PHYTOCHEMISTRY AND BIOLOGICAL ACTIVITY OF THE ROOT EXTRACT OF *MILLETTIA OBLATA*

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Phytochemistry and biological activity of the root extract of *millettia oblata*

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A thesis submitted in partial fulfilment for the degree of Master Science in Medicinal Chemistry in the Jomo Kenyatta University of Agriculture and Technology

2012

DECLARATION

This thesis is my original work and has not been presented for a degree in any other University

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DEDICATION

This thesis is dedicated to my beloved children Kamau and Njeri whose outstanding support, LOVE and encouragement steers me through my darkest moments.

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Glory, praise, thanks and honour to the Almighty God whose promises and blessings endure forever.

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ABBREVIATIONS AND SYMBOLS

ACT	Artemisinin Combination Therapy
AIDS	Auto Immune Deficiency Syndrome
ANOVA	Analysis of Variance
AP	Aerial Part
brs	Broad Singlet
CDs	Compact Disk
CH_2Cl_2	Dichloromethane
CIC	Critical Inhibitory Concentration
d	Doublet
dd:	Doublet of a Doublet
DDT	Dichlorodiphenyltrichloroethane
DEPT	Distortionless Enhanced Transfer
DMSO	Dimethylsulfoxide
FL	Flower
HEPES	Hydroxyethypiperazine Ethane Sulfonic acid
HW	Heartwood
Hz	Hertz
IC_{50}	Concentration for 50 percent inhibition
IP	Intraperitoneal
J	Coupling constant
KEMRI	Kenya Medical Research Institute.
L	Leaves

LD_{50}	Concentration that kills 50% of the animals
m	Multiple (multiplicity
MeOH	Methanol
MHA	Mueller Hinton Agar
MHz	Mega hertz
MIC	Minimum inhibitory concentration
mM	Millimoles
MSF	Malaria SYBR Green 1-based fluorescenc
$NaHCO_3$	Sodium bicarbonate
A±	Blood group type A positive or negative
QSAR	Quantitative Structure Activity Relationship
RB	Root bark
RBC	Red Blood Cells
RPMI	Rosewell Park Memorial Institute
RW	Root wood
s	Singlet
SAR	Structure Activity Relationship
SB	Stem bark
SD	Seeds
SDP	Seedpods
TLC	Thin Layer Chromatography
UV	Ultra violet

LBW	Very Low Birth Weight
WD	Wood
$US	United States Dollar
µl	Microlitre
^{13}C NMR	Carbon Nuclear Magnetic Resonance
^{1}H NMR	Proton Nuclear Magnetic Resonance
δ	Chemical shift
λ_{max}	Maximum wavelength of absorption

ABSTRACT

The genus *Millettia* belongs to Leguminoseae family, Tephroseae tribe and is known to elaborate prenylated flavonoids and isoflavonoids. In the search for bioactive principles *Millettia oblata* root was analysed. The dried and ground whole root of *Millettia oblata* was exhaustively extracted using dichloromethane: methanol (1:1) (CH_2Cl_2:MeOH (1:1)) followed by methanol by cold percolation. The CH_2Cl_2:MeOH (1:1) extract was then subjected to chromatographic isolation on normal silica gel and re-crystallisation leading to the isolation of five compounds. The structures of the isolated compounds were determined using spectroscopic methods including ^{1}H and ^{13}C NMR, comparison with literature and comparison with authentic samples. The isolated compounds included three isoflavones [isoerythrin A, 4'-(3-methylbut-2-enyl) ether **(1)**, calopogoniumisoflavone B **(2)**, 7,2'-dimethoxy-4',5'- methylene dioxyisoflavone **(4)**], a chalcone 4-hydroxyonchocarpin **(3)** and the commonly occurring triterpene lupeol **(5)**. This is the first report of these compounds from *Millettia oblata*.

In vitro anti-plasmodial activity of the crude extracts and isolated flavonoids was carried out against chloroquine sensitive D6 (CDC/Sierra Leone) and chloroquine resistant W2 (CDC/Rosewell Indochina III) strains of *Plasmodium falciparum*. The CH_2Cl_2:MeOH (1:1) crude extract showed anti-plasmodial activity against D6 and W2 *P. falciparum* strains with IC_{50} values of 8.26 ± 1.7 and 11.49 µg/ml, respectively. The methanol extract showed anti-plasmodial activity against the D6 strain only with IC_{50} value of 14.84 µg/ml. All the isolated and identified flavonoids showed anti-plasmodial activity against D6 and W2 *P.*

falciparum strains with the isoflavone isoerythrin A, 4'-(3-methylbut-2-enyl) ether **(1)** showing the highest potency with IC_{50} values of 6.61 ±2.8 and 15.10 ± 4.8 µM against D6 and W2, respectively.

Anti-bacterial activity of the crude extracts and isolated flavonoids was also carried out against gentamycin sensitive *Staphylococcus aureus* (NC 07447), *Bacillus pumilus* (NC 08241), and *Escherichia coli* (ATCC 25922). Anti-fungal activity of the crude extracts and isolated flavonoids was also carried out against nystatin sensitive *Candida albicans.* The crude extracts showed activity against the three bacteria but only the methanol extract showed anti-fungal activity against *Candida albicans*. Amongst the isolated compounds only the chalcone 4-hydroxyonchocarpin **(3)** showed anti-bacterial and anti-fungal activity. The critical inhibitory concentration (CIC) of the CH_2Cl_2):MeOH (1:1) crude extract and compound **3** were found to be below 6.45 and 1.53 mg/ml, respectively. The MICs (Minimum inhibitory concentration) of CH_2Cl_2:MeOH (1:1) crude extract and 4-hydroxyonchocarpin **(3)** were found to be 613 and 2.92 µg/ml, respectively against *Staphylococcus* aureas (NC07447), *Bacillus pumilus* (NC08241) and *Escherichia coli* (ATCC25922). The study has provided some flavonoids of *Millettia oblata* root as possible leads for the discovery, innovation and development of new anti-malarials and anti-bacterial agents. However, further bioassays including acute and chronic toxicity, pharmacokinetic and pharmacodynamic profiles should be carried out to fully establish the potential of *Millettia oblata* crude root extract and phytochemicals as safe and effective therapeutic agents.

CHAPTER ONE

1.0 INTRODUCTION

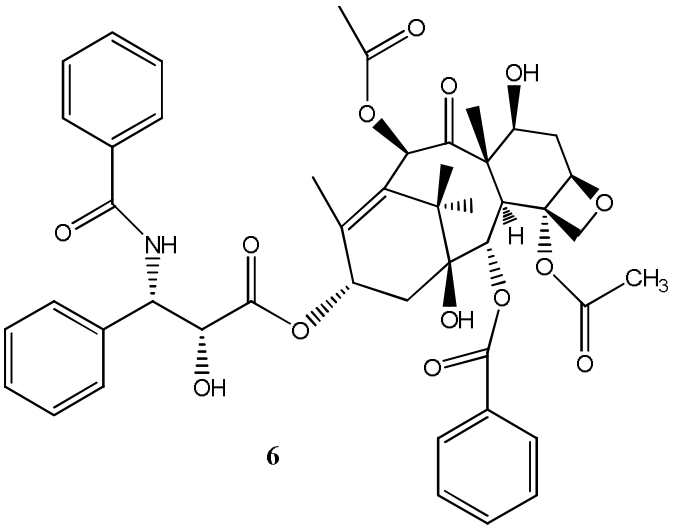
1.1 BACKGROUND INFORMATION

Medicinal plants have been used since ancient time for human healthcare and still remain the most widely used medication system in developing and least developed nations. There is no reliable figure for the total number of medicinal plants on earth, and numbers and percentages for countries and regions vary greatly (Schippmann *et al.*, 2002). There has been a continuous growth in demand for herbal medicines globally. The reliance of people on ethno-medicine has been for reasons of cost-effectiveness, acceptability, biomedical benefits and accessibility (Srivastava, 2000). The medicinal value of plants lies in some chemical substances that produce a definite physiological action on the human body. The most important of these bioactive compounds of plants are alkaloids, flavonoids, tannins and other phenolic compounds (Edeoga *et al.*, 2005).

Historical experiences with plants as therapeutic tools have helped to introduce single chemical entities in modern medicine. Plants, especially those with ethno-pharmacological uses, have been the primary sources of medicines for early drug discovery. The uses of 80% of 122 plant-derived drugs are related to their original ethno-pharmacological purposes (Fabricant and Farnsworth, 2001).

Current drug discovery from terrestrial plants has mainly relied on bioactivity-guided isolation methods, which, for example, have led to discoveries of the important anticancer

agents, paclitaxel **(6)** from *Taxus brevifolia* and camptothecin **(7)** from *Camptotheca acuminata* (Kinghorn, 1994) to mention just a few. Phytochemical and biological investigation of some *Millettia* species elaborated flavonoids with anti-plasmodial activity (Yenesew *et al*., 1998; 2003)



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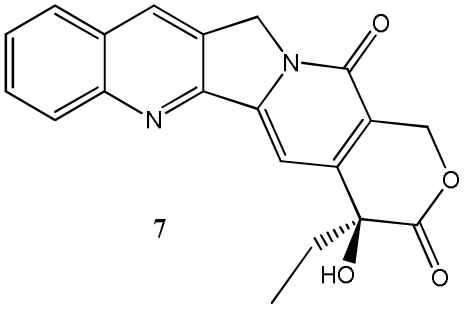

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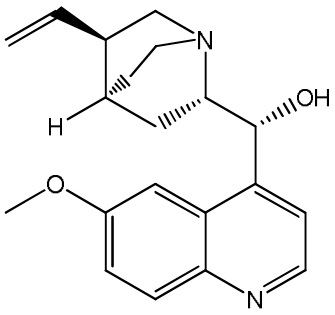
Several plant based drugs with diverse structures, varying mode and site of actions are currently available for treatment of infectious diseases including malaria.

Most of the anti-malarials including quinine **(8)** and artemisinin **(9)** are derived from plants used by indigenous societies in different parts of the world.



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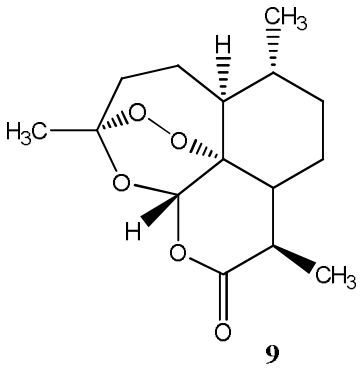

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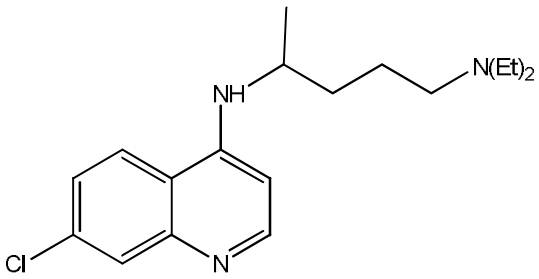
The medicinal use of quinine (**8**) dates back more than 350 years (Rocco, 2003) when it was used as the powdered Chinchona bark to treat fevers and tertian. Although quinine has been synthesised, the procedure is complex and hence it is still obtained from natural sources. Structure Activity Relationship (SAR) and Quantitative Structure Activity Relationship (QSAR) studies of the cinchona alkaloids provided the basis for the discovery of other anti-malarials such as chloroquine **(10)** and mefloquine **(11)** (Goodman and Gilman, 2006).



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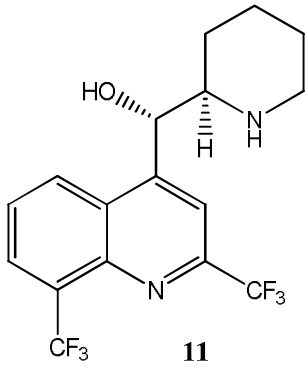

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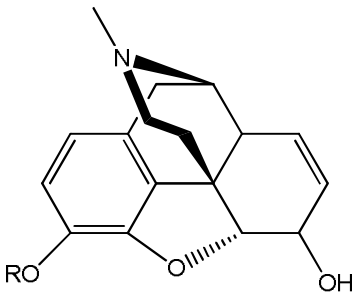
Plants provide the predominant ingredients of medicines in most medical traditions.

Plants have contributed hugely to Western medicine, through providing ingredients for drugs or having played central roles in drug discovery. Morphine **(12)**, codeine **(13)** noscapine **(14)**, and papaverine **(15)** isolated from *P. somniferum* were developed as single

chemical drugs and are still clinically used. Hemisuccinate carbenoxolone sodium **(16)**, a semi-synthetic derivative of glycyrrhetic acid **(17)** found in licorice is prescribed for the treatment of gastric and duodenal ulcers in various countries (Dewick, 2002). There are undoubtedly many more secrets still hidden in the world of plants (Mendelsohn and Balick, 1995).



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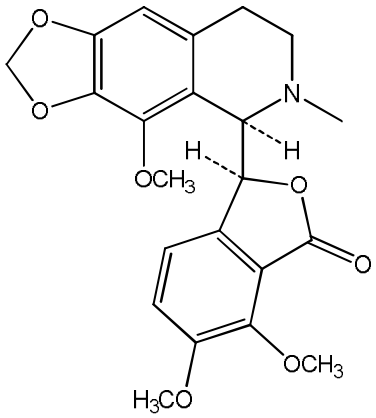

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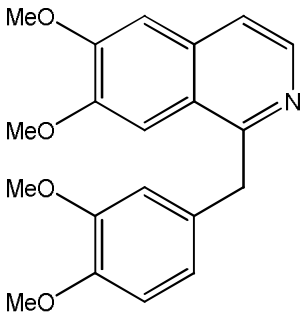

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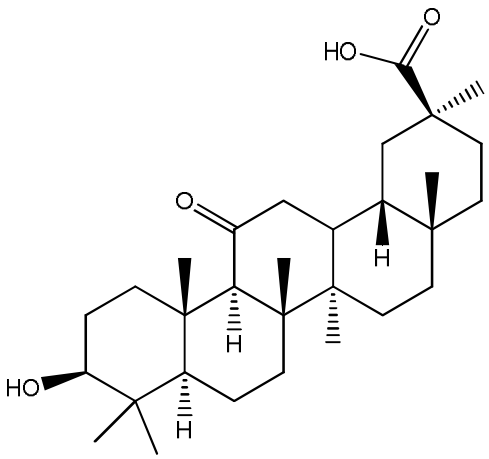

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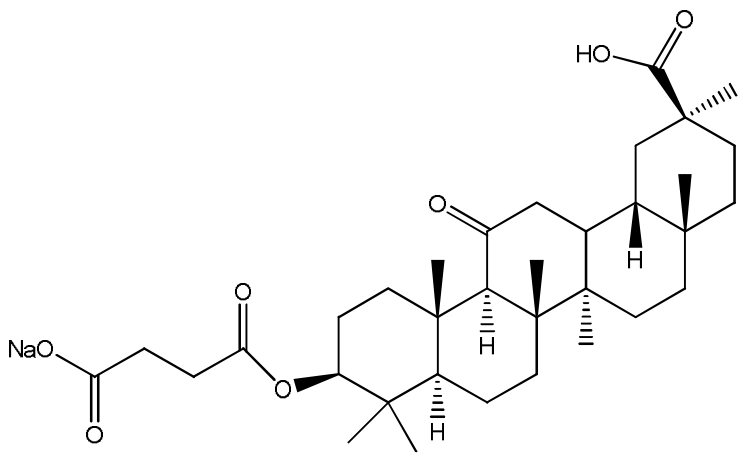

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Some *Millettia* plants are used traditionally for the treatment of bacterial and malarial infections (Desta, 1993; Khalid *et al.,* 1986; Anderson 1986). In developing countries, low-income people such as farmers, people of small isolated villages and native communities use folk medicine for the treatment of common infections (Rojas *et al*., 2006). The World Health Organization estimates that 80% of the people in developing countries of the world rely on traditional medicine (WHO, 1978) for their primary health care needs, and about 85% of traditional medicine involves the use of plant extracts. About 3.5 to 4 billion people in the world rely on plants as sources of drugs (Farnsworth *et al*., 1985).

Interest in medicinal plants as a re-emerging health aid has been fuelled by the increasing resistance towards existing drugs; rising costs of prescription drugs in the maintenance of personal health and well-being; and the bioprospecting of new plant-derived drugs (Hoareau and DaSilva, 1999) Although virtually everyone on Earth benefits from medicinal plants; it is the financially poorest who are typically most closely dependent on medicinal plants. The poor have little alternative to using herbal medicine, which, anyway,

they may prefer, at least for certain conditions (Marshall, 1998). Table 1.1 shows ethno-medical uses of some herbal medicinal products.

Table 1.1: Ethno-medical uses of some herbal medicinal products (HMPs) (Parmar, 2005; Leslie, 2000; Heinrich *et al*., 2004; Evans, 2009)

HMPs	**Botanical source and part of plant**	**Intended use**
Ginseng (Korean or Chinese)	*Panax ginseng* (root)	Relief of fatigue and general health
St John's wort	*Hyperricum perforatum* (Flowering tops including leaves, unopened buds and flowers)	Treatment of mood disorders, particularly depression
Ginkgo	*Ginkgo biloba* (leaves)	Treatment of cognitive deficiencies (often in the elderly), including impairement of memory and affective symptoms such as anxiety. Also used in circulatory disorders
Kava	*Piper methysticum*(roots)	Relief of anxiety and stress (tranquiliser properties) Safety concerns have resulted in the voluntary withdrawal of kava products from sale (2002)
Echinacea	*Echnacea angustifalia, E. purpurea* and *E.pallida* (roots and aerial parts)	Immune stimulant that helps increase resistance to colds, influenza, and other infections; wound healing
Feverfew	*Tonacetum parthenium* or *Chrysanthemum parthenium* (aerial parts)	Fever, rheumatism, migrane and menstrual problems

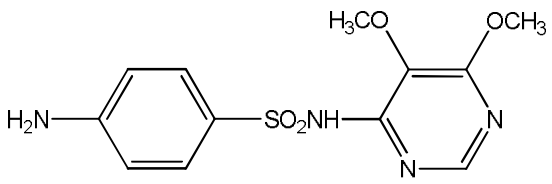
HMPs	**Botanical source and part of plant**	**Intended use**
Valerian	*Valeriana officinalis* (roots)	Sedative for the treatment of insomnia
Saw palmetto	*Serenoa repens* (fruit)	Treatment of benign prostatic hypertrophy
Garlic	*Allium sativum* (bulbs)	To lower cholesterol levels and blood pressure; prevention of heart attack and stroke
Ginger	*Zingiber officinale* (Rhizomes)	Nausea, vomiting cold, diarrhoea, aid to digestion, rheumatism and inflammation
Aloes,Barbados	*Aloe baradensis* and *Aloe ferux* (leaf exudate)	Laxative
Aloe vera	*Aloe baradensis* (leaf gel)	To heal wounds, burns, skin ulcers
Senna leaves and senna fruit	*Cassia senna* (leaves) and *Cassia anguistifolia* (fruit)	Stimulant laxative
Dong quai	*Angelica sinensis* (root)	Irregular menstruation, menopausal syndrome and blood deficiency
Cat's claw	*Uncaria tomentosa* and *U. guanensis* (roots,stem bark and leaves	Antirheumatic and to treat infections and tumours
Hawthorn	*Crataegus oxycanthoides* and *C. monogna* (flowers,leaves and berries)	For heart failure, hypertension, and angina pectoris
Pokeweed	*Phytolacca Americana* (roots and berries)	Antiinflammatory, antiviral and for tumours.Eating uncooked roots or berries may cause serious poisoning

Herbal remedies, like conventional medications, carry a risk of adverse reactions. Toxic effects of herbal medicines range from allergic reaction to cardiovascular, hepatic, renal,

neurologic, and dermatologic toxic effects (Parmar, 2005; Leslie, 2000). Occasionally serious adverse effects such as major potassium depletion and liver failure have been attributed to chronic ingestion of licorice (Connor *et al.*, 2003) and black cohosh, respectively (Lynch *et al.*, 2006). Factors contributing to the potential toxicity of herbs include misidentification of the plant, variability in the time and place of collecting the plant, use of the wrong part of the plant, incorrect storage, contamination during preparation, inconsistency in nomenclature and labelling of the final product and adulteration (Huxtable, 1990; 1992).

There is concern with respect to the numerous well-established interactions of herbs and drugs (Elvin-Lewis, 2001). With the expanding use of medicinal plant remedies, the risk of serious drug interactions increases. There is some information available on common interactions (D'Arcy, 1991; Stedman, 2002) but continued vigilance is required with the introduction of new medications.

Widespread drug resistance has hampered the effectiveness of most of the available cheap first line chemotherapeutic agents including anti-malarials such as chloroquine (Goodman and Gilman, 2006; Casteel, 1997). Malaria parasite resistance to other therapeutic drugs such as sulphadoxine **(18)** and pyrimethamine **(19)** has also increased significantly during the past two decades (Brooks *et al.*, 1994; Peterson *et al.*, 1988).



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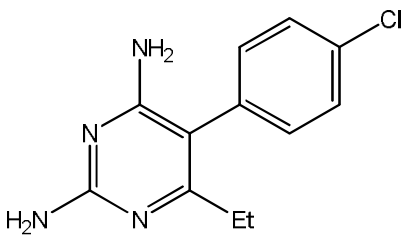

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Bacterial and anti-fungal drug resistance is also on the increase. As resistance to existing drugs is a globally occurring phenomenon and plants are a source of structurally diverse bioactive principles; investigation of plants for safe, effective and structurally diverse lead or/and therapeutic chemicals is necessary in order to continuously discover, innovate and develop more readily available and affordable superior drugs with higher effectiveness and lower toxicity.

1.2 PROBLEM STATEMENT

Drug resistance has become a major clinical and public health problem in the world today. Secondly, infectious diseases including malaria are highly prevalent and contribute largely to the global disease burden. Malaria is one of the most prevalent killer diseases in the tropical and sub-tropical region. It affects over three hundred million people annually, causing two million deaths of the affected persons (WHO, 2010).

Drug resistance is particularly serious in developing countries such as Kenya where rates of resistance are higher than in developed nations; fewer therapeutic options are available and most of the people are poor. The situation is especially dire in the least developed countries, which bear the heaviest burden of infectious diseases such as malaria,

tuberculosis (TB), and human immunodeficiency virus/ acquired immunodeficiency syndrome (HIV/AIDS) (Blum et al., 2006). Drug resistance has led to higher treatment costs, increased morbidity and mortality, and, in some cases permanent loss of specific drug therapies (Blum et al., 2006).

It is necessary to investigate plant extracts in order to validate their therapeutic use and to identify the active constituents which may act as lead compounds in drug discovery, innovation and development of safe, more effective, affordable and readily available antimalarial, anti-bacterial and anti-fungal agents.

1.3 JUSTIFICATION

Plants are important sources of potentially useful structures for development of new therapeutic agents. Most drug discoveries from plants are based on ethno-pharmacological approach. Some Millettia species such as M. thonningii (Khalid et al., 1986), M. ferruginea (Desta, 1993), M. Leptobotrya (Pei, 1985) are used traditionally for treatment of malaria and other infectious diseases. Previous phytochemical and biological investigation of Millettia usaramensis and M.dura, two species found in Kenyan elaborated flavonoids with anti-plasmodial activity (Yenesew et al., 1998; 2003; Derese 2004). Millettia oblata which is endemic to Taita hills, Kenya has not been previously studied. There is therefore reason to investigate Millettia oblata to determine its potential for new superior bioactive molecules to counteract increasing resistance and changing disease treads.

1.4 ALTERNATE HYPOTHESIS

Millettia oblata crude root extract and its pure phytochemicals exhibit anti-plasmodial, anti-bacterial and anti-fungal activity.

1.5 RESEARCH QUESTIONS

i. Does the *Millettia oblata* crude root extract have any anti-bacterial, anti-fungal and/or anti-plasmodial activity?

ii. Do specific pure phytochemicals from *Millettia oblata* root extract exhibit anti-bacterial, anti-fungal and/or anti-plasmodial activity?

1.6 STUDY OBJECTIVES

1.6.1 General objective

To identify bioactive principles from *Millettia oblata* root extract.

1.6.2 Specific objectives

i. To isolate and identify phytochemicals from *Millettia oblata* root extract.

ii. To determine anti-fungal, anti-bacterial and anti-plasmodial activity of *Millettia oblata* root crude extract.

iii. To determine anti-fungal, anti-bacterial and anti-plasmodial activity of the identified pure phytochemicals from *Millettia oblata* root extract

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 BACKGROUND INFORMATION

2.1.1 Malaria

Malaria is a life-threatening disease caused by parasites that are transmitted to people through the bites of infected mosquitoes. The *Plasmodium* parasites are highly specific, with man as the only vertebrate host and Anopheles mosquitoes as the vectors (WHO, 1987).

Malaria is generally endemic in the tropics, with extensions into the subtropics. Malaria in travellers arriving by air is now an important cause of death in non malarious areas (Weathersby and McCroddan, 1982) and this is not helped by the common ignorance or indifference of travelers to prophylaxis (WHO, 1980). In 1990, 80% of malaria cases were in Africa, with the remainder clustered in nine countries: India, Brazil, Afghanistan, Sri-Lanka, Thailand, Indonesia, Vietnam, Cambodia and China (Olliaro *et al*., 2001).

Currently, the best available treatment, particularly for *P. falciparum* malaria, is artemisinin-based combination therapy (ACT) (Rang *et al*., 2007). Insecticides such as DDT have long been available for malaria control through the vector.

2.1.2 History of malaria

Hippocrates was the first to clearly describe the different types of malaria depending upon the periodicity of the fever as tertian and quartan fever patterns. He also described septans and nonanes, as other malarial variants (Cheston *et al*., 2008).

The term malaria from the Italian mala (bad) and aria (air) was introduced to English by Horace Walpole in 1740 who described malaria as a horrid thing that killed (Kakklaya, 2006).

2.1.3 Malaria burden and economic impact

Malaria has prevented any economic development in vast regions of the earth and continues to be a huge social, economical and health problem especially in the tropical countries. Malaria traps people in poverty and undermines the development of some of the poorest countries in the world (WHO, 2008a)

The burden caused specifically by antimalarial drug resistance is more difficult to quantify **(**Phillips and Phillips-Howard, 1996). Estimates based on the best available data from Africa, suggest that the demise of chloroquine is one of the major factors that have contributed to the change in malaria specific mortality (Snow *et al.*, 2001) which has been estimated to have at least doubled over the last 15 years (Trape, 2001). Much of this burden falls on the poor, exacerbating already existing inequities, since the more expensive, effective anti-malarials are accessible only to patients affluent enough to obtain them through informal sources, and remain out of reach to the majority of the rural poor

who carry the largest burden of disease. Malaria thus, has disastrous social consequences and is a heavy burden on economic development (Casteel, 1997).

2.1.4 Malaria prevalence

Malaria is generally endemic in the tropics, with extensions into the subtropics.

In 1990, 80% of cases were in Africa, with the remainder clustered in nine countries: India, Brazil, Afghanistan, Sri-Lanka, Thailand, Indonesia, Vietnam, Cambodia and China (Olliaro *et al*., 2001).

Most malaria cases and deaths occur in sub-Saharan Africa. However, Asia, Latin America, and to a lesser extent the Middle East and parts of Europe are also affected (WHO, 2008b).

In 2008, Kenya ranked 5th after Nigeria, Democratic Republic of the Congo , Ethiopia, United Republic of Tanzania with over 11 million annual malaria cases (WHO, 2008b).

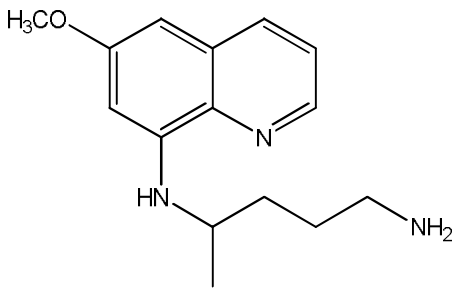
2.2 Structural classification of anti-malarials

Commonly used anti-malarials can be categorised according to their structure into various classes. Table 2.1 summarises the structural classification of anti-malarials.

Table 2.1: Structural classification of anti-malarials

(Rang *et al*., 2007; Goodman and Gilman, 2006)

Chemical class	**Examples**
Quinoline-methanols	Quinine **(8)** Mefloquine **(11)**
4-Aminoquinolines	Chloroquine **(10)**
8-Aminoquinolines	Primaquine **(20)**
Diaminopyrimidines	Pyrimethamine **(19)**
Sulphonamides	Sulfadoxine **(18)** and sulphamethoxypyrazine **(21)**
Sulphones	Dapsone **(22)**
Phenanthrene methanols	Halofantrine **(23)**
Hydroxynaphthoquinones	Atovaquone **(24)**
Tetrayclines	Tetracycline **(25)** and doxycycline **(26)**
Lincosamides	Clindamycin **(27)**
Aryl alcohols	Lumefantrine **(28)**
Sesquiterpene lactone endoperoxides	Artemisinin **(9)** and derivatives Dihydro artemisinin **(29)**,artesunate **(30)** and artemether **(31)**
Biquanides	Proquanil **(32)**
others	Tafenoquine **(33)**



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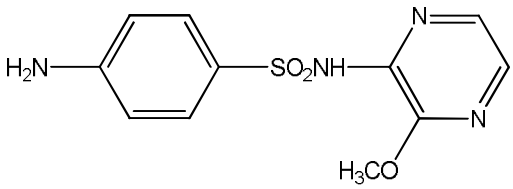

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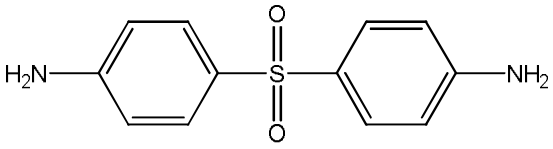

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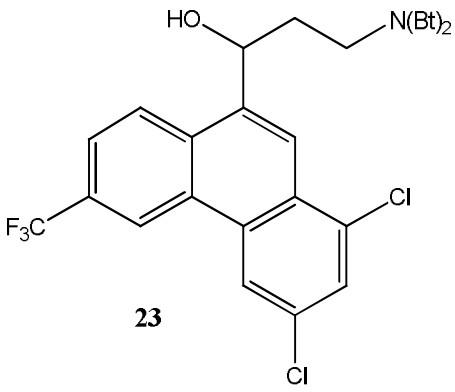

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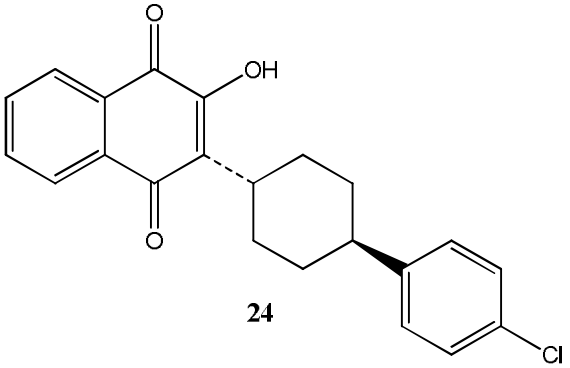

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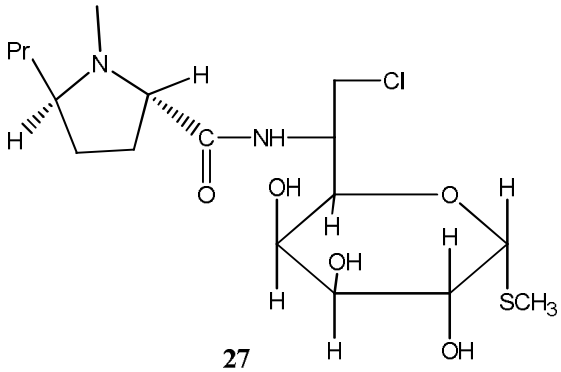

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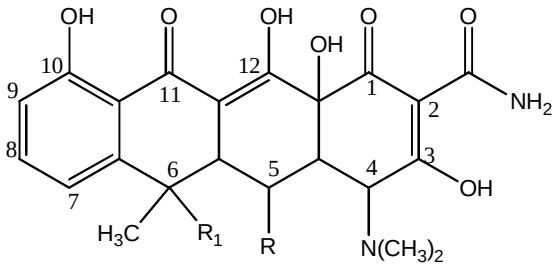
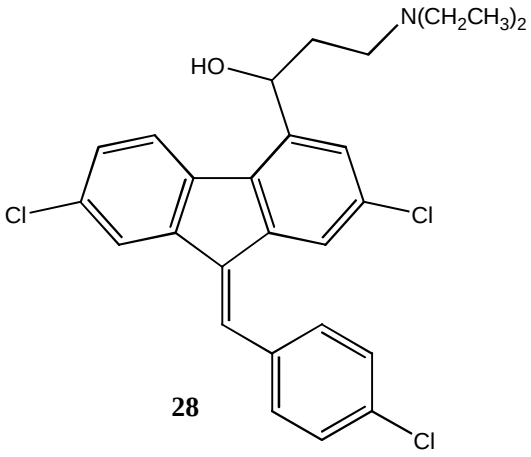


	R	R_1
25	**H**	**OH**
26	**OH**	**H**



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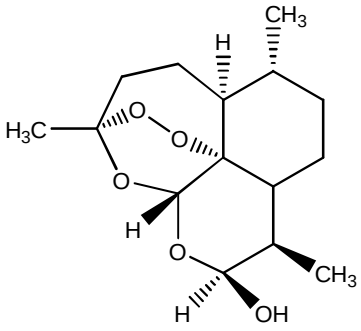

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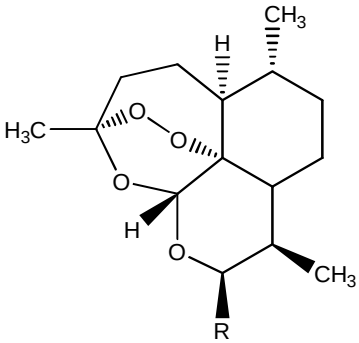
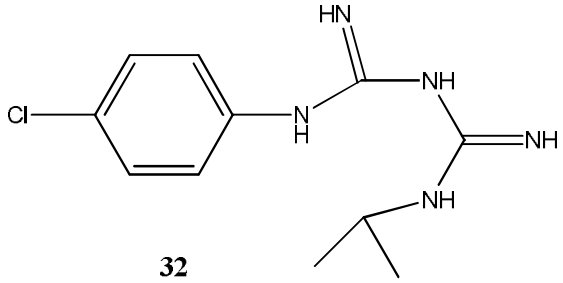


	R
30	**OCH_3**
31	**$OCO(CH_2)_2CO_2Na$**



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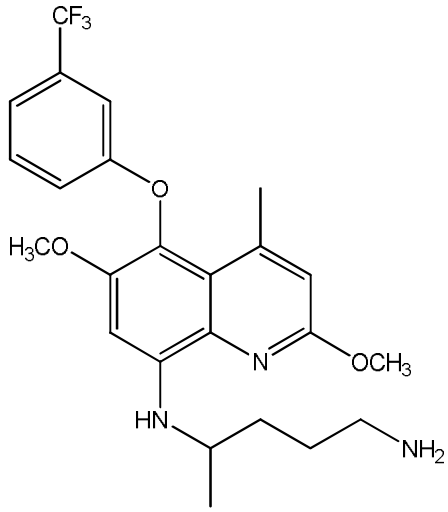

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2.3 RESISTANCE TO ANTI-MALARIAL DRUGS

Growing resistance to antimalarial medicines has spread very rapidly, undermining malaria control efforts. Monotherapy is the primary force behind the spread of artemisinin resistance. If resistance to artemisinin **(9)** develops and spreads to other large geographical areas, as has happened before with chloroquine **(10)** and sulphadoxine-pyrimethamine (SP), the public health consequences could be dire, as no alternative antimalarial medicines are available (WHO, 2010).

2.4 BACTERIAL AND FUNGAL INFECTIONS

A large number of human, animal and plant disease are caused by pathogenic microbes such as fungi, bacteria and algae. Infections due to fungi and bacteria have been a major cause of death in higher organisms.

Historically many of the new antibiotics were isolated from natural sources including soil microbes and plants. Many more were later synthesized and introduced in clinical practices (Atta-ur-Rahman *et al.,* 2005).

Human struggle against pathogenic microbes is far from over due to emergence of new pathogens, and remarkable abilities of microbes to develop resistance against used antimicrobials (Atta-ur-Rahman *et al.,* 2005). The increasing rate of antimicrobial resistance towards the broad spectrum agents is posing antimicrobial therapy challenges. These challenges are further compounded by the emergence of highly resistant opportunistic micro-organism especially in immune-compromised patients and the fact that relatively few new drugs are being developed, particularly those that treat resistant Gram-negative organism (Marcel, 2007).

Human fungal infections have increased dramatically in incidence and severity in recent years owing mainly to advances in surgery, cancer treatment, use of broad-spectrum antimicrobials and the HIV epidemic (Sheppard and Harry, 2007).

Candida albicans remains the most commonly encountered fungal pathogen among hospitalized patients, accounting for roughly 50-60% of all bloodstream fungal isolates (Michael, 2001). In addition, several reports have documented an increasing frequency with which non-albicans *Candida* species are isolated (Price *et al*., 1994; Wingard *et al*., 1993). Isolation of *Candida* species less susceptible to traditional therapies and recovery of increasingly resistant isolates during anti-fungal therapy is increasing (Michael**,** 2001).

2.4.1: Bacterial resistance

The enormous genetic flexibility of bacteria restricts the usefulness of currently available antimicrobials, and requires new approaches to antimicrobial agents' discovery and

development. Antimicrobial resistance can be acquired in a short time frame, both by genetic mutation and by direct transfer of resistance genes across genus and species boundaries (Marcel, 2007).

Over prescription of antibiotics by both qualified and unqualified medical practitioners is common in developing countries (D'Souza, 1999) and self-medication through the purchase of antibiotics from drug vendors and pharmacies is also widespread (Okeke, 2005; Graham, 2001). The overuse of antibiotics has increased resistance among common infectious disease causing bacteria, such as *Streptococcus pneumoniae, Staphylococcus aureus* and *Haemophilus influenza* among others. The former, though once universally sensitive to penicillin (Okeke, 2005) is currently highly resistant. Resistant strains of bacteria can quickly multiply and spread within a community where antibiotic use is common. The failure of first-line treatments prompts health-care workers to seek more-expensive and often less-available antibiotics. Consequently, antibiotic resistance often results in various societal costs, including increased drug costs, additional health-service costs (such as laboratory tests and hospitalizations), greater drug resistance-related morbidity and mortality, and decreased productivity (Yee-Wei *et al*., 2006).

2.5 BOTANICAL INFORMATION OF *MILLETTIA OBLATA*

2.5.1 Taxonomy of *Millettia oblata*

Some successful correlations have been established between plant taxonomy and the

occurrence of specific chemical constituents at different taxonomical levels of classification (Gershenzon, 1983; Harborne, 1984; Waterman, 1987). Figure 2.1 illustrates the taxonomy of *Millettia oblata.*

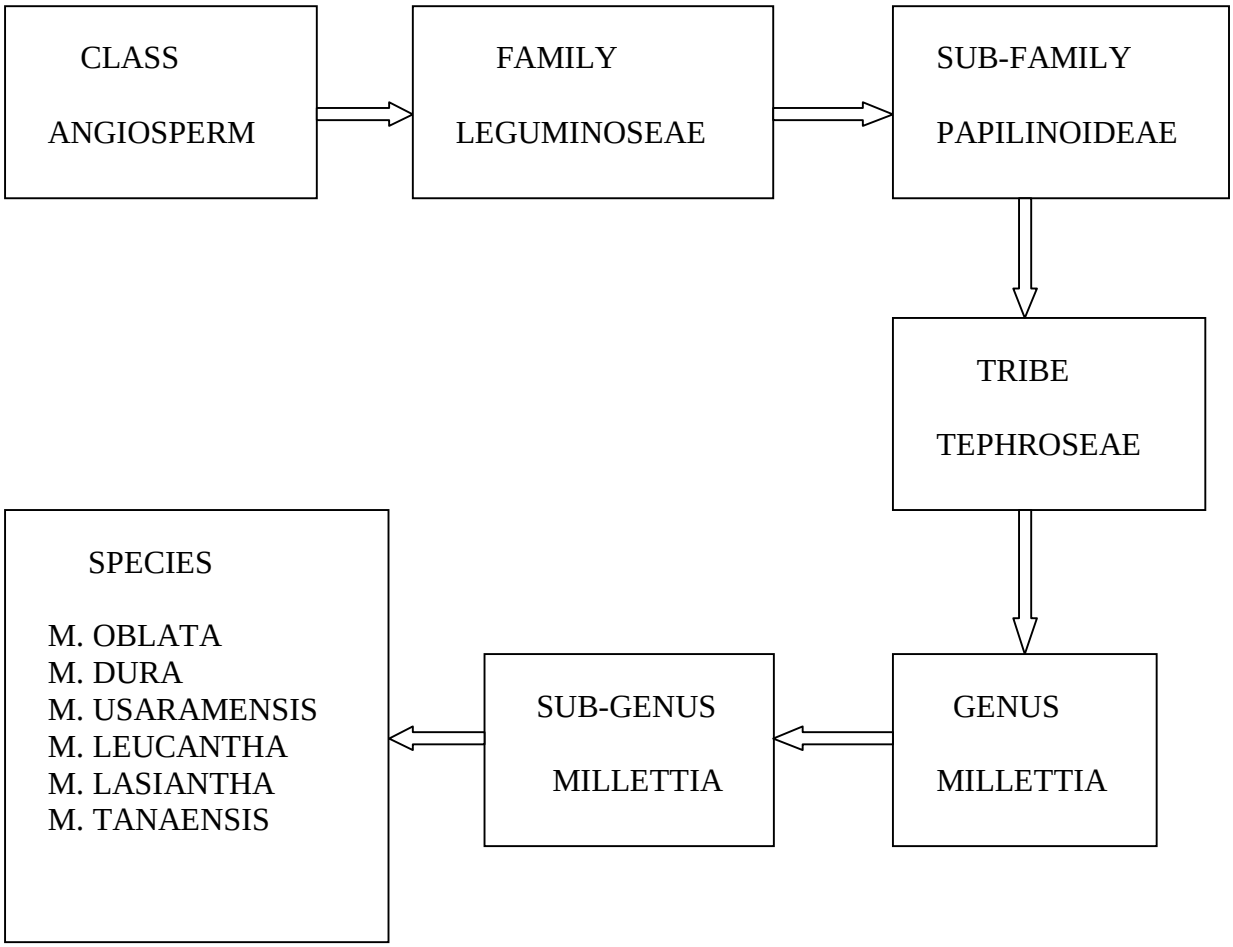


Figure 2.1: Flow chart of the taxonomy of Millettia oblata.

2.5.2 *Millettia oblata*

Millettia oblata (Figure 2.2) is a tree with a height of 3-21 m and has a brown corrugated bark. Its leaves have 9-21 leaflets which are either elliptic ovate or slightly obvate with the lowermost being the smallest. Its flowers which are 2 cm long are purple-blue in colour. Its

fruits are oblong with a downwards curved tip. It is endemic to Taita hills and remnants are found in moist evergreen forests. It is found in regions of altitude 1400-1850 m (Beentje, 1994).

Figure 2.2: *Millettia oblata* stem and leaves

2.6 ETHNO-MEDICAL INFORMATION

2.6.1 Ethno-medical uses of Leguminoceae family

Plants belonging to this family have been used traditionally in various communities for the treatment of various ailments. The root of *Sophora flavescens* Aiton, a well-known Chinese herbal medicine is used as a diuretic and for the treatment of diarrhoea, gastrointestinal haemorrhage and eczema (Woo *et al*., 1998). The roots of *Taverniera abyssinica*, known in Amharic as 'Dingetegna' (medicine for sudden illness), is widely used in Ethiopia as an effective remedy for sudden pain particularly of stomach and also to reduce high fever (Duddeck *et al*., 1987). Plants belonging to the genera *Derris*, *Lonchocarpus*, *Millettia*, *Mundulea* and *Tephrosia* of the family Fabaceae have long been used in Africa, Asia and South America as insecticides and fish poison. In Kenya, several plants of this family are used for the treatment of various ailments (Kokwaro, 1993). Table 2.2 lists some representative Kenyan Fabaceae species and their medicinal use.

Table 2.2: Ethno-medical uses of some Kenyan Fabaceae species (Kokwaro, 1993).

Plant species	**Method of use and disease treated**
Abrus precatorius	A decoction of the leaves and roots is taken as a remedy for gonorrhoea
Acacia albida	A decoction of the bark is drunk as a cure for coughs and diarrhoea.
Acacia mellifera	The bark is boiled and used as a remedy for stomach trouble, cleaning primary infection of syphilis, sterility, pneumonia and malaria.
Caesalpinia volkensii	The leaves are boiled in soup or tea and drunk to treat malaria.
Cassia abbreviata	A decoction of the roots is drunk to cure fever or malaria, stomach troubles and uterus complaints.
Cassia didymobotrya	A decoction of the leaves, stems, and roots is used as a purgative.
Dalbergia vacciniifolia	A decoction of the root is used as a purgative.
Erythrophleum suaveolens	The root decoction is used as antihelmintic.

2.6.2 Ethno-medical uses of genus *Millettia*

Plants of the genus *Millettia* have been widely used traditionally for the treatment of various ailments including malaria. Table 2.3 summarizes the traditional medicinal uses of some *Millettia* species.

Table 2.3: Ethno-medical uses of *Millettia*

Species	**Plant Part**	**Uses**	**Reference**
M. auriculata	Leaves Roots	Male infertility Fish poison Pesticide Vermicide	Choudhary *et al*., 1990 Jain *et al* ., 1994 ,, ,,
M. caerulea	Leaf + Stem	Reduce infection in cuts and burns	Anderson, 1986
M. dielsiana	Vine	Improve circulation and dissolve blood clots	Pong *et al.*,1981
M. dura	Entire Plant	Fish poison	Teesdale, 1954
M. elongatistyla	Roots	Treat Schistosomiasis	Hostettmann, 1984
M. extensa	Roots Root Bark	Stomach pain Prevent conception	Singh and Maheshwari 1994 ,,
M. ferruginea	Roots	Treat gonorrhoea	Desta, 1993
M. kitanja	Leaves	Treat diabetes	Mueller *et al* ., 1971
M. lasiantha	Roots	Aphrodisiac	Kokwaro, 1993
M. leptobotrya	Roots	Treat wounds	Pei, 1985
M. pachycarpa	Roots Seeds	Treat swelling Fish poison	Pei, 1985 Ramanujan and Ratha, 1980 Mukerjee and Tripathi, 1956
M. pervilleana	Seeds	Fish poison	Galeffi *et al.*,1997
M. reticulata	Roots	Inhibit blood coagulation	Kosuge *et al.*, 1984
M. stullmannii	Roots	Treat stomach-ache	Arnold and Gulumian, 1984
M. thonningii	Entire Plant Roots	Anti-malarial Anthelminthic	Khalid *et al*., 1986 Vasileva, 1969
M. usaramensis	Roots	Anti-venom	**S**elvanayahgam *et al.*, 1994

2.7 PHYTOCHEMISTRY OF FABACEAE

The family Fabaceae has been found to contain anthraquinones, alkaloids, terpenoids and flavonoids among others; with the flavonoids being the most comprehensively investigated. Isoflavonoids which have a limited distribution in the plant kingdom are almost exclusively restricted to the sub-family Papilinoideae of the Fabaceae family (Dewick, 1994). Previous phytochemical studies of extracts of *Millettia* species have led to the isolation of alkaloids, flavones, flavanones, chalcones, rotenoids, isoflavones and coumarins among others.

2.7.1 Phytochemical information of flavonoids

Flavonoids are classified into various subgroups according to the substitution pattern of ring C. Both the oxidation state of the heterocyclic ring and the position of ring B are important in the classification.

Figure 2.3 shows the basic skeleton and the numbering system of flavones, isoflavones and chalcones.

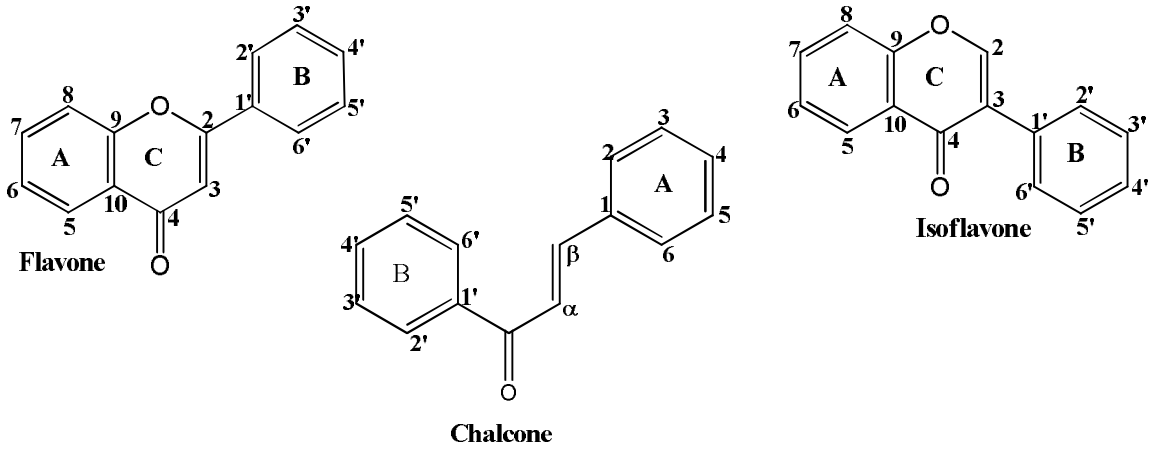


Figure 2.3: Basic skeleton of flavones, isoflavones and chalcones.

2.7.2 Compounds isolated from *Millettia*

Phytochemical investigations of *M. dura* and *M. usaramensis* have resulted in the isolation of a number of isoflavones, rotenoids and chalcones (Yenesew *et al*., 1996; 1997; 1998).

2.7.2.1 Isoflavones from *Millettia*

The genus *Millettia* is a rich source of isoflavonoids (Dewick, 1994). Isoflavones constitute the largest group of natural isoflavonoids. So far nearly one hundred isoflavones have been reported from the genus *Millettia*. Table 2.4 lists some of the isoflavones isolated from the genus *Millettia*.

Table 2.4: Isoflavones of *Millettia*

Isoflavone	**Source (plant part)**	**Reference**
Auricularin **(37)**	*M. auriculata* (RT)	Shabbir and Zaman, 1970
Auriculasin **(38)**	*M. auriculata* (LF) *M. auriculata* (SD) *M. taiwaniana* (SB)	Minhaj *et al*., 1976 Raju and Srimannarayana, 1978 Ito *et al*., 2004
Auriculatin **(39)**	*M. auriculata* (RT) *M. auriculata* (SD)	Shabbir and Zaman, 1970 Raju and Srimannarayana, 1978
Auriculin **(40)**	*M. auriculata* (RB)	Shabbir and Zaman, 1970
Aurmillone **(41)**	*M. auriculata* (SD)	Raju and Srimannarayana, 1978
2'-Deoxyisoauriculatin **(42)**	*M. auriculata* (RT)	Shabbir and Zaman, 1970
Isoauriculasin **(43)**	*M. auriculata* (LF)	Minhaj *et al*., 1976
Isoauriculatiin **(44)**	*M. auriculata* (RB)	Shabbir and Zaman, 1970
Isoaurmillone **(45)**	*M. auriculata* (SDP)	Gupta *et al*., 1983
2'-O-Methylisoauriculatin **(46)**	*M. auriculata* (RB)	Shabbir and Zaman, 1970

Isoflavone	**Source (plant part)**	**Reference**
Millettin **(47)**	*M. auriculata* (RB) *M. auriculata* (SD)	Shabbir and Zaman, 1970 Raju and Srimannarayana, 1978
Viridiflorin **(48)**	*M. brandisiana* (LF)	Pancharoen *et al*., 2008
robustigenin **(49)**	*M. brandisiana* (LF)	Pancharoen *et al*., 2008
Brandisianin A **(50)**	*M. brandisiana* (LF)	Pancharoen *et al*., 2008
7,4'-di-O-prenylgenistein **(51)**	*M. brandisiana* (LF)	Pancharoen *et al*., 2008
Conrauinones A **(52)**	*M. conraui* (SB)	Fuendjiep *et al*., 1998a
Conrauinones B **(53)**	*M. conraui* (SB)	Fuendjiep *et al*., 1998a
Conrauinones C **(54)**	*M. conraui* (SB)	Fuendjiep *et al*., 1998b
Conrauinones D **(55)**	*M. conraui* (SB)	Fuendjiep *et al*., 1998b
7-Hydroxy-6-methoxy-3',4'-methylenedioxyisoflavone **(56)**	*M. conraui* (SB)	Fuendjiep *et al*., 1998b
5-Methoxydurmillone **(57)**	*M. conraui* (SB) *M.ferruginea* (SB)	Fuendjiep *et al*., 1998b Dagne *et al*.,1989
Afrormosin **(58)**	*M. dielsiana* (SB) *M. reticulata* (SB) *M. nitida* (VS)	Rui et al.,1989 Chen *et al*., 1983 Xiang *et al*., 2009
Biochanin **(59)**	*M. dielsiana* (SB) *M. nitida* (VS)	Wang *et al*., 1990 Feng *et al*., 2007
Calycosin **(60)**	*M. dielsiana* (SB) *M. laurentii* (HW)	Rui *et al*., 1989 Kamnaing, 1999
Daidzein **(61)**	*M. dielsiana* (SB)	Rui *et al*., 1989
Formononetin **(62)**	*M. dielsiana* (SB) *M. nitida* (VS)	Rui *et al*., 1989 Xiang *et al*., 2009
Genistein **(63)**	*M. dielsiana* (SB) *M. nitida* (VS)	Wang *et al*., 1990 Feng *et al*., 2007
8-O-Methylretusin **(64)**	*M. dielsiana* (SB) *M. reticulata* (SB)	Rui *et al*., 1989 Chen *et al*, 1983

Isoflavone	**Source (plant part)**	**Reference**
Odoratin **(65)**	*M. dielsiana* (SB) *M. griffoniana* (RB)	Rui *et al*., 1989 Yankep *et al*., 1997
Pseudobaptigenin **(66)**	*M. dielsiana* (SB)	Wang *et al*., 1990
Calopogoniumisoflavone A **(67)**	*M. dura* (SB) *M. ferruginea* (SB)	Yenesew *et al*., 1996 Dagne *et al*., 1990a
Calopogoniumisoflavon A,6-methoxy **(68)**	*M. dura* (SDP)	Yenesew *et al.*, 1997b
6-Demethyldurallone **(69)**	*M. dura* (SDP)	Yenesew *et al*., 1996
7,2'-Dimethoxy-4',5'-methylenedioxyisoflavone **(3)**	*M. dura* (SB) *M. griffoniana* (RB) *M. griffoniana* (SD)	Dagne *et al*., 1991 Yankep *et al*., 1997 Ngamga *et al*., 2005
Durallone **(70)**	*M. dura* (SDP)	Yenesew *et al*., 1996
Durlettone **(71)**	*M. dura* (SD) *M. dura* (SD)	Ollis *et al*., 1967 Dagne *et al*., 1991
Durlmillone **(72)**	*M. dura* (SD) *M. ferruginea* (SB) *M. rubiginosa* (RB) *M. griffonianone* (RB)	Ollis *et al*., 1967 Dagne *et al*., 1989 Desai *et al*., 1977 Yankep *et al*., 1997
Isoerythrin A, 4'-(3-methylbut-2-enyl ether **(1)**	*M. dura* (SDP)	Yenesew *et al*., 1996
Jamaicin **(73)**	*M. dura* (SD) *M. ferruginea* (SB) *M. usaramensis* (SB) *M. griffonianone* (RB)	Yenesew *et al*., 1997b Dagne *et al*., 1989 Yenesew *et al*., 1998 Yankep *et al*., 1997
Maximaisoflavone B **(74)**	*M. dura* (SB)	Dagne *et al*., 1991

Isoflavone	**Source (plant part)**	**Reference**
Maximaisoflavone H **(76)**	*M. dura* (SB) *M. dura* (SB)	Dagne *et al*., 1991 Yenesew *et al*., 1996
Milldurone **(77)**	*M. dura* (SB)	Ollis *et al*., 1967
Predurallone **(78)**	*M. dura* (SDP)	Yenesew *et al*., 1996
Barbigerone **(79)**	*M. ferruginea* (SD) *M. usaramensis* (SB) *M. taiwaniana*	Dagne *et al*., 1990a Yenesew *et al*., 1998 Ito *et al*., 2004
Calopogonium isoflavone B **(2)**	*M. ferruginea* (SB) *M. griffonianone* (RB) *M. griffoniana* (SD)	Dagne *et al*., 1989 Yankep *et al*., 1997 Ngamga *et al*., 2005
Ferrugone **(81)**	*M. ferruginea* (SB,SD)	Dagne *et al*., 1991
7-O-Geranylformononetin **(82)**	*M. ferruginea* (RB) *M. griffonianone* (RB)	Dagne *et al*., 1990b Yankep *et al*., 1997
7-Hydroxy-5,6-dimethoxy-3',4'methylenedioxyisoflavone ..**(83)**	*M. ferruginea* (SB)	Dagne *et al*., 1989
Ichthynone **(84)**	*M. ferruginea* (SB) *M. rubiginosa* (RB)	Dagne *et al*., 1989 Desai *et al*., 1977
Isojamaicin **(85)**	*M. ferruginea* (SB) *M. usaramensis* (SB)	Dagne *et al*., 1989 Yenesew *et al*., 1998
Nordurlettone **(86)**	*M. ferruginea* (SB)	Dagne *et al*., 1990a
Prebarbigerone **(87)**	*M. ferruginea* (SB)	Dagne *et al*., 1990a
Predurmillone **(88)**	*M. ferruginea* (SB)	Dagne *et al*., 1990a
Preferrugone **(89)**	*M. ferruginea* (SB)	Dagne *et al*., 1990a

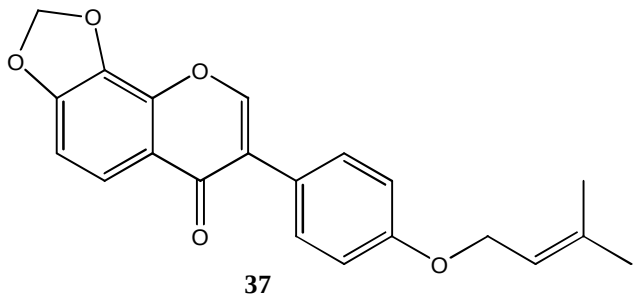
Isoflavone	**Source (plant part)**	**Reference**
Pre-5-methoxydurmillone **(90)**	*M. ferruginea* (SB)	Dagne *et al*., 1989
Griffonianone B **(91)**	*M. griffonianone* (RB)	Yankep *et al*., 2001
Griffonianone C **(92)**	*M. griffonianone* (RB)	Yankep *et al*., 2001
7-Hydroxy-6-methoxy-3',4'-methylene dioxyisoflavone **(93)**	*M. griffonianone* (RB)	Yankep *et al*., 2001
3',4'-Dihydroxy-7-O-[(E)-3,7-dimethylallyl-2,6-octadienyl] isoflavone **(94)**	*M. griffonianone* (RB)	Yankep *et al*., 1998
4'-methoxy-7-O-[(E)-3-methyl-7-hydroxy-2,6-octadienyl]isoflavone **(95)**	*M. griffonianone* (RB)	Yankep *et al*., 1998
7-O-Geranylpseudobaptigenin **(96)**	*M. griffonianone* (RB)	Yankep *et al*., 1997
Odorantin **(97)**	*M. griffonianone* (RB)	Yankep *et al*., 1997
Maximaisoflavone G **(98)**	*M. griffonianone* (RB) *M. usaramensis* (SB)	Yankep *et al*., 2001 Yenesew, 1997a
Pyrano[5'',6:6'',7]isoflavone,2',4',5'-trimethoxy-2'',2''-dimethyl **(99)**	*M. ichthyochtona* (LF)	Kamperdick *et al*., 1998
Gliricidin **(100)**	*M. laurentii* (HW)	Kamnaing *et al*., 1999
Hirsutissimiside B **(101)**	*M. nitida* (VS)	Xiang *et al*., 2009
Sphaerobioside **(102)**	*M. nitida* (VS)	
3'-O-methylorobol **(103)**	*M. nitida* (VS)	Feng *et al*., 2007

Isoflavone	**Source (plant part)**	**Reference**
4'-O-methylderrone **(104)**	*M. pachycarpa* (SD)	Singhal *et al*.,1981
6,8-Diprenylorobol **(105)**	*M. pachycarpa* (AP)	Singhal *et al*.,1981
5,7,4'-Trihydroxy-6,3'-diprenylisoflavone **(106)**	*M. pachycarpa* (AP)	Singhal *et al*.,1983
6,8-Diprenylgenistein **(107)**	*M. pachycarpa* (AP)	Singhal *et al*.,1983
6,8-Diprenylpratensin **(108)**	*M. pachycarpa* (SD)	Singhal *et al*.,1983
Pomiferin **(109)**	*M. pachycarpa* (SD)	Singhal *et al*.,1983
2'-Hydroxylupalbigenin **(110)**	*M. pulchra* (AP)	Baruah *et al*.,1984
2'-Methoxylupalbigenin **(111)**	*M. pulchra* (AP)	Baruah *et al*.,1984
Alpinumisoflavone **(112)**	*M. thonningii* (SD) *M. taiwaniana*	Olivares *et al*.,1982 *Ito et al*., 2004
O,O-Dimethylalpinumisoflavone **(113)**	*M. thonningii* (RB)	Asoamaning *et al*., 1999
3'-Hydroxy-4'-methoxy alpinumisoflavone **(114)**	*M. thonningii* (SD)	Olivares *et al*., 1982
5-Methoxyalpinumisoflavone **(115)**	*M. thonningii* (RW)	Asoamaning *et al*., 1999
4'-Methoxyalpinumisoflavone **(116)**	*M. thonningii* (SD)	Khalid and Waterman, 1983
5-O-Methyl-4'-O-(3-methyl-2-butenyl)-alpinumisoflavone **(117)**	*M. thonningii* (SD)	Asoamaning, 1995
Robustone **(118)**	*M. thonningii* (SD)	Khalid and Waterman, 1983
Thonninginisoflavone **(119)**	*M. thonningii* (RB)	Asoamaning *et al.*, 1995
Millewanins A **(120)**	*M. taiwaniana* (S)	Ito *et al*., 2004
Millewanins B **(121)**	*M. taiwaniana* (S)	Ito *et al*., 2004
Millewanins C **(122)**	*M. taiwaniana* (S)	Ito *et al*., 2004

Isoflavone	**Source (plant part)**	**Reference**
Millewanins D **(123)**	*M. taiwaniana* (S)	Ito *et al*., 2004
Millewanins E **(124)**	*M. taiwaniana* (S)	Ito *et al*., 2004
Warangalone **(125)**	*M. taiwaniana* (S)	Ito *et al*., 2004
8-γ,γ-dimethylallylwighteone **(126)**	*M. taiwaniana* (S)	Ito *et al*., 2004
5.7.4'-trihydroxy-3',5'-di-methylallylisoflavone **(127)**	*M. taiwaniana* (S)	Ito *et al*., 2004
Norisojamaicin **(128)**	*M. usaramensis* (SB)	Yenesew, 1997a
Toxicaroliisoflavone **(129)**	*M. usaramensis* (SB) *M. brandisiana* (LF)	Yenesew, 1997a Pancharoen *et al*., 2008

Key:

AP	Ariel Part	SB	Stem bark
HW	Heartwood	SD	Seeds
LF	Leaf	SDP	Seedpods
RB	Root bark	VS	Vine stem
RT	Root	S	Stem



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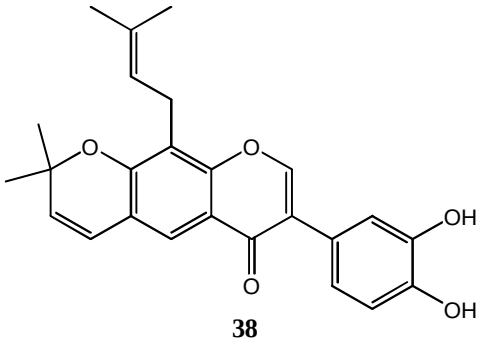

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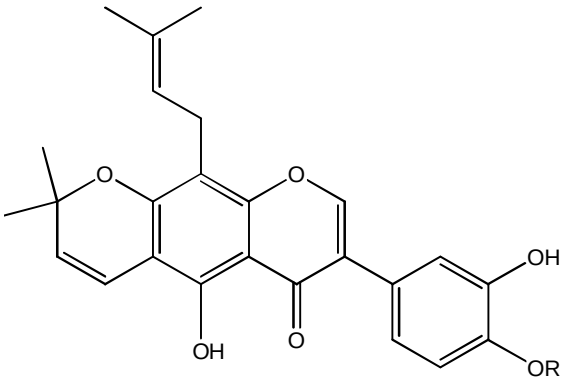
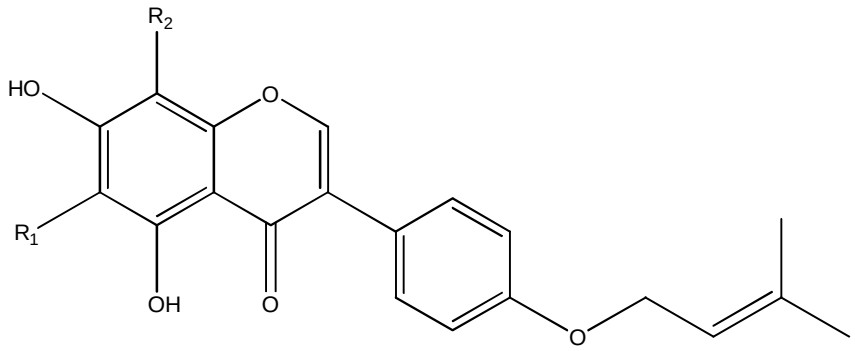
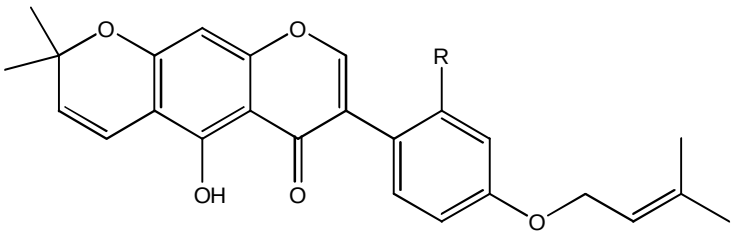


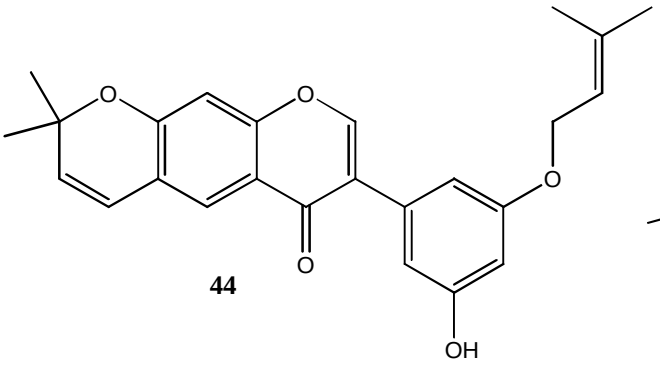
	R
39	**H**
40	**Me**



	R_1	R_2
41	**H**	**OMe**
45	**OMe**	**H**



	R
42	**H**
43	**OH**
46	**Me**



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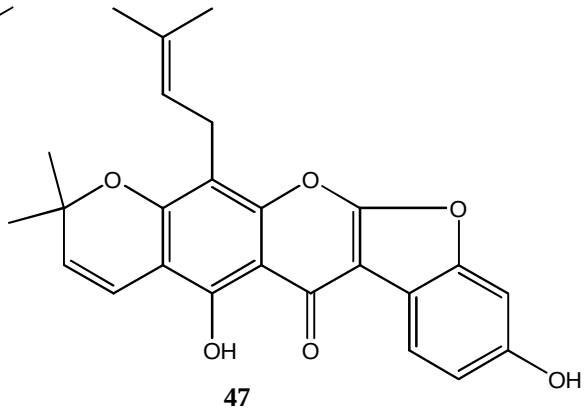

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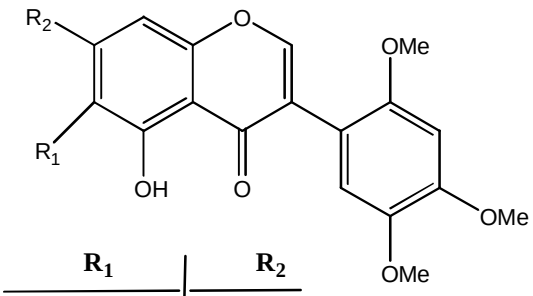
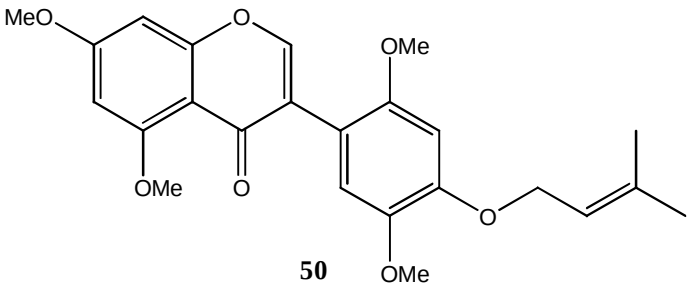


	R_1	R_2
48	**Prenyl**	**OH**
49	**H**	**OMe**



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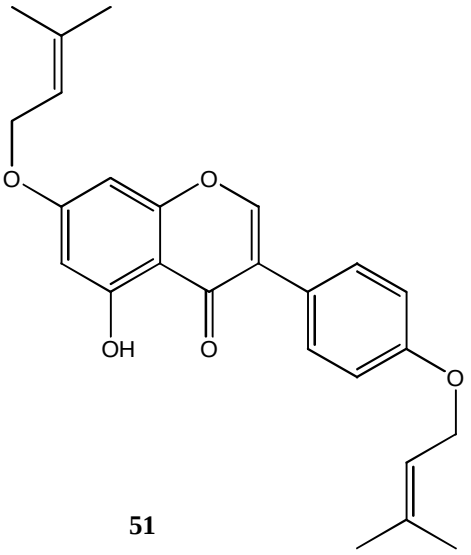

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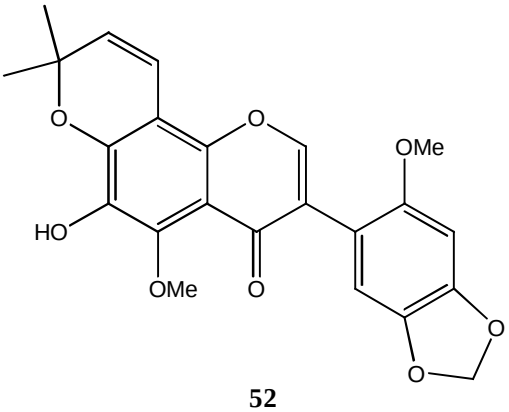

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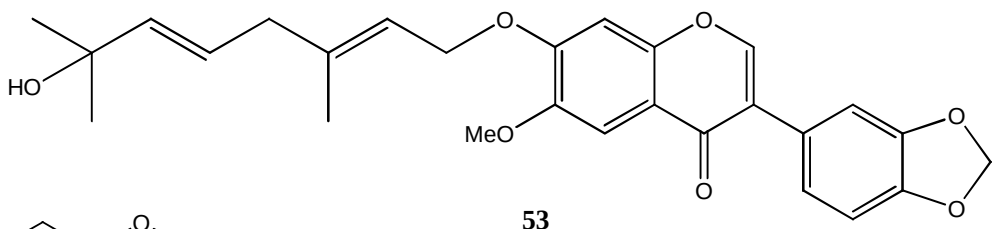

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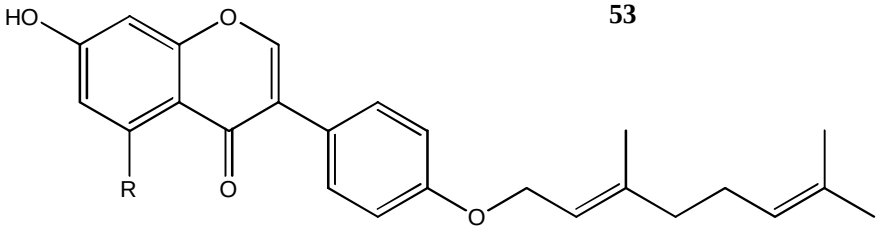
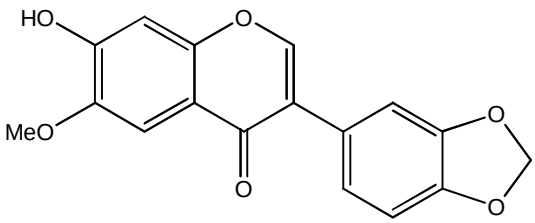


	R
54	**H**
55	**OH**



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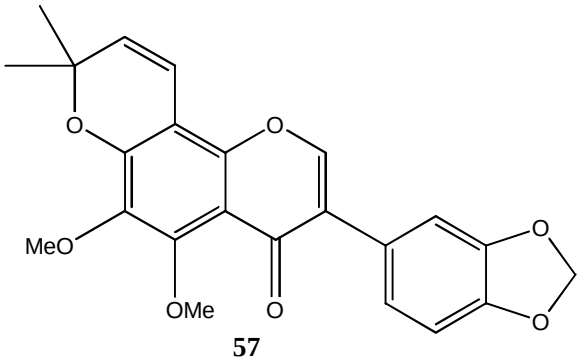

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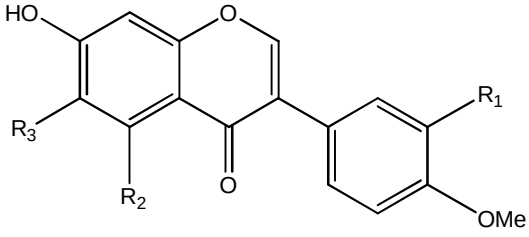
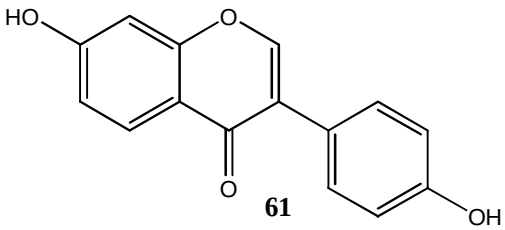


	R_1	R_2	R_3
58	**H**	**H**	**OMe**
59	**H**	**OH**	**H**
60	**OH**	**H**	**H**
65	**OH**	**H**	**OMe**



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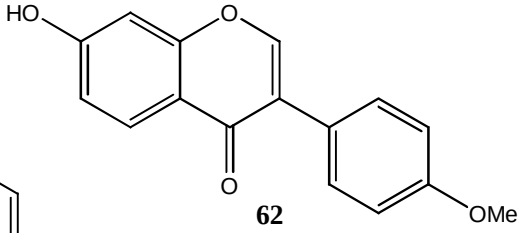

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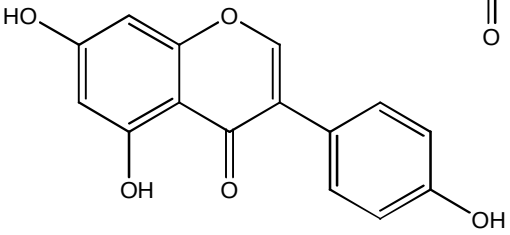

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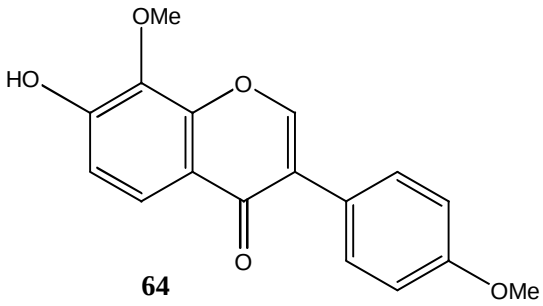

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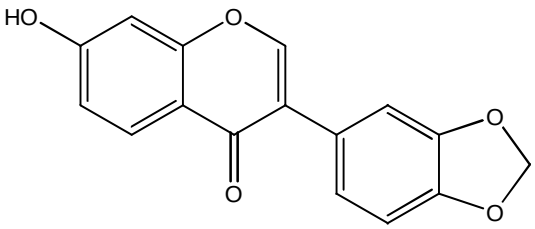

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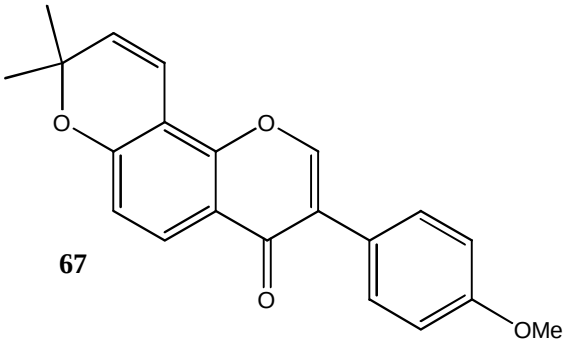

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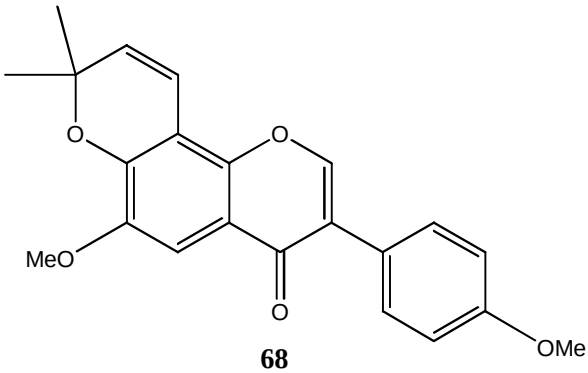

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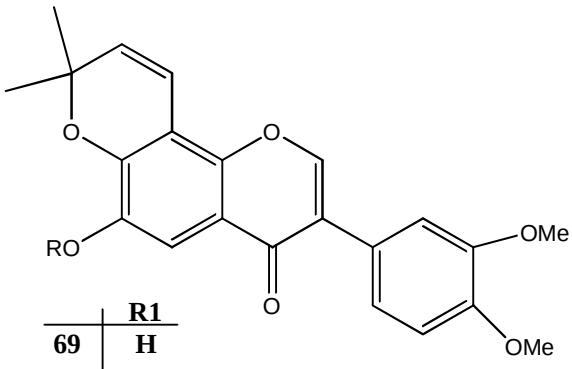
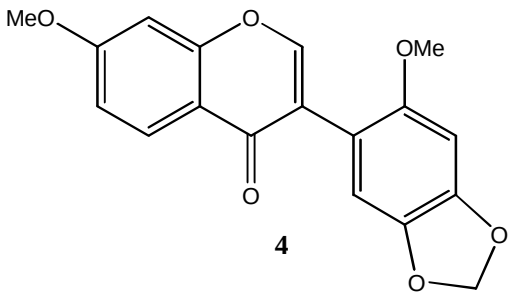


	R1
69	**H**
70	**Me**



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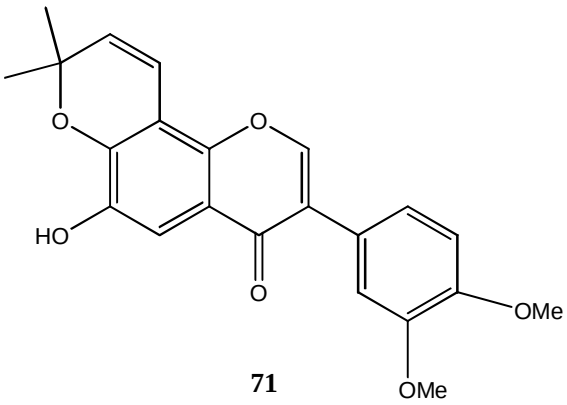

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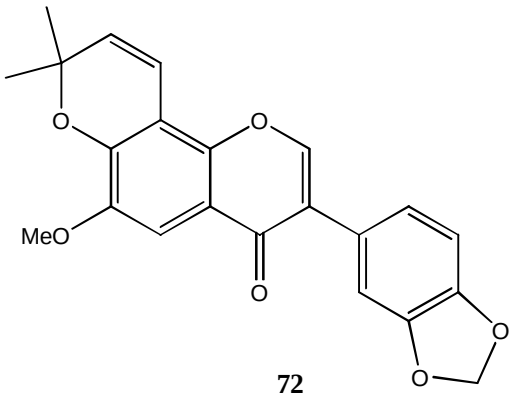

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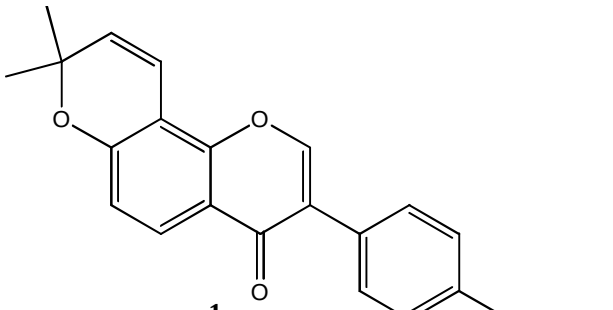

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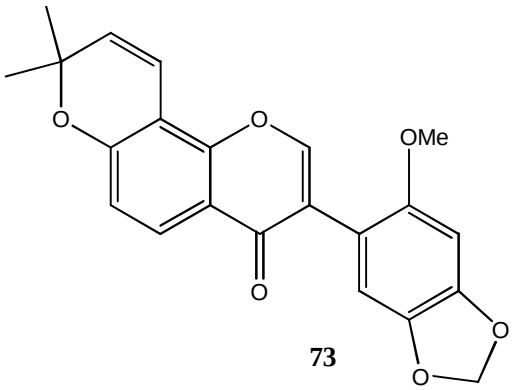

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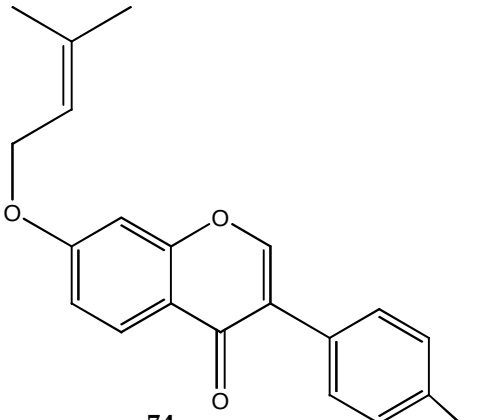

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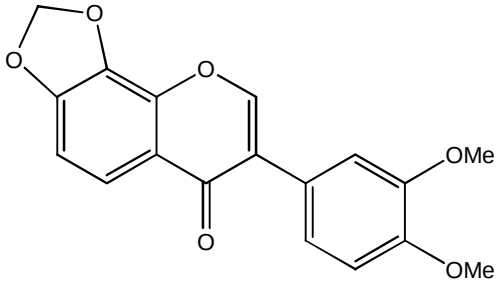

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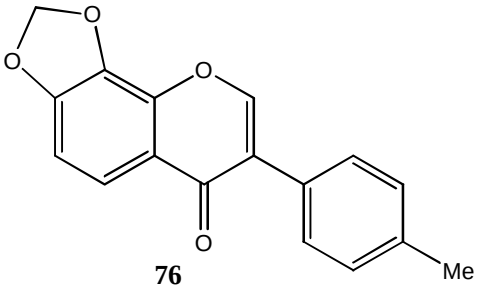

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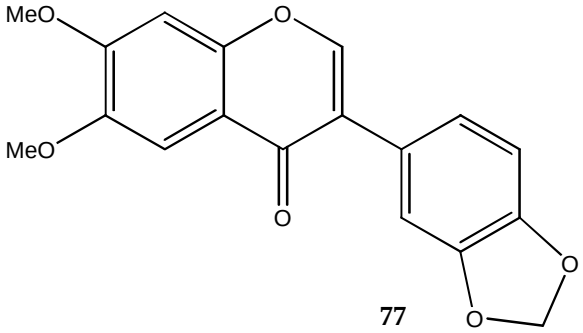

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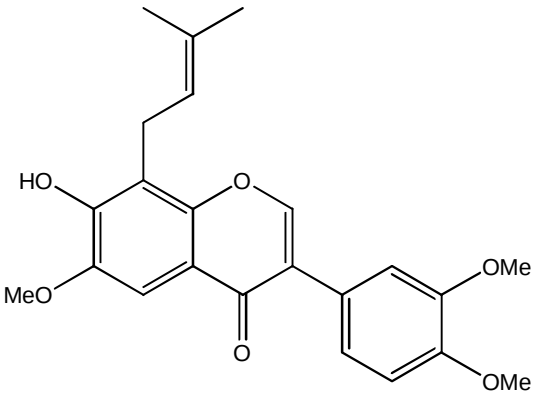

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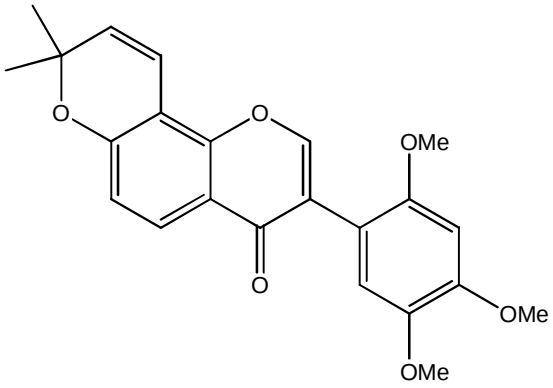

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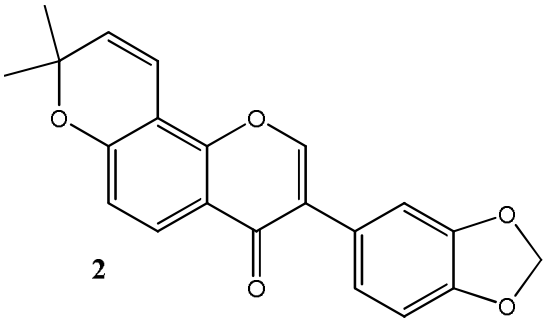

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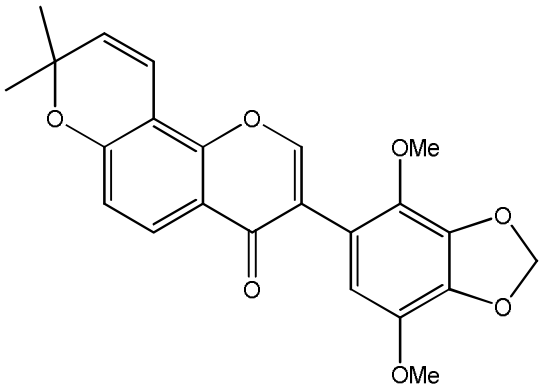

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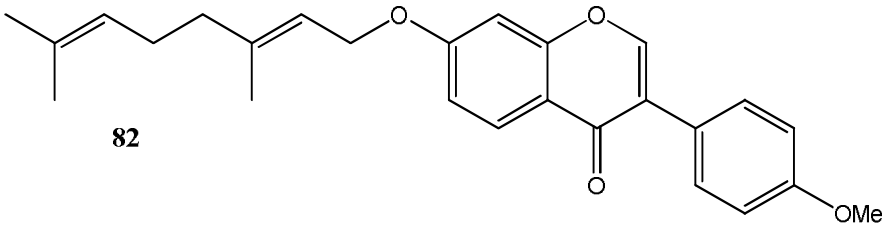

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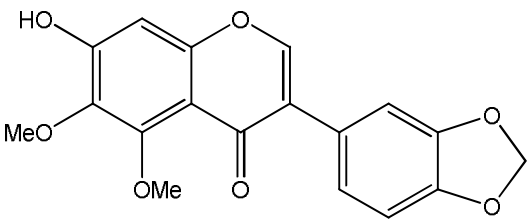

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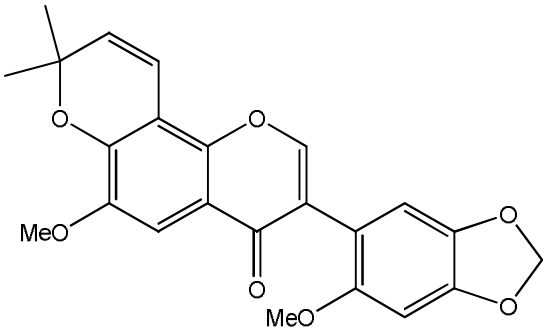

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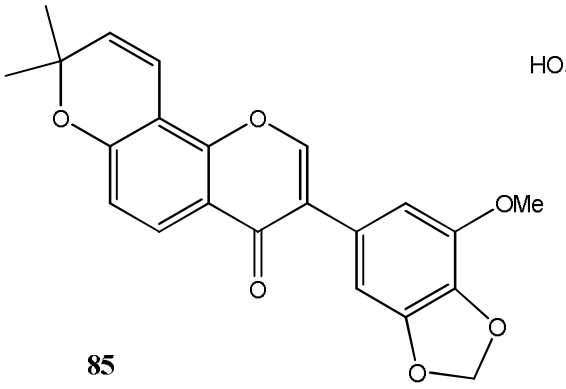

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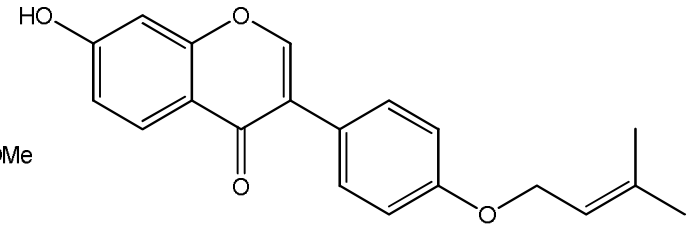

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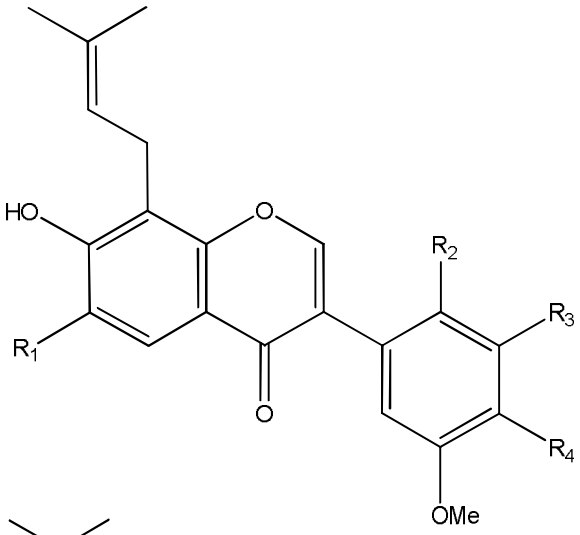
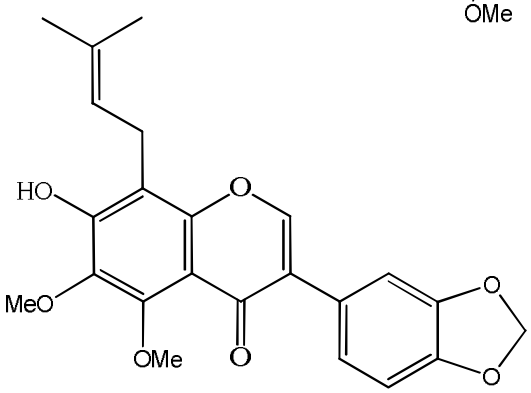


	R1	R2	R3	R4
87	**H**	**OMe**	**H**	**OMe**
88	**OMe**	**H**		**OCH_2O**
89	**H**	**OMe**		**OCH_2O**



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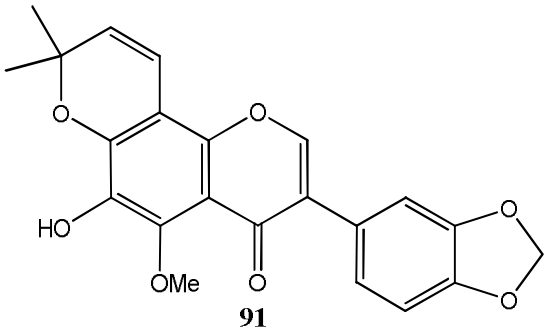

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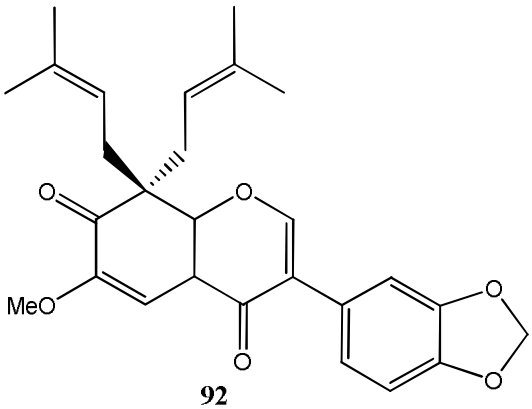

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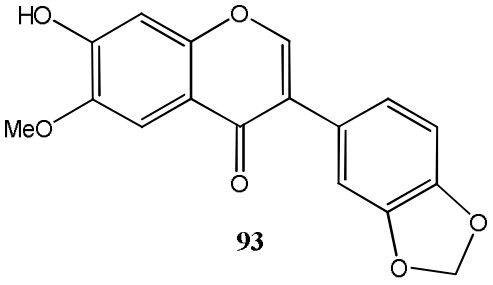

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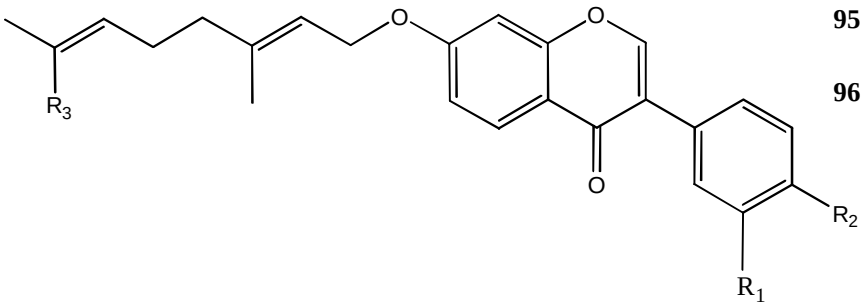
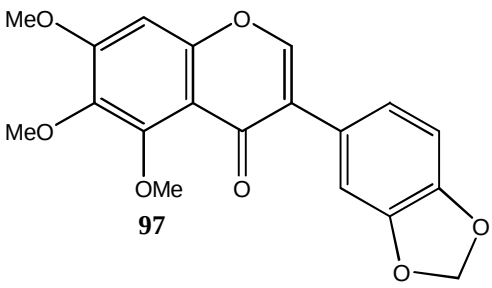


	R_1	R_2	R_3
94	**OH**	**OH**	**Me**
95	**H**	**OMe**	**CH_2OH**
96		**OCH_2O**	**H**



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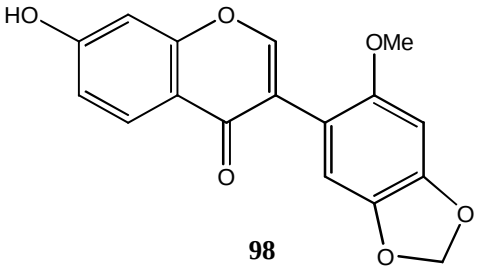

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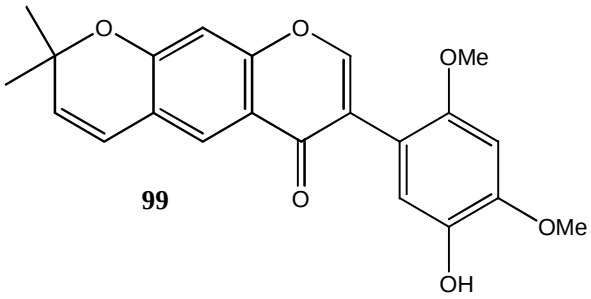

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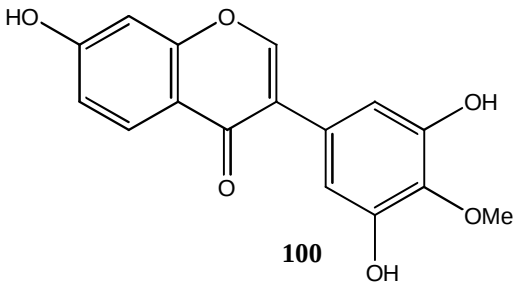

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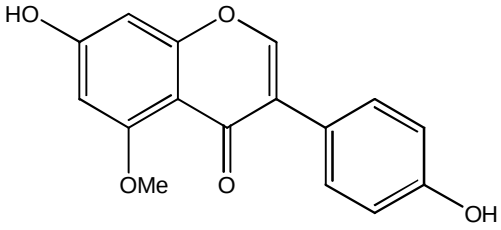

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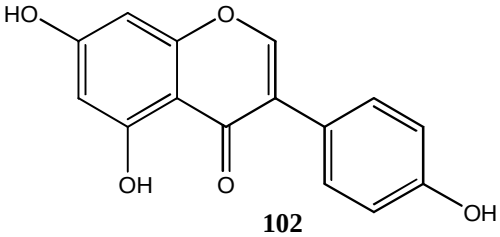

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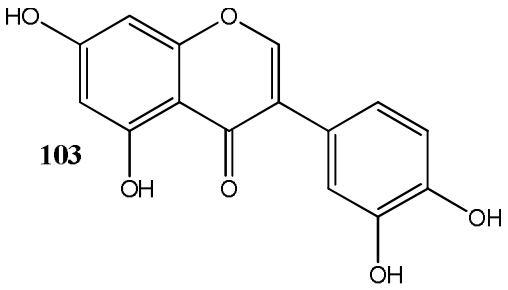

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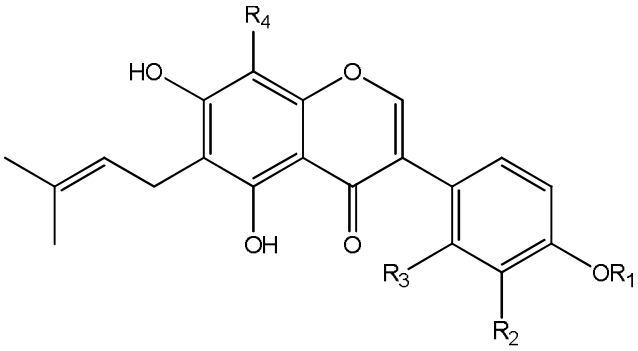
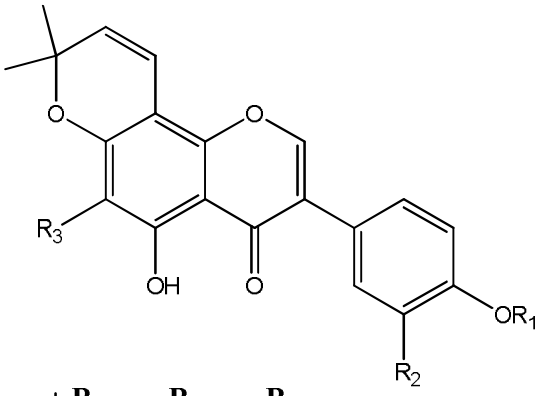
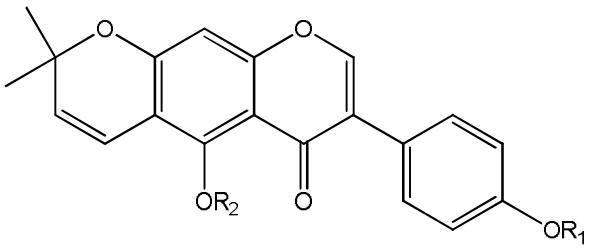


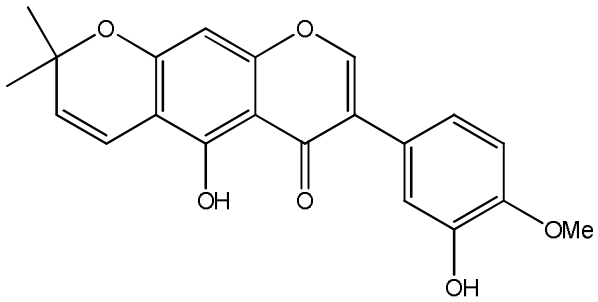


	R_1	R_2	R_3
104	**Me**	**H**	**H**
109	**H**	**OH**	**Prenyl**

	R_1	R_2	R_3	R_4
105	**H**	**OH**	**H**	**Prenyl**
106	**H**	**Prenyl**	**H**	**H**
107	**H**	**H**	**H**	**Prenyl**
108	**Me**	**OH**	**H**	**Prenyl**
110	**H**	**Prenyl**	**OH**	**H**
111	**H**	**Prenyl**	**OMe**	**H**



	112	**113**	**115**	**116**	**117**
R1	**H**	**Me**	**H**	**Me**	**Prenyl**
R2	**H**	**Me**	**Me**	**H**	**Me**



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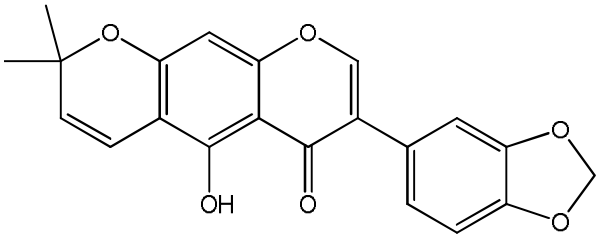

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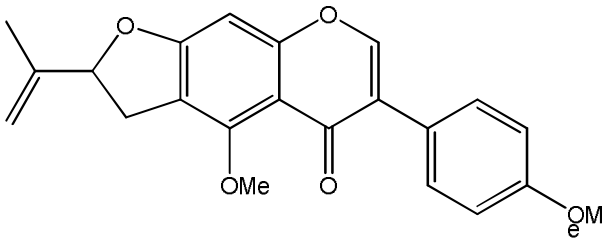

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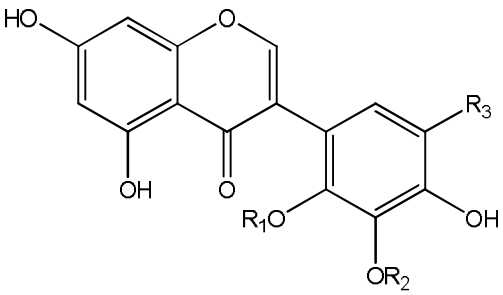
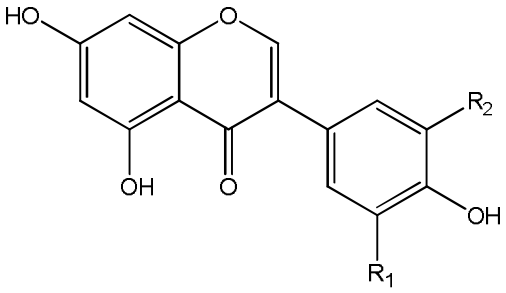
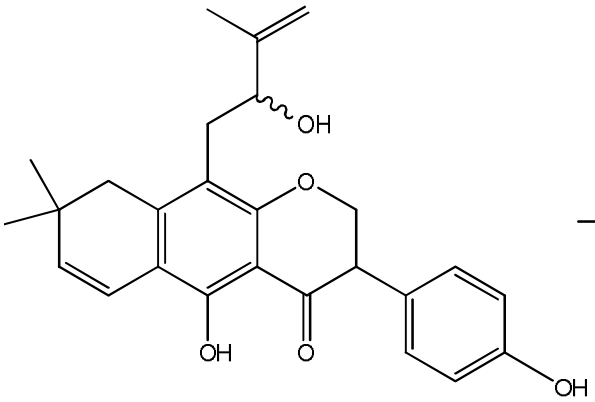


	R_1	R_2	R_3
120	**Prenyl**	**CH_3**	**Prenyl**
121	**Prenyl**	**CH_3**	**Geranyl**
122	**Prenyl**	**H**	**Geranyl**



	R_1	R_2
123	**Penyl**	**Geranyl**



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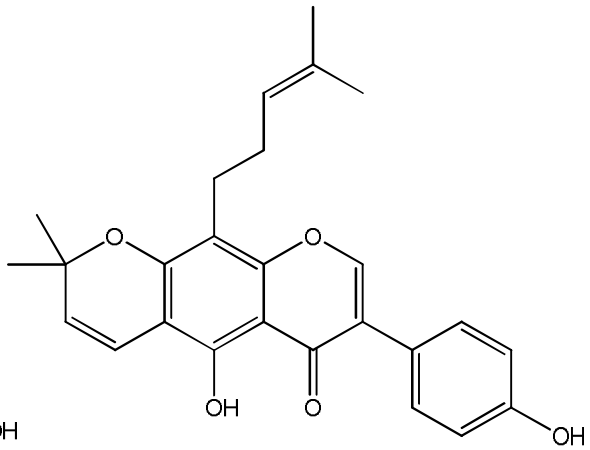

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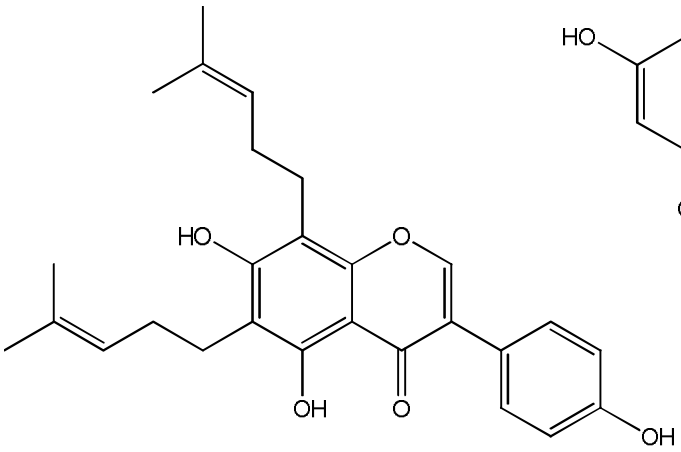

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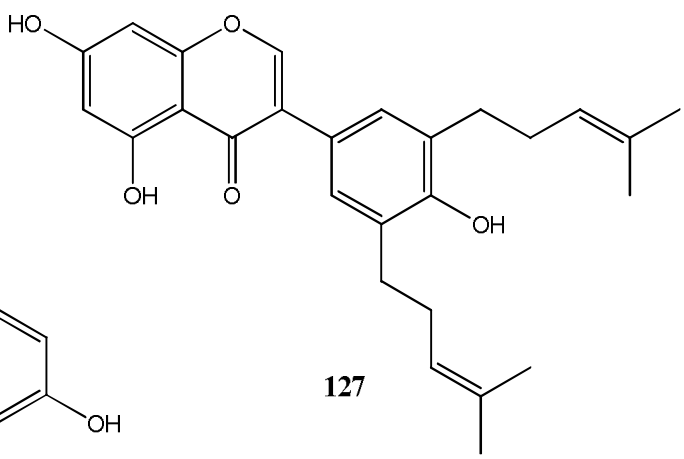

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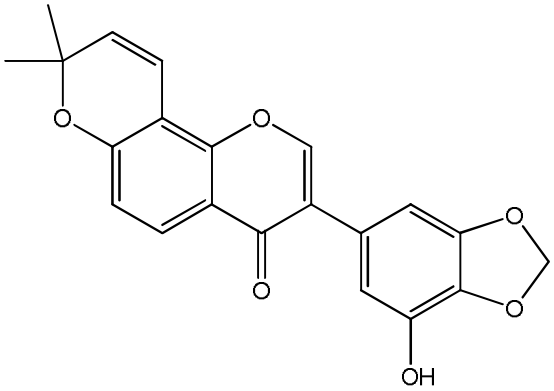

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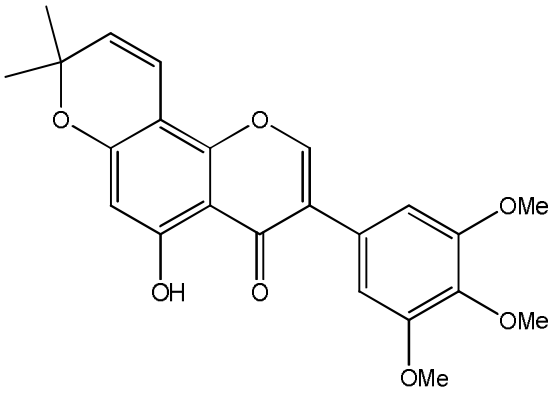

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2.8 FLAVONES AND ANTHOCYANINS OF *MILLETTIA*

Close to thirty flavones have so far been isolated from the genus *Millettia* and most of them posses a furan-ring, which is not a very common substituent in this genus. In all the cases the furan-ring is on ring A and mostly at 7, 8-position. The only exception to this is pongamol **(147)** from *M. penguensis* (Ganapatay *et al*., 1998) which has the furan-ring at 6, 7-position. Table 2.5 lists some of the flavones and anthocyanins of *Millettia*.

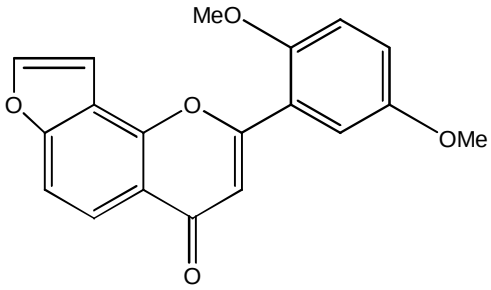
Table 2.5: Flavones and anthocyanins of *Millettia*

Compound	**Species**	**Reference**
Flavones		
Millettocalyxin C **(130)**	*M. erythrocalyx* (SB)	Sritularak *et al*., 2002a
Miilletttocalyxins A **(131)**	*M. erythrocalyx* (SB)	Sritularak *et al*., 2002a
Miilletttocalyxins B **(132)**	*M. erythrocalyx* (SB)	Sritularak *et al*., 2002a
Pongol methyl ether **(133)**	*M. erythrocalyx* (SB)	Sritularak *et al*., 2002a
Ovalifolin **(134)**	*M. erythrocalyx* (SB)	Sritularak *et al*., 2002a
Pongaglabrone **(135)**	*M. erythrocalyx* (SB)	Sritularak *et al*., 2002a
3',5'-dimethoxy-[2",3":7,8]-furanoflavones **(136)**	*M. erythrocalyx* (LF)	Kittisak et al., 2005
6,3′-dimethoxy-[2″,3″:7,8]-furanoflavones **(137)**	*M. erythrocalyx* (SD)	Sritularak and Kittisak, 2006
3,6-Dimethoxyfuranol[7,8:2'',3'']flavones **(138)**	*M. ichthyochtona* (LF)	Kamperdick *et al*., 1998
Laurentinol **(139)**	*M. laurenti* (FL)	Kamnaing *et al*., 1999
Karanjone **(140)**	*M. ovalifolia* (SD)	Gupta and Krishnamurti 1976a
Karanjin **(141)**	*M. ovalifolia* (SD)	Gupta and Krishnamurti 1976a
Lanceolatin **(142)**	*M. ovalifolia* (SD) *M. nitida* (VS)	Gupta and Krishnamurti 1976a Xiang et al., 2009

Compound	Species	Reference
Pongamol **(143)**	*M. penguensis* (LF)	Ganapatay *et al*., 1998
Pongaglabol **(144)**	*M. penguensis* (LF)	Ganapatay *et al*., 1998
Sanganone **(145)**	*M. sangana* (RB)	Mbafor *et al*., 1995.
5-Methoxyfurano[7,8:2'',3''] flavones **(146)**	*M. sangana* (LF)	Mbafor *et al*., 1995
Astragalin **(147)**	*M. Zechiana* (AP)	Parvez and Ogbeide, 1990
3-Hydroxy-4'-methoxyflavone **(148)**	*M. Zechiana* (AP)	Parvez and Ogbeide, 1990
3-0-α-L-rhamnosekampferol **(149)**	*M. Zechiana* (AP)	Parvez and Ogbeide, 1990
Quercitrin **(150)**	*M. Zechiana* (AP)	Parvez and Ogbeide, 1990
Isoquercitrin **(151)**	*M. Zechiana* (AP)	Parvez and Ogbeide, 1990
7-O-β-D-glucoside-8-hydroxyquercetin **(152)**	*M. Zechiana* (FL)	Ogbeide and Parvez, 1992
3-Methyletherquercetin **(153)**	*M. Zechiana* (FL)	Ogbeide and Parvez, 1992
Anthocyanins		
Cyanin **(154)**	*M. Zechiana* (AP)	Parvez and Ogbeide, 1990
3**,**5-Di-O-β-D-glucosidemalvidin **(155)**	*M. Zechiana* (AP)	Parvez and Ogbeide, 1990
3-0-α-L-rhamnosepelargonidin **(156)**	*M. Zechiana* (AP)	Parvez and Ogbide, 1990

Key:

AP	Ariel Part	SB	Stem bark
FL	Flower	SD	Seeds
LF	Leaf	RB	Root bark
VS	Vine stem		



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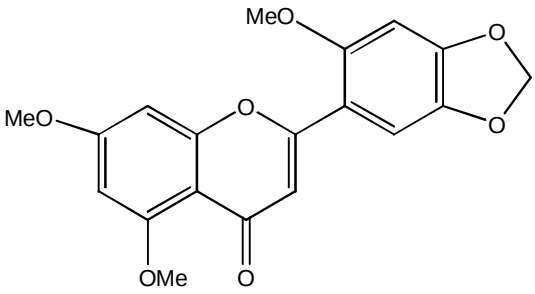

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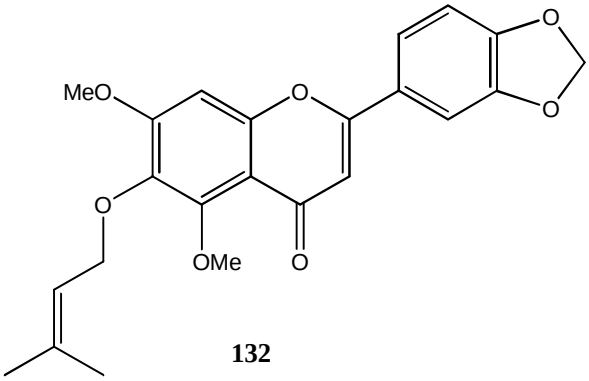

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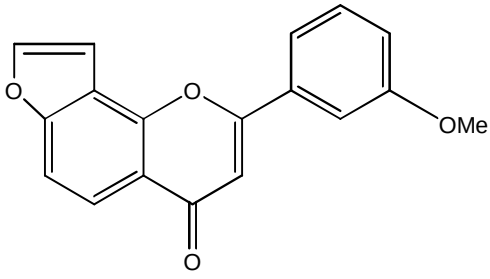

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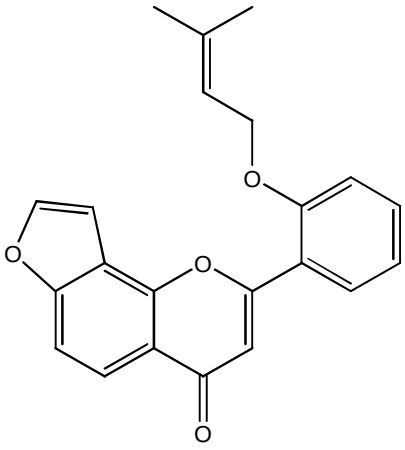

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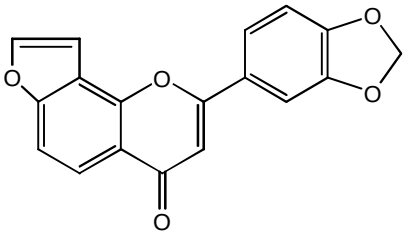

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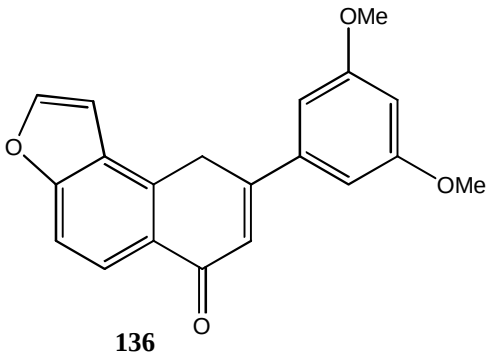

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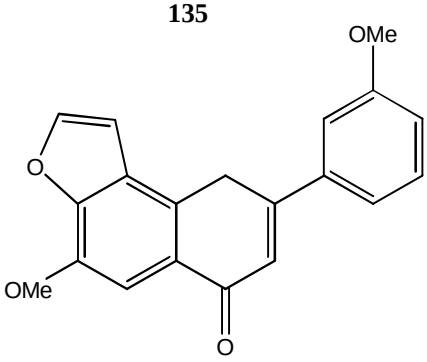

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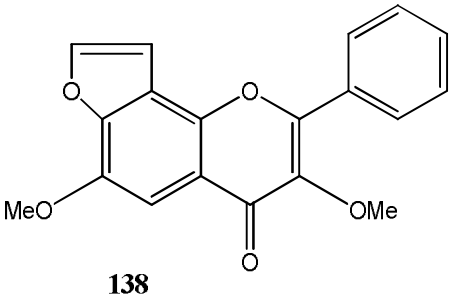

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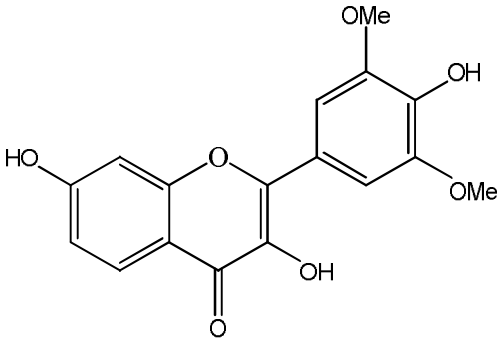

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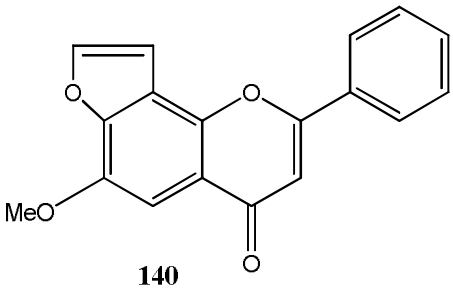

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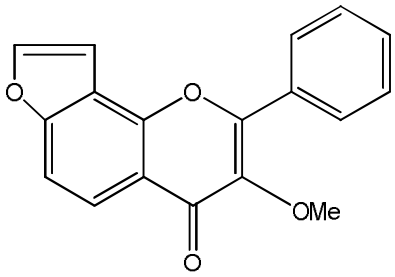

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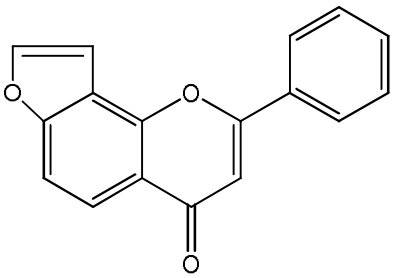

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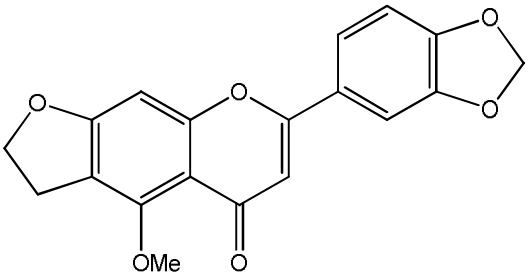

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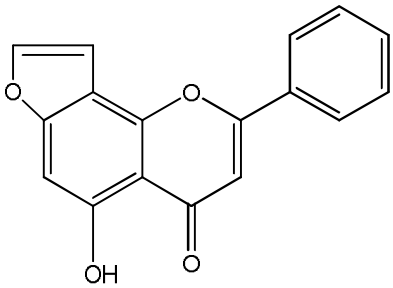

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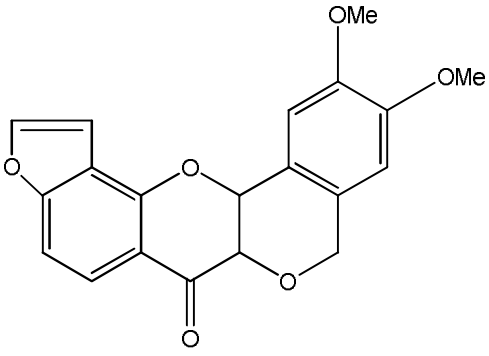

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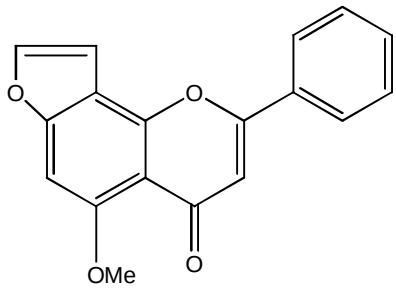

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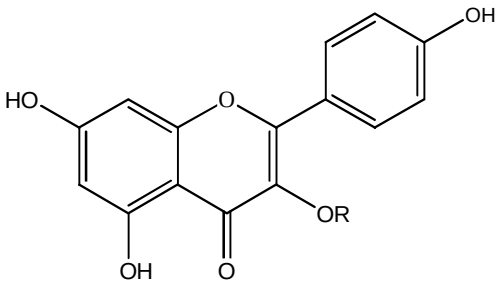
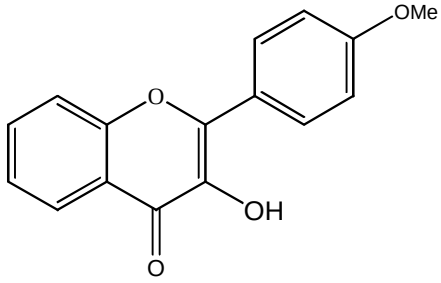


	R
147	**α-D-Glucose**
149	**α-L-Rhamnose**



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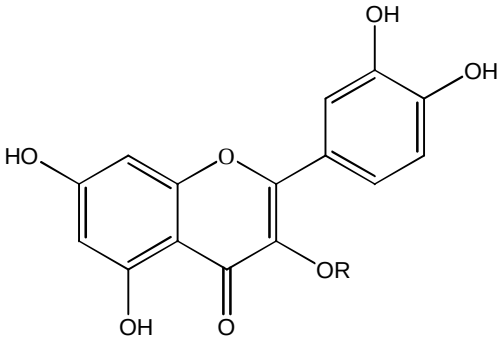
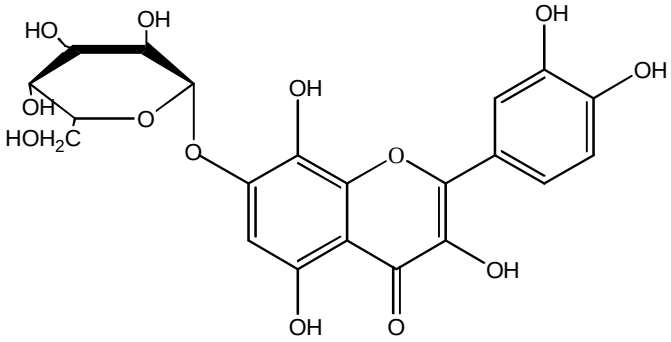


150 R=α-L-Rhamnose
151 R=β-D-Glucose



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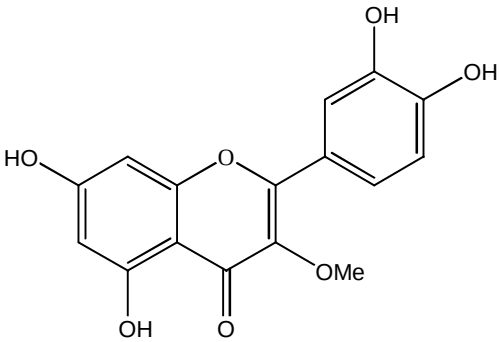

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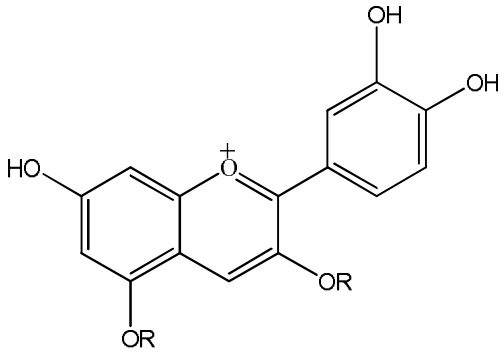
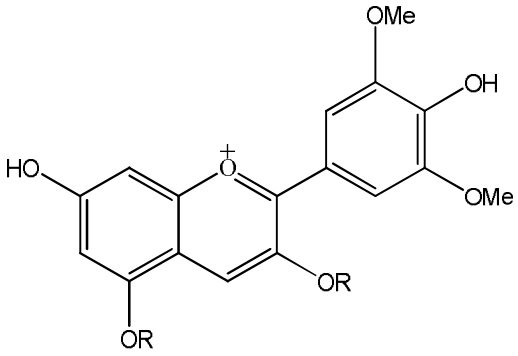
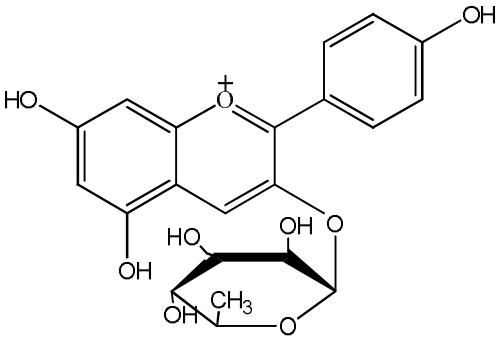


154 R=β-D-Glucose



155 R= β-D-Glucose



156

2.8.1 Flavanones of *Millettia*

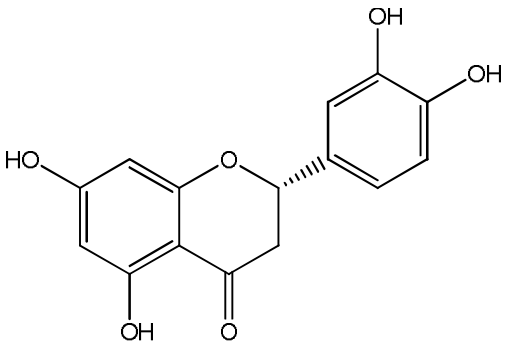
All the flavanones so far characterized from the genus *Millettia* are prenylated and lack oxygenation at C-5 position. This is considered to be typical of the flavonoids of the family Fabaceae (Hagnaeuer and Grayer-Barkmeijer, 1993). Table 2.6 lists some of the flavanones reported from *Millettia*.

Table 2.6: Flavanones reported from *Millettia*

Flavanones	**Source (plant part)**	**Reference**
Eriodictyol (**157)**	*M. duchesnei* (AP)	François *et al*., 2008
(−)-(2*S*)-6,3′,4′-trimethoxy-[2″,3″:7,8]-furanoflavanone **(158)**	*M. erythrocalyx* (SD)	Sritularak and Kittisak, 2006
6-Methoxy-[7,8:2'',3'']furanoflavanone **(159)**	*M. erythrocalyx* (RB)	Sritularak *et al*., 2002a
Ponganone **(160)**	*M. erythrocalyx* (RB)	Sritularak *et al*., 2002a
7-Prenyloxyflavanone **(161)**	*M. erythrocalyx* (RB)	Sritularak *et al*., 2002a
4'-Hydroxyisolonchocarpin **(162)**	*M. ferrugineae* (SB)	Dagne *et al*., 1989
Ovaliflavanone A **(163)**	*M. ovalifolia* (SD)	Gupta and Krishnamurti 1976a
Ovaliflavanone B **(164)**	*M. ovalifolia* (SD)	Gupta and Krishnamurti 1976a
Ovaliflavanone C **(165)**	*M. ovalifolia* (SD)	Islam *et al*., 1980
Ovaliflavanone D **(166)**	*M. ovalifolia* (SD)	Islam *et al*., 1980
7-Hydroxy-3',4'-methylenedioxyflavanone **(167)**	*M. ovalifolia* (SD)	Islam *et al*., 1980
ovalichromene **(168)**	*M. ovalifolia* (SD)	Gupta and Krishnamurti 1976b
Ovalichromene A **(169)**	*M. ovalifolia* (SD)	Gupta and Krishnamurti,1980
Ovalichromene B **(170)**	*M. ovalifolia* (SD)	Gupta and Krishnamurti,1980
Milletenin A **(171)**	*M. ovalifolia* (LF)	Khan and Zaman ,1974
Milletenin B **(172)**	*M. ovalifolia* (LF)	Khan and Zaman, 1974
Isolonchocarpin **(173)**	*M. ovalifolia* (SD)	Krishnamurti *et al*., 1987
Sophoranone **(174)**	*M. pulchra* (AP)	Baruah *et al*., 1984

Key:

AP	Ariel Part	SB	Stem bark
LF	Leaf	SD	Seeds
RB	Root bark		



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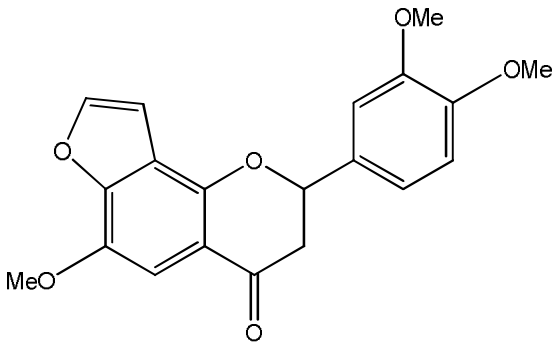

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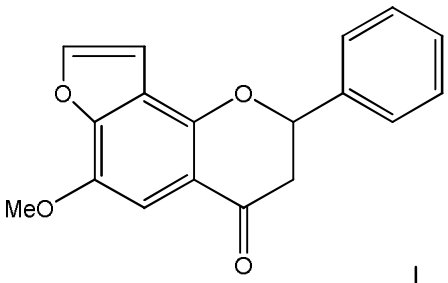

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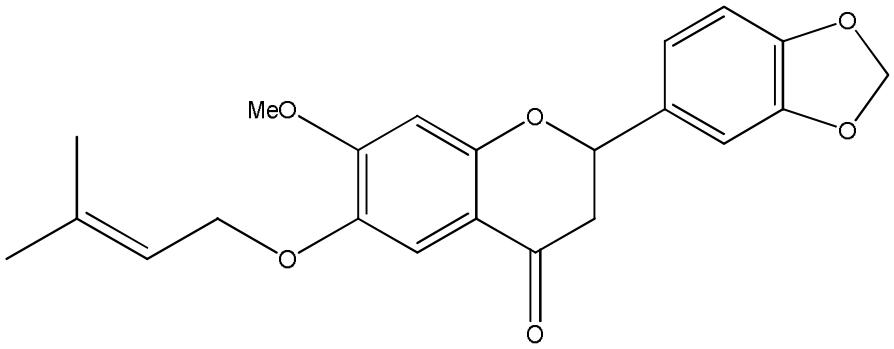

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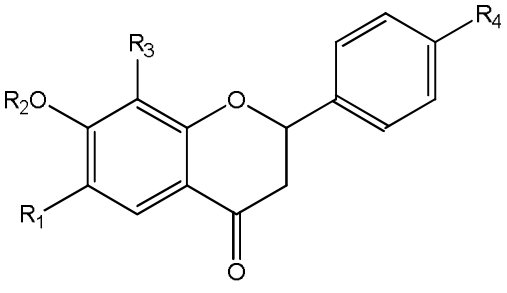
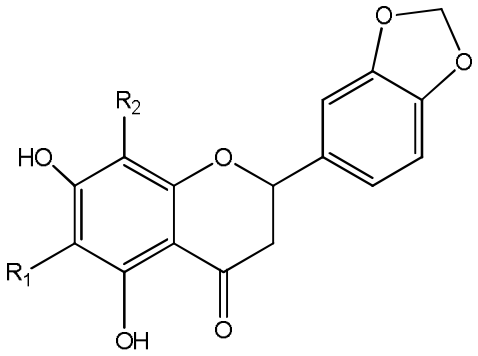
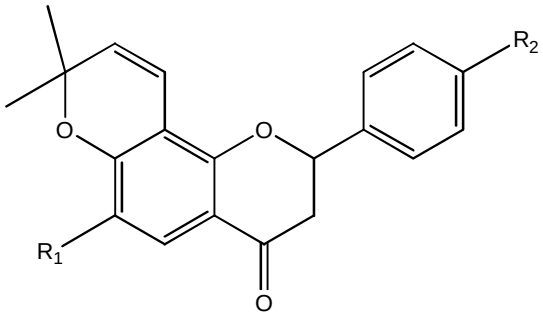


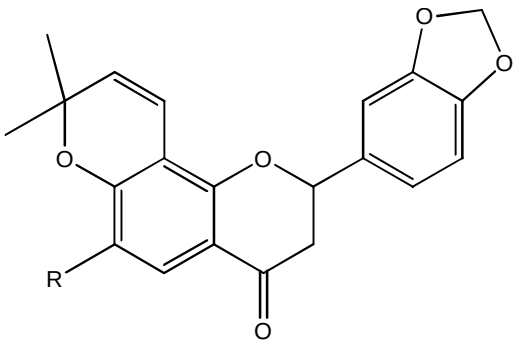
	R_1	R_2	R_3	R_4
161	**H**	**Prenyl**	**H**	**H**
163	**Prenyl**	**H**	**Prenyl**	**H**
164	**H**	**H**	**Prenyl**	**H**



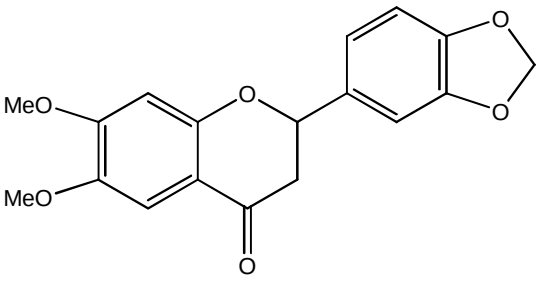
	165	**166**	**167**
R_1	**H**	**Prenyl**	**H**
R_2	**Prenyl**	**Prenyl**	**H**



	162	168	173
R_1	H	Prenyl	H
R_2	OH	OH	H



	R
169	OMe
170	H



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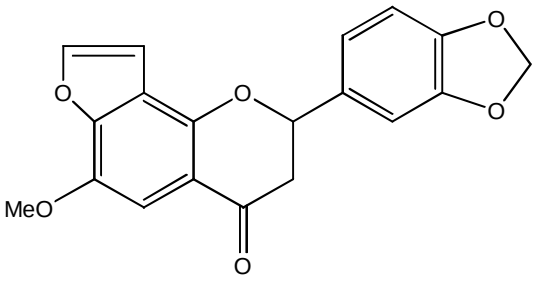

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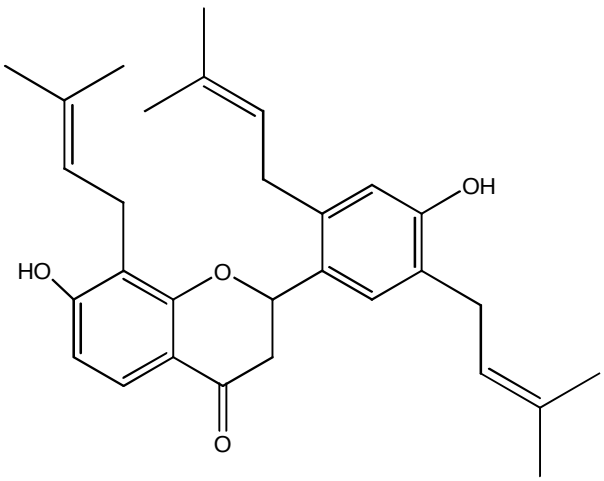
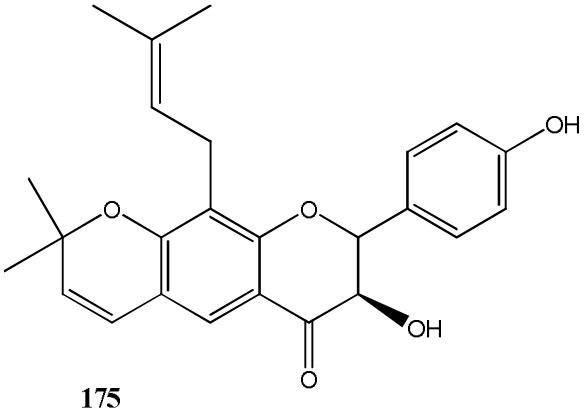


174

2.8.2 Flavanonols from *Millettia*

Three flavanonols namely Lupinifolol **(175)** from *M. pachycarpa* (Singhal *et al*., 1981), 7, 4'-Dihydroxy-8, 3', 6'-triprenyldihydroflavanol **(176)** and 7, 4'-Dihydroxy 8, 3', 5'-triprenyldihydroflavanol **(177)** from *M. pulchra* (*Baruah et al*., 1984) have been isolated from the *Millettia* genus.



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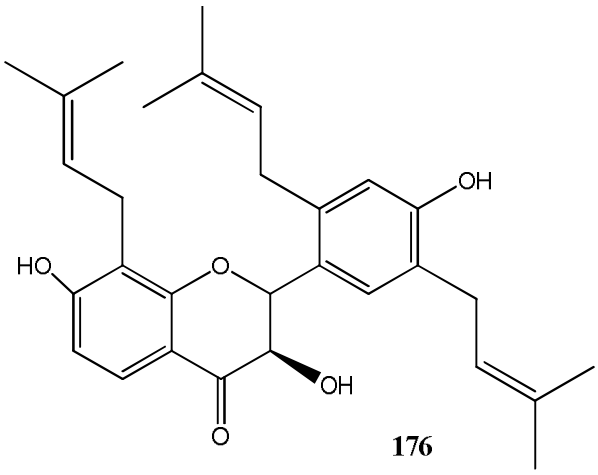

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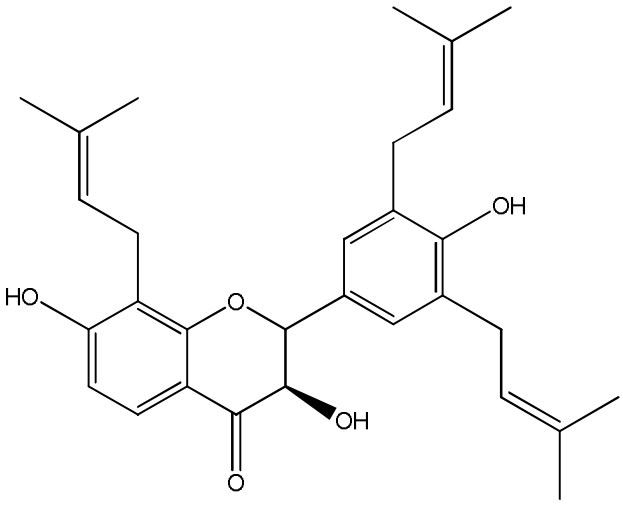


177

2.8.3 Chalcones of *Millettia*

Most chalcones isolated from M. ovalifolia have a methylenedioxy group incorporated in their structures. In addition several chalcones of this genus are prenylated (Gupta and Krishnamurti, 1977b; 1980). Table 2.7 lists some chalcones of Millettia species.

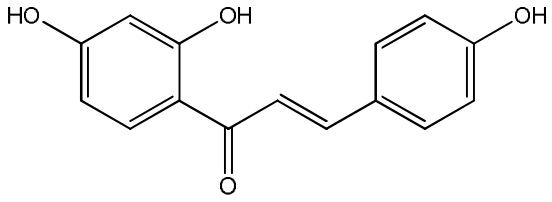
Table 2.7: Chalcones of *Millettia*

Chalcones	**Source (Plant part)**	**Reference**
2',4',4-Trihydroxychalcone **(178)**	*M. dielsiana* (SB)	Wang *et al*., 1990
4-Hydroxyderricidin **(179)**	*M. dielsiana* (SB)	Sritularak *et al*., 2002a
2′-hydroxy-3,4-dimethoxy-[2″,3″:4′,3′]-furanochalcone **(180)**	*M. erythrocalyx* (SDP)	Sritularak and Kittisak, 2006
2′,3-dihydroxy-4-methoxy-4′-γ,γ-dimethylallyloxychalcone (**181)**	*M. erythrocalyx* (SDP)	Sritularak and Kittisak, 2006
Derricidin **(182)**	*M. erythrocalyx* (RB)	Sritularak *et al*., 2002a
2'-Hydroxy-3,4-methylenedioxy-4'-γ,γ-dimethylallyloxychalcone **(183)**	*M. erythrocalyx* (RB)	Sritularak *et al*., 2002a
Ponganone (**184)**	*M. erythrocalyx* (RB)	Sritularak *et al*., 2002a
3,4-methylenedioxy-2',4'-dimethoxychalcone **(185)**	*M. erythrocalyx* (RB)	Sritularak *et al*., 2002b
Purperenone **(186)**	*M. erythrocalyx* (RB)	Sritularak *et al*., 2002b
4'-Hydroxylonchocarpin (**4)**	*M. ferruginea* (SB)	Dagne *et al*., 1989
4'-O-Geranylisoliquiritigenin **(188)**	*M. ferruginea* (RB) *M. griffoniana* (RB) *M. usaramensis* (SB)	Dagne *et al*., 1990b Yankep *et al*., 1997 Yenesew, 1997a
Dihydromilletenone,methyl ether **(189**)	*M. hemsleyana* (SB)	Mahmoud and Waterman, 1985
Dihydroisomilletenone,methylether **(190)**	*M. hemsleyana* (SB)	Mahmoud and Waterman, 1985

Chalcones	**Source (Plant part)**	**Reference**
Ovalichalcone **(191)**	*M. ovalifolia* (SD)	Gupta and Krishnamurti, 1977a
Ovalichalcone A **(192)**	*M. ovalifolia* (SD)	Gupta and Krishnamurti, 1980
Ovalitenin A **(193)**	*M. ovalifolia* (SD)	Gupta and Krishnamurti, 1977b
Ovalitenin B **194)**	*M. ovalifolia* (SD)	Gupta and Krishnamurti, 1977b
Ovalitenin C **(195)**	*M. ovalifolia* (SD)	Gupta and Krishnamurti 1980
Ovalitenone **(196)**	*M. ovalifolia* (SD)	Gupta and Krishnamurti 1977b
Pongamol **(197)**	*M. ovalifolia* (RB)	Saxena *et al*., 1987
Milletenone **(198)**	*M. ovalifolia* (SD) *M. ovalifolia* (LF)	Saxena *et al*., 1987
4-Methoxylonchocarpin **(199)**	*M. pachycarpa* (SD)	Singhal, 1983
4'-Geranyloxy-α,4,2'-trihydroxydihydrochalcone **(200)**	*M. usaramensis* (SB)	Yenesew, 1997a

Key:

LF	Leaf	SB	Stem bark
RB	Root bark	SD	Seeds
		SDP	Seedpods



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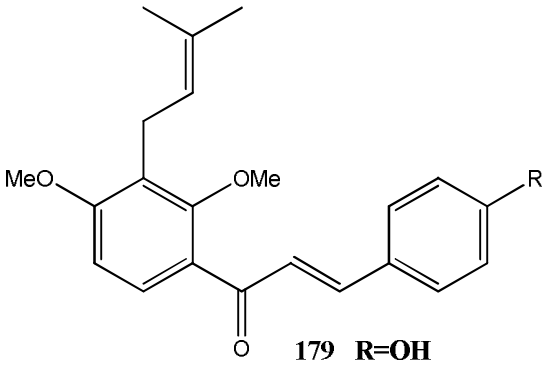
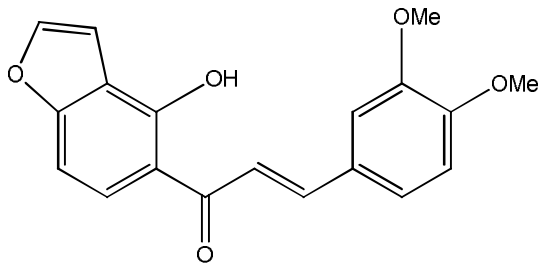


179 R=OH
182 R=H



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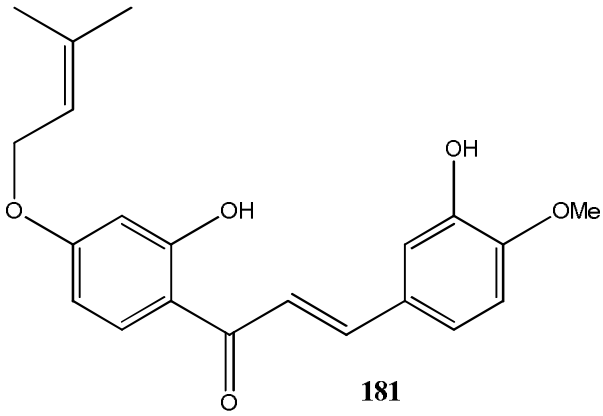

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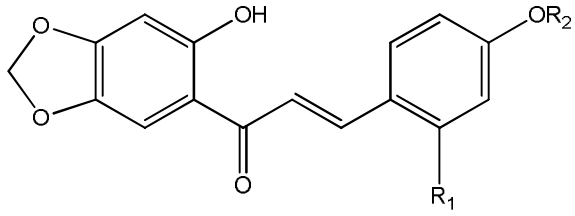
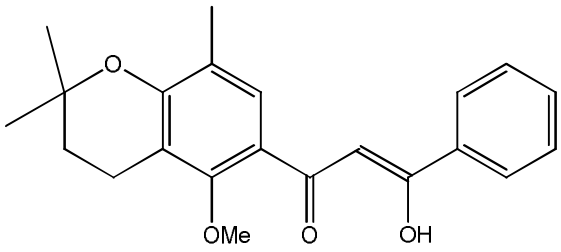
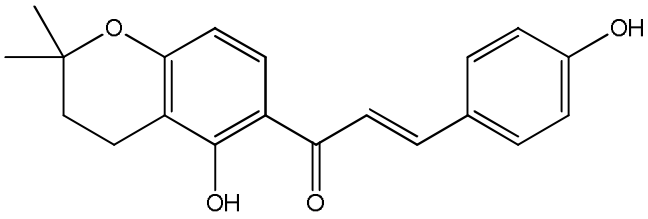


	183	185
R_1	H	Prenyl
R_2	OMe	H



	R
184	OMe
186	H



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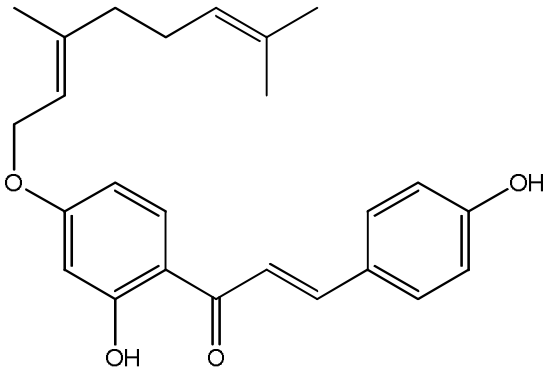

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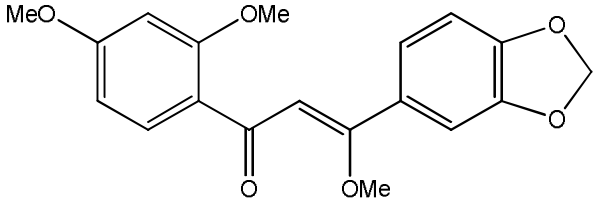

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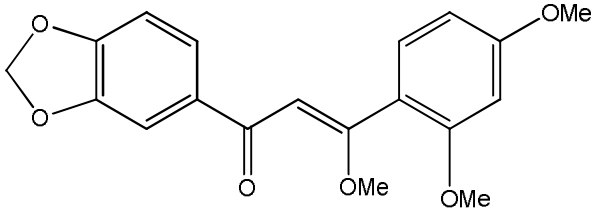

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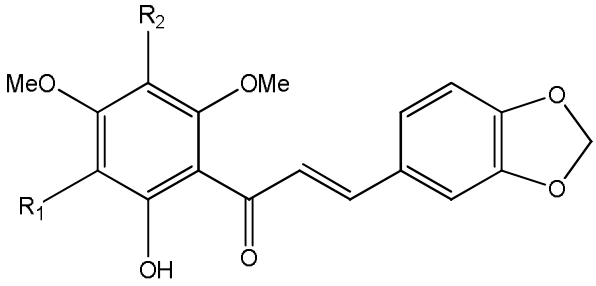
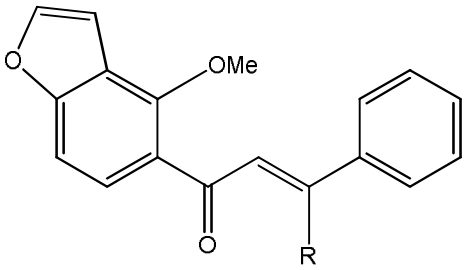
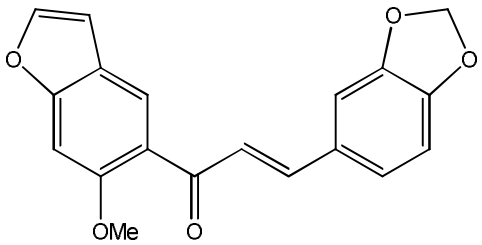


	R_1	R_2
191	**H**	**Prenyl**
192	**Prenyl**	**H**



	R
193	**H**
194	**OMe**



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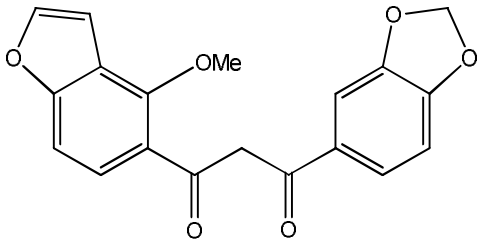

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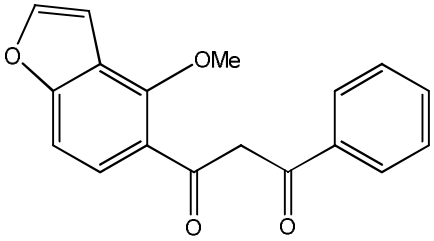

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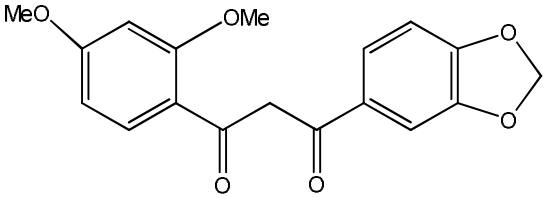

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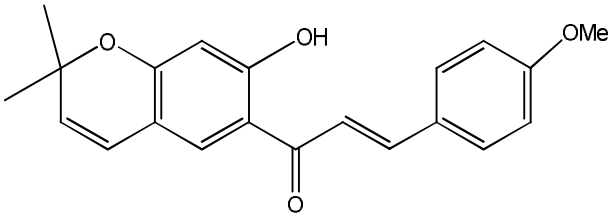

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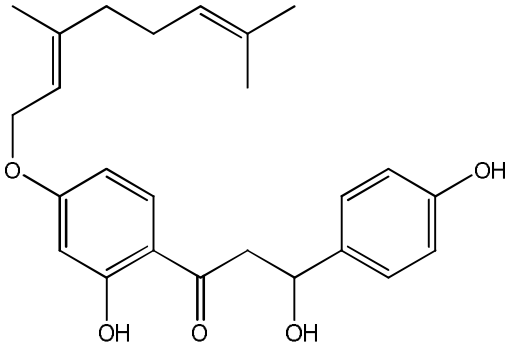

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2.8.4 Rotenoids of Millettia

Rotenoids mainly occur in the seeds of *Millettia* as opposed to isoflavones that occur in all plant parts. Most rotenoids previously characterized from this genus have a cis-B/C ring junction as demonstrated by rotenone (**220**) and 12a-hydroxyrotenone **(218)**. Nevertheless, rotenoids from the stem bark of *M. usaramensis* have a novel trans-B/C ring junction with a 6aR, 12aS configuration (Yenesew *et al.*, 1998). Rot-2'-enoic acid **(231)** has been shown to be an intermediate in the biosynthetic pathway of other rotenoids (Crombie *et al.*, 1979; 1982). Table 2.8 lists some rotenoids reported from the genus *Millettia.*

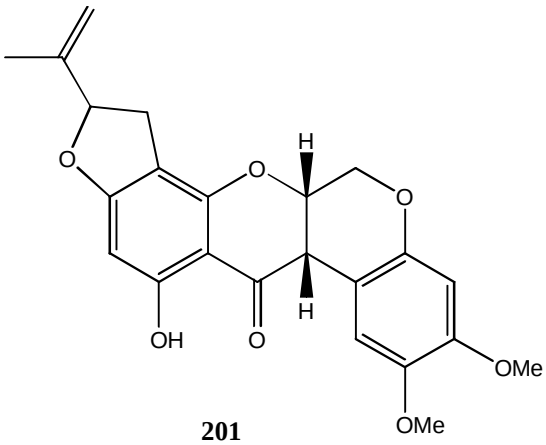
Table 2.8: Rotenoids of *Millettia*

Rotenoids	**Source (plant part)**	**Reference**
Sumatrol **(201)**	*M. auriculata* (SD)	Shabbir *et al*., 1968
α-Toxicarol **(202)**	*M. brandisiana* (LF) *M. taiwaniana* (S)	Pancharoen *et al*., 2008 Ito *et al*., 2004
Sermundone **(203)**	*M. brandisiana* (LF)	Pancharoen *et al*., 2008
12a-Hydroxy-α-toxicarol (**204)**	*M. brandisiana* (LF)	Pancharoen *et al*., 2008
6-Deoxyclitoriacetal **(205)**	*M. brandisiana* (LF)	Pancharoen *et al*., 2008
6a,12a-Dehydro-α-toxicarol **(206)**	*M. brandisiana* (LF)	Pancharoen *et al*., 2008
6a,12a-Dehydrosermundone **(207)**	*M. brandisiana* (LF)	Pancharoen *et al*., 2008
6-Hydroxy-6a,12a-dehydro-α-toxicarol **(208)**	*M. brandisiana* (LF)	Pancharoen *et al*., 2008
Usararotenoid C **(209)**	*M. brandisiana* (LF)	Yenesew *et al*., 2003
6a, 12a-Dehydromillettone **(210)**	*M. brandisiana* (LF)	Yenesew *et al*., 2003
Stemonal **(211)**	*M. brandiasa* (LF)	Pancharoen et al., 2008
Millettone **(212)**	*M. dura* (SD)	Ollins *et al*., 1967
Millettosin **(213)**	*M. dura* (SD)	Ollins *et al*., 1967
12a-Hydroxyrotenone (**214)**	*M. dura* (SD)	Ollins *et al*., 1967
Deguelin **(215)**	*M. dura* (SD) *M. ferruginea* (SD) *M. usaramensis* (SD) *M. taiwaniana* *M. pachycarpa* (SD)	Ollins *et al*., 1967 Dagne *et al*., 1991 Yenesew *et al*., 1997b Ito *et al*., 2004 Haoyu *et al*., 2008
Rotenone **(216)**	*M. dura* (SD) *M. ferruginea* (SD) *M. pachycarpa* (SD)	Ollins *et al*., 1967 Dagne *et al*., 1991 Singhal *et al*., 1982
6a,12a-Dehydrodeguelin **(217)**	*M. dura* (SD) *M. duchesnei* (AP) *M. pachycarpa* (SD)	Ollins *et al*., 1967 François *et al*., 2008 Haoyu *et al*., 2008

Rotenoids	**Source (plant part)**	**Reference**
Tephrosin (**218)**	*M. dura* (SD) *M. ferruginea* (SD) *M.usaramensis* (SD) *M. griffoniana* (SD) *M. taiwaniana* *M. pachycarpa* (SD)	Ollins *et al*., 1967 Dagne *et al*., 1991 Yenesew *et al*., 1997b Ngamga *et al*., 2005 Ito *et al*., 2004 Haoyu *et al*., 2008
Elliptol **(219)**	*M. duchesnei* (AP)	François *et al*., 2008
12-Deoxo-12α-methoxyelliptone **(220)**	*M. duchesnei* (AP)	François *et al*., 2008
6-Methoxy-6a,12a-dehydrodeguelin **(221)**	*M. duchesnei* (AP)	François *et al*., 2008
6-Hydroxy-6a,12a-dehydrodeguelin **(222)**	*M. duchesnei* (AP)	François *et al*., 2008
6-Oxo-6a, 12a-dehydrodeguelin **(223)**	*M. duchesnei* (AP)	François *et al*., 2008
Elliptone **(224)**	*M. duchesnei* (AP)	François *et al*., 2008
12a-Hydroxyelliptone **(225)**	*M. duchesnei* (AP)	François *et al*., 2008
Griffonianone A **(226)**	*M. griffoniana* (RB)	Yankep *et al*., 2001
Rot-2'-enoic acid **(227)**	*M. pachycarpa* (SD)	Singhal *et al*., 1982
12a-Hydroxy Rot-2'-enoic acid, *cis* **(228)**	*M. pachycarpa* (SD)	Singhal *et al*., 1982
12a-Epimillitosin **(229)**	*M. usaramensis*(SB)	Yenesew *et al*., 1998
(+)-Usararotenoid A **(230)**	*M. usaramensis*(SB)	Yenesew *et al*., 1998
(+)-12-Dihydrousararotenoid A **(231)**	*M. usaramensis*(SB)	Yenesew *et al*., 1998
(+)-Usararotenoid B **(232**	*M .usaramensis* (SB)	Yenesew *et al*., 1998

Key:

AP	Ariel Part	SB	Stem bark
LF	Leaf	SD	Seeds
RB	Root bark	S	Singlet



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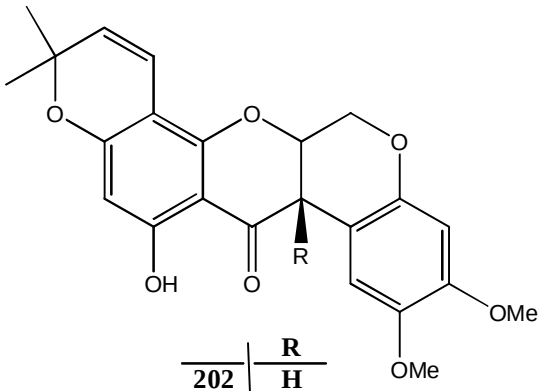
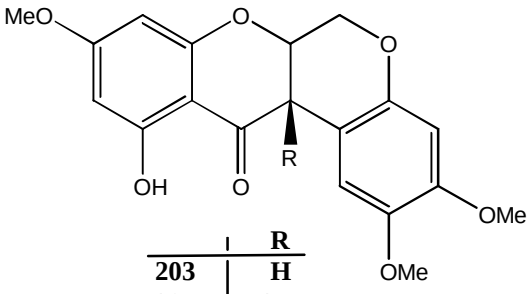
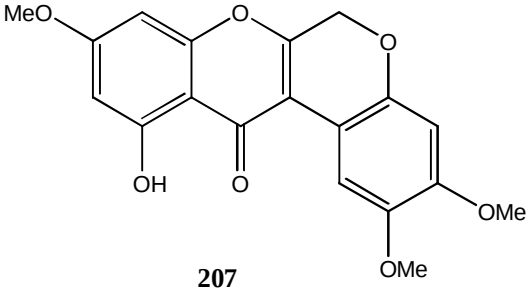


	R
202	**H**
204	**OH**



	R
203	**H**
205	**OH**



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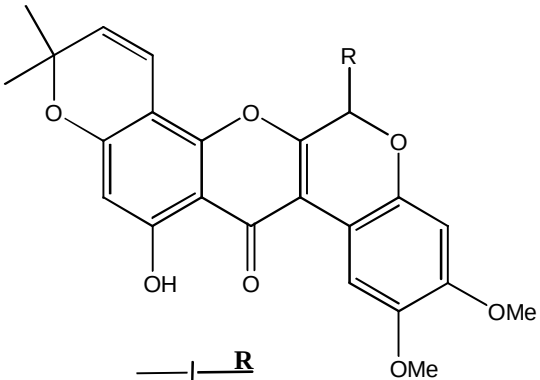
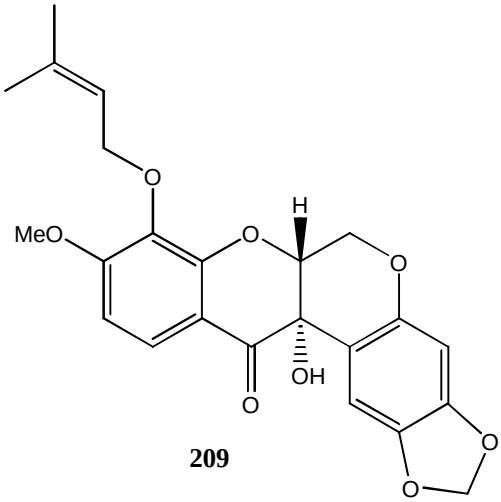


	R
206	**H**
208	**OH**



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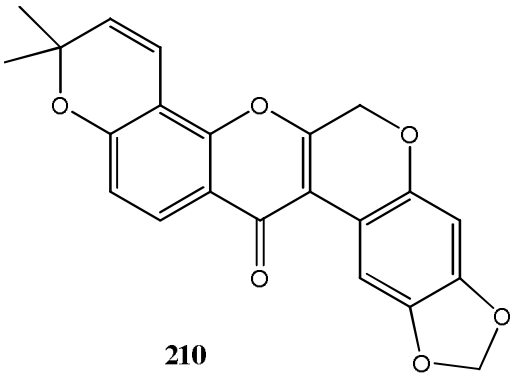

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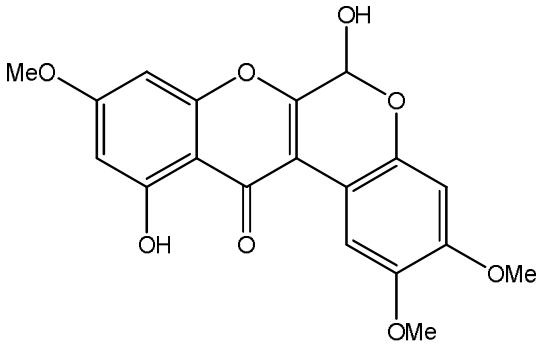

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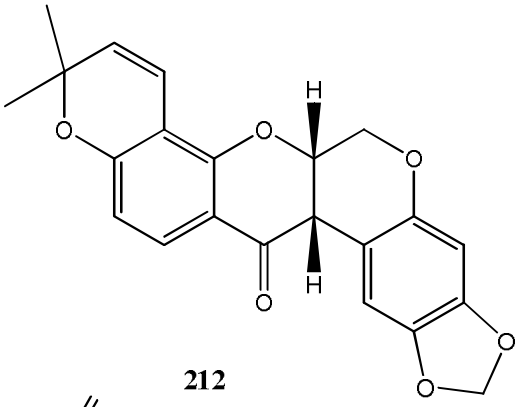

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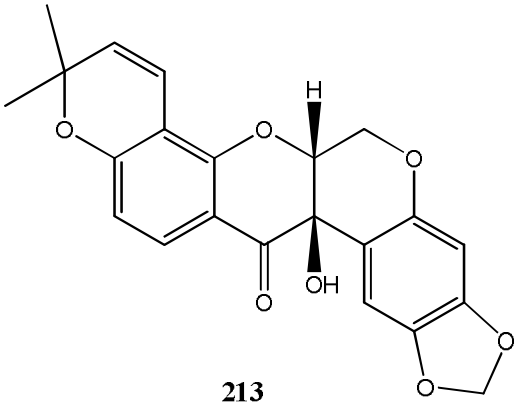

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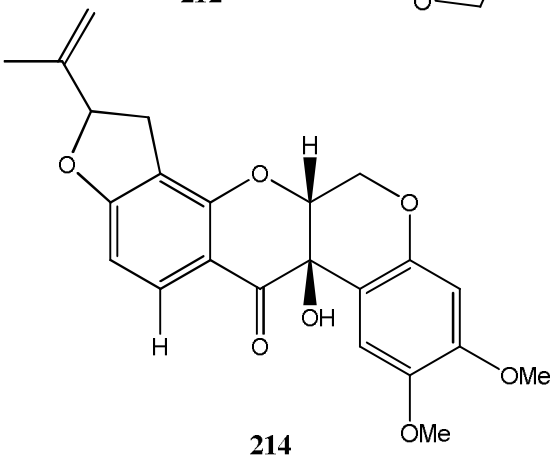

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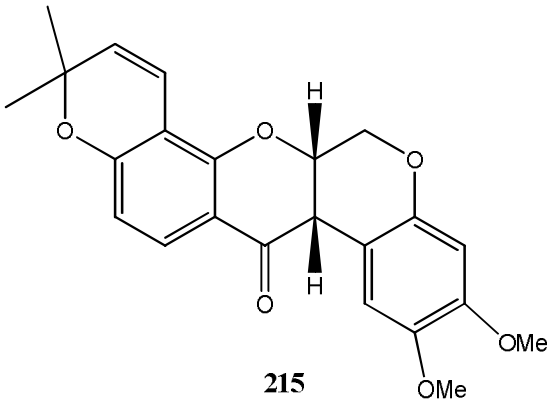

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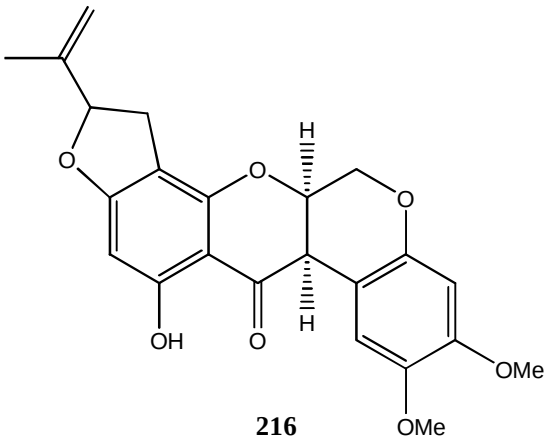

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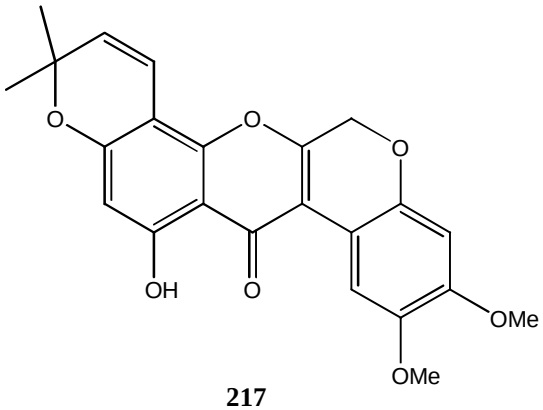

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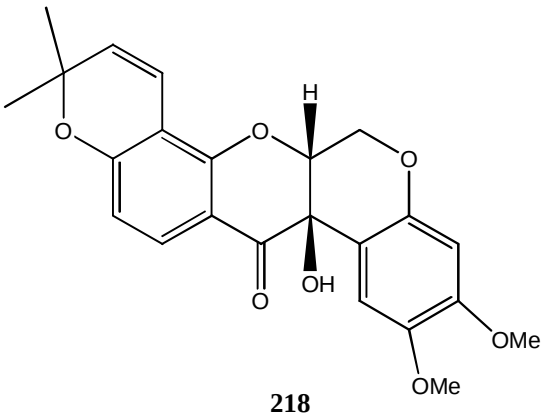

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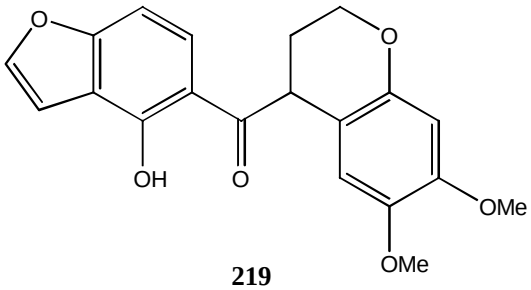

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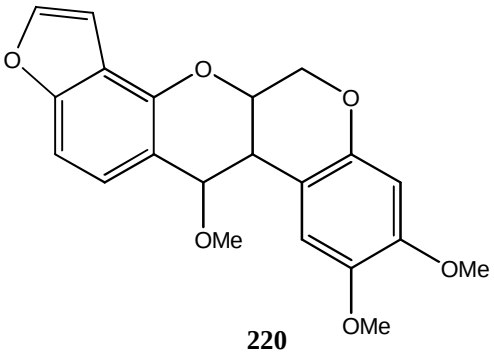

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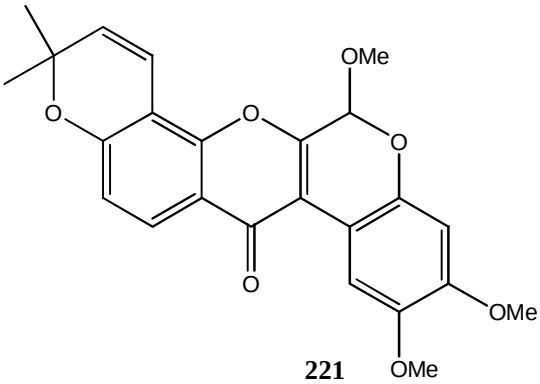

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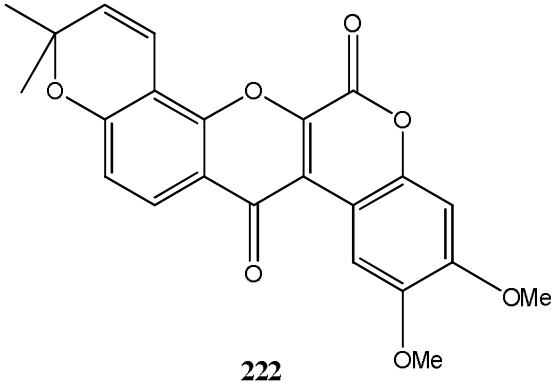

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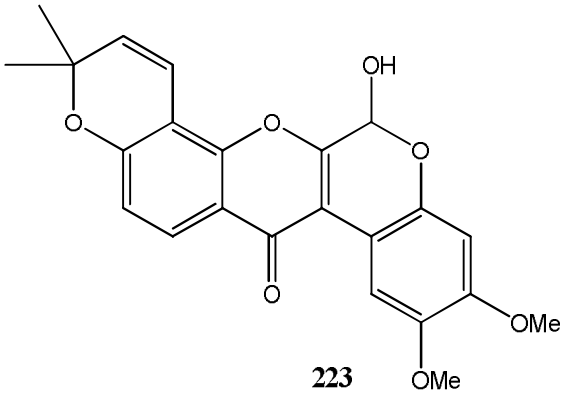

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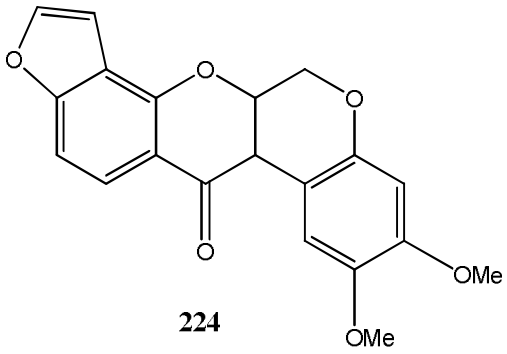

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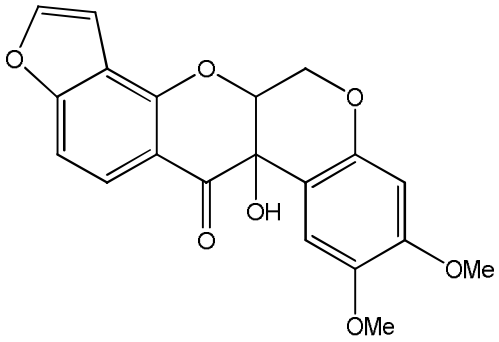

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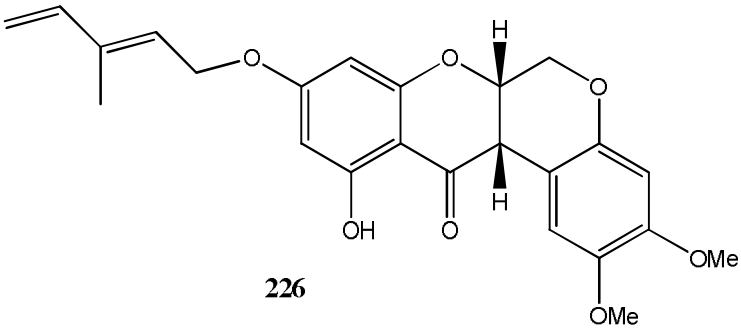

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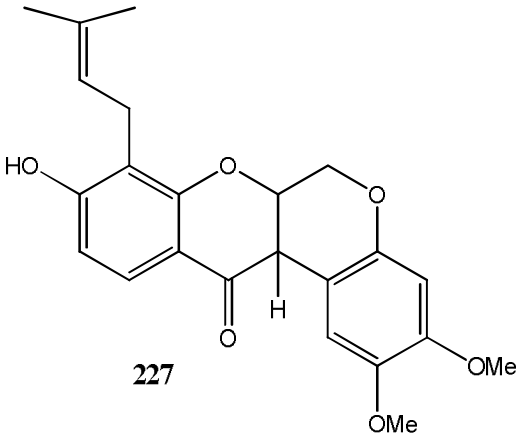

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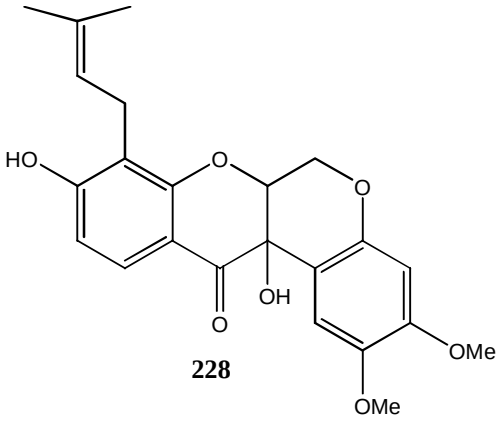

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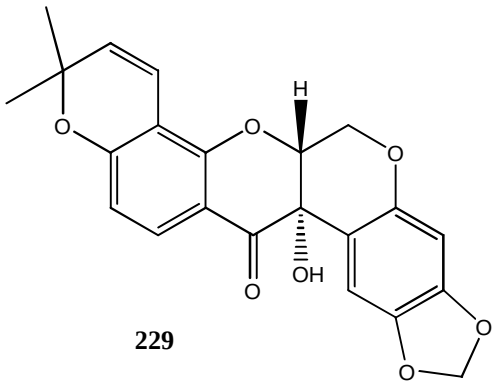

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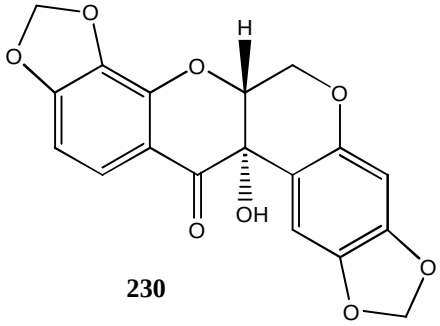

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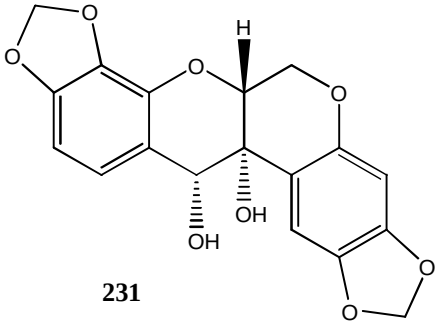

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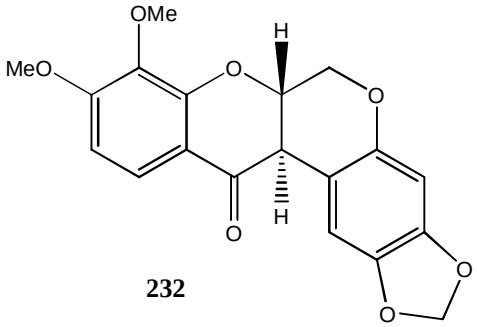


232

2.8.5 Minor compounds from the genus Millettia

Phytochemical investigation of the root bark and heart wood of some of the species belonging to the genus *Millettia* has led to the isolation of isoflavanones, isoflavans, flavan, pterocarpanoids, 3-phenylcoumarins, alkaloids as well as triterpenoids. Table 2.9 lists some of the minor compounds from the *Millettia* genus

Table 2.9: Minor compounds of *Millettia*

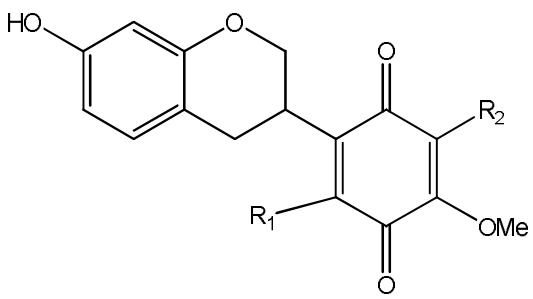
Compound	**Source (plant part**	**Reference**
Isoflavan-quinone		
Claussequinone **(233)**	*M. pendula* (HW)	Hayashi *et al*.,1978
Laurentiquinone **(234)**	*M. laurentii* (HW)	Kamnaing *et al*., 1999
Pendulone **(235)**	*M. pendula* (HW)	Hayashi *et al*.,1978
Isoflavanones		
Pervilleanone **(236)**	*M. pervilleana* (RB)	Galeffi *et al*., 1997
3'-O-Demethylpervilleanone (**237**)	*M. pervilleana* (RB)	Galeffi *et al*., 1997
Isoflavans		
Isomucronulatol **(238)**	*M. dielsiana* (SB)	Wang *et al*., 1990
Isosativan **(239)**	*M. dielsiana* (SB)	Wang *et al*., 1990
Vesttitol **(240)**	*M. dielsiana* (SB)	Wang *et al*., 1990
Laxifloran **(241)**	*M. racemosa* (HW)	Rao and Krupadanama, 1994
Isomillinol B **(242)**	*M. racemosa* (HW)	Rao and Krupadanama, 1994
Millinol **(243)**	*M. racemosa* (HW)	Kumar *et al*., 1989
Millinol B **(244)**	*M. racemosa* (HW)	Kumar *et al*., 1989
Cyclomillinol **(245)**	*M. racemosa* (HW)	Kumar *et al*., 1989
Millinolol **(246)**	*M. racemosa* (HW)	Rao *et al*., 1996
Neomillinol **(247)**	*M. racemosa* (HW)	Rao *et al*., 1996
Flavan		
2,5-Dimethoxy-4-hydroxy-(2'',3'':7,8)-furanoflavan **(248)**	*M. erythrocalyx* (RB)	Sritularak *et al*., 2002b
Pterocarpanoids		
Flemichapparin B **(249)**	*M. ferruginea* (SB)	Dagne *et al*., 1989
Emoroidocarpan **(250)**	*M. pervilleana* (RB)	Palazzino *et al*., 2003
Pervilline **(251)**	*M. pervilleana* (RB)	Palazzino *et al*., 2003
Pervillinine **(252)**	*M. pervilleana* (RB)	Palazzino *et al*., 2003
Maackiain **(253)**	*M. pulchra* (AP)	Baruah *et al*., 1984

Compound	Source (plant part	Reference
6-Methoxyhomopterocarpin **(254)**	*M. pulchra* (AP*)*	Baruah *et al*., 1984
6-Methoxypterocarpin **(255)**	*M. pulchra* (AP)	Baruah *et al*., 1984
Pterocarpin **(256)**	*M. pulchra* (AP)	Baruah *et al*., 1984
3-Phenylcoumarins		
4-Hydroxy-5,6,7-trimethoxy-3-(3',4'-methylenedioxy) phenylcoumarin (**257)**	*M. griffoniana* (RB)	Yankep *et al*., 1998
Pervilleanine **(258)**	*M. pervilleana* (RB)	Palazzino *et al*., 2003
Thonningine A **(259)**	*M. thonningii* (RW)	Khalid and Waterman, 1983
Thonningine B **(260)**	*M. thonningii* (RW)	Khalid and Waterman, 1983
Thonningine C **(261)**	*M. thonningii* (RW)	Asomaning *et al*., 1995
Robustic acid **(262)**	*M. thonningii* (RW)	Khalid and Waterman, 1983
Alkaloids		
Millaurine **(263)**	*M. laurentii*	Ngamga *et al*., 1993
O-acetylmillaurine **(264)**	*M. laurentii*	Ngamga *et al*., 1993
5a,9a-Dihydro-5a-hydroxymillaurine **(265)**	*M. laurentii*	Ngamga *et al*., 1994
Millettonine **(266)**	*M. laurentii*	Kamnaing *et al*., 1994
β-Sitosterol **(267)**	*M. brandiasa* (LF)	Pancharoen et al., 2008
3-*O*-[β-D-glucopyranosyl]-sitosterol **(268)**	*M. brandiasa* (LF)	Pancharoen et al., 2008
Stigmasterol **(269)**	*M. versicolor* (LF)	Ongoka *et al*., 2008
24-methylenecycloartan-3β-ol **(270)**	*M. versicolor*(LF)	Ongoka *et al*., 2008
22,23-dihydrostigmasterol **(271)**	*M. versicolor* (LF)	Ongoka *et al*., 2008
Stigmastan-3-ol **(272)**	*M. versicolor* (LF)	Ongoka *et al*., 2008
Tri terpenes		
Lupeol **(5)**	*M. versicolor* (LF)	Alphonse *et al*., 2006
Taraxasterol **(273)**	*M. versicolor* (LF)	Alphonse *et al*., 2006
β-Amyrin **(274)**	*M. versicolor* (LF)	Alphonse *et al*., 2006

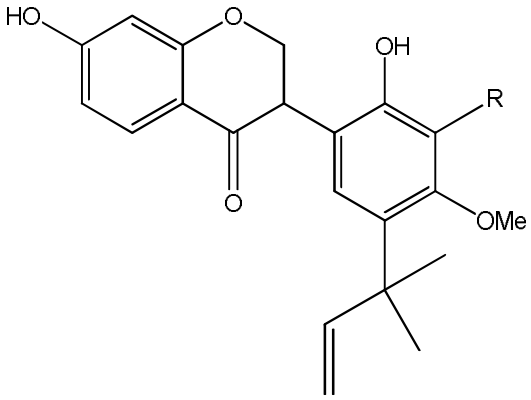
Others		
Compound	**Source (plant part**	**Reference**
Ononin **(275)**	*M. nitida*	Xiang *et al*., 2009
Odoratin-7-O-β-D-glucopyranoside **(276)**	*M. nitida*	Xiang *et al*., 2009

Key:

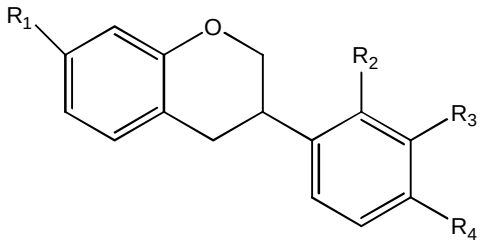
AP	Ariel Part	RB	Root bark
HW	Heartwood	RW	Root Wood
LF	Leaf	SB	Stem bark



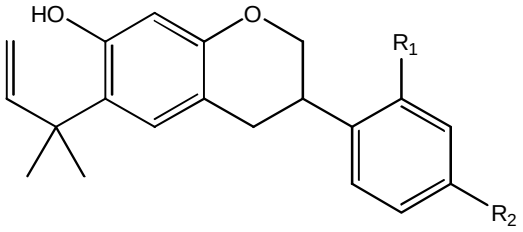
	R_1	R_2
233	**H**	**H**
234	**OCH_3**	**H**
235	**H**	**OCH_3**



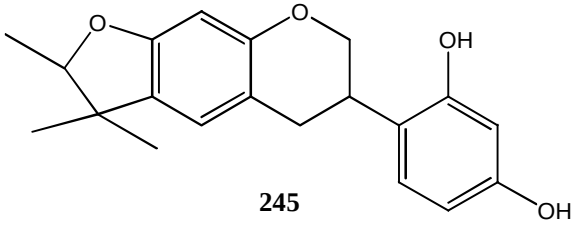
	R
236	**OMe**
237	**OH**



	R_1	R_2	R_3	R_4
238	**OH**	**OH**	**OMe**	**OMe**
239	**OMe**	**OH**	**OH**	**OMe**
240	**OH**	**OH**	**OH**	**OMe**
241	**OH**	**OMe**	**OMe**	**OH**



	R1	R2
242	**OH**	**OMe**
243	**OH**	**OH**
244	**OMe**	**OH**



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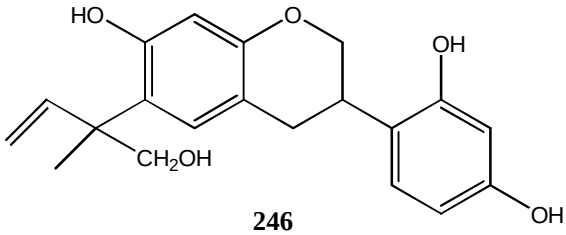

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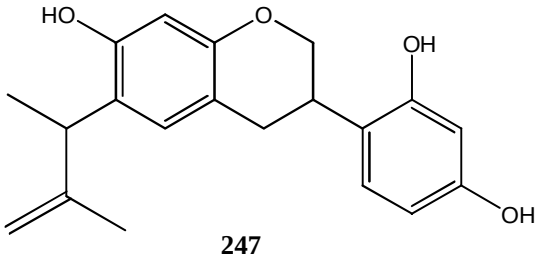

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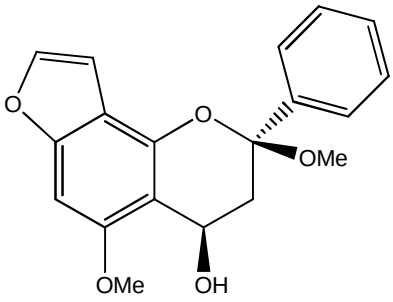

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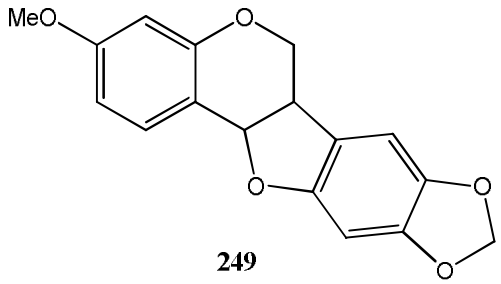

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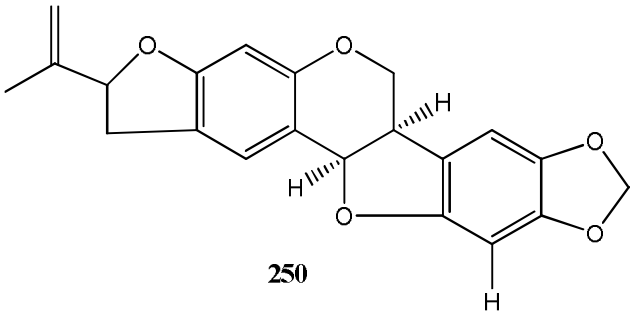

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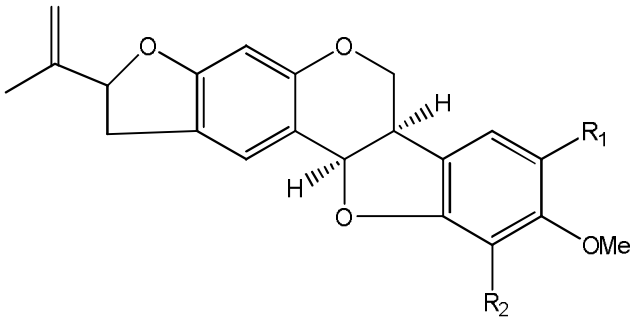
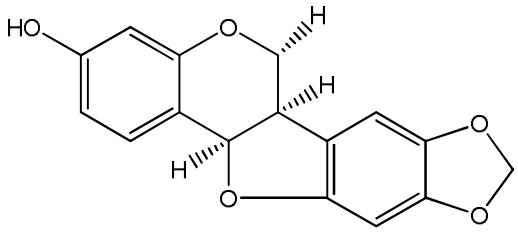


	R_1	R_2
251	**H**	**OH**
252	**OH**	**H**



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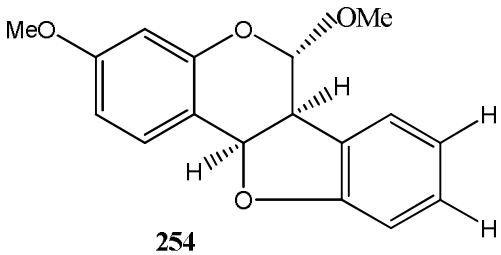

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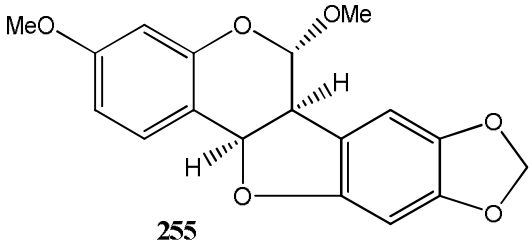

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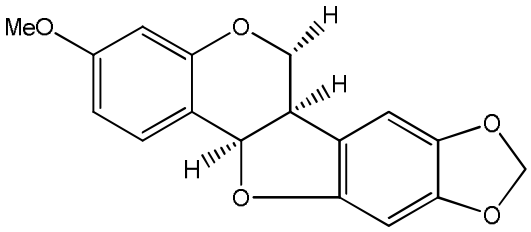

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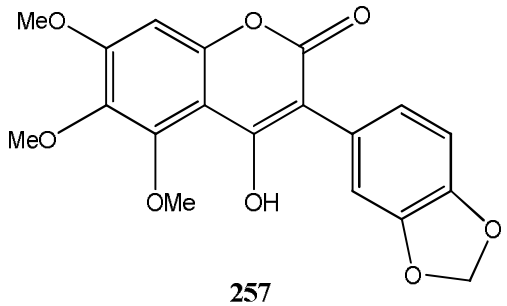

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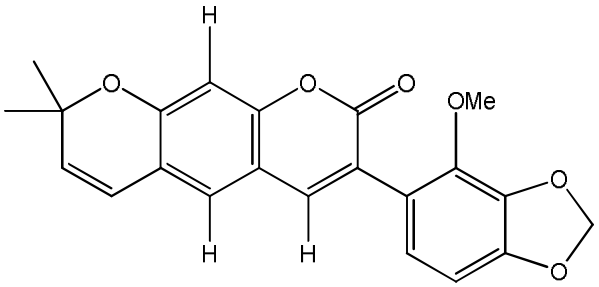

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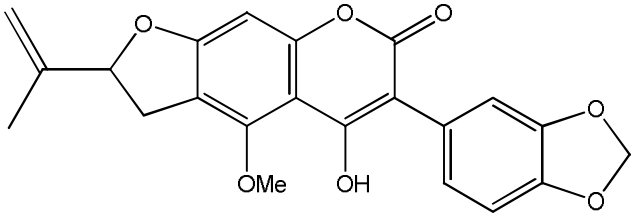

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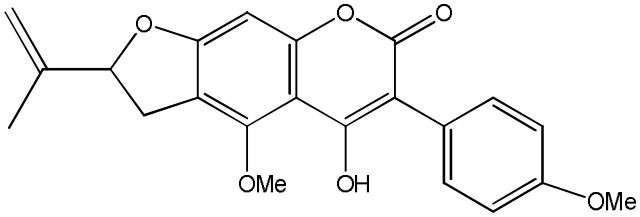

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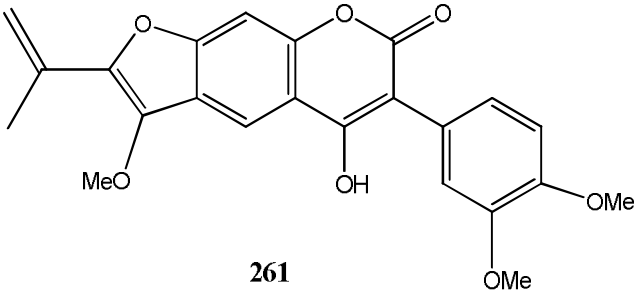

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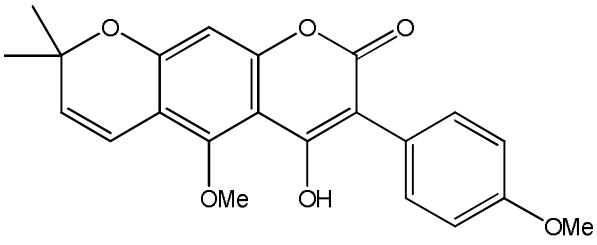

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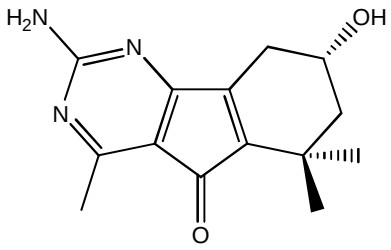

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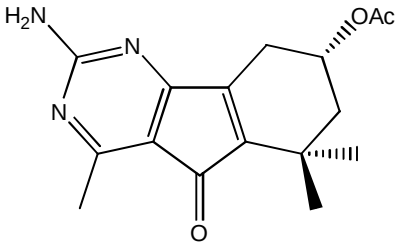

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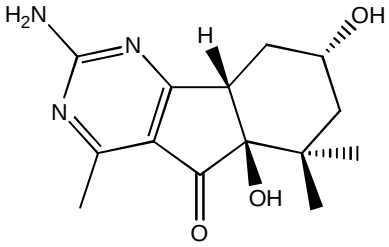

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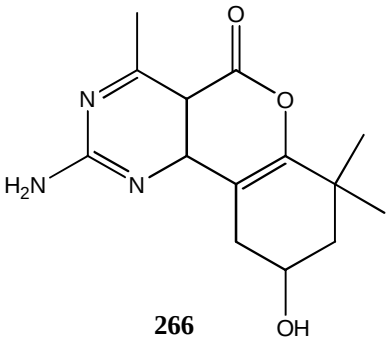

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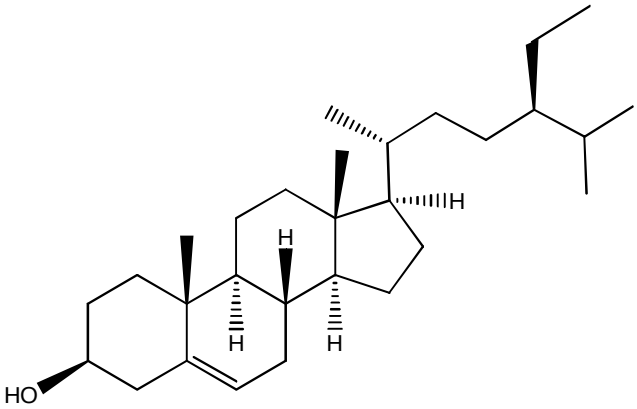

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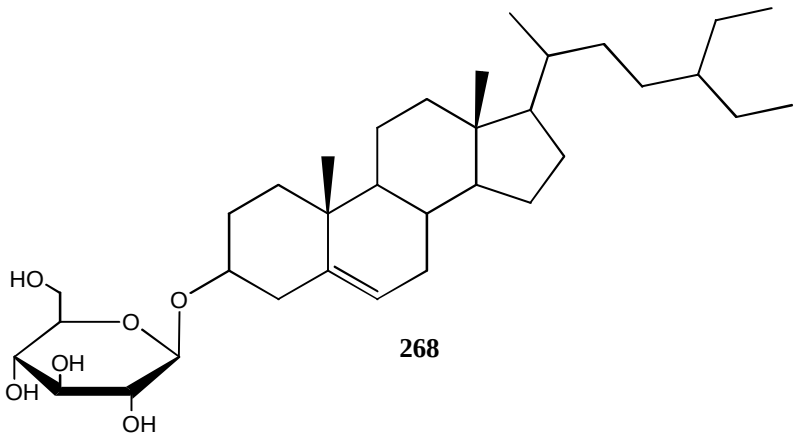

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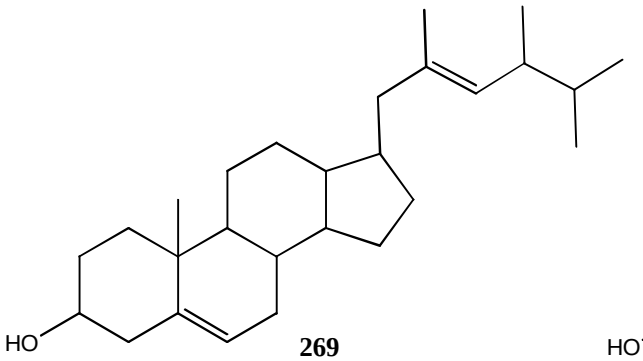

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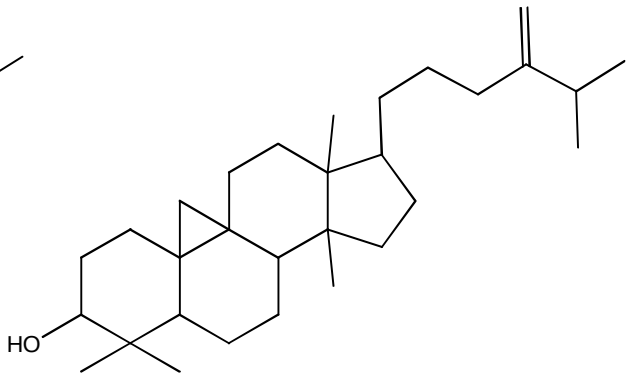

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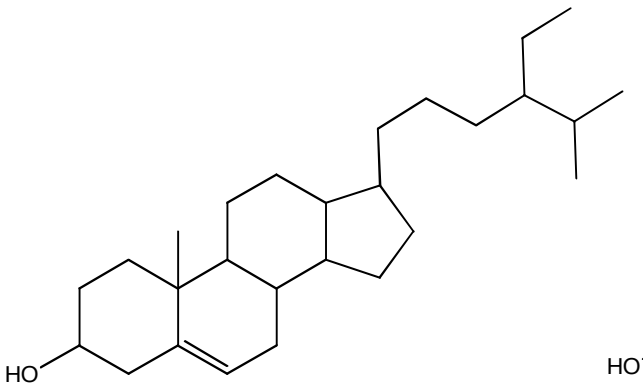

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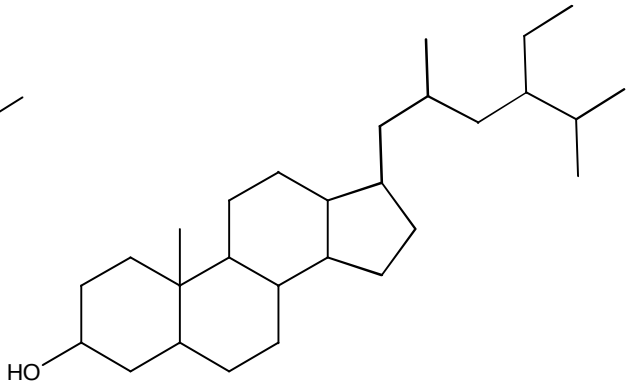

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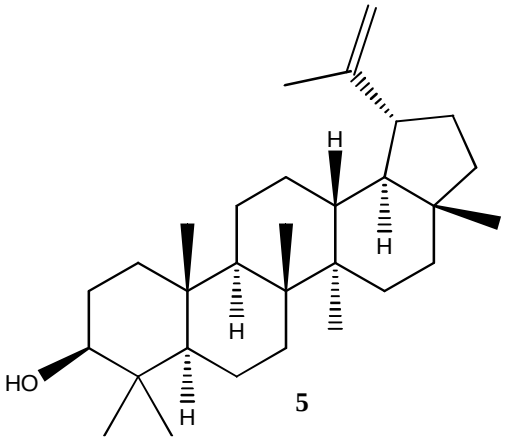

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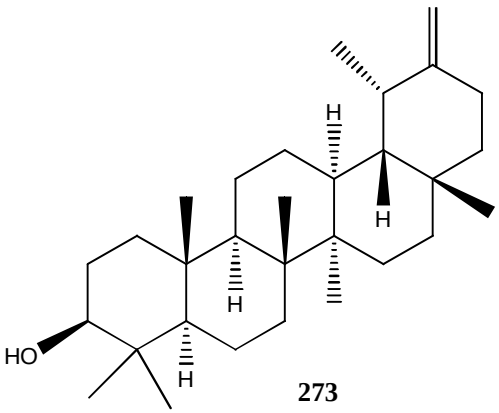

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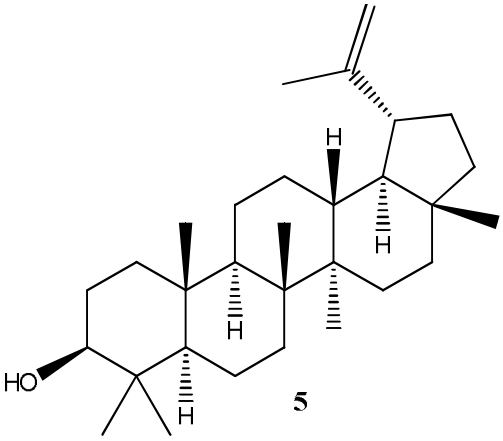

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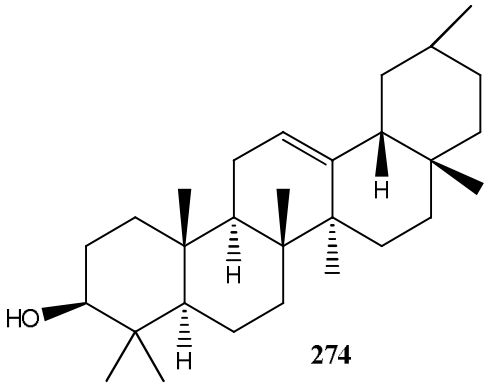

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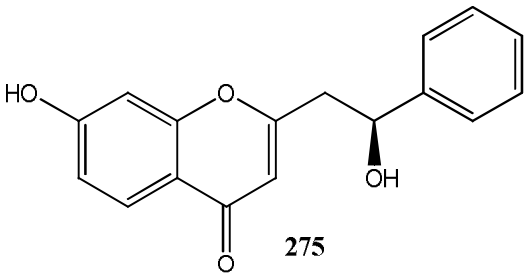

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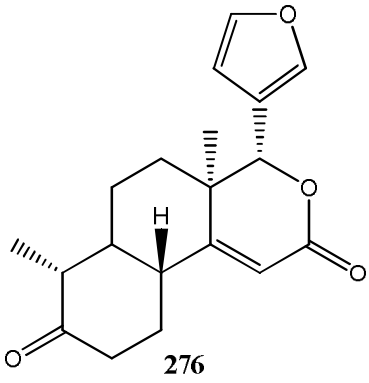

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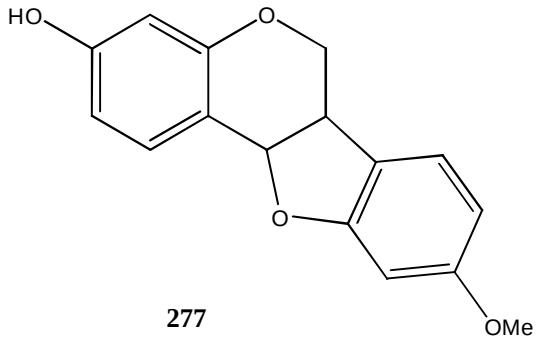
2.9 BIOLOGICAL ACTIVITY OF FABACEAE FAMILY

2.9.1 Anti-microbial isoflavonoids from Fabaceae

Isoflavonoids exclusively found in the sub-family Papilinoidea of the family Fabaceae, exhibit broad-spectrum anti-microbial activity and are believed to help the plant fight microbial disease (Dixon and Ferreira, 2002). Isoflavonoids have long been implied in the defence response to invading micro-organisms in these species (Etten and Pueppke, 1976; Nicholson and Hammerschmidt, 1992; Hahlbrock and Scheel, 1989). Isoflavonoids accumulate rapidly in most organs of the plants in response to pathogen attack. The same has been demonstrated during infection of alfalfa leaves with the fungal pathogen *Phoma*

medicaginis, where the level of the phytoalexins medicarpin (**277**) and sativan (**278**) increases from four hours post inoculation (Paiva *et al.*, 1994).



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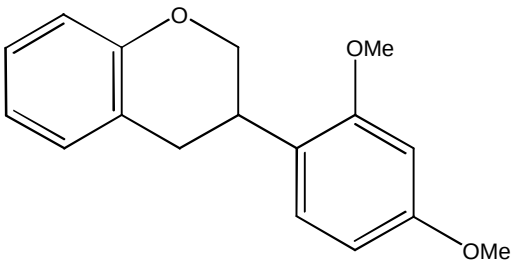

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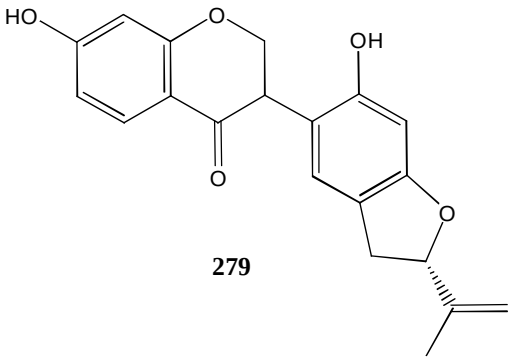
During microbial attack, the plants metabolise stored isoflavones into phytoalexins isoflavanones, isoflavans and pterocarpans which defend the plant from microbial attack. These compounds also inhibit spore germination of plant pathogens, and have been proposed for use against fungal pathogens of man (Harborne and Williams, 2000). Examples of some anti-microbial agents isolated from plants include crotamarin (**279**) from *Crotalaria madurensis* (Dewick, 1988) phaseollinisoflavan (**280**) a phytoalexin from the French bean *Phaseolous vulgaris* (O'Neil, 1986) and Hildecarpin (**281**) from the roots of *T. hildebrandtii* which also exhibited insect antifeedant activity against the legume pod-borer, *Maruca testulalis* as well as anti-fungal properties (Tarus *et al.*, 2002), (Lwande *et al.*, 1986). 4-Methoxymaackiain (**282**) which is a constituent of *T. bidwilli* shows anti-fungal activity (Tarus *et al.*, 2002).



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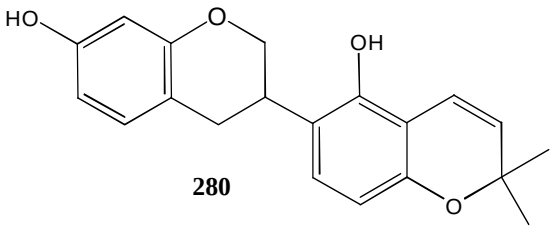

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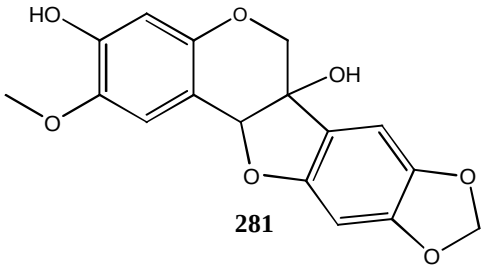

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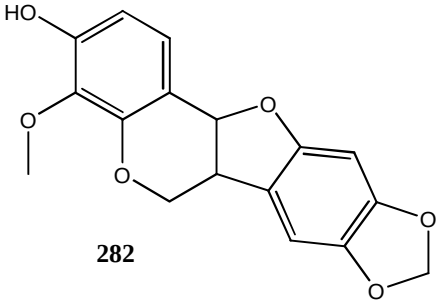

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SAR and QSAR studies of plant phytochemicals based on their important physiological roles in the plants could lead to isolation, discovery, innovation and development of superior chemotherapeutic agents.

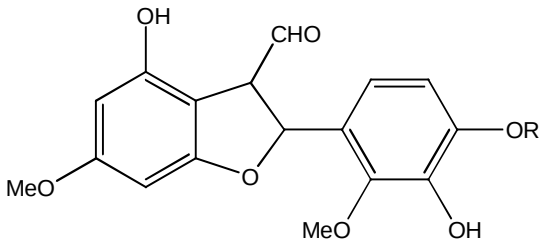
2.9.2 Anti-plasmodial activity of Fabaceae

Several phytochemicals especially isoflavonoids isolated from the Fabaceae family have been found to have anti-plasmodial activity. In addition some of the anti-plasmodial phytochemicals such as Licochalcone A **(288)** first isolated from *Glycrrhiza glabra* (Fabaceae) have been structurally modified and SAR studies carried out to discover, design, develop and synthesise chalcones and bischalcones as new antimalarial drugs (Ram *et al*., 2000; Neilsen *et al*., 1998; Liu *et al*., 2001). Among the flavonoids belonging to the

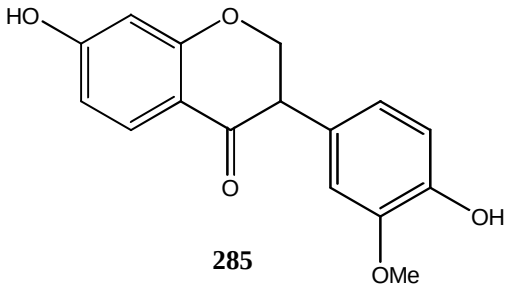
genus *Erythrina*, the flavanone abyssinone-IV (**290**) isolated from the roots of *E. abyssinica* showed high activity against both strains of *P. falciparum* (Yenesew *et al.*, 2003). Pterocarpans, pterocarpenes and isoflavenes have also been identified to represent new sub-classes of isoflavonoids with anti-plasmodial activities (Yenesew *et al.*, 2003). Anti-malarial chalcones are widely thought to inhibit the enzyme malarial cysteine protease which catalyses host haemoglobin degradation to provide malarial nutrients (Liu *et al*., 2001). Table 2.10 lists some of the anti-plasmodial Fabaceae phytochemicals.

Table 2.10: Anti-plasmodial Fabaceae phytochemicals

Species	**Compound**	**Reference**
Andira inermis	Andidermals A **(283)**	Schwikkard and van Heerden, 2002
	Andidermals C **(284)**	
	Calycosin **(285)**	
	Genistein **(63)**	
Bauhinia malabarcia	Racemosol **(286)**	
	Demethylracemosol **(287)**	
Glycyrrhiza glabra	Licochalcone A **(288)**	
Machaerium multiflorum	Machaeriol B **(289)**	Caniato and Puricelli , 2003
Erythrina abyssinica	abyssinone-IV**(290)**	Yenesew *et al.*, 2003.



	R
283	**Me**
284	**H**



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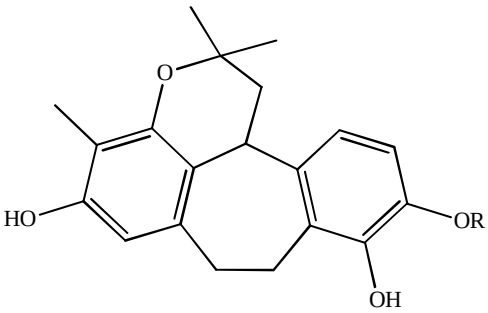
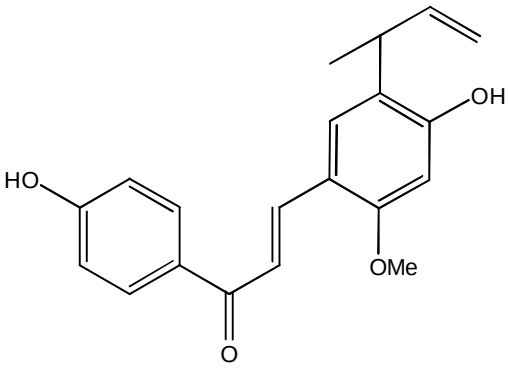


	R
286	**Me**
287	**H**



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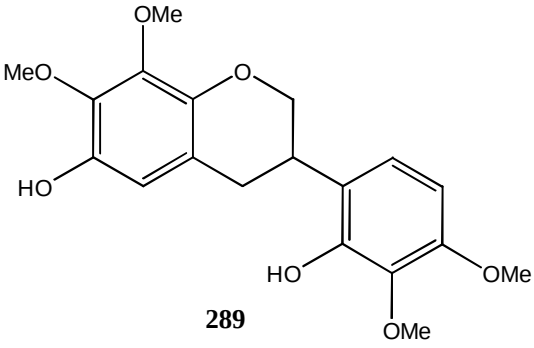

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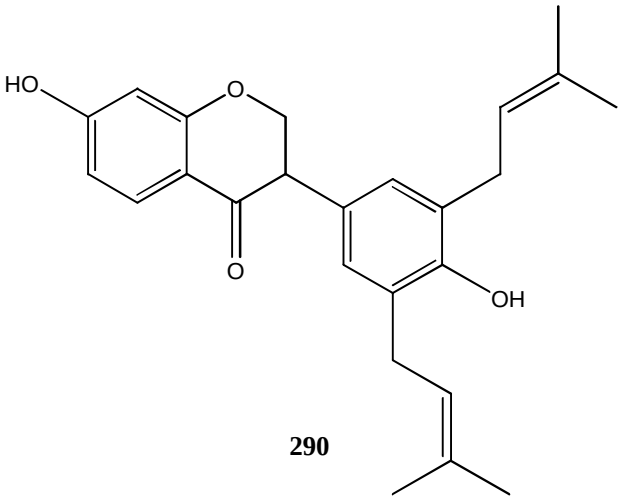

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2.10 BIOLOGICAL ACTIVITY OF *MILLETTIA*

Based on the wide ethno-medical use of *Millettia* genus, phytochemical and biological investigations have been carried out on some *Millettia* species and biologically active principles isolated. The same has authenticated some of the traditional medicinal uses of

these plants in treatment of various ailments. Some of these phytochemicals include isoflavones, rotenoids and chalcones. Phytochemical and biological investigation of *Millettia usaramensis,* subspecies *usaramensis* elaborated flavonoids with anti-plasmodial activity.

The rotenoids usaretonoid C (**209**), 12a-epimillettosin **229)** and 6a, 12a-dehydromillettone **(210)**; isoflavone barbigerone (**79)** and chalcone 4'-O-geranylisoliquiritigenin (**188**) were found to have anti-plasmodial activity (Yenesew *et al*., 1998; 2003). Table 2.11 summarises the biological activity observed from various *Millettia* species.

Table 2.11: Biological activity of some *Millettia* species

Plant species	**Plant part**	**Biological activity**	**Reference**
M. brandishing	Aerial	Anti-inflammatory	Pancharoen *et al*,2008
M. conraui	Stem bark	α-Glucosidase Inhibitors	Alembert *et al.*, 2007
M. erythrocalyx	N/S	Antiviral	Likhitwitayawuid *et al*., 2005
M. griffoniana	Root bark	Anti-inflammatory	Yankep *et al*., 2003
M. Laurentii	Stem bark	Insecticidal	Kamnaing *et al*., 1994
M. leucantha	Stem bark	Anti-inflammatory	Ampai *et al*., 2003
M. pachycarpa	Seeds	Insecticidal	Singhal *et al*., 1983
M. racemosa	Stem bark	Anti-bacterial	Rao and Krupadanam, 1994
M. taiwaniana	stem	Antitumor	Ito *et al*., 2004
M. thonningii	Seeds	Antischistosomal	Lyddiard *et al*., 2002
M. usaramensis	Stem bark	Anti-plasmodial	Yenesew *et al*., 2003
M. versicolor	Aerial Root	Anti-inflammatory Anthelminthic	Fotsing *et al*., 2003 Kasonia *et al*.,1989

2.11 ANTI-BACTERIAL AND ANTI-FUNGAL BIOASSAYS

2.11.1 Agar diffusion assays

As the chemical diffuses through the gel from the well its concentration falls steadily in that direction (Figure 2.4). The concentration in the region A to X is sufficiently high to prevent growth and is therefore an inhibitory concentration whereas the concentration between X and B is sub inhibitory and growth occurs. The concentration at X at the time the zone edge is formed is known as the critical inhibitory concentration (CIC). After incubation the gel between A and X is clear and that between X and B is opaque as a result of the microbial growth. The diameter of the zone of inhibition increases as the concentration of the chemical in the well increases (Norman *et al.,* 2007). Figure 2.3 shows the assessment of antimicrobial activity by agar diffusion.

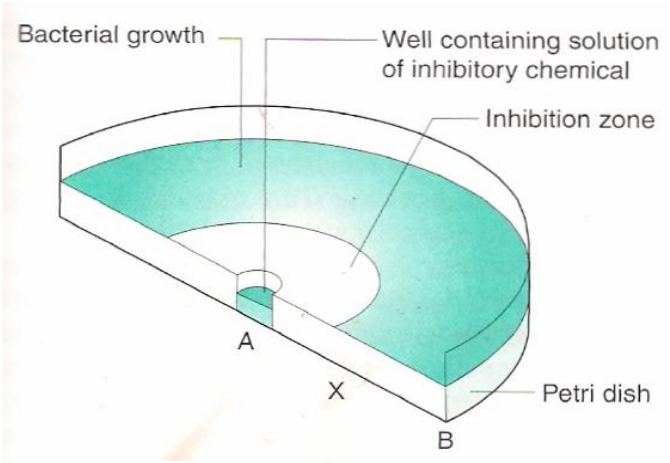


Figure 2.4: Assessment of antimicrobial activity by agar diffusion

2.11.2 Critical inhibitory concentration (CIC)

When agar diffusion method is used to identify the concentration that just fails to produce

an inhibition zone the same is known as critical inhibitory concentration and not MIC. This is the lowest concentration from a series of solutions of the test compound of progressively decreasing concentrations that fails to produce an inhibition zone using the agar diffusion method. The CIC usually exceeds the MIC value by a factor of 2-4 (Norman *et al.*, 2007).

2.11.3 Minimum inhibitory concentration (MIC)

MIC is the lowest concentration of an antimicrobial chemical found to inhibit the growth of a particular test organism; and is therefore a fundamental measure of the intrinsic antimicrobial activity (potency) of a chemical. An MIC is an absolute value which is not based upon a comparison with a standard or reference preparation as with antibiotic assays. This implies inadequate control of experimental conditions is particularly likely to have an adverse effect on the results and consequently result in discrepancies in MIC values measured in different laboratories or in the same laboratory at different times. Several factors including relative rate of diffusion of the chemical, growth of the test organisms, inoculum concentration and physiological state and gel strength which may influence the MIC value must be standardised. When MICs are conducted in agar there is no diffusion and no zones of growth inhibition and the result merely depends on the presence or absence of growth (Norman *et al.,* 2007).

2.12 ANTI-PLASMODIAL ACTIVITY

Blood schizonticides also known as drugs for suppressive or clinical cure act at the erythrocytic stage and are used to treat the acute attacks. These drugs include mefloquine, chloroquine, halofantrine, dapsone, pyrimethamine, Proquanil, atovaquone**,** artemisinin

and derivatives, tetracycline and doxycycline. Most of these drugs are used in combination for higher effectiveness and to reduce emergence of resistance (Rang and Dale, 2007). SYBR Green test measures the ability of compounds to inhibit the erythrocytic strains *in vitro*. SYBR Green 1 is a fluorescent DNA intercalating dye. The dye is highly fluorescent when intercalated into DNA but poorly flouresent when not intercalated. Laboratory growth of malarial parasites requires propagation in human red blood cells (RBC). The absence of DNA in RBC provides advantages for the use of SYBR Green 1 for malarial parasite growth assays. Quantification of growth inhibition is through determination of the fifty percent growth inhibitory concentration (IC_{50}). Comparative IC_{50}s are used in preliminary screens of anti-plasmodial compounds; however *in vitro* cytotoxicity should be carried out to determine the selective toxicity of the compounds.

In this study *Millettia oblata* root extract was studied to determine its phytochemicals and antimicrobial activity and safety.

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 STUDY DESIGN AND SITE

Extraction, isolation and identification of the various constituents and the bioassays were carried out through an experimental laboratory based design. Extraction and the subsequent isolation and identification were carried out at University of Nairobi, Chemistry Department, Chiromo, Nairobi; anti-fungal and anti-bacterial tests at University of Nairobi, Pharmacy Department, Nairobi; toxicity testing at KEMRI laboratory, Nairobi and anti-plasmodial activity at Walter Reed laboratory, KEMRI, Kisumu, Kenya.

3.2 CLEANING AND STERILIZING GLASSWARE

All reusable glassware (test tubes, conical flasks, round bottomed flasks, measuring cylinders, vials, beakers, volumetric flasks and teat pipettes) were soaked in hot water with liquid detergent before washing thoroughly and rinsing with distilled water. They were then dried in an electric oven at 105ºC for one hour and allowed to cool slowly to room temperature then rinsed with a mixture of distilled solvents to remove any organic impurities prior to use.

Glassware for bioassays were cleaned using tap water, distilled water, rinsed with alcohol, dried in an oven at 220ºC for two hours and sterilised through dry heat sterilization at 220ºC for four hours. Great care was taken to keep all materials sterile and the experiments were carried out in laminar flow.

3.3 EXTRACTION AND ISOLATION OF COMPOUNDS FROM THE *MILLETTIA OBLATA ROOT* EXTRACT

3.3.1 General procedures

The ^{1}H-NMR (200 MHz) and ^{13}C-NMR (50 MHz) spectra were recorded on Varian-Mercury spectrometer using residual solvent peak as reference. Chemical shifts were measured in parts per million (ppm) in (delta) δ values. Column and size exclusion chromatography were carried out using silica gel 60 (70-230 mesh) and Sephadex LH 20 respectively. Analytical TLC was done using Merck pre-coated silica gel 60 F_{254} plates. Chromatographic zones were detected under UV light at 254 λ max and/or exposing in some cases to iodine vapour. Purification through re-crystallisation was carried out by dissolving the sample in appropriate solvent/s covering with perforated foil and leaving it overnight. Any crystals or precipitate formed was then washed severally under suction using hexane.

3.3.2 Solvents

The general purpose grade (GPR) organic solvents methanol, dichloromethane, ethyacetate (EtOAc) and hexane (Kobian, Nairobi, Kenya) were distilled and stored in 2.5 litres reagent bottles to be used as analytical solvents.

3.3.3 Plant materials

The authenticated and ground *Millettia oblata* root previously collected from Taita Hills was provided by the University of Nairobi, Chemistry department, Chiromo courtsy of Dr. Solomon Derese.

3.3.4 Extraction and isolation procedure

The air dried and ground *Millettia oblata* root was extracted with CH_2Cl_2: MeOH (1:1) followed by 100% methanol solvent systems by cold percolation at room temperature. The solvent was removed under vacuum by use of a rotary evaporator at 40^oC. The CH_2Cl_2:MeOH (1:1) (70 grams) and the MeOH (2 grams) extracts obtained were brown and sticky. Sixty (60) grams of the CH_2Cl_2:MeOH (1:1) extract was subjected to gradient elution column chromatography (CC) on normal silica gel (350 g) eluting with hexane containing increasing percentage of ethyl acetate to a maximum of 50% EtOAc followed by 100% methanol. The fraction eluted with 3% EtOAc in n-hexane formed a white precipitate which was soluble in dichloromethane and insoluble in hexane. The precipitate was washed with hexane under suction to yield lupeol **(5)** whose identification was determined through co-spotting with an authentic sample. Latter fractions eluted with 3% EtOAc in n-hexane and pooled guided by analytical TLC (Merck pre-coated silica gel 60 F_{254} plates) formed a white precipitate which was washed in hexane to yield isoerythrin A, 4'-(3-methylbut-2-enyl) ether **(1)**. Early fraction eluted with 5% EtOAc in n-hexane and pooled appropriately formed a white precipitate which was dissolved in dichloromethane, the resultant solution concentrated, a small amount of hexane added and the fraction left overnight. A white precipitate was formed which on washing successively with hexane under suction yielded calopogonium isoflavone B **(2)**. A Latter fraction eluted with 5% EtOAc in n-hexane was further subjected to size exclusion chromatography on Sephadex LH-20 column (eluent CH_2Cl_2/MeOH, 1:1). Crystallisation occurred in one of the fractions/eluents forming large orange crystals of the chalcone 4-hydroxylonchocarpin **(3)** amongst an amorphous yellow /orange powder. Fractions eluted with 12% EtOAc in n-

hexane and pooled appropriately formed a precipitate in CH_2Cl_2:MeOH, 1:1 which on successive washing with hexane under suction yielded 7,2'-dimethoxy-4',5'- methylene dioxyisoflavone **(4)**.

3.4 BIOLOGICAL ACTIVITY ASSAYS

The crude extracts as well as isolated flavonoids were subjected to *in vitro* anti-plasmodial, anti-fungal and anti-bacterial bioassays.

3.4.1 Anti-plasmodial activity assay

3.4.1.1 Plasmodial parasites

Two strains consisting of chloroquine sensitive D6 (CDC/Sierra Leone) and the other chloroquine resistant W2 (CDC/Rosewell Indochina III) were available. For the D6 strain chloroquine and mefloquine had an IC_{50} < 45 ng/ml and >15 ng/ml respectively whereas for the W2 strain chloroquine and mefloquine had an IC_{50} > 45 ng/ml and < 15 ng/ml respectively. Parasite cultures were initiated from the stabilates preserved in liquid nitrogen (the level of parasitemia during storage is ≥10%). Following the initiation of a fresh culture, at least two full life cycles (96 h) were completed before parasites were used for assays. Prior to the assay initiation, the level of parasitemia of an aliquot of a stock culture was measured by light microscopy following Giemsa staining or by fluorescence-activated cell sorter analysis after staining with propidium iodide. The time that the stock culture was exposed outside a proper gas environment (5% CO_2, 5% O_2, and 90% N_2) was minimized (≤15 min).

W2 chloroquine-resistant and D6 chloroquine-sensitive *P. falciparum* strains were cultured continuously according to Trager and Jensen (1978) with modifications described by Van Huysenn and Rieckmann (1993). The parasites were routinely maintained in continuous long-term cultures in Rosewell Park Memorial Institute (RPMI) 1640 (Gibco, Inc., Grand Island, NY) medium supplemented with 5% washed human A+ erythrocytes (Valley Biomedical, Inc., Winchester, VA.) , 11 mM glucose, 25 mM hydroxyethylenepiperazine ethanesulfonic acid (HEPES), 32 nM sodium bicarbonate ($NaHCO_3$), 29 µM hypoxanthine, and 10% heat-inactivated A+ human plasma. The erythrocytes were washed several times with RPMI prior to use. The cultures were incubated at 37°C under an atmosphere of 5% CO_2 and 5% O_2, with a balance of N_2. Prior to performing the assays, the parasites were conditioned to the test culture condition for 3 to 4 days.

3.4.1.2 *In-vitro* anti-plasmodial activity assay procedure

The crude extract and pure compounds **1**, **2**, **3** and **4** were assayed for anti-plasmodial activity against chloroquine sensitive (D6) and chloroquine resistant (W2) cultured *P. falciparum* parasites. The IC_{50} of cultured *P. falciparum* parasites was determined using non radioactive MSF assay technique (Smilkstein *et al.*, 2004) with modifications. This invitro drug susceptibility method uses the fluorochrome called "SYBR Green 1", a non radioactive intercalating DNA marker that depicts *in vitro* parasite propagation. One hundred (100) microlitre culture volumes of *P. falciparum* strains in late-ring or early-trophozoite stages at an optimized starting parasitemia of 1% and hematocrit of 2% were used. Microtiter plate wells containing non infected erythrocytes in the absence of standard drug or plant extract served as negative controls, whereas parasitized erythrocytes in the

absence of standard drug or plant extract served as positive controls for parasite growth on each plate.

Ten twofold serial dilutions of chloroquine (1.953 to 1,000 ng/ml), mefloquine (0.488 to 250 ng/ml) and test sample (97.7–50,000 ng/ml) were prepared on a 96 well plate to be tested against the two strains of the *P. falciparum* The culture-adapted *P. falciparum* were added on to the plates containing dose range of drugs and test samples and incubated in gas mixture (5% CO_2, 5% O_2, and 90% N_2) at 37°C. The assay was terminated 72 hrs later by freezing at -80°C. After thawing, lysis buffer containing SYBR Green I were added directly to the plates and gently mixed by using the Beckman Coulter Biomek 2000 automated laboratory workstation (Beckman Coulter, Inc., Fullerton, CA). The plates were incubated for 5-15 minutes at room temperature in the dark. Parasite growth inhibition was quantified by measuring the per-well relative fluorescence units (RFU) of SYBR green 1 dye using the Tecan Genios Plus (Tecan US, Inc., Durham, NC) at excitation and emission wavelengths of 485 and 535 nm, respectively, and with the gain set at 60. Differential counts of relative fluorescence units (RFUs) were used in calculating IC_{50} for each drug or test sample using Prism 4.0 software for Windows (Graph pad Software, San Diego, CA). A minimum of three separate determinations was carried out for each sample at each concentration. Replicates had narrow data ranges hence presented as mean $\pm$ SD.

Overall growth inhibition was assessed by comparison of the growth in the treated wells with that in the control wells, to which no drug was added.

3.5 ANTI-BACTERIAL AND ANTI-FUNGAL ACTIVITY

3.5.1 Preparation of the medium

Fourty grams of Tryptone Soya Agar and 65 grams Sabouraud's dextrose agar (Oxoid LTD, Hampshire, England) were each separately suspended in 1 litre of distilled water and boiled to effect complete dissolution. Each broth was then sterilised by autoclaving at 121ºC for 15 minutes under one bar pressure.

3.5.2 Anti-bacterial and anti-fungal test micro-organism strains

Three bacteria *Staphylococcus aureus* (NC 07447), *Escherichia coli* (ATCC 25922) and *Bacillus pumillus* (NC08241) and one fungi *Candida albicans* (NCPF3179) were used. The micro-organisms were grown on Tryptone Soya Agar (TS Agar for bacteria) and Sabouraud's dextrose agar (SD Agar for fungi) obtained from Oxoid LTD, Hampshire, England. The-micro-organisms were maintained on nutrient agar slants stored at 4ºC. Micro-organisms were scooped using a loop from the stock culture and streaked onto the surface of the respective medium in a test tube. The inoculated medium was then incubated at 37ºC for bacteria and 30ºC for fungi. Microbial suspensions from one day old subcultures were prepared in sterile distilled water after washing and centrifugation. All procedures were carried out in the laminar flow equipment.

3.5.3 Standardisation of the inocula suspension

Aliquots of 0.5, 1.0, 1.5 and 2.0 ml of the one day old microbial suspension was added to different 100 ml of the molten agar and blended uniformly. These were allowed to solidify

in the plates and plated out at 0.35 and 0.175 mg/ml of getamycin and 0.3 and 0.15 mg/ml of nystatin in triplicate and all the plates incubated at optimum conditions overnight.

The developed zones of inhibition were examined for clarity, sharpness of zone edges, background growth and the zone of inhibition diameter read using the Wezu electronic digital calliper (Wezu Messzenge GmbH, Germany) to the nearest 0.1mm. Concentrations giving 18mm and 22mm for the low and high concentrations respectively, were accepted for further analytical work. These concentrations correspond with microscopy colony count of 10^{6}-10^{7} colony forming units (c.f.u).

3.5.4 Anti-bacterial activity assay

Five hundred (500) millilitres of the TS Agar was inoculated with 5 ml of the standardised inoculum and the contents of the tube swirled to effect even distribution. Twenty millilitres of the inoculated TS medium for bacteria was dispensed into twenty five 90 mm pre-sterilised Petri dishes to yield a uniform depth of 4 mm. The Petri dishes were then covered and allowed to cool at room temperature undisturbed until the inoculated culture medium hardened. Six wells of 7.00 mm diameter were made by cutting out the inoculated agar with a cork borer of a similar size. Two of the wells were used for the positive and negative controls and were filled with 50 ul of gentamycin 0.35mg/ml and 50 ul of 1% DMSO, respectively. The other four wells were each filled with 50 µl of (40-5) mg/ml of the pure compounds and (115-14.4) mg/ml of the extracts all prepared in twofold serial dillutions. The petri dishes were then incubated at 37^{o}C in an inverted position for 18 hours. Positive results (sensitivity) were established by the presence of clear zones of inhibition around active test samples which were measured using the Wezu electronic

digital calliper in millimetres. The experiment was repeated using two fold serial dilutions (12.25-1.53) mg/ml of the active pure compounds and (51.4-6.425) of the active crude extract. An inhibition zone of 14 mm or greater (including diameter of the well) was considered as high anti-bacterial activity (Ramzi and Ulrike, 2005).

3.5.5 Assay for anti-fungal activity

Two hundred milliliters of the SD Agar was inoculated with 2 ml of the standardised inoculum and the contents of the tube swirled to effect even distribution. Twenty millilitres of the inoculated SD Agar was dispensed into ten 90 mm pre-sterilised Petri dishes to yield a uniform depth of 4 mm. The Petri dishes were then covered and allowed to cool at room temperature undisturbed until the inoculated culture medium hardened. Six wells (per Petri dish) of 7.00 mm diameter were made by cutting out the inoculated agar with a cork borer of a similar size. Two of the wells were used for the positive and negative controls and were filled with 50 µl of nystatin 0.3 mg/ml and 50 µl of 1% DMSO, respectively. Two sets of four wells were each filled with 50 µl of (40–5) mg/ml of the pure compounds and (115-14.4) mg/ml of the extracts at two fold serial dillutions. The Petri dishes were then incubated at 30^{o}C in an inverted position for 24 hours. Positive results (sensitivity) were established by the presence of clear zone of inhibition around active test samples which were measured using the Wezu electronic digital calliper in millimetres.

3.5.6 Determination of the Minimum Inhibitory Concentration (MIC)

MIC determination was only carried out for 4-hydroxylonchocarpin **(3)** and the

CH_2Cl_2:MeOH (1:1) crude extract. The CH_2Cl_2:MeOH (1:1) crude extract had higher activity than the methanol extract and was also available in larger quantities while compounds **1**, **2** and **4** were inactive. Preliminary studies were carried out using *B.pumilus* and compound **3**. Definitive studies were carried out using the three bacteria (*S. aureus*, *E. coli* and *B. pumilus*), the CH_2Cl_2: MeOH (1:1) crude extract and compound **3**.

Two fold serial dilution was carried out to give final concentrations of (7.6-61.25) mg/ml from the extract and (36.5-291.6) μg/ml for 4-hydroxylonchocarpin (**3**).

Eight hundred (800) millilitres of the TS Agar was inoculated with 8 ml of the standardised inoculum and the contents of the tube swirled to effect even distribution. Nintynine millilitres of the inoculated culture TS Agar were dispensed into eight sterile screw-capped test tubes. One millilitre (per tube) of the respective test compound was added into the inoculated culture TS Agar in the test tubes. All the eight tubes were carefully swirled to effect even distribution. Swirling was preferred to shaking to prevent formation of bubbles. The tube contents were then poured into eight clean, dry, previously sterilised petri dishes; allowed to stand for 30 minutes to solidify and then incubated at 37º C for 18-24 hours. On completion of the incubation period the Petri dishes were examined visually and the lowest concentration that completely inhibited microbial growth noted.

CHAPTER FOUR

4.0 RESULTS AND DISCUSSION

4.1 PHYTOCHEMICAL PROFILES

Several UV-active (254 nm) and one non UV active but iodine active spot were detected for the methanol and CH_2Cl_2:MeOH (1:1) crude extracts. The CH_2Cl_2:MeOH (1:1) extract was found to have more UV active (254 nm) spots than the methanol extract and was therefore selected for further chromatographic analysis.

4.2 CHARACTERIZATION THE COMPOUNDS ISOLATED FROM *MILLETTIA OBLATA* ROOT EXTRACT

The crude *Millettia oblata* root extract afforded five compounds which were isoerythrin A 4'-(3-methylbut-2-enyl) ether (**1**), calopogonium isoflavone B (**2**), 4- hydroxylonchocarpin (**3**), 7,2'-dimethoxy-4',5'-methylenedioxyisoflavone (**4**) and lupeol (**5**).

4.2.1 Isoerythrin A 4'-(3-methylbut-2-enyl) ether (1)

The ^{1}H (δ 8.01, 1H, for H-2) (Markham, 1982) and ^{13}C NMR (δ 152 methine for C-2; 125.0 quaternary for C-3 and 176.2 carbonyl for C-4 (Agrawal, 1989) implied that compound **1** is an isoflavone derivative (Table 4.1 and 4.2).

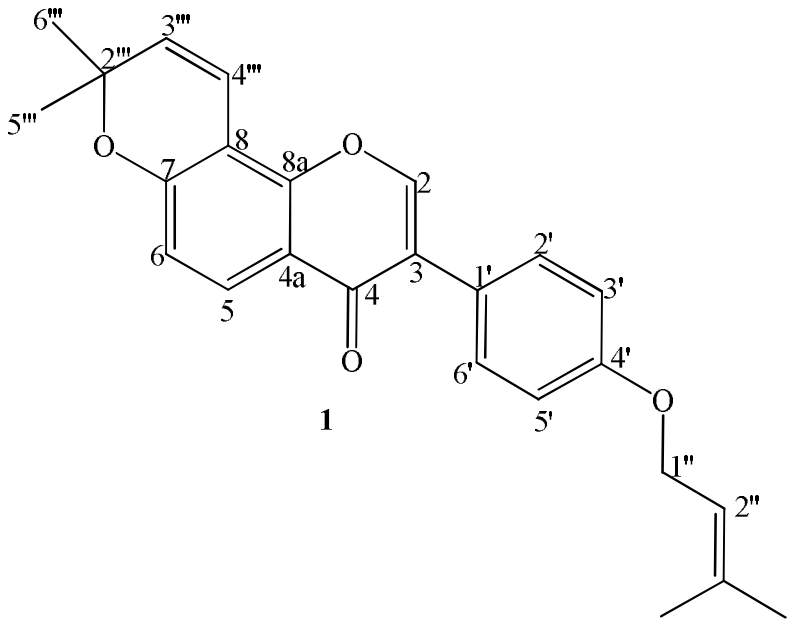
The ^{1}H NMR spectrum (Appendix 1a) of this compound also indicated the presence of a *cis*-oleffinic system consisting of two doublets at δ 5.76 and 6.88 (*d, J*=10 Hz) as well as two methyl groups (δ 1.57, *s,* 6H) indicating the presence of a 2,2–dimethylpyrano

substituent. The presence of this group was further supported by ^{13}C signal sets at δ 28.4, 77.3, 115.5 and 130.4 (Appendix 1b).

The ^{1}H NMR spectra (Appendix 1a) further indicated the presence of a prenyloxy group (δ 5.56 (1H, *t*, *J*=8.0 Hz); 4.54 (2H, *d*, *J*=8.0 Hz); 1.82 (3H, *s*) and 1.77(3H, *s*)). The resonance of the corresponding carbons at δ 64.8 (methyleneoxy), 119.5 (methine), 138.3 (quaternary), 28.8 (methyl) and 18.2 (methyl) as observed in the ^{13}C NMR spectra (Appendix 1b) further supported the presence of the prenyloxy group.

The ^{1}H NMR (Appendix 1a) showed an AA'XX' system at δ 7.02, 7.55 (2H, *d*, *J*=8.5 Hz) assigned to a mono-substituted ring B and AX spin system centered at δ 8.13 and 6.93 (1H, *d*, *J*=8.8 Hz) corresponding to ring A protons. This is consistent with placing the prenyloxy group at C-4' and the pyrano group in ring A at either 5/6 or 7/8 position with the biogenetically expected oxygenation at C-7. The fact that one of the AX spin system proton is highly deshielded, δ 8.13, indicated that this proton is close to the carbonyl group and therefore was assigned to H-5. This will unequivocally place the pyrano group at 7/8 position.

Based on this and comparison of the spectral data with that reported in literature (Derese, 2004) compound **1** was identified as isoerythrin A 4'-(3-methylbut-2-enyl) ether (**1**). This compound has been previously isolated, (Yenesew *et al.*, 1996; Derese, 2004) from the seed pod and stem bark of *Millettia dura*, respectively, but this is the first report of this compound from the root of *Millettia oblata*.



1

Table 4.1: ^{1}HNMR (200 MHz) data for Isoerythrin A 4'-(3-methylbut-2-enyl) ether (1) in chloroform $CDCl_3$

Position	**$\delta^{1}H_{obs}$, *m*, (*J* in Hz)**	**$\delta^{1}H_{lit}$ [a] , *m*, (*J* in Hz)**	**Position**	**$\delta^{1}H_{obs}$, *m*, (*J* in Hz)**	**$\delta^{1}H_{lit}$ [a], *m*, (*J* in Hz)**
2	8.00 *s*	7.94 *s*	2"	5.56 *t*	5.51 *t* (8.0)
5	8.13 *d* (8.8)	8.07 *d* (8.4)	3''		
6	6.93 *d* (8.8)	6.85 *d* (8.4)	4''-Me	1.82 *s*	1.82*s*
7			5"-Me	1.87 *s*	1.87*s*
2'	7.55 *d* (8.5)	7.50 *d* (10.0)	2"'		
3'	7.02 *d* (8.5)	6.98 *d* (10.0)	3"'	5.76 *d* (10.0)	5.72 d(10.0)
5'	7.02 *d* (8.5)	6.98 *d* (10.0)	4"'	6.88 *d* (10.0)	6.81 d(10.0)
6'	7.55 d (8.5)	7.50 *d* (10.0)	5'''-Me	1.57 *s*	1.77 *s*
1"	4.61 *d* (6.6)	4.54 *d* (8.0)	6'''-Me	1.57 *s*	1.77 *s*

a assignment according to Derese, 2004

Table 4.2: ^{13}C NMR data (50 MHZ) for isoerythrin A, 4'-(3-methylbut-2-enyl) ether (1) in $CDCl_3$

Position	**δ $^{13}C_{obs}$**	**δ $^{13}C_{lit}$ [a]**	**Position**	**δ $^{13}C_{obs}$**	**δ $^{13}C_{lit}$ [a]**
2	152.0	151.7	5'	115.0	114.6
3	125.0	124.7	6'	130.6	130.2
4	176.2	175.9	1"	65.1	64.8
4a	118.7	118.3	2"	119.9	119.5
5	127.0	126.7	3''	138.6	138.4
6	115.3	115.2	4''-Me	18.5	18.2
7	157.5	157.2	5"-Me	26.2	25.8
8	109.5	109.1	2"'	77.3	77.0
8a	152.7	152.3	3"'	130.4	130.1
1'	124.4	124.1	4"'	115.5	114.9
2'	130.6	130.2	5'''-Me	28.4	28.1
3'	115	114.6	6'''-Me	28.4	28.1
4'	159.2	158.8			

[a] assignment according to Derese, 2004

4.2.2 Calopogonium isoflavone B (2)

Isoflavone NMR spectral data (Table 4.1 and Table 4.2) characteristic features observed in compound **1** above were also present in compound **2** and therefore this compound was identified as an isoflavone derivative.

The ^{1}H NMR (Appendix 2a) indicated the presence of a 2, 2–dimethylpyrano system {δ 5.73 and 6.87 (1H, *d*, *J*=11Hz) for *cis* olefin protons; two methyl groups (δ 1.56, *s*, 6H)} while the corresponding carbons resonated at δ (131.0 (C-3'''),125.0 (C4''') and 28.4 (C-

5''' and C-6''') (Table 4.3;Appendix 2b) The NMR also showed the presence a methylenedioxy group ((δ_H 6.04 (*s*, 2H), δ_C (101.5)).

The ^{1}H NMR (Appendix 2a) further showed ABC aromatic protons (δ 6.92 (*d*, *J=8.1* Hz), 7.15 (*brs*) and 7.03 (*d*, *J=8.1* Hz)) and an AX (δ 8.12 and 6.92 (*d*, *J*=8.6 Hz)) spin systems. The highly deshielded proton, δ 8.12, was assigned to H-5 and therefore the AX protons were assigned to ring A, H-5 and H-6. This implied that the ABC protons are in ring B. Following the biogenetically expected oxygenation at position 4' these protons were attributed to H-2', H-5' and H- 6' of a 3'4' di-substituted ring B.

The methylenedioxy group and the pyrano ring can be at either C-7/C-8 or C-3'/C-4' positions. The methylenedioxy group was placed at C-3'/C-4' which was in agreement with the ^{13}C NMR data which indicated presence of two adjacent oxygenated aromatic carbons at position 3' and 4' (δ 147.95 (C-3') and 149.91 (C-4')). Placing the methylenedioxy at C-7/C-8 would result in three adjacent oxygenated aromatic carbons with one of them resonating at about 130 ppm. Based on this and comparison of the NMR data with literature (Table 4.3) compound 2 was identified as calopogonium isoflavone B (**2**) (Dagne *et al*., 1989; Murthy *et al*., 1985). Calopogonium isoflavone B (**2**) has been previously reported from *Millettia ferruginea* (Dagne *et al*., 1989) and *Tephrosia maxima* (root) (Murthy *et al*., 1985) but nevertheless it is the first report of calopogonium isoflavone B in *Millettia oblata*.

Table 4.3: ^{1}H and ^{13}C NMR (50MHZ) data for Calopogonium isoflavone B (3) in $CDCl_3$

Carbon position	**$\delta\ ^{1}H_{obs}$ (200 MHz), *m*, (*J* in Hz)**	**$\delta^{13}C_{obs}$**	**$\delta^{1}H_{lit}$[b] (250MHz), *m*, (*J* in Hz)**
2	7.98 *s*	152.2	7.93
3		118 56	
4		176.03	
4a		116.7	
5	8.12 *d* (8.6)	130.4	8.05 *d* (8.8)
6	6.92 *d* (8.6)	108.7	6.86 *d* (8.8)
7		157.6	
8		109.5	
8a		152.6	
1’		127	
2’	7.150 *brs*	110.1	7.09
3’		147.9	
4’		147.9	
5’	6.92 *d* (8.1)	115.2	6.86 *d* (8.0)
6’	7.03 *d* (8.1)	122.7	6.97 *d* (8.0)
2’’-OCH_2O	6.04 *s*	101.5	5.99
2”’		77.3	
3”’	5.73 *d* (11.4)	131.0	5.72 *d* (10.0)
4”’	6.87 *d* (11.4)	125.0	6.81*d* (10.0)
5’’’-Me	1.56 *s*	28.4	1.50
6’’’-Me	1.56 *s*	28.4	1.50

[b] assignment according to Dagne *et al*., 1989

4.2.3 4-Hydroxylonchocarpin (3)

Compound **4** was isolated as orange crystals that intensified on exposure to ammonia vapour and gave a yellow spot on TLC which changed to brown on exposure to iodine. The NMR spectral data of compound **4** (Table 4.4; appendix 3a and 3b)) was consistent with that of a chalcone ((δ_H 7.74 (*d*, *J*=15.4 Hz) for H-α and 7.87 (*d*, *J*=15.4 Hz) for H-β for *Trans* olefinic protons) and δ_C (117.4 for C-α; 144.9 for C-β) and 192.5 for C=O) (Agrawal, 1989).

The NMR spectra (Appendix 3a and 3b) further showed the presence of chelated hydroxyl group (δ 14.07, H–2') and a 2,2-dimethylpyrano-substituent (Table 4.4). The ^{1}HNMR (Appendix 3a) further indicated the presence of aromatic protons with an AX spin system δ 8.06 and 6.37 (1H, *d*, *J*=8.9 Hz) and an AA'XX' spin system δ 7.74 and 6.93 (*J*=2.0, 6.6 Hz). One of the AX protons was highly deshielded due to the fact that it lies in the deshielding zone of the carbonyl group and was therefore assigned H-6'. This positioned the AX and AA'XX' aromatic protons in ring B and A, respectively. In order to accommodate the two aromatic proton spin systems the 2, 2-dimethylpyrano group was placed in ring B. The presence of the AA'XX' spin system in ring A also indicated that this ring is para-substituted. Compound **4** was therefore identified as 4-hydroxylonchocarpin **(3)**. 4-Hydroxylonchocarpin **(3)** has been previously reported from *Millettia ferruginea* (Dagne *et al.*, 1989), however, this is its first report in *Millettia oblata* root.

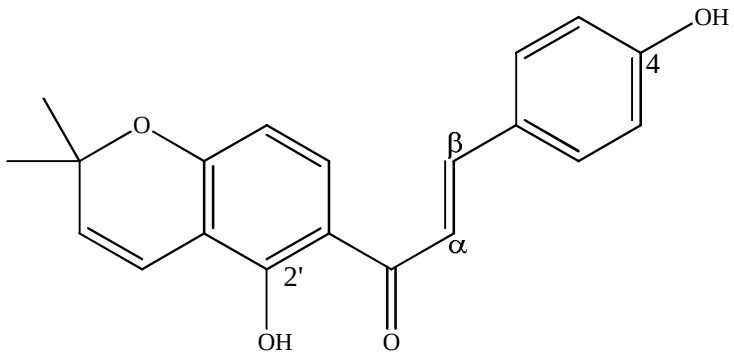


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Table 4.4: ^{1}H and ^{13}C NMR data for 4- Hydroxyonchocarpin (3) in acetone-d_6

Carbon position	**$\delta^{1}H_{obs}$ (200 MHz), m, (*J* in Hz)**	**$\delta^{13}C_{obs}$ (50MHz)**	**$\delta^{1}H_{lit}$[c] (250 MHz), m , (*J* in Hz)**	**δC_{lit}[c] (50 MHz)**
C=O		192.5		193.2
2	7.74 *dd* (2.0,6.6)	131.3	7.74 *d* (8.5)	131.9
3	6.94 *m*	116.7	6.92 *d* (8.5)	116.8
4		161.1		161.7
5	6.94 *m*	116.7	6.92 *d* (8.5)	116.8
6	7.74 *dd* (2.0,6.6)	131.3	7.74 *d* (8.5)	131.9
1’		114.3		115.0
2’	14.09 *s*	160.5	14.07 *s*	161.1
3’		108.2		108.8
4’		159.8		160.4
5’	6.37 *d* (8.9)	109.2	6.36 *d* (8.8)	110.0
6’	8.04 *d* (8.9)	128.6	8.06 *d* (9.0)	129.2
A	7.74 *d* (15.4)	117.4	7.70 *d* (15.4)	118.2
B	7.87 *d* (15.4)	144.9	7.86 *d* (15.4)	145.5
2’’		77.8		78
3”	5.69 *d* (9.9)	115.7	5.71 *d* (9.9)	116.3
4”	6.71 *d* (9.9)	131.6	6.69 *d* (9.9)	132.2
5”-Me	1.44 *s*	28.5	1.44 *s*	28.5
6”-Me	1.44 *s*		1.44 *s*	

[c] assignment according to Dagne *et al*., 1989

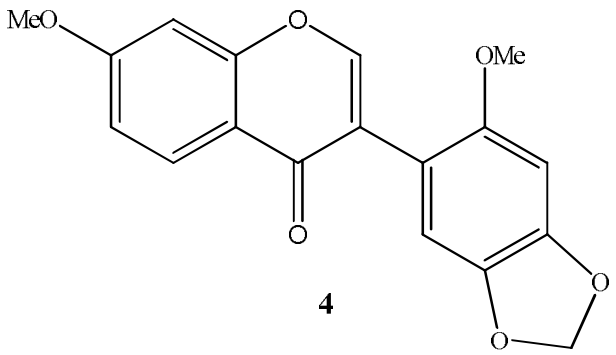
4.2.4 7, 2’-Dimethoxy-4’, 5’- methylenedioxyisoflavone (4)

The NMR spectra data (Table 4.5 and 4.6) indicated that this compound is an isoflavone derivative). The NMR spectra (Appendix 4a and 4b) also showed the presence of two methoxy groups {δ_H (3.71 and 3.81 (3H, s); δ_C 56.9 and 55.9)} and methylenedioxy substituent ((δ_H 5.96 (*s*, 2H), δ_C (101.4)) (Table 4.5 and 4.6)).

The ^{1}HNMR (Appendix 4a) further indicated the presence of aromatic protons with an AXY spin system (δ 8.19 (1H, *d, J*=8.4 Hz), 6.98 (1H, *dd, J*=2.6, 9.2 Hz)) and 6.85(1H, *d, J*=2.6 Hz)) and two siglets at δ 6.63 and 6.83 (1H, *s*).

One of the protons in the AXY spin system is highly deshielded and is therefore assigned H-5. This implies the AXY aromatic protons are in ring A and two singlet aromatic protons are in ring B. This places one methoxy group at C-7 which also agrees with the biogenetic oxygenation of this position. The second methoxy and methylenedioxy groups were consequently placed in ring B. In order to allow for the presence of two singlets and the biogenetic oxygenation of C-4', the methoxy and methylenedioxy groups were placed at C-2' and C-4'/C-5', respectively. This placement is further supported by ^{13}C NMR (Appendix 4b) δ_C (152.9, 148.3, and 141.4) which corresponds to three aromatic quaternary enolic carbons with a methoxy group *para* to one of the enolic carbons δ_C (141.4). This compound is thus identified as 7,2'-dimethoxy-4',5'-methylenedioxyisoflavone (**3**) was found to have similar spectral data as previously identified compound (Nigel e*t al* ., 2003).

7, 2'-dimethoxy-4', 5'- methylene dioxyisoflavone (**3**) has been previously reported from leaves of *Ateleia Herbert-smithii* (Nigel e*t al*., 2003), stem bark of *M. Dura* (Derese, 2004; Dagne *et al.*, 1991) and root bark of *M. griffonianone* (Yankep *et al.*, 1997). This is however it's first report in the root of *Millettia oblata*.



4

Table 4.5: ^{1}H NMR data for 7, 2'-dimethoxy-4',5'methylenedioxyisoflavone in $CDCl_3$

Position	**$\delta^{1}H_{obs}$ (200 MHz), *m*, (*J* in Hz)**	**$\delta^{1}H_{lit}$[d] (400 MHz), *m*, (*J* in Hz)**	**Position**	**$\delta^{1}H_{obs}$ (200 MHz), *m*, (*J* in Hz)**	**$\delta^{1}H_{lit}$[d] (400 MHz), *m*, (*J* in Hz)**
2	7.89 *s*	7.88 *s*	1'		
3			2'		
4			3'	6.63 *s*	6.62 *s*
4a			4'		
5	8.19 *d* (8.4)	8.19 *d* 8.8	5'		
6	6.98 *dd* (9.25,2.6,)	6.98 *dd* (8.8, 2.4)	6'	6.83 *s*	6.83 *s*
7			2''-OCH_2O	5.96 *s*	5.95 *s*
8	6.98 *d* (2.6)	6.85 *d* (2.4)	2'-OMe	3.73 *s*	3.73 *s*
8a			7-OMe	3.81 *s*	3.91 *s*

[d] assignment according to Nigel *et al*., 2003

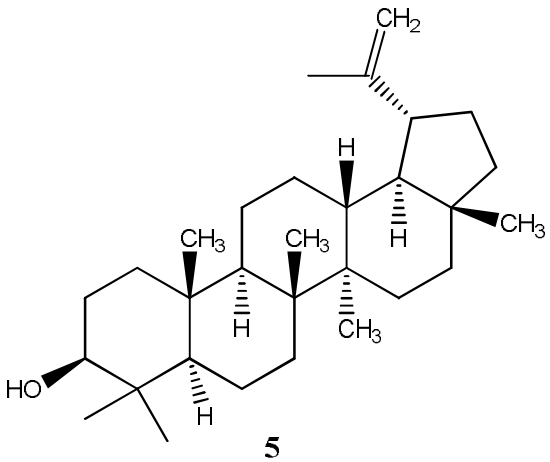
Table 4.6: ^{13}C NMR (50MHz) data for7, 2'-dimethoxy-4',5'- methylenedioxyisoflavone in $CDCl_3$.

Carbon position	**$\delta^{13}C_{obs}$**	**$\delta^{13}C_{lit}$[d]**	**Carbon Position**	**$\delta^{13}C_{obs}$**	**$\delta^{13}C_{lit}$[d]**
2	154.0	153.7	2'	152.9	153.1
3	122.1	122.3	3'	95.4	95.5
4	175.7	175.8	4'	148.3	148.5
4a	118.4	118.5	5'	141.4	141.3
5	127.8	127.7	6'	111.2	111.0
6	114.3	114.2	2''-OCH_2O	101.4	100.9
7	163.8	163.9	2'-OMe	56.9	57
8	100.1	100.1	7-OMe	55.7	55.9
8a	157.9	157. 9			
1'	112.2	112.9			

[d] assignment according to Nigel *et al*., 2003

4.2.5 Lupeol (5)

Compound **5** was isolated as non UV active white crystals and was detected on TLC using iodine. This compound was identified as lupeol by co-spoting with an authentic sample. Lupeol is ubiquitous in nature but this is the first report of this compound in this plant.



5

4.3 BIOLOGICAL ACTIVITIES

4.3.1 *In* vitro anti-plasmodial activity

4.3.1.1 *In-vitro* anti-plasmodial activity of crude extracts

Activity of crude extracts was considered high if IC_{50} ≤10 µg/ml, moderate between 11 and 50 µg/ml, low between 51 and 100 µg/ml and inactive when $1C_{50}$ was above 100 µg/ml (Muregi *et al*., 2003).

The CH_2Cl_2:MeOH (1:1) *Millettia oblata* root extract showed high (IC_{50} 8.26 ±1.7µg/ml) and moderate (IC_{50} 11.49 µg/ml) anti-plasmodial activity against chloroquine-sensitive (D6) and resistant (W2) strains of *Plasmodium falciparum,* respectively. The MeOH extract of *Millettia oblata* root showed moderate anti-plasmodial activity (IC_{50}14.84 µg/ml) against chloroquine-sensitive (D6) strain of *Plasmodium falciparum* but with no activity against the chloroquine resistant strain. The extracts exhibited higher activity against the chloroquine sensitive strain than the chloquine resistant.This is not always the case for all extracts (Derese *et al.,* 2004; Muregi *et al.,* 2003).The CH_2Cl_2:MeOH (1:1) extract also showed higer activity than the methanol extract.This may be due to the difference in the

phytochemical composition of the extracts resulting in the more lipophilic CH_2Cl_2:MeOH (1:1) exhibiting higher activity.

4.3.1.2 *In vitro* anti-plasmodial activity of pure compounds

Pure compounds were considered to have high activity at $IC_{50} \leq 2\mu M$, moderate at $2< IC_{50} \leq 11\mu M$ and low at $11< IC_{50} \leq 50\mu M$. IC_{50} exceeding 50μM were considered inactive (Bero *et al.*, 2009). All the flavonoids exhibited moderate to low anti-plasmodial activity with exception of 7,2'-dimethoxy-4',5'-methylenedioxyisoflavone (**4**) which showed no activity against chloroquine resistant (W2) strain. Previous reports have indicated that some flavonoids exhibit anti-plasmodial activity (Derese, 2004; Bero *et al*., 2009; Muiva *et al.*, 2009). Isoerythrin A 4'-(3-methyl but-2-enyl) ether (**1**) was found to have higher anti-plasmodial activity than the other tested flavonoids. Previous work has indicated that prenylated flavonoids have higher activity than non prenylated flavonoids due to their higher lipophilicity (Bero *et al*., 2009). However, unlike in earlier reports (Derese, 2004; Ngamga *et al*., 2005) all the compounds showed higher activity against the chloroquine sensitive (D6) *P. falciparum* strain than the chloroquine resistant (W2) strain. The *in vitro* IC_{50} values and anti-plasmodial activity of the pure compounds are summarized in table 4.7 and 4.8 respectively.

Table 4.7: *In vitro* IC_{50} values of the isolated flavonoids of *M. oblata root* against W2 and D6 strains of *Plasmodium falciparum*.

Compound	**IC_{50} in (μM)**	
	W2	**D6**
Isoerythrin A 4'-(3-methylbut-2-enyl) ether (**1**)	15.10 ± 4.8	6.61 ± 2.8
Calopogonium isoflavone B (**2**)	29.05	9.60 ± 1.15
4-Hydroxylonchocarpin (**3**)	42.36	17.35 ± 1.7
7,2'-Dimethoxy-4',5'-methylenedioxyisoflavone (**4**)	97.90	38.02
Chloroquine	0.29 ±0.1	0.019±0.002
Mefloquine	0.0037±0.02	0.0413

Table 4.8: *In vitro* activity of isolated flavonoids of *M. oblata root* against W2 and D6 strains of *Plasmodium falciparum*.

Compound	**Activity**	
	D6	**W2**
Isoerythrin A 4'-(3-methylbut-2-enyl) ether (**1**)	Moderate	Low
Calopogonium isoflavone B (**2**	Moderate	Low
4-Hydroxylonchocarpin (**3**)	Low	Low
7,2'-Dimethoxy-4',5'-methylenedioxyisoflavone (**4**)	Low	Inactive

4.3.1.3 Influence of the hydrocarbon proportion on anti-plasmodial activity

Elemental analysis of the anti-plasmodial flavonoids indicated that the higher the hydrocarbons proportion in comparison to oxygen the higher the anti-plasmodial activity with exception of the chalcone. The higher hydrocarbon proportion most probably resulted in higher lipid solubility of the compound and consequently better penetration of the red

blood cells as well as the parasites. This resulted in high test compound concentrations at the receptor site and consequently higher activity. The lower activity observed with the chalcone compared to calopogoniumisoflavone B despite the chalcone having a higher hydrocarbon proportion is most probably due to its lower lipid solubility accounted for by the hydroxyl groups. Table 4.9 shows the percentage elemental analysis of the *Millettia oblata* root flavonoids compared with their anti-plasmodial activity.

Table 4.9: Elemental analysis of the *Millettia oblata* flavonoids compared to their anti-plasmodial activity

Order of Activity	**Name of Compound**	**Molecular Formula**	**% Carbon**	**% Hydrogen**	**% Oxygen**
1	Isoerythrin A 4'-(3-methyl-2-butenyl) ether **(1)**	$C_{25}H_{24}O_4$	77.30	6.23	16.47
2	Calopogoniumisoflavone B (**2**)	$C_{21}H_{16}O_5$	72.41	4.63	22.96
3	4-Hydroxylonchocarpin **(3)**	$C_{20}H_{18}O_4$	74.52	5.63	19.85
4	7,2'-Dimethoxy-4',5'-methylenedioxyisoflavone (**4**)	$C_{18}H_{14}O_6$	66.26	4.32	29.42

Where (1-4) represents the anti-plasmodial activity in decreasing order

4.3.2 Anti-bacterial activity

4.3.2.1 Pre-liminary anti-bacterial activity evaluation

The size of the zone of inhibition was indicative of the anti-bacterial activity with inhibition diameters above 8 mm considered as positive results (activity). Exhibition of anti-bacterial activity by several plant extracts has been previously reported (Seyydnejad *et al*., 2010; Mahesh and Satish, 2008). The *Millettia oblata* root (CH_2Cl_2:MeOH (1:1) and

methanol) crude extracts and compound 3 were active while compounds **1**, **2**, and **4** were inactive. The CH_2Cl_2:MeOH (1:1) extract showed higher activity against all the three test bacteria compared to the methanol extract . The CH_2Cl_2:MeOH (1:1) extract also showed anti-bacterial activity at all the specified concentrations 0.72 - 5.75 mg/well. The methanol extract was inactive against *B. pumilus* and *S. aureus* at 0.72 mg/well and inactive against *E. coli* at concentrations ≤ 2.875 mg/well (Table 4.10). The higher resistance of *E.coli* to the methanoic extract is supported by Seyydnejad *et al*., 2010. The inhibition zones of the CH_2Cl_2:MeOH (1:1) *Millettia oblata* root extract against *Bacillus pumilus* (B.P) were not completely clear zones which may indicate presence of resistant strains whose growth was not effectively inhibited.

4-Hydroxylonchocarpin (**3**) the only active pure compound showed higher activity than the extracts against all the three test bacteria at all the specified concentrations. The results indicate that the various constituents in the extract were inhibitory rather than synergistic, a characteristic that is occasionally observed (Heinrich *et al*., 2008). Tables 4.10 and 4.11 show the inhibition zones (mm) of the crude extracts and 4-hydroxylonchocarpin (**3**) against the study bacteria, respectively. Figures 4.1 and 4.2 show the inhibition zones of CH_2Cl_2:MeOH (1:1) against *B. pumilus* and compound 3 against *S. aureus*, respectively.

4.3.2.2 Calculation of concentration in mg/well

Concentrations in mg/ml were converted into concentrations in mg/well which were indicative of the total amount of test compound in the well.

The concentration in mg/well was calculated by:

$$\text{Concentration in mg/well} = \frac{\text{Amount (mg) of test compound in 1 ml} * 50\mu l}{1000\ \mu l}$$

Where 50 µl is the volume of test compound in each well

.

Table 4.10: Anti-bacterial activity of ((CH_2Cl_2: MeOH, 1:1) and methanol) extracts and gentamycin (Std) against the study bacteria using agar diffusion method

		Inhibition zones (mm)					
		CH_2Cl_2: MeOH (1: 1)			**Methanol**		
Well No.	**Conc. mg/well**	***B.P***	***S.A***	***E.C***	***B.P***	***S.A***	***E.C***
1	5.75	13.78	10.98	9.79	9.69	10.23	9.17
2	2.875	12.26	10.89	9.11	8.49	9.45	7.70
3	1.43	11.13	10.16	9.07	8.38	9.07	7.70
4	0.72	8.96	9.54	8.89	7.70	7.70	7.70
Std	0.0175	27.19	25.35	23.05	26.06	24.86	22.25

Key:

B.P-Bacilus pumilus *S.A-Staphylococcus aureus* *E.C-Escherichia coli*

Table 4.11: Anti-bacterial activity of 4-hydroxylonchocarpin (3) and gentamycin (Std) against the study bacteria usig agar diffusion method.

		Inhibition zones (mm)		
Well No.	**Conc. mg/ well**	***B.P***	***S.A***	***E.C***
1	2	12.22	12.05	11.53
2	1	12.19	11.69	11.45
3	0.5	12.16	11.50	11.28
4	0.25	11.70	11.49	11.25
Std(Gen)	0.0175	25.47	24.90	21.57

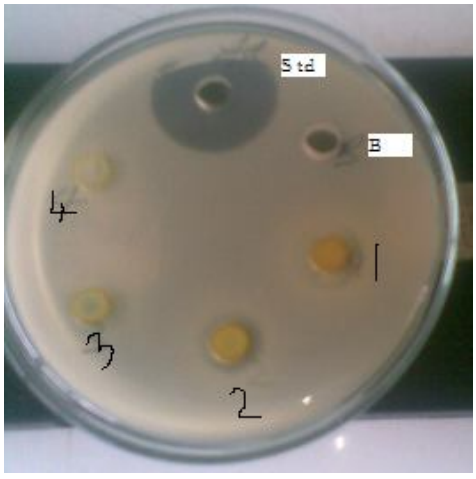


Figure 4.1: Growth inhibition zone of CH_2Cl_2:MeOH (1:1) (mm) against B.pumilus

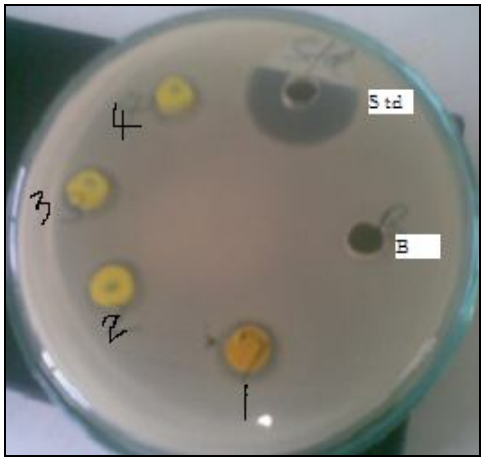


Figure 4.2: Growth inhibition zone of compound **3** against *B.Pumilus*

Key:

Std-Standard	Gen-Gentamicin
B-Blank (1% DMSO)	

4.3.2.3 Definitive anti-bacterial activity evaluation

The *Millettia oblata* CH_2Cl_2:MeOH (1:1) extract and 4-hydroxylonchocarpin **(3)** showed anti-bacterial activity against all the bacteria at all the specified concentrations.

The CH_2Cl_2:MeOH (1:1) extract as well as compound **3** showed higher activity against *E. coli* than *B. pumilus* and *S. aureus* (Tables 4.12 and 4.13). This may be an indication of better membrane penetration by the Gram negative micro-organisms compared to Gram positive micro-organisms. This may be due to possible differences in the structure of the cell membranes of the Gram negative and Gram positive micro-organisms (Seyydnejad *et al.*, 2010).

The *Millettia oblata* CH_2Cl_2: MeOH (1:1) extract showed its highest activity against all the study bacteria at 1.289 mg/well rather than at 2.57 mg/well. The anti-bacterial activity of 4-hydroxylonchocarpin against the study bacteria was higher at 0.306 mg/well than at 0.6125 mg/well (Tables 4.12 and 4.13). At high concentrations the molecules readily interact to form micelles resulting in a decrease in the rate and extent of diffusion and consequently decreased activity.

The CIC was below 6.45 and 1.53 mg/ml for the CH_2Cl_2:MeOH (1:1) extract and compound **3**, respectively. Table 4.12 and figures 4.3, 4.4 and 4.5 and table 4.13 and figures 4.6, 4.7 and 4.8 show the inhibition zones of the crude extract and compound **3**, respectively.

Table 4.12: *In vitro* anti-bacterial activity of CH_2Cl_2:MeOH (1:1) and gentamycin (Std) against the study bacteria using agar diffusion assay.

	Zone of inhibition and standard deviation (mm)				
	CH_2Cl_2: MeOH (1: 1) conc. (mg/well)				**Std (mg/well)**
Bacteria	**2.57(1)**	**1.29(2)**	**0.65 (3)**	**0.32(4)**	**0.018 (Std**
B.pumilus	9.47± 0.13	19.04± 0.02	14.75± 0.48	8.71± 0.07	25.49± 0.20
S.aureus	11.46 ± 3.15	17.28 ± 0.03	14.75± 0.48	8.80±0.35	25.43±0.49
E.coli	9.60± 0.09	20.32± 0.82	16.12± 0.47	9.53±0.13	26.27±0.82

Values are mean inhibition zone (mm) ± S.D of two replicates

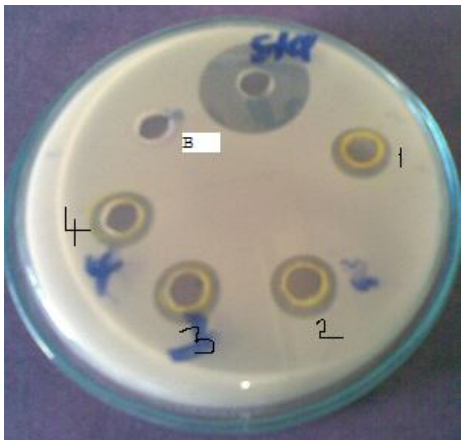


Figure 4.3: Growth inhibition zones of CH_2Cl_2:MeOH (1: 1) against *B. pumilus*

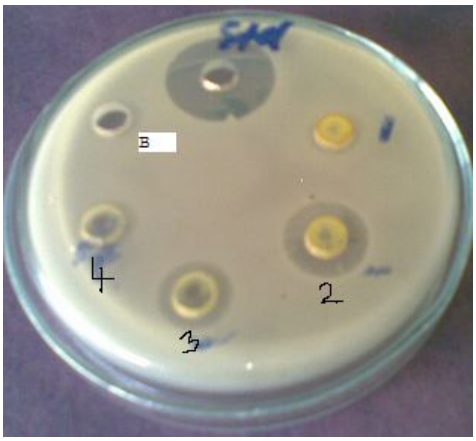


Figure 4.4: Growth inhibition zones of CH_2Cl_2:MeOH (1: 1) against *S. aureus*

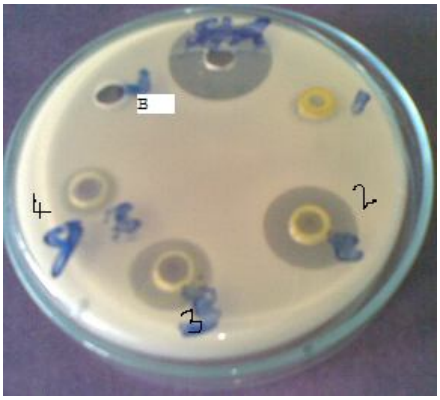


Figure 4.5: Growth inhibition zone of CH_2Cl_2: MeOH (1: 1) against *E. coli*

Table 4.13: Invitro anti-bacterial activity of compound 3 and gentamycin (Std) against the study bacteria using agar diffusion assay

	Zones of growth inhibition and standard deviation (mm)				
	Compound 3 Conc. (µg /well)				**Std (µg /well)**
Bacteria	**612.5**	**306.3**	**153.0**	**76.5**	**17.5**
B. pumilus	12.43±0.38	12.74±0.47	12.80±0.70	12.56±0.86	25.9±7.0
S. aureus	12.65±0.30	13.00±0.30	12.8±0.28	12.57±0.04	25.86
E. coli	13.05±4.25	16.98±0.66	14.13±0.66	13.56±3.56	25.80±0.39

Values are mean inhibition zone (mm) ± S.D of two replicates

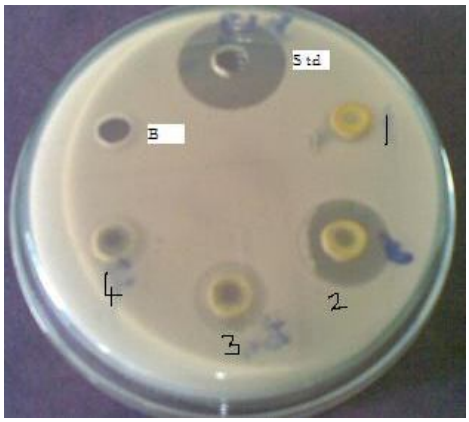


Figure 4.6: Growth inhibition zones of compound 3 against *B. pumilus*

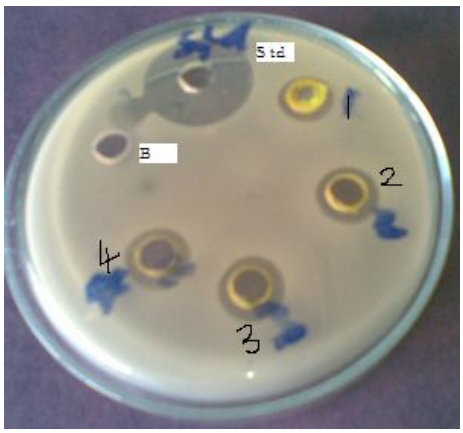


Figure 4.7: Growth inhibition zones of compound 3 against *S. aureus*

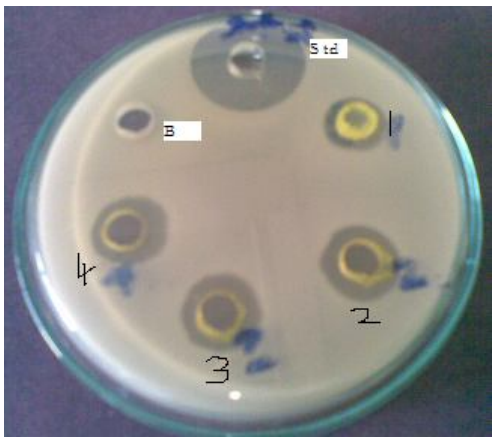


Figure 4.8: Growth inhibition zones of compound 3 against *E. coli*

4.3.3 *In-vitro* anti-fungal activity evaluation

The methanol extract and 4-hydroxylonchocarpin **(3)** showed anti-fungal activity while isoerythrin A 4'-(3-methylbut-2-enyl) ether (**1),** calopogoniumisoflavone B (**2**), 7,2'-dimethoxy-4',5'-methylenedioxyisoflavone (**4**) and the CH_2Cl_2:MeOH (1:1) were inactive. Anti-fungal activity of various plant extracts against *Candida albicans* and other fungi has been previously reported (Abdulmoniem, 2006; Samie *et al*., 2010). Exhibition of activity by the more polar methanol extract is in agreement with previous reports elsewhere using different plants (Abdulmoniem, 2006). The inhibition zones of the methanol extract against *Candida albicans* were not completely clear zones (hazy). This may be due to the presence of resistant strains whose growth was not inhibited by the methanol extract. Table 4.14 shows inhibition zones (mm) of compound **3** and methanol extract wheras figure 4.9 shows inhibition zones of methanol extract against *Candida albicans*.

Table 4.14: *In vitro* anti-fungal activity of *Millettia oblata* methanol extract, 4-hydroxylonchocarpin (3) and nystatin (Std) against *Candida albicans* using agar diffusion method

	Compound 3		**Methanol extract**	
Well No.	**Conc. mg/well**	**Inhibition zone (mm)**	**Conc. mg/well**	**Inhibition zone (mm)**
1	2	10.71±0.23	5.75	10.86±0.87
2	1	10.18±0.35	2.86	10.51±0.65
3	0.5	10.02±0.36	1.43	10.35±0.33
4	0.25	9.58±0.45	0.7	10.27±0.53
Std	0.015	15.21±1.23	0.015	16.97±0.74

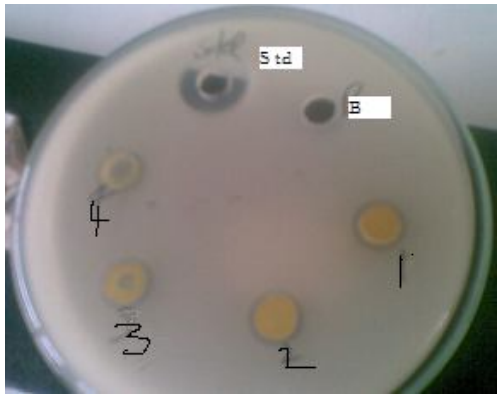


Figure 4.9: Growth inhibition zones of methanol extract against *Candida albicans*

4.3.4 Minimum Inhibitory Concentration Determination

The MIC for 4-hydroxylonchorcapin (**3**) was 2.92 µg /ml while that of the crude extract was 613 µg/ml against *S. aureus* (NC 0 7447), *E. coli* (ATCC 25922) and *B. pumilus* (NC08241). This implies that 4-hydroxylonchocarpin showed higher anti-bacterial potency than the crude extract. However, this is not always the case as mixtures of flavonoids in crude extracts are known to exhibit higher biological activity than the individual single flavonoid either through synergism or other mechanism (Heinrich *et al*., 2004). Figure 4.14 shows two representative Petri dishes A and B with A showing microbial growth and B showing growth inhibition corresponding to the minimum inhibitory concentration.

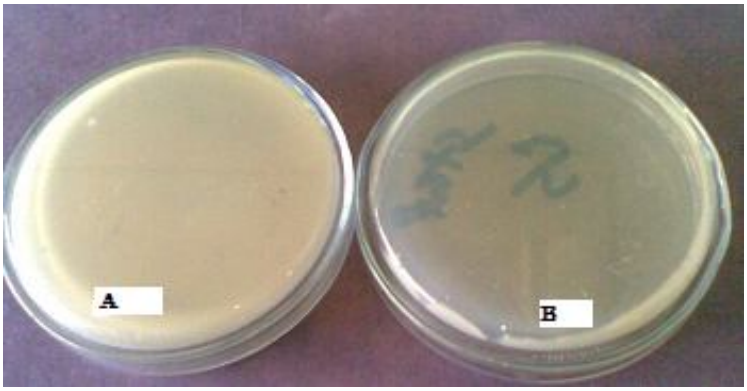


Figure 4.10: Microbial growth (A) and microbial inhibition (B)

CHAPTER FIVE

5.0 CONCLUSIONS AND RECOMMENDATIONS

5.1 CONCLUSIONS

1. Phytochemical investigation of the CH_2Cl_2:MeOH (1:1) *Millettia oblata* root extract led to the isolation and characterisation of three isoflavones (isoerythrin A, 4'-(3-methylbut-2-enyl) ether **(1)**, calopogonium isoflavone B **(2)**, 7,2'-dimethoxy-4'5'-methylene dioxyisoflavone **(4)**) one chalcone (4- hydroxyonchocarpin **(3)**) and a triterpene (lupeol **(5)**).
2. The flavonoids isolated from *Millettia oblata* root were found to have moderate to low anti-plasmodial activity against both D6 and W2 P. *falciparum* strain respectively.
3. Isoerythrin A, 4'-(3-methylbut-2-enyl) ether **(1)** was found to be the most potent anti-plasmodial compound
4. 4-Hydroxylonchorcapin (**3)** and the methanol extract were found to have anti-fungal activity.
5. 4-Hydroxylonchorcapin (**3)**, methanol and CH_2Cl_2:MeOH (1:1) extracts showed anti-bacterial activity.

5.2 RECOMMENDATIONS

1. Further phytochemical studies on *Millettia oblata* root should be carried out in order to fully isolate, identify and characterise all the isolable constituents.
2. *Invivo* anti-plasmodial, anti-bacterial and anti-fungal studies should be carried out on the active pure compounds and crude extracts to determine their potential for drug development and ethno-medical use respectively.

3. Cytotoxicity, acute and chronic toxicity studies should be carried out on the crude extracts and active pure compounds to determine their potential for safe use.

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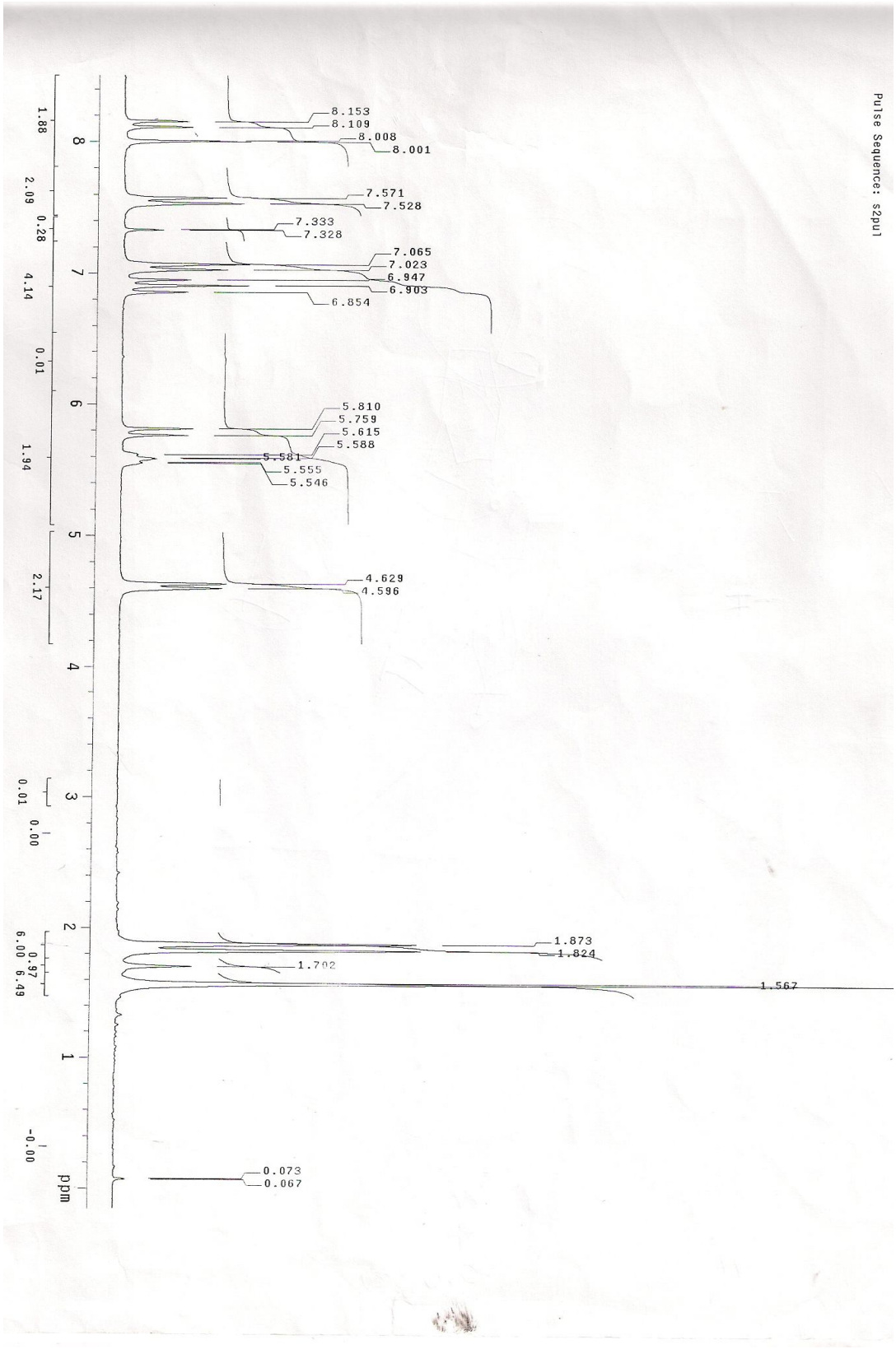
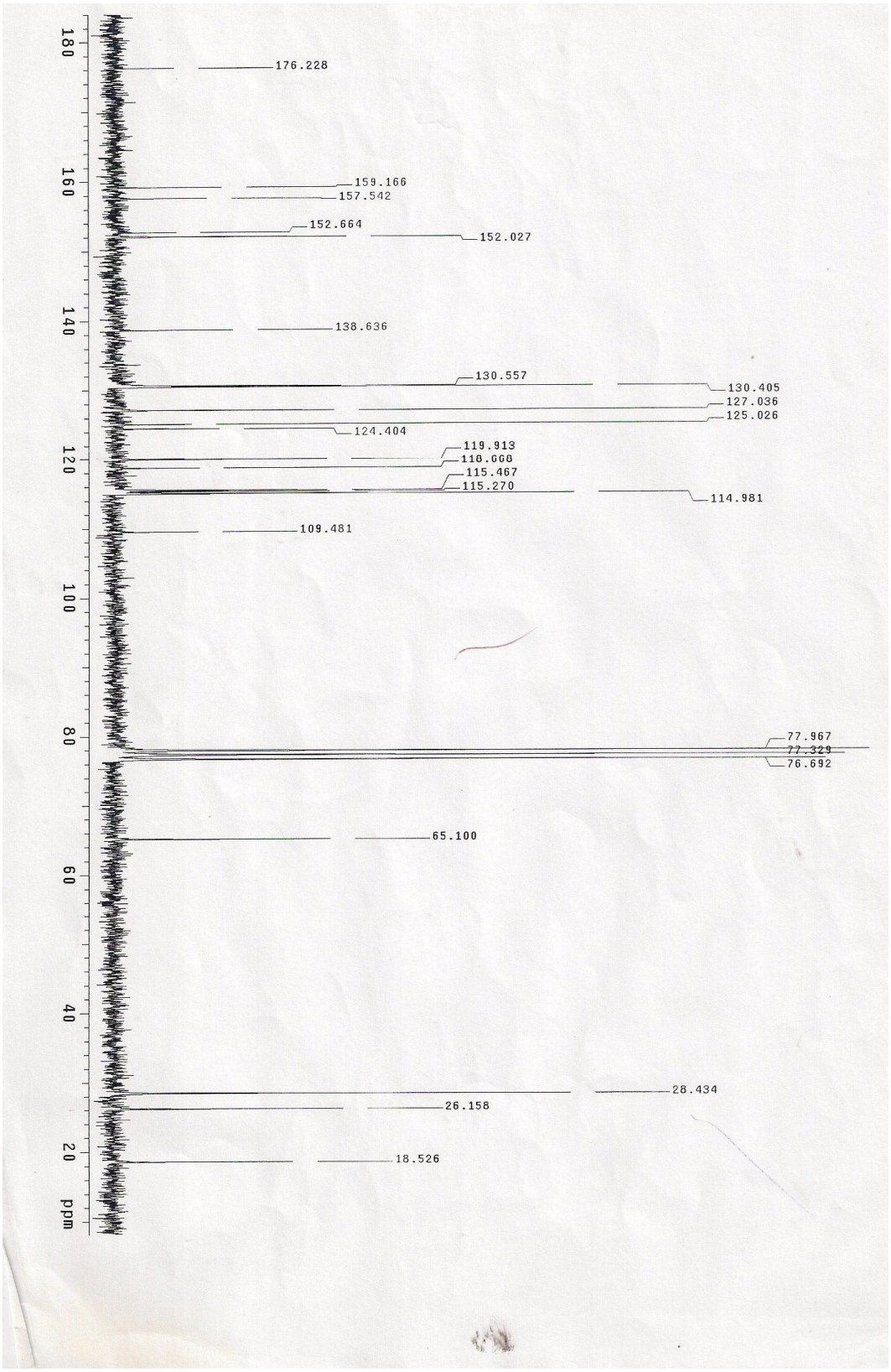
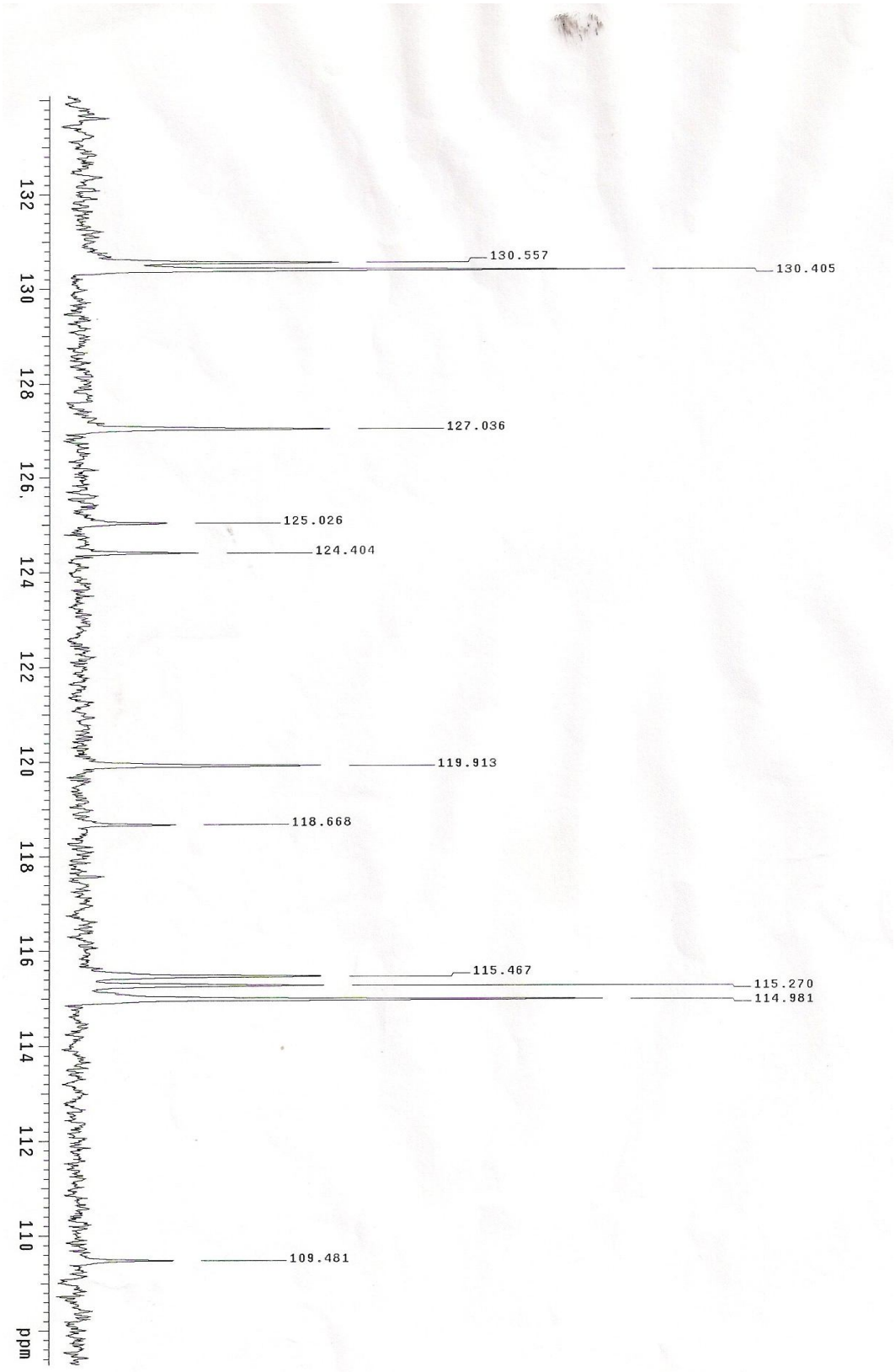
Appendix 1a: ^{1}HNMR SPECTRUM FOR COMPOUND 1



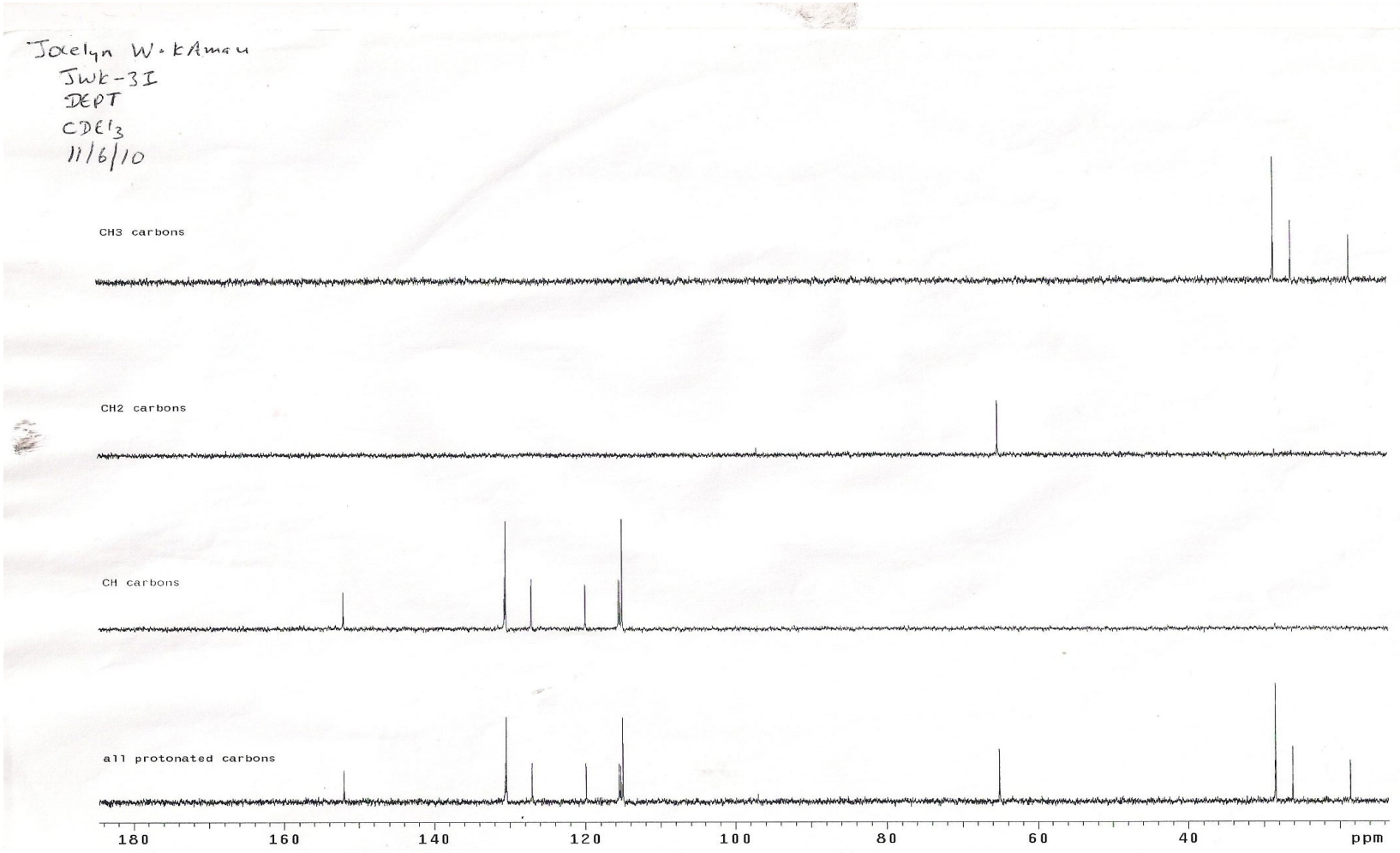
Appendix 1b: ^{13}C NMR SPECTRUM FOR COMPOUND 1



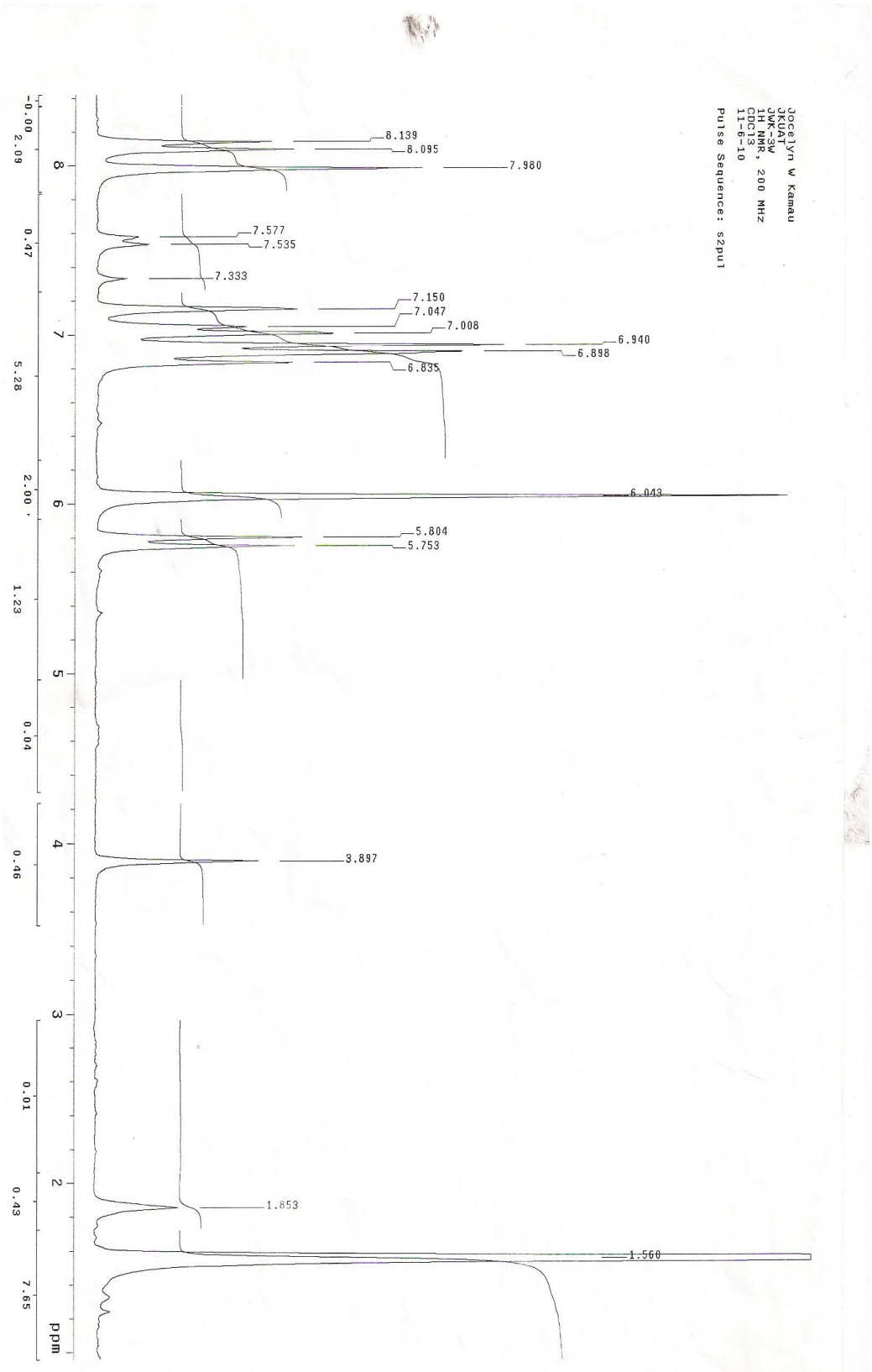
Appendix 1c: ^{13}C NMR SPECTRUM FOR COMPOUND 1



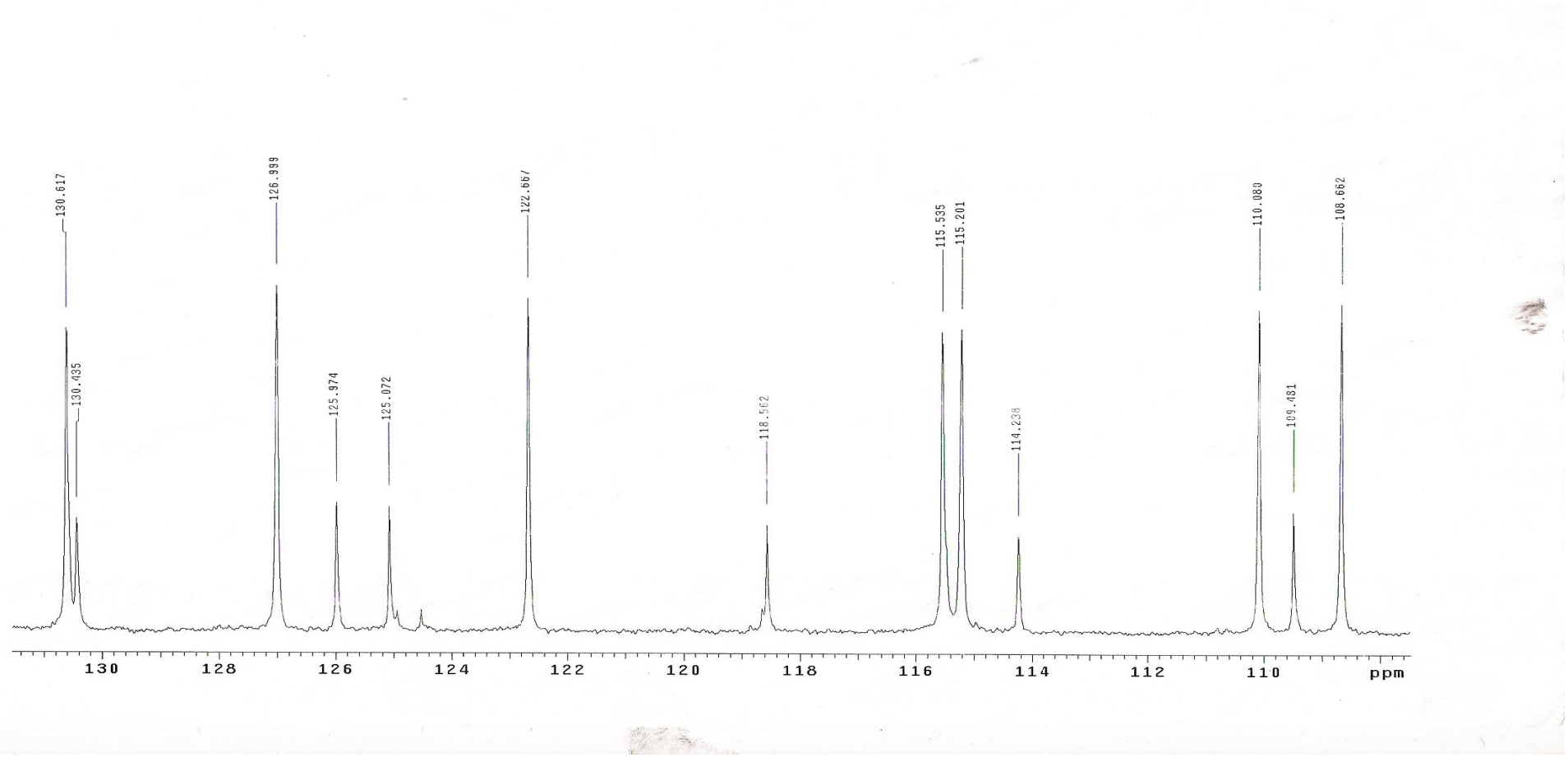
Appendix 1d: DEPT SPECTRUM FOR COMPOUND 1



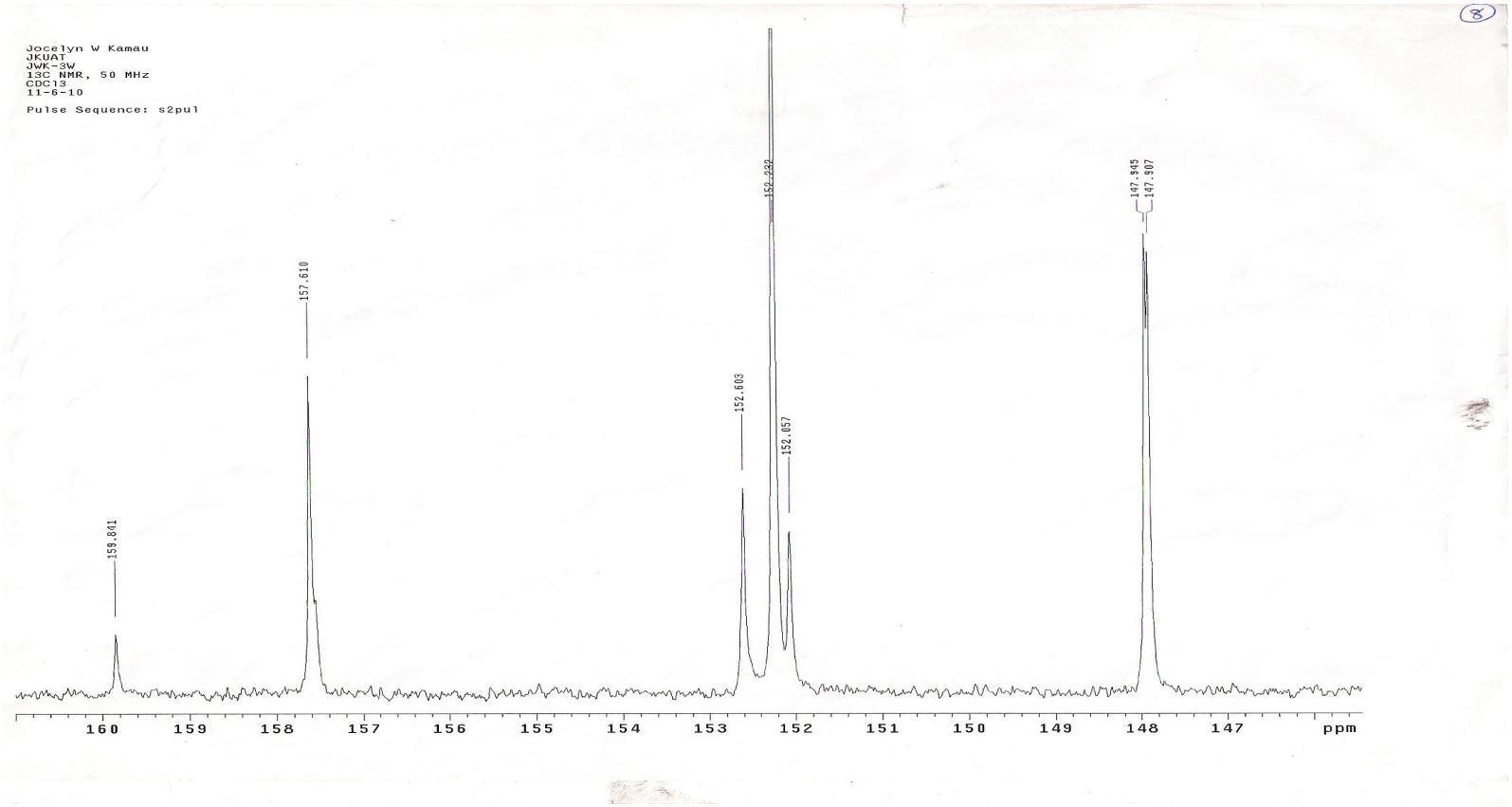
Appendix 2a: ^{1}HNMR SPECTRUM FOR COMPOUND 2



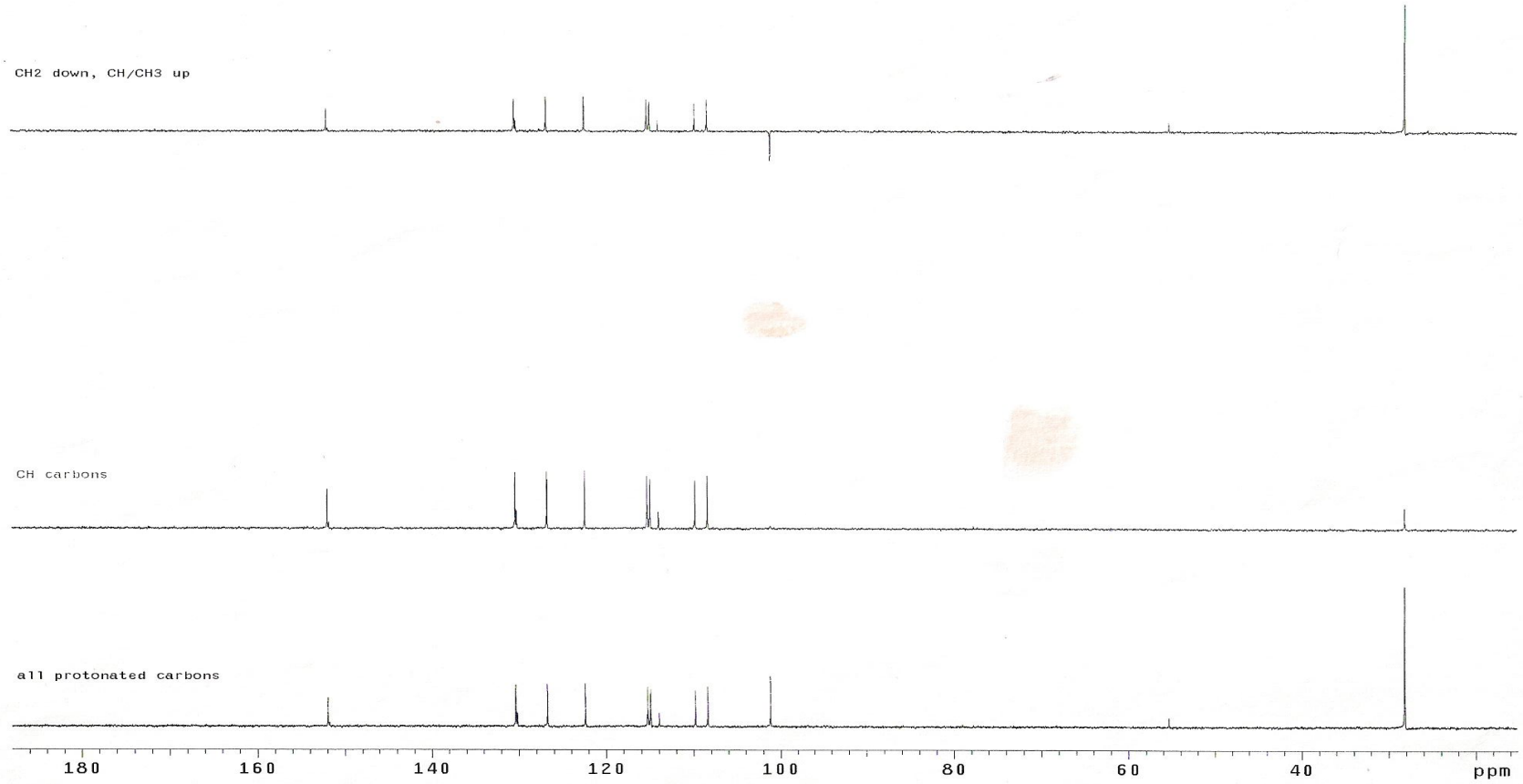
Appendix 2b: ^{13}C NMR FOR COMPOUND 2



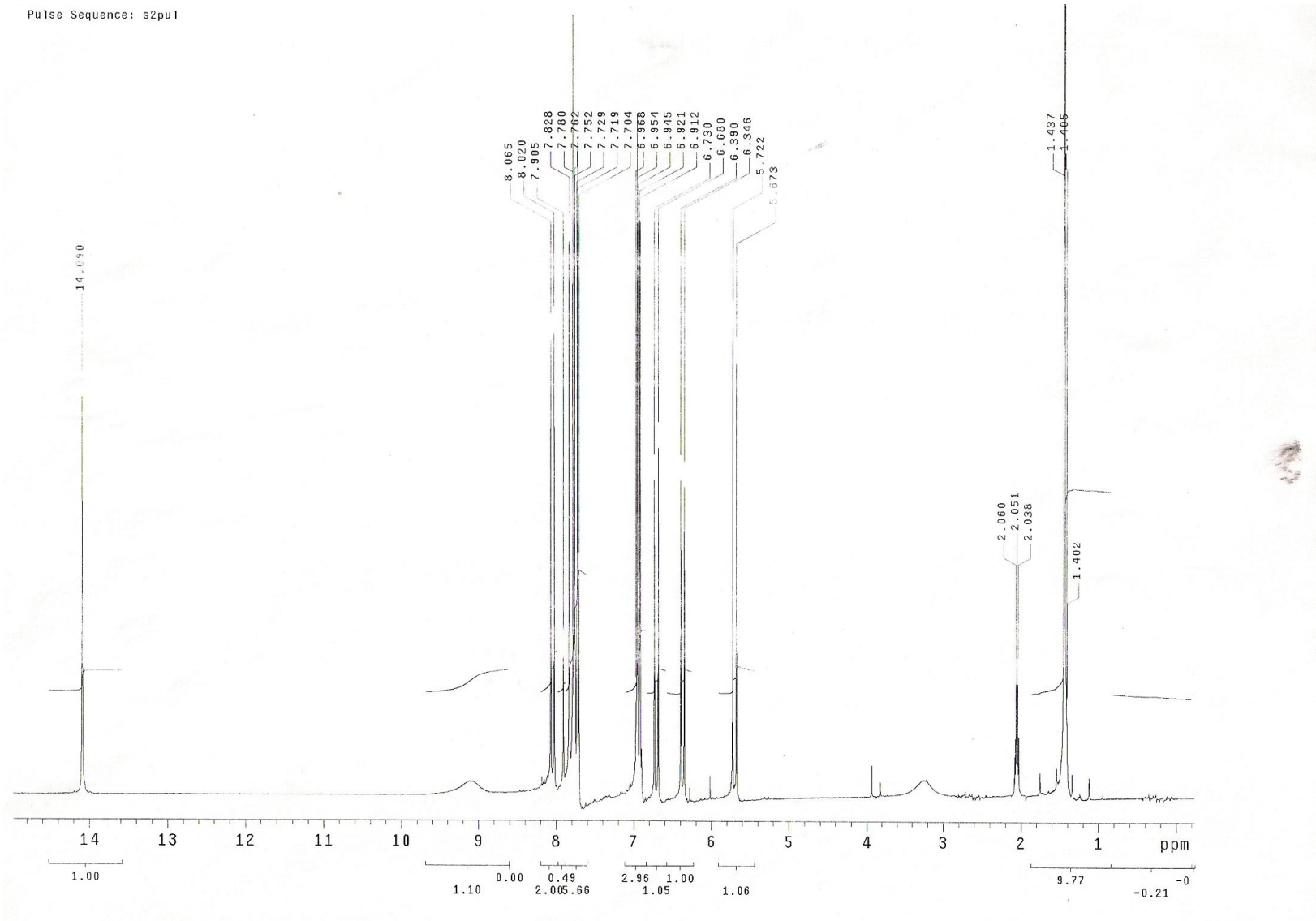
Appendix 2c: ^{13}C NMR FOR COMPOUND 2



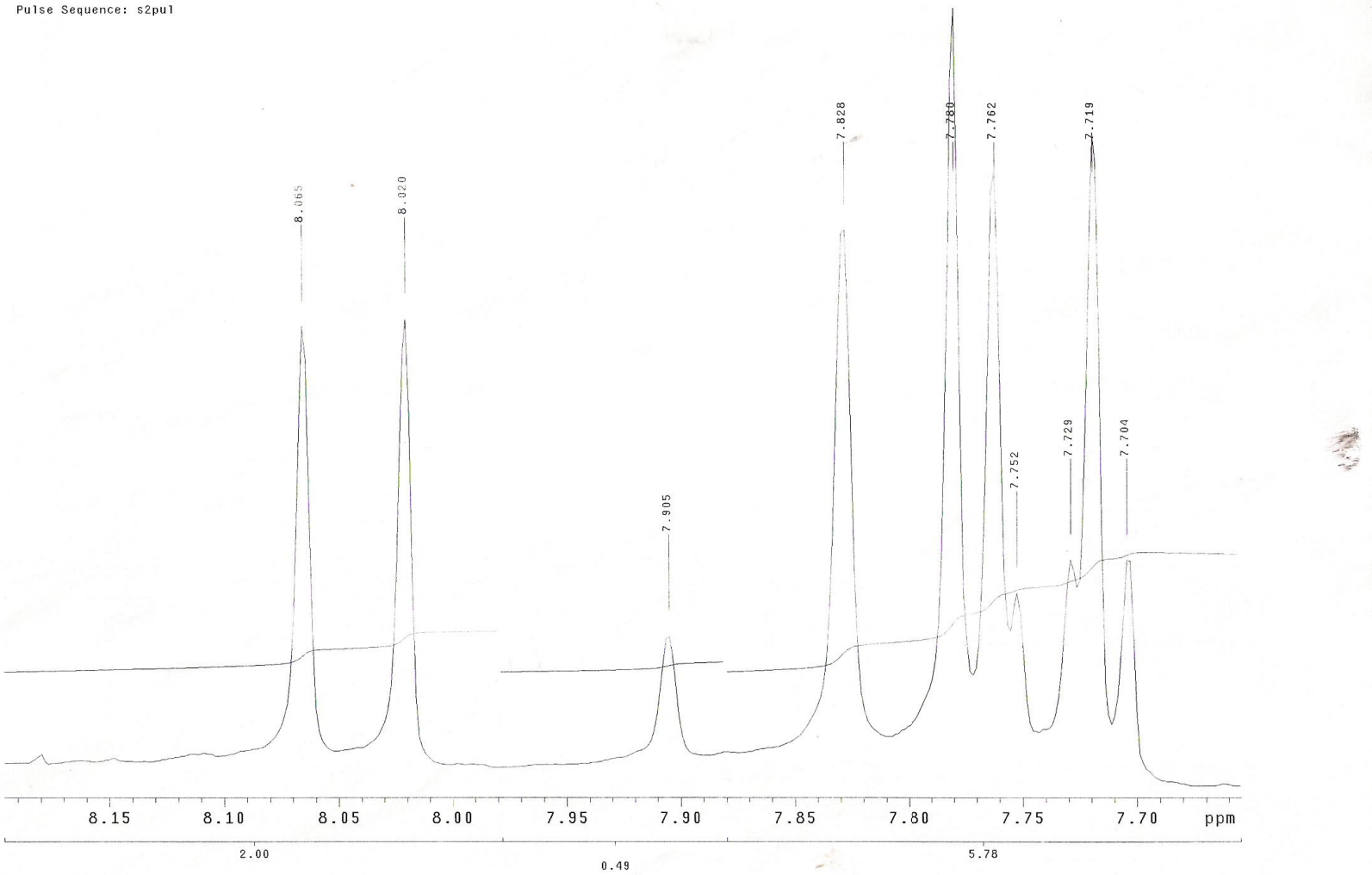
Appendix 2d: DEPT NMR FOR COMPOUND 2



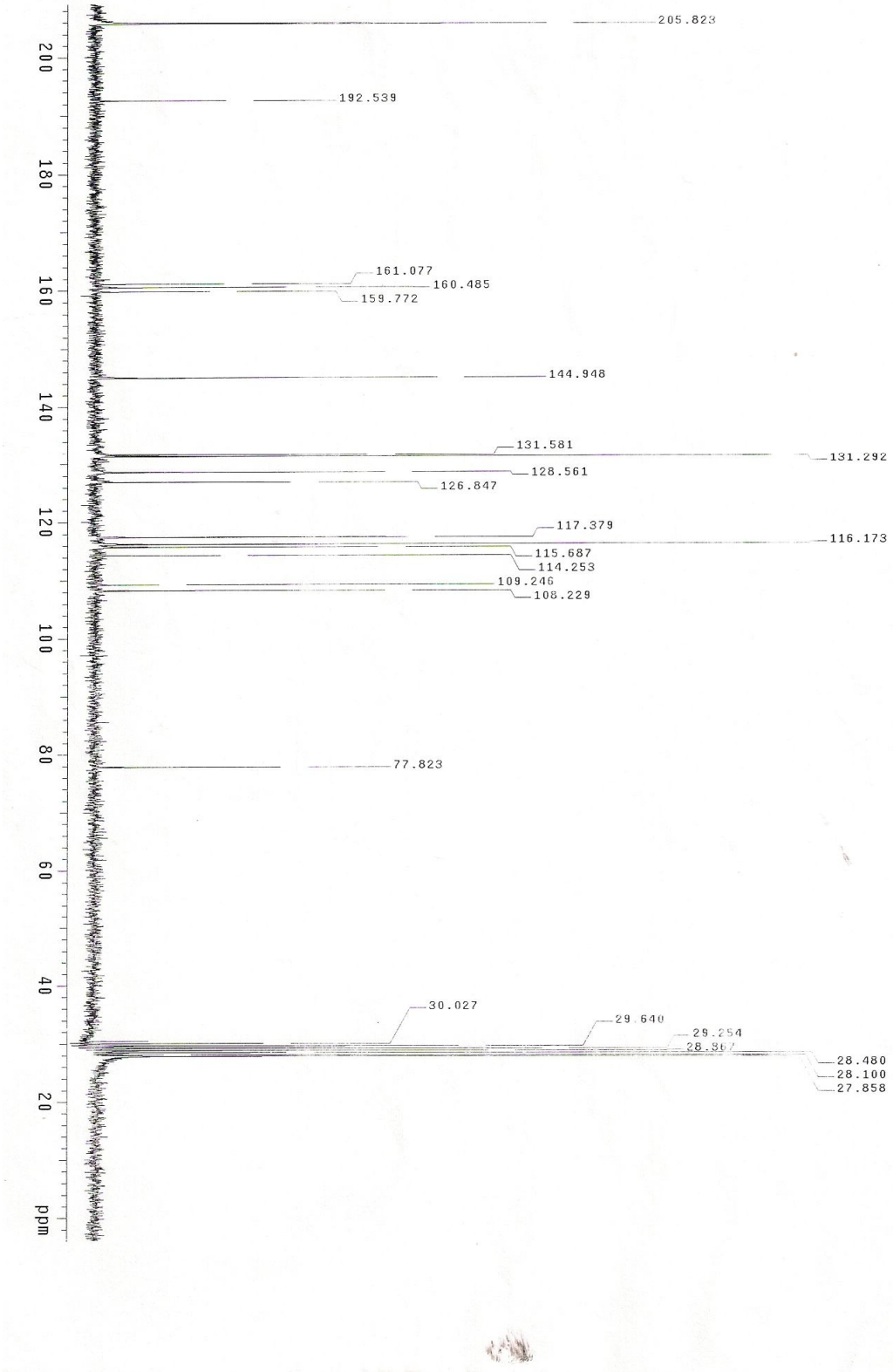
Appendix 3a: ^{1}H NMR SPECTRUM FOR COMPOUND 3



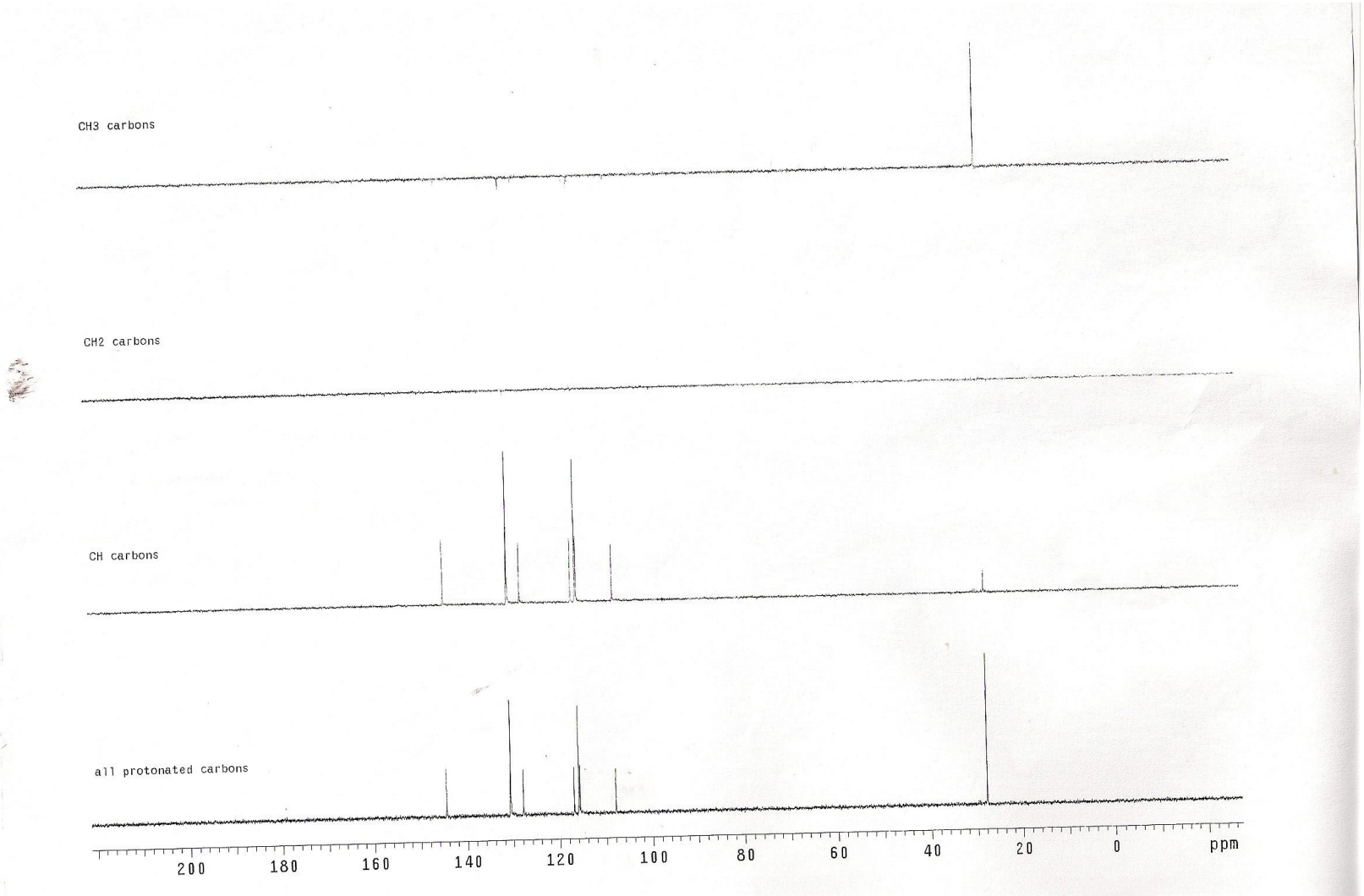
Appendix 3 b EXPANDED: ^{1}H NMR SPECTRUM FOR COMPOUND



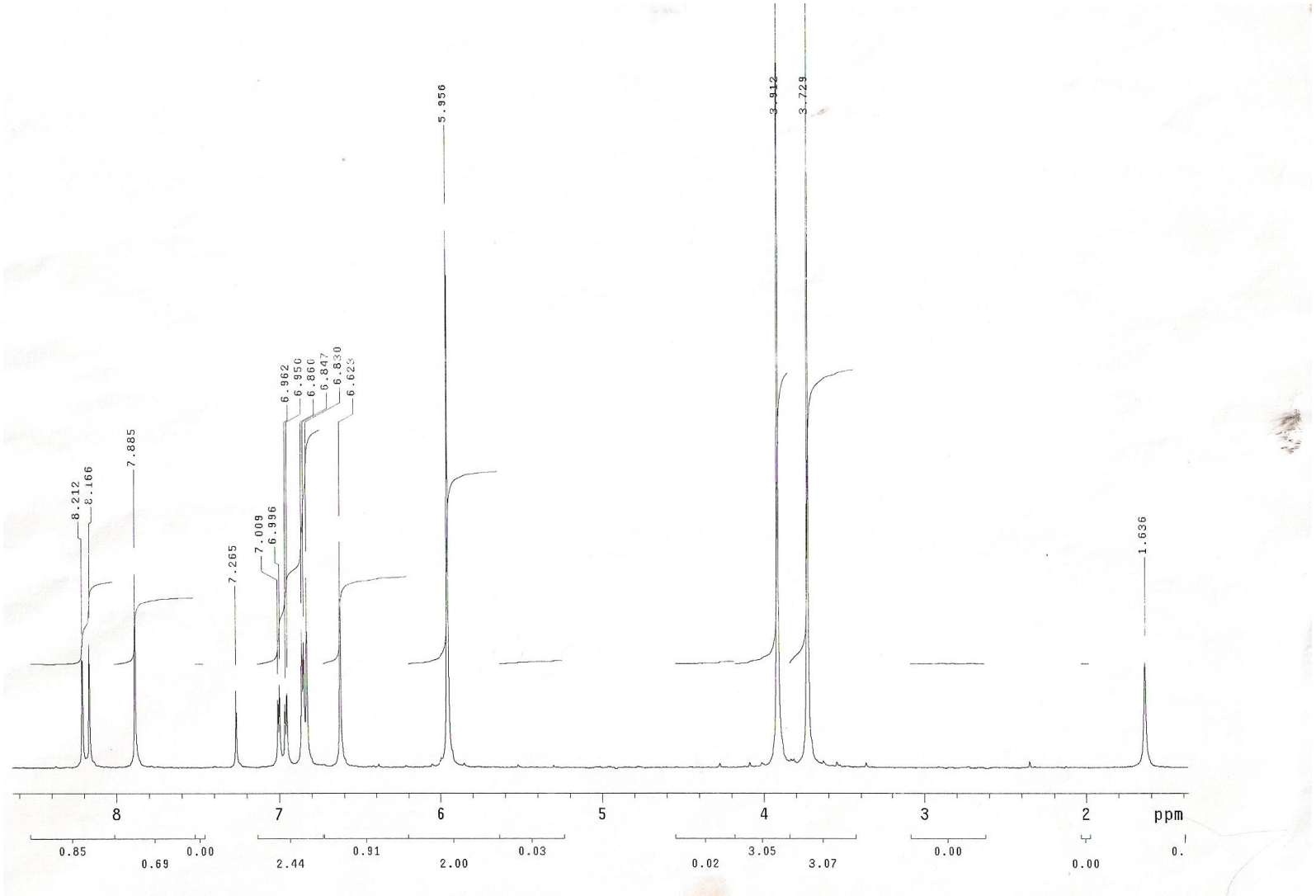
Appendix 3c: ^{13}C NMR SPECTRUM FOR COMPOUND 3



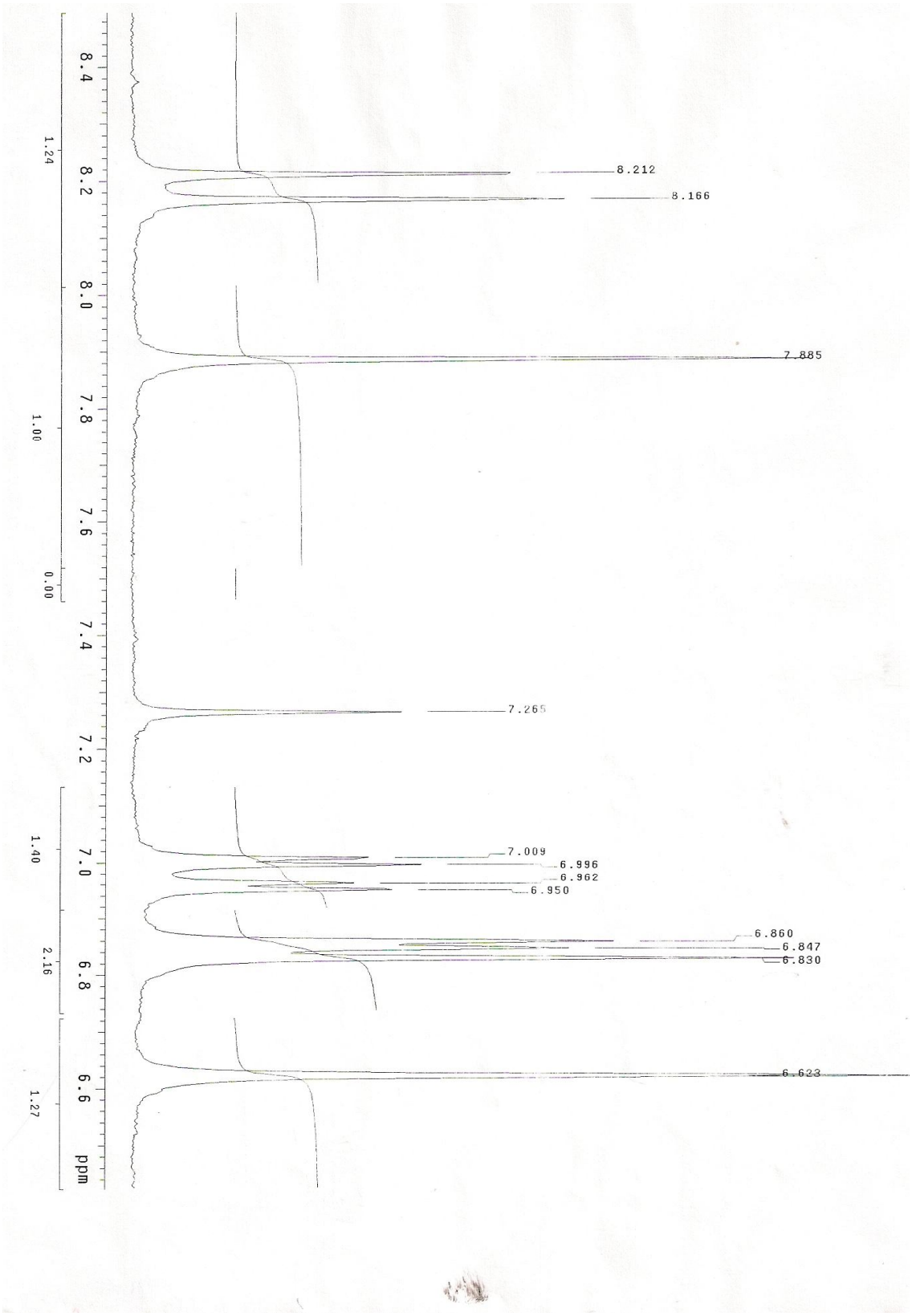
Appendix 3d: DEPTH NMR FOR COMPOUND 3



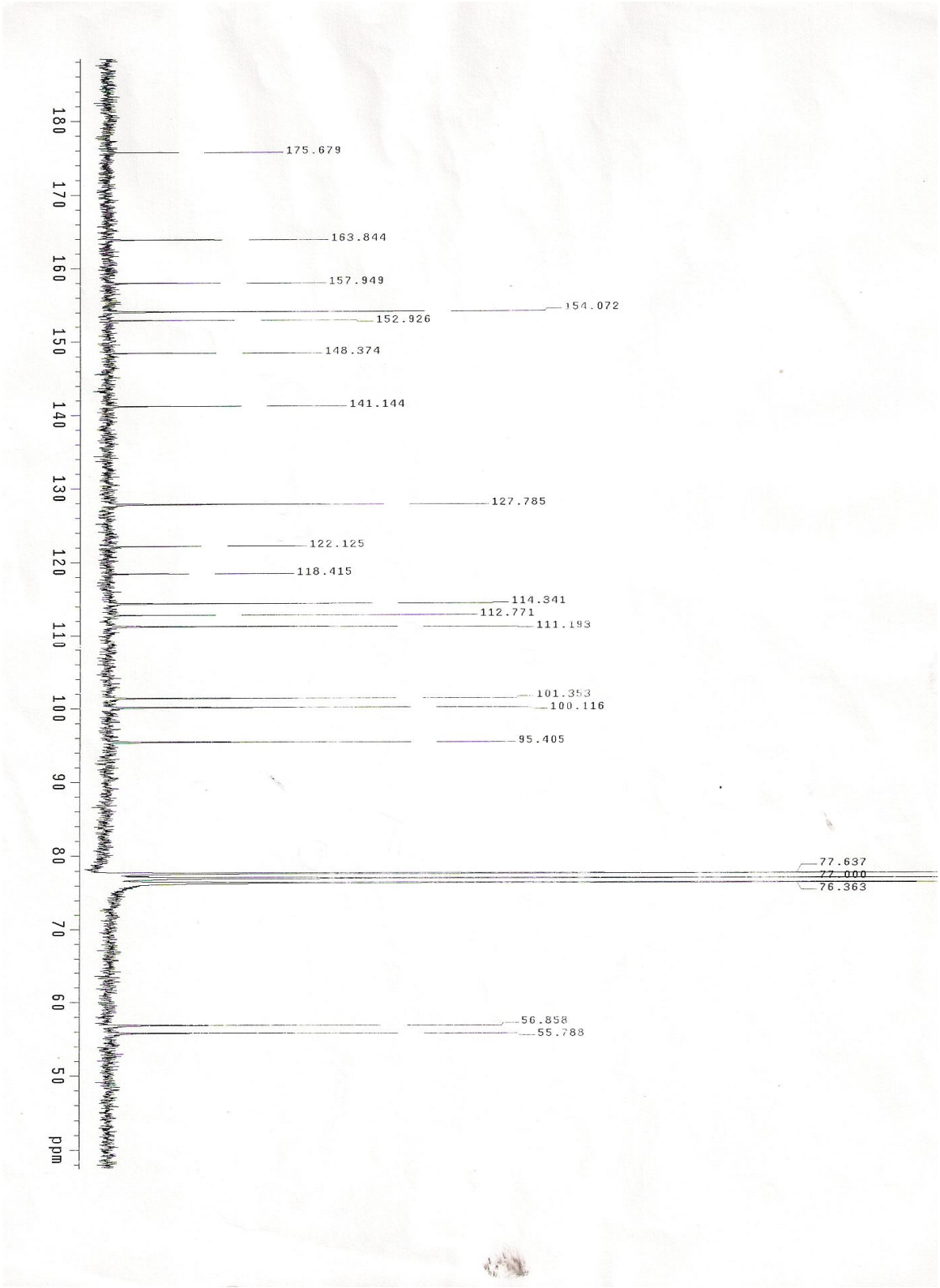
Appendix 4a: ^{1}H NMR SPECTRUM FOR COMPOUND 4



Appendix 4b: ^{1}H NMR SPECTRUM FOR COMPOUND 4



Appendix 4c: ^{13}C NMR SPECTRUM FOR COMPOUND 4



Appendix 4d: DEPT SPECTRUM FOR COMPOUND 4