PHYTOCHEMICAL AND ANTIPLASMODIAL EVALUATION OF THE ROOT BARK OF KENYAN *Warburgia stuhlmannii* Engl

BY

MULIANGA ALBERT MAKENZI

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MASENO UNIVERSITY

ABSTRACT

Malaria is a huge social, economic and health problem, particularly in tropical countries. Approximately 350-500 million malaria cases are reported annually, out of which 1-3 million die, majority of who are young children from sub-Saharan Africa. Malaria parasites have become resistant to almost every antimalarial drug currently available and most of the drugs in use have serious side effects. The genus Warburgia is valued for curing several ailments, including malaria. Phytochemical investigation of Warburgia species revealed presence of sesquiterpenes. Sesquiterpenes such as artemisinin are reported to be effective in treating malaria. *Warburgia stuhlmannii*, a species of Warburgia is used by communities in Kwale District Kenya, for malaria treatment and remedy for toothache and rheumatism. This work aimed at isolation and characterization of compounds from *W. stuhlmannii* root bark and evaluation of its extracts and isolates for antiplasmodial activity. The dried and ground root bark was extracted sequentially with ethyl acetate and methanol by cold percolation at room temperature. The extracts showed antiplasmodial activity against the chloroquine sensitive (D10) and chloroquine resistant (W2) strains of *Plasmodium falciparum*, with IC_{50} values of 32.5µg/ml and 38.4µg/ml respectively for the ethyl acetate extract but 80.5µg/ml and 95.35µg/ml respectively for methanol extract. Fractionation of *W. stuhlmannii* root bark ethyl acetate and methanol extracts led to the isolation of 10 compounds of farnesane-type sesquiterpenes. 6α,9α-dihydroxy-4(13),7,coloratadiene-11,12-dial **(52)** is being reported for the first time from this species. Fractionation of the ethyl acetate extract afforded the sesquiterpenes; mukaadial **(11),** ugandensidial **(12),** muzigadial **(14),** warburganal **(29),** polygodial **(30),** ugandensolide **(35),** deacetylugandensolide **(36)** cinnamolide **(37),** bemadienolide **(45),** and 6α,9α-dihydroxy-4(13),7,coloratadiene-11,12-dial **(52)** The methanol extract gave mukaadial **(11),** ugandensolide **(35)** and deacetylugandensolide **(36).** The isolated compounds were active against chloroquine sensitive (D10) and chloroquine resistant (W2) strains of *P. falciparum.* The antiplasmodial activities for warburganal **(29),** polygodial **(30),** deacetylugandensolide **(36)** and bemadienolide **(45)** are being reported for the first time. Mukaadial **(11)** had the highest antiplasmodial activity against both D10 and W2 strains of *P. falciparum*, with IC_{50} values of 4.3µM and 5.8µM, respectively, while muzigadial **(13)** though very effective on D10 strain (IC_{50} 5.6µM) was less effective against W2 strain (IC_{50} 16.4µM). The antiplasmodial activity of *W.* root bark extracts and compounds authenticates its ethno-pharmacological use in treatment of malaria.

CHAPTER ONE

1.0 BACKGROUND

Malaria has been a human infection for over 50,000 years, with Plasmodium as a human pathogen for the entire history of the Plasmodium species [Joy *et al.*, 2003). The disease has been responsible for economic decline of nations and military defeat by causing more casualties than weapons. It contributed to decline of Roman Empire (BBC News, January, 29, 2003); and caused death of sixty thousand American soldiers during North African and South Pacific campaigns (Joseph, 2008). Malaria continues to be a huge social, economic and health problem, particularly in tropical countries. It costs Africa $12 billion in lost gross domestic product (GDP) every year and consumes 40% of all public health spending (Sachs and Malaney, 2002). Malaria is widespread in tropical and sub tropical regions and is endemic in 91 countries, predominantly in Africa, Asia and Latin America, with about 40% of the world's population at risk (WHO, 2001). Approximately 350-500 million cases of malaria are reported annually (CDC, 2007). The disease kills between one and three million people, the majority of whom are young children in sub-Saharan Africa (Snow *et al.*, 2005) and indeed, in every thirty seconds a child dies from the disease, worldwide (WHO, 2009). In the year 2010 about 1,298,000 people died from the disease worldwide (Christopher *et al.*, 2012). In Kenya, 30-50% of outpatient treatments and 19% hospital admissions are malaria cases, accounting for 8-10 million treatments per year (Ochola, 2003).

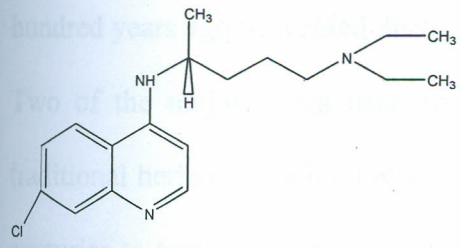
The protozoan parasites of the genus *Plasmodium*, cause malaria and the common species include; *Plasmodium falciparum*, *Plasmodium ovale*, *Plasmodium malariae*, *Plasmodium knowlesi* *Plasmodium berghei and Plasmodium falciparum* is responsible for 80% of all malaria cases and 90% of all death from malaria (Mendis *et al.*, 2001). The disease is transmitted by female anopheles mosquitoes that feed on blood meal at night. Male anopheles mosquitoes feed on plant juices, hence do not transmit malaria. Pregnant women are especially attractive to mosquitoes (Lindsay *et al.*,

2000). Global warming may lead to expansion of areas in which ambient temperature and climatic conditions are suitable for Plasmodium transmission (Minakawa *et al.*, 2002).

Efforts to control malaria include; attempts in development of effective vaccines, eradication of mosquito vectors and development of new drugs. Development of vaccines, have proven to be difficult and therefore, effective vaccine will probably not be available in the near future (Kilawa and Ntoumi, 2009). Malaria vector eradication involves larval control and insecticide use to reduce adult anopheles mosquitoes. In larval control, irrigation ditches and swamp lands are eliminated by in-filling while larvivorous fish are introduced in wells and reservoirs (Clyde, 1987). Natural pathogens such as viruses, bacteria, protozoa, fungi and nematodes may be used in attacking mosquito larvae as well as use of chemical larvicides such as petroleum oils, pyrethroids, chlorinated hydrocarbons and organophosphorus insecticides (Bruce-Chwatt, 1980). In control of adult anopheles vector, residual insecticide spraying of homes is employed. However, cost effectiveness of residual spraying is steadily decreasing as resistant anopheles strains emerge (Bruce-Chwatt, 1980).

Malaria parasites have demonstrated some level of resistance to most of antimalarial drugs currently available (CDC, 2007) and most of the drugs in use have serious side effects (Nosten *et al.*, 2000; Price *et al.*, 2004; Yam and Kwok, 2006; Pukrittayakamee *et al.*, 2006). Chloroquine **(1)**, for example, causes stomachache, headache, blurred vision and in fact strains of *Plasmodium falciparum* malaria parasites are resistant to the drug (Edrissian *et al.*, 1986). Quinine **(2)** causes blurred vision, ringing ears, headache, skin rashes, sweaty flushed skin, dizziness, dysphoria, nausea, vomiting and diarrhea. It has become ineffective against multidrug resistant strains of *Plasmodium falciparum* (Pukrittayakamee *et al.,* 2006). Quinidine **(3)** is associated with risk of cardio-toxic effects. Mefloquine **(4)** has encountered resistance as monotherapy, leading to drug failure and may cause acute brain syndrome (Nosten *et al.,* 2000; Price *et al.*, 2004). Halofantrine **(5)** causes abdominal pain, gastrointestinal disturbances, cough and sudden cardiac deaths (Nosten

et al., 1993). Artemisinin **(6)** causes headache, nausea, vomiting, abdominal-bleeding and has high rate of treatment failures when used in monotherapies (WHO, 2006). Though World Health Organization (WHO) recommends use of combination therapies due to high rate of recrudescence (return of parasite) (WHO, 2006), there is evidence of malaria strains that are resistant to the combination therapies, including artemisinin **(6)** combination therapies (Wongsrichanalai and Meshrick, 2008). There is therefore need to search for new possible malaria management drugs without these side effects.



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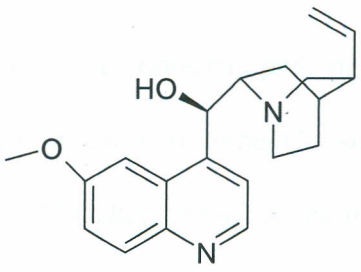

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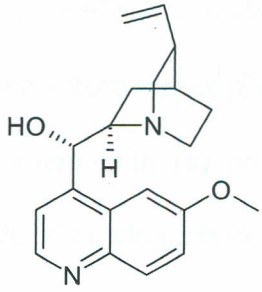

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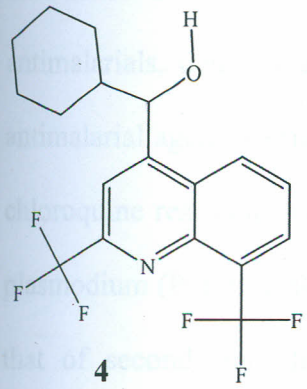

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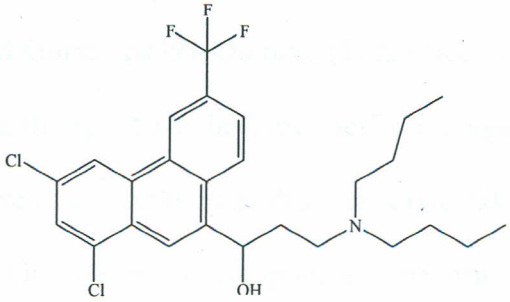

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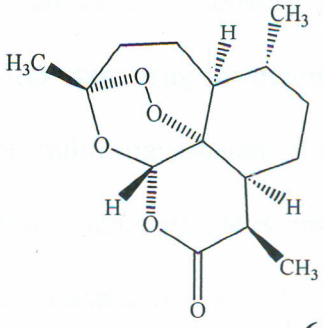

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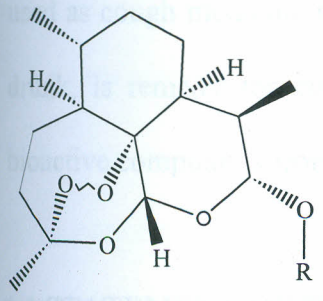
Treatment of malaria has become unaffordable due to rising cost of non-chloroquine drugs, high poverty levels and high prices of insecticide treated nets (ITN) (Guyatt *et al.*, 2002). This coupled with failure of mosquito eradication, the challenge of producing widely available vaccine that provide wide level of protection for sustainable period and the drug resistance phenomenon have

created urgent need to search for new drugs and alternative medicines for malaria and other diseases (Zowa *et al.*, 2003).

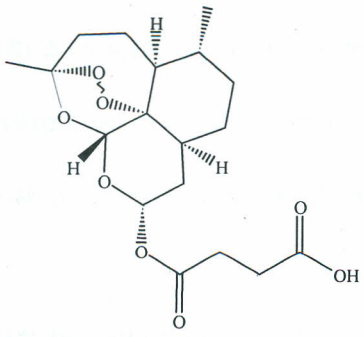
In the effort to find new anti malarial drugs, plants have been an alternative source of compounds containing novel structures. Plants present a major source of the discovery and development of new drugs. In developing countries, majority of people still rely on traditional herbal medicines for their primary healthcare (Mbwambo *et al.*, 2009). Traditional herbal remedies have been used to treat malaria for thousands of years and indeed 20% of patients use traditional herbal remedies for malaria, in endemic countries (Wilcox and Bodeker, 2004). Use of herbs in Africa for malaria treatment hundred years ago prevented destruction of the continent, proving their effectiveness (Elujoba, 2005). Two of the major drugs used to treat malaria, quinine **(2)** and artemisinin **(6)** originated from traditional herbal medicine (Wilcox and Bodeker, 2004). A tea made of cinchona bark was used for centuries to treat malaria fever in Peru and discovery of quinine **(2),** extracted from cinchona bark, about 4000 years ago was a major breakthrough in combating malaria (Tabuti, 2008). The long established use of quinine **(2)** and more recent introduction of arteminsin **(6)** as highly effective antimalarials, demonstrate that plant species may be an important resource for discovery of new antimalarial agents (Soni and Gupta, 2009). Quinine **(2)** has been an important drug for treatment of chloroquine resistant malaria though it has become ineffective against multidrug resistant strains of plasmodium (Pukrittayakamee *et al.*, 2006). The drug has toxic side effects hence its use is limited to that of second line drug for severe or complicated malaria (Pukrittayakamee *et al.*, 2003). Artemisinin **(6),** a sesquiterpene lactone, isolated from *Artemisia annua*, a herb described in Chinese traditional medicine, has been used by Chinese herbalists for more than one thousand years, in treatment of malaria and other diseases (Dewick, 2002). Synthetic derivatives of artemisinin **(6)** such as artemether **(7),** arteether **(8),** artesunate **(9),** and dihydroartemisinin **(10)** have been developed (Dewick, 2002). Reports indicate that artemisinin **(6)** and some of its combination therapies are

losing their potency due to drug resistant malaria (Noedl *et al.*, 2008). More research is therefore required for exploitation of active principles in plants to battle malaria.



7 R= Me

8 R=Et



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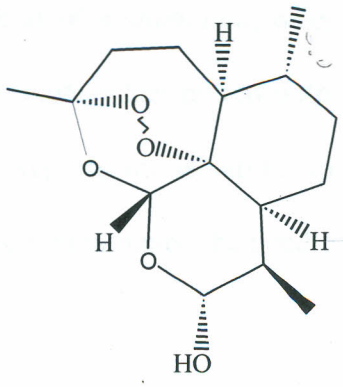

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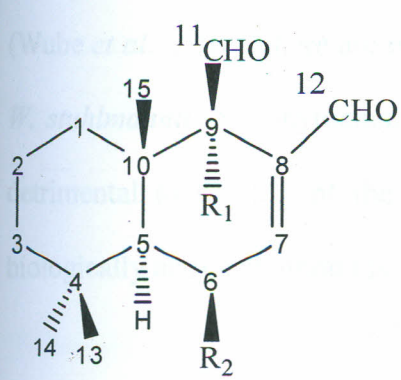
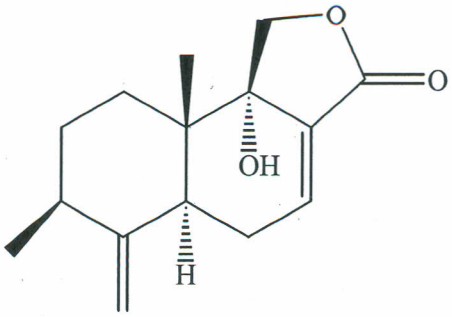
The genus *Warburgia* is valued for curing several ailments (Beentje, 1994; Watt and Breyer-Brandwijk, 1962). Phytochemical investigation of the *Warbugia* species revealed the presence of sesquiterpenes (Kioy *et al.*, 1990). Sesquiterpenes such as artemisinin **(6)** are effective in treating malaria. Sesquiterpenes isolated from the stem bark of *W. ugandensis* such as mukaadial **(11),** ugandensidial **(12),** muzigadiolide **(13)** and muzigadial **(14)** showed antiplasmodial activities against the chloroquine sensitive (3D7) and chloroquine resistant (K1) strains of *P. falciparum* (Wube *et al.*, 2010).



	R_1	R_2
11	**OH**	**OH**
12	**OH**	**OAc**



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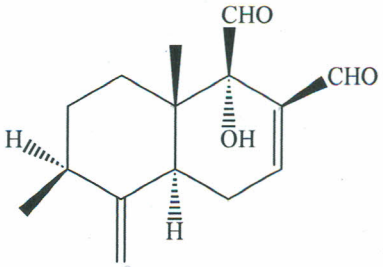

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The communities in Kwale District, Kenya, make use of the stem bark and root bark of *W. stuhlmannii* in treatment of malaria (Muthaura *et al.*, 2007) and as remedy for toothache and rheumatism (Beentje, 1994). The pulverized stem bark of *W. stuhlmannii,* when mixed with honey is used as cough medicine while exudates from the stem bark, when mixed with eggs then boiled and drunk, is remedy for constipation (Beentje, 1994). Reports on isolation and characterization of bioactive compounds from *W. stuhlmannii* root bark are scanty hence the need for the study.

1.1 STATEMENT OF THE PROBLEM

Malaria eradication has been unsuccessful due to spread of multi drug resistant *Plasmodium* strains (CDC, 2007) and high poverty levels in endemic areas. Most drugs in use have serious side effects (Nosten *et al.*, 2000; Price *et al.*, 2004; Yam and Kwok, 2006; Pukrittayakamee *et al.*, 2006). In the effort to find new anti malarial drugs, plants have been an alternative source of compounds containing novel structures. Plants present a major source of the discovery and development of new drugs. The root bark and stem bark of *W. stuhlmannii* have been used to treat malaria and related fevers (Muthaura *et al.*, 2007. Though some of the compounds responsible for antiplasmodial activity in the stem bark of the plant have been isolated, characterized and individual activities determined (Wube *et al.,* 2010), there are no reports on phytochemical and biological activity on the root bark of *W. stuhlmannii*. No phytochemical study has been done on the plant root possibly because this is detrimental to the life of the plant, but it is indeed logical to evaluate the plant root bark for biologically active compounds.

1.2 OBJECTIVES

1.2.1 GENERAL OBJECTIVE

To extract, isolate and characterize compounds from root bark of *W. stuhlmannii* and carry out antiplasmodial tests on the extracts and pure compounds

1.2.2 SPECIFIC OBJECTIVES

1. To isolate and characterize compounds from, root bark of *W. stuhlmannii.*

2. To perform *in vitro* bioassay investigation on the extracts and pure compounds of *W. stuhlmannii* root bark against *Plasmodium falciparum.*

1.3 0 HYPOTHESIS

1.3.1 NULL HYPOTHESIS

1. The root bark of *W. stuhlmannii* does not contain bioactive compounds.

2. The extracts and pure compounds of root bark of *W. stuhlmannii* are not biologically active against *P. falciparum.*

1.4 JUSTIFICATION OF THE RESEARCH

Development of resistance to antimalarial drugs such as chloroquine **(1),** quinine **(2),** artemisinin **(6)** and artemisinin **(6)** combination drugs have been reported and therefore, malaria continues to be a major health burden. Control of mosquitoes through use of insecticides has been unsuccessful. The challenges of producing widely available vaccines that provide high level of protection for sustainable period is yet to be met. The cost of available malaria control tools, exceed public health resources in malaria endemic areas of the world. These limitations necessitates search for new, effective antimalarial agents that are easily accessible and affordable, by the poor majority in local communities. *Warburgia stuhlmannii* stem bark and leaves extracts have been evaluated for their antimalaria activities but no study has been conducted on the root bark, in spite of its use in management of malaria by communities in Kwale district, Kenya.

The research will enable identification of anti-malarial compounds from *W. stuhlmannii,* to authenticate phyto-pharmaceutical value of the plant. Positive results from the research may provide cheap alternative of managing malaria from locally available medicinal plants. *W. stuhlmannii* is listed as vulnerable species, in the red list of threatened species, of the international union for conservation of nature and natural resources (IUCN, 2006). Positive results of antiplasmodial efficacy of the plant will justify the need to protect the plant and to possibly use it as an item of commerce.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 MALARIA TRANSMISION AND SYMPTOMS

. Malaria has been a human infection for over 50,000 years, with plasmodium as a human pathogen for the entire history of the species (Joy *et al.*, 2003). The disease has been responsible for economic decline of nations and military defeat by causing more casualties than weapons. It contributed to decline of Roman Empire (BBC News, January, 29, 2003); and caused death of sixty thousand American soldiers during North African and South Pacific campaigns (Joseph, 2008). Malaria continues to be a huge social, economic and health problem, particularly in tropical countries. It costs Africa $12 billion in lost gross domestic product (GDP) every year and consumes 40% of all public health spending (Sachs and Malaney, 2002). Malaria is widespread in tropical and sub tropical regions and is endemic in 91 countries, predominantly in Africa, Asia and Latin America, with about 40% of the world's population at risk (WHO, 2001). Approximately 350-500 million cases of malaria are reported annually (CDC, 2007). The disease kills between one and three million people, the majority of whom are young children in sub-Saharan Africa (Snow *et al.*, 2005) and indeed, in every thirty seconds a child dies from the disease, worldwide (WHO, 2009). In the year 2010 about 1,298,000 people died from the disease worldwide (Christopher *et al.*, 2012). In Kenya, 30-50% of outpatient treatments and 19% hospital admissions are malaria cases, accounting for 8-10 million treatments per year (Ochola, 2003). Plasmodium resistance to current anti malarial drugs is prevalent (CDC, 2007) and most drugs have serious side effects (Nosten *et al.*, 2000; Price *et al.*, 2004; Yam and Kwok, 2006; Pukrittayakamee *et al.*, 2006).

Malaria is caused by protozoan parasite, genus Plasmodium and *Plasmodium falciparum, Plasmodium malariae, Plasmodium ovale, Plasmodium vivax, Plasmodium berghei* and *Plasmodium knowlesi,* causes malaria in human (Mueller *et al.*, 2007; Singh *et al.*, 2004). *Plasmodium falciparum* is the major cause of the infection responsible for 80% of all malaria cases

and 90% deaths from malaria (Mendis *et al.*, 2001). Female anopheles mosquito, the primary hosts and transmission vectors of the parasites becomes infected by taking blood meal from infected human, who are secondary hosts. The parasite gametocyte differentiates into male and female gametes that fuse in mosquito gut, producing ookinete that forms oocyst in gut wall. The oocyst ruptures releasing sporozoites into mosquito's salivary glands. Sporozoites are injected alongside saliva, when the mosquito takes subsequent blood meal. Sporozoites in human body enter blood stream and migrate to liver then multiply into merozoites, which rupture liver cells and escape into blood stream (Bledsoe, 2005). Merozoites infect red blood cells and develop into trophozoites and schizonts that produce further merozoites and gametocytes, taken up by the mosquito (Bledsoe, 2005).

Malaria symptoms include fever, shivering, anthalgia (joint pain), vomiting, anaemia, haemoglobinuria, retinal damage and convulsions (Beare *et al.*, 2006). In pregnant women severe malaria causes still births, low birth weight, severe anaemia, infant mortality and sub-optimal growth and development (Van Geertruyden *et al.*, 2004). Untreated severe malaria causes coma and death (Trampuz *et al.*, 2003). In cerebral malaria, the sequestrated red blood cells breach the blood, brain barrier, leading to coma (Adam *et al.*, 2003). Efforts to eradicate anopheles mosquito vector has not been successful (Bruce-Chwatt, 1980).There is therefore need to search for new drugs and alternative medicines for malaria (Zowa *et al.*, 2003).

2.2 CURRENT MALARIA CHEMOTHERAPY AND PROPHYLAXIS

Antimalarial drugs are mainly applied in treatment and preventive chemoprophylaxis. Malarial treatment drugs are classified into, blood schintocides which kill erythrocytic stages in red blood cells and tissue schintocides that kill the liver stages of the parasite (Clyde, 1987) as summarized in Table 2.1. In *Plasmodium falciparum* malaria, no re-infection or relapse in liver occurs, hence single dose schizonticidal drug is sufficient treatment (Bruce-Chwat, 1980). In case of *Plasmodium*

ovale, Plasmodium vivax and *Plasmodium malariae* infections, combination of blood and tissue schintocides are used (Clyde, 1987).

Chloroquine **(1),** a 4-aminoquinoline is an effective antimalarial drug for treatment and prophylaxis and is potent schizonticidal drug against erythrocytic stage of all the four Plasmodium species but has no effect on sporozoites, hypnozoites or gametocytes. It has few side effects such as nausea, vomiting, headache, diarrhea and blurring vision. Chronic use of the drug could lead to toxicity in the eye (Yam and Kwok, 2006). Strains of *P. falciparum* malaria parasites are resistant to the drug (Wellens, 2002). Chloroquine resistant *P. vivax* malaria parasites have been reported (Alecrim, 1999; Schuurkamp, 1992). Quinine **(2)** is a blood schintocide that cures *P falciparum* malaria, but fails to cure or provide prophylaxis against *P. vivax* malaria. It destroys trophozoites in erythrocytes but has no effect on exo-erythrocytic stages that develop in liver (Rimchala *et al.*, 1996). The drug is recommended by WHO for treatment of malaria in pregnancy (Yeka *et al.,* 2009), but has considerable side effects and has become ineffective against multidrug resistant strains of *Plasmodium falciparum* (Pukrittayakamee *et al.,* 2007). Quinidine **(3)** is a stereoisomer of quinine **(2)** and is recommended for *P. falciparum* malaria treatment intravenously (CDC, 2007) but is associated with greater risk of cardio toxic effects (CDC, 2007). Mefloquine **(4)** is a quinoline methanol derivative antimalarial drug used prophylactically against and as a treatment for chloroquine resistant *Plasmodium falciparum* malaria. It is a blood schizonticidal of erythrocytic malaria as well as kills hypnozoites but causes giddiness, convulsions, insomnia and neuropsychiatric reactions (Hellgren *et al.*, 1997). The drug has encountered severe problems of resistance as monotherapy, leading to drug failure, even when used in combination with fast acting peroxide, artesunate **(9)** (Nosten *et al.,* 1993; Price *et al.,* 2004). It is also associated with higher rates of neurological and psychiatric symptoms (Jacquerioz and Croft, 2009), hence its use has declined due to the undesirable side effects. Halofantrine **(5)** is a phenathrene methanol, chemically related to quinine **(2)** and acts as a blood schintocide. It is effective against all Plasmodium

parasites. It however has high level of cardio toxicity and may cause nausea, abdominal pain, gastrointestinal disturbances, cough and sudden cardiac deaths (Nosten *et al.,* 1993). Artemisinin **(6),** a sesquitespene lactone posses most rapid action of all current drugs against *P. falciparum* malaria (White, 1997) and is increasingly used in *P. vivax* malaria (Douglas *et al.,* 2010). The drug has high rate of treatment failures when used in monotherapies hence its use in combination therapies (ACTS), to avoid recrudescence (WHO, 2006). Bioavailability of the drug has been improved by development of its derivatives; artemether **(7),** arteether **(8),** artesunate **(9)** and dihydroartemisinin **(10)** used in treatment of *vivax* malaria (Douglas *et al.*, 2010). Artemisinin **(6)** may be reduced to dihydroartemisinin **(10),** used for semi synthesis of analogues such as the acetals; artemether **(7)** arteether **(8),** water soluble salts of artelinic acid **(15)** and artesinic acid **(16),** which have increased activities than artemisinin **(6)** (Yeka *et al.*, 2009). Artelinic acid **(15)** is more stable than the rest of the analogues and is used in treatment of celebral malaria. Artemisinin **(6)** is however losing its potency (Noedl *et al.,* 2008) and indeed there is evidence of malaria parasites that are resistant to combination therapies that include artemisinins (Wongsrichanalai and Meshrick, 2008). Proguanil **(17)** is an antimalarial drug that works by stopping *P. falciparum* and *P. vivax* from reproducing once in red blood cells and is usually taken in combination with chloroquine **(1)** (Payen *et al.*, 2008). The drug may however lead to nausea, vomiting, abdominal pain, headache, diarrhea, appetite loss and dizziness. Pyrimethamine **(18)** and sulfadoxine **(19)** are antifolate drugs used in combination therapy (Sulfadoxine/pyrimethamine) for malaria treatment and prophylaxis. They inhibit enzymes which synthesize folic acid within the plasmodium parasites and have synergistic effect which out balances the use of exogenous folic acid by the parasites (Chulay *et al.,* 1983). *P. falciparum* resistance to pyrimethamine **(18)** is widespread (Gatton *et al.*, 2004).

Dapson **(20)** is used in combination with pyrimethamine **(18)** in malaria treatment (Akadi, 2007), however people treated with this combination suffer from haemolysis (Puavilai *et al.*, 1984). Dapson-containing antimalarial combination, Lapdap has been withdrawn from clinical use due to

severe haemolysis in those with glucose phosphate dehydrogenase (G6PD) deficiency (Luzzatto, 2010). Primaquine **(21)** is a tissue schizonticide that treats *P. vivax* and *P. ovale* malaria by destroying exo-erythrocytes in liver (hypnozoites). This prevents relapse and recrudescence, but, the drug causes nausea, vomiting, stomach cramps and haemolytic anaemia (Caymar *et al.*, 1952). The drug is also a casual prophylactic drug that targets blood stage of malaria and requires presence of chloroquine **(1)** or quinine **(2)** in order to be effective (Alving *et al.*, 1985). Doxycycline **(22)** is a semi synthetic tetracycline used as a suppressive prophylactic, in erythrocytic stage. It has no effect until the liver stage is complete and impairs progeny of apicoplast genes, resulting in their abnormal cell division (Dahl *et al.,* 2006). The drug is used in treatment plan with other agents such as quinine **(2)** (Lalloo *et al.*, 2007). Atovaquone **(23)** is a hydroxy-1,4-naphthoquinone used in combination with proguanil **(17)** in malaria treatment. This combination has few side effects such as vomiting, nausea and diarrhoea (Hughes, *et al.,* 1993), but malaria resistance to this combination has been observed (Famert *et al.,* 2003). The increasing resistance of *P. falciparum* to antimalarial drugs severely compromises efforts to control and treat malaria. Spread of multi-drug resistant strain of plasmodium and the adverse side effects of the existing antimalaria drugs have necessitated the search for novel well tolerated and more efficient antimalarial drugs (Bickii *et al.,* 2000). Search for new, more effective drugs for malaria treatment is currently a major concern. Compounds containing novel structures from natural origin such as plants, presents a major possible source for discovery and/or development of new antimalarial drugs. Medicinal plants may contain undiscovered antimalarial properties which can serve as a template for the production of affordable antimalarial drugs from indigenous plants (Odugbemi *et al.,* 2007)

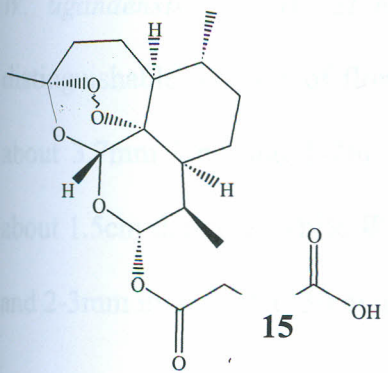
Table 2.1: Classification of anti-malarial drugs.

lass	rug	iological activity	
		lood schintocide	ssue schintocide
aminoquinolines	hloroquine (1)	+	
ryl amino alcohols	uinine (2)	+	
	uinidine (3)	+	
	efloquine (4)	+	
henathrene methanol	alofantrine (5)	+	
rtemisinin and derivatives	rtemisinin (6)	+	
	rtemether (7)	+	
	rtesunate (9)	+	
	ihydroartemisinin (10)	+	
nti-metabolites	roguanil (17)		
	yrimethamine (18)		
	ulfadoxine (19)		
minoquinolines	apson (20)		
	imaquine (21)		
tibiotics	oxycycline (22)		

(WHO, 2006)

KEY

++ .....Very effective. +.....effective. 0...not effective.



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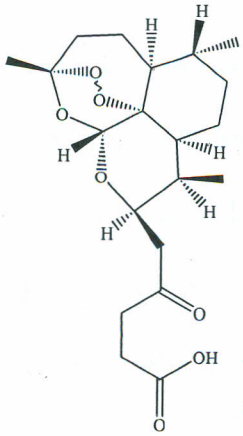

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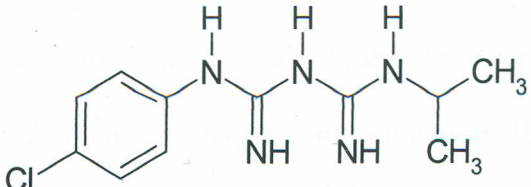

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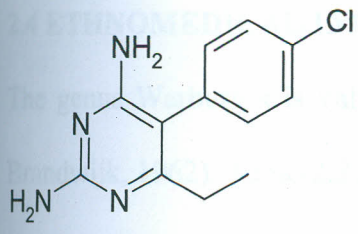

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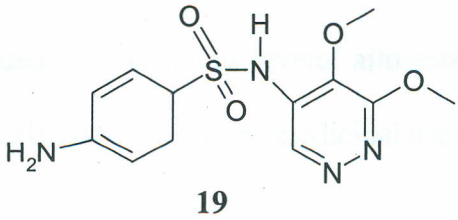

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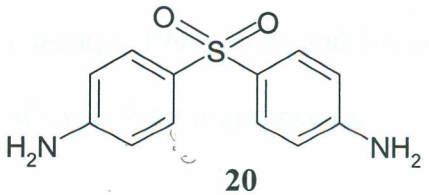

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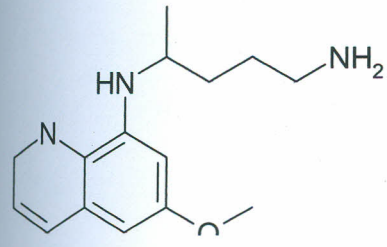

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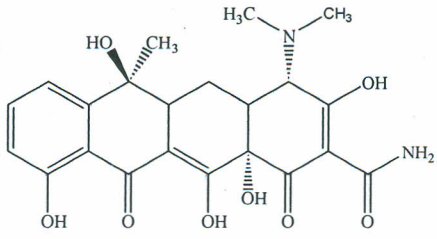

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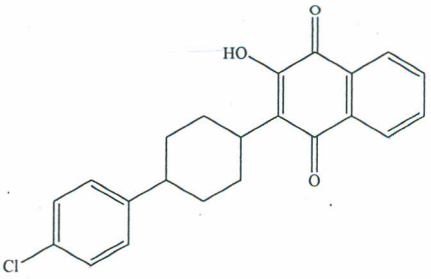

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2.3 THE GENUS *WARBURGIA*

Warburgia is a genus of plants in the family Canellacea, named after German botanist, Otto Warburg (Dale and Greenway, 1961). The species in this genus include; *W. salutaris* (Bertol F.) Chiov and *W. breyeri* (pott), found in Southern Africa, *W. ugandensis* (Sprague) endemic in East African highlands, *W. stuhlmannii* (Engl) wide spread at the East African coastline and *W. elongata* that grows in Tanzania (Muchugi *et al.*, 2009) The species are highly valued within traditional health systems, for managing stomach-ache, constipation, toothache, common cold, cough, fever, muscle pains, weak joints, measles and malaria (Kokwaro, 2009; Watt and Breyer-Brandwijk, 1962).

W. ugandensis and *W. stuhlmannii* are the *Warburgia* species found in Kenya .and are distinguishable by sizes of flowers and fruits. *W. stuhlmannii* has small flowers with staminal tube about 3.7mm long and 1-2mm diameter, ten ovules, anthers about 1.0mm long and small fruits about 1.5cm diameter while *W. ungandensis* has larger flowers with staminal tube about 5mm long and 2-3mm in diameter, 30 ovules, anthers 2.0 mm in diameter and larger fruit (Verdcourt, 1954).

2.4 ETHNOMEDICAL INFORMATION OF *WARBURGIA* GENUS

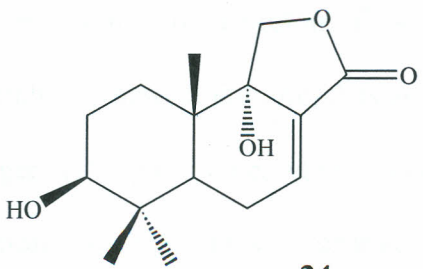
The genus Warburgia is valued for managing several ailments (Beentje, 1994; Watt and Breyer-Brandwijk, 1962). Table-2.2; gives a summary of medicinal uses of some Warburgia species.

Table 2.2; Ethnomedical information of the species in the genus *Warburgia*

Plant Species	Parts of the plant	Application	Region/ Country	References
W. ugandensis	Stem bark	Coughs, colds, fever, toothache, muscle pains, chest pains, malaria	East Africa	Kokwaro, 1976
	,,	Tuberculosis (T.B)	Ethiopia	Wube *et al.*, 2005
	,,	Malaria and trypanosomiasis.	Ethiopia	Wube *et al*, 2010
	,,		Tanzania	
	,,	HIV related opportunistic infections e.g. persistent malaria, flue diarrhoea. Sexually transmitted disease.	East Africa	Kayombo *et al.*, 2007
	Leaves	Throat infections, appetite loss, internal wound/ulcers.		Kokwaro *et al.*, 1976
	Roots	Malaria, skin diseases, diarrhoea	East Africa	Kokwaro *et al.*, 1976
W. stuhlmannii	Stem and root bark	Toothache, rheumatism, constipation.	Tanzania and Kenya	Beentje, 1994.
		Malaria	Kenya (Kwale)	Muthaura *et. al.*, 2007
W. salutaris	Stem bark and Roots	Sores and inflammations, headaches, influenza.	South Africa	Monhallal and Odhav, 2009.
		Coughs, colds, malaria, aphrodisiac, mouth and gastric ulcers, dermatological disorders, rheumatism, sexually transmitted diseases, bronchitis and clearing of sinuses and toothache.	South Africa	Rabe and Vanstaden, 2000

2.5 ANTIPLASMODIAL ACTIVITY OF *WARBURGIA* SPECIES.

The antiplasmodial activities of some *Warburgia* species have been demonstrated (Were *et al.*, 2010). Extracts of *W. ugandensis* were screened for *in vitro* antiplasmodial activity against *P. knowlesi*. Inhibitory concentrations (IC_{50}) values of 31.4µg/ml were obtained. There was 69% chemosupression of parasite growth and over 80% of treated mice survived (Were *et al.*, 2010). In the antiplasmodial investigation, sesquiterpenes isolated from the stem bark of *W. ugandensis* showed activities against the chloroquine sensitive (3D7) and chloroquine resistant (K1) strains of *P. falciparum* (Wube *et al.*, 2010). Isolated compounds including mukaadial **(11),** ugandensidial **(12),** muzigadiolide **(13)** and muzigadial **(14)** were found to be most potent against chloroquine resistant strain (K1) of *P. falciparum* with IC_{50} values 7.9µM, 11.0µM, 10.6µM and 7.3µM respectively (Wube *et al.*, 2010). For compounds tested with choroquine sensitive (3D7) strains of *P. falciparum* mukaadial **(11),** muzigadiolide **(13)** and 3β,9α-dihydroxycinnamolide **(24)** were most potent with IC_{50} values of 6.4µM, 7.2µM and 10.6µM, respectively (Wube *et al.*, 2010). In another antiplasmodial bioassay experiment, muzigadial **(14)** was the most potent sesquiterpene against chloroquine sensitive (D10) strain of *P. falciparum* with an IC_{50} value of 0.31µg/ml (Grace *et al.*, 2010).



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In an investigation of *W. stuhlmannii* stem bark, the water and methanol extracts were found to be most effective; IC_{50} < 10µg/ml when screened against chloroquine sensitive D6 and chloroquine resistant W2 *P. falciparum* clones (Muthaura *et al.*, 2007). The water extracts of *W. stuhlmannii* had higher chemosupression of parasitaemia than methanolic extract, *in vivo* (Muthaura *et al.*, 2007). Compounds isolated from *W. ugandensis* were evaluated for their antiplasmodial activity (Wube *et*

al., 2010) while those from *W. stuhlmannii* are yet to be evaluated, in spite of its traditional use in malaria treatment.

2.6 PHYTOCHEMISTRY AND BIOLOGICAL ACTIVITY OF SOME COMPOUNDS FROM *WARBURGIA* SPECIES

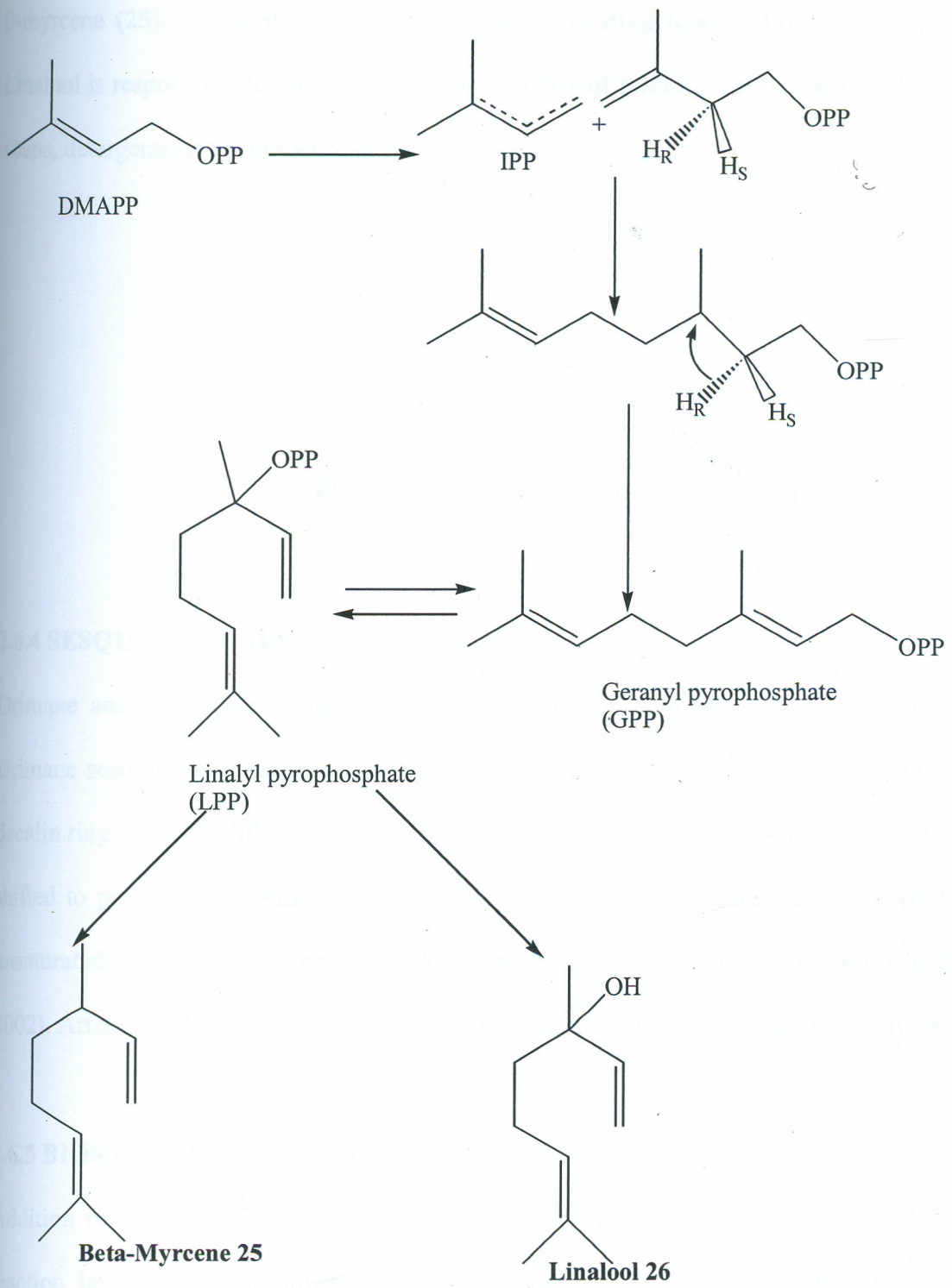
Previous phytochemical screening of the *Warburgia* species have led to isolation of monoterpenes, sesquiterpenes and flavonols, of which some are biologically active (Kioy *et al.*, 1990; Manguro *et al.*, 2003; Wube *et al.*, 2010; Opiyo *et al.*, 2011).

2.6.1 MONOTERPENES

Monoterpenes are mainly found as components of essential oils, mainly used in flavouring and perfumery.

2.6.2 BIOSYNTHESIS OF MONOTERPENES

Combination of DMAPP and IPP via the enzyme prenyl transferase yields geranyl diphosphate (GPP) (Scheme 3.1). This involves ionization of DMAPP to the allylic cation and addition to the double bond of IPP, followed by loss of a proton. This produces a monoterpenes diphosphate, geranyl diphosphate, in which the new double bond is *trans* (*E*). Linalyl diphosphate and neryl diphosphate are isomers of geranyl diphosphate, which can be formed from geranyl diphosphate by ionization to the allylic cation, that may allow a change in attachment of the diphosphate or a change in stereochemistry at the double bond (to *Z* in neryl pyrophosphate) (scheme 3.1). These three compounds, gives rise to a range of linear monoterpenes found as components of volatile oils used in flavouring and perfumery. The resulting compounds may be hydrocarbons, alcohols, aldehydes or esters, especially acetates (Dewick, 2002).

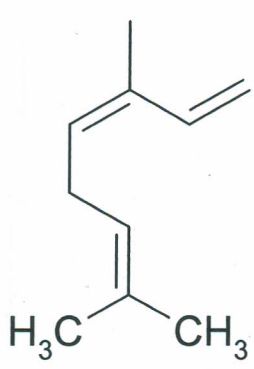


Scheme; 2.1- Biosynthetic pathway of monoterpenes (Dewick, 2002)

2.6.3 MONOTERPENES ISOLATED FROM WARBURGIA GENUS

Chemical analysis of steam volatile oils of *W. stuhlmannii* and *W. ugandensis* leaves revealed the presence of β-myrcene **(25)** as a major component, small amounts of linalool **(26)** while *cis*-β-ocimene **(27)** and *trans*-β-ocimene **(28)** as other major components (Kioy *et al.*, 1990). Essential oils containing

β-myrcene **(25),** are used in cosmetics and as flavouring food additives (Paumgatten *et al.*, 1998). Linalool is responsible for anti-inflammatory activity of essential oils (Peana *et al.*, 2004) and is used in soaps, detergents and lotions (Peana *et al.*, 2004).



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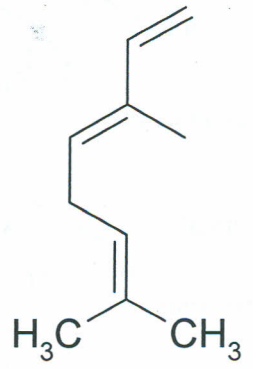

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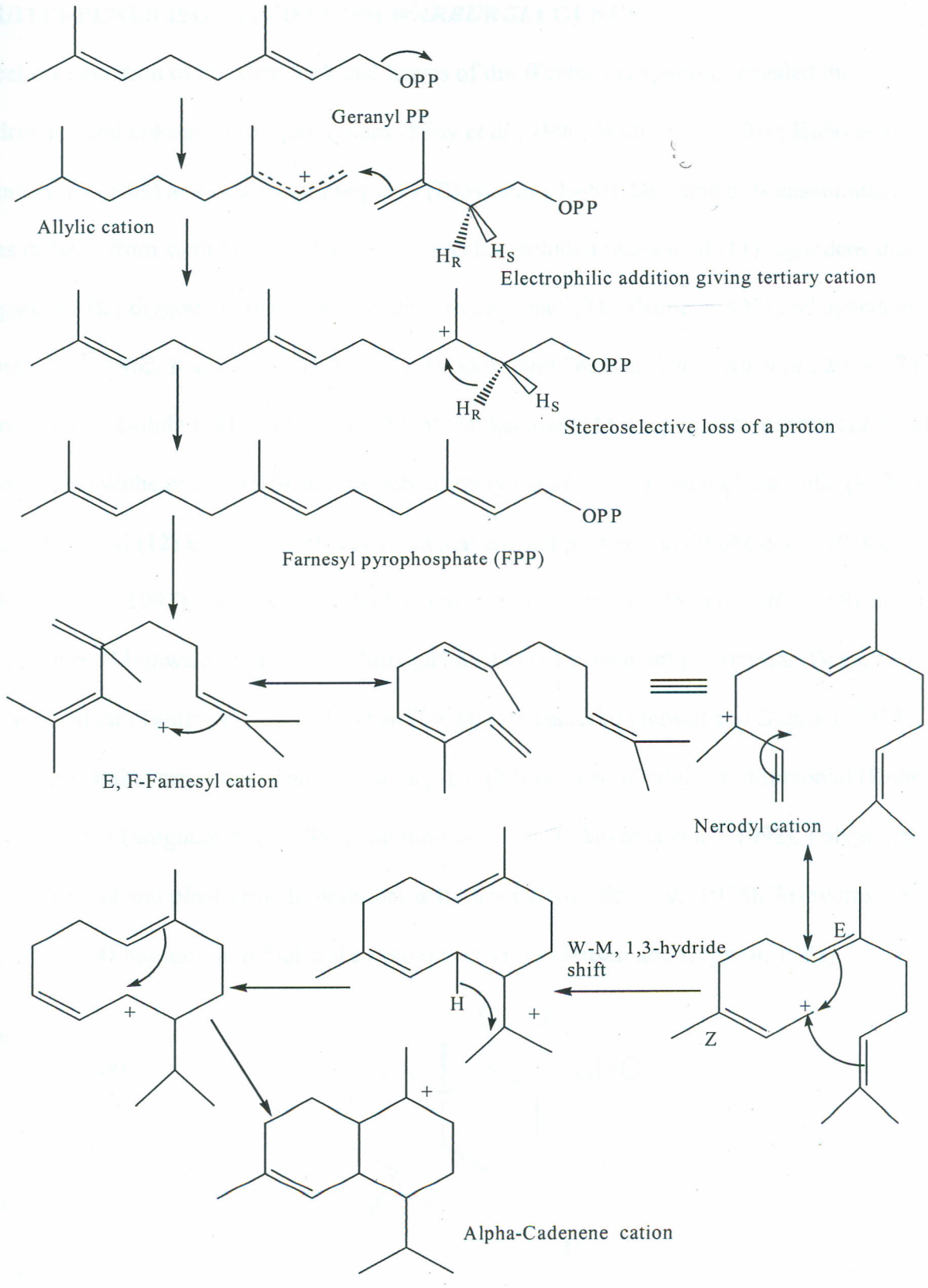
2.6.4 SESQUITERPENES

Drimane and coloratane sesquiterpenes are among the common naturally occurring sesquiterpenes. Drimane sesquiterpenes are characterised by α, β-unsaturated carbonyl chromophores around a trans-decalin ring system, while coloratanes are rearranged drimanes where one methyl group at position 4 is shifted to position 3, leaving an exocyclic methylene group (White *et al.,* 2004). The drimane α, β-unsaturated-1,4-dialdehydes and α, β-unsaturated lactones are known to be biologically active (Dewick 2002). Artemisinin is a sesquiterpene lactone whose antiplasmodial activity is well documented.

2.6.5 BIOSYNTHESIS OF SESQUITERPENES

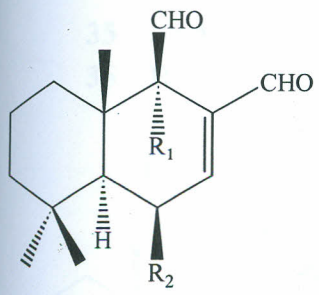
Addition of a further C5 IPP unit to geranyl diphosphate in an extension of the prenyl transferase reaction leads to the sesquiterpene precursor, farnesyl diphosphate (FPP) (Scheme 3.1). An initial ionization of GPP takes place. FPP can then give rise to linear and cyclic sesquiterpenes. Due to increased chain length and additional double bond, the number of possible cyclization modes is increased, and a range of mono-, bi-, and tri-cyclic structures results. The stereochemistry of the double bond nearest the diphosphate can adopt an *E* configuration (as in FPP), or a *Z* configuration via ionization, as found with geranyl/neryl PP (Scheme 3.2) (Dewick, 2002).



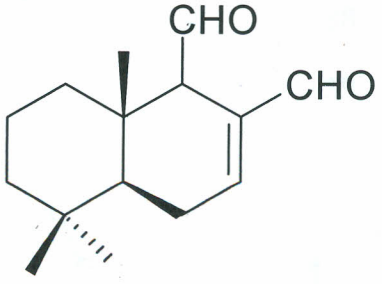
Scheme 2.2; Biosynthetic pathway for sesquiterpenes (Dewick, 2002)

2.6.6 SESQUITERPENES ISOLATED FROM *WARBURGIA* GENUS

Phytochemical investigation of the stem bark and leaves of the *Warburgia* species, revealed the presence of drimane and coloratane sesquiterpenes (Kioy *et al.*, 1990; Wube *et al.*, 2010; Kubo *et al.*, 1976; Manguro *et al.*, 2003) and other sesquiterpenes (Kioy *et al.*, 1990). Drimane α, β-unsaturated-1, 4-dialdehydes isolated from stem barks of *Warburgia* species included mukaadial **(11),** ugandensidial **(12),** warburganal **(29),** polygodial **(30),** isopolygodial (isotadeanal) **(31),** drimenin **(32)** and ugandenial A **(33)** (Barness *et al.*, 1962, Kubo *et al.*, 1976, 1977, Broooks and Draffan, 1969; Xu *et al.*, 2009). 7α-hydroxy-8-drimen-11,12-olide **(34)** (Wube *et al.*, 2005). Mukaadial **(11)** is a potent trypanomicidal and antiplasmodial agent (Wube *et al.*, 2010), antimicrobial (Opiyo *et al.*, 2011) and molluscicidal (Kubo *et al.*, 1983). Ugandensidial **(12)** is a potent trypanosomicidal and antiplasmodial (Wube *et al.*, 2010), antifeedant (Kubo *et al.*, 1997), molluscicidal (Kubo *et al.*, 1983), antifungal (Kubo *et al.*, 1988) and plant growth inhibitor (Meinwald *et al.*, 1978). Muzigadial **(14)** is a potent antiplasmodial (Grace *et al.*, 2010), insect antifeedant (Taniguchi *et al.*, 1984) antifungal, antibacterial (Jensen and Degroot, 1978) and anticomplement (Fukuyama *et al.*, 1982). Warburganal **(29)** is an antifeedant, antimicrobial (Kubo *et al.*, 1976), cytotoxic (Taniguchi *et al.*, 1984) and molluscicidal (Fukuyama *et al.*, 1992). Polygodial **(30)** exhibits antifeedant and plant growth inhibitory activities (Meinwald *et al.*, 1978). 7α-hydroxy-8-drimen-11,12-olide **(34)** has antimicrobial and cytotoxic activities (Jensen and Degroot, 1978).



	R_1	R_2
29	**OH**	**H**
30	**H**	**H**



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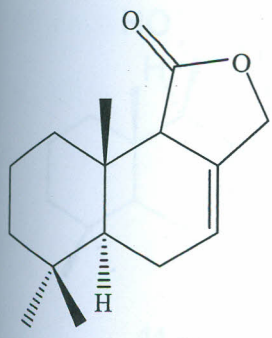

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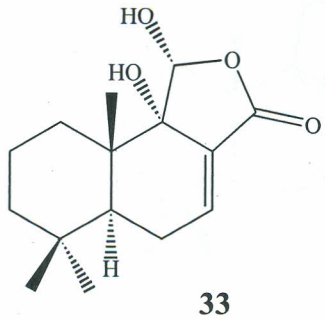

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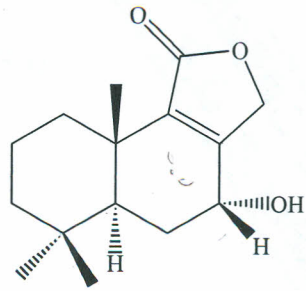

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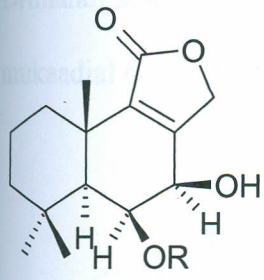
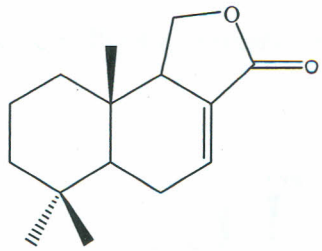
The drimane α, β-unsaturated sesquiterpene lactones that have so far been isolated from the Warburgia species include; muzigadiolide **(13),** ugandensolide **(35),** deacetylugandensolide **(36)**, cinnamolide **(37)**, 3β-hydroxycinnamolide **(38),** 3β-acetoxycinnamolide **(39),** 11α-hydroxy muzigadiolide **(40)** (Kioy *et al.,* 1990; Broooks and Draffan, 1966), drimenin **(32)** (Opiyo *et al.,* 2011), dendocarbin A **(41),** dendocarbin L **(42)** and dendocarbin M **(43)** (Xu *et al.*, 2009), warburgin **(44)** bemadienolide **(45)** and warburgiadione **(46)** (Broooks and Draffan, 1969),. 9α-hydroxy cinnamolide **(47),** and 9α,11α-dihydroxy-6β-acetyl cinnamolide **(48)** (Xu *et al.,* 2009).



35 R=Ac-

36 R=H



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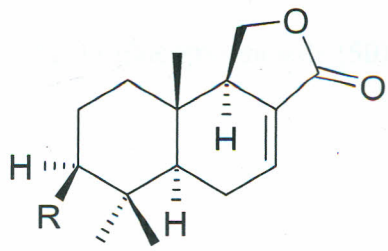
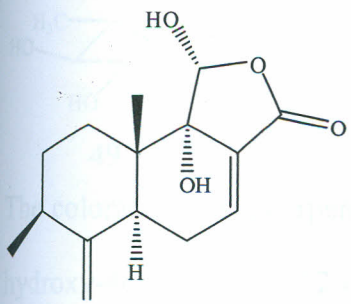


38 R=OH

39 R=OAc



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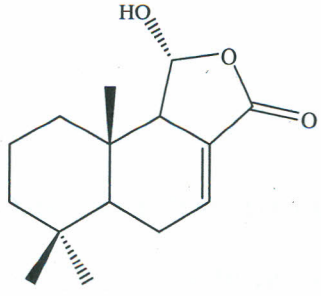

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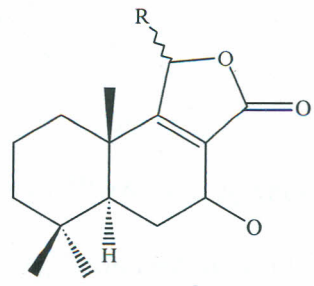
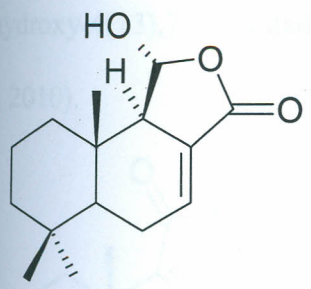


42 R=β OH

43 R=α OH



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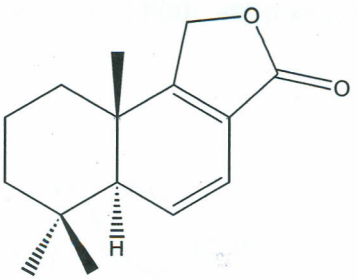

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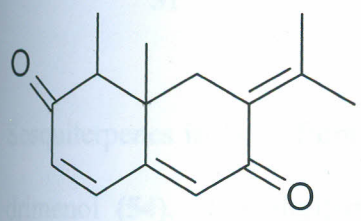

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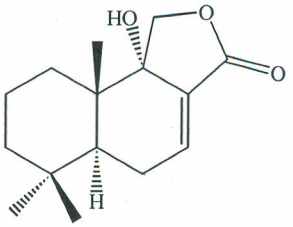

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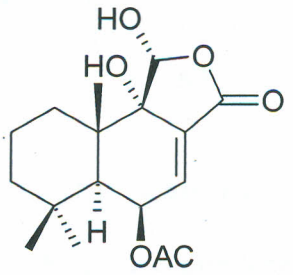
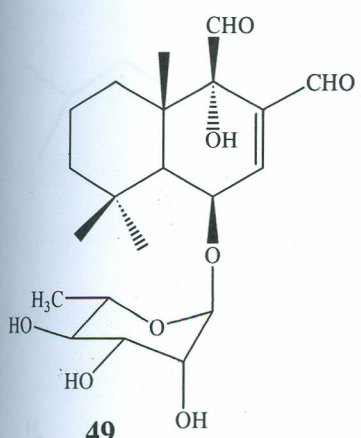


48

Ugandensolide **(35)** and cinnamolide-3β-acetate **(39)** are trypanosomicidal while 11α-hydroxy muzigadiolide **(40)** exhibit both antiplasmodial and anti trypanosomal activities (Wube *et al.*, 2010). Drimane sesquiterpene glycosides isolated from the leaves of *Warburgia stuhlmannii* included mukaadial 6-*O*-α-L-rhamnopyranoside **(49)** and mukaadial 6-*O*-*β*-D-glucopyranoside **(50)**.



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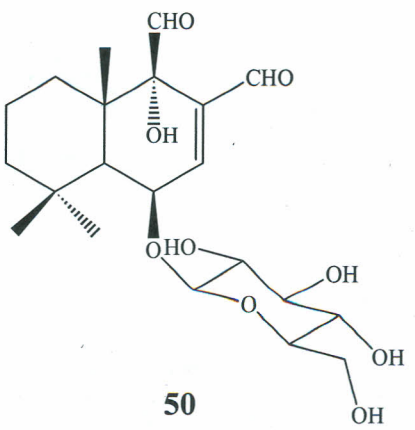
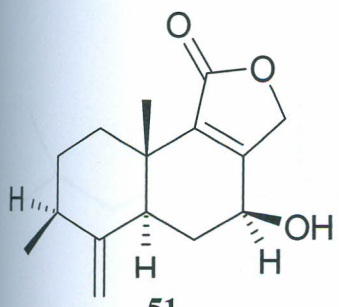


50

The coloratane sesquiterpenes isolated from stem bark extracts of *Warburgia ugandensis* included; 7β-hydroxy-4(13), 8-11, 12-olide **(51)**, 6α, 9α-dihydroxy-4(13),7-coloratadiene-11,12-dial **(52)** and 4(13),7-coloratadiene-12,11-olide **(53).** Among these compounds, 7β-hydroxy 4(13),7-coloratadiene-11,12-olide **(51)** and 4(13),7-coloratadiene-12,11-olide **(53)** are trypanosomicidal, while 6α,9α-

dihydroxy-4(13),7-coloratadiene-11,12-dial **(52),** is both antiplasmodial and trypanosomicidal (Wube *et al.*, 2010).



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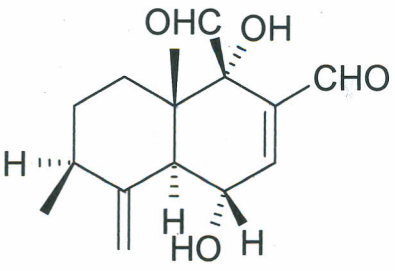

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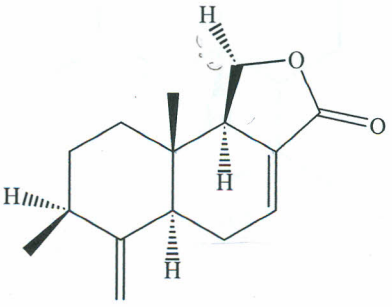

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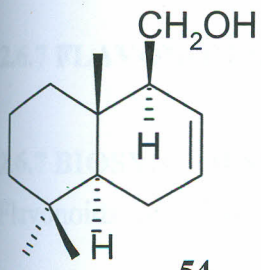
Sesquiterpenes isolated from steam volatile oils of *W. stuhlmannii* and *W. ugandensis* leaves included; drimenol **(54),** caryophyllene **(55),** *β*-cubebene **(56),** nerolidol **(57),** caryophyllene-4,5-oxide **(58),** copaene **(59),** α-humulene **(60),** *β*-farnesene **(61)** and δ-cadenene **(62)** (Kioy *et al.*, 1990). Some of these compounds have been subjected to biological activity studies while some have not.



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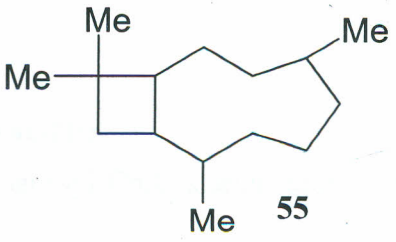

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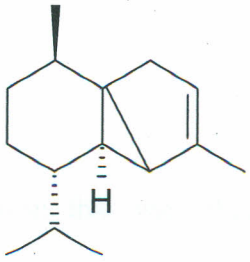

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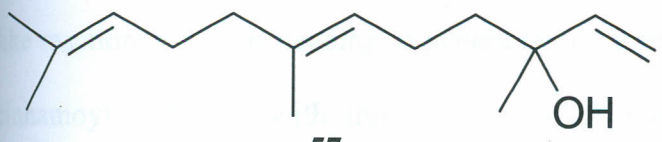

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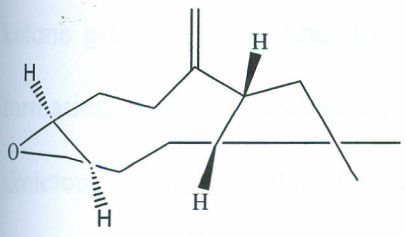

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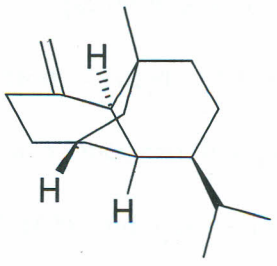

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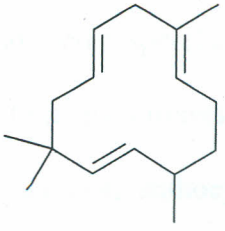

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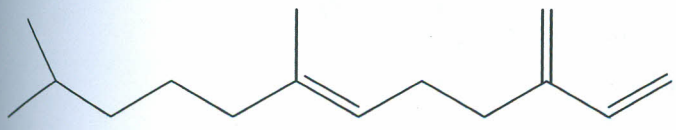

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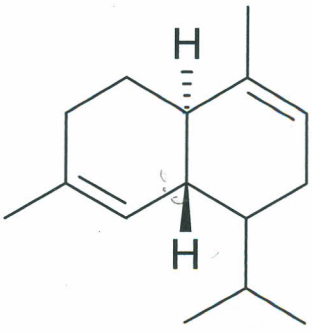


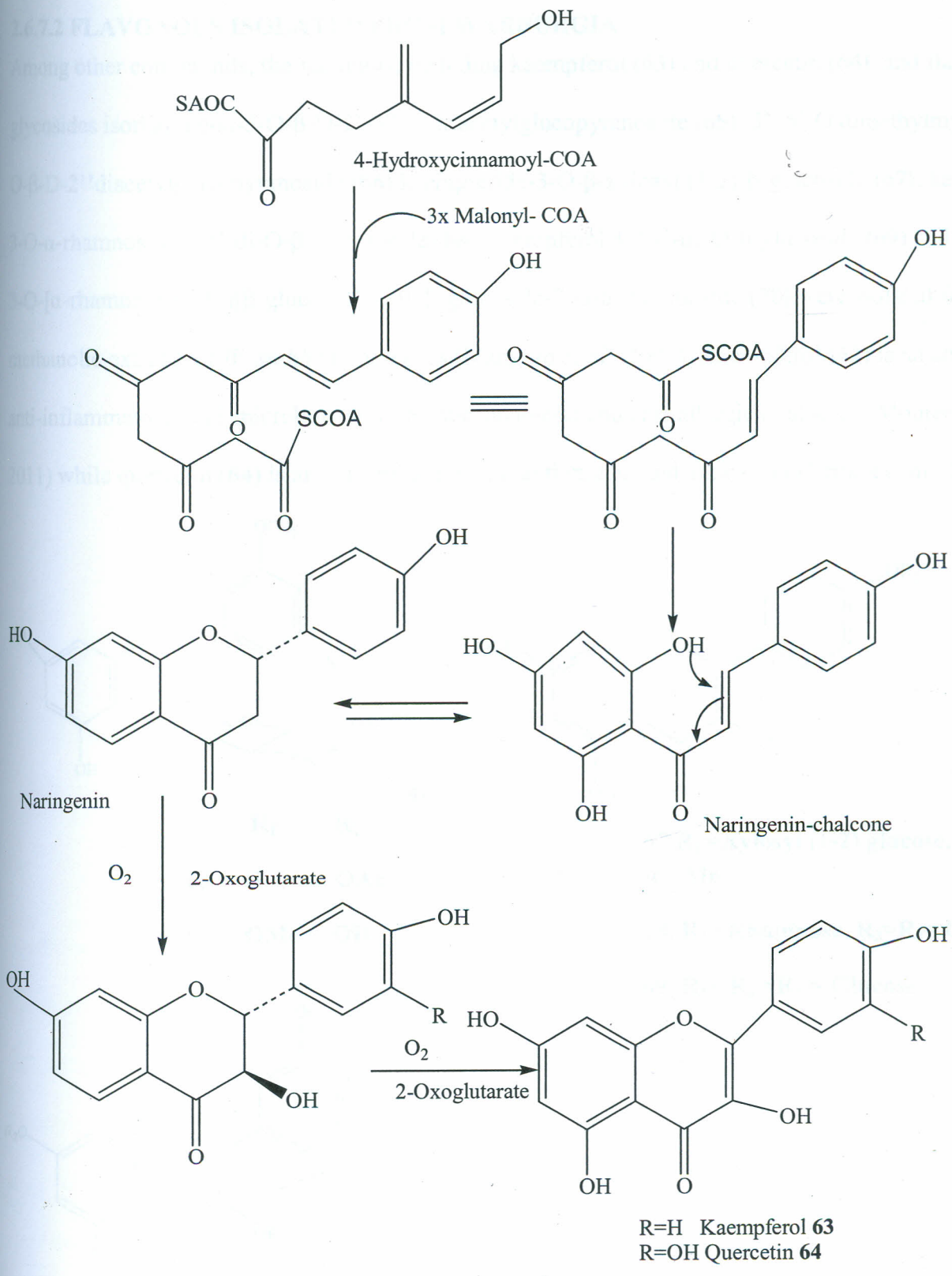
62

Caryophyllene **(55)** is an anti-inflammatory, antibiotic, antioxidant, anti-carcinogenic and a local anaesthetic (Leqault and Pichette, 2007), nerolidol **(57)** is antileishmanial (Arruda *et al.*, 2005), antifungal (Park *et al.*, 2009) and has inhibitory activities against *Escherichia coli* and *Staphylococcus aureus* (Piculo *et al.*, 2011), while α-humulene **(60)** has anti-inflammatory activity (Chaves *et al.*, 2008).

2.6.7 FLAVONOIDS

2.6.7 BIOSYNTHESIS OF FLAVONOIDS

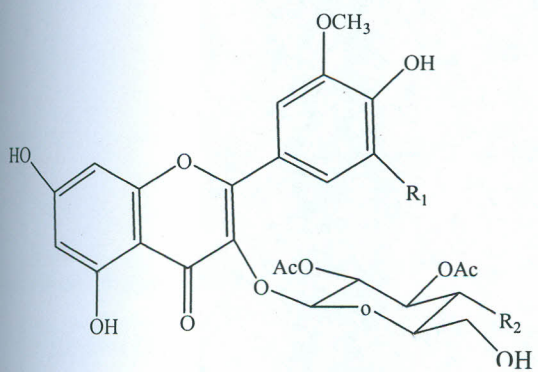
Flavonoids are formed from a cinnamoyl-CoA starter unit, with chain extension that uses three molecules of malonyl-CoA. This initially gives a polyketide (Scheme 3.3), which allow aldol or Claisen-like reactions to occur, leading to generation of aromatic rings. The enzyme chalcone synthase couple a cinnamoyl-CoA unit with three malonyl-CoA units giving chalcones, which act as precursors of flavonoid derivatives found throughout the plant kingdom. Most flavonoids contain a six-membered heterocyclic ring, formed by Michael-type nucleophilic attack of a phenol group on to the unsaturated ketone giving a flavanone. In nature the reaction is enzyme catalysed and stereo specific, resulting in formation of a single flavanone enantiomer. Flavanones can then give rise to many variants on this basic skeleton, e.g. flavonols such as kaempferol **(63)** and quercetin **(64)**, flavones, anthocyanidins and catechins (Dewick, 2002).



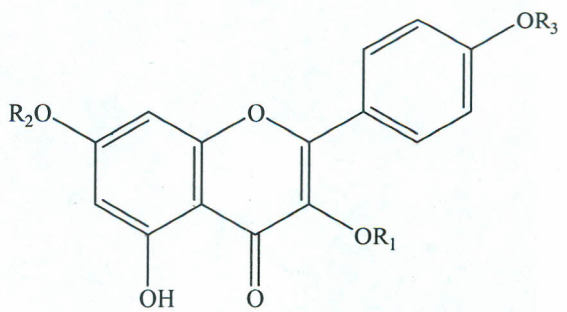
Scheme 2.3; The biosynthetic pathway for flavonoids (Dewick, 2002)

2.6.7.2 FLAVONOLS ISOLATED FROM WARBURGIA

Among other compounds, the flavonols; including kaempferol **(63)** and quercetin **(64).** and flavonol glycosides isorhamnetin-3-O-β-D-2",3",4"-triacetylglucopyranoside **(65)**, 3', 5'-O dimethylmyricetin-3-O-β-D-2''diacetylglucopyranoside **(66)** kaempferide-3-O-β-xylosyl (1-2)-β-glucoside **(67),** kaempferol-3-O-α-rhamnoside-7,4'-di-O-β-galactoside **(68),** kaempferol 3,7,4'-tri-O-β-glucoside **(69)** and quercetin 3-O-[α-rhamnosyl(1-6)][β-glucosyl(1-2)]–β-glucoside-7-O-α-rhamnoside **(70).**were isolated from methanolic extracts of *W. stuhlmannii* leaves (Manguro *et al.,* 2003). Kaempferol **(62)** is an antioxidant, anti-inflammatory, anti-microbial, anti-diabetic, analgesic and anti-allergic (Calderon–Montano *et al.*, 2011) while quercetin **(64)** is an anti-inflammatory, antioxidant and anticancer (Urmila *et al.*, 2011).



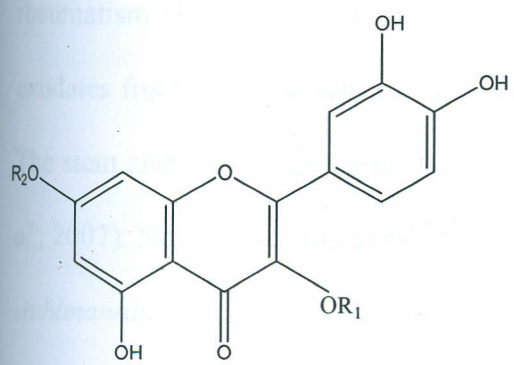
	R_1	R_2
65	**H**	**OAc**
66	**OMe**	**OH**



67 R_1 =Xylosyl (1-2) glucose, R_2=H, R_3=Me

68. R_1 =Rhamnose, R_2=R_3= Galactose

69. R_1= R_2 =R_3 = Glucose



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R_1 = [Rhamnosyl (1-6)][glucosyl (1-2)glucoside] **R_2 = rhamnose**

2.7 *WARBURGIA STUHLMANNII*

In Kenya, *W. stuhlmannii* known as mkaa or mkarambaki in Swahili, is endemic to the coastline, in Kaembeni-Dida, Kilifi District and Kinango, Kwale District of Coast Province. *Warburgia stuhlmannii* is a small ever green tree, 12-24m high, with a bole to 8m, girth 1.5m. It has yellow to greyish black bark, splitting into irregular flakes. Slash is blood red turning brown, leaves are very glossy above, elliptic, base cuneate, apex acute, 3.0-9.5cm by 1.3-3.3cm, petiole 3-5mm across, containing two or more seeds with an oily endosperm (Verdcourt, 1954).

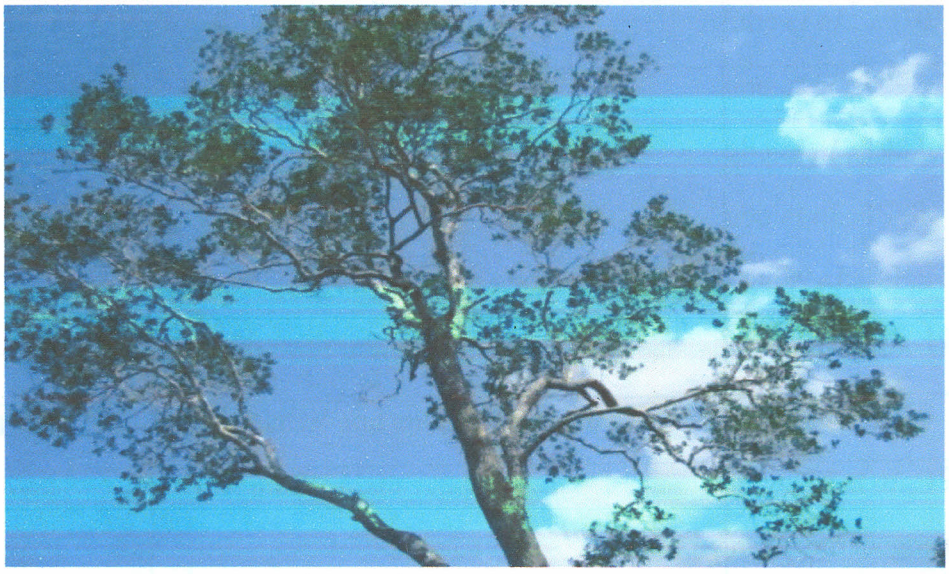


Plate 2.1 *Warburgia stuhlmannii*

Traditionally, the bark of *Warburgia stuhlmannii*, is used as spice, remedy for toothache and rheumatism (Beentje, 1994). Pulverised bark, mixed with honey, is used as cough medicine, while exudates from the bark, when mixed with egg, boiled and drunk manages constipation (Beentje, 1994). The stem and root barks are used by communities in Kwale District for malaria treatment (Muthaura *et al.*, 2007). No phytochemical and biological activity studies have been carried out on the root bark of *W. stuhlmannii.*

2.7.1 BIOLOGICAL ACTIVITY OF COMPOUNDS ISOLATED FROM *W. STUHLMANNII*

The biological activity of some compounds isolated from *W. stuhlmannii* stem bark were previously evaluated and the summary is given in Table-2.3. Evaluation of the other compounds needs to be undertaken.

Table 2.3; Biological activity of *Warburgia stuhlmannii* compounds

OMPOUND	CTIVITY	EFERENCE
lukaadial **(11)**	ntimalarial and ıtitrypanosomal	'ube *et al.*, 2010
gandensidial **(12)**	ntifeedant	ubo *et al.*, 1997.
	lolluscicidal	ubo *et al.*, 1983.
	ntifungal	ubo *et al.*, 1988.
	ntimalarial and antitrypanosomal	'ube *et al.*, 2010
	ant growth inhibition	leinwald *et al.*, 1978.
luzigadiolide **(13)**	ntimalarial and antitrypanosomal	'ube *et al.*, 2010
luzigadial **(14)**	sect anti feedant	aniguchi *et al.*, 1984.
	ntifungal, antibacterial	ınsen and Degroot, 1978.
	nticomplement	ıkuyama *et al.*, 1982.
	ntitrypanosomal	'ube *et al.*, 2010
	ntimalarial	race *et al.*, 2010
'arburganal **(29)**	ytotoxicity	aniguchi *et al.*, 1984
	ntifeedant and antimicrobial	ubo *et al.*, 1976; Opiyo *et* ., 2011
olygodial **(30)**	ntimicrobial	leinwald *et al.*, 1978
	ant growth inhibition	ıkuyama *et al.*, 1982
	ıd anti feedant	
	loluscicidal	ubo *et al.*, 1983
	ntifungal	ubo *et al.*, 1988

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 General experimental procedures

Optical rotations were taken on Perkin-Elmer 341 automatic recording polarimeter. Gallenkhamp melting point apparatus was used to determine melting points and the values are uncorrected. The UV spectra were determined using Perkin-Elmer Lambda 2 spectrophotometer, while IR data were obtained on Bruker Vector 22 spectrophotometer on KBr pellet. Electron ionization (70 eV) mass spectral data were obtained on a MAT 8200 311 A Varian Bremen instrument. The ^{1}H and ^{13}C NMR spectra were recorded on Bruker Avance 500 spectrometer operating at 500 and 125 MHz, respectively. Two dimensional (2D) NMR data including HMBC, HMQC, ^{1}H-^{1}H COSY and NOESY were obtained using the same equipment. Analytical thin layer chromatography was performed using aluminium precoated silica gel 60 F_{254} plates with solvent systems *n*-hexane-EtOAc (9:1; 4:1; 2:1 and 1:1) and CH_2Cl_2-MeOH (97:3; 95:5; 9:1 and 4:1). The chromatograms were visualised under UV light at 254 nm and 366 nm and also spraying with *p*-anisaldehyde-sulphuric acid mixture followed by heating at 100 ^{0}C. Isolation of compounds was achieved by phase liquid chromatography using silica gel 60G UV F_{254} (230-400 mesh). Solvents used for chromatography were distilled.

3.2 Collection of plant materials.

The root barks of *W. stuhlmannii* were collected from Kaembeni-Dida, Kilifi County, Coast province, Kenya (39^{0} 50' E, 3^{0} 20'S) in February 2010. Identification and authentification was done by Mr. Mashauri (taxonomist) of Kenya Forestry Research Institute (KEFRI), Gede Regional Centre, Malindi, Kenya and voucher specimens (leaves, twigs and fruits, voucher MKA-WS-2010-02) were identified after comparison with authentic samples at the Kenya Forestry Research Institute headquarter, Muguga, Nairobi.

3.3 Extraction of plant materials

The root bark was air dried under the shade then ground using a grinding mill into fine powder. Approximately 1kg of the powdered plant material was sequentially extracted by cold percolation with ethyl acetate and methanol (each 3 x 3L) for 48 hours each. The solvents were added to the material then vigorously shaken on an orbital shaker, then set aside for 48hrs, after which were filtered using Whatman No. 1 filter paper. Filtrates from ethyl acetate and methanol were separately combined, and concentrated in round bottomed flasks using rotary evaporator under reduced pressure (Kioy *et al.*, 1989) to give brownish-green and dark-brown extracts in the yields of 80 g and 150 g, respectively. The extracts were divided into two portions; for bioassay tests (approximately each 10 g) and the rest for isolation and characterization according to the procedure of Wube *et al.*, (2005).

3.4 Isolation of compounds from ethyl acetate extract of W. stuhlmannii root bark

Approximately 65g of the ethylacetate extract was subjected to column chromatography on silica gel (column size 5.0 x 60 cm, SiO_2 230-400 mesh ASTM, 500 g, pressure of about 1.0 bar) eluting with *n*-hexane (1.5 L) then *n*-hexane containing increasing percentage of ethylacetate (from 5% to 50%) and finally concluded with dichloromethane-methanol mixture (97:3; 95:5 and 9:1). A total of 85 fractions each 100 ml were obtained and their homogeneity monitored by use of TLC with solvent systems *n*-hexane-ethyl acetate (95:5, 9:1, 4:1, 2:1 and 1:1) and dichloromethane: methanol (99:1 and 93:7). Those fractions showing similar TLC profile were combined into five major fractions (1-V).

Fraction I (Fractions 1-6), eluted with *n* -hexane-EtOAc mixture, moved with the solvent front (eluent; *n* -hexane-EtOAc, 95:5) and were combined. Removal of solvent under reduced pressure gave sweet smelling yellow oil (10g).

Fraction II (Fractions 7-25) having been eluted with *n*-hexane-ethyl acetate (95:5 and 9:1) afforded five spots on TLC (solvent system, n-hexane-ethylacetate 9:1, 4:1, 2:1, 1:1) of R_f values 0.18, 0.22, 0.42, 0.56 and 0.67, respectively. From the combined fractions, crystallized out needle shaped white crystals,

which on re-crystallization (*n* -hexane-ethyl acetate, 9:1) afforded polygodial (**30**, R_f =0.56, 100 mg). Evaporation of the mother liquor under reduced pressure afforded yellow brown gummy material (7g) and further purification (SiO_2 200g, column size 3.0 x 50 cm, pressure ≈ 1.0 bar) using solvent systems; *n* -hexane-ethyl acetate (97:3, 95:5, 9:1 and 4:1), collecting 50 ml each afforded 30 fractions. The fractions were pooled together depending on TLC profiles into four pools (pools A-D). Pool B (fractions 17-22) afforded further polygodial (**30**, 75mg). Pool C (Fractions 25 and 26) contained a single spot on TLC (solvent system *n*-hexane-ethyl acetate, 95:5; 9:1) of R_f value 0.42 and was purified by crystallization in *n*-hexane-ethyl acetate (9:1) to give cinnamolide (**37**, R_f = 0.40, 40mg). Fractions 27-30 constituted pool D (3g) which afforded one major spot on TLC and were further purified by repeated medium pressure column chromatography (SiO_2 120g; eluent: *n*-hexane-ethyl acetate 9:1; pressure ≈ 1.0 bar, column size 2.0 x 50 cm) to give warburganal (**29**, R_f=0.18, 60 mg).

Fraction III (fractions 26-50, 15g) eluted mainly with *n*-hexane- ethyl acetate (7:3 and 2:1) gave three major spots (R_f values 0.40, 0.31 and 0.26) together with a minor one (R_f of 0.20) on TLC (solvent system, n-hexane-ethylacetate, 4:1, 2:1, 1:1). The fractions upon combining together followed by evaporation of the solvent crystallized out into colourless white needle like crystals which on filtration and further purification by re-crystallization (*n*-hexane-ethyl-acetate; 4:1) afforded more of warburganal (**29**) in 160 mg. The mother liquor was evaporated under reduced pressure to dryness, dissolved in a minimum amount of *n*-hexane-EtOAc (4:1) (approximately 10ml) and loaded on top of silica gel packed column (SiO_2 160g) using a pipette and eluted with *n*-hexane-ethyl acetate (17:1) followed by the same solvent systems in the ratios 9:1 and 4:1 to give further warburganal (**29**, 140 mg), bemadienolide (**45**, R_f=0.26, 55 mg), ugandensidial (**12**, R_f= 0.20, 24 mg) and cinnamolide (**37**).

Fraction 1V (fractions 52-73, approx. 13g) showed six spots of R_f values 0.56, 0.44, 0.35, 0.26, 0.18 and 0.14 on TLC using solvent system *n*-hexane-EtOAc (2:1) after the spraying of the TLC plate with anisaldehyde-sulphuric acid mixture followed by heating at 100^0 C over hot plate for two to three minutes. The fraction was similarly rechromatographed over a silica gel column (column size 3.5 x 60

cm, SiO_2 200g, pressure ≈1.5 bar) using solvent system *n*-hexane-EtOAc (17:1) followed by the same solvent system in the ratios 5:1, 4:1, 7:3 and 2:1, collecting 20 ml each. A total of 200 fractions were sampled and their compositions determined by TLC using *n*-hexane-EtOAc mixture of varying ratios. Fractions 20-30, eluted with *n* -hexane-EtOAc (4:1), contained mainly one spot of R_f value 0.56. They were combined, solvent evaporated and crystallized in *n*-hexane-EtOAc (4:1) to give further ugandensidial (**12**, 85 mg). Fractions 32-48 were also observed to contain one spot (R_f=0.26) and were similarly combined and upon removal of the solvent followed by crystallization in *n*-hexane-EtOAc (7:3) afforded further bemadienolide (**45**, 20 mg). Fractions 50-130 contained one major spot of R_f = 0.35 contaminated with other two minor ones. These were combined, solvent reduced using a rotary evaporator and the major compound crystallized out as white amorphous powder and was further purified by recrystallization from *n*-hexane-EtOAc (4:1) to give muzigadial (**14**, R_f = 0.35, 120 mg). The mother liquor upon evaporation of the solvent using rotary evaporator gave a residue of 3.5 g. This residue was further purified over silica gel column (column size 2.5 x 80 cm, SiO_2 100 g, pressure ≈1 bar) using *n*-hexane-EtOAc (3:1) and collecting 20 ml each (a total of 100 fractions were collected) to give further muzigadial (**14**), 150 mg and cinnamolide (**37**), 45 mg. Fractions 132-180 gave a mixture of compounds on TLC and were pooled together to give 5 g of semi purified extract. This portion was further subjected to medium pressure chromatography (column size 2.5 x 80 cm, SiO_2 160 g, pressure ≈1 bar) using *n*-hexane-ethyl acetate (3:1), collecting 10 ml each. A total of 200 fractions were collected and in the process fractions 10-80 afforded a single spot and were combined together. This upon evaporation of solvent and crystallization in *n*-hexane-ethyl acetate mixture gave a further muzigadial (**14**) in 75 mg. The sub-fractions 85-120 were combined and further subjected to medium pressure chromatography over silica gel column with *n*-hexane-EtOAc (3:1), collecting 10 ml each (a total of 70 fractions were collected). Fractions 15-36 showed single spots (solvent system: *n*-hexane-EtOAc, 2:1). These were combined, solvent evaporated and residue crystallized in *n*-hexane-EtOAc (4:1) to give further cinnamolide (**37**) in 20 mg.

Fraction V (fractions 74-85, 7.5 g) gave five spots of R_f values 0.33, 0.29, 0.23, 0.19 and 0.14 (solvent systems: *n*-hexane-EtOAc, 2:1 and CH_2Cl_2-MeOH, 97:3), all turned violet-bluish in colour after spraying with *p*-anisaldehyde-H_2SO_4 acid followed by heating indicating they are terpenoids. This fraction was further applied to silica gel column and elution with *n*-hexane-ethyl acetate mixture (3:2 and 1:1) followed by CH_2Cl_2-MeOH (97:3) led to the isolation of mukaadial (**11**, R_f=0.33, 150 mg), 6α, 9α-dihydroxy-4(13),7-coloratadiene-11,12-dial (**52**, R_f=0.29, 65 mg), ugandensolide (**35**, R_f =0.23, 34 mg) and deacetylugandensolide (**36,** R_f=0.14, 250 mg).

3.4.1 PHYSICAL AND SPECTRAL DATA OF COMPOUNDS ISOLATED FROM THE ETHYL ACETATE EXTRACT

3.4.1.1 Mukaadial (11)

White needle shaped crystals from *n*-hexane-EtOAc mixture, R_f = 0.33, mp 172 ^{0}C (Lit. 173 ^{0}C, Kioy *et al.*, 1990). $[\alpha]_D$ -28 ^{0} (c=0.05, methanol) (Lit. -30^0. Kioy *et al.*, 1989). UV λ_{max}. (MeOH) 228 nm. IR (KBr) ν_{max} 3450 (OH), 1725, 1660, 1630, 1450, 1400, 1370, 10 50 cm^{-1}. ^{1}H and ^{13}C NMR ($CDCl_3$ + drop DMSO-d_6): See Table 4.1. EIMS (70 eV) *m/z* (%) 266 $[M]^+$ (4), 248 $(M-H_2O]^+$ (25) 237$(M-CHO)^+$ (100), 109 (13), 95 (45), 83 (55), 55 (75), 45 (85).

3.4.1.2 Ugandensidial (cinnamodial) (12)

White needle shaped crystals from *n*-hexane-EtOAc mixture, R_f = 0.20, mp 136^0-139 ^{0}C (Lit. 137-140 ^{0}C, Kioy *et al.*, 1990). $[\alpha]_D$ –402^0 ($CHCl_3$, c= 1.0). (Lit. -398^0, Kioy *et al.*,1990). UV λ_{max} ($CHCl_3$) 216 nm. IR ν_{max} (KBr) 3425, 1745, 1720, 1690, 1240 cm^{-1}. ^{1}H and ^{13}C NMR ($CDCl_3$) δ: see Table 4:2. EIMS (70 eV) *m/z* (%): 308 $[M]^+$ (18), 279 (14), 278 (50), 248 (22), 237 (100), 220 (38), 205 (33), 124 (22), 95 (21), 91 (43), 43 (83). HREIMS: *m/z* 308.1611 $[M]^+$ (calcd for $C_{17}H_{24}O_5$, 308.1624).

3.4.1.3 Muzigadial (14)

White plate shaped crystals from *n*-hexane-EtOAc mixture, R_f = 0.35, mp 126-128 ^{0}C (Lit. 128-129 ^{0}C, Ying *et al.*, 1995). $[\alpha]_D$ -224^0 ($CHCl_3$, c= 1.0) (Lit. -193 ^{0}C, Kioy *et al.*, 1990). UV λ max (MeOH) 216

nm. IR ν_{max} (KBr) 3455, 2958, 2914, 2870, 1731, 1671, 1382, 1252, 1203, 1022, 899, 809 cm^{-1}. ^1H and ^{13}C NMR ($CDCl_3$) δ: see Table 4.3. EIMS (70 eV) *m/z* (%) 248 $[M]^+$ (2), 248 $[M]^+$ (5), 219 (100), 187 (13), 177 (21), 159 (170, 135 (42), 107 (40), 91 (38).

3.4.1.4 Warburganal (29)

White needle crystals from *n*-hexane-EtOAc mixture, R_f= 0.18, mp 132-134 ^{0}C [Lit. 112-113 ^{0}C, Kioy *et al.*, 1989]. $[\alpha]_D$ -218^0 (c=0.1, $CHCl_3$) [Lit. -216 ^{0}, Kioy *et al.*, 1989). UV λ_{max} ($CHCl_3$) 220 nm. IR ν_{max} (KBR) 3450, 2923, 2860, 1730, 1680, 1467, 1365, 1365, 1306, 1274, 1219, 1188, 1172, 1134, 1065, 1003, 965, 933, 861, 838, 809, 773, 715, 676, 650, 550 cm^{-1}. ^1H and ^{13}C NMR ($CDCl_3$) δ: see Table 4.4. EIMS (70 eV) *m/z* (%) 251 $[M+H]^+$ (12), 235 (10), 233(100) 215 (75), 220 (40), 187 (100), 149 (8), 145 (20), 133 (8), 124 (10), 109 (12), 95, (6), 91 (4). HREIMS:*m/z* 250.1563 $[M]^+$ (calcd. for $C_{15}H_{22}O_3$, 250.1563).

3.4.1.5 Polygodial (30)

White needle crystals from *n*-hexane-EtOAc (9:1), R_f= 0.56, mp 37-40^{0}C (Lit. 35-36 ^{0}C, Ying *et al.*, 1995). $[\alpha]_D$ -74 ^{0} (C=0.1, $CHCl_3$) (Lit. -73^0, Mashimbye *et al.*, 1999). UV λ_{max} ($CHCl_3$) 230 nm. IR ν_{max} (KBr) 2930, 2860, 1722, 1682, 1647, 1460, 1385, 960 cm^{-1}. ^1H and ^{13}C NMR ($CDCl_3$) δ: see Table 4.5. EIMS (70 eV) *m/z* (%) 234 $[M]^+$ (10), 219 (6), 206 (55), 191 (30), 173 (11), 163 (12), 145 (15),135 (12), 121 (62), 109 (63), 105 (30), 93 (30), 91 (35), 77(35), 69 (30), 55 (30), 43 (58), 41 (100).

3.4.1.6 Ugandensolide (35)

White needle shaped crystals from CH_2Cl_2-MeOH (95:5), R_f = 0.23, mp 212^{0}C (Lit. 210 ^{0}C, Kioy *et al.*, 1990). $[\alpha]_D$ + 25^0 (MeOH, c=1.0) (Lit. +26^0, Kioy *et al.*, 1990). UV λ max ($CHCl_3$) 218 nm. IR ν_{max} (KBr) 3451, 2993, 1760, 1750, 1733, 1642, 1459, 1398, 1265, 1150, 1097, 1043, 972, 742 cm^{-1}. ^1H and ^{13}C NMR ($CDCl_3$) δ: see Table 4.6. EIMS (70 eV) *m/z* (%): 308 $[M]^+$ (27) 266 (98), 248 (100), 233 (26), 215 (14), 179 (12) , 175 (21), 165 (36), 163 (30), 136 (15), 121 (20), 109 (11), 105 (13), 85 (11), 93 (10), 91(26). HREIMS 308.1421(Calcd for $C_{17}H_2O_2$, 308.1624).

3.4.1.7 Deacetylugandensolide (36)

White needle shaped crystals from *n*-hexane-ethyl acetate mixture, R_f = 0.14, mp 262-264^{0}C (Lit. 260-265^{o}C, Kioy *et al.*, 1990). $[\alpha]_D$ + 69^0 (MeOH, c=1.0) (Lit.+70, Kioy *et al.*, 1990). UV λ_{max} (MeOH) 220 nm. IR ν_{max} (KBr) 3340, 2930, 2850, 1750, 1740, 1385, 1360, 1250, 1225, 1170, 1100, 1050, 1025, 980, 860 cm^{-1}. ^{1}H and ^{13}C NMR ($CDCl_3$) δ: see Table 4.7. EIMS (70 eV) *m/z* (%) 266 $[M]^+$ (98), 248 (32), 236 (7), 230 (30), 215 (40), 202(32), 162 (35), 136 (70), 122 (40), 114(1152), 95 (40), 85 (52), 41 (100), 39 (23).

3.4.1.8 Cinnamolide (37)

Prism shaped crystals from *n*-hexane-ethyl acetate mixture, R_f = 0.40, mp 126-127^{0}C [Lit. 128-129 ^{0}C, Kioy *et al.*, 1990]. $[\alpha]_D$ -28^0 [$CHCl_3$ c=0.5] (Lit. -29^0, Kioy *et al.*, 1989). UV λ max ($CHCl_3$) 229 nm. IR ν_{max} (KBr) 2924, 2848, 1761, 1685, 1468, 1386, 1366, 1306, 1275, 1220, 1188, 1172, 1134, 1066, 1003, 966, 933, 862, 839, 809, 774, 743 cm^{-1}. ^{1}H and ^{13}C NMR ($CDCl_3$) δ: see Table 4.8. EIMS (70 eV) *m/z* (%) 235 $[M+1]^+$ (7) 234 $[M]^+$ (8), 219 (10), 201 (3), 149 (23), 137(8), 124 (90), 109 (100), 95 (10), 81 (20), 41 (21).

3.4.1.9 Bemadienolide (45)

White crystals from n-hexane-EtOAc mixture, R_f = 0.26, mp 123-125^{0}C (Lit, 124-126^{0}C, Opiyo *et al.*, 2011). $[\alpha]_D$ -28^0 [$CHCl_3$ c=0.5] (Lit. -29^0, Kioy *et al.*, 1989). IR ν_{max} (KBr) 2993, 1751, 1642, 1459, 1398, 1265, 1150, 1097, 1043, 972, 742 cm^{-1}. ^{1}H and ^{13}C NMR ($CDCl_3$) δ: see Tables 4.9. EIMS (70 eV) *m/z* (%) 232 $[M]^+$ (20), 217 (10), 203 (15), 176(15), 173(18), 161(26), 149(39), 147(100), 131 (20), 119(32), 91(28),56 (32), 41(28).

3.4.1.10 6α, 9α-Dihydroxy-4(13), 7-coloratadiene-11,12-dial (52)

White needle shaped crystals from n-hexane-EtOAc mixture, mp 138-140^{0}C (Lit. 137-139 ^{0}C, Wube *et al.*, 2005). $[\alpha]_D$ -45^0 ($CHCl_3$; c=0.5) (Lit.-44^0 Wube *et al.*, 1989). UV λ_{max} ($CHCl_3$) 236 nm. IR (KBr) ν_{max} 3364 (OH), 2921, 2865, 1723, 1665, 1642, 1453, 1381, 1320, 1169, 1135, 1030, 1028, 895, 831, 794 (exocyclic methylene), 672, 595 cm^{-1}. ^{1}H and ^{13}C NMR ($CDCl_3$) data see Table 4.10. EIMS (70 eV)

m/z (%) 246 $[M-H_2O]^+$ (6), 236 (26), 235 $[M-CHO]^+$ (100), 217 $[M-CHO-H_2O]^+$ (20), 203 (7), 189 $[M-CHO-H_2O-CO]^+$ (22), 175 $[M-CHO-OH-Me-CO]^+$ (9), 161 (10), 153 (29), 137 (150, 109 (24), 95 (18), 91 (21).

3.5 Isolation of compounds from methanol extract

The methanol extract (approx. 85g) was adsorbed onto silica gel and chromatographed over silica gel column (column size 5 x 60cm, SiO_2 500g, pressure= 1.5 bar) eluting with *n*-hexane-ethyl acetate in the ratios 3:2 and 1:1. The column was further eluted with increasing concentration of methanol in dichloromethane (2-30%) and finally with methanol to give 100 fractions of 100ml each. Fractions eluted with dichloromethane-methanol mixtures were monitored by CH_2Cl_2-MeOH (97:3, 95:5 and 9:1) while those eluted using *n*-hexane-EtOAc mixtures were monitored by *n*-hexane-EtOAc (2:1 and 1:1). Those showing similar TLC profiles were combined into four major fractions (I-IV). Fraction I (fractions 6-30 ,8g) eluted with *n*-hexane-ethyl acetate was further purified by repeated medium pressure column chromatography using *n*-hexane-ethylacetate (5:1, 4:1, 2:1 and 1:1) collecting 20 ml each to give further mukaadial **11** (20 mg), ugandensolide **35** (30 mg) and deacetylugandensolide **36** (50 mg).

Fraction II (fractions 35-70, 15 g) upon further purification over silica gel column (column size 3.5 x 80cm, SiO_2 320g, pressure=1.5 bar) using solvent system CH_2Cl_2-MeOH (97:3) followed by the same solvent system in the ratio 95:5 and collecting 20ml each afforded further mukaadial **(19)**, 16 mg.

Fraction III (fractions 73-89, 11g) upon further purification as described above over silica gel column using CH_2Cl_2-MeOH (95:5) followed by the same solvent system 9:1, collecting 10 ml each gave further ugandensolide **(35)** in 20 mg.

3.6 *In vitro* anti-plasmodial assay

3.6.1 Preparation of bioassay test samples

The stock solution of crude extracts and pure compounds were prepared by use of dimethyl sulphoxide (DMSO). Stock solutions of the standard drugs of same concentration, were prepared under sterile conditions. Test samples were stored at -20^{0}C, until use.

3.6.2 *Plasmodium falciparum* cultures

Two strains of laboratory adopted *P falciparum* cultures; chloroquine sensitive, Sierra-Leone (D10) and chloroquine resistant Indochina (W2) were used.

The medium of culture parasite was made of RPMI-1640, supplemented with 10% freshly frozen human serum in acid –citrate dextrose anticoagulant, haemocrit suspension of human 0^+ erythrocytes, 25Mm HEPES (hydroxyethylpiperazine-N-2-ethane sulphuric acid) and 5% $NaHCO_3$. The parasite culture was incubated at 37^{0}C in an atmosphere of 6% oxygen, 3% carbon dioxide and 91% nitrogen, cultured in KEMRI, Kisumu.

3.6.3 Bioassay and data computation

The test solutions were loaded on wells of sterile 96 well flat-bottom micro culture plates, followed by two fold serial dilutions, using Biomek automated laboratory work station and RPMI as diluents. *In vitro* semi-automated micro dilution assay technique that measures ability of extracts to inhibit incorporation of (G-^{3}H) hypoxanthine into malaria parasites, (Desjardins *et al,* 1979), was used. 200m/s of 1.4% haemocrit was applied to each well, giving rise to ring stage parasite with initial parasitaemia of 0.4.%, which was then used for susceptibility test. Both parasitized and non-parasitized erythrocytes were included in all tests. Plates were incubated at 37^{0}C for 48 hrs in an atmosphere of 3% CO_2, 6% O_2 and 91% N_2, then each well pulsed with 25µl of culture medium containing 0.5 µCi of G-^{3}H hypoxanthine and then plates incubated for further 18 hrs. Contents of each plate were harvested using an automated cell harvester then dried. Radioactivity in counts per minute (CPM) was measured by liquid scintillation. The concentration of drugs that cause 50% inhibition of G-^{3}H hypoxanthine uptake

(IC_{50}) was done by interpolation after logarithmic transformation uptake of both concentration and radioactivity in counts per minutes (CPM) values using the formula below (Sixsmith *et al.*, 1984).

The midpoint (Y_{50}) is given as;

$$Y_{50} = \frac{[(\text{PRBC-CPM value}) - (\text{VNPRBC-CPM value})]}{2}$$

$$IC_{50} = \text{Antilog}\left[\log X_1 + \frac{(\log Y_{50} - \log X_1)(\log X_2 - \log X_1)}{\log Y_2 - \log Y_1}\right]$$

IC_{50} = Concentration that causes 50% growth inhibition of culture parasites.

X_1=Lower concentration of sample

X_2 =higher concentration of sample

Y_1= CPM value which correspond to X_1

Y_2=CPM value, corresponding to X_2

CHAPTER 4

4.0 RESULTS AND DISCUSSION

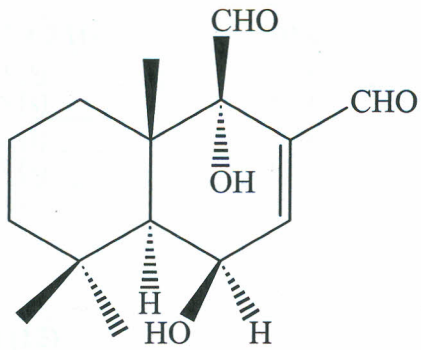
4.1 CHARACTERIZATION OF COMPOUNDS ISOLATED FROM ROOT BARK.

Column chromatography fractionation of *W. Stuhlmannii* root bark ethyl acetate and methanol extracts led to the isolation of ten compounds of farnesane-type sesquiterpenes which included; mukaadial **(11),** ugandensidial **(12),** muzigadial **(14),** warburganal **(29),** polygodial **(30),** ugandensolide **(35),** deacetylugandensolide **(36),** cinnamolide **(37),** bemadienolide **(45)** and 6α- hydroxymuzigadial **(52).** 6α, hydroxymuzigadial **(52).**is being reported for the first time in this species.

4.1.1 Mukaadial (11)

The compound crystallized out as white needles from n-hexane-ethyl acetate mixture, with melting point 172 ^{0}C and $[\alpha]_D$ -28 ^{0} (c=0.05, MeOH). It exhibited characteristic absorption band for hydroxyl (3450 cm^{-1}), C=O (1725 cm^{-1}) and C=C (1660 cm^{-1}) in the IR spectrum. The electron impact mass spectrum of the compound showed a molecular ion peak at *m/z* 266 $[M]^+$ corresponding to the molecular formula $C_{15}H_{22}O_4$. The ^{13}C NMR spectrum (Table 4.1) ($CDCl_3$) displayed a total of fifteen carbon signals assigned to three methyls, three methylenes, five methines and four quaternary carbons by DEPT experiments. In fact, the peaks at δ 192.0 and 201.4 with corresponding δ 9.67 and 9.44 values were assigned to 11-CHO and 12-CHO, respectively (Table 4.1). This signified that compound **11** is a drimane-type sesquiterpene derivative containing two aldehyde groups possibly at C-11 and C-12 positions (Kioy *et al*, 1990; Kioy *et al*., 1989). The presence of drimane skeleton was also demonstrated by three tertiary methyl groups at δ 1.08, 1.15 and 1.42 and an olefin functionality at δ 7.06 (d, *J*=2.4 Hz, H-7). The low field ^{1}H NMR peak at δ 9.67 was observed to become singlet from a doublet upon addition of D_2O, thus suggesting that the aldehyde is attached to a carbon bearing a hydroxyl group (Mahmoud *et al*., 1980). In fact, in the ^{1}H NMR of the compound, the proton of the hydroxyl group on C-9 was observed to absorb at about δ 4.47 as a doublet (*J*=1.5 Hz) and further supported by ^{13}C NMR signal at δ 76.8. The ^{13}C NMR data indicated that the hydroxyl is tertiary as per the peaks; δ 132.7 (C-

8), δ 42.5 (C-10) and δ 201.4 (C-11). The ^{1}H NMR spectrum also displayed a significant peak at δ 4.46 (dd, *J*=10.0, 2.3 Hz, H-6) suggesting that the proton is attached to a secondary carbon. The stereochemistry of the hydroxyl group at C-6 was established as β from the coupling constant *J*=10.0 Hz, which is due to spin-spin coupling between H-5α and H-6α (Kubo *et al.*, 1983; Kioy *et al.*, 1989). The ^{1}H and ^{13}C NMR data were in complete agreement with those previously reported for mukaadial isolated from *Canella winterana* (Kioy et al., 1990). Thus, on the basis of physical (melting point and optical rotation) and spectroscopic data, compound **11** was concluded as mukaadial.



11

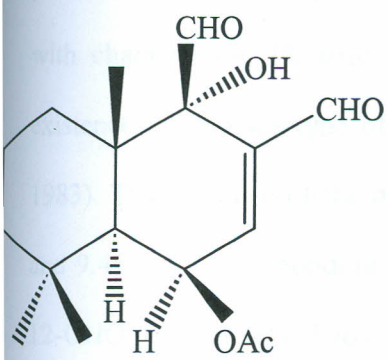
Table 4.1 ^{1}H and ^{13}C NMR chemical shift values for mukaadial (11).

	Data on isolated compound		Data from Literature (Kioy *et al.*, 1989)	
C	^{1}H NMR (*J* in Hz)	^{13}C	^{1}H (*J* in Hz)	^{13}C
1	1.92 (H_{ax}, m), 1.23-1.55 (H_{eq}, m)	41.7	.18 (H_{ax}, ddd, J=13.1, 13.1, 3.2 Hz), 1.24-1.55 (H_{eq}, m)	42.4
2	1.23-1.55 (2H, m)	31.8	1.55 (H_{ax}, m), 1.24 (H_{eq}, m)	32.6
3	1.23-1.55 (2H, m)	17.0	1.28 m, 1.24 m	17.0
4		31.8		32.6
5	2.58 (d, *J*=10.4 Hz)	47.2	2.51 (d, *J*=10.4 Hz)	47.9
6	4.46 (dd, *J*=10.0, 2.3 Hz)	66.6	.80 (ddd, *J*=10.4, 2.3 Hz)	67.2
7	7.06 (d, *J*=2.4 Hz)	158.0	7.33 (d, *J*=2.3Hz)	158.7
8		137.2		139.3
9		76.8		77.6
10		42.5		43.1
11	9.67 (d, *J*=1.2 Hz)	201.4	10.13 (s,)	202.5
12	9.44 (s)	192.0	9.57 (s)	192.3
13	1.15 (s)	21.9	1.12 (s)	21.9
14	1.42 (s)	38.6	1.47 (s)	35.8
15	1.08 (s)	17.1	1.18 (s)	17.1
9-OH	4.47		4.50	

4.1.2 Ugandensidial (12)

The compound was isolated from fractions eluted with *n*-hexane-ethyl acetate (7:3 and 2:1) and further purified by crystallization from *n*-hexane-ethylacetate (2:1) as white needles with melting point 137-139^{0}C. It showed characteristic absorption peaks at 1745, 1720 and 1690 cm^{-1} assigned to acetate, saturated aldehyde and conjugated aldehyde functional groups, respectively. The presence of an intramolecularly hydrogen bonded hydroxyl function was indicated by an IR band at 3425 cm^{-1}. The UV spectrum run in methanol showed λ_{max} 219 nm. The compound showed a high resolution molecular ion peak at *m/z* 308.1624 $[M]^{+}$ consistent with $C_{17}H_{24}O_5$ molecular formula. The other significant mass fragments at *m/z* 279, 248, 237 and 220 signified the presence of drimane skeleton containing two aldehyde groups and an acetoxy functionality (Mahmoud *et al.*, 1980, Kioy *et al.*, 1990). The presence of two aldehyde groups was confirmed by two proton resonances at δ 9.78 and 9.49 in the ^{1}H NMR spectrum (Table 4.2). The presence of three tertiary methyl groups (δ 1.03, 1.16 and 1.35) and an acetoxy group (δ 2.17) suggested a relationship with mukaadial (**11**) previously described (Kioy *et al.*,

1990). In fact, conjugated and non-conjugated aldehydes could then only be accommodated at C-8 and C-9 as in mukaadial (**11**). The acetoxy and hydroxyl groups were assigned as represented in structure **12** below (Mahmoud *et al.*, 1980). The H-6 appeared at δ 5.92 due to identical coupling constant of *J*=5.0 Hz with each of the H-5 (δ 2.03) and H-7 proton at δ 7.02 (d, *J*=4.8 Hz). The magnitude of the coupling constant between the protons at C-5 and C-6 made probable the assignment of the C-6 acetate as β (Kioy *et al*, 1990). Another striking feature observed in the ^{1}H NMR spectrum was a coupling of *J*=1.5 Hz between OH proton at δ 4.12 and the low field aldehyde group at δ 9.78. This seemed best explained by a coupling with the proton of the tertiary aldehyde function in the planar but "*non-w*" conformation (Fig. 4.1) which also accounted for the strong intramolecular hydrogen-bonding of the OH function. Based on spectroscopic data and comparison of the data with the already published data (Mahmoud *et al.*, 1980), compound **12** was concluded to be ugandensidial, a compound previously isolated from the stem bark of *W. stuhlmannii* and *W. ugandensis* (Kioy *et al.*, 1990).



12

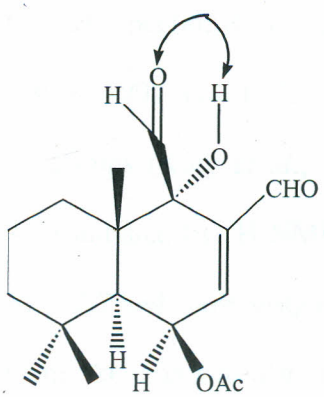


Figure 4.1: "Non-w" conformation accounting for intramolecular hydrogen-bonding of the OH function.of **12**

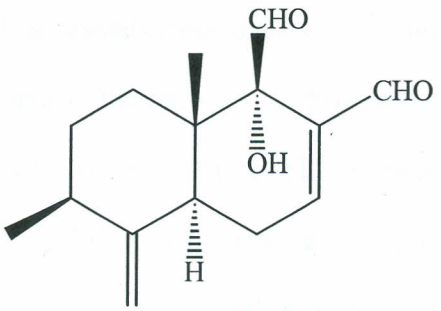
Table 4.2. ^{1}H and ^{13}C NMR data for ugandensidial (cinnamodial) (12)

	Data on isolated compound		Literature Data (Mahmoud *et al.*, 1980)	
C No	^{1}H NMR (J in Hz)	^{13}C NMR	^{1}H NMR(J in Hz)	^{13}C NMR
1	1.98 m, H_{ax}), 1.30-1.54 (m, H_{eq})	32.5	1.96 (m, H_{ax}), 1.28 (m, H_{eq})	31.9
2	1.30-1.54 (2H, m)	18.1	1.52 (m), 1.40 (m)	19.9
3	1.30-1.54 (2H, m)	44.4	1.46 (m), 1.35 (m)	44.2
4		32.4		34.0
5	2.03 (d, J=9.8 Hz)	45.3	2.04 (d, J=4.7 Hz)	45.2
6	5.90 (t, J=5.0 Hz)	66.4	5.87 (t, J=4.6 Hz)	66.2
7	7.02 (d, J=4.8 Hz)	149.0	7.04 (d, J=4.7 Hz)	148.5
8		141.3		141.3
9		77.4		77.5
10		42.0		41.7
11	9.78 (d, J=1.5 Hz)	201.5	9.75 (d, J= 1.4 Hz)	201.4
12	9.49 (s)	193.4	9.47 (s)	192.2
13	1.18 (s)	21.8	1.17 (s)	21.9
14	1.35 (s)	20.3	1.34 (s)	19.7
15	1.03 (s)	18.1	1.03 (s)	17.7
6-OAc	2.15 (s)	170.4, 23.2	2.14 (s)	170.8, 21.4
9-OH	4.10 (d, J=1.5 Hz)		4.10 (d, J=1.4 Hz)	

4.1.3 Muzigadial (14)

The compound was obtained as white plates, with melting point 126-128 ^{0}C. It showed a molecular ion peak at *m/z* 248, corresponding to the formula $C_{15}H_{20}O_3$. The UV spectral data λ_{max} 216 nm together with characteristic IR absorption peaks at 3455 (OH), 1730 (CHO) and 1671 cm^{-1} suggested the existence of drimane-type of sesquiterpenes with dialdehyde groups (Kioy *et al.*, 1990, Kubo *et al.*, 1983). The presence of the dialdehyde groups was further substantiated by ^{1}H NMR peaks at δ_H 9.65 and 9.45 with corresponding ^{13}C NMR peaks at δ_C 201.3 and 192.7 and were assigned to 11-CHO and 12-CHO, respectively (Kioy *et al.*, 1990). The presence drimane skeleton was also demonstrated by an olefinic proton at δ_H 7.26 (br t, J=3.1 Hz) which was supported by ^{13}C NMR peak at δ_C 156.7. As in the case of mukaadial **(11),** the low field ^{1}H NMR peak at δ_H 9.65 was observed to become singlet from doublet upon addition of D_2O suggesting that the aldehyde group is attached to a carbon bearing a hydroxyl group. The presence of a hydroxyl group was further supported by deuterium oxide exchangeable proton absorbing as a doublet at δ_H 4.1 (d, J=1.5 Hz) in the ^{1}H NMR spectrum (Mahmoud *et al.*, 1980) and further supported by the C-9 resonance at δ_C 78.0 in the ^{13}C NMR spectrum. The ^{13}C

NMR of compound **14** showed the presence of fifteen carbon signals sorted out by DEPT experiment into two methyls, four methylenes, five methines including two carbons containing an oxygen atom each and four quarternary carbons of which one carbon is holding a tertiary hydroxyl group. Of significance in the ^{1}H NMR spectrum of compound **14** were the presence of two broad singlet peaks at δ_H 4.94 (1H) and 4.77 (1H) attributed to a terminal double bond carbon and a doublet at δ_H 1.09 (J=6.6Hz) assigned to a methyl group on a quarternary carbon. Accordingly, the ^{13}C NMR confirmed the absence of gem-dimethyls in the compound. On the basis of the observed spectroscopic data, compound **14** was assumed to have two aldehyde groups, hydroxyl group and double bond as in warburganal **(29).** Also the absence of gem-dimethyl groups suggested the presence of a terminal double bond at C-4 further confirmed by ^{13}C NMR peaks at δ_C 151.6 (C-4) and 106.1 (C-13). Similarly, the presence of a peak at δ_H 1.09 as a doublet with coupling constant J=6.6 Hz signified that C-3 was substituted by a methyl group, a fact further supported by the ^{13}C NMR C-3 shift to δ_C 30.6 compared to that of warburganal (**29**) at δ_C 44.4. Furthermore, a comparative analysis of ^{1}H and ^{13}C NMR data (Table 4.3) with those of muzigadial previously isolated from *W. ugandensis* (Kioy *et al.*, 1990; Opiyo, 2011) were in complete agreement. Thus, compound **14** was confirmed to be muzigadial.



14

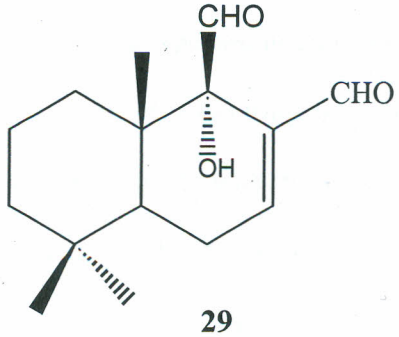
Table 4.3: ^{1}H NMR and ^{13}C NMR data for muzigadial (14)

Data on isolated compound			Literature Data (Kioy *et al.*, 1990)	
C	^{1}H NMR (*J* in Hz)	^{13}C NMR	^{1}H NMR (*J* in Hz)	^{13}C NMR
1	1.14-2.54 (m, 2H)	31.7		31.7
2	1.14-2.54 (m, 2H)	27.9		30.9
3	1.14-2.54 (m, 2H)	30.6		38.2
4		151.6		151.6
5	2.65 (m)	40.2	2.63 (m)	40.2
6		28.0		27.6
7	7.26 (br t, *J*=3.1Hz)	156.7	7.25 (t , *J*=3.7 Hz)	155.8
8		139.9		139.9
9		77.2		77.6
10		42.3		42.4
11	9.65 (d, *J*=1.5 Hz)	201.3	9.45 (s)	201.3
12	9.45 (s)	192.7	9.65 (s)	192.7
13	4.94 (s), 4.77 (s)	106.1	4.93(s), 4.76 (s)	106.1
14	1.07 (s),	18.8	0.88 (s)	18.4
15	1.09 (d, *J*=6.6 Hz)	15.48	1.08 (d, *J*=6.6 Hz)	15.1

4.1.4. Warburganal (29)

The compound was isolated as white crystals from silica gel column using n-hexane-ethyl acetate (4:1) followed by the same solvent system in the ratio 3:1. It showed R_f value of 0.18 with n-hexane-ethyl acetate (4:1) as the developing solvents. Its spot on TLC turned purple-bluish after spraying with anisaldehyde-sulphuric acid mixture followed by heating at approximately 100^0 C for two minutes. The physical property suggested that the compound is a terpenoid or a sterol (Kioy *et al.*, 1989). Compound **29** showed a molecular ion peak at *m/z* 250 in an electron impact mass spectrum measurement corresponding to $C_{15}H_{22}O_3$ molecular formula. Its proton NMR spectrum showed the presence of two low field peaks at δ_H 9.80 and 9.50 characteristic of drimane-type sesquiterpene with

aldehyde groups at C-11 and C-12 (Kioy *et al.*, 1990). These together with three tertiary methyl signals at δ_H 1.02, 1.14.and 1.41, and olefinic proton at δ_H 7.24 further suggested the presence of a drimane skeleton (Kubo *et al.,* 1983). In the ^{13}C NMR spectrum a peak at δ_C 76.6 relative to polygodial **(30)** suggested that C-9 contained a hydroxyl group. This was similarly confirmed by running its ^1H NMR spectrum when a drop of D_2O was added which showed the low field peak at δ_H 9.73 becoming singlet from doublet signifying that the aldehyde group was attached to a carbon bearing a hydroxyl group (Brooks and Draffan, 1969; Mahmoud *et al.*, 1980). The presence of a hydroxyl group was further supported by deuterium oxide exchangeable proton absorbing as a broad singlet at δ_H 4.00 in the ^1H NMR spectrum. The ^{13} C NMR spectrum of the compound indicated the presence of 15 carbon atoms in the molecule. Two olefinic and two aldehyde carbonyl resonances were evident as was one carbon attached to oxygen atom. Also the spectrum exhibited; three methyls, four methylenes, four methines and four quarternary carbons as shown by the DEPT spectrum. Similarly, the ^{13}C NMR confirmed the previous observation of the dialdehyde groups in **29** at approximately equivalent shifts to 11-CHO and 12-CHO in mukaadial **(11)** further supporting the OH groups to be at C-11. It should also be pointed out that compound **19** is 16 amu higher than that of warburganal (**29**) hence it can be argued out that compound **29** is monohydroxylated derivative of polygodial **(30).** Furthermore, a comparative analysis of both physical (mp and $[\alpha]_D$) and spectroscopic data (^1H, ^{13}C Table 4.4) and (ms) of compound **29** with those of warbuganal, previously isolated from *W. ugandensis* (Kioy *et al.,* 1990*)* were in complete agreement. Thus compound **29** was confirmed to be warburganal.



29

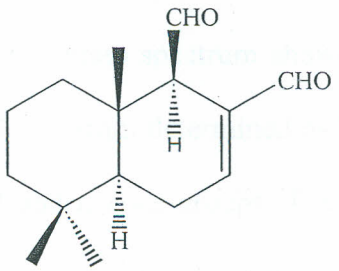
Table 4.4. ^{1}H and ^{13}C NMR data of warburganal (29)

	Data on isolated compound		Literature Data (Kioy *et al.*, 1990)	
	^{1}H NMR (*J* in Hz)	^{13}C NMR	^{1}H NMR (*J* in Hz)	^{13}C NMR
1	1.14-2.50 (m, 2H)	31.3	1.00-1.80 ((m, 2H)	31.2
2	1.14-2.50 (m, 2H)	17.7	1.00-1.80 (m, 2H)	17.7
3	1.14-2.50 (m, 2H)	41.5	1.00-1.80 (m, 2H)	41.3
4	1.08 (s, 3H)	33.0	1.09 (s, 3H)	33.0
5	2.65 (m)	40.6	.89 (dd, *J*=11.7, 5.0 Hz)	41.7
6	2.57 (dt)	25.9	.58 (dt, *J*=21.0, 5.0 Hz)	25.9
7	7.24 (br t, *J*=2.5 Hz)	157.6	.27 (dd , *J*=5.0, 2.6 Hz)	157.2
8		140.4		140.5
9		76.6		77.1
10		42.7		41.4
11	9.73(d, *J*=1.5 Hz)	202.3	9.73 (d, *J*=2.0 Hz)	202.0
12	9.41 (s)	192.7	9.41 (s)	192.5
13	1.09(s)	22.1	0.99 (s)	22.0
14	1.41 (s)	31.1	1.09 (s)	33.0
15	0.95 (s)	17.1	0.95 (s)	17.0
9-OH	4.00 (br s)			4.10 (br s)

4.1.5. Polygodial (30)

The compound was eluted from silica gel column with *n*-hexane-EtOAc mixture in a yield of 175 mg as white crystals. It had an R_f value of 0.56 with *n*-hexane-EtOAc (9:1) as a developing solvent system and its spot afforded bluish-purple coloration with *p*-anisaldehyde-sulphuric acid mixture followed by heating at 100 ^{0}C, indicating that it could be a terpenoid. The electron impact mass spectrum (EIMS, 70 eV) of the compound showed a molecular ion peak at *m/z* 235 $[M+H]^{+}$ which corresponded to $C_{15}H_{22}O_2$ molecular formula. Its proton NMR (Table 4.5) revealed the presence of two aldehyde groups (δ 9.67, d, *J*=1.3 Hz, 11-CHO and 9.49, s, 12-CHO) and confirmed by ^{13}C NMR spectrum peaks at δ 201.4 (C-11) and 192.0 (C-12), respectively. The other significant peaks observed in the ^{1}H NMR spectrum were trisubstituted conjugated olefinic bond at δ 7.06 (d, *J* =4.4 Hz, H-7) and a singlet at δ 2.56 (s, H-9). These together with three tertiary methyl groups at δ 1.03 (s, 15-Me), 1.17 (s, 13-Me) and 1.34 (s, 14-Me) revealed that the compound is a drimane-type sesquiterpene having aldehyde groups at C-11 and C-12 (Kubo *et al.*, 1983). Within the range of measuring accuracy, the chemical shifts and coupling constants of the protons in compound **30** coincide with those of polygodial previously isolated from *W. ugandensis* (Kioy *et al.*, 1990) and the ^{13}C NMR spectral data of compound **30** were in good agreement

with those reported for the latter. Therefore on the basis of spectroscopic data, the structure of **30** was determined to be polygodial.



30

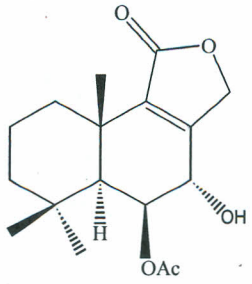
Table 4.5.^{1}H and ^{13}C NMR data for polygodial (30)

Data on isolated compound			Literature Data (Ying *et al.*, 1995)	
C	^{1}H NMR (*J* in Hz)	^{13}C NMR	^{1}H NMR(*J* in Hz)	^{13}C NMR
1	1.80 (m, H_{ax}), 1.20-1.51 (m, H_{eq})	41.7	1.82 (m, H_{ax}), 1.28 (m, H_{eq})	37.3
2	1.20-1.51 (2H, m)	16.9	1.79 (m), 1.30 (m)	18.5
3	1.20-1.51 (2H, m)	42.5	1.35 (m), 1.46 (m)	42.0
4		35.0		32.8
5	1.76 (m)	47.2	1.63 (m)	44.4
6	2.58 (m), 2.57 (m)	32.1	2.30- 2.55 (m, 2H)	26.00
7	7.05 (d, *J*= 4.4 Hz)	158.0	7.13 (dt, *J*=4.9Hz)	153.5
8		137.2		137.4
9	2.56 (br s)	66.6	2.84 (m)	60.4
10		39.6		37.8
11	9.67 (d, *J*=1.30Hz) s)	201.4	9.54 (d, *J*= 4.4 Hz)	202.4
12	9.44 (s)	192.0	9.46 (s)	192.8
13	1.17 (s)	21.7	0.95 (s)	21.5
14	1.34 (s)	22.7	0.93 (s)	21.9
15	1.03 (s)	31.6	0.90 (s)	32.7

4.1.6 Ugandensolide (35)

This compound was obtained as white needle shaped crystals (*n*–hexane-EtOAc), with melting point 212^{0}C and $[\alpha]_D$ +25 ^{0} (c=0.1, MeOH). The mass spectrum showed a molecular ion peak at *m/z* 308 indicating the formula $C_{17}H_{24}O_5$. The IR spectrum determined as KBr pellet showed absorption bands at 3451 and 1735cm^{-1} indicative of OH and acetate groups. The other significant peaks at 1750 and 1760 (shoulder of slightly weaker intensity) cm^{-1} were attributed to α, β-unsaturated lactone (Brooks and Draffan, 1969). In the UV region, the compound showed an absorption band at λ_{max} 214 nm and this value in conjunction with the IR data further suggested the existence of an α, β-unsaturated γ-lactone function (Fukuyama *et al.*, 1983; Kioy *et al.*, 1990). The ^{1}H NMR spectrum (Appendix 6.1) showed the presence of an acetate was indicated by a peak at δ_H 2.03 and this was confirmed by ^{13}C NMR (spectrum Appendix 6.3) resonances at δ_C 171.0 (-O-C-CH$_3$) and 23.1 (-O-C-CH$_3$). A broad singlet at δ_H 5.40 attributed to a proton on an oxygenated carbon was coupled with *J*=5.0 Hz to proton at δ_H 4.22 (H-7) which proved that the OH group is secondary. This was supported by ^{13}C NMR resonance at δ_C 73.8 and further confirmed by DEPT experiment. Like in the case of cinnamodial (**12**), three tertiary methyl groups were apparent from signals at δ_H 1.02, 1.05, and 1.47. The presence of a broadened singlet peak at δ_H 5.40 was assigned to the proton on carbon bearing the acetate (-CH-OAc). In fact, comparison of the compounds ^{1}H and ^{13}C NMR data with those reported in literature (Kioy *et al.*, 1990) suggested that the acetate is positioned at C-6. Similarly, two doublets at δ_H 4.94 (1 H) and δ_H4. 70 (1 H), which strongly coupled with coupling constant *J* = 17.0 Hz was assigned to the lactone methylene protons (O-CH$_2$-) evidently in a dissymmetric environment due to the OH group effect (Brooks and Draffan, 1969; Opiyo *et al.*, 2011). This was confirmed by the ^{13}C NMR and DEPT spectra which displayed a peak at δ_C 66.9. From the above spectroscopic data, compound **35** was proved to have neither olefinic proton nor the dialdehyde groups but instead was assumed to have a tetrasubstituted double bond. The presence of tetrasubstituted double bond in the molecule was confirmed by ^{13}C NMR peaks at δ_C 137.8 and 154.5 representing C-9 and C-8 respectively. The assignment of the acetate group in β-position was indicated by small coupling constant between H-5α

and H-6α protons, which are consistent with the dihedral angle of approximately 60^0 (Fukuyama *et al.*, 1983). Similarly the OH group at C-7 was assigned as α on basis of coupling constant between H-6α and H-7β. On this account, ugandensolide was suggested to have a trans- 6α, 7β-stereochemistry as already published in the literature (Books and Draffan, 1969). Thus on the basis of physical and spectroscopic data, compound **35** was identified as ugandensolide.



35

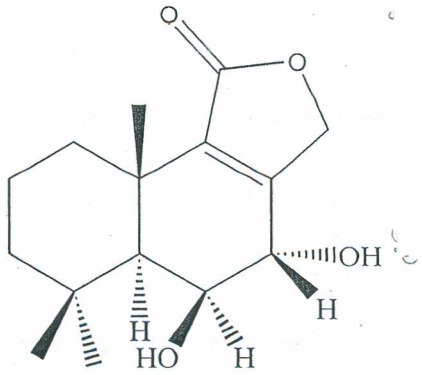
Table 4.6: ^{1}HNMR and ^{13}CNMR data for ugandensolide (35)

Data on isolated compound			Literature Data (Kioy *et al.*, 1990)	
C	^{1}H NMR (*J* in Hz)	C NMR	^{1}H NMR(*J* in Hz)	C NMR
1	2.55 (m), 1.20-190 (m)	33.1		33.1
2	1.20-1.90 (m, 2H)	20.7		20.8
3	1.20-190 (m, 2H)	43.0		43.1
4		35.3		35.4
5	1.63 (d, *J*=1.5 Hz)	49.2	1.61 (d, J=1.5 Hz)	49.3
6	3,60 (br s)	69.7	5.36 (dd, J= (5.5, 1.5Hz)	69.8
7	4.22 (d, *J*=5.0 Hz)	73.8	4.21 (d d, J= 5.5Hz)	73.8
8		154.5		154.5
9		137.8		137.9
10		36.3		36.5
11		171.9		172.1
12	.94 (d, *J*=17.0 Hz), 4.70 (d, *J*=17.0 Hz)	66.9	4.91 (d, *J*=17.2 Hz), 4.65 (d, *J*=17.2 Hz)	66.1
13	1.02 9 (s)	33.3	1.02 (s)	33.4
14	1.47 (s)	21.4	1.47 (s)	21.4
15	1.05 (s)	18.3	1.05 (s)	18.4
6-OAc	2.03(s)	171.0, 23.1	2.09 (s)	70.9, 23.1

4.1.7 Deacetylugandensolide (36)

It crystallized out as white needle shaped crystals from CH_2Cl_2-MeOH (97:3) with mp 262-264^{0}C. It exhibited characteristic absorption bands in IR spectrum for hydroxyl (3340cm^{-1}) and lactone (1750 and 1740cm^{-1}). The electron impact mass spectrum of compound **36** displayed a molecular ion peak at *m/z* 266 consistent with $C_{15}H_{22}O_4$ molecular formula. The ^{13}CNMR spectrum (Appendix 7.0) showed a total of fifteen carbon peaks, sorted out into three methyls, three methines, four methylenes and five quaternary carbons by DEPT spectrum. Similarly as in compound **35**, the UV absorption at λ_{max} 220

nm together with the IR data indicated above suggested the presence of an α, β- unsaturated γ-Lactone function. While differing substantially from ugandensolide **(35)** in melting point and specific rotation, the ^{1}H NMR data of the two compounds showed similarity with a major difference being the absence of an acetate group in compound **36**. In fact, the molecular weight of compound **36** is 43 amu less than that of compound **35**, thus further confirming lack of the acetyl group in the compound. In addition, the IR peaks at 1385 and 1360 cm^{-1} suggested the presence of gem-dimethyls and was confirmed by the ^{1}H NMR peaks at δ_H 1.19 and 1.49 as usual assigned to C-13 and C-14 methyls, respectively. Also, in the ^{1}H NMR spectrum, another methyl group appeared as a singlet at δ 1.03 and was assigned to 15-Me group. The close relationship of compound **36** and **35** was established by two broadened singlets at δ_H 4.93 and 4.65 attributed to the protons on carbons bearing the hydroxyl groups (-CH-OH). Also two doublets at δ_H 5.17 (IH) and 4.88 (IH) which strongly coupled with coupling constant *J*= 17.0 Hz were assigned to the lactone methylene protons (-O-CH_2-), evidently in dissymmetric environment (Brooks and Draffan, 1969; Kioy *et al*, 1990). Of significance was absence of any resonance attributed to the aldehyde groups and olefinic proton analogous to those appearing at δ_H 9.65, 9.45 and 7.46, respectively in muzigadial **(14).** On this rationale, the compound was also assumed to have a tetrasubstituted double bond as in the case of ugandensolide **(35).** This was confirmed by the presence of five double bond equivalents as implied by the formula $C_{15}H_{22}O_4$, further confirming that compound **35** is an acetate derivative of compound **36.** The assignment of the C-6 hydroxyl group as β was from the weak coupling constant of approximately *J*=2.8 Hz between H-5α and H-6α protons which is consistent with dihedral angle of about 60^0. The OH group at C-7 was deduced to be α orientation on the basis of spin–spin coupling between H-6α and H-7β which signified the *trans* 6β, 7α-stereochemistry in compound **36**. This agreed with the findings earlier reported (Kioy *et al.,* 1990). Therefore on the basis of physical and spectroscopic data and also comparison with literature data, compound **36** was concluded to be deacetylugandensolide.



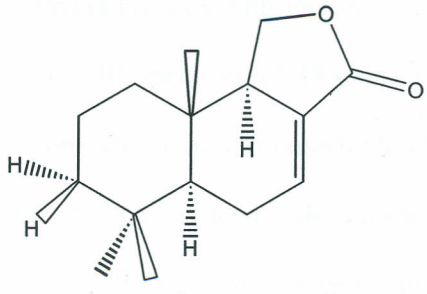
36

Table 4.7: ^{1}HNMR and ^{13}C NMR data for deacetylugandensolide (36)

Data on isolated compound			Literature Data (Kioy *et al.*, 1990)	
C	^{1}H NMR (*J* in Hz)	^{13}C NMR	^{1}H NMR(*J* in Hz)	^{13}C NMR
1	1.24-2.40 (m, 2H)	42.2		42.4
2	1.24-2.40 (m, 2H)	17.7		17.8
3	1.24-2.40 (m, 2H)	34.5		35.9
4		35.5		34.7
5	1.81 (d, *J*=2.2 Hz)	48.9	1.86 (d, *J*=1.2 Hz)	49.2
6	4.93 (d, *J*=2.8 Hz)	70.1	4.87 (br s)	71.0
7	4.65 (br s)	67.6	4.70 (br s)	68.8
8		155.9		156.9
9		135.6		135.7
10		35.6		33.6
11		171.5		172.6
12	5.17 (d, *J*=17.0 Hz), 4.88 (d, *J*=17.0 Hz)	69.0	5.20 (d, *J*=17.2 Hz), 4.79 (d, *J*=17.2 hz)	69.3
13	1.03 (s)	19.7	1.15 (s)	20.0
14	1.19 (s)	21.4	1.47 (s)	21.4
15	1.49 (s)	22.5	1.94 (s)	27.7

4.1.8. Cinnamolide (37)

Compound **37** was obtained as prism shaped crystals from n-hexane-ethylacetate mixture (4:1) with R_f value of 0.40. The compound was spotted on TLC plate after spraying with anisaldehyde-sulphuric acid mixture followed by heating at about 100 ^{0}C for two minutes. The compound showed a molecular ion peak at m/z 234 in an electron impact mass spectrum measurement corresponding to $C_{15}H_{22}O_2$. It had melting point of 126-127 ^{0}C and $[\alpha]_D$ -28 ($CHCl_3$, c=3.5) (Lit. -29, Kioy *et al.*, 1990). The compound exhibited characteristic absorption band in IR for lactone (1761 cm^{-1}) and double bond (1650 cm^{-1}). The UV absorption at λ_{max} 215 nm together with IR data indicated above suggested the presence of α, β unsaturated γ lactone. The ^{13}C NMR spectrum (appendix 8.2) displayed fifteen carbon signals assigned to three methyls, five methylenes, three methines and four quarternary carbons. 1HNMR (Table 4.8) had significant peaks at δ_H 0.88, and 0.90 assigned to the methyl protons at C-13 and C-14 respectively while the singlet at 0.92 assigned the C-15-Me group. The two broad singlets at δ_H 4.38 and 4.0 were assigned to the lactone methylene protons (-O-CH_2-). There were however significant peaks observed in the 1HNMR spectrum attributed to the trisubstituted conjugated olefinic peak at δ_H 6.88 and a broad singlet at δ_H 2.78 for H-9. On basis of physical and spectroscopic data and also comparison with literature data, compound **37** was concluded to be cinnamolide



37

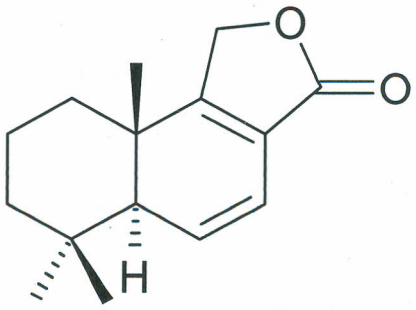
Table 4.8. ^{1}H NMR and ^{13}C NMR data for cinnamolide (37)

Data on isolated compound			Literature Data (Kioy *et al.*, 1990)	
C	^{1}H NMR (*J* in Hz)	^{13}C NMR	^{1}H NMR (*J* in Hz)	^{13}C NMR
1	1.61 (m), 1.25 (m)	42.1		41.6
2	1.20-1.60 (m, 2H)	18.3		17.9
3	1.20-1.60 (m, 2H)	32.8		31.3
4		33.0		32.7
5	1.72 (m)	50.8	1.38 (m)	50.5
6	2.50 (m), 2.20 (m)	24.9	2.40 (m), 2.10 (m)	25.3
7	6.88 (q *J*=5.50, 2.97Hz)	136.3	6.86 (m)	135.6
8		127.2		126.9
9	2.78 (br s)	49.7	2.80 (m)	49.2
10		39.5		37.6
11	4.38 (br s), 4.00 (brs))	67.2	38 (t, *J*=9.2 Hz), 4.04 (t, *J*=9.2 Hz)	66.8
12		170.2		169.6
13	0.88 (s)	33.1	0.81 (s)	27.3
14	0.90 (s)	13.4	0.92 (s)	15.8
15	0.92 (s)	21.3	0.94 (s)	21.9

4.1.9 Bemadienolide (45)

Compound **45** was obtained as colourless crystals with melting point 123–125^{0}C EIMS $[M]^{+}$ m/z 232 corresponding to the formula $C_{15}H_{20}O_2$. IR spectrum of **45** exhibited strong absorption bands at 1751 cm^{-1} and 1642 cm^{-1}, suggesting the presence of an α, β-unsaturated lactone, while the band at 1642 cm^{-1} indicated the presence of two olefinic groups. Indeed carbon resonances of δ_C 122.5, and the carbonyl carbon resonance at δ_C 171.8 ppm in ^{13}C NMR spectrum (Appendix 9.2) confirmed the presence of the α, β-unsaturated lactone. Three methyl signals were observed in ^{1}H NMR spectrum of which the signals at δ_H 1.04 was assigned to CH_3–15, the singlet at δ_H 1.02 to CH_3–14 and the singlet at δ_H 1.00 to CH_3–13. On the basis of the given information and comparison with data from literature (Table 4.9)

compound **45** was concluded to be bemadienolide, a compound previously isolated from the stem bark of *W. ugandensis* (Opiyo *et al.*, 2011). This is however the first report from *W. stuhlmannii.*



45

Table 4.9. ^{1}H NMR and ^{13}C NMR data for bemadienolide (45)

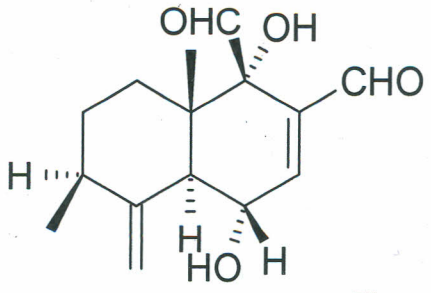
Data for isolated compound			Data from Literature (Opiyo et al., 2011)	
C	^{1}H NMR (J in Hz)	^{13}C NMR	^{1}H NMR (J in Hz)	^{13}C NMR
1	1.20-1.70 (m, 2H)	33.7		33.5
2	1.20-1.70 (m, 2H)	18.0		17.9
3	1.20-1.70 (m, 2H)	40.6		40.5
4		32.8		32.7
5	2.22 (br s)	52.5	2.22 (m)	52.3
6	6.08 (d, *J*=10.0 Hz))	117.5	6.01 (d, J=10.1 Hz)	117.6
7	6.31(d, J=11.0 Hz)	131.7	6.32 (d, J=10.0 Hz)	131.7
8		122.5		122.3
9		171.7		171.7
10		36.9		36.8
11	4.82 (dd, *J*=17.1 Hz)	67.7	4.38 (d, *J*=17.2Hz) 4.77 (d, *J*=17.1Hz)	67.7
12		170.2		170.2
13	1.00 (s)	15.1	0.99 (s)	14.7
14	1.02 (s)	22.6	1.01(s)	22.5
15	1.04 (s)	32.4	1.04 (s)	32.3

4.1.10 6α, 9α-Dihydroxy-4(13), 7-coloratadiene-11,12-dial (6α-hydroxymuzigadial) (52)

The compound was isolated as white crystals from *n*-hexane-ethyl acetate mixture. It gave R_f. value of 0.29 using *n*-hexane-ethyl acetate (7:3) after spraying the TLC plate with anisaldehyde-sulphuric acid mixture followed by heating at approximately 100^0C for 2 minutes. It showed significant IR absorption peaks at 3384, 1722, 1666 and 1642 cm^{-1} corresponding to hydroxyl, saturated aldehyde, conjugated aldehyde and double bond, respectively. The compound showed molecular ion peak at *m/z* 264 $[M]^+$ which is consistent with $C_{15}H_{20}O_4$ molecular formula. The ^{13}C NMR spectrum (Appendix 10.2) displayed a total of fifteen distinct carbon signals representing two methyls, three methylenes

including a terminal double bond, six methines including three carbons containing an oxygen atom each and four quarternary carbons out of which one carbon is holding a tertiary hydroxyl group. Comparing the EIMS molecular ions of compound **52** and that of muzigadial **(14)** it was observed that compound **52** had a molecular weight which was 16 amu higher than that of muzigadial **(14).** While differing substantially from muzigadial (**14**) in physical properties including melting point and specific rotation, the ^{1}H NMR data of the two compounds showed close similarity with a notable difference being the presence of a peak at δ_H 4.66 (d, J=11.0, 2.6 Hz) representing a proton on carbon bearing a hydroxyl group (-CH-OH). A comparative analysis of the compound ^{1}H and ^{13}C NMR data with those reported in literature (Wube *et al.,* 2005) suggested that the hydroxyl group is positioned at C-6. Like in the case of compound **14,** the ^{1}H NMR of compound **52** (Appendix 10.1) showed peaks attributed to secondary methyl group (δ_H 1.11, d, J = 6.5 Hz, 3-Me), dialdehydes (δ_H 9.63, 11-CHO and δ_H 9.47 12-CHO) a trisubstituted olefinic bond (δ_H 7.10, d, J=2.5 Hz, H-7) and hydroxyl proton (4.29, d, br s, 9-OH).

On the above rationale, the A ring substitution in **52** was assumed to be similar to that of muzigadial (**14**), leaving the second hydroxyl group to be positioned at C-6 and confirmed by HMBC correlation between H-6 and C-7 ((δ_C 154.4). The assignment of 6-OH as α was confirmed by spin-spin coupling between H-5α and H-6β which afforded coupling constant J=11.0 Hz which is in agreement with value reported for the compound by Wube *et al.*, (2005). Therefore on the basis of spectroscopic data and in comparison with data already reported previously on compound **52** it was established as 6α-hydroxymuzigadial.



52

Table 4.10. ^{1}H NMR and ^{13}C NMR data for 6α-hydroxymuzigadial (52)

Data on isolated compound			Data from Literature (Wube *et al*, 2005)	
C	^{1}HNMR (*J* in Hz)	^{13}C NMR	^{1}HNMR (*J* in Hz)	^{13}C NMR
1	1.02 (m), 2.02 (m)	31.6	2.05 (m) , 1.02 (d, J=13.5, 4.0 Hz)	31.7
2	1.02-1.20 (m), 1.92-2.10 (m)	31.6	1.12 (m), 1.73 (m)	31.8
3	2.0 (m), 1.02-1.20 (m)	38.6	2.00 (m)	38.8
4		148.7		149.1
5	2.65 (d, *J*=10.5 Hz)	50.1	2.65 (d, *J*=10.0Hz)	50.4
6	4.66 (dd, *J*=11.0, 2.6 Hz)	65.9	4.70 (dd, *J*= 10.0, 2.5 Hz)	66.1
7	7.10 (d, *J*=2.5 Hz)	154.4	7.10 (d, *J*=2.5 Hz)	153.7
8		139.0		139.3
9		77.0		77.6
10		44.1		44.1
11	9.63 (s)	200.6	9.65 (s)	200.5
12	9.47 (s)	192.7	9.50 (s)	192.6
13	5.12 (s), 5.04 (s)	106.9	5.13 (s)	106.7
14	1.11 (*d*, *J*=6.5 Hz)	18.2	1.11 (d, *J*=6.5 Hz)	18.2
15	0.94 (s)	15.8	0.96 (s)	15.8
9-OH	4.29 (br s)		4.07 (br s)	
6-OH	2.70 (br s)		1.59 (br s)	

4.2. BIOLOGICAL ACTIVITIES OF THE CRUDE EXTRACTS AND PURE ISOLATES OF *WARBURGIA STUHLMANNII ROOT BARK*

4.2.1 ANTI-PLASMODIAL TESTS

The crude extracts of *Warburgia stuhlmannii* (root bark) were tested for antiplasmodial activity against *Plasmodium falciparum*. The strains were the chloroquine sensitive (D10) and the chloroquine resistant (W2) strains. Artesunate was used as positive control. The ethyl acetate and methanol extracts of the root bark of *W. stuhlmannii* showed antiplasmodial activity against the chloroquine sensitive (D10) and chloroquine resistant (W2) strains of *P. falciparum* with IC_{50} values of 32.5µg/ml and 38.4µg/ml respectively for the ethyl acetate extract while the methanol extract had values of 80.5µg/ml and 95.3µg/ml respectively (Table 4.11). This explains the traditional uses of the plant to treat malaria at the Kenyan Coast. (Muthaura *et al.*, 2007).

Among the isolated compounds, mukaadial **(11)** was the most potent against both **D10** and **W2** strains of *Plasmodium falciparum* with IC_{50} values of 4.3µM and 5.8 µM respectively which is in agreement with the results of Wube *et al.*, (2010), given as 6.4 µM for choroquine sensitive and 7.9 µM for chloroquine resistant strains of *P. falciparum.* Muzigadial **(14)**, was more active against the **D10** strain 5.6 µM than against **W2** strain of *P. falciparum*, IC_{50}, 16.4µM. This agrees well with results of Grace *et al.*, (2010); 0.31µg/ml and 1.18µg/ml respectively for muzigadial **(14).** Ugandensidial **(12)** was more active against the **W2** strain than the **D10** strain, with IC_{50} values of 8.2µM and 30.2µM respectively, similar to the results of Wube *et al.*, (2010), given as 7.6 µM for chloroquine resistant and 29.8 µM for chloroquine sensitive strains of *P falciparum*. The coloratane sesquiterpene lactone, 6α, 9α-dihydroxy 4(13) coloratadiene -11, 12-dial **(52)** was more active (IC_{50} 12.2µM) against the **W2** strain than the **D10** strain IC_{50} 29.8µM which compares well with the results of Wube *et al.*, (2010), 11.0 µM for the chloroquine resistant strain and 32.9 µM for the chloroquine sensitive strains. The drimane sesquiterpene lactones; ugandensolide **(35)** and bemadienolide **(45),** showed low activity against both the **D10** and **W2** strains of *Plasmodium falciparum* (Table 4.11). The activity of some of these compounds, against strains of *Plasmodium falciparum* could be enhanced by the hydroxyl group

at position C-9. (Wube *et al.*, 2010; Grace *et al.*, 2010). Tendency to increase the antiplasmodial activity increases with the number of hydroxyl groups in the molecule for the compounds with lactone moiety (Wube *et al.*, 2010). Sesquiterpenes without hydroxyl groups shows low activity against the strains of *Plasmodium falciparum* (Wube *et al.*, 2010)

Table 4.11; Anti-plasmodial activity of extracts and compounds isolated from root bark of *Warburgia stuhlmannii.*

IC_{50} for extracts (µg/ml)		
Tested substance	D10	W2
EtOAc extract	32.5	38.4
Methanol extract	80.5	95.3
IC_{50} for isolated compounds (µM)		
Tested compound	D10	W2
Mukaadial **(11)**	4.3±1.1	5.8±1.6
Ugandensidial **(12)**	30.2±2.2	8.2±1.8
Muzigadial **(14)**	5.6±1.6	16.4±1.8
Warburganal **(29)**	6.4±1.3	18.6±1.4
Polygodial **(30)**	>102.5	>102.5
Ugandensolide **(35)**	>98.4	>98.4
Deacetyl ugandensolide **(36)**	63.5±1.5	50.6±1.3
Cinnamolide **(37)**	42.5±1.5	22.3±1.2
Bemadienolide **(45)**	>92.3	>92.3
6α,9α-dihydroxy-4(13),7-coloratadien-11,12-dial **(52)**	29.8±1.2	12.2 ± 1.4
Artesunate	0.008	0.005

CHAPTER FIVE

5.0 SUMMARY, CONCLUSIONS AND RECOMMENDATIONS

5.1 SUMMARY

1. Ethylacetate and methanol extracts of *W. stuhlmannii* root bark were obtained.

2. The ethylacetate and methanol extracts were moderately active against the chloroquine sensitive (D10) and the chloroquine resistant W2 strain of *Plasmodium falciparum*.

3. Ten compounds of farnesane-type sesquiterpenes which included, mukaadial **(11),** ugandensidial **(12),** muzigadial **(14),** warburganal **(29)**, polygodial **(30),** ugandensolide **(35),** deacetylugandensolide **(36)** cinnamolide **(37),** bemadienolide **(45)**, and 6α,9α-dihydroxy-4(13),7,coloratadiene-11,12-dial **(52)** were isolated. Compound **(52)** is being reported for the first time from this species while the rest of the compounds were previously isolated from the stem bark of the plant.

4. The pure isolates were found to be moderately active against the D10 and W2 strains of *Plasmodium falciparum*. Mukaadial **(11)** had the highest antiplasmodial activity against both D10 and W2 strains of *P. falciparum*, with IC_{50} values of 4.3 μM and 5.8 μM, respectively, while muzigadial **(13)** though very effective on D10 strain (IC_{50} 5.6 μM) was less effective against W2 strain (IC_{50} 16.4 μM). Polygodial **(30)** had the lowest antiplasmodial activity against both D10 and W2 strains with IC_{50} values of >102.5.

5.2 CONCLUSIONS.

The extracts and pure isolates were tested for antiplasmodial activity. The following conclusions were drawn from this study;

(1) Ten compounds were isolated and characterised from the root bark of *W. stuhlmannii*.

(2). Compounds isolated were of farnesane-type sesquiterpenes. One of the sesquiterpenes Compound **52** is being reported for the first time from this species.

(3).The ethyl acetate extracts of *W. stuhlmannii* root bark had higher activity than the methanol extracts against the chloroquine sensitive (D10) and chloroquine resistant (W2) strains of *P. falciparum*.

(4). Antiplasmodial activity of the sesquiterpenes from this plant were tested and found that, all were active against the chloroquine sensitive (D10) and chloroquine resistant (W2) strains of *P. falciparum.* The sesquiterpenes with hydroxyl group at C-9 were more potent while an additional hydroxyl in the molecule of sesquiterpenes with lactone moiety increased the potency.

5.2 RECOMMENDATIONS

1. Traditional healers are encouraged to use the root bark of *W. stuhlmannii* in management of malaria at pre-determined dosages.

2. The active pure isolates may be verified by *in vivo* experiments, hence be used directly as antimalarial drugs or as templates in development of new, more effective and cheaper antimalarial drugs.

5.4 SUGGESTION FOR FUTURE STUDIES

1. Other strains of *P. falciparum* should be used to test the antiplasmodial activity of compounds isolated in this study.

2. Other methods of extraction using different solvents should be employed to see whether more compounds can be isolated from the root bark of *Warburgia stuhlmannii.*

3. Synergism effect of the compounds to be studied

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