence of the disease, including those which relate to climatic change like the *El Nino* weather phenomenon, global warming and human interference with the environment.[12,13] Changes in land use linked to developmental activities, such as road construction, agricultural and irrigation schemes, mining and logging, have provided habitats for *Anopheles* mosquitoes and this has resulted into "man-made malaria".[12,13] The extension of urban areas has led to epidemics in the peripheries of the growing cities. Mass migration of non-immune populations into endemic areas for political or other reasons have also led to

increased transmission of the disease.[12,13] At the same time, poverty, war and political instability have weakened public health systems in many developing countries. And most experts agree that the resurgence of the disease is due in large part to the fact that malaria parasites and their mosquito vectors are increasingly developing resistance to the drugs and insecticides used to control them.[14] Therefore, this has continued to prompt extensive research efforts to establish new, more viable antimalarial and mosquitocidal agents.

1.2.1 Malaria in Tanzania

Malaria is the commonest communicable disease in Tanzania. It is a leading health problem and a threat to every one of the estimated 32 million people (94 % of the population) living in areas where transmission is possible.[15] In Tanzania malaria kills about 100,000 people every year, which is like killing a person every five minutes.[15-18] It is a major cause of infant morbidity and mortality, and is the only insect borne parasitic disease comparable in impact to other major killer transmissible diseases like diarrhoea, acute respiratory infections, tuberculosis and AIDS.[15] Each year, 70,000 children are lost due to malaria - that is in every 10 deaths 7 are children.[18] Major victims are children aged less than five years, particularly those who are below two years, and creates a huge burden of disease, due to anaemia, especially in pregnant women.[15-18]

Tanzania is probably one of the worst hit malaria endemic countries in Africa, having areas with highest transmission (over 1 infective bite per person per night).[15] The distribution of malaria endemicity in the country is not homogenous (Table 1.2 and Fig. 1.2). The variations in endemicity are conventionally classified as: Unstable

seasonal malaria, stable malaria with seasonal variations, and stable perennial malaria.[15] The predominant vector in areas with stable perennial transmission is *Anopheles gambiae* complex (*Anopheles gambiae sensu stricto* and *An. arabiensis*). *An. funestus* is found particularly in humid areas and around permanent water bodies, such as rivers, lakes and dams, often coexisting with *Anopheles gambiae.*[15]

Table 1.2 Stratification of Malaria Transmission in Tanzania[15]

Zone	Population	Transmission and Endemicity
Coastal		
Extending inland as far as 160 – 240 km Have temperatures of 24 – 32 °C year round Mean vapour pressures 26-29 millibars	14,000,000 (42 %)	Over 6 months (stable perennial malaria) Most age groups have considerable immunity increasing with age
Central		
High altitude plains Temperatures of 15 °C Mean vapour pressures 10 – 20 millibars	11,300,000 (33 %)	4 – 6 months (stable malaria with seasonal variations) People have weak immunity in all age groups, and therefore susceptible to severe malaria
Fringe Highlands, Rift Valley		
Mountains with altitude up to 2,000 metres Temperatures up to 20 °C Mean vapour pressures 13 – 15 millibars	2,600,000 (8 %)	1 – 3 months (strongly seasonal or epidemic) People have little immunity, therefore are susceptible to severe malaria in all age groups
Highlands		
	5,800,000 (17 %)	Less than 1 month (epidemic potential or no malaria) People have little immunity, therefore are susceptible to severe malaria in all age groups

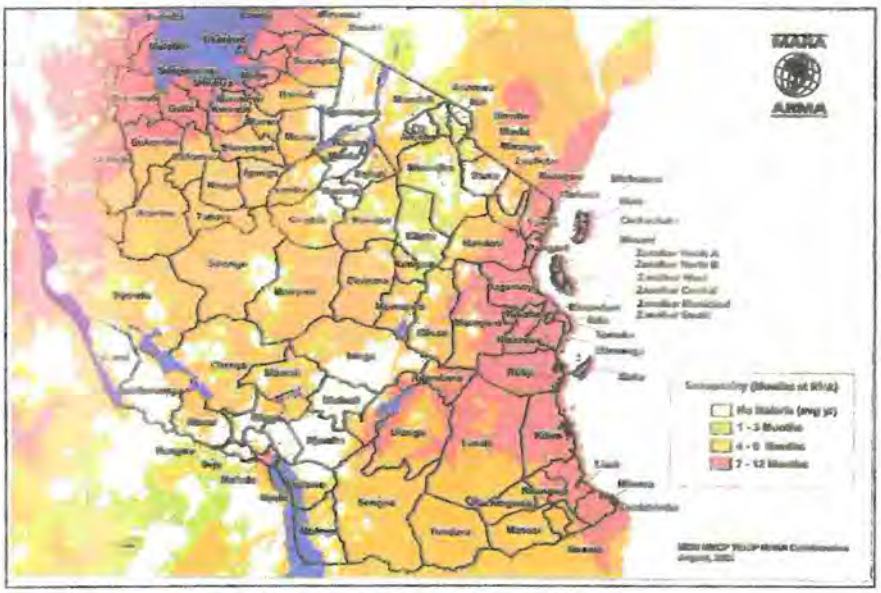


Fig 1.2 Malaria Transmissions in Tanzania[15]

The treatment and prevention of malaria victims cost the country more than 121 billion Tanzanian shillings per annum, or 3.4 per cent of the national GDP.[16,19] The cost for outpatient care per case is estimated to be Tsh 1,600, that of inpatient care per case is Tsh 20,000 and the national expenditure per person per annum is Tsh 2,200 (US$ 2.14). This represents almost 39 % of the total health sector expenditures and 1.1 % of the country's GDP; 71 % of the total spending is from private sources (i.e. household expenditures) in the formal and informal private sector, with the Government of Tanzania representing a further 20 % and the remainder attributed to external funding.[16,19]

Malaria control strategies in Tanzania were first initiated in 1901 on the mainland (then called German East Africa and later Tanganyika). An attempt was made to control malaria in the coastal areas, Tanga and Dar es Salaam by mass prophylactic treatment of the population with quinine (**1.1**).[20] This programme was drawn up to ensure that ponds, vessels, coconut shells and the like should be emptied of water at

least once every four days by the owners. Failure to abide by these regulations was punishable by a fine or in default, imprisonment for a period not exceeding ten days.[21] These two programs failed, mainly due to the emergence of the First World War.[22] A third and more programs were the result of the 8th WHO Assembly held in 1955, which advocated worldwide malaria eradication.[23] However, this was not possible to achieve in Tanzania as well as in most parts of Africa. One major reason for the failure was lack of supporting health infrastructure.[22] Some trials were however carried out with chloroquinised salt[24] and vector control measures were used for a short time in Dar es Salaam involving spraying of larvicides and insecticides in potential breeding places, but again without any major success.[25]

Several research programmes aimed at establishing alternative malaria control measures have been conducted in Tanzania. The establishment of the Amani Biological Institute in 1890 in the Usambara Mountains near Tanga was one of the efforts by the German East Africa Administration towards establishing stations in the country that would facilitate research in various pertinent areas including insect borne disease control. This station was later developed into the East African Malaria and Vector Borne Diseases Institute during the British colonial administration.[20,26]

The Amani centre is still an active place for malaria research in Tanzania and constitutes one of the subunits of the National Institute for Medical Research (NIMR). Thus, during the First World War, quinine (**1.1**) from the *Cinchona* tree for malaria treatment was even produced at Amani.[27] Quinine was the first antimalarial drug to be isolated from plants and up till now it continues to be one of the best treatments for malaria. The drug was not recognised in western medicine until the

beginning of the 19th century although *Cinchona* extract had been widely known by Latin American Indians for centuries.

The usefulness of *Cinchona* tree bark for malaria treatment was discovered by accident. Legend has it that a boy suffering from fever had got lost in the high jungles of the Andes Mountains in Latin America. Accidentally, he landed in an area rich with *Cinchona* trees. As he sought something to quell his thirst he came across a stagnant pool of water.[28] The water tasted bitter and the boy immediately realised that it was tainted with an extract from the bark of the *Cinchona* trees (referred by the Latin American Indians as *quiana-quiana*), which was believed to be poisonous. However, because of the severe fever the boy went ahead to the risk of drinking the bitter water. Contrary to expectations, the boy survived and his health improved. He regained strength and was able to find the way back to his village where he disclosed to family and friends of his discovery. This marked the beginning of the treatment of malaria fevers with *Cinchona* tree bark extracts. The treatment thereafter spread in other Latin American countries where *Cinchona* trees grew. Therefore, when quinine was isolated in its pure form in 1822 it was was named after the Latin American Indian name for *Cinchona* trees, *viz. quiana-quiana*.[28]

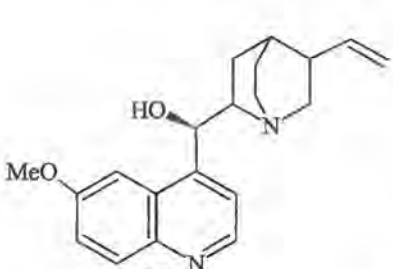
Accounts of the origin of malaria chemotherapy using *Cinchona* bark include an apocryphal story about the countess of *Cinchon* in Spain who lived in Lima, Peru during the 17th century AD. It is recollected that the countess had recovered dramatically from a severe attack of malaria when she took a remedy given by a local Spanish official, having been prepared from a bark of a native medicinal tree. It is alleged further that the celebrity status and charitable nature of the countess

prompted the widespread use of this remedy. It is believed that missionary priests returning from Peru to Spain introduced the Peruvian bark into Europe in the mid 16th century AD. The bark gained its curative reputation when it was used to treat the king of England, the Dauphin of France (1682 AD) and the Emperor of China (1692 AD) who were suffering from intermittent fevers. In 1749 AD the famous Swedish botanist Linnaeus named the Peruvian tree *Cinchona* in deference to the countess of Cinchon.[28] After the isolation of quinine from the barks of *Cinchona* trees in 1822 by French Chemists and its subsequent establishment as a standard drug for malaria treatment, the cultivation of *Cinchona* trees became an economic venture in many parts of the world. Colonial powers spread this plant in colonies where climatic conditions were favourable for its growth. Therefore, the Dutch established *Cinchona* plantations in the East Indies while the Germans did the same in German East Africa, now Tanzania. In Tanzania, *Cinchona* cultivation was confined to the west Usambara Mountains at Mazumbai where climatic conditions allowed the trees to thrive. However, after the establishment of efficient synthesis of quinine and other synthetic antimalarial agents, these plantations have now been literally abandoned.[28]

Apart from research and other past methods, fighting malaria is now one of the priorities of the Ministry of Health and Social Welfare (MOHSW) in Tanzania. Thus, in June 2000, the MOHSW officially announced a change in the national malaria treatment protocol, to be implemented in 2001. With resistance to chloroquine (1.2) ranging from 28 to 72% among regions, the Government decided to switch to Fansidar® (sulphadoxine-pyrimethamine (SP)) and Amodiaquine as the first and second line treatment respectively.[15] However, increasing parasite resistance within the initial five years after introduction of SP and Amodiaquine, made the Ministry of

Health and Social Welfare to decide to change malaria treatment guidelines from monotherapy to Artemisinine based combination treatment in 2006.[29] Of equal importance was the need to move to more effective combined antimalarial treatment with the aim of improving clinical cure and delaying the development of drug resistance. Under this programme the following antimalarial drugs were recommended in Tanzania:

- The first line drug is Artemether/Lumefantrine (ALu).
- The second line drug is Quinine, where Artemether/Lumefantrine has failed or is contraindicated.
- The drug of choice for treatment of severe malaria is Quinine.
- The first line drug for pregnant women during first trimester and children under 5 kg is quinine.[29]



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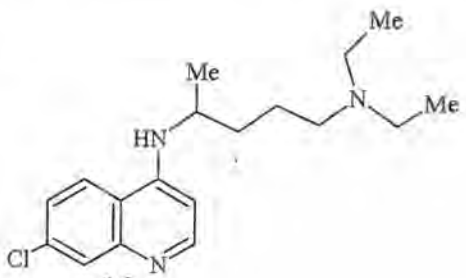


1.2

1.3 Worldwide Malaria Control Efforts

Control of malaria represents one of the world's greatest public health challenges, especially in sub-Saharan Africa where most of the disease occurs. In the past decades, efforts to control malaria have been met with mixed success.[30] The strategy for malaria control is based on breaking the chain of transmission of the parasites between humans and mosquitoes. It therefore involves three living beings: Humans

(host) by treating the affected and protect the unaffected; *Plasmodia* (agent) by killing the asexual and sexual forms as well as ensuring full treatment; and *Anopheles* mosquitoes (vectors) by preventing breeding, entry and bites.[31]

There are presently two approaches that are important and vital for the elimination or control of malaria. These involve control of parasites by chemical drugs known as chemotherapy, chemoprophylaxis and/or vaccination, and the control of vector populations by chemical insecticides, repellents, screens such as bed nets, window gauzes and curtains, or environmental management.[32] Discoveries of chemical insecticides and drugs have provided a powerful weapon against malaria and their use has played a major role in the control of this disease.[32]

1.3.1 Mosquito Control

Since the discovery of the connection between *Anopheles* vectors and malarial transmission in 1897, vector control strategies have been the most widely used malaria transmission management measures.[30] Vector control is assumed to be one of the effective methods for management of the transmission of malaria. This is because it is easier to control mosquito populations within a given geographical area than giving vaccines for protection, or administration of prophylactic drugs to individual persons. The principal objective of mosquito control is the reduction of malaria morbidity and mortality by reducing the levels of transmission *via* mosquito population manipulation (Fig. 1.2).[33] This includes activities that reduce the number of infective or infectious bites of the vector by reducing the vector density, longevity and preventing human-vector contact.[33]

Fig. 1.2 Mosquito Life Span
(http://www.mosquitoes.org/LifeCycle.html, 2007)

Before World War II vector control measures included environmental sanitation through drainage and landfills to eliminate the larval mosquito habitat; biological control through the use of larvivorous fish in ponds; and larviciding with oil and Paris green. All these methods were effective, especially in Europe, but malaria continued to be a problem on a global scale.[34] The availability of dichlorodiphenyltrichloroethane [DDT (1.3)] and other insecticides (Table 1.6) marked a new era for global malarial control. The effectiveness of DDT against indoor mosquito resting sites led to the adoption of the Global Eradication Programme of Malaria in 1955, which was coordinated and supported by the WHO.[35] For the first 10 years (1957–66) the results were spectacular; malaria was completely eradicated in the United States as well as in the former Soviet Union and other European countries. Disease incidence was also significantly reduced in many countries in the tropical region of South-East Asia, India and Latin America.[36] However, gains made in some of the countries, particularly in the tropical regions, could not be sustained and there were reverses due to financial, administrative or operational problems, resistance or behaviour of vectors, or to the inadequate

development of basic health care services.[37] The time-limited eradication policy was eventually abandoned in 1969 and replaced by a long-term Global Malaria Control Strategy in 1992.

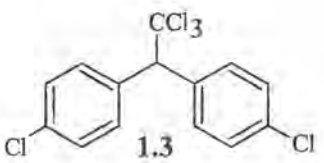
Table 1.5 List of Chemical Insecticides Used for Mosquito Control[30]

Active Ingredient	Use[a]	WHO Toxicity Class[b]	Chemical Type[c]
DDT (**1.3**)	A	I B	OC
Methoxychlor (**1.4**)	A	III N	OC
Chlorpyrifos (**1.5**)	L, A	II	OP
Chlorpyrifos-methyl (**1.6**)	L	II	OP
Diazinon (**1.7**)	L, A	II	OP
Dichlorvos (**1.8**)	L, A*	I B	OP
Fenitrothion (**1.9**)	L, A	II	OP
Fenthion (**1.10**)	L, A	I B	OP
Pirimiphos-methyl (**1.11**)	L, A	III	OP
Malathion (**1.12**)	L, A	III	OP
Naled (**1.13**)	L, A	II	OP
Temephos (**1.14**)	L	III N	OP
Bendiocarb (**1.15**)	L	II	C
Propoxur (**1.16**)	A	II	C
Jodfenphos (**1.17**)	L, A	III N	OP
Cypermethrin (**1.18**)	A	III A	PY
Deltamethrin (**1.19**)	L, A	II	PY
Resmethrin (**1.20**)	A	III	PY
Pyrethrins (**1.21**)	A	II	NO
Diflubenzuron (**1.22**)	L	III N	IGR
Methoprene (**1.23**)	L	III N	IGR

[a]L = larvicide; A = adulticide

[b]IGR = Insect Growth Regulator; OC = Chlorinated hydrocarbons; OP = organophosphates; C = carbamates; PY = pyrethroids; NO = natural organic

IA = Extremely hazardous, IB = Highly hazardous, II = Moderately hazardous, III = Slightly hazardous, IIIN = Negligible hazard



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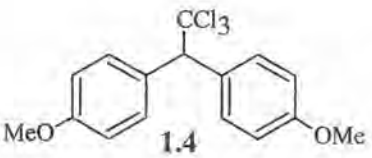

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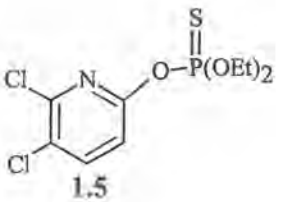

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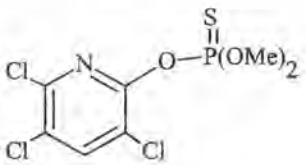

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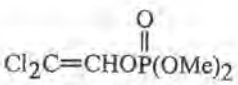

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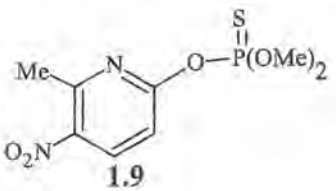

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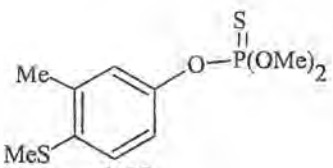

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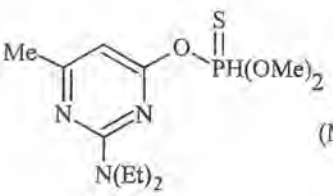

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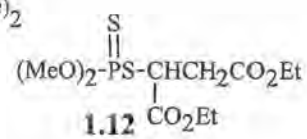

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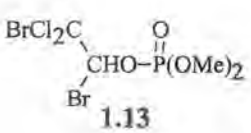

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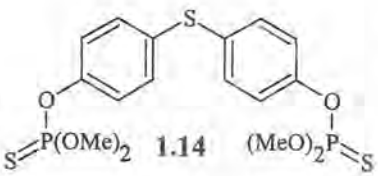

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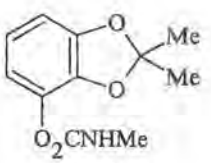

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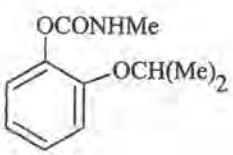

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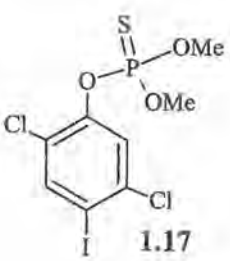

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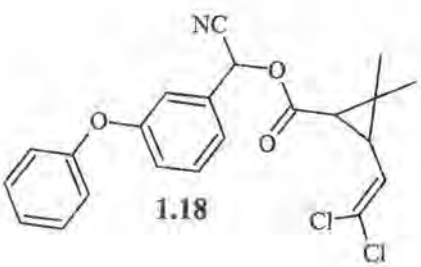

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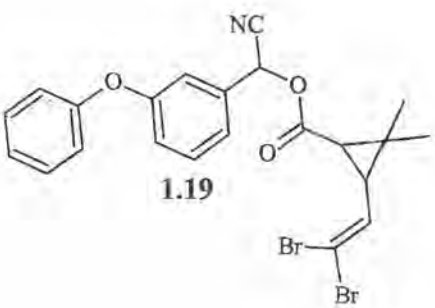

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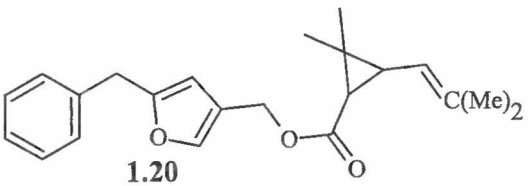

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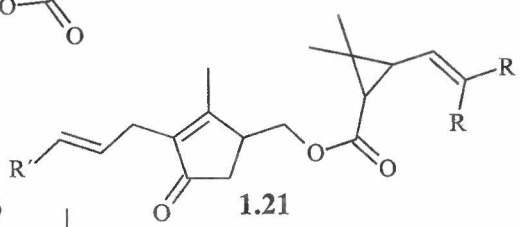

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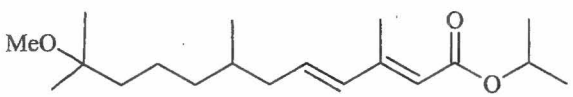

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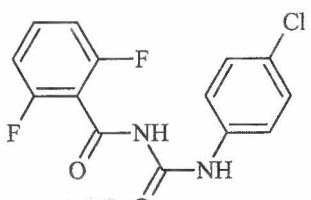


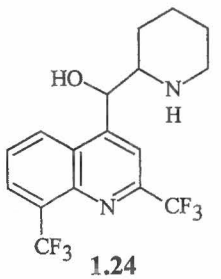
1.22

	R	R′
Pyrethrin I	Me	$CHCH_2$
Pyrethrin II	CO_2Me	$CHCH_2$
Jasmolin I	Me	Et
Jasmolin II	CO_2Me	Et
Cinerin I	Me	Me
Cinerin II	CO_2Me	Me

1.3.2 Parasite Control

Development of an effective malaria vaccine has been a goal of researchers for more than 30 years now. The three main types of vaccines being developed are anti-sporozite,[38] anti-asexual blood stage and transmission-blocking vaccines.[39] The vaccines are designed to prevent infection, reduce severe and complicated manifestation of the disease and arrest the development of the parasite in the mosquito, respectively.[39] The chemically synthesized vaccine, SPf66, was tested in Tanzania but gave insignificant protection with serious side effects.[40] Peptide based vaccines have been successfully employed but face the challenge of toxicity.[40,41] Other candidate trial vaccines include nucleic acids that target asexual and pre-erythrocytic stages of malaria parasites.[41] Besides vaccines, chemoprophylaxis and chemotherapy have been used to minimise disease prevalence.

A number of diverse single compound drugs have been found to be effective against malaria. However, in many cases, the initial success in treating this disease is followed by total failure. Drugs which worked initially became totally ineffective after a period of time. An initial period of remission is often followed by a period of frustration during which nothing seems to be effective against the disease. Death becomes inevitable. Such a phenomenon is often referred to as multi-drug resistance. A material cell which initially responds to treatment by one or more drugs becomes resistant to treatment by not only the drug previously used, but any malarial treatment drug. Thus, since the 1950s the treatment of malaria has principally relied on chloroquine (**1.2**), which is safe for use in pregnancy, inexpensive, widely available, and once a highly effective treatment.[42] However, resistance to chloroquine (**1.2**) is widespread and that has restricted its use.[43-45] The loss of this drug has been a major setback to the effective treatment and control of malaria. Furthermore, resistance to other anti-malarials such as mefloquine (**1.24**), dapsone (**1.25**) and pyrimethamine (**1.26**) has also been reported.[45,46] Resistance to drugs like quinine (**1.1**),[47] halofantrine (**1.27**) and amodiaquine (**1.28**) have also been observed or demonstrated *in vitro*.[48] Halofantrine (**1.27**) exhibits serious toxicity at dosages required for treatment of resistant strains.[49] A limited number of single drugs for treatment of malaria including atovaquone (**1.29**) and proguanil (Paludrine®, **1.30**) are still available today. In view of the drug resistance that has developed among *Plasmodium falciparum* malaria parasite strains, more attention is particularly being focused on the development of anti-malarial agents that do not possess the quinoline skeleton found in chloroquine and related synthetic anti-malarial drugs, in order to avoid the emergence of parasite cross resistance to the new drugs.[50]



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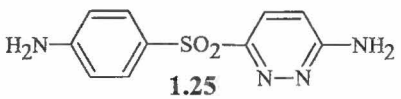

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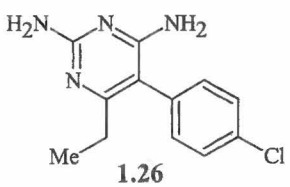

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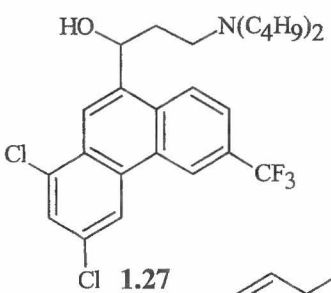

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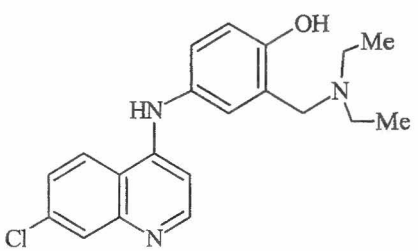

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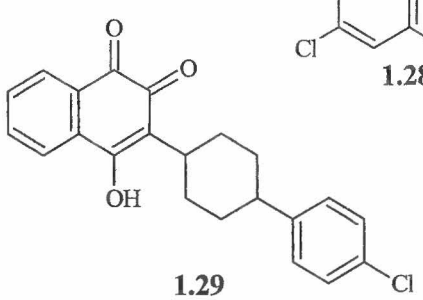

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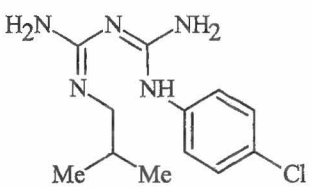

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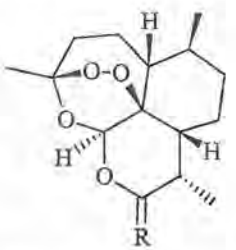
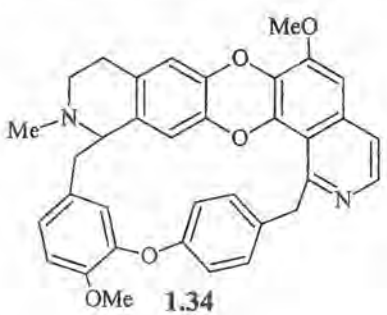
One of the most spectacular discoveries of plant based drugs in recent years is the isolation and characterisation of artemisinine (**1.31**) in the 1970s, which was later established to be an effective antimalarial drug.[51] Artemisinine was obtained from a Chinese antimalarial herb, A*rtemisia annua* (Asteraceae), whose extract had been used for the treatment of malaria in China since medieval times. The compound and its derivatives like artesunate (**1.32**) and artemether (**1.33**) are now commercial anti-malarial drugs[50] and have been known to show no cross-resistance.[45,46] Presently, artemisinine is used for the treatment of malaria in many countries including Tanzania, where the cultivation of *Artemisia annua* for local production of artemisinine is currently being practised. *Artemisia* growing is likely to become a major economic venture for Tanzania and other African countries with a dual

advantage of poverty alleviation and contributing to the fight against malaria. This is particularly the case because the use of artemisinine for the treatment of malaria has been extended to combination therapies, whereby the compound or its derivatives are used in combination with other, less effective agents like pyrimethamine. It is therefore expected that the local cultivation of *Artemisia annua* will lead to the production of *Artemisia* drugs locally, such that the anti-malarial agents would be cheaper and easily accessible by the Tanzanian population.[50]

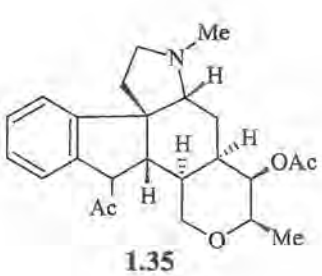


1.31: R = O
1.32: R = $CHOCH_3$
1.33: R = $CHCH_2CO_2(CH_2)_2CO_2H$

The discovery of artemisinine prompted enormous efforts to search for other anti-malarial agents from medicinal plants growing in tropical and subtropical countries. To date extensive investigations on anti-malarial plants from Africa, Latin America and South East Asia have been carried out and several alkaloids, terpenoids and other compounds with antimalarial efficacy have been isolated.[50] These include the *bis* benzylisoquinoline alkaloid tetrandrine (**1.34**) and related compounds isolated from the Chinese plant *Stephania tetrandra* (Menispermaceae),[52] and malagashanine (**1.35**) that has been isolated from a Madagascan *Strychnos* species.[50]



1.34



1.35

Following the rapid development of resistance to single line therapy, combination therapies are also targeted and have proved useful in malaria treatment. Fansidar® (sulfadoxine and pyrimethamine), Malarone® (proguanil and atovaquone) and Coartem® (artemether and lumefantrine) are available today.[53] The combination of an oral artemisinine derivative (usually artesunate) and mefloquine has become standard treatment for multidrug-resistant falciparum malaria.[54] However, resistance is already being reported for combinations like Fansidar®.[53] Thus, the looming failure of two-compound antimalarial drug combinations leaves triple therapy as a viable approach to fighting drug resistant *P. falciparum* malaria. Three-compound combination slows down development of resistance against the individual drugs.[55] This approach is likely to promote the use of the currently withdrawn cheap antimalarial agents like chloroquine in the treatment of drug resistant malaria using combination therapy regimes.[50] Thus, chloroquine plus SP and amodiaquine in combination with SP have been used.[56] Large parts of the world may soon be without any effective anti-malarial drug and therefore vulnerable to devastating malaria epidemics. Other alternative malaria control strategies therefore need to be developed since chemotherapy appears to be failing to achieve the desired goal in malaria eradication. Besides chemotherapy, vector control strategies have also been employed.[46] This latter aspect is the basis for investigations whose results are reported in this Thesis.

1.4 LITERATURE SURVEY

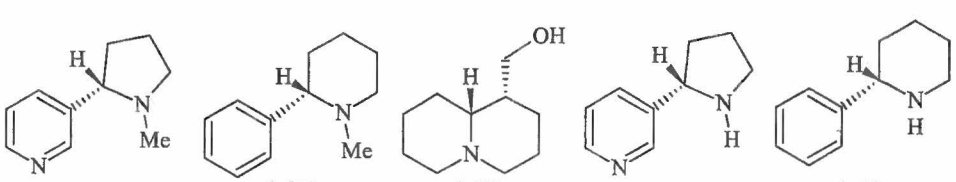
Plants are highly vulnerable to predation by herbivorous animals, insects, fungi and other microorganisms within the ecosystem. Therefore, plants synthesize a variety of chemically diverse secondary metabolites, at least in part in response to selection pressures against predation and/or attack by herbivores and other microorganisms. In this regard plants found in the tropical climates have been considered to be viable sources of chemical agents with effective properties and hence would be used to protect the plants against predation. This is believed to be the case since tropical conditions are quite challenging to the plants' survival, as the plants are threatened by a myriad of organisms that thrive under tropical climatic conditions.[50] Therefore, through evolutionary transformations, tropical plants must have evolved the ability to metabolise toxic compounds that can interfere with the basic biochemistry of the predatory or harmful organisms, such compounds are therefore suitable candidates for the development of medicinal and/or pest control agents.[50,57] In this regard, plants and plant products having insecticidal or acaricidal properties have been used traditionally by local communities in different areas of the world against medically or agriculturally harmful insects, ticks and mites even before the advert of synthetic chemicals.[57,58] Ancient peoples used smoke from burning cattle or goat dung to drive out mosquitoes from their caves or huts before sleeping. Later on, certain herbs of barks of some trees were added to the smoldering fire to enhance the repellent action of smoke. The Ainu people of Hokkaido, Japan and Micmac Indians of Newfoundland wore legginings of sedge, bark or cloth to reduce insect biting nuisance, which is concentrated around the lower legs. The Chinese had many prescriptions of repellents against mosquitoes, among other blood-sucking flies.[59]

During World War II, the military used aromatic oils like citronella, bergamot, eucalyptus, peppermint, turpentine and spirit of camphor in various formulations to repel biting insects. However, several plants and products that are traditionally used to kill or repel mosquitoes and other blood sucking insects[59] lack scientifically reliable data that would demonstrate their efficacy under uncontrolled field conditions. Many plants produce chemicals with anti-feedant, insecticidal or IGR properties, which could potentially be exploited to provide insecticides that are readily biodegradable, as substitutes to synthetic chemicals like DDT, that are now being withdrawn from the market or their use has been restricted because of persistency in the environment, and therefore toxic to humans and other organisms.[60] Generally many rural communities in developing countries in Africa, Asia and Latin America continue to rely on herbal extracts for their daily healthcare provisions and insecticidal applications. In part this situation has been prompted by limited supply of conventional medicines and insecticides in these areas but partly because of the belief in the therapeutic capacity of these extracts. Subsequently, the search for medicinal and insecticidal agents from tropical flora has continued to be a subject of great research interest worldwide.

The most successful example of plant produced insecticides is that of the pyrethroids that occur in the flowering heads of pyrethrum (*Chrysanthemum cinerariaefolium* and *C. coccineum,* family Compositae = Asteraceae). Insecticidal properties of several *Chrysanthemum* species were known for centuries in Asia. Even at present powders of dried flowers of these plants are sold as insecticides. The insecticidal properties of pyrethrum are due to six terpenoids (**1.21**, page 18). These compounds

have quite high knockdown effects against flying and crawling insects, but are highly unstable to light, air, moisture and alkali. Knowledge of their structure and their high cost stimulated structural optimization of synthetic analogues, having activity found in the natural pyrethroids being either retained or enhanced. This has resulted in reduced costs, wide affordability and acceptability of pyrethroid analogues.[61-63] Examples of these synthetic pyrethroids include permethrin (**1.18**) and deltamethrin (**1.19**). Although synthetic pyrethroids offer improved selectivity (over the other synthetic insecticides) and lower mammalian toxicity, incidences of mosquito resistance to these insecticides have however been recently reported.[64]

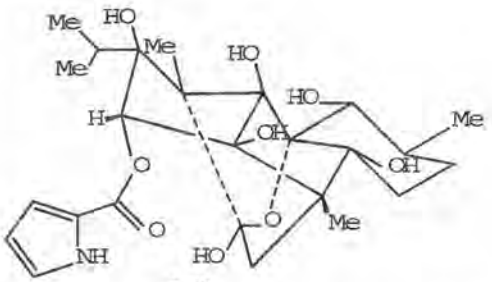
One of the earliest reports of the extensive use of natural insecticides (even before the discovery of synthetic organic insecticides) against mosquito larvae involved the plant alkaloids like nicotine (**1.36**), methylanabasine (**1.37**) and lupinine (**1.38**) extracted from the Russian weed *Anabasin aphylla* that killed larvae of *Culex pipiens, Cx. Quinquefasciatus* and *Cx. territans*.[62,65] Nicotine is also found in the leaves of *Nicotiana tobacum, N. rustica, Duboisa hopwoodii* and *Aesclepias syriaca*. It occurs as the main alkaloid along with small amounts of twelve other alkaloids of which *nor*-nicotine (**1.39**) and anabasine (**1.40**) are of insecticidal importance.[62, 65]



1.36 **1.37** **1.38** **1.39** **1.40**

Synthetic analogues of nicotine, like 5'–methyl-*nor*-nicotine have been demonstrated to be effective insecticides. Ryanodine (**1.41**), an alkaloid from the tropical shrub, *Ryania speciosa,* has been used as a commercial insecticide against the European

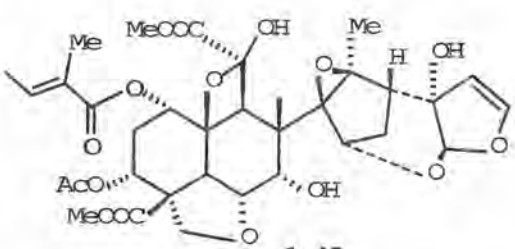
corn borer. The high cost, toxicity to mammals and limited efficacy has limited the use of natural alkaloids as insecticides.[61]



1.41

Neem (*Azadirachta indica*) is a fast growing tree originally from the Indian sub-continent but now found in most parts of Africa and Asia. It contains several insecticidal compounds, the main one being azadirachtin (**1.42**), which deters and kills many insect pests and vectors. The seeds of neem keep their killing potency for a year if kept in darkness.[65]

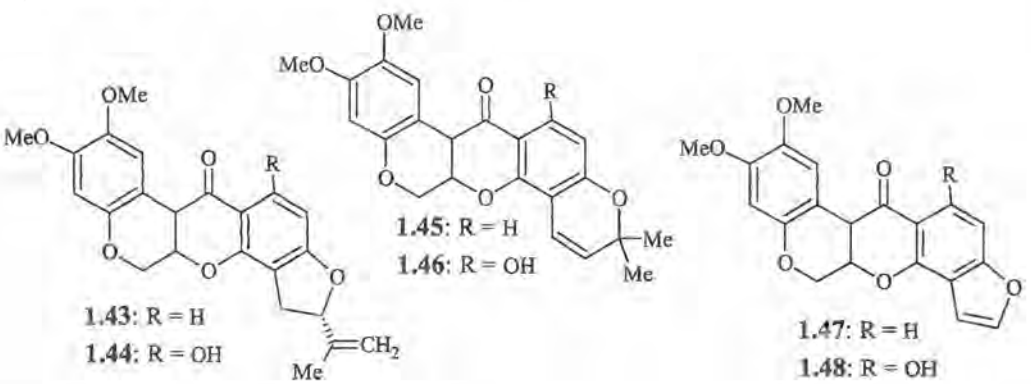
Insect antifeedants, such as azadirachtin are highly toxic natural products and probably these compounds constitute the main defence mechanism that plants have evolved against insect attacks. The compound also blocks insect's reproduction and release of hormones that control metamorphosis.[50] The main disadvantage is the instability of neem extracts to light.



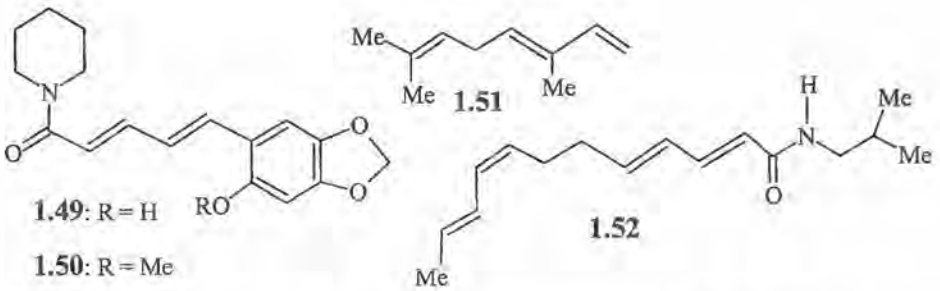
1.42

Rotenoids like rotenone (**1.43**), sumatrol (**1.44**), deguelin (**1.45**), L-α-toxicarol (**1.46**), elliptone (**1.47**) and malaccol (**1.48**), which are found in the family Leguminosae, have been used as insecticides.[66] The genera *Derris, Lonchocarpus,*

Milletia and *Tephrosia* are known to be rich in rotenone (**1.43**) from their roots. Root extracts of plants in these genera were commercially used as insecticides in the 1930's. The use of rotenone – yielding roots as insecticides in the USA was developed as a result of Federal laws against high residues of lead, arsenic and fluorine in edible products. Rotenone is harmless to plants, highly toxic to insects, relatively toxic to fish, pigs and honey bees. Twenty one species of *Tephrosia*, twelve of *Derris*, twelve of *Lonchocarpus*, ten of *Milletia* and several of *Mundulea*, have been reported to contain rotenoids.[62]



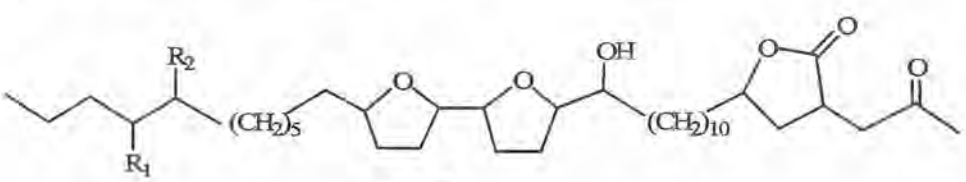
Limonoids, quinones, alkaloids, flavonoids, terpenoids, polyacetylenes and butyl-amides extracted from plants show a degree of larvicidal activity against mosquito larvae.[67] For instance, piperine (**1.49**) and wisanine (**1.50**) are alkaloids that were isolated from *Piper guineense* and found to be very active against *Aedes aegypti* larvae.[68] Terpenoids such as 5-(*E*)-ocimenone (**1.51**) from *Tagetes minuta* have been reported to possess larvicidal activity against mosquito larvae.[69] Larvicidal activity of long chain fatty amides such as *N*-isobutyl-2*E*,4*E*,8*Z*,10*Z*-dodeca-2,4,8,10-tetraenamide (**1.52**) isolated from *Spilanthes mauritiana* have been reported.[70] The amides from *Zanthoxylum gilleti* (*Fagara macrophylla*) have also been reported as larvicides against *Culex* species.[67]



1.49: R = H

1.50: R = Me

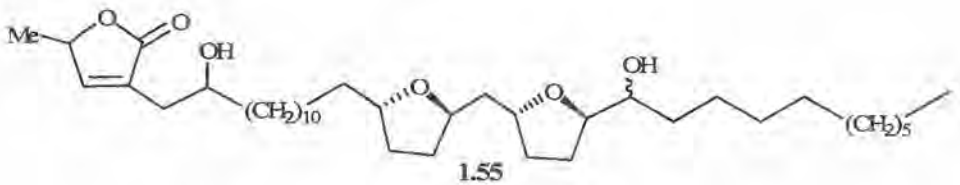
A particular type of acetogenins occurring in plant species of the family Annonaceae have been shown to inhibit adenosine triphosphate (ATP) production at a similar site of action and higher levels of insecticidal potency as the natural insecticidal agent rotenone (**1.43**), i.e at NADH-ubiquinone oxido-reductase complex I of the mitochondrial electron transport chain.[71] The strong biological activity of the acetogenins as larvicides, pesticides and antitumourals is dependent on the functional group variations in which the degree of hydroxylation is particularly important for these effects.[71] Some of the annonaceous acetogenins include two bullatacinones 31-OH (**1.53**) and 30-OH (**1.54**) isolated from *Annona bullata*. The compounds are very active insecticides, being slightly more active than rotenone (**1.43**).[71]



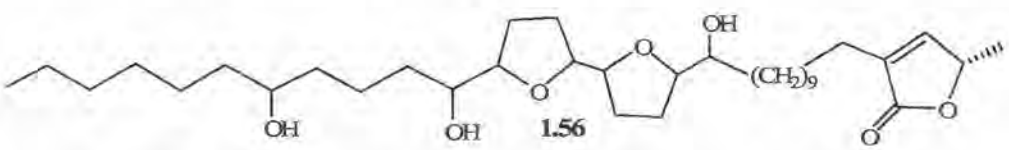
	R_1	R_2
1.53:	OH	H
1.54:	H	OH

Asimicin (**1.55**) isolated from the fruits of *Rollinia sylvastica*, was found to be active against mosquito larvae as well as aphids, mites, the Mexican bean beetle, nematodes and spiders.[72] Another acetogenin, ophrypetalin (**1.56**) isolated from the leaves of *Ophrypetalum odoratum*[73] exhibited cytotoxic and trypanocidal activities. It is also expected to have insecticidal activity owing to its strong activity in the brine shrimp

test.[73]

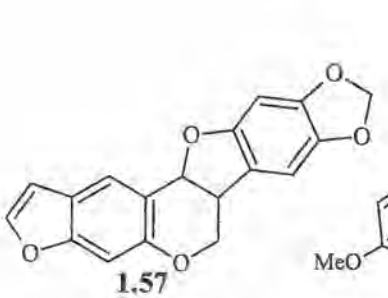


1.55

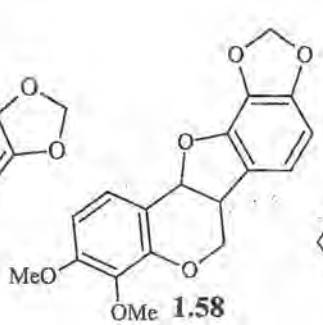


1.56

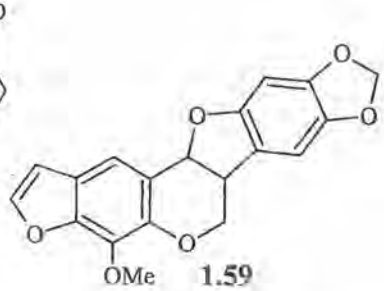
In the late 1990s several Tanzanian plant species were investigated at the Chemistry Department of the University of Dar es Salaam for their anti-mosquito properties. In these investigations several mosquitocidal compound were obtained from different plant species. Thus, *Neorautanenia mitis,* known as *Lidupala* by Hehe tribe in Iringa region, yielded four mosquitocidal flavonoids **1.57-1.60**. Compounds **1.57** and **1.58** were found to be active against larvae as well as adult *An. gambiae* mosquitoes, while the mixture of compounds **1.59** and **1.60** was found to be even more active, thus showing some kind of synergistic effects between the two compounds.[74,75] 2',3'-Epoxyasteranthine (**1.61**) and 2',3'-dihydroxyasteranthine (**1.62**) obtained from *Asteranthe lutea,* cleistenolide (**1.63**) from *Cleistochlamys kirkii* and the monoterpene espintanol (**1.64**) from *Uvaria scheffleri* also exhibited mosquitocidal activity.[76-78]



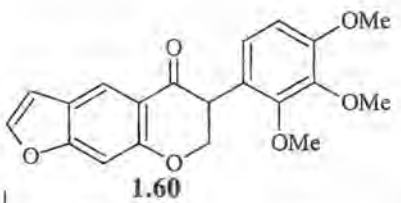
1.57



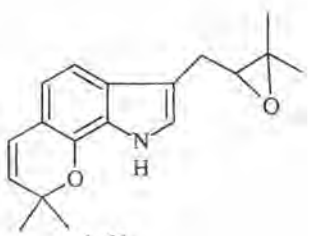
1.58



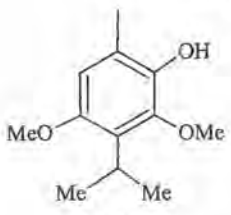
1.59



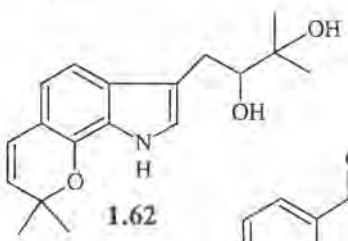
1.60



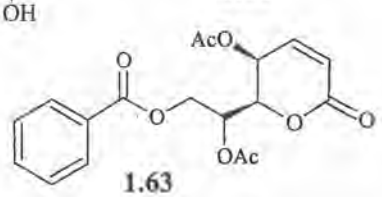
1.61



1.64

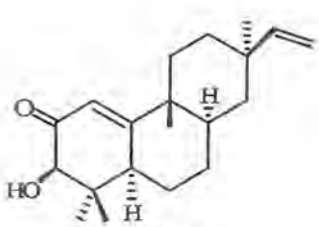


1.62

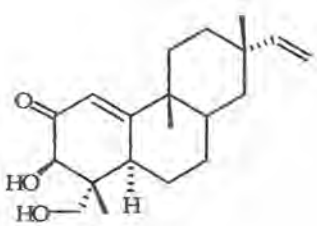


1.63

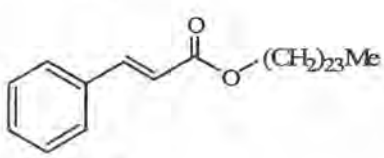
Phytochemical investigations of the dichloromethane and ethanol extracts of the cytotoxic root barks of *Hugonia castaneifolia* and *H. busseana* (Linaceae) yielded the rosane diterpenoids hugorosenone (**1.65**) and 18-hydroxyhugorosenone (**1.66**), and tetracosyl-(*E*)-ferrulate (**1.67**) and a sesquiterpenoid 4-methoxy-5,9-oxahimachal-9-ene (**1.68**), respectively that exhibited mosquito larvicidal activity against *An. gambiae* after 24 and 48 h exposure.[79-81]



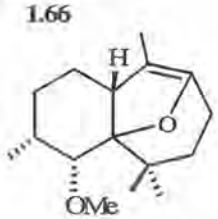
1.65



1.66



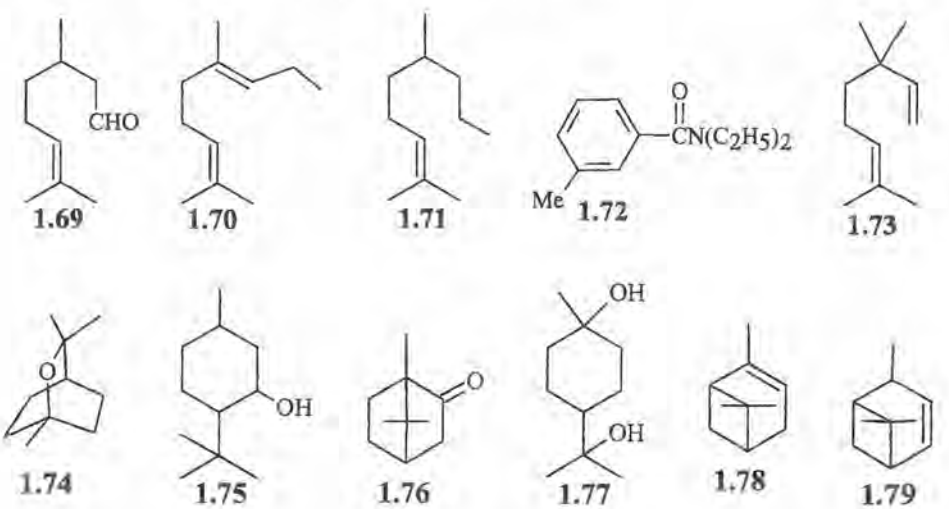
1.67



1.68

Essential oils from several plant species have been reported to have repellent activity, including citronella, verbena, pennyroyal, geranium, lavender, pine, cajeput, cinnamon, rosemary, basil, thyme, allspice, garlic, and peppermint.[82] Most of the

essential oils tend to give transient protection (2 hours).[83] Readily available plant-derived repellents include citronellal (**1.69**), which is the active ingredient of most natural (herbal) insect repellents. Citronella oil, which contains citronellal (**1.69**), geraniol (**1.70**) and citronellol (**1.71**), was originally extracted from lemon grass (*Cymbopogon nardus)* and shown to be an effective repellent with shorter protection time than DEET [*N,N*-diethyl-3-methylbenzamide (**1.72**)].[84] Citronellal-based commercial products include Natrapel®, Avon Skin-So-Soft® and Buzz Away®.[82] Citronella candles have also been promoted as effective mosquito repellents in the backyard.[84] Bite Blocker®, a plant-based repellent, is a combination of soybean oil, geranium oil and coconut oil in a formulation that has been available in Europe for many years.[85] Other essential oils that have been extracted from some plants with repellent activity include linalool (**1.73**), 1,8-cineole (**1.74**), *p*-menthane-3,8-diol (**1.75**), camphor (**1.76**), 1,8-menthanediol (**1.77**), α-pinene (**1.78**) and β-pinene (**1.79**).[86,87] Pyrethrins, which are known for their repellent and insecticide activity, have also been used in keeping away mosquitoes.[88]



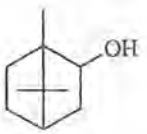
1.69 **1.70** **1.71** **1.72** **1.73**

1.74 **1.75** **1.76** **1.77** **1.78** **1.79**

Quwenling, from the waste distillate of lemon eucalyptus (*Eucalyptus maculata citriodon*) extract is a repellent against mosquitoes and has replaced dimethyl phthalate (DMP) in China.[86] The principal active component is *p*-menthane-3,8-diol (**1.75**).[89] It is used in a formulation which is principally 50% of compound **1.75** with additional isopugenol (**1.80**) and citronellol (**1.71**), the repellent effect of which is more persistent than citronella and nearly as effective as DEET.[90] Field tests of **1.80** against *Anopheles* spp. in Tanzania showed 6-7 h repellency, which was comparable with DEET.[91]

An essential oil extracted from the seeds of the neem tree, *Azadirachta indica* A. Juss (Meliaceae) is known for its insecticidal and repellent properties.[92,93] Thus, Neem oil formulation (2%) in coconut oil prevented bites by anopheline mosquitoes inside dwellings in some villages in India for 12 h.[94] Using a mixture of neem oil and kerosene in lamps has been shown to provide personal protection from the bites of some anopheline mosquitoes.[95,96] Neem oil contains several terpenoids, steroids, alkaloids, flavonoids and glycosides.[97] Neem extract contains non-volatile insecticidal compounds with the most active component being azadirachtin (**1.42**). The main disadvantage of neem extracts is their instability to light.[98]

Burning of certain herbs such as *Artemisia* and *Calamus* species is still practised in some villages in China to repel mosquitoes.[98] An essential oil of *Artemisia vulgaris* has linalool (**1.73**), camphor (**1.76**), borneol (**1.81**) and terpinen-4-ol (**1.82**) as the main repellent constituents. Terpinen-4-ol was found to be the most active and as effective as DMP.[99]



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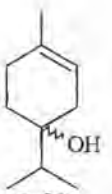


1.82

1.5 Objective of This Study

The understanding of the ecology and epidemiology of malaria and the availability of tools to combat it have changed dramatically in recent years. In Africa especially, the rapid spread of resistance first to chloroquine and now to sulfadoxine-pyrimethamine has greatly increased the cost and difficulty of management of malaria case management. Likewise, other major problems are absence of a viable malaria vaccine, vector resistance to insecticides, high and rapidly increasing costs of modern synthetic chemical insecticides, and environmental quality and effects of the insecticides on non-targeted organisms. Therefore, renewed emphasis must be given to find alternative malaria control methods, which can save the society from illness and the side effects of the currently used malaria control strategies. From **Section 1.3.1** it has been shown that there have been very successful past efforts for the control of malaria through the use of insecticides, which were coordinated and supported by the WHO. In this regard, it appears that reducing transmission by control of vector populations through chemical insecticides, repellents, screens such as bed nets, window gauzes and curtains, or environmental management may be more important and more useful than the currently emphasized malaria control strategies. Therefore insecticides, particularly plant based insecticides, have become an important part of this process. And as revealed in **Section 1.4,** plants are rich sources of phytochemicals that have varied biological applications, including

insecticidal use. Thus, it is an opportune time to look back and review the vast literature on plant species having active chemicals against mosquitoes and those used in traditional insect control techniques, as a foundation for developing integrated malaria management strategies. The general objective of investigations reported in this thesis, therefore, was to establish the mosquitocidal properties of the extracts and pure compounds from the selected plant species and to identify the mosquitocidal constituents that have the potential of being established as simple mosquito control agents. This would be achieved through carrying out different mosquitocidal bioassays (i.e larvividal, tarsal contact and repellent assays) for the crude extracts, and isolation processes in order to establish the phytochemical constituents of the studied plants, some of which would be active against larvae or adult mosquitoes, while these or others would have new chemical structures. The investigated plant species included *Annona squamosa, Asteranthe asterias, Croton sylvaticus, Hoslundia opposita, Lettowianthus stellatus, Polyalthia tanganyikensis, Tessmannia densiflora, T. martiniana* var *pauloi, T. martiniana* var *martiniana, Uvaria lungonyana, U. scheffleri, U. faulknerae, U. leptocladon, U. kirkii, Uvariodendron usambarense,* and *U. pycnophyllum*. These plants belong to the families Annonaceae and Leguminosae, which have already been indicated to contain phytochemicals having potential insecticidal and other biological properties.

CHAPTER TWO

ACTIVITY OF SOME TANZANIAN PLANT EXTRACTS AGAINST *ANOPHELES GAMBIAE* S.S GILES MOSQUITO LARVAE

Abstract

This Chapter reports on the larvicidal activity of sixteen Tanzanian plant species against the endophilic malaria vector, *Anopheles gambiae* s.s Giles, larvae. The crude extracts from the leaves, stem and root barks of *Annona squamosa, Asteranthe asterias, Croton sylvaticus, Hoslundia opposita, Lettowianthus stellatus, Polyalthia tanganyikensis, Tessmannia densiflora, T. martiniana* var *pauloi, T. martiniana* var *martiniana, Uvaria lungonyana, U. scheffleri, U. faulknerae, U. leptocladon, U. kirkii, Uvariodendron usambarense,* and *U. pycnophyllum* obtained by solvent extraction and then bioassayed following WHO protocols showed LC_{50} values ranging from 10 to 400 ppm after 24 h exposure. The most active extracts were those from the stem and root barks of *Annona squamosa, Uvaria faulknerae, U. kirkii* and *Uvariodendron pycnophyllum,* all of which had LC_{50} values between 10 and 100 ppm. Long term exposure beyond 24 h also showed more susceptibility of the larvae to the extracts. The most active extracts were fractionated by vacuum liquid chromatography and then bioassayed against the larvae for an exposure period beyond 120 h. Larvae deformities by formation of tailed tadpole like creatures were observed for the methanol extracts of *Tessmannia martiniana* var *pauloi*. The results suggest that the investigated plant extracts are promising as larvicides against *An. gambiae* s.s Giles mosquito and could be useful leads in the search for new and biodegradable botanical larvicidal natural products.

2.1 Introduction

For quite sometime now, chemicals have been commonly used for controlling mosquitoes in many parts of the world.[100,101] Initially, the use of these chemicals was for either repelling or killing. However, the emergence of mosquito resistance against conventional insecticides, together with public concerns over the safety and availability of the insecticides, have continued to necessitate the search for new alternative insect control agents that are environmentally acceptable. They should also be cost effective by requiring no sophisticated technology for their dispensation but giving excellent results.[102,103] Therefore interest in this field continues to increase since the latter category of insecticides are considered less deleterious to the environment.[104,105]

Five approaches have been considered in the selection of plant species for pharmacological and insecticidal screening. These include:

- Random screening which involves collection of all plant species from a particular study area.
- Phytochemical targeting aimed at the collection of all members of the plant's family known to be rich in bioactive compounds.
- Ethnobotanical survey that is based on traditional uses of the plant(s).
- Chemotaxonomic approach that is based on plants having similar constituents which may be in different families.
- Targeted screening of specific parts of a plant such as the seeds, barks, roots, leaves and other plant parts.

Among these approaches, it is generally acknowledged that sampling based on ethnobotanical approach gives a greater percentage yield of bioactive compounds

over the other methods, although targeted and random screening of plants and their extracts for activity have also yielded excellent results.[106, 107]

About 3000 plant species have been listed in the literature as having potential insecticidal and repellent value.[108] However, only a fraction of these species have been investigated chemically or biologically. Furthermore, many of these plant species are used in traditional practices for preventing mosquito bites. Therefore, these are considered to be appropriate candidates for the control of malaria transmitting mosquitoes. Again, most such plant species continue to remain uninvestigated and hence their bioactive constituents are not yet known.[109] Unfortunately, despite the above stated facts, the rapidly increasing human population continues to consume plant biota much more rapidly than it is being replaced. Therefore, based on such trend there is an imminent threat that most valuable plant species may soon become extinct without having harnessed their valuable chemical constituents. Meanwhile, remnants of such genetic resources continue to be preserved in forest reserves and national parks in some countries including Tanzania, a practice that needs to be continuously safeguarded through proper conservation management strategies. However, worldwide, about half of the original forests in developing countries are being replaced by cities and farms, or are destroyed through other human activities. A big proportional of the rain forests in Africa and other tropical regions have continued to undergo destruction through what is called "slash- and –burn" agricultural practices, in which forests are cleared (Fig. 2.1) for the land to be used for farming for a few years until soil nutrients are depleted, and then the land is abandoned for another "virgin" spot of forest. It is

estimated that under the current deforestation trend between 5 and 40 percent of all plant species may become extinct by the year 2020, and this will include many species that could be containing biologically useful compounds of incalculable value. It is presently estimated that about 20% of all tropical and semi- tropical plant species might have already been extinct between 1952 and 1992.[110] Indeed the depletion rate of genetic resources particularly in tropical virgin forests continues to prevail at an alarmingly high proportion, yet little is known about the medicinal and other values of most of the plant species in these ecosystems. It is in this respect that concerted efforts are urgently needed to survey, scientific analyse and document the bioactive plant flora of indigenous communities before disappearance of the former.[110]

Plants which are known to be efficacious and frequently prescribed in folklore medicines and insect management protocols and some of them indeed contain compounds that could be useful medicinal and/or insecticidal substances and as such would be potential candidates for further scientific investigations. This latter overview is the basis for carrying out investigations for larvicidal properties of sixteen plant species growing in Tanzania, belonging to the families Annonaceae,

(a)

(b)

Figure 2.1 Rain forest destruction. The remnants of slash- and – burn agriculture in Muheza, Tanga (a) stand in stark contrast to (b)an undisturbed rain forest in Amani Nature Reserve (Photo: C Kihampa)

Eurphobiaceae, Lamiaceae and Leguminosae. *Anopheles gambiae* s.s Giles mosquito larvae were specifically targeted in these investigations because the mosquitoes are important vectors for the transmission of malaria in East Africa. The screened plant species were *Uvaria lungonyana, U. scheffleri, U. faulknerae, U. leptocladon, U. kirkii, Uvariodendron usambarense, U. pycnophyllum, Annona squamosa, Lettowianthus stellatus, Polyalthia tanganyikensis, Asteranthe asterias, Tessmannia densiflora, T. martiniana* var *pauloi, T. martiniana* var *martiniana, Croton sylvaticus* and *Hoslundia opposita.*

2.2 MATERIALS AND METHODS

2.2.1 Selection and Collection of Plant Materials

Ethnobotanical information about plant species selected for these investigations was obtained through field interviews of either traditional healers or villagers, or through analysing ethnobotanical literature regarding anti-mosquito plant species (Scheme 2.1). Chemotaxonomic approach was also deployed especially for the plant species belonging to the families Annonaceae, Leguminosae and Euphorbiaceae. The stem and root barks of the investigated plant species (Table 2.1) were collected from Tanga, Dar es Salaam and Coast regions in Tanzania. The plant species were authenticated at the Herbarium of the Department of Botany, University of Dar es Salaam in Tanzania where voucher specimens are preserved.

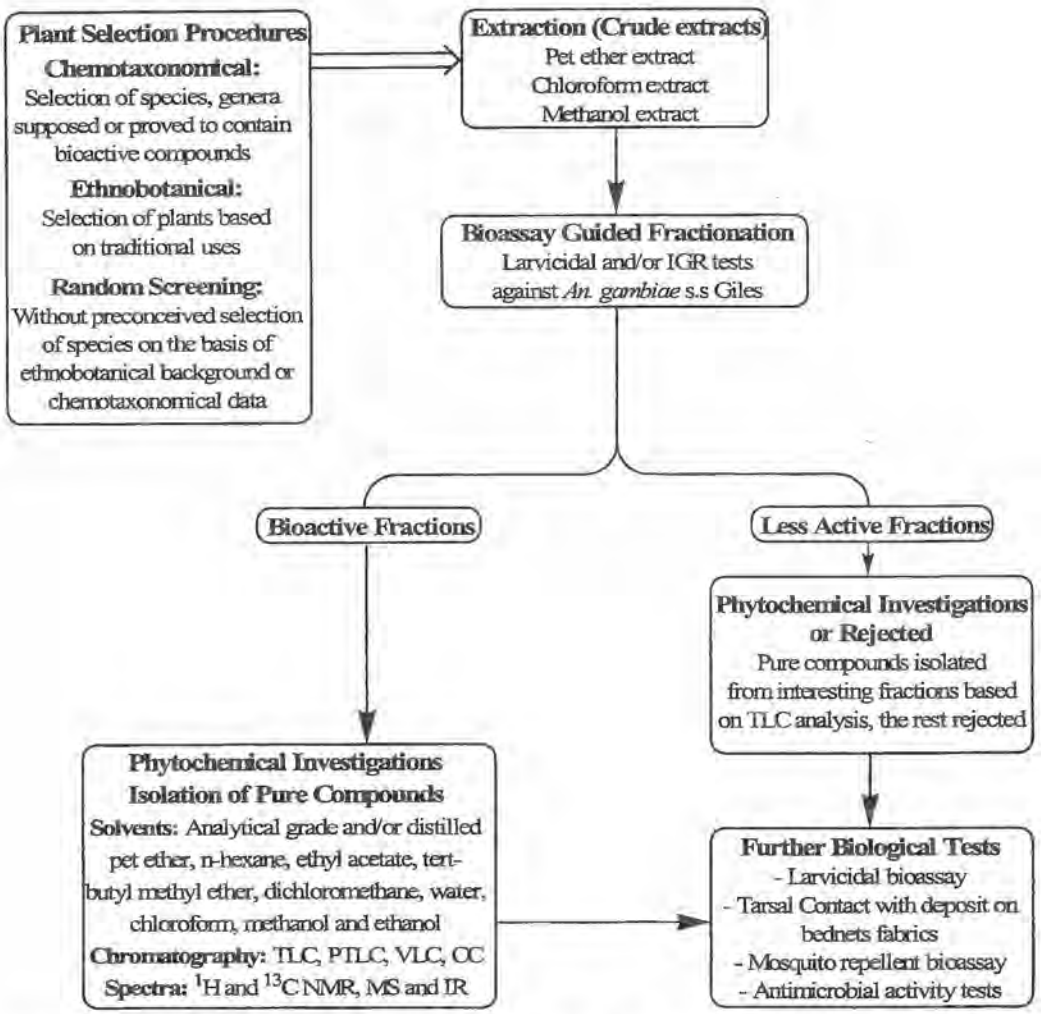
Table 2.1 Investigated Plant Species

Name of Plant	Family	Parts	Locality	Criteria for Selection
Uvaria lungonyana Vollesen	AN	SB, RB	Selous Game Reserve	*Family activity, **Endemic plant species
U. scheffleri Diels.	AN	SB, RB	Maramba, Muheza District	*Family activity
U. faulknerae Verdc	AN	SB, RB	Handeni, Pangani District	,,
U. leptocladon Oliv.	AN	SB	Korogwe District	,,
U. kirkii Hook. F.	AN	SB, RB	UDSM Main Campus	,,
Uvariodendron usambaranse Diels.	AN	SB, RB	Amani Nature Reserve	*Family activity
U. pycnophyllum (Diels) R.E Fr.	AN	SB, RB	Amani Nature Reserve	*Family activity, **Endemic plant species
Lettowianthus stellatus Diels	AN	SB	UDSM Main Campus (Planted)	*Family activity, Literature reports
Annona squamosa L.	AN	SB, RB	Kibanda village, Muheza district	*Family activity, Literature reports
Polyalthia tanganyikensis Vollesen	AN	RB	Kichi hills, Utete, Rufiji district	,,
Asteranthe asterias (S. Moore) Engl. & Diels	AN	LS, SB	Chalinze, Coast Region	Literature reports
Tessmannia densiflora Harms	LG	SB, RB	Selous Game Reserve	Family activity, **Endemic plant species
T. martiniana var *pauloi* Harms	LG	SB, RB	Pugu forest, Coast region	,,
T. martiniana var *martiniana* Harms	LG	SB, RB	Zaraninge Forest Reserve	,,
Croton sylvaticus L	EB	SB, RB	Selous Game Reserve	Family and genus activity
Hoslundia opposita Vahl	LM	LS, RB	UDSM Main Campus	Ethnobotanical information

AN = Annonaceae; LG = Leguminosae; EB = Euphorbiaceae; LM = Lamiaceae; SB = Stem Bark; RB = Root Bark; LS = Leaves; UDSM = University of Dar es Salaam

*The family Annonaceae is known to have compounds with pharmacological, insecticidal, antimicrobial and antiprotozoal activities

** The plant is endemic to Tanzania and neither chemical nor biological investigations have been carried out



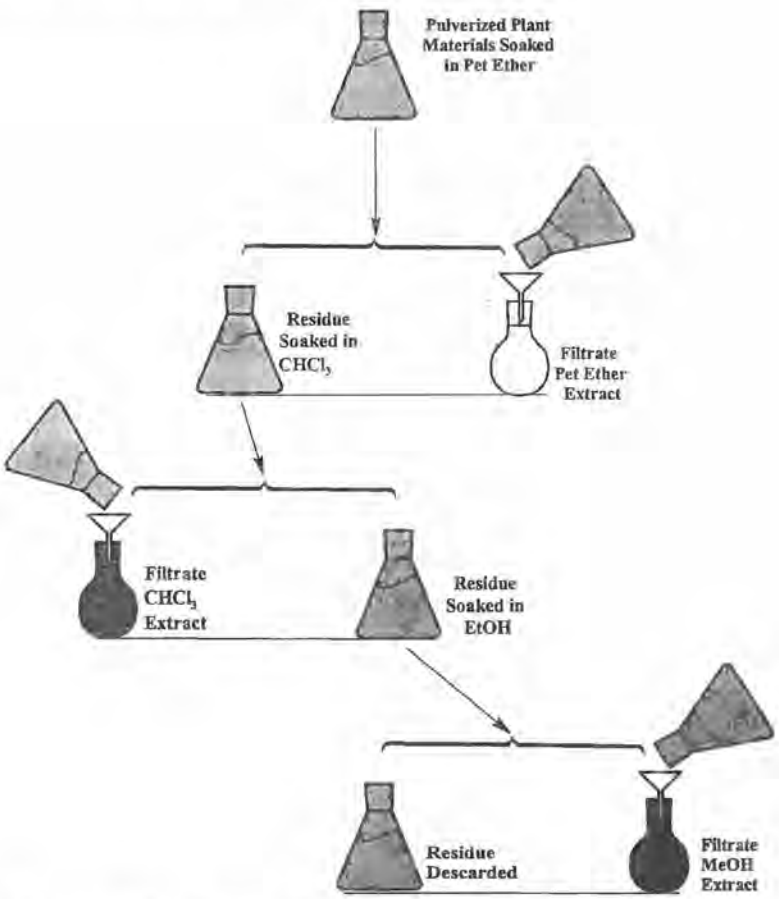
TLC = Thin layer chromatography; PTLC = Preparative thin layer chromatography; VLC = Vacuum liquid chromatography; CC = Column chromatography; IGR = Inhibition growth rate

Scheme 2.1 Plant Selection, Extraction, Isolation and Bioassay Procedures

2.2.2 Extraction

The plant materials (i.e. stem or root barks) were air-dried, pulverized and soaked sequentially in three solvents of increasing polarity (2 x 48 h for each solvent, Scheme 2.2) in the following order: Pet ether, chloroform and methanol. The extracts were concentrated under reduced pressure in a rotary evaporator while maintaining the water bath temperature below 40°C, in order to avoid decomposition of thermally

labile compounds. The resulting crude extracts were kept at -20 ^{o}C until bioassay and isolation processes were undertaken.



Scheme 2.2 Extraction Scheme for Crude Extracts

2.2.3 Mosquitocidal Bioassays

The bioassays were carried out at Amani Research Centre that belongs to the Tanzanian National Institute for Medical Research (NIMR), located in Muheza, Tanga Region, which has been active in malaria research since 1901.

2.2.3.1 Test Insects

Anopheles gambiae s.s Giles mosquito larvae were used in all the tests. To ensure a constant supply of the test insects, larvae at different instars used in the bioassays were reared in the laboratory at room temperature (28 ± 4 °C) and relative humidity of 80 ± 5 %. The larvae were fed with Tetramin® fish food and adults with 10% glucose solution soaked in cotton on petri dishes. In addition to glucose feeding, the female mosquitoes were regularly fed with rabbit blood twice a week.

2.3.3.2. Larvicidal and IGR Effects

Laboratory reared 3rd and/or early 4th instar larvae of *An. gambiae* s.s Giles mosquitoes were treated with aqueous solution at different concentrations. The test samples of the crude extracts from the sixteen plant species (Table 2.1) were prepared following standard WHO experimental protocols.[64] All experiments were conducted at room temperature (27-28 °C), and at different concentrations, *viz.* 0 (control), 7, 15, 32, 62, 125, 250 and 500 ppm in water, having beeen prepared from the stock solution obtained by dissolving the crude extract (250 mg) in dimethyl sulfoxide (DMSO, 10 ml) to make 25 mg/ml solution concentration. For each of the concentrations at least 3 replicates, each having 25 late 3rd and/or early 4th instar mosquito larvae in beakers fed with Tetramin® fish food were set. In control experiments only DMSO dissolved in water was used and then following the experimental procedure stated above. The mortality and deformities of the larvae was recorded after every 24 h of continuous exposure and expressed as percent mortality,[64,111] which was later analysed for toxicity and comparison was made using the WHO statistical package POLO PLUS.

2.3 RESULTS AND DISCUSSION

The results pertaining to susceptibility of the *An. gambiae* larvae to crude extract samples of the investigated plant species are presented in Table 2.2. Sixteen plant species belonging to six genera of the family Annonaceae, one genus each of the families Leguminosae, Lamiaceae and Euphorbiaceae were collected and investigated. The genus *Uvaria* was represented by five species, followed by the genera *Uvariodendron* and *Tessmannia* that had at least two species each.

All the crude extracts were effective against the late 3rd and/or early 4th instar *An. gambiae* s.s Giles larvae. The results showed that mortality of larvae increased with concentration of the samples. The same activity trend was also observed in the case of time elapsed mortality. The sluggish movement and perculiar coiling of treated larvae that was observed suggested that neural or mascular disturbance by some active principles could have taken place, which might have been the cause of the registered acute lethal effects. The delayed lethal effects related to the duration of exposure to the test samples may be considered more likely to be a result of disturbance of the endocrine mechanisms that regulate moulting and metamorphosis, as previously postulated for neem seed kernel extracts.[112]

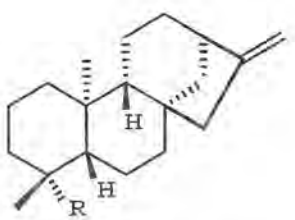
The larvae mortality of the crude extracts (Table 2.2) were recorded after every 24 h and the results were categorised as follows: 10-100 ppm = strong activity; 101-500 ppm = active; beyond 500 ppm = mild or weak activity. This criterion was used in the selection of the active extracts which were further separated by VLC (Tables 2.3-2.10) into simplified fractions and ultimate isolation of their constituents using different chromatographic techniques.

The crude pet ether, chloroform and methanol extracts of the root and stem barks of *Annona squamosa* were the most active among all the investigated plant species. The extracts displayed LC_{50} values of 44, 13, 21 and 50, 17, 24 ppm respectively, after 24 h exposure. TLC analysis of all the crude extracts, except the chloroform extract of the root bark revealed the presence of unstable complex mixtures of compounds which decomposed on TLC. Therefore, only the chloroform extract of the root bark which did not show decomposition on TLC was separated into VLC fractions and then each fraction was tested for larvicidal activity (Table 2.3). There was significant difference in activity between the crude extracts and VLC fractions after 24 h exposure (Table 2.2 and 2.3). The crude extract was the most active, the activity being comparable with that observed for VLC fractions 1-6 with the activity level increasing for VLC fraction 6.

In the literature no studies describing insecticidal activity of *A. squamosa* extracts against *An. gambiae* mosquitoes have been reported. However, literature reports indicate that annonaceous acetogenins are the most active insecticidal components of *A. squamosa*.[113] The acetogenins have been reported to have very potent bioactivities, such as *in vivo* antitumour, antimicrobial, antimalarial, pesticidal, antiparasitic, antihelmintic, cytotoxic, antifeedant and immunosuppressant activities.[113] For this reason a number of annonaceous acetogenins have been patented.[114]

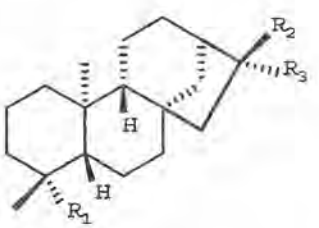
During investigations whose results are hereby being reported, acetogenins were neither isolated nor detected in the crude extracts of *A. squamosa*. This indicates that compounds other than acetogenins were responsible for the detected larvicidal

activity. Other classes of compounds known to occur in this plant species are *ent*-kaurane diterpenoids and flavonoids.[115,116] Some compounds of the former class are known to posses significant inhibition of HIV replication in H9 lymphocytic cells,[115] while the latter are known to have antimicrobial and pesticidal properties.[116] Thus, phytochemical analyses of the fruits of *Annona squamosa* yielded 14 known kaurane derivatives **2.1**-**2.25**. Compound **2.2** showed significant activity against HIV replication in H9 lymphocyte cells, having an EC_{50} value of 0.8 μg/ml.[115]



2.1: R = COOH

2.2: R = CH_2OH

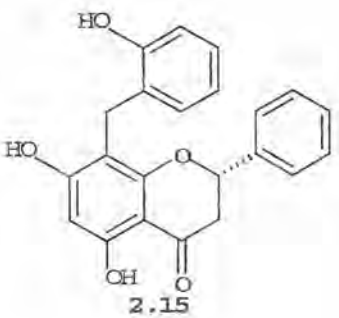


	R_1	R_2	R_3
2.3:	COOH	OH	CH_2OH
2.4:	CH_2OH	OH	CH_2OH
2.5:	COOH	H	CH_2OH
2.6:	COOH	CH_2OH	H
2.7:	COOH	CH_2OH	OH
2.8:	COOH	H	CH_2OAc
2.9:	CHO	H	COOH
2.10:	CHO	OH	CH_2OH
2.11:	CHO	H	CH_2OH
2.12:	CHO	OH	CH_2OAc
2.13:	OH	H	COOH
2.14:	OH	OH	CH_2OH

In the present investigations only *ent*-kaurane diterpenoids were isolated from the chloroform extract of the root bark. The isolation and larvicidal assay results of the isolated compounds are reported in Chapters 5 and 6. The absence of annonaceous acetogenins and flavonoids in *A. squamosa* as hereby being reported and which contradicts earlier reports suggests seasonal variations in the metabolism of such compounds by the plant species. Other reasons could be geographical variation or different in technique.

Extracts from plants of the genus *Uvaria* (Annonaceae) was represented by five species, namely *U. lungonyana, U. faulknerae, U. kirkii, U. scheffleri* and *U. leptocladon* displayed larvicidal activity at varying levels. Thus, the crude extracts from *Uvaria faulknerae* were among those that showed strong larvicidal activity, displaying LC_{50} values of 27, 24, 165 and 162, 33, 82 ppm after 24 h exposure for the petroleum ether, chloroform and methanol extracts of the stem and root barks, respectively (Table 2.2).

Although there are no literature reports on investigations for chemical constituents of *U. faulknerae,* a recently published M.Sc thesis at the University of Dar es Salaam indicate the isolation of the C-benzylflavanones chamanetin (**2.15**), dichamanetin (**2.16**), uvarinol (**2.17**) and isouvarinol (**2.18**) from the root and stem barks.[117] Furthermore, unreported investigations in the Chemistry Department at the University of Dar es Salaam indicate the presence of the cyclohexane epoxide (+)-β-senepoxide (**2.19**) in the root bark, besides chamanetin and dichamanetin. In the literature, compounds occurring in other *Uvaria* species are reported to have cytotoxic, antimicrobial as well as antimalarial activities.[118] However, it is difficult at this stage to conclude whether or not any of compounds **2.15–2.19** occurring in *U. faulknerae* or a combination thereof was responsible for the observed larvicidal activity of the crude extract.



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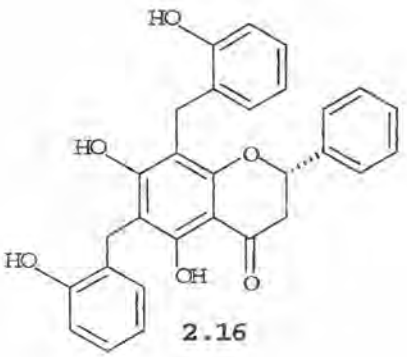

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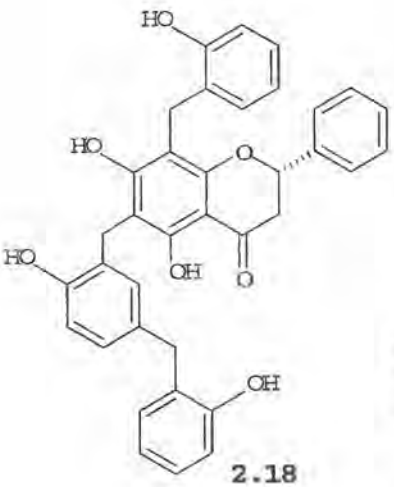

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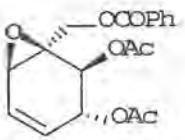

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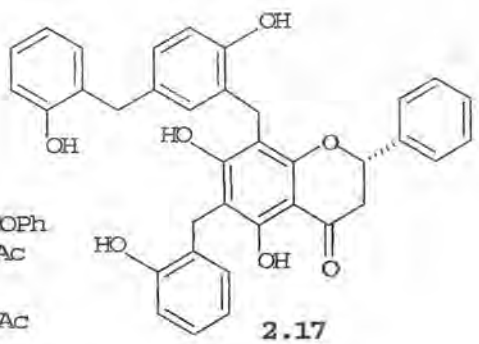


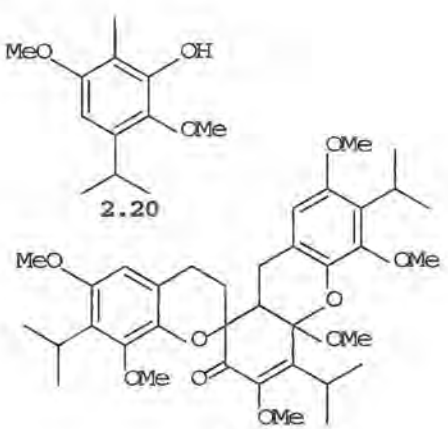
2.17

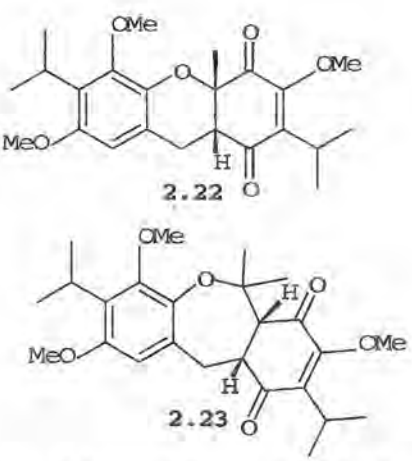
The crude extracts from the other three *Uvaria* species, namely *U. lungonyana, U. scheffleri* and *U. leptocladon* displayed almost similar activity results, with the exception of the chloroform extracts of the stem and root barks of *U. lungonyana* and *U. leptocladon* which showed stronger activity (LC_{50} value of 93 and 88 ppm after 24 h exposure, respectively). The other crude extracts displayed activity with LC_{50} values ranging between 100-500 ppm.

This is the first time for the crude extracts of *U. scheffleri* to have been assayed for activity against *An. gambiae* larvae. In Tanzania *U. scheffleri* is used as a traditional medicine for the treatment of fevers by local people in Tanga region.[119] Previous phytochemical investigations of the root bark yielded the aromatic monoterpene

espintanol (**2.20**) together with its apparent trimeric and dimeric derivatives schefflone (**2.21**), schefloquinone A (**2.22**) and schefloquinone B (**2.23**).[76] The compounds were tested against *An. gambiae* mosquito larvae and adults. Espintanol and schefflone displayed activity against the larvae, with schefflone being less active as compared to espintanol.[77] Espintanol also showed strong activity against adult mosquitoes while the two compounds also showed *in vitro* antifungal and antibacterial activity.[76,77]

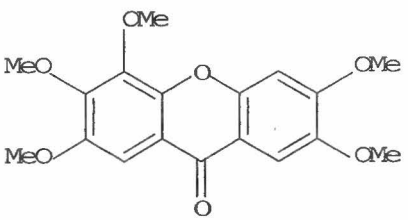
The low activity observed for the crude extracts of *U. scheffleri* in these investigations, could be due to the absence of the previously reported compounds in *U. scheffleri* materials hereby investigated as a result of seasonal and geographical variations.



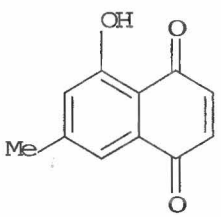


The crude extracts from *U. kirkii* also showed larvicidal activity (Table 2.2), displaying LC_{50} values below 100 ppm. This activity may be associated with the type of compounds known to be found in this plant species. In reported investigations which focused on the antitumour and antibacterial agents from some *Uvaria* species, six compounds including 1,2,3,4,6,7-hexamethoxyxanthone (**2.24**), 7-methyljuglone

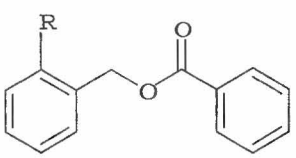
(**2.25**), benzyl benzoate (**2.26**), 2-methoxybenzyl benzoate (**2.27**) and diuvaretin (**2.28**) were isolated,[120] some of which like **2.27** and **2.28** are known to exhibit some insecticidal activities.[121]



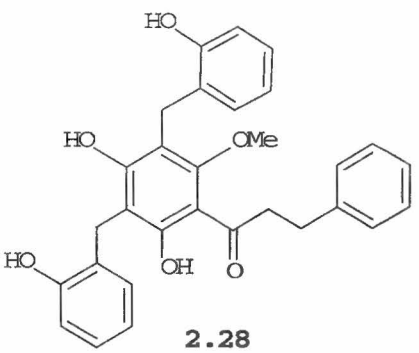
2.24



2.25

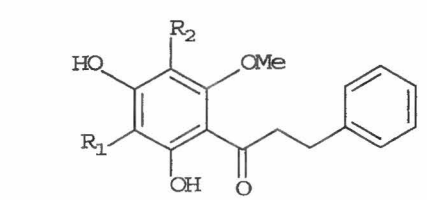


2.26: R = H
2.27: R = OMe

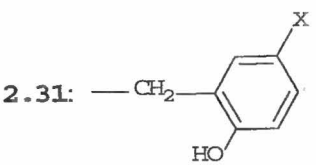


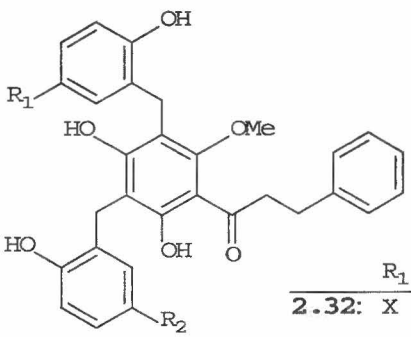
2.28

U. leptocladon is used in traditional medicine for the treatment of cerebral malaria.[122] In these reported investigations, the plant species was found to contain the C-benzylated dihydrochalcones uvaretin (**2.29**), isouvaretin (**2.30**), diuvaretin (**2.28**), angoluvarin (**2.31**), triuvaretin (**2.32**) and isotriuvaretin (**2.33**).[122] These compounds have been reported to exhibit different biological activities, including insecticidal, larvicidal, antimicrobial, antitumour, antimycotic and antiprotozoal activities.[123] The larvicidal activities observed in investigations whose results are hereby being reported could be associated with the compounds mentioned above. This is because compounds having antitumour and insecticidal activity have been reported to elicit similar mode of cell action by blocking the cellular oxygen transport system.[124]

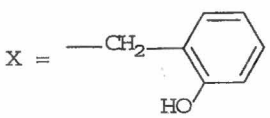


	R_1	R_2
2.29:	X	H
2.30:	H	X





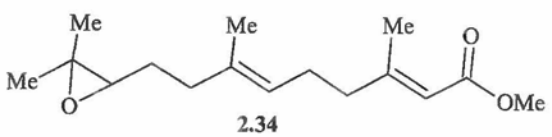
	R_1	R_2
2.32:	X	H
2.33:	H	X



U. lungonyana is endemic to Tanzania[125] and so far neither chemical nor biological investigations have been reported for this plant species. Despite the crude extracts of the plant species displaying moderate activity, it was considered reasonable to select it for further analysis so as to isolate the active constituents because of its restricted occurrence. Table 2.2 shows activity of the crude chloroform extract of the root bark while Table 2.4 displays results for VLC fractions in which there is enhanced activity for the VLC fractions as compared to the crude extracts. The high activity for the VLC fractions appeared to be concentrated in the least polar and medium polar compounds. The isolation and biological activity of the isolated compounds is discussed in Chapters 5 and 6 of this Thesis.

The crude pet ether, chloroform and methanol extracts from *L. stellatus* displayed larvicidal activity at varying levels, having LC_{50} values of 93, 256 and 355 ppm after 24 h exposure, respectively. Previous phytochemical investigations of *L. stellatus* indicated the crude extracts from the stem and root barks to have *in vitro* antimalarial activity, as well as weak toxicity against brine shrimp (BST) larvae.[126,127] Among the compounds isolated from the extracts was insect juvenile hormone III (**2.34**) which

was previously isolated from this and other plant species.[126,127] The occurrence of insect juvenile hormone III in plants has been quite intriguing since normally the compound is metabolised by insects in order to regulate their developmental processes (metamorphosis). Therefore, the compound metabolised by plants may have similar roles, suggesting that the plants would be producing the compound in order to deter insect accumulation since insects would not prefer to acquire additional JH III doses beyond what is required for metabolism. Accumulation of this compound beyond biochemically allowable levels would disrupt the insects' development process, thereby the compound acting as a bio-insecticide. The presence of this compound in plants would make the extracts deployable as environmentally friendly larvicides as the constituents are readily biodegradable.[126,127] However, during these investigations no sign of growth disruption was noticed, either in the larvae or adult stage of the insect. This could have been attributed to either small amount of the compound present in the extract or due to absence of the compound in the investigated extract as a result of seasonal and/or geographical location.

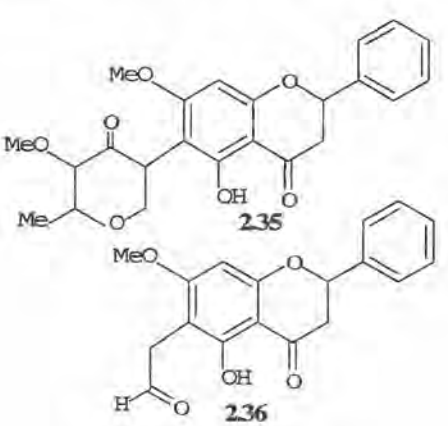


2.34

Crude extracts from *H. opposita* also showed some larvicidal activity, having LC_{50} values in the concentration range of 100-500 ppm. In the previous studies such extracts showed no larvicidal activity.[76] These inconsistencies in activity may be attributable to seasonal fluctuations in the biosynthesis of the active components by the plant. They may also be related to differences in extraction methods, photosensitivity of some of the compounds in the extract or geographical origin of

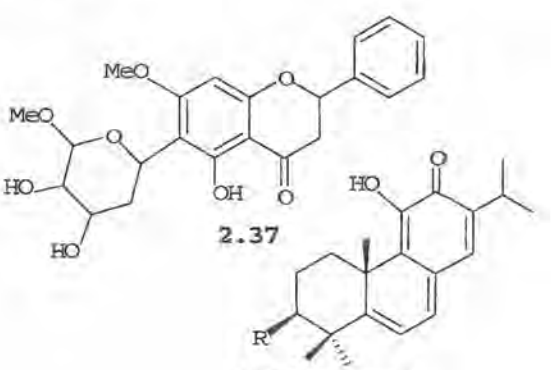
the plant. Apparently, in previous studies *H. opposita* plant materials were collected from Kwamngumi forest reserve in Muheza District, while in the present studies the materials were collected from around the Main Campus of UDSM. Therefore, the origin of the plant materials and therefore difference in chemical composition could be the cause for the difference in larvicidal activity stated above, although seasonal variations could also be a factor. However, the two assumptions were not pursued further for verification in these investigations.

In previous studies, the twigs of *H. opposita* collected from Cameroon were reported to contain the flavanones hoslundin (**2.35**), hoslundal (**2.36**) and hoslunddiol (**2.37**) while the root bark extract of plant materials from Tanzania were found to contain 3-*O*-benzoyl or 3-*O*-cinnamoylabiatane diterpenoids such as **2.38** and **2.39**.[128] Some of such compounds are known to exhibit antitumour, insect antifeedant, antimicrobial and allelochemical activites.[129] Indeed, since the physiological effects of the crude extracts on the tested larvae were not investigated, it could have been quite possible that the observed larval toxicity was due to such effects being exerted by some of the constituent compounds acting as allelochemicals.



2.35

2.36



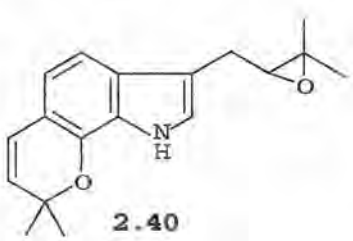
2.37

2.38: R = benzoyl

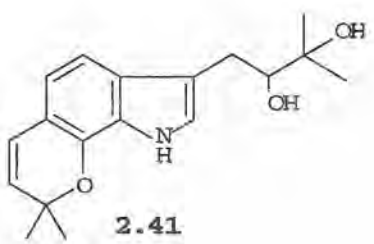
2.39: R = Cinnamoyl

Crude extracts from *Asteranthe asterias* exhibited weak larvicidal activity, having LC_{50} values in the concentration range of 200-500 ppm after 24 h exposure, for both leaves and stem bark extracts. Previous phytochemical studies on the stem and root barks of *A. asterias* showed the presence of the diprenylated indoles 2',3'-epoxyasteranthine (**2.40**) and 2',3'-dihydroxyasteranthine (**2.41**) that showed antimycotic activity against *Saprolegnia* and *Rhizoctonia* species.[130] The crude stem and root bark extracts were found to be active against *Plasmodium falciparum* and *Trypanosoma brucei rhodesiense* malaria and trypanosomiasis parasites respectively.[130]

This is the first time for the crude extracts from *A. asterias* to be subjected to larvicidal assays. However, in reported mosquitocidal investigation compounds **2.40** and **2.41** isolated from another *Asteranthe* species, namely *A. lutea* displayed strong larvicidal activity against *An. gambiae* larvae.[76] Therefore, the larvicidal activity of the crude extracts observed for *A. asterias* could be associated with the above mentioned compounds.



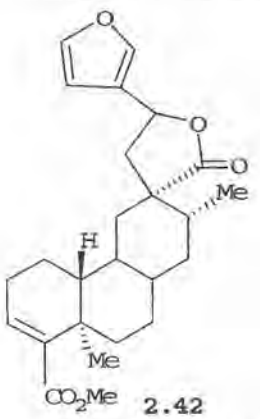
2.40



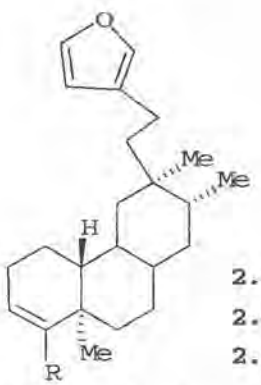
2.41

Crude extracts from *Croton sylvaticus* displayed larvicidal activity, having LC_{50} value in the concentration range of 100-300 ppm after 24 h exposure, which is a fairly good activity. In the literature the genus *Croton* is well known to constitute toxic plant species. Thus, *Croton* species are used in Africa as sources of poison for hunting and fishing.[130] Previous phytochemical work on the genus *Croton* indicated the presence of cleorodane diterpenes, such as hardwickic (**2.42**) and 3,4-*seco*-

trachybanoic acid (**2.43-2.45**) isolated from the extract of the root bark of *Croton sonderianus*.[131] Compounds **2.43-2.45** displayed strong antimicrobial activity.[131] Therefore, it is quite possible that similar compounds could have been responsible for the observed larvicidal activity.



2.42



2.43: R=CO_2H
2.44: R=CO_2Me
2.45: R=CH_2OH

Some of the plant species included in these investigations were chosen because they are threatened species. Plant species in this category also belonged to families or genera that are known to constitute members having insecticidal properties.[108,109] Such plant species included *Uvariodendron pycnophyllum* that occur in the Amani Nature Reserve in Tanga region. Other plant species in this category were those belonging to the genus *Tessmannia*, which consists of four species that are endemic to Tanzania, namely *Tessmannia densiflora* Verd., *T. martiniana* var *pauloi* Harms, *T. martiniana* var *martiniana* Harms and *T. burtii* Harms.[132] However, *T. burtii* Harms was not investigated due to time constraint and difficulty in accessing it. On the other hand, only very few trees of *T. martiniana* var *pauloi* Harms were found in Pugu Forest, whose depletion is taking place rather quickly due to charcoal production, and uncontrolled harvesting of firewood and building poles for the ready Dar es Salaam market. Therefore, there is no doubt that within the coming few years the plant species may disappear if the current trend in depletion of Pugu forest continues unabaited.

Table 2.2 Activity of Crude Extracts Against 3rd/4th Instar Larvae of *Anopheles gambiae* s.s Giles After 24 h Exposure (ppm)

Botanical Name	Plant Part	PE		CH		ME	
		LC_{50}	95% CL	LC_{50}	95% CL	LC_{50}	95% CL
Uvaria lungonyana	SB	ne	ne	245	185-337	373	260-781
	RB	ne	ne	93	22-155	161	116–227
U. scheffleri	SB	130	84–189	224	150-355	250	153-545
	RB	209	135–339	363	243–880	164	104–252
U. faulknerae	SB	162	109-239	33	23-48	82	61-111
	RB	27	17-46	24	17-34	165	122–222
U. leptocladon	SB	153	98–228	88	26–142	393	263–1085
U. kirkii	SB	48	34–66	52	38–75	70	52–97
	RB	76	51–113	95	64–139	129	83–209
Uvariadendron usambarense	SB	188	137-262	188	29-318	357	258-554
	RB	439	334–707	150	106–207	494	394-775
U. pycnophyllum	SB	ne	ne	56	34–87	109	66–192
	RB	ne	ne	56	34 – 86	56	35–86
Lettowianthus stellatus	SB	93	65–127	256	149–708	355	265–500
Annona squamosa	SB	50	38–67	17	9–25	24	10–40
	RB	44	29–66	13	8–18	21	38-48
Polyalthia tanganyikensis	RB	96	69–132	133	90–199	70	50–100
Asteranthe asterias	LS	444	319-898	267	186-384	494	394-775
	SB	238	150 – 349	439	334 – 707	294	220–405
Tessmannia densiflora	SB	ne	ne	104	60 – 150	192	130–285
	RB	ne	ne	162	113–232	383	243–402
T. martiniana var *pauloi*	SB	ne	ne	83	40 – 120	122	82 – 173
	RB	ne	ne	ne	ne	114	44–186
T. martiniana var *martiniana*	SB	ne	ne	256	149–708	353	213–402
	RB	ne	ne	204	133–340	148	74–254
Croton sylvaticus	SB	246	195–372	232	170–342	238	184–354
	RB	110	76–157	163	117–232	164	115-239
Hoslundia opposita	LS	171	6–288	369	257–659	191	69–301
	RB	375	276–583	439	334–707	368	276-537

SB = Stem bark, RB = Root bark, LS = Leaves, PE = Pet ether, CH = Chloroform, ME = Methanol, ne = not extracted

So far no information about the medicinal uses of plant species of the genus *Tessmannia* have been reported in the literature. Furthermore, even local people did not report any traditional uses of *Tessmannia* species as recorded in interviews during plant collection expeditions.

Tables 2.5-2.9 give larvicidal results for VLC fractions of the active extracts from the three *Tessmannia* species (*T. densiflora, T. martiniana* var *martiniana* and *T. martiana* var *pauloi*). The results show that there is no significant difference in activity between the crude extracts of the three species compared to their respective VLC fractions. Thus, activities for the crude extracts ranged between 101-500 ppm which was the same range of activity for VLC fractions. The activity increased with longer exposure time. Interesting was the larvae deformities caused by VLC fractions 3 and 4 of the crude *T. martiniana* var *pauloi* root bark methanol extract, while the two VLC fractions were least active (Table 2.3, Fig. 2.3) after 24 h exposure. VLC fraction 3 exhibited 50% mortality at the highest concentration of 500 ppm and no activity was observed at lower concentrations. On the other hand VLC fraction 4 exhibited 40 and 10% mortality at 500 and 250 ppm, respectively. However, like VLC fraction 3, it showed no activity at lower concentration after 24 h exposure. Furthermore, tail-like structural abnormalities for the larvae appeared after 24 h exposure. The tail-like structures began to emerge after 24 h of exposure, and continued to grow until reaching their peak after 48 h, where the length of the larvae was equal to the length of the tail-like structures. The larvae also attained dark brown colouration and shaded during 48-72 h post exposure. Microscopic analysis

suggested the tail-like structures to be part of the gut that had been elongated and extruded through the anal cavity.

The larvae that shaded the tail and continued to survive were reared and monitored through their life cycle until adulthood. The morphological features of the emerged adults were normal as those from the control experiments. The males and females were allowed to mate and then fed with human blood. However, the females could not produce any batch of eggs. This observation suggest that the larvae deformities could have interfered with the reproduction system in the adult mosquitoes. This process was repeated until the entire mosquito colony died.

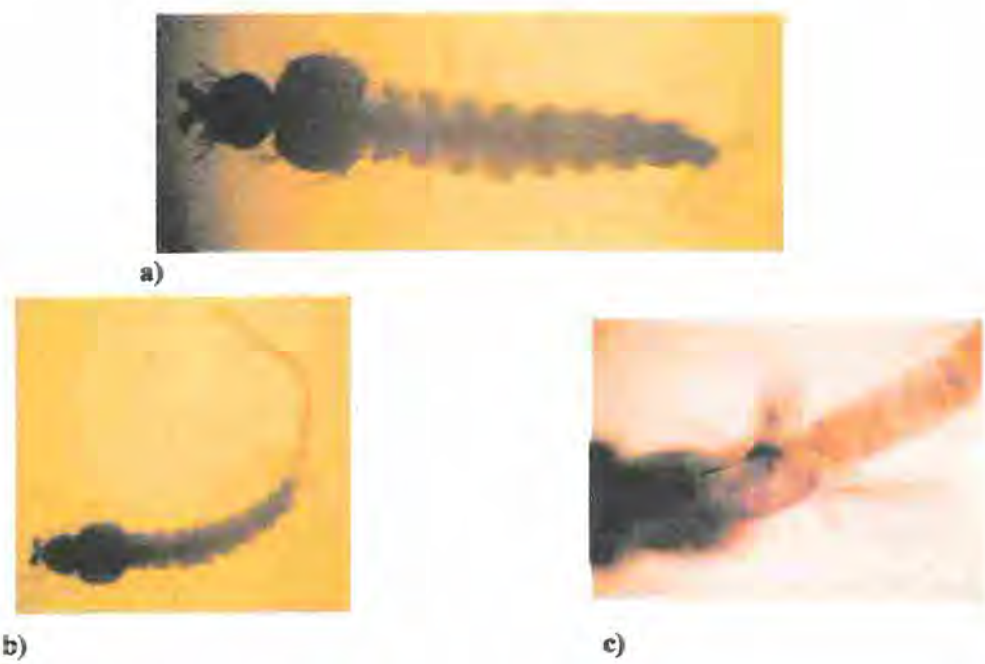
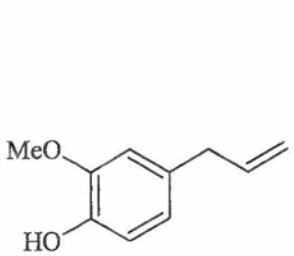


Fig. 2.3 Larvae Deformities due to VLC Fractions 3 and 4 of *T. martiniana* var *pauloi* Root Bark Methanol Extract

a) Normal larvae b) Tailed larvae and c) Closer look of larvae abdomen with tail connected

Crude extracts from *U. pycnophyllum* and *U. usambaranse* displayed significant difference in larvicidal activity (Table 2.2). The former plant species showed higher activity than the latter, displaying LC_{50} values of 56 and 109, and 56 and 56 ppm after 24 h exposure for the chloroform and methanol extracts of the stem and root barks, respectively. So far there are no any investigations describing the larvicidal or any biological activity for either of the two plant species. However, phytochemical investigations already done on other members of the genus *Uvariodendron*, namely *U. gorgonis* from Tanzania and *U. connivens* that is found in Nigeria and Cameroon revealed the presence of the phenylpropanoids eugenol (**2.46**), 3,4-dimethoxycinnamylalcohol (**2.47**), dehydrodieugenol (**2.48**), elemicin (**2.49**), 3,4,5-trimethoxycinnamaldehyde (**2.50**) and 3,4,5-trimethoxycinnamylalcohol (**2.51**).[133,134] The phenylpropanoid type of compounds are known to be toxic and most of them have been documented to be insecticidal, fungicidal, nematocidal and bacteriocidal.[133,134]

Since *U. pycnophyllum* displayed strong activity among other investigated extracts, it was further subjected to VLC fractionation. Table 2.10 shows that there was significant difference between the activities of the VLC fractions and the respective crude chloroform extract of the stem bark. Detailed phytochemical results for the *U. pycnophyllum* extracts and larvicidal properties of the isolated compounds are discussed in Chapters 5 and 6 of this Thesis.



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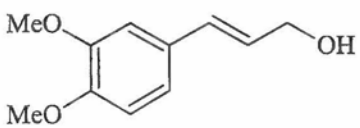
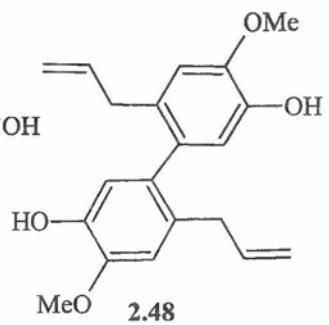


2.47



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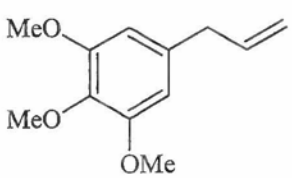

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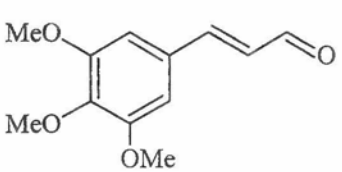
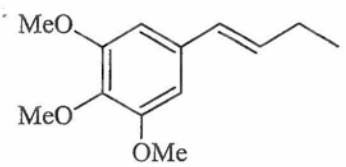


2.50



2.51

2.4 CONCLUSION

From the results discussed in this Chapter, it can be concluded that all the plant species tested displayed activity against *An. gambiae* larvae at different concentration levels. Some of the plant extracts showed high toxicity to the larvae. In other cases the larvae developed deformities and impairment of colony sustainability as the female adults that had emerged from deformed larvae failed to lay eggs. This indicates the potential of the studied plant extracts towards eventual elimination of malaria mosquito colonies. Furthermore, for some of the tested extracts the high rates of larval mortality observed at lower concentrations after long-term exposure (beyond 24 h) indicated a useful residual effect of the extracts on the tested larvae, a factor which again suggested the potential of the extracts in malaria mosquito control endeavours. Therefore, the results have given indicative clues on the insecticidal potency of the traditional use of plant species in the crude form. This may be attractive since at community level in Africa isolation of the

active compounds might be expensive and technically not feasible, and hence the crude extracts could be employed as such in larviciding without requiring isolation of active constituents.

Table 2.3 Activity of the Chloroform VLC Fractions of *Annona squamosa* Root Bark Against 3rd/4th Instar Larvae of *An. gambiae* s.s Giles

VLC	Time (h)	Percentage mortality				LC_{50} (ppm)	95% CL
		62.5	125	250	500		
1	24	0 ± 0	6.7 ± 3.3	50 ± 5.7	93.3 ± 3.3	292	221 - 392
	48	16.7 ± 3.3	40 ± 5.7	86.7 ± 3.3	100 ± 0	155	88 - 237
	72	20 ± 0	40 ± 0	100 ± 0	100 ± 0	126	88 - 185
2	24	0 ± 0	6.7 ± 3.3	30 ± 5.7	70 ± 5.7	385	293 - 359
	48	10 ± 0	20 ± 0	53.3 ± 3.3	96.7 ± 3.3	267	186 - 384
	72	13.3 ± 3.3	26.7 ± 3.3	83.3 ± 3.3	100 ± 0	174	124 - 249
3	24	3.3 ± 3.3	0 ± 0	0 ± 0	26.7 ± 17.6		
	48	56.7 ± 6.7	70 ± 0	80 ± 5.7	100 ± 0		
	72	76.7 ± 3.3	100 ± 0	100 ± 0	100 ± 0		
4	24	36.7 ± 3.3	50 ± 5.7	83.3 ± 3.3	100 ± 0		
	48	100 ± 0	100 ± 0	100 ± 0	100 ± 0		
	72	100 ± 0	100 ± 0	100 ± 0	100 ± 0		
5	24	20 ± 0	50 ± 5.7	60 ± 5.7	100 ± 0	157	70 - 246
	48	30 ± 0	80 ± 5.7	90 ± 0	100 ± 0		
	72	50 ± 0	80 ± 0	100 ± 0	100 ± 0		
6	24	100 ± 0	100 ± 0	100 ± 0	100 ± 0		
	48	100 ± 0	100 ± 0	100 ± 0	100 ± 0		
	72	100 ± 0	100 ± 0	100 ± 0	100 ± 0		

Table 2.4 Activity of the Chloroform VLC Fractions of *Uvaria lungonyana* Root Bark Against 3rd/4th Instar Larvae of *An. gambiae* s.s Giles

VLC	Time (h)	Percentage mortality					LC_{50} (ppm)	95% CL
		7.5	15.6	31.2	62.5	125		
1	24	35 ± 5	70 ± 10	85 ± 5	90 ± 0	100 ± 0		
	48	46.7 ± 3.3	80 ± 5.7	93.3 ± 3.3	100 ± 0	100 ± 0		
	72	50 ± 0	83.3 ± 3.3	100 ± 0	100 ± 0	100 ± 0		
2	24	6.7 ± 3.3	36.7 ± 3.3	73.3 ± 6.7	86.7 ± 3.3	90 ± 0	40	13 - 68
	48	10 ± 0	40 ± 5.7	70 ± 5.7	100 ± 0	100 ± 0	21	14 - 32
	72	20 ± 0	66.7 ± 3.3	90 ± 0	100 ± 0	100 ± 0	14	6 - 21
3	24		6.7 ± 3.3	20 ± 0	50 ± 0	90 ± 0	32	22 - 46
	48		6.7 ± 3.3	30 ± 0	86.7 ± 3.3	100 ± 0	21	15 - 30
	72		6.7 ± 3.3	46.7 ± 3.3	96.7 ± 3.3	100 ± 0	17	11 - 23
4	24	0 ± 0	0 ± 0	0 ± 0	93.3 ± 3.3	93.3 ± 3.3		
	48	20 ± 0	43.3 ± 3.3	60 ± 0	100 ± 0	100 ± 0	22	12 - 34
	72	50 ± 0	60 ± 0	70 ± 5.7	100 ± 0	100 ± 0		
5	24		0 ± 0	40 ± 0	80 ± 0	90 ± 0		
	48		40 ± 0	80 ± 11.5	100 ± 0	100 ± 0		
	72		80 ± 11.5	80 ± 11.5	100 ± 0	100 ± 0		
6	24	0 ± 0	0 ± 0	6.7 ± 3.3	20 ± 0	30 ± 0	408	307-629
	48	0 ± 0	0 ± 0	0 ± 0	50 ± 11.5	100 ± 0		
	72	0 ± 0	0 ± 0	0 ± 0	60 ± 11.5	100 ± 0		

Table 2.5 Activity of the Chloroform VLC Fractions of *Tessmannia densiflora* Root Bark Against 3rd/4th Instar Larvae of *An. gambiae* s.s Giles

VLC	Time (h)	Percentage mortality					LC_{50} (ppm)	95% CL
		31.2	62.5	125	250	500		
1	24	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0		
	48	0 ± 0	23.3 ± 3.3	23.3 ± 3.3	50 ± 0	80 ± 5.7	229	146 - 454
	72	20 ± 0	46.7 ± 3.3	80 ± 0	90 ± 0	100 ± 0	67	40 - 100
2	24	0 ± 0	0 ± 0	30 ± 0	50 ± 0	70 ± 0	265	176 - 493
	48	0 ± 0	36.7 ± 3.3	63.3 ± 3.3	83.3 ± 3.3	100 ± 0	99	65 - 145
	72	60 ± 5.7	66.7 ± 3.3	86.7 ± 3.3	100 ± 0	100 ± 0	37	10 - 61
3	24	0 ± 0	20 ± 0	50 ± 0	80 ± 0	100 ± 0	122	85 - 175
	48	3.3 ± 3.3	76.7 ± 3.3	83.3 ± 3.3	100 ± 0	100 ± 0	45	21 - 67
	72	3.3 ± 3.3	80 ± 0	86.7 ± 6.7	100 ± 0	100 ± 0	40	19 - 58
4	24	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0		
	48	0 ± 0	0 ± 0	0 ± 0	0 ± 0	3.3 ± 3.3		
	72	0 ± 0	0 ± 0	0 ± 0	6.7 ± 3.3	10 ± 10		
5	24	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0		
	48	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0		
	72	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0		
6	24	0 ± 0	0 ± 0	0 ± 0	0 ± 0	30 ± 10		
	48	0 ± 0	0 ± 0	10 ± 0	43.30 ± 3.3	73.3 ± 3.3	321	222 - 585
	72	6.7 ± 3.3	6.7 ± 3.3	30 ± 0	50 ± 5.7	90 ± 5.7	177	113 - 311

Table 2.6 Activity of the Methanol VLC Fractions of *Tessmannia martiniana* var *pauloi* Root Bark Against 3rd/4th Instar Larvae of *An. gambiae* s.s Giles

VLC	Time (h)	Percentage mortality					LC_{50} (ppm)	95% CL
		31.2	62.5	125	250	500		
1	24	0 ± 0	0 ± 0	0 ± 0	10 ± 5.7	50 ± 5.7	493	395 - 747
	48	0 ± 0	10 ± 0	20 ± 0	30 ± 5.7	100 ± 0	266	203 - 378
	72	0 ± 0	13.3 ± 3.3	50 ± 0	93.3 ± 3.3	100 ± 0	143	108 - 195
2	24	0 ± 0	0 ± 0	0 ± 0	0 ± 0	50 ± 5.7		
	48	0 ± 0	0 ± 0	3.3 ± 3.3	10 ± 5.7	90 ± 5.7	340	268 - 446
	72	0 ± 0	0 ± 0	40 ± 5.7	70 ± 5.7	100 ± 0		
3	24	0 ± 0	0 ± 0	0 ± 0	0 ± 0	50 ± 5.7		
	48	0 ± 0	13.3 ± 3.3	20 ± 0	33.3 ± 3.3	73.3 ± 3.3	352	258 - 545
	72	30 ± 5.7	30 ± 0	30 ± 5.7	86.7 ± 3.3	100 ± 0	140	81 - 225
4	24	0 ± 0	0 ± 0	0 ± 0	10 ± 5.7	40 ± 5.7		
	48	0 ± 0	0 ± 0	20 ± 0	30 ± 0	50 ± 0	459	334 - 870
	72	10 ± 0	50 ± 5.7	70 ± 5.7	96.7 ± 3.3	100 ± 0	93	36 - 147

Table 2.7 Activity of the Chloroform VLC Fractions for *Tessmannia martiniana* var *pauloi* Stem Bark Against 3rd/4th Instar Larvae of *An. gambiae* s.s Giles

VLC	Time (h)	Percentage mortality					LC_{50} (ppm)	95% CL
		31.2	62.5	125	250	500		
1	24	0 ± 0	0 ± 0	3.3 ± 3.3	60 ± 5.7	96.7 ± 3.3	308	240 - 408
	48	0 ± 0	10 ± 0	30 ± 5.7	70 ± 5.7	100 ± 0	182	136 - 263
	72	0 ± 0	13.3 ± 3.3	40 ± 5.7	93.3 ± 3.3	100 ± 0	143	108 - 195
2	24	0 ± 0	6.7 ± 6.7	6.7 ± 6.7	6.7 ± 6.7	53.3 ± 18.5	402	314 - 562
	48	0 ± 0	16.7 ± 3.3	26.7 ± 8.8	36.7 ± 14.5	93.3 ± 3.3	252	178 - 369
	72	20 ± 0	30 ± 0	40 ± 5.7	70 ± 5.7	100 ± 0	154	88 - 248
3	24	0 ± 0	0 ± 0	3.3 ± 3.3	10 ± 5.7	40 ± 0		
	48	0 ± 0	0 ± 0	13.3 ± 3.3	70 ± 5.7	100 ± 0	205	162 - 274
	72	0 ± 0	13.3 ± 3.3	20 ± 0	83.3 ± 3.3	100 ± 0	183	142 - 250
4	24	0 ± 0	0 ± 0	6.7 ± 3.3	16.7 ± 8.9	50 ± 0	481	363 - 826
	48	10 ± 0	10 ± 0	10 ± 0	30 ± 0	90 ± 5.7	307	229 - 440
	72	10 ± 0	10 ± 0	30 ± 0	33.3 ± 3.3	100 ± 0	183	142 - 250
5	24	0 ± 0	13.3 ± 3.3	50 ± 5.7	83.3 ± 8.8	100 ± 0	152	108 - 219
	48	0 ± 0	40 ± 0	76.7 ± 3.3	96.7 ± 3.3	100 ± 0	131	90 - 190
	72	0 ± 0	76.7 ± 3.3	90 ± 5.7	100 ± 0	100 ± 0	72	51 - 99
6	24	0 ± 0	0 ± 0	3.3 ± 3.3	13.3 ± 8.8	76.7 ± 8.8	354	277 - 476
	48	10 ± 0	3.3 ± 3.3	6.6 ± 3.3	13.3 ± 8.8	90 ± 10	288	216 - 402
	72	0 ± 0	13 ± 3.3	40 ± 5.7	86.7 ± 3.3	100 ± 0	101	4 - 176

Table 2.8 Activity of the Chloroform VLC Fractions for *Tessmannia martiniana* var *martiniana* Root Bark Against 3rd/4th Instar Larvae of *An. gambiae* s.s Giles

VLC	Time (h)	Percentage mortality					LC_{50} (ppm)	95% CL
		31.2	62.5	125	250	500		
1	24	0 ± 0	10 ± 5.7	13.3 ± 3.3	23.3 ± 3.3	50 ± 5.7	493	353 - 1030
	48	26.7 ± 3.3	73.3 ± 6.7	73.3 ± 8.8	83.3 ± 3.3	86.7 ± 3.3		
	72	63.3 ± 3.3	83.3 ± 3.3	86.7 ± 6.7	96.7 ± 3.3	100 ± 0		
2	24	0 ± 0	0 ± 0	23.3 ± 3.3	60 ± 0	80 ± 5.7	312	236 - 431
	48	6.7 ± 3.3	20 ± 0	70 ± 5.7	80 ± 5.7	96.7 ± 3.3	155	106 - 233
	72	30 ± 5.7	76.7 ± 6.7	86.7 ± 3.3	100 ± 0	100 ± 0		
3	24	0 ± 0	10 ± 0	20 ± 5.7	43.3 ± 3.3	73.3 ± 3.3	352	258 - 545
	48	0 ± 0	43.3 ± 3.3	80 ± 5.7	90 ± 5.7	100 ± 0		
	72	0 ± 0	80 ± 5.7	100 ± 0	100 ± 0	100 ± 0		
4	24	10 ± 0	13.3 ± 3.3	30 ± 5.7	43.3 ± 3.3	100 ± 0	235	170 - 346
	48	50 ± 0	50 ± 0	100 ± 0	100 ± 0	100 ± 0		
	72	53.3 ± 3.3	86.7 ± 3.3	100 ± 0	100 ± 0	100 ± 0		

Table 2.9 Activity of the Chloroform VLC Fractions for *Tessmannia martiniana* var *martiniana* Stem Bark Against 3rd/4th Instar Larvae of *An. gambiae* s.s Giles

VLC	Time (h)	Percentage mortality					LC_{50} (ppm)	95% CL
		31.2	62.5	125	250	500		
1	24	0 ± 0	0 ± 0	0 ± 0	80 ± 5.7	100 ± 0		
	48	26.7 ± 3.3	70± 5.7	86.7 ± 3.3	96.7 ± 3.3	100 ± o		
	72	60 ± 0	86.7 ± 3.3	100 ± 0	100 ± 0	100 ± 0		
2	24	0 ± 0	0 ± 0	0 ± 0	0 ± 0	83.3 ± 6.7		
	48	6.7 ± 3.3	13.3 ± 6.7	43.3 ± 3.3	86.7 ± 3.3	96.7 ± 3.3	249	178 - 359
	72	23.3 ± 3.3	40 ± 5.7	63.3 ± 3.3	100 ± 0	100 ± 0	89	50 - 141
3	24	0 ± 0	3.3 ± 3.3	20 ± 5.7	70 ± 5.7	96.7 ± 3.3	272	203 - 380
	48	0 ± 0	33.3 ± 3.3	60 ± 5.7	96.7 ± 3.3	100 ± 0	131	90 - 190
	72	0 ± 0	50 ± 5.7	76.7 ± 3.3	100 ± 0	100 ± 0	99	77 - 135
4	24	0 ± 0	10 ± 0	10 ± 0	23.3 ± 3.3	93.3 ± 3.3	330	255 - 453
	48	0 ± 0	20 ± 5.7	30 ± 0	100 ± 0	100 ± 0	133	102 - 194
	72	6.6 ± 3.3	33.3 ± 3.3	56.7 ± 8.8	100 ± 0	100 ± 0	99	69 - 155

Table 2.10 Activity of the Chloroform VLC Fractions of *Uvariodendron pycnophyllum* Stem Bark Against 3rd/4th Instar Larvae of *An. gambiae* s.s Giles

VLC	Time (h)	Percentage mortality				LC_{50} (ppm)	95% CL
		62.5	125	250	500		
1	24	20 ± 0	40 ± 5.7	73.3 ± 3.3	100 ± 0	167	91 - 261
	48	53.3 ± 3.3	86.7 ± 3.3	96.7 ± 3.3	100 ± 0		
	72	66.7 ± 6.7	100 ± 0	100 ± 0	100 ± 0		
2	24	0 ± 0	6.7 ± 3.3	20 ± 0	23.3 ± 3.3		
	48	0 ± 0	16.7 ± 3.3	56.7 ± 3.3	96.7 ± 3.3		
	72	30 ± 0	43.3 ± 8.8	70 ± 5.7	100 ± 0		
3	24	3.3 ± 3.3	100 ± 0	100 ± 0	100 ± 0		
	48	33.3 ± 3.3	100 ± 0	100 ± 0	100 ± 0		
	72	50 ± 5.7	100 ± 0	100 ± 0	100 ± 0	154	56 - 247
4	24	0 ± 0	0 ± 0	20 ± 5.7	93.3 ± 3.3	337	266 - 433
	48	0 ± 0	0 ± 0	26.7 ± 3.3	100 ± 0		
	72	10 ± 0	10 ± 0	53.3 ± 3.3	100 ± 0	242	181 - 352
5	24	0 ± 0	0 ± 0	0 ± 0	36.7 ± 3.3		
	48	0 ± 0	0 ± 0	0 ± 0	46.7 ± 3.3		
	72	0 ± 0	0 ± 0	0 ± 0	83.3 ± 3.3		

CHAPTER THREE

NOVEL *NOR*–HALIMANE DITERPENES, *ISO*COUMARINS, AND OTHER CONSTITUENTS OF *TESSMANNIA DENSIFLORA*

Abstract

This chapter describes the isolation, structural determination of constituents of larvicidal extracts of *Tessmannia densiflora* Harms (Leguminosae, sub family Caesalpinioideae) that is endemic to Tanzania. Two diterpenoids distereomeric to the previously isolated halimane diterpenoids *seco*-chiliotrin and methyl *seco*-chiliotrinoate, and 2-methylpropyl- and 1-methylbutyl-tessmannoate were obtained from the larvicidal chloroform extracts of the root and stem barks. A new isocoumarin, chlotessmin, together with a known isocoumarin 8-hydroxy -6-methoxy-3-pentylisocoumarin and other compounds were isolated from the chloroform extract of the stem bark, and the structures and stereochemistry of the isolated compounds were established on the basis of spectroscopic data. The diterpenoids are regarded as halimane derivatives derived from oxidoreductive cleavage of the less substituted cyclohexane ring.

3.1 INTRODUCTION

Tessmannia is one of about 700 genera of the Leguminosae family (sub family Caesalpinioideae). The family comprises herbs, shrubs and trees that occur in a variety of habitats, including aquatic, xerophytes and climbing plants. Approximately 700 genera and 1700 species of Leguminosae have a cosmopolitan distribution in the tropical, subtropical and temperate zones, where they provide many articles of food, fodder, dyes, gums, resins and oils. Over 140 species of Leguminosae genera are grown domestically as ornamental plants.[135,136] A number of Leguminosae species are also poisonous and most of their seeds may contain a cyanogenetic glycoside that produces hydrocyanic acid.[136]

The genus *Tessmannia* consists of 11 species which are either small or big trees, being mainly found in the rain forest regions of Central and West Africa. In Tanzania only four *Tessmannia* species are known, which iclude *Tessmannia densiflora* Harms, *T. martiniana* var *pauloi* Harms, *T. martiniana* var *martiniana* Harms and *T. burttii* Harms. All the four species are endemic and have not been phytochemically investigated.

Tessmannia densiflora Harms (*Mundeu* in *Kindengeleko*, Fig. 3.1) grows up to 14 m tall and is known to occur only at Kichi Hills Forest on the edge of the Selous Game Reserve in Rufiji District, Coast Region. The plant materials for the reported investigations were found abundantly at the above mentioned area. Neither bioactivity studies are reported nor traditional uses are known for this plant species. Furthermore, neither medicinal nor insecticidal uses were mentioned upon interviewing local communities during field work in these studies. However, the

plant species was included in investigations whose results are hereby being reported because it belongs to the Leguminosae family that is known to constitute plant species having insecticidal properties.

Fig. 3.1 Aerial Parts of *Tessmannia densiflora* Harms (Photo by Stephen Samwel, PhD student in the Chemistry Department at the University of Dar es Salaam)

3.2 RESULTS AND DISCUSSION

3.2.1 Phytochemical Results

The pulverized air-dried stem and root barks of *Tessmannia densiflora* Harms were sequentially extracted with chloroform and methanol. The resulting extracts were then assayed for activity against *An. gambiae* s.s. Giles mosquito larvae as reported in Chapter 2. The chloroform extract displayed strong larvicidal activity (Chapter 2). Therefore, it was decided to carry out detailed phytochemical analysis for the active chloroform extracts.

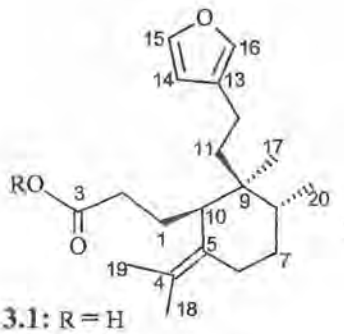
Partitioning of the chloroform extract from the root bark by vacuum liquid chromatography (VLC) and then bioassaying the resulting fractions, yielded non-

polar inactive fractions which also consisted of minor constituents as complex mixtures (TLC). The more polar fractions were also inactive and in addition they showed extensive decomposition on TLC. Therefore, it was decided not to work them up any further.

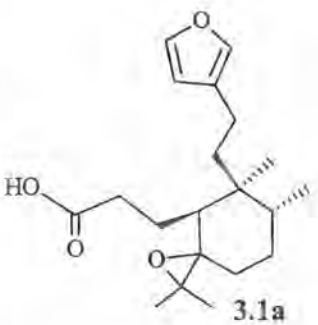
The medium polar VLC fractions were the ones that exhibited larvicidal activity and these were therefore combined and TLC analysis of the combined fractions indicated the presence of only two major compounds which were ultimately isolated as the larvicidal constituents as discussed here below.

3.2.1.1 The *nor*-Halimane Diterpenoids Tessmannic acid (3.1) and Methyltessmannoate (3.2)

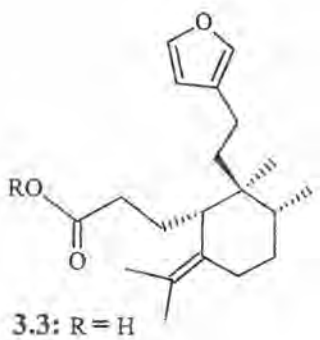
Repeated chromatography of the combined VLC fractions that showed larvicidal activity yielded two compounds as oils whose structures **3.1** and **3.2** were identified based on spectroscopic data, representing *nor*-halimane diterpenoids **3.1** and **3.2** similar to *seco*-chiliotrin (**3.3**) and its methyl ester **3.4** that were previously reported from *Chilliotrichium rosmarinifolium* Less (Compositae = Asteraceae).[137] However, the fact that the reported compounds **3.3** and **3.4** had negative optical rotation values as opposed to the positive values and different magnitudes observed for the isolated compounds indicated that the two sets of compounds **3.1** and **3.2**, and **3.3** and **3.4** were diastereomeric, as it will be discussed later in this section.



3.1: R = H
3.2: R = Me



3.1a



3.3: R = H
3.4: R = Me

Structures **3.1** and **3.2** for the isolated compounds were established based on analysis of spectral data (IR, ^{1}H and ^{13}C NMR, and mass spectra), which closely resembled those previously reported for methyl *seco*-chiliotrinoate **3.4**.[137] Thus, the MS of compound **3.1** exhibited the molecular ion peak at *m/z* 318, which corresponds to the molecular formula $C_{20}H_{30}O_3$. The presence of the mono-substituted furanoid unit was indicated by the appearance of ^{1}H NMR signals at δ 6.23 (1H, d, J = 0.9 Hz), 7.17 (1H, dd, J = 1.5, 0.9 Hz) and 7.32 (1H, dd, J = 1.8, 1.5) whose small ^{2}J values (< 2 Hz) was consistent with a furanoid proton coupling constant.[138] The ^{1}H NMR spectrum also consisted of signals due to a quaternary and a secondary methyl group (δ 0.97, 3H, s and 0.79, 3H, d, J = 6.8 Hz), and a vinyl gem-dimethyl unit (δ 1.68, 3H, s and 1.69, 3H, s). These are in conformity with structure **3.1**.

In the low field region the ^{13}C NMR spectrum consisted of signals due to the furanoid C-atoms and two other unsaturated carbons, the latter being due to the fully substituted C atoms of the exocyclic double bond in **3.1**. The spectrum also consisted of the lowest field signal at δ 179.9, this indicating the presence of a carboxylic group in **3.1**, as further corroborated by the IR spectrum (ν_{CO} = 1708 cm^{-1}).

Both the ^{1}H and ^{13}C NMR spectra of compound **3.1** exhibited signals due to two saturated methine groups (δ_H = 1.79, s and 2.67, dd, J = 11.3, 4.0 Hz; δ_C = 36.4 and 44.1), which on the basis of H/H COSY and C/H HMBC interactions were found to correspond to the C-8 and C-10 methine units, respectively. The ^{13}C NMR spectrum in addition consisted of signals due to six methylene C–atoms and a saturated quaternary carbon, whose assignment in structure **3.1** was facilitated by considering the single bond and long range H/C interactions observed in the HMQC and HMBC plots (Figs. 3.8 and 3.9) respectively. The latter interactions as well as H/H correlations observed in the COSY plot enabled complete assignment of structure **3.1** for the isolated diterpenoid, the spatial arrangement of substituents being derived from H/H interactions observed in the NOESY spectrum (Fig. 3.7), and on biogenetic considerations as discussed later.[139]

The MS of compound **3.2** exhibited the molecular ion peak at *m/z* 332, which corresponds to the formula $C_{21}H_{32}O_3$. Except for the appearance of resonances due to a methoxy moiety (δ_H = 3.64, s and δ_C = 51.4 ppm) and the upfield shift of the carboxylic carbon resonance (δ_{CO} = 174.8 ppm), the ^{1}H and ^{13}C NMR spectral features were very similar to those observed for compound **3.1**. This indicated that the two compounds **3.1** and **3.2** consisted of the same basic skeleton, except that **3.2** was a methyl ester of the metabolite **3.1**. Therefore, this indicated that unlike in the previous investigations where the methyl ester of the acid **3.3** was prepared as an *ex-situ* derivative, the carboxylic acid **3.1** occurs in *Tessmannia densiflora* together with its methyl ester, both as natural products.

Contrary to the previously reported compounds **3.3** and **3.4** whose optical rotation values had a negative signal, both compounds **3.1** and **3.2** exhibited optical rotation values with positive signals and the magnitudes being different from those reported for **3.3** and **3.4**. This suggested that compounds **3.1** and **3.3**, and **3.2** and **3.4** constituted sets of diastereomers. Indeed, close analysis of the ^{1}H and ^{13}C NMR spectra of **3.1** and **3.2** versus those reported for the diterpenoid **3.4** indicated dinstictive differences with respect to resonances of some nuclei of the cyclohexyl skeleton and some of those adjacent to the cyclohexyl ring. The major differences were observed for the H-7, H-8 and H-10, and C-6, C-10 and C-11 signals (Tables 3.1 and 3.2). It was therefore conceived that the differences were due to stereochemical factors. Thus, considering the signs and magnitudes of the optical rotation values discussed above, it was concluded that the isolated compounds **3.1** and **3.2** were diastereomers of the previously reported diterpenoids **3.3** and **3.4** respectively.

Besides the M^{+} and $[M+1]^{+}$ peaks at *m/z* 318 and 333 for compounds **3.1** and **3.2** respectively, the MS (Fig. 3.11 and 3.12) also exhibited other prominent ion peaks as shown in Schemes 3.2 and 3.3, all of which were consistent with structures **3.1** and **3.2** respectively. The MS of compound **3.1** also indicated a peak at *m/z* 334 which was due to the epoxide **3.1a** that was present as a minor impurity since both the ^{1}H and ^{13}C NMR spectra did not exhibit any signals for that compound. Epoxide **3.1a** could have been formed through air oxidation of **3.1** during storage.

Biogenetically, the diterpenoids **3.1–3.4** would be envisioned to be formed *via* the *ent*-halimane skeleton such as in **3.5**, through base induced cleavage of ring A

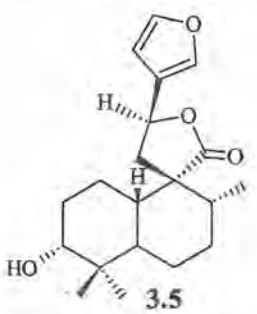
followed by protonation, as shown in Scheme 1. This process would retain the stereochemical integrity at the two chiral centres C-8 and C-9, which, apparently is the biogenetic structural feature of halimane diterpenoids.[139] However, the new chiral centre at C-10 would possess either the stereochemistry shown in the isolated metabolites **3.1** and **3.2**, or that in the previously reported compounds **3.3** and **3.4**. Indeed, this stereochemical difference would explain the observed disparities in the chemical shift of H-7, H-8 and H-10, and C-6, C-10 and C-11 between compounds **3.2** and **3.4** (Tables 3.1 and 3.2) as discussed above.

The spatial disposition of H-10 and the C-9 methyl group being on the same side of the cyclohexane ring was deduced from H/H interactions observed in the NOESY spectrum (Fig. 3.7). The lower field position of the H-10 signal in compound **3.1** and **3.2** relative to the chemical shift values reported for the diterpenoid **3.4**[140] indicated a near coplanarity disposition of H-10 and the C-5 exocyclic double bond, resulting into the availability of a suitable obital overlap situation that would cause the deshielding effect.[140] This indicated that H-10 occupied an equatorial position in compounds **3.1** and **3.2**, while it had an axial configuration in the diterpenoids **3.3** and **3.4**.

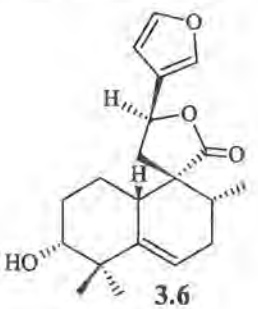
The fact that the previously reported compounds **3.3** and **3.4** were isolated from the family Asteraceae (Compositae) and not the presently investigated family (Leguminosae) might be the reason for the stereochemical differences of the two pairs of compounds. Thus, it would be envisioned that the corresponding plant species possessed specific enzymes for the biosynthesis of metabolites having specific stereochemical configurations.[139]

It has been observed that the nomenclature of the previously reported carboxylic acid **3.3** is somewhat awkward, since it was named against normal practice of adding "acid" at the end of the name. This made it difficult to generate a generic name for the methyl ester **3.4**. Moreover, the use of the prefix "*seco*" would have been properly applied if the compounds were derived from the corresponding name of the diterpenoid skeleton. Therefore, it has been decided to give different trivial names of the isolated compounds **3.1** and **3.2**, instead of basing them on the names of the previously isolated compounds **3.3** and **3.4**. The isolated compounds have been given the trivial names tessmannic acid (**3.1**) and methyltessmannoate (**3.2**).

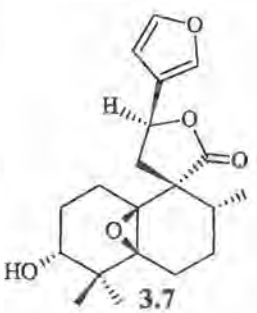
Besides the *nor*-halimanes **3.3** and **3.4** the aerial parts of *Chilliotrichium rosmarinifolium* were also reported to have yielded the halimanolides **3.5-3.9** and the *nor*-halimanolides **3.10** and **3.11**.[137] However, so far all these compounds as well as **3.3** and **3.4** have not been assayed for any biological activity.



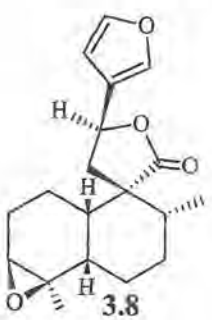
3.5



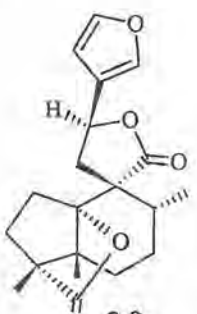
3.6



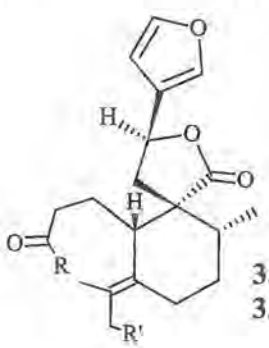
3.7



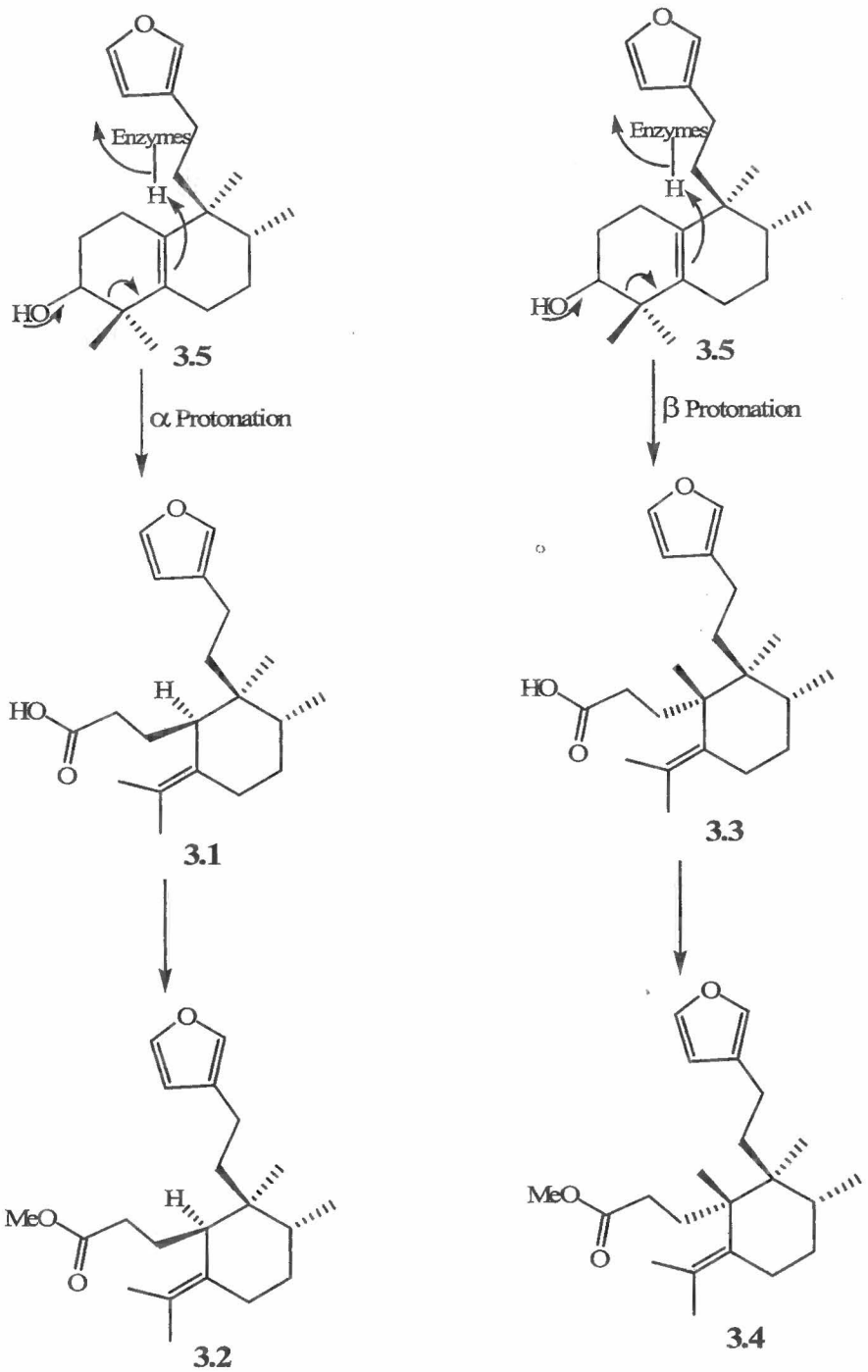
3.8



3.9



	R	R'
3.10:	H	H
3.11:	OH	H



Scheme 1 Biosynthetic Pathway of Diterpenoids **3.1–3.4**

Table 3.1 ^{1}H NMR Spectral Data for Tessmannic acid (3.1) and Methyltessmannoate (3.2)

H	Observed				Reported[137]	
	$\delta_{3.1}$	*J* (Hz)	$\delta_{3.2}$	*J* (Hz)	$\delta_{3.4}$	*J* (Hz)
1 a	1.84	m	1.84	m	1.96	dddd, 13, 9.5, 6.5, 3
1 b	1.79	m	1.84	m	1.79	ddd, 13, 12.5, 9, 5.5
2 a	2.21	m	2.18	m	2.21	ddd, 16
2 b	2.15	m	2.11	m	2.09	ddd, 16
6 a	1.80	m	1.80	m	1.98	m, 4
6 b	2.48	m	2.46	d, 12.6	2.34	m, 4
7 a	1.24	m	1.20	m	1.66	m, 13.5, 4
7 b	1.41	m	1.41		1.79	dddd, 13.5, 4, 4
8	1.79	m	1.80	m	1.52	ddq, 4
10	2.67	dd, 11.3, 4.0	2.64	d, 11.5	2.35	dd, 12.5, 3
11 a	1.50	m	1.51	m	1. 58	m
11 b	1.42	m	1.41	m	1. 42	m
12	2.30	m	2.30	br d, 8.01	2.28	br dd
14	6.23	d, 0.9	6.21	s	6.21	br s
15	7.32	dd, 1.8, 1.5	7.33	s	7.31	dd
16	7.17	dd, 1.5, 0.9	7.17	s	7.15	br s
17	0.97	s	0.90	s	0.97	s
18	1.68*		1.66*	d, 1.7	1.60	d
19	1.69*	s	1.68*	br s	1.68	br s
20	0.79	d, 6.8	0.78	d, 6.9	1.03	d
OMe	-	-	3.64	s	3.63	s

* interchangeable assignments

Table 3.2 ^{13}C NMR Spectral Data for Tessmannic acid (3.1) and Methyltessmannoate (3.2)

C	Observed		Reported	C	Observed		Reported[137]
	$\delta_{3.1}$	$\delta_{3.2}$	$\delta_{3.4}$		$\delta_{3.1}$	$\delta_{3.2}$	$\delta_{3.4}$
1	22.5	24.7	25.2	11	32.8	32.4	42.0
2	32.2	32.2	33.3	12	19.5	19.3	19.6
3	179.9	174.8	180.0	13	125.1	1246	124.8
4	126.6	126.2	125.8	14	111.4	111.0	111.0
5	131.0	130.8	131.2	15	142.8	142.6	142.5
6	25.0	24	20.8	16	138.7	138.4	138.3
7	31.2	30.9	29.5	17	24.0	22.4	21.0
8	36.4	36.1	35.6	18	20.2*	20.2*	20.7
9	39.4	39.2	39.9	19	20.9*	20.7*	20.3
10	44.1	43.8	46.1	20	15.6	15.5	17.2
				OMe	-	51.4	

* interchangeable assignments

Fig. 3.1 ^{1}H NMR Spectrum of Tessmannic acid (3.1)

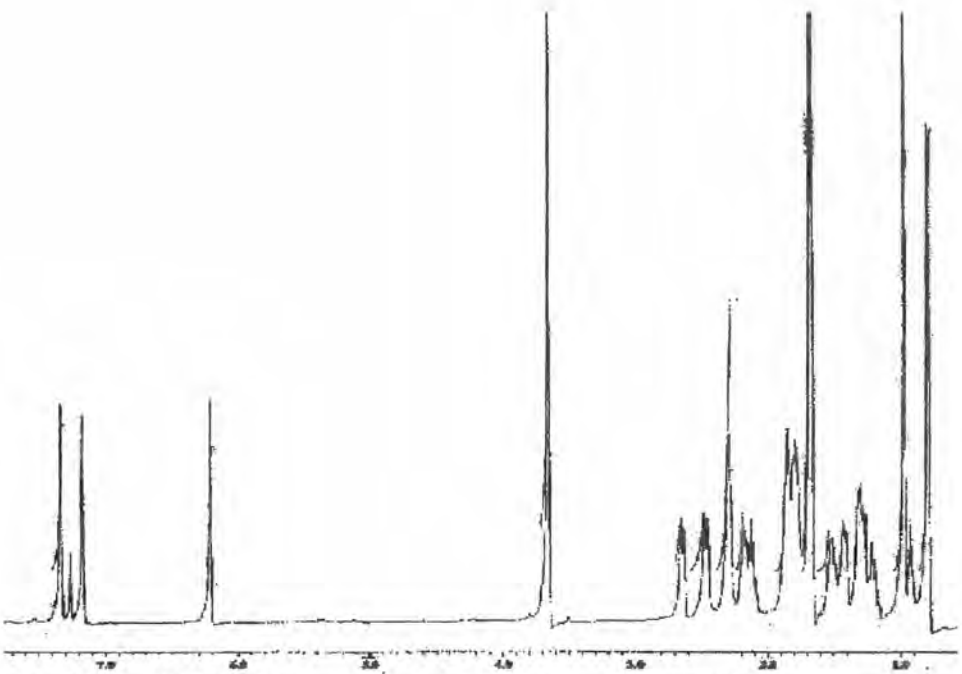


Fig. 3.2 **^{1}H NMR Spectrum of Methyltessmannoate (3.2)**

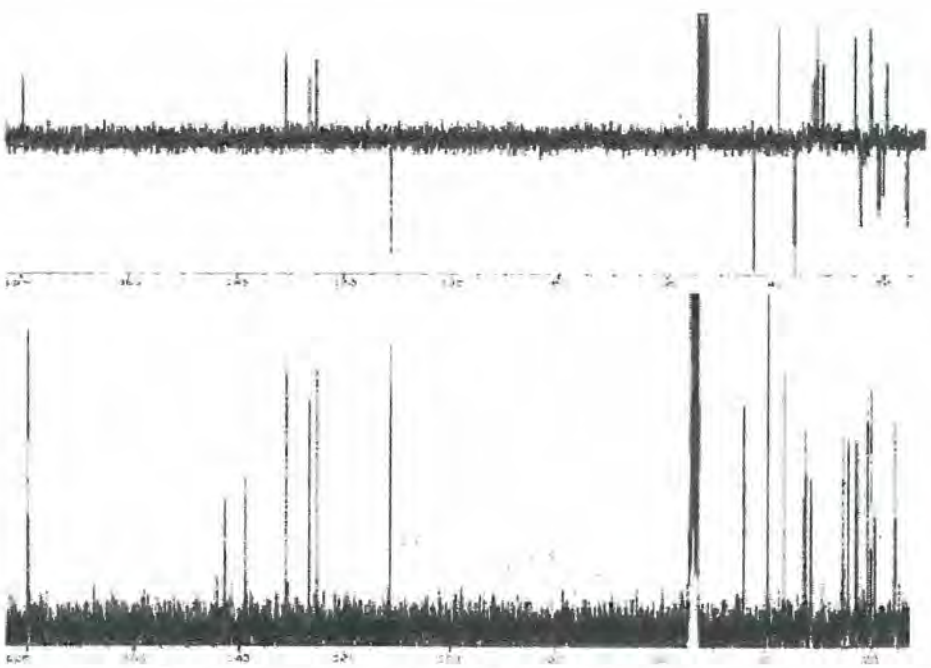


Fig. 3.3 **^{13}C NMR (with APT Experiment) Spectrum of Tessmannic acid (3.1)**

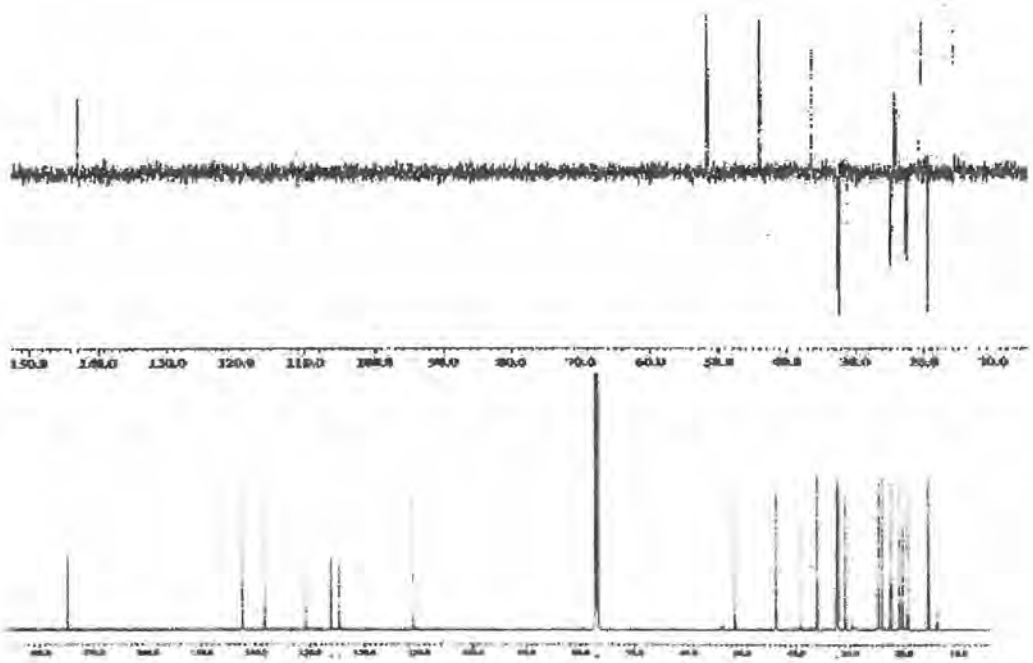


Fig. 3.4 ^{13}C NMR (with DEPT Experiment) Spectrum of Methyltessmannoate (3.2)

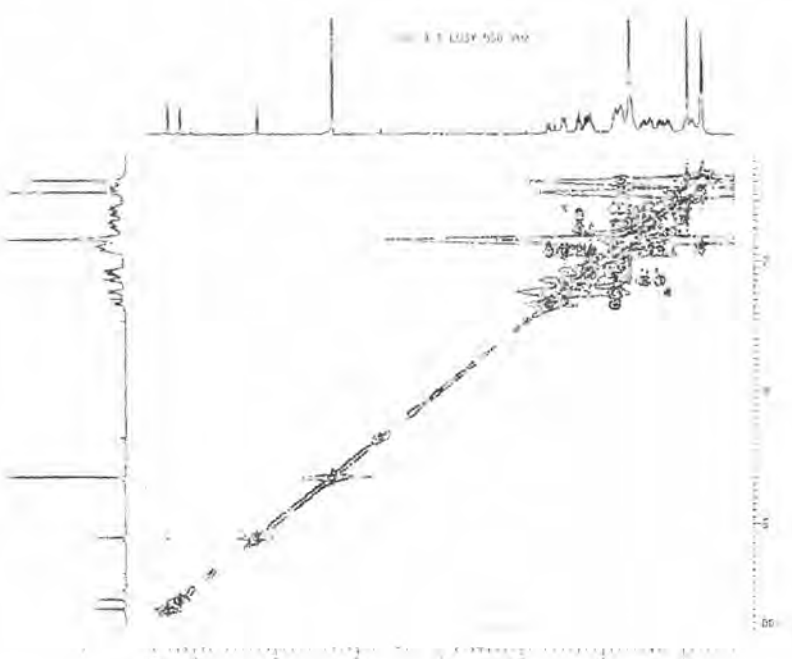


Fig. 3.5 H/H COSY Spectrum of Tessmannic acid (3.1)

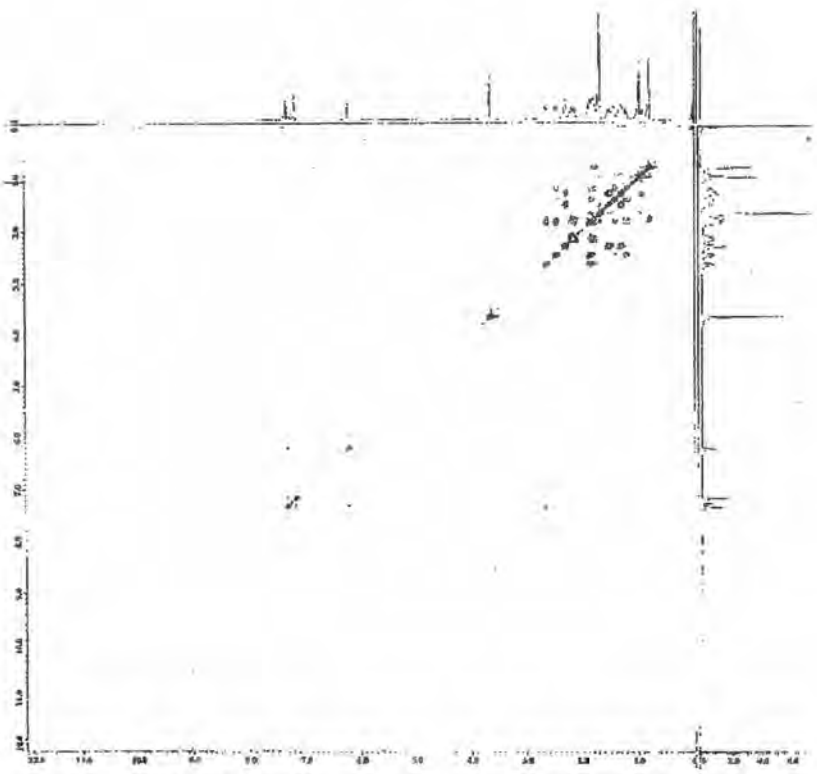


Fig. 3.6 H/H COSY Spectrum of Methyltessmannoate (3.2)

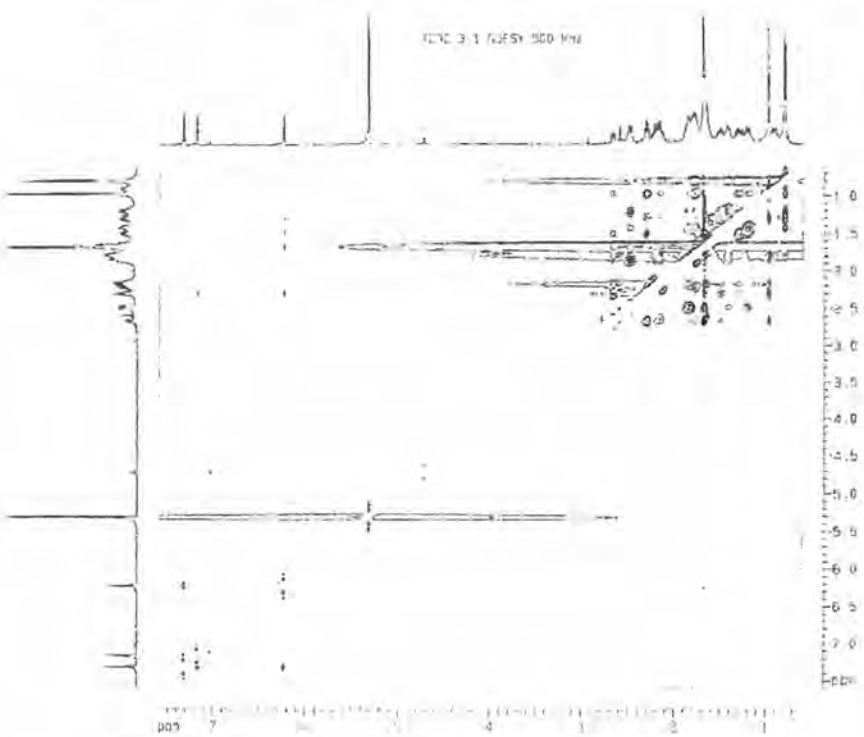


Fig. 3.7 H/H NOESY Spectrum of Tessmannic acid (3.1)

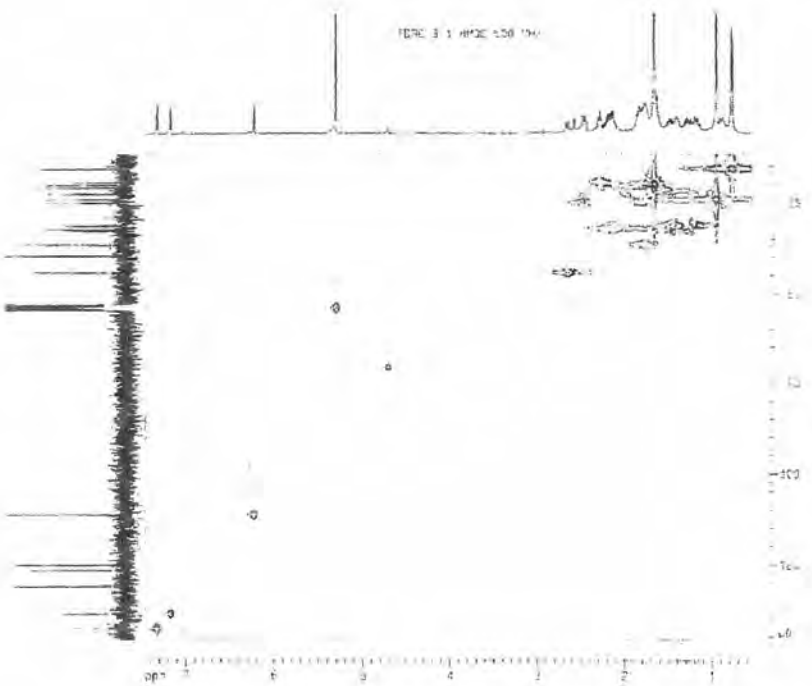


Fig. 3.8 HMQC Spectrum for Tessmannic acid (3.1)

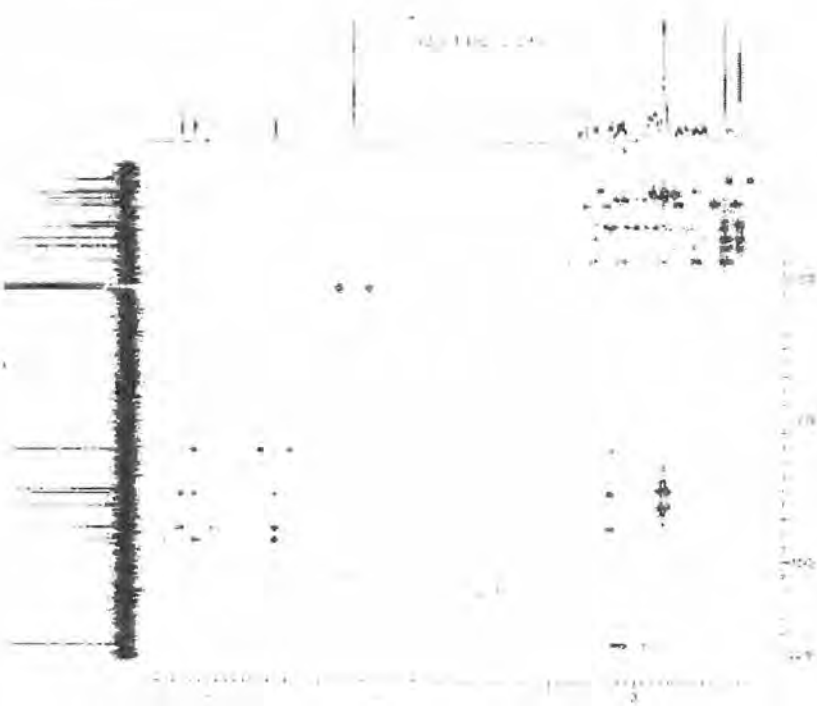


Fig. 3.9 HMBC Spectrum for Tessmannic acid (3.1)

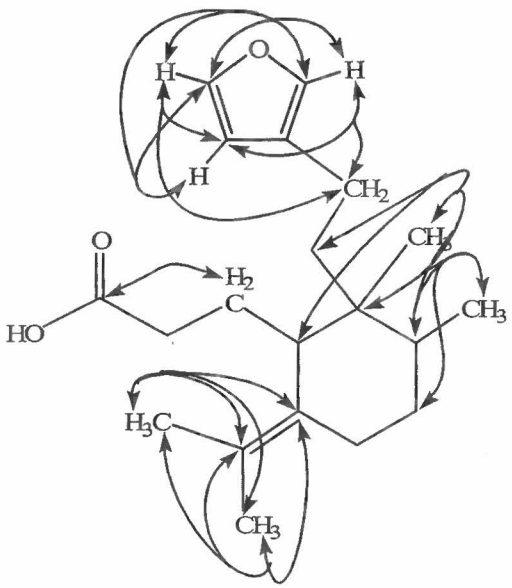


Fig. 3.9a Important Long Range H/C HMBC Correlations for Compound 3.1

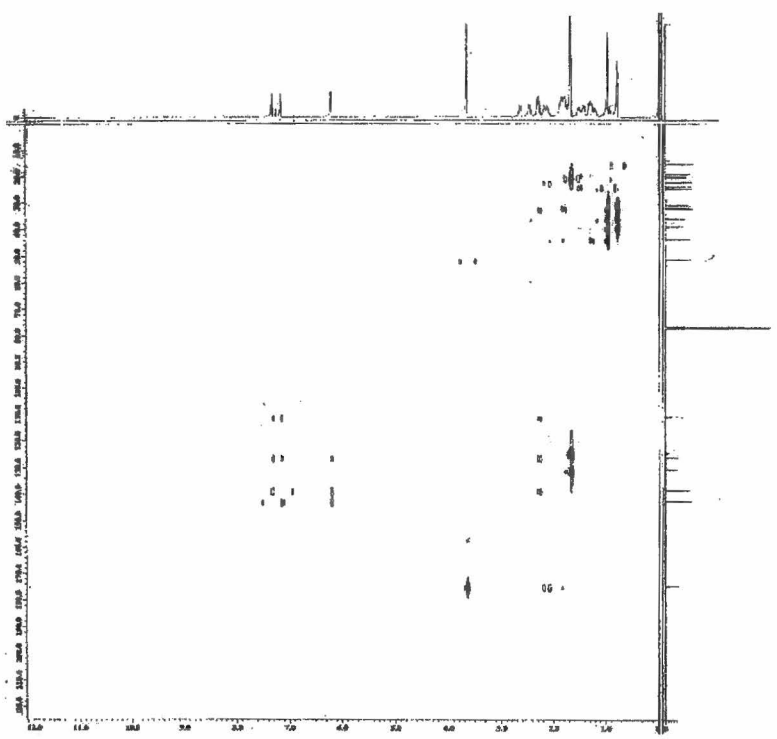


Fig. 3.10 HMBC Spectrum for Methyltessmannoate (3.2)

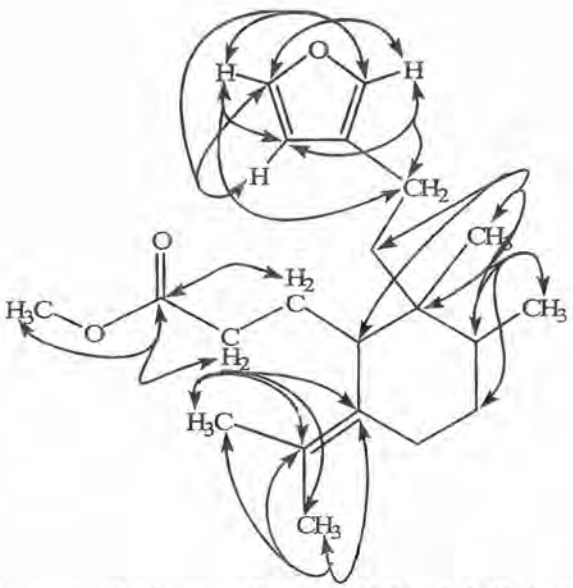


Fig. 3.10a Important Long Range H/C HMBC Correlations for Compound 3.2

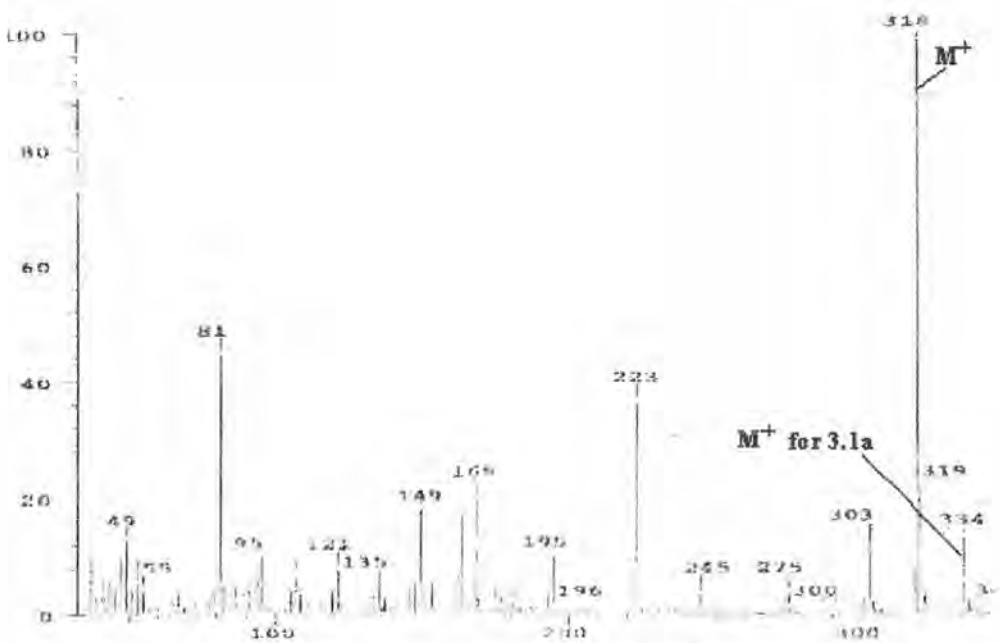
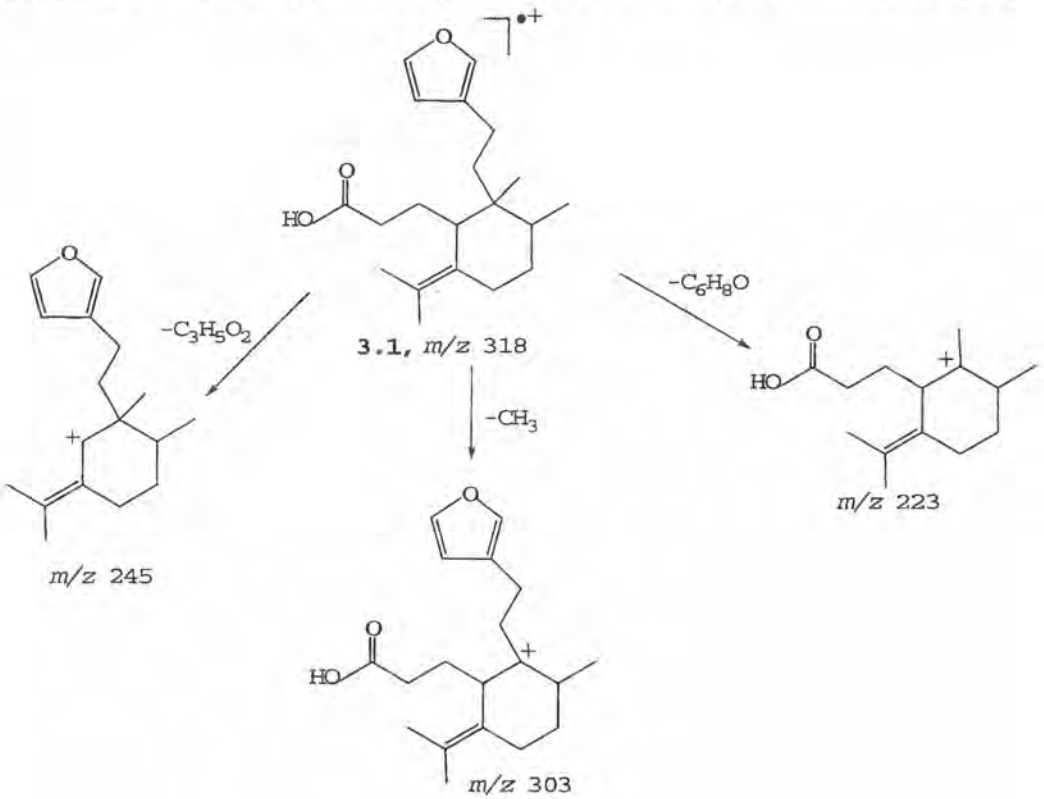


Fig. 3.11 Mass Spectrum of Tessmannic acid (3.1)

Scheme 3.2 Mass Spectral Fragmentation Pattern for Tessmannic acid (3.1)



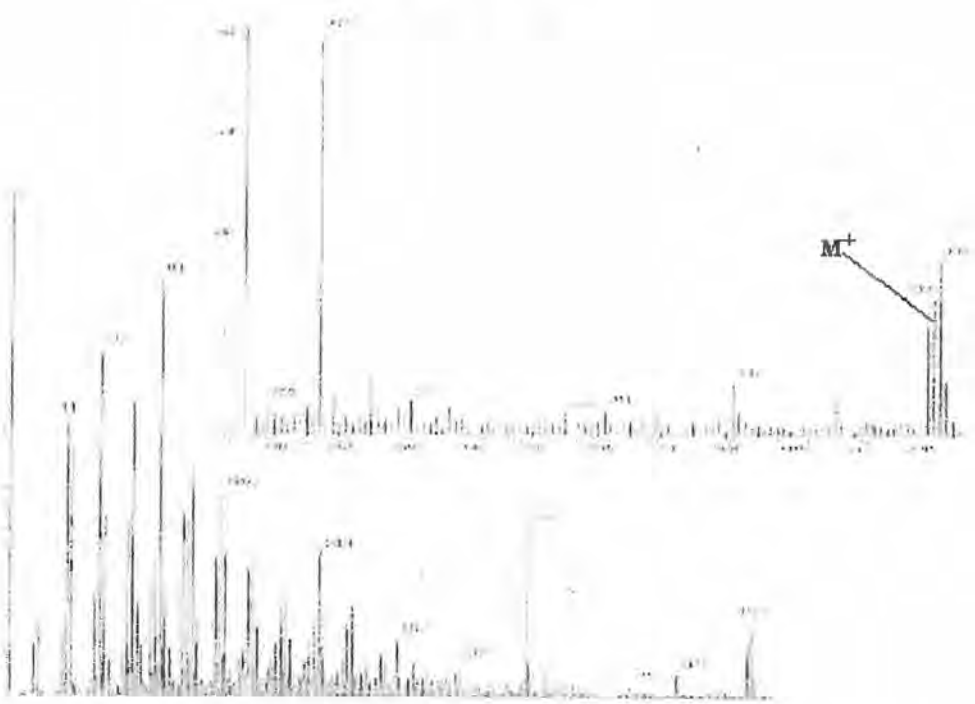
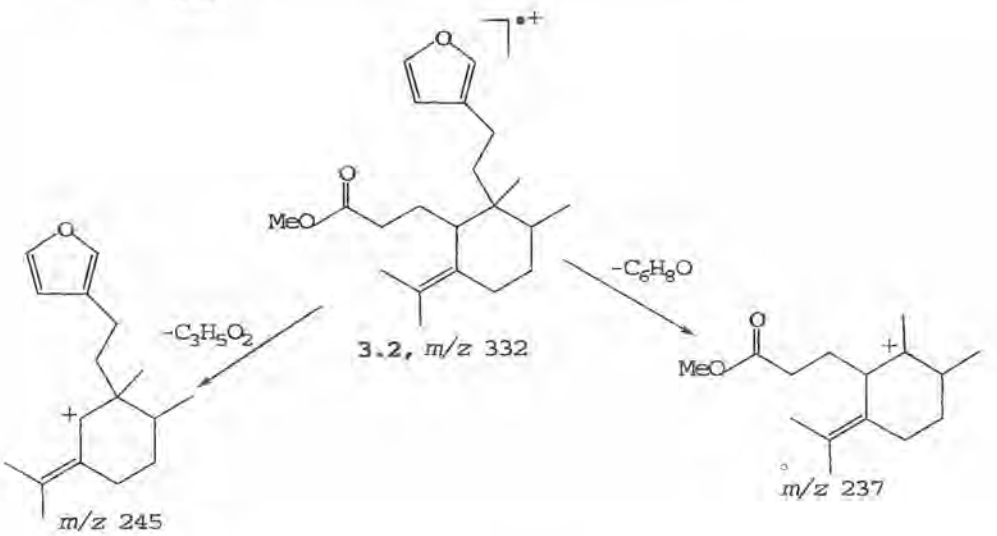


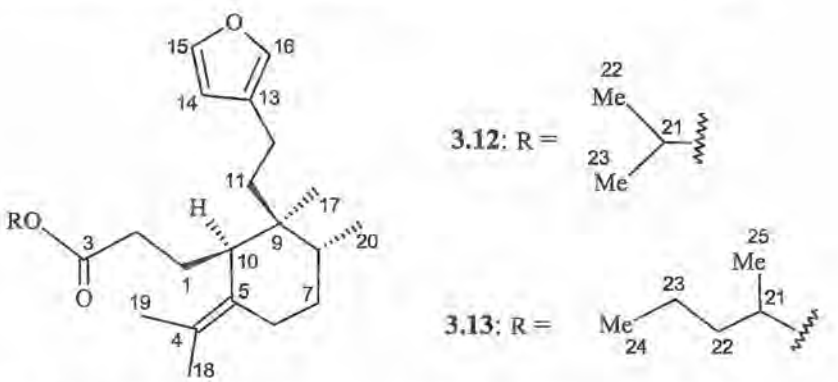
Fig. 3.12 Mass Spectrum of Methyltessmannoate (3.2)

Scheme 3.3 Mass Spectral Fragmentation Pattern for Methyltessmannoate (3.2)



3.2.1.2 2-Methylpropyltessmannoate (3.12) and 1-Methylbutyltessmannoate (3.13)

Repeated chromatography of VLC fraction 2 from the crude chloroform extract of the stem bark of *Tessmannia densiflora* yielded two compounds as oils whose structures **3.12** and **3.13** were established based on spectroscopic data.



Thus, the ^{1}H NMR spectrum of compound **3.12** exhibited signals for protons due to two isopropyl methyl groups at δ = 1.21 (d, J = 6 Hz) and the corresponding isopropyl methine proton signal at δ 4.03 (septet, J = 6 Hz) whose chemical shift

position indicated that the isopropyl group was attached to an oxygen atom. The above spectral data and the appearance of a carbonyl carbon signal at $\delta = 179.6$ in ^{13}C NMR spectrum due to an ester carbonyl indicated presence of an isopropyl ester group in the isolated compound, instead of a methyl ester group found in **3.2**. This was further confirmed from H/H COSY and C/H HMBC interactions (Figs. 3.17 and 3.21) which also enabled complete assignment of structure **3.12**.

Presence of a 1-methylbutyl ester in compound **3.13** was indicated by the appearance of ^{1}H NMR signals for the corresponding protons at $\delta = 2.90$ (m), 1.73 (m), 1.38 (d, $J = 7.2$), 0.97 (d, J=7.2), 1.23 (d) and a ^{13}C NMR signal at $\delta = 182.4$ due to a relatively deshielded carbonyl group as a result of the presence of the four – carbon alkyl group substituted to the oxycarbonyl group. As for compound **3.12** presence of the 1-methylbutyl group and complete assignment of structure **3.13** was deduced from H/H COSY and C/H HMBC interactions (Figs. 3.18 and 3.22).

The MS (Figs. 3.23 and 3.24) of compounds **3.12** and **3.13** did not show the molecular ion peaks. This is normally the case where the ester molecule is large, and hence the molecular ion peak intensity become very small. This may be the case as a result of the presence of a proton which is in a γ position relative to the carbonyl carbon that would allow McLaferty rearrangement to form an acid. In this case the acid fragment appeared in the MS, which in fact made the two compounds **3.12** and **3.13** to constitute MS fragmentation patterns (Figs. 3.23 and 3.24, Schemes 3.4 and 3.5) similar to those observed for tessmannic acid (**3.1**). Only the HRMS was done for **3.12**, which showed the M^+ peak at *m/z* 360.2645 corresponding to the molecular formula $C_{23}H_{36}O_3$.

Table 3.3 ^{1}H and ^{13}C NMR Spectral Data for 2-Methylpropyltessmannoate (3.12) and 1-methylbutyltessmannoate (3.13)

H/C	3.12			3.13		
	δ_H	*J* (Hz)	δ_C	δ_H	*J* (Hz)	δ_C
1 a	1.85	m	22.4	1.84	m	22.5
1 b	1.82	m		1.84	m	
2 a	2.23	dd, 14.1, 9.0	32.2	2.45	m	32.2
2 b	2.29	dd, 14.1, 12.3		2.45	m	
3	-	-	179.6	-	-	182.4
4	-	-	126.2	-	-	126.2
5	-	-	130.7	-	-	130.8
6 a	2.07	m	25.3	2.25	m	24.7
6 b	2.20	m		2.27	m	
7 a	1.24	m	30.9	1.25	m	30.9
7 b	1.40	m		1.45	m	
8	1.79	m	36.1	1.78	m	36.1
9	-	-	39.2	-	-	39.2
10	2.65	dd, 7.5, 4.9	43.8	2.64	d,d	43.9
11 a	1.77	m	32.5	1.51	m	32.4
11 b	1.73	m		1.41	m	
12	2.46	dt, 12.0, 2.1	19.3	2.30	m	20.8
13	-	-	124.8	-	-	124.6
14	6.21	d, 0.9	111.0	6.21	d, 0.9	111.0
15	7.33	t, 1.8	142.6	7.33	t, 1.8	142.6
16	7.17	d, 0.6	138.4	7.17	s	138.4
17	0.97	s	24.0	0.97	s	24.0
18	1.68*	s	20.2	1.68*	s	20.2*
19	1.68*	s	20.8	1.68*	s	20.2*
20	0.78	d, 6.6	15.6	0.78	d, 6.9	15.6
21	4.03	septet, 6	64.5	2.90	m	52.0
22	1.21	d, 6	24.7	1.73	m	25.3
23	1.21	d, 6	24.7	1.38	q, 7.2	20.8
24	-	-		0.97	t, 7.2	13.6
25	-	-		1.23	s	29.7

* interchangeable assignments

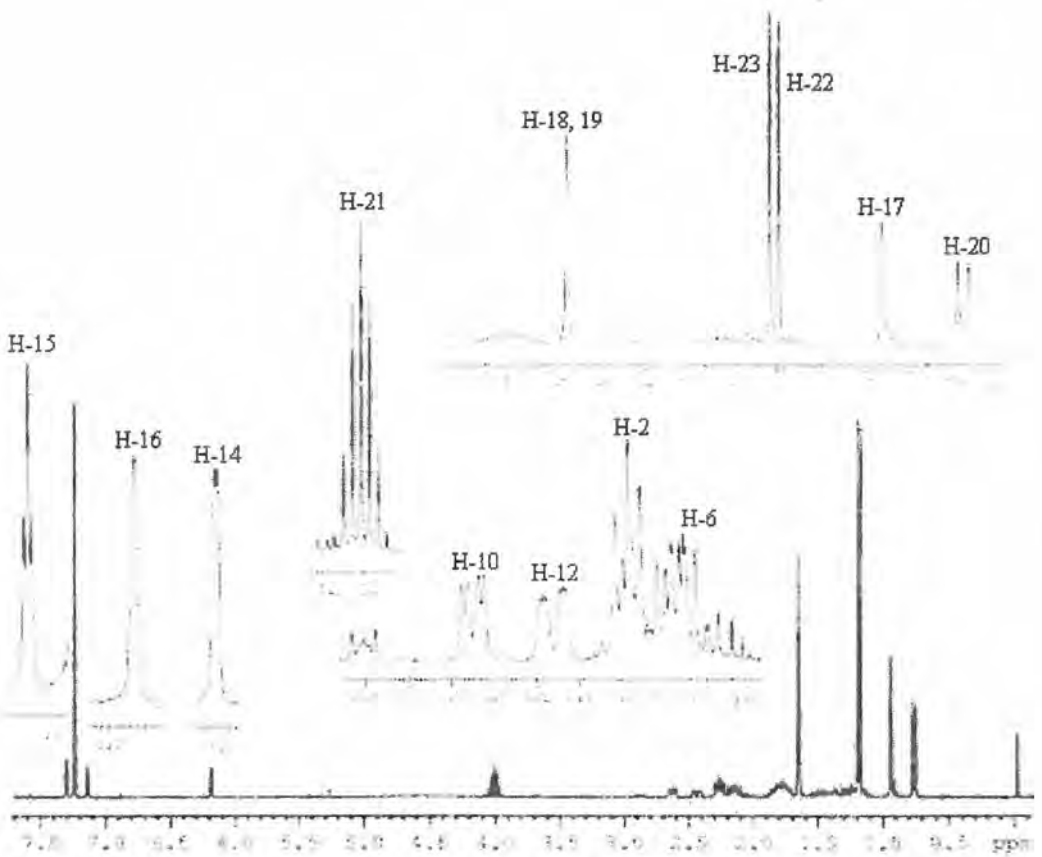


Fig. 3.13 ^{1}H NMR Spectrum of 2-Methylpropyltessmannoate (3.12)

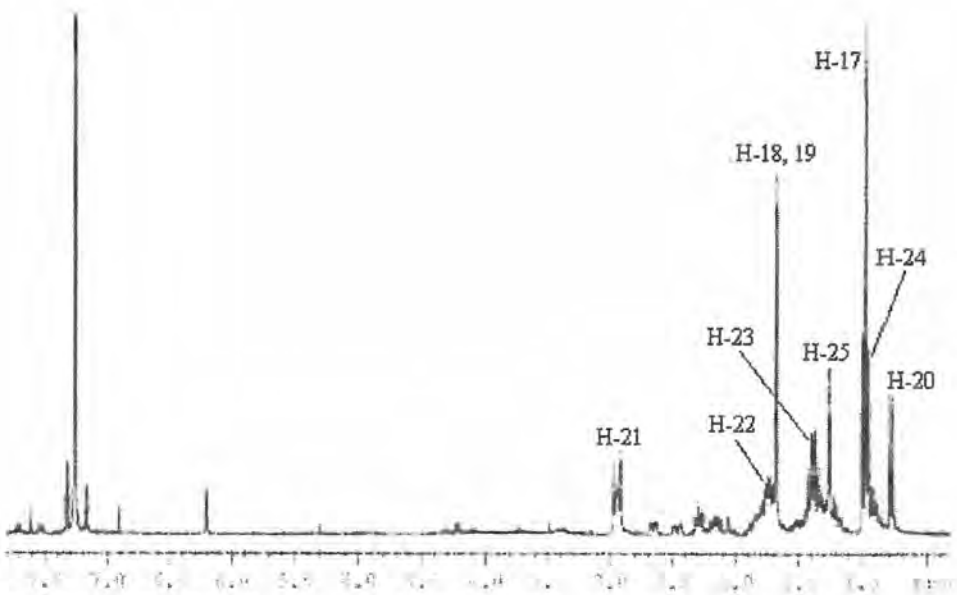


Fig. 3.14 ^{1}H NMR Spectrum of 1-Methylbutyltessmannoate (3.13)

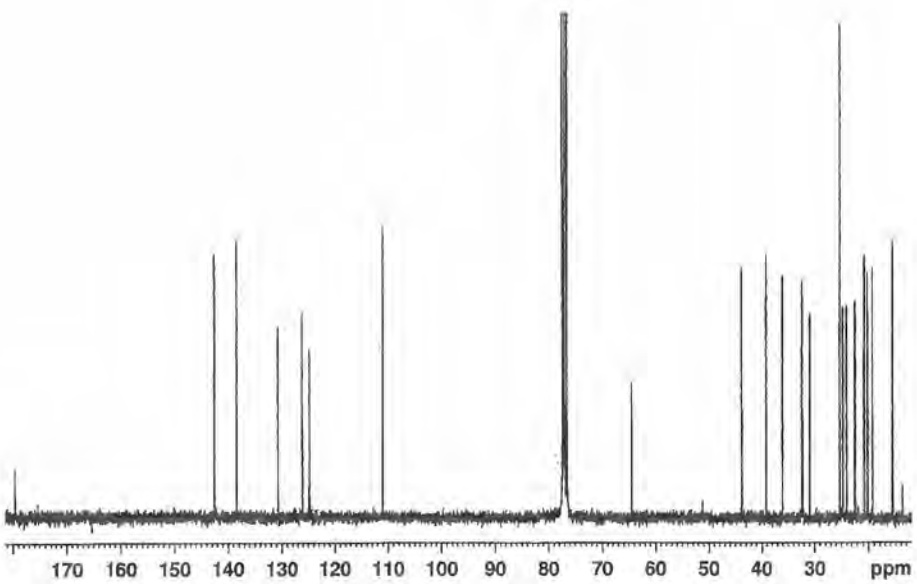


Fig. 3.15 ^{13}C **NMR Spectrum of 2-Methylpropyltessmannoate (3.12)**

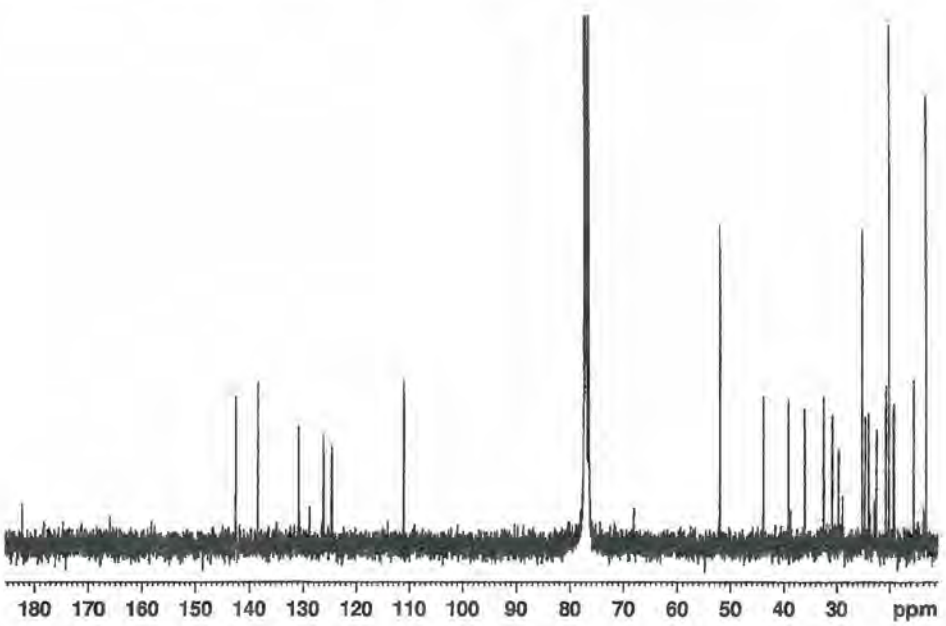


Fig. 3.16 ^{13}C **NMR Spectrum of 1-Methylbutyltessmannoate (3.13)**

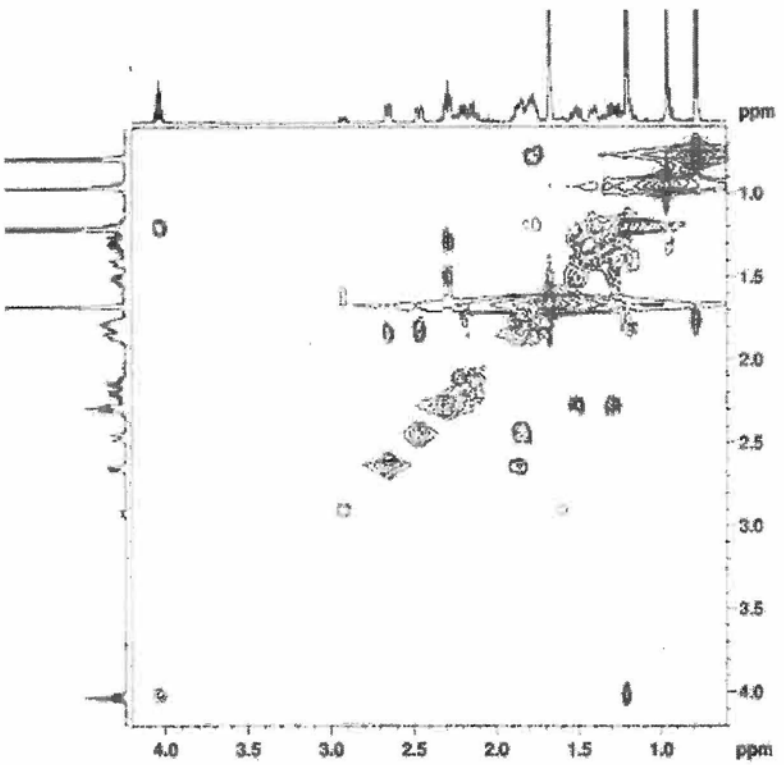


Fig. 3.17 COSY Spectrum of 2-Methylpropyltessmannoate (3.12)

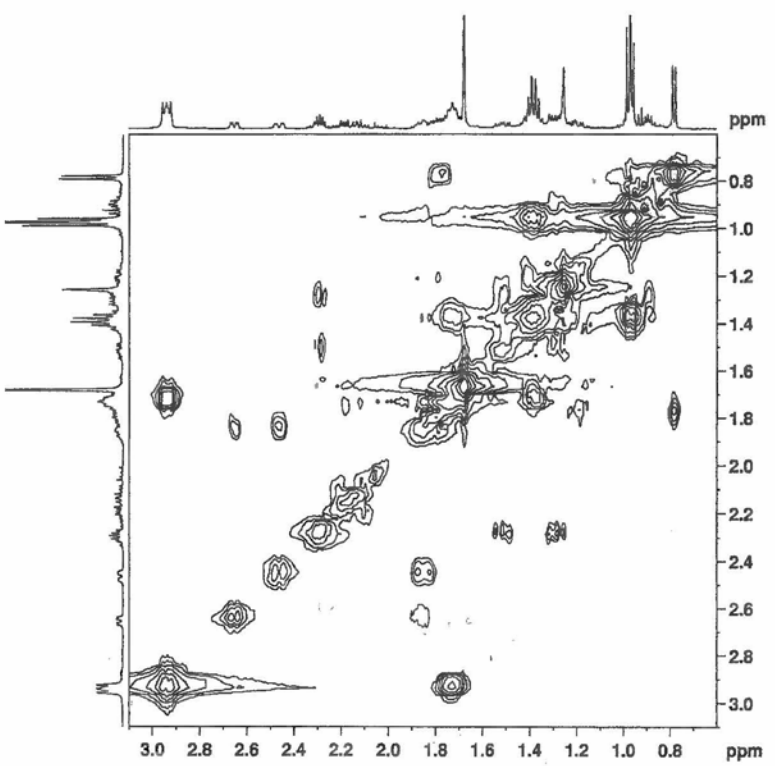


Fig. 3.18 COSY Spectrum of 1-Methylbutyltessmannoate (3.13)

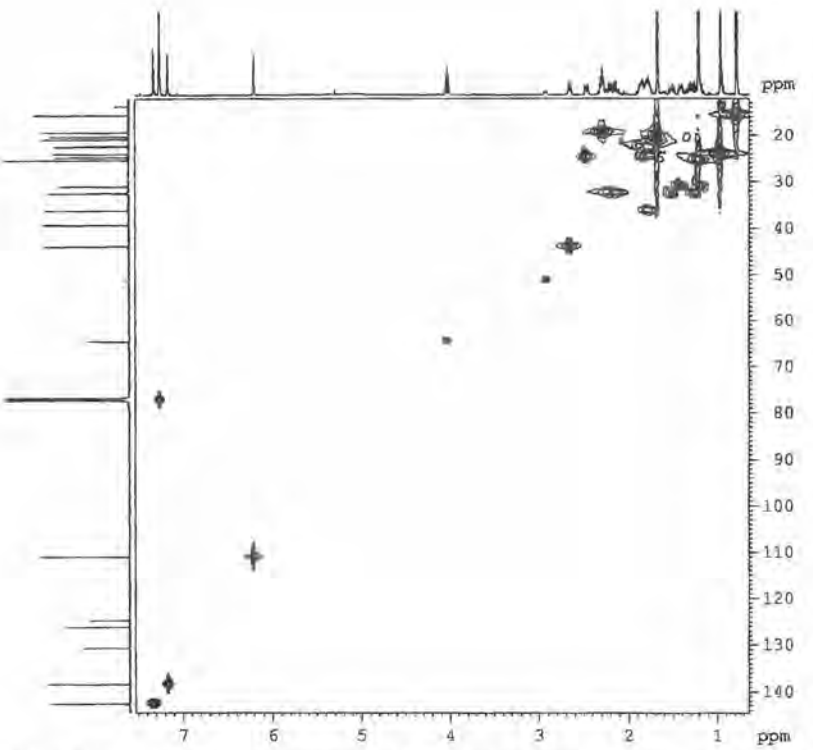


Fig. 3.19 HMQC Spectrum of 2-Methylpropyltessmannoate (3.12)

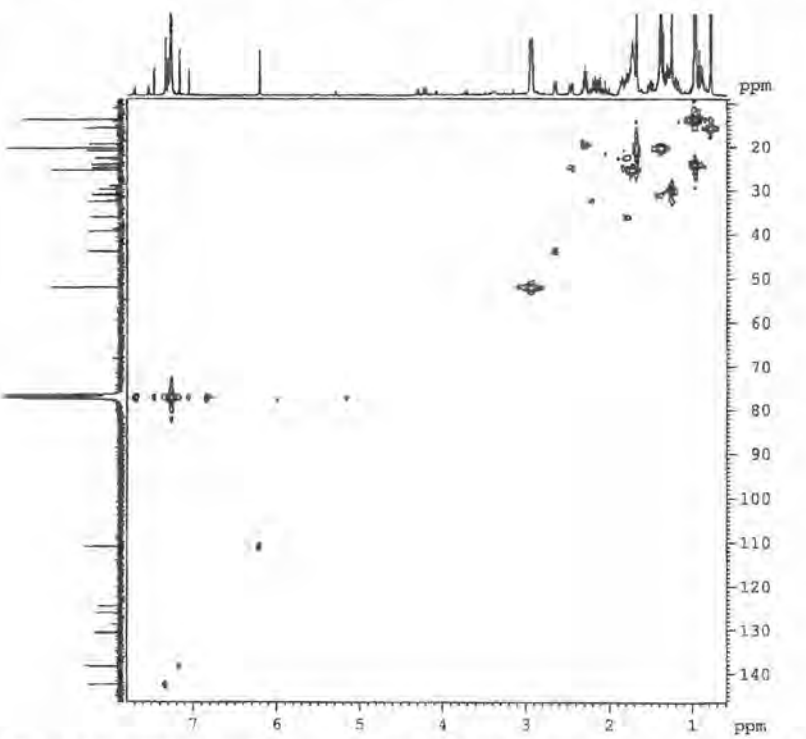


Fig. 3.20 HMQC Spectrum of 1-Methylbutyltessmannoate (3.13)

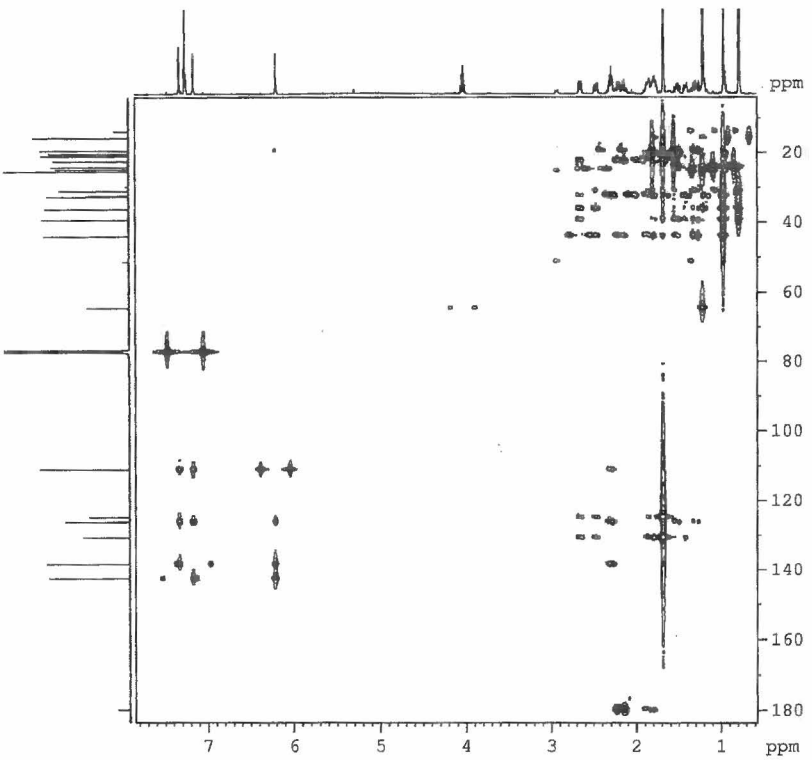


Fig. 3.21 HMBC Spectrum of 2-Methylpropyltessmannoate (3.12)

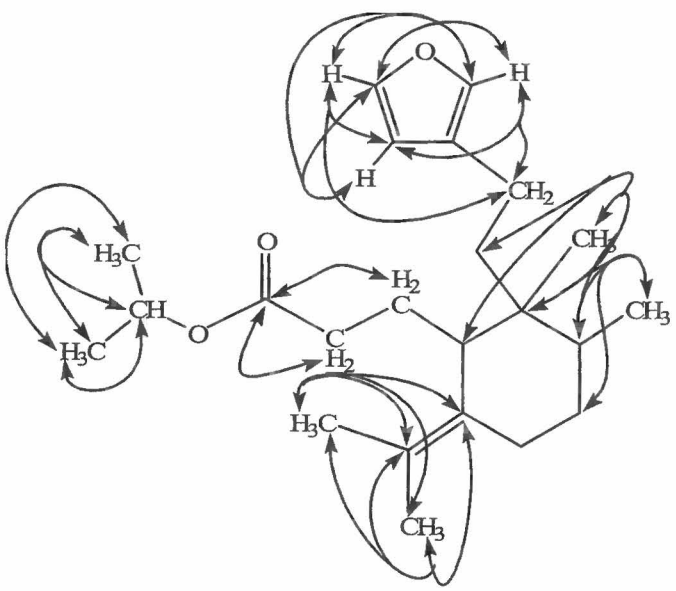


Fig. 3.21a Important Long Range H/C HMBC Correlations for Compound 3.12

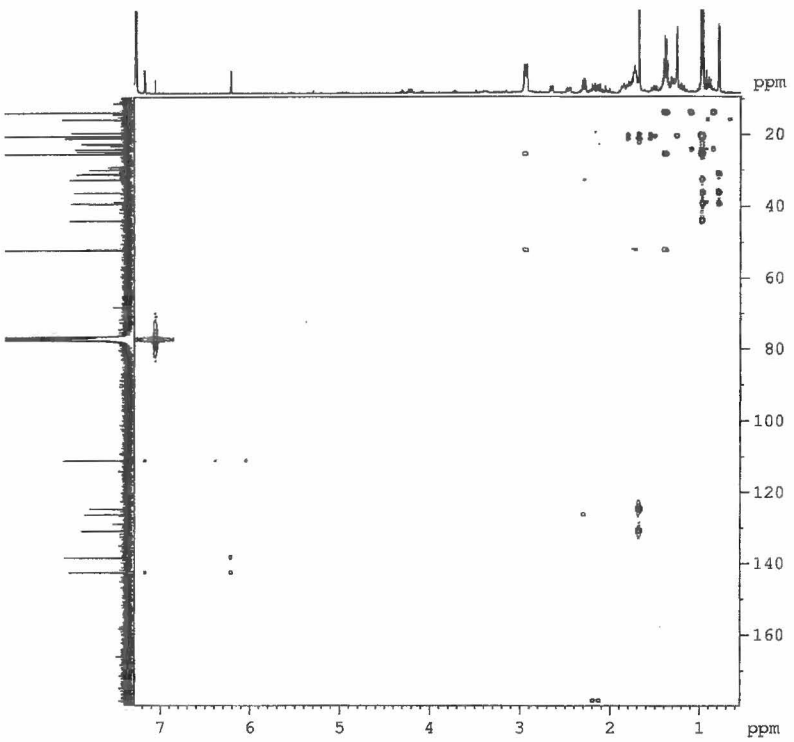


Fig. 3.22 HMBC Spectrum of 1-Methylbutyltessmannoate (3.13)

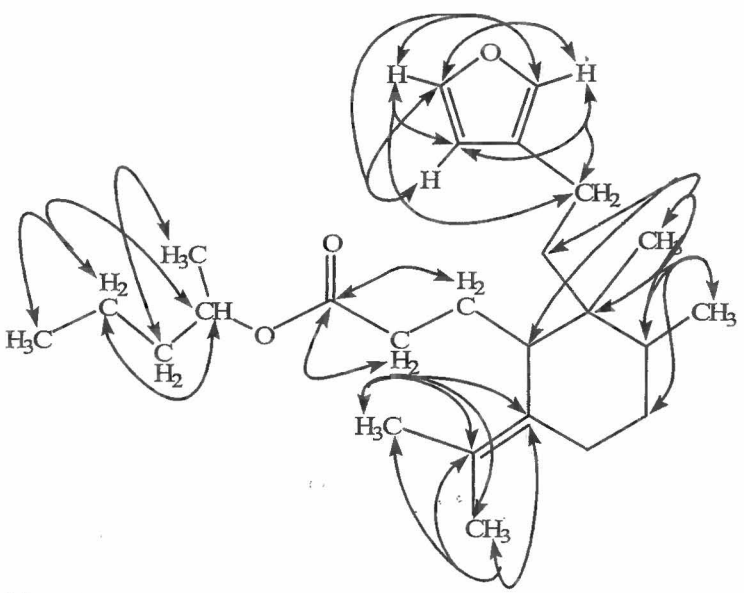


Fig. 3.22a Important Long Range H/C HMBC Correlations for Compound 3.13

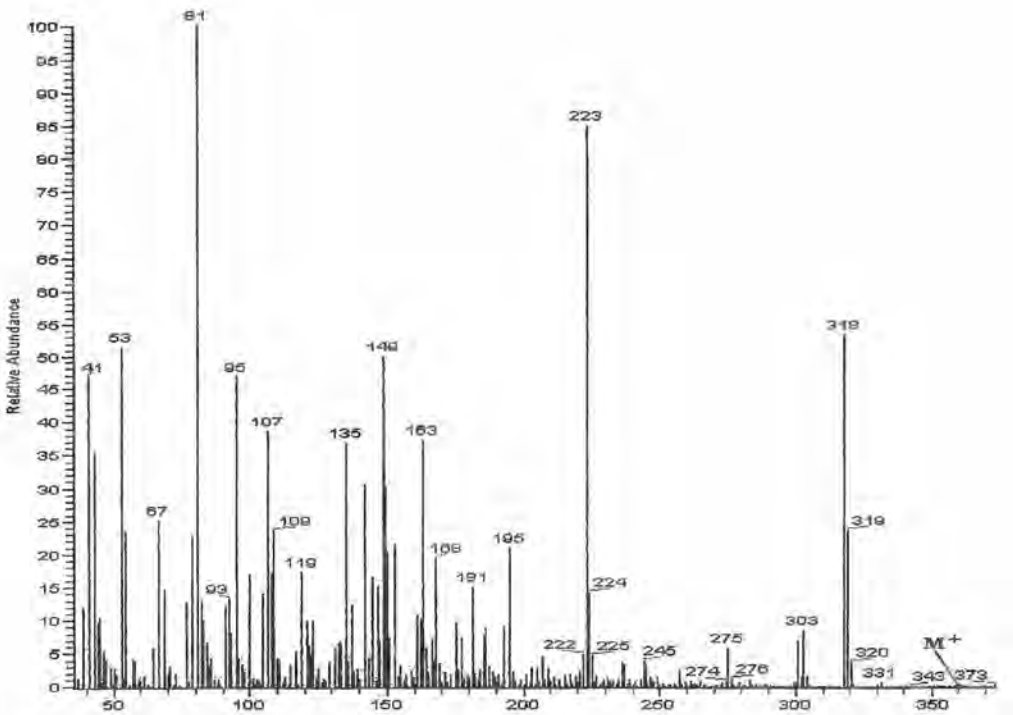
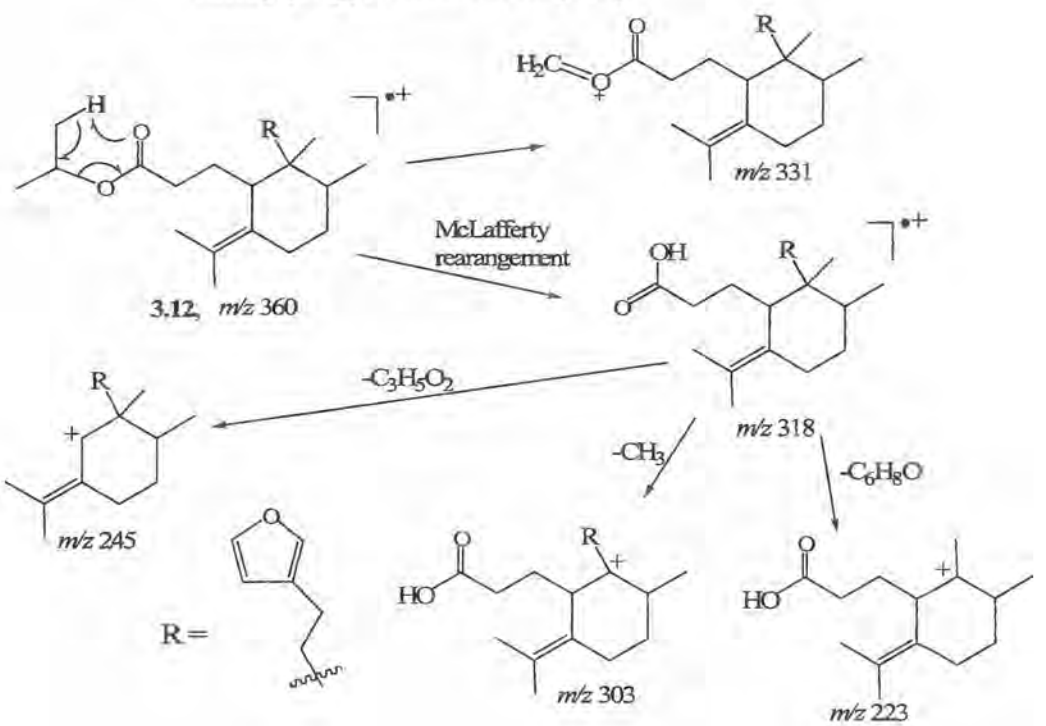


Fig. 3.23 Mass Spectrum of 2-Methylpropyltessmannoate (3.12)

Scheme 3.4 Mass Spectral Fragmentation Pattern for 2-Methylpropyltessmannoate (3.12)



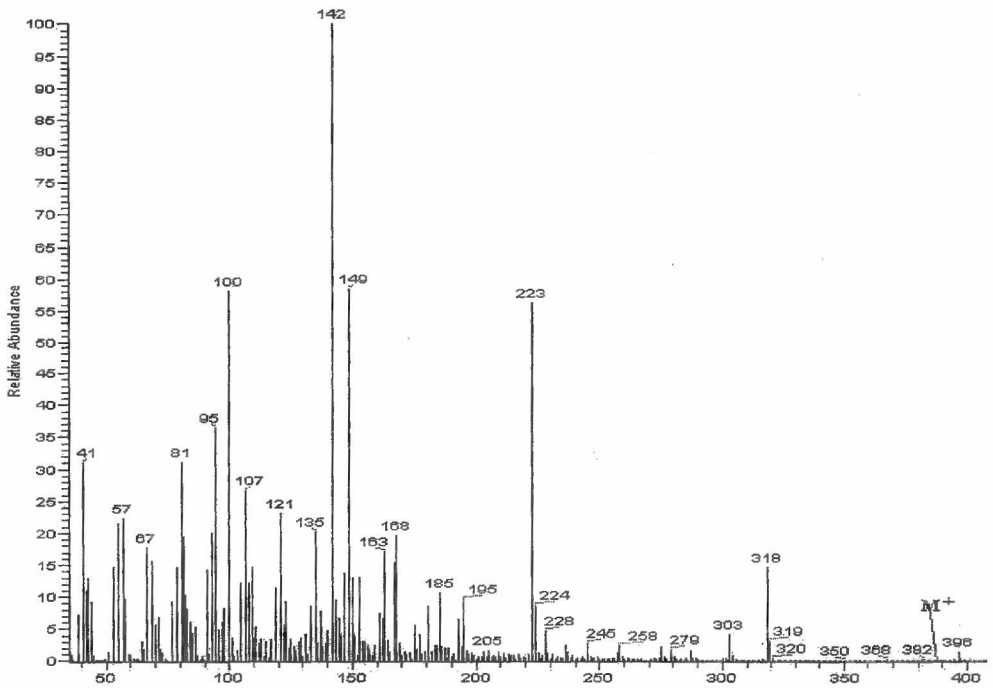
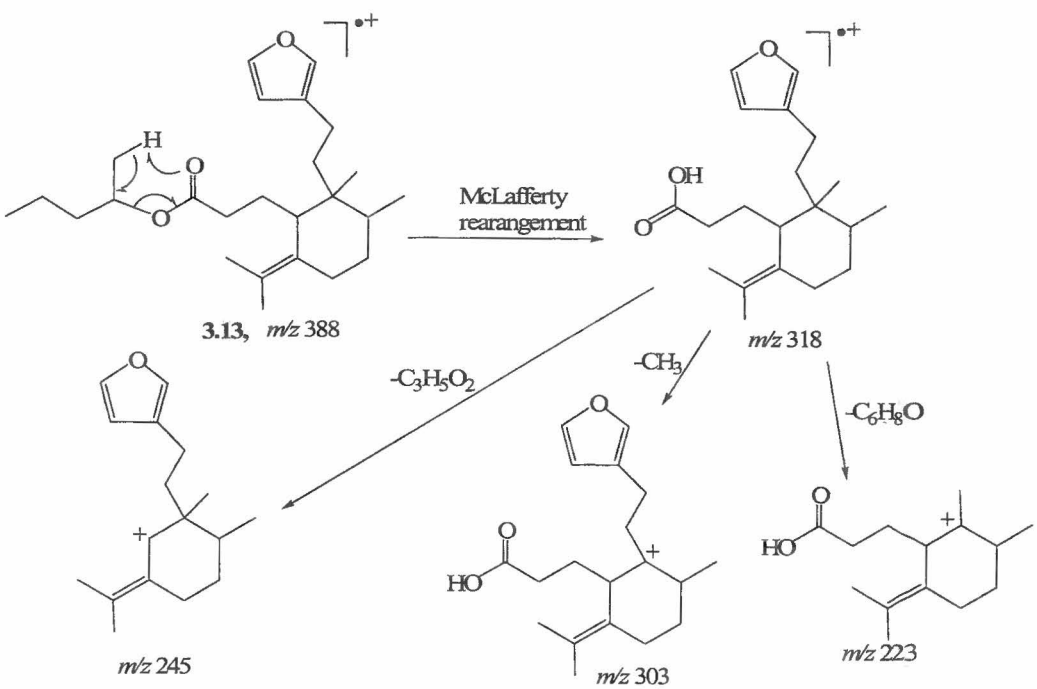


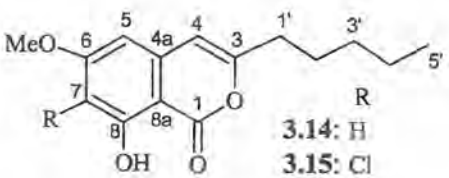
Fig. 3.24 Mass Spectrum of 1-Methylbutyltessmannoate (3.13)

Scheme 3.5 Mass Spectral Fragmentation Pattern for 1-Methylbutyltessmannoate (3.13)



3.2.1.3 8-Hydroxy-6-methoxy-3-pentylisocoumarin (3.14) and 7-Chloro-8-hydroxy-6-methoxy-3-pentylisocoumarin or Chlotessmin (3.15)

Repeated chromatography on silica gel of VLC fraction 5 of the chloroform extract of the stem bark of *Tessmannia densiflora* yielded two compounds as white plates whose structures **3.14** and **3.15** were established on the basis of their spectroscopic properties and upon comparison of these properties with those reported in the literature for compound **3.14** that was previously isolated from the stem bark of *Knema tenuineervia* ssp. *setosa* (Myristicaceae).[141] Thus, the MS (Figs. 3.36 and 3.37) showed molecular ion peaks at *m/z* 262.12 and 296.08, which corresponded to the molecular formulae $C_{15}H_{18}O_4$ and $C_{15}H_{17}O_4Cl$ for **3.14** and **3.15** respectively.



The ^{13}C NMR spectra of both compounds **3.14** and **3.15** consisted of fifteen signals, 9 of which were due to unsaturated carbons including a carbonyl C atom, which was consistent with the presence of a coumaryl unit in each of the two compounds. The other ^{13}C NMR signals appeared at the high field region, these being due to a methoxy carbon (δ 55.6 for **3.14** and 56.6 for **3.15**), and a C_5 side chain (Table 3.4). The substitution pattern of the coumaryl unit in each of the two compounds was deduced from the ^1H NMR signals as well as H/H COSY and C/H HMBC interactions. Thus, the ^1H NMR spectrum of compound **3.14** exhibited signals due to two *meta* coupling aromatic protons at δ 6.31 and 6.46, each d, $J \approx 2$ Hz,[142] one isolated vinylic proton (δ 6.17, s) and an OH proton signal at δ 11.2, s whose downfield position indicated that the corresponding OH group was chelated. This

indicated the proximity of the OH group to the coumaryl carbonyl group. Therefore, considering the high field positions of *meta* coupling aromatic protons as stated above, it was concluded that the methoxy group indicated by both the ^{1}H and ^{13}C NMR spectra (δ_H 3.86, δ_C 55.6) was at a *meta* position relative to the OH group. The vinylic proton signal appearing at a relatively high field (δ 6.17) indicated that the corresponding proton was β to the lactonyl oxygen, hence being devoid of anisotropic deshielding effects. Therefore, having established the above substitution pattern it followed that the pentyl group was attached to the carbon atom α to the lactonyl oxygen, as further indicated by HMBC interactions which also confirmed the entire structure **3.14**.

Structure **3.14** was also consistent with the fragmentation pattern observed in the MS (Fig. 3.36). Thus, besides the M^+ peak (*m/z* 262.1) the MS exhibited fragment ion peaks due to extrusion of a molecule of carbon dioxide from the molecular ion (*m/z* 218.9) and a base peak at *m/z* 164.0 being due to rearrangement involving cleavege of the coumaryl lactone ring (Scheme 3.6).

As for **3.14** the substitution pattern shown in structure **3.15** was deduced from ^{1}H and ^{13}C NMR, and mass spectra. The appearance of only two singlets instead of three signals due to vinylic protons observed in the ^{1}H NMR spectrum of **3.14** indicated that one of the three unsubstituted sp^2 carbons in **3.14** was substituted in **3.15**. Since the ^{1}H NMR spectrum of **3.15** lacked a signal that was assigned to H-7 in **3.14** it was concluded that C-7 was substituted in compound **3.15**, as further indicated by the presence of cross-peaks in the HMBC plot (Fig. 3.35) that indicated H-4 and H-5 being unsubstituted. The substitution pattern was also indicated by the

NOEDIFF spectrum (Fig. 3.31) which showed the relative positions of the pentyl, OH and OMe group on the coumaryl unit.

At first instance, the nature of the substituent group at C-7 became intriguing until after having made a close analysis of the EIMS, which indicated the $[M+1]^+$ and $[M+3]^+$ peaks at *m/z* 297.1 and 299.0, appearing in the abundance ratio of 3:1. This indicated the presence of a chlorine nucleus in **3.15**,[143] as further confirmed by the HRMS which exhibited the M^+ peak at *m/z* 296.0774 (calculated for $C_{15}H_{17}O_4Cl$ = 296.08154), and an $[M+2]^+$ ion appeared at *m/z* 298.0794, the two peaks being in the 3:1 ratio of abundance corresponding to the two isotopes of chlorine. That the Cl unit was substituted on the benzene ring of the coumaryl unit was shown by the appearance of the MS fragment ion peak corresponding to the benzene ring at *m/z* 198.0050, upon cleavage of the lactonyl ring (Scheme 3.7, calculated for $C_9H_7O_3Cl$ = 198.00837).

Compound **3.15** is hereby being reported for the first time. It has therefore given the trivial name chlotessmin.

Table 3.4 ^{1}H NMR Spectral Data for 8-Hydroxy-6-methoxy-3-pentylisocoumarin (3.14) and Chlotessmin (3.15)

H/C	3.14			3.15			3.14[141]		
	δ_H	*J* (Hz)	δ_C	δ_H	*J* (Hz)	δ_C	δ_H	*J* (Hz)	δ_C
1	-	-	166.8	-	-	166.1	-	-	166.8
3	-	-	158.1	-	-	158.7	-	-	158.1
4	6.17	s	103.9	6.22	s	103.7	6.18	s	103.9
4a	-	-	139.1	-	-	137.4	-	-	139.5
5	6.31	d, 2.1	101.1	6.37	s	97.9	6.31	d, 2.3	101.0
6	-	-	166.5	-	-	161.9	-	-	166.6
6-OMe	3.86	s	55.6	3.99	s	56.6	3.86	s	55.6
7	6.46	d, 2.4	100.2	-	-	107.7	6.46	d, 2.3	101.2
8-OH	11.12	s	163.7	11.61	s	158.3	11.13	s	163.5
8a	-	-	100.0	-	-	100.7	-	-	99.9
1'	2.49	t, 4.5	33.3	2.51	t, 4.5	33.3	2.49	t, 7.5	33.3
2'	1.69	quintet, 3.0	26.4	1.69	quintet, 4.5	26.4	1.65	m	26.5
3'	1.37	quintet, 3.6	31.1	1.37	quintet, 3.6	31.1	1.34	m	31.1
4'	1.32	sextet, 3.6	22.3	1.35	sextet, 3.6	22.3	1.34	m	22.3
5'	0.91	t, 4.8	13.9	0.91	t, 2.7	13.9	0.9	t, 6.5	13.9

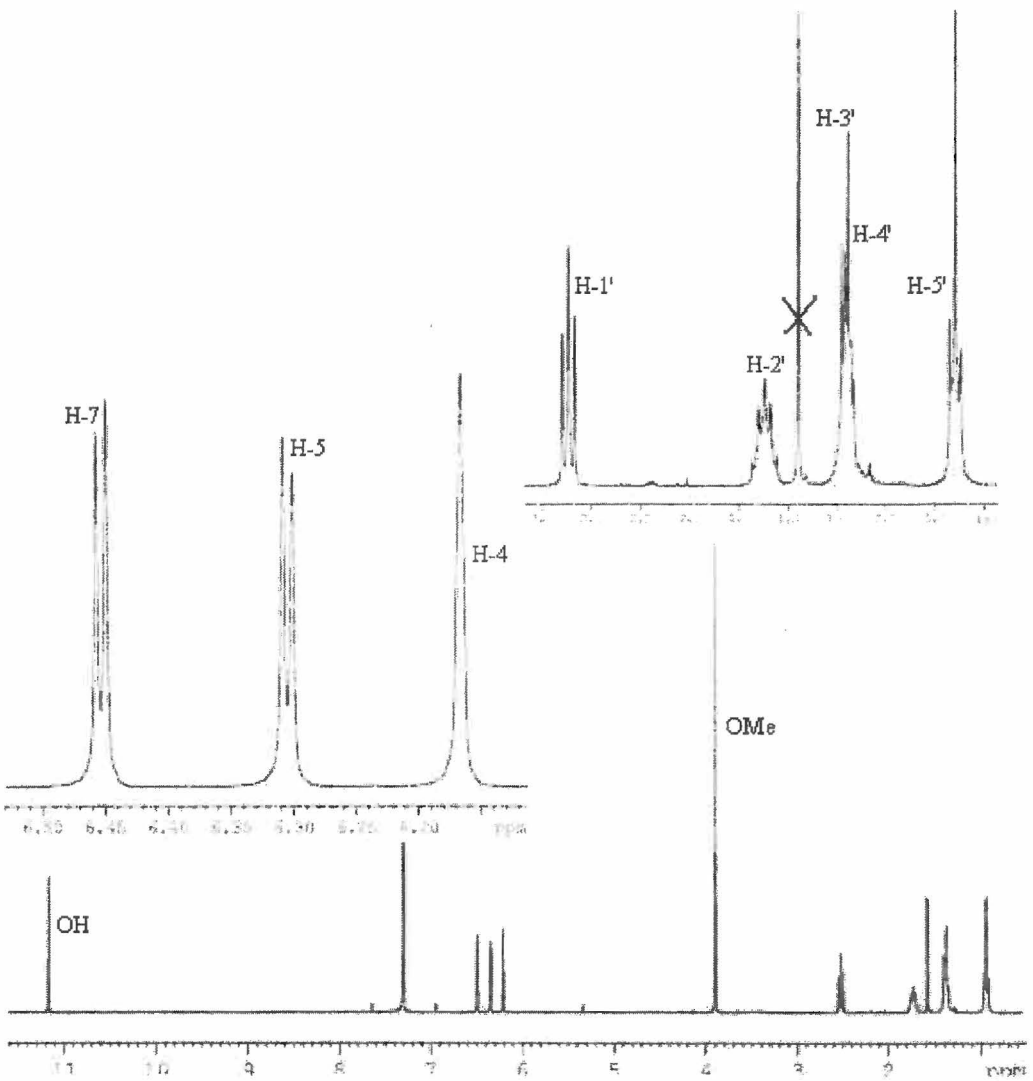


Fig. 3.25 ^{1}H NMR Spectrum of 8-Hydroxy-6-methoxy-3-pentylisocoumarin (3.14)

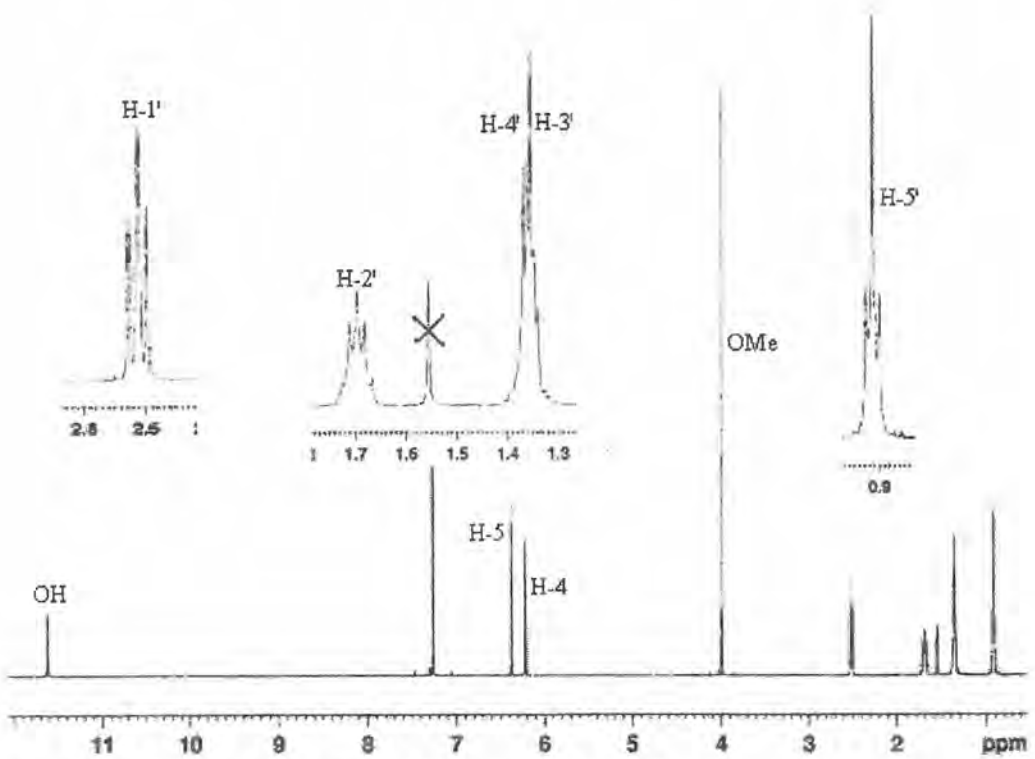


Fig. 3.26 ^{1}H NMR Spectrum of Chlotessmin (3.15)

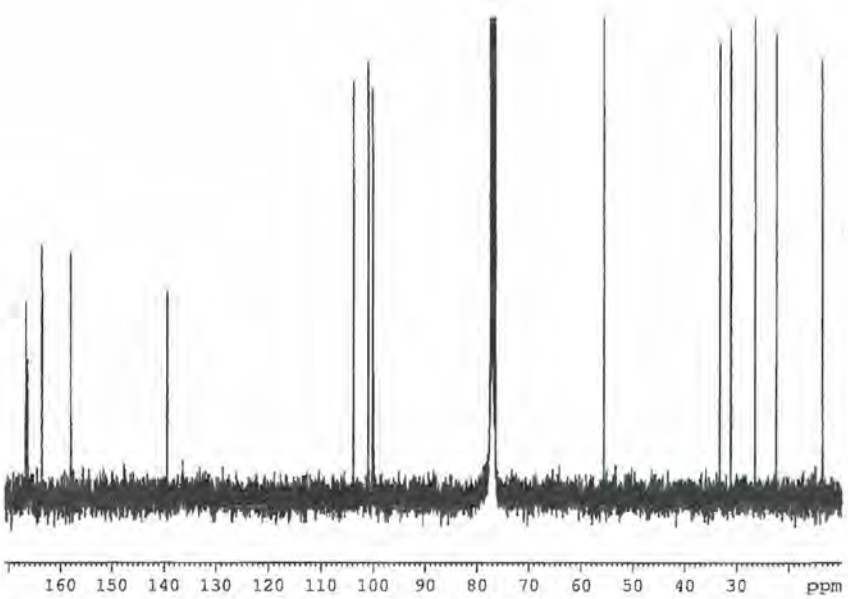


Fig. 3.27 ^{13}C NMR Spectrum of 8-Hydroxy-6-methoxy-3-pentylisocoumarin (3.14)

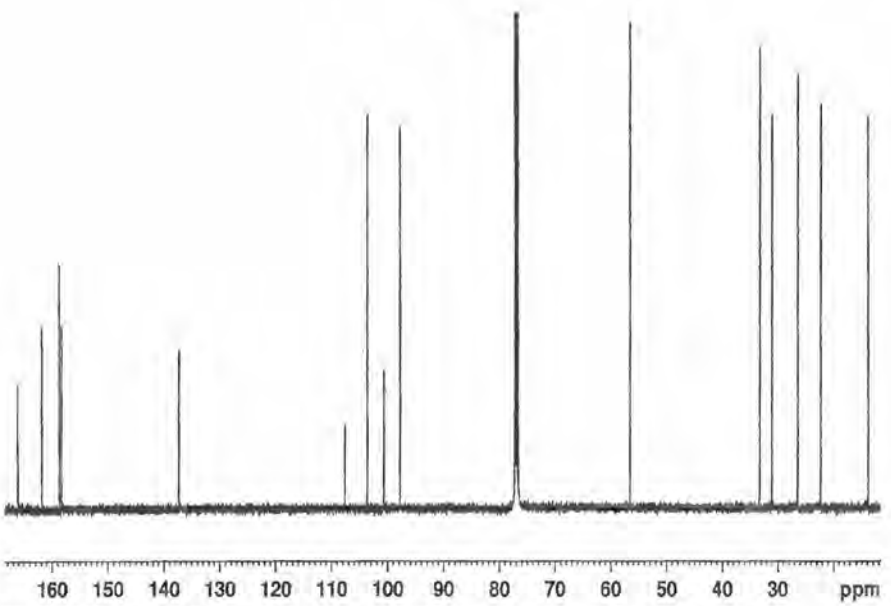


Fig. 3.28 ^{13}C NMR Spectrum of 7 Chlotessmin (3.15)

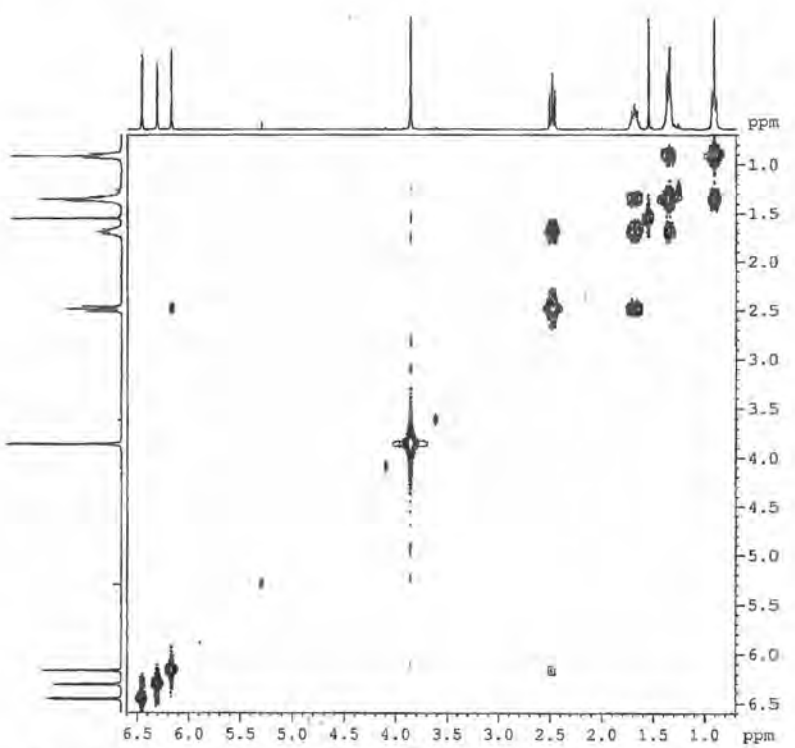


Fig. 3.29 COSY Spectrum of 8-Hydroxy-6-methoxy-3-pentylisocoumarin (3.14)

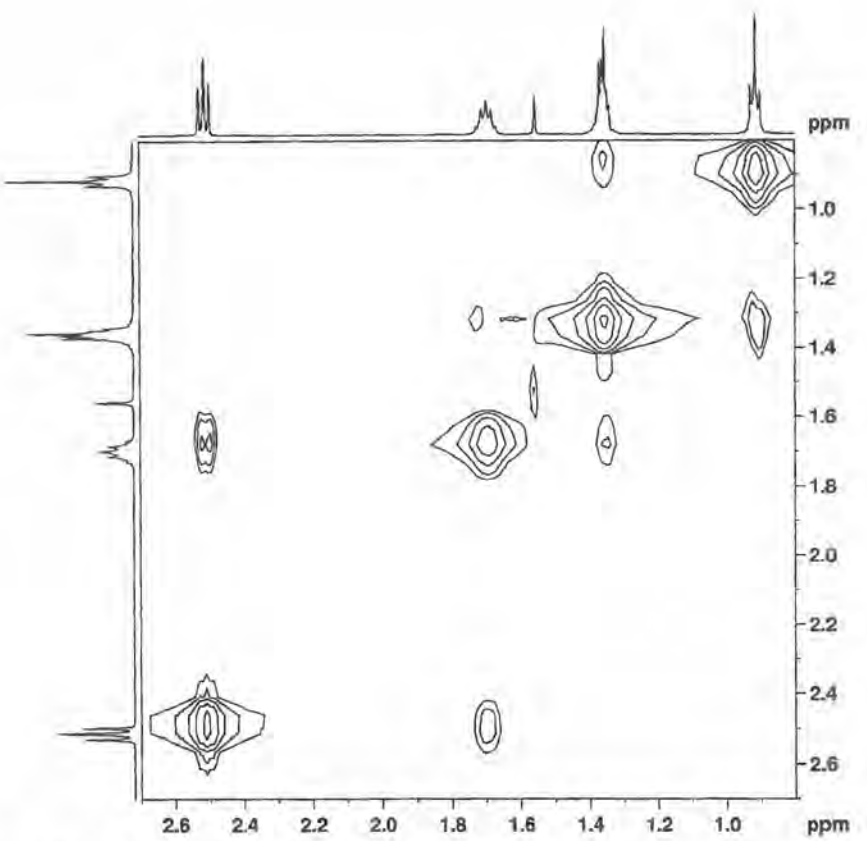


Fig. 3.30 COSY Spectrum of Chlotessmin (3.15)

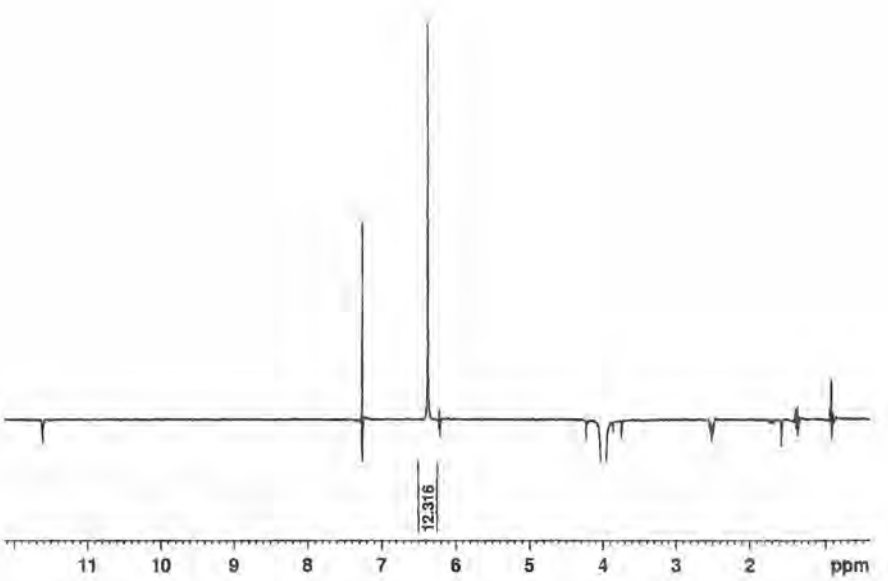


Fig. 3.31 NOEDIFF Spectrum of Chlotessmin (3.15)

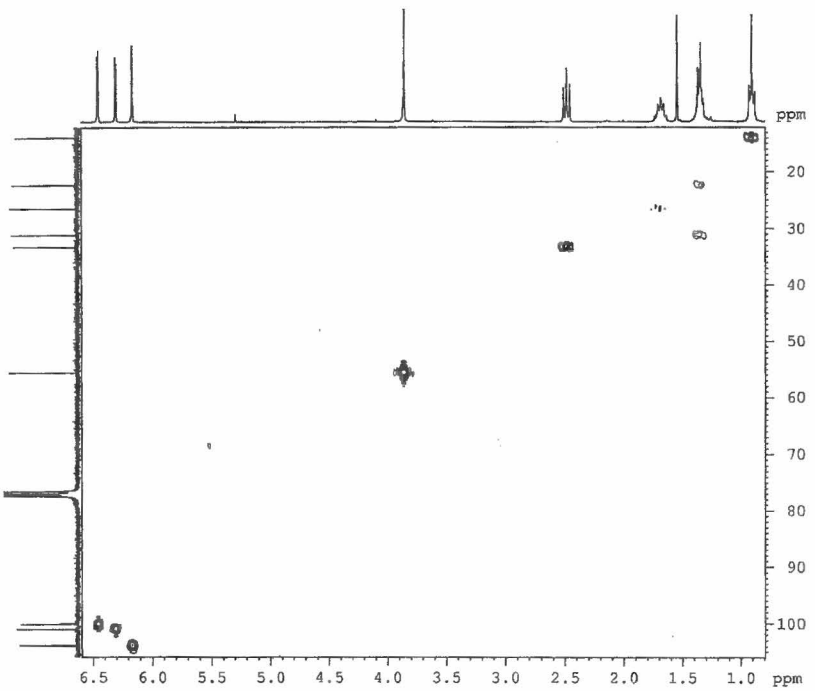


Fig. 3.32 HMQC Spectrum of 8-Hydroxy-6-methoxy-3-pentylisocoumarin (3.14)

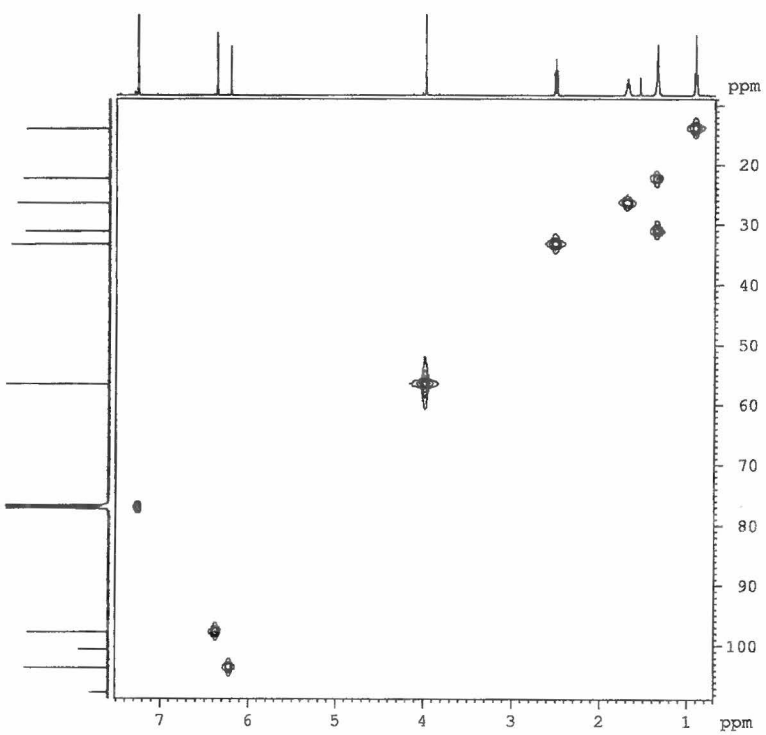


Fig. 3.33 HMQC Spectrum of Chlotessmin (3.15)

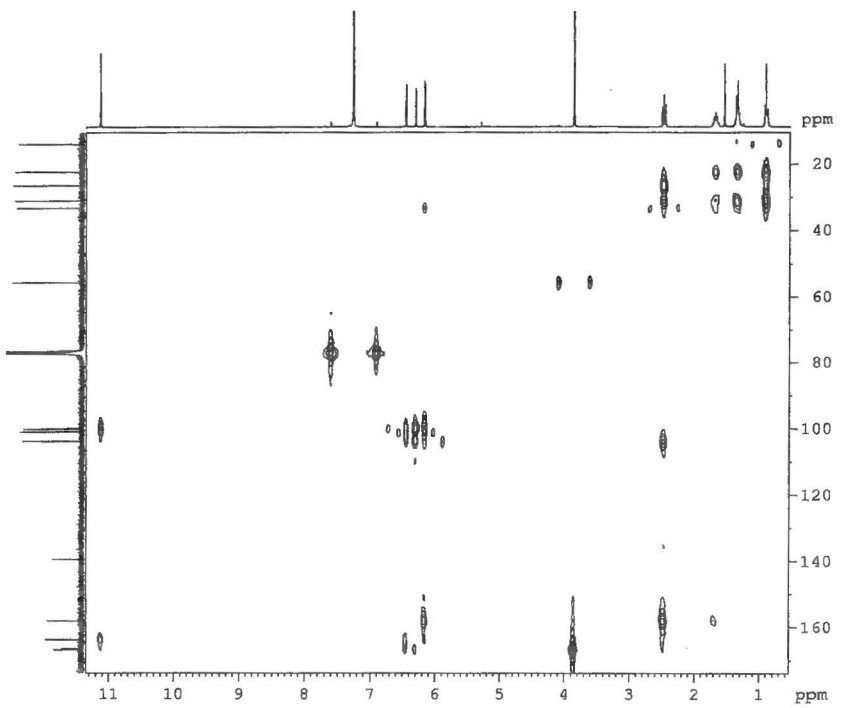


Fig. 3.34 HMBC Spectrum of 8-Hydroxy-6-methoxy-3-pentylisocoumarin (3.14)

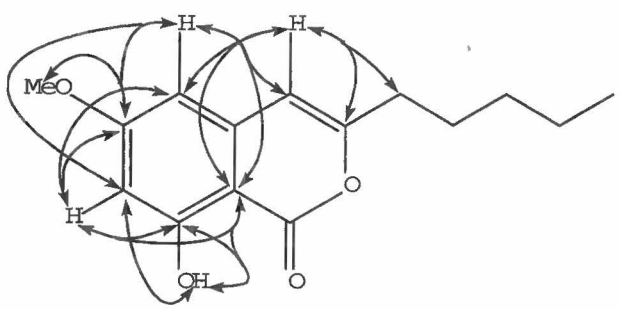


Fig. 3.34a Important Long Range H/C HMBC Correlations for Compound 3.14

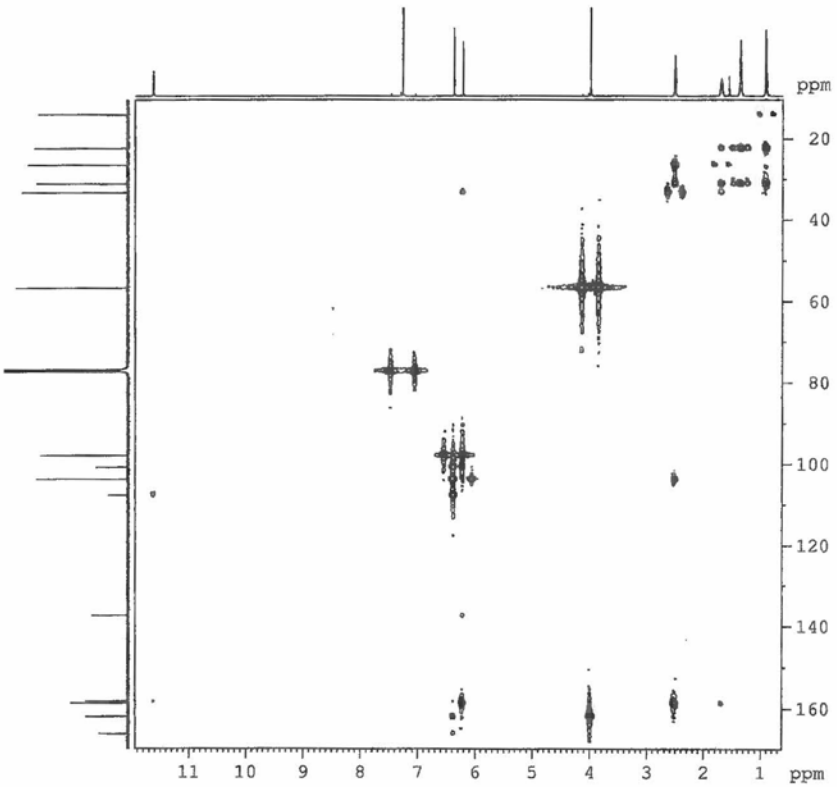


Fig. 3.35 HMBC Spectrum of Chlotessmin (3.15)

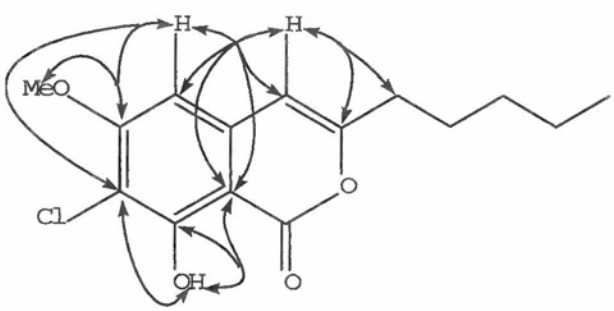


Fig. 3.35a Important Long Range H/C HMBC Correlations for Compound 3.15

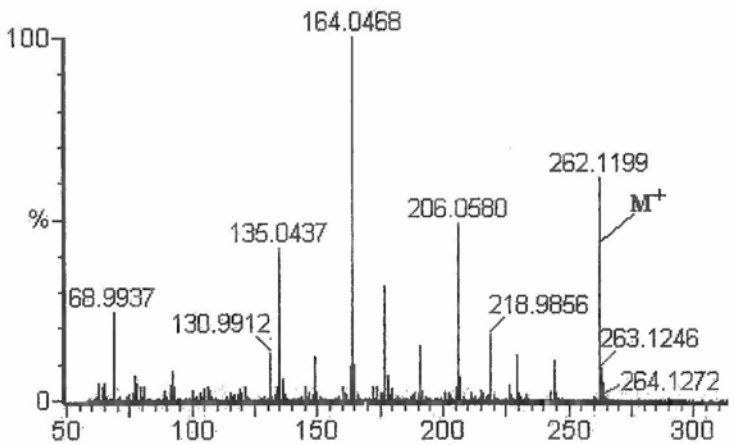
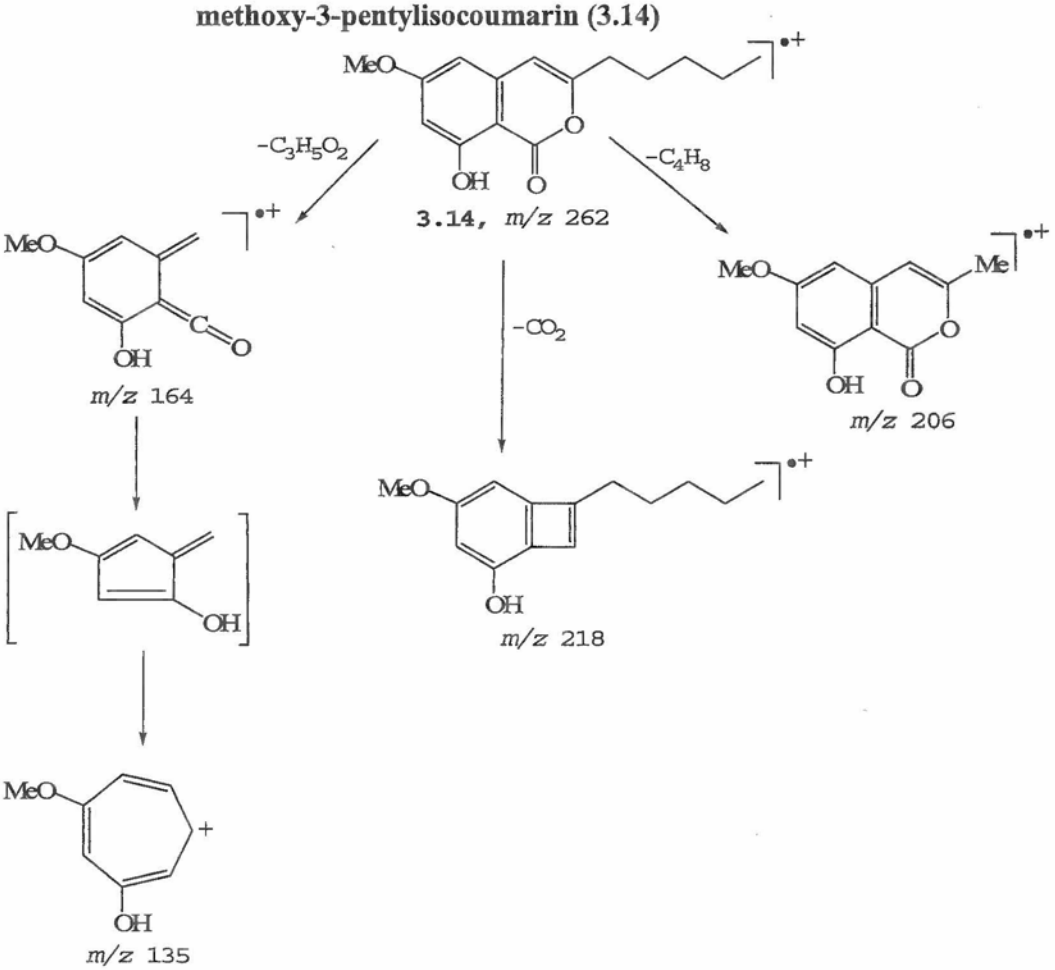


Fig. 3.36 Mass Spectrum of 8-Hydroxy-6-methoxy-3-pentylisocoumarin (3.14)

Scheme 3.6 Mass Spectral Fragmentation Pattern for 8-Hydroxy-6-methoxy-3-pentylisocoumarin (3.14)



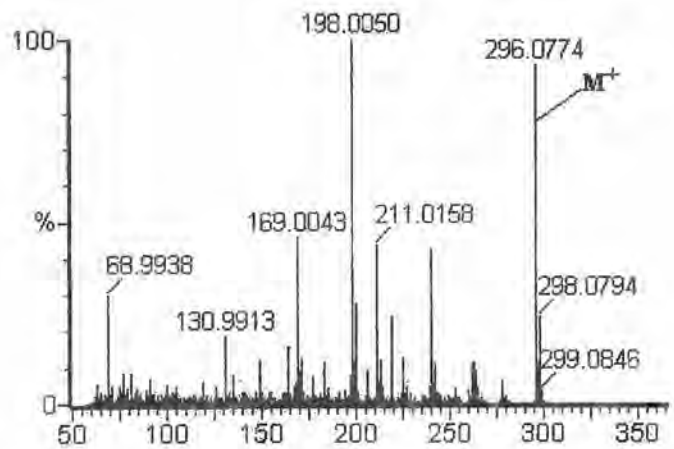
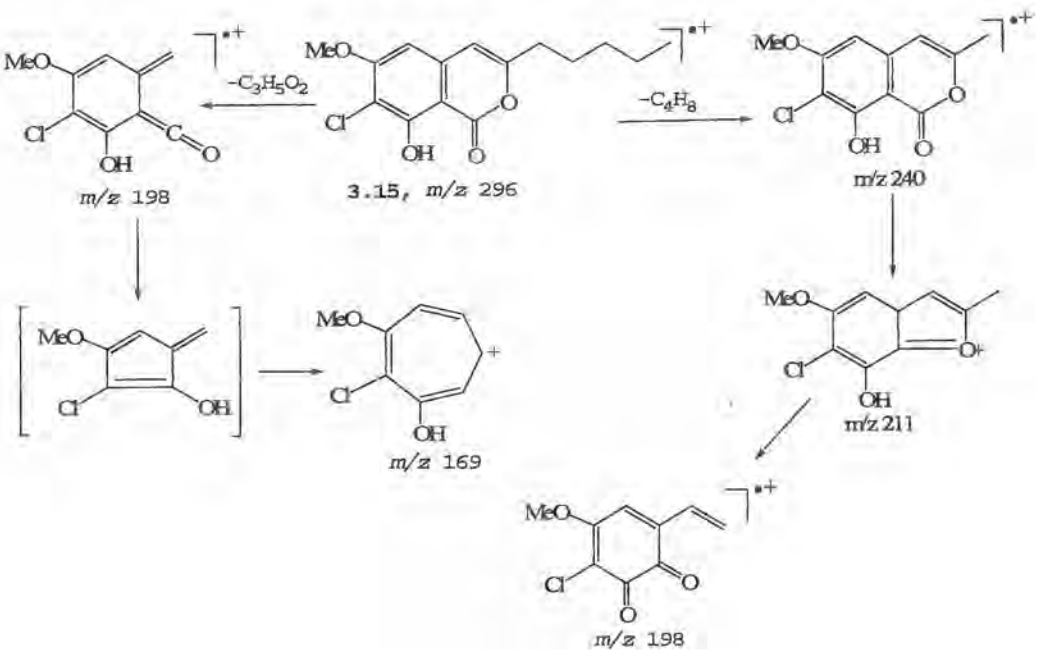


Fig. 3.37 Mass Spectrum of Chlotessmin (3.15)

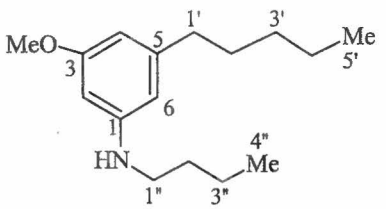
Scheme 3.7 Mass Spectral Fragmentation Pattern for Chlotessmin (3.15)



3.2.1.4 5-Pentyl-3-methoxy-*N*-butylaniline (3.16)

Repeated chromatography of the VLC fraction 5 of the chloroform extract of the stem bark of *Tessmannia densiflora* yielded a yellow oil, whose structure **3.16** was deduced upon analysis of its spectroscopic data (^{1}H and ^{13}C NMR, and MS). Thus, the MS (Fig. 3.43 and Scheme 3.8) exhibited the base peak at *m/z* 138 which is

consistent with the molecular formula $C_{16}H_{27}NO$. The ^{13}C NMR spectrum exhibited only six signals in the aromatic region, which, together with the presence of three ^{1}H NMR resonances in that region of the spectrum indicated that the compound consisted of a benzene ring, having three of the six C-atoms unsubstituted (δ_H 6.25, t, 1H, J = 2.1 Hz; 6.28, t, 1H, J = 2.1 Hz and 6.31, t, 1H, J = 2.1 Hz; and δ_C 98.7, 108.0 and 106.5 respectively, Table 3.5). The ^{1}H NMR spectrum (Fig. 3.36) further showed a methoxy proton signal at δ 3.76, with the corresponding carbon absorption appearing at δ 160.8 in the ^{13}C NMR spectrum (Fig. 3.39), thus indicating the presence of a methoxy group on the benzene ring.



3.16

The ^{1}H NMR spectrum also showed resonances due to a proton attached to a nitrogen atom at δ_H 2.85 (s, 1H), this being superimposed on a signal due to one of the methylene protons of the side chain that was also connected to the nitrogen atom (Table 3.5).

That the compound consisted of a butyl and pentyl chain attached to the benzene ring and an aromatic amino nitrogen was deduced from the corresponding ^{1}H and ^{13}C NMR signals for the two units (Table 3.5) as further indicated by H/H COSY and HMBC interactions, which also established the relative positions of all the substituents on the benzene ring.

Compound **3.16** is hereby being reported for the first time.

Table 3.5 ^{1}H NMR Spectral Data for 5-Pentyl-3-methoxy-*N*-butylaniline (3.16)

H/C	δ_H	*J* (Hz)	δ_C	H/C	δ_H	*J* (Hz)	δ_C
1	-	-	156.8	3a'	1.29-1.38	m	22.5
2	6.25	t, 2.1	98.7	3b'	2.03	s	
3	-	-	160.8	4'	1.64-1.67	m	25.9
3-OMe	3.76	s	55.2	5'	0.89	t, 7.0	13.7
4	6.28	t, 2.1	108.0	1"	2.85	t, 8.1	52.1
5	-	-	145.7	2"	1.28-1.36	m	31.4
6	6.31	t, 2.1	106.5	3"	1.30-1.44	m	20.3
1'	2.51	t, 8.1	36.0	4"	0.94	t, 7.5	14.0
2'	1.54-1.64	m	30.8	NH	2.85	br s	

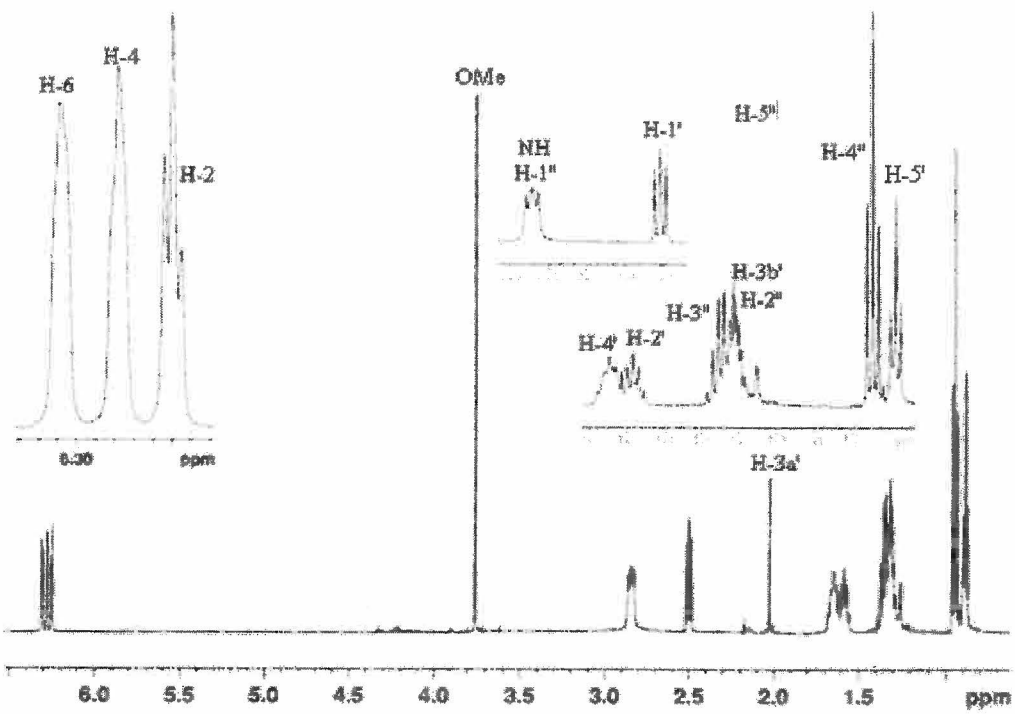


Fig. 3.38 ^{1}H NMR Spectrum of 5-Pentyl-3-methoxy-*N*-butylaniline (3.16)

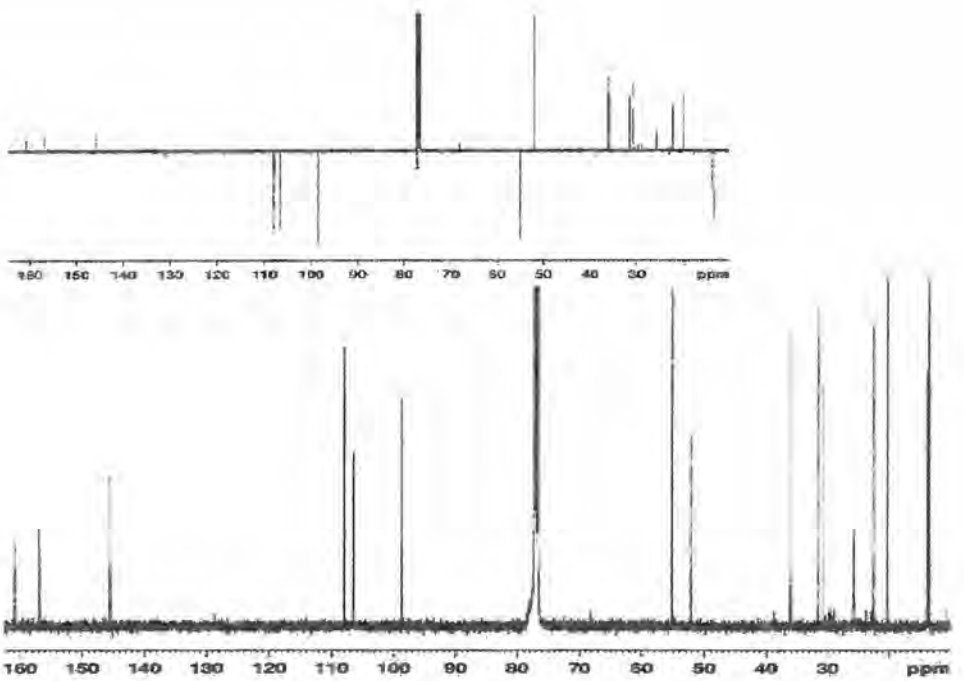


Fig. 3.39 ^{13}C NMR Spectrum of 5-Pentyl-3-methoxy-*N*-butylaniline (3.16)

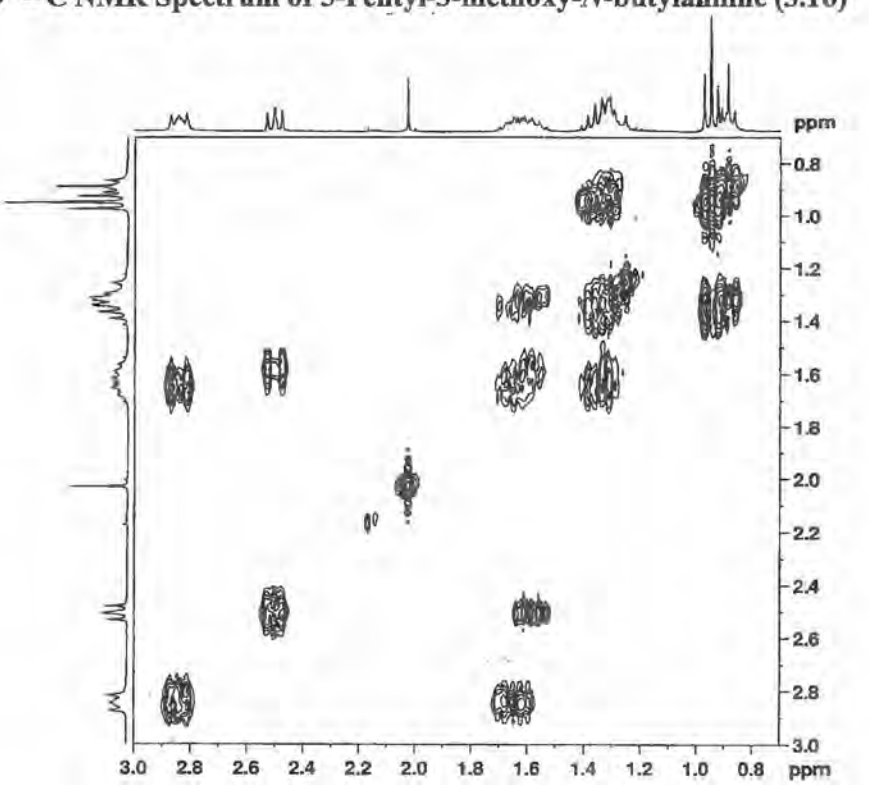


Fig. 3.40 COSY Spectrum of 5-Pentyl-3-methoxy-*N*-butylaniline (3.16)

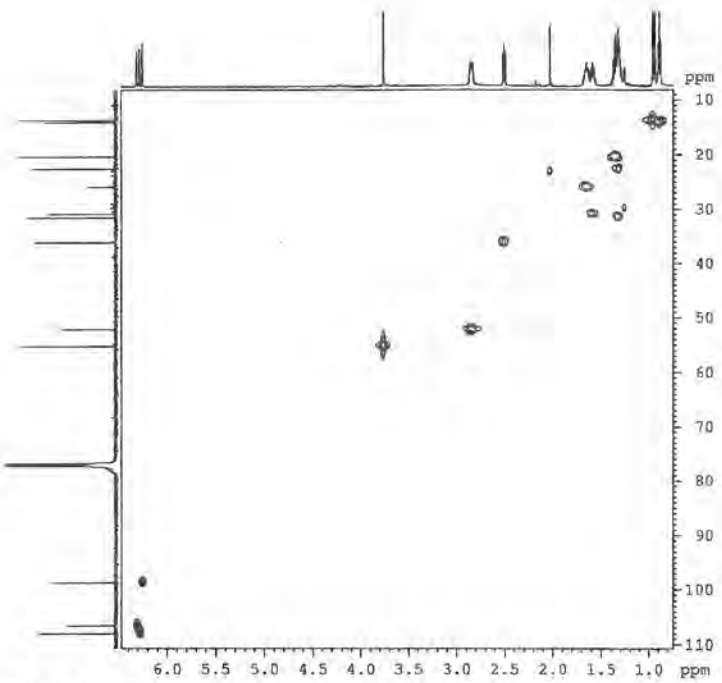


Fig. 3.41 HMQC Spectrum of 5-Pentyl-3-methoxy-*N*-butylaniline (3.16)

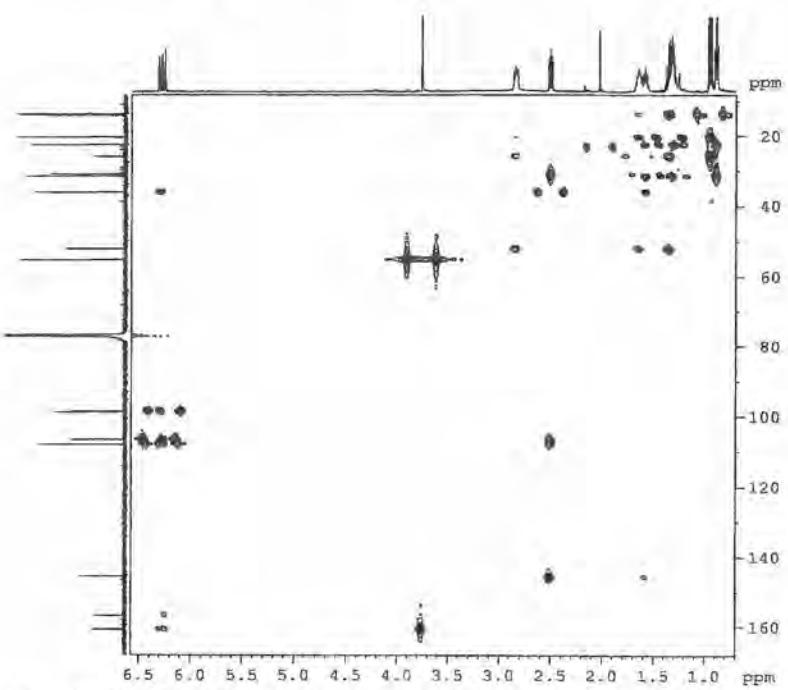


Fig. 3.42 HMBC Spectrum of 5-Pentyl-3-methoxy-*N*-butylaniline (3.16)

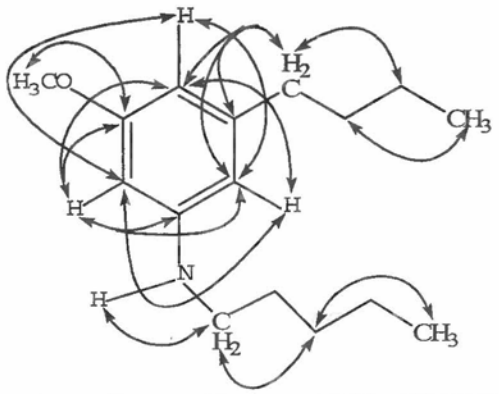


Fig. 3.42a Important Long Range H/C HMBC Correlations for Compound 3.16

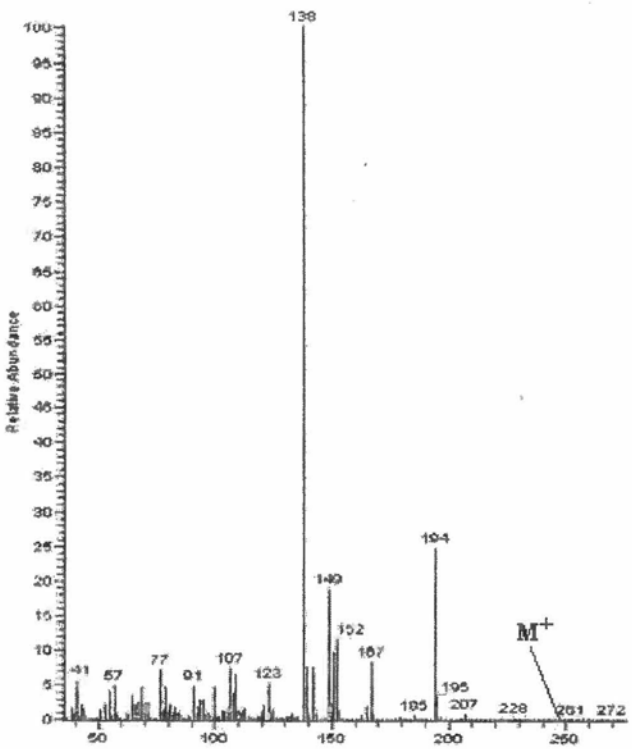
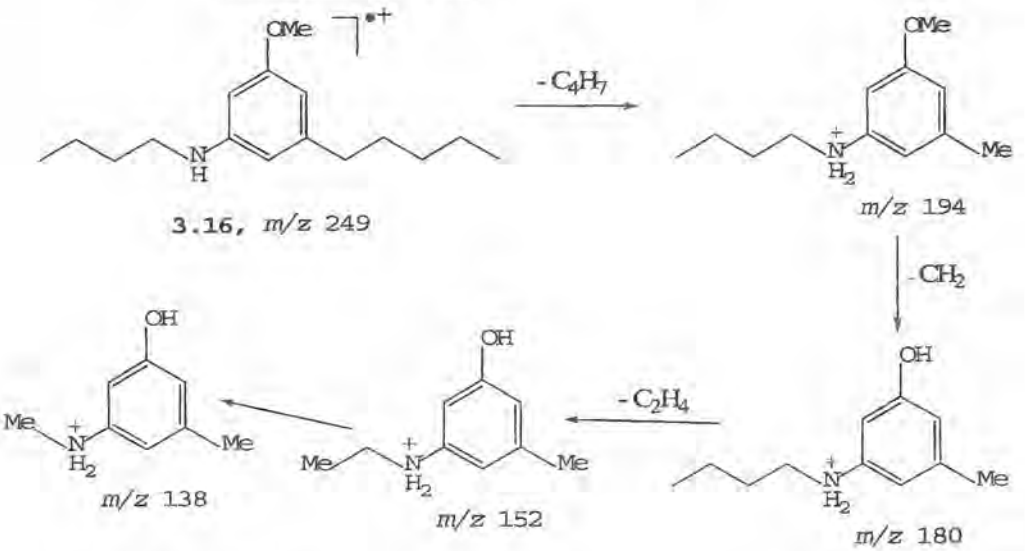


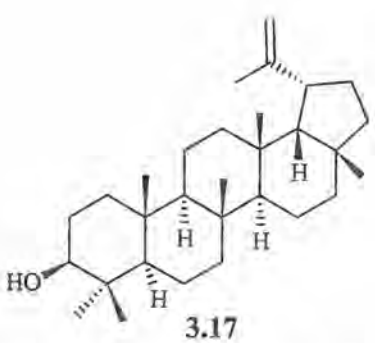
Fig. 3.43 Mass Spectrum of 5-Pentyl-3-methoxy-*N*-butylaniline (3.16)

Scheme 3.7 **Mass Spectral Fragmentation Pattern for 5-Pentyl-3-methoxy-*N*-butylaniline (3.16)**



3.2.1.5 Lupeol (3.17)

Repeated chromatography of VLC fraction 5 of the chloroform extract of the stem bark of *Tessmannia densiflora* yielded lupeol (**3.17**) as white powder, m.p. 197-198 °C (Lit.[145] m.p. 206-208 °C) whose spectral properties (^{1}H and ^{13}C NMR, COSY, HMQC, HMBC and MS) were identical with those previously reported for lupeol.[144,145]



3.17

Since lupeol is a well known natural products, it was not found necessary to give extensive spectral description so as to confirm the structure of this compound which previously had been isolated from a number of higher plants, including *Euclea*

natalensis (Ebenaceae),[146] and some *Diospyros* species,[147] *Gossampinus malabarica* (Bombacaceae),[148] and *Uvaria scheffleri* (Annonaceae).[149] The compound has been reported to exhibit antiinflamatory[150] as well as anti-leukemia activity by inducing apoptosis in human leukemia HL-60 cells.[144]

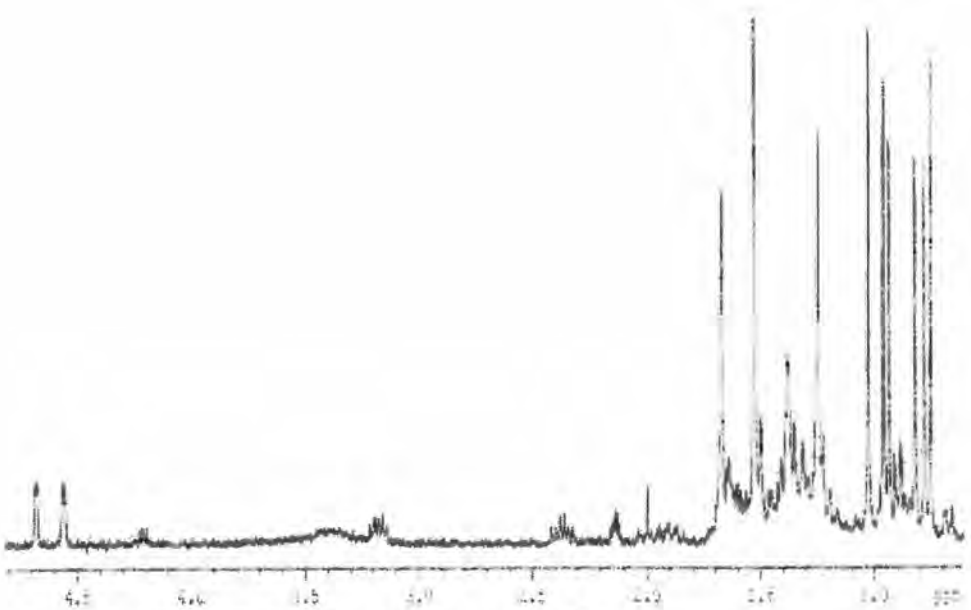


Fig. 3.44 ^{1}H NMR Spectrum of Lupeol (3.17)

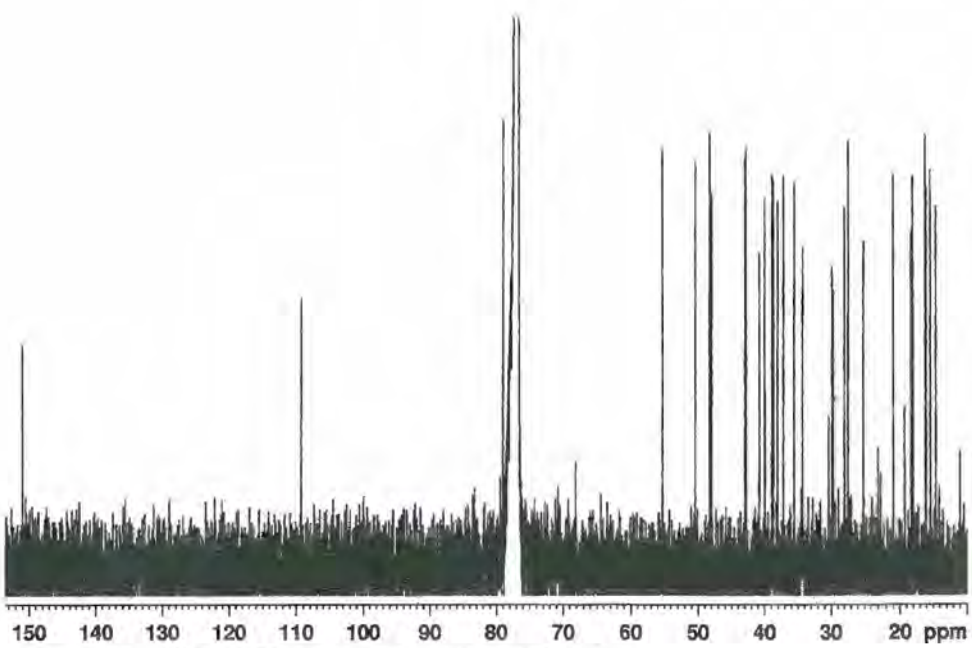


Fig. 3.45 ^{13}C NMR Spectrum of Lupeol (3.17)

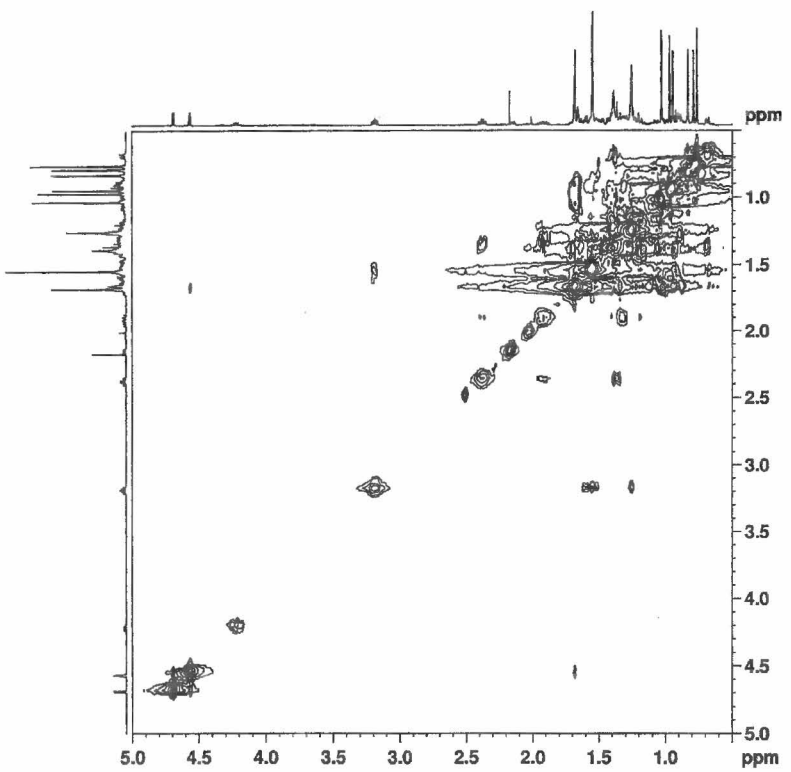


Fig. 3.46 COSY Spectrum of Lupeol (3.17)

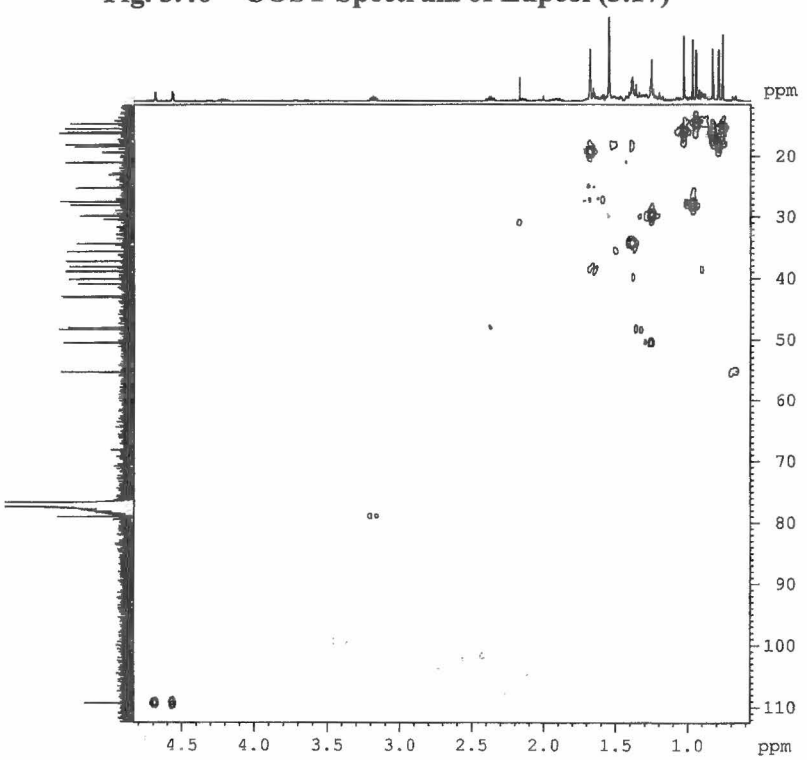


Fig. 3.47 HMQC Spectrum of Lupeol (3.17)

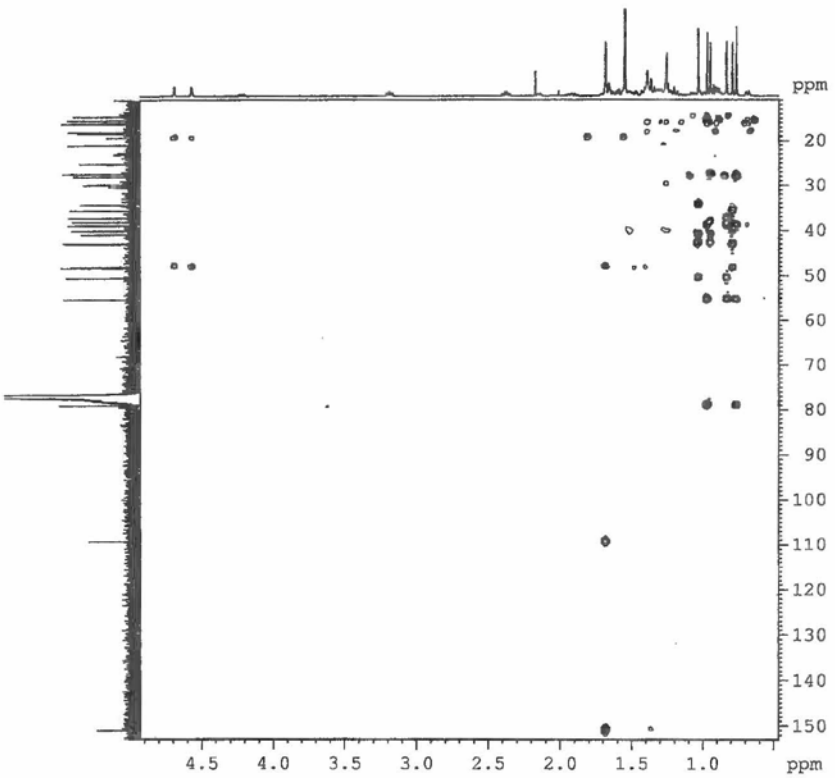


Fig. 3.48 HMBC Spectrum of Lupeol (3.17)

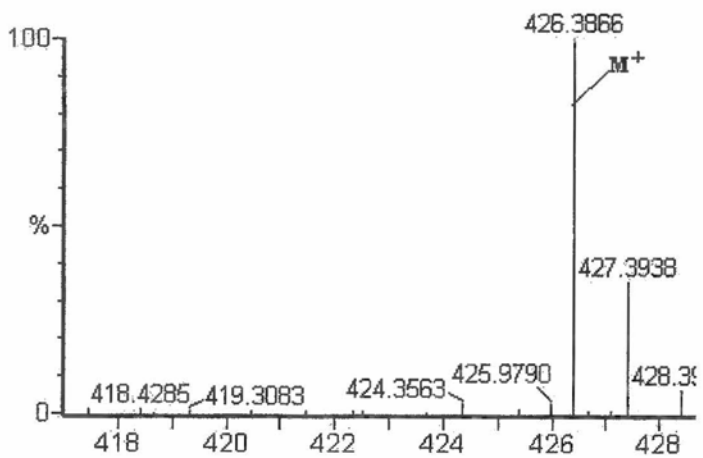
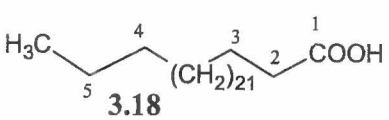


Fig. 3.49 Mass Spectrum of Lupeol (3.17)

3.2.1.6 Heptacosanoic acid (3.18)

Repeated chromatography of VLC fraction 2 from the chloroform extract of the stem bark of *Tessmannia densiflora* yielded white crystals, m.p. 87-90 °C which were shown to constitute a long chain fatty acid whose structure **3.18** was established upon analysis of ^{1}H and ^{13}C NMR, and mass spectral data (Table 3.5, Figs. 3.46, 3.47 and 3.48). Thus, both ^{1}H and ^{13}C NMR spectra showed resonances due to terminal methyl protons at δ_H 0.88 (t, 3H, J = 6.9) and δ_C 14.1. The NMR spectrum also displayed signals due to two sets of methylene protons next to the terminal methyl group at δ_H 1.25 (m, 2H) and δ_C 31.9, and δ_H 1.25 (m, 2H) and δ_C 22.9. The ^{13}C NMR spectrum further showed a signal due to a carboxylic group at δ_C 179.2. Signals due to two sets of methylene group at δ_H 2.35 (t, 2H, J = 7.5) and δ_C 33.9, and δ_H 1.63 (quintet, 2H, J = 7.5) and δ_C 24.7 were assigned to α and β methylene groups relative to carbonyl carbon. The ^{1}H and ^{13}C NMR spectra further showed signals due to several sets of methylene groups at δ_H 1.25 (m, 2H) and δ_C 26.0-29.7, the exact number of the methylene groups was established on the basis of the MS (Fig. 3.52).



3.18

Table 3.5 ^{1}H NMR Spectral Data for Heptacosanoic acid (3.18)

H/C	δ_H	J (Hz)	δ_C
1	-	-	179.2
2	2.35	t, 7.5	33.9
3	1.63	quintet, 7.5	24.7
$(CH_2)x$	1.25	m	26.0-29.7
4	1.25	m	31.9
5	1.25	m	22.9
Me	0.88	t, 6.9	14.1

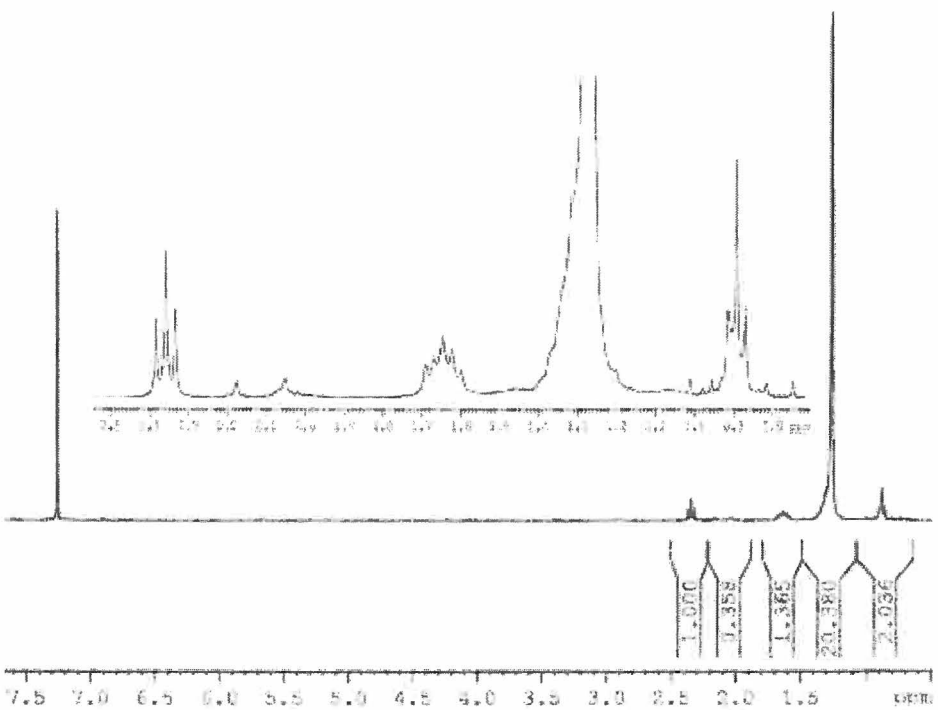


Fig. 3.50 ^{1}H NMR Spectrum of Heptacosanoic acid (3.18)

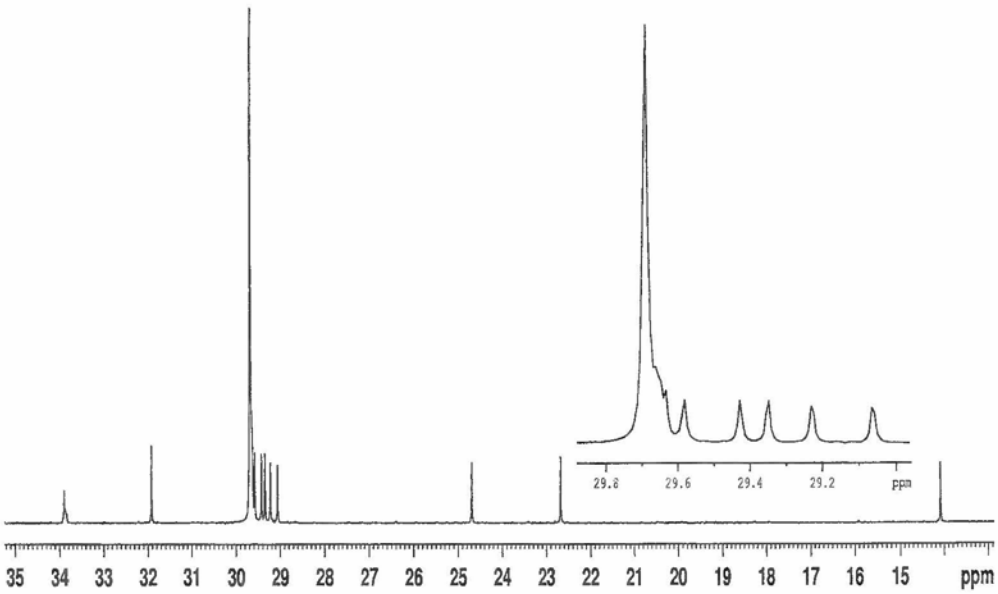


Fig. 3.51 ^{13}C NMR Spectrum of Heptacosanoic acid (3.18)

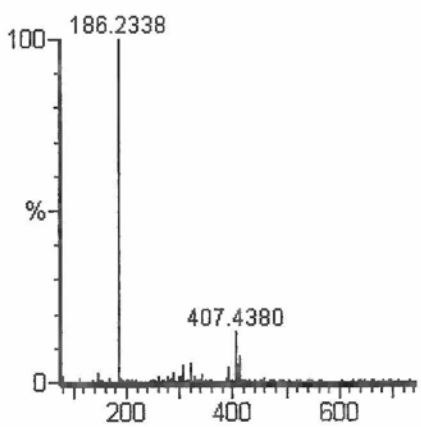


Fig. 3.52 Mass Spectrum of Heptacosanoic acid (3.18)

3.3 EXPERIMENTAL

3.3.1 General

Solvents: Analytical grade and/or distilled solvents were used throughout the investigations. These included petroleum ether (b.p. 40-60°C), n-hexane, toluene, ethyl acetate, *tert*-butyl methyl ether, dichloromethane, chloroform, methanol and

ethanol. Distilled water was also used in reversed phase column chromatography and HPLC.

Spectra: Infrared (IR) spectra, taken for chloroform solutions, were recorded on a Shimadzu Model IR-435 spectrophotometer with absorptions given in wave numbers (cm^{-1}). ^{1}H NMR Spectra were recorded on Bruker AVANCE 300 or 500 spectrometers operating at 300 and 500 MHz, using CD_2Cl_2, $CDCl_3$ or CD_3OD as solvents. Chemical shifts are given in δ values relative to internal standard TMS (δ = 0 ppm). Absorptions are designated as *s* for singlet, d for doublet, t for triplet, q for quartet, m for multiplet, etc. Coupling constants (*J*) are given in Hz. ^{13}C NMR Spectra were also recorded on a Bruker AVANCE 300 or 500 Spectrometers operating at 75 or 125 MHz and CD_2Cl_2, $CDCl_3$ or CD_3OD were used as solvents. Chemical shifts are given in δ values relative to the internal standard TMS, δ [TMS = δ ($CDCl_3$) +76.9 ppm]. Mass spectra were determined on DSQII (Axel Semrau GmbH), GC-TOF (micromass), and Q-TOF micro (micromass) equipment. ^{1}H and ^{13}C NMR and mass spectra were recorded at the University of Potsdam in Germany.

Chromatography: Thin layer chromatography (TLC) was carried out using both plastic and aluminium sheets pre-coated with silica gel (60 F_{254}, Merck) with layer thickness of 0.2 mm. In some cases reversed phase plates were used. After eluting with a proper solvent system, the plates were visualised under UV light (at 254 and 366 nm). This was followed by placing them in an iodine tank, or spraying with an anisaldehyde reagent (prepared by mixing 95 ml of absolute ethanol, 5 ml of glacial acetic acid, 5 ml of anisaldehyde and 5 ml of concentrated sulphuric acid) then heated at about 100°C for a few minutes.

Vacuum liquid chromatography (VLC) was performed using normal phase silica gel of particle size 230-400 mesh ASTM (Merck). The vacuum was generated by a vacuum pump. Preparative thin layer chromatography was done either by using normal or reversed phase silica gel (F_{254}, Merck) pre-coated on glass plates (20 x 20 cm) with a layer thickness of 0.25, 0.5, 1.0 and 2.0 mm. Detection was carried out under UV light at 254 or 366 nm wavelength.

Several chromatographic techniques were used, including gravitational column chromatography which was carried out using glass columns wet packed with silica gel of particle size of 230 or 400 Mesh ASTM (Merck). Gel filtration chromatography was performed using Sephadex® LH-20 (Pharmacia), eluting with methanol or a 1:1 mixture of methanol and chloroform.

Melting points were determined on an Electrothermal melting point apparatus and are uncorrected. Optical rotation values were recorded from PerkinElmer model 343 polarimeter.

3.3.2 Biological Tests

These were conducted as described in Chapters 2 and 6.

3.3.3 Plant Materials

The root and stem barks of *Tessmannia densiflora* Harms were collected from Kichi Hills near Selous Game Reserve, Rufiji District, Coast Region in Tanzania, in January 2004 and March 2007. The plant species was authenticated at the Herbarium of the Department of Botany, University of Dar es Salaam, Tanzania where a voucher specimen is preserved (Mbago: FMM 3440).

3.3.4 Extraction and Isolation of Compounds

3.3.4.1 Chloroform Extract of the Root Bark of *Tessmannia densiflora* Harms

The air-dried and pulverized root bark was extracted sequentially using chloroform and methanol, 2 x 48 h for each solvent. The crude extracts were dried by concentrating under reduced pressure in a rotary evaporator while maintaining the water bath temperature below 40 °C in order to avoid decomposition of thermally labile compounds. The resulting chloroform and methanol extracts were kept at -20 °C until the isolation process was undertaken.

The crude chloroform extract (18 g) was fractionated by vacuum liquid chromatography (VLC) on silica gel, eluting with petroleum ether, and then petroleum ether containing increasing amounts of ethyl acetate. The first three fractions consisted of least and medium polar compounds which were active against *An. gambaiae* larvae, the activity increasing with time. Fraction three was the most active, showing LC_{50} values of 122, 45 and 40 ppm after 24, 48 and 72 h exposure, respectively. LC_{50} values for fractions one and two ranged between 37–265 ppm after 24, 48 and 72 h (Table 2.2, Chapter 2). The remaining fractions were not active and TLC analysis indicated them to consist of complex mixtures of polar compounds. Therefore, no further analysis was done for these fractions, either to isolate compounds or any other biological assay.

Silica gel chromatography of the combined active fractions, eluting with a mixture of ethyl acetate in petroleum ether (3:7 v/v) yielded 23 fractions. The fractions were combined on the basis of TLC profiles to give six combined fractions 1.1, 1.2, 1.3, 1.4, 1.5, 1.6 and 1.7. The combined fraction 1.1 consisted of a complex mixtures of

non-polar compounds, while sub fractions 1.3 and 1.6 were obtained as mixture and in small quantities. Therefore, these were not analysed further. Fraction 1.2 was obtained as greenish oil which was found to constitute compound **3.2**, while compound **3.1** was obtained from fraction 1.7 as UV negative green oil. The compound was also obtained in larger quantity from VLC fractions 2 and 3 upon repeated CC on silica gel eluting with petroleum ether and then petroleum ether containing increasing amount of ethyl acetate. An overview of the chromatographic separation of the chloroform extract is given in Chart 3.1.

3.3.4.2 Chloroform Extract of the Stem Bark of *Tessmannia densiflora* Harms

The air dried and pulverized stem bark of *Tessmannia densiflora* was extracted sequentially with chloroform and methanol, 2 x 48 h for each solvent. The crude extracts were dried by concentrating under reduced pressure in a rotary evaporator while maintaining the water bath temperature below 40 °C in order to avoid decomposition of thermally labile compounds. The resulting chloroform and methanol extracts were kept at -20 °C until the isolation process was undertaken.

The crude chloroform extract (30 g) was fractionated by VLC on silica gel, eluting with petroleum ether, and then petroleum ether containing increasing amounts of ethyl acetate, and then ethyl acetate containing increasing amounts of methanol. Nineteen fractions were obtained and TLC analysis led to combination of these fractions into five series as follows: Fractions 1, 2 (2-4), 3 (5-8), 4 (9-14) and 5 (15-19). The first, third and fourth combined series consisted of complex mixture of non- and medium polar compounds which decomposed on TLC analysis. Therefore these were not analysed any further.

Silica gel chromatography of the second series (combined fractions 2-4), eluting with 5% ethyl acetate in petroleum ether yielded thirty four fractions. The fractions were combined on the basis of TLC analysis to give six combined fractions 2.1 (1-3), 2.2 (4-11), 2.3 (12-16), 2.4 (17-22), 2.5 (23-25) and 2.6 (26-34). Combined fraction 2.4 formed white crystals that were identified as constituting compound **3.18**. The resulting filtrate upon removal of **3.18** was worked up on Sephadex® LH-20 chromatography eluting with a mixture of methanol and chloroform (1:1 v/v) and gave thirteen fractions which were combined on the basis of TLC analysis to yield five combined fractions 2.4.1 (1-3), 2.4.2 (4-7), 2.4.3 (8-9), 2.4.4 (10-11) and 2.4.5 (12-13). Fraction 2.4.2 on repeated chromatography yielded compound **3.17** which crystallized out as white crystals upon standing of the fraction.

Silica gel chromatography of the combined fraction 2.5 eluting with 5% ethyl acetate in petroleum ether yielded twenty four fractions, which were then combined into five fractions based on TLC analysis as follows: 2.5.1 (1-2), 2.5.2 (3-7), 2.5.3 (8-15), 2.5.4 (16-20) and 2.5.5 (21-24). Combined fractions 2.5.2 and 2.5.4 were found to constitute compounds **3.12** and **3.13** respectively.

The combined VLC fraction 5 was also subjected to silica gel column chromatography eluting with 10% ethyl acetate in petroleum ether and 21 fractions that were obtained were combined to 4 fractions based on TLC analysis, as 5.1 (1-10), 5.2 (11-15), 5.3 (16-17) and 5.4 (18-21). The combined fractions 5.1 was an oil which displayed a single spot on TLC analysis and later crystallized into white plates of compounds **3.14.** Combined fraction 5.2 formed white plates which were filtered and washed with petroleum ether to yield compound **3.15.** The resulting filtrate

consisted of compounds that decomposed on both silica gel and Sephadex® LH-20. Thus, normal phase preparative TLC was used to isolate compound **3.16**. All other combined fractions in the first fractionation process could not be worked up due to their complexity and decomposition during chromatographic separation.

An overview of the chromatographic separation of the chloroform extract is given in Chart 3.2.

Chart 3.1 Isolation of Compounds 3.1, 3.2 and 3.3 from the Root Bark of *Tessmannia densiflora* Harms

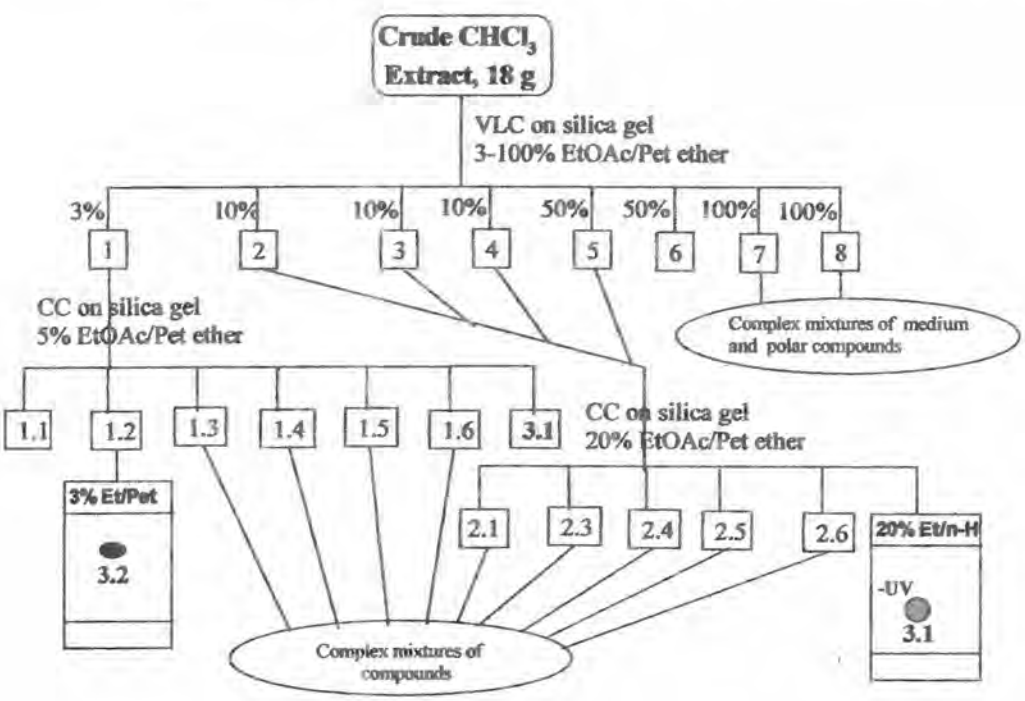
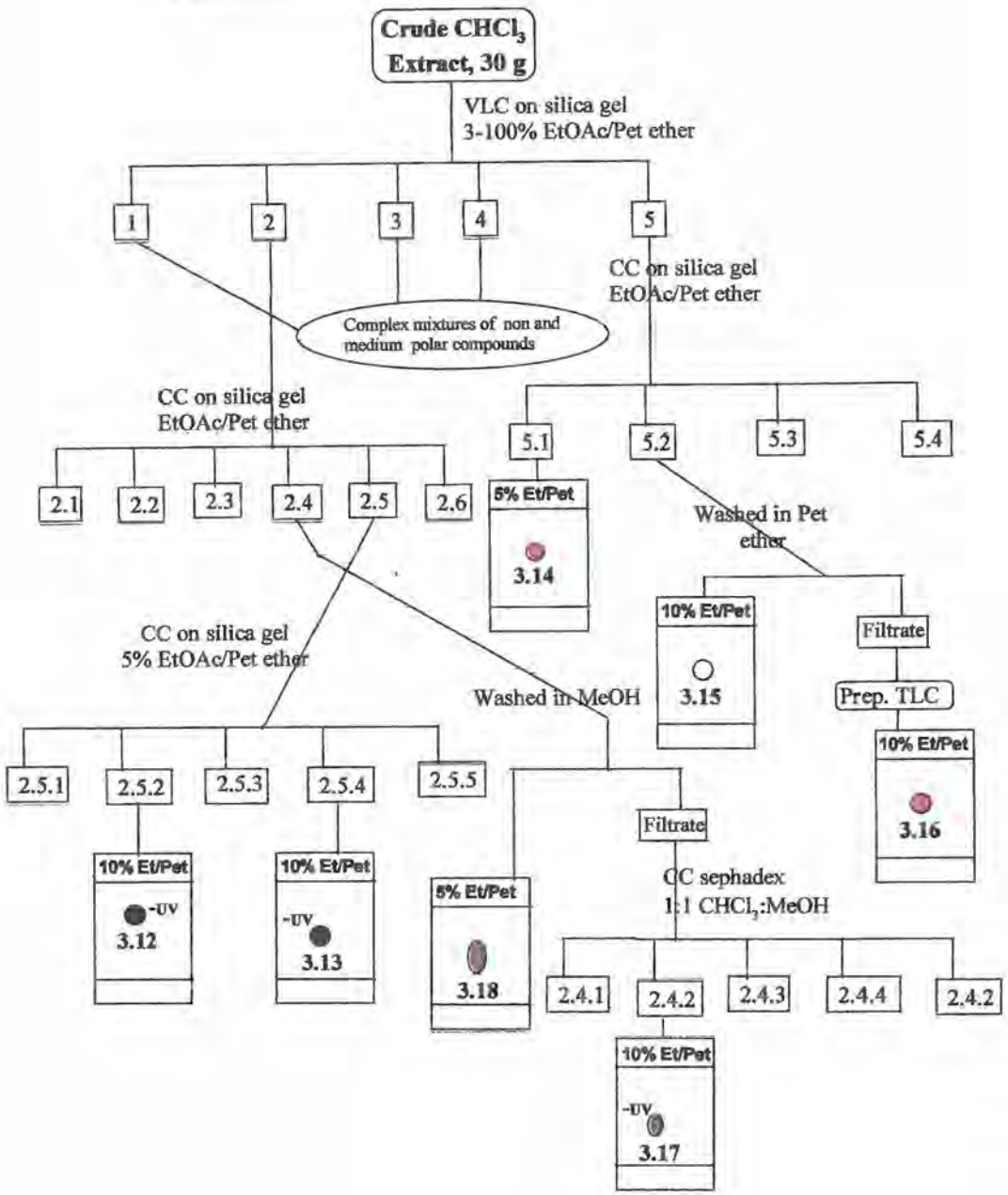


Chart 3.2 **Isolation of Compounds 3.1, 3.2 and 3.3 from the Stem Bark of *Tessmannia densiflora* Harms**



Tessmannic acid **(3.1)**. Greenish oil; UV negative; yield, 490 mg; anisaldehyde: green; $[\alpha]_D^{20}$ = +14° ($CHCl_3$, c = 0.07); IR, ν_{max} cm^{-1}: 3434 (OH), 1708 (CO) and 755 (furan); MS, *m/z* (% rel. int.) 318 (M^+, 100), 303 (18), 245 (5) and 223 (35); ^1H and ^{13}C NMR: See Table 3.1 and 3.2.

Methyltessmannoate **(3.2)**. Greenish oil; UV negative; yield, 575 mg; anisaldehyde: green; $[\alpha]_D^{20} = +18°$ ($CHCl_3$, c = 0.16); MS, *m/z* (% rel. int.) 332 (M^+, 13), 245 (5) and 237 (48); ^1H and ^{13}C NMR: See Table 3.1 and 3.2.

2-Methylpropyltessmannoate **(3.12)**. Oil; UV negative; yield, 20 mg; anisaldehyde: dark green; $[\alpha]_D^{20} = +14°$ ($CHCl_3$, c = 0.038); MS, *m/z* (% rel. int.) 318 ($[M-42]^+$, 55), 303 (9), 245 (5) and 223 (85); ^1H and ^{13}C NMR: See Table 3.3.

1-Methylbutyltessmannate **(3.13)**. Oil; UV negative; yield, 5 mg; anisaldehyde: dark green; $[\alpha]_D^{20} = +18°$ ($CHCl_3$, c = 0.03); MS, *m/z* (% rel. int.) 318 ($[M-70]^+$, 16), 303 (5), 245 (3) and 223 (56); ^1H and ^{13}C NMR: See Table 3.3.

8-Hydroxy-6-methoxy-3-pentylisocoumarin **(3.14)**. White crystals; m.p. 79-80 °C; yield, 10 mg; anisaldehyde: pink; MS, *m/z* (% rel. int.) 262 (M^+, 60), 218 (18), 206 (50), 164 (100) and 135 (42); ^1H and ^{13}C NMR: See Table 3.4.

7-Chloro-8-Hydroxy-6-methoxy-3-pentylisocoumarin **(3.15)**. White crystals; m.p. 170-173 °C; yield, 21 mg; anisaldehyde: no reaction; MS, *m/z* (% rel. int.) 296 (M^+, 90), 211 (40), 198 (100) and 169 (47); ^1H and ^{13}C NMR: See Table 3.4.

5-Pentyl-3-methoxy-N-butylaniline **(3.16)**. Oil; yield, 5 mg; anisaldehyde: orange; MS, *m/z* (% rel. int.) 194 ($[M-45]^+$, 25), 152 (12) and 138 (100); ^1H and ^{13}C NMR: See Table 3.5.

Lupeol **(3.17)**. White crystals; m.p. 196-197 °C (Lit.[144] 206-208 °C); UV negative; yield, 5 mg; anisaldehyde: light blue; MS, *m/z* (% rel. int.) 426 (M^+, 11); ^1H and ^{13}C NMR: See Figs. 3.1 and 3.2.

Heptacosanoic acid **(3.18)**. White crystals, m.p. 92-93 °C; yield, 21 mg; anisaldehyde: purple; MS: see Fig. 3.52; ^1H and ^{13}C NMR: See Table 3.6.

CHAPTER FOUR

NOVEL CLERODANE DITERPENES AND A CHLORINATED BENZENOID FROM *TESSMANNIA MARTINIANA* VAR *PAULOI* HARMS AND *T. MARTINIANA* VAR *MARTINIANA* HARMS

Abstract

This chapter discusses the isolation and structural determination of the clerodane diterpenoids *cis*-kalavenolic acid, 18-oxocleroda-3,13(*E*)-dien-15-oic acid, *ent*-(18-hydroxycarbonyl)-cleroda-3,13(*E*)-dien-15-oate, (9-*epi*)-*ent*-(18-hydroxycarbonyl)-cleroda-3,13(*E*)-dien-15-oate, 2-oxo-*ent*-cleroda-3,13(*Z*)-dien-15-oic acid, *cis*-2-oxo-*ent*-cleroda-13(*Z*)-en-15-oic acid, and *O*-(3-hydroxy-4-hydroxycarbonyl-5-pentylphenyl)-3-chloro-4-methoxy-5-pentyl-2-oxybenzoic acid from the two Tanzanian *Tessmannia* species (Leguminosae) *T. martiniana* var *pauloi* and *T. martiniana* var *martiniana*. Some of the compounds exhibited antimosquito, antifungal and antibacterial activity, results of which are reported in chapter 6 of this thesis. Structural determination was achieved based on interpretation of spectroscopic data, which also revealed the relative stereochemistry for the isolated compounds. *cis*-Kolavenolic acid, *cis*-2-oxo-*ent*-cleroda-13(*Z*)-en-15-oic acid and *O*-(3-hydroxy-4-hydroxycarbonyl-5-pentylphenyl)-3-chloro-4-methoxy-5-pentyl-2-oxybenzoic acid are hereby being reported for the first time.

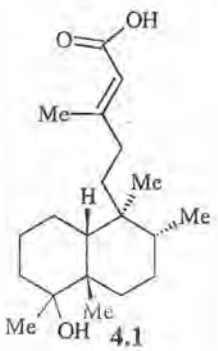
4.1 INTRODUCTION

Tessmannia martiniana var *pauloi* Harms and *T. martiniana* var *martiniana* Harms (Fig. 4.1 and 4.2) are among the four species in the genus *Tessmannia* which are endemic to Tanzania (more details in section 3.1), none of which has been investigated for chemical constituents. Based on the preliminary larvicidal assay whose results are reported in Chapter 2 the two plant species were investigated for their active constituents, and the six *ent*-clerodane diterpenoids *cis*-kolavenolic acid (**4.1**), 18-oxocleroda-3,13(*E*)-dien-15-oic acid (**4.2**), *ent*-(18-hydroxycarbonyl)-cleroda-3,13(*E*)-dien-15-oate (**4.3**), (9-*epi*)-*ent*-(18-hydroxycarbonyl)-cleroda-3,13(*E*)-dien-15-oate (**4.4**), 2-oxo-*ent*-cleroda-3,13(*Z*)-dien-15-oic acid (**4.5**), *cis*-2-oxo-*ent*-cleroda-13(*Z*)-en-15-oic acid (**4.6**) and *O*-(3-hydroxy-4-hydroxycarbonyl-5-pentylphenyl)-3-chloro-4-methoxy-5-pentyl-2-oxybenzoic acid (**4.7**) were

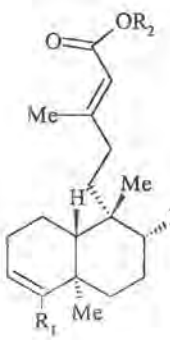
Fig. 4.1 Branch of *T. martiniana* var *pauloi* Harms (Photo: C. Kihampa)

Fig. 4.2 *T. martiniana* var *martiniana* Harms (Photo: C. Kihampa)

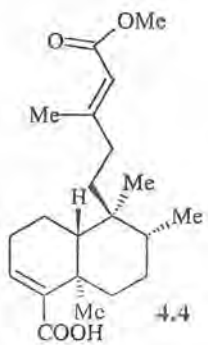
isolated from the larvicidal extracts. *cis*-Kolavenolic acid (**4.1**), *cis*-2-oxo-*ent*-cleroda-13(*Z*)-en-15-oic acid (**4.6**) and *O*-(3-hydroxy-4-hydroxycarbonyl-5-pentylphenyl)-3-chloro-4-methoxy-5-pentyl-2-oxybenzoic acid (**4.7**) are hereby being reported for the first time.



4.1



	R_1	R_2
4.2:	CHO	H
4.3:	COOH	Me



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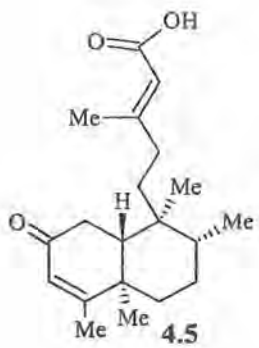

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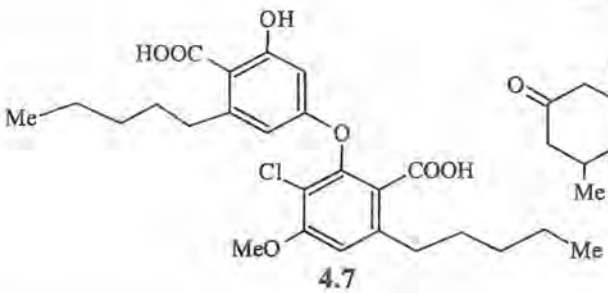
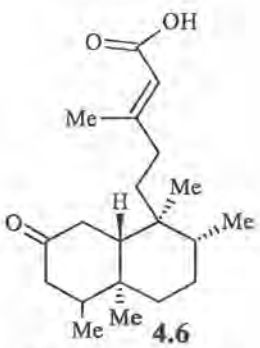


4.7



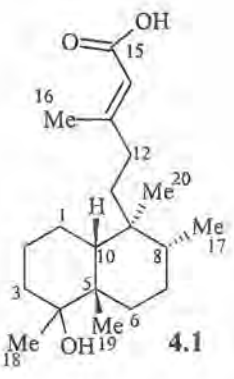
4.6

4.2 RESULTS AND DISCUSSION

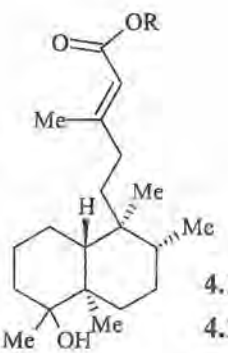
4.2.1 *cis*-Kolavenolic acid (4.1)

Compound **4.1** was obtained from the combined less polar VLC fractions of the methanol extract of the root bark of *Tessmannia martiniana* var *pauloi* as yellow oil whose structure was established on the basis of ^{1}H and ^{13}C NMR, and mass spectral properties upon comparison of these properties with spectral data reported in the literature for the *ent*-clerodanoid diterpene kolavenolic acid (**4.1a**).[151] The MS (Fig.

4.6) exhibited an M+1 peak at *m/z* 323, which is consistent with the molecular formula $C_{20}H_{34}O_3$. The double bond equivalence of 4 derived from the molecular formula was also consistent with structure **4.1**. The ^{1}H NMR spectrum showed three signals at δ 0.74 (*s*, 3H), 1.03 (*s*, 3H) and 1.29 (*s*, 3H), due to protons of the quaternary methyl groups, the signal appearing at δ 1.23 (s) being suggestive of the corresponding methyl group to be substituted at a carbinol carbon. Two other methyl resonances were observed at δ 0.81 (d, J = 5.3 Hz, 3H), due to a secondary methyl group, and at δ 2.17 (br s, 3H) due to an allylic methyl group, coupling within the vinylic proton resonating at δ 5.69 (br s, 1H). The ^{1}H NMR spectrum also revealed signals due to methylene protons at δ 2.04 (td, J = 7.2, 2.7 Hz) and 1.93 (td, J = 7.5, 3 Hz), the corresponding methylene group being adjacent to a quaternary sp^2 carbon and cross peaks in the COSY spectrum showing coupling with a methine proton resonating at δ 5.69 (br s, 1H) and the two non-equivalent methylene protons whose signals appeared at δ 1.41 (m) and 1.36 (m) (Table 4.1). These protons were in turn shown to be connected to the carbon atom which was attached to a quaternary carbon that was substituted by a methyl group whose proton signal appeared at δ 0.74 (s). The assignment of the ^{1}H and ^{13}C NMR spectral data (Table 4.1) was achieved with the help of 1D and 2D NMR data (COSY, HMBC and HMQC, Figs. 4.3, 4.4 and 4.5). HMQC allowed the assignment of all but the quaternary carbon signals, while long range H/C correlations (HMBC) in addition enabled the assignment of the quaternary carbons, thereby establishing the substitution pattern of the compound and hence structure **4.1**.



4.1



4.1a: R = H

4.1b: R = Me

The ^{13}C NMR spectrum (Fig. 4.2 and Table 4.1) exhibited signals due to 20 carbon atoms distributed as five methyls, seven methylenes, three methines, three quaternary, one carbonyl and one substituted vinyl carbon atom. The ^{13}C NMR spectral data were similar to those reported for this type of compounds, with the exception of the chemical shift of C-19, which for the *trans* compound **4.1a** was expected to appear at around δ 19, but in compound **4.1** it was observed at δ 14.7. This indicated the stereochemical difference between the two clerodane diterpenes **4.1** and **4.1a**. Thus compound **4.1** had a *cis* relationship for the A/B rings, while the two rings in **4.1a** are *trans* fused;[152-155] the two compounds are epimeric at C-5.

Besides the M+1 peak at *m/z* 323, the MS (Fig. 4.6) also exhibited other prominent fragment ion peaks as shown in Scheme 4.1, all of which were consistent with structure **4.1**.

The compound *trans*-kolavenolic acid (**4.1a**) was previously isolated from *Hardwickia pinnata* Roxb[151] but the *cis*-isomer **4.1** is being reported for the first time in these investigations. Compound **4.1** has been found to have mosquitocidal and antimicrobial activity (Chapter 6).

Table 4.1 NMR Spectral Data for *cis*-Kolavenolic acid (4.1)

H/C	δ_H	*J* (Hz)	δ_C	H/C	δ_H	*J* (Hz)	δ_C
1a	1.42	m	21.1	10	1.32	m	43.2
1b	1.31	m		11a	1.41	m	36.6
2a	1.72	d, 5.4 or m	23.3	11b	1.36	m	
2b	1.32	m		12a	1.93	td, 7.5, 3	35.0
3a	1.72	m	36.9	12b	2.04	td, 7.2, 2.7	
3b	1.50	m		13	-	-	163.9
4	-	-	76.2	14	5.69	br s	115.0
5	-	-	42.0	15	-		171.6
6a	1.50	m	31.9	16	2.17	d, 2	19.4
6b	1.39	m		17	0.81	d, 5.3	15.9
7	1.40	m	27.1	18	1.29	s	23.3
8	1.40	m	36.8	19	1.03	s	14.7
9	-		39.0	20	0.74	s	18.1

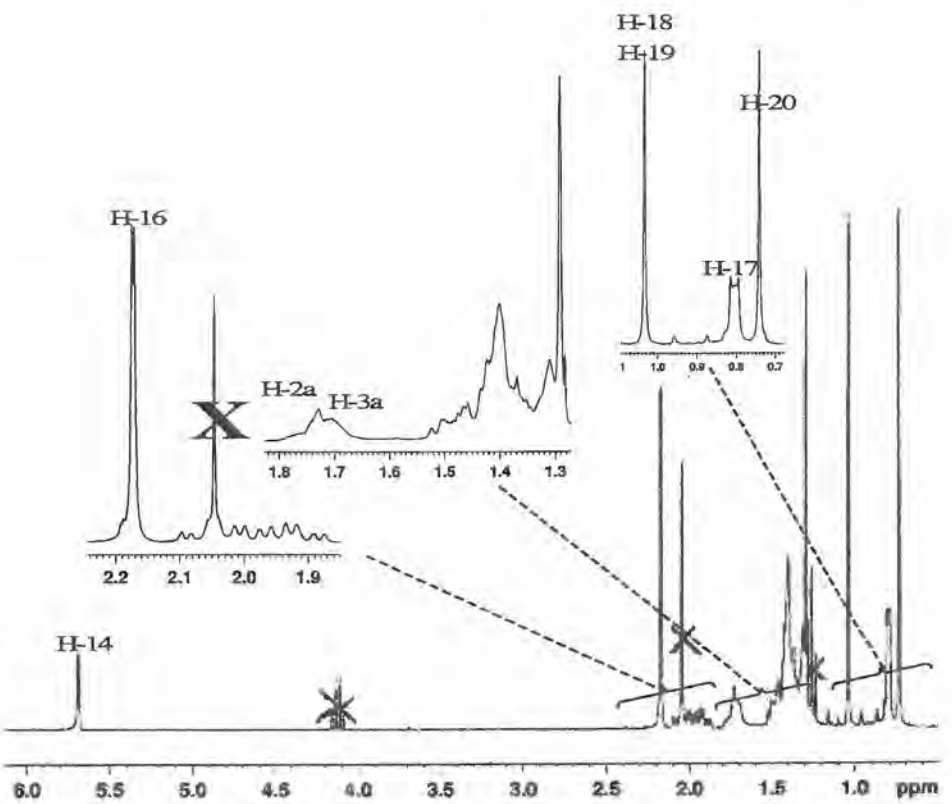


Fig. 4.1 ^{1}H NMR Spectrum of *cis*-Kolavenolic acid (4.1)

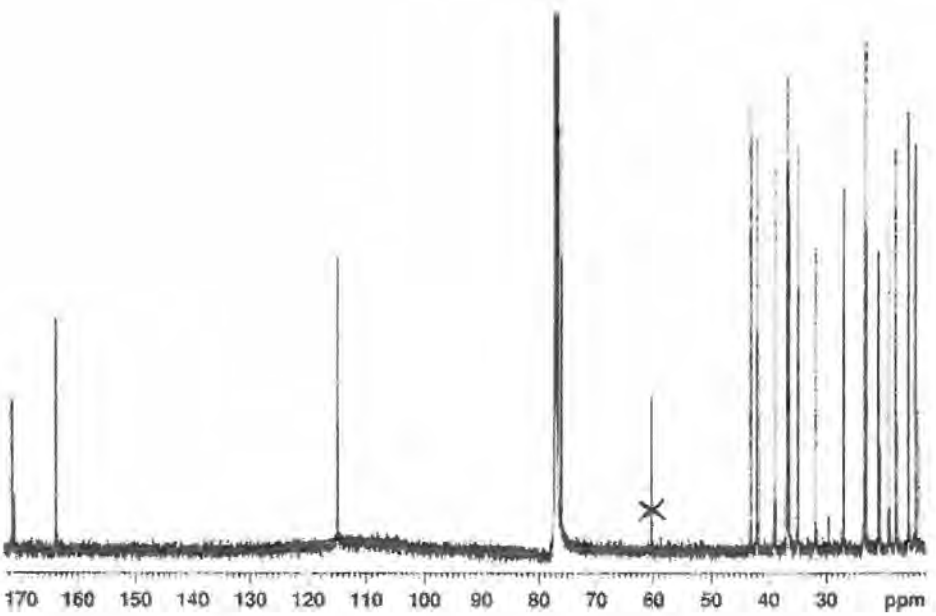


Fig. 4.2 ^{13}C NMR Spectrum of *cis*-Kolavenolic acid (4.1)

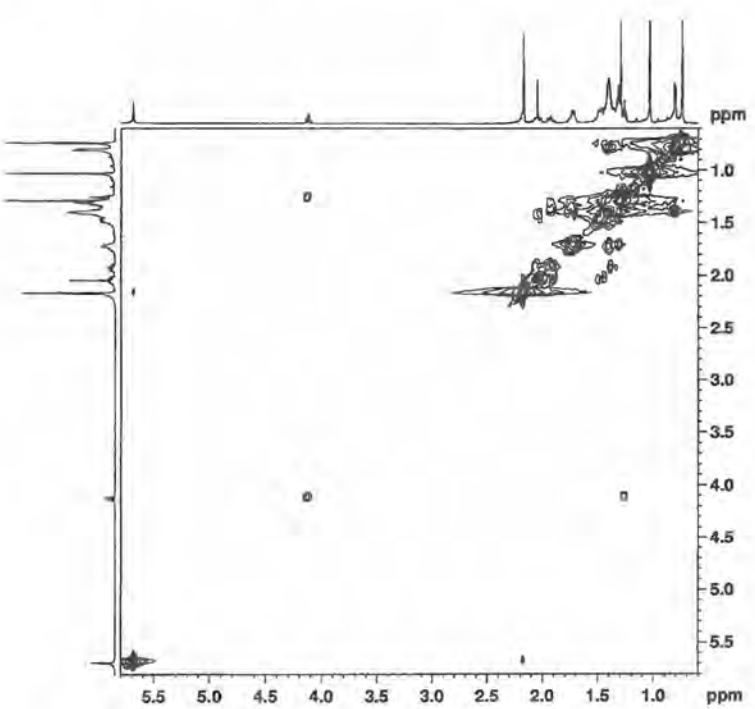


Fig. 4.3 COSY Spectrum of *cis*-Kolavenolic acid (4.1)

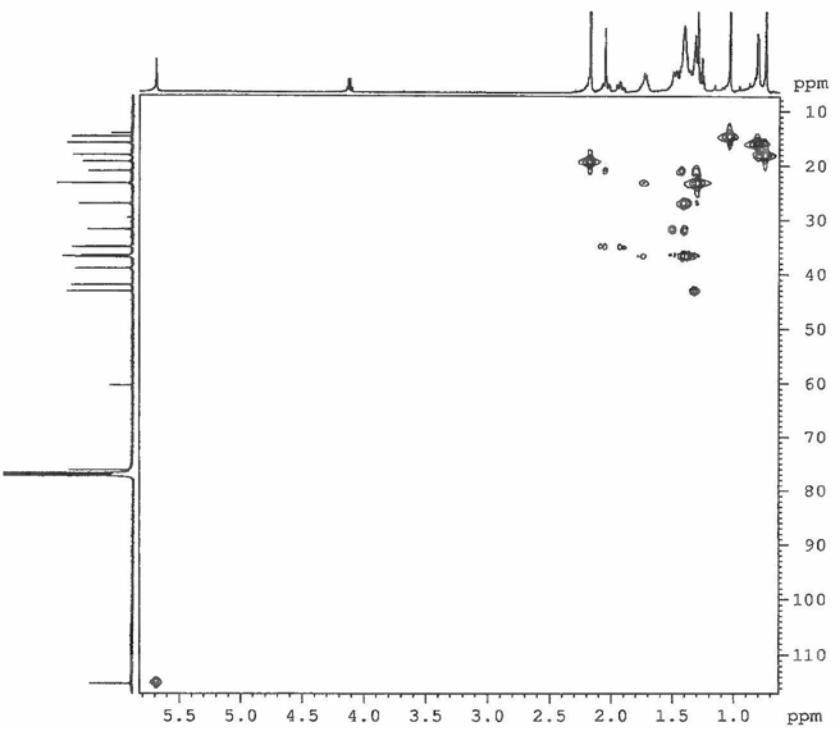


Fig. 4.4 HMQC Spectrum of *cis*-Kolavenolic acid (4.1)

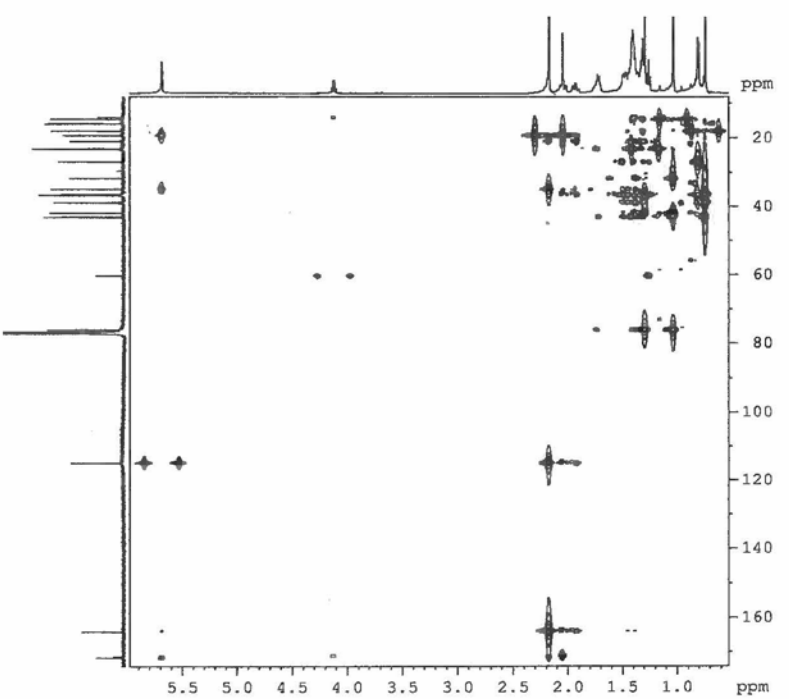


Fig. 4.5 HMBC Spectrum of *cis*-Kolavenolic acid (4.1)

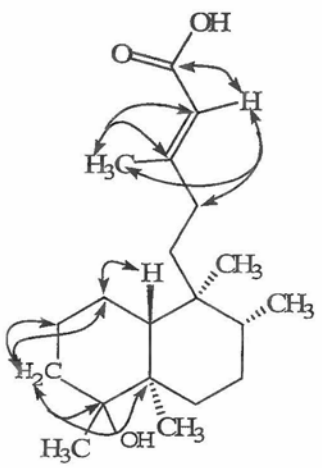


Fig. 4.5a Important Long Range H/C HMBC Correlations for Compound 4.1

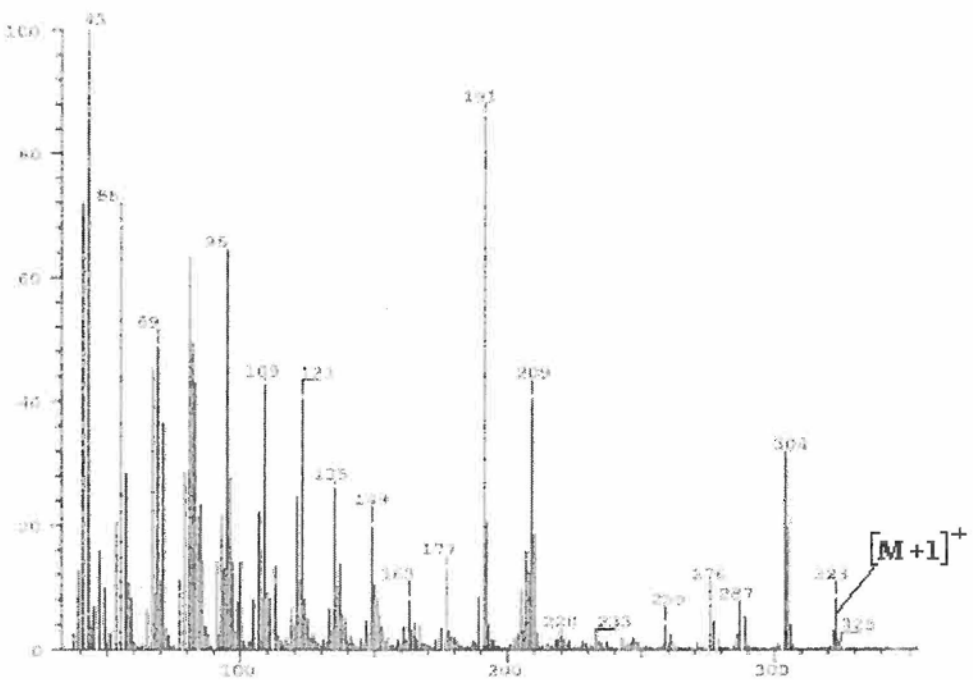
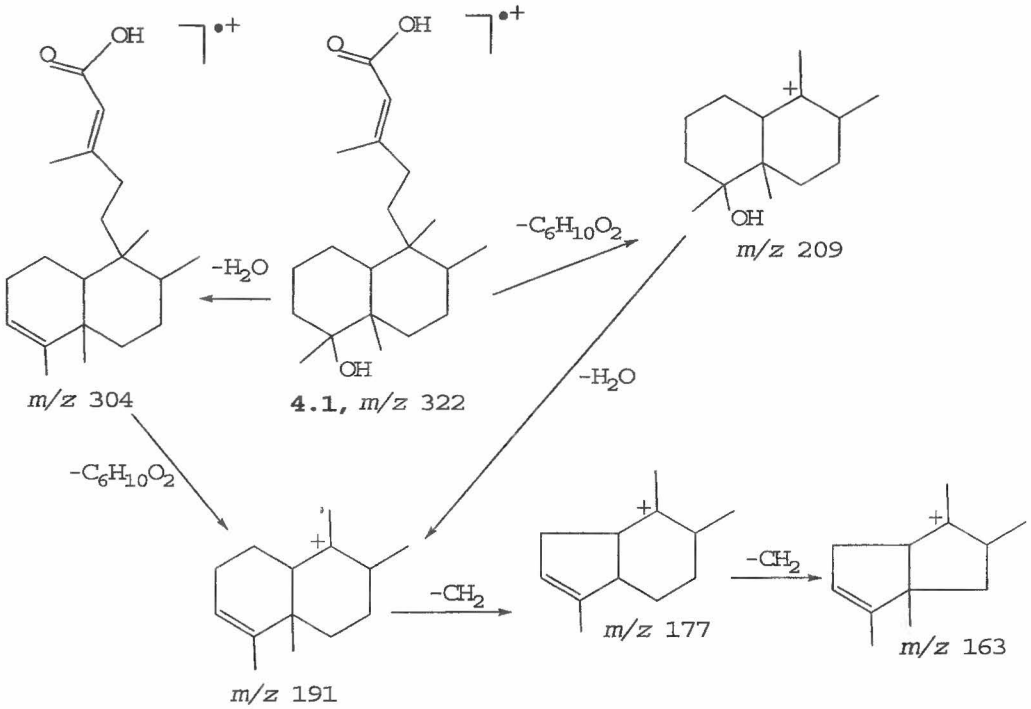


Fig. 4.6 Mass Spectrum of *cis*-Kolavenolic acid (4.1)

Scheme 4.1 Mass Spectral Fragmentation Pattern for *cis*-Kolavenolic acid (4.1)

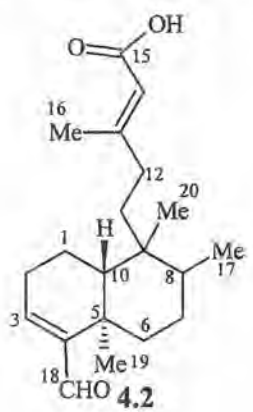


4.2.2 18-Oxocleroda-3,13(*E*)-dien-15-oic acid (4.2)

Compound **4.2** was obtained from the combined larvicidal VLC fraction 1 of the methanol extract of the root bark of *T. martiniana* var *pauloi* as a yellow oil and its structure was established on the basis of ^{1}H and ^{13}C NMR, and mass spectral features (Figs. 4.7, 4.8 and 4.12) upon comparison of these properties with those reported in the literature for this compound, as well as those observed for *cis*-kolavenolic acid (**4.1**) as discussed in section 4.2.1. Thus, the MS exhibited the M-2 ion peak at *m/z* 316 which corresponds to the molecular formula $C_{20}H_{30}O_3$ for the parent compound.

The ^{1}H and ^{13}C NMR spectral features for the two compounds **4.1** and **4.2** were closely related, except for the absence of a C-18 methyl proton signal at δ 1.29 (3H, s) and presence of a new aldehydic proton resonance at δ 9.31 (1H, s). This

suggested that the C-18 methyl group in **4.1** had been transformed into an aldehyde group. Furthermore, the ^{1}H NMR spectrum exhibited a signal at δ 6.42 (t, J = 13.2 Hz) due to a vinylic proton, besides H-14 and this indicated the presence of one additional double bond in **4.2** as compared to compound **4.1**. The position of the additional double bond at C-3/C-4 was indicated from 2D spectra (COSY and HMBC) which showed coupling between the vinylic proton and H-2, and long range coupling with the aldehyde carbon. This, thus explained the downfield position of the ^{1}H and ^{13}C NMR signals due to H-3 and C-3 (δ 152.0) respectively, resulting from anisotropic effects of the α,β-unsaturated carbonyl system involving C-3/C-4/C-18. Complete assignment of structure **4.2** was achieved upon considering H/H COSY and long range C/H HMBC interactions (Figs. 4.9 and 4.11).



4.2

The MS fragmentation pattern of **4.2** as shown in Scheme 4.2 followed a similar trend as that explained for compound **4.1**, and hence supporting the structural similarity of the two compounds **4.1** and **4.2**.

Previously, compound **4.2** was isolated from *Detarium microcarpum* and was subsequently shown to have antifeedant activity against termites.[156] The

mosquitocidal and antimicrobial activity of this compound as determined in these investigations is reported in Chapter 6 of this Thesis.

Table 4.2 NMR Spectral Data for 18-Oxocleroda-3,13(*E*)-dien-15-oic acid (4.2)

H/C	Observed			Reported[156]		
	δ_H	*J* (Hz)	δ_C	δ_H	*J* (Hz)	δ_C
1a	1.47	m	17.5			26.6
1b	1.68	dd, 12.9, 6.6				
2a	2.34	ddd, 11.1, 7.2, 3	28.6			28.7
2b	2.50	dt, 20.1, 4.8				
3	6.58	t, 13.2	152.0			157.9
4	-	-	139.9			137.5
5	-	-	37.6			38.2
6a	1.10	td, 11.4, 3.9	35.2			35.3
6b	2.64	td, 13.2				
7	1.49	m	27.1			27.6
8	1.49	m	36.3			36.6
9	-	-	38.8			38.5
10	1.28	m	46.8			49.0
11	1.49	m	36.4			34.5
12a	1.9	td, 11.7, 5.4	34.9			37.8
12b	2.05	td, 11.1, 5.4				
13	-	-	164.0	-	-	164.3
14	5.69	d,	114.6	5.66	br s	114.8
15	9.31	s	170.8			170.5
16	2.18	d, 1.2	19.4	2.15	d, 1	18.5
17	0.83	dd, 6.6, 2.7	15.9	0.82	d, 6.5	15.5
18	9.31	s	194.1	9.96	s	189.1
19	1.16	s	20.2	1.06	s	19.9
20	0.76	s	18.4	0.75	s	17.6

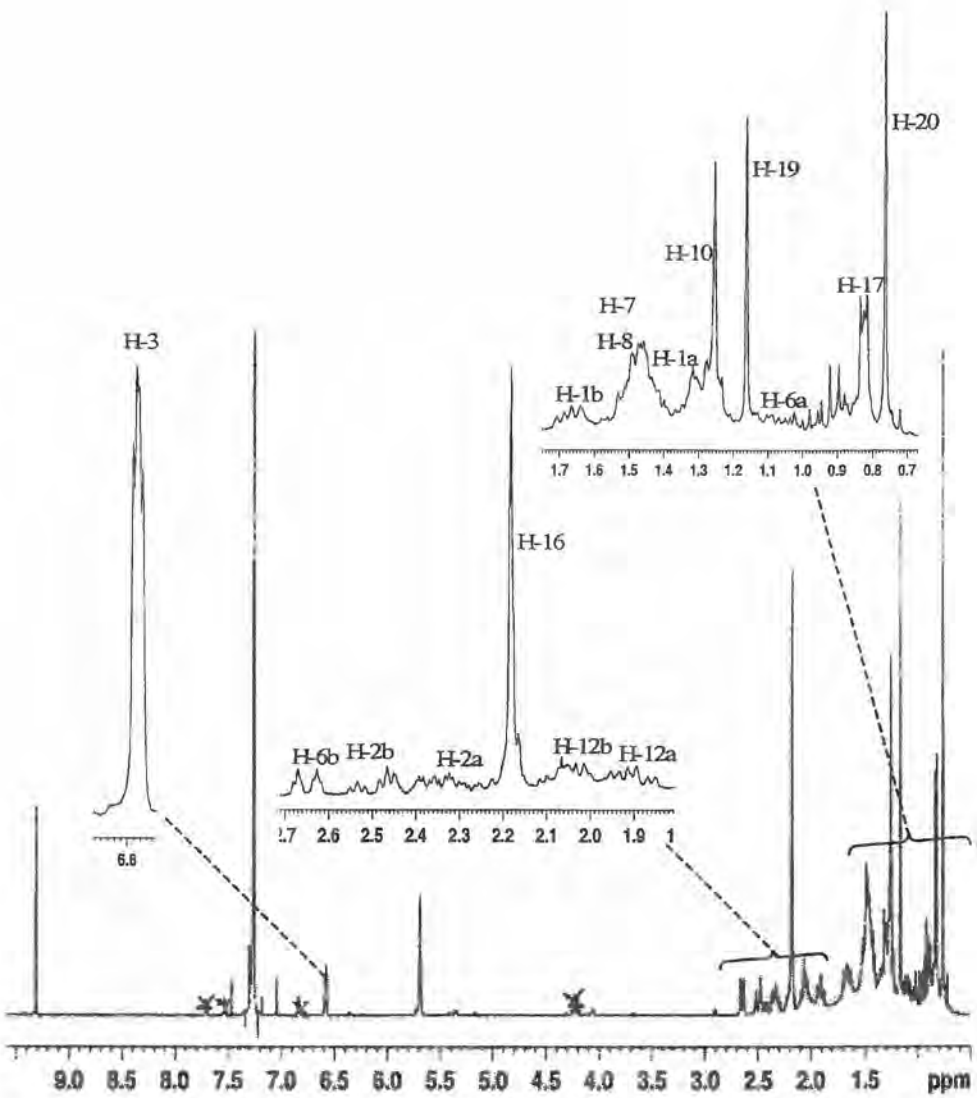


Fig. 4.7 ^{1}H NMR Spectrum of 18-Oxocleroda-3,13(*E*)-dien-15-oic acid (4.2)

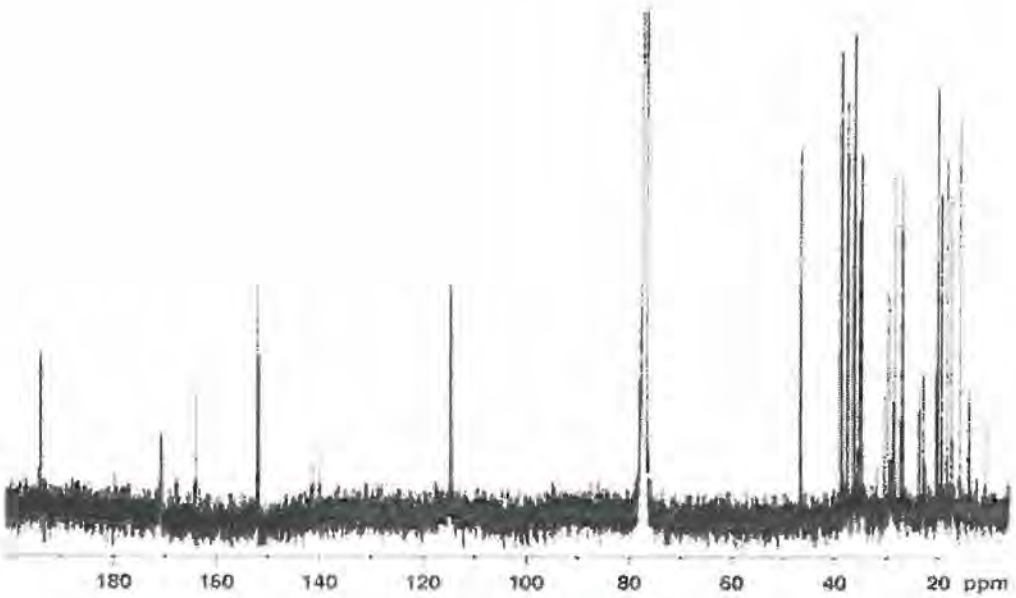


Fig. 4.8 ^{13}C NMR Spectrum of 18-Oxocleroda-3,13(*E*)-dien-15-oic acid (4.2)

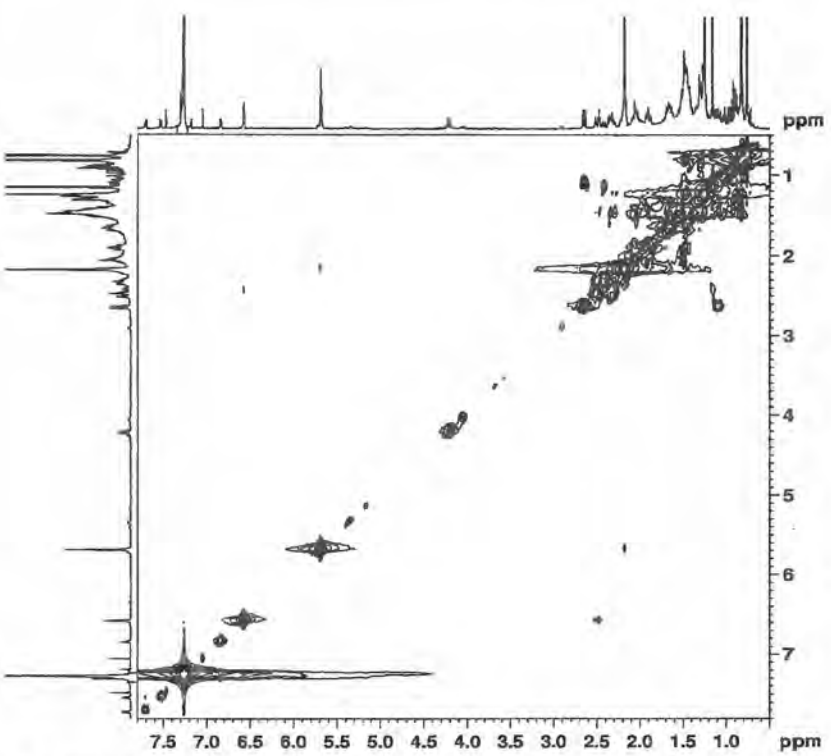


Fig. 4.9 COSY Spectrum of 18-Oxocleroda-3,13(*E*)-dien-15-oic acid (4.2)

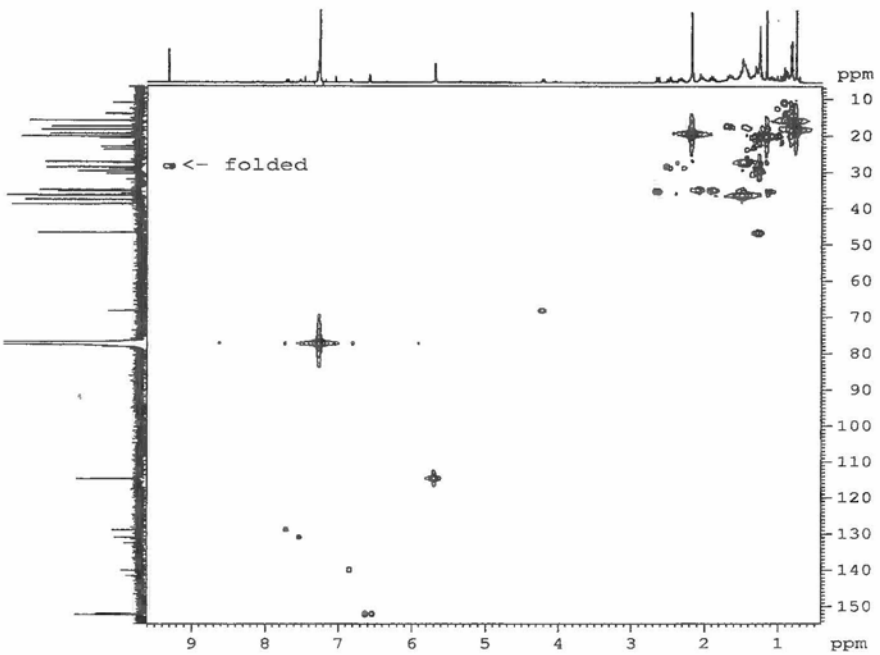


Fig. 4.10 HMQC Spectrum of 18-Oxocleroda-3,13(*E*)-dien-15-oic acid (4.2)

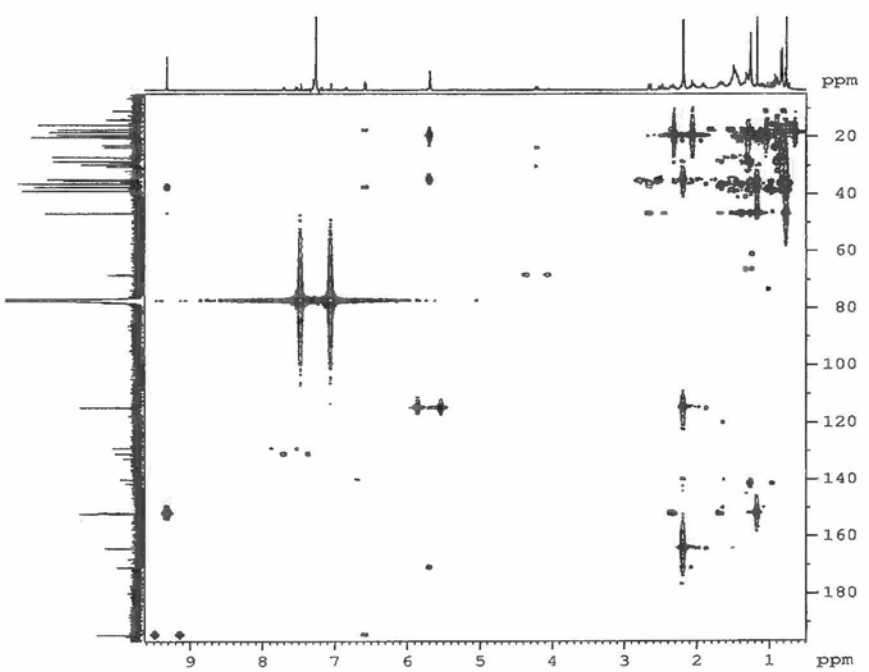


Fig. 4.11 HMBC Spectrum of 18-Oxocleroda-3,13(*E*)-dien-15-oic acid (4.2)

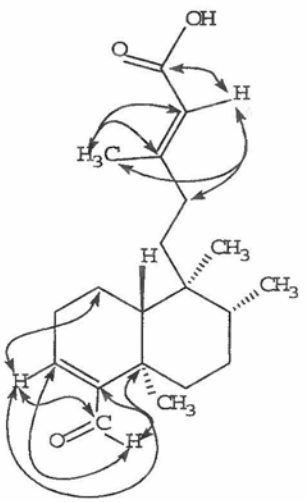


Fig. 4.11a Important Long Range H/C HMBC Correlations for Compound 4.2

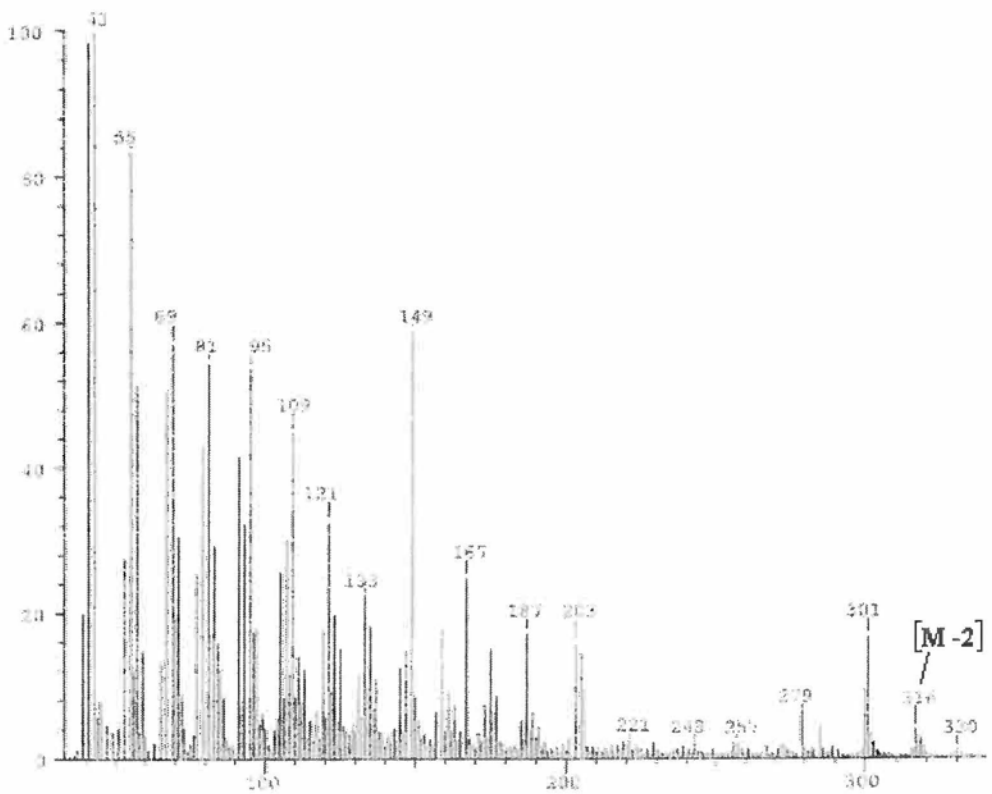
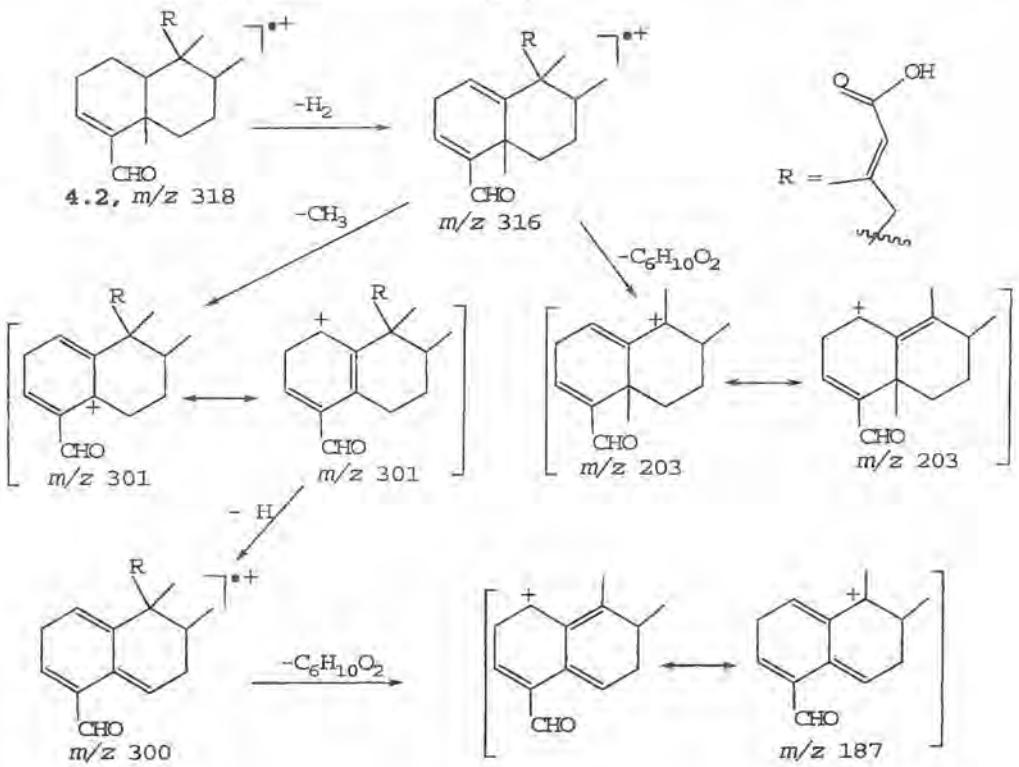


Fig. 4.12 Mass Spectrum of 18-Oxocleroda-3, 13(*E*)-dien-15-oic acid (4.2)

Scheme 4.2 Mass Spectral Fragmentation Pattern for 18-Oxocleroda-3,13(*E*)-dien-15-oic acid (4.2)



4.2.3 *ent*-(18-Hydroxycarbonyl)-cleroda-3,13(*E*)-dien-15-oate (4.3)

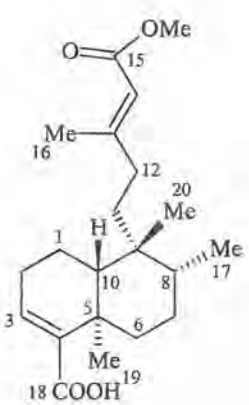
Upon standing at room temperature the combined VLC fraction 3 from the chloroform extract of the root bark of *Tessmannia martiniana* var *martiniana* yielded white crystals, m.p. 119-120 °C, $[\alpha]_D^{20}$ = -132° ($CHCl_3$, c = 0.2), constituting a compound whose structure **4.3** was established on the basis of spectral features (^1H and ^{13}C NMR, and mass spectrum, Figs. 4.13, 4.14 and 4.19), which were almost identical with those already reported for the clerodane diterpenoid **4.8** previously isolated as an antitrypanosomal constituent of the stem bark of *Entada abyssinica*, a plant species used traditionally for the treatment of sleeping sickness.[156,157] Thus, the MS of the isolated compound exhibited an M+1 peak at *m/z* 349, which was consistent with the molecular formula $C_{21}H_{28}O_4$. Except for the

absence of the aldehydic proton signal at δ 9.31 (1H, s) observed in the ^{1}H NMR spectrum of 18-oxocleroda-3,13(*E*)-dien-15-oic acid (**4.2**) and the presence of a carboxylic carbon resonance in the ^{13}C NMR spectrum of the isolated compound (δ 172.6), the ^{1}H and ^{13}C NMR spectra of the latter and compound **4.2** were similar. This indicated close structural similarities of the two compounds and hence the basic diterpenoid skeleton of the two compounds was assumed to be identical, save for the presence of a C-18 carboxylic group in the isolated compound and not an aldehyde unit in **4.2**, as further indicated by H/C interactions observed between the vinylic proton (δ 6.85, dd, J = 4.2, 3.0 Hz) and the carboxylic carbon (δ 172.6), the former having been appreciably deshielded as a result of its β position relative to the carboxylic carbon. Another feature present in the ^{1}H and ^{13}C NMR spectra of the isolated compound that was not observed in the spectra of compound **4.2** was the appearance of signals due to a carboxymethyl unit (δ_{OMe} 3.69 and 50.8; δ_{CO} 167.2), the methoxy group being attached to C-15, as established from H/C HMBC interactions (Fig. 4.18), which indicated long range coupling between H-15 and C-14, and between H-14 and C-15.

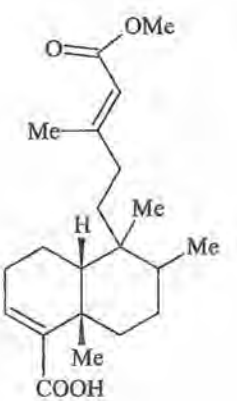
Complete assignment of structure **4.3** for the isolated compound was made based on the above discussion as well as upon analysis of H/H COSY, and H/C HMQC and HMBC interactions (Fig. 4.15, 4.17 and 4.18). The *transoid* stereochemical configuration of the C-5/C-10 ring junction was deduced from the ^{13}C NMR resonance for C-19 appearing at δ 20.5 and not at *ca.* δ 14 as expected for a *cisoid* ring junction.[152-155] Furthermore, close scrutiny of the NOESY spectrum indicated the C-8 methyl and the C-11 methylene groups to be on the same side of the

molecule, thereby establishing the relative stereochemistry of the C-11 methylene and C-17, as shown in structure **4.3**.

The mass spectrum (Fig. 4.19) in addition to the M+1 peak at *m/z* 349, exhibited other prominent fragment ion peaks that are consistent with the MS fragmentation pattern expected for compound **4.3**. The most facile fragmentation process would involve an initial loss of a hydrogen molecule, then followed by cleavage of a methyl unit to form a fragment ion peak at *m/z* 331 that would be stabilized by resonance. Subsequent fragmentation of the latter fragment ion would then involve cleavage of a hydrogen atom and then loss of either a methyl unit or the side chain alkyl group to form fragment ion peaks at *m/z* 315 and 203, respectively (Scheme 4.3).



4.3



4.8

ent-(18-Hydroxycarbonyl)-cleroda-3,13(*E*)-dien-15-oate (**4.3**) is being reported for the first time in these investigations, and it displayed mosquitocidal and antimicrobial activity as detailed in Chapter 6 of this Thesis.

Table 4.3 NMR Spectral Data for *ent*-(18-Hydroxycarbonyl)-cleroda-3,13(*E*)-dien-15-oate (4.3)

	4.3			**4.8**[157]		
H/C	δ_H	*J* (Hz)	δ_C	δ_H	*J* (Hz)	δ_C
1a	1.44	m	17.4			17.5
1b	1.64	dd, 13.3, 7.1				
2a	2.21	ddd, 11.0, 7.7, 3.0	27.4*	2.15		27.8
2b	2.34	dt, 19.9, 5.3		2.30		
3	6.85	dd, 4.2, 3.0	140.1	6.80		140.3
4	-	-	141.5	-	-	142.1
5	-	-	37.6	-	-	38.0
6a	1.14	td, 12.5, 4.1	35.8			38.1
6b	2.42	td, 13.4, 2.5				
7	1.44	m	27.2*	1.40		25.8
8	1.44	m	36.2	1.60		35.2
9	-	-	38.8	-	-	38.0
10	1.28	d, 11.9	46.7	1.45		45.5
11	1.44	m	36.5	2.20		29.57
12a	1.88	td, 13.0, 4.8	34.5	2.08		34.80
12b	2.02	td, 12.9, 4.2				
13	-	-	161.2	-	-	161.9
14	5.66	q, 1.0	115.0	5.68		115.2
15	-	-	167.2	-		167.6
16	2.17	d, 1.3	19.1	2.18		19.6
17	0.82	d, 5.7	15.8	0.93		15.1
18	-	-	172.6	-	-	172.8
19	1.25	s	20.5	0.95		20.7
20	0.75	s	18.3	1.3		21.7
15-OMe	3.69	s	50.8	3.68	s	51.2

* interchangeable signals

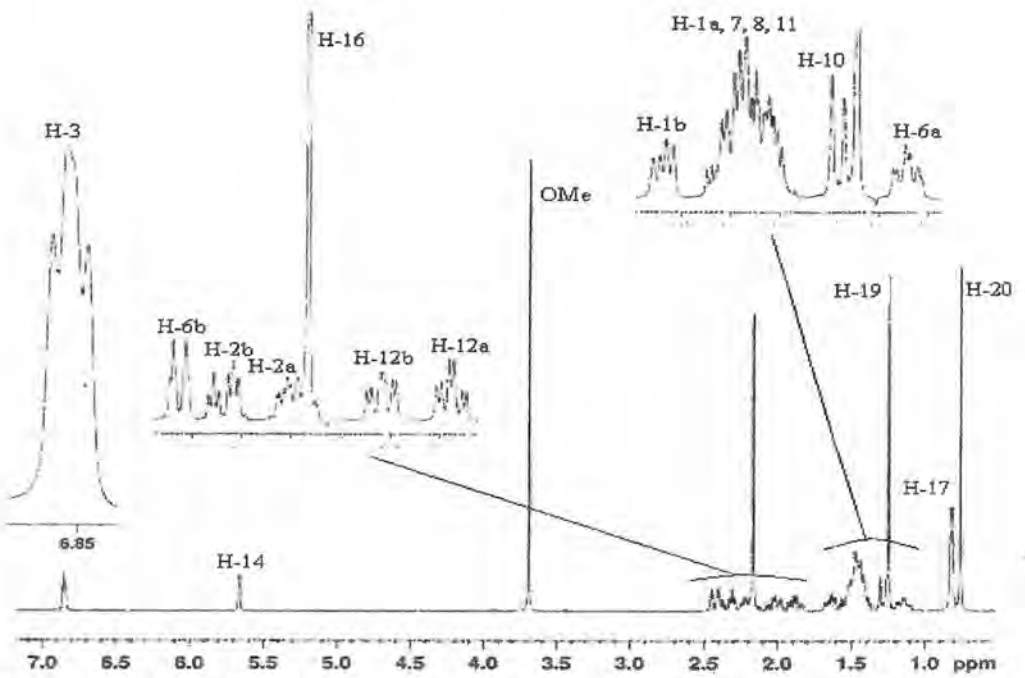


Fig. 4.13 ^{1}H NMR Spectrum of *ent*-(18-Hydroxycarbonyl)-cleroda-3,13(*E*)-dien-15-oate (4.3)

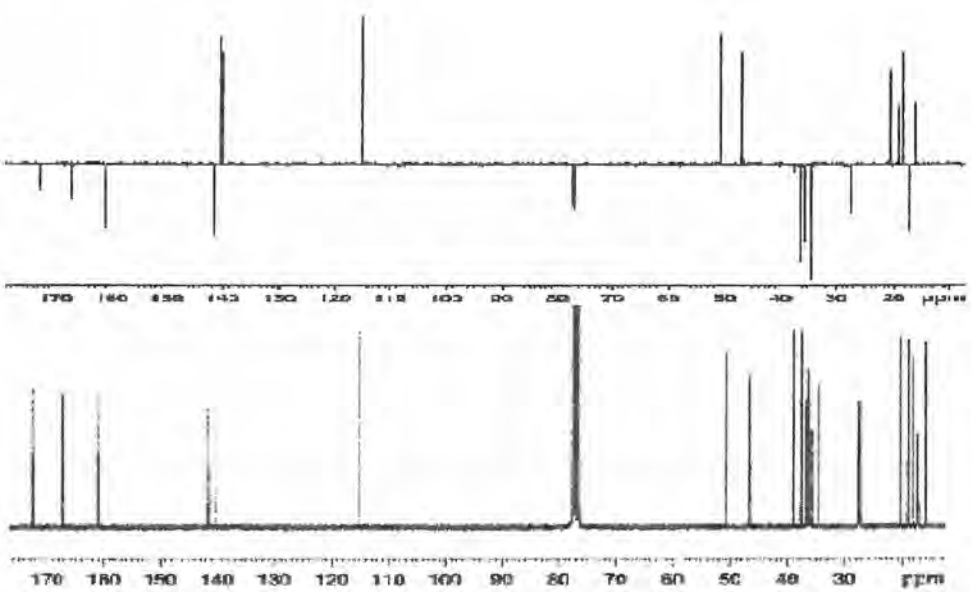


Fig. 4.14 ^{13}C NMR Spectrum of *ent*-(18-Hydroxycarbonyl)-cleroda-3,13(*E*)-dien-15-oate (4.3)

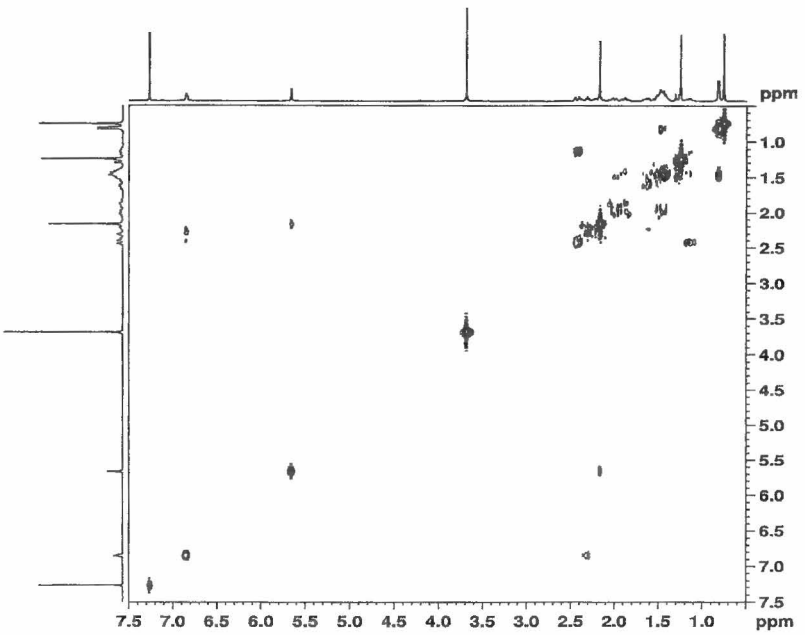


Fig. 4.15 COSY Spectrum of *ent*-(18-Hydroxycarbonyl)-cleroda-3,13(*E*)-dien-15-oate (4.3)

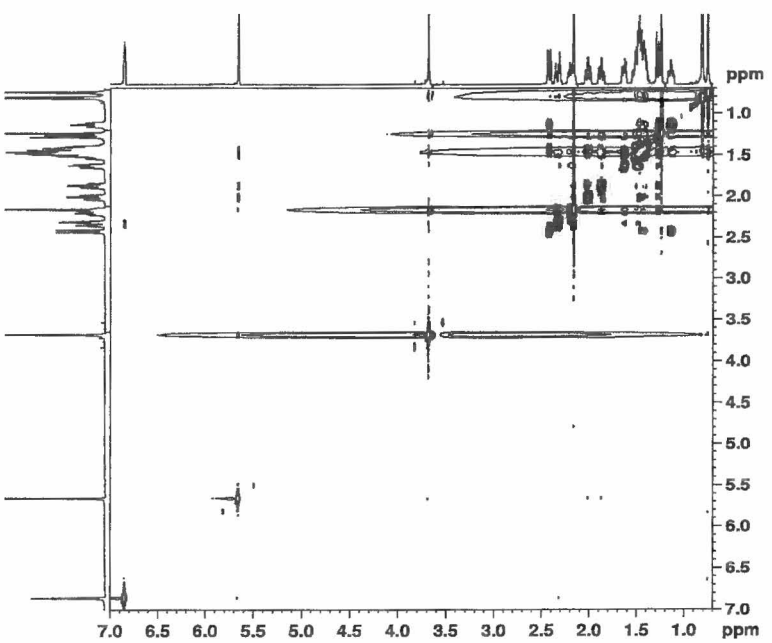


Fig. 4.16 NOESY Spectrum of *ent*-(18-Hydroxycarbonyl)-cleroda-3,13(*E*)-dien-15-oate (4.3)

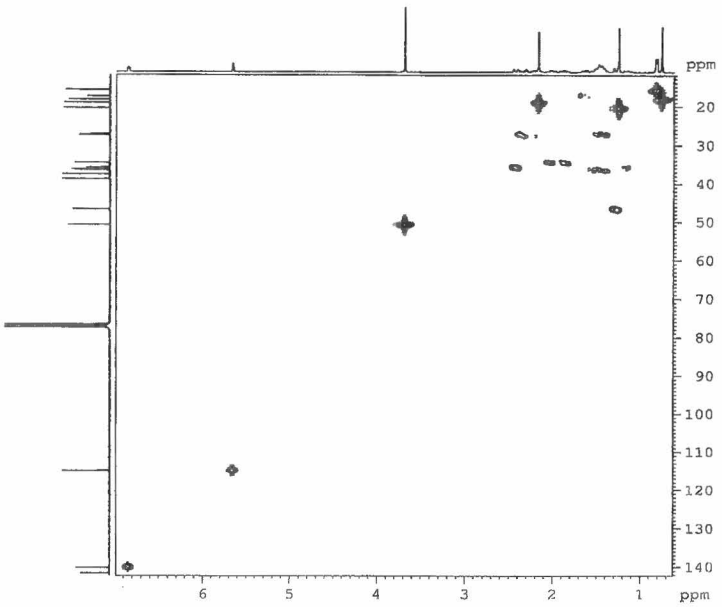


Fig. 4.17 HMQC Spectrum of *ent*-(18-Hydroxycarbonyl)-cleroda-3,13(*E*)-dien-15-oate (4.3)

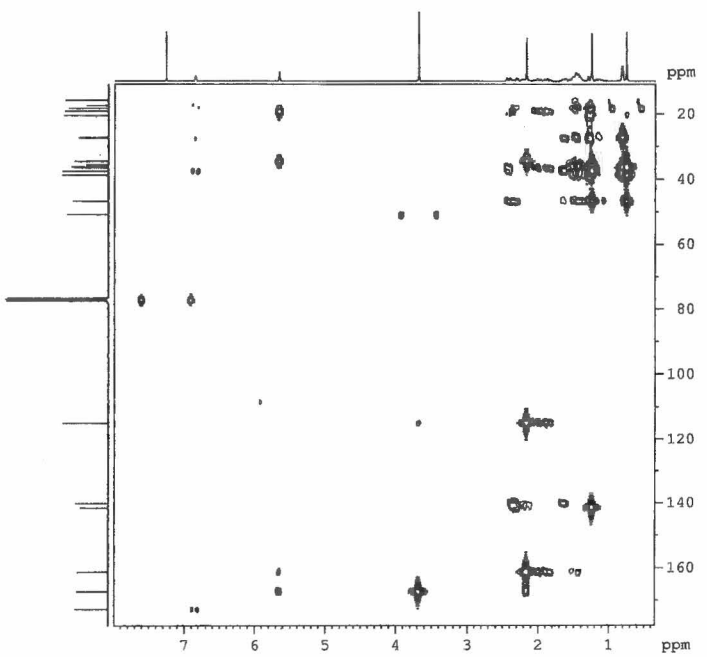


Fig. 4.18 HMBC Spectrum of *ent*-(18-Hydroxycarbonyl)-cleroda-3,13(*E*)-dien-15-oate (4.3)

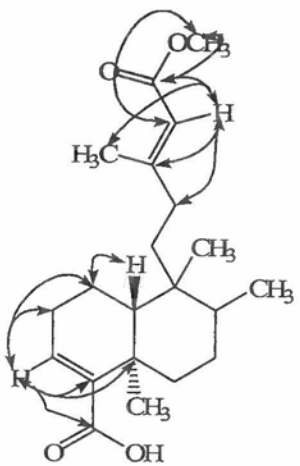


Fig. 4.18a Important Long Range H/C HMBC Correlations for Compound 4.3

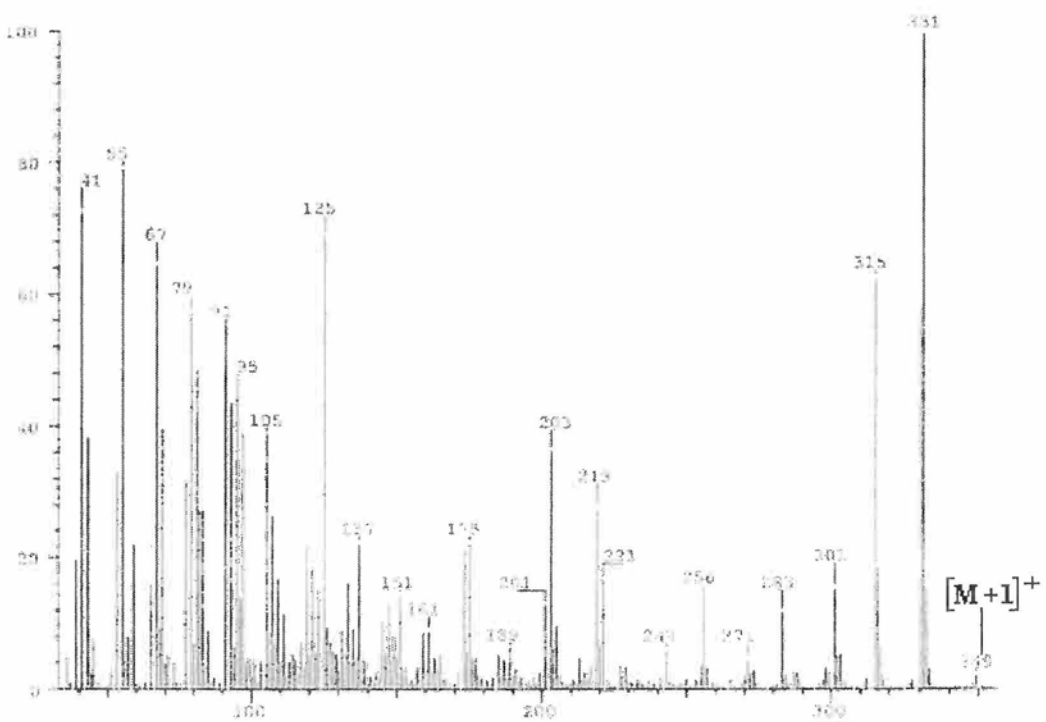
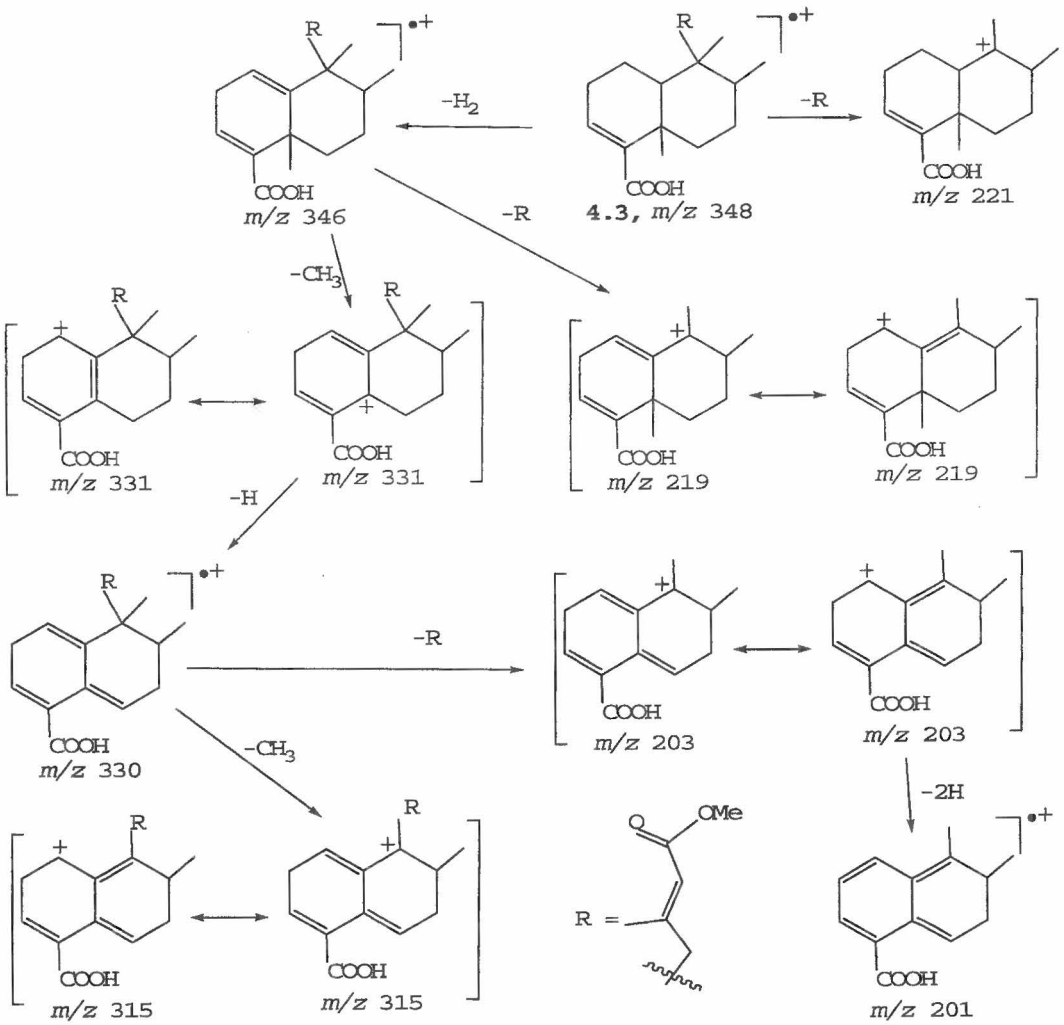


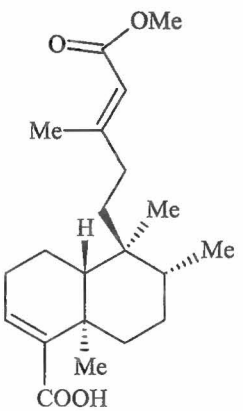
Fig. 4.19 Mass Spectrum of *ent*-(18-Hydroxycarbonyl)-cleroda-3,13(*E*)-dien-15-oate (4.3)

Scheme 4.3 Mass Spectral Fragmentation Pattern for *ent*-(18-Hydroxycarbonyl)-cleroda-3,13(*E*)-dien-15-oate (4.3)



4.2.4 (9-*epi*)-*ent*-(18-Hydroxycarbonyl)-cleroda-3,13(*E*)-dien-15-oate (4.4)

The VLC fraction 1 of the methanol extract of the root bark of *Tessmannia martiniana* var *pauloi* yielded a yellow oil that constituted a compound whose structure **4.4** was deduced from analysis of its spectroscopic data that were found to be virtually identical to those observed for *ent*-(18-Hydroxycarbonyl)-cleroda-3,13(*E*)-dien-15-oate (**4.3**) as discussed in the previous section. However, apart from the fact that while compound **4.3** was isolated as a solid, the metabolite from *T. martiniana* var *pauloi* was an oil, and the magnitude of the specific rotation values at the same concentration (0.2 in $CHCl_3$; **4.3**: $[\alpha]_D^{20} = -132°$; **4.4**: $[\alpha]_D^{20} = -126°$) for the two compounds were also slightly different. Furthermore, in the mosquito larvicidal assays compound **4.3** exhibited higher activity than metabolite **4.4**. These bioassay results and the physical properties discussed above led to the conclusion that the two metabolites from *T. martiniana* var *martiniana* and *T. martiniana* var *pauloi* represented different compounds, but having the same molecular framework. The fact that the two compounds exhibited virtually the same spectral properties suggested that the metabolites could have been epimeric, differing by the stereochemistry at either C-8 or C-9. Scrutiny of the spectroscopic and other physical properties of a series of similar compounds as reported in the literature[151,156-161] indicated that the compound from *T. martiniana* var *pauloi* differed from **4.3** in the relative stereochemical configuration around the C-8/C-9 bond, the latter metabolite being epimeric to **4.3** at C-9.



4.4

Analysis of the spectroscopic properties of compound **4.8** (which is similar to **4.3** and **4.4**), that was reported from *Entada abyssinica,* indicated that the available spectroscopic and other physical data for that compound were insufficient to unambiguously establish its structure, considering the variety of stereochemical options available for this series of compounds.[157] Thus, while the optical rotation value for compound **4.8** was not given, the indicated *cisoid* stereochemistry of the ring fusion contradicted the reported C-19 chemical shift (δ 21.7). Thus, while the reported *cisoid* stereochemistry would have required C-19 to resonate at *ca.* δ 14, the observed chemical shift value for that C-atom was δ 21.7 which meant *transoid* ring fusion. Furthermore, the authors of the article describing compound **4.8** did not indicate the relative stereochemical configuration at C-8 and C-9,[157] which was crucial in discussing the reported bioactivity results, as it was observed in investigations hereby being reported (Chapter 6), whereby pinning down the actual stereochemical structure of the active compound is necessary.

Table 4.4 NMR Spectral Data for (9-*epi*)-*ent*-(18-Hydroxycarbonyl)-cleroda-3,13(*E*)-dien-15-oate (4.4)

H	4.4			4.8[157]		
	δ_H	*J* (Hz)	δ_C	δ_H	*J* (Hz)	δ_C
1a	1.44	m	17.4			17.5
1b	1.64	dd, 13.3, 7.1				
2a	2.21	ddd, 11.0, 7.7, 3.0	27.4*	2.15		27.8
2b	2.34	dt, 19.9, 5.3		2.30		
3	6.85	dd, 4.2, 3.0	140.1	6.80		140.3
4	-	-	141.5	-	-	142.1
5	-	-	37.6	-	-	38.0
6a	1.14	td, 12.5, 4.1	35.8			38.1
6b	2.42	td, 13.4, 2.5				
7	1.44	m	27.2*	1.40		25.8
8	1.44	m	36.3	1.60		35.2
9	-	-	38.8	-	-	38.0
10	1.28	d, 11.9	46.7	1.45		45.5
11	1.44	m	36.5	2.20		29.57
12a	1.88	td, 13.0, 4.8	34.5	2.08		34.80
12b	2.02	td, 12.9, 4.2				
13	-	-	161.2	-	-	161.9
14	5.66	q, 1.0	115.0	5.68		115.2
15	-	-	167.2	-		167.6
16	2.17	d, 1.3	19.1	2.18		19.6
17	0.82	d, 5.7	15.8	0.93		15.1
18	-	-	172.6	-	-	172.8
19	1.25	s	20.5	0.95		20.7
20	0.75	s	18.3	1.3		21.7
15-OMe	3.69	s	50.8	3.68	s	51.2

* interchangeable signals

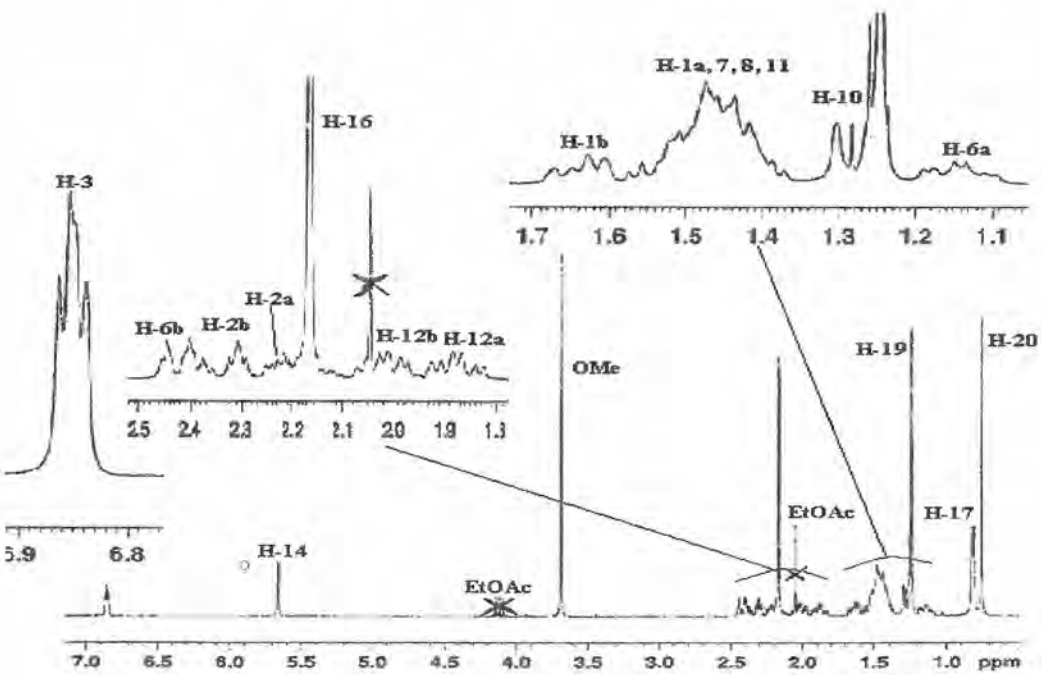


Fig. 4.20 ^{1}H NMR Spectrum of (9-*epi*)-*ent*-(18-Hydroxycarbonyl)-cleroda-3,13(*E*)-dien-15-oate (4.4)

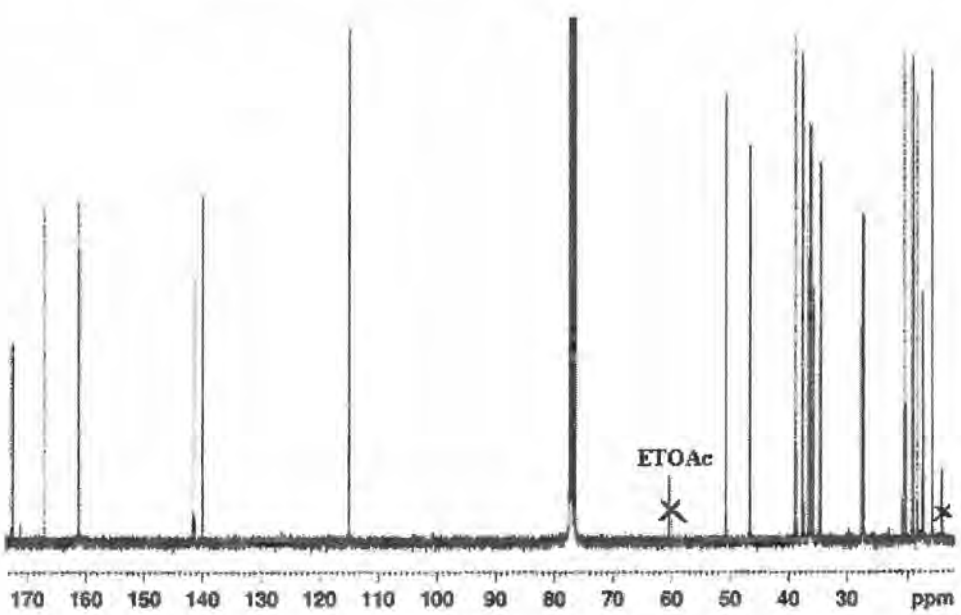


Fig. 4.21 ^{13}C NMR Spectrum of (9-*epi*)-*ent*-(18-Hydroxycarbonyl)-cleroda-3,13(*E*)-dien-15-oate (4.4)

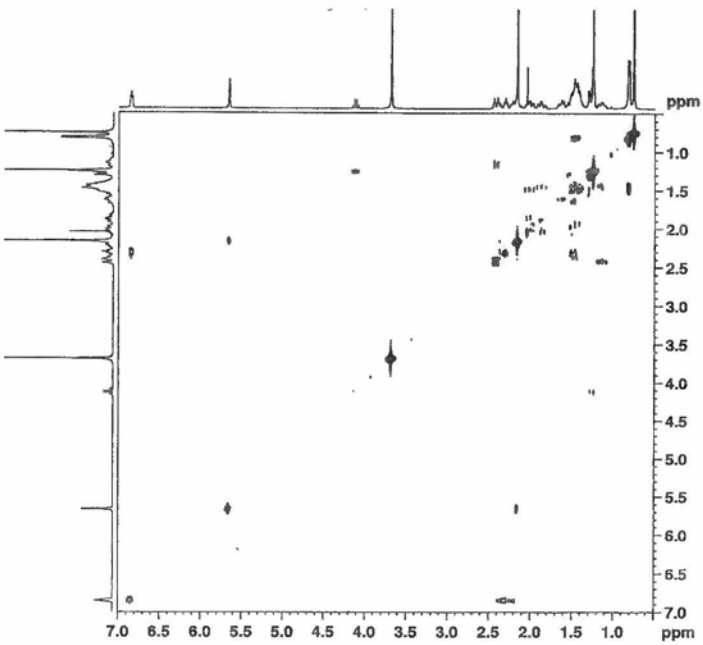


Fig. 4.22 COSY Spectrum of (9-*epi*)-*ent*-(18-Hydroxycarbonyl)-cleroda-3,13(*E*)-dien-15-oate (4.4)

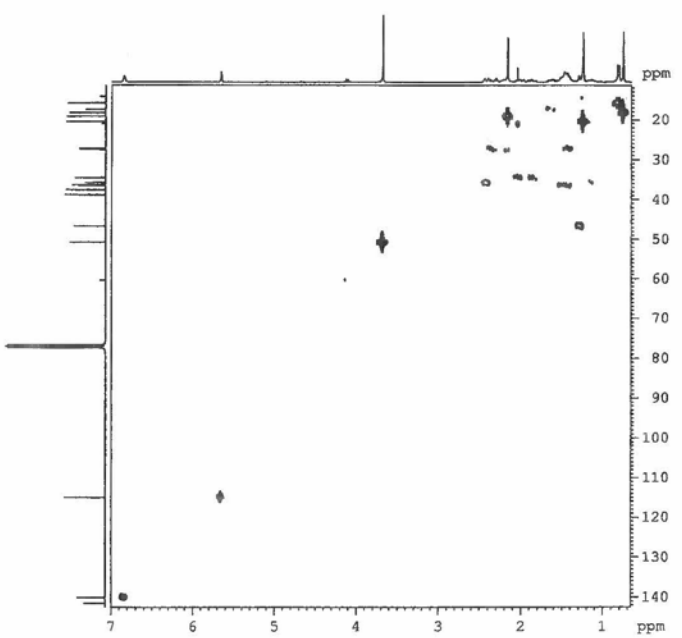


Fig. 4.23 HMQC Spectrum of (9-*epi*)-*ent*-(18-Hydroxycarbonyl)-cleroda-3,13(*E*)-dien-15-oate (4.4)

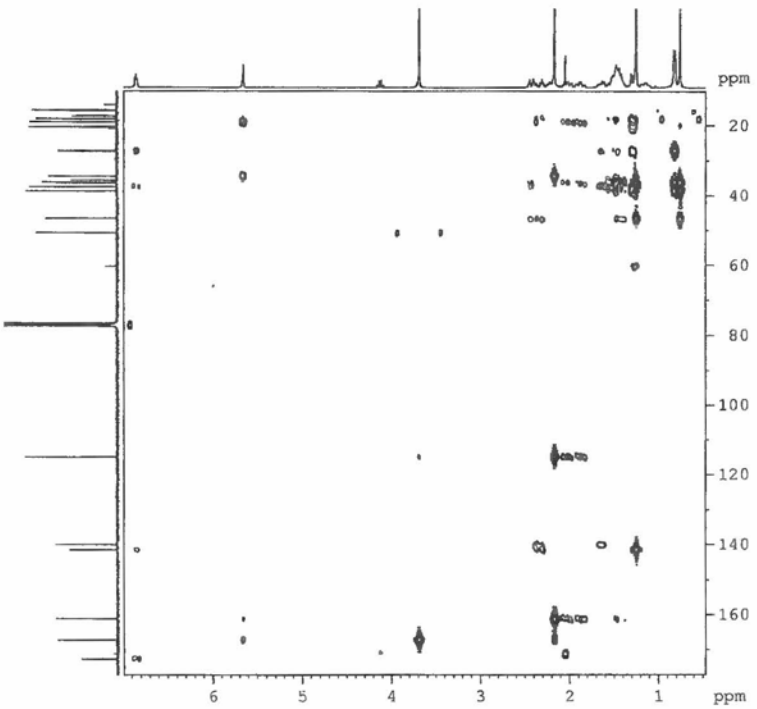


Fig. 4.24 HMBC Spectrum of (9-*epi*)-*ent*-(18-Hydroxycarbonyl)-cleroda-3,13(*E*)-dien-15-oate (4.4)

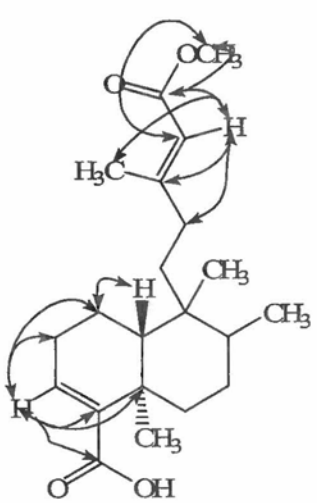


Fig. 4.24a Important Long Range H/C HMBC Correlations for Compound 4.4

4.2.5 2-Oxo-*ent*-cleroda-3,13(*Z*)-dien-15-oic acid (4.5)

Upon standing, the combined VLC fraction 3 from the chloroform extract of the root bark of *T. martiniana* var *martiniana* yielded white crystals of *ent*-(18-Hydroxycarbonyl)-cleroda-3,13(*E*)-dien-15-oate (**4.3**) which were recrystallised from chloroform, m.p. 119-120 °C, $[\alpha]_D^{20}$ = -132° ($CHCl_3$, c = 0.2). Repeated column chromatography of the filtrate yielded an oil, whose structure **4.5** was established on the basis of spectral features (^1H and ^{13}C NMR, and Mass Spectrum, Figs. 4.25, 4.26 and 4.30) upon comparison of these properties with those reported for 2-oxo-*ent*-cleroda-3,13(*Z*)-dien-15-oic acid. Thus, the MS exhibited the molecular ion peak at *m/z* 318, which corresponds to the molecular formula $C_{20}H_{30}O_3$ (exact mass, *m/z* 318.2218, calculated for $C_{20}H_{30}O_3$ = 318.4504). The ^1H and ^{13}C NMR spectra displayed signals similar to those observed for 18-oxo-3,13(*E*)-clerodien-15-oic acid (**4.2**) (section 4.2.2) that was isolated from the root bark of *T. martiniana* var *pauloi*. The major difference in the spectral properties for the two compounds was the absence of aldehydic signals due to C-18 found in the spectra of **4.2** and presence of C-18 methyl signals (δ_H 2.15, d, J = 0.9 Hz and δ_C 18.3) in the spectra for compound **4.5**. Another important signal observed in the ^{13}C NMR spectrum of **4.5** was at δ 200.2 being due to a carbonyl carbon, and the absence of a C-2 signal at δ 28.5. This, therefore suggested that C-2 in compound **4.5** constituted a carbonyl group. Other spectra features were virtually the same as those expected for this compound and it was therefore concluded that the isolated compound had structure **4.5**.

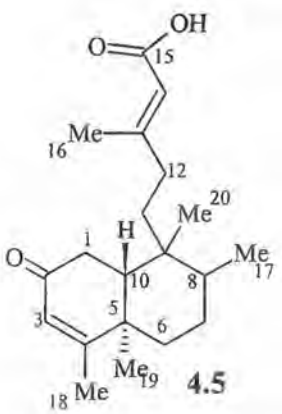
Besides the M^+ peak at *m/z* 318 the MS (Fig. 4.30) also exhibited other prominent ion peaks as shown in Scheme 4.4 all of which were consistent with structure **4.5**. The MS also indicated a peak at *m/z* 320 which was due to the *cis*-2-oxo-*ent*-cleroda-

13(*Z*)-dien-15-oic acid (**4.6**) that was present as an impurity, whose presence was also shown by the ^{13}C NMR spectrum.

18-Oxo-3,13(*E*)-clerodien-15-oic acid (**4.2**) was once isolated from the root bark of *Ageratina ixiocladon*.[158] The mosquitocidal and antimicrobial activity of this compound as determined in these investigations is reported in Chapter 6 of this Thesis.

Table 4.5 NMR Spectral Data for 2-Oxo-*ent*-cleroda-3,13(*Z*)-dien-15-oic acid (4.5)

C/H	Observed			Reported[158]		
	δ_H	*J* (Hz)	δ_C	δ_H	*J* (Hz)	δ_C
1a	1.47	m	35.5	2.48	dd, 18, 4	35.9
1b	1.68	dd, 12.9, 6.6		2.36	dd, 18, 14	
2	-	-	200.2	-		200.5
3	5.74	d, 0.6	125.5	5.73	br s	126.3
4	-	-	171.4			171.8
5	-	-	39.8			40.3
6a	1.10	td, 11.4, 3.9	34.9			35.4
6b	2.42	td, 13.2				
7	1.49	m	27.1			27.1
8	1.49	m	36.0	1.61	ddq, 6.5	36.4
9	-	-	38.7			39.2
10	1.28	m	45.7	1.96	dd, 14, 4	46.2
11a	1.49	m	34.3	1.52	m, 12, 12, 5	34.7
11b				1.40	dt, 12, 5	
12a	2.16	td, 11.7, 0.9	35.5	2.64	dt, 12, 12	36.0
12b	2.05	td, 11.1, 5.4		2.24	dt, 12, 5	
13	-	-	163.2	-	-	163.6
14	5.67	d, 0.9	115.1	5.62	br s	114.9
15	-	-	172.7	-	-	172.8
16	1.89	d, 1.2	19.0	1.87	d, 1.5	19.4
17	1.11	br s	15.7	0.88	d, 6.5	16.2
18	2.15	d, 0.9	18.3	1.88	d, 1	18.8
19	1.12	s	19.5	1.10	s	19.9
20	0.82	s	17.8	0.79	s	18.3



4.5

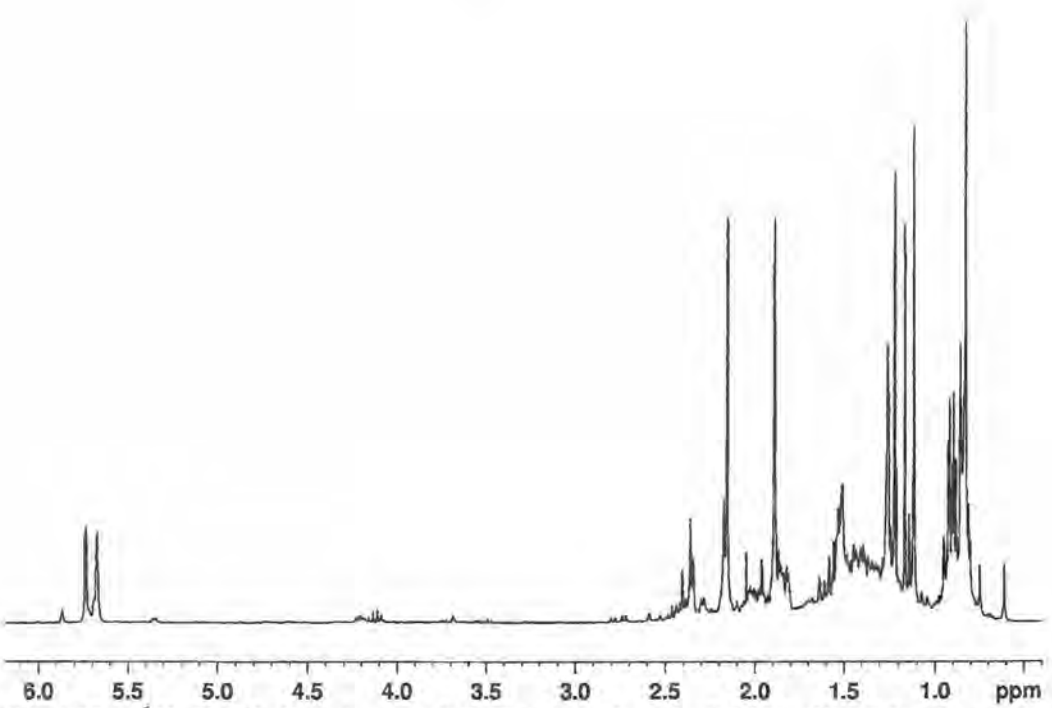


Fig. 4.25 ^{1}H NMR Spectrum of 2-Oxo-*ent*-cleroda-3,13(*Z*)-dien-15-oic acid (4.5)

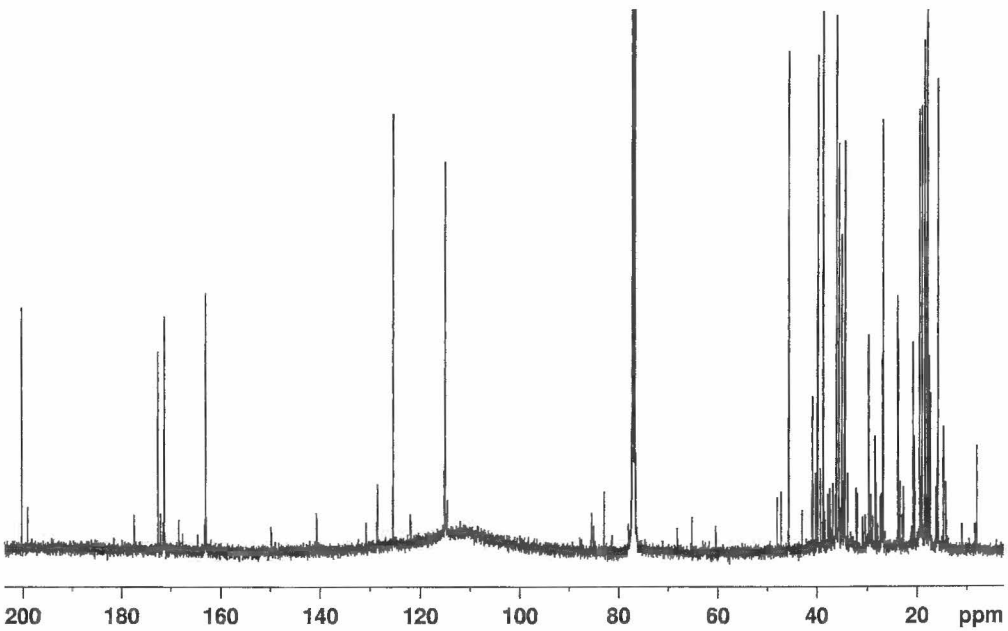


Fig. 4.26 ^{13}C NMR Spectrum of 2-Oxo-*ent*-cleroda-3,13(*Z*)-dien-15-oic acid (4.5)

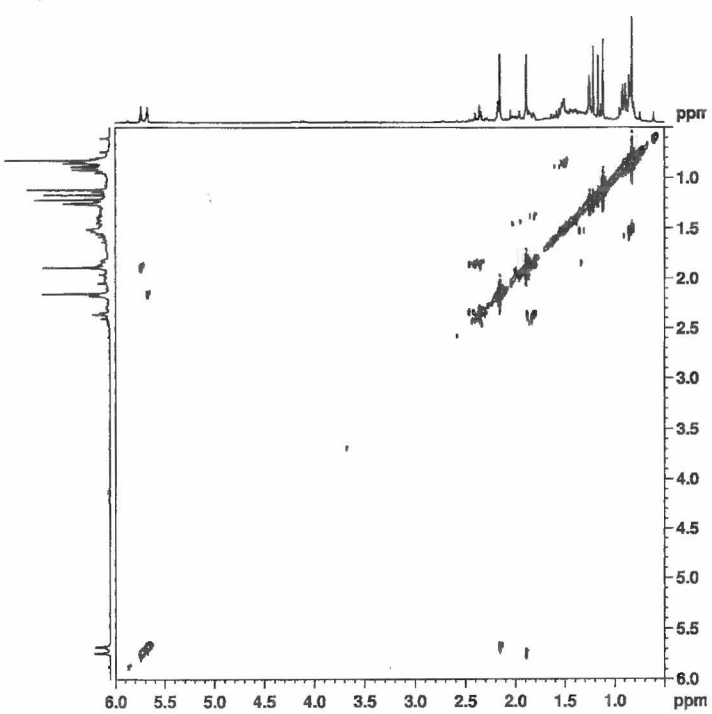


Fig. 4.27 COSY Spectrum of 2-Oxo-*ent*-cleroda-3,13(*Z*)-dien-15-oic acid (4.5)

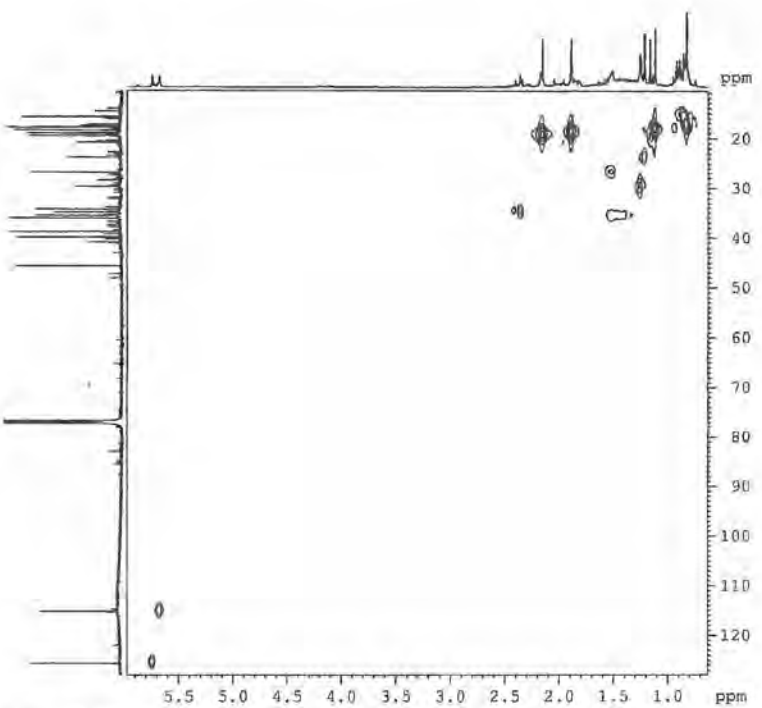


Fig. 4.28 HMQC Spectrum of 2-Oxo-*ent*-cleroda-3,13(*Z*)-dien-15-oic acid (4.5)

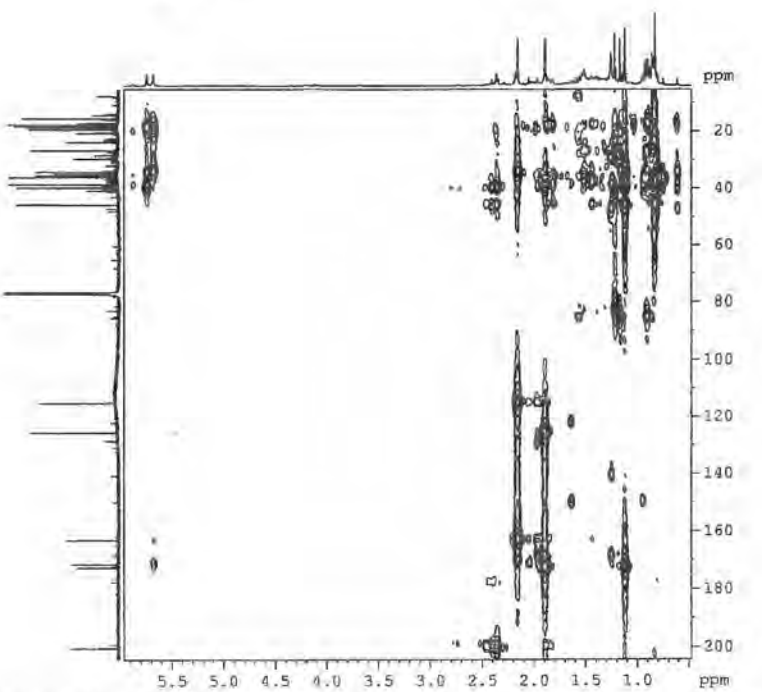


Fig. 4.29 HMBC Spectrum of 2-Oxo-*ent*-cleroda-3,13(*Z*)-dien-15-oic acid (4.5)

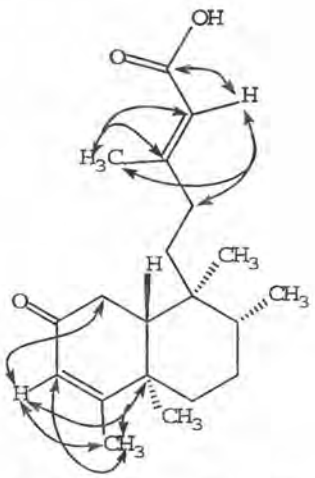


Fig. 4.29a Important Long Range H/C HMBC Correlations for Compound 4.5

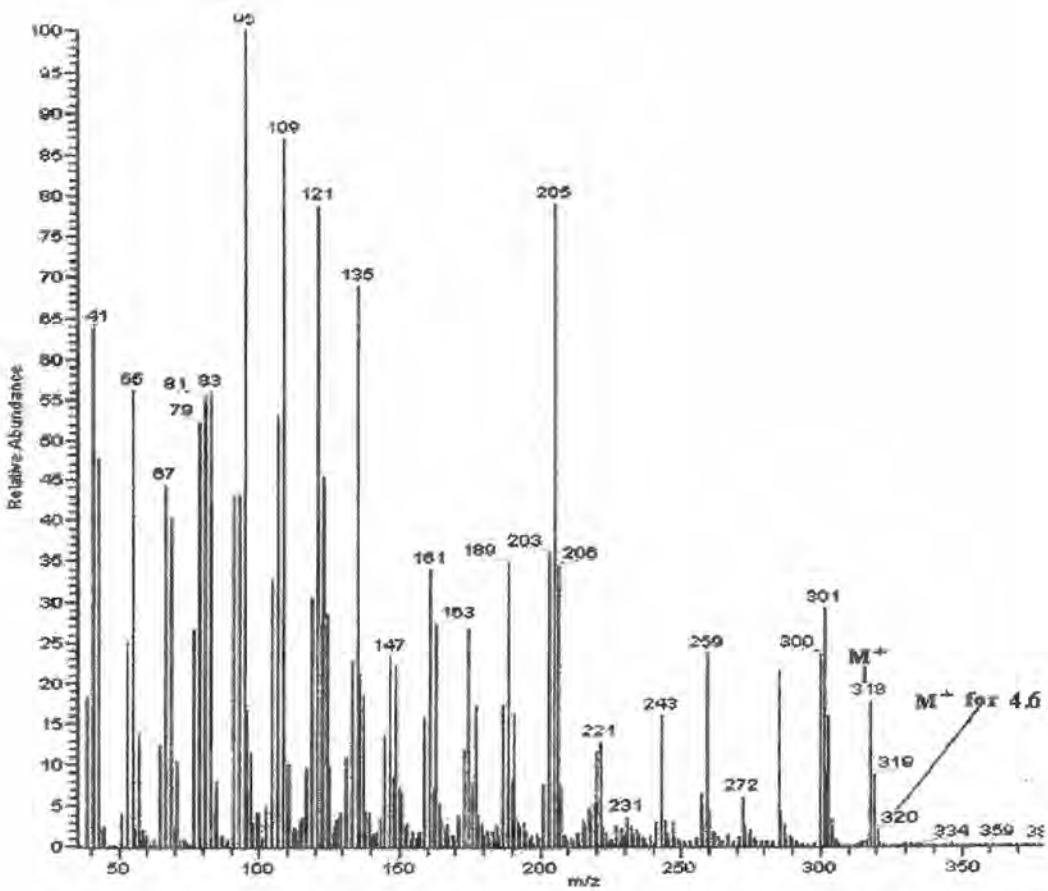
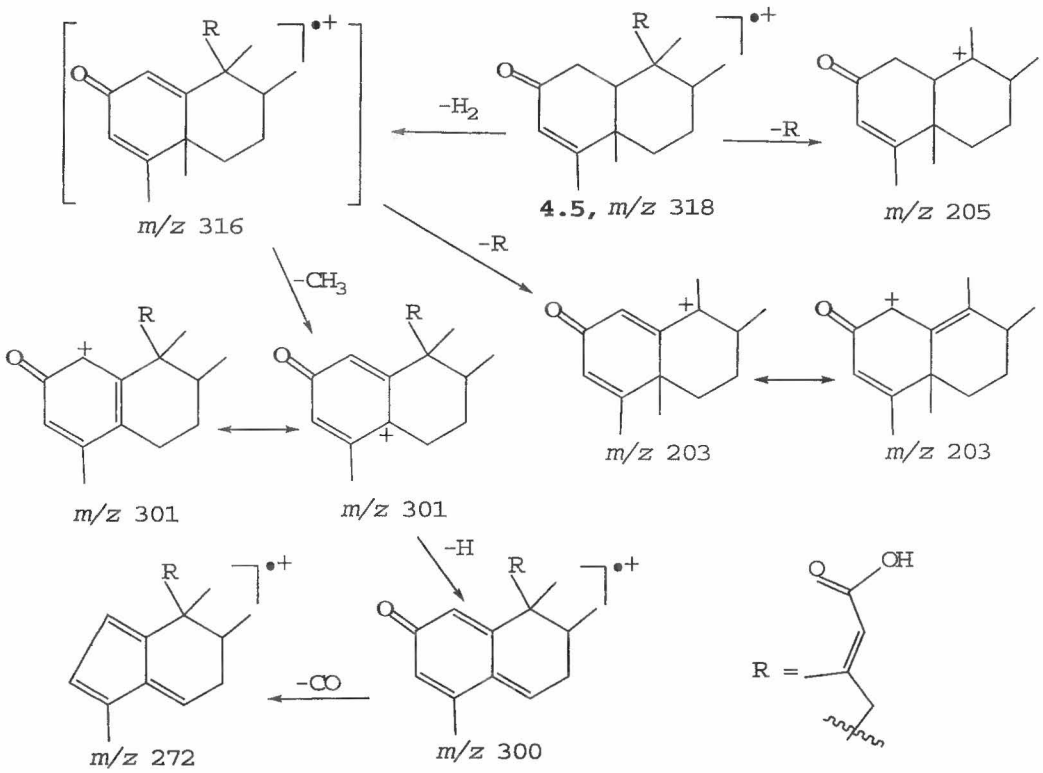


Fig. 4.30 Mass Spectrum of 2-Oxo-*ent*-cleroda-3,13(*Z*)-dien-15-oic acid (4.5)

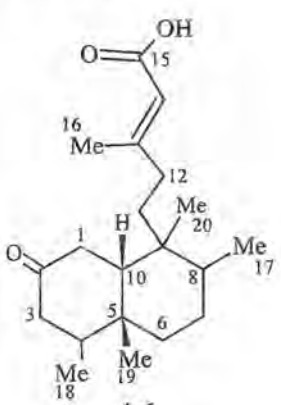
Scheme 4.4 Mass Spectral Fragmentation Pattern for 2-Oxo-*ent*-cleroda-3,13(*Z*)-dien-15-oic acid (4.5)



4.2.6 *cis*-2-Oxo-*ent*-cleroda-13(*Z*)-dien-15-oic acid (4.6)

Repeated column chromatography of the filtrate obtained from the combined larvicidal VLC fraction 3 of the chloroform extract from the root bark of *T. martiniana* var *martiniana* yielded an oil whose structure was established on the basis of the ^{1}H and ^{13}C NMR, and mass spectral features (Fig. 4.31, 4.32 and 4.36, Table 4.6) upon comparison of these properties with those observed for 2-oxo-3,13(*Z*)-dien-15-oic acid (**4.5**) (Section 4.2.5). Thus, the MS exhibited the molecular ion peak at *m/z* 320, which corresponds to the molecular formula $C_{20}H_{32}O_3$ (exact mass, *m/z* 320.2318, calculated for $C_{20}H_{30}O_3$ = 320.4663). The ^{1}H and ^{13}C NMR spectral features for the two compounds **4.5** and **4.6** closely resembled each other,

except for the absence of the signal due to the C-3/C-4 unsaturation in the spectra for **4.6** and appearance of an additional secondary methyl proton signal in the ^{1}H NMR spectrum (δ 2.17, d, J = 0.6 Hz) which was consistent with structure **4.6**, and a carbonyl carbon resonance at δ 200.1. HMBC interactions (Fig. 4.35a) indicated the carbonyl group being at C-2, as in structure **4.6**. Full assignment of the ^{1}H and ^{13}C NMR spectral data was achieved with the help of H/H COSY and C/H HMBC and HMQC interactions, respectively (Figs. 4.33, 4.34 and 4.35). HMQC enabled the assignment of all but the quaternary carbon signals, while long range H/C correlations (HMBC) in addition allowed the assignment of the quaternary carbons and the corresponding connectivities, thereby establishing the substitution pattern of the compound and this confirmed that the compound had structure **4.6**. Unfortunately, as it was noted in the ^{1}H and ^{13}C NMR spectra, compound **4.6** contained some impurities which could not be removed during the purification process. The major impurity appeared to be a compound closely related to **4.6**, probably possessing a hydroxyl group at C-2, instead of a carbonyl moiety, as well as a quaternary carbinol carbon (^{13}C NMR).



4.6

Besides the M^+ peak at *m/z* 320 the MS (Fig. 3.36) also exhibited other prominent ion peaks as shown in Scheme 4.5, all of which were consistent with structure **4.6**. The mosquitocidal activity of this compound as determined in these investigations is reported in Chapter 6.

Table 4.6 NMR Spectral Data for *cis*-2-Oxo-*ent*-cleroda-13(*Z*)-dien-15-oic acid (4.6)

	4.6			**4.5**[158]		
	δ_H	*J* (Hz)	δ_C	δ_H	*J* (Hz)	δ_C
1a	1.47	m	36.8	2.48	dd, 18, 4	35.9
1b	1.68	dd, 12.9, 6.6		2.36	dd, 18, 14	
2	-	-	200.1	-		200.5
3a	1.72	m	42.3	5.73	br s	126.3
3b	1.50	m				
4	1.35-1.45	m	27.0			171.8
5	-		31.6			40.3
6a	1.10	td, 11.4, 3.9	36.9			35.4
6b	2.64	td, 13.2				
7	1.49	m	21.0			27.1
8	1.49	m	34.8	1.61	ddq, 6.5	36.4
9	-	-	39.8			39.2
10	1.28	m	43.2	1.96	dd, 14, 4	46.2
11a	1.49	m	23.3	1.52	m, 12, 12, 5	34.7
11b				1.40	dt, 12, 5	
12a	2.16	td, 11.7, 0.9	36.6	2.64	dt, 12, 12	36.0
12b	2.05	td, 11.1, 5.4		2.24	dt, 12, 5	
13	-	-	164.2	-		163.6
14	5.67	d, 0.9	114.9	5.62	br s	114.9
15	-	-	171.4	-		172.8
16	1.78	br s	11.4	1.87	d, 1.5	19.4
17	1.31	d	23.3	0.88	d, 6.5	16.2
18	2.17	d, 0.6	19.3	1.88	d, 1	18.8
19	1.03	s	14.5	1.10	s	19.9
20	0.74	s	18.1	0.79	s	18.3

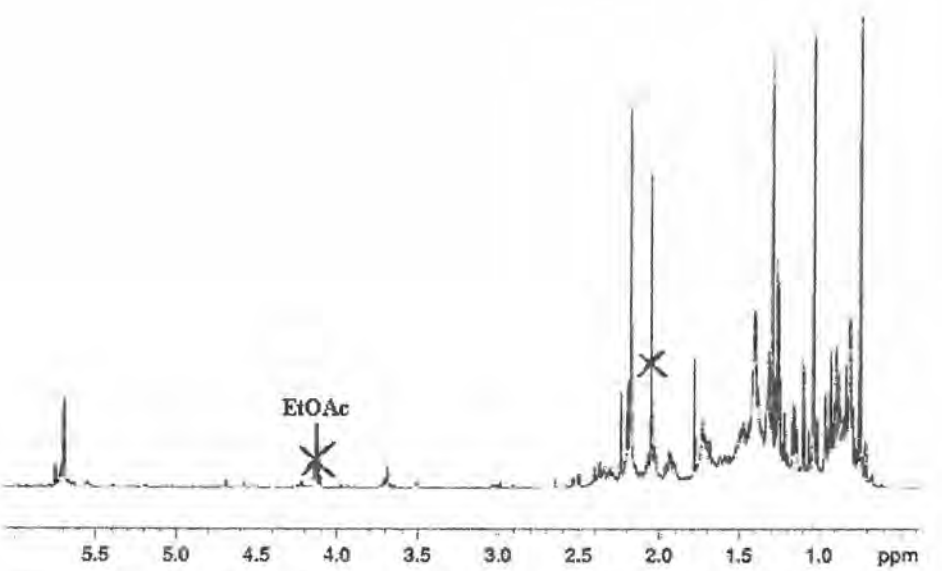


Fig. 4.31 ^{1}H NMR Spectrum of *cis*-2-Oxo-*ent*-cleroda-13(*Z*)-dien-15-oic acid (4.6)

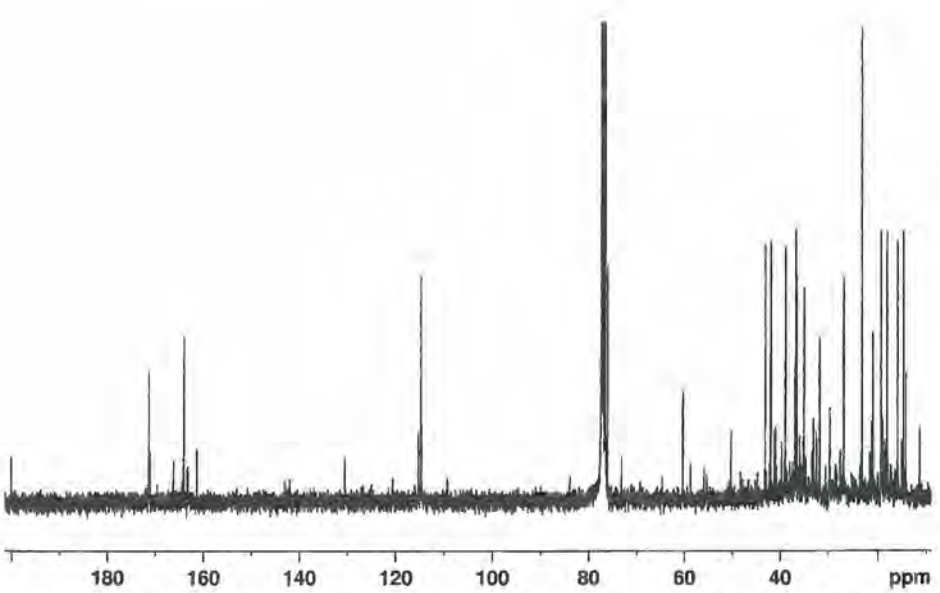


Fig. 4.32 ^{13}C NMR Spectrum of *cis*-2-Oxo-*ent*-cleroda-13(*Z*)-dien-15-oic acid (4.6)

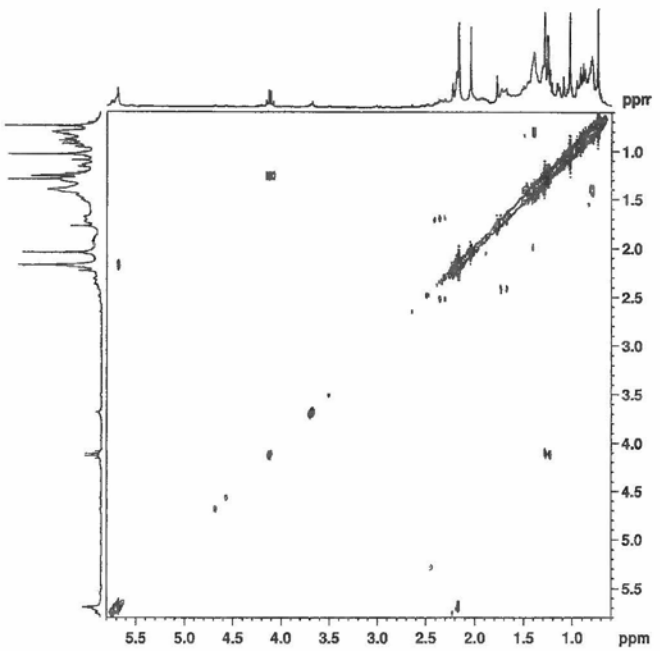


Fig. 4.33 COSY Spectrum of *cis*-2-Oxo-*ent*-cleroda-13(*Z*)-dien-15-oic acid (4.6)

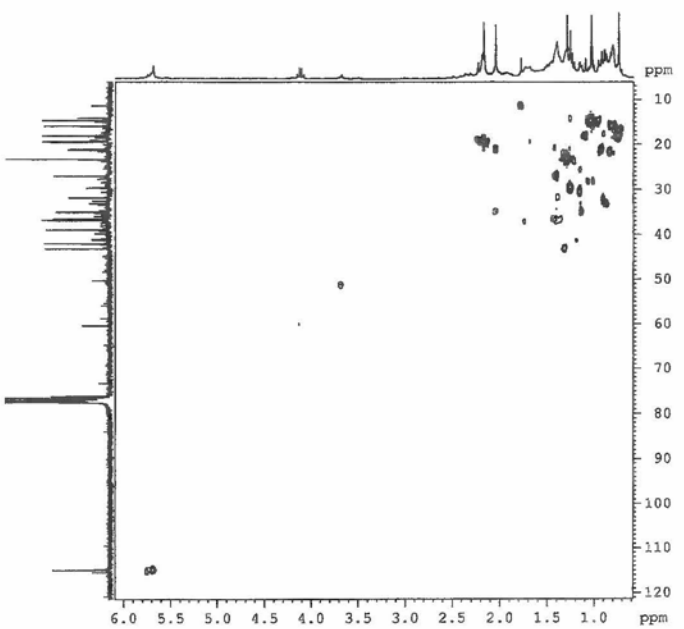


Fig. 4.34 HMQC Spectrum of *cis*-2-Oxo-*ent*-cleroda-13(*Z*)-dien-15-oic acid (4.6)

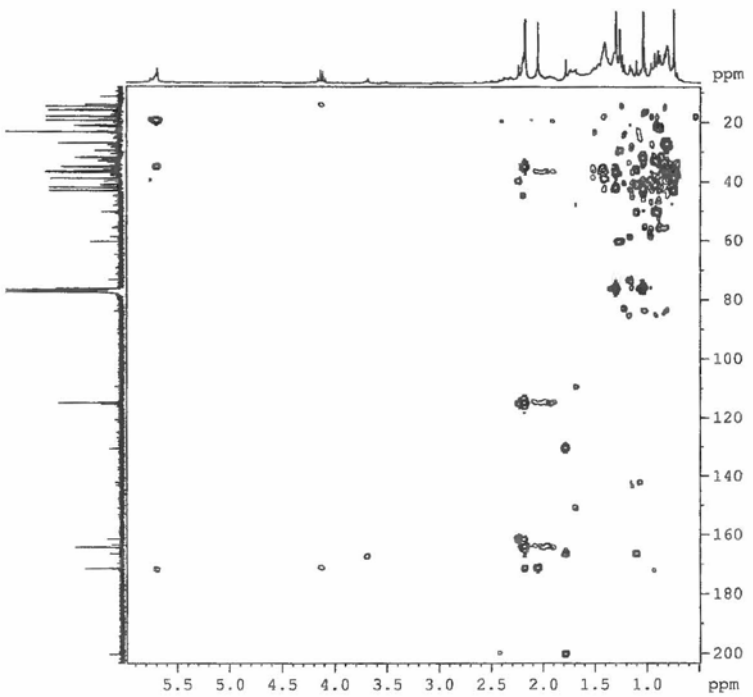


Fig. 4.35 HMBC Spectrum of *cis*-2-Oxo-*ent*-cleroda-13(*Z*)-dien-15-oic acid (4.6)

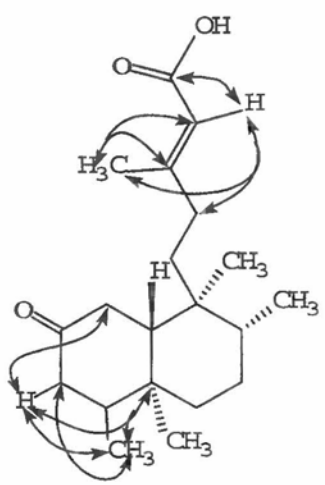


Fig. 4.35a Important Long Range H/C HMBC Correlations for Compound 4.6

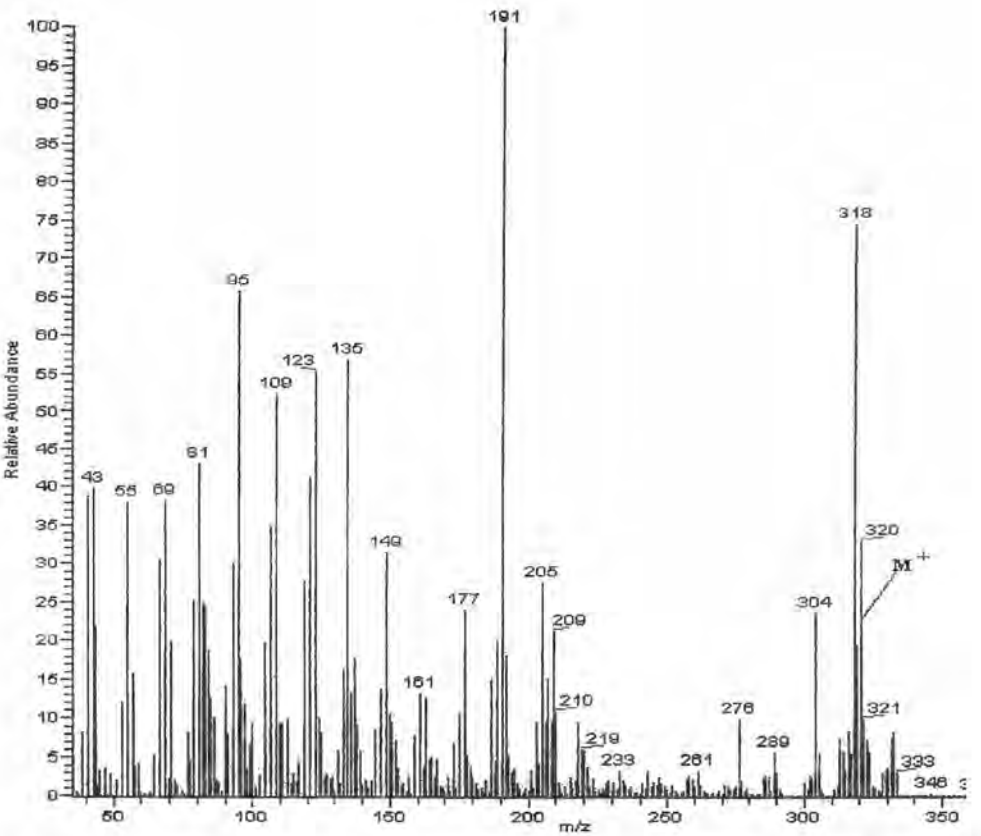
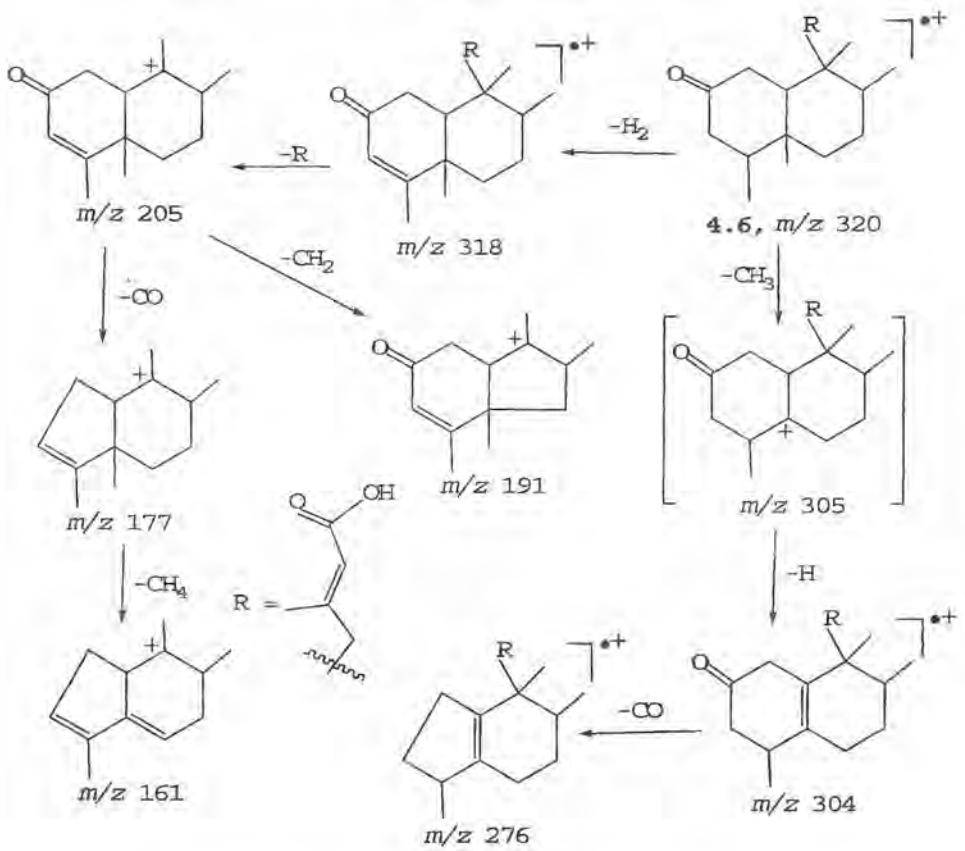


Fig. 4.36 Mass Spectrum of *cis*-2-Oxo-*ent*-cleroda-13(*Z*)-dien-15-oic acid (4.6)

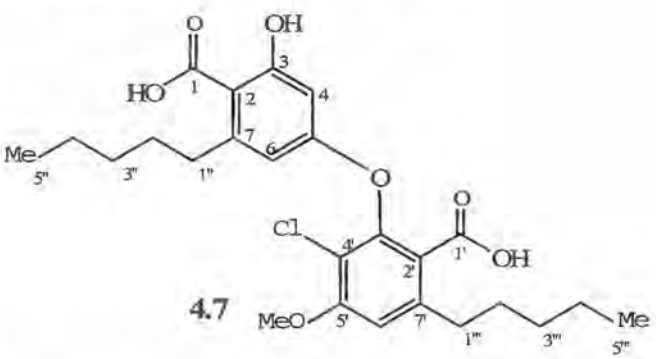
Scheme 4.5 Mass Spectral Fragmentation Pattern for *cis*-2-Oxo-*ent*-cleroda-13(*Z*)-dien-15-oic acid (4.6)



4.2.7 *O*-(3-Hydroxy-4-hydroxycarbonyl-5-pentylphenyl)-3-chloro-4-methoxy-5-pentyl-2-oxybenzoic acid (4.7)

The 4th VLC fraction of the chloroform extract of the stem bark of *T. martiniana* var *martiniana* on repeated chromatography yielded white crystals, m.p 149 °C, whose structure **4.7** was deduced on the basis of its ^{1}H and ^{13}C NMR, and mass spectral features (Figs. 4.37, 4.38 and 4.42) upon comparison of these properties with spectral data for compounds **3.14** and **3.15** that were isolated from *Tessmannia densiflora* (Section 3.2.1.5). Compound **4.7** was found to constitute two units derived from **3.14** and **3.15** having been linked through an oxygen bridge. Thus, the

MS showed the M-1 peak at 477.2 which corresponds to the molecular formula $C_{25}H_{31}O_7Cl$ (exact mass, m/z 478.1758, calculated for $C_{25}H_{31}O_7Cl$ = 478.9624). Both the ^1H and ^{13}C NMR spectra (Figs. 4.37 and 4.38) consisted of signals expected for structure **4.7**. Thus, the ^{13}C NMR spectrum revealed 14 signals in the low field region, which, compared with the ^1H NMR spectrum, indicated the presence of two substituted benzene rings, one of which being methoxylated at a carbon atom flanked by only one substituted *ortho* position (δ_H 3.99, s, δ_{OCH3} 56.3).[162] Furthermore, the ^1H NMR spectrum revealed the presence of two methylene groups (δ_H 3.01, m) each being substituted to a benzene ring and whose ^1H NMR signal appeared at the same position. The spectrum also showed another superimposed signal at δ_H 1.68 (m) due to protons of two methylene groups, then a signal due to protons of four methylene groups at δ 1.34 (m), and signals at δ 0.91 and 0.88 as a superimposed multiplet due to two terminal methyl groups. These spectral features suggested that the isolated compound consisted of two C-5 chains attached to either one or each of the benzene rings.



4.7

In the low field region, the ^1H NMR spectrum consisted of signals due to two *meta* coupling aromatic protons (δ 6.63, d, J = 2.4 Hz and 6.75, d, J = 2.1 Hz), one isolated aromatic proton (δ 6.44, s) and signals due to chelated OH protons at δ 11.3 and 11.7,

the former being broadened and was therefore assumed to be due to a carboxylic OH group. Indeed, the presence of two carboxylic groups would account for the observed signals at δ_C 174.7 and 169.2 in the ^{13}C NMR spectrum. Full assignment of the ^{1}H and ^{13}C NMR signals were facilitated by considering the single bond and long range H/C interactions observed in the HMQC and HMBC plots respectively (Figs. 4.40 and 4.41). These interactions as well as H/H correlations observed in the COSY plot (Fig. 4.39) enabled complete assignment of all protons and most of the C atoms in structure **4.7**.

The presence of a chlorine unit was deduced from the high resolution EIMS which showed the presence of two peaks at *m/z* 477.1626 and 479.1635 that are separated by 2 atomic mass units and being in the ratio of 3:1, which corresponds to the natural abundance of the two isotopes of the chlorine atom (Cl:35, 75% and Cl:37, 25%).[142] The position of the Cl atom in structure **4.7** was deduced based on analysis of HMBC interactions that indicated the relative positions of all substituents in structure **4.7**.

Besides the M-1 peak, the MS (Fig. 4.42) exhibited several other peaks that were consistent with the fragmentation pattern shown in Schemes 4.6, which is in agreement with structure **4.7**.

As no compound with structure **4.7** was found in the literature, this is therefore the first time that compound **4.7** is being reported.

Table 4.7 NMR Spectral Data for *O*-(3-Hydroxy-4-hydroxycarbonyl-5-pentylphenyl)-3-chloro-4-methoxy-5-pentyl-2-oxybenzoic acid (4.7)

H/C	δ_H	*J* (Hz)	δ_C	H/H COSY	H/C HMBC
1	-		174.7		
2	-		108.6		
3	-		165.2		
4	6.75	d, 2.1	108.6	H-6	C-2, C-3, C-5, C-6
5	-		154.7		
6	6.63	d, 2.4	115.9	H-4	C-2, C-4, C-5, C-1"
7	-		146.8		
1'	-		169.2		
2'	-		105.2		
3'	-		160.5		
4'	-		107.9		
5'	-		159.8		
6'	6.44	s	106.5		C-2', C-4', C-5', C-1"'
7'	-		150.1		
1"	3.01	m	36.5	H-2"	C-2, C-6, C-7, C-2", C-3"
2"	1.68	m	32.0	H-1", H-3"	C-1", C-4"
3"	1.34	m	31.3	H-2", H-4"	C-2", C-4"
4"	1.34	m	22.5	H-3", H-5"	C-2", C-4", C-5"
5"	0.91	d, 7.5	14.0	H-4"	C-2", C-3", C-4"
1"'	3.01	m	37.6	H-2"'	C-2', C-6', C-7', C-2"', C-3"'
2"'	1.68	m	32.1	H-1"', H-3"'	C-1"', C-4"'
3"'	1.34	m	31.9	H-2"', H-4"'	C-2"', C-4"'
4"'	1.34	m	22.4	H-3"', H-5"'	C-2"', C-4"', C-5"'
5"'	0.88	d, 7.0	14.0	H-4"'	C-2"', C-3"', C-4"'
OMe	3.99	s	56.3		C-5'
COOH	11.30	br s			
OH	11.70	br s			

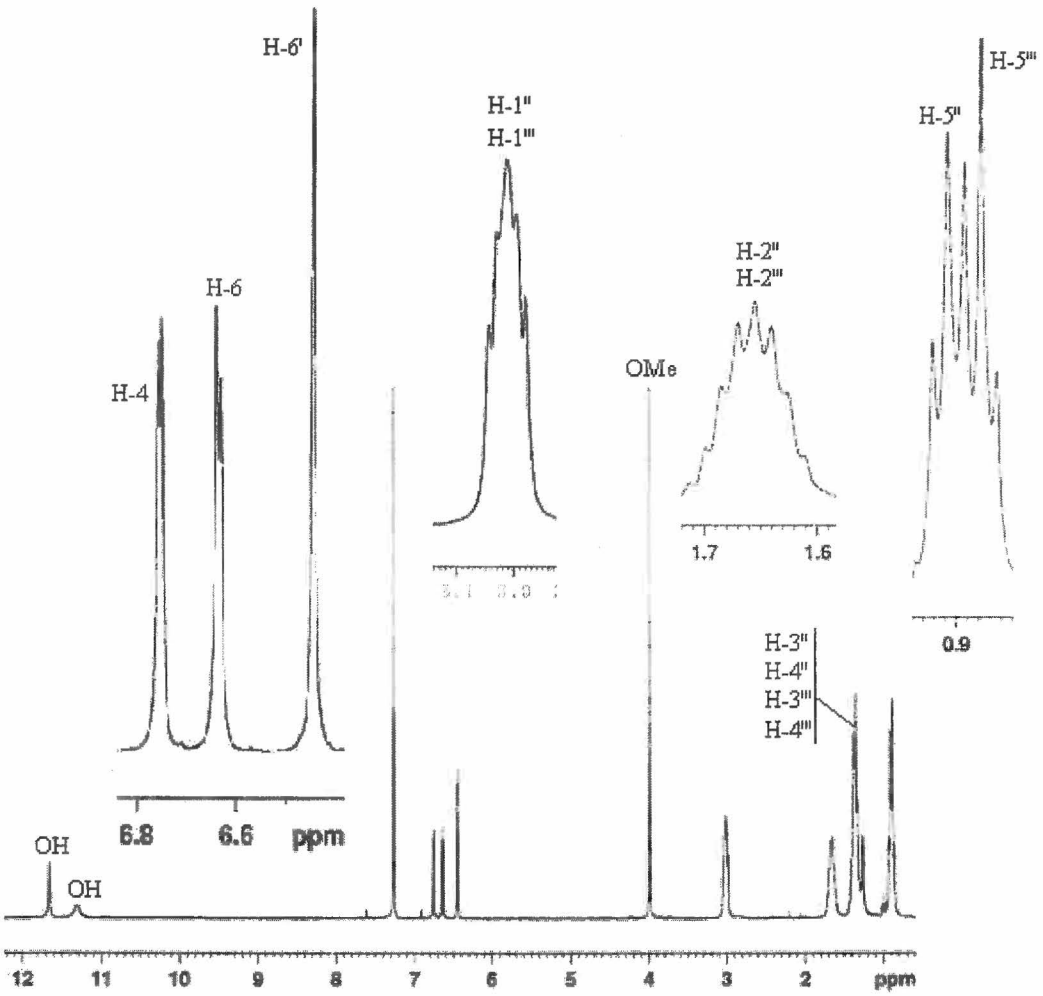


Fig. 4.37 ^{1}H NMR Spectrum of *O*-(3-Hydroxy-4-hydroxycarbonyl-5-pentylphenyl)-3-chloro-4-methoxy-5-pentyl-2-oxybenzoic acid (4.7)

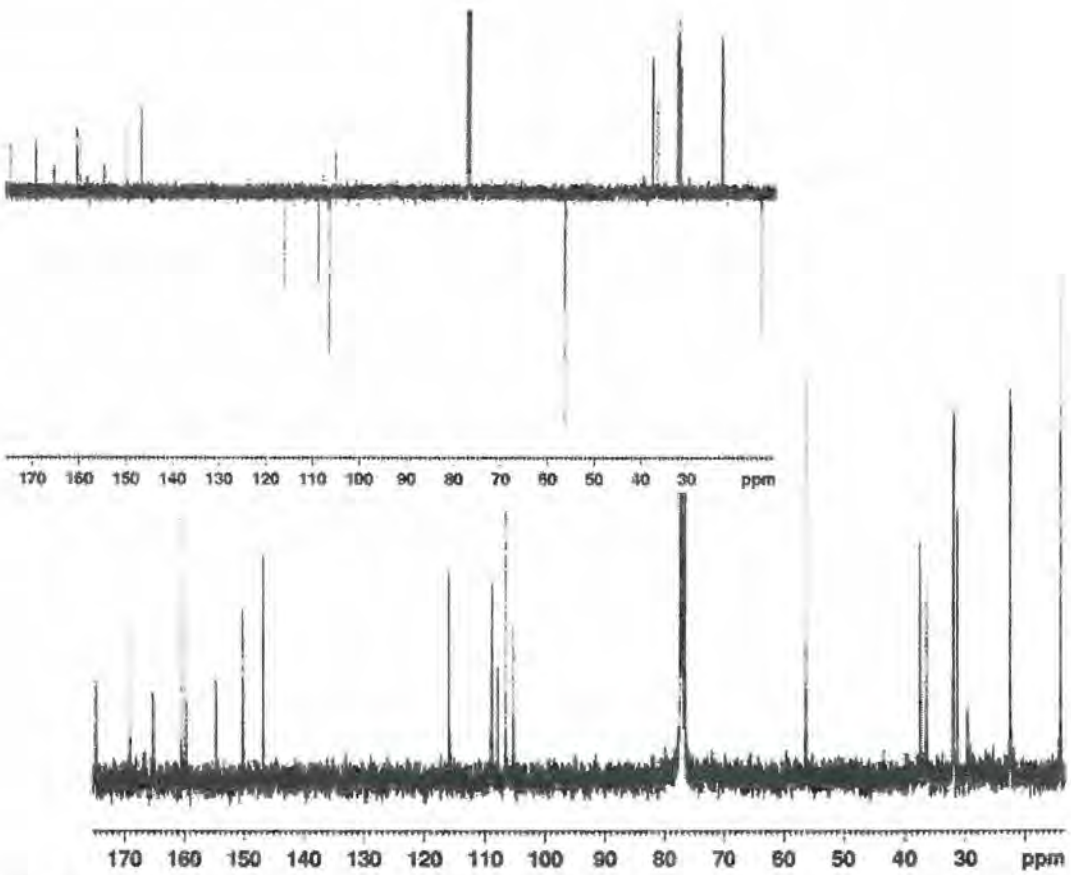


Fig. 4.38 ^{13}C NMR Spectrum (With APT Experiment) of *O*-(3-Hydroxy-4-hydroxycarbonyl-5-pentylphenyl)-3-chloro-4-methoxy-5-pentyl-2-oxybenzoic acid (4.7)

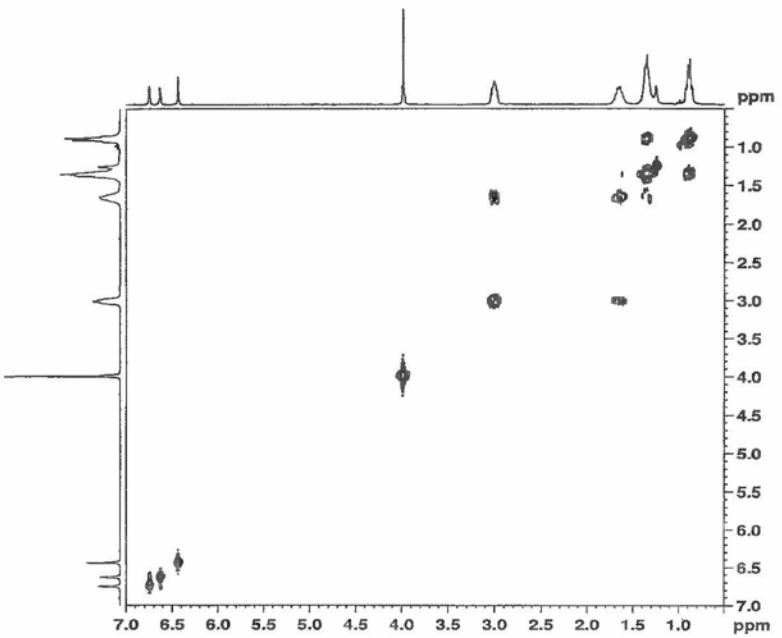


Fig. 4.39 COSY Spectrum of *O*-(3-Hydroxy-4-hydroxycarbonyl-5-pentylphenyl)-3-chloro-4-methoxy-5-pentyl-2-oxybenzoic acid (4.7)

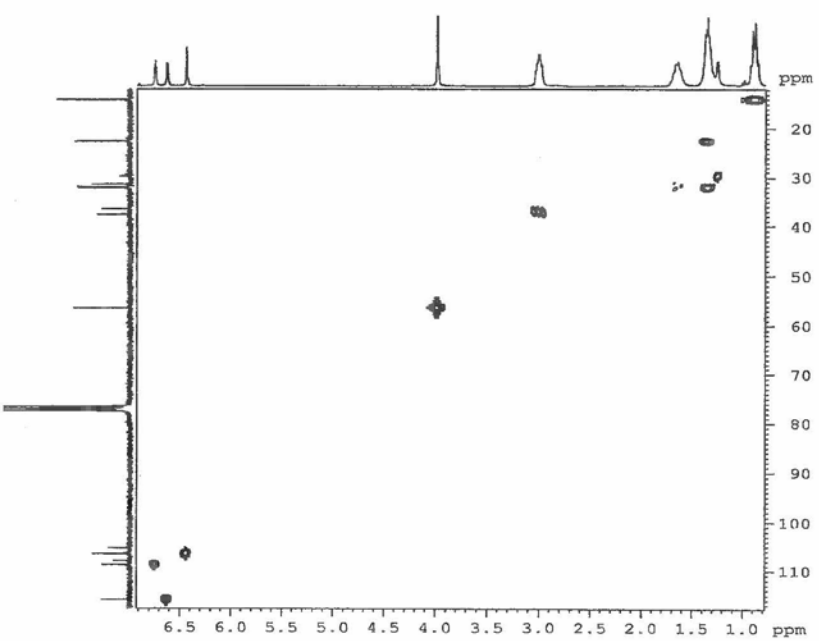


Fig. 4.40 HMQC spectrum of *O*-(3-Hydroxy-4-hydroxycarbonyl-5-pentylphenyl)-3-chloro-4-methoxy-5-pentyl-2-oxybenzoic acid (4.7)

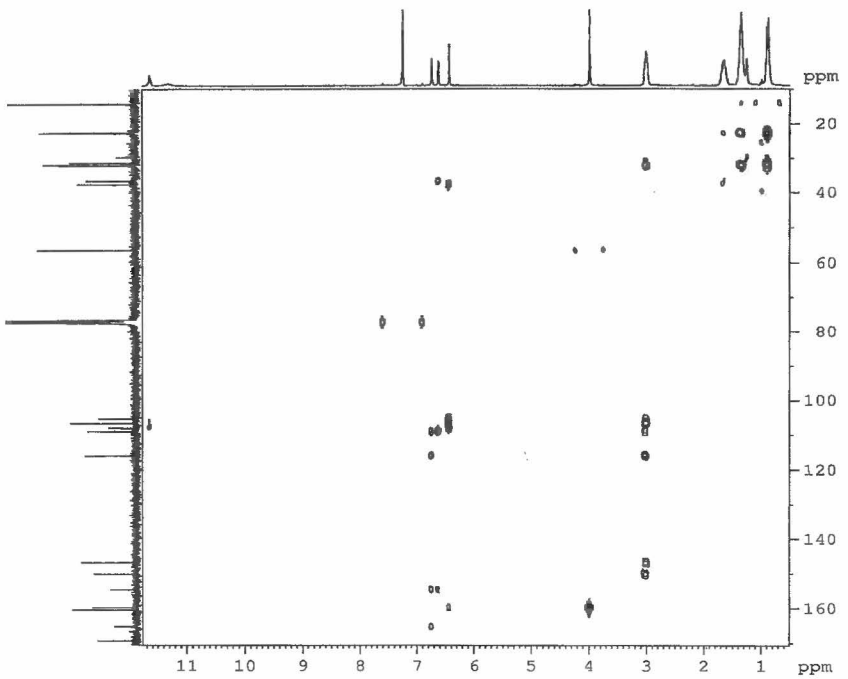


Fig. 4.41 HMBC Spectrum of *O*-(3-hydroxy-4-hydroxycarbonyl-5-pentylphenyl)-3-chloro-4-methoxy-5-pentyl-2-oxybenzoic acid (4.7)

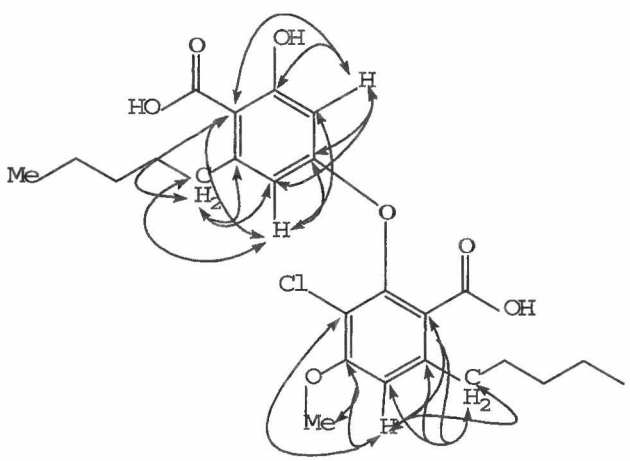


Fig. 4.41a Important Long Range H/C HMBC Correlation for Compound 4.7

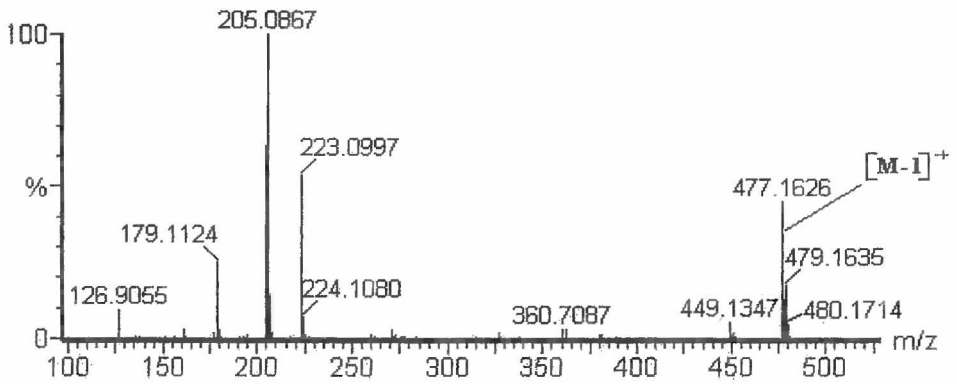
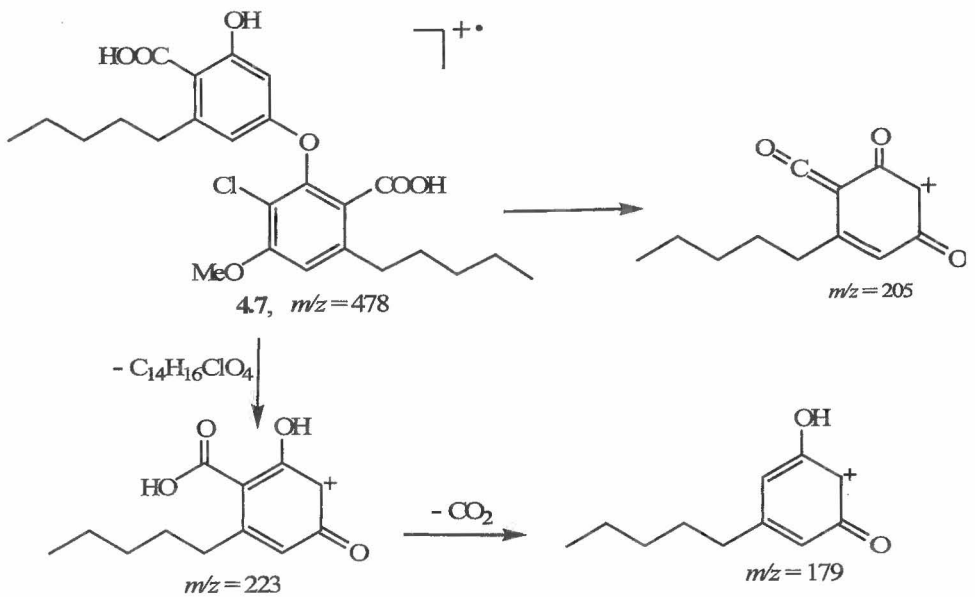


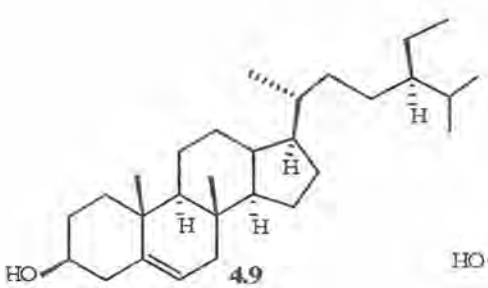
Fig. 4.42 Mass Spectrum of *O*-(3-Hydroxy-4-hydroxycarbonyl-5-pentylphenyl)-3-chloro-4-methoxy-5-pentyl-2-oxybenzoic acid (4.7)

Scheme 4.6 Mass Spectral Fragmentation Pattern for *O*-(3-hydroxy-4-hydroxycarbonyl-5-pentylphenyl)-3-chloro-4-methoxy-5-pentyl-2-oxybenzoic acid (4.7)

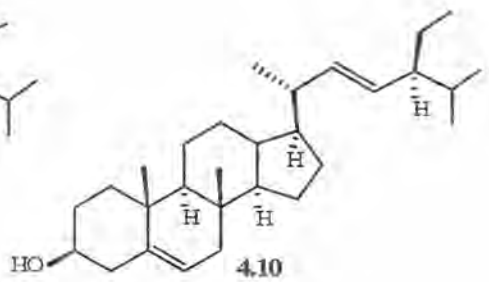


4.2.8 β-Sitosterol (4.9) and Stigmasterol (4.10)

The two commonly occurring steroids β-sitosterol (**4.9**) and stigmasterol (**4.10**) were isolated from the stem bark of *T. martiniana* var *martiniana* as white crystals and their structures were established based on spectral properties.



4.9



4.10

4.3 EXPERIMENTAL

4.3.1 General

These were similar to those described in Chapter 3 (Sections 3.2.1).

4.3.2 Biological Tests

These were carried out as described in Chapters 2 and 6.

4.3.3 Plant Materials

The root and stem barks of *Tessmannia martiniana* var *pauloi* and *T. martiniana* var *martiniana* were collected from Pugu and Zaraninge Forest Reserves, Kisarawe and Bagamoyo District, respectively, Coast Region in Tanzania, in March 2006. The plant species was authenticated at the Herbarium, Department of Botany, University of Dar es Salaam, Tanzania where voucher specimens are preserved (Mbago: FMM1321, 3410 and 3374).

4.3.4 Extraction and Isolation of Compounds

The air-dried and pulverized plant materials were extracted sequentially with chloroform and methanol, each extraction lasting for 48 h for two rounds of extraction for each solvent. The concentrated (*vacuo*) crude extracts were then separated into pure compounds using different chromatographic techniques.

4.3.4.1 Methanol Extract of the Root Bark of *Tessmannia martiniana* var *pauloi*

The crude methanol extract (20 g) from the root bark of *T. martiniana* var *pauloi* was obtained by direct total extraction using methanol, due to the paucity of the plant materials. The extract was screened for activity against *An. gambiae* mosquito larvae and then fractionated by vacuum liquid chromatography (VLC) on silica gel, eluting with pet ether, then pet ether containing increasing amounts of ethyl acetate, and later ethyl acetate containing increasing amounts of methanol. Seventeen fractions were obtained which on the basis of TLC analysis were combined into five series as follows: Fractions 1(1-4), 2(5-7), 3(8-11), 4(12-14) and 5(15-17). The combined VLC fractions were then screened for larvicidal activity. There was no significance difference in activity in terms of mortality among the combined VLC fractions after 24 h exposure (Table 2.7 Chapter 2). However the combined VLC fractions 3 and 4 displayed interesting larvae deformities (Fig. 2.3) which formed after 24 h exposure and reached the peak after 72 h exposure. Despite the interesting activity shown by these fractions, they could not be worked up further to get their constituents, due to the fact that they consisted of complex mixtures of non-, medium and highly polar compounds which decomposed on TLC analysis and therefore isolation on silica gel and Sephadex® LH-20 chromatography was not possible.

Silica gel chromatography of the first combined VLC fractions, eluting with a mixture of ethyl acetate/and pet ether (5–10 %) yielded 34 fractions which were also combined on the basis of TLC analysis and further chromatography of the latter combined fraction yielded compounds **4.2** and **4.4**. The isolation procedure is summerised in **Chart 4.1**.

4.3.4.2 Chloroform Extract of the Root Bark of *Tessmannia martiniana* var *martiniana*

The crude chloroform extract (25 g) from the root bark of *T. martiniana* var *martiniana* was fractionated by vacuum liquid chromatography (VLC) on silica gel, eluting with pet ether and then pet ether containing increasing amounts of ethyl acetate followed by increasing amounts of methanol. Fourteen fractions were obtained and TLC analysis led to the combination of these fractions into six series as follows: Fractions 1(1-2), 2(3-6), 3(7-8), 4(9-10), 5(11-13) and 6(14). The second and sixth series consisted of complex mixtures of non polar, medium and highly polar compounds which decomposed during further separation on silica gel and Sephadex® LH-20. Therefore, these were not analysed further. The first series of the combined VLC fractions, was subjected to silica gel chromatography (5 % EtOAc/petroleum ether) yielding compound **4.3**, which was recrystallized from the third combined series and on standing deposited crystals which on recrystallization (pet ether) yielded more of **4.3**. The filtrate upon silica gel chromatography (20 % ethyl acetate/pet ether) yielded compound **4.6** as oil. Series 5 combined fraction on silica gel chromatography (20-50 % ethyl acetate/pet ether) gave compound **4.5**. The isolation procedure for compounds from the root bark of *T. martiniana* var *martiniana* is summarised in **Chart 4.2**.

4.3.4.3 Chloroform Extract of the Stem Bark of *Tessmannia martiniana* var *martiniana*

The chloroform extract (25 g) from the root bark of *T. martiniana* var *martiniana* obtained after sequential soaking twice in chloroform and methanol of the air-dried

pulverized plant materials and each soaking lasting 48 h, was fractionated by VLC on silica gel, eluting with pet ether, and then pet ether containing increasing amounts of EtOAc (Scheme 4.3). Eleven fractions were obtained and TLC analysis led to the combination of these fractions into five series as follows: Fractions 1(1), 2(2-3), 3(4), 4(5-8) and 5(9-11). The first combination series consisted of a complex mixture of non-polar compounds and hence this was not analysed further. Combination series 2 and 3 showed almost a similar composition upon TLC analysis. Therefore, these were combined and subjected to silica gel chromatography (pet ether/EtOAc gradient) and compounds **4.8** and **4.9** were obtained as white crystals. The combined series 4 upon standing at room temperature formed crystals of **4.7** which were recrystalised from a mixture of pet ether and EtOAc (2:1 v/v). The isolation process for compounds **4.7-4.9** is summarized in **Chart 4.3**.

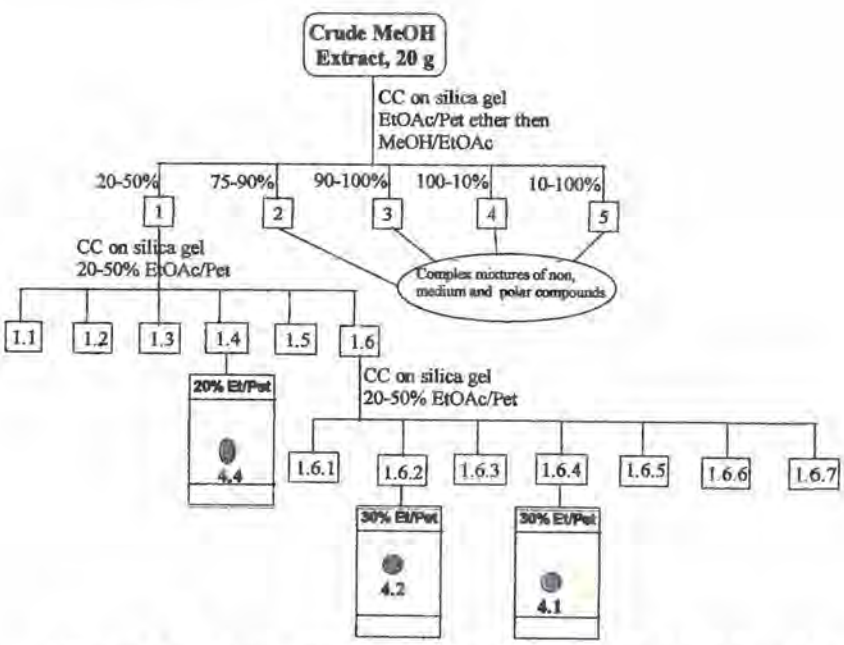


Chart 4.1 Isolation Scheme for Compounds 4.1, 4.2 and 4.4

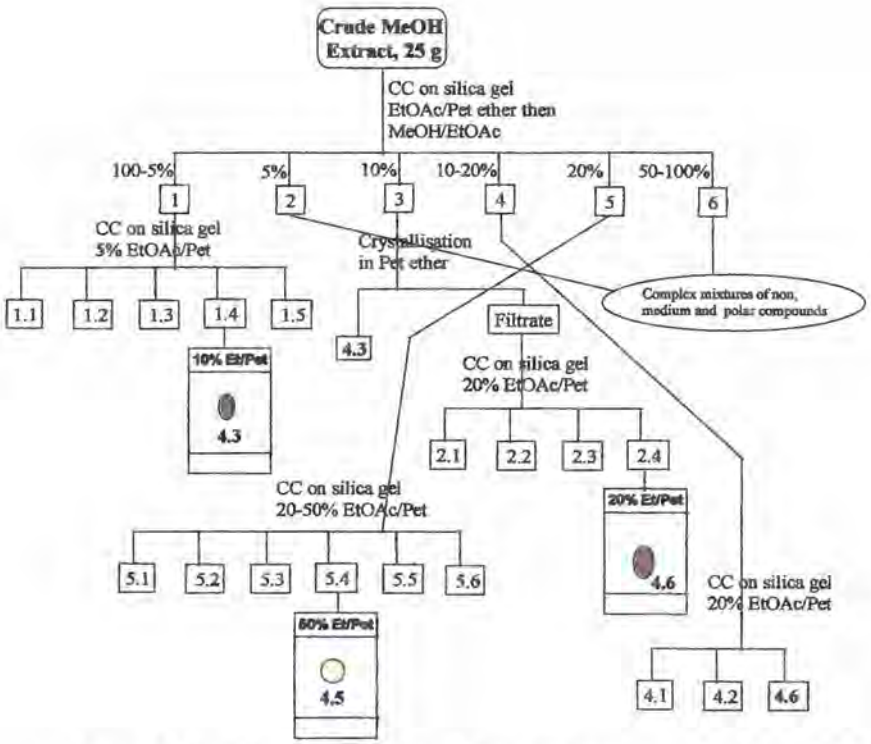


Chart 4.2 Isolation Scheme for Compounds 4.3, 4.5 and 4.6

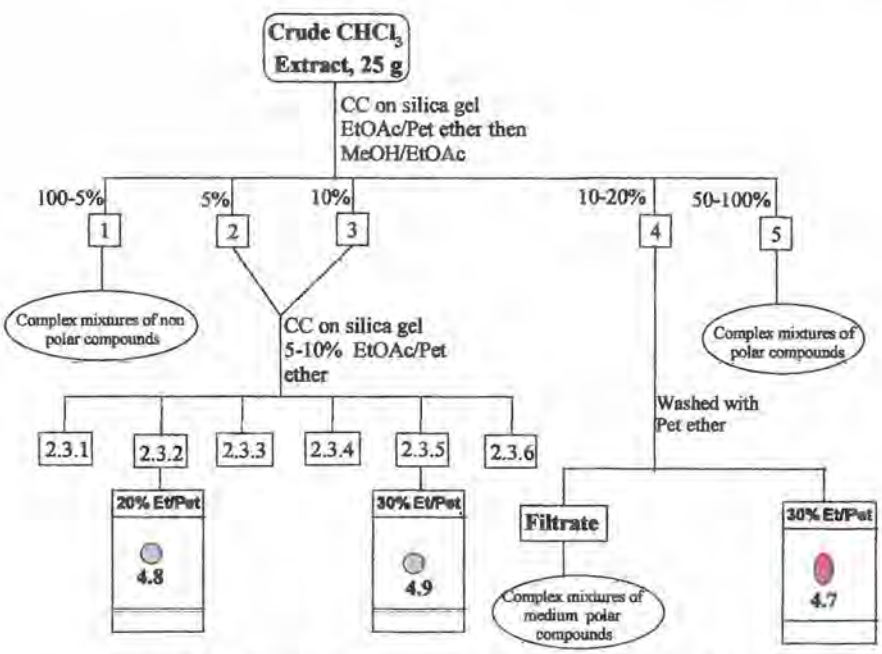


Chart 4.3 Isolation Scheme for Compounds 4.7-4.9

cis-Kolavenolic acid **(4.1)**. Yellow oil; yield, 36 mg; anisaldehyde: pale-blue; MS, *m/z* (% rel. int.) 323 ($[M+1]^+$, 8), 304 (33), 209 (40), 191 (88), 177 (10) and 163 (8); ^1H and ^{13}C NMR: See Table 4.1.

18-Oxo-3,13E-clerodien-15-oic acid **(4.2)**. Yellow Oil; yield, 7 mg; anisaldehyde: brown; MS, *m/z* (% rel. int.) 316 ($[M-2]^+$, 4), 301 (18), 300 (8), 203 (16) and 187 (20); ^1H and ^{13}C NMR: See Table 4.2.

ent-(18-Hydroxycarbonyl)-cleroda-3,13(E)-dien-15-oate **(4.3)**. White crystals; yield, 1.5 g; anisaldehyde: puple; m.p. 119-120 °C (Lit.[157] m.p. 162-163 °C); $[\alpha]_D^{20} = -132°$ ($CHCl_3$, c = 0.2); MS, *m/z* (% rel. int.) 349 ($[M+1]^+$, 4), 331 (100), 330 (58), 315 (65), 221 (16), 219 (28) and 201 (15); ^1H and ^{13}C NMR: See Table 4.3.

(9-epi)-ent-(18-Hydroxycarbonyl)-cleroda-3,13(E)-dien-15-oate **(4.4)**. Oil; yield, 2.102 g; anisaldehyde: purple; $[\alpha]_D^{20} = -126°$ ($CHCl_3$, c = 0.2); ^1H and ^{13}C NMR: See Table 4.4.

2-Oxo-ent-cleroda-3,13(Z)-dien-15-oic acid **(4.5)**. Oil; yield, 106 mg; anisaldehyde: yellow; $[\alpha]_D^{20} = -132°$ ($CHCl_3$, c = 0.2); MS, *m/z* (% rel. int.) 318 (M^+, 18), 301 (300), 300 (24), 272 (6), 205 (79) and 203 (35); ^1H and ^{13}C NMR: See Table 4.5.

cis-2-Oxo-ent-cleroda-13(Z)-dien-15-oic acid **(4.6)**. Oil; yield, 478 mg; anisaldehyde: puple/brown; MS, *m/z* (% rel. int.) 320 (M^+, 33), 318 (75), 304 (24), 276 (8), 205 (28), 191 (100), 177 (25) and 161 (13); ^1H and ^{13}C NMR: See Table 4.6.

O-(3-Hydroxy-4-hydroxycarbonyl-5-pentylphenyl)-3-chloro-4-methoxy-5-pentyl-2-oxybenzoic acid **(4.7)**. Oil; yield, 4 mg; anisaldehyde: red; m.p. 149 °C. MS, m/z (% rel. int.) 479 ($[M+1]^+$, 15), 477 ($[M-1]^+$, 45), 223 (55), 205 (100) and 179 (25); ^1H and ^{13}C NMR: See Table 4.7.

CHAPTER FIVE

A NOVEL TRIHYDROXYAROMADENDRENOID, KAURANOIDS AND OTHER CONSTITUENTS OF THREE TANZANIAN ANNONACEAE SPECIES

Abstract

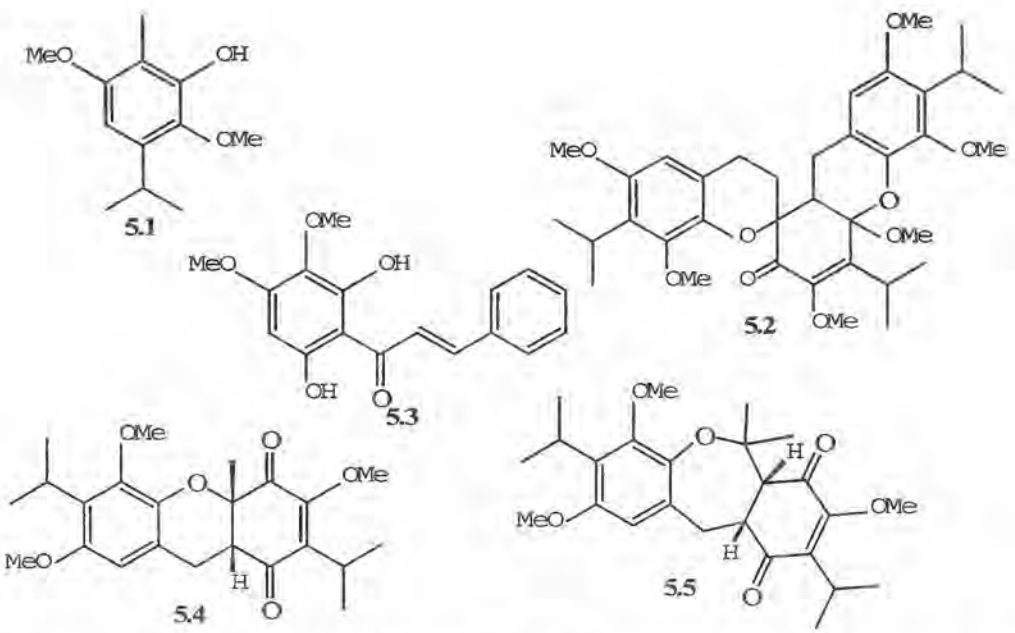
This chapter describes bioassay guided isolation and structural determination of larvicidal, mosquitocidal and mosquito repellent constituents of extracts from the stem and/or root barks of the Annonaceae species *Uvaria lungonyana*, *Polyalthia tanganyikensis*, *Uvariodendron pycnophyllum* and *Annona squamosa*. The structures of the 16 compounds that were isolated from the four plant species were established on the basis of analysis of spectroscopic data. Among the isolated compounds a trihydroxylated aromadendrene sesquiterpenoid tanganyikenol, and 17-acetoxy-*ent*-kauran-19-al are hitherto reported for the first time. Results from bioassays for larvicidal, mosquitocidal and mosquito repellency activities of the isolated compounds against the malaria transmitting *Anopheles gambiae* s.s Giles mosquitoes were performed according to WHO protocols. Antimicrobial activities against the Gram positive and/or Gram negative bacteria, and fungi, were also carried out as reported in Chapter 6.

5.1 INTRODUCTION

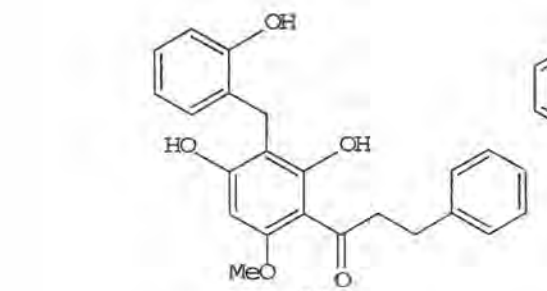
5.1.1 The Genus *Uvaria*

The genus *Uvaria* is among about 130 plant genera that belong to the family Annonaceae, consisting of at least 100 species which are distributed in tropical and sub tropical regions. About 63 of them being confined to tropical Africa and at least 16 species occurring in Tanzania.[125] Several *Uvaria* species are used in folk medicines and a few of them have been found to contain insecticidal and/or larvicidal compounds.[77,125,163,164]

In the literature, most *Uvaria* species are known to be rich sources of several natural products of varied structures, some of which posses interesting bioactivities, including anti-parasitic and insecticidal properties.[71,165] *Uvaria* natural products include *C*-benzyl dihydrochalcones and flavanones, variously substituted flavonoids, annonaceous acetogenins, benzopyranyl sesquiterpenes, prenyl-, farnesyl- and benzylindoles, triterpenes and several others.[166] Among the *Uvaria* species occurring in Tanzania is *U. scheffleri* Diels that grows around the Soni Valley, Daluni and Kwamngumi Forest Reserve in Tanga Region. Recent investigations of this plant species yielded the larvicidal and insecticidal monoterpene espintanol (**5.1**), together with its trimeric derivative schefflone (**5.2**), 2',6'-dihydroxy-3',4'-dimethoxychalcone (**5.3**) and two presumed dimers of **5.1**, namely scheffloquinone A (**5.4**) and scheffloquinone B (**5.5**).[76,77] *U. scheffleri* is used as a source of traditional medicine for the treatment of fevers and previous studies indicated that is crude extracts therefrom to have strong *in vitro* antimalarial activity.[77]



The genus *Uvaria* has also been shown to be a source of several compounds that possess antimalarial, antimicrobial, antitumour, antimycotic and insecticidal activities.[167] Antitumour activity may be correlated with insecticidal potency due to the fact that both bioactivities are associated with the active compounds being able to inhibit cellular uptake of respiratory oxygen that is required for energy production through oxidative phosphorylation.[167,168] Therefore, the antitumour dihydrochalcones from *Uvaria* species can be considered potential insecticidal agents. This class of compounds includes the dihydrochalcones uvaretin (**5.6**), isouvaretin (**5.7**), diuvaretin (**5.8**), angoluvarin (**5.9**), chamuvaritin (**5.10**), triuvaretin (**5.11**) and isotriuvaretin (**5.12**), all or individually having been isolated from *U. accuminata, U. chamae, U. leptocladon, U. puguensis* and *U. tanzaniae*.[73]



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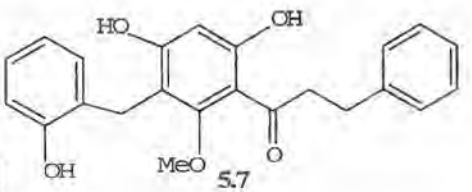

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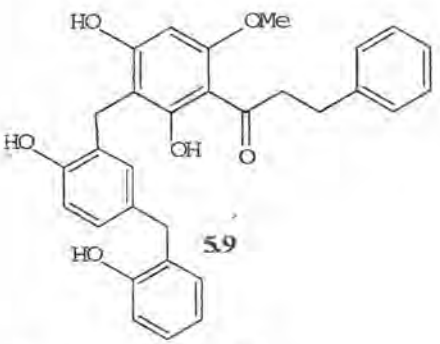

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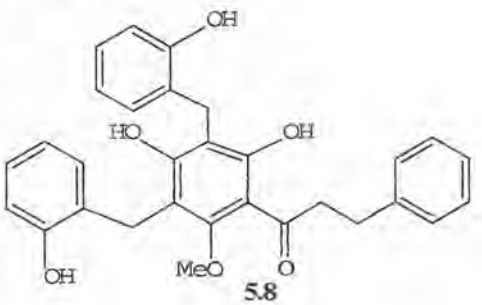

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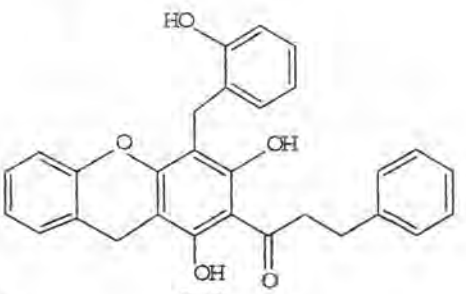

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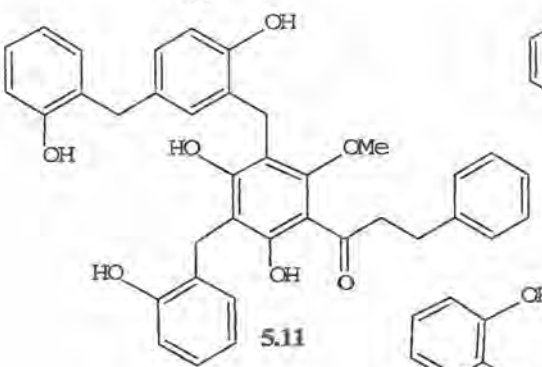

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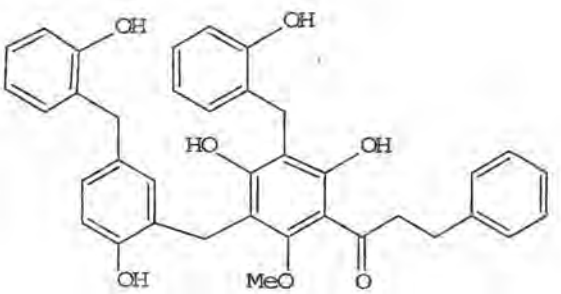


5.12

Flavanoids from the genus *Uvaria* which contain *ortho*-hydroxybenzyl groups similar to dihydrochalcones include chamanetin (**5.13**), dichamanetin (**5.14**), uvarinol (**5.15**) and isouvarinol (**5.16**).[169] These compounds were obtained from *U. faulknerae, U. chamae* and *U. lucida* ssp. *lucida*.[170] As for dihydrochalcones, these compounds displayed cytotoxicity that may be correlated with potential insecticidal properties.[170]

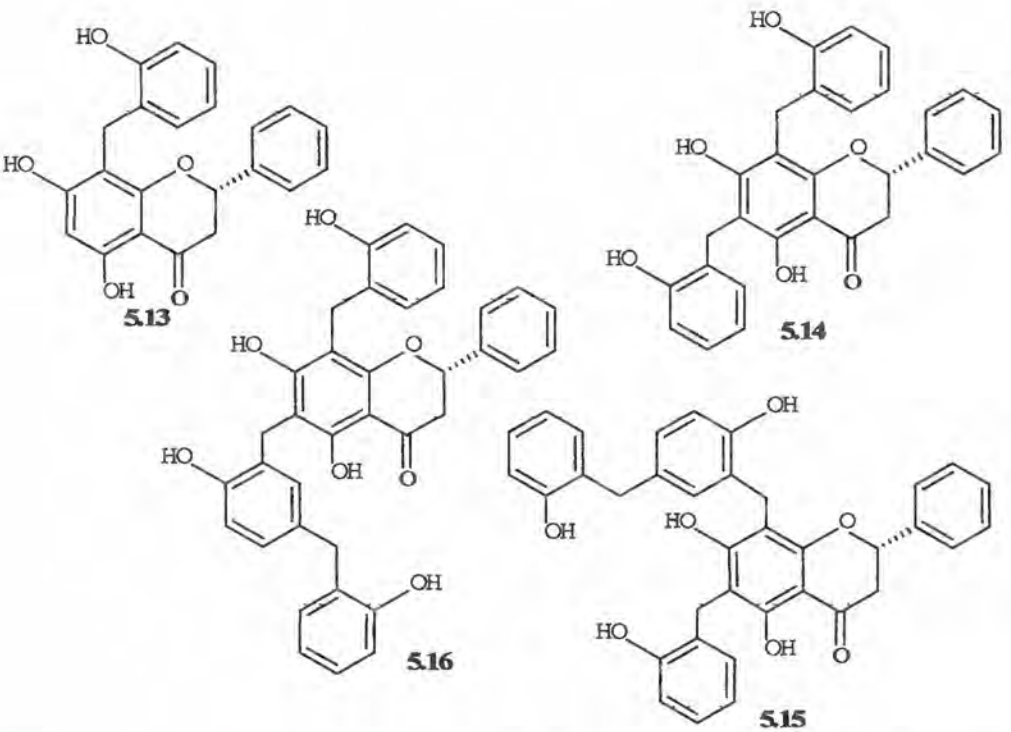


Figure 5.1 A branch of *U. lungonyana* Vollesen (Photo: C. Kihampa)

Uvaria lungonyana Vollesen (Fig. 5.1), which was chosen for investigations whose results are hereby being reported is a shrub of up to 10 m tall, being endemic to Tanzania. It is reported to be found only in the Selous Game Reserve, Rufiji District, Coast Region. In the present study, the plant species was abundantly found at the above mentioned area and it was growing along Lungonya valley.

No information is available about the use of this plant species by local people in traditional medicine. Furthermore, neither phytochemical nor biological investigations for the whole plant or its chemical constituents has been done. This

might be due to the fact that it is only found within the protected Selous Game Reserve area that is not easily accessible.

5.1.2 The Genus *Uvariodendron*

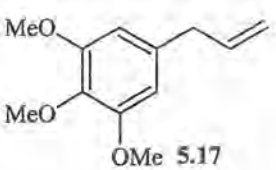
The genus *Uvariodendron* is a small genus comprising about 16 species which are either shrubs or trees, the genus being restricted to tropical Africa and about 6 species are confined to East Africa.[125] According to the Flora of Tropical East Africa that was published in 1971, four *Uvariodendron* species, namely *U. kirkii, U. gorgonis, U. pycnophyllum* and *U. usambarense* were at that time known to occur in Tanzania.[125]

U. pycnophyllum (Fig. 5.2) is a tree of up to 7 m tall which is endemic to Tanzania and reported to be found only in Sigi Valley Amani Nature Reserve in West Usambara Mountains, Muheza District, Tanga Region.[125] In the present study, the materials of this plant species were collected from Sigi valley, 3 km from Kisiwani along the road to Bombani village in Muheza District. So far there is no information regarding the use of *U. pycnophyllum* by local people in traditional medicine. Furthermore, neither phytochemical nor biological investigations are reported for *U. pycnophyllum*.

Fig. 5.2 A branch of *U. pycnophyllum* (Diels) R. E. Fries (Photo: C. Kihampa)

Previous phytochemical investigations of *Uvariodendron* species indicated the presence of arylalkenes,[133,134] mostly phenylpropenes that are also found in high

concentration (up to 90%) in many essential oil-bearing plants where they appear as isomeric mixtures of *trans* (α), *cis* (β) and *gamma* (γ) isomers. Thus, phytochemical investigations of the ripe seeds of *Uvariodendron connivens* (Benth.) R.E. Fries found in Nigeria and Cameroon yielded the phenylpropanoid derivatives elemicin (**5.17**), 3,4,5-trimethoxycinnamaldehyde (**5.18**) and 3,4,5-trimethoxycinnamyl alcohol (**5.19**).[133] Compound **5.18** is also reported from *Zanthoxylum procerum* which belongs to the family Rutaceae.[133,171] Separation of the isomers into single isomers by column chromatography has been reported to be tedious.[171] *Cis*–arylalkenes are known to be carcinogenic and toxic and this factor restricts the market potential of any essential oil that is rich in the *cis*-isomer.[171]



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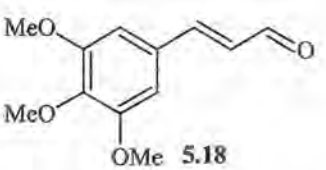
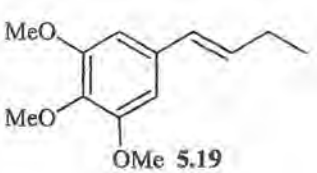
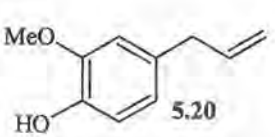


5.18



5.19

In another study in the Department of Chemistry at the University of Dar es Salaam, the stem bark of *U. gorgonis* yielded the phenylpropanoids eugenol (**5.20**), *O*-methyleugenol (**5.21**), 3,4-dimethoxycinnamylalcohol (**5.22**) and dehydrodieugenol (**5.23**), together with a mixture of β-sitosterol (**5.24**) and stigmasterol (**5.25**).[134]



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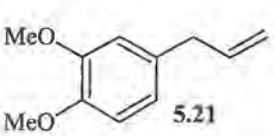

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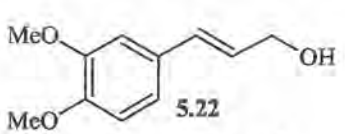

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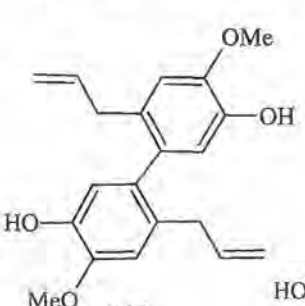

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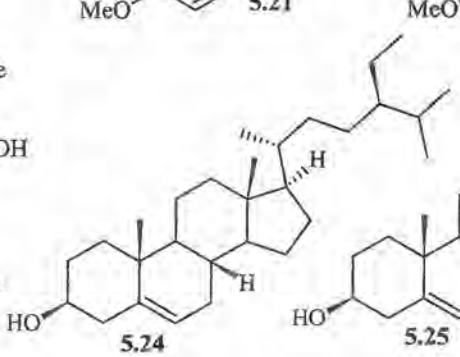

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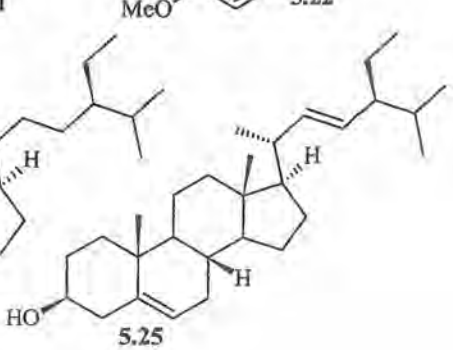


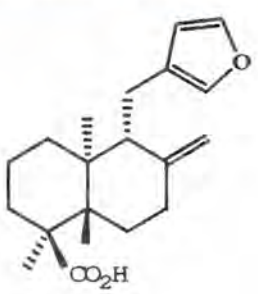
5.25

5.1.3 The Genus *Polyalthia*

The genus *Polyalthia* includes more than 150 plant species that belong to the family Annonaceae.[172,173] *Polyalthia* is considered to be one of the most unusual genera of the family Annonaceae, with its species occurring in the tropical zones of Africa, Asia and Oceania.[172,173] Plant species of this genus have been established as a sources of different types of metabolites, some of which possess interesting bioactivities, including alkaloids, triterpenes, flavonoids and clerodane diterpenes.[174] Thus, the stem bark of *P. fragrans* was shown to contain the labdane diterpenoid polyalthic acid (**5.26**) which, however, was not assayed for biological activity.[174]

Figure 5.3 A branch of *P. tanganyikaensis* Vollesen (Photo: C. Kihampa)

The zinc containing natural product, zincpolyanemine (**5.27**), was isolated from *P. nemoralis*, a medicinal herb that is used for the treatment of hepatitis and malaria.[175] However, compound **5.27** was not assayed for either antimalarial properties or for activity against hepatitis causing viruses.



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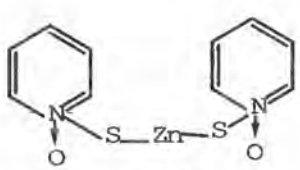
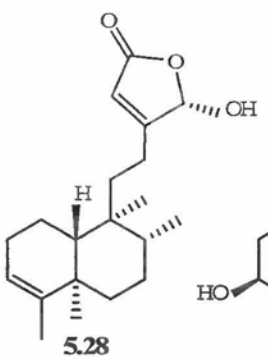


5.27

Chemical analysis and cytotoxicity assay of *P. barnesii* yielded three cytotoxic clerodane diterpenes 16α-hydroxycleroda-3,13(*Z*)-dien-15,16-olide (**5.28**), 3β,16α-dihydroxycleroda-4(18),13(*Z*)-dien-15,16-olide (**5.29**) and 4β,16α-dihydroxyclerod-13(*Z*)-en-15,16-olide (**5.30**).[176] From *P. longifolia* that is used as a febrifuge, three clerodane diterpenes **5.31-5.33** and six *ent*-halimane diterpenes **5.34-5.39** were isolated,[177] as well as the flavonoid leucocyanidin (**5.40**), its trimeric derivative procyanidin (**5.41**) and β-sitosterol (**5.24**).[178]



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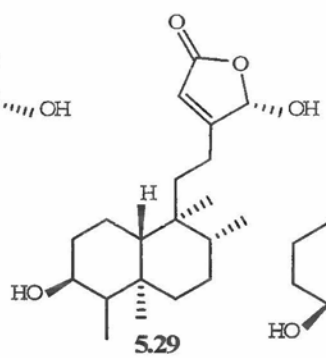

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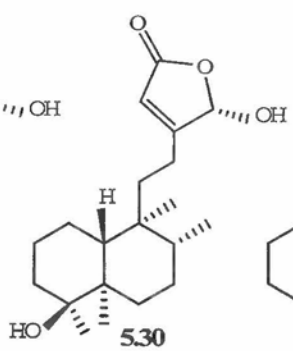

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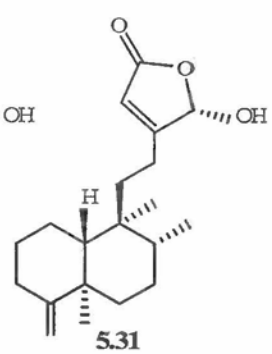

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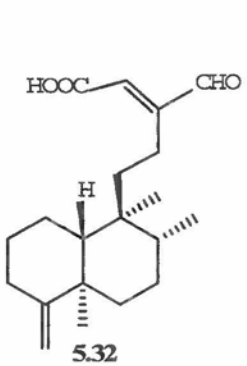

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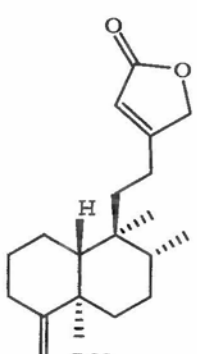

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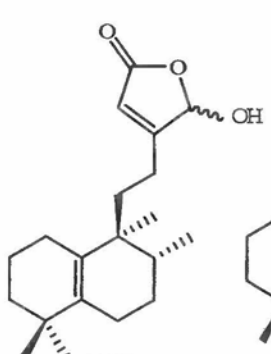

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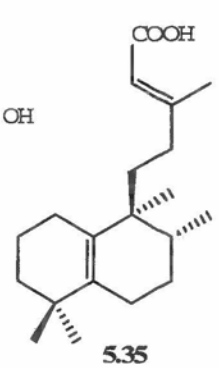

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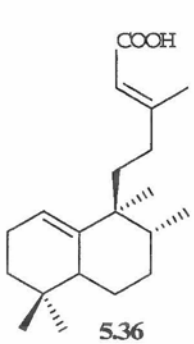

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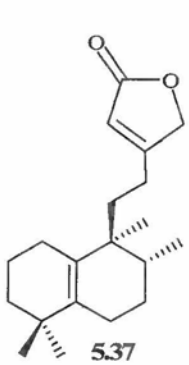
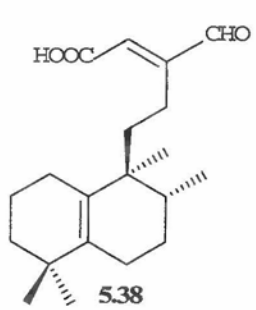


5.37



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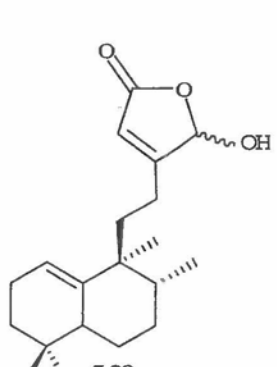

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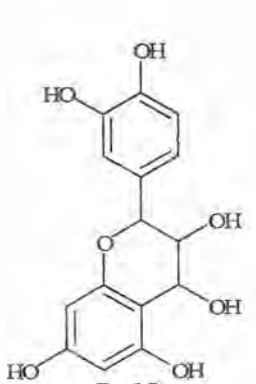
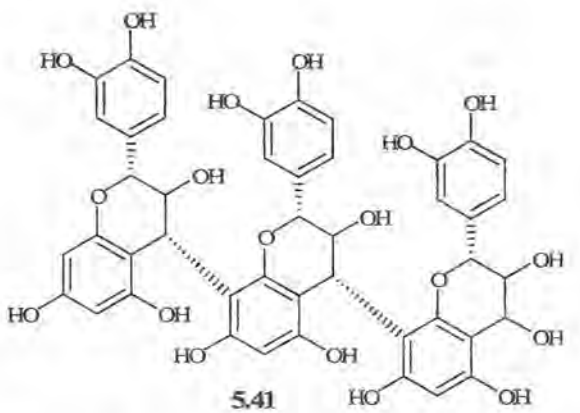
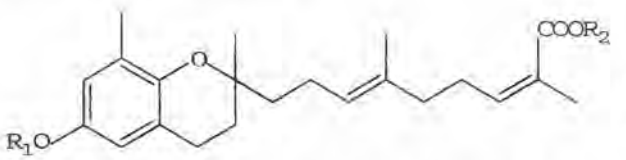


5.40



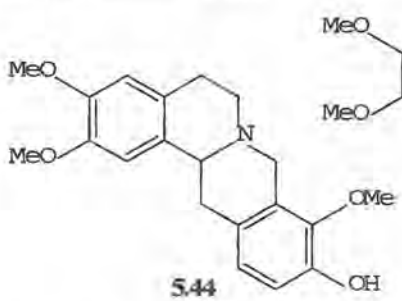
5.41

The stem bark of *P. cerasoides* yielded two novel benzopyranyl sesquiterpenes polycerasoidin (**5.42**) and polycerasoidal (**5.43**).[174] These compounds have not been assayed for any biological activity.

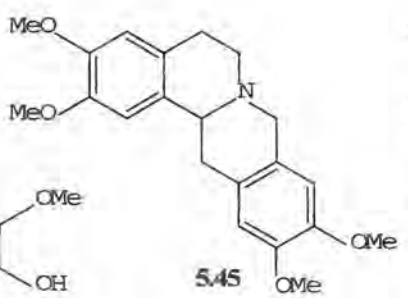


	R_1	R_2
5.42:	Me	H
5.43:	H	H

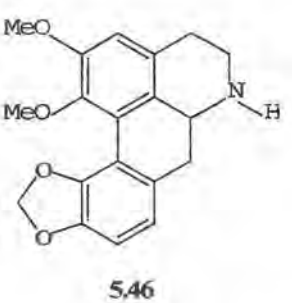
P. oligosperma yielded the tetrahydroprotoberberine alkaloids kikemanine (schefferine) (**5.44**) and xylopinine (**5.45**), and the aporphine alkaloid polygospermine (**5.46**).[179]



5.44



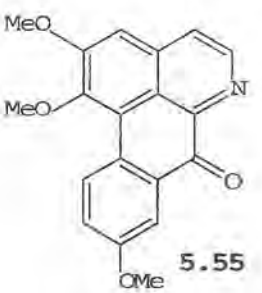
5.45



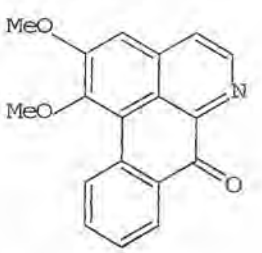
5.46

A number of 7-substituted aporphines including guatterine (**5.47**), noroliverinel (**5.48**), oliveridine (**5.49**), oliverine (**5.50**), oliveroline (**5.51**), pachypodanthine (**5.52**), polyalthine (**5.53**) and polysuavine (**5.54**) have been isolated from *P. suaveolens*, together with the oxoaporphine alkaloids lysicamine (**5.55**) and

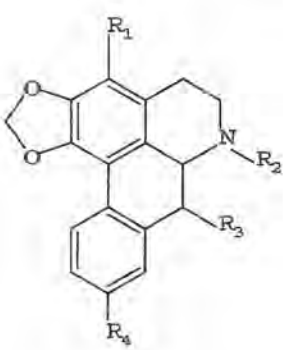
oxostephanine (**5.56**),[180] while *N*-methylpachypodanthine-*N*-oxide (**5.57**), *nor*-oliveridine (**5.58**), oliveroline-*N*-oxide (**5.59**), lanuginosine (**5.60**) and liriodenine (**5.61**) were obtained from *P. olivera*.[181] Although none of the aporphinoids from *Polyalthia* species has been assayed for biological activity, several related compounds have been reported in the literature to have antitumour, antimicrobial and other biological activities.[180,181] On the other hand some protoberberine alkaloids like palmatine (**5.62**), are known to have antiprotozoal activity.[180,181]



5.55

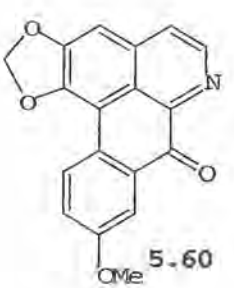


5.56

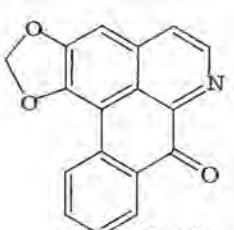


	R_1	R_2	R_3	R_4
5.47:	OMe	Me	OH	-
5.48:	-	H	OMe	OMe
5.49:	-	Me	OH	OMe
5.50:	-	Me	OMe	OMe
5.51:	-	Me	OH	-
5.52:	-	H	OMe	-
5.53:	OMe	Me	OH	OMe
5.54:	-	Me	OMe	OH
5.57:	-	N-oxy	OMe	-
5.58:	-	H	OH	OMe
5.59:	-	N-oxy	OH	-

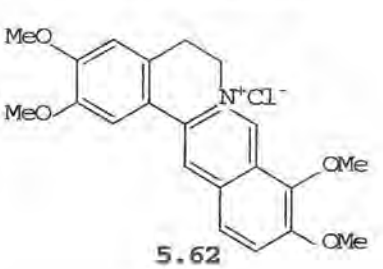
R_3 has a β configuration in all the compounds



5.60

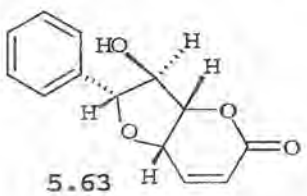


5.61

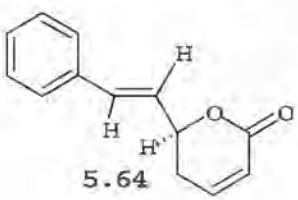


5.62

The tetrahydrofuropyran-5-one altholactone (**5.63**) was obtained from the bark of an un-named *Polyalthia* species.[182] The compound is biogenetically related to a number of α-pyrones that have been isolated from plant species of the families Annonaceae, Lauraceae and Piperaceae.[182] In formal terms, altholactone can be regarded as an oxygenated and cyclized derivative of the lactone goniothalamin (**5.64**).[182]

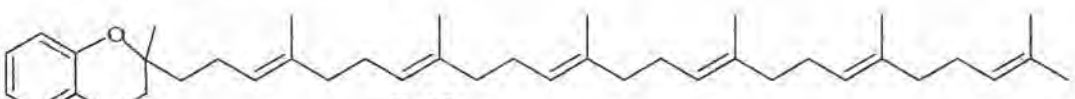


5.63

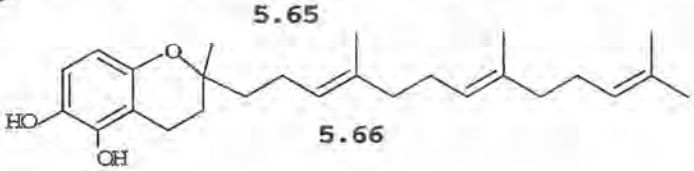


5.64

Previous phytochemical analysis of *P. tanganyikensis* in the Department of Chemistry at the University of Dar es Salaam yielded a mixture of C_{35} and C_{20} terpenyl benzopyrans **5.65** and **5.66**.[183]



5.65



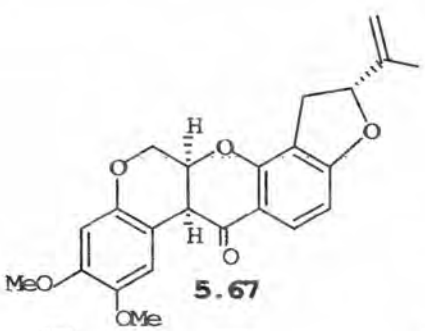
5.66

5.1.4 The Genus *Annona*

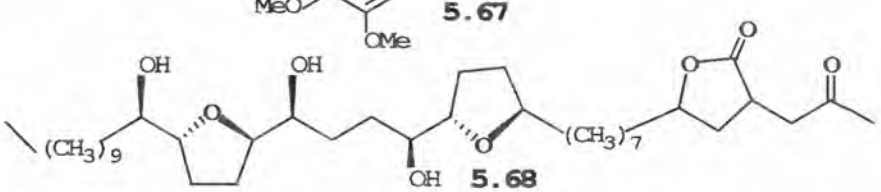
The genus *Annona* (Annonaceae) is large, comprising about 100 – 150 species that mostly constitute neotropical trees and shrubs. Many *Annona* species have significant agricultural, medicinal, pharmaceutical and other uses.[184] In the literature the genus *Annona* is known to be a rich source of annonaceous acetogenins that have been reported to possess very potent bioactivities, such as *in vivo* antitumour, antimicrobial, antimalarial, pesticidal, antiparasitic, antihelmintic, cytotoxic, antifeedant and immunosuppressant activities.[165,185,186] For this reason a

Figure 5.3 A branch of *A. squamosa* Vollesen (Photo: C. Kihampa)

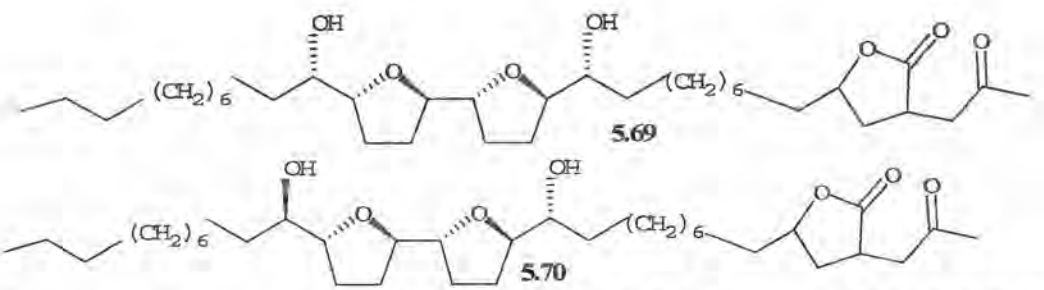
number of annonaceous acetogenins have been patented.[186] Annonaceous acetogenins have also been shown to inhibit adenosine triphosphate (ATP) production at a similar site of action and higher levels of potency as the natural insecticidal agent rotenone (**5.67**), that is at the NADH-ubiquinone oxido-reductase site of complex I of the mitochondrial electron transport chain.[71] The strong biological activity of annonaceous acetogenins as larvicides, pesticides and antitumorals is dependent on the nature of the functional groups, the degree of hydroxylation being particularly important for these effects.[71] Thus, the roots of *Annona cherimolia* which is used in traditional medicine as an insecticide and parasiticide, yielded three annonaceous acetogenins isocherimolin-1 (**5.68**) and isomolvizarins-1 and 2 (**5.69** and **5.70**).[188] *A. cherimolia* is cultivated in Spain for its edible fruits.[188]



5.67



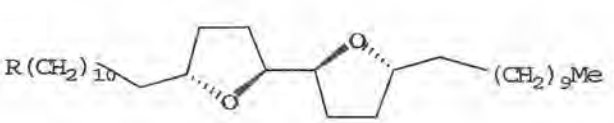
5.68



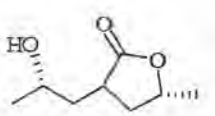
5.69

5.70

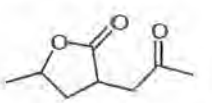
Two cytotoxic compounds bullatacin (**5.71**) and bullatacinone (**5.72**) that had selective cytotoxicities in human tumour cell lines and certain susceptible cells were isolated from *A. bullata*.[189] Compound **5.71** also showed insecticidal activity against *Aedes aegypti* mosquito larvae while **5.72** was inactive.[189,190] The acetogenin **5.71** was also active against a cotton aphid (*Aphis gossypi*) when applied by foliage spraying.[190]



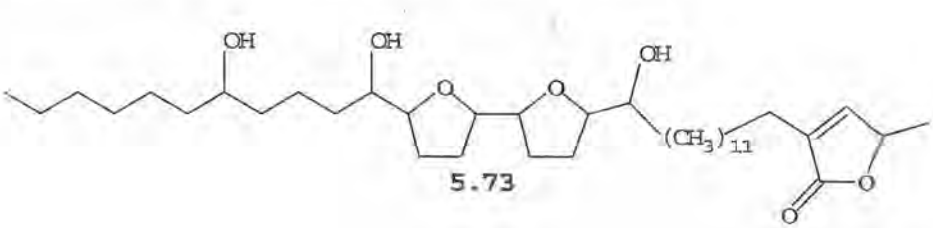
5.71: R=



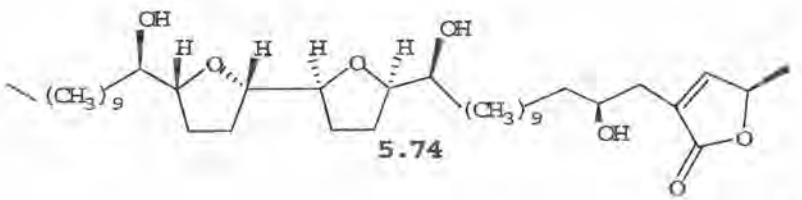
5.72: R=



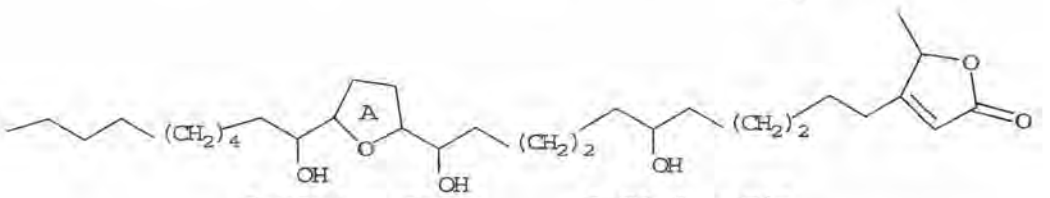
In Tanzania, *Annona squamosa* (Mtopetope in Kiswahili) is grown as a backyard plant whose fruits are edible. In the literature *A. squamosa* has been shown to be a rich source of annonaceous acetogenins, including squamocin (**5.73**), rolliniastatin I (**5.74**), 4-deoxyannoreticuin (**5.75**), *cis*-4-deoxyannoreticuin (**5.76**) and (2,4-*cis* and *trans*)-squamoxinone (**5.77**).[191] The compounds showed moderate but significant cytotoxicity against a panel of 6-human tumour cell lines, compound **5.77** showing the most promising selectivity against pancreatic cell line.[191]



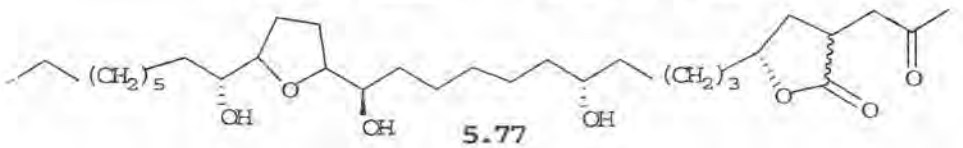
5.73



5.74



5.75: A = *Trans* 5.76: A = *Cis*



5.77

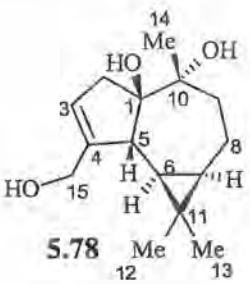
Besides acetogenins, *A. squamosa* has also been found to contain kaurane diterpenoids, as it is presented in Chapter 2 (section 2.3).

5.2 RESULTS AND DISCUSSION

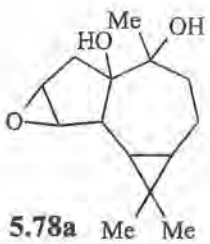
5.2.1 Tanganyikenol (5.78): A Trihydroxyaromadendrenoid from *Polyalthia tanganyikensis*

Repeated chromatography of the pet ether extract of the root bark of *P. tanganyikensis* yielded a pure compound as a gum whose spectroscopic properties indicated it to be a sesquiterpene having an aromadendrane skeleton **5.79**,[192] constituting structure **5.78**. Thus, the MS (Fig. 5.6) exhibited the M-1 peak at *m/z* 251, which was consistent with the molecular formula $C_{15}H_{24}O_3$. The ^{1}H NMR spectrum (Fig. 5.1, Table 5.1) consisted of signals due to cyclopropyl protons as expected for an aromadendrane sequiterpene (δ 0.43, dd, J = 10, 10 Hz and 0.53, m). Furthermore, the ^{1}H NMR spectrum exhibited resonances due to three, instead of four methyl groups present in the aromadendrane skeleton, appearing at δ 1.03, 1.05

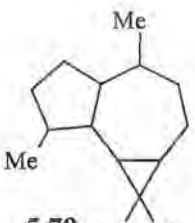
and 1.07 (each s). This indicated that the fourth methyl group in skeleton **5.79** had been functionalized in **5.78**, and that each of the remaining three methyl groups was quaternary. Indeed, the ^{1}H NMR spectrum exhibited signals due to protons of an oxygenated methylene group (δ_H 3.51 and 3.81, ABq, J_{AB} ≈11 Hz, δ_C 69.7), whose splitting pattern indicated that the corresponding methylene carbon was attached to a fully substituted carbon. That the former was substituted to an sp^2 carbon was deduced from H/C HMBC interactions (Figs. 5.5 and 5.5a), which also indicated the corresponding unsaturation to be at C-3/C-4 ($\delta_{H\text{-}3}$ 5.43, d, J = 3 Hz, $\delta_{C\text{-}3}$ 123.0, $\delta_{C\text{-}4}$ 151.7).



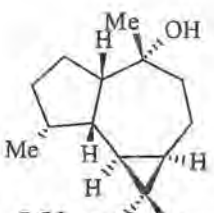
5.78



5.78a



5.79



5.80

That the bridgehead C-1 was substituted was deduced from the fact that the ^{1}H NMR signal due to H-5 appeared as a dt instead of a more complex resonance, indicating that H-5 coupled only with H-6 (large J value) and with the methylene protons H-2, the latter through long range coupling, as further indicated in the COSY spectrum (Fig. 5.3). The large $J_{5,6}$ value also indicated a β configuration for H-5 since in an aromadendrane skeleton, the C-6/C-7 cyclopropane ring is β,[193] and hence the corresponding protons H-6 and H-7 being α disposed.

The presence of hydroxyl substituents at C-1 and C-10 was deduced from the appearance of two quaternary carbinol C resonances in the ^{13}C NMR spectrum

(δ 73.7 and 70.2), and the positions of the corresponding C-atoms being ascertained from H/C HMBC interactions (Figs. 5.5 and 5.5a). Considering the close similarity of the C-10 resonance in the ^{13}C NMR spectrum of **5.78** (δ 73.7) and that reported for compound **5.80** (δ 74.6) it was concluded that the relative stereochemistry around the C-1/C-10 bond was the same for the two compounds **5.78** and **5.80**. Accordingly, the C-1 OH group was deduced to be β disposed, implying a *cisoid* C-1/C-5 ring junction. However, this stereochemical configuration needs further confirmation by additional NMR experiments, particularly NOE measurements.

The MS (Fig. 5.6) indicated the presence of trace amounts of the epoxide **5.78a** and its methylated product [M-1 peaks at *m/z* 267 and 281, respectively). The MS also depicted a fragmentation process consistent with structure **5.78** (Scheme 5.1), involving cleavage of the cyclopropane ring to form a fragment ion at *m/z* 236, followed by cleavage of a hydroxyl unit giving a fragment ion peak at *m/z* 219, which was then followed by subsequent cleavage of a hydrogen radical and then a methylene unit to form fragment ion peaks at *m/z* 218 and 205 (base peak), respectively. For the other type of fragmentation process, cleavage began with the loss of a water molecule to form a fragmentation ion peak at *m/z* 234, which was followed by extrusion of another water molecule and a hydroxyl unit to form fragment ion peaks at *m/z* 216 and 199, respectively (Scheme 5.1).

Compound **5.78** whose mosquito larvicidal and insecticidal activity results are presented in Chapter 6 has so far not been reported in the literature, even where the stereochemical configuration shown would be ignored.

Table 5.1 ^{1}H and ^{13}C NMR Spectral Data for Tanganyikenol (5.78)

H/C	5.78			5.80[193]	
	δ_H	*J* (Hz)	δ_C	δ_H	δ_C
1	-	-	70.2	2.06	53.9
2a	2.24	d, 3	38.2	1.88	24.7
2b	2.04	d, 3		1.67	
3a	5.42	d, 3	123.0	1.68	30.9
3b	-	-	-	1.27	
4	-	-	151.7	1.96	38.5
5	2.44	dt, 12.5, 2	44.2	1.78	40.9
6	0.43	t, 10	24.1	0.31	23.5
7	0.53	m	25.1	0.69	25.1
8a	1.76	m	18.9	1.80	20.4
8b	1.51	m		1.20	
9a	1.85	m	37.3	1.81	39.3
9b	1.37	m		1.67	
10	-	-	73.7	-	74.6
11	-	-	20.3	-	19.2
12	1.07	s	28.7	1.01	28.7
13	1.05	s	15.1	0.96	15.4
14	1.03	s	25.1	1.12	30.5
15a	3.81	d, 10.5	69.7	0.91	16.0
15b	3.51	d, 11		-	

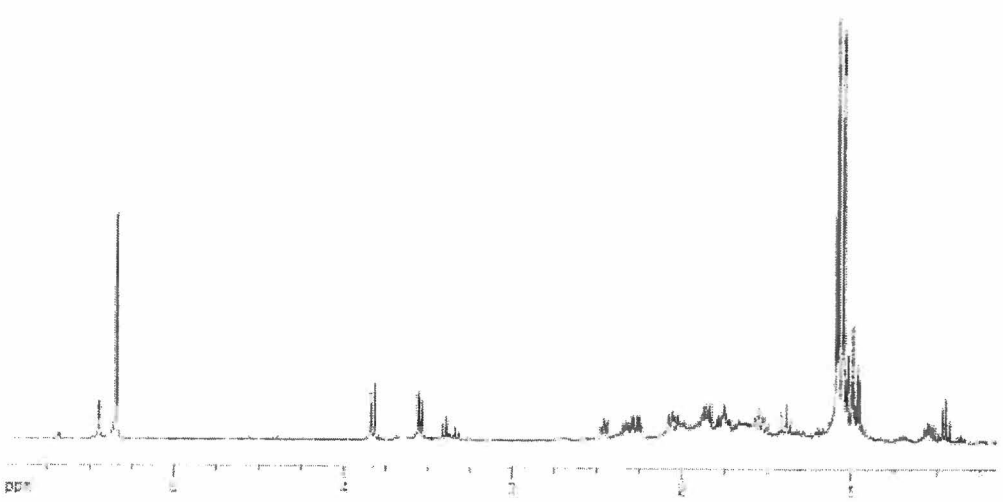


Fig. 5.1 ^{1}H NMR Spectrum of Tanganyikenol (5.78)

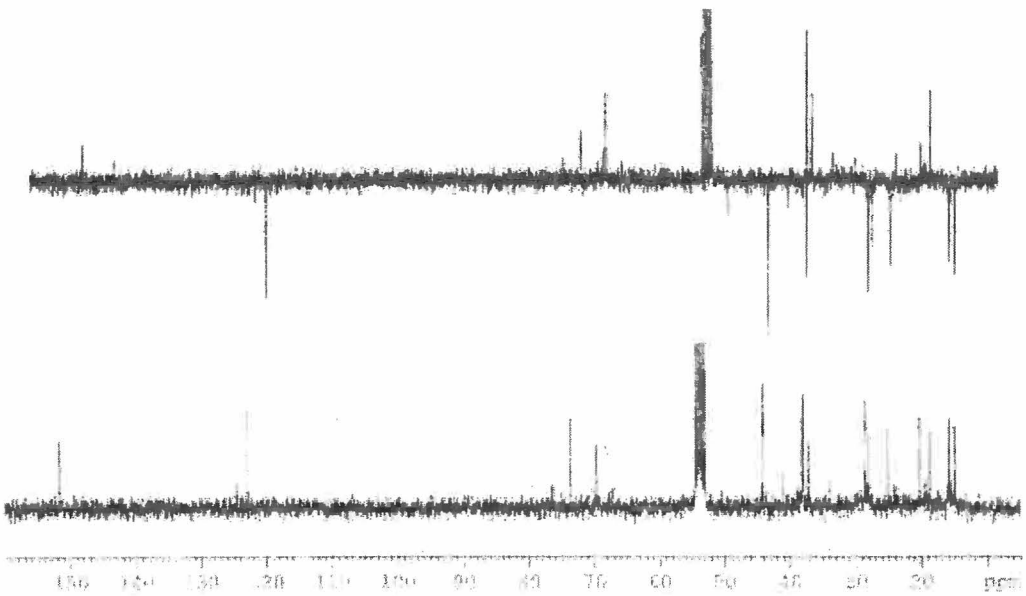


Fig. 5.2 ^{13}C NMR Spectrum of Tanganyikenol (5.78)

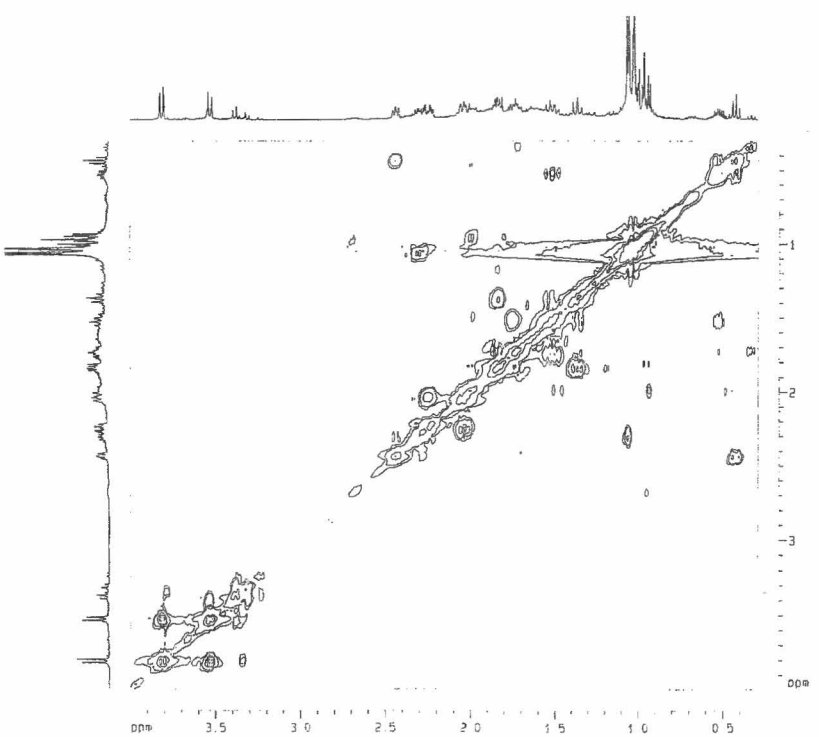


Fig. 5.3 H/H COSY Spectrum of Tanganyikenol (5.78)

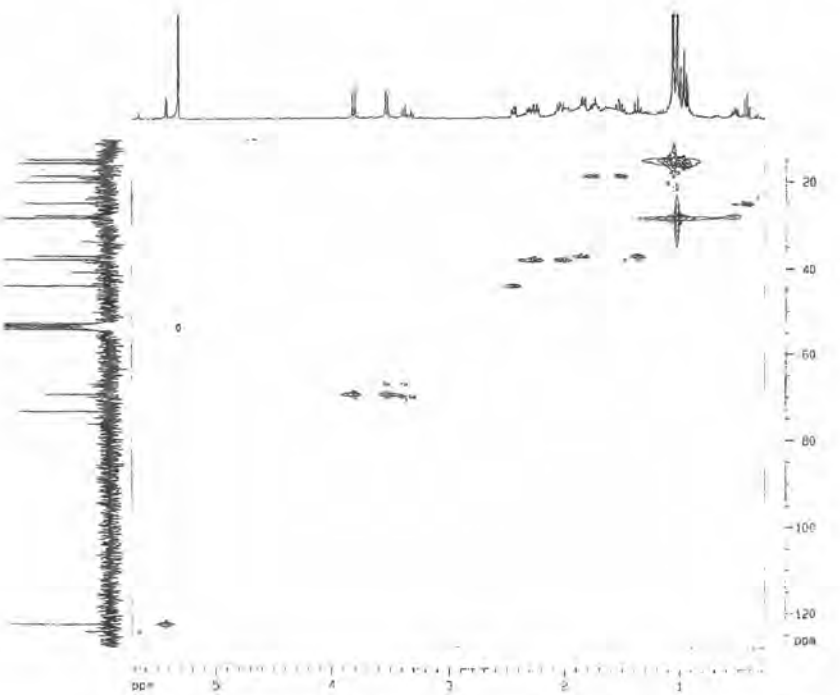


Fig. 5.4 HMQC Spectrum for Tanganyikenol (5.78)

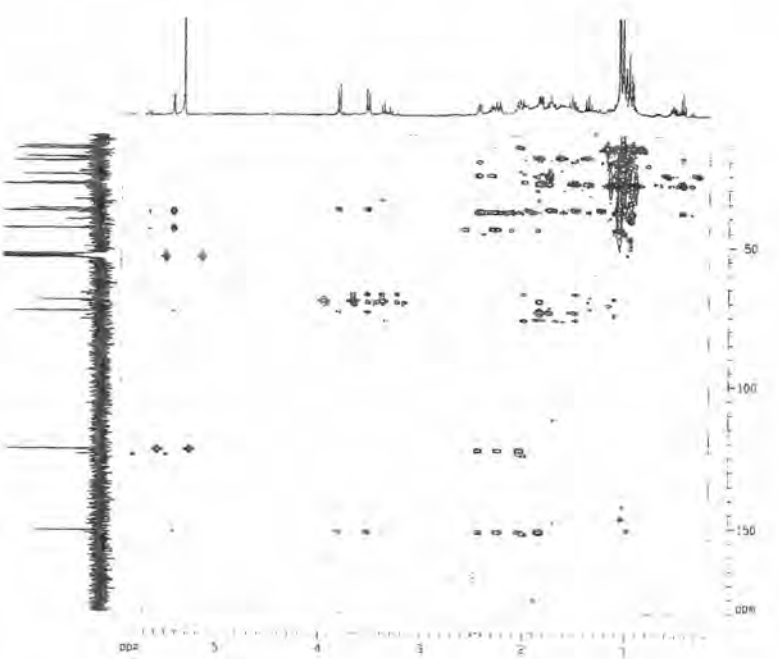
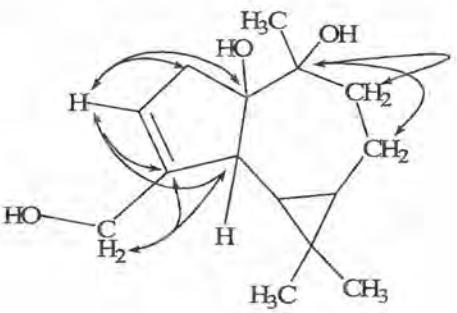


Fig. 5.5 HMBC Spectrum for Tanganyikenol (5.78)

Fig. 5.5a Important Long Range H/C Interactions in Compound 5.78 as Deduced from the HMBC Plot



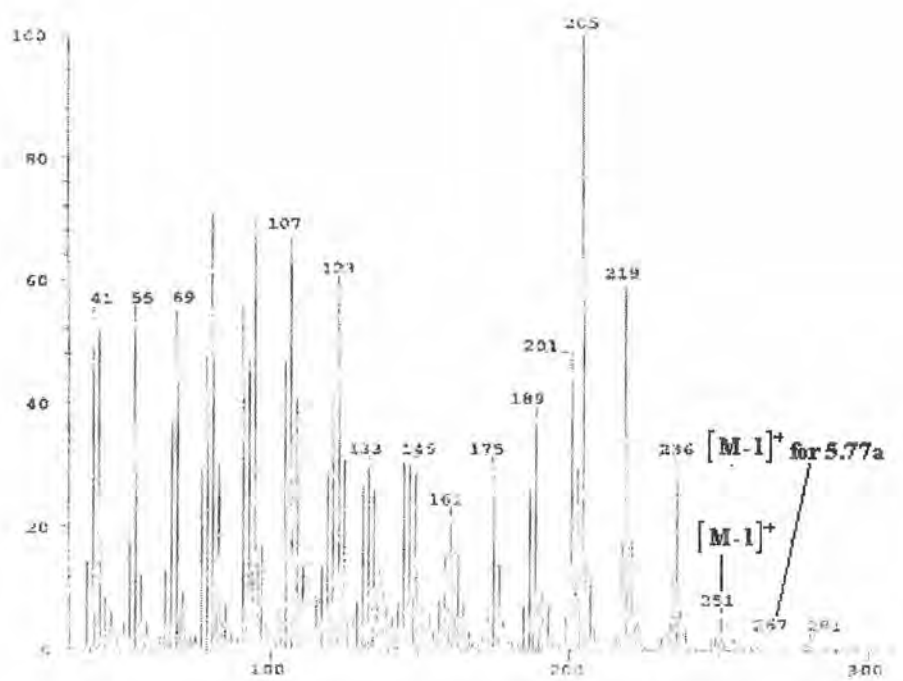
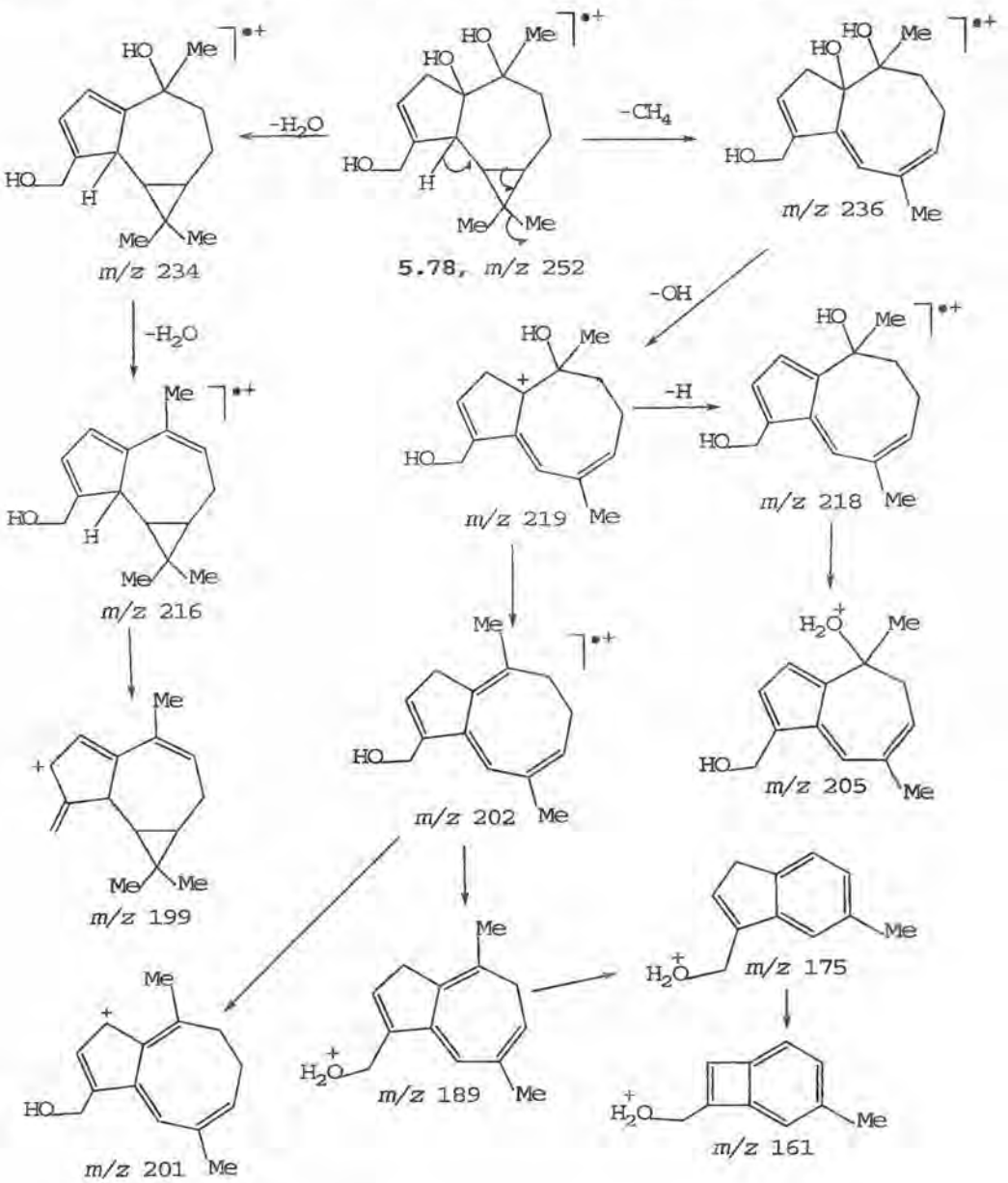


Fig. 5.6 Mass Spectrum of Tanganyikenol (5.78)

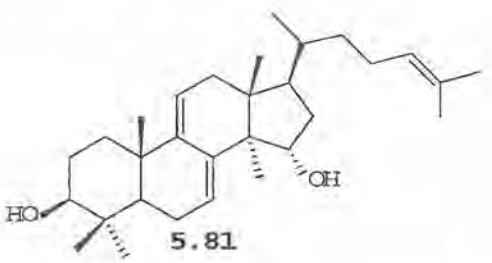
Scheme 5.1 MS Fragmentation Pattern for Tanganyikenol (5.78)



5.2.2 Polycarpol (5.81) from *Polyalthia tanganyikensis*

Repeated chromatography of the root bark extract of *Polyalthia tanganyikensis* yielded white crystals whose structure **5.81** was established upon comparison of its ^{1}H and ^{13}C NMR, and mass spectra with those reported in the literature for polycarpol.[194,195] Compound **5.81** occurs in several species of the family

Annonaceae, being exclusively found in this family. Its presence constitutes a useful chemotaxonomical marker of the family. It was previously isolated from several other Annonaceae species, such as *Polyalthia oliveri, Meiocarpidium lepidotum.*[196] *Greenwayodendron suaveolens* ssp. *usambaricum,*[197] *Polyceratocarpus scheffleri,*[198] *Hexalobus crispiflora*[199] and *Cleistoclamys kirkii.* As such, since its spectral properties have been extensively discussed elsewhere, it was found unnecessary to repeat such discussion in this Thesis.



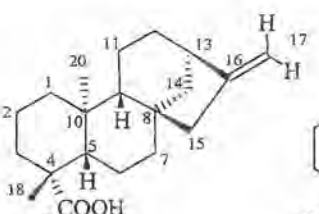
5.81

The compound exhibited mild larvicidal activity against *An. gambiae* mosquitoes, displaying an LC_{50} value of 393 ppm after 24 h exposure. It also exhibited antibacterial activity against *P. aeruginosa* and *B. subtilis,* and antifungal activity against *A. niger*, at a minimum inhibitory concentration (MIC) of 10 mg/ml.

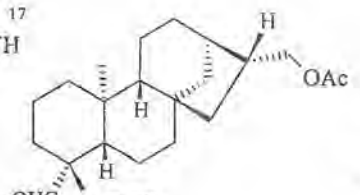
5.2.3 17-Acetoxy-*ent*-kauran-19-al (5.83): A Kaurane Diterpenoids from *Annona squamosa*

Extracts from the stem and root barks of *A. squamosa* showed strong activity against *An. gambiae* s.s Giles mosquito larvae after 24 h exposure (Chapter 2). TLC analysis of the stem bark extracts revealed the presence of complex mixtures of compounds which decomposed during CC on silica gel and on Sephadex® LH 20. Therefore, these were not analysed further. The petroleum ether and methanol extracts of the root bark were obtained in small quantities which also consisted of complex mixtures

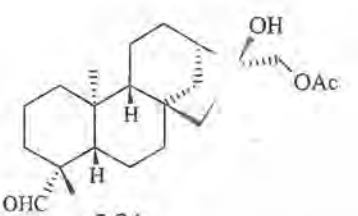
of polar and non-polar compounds. Therefore, these were also not further analysed. The chloroform extract of the root bark which displayed high larvicidal activity among the *A. squamosa* extracts was separated chromatographically and *ent*-kaur-16-en-19-oic acid (**5.82**) and 17-acetoxy-*ent*-kauran-19-al (**5.83**) were isolated. Structure **5.82** for *ent*-kaur-16-en-19-oic acid was established based on analysis of its spectroscopic data (^{1}H and ^{13}C NMR and MS) upon comparison of the data with those reported in the literature for the compound.[116]



5.82



5.83



5.84

The ^{1}H and ^{13}C NMR spectra (Figs. 5.7 and 5.8, Table 5.2 and 5.3) of compound **5.83** were comparable to those observed for **5.82**. However, signals due to an aldehydic and acetoxy protons were observed in both the ^{1}H and ^{13}C NMR spectra and this was an indication that the structure of the isolated compound was similar to compound **5.84** previously isolated from *A. squamosa*, rather than **5.82**. Thus, the ^{1}H NMR spectrum exhibited signals due to three tertiary methyl protons (δ 0.88, 0.99 and 1.2) and one aldehydic proton (δ 9.74), which was typical of an equatorial C-18, axial C-20 and C-22 methyl groups of an *ent*-kaurane diterpene having a C-19 (axial) aldehyde group.[116] Furthermore, the ^{1}H-NMR spectrum consisted of an ABX system (δ 5.10, J = 6.6, 4.5 Hz and 4.19, J = 6.3, 5.1 Hz), suggesting the presence of an acetoxymethylene group attached to a methine carbon, which would be achieved only if C-17 was the acetoxy methylene carbon, as in structure **5.83**.

The ^{13}C NMR spectrum (Table 5.3) indicated signals due to 22 carbons, consisting of three methyl, 11 methylene (including an acetoxy bearing carbon at δ 65.8), three methine, four quaternary and one aldehyde carbon. Comparison of these carbon resonances with those reported in the literature for the related kauranoid diterpene 16β-hydroxy-17-acetoxy-*ent*-kauran-19-al (**5.84**) (Table 5.3)[116] suggested that compound **5.83** possessed the same *ent*-kaurane type skeleton as **5.84**. The presence of a C-17 acetoxy group in **5.83** was shown by the appearance of carbon resonances at δ 21.0 (Me) and 171.4 (carbonyl). Complete assignment of structure **5.83** for the isolated compound was made based on H/H and H/C interactions observed in the COSY and HMBC spectra (Figs. 5.8 and 5.9).

The MS (Fig. 5.12) exhibited the molecular ion peak at *m/z* 346 corresponding to the molecular formula $C_{22}H_{34}O_3$. Besides the molecular ion peak, the MS also showed other prominent ion peaks that were consistent with structure **5.83**. The MS fragmentation pattern is summerized in Scheme 5.2.

The mosquito larvicidal and insecticidal activity of compound **5.83** is presented in Chapter 6. The compound has so far not been reported in the literature.

Table 5.2 ^{1}H NMR Spectral Data for *ent*-Kaur-16-en-19-oic acid (5.82) and 17-Acetoxy-*ent*-kauran-19-al (5.83)

H	$\delta_{5.82}$	*J* (Hz)	$\delta_{5.83}$	*J* (Hz)	H	$\delta_{5.82}$	*J* (Hz)	$\delta_{5.83}$	*J* (Hz)
1α	1.64*	m	1.61	m	11β	1.78*	m	1.88	m
1β	1.54*	m	1.52	m	12α	1.9*	m	1.54	m
2α	1.54*	m	1.52	m	12β	1.86*	m	1.44	m
2β	1.45*	m	1.45	m	13	2.62	bs	-	-
3α	1.98*	m	1.89	m	13-CH_3			1.26	s
3β	1.83*	m	1.81	m	14α, β	1.0 – 1.15	m	1.1 – 1.16	m
4	-	-	-	-	15α	2.18*	bs	1.64	m
5	2.0	bs	1.52	m	15β	2.13*	bs	1.59	m
6α	2.0*	m	2.0	m	16	-	-	2.28	m
6β	1.98*	m	1.98	m	17a	4.8*	d, 1.5	4.19	dd, 6.6, 4.8
7α	1.54*	m	1.54	m	17b	4.7*	bs	4.10	dd, 6.3, 5.1
7β	1.45*	m	1.45	m	18	1.24	s	0.99	s
8	-	-	-	-	19	-	-	9.74	s
9	1.6	m	1.6	m	19-OH	12	s	-	-
10	-	-	-		20	0.95	s	0.86	s
11α	1.79*	m	1.81	m	Ac	-	-	2.04	s

* interchangeable assignments

Table 5.3 ^{13}C NMR Spectral Data for *ent*-Kaur-16-en-19-oic acid (5.82) and 17-Acetoxy-*ent*-kauran-19-al (5.83)

H	Observed		Reported[116]		H	Observed		Reported[116]	
	$\delta_{5.82}$	$\delta_{5.83}$	$\delta_{5.82}$	$\delta_{5.84}$		$\delta_{5.82}$	$\delta_{5.83}$	$\delta_{5.82}$	$\delta_{5.84}$
1	41.6	41.9	41.8	41.8	12	33.5	25.8	33.6	26.5
2	19.5	18.3	19.6	18.7	13	44.3	40.5	44.3	41.3
3	38.2	34.2	38.3	34.3	14	40.1	39.1	40.2	38.4
4	44.6	48.4	44.2	48.4	15	49.4	37.3	49.4	52.4
5	57.5	56.6	57.5	56.5	16	156.3	44.2	156.4	78.6
6	22.2	18.9	22.3	19.7	17	103.4	65.8	103.5	71.2
7	41.1	39.8	41.8	39.8	18	29.4	24.2	29.5	24.3
8	44.1	43.7	44.7	43.6	19	184.1	205.9	185.0	205.9
9	55.5	55.8	55.6	55.5	20	16.0	16.4	16.1	16.3
10	40.1	39.3	41.2	39.8	CH_3	-	21.0	-	21.0
11	18.8	20.1	18.9	18.3	CO	-	171.4	-	171.4

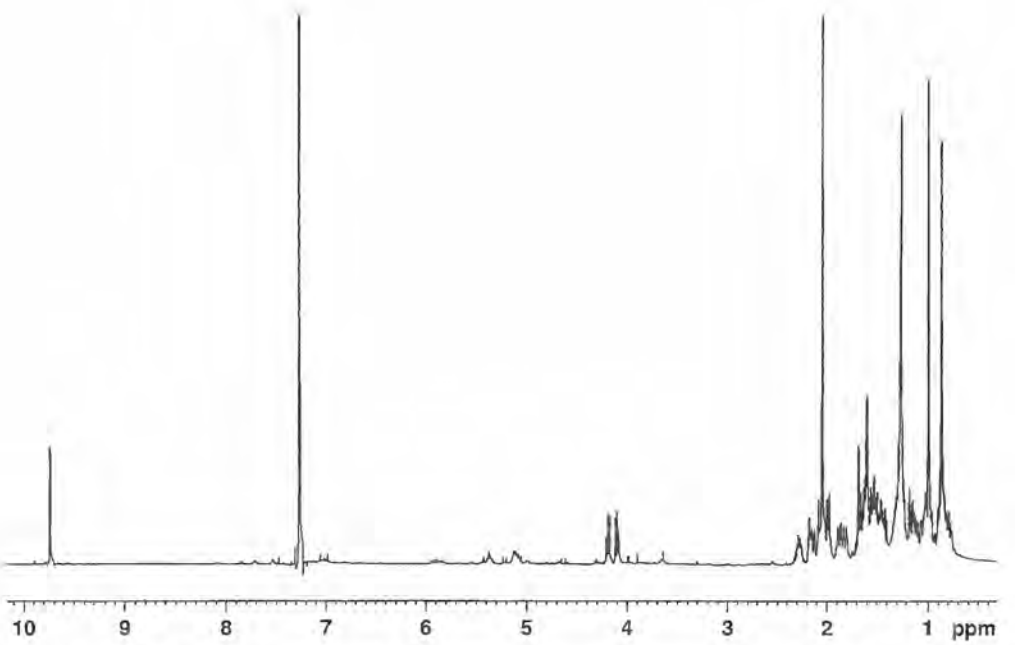


Fig. 5.7 ^{1}H **NMR Spectrum of 17-Acetoxy-*ent*-kauran-19-al (5.83)**

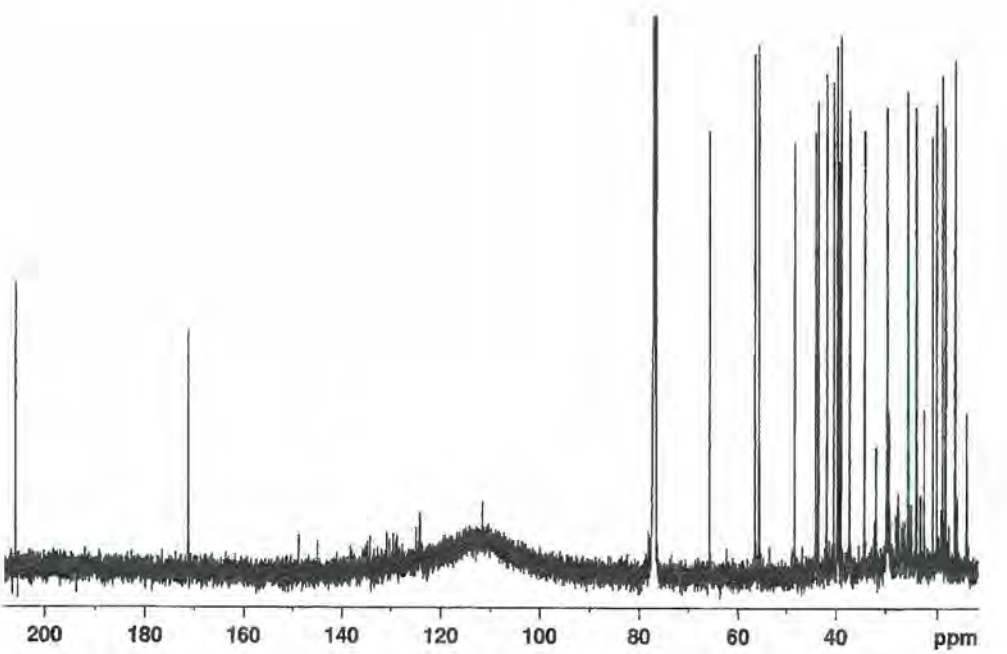


Fig. 5.8 ^{13}C **NMR Spectrum of 17-Acetoxy-*ent*-kauran-19-al (5.83)**

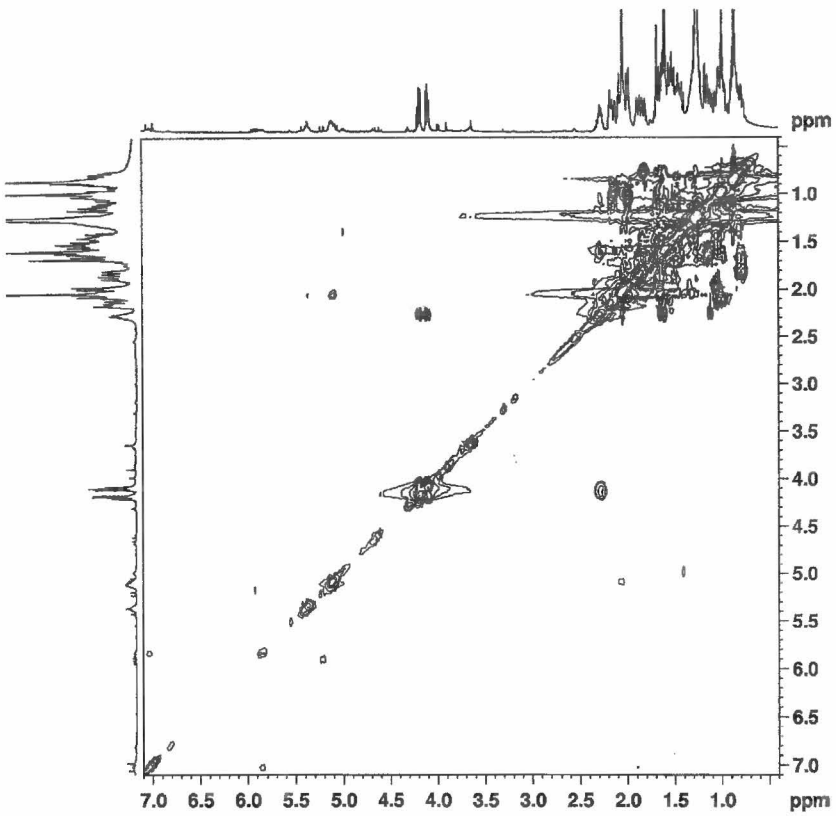


Fig. 5.9 H/H COSY Spectrum of 17-Acetoxy-*ent*-kauran-19-al (5.83)

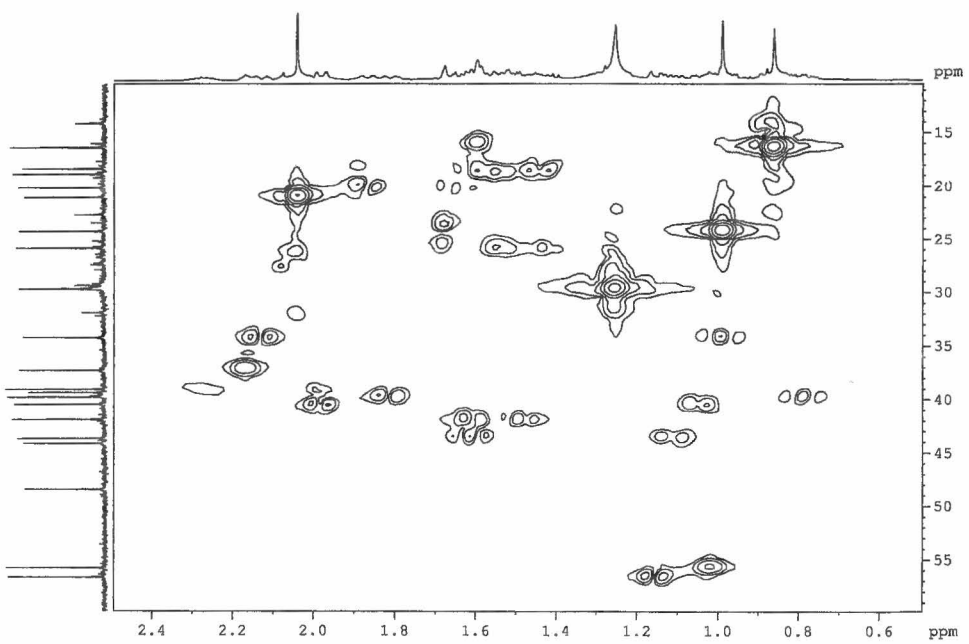


Fig. 5.10 HMQC Spectrum for 17-Acetoxy-*ent*-kauran-19-al (5.83)

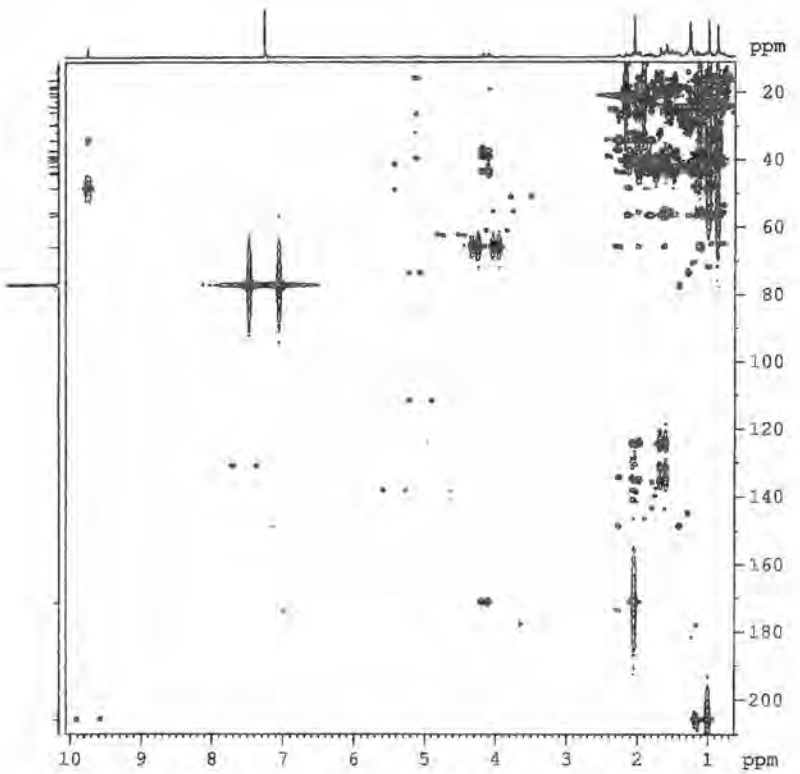
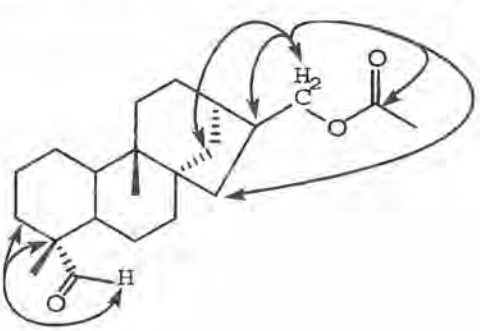


Fig. 5.11 HMBC Specrtum for 17-Acetoxy-*ent*-kauran-19-al (5.83)

Fig. 5.11a Important Long Range H/C Interactions in Compound 5.83 as Deduced from the HMBC Plot



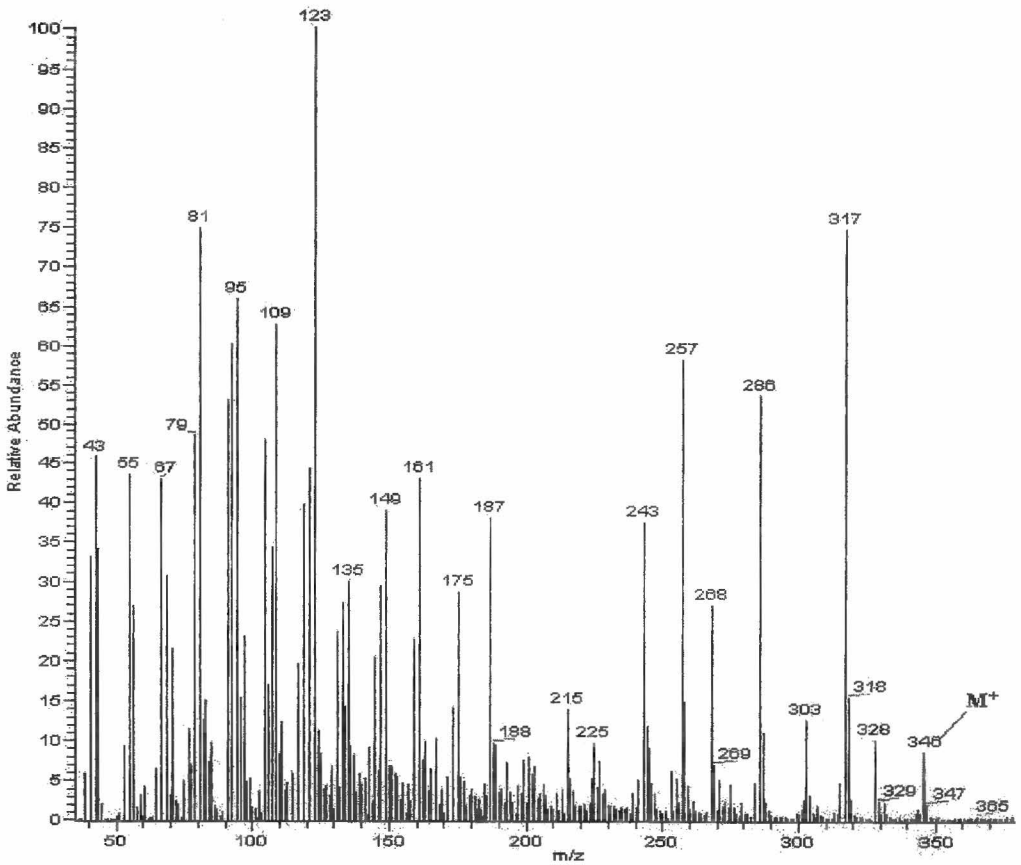
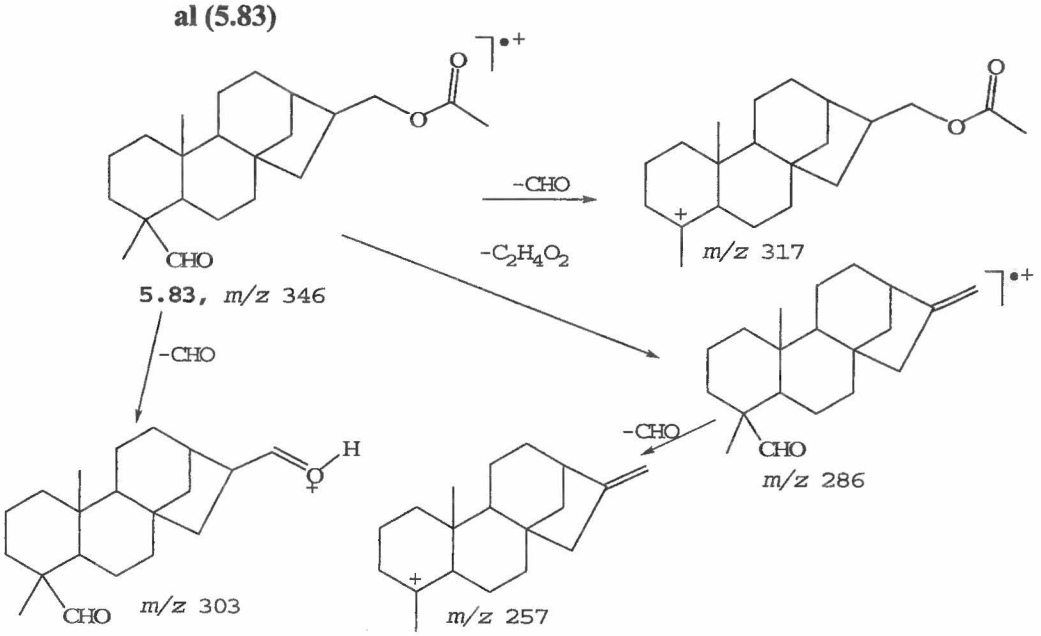


Fig. 5.12 Mass Spectrum of 17-Acetoxy-*ent*-kauran-19-al (5.83)

Scheme 5.2 MS Spectral Fragmentation Pattern for 17-Acetoxy-*ent*-kauran-19-al (5.83)

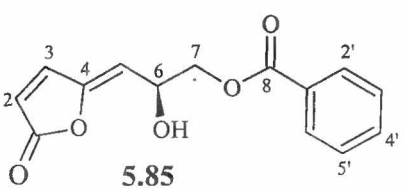


5.2.4 Melodorinol (5.85) and Acetylmelodorinol (5.86) from *Uvaria lungonyana*

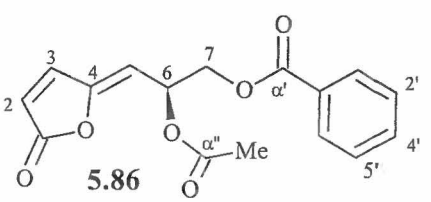
Repeated chromatography of the larvicidal VLC fraction 1 of the dichlomethane extract of the root bark of *U. lungonyana* yielded a yellow gum which was identified to constitute the previously known heptanolide melodorinol (**5.85**), while the methanol extract after repeated chromatography gave a solid which was identified to be acetylmelodorinol (**5.86**).[117,194,196,200,201] Structures **5.85** and **5.86** for the two compounds were deduced on the basis of spectral properties, which were virtually the same as those already reported in the literature.[117,194,196,200,201] The IR ($CHCl_3$) spectrum of **5.85** indicated that the compound consisted of a hydroxyl functional group (ν_{OH} 3448 cm^{-1}) and lactonyl and benzoyl carbonyl groups (ν_{CO} 1779 and 1719 cm^{-1}), similar absorptions being observed for compound **5.86** at ν_{OH} 3448, and ν_{CO} 1790 and 1728 cm^{-1} respectively, which was in conformity with the deduced structures. Furthermore, both the ^{1}H and ^{13}C NMR spectra (Figs. 5.5, 5.6, 5.7 and 5.8, and Tables 5.1 and 5.2) of compounds **5.85** and **5.86** exhibited signals due to the benzoyl and heptanolide moieties and for **5.86** also an acetyl unity.

Complete assignment of structures **5.85** and **5.86** was achieved on the basis of a combination of spectral measurements, especially HMBC and HMQC plots. The H-2 resonance appeared as a doublet of doublets at δ 6.24 (J = 5.4, 0.3 Hz) and a doublet at δ 6.27 (J = 5.4 Hz) for **5.85** and **5.86**, respectively, showing coupling with H-3 without an extra long range coupling with H-5, thus confirming the *cisoid* configuration for the exocyclic butenolide double bond in structures **5.85** and **5.86**, respectively.

The EIMS (Figs. 5.15 and 5.16) exhibited the molecular ion peaks at *m/z* 260 and 302 which were consistent with the molecular formulae $C_{14}H_{12}O_5$ and $C_{16}H_{14}O_6$ for **5.85** and **5.85**, respectively. Both the mass spectra also showed base peaks at *m/z* 105 $[C_7H_5O]^+$ due to a benzoyl fragment ion formed from the molecular ion. The mass spectral fragmentation patterns are summarized in Schemes 5.1 and 5.2 and are in consistent with structures **5.85** and **5.86**, respectively.



5.85



5.86

Table 5.4 ^{1}H NMR Spectral Data for Melodorinol (5.85) and (*Z*)-Acetylmelodorinol (5.86)

H	Observed				Reported[194]			
	5.85		**5.86**		**5.85**		**5.86**	
	δ	*J* (Hz)	δ	*J* (Hz)	δ	*J* (Hz)	δ	*J* (Hz)
2	6.24	dd, 5.4, 0.3	6.27	d, 5.4	6.22	dd, 5.5	6.26	d, 5.5
3	7.42	d, 5.7	7.37	d, 5.4	7.37	d, 5.5	7.35	d, 5.5
5	5.41	d, 8.1	5.32	d, 8.1	5.39	d, 8.1	5.31	d, 8.0
6	5.15	ddd, 8.1, 6.0, 4.2	6.14	ddd, 7.8, 5.7, 4.2	5.15	ddd, 8.1, 6.1, 4.0	6.12	dt, 8, 5.8, 4.5
6-OH	2.79	br	-	-	2.96	br	-	-
7α	4.46	dd, 11.4, 4.2	4.57	dd, 12, 4.5	4.45	dd, 11.4, 4.0	4.55	dd, 11.8, 4.5
7β	4.41	dd, 11.4, 6.3	4.52	dd, 11.7, 6	4.42	dd, 11.4, 6.0	4.50	dd, 11.5, 5.8
2', 6'	8.04	ddd, 8.4, 1.5, 0.9	8.03	ddd, 7.8, 1.5, 0.9	8.02	ddd, 8.2, 1.3, 0.5	8.01	m
3', 5'	7.46	tt, 7.8, 1.2	7.45	t, 7.8	7.42	tt, 8.2, 1.3	7.43	m
4'	7.59	tt, 7.5, 1.2	7.58	tt, 7.2, 1.2	7.55	tt, 8.2, 1.3	7.56	tt, 7.5, 1.4
2''	-	-	2.10	s	-	-	2.08	s

Table 5.5 ^{13}C NMR Spectral Data for Melodorinol (5.85) and (Z)-Acetylmelodorinol (5.86)

C	Observed		Reported[55]		C	Observed		Reported[194]	
	5.85	**5.86**	**5.85**	**5.86**		**5.85**	**5.86**	**5.85**	**5.86**
	δ	δ	δ	δ		δ	δ	δ	δ
1	169.27	168.82	168.80	168.45	8	166.82	166.42	166.66	165.99
2	121.29	121.27	121.00	121.59	1'	130.13	130.11	129.71	129.49
3	144.15	143.66	143.58	143.29	2', 6'	129.97	129.96	129.48	129.68
4	150.56	151.12	150.01	150.66	3', 5'	128.86	128.87	128.45	128.46
5	113.28	109.25	113.06	108.85	4'	133.65	133.68	133.30	133.28
6	66.16	67.71	67.48	67.26	1''	-	21.26	-	20.89
7	67.88	65.01	65.88	64.60	2''	-	170.16	-	169.77

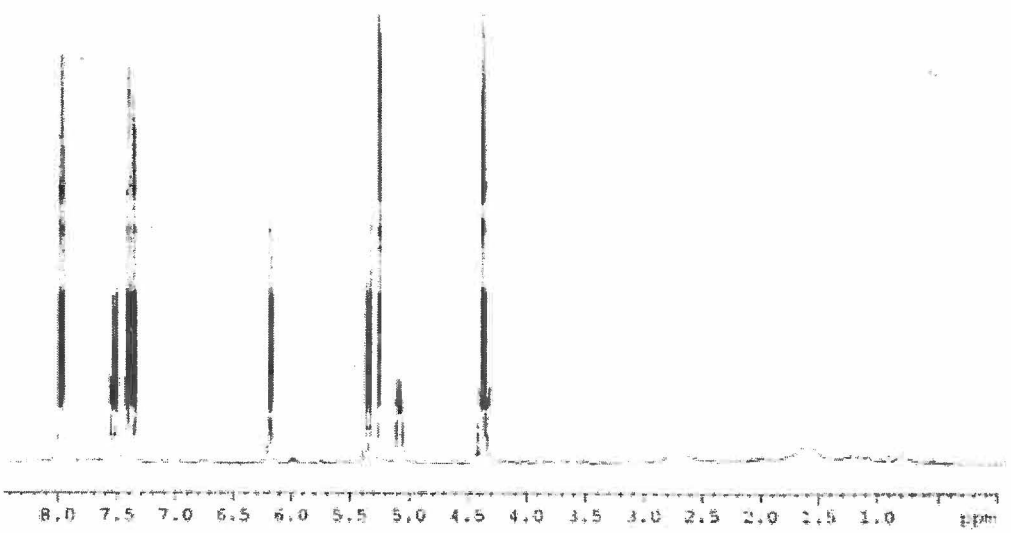


Fig. 5.13 ^{1}H NMR Spectrum of Melodorinol (5.85)

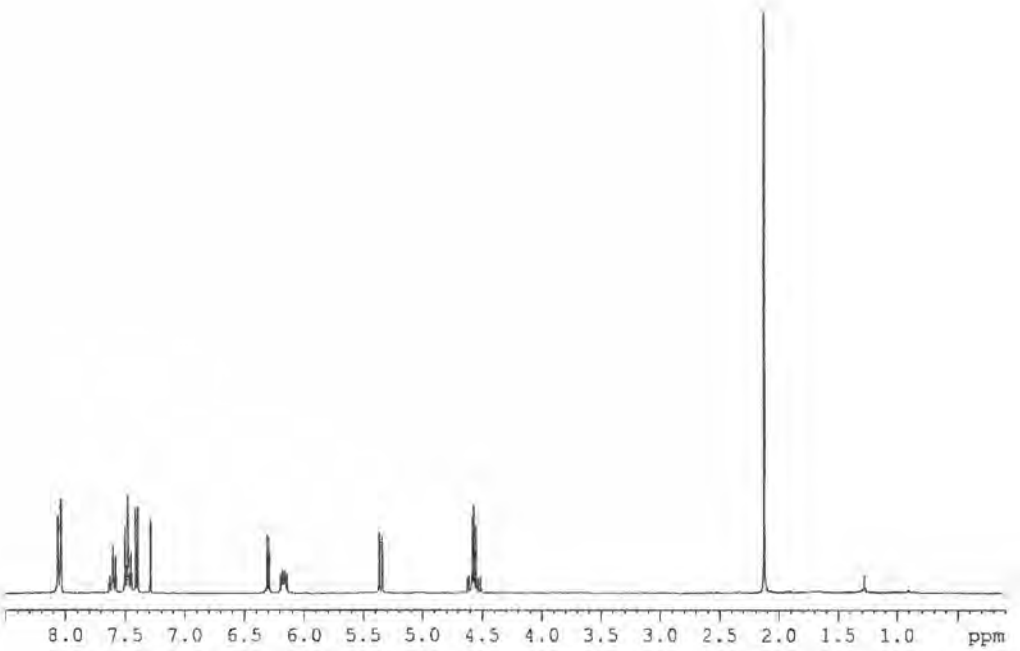


Fig. 5.14 ^{1}H NMR Spectrum of (*Z*)-Acetylmelodorinol (5.86)

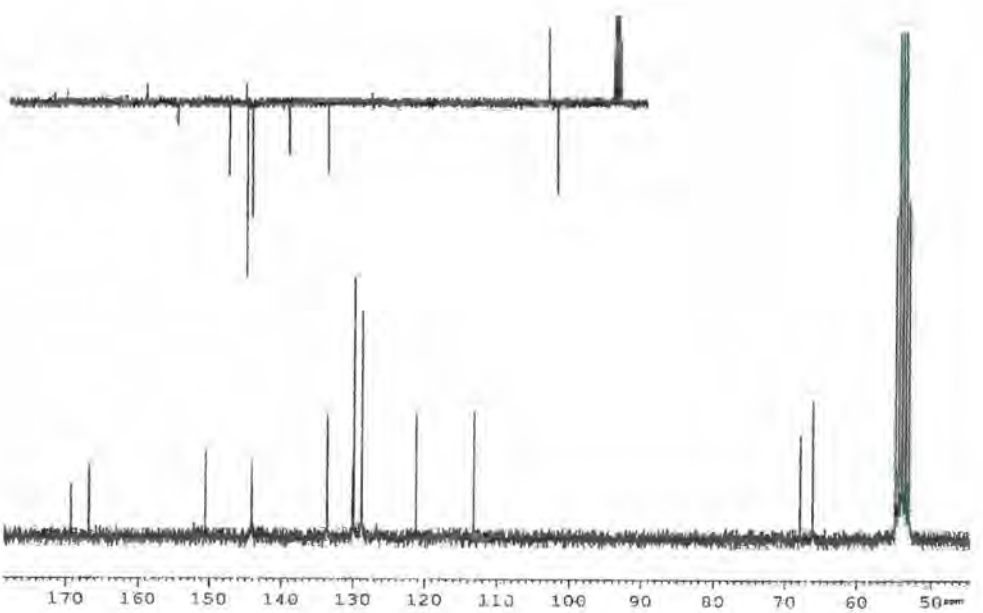


Fig. 5.15 ^{13}C NMR with APT Spectrum of Melodorinol (5.85)

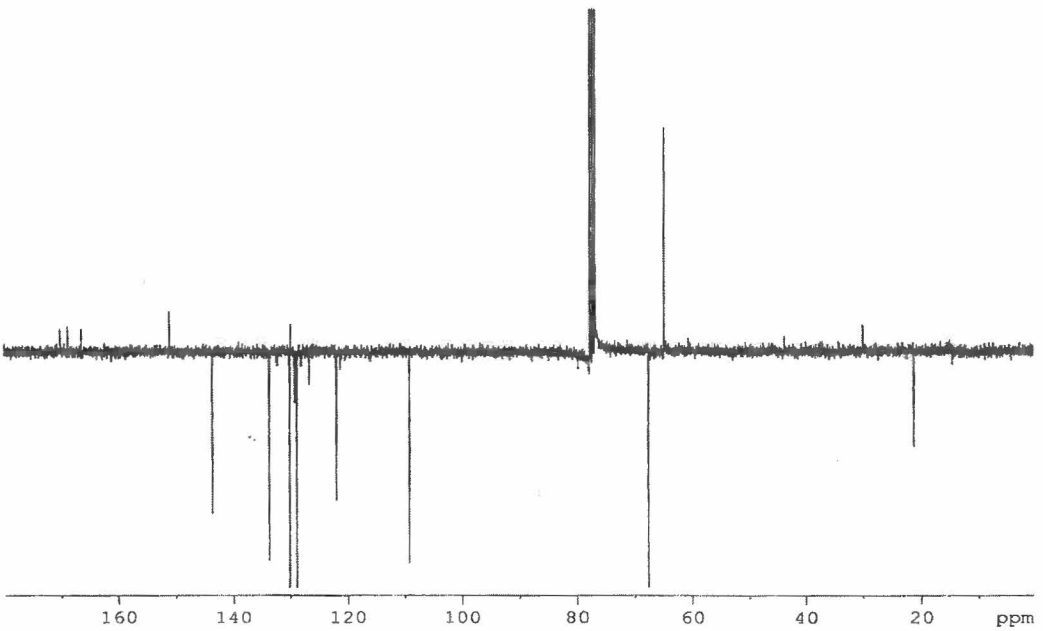


Fig. 5.16 ^{13}C NMR APT Spectrum of (*Z*)-Acetylmelodorinol (5.86)

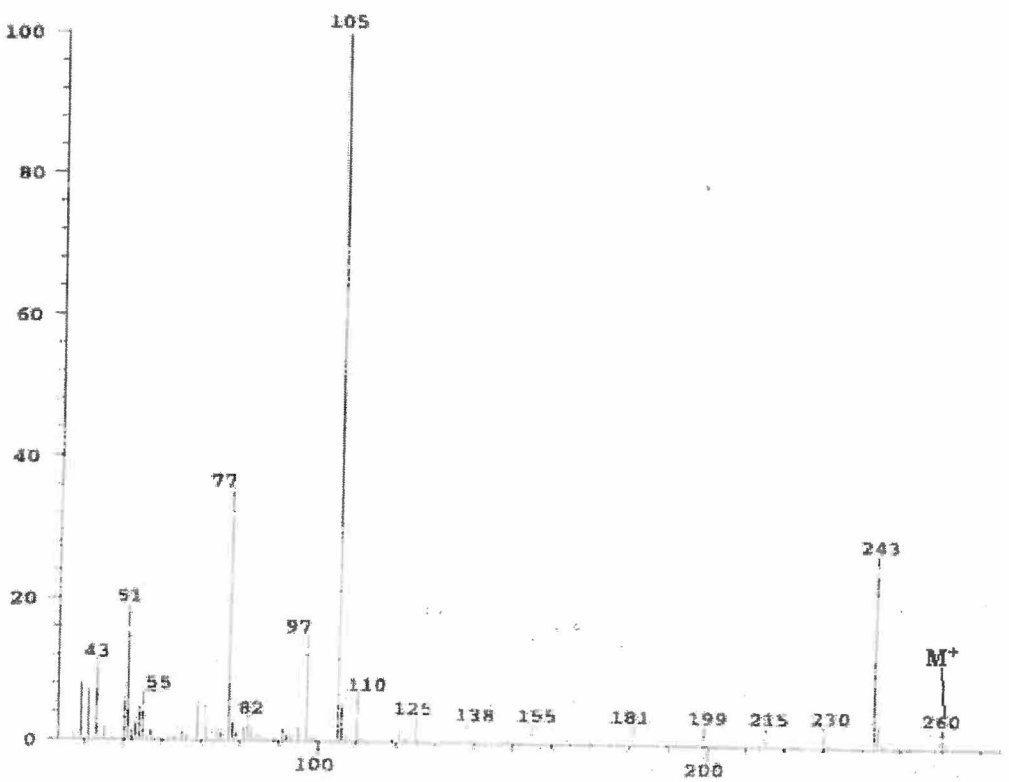


Fig. 5.17 Mass Spectrum of Melodorinol (5.85)

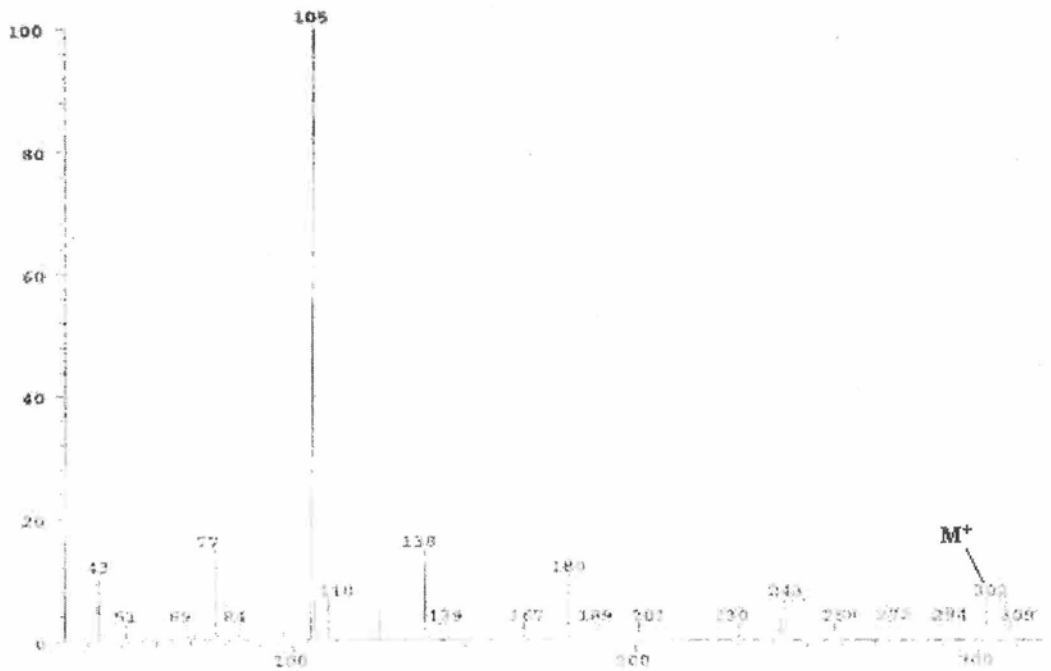
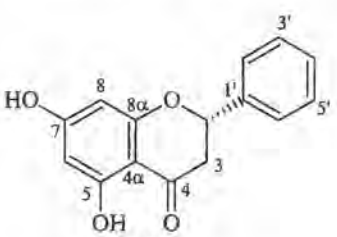


Fig. 5.18 Mass Spectrum of (*Z*)-Acetylmelodorinol (5.86)

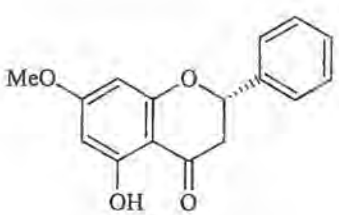
Heptanolides such as **5.85** and **5.86** belong to a series of heptene derivatives possessing cytotoxic activity against breast, lung, colon and other carcinomas.[196,200] In this study compound **5.85** exhibited larvicidal activity against *An. gambiae* mosquitoes (LC_{50} values of 21 and 16 ppm after 48 and 72 h exposure, respectively). It also exhibited antibacterial activity against *P. aeruginosa* and antifungal activity against *A. niger* at a minimum inhibitory concentration (MIC) of 10 mg/ml.

5.2.5 Pinocembrin (5.87) and 5–Hydroxy–7–methoxyflavanone (5.88) from *Uvaria lungonyana*

Repeated column chromatography of VLC fraction 5 of the chloroform extract of the stem bark of *U. lungonyana* yielded white crystals which were shown to constitute pinocembrin (**5.87**) based on comparison of the m.p. of 188-190 °C (Lit.[195] m.p. 189-192 °C) and spectral features (^{1}H and ^{13}C NMR, and MS, Figs. 5.19, 5.21 and 5.23) with those reported in the literature.[195,196,202,203] The VLC fraction 5 also yielded white crystals whose spectral features were identical to those reported for 5-hydroxy-7-methoxy-flavanone (**5.88**).[200] It was therefore concluded that the isolated compound had structure **5.88**. The structures **5.87** and **5.88** were both confirmed on the basis of C/H HMBC interactions.



5.87



5.88

Compound **5.87** which was previously isolated from *Melodorum fruticosum*[195,196] and *Uvaria chamae*[194] is reported to have *in vitro* antifungal and antibacterial activity,[196,202] local anaesthetic effects in rabbits,[204] cytotoxicity to chemically induced murine lymphocytic leukemia[196] and inhibitory effects against protein tyrosine kinase.[205]

Table 5.6 ^{1}H NMR Spectral Data for Pinocembrin (5.87) and 5-Hydroxy-7-methoxyflavanone (5.88)

H	Observed				Reported[196]			
	$\delta_{5.87}$	*J* (Hz)	$\delta_{5.88}$	*J* (Hz)	$\delta_{5.87}$	*J* (Hz)	$\delta_{5.88}$	*J* (Hz)
2	5.44	dd, 12.6, 3	5.45	dd, 12.9, 3.1	5.49	dd, 11.5, 4.5	5.45	dd, 13.0, 3.1
3α	3.08	dd, 17.1, 12.6	3.11	dd, 17.2, 12.9	3.16	dd, 17, 11.5	3.12	dd, 17.2, 13.0
3β	2.76	dd, 17.1, 3.3	2.84	dd, 17.2, 3.1	2.73	dd, 17, 4.5	2.85	dd, 17.2, 3.1
6	5.90	d, 2.4	6.11	d, 2.3	5.98	s	6.10	d, 2.3
8	5.94	d, 2.4	6.08	d, 2.3	5.98	s	6.09	d, 2.3
5-OH	12.04	br s	12.04	s	12.71	br s	12.05	s
7-OH	12.04	br s	-	-	9.61	s	-	-
7-MeO	-	-	3.84	s	-	-	3.84	s
2' - 6'	7.47	m	7.47	m	7.43	s	7.49	m

Table 5.7 ^{13}C NMR Spectral Data for Pinocembrin (5.87) and 5-Hydroxy-7-methoxyflavanone (5.88)

C	Observed		Reported[196]		C	Observed		Reported[196]	
	$\delta_{5.87}$	$\delta_{5.88}$	$\delta_{5.87}$	$\delta_{5.88}$		$\delta_{5.87}$	$\delta_{5.88}$	$\delta_{5.87}$	$\delta_{5.88}$
2	79.4	79.2	79.6	79.6	8	95.2	94.2	95.9	95.6
3	43.2	43.5	43.5	43.8	8α	163.7	163.7	163.8	168.4
4	196.3	196.3	196.3	196.2	1'	139.4	139.4	139.7	138.8
4α	102.4	102.4	103.4	103.6	2',6'	126.3	126.0	127.0	126.6
5	164.8	164.8	165.1	164.6	3',5'	128.7	128.8	129.2	129.3
6	96.2	95.1	97.0	94.7	4'	128.6	128.6	129.1	129.3
7	167.4	167.4	167.2	163.2	MeO	-	56.0	-	56.1

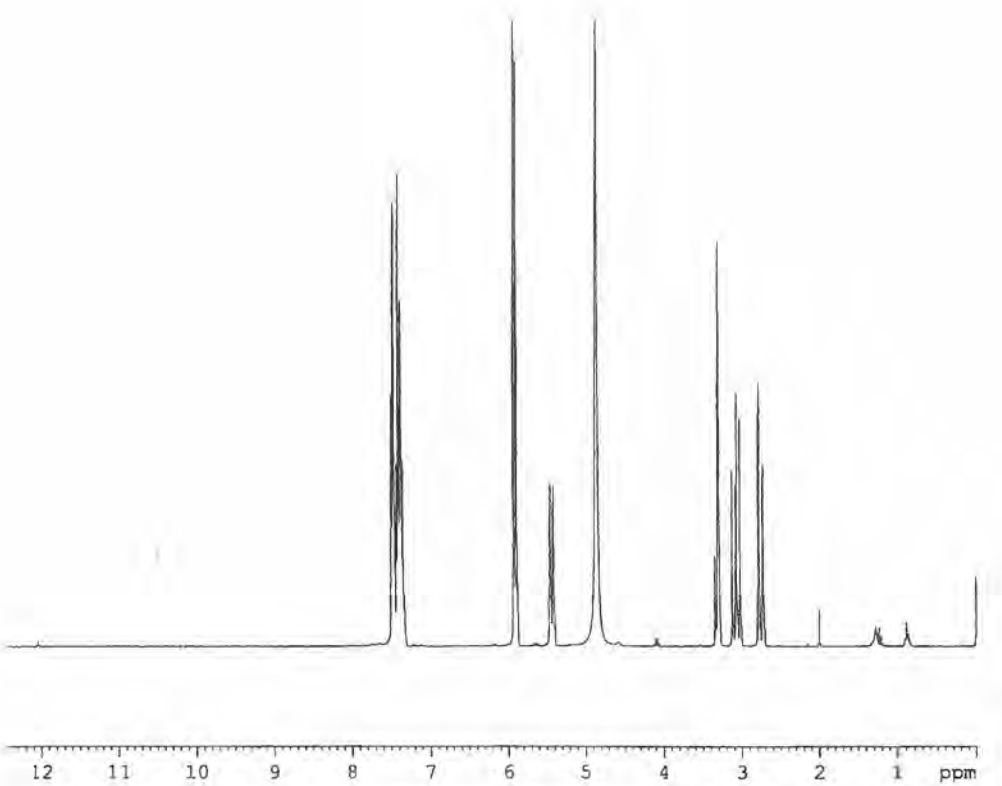


Fig. 5.19 ^{1}H NMR Spectrum of Pinocembrin (5.87)

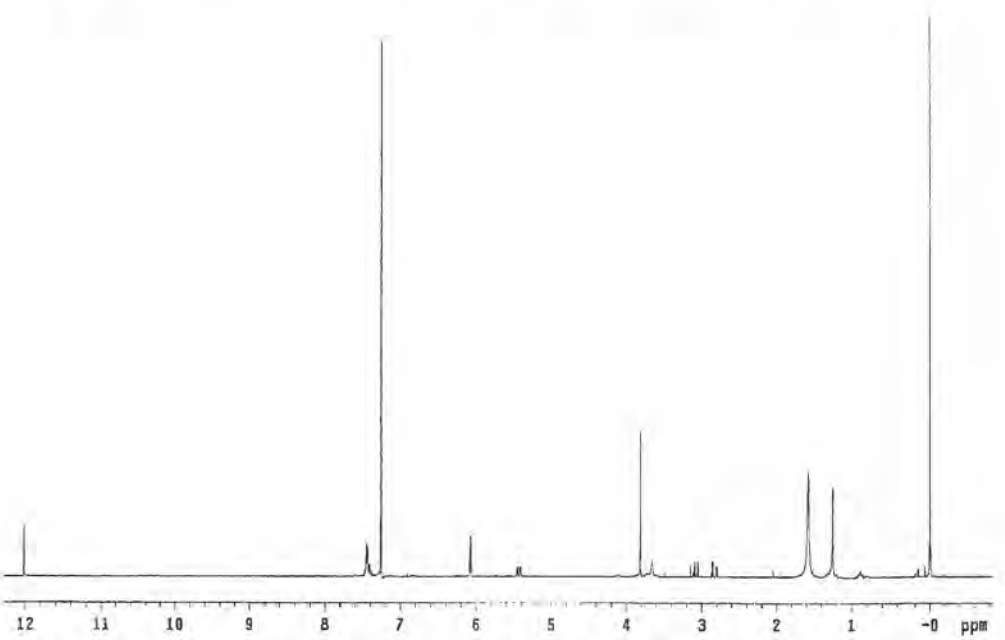


Fig. 5.20 ^{1}H NMR Spectrum of 5–Hydroxy–7–methoxyflavanone (5.88)

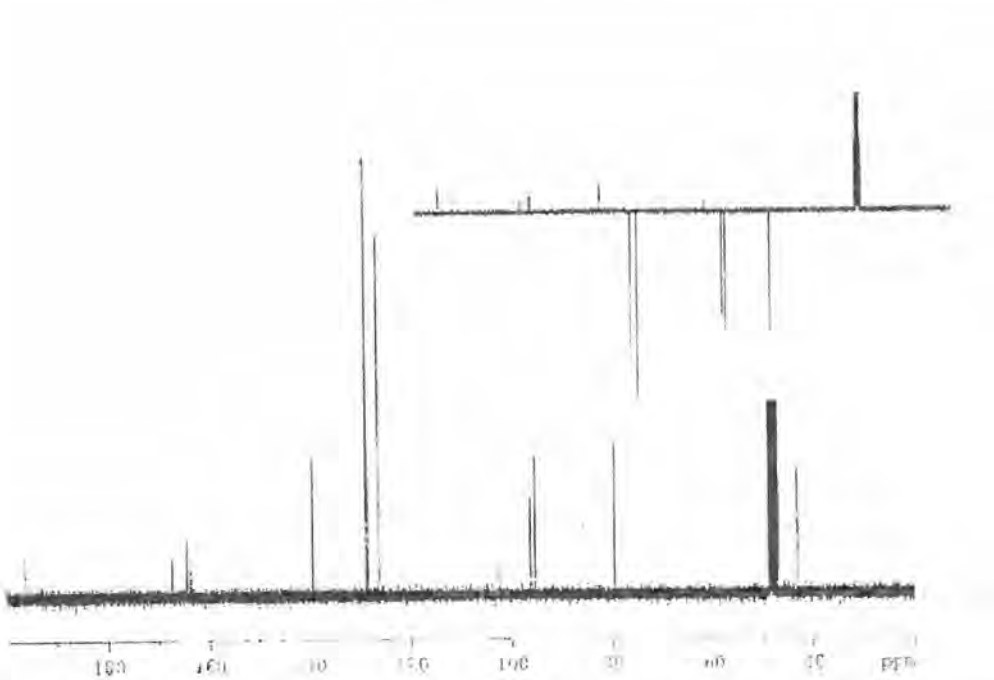


Fig. 5.21 ^{13}C NMR Spectrum (with APT Experiment) of Pinocembrin (5.87)

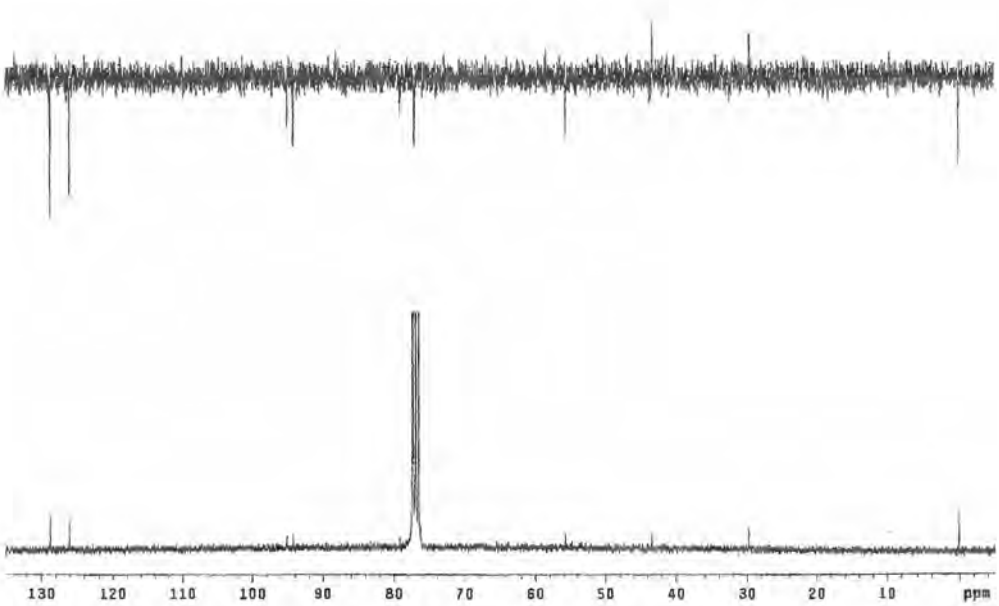


Fig. 5.22 ^{13}C NMR Spectrum (with DEPT 135 Experiment) of 5–Hydroxy–7–methoxyflavanone (5.88)

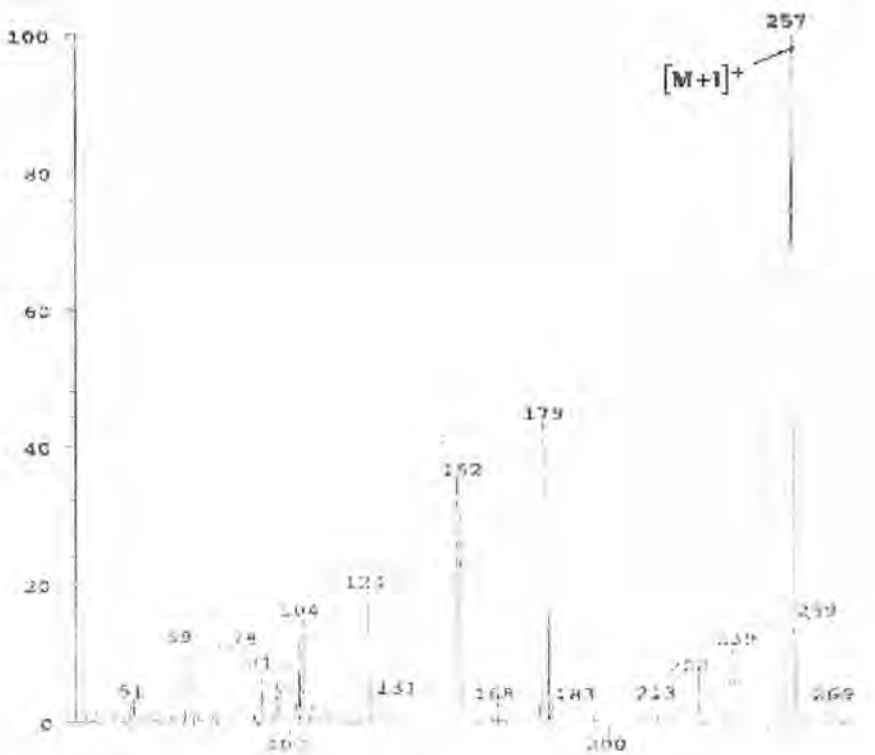
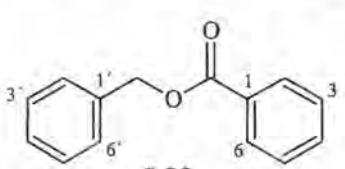


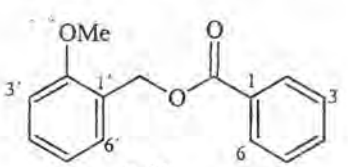
Fig. 5.23 Mass Spectrum of Pinocembrin (5.87)

5.2.6 Benzyl benzoate (5.89) and 2–Methoxybenzyl benzoate (5.90) from *Uvaria lungonyana*

The crude chloroform extract of the root bark of *Uvaria lungonyana* was fractionated by VLC separation into eight simpler fractions. Repeated chromatography of the first VLC fraction yielded benzyl benzoate (**5.89**) and 2-methoxybenzyl benzoate (**5.90**) whose structures were established on the basis of ^{1}H and ^{13}C NMR spectra (Figs. 5.24-5.27, Tables 5.8 and 5.9) and MS (Figs. 5.28-5.29) and on comparison of the spectral data with those previously reported for benzyl benzoate.[121]



5.89



5.90

Table 5.8 **^{1}H NMR Spectral Data for Benzyl benzoate (5.89) and 2-Methoxybenzyl benzoate (5.90)**

H	$\delta_{5.89}$	*J* (Hz)	$\delta_{5.90}$	*J* (Hz)
2, 6	8.07	ddd, 7.8, 1.5, 0.6	8.08	d, 7, 3
3, 5	7.45	m	6.90–6.99	m
4	7.86	tt, 7.2,1.5	7.43	t, 7.7
MeO	-	-	3.86	s
CH_2	5.36	d, 12	5.42	s
2'	7.36	m	-	-
3', 5'	7.47	m	7.55	t, 7.7
6'	7.36	m	7.32	td, 7.7, 3
4'	7.42	m	6.90–6.99	m

Table 5.9 **^{13}C NMR Spectral Data for Benzyl benzoate (5.89) and 2–Methoxybenzyl benzoate (5.90)**

C	$\delta_{5.89}$	$\delta_{5.90}$	C	$\delta_{5.89}$	$\delta_{5.90}$
1	130.61	124.28	CH_2	67.08	62.19
2	130.12	157.31	C=O	166.83	166.36
3	128.64	110.36	1'	136.51	132.73
4	133.41	130.26	2', 6'	129.01	129.34
5	128.64	120.31	3', 5'	128.77	128.18
6	130.12	129.57	4'	128.56	129.28
MeO	-	55.46			

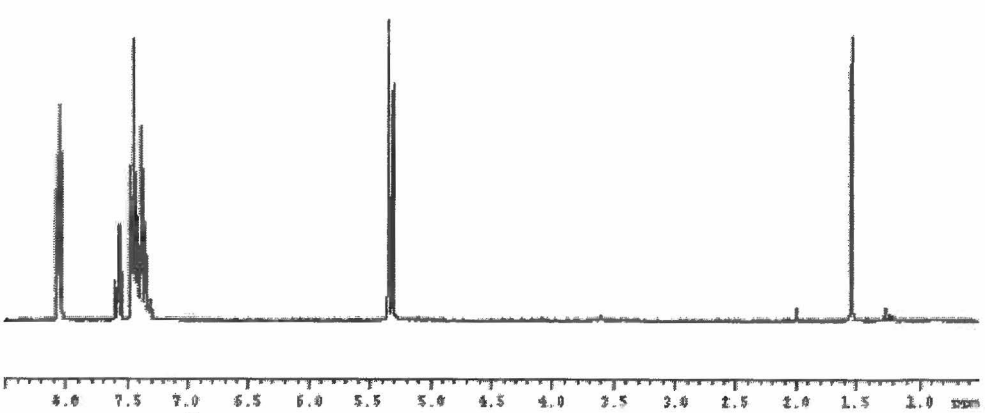


Fig. 5.24 **^{1}H NMR Spectrum of Benzyl benzoate (5.89)**

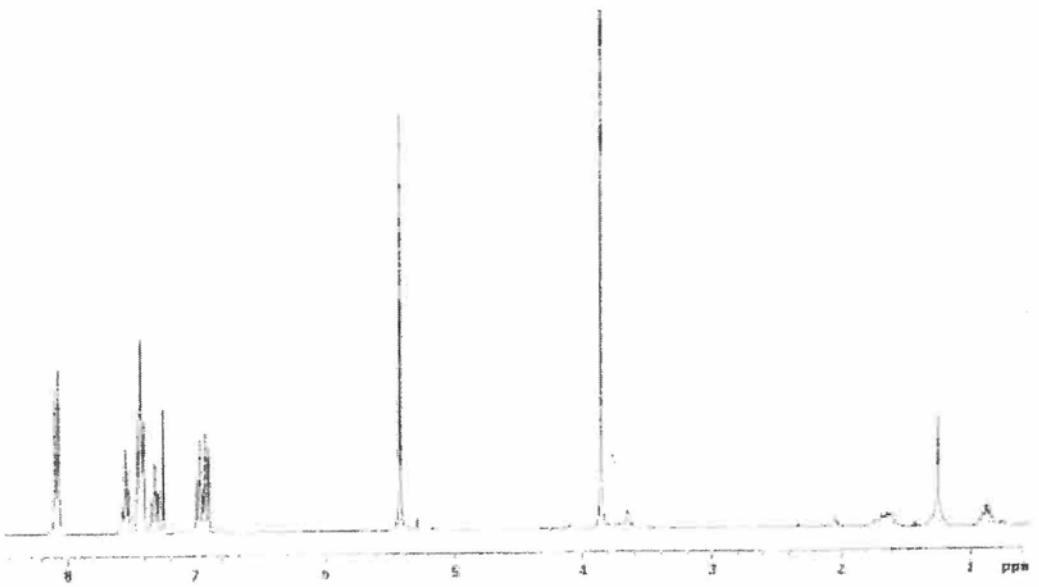


Fig. 5.25 ^{1}H **NMR Spectrum of 2–Methoxybenzyl benzoate (5.90)**

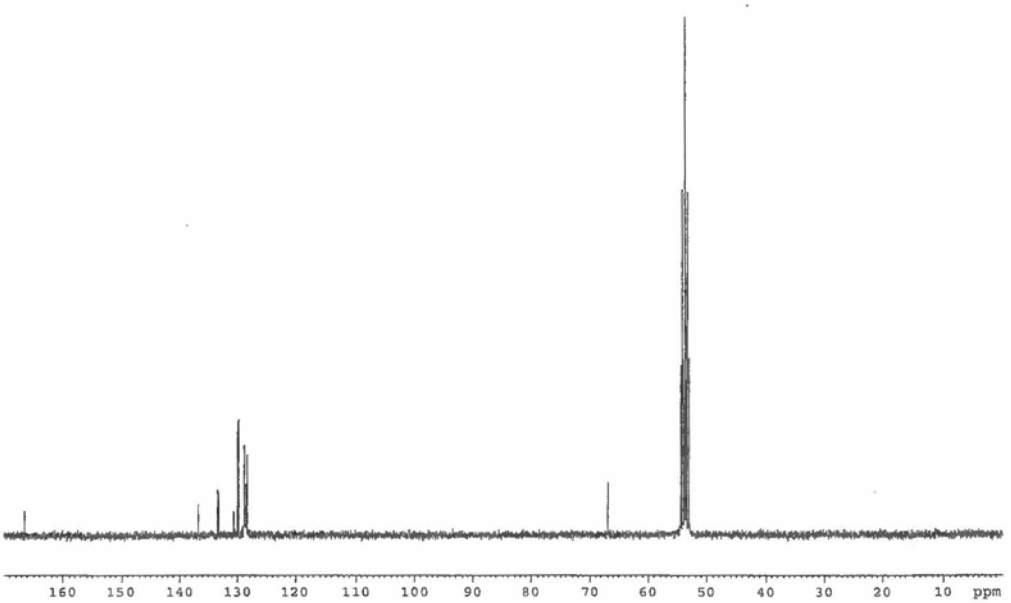


Fig. 5.26 ^{13}C **NMR Spectrum of Benzyl benzoate (5.89)**

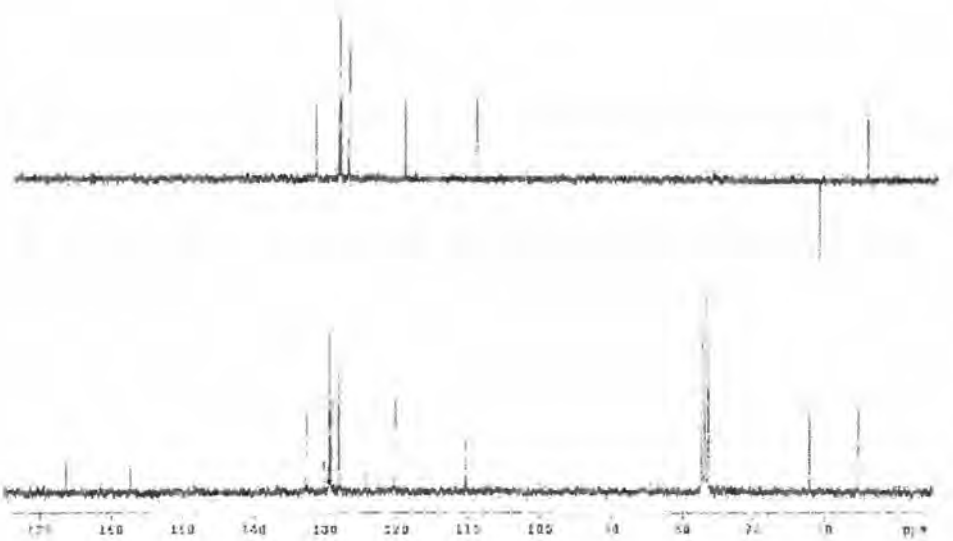


Fig. 5.27 ^{13}C NMR Spectrum (with APT Experiment) of 2–Methoxybenzyl benzoate (5.90)

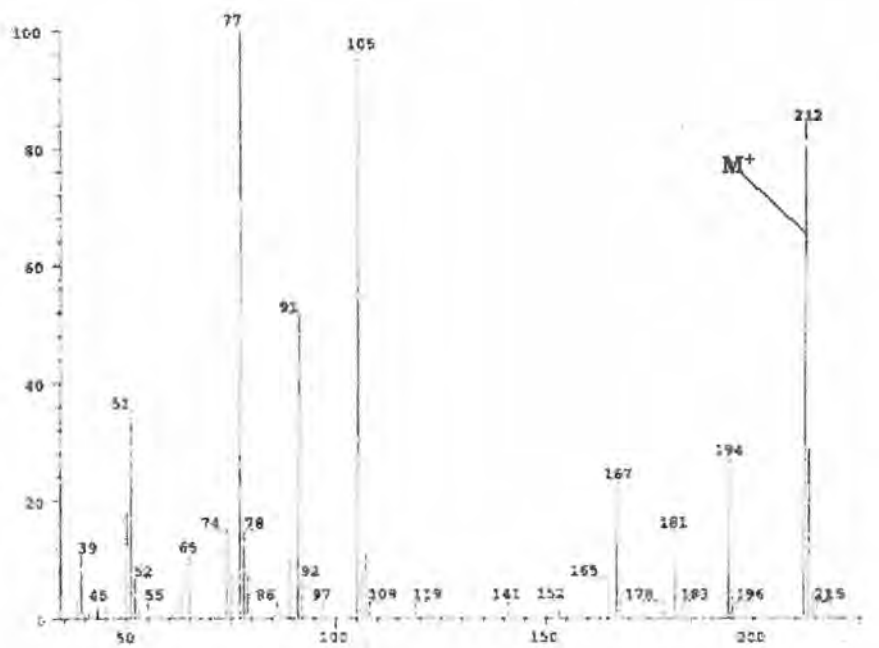


Fig. 5.28 Mass Spectrum of Benzyl benzoate (5.89)

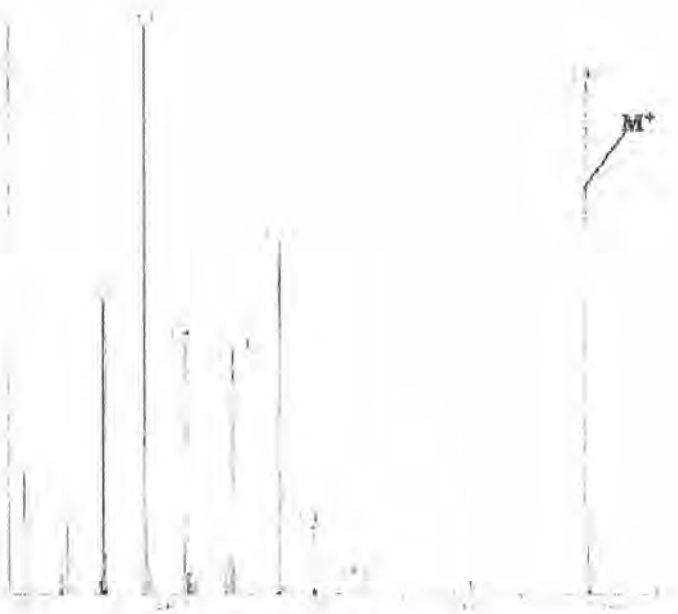


Fig. 5.29 Mass Spectrum of 2–Methoxybenzyl benzoate (5.90)

5.2.7 Polycarpol (5.81) and a Mixture of Chamanetin (5.91) and Dichamanetin (5.92) from *Uvaria lungonyana*

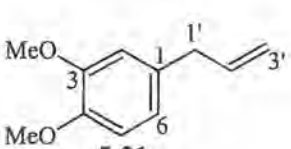
Repeated chromatography of VLC fraction 7 of the pet ether extract of the root bark of *U. lungonyana* yielded polycarpol **5.81** while a mixture of the isomeric C-benzyl flavanones chamanetin **5.91** and dichamanetin **5.92** was also isolated from the root bark of *Uvaria lungonyana* as gum and the structures of the two compounds were established upon comparison of spectral properties of the mixture (^{1}H and ^{13}C NMR, MS) with those reported in the literature for the individual compounds.[117]

The mixture of compound **5.91** and **5.92** exhibited larvicidal activity against *An. gambiae* mosquito larvae (LC_{50} value of 122 ppm after 24 h exposure) and antifungal activity against *A. niger* (MIC = 10 mg/ml).

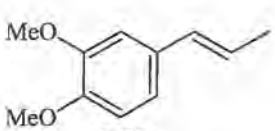
5.2.8 *O*-Methyleugenol (5.21), *O*-Methylisoeugenol (5.93) and 2,3-Dimethoxycinnamaldehyde (5.94): Phenylpropenoids from *Uvariodendron pycnophyllum*

Extracts from the stem and root barks of *U. pycnophyllum* when assayed for activity against *An. gambiae* s.s Giles mosquito larvae showed very strong activity as reported in Chapter 2. Hence it was decided to carry out detailed phytochemical analysis of the chloroform extract of the stem bark and the two extracts from the root bark. Repeated chromatography of the $CHCl_3$ extract from the stem bark, and $CHCl_3$ and MeOH extracts from the root bark yielded *O*-methyleugenol (**5.21**) and *O*-methylisoeugenol (**5.93**) as the major constituents, in addition to two other minor compounds 2,3-dimethoxycinnamaldehyde (**5.94**) and stigmasterol (**5.25**) that were obtained from the chloroform extract of the root bark. Structures of the isolated compounds were identified based on analysis of spectroscopic data (Figs. 5.30-5.38), upon comparison of the data with those reported in the literature.[133,134]

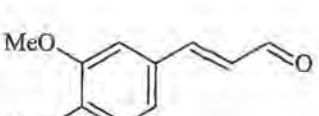
Phenylpropanoids type of compounds like **5.21**, **5.93** and **5.94** are reported to possess some insecticidal, fungicidal, nematocidal and bacterial activities.[133, 134] The mosquitocidal and antimicrobial activity of these compounds compounds as determined in these investigations are reported in Chapter 6 of this Thesis.



5.21



5.93



5.94

Table 5.10 ^{1}H NMR Spectral Data for *O*-Methyleugenol (5.21), *O*-Methylisoeugenol (5.93) and 2,3-Dimethoxycinnamaldehyde (5.94)

H	Observed						Reported[133,134]	
	5.21		**5.93**		**5.94**		**5.21**	
	δ_H	*J* (Hz)	δ_H	*J* (Hz)	δ_H	*J* (Hz)	δ_H	*J* (Hz)
2	6.78	s	6.88	d, 1.9	7.10	d, 2.1	6.72	br s
3 - OMe	3.80	s	3.82	s	-	-	3.87	s
4 - OMe	3.79	s	3.80	s	-	-	3.87	s
5	6.81	dd, 8.4, 1.5	6.78	d, 8.4	6.99	d, 8.5	6.81	d, 7.8
6	6.70	dd, 8.4, 1.7	6.84	dd, 8.4, 1.9	7.17	dd, 8.5, 2.1	6.73	dm, 7.8
1'	3.32	d, 6.6	6.33	dq, 15.8, 1.7	7.43	d, 15.8	3.34	d, 6.6
2'	5.95	ddt, 17.1, 10.2, 6.6	6.11	dq, 15.6. 6.5	6.60	dd, 15.8, 7.7	5.94	ddt, 16.8, 9.9, 6.6
3'	5.10 *trans*	dd, 17.1, 1.8	1.84	dd, 6.6, 1.7	9.64	d, 7.7	5.08	dm, 16.8
3'	5.04 *cis*	dd, 10.2, 1.8					5.06	dm, 9.9

Table 5.11 ^{13}C NMR Spectral Data for *O*-Methyleugenol (5.21), *O*-Methylisoeugenol (5.93) and 2,3-Dimethoxy-cinnamaldehyde (5.94)

C	Observed			Reported[133,134]
	5.21 δ_C	**5.93** δ_C	**5.94** δ_C	**5.21** δ_C
1	133.16	131.4	127.4	137.2
2	112.57*	109.1	110.5	111.3
3 – Ome	55.84	56.0*	56.2	55.3
3	148.83	149.5*	152.6	148.4
4 - OMe	55.84	56.1*	56.2	55.16
4	148.02	148.7*	149.9	146.9
5	115.52	111.8	111.6	110.7
6	121.26*	118.9	123.6	119.9
1'	40.09	130.8	152.9	39.2
2'	138.28	124.0	127.0	132.1
3'	111.98*	18.4	193.6	115.1

* interchangeable assignments

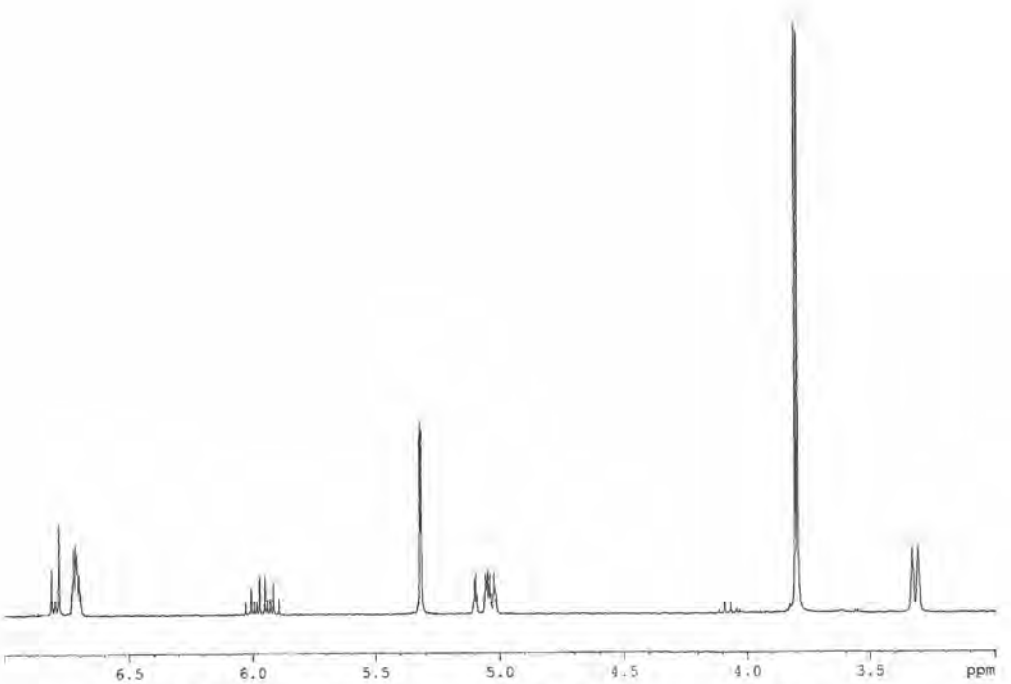


Fig. 5.30 ^{1}H NMR Spectrum of *O*-Methyleugenol (5.21)

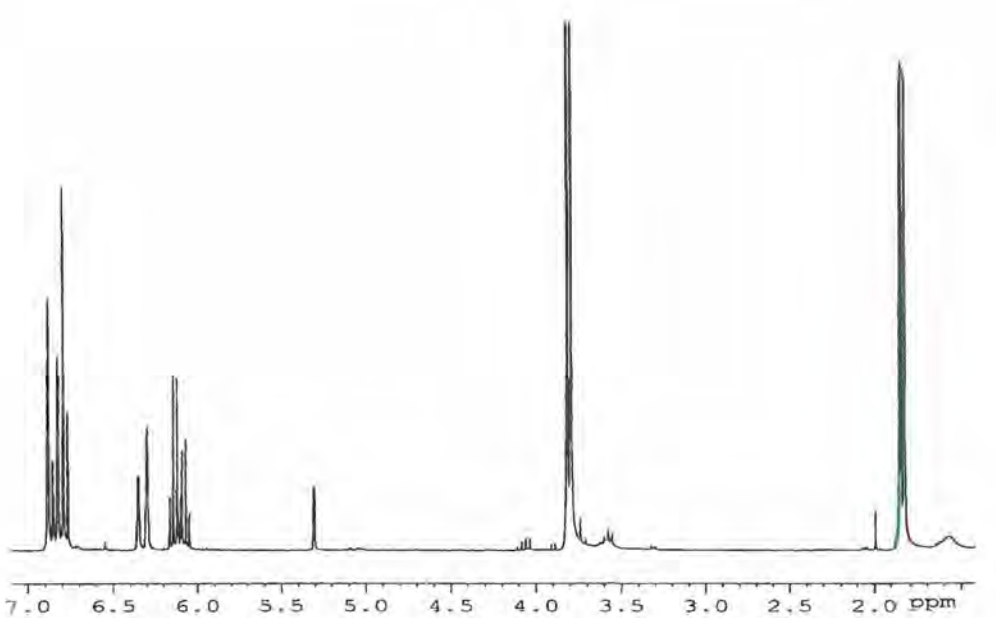


Fig. 5.31 ^{1}H NMR Spectrum of *O*-Methylisoeugenol (5.93)

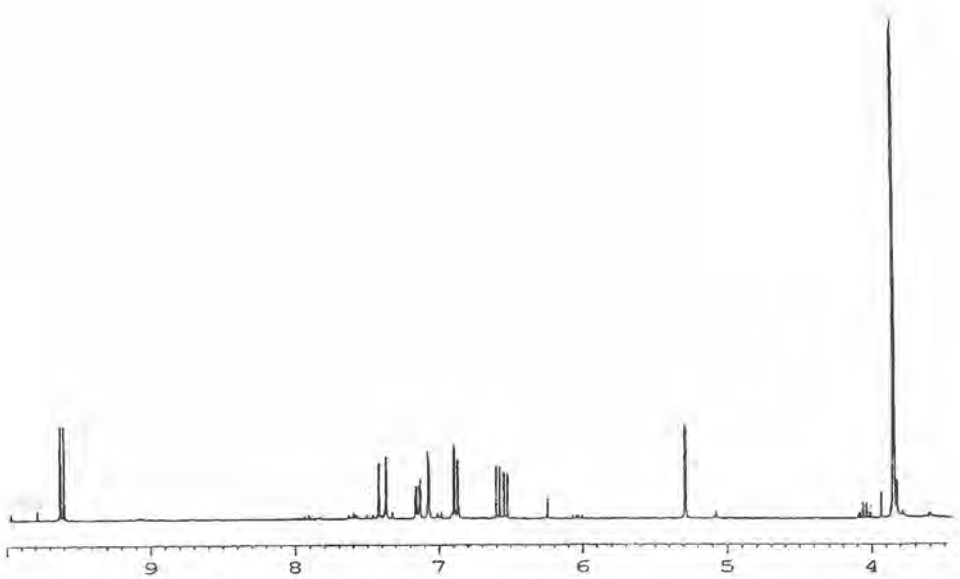


Fig. 5.32 ^{1}H NMR Spectrum of 2, 3-Dimethoxycinnamaldehyde (5.94)

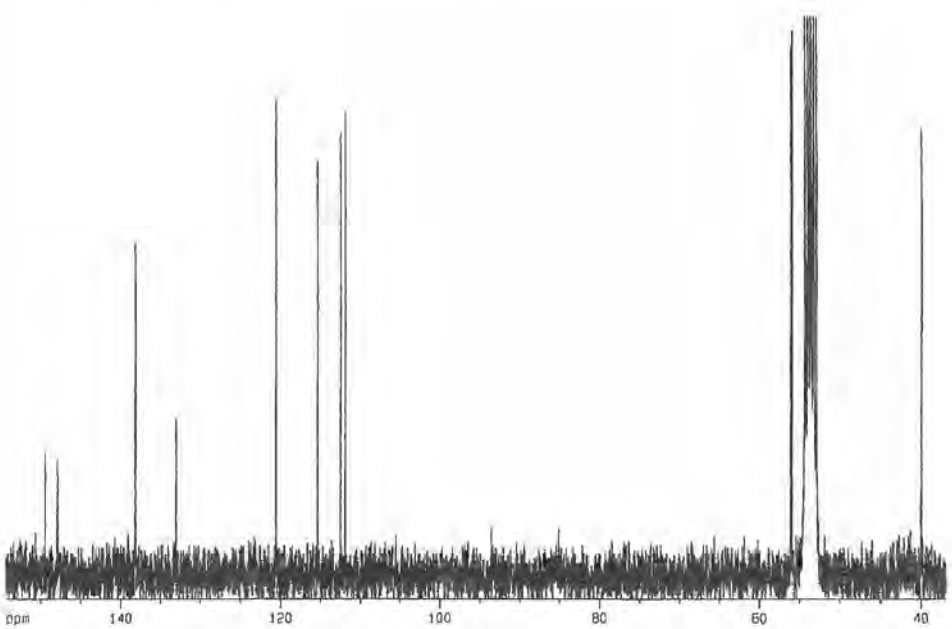


Fig. 5.33 ^{13}C NMR Spectrum of *O*-Methyleugenol (5.21)

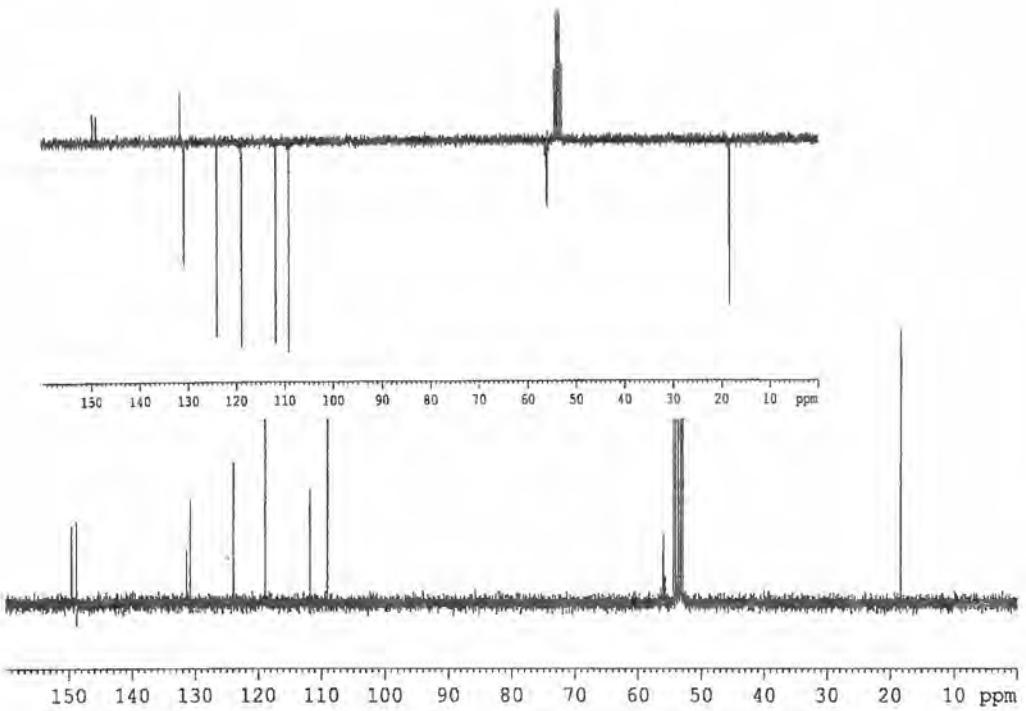


Fig. 5.34 ^{13}C NMR Spectrum of *O*-Methylisoeugenol (5.93)

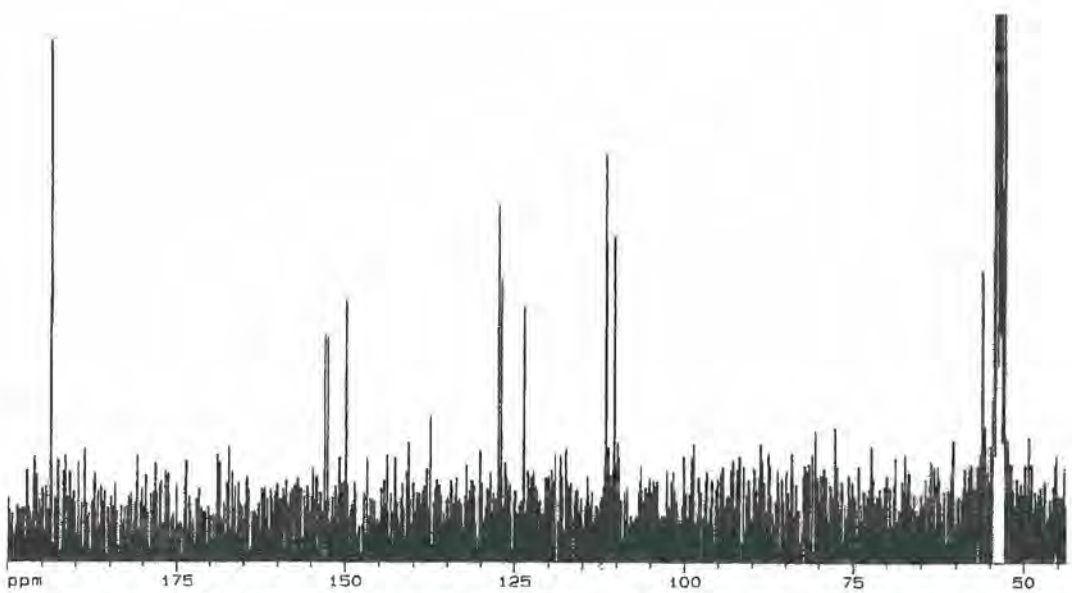


Fig. 5.35 ^{13}C NMR Spectrum of 2, 3-Dimethoxycinnamaldehyde (5.94)

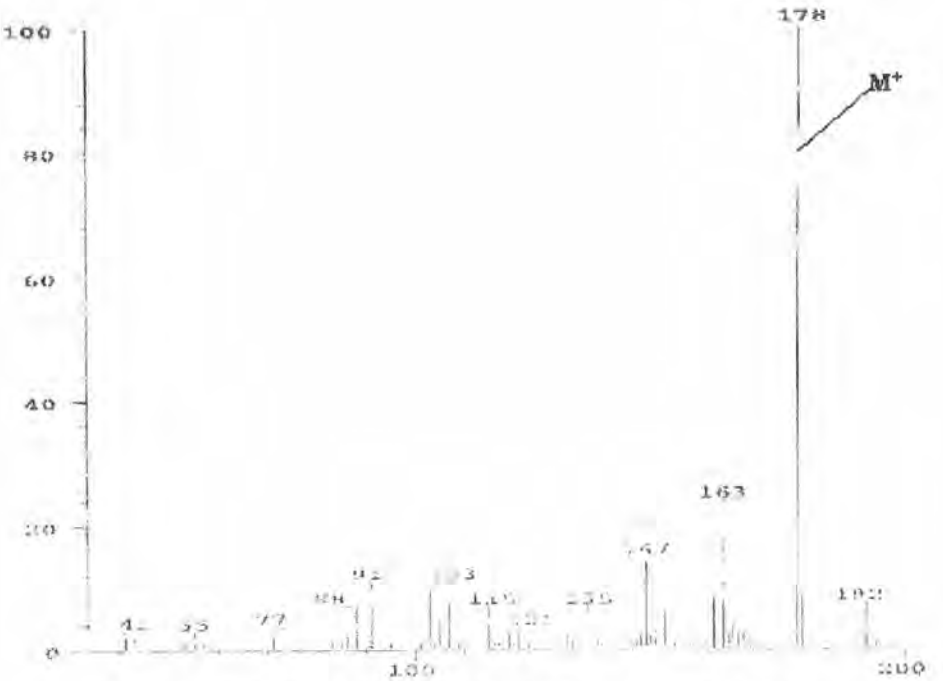


Fig. 5.36 Mass Spectrum of *O*-Methyleugenol (5.21)

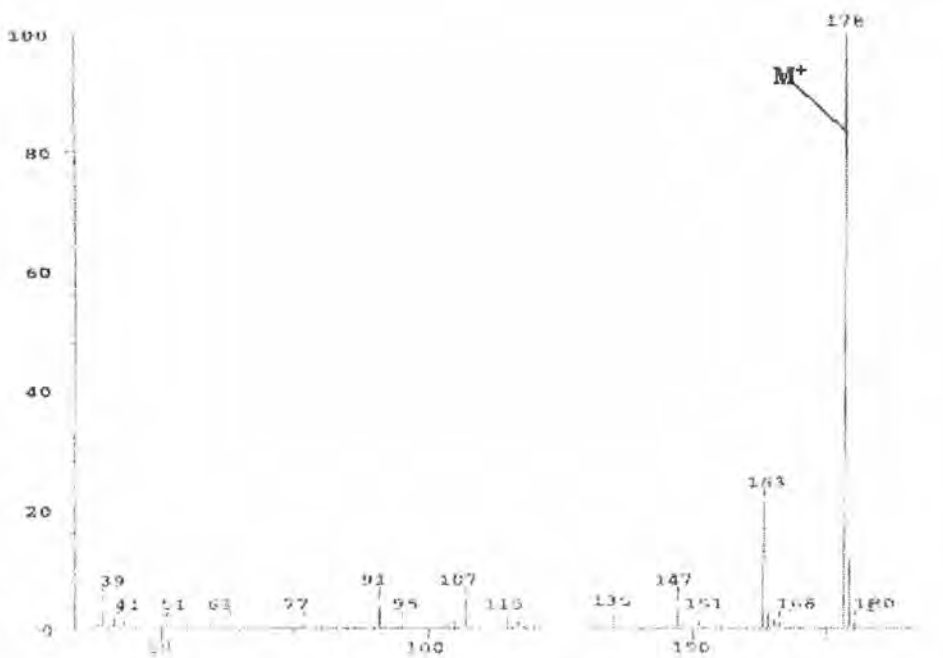


Fig. 5.37 Mass Spectrum of *O*-Methylisoeugenol (5.93)

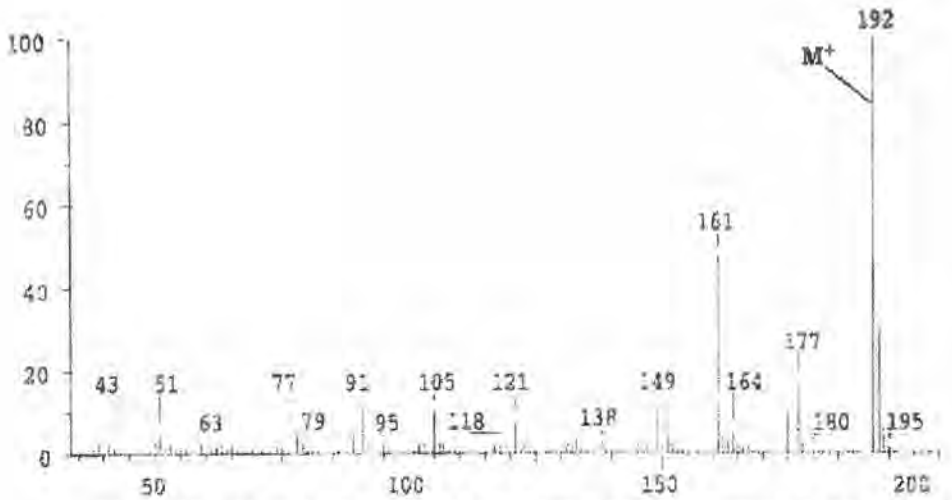


Fig. 5.38 Mass Spectrum of 2, 3-Dimethoxycinnamaldehyde (5.94)

5.3 EXPERIMENTAL

5.3.1 General

The general experimental procedures were the same as described in Chapter 3.

5.3.2 Biological Tests

These were conducted as described in Chapter 3.

5.3.3 Plant Materials

The root and stem barks of *Polyalthia tanganyikensis* and *Uvaria lungonyana* were collected from Rufiji District, Coast Region, Tanzania, in January 2004, the former from Kichi Hill forest at the boundary of the Selous Game Reserve, while the later inside the Game Reserve. Materials of *Uvariodendron pycnophyllum* were collected from Sigi Valley, 3 km from Kisiwani Village along the road to Bombani Village in the Amani Nature Reserve in East Usambara Mountains, Muheza District, Tanga Region in April 2005, and those of *A. squamosa* were collected from Kibanda village, Muheza District, Tanga Region in April 2005. All the plant species were

authenticated at the Herbarium, Department of Botany, University of Dar es Salaam, Tanzania where voucher specimens are preserved.

5.3.4 Extraction and Isolation

The air-dried and pulverized plant materials of *U. lungonyana, U. pycnophyllum, P. tanganyikensis* and *A. squamosa* were extracted at room temperature (25–30 °C), sequentially with pet ether, chloroform and ethanol, each extraction lasting for 48 h for two rounds of extractions. The concentrated (*vacuo*) crude extracts were then separated into pure compounds using different chromatographic processes.

5.3.4.1 Pet ether Extract of the Root Bark of *Polyalthia tanganyikensis*

The crude pet ether extract (15 g) from the root bark of *P. tanganyikensis* was screened for activity against *An. gambiae* mosquito larvae, after which it was then fractionated by vacuum liquid chromatography (VLC) on silica gel, eluting with pet ether, then pet ether containing increasing amounts of ethyl acetate. Thirteen fractions were obtained which, on the basis of TLC analysis were combined into six series as follows: Fractions 1, 2, 3, 4(4-5), 5(6-8) and 6(9-13). The combined VLC fractions 1-4 consisted of complex mixtures of non- and medium polar compounds which decomposed on TLC, hence further chromatographic work up was not carried out for the mixtures.

The combined VLC fractions 5 and 6 were recrystallised from pet ether to give compound **5.81** as white crystals. Silica gel chromatography of the filtrate, eluting with a mixture of ethyl acetate (5–40 %) in n-heaxane yielded compound **5.78** as an oil. The isolation procedure is summerised in **Chart 5.1**.

5.3.4.2 Chloroform Extract of the Root Bark of *Annona squqmosa*

The chloroform extract (19 g) from the root bark of *A. squamosa* obtained after sequential soaking in chloroform and methanol (2 x) of the air-dried pulverized plant materials, each soaking lasting 48 h, was fractionated by VLC on silica gel eluting with pet ether, and then pet ether containing increasing amounts of EtOAc (Chart 5.2). Fourteen fractions were obtained and TLC analysis led to the combination of these fractions into six series as follows: Fractions 1(1-2), 2(3-4), 3(5-7), 4(8-9), 5(10-11) and 6(12-14). The combined VLC fractions 1-4 consisted of a complex mixture of non-polar compounds and hence this was not analysed further. Combination fractions 5 and 6 when subjected to silica gel and Sephadex® LH-20 chromatography yielded compounds **5.82** and **5.83**. The isolation process for compounds **5.82** and **5.83** is summarized in **Chart 5.2**.

5.3.4.3 Chloroform Extract of the Root Bark of *Uvaria lungonyana*

The crude chloroform extract (22 g) was fractionated by VLC (pet ether, and then pet ether containing increasing amounts of ethyl acetate) and fourteen fractions were obtained which on TLC analysis were combined into six series as follows: Fractions 1(1), 2(2–4), 3(5–7), 4(8–10), 5(11–13) and 6(14). The combined VLC fractions 3 and 5 constituted complex mixtures of unstable compounds, which decomposed on TLC analysis. Therefore, these were not worked up further. Repeated chromatography of the combined VLC fractions 1, 2, 4 and 6 yielded compounds **5.85-5.92**. The isolation process for these compounds is summarized in **Chart 5.4**.

5.3.4.4 Chloroform Extract of the Root Bark of *Uvariodendron pycnophyllum*

The crude chloroform extract (15 g) from the root bark of *U. pycnophyllum* was fractionated by silica gel chromatography (n-hexane, and then n-hexane containing increasing amounts of ethyl acetate: 5, 10, 20 and 40%). Forty-two fractions were obtained and TLC analysis led to the combination of these fractions into six series as follows: Fractions 1(1-4), 2(5-9), 3(10-14), 4(15-18), 5(19-32) and 6(33-42). The first two series consisted of complex mixtures of non-polar compounds, hence these were not analysed further. Silica gel chromatography of fractions 3 and 5 (ethyl acetate/n-hexane) followed by preparative TLC and then recrystallization yielded compounds **5.21**, **5.25**, **5.93** and **5.94**. The isolation process is summerised in **Chart 5.3**.

Chart 5.1 Isolation of Compounds 5.78 and 5.81 From the Root Bark of *Polyalthia tanganyikensis*

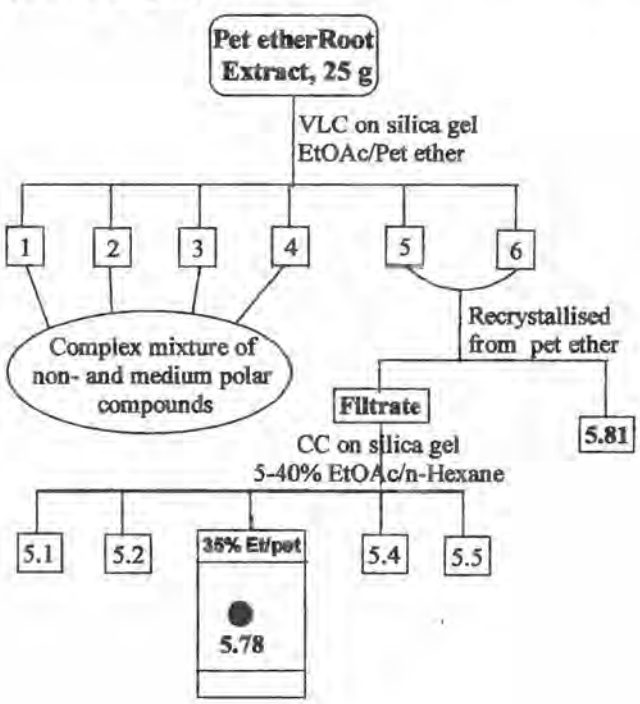


Chart 5.2 Isolation of Compounds 5.82 and 5.83 From the Root Bark of *Annona squamosa*

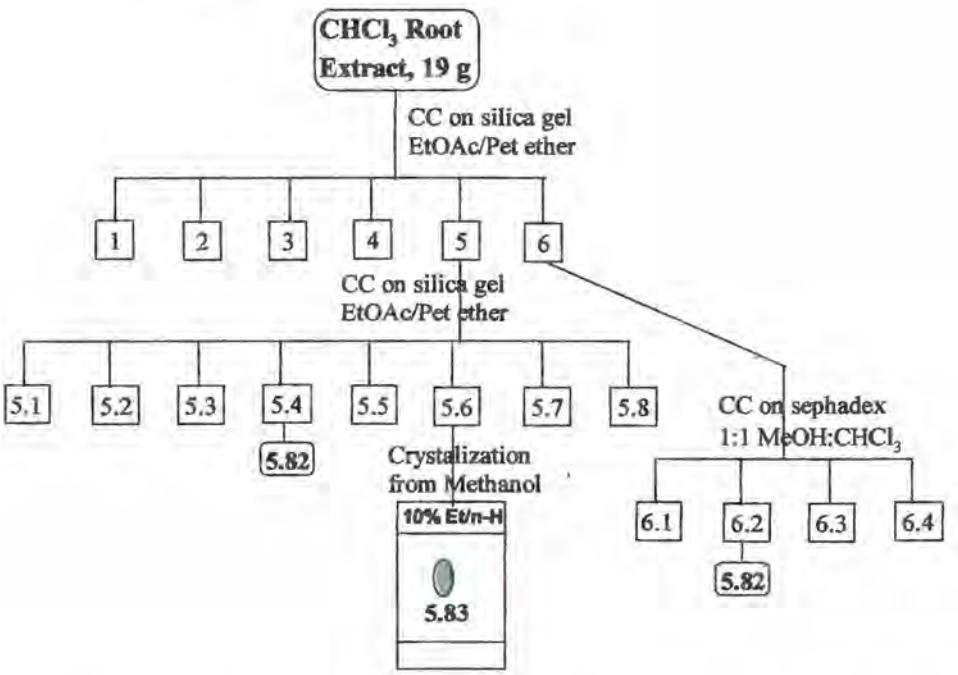


Chart 5.3 Isolation of Compounds 5.21, 5.25, 5.93 and 5.94 From the Root Bark of *Uvariodendron pycnophyllum*

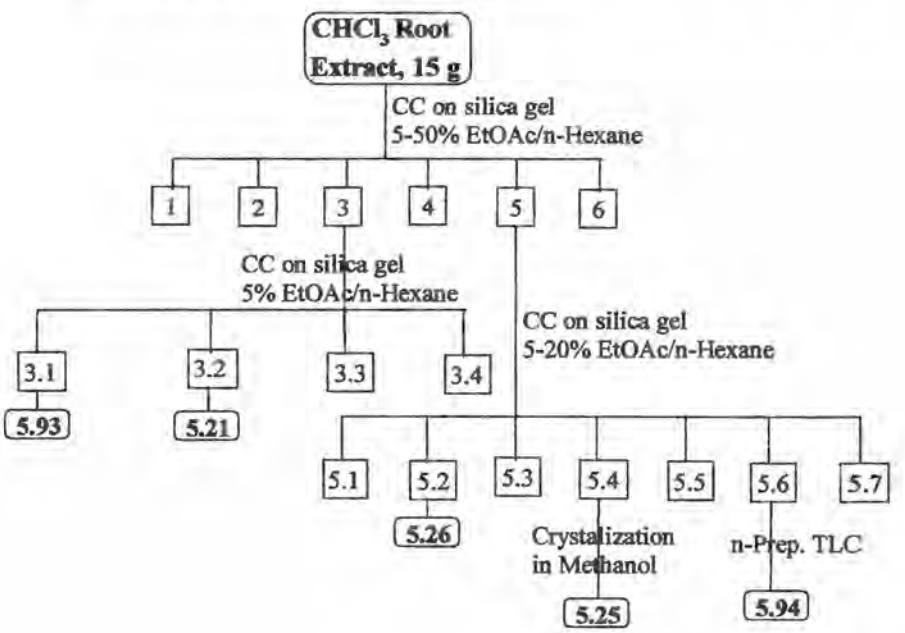
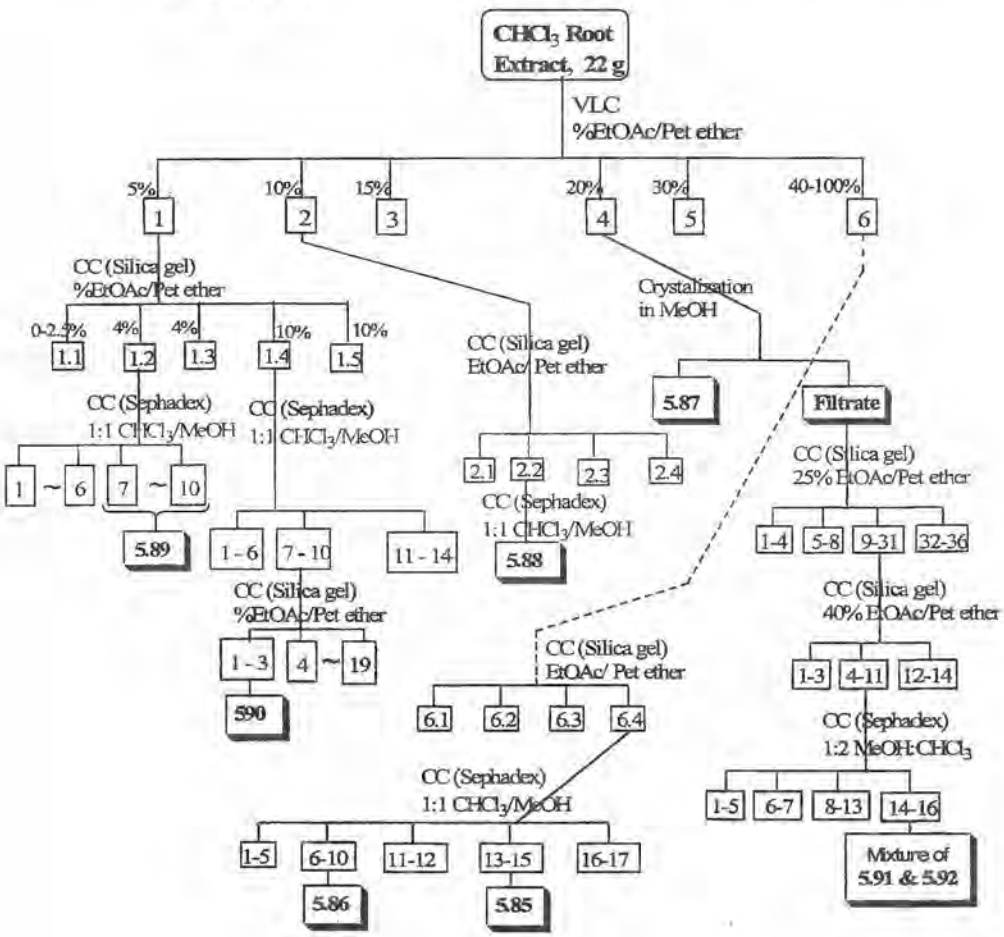


Chart 5.4 Isolation of Compounds 5.85-5.92 From the Root Bark of *Uvaria lungonyana*



***Tanganyikenol* (5.78)**. Gum; yield, 265 mg; anisaldehyde: dark green; MS, *m/z* (% rel. int.) 251 ($[M-1]^+$, 5), 236 (25) and 219 (53); ^1H and ^{13}C NMR: See Table 5.1.

***Polycarpol* (5.81)**. White crystals; yield, 775 mg; m.p 174-176 °C (Lit. m.p. 172-179 °C); anisaldehyde: light blue; MS, *m/z* (% rel. int.) 440 (M^+, 30), 407 (35), 327 (10), 273 (18), 227 (24), 197 (35), 171 (65), 157 (60), 119 (52), 105 (38), 69 (100) and 55 (38).

ent-Kaur-16-en-19-oic acid **(5.82)**. Needle like white crystals; yield, 235 mg; anisaldehyde: green; MS, *m/z* (% rel. int.) 301 ($[M+1]^+$, 100), 285 (35), 197 (90), 181 (15), 153 (3), 131 (5) and 103 (3); ^1H and ^{13}C NMR: See Table 5.2.

17-Acetoxy-ent-kauran-19-al **(5.83)**. Oil; yield, 196 mg; anisaldehyde: green; MS, *m/z* (% rel. int.) 346 (M^+, 9), 317 (75), 303 (13), 286 (55) and 257 (60); ^1H and ^{13}C NMR: See Table 5.2.

Melodorinol **(5.85)**. Oil; yield, 325 mg; anisaldehyde: green; $[\alpha]_D^{20} = +62°$ ($CHCl_3$, c = 0.1); MS, *m/z* (% rel. int.) 260 (M^+, 3), 243 (20), 230 (5), 138 (3), 105 (100), 77 (30) and 51 (15); ^1H and ^{13}C NMR: See Table 5.3.

Acetylmelodorinol **(5.86)**. Oil; yield 12 mg; anisaldehyde; green; MS, *m/z* (% rel. int.) 302 (M^+, 5), 243 (8), 180 (10), 138 (12), 105 (100), 77 (8) and 51 (3); ^1H and ^{13}C NMR: See Table 5.3.

Pinocembrin **(5.87)**. White crystals; m.p 188-190 °C (Lit.[195] m.p. 189-192 °C); yield, 7 mg; anisaldehyde: orange; MS, *m/z* (% rel. int.) 257 ($[M+1]^+$, 100), 179 (40), 152 (47), 124 (15), 104 (10) and 69 (8); ^1H and ^{13}C NMR: See Table 5.4.

5–Hydroxy–7–methoxyflavanone **(5.88)**. White crystals; m.p. 105-109 °C (Lit.[117] 108 °C); yield, 9 mg; anisaldehyde: pink; ^1H and ^{13}C NMR: See Table 5.4.

Benzyl benzoate **(5.89)**. Gum; yield, 10 mg; anisaldehyde: no reaction; MS, *m/z* (% rel. int.); 212 (M^+, 80), 105 (95), 91 (43), 77 (100) and 51 (33); ^1H and ^{13}C NMR: See Table 5.5.

2–Methoxybenzyl benzoate **(5.90)**. Gum; yield, 7 mg; anisaldehyde: red; MS, *m/z* (% rel. int.) 242 (M^+, 90), 137 (55), 121 (43), 105 (46), 91 (100) and 77 (50); ^1H and ^{13}C NMR: See Table 5.5.

A Mixture of Chamanetin **(5.91)** and ***Dichamanetin*** **(5.92)**. Brown gum; yield, 412 mg; anisaldehyde: Orange.

O-Methyleugenol **(5.21)**. Oil; yield, 1.5 g mg; anisaldehyde: orange; MS, *m/z* (% rel. int.) 178 (M^+, 100), 163 (23), 147 (10) and 135 (5); ^1H and ^{13}C NMR: See Table 5.6.

O-Methylisoeugenol **(5.93)**. Oil; yield, 1.3 g; anisaldehyde: green; MS, *m/z* (% rel. int.) 178 (M^+, 100), 163 (20), 151 (2), 135 (5) and 107 (7); ^1H and ^{13}C NMR: See Table 5.6.

2,3–Dimethoxycinnamaldehyde **(5.94)**. Gum; yield, 5 mg; anisaldehyde: dark green; MS, *m/z* (% rel. int.) 192 (M^+, 100), 177 (19) and 149 (7); ^1H and ^{13}C NMR: See Table 5.6.

CHAPTER SIX

MOSQUITOCIDAL AND ANTIMICROBIAL ACTIVITIES OF *NOR* – HALIMANOIDS, *ENT*-CLERODANOIDS, AND OTHER COMPOUNDS FROM THREE *TESSMANNIA* AND SOME ANNONACEAE SPECIES

Abstract

This Chapter reports on the mosquitocidal and antimicrobial activity of *nor*-halimanoids, *ent*-clerodanoids and other natural products isolated from stem and root bark extract of the Tanzanian *Tessmannia densiflora, T. martiniana* var *pauloi* and *T. martiniana* var *martiniana* (Leguminosae), and *Polyalthia tanganyikensis, Annona squamosa, Uvaria lungonyana and Uvariodendron pycnophyllum* (Annonaceae) against the larvae and adult *An. gambiae* mosquitoes, as well as against different Gram positive and Gram negative bacteria, and fungal species. The activity of the compounds was found to increase with long term exposure of an organism to the test sample. The activity of compounds with related structures such as tessmannic acid and methyltessmannoate, and *ent*-(18-Hydroxycarbonyl)-cleroda-3,13(*E*)-dien-15-oate, (9-*epi*)-*ent*-(18-Hydroxycarbonyl)-cleroda-3,13(*E*)-dien-15-oate, 2-oxo-*ent*-cleroda-3,13(*Z*)-dien-15-oic acid and *cis*-2-oxo-*ent*-cleroda-13(*Z*)-en-15-oic showed some reactivity trends. The insecticide Fendona® and mosquito repellent DEET, as well as the antimicrobial agents Gentamycin, Ampicillin, Clotrimazole and Fluconazole were used as standards.

6.1 INTRODUCTION

Insecticide and drug resistance is not a new phenomena (Chapters 1 and 2) as it has continued to be among the main factors that hinder efforts to effectively control the proliferation of malaria in various parts of the world. Other bottlenecks in this regard have been associated with limited access to essential resources (human, capital and equipment) and these affect conventional use of malaria control methods. Furthermore, environmental concerns and serious side effects caused by most antimalarial drugs have also continued to be recognised.[206] Likewise bottlenecks towards the control of microbial infections that to a large extent have been associated with drug resistance are a major concern in the health sector. As a matter of fact, drug resistance began to emerge not long after the discovery of antibiotics about 50 years ago. Thus, just four years after the start of mass-production of penicillium in 1943, it was noted that the drug that had become widely available during the Second World War and rapidly defeated the biggest wartime killer infected wounds began to be resisted by some microbial strains.[207] Hence, by 1995 plague infection failed to respond to the usual antibiotic treatements.[208]

On the other hand opportunistic fungal infections resistant to antifungal agents have increasingly been noted in recent years and their frequency will most likely continue to increase in the near future.[209] The situation has become even more complicated due to the emergence of the global human immunodeficiency virus (HIV) epidemic which has resulted in an increase in reports of drug resistant fungal infections. Fungal infections are common in severely immuno-compromised and/or critically ill patients, mostly HIV-infected patients. *Candida* spp, *Cryptococcus neoformans* and

Aspergillus spp are among the leading fungi responsible for such invasive infections.[210]

The above mentioned problems have therefore prompted an urgent need to search, develop and establish new insecticides, and antiparasitic and antimicrobial agents, particularly based on easily available plant resources, in order to deal with the insects and pathogens that are developing resistance to the presently available insecticides and drugs. This is the essence of evaluating the compounds isolated in these investigations for their mosquitocidal and antimicrobial properties. The investigated compounds were isolated from the Annonaceae species *Uvaria lungonyana, Uvariodendron pycnophyllum, Polyalthia tanganyikensis* and *Annona squamosa,* and the Leguminosae species *Tessmannia densiflora, T. martiniana* var *pauloi* and *T. martiniana* var *martiniana*. In these studies the larvicidal, mosquitocidal and insect repellent properties of the isolated compounds as well as their antibacterial and antifungal activities were determined following standard bioassay protocols, results from which are hereby being reported.

6.2 MATERIALS AND METHODS

6.2.1 Plant Materials and Isolation of the Test Compounds

The root and/or stem barks of *Tessmannia densiflora, T. martiniana* var *pauloi, T. martiniana* var *martiniana, Uvaria lungonyana, Uvariodendron pycnophyllum, Polyalthia tanganyikensis* and *Annona squamosa* were collected from Rufiji, Kisarawe, Bagamoyo and Muheza Districts in Coast and Tanga Regions, Tanzania. Detailed information about plant collection and isolation of the assayed compounds is described in Chapters 2, 3, 4 and 5.

6.2.2 Larvicidal Bioassay

This was conducted as described in Chapter 2 (Sections 2.2.3.1). In order to evaluate the larvicidal activity of the isolated compounds against *An. gambiae* mosquitoes, the compounds were subjected to larvicidal bioassays against *An. gambiae* larvae at different sample concentrations, *viz.* 0 (control), 7.5, 15.6, 31.2, 62.5, 125, 250 and 500 ppm after diluting with a stock solution made with dimethyl sulfoxide (DMSO). The prepared doses were triplicates in beakers with larvae food medium, each having 10 late 3rd and early 4th instar *An. gambiae* mosquito larvae. The mortality of the larvae were assessed after every 24 h, until the larvae pupated or 100 % larvae mortality was recorded. The recorded results in each experiment were analysed into their percentage mortality and LC_{50} values were obtained using Excel spreadsheet and POLO PLUS computer programs. The results are pooled in Tables 6.1-6.6 and in the histograms presented in Figs 6.1 – 6.4.

6.2.3 Mosquitocidal Assay by Tarsal Contact Bioassay

Anopheles gambiae s.s Giles adult mosquitoes were obtained from the laboratory colony (mosquito insectary) at the Amani Research Centre of the National Institute for Medical Research, in Muheza, Tanga region. For the bioassay for a given dose, a solution of the test sample was impregnated into three mosquito nets (1 m^2) that were placed in three cones ("treated sets"). Three other mosquito nets to which only solvent had been impregnated were used as controls. After the solvent had dried from the mosquito nets, five non-blood fed female *Anopheles gambiae* mosquitoes were placed into each of the cones through a hole. The mosquitoes were allowed to be in contact with the nets for 3 min, then transferred into paper cups and observed for 1 h.

after which a glucose solution was applied into each paper cup to sarve as food and the mosquitoes were observed after 24 h. The knockdown effect and mortality of the mosquitoes was recorded and the results were analysed graphically.

6.2.4 Mosquito Repellency Bioassays

The bioassays were carried out in a dark room with red light as the only source of illumination. The room temperature and humidity were artificially set using a heater and humidifier to mimic the host feeding conditions for the female *An. gambiae* mosquitoes (Temp. 27-35 °C and relative humidity > 65%). All the repellency tests were carried out on 5-7 days old mosquitoes that had been starved overnight, but previously fed on 6 % glucose solution.

Six human volunteers were used in the repellency bioassays. They were not allowed to use lotions, perfumes, oils or perfumed soaps on the day of the bioassay. Various test solutions of the compounds were prepared in acetone. The highest concentration was 1% (0.01 g/ml). Therefore, the bio-assayed doses were of 0.000015, 0.00015, 0.0015, 0.015 and 0.15 mg/cm^2. A test solution (0.5 ml) was dispensed on the right forearm of a volunteer from the wrist to the elbow. The rest of the hand was covered with glove to make it unattractive to the mosquitoes. Acetone (0.5 ml) was dispensed on the left forearm, to act as control. The arms were swapped regularly to eliminate any bias. The control arm was introduced into the cage immediately after releasing the 25 insects and kept there for 3 min. The mosquitoes that had landed on that arm during the test duration were recorded. The treated arm was then introduced into the cage and kept there for 3 min. The number of mosquitoes that landed on the treated arm was also recorded. The screening was done sequentially starting with the lowest

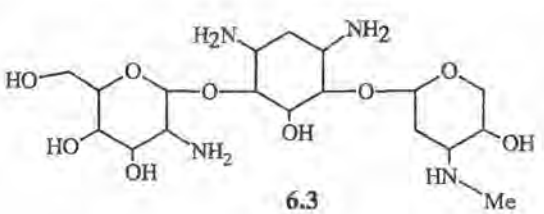
dose (0.001%) and ending with the highest one (1%). Each concentration was screened using a fresh batch of mosquitoes. After the bioassay of each concentration, the arms were washed with bar soap, rinsed well with tap water and then allowed to dry for 15-20 min, before application of the next dose of the test sample. The % protective efficacy (PE) was calculated as follows:

$$PE = \left(\frac{PCM - PTM}{PCM} \right) \times 100\%$$

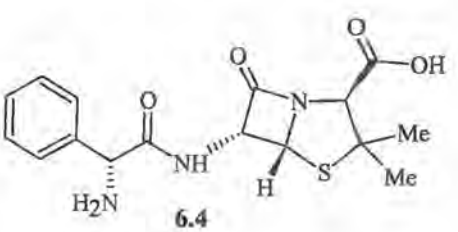
Where PCM is the percent control mean and PTM is the percent test mean of mosquitoes landing on the control and treated arms respectively. The results of the repellency bioassay of the pure compounds are summerised in Tables 6.8.

6.2.5 Antibacterial Assay

In order to evaluate the antimicrobial activity the isolated compounds were tested for antibacterial efficacy against Gram-positive and Gram-negative bacteria using Gentamycin (**6.3**) and Ampicilin (**6.4**) as the standards.



6.3



6.4

Seven bacteria species were used in this test, namely *Staphylococcus aureus* and *Bacillus anthracis* as Gram positive bacteria, while *Escherichia coli*, *Pseudomonas aeruginosa*, *Streptococus faecalis*, *Klebsiella pneumoniea* and *Salmonella typhimurium* were used as Gram negative bacteria. All organisms were obtained from the Department of Microbiology and Immunology at the then Muhimbili University College of Health Sciences (MUCHS) now a full fledged university known as

Muhimbili University of Health and Allied Sciences (MUHAS). The disc diffusion method was used to test for activity against the bacteria. In that bioassay sterilized Mueller Hinton agar was used and it was aseptically aliquoted at volumes of 25 ml into Petri dishes and left to congeal. The agar was then inoculated aseptically with test organisms by the streaking method. Eight discs (5 mm in diameter) were placed on the agar at approximately equidistant intervals using sterile forceps, six discs for the extract/VLC fraction/compound, one for the standard drug and one disc for the solvent (blank). The discs were first prepared by placing 10 µl of each of the solvents, extracts and standard drugs onto them and left to dry at 37 °C for 2 h. The plates were then incubated overnight at 37 °C. All extracts/compounds were dissolved in DMSO to make a concentration of 10 mg/ml. The zones of inhibition were measured in mm, the diameter being taken as a measure of antibacterial activity of the extract/compound. In each case a seeded plate to which neither extracts, pure compounds, standard drug nor solvent had been applied was also incubated under the same conditions. Each test was done in triplicate.

6.2.6 Bioassay Against Yeast – Like Fungi

The disc diffusion method was used and a *Candida albicans* strain which was obtained from the Department of Microbiology and Immunology, MUCHS was assayed as the representative organism. Sterilized Mueller Hinton agar was aseptically aliquoted at volumes of 25 ml to Petri dishes and left to congeal. The agar was then inoculated aseptically with the test organisms using the streaking method. Eight discs were placed on the agar at approximately equidistant intervals using sterile forceps, six discs for the extract/compound, one for the standard drug and one

disc for the solvent (blank). The discs were first prepared by adding 10 µl of the solvent (blank), and each of the extract/compound and standard drug onto the disc and left to dry at 37 °C for 2 h. The plates were then incubated overnight at 37 °C. All extracts/compounds were dissolved in DMSO to make a concentration of 10 mg/ml. The zones of inhibition were measured in mm and the diameter was taken as a measure of the degree of antifungal activity of the test extract/compound. In each case a seeded plate to which neither extract, pure compound, standard drug nor solvent had been applied was also incubated under the same conditions. Each test was done in triplicate.

6.2.7 Bioassay Against Filamentous Fungi

Isolates of *Aspergillus fumigatus* and *A. niger* were obtained from the Department of Microbiology and Immunology at MUCHS. The disc method was used for the antifungal tests. A standardized suspension of fungi (*A. niger* or *A. fumigatus* species, 5 ml) was prepared to make up a 0.5 MacFarland solution. Sterile Saboraud's dextrose agar (SDA) was poured into pre-sterilised Petri dishes and allowed to congeal. A colony-forming unit was sampled from an overnight culture and dispersed in 5 ml of the sterile distilled water by vortexing. A cotton swab was dipped in the suspension and after soaking it was squeezed on the sides of the tube to remove water. The swab was then streaked on the surface of congealed SDA. After congealing, the test discs were placed into the seeded agar using sterile forceps, 10 µl of plant extract/compounds were each placed on the disc. Eight discs were placed on the agar at approximately equidistant interval using a sterile forceps, six discs for the extract/compound, one for the standard drug and one disc for the solvent (blank). The discs were first prepared by adding 10 µl of solvents, each of the extracts/compound

and standard drug onto the disc and left to dry at 37 °C for 2 h. The agar plates were then incubated at 30 °C for 72 h. After incubation the inhibition zones were measured in mm. Discs soaked in solutions of extracts/pure compound and standard drug were dried, placed on unseeded Petri dishes and then incubated and these served as controls for the purpose of ensuring sterility. A blank (disc with no sample of standard solution) was used as another control to ensure the validity of the test. The test for each sample was done in triplicate.

6.3 RESULTS AND DISCUSSION

The *nor*-halimanoid diterpenes tessmannic acid (**3.1**) and methyltessmannoate (**3.2**) from *Tessmannia densiflora*, 18-oxocleroda-3,13(*E*)-dien-15-oic acid (**4.2**) and (9-*epi*)-*ent*-(18-hydroxycarbonyl)-cleroda-3,13(*E*)-dien-15-oate (**4.4**) from *Tessmannia martiniana* var *pauloi*, *ent*-(18-hydroxycarbonyl)-cleroda-3,13(*E*)-dien-15-oate (**4.3**), 2-oxo-*ent*-cleroda-3,13(*Z*)-dien-15-oic acid (**4.5**), *cis*-2-oxo-*ent*-cleroda-13(*Z*)-en-15-oic acid (**4.6**) and *O*-(3-hydroxy-4-hydroxycarbonyl-5-pentylphenyl)-3-chloro-4-methoxy-5-pentyl-2-oxybenzoic acid (**4.7**) from *Tessmannia martiniana* var *martiniana*, polycapol (**5.81**), mixture of chamanetin (**5.91**) and dichamanetin (**5.92**), and melodorinol (**5.85**) from *Uvaria lungonyana*, *O*-methyleugenol (**5.21**) and *O*-methylisoeugenol (**5.93**) from *Uvariodendron pycnophyllum*, the sesquiterpenoid tanganyikenol (**5.78**) from *Polyalthia tanganyikensis*, and *ent*-kaur-16-en-19-oic acid (**5.82**) and 17-acetoxy-*ent*-kauran-19-al (**5.83**) from *Annona squamosa*, were assayed for larvicidal, mosquitocidal, antibacterial and antifungal activity. Results are discussed in the following sections.

6.3.1 Larvicidal Activity of *nor*-Halimanoids from *Tessmannia densiflora*

Table 6.1 shows the larvicidal activity results for the *nor*-halimanoid diterpenoids tessmannic acid (**3.1**) and methyltessmannoate (**3.2**). The LC_{50} value for compound **3.1** was 93 ppm after 24 h exposure of the larvae, indicating strong activity. The results indicated that there was no significant difference between the activity of the crude chloroform extract of the root bark (LC_{50} = 162 ppm), refined fraction containing compound **3.1** as a major constituent (LC_{50} = 122 ppm) and the isolated compound **3.1**. However, the results indicated that there was a significant difference in activity between the two diterpenoids 3.1 and 3.2, since the latter compound exhibited an LC_{50} value of 244 ppm after 24 h of larvae exposure. Thus, the larvicidal activity of the acid **3.1** was nearly 3 times higher than that displayed by its methyl ester **3.2**. This activity trend can be explained based on enhanced reactivity generally expected for carboxylic acids as compared to their esterification derivatives.

Table 6.1 Larvicidal Activity of Tessmannic acid (3.1) and Methyltessmannoate (3.2) Against *An. gambiae* s.s Giles

Comp.	T (h)	Concentration (ppm)					LC50 (ppm)	95% CL
		31.5	62.5	125	250	500		
3.1	24	6.6 ± 3.3	36.6 ± 3.3	60 ± 0	76.6 ± 3.3	100 ± 0	93	57-140
	48	46.7± 3.3	66.7 ± 3.3	90 ± 0	100 ± 0	100 ± 0	34	08-54
	72	100 ± 0	100 ± 0	100 ± 0	100 ± 0	100 ± 0		
3.2	24	0 ± 0	10 ± 0	46.7 ±3.3	60 ± 0	80 ± 5.8	244	148– 397
	48	43.3 ±3.3	46.7 ± 3.3	80 ± 0	86.7 ± 3.3	100 ± 0	92	15 - 155
	72	80 ± 0	90 ± 0	100 ± 0	100 ± 0	100 ± 0	~ 4	

In order to display the larvicidal activity trend for the crude extract, VLC fractions and the isolated compounds **3.1** and **3.2**, within a long-time exposure (24 – 72 h

exposure) a histogram was constructed, as shown in Fig. 6.1. The histogram showed clearly the increase of activity with increased exposure time. After 72 h exposure compound **3.1** exhibited quite strong activity, displaying 100 % mortality at 31.5 ppm (Table 6.1), and therefore its LC_{50} value could not be determined, and hence it is not appearing in Fig. 6.1.

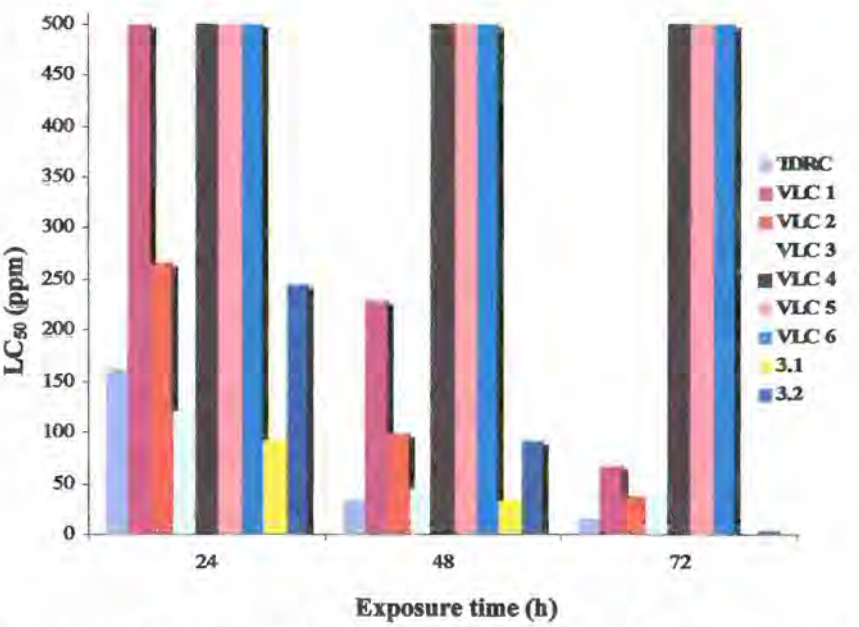


Fig. 6.1 Variation of Activity Between the Crude Extract from the Root Bark of *Tessmannia densiflora*, VLC Fractions and Their Constituents

6.3.2 Larvicidal Activity of *ent*-Clerodanoids from *Tessmannia martiniana* var *pauloi* and *Tessmannia martiniana* var *martiniana*

Table 6.2 shows the larvicidal activity of the *ent*-clerodanoids 18-oxocleroda-3,13(*E*)-dien-15-oic acid (**4.2**) and (9-*epi*)-*ent*-(18-hydroxycarbonyl)-cleroda-3,13(*E*)-dien-15-oate (**4.4**) obtained from VLC fraction 1 of the methanol extract from the root bark of *T. martiniana* var *pauloi*. The results indicated no significant difference

in activity between the two compounds after 24 – 72 h exposure. The activity was moderate after 24 h but it was very strong after prolonged time of exposure (48 and 72 h). Comparing the activity of the compounds, refined VLC fraction 1 and crude extract, the results indicated no significant difference in activity between the two compounds **4.2** and **4.4**, and the crude extract, while there was a significant difference of the activity between the compounds and the VLC fraction 1 from which the compounds were isolated. The activity of the VLC fraction 1 was found to be lower than that shown by the compounds and the crude extract.

It is difficult at this point to conclude that the activities of the two compounds **4.2** and **4.4** reflected the activity of the crude extract, or that the activity of the crude extract was due to compounds **4.2** and **4.4**. This is because apart from compounds **4.2** and **4.4**, VLC fraction 1 also yielded *cis*-kolavenolic acid (**4.1**) which was not assayed for larvicidal properties due to paucity of the isolated sample. In addition, other VLC fractions such as VLC fractions 3 and 4 decomposed on TLC analysis and they could not be analysed further for constituents. However, the extracts displayed very interesting IGR and larvicidal effects. It can therefore be speculated that whether in crude extract form, refined fractions or pure compounds, the root bark of *T. martiniana* var *pauloi* can be considered useful for mosquito larvae control, for which further work is recommended.

Table 6.2 Larvicidal Activity of 18-Oxocleroda-3,13(*E*)-dien-15-oic acid (4.2) and (9-*epi*)-*ent*-(18-Hydroxycarbonyl)-cleroda-3,13(*E*)-dien-15-oate (4.4) Against *An. gambiae* Larvae

Sample	T (h)	Concentration (ppm)				LC_{50} (ppm)	95% CL
		62.5	125	250	500		
4.2	24	33.3 ± 3.3	46.7 ± 3.3	80 ± 5.7	90 ± 5.7	125	57-204
	48	53.3 ± 8.8	63.3 ± 3.3	96.7 ± 3.3	96.7 ± 3.3	~ 57	
	72	70 ± 5.7	90 ± 5.7	100 ± 0	100 ± 0	~ 11	
4.4	24	13.3 ± 3.3	33.3 ± 3.3	73.3 ± 3.3	96.7 ± 3.3	177	116-269
	48	40 ± 5.7	60 ± 5.7	90 ± 5.7	100 ± 0	86	35-128
	72	80 ± 5.7	80 ± 5.7	100 ± 0	100 ± 0	~ 6	

Fig. 6.2 consists of histograms which show the trend of larvicidal activity for the crude extract, VLC fractions and compounds **4.2** and **4.4** after long time exposure (24 – 72 h). From the histogram the variation of the increase in activity with increased exposure time and comparability of the activity of the pure compounds and that of the crude extract can be observed.

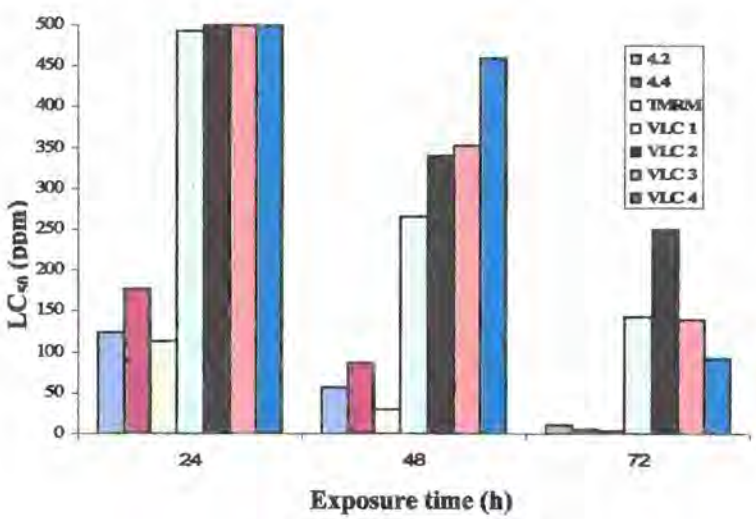


Fig. 6.2 Variation of Activity Between Crude extract from the Root Bark of *Tessmannia martiniana* var *pauloi*, VLC 1 and Compounds 4.2 and 4.4

Table 6.3 also shows the larvicidal activity of *ent*-clerodane diterpenes *ent*-(18-hydroxycarbonyl)-cleroda-3,13(*E*)-dien-15-oate (**4.3**), 2-oxo-*ent*-cleroda-3,13(*Z*)-

dien-15-oic acid (**4.5**) and *cis*-2-oxo-*ent*-cleroda-13(*Z*)-en-15-oic acid (**4.6**), and *O*-(3-hydroxy-4-hydroxycarbonyl-5-pentylphenyl)-3-chloro-4-methoxy-5-pentyl-2-oxybenzoic acid (**4.7**) from the chloroform extracts of the root and stem barks, respectively of *T. martiniana* var *martiniana*. The results showed significant difference in activity after 24 – 72 h exposure among all the three compounds **4.3**, **4.5-4.6** isolated from the root bark, with compound **4.3** being the most active (LC_{50} values of 48 and 19 ppm after 24 and 48 h exposure respectively). Compound **4.3** was also very active compared to the crude extract and VLC fractions 1 and 3 from which it was obtained, the crude extract displaying an activity corresponding to an LC_{50} value of 159 ppm after 24 h exposure, while VLC fractions 1 and 3 exhibited LC_{50} values of 493 and 254 ppm after 24 h exposure respectively. This implied that compound **4.3** was nearly three times more active than the crude extract after 24 h exposure, and ten and five times more active than VLC fractions 1 and 3 after 24 h exposure, respectively.

Compound **4.5** displayed an activity which was comparable to that of the crude extract and VLC fraction 3 from which it was obtained, while compound **4.6** was the least active among the isolated compounds, being even less active than the crude extract and VLC fractions 3 and 4 from which it was obtained. Compound **4.6** displayed an activity which was nearly four times less potent than that shown by the crude extract, and two times less active than the VLC fractions 3 and 4 after 24 h exposure, respectively.

The observed difference in activity of compounds **4.3**, **4.5-4.6** revealed structure-activity relationship of these compounds. The activity of the compounds could be

attributed to the decalin unit as well as the exocyclic side chain of the molecules. The observed high activity of the compound **4.3** and **4.5** could be attributed to the presence of an α, β-unsaturated carbonyl system which could be acting as a electrophile to the DNA bases of the test organism cells.[211] Furthemore, the high activity of compound **4.3** than **4.5** seems to indicate that the activity is also associated with the exocyclic C-18 unit, in this regard the activity of the C-18 carboxylic acid is expected to be higher compared to the C-18 methyl group, which was indeed the case.

Table 6.3 Larvicidal Activity (%) of *ent*-(18-Hydroxycarbonyl)-cleroda-3,13(*E*)-dien-15-oate (4.3), 2-Oxo-*ent*-cleroda-3,13(*Z*)-dien-15-oic acid (4.5), 2-Oxo-*ent*-cleroda-13(*Z*)-dien-15-oic acid (4.6) and *O*-(3-hydroxy-4-hydroxycarbonyl-5-pentylphenyl)-3-chloro-4-methoxy-5-pentyl-2-oxybenzoic acid (4.7) Against *An. gambiae* Larvae

Sample	T (h)	Concentration (ppm)						LC_{50} (ppm) 95% CL
		15.62	31.25	62.5	125	250	500	
4.3	24	16.7±3.3	30±5.7	50±0	83.3±3.3	100±0	100±0	48 (29-73)
	48	46.7±3.3	70±5.7	90±5.7	100±0	100±0	100±0	19 (7-29)
	72	83.3±3.3	86.7±3.3	100±0	100±0	100±0	100±0	~1
4.5	24			20±5.7	33.3±3.3	46.7±3.3	80±5.7	212 (114-520)
	48			50±5.7	70±0	73.3±3.3	100±0	~55
	72			80±5.7	100±0	100±0	100±0	~22.
4.6	24			10±1	16.7±6.7	20±5.7	36.7±3.3	~737
	48			16.7±8.8	33.3±8.8	36.7±12	66.7±3.3	~345
	72			20±5.7	43.3±12	36.7±12	80±5.7	256 (149-708)
4.7	24	16.7±3.3	50±0	53.3±3.3	60±5.7	70±5.7	100±0	62 (30-111)
	48	50±5.7	83.3±3.3	100±0	80±5.7	100±0	100±0	15 (2-26)
	72	60±5.7	100±0	100±0	100±0	100±0	100±0	~3

Fig. 6.3 shows histograms indicating the variation of the activity between the crude extract, VLC fractions and pure compounds **4.3, 4.5-4.6** after long time exposure (24 – 72 h). The bars show that the activity increased with time, indicating that the substances were acting slowly, which is very essential for avoidance of resistance development.

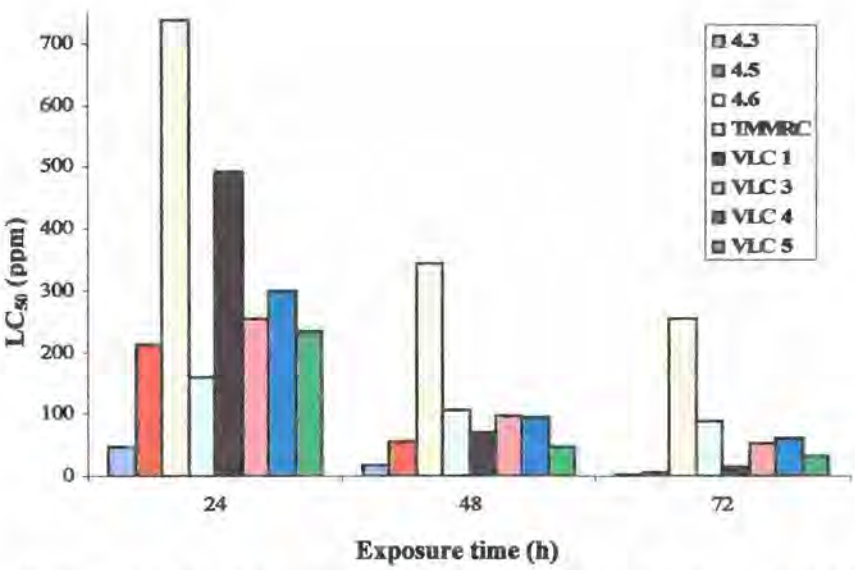


Fig. 6.3 Variation of Activity Between Compounds 4.3, 4.5-4.6, Crude and Semipurified VLC Fractions from the Root Bark of *Tessmannia martiniana* var *martiniana*

The results for the two diastereomers **4.3** and **4.4** (Table 6.2 and 6.3) obtained from *T. martiniana* var *martiniana* and *T. martiniana* var *pauloi* resepectively, showed that compound **4.3** was more active than **4.4**. Thus, Tables 6.2 and 6.3 show significant difference in activity between **4.3** and **4.4** after 24-72 h exposure time. Compound **4.3** showed LC_{50} values of 48, 19 and *ca.* 1 ppm after 24, 48 and 72 h respectively, while compound **4.4** displayed LC_{50} values of 177, 86 and *ca.* 6 ppm after 24, 48 and 72 h respectively. Such difference in activity trend is usually shown by isomeric compounds, such as **4.3** and **4.4**, whereby while one isomer would be

biologically active and the other one could either be weakly active or could be completely inactive.

Table 6.3 presents the larvicidal activity of *O*-(3-hydroxy-4-hydroxycarbonyl-5-pentylphenyl)-3-chloro-4-methoxy-5-pentyl-2-oxybenzoic acid (**4.7**), isolated from the chloroform extract of the stem bark of *T. martiniana* var *martiniana*. Compound **4.7** was the only interesting constituent isolated from that source, since the other compounds that were obtained were β-sitosterol and stigmasterol, both of which are common plant constituents. The results in Tables 2.2 (Chapter 2) and 6.3 indicate a significance difference between the activity of the crude extract and compound **4.7** (LC_{50} values of 256 and 62 ppm, respectively). However, no significance difference was observed between the activity of the crude extract and VLC fraction 4 (LC_{50} value = 330 ppm) from which compound **4.7** was obtained. Other VLC fractions (1-3) also displayed LC_{50} values which were similar to those shown by the crude extract and VLC fraction 4. It may therefore be concluded from these results that the activity of compound **4.7** was not displayed in the crude extract and VLC fraction 4 because of the minute abundance of the compound in the extract. The sample isolated from the crude extract constituted only 0.2 % yield.

The histograms in Fig. 6.4 display the activity trend of the crude extract, VLC fractions and compound **4.7**.

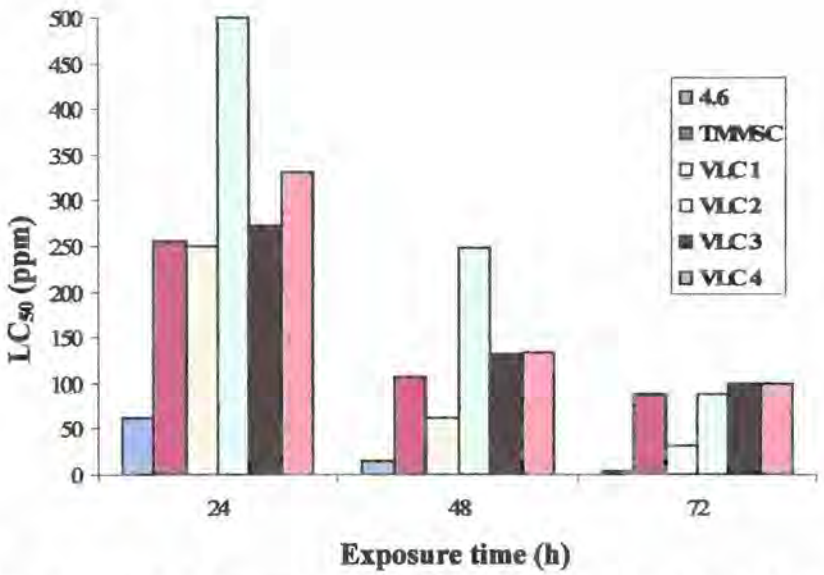


Fig. 6.4 Variation of Activity Between Compound 4.7, Crude and Semipurified VLC Fractions from the Stem Bark of *Tessmannia martiniana* var *martiniana*

6.3.3 Larvicidal Activity of Compounds from *U. lungonyana, U. pycnophyllum, P. tanganyikensis* and *A. squamosa*

The results in Table 6.4 show the larvicidal activity of polycarpol, a mixture of chamanetin and dichamanetin, and melodorinol, a mixture of chamanetin and dichamanetin being the most active compound (LC_{50} = 22 ppm) followed by melodorinol (LC_{50} = 80 ppm) while polycarpol had the least activity (LC_{50} = 393 ppm). It is not yet known if the activity of the mixture of compounds **5.91** and **5.92** is due to synergistic effects or due to one specific compound, since the compounds were obtained as a 1:1 mixture.

The results indicated no significant difference between the activity of melodorinol and the mixture of chamanetin and dichamanetin after 24 h exposure. Long term exposure showed significant increase in activity, as could be observed for LC_{50}

values after 48 and 72 h exposure being greatly enhanced (Table 6.4). The chloroform extract from the root bark of *U. lungonyana* from which the above compounds were obtained displayed an LC_{50} value of 93 ppm (Table 2.2) after 24 h exposure, this activity being comparable with that shown by melodorinol and the mixture of chamanetin and dichamanetin.

Comparison of the activities of the compounds, crude chloroform extract and semipurified VLC fractions from which the compounds were obtained showed that out of the six VLC fractions, it was only VLC fraction 6 which was the least active. However, melodorinol which was among the most active compounds from that source, was isolated from the least active VLC fraction 6. This could indicate that the efficacy of the active ingredient could have been masked by other more abundant inactive constituents.

The mixture of compounds **5.91** and **5.92,** together with polycarpol were obtained from the active VLC fraction 4, the postulated LC_{50} value for the VLC fraction 4 being below 62.5 ppm. This indicated that the VLC fraction could have contained more active compounds than those that were isolated. However, complex as it was, attempts to isolate compounds from this source proved futile. Indeed, the high larvicidal activity of VLC fraction 4 could be presumed to be a result of the combination of the compounds presented in the mixture. However, the activity could as well have been due to other unstable compounds that decomposed during the separation process. Although VLC fraction 1 (Table 2.4, Chapter 2) was the most active among the fractions from the crude extract, the compounds isolated from it could not be tested due to their paucity. However, benzylbenzoate and 2-methoxybenzylbenzoate that were obtained from that fraction have been reported

elsewhere to have insecticidal activity.[121] Therefore, these compounds could have been responsible for the high activity observed for fraction 1. Other compounds isolated from the active VLC fractions that were not tested due to insufficient amounts included acetylmelodorinol, pinocembrin and 5-hydroxy-7-methoxyflavanone.

Table 6.4 Larvicidal Activity (% mortality) of Polycapol (5.81), Mixture of Chamanetin (5.91) and Dichamanetin (5.92), and Melodorinol (5.85) Against *An. gambiae* Larvae

Sample	T	Concentration (ppm)						LC_{50} (ppm)
	(h)	15.62	31.25	62.5	125	250	500	95% CL
5.81	24			0 ± 0	10 ± 0	40 ± 0	50 ± 0	393 (263-1085)
	48			16.6±3.3	40 ± 0	66.6 ± 3.3	90 ± 0	150 (83-242)
5.91 & 5.92	24			50 ± 0	60 ± 5.7	90± 0	100 ± 0	122 (44-209)
	48			83.3±8.8	93.3±3.3	100 ± 0	100 ± 0	
	72			100 ± 0	100 ± 0	100 ± 0	100 ± 0	
5.85	24	6.7±3.3	10 ± 10	50 ± 0	60 ± 5.7			80 (52-191)
	48	20±5.7	26.7±3.3	66.7±3.3	93.3±3.3			21 (14-33)
	72	30 ± 5.7	33.3±6.5	20 ± 0	100 ± 0			16 (11-22)

Table 6.5 shows the larvicidal activity of the phenylpropenoids **5.21** and **5.93**, and stigmasterol, all of which were obtained from *Uvariodendron pycnophyllum*. The results indicated that there was no significant difference between the activity of *O*-methyleugenol, *O*-methylisoeugenol and stigmasterol after 24 h of larvae exposure. The LC_{50} values for the compounds after 24 h exposure were 43, 59 and 46 ppm for **5.21, 5.26** and **5.25**, respectively. It was further observed that there was no significant difference between the activity of the three compounds and that exhibited by the

crude chloroform extracts from the stem and root barks, as well as that of the methanol extract from the root bark (Tables 2.2 and Table 6.5). The LC_{50} value for the stem and root bark chloroform extract were both 56 ppm, and that for the methanol extract from the root bark was 56 ppm. Long term exposure of up to 120 h showed significant increase in larvicidal activity as it could be deduced from the LC_{50} value after 48 h larvae exposure for **5.26** and **5.25**, which were 17 and 22 ppm respectively, while the LC_{50} value for compound **5.21** could not be evaluated but indicative results showed more prominent activity, postulated to an LC_{50} value below 15.62 ppm. All the three compounds displayed complete larval mortality at the lowest dose tested after 72 h exposure.

Apart from **5.21** and **5.26**, another phenylpropenoid 2,3-dimethoxycinnamaldehyde was obtained from the crude extract but it was in very minute amount that was not enough for any bioassay.

Table 6.5 Larvicidal Activity for *O*-Methyleugenol (5.21), *O*-Methylisoeugenol (5.93) and Stigmasterol (4.25) Against *An. gambiae* Larvae

Sample	T (h)	Concentration (ppm)						LC_{50} (ppm)
		15.62	31.25	62.5	125	250	500	95% CL
5.93	24	23.3± 3.3	30 ± 5.7	36.7 ±8.8	70 ± 0	90 ± 0	100 ± 0	59 (34 – 95)
	48	53.3± 6.7	66.7 ± 3.3	93.3 ±3.3	100 ± 0	100 ± 0		17 (04 – 26)
	72	100 ± 0	100 ± 0	100 ± 0				
5.21	24	23.3 ±6.7	53.3 ± 8.8	53.3 ±6.7	76.7± 3.3	100 ± 0	100 ± 0	43 (23 – 69)
	48	80 ± 5.7	80 ± 5.7	100 ± 0	100 ± 0			
	72	100 ± 0	100 ± 0					
4.9	24	10 ± 5.7	16.7 ± 3.3	66.7 ±3.3	96.7 ±3.3	100 ± 0	100 ± 0	46 (31 – 66)
	48	30 ± 5.7	73.3 ± 13	90 ± 5.7	100 ± 0			22 (11 – 32)
	72	100 ± 0	100 ± 0	100 ± 0				

Table 6.6 shows mosquito larvicidal activity of tanganyikenol (**5.78**), a sesquiterpenoid isolated from the pet ether extract of the root bark of *P. tanganyikensis*. The compound displayed an LC_{50} value of 139 ppm, this activity not being significantly different from that observed for the crude pet ether extract, which displayed an LC_{50} value of 96 ppm. The chloroform extract was moderately active, while the methanol extract showed higher activity (LC_{50} of 70 ppm). However, the extract consisted of unstable compounds which decomposed during the isolation process. As the compound was isolated directly from the column chromatography of the crude extract, there were no fractions to be tested.

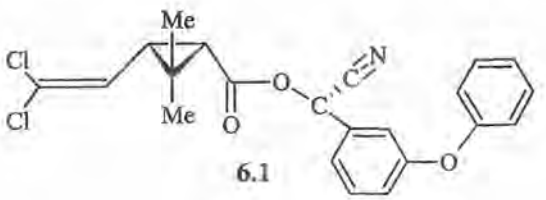
ent-Kaur-16-en-19-oic acid (**5.82**) and 17-acetoxy-*ent*-kauran-19-al (**5.83**) obtained from the chloroform extract of *Annona squamosa* root bark showed strong larvicidal activity (LC_{50} values of 61, and 20 ppm after 24 and 48 h exposure respectively, Table 6.6). 17-Acetoxy-*ent*-kauran-19-al displayed moderate activity (LC_{50} values of 173 and 130 ppm after 48 and 72 h exposure, respectively). The crude pet ether, chloroform and methanol extracts of *A. squamosa* displayed strong activity (LC_{50} values below 44 ppm after 24 h exposure). This showed that there could have been a synergistic effect between the compounds present in the crude extracts, most of which decomposed during the isolation process.

Table 6.6 Larvicidal Activity (% mortality) of Tanganyikenol (5.78), *ent*-Kaur-16-en-19-oic acid (5.82) and 17-Acetoxy-*ent*-kauran-19-al (5.83) Against *An. gambiae* Larvae

Sample	T	Concentration (ppm)						LC_{50} (ppm)
	(h)	15.62	31.25	62.5	125	250	500	95% CL
5.78	24	10 ± 0	20 ± 5.7	63.3 ±3.3	66.7 ±3.3			139 (81 – 318)
	48	23.3 ±3.3	50 ± 5.7	83.3 ±3.3	100 ± 0			68 (40 – 98)
	72	73.3 ±3.3	86.7±3.3	100 ± 0	100 ± 0			
5.82	24	0 ± 0	30 ± 5.7	50.7 ±5.7	70 ± 0	90 ± 0	100 ± 0	61 (29 – 95)
	48	43.3 ±6.7	73.3±3.3	80 ± 5.7	93.3 ±3.3	100 ± 0	100 ± 0	20 (05 – 33)
	72	100 ± 0	100 ± 0	100 ± 0	100 ± 0	100 ± 0	100 ± 0	
5.83	24			0 ± 0	0 ± 0	0 ± 0	0 ± 0	
	48			10 ± 0	20 ± 0	70 ± 0	100 ± 0	173 (123 – 247)
	72			20 ± 0	43.3 ±6.7	83.3 ± 3.3	100 ± 0	130 (84 – 189)

6.3.4 Mosquitocidal Activity

In order to evaluate the insecticidal properties of the isolated metabolites, compounds **3.1**, **3.2**, **4.3**, **4.4**, **5.21**, **5.93**, **5.81**, **5.91** and **5.92**, and **5.78**, female *An. gambiae* mosquitoes were exposed to the compounds following the Tarsal Contact Methods,[111] in which the test compounds at different concentrations (100, 125, 150, 175 and 200 mg/m^2) were impregnated onto bednet fabric. The knock down effect and mortality was then recorded as a percentage. Fendona® [15 SC an insecticide containing 15 g/l of *alpha*-cypermethrin (**6.1**)] was used as a standard for comparison purposes. Results are summarized in Table 6.4 and in the histograms presented as Figs 6.5-6.10.



6.1

Tables 6.7a and 6.7b show that all the compounds as well as the standard insecticide Fendona® were inactive against *An. gambiae* mosquitoes at all the tested concentrations after 3 min exposure of the mosquitoes to the test samples. Only after 1 h holding did the isolated compounds exhibit noticeable knock down effect at the highest concentration tested (200 mg/m^2). At the same time interval (1 h) the standard insecticide Fendona® showed strong activity even at the lowest concentration tested (100 mg/m^2), whereby the knock down effect was 80 %. At the highest concentration tested (200 mg/m^2) the insecticide exhibited complete knock down effect after 1 h holding. It is interesting to note that while mortality caused by the standard insecticide was decreasing with extension of holding time, that for the isolated compounds showed an opposite trend. Therefore, it would be interesting to follow up the mosquitocidal activity for much longer exposure time beyond 48 h so as to establish the optimum insecticidal viability time for these compounds. If the activity trend observed in these investigations with extended exposure time would persist, then the isolated compounds, and especially tessmannic acid, *O*-methyleugenol, *O*-methylisoeugenol, polycarpol and tanganyikenol would be interesting candidates for the development of insecticides for bednet impregnation. The results pooled in Tables 6.7a and 6.7b are amplified in the histograms in Figs. 6.5-6.10.

Table 6.7a Insecticidal Activity of Compounds from Some Tanzanian *Tessmannia* Species

Sample	Plant source	Time	% Mortality/Concentration (mg/m^2)				
			100	125	150	175	200
3.1	*T. densiflora*	3 min	0±0	0±0	0±0	0±0	0±0
		1 h	0±0	0±0	0±0	0±0	40±0
		24 h	0±0	0±0	0±0	20±0	20±11.5
		48 h	0±0	0±0	0±0	0±0	46.7±6.7
3.2	"	3 min	0±0	0±0	0±0	0±0	0±0
		1 h	0±0	0±0	0±0	0±0	6.6±3.3
		24 h	0±0	6.6±3.3	10±5.8	20±5.8	30±11.5
		48 h	0±0	30±0	30±5.8	30±5.8	40±0
4.4	*T. martiniana*	3 min	0±0	0±0	0±0	0±0	0±0
	var *pauloi*	1 h	0±0	0±0	0±0	10±0	20±0
		24 h	0±0	0±0	0±0	10±0	60±3.3
		48 h	0±0	0±0	0±0	60±0	60±5.8
4.3	*T. martiniana*	3 min	0±0	0±0	0±0	0±0	0±0
	var *martiniana*	1 h	0±0	0±0	0±0	20±0	10±0
		24 h	0±0	0±0	0±0	0±0	10±0
		48 h	0±0	0±0	0±0	0±0	10±0

Table 6.7b Insecticidal Activity of Compounds from Three Annonaceae Species

Sample	Plant source	Time	% Mortality/Concentration (mg/m^2)				
			100	125	150	175	200
5.81	*U. lungonyana*	3 min	0±0	0±0	0±0	0±0	0±0
		1 h	0±0	0±0	0±0	0±0	20±0
		24 h	0±0	0±0	0±0	20±0	40±11.6
		48 h	20±0	20±0	20±0	40±0	60±11.6
5.91 & 5.92	"	3 min	0±0	0±0	0±0	0±0	20±0
		1 h	0±0	0±0	0±0	0±0	20±5.8
		24 h	0±0	13.4±3.3	20±0	20±0	20±5.8
		48 h	0±0	20±0	40±11.6	40±11.6	60±0
5.21	*U. pycnophyllum*	3 min	0±0	0±0	0±0	0±0	20±0
		1 h	0±0	0±0	0±0	0±0	30±3.3
		24 h	0±0	10±3.3	20±0	30±0	40±5.8
		48 h	0±0	10±0	20±5.8	20±5.8	30±0
5.93	"	3 min	0±0	0±0	0±0	0±0	0±0
		1 h	0±0	0±0	0±0	0±0	10±0
		24 h	0±0	10±0	20±0	20±0	30±5.8
		48 h	10±0	40±0	40±0	40±0	50±0
5.78	*P. tanganyikensis*	3 min	0±0	0±0	0±0	0±0	0±0
		1 h	0±0	0±0	0±0	0±0	40±0
		24 h	0±0	0±0	0±0	0±0	40±0
		48 h	0±0	0±0	20±0	20±0	60±0
Fendona®		3 min	0±0	0±0	0±0	0±0	0±0
		1 h	80±0	80±11.5	80±11.5	100±0	100±0
		24 h	46.7±6.7	80±11.5	80±0	80±0	100±0
		48 h	30±0	66.7±6.7	80±0	80±0	80±0

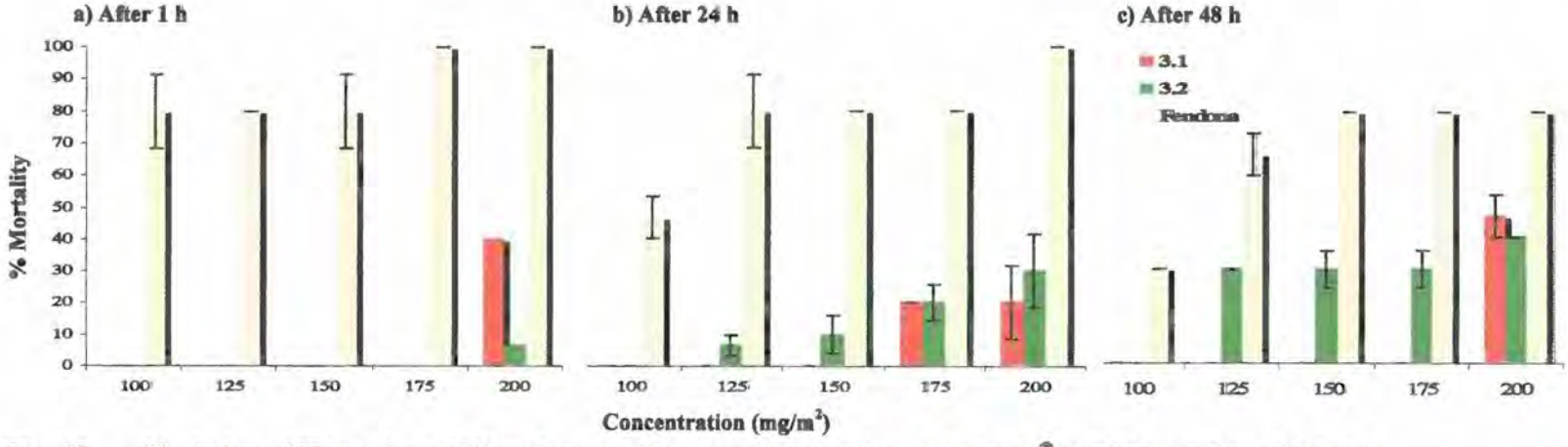


Fig 6.5 Variation of Knockdown Effect Between Compounds 3.1, 3.2 and Fendona® at Different Time Intervals

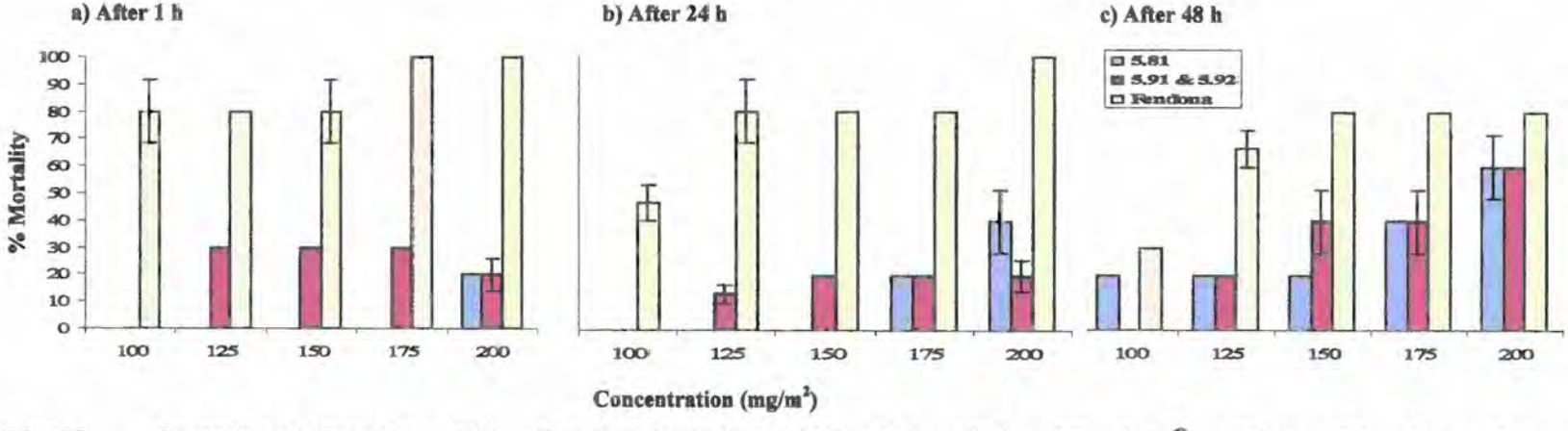


Fig 6.8 Variation of Knockdown Effect Between Compounds 5.81, 5.91 and 5.92 and Fendona® at Different Time Intervals

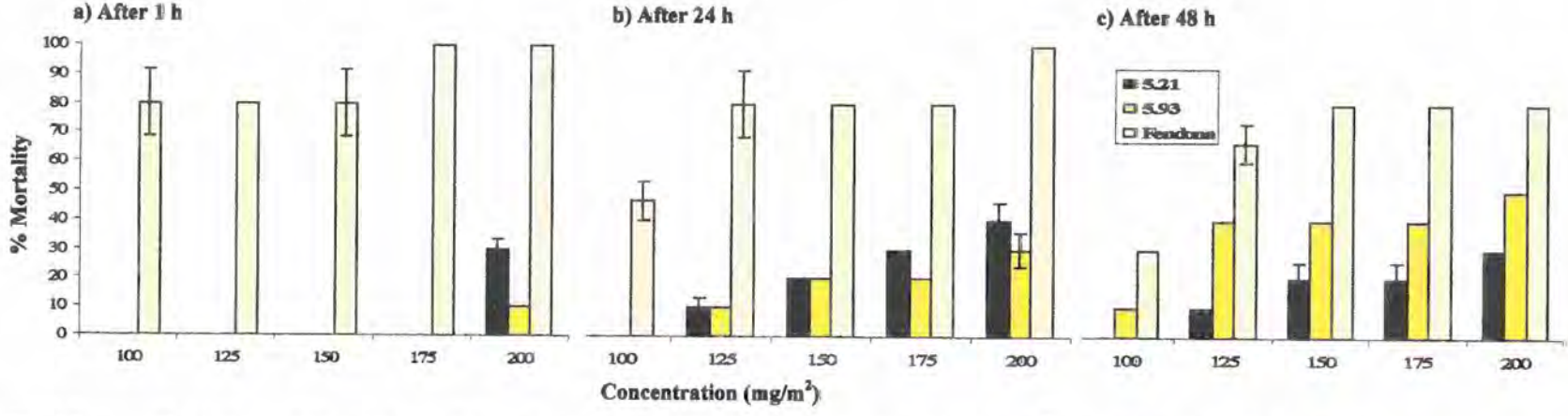


Fig 6.9 Variation of Knockdown Effect Between Compounds 5.21, 5.93 and Fendona® at Different Time Intervals

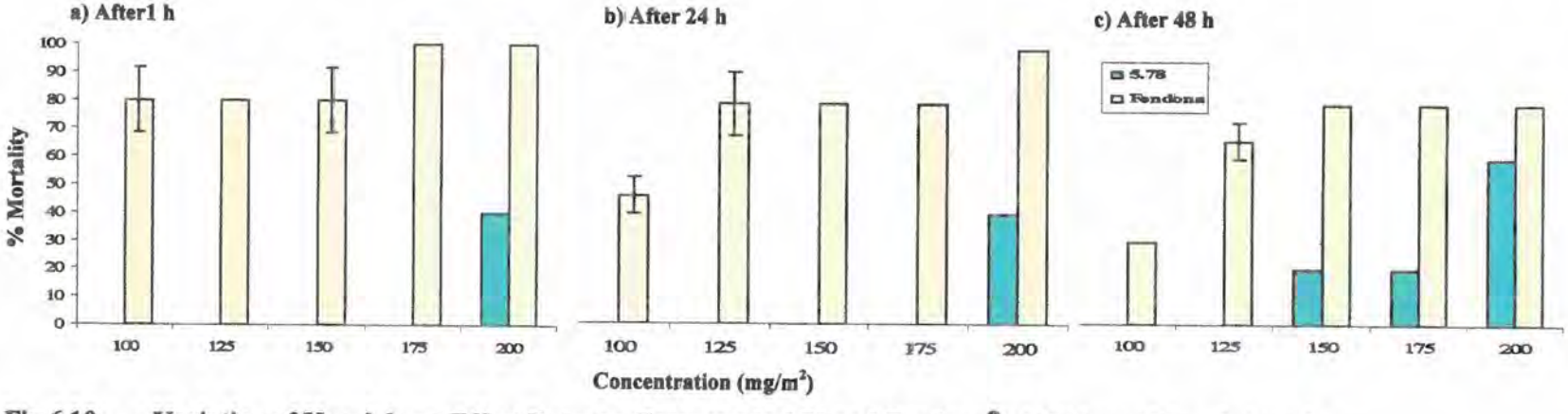
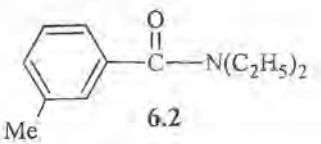


Fig 6.10 Variation of Knockdown Effect Between Compounds 5.78 and Fendona® at Different Time Intervals

6.3.5 Mosquito Repellency Activity

In order to evaluate the repellent properties of compounds **3.1, 3.2, 5.21, 5.26, 4.3** and **4.4**, the WHO procedure was followed (Section 6.2.4), DEET (**6.2**) being used as the standard repellent agent for comparison purposes. The recorded results in each experiment were analysed as percentage mortalities and RC_{50} values using the Excel Spreadsheet and POLO PLUS Computer programs. Results are summarized in Table 6.8 and in the histograms presented as Figs 6.12 – 6.15.



6.2

Table 6.8 shows the results of the repellency activity of the six compounds isolated from *Tessmannia densiflora* (**3.1** and **3.2**), *Tessmannia martiniana* var *pauloi* **4.4**, *Tessmannia martiniana* var *martiniana* **4.3** and *Uvariodendron pycnophyllum* **5.21** and **5.93**, indicating that tessmannic acid (**3.1**) was more active ($RC_{50} = 10^{-7}$ mg/ml) than the ester, methyltessmannoate (**3.2**). Comparing the activity with that for the standard repellent DEET, the compounds exhibited high repellent activity than DEET. The results are pooled in the graph shown in Fig. 6.12 for visual comparisons. The graph shows that the two *nor*-halimanoids **3.1** and **3.2** had good activity at lower dose than the standard repellent DEET. Compounds **3.1** and **3.2** attained an average repellency above 50 % at lower doses (10^{-5} to 10^{-4} mg/ml) while DEET exhibited an activity which was below 50 % at the same concentrations range. This, therefore, implied that these compounds could be good repellent agents even at lower concentration levels.

Table 6.8 also shows the repellency activity of compounds **4.3** and **4.4**. The compounds exhibited very low activity compared to DEET. The graph (Fig. 6.13) shows that the average activity of the two compounds fell below 50 % in all the doses used, with compound **4.4** being the least active metabolite. This activity, therefore, indicated that compound **4.3** and **4.4** are not suitable as a repellent agents compared to DEET.

The phenylpropenoids *O*-methyleugenol (**5.21**) and *O*-methylisoeugenol (**5.93**) were also evaluated for repellency activity as presented in Table 6.8 and Fig. 6.14. The graph in Fig. 6.14 shows that the activity trend of the two compounds was almost linear, indicating that an increase in concentration contributed to an almost the same increase in activity. Compound **5.21** exhibited very high repellency activity (RC_{50} = 5.84 x 10^{-6} mg/ml) much higher than that shown for **5.93** (RC_{50} = 1.58 x 10^{-4} mg/ml) and the two compounds had an activity higher than that displayed by the standard repellency DEET.

Table 6.8 Repellency Activity of the Isolated Compounds and DEET

Compound	Percentage Repellency/Concentration (mg/ml)					RC_{50} (mg/cm^2)
	10^{-5}	10^{-4}	10^{-3}	10^{-2}	10^{-1}	
3.1	64±1.7	67±1.2	73±0	80±0	88±0	1 x 10^{-7}
3.2	52±1.7	52±1.2	56±0	61±0	78±0	2.7 x 10^{-5}
4.4	0±0	25±1.2	28±1.7	31±0.6	40±0.6	1.26
4.3	0±0	40±0	51±15.1	46±12.8	52±3.3	1.4 x 10^{-2}
5.21	45±1.7	71±1.2	91±0	100±0	100±0	5.84 x 10^{-6}
5.93	25±1.2	46±0	74±0.6	85±0.6	92±0	1.58 x 10^{-4}
DEET	0±0	41±5.8	46±0	98±5.8	100±0	6.12 x 10^{-4}

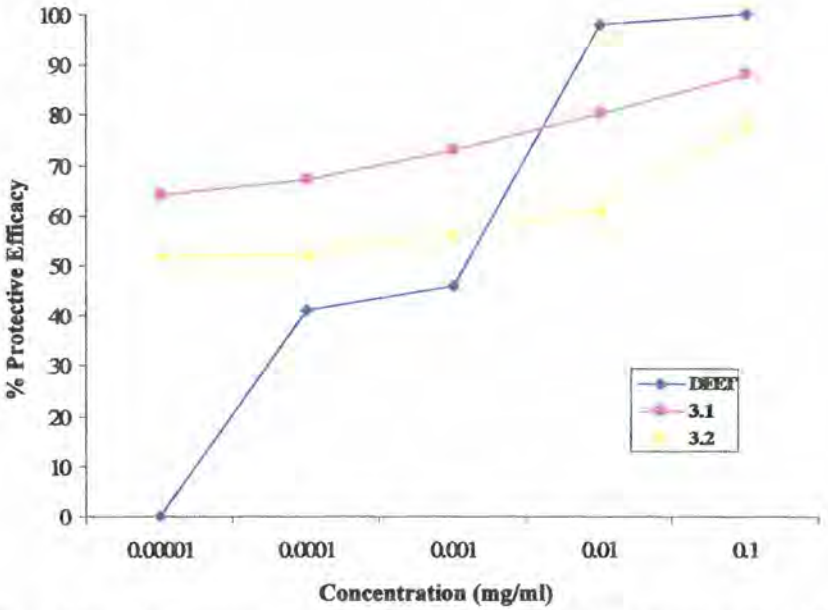


Fig. 6.12 **Percentage Variation of Mosquito Repellency Efficacy of Tessmannic acid (3.1), Methyltessmannoate(3.2) and DEET (6.2) Against *An. gambiae* Mosquitoes**

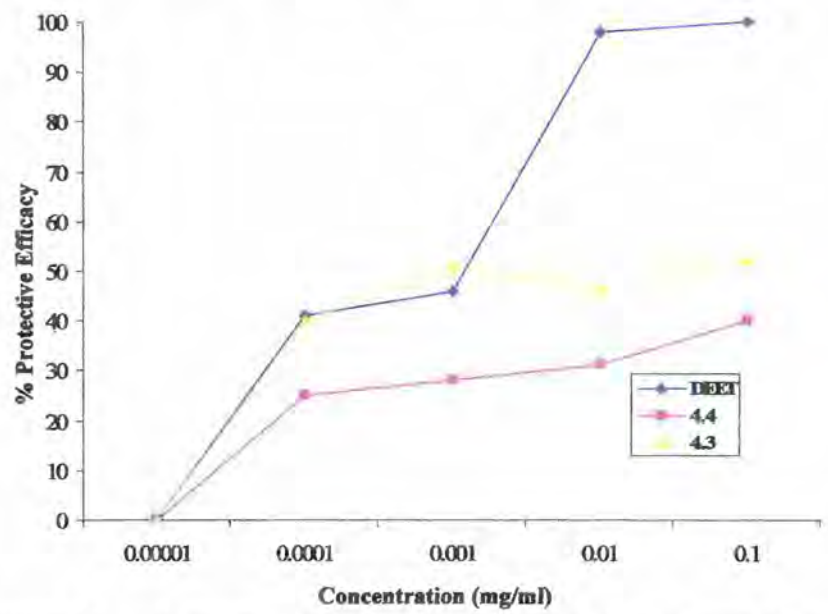


Fig. 6.13 **Percentage Variation of Mosquito Repellency Efficacy of *ent*-(18-Hydroxycarbonyl)-cleroda-3,13(*E*)-dien-15-oate (4.3), (9-*epi*)-*ent*-(18-Hydroxycarbonyl)-cleroda-3,13(*E*)-dien-15-oate (4.4) and DEET (6.2) Against *An. gambiae* Mosquitoes**

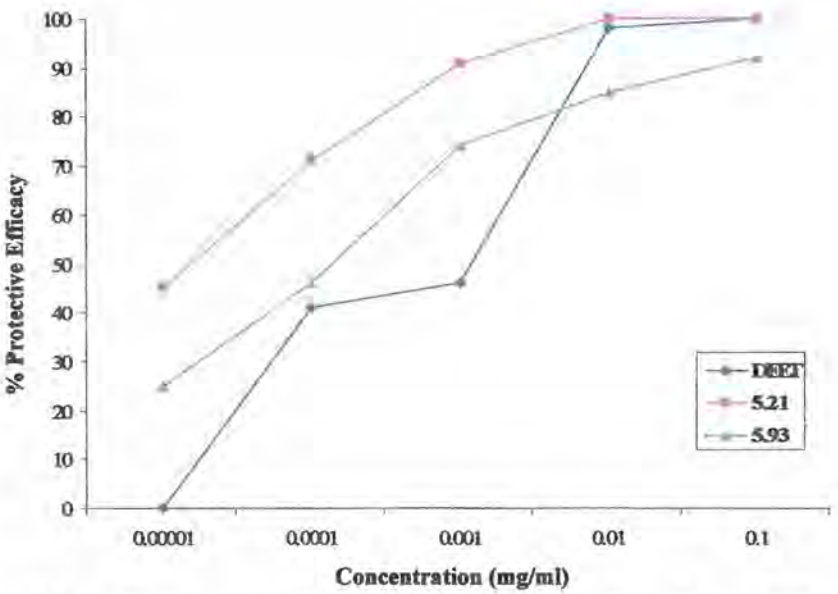


Fig. 6.14 **Percentage Variation of Mosquito Repellency Efficacy of *O*-Methyleugenol (5.21), *O*-Methylisoeugenol (5.93) and DEET (6.2) Against *An. gambiae* Mosquitoes**

6.3.6 Antibacterial and Antifungal Activity of *Tessmannia densiflora* Extract and Its Constituent Diterpenoid 3.1

The crude chloroform extract from the root bark of *Tessmannia densiflora* which exhibited mosquito larvicidal activity, its VLC fractions and compound **3.1** were also tested for antimicrobial activity. The results from the antibacterial assay are given in Table 6.9, and indicated that the crude extract exhibited some activity against the two Gram-positive bacteria species tested, the activity being much weaker than that shown by Gentamycin (**6.3**) which was used as the standard antibiotic. Against *B. anthracis* the extract exhibited the same activity as Ampicillin (**6.4**), which was also used as another standard antibiotic. For *S. aureus* the activity of the crude extract was weaker as compared to that displayed by the two standard antibiotics. As for insecticidal activity it was the medium polar VLC fractions that showed appreciable

activity. To a great extent this activity would have been due to the constituent diterpenoid **3.1**, which showed the same activity level as that exhibited by the most active VLC fractions, against both the G+ve bacteria species *S. aureus* and *B. anthracis*. It was interesting to note that against *S. aureus* tessmannic acid (**3.1**) showed the same level of activity as that observed for Gentamycin and that of Ampicillin against *B. anthracis*.

For G-ve bacterial strains only *E. coli, V. cholera* and *P. mirabilis* showed susceptibility to the crude extract. The medium polar VLC fractions again showed some activity against all the tested G-ve bacteria strains. Tessmannic acid (**3.1**) was shown to be an active constituent of these fractions since it was active at different levels against all the tested G-ve bacteria species, although that activity was generally weaker than that shown by the standard antibiotics Ampicillin and Gentamycin, except for *V. cholera* where compound **3.1** had the same level of activity as Ampicilllin. It was interesting to note that whereas compound **3.1** was appreciably active against *P. mirabilis*, the standard antibiotic Ampicillin was inactive against this G-ve bacteria strain.

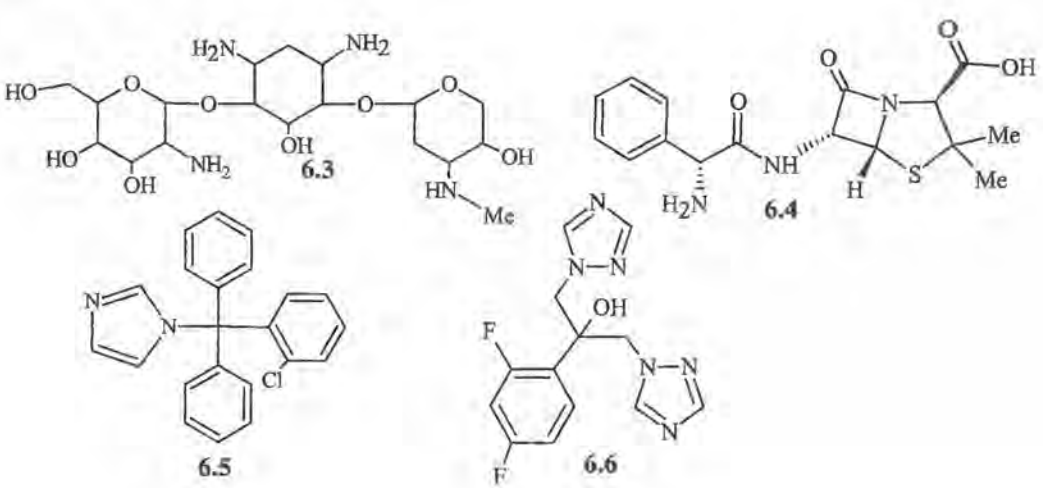


Table 6.9 **Anti-microbial Activity of *Tessmannia densiflora* Crude $CHCl_3$ Extract, VLC Fractions and Their Constituent Compound 3.1**

	Bacterial species									Fungal species				
Sample/VLC	1	2	3	4	5	6	7	8	9	I	II	III	IV	V
TDRC	++	-	-	wet	-	+	++	+	+	-	-	-	-	+
1	-	+	-	wet	-	-	-	+	-	-	-	-	-	-
2	+	+	+	wet	-	+	-	+	-	-	-	-	-	-
3	+	+	+	wet	-	++	++	+	+	-	-	-	-	+
4	+	+	+	wet	+	++	++	+	+	-	-	-	-	+
5	+	+	+	++	+	++	+	+	+	-	-	-	-	-
6	+	+	+	++	+	++	+	+	+	-	-	-	-	-
7	-	+	+	++	-	+	+	+	-	-	-	-	-	-
8	-	-	-	+	-	+	-	-	-	-	-	-	-	-
3.1	+	++	++	+	+	++	++	++	+	-	-	-	-	+
Ampicillin	+++	+++	off	wet	++	+++	++	-	+					
Gentamycin	+++	+++	+++	++	+++	++	+++	+++	+++					
										++	++	++	++	++
Clotrimazole										+	+	+	+	+

Key: 1 - *E. coli*, 2 - *S. typhi*, 3 - *S. dysenteriae*, 4 - *K. pneumoniae*, 5 - *P. aeruginosa*, 6 - *S. aureus*, 7 - *V. cholera*, 8 - *P. mirabilis*, 9 - *B. anthracis*, I - *C. albicans*, II - *C. neoformans*, III - *A. fumigatus*, IV - *A. niger*, V - *A. flavus*

- = < 6 mm no zone of inhibition, + = 6 – 10, ++ = 11 – 20, +++ = 21 – 30 mm

The crude extract, VLC fractions and tessmannic acid (**3.1**) were inactive against all the tested fungi (Table 6.10) except *A. flavus* which was mildly susceptible to the crude extract, some of the medium polar fractions and tessmannic acid (**3.1**), the activity being much weaker than that displayed by the standard antifungal agent Clotrimazole (**6.5**).

6.3.7 Antibacterial and Antifungal Activity of Constituents of *Tessmannia martiniana* var *pauloi* and *Tessmannia martiniana* var *martiniana*

Table 6.10 shows the antimicrobial results of 18-oxocleroda-3,13(*E*)-dien-15-oic acid (**4.2**) and (9-*epi*)-*ent*-(18-hydroxycarbonyl)-cleroda-3,13(*E*)-dien-15-oate (**4.4**) isolated from *T. martiniana* var *pauloi*, *ent*-(18-hydroxycarbonyl)-cleroda-3,13(*E*)-dien-15-oate (**4.3**) and *cis*-2-oxo-ent-cleroda-13(*Z*)-en-15-oic acid (**4.6**) from *T. martiniana* var *martiniana*. The results indicate that the compounds were active at different levels against both G+ve and G-ve bacteria as well as the tested fungal species, with compound **4.2** being the least active. Compound **4.2** was only active against the G+ve bacteria species *B. subtilis* and the filamentous fungus *A. niger*, the activity being lower than the standard antibiotic Ampicillin and standard antifungal agent Fluconazole (**6.6**) respectively.

Compound **4.6** was active against both the G-ve and G+ve bacteria *P. aeruginosa*, *S. aureus* and *B. subtilis*, the activity being lower than that observed for the standard antibiotics Ampicillin and Gentamycin. Howerver, compound **4.6** exhibited strong activity against the filamentous fungi *A. niger*, the activity being comparable with that observed for the standard antifungal agent Fluconazole. The diastereomers **4.3** and **4.4** displayed some differences in activity. While compound **4.4** was active against the three bacteria species *P. aeruginosa*, *S. aureus* and *B. subtilis*, its isomer **4.3** was not active against *S. aureus*. In addition to that the activity of compound **4.4** against *B. subtilis* was comparable to that of the standard antibiotic Ampicillin. Both the two isomeric compounds showed the same level of activity against the filamentous fungi *A. niger*.

Table 6.10 Antimicrobial Activity of Compounds from *T. martiniana* var *pauloi and T. martiniana* var *martiniana*

Compound	Bacterial species				Fungi species		
	1	2	3	4	I	II	III
4.2	-	-	-	+	-	-	+
4.6	-	+	+	+	-	-	++
4.4	-	+	+	++	-	-	+
4.3	-	+	-	+	-	-	+
Ampicillin			++	++			
Gentamycin	++	++					
Fluconazole					+++	++	++

Key: 1 - *E. coli*, 2 - *P. aeruginosa*, 3 - *S. aureus*, 4 - *B. subtilis*, I - *C. albicans*, II - *C. neoformans*, III - *A. niger*

\- =< 6 mm no zone of inhibition, + = 6 – 10, ++ = 11 – 20, +++ = 21 – 30 mm

6.3.8 Antibacterial and Antifungal Activity of Constituents of *U. lungonyana, U. pycnophyllum, P. tanganyikensis* and *A. squamosa*

Polycapol, a mixture of chamanetin and dichamanetin, and melodorinol obtained from *U. lungonyana*, *O*-methyleugenol and *O*-methylisoeugenol obtained from *U. pycnophyllum*, and tanganyikenol obtained from *P. tanganyikensis* were all tested for antibacterial and antifungal activity against *E. coli*, *P. aeruginosa*, *S. aureus* and *B. subtilis*, and *C. albicans*, *C. neoformans* and *A. niger* respectively. Results are given in Table 6.11, and they indicate that all the compounds were inactive against the G-bacterium *E. coli,* except that polycarpol and melodorinol exhibited strong activity against the G- bacterium *P. aeruginosa,* the activity being comparable with that observed for the standard drug Gentamycin. *O*-Methyleugenol and *O*-methylisoeugenol were also active against *P. aeruginosa* but the activity was somehow weaker compared to that shown by the standard antibiotic Gentamycin.

Against the G+ve bacterium *S. aureus*, *O*-methyleugenol and *O*-methylisoeugenol displayed weak activity compared to the standard antibiotic Ampicillin, while compound **5.78** showed an activity which was comparable to that observed for Ampicilin. Compound **5.93** as well as **5.81** and **5.85** further exhibited weak activity against the G+ve bacteria species *B. subtilis*, the activity being lower than that shown by the antiobiotic Ampicilin.

Compounds **5.93** and **5.78** also exhibited antifungal activity against *A. niger*, and compound **5.78** showed strong activity which was comparable to that observed for the standard antifungal agent Fluconazole. Polycarpol (**5.81**) and melodorinol (**5.85**) exhibited strong activity against the G+ve bacterium *S. aureus*, the activity being comparable to that observed for the standard antibiotic Gentamycin. The two compounds **5.81** and **5.85** also displayed antifungal activity against *A. niger*, with compound **5.85** showing an activity which was comparable to that observed for the standard antifungal agent Fluconazole. A mixture of chamanetin and dichamanetin was inactive against all G+ve and G-ve bacteria species tested, but exhibited strong antifungal activity that was comparable to the standard antifungal agent Fluconazole against *A. niger*.

6.4 Conclusion

The bioactivity results discussed in this Chapter indicated that some of the isolated compounds possess different levels of mosquitocidal and antimicrobial activities. An interesting observation was made regarding the increase in activity after long term exposure of the organisms to the test samples, as it was observed for larvae mortality and mosquitocidal activity in the tarsal contact assay. Such results would imply that

only minimal amount of the insecticide in a single application could stay for a reasonable period on the test site, and this would reduce multiple applications. One major advantage of delaying mortality is the difficulty for the insect strains to generate resistance against the test agents. It is also cost effective since only a small amount of the insecticidal agent would be used. Such long-acting insecticides are also environmentally advantageous, since their application would not involve large amounts that would be at alarming levels with respect to environmental pollution. After all, the studied substances being of botanical origin are easily biodegradable and hence posing no threat to the environment should they be developed as useful insecticides for the centre of malaria transmitting mosquitoes.

Table 6.11 Antimicrobial Activity of Compounds from *U. lungonyana, U. pycnophyllum, P. tanganyikensis* and *A. squamosa*

Compound	Bacterial species				Fungi species		
	1	2	3	4	5	6	7
5.81	-	++	-	+	-	-	+
5.85	-	++	-	+	-	-	++
5.91 & 5.92	-	-	-	-	-	-	++
5.21	-	+	+	-	-	-	-
5.93	-	+	+	+	-	-	+
5.78	-	-	++	-	-	-	++
Ampicillin			++	++			
Gentamycin	++	++					
Fluconazole					+++	++	++

Key: 1 - *E. coli*, 2 - *P. aeruginosa*, 3 - *S. aureus*, 4 - *B. subtilis*, 5 - *C. albicans*, 6 - *C. neoformans*, 7 - *A. niger*

- = < 6 mm no zone of inhibition, + = 6 – 10, ++ = 11 – 20, +++ = 21 – 30 mm

CHAPTER SEVEN

CONCLUSION AND RECOMMENDATIONS

The phytochemical investigations and biological assay results described in this Thesis were carried out following a synergistic research approach involving an interdisciplinary team of collaborators. This has facilitated achievement of the objectives of the research. Thus, bio-prospecting for mosquito larvicidal, mosquitocidal, mosquito repellent and antimicrobial constituents of different plant species that was undertaken yielded bioactive and other natural products, some of which were novel compounds. During these studies, some extracts from the leaves, stem and root barks of sixteen Tanzanian plant species, namely *Annona squamosa, Asteranthe asterias, Croton sylvaticus, Hoslundia opposita, Lettowianthus stellatus, Polyalthia tanganyikensis, Tessmannia densiflora, T. martiniana* var *pauloi, T. martiniana* var *martiniana, Uvaria lungonyana, U. scheffleri, U. faulknerae, U. leptocladon, U. kirkii, Uvariodendron usambaranse,* and *U. pycnophyllum* showed activity against *An. gambiae* mosquito larvae. The investigations formed part of long term phytochemical studies to search for botanical insecticides for the control of malaria transmitting mosquitoes, which would be affordable to the local community and environmentally friendly, being carried out in the Department of Chemistry at the University of Dar es Salaam, in collaboration with the International Centre of Insect Physiology and Ecology (ICIPE) in Nairobi, Kenya and the Tanzanian National Institute for Medical Research (NIMR). Collaboration with the Institute of Traditional Medicine at MUCHS was also pursued, in establishing antimicrobial activity of the compounds, as reported in this Thesis. Some of the extracts from *Annona squamosa, Polyalthia tanganyikensis, Tessmannia densiflora, T. martiniana*

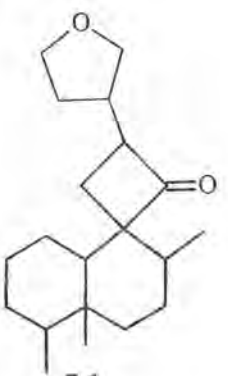
var *pauloi*, *T. martiniana* var *martiniana*, *Uvaria lungonyana*, and *Uvariodendron pycnophyllum* were further analysed in order to isolate the active constituents. Thus, 33 compounds were obtained and their chemical structures characterized upon analysis of spectroscopic properties. The compounds consisted of ten novel metabolites, most of which were *nor*-halimanoid and *ent*-clerodanoid diterpenes, and isocoumarins, while the rest of the compounds were known natural products. The novel compounds include the *nor*-halimanoids tessmannic acid (**3.1**), methyltessmannoate (**3.2**), 2-methylpropyltessmannoate (**3.12**) and 1-methylbutyltessmannoate (**3.13**), the new clerodane diterpenoid *cis*-kolavenolic acid (**4.1**), *ent*-(18-hydroxycarbonyl)-cleroda-3,13(*E*)-dien-15-oate (**4.3**) and (9-*epi*)-*ent*-(18-hydroxycarbonyl)-cleroda-3,13(*E*)-dien-15-oate (**4.4**), and the isocoumarin chlotessmin (**3.15**) and other new metabolites 5-pentyl-3-methoxy-*N*-butylaniline (**3.16**), *O*-(3-hydroxy-4-hydroxycarbonyl-5-pentylphenyl)-3-chloro-4-methoxy-5-pentyl-2-oxybenzoic acid (**4.7**), the aromadendrane sesquiterpenoid tanganyikenol (**5.78**) and 17-acetoxy-*ent*-kauran-19-al (**5.83**). The bioassay results for the crude extracts as well as the isolation and structural determination of all the natural products obtained in these studies are discussed in Chapters 2-5, while results from the bioassays of the isolated compounds are presented in Chapter 6.

So far, the three *Tessmannia* species analysed in these investigations are reported to occur only in Tanzania and neither phytochemical nor biological investigations have been carried out on these plant species. The root and stem barks of the apparently endangered plant species (*T. martiniana* var *pauloi*) that has been depleted in Pugu Forest near Dar es Salaam due to charcoal production, yielded a series of *ent*-clerodanoid diterpenes, including 18-oxocleroda-3,13(*E*)-dien-15-oic acid (**4.2**), the

new clerodanoid *cis*-kolavenolic acid (**4.1**) and (9-*epi*)-*ent*-(18-hydroxycarbonyl)-cleroda-3,13(*E*)-dien-15-oate (**4.4**). *T. densiflora* yielded a series of *nor*-halimanoids and isocoumarins, while *T. martiniana* var *martiniana* also yielded a series of *ent*-clerodanoid diterpenes. Compounds from the former species were tessmannic acid (**3.1**), methyltessmannoate (**3.2**), 2-methylpropyltessmannoate (**3.12**), 1-methylbutyltessmannoate (**3.13**), 8-hydroxy-6-methoxy-3-pentylisocoumarin (**3.14**), chlotessmin (**3.15**) and 5-pentyl-3-methoxy-*N*-butylaniline (**3.16**). Those from the latter plant species were *ent*-(18-hydroxycarbonyl)-cleroda-3,13(*E*)-dien-15-oate (**4.3**), 2-oxo-*ent*-cleroda-3,13(*Z*)-dien-15-oic acid (**4.5**), *cis*-2-oxo-*ent*-cleroda-13(*Z*)-dien-15-oic acid (**4.6**) and *O*-(3-hydroxy-4-hydroxycarbonyl-5-pentylphenyl)-3-chloro-4-methoxy-5-pentyl-2-oxybenzoic acid (**4.7**). The *ent*-clerodanoids and *nor*-halimanoids are related diterpenoids whose biosynthetic relationship would be conceived to involve cyclization of the side chain alkanoyl unit and the exocyclic propenyl moiety in the halimanoids, followed by methyl migration and then opening of the furanoid ring.

Although the three *Tessmannia* species are not used in traditional medicine, the larvicidal and antimicrobial activity shown by the extracts and the type of compounds isolated (*nor*-halimanoids and *ent*-clerodanoids) revealed the potential of this genus as a source of natural products that could influence the behaviour, development, reproduction and fitness of insects in various ways. Most of the diterpenes such as those reported in this Thesis have been reported to possess a number of bioactivities, including insecticidal, antitrypanosomal and antifeedant activity. Of particular interest among the biological activities that were observed in these investigations when mosquito larvae were exposed to the methanol extract of *T. martiniana* var

pauloi is larvae developing deformities by forming tail like-structures. The compound or compounds responsible for the observed deformities was not obtained, as the particular chromatographic fraction that exhibited the activity when further worked up only led to decomposition. However, these larval deformities could be envisioned to have been caused by some of the clerodane diterpenes occurring in the plant species, such as the ajugarins like **7.1**, that have been reported in several other studies to exhibit such biological activity.[212]



7.1

Furthermore, the series of compounds isolated from *T. martiniana* var *martiniana*, namely *ent*-(18-hydroxycarbonyl)-cleroda-3,13(*E*)-dien-15-oate (**4.3**), 2-oxo-*ent*-cleroda-3,13(*Z*)-dien-15-oic acid (**4.5**) and *cis*-2-oxo-*ent*-cleroda-13(*Z*)-dien-15-oic acid (**4.6**) that exhibited larvicidal and antimicrobial activity, tend to suggest that the active site in these compounds could have been the α, β-unsaturated carbonyl moiety, as previously reported in other studies, thereby indicating the importance of α, β-unsaturated carbonyl groups as bioactive sites in natural products. However, other diterpenoids as well as the isocoumarins from *T. densiflora* and *T. martiniana* var *pauloi* were not tested for bioactivity due to the paucity of the isolated samples.

Hence, at this stage the structure activity relationship for all the isolated compounds may not be conclusively stated.

Chemical investigations of the pet ether extract of the root bark of *Polyalthia tanganyikensis* yielded a new aromadendrane sesquiterpenoid that has been named tanganyikenol (**5.78**) whose isolation and structural determination is described in Chapter 5 of this Thesis. In addition to this compound polycarpol (**5.81**) which seems to be exclusively found in the family Annonaceae was also obtained. The compounds exhibited mosquitocidal activity against *An. gambiae* larvae and adult mosquitoes. The mosquitocidal activity of polycarpol (5.81) would suggest that the compound, just as for its antitumour efficacy, acts through interference with oxidative phosphorylation in insect cells by binding to complex I of the mitochondrial electron transport system, as previously reported for annonaceous acetogenins.

A new *ent*-kaurane diterpenoids, 17-acetoxy-*ent*-kauran-19-al (**5.83**), and a known compound *ent*-kaur-en-19-oic acid (**5.82**) were obtained from the chloroform extract of the root bark of *Annona squamosa* that showed activity and the isolated compounds exhibited larvicidal activity against *An. gambiae* mosquito larvae. Although *A. squamosa* is known to be a rich source of annonaceous acetogenins, in these investigations such acetogenins were not found, probably this being a result of either seasonal of geographical variation in the metabolism of these compounds by the investigated plant species.

Until now *Uvaria lungonyana* and *Uvariodendron pycnophyllum* are reported to be found only in Tanzania, the former at Lungonya valley within the Selous Game Reserve in Rufiji district and the latter at Kisiwani village, within the Amani Nature

Reserve in Muheza district. Chemical investigations of *Uvaria lungonyana* and *Uvariodendron pycnophyllum* yielded the usual compounds reported to occur in the genera *Uvaria* and *Uvariodendron*, these being flavonoids in the former and phenylalkenes (eugenol derivatives) in the latter plant species respectively. However, *Uvaria lungonyana* also yielded the heptanolides melodorinol (**5.85**) and acetylmelodorinol (**5.86**). These findings are interesting because the occurrence of the heptanolides has never been reported from the genus *Uvaria*, and as such the phytochemical results tend to indicate a (chemo) taxonomical relationship between the genera *Sphaerocoryne*, *Cleistochlamys* and some species of the genus *Uvaria*.

Apparently, it has been established in these studies that the occurrence of *Uvaria faulkenerae* in Tanzania is not restricted to Pangani District, Tanga Region, as it was previously reported, but the distribution of the plant also extends to Handeni and in Kimboza forest reserve in Morogoro District, respectively.

The bioassay results in Chapter 2 indicate that some of the crude extracts exhibited good larvicidal activity, and this was the basis for choosing the extracts for further analysis in order to isolate the constituent compounds, some of which were expected to be responsible for the observed activity. The biological activities of the crude extracts from the sixteen plant species as discussed in Chapter 2 have given clues on the insecticidal potency of the traditional use of the plant species in the crude form. The high activity shown by some extracts conforms to the suggestion that plants never defend themselves against insects with a mono-component system. Normally, for this purpose plants are said to use numerous constituent compounds, whether biosynthetically related or unrelated. Individual plant species are known to produce a variety of biosynthetically distinct metabolites and usually insects would perceive

and react to these compounds as mixtures rather than as individual compounds. This could also be supported by some other evidence that natural mixtures do act synergistically. However, the high bioactivities shown by some pure compounds as discussed in Chapter 6 also tend to suggest that the natural products were in fact the active components in the crude extracts. However, where the crude extracts were less active than the pure compounds, such phenomenon could be explained by assuming that the activity of the pure compounds in the crude mixtures was masked by the other, less active or completely inactive minor constituents. These studies have therefore considered these findings as challenges which have to be clarified when considering using a particular plant in crude form or as semi purified fractions.

Generally, the bioactivity trends in plant extracts versus the constituent natural products is a subject of many phenomena. Thus, apart from the masking effects discussed above the activity and hence the presence of the active constituents would also be determined by the plant part investigated, the nature of the test organism, the method and time of collection of plant materials, the method of extraction and the solvent used, and geographic location of the plant. In this regard, therefore, proper screening methods are of paramount importance in obtaining promising insecticides from plants.

As it has been observed before, Chapter two indicates that plant ecotypes in different geographical regions may differ in the accumulation of their active ingredients. For instance the extracts of the plant materials of *H. opposita* collected around the University of Dar es Salaam Main Campus were strongly active against *An. gambiae* mosquito larvae, while the extracts from the same plant species collected from Kwamngumi forest reserve in Muheza district were not active. Furthermore, different

collection seasons may also yield different constituents, as it could be deduced from the fact that plant materials of *T. densiflora* collected in January 2004 from Kichi Hills in Rufiji district, had the chloroform extract constituting only two major compounds, tessmannic acid (**3.1**) and methyltessmannoate (**3.2**), while extracts from the same plant species collected from the same locality in April 2007 were found to constitute different compounds, namely 2-methylpropyltessmannoate (**3.12**), 1-methylbutyltessmannoate (**3.13**), 8-hydroxy-6-methoxy-3-pentylisocoumarin (**3.14**), chlotessmin (**3.15**), 5-pentyl-3-methoxy-*N*-butylaniline (**3.16**), lupeol (**3.17**) and heptacosanoic acid (**3.18**). Therefore, in bioprospecting initiatives for active plant constituents, it is important to take the seasonal variations of the constituents into consideration in order to maximize the yield of such compounds at the time when they are metabolized in abundance. Consideration must also be given to the fact that most active principles in plants would show low stability, and as such they are usually lost during the extraction and purification process. Therefore, again this factor needs to be recognised when embarking onto bioprospecting ventures for bioactive compounds.

In general, plant species of the same or closely related genera would contain the same or similar active principles. Consequently, a screening system which was successful with one species should also be attempted with closely related species. This applied, for instance, for the genus *Tessmannia*, which in these investigations was represented by three species, namely *T. densiflora*, *T. martiniana* var *pauloi* and *T. martiniana* var *martiniana*. The three *Tessmannia* species were found to be a rich source of diterpenoids, especially *nor*-halimanoid and *ent*-clerodanoid diterpene. As such it

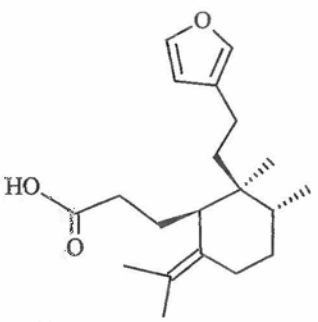
proved useful to invoke the same separation techniques for compounds from each of the three species.

Several other major challenges were encountered when conducting these studies, that included the isolation of small amounts of pure compounds which were not sufficient to conduct bioassay tests. Such compounds included some of the natural products that had novel chemical structures, like 2-methylpropyltessmannoate (**3.12**), 1-methylbutyltessmannoate (**3.13**), 8-hydroxy-6-methoxy-3-pentylisocoumarin (**3.14**), 7-chloro-8-hydroxy-6-methoxy-3-pentylisocoumarin (**3.15**), 5-pentyl-3-methoxy-*N*-butylaniline (**3.15**), *cis*-kolavenolic acid (**4.1**) and 18-oxocleroda-3,13(*E*)-dien-15-oic acid (**4.5**). Likewise, limits in the availability of essential facilities hampered the ability to carry out spectroscopic experiments such as one- and two-dimensional NMR spectroscopy, and MS and X-ray diffraction analysis for structural elucidation and stereochemical and conformational assignments, particularly of enantiomeric structures such as *ent*-(18-hydroxycarbonyl)-cleroda-3,13(*E*)-dien-15-oate (**4.3**) and (9-*epi*)-*ent*-(18-hydroxycarbonyl)-cleroda-3,13(*E*)-dien-15-oate (**4.4**).

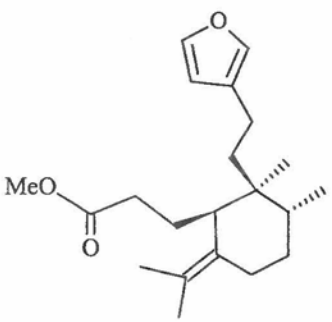
In summary, as investigations in natural products research continue to develop and proceed towards the discovery of novel natural products with pesticidal or other activities, future studies should aim at examining and evaluating the influence of the bioactive compounds on a variety of biological screens and improve strategies of evaluating extracts, re-collect, re-isolate and/or synthesize authentic samples of natural product(s) and their derivatives to afford gram quantities for follow-up bioassay screens so as to update the available chemical and biological data.

COMPOUNDS ISOLATED IN THESE INVESTIGATIONS

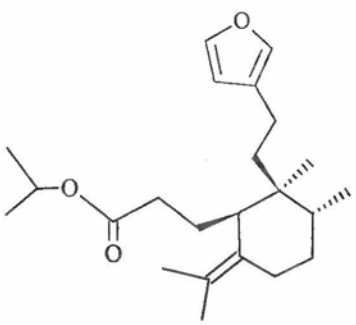
A. COMPOUNDS FROM *TESSMANNIA DENSIFLORA*



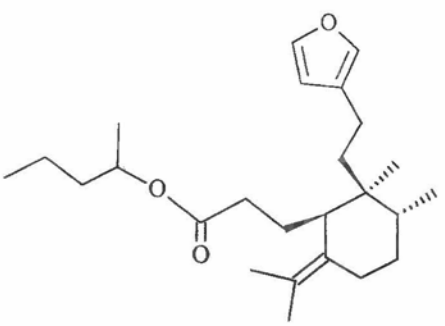
Tessmannic acid (**3.1**, NEW)



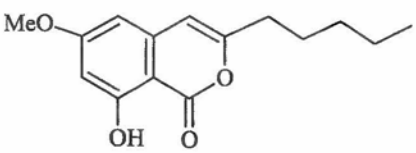
Methyltessmannoate (**3.2**, NEW)



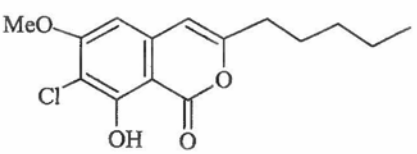
2-Methylpropyltessmannoate (**3.12**, NEW)



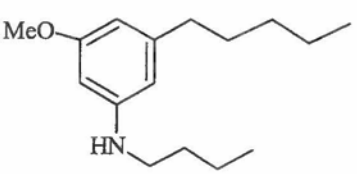
1-Methylbutyltessmannoate (**3.13**, NEW)



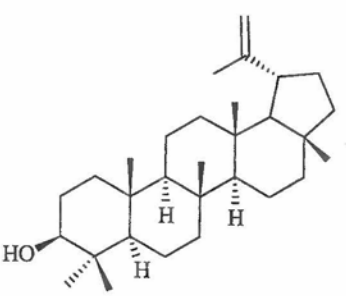
8-Hydroxy-6-methoxy-3-pentylisocoumarin (**3.14**)



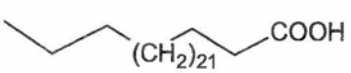
Chlotessmin or 7-Chloro-8-Hydroxy-6-methoxy-3-pentylisocoumarin (**3.15**, NEW)



5-Pentyl-3-methoxy-*N*-butylaniline (**3.16**, NEW)

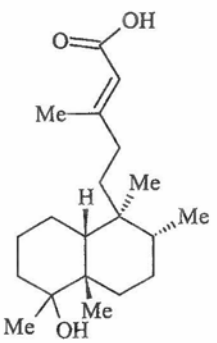


Lupeol (**3.17**)

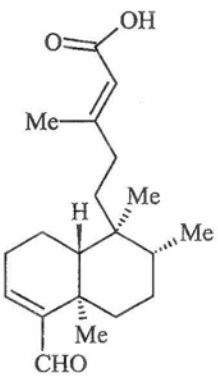


Heptacosanoic acid (**3.18**)

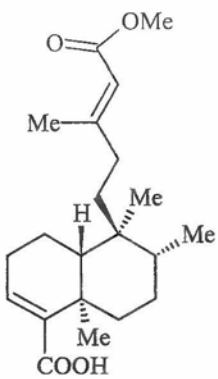
B. COMPOUNDS FROM *TESSMANNIA MARTINIANA* VAR *PAULOI*



cis-Kolavenolic acid (**4.1**, NEW)

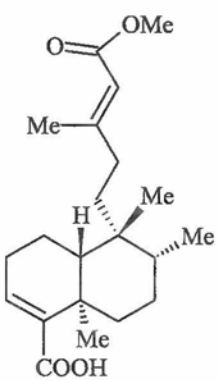


18-Oxocleroda-3,13(*E*)-dien-15-oic acid (**4.2**)

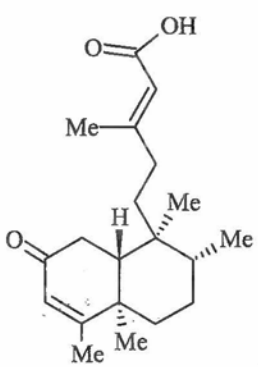


(9-*epi*)-*ent*-(18-Hydroxycarbonyl)-cleroda-3,13(*E*)-dien-15-oate (**4.4**, NEW)

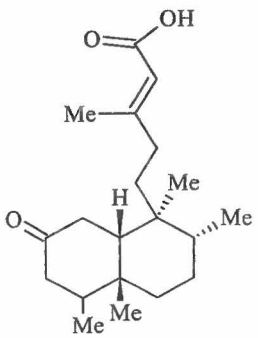
C. COMPOUNDS FROM *TESSMANNIA MARTINIANA* VAR *MARTINIANA*



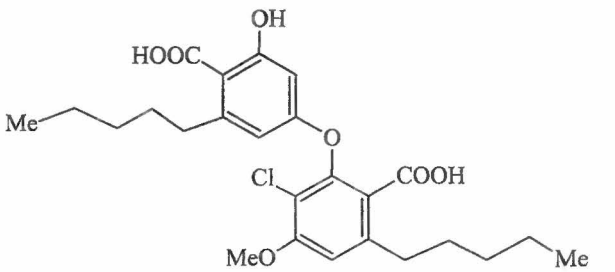
ent-(18-Hydroxycarbonyl)-cleroda-3,13(*E*)-dien-15-oate (**4.3**, NEW)



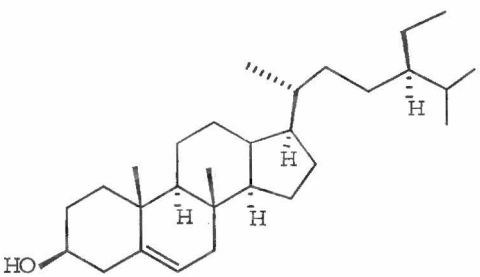
2-Oxo-*ent*-cleroda-3,13(*Z*)-dien-15-oic acid (**4.5**)



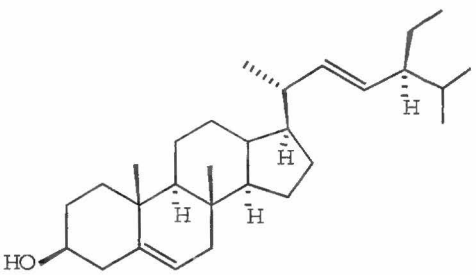
cis-2-Oxo-*ent*-cleroda-13(*Z*)-en-15-oic acid (**4.6**)



O-(3-Hydroxy-4-hydroxycarbonyl-5-pentylphenyl)-3-chloro-4-methoxy-5-pentyl-2-oxybenzoic acid (**4.7**, NEW)

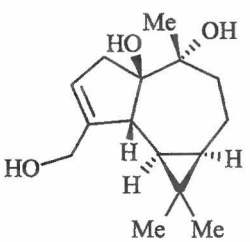


β-Sitosterol (**4.8**)

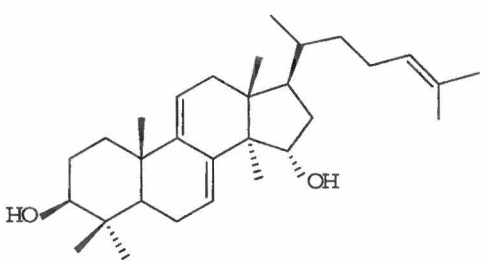


Stigmasterol (**4.9**)

D. COMPOUNDS FROM *POLYALTHIA TANGANYIKENSIS*

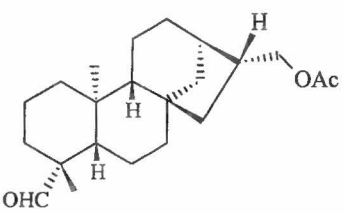


Tanganyikenol (**5.78**, NEW)

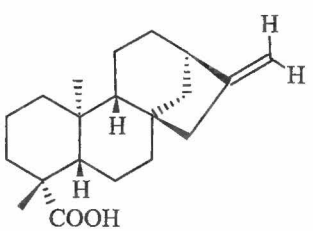


Polycarpol (**5.81**)

E. COMPOUNDS FROM *ANNONA SQUAMOSA*

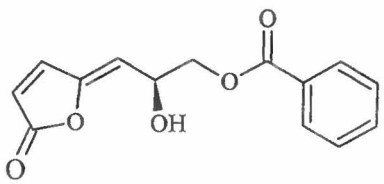


17-Acetoxy-*ent*-kauran-19-al (**5.83**, NEW)

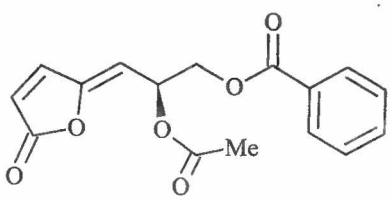


ent-Kaur-16-en-19-oic acid (**5.82**)

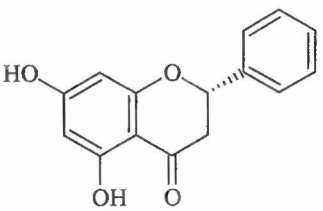
F. COMPOUNDS FROM *UVARIA LUNGONYANA*



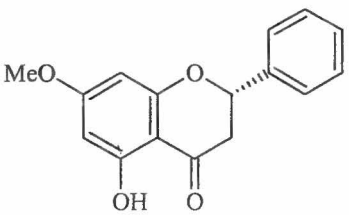
Melodorinol (**5.85**)



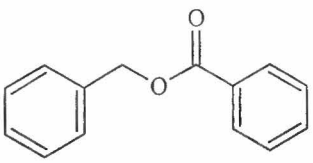
Acetylmelodorinol (**5.86**)



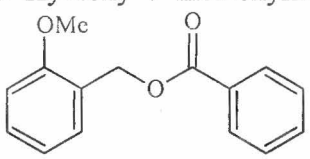
Pinocembrin (**3.1**)



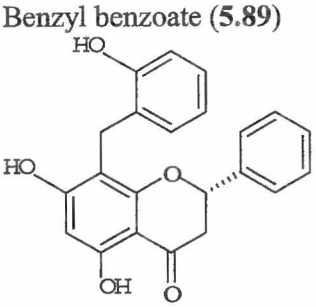
5–Hydroxy–7–methoxyflavanone (**3.1**)



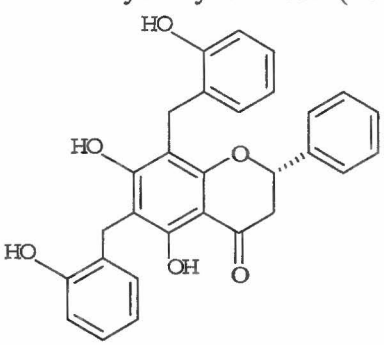
Benzyl benzoate (**5.89**)



2-Methoxybenzyl benzoate (**5.90**)

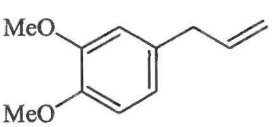


Chamanetin (**5.91**)

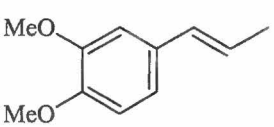


Dichamanetin (**5.92**)

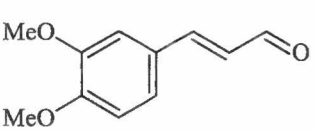
G. COMPOUNDS FROM *UVARIODENDRON PYCNOPHYLLUM*



O-Methyleugenol (**5.21**)



O-Methylisoeugenol (**5.93**)



2,3-Dimethoxycinnamaldehyde (**5.94**)

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