# PHYTOCHEMICAL INVESTIGATION OF UNDERGROUND PART OF *Ajuga remota* Benth. FOR ANTI-PLASMODIAL, LARVICIDAL AND ANTI-OXIDANT ACTIVITIES


BY

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## ABSTRACT

The fight against malaria has been challenged by malaria parasites and vectors becoming resistant to anti-malaria drugs and insecticides respectively. Malaria accompanying complications like cerebral and pulmonary oedema, have been linked to oxidative stress. This necessitates the continued search for alternative effective, safe and affordable anti-malarial agents that can also act as anti-oxidants. The purpose of this study was to search for compounds with anti-plasmodial, larvicidal and anti-oxidant activities from *Ajuga remota*, an erect rhizomatous pubescent herb found growing in East Africa, ethnomedically used to manage malaria. The underground part of *A. remota* was sampled, dried in the shade, milled into fine powder and extracted with organic solvents of varying polarities (*n*-hexane, EtOAc, and MeOH). Extracts were subjected to antiplasmodial, larvicidal and antioxidant assays. Chromatographic fractionation of extracts produced twelve compounds which were characterized using spectroscopic techniques ($^{1}$H NMR, $^{13}$C NMR, UV, IR and MS). Seven compounds, betulinic acid **(40),** β-sitosterol **(69),** 7β-hydroxylup-5,20(29)-diene-3-one **(124),** arjungenin **(125),** quercetin 3-*O*-β-galactoside **(127),** quercetin 3-*O*-β-(6-*O*-α-rhamnosyl)galactoside **(128),** and myricetin 3-*O*-α-rhmnoside **(129)** are reported for the first time in this plant. EtOAc extract was the most potent against *Plasmodium falciparum* with $IC_{50}$ values 15.4 and 19.4 µg/ml against chloroquine-resistant (W2) and chloroquine-sensitive (D6) strains respectively. The extract was the most active against 2$^{nd}$ instar larvae of *Aedes aegpti* with $LC_{50}$ value 5.7 µg/ml at 24 hours. Compound **40** was the most active against W2 and D6 strains of *P. falciparum* with $IC_{50}$ values 9.4 and 10.0 µM, respectively and 2$^{nd}$ instar larvae of *A. aegpti* with $LC_{50}$ value 4.8 µM. Preliminary assay for radical scavenging activity using DPPH free radical as spray reagent on TLC plates indicated radical scavenging compounds in MeOH extract. UV-VIS method indicated the extract had radical scavenging activity with an $IC_{50}$ value of 10.4 µg/ml. Myricetin **(113)** with $IC_{50}$ value 17.6 µM was the most active antioxidant. This study has justified the use of *A. remota* for management of malaria, exhibited potential use of *A. remota* for small scale control of mosquitoes from the larval stage in Kenyan rural communities where malaria is endemic and confirmed potential use of phenolic compounds of the underground part of *A. remota* as antioxidants. Proper validation of the metabolites *in vivo* could lead to their direct use or templates for antimalarials, larvicides and antioxidant agents which may help in management of malaria burden to economies of tropical and sub-tropical countries where malaria is endemic.

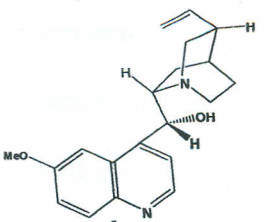
# CHAPTER ONE: INTRODUCTION

## 1.1 Background Information

Despite more than a century of efforts to eradicate and control malaria, it still remains a major risk to public health and economies of countries in the tropical and subtropical regions of the world. About 300 to 660 million clinical attacks of malaria occur globally every year (Geissbühler *et al.*, 2007) and result in over a million deaths (Hatzel *et al.*, 2007), with over 80% of these deaths occurring in Africa (Geissbühler *et al.*, 2007). In Kenya, about 4.6 million people are at risk of contracting malaria (WHO, 2011). In 2010 there were about 900000 confirmed *Plasmodium falciparum* malaria cases, about 200000 inpatient cases and about 26000 deaths were reported (WHO, 2011). This is occurring mainly because the parasite frequently develops resistance to current drugs in use. This necessitates continued search for alternative drugs to commonly used anti-malarial drugs, which are now resisted by *P. falciparum*, the most deadly malaria parasite (Muthaura *et al.*, 2007a). The second obstacle is limited availability coupled with higher cost and greater toxicity of alternative drugs (Saidu *et al.*, 2000).

Plants have been used over the years to alleviate suffering from diseases, and are still used for the same purpose. About 80% of the world population, primarily those in rural areas of developing countries, depend on traditional medicine for their primary health care needs (Gurib-Fakim, 2006). The use has been justified by isolation of several compounds from plants that are active against the causative agents of the disease to different degrees of efficacy. Some of these compounds have been developed into top therapeutic drugs. The significance of plant isolates in medicine is evident from the fact

that 75% of drugs used against infectious diseases are either plant natural products or their derivatives (McChesney *et al.*, 2007). Anti-malarial drugs of plant origin have been known for decades. The continuing clinical use of quinine **(1)** isolated from *Cinchona succiriba* and artemisinin **(2)** from Chinese herb *Artemisia annua*, demonstrates the supremacy of plant isolates in treatment of malaria (Bickii *et al.*, 2007).

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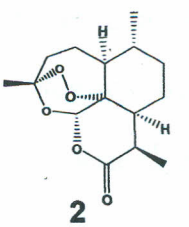


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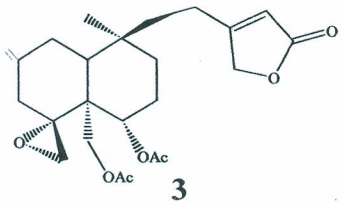
Extracts from species belonging to genus *Ajuga* have been used in folk medicine as antimalarial agents (Kokwaro, 2009). The focus of this study was upon *A. remota*, an erect rhizomatous pubescent herb found growing in East Africa. It belongs to the family Labiatae. Its leaves are most frequently prescribed for malaria treatment by herbalists (Kuria *et al.*, 2001). The whole plant is used for treatment of malaria, among the Kikuyu, Mbere and Embu (Gachathi, 1989; Kareru *et al.*, 2007). Arjugarin I **(3)** isolated from the aerial part of *A. remota* and ergosterol-5,8-endoperoxide **(4)** showed anti-plasmodial activity (Kuria *et al.*, 2002). However, the antiplasmodial principles from the underground part of *A. remota* are not known. Finding clinically useful antiplasmodial compounds in underground part extracts of *A. remota* could provide lead compounds or/and templates for development of therapeutic drugs for malaria treatment leading to significant medical and economic benefits.

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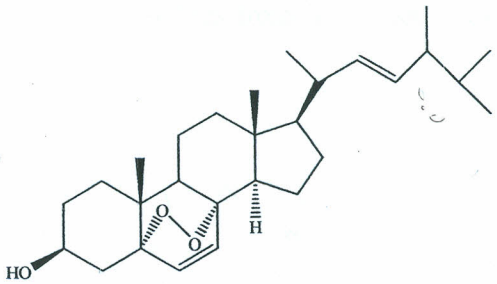


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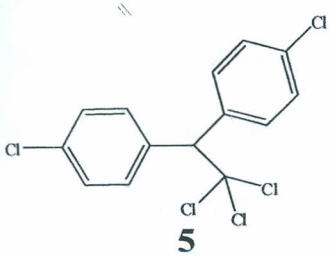
Efforts to overcome malaria have also been hindered by resistance of the vector *Anopheles* mosquito to insecticides (Bilia, 2006). There is also environmental concern on the use of synthetic insecticides, in particular halogenated compounds such as *p,p'*-dichlorodiphenyltrichloroethane (DDT) **(5)**, which has resulted in high prevalence of malaria in Africa (Gikonyo *et al.*, 1988; Mwangi and Mukiama, 1988). Treatment of malaria in Kenya has become unaffordable due to high poverty levels (Guyatt, *et al.*, 2002). Aiming for the discovery of cost effective alternatives for the control of *Anopheles* mosquito, plant extracts and pure compounds have been tested for larvicidal activities (Gikonyo *et al.*, 1988). Petroleum-ether extract of aerial part of *A. remota* was active against the larvae of *Anopheles stephensi* while carbon-tetra-chloride extract was active against the larvae of *Culex quinquefasciatus* (Sharma *et al.*, 2004). The active metabolites from these extracts are not known. EtOAc and MeOH extracts from the underground part of *A. remota* and isolated compound **(4)** from EtOAc exhibited good larvicidal activity, 6'-*O*-rhamnosylharpagide **(6)** and 6-*O*-xylosyharpagoside-B **(7)** isolated from the MeOH extract exhibited low activity against second instar *Aedes aegypti* larvae (Manguro *et al.*, 2011). Moreover, the larvicidal activity of isolates from MeOH extract were lower than the extract raising the possibility of synergistic activity of compounds in the extract or presence of other more active compounds that were not isolated in the study. Moreover,

larvicidal activity of *n*-hexane extract and metabolites from it has not been established. (Manguro *et al.*, 2006)

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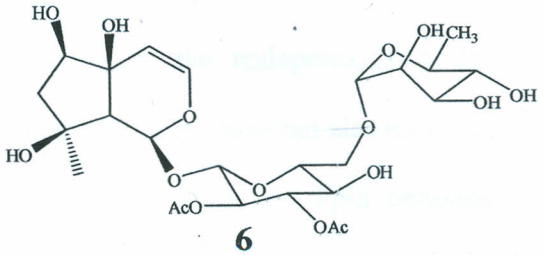


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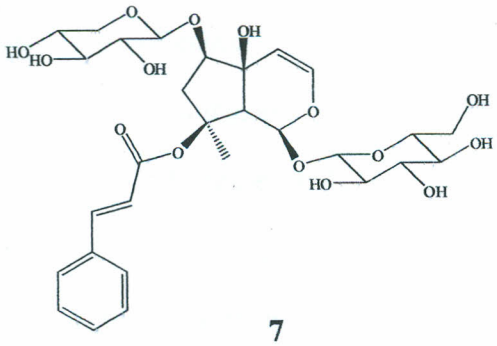


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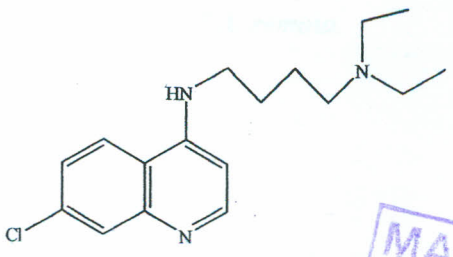
Malaria complications such as cerebral and pulmonary oedema, and poor eyesight have been linked to oxidative stress which is an imbalance between pro-oxidants and anti-oxidants (Taoufiq *et al.*, 2006). Reactive oxygen species (ROS) are important pro-oxidants in malaria. They are produced during malaria infection *via* two mechanisms. The first mechanism involves the production of ROS from haemoglobin degradation by the intracellular parasite. In this case $Fe^{2+}$ is oxidized to $Fe^{3+}$ after heme is separated from globin and the electron produced during this process reacts with molecular oxygen to form ROS (Foley and Tiley, 1998). The second mechanism involves the host's immune system, which is activated and as a response it produces cytokines, tumor necrosis factor-alpha (TNF-α) and interferon-γ (IFN-γ) which increase respiratory burst on phagocytes (Dockrell and Playfair, 1984). During acute non complicated *P. falciparum* or *P. vivax*

malaria, high oxidative stress is observed in the patients (Pabon *et al.*, 2003). This problem can be aggravated by some anti-malarials like artemisinins **(2)** and chloroquine **(8)** (Oliaro *et al.*, 2001; Schuller, 2002). The mechanism of action of artemisinin and its derivatives involves heme-mediated decomposition of the endoperoxide bridge to produce carbon centered free radicals which act against the parasite but also alkylates the heme and proteins hence damaging some intracellular targets by lipid peroxidation (Schuller, 2002). Since free radicals and lipid peroxidation have putative role in the aetiology of cancer, atherosclerosis and anaemia, there is need for free radical control during malaria therapy. ROS are normal intermediates of metabolic processes in aerobic organisms. If these molecules are produced in excess, tissue damage can result (Athanasas *et al.*, 2004). Most of the known antioxidants are derived from plants, probably due to their increased capacity to defend themselves from various sources of stress (Athanasas *et al.*, 2004). Aqeous extract of the whole plant of *A. remota* exhibited antioxidant activity (Ohsugi *et al.*, 1999; Israili and Lyoussi, 2009), although the compounds responsible for this activity were not determined. Aqeous MeOH extract from leaves of *A. remota* exhibited DPPH radical scavenging activity (Nasser *et al.*, 2010). The antioxidant activity of crude extracts and pure isolates of *A. remota* has not been determined.

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## 1.2 Statement of the Problem

Efforts to combat malaria are hampered by growing resistance of malaria parasites to the readily available drugs and *Anopheles* mosquito resistance to conventional insecticides. Alternative drugs are also unaffordable to the poor. It is therefore necessary to search for plant extracts and compounds that are cost effective in management of malaria and controlling malaria disease vector insects. ROS are normal intermediates of metabolic processes in aerobic organisms, however, if these molecules are produced in excess, which happens during malaria chemotherapy may cause damage to the central nervous system and ROS are implicated in the pathogenesis of various neurodegenerative disorders. There is need to search for plant extracts and metabolites which can be used to increase antioxidant activity at cellular level during malaria therapy. The phytochemical information of the aerial part of *A. remota* is well documented. However, that for the underground part is not very well investigated. In this study the underground part of *A. remota* was targeted for phytochemical and bioassay investigation to identify potential antiplasmodial, larvicidal and antioxidant metabolites.

## 1.3 Objectives

### 1.3.1 General objective

The general objective was to isolate and characterize anti-plasmodial, larvicidal and anti-oxidant compounds from underground part of *A. remota.*

### 1.3.2 Specific objectives

The specific objectives were:-

- To isolate and characterize compounds from underground part of *A. remota.*

- To determine the antiplasmodial activities of extracts and pure isolates from underground part of *A. remota* against chloroquine-sensitive and chloroquine-resistant strains of *P. falciparum*.
- To determine larvicidal activities of extracts and pure isolates from underground part of *A. remota* against *A. aegypti* 2nd instar larvae.
- To determine antioxidant activities of extracts and pure isolates from underground part of *A. remota*.

### 1.4 Null hypotheses

- Extracts and isolates of underground part of *A. remota* do not exhibit antiplasmodial activities against chloroquine-sensitive and chloroquine-resistant *P. falciparum*.
- Extracts and isolates of underground part of *A. remota* do not exhibit larvicidal activities.
- Extracts and isolates of underground part of *A. remota* do not exhibit anti-oxidant activities.

### 1.5 Justification of the Research

There is growing concern about malaria parasites and vectors becoming resistant to anti-malaria drugs and insecticides respectively. This calls for continued search for plant metabolites which can be developed into top class anti-malarials and insecticides. Increased oxidative stress, which is aggravated during malaria chemotherapy is associated with some pathological phenomenon such as celebral and pulmonary oedema, poor eye sight, atherosclerosis and cancer among others. Therefore, there is need for free radical control during malaria therapy by increasing antioxidant agents at cellular level. Finding compounds with antiplasmodial, insecticidal and antioxidant activities could

prove valuable in enhancing malaria control strategies and thus reducing the burden of malaria to economies of countries where malaria is endemic.

# CHAPTER TWO: LITERATURE REVIEW

## 2.1 Background on Malaria

### 2.1.1 Epidemiology of malaria

Malaria is endemic throughout most of the tropics. Of the approximately three billion people living in 108 countries who are exposed, approximately 243 million will develop symptomatic malaria annually, about one million deaths are caused by malaria each year; over 80 percent of the deaths that occur among children in sub-Saharan Africa are also due to malaria (WHO, 2008). Malaria occurs throughout most of the tropical regions of the world, with *P. falciparum* causing the largest burden of disease, followed by *P. vivax* (Snow *et al.*, 2005; Price *et al.*, 2007; Guerra *et al.*, 2008). *P. falciparum* predominates in Africa, New Guinea, and Hispaniola (Haiti and the Dominican Republic); *P. vivax* is more common in the Americas and the western Pacific (Breman, 2009). The prevalence of these two species is approximately equal in the Indian subcontinent, Eastern Asia, and Oceania (Breman, 2009). *P. malariae* is uncommon and is found in most endemic areas, especially in sub-Saharan Africa. *P. ovale*, though less common, is relatively unusual outside of Africa and, where it is found, comprises less than 1% of isolates. *P. knowlesi*, with similar morphology to *P. malariae*, has been identified by molecular methods in patients in Malaysia, the Philippines, Thailand, and Myanmar (Smith *et al.*, 2006).

### 2.1.2 Malaria transmission

Malaria is transmitted via the bite of a female *Anopheles spp*, which occurs mainly between dusk and dawn (Filler *et al.*, 2003). Mosquitoes are found throughout the entire world except in Antarctica. These two winged insects belong to the order dipterans. Members of the genera *Anopheles*, *Culex* and *Aedes* are most commonly responsible for

bites in humans. There are over 2500 different species of mosquito throughout the world (Marks and Fradin, 1992). Malaria is transmitted from man to man by the female *Anopheles* mosquito, one of the most capable vectors of human disease. Various species are vectors in different parts of the world. *A. gambiae* complex is the chief vector in Africa and *A. freeborni* in N. America. Nearly 45 species of the mosquito have been found in India and *A. culicifacies, A. fluviatilis, A. minimus, A. philippinensis, A. stephensi, A. sundaicus,* and *A. leucosphyrus* have been implicated in the transmission of malaria (Filler *et al.*, 2003). The areas of distribution are different for these mosquitoes: *A. fluviatilis, A. minimus* are found in the foot-hill regions, *A. stephensi, A. sundaicus* are found in the coastal regions, *A. culicifacies* and *A. philippinensis* are found in the plains. Species like *A. stephensi* are highly adaptable and are found to be very potent vectors of human malaria (Filler *et al.*, 2003). Other comparatively rare mechanisms for malaria transmission include: congenitally-acquired disease, blood transfusion, sharing of contaminated needles, and organ transplantation (Filler *et al.*, 2003).

### 2.1.3 Life cycle of *Plasmodium*

The life cycle of the *Plasmodia* causing malaria is divided into the sexual and asexual phases. The sexual phase takes place in the female mosquitoe while the asexual phase occurs in man. When man is infected with the sporozoite from the saliva of the mosquito, the sporozoite leaves the blood stream after 30–60 minutes and enters the liver cell. After invading the hepatocyte, the parasite undergoes asexual replication. This replicative stage is often called exoerythrocytic schizont stage. The replicated cells are then released into the circulatory system as merozoites following rupture of the host hepatocyte (Figure 2.1). The merozoite released recognizes specific proteins on the surface of the erythrocyte and

actively invades the cell. After entering the erythrocyte, the parasite undergoes a trophic period followed by an asexual replication. The young trophozoite is often called a ring form. As the parasite increases in size, the ring morphology disappears. During the trophic period, the parasite ingests the host cell cytoplasm and breaks down the haemoglobin into amino acids. A by-product of the haemoglobin digestion is the malaria pigment or hemozoin. The gametocytes stage, when it occurs does not cause pathology in the human host and when the gametocyte is ingested by the mosquito, it tends to initiate the sexual stage of the *Plasmodium* life cycle. The two phases of the gametocytic stage are gametocytogenesis, occuring in the blood stream of the host (man) and the gametogenesis taking place in the mosquito gut. For *P. vivax* and *P. falciparum,* the exoerythrocytic stage lasts for 10-14 days, while for *P. malariae*, it lasts for 18 days to 6 weeks. The erythrocytic stage on the other hand lasts for 48 hours in *P. falciparum, P. vivax* and *P. ovale* and 72 hours in *P. malariae.* (Ibezim and Odo, 2008).

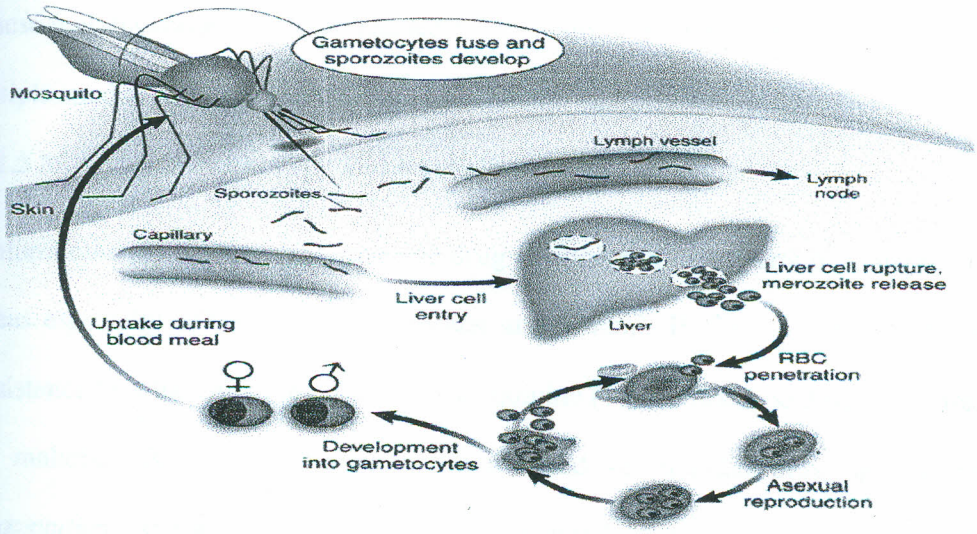



Figure 2.1: **Life Cycle of *Plasmodium falciparum* (Batista *et al.*, 2009)**

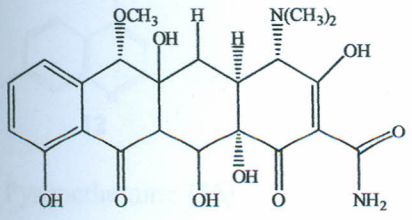
### 2.1.4 Malaria Control

Important components that have been applied for reducing the burden of malaria morbidity and mortality in malaria endemic countries include more sensitive diagnostic tools, effective administration of antimalarial drugs especially to vulnerable groups such as pregnant women and improved personal protection and mosquito control. The approach to elimination or control of malaria includes these basics, along with improvements in tracking of human illness and parasite surveillance, and effective resource delivery so that other health priorities are not compromised (WHO, 2008). However, these efforts are hindered by parasite *P. falciparum* drug resistance and vector *Anopheles* insecticide resistance (Bilia, 2006), inadequate health infrastructure and lack of education for the affected inhabitants. Drug resistance of *P. falciparum* and mosquito resistance to insecticides has compromised the efficacy of intervention resulting in the use of alternative drugs that are often more costly, less safe and less easy to administer. These malaria control problem has led to continued efforts by scientists to develop new antiplasmodial lead compounds from both natural and synthetic origins.

### 2.1.5 Malaria chemotherapy and its challenges

Chloroquine **(8)** has been the first line drug of choice in most parts of malarial endemic areas especially sub-Sahara Africa (Slater and Cerami, 1992). However, *P. falciparum* resistance to it has led to reintroduction of quinine **(1)** as a first line drug in the treatment of malaria, which like chloroquine is a blood schizontidal drug against the four *Plasmodium* species. It should be supplemented orally with tetracycline **(9)** and clindamycin **(10)** when the patient recovers (WHO, 1990). However, there has been poor compliance with oral quinine **(1)** due to its bitter taste and consistent adverse effects

comprising nausea, dysphoria, tinnitus and high tone deafness. Quinidine **(11)**, a stereoisomer of quinine **(1)** is used in the United States, has greater efficacy, but is toxic (WHO, 1997)

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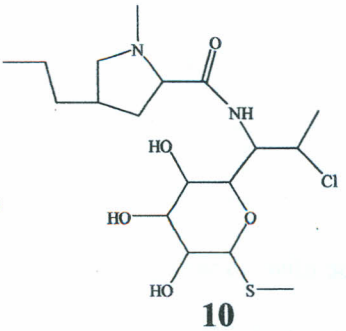


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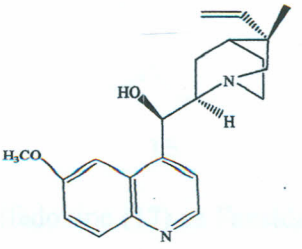


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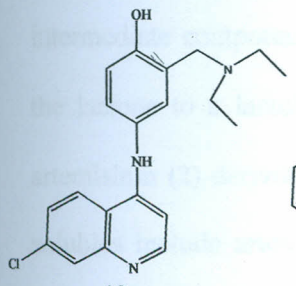
Amodiaquine **(12)** was synthesized and developed as a response to quinine **(1)** and chloroquine **(8)** having little effect on sporozoites (pre-erythrocytic forms), hyponozoites (resting stage sporozoites in the liver cells) or gametocytes which makes them not to clear malaria parasites in all stages. However, its toxicity led to development of mefloquine **(13)**, which is used for prophylaxis and oral treatment of uncomplicated malaria. It has a long terminal half-life of 14-21 days that probably contributes to its resistance by some strains of parasites. However, the combination of artesunate **(14)** and mefloquine **(13)** in Thailand slowed down further development of mefloquine **(13)** resistance (Price *et al.*, 1998). Halofantrine **(15)** is better tolerated and is effective in the treatment of chloroquine-resitant *P. falciparum* malaria. However, when halofantrine **(15)** is taken with food, it may increase the risk of irregular heart beat, hence it is advised that it be taken an hour before a meal or two hours after a meal. In rare cases, halofantrine **(15)** may affect the heart, causing irregular heartbeats that could result in

death. It is contraindicated in patients with a history of irregular heartbeat (Ibezim and Udo, 2008).

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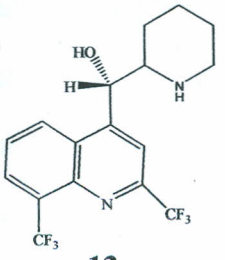


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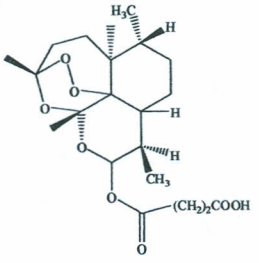


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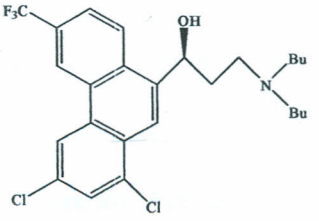


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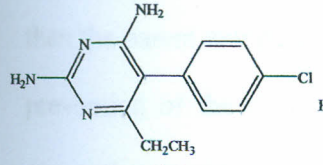
Pyrimethamine **(16)** is used in synergistic combination with sulfadoxine **(17)** as Fansidar or with sulfalene **(18)** as Metakelfin. This sulfadoxine-pyrimethemine (SP) drug combination emerged as a highly effective synergistic drug against chloroquine resistant strains of *P. falciparum* (Oketch-Rabah, 1996), there are also reports of resistance to sulphadoxine-pyrimethamine (Watkins and Masobo 1993; JSCPR, 2002). However, SP drugs have been adopted as national policy for intermittent preventive treatment for pregnant women in sub-Sahara Africa, and also Papua New Guinea (WHO, 2011).

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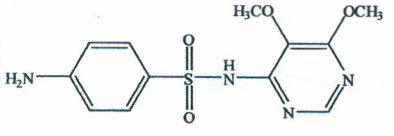


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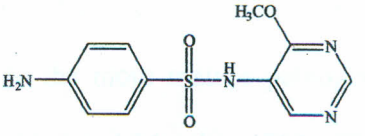


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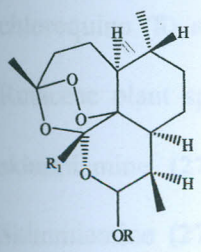
Negative side effects of synthetic antimalarials and development of resistance of malaria parasites towards them resulted in the change of course to searching for antimalarial drugs from natural sources such as artemisinin **(2)**, a sesquiterpene lactone endoperoxide whose antimalarial principle is extracted from the herb *Artemisia anuua* L, first discovered in China (Wayman, 1995). It is a 15-C atom structure with a trioxane ring and a lactone ring and has a molecular weight of 282 (Webster and Lehnert, 1994).

Artemisinin **(2)** is sparingly soluble in water and oils. This led to the search for and discovery of its derivatives, which are more water-soluble. Dihydroartemisinin **(19)** is an intermediate compound formed after breakdown of artemisinin **(2)**, by the reduction of the lactone to a lactol and other active derivatives (Looareesuwan *et al.*, 1996). The artemisinin **(2)** derivatives can be grouped as water soluble and lipid soluble, the water solubles include artesunate **(14)** and artelinic acid **(20)** and are currently available as tablets, intravenous injections and suppositories. The lipid soluble types include artemether **(21)** and arteether **(22)** (Basco and Lebras, 1997). Artelinnic acid **(20)** is still undergoing trials for transdermal administration. Artemisinin **(2)** and its derivatives have clinically been used in the treatment of uncomplicated malaria. In general, the oral formulations of these drugs are rapidly incompletely absorbed and their bioavailability is low (Bethel *et al.*, 1997). There is good evidence that they undergo extensive first pass metabolism in the liver. Both artesunate **(14)** and artemether **(21)** are rapidly transformed into dihydroartemisinin **(19)** so that the metabolite is generally present at higher levels than the parent compound. The major mechanism of action of all artemisinin drugs is the prevention of the development of ring stage parasites to the more mature pathogenic stages that resettle and cytoadhere in the capillaries (Watkins and Masobo, 1993). They act essentially as blood schizonticides, and the presence of the endoperoxide bridge appears to be essential for antimalarial activity. It is also suggested that they cause a marked diminution of nucleic acid synthesis. The artemisinin derivatives have equally been observed to produce a faster relief of clinical symptoms and clearance of parasites from the blood than other antimalarial drugs (DeVires and Dien, 1996). The only problem encountered in the use of artemisinin drugs is that when used as monotherapy

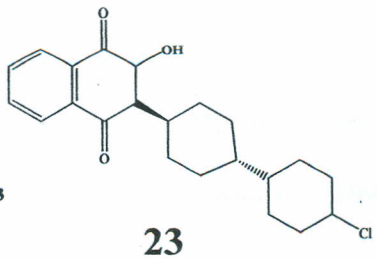
within a short period, that is, less than 5 days, clearance of parasitemia from the blood is only temporary in up to 50% of patients. This has been attributed to the short time it takes for artemisinin drugs to be eliminated from the body. To reduce chances of development of resitance they should always be administered with another unrelated anti-malarial drug such as mefloquine **(13)** (Meshnick, 2003). Artemisinin **(2)** has been widely employed in the treatment of uncomplicated multidrug resistant *falciparum* infections (Price *et al.*, 1997). Compared to quinine **(1)** in the treatment of severe malaria however, patients treated with artemether **(21)** had an equal chance of survival as a patient treated with quinine **(1)**, while artesunate **(14)** and artemether **(21)** are easier to use than quinine **(1)**, and do not induce hypoglycemia (Van Hensbroak *et al.*, 1996). Artemisinin **(2)** and its derivatives have a significant effect on gametocytogenesis (Price *et al.*, 1998). Most common side effects of artemisinin drugs are headache, nausea, abdominal pain, vomiting and occasionally diarrhoea (Brewer *et al.*, 1994).

Oral dihydroartemisinin **(19)** has been shown to be effective in the treatment of multidrug resistant uncomplicated *P. falciparum* infection. Dihydroartemisinin **(19)** does not have activity against hypnozoites, so it cannot be used in their eradication from the liver but it has an effect on gametocytocytic stage. It has a half-life of more than 10 hours and has currently been discovered that dihydroartemisinin **(19)** appears to offer no advantage in the treatment of uncomplicated malaria or severe malaria. It is not also recommended for the treatment of malaria caused by *P. vivax, P. ovale* and *P. malariae* (Ibezim and Udo, 2008). The newest antimalarial drug is atovaquone **(23)** which is used in combination with proguanil **(24)** (Looareesuwan *et al.* 1999), it is effective for the treatment of

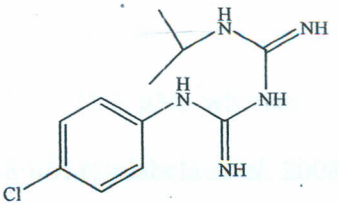
malaria; however, the high cost of this drug precludes its wide-scale use in many malaria endemic countries (Fidock *et al.* 2004).



**19** R = H $R_1$ = H
**20** R = $CH_2C_6H_4COOH$ $R_1$ = $CH_3$
**21** R = $CH_3$ $R_1$ = $CH_3$
**22** R = $CH_2CH_3$ $R_1$ = $CH_3$



**23**



**24**

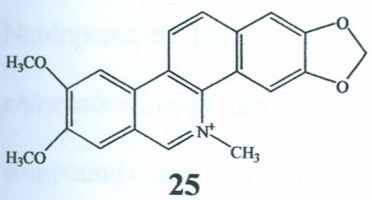
**2.2 Plants in the management of malaria**

Plants remain an ever evolving source for compounds of medicinal importance. The use of medicinal plants for the treatment of parasitic diseases is well known and documented since ancient times. For example, use of *Cinchona succiruba* (Rubiaceae) for the treatment of malaria infection is known for centuries (Kaur *et al.*, 2009). Classes of plant metabolites that are recognized for anti-malarial activity include alkaloids, terpenes, quassinoids, flavonoids, limonoids, chalcones, peptides, xanthones, and coumarins (Batista *et al.*, 2009). Alkaloids, quassinoids and sesquiterpene lactones are the most important with diverse biopotency (Batista *et al.*, 2009).

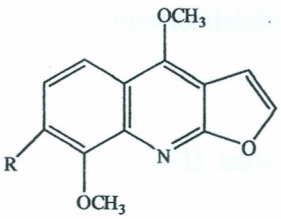
**2.2.1 Alkaloids**

Alkaloids are one of the most important classes of natural products providing drugs since ancient times. Quinine **(1)** from *Cinchona succiruba* (Rubiaceae) has been used for the treatment of malaria for more than three centuries. Benzofenantridine alkaloid, nitidine **(25)** was isolated by biossay-guided fractionation of extracts from *Toddalia asiatica*, a Rutaceae used by the Pokot tribe of Kenya as the major antimalarial component (Gakunju

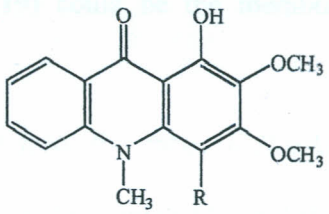
*et al.* 1995). Fractions containing nitidine **(25)** showed $IC_{50}$ values against *P. falciparum* in the range of 9 - 108 µg/ml. Moreover, no cross-resistance was observed between chloroquine **(8)** and nitidine **(25)** (Gakunju *et al.* 1995). From *Esenbeckia febrifuga*, a Rutaceae plant species popularly used in Brazil to treat malaria, γ –fagarine **(26)** and skimmiamine **(27)** were the most active of the five alkaloids that were isolated. Skimmiamine **(27)** was potent against the W2 strain with $IC_{50}$ 75.3 µM, whereas γ – fagarine **(26)** was active against the 3d7 strain with $IC_{50}$ 109.8 µM (Dolabela *et al.* 2008). Moreover, ethanol extract from the stems was more active than either of these alkaloids $IC_{50}$ value 15.5 µg/ml against W2 strain which indicated the existence of more active, non-isolated compounds or synergism between the various constituents (Dolabela *et al.*, 2008). Normelicopicine **(28)** and arborinine **(29)** isolated from *Toddalia trichocarpa* from Kenya displayed *in vitro* activity against *P. falciparum* strains (HB3 and K1) (Muriithi *et al.*, 2002). Normelicopicine **(28)** was also shown to be active against *P. berghei*-infected mice (32% suppression of parasitaemia at a dose of 25 mg/kg/day) in addition to showing *in vitro* KB cell cytotoxicity $IC_{50}$ > 328 µM (Muriithi *et al.*, 2002). Alkaloids have been isolated from *A. parviflora* (Nawaz *et al.*, 2000). However, they have not been reported in *A. remota*.




**25**




**26** R= H
**27** R= $OCH_3$




**28 R = $OCH_3$**
**29 R = H**

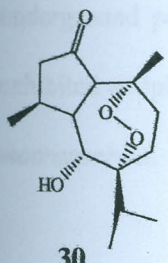
### 2.2.2 Terpenes

#### 2.2.2.1 Sesquiterpenes

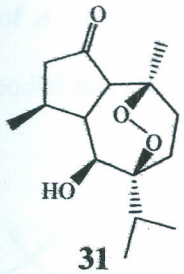
Artemisinin **(2)** is a sesquiterpene lactone endoperoxide whose extracted from the herb *Artemisia anuua* (L.), first discovered in China (Wayman, 1995). It is a 15-C atom structure with a 1,2,4-trioxane ring unique in nature, essential for its activity (Wayman, 1995). After being opened in the parasite it liberates singlet oxygen and forms a free radical which is a cytotoxin (Webster and Lehnert, 1994). Over 2000 years ago, it was discovered and used as an antipyretic (DeVires and Dien, 1996). In 1972, however, its antimalarial component was discovered and it produced more rapid resolution of fever and parasitaemia than all known antimalarial agents and was more potent schizontocide (DeVires and Dien, 1996). Dehydroartemisinin **(19)** is the active metabolite of artemisinin **(2)** and its derivatives are the most potent antimalarial of this group of compounds, though the least stable (Ibezim and Udo, 2008). Its mode of action or pharmacodynamics is the same with that of artemisinin. The two have peroxide functional group essential for antimalarial activity. When the peroxide bond breaks up, it generates singlet oxygen and free radicals and studies have shown that the presence of free radicals has resulted in morphological changes of the parasitic membranes and this shows that the site of action of dihydroartemisinin **(19)** could be the membranous structure (Ibezim and Udo, 2008).

Nardoperoxide **(30)** and isonardoperoxide **(31)** isolated from the roots of *Nordostchy chinensis* showed strong antiplasmodial effects comparable to quinine **(1)**, and the two compounds are seen to be the promising lead compounds for anti-malarial drugs (Sudhanshu *et al.*, 2003). Germacranolide sesquiterpene, neurolenin B **(32)** with $IC_{50}$

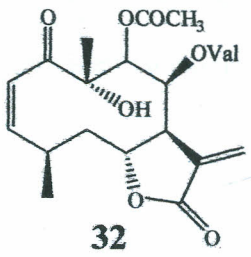
value 0.62 μM against *P. falciparum* was more potent than the furanoheliangolide, lobatin B **(33)** with $IC_{50}$ value 16.51 μM (Francois, *et al.*, 1996). The sesquiterpene lactones were isolated from *Neurolaena lobata* (Francois, *et al.*, 1996). Among the germacranolides, the shift of the double bond from the 2,3-position (neurolenin B) to the 3,4-position led to dramatic decrease in the activity suggesting that one of the structural requirements is the presence of α/β-unsaturated keto function (Francois, *et al.*, 1996). Additionally, a free hydroxyl group at C-8 increased the antiplasmodial activity, while a free hydroxyl group at C-9 decreased the activity (Francois, *et al.*, 1996). This class of compounds has not been isolated from the genus *Ajuga*. However, this could help in the improvement of the antiplasmodial activity of compounds isolated from *A. remota* by introducing an endoperoxide bridge or α/β-unsaturated keto functional group.



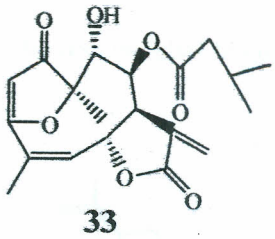

**30**




**31**




**32**

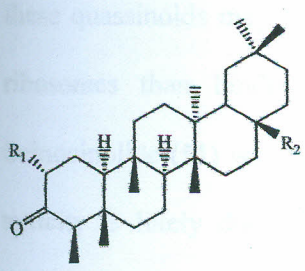



**33**

### 2.2.2.2 Triterpenoids

Triterpenoids endodesmiadiol **(34)**, friedelin **(35)**, canophyllol **(36)**, canophyllal **(37)**, cerin **(38)**, acetoxyoleanolic acid **(39)**, isolated from the EtOAc extract of *Endodesmia calophylloides* (Guttiferae), showed antiplasmodial activity against the W2 chloroquine-resistant strain of *P. falciparum* with $IC_{50}$ values ranging from 7.2 to 23.6 μM (Ngouamegne *et al.*, 2008). From these results, it was thought that friedelane derivatives might be interesting sources for new potential antimalarial leads (Ngouamegne *et al.*, 2008). Betulinic acid **(40)** and its derivatives betulonic acid **(41)**, betulinic acid acetate

(**42**), betulinic acid methyl ester (**43**) and betulinic acid methyl ester acetate (**44**), showed antiplasmodial activity against W2 chloroquine-resistant *P. falciparum* parasites *in vitro*, with $IC_{50}$ values of 9.89, 10.01, 5.99, 51.58 and 45.79 µM, respectively (De Sa *et a.l*, 2009). Moreover, since betulinic acid acetate (**42**) displayed the best selectivity index among all substances tested, this compound was administered by intraperitoneal route to mice infected with *P. berghei*, causing a dose-dependent reduction of parasitemia of 70%, while mice treated with chloroquine had undetectable parasitemia (De Sa *et a.l*, 2009). These results indicate that betulinic acid and its derivative compounds might be considered as potential lead compounds for the development of new antimalarial drugs (De Sa *et a.l*, 2009). Some of this group of compounds have been isolated from the genus *Ajuga*, for example, ergosterol 5,8-endoperoxide (**4**) from both the aerial and underground part of *A. remota* (Kuria *et al.*, 2002; Manguro *et al.*, 2011) which has exhibited antiplasmodial and larvicidal activities. Betulinic acid (**40**) was isolated from *A. macrosperma*.



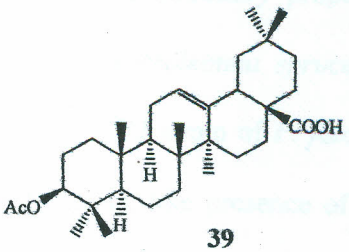

**34** $R_1$ = OH $R_2$ = $CH_2OH$

**35** $R_1$ = H $R_2$ = $CH_3$

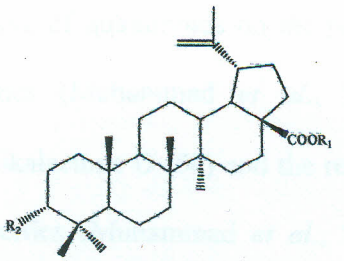
**36** $R_1$ = H $R_2$ = $CH_2OH$

**37** $R_1$ = H $R_2$ = CHO

**38** $R_1$ = OH $R_2$ = $CH_3$




**39**




**40** $R_1$ = H $R_2$ = OH

**41** $R_1$ = H $R_2$ = =O

**42** $R_1$ = H $R_2$ = OAc

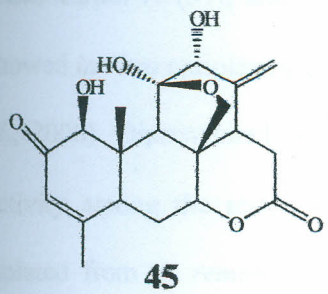
**43** $R_1$ = $CH_3$ $R_2$ = OH

**43** $R_1$ = $CH_3$ $R_2$ = OAc

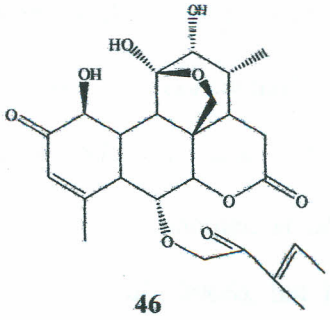
### 2.2.3 Quassinoids

Quassinoids are a group of degraded triterpenes found in the family Simaroubaceae. The majority of isolated quassinoids have a C-20 skeleton and δ-lactones, while the few C-19 skeletal type are γ-lactones. The lactonic linkage may be at C-12 or at C-7. Antiplasmodial activity has been reported for ailanthone **(45)**, $IC_{50}$ value 0.003 µg/ml and 6a-tigloyloxychaparrinone **(46)**, $IC_{50}$ value 0.061 µg/ml isolated from *Ailanthus altissima* (Okunade *et al.*, 2003). Pasakbumin B **(47)**, pasakbumin C **(48)** and eurycomanone **(49)** possessing antiplasmodial activity were isolated from *E. longifolia* with $IC_{50}$ values 22.6, 93.3 and 40.0 ng/ml, respectively (Chan *et al.*, 2004; Kuo *et al.*, 2004). Simalikalactone D **(50)** and orinocinolide **(51)** equally potent against D6 and W2 strains ($IC_{50}$ = 3.0 and 3.67 ng/ml vs 3.2 and 8.5 ng/ ml, respectively) were isolated from the roots of *Simaba orinocensis* (Muhammad *et al.*, 2004), these quassinoids inhibited protein biosynthesis *in vitro* in translation system from the Krebs cells. During intraerythrocytic proliferation, the malaria parasite makes its own ribosome, and the selective antiplasmodial action of these quassinoids may be explained by stronger binding of quassinoids on the parasite ribosomes than binding to the host cell ribosomes (Muhammad *et al.*, 2004). Orinocinolide **(51)** was four fold less toxic than Simalikalactone D **(50)** and the reduced toxicity is likely due to its reduced inhibitory properties (Muhammad *et al.*, 2004). Neosergeolide **(52)** isolated from *Picrolemma sprucei*, showed potent antiplasmodial activity $IC_{50}$ value 2.0 nM against W2 strain of *P. falciparum* (De Andrade-Nato *et al.*, 2007). Some structural requirements, like presence of α,β-unsaturated ketone in the A ring, an epoxymethylene bridge in the C ring and ester functions in C-15 were considered essential for the antiplasmodial activity (De Andrade-Nato *et al.*, 2007). Quassinoids

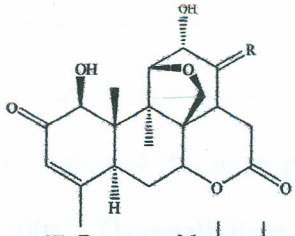
have not been isolated from the genus *Ajuga*. Never the less, their structural activity relationship could be utilized in improving the antiplasmodial activity of triterpenes by introducing α,β-unsaturated ketone or an epoxymethylene bridge.



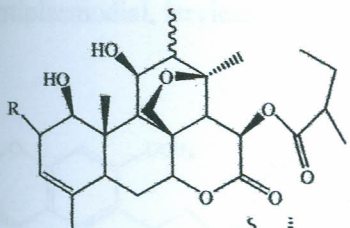

**45**



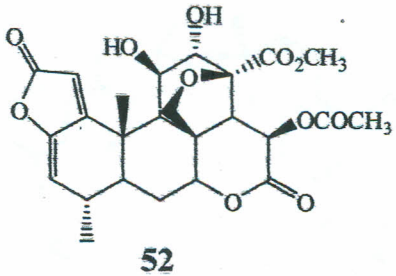

**46**




**47** R = epoxide
**48** R = $CH_3$
**49** R = $CH_2$




**50** R = =O
**51** R = OH

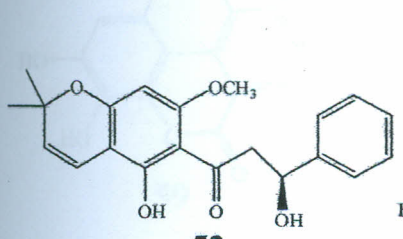



**52**

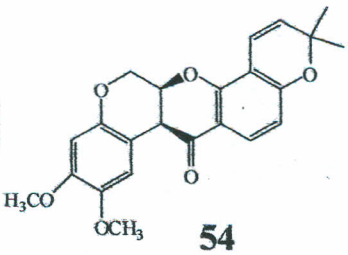
### 2.2.4 Flavonoids and related compounds

A new β-hydroxydihydrochalcone (**53**) was isolated, along with the known flavonoids deguelin (**54**) and obovatin (**55**) from the seedpods of *Tephrosia elata* (Fabaceae) (Muiva *et al.*, 2009). The crude MeOH-$CH_2Cl_2$ (1:1) extract of *T elata* showed antiplasmodial activities against chloroquine-sensitive (D6) $IC_{50}$ value 8.4 µg/ml and chloroquine-resistant (W2) $IC_{50}$ value 8.6 µg/ml strains of *P. falciparum* (Muiva *et al.*, 2009). Compound (**53**) exhibited the highest antiplasmodial activity with $IC_{50}$ values 8.2 and 16.3 µM against D6 and W2 strains, respectively, while **54** and **55** showed $IC_{50}$ values ranging from 12.4 to 27.6 µM against these strains. This compound along with the other flavonoids appeared to be responsible for the activities observed in the crude extract

(Muiva *et al.*, 2009). The EtOAc extract of the stem bark of *Erythrina fusca* Lour showed significant antiplasmodial activity against the multi-drug-resistant *P. falciparum* (K1 strain) $IC_{50}$ value 7.5 µg/ml (Khaomek *et al.*, 2008). The flavonoids, citflavanone (**56**), lonchocarpol A (**57**), and 8-prenyldaidzein (**58**) which were isolated from the extract, showed *in vitro* antiplasmodial activity at a concentration less than 12.5 µM (Khaomek *et al.*, 2008). Diprenylated flavanone **57**, $IC_{50}$ value 3.9 µM, displayed the most potent activity among the tested compounds (Khaomek *et al.*, 2008). Flavonols have been isolated from *A. remota* (Manguro *et al.*, 2006), but it is not known if they exhibit antiplasmodial, larvicidal and antioxidant activities.




**53**

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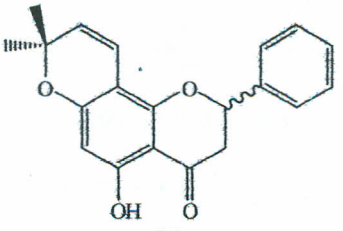


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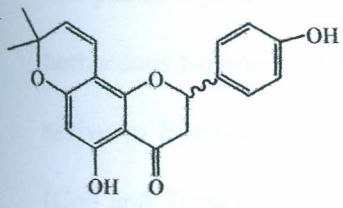
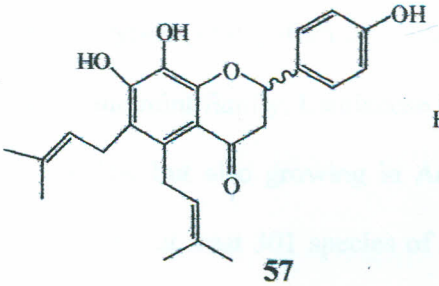
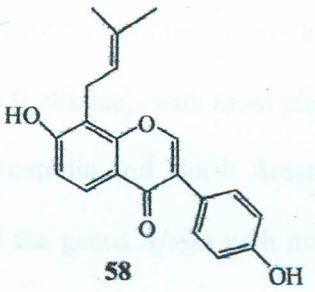



**56**




**57**

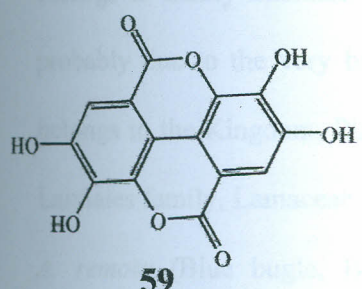



**58**

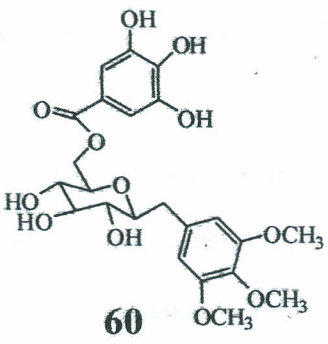
### 2.2.5 Miscellaneous Compounds

Evaluation of plants from New Caledonia with anti-elastase activity for anti-plasmodial activity, *Tristaniopsis spp.* inhibited parasite growth *in vitro* (Verotta *et al.*, 2001). Ellagic acid **(59)** and a glycoside A3A **(60)** were identified as active constituents with $IC_{50}$ values 0.5 and 3.2 µM, respectively. Reduced activity of **60** was attributed to

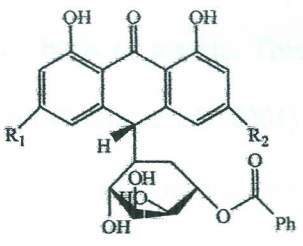
bioavailability (Verotta *et al.*, 2001). Since gallic acid was inactive it appeared that galloyl and glucose moieties are devoid of activity and only the trimethoxyphenol group contributed to the observed effect (Verotta *et al.*, 2001). Anthrone glycosides uveoside **(61)** and 10-epi-uveoside **(62)** isolated from *Picramnia antidesma* were reported to exhibit good antimalarial activity $IC_{50}$ values between 2.4–5.1 µM, but lacked selectivity (Hernadez-Medal and Pereda-Miranda, 2002). This class of compounds has not been isolated from the genus *Ajuga*.




**59**




**60**




**61** $R_1 = H$ $R_2 = CH_3$

**62** $R_1 = CH_3$ $R_2 = H$

**2.3 The genus *Ajuga***

The plants of genus *Ajuga* are evergreen, clump-forming rhizomatous annual or perennial herbaceous flowering species in the mint family, Lamiaceae (Labiatae), with most plants native to Europe, Asia, and Africa, but also growing in Australia and North America (Coll and Tendrón, 2008). There are at least 301 species of the genus *Ajuga* with many variations. *Ajuga* is one of the 266 genera of the family Lamiaceae. The *Ajuga* plants grow to 5-50 cm tall, with opposite leaves, which are attractive. The flowers are two lipped and tubular, and mostly blue, purple or yellow in color. Many *Ajuga* plants are used in horticulture as ground cover or border, and in rock gardens, but some are regarded as weeds. Some *Ajuga* species have a large number of varieties (cultivars), which are used in gardens because of their varied blooms of different colors. Many plants

of the genus *Ajuga* and some compounds isolated from these plants have medicinal value and of ecological and economic importance. Extracts and compounds from this genus show antimalarial, larvicidal and antioxidant properties (Israili and Lyoussi, 2009). These extracts and compounds are either from the whole plant or the aerial part. However, it is not known if extracts and compounds from the underground part exhibit these properties.

### 2.4 *Ajuga remota* Wall. ex Benth.

*A. remota* is an erect rhizomatous pubescent herb found growing in East Africa. It belongs to family Labiatae. The herb is not eaten by animals, birds or insects. This is probably due to the very bitter taste of almost all of its parts (Kuria *et al.,* 2001). It belongs to the Kingdom, Plantae, division, Magnoliophyta, class, Magnoliopsida, order, Lamiales family, Lamaceae subfamily, Ajugoideae genus, *Ajuga*, Species, *Ajuga remota.* *A. remota* (Blue bugle, Bugleherb, Bugleweed, Carpetweed, Common bugle) is a herbaceous flowering plant native of Africa (Wainaina, 2008). Vernacular names for the plant include Salala (Dorobo), Chebonurar (Marakwet), Akech (Luo), Mataliha, Imbuli yu mudaka (Luhya), Karaci, Wanjiru wa rucii (Kikuyu), Wanjiru-wa-rurii (Embu), Kirurite (Meru), Imenang (Samburu) and Chemogong (Kipsigis) (Kokwaro, 2009).

### 2.5 Ethinomedical/folklore information on *Ajuga remota*

*A. remota* is used as a medicinal plant in most parts of Kenya. Infusions of the plant are used to treat fever, paludism, gonorrhoea and cold among the Marakwet, Luhya and Kikuyu (Githinji and Kokwaro, 1993). The whole plant is used for treatment of malaria, pneumonia, stomach and liver ailments among the Kikuyu, Mbere and Embu (Gachathi, 1989; Kareru *et al.*, 2007). The leaves of *A. remota* are known to relieve toothache, while

a decoction or infusion from leaves is prescribed by Kenyan herbalists for severe stomachache and treatment of malaria (Kuria *et al.*, 2001). Among the Luhya and Marakwet, juice from pound *A. remota* leaves is used to cure rash, dermatitis and itch (Kokwaro, 1987). Juice from leaves is used to treat eczema among the Marakwet (Lindsay and Hepper, 1978). Infusion in cold or hot water of the whole plant is used to treat paludism among the Meru (Muthaura, *et al.* 2007b). Leaves, roots and bulb of *A. remota* are used as a remedy for diarrhoea (Njoroge and Kibunge, 2007), decoction from boiled leaves are used in the management of common cold, coughs, flu, stomach upsets, helminthiasis and anaplasmosis (Njoroge *et al.*, 2004; Njoroge and Bussmann, 2006). Crushed leaves or hot decoction with soup is utilized in the treatment of gastrointestinal disorders, mastitis, retained afterbirth and anaplasmosis among the Samburu (Nanyingi *et al.*, 2008). From this section it is seen that either the whole plant or the aerial part is used in the management of malaria. The antiplasmodial principles from the aerial part are known (Kuria *et al.*, 2002). However, it is not known whether extracts and isolates from the underground part exhibit antiplasmodial activity.

### 2.6 Biological activities of extracts of *Ajuga remota*

Biological activities of *A. remota* extracts have been reported ranging from their chemotherapeutic, antifeedant activity to their effects on disease vectors. Table 2.1 summarises these activities. Activities of interest to this study are the antiplamodial, larvicidal and antioxidant activities. Antiplasmodial activities of extracts from the aerial part and compounds from these extracts are known (Kuria *et al.*, 2001 & 2002; Muregi *et al.*, 2007). However, the antiplasmodial activity of extracts and compounds from underground part are not known despite the fact that the whole plant is used in the

management of malaria by some communities in Kenya. Larvicidal activities of Pet-ether & $CCl_4$ extracts (*in vitro*) (Sharma *et al.*, 2004) are known, but the active principles are not known. Antioxidant activity of aqeous MeOH extract of whole has been reported (Ohsugi *et al.*, 1999; Israili and Lyuossi, 2009; Nasser *et al.* 2010). Never the less, the compounds responsible for these activities are not known and it has not been validated whether they work synergistically or in isolation.

**Table 2.1: Biological activity of extracts of *Ajuga remota***

| Biological activity | Plant extracts | Reference |
|---|---|---|
| Analgesic | Aqeous. and EtOH extract (mice) | Debella *et al.*, 2003 |
| Antihypertensive | Aqeous extract (rat) | Odek-Ogunde *et al.*, 1993 |
| Anti-inflammatory effect | Aqeous,MeOH, & hexane extracts (*in vitro*) | Debella *et al.*, 2003; |
| Antimalarial/antipl asmodial | Aqeous , EtOH & MeOH plant extracts (mice & *in vitro*) | Kuria *et al.*, 2001; Muregi *et al.*, 2007 |
| Antioxidant /oxygen scavenging | Aqeous extract of whole plant; aqeous MeOH extract (*in vitro*) | Ohsugi *et al.*, 1999; Israili and Lyuossi, 2009; Nasser *et al.* 2010. |
| Antipyretic activity | Aqeous extract | Debella *et al.*, 2005 |
| Cardiac stimulant | Plant extract (*in vivo*) | Kuria and Muriuki, 1984 |
| Larvicidal (mosquito) | Pet-ether & $CCl_4$ extracts (*in vitro*) | Sharma *et al.*, 2004 |

## 2.7 Antiplasmodial activity of *Ajuga* species

The two *Ajuga* species that are used ethnomedically for treatment of malaria with antiplasmodial activities are *A. remota* and *A. bracteosa* (Kuria *et al.*, 2001; Chandel and Bagai, 2011). Infusion or decoction of the whole plant of *A. remota* is used for treatment of malaria among the Kikuyu, Meru and Embu (Kareru *et al.*, 2007). The MeOH extract

of the aerial part presented *in vitro* antiplasmodial activity $IC_{50}$ value 21.6 µg/ml (Muregi *et al.*, 2007). Earlier Kuria *et al.*, (2002) was able to establish the active antiplasmodial principles from the aerial part. However, the antiplasmodial principles of the underground part of *A. remota* have not been established despite the whole plant being utilized for treatment of malaria. The MeOH leaf extract of *A. bracteosa* possesses (Chandel and Bagai, 2011) *in vitro* antiplasmodial efficacy with an $IC_{50}$ value 10.0 µg/ml (Chandel and Bagai, 2011). Thus, the extract was further evaluated for its *in vivo* schizontocidal activity and efficacy in terms of survival time in *P. berghei* infected BALB/c mice. The extract at 250, 500, and 750 mg/kg/day exhibited significant ($p < 0.0001$) blood schizontocidal activity during established infection with enhanced mean survival time comparable to that of standard drug chloroquine, 5 mg/kg/day (Chandel and Bagai, 2011). The significant schizontocidal activity and enhanced mean survival time of mice stress the need to identify and characterize active antiplasmodial principle from this plant.

**2.8 Phytochemistry of the genus *Ajuga***

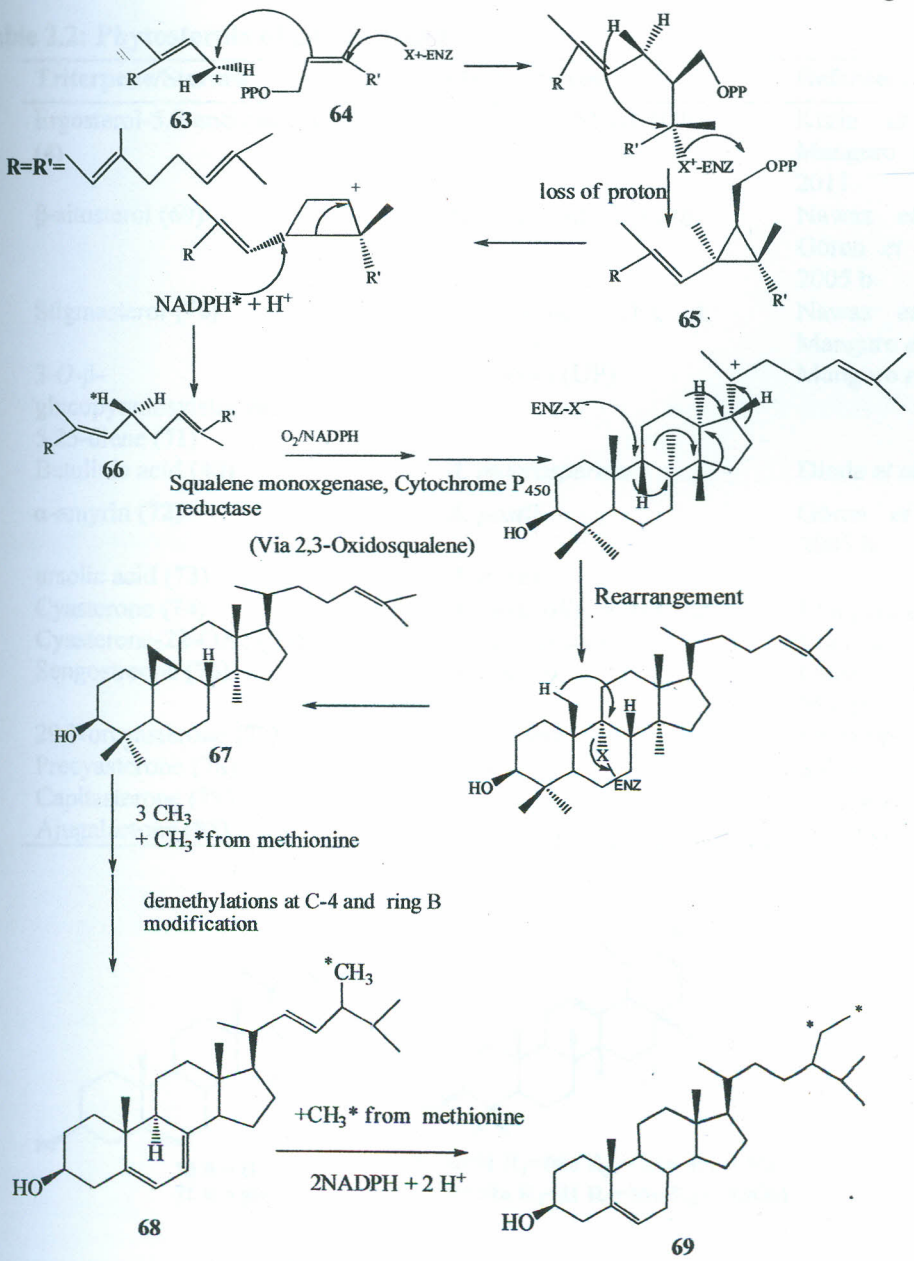
The *Ajuga spp.* has several classes of compounds which including, phytoecdysteroids, *neo*-clerodane-diterpenes and diterpinoids, triterpenes, anthocyanidin-glucosides, iridoid glycosides, flavonoids, triglycerides and essential oils (Israili and Lyoussi, 2009). These classes of compounds possess a wide variety of biological, pharmacological and medicinal properties, such as analgesic (Debella *et al.*, 2003), anti-arthritic (Ono *et al.*, 2008) antibacterial (Shafi *et al.*, 2004) antiestrogenic (Hostettmann *et al.*, 1999), antifungal (Shafi *et al.*, 2004), anti-inflammatory (Debella *et al.*, 2003), antihypertensive (Odek-Ogunde *et al.*, 1993), antileukemic (Akbay *et al.*, 2002), antimalarial (Kuria *et al.*,

2002) antimycobacterial (Cantrell *et al.*, 1999), antioxidant (Ohsugi *et al.*, 1999), antipyretic (Debella *et al.*, 2005), as well as antifeedant and insect growth-inhibitory properties (Israili and Lyoussi, 2009) among others. Some of the compounds isolated are reviewed herein.

### 2.8.1 Steroids and triterpenoids of the genus *Ajuga*

Before going into the details of steroids and triterpeniods of this genus, it is important to review the biosynthesis of steroids and triterpenoids. The key to understanding the biosynthesis of triterpenoids arose initially from the discovery of large amounts of $C_{30}$ compound squalene **(66)** in the shark liver oil (Harrison, 1990). This structure clearly contained two fernosyl moieties, but joined in a tail-to-tail fashion rather than in a head-to-tail fashion observed in the smaller terpenoids. The actual mechanism by which these two $C_{15}$ units condense proceeds via the formation of cyclopropane pre-squalene pyrophosphate **(65).** The importance of the electrophilic allyl cation is evident here (Scheme I). Then the phytosterols found in plants and yeast is produced via the pathway that proceeds through cyclopropyl intermediate cycloartenol **(67).** Some of these phytosterols contain extra carbon atoms in the side chain and these are added via the squalene sequence shown in scheme I. In this way the $C_{27}$ precursor is converted to the $C_{28}$ and $C_{29}$ phytosterols like ergosterol **(68)** and β-sitosterol **(69).** The genus *Ajuga* has been realized to biogenetically modify these phytosterol precursors further to several derivatives. Table 2.2 summarizes some of the phytosterols isolated from the genus *Ajuga*. Some of these phytosterols exhibit antiplasmodial and larvicidal activity, for example, ergosterol-5,8-endoperoxide **(4)** isolated from both the aerial and underground part of *A. remota* (Kuria *et al.*, 2002; Manguro *et al.*, 2006). Betulinic acid **(40)** isolated from *A. macrosperma* (Dinda *et al.*, 1997), α-amyrin **(72)** and ursolic acid

(73) isolated from *A. postii* (Gören *et al.*, 2005 a; 2005 b) exhibit antiplasmodial activity. However, these compounds have not been isolated from *A. remota*.

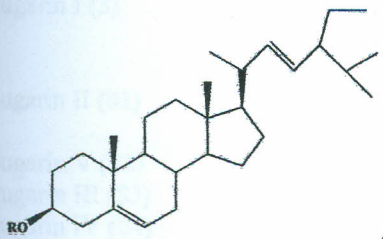



Scheme I: **Biosynthesis of phytosterols** (adopted from Harrison, 1990)

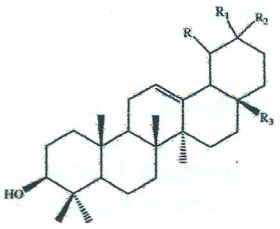
Scheme I: **Biosynthesis of phytosterols** (adopted from Harrison, 1990)

**Table 2.2: Phytosterols of genus *Ajuga***

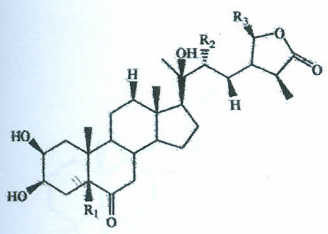
| Triterpene/Steroid | Plant sources | Reference |
|---|---|---|
| Ergosterol-5,8-endoperoxide **(4)** | *A. remota* (AP) & (UP) | Kuria *et al.*, 2002; Manguro *et al.*, 2006, 2011 |
| β-sitosterol **(69)** | *A. bracteosa; A. postii* | Nawaz *et al.*, 1999; Gören *et al.*, 2005 a; 2005 b |
| Stigmasterol **(70)** | *A. remota* (UP), *A. bracteosa* | Nawaz *et al.*, 1999; Manguro *et al.*, 2011 |
| 3-*O*-β-glucopyranosylstigmasta-5,25-diene **(71)** | *A. remota* (UP) | Manguro *et al.*, 2011 |
| Betulinic acid **(40)** | *A. macrosperma* | Dinda *et al.*, 1997 |
| α-amyrin **(72)** | *A. postii* | Gören *et al.*, 2005a; 2005 b |
| ursolic acid **(73)** | *A. postii* | ,, |
| Cyasterone **(74)** | *A. austrialis; A. remota* | Manguro *et al.*, 2011 |
| Cyasterone-22-OAc **(75)** | *A. taiwanensis* | Liu *et al.*, 1995 |
| Sengostreone **(76)** | *A. remota* | Okuzumi *et al.*, 2003; Manguro *et al.*, 2011 |
| 29-Norcyasterone **(77)** | *A. reptans* | Okuzumi *et al.*,, 2003 |
| Precyasterone **(78)** | *A. iva* | Sabri *et al.*, 1981 |
| Capitasterone **(79)** | *A. reptans* | Camps *et al.*, 1990 |
| Ajugalactone **(80)** | *A. reptans* | Camps *et al.*, 1990 |



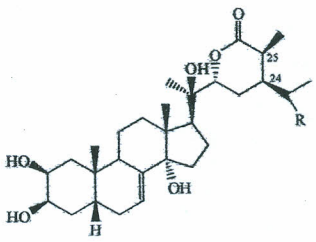

**70 R = H**
**71 R = glc**




**72 R=H $R_1$=Me $R_2$ = Me $R_3$ = Me**
**73 R=Me $R_1$=H $R_2$=Me $R_3$=COOH**



| | $R_1$ | $R_2$ | $R_3$ |
|---|---|---|---|
| 74 | H | OH | $CH_3$ |
| 75 | H | OAc | $CH_3$ |
| 76 | OH | OH | $CH_3$ |
| 77 | H | OH | H |



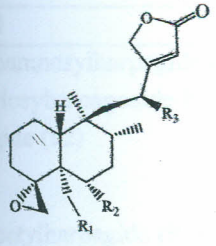
| | R | $C^{24}$- $C^{25}$ |
|---|---|---|
| 78 | OH | $CH_2$-$CH_2$ |
| 79 | H | $CH_2$-$CH_2$ |
| 80 | H | CH = CH |

### 2.8.2 *Neo*-clerodane diterpenoids of the genus *Ajuga*

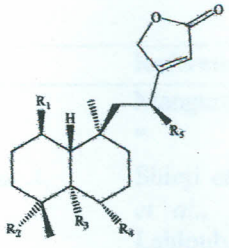
The *neo*-clerodane diterpenoids are a large group of secondary metabolites reported in the genus *Ajuga*. Table 2.3 gives a summary of some of *neo*-clerodane diterpenoids isolated among the *Ajuga spp*. Ajugarin I **(3)** isolated from the aerial part of *A. remota* exhibited antiplasmodial activity (Kuria *et al.*, 2002). However, it is not known if it is present in the underground part and if it exhibits larvicidal activity. It is also not known if the other ajugarins exhibit antiplasmodial and larvicidal activities.

**Table 2.3: *Neo*-clerodane diterpenoids of the genus *Ajuga*.**

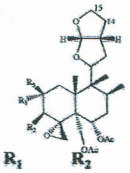
| *Neo*-clerodane diterpenoid | Plant source | Reference |
|---|---|---|
| Ajugarin I **(3)** | *A. remota, A. parviflora, A. bracteosa* | Cantrell *et al.*, 1999; Coll and Tandrón, 2005; Nawaz *et al.*, 2000 |
| Ajugarin II **(81)** | *A. remota, A. parviflora, A. bracteosa* | Coll and Tandon, 2005; Nawaz *et al.*, 2000 |
| Ajugarin V **(82)** | *A. remota, A. bracteosa* | Coll and Tandrón, 2005 |
| Ajugarin III **(83)** | *A. remota,, A. bracteosa* | Coll and Tandrón, 2005 |
| Ajugarin IV **(84)** | *A. remota, A. bracteosa* | Coll and Tandrón, 2005 |
| Deacetylajugarin IV **(85)** | *A. remota* | Coll and Tandrón, 2005 |
| Clerodin **(86)** | *A. remota* | Coll and Tandrón, 2005 |
| Dihydroclerodin **(87)** | *A. remota* | Coll and Tandrón, 2005 |
| Ajugapitin **(88)** | *A. remota* | Coll and Tandrón, 2005 |
| 14-hydro-15-hydroxyclerodin **(89)** | *A. remota* | Coll and Tandrón, 2005 |
| 14-hydro-15-hydroxyajugapitin **(90)** | *A. remota* | Coll and Tandrón, 2005 |
| Lupulin **(91)** | *A. lupulina* | Chen *et al.*, 1996 |



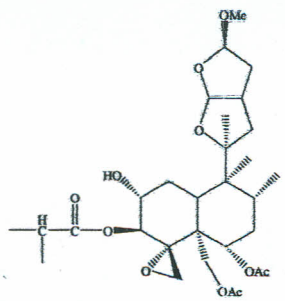
| | $R_1$ | $R_2$ | $R_3$ |
|---|---|---|---|
| 81 | OAc | OH | H |
| 82 | H | OAc | H |



| | $R_1$ | $R_2$ | $R_3$ | $R_4$ | $R_5$ |
|---|---|---|---|---|---|
| 84 | H | $CH_2OH$ | OAc | OAc | H |
| 85 | H | $COOCH_3$ | H | OAc | H |
| 86 | H | $COOCH_3$ | H | OH | H |



| | $R_1$ | $R_2$ | $R_3$ | $R_4$ | $C_{14}$-$C_{15}$ |
|---|---|---|---|---|---|
| 86 | H | H | H | H | CH = CH |
| 87 | H | H | H | H | -CH - $CH_2$ |
| 83 | H | OH | EtCH(Me)COO | H | CH = CH |
| 89 | H | H | H | OH | -CH - $CH_2$ |
| 90 | H | OH | EtCH(Me)COO | OH | -CH - $CH_2$ |



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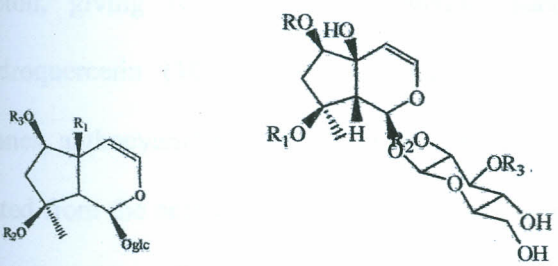
### 2.8.3 Iridoid glycosides of genus *Ajuga*

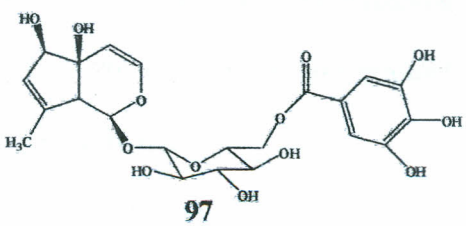
Iridoids are a class of secondary metabolites found in a wide variety of plants. They are monoterpenes biosynthesized from isoprene and are often intermediates in the biosynthesis of alkaloids (Israili and Lyoussi, 2009). Iridiods found in the genus *Ajuga* contain glycoside moieties (Israili and Lyoussi, 2009). Some Iridoid glycosides reported from *Ajuga* species are summarized in Table 2.4. 6'-*O*-rhamnosylharpagide **(6)** and 6-*O*-xylosyharpagoside-B **(7)** isolated from the MeOH extract of *A. remota* exhibited low larvicidal activity (Manguro *et al.*, 2011). Moreover, the larvicidal activity of isolates from MeOH extract were lower than that of the extract raising the possibility of presence of synergistic activity of compounds in the extract or presence of other more active compounds that were not isolated in the study.

**Table 2.4: Iridoids of the genus *Ajuga***

| Iridoid | Plant source | Reference |
|---|---|---|
| 6'-*O*-rhamnosylharpagide **(6)** | *A. remota* | Manguro *et al.*, 2011 |
| 6-*O*-xylosyharpagoside-B **(7)** | " | " |
| Harpagide **(92)** | *A. reptans, A. iva, A. remota* | Shioji *et al.*, 1992; Breschi *et al.*, 1992; Assaad and Lahloub, 1998 |
| 8-*O*-Acetylharpagide **(93)** | *A. reptans, A. remota* | Breschi *et al.*, 1992; Manguro *et al.*, 2006 |
| 6,8-Diacetylharpagide **(94)** | *A. remota* | Manguro *et al.*, 2006 |
| 6,8-Diacetylharpagide-1-*O*-β-(2',3'-diacetylglucoside) **(95)** | " | " |
| 2',3'-Diacetylharpagide **(96)** | " | " |
| *O*-galloy-7,8-dehydroharpagide **(97)** | " | Manguro *et al.*, 2011 |




| | $R_1$ | $R_2$ | $R_3$ | | R | $R_1$ | $R_2$ | $R_3$ |
|---|---|---|---|---|---|---|---|---|
| 92 | OH | H | H | 95 | Ac | Ac | Ac | Ac |
| 93 | OH | Ac | H | | | | | |
| 94 | OH | Ac | Ac | 96 | H | H | Ac | Ac |

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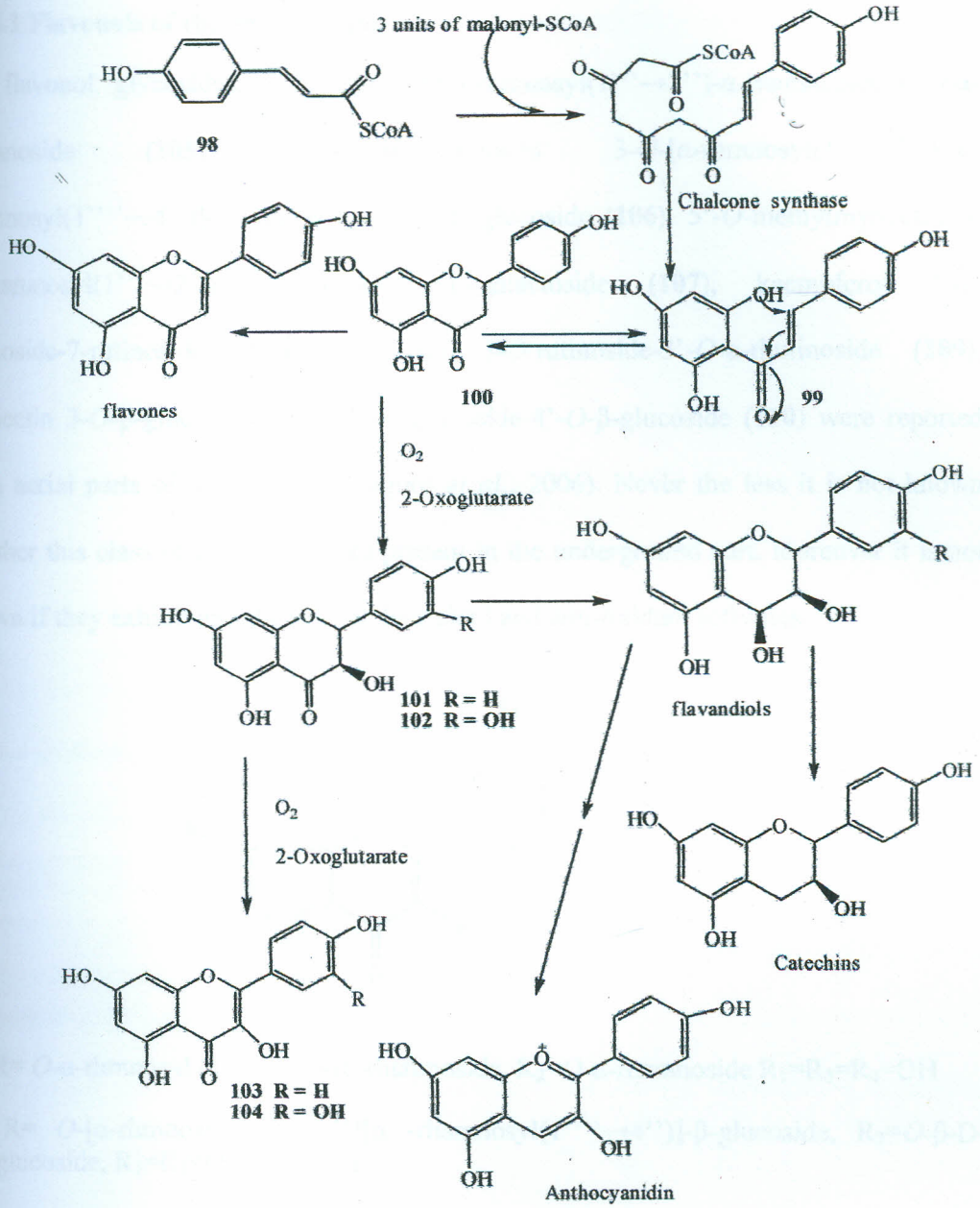
### 2.8.4 Flavonoids of genus *Ajuga*

Previous studies (Terahara *et al.*, 1996; Manguro *et al.*, 2006) on extracts of *Ajuga* species have led to isolation of flavonols and anthocyanins. It is important to review the biosynthesis of flavonoids. Scheme II summarises the biosynthesis of flavonoids. Flavonoids are products from a cinnamoyl-SCoA **(98)** starter unit, with chain extension using three molecules of malonyl-SCoA. This initially gives a polyketide. These allow aldol or Claisen-like reactions to occur, generating aromatic rings (Dewick, 2002). The enzyme chalcone synthase couples a cinnamoyl-SCoA unit with three malonyl-SCoA units giving a chalcone **(99)**. This structure illustrates the different characteristic

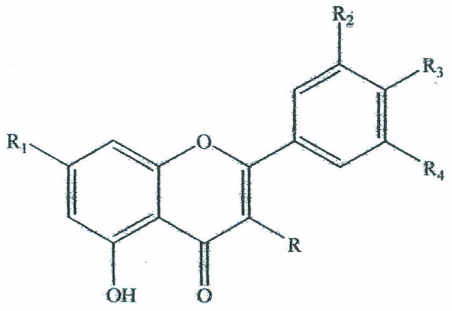
oxygenation patterns in aromatic rings derived from the acetate or shikimate pathways. Chalcones act as precursors for a vast range of flavonoid derivatives found throughout the plant kingdom. Most contain a six-membered heterocyclic ring, formed by Michael-type nucleophilic attack of a phenol group on to the unsaturated ketone giving a flavanone **(100)**. The isomerization can occur chemically, acid conditions favouring the flavanone **(100)** and basic conditions the chalcone **(99)**, but in nature the reaction is enzyme catalysed and stereospecific, resulting in formation of a single flavanone enantiomer (Dewick, 2002). Flavanones can then give rise to many variants on this basic skeleton, giving rise to dihydroflavonols such as dihydrokaempferol **(101)** and dihydroquercetin **(102)**, flavonols such as kaempferol **(103)** and quercetin **(104)**, flavones, anthocyanidins, and catechins (Dewick, 2002). It is not known if the flavonols isolated from the aerial part of *A. remota* (Manguro *et al.*, 2006) are in the underground part and exhibit antiplasmodial, larvicidal and antioxidant activities.




Scheme II: **Biosynthesis of flavonoids** (adopted from Dewick, 2002)

### 2.8.4.1 Flavonols of the genus *Ajuga*

Six flavonol glycosides, myricetin 3-*O*-α-rhmnosyl(1'''→2'')-α-rhamnoside-3'-*O*-α-rhamnoside **(105)**, 5'-*O*-methylmyricetin 3-*O*-[α-rhmnosyl(1'''→2'')][α-rhamnosyl(1''''→4'')]-β-glucoside-3'-*O*-β-D-glucoside **(106)**, 5'-*O*-methylmyricetin 3-*O*-rhamnosyl(1'''→2'')-α-rhmnoside-3'-*O*-D-galactoside **(107)**, kaempferol 3-*O*-rutinoside-7-rutinoside **(108)**, myricetin 3-*O*-rutinoside-3'-*O*-α-rhutinoside **(109)**, myricetin 3-*O*-β-glucosyl-(1'''→2'')-β-glucoside-4'-*O*-β-glucoside **(110)** were reported from aerial parts of *A. remota* (Manguro *et al.*, 2006). Never the less it is not known whether this class of compounds are present in the underground part, moreover it is not known if they exhibit antiplasmodial, larvicidal and anti-oxidant activities.




**105** R= *O*-α-rhmnosyl (1'''→2'')-α -rhamnoside, $R_2$=*O*-α-rhamnoside $R_1$=$R_3$=$R_4$=OH

**106** R= *O*-[α-rhmnosyl(1'''→2'')][α -rhamnosyl(1''''→4'')]-β-glucoside, $R_2$=*O*-β-D-glucoside, $R_1$=$R_3$=OH, $R_4$=OMe.

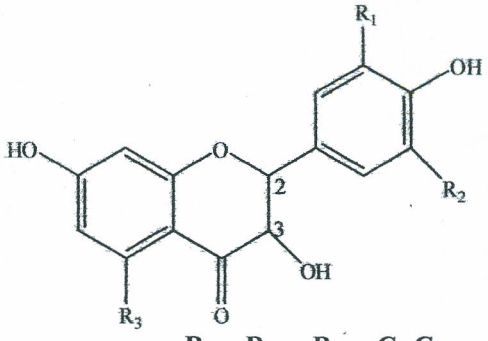
**107** R=*O*-α-rhamnosyl(1'''→2'')-α-rhmnoside, $R_2$=*O*-β-D-galactoside $R_1$=$R_3$=OH, $R_4$=OMe.

**108** R=$R_1$=*O*-rutinoside, $R_2$=$R_3$=$R_4$=OH

**109** R= *O*-rutinoside, $R_2$= *O*-α-rutinoside $R_1$=$R_3$=$R_4$=OH

**110** R= *O*-β-glucosyl-(1'''→2'')-β-glucoside, $R_3$=*O*-β-glucoside, $R_1$=$R_2$=$R_4$=OH

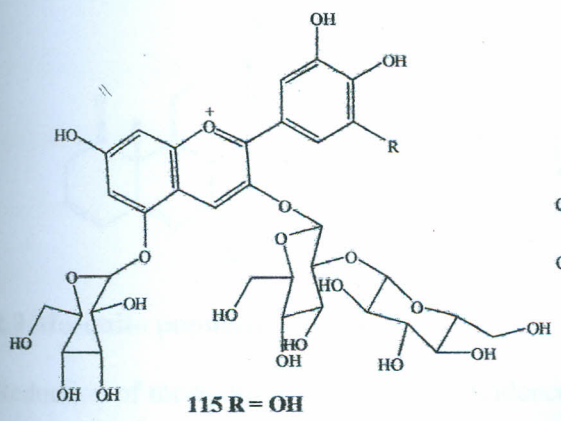
Flavonols including, dihydrokaempferol (**101**), dihydroquercetin (**102**) kaempferol (**103**), quercetin (**104**), 5-methylkaempferol (**111**), 5-methylmyricetin (**112**), myricetin (**113**), dihydromyricetin (**114**), were reported from *Ajuga* species (Israili and Lyoussi, 2009).




| | $R_1$ | $R_2$ | $R_3$ | $C_2$-$C_3$ |
|---|---|---|---|---|
| 111 | H | H | $CH_3$ | C=C |
| 112 | OH | OH | $CH_3$ | C=C |
| 113 | OH | OH | OH | C=C |
| 114 | OH | OH | OH | C-C |

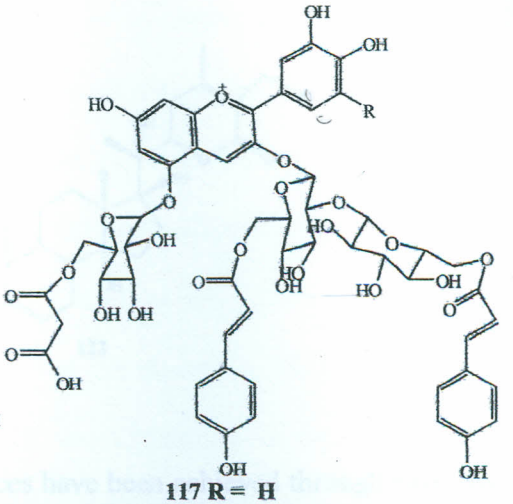
### 2.8.4.2 Anthocyanidins of genus *Ajuga*

Anthocyanidins reported from flowers of *A. reptans* include delphinidin 3 sophoroside-5-glucoside (**115**), cyanidin 3-sophoroside-5-glucoside (**116**), delphinidin 3-(di-*p*-coumaryl)sophoroside-5-malonylglucoside (**117**), cyanidin 3-(di-*p*-coumaryl)sophoroside-5-malonylglucoside (**118**), (Terahara *et al.*, 1996). However, these metabolites have not been isolated from *A. remota* and it is not known if they exhibit antiplasmodial, larvicidal and antioxidant activities.



**115 R = OH**

**116 R = H**

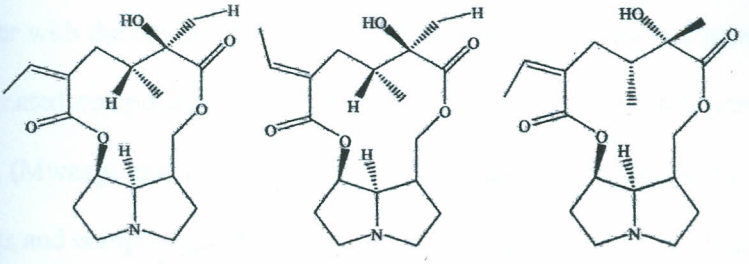


**117 R = H**

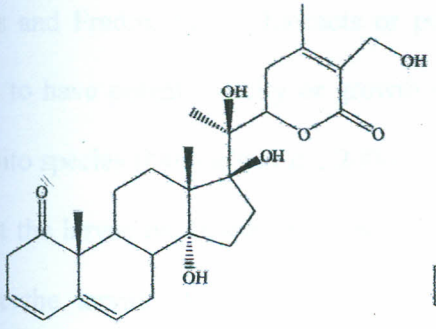
**118 R = OH**

### 2.8.5 Other classes of compounds of genus *Ajuga*

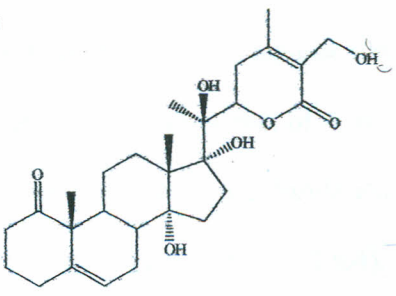
Other classes of compounds reported from the genus *Ajuga* include alkaloids and withanolides among others, examples of alkaloids include senecionine **(119)**, integerrimine **(120)**, ligularine **(121)** isolated from the whole plant of *A. parviflora* (Nawaz *et al.*, 2000). Withanolides reported from the whole plant of *A. parviflora* include ajugin E **(122)** and ajugin F **(123)** (Nawaz *et al.*, 1999). These compounds have not been reported from *A. remota*. Moreover, It is not known if they pocess antiplasmodial, larvicidal and antioxidant activities.



**119** **120** **121**




122




123

## 2.9 Mosquito population control

Reduction of mosquito borne diseases incidences have been achieved through two ways, which include habitat control using chemical and biological control, and personal protection in the form of repellants (Marks and Fradin, 1992). Malaria infection is prevented when *Plasmodium* carrying *Anopheles* mosquitoes are prevented from bitting humans, achieved through destruction of larval breeding sites, use of insecticide-treated bed nets (ITN) combined with vector control and personal protection (JSCPR, 2002). The intervention can be conducted by the communities themselves and has become a major intervention in malaria control. The most effective way to reduce the local population of mosquitoes is to eliminate sources of mosquito breeding zones of standing waters (JSCPR, 2002). Emergence of resistance of *Anopheles* to conventional insecticides together with the environmental concern on the use of synthetic insecticides, in particular halogenated compounds such as DDT **(5)**, has resulted in high prevalence of malaria in Africa (Mwangi and Mukiama, 1988). Consequently this calls for continued search for extracts and compounds which can help in the fight against the malaria vector.

Plant products may be used in malarial control in two ways, as repellants or sources of repellant material to reduce vector contact and in control of mosquito larva and/or adults

(Marks and Fradin, 1992). Extracts or pure isolates of a number of plants have been shown to have potent toxicity or growth inhibitory activities against larvae of different mosquito species (Ndung'u *et al.*, 2004). Petroleum-ether extract of *A. remota* was active against the larvae of *Anopheles stephensi* while carbon-tetra-chloride extract was active against the larvae of *Culex quinquefasciatus* (Sharma *et al.*, 2004). However, the larvicidal pure isolates from this extracts were not determined to verify whether the isolates were individually active or their activity was synergistic in the crude extract. EtOAc extract of the underground part of *A. remota* and ergosterol-5,8-endoperoxide **(4)** associated with the extract were found to be active against 2nd instar larvae of *A. aegypti* (Manguro *et al.*, 2011). Moreover, the larvicidal activity of isolates from MeOH extract were lower than the extract raising the possibility of absence of synergistic activity of compounds in the extract or presence of other more active compounds that were not isolated in the study. The activity of *n*-hexane extract was not established, furthermore, isolation of compounds from this extract was not done.

**2.10 Oxidative stress and antioxidants**

Reactive oxygen species (ROS) are produced by normal and physiological processes in the body as well as the influence of exogenous chemicals. They are usually counteracted by numerous enzymatic and non enzymatic antioxidant systems in cells and biologic fluids that prevent their adverse effects. Under condition of elevated radical production and/or decreased radical defenses as in the case of malaria patients, oxidative tissue damages may occur leading to oxidative stress associated with some pathological phenomenon such as cerebral and pulmonary oedema, poor eyesight, atherosclerosis, cardiac ischemies, rheumatic diseases and cancer (Bahorun *et al.*, 1996). Antioxidants act

by suppressing enzymes responsible for superoxide production, chelating metal ions that induce free radicals and reduction of radicals by donation of hydrogen atoms, as in Figure 2.2 (Pieta, 2000).

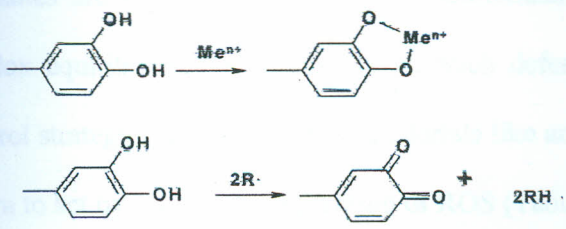



**Figure 2.2:** Mechanisms of anti-oxidants (Pieta, 2000)

Therefore increased antioxidant concentration at the cellular level could provide considerable protection against ROS. Most of the known antioxidants are derived from plants, probably due to their increased capacity to defend themselves from various sources of stress (Athanasas *et al.*, 2004). Aqeous extract of the whole plant of *A. remota* exhibited antioxidant activity (Ohsugi *et al.*, 1999; Israili and Lyoussi, 2009), although the compounds responsible for this activity were not isolated. Aqeous methanolic extract from leaves of *A. remota* exhibited DPPH radical scavenging activity $IC_{50}$ value 8.8 µg/ml (Nasser *et al.* 2010). However, the actual principles responsible for this activity are not known.

### 2.11 Malaria and oxidative stress

As malaria parasites proliferate in a patient, large quantities of ROS are generated which damage macromolecules leading to several complications. The major source of ROS is heme, a byproduct of haemoglobin digestion degradation by *Plasmodium*, whereby the iron II in heme is oxidized to iron III releasing an electron that is used to ionize oxygen

molecules and subsequently forming peroxides which are easily broken to radicals. ROS are also produced by host immune system for the purpose of eradicating the malaria parasite, as the parasite is highly susceptible to oxidative burden (Kawazu *et al.*, 2008). Malaria parasites are therefore equipped with antioxidant defenses that are meant to establish redox equilibrium for their survival. Such defenses are the ones targeted in malaria control strategies. Some of the antimalarials like artemisinin **(2)** and chloroquine **(6)** are known to act by increasing production of ROS (Taoufiq *et al.*, 2006).

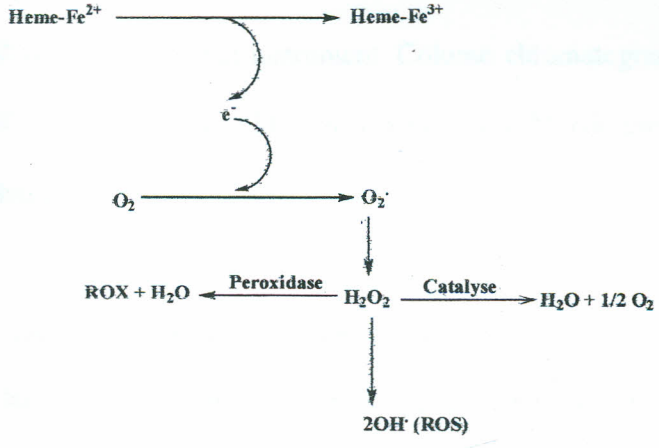



**Figure 2.3**: Generation of ROS from heme (Kawazu *et al.*, 2008)

The effect of ROS to malaria patient, though beneficial as it suffocates the parasite, it is also detrimental since it leads to the damage of some cells and hence bringing about more complications to the patient. It is therefore important to search for plant metabolites that have antioxidant properties to reduce the risk of malaria patients encountering complications associated with elevated oxidative stress encountered in the course of malaria chemotherapy. Finding antioxidant metabolites from the underground part of *A. remota* could go a long way in reducing oxidative stress during malaria therapy.

## CHAPTER THREE: MATERIALS AND METHODS

### 3.1 General

Melting points were determined using Gallenkamp melting point apparatus. The values obtained were uncorrected. The ultraviolet-visible spectra were recorded on Hewlett-Packard Array 8452A spectrophotometer, while the infrared spectra were recorded on Perkin-Elmer FT-IR 600 Series Spectrophotometer. The NMR spectra were recorded in DMSO-$d_6$, $CDCl_3$, and $CDCl_3$-DMSO-$d_6$ on a Bruker NMR advanced Ultrashield TM spectrophotometer operating at 500, 300, 125 and 75 MHz. The EIMS were recorded on a 70 eV MAT 8200 A Varian Bremen instrument. Column chromatography was carried out using Merck 40 (230-400 mesh). TLC was done using Merck pre-coated 60 $F_{254}$ commercial TLC plates

### 3.2 Plant material

Whole plants were collected at Donholm estate, Nairobi (1° 17' S, 36° 49' E) in August 2009 under the guidance of a taxonomist to confirm the authenticity of the plant. Voucher specimen number LOM-AR-2009-05 for *Ajuga remota* was deposited at Kenya National Museum.

### 3.3 Extraction of plant material

The underground part was separated from the aerial part and air dried for one week under shade, ground using a grinding mill to a fine powder. One kilogram of the powder was transferred into a five liter conical flask. Cold extraction was done sequentially using *n*-hexane, EtOAc and MeOH (3 x 3 L each). The extracts were concentrated using a rotary evaporator under reduced pressure to give a light brown *n*-hexane extract, 22.2 g and brown extracts for both EtOAc and MeOH with yields of 34.4 and 82.9g respectively.

The crude extracts were subjected to phytochemical, antiplasmodial, larvicidal and antioxidant activity tests.

### 3.4 Isolation of Compounds

#### 3.4.1 Isolation of components from *n*-hexane extract

The TLC profile of the *n*-hexane extract of the underground part showed components with very close $R_f$ values in (*n*-hexane-EtOAc (1:9, 1:4, 3:7 and 1:1) and ($CH_2Cl_2$-MeOH (1:99, 5:95, 1:9)) solvent systems. An attempt to separate these components using column chromatography was not possible.

#### 3.4.2.1 Preliminary analysis of EtOAc extract

Thin layer chromatography was performed on the EtOAc extract to reveal the components in this extract. Four brown/orange spots were detected upon spraying the analytical TLC plates with concentrated sulphuric acid and then heating at 110 $^0$C. The spots were also exhibited when the TLC plate was exposed to iodine vapour. This indicated that there were four compounds that could be isolated from this extract.

#### 3.4.2.2 Isolation of components from EtOAc extract

Approximately 20.0g of the crude extract was mixed with silica gel in a minimum amount of $CH_2Cl_2$ in a round bottomed flask. The solvent was removed under pressure using a rotary evaporator. The dried material was subjected to flash chromatography using 240 g of silica gel in a 3.0 x 60 cm column at a pressure of 1.5 bar using *n*-hexane-EtOAc and $CH_2Cl_2$-MeOH stepwise gradient elution with increasing polarities, collecting 310 fractions 20 ml each. The homogeneity of the fractions was monitored by TLC. Out of the 310 eluates, fractions 5-215 had similar TLC profile and were pooled together, to give pool I (5.3 g) upon removal of the solvent.

This was rechromatographed over silca gel column using 10% EtOAc in *n*-hexane to give 30 fractions 20 ml each. Fractions 1-12 gave β-Sitosterol **(69)** a white crystalline solid (118.5mg) on crystallization (*n*-hexane-EtOAc (9:1)). $R_f$ value 0.71 (*n*-hexane-EtOAc (9:1)) and a melting point in the range of 142-143 $^0$C.

Fractions 13-30 gave stigmasterol **(70)**.white crystalline needles (102.4 mg) upon removal of solvent and crystallization, which was filtered under pressure, its melting point determined to be in the range of 144-145 $^0$C indicating that it was a pure compound. It had an $R_f$ value 0.64 (EtOAc-*n*-hexane (1:9)).

Similarly, fractions 216-310 had similar TLC profile and were pooled together to give pool II (8.3 g), which was fractionated over silca gel column (4.0x60cm, $SiO_2$ 160g) collecting 95 fractions 20 ml each. Fractions 1-42 gave one spot and were pooled together, solvent removed and on crystallization gave a white crystalline powder (85.4 mg) in 5% MeOH in $CH_2Cl_2$ with $R_f$ value 0.61 (MeOH-$CH_2Cl_2$ (1:99)) with a melting point 173-174 $^0$C. Spectroscopic experiments led to the deduction of this compound as 7β-hydroxylup-5,20(29)-diene-3-one **(124)**.

Fractions 43-87 had similar TLC profiles and were pooled together, and on crystallization ($CH_2Cl_2$-MeOH, (9:1)) gave white crystals (66.3 mg) with an $R_f$ value of 0.43 (MeOH-$CH_2Cl_2$ 1:99)) and a melting point in the range 279-280 $^0$C which was further deduced to be betulinic acid **(40)** with the aid of spectroscopic experiments.

### 3.4.2.3 Physical and spectroscopic properties of compounds isolated from the EtOAc extract.

**β –Sitosterol (69)**

White crystalline needles, mp 143-144 °C (Lit. 144-146, Pateh *et al.*, 2008), $[\alpha]_D^{25} = -37^\circ$ ($CHCl_3$, c 2.0). $R_f$ value 0.71 (*n*-hexane-EtOAc (9:1)) $^1H$ NMR and $^{13}C$ NMR (See Table 4.1) EIMS *m/z* (%): 414.36 (25) $[M^+]$, 396 (60) $[M^+-H_2O]$, 329 (10) $[M^+-C_6H_{13}]$, 351 (20) $[M^+-C_4H_9+H_2O]$, 273 (25) $[M^+-C_{10}H_{21}]$, 255 (70) $[M+-C_{10}H_{21}-H_2O]$ (Habib *et al.*, 2007; Pateh *et al.*, 2009; Kamboj and Saluja, 2011)

**Stigmasterol (70)**

White crystalline powder, mp 144-145 °C (Lit. 144-146 °C, Kamboj and Saluja, 2011), $[\alpha]_D^{20} = -52^\circ$ ($CHCl_3$, c 2.0), $R_f$ value 0.64 (*n*-hexane-EtOAc (9:1)). UV $\lambda_{max}$($CH_3CN$); 192.5nm. IR $\upsilon_{max}$(KBr) $cm^{-1}$; 3424-3073 (OH), 1644-1456 (C=C). $^1H$ NMR and $^{13}C$ NMR (See Table 4.2). EIMS *m/z* (%) $M^+$; 412.5 (100), 394.6 (9), 329 (22), 355 (1), 256.2 (40) (Habib *et al.*, 2007; Pateh *et al.*, 2009; Kamboj and Saluja, 2011).

**7β-Hydroxylup-5,20(29)-diene-3-one (124)**

White crystalline powder, mp 173-174 ° C. $R_f$ value 0.61 ($CH_2Cl_2$-MeOH (99:1)) $^1H$ NMR and $^{13}C$ NMR (See Table 4.3)

**Betulinic acid (40)**

White crystals, mp 279-280 °C (Lit., mp 279-280 °C, Igoli and Gray, 2008), $[\alpha]_D^{25} = +10.12^\circ$ ($C_5H_5N$, c 0.4). $R_f$ value of 0.43 in ($CH_2Cl_2$-MeOH (99:1)). IR $\upsilon_{max}$ (KBr disc) $cm^{-1}$: 2911 (C-H), 1452 (C=C), 1041 (C-O) 3550-3100 (OH). $^1H$ NMR and $^{13}C$ NMR (See Table 4.4) (Igoli and Gray, 2008; Sharma *et al.*, 2010).

### 3.4.3.1 Isolation of components from MeOH extract

Approximately 40.0 g of the MeOH extract was mixed with silica gel in a minimum amount of MeOH and dried under pressure using a rotary evaporator to give a free flowing mixture. The whole product was loaded onto a silca gel column (3.0 x 60 cm, $SiO_2$ 400g) and elution started with 10% and 20% EtOAc in *n*-hexane followed by 5% and 20% MeOH in $CH_2Cl_2$, elution was concluded with 20% MeOH in EtOAc. 152 fractions 20 ml each were collected. The homogeneity of the fractions was monitored by TLC using the same solvent system as in elution. Fractions showing similar TLC profiles were grouped together to give four pools.

Pool I consisted of fractions 1-25 which gave 3.6 g of a white solid after removal of the solvent and was further purified by flash chromatography. Elution with 5% MeOH in $CH_2Cl_2$ afforded 37 mg of 8-*O*-acetylharpagide **(93)**, $R_f$ value 0.44 ($CH_2Cl_2$-MeOH (99;1)), while elution with 10% MeOH in $CH_2Cl_2$ gave 45.5 mg of 6,8-diacetylharpagide-1-*O*-β-(2',3'-diacetylglucoside) **(95)**, $R_f$ value 0.23 ($CH_2Cl_2$-MeOH (99;1)) and 65.3 mg of arjungenin **(125)**, $R_f$ value 0.19 ($CH_2Cl_2$-MeOH (99;1)).

Pool II consisted of fractions 26-52 which gave 2.5 g upon removal of solvent which was subjected to flash chromatography (4.0x60cm, $SiO_2$ 60g) elution with 5% and 10% MeOH in $CH_2Cl_2$ gave 85 fractions 20ml each. Fractional crystallisation of fractions 5-31 yielded 83.5 mg of kaempferol 3-*O*-β-rhamnoside **(126)** $R_f$ value 0.72. Fraction 32-52 afforded 104 mg quercetin 3-*O*-β-galactoside **(127)** $R_f$ value 0.65. Fractions 33-83 gave 92.5 mg of quercetin 3-*O*-β-(6-*O*-α-rhamnosyl) galactoside **(128)** $R_f$ value 0.61.

Pool III (fraction 53-78), 5.6g was subjected to further purification by repeated flash chromatography (4.0 x 60 cm, $SiO_2$ 100g), eluent 20 and 30% MeOH in $CH_2Cl_2$

collecting 65 fractions 20 ml each. Fractional crystallization of fraction 3-35 gave 86.5 mg of myricetin **(113)** $R_f$ value 0.58. While, fractions 37-63 gave 110 mg of myricetin 3-*O*-α-rhamnoside **(129)** $R_f$ value 0.46.

Pool IV consisting of fractions 79-152 (7.8 g) gave two components when subjected to repeated flash chromatography (4.0x60cm, $SiO_2$ 80g), eluent 20% MeOH in EtOAc. However upon exposure of these components to the atmosphere, they dissolved in the solvent indicating that they were highly hygroscopic.

**3.4.3.2 Physical and spectroscopic properties of compounds isolated from the MeOH extract.**

**8-*O*-Acetyharpagide (93)**

Colourless crystals, $R_f$ value 0.44 ($CH_2Cl_2$-MeOH (99:1)) mp 227-228 °C (Lit, 225-228 °C, Kuria *et al.*, 2002). The UV $\lambda_{max}$ ($CH_3CN$); 200nm. IR $\upsilon_{max}$ (KBr) $cm^{-1}$; 3439-3062 (OH), 1711 (ester), 1653, 1040-1010 (C-O). $^1H$ NMR and $^{13}C$ NMR (See Table 4.5) (Kuria *et al.*, 2002; Manguro *et al.*, 2011).FABMS *m/z* (%); 405 (24) $[M-H]^-$, 451 (100) $[M+HCO_2]^-$, 811 (44) $[2M-H]^-$, 856.7 (70) $[2M+ HCO_2]^-$.

**6,8-Diacetylharpagide-1-*O*-β-(2',3'-diacetylglucoside) (95)**

Colourless Crystals $R_f$ value 0.23 ($CH_2Cl_2$-MeOH (99:1)), mp 174-175 °C (Lit. 174-176 °C, Manguro *et al.*, 2006), $[\alpha]_D^{25} = +92^o$ ($CH_2Cl_2$, c 1.5). $^1H$ NMR and $^{13}C$ NMR (See Table 4.6) (Manguro *et al.*, 2006)

**Arjungenin (125)**

White solid, $R_f$ value 0.23 ($CH_2Cl_2$-MeOH (99:1)), mp 291-293 °C (Lit, mp 292-294 °C, Ullah *et al.*, 2007)., $[\alpha]_D^{22} = +39^o$ (MeOH, c 0.6). $^1H$ NMR and $^{13}C$ NMR (See Table

4.7). ESIMS *m/z* $[M+ Na]^+$ 527.3. (Nandy *et al.*, 1989; Jossang *et al.*, 1995; Ullah *et al.*, 2007).

**Kaempferol 3-*O*-β-rhmnoside (126)**

Yellow needles, $R_f$ value 0.72 ($CH_2Cl_2$-MeOH (95:5)). $^1H$ NMR and $^{13}C$ NMR (See Table 4.8) (Lin and Lin, 1999)

**Quercetin 3-*O*-β-galactoside (127)**

Yellow powder, $R_f$ value 0.65 ($CH_2Cl_2$-MeOH (95:5)), mp 230-231 °C (Lit, 231-232 °C, Liu *et al.*, 2008). $[\alpha]_D^{20} = -83°$ ($C_5H_5N$, c 0.2). UV $\lambda_{max}$ (MeOH) 358, 300, 258; (MeOH + $AlCl_3$) 436, 274; (MeOH + $AlCl_3$/HCl) 406, 300, 270; (MeOH + NaOMe) 408, 328, 272; (MeOH + NaOAc) 374, 322, 274; (MeOH + NaOAc/$H_3BO_3$) 378, 262 nm. $^1H$ NMR and $^{13}C$ NMR (See Table 4.9). EIMS *m/z* (%) $M^+$; 303 (10), 302 (100), 285 (11), 275 (10), 229 (10), 217 (4), 153 (10), 137 (13) (Tsimogiannis *et al.*, 2007).

**Quercetin 3-*O*-β-(6-*O*-α-rhamnosyl) galactoside (128)**

Yellow amorphous powder, $R_f$ value 0.61 ($CH_2Cl_2$-MeOH (9:1)), mp 181-182 °C (Lit, 180-182 °C, Liu *et al.*, 2008), $[\alpha]_D^{23} = -39.3°$ ($C_5H_5N$, c 0.2). UV $\lambda_{max}$ (MeOH) 358, 300, 258; (MeOH + $AlCl_3$) 436, 304, 274; (MeOH + NaOMe) 410, 330, 272; (MeOH + NaOAc) 384, 324, 272; (MeOH + NaOAc/$H_3BO_3$) 378, 262 nm.$^1H$ NMR and $^{13}C$ NMR (See Table 4.10). EIMS *m/z* (%) $M^+$; 302 (100), 275 (10), 247 (10), 229 (10), 153 (28), 137 (25) (Tsiamogiannis *et al.*, 2007).

**Myricetin (113)**

Yellow needles from $CH_2Cl_2$-MeOH, $R_f$ value 0.58 ($CH_2Cl_2$-MeOH (4:1)), mp 229-230 °C (Lit, mp 230-231 °C, David *et al.*, 1996). UV $\lambda_{max}$(MeOH) 376, 304, 254; (MeOH + $AlCl_3$) 462, 312, 270; (MeOH + $AlCl_3$/HCl) 432, 362, 308, 266; (MeOH + NaOMe) 318,

286; (MeOH + NaOAc) 388, 318, 274; (MeOH + $NaOAc/H_3BO_3$) 392, 304, 258 nm.$^1$H NMR and $^{13}$C NMR (See Table 4.11). EIMS *m/z* (%) $M^+$; 318 (100), 289 (5), 243 (10), 165 (1), 153 (16), 136 (4) (Tsiamogiannis *et al.*, 2007).

**Myricetin 3-*O*-α-rhamnoside (129)**

Pale yellow armorphous powder from $CH_2Cl_2$-Methanol mixture, $R_f$ ($CH_2Cl_2$-MeOH (4:1)) mp 195-197 °C (lit. 197-201 °C, Zhong *et al.*, 1997) $[\alpha]_D^{21}$ = -152.8° (MeOH, c 0.89). UV $\lambda_{max}$(MeOH) 354, 302, 258; (MeOH + $AlCl_3$) 436, 312, 270; (MeOH + $AlCl_3$/HCl) 400, 358, 308, 272; (MeOH + NaOMe) 394, 322, 270; (MeOH + NaOAc) 370, 316, 272; (MeOH + $NaOAc/H_3BO_3$) 374, 302, 260 nm.$^1$H NMR and $^{13}$C NMR (See Table 4.12). EIMS *m/z* (%) $M^+$; 319 (30), 318 (100), 289 (12), 261 (38), 244 (10), 166 (6), 153 (40), 136 (20) (Tsiamogiannis *et al.*, 2007).

**3.5 Acid hydrolysis**

About 5 mg of Compound **93**, **95**, **126**, **127**, **128** and **129**, each in a mixture of 8% HCl (2ml) and methanol were separately refluxed for 2 hours. The reaction mixtures were reduced *in vacuo* to dryness, dissolved in 2 ml of water and neutralized with NaOH. The neutralized products were subjected to TLC analysis (EtOAc-MeOH-$H_2O$-HOAc, (6:2:1:1)) and PC (*n*-BuOH-HOAc-$H_2O$, (4:5:1)) and ($C_6H_6$-*n*-BuOH-$H_2O$-pyridine, (1:5:3:3)). The chromatograms were sprayed with aniline hydrogen phthalate followed by heating. The sugars were identified after comparison with authentic samples (Manguro *et al.*, 2006).

**3.6 Evaluation of *in vitro* antiplasmodial activity**

The objective of this testing was to determine the biopotency of crude extracts and pure isolates from underground part of *A. remota* against two clones of laboratory adopted *P.*

*falciparum* cultures Sierra-Leone chloroquine-sensitive (D6) and Indochina Chloroquine-resistant (W2) strains.

### 3.6.1 Preparation of bioassay test samples

200 µg/ml stock solutions of extracts and pure isolates were made in DMSO followed by subsequent dilutions to lower concentrations. Stock solutions of Reference drugs, chloroquine **(8)**, quinine **(1)** and mefloquine **(13)** were similarly prepared, and all solutions stored at -20°C until used.

### 3.6.2 Bioassay and Computation of data

*In vitro* antiplasmodial activities were done at Kenya Medical Research Institute (KEMRI). The semi automated microdilution assay technique was used to determine the $IC_{50}$ (concentration of crude extract/compound required to induce 50% growth inhibition of cultured parasites) (Desjardins *et al.*, 1979; Muthaura *et al.*, 2007a).

The samples were serially diluted across a 96 well microliter plate to provide a range of concentrations used to determine the $IC_{50}$ values. A constant volume of parasitized blood was added and plates incubated for 24 hours in mixed gas (91% $N_2$, 6% $O_2$, 3% $CO_2$) incubator. A radiolabelled nucleic acid ($^3H$) hypoxanthine was added and the parasites allowed growth for an additional 18 hours. After freezing at -20°C and thawing at 20°C, the cells were processed on a plate harvester (Filtermate) onto filter mats. The filter mats were then counted on a liquid scintillation counter and luminescence counter. The raw data from the scintillation counter in form of Excel spreadsheet were imported into Data Aspects Plate Manager Program to determine the $IC_{50}$ values. A minimum of three replicates for each sample were done (Muthaura *et al.*, 2007a).

### 3.7 Evaluation of larvicidal activities

The objective of this testing was to determine the inherent biopotency of the crude extracts and pure isolates of underground part of *A. remota* against second instar larvae of *A. aegypti*. The larvae were exposed for 24 hours in water treated with larvicides at various concentrations within their activity range and mortality was recorded.

#### 3.7.1 Preparation of test solutions

20 mg of compound/extract dissolved in 1 ml DMSO were added to dechlorinated water in a container and the final volume adjusted to make 200 µg/ml of stock solution. Six-fold dilutions of stock solution were prepared to provide working concentration range (100, 50, 25, 12.5, 6.25 and 3.13) µg/ml. Positive control used commercial malathion prepared in a similar manner as the samples. Negative control was prepared by adding 1 ml of DMSO in 100 ml of dechlorinated water and making six-fold dilutions as before (de Omena *et al.*, 2007).

#### 3.7.2 Bioassay

Larvicidal activities were conducted following standard WHO procedures (de Omena *et al.*, 2007) using larvae of *A. aegypti*. Eggs of *A. aegypti* were hatched by submerging them in dechlorinated tap water at temperatures in the range 25-27°C. Twenty-second instar larvae (larvae in the second larval stage of mosquito life circle) were transferred by means of droppers to small disposable vails each containing 10ml of water in each of the six containers with different concentrations. Small unhealthy or damaged larvae were removed and replaced. During the assays, proprietary cat food was offered to the larvae. Three replicates were set up for each concentration and the controls. The assay was conducted in darkness (except during counting at time = 0 hours and time = 24 hours

when the larvae were briefly illuminated by light) at temperatures of 25-27°C. Mortality was observed after twenty four hours. Moriband larvae were counted and added to living larvae for the purpose of calculating percentage mortality. Moriband larvae were those incapable of rising to the surface or not showing the characteristic diving reaction when the water is disturbed. Dead larvae were those which if induced to move by probing with a needle at the siphon or the cervical region could not move.

### 3.7.3 Data Analysis

$LC_{50}$ values (concentration required to kill 50% of larvae, after twenty four hours) were calculated from an average of three replicates using Finney's probit analysis for quantal data (Mclaughlin *et al.*, 1991) using SPSS computer software program.

### 3.8 Antioxidant activity test

This test was aimed at determining, 1,1-diphenyl-2-picryhydrazyl (DPPH) radical scavenging activity of the crude extracts and pure isolates of the underground part of *A. remota*.

### 3.8.1 Radical scavenging activity

The tests were performed using two methods; TLC method and UV-VIS spectrometry method. The TLC method gave preliminary qualitative results and the UV-VIS spectrometry method generated quantitative data. Both methods employed a stable 1,1-diphenyl-2-picryhydrazyl (DPPH) radical (Hou *et al.*, 2002; Zou *et al.* 2004).

The TLC method required preparation of DPPH solution by dissolving 20 mg of DPPH in 100ml of methanol to make 200 µg/ml of the solution. This solution was sprayed on dry TLC spotted with compounds/extracts and developed on appropriate solvent. Activity

was observed through appearance of yellowish or white spots on purple sprayed TLC plate.

The UV-VIS spectrometry method was adopted from Zou *et al.*, (2004) with minor changes to determine scavenging effect of different concentrations of pure isolates/extracts on DPPH. This method was modified as follows, 3ml of DPPH (0.0002M) was mixed with equivalent aliquot of 3ml of different concentrations of the samples (100, 50, 25, 12.5, 6.25 and 3.13) µg/ml, and after standing in the dark for 30 minutes, absorbance at 517nm was measured. DPPH having zero concentration of sample was used as negative control (blank) and quercetine as the positive control. The absorbance measured at each of these intervals was converted into percentages of scavenged DPPH radicals using the following equation

$$\% \text{ of scavenged DPPH} = \left( \frac{A_{blank} - A_{sample}}{A_{blank}} \right) \times 100$$

Where $A_{blank}$ is the absorbance of DPPH solution having zero concentration of the sample. In all cases the mean values were used for triplicate experiments.

**3.8.2 Data Analysis**

$IC_{50}$ values were calculated using Finney's probit analysis for quantal data (Mclaughlin *et al.*, 1991), using SPSS computer software program.

# CHAPTER FOUR: RESULTS AND DISCUSSION

## 4.1 Components in *n*-hexane extract

It was not possible to separate components in the *n*-hexane extract using column chromatography because they had very close $R_f$ values in the range between 0.1 to 0.7 in (EtOAc-*n*-hexane (1:9, 1:4, 3:7 and 1:1)) and (MeOH-$CH_2Cl_2$ (1:99, 19:1, 9:1)) solvent systems.

## 4.2 Compounds Isolated from EtOAc extract

Isolation of components from the EtOAc crude extracts was achieved by flash chromatography using silica gel and eluting with mixtures of *n*-hexane-EtOAc and $CH_2Cl_2$- MeOH successively. A total of four compounds were realized. Their structures were determined by their $R_f$ values, melting points and spectroscopic techniques such as IR, UV, mass spectrometry (MS), $^1H$ NMR and $^{13}C$ NMR. Detailed spectroscopic data for compounds isolated are summarized in their Tables 4.1-4.4 and their spectra are shown in the appendices section.

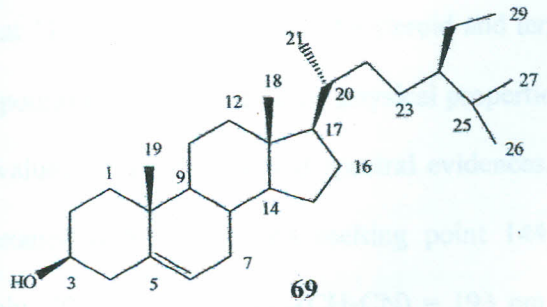
### 4.2.1 β-Sitosterol (69)

Compound **69** was isolated as white crystalline needles, with mp 143-144 °C (Lit. 144-146, Pateh *et al.*, 2009) with $R_f$ value 0.71 in (*n*-hexane-EtOAc (9:1)) mixture. It gave brown/orange spots on silica gel TLC plate when exposed to iodine and also when sprayed with concentrated sulphuric acid, then heated at 110 $^0$C. It gave positive test to Liberman-Buchard reagent for steroid and triterpenoid compounds (Pateh *et al.*, 2009). The EIMS (Appendix 1C) showed a molecular ion peak at *m/z* 414.36 corresponding to $C_{29}H_{50}O$. The fragmentation ion at *m/z* 396.35 was attributed to loss of water from the molecular ion. It showed another fragmentation ion at *m/z* 329.31 attributed to probable

fragmentation between C-23 and C-24, a characteristic of phytosterols, fragment at *m/z* 273 due to loss of $C_{10}H_{21}$ from molecular ion and *m/z* 255 due to dehydration of fragment at *m/z* 273 (Habib *et al.*, 2007; Pateh *et al.*, 2009; Kamboj and Saluja, 2011). The $^{13}C$ NMR spectrum (Appendix 1B) of compound **69** showed signals at δ 140.8 and δ 121.7 which were assigned to C-5 and C-6 vinyl carbon atoms respectively as in $\Delta^5$ spirostene (Agrawal *et al.*, 1985; Pateh *et al.*, 2009). The signal at δ 71.8 was attributed to hydroxylated C-atom at C-3 (Pretsch and Affolter, 2000), while peaks at δ 19.4 and δ 11.8 were attributed to C-19 and C-18.respectively. The value for C-18 is lower due to γ-gauche interaction that increases the screening of the C-18 hence a lower chemical shift (Smith, 1978). However, the loss of H in C-6 results in decrease in the screening of C-19 leading to increase in the $^{13}C$ chemical shift to a higher frequency (Smith, 1978). The spectra further revealed four additional methyl resonances at 18.8 (C-21), 19.8 (C-26), 19.0 (C-27) and 12.0 (C-29).

The $^{1}H$ NMR spectrum (Appendix 1A) of compound **69** corroborated the $^{13}C$ NMR spectrum results. A multiplet at δ 5.36 led to the conclusion that there was a single carbon-carbon double bond and this multiplate was attributed to to the vinylic proton at H-6. Another multiplate at δ 3.51 was assigned to the hydroximethine proton at C-3. The β-sitosterol structure was further supported by two intense singlets integrating for three protons shifts which appeared as singlets at δ 0.68 and δ 0.87 due to tertiary methyl groups at C-18 and C-19 respectively. An overlapped doublet at δ 0.92 was attributed to secondary methyl protons at C-21. Doublets at δ 0.81 with a coupling constant 7 Hz were assigned to peaks for two equivalent methyl protons at C-26 and C-27. Consequently, basing on spectroscopic data and comparison with literature (Pateh *et al.*, 2009)

compound **69** was confirmed to be β-sitosterol, isolated previously from *A. bracteosa* (Nawaz *et al.*, 1999) and *A. postii* (Gören *et al.*, 2005 a; 2005 b) but this is the first report of this compound from *A. remota*.




**69**

**Table 4.1: $^{1}$H (500 MHz, $CDCl_3$) and $^{13}$C NMR (125 MHz, $CDCl_3$) data for β-sitosterol (69)**

| ATOM | $^{1}$H *m* (*J* in Hz) | $^{13}$C | $^{1}$H *m* (*J* in Hz)* | $^{13}$C** |
|---|---|---|---|---|
| 1 | | 37.3 | | 37.3 |
| 2 | | 31.7 | | 31.6 |
| 3 | 3.51 *m* | 71.8 | 3.52 *m* | 71.8 |
| 4 | | 42.3 | | 42.2 |
| 5 | | 140.8 | | 140.8 |
| 6 | 5.36 *m* | 121.7 | 5.36 *brs* | 121.7 |
| 7 | | 31.9 | | 31.9 |
| 8 | | 31.9 | | 31.9 |
| 9 | | 50.1 | | 51.2 |
| 10 | | 36.5 | | 36.5 |
| 11 | | 21.1 | | 21.1 |
| 12 | | 39.8 | | 39.9 |
| 13 | | 42.3 | | 42.3 |
| 14 | | 56.8 | | 56.8 |
| 15 | | 24.3 | | 24.3 |
| 16 | | 28.2 | | 28.3 |
| 17 | | 56.1 | | 56.0 |
| 18 | 0.68 *s* | 11.8 | 0.68 *s* | 11.9 |
| 19 | 0.87 *s* | 19.4 | 1.01 *s* | 19.4 |
| 20 | | 36.1 | | 36.2 |
| 21 | 0.92 *d* (6.9) | 18.8 | 0.92 *d* (6.4) | 18.8 |
| 22 | | 34.0 | | 33.9 |
| 23 | | 26.1 | | 26.1 |
| 24 | | 45.8 | | 45.9 |
| 25 | 0.84 *d* (7.0) | 29.2 | 0.83 *d* (6.5) | 29.2 |
| 26 | 0.81 *d* (7.0) | 19.8 | 0.81 *d* (6.5) | 19.8 |
| 27 | 0.81 *d* (7.0) | 19.0 | | 19.3 |
| 28 | 0.85 *t* (7.3) | 23.1 | 0.85 *t* (7.5) | 23.1 |
| 29 | | 12.0 | | 12.2 |

* $^{1}$H NMR & ** $^{13}$C NMR (400 MHz, $CDCl_3$) for β-sitosterol (Pateh *et al.*, 2009)

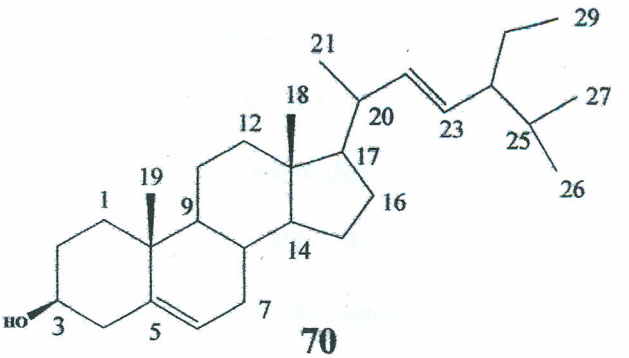
### 4.2.2 Stigmasterol (70)

Compound **70** was isolated as white crystalline needles. It gave brown/orange spots on silica gel TLC plate exposed to iodine and also by spraying with concentrated sulphuric acid, then heated at 110 $^{0}$C. It responded to the steroid and terpenoid test reagents in the same way as compound **69,** difference in the physical properties of these two compounds were in their $R_f$ values, melting point and spectral evidences. Compound **70** had an $R_f$ value 0.64 (*n*-hexane-EtOAc (9:1)) and melting point 144-145°C (Lit. 144-146 °C, Kamboj and Saluja, 2011). The UV $\lambda_{max}(CH_3CN)$ = 193 nm (Appendix 2A) indicating presence of carbon-carbon double bonds. The IR (KBr disc) spectrum (Appendix 2B) 3424-3073 and 1644-1456 $cm^{-1}$ suggested the presence of OH and –C=C- bonds respectively. The EIMS (Appendix 2E) of the compound showed a molecular ion peak at *m/z* 412.5 corresponding to $C_{29}H_{48}O$ with a fragmentation pattern characteristic of sterols. The fragment ion at *m/z* 394.6 corresponded to loss of water from the molecular ion peak. A peak at *m/z* 329 for [$M^+$-methylpentyl] and *m/z* 355 for [$M^+$-isopropyl-$H_2O$] in the mass spectrum comfirmed presence of unsaturation between C-22 and C-23. A fragment ion at *m/z* 256.2 for loss of side chain and water confirmed unsaturation between C-5 and C-6 (Habib *et al.*, 2007; Pateh *et al.*, 2009; Kamboj and Saluja, 2011).

The $^{13}$C and DEPT NMR spectra had 29 distinct signals which can be accounted for by six methyl, nine methylene, eleven methine and three quarternary carbon atoms. The $^{13}$C spectrum showed two sets of olefinic carbon chemical shifts at δ140.4 (C-5) and δ121.1 (C-6) due to trisubstituted functionality, which undoubtedly proved the presence of an endocyclic double bond carbon atoms (Habib *et al.*, 2007), whereas, the chemical shift at δ 136.9 and δ 129.5 were attributed to C-22 and C-23 respectively. The spectrum further

exhibited the presence of six methyl C-atoms at δ 11.6, δ 11.9, δ 20.6, δ 20.6, δ 19.0 and δ 11.9 for C-18, C-19, C-21, C-26, C-27, and C-29 respectively. It also showed the presence of a hydroxylated methine carbon at δ 70.1 for C-3 which signified the presence of a cholestane skeleton (Habib *et al.*, 2007).

The $^{1}H$ NMR spectrum (Appendix 2C) corroborated the $^{13}C$ NMR results. The broad singlet at δ 5.33 (H-6) and a multiplet at δ 3.42 (H-3) are characteristic features of sterols (Guyot *et al.*, 1982). The spectrum further showed a two proton multiplate at δ 5.21 consistent with the existence of a double bond between C-22 and C-23 which led to the suggestion that the compound is stigmasterol (Habib *et al.*, 2007). The Stigmasterol structure was also supported by proton chemical shifts, in which tertiary methyls appeared as singlets at δ 0.65 and δ 0.98. Signals of secondary methyl groups at δ 1.04, δ 0.77 and δ 0.88, and the chemical shift at δ 0.81 attributed to a primary methyl group. $^{1}H$-$^{1}H$ COSY indicated that the broad singlet at δ 5.33 for H-6 was coupled to a multiplet at δ 1.90 for the methylene protons at C-7. Furthermore, HSQC (Appendix 2G) experiments confirmed the olefinic bond between C-22 and C-23. Consequently, on the basis of physical and spectroscopic data, and comparison with literature (Pateh *et al.*, 2009) compound **70** was confirmed to be stigmasterol.




**70**

**Table 4.2: $^{1}H$ (500 MHz, DMSO) and $^{13}C$ (125 MHz, DMSO) NMR data for Stigmasterol (70)**

| ATOM | $^{1}H$ *m* (*J* in Hz) | $^{13}C$ | $^{1}H$ *m* (*J* in Hz)* | $^{13}C$** |
|---|---|---|---|---|
| 1 | | 38.3 | | 37.3 |
| 2 | | 31.3 | | 31.6 |
| 3 | 3.42 *m* | 70.1 | 3.52 *m* | 71.8 |
| 4 | | 41.8 | | 42.3 |
| 5 | | 140.4 | | 140.8 |
| 6 | 5.33 *brs* | 121.1 | 5.36 *brs* | 121.7 |
| 7 | 1.90 *m* | 31.4 | | 31.9 |
| 8 | | 31.4 | | 31.9 |
| 9 | | 51.2 | | 51.2 |
| 10 | | 36.8 | | 36.5 |
| 11 | | 20.5 | | 21.1 |
| 12 | | 39.8 | | 39.7 |
| 13 | | 41.7 | | 42.3 |
| 14 | | 56.2 | | 56.9 |
| 15 | | 23.8 | | 24.4 |
| 16 | | 28.6 | | 28.4 |
| 17 | | 56.1 | | 56.1 |
| 18 | 0.65 *s* | 11.8 | 0.70 *s* | 11.0 |
| 19 | 0.98 *s* | 20.6 | 1.01 *s* | 21.2 |
| 20 | | 40.3 | | 40.5 |
| 21 | 1.04 *d* (8.1) | 20.6 | 1.02 *d* (7.5) | 21.2 |
| 22 | 5.21 *m* | 136.9 | | 138.3 |
| 23 | 5.21 *m* | 129.5 | | 129.3 |
| 24 | | 51.2 | | 51.2 |
| 25 | | 31.4 | | 31.9 |
| 26 | 0.77 *d* (4.2) | 20.6 | 0.80 *d* (6.5) | 21.2 |
| 27 | 0.88 *d* (6.3) | 19.0 | 0.85 *d* (6.5) | 19.0 |
| 28 | | 25.9 | | 25.4 |
| 29 | 0.81 *t* (3.3) | 11.9 | 0.80 *t* (7.5) | 12.1 |

* $^{1}H$ NMR & ** $^{13}C$ NMR (400 MHz, $CDCl_3$) for stigmasterol (Pateh *et al.*, 2009)

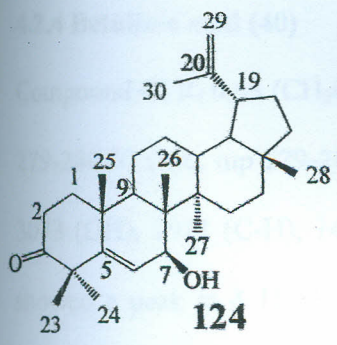
### 4.2.3 7β-Hydroxylup-5,20(29)-diene-3-one (124)

Isolated as white crystalline powder $R_f$ 0.61 ($CH_2Cl_2$: MeOH (99:1)) mixture. The $^{1}H$ NMR spectrum (Appendix 3A) of this compound displayed signals typical of pentacyclic triterpenoid methyl groups at δ 0.83, 0.92, 1.00, 1.01, 1.08. This was in agreement with the $^{13}C$ NMR spectrum (Appendix 3B) which had signals at δ 14.8 (C-28), δ 18.0 (C-25), δ 16.9 (C-27), δ 21.3 (C-24), δ 26.2 (C-23) and δ 17.7 (C-26) (Pech *et al.*, 2002). The

presence of a methyl broad singlet at δ 1.69 and two vinyl proton signals at δ 4.68 *d* (*J* =2.0 Hz) and δ 4.55 *dd* (*J* =3.5, 1.5 Hz) with $^{13}C$ peaks at δ 19.3 for C-30, δ 150.8 for C-20 and δ 109.4 for C-29 which were confirmed by HSQC experiments are characteristic of isopropenyl group of triterpenes of the lupene type (Pech *et al.*, 2002).

$^{1}H$ NMR spectrum had a doublet at δ 4.46 *d* (*J*= 6.5 Hz) due to a hydroxymethine at δ 69.7 for C-7. The multiplicity and coupling constant values indicated an axial orientation of the proton which limited the locations to C-7, C-15 or C-16. Positions C-15 and C-16 were eliminated by looking at the HMBC spectrum which showed long range coupling between the hydroxyl carbon at δ 69.7 for C-7 and methyl protons at δ 1.08 for H-26 and δ 1.65 for H-13. Comparing the $^{13}C$ NMR data of this compound with those of lupenone were in agreement with the presence of the keto group at C-3. HMBC experiments confirmed the presence of the carbonyl group at C-3 and the hydroxyl group at C-7. Long range couplings were observed between the carbonyl signal at δ 216.7 and the gem-dimethyl proton signals at δ 1.08 and δ 1.01.

The $^{13}C$ NMR spectrum had two olefinic signals at δ 142.6 for C-5 and δ 130.0 for C-6 which proved the presence of endocyclic olefinic carbon atoms. This was corroborated by $^{1}H$ NMR spectrum which had a singlet at δ 4.84 for H-6. This was absent in the $^{1}H$ NMR data for 16β-hydroxylup-20(29)-ene-3-one (Pech *et al.*, 2002). The β-orientation of the hydroxyl group was shown by the large coupling constant of 6.5 Hz at δ 4.46 for H-7. $^{1}H$-$^{1}H$ COSY confirmed that the double doublet at δ 4.55 (H-29a) was coupled to the broad singlet at δ 1.69 (H-30) which was also coupled to the doublet at δ 4.68 (H-29b). Therefore the structure of this compound **124** was determined as 7β-Hydroxylup-5,20(29)-diene-3-one a compound isolated for the first time from *A. remota.*



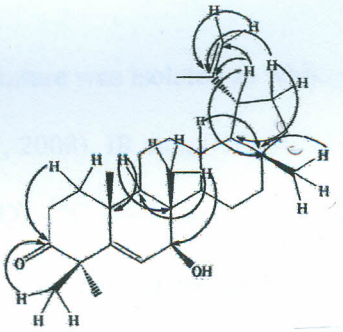



**Figure 4.1**: Significant correlations in the HMBC spectrum of compound **124**

**Table 4.3: $^{1}H$ (500 MHz, $CDCl_3$) and $^{13}C$ (125 MHz, $CDCl_3$) NMR data for 7β-hydroxylup-5,20(29)-diene-3-one (124)**

| ATOM | $^{1}H$ *m* (*J* in Hz) | $^{13}C$ | $^{1}H$ *m* (*J* in Hz)* | $^{13}C$** |
|---|---|---|---|---|
| 1 | 1.38 *m* | 39.9 | a: 1.38 *m*, b:1.90 *m* | 39.6 |
| 2 | a: 2.37 *m* b: 2.38 | 34.4 | a: 2.40 *m*, b: 2.49 *m* | 34.2 |
| 3 | | 216.7 | | 218.1 |
| 4 | | 47.9 | | 47.3 |
| 5 | | 142.6 | 1.30 *m* | 54.9 |
| 6 | 4.84 *s* | 130.0 | 1.48 *m* 2H | 19.6 |
| 7 | 4.46 *d* (6.5) | 69.7 | 1.43 *m* | 33.5 |
| 8 | | 40.0 | | 40.9 |
| 9 | 1.38 *m* | 49.0 | 1.36 *m* | 49.4 |
| 10 | | 36.8 | | 36.8 |
| 11 | 1.43*m* | 21.4 | a: 1.26 *m*, b: 1.42 *m* | 21.4 |
| 12 | | 25.0 | a : 1.04, b: 1.70 *brd* | 24.8 |
| 13 | 1.65 *m* | 37.3 | 1.64 m | 37.4 |
| 14 | | 44.1 | | 44.1 |
| 15 | 1.32 *m* | 36.8 | a : 1.30 *m* ,b: *t* (12.5) | 36.8 |
| 16 | 1.43 *m* | 33.3 | 3.62 *dd*(11.0, 4.5) | 76.9 |
| 17 | | 48.3 | | 48.6 |
| 18 | 1.41 *m* | 43.1 | 1.40 *m* | 47.7 |
| 19 | 2.38 *m* | 42.9 | 2.50 *td*(11.5, 5.5) | 47.6 |
| 20 | | 150.8 | | 149.9 |
| 21 | 1.39 *m* | 29.8 | a: 1.40 *ddd* (12.0, 8.5, 1.5) b: 1.98 *m* | 29.9 |
| 22 | 1.66 *m* | 37.6 | a: 1.28 *m* b: 1.65 *m* | 37.7 |
| 23 | 1.08 *s* | 26.2 | 1.07 *s* | 26.6 |
| 24 | 1.01 *s* | 21.3 | 1.02 *s* | 21.0 |
| 25 | 0.92 *s* | 18.0 | 0.94 *s* | 16.1 |
| 26 | 1.08 *s* | 17.7 | 1.08 *s* | 15.8 |
| 27 | 1.00 *s* | 16.9 | 1.01*s* | 15.9 |
| 28 | 0.83 *s* | 14.8 | | 11.7 |
| 29 | a: 4.55 *dd* (3.5, 1.5) b: 4.68 *d* (2) | 109.4 | a: 4.60 *dq* (2.0, 1.5) b: 4.71 *brd* (2.5) | 109.9 |
| 30 | 1.69 *brs* | 19.3 | 1.68 *brs* | 19.3 |

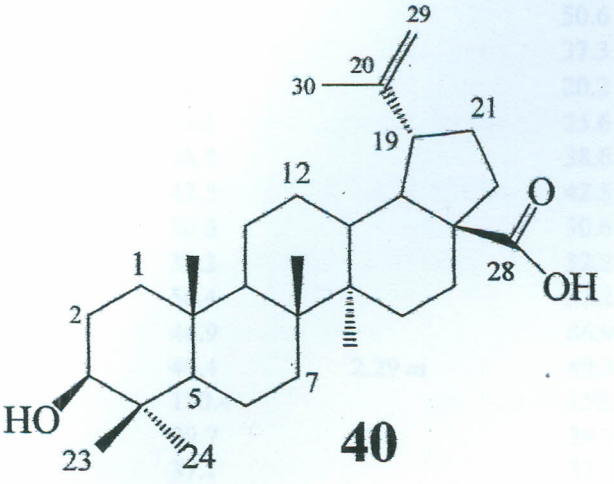
*$^{1}H$ NMR (500 MHz, $CDCl_3$) & **$^{13}C$ NMR (125 MHz, $CDCl_3$) data for 16 β-Hydroxylup-20(29)-ene-3-one (Pech *et al.*, 2002)

### 4.2.4 Betulinic acid (40)

Compound **40** $R_f$ 0.43 ($CH_2Cl_2$: MeOH (99:1)) mixture was isolated as white crystals, mp 279-280 °C (Lit., mp 279-280 °C, Igoli and Gray, 2008). IR $\upsilon_{max}$ (KBr disc) $cm^{-1}$: 3350–3073 (OH), 2911 (C-H), 1452 (C=C), 1041 (C-O). $^{13}C$ NMR spectrum (Appendix 4C) showed a peak at δ 180.6 which is typical of $^{13}C$ signals of the carbonyl carbon of a carboxylic acid, attributed to C-28 (Gohar *et al.*, 2008; Sharma *et al.*, 2010). The spectrum had two signals at δ 150.4 (C-20) and 109.7 (C-29) which are typical of exocyclic olefinic carbon atoms. It had a signal for hydroxylated carbon at δ 79.1 which was attributed to C-3 (Sharma *et al.*, 2010). The spectrum further showed a peak at δ 49.4 which was attributed to C-19 which had a resonance in the lowfield region as compared to δ 46.9 (C-18) because of being closer to an $sp^2$ carbon at C-20 which has an electron withdrawing effect. The spectrum had five methyl carbon signals typical of lupane compounds at δ 28.0 (C-23), δ 15.3 (C-24), δ 16.0 (C-25), δ 16.1 (C-26), δ 14.7(C-27) (Igoli and Gray, 2008; Sharma *et al.*, 2010).

The $^{13}C$ NMR results were corroborated by the $^1H$ NMR spectrum (Appendix 4B) which had two singlets at δ 4.60 and δ 4.72 which was attributed to the olefinic protons at C-29. The multiplet at δ 2.98 was attributed to H-28, while the multiplet at δ 2.28 was attributed to H-19. The spectrum showed a multiplet at δ 3.17 for H-3 as a result of being connected to a hydroxylated carbon. The multiplet between δ 0.73 and 1.61 was attributed to the methyl protons connected to C-23, C-24, C-25, C-26 and C-27 (Igoli and Gray, 2008). $^1H$-$^1H$ COSY confirmed that the singlets at δ 4.60 and δ 4.72 for the vinyl protons at C-29 were coupled to a singlet at δ 1.67 integrating for three protons at C-30. It confirmed further that the multiplet at δ 3.17 (H-3) was coupled to the multiplet between δ 0.73-

1.61. HSQC experiments (Appendix 4E) confirmed the connectivity of protons to carbon atoms. Consequently basing on physical, spectroscopic data and comparison with literature (Igoli and Gray, 2008) compound **40** was confirmed to be betulinic acid isolated for the first time from this plant. This compound had been isolated from *Ajuga microsperma* (Dinda *et al.*, 1997)




**40**

**Table 4.4: $^{1}H$ (500 MHz, $CDCl_3$) and $^{13}C$ (125 MHz, $CDCl_3$) NMR data of betulinic acid (40)**

| ATOM | $^{1}H$ *m* (*J* in Hz) | $^{13}C$ | $^{1}H$ *m* (*J* in Hz)* | $^{13}C$** |
|---|---|---|---|---|
| 1 | | 38.8 | | 38.8 |
| 2 | | 27.4 | | 27.4 |
| 3 | 3.17 *m* | 79.1 | 3.17 *m* | 79.1 |
| 4 | | 38.9 | | 38.9 |
| 5 | | 55.4 | | 55.4 |
| 6 | | 18.3 | | 18.3 |
| 7 | | 34.4 | | 34.4 |
| 8 | | 40.8 | | 40.8 |
| 9 | | 50.6 | | 50.6 |
| 10 | | 37.3 | | 37.3 |
| 11 | | 20.9 | | 20.2 |
| 12 | | 25.6 | | 25.6 |
| 13 | | 38.5 | | 38.6 |
| 14 | | 42.5 | | 42.5 |
| 15 | | 30.6 | | 30.6 |
| 16 | | 32.2 | | 32.2 |
| 17 | | 56.4 | | 57.2 |
| 18 | | 46.9 | | 46.9 |
| 19 | 2.28 *m* | 49.4 | 2.29 *m* | 49.3 |
| 20 | | 150.4 | | 150.4 |
| 21 | | 29.7 | | 29.7 |
| 22 | | 37.1 | | 37.1 |
| 23 | 0.73-1.61 *m* | 28.0 | 0.75-1.61 *m* | 28.0 |
| 24 | 0.73-1.61 *m* | 15.3 | 0.75-1.61 *m* | 15.4 |
| 25 | 0.73-1.61 *m* | 16.0 | 0.75-1.61 *m* | 16.1 |
| 26 | 0.73-1.61 *m* | 16.1 | 0.75-1.61 *m* | 16.2 |
| 27 | 0.73-1.61 *m* | 14.7 | 0.75-1.61 *m* | 14.7 |
| 28 | | 180.6 | | 180.4 |
| 29 | a:4.60 *s* b: 4.72 *s* | 109.7 | 4.68 *d* (32) 2H | 109.7 |
| 30 | 1.67 *s* (3H) | 19.4 | 1.69 s (3H) | 19.4 |

*$^{1}H$ NMR and **$^{13}C$ NMR (400 MHz, $CDCl_3$) data for betulinic acid (Igoli and Gray, 2008)

**4.3 Compounds isolated from MeOH extract**

Isolation of compounds from the MeOH crude extracts was achieved by flash chromatography using silica gel eluting with mixtures of *n*-hexane-EtOAc, $CH_2Cl_2$-MeOH and EtOAc-MeOH successively which led to isolation of eight compounds which were characterized by their $R_f$ values, melting points and spectroscopic techniques which

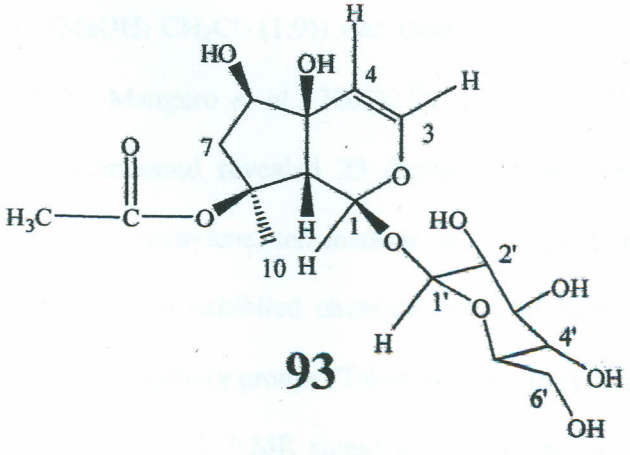
are discussed herein. Detailed spectroscopic data for compounds isolated are summarized in Tables 4.5-4.12 and their spectra are shown in the appendix section.

**4.3.1 8-*O*-Acetylharpagide (93)**

Compound **93,** $R_f$ value 0.44 (MeOH: $CH_2Cl_2$ (1:9)) was isolated as colourless crystals, mp. 227-228°C (Lit., 225-228 °C, Kuria *et al.*, 2002). The UV $\lambda_{max}(CH_3CN)$ = 200nm indicating a single carbon-carbon double bond. IR $\upsilon_{max}$ (KBr) $cm^{-1}$: 3439-3062 (OH), 2959 (C-H), 1711 (ester), 1653, 1463-1405 (C=C), 1040-1010 (C-O). The ESIMS (Appendix 5F) of compound **93** showed a pseudomolecular ion peak at *m/z* 429.1 $[M+Na]^+$ corresponding to $C_{17}H_{26}O_{11}$. $^{13}C$ and DEPT NMR spectra (Appendix 5D & E) of this compound indicated 17 distinct chemical shifts accounted for by two methyl, two methylene, ten methine and three quartenary carbon atoms.

The $^{13}C$ NMR spectrum had a methine peak at δ 94.5 (C-1) which indicated the presence of a sugar moiety, further confirmed by $^1H$ NMR signal at δ 4.58 (*d*, *J*=7.8Hz) for the anomeric proton (H-1'), with a large coupling constant futher indicating that the glucose unit was in β-configuration (Kuria *et al.*, 2002). $^{13}C$ NMR spectrum (Appendix 5D) also indicated a set of olefinic carbon chemical shifts at δ 143.9 (C-3) and δ 106.9 (C-4), C-3 appeared downfield due to inductive effect of the neighbouring oxygen atom. The spectrum exhibited six peaks of hydroxylated carbon atoms at δ 74.5 (C-5), δ 77.6 (C-6), δ 73.3 (C-2'), δ 78.2 (C-3'), δ 71.5 (C-4'), δ 77.7 (C-6'), $^{13}C$ NMR spectrum also showed two peaks at δ 24.3 (C-10) and δ 24.5 for the methyl attached to the acetoxy group, and a methylene peak at δ 46.0 for C-7. This was consistent with the proton chemical shifts in the $^1H$ NMR spectrum which showed a set of olefinic protons at δ 6.38 (*d*, *J* = 6.0 Hz) and δ 4.91 (*d*, *J*= 6.6 Hz) for H-3 and H-4 respectively. The relatively highfield broad

singlet at δ 3.86 suggested the presence of a hydroxyl group, which informed the assignment of this peak to H-6. The presence of a singlet at δ 2.84 (H-9) and absence of long range coupling to H-3 gave credence to the fact that C-5 was hydroxylated. The unshielded C-8 methyl at δ 1.44 and the presence of an acetoxy group methyl at δ 2.01 informed the assignment of the acetate group to C-8 (Belofsky *et al.*, 1989; Kuria *et al.*, 2002; Manguro *et al.*, 2006). $^{1}$H-$^{1}$H COSY confirmed that the doublet at δ 6.38 (H-3) was coupled to the doublet at δ 4.91 (H-4), the singlet at δ 6.06 (H-1) was coupled to a singlet at δ 2.84 (H-9) and further confirmed the substitution pattern on the sugar moiety, the doublet at δ 4.58 (H-1') was coupled to the multiplet at δ 3.35 (H-2') which was further coupled to the multiplet at δ 4.10 (H-3'). The HSQC experiments confirmed that the double bond was between C-3 and C-4. HMBC spectrum indicated that there was long range coupling between H-1 and H-4 to C-3 and H-3 to C-4.Thus, on the basis of physical, spectroscopic data, and comparison with literature (Kuria *et al.*, 2002) compound **93** was confirmed to be 8-*O*-acetylharpagide. This has been isolated from the aerial part of *A. remota* (Kuria *et al.*, 2002; Manguro *et al.*, 2006).




**93**

**Table 4.5: $^{1}H$ (500 MHz, $CDCl_3$) and $^{13}C$ (125 MHz, $CDCl_3$) NMR data for 8-*O*-acetylharpagide(93)**

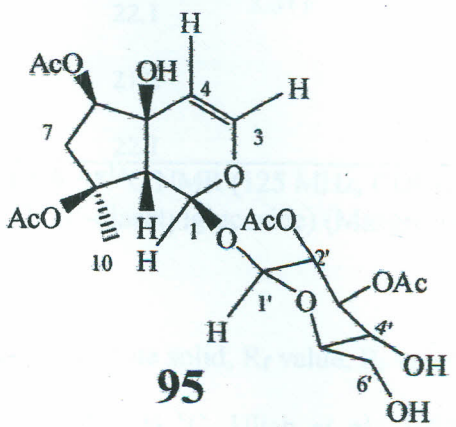
| ATOM | $^{1}H$ *m* (*J* in Hz) | $^{13}C$ | $^{1}H$ *m* (*J* in Hz)* | $^{13}C$** |
|---|---|---|---|---|
| 1 | 6.06 *s* | 94.5 | 6.07 *d* (0.80) | 96.6 |
| 3 | 6.38 *d* (6.6) | 143.9 | 6.46 *d* (6.4) | 145.3 |
| 4 | 4.91 *d* (1.8) | 106.9 | 5.01 *dd*(6.4, 1.5) | 107.6 |
| 5 | | 74.5 | | 75.0 |
| 6 | 3.86 *brs* | 77.6 | 3.84 *d* (4.1) | 79.0 |
| 7 | a: 2.19 *brs* b: 2.13 *brs* | 46.0 | a: 2.17 *d*(15.9) b: 2,03 *dd*( 15.9, 4.2) | 47.0 |
| 8 | | 88.6 | | 90.6 |
| 9 | 2.84 *s* | 55.4 | 2.86 *s* | 55.8 |
| 10 | 1.44 *s* (Me) | 24.3 | 1.45 *s* (Me) | 24.0 |
| 1' | 4.58, *d* (7.8) | 99.9 | 4.75 *d*(8.3) | 101.4 |
| 2' | 3.35 *m* | 73.3 | 3.33 *t* (8.7) | 75.2 |
| 3' | 4.10 *m* | 78.2 | 3.51 *t* (9.0) | 78.2 |
| 4' | 3.30 *m* | 71.5 | 3.41 *t* (9.2) | 72.3 |
| 5' | 3.38 *m* | 77.7 | 3.49 *dd* (2 and obs) | 78.8 |
| 6' | a: 3.90 *brs* b: 3.71 *d* (4.5) | 62.8 | a: 3.94 *dd* ( 12.6,2.0) b: 3.75 *dd* (12.6, 5.9) | 63.4 |
| OAc | | 173.3 | | 176.8 |
| Me-Ac | 2.01 *s* (Me) | 24.5 | 2.06 *s* (Me) | 24.4 |

* $^{1}H$ NMR (500 MHz, $D_2O$) & ** $^{13}C$ NMR (125 MHz, $D_2O$) data for 8-*O*-Acetylharpagide (Kuria *et al.*, 2002)

### 4.3.2 6,8-Diacetylharpagide-1-*O*-β-(2',3'-diacetylglucoside) (95)

Compound **95** $R_f$ value 0.23 (MeOH: $CH_2Cl_2$ (1:9)) was isolated as colourless crystals, mp 174-175°C (Lit. 174-176 °C, Manguro *et al.*, 2006). $^{13}C$ and DEPT NMR spectra (Appendix 6B & C) of this compound revealed 23 distinct signals which can be accounted for by five methyl, two methylene, ten methine and six quarternary carbon atoms. Its NMR spectra (Appendix 6) exhibited chemical shifts resembling those of compound **93** except for 3 additional acetoxy groups (Takaeda *et al.*, 1987; Shimomura *et al.*, 1987; Manguro *et al.*, 2006). The $^{13}C$ NMR signal at δ 71.9 was hydroxilated as shown by a doublet at δ 3.00 for H-9 and the absence of long range coupling to H-3

(Manguro *et al.*, 2006). Other peaks which exhibited hydroxylation at δ 70.9 (C-4') and δ 61.7 (C-6'). On further examination of the $^{1}$H NMR decoupling experiment, the lowfield doublet at δ 5.26 assigned to H-6 showed that the OH at C-6 was acylated. On the other hand, the unshielded C-8 methyl at δ 1.41 and the presence of an acetoxy group at δ 2.05 informed the placement of the acetate group at C-8 (Shimomura *et al.*, 1987; Belofsky *et al.*, 1989; Manguro *et al.*, 2006). This led to the conclusion that the remaining acetyl groups were attached to the sugar residue, a fact corroborated by the diagonostic downfield shifts for δ 5.03 for H-2' and δ 5.27 for H-3'. The $^{1}$H-$^{1}$H COSY assisted in confirming the substitution pattern on the glucose moiety, which showed that the chemical shift at δ 5.03 (H-2') was coupled with a multiplet at δ 5.27 (H-3'), which was further coupled to another multiplet at δ 3.75 (H-4'). The doublet at δ 4.77 ($J$ = 8.4 Hz) collapsed to a singlet when δ 5.03 (H-2') was irradiated. HSQC experiments confirmed the connectivity of protons to carbon atoms. Consequently, **95** was confirmed to be 6,8-diacetylharpagide-1-*O*-β-(2',3'-diacetylglucoside). Has been isolated from the aerial part of *A. remota* (Manguro *et al.*, 2006)




**95**

**Table 4.6: $^{1}H$ (500 MHz, $CDCl_3$) and $^{13}C$ (125 MHz, $CDCl_3$) NMR data for6,8-diacetylharpagide-1-*O*-β-(2',3'-diacetylglucoside) (95).**

| ATOM | $^{1}H$ *m* (*J* in Hz) | $^{13}C$ | $^{1}H$ *m* (*J* in Hz)* | $^{13}C$** |
|---|---|---|---|---|
| 1 | 6.00 *d* (1.8) | 94.0 | 5.56 *d* (1.1) | 95.4 |
| 3 | 6.23 *d* (6.6) | 141.7 | 6.44 *d* (6.3) | 143.7 |
| 4 | 4.95 *dd* ( 6.6, 1.2) | 107.0 | 4.94 *dd* (6.3, 1.3) | 107.2 |
| 5 | | 71.4 | | 73.3 |
| 6 | 5.26 *d* (9.6) | 77.6 | 5.30 *d* (4.3) | 80.0 |
| 7 | a: 2.18 *d* (15.6) | 43.3 | a : 2.30 *d* (15.5) | 46.9 |
| | b:2.06 *dd* ( 15.6, 1.1) | | b : 2.14 *dd* (15.5, 1.2) | |
| 8 | | 86.1 | | 89.0 |
| 9 | 3.00 *d* (9.3) | 54.5 | 2.84 *d* (1.8) | 55.2 |
| 10 | 1.41 *s* (Me) | 20.6 | 1.36 *s* (Me) | 22.8 |
| 1' | 4.77 *d* (8.4) | 96.4 | 4.60 *d* (7.8) | 98.9 |
| 2' | 5.03 *m* | 71.9 | 5.02 *t* (8.8) | 74.0 |
| 3' | 5.27 *m* | 76.6 | 5.10 *t* (9.0) | 76.6 |
| 4' | 3.75 *m* | 70.9 | 3.50 *m* | 71.2 |
| 5' | 4.57 *m* | 77.0 | 3.55 *m* | 76.9 |
| 6' | a: 4.36 *dd* (12.4, 2.4) | 61.7 | a: 4.01 *dd* (12.4, 2.2) | 62.4 |
| | b; 4.07 *dd* (12.4, 5.1) | | b: 3.81 *dd* (12.4, 5.4) | |
| AcO | | 169.4 | | 169.0 |
| | | 170.1 | | 170.0 |
| | | 170.4 | | 170.1 |
| | | 170.7 | | 171.1 |
| Me-Ac | 2.15 *s* | 22.0 | 2.15 s | 25.0 |
| | 2.05 *s* | 22.1 | 2.04 s | 25.4 |
| | | 21.2 | | 24.9 |
| | | 22.1 | | 25.0 |

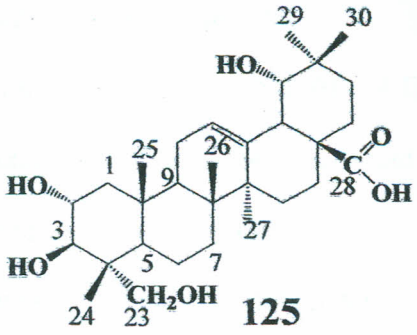
*$^{1}H$ NMR (500 MHz, DMSO-$d_6$) & **$^{13}C$ NMR (125 MHz, $CDCl_3$-DMSO-$d_6$) data for 6,8-Diacetyleharpagide-1-*O*-β-(2', 3'-diacetylglucoside) (Manguro *et al.*, 2006)

### 4.3.3 Arjungenin (125)

Compound **125** was isolated as a white solid, $R_f$ value, $R_f$ value 0.19 in ($CH_2Cl_2$-MeOH (99:1)), mp 291-293 °C (Lit, 292-294 °C, Ullah *et al.*, 2007). The ESIMS spectrum (Appendix 7) showed a pseudomolecular ion peak at *m/z* $[M+Na]^+$ 527.3 corresponding to the molecular formular $C_{30}H_{48}O_6$. $^{13}C$ and DEPT NMR spectra (Appendix 7B & C) of

this compound indicated 30 distinct carbon chemical shifts accounted for by six methyl, ten methylene, six methine and eight quartenary carbon atoms.

$^{13}C$ NMR spectrum (Appendix 7B) indicated chemical shifts for a hydroxymethylene carbon at δ 63.8 for C-23 and three hydroxymethines at δ 83.8, 80.1, 67.0 for C-19, C-3, and C-2 respectively (Nandy *et al.*, 1989; Jossang *et al.*, 1996; Ullah *et al.*, 2007). The spectrum also indicated a set of endocyclic olefinic chemical shifts at δ 143.5 and δ 122.1 for C-13 and C-12 respectively. The spectrum had a peak at δ 179.2 which is typical of a carboxylic acid carbonyl carbon, which was attributed to C-28. The spectrum further showed six methyl carbon shifts typical of pentacyclic triterpenes at δ 27.2, 23.4, 23.4, 16.6, 16.4 and 14.1 for C-29, C-30, C-27, C-26, C-25 and C-24 respectively. $^{13}C$ NMR spectrum was corroborated by the $^{1}H$ NMR spectrum (Appendix 7A) which had doublets at δ 4.49 ($J$ = 3.3 Hz) and 4.03 ($J$ =4.2 Hz) which were attributed to H-2 and H-3 respectively. The spectrum had a doublet at δ 3.29 ($J$ = 6.6 Hz) which was attributed to H-19. This spectrum had a triplet-like peak at δ 5.23 for H-12 (Nandy *et al.*, 1989). The spectrum further indicated methyl proton chemical shifts at δ 1.23, 1.09, 0.90, 0.88, 0.84 and 0.65 which appeared as singlets. Therefore, basing on physical, spectroscopic data and comparison with literature (Nandy *et al.*, 1989) compound **125** was confirmed to be arjungenin isolated for the first time from *A. remota*. However, it has been reported in *Terminalia bellerica* (Nandy *et al.*, 1989), *Davilla flexuosa* (Jossang *et al.*, 1996) and *Rhododendron collettianum* (Ullah *et al.*, 2007)




125

Table 4.7: $^{1}H$ (500 MHz, $CDCl_3$) and $^{13}C$ (125 MHz, $CDCl_3$) NMR data of arjungenin (125)

| ATOM | $^{1}H$ *m* (*J* in Hz) | $^{13}C$ | $^{1}H$ *m* (*J* in Hz)* | $^{13}C$** |
|---|---|---|---|---|
| 1 | | 47.4 | | 47.4 |
| 2 | 4.50 *d* (3.3) | 67.0 | 5.15 *td* (12, 4) | 68.9 |
| 3 | 4.03 *d* (4.2) | 80.1 | 5.06 *d* (12) | 78.4 |
| 4 | | 43.1 | | 43.3 |
| 5 | | 59.7 | | 48.2 |
| 6 | | 18.9 | | 18.8 |
| 7 | | 32.8 | | 33.6 |
| 8 | | 41.0 | | 40.1 |
| 9 | | 55.5 | | 48.5 |
| 10 | | 37.6 | | 36.6 |
| 11 | | 24.5 | | 28.8 |
| 12 | 5.23 *t* (3.9) | 122.1 | 5.42 *t-like* | 123.5 |
| 13 | | 143.5 | | 144.0 |
| 14 | | 42.9 | | 42.2 |
| 15 | | 28.4 | | 29.2 |
| 16 | | 27.9 | | 28.2 |
| 17 | | 46.8 | | 46.0 |
| 18 | 3.11 *t* (5.4) | 44.7 | 3.13 *brs* | 44.8 |
| 19 | 3.29 *d* (6.6) | 83.8 | 3.33 *brs* | 82.1 |
| 20 | | 34.9 | | 35.7 |
| 21 | | 28.1 | | 28.4 |
| 22 | | 32.3 | | 33.1 |
| 23 | 3.74 *d* (6.6) | 63.8 | 3.75 *d* (12.0) | 66.8 |
| 24 | 0.65 *s* | 14.1 | 0.66 *s* | 14.2 |
| 25 | 0.84 *s* | 16.6 | 0.88 *s* | 17.7 |
| 26 | 0.88 *s* | 16.4 | 0.96 *s* | 17.3 |
| 27 | 0.90 *s* | 23.4 | 0.96 *s* | 24.9 |
| 28 | | 179.1 | | 180.0 |
| 29 | 1.23 *s* | 23.4 | 1.22 *s* | 24.9 |
| 30 | 1.09 *s* | 23.4 | 1.06 *s* | 24.9 |

*$^{1}H$ NMR (300 MHz, $CDCl_3$) & **$^{13}C$ NMR (75 MHz, pyridine-$d_5$) for Arjungenin (Nandy *et al.*, 1989)

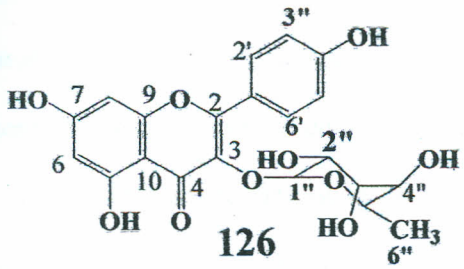
### 4.3.4 Kaempferol 3-*O*-β-rhamnoside (126)

Compound **126** $R_f$ value 0.72 ($CH_2Cl_2$: MeOH (19:1) was isolated as yellow needles in $CH_2Cl_2$-MeOH mixture, mp 231-234 °C. Acid hydrolysis gave the aglycone characterized as kaempferol and a sugar moiety which was identified as β-L-rhamnose by comparison with authentic samples of β-L-rhamnose through silica gel TLC (EtOAc-MeOH-$H_2O$-HOAc (6:2:1:1)).

$^{13}C$ NMR spectrum (Appendix 8B) showed the shielding for C-3 at δ 133.6 and deshielding for δ 177.7 (C-4) and δ 157.0 (C-2) indicating glycosation at the C-3 position (Markharm, 1982). This was further supported by the $^1H$ MMR spectrum (Appendix 8A) which had expected signals in the aromatic region of kaempferol (Lin and Lin, 1999). The multiplate at δ 7.52 for H-2' and the orthor-orthor coupled doublet at δ 6.84 (2H, $J$ = 8.0 Hz) for H-3' and H-5' together with the shielded meta-coupled doublet at δ 6.19 (*d*, $J$ =2.0 Hz) for H-6 and δ 6.39 (*d*, $J$ = 2.5) for H-8 indicated that no sugar was attached at positions 7, 5 and 4'. This spectrum further had a doublet at δ 5.31 ($J$ = 7.5 Hz) attributed to the anomeric proton H-1'' of the inner β-rhamnose, thus confirming the linkage at C-3 of the aglycone, it was in contrast to the α-D-rhamnose assignment for C-3 in kaempferol 3-*O*-α-D-rhmnoside made by Lin and Lin, (1999) which had a doublet at δ 5.28 with a coupling constant of 1.4 Hz. This also indicated that the sugar residue was linked to the aglycone in a β configuration (Kuria *et al*., 2002).

HSQC spectrum confirmed that the multiplet at δ 7.52 for H-2' was linked to C-2'. It also confirmed the assignment of the carbon peaks for the sugar residue. It further confirmed the linkage of a doublet for H-6 with δ 6.19 ($J$ = 2.0) to C-6. The HMBC spectrum

(Appendix E) indicated long range coupling of H-6 and H-8 to the carbonyl carbon, C-4. These protons were also coupled to C-7. Compound **126** was confirmed to be kaempferol 3-*O*-β-rhamnoside. It has also been isolated from the aerial part of *A remota* (Manguro *et al.*, 2006)




**126**

**Table 4.8: $^{1}$H (500 MHz, DMSO-d$_6$) and $^{13}$C (125 MHz, DMSO-d$_6$) NMR data for Kaempferol 3-*O*-β-rhamnoside (126)**

| ATOM | $^{1}$H *m* (*J* in Hz) | ATOM | $^{1}$H *m* (*J* in Hz)* | $^{13}$C** |
|---|---|---|---|---|
| 2 | | 157.0 | | 157.6 |
| 3 | | 133.6 | | 134.6 |
| 4 | | 177.7 | | 177.9 |
| 5 | 12.55 *s* (1H) | 161.5 | 12.61 *s* (1H) | 161.3 |
| 6 | 6.19 *d* (2.0) (1H) | 99.0 | 6.20 *d* (2.1) (1H) | 99.2 |
| 7 | | 164.3 | | 164.9 |
| 8 | 6.39 *d* (2.5) 1H | 94.0 | 6.40 *d* (2.1) 1H | 94.3 |
| 9 | | 156.8 | | 157.0 |
| 10 | | 104.3 | | 104.3 |
| 1' | | 121.5 | | 121.0 |
| 2' | 7.52 *m* | 122.0 | 7.74 *d* (8.8) | 131.0 |
| 3' | 6.84 *d* (8.0) 1H | 115.5 | 6.90 *d* (8.8) 2H | 115.7 |
| 4' | | 156.8 | | 160.1 |
| 5' | 6.84 *d* (8.0) 1H | 115.5 | 6.90 *d* (8.8) 2H | 115.7 |
| 6' | 7.52 *m* | 122.0 | 7.74 *d* (8.8) | 131.0 |
| 1'' | 5.31 *d* (7.5) | 101.4 | 5.28 *d* (1.4) | 102.1 |
| 2'' | | 70.6 | | 70.5 |
| 3'' | | 70.8 | | 71.0 |
| 4'' | | 72.1 | | 71.3 |
| 5'' | | 70.3 | | 70.3 |
| 6'' | 5.15 *s* | 17.9 | 5.28 *d* (1.4) | 17.7 |

*$^{1}$H NMR (300 MHz, DMSO-d$_6$) and **$^{13}$C NMR (75 MHz, DMSO-d$_6$) data for Kaempferol 3-*O*-α-D-rhmnoside (Lin and Lin, 1999)

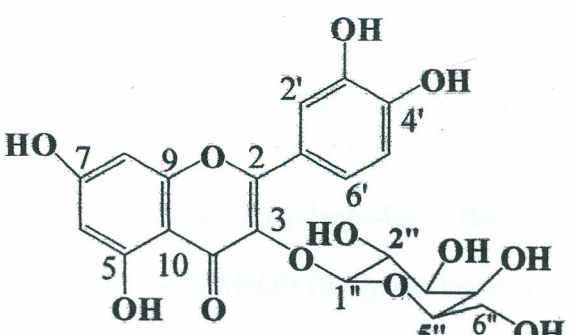
### 4.3.5 Quercetin 3-*O*-β-galactoside (127)

Compound **127** $R_f$ value 0.65 ($CH_2Cl_2$-MeOH (19:1)), was isolated as a yellow powder, mp 230-231 °C (Lit, 231-232 °C, Liu *et al.*, 2008). When exposed to ammonia on TLC (silca gel) appeared as dark UV absorbing spots, which is a characteristic of flavonols with substituted 3-OH and free 5-OH groups (Markham, 1982). Acid hydrolysis of the compound yielded the aglycone, characterized as quercetin and a sugar moiety which was identified as β-galactose by comparison with authentic samples of glucose and galactose through silica gel TLC (EtOAc-MeOH-$H_2O$-HOAc (6:2:1:1)). The UV spectra (Appendix 9A) in methanol and shift reagents indicated the presence of ortho-dihydroxy groups in B-ring exhibited by the absorption spectrum in NaOAc/$H_3BO_3$ which showed a bathochromic shift of band I (20 nm) relative to the spectrum in methanol ( Markham, 1982). This was further supported by the absorption in NaOMe which gave a bathochromic shift (50 nm) with an increase in intensity relative to the spectrum in methanol indicating the presence of 4'-OH group (Markham, 1982). Similarly a bathochromic shift of band II found in NaOAC for flavonols containing free 7-OH group (Markham, 1982) and presence of a free 5-OH group indicated by a bathochromic shift of (60nm) found in $AlCl_3$/HCl were observed.

EIMS (Appendix 9C) showed a molecular ion peak at *m/z* 303 corresponding to $C_{15}H_{10}O_7$ consistent with quercetin aglycone indicating the loss of galactose from the molecule. It also exhibited fragmentation pattern characteristic of flavonols. The fragment ion at *m/z* 285 corresponded to loss of water from the molecular ion. A peak at *m/z* 275 corresponded to a loss of one molecule of carbon monoxide. A peak at *m/z* 229 was caused by loss of one molecule of water and two molecules of carbon monoxide. Peaks at

*m/z* 153 and 137 were attributed to retro Diels-Alders fission resulting in the cleavage of bonds in the C-ring of quercetin (Tsimogiannis *et al.*, 2007).

The $^{13}C$ NMR spectrum corroborated the acid hydrolysis results by showing 21 carbon atoms in the molecule of which six carbon peaks in the glycosidic region corresponding to a hexose moiety at δ 101.9 and the comparison of the chemical shift of the sugar carbon signals with reported data confirmed the presence of galactose (Wei *et al.*, 2009). The upfield chemical shift at δ 133.4 for (C-3) indicated the linkage of a sugar moiety at this point. Furthermore, $^{1}H$ NMR spectrum (Appendix 9B) of compound showed the anomeric proton resonance as a doublet at δ 5.40. The coupling constant $J$ = 7.6 Hz indicated that the galactose moiety was in a β-configuration (Kuria *et al.*, 2002). The spectrum further showed singlets at δ 12.63 (5-OH), δ 10.87 (7-OH), δ 9.70 (3'-OH) and δ 9.20 (4'-OH). Thus, basing on physical and spectroscopic data, compound **127** was concluded to be quercetin 3-*O*-β-galactoside, isolated for the first time from this plant.




**127**

**Table 4.9: $^1H$ (300 MHz, DMSO-$d_6$) and $^{13}C$ (75 MHz, DMSO-$d_6$) NMR data for quercetin 3-*O*-β-galactoside (127).**

| ATOM | $^1H$ *m* (*J* in Hz) | $^{13}C$ | $^1H$ *m* (*J* in Hz)* | $^{13}C$** |
|---|---|---|---|---|
| 2 | | 156.4 | | 156.3 |
| 3 | | 133.4 | | 133.5 |
| 4 | | 177.7 | | 177.4 |
| 5 | | 161.5 | | 161.4 |
| 6 | 6.20 *d* (1.9) | 98.7 | 6.19 | 98.7 |
| 7 | | 164.4 | | 164.1 |
| 8 | 6.38 *d* (1.9) | 93.4 | 6.40 | 93.6 |
| 9 | | 156.4 | | 156.3 |
| 10 | | 104.0 | | 104.0 |
| 1' | | 121.2 | | 121.1 |
| 2' | 7.50 *d* (2.0) | 116.1 | 7.53 | 115.2 |
| 3' | | 145.0 | | 144.8 |
| 4' | | 148.7 | | 148.4 |
| 5' | 6.80 *d* (8.5) | 115.3 | 6.81 | 116.0 |
| 6' | 7.70 *dd* (8.5, 2.0) | 122.1 | 7.63 | 121.9 |
| 5-OH | 12.63 *s* | | | |
| 7-OH | 10.87 *s* | | | |
| 3'-OH | 9.70 *s* | | | |
| 4'-OH | 9.20 *s* | | | |
| 1" | 5.40 *d* (7.6) | 101.9 | 5.35 | 101.9 |
| 2" | 4.50-3.10 *m* | 71.3 | | 71.3 |
| 3" | 4.50-3.10 *m* | 73.2 | | 73.3 |
| 4" | 4.50-3.10 *m* | 69.9 | | 68.0 |
| 5" | 4.50-3.10 *m* | 75.9 | | 75.9 |
| 6" | 4.50-3.10 *m* | 60.1 | | 60.2 |

* $^1H$ NMR (500 MHz, DMSO-$d_6$) & ** $^{13}C$ NMR (500 MHz, DMSO-$d_6$) for quercetin 3-*O*-β-galactoside (Wei *et al*, 2009).

### 4.3.6 Quercetin 3-*O*-β-(6-*O*-α-rhamnosyl) galactoside (128)

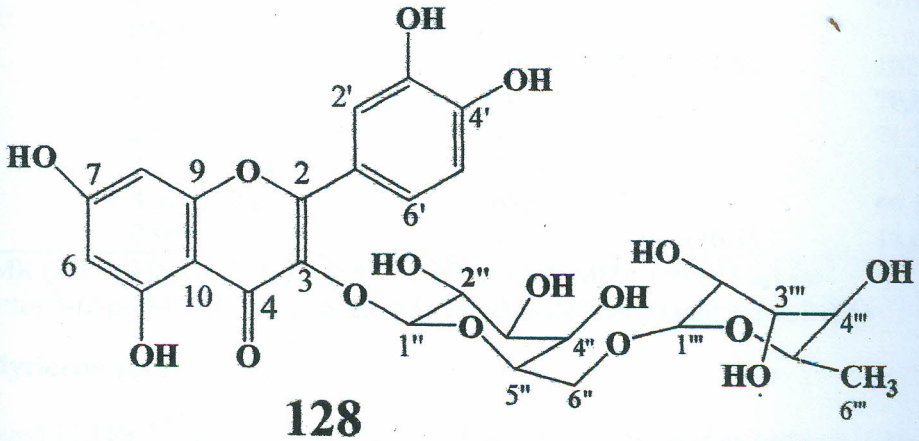
Compound **128** $R_f$ value 0.61 ($CH_2Cl_2$-MeOH (9:1)) was isolated as a yellow armorphous powder, mp 181-182 °C (Lit, 180-182 °C, Liu *et al.*, 2008). On TLC under UV light, it showed a deep purple colour which turned yellow with concentrated ammonia which indicated that it could be probably a flavonol glycoside with a substituted 3-OH ( Markham, 1982; Arya *et al.*, 1992). Its IR spectrum determined in nujol exhibited absorption bands due to polyhydroxyl groups at 3400-3100 $cm^{-1}$ and α,β-unsaturated ketone at 1670, 1600 $cm^{-1}$. The UV spectra (Appendix 10A) indicated that the compound

was substituted at C-3 as shown by UV spectral data on addition of diagonostic shift reagents (Mabry *et al.*, 1970; Markham, 1982). Acid hydrolysis (8% HCl) galactose was detected (TLC, eluent EtOAc-MeOH-$H_2O$-HOAc (6:2:1:1)). The FABMS showed a pseudo-molecular ion peak at m/z 611 corresponding to $C_{27}H_{30}O_{16}$. The other intense peak at *m/z* 302 indicated the loss of galactose and rhamnose units from the molecule. EIMS showed a molecular ion peak at *m/z* 303 corresponding to $C_{15}H_{10}O_7$. It also exhibited fragmentation pattern characteristic of flavonols. A peak at *m/z* 275 corresponded to a loss of one molecule of carbon monoxide from the molecular ion. A peak at *m/z* 247 corresponded to a loss of two carbon monoxide molecules. A peak at *m/z* 229 was caused by loss of a molecule of water and two molecules of carbon monoxide. Peaks at *m/z* 153 and 137 were attributed to retro Diels-Alders fission resulting in the cleavage of bonds in the C-ring of quercetin (Tsimogiannis *et al.*, 2007).

$^1$H NMR spectrum (Appendix 10B) showed the characteristic feature of the aglycone pattern of quercetin by exhibiting a 2H AX and a 3H ABX system in the spectrum. The spectrum further supported quercetin 3-*O*-disaccharide with rhamnose as the terminal sugar a fact which is corroborated by the $^{13}$C NMR spectrum (Appendix 10C) in which all the corresponding signals of quercetin consistent with 3-*O*-glycosidation were observed (Markham *et al.*, 1978). The spectrum further displayed aglycone peaks at δ 12.60 (5-OH), δ 10.80 (7-OH), δ 9.70 (3'-OH) and δ 9.20 (4'-OH), confirming the UV spectral data and are consistent with quercetin moiety with 3-OH substituted (Mabry *et al.*, 1970). The presence of an inner anomeric proton with a doublet at δ 5.03 with a coupling constant 7.8 Hz commonly found at δ 5.20 for a proton attached to C-7 was evidence that the rhamnosylgalactose was attached at C-3 (Mizuno *et al.*, 1991;

Fathiazadi *et al.*, 2006). This was further supported by $^{13}C$ NMR which gave a spectrum similar to that of quercetin 3-*O*-rutinoside (Mendez, 1978; Allais *et al.*, 1991). On the other hand, the $^{1}H$ NMR spectrum determined in methanol because of disappearance of the OH groups displayed well resolved resonances for quercetin 3-*O*-rutinoside (Mendez, 1978; Allais *et al.*, 1991). Chemical shifts at δ 4.40 for H-1''' and the methyl of rhamnose ar δ 0.90 clearly indicated the presence of a disaccharide (Arya *et al.*, 1992; Fathiazadi *et al.*, 2006).

$^{13}C$ NMR spectrum (Appendix 10C) confirmed the interglycosidic linkage as galactose (6''→1''') rhamnose as shown by δ 68.2 for C-6'' of galactose. In fact the C-6'' of galactose underwent a downfield shift of approximately δ 8.0 compared to the $^{13}C$ NMR spectrum of quercetin 3-*O*-galactose. Thus, on the basis of physical, chemical, spectroscopic data and comparison with literature (Fathiazadi *et al.*, 2006), compound **128** was characterized as quercetin 3-*O*-β-(6-*O*-α-rhamnosyl)-galactose isolated for the first time from this plant.




**128**

**Table 4.10: $^{1}H$ (300 MHz, $CDCl_3$-DMSO-$d_6$) and $^{13}C$ (75 MHz, $CDCl_3$) NMR data for Quercetin 3-*O*-β-(6-*O*-α-rhamnosyl)-galactoside (128).**

| ATOM | $^{1}H$ *m* (*J* in Hz) | $^{13}C$ | $^{1}H$ *m* (*J* in Hz)* | $^{13}C$** |
|---|---|---|---|---|
| 2 | | 156.6 | | 157.3 |
| 3 | | 133.3 | | 134.1 |
| 4 | | 177.4 | | 178.2 |
| 5 | | 161.2 | | 157.5 |
| 6 | 6.20 *d* (2.0) | 98.7 | 6.21 *d* (2.0) | 99.5 |
| 7 | | 164.1 | | 164.9 |
| 8 | 6.40 *d* (1.9) | 93.4 | 6.40 *d* (2.0) | 94.5 |
| 9 | | 156.4 | | 162.1 |
| 10 | | 104.0 | | 104.8 |
| 1' | | 121.2 | | 122.5 |
| 2' | 7.55 *d* (2.3) | 116.3 | 7.55 *d* (2.0) | 116.1 |
| 3' | | 144.8 | | 145.6 |
| 4' | | 148.4 | | 149.3 |
| 5' | 6.80 *d* (8.5) | 115.2 | 6.86 *d* (9.0) | 117.1 |
| 6' | 7.50 *dd* (11.0) | 121.6 | 7.56 *dd* (9.0, 2.1) | 122.0 |
| 5-OH | 12.60 *s* | | | |
| 7-OH | 10.80 *s* | | | |
| 3'-OH | 9.70 *s* | | | |
| 4'-OH | 9.20 *s* | | | |
| 1" | 5.03 *d* (7.8) | 101.2 | 5.35 *d* (7.4) | 101.6 |
| 2" | | 74.0 | | 74.9 |
| 3" | | 76.5 | | 77.3 |
| 4" | | 70.6 | | 72.7 |
| 5" | | 75.2 | | 76.7 |
| 6" | a:3.50-2.9 *m*, b: 3.77 *dd* (9.0) | 68.2 | | 67.9 |
| 1"' | 4.40 *d* (0.9) | 100.7 | 5.12 *d* (1.9) | 102.2 |
| 2"' | 3.50-2.90 *m* | 70.0 | | 70.8 |
| 3"' | 3.50-2.90 *m* | 70.4 | | 71.2 |
| 4"' | 3.50-2.90 *m* | 71.9 | | 71.4 |
| 5"' | 3.50-2.90 *m* | 67.0 | | 69.1 |
| 6"' | 0.90 *d* (6.7) | 17.7 | 1.00 *d* (6.1) | 18.6 |

*$^{1}H$ NMR (200 MHz, DMSO-$d_6$) & **$^{13}C$ NMR (500 MHz, DMSO-$d_6$) for Quercetin 3-*O*-β-D-(6-*O*-α-L- rhamnosyl)-galactoside (Fathiazadi *et al.*, 2006)

### 4.3.7 Myricetin (113)

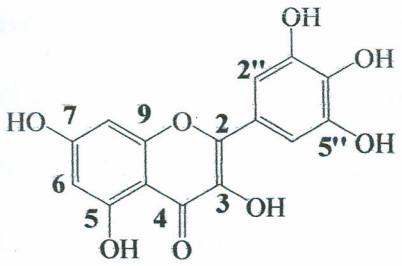
Compound **113** $R_f$ value 0.58 ($CH_2Cl_2$-MeOH (4:1)) was obtained as yellow needles from $CH_2Cl_2$-MeOH mixture, mp 229-230 °C (Lit, mp 230-231 °C, David *et al.*, 1996). Addition of concentrated ammonia and concentrated sulphuric acid, exhibited a dark

yellow colour on TLC a characteristic feature of a flavonol with 3-OH unsubstituted (Markham *et al.*, 1984).

UV $\lambda_{max}$ (MeOH): 376 (band I), 254 (band II) nm which gave a bathochromic shift of band I with $AlCl_3$/HCl (56 nm) indicating a 3-*O*-unsubstituted flavonol (Markham, 1982). A bathochromic shift of band II (14 nm) with NaOAc was an indication of unsubstituted hydroxyl group at C-7. In addition a bathochromic shift of band I (12 nm) found in NaOAc/$H_3BO_3$ for flavonols containing 3' and 4' hydroxyl group was observed (Markham, 1982). The presence of 3', 4', 5' hydroxyl groups which are alkali sensitive were indicated by the UV absorption spectrum in NaOMe which showed rapid decrease of the spectrum with time (Agrawal and Rastogi, 1981). The EIMS (Appendix 11C) showed a molecular ion peak at *m/z* 318 corresponding to $C_{15}H_{10}O_8$. Other significant fragmentation peaks in the spectrum at m/z 289 was due to a loss of carbon monoxide from the parent molecular ion, a peak at m/z 243 atttibuted to loss of water and two carbon monoxide molecules and peaks at *m/z* 165, 153 and 136 attributed to retro Diels-Alders fission resulting in the cleavage of bonds in the C-ring of myricetin (Tsimogiannis *et al.*, 2007).

Above data was further confirmed by $^1$H NMR spectrum (Appendix 11B) which exhibited singlet chemical shifts in the downfield region at δ 12.00 (5-OH), δ 10.08 (7-OH), δ 9.70 (3-OH), δ 8.11 (3',5'-OH) and δ 7.74 (4'-OH) these hydroxyl groups are deuterium exchangeable (Agrawal, 1989; David *et al.*, 1996). A singlet at δ 7.41 which on integration corresponded to two protons was attributed to H-2' and H-6' in ring B. Two meta-coupled doublets at δ 6.42 ($J$ = 2.1 Hz) and δ 6.29 ($J$ = 2.0 Hz) were assigned to H-8 and H-6 respectively. Consequently from physical, chemical, spectroscopic data

and comparison with literature (David et al., 1996), compound **113** was concluded to be myricetin. It has been reported in *Davilla flexuosa* (David *et al.*, 1996) and *Embelia keniensis* (Manguro *et al.*, 2005).




**113**

**Table 4.11: $^{1}$H (300 MHz, $CDCl_3$-DMSO-$d_6$) and $^{13}$C (75 MHz, $CDCl_3$) NMR data for Myricetin (113)**

| ATOM | $^{1}$H *m* (*J* in Hz) | $^{13}$C | $^{1}$H *m* (*J* in Hz)* | $^{13}$C** |
|---|---|---|---|---|
| 2 | | 147.8 | | 147.7 |
| 3 | | 137.0 | | 136.9 |
| 4 | | 178.2 | | 177.0 |
| 5 | | 104.6 | | 104.4 |
| 6 | 6.29 *d* (2.0) | 157.2 | 6.19 *d* (2.1) | 158.2 |
| 7 | | 98.7 | | 99.7 |
| 8 | 6.42 *d* (2.1) | 164.5 | 6.49 *d* (2.1) | 165.7 |
| 9 | | 94.8 | | 95.0 |
| 10 | | 164.3 | | 162.5 |
| 1' | | 119.9 | | 123.2 |
| 2' | 7.41 *s* | 108.2 | 7.37 *s* | 108.9 |
| 3' | | 146.1 | | 146.9 |
| 4' | | 136.7 | | 137.2 |
| 5' | | 146.1 | | 146.9 |
| 6' | 7.41 *s* | 108.2 | 7.37 *s* | 108.9 |
| 3-OH | 9.70 *s* | | | |
| 5-OH | 12.00 *s* | | | |
| 7-OH | 10.08 *s* | | | |
| 3'-OH | 8.11 *s* | | | |
| 4'-OH | 7.74 *s* | | | |
| 5'-OH | 8.11 *s* | | | |

* $^{1}$H NMR (300 MHz, $Me_2CO$-$d_6$/$D_2O$ (1:1)) & ** $^{13}$C NMR (75 MHz, $Me_2CO$-$d_6$/$D_2O$ (1:1)) data for myricetin (David *et al.*, 1996)

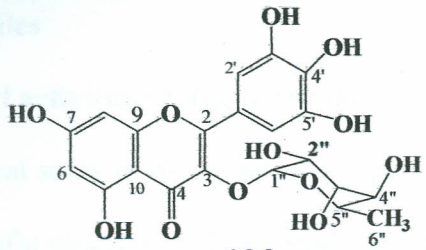
### 4.3.8 Myricetin 3-*O*-α-rhamnoside (129)

Compound **129** $R_f$ value 0.46 ($CH_2Cl_2$-MeOH (4:1))was obtained as a pale yellow amorphous powder from $CH_2Cl_2$-Methanol mixture, mp 195-197 °C (lit. 197-201 °C, Zhong *et al.*, 1997). It gave a yellow colour on addition of concentrated ammonia and sulphuric acid solution, a characteristic feature of flavonoid compounds. It appeared deep purple on TLC chromatogram under UV light suggesting that it could be flavonol glycoside with substituted 3-OH ( Anderson *et al.*, 1993). Complete acid hydrolysis with 8% aqueous HCl yielded myricetin and rhamnose (confirmed by TLC with authentic samples).

The UV spectral data (Appendix 12A) revealed the rhmnosyl moiety to be attached at C-3, in pyranose form (Alton *et al.*, 1980). UV $\lambda_{max}$ (MeOH): 354 nm (band I), 258 nm (band II) which gave a bathochromic shift of band I with $AlCl_3$ (52nm) indicating a 3-*O*-substituted flavonol (Markham, 1982). A bathochromic shift of band II (16 nm) with NaOAc indicated an unsubstituted OH group at C-7. Similarly a bathochromic shift of band I (20 nm) found in NaOAc/$H_3BO_3$ mixture for flavonols containing free 3' and 4' -OH groups was also observed (Markham, 1982). The presence of alkali sensitive 3', 4', 5' OH groups in the molecule was indicated by the absorption spectrum of this compound in NaOMe which exhibited a rapid decrease of the spectrum with time.The EIMS (Appendix 12D) of compound showed a molecular ion peak at *m/z* 318 corresponding to $C_{15}H_{10}O_8$ (myricetin) due to loss of rhmnose unit. Fragmentation pattern characteristic of flavonols were observed in the spectrum. The fragment ion at *m/z* 301 corresponded to a loss of water from the parent molecular ion. A peak at *m/z* 289 attributed to loss of carbon monoxide. A peak at *m/z* 261 corresponded to loss of two

carbon monoxide molecules. A fragment at *m/z* 244 due to loss of water and two molecules of carbon monoxide, and fragment ions at *m/z* 166, 153 and 136 considered to originate from retro Diels-Alder fission due to cleavage of the C-ring bonds of myricetin (Tsimogiannis *et al.*, 2007). The $^{13}C$ NMR spectrum of compound showed 21 carbon chemical shifts in the off resonance decoupled spectrum, the position of the rhamnose attachment to the aglycone was confirmed by the upfield shift of δ 2.4 of C-3 with respect to the same chemical shifts in the $^{13}C$ NMR of myricetin (Agrawal, 1989).

$^{1}H$ NMR spectrum (Appendix 12B) exhibited eight signals for the myricetin aglycone in the downfield region of the spectrum. Singlets at δ 12.67, δ 10.24, δ 8.60 and δ 8.20 were attributed to the hydroxyl functional groups at C-5, C-7, C-3' and C-4' respectively. A singlet at δ 7.03, which on integration corresponded to two protons was assigned to B ring, H-2' and H-6' protons. In the same spectrum, characteristic meta-coupled doublets at δ 6.36 (*J* = 2.1 Hz) and δ 6.24 (*J* =2.1 Hz) were assigned to H-8 and H-6 respectively. Furthermore, the $^{1}H$ NMR spectrum showed an anomeric proton chemical shift which appeared considerably downfield compared to sugar proton resonances (Markham and Mabry, 1975) and thus the doublet at δ 5.44 (*J* = 0.80 Hz) together with integration data defined myricetin-rhmnose ratio of 1:1. The coupling constant (*J* = 0.80 Hz) was in accordance with equatorial coupling between protons on C-1'' and C-2'' in α-linked rhamnose (Markham and Mabry, 1975; Anderson *et al.*, 1991). Other signals in the relatively higher part of the spectrum at δ 4.24 *m*, δ 3.85 (*dd*, *J* = 9.2, 3.9 Hz), δ 3.35 (*dd*, *J* = 9.3, 1.4 Hz) and δ 3.51 corresponding to one proton was assigned to C-2", C-3", C-4" and C-5" sugar protons respectively, whereas the rhamnosyl methyl protons appeared as a doublet at δ 0.98 (*J* = 6.0 Hz). Consequently, basing on physical, chemical, spectroscopic data and comparison with literature (Duan *et al.*, 2009), compound **129** was concluded to be myricetin 3-O-α-rhamnoside, which has been isolated from this plant for the first time. However, it has been reported in *E. keniensis* (Manguro *et al.*, 2005) and *Metasequoia glyptostroboids* (Duan *et al.*, 2009)



129

**Table 4.12: $^{1}H$ (300 MHz, $CDCl_3$-DMSO-$d_6$) and $^{13}C$ (75 MHz, DMSO-$d_6$) NMR data for Myricetin 3-*O*-α-rhamnoside (129).**

| ATOM | $^{1}H$ *m* (*J* in Hz) | $^{13}C$ | $^{1}H$ *m* (*J* in Hz)* | $^{13}C$** |
|---|---|---|---|---|
| 2 | | 158.7 | | 157.3 |
| 3 | | 134.6 | | 135.1 |
| 4 | | 179.9 | | 178.5 |
| 5 | | 163.4 | | 162.0 |
| 6 | 6.24 *d*(2.1) | 98.9 | 6.19 *d* (2.0) | 98.6 |
| 7 | | 166.1 | | 164.6 |
| 8 | 6.36 *d* (2.1) | 94.7 | 6.35 *d* (2.0) | 93.5 |
| 9 | | 158.7 | | 158.2 |
| 10 | | 103.7 | | 104.7 |
| 1' | | 122.1 | | 120.7 |
| 2' | 7.03 *s* | 109.7 | 6.95 *s* | 108.4 |
| 3' | | 147.0 | | 145.6 |
| 4' | | 136.5 | | 136.7 |
| 5' | | 147.0 | | 145.6 |
| 6' | 7.03 *s* | 109.7 | 6.95 *s* | 108.4 |
| 5-OH | 12.67 | | | |
| 7-OH | 10.24 | | | |
| 3'-OH | 8.60 | | | |
| 5'-OH | 8.60 | | | |
| 4'-OH | 8.20 | | | |
| 1" | 5.44 *d* (0.8) | 106.0 | 5.31 *d* (1.6) | 102.2 |
| 2" | 4.24 *m* | 71.9 | 4.22 *dd* (3.6, 1.6) | 70.8 |
| 3" | 3.85 *dd* (9.2, 3.9) | 72.1 | 3.87 *dd* (9.6, 3.6) | 70.5 |
| 4" | 3.35 *dd* (9.3, 1.4) | 73.4 | 3.35 *dd* (9.6, 1.6) | 71.6 |
| 5" | 3.51 *m* | 72.2 | 3.50 *m* | 70.7 |
| 6" | 0.98 *d* (.6.0) | 17.6 | 0.96 *d* (6.4) | 17.7 |

*$^{1}H$ NMR (400 MHz, $CD_3OD$) & **$^{13}C$ NMR (100 MHz, $CD_3OD$) data formyricetin 3-*O*-α-L-rhamnoside (Duan *et al.*, 2009)

## 4.4 Biological activities

### 4.4.1 Antiplasmodial activities of *Ajuga remota*

Besides phytochemical study of the underground part of *A. remota,* antiplasmodial study was one of the specific objectives of this study to validate the ethinomedical use of *A. remota* in the management of malaria and its complications. Consequently, crude and pure isolates were assayed using automated microdilution technique to determine 50% growth inhibition of cultured parasites. Crude extracts and pure isolates were tested for antiplasmodial activities against chloroquine-resistant (W2) and chloroquine-sensitive (D6) strains of *P. falciparum* using (G-$^{3}$H) hypoxanthine uptake assay (Desjardins *et al.*, 1979; Muthaura *et al.*, 2007a). Antiplasmodial activities of crude extracts and pure isolates are summarized in Table 4.13. EtOAc extract was the most active. It showed moderate activities with $IC_{50}$ value 15.4 and 19.4 µg/ml against W2 and D6 respectively. The MeOH extract had moderate activities with $IC_{50}$ values 31.3 and 32.5 µg/ml against W2 and D6 respectively. The *n*-hexane extract showed low activity of $IC_{50}$ value 55.6 and 62.4 µg/ml against W2 and D6 respectively. It was noted that this extracts were consistently more active against the W2 strain than D6 strain. The activity noted for the crude extracts might be the reason why the plant is used by herbalists to manage malaria and complications associated with it.

Among compounds isolated from the EtOAc extract betulinic acid **(40),** β-sitosterol **(69),** stigmasterol **(70)** exhibited good activity against W2 and D6 with $IC_{50}$ values 9.4 and 10.0, 17.3 and 17.7, 14.9 and 16.8 µM respectively this was attributed to OH group attached to C-3 (De Sa *et al.*, 2009), while 7β-hydroxylup-5,20(29)-diene-3-one **(124)** showed moderate activity with $IC_{50}$ values of 21.6 and 22.0 µM against W2 and D6

respectively. The activity of compound **124** was attributed to the keto group at C-3 which binds to the protein of the parasite. These results were however statistically not significant compared to the controls, never the less these activities were noted to be responsible for the antiplasmodial potential of the EtOAc extract.

Flavonols isolated in this study showed moderate antiplasmodial activity. For instance, myricetin 3-*O*-α-rhmnoside **(129)** had activities of 22.2 and 27.6 μM against W2 and D6 respectively and, quercetin3-*O*-β-galactoside **(127)** had activities of 35.3 and 37.9 μM against W2 and D6 respectively. The moderate activity noted for the flavonols could be probably due to the free phenolic hydroxyl groups in ring B, which happen to form very stable radicals due to extended conjugation up to C-4 of the keto group. These moderate activities were noted to be responsible for the moderate activity of the MeOH extract. 8-*O*-Acetylharpagide **(93)** and 6,8-diacetylharpagide-1-*O*-β-(2',3'-diacetylglucoside) **(95)** were noted to be inactive against the two strains of *P. falciparum*.

Generally, it was noted that the antiplasmodial activity of some of the compounds isolated in this study were relatively low compared to the standards. This could be explained by the way in which plants are used in the management of malaria, not for their anti-plasmodial effects but also because of other activities with therapeutic value to a malaria patient. For example, apart from treatment of malaria *A. remota* exhibits analgesic properties (Debella *et al.*, 2003) and is also used to relieve fever in some communities (Githinji and Kokwaro, 1993). In addition, traditional healers give a mixture of plants for treatment of diseases. Mixtures could be active because of synergistic activity of crude mixture metabolites.

Table 4.13: *In vitro* $IC_{50}$ values of extracts and compounds of *A. remota* against W2 and D6 strains of *P. falciparum*

| Extract | W2 (µg/ml) | D6 (µg/ml) |
|---|---|---|
| *n*-Hexane | 55.6 ± 2.0 | 62.4 ± 0.2 |
| EtOAc | 15.4 ± 1.1 | 19.4 ± 1.9 |
| MeOH | 31.3 ± 0.1 | 32.5 ± 0.6 |
| **Compound** | **W2 (µM)** | **D6 (µM)** |
| β-sitosterol**(69)** | 17.3 ± 0.1 | 17.7± 0.4 |
| Stigmasterol **(70)** | 14.9 ± 0.1 | 16.8 ± 1.4 |
| 7β-hydroxylup-5,20(29)-diene-3-one **(124)** | 21.6 ± 0.5 | 22.0 ± 0.8 |
| Betulinic acid **(40)** | 9.4 ± 0.4 | 10.0 ± 1.1 |
| 8-*O*-Acetylharpagide **(93)** | 241.6 ± 1.0 | 245.6 ± 2.5 |
| 6,8-Diacetylharpagide-1-*O*-β-(2',3'-diacetylglucoside) **(95)** | 179.7± 1.5 | 183.5± 0.5 |
| Myricetin **(113)** | 38.4 ± 1.3 | 45.6 ± 1.5 |
| Arjungenin **(125)** | 36.9 ± 0.3 | 38.7 ± 0.2 |
| Kaempferol 3-*O*-β-rhamnoside **(126)** | 68.1± 0.5 | 74.8±0.1 |
| Quercetin 3-*O*-β-galactoside **(127)** | 35.3 ± 3.5 | 37.9 ± 2.3 |
| Quercetin 3-*O*-β-(6-*O*-α-rhamnosyl)galactoside **(128)** | 38.2 ± 0.4 | 39.8 ± 0.6 |
| Myricetin 3-*O*-α-rhmnoside **(129)** | 22.2 ± 0.4 | 27.6 ± 1.8 |
| Chloroquine | 0.20 ± 0.005 | 0.15 ± 0.001 |
| Quinine | 0.05 ± 0.002 | 0.10 ± 0.001 |
| Mefloquine | 0.03 ± 0.004 | 0.04 ± 0.004 |

### 4.4.2 Larvicidal activities of *Ajuga remota*

The larvicidal test involved second instar lavae of *Aedes aegypti* which could lead to a possibility of larval source reduction by habitat modification or use of larval control agents which are target specific and environmentally benign botanicals which could be incorporated in larval control for malaria management programmes. Crude extracts and pure isolates were tested for larvicidal activities against second instar lavae of *A. aegypti*.The larvicidal activities are summarized in Table 4.14. The crude extracts showed a potent and dose dependent larvicidal activity that were statistically significant ($p \leq 0.05$) compared with the control. The EtOAc extract showed a good activity with $LC_{50}$ value 5.7µg/ml at 24 hours. The *n*-hexane extract had a moderate activity with an $LC_{50}$ value

32.5µg/ml. While, the MeOH extract exhibited a low activity with $LC_{50}$ value 62.8 µg/ml at 24 hours.

Betulinic acid **(40)** isolated from the EtOAc extract exhibited good activity with an $LC_{50}$ value 4.8 µM. 7β-hydroxylup-5,20(29)-diene-3-one **(124)** exhibited a moderate activity with an $LC_{50}$ value 22.5 µM. β-Sitosterol **(69)** and stigmasterol **(70)** were inactive against second instar lavae of *A. aegypti* at 24 hours because they exhibited toxicity with $LC_{50}$ values greater than 200 µM. This indicated that the activity of the EtOAc extract is largely due to betulinic acid **(40)** and to some extent 7β-hydroxylup-5,20(29)-diene-3-one **(124)**. Arjungenin **(125)**, Kaempferol 3-*O*-β-rhamnoside **(126)**, Quercetin3-*O*-β-galactoside **(127)**, Quercetin 3-*O*-β-(6-*O*-α-rhamnosyl)galactoside **(128)** showed moderate toxicity with $LC_{50}$ value of 44.6, 75.2, 80.0, 73.0 µM respectively. These activities were better than that of crude MeOH extract from which they were isolated this indicated that there was no synergistic activity of compounds in the crude extract. Myricetin **(113)** and Myricetin 3-*O*-α-rhmnoside **(129)** exhibited low activity with $LC_{50}$ values of 134.6 and 118.3 µM respectively. It was observed that with the introduction of a sugar moiety at C-3 improved the activity as illustrated by compound **113** and **129**. It was observed further that an increase in the molecular mass of the sugar moiety also improved activity as illustrated by compound **124** and **125**. 8-*O*-acetylharpagide **(93)**, 6,8-diacetylharpagide-1-*O*-β-(2',3'-diacetylglucoside) **(95)** were inactive because they exhibited $LC_{50}$ values greater than 200 µM.

**Table 4.14: $LC_{50}$ values of extracts and compounds of *A. remota* on the 2nd instar larvae of *A. aegypti* at 24 hours after exposure**

| Extract | $LC_{50}$ (µg/ml) in 24 hours |
|---|---|
| *n*-Hexane | 32.5 ± 0.34 |
| EtOAc | 5.7 ± 0.32 |
| MeOH | 62.8 ± 0.24 |
| **Compound** | **$LC_{50}$ (µM) in 24 hours** |
| β-sitosterol **(69)** | 262.8 ± 0.46 |
| Stigmasterol **(70)** | 249.8 ± 0.30 |
| 7β-hydroxylup-5,20(29)-diene-3-one **(124)** | 22.5 ± 0.33 |
| Betulinic acid **(40)** | 4.8 ± 0.48 |
| 8-*O*-acetylharpagide **(93)** | 290.4 ± 0.28 |
| 6,8-diacetylharpagide-1-*O*-β-(2',3'-diacetylglucoside) **(95)** | 210.9 ± 0.48 |
| Myricetin **(113)** | 134.6 ± 0.25 |
| Arjungenin **(125)** | 44.6 ± 0.31 |
| Kaempferol 3-*O*-β-rhamnoside **(126)** | 75.2 ± 0.23 |
| Quercetin3 -*O*-β-galactoside **(127)** | 80.0 ± 0.24 |
| Quercetin 3-*O*-β-(6-*O*-α-rhamnosyl)galactoside **(128)** | 73.0 ± 0.33 |
| Myricetin 3-*O*-α-rhmnoside **(129)** | 118.3 ± 0.24 |
| **Control** | |
| Malathion | 0.02 ± 0.004 |
| DMSO | 2678.4 ± 0.43 |

### 4.4.3 Antioxidant activities of *Ajuga remota*

This study involved two methods, TLC which gave qualitative data on radical scavenging activity and UV-VIS spectrometry method which gave quantitative data, they both involved a stable DPPH radical. This was to identify antioxidant compounds which could be incorporated in the treatment of malaria to help in control of production of reactive oxygen species during malaria chemotherapy. The antioxidant activities are summarized in Table 4.15. Preliminary scavenging activities using DPPH free radical on TLC plates of the crude extracts indicated that *n*-hexane and EtOAc extracts had no radical scavenging activity. However, the MeOH extract showed radical scavenging activity.

Isolates from the MeOH extract were also subjected to preliminary radical scavenging activities which showed that 8-*O*-acetylharpagide **(93)**, 6,8-diacetylharpagide-1-β-(2',3'-diacetylglucoside) **(95)** and arjungenin **(125)** were inactive, while, myricetin **(113)**, kaempferol 3-*O*-β-rhamnoside **(126)**, quercetin3-*O*-β-galactoside **(127)**, quercetin3-*O*-β-(6-*O*-α-rhamnosyl)galactoside **(128)** and myricetin 3-*O*-α-rhmnoside **(129)** showed radical scavenging activities. This showed that phenolic groups are involved in the radical scavenging process.

Consequently, the active extract and isolates were subjected to the UV-VIS spectrometry. All the samples showed a potent and dose dependent radical scavenging activity towards DPPH radical that were statistically significant ($p \leq 0.05$) compared to the control. The MeOH extract had an $IC_{50}$ value 10.4 µg/ml. Compound **113** showed a DPPH radical scavenging activity with $IC_{50}$ value 17.6 µM, which has six hydroxyl groups was the most effective and was even a better radical scavenger than the standard quercetin ($IC_{50}$ value 18.4. µM), which indicated that the more the hydroxyl groups in ring B the better the scavenging activity. Compound **129** had an $IC_{50}$ value 19.4 µM which was lower than compound **113** which showed that the presence of a sugar residue resulted in a decrease in the antioxidant activity for myricetin (Zou *et al.*, 2004). Activity decreased even further when the molecular mass of a sugar residue increased as evidenced by compound **127** ($IC_{50}$ value 19.2 µM) and **128** ($IC_{50}$ value 20.3 µM) (Zou *et al.*, 2004). Compound **126** exhibited insignificant DPPH free radical scavenging activity, this was attributed to the one hydroxyl group at C-4' as compared to the other isolates which have at least two hydroxyl groups in ring B of the flavonols.

**Table 4.15: Radical scavenging activities of extracts and compounds from *A. remota* on DPPH**

| Extract | TLC Assay Results | $IC_{50}$ (µg/ml) |
|---|---|---|
| *n*-hexane | - | NT |
| EtOAc | - | NT |
| MeOH | + | 10.4 ± 0.13 |
| **Compound** | | **$IC_{50}$ (µM)** |
| 8-*O*-Acetylharpagide **(93)** | - | NT |
| 6,8-Diacetylharpagide-1-β-(2',3'-diacetylglucoside) **(95)** | - | NT |
| Myricetin **(113)** | + | 17.6 ± 0.16 |
| Arjungenin **(125)** | - | NT |
| Kaempferol 3-*O*-β-rhamnoside **(126)** | + | 1074.3 ± 0.12 |
| Quercetin 3-*O*-β-galactoside **(127)** | + | 19.2 ± 0.12 |
| Quercetin 3-*O*-β-(6-*O*-α-rhamnosyl)galactoside **(128)** | + | 20.3 ± 0.13 |
| Myricetin 3-*O*-α-rhmnoside **(129)** | + | 19.4 ± 0.16 |
| Quercetin(Control) | + | 18.4 ± 0.13 |

NT- Not Tested

## CHAPTER FIVE: Summary, Conclusions and Recommendations

### 5.1 Summary

- Sequential extraction of 1 Kg powder of underground part gave 22.2g of a light brown *n*-hexane extract, and brown extracts for both EtOAc and MeOH with yields of 34.4 and 82.9g respectively.
- Two harpagides; 8-*O*-acetylharpagide **(93)** and 6,8-diacetylharpagide-1-*O*-β-(2',3'-diacetylglucoside) **(95),** five flavonols; Myricetin **(113),** Kaempferol 3-*O*-β-rhamnoside **(126),** quercetin 3-*O*-β-galactoside **(127),** quercetin 3-*O*-β-(6-*O*-α-rhamnosyl)galactoside **(128)** and myricetin 3-*O*-α-rhmnoside **(129)** and five triterpenes; betulinic acid **(40),** β-sitosterol **(69),** stigmasterol **(70),** 7β-hydroxylup-5,20(29)-diene-3-one **(124),** and arjungenin **(125)** were isolated from underground part of *A. remota*.
- EtOAc extract was the most potent extract against the strains of the *P. falciparum* tested. This was also exhibited by its pure isolates. It was followed by the MeOH extract which also gave isolates with moderate activity. The *n*-hexane extract was the least active.
- EtOAc extract was the most active against 2$^{nd}$ instar lavae of *A. aegpti* after 24 hours of exposure. Betulinic acid **(40)** was the most active compound followed by 7β-Hydroxylup-5,20(29)-diene-3-one **(124).** Some of the isolates had moderate activity. However, the harpagides and sterols had insigficant activity.
- The *n*-hexane extract exhibited moderate activity with an $LC_{50}$ value 32.5 µg/ml against 2$^{nd}$ instar lavae of *Aedes aegypti* at 24 hours. Though isolation of metabolites from it was not possible using column chromatography.

- The MeOH extract had the DPPH radical scavenging activity among the crude extracts. Whereas the *n*-hexane and EtOAc extracts had no activity. Myricetin **(113)** was the most potent among the pure isolates.

## 5.2 Conclusions

- Betulinic acid **(40)**, β-sitosterol **(69)**, 7β-hydroxylup-5,20(29)-diene-3-one **(124)**, arjungenin **(125)**, quercetin 3-*O*-β-galactoside **(127)**, quercetin 3-*O*-β-(6-*O*-α-rhamnosyl)galactoside **(128)**, Myricetin 3-*O*-α-rhmnoside **(129)** have been reported for the first time from this plant.
- Among the pure isolates tested betulinic acid **(40)**, β-sitosterol **(69)**, stigmasterol **(70)** exhibited good activity against the strains of *P. falciparum* tested. 7β-hydroxylup-5,20 (29)-diene-3-one **(124)** and flavonols had moderate activity. While the harpagides isolated had insigficant activity.
- The underground part of *A. remota* has the potential use for small scale control of mosquitoes from the larval stage in Kenyan rural communities where mosquito transmitted diseases such as malaria is endemic.
- There is potential use of the phenolic compounds of the underground part of *A. remota* as radical scavengers.

## 5.3 Recommendations

- Herbalists may be advised to use the underground part of *A. remota* to manage malaria and its related complications.
- The underground part of *A. remota* may be used for contol of mosquitoes from the larval stage. Structural-activity relationship and modification of the pure isolates be carried out with a view of improving the efficacy of these isolates.

## 5.4 Suggestions for future studies

- EtOAc and MeOH extracts be subjected to other methods of separation such as preparative high performance liquid chromatography and reversed phase column chromatography to establish if more compounds can be isolated.
- Structural modifications of the compounds should be done to improve their efficacy followed by *in vitro* anti-plasmodial & larvicidal tests. Then *in vivo* anti-plasmodial tests carried out, together with toxicological studies.
- The ability of the active antioxidants isolated in this study to scavenge various oxidizing species such as superoxide ion ($O_2^{\cdot}$), hydroxyl radical ($HO^{\cdot}$) and peroxy radical ($ROO^{\cdot}$), alkoxy radical ($RO^{\cdot}$) and nitric oxide be investigated.

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