UNIVERSITY OF NAIROBI

COLLEGE OF BIOLOGICAL AND PHYSICAL SCIENCES

DEPARTMENT OF CHEMISTRY

PHYTOCHEMICAL INVESTIGATION OF THREE LEGUMINOSAE PLANTS FOR LARVICIDAL ACTIVITY AGAINST *AEDES AEGYPTI*

BY

MARCO MAKUNGU
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A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE AWARD OF THE DEGREE OF MASTERS OF SCIENCE IN CHEMISTRY OF THE UNIVERSITY OF NAIROBI

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DECLARATION

This thesis is composed of my original work, and contains no material previously published or written by another person except where due reference has been made in the text. It has not been submitted to qualify for the award of any other degree or diploma in any university or other tertiary institution. The research was carried out in the Department of Chemistry of the University of Nairobi.

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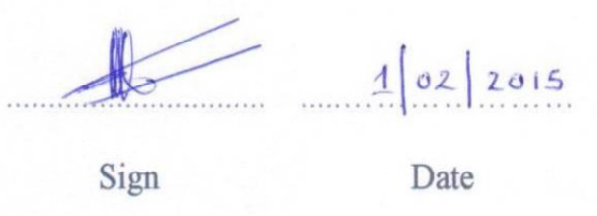

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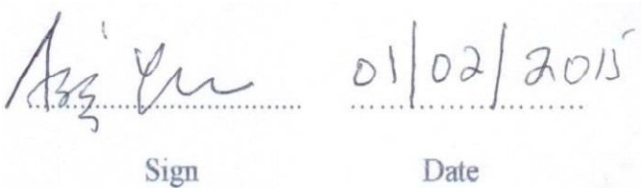

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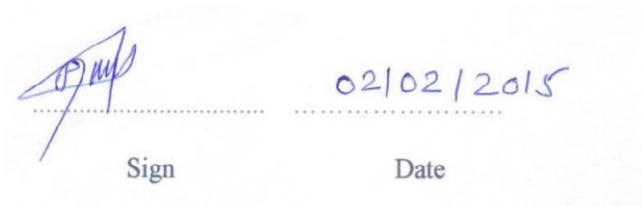

DEDICATION

This thesis is dedicated to my wife Nakaniwa (Kekuu) and my children Jenipha Modest Makungu and Jossia Madi Makungu

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LIST OF ABBREVIATIONS AND ACRONYMS

ANVR African Network for Vector Resistance
1D One Dimension
2D Two Dimensions
br s *Broad singlet*
CC Column Chromatography
CHI Chalcone Isomerase
CHS Chalcone Synthase
CoA Co-enzyme A
COSY Correlation Spectroscopy
CD Circular Dichroism
d *doublet*
DCM Dichloromethane
DCPP Disease Control Priorities Project
dd *double doublet*
DHF Dengue Hemorrhagic Fever
DMSO Dimethylsulphoxide
DSS Dengue Shock Syndrome
ED_{50} Effective Dose at 50%
EI-MS Electron Impact Ionization Mass Spectrometry
ESI Electrospray Ionization
EtOAc Ethyl acetate
HMBC Heteronuclear Multiple Bond Correlation
HMQC Heteronuclear Multiple Quantum Coherence
HSQC Heteronuclear Single Quantum Correlation
Hz Hertz
IC_{50} Inhibitory Concentration at 50%
IFS Isoflavone Synthase
IMM Integrated Mosquito Management
IUCN International Union for Conservation of Nature
J Coupling constant
LC_{50} Concentration causing 50% Lethality
m *multiplet*
m/z Mass to charge ratio
MeOH Methanol
MHz Mega Hertz
NADH Nicotinamide Adenine Dinucleotide Dehydrogenase
NaTHNac National Travel Health Network and Care
NIAID National Institute of Allergy and Infectious Diseases
NMR Nuclear Magnetic Resonance
NOESY Nuclear Overhauser and Exchange Spectroscopy
NICD-NHLS. National Institute for Communicable Diseases -Division of the National Health Laboratory Service
SPRTTD Special Programme for Research and Training in Tropical Diseases
t *triplet*
SD Standard Deviation
TDNP Tanzania Daima News Paper
UNICEF United Nation Children Fund
UV Ultra-Violet
ORD Optical Rotatory Dispersion

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ABSTRACT

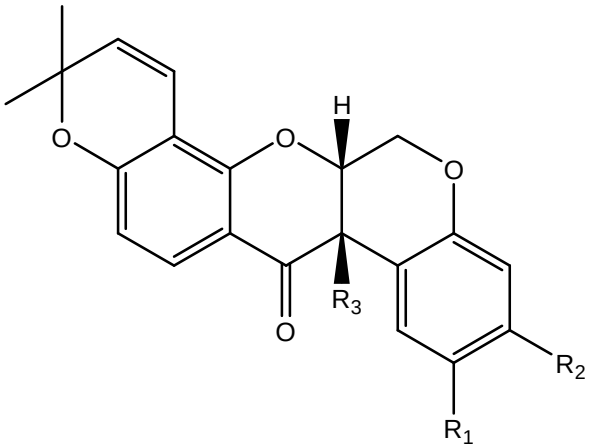
The family Leguminosae is known to synthesize a range of flavonoids and isoflavonoids that possess wide varieties of biological activities. Among these, larvicidal activity is associated with the rotenoids; mainly found in the genera *Tephrosia*, *Derris*, *Millettia* and *Lonchocarpus.* Investigation of these plants could result in the identification of biodegradable and cost effective larvicidal agents to control mosquito borne diseases such as malaria and dengue fever.

Three plants from this family namely *Millettia oblata* spp. *teitensis* (leaves), *Millettia micans* (root bark) and *Derris trifoliata* (leaves) have been phytochemically investigated. A total of seven compounds have been isolated from these plants. The characterization of the isolated compounds was based on spectroscopic evidence including 1D NMR, 2D NMR, UV, ORD, CD, EI-MS and ESI. In this study, the larvicidal activity of the crude extracts and four compounds have also been investigated.

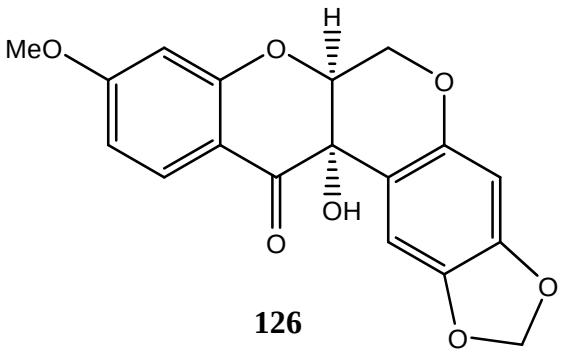
The crude extract (DCM/MeOH, 1:1) of *M. oblata* spp. *teitensis* was tested against the 3rd instar larvae of *Aedes aegypti* and showed moderate larvicidal activity with LC_{50} value of 84.6±7.8 µg/mL, at 24 hr. This crude extract was subjected to column chromatography over silica gel eluting with increasing polarities of mixtures of *n*-hexane and ethyl acetate (100:0 to 0:100) and led to the isolation of four compounds. These were identified as the rotenoids; tephrosin (**125**), oblatarotenoid A **(126)**, and millettone (**128**) and the steroid stigmasterol (**127**). Among these, oblatarotenoid A (**126**) is a new compound. Tephrosin (**125**) showed high larvicidal activity with LC_{50} value of 1.4±0.2 µg/mL, while oblatarotenoid A (1**26**) was inactive (LC_{50} > 100 µg/mL). This finding is consistent with the previous suggestion that the two methoxyl groups in ring A of tephrosin are important for larvicidal activity.

The crude extract (DCM/MeOH, 1:1) of *M. micans* (root bark), a plant with no phytochemical report; showed weak larvicidal activity against the 3rd instar larvae of *Aedes aegypti* with LC_{50} value of 132±14.4 µg/mL at 24 hr. The crude extract was then subjected to CC and led to isolation of one compound. This compound was characterized as a new pterocarpan, for which the trivial name micanspterocarpan A (**129**) is assigned. The new pterocarpan was tested against the 3rd instar larvae of *Aedes aegypti* and it was inactive (LC_{50} > 100 µg/mL).

The crude extract (DCM/MeOH, 1:1) of *D. trifoliata* (leaves) showed weak larvicidal activity against the 3rd instar larvae of *Aedes aegypti* with LC_{50} value of 367.7±162.8 µg/mL at 24 hr. This crude extract was also subjected to CC and led to isolation of two known flavanones namely: lupinifolin (**130**) and lupinifolin 4'-methyl ether (**131**). Lupinifolin (**130**) which is the major compound of the extract did not show significant larvicidal activity (LC_{50} > 100 µg/mL). However, good antiplasmodial activity has been reported for this compound.



125 R_3 = OH R_1= R_2 = OMe
128 R_3 = H R_1, R_2 = OCH_2O



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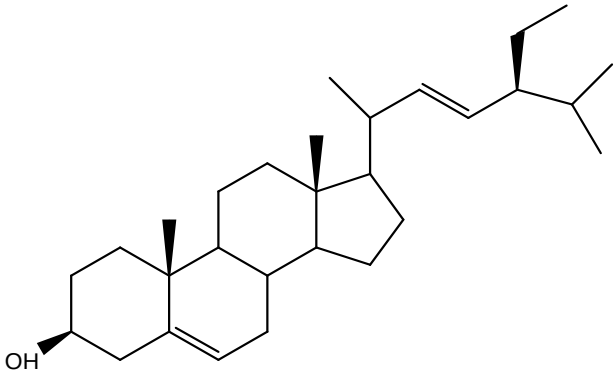

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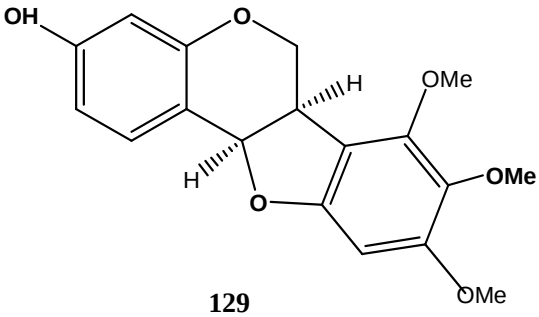

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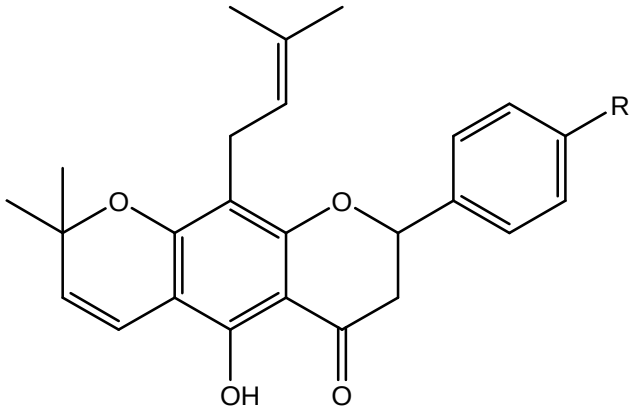


130 R = OH
131 R = OMe

CHAPTER ONE

INTRODUCTION

1.1 Background

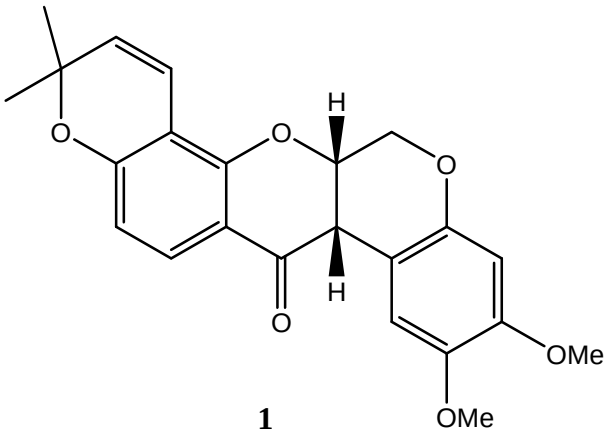
Plants have been a source of medicinal agents for thousands of years and an impressive number of modern drugs have been isolated from natural resources which are often considered to have fewer side effects (Abolfazl *et al.,* 2014). Consequently, over centuries of research, several medicinal plants have been proved to be effective in the treatment of infectious diseases (Iwu, 2014). In Africa, it is estimated that 75% of the rural population rely on herbal medicine for their primary healthcare (Ogoche, 2014). Herbal remedies have the advantage of being readily available, cheaper, and the process of thier preparation is often easier than formulating and producing synthetic drugs (Ogoche, 2014).

Mosquito-borne diseases cause significant morbidity, mortality and economic burden to society (Lima *et al.,* 2011). They are complex and their occurrence depends on the interaction of various biological, ecological, social and economic factors (Boratne *et al.,* 2010). People infected with these diseases may not show symptoms immediately after infections; and when symptoms appear, they may be mild (in the form of fever, headache, tiredness, body aches, rash and swollen lymph nodes) or severe (in the form of high fever, headache, stiff neck, muscle weakness, tremors, paralysis, confusion, brain swelling and coma). Mosquito-borne diseases (such as malaria, dengue, chikungunya and filaria) are transmitted by mosquitoes belong to genera *Anopheles, Aedes*, and *Culex.*

In an effort to address the problem of infectious diseases and its associated complications, including mosquito-borne diseases, the WHO developed an international programme which *interalia* reviews available scientific data relating to the efficacy of medicinal plants (Oliver-Bever, 1986). In order to attain this, considerable research works have been done on the pharmacological and phytochemical aspects of plants by finding preventive measures that will effectively control the population of the vector species namely *Anopheles, Aedes*, and *Culex*.

The genus *Millettia* is known to elaborate isoflavonoids including rotenoids such as deguelin (**1**), tephrosin (**2**), and rotenone (**3**) with larvicidal activities (Yenesew *et al*., 2003a). The genus is also known for its antiprotozoal activities (Rajemiarimiraho *et al*., 2014). Majority of antiprotozoal compounds isolated from various *Millettia* species are flavonoids belonging to the subclasses of isoflavans, isoflavones, rotenoids and pterocarpans (Rajemiarimiraho *et al*., 2014). The African plant, *M. griffoniana* (seeds) had trypanocidal activity due to griffonianone E (**4**) (IC_{50} = 8.6 and 6.35 µg/mL against *T. b. brucei* and *T. b. rhodesiense,* respectively) and antiplasmodial activity due to the presence of griffonianone E (**4**), 7-methoxyebenosin (**5**) and prenylated isoflavonoids, with IC_{50} ranging from 28.3 to 36.7 µg/mL (Ngamga *et al*., 2005). *M. usaramensis* ssp. *usaramensis* (stem bark) showed also antiplasmodial properties (IC_{50} = 21 µg/mL) with its most active compound (**6**) showing an IC_{50} value of 8.7 µg/mL against W_2 strain of *P. falciprum* (Yenesew *et al*., 2003b). The stem bark of *M. zechiana* and *M. versicolor* showed moderate antiplasmodial activities (IC_{50} = 16.1 and 33 µg/mL, respectively) with low cytotoxicity (Rajemiarimiraho *et al*., 2014). Antileishmanial properties (IC_{50} = 32 µM for 6,7-dimethoxy-3',4'-methylenedioxyisoflavone (**7**) against *L. infantum*) and low cytotoxicity (IC_{50} = 43 µM against MRC-5 cells) were also reported for *M. puguensis* (roots) (Rajemiarimiraho *et al*.,

2014). The Asian plant *M. diptera* (stem bark) showed antiplasmodial activity with IC_{50} value of 6.2 and 5.5 µg/mL for dichloromethame and cyclohexane extracts, respectively (Rajemiarimiraho *et al*., 2014). Also *M. pendula* (timber heart wood) had shown potent leishmanicidal activity; pendulone (**8**), an isoflavan extracted from this plant being the most active compound, IC_{50} = 0.07 µg/mL against *L. major* (Rajemiarimiraho *et al*., 2014).



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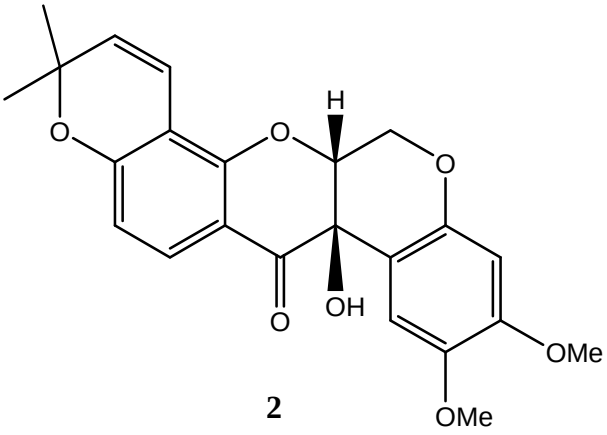

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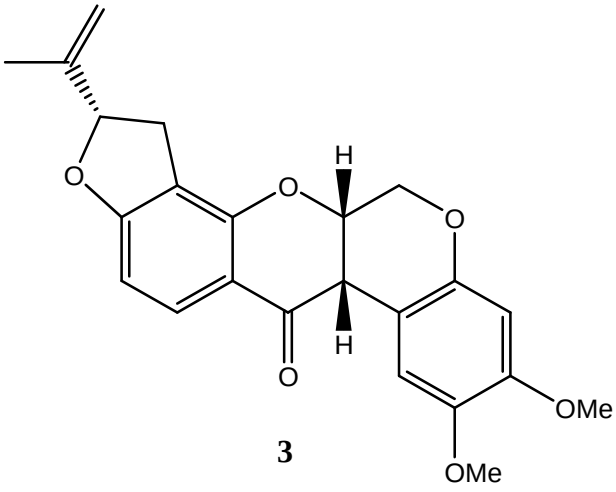

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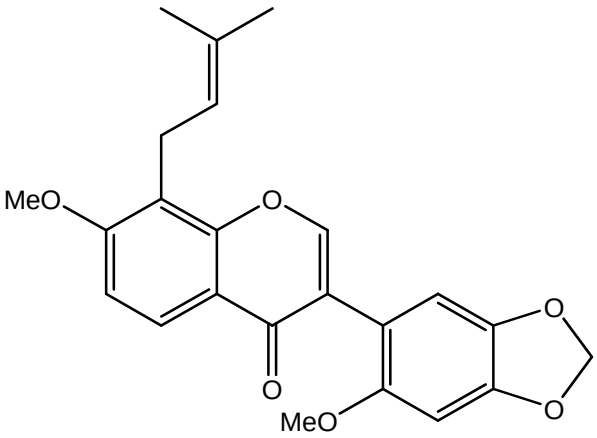

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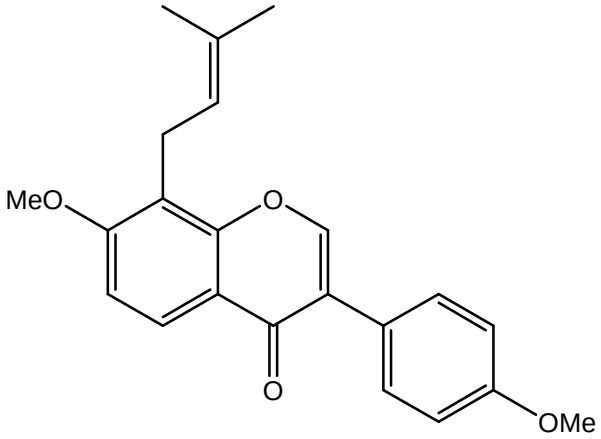

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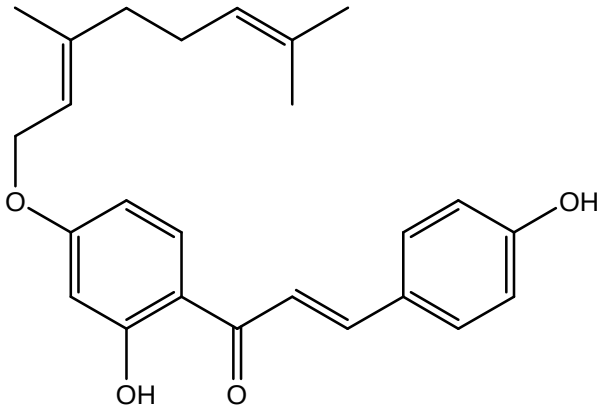

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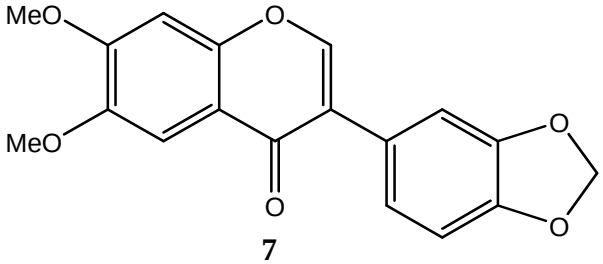

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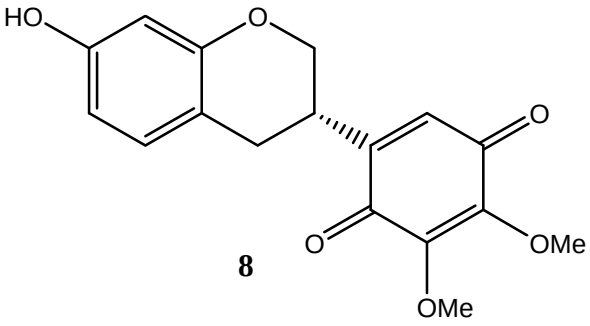

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An alternative in combating mosquito-borne diseases is to control the population of mosquito vectors at the larval stages (Shivakumar *et al*., 2013). Larval control focuses target-specific agents in definable aquatic breeding sites and has three key components: environmental management, biological control, and chemical control (including plant extractives) (McCormick and Whitney, 2013). Control of mosquito larvae and pupae prevents mosquitoes from becoming biting female adults capable of transmitting disease and producing another generation of mosquitoes (McCormick and Whitney, 2013).

In line with vector control at larval stage, different *in vitro* tests have been carried out. For example, the crude extract of the seeds of *Millettia dura* and the rotenoids, deguelin (**1**) and

tephrosin (**2**) isolated from this extract showed potent larvicidal activity against the larvae of *Aedes aegypti* with LC_{50} values of 3.5, 1.6 and 1.4 µg/mL at 24 h, respectively (Yenesew *et al*., 2003a). Furthermore, the crude extract of the roots of *Derris trifoliata* showed potent larvicidal activity against the second instar larvae of *Culex quinquefasciatus* and *Aedes agypti* with LC_{50} values of 0.45 and 0.74 µg/mL at 24 hr, respectively (Yenesew *et al*., 2005). The isolates of this extract, deguelin (**1**) and rotenone (**3**) among others were tested for larvicidal activity. Rotenone (**3**) showed LC_{50} of 0.52 and 0.45 µg/mL, while deguelin (**1**) showed 1.6 and 1.8 µg/mL against both *Aedes agypti* and *Culex quinquefasciatus,* respectively (Yenesew *et al*., 2005).

In another study, the seeds of *Millettia usaramensis* subspecies *usaramensis* showed very good activity against the second instar larvae of *Aedes aegypti* and *Culex quinquefasciatus* with LC_{50} value of 3.5 and 3.2 µg/mL, respectively (Yenesew *et al*., 2005). The extract elaborated many compounds that were tested for larvicidal activity with deguelin (**1**) and tephrosin (**2**), among others, showed good larvicidal activity (Yenesew *et al*., 2005).

In this study, in the search for biodegradable larvicidal compounds, three plants belonging to the family Leguminosae, namely *Millettia oblata* spp. *teitensis*, *Millettia micans* and *Derris trifoliata* were phytochemically investigated.

1.2 Problem Statement

Protozoan parasites in general have more complex genomes, metabolisms and life cycles making them a difficult target for interventions by drugs and vaccines because the parasite's shape-shifting ways allow it to evade chemical and immunological defenses (Petersen and Hadler,

2013). They pose a moving target as well, intentionally changing their outer coating during each phase of their life cycle, and creating a diverse antigenic and metabolic wardrobe through sexual recombination (Petersen and Hadler, 2013).

Currently, one of the methods for preventing vector-borne diseases is vector control by using synthetic insecticides and destroying the breeding sites. However, host mosquitoes have also developed resistance to conventional synthetic insecticides. In addition, synthetic insecticides are toxic to non-target organisms and also affect the environment by contaminating soil, water and air.

1.3 Objectives

1.3.1 Overall Objective

To identify the larvicidal constituents from some selected *Millettia* and *Derris* species.

1.3.2 Specific Objectives

(i) To determine the larvicidal activity of the crude extracts of *Millettia micans* (root bark), *M. oblata* spp. *teitensis* (leaves) and *Derris trifoliata* (leaves);

(ii) To isolate and characterize the constituents from these plants;

(iii) To establish the larvicidal activity of the isolated compounds.

1.4 Justification of the Research

The utilization of plant extracts or isolated phytoconstituents is a promising method for controlling immature forms of mosquitoes (Medeiros *et al.,* 2013). The best stage to target mosquito vector is at the larval stage rather than the adult stage. The advantage of this is that

they are localized at their breeding sites until the adult emergences and hence the overall usage of pesticide is low as compared to control of adults by aerial application of adulticidal chemicals (Shivakumar *et al*., 2013).

Previous investigations of the phytochemical profile of the genera *Millettia* and *Derris* have resulted in the isolation of several compounds with potent larvicidal activities. Therefore, this has left a room for further studies of other species of these genera. For example, *Millettia micans* (root bark), *Millettia oblata* spp. *teitensis* (leaves) *and Derris trifoliata* (leaves) which have no phytochemical reports were studied under this research in order to identify natural larvicidal constituents which may be cheap, less toxic and more efficacious in combating mosquito-borne diseases.

CHAPTER TWO

LITERATURE REVIEW

2.1 Mosquito-Borne Diseases

Mosquitoes are arthropods, and are the major vectors responsible for the transmission of diseases. They transmit both protozoan and viral diseases (Shivakumar *et al.,* 2013). They are vectors for various diseases including malaria, yellow fever, dengue fever, filariasis, Japanese encephalitis and chikungunya (Shivakumar *et al.,* 2013). These mosquito-borne infections which kill millions of people each year are found in tropical and sub-tropical regions of the world, including urban and semi-urban areas where the hot humid climate favors the proliferation of many different mosquito species (Medeiros *et al.,* 2013). The vectors for these diseases belong to genera *Aedes*, *Anopheles* and *Culex*. The mosquito *Aedes aegypti* (Figure **2**.**1**) is the major vector of yellow fever, dengue and dengue hemorrhagic fever (Lima *et al.,* 2011). The *Anopheles* mosquito (Figure **2**.**1**) is the vector for malaria, an acute or chronic infectious disease while *Culex* mosquito is a vector for filariasis. Malaria and dengue are more commonly found in tropical countries and there is no vaccine for these diseases (Medeiros *et al.,* 2013). Thus, vector control becomes indispensible for preventing malaria, dengue and other vector-borne diseases (Medeiros *et al.,* 2013). The control of these vectors is based on the destruction of breeding sites and using larvicides that target the mosquito at the larval stage (Lima *et al.,* 2011).

Therefore, there is need to investigate new larvicidal substances which are non-toxic, easily available at affordable prices, biodegradable and with broad-spectrum target-specific activities against different species of vector mosquitoes. Ideally, these substances would be able to

interfere with vector development and keep vectors under control, that is, at very low population level.

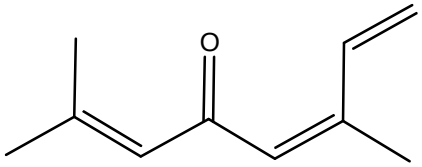
a) *Anopheles* mosquito (NIAID, 2007) b) *Aedes aegypti* (TDNP, 12/05/2014**)**

Figure 2.1: Malaria (a) and dengue (b) fever vectors

Application of phytochemicals as mosquito larvicide is an essential component of Integrated Mosquito Management (IMM) whose major purpose is to protect public health from diseases transmitted by mosquitoes, maintain healthy environment through proper use and disposal of pesticides and improve the overall quality of life through practical and effective pest control strategies (Ghosh *et al*., 2012). The efficacy of phytochemicals against mosquito larvae can vary significantly depending on plant species, plant parts used, age of plant parts (young, mature or senescent), solvent used during extraction (Ghosh *et al*., 2012). Larvicidial approach is more proactive, pro-environment, target specific and safer approach than controlling adult mosquitoes (Ghosh *et al*., 2012).

The use of larvicides from botanical origin was extensively studied as an essential part of IMM, and various mosquito control agents such as rotenone (**3**), ocimenone (**9**), goniothalamin (**10**),

eugenol (**11**), thymol (**12**), quassin (**13**), neolignans (**14**) and arborine (**15**) were developed (Ghosh *et al*., 2012).



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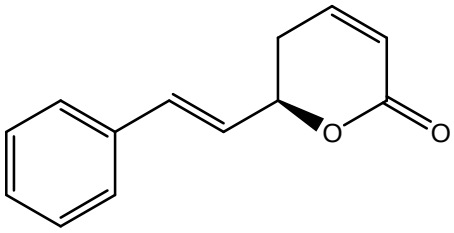

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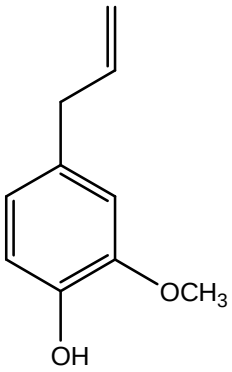

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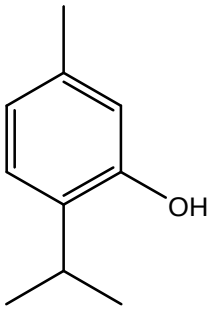

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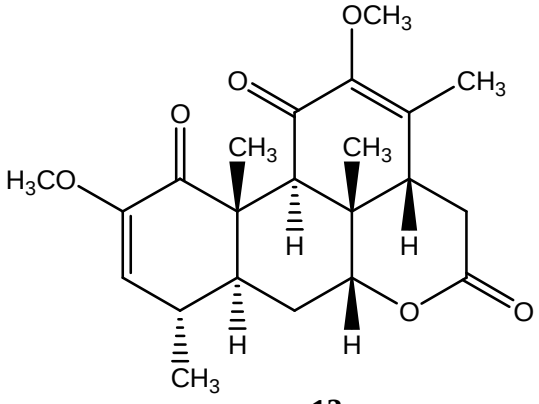

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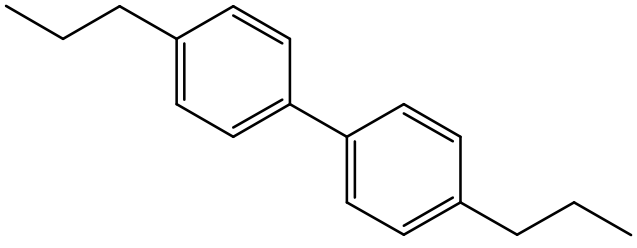

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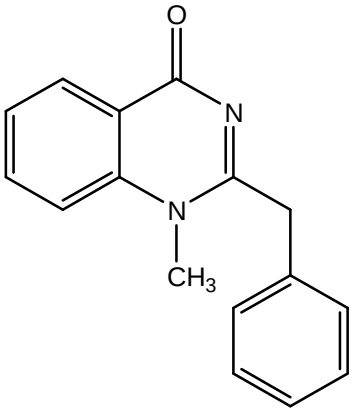

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2.1.1 Malaria

Malaria is a mosquito-borne infectious disease caused by one of four protozoan species of the genus *Plasmodium* namely: *P. falciparum*, *P. vivax*, *P. ovale*, and *P. malariae*. It is transmitted from person to person by female *Anopheles* mosquitoes which usually bite at night. Occasionally, transmission occurs by blood transfusion, needle-sharing, organ transplantation or congenitally from mother to foetus. Malaria is characterized by its biological diversity that depends mostly on the vector species that are involved in transmission in terms of their distribution, behavior and capacity. Also this diversity depends on seasonality of transmission, pathoneginicity of parasites species and by immune response of human hosts (ANVR, 2005). The geographical distribution of vector species belonging to the female *Anopheles* mosquito has been developed in Africa as seen from Figure **2.2**.

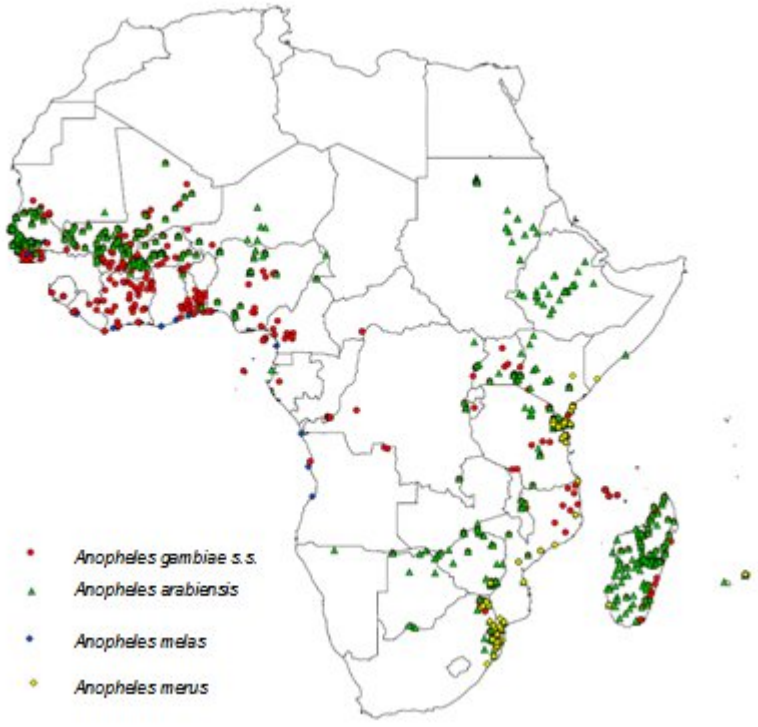


Figure 2.2: Distribution of malaria vectors in Africa (ANVR, 2005)

Malaria is endemic in about 100 developing countries (WHO, 2001). In 2010, there were 219 million malaria cases leading to approximately 660,000 deaths, mostly among African children (UNICEF, 2013). An estimated 90% of all malaria deaths occur in Africa, the majority of which being children under five (Figure **2.3**) and pregnant women (UNICEF, 2013). Globally, 80% of malaria deaths occur in just 14 African countries (UNICEF, 2013). Malaria still kills a child every minute (UNICEF, 2013). It remains one of the leading public health problems in Sub-Saharan Africa (Denis *et al.,* 2011).

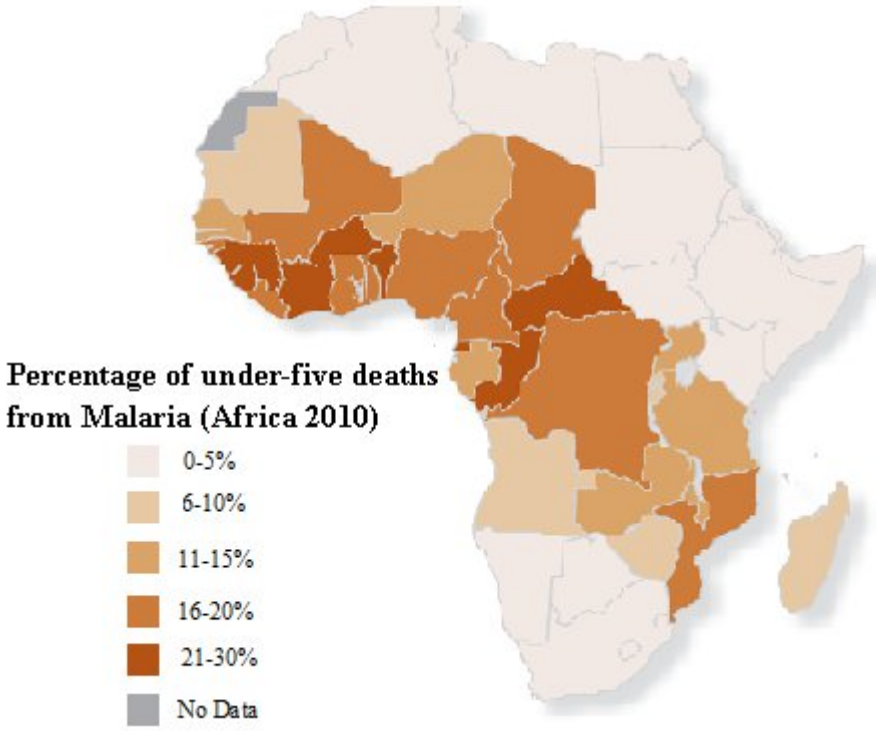


Figure 2.3: Malaria deaths among children under five in Africa (%)-(UNICEF, 2013).

2.1.2 Dengue Fever

Dengue fever is an arthropod borne disease caused by virus of the genus *Flavivirus*, and within the family Flaviviridae (NaTHNac, 2003). There are four distinct serotypes of dengue virus (DEN 1, DEN 2, DEN 3 and DEN 4) all of which have the potential to cause either classic dengue fever or the more serious form of the disease, dengue haemorrhagic fever (NaTHNan, 2003). One can be infected by at least two if not all four types at different times during lifetime, but only once by the same type.

Dengue is an acute viral illness characterised by fever, muscular pain, rash, headache and sometimes thrombocytopoaenia (Abedayo *et al.,* 2013). More severe forms of dengue infection such as Dengue Hemorrhagic Fever (DHF) and Dengue Shock Syndrome (DSS) have a high mortality rate (Abedayo *et al*., 2013). Dengue fever is transmitted to humans by the bite of an infected mosquito (Knowlton *et al*., 2009; Bhatt *et al*., 2013). The mosquito is attracted by the body odor, carbondioxide and heat emitted from the animal or humans. The virus incubates for 3 to 14 days before symptoms appear with an average symptom onset at four to seven days. There are two species of mosquito that can transmit dengue fever, namely *Aedes aegypti* and *Aedes albopictus*. The principal vector being *Aedes aegypti*, which is also the vector of yellow fever (Abedayo *et al*., 2013).

Dengue fever is found mostly during and shortly after the rainy season in tropical and subtropical areas of the Caribbean and Central and South America, Africa, Southeast Asia and China, India, the Middle East, Australia and the South and Central Pacific (Abedayo *et al*., 2013). Worldwide, 50 to 100 million cases of dengue infections occur each year (SPRTTD-WHO, 2009; Malavige *et al*., 2004). In 2010, there were an estimate of 96 million apparent dengue infections

worldwide, of which Africa's dengue burden being 16%, representing a significantly larger burden than previous estimate (Figure **2.4**) (Bhatt *et al.*, 2013; NICD-NHLS, 2014). This disparity supports the notion of a largely hidden African dengue burden, being masked by symptomatically similar illnesses, under-reporting and highly variable treatment-seeking behavior (Bhatt *et al.*, 2013). Currently, there is no specific treatment for classic dengue fever (Malavige *et al.,* 2004; Medeiros *et al.,* 2013).

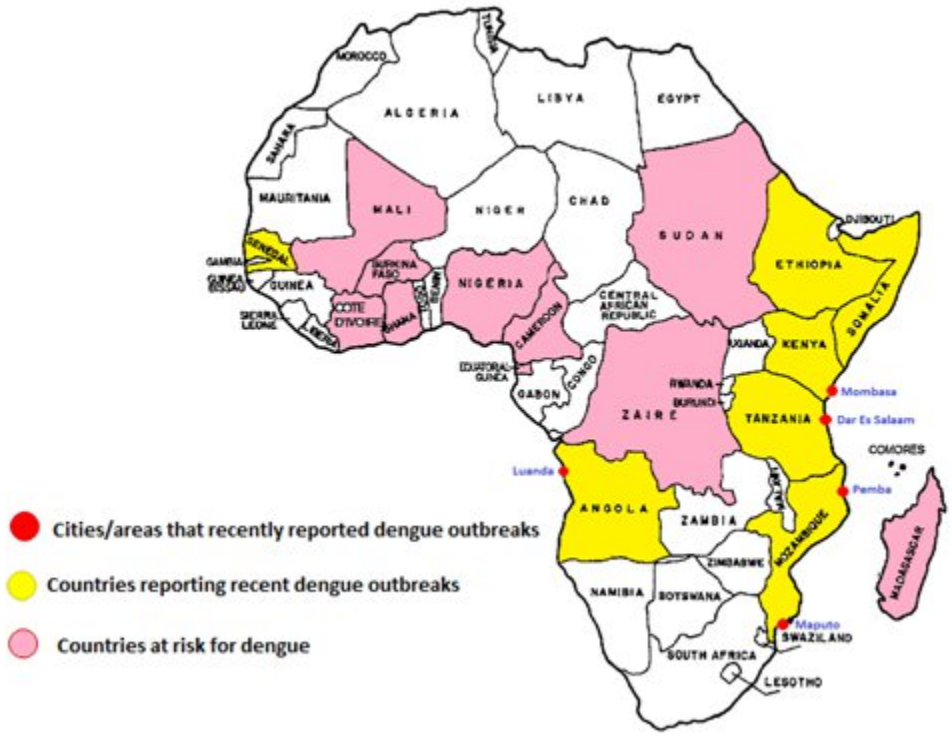


Figure 2.4: Map showing distribution of dengue fever in Africa (NICD-NHLS, 2014)

2.2 Plants as Sources of Larvicidal Agents

Plants produce diverse types of bioactive molecules. Recent studies reveal that plants possess many subtle defenses strategies that interfere with insect growth, development and behavior without toxicity to higher animals (Azmathullah *et al.,* 2011). Ethnobotanical and laboratory based studies have revealed the existence of insecticidal plants belonging to different families in different parts of the world (Tomass *et al*., 2011). For example, crude extracts of plant parts belonging to different families, essential oils or their chromatographic fractions are shown to have various levels of bioactivity against different developmental stages of mosquitoes (Tomass *et al* ., 2011). In line with this, more than 2,000 plant species from different plant families such as Leguminosae, Solanaceae, Asteraceae, Cladophoraceae, Labiatae, Miliaceae, Oocystaceae and Rutaceae have been evaluated for larvicidal activities (Ghosh *et al* ., 2012). In addition, recent research has shown the effectiveness of plant derived compounds, such as saponine, steroids, isoflavonoids, essential oils, alkaloids and tannins as mosquito larvicides (Shivakumar *et al*., 2013). Therefore, the search for eco-safe, low cost and a highly potent larvicide for the control of mosquitoes need the preliminary screening of plants to evaluate their insecticidal activities.

2.2.1 Larvicides from the Family Leguminosae

Plants of the family Leguminosae are distributed in tropical region where malaria vectors are found. Utilization of these plants in search for biodegradable and cost effective larvicidal agents would help to decrease the problem of mosquito borne diseases. Although, insecticide-based control programmes targeting the vector have been the most successful method of controlling the disease, the effectiveness has been greatly reduced by insect resistance and the growing environmental concern (Ghosh *et al*., 2012).

Rotenone (**3**) found in some genera of the Leguminosae (e.g. *Derris* or *Lonchocarpus*), is an excellent biodegradable insecticide and is used as such either in pure or powdered plant form (Dewick, 2009). Rotenone and other rotenoids have been tested for insecticidal activities (Yenesew *et al*., 2006; Derese, 2004). They are powerful insecticidal and piscicidal (fish poison) agents, interfering with oxidative phosphorylation in animal mitochondria by blocking transfer of electrons to ubiquinone by complexing with NADH:ubiquinone oxidoreductase of the respiratory electron transport chain (Dewick, 2009). This effect is responsible for insecticidal and piscicidal properties of this group of compounds (Williams and Harborne, 1989). They are relatively harmless to mammals unless they enter the bloodstream, being metabolized rapidly upon ingestion; Insects lack this rapid detoxification. Structurally, the presence of *cis* B/C ring junction and also presence of methoxy groups (one or two) at C-2 and C-3 is a requirement for larvicidal activity (Yenesew *et al.,* 2003b).

2.3 Botanical Information

2.3.1 The Family Leguminosae

The legume family (Leguminosae) is the third largest of the flowering plants with around 650 genera and 18,000 species of herbs, shrubs, trees, and climbers (Kass and Wink, 1997) with a long history of use in agriculture. This large family is divided into three subfamilies namely Mimosoideae, Caesalpinoideae and Papilionoideae. The later being the largest and the most diverse of the three sub families (Kass and Wink, 1997). They are all economically important providing food, fodder, timber, fibres, gums, resins, tannins, medicine and ornamentals (Bisby *et al*., 1994). Also the plants in this family are important in replenishment of nitrogen removed by agricultural processes from soil through symbiotic associations (Bisby *et al*., 1994). Plants in the Leguminosae family have characteristic leaves and pods that help to identify them as legumes.

The leaves are usually alternate and compound. They may be pinnate or trifoliate. All legumes have similar fruits, called 'pods' (Burgess *et al*., 2000).

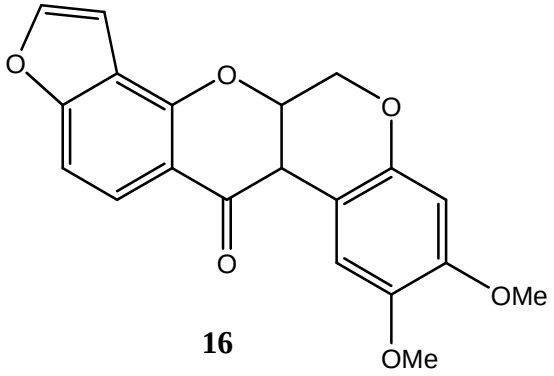
The Leguminosae family is also known for elaborating a wide range of flavonoids and isoflavonoids, the latter being more or less restricted to the subfamily Papilinoideae (Yenesew *et al*., 1997b; Bisby *et al*., 1994). Isoflavonoids are known to possess a wide range of biological activities, the most prominent of which being insecticidal and antimicrobial properties (Yenesew *et al.,* 1997b; Patra, 2012). The genera *Millettia* and *Derris* belong to the subfamily Papilinoideae.

2.3.1.1 The Genus *Derris*

The genus *Derris* was established by Joao de Loureiro in 1790 to accommodate two species: *Derris pinnata* Lour and *D. trifoliata* (Sirichamorn, 2013). *Derris* consists of about 60 species found throughout the Old World tropics (*de* Padua *et al*., 1999). Most species (approximately 50) are found in South-East Asia, 3 in Australia and one species extends from Asia to eastern Africa (*de* Padua *et al.,* 1999).

Derris is placed in the tribe *Millettieae* within the subfamily Papilionoideae (*de* Padua *et al*., 1999; Sirichamorn, 2013). It seems to be closely related to *Millettia* and *Lonchocarpus*, and to *Aganope*. The genus is often subdivided into 3 sections: section *Derris* with about 50 species including *Derris elegans* and *Derris trifoliolata*; section *Brachypterum* (Wight and Arn.) Benth. with 3 species including *Derris robusta* and *Derris scandens*; and section *Paraderris* Miq. with 6 species including *Derris elliptica* and *Derris malaccensis (de* Padu*a et al.,*1999).

Derris species have several characters that deviate from the other genera, for example, the inflorescence, adhesion of wings (winged sutures of the pods) and keel petals and upper filaments uniting with others. *Derris* lianas may climb over trees and other vegetation forming a thick cover and thus act as a serious weed in forest plantations (*de* Padua *et al*., 1999; Sirichamorn, 2013). *Derris* species are further characterized by the presence of toxic rotenoids, mainly rotenone (**3**) (0.3-12% in the root) which is the most toxic compound, followed by deguelin (**1**) (0.2-2.9%), elliptone (**16**) (0.4-4.6%) and toxicarol (**17**) (0-4.4%). These compounds are effective respiratory poisons (*de* Padua *et al*., 1999).



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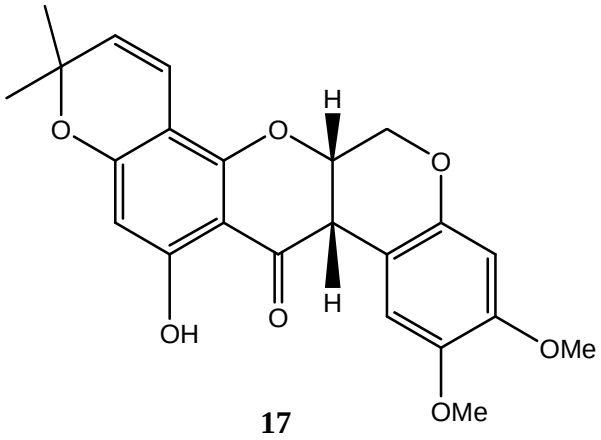

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The genus *Derris* has received much attention from phytochemical viewpoint because of their plentiful production of flavonoids. In Kenya, this genus is represented by *Derris trifoliata* (Beentje *et al*., 1994).

2.3.1.1.1 *Derris trifoliata*

Derris trifoliata Lour (Figure **2.5-2.6**) is mainly distributed in humid environment of subtropical and tropical regions. It grows near the coast in swampy scrub vegetation and forest behind beaches, on muddy foreshores and estuaries, and in edges of mangroves (Orwa *et al.,* 2009). It is a perennial climber, or a much branched climbing shrub, reaching a length of 8 meters or less. The plant has 12.5-20 cm long odd-pinnate compound leaves with 3- 7 leaflets (5.7-10 cm by 3.2-5 cm) and 7.5-15 cm long white flowers that are fascicled in axillary racemes (*de* Padua *et al*., 1999). It is one of the few climbers in the local mangroves (*de* Padua *et al*., 1999). In Kenya, *Derris trifoliata* is found near the mouths of Tana, Mwena and Sabaki rivers (Beentje *et al*., 1994).

Figure 2.5: Leaves of *Derris trifoliata* (Sirichamorn, 2013**).**

Figure 2.6: Flowers of *Derris trifoliata* (Sirichamorn, 2013).

2.3.1.2 The Genus *Millettia*

The genus *Millettia* appears in the Africa Pharmacopeia since centuries (Banzouzi *et al*. 2008). It is named after Charles Millet of Canton – China in 1830 who was the plant collector and an official with the East India Company after collecting samples of *Millettia* (Mutshinyalo, 2010). The genus is further subdivided into two sub-genera; namely sub-genus *Millettia* and sub-genus *Otosema*. In Tanzania, 25 species represent the genus *Millettia*; these include: *M. sericantha* (endemic), *M. semseii* (endemic), *M. schliebenii* (endemic), *M. micans* (endemic) and *M. eriocarpa* (endemic) among others. In Kenya, this genus is represented by 6 species. These are: *M. dura, M. leucantha, M. usaramensis* ssp. *usaramensis, M. oblata* ssp. *teitensis, M. lasiantha* and *M. tanaensis* (Beentje *et al*., 1994).

The genus *Millettia* is distributed in the tropical and subtropical countries. They are widely distributed throughout Burma, China, India, Japan, Malaysia, equatorial Africa, and Madagascar (Lock, 1989). The plants usually occur in grasslands, along river banks, and in secondary, fringe, coastal, and humid forests. The flowers of all species are beautifully tinted and are borne in drooping panicles (Karunamoorthi *et al*., 2009; Lock, 1989). The genus *Millettia* comprises of approximately 260 species divided mainly between Africa (139 species) and Asia (121 species) (Banzouzi *et al*., 2008). *Millettia* can be found in nearly every country of Sub-Saharan Africa, but 60% of the species grow in the Guineo-Congolian area, with 52 species in Democratic Republic of Congo (DRC), 27 in Gabon and 20 in Cameroon. East Africa accounts for approximately 24% of the African *Millettia* with 25 species found in Tanzania (Banzouzi *et al.,* 2008).

The distribution area of *Millettia* covers 4 out of 8 regional centres of endemism described by Whites in 1983 in continental Africa (Figure **2.7**) (Banzouzi *et al.*, 2008). The highest endemism rate is observed in the Malagasy area (100%), followed by the Somalia/Masai area (75% in Ethiopia), the Guineo-Congolian area (63% in DRC, 41% in Gabon) and in the third position the Zambesian area (44% in Tanzania). However, the distribution of *Millettia* species for East Africa countries is as follows: Kenya 19% (6 species), Tanzania 59.5% (25 species), Uganda 0.095% (4 species), Rwanda 0.095% (4 species) and Burundi 0.024% (1 species). Most of these species grow in forest (80%), or in woodland (8%), bush land (7%) and shrub land (5%), (Banzouzi *et al.*, 2008).

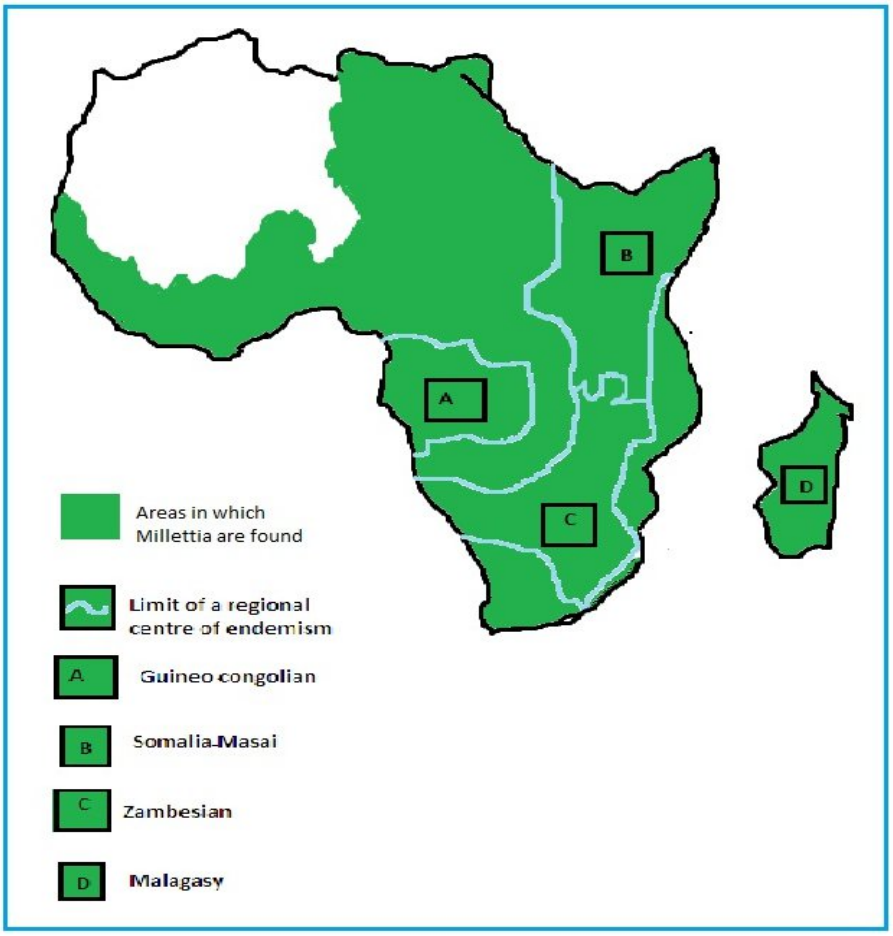


Figure 2.7: Distribution map of African *Millettia* species (Banzouzi *et al*., 2008).

2.3.1.2.1 *Millettia micans*

Millettia micans Taub (Figure **2**.**8**) is endemic to Tanzania (IUCN, 2006). It is found in East and South-East Tanzania, specifically in Pwani (Kisarawe) and Selous Game Reserve respectively (IUCN, 2006). It is a small tree restricted to areas of dry coastal forest and open wood land.

Figure 2.8: Collection of *Millettia micans*: (Picture taken by Marco M, 2013)

2.3.1.2.2 *Millettia oblata*

Millettia oblata Dunn (Figure **2**.**9**) is a threatened small tree with perennial lifespan. There are five subspecies of *Millettia oblata* Dunn. They are distributed in Tanzania and Kenya. Among these, *Millettia oblata* ssp. *teitensis* J.B.Gillet is found only from Taita Hills forest, Kenya (Collins *et al*., 1985).

Figure 2.9: *Millettia oblata* spp. *teitensis* (Catalogue of life, 2009)

2.4 Ethno-Medical and Pharmacological Information of the Genera *Millettia* and *Derris*

2.4.1 Ethno-Botanical Uses of the Genus *Millettia*

Plants of the genus *Millettia* have been used traditionally in various communities for the treatment of different ailments. However, the presence of poisonous principles in the plant tissue has been the primary reason for interest in this genus (Thasana *et al.,* 2001; Kumar *et al,* 1989). These toxic substances have served native uses as arrow poisons, fish poisons, insecticides, vermifuges, and febrifuges (Thasana *et al.,* 2001; Kumar *et al,* 1989). Several examples on the uses of *Millettia* are summarized in Table 2.**1.**

Table 2.1: Ethno-botanical uses of the genus *Millettia*

Species	**Plant part (s)**	**Uses**	**Reference (s)**
M. makondesis	Leaves	Treat toothache	Kokwaro, 1976
M. auriculata	Leaves Roots	Treat male infertility Used as fish poison, pesticide and vermicide	Choudhary *et al*., 1990
M. bicolor	Leaves Roots	Treat filaires (in eye), otitis(ear), teeth ache, leucorrhoea and vaginal diseases Treat heart aches	Lock, 1989
M. elongatistyla	Roots	Treat schistosomiasis	Hostettmann, 1984
M. dura	Roots and leaves	Treat hernias, diarrheas and painful menstruation	Lock, 1989

M. puguensis	Roots	Treat umbilical hernia	Lock, 1989;
M. oblata	Root bark Roots	Treat stomach aches and cough Treat swollen parts and bladder problems	Lock, 1989
M. usaramensis	Roots Roots pulp	Fish poison and treat convulsion Snake bite	Lock, 1989
M. extensa	Roots Root bark	Treat stomach pain Prevent conception	Singh *et a*l., 1994

The genus *Millettia* has numerous therapeutic indications, such as antitumor, anti-inflammatory, antiviral, bactericidal, insecticidal and pesticidal (Banzouzi *et al.*, 2008). The various uses of *Millettia* species in traditional medicine have been confirmed by pharmacological studies in laboratory and confer an interest in phytochemical research of active compounds (Banzouzi *et al.*, 2008). Table **2.2** below shows some biological activities of some *Millettia* species.

Table 2.2: Biological activities of some *Millettia* species

Plant species	**Plant part (s)**	**Biological activity**	**Reference (s)**
M. usaramensis	Stem bark	Antiplasmodial	Yenesew *et al*., 2003b
M. griffoniana	Root bark	Antiinflammatory	Yankep *et al*., 2003
M. leucantha	Stem bark	Antiinflammatory	Ampai *et al*., 2003
M. taiwaniana	Stem bark	Antitumor	Ito *et al*., 2004
M. versicolor	Root bark	Antiplasmodial,	Banzouzi *et al.*, 2008
M. duchesnei	Stem bark	Insecticidal	Banzouzi *et al.*, 2008
M. concraui	Root bark	α-glucosidase inhibitory	Banzouzi *et al.*, 2008
M. puguensis	Root bark	Anti-leishmanial	Banzouzi *et al.*, 2008
M. brandisiana	Leaves	Antiinflammatory	Pancharoen *et al*., 2008
M. aboensis	Root bark	Hepatpprotective	Ugwueze and Attama, 2013
M. thonningii	Leaves	Molluscicidal	Banzouzi *et al.*, 2008
M. griffoniana	Seeds	Antiparasitic	Banzouzi *et al.*, 2008
M. ferruginea	Leaves	Insecticidal	Banzouzi *et al.*, 2008
M. congolensis	Root bark	Antiviral	Banzouzi *et al.*, 2008

2.4.2 Ethno-Botanical Uses of the Genus *Derris*

Derris species are poisonous plants used locally for catching fish. These plants are also used extensively as pesticides as they elaborate large amounts of rotenoids, particularly rotenone (*de* Padua *et al*., 1999). Different parts of *Derris* species are used in traditional medicine for treatment of wounds, calculus, rheumatism and dysmenorrheal and asthma (*de* Padua *et al*., 1999). *Derris elliptica* and *Derris malaccensis* are used as fish poison throughout Southern Asia and Pacific (*de* Padua *et al*., 1999). The pounded roots are considered as the strongest fish poison in South East Asia. In addition, *Derris elliptica* is traditionally used for antisepsis and applied to abscesses and against leprosy and itch, and sometimes as an abortifacient (*de* Padua *et al*., 1999). The stems are sometimes used for rough cordage. The roots of *Derris scandens* are used in India to increase milk secretion after childbirth; it is crushed with or without water and the juice is given orally (*de* Padua *et al*., 1999). The whole plant of *Derris trifoliata* is used in India as a stimulant, anti-spasmodic and counter-irritant while the bark is used to treat rheumatism, chronic paralysis and dysmenorrhoea. In Papua-New Guinea, a decoction of the roots is applied externally against fever and internally against sores (*de* Padua *et al*., 1999). A solution of crushed leaves of *Derris elegans* is used to wash snake bites in Papua-New Guinea (*de* Padua *et al*., 1999). The stems are sometimes used for rough cordage. The wood of *Derris robusta* trees is sometimes used for tea chests and implements (*de* Padua *et al*., 1999).

Biological Activity of *Derris* Species

Extracts and metabolites from *Derris* species have been found to possess significant larvicidal, pesticidal, cytotoxic, antifungal, anti-inflammatory, antimicrobial, nitric oxide inhibitory, and cancer chemopreventive activities (*de* Padua *et al*., 1999). For example, *Derris elliptica* has been reported for antitumor (roots) and antimicrobial (leaves) activities. The roots of *Derris*

elliptica contain the rotenoid elliptinol and tubaic acid (0.01% of air-dried roots) while the leaves contain the amino-alcohol 2,5-dihydroxymethyl-3,4-dihydroxypyrrolidine (0.1% of fresh leaves) (*de* Padua *et al*., 1999). Table **2.3** below shows some biological activities of different *Derris* species.

Table 2.3: Biological activities of different *Derris* species

Plant species	Part (s)	Biological activity	Reference (s)
D. *brevipes van brevipes*	leaves	Antibacterial, antifungal, antitumor	Yuvaraj *et al*., 2010
D. trifoliata	Roots	Anti-oxidant, antibacterial	Sharief *et al.,* 2014
D. trifoliata	leaves	Anti-oxidant, analgesic	Sarkar *et al*., 2012
D. robusta	Seed shells	Insecticidal, pisicidal	Chibber and Sharma, 1979b
D. robusta	Roots	Insecticidal, pisicidal	Chibber and Sharma, 1979a
D. scanden	Stems	Immunostimulant, antiinflamatory	Rukachaisirikul *et al.*, 2002
D. amazonica	Roots	Pisicidal	Munoz *et al*., 2000
D. malaccensis	Roots	Insecticidal	Toxopeus *et al*., 1992

2.5 Phytochemistry of the Family Leguminosae

The family Leguminosae has been extensively investigated for its secondary metabolites and this has resulted in the isolation of several secondary metabolites including alkaloids, flavonoids, isoflavonoids, terpenoids, anthraquinones and anthocyanin (Bisby *et al*., 1994). The family is reported to be rich in flavonoids and isoflavonoids, producing about 28% of all known flavonoids and 95% of all isoflavonoid aglycones (Bisby *et al*., 1994; Dewick, 1994). The isoflavonoids are almost exclusive found to the subfamily Papilionoideae of the Leguminosae (Dewick, 1994). The flavonoids isoliquiritigenin and liquiritigenin are considered as characteristic constituents of the family (Bisby *et al*., 1994). Furthermore, a variety of plant alkaloids has been recorded in this family but the most wide spread and characteristic of the family are the quinolizidine and lupine alkaloids (Bisby *et al*., 1994). In addition, a wide range of

terpenoids has been reported, the most characteristic constituents being diterpene acids (Bisby *et al*., 1994).

2.5.1 Flavonoids

Flavonoids are secondary metabolites with a C_6-C_3-C_6 arrangement (Figure **2**.**10**) found across the plant kingdom with over 9,000 structural variants known (Buer, 2010). The chemical diversity, size, three-dimensional shape, and physical and biochemical properties of flavonoids allow them to interact with targets in different subcellular locations to influence biological activity in plants, animals, and microbes (Buer, 2010).

Flavonoids are produced from two main primary biosynthetic pathways- the acetic acid pathway and the shikimate pathway (Scheme **2**.**1**). Phenylalanine and tyrosine from the shikimate pathway are converted to cinnamic acid and para-hydroxycinnamic acid, respectively, by the action of phenylalanine and tyrosine ammonia lyases (Harrison *et al*., 2011). Either one of these can be extended with three molecules of malonyl CoA in a head-to-tail manner in order to form a tetraketide intermediate (Dewick, 1994). The process is catalyzed by the enzyme chalcone synthase (CHS). The intermediate then folds and condenses further to give two chalcones (naringeninchalcone and isoliquiritigenin) under the catalyzation of CHS in the first case and CHS together with NADPH in the second case, respectively (Stevens *et al.,* 1998). The chalcone isomerase (CHI) catalyzes the stereospecific intramolecular cyclization of isoliquiritigenin (chalcone) and naringeninchalcone (chalcone) into a (2*S*)-liquiritigenin (7,4'-dihyroxyflavanone) and (2*S*)-naringenin (5,7,4'-trihyrdoxyflavanone), respectively (Dewick, 1994; Stevens *et al.,* 1998). These two flavanones are the precursors of flavonoids in general and all sub-classes of

flavonoids are derived from these by a variety of routes. For example, further reactions such as oxidation, reduction, hydroxylation, methylation, prenylation and gycosylation leads to flavan, flavanone, dihydroflavonol, flavonol, flavone, flavone-3-ol and flavone-3, 4-diol and their glycosides (Samanta *et al*., 2011., Stevens *et al*., 1998). These reactions give rise to the enormous structural diversity of natural flavonoids as shown on Figure **2**.**10**.

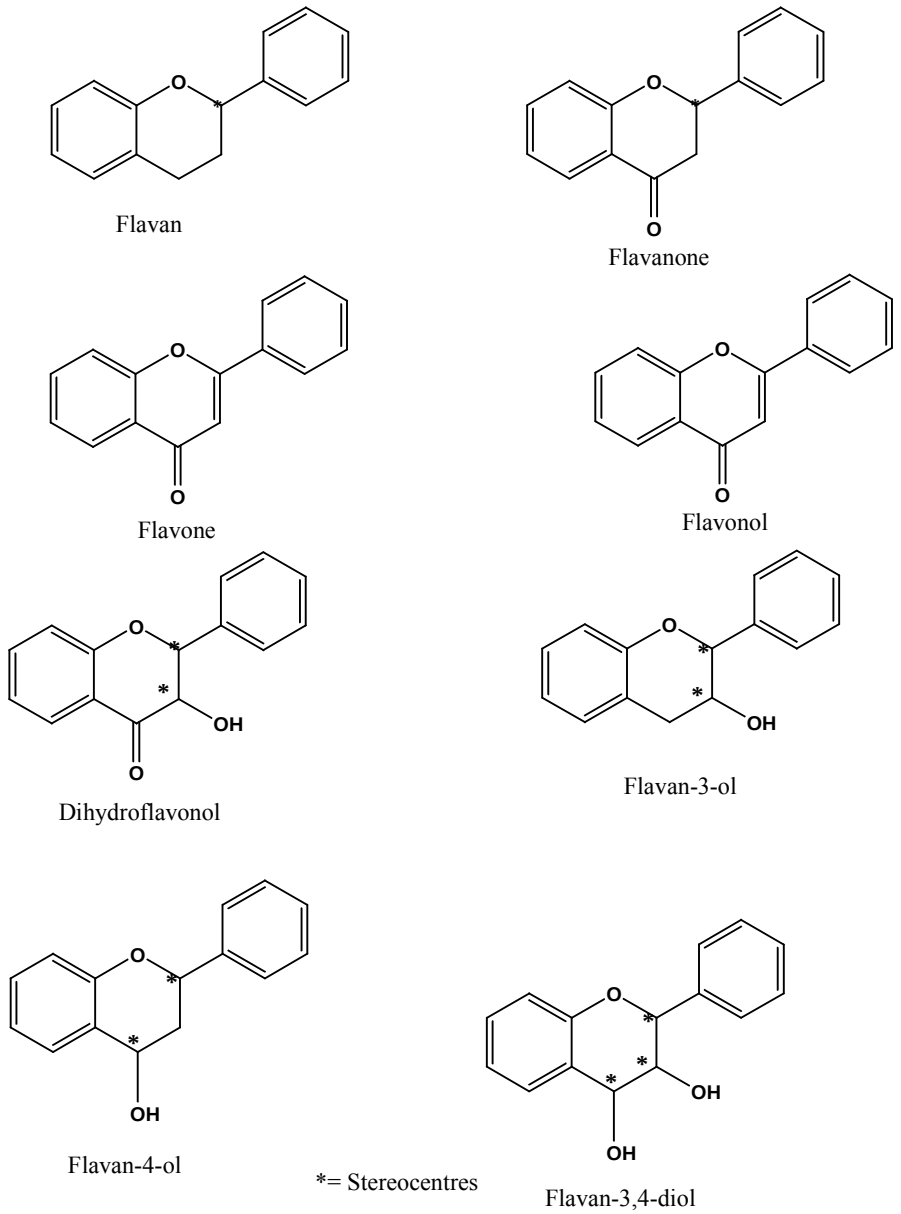
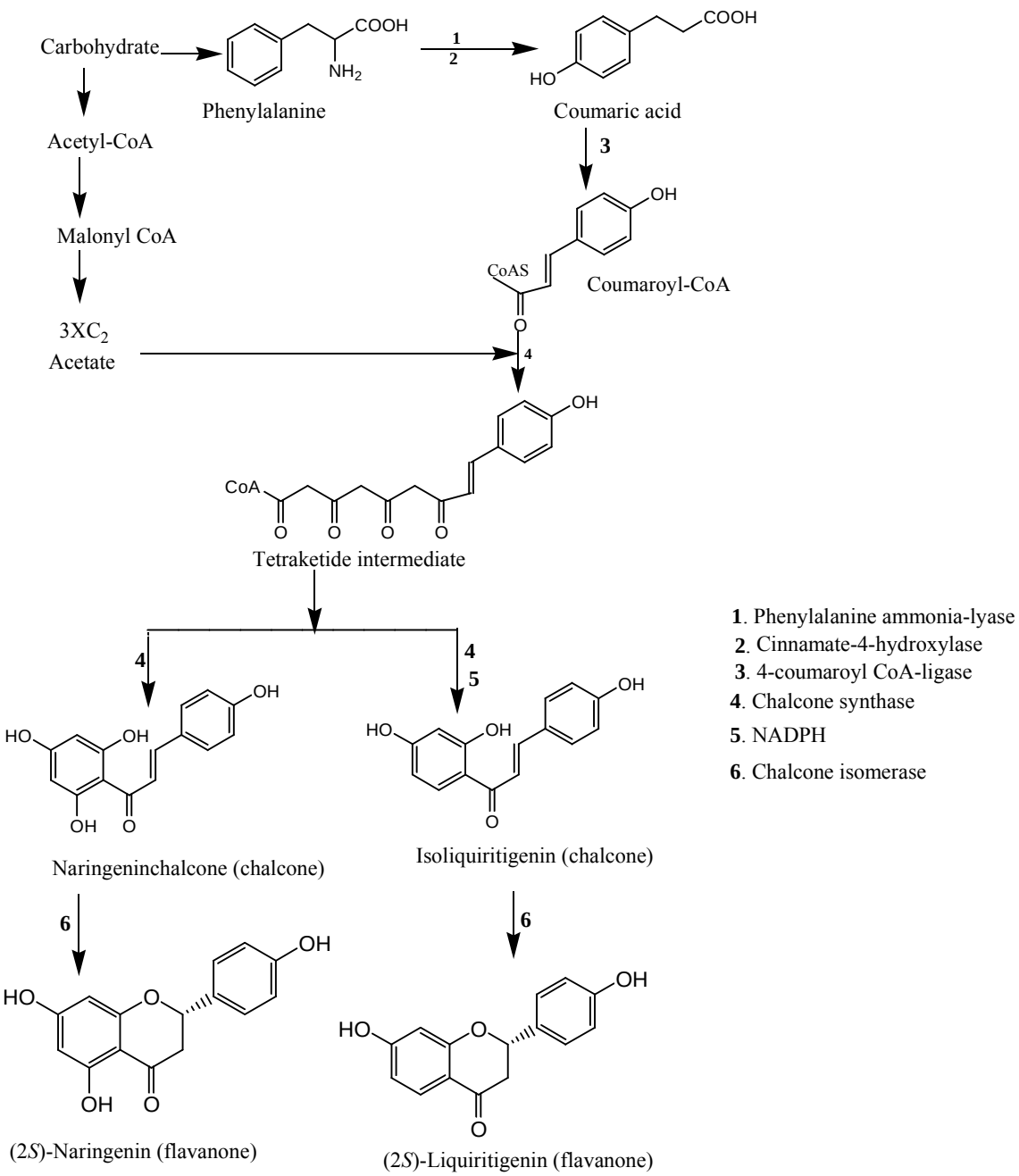


Figure 2.10: Structural diversity of natural flavonoids (Grotewold, 2006)



Scheme 2.1: Biosynthetic route to flavonoids (Dewick, 1994; Harrison *et al*., 2011)

2.5.2 Isoflavonoids

The isoflavonoids belong to a large and very distinctive subclass of flavonoids. These compounds possess a 3-phenylchroman skeleton that is biogenetically derived by rearrangement of the flavonoid 2-phenylchroman system by means of a 1,2-aryl migration (Harborne, 1994). The isoflavonoid pathway begins by the abstraction of a hydrogen radical at C-3, followed by ring B migration from C-2 to C-3 and subsequent hydroxylation of the resulting C-2 radical. This reaction is catalyzed by isoflavone synthase (IFS) or 2-hydroxyisoflavanone synthase regioselectively. The resulting 2-hydroxyisoflavanone is unstable and undergoes dehydration to form isoflavone. The dehydration reaction can take place non-enzymatically. Consequently, daidzein is formed from liquiritigenin and genistein from naringenin as shown in scheme **2.2** (Heinonen, 2006).

Depending upon the degree of oxidation and saturation in the heterocyclic ring, isoflavonoids are further divided into the subgroups: isoflavan, isoflavone, isoflavanone, isoflavan-3-ene, isoflavanol, rotenoids, coumestane, 3-arycoumarin, coumaronochromone and pterocarpans (Samanta *et al*., 2011). The structural diversity of natural isoflavonoids is shown in Figure **2.11**.

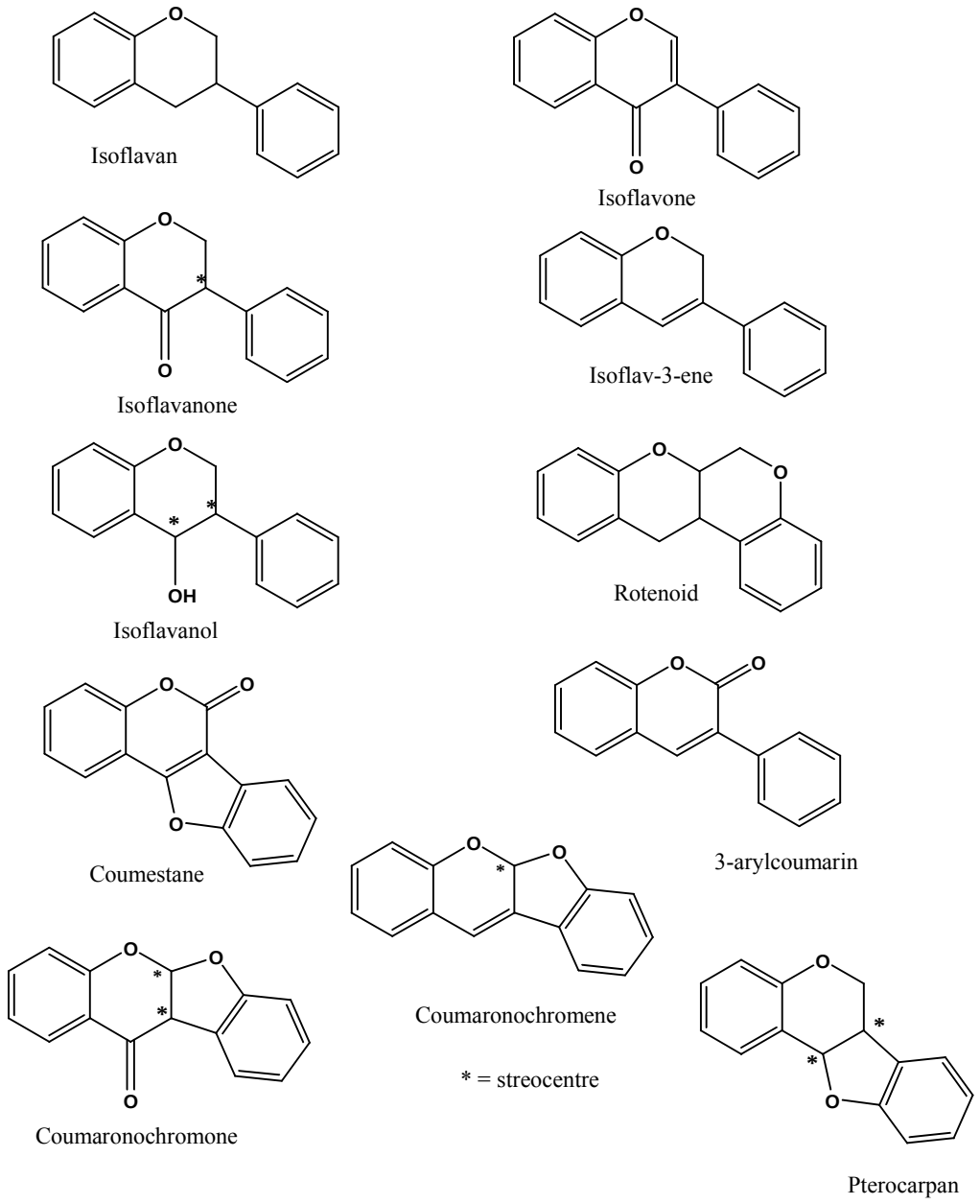
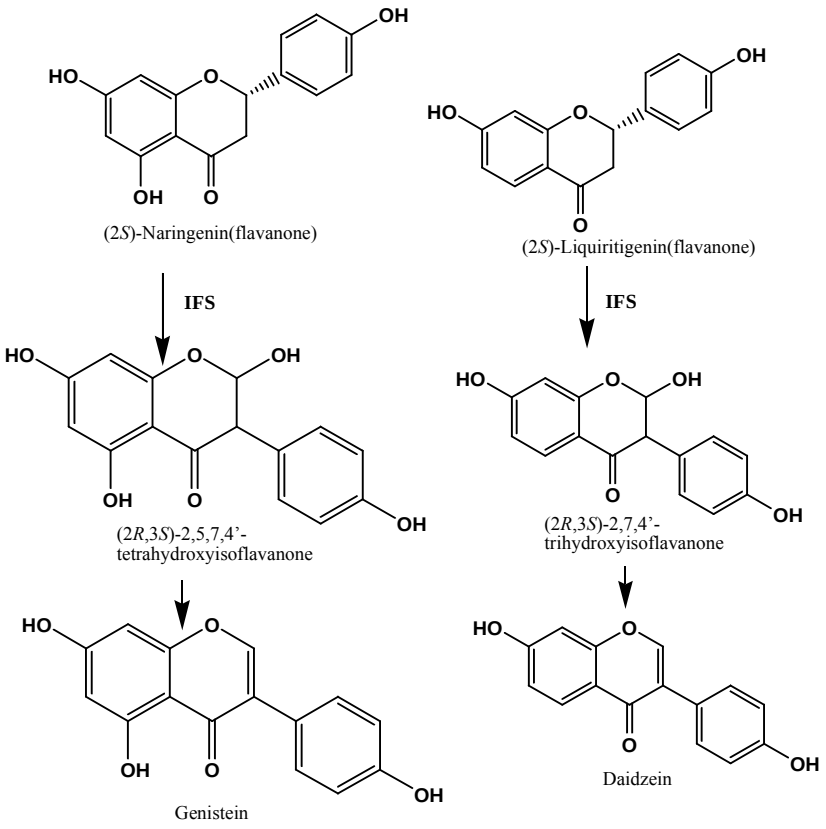


Figure 2.11: Structural diversity of natural isoflavonoids (Grotewold, 2006)

In majority of the cases, the isoflavonoids found in these genera contain 3-methylbut-2-enyl (isoprenyl) or 2,2-dimethylpyrano substituent on ring A. Hydroxylation, methoxylation and methylenedioxy formation are common in ring B. Some species have the ability to deoxygenate the C-5 position and on the other hand additional oxygenation at C-6 has been observed in some

species, e.g. isoflavones from *Millettia dura* (Ollis *et al*., 1967) and *Millettia ferruginea* (Dagne *et al*., 1989).



Scheme 2.2: Biosynthetic route to isoflavonoids (Heinonen, 2006).

2.5.3 Some Compounds of the Genus *Millettia*

Previous studies on different species of the genus *Millettia* revealed the presence of chalcones, isoflavones, rotenoids (Dagne *et al*., 1989; Yenesew *et al*., 1998a, b), isoflavans (Khalid *et al*., 1983), flavanones, isocoumarins (Baruah *et al.,* 1984) and pterocapans (Sritularak *et al*. ,2002a).

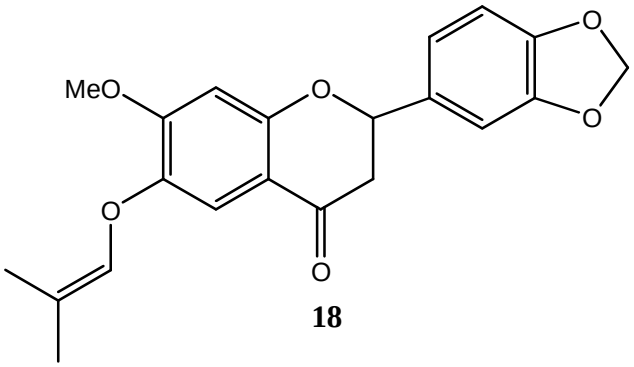
The presence of isoflavonoids and rotenoids is compatible with various claims that some *Millettia* species show insecticidal and piscicidal activities (Ollis, 1967).

2.5.3.1 Flavanones

Flavanones from genus *Millettia* are prenylated and lack oxygenation at C-5 position. They possess a stereocenter at the C-2 which potentially leads to the existence of two stereoisomeric forms (Maltese *et al*., 2009). Most of the known flavanones have the 2*S* configuration, but racemization can occur during extraction and/or chromatography. Some of the flavanones of the genus *Millettia* are listed in Table **2.4**.

Table 2.4: Some selected flavanones of the genus *Millettia*.

Flavanone	**Source**	**Reference**
Ponganone (**18**)	*M. erythrocalyx* (root bark)	Sritularak *et al*., 2002b
Ovaliflavanone A (**19)**	*M. ovalifolia* (seed)	Gupta *et al*., 1976a
Eriodictyol (**20**)	*M. duchesnei* (aerial parts)	Ngandeu *et al*., 2008
Ovalichromene B (**21**)	*M. ovalifolia* (seed)	Gupta *et al*., 1976b
Sophoranone (**22**)	*M. pulchra* (aerial parts)	Baruah *et al*., 1984
7-Prenyloxyflavanone (**23)**	*M. erythrocalyx* (root bark)	Sritularak *et al*., 2002b
4'-HydroxyIsolonchocarpin (**24**)	*M. ferrugineae* (stem bark)	Dagne *et al*., 1989
Milletenin B (**25)**	*M. ovalifolia* (leaf)	Khan *et al*., 1974



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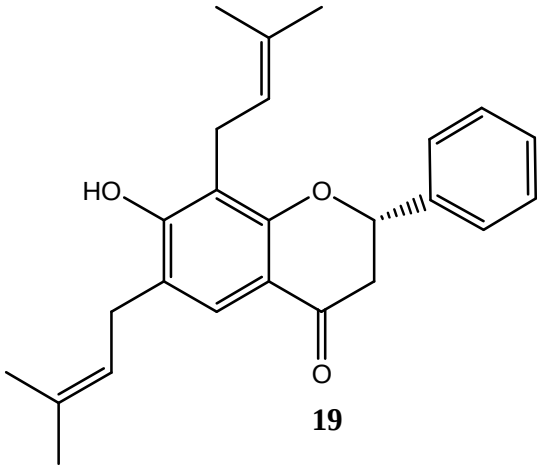

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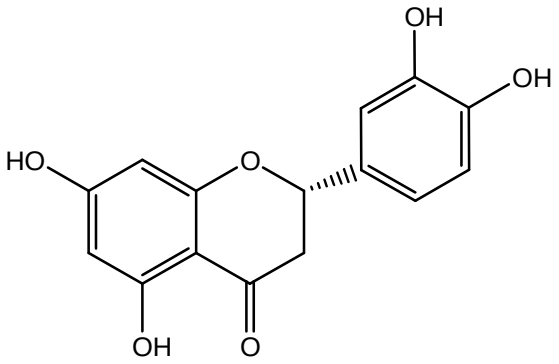

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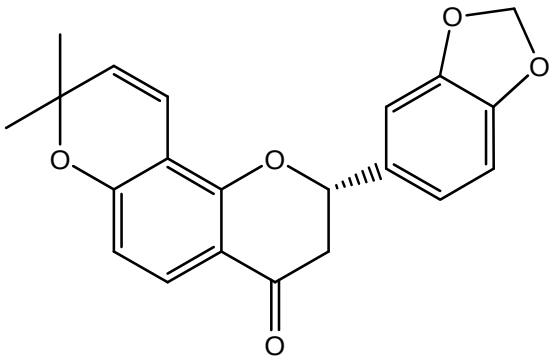

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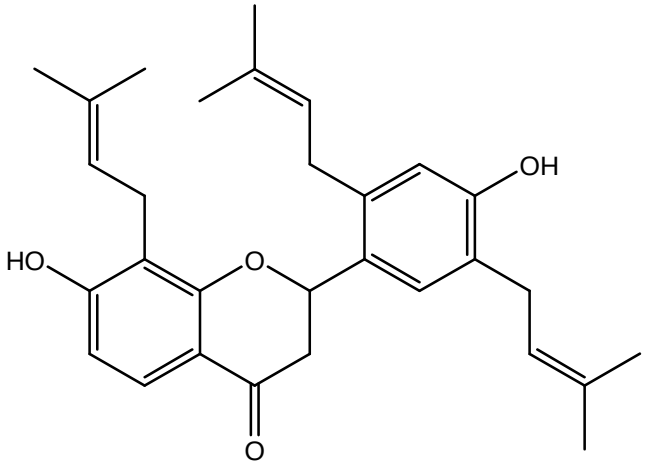

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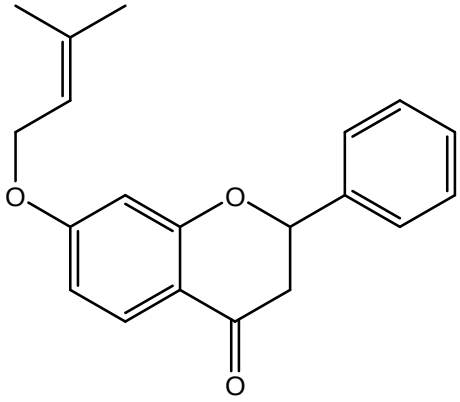

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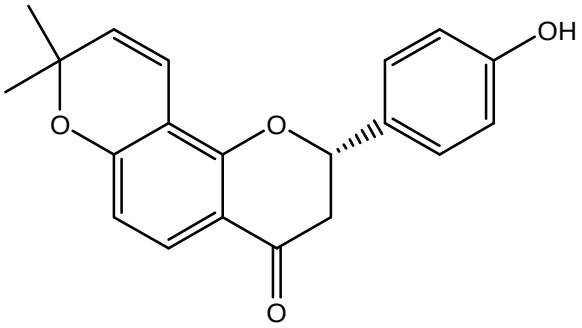

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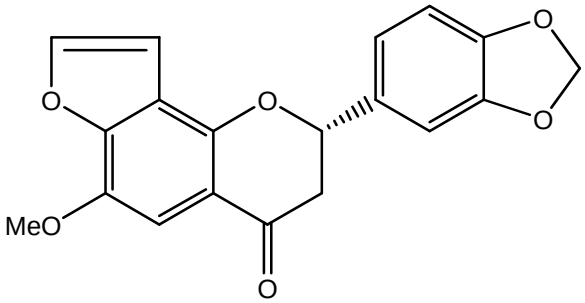

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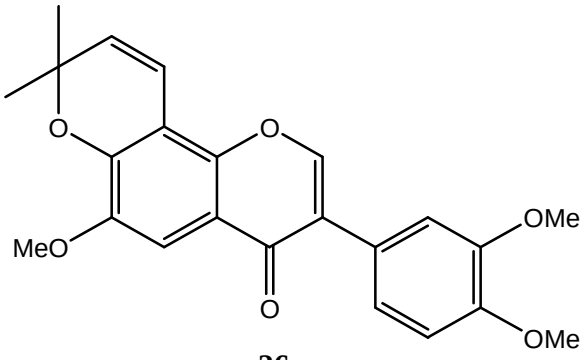
2.5.3.2 Isoflavones

Isoflavones constitute the largest group of natural isoflavonoids (Dewick, 1994). Some of these isoflavones lack oxygenation at C-5 (C-5 deoxygenated) (which is a feature unique to Leguminosae), or are prenylated which in some cases are modified to furano/pyrano ring while others are both prenylated and C-5 deoxygenated. Some isoflavones reported from the genus *Millettia* are listed in Table **2.5**.

Table 2.5: Some selected isoflavones of the genus *Millettia*

Isoflavone	**Source (s)**	**Reference (s)**
Durallone (**26**)	*M. dura* (Seed pod)	Yenesew *et al*., 1996
Durlettone (**27**)	*M. dura* (Seed)	Ollis *et al.,* 1967
Durlmillone (**28**)	*M. griffonianone* (Root bark)	Yankep *et al*., 1997
Isoaurmillone (**29**)	*M. auriculata* (Seed pod)	Gupta *et al*.,1983
Isojamaicin (**30**)	*M. ferruginea* (Stem bark) *M. usaramensis* (Stem bark)	Dagne *et al*., 1989 Yenesew *et al*., 1998b
Formononetin (**31**)	*M. dielsiana* (Stem bark)	Rui *et al* ., 1989
5-Methoxydurmillone (**32**)	*M. conraui* (Stem bark) *M. ferruginea* (Stem bark)	Fuendjiep, *et al*., 1998 Dagne, 1989
Millettin (**33**)	*M. auriculata* (Root bark) *M. auriculata* (Seed)	Shabbir, 1970 Raju, 1978
Prebarbigerone (**34**)	*M. ferruginea* (Root bark)	Dagne, 1990
Milldurone (**35**)	*M. dura* (Seed)	Ollis, 1967
Aurimillone (**36**)	*M. auriculata* (Seed)	Raju, 1978
Odoratin (**37**)	*M. griffonianone* (Root bark)	Yankep *et al*., 1997



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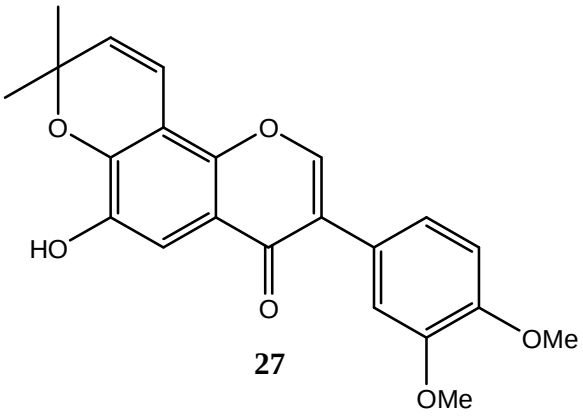

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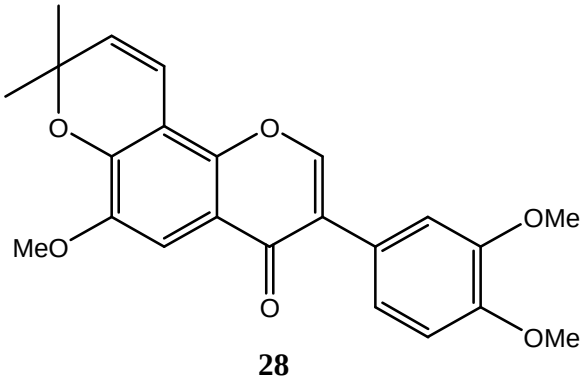

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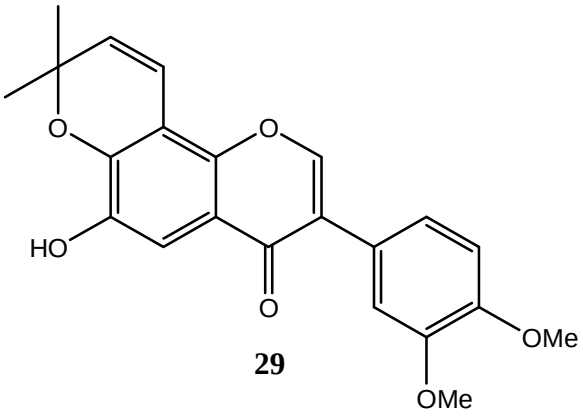

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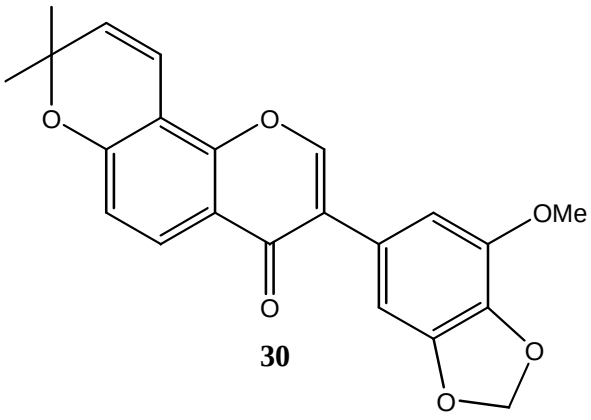

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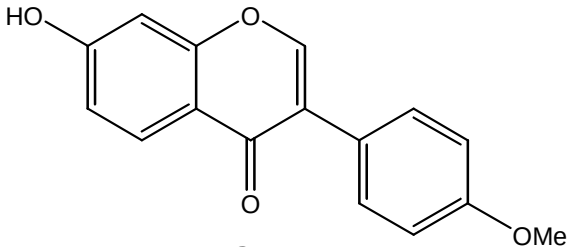

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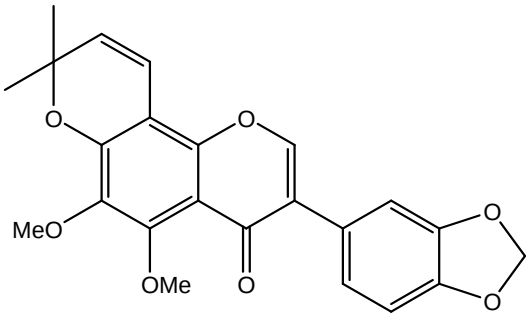

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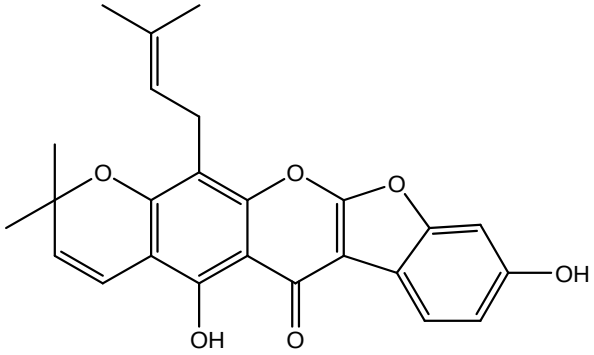

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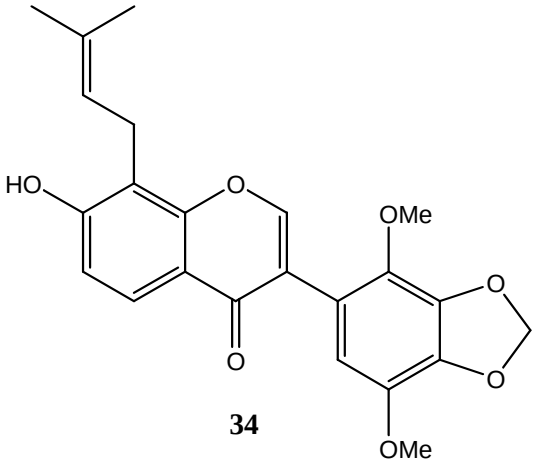

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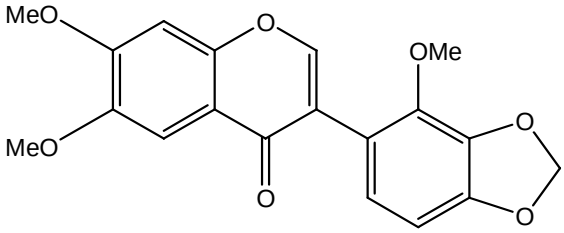

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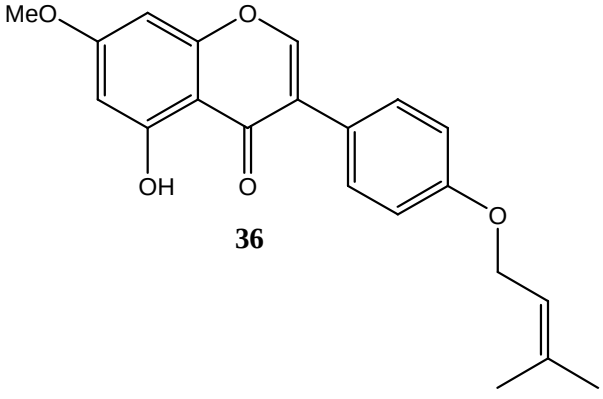

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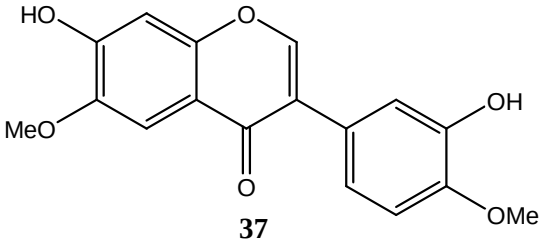

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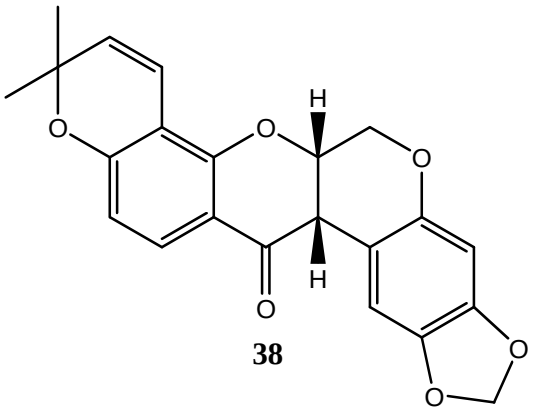
2.5.3.3 Rotenoids

Rotenoids are characterized by inclusion of an extra carbon atom into a heterocyclic ring. Most of the known rotenoids (Table **2**.**6**) contain an isoprenoid substituent and are noted for their insecticidal, piscicidal and antiviral activities. For example, rotenone contains an isopropenylfurano ring formed from cyclization of a C_5 isoprene unit (as do most natural rotenoids) introduced to demethylmunduserone (Scheme **2**.**3**). It has been shown that the isopropenylfurano system of rotenone and even the dimethylpyrano of deguelin are formed *via* rotenonic acid without any detectable epoxide or hydroxy intermediates (Scheme **2**.**3**).

Table 2.6: Some selected rotenoids of the genus *Millettia*

Rotenoids	**Source**	**Reference**
Millettone (**38**)	*M. dura* (seed)	Ollis *et al.,* 1967
Deguelin (**1**)	*M. dura* (seed)	Yenesew *et al*., 1997b
Tephrosin (**2**)	*M. usaramensis* (seed)	Yenesew *et al*., 1997b
Sumatrol (**39**)	*M. auriculata* (seed)	Shabbir *et al*., 1968
Stemonal (**40**)	*M. brandiasa* (leaves)	Pancharoen *et al*., 2008
Rot-2'–enoic acid (**41**)	*M.pachycarpa (seed)*	Singhal, 1982
Millettosin **(42**)	*M. dura* (seed)	Ollis *et al.,* 1967
(+)-Usararotenoid-A (**43**)	*M. usaramensis* (stem bark)	Yenesew *et al*., 1998b
(+)-Usararotenoid-B **(44**)	*M. usaramensis* (stem bark)	Yenesew *et al*., 1998b
12a-Epimillitosin (**45**)	*M. usaramensis* (stem bark)	Yenesew *et al*., 1998b



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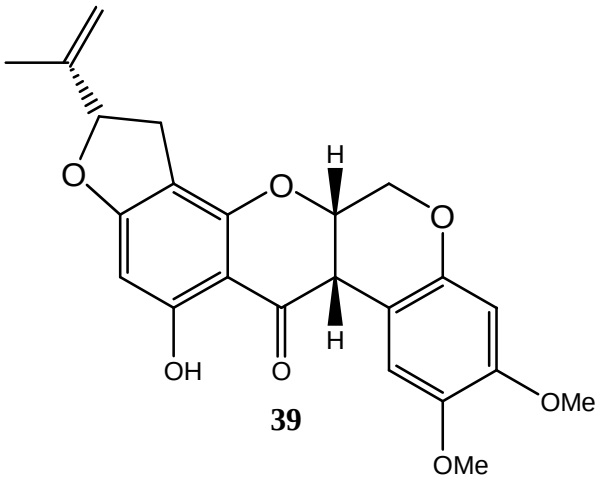

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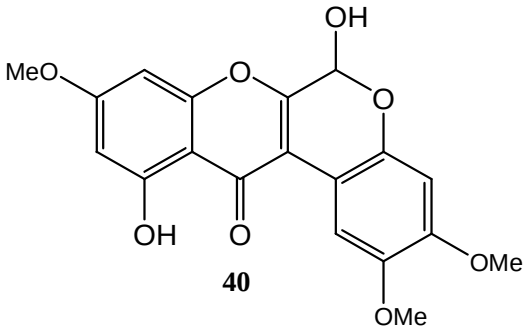

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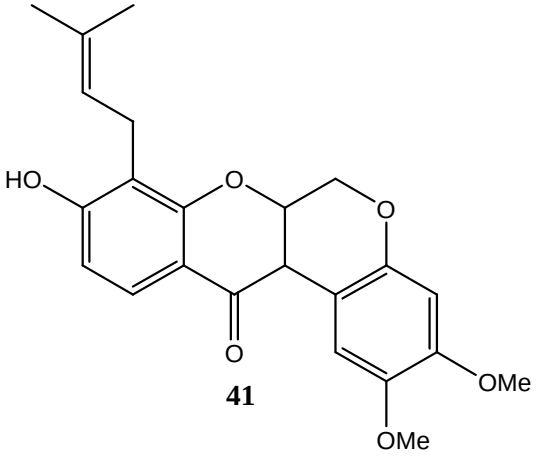

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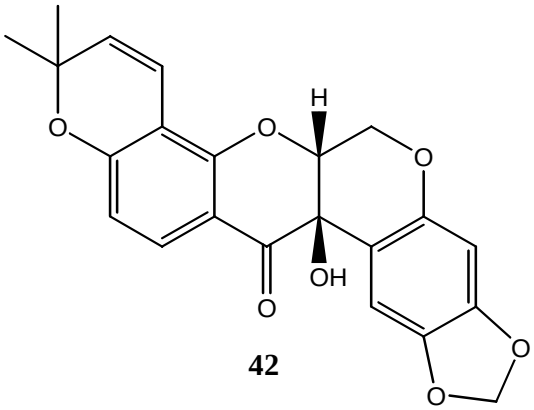

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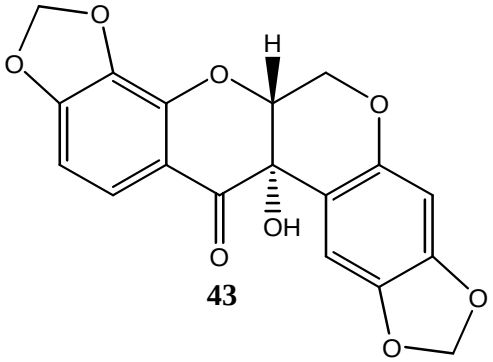

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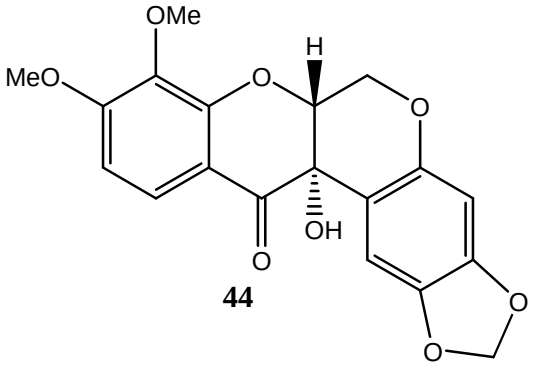

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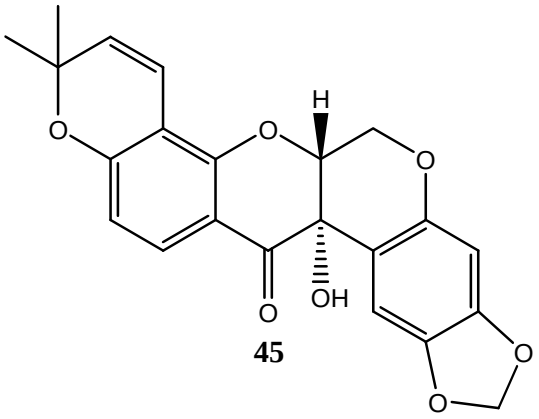

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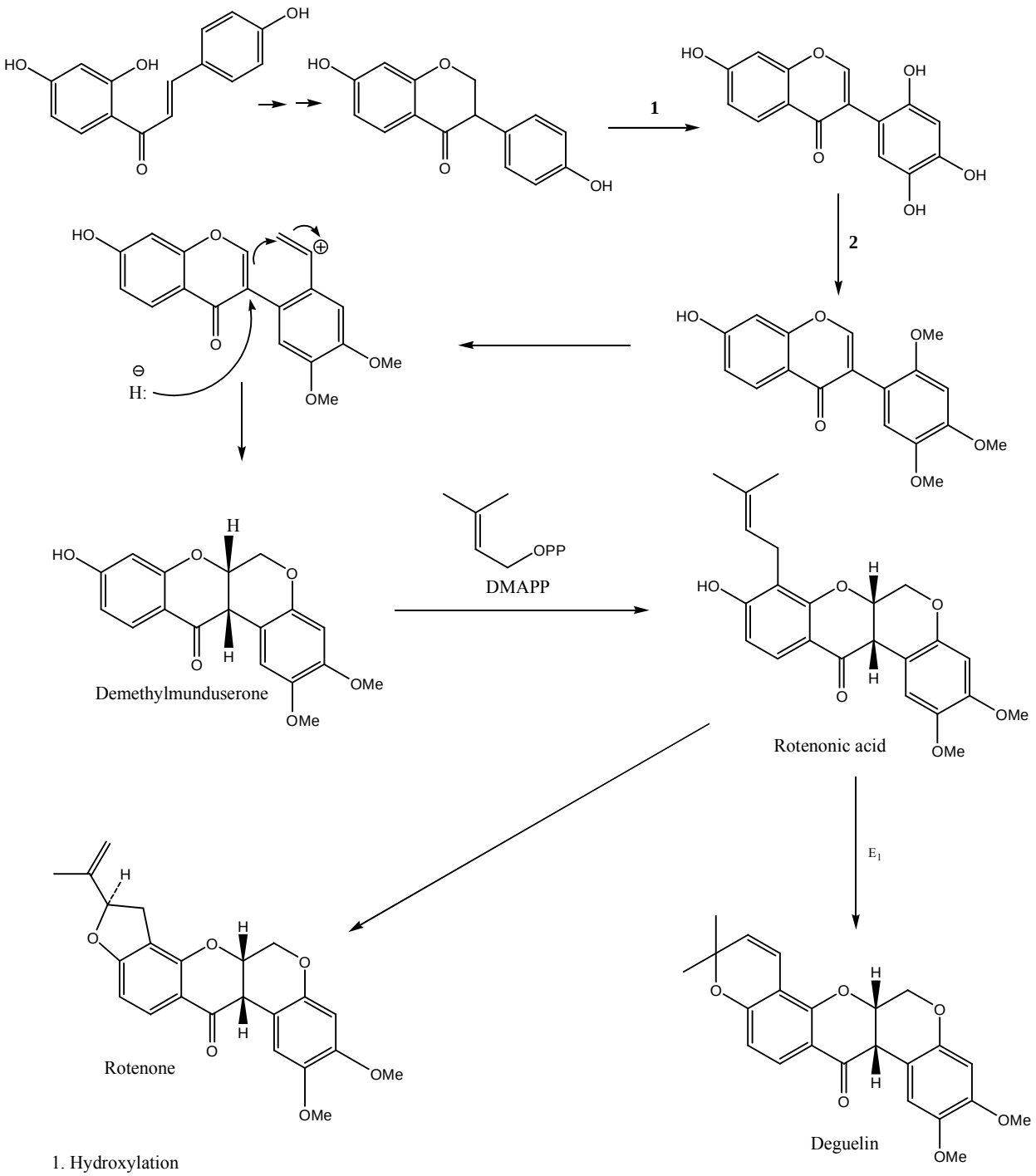


1. Hydroxylation

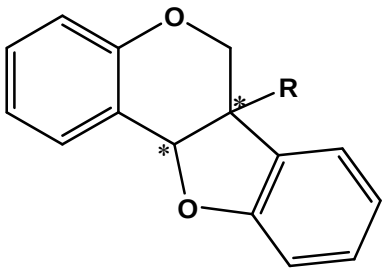
2. Methylation with Methionine

E_1: Deguelin cyclase

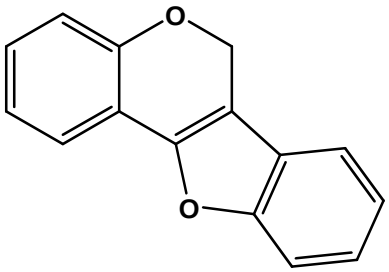
Scheme 2.3: Biosynthetic pathway to rotenone and deguelin (Dewick, 2009)

2.5.3.4 Pterocarpanoids

Pterocarpanoids are the second largest group of isoflavonoids after isoflavones produced by leguminous plants (Sobolev *et al*., 2010). They have a C_6-C_3-C_6 fundamental ring system of either coumarano [3, 2-C] chroman (**46**, **47**) or oxidized forms of pterocarpene (**48**) and coumestone (**49**).

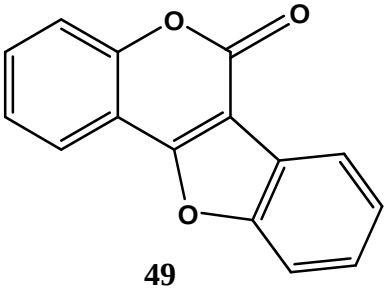


* stereo centre



48

46 R = H, Pterocarpan
47 R = OH, 6a-Hydroxypterocarpan



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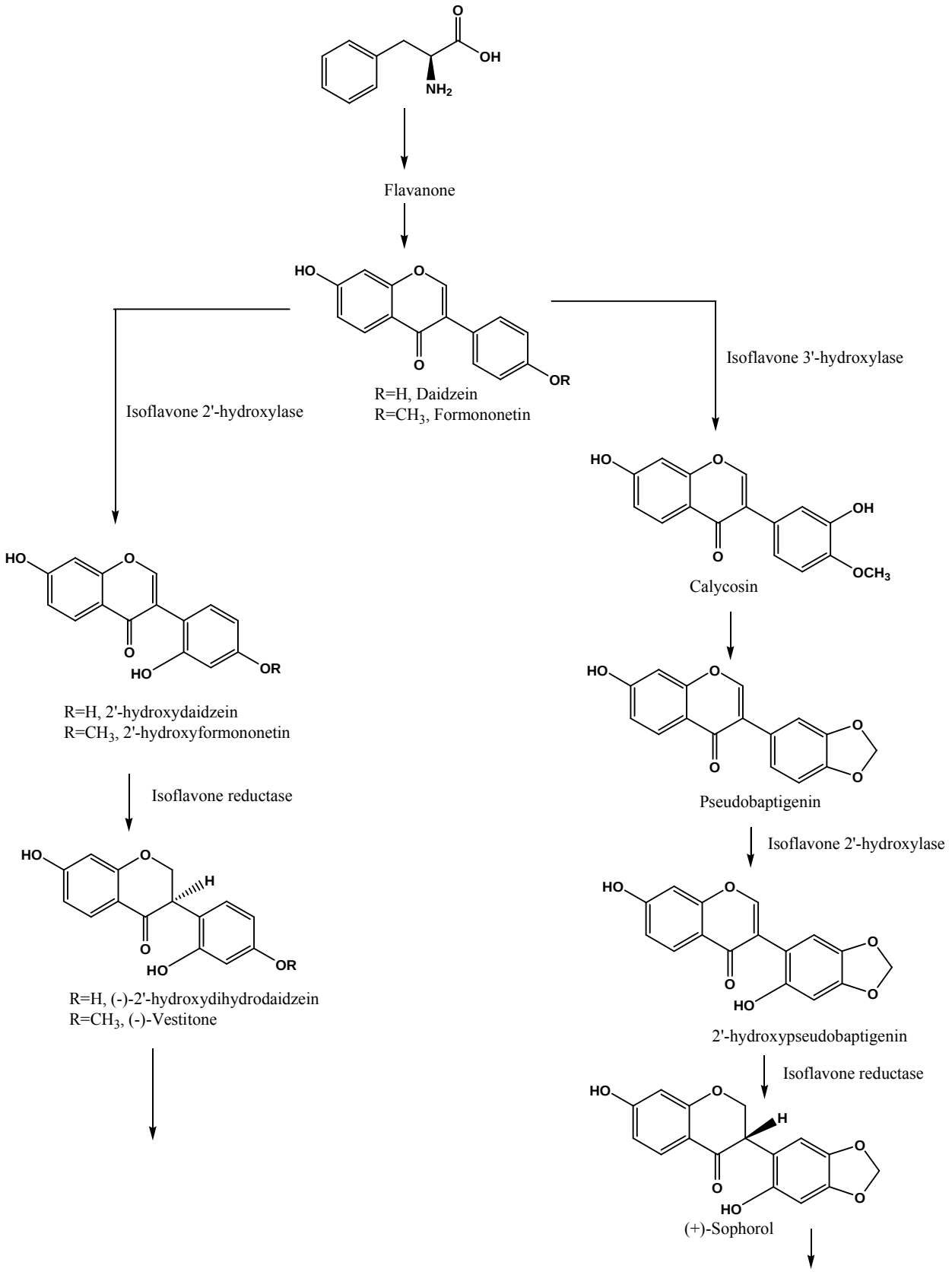
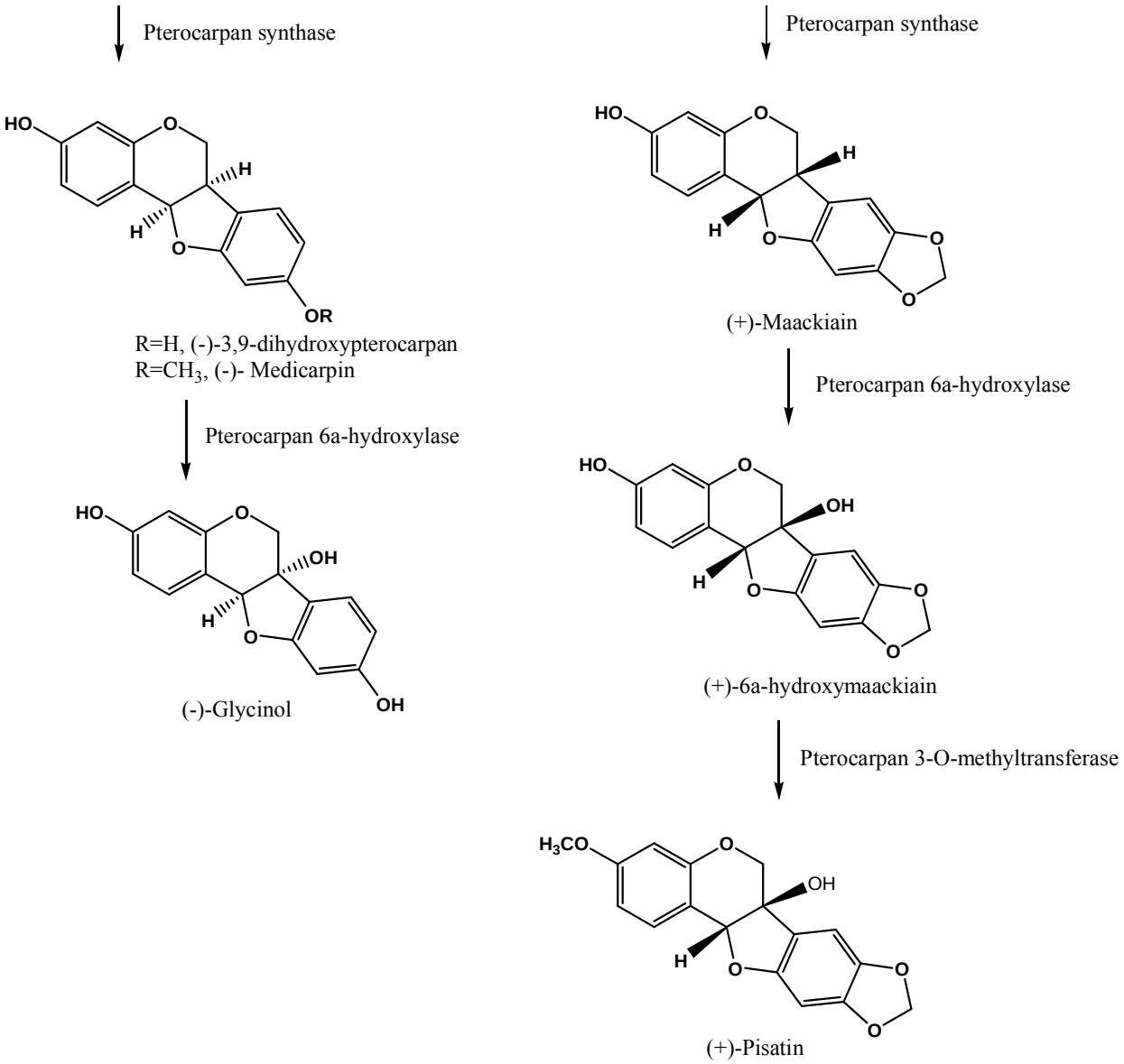
Pterocarpanoids have asymmetric centers at C-6a and C-11a, as a result of which four isomers are possible. They are produced in plants during infection by fungi, bacteria or viruses (Kiss *et al*., 2002). They are divided into four groups depending on the level of oxidation in ring B and at B/C ring junction. These are pterocarpans, 6a-hydroxypterocarpans, pterocarpenes and coumestones.

Pterocarpans constitute the largest sub-group among pterocarpanoids. They possess a 6a,11a-dihydro-6*H*-benzofuro-benzopyran skeleton (Kiss *et al*., 2002). They contain a tetracyclic ring system that is derived from the basic isoflavonoid skeleton. Pterocarpans are isoflavans in which a furan ring is formed through generation of an ether link between the chromane and the 3-phenyl unit. Pterocarpans are derived from the 5-deoxy derivatives of isoflavanones and their precursor is daidzein (Diego *et al*., 2002). Scheme **2.4** shows the pathway of 6a-hydroxypterocarpans from 5-deoxyflavanones (Left column: via 3*R*-isoflavanones, Right column: via 3*S*-isoflavanones).


Flavanone
R=H, Daidzein
R=CH3, Formononetin
Isoflavone 2'-hydroxylase
Isoflavone 3'-hydroxylase
R=H, 2'-hydroxydaidzein
R=CH3, 2'-hydroxyformononetin
Isoflavone reductase
R=H, (-)-2'-hydroxydihydrodaidzein
R=CH3, (-)-Vestitone
Calycosin
Pseudobaptigenin
Isoflavone 2'-hydroxylase
2'-hydroxypseudobaptigenin
Isoflavone reductase
(+)-Sophorol

Cont'd



Scheme 2.4: Biosynthesis of 6a-hydroxypterocarpans from 5- deoxyflavanone (Kumar, 2010; Seigler, 1998; Stafford, 1990).

Pterocarpans possess the highest antifungal activity among the phytoalexins in the flavonoid-based group of compounds (Sobolev *et al*., 2010). Figure **2.12** below shows a pterocarpan skeleton.

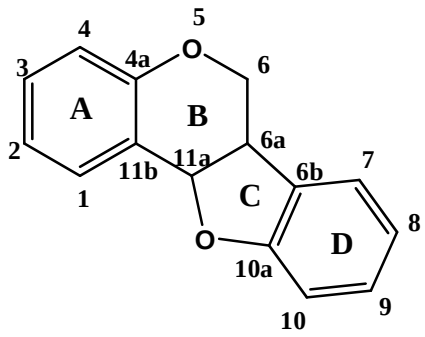


Figure 2.12: Pterocarpan skeleton and its numbering

Pterocarpans contain two stereocenters, this possibly leads to four stereoisomers, two *cis*, (-)-(6a*R*,11a*R*) and (+)-(6a*S*,11a*S*) and two *trans*, (-)-(6a*R*,11a*S*) and (+)-(6a*S,*11a*R*). Only two stereoisomers, 6a*R*,11a*R,* and 6a*S*,11a*S* configurations are energetically stable (Harborne, 1994). Dextrorotatory and racemic pterocarpans are the ones known in nature, and most of the chiral compounds have 6a*R*, 11a*R* absolute configuration (Kiss *et al*., 2002). Computational studies agree in that the *trans*-fused *B/C*-ring system (6a*R*,11a*S* and 6a*S*,11a*R*) is much less favorable than the *cis*-fused one, observed in all natural pterocarpans (Kiss *et al*., 2002). The *cis*-(6a,11a)-pterocarpan skeleton in which O-11 is *pseudo*-axially oriented with respect to the hydrogen at C-6 is the most stable conformation (Alagona *et al*., 2005; 2006).

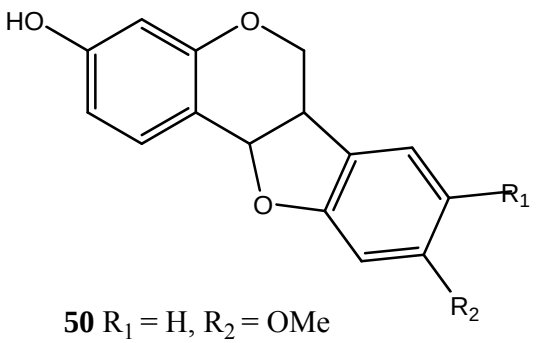
As the pterocarpans have two asymmetric centres, C-6a and C-11a, coupling constant ($J_{6aH,\ 11aH}$) of the aliphatic protons (6a H and 11a H) decides the conformation of the heterocyclic rings. The coupling constant ($J_{6aH,\ 11aH}$) for *cis*-conformation of the heterocyclic ring of pterocarpans ranges

between 5.0-7.0 Hz while for the *trans*-conformation ranges between 13.0-14.0 Hz (Pachler *et al*., 1967; Suginome, 1962).

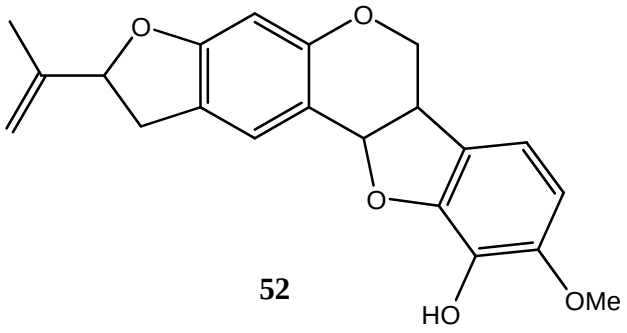
The structures of pterocarpans have been assigned by UV, NMR spectroscopy, mass spectrometery and circular dichroism. The UV spectrum of pterocarpans shows three characteristic absorption bands at 281, 287 and 311 nm (Suginome, 1959; 1962). In addition, pterocarpans are stable to electron impact ionization and they show intense molecular ion peaks in their mass spectra (Piccinelli *et al.,* 2005; Poster *et al.,* 1970). Some of the pterocarpans isolated from *Millettia* species are listed in Table **2**.**7**.

Table 2.7: Some selected pterocarpans of the genus *Millettia*

Pterocarpans	**Source (s)**	**Reference (s)**
Medicarpin (**50**)	*M. leptobotrya* Dunn (twigs)	Na *et al.,* 2013
Maackiain (**51**)	*M .leptobotrya* Dunn (twigs)	Na *et al.*, 2013
Pervilline (**52**)	*M. pervilleana* (root bark)	Palazzino *et al*., 2003
Pervillinine (**53**)	*M .pervilleana* (root bark)	Palazzino *et al*., 2003
4-Hydroxy-3-methoxy-8,9-methylenedioxypterocarpan **(54)**	*M. leucantha* (wood)	Rayanil *et al*., 2011



50 R_1 = H, R_2 = OMe
51 R_1+R_2 = OCH_2O



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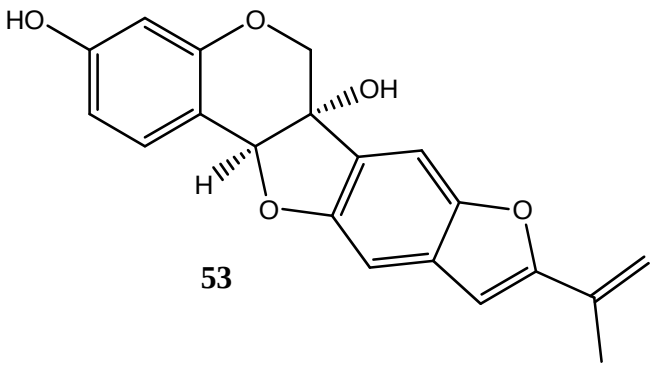

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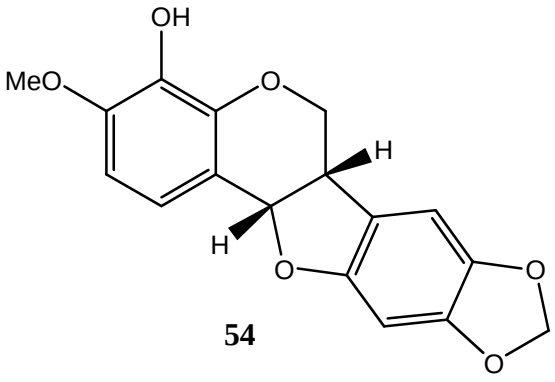

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2.5.4 Some Compounds of the Genus *Derris*

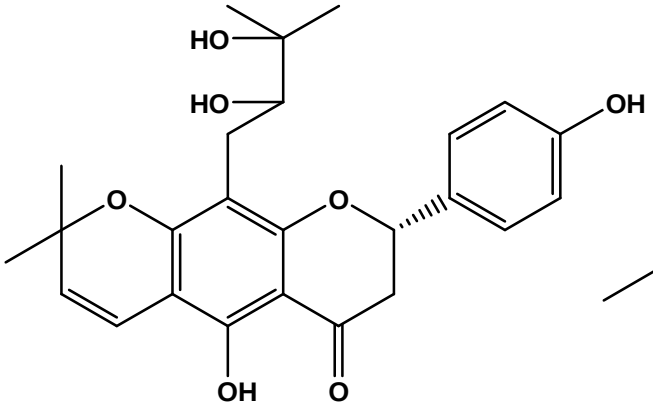
Derris species are noted for being a profuse source of many classes of phytochemicals including rotenoids, chalcones, coumarins, flavans, flavanones, flavones, glycosides, isoflavones, pterocarpans, quinoids, steroids, stilbenes aurones and terpenoids among others (Kongjinda, 2004). Among these, the flavanones, isoflavones, chalcones and rotenoids occur more widely. Representative examples are presented in the following sections.

2.5.4.1 Flavanones

Flavanones are the second largest group of flavonoids in this genus. The main features of flavanones of this genus is the oxygenation at C-5 in ring A and at C-4' in ring B. Prenylation mostly occurs at C-6 and at C-8. The other notable feature is the cyclization of the prenyl group at C-6 with the hydroxyl at C-7 to form the pyran ring. Table **2.8** shows some flavanones of this genus.

Table 2.8: Some selected flavanones of genus *Derris*

Flavanone	**Source (s)**	**Reference (s)**
2",3"-Dihydroxylupinifolin.**(55)**	*D.reticulata* (stem bark)	Mahidol *et al*., 2002
4',5-Dihyroxy-8-hydromethyl-6",6"-dimethylpyranol (2",3":7,6) flavanone **(56)**	*D.reticulata* (stem bark)	Mahidol *et al*., 2002
Dereticulatin **(57)**	*D. reticulata* (stem bark)	Mahidol *et al*., 1997
Derriflavanone **(58)**	*D. laxiflora* (root bark)	Lin *et al*., 1992
5,7-Dihydroxy-6-prenylflavanone **(59)**	*D. florobunda* (root bark)	Filho *et al*., 1975a
Epiderriflavanone **(60)**	*D. laxiflora* (root bark)	Lin *et al.,* 1992
2''',3'''- Epoxylupinifolin **(61)**	*D. reticulata* (stem bark)	Mahidol *et al*., 1997
5-Hydroxy-7-methoxy-6-prenyl flavanone (**62**)	*D. rariflora* (wood)	Filho *et al*., 1975b
3'-Methoxylupinifolin **(63)**	*D. laxiflora* (root bark)	Lin *et al*., 1992
Senegalensein (**64**)	*D. scandens* (stem bark)	Chuankamnerdkam *et al*., 2002



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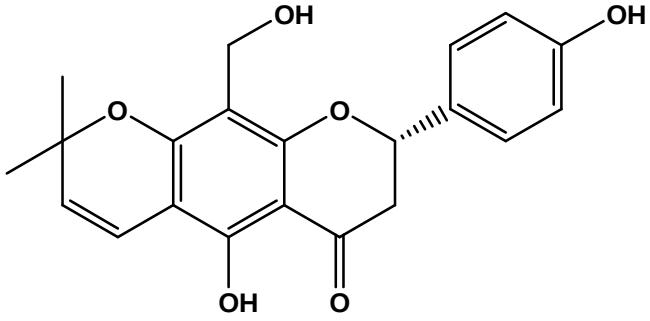

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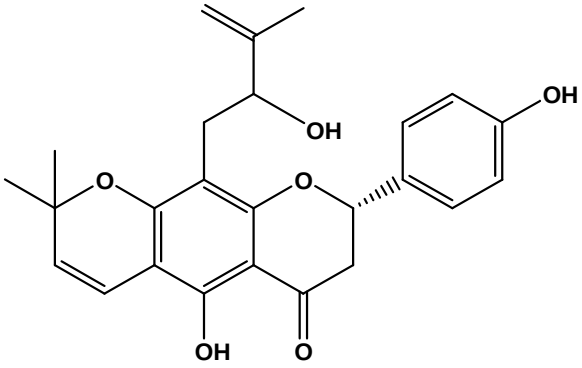

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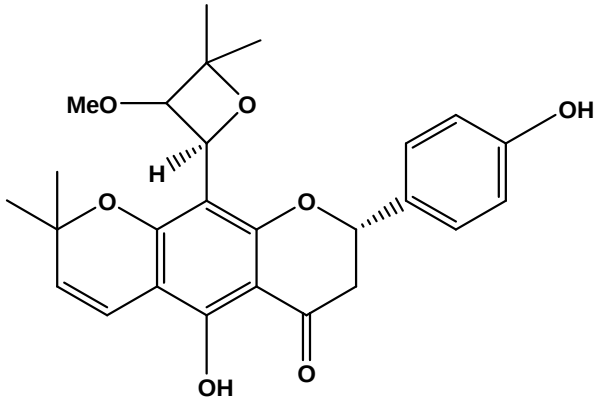

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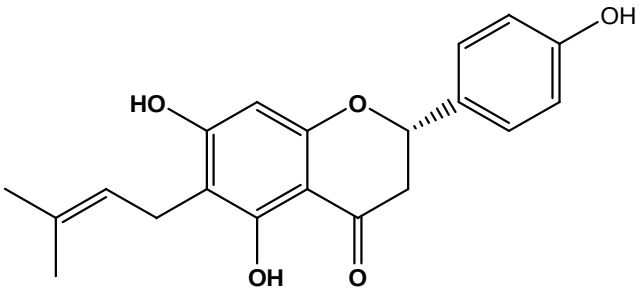

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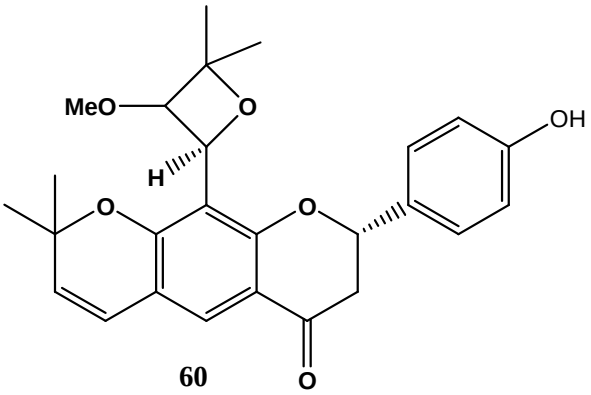

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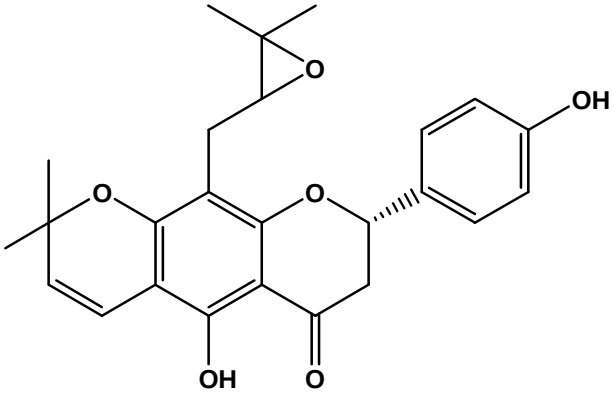

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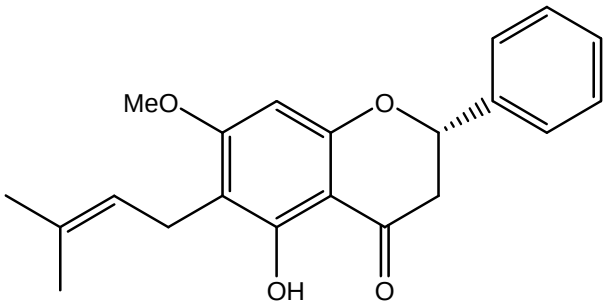

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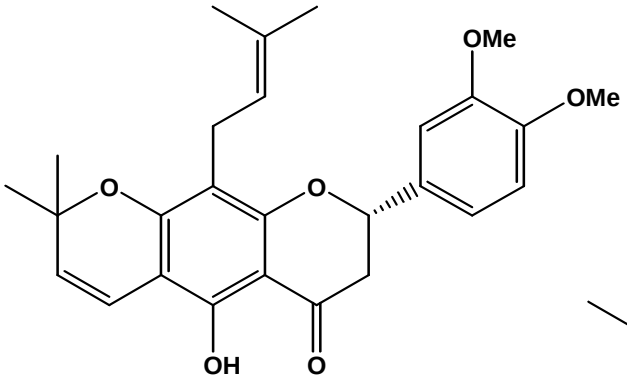

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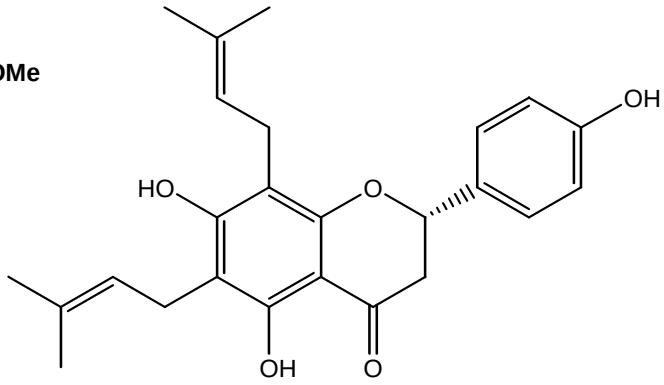


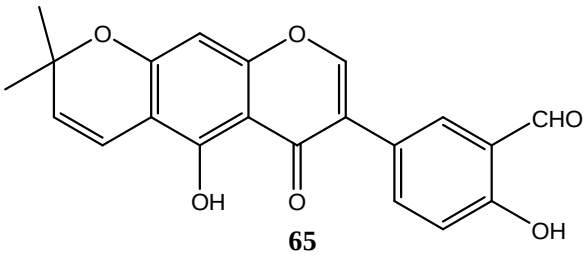
64

2.5.4.2 Isoflavones

Most of the isoflavones of this genus are prenylated and this occurs mainly at C-6 and/or C-8 in ring A and C-3' and/or C-5' in ring B. Presence of a furano ring in isoflavones of *Derris* is rare. Some representative of isoflavones from *Derris* species are presented in Table **2**.**9**

Table 2.9: Some selected isoflavones of genus *Derris*

Isoflavone	**Source (s)**	**Reference (s)**
3'-Formylalpinumisoflavone (**65**)	*D. scandens* (stem bark)	Chuankamnerdkarn *et al*., 2002
Lupinisol (**66**)		Rukachaisirikul *et al*., 2002
Lupalbigenin (**67**)		Chuankamnerdkarn *et al*., 2002
Senegalensin (**68**)		
Lupinisoflavone G (**69**)		
2,3-Dihyro-3-hyrdo-2-1(1-hydroxy-1-methylethyl) furanoalpinumisoflavone (**70**)		
Derriscandenoside B (**71**)		Rukachaisirikul *et al*., 2002
Derriscandenoside C (**72**)		
Derriscandenoside A (**73**)		
Ononin (**74**)		
Chandalone (**75**)	*D. scandens* (root bark)	Falshaw *et al*., 1969
Diadzein (**76**)	*D. oblonga* (root bark)	Lin and Kuo, 1995
Derrisisoflavone A (**77**)	*D. scandens* (stem bark)	Sekine *et al*., 1999
Derrisisoflavone B (**78**)		
Derrisisoflavone C (**79**)		
Derrisisoflavone D (**80**)		
Derrisisoflavone E (**81**)		
Derrisisoflavone F (**82**)		
Derrone (**83**)	*D. robusta* (seed)	Chibber and Sharma, 1980
Derrubone (**84**)	*D. robusta* (root bark)	East *et al*.,1969
Derrugenin (**85**)	*D. robusta* (seed shells)	Chibber and Sharma, 1979c
Derrustone (**86**)	*D. robusta* (root bark)	East *et al*., 1969



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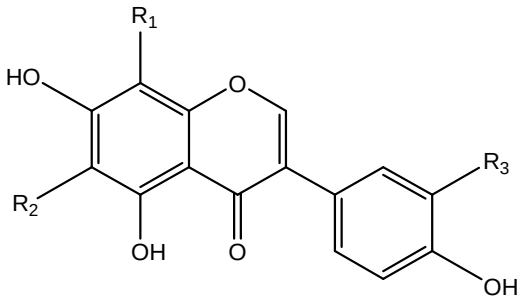
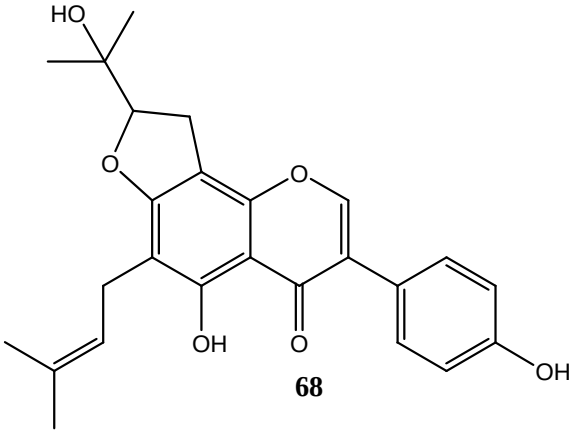


66 R_1= H, R_2 = $CH_2CH(OH)CCH_2CH$, R_3 = Isoprenyl

67 R_1= H, R_2 = R_3 = Isoprenyl



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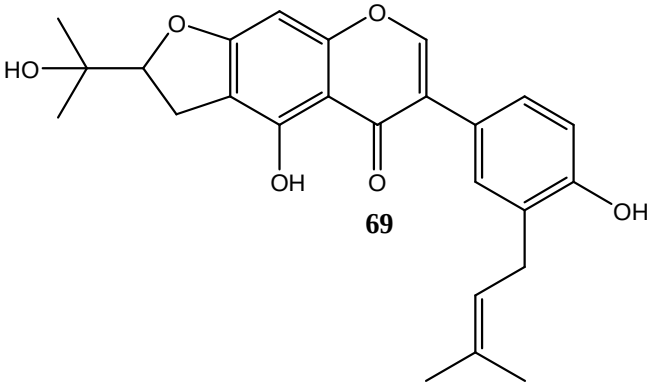

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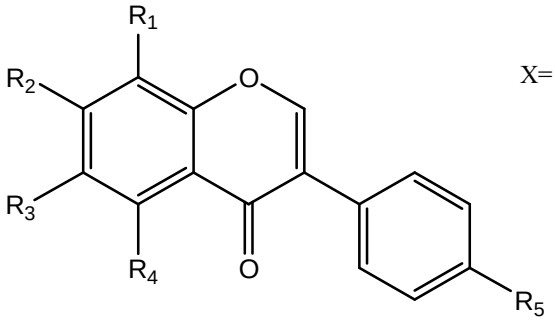
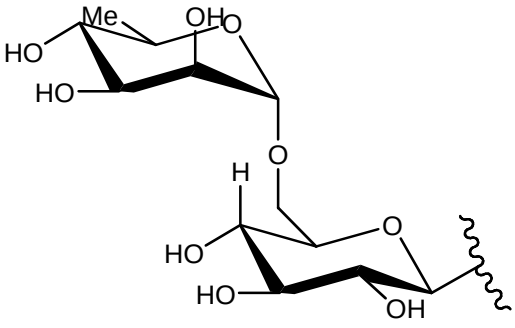
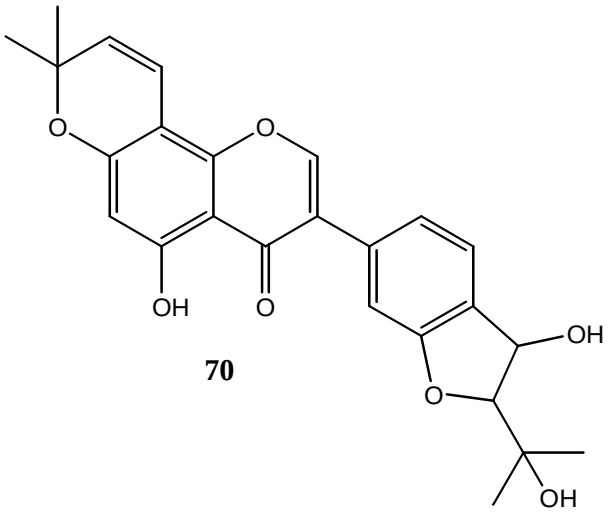


X=



71 R_1= OH, R_2= OX, R_3= R_4= H R_5= OMe

72 R_1= OX, R_2= R_5= OMe, R_3= R_4 =H



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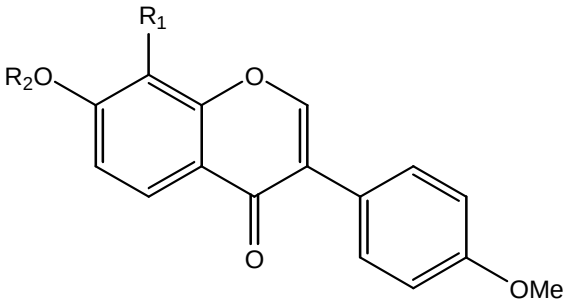
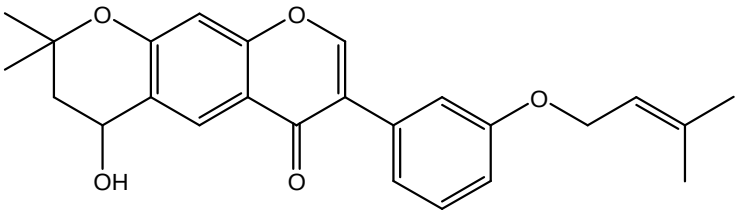


73 R_1= *O*-*β*-D-glucosy, R_2 = H
74 R_1 =H, R_2 = *β*-D-glucosy



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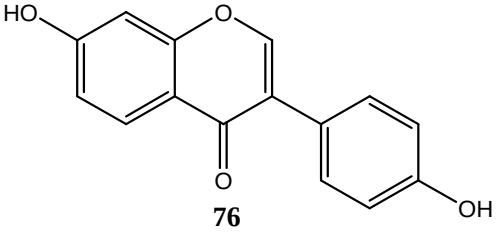

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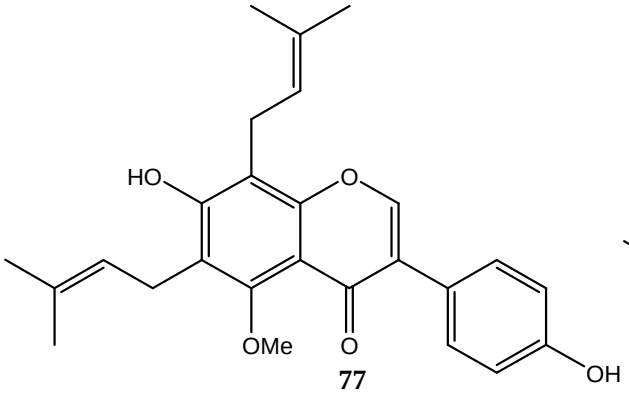

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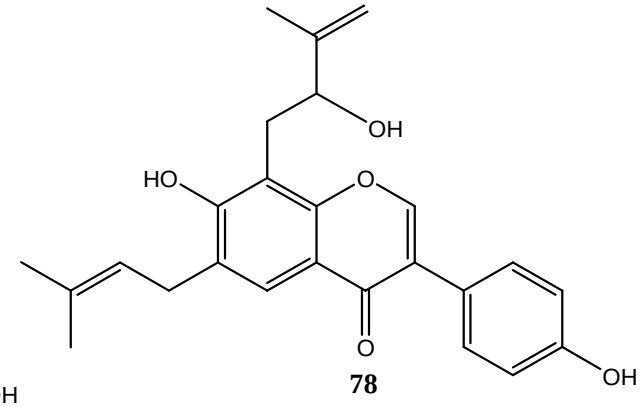

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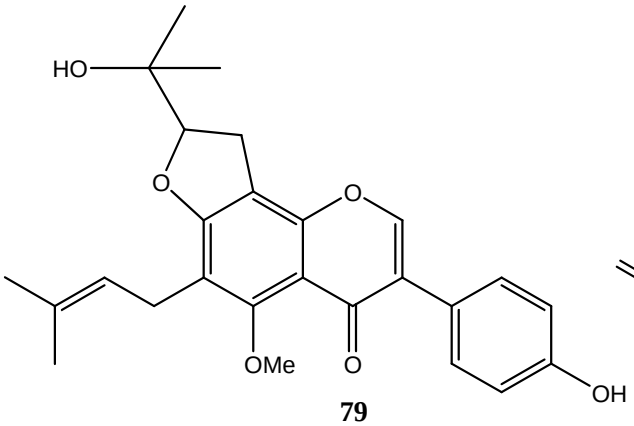

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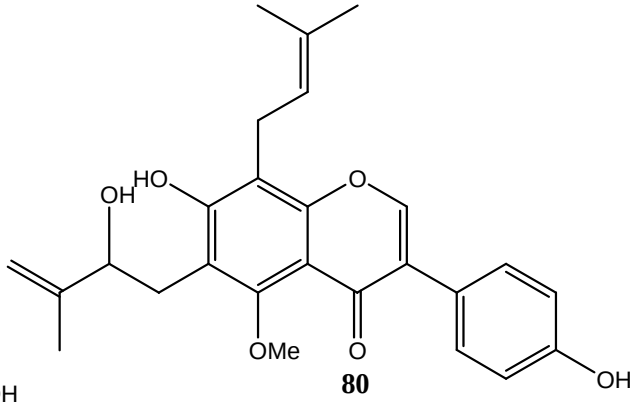

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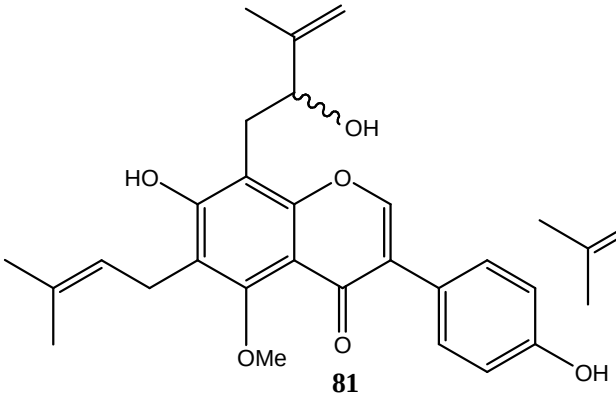

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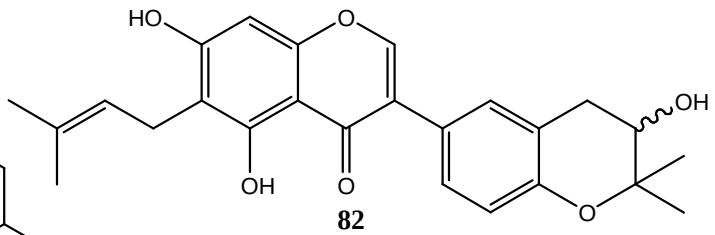

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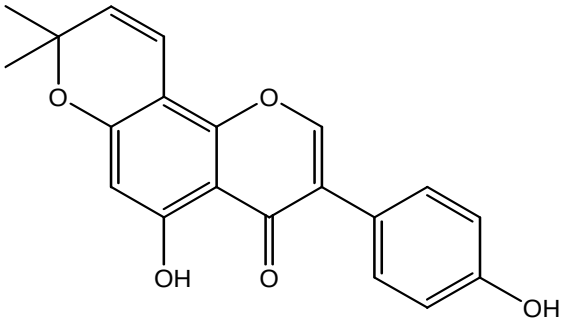

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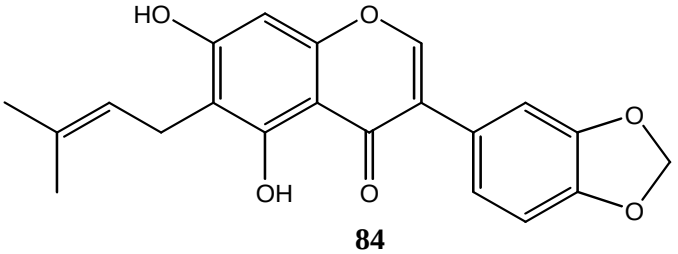

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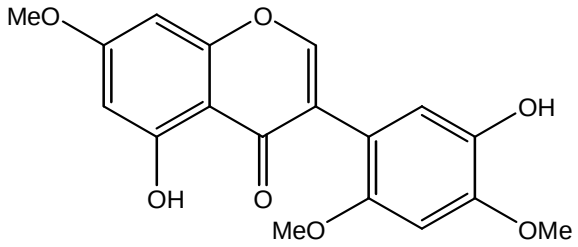

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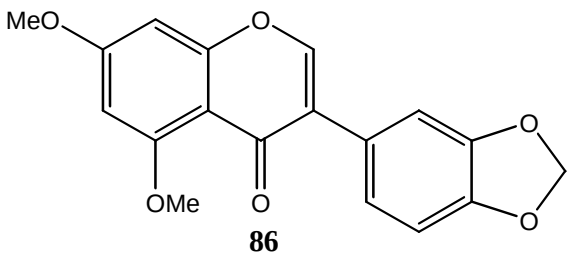

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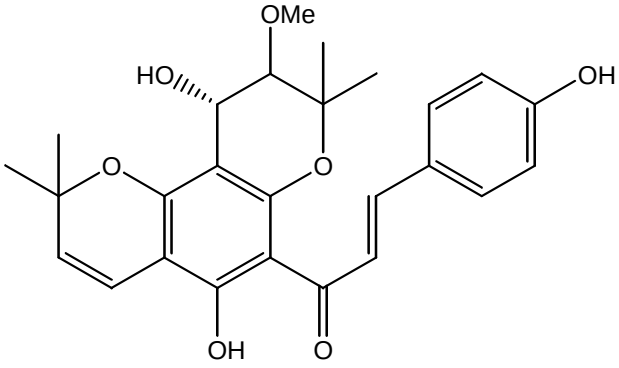
2.5.4.3 Chalcones

The chalcones of the genus *Derris* are oxygenated at C-4, -2', -4' and C-6'. Prenylation is common, which occurs at C-3' and C-5'. Table **2**.**10** shows some chalcones of this genus.

Table 2.10: Some selected chalcones of the genus *Derris*

Chalcone	Source (s)	Reference (s)
Derrichalcone (**85**)	*D. obtusa* (root bark)	Lin *et al.,* 1992
Derricidin (**86**)	*D. floribunda* (root)	Filho *et al*., 1975c
Derricin (**87**)	*D. sericea* (root bark)	Nascimento and Mors, 1972
4-Hydroxylonchocarpin (**88**)	*D. floribunda* (root bark)	Filho *et al.,* 1975a
Laxichalcone (**89**)	*D. floribunda* (root bark)	Lin *et al*., 1991a
Isocordion (**90**)	*D. floribunda* (root bark)	Filho *et al.,* 1975c
Lonchocarpin (**91**)	*D .floribunda* (root bark)	Filho *et al.,* 1975a
Methylenedioxy-(3,4)-5'-hydroxy-2',3'-methoxyfurano-(3',4',2",3")-dihydrochalcone (**92**)	*D. araripensis* (root bark)	Nascimento and Mors, 1981
Isobavachromene (**93**)	*D. floribunda* (root bark)	Braz *et al*., 1975a
Methylenedioxy-(3,4)-5'-hydroxy-2'-methoxyfurano-(3',4'2",3")-chalcone (**94**)	*D. obtusa* (root bark)	Nascimento *et a*l., 1976
4,4'-Dihydroxy-2'-methoxychalcone (**95**)	*D. oblonga* (root bark)	Nascimento *et a*l., 1976



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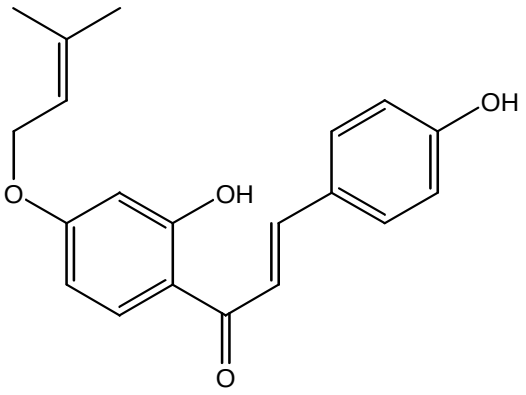

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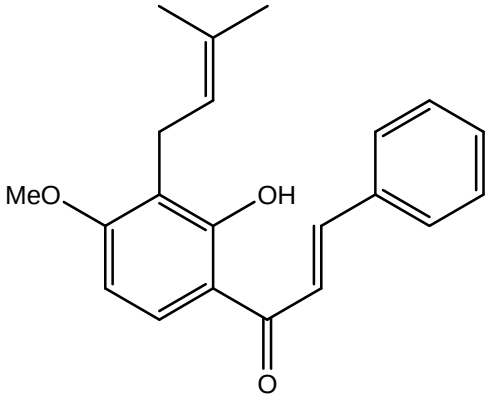

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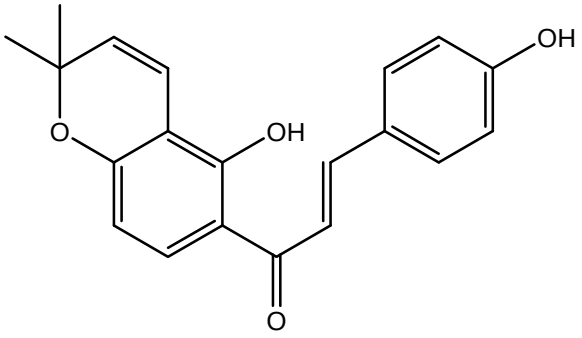

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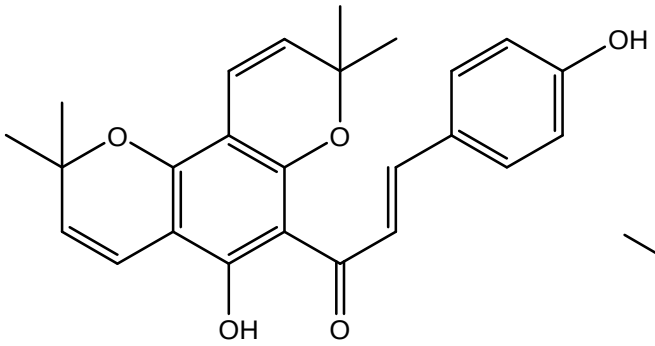

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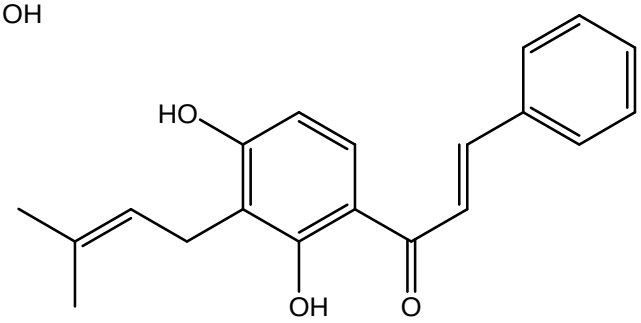

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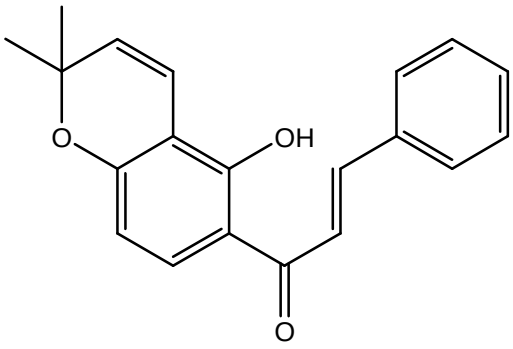

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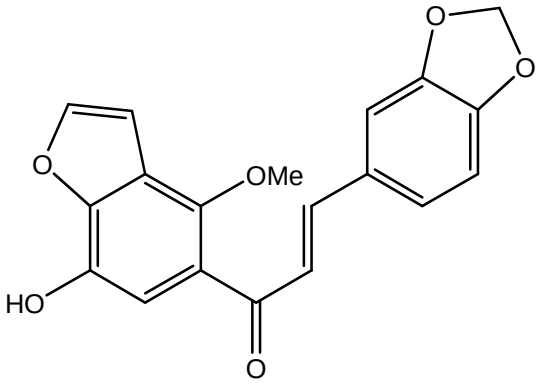

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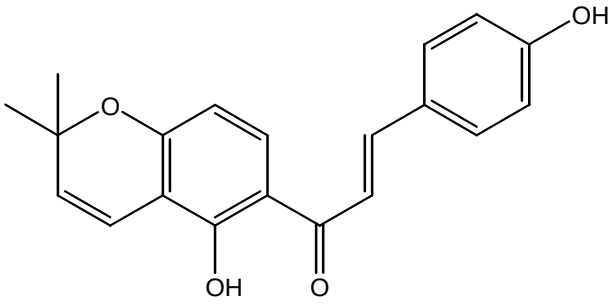

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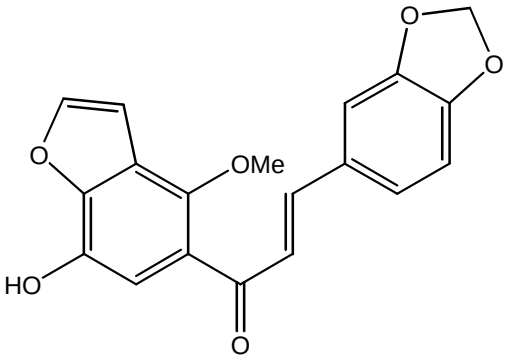

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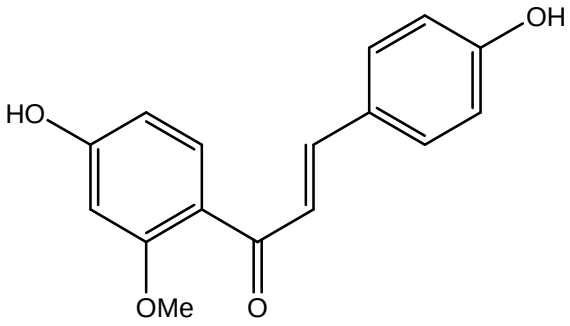

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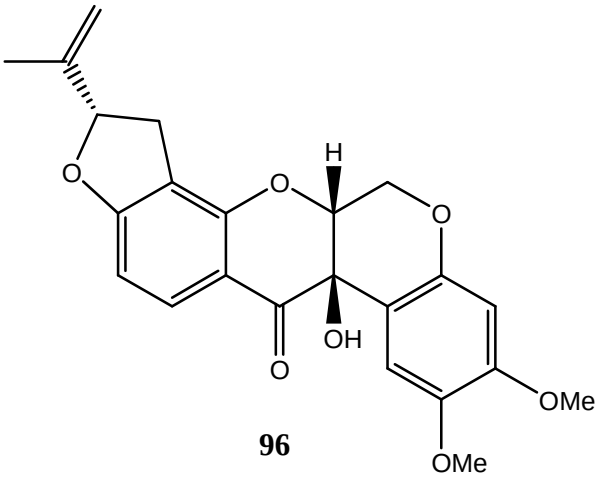
2.5.4.4 Rotenoids

The genus *Derris* is known for the presence of insecticidal rotenoids including rotenone (**3**). The most common rotenoids isolated from this genus are given in table **2**.**11**.

Table 2.11: Some selected rotenoids of the genus *Derris*

Rotenoids	**Source (s)**	**Reference (s)**
Deguelin (**1**)	*D. trifoliata* (stem)	Cheenpracha *et al*., 2007
Tephrosin (**2**)	*D. trifoliata* (stem)	Cheenpracha *et al*., 2007
Rotenone (**3**)	*D. trifoliata* (stem)	Cheenpracha *et al*., 2007
12a-Hydroxyrotenone (**96**)	*D. trifoliata* (stem)	Cheenpracha *et al*., 2007
12a-Hydroxyelliptone (**97**)	*D. trifoliata* (root),	Thasana *et al*., 2001
12-Deoxo-12α-acetoxyelliptone (**98**)	*D. malacensis* (stem)	Thasana *et al*., 2001
6a,12a-Dehydrorotenone (**99**)	D. *oblonga* (root)	Lin and Kuo, 1995
6a,12a-Dehydro-α-toxicarol (**100**)	*D. oblonga* (root)	Lin and Kuo, 1993
12-Deoxo-12α-acetoxyelliptone (**101**)	*D. malaccensis* (stem)	Thasana *et al*., 2001
Derrisin (**102**)	*D. malaccensis* (root)	Takashima *et al*., 2002
Elliptinol (**103**)	*D. elliptica* (root)	Ahmed *et al*., 1989

Elliptone (**16**)	*D. malaccensis* (root)	Takashima *et al*., 2002
12a-Hydroxyelliptone (**104**)	*D. malaccensis* (stem)	Thasana *et al*., 2001
6a,12a-Dehydro-β-toxicarol (**105**)	*D. oblonga* (root)	Lin and Kuo, 1993
Sumatrol (**106**)	*D. oblonga* (root)	Lin and Kuo, 1995
Villosol (**107**)	*D. oblonga* (root)	Lin and Kuo, 1995
Toxicarol (**17**)	*D. malaccensis* (root)	Takashima *et al*., 2002
6a,12a-Dehydrodeguelin (**108**)	*D. malaccensis* (root)	Takashima *et al*., 2002
6-Hydroxy-6a,12a-dehydro-α-toxicarol (**109**)	*D. oblonga* (root)	Lin and Kuo, 1995



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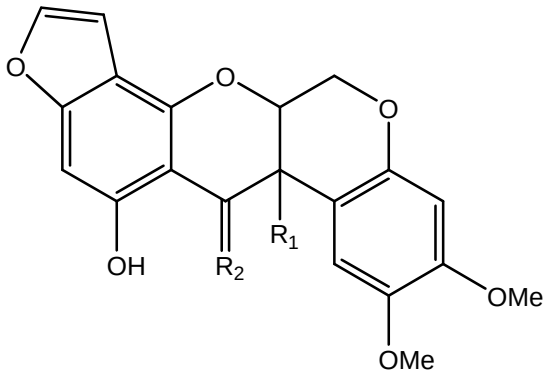
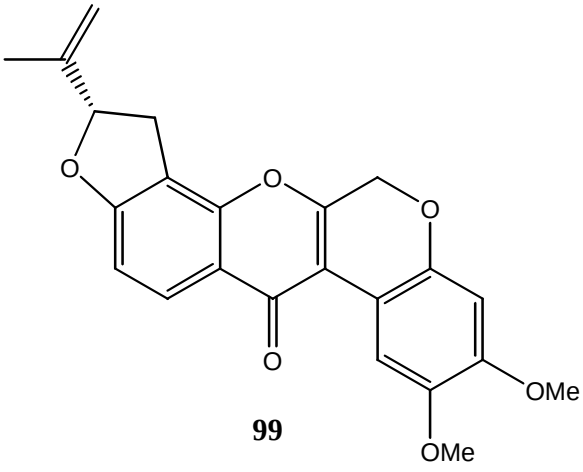


97 R_1= OH R_2 = O
98 R_1= H R_2 = OAc



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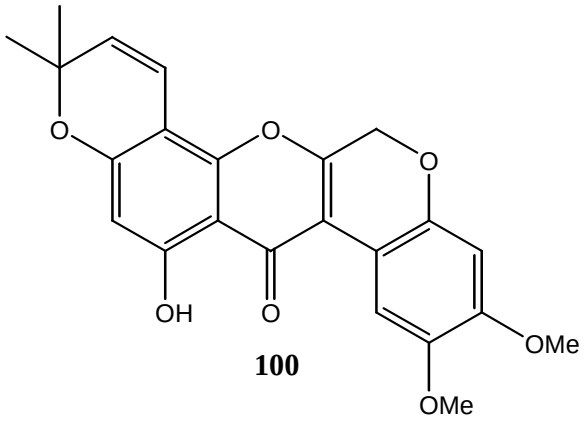

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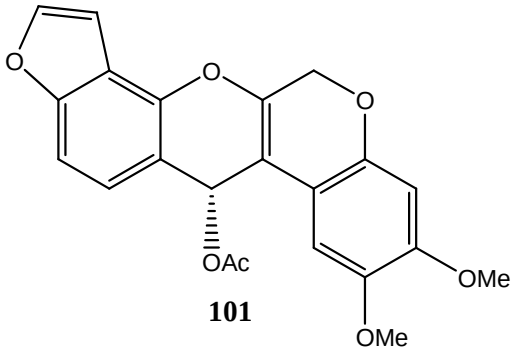

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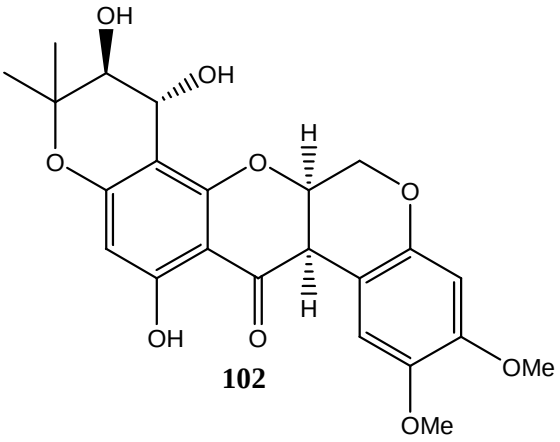

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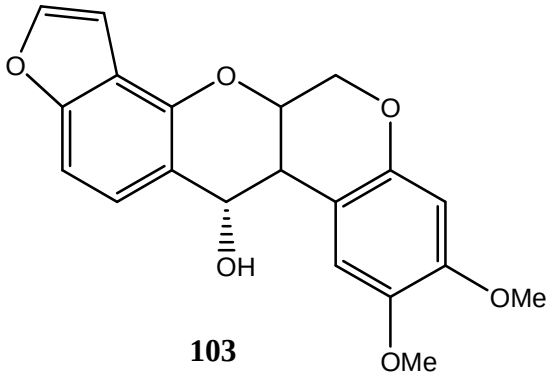

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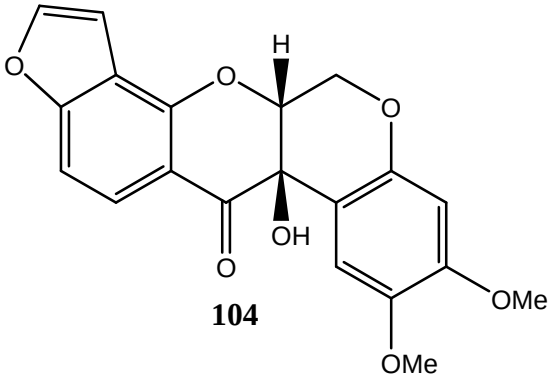

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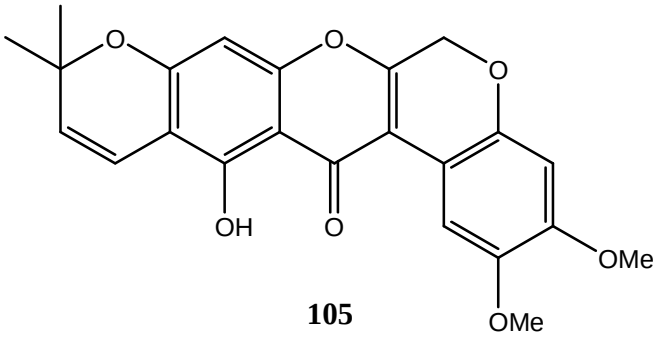

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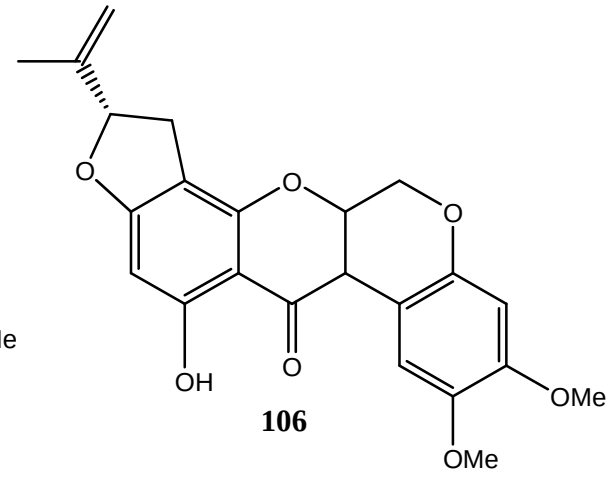

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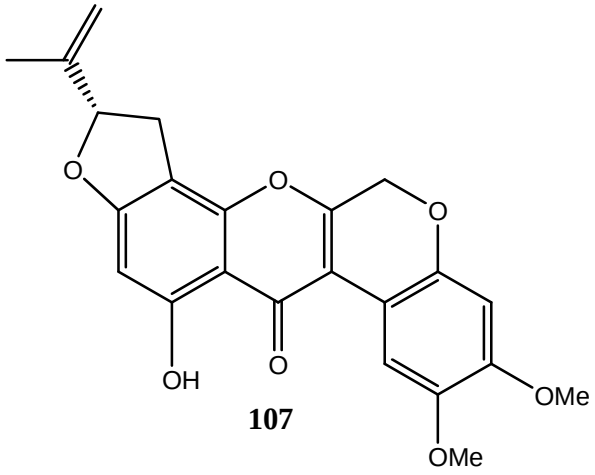

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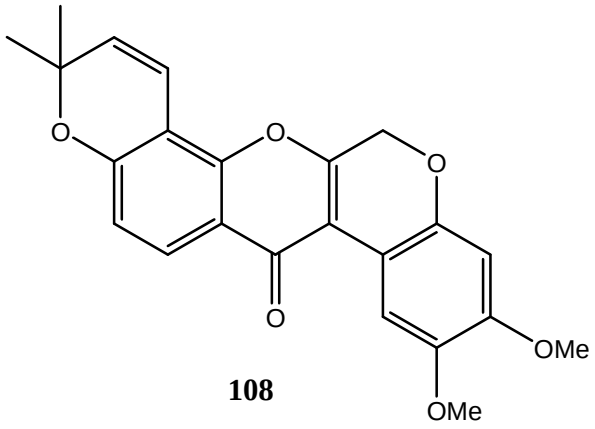

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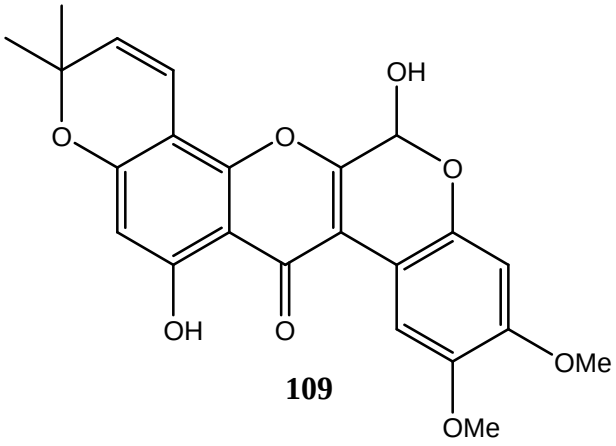

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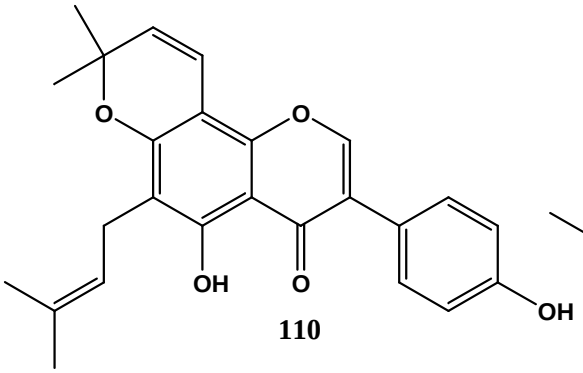
2.6 Biological Activities of Flavonoids and Isoflavonoids of the Family Leguminosae

Flavonoids and isoflavonoids from this family have been reported to show diverse biological activities such as insecticidal, antimicrobial and recently antitumor activities (Banzouzi *et al*. 2008). Recent studies reveal that many plants possess subtle defenses that interfere with pest growth, development and behavior with low toxicity to higher animals (Azmathullah *et al*., 2011). Nowadays the control of pests is becoming more and more difficult because of increasing resistance to synthetic pesticides (Azmathullah *et al*., 2011). An alternative, safe and eco-friendly approach to control these pests is the use of plant extracts or phyto-constituents isolated from

these extracts. For example, in the studies conducted to confirm whether the mode of toxicity is confined to contact effect, the compounds isolated from *Derris scandens* Benth were topically applied to the larvae *T. castaneum* of red flour beetle and *C. cephalonica* of the rice moth. Osajin (**110**)**,** lupalbigenin (**111**), scandinone (**112**)**,** genistein (**113**) and sphaerobioside (**144**) produced significant mortality with the LC_{50} being 4.5-6.9 µg/larva and 5.7-8.9 µg/larva to *T. castaneum* and *C. cephalonica* larvae, respectively, after 14 days of treatment (Rani *et al*., 2013).



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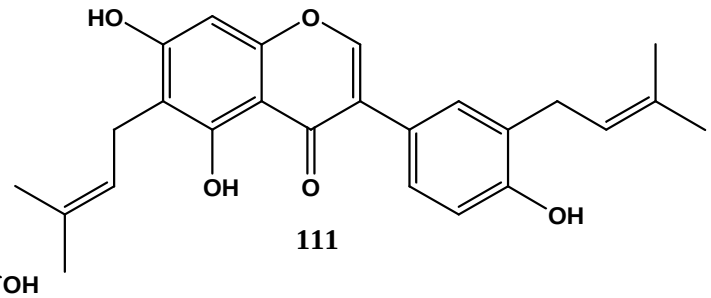

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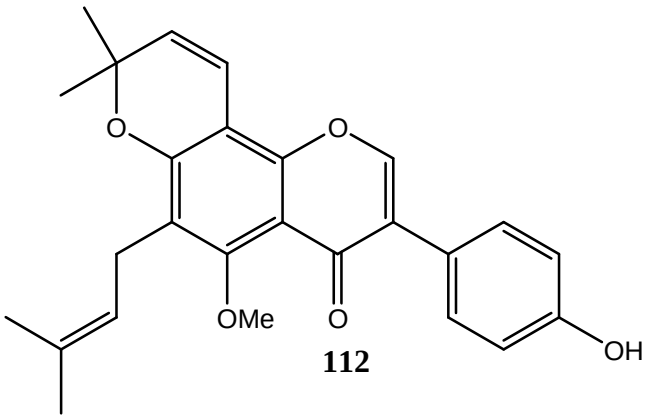

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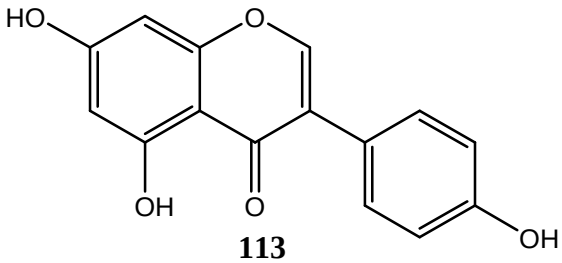

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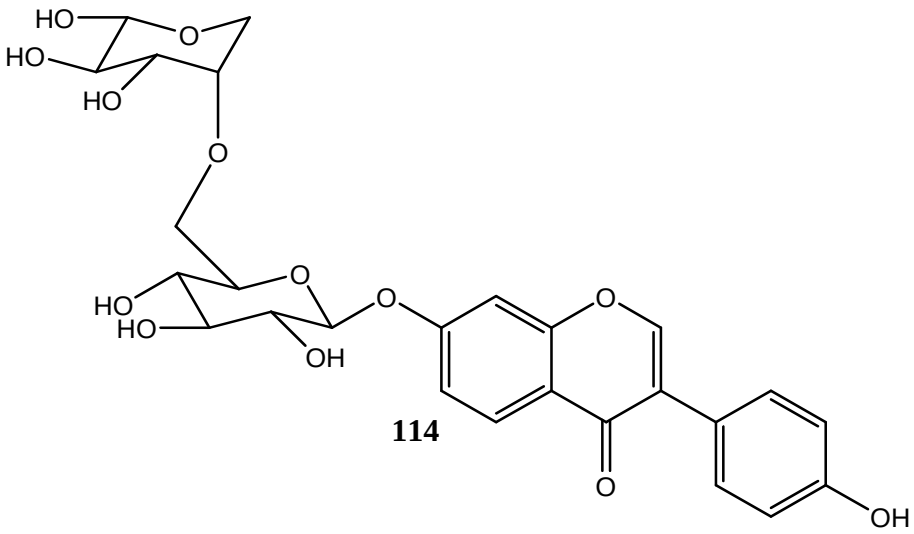

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The rotenoids, in particular, have been reported for high insecticidal activities. Rotenone (**3**) was found to be extremely potent against many insects (Dewick, 2009). Rotenoids-bearing plants such as *Derris, Lonchocarpus, Tephrosia* and *Mundulea* of the sub-family Papilionoideae of Leguminosae have been used in Asia, Africa and South America as insecticides (Yenesew, 1997b). However, it must be stressed that hydroxylation of C-12a, the presence of another hydroxyl function at C-11 and lack of unsaturation at the B/C-ring junction had subtle effects on insecticidal activity. Nonetheless, the importance of the lipophilicity is also another factor that contributes to high efficiency of rotenoids (Vasconcelos *et al*., 2012).

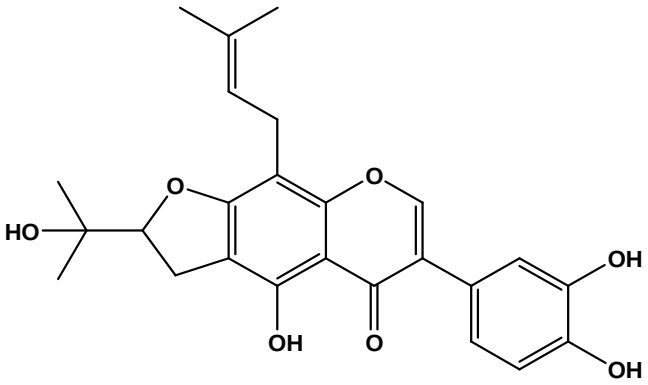
Flavonoids and isoflavonoids are powerful anti-oxidants by acting as free-radical scavengers (Kumar *et al.,* 2011; Kumar and Pandey, 2013; Kumar *et al*., 2012; Kumar *et al*., 2013a; Taie *et al.,* 2008; Rice-Evans and Packer, 1998). Anti-oxidants protect cells against the damaging effects of reactive oxygen species, such as singlet oxygen, superoxide, peroxyl radicals, hydroxyl radicals and peroxynitrite (Packer, 2001). An imbalance between anti-oxidants and reactive oxygen species results in oxidative stress, leading to cellular damage (Packer, 2001). Oxidative

stress has been linked to cancer, aging, atherosclerosis, ischemic injury, inflammation and neurodegenerative diseases such as Parkinson's and Alzheimer's diseases. Flavonoids and isoflavonoids may help to provide protection against these diseases by contributing, along with anti-oxidant vitamins and enzymes, to the total anti-oxidant defense system of the human body (Packer, 2001; Taie *et al* 2008; Rice-Evans and Packer, 1998). This activity is attributed to their *m*-hydrogen-donating ability (Kumar *et al.,* 2011). For example, phenolic groups of flavonoids serve as a source of a readily available ‘‘H” atoms such that the subsequent radicals produced can be delocalized over the flavonoid structure. In general, the capacity of flavonoids and isoflavonoids to act as anti-oxidants depends upon their molecular structure, for example, the position of hydroxyl groups (Kumar *et al.,* 2011). Functional hydroxyl groups mediate their anti-oxidant effects by scavenging free radicals and/or by chelating metal ions (Kumar and Pandey, 2013; Kumar *et al*., 2012; Kumar *et al*., 2013b).

The family Leguminosae is a rich source of flavonoids and isoflavonoids (Dewick, 1994). These compounds have been reported to possess anticarcinogenic effects since they can interfere with the initiation, development and progression of cancer by the modulation of cellular proliferation, differentiation, apoptosis, angiogenesis and metastasis (Kumar *et al.,* 2011). They have emerged as potential chemopreventive candidates for cancer treatment due to their ability to induce apoptosis. For example, genistein (**113**), an isoflavone isolated from *Millettia reticulata* was shown to have strong inhibitory effect with an IC_{50} value of 16.23 μM on SK-Hep-1 human hepatocellular carcinoma cells by inducing apoptosis (Fang *et al*., 2010). Thhe isoflavones furowanin-A (**115**), warangalone (**116**) and isoerysenegalensein-E (**117**) isolated from the leaves of *Milettia taiwaniana* are reported to induce apoptosis in human leukemia HL-60 cells (Ito *et al.,* 2006). Subsequently, millepachine (**118**), a compound for the first time isolated from seeds

of *Millettia pachycarpa* Benth (Leguminosae), induced cell cycle arrest and apoptosis in human hepatocarcinoma cells *in vitro* and *in vivo* and has showed strong antiproliferation activity in several human cancer cell lines, especially in HepG2 cells with an IC_{50} value of 1.51 μM. (Wu *et al*., 2013). In addition, previous study on *M. pachycarpa* has revealed that two of its major components, barbigerone (**119**) and deguelin (**1**) showed significant antitumor activity (Wu *et al*., 2013).

Furthermore, previous studies on deguelin (**1**), tephrosin (**2**), rotenone (**3**), 12a-hydroxyrotenone (**96**), 12a-hydroxyelliptone (**104**), 6,7-dimethoxy-2,3-dihydro-4H-chromen-4-one (**120**) and trifolinone B (**121**) isolated from the stem back of *Derris trifoliata* have showed these compounds to exhibit significant cytotoxic effect with ED_{50} value ranging from 4.17 to 0.02 μg/mL (Cheenpracha *et al*., 2007).



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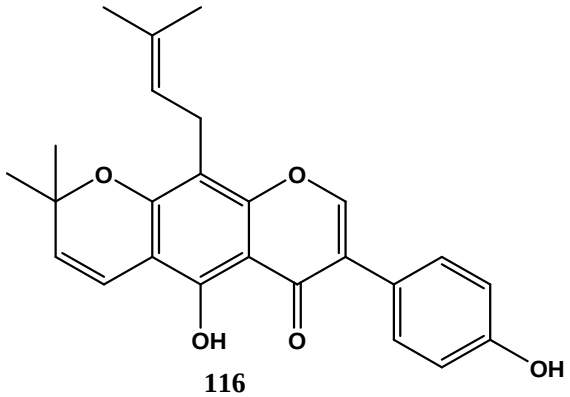

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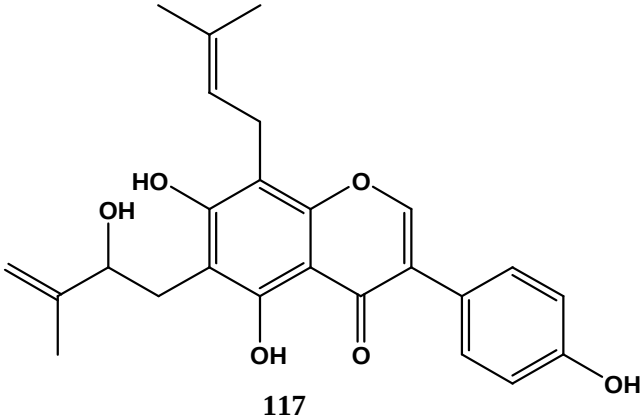

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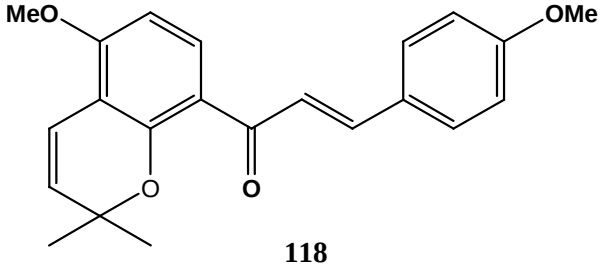

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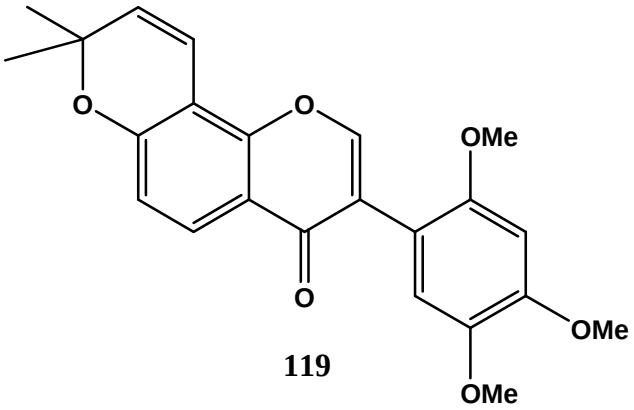

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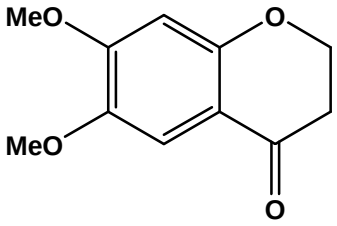
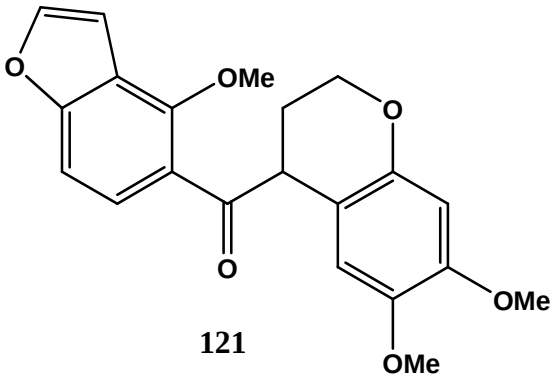


120

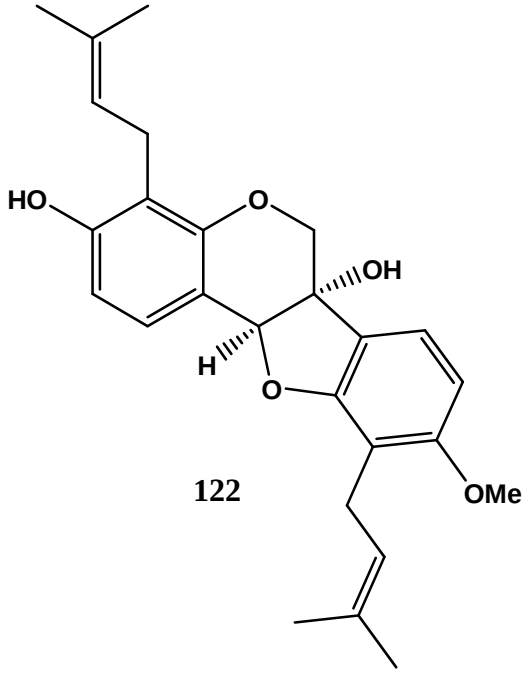


121

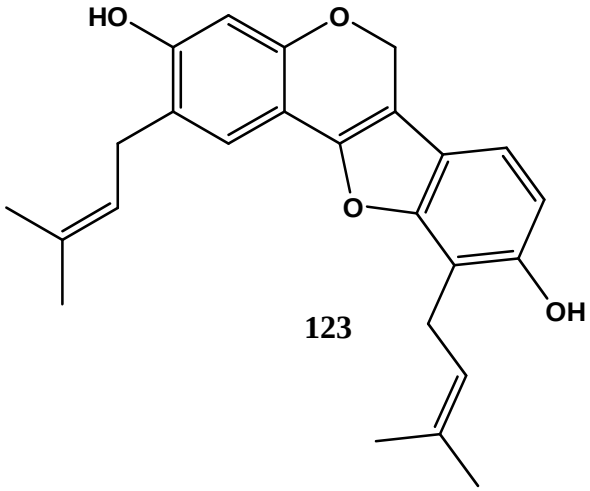
Flavonoid and isoflavonoid rich plant extracts from family Leguminosae have been reported to possess antibacterial activity (Kumar and Pandey, 2013; Kumar *et al*., 2012; Kumar *et al*., 2013). Several flavonoids and isoflavonoids including apigenin and galangin have been shown to possess potent antibacterial activity (Kumar and Pandey, 2013). Antibacterial flavonoids and isoflavonoids have multiple cellular targets, rather than one specific site of action. One of their molecular actions is to form complex with proteins through nonspecific forces such as hydrogen bonding and hydrophobic interaction, as well as by covalent bond formation (Kumar and Pandey, 2013; Kumar *et al*., 2012; Kumar *et al*., 2013). Thus, their mode of antimicrobial action may be related to their ability to inactivate microbial adhesins, enzymes and cell envelope transport proteins (Kumar and Pandey, 2013). The differences in core structures (pterocarpan, flavanone and isoflavone), as well as the presence and the position of substituent groups in the molecules, play an important role in the antibacterial activity (Rukachaisirikul *et al*., 2007). The presence of prenyl groups at C-2 or C-4 and C-10, hydroxyl groups at C-3 and C-9 and/or a hydroxyl group at C-6a, in pterocarpans enhance activity (Rukachaisirikul *et al*., 2007). On the other hand, replacement of the hydroxyl group at C-9 with the methoxyl group reduced the antibacterial activity (Rukachaisirikul *et al*., 2007). However, with regard to flavanones, the presence of a hydroxyl group at C-5 and more bulky substituents in the ring B might either enhance or reduce activity depending on strains tested, respectively (Rukachaisirikul *et al*., 2007).

A number of isoflavonoids identified as phytoalexins (pterocarpanoids and isoflavones) has shown their antibacterial activity to be selective to Gram-positive bacteria (Gnanamanickman and Mansfield, 1981). For example, compound **122, 123, 124** and **125** isolated from *Erythrina*

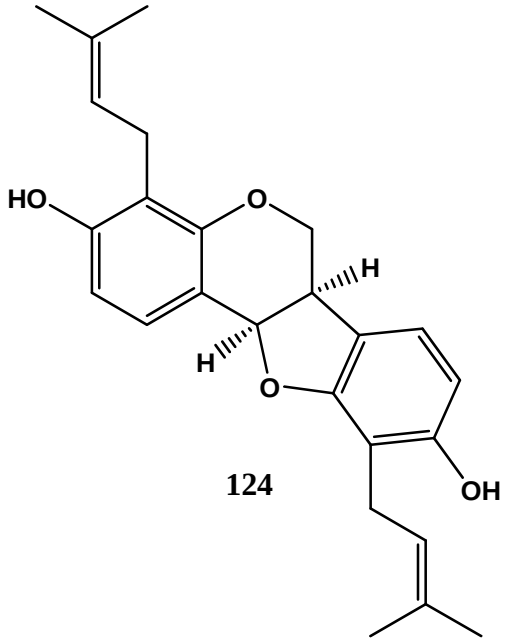
subumbrans were found to be inhibitory towards the Gram-positive human pathogens, *Staphylococcus* strains and *Streptococcus* strains. Erybraedin A (**124**) and erythrabyssin II (**125**) showed the highest level of activity against *Streptococcus* strains, with an MIC range of 0.78–1.56 µg/mL, followed by erystagallin A (**122**) and erycristagallin (**123**) (0.78–3.13 µg/mL), while the most active compounds against *Staphylococcus* strains were erycristagallin (**123**) (0.39–1.56 µg/mL), erythrabyssin II (**125**) (0.78–3.13 µg/mL), erybraedin A (**124**) (0.78–6.25 µg/mL) and erystagallin A (**122**) (0.78–12.5µg/mL) in that order (Rukachaisirikul *et al*., 2007).



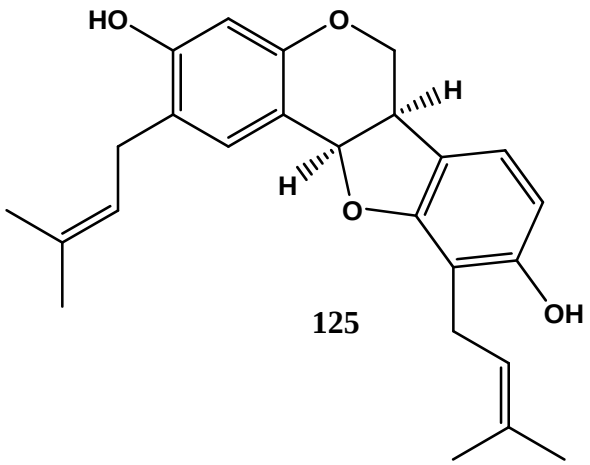
122



123



124



125

Moreover, isoflavonoids (phytoalexins) are generally recognized as fungitoxic compounds since they are mostly synthesized by plants in response to fungal attack (Gnanamanickman and Mansfield, 1981). Isoflavonoids are considered to be multi-site toxicants, with fungal membrane being one of the sites for disruption. Among the phytoalexins with the highest antifungal activity is the isoflavonoid based group of pterocarpans (Jimaenez-Gonzaalez *et al*., 2008). While some pterocarpans have antifungal, antitubercular and oestrogenic activity, others have been reported to inhibit HIV-1 in cell cultures (Kiss *et al*., 2002). In addition to their defensive antifungal functions, pterocarpans display other diverse biological effects, such as antibacterial, anti-inflammatory, antitumor, anti-oxidant and antiallergenic and antiparasitic activities, as well as activity against *Anopheles gambiae* adult mosquitoes and common cutworm, *Spodoptera litura* (Sobolev *et al*., 2010). Furthermore, Nakanishi and co-workers have demonstrated that the pterocarpans are antagonists against some snake and spider venoms (Kiss *et al*., 2002).

CHAPTER THREE

METHODOLOGY

3.1 Instrumentation

Column chromatography was carried out using Merck silica gel 40 (70-230 mesh) and Sephadex LH-20. Analytical TLC was done using Merck pre-coated 60 PF_{254} while PTLC was done on silica gel. Chromatographic zones were detected under UV lamp (254 or 365nm) light and/or exposing in some cases to iodine vapour. Distilled solvents were used for extraction and chromatographic separation. The plant material was ground using Willy Mill grinding machine. The 1D and 2D NMR data were run on Bruker Avance (600 MHz) or Bruker BioSpin instrument (500 MHz). The chemical shifts were measured in ppm in δ values relative to the internal standard tetramethyl silane (TMS). EI-MS spectra were recorded on SSQ 710 Finnigan MAT spectrometer at 70 eV while ESI spectra were recorded with Finnigan LC-Q mass spectrometer. UV spectra were obtained using SP8 150 UV/VIS spectrophotometer and ORD were obtained using a JASCO-1020 polarimeter.

3.2 Collection of Plant Material

The leaves of *Millettia oblata* spp. *teitensis* (LM-03/2013) were collected from Taita Hills forest, Kenya on September 2013 by Mr. P. Mutiso while the leaves of *Derris trifoliata* (AYT-020/2005) were collected at the mouth of river Sabaki in Malindi, Coast Province, Kenya in July 2005 by Mr. S. Mathenge, both are botanists from School of Biological Science, University of Nairobi. The specimens are kept at the University Herbarium, School of Biological Science. The roots of *Millettia micans* (FMM 3591/2013) were collected from Kisarawe, Tanzania in June 2013 by Mr. F. Mbago, a botanist from the Department of Botany, University of Dar es Salaam,

Tanzania and the specimen is kept at the Institute of traditional medicine, Muhimbili University of Health and Allied Science.

3.3 Extraction and Isolation of Compounds

3.3.1 Extraction and Isolation of Compounds from Leaves of *Millettia oblata* spp. *teitensis*

Dried and ground leaves (1.3 Kg) of *Millettia oblata* spp. *teitensis* was extracted with DCM/MeOH (1:1) exhaustively three times for 24 hours each. The extracts were filtered and concentrated using rotary evaporator to give a black crude extract of 116.0 g. A 100 g portion of the crude extract was subjected to column chromatography on silica gel (500 g) and eluted with the mixture of n-hexane and ethyl acetate with increasing polarities. The eluates were collected in approximately 250mL per fraction. The collected fractions were combined on the basis of their TLC pattern after removal of the solvent by rotary evaporator. The fractions were further purified by CC on Sephadex LH-20, preparative TLC and recrystallization to give pure compound. The fraction which eluted with 4% EtOAc in n-hexane afforded compound **126** that weighed 401.9 mg after being purified using Sephadex LH-20 and then preparative TLC.

The fractions which were eluted with 6% EtOAc in n-hexane afforded two compounds (**127**, 4.3 mg) and (**125,** 5.6 mg) which were separated by preparative TLC and finally purified using Sephadex LH-20. The fractions which were eluted at 10% EtOAc in n-hexane afforded a mixture of two compounds **126** and **128** Purification with preparative TLC and finally Sephadex LH-20 yielded 3.2 mg as a mixture of compound **126** and **128**.

3.3.2 Extraction and Isolation of Compounds from Roots of *Millettia micans*

Dried and ground root bark (0.9 Kg) of *Millettia micans* was extracted with DCM/MeOH (1:1) exhaustively three times 24 hours each. The extract was filtered and concentrated using rotary

evaporator to give a brown crude extract (38 g). A 35 g portion of the crude extract was subjected to column chromatography on silica gel (150 g) and eluted with the mixture of n-hexane and ethyl acetate with increasing polarities. The fractions which were eluted with 6% EtOAc in n-hexane yielded compound **129** (40.4 mg) on purification by Sephadex LH-20 (eluent DCM/MeOH; 1:1) and recrystallization (n-hexane/dichloromethane).

3.3.3 Extraction and Isolation of Compounds from Leaves of *Derris trifoliata*

Dried and ground leaves (0.78 Kg) of *Derris trifoliata* was extracted with DCM/MeOH (1:1) exhaustively three times for 24 hours each. The extracts were filtered and concentrated using rotary evaporator to give 112.6 g of black crude extract. Two portions of the crude extract were prepared. A 105 g portion of the crude extract subjected to column chromatography on silica gel (540 g) and eluted with the mixture of n-hexane and ethyl acetate with increasing polarities. The fraction which eluted with 4% EtOAc in n-hexane yielded compound **130** (2.4 g) on crystallization (n-hexane/dichloromethane) while the fraction that eluted at 6% EtOAc in n-hexane yielded compound **131** (8.5 mg) on purification by Sephadex LH-20 followed by recrystallization in methanol.

3.3.4 Physical and Spectroscopic Data for the Isolated Compounds

3.3.4.1 *Tephrosin (125)*

Colourless oil. **^{1}H NMR** (CD_2Cl_2, 600 MHz): δ 6.52 (1H, *s*, H-1), 6.45 (1H, *s*, H-4), 4.63 (1H, *dd*, *J* = 2.4, 12.0 Hz, H-6ax), 4.50 (1H, *dd*, *J* = 0.9, 12.0 Hz, H-6eq), 4.57 (1H, *dd*, *J* = 0.9, 2.4 Hz, H-6a), 6.60 (1H, *d*, *J* = 10.0 Hz, H-4'), 5.55 (1H, *d*, *J* = 10.0Hz, H-5'), 1.45 (3H, *s*, H-7'), 1.39 (3H, *s*, H-8'), 6.47 (1H, *d*, *J* = 9.0 Hz, H-10), 7.73 (1H, *d*, *J* = 9.0 Hz, H-11), 4.40 (1H, *brs*, OH-12a), 3.81 (3H, *s*, OCH_3-3), 3.78 (3H, *s*, OCH_3-2).

^{13}C NMR (CD_2Cl_2, 150 MHz): δ 109.8 (C-1), 101.3 (C-4), 64.0 (C-6), 76.5 (C-6a), 115.5 (C-14), 128.9 (C-15), 28.4 (C-18), 28.7 (C-17), 112.0 (C-10), 128.7 (C-11), 67.6 (C-12a), 56.5 (OCH_3-3), 56.0 (OCH_3-2), 144.2 (C-2), 151.4 (C-3), 148.6 (C-4a), 156.8 (C-7a), 109.3 (C-8), 78.1 (C-16), 160.9 (C-9), 11.3 (C-11a), 191.5 (C-12), 108.8 (C-1a).

3.3.4.2 *Oblatarotenoid A (126)*

Colourless oil. **UV/Vis** λ_{max} (MeOH): 269.3 nm. **ORD**: $[\alpha]_D$ = +22.2 (MeOH) **CD**: λ nm 232 min (-13.27), 305 max (+13.67) and 335 min (-17.15).. **EI-MS** (70 eV, rel.int.): $[M]^+$ at *m/z* =342. **HRESI-MS**: $[M+Na]^+$ found *m/z* =365.0645 for $C_{18}H_{14}NaO_7$.

^{1}H NMR (CD_2Cl_2, 600 MHz): δ 6.51 (1H, *s*, H-1), 6.42 (1H, *s*, H-4), 4.44 (1H, *dd*, *J* = 12.1, 1.0 Hz, H-6ax), 4.56 (1H, *dd*, *J* = 12.1, 2.5Hz, H-6eq), 4.58 (1H, *dd*, *J* = 2.5, 1.0 Hz, 6a), 6.35 (1H, *d, J* = 2.2 Hz, H-8), 6.56 (1H, *dd, J* = 8.9, 2.3 Hz, H-10), 7.78 (1H, *d, J* = 8.9 Hz, H-11), 3.75 (3H, *s*, OMe-9), 5.81 (1H, *d*, *J* = 1.3 Hz, OCH_2O), 5.78 (1H, *d*, *J* = 1.3 Hz, OCH_2O).

^{13}C NMR (CD_2Cl_2, 150 MHz): δ 106.0 (C-1), 98.9 (C-4), 64.1 (C-6), 76.1 (C-6a), 100.7 (C-8), 111.1 (C-10), 129.4 (C-11), 67.9 (C-12a), 142.4 (C-2), 149.5 (C-3), 149.6 (C-4a), 162.6 (C-7a), 167.3 (C-9), 111.3 (C-11a), 191.4 (C-12), 109.9 (C-12b), 56.0 (OMe-9), 101.7 (OCH_2O).

3.3.4.3 *Stigmasterol (127)*

White powder. **^{1}H NMR** (CD_2Cl_2, 600 MHz): δ 1.08 (1H, *dd*, *J* = 13.3, 4.8 Hz, H-1ax), 1.82 (1H, *dt, J* = 13.2, 3.6 Hz, H-1eq), 1.78 (1H, *m*, H-2eq), 1.49 (1H, *m*, H-2ax), 3.53 (1H, *tdd, J* = 4.5, 4.2, 3.8 Hz, H-3), 2.5 (1H, *ddd*, 13.2, 4.8, 2.4 Hz, H-4ax). 2.19 (1H, *tm, J* = 13.2Hz, H-4eq), 5.31 (1H, *t, J* = 6.1, H-6), 1.97 (1H, *m*, H-7ax), 1.97 (1H, *m*, H-7eq), 1.47 (1H, *m*, H-8), 0.93 (1H, *m*, H-9), 1.53 (1H, *m*, H-11ax), 1.53 (1H, *m*, H-11eq), 1.97 (1H, *m*, H-12eq), 1.97 (1H, *m*, H-12ax), 1.02 (1H, *m*, H-14), 1.56 (1H, *m*, H-15ax), 1.56 (1H, *m*, H-15eq), 1.72 (1H, *m*, H-

16ax), 1.72 (1H, *m*, H-16eq), 1.16 (1H, *m*, H-17), 1.03 (1H, *s*, H-18), 0.71 (1H, *s*, H-19), 2.05 (1H, *m*, H-20), 0.19 (1H, *d*, *J* = 6.2 Hz, H-21), 4.98 (1H, *m*, H-22), 5.14 (1H, *m*, H-23), 1.53 (1H, *m*, H-24), 1.54 (1H, *m*, H-25), 0.82 (1H, *d*, *J* = 6.6 Hz, H-26), 0.80 (1H, *d, J* = 6.6 Hz, H-27), 1.22 (1H, *m*, H-28a), 1.56 (1H, *m*, H-28b), 0.83 (1H, *t*, *J* = 7.1 Hz, H-29).

^{13}C NMR (CD_2Cl_2, 150 MHz): δ 36.5 (C-1), 33.9 (C-2), 71.7 (C-3), 42.2 (C-4), 121.4 (C-5), 141.0 (C-6), 31.7 (C-7), 31.9 (C-8), 50.2 (C-9), 36.1 (C-10), 20.8 (C-11), 37.3 (C-12), 42.2 (C-13), 56.8 (C-14), 24.2 (C-15), 28.2 (C-16), 56.1 (C-17), 12.00 (C-18), 18.8 (C-19), 39.8 (C-20), 21.1 (C-21), 138.4 (C-22), 129.3 (C-23), 45.9 (C-24), 29.2 (C-25), 19.5 (C-26), 19.2 (C-27), 26.0 (C-28), 11.8 (C-29).

3.3.4.4 *Millettone (128)*

Colourless needles. **^{1}H NMR** (CD_2Cl_2, 600 MHz): δ 6.53 (1H, *s,* H-1), 6.45 (1H, *s,* H-4), 4.60 (1H, *dd, J* = 0.9, 12.0 Hz, H-6eq), 4.15 (1H, *dd, J* = 2.4, 12.0 Hz, H-6ax), 4.92 (1H, *dd, J* = 0.9, 2.4, Hz, H-6a), 6.58 (1H, *d, J* = 9.0 Hz, H-10), 7.68 (1H, *d, J* = 9.0 H-11), 3.80 (1H, *d, J* = 9.6 Hz, H-12a), 5.56 (1H, *d,* H-4'), 6.64 (1H, *d,* H-5'), 1.48 (3H, *s,* H-8'), 1.46 (3H, *s,* H-7'), 5.75 (1H, *d,* OCH_2O), 5.78 (1H, *d,* OCH_2O).

^{13}C NMR (CD_2Cl_2, 150 MHz): δ 106.7 (C-1), 142.2 (C-2), 147.8 (C-3), 98.7 (C-4), 149.3 (C-4a), 66.4 (C-6), 72.1 (C-6a), 157.0 (C-7a), 109.7 (C-8), 160.7 (C-9), 111.7 (C-10), 128.4 (C-11), 111.7 (C-11a), 188.9 (C-12), 44.8 (C-12a), 105.8 (C-12b), 128.4 (C-14), 115.0 (C-15), 78.0 (C-16), 27.9 (C-17), 28.2 (C-18), 101.4 (OCH_2O).

3.3.4.5 *Micanspterocarpan A (129)*

White powder. **UV** λ_{max} (MeOH): 281.0 nm. **EI-MS** (70 eV, rel.int): $[M]^+$ at m/z =330. **ORD**: $[\alpha]_D$= -134.3^0 CD: λ nm 238 min (-31.47) and 287 max (+12.83)., HRMS $[M]^+$ found at 330.1115 for $C_{18}H_{18}O_6$ (Theoretical value is 330.1103).

^{1}H NMR (CD_2Cl_2, 600 MHz): δ 7.25 (1H, *d*, *J* = 8.4 Hz, H-1), 6.46 (1H, *dd*, *J* = 2.4, 8.4 Hz, H-2), 6.32 (1H, *d*, *J* = 2.4 Hz, H-4), 4.22 (1H, *dd*, *J* = 10.9, 5.0 Hz, H-6ax), 3.61 (1H, *dd*, *J* = 5.0, 6.8 Hz, H-eq), 3.56 (1H, *m*, H-6a), 6.14 (1H, *s*, H-10), 5.35(1H, *d*, *J* = 6.8 Hz, H-11a), 4.18 (1H, *br*s, OH-3), 3.66 (3H, *s*, OCH_3-9), 3.89 (3H, *s*, OCH_3-7), 3.78 (3H, *s*, OCH_3-8).

^{13}C NMR (CD_2Cl_2, 150 MHz): δ 132.1 (C-1), 109.8 (C-2), 157.1 (C-3), 103.4 (C-4), 156.8 (C-4a), 65.8 (C-6), 39.1(C-6a), 109.5 (C-6b), 150.6 (C-7), 154.8 (C-8), 135.5 (C-9), 90.6 (C-10), 155.8 (C-10a), 78.1 (C-11a), 112.8 (C-11b), 56.1 (OCH_3-9), 60.7 (OCH_3-7), 60.8 (OCH_3-8).

3.3.4.6 *Lupinifolin (130)*

Viscous oil. **UV** λ_{max} (MeOH): 271.2 nm.

^{1}H NMR (CD_2Cl_2, 600 MHz): δ 5.33 (1H, *dd*, *J* = 3.3, 13.3 Hz, H-2), 2.80 (1H, *dd*, *J* = 17.5, 13.0 Hz, H-3ax), 3.04 (1H, *dd*, *J* = 17.5, 13.0 Hz, H-3eq), 7.31 (2H, *d*, *J* = 8.5 Hz, H-2'/6'), 6.87 (2H, *d*, *J* = 8.5 Hz, H-3'/5'), 5.50 (1H, *d*, *J* = 10.2 Hz, H-3''), 6.63 (1H, *d*, *J* = 10.2 Hz, H-4''), 1.43 (1H, *s*, H-5''), 1.45 (1H, *s*, H-6''), 3.21 (1H, *brd*, *J* = 7.2 Hz, H-1'''), 5.16 (1H, *t*, *J* = 7.2 Hz, H-2'''), 1.65 (1H, *s*, H-4'''), 1.65 (1H, *s*, H-5'''), 12.23 (1H, *s*, OH-5).

^{13}C NMR (CD_2Cl_2, 150 MHz): δ 78.5 (C-2), 43.1 (C-3), 196.5 (C-4), 102.6 (C-4a), 156.5 (C-5), 102.8 (C-6), 159.9 (C-7), 108.6 (C-8), 159.4 (C-8a), 130.8 (C-1'), 127.6 (C-2'/6'), 115.5 (C-3'/5'), 156.0 (C-4'), 78.1 (C-2''), 125.9 (C-3''), 115.6 (C-4''), 28.4 (C-6''), 28.3 (C-5''), 21.4 (C-1'''), 122.6 (C-2'''), 131.1 (C-3'''), 25.8 (C-4'''), 17.8 (C-5''').

3.3.4.7 *Lupinifolin 4'-methyl ether (131)*

Viscous oil. **^{1}H NMR** (CD_2Cl_2, 600 MHz): δ 5.33 (1H, *dd*, *J* = 3.3, 13.0 Hz, H-2), 2.80 (1H, *dd*, *J* = 17.0, 13.0 Hz, H-3ax), 3.05 (1H, *dd*, *J* = 17.0, 13.0 Hz, H-3eq), 7.37 (2H, *d*, *J* = 8.4 Hz, H-2'/6'), 6.94 (2H, *d*, *J* = 8.4 Hz, H-3'/5'), 5.49 (1H, *d*, *J* = 10.0 Hz, H-3''), 6.63 (1H, *d*, *J* = 10.0 Hz, H-4''), 1.43 (1H, *s*, H-5''), 1.45 (1H, *s*, H-6''), 3.21 (1H, *brd*, *J* = 7.2 Hz, H-1'''), 5.16 (1H, *t*, *J* = 7.2 Hz, H-2'''), 1.65 (1H, *s*, H-4'''), 1.65 (1H, *s*, H-5'''), 12.26 (1H, *s*, OH-5), 3.84 (3H, *s*, OCH_3-4).

^{13}C NMR (CD_2Cl_2, 150 MHz): δ 78.5 (C-2), 43.1 (C-3), 196.4 (C-4), 102.6 (C-4a), 156.5 (C-5), 102.8 (C-6), 159.8 (C-7), 108.6 (C-8), 159.4 (C-8a), 130.9 (C-1'), 127.6 (C-2'/6'), 114.1 (C-3'/5'), 159.8 (C-4'), 78.0 (C-2''), 125.9 (C-3''), 115.7 (C-4''), 28.4 (C-6''), 28.3 (C-5''), 21.5 (C-1'''), 122.6 (C-2'''), 131.6 (C-3'''), 25.8 (C-4'''), 17.8 (C-5'''), 55.3 (OCH_3-4).

3.4 Larvicidal Activity

3.4.1 Rearing of Mosquito Larva

Larvae of *Aedes aegypti* used in this investigation were obtained from the Department of Zoology, University of Nairobi. The larvae were kept in enamel larval trays until adult emergence. Cyclic generations of *Aedes aegypti* were maintained in cages. The adult mosquitoes were fed 10% glucose solution and ovitraps were placed inside the cages for egg laying. The eggs laid were then transferred to enamel larval trays maintained in the larval rearing chamber. The eggs were flooded with 0.08% NaCl solution and left to hatch at 28^0C. The larvae were fed with larval food (quaker oat and yeast). The 3rd instar larvae were then picked for larvicidal bioassay.

3.4.2 Sample preparation

A 20 mg each of the methanol extracts were solublized in 2 mL of dimethylsulphoxide (DMSO). A 200 µL portion of the solublized extracts were then diluted to 20 mL with distilled water to obtain a stock solution of 0.1 mg/mL. From the stock, graded concentrations of 100 µg/mL, 50. µg/mL, 25 µg/mL, 12.5 µg/mL and 6.25 µg/mL were then obtained. Three replicates of graded concentration were prepared. Subsequently, 5 mg of each of the pure compounds were solublized in 1 mL of DMSO. A 200 µL portion was then diluted in 20 mL of distilled water to obtain a stock solution of 0.05 mg/mL. A serial dilution of 50, 25, 12.5, 6.25 and 3.25 µg/mL was prepared from this stock solution. Three replicates of graded concentration were made also. Control larvae in all cases received 50.00 µL of DMSO.

3.4.3 Larvicidal Activity Assay

Larvicidal bioassay of individual plant extracts was tested against 3rd instar larvae of *A. aegypti.* The tests were conducted in Petri dishes, in accordance with Mwangi and Rembold protocol with slight modification (1988). Three replicates and a control were run simultaneously during each trial. Ten healthy larvae were released in each Petri dish and mortality was observed at 24, 48 and 72 hrs after treatment with extract concentrations of 100, 50, 25, 12.5, and 6.25 µg/mL. Likewise the same procedures applied to the pure compounds concentrations of 50 µg/mL, 25, 12.5, 6.25 and 3.25 µg/mL. The treatments were maintained at room temperature. Larvicidal activity of each extract or pure compound was determined, by counting the number of dead larvae on daily basis (24 hrs interval). Dead larvae in the three replicates were expressed as percentage mortality for each concentration. Dead larvae were recorded when they failed to move after probing with a needle. The percentage mortality was calculated and analysis of data was carried out by employing probit analysis (Dibua *et al.,* 2013).

CHAPTER FOUR

RESULTS AND DISCUSSION

4.1 Preliminary Tests Results

The crude extracts of *Millettia oblata* spp. *teitensis* (leaves), *Milletia micans* (root bark) and *Derris trifoliata* (leaves) were tested for larvacidal activity. The crude extract of *Millettia* spp. *teitensis* (leaves) showed moderate larvicidal activity with LC_{50} value of 84.6 ± 7.8 µg/mL at 24 hrs against the 3rd instar larvae of *Aedes aegypti* while *Derris trifoliata* (leaves) and *Milletia micans* (root bark) showed weak activities with LC_{50} value of 367.7 ± 162.8 µg/mL and 132 ± 14.4 µg/mL at 24 hrs, respectively (Nguta *et, al*., 2012; Devaraj, *et al*., 2013). TLC analyses of the crude extracts obtained from these plants revealed the presence of several UV (254 nm) sensitive components. The crude extracts were subjected to CC that led to the isolation of seven compounds. Some of the pure compounds were tested for larvicidal activity. The identities of the isolated compounds were established by the use of a combination of spectroscopic analyses including 1D NMR, 2D NMR, UV, HRESI, ORD and EI-MS. The characterization of the compounds is discussed below.

4.2 Characterization of the Isolated Compounds

4.2.1 Characterization of Compounds from *Millettia oblata* spp. *teitensis*

The dried powdered leaves of *Millettia oblata* spp. *teitensis* was extracted with MeOH/CH_2Cl_2 (1:1) and MeOH successively. Silica gel chromatography of the combined extract using gradient elution from hexane to ethyl acetate yielded four compounds, namely tephrosin (**125**), oblatarotenoid A (**126**), stigmasterol (**127**), and millettone (**128**).

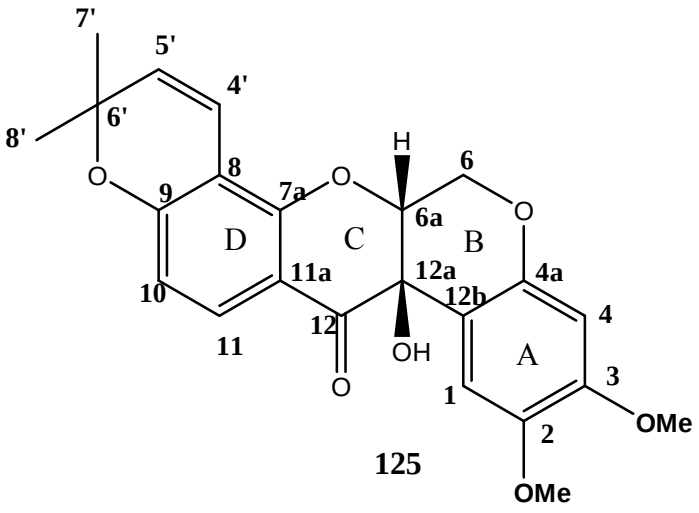
4.2.1.1 Tephrosin (125)

Compound **125** was isolated as colourless oil. This compound was identified to be a 12a-hydroxyrotenoid derivative based on ^{1}H and ^{13}C NMR data [δ_H 4.50 (1H, *dd*, *J* = 0.9, 12.0 Hz, H-6eq), 4.63 (1H, *dd*, *J* = 2.4, 12.0 Hz, H-6ax), 4.57 (1H, *dd*, *J* = 0.9, 2.4 Hz, H-6a) and 4.40 (1H, *brs*, OH-12a); δ_C 64.0 (C-6); 76.5 (C-6a), 67.7 (C-12a) and 191.5 (C-12)] (Yenesew *et al*., 1997a; 2003; Agrawal, 1989). The ^{1}H NMR spectrum (Table **4.1**) further revealed the presence of two methoxy [δ_H 3.81 (3H, *s*, OCH_3) and 3.78, (3H *s*, OCH_3)] and a 2,2-dimethylpyran [δ_H 1.45 (3H, *s*, CH_3), 1.39 (3H, *s,* CH_3), 5.5 (1H, *d*, *J* = 10.0 Hz) and 6.60 (1H, *d*, *J* = 10.0 Hz)] substituents.

The ^{1}H NMR spectrum (Table **4.1**), further showed two *ortho*-coupled [δ_H 6.47 (1H, *d*, *J* = 9.0 Hz) and 7.73 (1H, *d*, *J* = 9.0 Hz)] and two *para*-oriented singlets [δ_H 6.52 6.45 (1H, *s*) and 6.45 (1H, *s*)] aromatic protons assignable to ring A and D protons. The fact that one of *ortho*-coupled protons is deshielded (δ_H 7.73) due to the peri-effect of the carbonyl carbon at C-12 led to its assignment to H-11 and thus together with its coupling partner (H-10, δ_H 6.47) belong to ring D. As a result, the two *para* protons are placed in ring A (H-1 and H-4).

The chromene ring and the two methoxy groups can be either in ring A or D at positions C-8 and C-9 or C-2 and C-3. The placement of the methoxyl groups in ring A was established by three bond HMBC correlation between OCH_3-2 [δ_H 3.78 (3H, s)] and C-2 (δ_C 144.2) and OCH_3-3 [δ_H 3.81 (3H, *s*)] and C-3 (δ_C 151.4). This was further confirmed by the correlations of H-1 [δ_H 6.52 (1H, *s*)] with C-2 (δ_C 144.2), C-3 (δ_C 151.4), C-4a (δ_C 148.6), C-12a (δ_C 67.6) and C-12b (δ_C 108.8) and H-4 [δ_H 6.45 (1H, *s*)] with C-2 (δ_C, 144.2), C-3 (δ_C 151.4), C-4a (δ_C 148.6) and C-12b

(δ_C 108.8). Moreover, the location of these methoxy groups in this ring A was indicated by the observation of NOEs between a methoxy at δ_H 3.81 (3H, *s*, OCH_3-3) and aromatic singlet proton at δ_H 6.45 (1H, *s*, H-4) and between another methoxy at δ_H 3.78, (3H *s*, OCH_3-2)] and an aromatic singlet proton at δ_H 6.52 (1H, *s*, H-1). As a result the chromene ring is placed in ring D. The presence of the chromene group was further confirmed from the ^{13}C NMR spectrum (Table **4.1**) that showed the characteristic five resonances at δ_C 115.5, 128.9, 78.1, 28.7 and 28.4 (Marzouk *et al.*.2008). The attachment of this group to C-8 and C-9 was also proven by the downfield shift of C-8 to δ_C 102.1 (Δ + 6.2) and the upfield shift of C-9 to δ_C 163.6 (Δ - 5.1) (Marzouk *et al*., 2008).



125

The characteristic *J*-values (Table **4.1**) of the germinal CH_2-6 protons with H-6a were an evidence for the equatorial orientation (*β* configuration) of H-6a (Marzouk *et al.,* 2008). Furthermore, the chemical shift of H-1 (δ_H 6.52, *s*) is indicative of a *cis*-B/C ring junction (Yenesew *et al.,* 1997b). Based on these and by comparison of the data with literature (Yenesew *et al.,* 2003b and Vasconcelos *et al*., 2012), compound **125** was identified as tephrosin (Ollis *et*

al., 1967) which has earlier been isolated from seeds of *Millettia dura* (Yenesew *et al*., 2003a) and *Tephrosia* species (Marzouk *et al.*, 2008).

Table 4.1: ^{1}H (600 MHz) and ^{13}C (150 MHz) NMR data of tephrosin (**125**) in CD_2Cl_2.

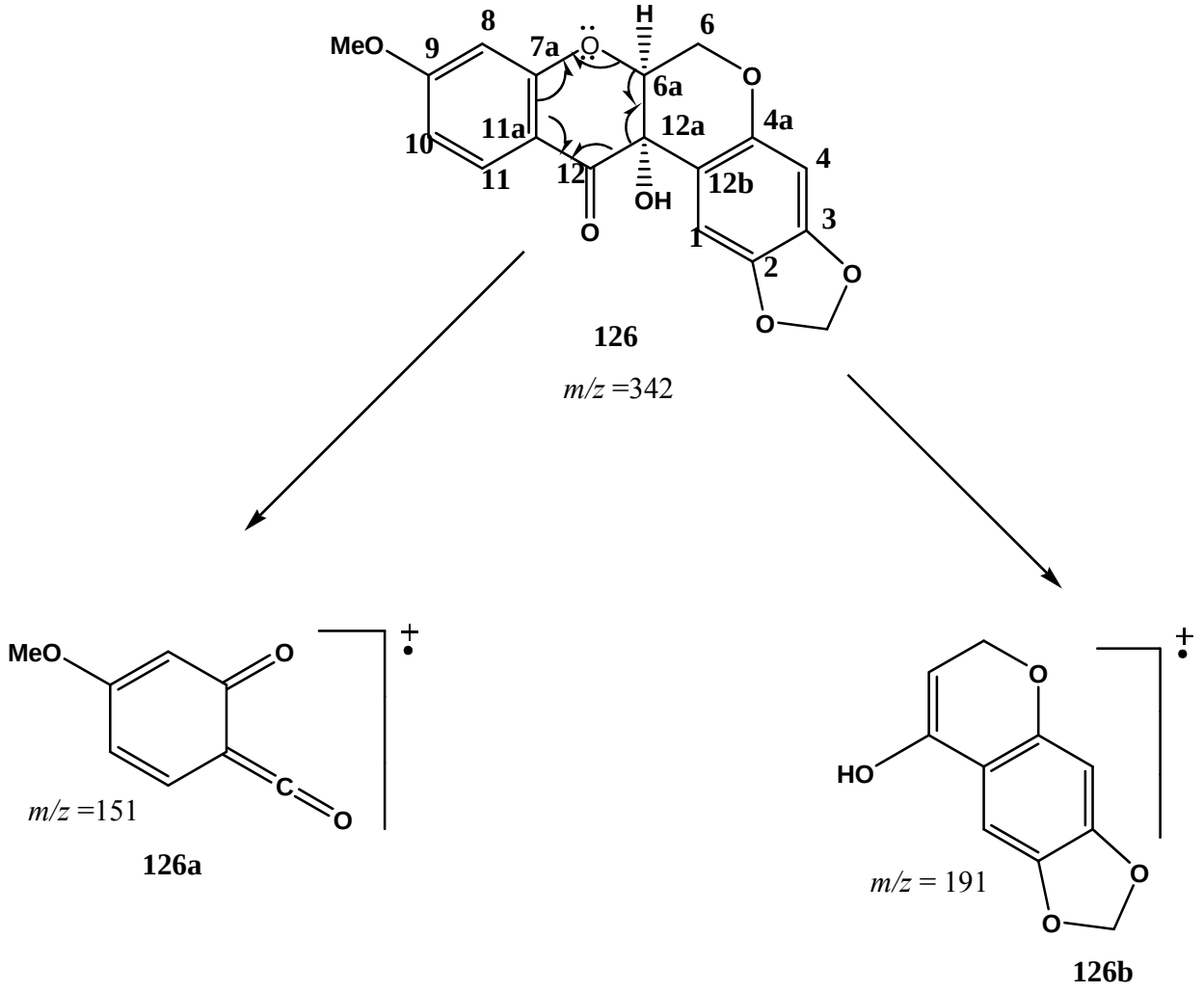
Position	^{1}H, δppm (*m, J* in Hz)	^{13}C, δppm	HMBC	
			^{2}J	^{3}J
1	6.52 (*s*)	109.8	C-2, C-12b	C-4a, C-3, C-12a
2		144.2		
3		151.4		
4	6.45 (*s*)	101.3	C-3, C-4a	C-2, C-12b
4a		148.6		
6_{ax}	4.63 (*dd*, 2.4, 12.0)	64.0		C-12a, C-4a
6_{eq}	4.50 (*dd,* 0.9,12.0)			C-12a, C-4a
6a	4.57 (*dd*, 0.9,2.4)	76.5		C-12
7a		156.8		
8		109.3		
9		160.9		
10	6.47 (*d*, 9.0)	112.0		C-8, C-11a
11	7.73 (*d,* 9.0)	128.7		C-12, C-9, C-7a
12		191.5		
12a (OH)	4.40 (*s*)	67.6	C-6a, C-12	
12b		108.8		
11a		111.3		
4’	6.60 (*d,* 10.0)	115.5		C-16, C-7a
5’	5.55 (*d,* 10.0)	128.9		C-8, C-17, C-18
6’		78.1		
7’	1.45 (*s*)	28.4		C-15
8’	1.39 (*s*)	28.7		C-15
OCH_3-3	3.81 (*s*)	56.5		C-3
OCH_3-2	3.78 (*s*)	56.0		C-2

4.2.1.2 Oblatarotenoid A (126)

Compound 1**26** was isolated as a colourless oil whose UV spectrum showed absorption maxima at 236, 250 and 269 nm. The high resolution electrospray ionisation (HRESI) mass spectrum showed a *pseudo*-molecular ion peak, $[M+Na]^+$, at *m/z* 365.0645 corresponding to the molecular formula $C_{18}H_{14}O_7$. On the basis of ^{1}H NMR [δ_H 4.56 (1H, *dd*, *J* = 12.1, 2.5 Hz, H-6eq), 4.44 (1H, *dd*, *J* = 12.1, 1.0 Hz, H-6ax) and 4.58 (1H, *dd*, *J* = 2.5, 1.0 Hz, 6a); and ^{13}C NMR [δ_C 64.1 (C-6); 76.1 (C-6a), 67.9 (C-12a) and 191.4 (C-12)] compound **126** was assumed to be a 12a-hydroxyrotenoid derivative (Yenesew *et al*., 1997b; 2003b; Agrawal, 1989). The presence of hydroxyl group at C-12a was indicated by HMBC spectrum which showed a significant C/H three bond correlation between a carbonyl carbon at δ_C 191.4 (C-12) and a hydroxyl proton at δ_H 2.66. The NMR spectra (Table **4.2**) also showed the presence of a methoxy (δ_H 3.75; δ_C 56.0) and methylenedioxy substituents (δ_H 5.78 and 5.81; δ_C 101.7) on the 12a-hydroxyrotenoid skeleton.

The ^{1}H, ^{1}H-COSY revealed the presence of three mutually coupled aromatic protons showing AXY spin system [δ_H 7.78 (*d*, *J* = 8.9 Hz), 6.56 (*dd*, *J* = 8.9, 2.3 Hz) and 6.35 (*d*, *J* = 2.2 Hz)] and two singlets aromatic protons (δ_H 6.51 and 6.42). The AXY spin system is assigned to ring D (with methoxyl at C-9) due to the HMBC correlation of the de-shielded (as a result of *peri*-effect) proton (δ_H 7.78, H-11) with C-12 (δ_C 191.4). The singlets are then placed in ring-A with the methylenedioxy group being at C-2/C-3. The substitution pattern is confirmed by HMBC spectrum (Table **4.2**). Placement of the methoxy at C-9 of ring D and the methylenedioxy in ring A was further supported by EI-MS fragmentation (Scheme **4.1**) that showed fragment ions at *m/z* 151 (1**26a**) and *m/z* 191 (1**26b**) (base peak) caused by *retro*-Diels Alder cleavage of ring C.

The chemical shift of H-1 (δ_H 6.51, 1H, *s*) is consistent with *cis*-B/C ring junction as most rotenoids of this family (Yenesew, 1997a). A positive optical rotation ($[\alpha]_D$ = +22.24 and negative Cotton effect at 334.9 nm in the CD spectrum are consistent with a 6a*S*:11a*S* absolute configuration (Tahara *et al*., 1990). Furthermore, the equatorial orientation of H-6a was established by small coupling constant observed for coupling of H-6a with both methylene protons at C-6 (Yenesew, 1997b). This new compound was therefore identified as (6a*S,* 12a*S)*-9-methoxy-2, 3-methylenedioxy-12a-hydroxyrotenoid and the trivial name oblatarotenoid A is given.



Scheme 4.1: EI-MS fragmentation in compound **126**

Table 4.2: ^{1}H (600 MHz) and ^{13}C (150 MHz) NMR data of oblatarotenoid A (**126**) in CD_2Cl_2

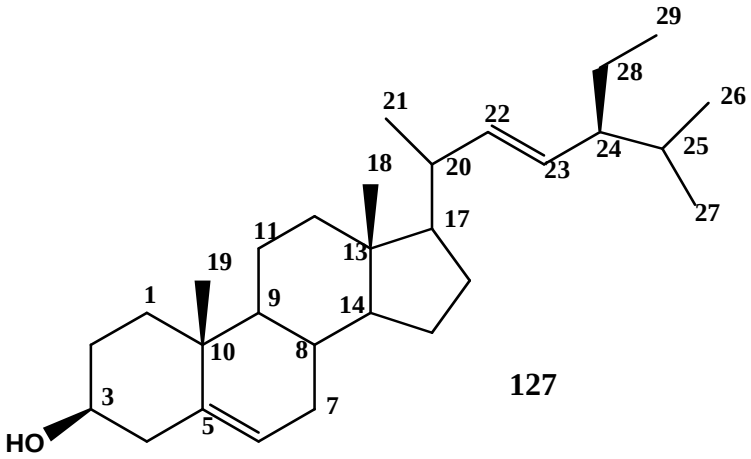
Position	^{1}H, δ ppm (*m*, *J* in Hz)	^{13}C, δ ppm	HMBC	
			^{2}J	^{3}J
1	6.51 (*s*)	106.0		C-12a, C-3, C-4a
2		142.4		
3		149.5		
4	6.42 (*s*)	98.9	C-3, C-4a	C-12b
4a		149.6		
6_{ax}	4.56 (*dd*, 12.1, 2.5)	64.1	C-6a	C-4a
6_{eq}	4.44 (*dd*,12.1, 1.0)		C-6a	C-4a
6a	4.58 (*dd*, 2.5,1.0)	76.1	C-12a	C-12b
7a		162.6		
8	6.35 (*d*, 2.2)	100.7	C-7a, C-9	C-11a
9		167.3		
10	6.56 (*dd*, 8.9, 2.3)	111.1	C-9	C-11a
11	7.78 (*d*, 8.9)	129.4	C-10, C-11a	C-7a, C-9, C-12
11a		111.3		
12		191.4		
12a(OH)		67.9		
12b		109.9		
OMe-9	3.75 (*s)*	56.0		C-9
OCH_2O	5.81 (*d*, 1.3)	101.7		C-2
	5.78 (*d*, 1.3)			C-3

4.2.1.3 Stigmasterol (127)

Compound **127** was isolated as a white powder. It is not UV (254 nm) sensitive but turned brown upon exposure to iodine vapour. The ^{13}C NMR spectrum (Table **4.3**) showed 29 signals indicating that it could be a steroid. Among these signals, there are six methyl carbons [δ_C 12.0 (C-18), 18.8 (C-19), 19.2 (C-27), 19.5 (C-26) and 21.1 (C-21)], nine methylene carbons [δ_C 36.5 (C-1), 33.9 (C-2), 42.2 (C-4),31.7 (C-7), 20.8 (C-11), 37.3 (C-12), 24.2 (C-15) 28.2 (C-16) and 26.0 (C-28)], eleven methine carbons [δ_C 71.7 (C-3), 141.0 (C-6), 32.0 (C-8), 50.2 (C-9), 56.8 (C-14), 56.1 (C-17), 39.8 (C-20), 138.4 (C-22), 129.3 (C-23), 45.9 (C-24) and 29.2 (C-25)] and three quaternary carbons [δ_C 121.4 (C-5), 36.1 (C-10) and 42.2 (C-13)]. Four of these carbons

are olefinic [δ_C 141.0 (C-6), 138.4 (C-22), 129.3 (C-23) and 121.4 (C-5)] and the rest are sp^3 carbons, one of them is oxygenated [δ_C 71.7 (C-3)]. In addition, the ^{1}H NMR spectrum (Table **4.3**) revealed a one-proton multiplet at δ_H 3.53. The position and multiplicity of which was indicative of the hydroxymethine proton at C-3 of the steroidal nucleus. The typical signal for olefinic H-6 of the steroidal skeleton was evident from a multiplet at δ_H 5.31 intergrating for one proton.

Furthermore, the ^{1}H NMR spectrum also indicated the presence of two olefinic protons (H-22 and H-23) which appeared as characteristics downfield signals at δ_H 4.98 and 5.14, respectively. Each of the signals was observed as multiplet which indicated coupling with the neighbouring methine protons. The spectrum further revealed signals for six methyl at δ_H [(1.03, *s,* H-18), (0.71, *s,* H-19), (0.80, *d*, *J* = 6.6 Hz, H-27), (0.82, *d*, *J* = 6.6 Hz, H-26), (0.83, *t*, *J* = 7.1 Hz, H-29) and (0.19, *d*, *J* = 6.2, H-21)]. These spectral features are in close agreement to those reported for stigmasterol, a compound widely occurs in higher plants (Chaturvedula and Prakash, 2012). On this basis, the identity of compound **127** was confirmed as stigmasterol (Muhit *et al*., 2010).



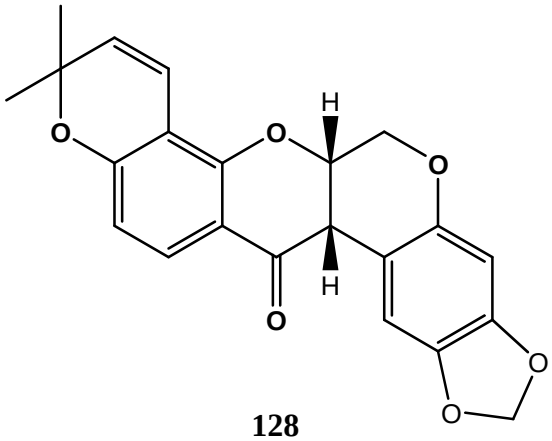
127

Table 4.3: ^{1}H (600 MHz) and ^{13}C (150 MHz) NMR data of stigmasterol (**127**) in CD_2Cl_2

Position	^{1}H, δ ppm (*m*, *J* in Hz)	^{13}C,δppm	HMBC
1	1.08 (*dd*, 13.3, 4.8)	36.5	
	1.82 (*dt*, 13.2, 3.6)		
2	1.78 (*m)*	33.9	
	1.49 (*m*)		
3	3.53 (*tdd*, 4.5, 4.2, 3.8)	71.7	
4	2.5 (*ddd,* 13.2, 4.8, 2.4)	42.2	
	2.19 (*tm,* 13.2)		
5		121.4	
6	5.31 (*t* ,6.1)	141.0	C-4
7	1.97 (*m)*	31.7	
	1.97 (*m)*		
8	1.47 (*m)*	31.9	
9	0.93 (*m)*	50.2	
10		36.1	
11a	1.53 (*m)*	20.8	
11b	1.53 (*m)*		
12a	1.97 (*m)*	37.3	
12b	1.97 (*m)*		
13		42.2	
14	1.02 (*m)*	56.8	
15	1.56 (*m)*	24.2	
	1.56 (*m)*		
16	1.72 (*m)*	28.2	
	1.72 (*m)*		
17	1.16 (*m)*	56.1	
18	1.03 (*s*)	12.0	C-14
19	0.17 (*s*)	18.8	C-5
20	2.05 (*m)*	39.8	
21	0.19 (*d*, 6.2)	21.1	
22	4.98 (*m*)	138.4	C-17
23	5.14 (*m*)	129.3	
24	1.53 (*m*)	45.9	
25	1.54 (*m*)	29.2	
26	0.82 (*d,* 6.6)	19.5	C-25
27	0.80 (*d*, 6.6)	19.2	C-25
28a	1.22 (*m*)	26.0	
28b	1.56 (*m*)		
29	0.83 (*t*, 7.1)	11.8	C-22

4.2.1.4 Millettone (128)

Compound **128** was isolated along with compound **126** as a mixture. The ^{1}H [δ_H 4.60 (1H, *dd*, *J* = 0.9, 12.0 Hz, H-6eq), 4.15 (1H, *dd*, *J* = 2.4, 12.0 Hz, H-6ax), 4.92 (1H, *dd*, *J* = 0.9, 2.4 Hz, H-6a) and 3.80 (1H, *brs*, H-12a) and ^{13}C [δ_C 66.4 (C-6); 72.1 (C-6a), 44.8 (C-12a) and 188.9 (C-12)] NMR spectra suggested the compound to be a rotenoid derivative (Yenesew *et al*., 1997b; 2003; Agrawal, 1989). The NMR spectral data (Table **4.4**) of compound **128** also showed the presence of a methylenedioxy and 1,1-dimethylchromene substituents on the rotenoid skeleton. The presence of two *para*-oriented aromatic protons at δ_H 6.53 (1H, *s*, H-1) and 6.45 (1H, *s*, H-4) and the chemical shift values of oxygenated carbon atoms in ring A, confirmed the presence of methylenedioxy group at C-2/C-3 as in compound **126**. In ring D, the presence of two *ortho*-coupled protons at δ_H 7.68 (*d, J* = 9.0 Hz, H-11) and 6.58 *d, J* = 9.0 Hz) is consistent with the placement of the 1,1-dimethylchromene ring at C-8/C-9. Thus compound **128** was identified as millettone, a compound that has been reported from the seed pods of *Millettia dura* (Yenesew, 1997b; 1998a).



128

Table 4.4: ^{1}H (600 MHz) and ^{13}C (150 MHz) NMR data of millettone (**128**) in CD_2Cl_2

Position	^{1}H, δ ppm (*m, J* in Hz)	^{13}C, δppm
1	6.53 (*s)*	106.7
2		142.2
3		147.8
4	6.45 (*s)*	98.7
4a		149.3
6	4.60 (*dd,* 0.9,12.0)	66.4
	4.15 (*dd*, 2.4, 12.0)	
6a	4.92 (*dd*, 0.9, 2.4)	72.1
7a		157.0
8		109.7
9		160.7
10	6.58 (*d,* 9.0)	111.7
11	7.68 (*d,* 9.0)	128.4
11a		111.7
12		188.9
12a	3.80 (*d,* 9.6)	44.8
12b		105.8
4’	5.56 (*d,*9.8)	128.4
5’	6.64 *(d,*9.7)	115.0
6’		78.0
8’	1.48 (*s)*	27.9
7’	1.46 (*s)*	28.2
OCH_2O	5.75 (*d,*1.3)	101.4
	5.78 (*d,*1.3)	

4.2.2 Characterization of a Pterocarpan from *Millettia micans*

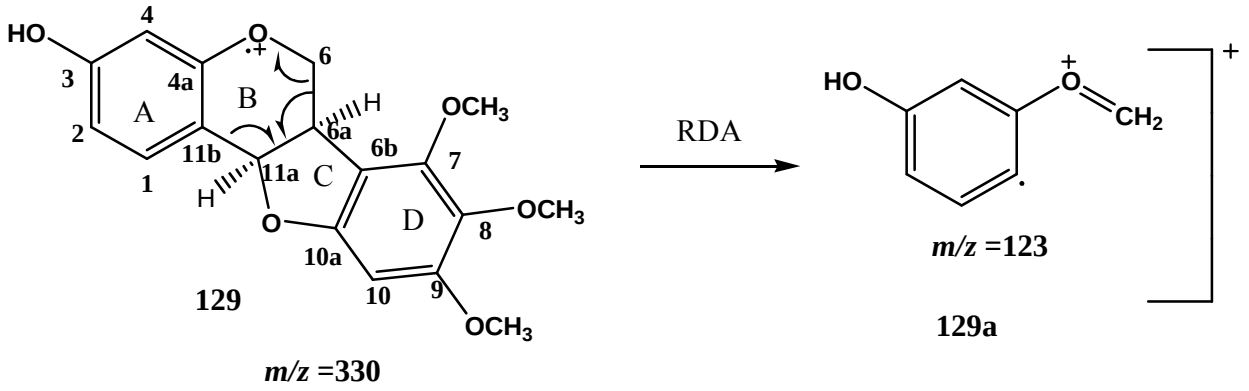
4.2.2.1 Micanspterocarpan A (129)

Compound **129** was obtained as a white powder. The HREI mass spectrum of compound **129** exhibited a molecular ion peak, $[M]^{+}$, at *m/z* 330.1115 corresponding to the molecular formula $C_{18}H_{18}O_6$. The UV (λ_{max} 268, 269 and 281 nm), ^{1}H NMR [δ_H 4.22 (*dd*, *J* = 10.89 Hz, *J* = 5.0 Hz, H-6*ax)*, 3.61 (*dd*, *J* = 5.0 Hz, *J* = 6.76 Hz, H-6*eq*), 3.56 (*m,* H-6a) and 5.35 (*d*, *J* = 6.76 Hz, H-11a)] and ^{13}C NMR [δ_C 39.1 (C-6a), 65.8 (C-6) and 78.1 (C-11a)] spectra are consistent with a pterocarpan skeleton (Suginome, 1959; 1962; Schonig *et al*., 1989; 1990 and Agrawal, 1989). In the mass spectrum of compound **129**, an intense molecular ion peak at *m/z* 330 is also in

agreement with the compound being a pterocarpan derivative, as pterocarpans are known to be stable to electron impact fragmentation (Poster *et al*., 1970; Piccinelli *et al*., 2005). The ^{1}H NMR spectrum showed the presence of three methoxyl substituents at δ_H 3.66, 3.89, and 3.78 with the corresponding carbon signals appearing at δ_C 56.1, 60.7 and 60.8, respectively (Table **4.5**).

The ^{1}H NMR spectrum also revealed the presence of an AXY spin system [at δ_H 7.25 (1H, *d*, *J* = 8.4 Hz), 6.46 (1H, *dd*, *J* = 8.4, 2.4 Hz) and 6.32 (1H, *d*, *J* = 2.4 Hz)], and a singlet aromatic proton (δ_H 6.14), showing that the compound is tetra-substituted (three methoxyl and one hydroxyl) pterocarpan. In the HMBC spectrum, H-11a (δ 5.35) showed correlation with the doublet at δ 7.25 allowing the assignment of this signal to H-1, and hence the AXY system to ring A, *vis a vis* δ_H 7.25 (1H, *d*, *J* = 8.4 Hz, H-1), 6.46 (1H, *dd*, *J* = 8.4, 2.4 Hz, H-2) and 6.32 (1H, *d*, *J* = 2.4 Hz, H-4). The singlet aromatic proton (δ_H 6.14) is then placed in ring D which otherwise is fully substituted. HMBC correlation of this singlet aromatic proton (δ_H 6.14) with C-6b (δ_C 109.5), C-8 (δ_C 154.8), C-9 (δ_C 135.5) and C-10a (δ_C 155.8) allowed its assignment to H-10, with oxygenation at C-7, -8 and -9. The ^{13}C NMR chemical shift values for ring D carbon atoms are in agreement with such substitution pattern (Table **4.5**). Two of the methoxy groups are de-shielded (δ_C 60.7 and 60.8) showing that they are di-*ortho* substituted and hence placed at C-7 and C-8. The third methoxy group (δ_C 56.1; δ_H 3.66, which appeared in the normal range) was placed at C-9 based on HMBC correlation (H-10 with C-9; OMe-9 with C-9), which was supported by NOE correlation between this methoxyl protons and H-10. EI-MS fragment at *m/z* 123 (**129**a) (Scheme **4.2**) resulting from RDA cleavage of ring B supports that ring A is substituted with hydroxyl and hence the three methoxy groups are placed in ring D.

The coupling constant (6.7 Hz) for $J_{6aH,11aH}$ is typical of the energetically more favorable (Poster *et al*., 1970) *cis* B/C ring junction (Schonig *et al*., 1989; 1990). A large negative optical rotation ($[\alpha]_D$ = -134.3°) and negative Cotton effect at 232.6 nm in the CD spectrum are consistent with a 6a*R*:11a*R* absolute configuration (McKee *et al.,* 1997; Yenesew *et al*., 1998b). Therefore this new compound is characterized as (6a*R,* 12a*R*)-7,8,9-trimethoxy-3-hydroxypterocarpan (**129**) for which the trivial name micanspterocarpan A is assigned.



Scheme 4.2: EI-MS fragmentation in compound **129**

Table 4.5: ^{1}H (600 MHz) and ^{13}C (150 MHz) NMR data of micanspterocarpan A (**129**) in CD_2Cl_2

Position	^{1}H, δ ppm (*m*, *J* in Hz)	^{13}C, δ ppm	HMBC
1	7.25 (*d*, *J* = 8.4 Hz)	132.1	C-11a, C-4a, C-3
2	6.46 (*dd*, *J* = 2.4 Hz, 8.4 Hz)	109.8	C-4, C-11b
3		157.1	
4	6.32 (*d*, *J* = 2.4 Hz)	103.4	C-2, C-11b
4a		156.8	
6	4.22 (*dd*, *J* = 10.9 Hz, *J* = 5.0 Hz, H-6_{ax})	65.8	C-4a, C-11a, C-C-6b, 6a
	3.61 (*dd*, *J* = 5.0 Hz, *J* = 6.8 Hz, H-6_{eq})		
6a	3.56 (*m*)	39.1	C-6, C-6b, C-10a
6b		109.5	
7		150.6	
8		154.8	
9		135.5	
10	6.14 (*s*)	90.6	C-8, C-6b, C-9, C-10a
10a		155.8	
11a	5.35 (*d*, *J*= 6.8 Hz)	78.1	C-6, C-1, C-4a, C-11b
11b		112.8	
3-OH	4.18 (*br s*)		
9-OCH_3	3.66 (*s*)	56.1	C-9
7-OCH_3	3.89 (*s*)	60.7	C-7
8-OCH_3	3.78 (*s*)	60.8	C-8

4.2.3 Characterization of Compounds from *Derris trifoliata*

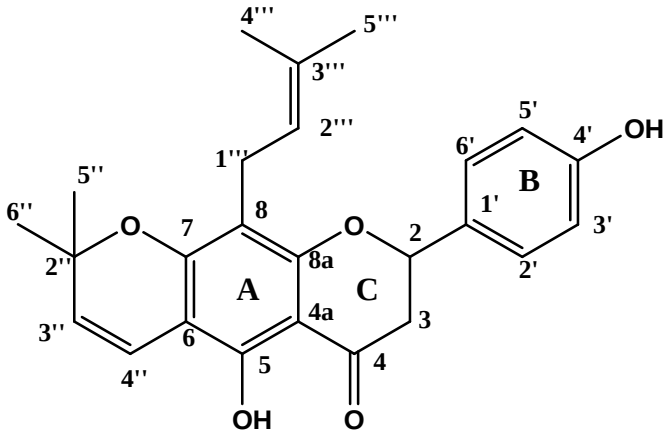
4.2.3.1 Lupinifolin (130)

Compound **130** was obtained as viscous oil whose UV spectrum showed absorption maxima at 247, 256, 265 and 271.2 nm. The ^{1}H NMR spectrum showed resonances for an AXY system at δ_H 3.04 (1H, *dd*, *J* = 17.5, 13.0 Hz, H-3β), 2.80 (1H, *dd*, *J* = 17.5, 3.0Hz, H-3α) and 5.33 (1H, *dd*, *J* = 3.3, 13.3Hz, H-2β) and ^{13}C NMR (δ_C 78.5 (C-2), 43.1 (C-3) and 196.5 (C-4) which together with a proton at δ_H 12.23 (Table **4.6**) for a chelated hydroxyl group indicate this compound has a 5-hydroxyflavanone nucleus (Yenesew *et al*., 2009).

The NMR spectrum also revealed the presence of a prenyl (δ_H 3.21 [2H, *br d*, *J* = 7.2 Hz, H-1'''), 5.16 (1H, *t*, *J* = 7.2 Hz, H-2'''), (2H, *brd*, *J* = 7.2 Hz, H-1''') and 1.65 (s, 6H); δ_C 21.4 (C-1'''), 122.6 (C-2'''), 131.1 (C-3''') and 25. 8 (C-4''') and 17.7 (C-5''')] and a 2,2-dimethylpyran [δ_H 5.50 (1H, *d*, *J* = 10.2 Hz, H-3''), 6.63 (1H, *d*, *J* = 10.2 Hz, H-4''), 1.43 (3H, *s*, H-5'') and 1.45 (3H, *s*, H-6''); δ_C 78.1 (C-2''), 125.9 (C-3''), 115.6 (C-4''), 28.3 (C-5'') and 28.4 (C-6'')] substituents.

The ^{1}H NMR spectrum showed an AA'XX' spins system centered at δ_H 7.31 (H-2'/6') and 6.87 (2H, *d*, *J* = 8.5 Hz, (H-3'/5')) which are assigned to a 4'-oxygenated ring B. Thus, the prenyl and the 2,2-dimethylpyran groups are located in ring A. The prenyl group is placed at C-8 due to HMBC correlation of H-1''' (δ_H 3.21) with C-7 (δ_C 159.9), C-8 (δ_C 108.6) and C-8a (δ_C 159.4). This allows the placement of the 2,2-dimethylpyran ring between C-6 and C-7 with the oxygen being at C-7.

Based on this and comparing of the NMR data with literature, compound **130** is identified as lupinifolin (Mahidol, *et al*., 1997; Yenesew *et al*., 2009). Lupinifolin (**130**) has been previously isolated from *Derris trifoliata* seeds (Yenesew *et al*., 2009) and *Derris reticulata* (Mahidol *et al*., 1997). This however is the first report on the occurrence of this compound from the leaves of this plant.



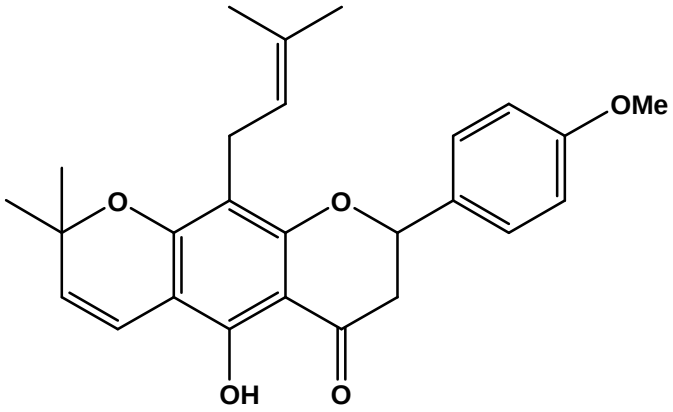
130

Table 4.6: ^{1}H (600 MHz) and ^{13}C (150 MHz) NMR data of lupinifolin (**130**) in CD_2Cl_2

Position	^{1}H, δppm (*m, J* in Hz)	^{13}C, δ ppm	HMBC
2	5.33 (*dd*, *J* = 3.3, 13.3)	78.5	C-4, C-1', C-2'
3	2.80 (*dd*, *J* = 17.5, 3.0)	43.1	C-4, C-2, C-1'
	3.04 (*dd*, *J* = 17.5,13.0)		C-4, C-2, C-1'
4		196.5	
4a		102.6	
5		156.5	
6		102.8	
7		159.9	
8		108.6	
8a		159.4	
1'		130.8	
2'/6'	7.31 (*d*, *J* = 8.5)	127.6	C-2, C-4', C-6'/2'
3'/5'	6.87 (*d*, *J* = 8.5)	115.5	C-1', C-4', C-5'/3'
4'		156.0	
2''		78.1	
3''	5.50 (*d*, *J* = 10.2)	125.9	C-6, C-2'', C-5'', C-6''
4''	6.63 (*d*, *J* = 10.2)	115.6	C-5, C-6, C-7, C-2''
5''	1.43 (*s*)	28.3	C-2'', C-3'', C- 6''
6''	1.45 (*s*)	28.4	C-2'', C-3'', C-5''
1'''	3.21 (*br d, J* = 7.2)	21.4	C-7, C-8, C-8a, C-2''', C-3'''
2'''	5.16 (*t, J*=7.2)	122.6	C-8, C-1''', C-4''', C-5'''
3'''		131.1	
4'''	1.65 (*s*)	25.8	C-8, C-2''', C-3''', C-5'''
5'''	1.65 (*s*)	17.8	C-8, C-2''', C-3''', C-4'''
5-OH	12.23 (*s*)		C-4a, C-5, C-6

4.2.3.2 Lupinifolin 4-methyl ether (131)

Compound **131** was obtained as viscous oil. The NMR spectra (Table **4.7**) of compound **131** are similar to that of compound **130** except for the appearance of a signal for a methoxyl group [δ_H 3.84 (3H, s,); δ_C 55.3] in compound **131**. Based on this, compound **131** is identified as a methoxyl derivative of compound **130**. The complete analysis of ^{13}C and ^{1}H NMR spectral data of this compound were assigned with information provided from HMBC, HSQC and COSY spectrum, along with comparison of spectral data of compound **130**. The methoxy group is placed at C-4' due to its HMBC correlation with C-4' (δ_C 159.8). Based on this and comparing the NMR data with literature, compound **131** is identified as lupinifolin 4'-methyl ether (Yenesew *et al*., 2009). Lupinifolin 4'-methyl ether (**131**) has been previously isolated from *Derris trifoliata* seeds (Yenesew *et al*., 2009).



131

Table 4.7: ^{1}H (600MHz) and ^{13}C (150 MHz) NMR data of lupinifolin 4'-methyl ether (**131**) in CD_2Cl_2

Position	^{1}H, δppm (*m, J* in Hz)	^{13}C, δ ppm	HMBC
2	5.35 (*dd*, *J* = 3.0, 13.0)	78.5	C-4, C-1',C-2'
3	2.80 (*dd*, *J* = 17.0, 3.0)	43.1	C-4, C-2, C-1'
	3.05 (*dd*, *J* = 17.0,13.0)		C-4, C-2, C-1'
4		196.4	
4a		102.6	
5		156.6	
6		102.8	
7		159.8	
8		108.6	
8a		159.4	
1'		130.9	
2'/6'	7.37 (*d*, *J* = 8.4)	127.6	C-2, C-4', C-6'/2'
3'/5'	6.94(*d*, *J* = 8.4)	114.1	C-1', C-4', C-5'/3'
4'		159.8	
2''		78.0	
3''	5.49 (*d*, *J* = 10.0)	125.9	C-6, C-2'', C-5'', C-6''
4''	6.63 (*d*, *J* = 10.0)	115.7	C-5, C-6, C-7, C-2''
5''	1.43 (*s*)	28.3	C-2'', C-3'', C- 6''
6''	1.45 (*s*)	28.4	C-2'', C-3'', C-5''
1'''	3.21 (*br d, J* = 7.2)	21.5	C-7, C-8, C-8a, C-2''', C-3'''
2'''	5.16 (*t, J* = 7.2)	122.6	C-8, C-1''', C-4''', C-5'''
3'''		131.6	
4'''	1.65 (*s*)	25.8	C-8, C-2''', C-3''', C-5'''
5'''	1.65 (*s*)	17.8	C-8, C-2''',C-3''', C-4'''
5-OH	12.26 (*s*)		C-4a, C-5, C-6
OCH_3	3.84 (*s*)	55.3	C-4'

4.3 Larvicidal Test Results

Three crude extracts and three compounds were tested against 3rd instar larvae of *A. aegypti.* The results are tabulated in table **4.8** below.

Table 4.8: Larvicidal test results

Sample tested	**LC_{50} (µg/mL ± SD) at 24 hr.**
M. oblata spp *teitensis* (DCM/MeOH crude leaves extract)	84.6± 7.8
M. micans (DCM/MeOH crude root bark extract)	132± 14.4
D. trifoliata (DCM/MeOH crude leaves extract)	367.7±162.8
Tephrosin **(125)**	1.4±0.2
Oblatarotenoid A (1**26**)	>100
Micanspterocarpan A (**129**)	>100
Lupinifolin (**130)**	>100

The crude of *M. oblata* spp. *teitensis* and *M. micans* showed larvicidal activity with the LC_{50} value of 84.6 ± 7.8 µg/mL (moderate) and 132 ±14.4 µg/mL (weak) at 24hr respectively. (Nguta *et, al*., 2012; Devaraj, *et al*., 2013). Tephrosin **(125)** isolated from the extract of leaves of *M.* spp. *teitensis* showed a moderate activity with LC_{50} value of 1.4 ± 0.2 µg/mL while oblatarotenoid A **(126)** was inactive with LC_{50} value greater than 100 µg/mL at 24hr (Rahman, 2012).

Previous studies on structure-activity relationship have shown that the larvicidal activity of rotenoids depends on the presence of methoxy groups at C-2 and C-3 in the ring A and β-orientation at both 6a and 12a carbons (Yenesew *et al*., 2003b). Furthermore, it must be stressed that hydroxylation of C-12a, the presence of another hydroxyl group at C-11 and lack of unsaturation at the B/C-ring junction had subtle effects on larvicidal activity. The importance of the lipophilicity is also another factor that contributes to high efficiency of rotenoids (Vasconcelos *et al*., 2012). Therefore, the findings on tephrosin (**125**) is consistent with some of

the above suggestions that the presence of the methoxy groups at C-2 and C-3, *β*-orientation at C-6a and C-12a, lack of unsaturation at B/C- ring junction and hydroxylation at C-12a are important for larvicidal. However, inactiveness of oblatarotenoid A (**126**) might be due to lack of functional groups responsible for larvicidal activity such as the two methoxyl groups in ring A. Furthermore, tephrosin (**125**) might be responsible for the activity of the crude extract of *M. oblata* spp. *teitensis.*

The crude extract of *D. trifoliata* (leaves) showed weak larvicidal activity against the 3rd instar larvae of *Aedes aegypti* with LC_{50} value of 367.7 ± 162.8 µg/mL at 24 hr (Nguta *et, al*., 2012; Devaraj, *et al*., 2013). Similarly, lupinifolin (**130**) which is the major compound of the crude leaves extract of *D. trifoliata* was also inactive with LC_{50} value greater than 100 µg/mL at 24hr (Rahman, 2012). However, good antiplasmodial and anticancer activities have been reported for this compound (Yenesew *et al*., 2009). Subsequently, micanspterocarpan A (**129**), a compound isolated from *M. micans* (root bark) was also inactive with LC_{50} value greater than 100 µg/mL at 24hr (Rahman, 2012).

CHAPTER FIVE

CONCLUSIONS AND RECOMMENDATIONS

5.1 Conclusions

In this study, three plants (*Millettia oblata* spp. *teitensis, Millettia micans* and *Derris trifoliata*) were investigated and seven compounds were isolated and characterized. The study showed that these plants are sources of flavanones, rotenoids, pterocarpans and steroids. The crude extracts and four compounds were tested for larvicidal activity against the third instar larvae of mosquito of *Aedes aegypti.*

Thus, from the leaves of *M. oblata* spp. *teitensis*, four compounds were isolated and characterized. These were identified as the rotenoids tephrosin (**125**), oblatarotenoid A **(126)**, and millettone (**128**), and the steroid stigmasterol (**127**). Oblatarotenoid A (**126**) is new compound and isolated as the major compound of this extract. The crude extract of *M. oblata* spp. *teitensis* showed potent larvicidal activity against the third instar larvae of *A.aegypti*. Two compounds, tephrosin (**125**) and oblatarotenoid A (1**26**) were also tested and the activity of the crude is mainly due to tephrosin.

From the roots of *M. micans*, a new pterocarpan, named micanspterocarpan A (**129**), was isolated and characterized. The crude extract and micanspterocarpan A (**129**), were also tested for larvicidal activity. Both of them were inactive against the third instar larvae of *A.aegypti*. In similar phytochemical study on the leaves of *D. trifoliata*, two compounds were isolated and characterized. These were identified as the flavanones lupinifolin (**130**) and lupinifolin 4'-methyl

ether (**131**). The crude extract of *D. trifoliata* and the major compound of the extract, lupinifolin (**130**), did not show significant larvicidal activity against the third instar larvae of *A.aegypti*.

5.2 Recommendations.

1. Further phytochemical investigation of *M. micans* and *M. oblata* (leaves) should be carried out in order to establish the complete phytochemical profile of this plant.
2. Antimicrobial activities should be carried on *Derris trifoliata* and *Millettia micans.*
3. Derivatization of the isolated compounds should be carried out in order to determine the functional groups necessary for larvicidal activity. Comprehensive study should be carried out on structure-activity-relationship in order to determine the properties responsible for larvicidal agents.

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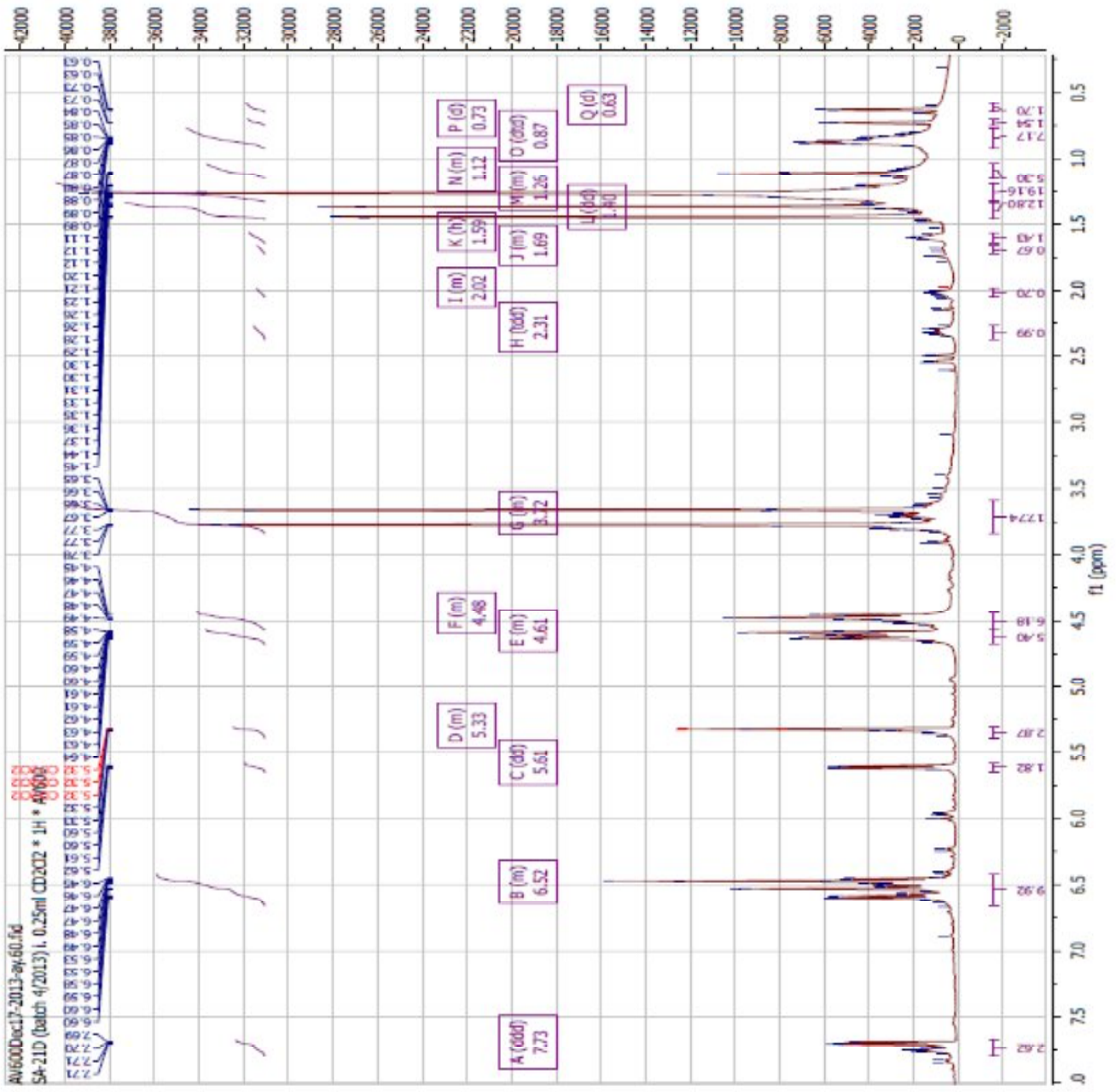
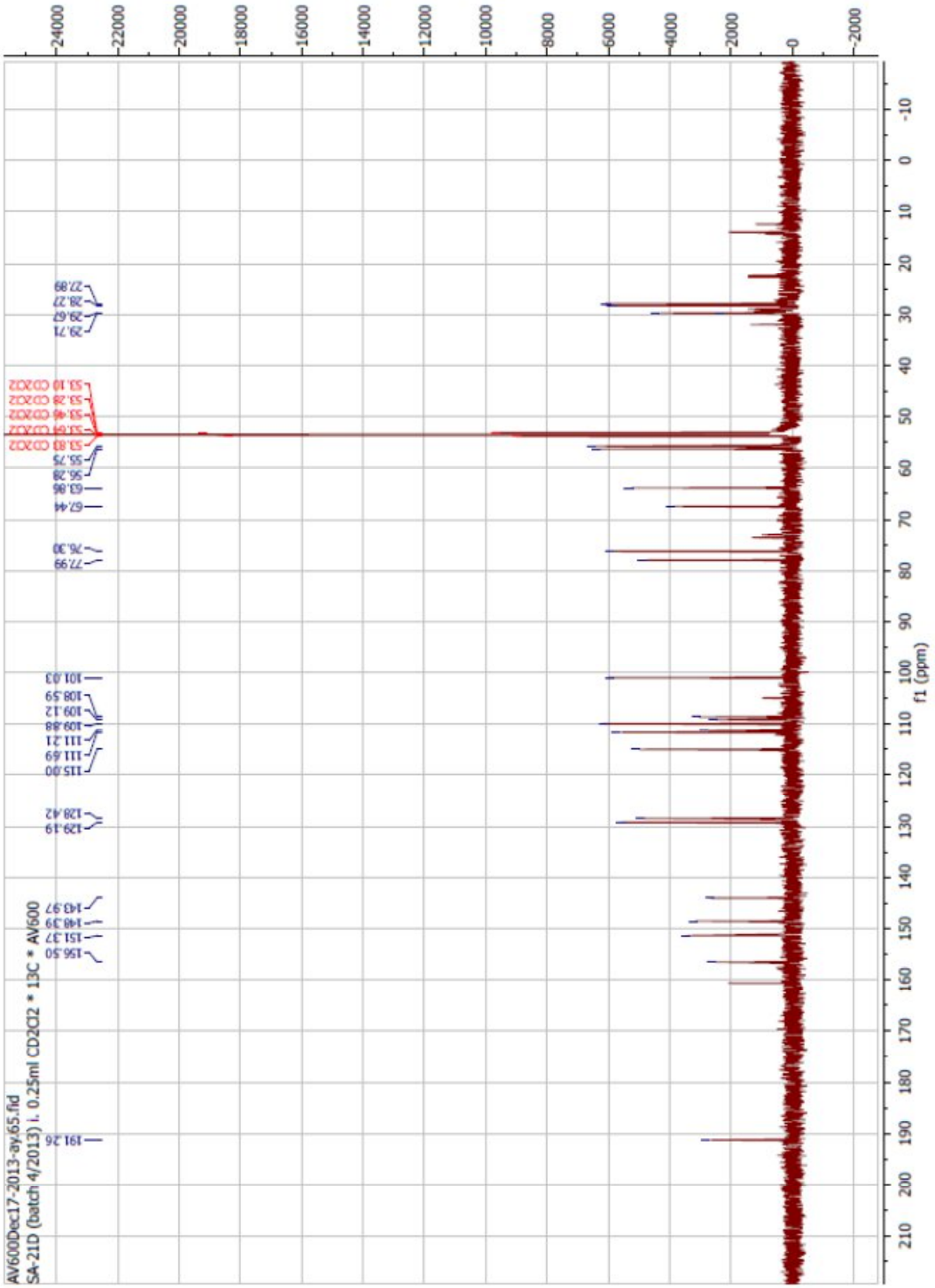
APPENDICES

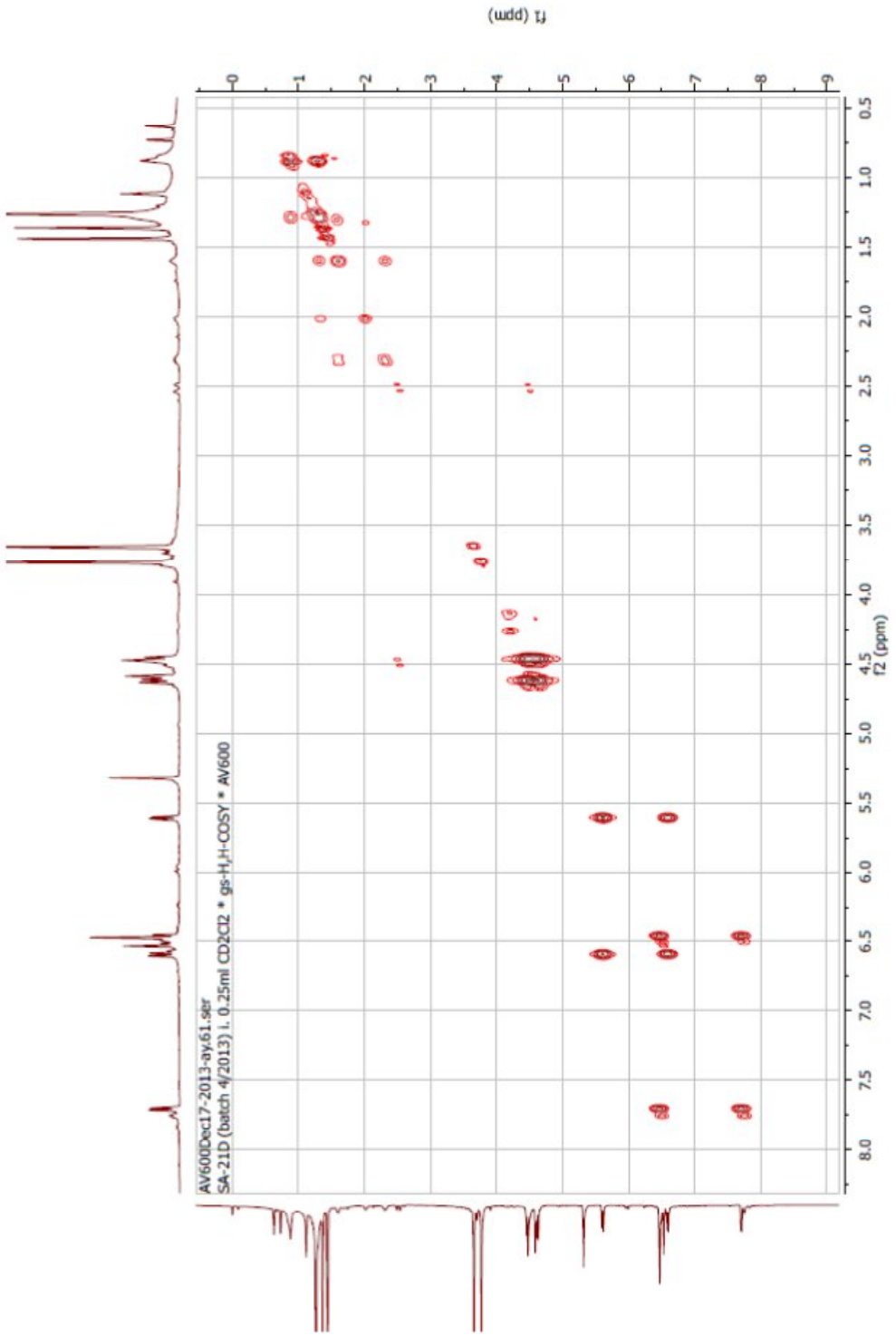
Appendix 125A: ^{1}H (CD_2Cl_2, 600 MHz) NMR spectrum of tephrosin (**125**)



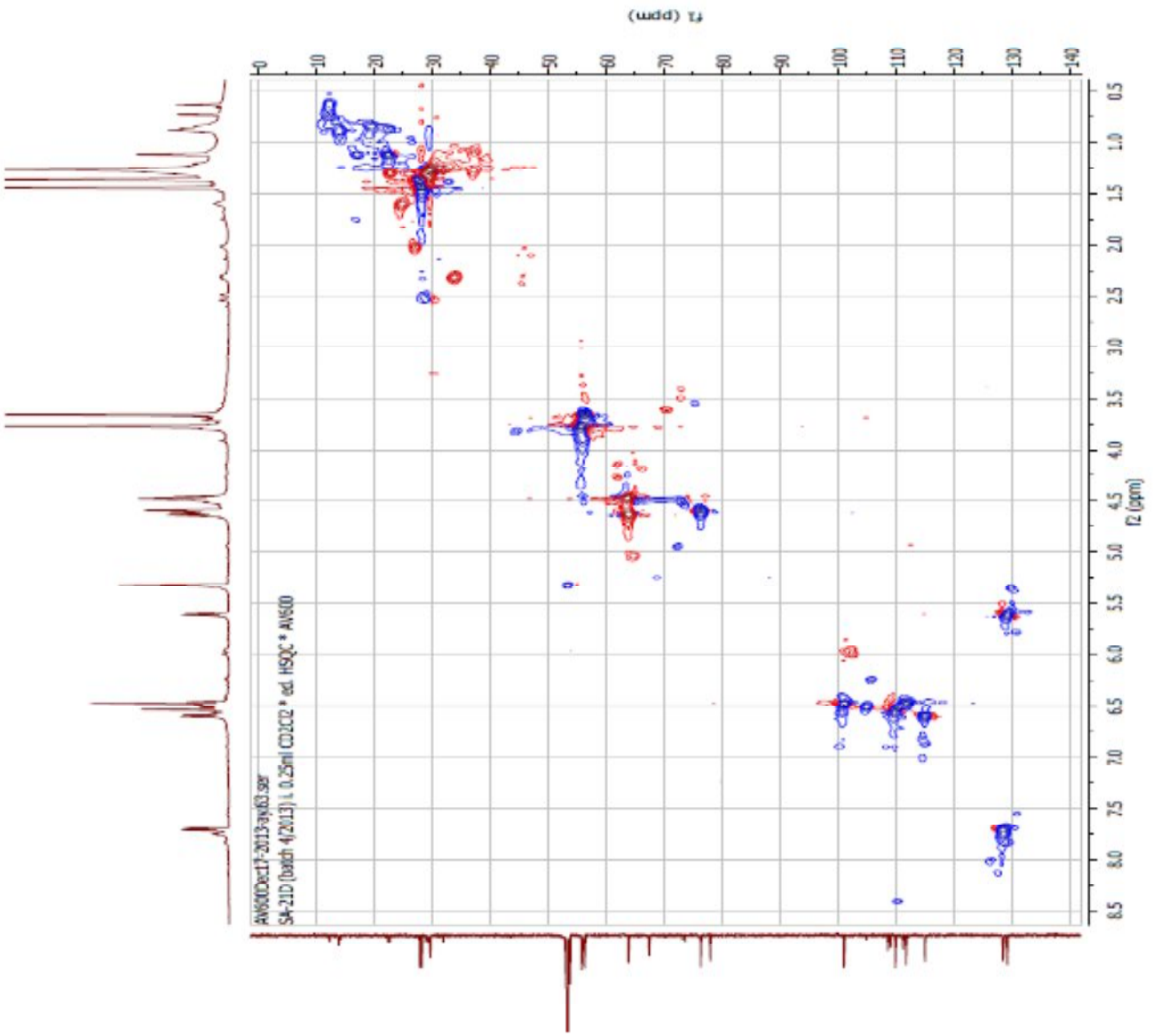
Appendix 125B: ^{13}C (CD_2Cl_2, 150 MHz) NMR spectrum of tephrosin (**125**)



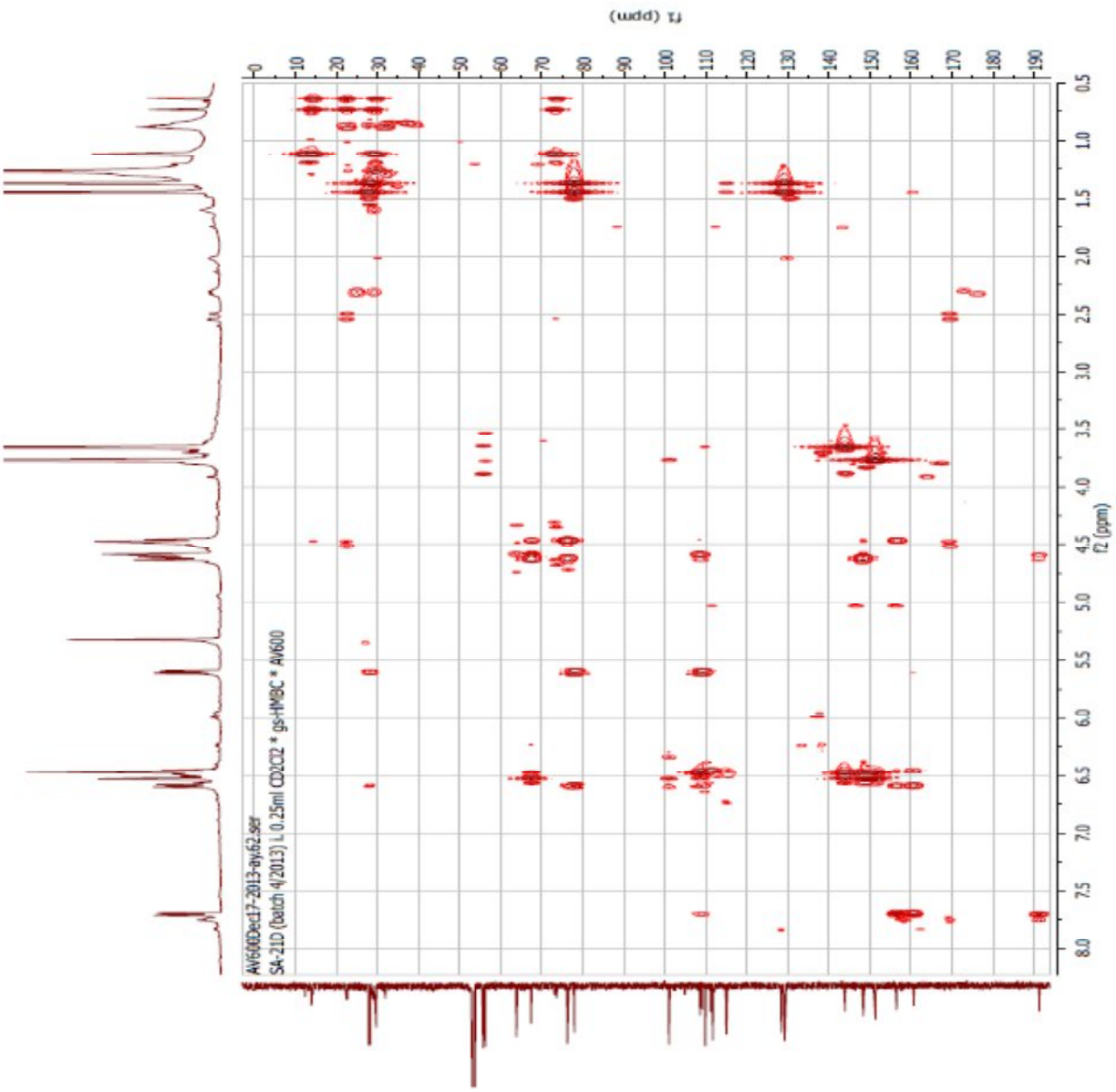
Appendix 125C: ^{1}H-^{1}H COSY (CD_2Cl_2, 600 MHz) NMR spectrum of tephrosin (**125**)



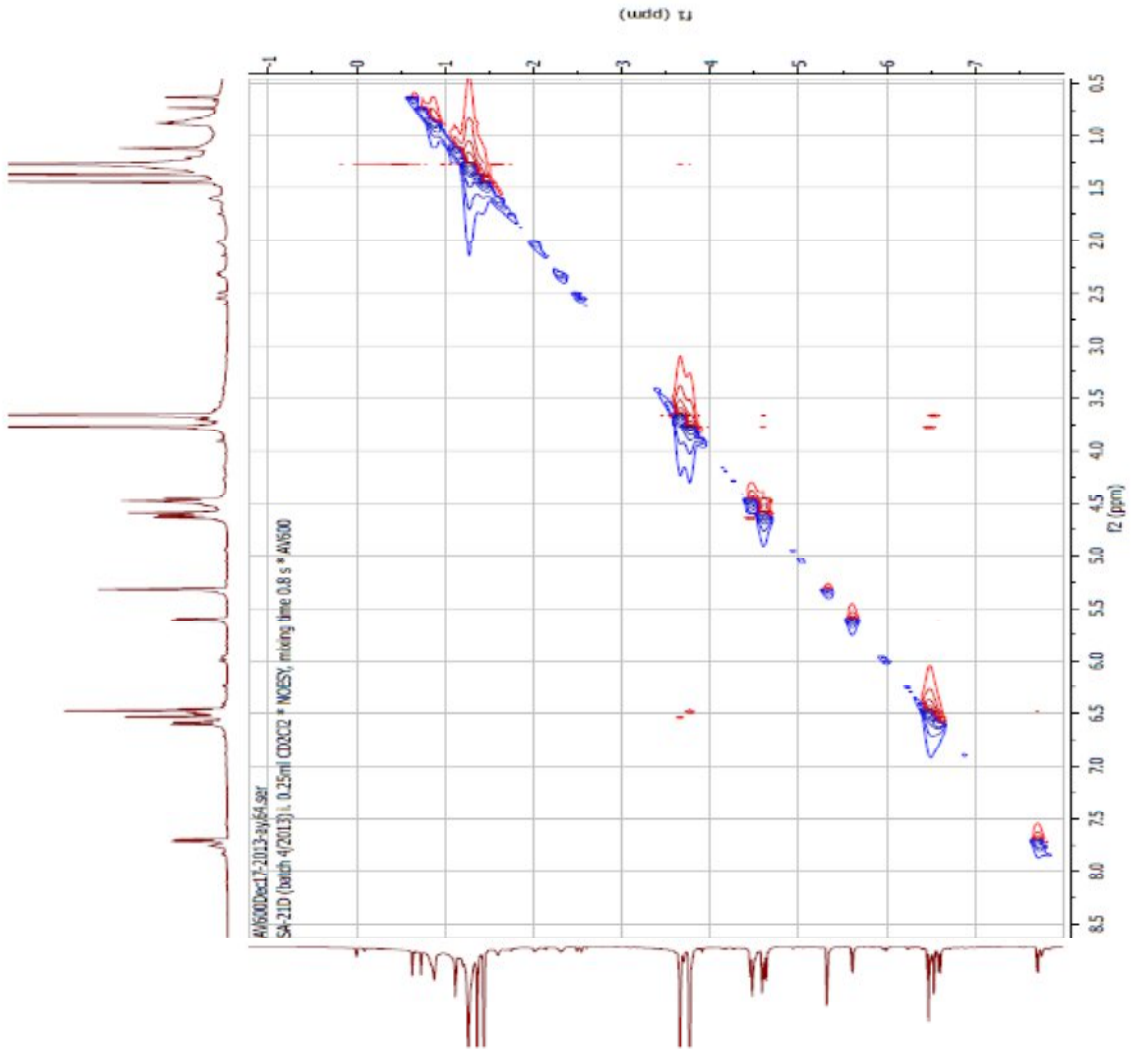
Appendix 125D: gHSQC(CD_2Cl_2, 600 MHz) NMR spectrum of tephrosin (**125**)



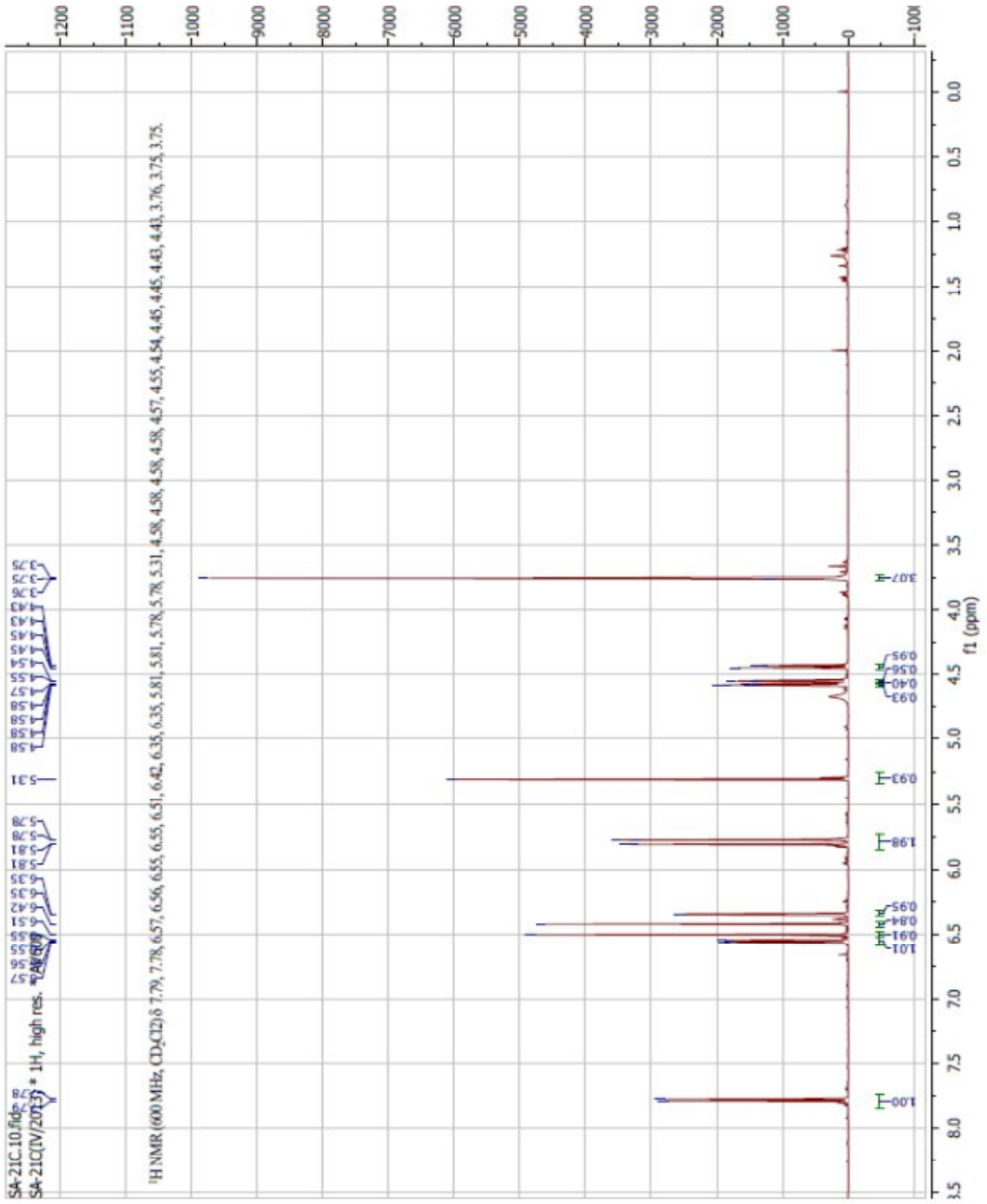
Appendix 125E: gHMBC (CD_2Cl_2, 600 MHz) NMR spectrum of tephrosin (**125**)



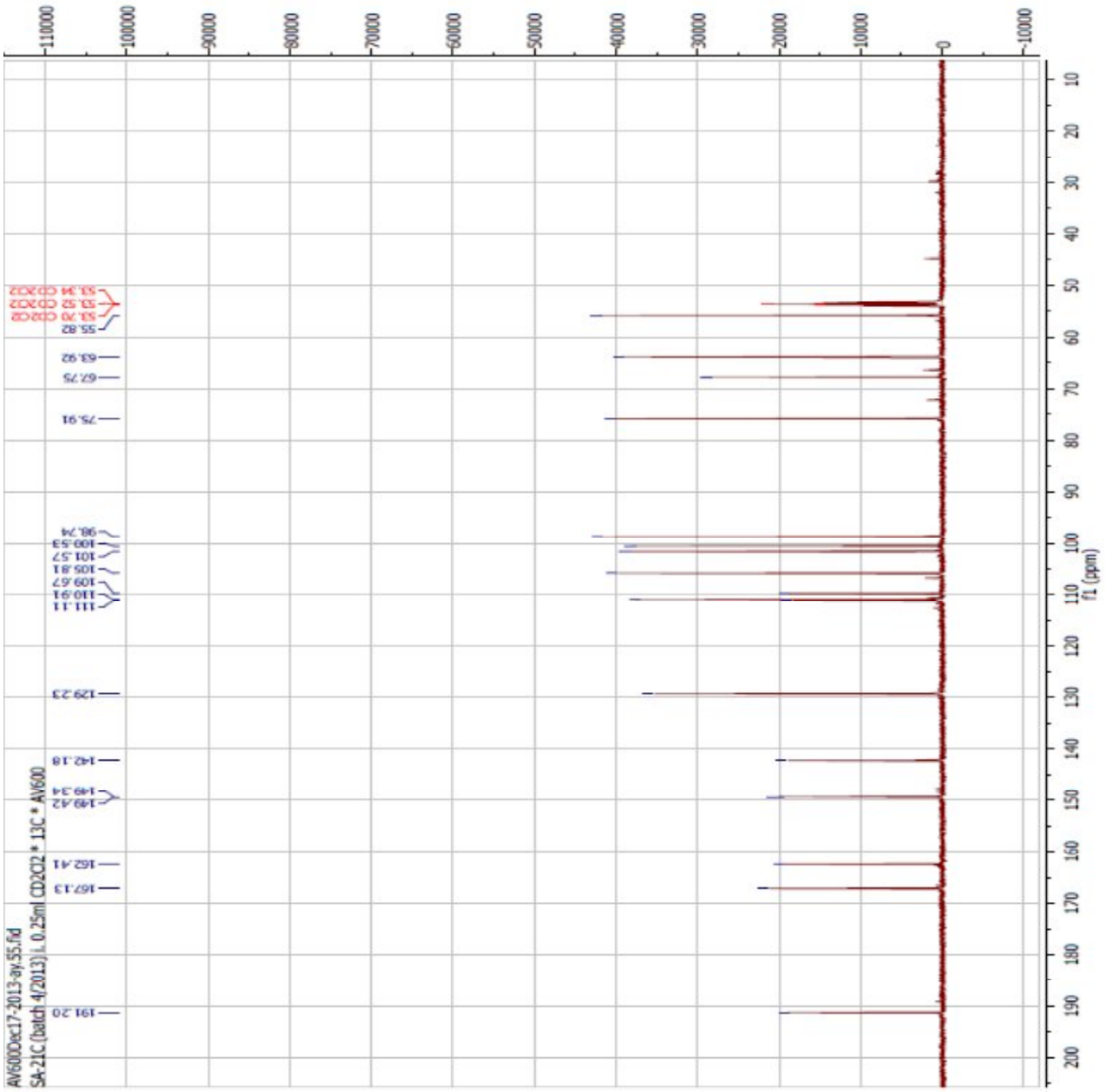
Appendix 125F: gNOESY (CD_2Cl_2, 600 MHz) NMR spectrum of tephrosin (**125**)



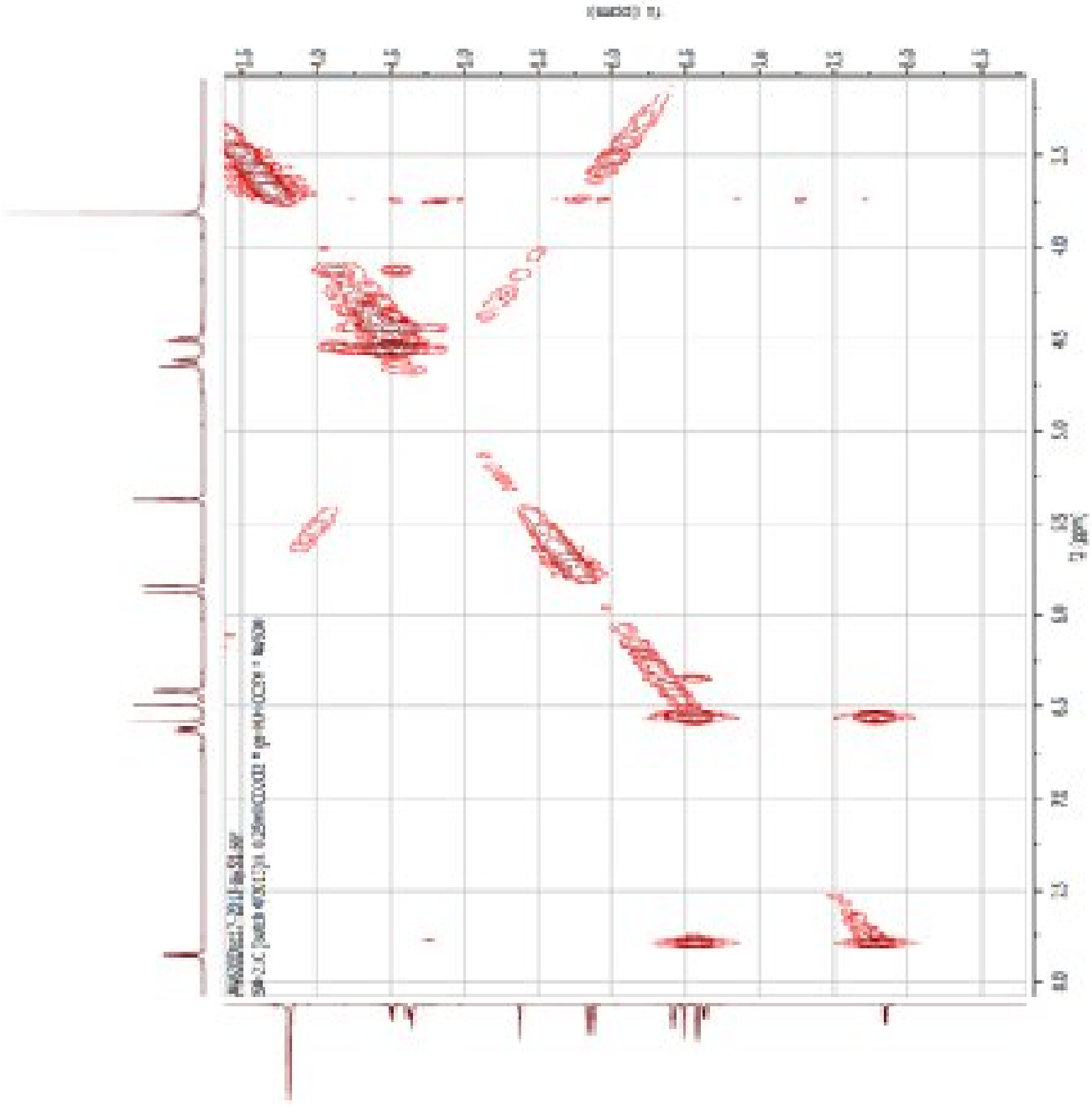
Appendix 126A: ^{1}H (CD_2Cl_2, 600 MHz) NMR spectrum of oblatarotenoid A (**126**)



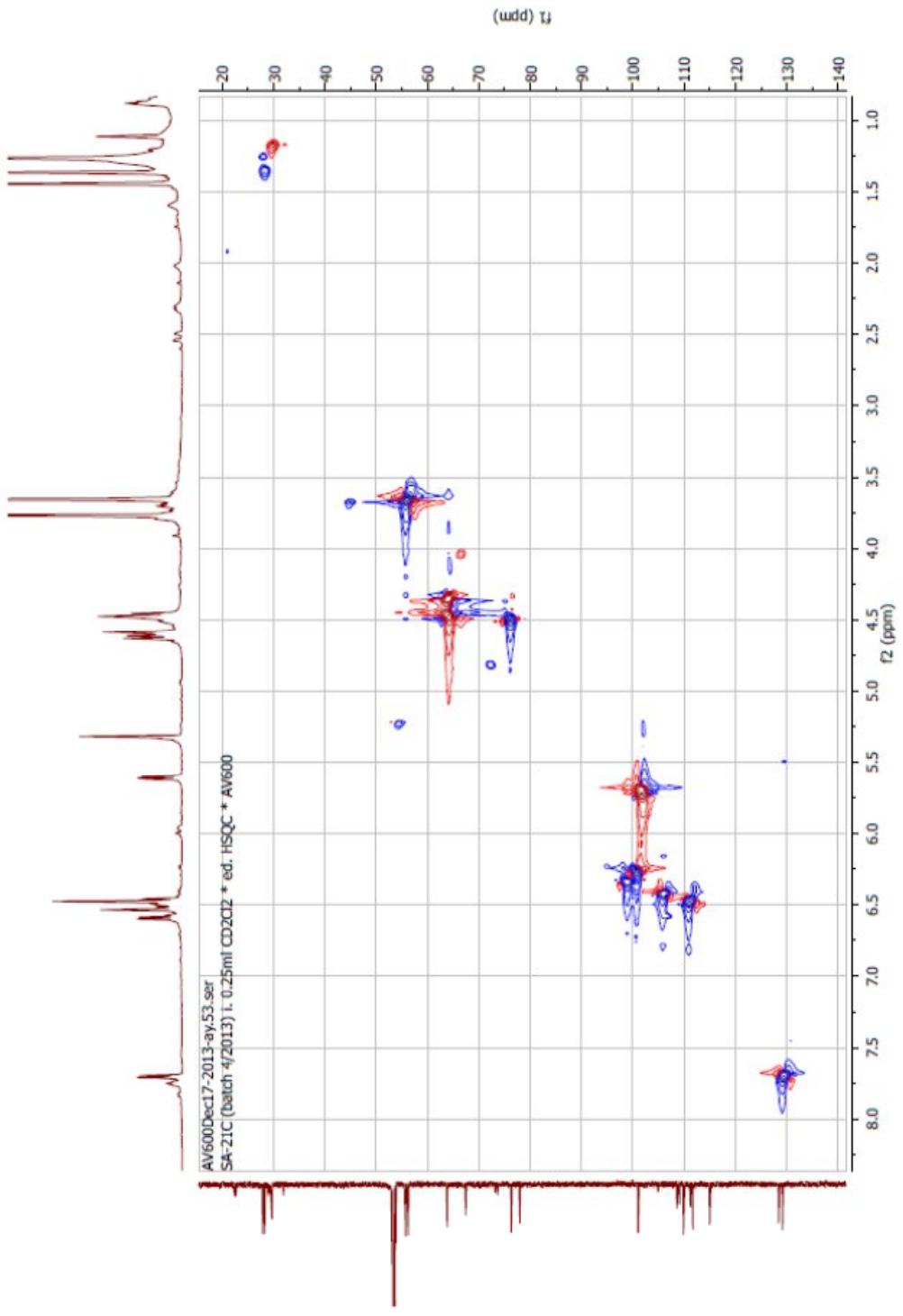
Appendix 126B: ^{13}C (CD_2Cl_2, 600 MHz) NMR spectrum of oblatarotenoid A (**126**)



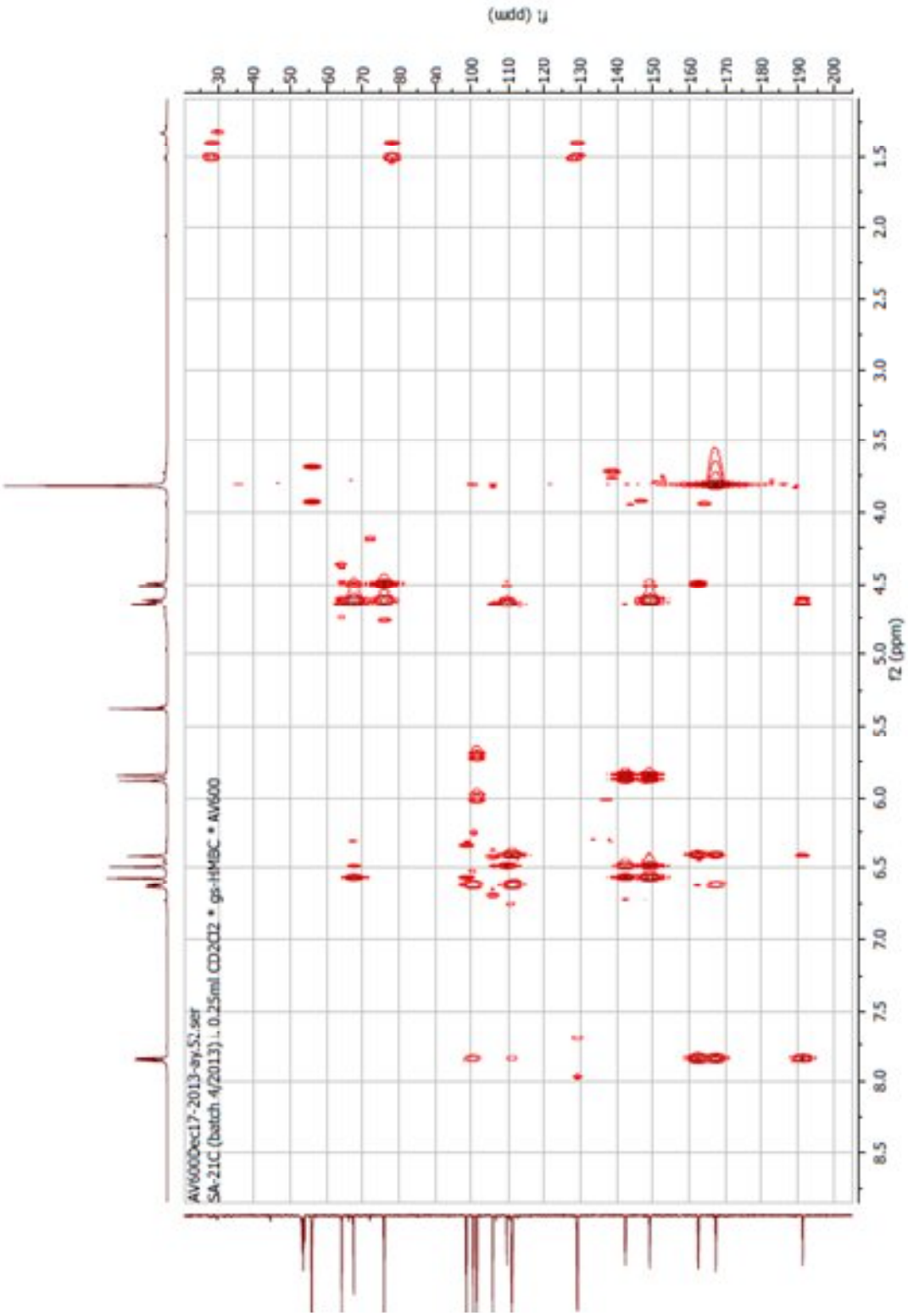
Appendix 126C: ^{1}H-^{1}H COSY (CD_2Cl_2, 600 MHz) NMR spectrum of oblatarotenoid A (**126**)



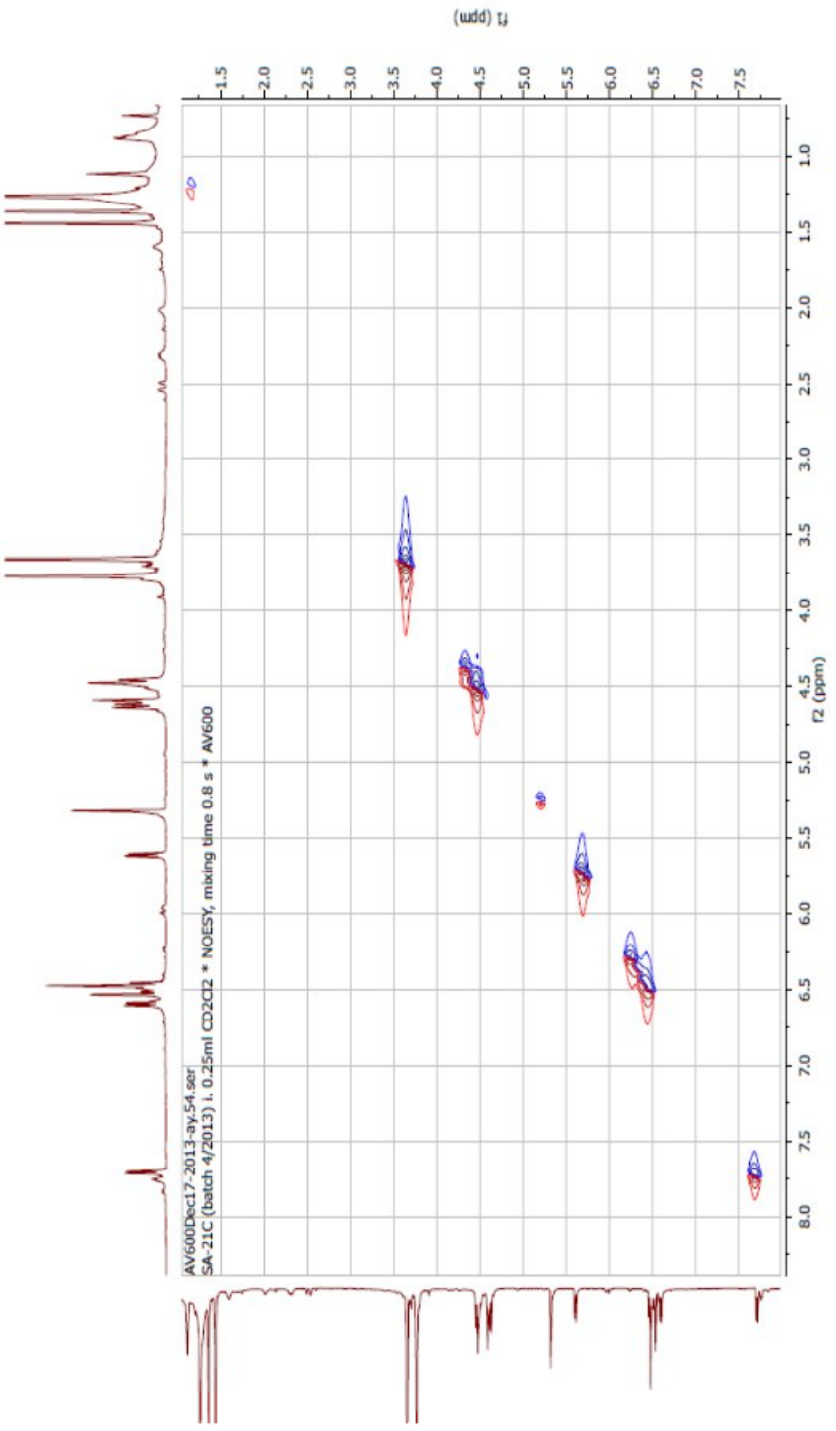
Appendix 126D: gHSQC (CD_2Cl_2, 600 MHz) NMR spectrum of oblatarotenoid A (**126**)



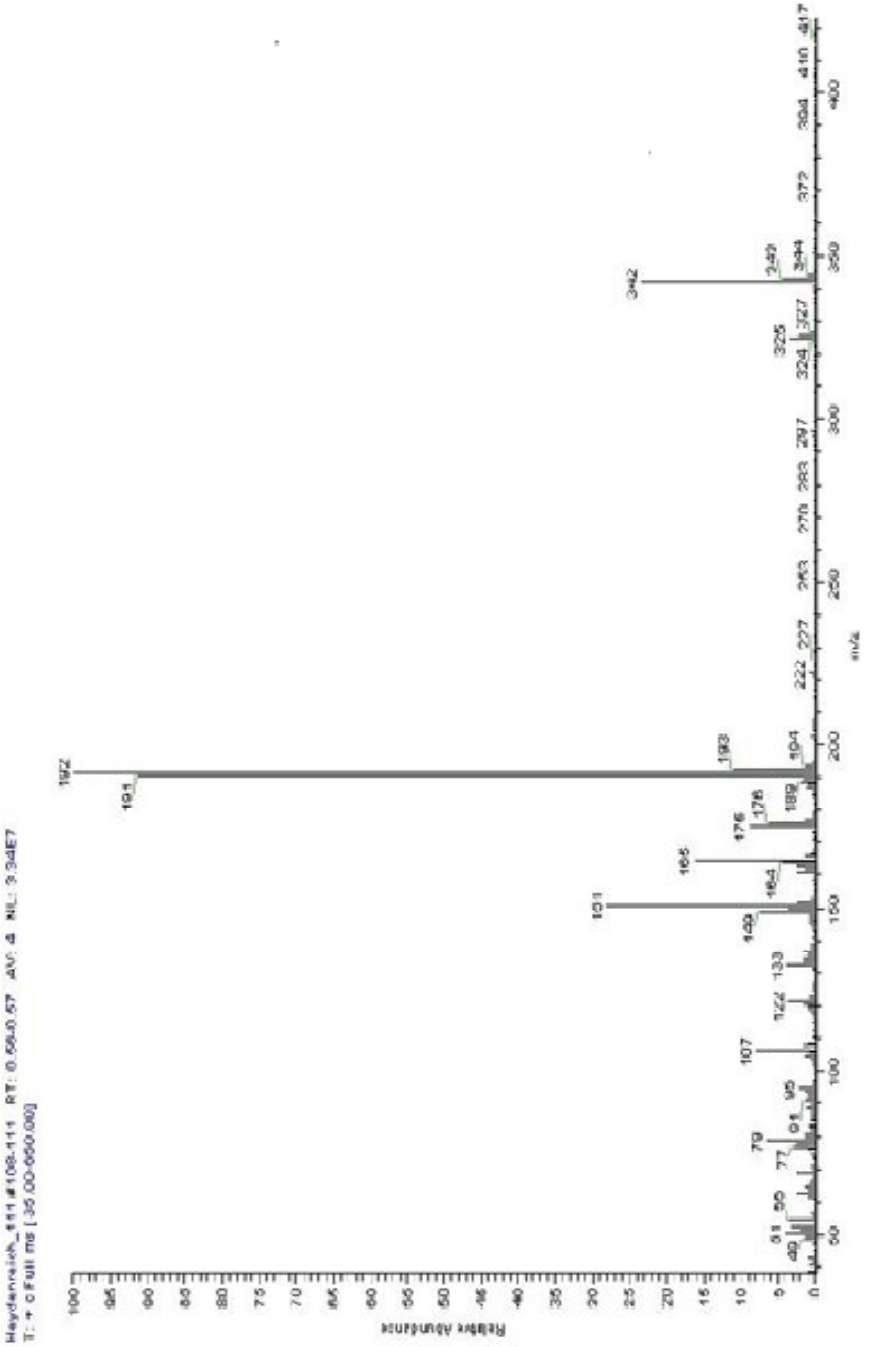
Appendix 126E: gHMBC (CD_2Cl_2, 600 MHz) NMR spectrum of oblatarotenoid A (**126**)



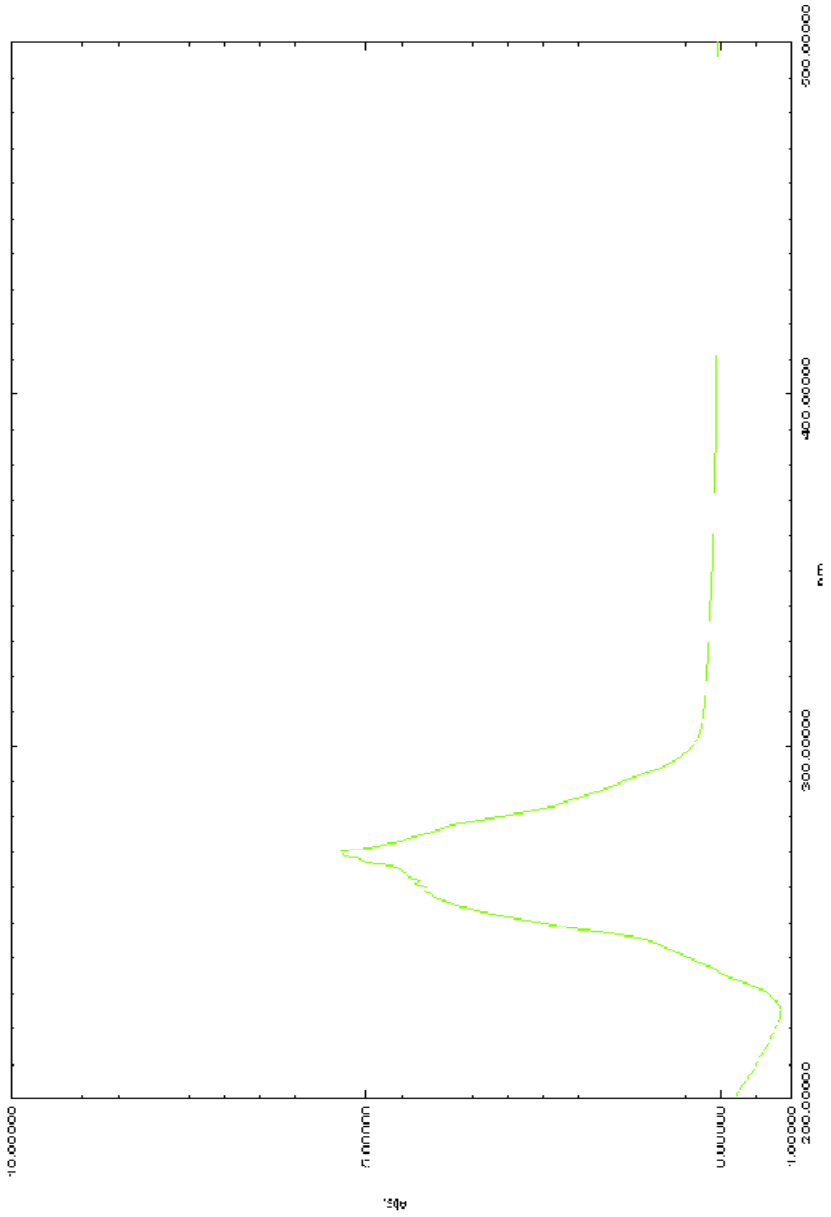
Appendix 126F: gNOESY (CD_2Cl_2, 600 MHz) NMR spectrum of oblatarotenoid A (**126**)



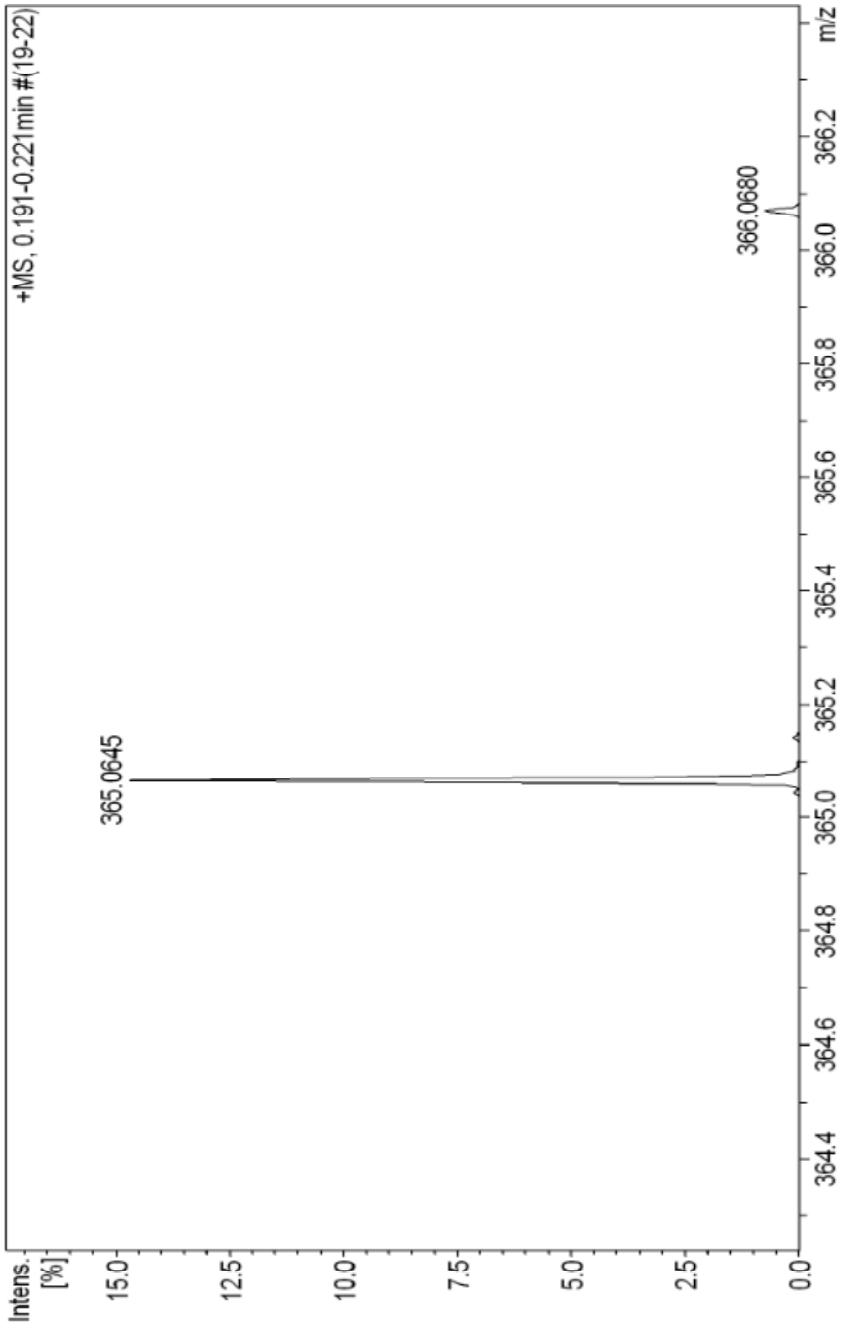
Appendix 126G: EI-MS of oblatarotenoid A (**126**)



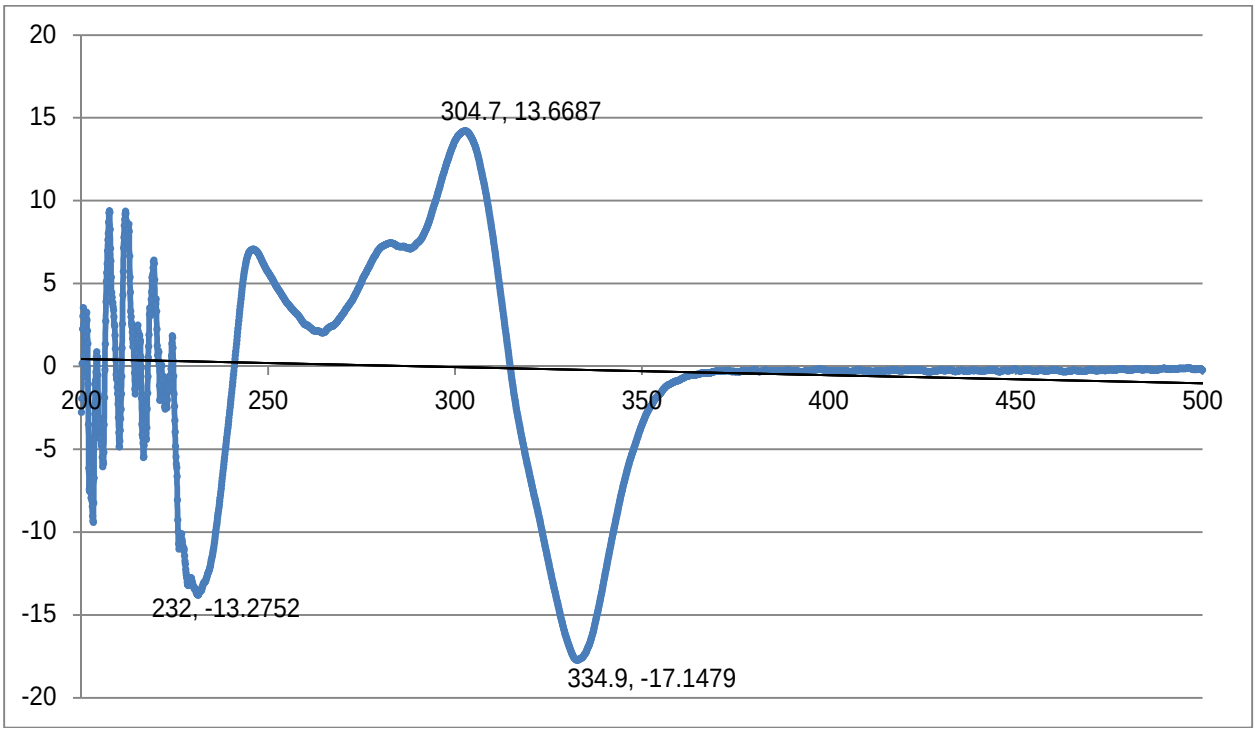
Appendix 126H: UV-VIS of oblatarotenoid A (**126**)



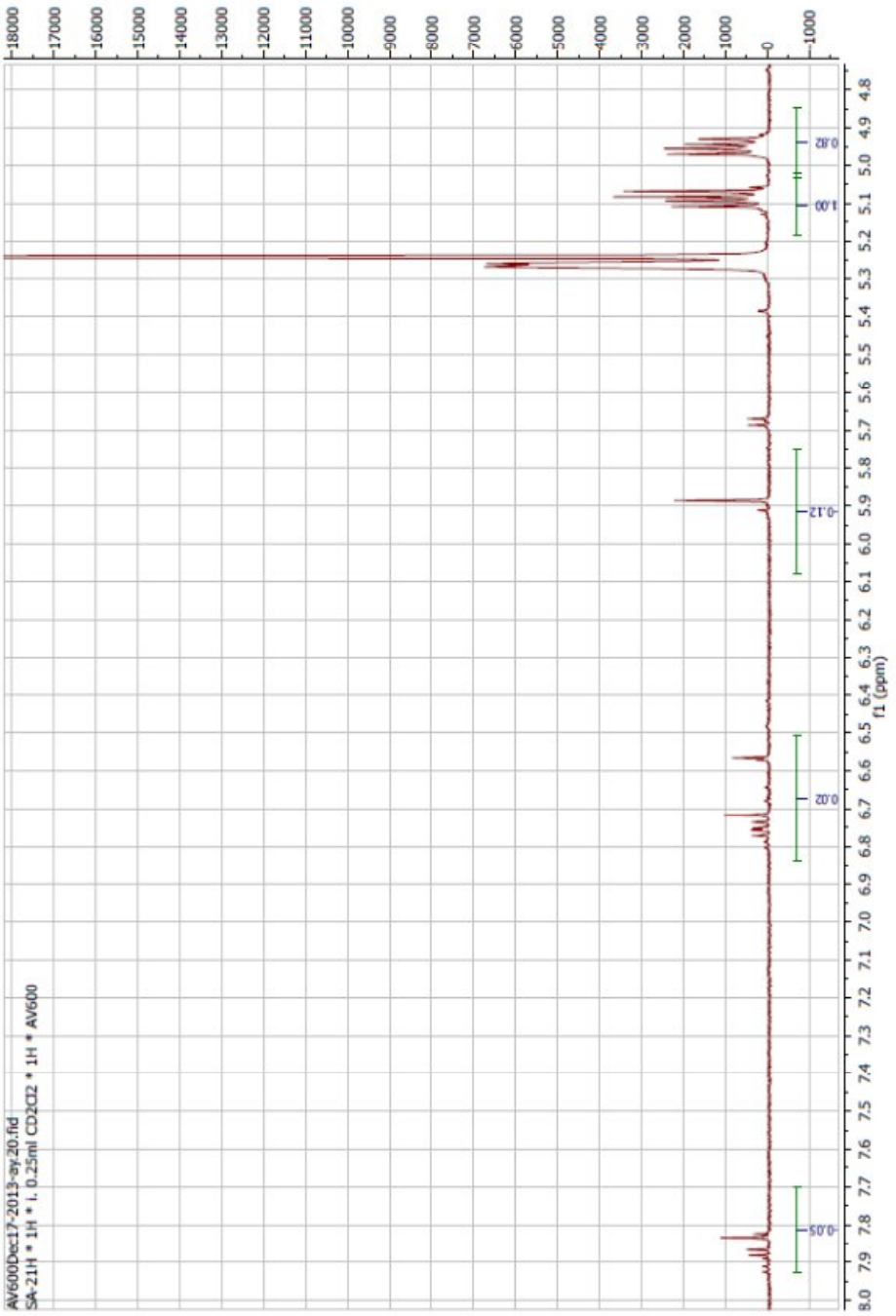
Appendix 126I: ESI spectrum of oblatarotenoid A (**126**)



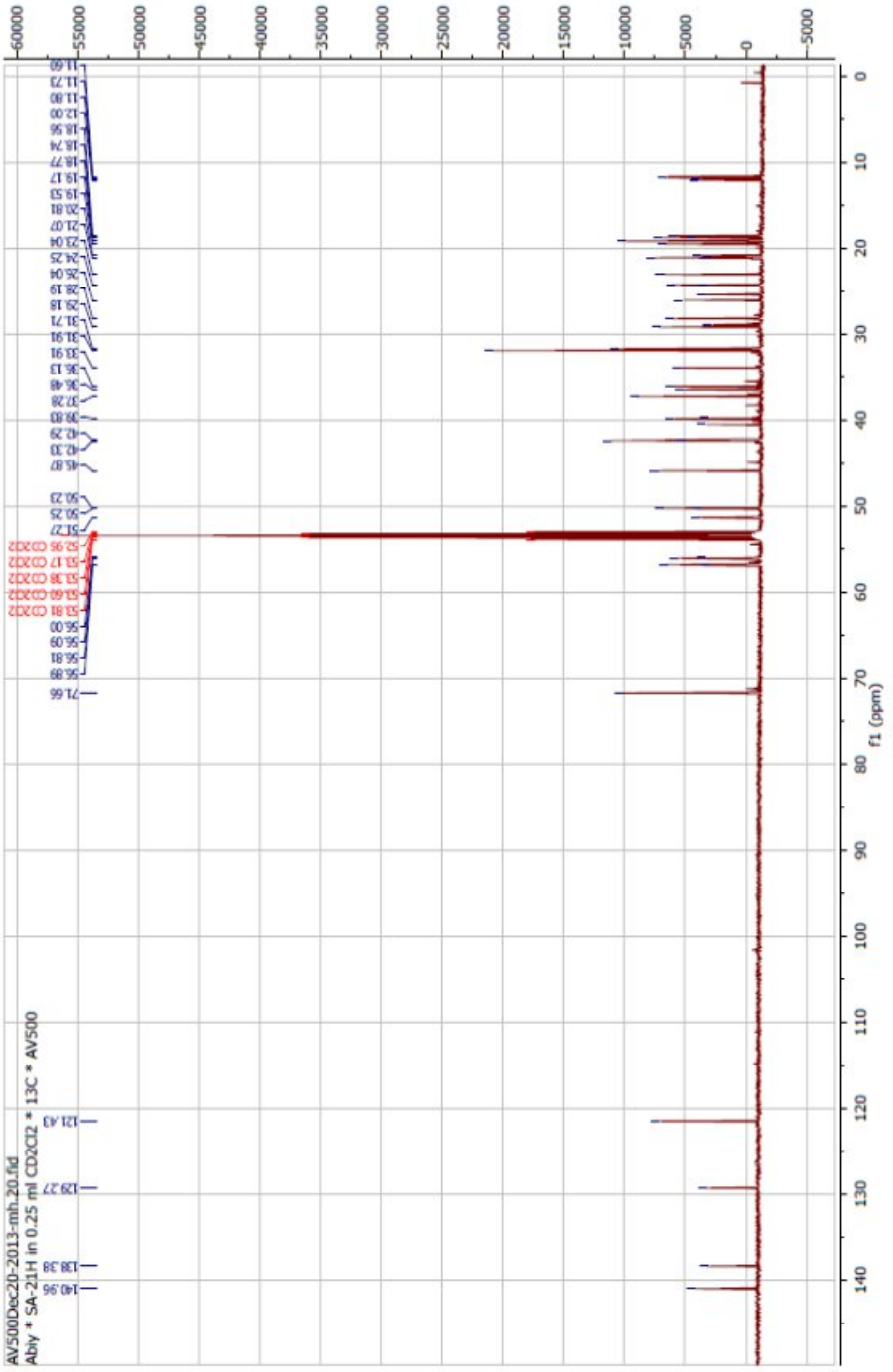
APPENDIX 126J: CD spectrum of oblatarotenoid A (**126**)



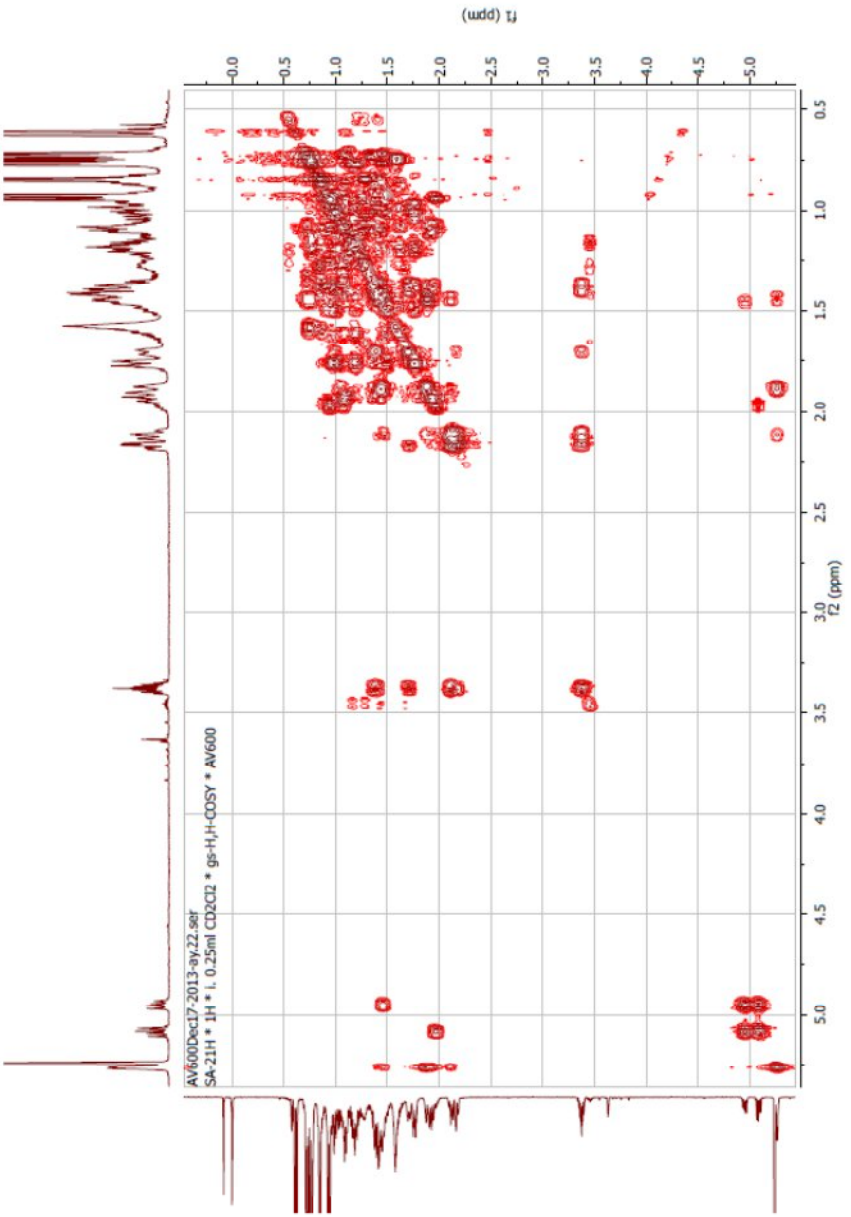
Appendix 127A: ^{1}H (CD_2Cl_2, 600 MHz) NMR spectrum of stigmasterol (**127**)



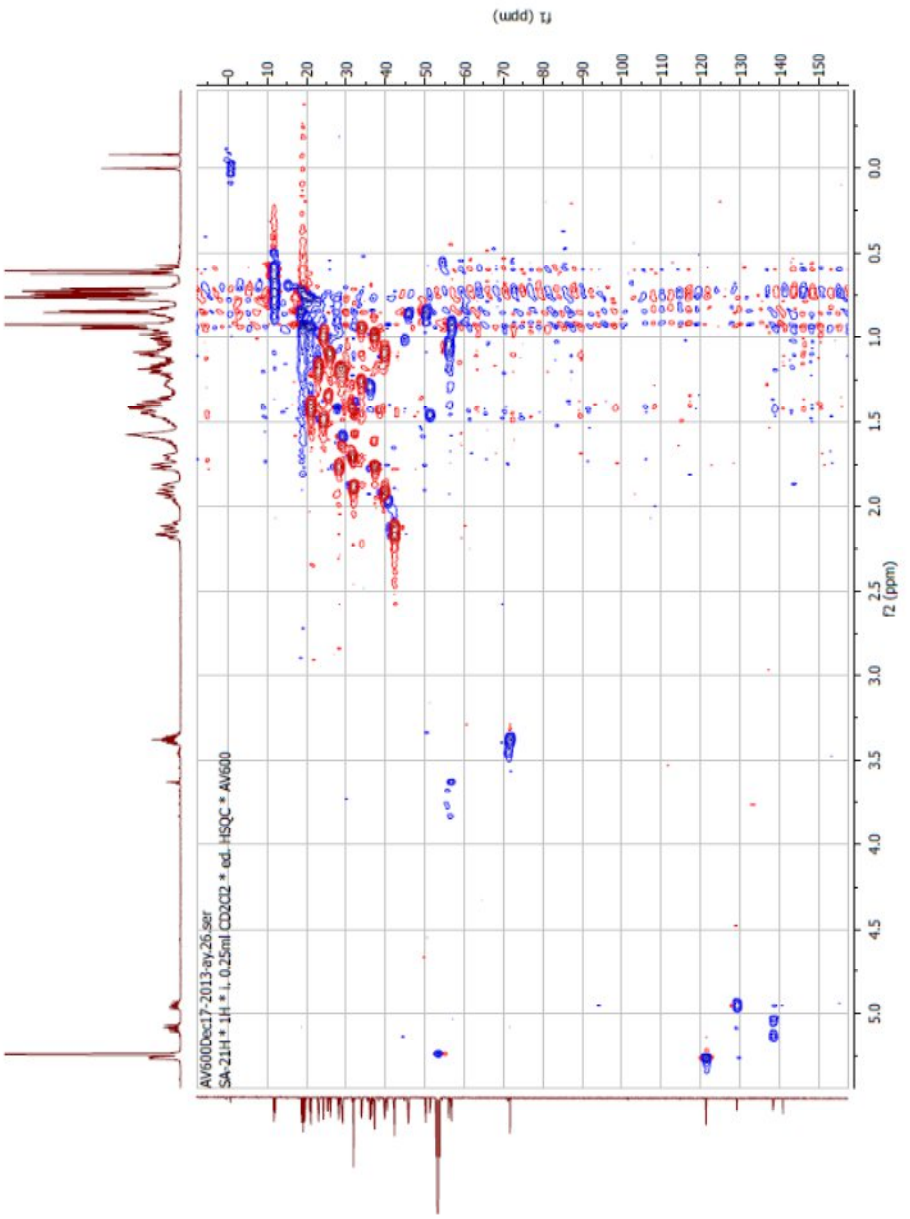
Appendix 127B: ^{13}C (CD_2Cl_2, 150 MHz) NMR spectrum of stigmasterol (**127**)



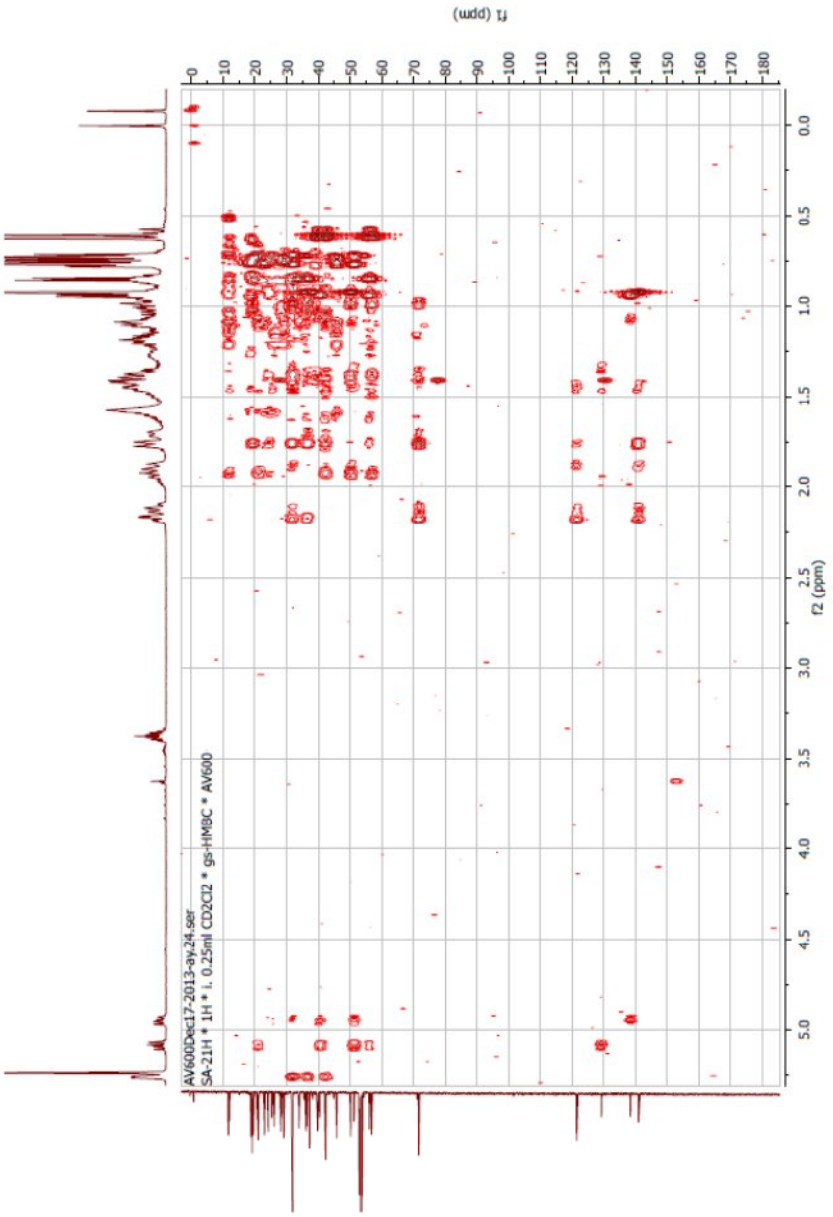
Appendix 127C: ^{1}H-^{1}H COSY (CD_2Cl_2, 600 MHz) NMR spectrum of stigmasterol (**127**)



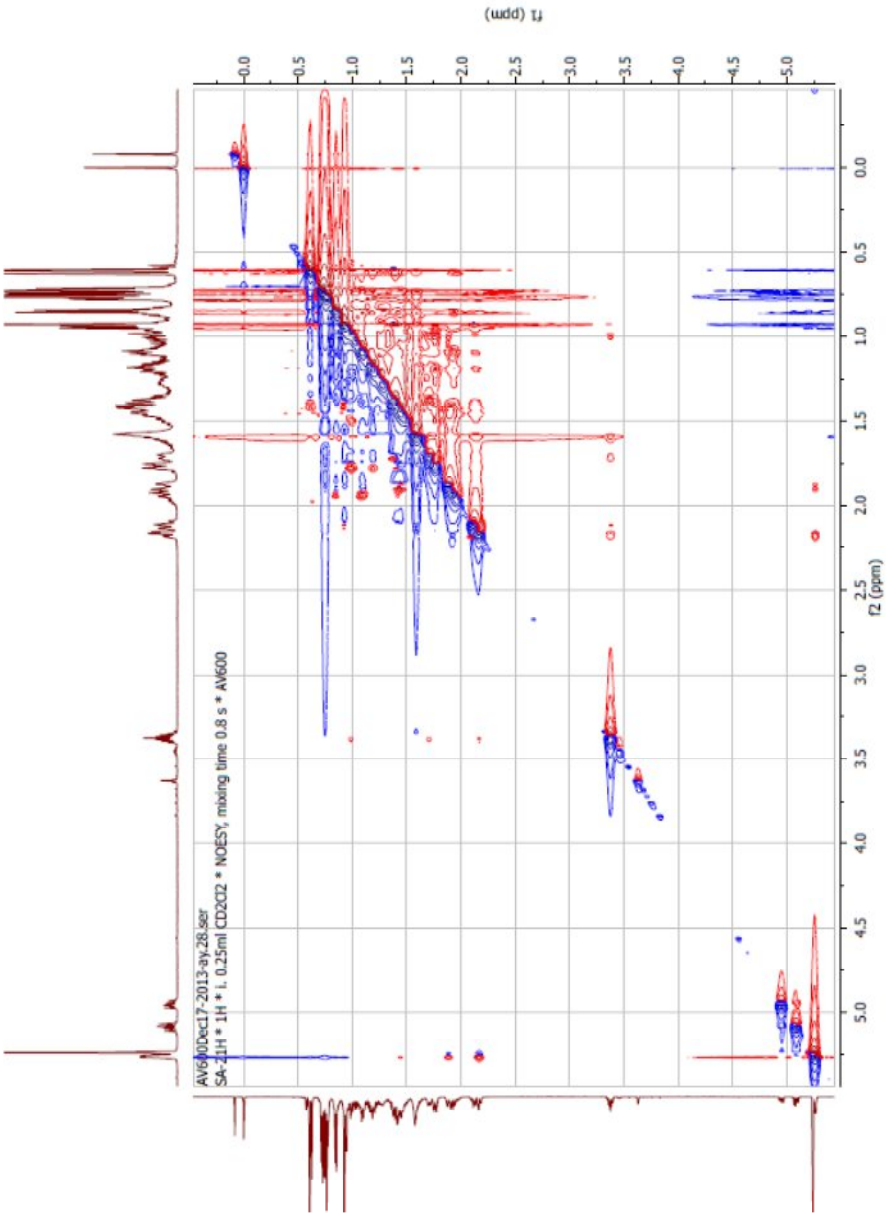
Appendix 127D: gHSQC (CD_2Cl_2, 600 MHz) NMR spectrum of stigmasterol (**127**)



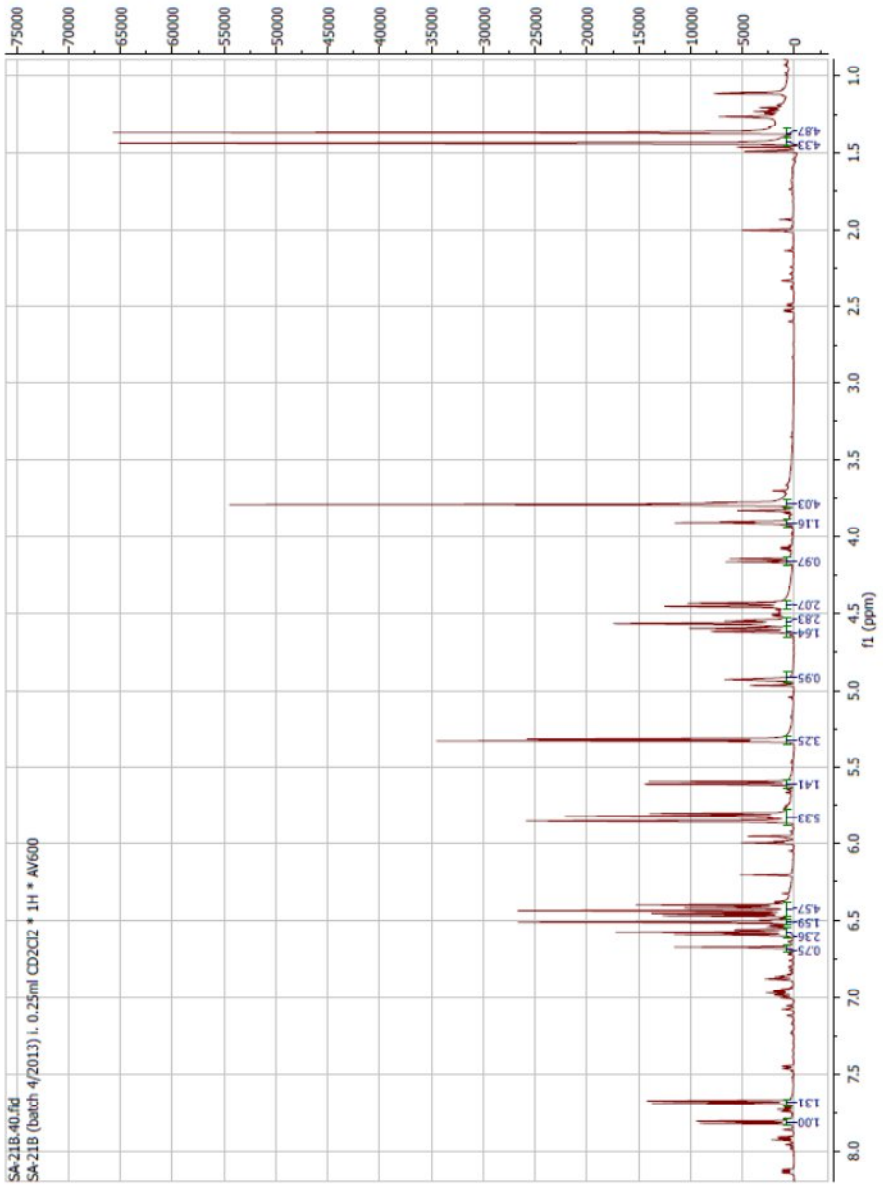
Appendix 127E: gHMBC (CD_2Cl_2, 600 MHz) NMR spectrum of stigmasterol (**127**)



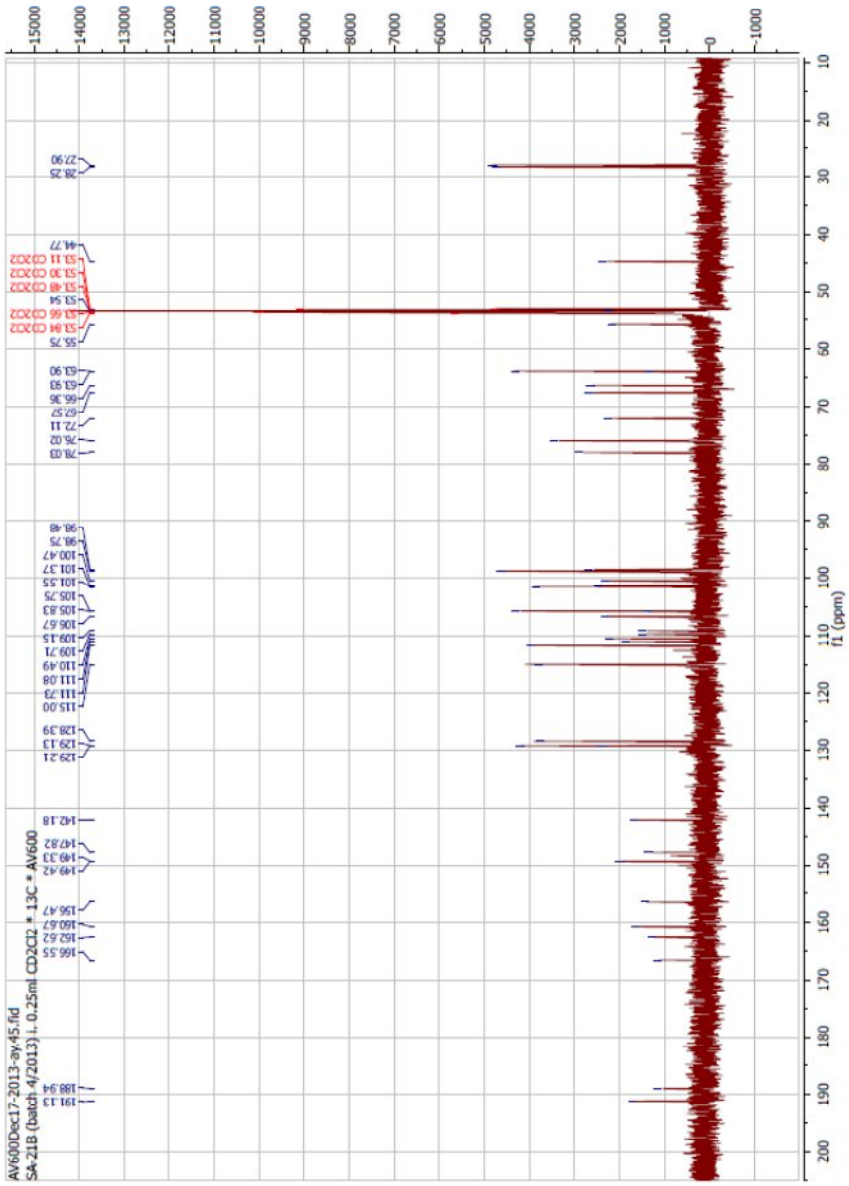
Appendix 127F: gNOESY (CD_2Cl_2, 600 MHz) NMR spectrum of stigmasterol (**127**)



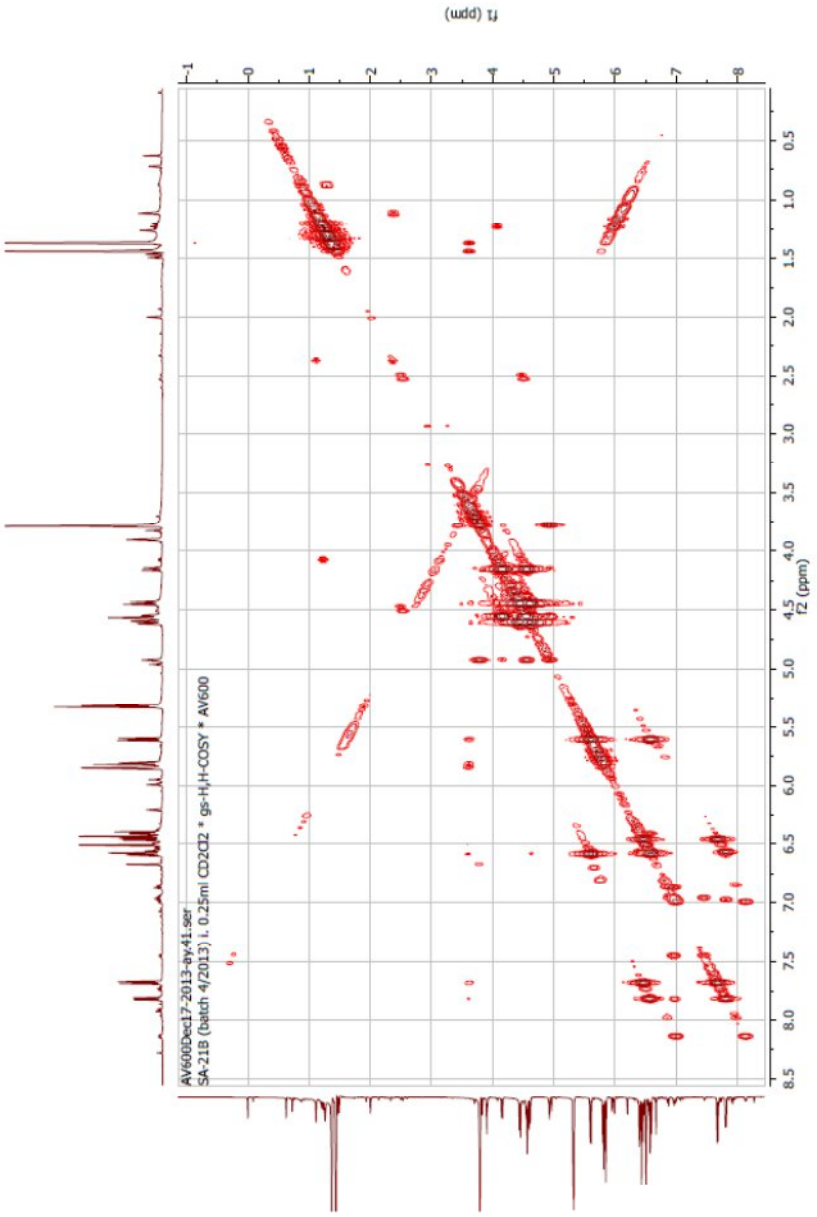
Appendix 128A: ^{1}H (CD_2Cl_2, 600 MHz) NMR spectrum of millettone (**128**)



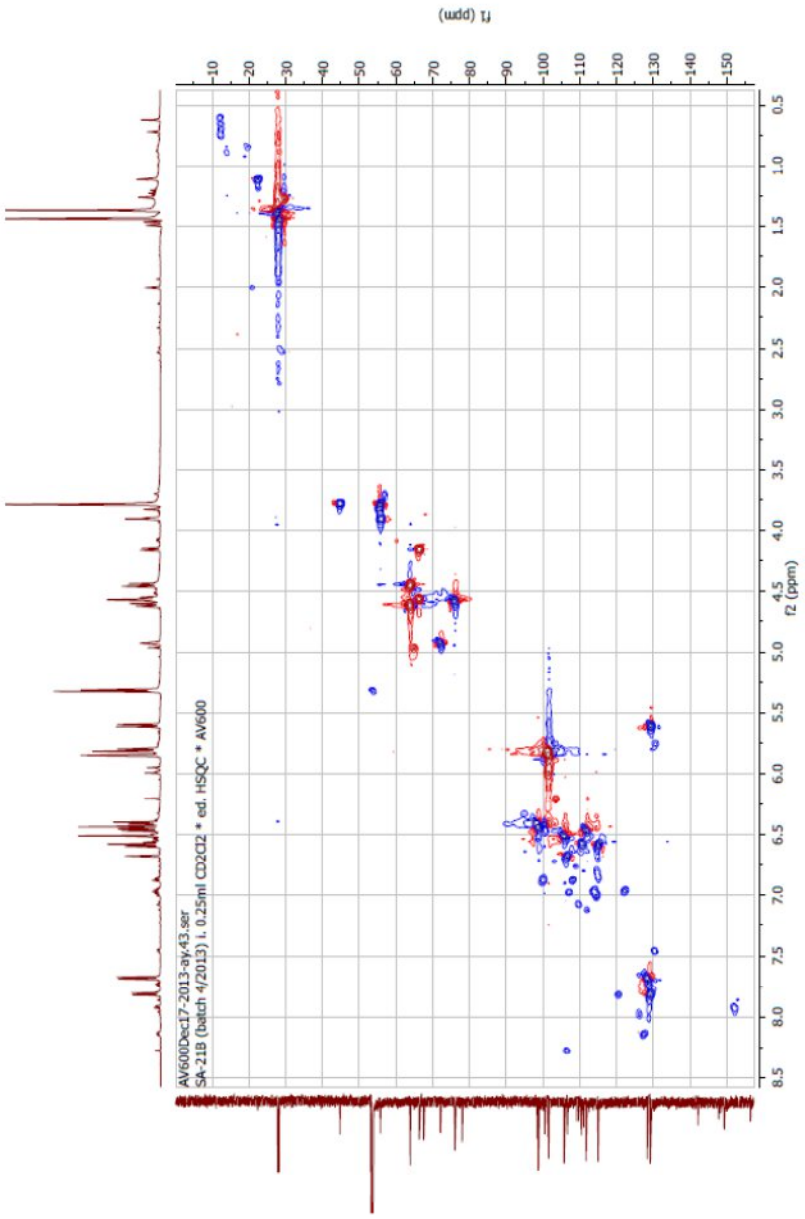
Appendix 128B: ^{13}C (CD_2Cl_2, 150 MHz) NMR spectrum of millettone (**128**)



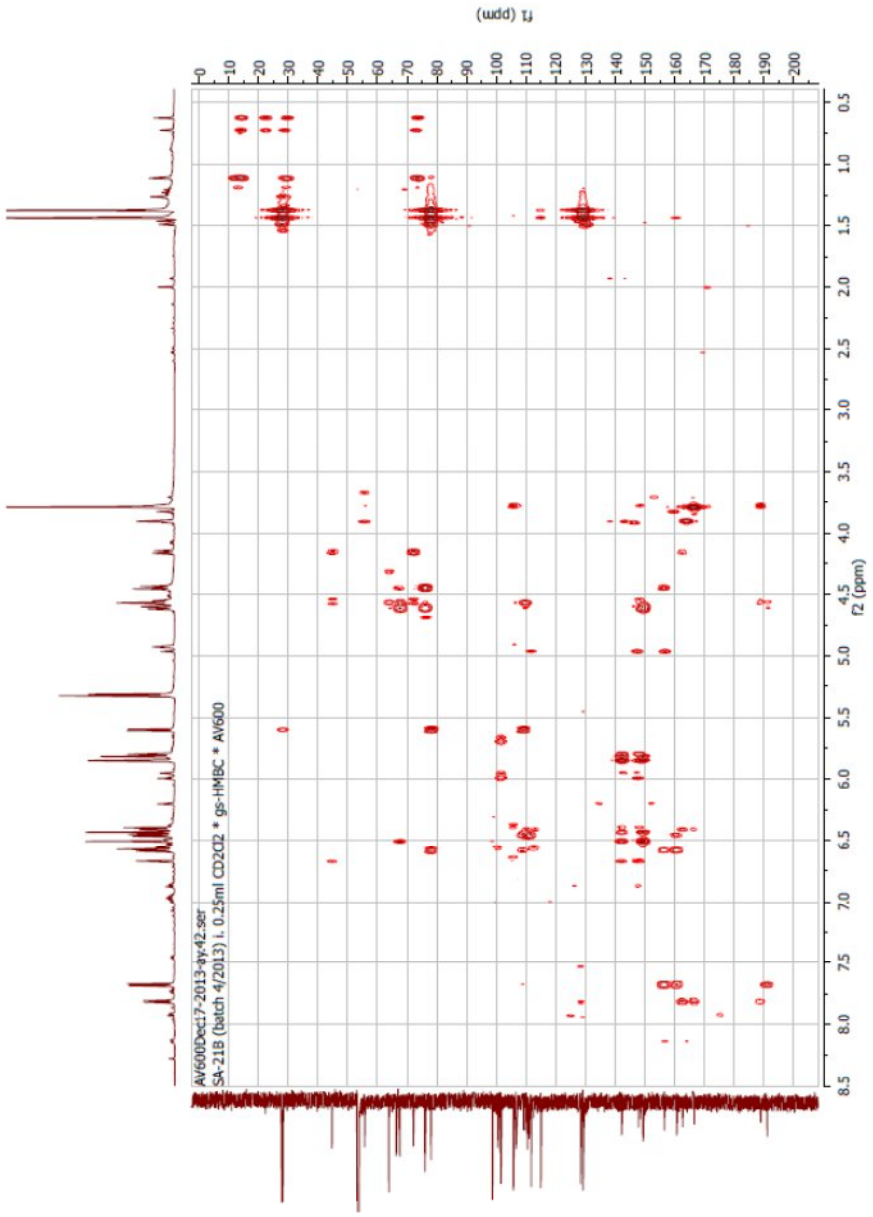
Appendix 128C: ^{1}H-^{1}H COSY (CD_2Cl_2, 600 MHz) NMR spectrum of millettone (**128**)



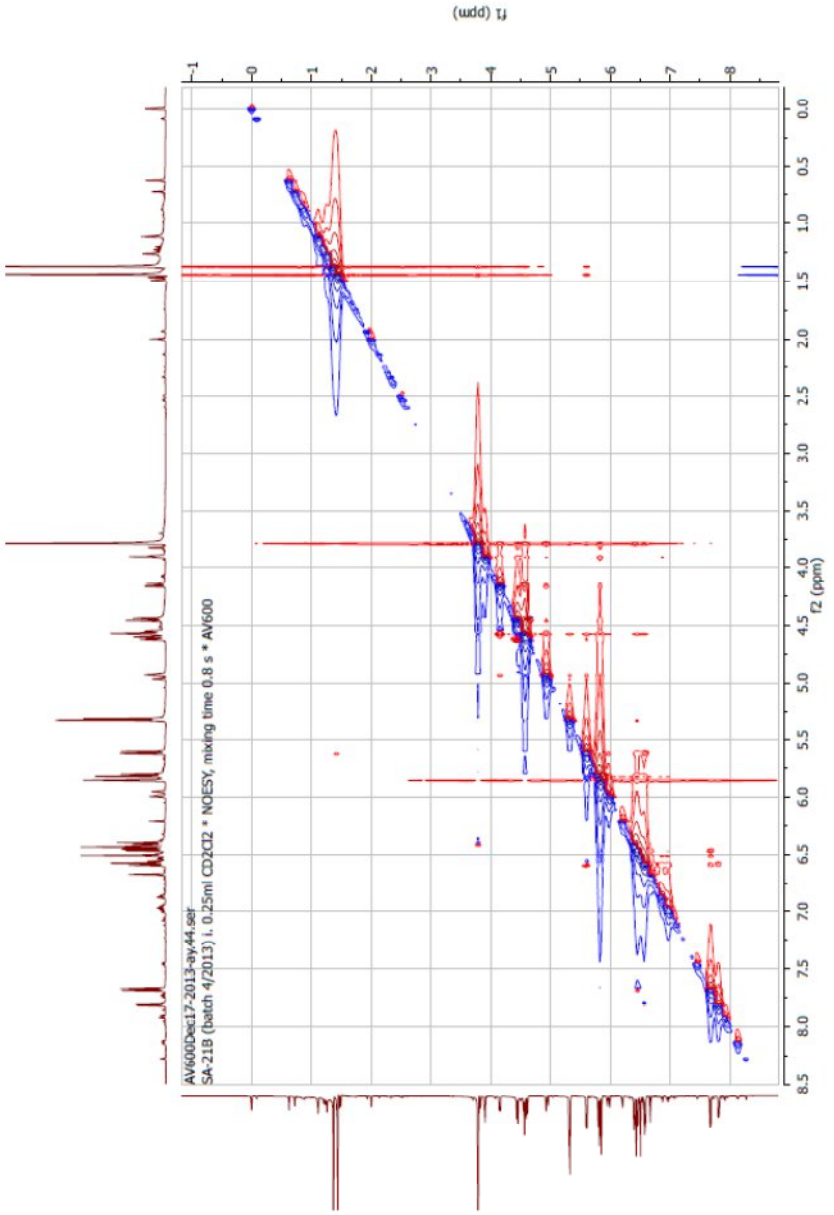
Appendix 128D: gHSQC (CD_2Cl_2, 600 MHz) NMR spectrum of millettone (**128**)



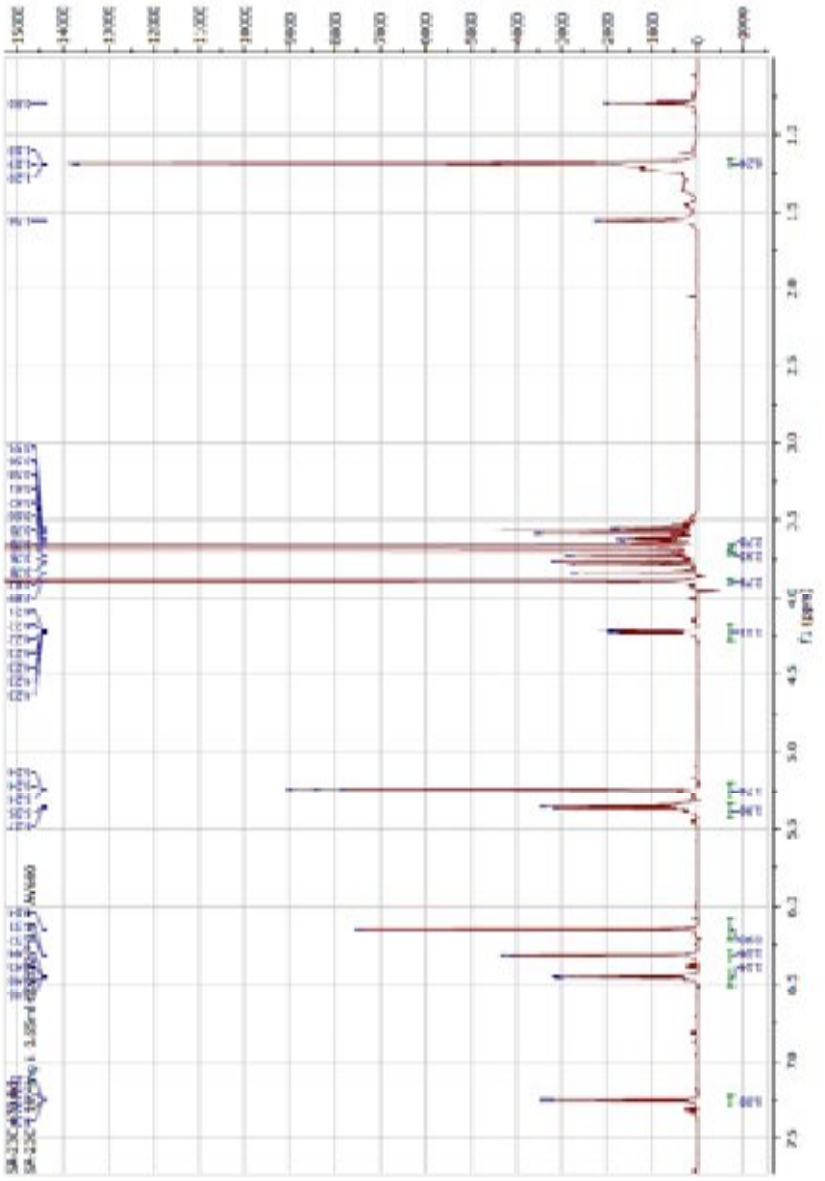
Appendix 128E: gHMBC (CD_2Cl_2, 600 MHz) NMR spectrum of millettone (**128**)



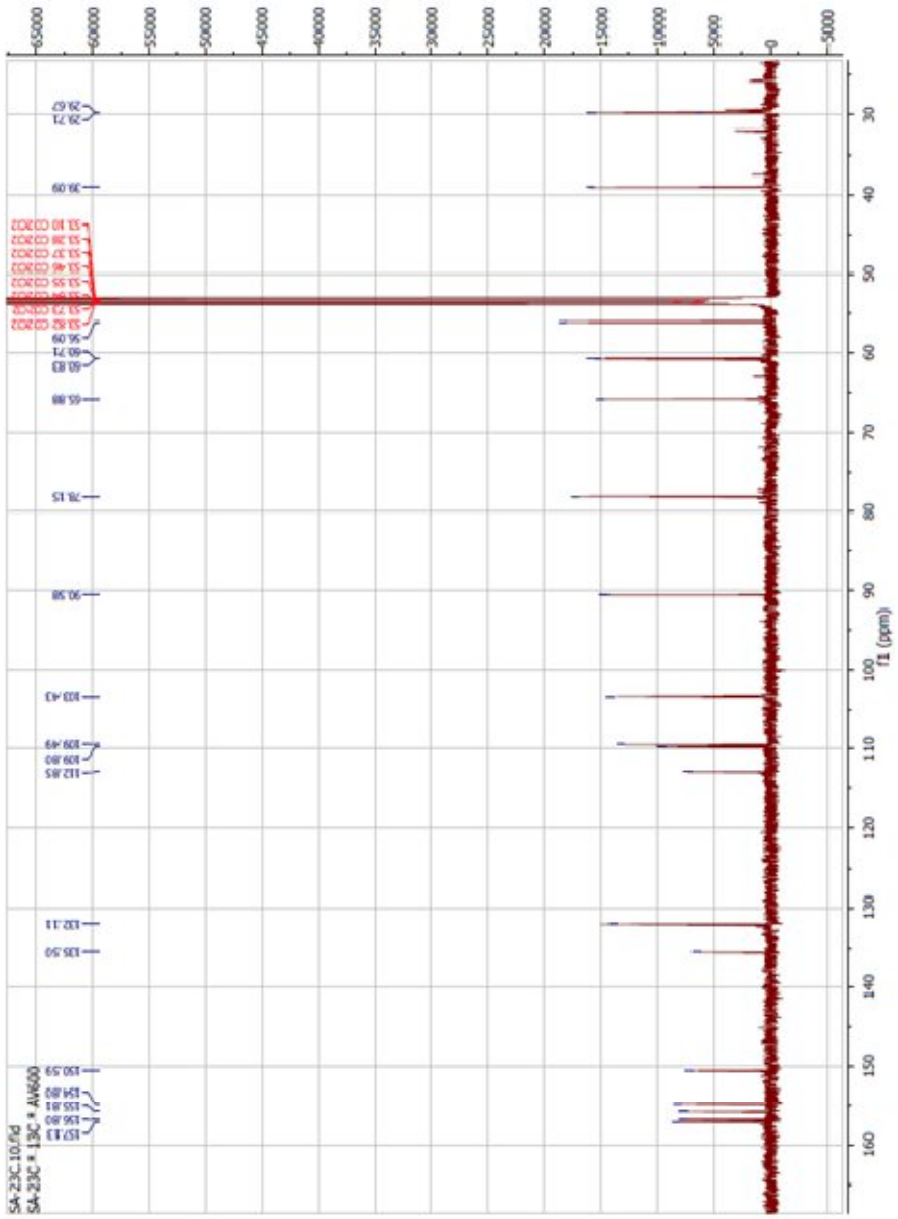
Appendix 128F: gNOESY (CD_2Cl_2, 600 MHz) NMR spectrum of millettone (**128**)



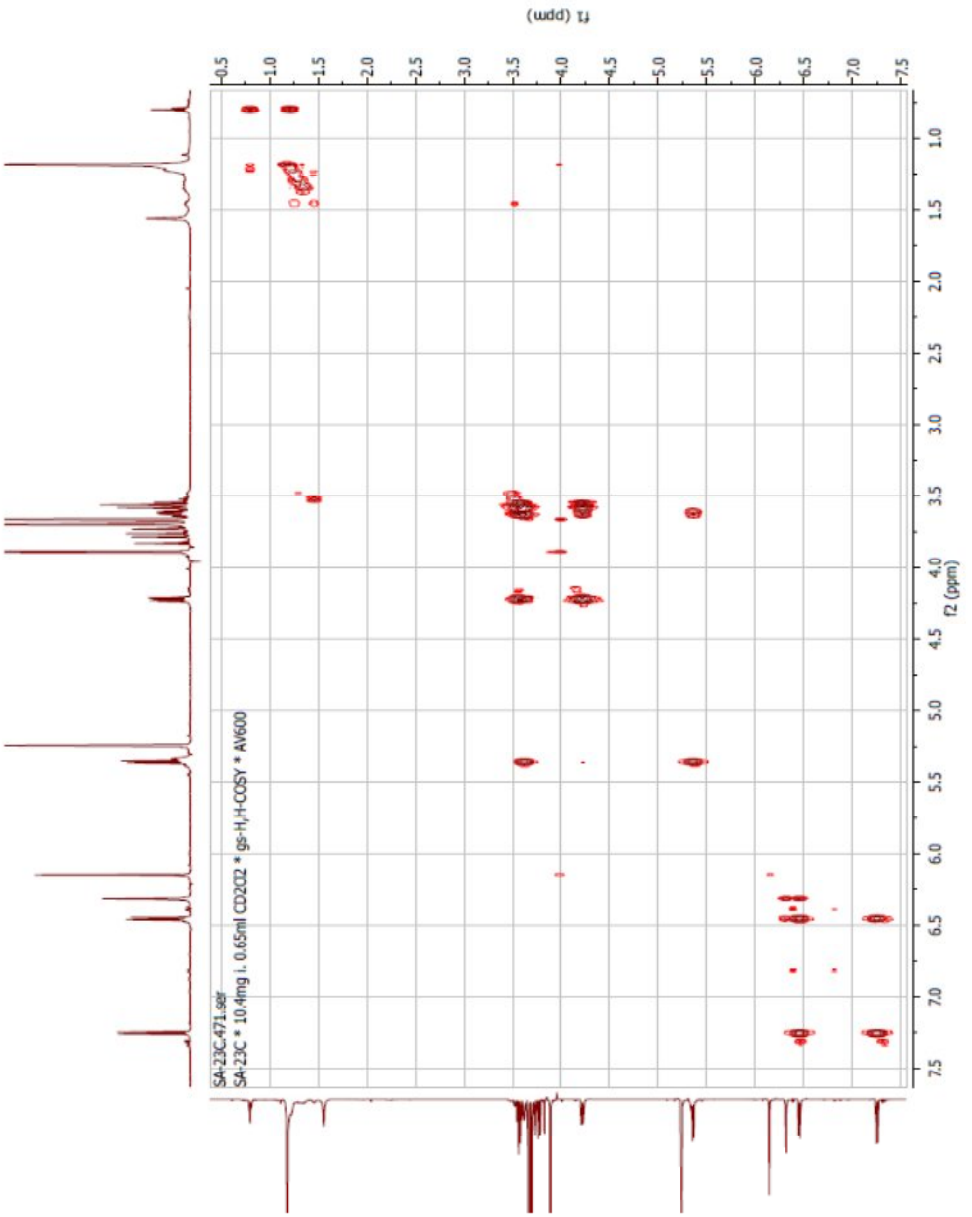
Appendix 129A: ^{1}H (CD_2Cl_2, 600 MHz) NMR spectrum of micanspterocarpan A (**129**)



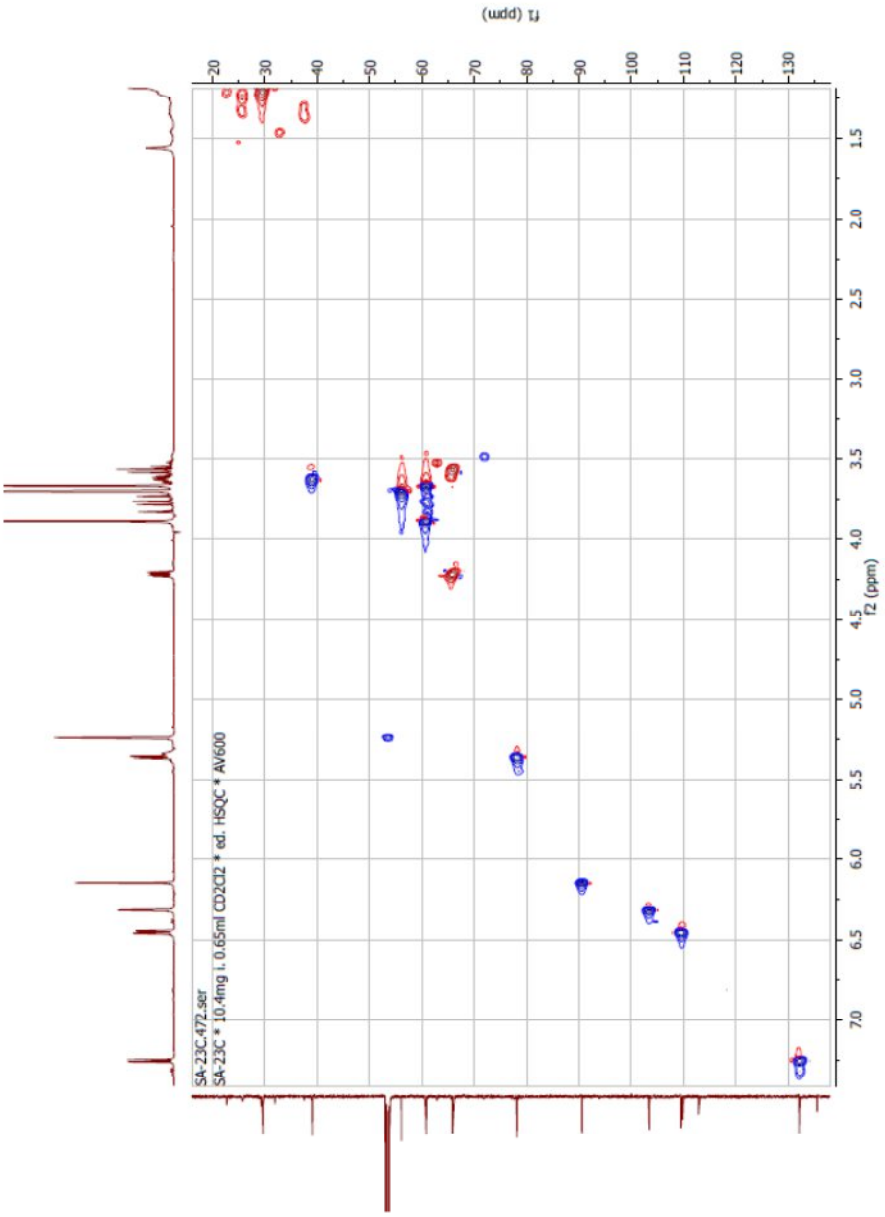
Appendix 129B: ^{13}C (CD_2Cl_2, 150 MHz) NMR spectrum of micanspterocarpan A (**129**)



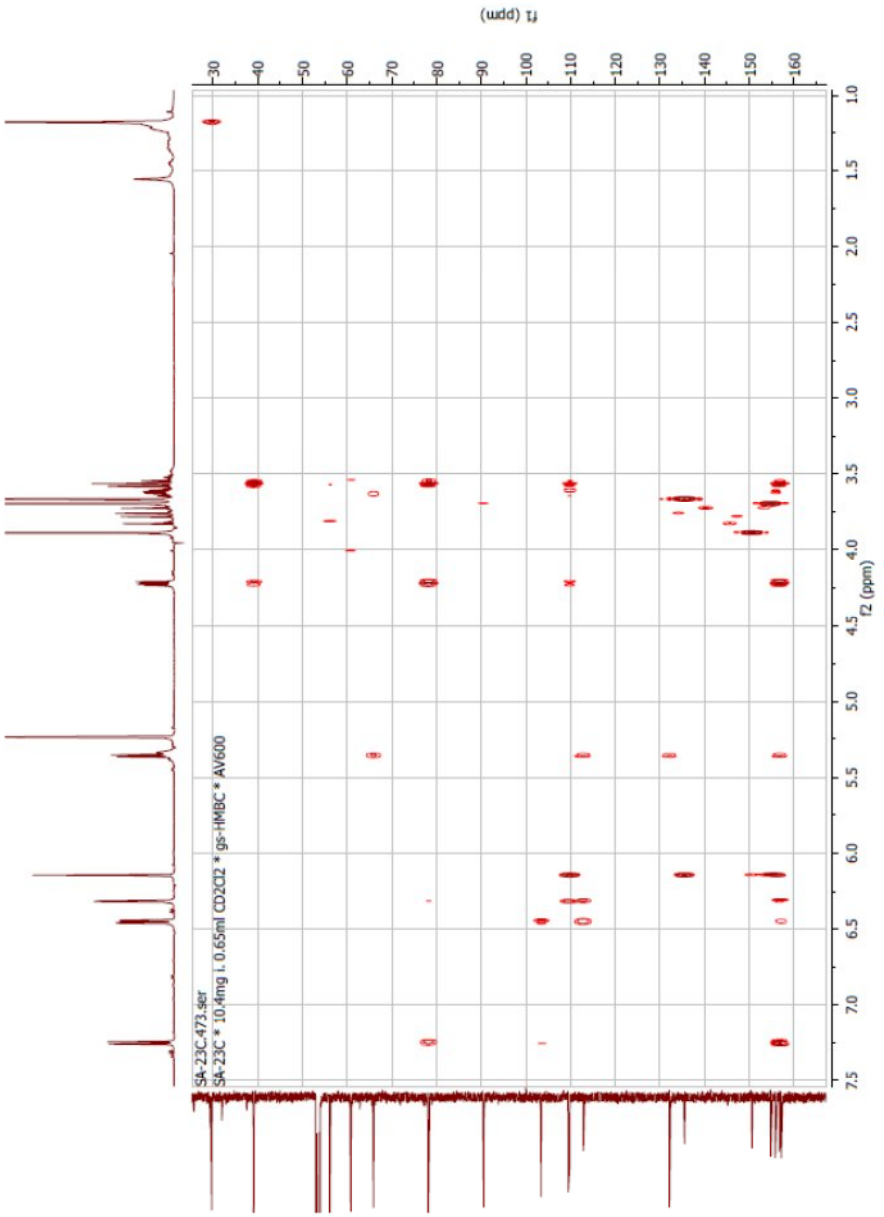
Appendix 129C: ^{1}H-^{1}H COSY (CD_2Cl_2, 600 MHz) NMR spectrum of micanspterocarpan A (**129**)



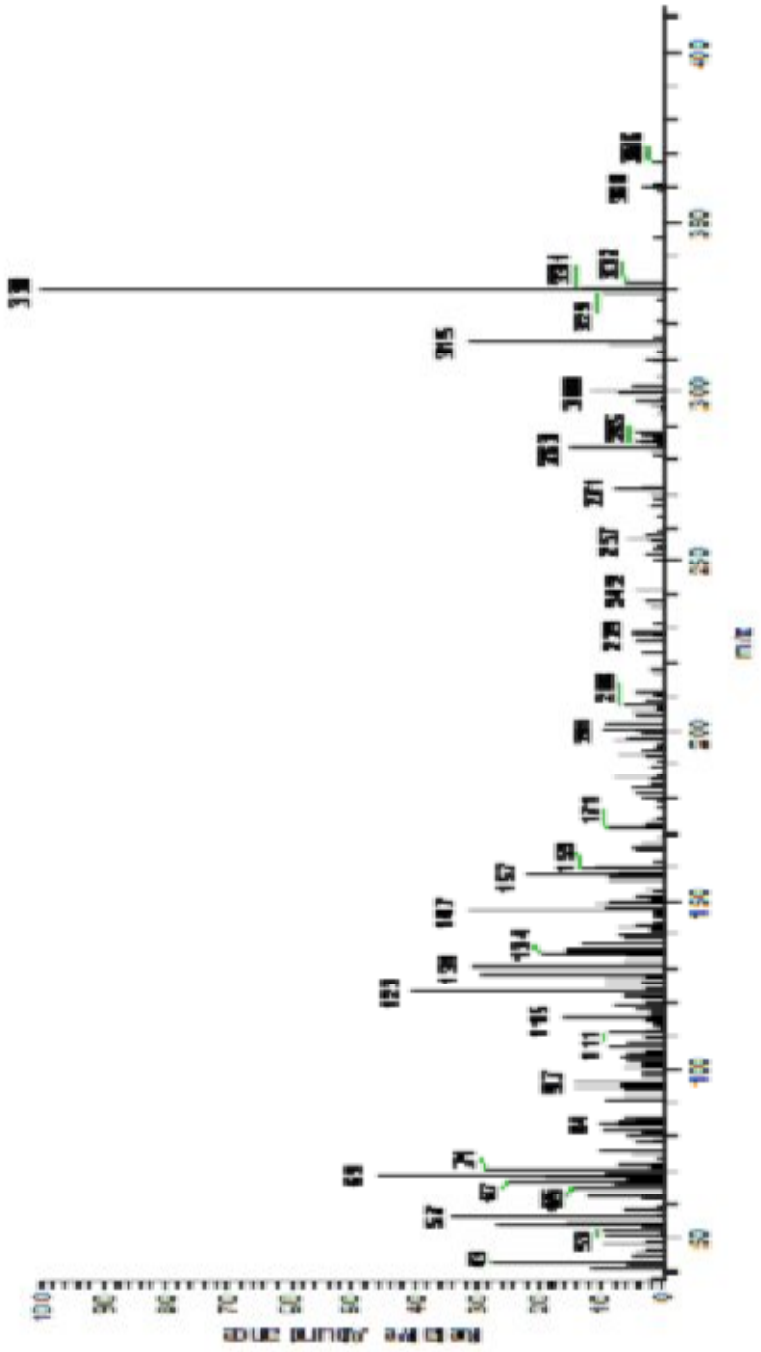
Appendix 129D: gHSQC (CD_2Cl_2, 600 MHz) NMR spectrum of micanspterocarpan A (**129**)



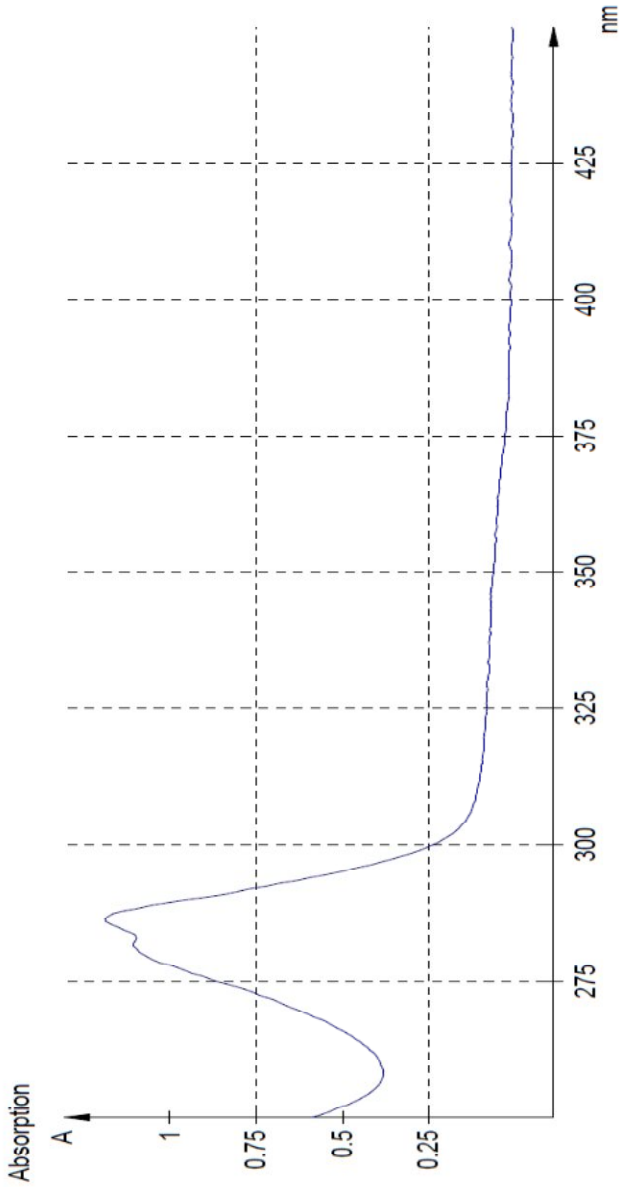
Appendix 129E: gHMBC (CD_2Cl_2, 600 MHz) NMR spectrum of micanspterocarpan A (**129**)



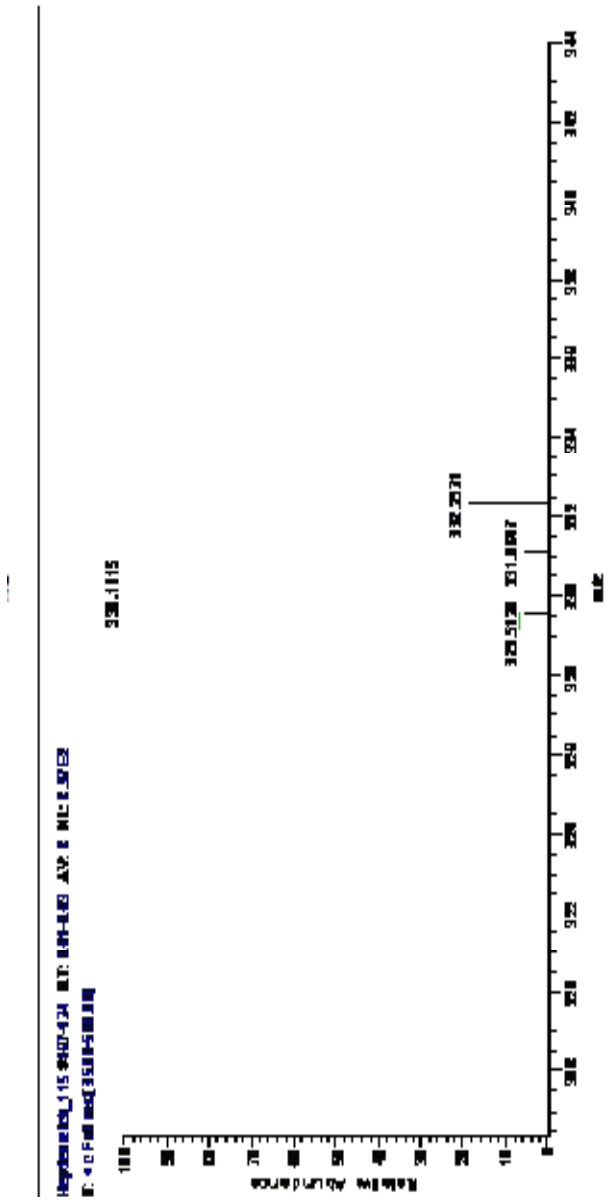
Appendix 129F: EI-MS of micanspterocarpan A (**129**)



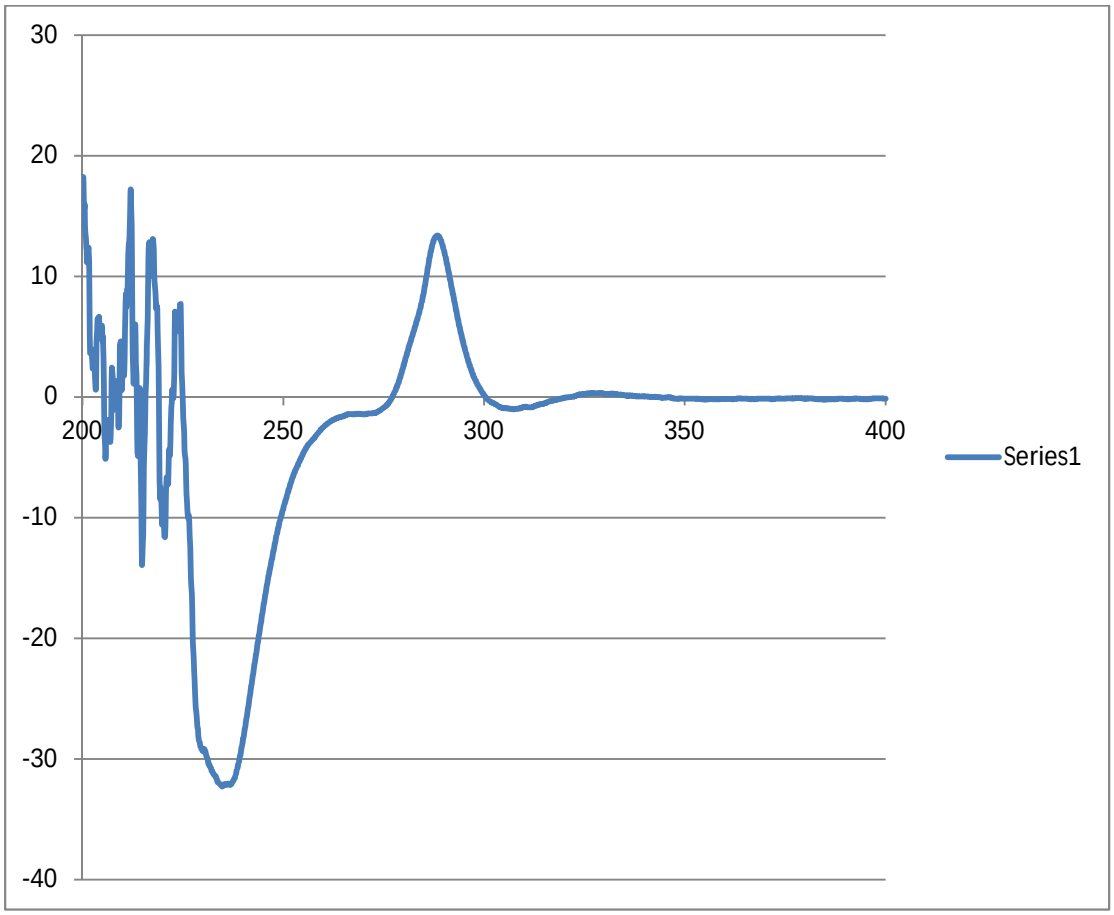
Appendix 129G: UV-VIS of micanspterocarpan A (**129**)



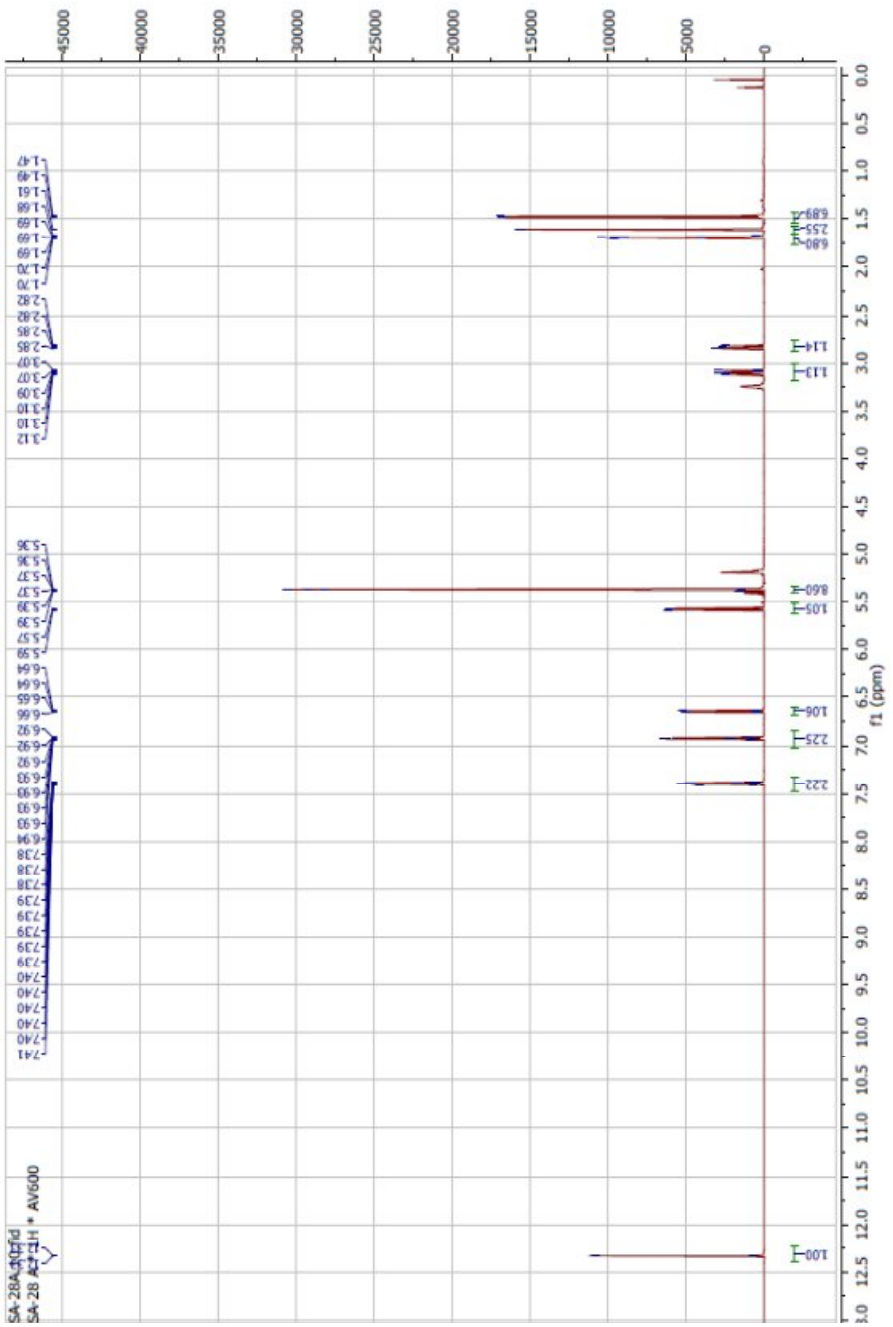
APPENDIX 129H: HREIMS of micanspterocarpan A (**129**)



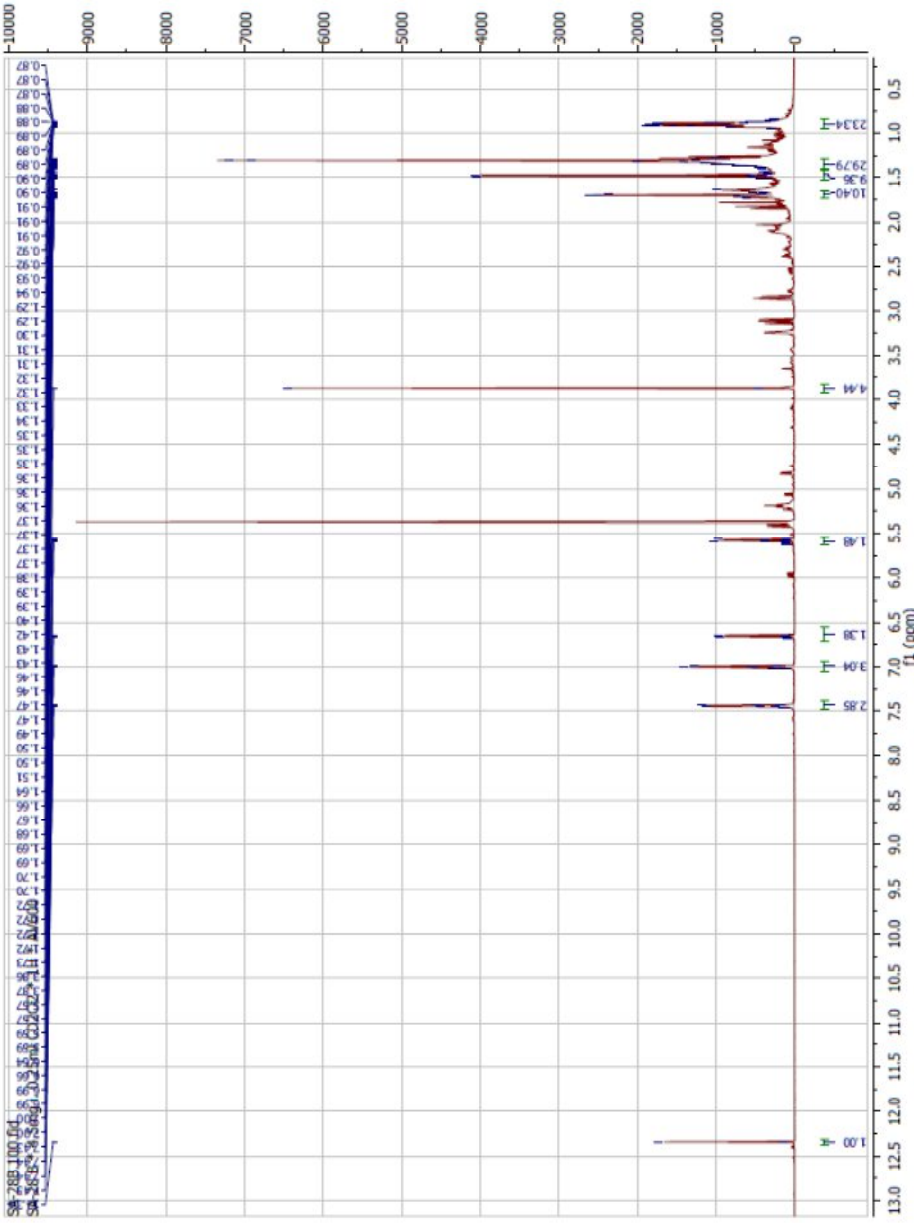
APPENDIX 129I: CD spectrum of micanspterocarpan A (**129**)



Appendix 130A: ^{1}H (CD_2Cl_2, 600 MHz) NMR spectrum of lupinifolin (**130**)



Appendix 131A: ^{1}H (CD_2Cl_2, 600 MHz) NMR spectrum of lupinifolin 4'-methyl ether (**131)**



Appendix 131B: ^{13}C (CD_2Cl_2, 150 MHz) NMR spectrum of lupinifolin 4'-methyl ether (**131**)