PHYTOCHEMICAL INVESTIGATION OF THE STEM BARK OF
MILLETTIA OBLATA SSP. TEITENSIS
FOR
ANTIPLASMODIAL AND LARVICIDAL PRINCIPLES

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

2011
DECLARATION

This thesis is my original work and has not been presented for a degree in any other university. The research is carried out in the Department of Chemistry of the University of Nairobi.

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This thesis has been submitted for examination with my approval as a University supervisor.

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UNIVERSITY OF NAIROBI
DEDICATION

This thesis is dedicated to my late beloved mum; Esther Nyongesa. Thank you for your
great love and for giving me direction in life. My love for you is immeasurable.
I would like to express my sincere gratitude to my supervisors Dr. Solomon Derese and Prof. Abiy Yenesew for their prompt guidance, support and inspiration throughout my MSc work.

I am deeply indebted to Prof. Martin G. Peter and Dr. Matthias Heydenreich for organizing and analyzing the samples on high resolution NMR and MS. Mr. Akala, H., Waters, N. C and Ms. Liyala, P. of Kenya Medical Research Institute and United States Army Medical Research Unit – Kenya; are greatly acknowledged for their collaboration in carrying out the antiplasmodial tests described in this work.

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thanked for their endless support and encouragement. Lastly, I wish to thank the almighty God for giving me life, strength and protection during the entire study period.
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>m/z</td>
<td>Mass to charge ratio</td>
</tr>
<tr>
<td>CHS</td>
<td>Chalcone synthase</td>
</tr>
<tr>
<td>PKR</td>
<td>Polyketide reductase</td>
</tr>
<tr>
<td>IC₅₀</td>
<td>Concentration of 50% inhibition</td>
</tr>
<tr>
<td>COSY</td>
<td>Correlated spectroscopy</td>
</tr>
<tr>
<td>HMBC</td>
<td>Heteronuclear multiple bond correlation (²JCH, ³JCH)</td>
</tr>
<tr>
<td>NMR</td>
<td>Nuclear magnetic resonance</td>
</tr>
<tr>
<td>UV</td>
<td>Ultra violet</td>
</tr>
<tr>
<td>MS</td>
<td>Mass spectroscopy</td>
</tr>
<tr>
<td>Hz</td>
<td>Hertz</td>
</tr>
<tr>
<td>d</td>
<td>Doublet</td>
</tr>
<tr>
<td>t</td>
<td>Triplet</td>
</tr>
<tr>
<td>dd</td>
<td>Doublet of a doublet</td>
</tr>
<tr>
<td>AP</td>
<td>Aerial parts</td>
</tr>
<tr>
<td>HW</td>
<td>Heart wood</td>
</tr>
<tr>
<td>RB</td>
<td>Root bark</td>
</tr>
<tr>
<td>SB</td>
<td>Stem bark</td>
</tr>
<tr>
<td>SDP</td>
<td>Seedpods</td>
</tr>
<tr>
<td>DCM</td>
<td>Dichloromethane</td>
</tr>
<tr>
<td>[M]⁺</td>
<td>Molecular ion</td>
</tr>
<tr>
<td>CHI</td>
<td>Chalcone isomerase</td>
</tr>
<tr>
<td>EIMS</td>
<td>Electron ionization mass spectroscopy</td>
</tr>
<tr>
<td>LC₅₀</td>
<td>Concentration of 50% lethality</td>
</tr>
<tr>
<td>DEPT</td>
<td>Distortionless enhanced polarization transfer</td>
</tr>
<tr>
<td>HMQC</td>
<td>Heteronuclear multiple quantum coherence (¹JCH)</td>
</tr>
<tr>
<td>λₘₐₓ</td>
<td>Maximum wavelength of absorption</td>
</tr>
<tr>
<td>δ</td>
<td>Chemical shift</td>
</tr>
<tr>
<td>J</td>
<td>Coupling constant</td>
</tr>
<tr>
<td>MHz</td>
<td>Mega hertz</td>
</tr>
<tr>
<td>S</td>
<td>Singlet</td>
</tr>
<tr>
<td>M</td>
<td>Multiplet (multiplicity)</td>
</tr>
<tr>
<td>TLC</td>
<td>Thin layer chromatography</td>
</tr>
<tr>
<td>FL</td>
<td>Flowers</td>
</tr>
<tr>
<td>L</td>
<td>Leaves</td>
</tr>
<tr>
<td>RW</td>
<td>Root wood</td>
</tr>
<tr>
<td>SD</td>
<td>Seeds</td>
</tr>
<tr>
<td>WD</td>
<td>Wood</td>
</tr>
<tr>
<td>Mp</td>
<td>Melting point</td>
</tr>
</tbody>
</table>
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ABSTRACT

The genus *Millettia* is rich in flavonoids and isoflavonoids. In the search for compounds with antiplasmodial and larvicidal activities from medicinal plants, the stem barks of *Millettia oblata* ssp. *teitensis* were analysed. The dried and ground stem barks were extracted with the CH$_2$Cl$_2$/MeOH (1:1) by cold percolation for 24 hours at room temperature. The crude extracts showed significant antiplasmodial activities with IC$_{50}$ values of 10.0±2.3 and 12.0±1.2 μg/ml against chloroquine-resistant (W2) and chloroquine-sensitive (D6) strains of *Plasmodium falciparum*, respectively. Chromatographic separation of the extract led to the isolation of eight compounds. These were identified as durmillone (1), 4'-prenyloxyderrone (2), maxamaisoflavone H (3), 8-O-methylretusin (4), maxamaisoflavone J (5), maxamaisoflavone B (6), tephrosin (7) and lupeol (8). 4'-Prenyloxyderrone (2) is a novel compound. The remaining compounds are reported here for the first time from *Millettia oblata* ssp. *teitensis*. The identification of these compounds was based on spectroscopic techniques (1H NMR, 13C NMR, HMBC, HMQC, COSY, DEPT, UV and MS).

The novel compound, 4'-prenyloxyderrone (2) showed antiplasmodial activity with IC$_{50}$ of 6.0±0.9 and 5.4±1.0 μg/ml against (W2) and (D6), respectively. Durmillone (1) showed activity against (W2) and (D6) strains of *Plasmodium falciparum* with IC$_{50}$ value of 9.8±0.1 and 6.0±1.2 μg/ml, respectively. Lupeol (8) showed antiplasmodial activity with IC$_{50}$ value of 13.9±0.9 μg/ml against (D6). Tephrosin (7) showed antiplasmodial activity with IC$_{50}$ value of 11.5±0.3 and 12.7±0.9 μg/ml against (W2) and (D6) respectively. A mixture of Maxamaisoflavone J (5) and Maxamaisoflavone B (6) showed activity against (W2) and (D6) strains of *Plasmodium falciparum* with IC$_{50}$ value of 16.5±3.5 and 4.6±0.5 μg/ml, respectively.
In addition to anti-plasmodial activity tests, the methanol extracts of the stem barks, seeds and seedpods were tested against second instar larvae of the mosquito, *Aedes aegypti* for larvicidal activities. The stem bark extract showed activities with the LC$_{50}$ values of 11.2±1.9 μg/ml after 24 hours. The seed extract showed potent larvicidal activity with LC$_{50}$ value of 1.4±0.2 μg/ml after 24 hours. The seedpod extracts were inactive and after 10 days the larvae turned into adult mosquitoes. The observed activity of the seeds and the stem barks of *Millettia oblata* ssp. *teitensis* should mainly be due to the presence of rotenoids in these extracts.
CHAPTER ONE

1.0 INTRODUCTION

1.1 GENERAL
Plants have been used and are still in use by many people for the treatment of various
diseases including malaria. The medicinal properties of plants depend upon the presence
of certain active principles which vary from plant to plant. The use of medicinal plants
varies from species to species, from disease to disease, from tribe to tribe and even from
person to person (Kokwaro, 2009).

Although the use of bioactive natural products as herbal drug preparations dates back
hundreds of years ago, their application as isolated and characterized compounds to
modern drug discovery and developments started in the 19th century, the dawn of
chemotherapy era (Bankova, 2007). The searches for new biologically active compounds
are most often based on hints coming from ethnobotany but there are still a huge number
of unstudied plants (Bankova, 2007).

There is still a great potential for plants in the development of new drugs especially from
African plants, because the continent has an immensely rich biodiversity and knowledge
in using plants to treat various ailments. In fact the WHO estimates that 80% of Africans
below the Sahara depend solely on traditional medicine from plants for their primary
health care needs (Bankova, 2007). These resources, however, are hardly scientifically
investigated.
Some African scientists have made efforts to document medicinal plants and their mode of use; for example, Kokwaro (2009) and Sofowora (1982) have listed some of the traditional medicinal plants used in East and West Africa, respectively. Kokwaro (2009) listed close to 1400 East African medicinal plants and the diseases they treat. Among those listed by the author to have wide traditional medicinal uses are *Millettia* species. They have various traditional medicinal uses in many communities including antimalarial uses. Investigation of the phytochemistry of some of these plants indicates that the plants contain mainly isoflavonoids most of which are prenylated. Prenylated isoflavonoids have been shown to have various biological activities including antiplasmodial, anti-microbial, anti-cancerous and anti-oxidant (Yenesew, 1997 and Derese, 2004).

Pharmaceutical research in natural products represents a major strategy for discovering and developing new drugs. The discovery of quinine (9) from *Cinchona succiruba* Vahl. (Rubiaceae) (Rang *et al.*, 2003; Trease and Evans, 2002) and artemisinin (10) from *Artemisia annua* L. (Asteraceae) followed by their subsequent development as antimalarial drugs provided impetus to the management of malaria (Taylor and Triggle, 2007; Rang *et al.*, 2003).
Malaria is a vector-borne infectious disease caused by protozoan parasites. It is spread in tropical and sub-tropical regions. Each year, there are approximately 350-500 million cases of malaria, killing between one and three million people, the majority being young children in Sub-Saharan Africa (Johnson et al., 2007; Taylor and Triggle, 2007). Malaria is commonly associated with poverty, but is also a cause of poverty and a major hindrance to economic development.

The disease is caused by protozoan parasites of the genus *Plasmodium*. Five species of the *Plasmodium* parasite can infect humans. The most deadly form of the disease is caused by *Plasmodium falciparum*. The parasite is transmitted to humans via the bites of infected female mosquitoes. In the human body, the parasite multiplies in the liver, and then infects red blood cells.

Malaria transmission can be reduced by the use of mosquito nets and insect repellents to prevent mosquito bites, or by mosquito control measures such as spraying insecticides inside houses and draining standing water where mosquitoes lay their eggs. Prevention by
vaccine is theoretically possible; however, work on the development of malaria vaccines has not been successful so far.

In Kenya, malaria is the leading cause of morbidity and mortality, accounting for 30-50% of the outpatient attendance and 20% of all admissions to health facilities (Kigondu, 2007; Kirira et al., 2006).

Kenya has revised its treatment policy by adopting artemisinin (10) combination therapy (ACT) as the first line drugs for treatment of uncomplicated malaria. Spread of multidrug resistant (MDR) strains of *Plasmodium* and the adverse side effects of the existing antimalarial drugs have necessitated the search for novel, well tolerated and more efficient antimalarial drugs. Indigenous plants are important sources of biologically active compounds that have potential for the development into novel antimalarial drugs (Yenesew, 1997 and Derese, 2004).

The increasing prevalence of strains of *Plasmodium falciparum* that are resistant to chloroquine (11), a blood schizontocide which had been efficacious, safe, accessible and affordable, poses a serious problem for malaria control, predisposing Africa to an unprecedented situation since the only affordable treatment options are rapidly losing therapeutic efficacy (Frankish, 2002). Drug resistant strains of *Plasmodium falciparum* are endemic in many areas of the world and the majority of conventional antimalarial drugs have been associated with treatment failure (Johnson et al., 2007). These developments and the difficulty of creating efficient vaccines, coupled with adverse
reactions to chemotherapy, underline the urgent need for novel, cheap, safe and efficacious antimalarial drugs. It is estimated that 80% of people worldwide use herbal remedies, due to limited access to modern medicine because of low income and the shortage of efficient health care facilities. There is a dearth of evidence on the efficacy and safety of these remedies, despite the fact that validation of traditional practices could lead to innovative strategies in malaria control (Vishnu et al., 2000).

![Chemical Structure](image)

Plants of the genus *Millettia* are used traditionally in different cultures globally. These plants have a wide range of biological activities such as anti-tumoral, anti-inflammatory, anti-viral, bactericidal and insecticidal. Phytochemical investigations of roots, stems, leaves, seeds and seedpods have been done on *Millettia dura* and *Millettia usaramensis* (Yenesew, 1997 and Derese, 2004). Flavonoids from these *Millettia* species have shown appreciable anti-plasmodial activities. These plants are known to contain several classes of isoflavonoids. In this work the anti-malarial activity of the crude extract of the stem and the isoflavonoids of *Millettia oblata* ssp. teitensis were investigated in order to determine their potential use as anti-malarials.

### 1.3 Biodegradable larvicides

Rotenone (12) is one of the extensively used natural insecticides (Abe et al., 1985). The insecticidal activities of rotenone and other rotenoids against a variety of insect species
are well known, (Derese, 2004). Commercially, rotenone (12) is mainly extracted from the *Derris* and *Lonchocarpus* species from Asia and South America respectively. *Millettia* plants have not been exploited commercially as a source of rotenoids, even if the seeds of these plants are known in traditional practice for their insecticidal and piscicidal properties (Dagne *et al.*, 1991).

![Chemical structure of rotenone](image)

In the search for biodegradable compounds with larvicidal and pesticidal activities from plants, the larvicidal activities of rotenoids isolated from the seeds of *Millettia usaramensis* ssp *usaramensis* have been reported (Yenesew *et al.*, 2003). Insect resistance to most conventional insecticides have resulted in high prevalence of mosquito transmitted diseases in Africa. Aiming for the discovery of cost effective alternatives for the control of vector disease insects, extracts from plants have been tested for larvicidal activities, (Yenesew *et al.*, 2003). In this study, the larvicidal activities of the extract of *Millettia oblata* ssp. *teitensis* were tested against the larvae of *Aedes aegypti*.

### 1.4 Problem statement

Malaria is a major contributor to the global burden of disease and a significant impediment to socio-economic development in poor countries. Malaria remains one of the deadliest diseases on this planet, which accounts for 350-500 million clinical cases and up to 2.7
million deaths each year (Taylor and Triggle, 2007). It is the leading cause of the rising morbidity and mortality rates in most countries in Sub-Saharan Africa. At least one child dies of malaria every 40 seconds in the world and this shows the devastating effect of this disease (Geissböhler et al., 2007; Taylor and Triggle, 2007). Efforts to combat the disease are hampered by growing resistance of malaria parasites to the available drugs. Furthermore the cost of drugs is a sizeable proportion of the total health expenditure in most developing countries. Drug related expenses in these countries account for between 30-50% of the total cost of healthcare needs (Taylor and Triggle, 2007; WHO, 2007). Toxicity of the available antimalarial drugs to many of the patients is another important issue yet to be addressed conclusively. Because of these reasons, many patients turn to herbal medicines which may end up being more detrimental to their health, since little or no scientific research has been done on these herbal remedies to establish their safety and efficacy. Due to the increasing prominence of herbal remedies, additional contributions describing scientific investigations of a rigorous nature are most welcomed. In addition, continuous research is desperately needed in order to come up with compounds which can be developed into new, cheap, less toxic and more efficacious antimalarial drugs for combating the disease.

1.5 Justification of the Research

Various attempts have been made in the control of malaria including, selective application of vector control, early diagnosis and effective and prompt treatment of malarial disease and early detection or forecasting of epidemics and rapid application of control measures. Since malaria is transmitted by the female anopheles mosquito, a major strategy of control is to attack the vector with adulticides and larvicides. Extended use, however, has led to emergence of insecticide-resistant mosquitoes (Campbell, 1997). The use of *Millettia*
species of Kenyan origin for control of mosquitoes at larvae stage may provide a useful deterrent against proliferations of these disease vectors. Previous investigations of the phytochemical profile of the genus *Millettia* have resulted in the isolation and identification of compounds such as isoflavonoids, chalcones, rotenoids among many others. Rotenone (12) and tephrosin (7) have showed potent larvicidal activity against 2\textsuperscript{nd} instar larvae of *Aedes aegypti* (Yenesew et al., 2005). The study of *Millettia oblata* ssp. *teitensis* may lead to the isolation, identification and characterization of cheap, less toxic and more efficacious natural larvicidal compounds. Furthermore, the antiplasmodial activities of several flavonoids have been reported (Yenesew 1997 and Derese 2004).

The identification of antiplasmodial and larvicidal compounds, which are biodegradable and environmentally friendly, that can be used as an alternative to synthetic antimalarial drugs and larvicides will contribute significantly to safe guarding the health of the people especially those living in malaria endemic rural areas. It is worth to note that the medicinal plant industry plays a critical role in empowering large numbers of rural population in many African countries (Anthonia and Benjamin, 2003).

Thus, in this study, the antiplasmodial principles of the crude extracts and isolated compounds from the stem bark of *Millettia oblata* ssp. *teitensis* were tested against chloroquine-sensitive (D6) and chloroquine-resistant (W2) strains of *Plasmodium falciparum*. Furthermore the larvicidal principles of the crude extracts of the stem bark, seeds and seedpods were tested against the larvae of *Aedes aegypti*, in order to establish the potential use of the *Millettia oblata* ssp. *teitensis* in the treatment and control of malaria.
1.6.0 Objectives

1.6.1 General Objective
To isolate, identify and characterize the compounds of *Millettia oblata* ssp. *teitensis* and establish their antiplasmodial and larvicidal activity.

1.6.2 Specific Objectives
  - To determine the antiplasmodial activities of the crude extracts of the stem bark of *Millettia oblata* ssp. *teitensis*
  - To determine the larvicidal activities of the crude extracts of the stem barks, seeds and seedpods of *Millettia oblata* ssp. *teitensis*
  - To isolate and characterize the constituents of the stem barks of *Millettia oblata* ssp. *teitensis*
  - To determine the antiplasmodial activities of the isolated compounds.
CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Botanical information

2.1.1 The family Fabaceae

The genus *Millettia* belongs to the family Fabaceae (also known as Leguminosae). This family comprises of 657 genera and about 20,000 species of trees, shrubs and herbs. These plants are known for their ability of nitrogen fixation in the soil. They are widely distributed in the temperate as well as tropical regions of the world (Heguaurer and Grayer-Barkmeijer, 1993).

The family is divided into three sub-families; Mimosoideae, Papilionoideae and Caesalpinioideae. The genus *Millettia* belongs to the sub-family Papilionoideae.

2.1.1.1 The sub-family Papilionoideae

Papilionoideae is the largest of the three sub-families with over 630 genera and 18,000 species of trees, shrubs and herbs. This sub-family has 32 tribes and is distinguished from the two other subfamilies by the presence of papilionoid flowers, a hilar valve of seeds and by their ability to synthesize quinolizidine alkaloids and isoflavonoids (Polhill, 1981).

2.1.1.2 The genus *Millettia*

The genus *Millettia* belongs to the tribe Tephrosiae that is known to synthesize prenylated flavonoids and isoflavonoids, (Derese, 2004). The genus has over 323 species
worldwide and found widely distributed in tropical Africa, Asia and Australia. *Millettia* plants are trees, shrubs or lianas, or rarely semi-herbaceous plants with woody rootstocks, (Derese, 2004).

In Kenya, the genus *Millettia* is represented by six species, which are *Millettia dura, Millettia lasiantha, Millettia leucantha, Millettia oblata, Millettia tanaensis* and *Millettia usaramensis*, (Beentje, 1994).

2.1.1.2.1 *Millettia oblata* Dunn.

Table 2.1: Distribution of *Millettia oblata* Dunn subspecies

<table>
<thead>
<tr>
<th>Subspecies</th>
<th>Distribution</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>teitensis</em> J. B. Gillett</td>
<td>Threatened small tree with perennial lifespan found only from Taita Hills forest, Kenya.</td>
<td>Catalogue of Life, 2009</td>
</tr>
<tr>
<td><em>oblata</em> Dunn</td>
<td>A small tree found in tropical areas of Tanzania, with perennial lifespan</td>
<td>Catalogue of Life, 2009</td>
</tr>
<tr>
<td><em>intermedia</em> J. B. Gillett</td>
<td>A small tree distributed in tropical areas of Tanzania, with perennial lifespan</td>
<td>Catalogue of Life, 2009</td>
</tr>
<tr>
<td><em>stolzii</em> J. B. Gillett</td>
<td>A small tree distributed in tropical areas of Tanzania, with perennial lifespan</td>
<td>Catalogue of Life, 2009</td>
</tr>
<tr>
<td><em>burttii</em> J. B. Gillett</td>
<td>A shrub or small tree distributed in tropical areas of Tanzania, with perennial lifespan</td>
<td>Catalogue of Life, 2009</td>
</tr>
</tbody>
</table>

Figure 2.1: *Millettia oblata* ssp. *teitensis* (Catalogue of life, 2009)

2.1.2 The genus *Millettia* as potential sources of anti-malarials.

Research on the Kenyan *Millettia* species as potential anti-malarial drugs has been going on since late 1990’s at the University of Nairobi, Kenya. Phytochemical investigations of roots, stems, leaves, seeds and seedpods have been done widely on *Millettia dura* and
Millettia usaramensis, (Yenesew, 1997 and Derese, 2004). Flavonoids from these Millettia species have shown appreciable anti-plasmodial activities. However, there are no records of the use of these plants as anti-malarials in traditional medicine. Table 2.2 gives a summary of anti-plasmodial activities of flavonoids from Kenyan Millettia species.

Table 2.2: Anti-plasmodial activities of flavonoids from Kenyan Millettia species

<table>
<thead>
<tr>
<th>Flavonoids</th>
<th>IC$_{50}$ in (μM)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Maximaisoflavone H (3)</td>
<td>38.7±0.6</td>
<td>Derese 2004</td>
</tr>
<tr>
<td>Maximaisoflavone B (6)</td>
<td>58.9±1.5</td>
<td></td>
</tr>
<tr>
<td>Jamaicin (13)</td>
<td>45.6±2.3</td>
<td></td>
</tr>
<tr>
<td>7,2′-Dimethoxy-4′,5′-methylenedioxyisoflavone (14)</td>
<td>49.4±0.2</td>
<td></td>
</tr>
<tr>
<td>Mildurone (15)</td>
<td>40.7±2.2</td>
<td></td>
</tr>
<tr>
<td>Calopogoniumisoflavone A (16)</td>
<td>81.4</td>
<td></td>
</tr>
<tr>
<td>Durmillone (1)</td>
<td>25.1±1.6</td>
<td></td>
</tr>
<tr>
<td>Isoerythrin A 4′-(3-methyl-2-butenyl)ether (17)</td>
<td>21.8±0.6</td>
<td></td>
</tr>
<tr>
<td>Isojamaicin (18)</td>
<td>39.0±0.8</td>
<td></td>
</tr>
<tr>
<td>Nordurlettone (19)</td>
<td>51.4±1.7</td>
<td></td>
</tr>
<tr>
<td>7,3′-Dimethoxy-4′,5′-methylendioxyisoflavone (20)</td>
<td>56.3±0.8</td>
<td></td>
</tr>
<tr>
<td>Durallone (21)</td>
<td>49.9±2.4</td>
<td></td>
</tr>
<tr>
<td>6-Methoxycalopogonium isoflavone A (22)</td>
<td>35.4±1.9</td>
<td></td>
</tr>
<tr>
<td>Deguelin (23)</td>
<td>21.1</td>
<td></td>
</tr>
<tr>
<td>Millettone (24)</td>
<td>64.1</td>
<td></td>
</tr>
<tr>
<td>Usararotenoid-A (25)</td>
<td>66.6</td>
<td>Yenesew et al, 2003</td>
</tr>
<tr>
<td>12a-Epimillettosin (26)</td>
<td>22.2</td>
<td></td>
</tr>
<tr>
<td>4′-O –Geranyloxyisoliquiritigenin. (27)</td>
<td>8.7</td>
<td></td>
</tr>
<tr>
<td>Barbigerone (28)</td>
<td>27.0</td>
<td></td>
</tr>
<tr>
<td>Usararotenoid C (29)</td>
<td>25.8</td>
<td></td>
</tr>
<tr>
<td>6a,12a-Dehydromillettone (30)</td>
<td>33.3</td>
<td></td>
</tr>
<tr>
<td>Chloroquine (11)</td>
<td>0.094</td>
<td></td>
</tr>
<tr>
<td>Quinine (9)</td>
<td>0.209</td>
<td></td>
</tr>
</tbody>
</table>
2.1.3 The Kenyan *Millettia* plants as potential larvicides

Research on *Millettia* plants species at the University of Nairobi has revealed that the seeds of these plants show larvicidal activity. The phytochemical investigation of the seeds of *Millettia dura* and *Millettia usaramensis* have led to the isolation of rotenoids as the main components of the seeds, (Yenesew *et al.*, 2003, Derese, 2004). The rotenoids
are believed to be responsible for the larvicidal activity. Table 2.3 below gives the summary of the larvicidal activity as LC$_{50}$ values of the rotenoids after 24 hours against the 2$^{\text{nd}}$ instar larvae of *Aedes aegypti*.

Table 2.3: The larvicidal activity, LC$_{50}$, of the rotenoids after 24 hours on 2$^{\text{nd}}$ instar larvae of *Aedes aegypti*.

<table>
<thead>
<tr>
<th>Rotenoid/ Crude Extract</th>
<th>LC$_{50}$ in µg/ml</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rotenone (12)</td>
<td>0.47</td>
<td>Yenesew <em>et al.</em>, 2003</td>
</tr>
<tr>
<td>Tephrosin (7)</td>
<td>1.40</td>
<td></td>
</tr>
<tr>
<td>Deguelin (23)</td>
<td>1.60</td>
<td>Derese, 2004</td>
</tr>
<tr>
<td>Seed-extract of <em>Millettia dura</em></td>
<td>0.90</td>
<td>Derese, 2004</td>
</tr>
</tbody>
</table>

2.1.4 Ethno-medical uses of some Kenyan *Millettia* species

*Millettia* species are used traditionally in different cultures globally. These plants have a wide range of biological activities such as anti-tumoral, anti-inflammatory, anti-viral, bactericidal and insecticidal. Table 2.4 below summarizes the reported uses of the Kenyan *Millettia* species (Gillet *et al.*, 1971).
Table 2.4: Traditional uses of some Kenyan *Millettia* species.

<table>
<thead>
<tr>
<th>Species</th>
<th>Plant part</th>
<th>Uses</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>M. lasiantha</em></td>
<td>Roots</td>
<td>Decoctions of roots drunk as aphrodisiac (Kenya).</td>
<td>Gillet <em>et al.</em>, 1971</td>
</tr>
<tr>
<td><em>M. oblata</em></td>
<td>Barks</td>
<td>Treat stomach-aches</td>
<td>Gillet <em>et al.</em>, 1971</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Remedy against cough</td>
<td></td>
</tr>
<tr>
<td><em>M. usaramensis</em></td>
<td>Roots root pulp</td>
<td>Fish poison in Kenya Trements of snake bite</td>
<td>Gillet <em>et al.</em>, 1971</td>
</tr>
<tr>
<td><em>M. dura</em></td>
<td>Roots</td>
<td>Decoctions employed to treats swollen parts of the body. Treats bladder problems</td>
<td>Gillet <em>et al.</em>, 1971</td>
</tr>
</tbody>
</table>

2.2 Phytochemistry of the genus *Millettia*

Previous studies of extracts of *Millettia* species have led to the isolation of alkaloids, flavones, chalcones, rotenoids, isoflavones and coumarins among others. Phytochemical investigation of the Kenyan *Millettia* species at the University of Nairobi have led to the isolation of flavanones, chalcones, rotenoids, isoflavones and terpenes, among others, (Yenesew, 1997, Derese, 2004), of which isoflavonoids are the most reported.

2.2.1 Rotenoids of the genus *Millettia*

Rotenoids mainly occur in the seeds of *Millettia* plants. These compounds, especially rotenone (12), are considered to be responsible for insecticidal and piscicidal activities observed in the *Millettia* species, (Ollis *et al.*, 1967, Yenesew *et al.*, 2003). Most of the rotenoids previously characterized from these plants have a cis-B/C junctions as in rotenone. However, rotenoids of the stem bark of the Kenyan *Millettia usaramensis* have a novel trans-B/C ring junction with a 6aR, 12aS configuration (Derese, 2004).
A recent investigation in Thailand isolated four rotenoids from the flowers of *Millettia brandisiana* Kurz (Orasa *et al.*, 2007). These four rotenoids had not been isolated previously from other *Millettia* species but from different leguminous plants; *Tephrosia* species and *Clitoria* species (Orasa *et al.*, 2007). These four rotenoids are: α - toxicarol (31), 12α - hydroxyl - α - toxicarol (32), 6 - deoxyclitoriacetal (33) and 6α, 12α - dehydro - α - toxicarol (34).

![Chemical structures of rotenoids](image-url)
From the *Millettia duchesnei* plant, three new rotenoids have been reported from the twigs (Ngandeu *et al.*, 2008). These are; elliptol (35), 12-deoxo-12α-methoxyelliptone (36) and 6-methoxy-6α, 12α-dehydrodeguelin (37).

2.2.2 Isoflavones of the genus *Millettia*

Isoflavones form the largest group of natural isoflavonoids. By 2004, seventy isoflavones had been reported from the genus. About 37 of them, lacked oxygenations at C-5, 54 were prenylated or with furanol/pyrano ring while 28 were both prenylated and C-5 deoxygenated, (Derese, 2004). Eight new isoflavones have been reported, (Chihiro *et al.*, 2004 and 2006). Table 2.5 gives the summary of isoflavones reported.
Table 2.5: A summary of isoflavones of *Millettia* reported.

<table>
<thead>
<tr>
<th>Isoflavone</th>
<th>Source (plant part)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Willewanin A (38)</td>
<td><em>Millettia taiwaniana</em> (SB)</td>
<td>Chihiro <em>et al.</em>, 2004</td>
</tr>
<tr>
<td>Willewanin C (40)</td>
<td><em>Millettia taiwaniana</em> (SB)</td>
<td>Chihiro <em>et al.</em>, 2004</td>
</tr>
<tr>
<td>Willewanin D (41)</td>
<td><em>Millettia taiwaniana</em> (SB)</td>
<td>Chihiro <em>et al.</em>, 2004</td>
</tr>
<tr>
<td>Willewanin E (42)</td>
<td><em>Millettia taiwaniana</em> (SB)</td>
<td>Chihiro <em>et al.</em>, 2004</td>
</tr>
<tr>
<td>Willewanin G (43)</td>
<td><em>Millettia pachycarpa</em> (L)</td>
<td>Chihiro <em>et al.</em>, 2006</td>
</tr>
<tr>
<td>Willewanin H (44)</td>
<td><em>Millettia pachycarpa</em> (L)</td>
<td>Chihiro <em>et al.</em>, 2006</td>
</tr>
<tr>
<td>Furowanin B (45)</td>
<td><em>Millettia pachycarpa</em> (L)</td>
<td>Chihiro <em>et al.</em>, 2006</td>
</tr>
</tbody>
</table>

![Isoflavone Structure](image)

<table>
<thead>
<tr>
<th></th>
<th>R&lt;sub&gt;1&lt;/sub&gt;</th>
<th>R&lt;sub&gt;2&lt;/sub&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>38</td>
<td>Me</td>
<td>H</td>
</tr>
<tr>
<td>39</td>
<td>Me</td>
<td>Prenyl</td>
</tr>
<tr>
<td>40</td>
<td>H</td>
<td>Prenyl</td>
</tr>
</tbody>
</table>
2.2.3 Chalcones of the genus *Millettia*

By 2004, 18 chalcones had been isolated from the genus *Millettia*, (Derese, 2004). Two new chalcones have been reported from the seedpods of *Millettia erythrocalyx* Gagnep
(Sritularak et al., 2006). This include 2’- hydroxy-3, 4-dimethoxy-[2”, 3”: 4’, 3’]-furaochalcone (46) and 2’, 3-dihydroxy-4- methoxy-4’-γ, γ-dimethylallyloxychalcone (47).

Also four chalcones derivatives have been reported from the stem bark of Millettia leucanthe Kurz, (Ampai et al., 2003). These are; 2’,4’-dimethoxy-3,4- methylenedioxychalcone (48), 2’,4’,6’-trimethoxy-3,4-methylenedioxydihydrochalcone (49), 2,4,6,β-tetramethoxy-3’,4’-methylenedioxychalcone (50) and 2’,4’,6’-trimethoxy-3,4-methylenedioxychalcone (51).
2.2.4 Flavanones of the genus Millettia

By 2004, all the flavanones that had been characterized were prenylated and lacked oxygenation at C-5 positions (Derese, 2004). A new flavanone, (-)-(2S)-6, 3', 4'-trimethoxy-[2'', 3'': 7, 8]-furanoflavanone (52) has been reported from the seedpods of Millettia erythrocalyx Gagnep, (Sritularak et al., 2006).

![Flavanone 52](image)

2.2.5 Flavones and Anthocyanins of the genus Millettia

Twenty five flavones had been isolated from the genus Millettia by 2004. 60% of them possessed a furan-ring, which is not a common substituent in the genus. In all the cases, the furan-ring is on ring A (Derese, 2004).

A novel flavonol; 3', 4'-methylenedioxy-[2'', 3'': 7, 8]-furanoflavonol (53) and flavone; 6, 3'-dimethoxy-[2'', 3'': 7, 8]-furanoflavone (54) have been reported from the seedpods of Millettia erythrocalyx Gagnep in Thailand (Sritularak et al., 2006) and one new flavonol triglycoside; millettiaspecoside D (55) reported from Millettia speciosa Champ in China (Ting et al., 2010).
2.2.6 Alkaloids of the genus *Millettia*

Apart from flavonoids, alkaloids have also been reported from this genus. They are reported only from the species *Millettia laurentii* (Derese, 2004). Millaurine A (56), a new guanidine alkaloid has been isolated from a Camerounian plant *Millettia laurentii* (Ngamga et al., 2007).
2.2.7 Other Phenolic compounds of the genus *Millettia*

Phenolic compounds from the stem wood of *Millettia leucantha* Kurz in Thailand have been reported (Sritularak *et al.*, 2010). The isolated compounds were identified as (−)-maackiain (57), syringic acid (58), 4-hydroxyl-3-methoxybenzoic acid (59), (−)-balanocarpol (60) and (+)-diptoindonesin D (61).

It should be noted that both (−)-balanocarpol (60) and (+)-diptoindonesin D (61) could be considered as biogenetically derived from two units of the stilbene resveratrol (62) (Sritularak *et al.*, 2010). Prior to this study, no stilbene – related compounds were identified from the genus *Millettia* (Sritularak *et al.*, 2010).
CHAPTER THREE

3.0 METHODOLOGY

3.1 General

3.1.1 Instrumentation
The $^{13}$C NMR (125 or 50 MHz) and $^1$H NMR (300 or 200 MHz) were run on Bruker or Varian-Mercury spectrometers using residual solvent signal as reference. Homonuclear correlation spectroscopy (COSY), Nuclear Overhauser Enhancement spectroscopy (NOESY), Heteronuclear correlation spectroscopy (HETCOR) including HMBC ($^2$J$_{CH}$, $^3$J$_{CH}$) and Heteronuclear multiple quantum coherence [HMQC ($^1$J$_{CH}$)] were acquired using standard Bruker software. UV/VIS spectra were recorded using a Pye-Unicam SPS-150 Spectrophotometer. The plant material was grounded using Willymill.

3.1.2 Collection of Plant Material
The stem barks of *Millettia oblata* ssp. *teitensis* were collected from Taita Hill forest, Kenya and identified voucher specimen is deposited at the University Herbarium, School of Biological Sciences.

3.2 Extraction and Isolation of Compounds
Air dried and ground stem bark of *Millettia oblata* ssp. *teitensis* (449 g) were extracted with CH$_2$Cl$_2$/MeOH (1:1) by cold percolation. The extract was evaporated under reduced pressure to yield 19.9 g (4.4 %) crude extract. The 19.9 g extract was subjected to column chromatography on Silica gel (230 g) eluting with hexane containing increasing
percentage of ethyl percentage (1%, 3%, 5%, 7%, 9%, 12%, 15%, 20%, 25%, 30% and 40%, ethyl acetate in hexane each of ca 1L). 26 fractions labelled A to Z were collected. 

Crystallization of the combined fractions A to D (which was eluted with 3%EtOAc in hexane) in dichloromethane/methanol gave rise to a new compound 1, 4'-prenyloxyderrone (56 mg). Crystallization of the combined fractions E to H (which were eluted with 12% EtOAc in hexane) in dichloromethane/methanol gave rise to compound 2, durmillone (400 mg) (Ollis et al., 1967). Crystallization of the combined fractions I to K in dichloromethane/methanol gave rise to compound 3, lupeol (40 mg) (Furukawa et al., 2002). Crystallization of the combined fractions L to N (which were eluted with 20% EtOAc in hexane) in dichloromethane/methanol gave rise to a mixture of two compounds (4) and (5); maximaisoflavone J, this is the first report on this plant and maximaisoflavone B (180 mg) (Dagne et al, 1991).

Combined fractions of P to Q (which were eluted with 30% EtOAc in hexane) was subjected to Sephadex LH-20 [CH2Cl2/CH3OH (1:1)] to give rise to compound 6, maximaisoflavone H (10 mg) (Dagne et al., 1991 and Yenesew et al., 1996).

Combined fractions of R to V (which were eluted with 30% EtOAc in hexane) was subjected to small column chromatography on Silica gel (27g) using hexane containing increasing amounts of ethyl acetate to realize compound 7, tephrosin (78 mg) (Ollis et al., 1967) and compound 8, 8-O-methylretusin (5 mg) (Chen et al., 1983 and Rui et al., 1989).
3.3 Physical and Spectroscopic Data for the Isolated Compounds.

3.3.1 DURMILLONE (1)

White crystals; \( R_f = 0.4 \) (n-hexane/EtOAc, 7:3), mp 179 – 181 °C. \(^1\)H NMR (CD\(_2\)Cl\(_2\), 600 MHz): \( \delta 7.95 \) (1H, s, H-2), 7.49 (1H, s, H-5), 7.08 (1H, d, \( J=1.8 \) Hz, H-2'), 6.97 (1H, dd, \( J=1.8, J=7.8 \) Hz, H-6'), 6.87 (1H, d, \( J=7.8 \) Hz, H-5'), 6.82 (1H, d, \( J=9.6 \) Hz, H-4''), 5.99 (2H, s, O-CH\(_2\)-O), 5.77 (1H, d, \( J=9.6 \) Hz, H-3''), 3.93 (3H, s, OMe-6), 1.64 (3H, s, Me-2'') and 1.52 (3H, s, Me-2''). \(^1\)C NMR (CDCl\(_3\), 50 MHz): \( \delta 175.7 \) (C-4), 152.0 (C-2), 147.9 (C-6'), 147.8 (C-3'), 147.6 (C-8a), 147.5 (C-7), 147.4 (C-6), 130.6 (C-3''), 126.1 (C-1'), 124.5 (C-3), 122.6 (C-6'), 117.8 (C-4a), 115.4 (C-4''), 110.4 (C-8), 110.0 (C-2'), 108.6 (C-5'), 105.3 (C-5), 101.4 (OCH\(_2\)O), 78 (C-2''), 56.6 (6-OMe) and 28.2 (2''-Me\(_2\)). (Appendix A).

3.3.2 4'-PRENYLOXYDERRONE (2)

White crystals; \( R_f = 0.4 \) (n-hexane/EtOAc, 7:3), mp 130 – 132 °C, ElMS (m/z 404, C\(_{25}\)H\(_{34}\)O\(_3\)); \(^1\)H NMR (CD\(_2\)Cl\(_2\), 600 MHz): \( \delta 12.94 \) (s, 5-OH), 7.94 (1H, s, H-2), 7.44 (2H, d, \( J=9.0 \) Hz, H-2' and H-6'), 6.96 (2H, d, \( J=9.0 \) Hz, H-3' and H-5'), 6.71 (1H, d, \( J=10.2 \) Hz, H-4''), 6.25 (1H, s, H-6), 5.62 (1H, d, \( J=10.2 \) Hz, H-3''), 5.49 (1H, t, \( J=6.6 \) Hz, H-2'''), 4.55 (2H, d, \( J=6.6 \) Hz, H-1'''), 1.80 (3H, s, Me-3''), 1.76 (3H, s, Me-3''), 1.53 (3H, s, Me-2'') and 1.47 (3H, s, Me-2''). \(^1\)C NMR (CD\(_2\)Cl\(_2\), 150 MHz): \( \delta 181.3 \) (C-4), 162.5 (C-5), 159.8 (C-4''), 159.4 (C-7), 152.9 (C-2), 152.5 (C-8a), 138.4 (C-3''), 130.4 (C-2' and C-6'), 127.8 (C-3''), 123.8 (C-3), 123.1 (C-1'), 119.9 (C-2'''), 114.9 (C-3' and C-5'), 114.7 (C-4''), 106.3 (C-4a), 101.5 (C-8), 100.2 (C-6), 78.4 (C-2''), 29.9 (2''-Me), 28.2 (2''-Me), 25.7 (3'''-Me) and 18.2 (3'''-Me). (Appendix B).
3.3.3 MAXIMAISOFLAVONE H (3)

White crystals; \( R_f = 0.7 \) (n-hexane/EtOAc, 7:3), mp 190-192 °C, \(^1\)H NMR (CD\(_2\)Cl\(_2\), 600 MHz): \( \delta \) 7.92 (1H, s, H-2), 7.83 (1H, d, \( J = 8.4 \) Hz, H-5), 7.46 (2H, d, \( J = 9.0 \) Hz, H-2' and H-6'), 6.98 (2H, d, \( J = 9.0 \) Hz, H-3' and H-5'), 6.96 (1H, d, \( J = 8.4 \) Hz, H-6), 6.21 (2H, s, -OCH\(_2\)O-) and 3.84 (3H, s, 4'-OMe). (Appendix C).

3.3.5 8-O-METHYLRETUSIN (4)

White crystals; \( R_f = 0.7 \) (n-hexane/EtOAc, 6:4), mp 230-232°C \(^1\)H NMR (CD\(_2\)Cl\(_2\), 600 MHz): \( \delta \) 8.00 (1H, s, H-2), 7.91 (1H, d, \( J = 9.0 \) Hz, H-5), 7.49 (2H, d, \( J = 8.4 \) Hz, H-2' and H-6'), 7.04 (1H, d, \( J = 9.0 \) Hz, H-6), 6.97 (2H, d, \( J = 8.4 \) Hz, H-3' and H-5'), 4.07 (3H, s, 8-OCH\(_3\)) and 3.83 (3H, s, 4'-OCH\(_3\)). \(^1^3\)C NMR (CD\(_2\)Cl\(_2\), 150 MHz): \( \delta \) 175.7 (C-4), 159.8 (C-4'), 153.1 (C-6), 150.3 (C-5), 150.1 (C-7 and C-8a), 134.2 (C-8), 130.3 (C-3' and C-5'), 124.5 (C-3), 124.3 (C-1'), 115.0 (C-4a), 113.8 (C-2' and C-6'), 56.6 (8-OMe) and 56.6 (4'-OMe). (Appendix D).

3.3.5 MAXIMAISOFLAVONE J (5)

White crystals; \( R_f = 0.5 \) (n-hexane/EtOAc, 7:3), mp 138-140 °C; \(^1\)H NMR (CD\(_2\)Cl\(_2\), 600 MHz): \( \delta \) 8.10 (1H, d, \( J = 8.4 \) Hz, H-5), 7.92 (1H, s, H-2), 7.47 (2H, d, \( J = 8.4 \) Hz, H-2' and H-6'), 6.96 (2H, d, \( J = 8.4 \) Hz, H-3' and H-5'), 6.87 (1H, d, \( J = 2.4 \) Hz, H-8), 5.49 (1H, t, \( J = 6.6 \) Hz, H-2''), 4.62 (2H, d, \( J = 6.6 \) Hz, H-1''), 3.83 (3H, s, 4'-OMe), 1.81 (3H, s, 3''-Me) and 1.77 (3H, s, 3'''-Me). \(^1^3\)C NMR (CD\(_2\)Cl\(_2\), 150 MHz): \( \delta \) 175.8 (C-4), 163.6 (C-5), 159.8 (C-7 and C-4'), 158.2 (C-8a), 152.6 (C-2), 139.4 (C-3''), 130.4 (C-2' and C-6'), 125.0 (C-3), 124.7 (C-1'), 118.9 (C-2''), 115.2 (C-3' and C-5'), 109.9 (C-4a and C-8), 31
101.6 (C-6), 65.8 (C-1"'), 55.5 (4'-OMe), 25.7 (3"'-Me) and 18.2 (3"'-Me). (Appendix E).

### 3.3.6 MAXIMAISOFLAVONE B (6)

White crystals; $R_f=0.5$ (n-hexane/EtOAc, 7:3), mp 126-128 °C; $^1$H NMR (CD$_2$Cl$_2$, 600 MHz): $\delta$ 8.10 (1H, d, $J$=8.4 Hz, H-5), 7.92 (1H, s, H-2), 7.10 (1H, d, $J=2.4$ Hz, H-2'), 6.96 (1H, $dd$, $J=2.4$, 8.4 Hz, H-6'), 6.87 (1H, $d$, $J=2.4$ Hz, H-8), 6.86 (1H, $d$, $J=8.4$ Hz, H-5'), 5.99 (2H, s, OCH$_2$O), 5.49 (1H, $t$, $J=6.6$ Hz, H-2''), 4.62 (2H, $d$, $J=6.6$ Hz, H-1''), 1.81 (3H, s, 3'-'Me) and 1.77 (3H, s, 3'-'Me). $^{13}$C NMR (CD$_2$Cl$_2$, 150 MHz): $\delta$ 175.8 (C-4), 163.6 (C-5), 159.8 (C-7 and C-4'), 158.2 (C-8a), 152.6 (C-2), 147.9 (C-4'), 147.8 (C-3'), 139.4 (C-3''), 125.0 (C-3), 124.7 (C-1'), 122.6 (C-6'), 118.9 (C-2''), 109.9 (C-2', C-4a and C-8), 108.4 (C-5'), 101.1 (OCH$_2$O), 101.6 (C-6), 65.8 (C-1''), 25.7 (3"'-Me) and 18.2 (3"'-Me). (Appendix F).

### 3.3.7 TEPHROSIN (7)

Yellow oil; $R_f=0.5$(n-hexane/EtOAc, 6:4), mp 197-198 °C $^1$H NMR (CD$_2$Cl$_2$, 600 MHz): $\delta$ 7.79 (1H, $d$, $J=8.4$ Hz, H-11), 7.31 (1H, s, H-1), 6.56 (1H, $d$, $J=10.4$ Hz, H-4'), 6.53 (1H, $d$, $J=8.4$ Hz, H-10), 6.01 (1H, s, H-4), 5.51 (1H, $d$, $J=10.4$ Hz, H-3'), 4.70 (1H, $dd$, $J=2.4$, 12.0 Hz, H-6a), 4.65 (2H, $dd$, $J=2.4$, 12.0 Hz, H-6), 3.85 (3H, s, OMe), 3.76 (3H, s, OMe), 1.49 (3H, s, 2'-Me) and 1.42 (3H, s, 2'-Me). $^{13}$C NMR (CDCl$_3$, 50 MHz): $\delta$ 191.7 (C-12), 161.0 (C-9), 156.9 (C-7a), 151.4 (C4a), 148.7 (C-2), 144.2 (C-3), 129.1 (C-11), 128.8 (C-3'), 115.7 (C-4'), 112.2 (C-1), 111.4 (C-11a), 109.7 (C-10), 109.4 (C-8), 108.9 (C-12b), 101.4 (C-4), 78.3 (C-2'), 76.5 (C-6a), 67.7 (C-12a), 64.1 (C-6), 56.6 (OMe), 56.1 (OMe), 28.8 (2'-Me) and 28.6 (2'-Me). (Appendix G).
3.3.8 LUPEOL (8)

White crystals; $R_f=0.7$ (n-hexane/EtOAc, 9:1), mp 213-215 °C. $^1$H NMR (CD$_2$Cl$_2$, 600 MHz), $\delta$ 0.91 (H-1a), 1.68 (H-1e), 1.54 (H-2a), 1.61 (H-2e), 3.18 (H-3), 0.69 (H-5), 1.39 (H-6a), 1.54 (H-6e), 1.41 (H-7), 1.28 (H-9), 1.25 (H-11a), 1.42 (H-11e), 1.07 (H-12a), 1.68 (H-12e), 1.67 (H-13), 1.01 (H-15a), 1.71 (H-15e), 1.38 (H-16a), 1.49 (H-16e), 1.37 (H-18), 2.39 (H-19), 1.33 (H-21), 1.93 (H-21), 1.20 (H-22), 1.42 (H-22), 0.98 (Me-23), 0.77 (Me-24), 0.84 (H-25), 1.04 (Me-26), 0.97 (Me-27), 0.79 (Me-28), 4.56 (H-29), 4.69 (H-29), 1.69 (H-30). $^{13}$C NMR (CDCl$_3$, 50 MHz): $\delta$ 38.9 (C-1), 28.2 (C-2), 79.2 (C-3), 39.1 (C-4), 55.5 (C-5), 16.2 (C-6), 34.5 (C-7), 41.1 (C-8), 50.7 (C-9), 37.4 (C-10), 19.5 (C-11), 25.4 (C-12), 38.3 (C-13), 43.1 (C-14), 27.7 (C-15), 35.8 (C-16), 43.2 (C-17), 48.5 (C-18), 48.2 (C-19), 151.2 (C-20), 30.0 (C-21), 40.0 (C-22), 21.2 (C-23), 21.2 (C-24), 15.6 (C-25), 18.2 (C-26), 14.8 (C-27), 16.3 (C-28), 109.5 (C-29), 18.5 (C-30).

(Appendix H).

3.4 Biological Activity Assays

3.4.1 In-vitro antiplasmodial activity assays

The crude extract and pure compounds were assayed using a non radioactive assay technique (Smilkstein et al., 2004) with modifications to determine 50% growth inhibition of cultured parasites. This is an accepted method for assaying in-vitro drug susceptibility using the fluorochrome called ‘SYBR Green I’, a non-radioactive intercalating DNA marker that accurately depicts in vitro parasite replication. This test replaces the older, $^3$H-hypoxanthine uptake assay, is fully endorsed by the WHO.
Briefly, two different strains, chloroquine-sensitive Sierra Leone I (D6) and chloroquine-resistant Indochina I (W2), of *Plasmodium falciparum* were grown as described in the literature [Johnson *et al.*, 2007]. Concurrently, twofold serial dilutions of the drugs 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. The culture-adapted *Plasmodium falciparum* were added on to the plate containing dose range of drugs 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 (1x final concentration) 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 I dye using the Tecan Genios Plus (Tecan US, Inc., Durham, NC) with excitation and emission wavelengths of 485 nm and 535 nm, respectively, and with the gain set at 60. Differential counts of relative fluorescence units (RFUs) were used in calculating IC$_{50}$’s for each drug using prism 4.0 software for Windows (Graphpad Software, San Diego, CA). A minimum of three separate determinations was carried out for each sample. Replicates had narrow data ranges hence presented as mean ± SD.

3.4.2 Larvicidal activity assays

The eggs of *Aedes aegypti* L. (Diptera: Culicidae) were obtained from the Department of Zoology, University of Nairobi. The eggs were flooded with 0.08% NaCl solution and
left to hatch at 28°C. 20 2nd instar larvae were transferred into a Petri-dish containing 500ml of 0.08% NaCl solution. The larvae were treated with the test extracts according to Mwangi and Rembold (1998). Every 20 milligrams of test samples were dissolved in 2 ml of DMSO. From the stock solution different concentrations were prepared by serial dilution and the larvae were tested for mortality at 20, 10, 5, 2.5 and 1.25 µg/ml of sample solutions. Control larvae in all cases received 50µl of DMSO as in the test larvae. The rotenone (12) was used as the standard. Mortality was checked after 24 hours. LC50 values were calculated from the average of three observations for each concentration using Finney’s probit analysis for quantal data (McLaughlin et al., 1991: Finney, 1971)
CHAPTER FOUR

RESULTS AND DISCUSSION

4.0 PRELIMINARY TEST RESULTS

TLC analyses of the crude extracts obtained from the stem bark of *Millettia oblata* ssp. *teitensis* showed the presence of UV (254 nm) active compounds. From biogenetic considerations most of these compounds were considered to be isoflavonoids. The extracts were subjected to chromatography which led to the isolation of eight compounds. The isolated compounds were characterized using 1D (\(^1\)H and \(^13\)C), 2D (COSY, HMBC and HMQC) NMR, and MS. In the following sections the isolations, structural elucidation and biological activities of compounds of this plant will be discussed.

4.1 COMPOUNDS FROM THE STEM BARK OF *MILLETTIA OBLATA* SPP. *TEITENSIS*

4.1.1 DURMILLONE (1)

Compound 1 was isolated as white crystals. The \(^1\)H (\(\delta\) 7.95 for H-2) and \(^13\)C (\(\delta\) 152.0 for C-2, 124.5 for C-3 and 175.7 for C-4) NMR spectra (Table 4.1) indicated that compound 1 is an isoflavone derivative (Yenesew *et al.*, 1996). Furthermore, the \(^1\)H and \(^13\)C NMR spectra showed the presence of a methylenedioxy (\(\delta\) 5.99, s, in \(^1\)H and 101.4 in \(^13\)C NMR) and a methoxyl (\(\delta\) 3.93 in \(^1\)H and 56.6 in \(^13\)C NMR) groups. The \(^1\)H NMR spectrum also showed a cis-olefinic protons consisting of two doublets at \(\delta\) 6.82 and 5.77 (d, \(J = 9.6\) Hz), which together with two singlets at \(\delta\) 1.52 and 1.64 each integrating for three protons, suggesting the presence of a 2, 2-dimethylpyrano substituent.
The $^1$H NMR spectrum in addition showed a deshielded aromatic proton at $\delta$ 7.49 and an AMX spin system aromatic protons [$\delta$ 7.08 ($d, J = 1.8$), 6.87 ($d, J = 7.8$), 6.97 ($dd, J = 1.8, 7.8$ Hz)].

The placement of the methylenedioxy group at C-7/C-8 will result in two highly shielded oxygenated aromatic carbons (C-7 and C-8) which normally resonate at $\delta_{C}$ 130 (Agrawal 1989). In compound 1 these carbons are resonating at around $\delta$ 147 and therefore, the methylenedioxy group is at C-3'/C-4' position and hence the pyran ring at C-7/C-8 position. The deshielded aromatic proton at $\delta$ 7.49 is assigned to H-5 of ring A and hence the methoxyl is at C-6. Based on these and by comparison of the data with literature, compound 1 was identified as durmillone (1). Durmillone has been previously isolated from Millettia dura (Ollis et al., 1967). This is, however, the first report of durmillone from this plant.
Table 4.1: $^1$H (CD$_2$Cl$_2$, 600 MHz) and $^{13}$C (CDCl$_3$, 50 MHz) - NMR Chemical Shift Values for Compound 1

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<thead>
<tr>
<th>Position</th>
<th>$^1$H NMR δppm (m, J in Hz)</th>
<th>$^{13}$C NMR δppm</th>
<th>Position</th>
<th>$^1$H NMR δppm (m, J in Hz)</th>
<th>$^{13}$C NMR δppm</th>
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4.1.2 4'-PRENYLOXYDERRONE (2)

Compound 2 was isolated as white crystals; mp 130-132 °C. The HRMS of compound 2 showed a [M]$^+$ at m/z 404.1603 corresponding to the molecular formula C$_{25}$H$_{24}$O$_5$. The $^1$H (δ 7.93 for H-2) and $^{13}$C (δ 152.9 for C-2, 123.8 for C-3 and 181.3 for C-4) NMR spectra (Table 4.2) indicated that compound 2 is an isoflavone derivative (Yenesew et al., 1996).
The $^1$H NMR spectrum showed a cis-olefinic system consisting of two doublets at $\delta$ 6.71 and 5.62 ($d, J = 10.2\text{Hz}$), which together with 6H singlet at $\delta$ 1.47 and 1.53 suggested the presence of a 2, 2-dimethylpyrano substituent. The $^1$H NMR spectrum further indicated the presence of a prenyloxy group ($\delta$ 5.49, 1H, $t, J = 6.6\text{ Hz}$ for H-2$'''$; 4.55, 2H, $d, J = 6.6\text{ Hz}$ for H-1$'''$; 1.76, 3H, $s$, for H-4$'''$ and 1.80, 3H, $s$, for H-5$'''$). The corresponding carbons resonated at $\delta$ 65.1 (C-1$'''$), 119.9 (C-2$'''$), 138.4 (C-3$'''$), 18.2 (C-4$'''$) and 25.7 (C-5$'''$) in the $^{13}$C NMR, respectively. This was further confirmed by the presence in the EIMS of an intense peak at $m/z$ 321 [(M-15)-C$_5$H$_9$]$^+$ due to the loss of a prenyl (Figure 4.1).

The $^1$H NMR revealed that the presence of an AA'XX' spins system centred at $\delta$ 7.44 and 6.96 ($d, J = 9\text{ Hz}$) indicating that ring B is substituted at C-4' with the prenyloxy group. The presence of the fragment at $m/z$ 203 (2b in Figure 4.1) in the HRMS resulting from retro-Diels-Alder cleavage of ring C is consistent with the placement of the prenyloxy group in ring-B and, the hydroxyl and 2, 2-dimethyl group in ring A. A singlet aromatic proton at $\delta$ 6.25 and a chelated OH ($\delta$ 12.94) in ring A require that the pyran group is placed at either C-5/C-6 or C-7/C-8 positions. HMBC correlation of the singlet proton at $\delta$ 6.25 with C-5 (Figure 4.2) places this proton at H-6 and hence the pyran group at C-7/C-8 positions. On this basis, compound 2 was characterized as 7-hydroxy-7,8-(2,2-dimethylpyrano)-4'-prenyloxyisoflavone for which the trivial name 4'-prenyloxyderrone is suggested. This is the first report of this compound in nature. The identity of this new compound was further confirmed through HMQC and HMBC experiments.
Figure 4.1: EIMS fragmentation pattern of compound 2
Table 4.2: $^1$H (CD$_2$Cl$_2$, 600 MHz) and $^{13}$C (CD$_2$Cl$_2$, 150 MHz) -NMR Chemical Shift Values for Compound 2

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<th>$^1$H NMR δppm (m, J in Hz)</th>
<th>$^{13}$C NMR δppm</th>
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41
4.1.3 MAXIMAISOFLAVONE H (3)

Compound 3 was isolated as white crystals. The $^1$H ($\delta$ 7.92 for H-2) NMR spectra (Table 4.4) reveal that compound 3 is an isoflavone derivative (Yenesew et al., 1996). The $^1$H NMR spectrum further revealed the presence of a methylenedioxy ($\delta$ 6.21, s) and a methoxyl ($\delta$ 3.84, s) groups.

The $^1$H NMR spectrum showed an AA'XX' spin system centred at $\delta$ 7.46 and 6.98 ($d, J = 9.0$ Hz), indicating that ring B is substituted at C-4' with the methoxyl group. In ring A, the methylenedioxy group was placed at C-7/C-8 position because of AX spins system at $\delta$ 7.83 and 6.96 ($d, J = 8.4$ Hz) for H-5 and H-6. Based on these and by comparison of the data with literature, compound 3 was identified as maximaisolavone H (3), a compound
which has been previously isolated from *Millettia dura* (Dagne et al., 1991; Yenesew et al., 1996). This is, however, the first report of maximaisoflavone H from this plant.

![Chemical structure of compound 3](image)

**Table 4.3**: $^1$H (CD$_2$Cl$_2$, 600 MHz) -NMR Chemical Shift Values for Compound 3

<table>
<thead>
<tr>
<th>Position</th>
<th>$^1$H NMR $\delta$ ppm ($m$, $J$ in Hz)</th>
<th>Position</th>
<th>$^1$H NMR $\delta$ ppm ($m$, $J$ in Hz)</th>
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<td>7.46 (d, $J$=9.0)</td>
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4.1.4: 8-β-O-METHYLRETUSIN (4)
Compound 4 was isolated as white crystals. The \( ^1H \) (δ 8.00 for H-2) NMR spectra (Table 4.5) reveal that compound 4 is an isoflavone derivative (Yenesew et al., 1996). The \( ^1H \) NMR spectrum further revealed two methoxyl (δ 3.83 and 4.07 ppm) substituents.

The \( ^1H \) NMR spectrum (Table 4.5) showed an AA'XX' spin system centred at δ 7.49 and 6.97 (\( d, J = 9.0 \) Hz), indicating that ring B is substituted at C-4' with either a methoxyl or a hydroxyl group. The \( ^1H \) NMR spectrum also revealed an AX spins system at δ 7.91 and 7.04 (\( d, J = 9.0 \) Hz) which were readily assigned to H-5 and H-6.

From the HMBC spectrum (Figure 4.3), the methoxyl group at δ 4.07 was correlating with C-8 and the methoxyl at δ 3.83 correlating with C-4'. The hydroxyl group was then placed at C-7. Based on these and by comparison of the data with literature, compound 4 was identified as 8-β-O-methylretusin (4), a compound which has been previously isolated from Millettia dielsiana (Rui et al., 1989) and Millettia reticulata (Chen et al., 1983). This is, however, the first report of 8-β-O-methylretusin from this plant.

![Figure 4.3: HMBC Correlation in Compound 4](image-url)
Table 4.4: $^1$H (CD$_2$Cl$_2$, 600 MHz)-NMR Chemical Shift Values for Compound 4

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</table>

**4.1.5: MAXIMAISOFлавONE J (5)**

Compound 5 was isolated as white crystals. The $^1$H ($\delta$ 7.92 for H-2) and $^{13}$C ($\delta$ 152.6 for C-2, 125.0 for C-3 and 175.8 for C-4) NMR spectra (Table 4.7) indicated that compound 5 is an isoflavone derivative (Yenesew et al., 1996). The $^1$H NMR spectrum further indicated the presence of a methoxyl ($\delta_H$ 3.83; $\delta_C$ 55.5) and a prenyloxy group ($\delta$ 5.50, 1H, $r, J = 6.6$ Hz for H-2''); 4.62, 2H, $d, J = 6.6$ Hz for H-1''; 1.77, 3H, $s$, for H-4'' and 1.81, 3H, $s$, for H-5''). The corresponding carbons resonated at $\delta$ 65.8 (C-1''), 118.9 (C-2''), 139.4 (C-3''), 18.2 (C-4'') and 25.7 (C-5'') in the $^{13}$C NMR.
The $^1$H NMR revealed an AA'XX' spins system centred at $\delta$ 7.47 and 6.96 ($d, J = 8.4$ Hz) indicating that ring B is substituted at C-4' with either methoxyl or prenyloxyl group. The position of methoxyl group was determined from NOESY interactions between the methoxyl group at $\delta$ 3.83 and the doublet at $\delta$ 6.96, hence the methoxyl group was placed at C-4' and prenyloxyl group at C-7 of monosubstituted ring A where H-5, H-6 and H-8 appeared at $\delta_H$ 8.10 ($d, J = 8.4$ Hz, H-5), 6.96 ($d, J = 8.4$ Hz, H-6) and 6.87 ($d, J = 2.4$ Hz, H-8). NOE interaction of CH$_2$-1’’ with H-6 and H-8 is consistent with the placement of the prenyloxyl group at C-7. Based on these and by comparison of the data with literature (Rao et al., 1994), compound 5 was identified as maximaisolavone J (5). This is the first report of maximaisolavone J from the genus *Millettia*.
Table 4.5: $^1$H (CD$_2$Cl$_2$, 600 MHz) and $^{13}$C (CD$_2$Cl$_2$, 150 MHz) -NMR Chemical Shift

Values for Compound 5

<table>
<thead>
<tr>
<th>Position</th>
<th>$^1$H NMR δppm (m, J in Hz)</th>
<th>$^{13}$C NMR δppm (m, J in Hz)</th>
<th>Position</th>
<th>$^1$H NMR δppm (m, J in Hz)</th>
<th>$^{13}$C NMR δppm (m, J in Hz)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>7.92 (s)</td>
<td>152.6</td>
<td>3'</td>
<td>6.96 (d, J=8.4)</td>
<td>115.2</td>
</tr>
<tr>
<td>3</td>
<td>125.0</td>
<td>175.8</td>
<td>4'</td>
<td>159.8</td>
<td>175.8</td>
</tr>
<tr>
<td>4</td>
<td>125.0</td>
<td>109.9</td>
<td>5'</td>
<td>6.96 (d, J=8.4)</td>
<td>115.2</td>
</tr>
<tr>
<td>4a</td>
<td></td>
<td></td>
<td>6'</td>
<td>7.47 (d, J=8.4)</td>
<td>130.4</td>
</tr>
<tr>
<td>5</td>
<td>8.10 (d, J=8.4)</td>
<td>163.6</td>
<td>1''</td>
<td>4.62 (d, J=6.6)</td>
<td>65.8</td>
</tr>
<tr>
<td>6</td>
<td>6.96 (d, J=8.4)</td>
<td>101.6</td>
<td>2''</td>
<td>5.49 (t, J=6.6)</td>
<td>118.9</td>
</tr>
<tr>
<td>7</td>
<td></td>
<td>159.8</td>
<td>3''</td>
<td></td>
<td>139.4</td>
</tr>
<tr>
<td>8</td>
<td>6.87 (d, J=2.4)</td>
<td>109.9</td>
<td>4''-Me</td>
<td>1.77 (s)</td>
<td>18.2</td>
</tr>
<tr>
<td>8a</td>
<td></td>
<td>158.2</td>
<td>5''-Me</td>
<td>1.81 (s)</td>
<td>25.7</td>
</tr>
<tr>
<td>1'</td>
<td></td>
<td>124.7</td>
<td>6-OMe</td>
<td>3.83 (s)</td>
<td>55.5</td>
</tr>
<tr>
<td>2'</td>
<td>7.47 (d, J=8.4)</td>
<td>130.4</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

4.1.6: MAXIMAISOFLAVONE B (6)

Compound 6 was isolated as white crystals. The $^1$H (δ 7.92 for H-2) and $^{13}$C (δ 152.6 for C-2, 125.0 for C-3 and 175.8 for C-4) NMR spectra (Table 4.8) indicated that compound 6 is an isoflavone derivative (Yenesew et al., 1996). The $^1$H NMR spectrum further indicated the presence of a methylenedioxy (δ 5.99, s, in $^1$H and 101.1 in $^{13}$C NMR) and a prenyloxy group (δ 5.50, 1H, t, J = 6.6 Hz for H-2''; 4.62, 2H, d, J = 6.6 Hz for H-1''; 1.77, 3H, s, for H-4'' and 1.81, 3H, s, for H-5''). The corresponding carbons of the prenyloxy group resonated at δ 65.8 (C-1''), 118.9 (C-2''), 139.4 (C-3''), 18.2 (C-4'') and 25.7 (C-5'').

The $^1$H NMR spectrum in addition showed an AMX spin system for aromatic protons [δ 7.10 (d, J = 2.4, H-2''), 6.86 (d, J = 8.4, H-5'), 6.96 (dd, J = 8.4, 2.4, H-6')] in ring B. The position of the prenyloxyl group was determined from NOESY interactions between the methylene protons at δ 4.62 and the doublet at δ 6.87 (d, J = 2.4, H-8) and δ 6.96 (d, J =
8.4, H-6) in ring A, hence the prenyloxylo group was placed at C-7 and methylenedioxy group at C-3'/'C-4' in agreement with the biogenetic expectation of oxygenation at C-7 and C-4'. Based on these and by comparison of the data with literature (Rao et al., 1984), compound 6 was identified as maximaisoflavone B (6), a compound which has been previously isolated from *Millettia dura* (Dagne et al., 1991). This is the first report of maximaisoflavone B from this plant.

Table 4.6: $^1$H (CD$_2$Cl$_2$, 600 MHz) and $^{13}$C (CD$_2$Cl$_2$, 150 MHz) NMR Chemical Shift Values for Compound 6

<table>
<thead>
<tr>
<th>Position</th>
<th>$^1$H NMR $\delta$ppm ($m, J$ in Hz)</th>
<th>$^{13}$C NMR $\delta$ppm</th>
<th>Position</th>
<th>$^1$H NMR $\delta$ppm ($m, J$ in Hz)</th>
<th>$^{13}$C NMR $\delta$ppm</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>7.92 (s)</td>
<td>152.6</td>
<td>3'</td>
<td>147.8</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>125.0</td>
<td>4'</td>
<td>6'</td>
<td>147.9</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>175.8</td>
<td>5'</td>
<td>6.86 ($d, J=8.4$)</td>
<td>108.4</td>
<td></td>
</tr>
<tr>
<td>4a</td>
<td>-109.9</td>
<td>6'</td>
<td>6.96 ($dd, J=2.4, 8.4$)</td>
<td>122.6</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>8.10 ($d, J=8.4$)</td>
<td>163.6</td>
<td>1''</td>
<td>4.62 ($d, J=6.6$)</td>
<td>65.8</td>
</tr>
<tr>
<td>6</td>
<td>6.96 ($d, J=8.4$)</td>
<td>101.6</td>
<td>2''</td>
<td>5.49 ($t, J=6.6$)</td>
<td>118.9</td>
</tr>
<tr>
<td>7</td>
<td>159.8</td>
<td>3'''</td>
<td>139.4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>6.87 ($d, J=2.4$)</td>
<td>109.9</td>
<td>4''-Me</td>
<td>1.77 (s)</td>
<td>18.2</td>
</tr>
<tr>
<td>8a</td>
<td>158.2</td>
<td>5''-Me</td>
<td>1.81 (s)</td>
<td>25.7</td>
<td></td>
</tr>
<tr>
<td>1''</td>
<td>124.7</td>
<td>-OCH$_2$O-</td>
<td>5.99 (s)</td>
<td>101.1</td>
<td></td>
</tr>
<tr>
<td>2'</td>
<td>7.10 ($d, J=2.4$)</td>
<td>109.9</td>
<td>49.6</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
4.1.7 TEPHROSIN (7)

Compound 7 was isolated as yellow oil. The $^1$H-NMR [δ 4.65 ($dd, J = 12.0, 2.5$ Hz, H-6), 4.58 ($dd, J = 2.5, 0.8$ Hz, H-6), 4.70 ($dd, J = 12.0, 0.8$ Hz, H-6a)] and $^{13}$C-NMR [δ 64.1 (C-6), 76.5 (C-6a)] showed peaks which are characteristic of a 12α-hydroxyrotenoid skeleton (Yenesew et al., 2003). The $^1$H-NMR (Tabe 4.7) further revealed two methoxy (δ 3.77, 3.66) and a 2, 2-dimethylpyran δ 1.42 (2’-Me), 1.49 (2’-Me), 5.51 (d, $J = 10.4$ Hz, H-3’), 6.56 (d, $J = 10.4$ Hz, H-4’) substituents.

In the $^1$H-NMR spectrum revealed two para-oriented aromatic protons at δ 7.31 (H-1) and 6.47 (H-4) which are in agreement with the placement of the methoxyls at C-2 and C-3 in ring A. The presence of AX doublets at δ 6.51 for H-10 and 7.77 for H-11, would place the 2,2-dimethylpyran group at C-8/C-9, with oxygen at C-9 in ring D. Based on these and by comparison of the data with literature (Yenesew et al., 2003), compound 7 was identified as tephrosin (7) (Ollis et al., 1967) which has earlier been isolated from the seeds of Millettia dura (Yenesew et al., 2003). This is the first report of tephrosin from this plant.
Table 4.7: $^1$H (CD$_2$Cl$_2$, 600 MHz) and $^{13}$C (CDCl$_3$, 50 MHz) -NMR Chemical Shift

Values for Compound 7

<table>
<thead>
<tr>
<th>Position</th>
<th>$^1$H NMR, $\delta$ppm ($m$, $J$ in Hz)</th>
<th>$^{13}$C NMR, $\delta$ppm</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>7.31 (s)</td>
<td>112.2</td>
</tr>
<tr>
<td>1a</td>
<td></td>
<td>108.9</td>
</tr>
<tr>
<td>2</td>
<td></td>
<td>148.7</td>
</tr>
<tr>
<td>3</td>
<td></td>
<td>144.2</td>
</tr>
<tr>
<td>4</td>
<td>6.01 (s)</td>
<td>101.4</td>
</tr>
<tr>
<td>4a</td>
<td></td>
<td>151.4</td>
</tr>
<tr>
<td>6</td>
<td>4.65 ($dd$, $J$=2.4, 12.0)</td>
<td>64.1</td>
</tr>
<tr>
<td>6a</td>
<td>4.70 ($dd$, $J$=0.8, 12.0)</td>
<td>76.5</td>
</tr>
<tr>
<td>7a</td>
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<td>156.9</td>
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<tr>
<td>8</td>
<td></td>
<td>109.4</td>
</tr>
<tr>
<td>9</td>
<td></td>
<td>161.0</td>
</tr>
<tr>
<td>10</td>
<td>6.53 ($d$, $J$=8.4)</td>
<td>109.7</td>
</tr>
<tr>
<td>11</td>
<td>7.79 ($d$, $J$=8.4)</td>
<td>129.1</td>
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<td>191.7</td>
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<td>67.7</td>
</tr>
<tr>
<td>2'</td>
<td></td>
<td>78.3</td>
</tr>
<tr>
<td>3'</td>
<td>5.51 ($d$, $J$=10.4)</td>
<td>128.8</td>
</tr>
<tr>
<td>4'</td>
<td>6.56 ($d$, $J$=10.4)</td>
<td>115.7</td>
</tr>
<tr>
<td>2'-Me$_2$</td>
<td>1.49 (s)</td>
<td>28.8</td>
</tr>
<tr>
<td></td>
<td>1.42 (s)</td>
<td>28.6</td>
</tr>
<tr>
<td>OMe-2</td>
<td>3.77 (s)</td>
<td>56.6</td>
</tr>
<tr>
<td>OMe-3</td>
<td>3.66 (s)</td>
<td>56.1</td>
</tr>
</tbody>
</table>

4.1.8 LUPEOL (8)

Compound 8 was obtained as non-UV active white crystals (from MeOH). The $^{13}$C NMR and DEPT of this compound revealed the presence of 30 carbons indicating that it could be a triterpene. The characteristic peaks at $\delta$ 79.2 for the oxygenated C-3, the quarternary carbon peaks (at $\delta$ 39.1, 37.4, 41.1, 43.1 and 43.2) and olefinic carbons ($\delta$ 151.2 and 109.5) showed that compound 8 is lupeol (Furukawa et al., 2002). In agreement with this, the $^1$H NMR revealed the presence of seven singlet methyl protons ($\delta$ 0.98, 0.77, 0.84,
1.04, 0.97, 0.79, and 1.69) and methylene protons (δ 4.56 and 4.69) as well as a double doublet at δ 3.18.

In the 1H-NMR spectrum, a double doublet at δ 3.18 was assigned to H-3, 0.98 for Me-23, 0.77 for Me-24 and 0.84 for Me-25 in ring A. In ring B, the singlet methyl protons δ 1.04 was assigned Me-26 and 0.97 for Me-27 in ring C. In ring D, the singlet methyl protons δ 0.79 was assigned Me-28 and 1.69 for Me-30 in ring E. The methylene protons (δ 4.56 and 4.69) were assigned at H-29a and H-29b. This is the first report of Lupeol from this plant.

4.2 Biological Activities of the Isolated Compounds

4.2.1 Anti-plasmodial activities of compounds from the stem bark of *Millettia oblata* ssp. *teitensis*

The crude extract (CH2Cl2/MeOH, 1:1) of the stem bark of *Millettia oblata* ssp. *teitensis* was tested for antiplasmodial activities against two different strains of the malaria parasite. Moderate antiplasmodial activities against both W2 and D6 strains of
*Plasmodium falciparum* was observed with an IC50 value of 10.0±2.3 and 12.0±1.2 μg/ml respectively. The crude extract of the stem bark of *Millettia oblata* ssp. *teitensis* yielded eight compounds, of which five were tested against the W2 and D6 strains of *Plasmodium falciparum*, and showed anti-plasmodial activities. The results are summarised in Table 4.8.

Table 4.8: *In vitro* IC50 values of some compounds of *Millettia oblata* ssp. *teitensis* against W2 and D6 strains of *Plasmodium falciparum*.

<table>
<thead>
<tr>
<th>Tested compound</th>
<th>IC50 in μg/ml (± SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>W2</td>
</tr>
<tr>
<td>Durmillone (1)</td>
<td>9.8±0.1</td>
</tr>
<tr>
<td>4'-Prenyloxyderrone (2)</td>
<td>6.0±0.9</td>
</tr>
<tr>
<td>A mixture of maximaisoflavone J (5) and maximaisoflavone B (6)</td>
<td>16.5±3.5</td>
</tr>
<tr>
<td>Tephrosin (7)</td>
<td>11.5±0.3</td>
</tr>
<tr>
<td>Lupeol (8)</td>
<td>NT</td>
</tr>
<tr>
<td>Chloroquine</td>
<td>0.0699±0.0102</td>
</tr>
<tr>
<td>Mefloquine</td>
<td>0.0024±0.0004</td>
</tr>
</tbody>
</table>

NT- Not tested

The activities observed for the crude extract is within the range reported for isoflavones (Yenesew, 1997 and Derese, 2004). It is worth noting that the crude extract is more active against the chloroquine-resistant strain than the chloroquine-sensitive strain.

Durmillone (1) showed good antiplasmodial activity with the activity being more against the chloroquine-sensitive D6 stains than the chloroquine-resistant W2 strains. The new compound 4'-prenyloxyderrone (2) showed good activity against both strains. Lupeol (8)
showed moderate antiplasmodial activity, while tephrosin (7) and the mixture of maximaisoflavone J (5) and maximaisoflavone B (6) showed some appreciable amount of antiplasmodial activity.

4.2.2 Larvicidal activity tests

The MeOH/CH$_2$Cl$_2$ (1:1) extracts of the stem, seeds and seedpods of *Millettia oblata* ssp. *teitensis* were partitioned between hexane and methanol. The purpose of partitioning was to remove the oil part which is the hexane layer. The methanol layer of the stem, seeds and seedpods were tested against the second instar larvae of *Aedes aegypti*. The stem bark extract showed larvicidal activity with the LC$_{50}$ value of 11.2±1.9 µg/ml after 24 hours. The seed extract showed potent larvicidal activity with LC$_{50}$ value of 1.4±0.2 µg/ml after 24 hours. The seedpod extracts were inactive even at 20.0 µg/ml concentration of the sample and after 10 days the larvae turned into adult mosquitoes. The observed activity of the seeds and the stem bark of *Millettia oblata* ssp. *teitensis* could be due to the presence of rotenoids in their composition.

Rotenoids are known to occur widely in some genera of the family leguminosae, such as *Millettia*, *Lonchocarpus*, *Tephrosia* and *Derris* (Dewick, 1994). These plants are distributed in tropical region of the world where there are mosquitoes. Utilization of such plants as larvicidal agents will decrease the population of malaria vector and help to control epidermic caused by the vector. Isoflavones are reported to be inactive for larvicidal activity (Yenesew, 1997), that is why larvicidal activities of the pure compounds were not carried out.
CHAPTER FIVE
CONCLUSIONS AND RECOMMENDATIONS

5.1 CONCLUSIONS

In this study, the stem bark of *Millettia oblata* ssp. *teitensis* was investigated and eight compounds were isolated and characterized. The conclusions drawn from this study are outlined below:

1. The phytochemical study of the stem bark of *Millettia oblata* ssp. *teitensis* led to the isolation and characterization of eight compounds. These include six isoflavones: durmillone (1), 4'-prenyloxyderrone (2), maximaisoflavone-H (3), 8-O-methylretusin (4), maximaisoflavone J (5) and maximaisoflavone B (6), one rotenoid tephrosin (7) and a triterpene lupeol (8). Of these, the isoflavone 4'-prenyloxyderrone is a new compound.

2. The antiplasmodial activities of the crude extracts and some of the isolated compounds were tested against chloroquine-resistant strain (W2) and the chloroquine-sensitive strain (D6) of *Plasmodium falciparum* parasite for malaria. The results showed that some compounds had moderate antiplasmodial activities with the new compound showing the highest activity with IC$_{50}$ values of 6.0±0.9 for W2 and 5.4±1.0 for D6. The crude extract of stem, seeds and seedpods were tested for larvicidal activities and the seed extract had the highest larvicidal activity with LC$_{50}$ value of 1.4±0.2 µg/ml after 24 hours.
5.2 RECOMMENDATIONS

Although the results of the study demonstrated moderate antiplasmodial activities of the extract and compounds isolated from the stem bark of *Millettia oblata* ssp. *teitensis*, more information on toxicity and efficacy is required as a means of developing them into therapies for human use. In order to fulfill some of these requirements, the following recommendations are put forward:

1. Further phytochemical investigation of the stem bark, root, seeds and seedpods of *Millettia oblata* ssp. *teitensis* should be carried out in order to determine fully all the major and isolable compounds that are biosynthesized by this medicinal plant.

2. *In vivo* antiplasmodial activity tests should be carried out on the extract and isolated compounds from this medicinal plant in order to establish their potency and efficacy.
REFERENCES


APPENDICES
APPENDIX A: SPECTRA FOR COMPOUND 1
$^1$H NMR SPECTRUM FOR COMPOUND 1 (600 MHz, CD$_2$Cl$_2$)
$^1$H NMR SPECTRUM EXPANSION FOR COMPOUND 1

ppm

0.856
0.960
1.001
1.033
2.006
5.994
5.776
6.080
6.977
6.974
6.964
6.961
6.875
6.862
5.817
5.801
4.851.380
4.849.699
4.848.055
4.86.254
4.80.072
4.78.271
4.776
4.760
$^{13}$C NMR SPECTRUM FOR COMPOUND 1 (50 MHz, CDCl$_3$)

- 175.659
- 162.004
- 147.869
- 147.788
- 147.558
- 147.456
- 147.384
- 130.633
- 126.134
- 124.533
- 122.576
- 117.766
- 115.368
- 110.422
- 110.027
- 108.594
- 105.324
- 101.379
DEPT NMR SPECTRUM FOR COMPOUND 1

- **CH3 carbons**

- **CH2 carbons**

- **CH carbons**

- **All protonated carbons**

The spectrum is plotted on a scale from 180 to 0 ppm.
APPENDIX B: SPECTRA FOR COMPOUND 2
$^1$H NMR SPECTRUM FOR COMPOUND 2 (600 MHz, CD$_2$Cl$_2$)
$^1$H NMR SPECTRUM EXPANSION FOR COMPOUND 2
$^1$H NMR SPECTRUM EXPANSION FOR COMPOUND 2
$^1$H NMR SPECTRUM FOR COMPOUND 2 (150 MHz, CD$_2$Cl$_2$)
$^{13}$C NMR SPECTRUM EXPANSION FOR COMPOUND 2

153 ppm

1 3

C NMR SPECTRUM EXPANSION FOR COMPOUND 2

163
162
161
160
159
158
157
156
155
154
153

159.799
159.400
162.928
152.917
152.538
$^{13}C$ NMR SPECTRUM EXPANSION FOR COMPOUND 2

130.407
127.838
123.781
123.161
119.890
114.875
114.679
$^{13}$C NMR SPECTRUM EXPANSION FOR COMPOUND 2
$^1\text{H} - ^1\text{H COSY SPECTRUM FOR COMPOUND 2}$
$^1$H – $^1$H COSY SPECTRUM EXPANSION FOR COMPOUND 2
HMOC SPECTRUM FOR COMPOUND 2
HMOC SPECTRUM EXPANSION FOR COMPOUND 2
HMQC SPECTRUM EXPANSION FOR COMPOUND 2
HMBC SPECTRUM FOR COMPOUND 2
HMBC SPECTRUM EXPANSION FOR COMPOUND 2
HMBC SPECTRUM EXPANSION FOR COMPOUND 2
HMBC SPECTRUM EXPANSION FOR COMPOUND 2
HMBC SPECTRUM EXPANSION FOR COMPOUND 2
EI-MS SPECTRUM FOR COMPOUND 2
APPENDIX C: SPECTRA FOR COMPOUND 3
\(^1\)H NMR SPECTRUM FOR COMPOUND 3 (600 MHz, CD\(_2\)Cl\(_2\))
\[ ^1H \text{ NMR SPECTRUM EXPANSION FOR COMPOUND 3} \]
APPENDIX D: SPECTRA FOR COMPOUND 4
$^1$H NMR SPECTRUM FOR COMPOUND 4 (600 MHz, CD$_2$Cl$_2$)
1H NMR SPECTRUM EXPANSION FOR COMPOUND 4

ppm

8.0 7.9 7.8 7.7 7.6 7.5 7.4 7.3 7.2 7.1 7.0 6.9 6.8 6.7 6.6 6.5 6.4 6.3 6.2 6.1 5.9 5.8 5.7 5.6 5.5 5.4 5.3 5.2 5.1 5.0 4.9 4.8 4.7 4.6 4.5 4.4 4.3 4.2 4.1 4.0 3.9 3.8 3.7 3.6 3.5 3.4 3.3 3.2 3.1 3.0 2.9 2.8 2.7 2.6 2.5 2.4 2.3 2.2 2.1 2.0 1.9 1.8 1.7 1.6 1.5 1.4 1.3 1.2 1.1 1.0

APPENDIX E: SPECTRA FOR COMPOUND 5 AND 6
$^1$H NMR SPECTRUM FOR COMPOUND 5 AND 6 (600 MHz, CD$_2$Cl$_2$)
\textbf{\textsuperscript{1}H NMR SPECTRUM EXPANSION FOR COMPOUND 5 AND 6}

\begin{figure}
\centering
\includegraphics[width=\textwidth]{nmmr_spectrum_expansion.png}
\end{figure}
'H NMR SPECTRUM EXPANSION FOR COMPOUND 5 AND 6
$^{13}$C NMR SPECTRUM FOR COMPOUND 5 AND 6 (150 MHz, CD$_2$Cl$_2$)
$^{13}$C NMR SPECTRUM EXPANSION FOR COMPOUND 5 AND 6
$^{13}$C NMR SPECTRUM EXPANSION FOR COMPOUND 5 AND 6
$^{13}$C NMR SPECTRUM EXPANSION FOR COMPOUND 5 AND 6

- 130.392
- 127.595
- 126.236
- 124.954
- 124.867
- 124.713
- 122.564
- 118.901
- 118.879
- 118.486
- 118.411
- 115.241
- 115.174
- 113.985
- 109.906
- 108.386
- 101.632
- 101.106
$^1\text{H} - ^1\text{H COSY SPECTRUM FOR COMPOUND 5 AND 6}$
HMBC SPECTRUM FOR COMPOUND 5 AND 6
HMBC SPECTRUM EXPANSION FOR COMPOUND 5 AND 6
HMBC SPECTRUM EXPANSION FOR COMPOUND 5 AND 6
HMBC SPECTRUM EXPANSION FOR COMPOUND 5 AND 6
HMOC SPECTRUM FOR COMPOUND 5 AND 6
HMOC SPECTRUM EXPANSION FOR COMPOUND 5 AND 6
NOESY SPECTRUM FOR COMPOUND 5 AND 6
NOESY SPECTRUM FOR COMPOUND 5 AND 6
APPENDIX F: SPECTRA FOR COMPOUND 7
\textbf{'H NMR SPECTRUM FOR COMPOUND 7 (600 MHz, CD$_2$Cl$_2$)}
$^1$H NMR SPECTRUM EXPANSION FOR COMPOUND 7
$^1$H NMR SPECTRUM EXPANSION FOR COMPOUND 7
$^1$H NMR SPECTRUM EXPANSION FOR COMPOUND 7
\(^{13}\)C NMR SPECTRUM FOR COMPOUND 7 (50 MHz, CDCl\(_3\))

Pulse Sequence: s2pul
\(^{13}\text{C} \text{ NMR SPECTRUM EXPANSION FOR COMPOUND 7}\)
$^{13}$C NMR SPECTRUM EXPANSION FOR COMPOUND 7
$^{13}$C NMR SPECTRUM EXPANSION FOR COMPOUND 7
$^{13}$C NMR SPECTRUM EXPANSION FOR COMPOUND 7
DEPT NMR SPECTRUM FOR COMPOUND 7

CH₃ carbons

CH₂ carbons

CH carbons

All protonated carbons
DEPT NMR SPECTRUM FOR COMPOUND 7
APPENDIX G: SPECTRA FOR COMPOUND 8
\( ^1H \text{ NMR SPECTRUM FOR COMPOUND 8 (600 MHz, CD}_2\text{Cl}_2) \)
$^1$H NMR SPECTRUM EXPANSION FOR COMPOUND 8
$^{13}$C NMR SPECTRUM FOR COMPOUND 8 (50 MHz, CDCl$_3$)
$^{13}$C NMR SPECTRUM EXPANSION FOR COMPOUND 8
DEPT NMR SPECTRUM FOR COMPOUND 8

- CH₃ carbons
- CH₂ carbons
- CH carbons
- all protonated carbons