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KUMASI, GHANA.

ANTIPLASMODIAL EVALUATION OF EXTRACTS OF SELECTED GHANAIAN MEDICINAL PLANTS AND OTHER BIOACTIVITIES OF ISOLATES OF *POLYALTHIA LONGIFOLIA* VAR. PENDULA (ANNONACEAE)

BY

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DECLARATION AND CERTIFICATION

I, Stephen Yao Gbedema, do hereby declare that this submission is my own PhD research work and that, to the best of my knowledge, it contains no material previously published by another person nor material which has been accepted for the award of any degree of the University, except where due acknowledgement has been made in the text.

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A DEDICATION

TO

MY WIFE; JULIET GBEDEMA

AND

KIDS; SETORNAM & SENAM GBEDEMA

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It would not have been possible to write this thesis without the strength of the Almighty God; His goodness and mercies were incessantly abundant towards me in the course of this work; I am most grateful to Him.

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ABSTRACT

Malaria is one of the most important tropical infectious diseases. About half of the world's population is at risk of developing malaria. It is a major public health challenge as well as a significant economic burden on many developing countries, especially in the African region. Most malaria patients are usually anaemic and also experience reduced or compromised immune systems which therefore predispose them to secondary bacterial and fungal infections. People living in extreme poverty are the most vulnerable to these infectious diseases simultaneously. This affects their ability to make a living and move out of poverty. Many of these people rely on herbs for treatment of the disease due to lack of access to efficacious antimalarial drugs against, especially the fast spreading multidrug-resistant *Plasmodium falciparum*. However, very few of these folklore herbs have been scientifically investigated and authenticated for used in the management of malaria and other infectious diseases. This study therefore sought to investigate and validate or otherwise the traditional uses of some Ghanaian plants for treating malaria. The ethanolic extracts of ten plant species which were selected based on their traditional medical application were screened against the multidrug resistant *P. falciparum* (K1 strain) by the parasite lactate dehydrogenase (pLDH) assay. Seven of the plant species (*Adenia cissampeloides* Planch. ex Hook. *Anthocleista nobilis* G. Don, *Elaeis guineensis* Jacq., *Entandrophragma angolense* (Welw.) CDC, *Mallotus oppositifolius* (Geisel.) Müll. Arg., *Sarcocephalus latifolius* (J.E.Sm) E. A Bruce and *Polyalthia longifolia* var. pendula) showed potent antiplasmodial activity with IC_{50} < 50 µg/ml. The extracts *P. longifolia* exhibited the most potent activity (IC_{50} < 23 µg/ml). Bioassay guided fractionation of *P. longifolia* extracts yielded four clerodane diterpenes [16-hydroxycleroda-3,13-dien-16,15-olide (**1**) and its acetylated derivative acetyl-16-oxycleroda-3,13-dien-16,15-olide (**1a**), 16-oxocleroda-3,13E-dien-15-oic acid (**2**) and 3,16-dihydroxycleroda-4(18),13(14)Z-dien-15,16-olide (**3**)], a steroid [β-Stigmasterol (**4**)] and two

alkaloids [Darienine (**5**) and L-Stepholidine (**6**)]. Even though the steroid and the alkaloids showed weak antiplasmodial activity (IC_{50}: 22 -105 µg/ml), the clerodane diterpenes exhibited significantly ($p < 0.005$) potent blood schizonticidal activity (IC_{50}: 3 - 6 µg/ml) against the multidrug resistant malaria parasite. Although the isolated clerodane diterpenes **1a, 2** and **3** are known compounds, this is the first report of their antiplasmodial activity especially against the multi-drug resistant *Plasmodium falciparum*. The clerodane diterpenes also displayed potent antibacterial and antifungal activities (MIC ranged between 3.13 and 50.00 µg/ml) against *Staphylococcus aureus* (ATCC 25923), *Streptococcus pneumoniae* (clinical isolate), *Bacillus subtilis* (NCTC 10073), *Pseudomonas aeruginosa* (ATCC 4853), *Salmonella typhi* (NCTC 8385), *Escherichia coli* (NCTC 25922) and *Candida albicans* (NCPF 3179). The study therefore appeared to lend support to the traditional uses of these plants as remedies for malaria in Ghana and formulations containing especially *P. longifolia* extracts will be highly beneficial in treating mixed infections associated with malaria.

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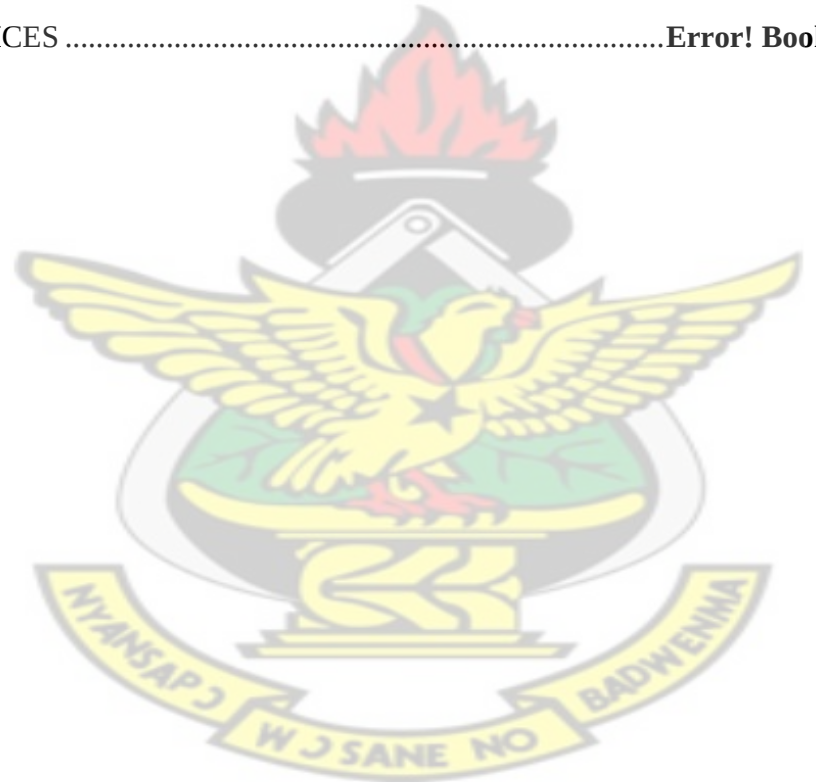

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Chapter One

1 INTRODUCTION

1.1 General Background

Throughout history, man has suffered from infectious diseases caused by pathogenic microorganisms such as viruses, bacteria, fungi and parasites. Infectious diseases comprise clinically evident illness resulting from the presence and growth of the pathogenic microorganisms in the individual host (Neelavathi *et al*., 2013). They are transmitted in the host population, often resulting in epidemics that endanger people's health and hamper national economies. The eminence of infectious diseases can be established from records of human suffering and death caused by diseases such as example smallpox and tuberculosis. Even though the era of anti-infective chemotherapy and vaccines development has tremendously helped to curb the impact or eradicate most of these diseases, they are still a major public health burden especially in the third world countries (Bayor, 2007). A notable example of these infectious diseases, which is a major public health and developmental challenge in Ghana and many other African countries, is malaria.

Malaria, a parasitic infection caused by a protozoan of the *Plasmodium* genus, contributes substantially to the poor health situation in Africa. About 90% of the world's 216 million cases and 655 000 annual deaths of malaria occur in the sub-Saharan African region (WHO, 2012). In Ghana more than 3 million cases are reported annually. A significant proportion (900,000) of such cases occurs in children under the age of five (PMI, MOP for 2011). Malaria has an immense effect on people of all ages but children under five years, pregnant women and immigrants from non-endemic regions are the most vulnerable because of their low immunity. It is a major cause

of absenteeism from school in endemic countries and frequent episodes of severe malaria in young children may negatively impact on their learning abilities and educational attainment. According to WHO/UNICEF (2003), about 2% of children who recover from cerebral malaria suffer brain damage including epilepsy. In pregnant women, malaria can cause anaemia, miscarriages, stillbirths, underweight babies and maternal deaths. Malaria is therefore, a threat to human capital accumulation, which constitutes a key factor in economic development (Asante & Asenso-Okyere, 2003). The devastating impacts of malaria on adult victims are also very much disturbing.

It causes considerable pain and weakness in the victims which translate into reduced working abilities. A single bout of the disease in a young adult costs an equivalent of 10 working days. It has also put an unbearable strain on household resources as malaria care can cost up to 34 % of a poor household's income (Asante & Asenso-Okyere, 2003). This adversely and substantially impact on gross domestic product. In endemic countries, malaria causes a negative effect on growth of tourism, investments and trade and loss of productivity on the major sectors of their economies (Perkins *et al*., 2011). In Africa, the low productivity and high mortality resulting from malaria has been estimated to cost US$ 12 billion in lost Gross Domestic Product (GDP) and has also slowed economic growth by 1.3 % every year.

Malaria control in the sub-region is key to achieving five of the eight millennium development goals (i.e. Eradicating extreme poverty and hunger, Achieving universal primary education, Reducing child mortality rates, Improving maternal health, combating HIV/AIDS, malaria, and other diseases). Control measures of the disease include prevention of infection, treatment of infected people and control of the mosquito vector.

Great efforts have been made to eradicate malaria all over the world. The WHO in 1955 launched the Global Malaria Eradication Programme which aggressively employed effective treatment of the infection (with chloroquine) and control of the mosquito vectors (with DDT insecticide) (Mendis *et al*., 2009; Stapleton, 2004). Even though this programme helped to eradicate the disease in nations with temperate climates and seasonal malaria transmission, many other nations such as Indonesia, Haiti, Afghanistan and Nicaragua, recorded negligible achievements. The hyperendemic countries in the sub-Saharan Africa region were however completely excluded from this eradication campaign (WHO, 1978).

In Ghana, malaria control has been high on the public health agenda as far back as pre-independence with preventive interventions including indoor residual and aerial spraying with insecticides and the addition of Pyrimethamine to table salt (Adams *et al*., 2004). However, the widespread mosquito resistance to insecticides, inaccessibility of health services in the rural areas, proliferation of fake and substandard antimalarial drugs in the sub-region and the emergence of multidrug-resistant malaria parasites, have hampered the goal of these control measures. Treatment of malaria with potent, effective, available and affordable drugs nevertheless, remain crucial to the control and the eventual eradication of the disease in Ghana and the sub-region as a whole. Many antimalarial chemotherapeutic agents have been used to treat the infection but most of these agents are now not effective due to the widespread of multidrug-resistant malaria parasites (Asante & Asenso-Okyere, 2003).

Though the WHO reported a 50 % decrease in malaria cases and malaria related deaths in 11 African countries (attributed to extensive control measures such as insecticide treated nets), these achievements are mainly concentrated within the southern and middle belts of Africa (WHO Malaria Report, 2010). Effective malaria control is still complicated by poor availability, distribution and choice of antimalarials at medicine outlets (Buabeng *et al*., 2008) in the sub-

Saharan African region. The WHO report also highlighted the wide distribution of fake and ineffective malaria therapies in the region coupled with the rampant and fast spreading of both drug resistant *P. falciparum* and insecticide resistant anopheles mosquito.

The way forward for malaria is a safe, available, reliable, affordable, and effective alternative therapy. Whilst attempts at eradicating the disease are necessary in the long term, a recent report has highlighted the importance of controlling the disease given that it is largely preventable and treatable (Feacham *et al*., 2010; Tatem *et al*., 2010). There is therefore the need to continue the search for potent and more effective antimalarial agents for the treatment of the infection.

Historically, plants have played a vital role in malaria treatment: the Peruvian *Cinchona* tree bark and the Chinese herb *Artemisia annua* are typical examples. A large proportion of our rural population in Ghana and many other communities in Africa continue to depend on plants as accessible first line and cost effective therapy for malaria due to the high cost of conventional drugs and the absence of health facilities in these areas (Asase *et al*., 2005; Mshana *et al*., 2001; Dokosi, 1998; Asase & Oppong-Mensah, 2009). Even in the urban centres where the health facilities are sited, many of the people still rely on these traditional herbal therapies due to the high cost of the conventional treatment and the believe that herbs being organic are safer with little or no adverse effects (Asase *et al*., 2010; WHO 2004b). Moreover, the high surge in the spread of the resistant malaria parasites which has led to high rates of recorded treatment failures (WHO, 2001) in the sub-region necessitates the urgency for the search of new treatment options. Plants still appear to hold the potential as a reservoir of largely untapped chemical compounds possibly unrelated structurally to the existing antimalarial agents which may be active through new, independent mechanisms (Phillipson, 1994) including drug-resistance reversing (Gibbons

2005), that will withstand the problem of resistance and cross-resistance being encountered among the current antimalarial agents.

1.2 The aims and objectives of the study

This research project was designed to:

- make a selection of some indigenous plant species on the basis of folkloric use in malaria treatment.
- evaluate the extracts and fractions of these plants for antiplasmodial properties.
- investigate promising species through isolation, purification, characterization, identification and retesting of the constituents.

It is anticipated that this might reveal potent antimalarial drugs or lead compounds for the development of new and potent drugs for the treatment of drug resistant *P. falciparum* infections.

In Ghana and many other developing countries, bacterial and fungal infections are still among our worst hit diseases (Bayor, 2007). Most severe malaria victims are usually anaemic with reduced or compromised immune system which predispose them to secondary bacterial and fungal infections (Dasari *et al*., 2011). As such, in many poor rural settings, malaria victims most often suffer microbial infection simultaneously. Therefore, research into this area is still necessary and the evaluation of interesting bioactive plant extracts and phytochemical compounds for antibacterial and antifungal properties is also central in our drug development agenda.

Natural products in general have been found to exhibit more than one bioactivity. It is therefore likely that compounds obtained from the selected plants could have other bioactivities in addition to or rather than the antimalarial activity. In view of the possibility of "multiple bioactivity" and

having identified microbial infections amongst our major infectious diseases and of which most severe malaria patients are highly predisposed to, the extracts and compounds of the most active plant were to be evaluated for antibacterial and antifungal properties in order to establish or otherwise their usefulness as broad spectrum anti-infective remedies.

Chapter Two

2 LITERATURE REVIEW

2.1 Malaria

Among the parasitic infectious diseases, malaria is the most serious public health problem in tropical and sub-tropical regions of the world. Malaria ranks high among the WHO listing of 'Infectious diseases of poverty' and remains a major cause of morbidity and mortality world-wide. Malaria is known to exert a huge negative impact on population health and economic development in countries where the disease is endemic (WHO, 2011; Sachs & Malaney, 2002). Over the years, significant efforts have been made at both national and international levels to reduce the burden of malaria disease and its impact on global health (Miller *et al*., 1994).

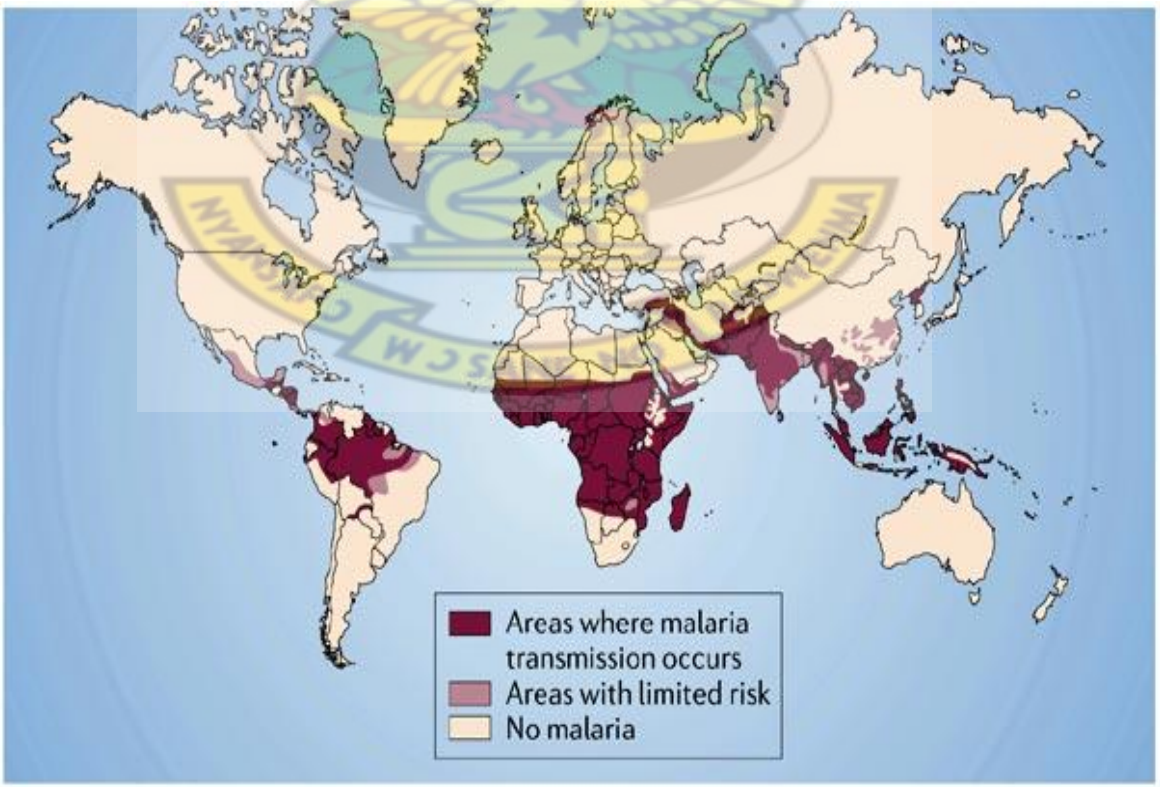


Figure 2.1 Geographical distribution of malaria burden worldwide.

(Source: WHO, 2011)

However, whereas significant improvement has been made in Latin America and Southern Africa to eliminate malaria, the situation in the rest of Africa is getting worse especially in the Sub-Saharan regions where majority (90%) of the disease burden is found (Figure 2.1). In 1998, sub-Saharan Africa was estimated to account for 34 million out of the world-wide figure of more than 39 million future life-years lost from disability and premature death (Perkins *et al*., 2011). The disease continues to spread to previously non-endemic or low-transmission areas of eastern and southern parts of the continent (WHO, 1999). The climatic conditions existing in many parts of sub-Saharan Africa favours high transmission of the disease (Snow *et al*., 1999). Furthermore, *Plasmodium falciparum* which is associated with high virulence, severe morbidity and high mortality is the predominant malaria parasite species in this region.

In Ghana, the malaria situation is typical of the sub-Saharan African pattern. Transmission of the disease is all-year-round with prevalence rates in the forest ecological zones being higher (51.3%) than the coastal savannah (36.6%) (Afrane et al., 2004). From the Ghana Health Service National Malaria Control Programme (NMCP) annual report, malaria has been a major cause of poverty and low productivity accounting for about 32.5% of all OPD attendances and 48.8% of children under five years hospital admissions in the country (NMCP, 2009). The attempt to control malaria in Ghana began in the 1950s. It was aimed at reducing the malaria disease burden until it is no longer of public health significance. It was also recognized that malaria cannot be controlled by the health sector alone; therefore multiple strategies were pursued with other health related sectors. In view of this, interventions were put in place to help in the control of the deadly disease. Some of the interventions applied at the time included residual insecticide application against adult mosquitoes, mass chemoprophylaxis with Pyrimethamine medicated salt and improvement

of drainage systems. But malaria continued to be the leading cause of morbidity in Ghana (NMCP, 2009).

Malaria is really a threat to the whole world as imported malaria (infection acquired outside and brought into a national territory of a malaria-free country) and local transmission from these cases has often been reported in many countries including France, Spain, USA and UK since 2001 (WHO, 2011).

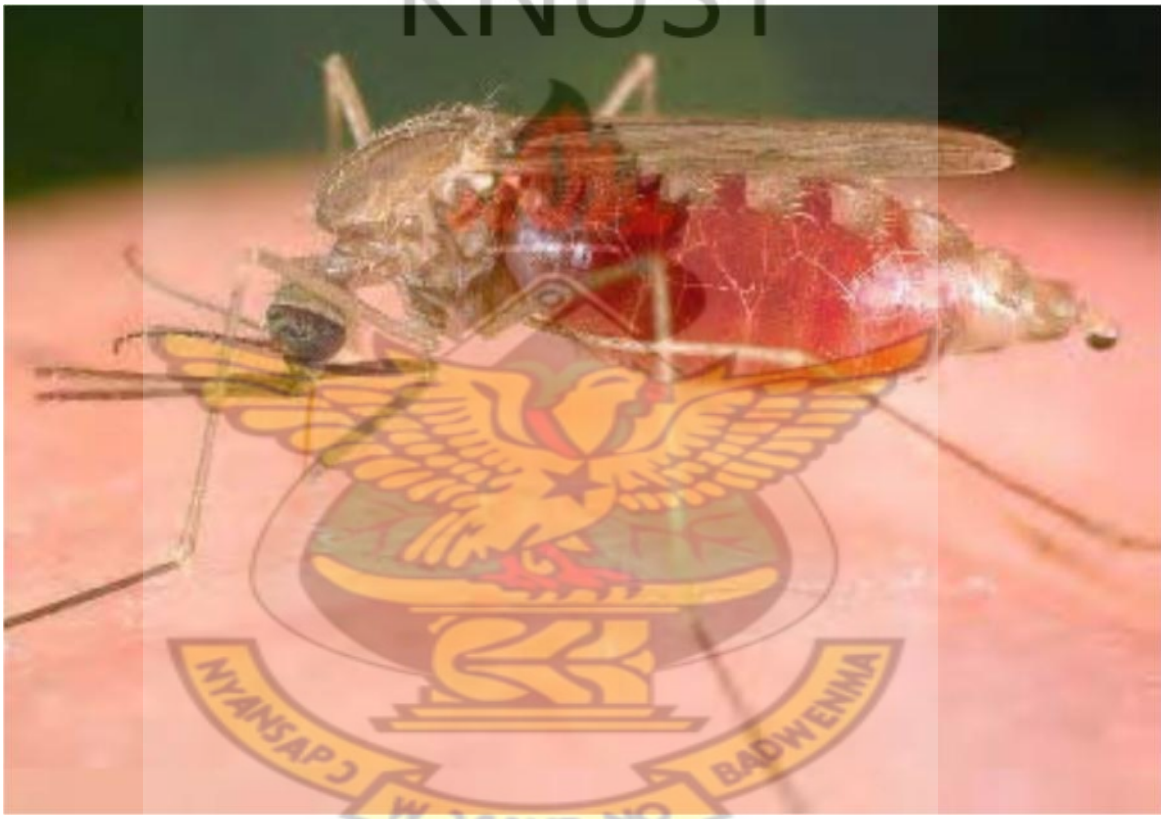


Figure 2.2. An *Anopheles* mosquito, the vector of *Plasmodium falciparum*.

(Source: Filou, 2013)

Malaria is a potentially deadly parasitic disease caused by infection of liver and red blood cells by a protozoan of the genus *Plasmodium*. It is transmitted to humans following a bite from its vector, various species of the *Anopheles* mosquito (Figure 2.2) or by a contaminated needle or

transfusion. Five mains species of *Plasmodium* can cause malaria in humans; *P. vivax* and *P. ovale*, both causing benign tertian malaria, characterised by fever that occurs every third day; *P. malariae*, which causes malariae or quartan malaria, the fever classically recurs every fourth day; *P. falciparum*, responsible for malignant tertian malaria; and *P. knowlesi* which is the most recently implicated species in human malaria (White, 2008). So far, the most virulent of the parasites is *P. falciparum*, which also accounts for the majority of severe illnesses, complications and deaths from malaria. Falciparum or malignant tertian malaria is also the most prevalent in sub-Saharan Africa, where the disease burden is highest globally (Baniecki *et al.*, 2007; Perkins *et al.*, 2011; Winstanley *et al.*, 2004).

The symptoms of malaria include cycles of chills, fever, sweats, muscle aches and headache that recur every few days. There can also be vomiting, diarrhoea, coughing, and yellowing (jaundice) of the skin and eyes. Persons with severe falciparum malaria can develop bleeding problems, shock, kidney and liver failures, central nervous system problems, coma and death.

Malaria transmission occurs primarily between dusk and dawn because of the nocturnal feeding habits of the Anopheles mosquitoes.

2.1.1 History of Malaria

Malaria is probably one of the oldest diseases known in history and it appears to have evolved together with humans as it has been known for millennia. Description of malaria dates back to the earliest medical writings in Egypt, China and India where it was mostly attributed to evil spirits. Hipocrates, in the 5th century, tried describing the clinical symptoms of the disease and by so doing differentiated quotidian and tertian fevers. Vapours and mists arising from swamps and marshes were the proposed causative agents and hence the name *mal aria* (bad air).

2.1.2 Life cycle of *Plasmodium*

Malaria is mainly transmitted through the bite of an infected female *Anopheles* mosquito. This species of mosquito needs blood for egg production. During a blood meal, the infected mosquito releases mature forms of the developing parasite, the sporozoites into the capillary bed of the human skin (Figure 1.2). On reaching humans, the sporozoites rapidly move into the parenchymal cells of the liver where they asexually develop and replicate (exoerythrocytic schizogony) into merozoites. Each merozoite when released from the liver can invade a red blood cell. The pathology and symptoms of the disease starts with the invasion and destruction of the red blood cells (Dondorp, 2005; Buffet *et al.*, 2011). With respect to *P vivax* and *P ovale* infections, some of the liver forms of the parasite (hypnozoites) could remain dormant and reactivate later to cause the disease, several months or years after an infective bite from a mosquito. In the rbcs, repeats of asexual replication (erythrocytic schizogony; Figure 1.3) occur and yield around 20 merozoites per mature parasite (Guerin *et al.*, 2002; Hotez & Kamath, 2009).

A small proportion of the merozoites do not undergo this asexual replication. These merozoites differentiate into the sexual forms referred to as macro- (female) and micro- (male) gametocytes which are enclosed in protective erythrocyte membranes. When drawing blood for egg production, these mature gametocytes can be taken by the mosquito. The protective membranes are shed in the mid-gut of the mosquito and gametogenesis is initiated. The macrogametocyte becomes the macrogamete while the microgametocyte exflagellates into 8 sperm-like microgametes and each of these is capable of fertilizing the macrogamete. When fertilization occurs, a spherical shaped zygote is formed which transforms within 12 to 18 hours into an oval leaf-shaped ookinete. The ookinete crosses the gut wall and settles in mid-gut of the mosquito

where it develops into a round oocyst which matures over a period of 10 to12 days to the infective form, (the sporozoites) in an enclosed oocyst capsule. On release from the oocyst, the sporozoites reach and penetrate the salivary gland of the mosquito. The time taken to complete the sexual phase (sporogony; Figure 2.3) is dependent on external environmental factors such as temperature and humidity (Eling *et al*., 2001).

The factors that determine the extent and intensity of malaria transmission are known to include those that influence the life cycle or development of the parasite in the mosquito vector and also in humans. These include the warm climatic conditions that promote breeding of mosquitoes, the

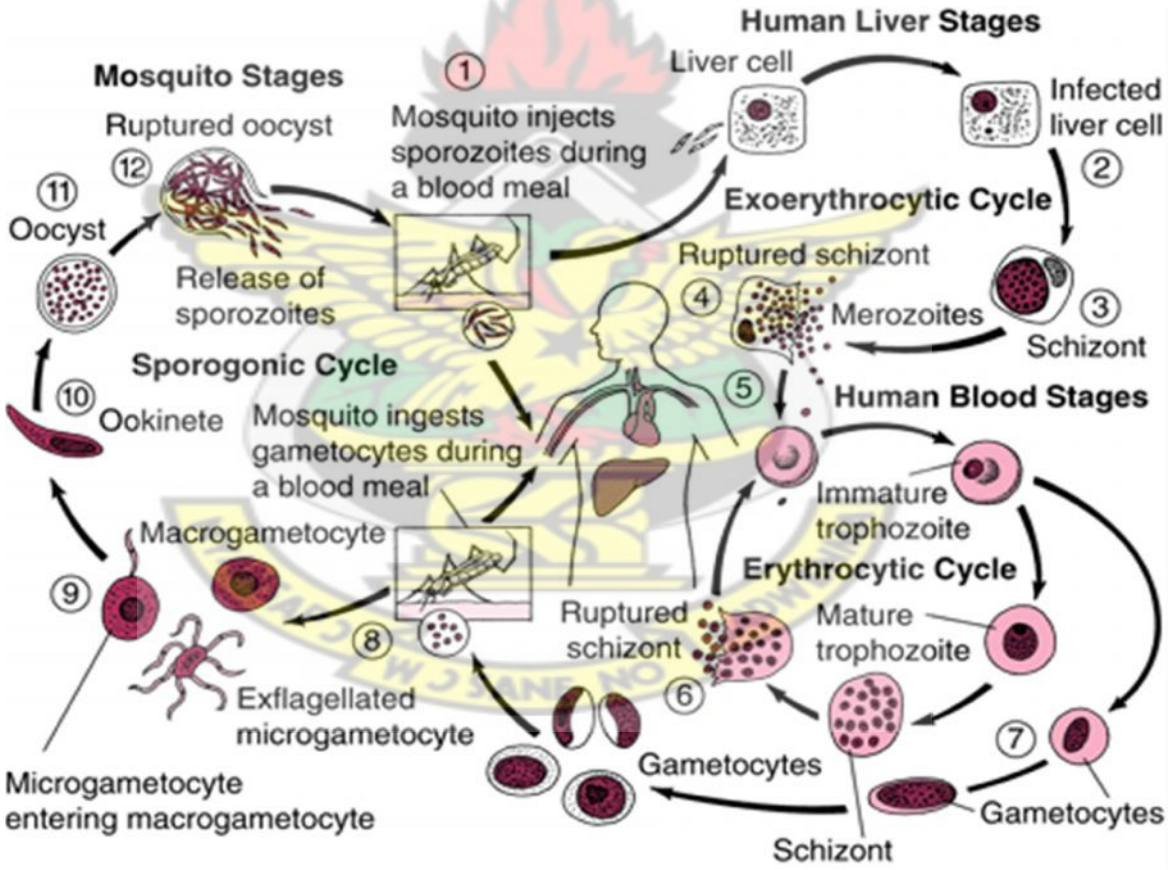


Figure 2.3 Life Cycle of *Plasmodium* species

The malaria parasite life cycle involves 2 hosts. During a blood meal, a malaria-infected female Anopheles mosquito inoculates sporozoites into the human host.
Sporozoites infect liver cells.
There, the sporozoites mature into schizonts.
The schizonts rupture and release merozoites. This initial replication in the liver is called the exoerythrocytic cycle.

Merozoites infect rbcs. There, the parasite multiplies asexually. The merozoites develop into ring-stage trophozoites. Some then mature into schizonts.
The schizonts rupture, releasing merozoites.
Some trophozoites differentiate into gametocytes.
During a blood meal, an Anopheles mosquito ingests the male and female gametocytes beginning the sporogonic cycle.
In the mosquito's stomach, the microgametes penetrate the macrogametes, producing zygotes.
The zygotes become motile and elongated, developing into ookinetes.
The ookinetes invade the midgut wall of the mosquito where they develop into oocysts.
The oocysts grow, rupture, and release sporozoites, which travel to the mosquito's salivary glands. Inoculation of the sporozoites into a new human host perpetuates the malaria life cycle.

(Source: CDC, 2012)

immune status of the human host, unavailability of resources to fight the disease and inefficient implementation of proven effective interventions for malaria control (Winstanley *et al*., 2004; Snow et al. 2005). The incubation period, which is the time between infective mosquito bites to observation of symptoms in the human host, varies from 7 to 30 days. Shorter incubation periods are observed frequently with *P. falciparum* and longer periods with *P. malariae*.

In some rare cases, malaria parasites could also be transmitted from human to human through blood transfusion or from an infected pregnant mother to the unborn child (congenital malaria) (Buabeng 2010; Winstanley *et al*., 2004).

2.1.3 Clinical (Presumptive) Diagnosis

Although reliable diagnosis cannot be made on the basis of signs and symptoms alone because of the non-specific nature of clinical malaria, clinical diagnosis of malaria is common in many malarious areas (WHO, 1997). In many of the malaria-endemic countries, resources and trained health personnel are so scarce that presumptive clinical diagnosis is unavoidable (Smith *et al*., 1994). Clinical diagnosis offers the advantages of ease, speed, and low cost. In areas where malaria is prevalent, clinical diagnosis usually results in all patients with fever and no apparent other cause being treated for malaria. This approach can identify most patients who truly need antimalarial treatment, but it is also likely to misclassify many who do not (Bloland, 2001). Over-

diagnosis can be considerable and contributes to misuse of antimalarial drugs (Ogungbamigbe *et al.*, 2005). Considerable overlap exists between the signs and symptoms of malaria and other frequent diseases, especially acute lower respiratory tract infection (ALRI), and can greatly increase the frequency of misdiagnosis and mistreatment (Redd *et al.*, 1992).

Attempts to improve the specificity of clinical diagnosis for malaria by including signs and symptoms other than fever or history of fever have met with only minimal success (Smith *et al.*, 1994). The Integrated Management of Childhood Illnesses (IMCI) programme defined an algorithm that has been developed in order to improve diagnosis and treatment of the most common childhood illnesses in areas relying upon relatively unskilled health care workers working without access to laboratories or special equipment. With this algorithm, every febrile child living in a "high-risk" area for malaria should be considered to have, and be treated for, malaria. "High risk" has been defined in IMCI Adaptation Guides as being any situation where as little as 5% of febrile children between the ages of 2 and 59 months are parasitaemic (WHO, 1997), a definition that will likely lead to significant over-diagnosis of malaria in areas with low to moderate malaria transmission.

2.1.4 Laboratory Diagnosis of Malaria

Laboratory diagnosis of malaria involves identification of malaria parasite or its antigens/products in the blood of the patient.

2.1.4.1 Microscopy

Microscopy is gold standard for laboratory confirmation of malaria. A drop of the patient's blood is collected by finger-prick, or from a larger venous blood specimen. It is then spread on a glass

slide (blood smear), dipped in a reagent that stains the malaria parasites (Giemsa stain), and examined under a microscope at a 1000-fold magnification. Malaria parasites are recognizable by their physical features (Table 1.0) and by the appearance of the red blood cells that they have infected.

Advantages: Microscopy is an established, relatively simple technique that is familiar to most laboratorians.

Disadvantages: In many developing countries, microscopy is not reliable because the microscopists are insufficiently trained and supervised and are overworked, the microscopes and reagents are of poor quality, and often the supply of electricity is unreliable. Conversely in non-endemic countries, laboratory technicians are often unfamiliar with malaria and may miss the parasites.

2.1.4.2 Morphological species and stage diagnosis

Complete knowledge of the morphological features (Table 2.1) of the different blood stages of the different *Plasmodium* species represents the essential basis of a correct laboratory diagnosis confirmation of malaria infection. It is to be stressed that a correct diagnosis may be done only after attentive and careful observation of a number of microscopic fields (at least 100 microscopic fields should be observed before a thick film may be classed as negative) and of a number of different morphologic characteristics that draw a well-defined picture of the species. A crescent-shaped gametocyte in peripheral blood does not obligatorily mean that a *P. falciparum* infection is the cause of the actual clinical complaint and, however, does not rule out the possibility of mixed infection.

Table 2.1 Morphological features of different stages of Plasmodia in stained thin blood films

	P. falciparum	*P. vivax*	*P. ovale*	*P. malariae*
Trophozoites	Always present in peripheral blood. Ring-shaped, small to medium size in dimension (Æ = 2-4 mm) depending on maturation. Young form may lay in marginal position. Polyparasitism and double chromatin dots possible.	Polymorphous in shape from large ring forms (younger forms) to ameboid mass occupying the entire red blood cell (mature forms).	Polymorphous in shape from ring forms often showing a central clear vacuole surrounded by regular cytoplasm (younger forms) to large ameboid masses (mature forms). Their dimensions are slightly inferior to *P. vivax.*	Ring form, small and regular in shape, with no pseudopodes. Older forms may be large, with vacuole Occasionally, equatorial band form present
Schizonts	Solely present in more severe infections. Small and compact, containing 15 to 30 merozoites and a dense dark brown pigmented residual body.	Normally present in peripheral blood. Large (Æ = 12-16 mm), round bodies containing 12 to 24 merozoites and loose golden brown residual pigmentation	Normally present in peripheral blood. Large (Æ = 10-12 mm), round bodies containing 4 to 12 merozoites and dark pigmentation	Compact, rosetta-like forms with 8-10 merozoites surrounding a central pigmented area
Gametocytes	Present in the second phase of the erythrocytic cycle. Crescent-shaped with coarse rice-like granules and pigment. The female is blue in colour and granules are in central position, while the male form is violet and granules are scattered over the parasite	Round regular bodies with a single voluminous nucleus (dense and red purple in female gametocytes, loose and pink in male forms).	Round regular bodies with a single voluminous nucleus (dense and red purple in female gametocytes, loose and pink in male forms). Their dimensions are usually inferior than in *P. vivax*	Compact large single dense purple nucleus (female form) or loose violet nucleus (male form). Scattered coarse pigment granules are present
Parasitic density	may be very high (average 20-500.000, max 2.000.000)	intermediate level (average 20.0000, max 50.000)	usually moderate (average 9.000, max 30.000)	usually very low (average 6.000, max 20.000)

(Source: Nizamuddin, 2009).

2.1.4.3 Peripheral Blood Smear Examination for Malarial Parasite

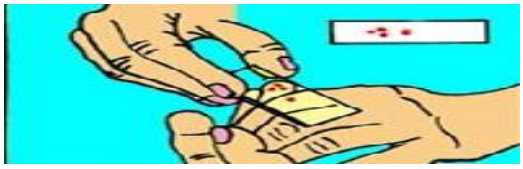
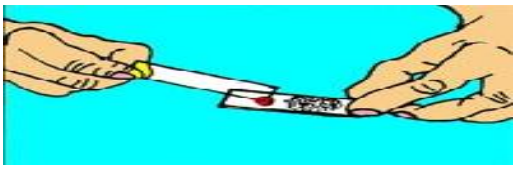
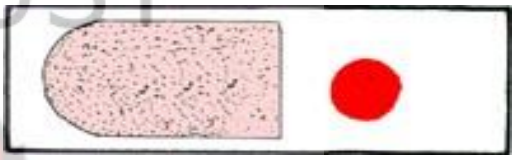
Peripheral smear examination for malaria parasite is the gold-standard in confirming the diagnosis of malaria. Thin and thick smears prepared from the peripheral blood are used for the purpose. Microscopic examination of the peripheral blood smear provides comprehensive information on the species, the stages and the density of parasitaemia with a sensitivity of 5 to 10 parasites/µl of blood. The efficiency of the test depends on the quality of the equipment and reagents, the type and quality of the smear, skill of the technician, the parasite density and the time spent on reading the smear. The test takes about 20 to 60 minutes depending on the factors mentioned above. It is estimated to cost about 50 to 90 US cents per slide in the endemic countries.

2.1.4.4 Preparation of the Blood Smears

The peripheral blood smears are prepared as illustrated in Table 2.2. Universal precautions are used while preparing the smears for malaria parasites (use of gloves and only disposable needles/lancets; hand washing; careful handling and disposal of the sharp instruments and other materials contaminated with blood to avoid injury).

Table 2.2 Steps involved in smear preparation for microscopic diagnosis of malaria.

Step1. Hold the third finger of the left hand and wipe its tip with spirit/Savlon swab; allow to dry. 	Step 2. Prick the finger with disposable needle/lancet; allow the blood to ooze out 

Step 3. Take a clean glass slide. Take 3 drops of blood 1 cm from the edge of the slide, take another drop of blood one cm from the first drop of blood. 	**Step 4**. Take another clean slide with smooth edges and use it as a spreader….. 
Step 5. ...and make thick and thin smears. Allow it to dry 	**Step 6.** Prepared smear (Slide number can be marked on the thin smear with a lead pencil.) 

(Source: WHO, 1991)

Thin Blood Smears - The thin smear is air-dried for 10 minutes and then fixed in methanol, by either dipping the thin smear into methanol for 5 seconds or by dabbing the thin smear with a methanol-soaked cotton ball.

Thick Blood Smears - A thick blood smear of correct thickness is the one through which newsprint is barely visible. It is dried for 30 minutes and not fixed with methanol. This allows the red blood cells to be haemolyzed and leukocytes and any malaria parasites present will be the only detectable elements. However, due to the haemolysis and slow drying, the plasmodia morphology can get distorted, making differentiation of species difficult. Thick smears are therefore used to detect infection and to estimate parasite concentration. Below are the steps involved in smear preparation:

2.1.4.5 Staining

A number of Romanowsky stains like Field's, Giemsa's, Wright's and Leishman's are suitable for staining the smears. Thick films are ideally stained by the rapid Field's technique or Giemsa's

stain for screening of parasites. The sensitivity of a thick blood film is 20 parasites/µl (0.0004%) parasitaemia. Thin blood films stained by Giemsa's or Leishman's stain are useful for specification of parasites and for the stippling of infected red cells and have a sensitivity of 200 parasites/µl (0.004%). The optimal pH of the stain is 7.2. The slides should be clean and dry and it is better to use neutral distilled water.

2.1.4.6 Giemsa Staining of Thin Blood Films

The fixed thin blood film is covered with Giemsa (diluted 1 in 10 with buffered distilled water pH 7.2) and left for 30 minutes. After that the film is washed with distilled water, drained, dried and examined.

2.1.4.7 Giemsa Staining of Thick Blood Films

The thick film is first dehaemoglobinized in water and then stained with Giemsa.

Rapid Giemsa: A 10% Giemsa is prepared in buffered water at pH 7.1. The slide is then immersed in the stain for 5 minutes. After that, it is rinsed gently for 1 or 2 seconds in a jar of tap water, drained, dried and then examined.

Standard Giemsa: A 4% Giemsa is prepared in buffered solution at pH 7.1. The slide (at least 12 hours old) is immersed in the stain for 30 minutes, rinsed with fresh water, drained, dried and then examined.

2.1.4.8 Field's Staining of Thick blood Films

Field's Stain is made from two components. Field's A is a buffered solution of azure dye and Field's B is a buffered solution of eosin. The unfixed slide is dipped into Field's stain A for three seconds. The slide is then dipped into tap water for three seconds and gently agitated. The slide

is dipped into Field's stain B for three seconds and washed gently in tap water for a few seconds until the excess stain is removed. The slide is drained vertically and left to dry.

Field's Solution A.

Methylene blue ..... 0.80 g

Azure B (American stain) ..... 0.50 g

Sodium phosphate, dibasic, anhydrous ..... 5.00 g

Potassium phosphate, monobasic, anhydrous…. 6.25 g

Distilled water, q.s ..... 500.00 ml

Field's Solution B.

Eosin ..... 1.00 g

Sodium phosphate, dibasic, anhydrous ..... 5.00 g

Potassium phosphate, monobasic, anhydrous… 6.25 g

Distilled water, q.s ..... 500.00 ml

2.1.4.9 Advantages and disadvantages of Malaria Rapid Diagnostic Tests

High quality malaria microscopy is not always immediately available in every clinical setting where patients might seek medical attention. Although this practice is discouraged, many healthcare settings either save blood samples for malaria microscopy until a qualified person is available to perform the test, or send the blood samples to commercial or reference laboratories. These practices have resulted in long delays in diagnosis. The laboratories associated with these healthcare settings may now use an RDT to more rapidly determine if their patients are infected with malaria.

The use of the RDT does not eliminate the need for malaria microscopy. The RDT may not be able to detect some infections with lower numbers of malaria parasites circulating in the patient's

bloodstream. Also, there is insufficient data available to determine the ability of this test to detect the 2 less common species of malaria, *P. ovale* and *P. malariae*. Therefore all negative RDT's must be followed by microscopy to confirm the result.

In addition, all positive RDTs should also be followed by microscopy. The currently approved RDT (Figure 2.9) detects 2 different malaria antigens; It uses a pair of monoclonal and polyclonal antibodies to *P. falciparum* specific protein, Histidine Repeat Protein II (pHRP-II) and a pair of monoclonal antibodies to *Plasmodium* Lactate Dehydrogenase (pLDH), a protein produced by the four species of the *Plasmodium*. This enables simultaneous detection and differentiation of the infection with *P. falciparum* and any of the other 3 species (Kakkilaya, 2003; Gillet, 2011; Murray *et al*., 2003). It can be performed by minimally skilled personnel without laboratory equipment.

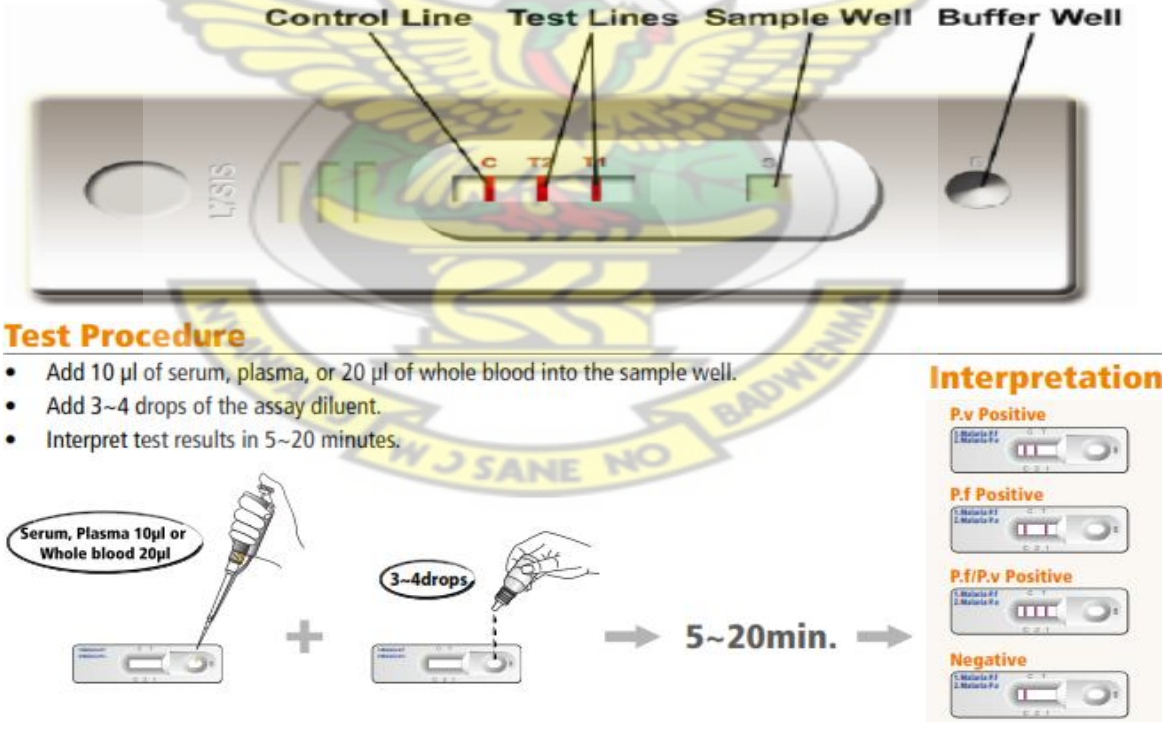


Figure 2.9 Malaria Rapid Diagnostic Test kit and procedure

(Source: Standard Diagnostics Inc, Giheung-ku, Republic of Korea).

2.1.5 Polymerase Chain Reaction

Using the non-isotopically labelled probe following PCR amplification, it is possible to detect malaria parasites. In travelers returning to developed countries, studies based on PCR have been found to be highly sensitive and specific for detecting all four species of *Plasmodium*, particularly in cases of low level parasitaemia and mixed infections. The PCR test is reportedly 10-fold more sensitive than microscopy, with one study reporting a sensitivity to detect 0.38 to1.35 parasites/µL for *P. falciparum* and 0.12 parasites/µL for *P. vivax* (Morassin *et al.*, 2002). The PCR test has also been found useful in unravelling the diagnosis of malaria in cases of undiagnosed fever (Iqbal *et al*., 1999).

2.2 Antimalarials in clinical use and drug resistance

In all the malaria elimination efforts the world over, antimalarial drugs remain very crucial with the use of insecticide treated nets (ITN) and indoor residual spraying (IRS) of insecticides to strengthen the efforts against combating malaria. The fact of the case is that with all the efforts to eliminate malaria, people in hyper endemic regions such as the sub-Saharan Africa will still contract the disease and without the availability of affordable and effective drugs, many people will succumb to the devastating effect of malaria.

The problem is further aggravated by the rampant resistance development of *P. falciparum* to the antimalarials in current use. Antimalarial drug resistance can be defined as the ability of the malaria parasite to survive and/or multiply despite the administration and absorption of a drug given in doses equal to or higher than those usually recommended but within tolerance of the subject (Bruce-Chwatt *et al*., 1986; WHO, 1972). A modification of this definition to specify that the drug in question must gain access to the parasite or the infected red blood cell for the duration

of the time necessary for its normal action has been reported (Wernsdorfer & Wernsdorfer, 1995). Most researchers interpret this as referring only to persistence of parasites after treatment doses of an antimalarial rather than prophylaxis failure, although the latter is a useful tool for early warning of the presence of drug resistance (Lobel & Campbell, 1986). Resistance generally appears to occur through spontaneous mutations that confer reduced sensitivity to a given drug or class of drugs. For some drugs, just a single point mutation is required to confer resistance while for others multiple mutations appear to be required. Provided the mutations are not deleterious to the survival or reproduction of the parasite, drug pressure will remove susceptible parasites while resistant parasites survive (Thaithong, 1983; Sridaran *et al.,* 2010; Mayfong Mayxay *et al.,* 2007).

The spread of drug and insecticide resistance is on the increase due to the evolutionary pressure being put on both the *Plasmodium* parasite and the mosquito. The Thai-Cambodia border continues to be the historical site for emergence of resistance to antimalarial drugs as the initial signs of resistance to both the older drugs such as chloroquine and the newer artemisinins have all been first reported from this area (Satimai *et al*., 2012; Maude *et al*., 2009; Noedl *et al*., 2010). With the exception of the antifolates, the modes of action and resistance mechanisms of available antimalarials are not clearly understood. However, the resistance mechanisms appear to involve among others increased elaboration of inhibited (or targeted) enzymes, modification of drug transport systems, increase production of enzymes that inactivate the drugs as well as alternative metabolic pathways (Petersen *et al*., 2011).

Currently, the available antimalarial drugs are basically derivatives of certain core structures which can be grouped into three main classes; quinolones (comprising the aryl amino alcohols, aminoquinolones and the bisquinolone), anti-folates and the artemisinins (Foley & Tilley, 1998;

Na-Bangchang & Karbwang, 2009; Achan *et al*., 2011). Some antibiotics have also exhibited antimalarial properties and these include the tetracyclines and clindamycin.

2.2.1 Aryl amino alcohols

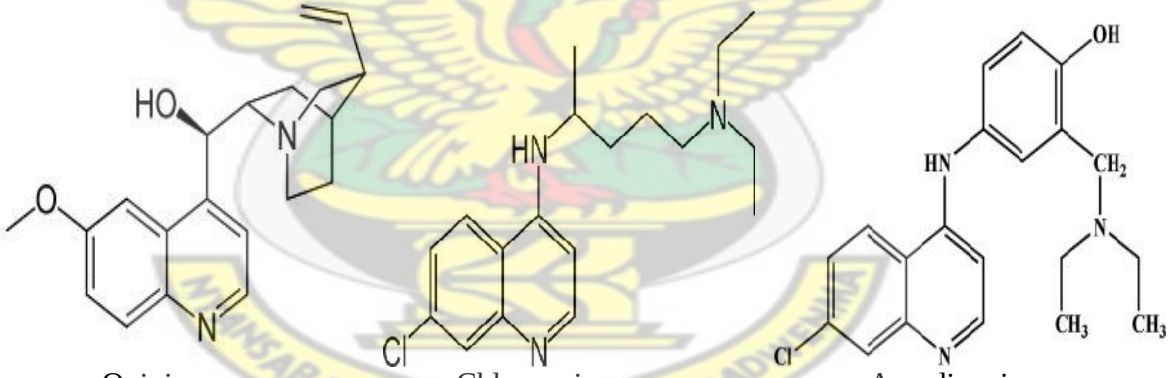
The aryl amino alcohol antimalarials include the *Cinchona* alkaloids (quinine, quinidine), mefloquine, and halofantrine and its derivatives (Figure 2.10).

2.2.1.1 Cinchona alkaloids

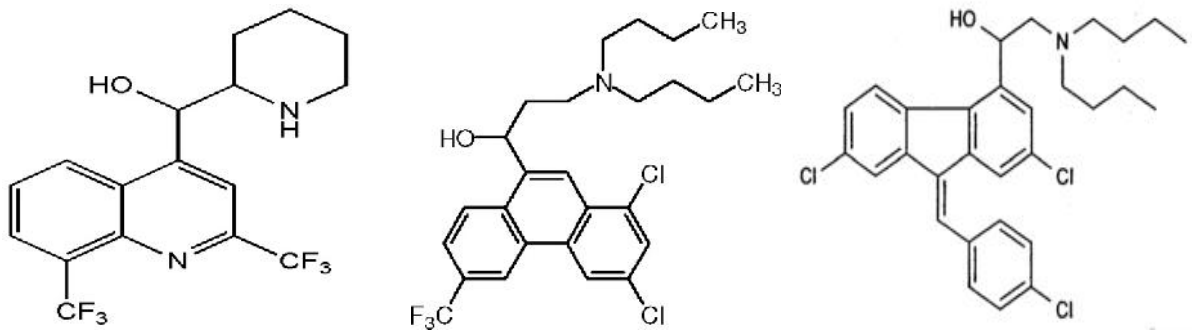
Quinine and quinidine are among the cinchona alkaloids used as antimalarials. The cinchona alkaloids are the oldest antimalarial preparation used originally by the natives of Peru for centuries in the form of *Cinchona* bark extract to successfully treat fevers. Quinine was the first extracted drug from the back of *Cinchona pubescens* in 1820 (Foley & Tilley, 1998). Quinidine is not often used due to its very high cardio-toxic effect compared to quinine. Quinine remains an important antimalarial drug almost 400 years since Jesuit priests first documented its effectiveness. Quinine is extremely basic and hence always presented as a salt. Quinine is rapidly schizonticidal against the intraerythrocytic stage of all the malaria parasites. It is also known to have gametocytocidal action on *P. vivax* and *P. malariae*. The antimalarial mechanism of action of quinine is still not clearly understood (Achan *et al.*, 2011).

Quinine has traditionally been used to treat severe cases of malaria despite its toxic effects (central nervous system or cardiovascular toxicity) upon intravenous administration. The oral administration is usually associated with non-compliance since it must be taken three times daily for seven days therefore resulting in resistance development by the parasites (Na-Bangchang & Karbwang, 2009). *Plasmodium* resistance to quinine has been reported since 1910 (Andriantsoanirina *et al*., 2010). However, the resistance has been of a low grade, with the drug retaining some activity but having its action delayed or diminished. The low grade resistance seen

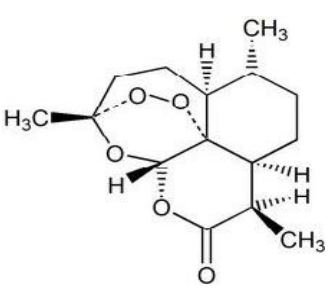
with quinine could be due to the fact that its use has been drastically reduced since the introduction of the newer synthetic antimalarials such as chloroquine which has relatively fewer and milder side effects. In its partially understood mechanism of action, quinine has been found to accumulate in the parasite's DV whereby it appears to inhibit the detoxification of haem; an important biochemical process which the parasite undertakes for survival (Petersen *et al*., 2011). Studies have shown that the genetic basis for resistance to quinine is complex and multiple genes influence susceptibility. Three types of genes have been identified of being associated with quinine resistance. These genes, all of which encode for transporter proteins, include *pfcrt* (*P. falciparum* chloroquine resistance transporter), *pfmdr1* (*P. falciparum* multidrug resistance transporter 1) and *pfnhe1* (*P. falciparum* sodium/proton exchanger 1) (Sinou *et al*., 2011).

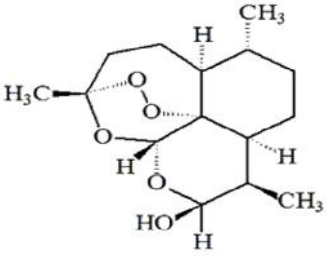


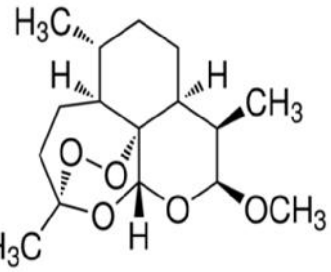
Quinine Chloroquine Amodiaquine



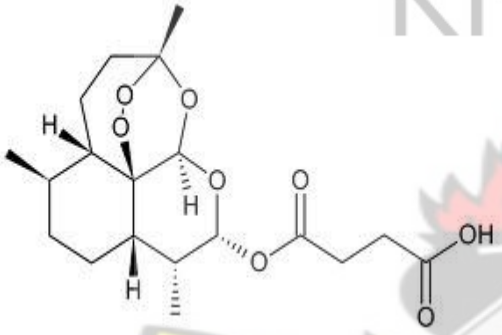
Mefloquine Halofantrine Lumefantrine

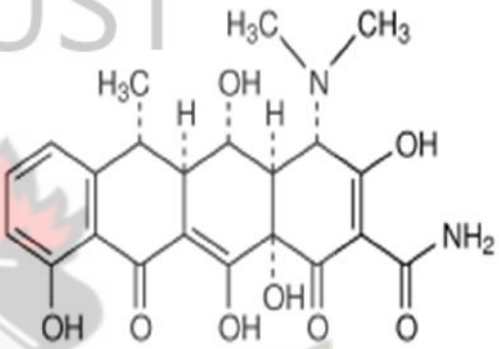




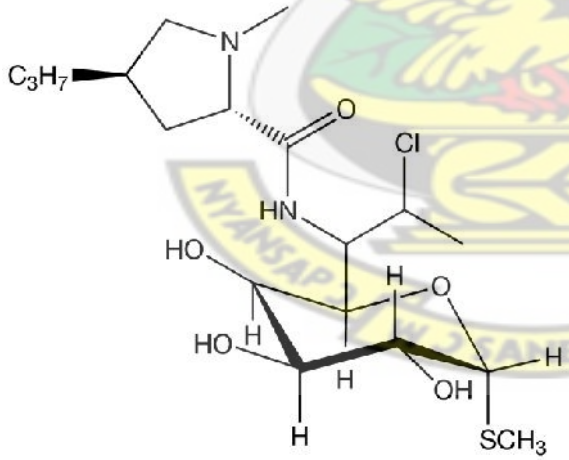


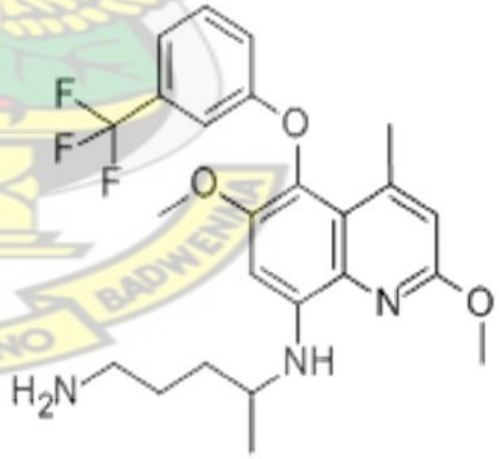
Artemisinin Dihydroartemisinin Artemether





Artesunate Doxycycline





Clindamycin Tafenoquine

Figure 2.10 Some common antimalarial drugs that target the blood stages.

2.2.1.2 **Mefloquine**

Mefloquine was first introduced in clinical use in the 1970s. Like chloroquine, mefloquine acts primarily on the intraerythrocytic asexual stages of the parasite (Foley & Tilley, 1998; Farooq & Mahajan, 2004). Mefloquine's exact mechanisms of action are also not known but studies have shown that it interferes in the haem detoxification process of the parasite. Resistance to mefloquine is mediated by amplification of *pfmdr1*, which leads to overexpression of this DV membrane transporter (Farooq & Mahajan, 2004; Mayxay *et al*., 2007). However, on studies with transgenic parasites expressing different *pfmdr1* copy numbers, Sidhu *et al*., (2006) observed a reduced parasite susceptibility to mefloquine with increased PfMDR1-mediated import into the DV, suggesting another mode of action outside of the DV (Folley & Tilley, 1998). Mefloquine's mode of action has also been suggested to target the PfMDR1 transport function itself due to the fact that it inhibited the import of other solutes into the DV (AlKadi, 2007; Ginsburg, Famin, Zhang, & Krugliak, 1998).

2.2.1.3 **Halofantrine**

Halofantrine, a phenanthrene-methanol derivative of the aryl amino alcohols was first marketed in 1988. The mode of antimalarial action of halofantrine appears to be similar to chloroquine in interfering in hemozoin formation process. Halofantrine has been shown to bind to plasmepsin, a haemoglobin degrading enzyme unique to the malarial parasites (Friedman & Caflisch, 2009). Serious cardiotoxicity side effects have been reported with the use of halofantrine, which has drastically limited its use. Lumefantrine, which is closely related to halofantrine is now being used in malaria treatment in combination therapy with artemisinin base. Lumefantrine appears to have fewer side effects but polymorphism in PfMDR1 producing strains of the parasite have been reported.

2.2.2 4-aminoquinolines

Chloroquine and amodiaquine (Figure 1.9) are the most important and widely used members of the 4-aminoquinolines.

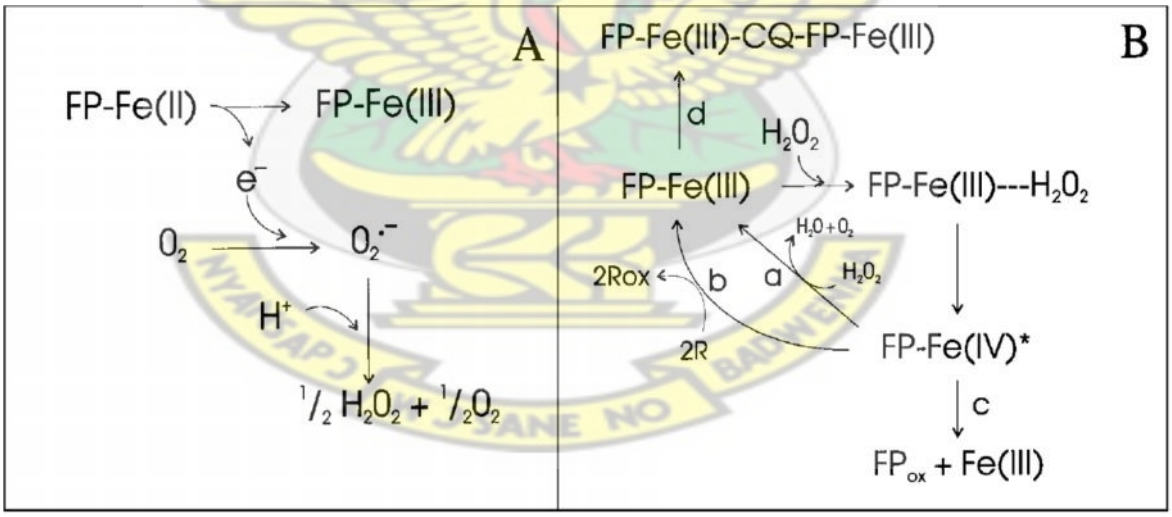
2.2.2.1 Chloroquine

Chloroquine was first synthesized in 1934 and named Resochin (Bayer Laboratories, Germany). It was initially thought to be too toxic for clinical use until after ten years of synthesis when its safety at therapeutic concentrations was realised (Foley & Tilley, 1998). Since the late 1940s chloroquine has been used massively for treatment and prevention of malaria. It was the gold standard of malaria treatment for many years (Alkadi, 2007), due to efficacy, affordability and low risk of side effects when used in the prescribed dosages. Chloroquine is schizonticidal against all the species of *Plasmodium* and also gametocitocidal to *P. vivax* and *P. malariae* but not *P. falciparum* (Loria *et al.*, 1999; Ecker *et al*., 2012). The main target of chloroquine's mechanism of action is the haem detoxification pathway in the DV. Chloroquine accumulates in the DV and thereby prevents the formation of the bio-crystals of hemozoin. This leads to a build-up of toxic haem which permeabilizes the membrane resulting in eventual death of the parasite (Rohrbach *et al.*, 2006; Petersen *et al.*, 2011).

Secondly, due to the acidic conditions of the food vacuole, haem, ferriprotoporphyrin IX (FP), is rapidly oxidized to the Fe (III) state with the transfer of an electron to oxygen to generate a superoxide radical. The superoxide is spontaneously converted to H_2O_2 (Figure 2.11A). H_2O_2 oxidizes the porphyrin ring of haem to produce an activated intermediate [FP-Fe(IV)*] that can participate in a catalase-like reaction (Figure 1.10B; reaction a) or peroxidase-like reactions (reaction b). In addition, peroxidative cleavage reactions can destroy the porphyrin ring (reaction c). Chloroquine appears to form a tight complex with haem (reaction d) which interferes with

these reactions (Loria *et al*., 1999; Sullivan, 2002). The free radicals therefore build up and complement the effects of the soluble haem in killing the parasite.

Unfortunately chloroquine has not always been used wisely and this led to development of resistant parasites after 10 years of introduction to clinical use. PfCRT protein (enzyme) has been found to be the main determinant of chloroquine resistance in addition to PfMDR1 in some strains (Nkhoma *et al.*, 2007; Nkrumah *et al.*, 2009). These transporter proteins operate in similar manner as in the case of quinine by rapidly effluxing the drug out of the parasite which prevents the drug from reaching therapeutic concentrations in the parasite DV. Some compounds including verapamil (a calcium channel blocker), vinblastine and daunomycin have been shown to reduce this trans membrane efflux of chloroquine leading to increased accumulation within the resistant parasite (Menezes *et al.*, 2003; Ecker *et al*., 2012; van Schalkwyk *et al*., 2001).



(`R' may be glutathione, protein or lipid).

Figure 2.11 (A) Generation of H_2O_2 in the food vacuole and (B) putative mechanism for the catalase- and peroxidase-like activities of haem.

(Source: Anon., no date)

2.2.2.2 Amodiaquine

Amodiaquine has been used for more than 50 years (Stepniewska & White, 2008) and is closely related to chloroquine, differing only by having a p-hydroxyanilino aromatic ring in its side chain (Figure 2.10). Based on this structural similarity, amodiaquine is hypothesized to act by inhibiting haem detoxification and has been shown to accumulate within the DV as well as binding to haem *in vitro*; however it is rapidly metabolised *in vivo* to its desethyl derivative which has a significantly reduced activity (Adjei *et al.*, 2008). Resistance to amodiaquine has inevitably followed the path of chloroquine resistance (Bjorkman & Philips-Howard, 1990). However, this cross-resistance is incomplete, as some chloroquine resistant strains of *Plasmodium* remain susceptible to amodiaquine (Winstanley *et al.*, 2004).

2.2.3 8-aminoquinoline.

Primaquine is a member of the 8-aminoquinoline antimalarials and is currently the only approved chemotherapeutic agent for eradicating the refractory, liver reservoir hypnozoites of *P. vivax* and *P. ovale* (Foley & Tilley, 1998). Primaquine is also gametocytocidal. The mode of action of primaquine appears to be related to the action of atovaquone (a naphthoquinone compound; Figure 2.14), which has been demonstrated to inhibit the cytochrome bc1 complex of the mitochondrial respiratory chain, collapsing the mitochondrial membrane potential (Fry & Pudney, 1992). The metabolites of primaquine undergo redox cycling in the erythrocyte leading to an oxidative stress that is poorly tolerated by glucose-6-phosphate dehydrogenase deficient (G6PD) cells; hence the drug is contraindicated in individuals with certain subclasses of glucose-6-phosphate dehydrogenase deficiency (Fletcher *et al.*, 1988).

2.2.4 Bisquinoline

Piperaquine (Figure 2.12), a bisquinoline antimalarial drug was first synthesized in 1960s and used extensively especially in China and Indonesia until resistant strains started to emerge after about two decades of its introduction into clinical use (Davis *et al*., 2005). Piperaquine is relatively more potent and even active against some chloroquine-resistant *Plasmodium* strains (Raynes, 1999). There are reports that piperaquine may be inhibiting the PfCRT and PfMDR1 transporters that efflux chloroquine from the parasite DV (Vennerstrom *et al*., 1992; O'Neill *et al*., 1998). The chemical structure of piperaquine contains the 7-chloro-4-aminoquinoline moiety present in chloroquine and hence believed to act on similar targets (example haemoglobin degradation) as chloroquine but the bulky bisquinoline structure may be important for activity against the chloroquine-resistant parasites (Vennerstrom *et al*., 1992).

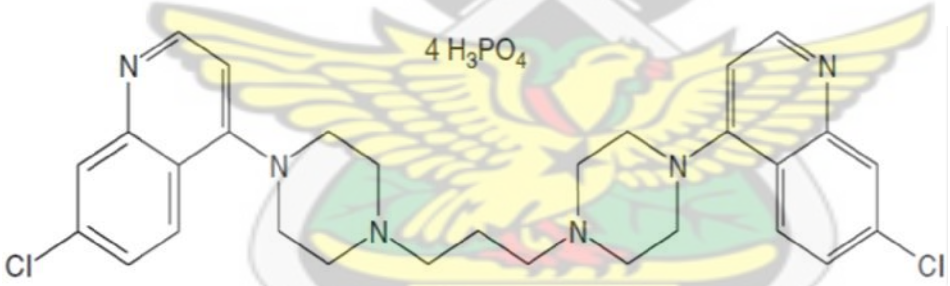


Figure 2.12 Structure of Piperaquine

2.2.5 Anti-folates

The anti-folates were rationally designed following an antimetabolite approach. Malaria parasites need folate to synthesise tetrahydrofolate for methylation reactions necessary in DNA replication. They cannot salvage this cofactor and hence synthesize it *de novo* through a system of enzymatically catalysed reactions. The anti-folates inhibit two key enzymes in this biosynthetic pathway and thus denies the parasite folate. Figure 2.13 below schematically depicts these conversions: dihydropteroate synthase (DHPS) converts 7,8-dihydro-6-hydroxymethylpterin

pyrophosphate (DHPt-PP) to 7,8-dihydropteroate (DHP) and dihydrofolate reductase (DHFR) catalyzes the reduction of 7,8-dihydrofolate (DHF) to 5,6,7,8-tetrahydrofolate (THF).

DHPS inhibitors (Type I anti-folates) are the sulphonamides and sulphone which include sulphadoxine and dapsone (Figure 2.14). The type II anti-folates are the DHFR inhibitors which also include biguanides (like proguanil and chloroproguanil) and diaminopyrimidine (like pyrimethamine). Anti-folates are schizonticidal against all stages of the malaria parasite during erythrocytic schizogony. They can also inhibit the early developmental stage of the parasite in the liver and mosquito (Gregson & Plowe, 2005; Delfino *et al*., 2002; Mharakurwa *et al*., 2011).

The anti-DHPS are usually used in combination with the DHFR inhibitors in malaria treatment (example is Fansidar) as they are found to synergize with the type II. Malaria parasites have developed resistance to antifolates through point mutations in the genes for DHPS enzyme (Type I antifolates) as well as DHFR enzyme (type II antifolates) (Nzila, 2006).

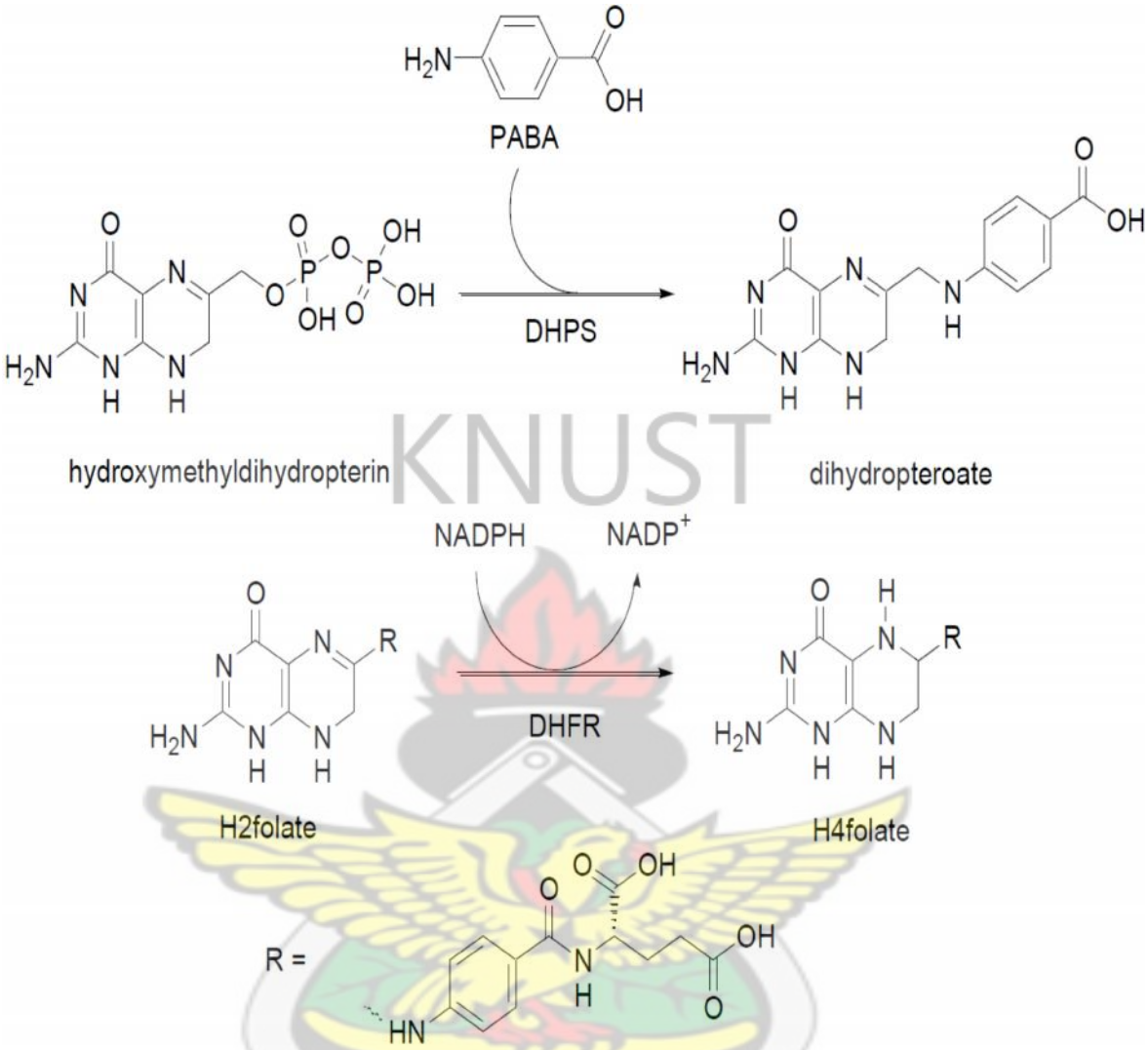


Figure 2.13 The conversion of hydroxymethyldihydropterin to dihydropteroate facilitated by DHPS (*above*). The conversion of dihydrofolate to tetrahydrofolate via the NADPH dependent DHFR enzyme (*below*).

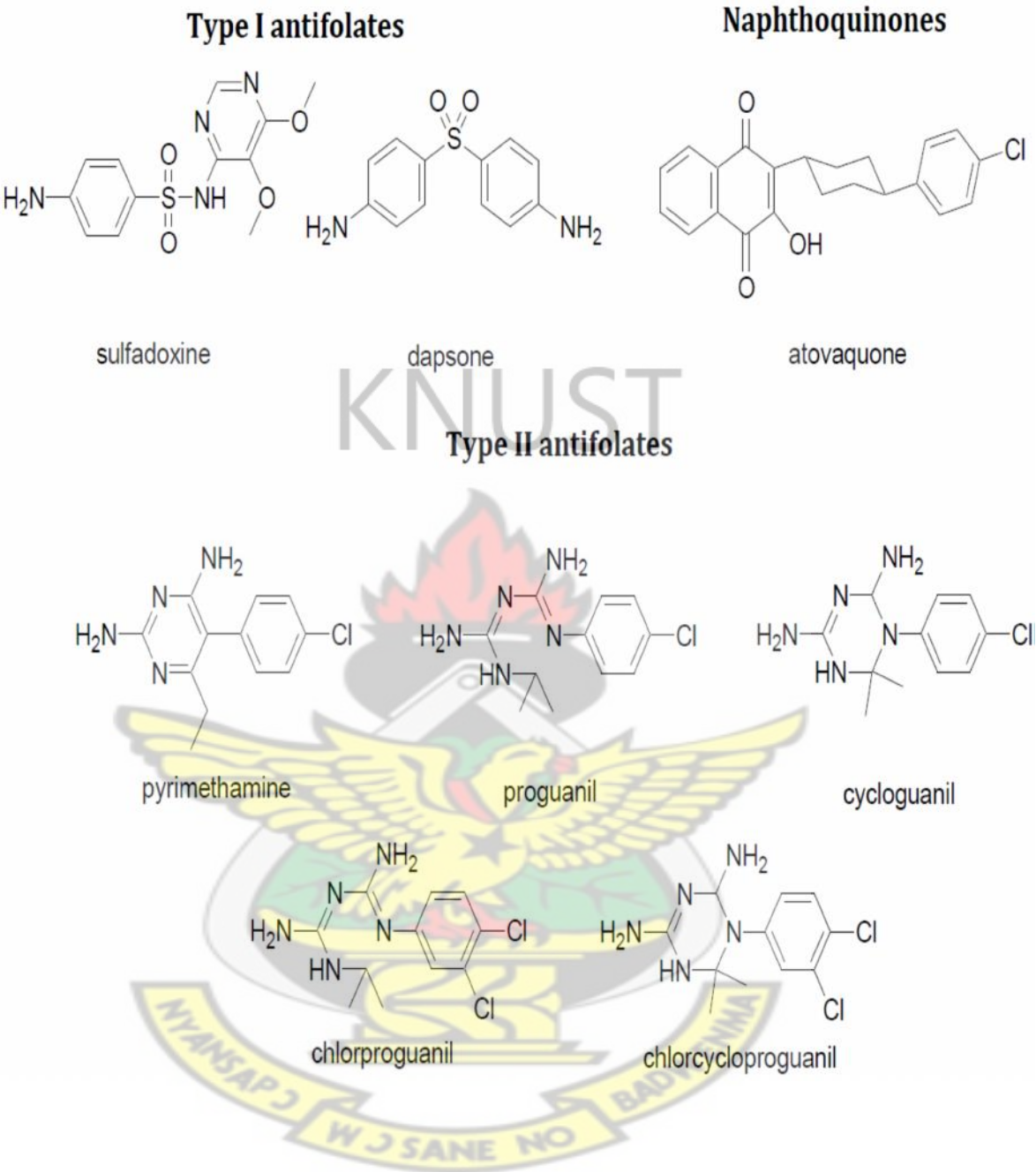


Figure 2.14 Structure of piperaquine and nucleic acid inhibitors used as antimalarials.

2.2.6 Artemisinin and its derivatives

Artemisinin is a natural endoperoxide of the sesquiterpene lactone class isolated from the Chinese herb, *Artemisia annua* ('Qinghao' or sweet wormwood) in 1972. 'Qinghao' is documented in traditional Chinese Pharmacopeia as remedy for fever for at least two millennia, which showed

that qinghao has a long history of use in humans (Mueller *et al*., 2000; Brown 2010; Wright *et al*., 2010; Carmargo *et al*., 2009). Artemisinin is poorly soluble in water which results in poor absorption and bioavailability when administered. However, various modifications of the artemisinin chemical structure have yielded very important derivatives with such as artesunate, artemether, arteether and dihydroartemisinin (figure 2.10), which have improved pharmacokinetic properties as compared to the parent compound (Meshnick, 2002). The artemisinins are fast acting drugs with rapid onset of action and hence very useful as first-line drugs in management of severe malaria. They are schizonticidal against all forms of the erythrocytic stages as well as gametocytocidal to the *Plasmodium* parasites but are not active on the liver stage. The exact mode of action of artemisinin remains elusive. However, probable multi-targeted mechanisms of action have been proposed: Artemisinin inhibits an essential calcium adenosine triphosphatase enzyme (the sarco/endoplastic reticulum calcium-dependent ATPase: *Pf*ATP6) of *P. falciparum,* disrupting calcium homeostasis, ultimately resulting in parasite death (Eckstein-Ludwig *et al*., 2003; Krishna *et al*., 2006). Also, the Endoperoxide Bridge in artemisinin structure interacts with iron in the infected erythrocyte to form free toxic radicals which through alkylation process destroy proteins that are crucial to the parasite's survival, leading to demise of the parasite (De Ridder *et al*., 2008; Vangapandu *et al*., 2007). This multi-targeted attack on the parasite by artemisinin may even be responsible for inhibiting proteolytic activity of some of the haemoglobin digesting enzymes.

Artemisinins also have comparatively short half-lives (example artesunate, $t_{½}$ is 0.5 h), and therefore unlikely resistance development, although a sign of resistance to artemisinins has already been reported in Western Cambodia (Noedl *et al*., 2008; Satimai *et al*., 2012). Recrudescence in patients receiving artemisinin mono-therapy has also been observed in Cambodia and North-Western parts of Thailand (Dondorp *et al*., 2009).

2.2.7 Antibiotics

Some antibiotics have either shown potent activity against the *Plasmodium* parasites or synergize the activity of other antimalarials. Tetracycline and its derivatives such as doxycycline are very potent antimalarials and are used for both treatment and prophylaxis (Kremsner *et al*., 1994). In regions where response to quinine has deteriorated, tetracyclines are often used in combination with quinine to improve cure rates. Clindamycin has been used but offers only limited advantage when compared to other available antimalarial drugs as parasitological response is slow and recrudescence rates are high (Kremsner *et al*., 1989).

2.2.7.1 Doxycycline

Doxycycline is a tetracycline compound derived from oxytetracycline. The tetracyclines are still used widely in many types of infection even though they were one of the earliest groups of antibiotics to be developed. Doxycycline is a bacteriostatic agent that interferes with protein synthesis process by binding to the 30S ribosomal subunit thus preventing the 50s and 30s units from bonding (Kremsner *et al*., 1989). Doxycycline is primarily employed for chemoprophylaxis in regions where quinine resistance exists. It is also used in resistant cases of uncomplicated *P. falciparum* but has a very slow action in acute malaria. The common side effects include permanent enamel hypoplasia, transient depression of bone growth, gastrointestinal disturbances and some increased levels of photosensitivity. Due to its effect on bone and tooth growth it is not used in children under 8, pregnant or lactating women and those with a known hepatic dysfunction (Kremsner *et al*., 1994). Tetracycline is only used in combination for the treatment of acute cases of *P. falciparum* infections due to its slow onset. Unlike doxycycline, tetracycline is not used in chemoprophylaxis. Oesophageal ulceration, gastrointestinal upset and interferences with the process of ossification and depression of bone growth are known to occur. The majority of side effects associated with doxycycline are also experienced.

2.2.7.2 Clindamycin

Clindamycin, a derivative of Lincomycin, has a slow action against blood schizonticides. It is only used in combination with Quinine in the treatment of acute cases of resistant *P. falciparum* infections and not as a prophylactic (Kremsner *et al*., 1989; 1994).

2.3 New Approaches to Malaria Chemotherapy

2.3.1 Drug Combination therapy

In weighing the problem of drug-resistance development of the malaria parasite against the essential goal of anti-malarial care of reducing morbidity and mortality, a balance must be reached that attempts to achieve both goals whilst not compromising either too much. Administration of drug combination therapy (CT) has so far been the most successful attempt. CT can be defined as, the simultaneous use of two or more blood schizonticidal drugs with independent modes of action and different biochemical targets in the parasite (Bacon *et al*., 2007; Kindermans *et al*., 2007). Some important potential advantages are offered by the combination therapy. First, the combination should improve antimalarial efficacy, providing additive or, ideally, synergistic antiparasitic activity. A typical example of this is proguanil/atovaquone combination; both drugs exhibited failure rates when used individually, but their CT dosage regimen is a potent antimalarial.

Table 2.3 Fixed-dose Artemisinin Combination Therapies available or in late-stage development.

Compound	Lunch date	Key strengths	Key weaknesses	Formulation
Arthemether-lumefantrine (Coartem/Coartem-D*; Norvatiz/ Chinese Academy of Medical Military Science/MMV)	First quarter (Q1) of 2001 (Coartem) and Q1 of 2009 (Coartem-D)	-Market leader with over 200million treatments given -Excellent safety data. -Paediatric formulation. -First-line therapy in most of anglophone sub-Saharan Africa	-Twice per day treatment. -Low bioavailability and potentially causes a dependency on fatty foods. -Second dose 6 hours after first dose -Pharmacokinetic mismatch	-Tablet (adult) -Dispersible (child)
Dihydroartemisinin-piperaquine (eseuartesim*; Sigma-Tau/MMV/Pfizer)	Q3of 2010	-Once per day therapy -Long terminal half-life of piperaquine: 80% of patients are protected against re-infection for 42 days -Potential for IPTi -Excellent safety data of non-GMP product	-Dihydroartemisinin is the least stable of the artemisinins -The two component drugs interact -Paediatric formulation is still indevelopment -Pharmacokinetic mismatch	-Tablet (adult) -Crushed tablet (child) -Dispersible (child, 2012)
Artesunate-pyronaridine (known as pyramax: Shin Paang/MMV	Q1 of 2011	-Once per day therapy -Clinical data and registration also for *P. vivax* malaria -Potential to combine with primaquine for radical cure -Excellent safety of pyronaridine monotherapy.	-Limited safety data on combination (3000 patients in clinical dossier) -Pharmacokinetic mismatch.	-Tablet (adult) -Sachet (child)
Artesunate-amodiaquine (Coarsucam: Senofi-Aventis/ DNDi/MMV	Q4 of 2008	-Once per day dosing -First-line therapy in francophone Africa	-Resistance (to amodiaquine) can compromise efficiency -Increased nausea -No approval by stringent regulatory authority -Pharmacokinetic mismatch	Dispersible tablet for all ages
Artesunate-mefloquine (ASMQ: Farmanguinhos/ DNDi/Cipla)	Q2 of 2008	-Once per day therapy -satisfactory safety record in Thailand of non-fixed-dose combination -Successful treatment of *P. vivax* malaria in chloroquine resistant areas	-Mixture of toe diastereoisomers -Psychiatric and gastrointestinal adverse events -No approval by stringent regulatory authority -No pre-qualification by the WHO -Pharmacokinetic mismatch -Expensive	Crushed tablet for all

*Coartem-D is a child-friendly dispersible form of artemether–lumefantrine, launched this year.
‡Dihydroartemisinin–piperaquine is sold by Chongqing Holley as Artekin; this has not been prequalified by the World Health Organization (WHO) or approved by a stringent regulatory authority. DNDi, Drugs for Neglected Diseases initiative; GMP, good manufacturing practice; IPTi, intermittent preventive treatment in infants; MMV, Medicines for Malaria Venture. (Source: Wells *et al*., 2009)

Another and most important reason is that the CT use should slow the progression of parasite resistance to the agents in the combination. In the design of CT, both drugs should show little or no resistance to the parasite and have similar pharmacokinetics.

Similarity in pharmacokinetics diminishes the chances of having low concentration of either drug being present in the bloodstream, reducing the likelihood of drug resistance development (Wells *et al*., 2009; Mharakurwa *et al*., 2011).

However, the employment of drugs with different pharmacokinetics is also possible but should include in the combination, a potent short-acting drug and a longer-acting drug. This has only proven effective if the short-acting drug is potent enough to sufficiently overcome the parasite-burden such that there are limited parasites to develop resistance to the long-acting drug (Winstanley *et al*., 2004; Petersen *et al*., 2011).

The use of CT may slow the progression of resistance to any newly developed antimalarials. The artemisinins are the only available drug in clinical use with relatively limited reported resistance. As a protection, the WHO in 2006 recommended among others, Artemisinin-based combination therapies (ACTs) in the treatment of uncomplicated *P. falciparum* malaria (Greenwood *et al*., 2008; Wells & Gutteridge, 2011; Young, 2011).

The gold standard has therefore become fixed-dose ACTs, with both drugs in the same tablet. Examples of such dosage forms are shown in Table 2.3. It may be important to consider the use of CT when designing drugs. Developed drugs may show little promise as a single antimalarial agent but may be far more effective if used in a combination therapy.

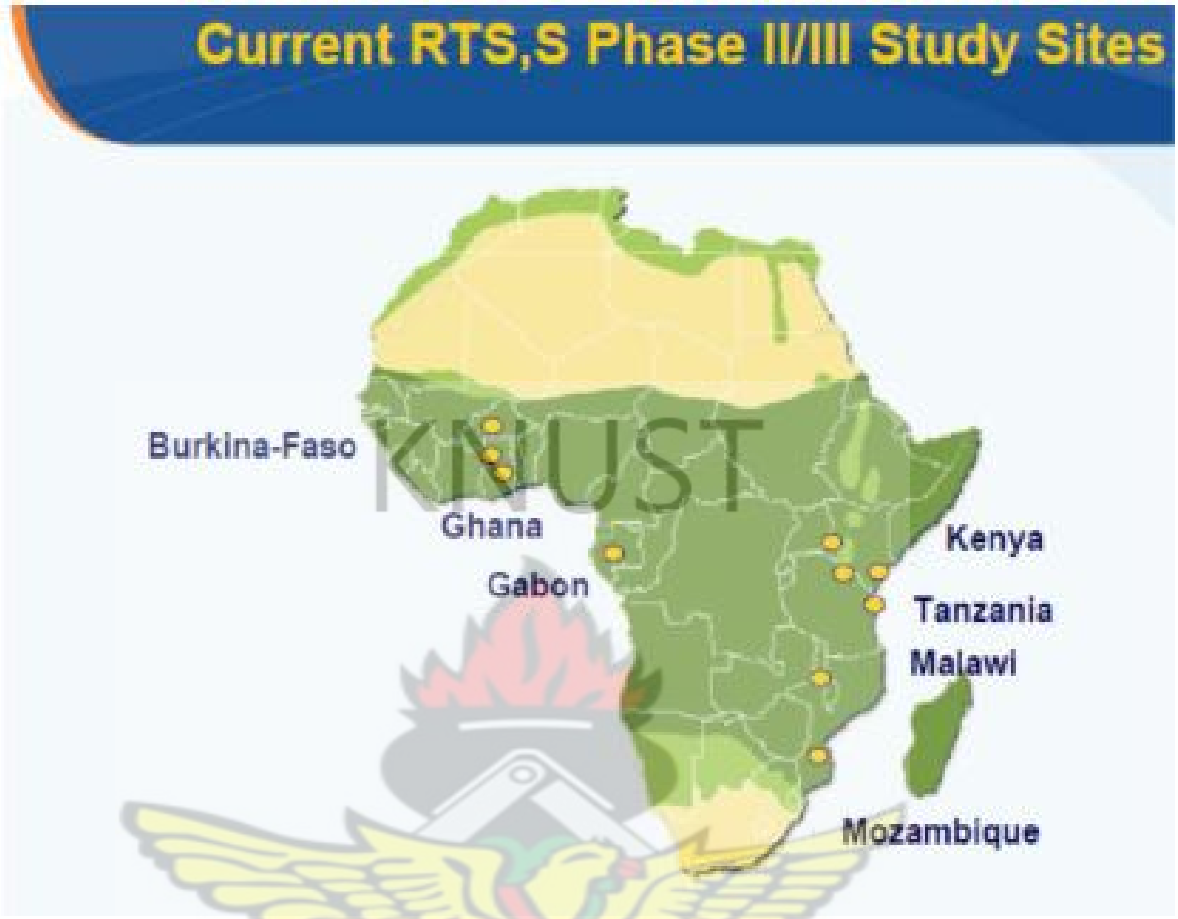


Figure 2.15 Current RTS, S phase III study sites

(Source: Otarigho, 2012).

2.3.2 Natural products

Throughout the ages, natural products have been the most consistently successful source of antimalarial drugs and lead compounds for antimalarial drug development. As previously stated, the *Cinchona* bark extract was the first malaria treatment option discovered and quinine was one of the alkaloids isolated from this extract. Despite the adverse effects and the resistance developed by some of the malaria parasites to it, quinine is still a very important chemotherapeutic agent

and is used even in severe and complicated conditions whenever all the other drugs fail to provide therapeutic response.

The development of artemisinin form the Chinese herb *Artemisia annua* is another typical example of natural product source of potent antimalarial (Dalrymple, 2012).

Natural products have also provided structural pharmacophores for the development of effective antimalarials. The synthetic antimalarials such as chloroquine, amodiaquine and primaquine, are but structural analogues of quinine. The prenylated naphthoquinone lapachol from *Tabebuia* species (Bignoniaceae) provided the pharmacophore that led ultimately to the development of the antimalarial atovaquone, a synthetic 2-alkyl-3-hydroxy-1,4-naphthoquinone (Weiss *et al.*, 2000; Wells & Gutteridge, 2011; Vianal *et al*., 2003). Further discussion on plant natural products as drugs and or lead compounds for the anti-infective drug development is in section 1.6 of this thesis.

2.4 Antiplasmodial Assays Methods

The traditional antiplasmodial bioassay tests are all based on the measurement of the effect of the compound or extract on the growth and development of malaria parasites. The antiplasmodial effect (endpoint) is generally characterized by inhibition of parasite growth and, consequently, their multiplication; these are parameters that can be measured *in vitro* in several different ways (Figure 2.16). The parasites are mixed with medium (usually RPMI 1640 culture medium) and the growth in test cultures is measured relative to a drug-free control. Variations in parasite density, haematocrit and the stage of the malaria parasite in its life cycle may have a significant impact on the outcome of these tests (Duraisingh *et al.*, 1999; Zhang *et al.*, 1986) . It is therefore

necessary to control the culture for parasite density, hematocrit and developmental stage of the parasites as far as possible. The assays usually result in sigmoid dose–response curves when performed with serial dilutions of the compound or extract. For the *in vivo* methods, the malaria infection is induced in laboratory animals which are then treated with varying concentrations or preparations of the compound. In the course of treatment, parasitaemia levels are determined by analysing blood samples taken from the animals. The antiplasmodial effect in both cases is usually measured as the lowest concentration of the compound that produces 50% inhibition of the parasite growth. The most commonly used methods for determining the antiplasmodial effect of compounds and extracts are the WHO micro-test, the isotopic test and measurement of pLDH or HRP2 (Desjardins *et al.*, 1979).

2.4.1 WHO micro-test

In the WHO micro-test, thin blood smears of the incubated cultures are prepared, fixed and stained and then observed under a light microscope. Growth inhibition due to the compound or extract defined as the difference between the percentage parasitaemia of test culture, and the corresponding positive control (culture without test compound or extract) is calculated as follows (Ene *et al.*, 2009):

$$\%\ \text{Growth inhibition} = \frac{(\%\ \text{Parasitemia CIRBC} - \%\ \text{parasitemia DIRBC}) \times 100\%}{\%\ \text{parasitemia CIRBC}}$$

Where CIRBC is the percentage parasitemia of infected RBC without extracts (i.e. control); DIRBC is the percentage parasitemia of infected RBCs incubated with the test compound. The Percentage growth inhibition is plotted against the log concentration of the test compound to get the sigmoid curve.

2.4.2 Isotopic assay

The in vitro isotopic antiplasmodial bioassay is based on the incorporation of tritium-labelled hypoxanthine into the growing parasite (Desjardins *et al.*, 1979). This involves the initial cultivation of the parasites in micro-plates for a period of 24 hours after which the hypoxanthine isotope prepared in culture medium is added to each well. The plates are then re-incubated for an additional 18 h. At the end of the second incubation period, each plate is harvested on an automated cell harvester. This instrument aspirates and deposits the particulate contents of each of the wells onto small disks of filter paper which are then washed thoroughly with distilled water.

The disks are dried and placed in a glass scintillation vial containing 10 ml of a toluene- based scintifluor for counting. The vials, corresponding to the wells of the micro-plate, are counted in a liquid scintillation spectrometer. The mean values for parasite control uptake and non-parasitized erythrocyte control uptake of the isotopic hypoxanthine are calculated. Ethanolamine can alternatively be used as the radiolabelled isotope material with the advantage that the culture medium can be supplemented with hypoxanthine, resulting in improved parasite growth (Elabbadi *et al.*, 1992). These assays allow a fairly high degree of automatization and are therefore considerably faster to perform than the tests based on the morphological assessment of parasite growth. The limitations of this technique include restrictions on handling of radioactive materials and the high purchase cost of the equipment, such as liquid scintillation counters and harvesting machines.

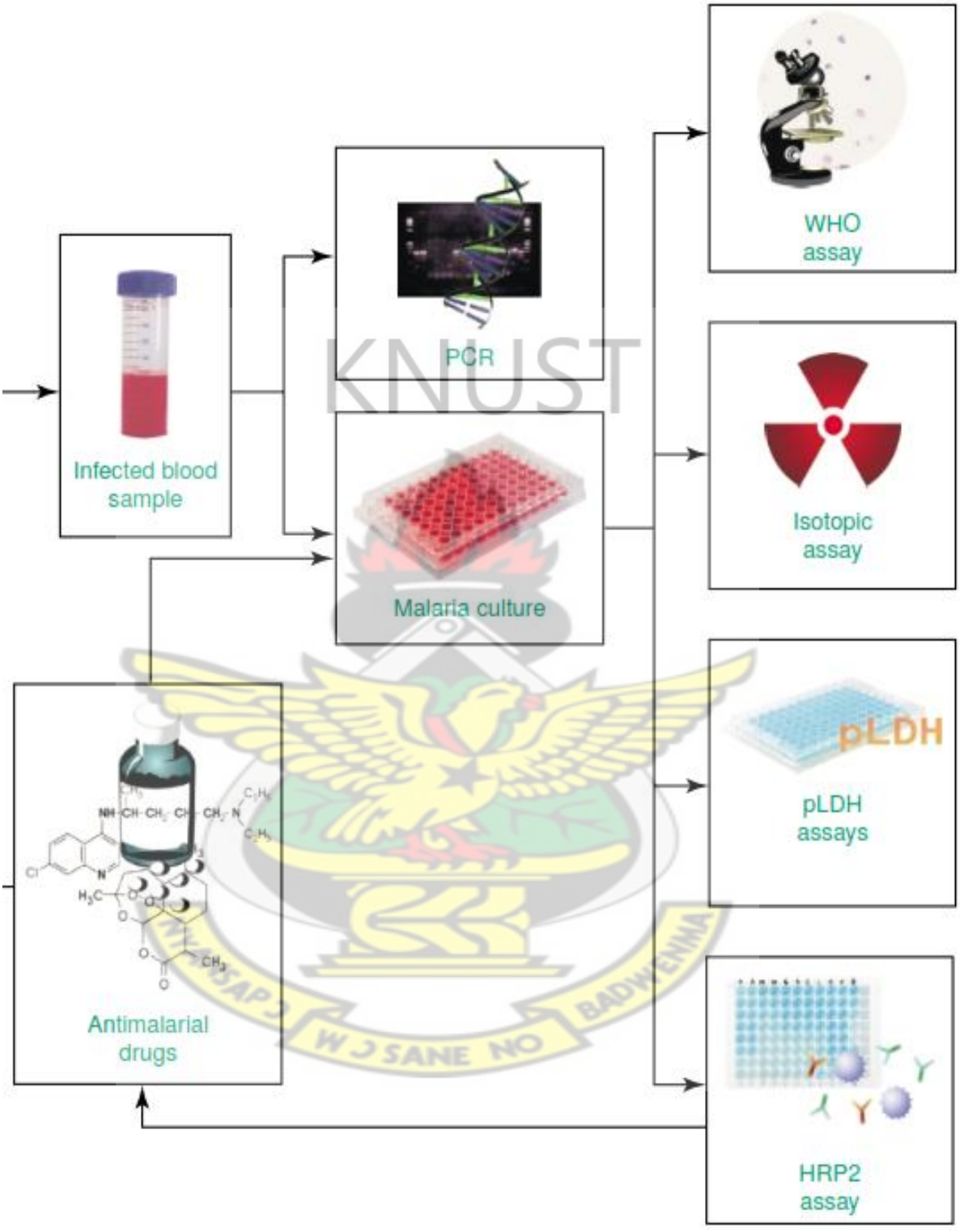


Figure 2.16 Different bioassay methods for assessing antiplasmodial activity of compounds.

(Source: Zhang *et al.*, 1986)

2.4.3 Parasite Lactate Dehydrogenase (pLDH) Assay

Plasmodium species obtain energy by way of the Embden Meyerhoff pathway with pyruvate as the end product (Jensen *et al.*, 1999). The pyruvate is converted to lactate, a process that causes NADH produced during glycolysis to be oxidized (Figure 2.17). It has been found that when NAD is replaced by 3-acetyl pyridine adenine dinucleotide (APAD), the equilibrium of NAD-dependent enzyme reactions is altered. The more positive redox potential of the APAD/APADH system in comparison to the NAD/NADH system displaces the equilibrium in favour of substrate oxidation (Piper *et al.*, 2011; Orjuela-Sánchez *et al.*, 2012; Sherman, 1966a). It was also reported that *P. lophurae* LDH could be distinguished from that of its duck host by APAD (Sherman, 1966b; Sherman, 1977). Makler and Hinrichs (1993) demonstrated that APAD also differentiated *P. falciparum* LDH (*pf*LDH) from human LDH, and this has been developed into a drug sensitivity test (Bayor, 2007). The parasite lactate dehydrogenase (pLDH) assay enjoyed some advantages over the other methods such as speed, lower cost and safety and because it is a colorimetric method its results could be interpreted visually without the aid of a plate reader (Makler *et al.*, 1998; Piper *et al.*, 2011).

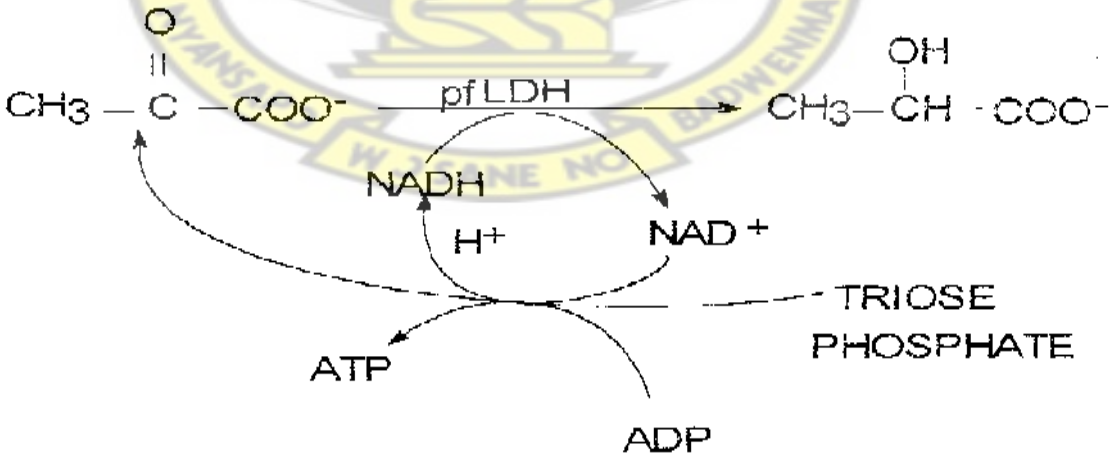


Figure 2.17 Schematic outline of energy production and pyruvate oxidation in *P. falciparum*.

2.4.4 Histidine-Rich Protein II Assay

The antiplasmodial effect of compounds and extracts can also be determined by measuring the Histidine-Rich protein II (HRP2) content of the *P. falciparum* cultures. The HRP2 assay is more sensitive than the isotopic assays and does not require very sophisticated technical equipment. HRP2 levels are closely associated with parasite density and development (Noedl *et al.*, 2002; Howard *et al*., 1986; Desakorn *et al*., 1997). Longer culture incubation periods (72 to 96 hours) are needed for HRP2 assay and hence ideal for testing even slow-acting compounds without changes in the protocol. Parasite growth and development is assessed by measuring the production of HRP2 in a simple, commercially available, double-site sandwich, ELISA test kit (or essentially any ELISA that is specific to HRP2). The HRP2 is highly stable and persistent creating a background during assay (a major problem in predicting clinical treatment outcome using malaria rapid-diagnostic dipstick tests). This background can be excluded by subtracting a control value taken after 24 h from all results, thereby limiting the measurement of parasite growth to the later part of the culture (Mayxay *et al.*, 2007; Mayxay *et al.*, 2001).

2.5 Selected plant species for antiplasmodial activity screening

2.5.1 *Adenia cissampeloides* (Planch. ex Hook.) Harms

Description: *Adenia cissampeloides* (synonyms: *A. gracilis, A. gummifera, A. guineensis*), commonly called the monkey rope or snake climber belongs to the family Passifloraceae. It is locally called *ɛkyɛm* (Twi) in Ghana. *A cissampeloides* is widespread in primary forests and secondary jungle formations along the coast of West Africa and extends through Central to East and southern Africa (Burkill, 1997). It is a robust semi-woody climber or liane, up to about 30 m

long and the stem to 10 cm thick at the base. The stems are striped bluish-green and older stems are often with whitish powder. The leaves are mostly shallowly three-lobed grey-green and distinctly three-veined from the base. *A cissampeloides* produces unisexual flowers on different plants in axillary clusters which are pale to greenish in colour. The male flowers are much larger with denser clusters than the female ones. The fruits are usually light green oval capsules consisting of 5 valves.

Figure 2.21 *Adenia cissampeloides* (Planch. ex Hook.) Harms

(Source: A picture of the plant on KNUST campus)

Folklore uses: Throughout tropical Africa, the infusion and decoction of the root, stem or leaves of *A. cissampeloides* are used for the treatment of gastro-intestinal complaints, such as stomach-ache, constipation, diarrhoea and dysentery as well as various inflammatory ailments, and for pain relief, particularly against headache and back pain. The leaves or root decoction is used to treat intestinal worms, malaria fever, cholera, liver ailments, excessive menstruation and as a diuretic. Infusions of the root or aerial parts are taken to treat sexually transmitted infections.

Poultice of the pounded roots is applied to wounds and sores while the decoction of the leaves is applied to leprosy sores in addition to drinking of the root decoction. The ashes of the bark or root, mixed with castor oil are used to treat scabies. Bronchitis and other lung ailments are treated with the leaves, eaten raw with salt and palm oil or cooked as a vegetable with meat or fish. An infusion of the leaf is used to treat depression and insanity and as a stimulant. Roots are chewed to treat snakebites and are used as antidote for *Acokanthera* arrow poison. The crushed stems, leaves and roots are used as fish or arrow poisons while the smoke of the burning roots is also used to calm bees before harvesting the honey (Neuwinger, 2000).

Phytoconstituents and bioactivity: The stem, leaves, fruit and roots of *A. cissampeloides* contain cyanogenic glycosides. The leaves also contain gummiferol, a cytotoxic polyacetylenic di-epoxide, which has shown *in vitro* anti-cancer activity. Excessive use of *A. cissampeloides* has been associated with liver complaints, and *in vivo* tests have confirmed the presence of compounds causing liver damage. The leaves and root bark are rich in iron. The aqueous extracts are reported to have a dose dependant depressing effect on the blood pressure of cats which was neutralized by small doses of atropine. A significant larvicidal effect has also been reported. An anaesthetic against the African honey bee (*Apis mellifera adansonii*) has been formulated from the diethyl-ether extract of the bark (Grace & Fowler, 2007).

2.5.2 *Anthocleista nobilis* G. Don

Description: *Anthocleista nobilis* belongs to the family Loganiaceae. It is a lower canopy tree found in transitional and secondary forests in West Africa. It is sparsely but fairly evenly distributed, even in the drier parts of the semi-deciduous forests. In many communities in Ghana, it is often left standing on farms because of its medicinal properties. *A. nobilis* grows up to about 18 m high and 45 (or even 90) cm thick. The stem is usually a light grey pole with smooth bark

slashed with cream-yellow granules, free of branches up to 15 m which is crown with small ascending, hollow branches and twigs with two divergent spines above the leaf axils. The leaves are crowded at the apices of the branchlets, opposite, subequal, sessile or shortly petiolate with petioles up to 1/6th as long as the oblong-elliptical blades. *A. nobilis* bears numerous seeded, ellipsoid dark brown, berry fruits which are usually about 2.5 cm in diameter with persistent calyx (Madubunyi *et al*., 1994; Lutterodt, 1976; Leeuwenberg, 1961).

Figure 2.22 Anthocleista nobilis G. Don

(Source: A picture of the plant at Ejisu-Besease, near Kumasi)

Folklore uses: In Ghana, *A. nobilis* stem or root bark decoction is taken orally to treat piles, jaundice, intestinal infections and worm infestation, gonorrhoea, leprosy, malaria, liver diseases and as an antidote to poisoning (Madubunyi *et al*., 1994). It is also used as a strong purgative and diuretic. The root decoction is used to treat constipation, regularise menstruation and as abortifacient. The bark infusion when exposure to sun in an open bottle for a period is used to treat gonorrhoea. The pulped bark is used an enema for treating thread worm and other intestinal

parasitic infestation in children. Poultice made from the young green shoots are applied to treat ulcerous wounds and abscesses (Walker, 1953). A leaf decoction is taken with lemon for abdominal pains of uterine origin (Daziel, 1936). The bark of *A. nobilis* (together with those of *Bosqueia angolensis* and *Spathodea campanulata*) are boiled and taken orally once a week for birth control of family planning; part of the liquid is also applied as enema.

General Phytoconstituents and bioactivity: Alkaloids (example brucine and loganine), xanthones and secoiridoids (such as anthocleistol) in addition to glycosides, saponins and steroids have been reported to occur in *A. nobilis* (Burkill, 1997). Alcohol extract of the bark is found to possess antihepatotoxic, antimicrobial, anti-inflammatory, hypotensive and hypoglycaemic properties.

2.5.3 *Calliandra portoricensis* (Jacq) Benth

Description: *Calliandra portoricensis* (Jacq) Benth (syn. *Zapoteca portoricensis*), locally called *Hwentewitente* (twi) belongs to the fabaceae family. This plant is native to Central America. It is found cultivated in most countries in West Africa, from Cote d'Ivore up to Nigeria. *C. portoricensis* is a shrub of up to about 3 m high, with thin branches. The plant has 2 - 7 pairs of pinnate leaves with overlapping leaflets and bears white flowers in solitary heads with stamens of about 2 cm long (Mshana *et al*., 2000).

Folklore uses**:** *C. portoricensis* is cultivated in many countries to check soil erosion. The leaves of *C. portoricensis* are employed in the form of poultice to treat snakebite and haemorrhoid. The leaf decoctions are used in treating wounds and skin infections. The decoction is also drunk for

fever (personal communication). The roots are boiled and used to manage asthma and lumbago (Mshana *et al.*, 2000).

Fig 2.23 Calliandra portoricensis (Jacq) Benth

(Source: Picture of the plant on KNUST campus, Kumasi)

***Phytoconstituents and bioactivity*:** Tannins, saponins, glycosides, polyphenols and flavonoids have been reported present in the *C. portoricensis* (Ebong *et al*., 2012). 2-Hydroxy-4-methoxybenzoic acid isolated from the leaves has been suspected to the active compound responsible for the antivenin property of the plant (Onyeama *et al.,* 2012).

2.5.4 *Elaeis guineensis* Jacq.

Description: *Elais guineensis* commonly called the African oil palm of the Arecaceae family is native to west and southwest Africa, occurring between Angola and Gambia. Malaysia currently holds the largest oil palm plantations in the world. *E. guineensis* is single-stemmed monoecious plant, with the trunk up to 20 m tall and 30 cm in diameter. It has pinnate leaves that reach between 3-5 m long and produces dense clustered flowers which are individual small with three

sepals and three petals. *E. guineensis* fruits grow in large bunches and take five to six months to mature into reddish nuts which are rich in oil (Rajoo *et al*., 2010).

Folklore uses: In traditional medicine, the leaf of *E. guineensis* is squeezed and the juice applied unto wounds to promote healing (Sasidharan *et al*., 2012). The sap is employed as a laxative while the partially fermented palm wine is taken by nursing mothers for lactation improvement. Soap prepared with the fruit-husk ash and the fruit mesocarp oil or the kernel oil is applied to treat skin infections. A root decoction is used for headaches. The pulverized roots are added to drinks for gonorrhea, menorrhagia and as a cure for bronchitis (Rajoo *et al*., 2010). The fruit mesocarp oil and palm kernel oil are administered as poison antidote and used externally with several other herbs as lotion for skin diseases. The palm kernel oil is also used topically to regulate body temperature in convulsant children. Other folk remedies of *E. guineensis* include treatment for cancer, headache and rheumatism and as an aphrodisiac, diuretic and liniment (Chong *et al*., 2008).

Phytoconstituents and bioactivity: *E. guineensis* fresh sap contains 4.3 g/100 ml of sucrose and 3.4 g/100 ml of glucose which ferments into wine. It is an important source of Vitamin B complex. Palm oil contains saturated palmitic acid, oleic acid and linoleic acid and also rich in carotene (Farombi, 2003). Proteins, fats, Ca, P, Fe, riboflavin, thiamin, niacin, ascorbic acid and many other nutrients and or antioxidant phytoconstituents such as flavonoids and phenolic acids as well as saponins have been reported present in the oil palm. The rich antioxidant constituents are reported to confer hepatoprotective properties on paracetamol-induced hepatic damaged mice (Sasidharan *et al*., 2012). Antimicrobial properties have also been reported in the leaf extract (Gbedema *et al*., 2010).

Figure 2.24 ***Elaeis guineensis*** **Jacq**

(Source: A picture of the plant at Ejisu-Besease, near Kumasi)

2.5.5 *Entandrophragma angolense* (Welw.) CDC

Description: *Entandrophragma angolense* (synonyms *E. macrophyllum, E. rederi and E. leplaei*) of family Meliaceae, is widespread. It occurs in the coastal countries of West Africa and Guinea, Sudan, Uganda, Kenya, DR Congo and Angola. It is a deciduous dioecious large forest tree that grows to about 60 m high with straight and cylindrical branchless bole of up to 40 m long and 200 cm thick. It stands on blunt buttresses of up to 6 m high which often extend into the surface roots. The stem bark surface of *E. angolense* is pale greyish brown to orange-brown; initially smooth but becomes scaly as the plant matures. The inner bark is finely fibrous and pinkish red in colour with whitish streaks. The plant is dome-shaped crowned and has glabrous twigs which are marked with large leaf scars and lenticels. The leaves are alternate and paripinnately compound with up to 22 leaflets that cluster near the ends of the twigs. E angolense bears regular

5-merous unisexual flowers. The fruits are cylindrically pendulous capsules of up to 22 cm long and 5 cm thick, brown to black in colour and with many small lenticels dehiscing from the base (Kokwaro, 1976; Akubue & Mittal, 1982; Falodun, 2010).

a.  b. 

Figure 2.25 *Entandrophragma angolense*. (a) Stem bark and (b) leaves, fruits and seeds. (Source: Tchinda, 2008)

***Folklore uses*:** The bark decoction of *E. angolense* is widely used in ethnomedical treatment of various gastrointestinal disorders (including gastric ulcers), malaria and fever (Bickii *et al*., 2007; Orisadipe *et al*., 2005; Orisadipe *et al.*, 2001).

Phytoconstituents and bioactivity: Limonoids (Bickii *et al*., 2007) and triterpenoids, such as methyl angolensate which appears to be responsible for the antiulcer activity (Orisadipe *et al*., 2001) of the plant, have been isolated. Other compounds reported present in E. angolense include entandrolide, gedunin and beta-sitosterol.

2.5.6 *Mallotus oppositifolius* (Geisel.) Müll. Arg.

Description: Mallotus oppositifolius (family Euphobiaceae) is an open shrub or small tree up to 10 m tall. It is common in drier types of forest and in secondary regrowth throughout the West African region and widespread across tropical Africa to Madagascar. It is a common weed of rice-fields, maize and cassava farms. M. oppositifolius is often a sarmentose-dioecious plant with smooth to slightly roughened and flaky, whitish- or greenish-grey or brown bark. The young shoots are fulvous-stellate-pubescent while the old twigs are glabrescent. The leaves occur in pairs and opposite with long petioles (Irvine, 1961; Burkill, 1997; Drokosi, 2002).

Folklore uses: The leaves are taken in cold infusion for tape-worm well the decoction is a general vermifuge. Crushed or chewed leaves are used as a haemostatic on fresh wounds and sores to arrests bleeding. The powdered or crushed leaves are also mixed with ointments for application to sores and ulcers. The leaves are boiling and the steam inhaled for headache and malaria fever. The leaf-sap is also instilled up the nostrils for headache and also applied on inflamed eyes. The roots with guinea-grains are prepared as an enema for lumbago. All parts of the plant are used for dysentery. Other conditions for which *M. oppositifolius* is employed to treat are pneumonia, kidney infections, tinea infection, venereal diseases, leprosy, chicken-pox, urinary troubles during pregnancy, anaemia, epilepsy, convulsion in children, paralysis, general fatigue and female sterility and as a diuretic. The stem is used as chew-sticks for personal oral hygiene and the root is used as an aphrodisiac (Burkill, 1985; Drokosi, 2002).

Phytoconstituents and bioactivity: Anti-inflammatory (Nwaehujor *et al*., 2012; Farombi *et al*., 2001) and antimicrobial effects of *M. oppositifolius* (Adekunle & Ikumapayi, 2006; Gbedema *et al*., 2010) of the plant have been reported. Its CNS depressant, analgesic and anticonvulsant

effects have also been reported (Kukuia *et al*., 2012)). Alkaloids, saponins, sterols and glycosides have been detected in the plant (EFarombi *et al*., 2001; Adekunle & Ikumapayi, 2006).

Figure 2.26 ***Mallotus oppositifolius*** **(Geisel.) Müll. Arg.**

(Source: A picture of the plant at Apeadu, Kumasi)

2.5.7 *Petersianthus macrocarpus* (P Beauv.) Liben

Description: *Petersianthus macrocarpus* (synonyms *Combretodendron africanum* and *Combretodendron macrocarpum*) commonly referred to as the 'stinkwood tree' or 'soap tree' belonging to the Lecythidaceae family is native to the West African sub-region (from Liberia through Cote d'Ivoire, Ghana and to Sierra Leone). It is a large evergreen tree of up to 50 m high with straight, cylindrical or tapering, unbuttressed bole or with small spur roots of about 1 m in diameter, bearing a well-developed crown (Burkill, 1997).

Figure 2.27 ***Petersianthus macrocarpus*** **(P. Beauv.) Liben**

(Source: Hawthorne & Gyakari, 2006)

Folklore uses: Almost every part of this plant a folklore use. Decoctions of leaves, twigs and root and stem barks are employed for managing conditions such as arthritis, rheumatism, heart palpitation, pulmonary troubles and as general pain-killer. The decoctions are used either orally or by enemas for regularising abnormal menstrual cycles, haemorrhoids, paralysis and ulcerative incurable wounds, and also as emetic, laxative, ecbolic and abortifacient. The wood is a good building materials and also for fuel and lighting (Line-Edwigea *et al*., 2009; Olugbade *et al*., 2000; Irvine, 1961).

Phytoconstituents and bioactivity: The chemical reported present in *P. marcrocapus* include glycosides, saponims, steroids and tannins. The decoctions are reported to have astringent, hypotensive and antimicrobial properties (Kouitcheu Mabeku *et al.*, 2011; Burkill, 1997; Olugbade *et al*., 2000).

2.5.8 *Polyalthia longifolia* var. pendula

Description: *Polyalthia longifolia* popularly called the "Mast Tree" of the family Annonaceae is native to the drier regions of India and Sri Lanka. It is commonly cultivated in Pakistan (Katkar *et al*., 2010; Verma *et al*., 2008) and many other tropical countries around the world. The Annonaceae, on the basis of source of edible fruits and oils, is of very significant economic importance. *Polyalthia* is a large genus of shrubs and trees consisting of about 120 species which are distributed in Asia and tropical Africa. Few of these species are *P. cerasoides, P. suberosa, P. nemoralis, P. barnesii, P. viridis, P. acuminata, P. macropoda* and *P. oliveri*.

In Ghana and other West African countries *P. longifolia* is usually cultivated in residential areas, especially along streets and around houses as an ornamental plant and a wind-break. *P. longifolia* is an evergreen, tall and slender tree (up to12 meter) that grows symmetrically and produces fresh and shining green foliage. The entire length of the plant is covered by long and wavy leaves. The beautiful contrast of new golden and coppery brown leaves against old dark-green leaves make a spectacular show.

There are two varieties of *P. longifolia* and both are very commonly cultivated in Ghana; *P. longifolia* Thwaites and *P longifolia* var. pendula. The Thwaites variety is also a tall handsome evergreen tree with straight bole and all other feature and uses just like the pendula except that Thwaites has horizontal branches while pendula is of dropping-branching style, a habit that gives it a very beautiful narrow columnar shape.

Folklore uses: Almost all parts of the plant are used in many traditional systems of medicine all over the world for the treatment of various ailments in humans and animals. These include treatment of skin diseases, cancer, fever, diabetes, helminthiasis and hypertension (Ichino *et al.*,

2006; Verma *et al*., 2008). Aside the medicinal uses, *P. longifolia* serves as an ornamental plant and a wind-break.

Figure 2.28 ***Polyalthia longifolia*** **variety pendula**

(Source: A picture of the plant on KNUST campus)

Phytoconstituents and bioactivity: Reports indicate the presence of phytochemicals such as aporphine, azafluorene, and protoberberine alkaloids, clerodane diterpenes, ent-halimanes, altholactone, proanthocyanidin, quercetin and its glycosides and steroids *in Polyalthia longifolia*. Other constituents present include saponins, flavanoids, and terpenoids in addition to various amino acids such as proline, glutamic acid and methionine. All parts of the plant have been reported to exhibit various bioactivities including antibacterial, antifungal, antitumour, antiulcer, antioxidant, antiparasitic, antifeedant, antipyretic, hypoglycaemic, antidyslipidemic, and antihypertensive properties (Faizi *et al*., 2008; Bhatta *et al*., 2011; Chang *et al*., 2006; Prachayasittikul *et al*., 2009; Nair & Chanda, 2006; Katkar *et al*., 2010).

2.5.9 *Sarcocephalus latifolius* (J.E. Sm) E.A. Bruce

Description*:** *Sarcocephalus latifolius* (synonym: ***Nauclea latifolia) of the Rubiaceae family is commonly called the pin cushion tree, African peach, Guinea peach or the Sierra Leone peach. *S. latifolius* is an evergreen multi-stemmed shrub that grows up to an altitude of 200 m. It is widespread in the humid tropical rainforest zone or in savannah woodlands of West and Central Africa. There are three other related species: *N. pobeguini*, *N. diderichii,* and *N. vanderguchtii*. *N. latifolia* has an open canopy and terminal spherical head lined cymes of white flowers which are joined with their calyces. It flowers from April to June and bears syncarp fruits that ripen from July to September (Irvine, 1961).

***Folklore uses*:** In West and South Africa infusions and decoctions of the root, stem, stem bark and leaves (along or in addition to other plant ingredients) are used for the treatment of stomach pains, fever, diarrhoea, tuberculosis, malaria and helminth infestation in humans and animals (Okwori *et al*., 2008) as well as prolong menstrual flow. The wood is also used as a chewing stick in Nigeria. The aqueous decoctions are also used as tonic and antiseptic medicines (Atta-Agyeman, 1980). A concoction of *S. latifolius* with *Anthocliesta adjalonensis* and *Uvaria afzalii* is employed in the treatment of sexually transmitted infections (STI), toothahes, dental caries and oral sepsis (Keay *et al*., 1960). Decoction of dried fruits is used to treat dysentery and piles while stem bark is used as analgesic, homeostatic and diuretic as well as in treating skin infections (Atta-Agyeman, 1980; Abbiw, 1990).

Phytoconstituents and bioactivity: All the plant parts of *S. latifolius* and the related species are a rich source of monoterpene indol alkaloids (such as naucleamides A – E), glycosides (example swerosid and loganin), saponins (example quinovic acid), polyphenols and carbohydrates (Abbiw, 1990).

Figure 2.29 ***Sarcocephalus latifolius*** **(J.E. Sm) E.A. Bruce**

(Source: A picture of the plant at Haatso, Accra)

2.5.10 *Terminalia ivorensis* A. Chev

Description: *Terminalia ivorensis* A. Chev popularly called 'Black Afara' (family Combretaceae) is native to the West African tropical forest. It is an also exotic plant found in countries including Fiji, Solomon Islands, South Africa, Tanzania, Trinidad and Tobago, Uganda, Zambia and Zimbabwea. *T ivorensis* is a large deciduous forest tree ranging in height from 15 to 46 m, branchless for up to 30 m. The bole is clean and very straight with small buttresses which are sometimes fluted. The mature trees are flat-topped with a wide horizontal canopy of evenly distributed foliage arising from the apex of the straight bole. The bark is smooth and light grey to dark brown when young and on branchlets but in mature trees it is often blackish with deep longitudinal fissures that flakes off in long thin strips. The leaves (6.4-12.7 x 2.5-6 cm) are whorled, simple, oval and blunt tipped with orange-brown hairs below and on veins above and on the short stalks. *T ivorensis* produces bisexual flowers in axillary spikes (7.6-10.2 cm long).

The fruits are winged and somewhat variable in size, especially in the width of the wings. When ripe it is reddish-brown and woody, frequently having a weevil hole and oval seed in the centre (Irvine, 1961).

Folklore uses: In the West African region, the bark, root and leaves of *T. ivorensis* preparations (such as decoctions, infusions, tinctures and lotions) are employed for the treatment of various ailments including treatment of ulcerated wounds, sores and cuts, skin infections and rheumatism. The wood is also used as timber and firewood or for charcoal making (Iwu & Anyanwu, 1982; Abbiw, 1990; Irvine, 1961).

Figure 2.30 *Terminalis ivorensis* A. Chev

(Source: A picture of the plant in the Bobiri Forest near Kumasi)

Phytochemistry and bioactivity: The chemical constituents reported present in *T. ivorensis* include saponins, glycosides, flavonoids, terpenoids, steroids and anthraquinones (Iwu &

Anyanwu, 1982; Irvine, 1961). *T. ivorensis* extracts are found to be effective against *Trypanosoma brucei rhoderienses* (Iwu &Anyawu, 1982).

Chapter Three

3 ANTIPLASMODIAL SCREENING OF SELECTED PLANT SPECIES

3.1 The plant species used in the study

The plants selected and investigated for antiplasmodial activity were *Adenia cissampeloides* (Planch. ex Hook.), *Anthocleista. nobilis* G. Don, *Elaeis guineensis* Jacq., *Entandrophragma angolense* (Welw.) CDC, *Mallotus oppositifolia* (Geisel.) Müll. Arg., *Petersianthus macrocarpus* (P Beauv.) Liben, *Polyalthia longifolia* var. pendula, *Sarcocephalus latifolius* (J.E.Sm) E.A. Bruce and *Terminalia ivorensis* A. Chev. These plants were selected after a thorough search through ethnobotanical survey reports by several researchers including Addae-Kyereme *et al*. (2001), Zofou *et al.* (2011), Nogueira & Lopes (2011), Batista *et al.* (2009), Asase *et al*. (2010), Agyare *et al*. (2009), Asase & Oppong-Mensah (2009) and personal communications with Traditional healers and herbalists in Ghana.

3.2 The Plant Extracts

3.2.1 Collection and authentication of plant materials

The parts of the various plant species used in the study are as indicated in Table 3.1. The plant materials were collected between the months of January and April 2011 by Mr Asare, a herbal practitioner attached to the Department of Herbal Medicine, Kwame Nkrumah University of Science and Technology, Kumasi and Mr Batsa of the Centre For Scientific Research into Plant

Medicine (CSRPM), Akwapim-Mampong, Ghana. They were authenticated in the Department of Pharmacognosy, KNUST, Kumasi and the voucher specimens are kept in their herbarium.

3.2.2 Pre-treatment of the plant material

The plant materials were initially screened for foreign materials such as sand, insects, organic and inorganic materials as well as morphological parts of the same plant other than those needed. A stiff brush was used to clean off the dirt from the roots and stems. Each plant material was then washed thoroughly in cold water to remove any trace of unwanted foreign matter. The materials were cut into smaller pieces and dried at room temperature for 48 hours followed by oven drying at 40^{o}C for further 48 to 72 hours. The dried plant materials were powdered using a hammer mill and kept in sealed and labelled polythene bags until needed.

Table 3.1 The plant parts used in the study and their voucher numbers

Plant species	Part used	Voucher number
Adenia cissampeloides (Planch. ex Hook.)	Stem	AC-01/11/024
Anthocleista. nobilis G. Don	Stem bark	AN-01/11/025
Calliandra portoricensis (Jacq) Benth	Stem bark	CP-07/12/001
Elaeis guineensis Jacq.	Leaves	EG-01/110/26
Entandrophragma angolense (Welw.) CDC	Stem bark	EA-04/11/027
Mallotus oppositifolia (Geisel.) Müll. Arg.	Aerial part	MO-01/11/028
Petersianthus macrocarpus (P Beauv.) Liben	Stem bark	PM-03/11/030
Polyalthia longifolia var. pendula	Stem bark	PL-01/11/031
Sarcocephalus latifolius (J.E.Sm) E.A. Bruce	Root	SL-04/11/029
Terminalia ivorensis A. Chev	Stem bark	TI-02/11/032

3.2.3 Preliminary total extraction

The methanolic extracts of the powdered plant materials were obtained by cold maceration. One hundred grams (100 g) of each plant material was weighed into a 1 litre flat bottom conical flask and cold macerated with 450 ml of 70% ethanol for 48 hours, with regular shaking. It was then filtered using Whatman filter paper number 1 and the filtrate concentrated to a syrupy mass under reduced pressure in a rotary evaporator. These extracts were then dried completely over silica gel in a vacuum chamber at room temperature. All the dried extracts were stored in a refrigerator until required for use. Also solutions made of these extracts were frozen until required for use.

3.2.4 Preliminary alkaloid extraction

Two hundred grams (200 g) each of the powdered plant materials was macerated with 150 ml of ammoniacal-alcohol (9 parts of ethyl alcohol and 1 part of liquid ammonia) for 24 hours and filtered. The filtrate was concentrated to a syrupy mass in a rotary evaporator, acidified with enough dilute sulphuric acid (1% v/v) and allowed to stand for about 10 minutes after which the supernatant aqueous acidified solution was decanted from the precipitates that formed. The acidified solution was basified with dilute ammonia and then extracted with chloroform by means of a separation funnel. Molecules of water present in the separated chloroform fraction were removed using sodium sulphate anhydrate. The chloroform was evaporated off resulting in a dried extract.

3.3 Antiplasmodial Activity Evaluation of Extracts

The *in vitro Plasmodium falciparum* parasite cultivation was based on the method described by Jensen and Trager (Trager & Jensen, 2005; Trager & Jensen, 1997; Trager & Jensen, 1977; Trager & Jensen, 1976) and modified by many researchers (Zolg *et al*., 1982; Reber-Liske, 1983;

Fairlamb *et al*., 1985; Jensen, 2005). The *in vitro* antiplasmodial assays were based on the parasite lactate dehydrogenase (pLDH) assay method developed by Makler and Hinrichs (1993) and as optimised and revised by other scientists which eliminated the transfer of very small volumes into new wells and also replaced the Malstat reagent with an APAD reagent (Ferrier, 1990; Knobloch & Henk, 1995; Jelinek *et al*., 1996; Malik *et al.*, 2004). All operations involving parasite cultures were carried out aseptically in a Cytox II Laminar flow chamber (Envair UK). To minimize microbial contamination only sterile disposable tubes, transfer pipettes and flasks were used throughout this work.

3.3.1 Malaria Parasite

The multidrug resistant *P. falciparum* (K1 strain) culture obtained from Professor D.C. Warhurst of the London School of Hygiene and Tropical Medicine (LSHTM) was used in the experiment.

3.3.2 Preparation of Red Blood Cells and Serum

About 300 ml of packed non-immuned fresh human blood (group A^+) that had tested negative for HIV and hepatitis B antibodies was purchased from the Yorkshire Blood Transfusion Centre (YBTC), Leeds, UK. The blood was transferred into 50 ml sterile centrifuge tubes (with tight lids) and centrifuged for 10 minutes at 2500 rpm in a bench top centrifuge. This caused separation of the blood into three layers: the red blood cells at the bottom, separated from the yellow layer of plasma (on top) by the buffy coat of white blood cells (in the middle) (Figure 3.1). By means of sterile pasteurs pipettes, the plasma was aseptically transferred into a new sterile tube with tight lid for further treatment while the buffy coat was discarded into a disinfectant.

Red blood cells **(rbcs)** - An equal volume of phosphate buffer saline (PBS, Apendix) was added to the bottom layer red cells in the tube and mixed well. This was centrifuged as above and the clear supernatant together with remnants of white cells on top of the packed red cells were

removed and discarded. This process of washing the red cells with PBS was repeated severally to remove all the buffy coat of white blood cells and any preservatives added on donation of the blood. The thoroughly washed rbcs were finally suspended in Roswell Park Memorial Institute (RPMI) 1640 medium (to give a haematocrit of 50 %) and stored at 4°C (Trager & Jensen, 1977).

Red blood cells (lower layer) and serum (upper layer)

Figure 3.1 Separation of Red blood cells from serum.

Serum – To every 40 ml of the plasma obtained from the above procedure was added 400 µl of Ca-Kaolin Clotting Reagent (Appendix) and placed in a water bath at 37ºC for 1 hour or until complete clotting is observed. This was vigorously shaken and then centrifuged at 2500 rpm for 30 minute to separate the clot from the serum. The supernatant serum was aseptically transferred into a sterile tube and stored at -20ºC. The useful life span of this frozen serum is about 6 months (Read & Hyde, 1993). The frozen serum was thawed and used whenever necessary.

3.3.3 Growth Medium

The medium for parasite cultivation was prepared by dissolving 10.4 g lyophilised Roswell Park Memorial Institute (RPMI) 1640 medium in about 800 m1 distilled water. To this solution were

added 2 g D-glucose, 2.33 g sodium bicarbonate and 9.2 g TES buffer (2-[[1,3-dihydroxy-2-(hydroxymethyl)propan-2-yl]amino]ethane sulfonic acid) as well as 40 mg gentamicin. All the ingredients were obtained from Sigma-Aldrich, St. Louis, MO, UK. A magnetic stirrer was employed to achieve complete dissolution. The solution was made up to a litre with distilled water and the pH adjusted to about 7.3 with 5 M sodium hydroxide. This stock RPMI 1640 medium was sterilized by filtration and stored in 225 m1 quantities at -20 °C. For culturing parasites, the medium was made up by mixing 2.5 ml of serum with 20 m1 of thawed RPMI 1640 (about 12 %) (Sutar *et al*., 1992). This mixture (herein called complete medium) was stored at 4° C and used within seven days.

3.3.4 Initiating malaria parasite cultures from frozen stocks

Frozen cryopreserved culture of *P. falciparum* was quickly thawed in warm water (37°C), pipetted into 15 ml centrifuge tube after the vial was wiped with 70% ethanol to reduce contamination. An equal volume of sterile 3.5 % (w/v) sodium chloride (Sigma, USA) was aseptically added. After centrifuging it at 1500 rpm for 5 minutes, the supernatant was removed. The washing process was repeated thrice with 5 ml quantities of RPMI complete medium. The washing was done to remove the cryopreservative from the culture to the barest minimum. The cells were then suspended in complete medium (5 % haematocrit) and the culture made up to 5 ml with fresh uninfected rbcs (5 % haematocrit). The culture was placed (laid on the side) in an incubator at 37^{o}C after it was transferred into a 50 ml tissue culture flask (Corning, NY, USA) and flushed with pre-filtered gas mixture (of 3 % O_2, 4 % CO_2 and 93 % N_2) for 1 minute.

3.3.5 Cultivation and maintenance of *Plasmodium falciparum* culture

The malaria parasite *P. falciparum* (K1 strain) *in vitro* cultivation was done in 50 m1 tissue culture flasks. The cultures were limited to 5 m1 and 5 % haematocrit. Starting a new culture

involved transferring a calculated volume of parasitized cells into a tube and making it up with the needed volume of uninfected rbcs (5 % haematocrit). The resultant suspension was aseptically pipetted into a culture flask and flushed with gas for 1 minute. The flask was quickly sealed and placed in an incubator at 37°C as shown in figure 3.2 (Trager & Jensen, 1997; Petmitr *et al*., 1995).

The culture medium was regularly changed every 48 hours when the level of parasitaemia is low (less than 2%) or 24 hours at high parasitaemia levels (Fairlamb *et al*., 1985). In this process, the culture flask was inclined at an angle of about 45 degrees for about an hour to allow the cells to settle. An alternative procedure of transferring the culture into 15 ml centrifuge tube which was then spun at 1500 rpm to separate the cells from the spent medium was also employed whenever it was necessary. In both cases, the spent medium (top layer) was aseptically pipetted out and the cells re-suspended in an equal volume of pre-warmed complete RPMI 1640 medium. The culture in the flask was then flashed with gas and placed back in the incubator.

In many drug assays, 2 – 5 % parasitaemia mostly at the ring stages of the life cycle with few or no gametocytes are suitable. Because *P. falciparum* reproduces every 48 hours it was necessary to maintain the cultures at reasonable parasitaemia levels (i.e. not exceeding 10% parasitaemia) for healthy growth. Sub-culturing was therefore necessary whenever parasitaemia approaches 10 %. This was done by aseptically splitting the overgrown culture (after it was re-suspended in fresh complete medium) into sterile flasks which were then diluted to 5 ml with calculated volumes of 5% haematocrit uninfected rbcs. The sub-culturing was usually performed to reduce parasitaemia of the new cultures to below 2%. The new cultures were then incubated after they were gassed and the flasks tightly closed with caps.

Parasitaemia was daily monitored by making thin blood film smears. About 500 µl aliquots of the cultures were aseptically withdrawn into 1.5 ml eppendorf tube and centrifuging for 2

minutes. The supernatant was then removed to a level leaving a pellet of cells and an equal volume of medium to give a 50% haematocrit on re-suspension. A drop of this was spotted on a glass slide, and using another glass slide, a thin film of blood smear was formed. The smear was dried in air and fixed with methanol. This was stained with freshly prepared 10 % Giemsa's stain (BDH, UK) in phosphate buffer saline (pH 7.2) for 20 minutes. The stained slide was rinsed in a gently flowing tap water and dried in air at room temperature. By means of a light microscope the cells were observed using an oil immersion objective lens at 100 x 10 magnifications. A minimum of 10 fields were viewed. The number of infected rbcs and the total number of rbcs were counted and used in calculating the percentage parasitaemia. Microscopy was aided by the use of a hand held counter.

Figure 3.2 Cultivation of *P. falciparum* cultures in an incubator

3.3.6 Synchronization of malaria parasite culture

For *in vitro* antimalarial assays, synchronized parasite cultures produce reproducible results. Hence highly synchronized malaria parasite cultures were used in this thesis. The two basic techniques employed were D-sorbitol (5 %) and Percoll (60 %). The choice of either was depended upon the predominating stage of the parasite's life cycle in the culture.

When the parasites are predominantly early rings, 5 % (w/v) D-sorbitol is used because it kills all other stages leaving only rings. However, if the parasites are predominantly schizonts, then 60 % Percoll is used because it purifies the schizonts into a distinct layer.

3.3.6.1 D-Sorbitol Synchronization

Sorbitol synchronization technique selectively lyses the mature forms of the malaria parasite which are more permeable to sorbitol. Sorbitol enters the trophozoite and schizont cells and produce osmotic pressure which causes them to swell and eventually lyse and die, leaving the young ring stages unaffected (Zolg *et al*., 1982; Petmitr *et al*., 1995; Lambros & Vanderberg, 1979).

The D-sorbitol synchlonization method was employed for cultures with high proportion of the parasites in the ring stage. The culture was aseptically transferred into sterile 15 ml centrifuge tube and spun at 1500 rpm for 5 minutes at room temperature. The supernatant was removed and the cell pellet (of up to 200 µl volume) was re-suspended in 10 ml of sterile 5 % (w/v) D-sorbitol solution. The suspension was centrifuged at 1500 rpm for 5 minutes after it was allowed to stand in a water bath at 37^{o}C for about 15 minutes. The parasitized cell pellet was then washed repeatedly in 10 ml of complete medium and finally suspended to form 5% haematocrit. The synchronized culture was transferred into a culture flask, cultivated for 48 hours and then used for the intended purpose. The procedure was repeated after 48 hours whenever the results were not satisfactory (Schuster, 2002).

3.3.6.2 Percoll Synchronization

The percoll synchronization technique is based on density gradient coupled with differential centrifugation (Kramer *et al*., 1982). It was employed for cultures predominantly in the late trophozoite/early schizont stage. Into a 15 ml sterile centrifuge tube was aseptically placed 5 ml

of 65 % Percoll solution (prepared and sterilized by filtration) and warmed in a water bath at 37ºC prior to use. The parasite culture was transferred into a 15 ml tube and centrifuged at 1500rpm for 5 minutes and the supernatant was aspirated leaving just enough for re-suspending the cell pellet to about 50% haematocrit. The re-suspended culture was carefully and slowly overlaid on the 65% percoll solution without mixing it. A brown layer of late trophozoites and schizonts formed between the medium and the percoll solution after the setup was centrifuged at 3000 rpm for 5 minutes. The medium was aspirated and the trophozoite brown layer transferred into another 15 ml tube. The percoll was washed out of the cells by filling the tube with RPMI medium. After mixing by gently inverting it, the tube was centrifuged for 5 minutes at 1500 rpm and the supernatant aspirated. The washing was repeated two times in complete RPMI medium to ensure adequate removal of the percoll. Depending on the volume of the pellet, 200 – 600 µl of 50% washed rbcs was added followed by the complete medium to bring the suspension to 5 % haematocrit. The culture was then maintained as normal.

3.3.7 Cryopreservation

The ring stages of parasites in fresh rbcs with parasitaemias of between 3 % and 5 % produce the most viable frozen cultures. A culture to be cryopreserved was pipetted into a 10 ml centrifuge tube and spun for 5 minutes at 2500 rpm. The supernatant was replaced with the cryopreservation solution equal to the volume of the pellet to give a 50 % haematocrit. The cells were resuspended and transferred into a labelled 1.8 ml screw-capped cryotube (Nunc). The tube was placed in an ampoule cane in a plastic sleeve and gently lowered into liquid nitrogen at -196°C (Srinivasa *et al.*, 1988).

3.3.7.1 Cryopreservation Solution

The cryopreservation solution is an isotonic solution prepared by dissolving 0.65 g NaCl, 3.024 g sorbitol and 28 ml glycerol in distilled water to form 100 ml solution. It was sterilized by filtration using a 0.2 μm-acrylic filter. All the chemicals were procured from Sigma, and were cell culture tested.

3.3.8 Theory of Parasite Lactate Dehydrogenase Assay

The activity of the various plant extracts and compounds as well as the reference antimalarial drugs used in these studies were tested against the multi-drug resistant *P. falciparum* (K1). An adaption of the method reported by Makler and Hinrichs (1993) was used to assess parasite viability after treatment with the various agents. In the *in vitro* pLDH assay, the conversion of lactate to pyruvate by *P. falciparum* is catalysed by pfLDH. 3-acetyl pyridine adenine dinucleotide (APAD) (analogue of NAD) serves as the coenzyme and is reduced to APADH.

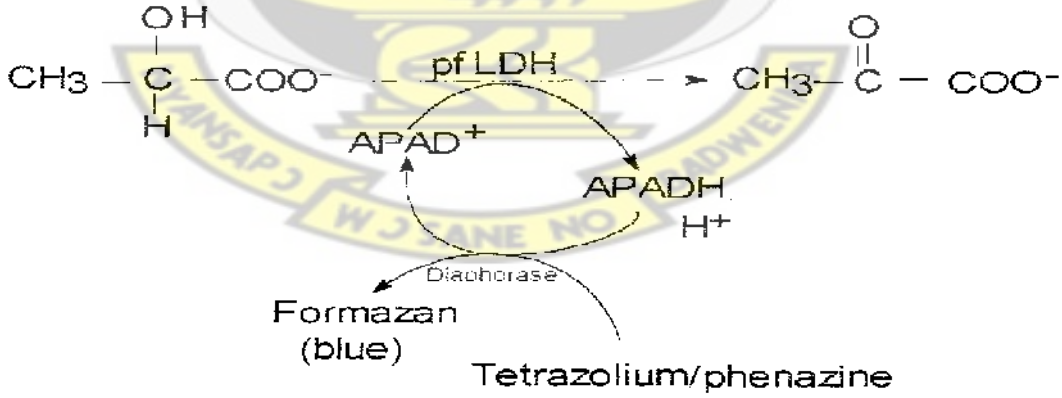


Figure 3.3 Schematic illustration of the basis of the pLDH assay

The amount of APADH formed is measureable using a mixture of nitroblue tetrazolium and phenazine ethosulphate. APADH is re-oxidized to APAD in a reaction that is catalysed by diaphorase in the presence of the nitroblue tetrazolium/phenazine ethosulphate electron acceptor system (Figure 3.3). Nitroblue tetrazolium is reduced to blue formazan which has a λmax at 550 nm (Malik *et al.*, 2004).

3.3.9 Preparation of plates, incubation and assessment of parasite growth

The solutions of plant extracts (dissolved in complete medium) were two-fold serially diluted in sterile 96 well flat-bottomed micro-titre plates:

- 50 µl of complete medium was placed in each well of the 96-microtiter plate except the first column.
- 50 µl of extract/compound solution was placed in columns 1 and 2 (two rows for each extract/compound).
- A two-fold serial dilution was made from column 2 to 12 in each row.
- 50 µl of parasitized rbcs (1.5 % parasitaemia and 5 % haematocrit) was added to each well.
- A row for control and another for blank were included.

In the preliminary screening, the starting concentration of the extracts was 500µg/ml. With some samples, initial dissolution in either ethanol or DMSO was necessary before they were made up in complete medium. The final concentration of either ethanol or DMSO was never more than l% in each well (concentrations of 1% or lower were found to be harmless to the parasites). The content of each well was mixed (the haematocrit then reduced to 2.5 %). The control wells had no test substances and the blank wells had only uninfected rbcs. Chloroquine diphosphate and artemether were used as standard drugs in the evaluation.

Figure 3.4 Picture of prepared microtiter plates in a modular incubator chamber.

The plates were covered, placed in a modular incubator chamber (Figure 3.4; Billups-Rothenberg) (the chamber contained a dish of cotton wool soaked in sterile water to reduce evaporation from the wells) and flushed with gas for 5 minutes. The chamber was quickly sealed and gently placed in an incubator (37°C) for 48 hours. The plates were removed after incubation and to each well was added 50 µl of freshly prepared APAD reagent (Appendix II) and 50 µl of a 20:1 mixture of nitroblue tetrazolium (1 mg/ml) and phenazine ethosulphate (0.05 mg/ml). The reagent solutions were protected from light by covering with metal foil. The well contents were carefully mixed. Any bubbles that had formed were removed by a quick blow-over of hot air from a hairdryer. The plate was kept (covered) in a dark, cool place for 15 minutes after which the optical densities (OD) of the wells were measured on a plate reader (MRX Dynatech Laboratories; Figure 3.5) and used to calculate % inhibitions as shown in Table 3.2. By means of Microsoft Excel 2013, log dose-response curves were plotted and the IC_{50} values for test samples

against *P. falciparum* were determined through application of regression analysis to the linear section (20-80 % range) of the curves.

Figure 3.5 MRX Dynatech Laboratories microtiter plate reader and printer

It was possible to estimate quite accurately by visual inspection the range of IC_{50} values of test substances. These values were indicated by the points at which the colour changed from straw to deep blue (Figure 3.6). The tests were carried out in duplicates on each microtiter plate and at least three series of independent tests were conducted for each compound or sample.

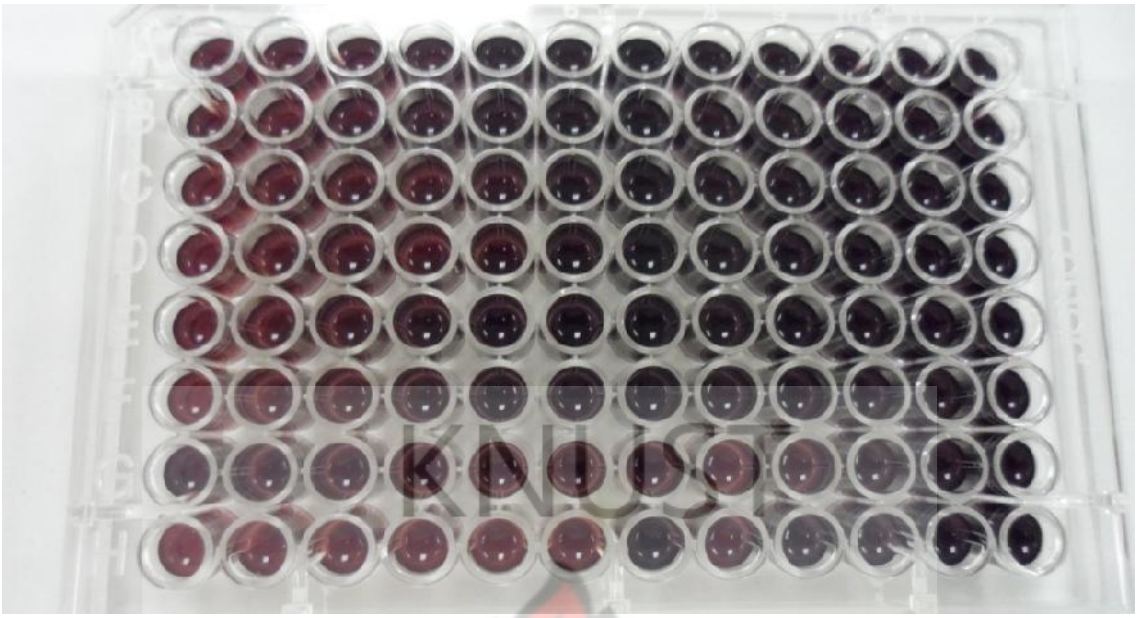


Figure 3.6 A microtiter plate showing the results of a pLDH assay

Table 3.2 Calculation of %Inhibition and IC_{50} values

Concentration of sample	Mean test absorbance	Corrected absorbance (Growth)	%Growth	%Inhibition of growth
µg/ml	A_{test}	$G = A_{test} - A_{un}$	%G=(G/Tg)x100	100 - %G

A_{un} = The average absorbance of the blank/un-infected control. Tg = The total growth of parasite obtained as the difference between the average absorbance of infected control and blank.

3.3.10 Results of antiplasmodial activity of the plants extracts

The log dose-response curves of the various extracts and the reference drugs tested against the multi-drug resistant *P. falciparum* (K1 strain) were plotted (Appendix 2) and their antiplasmodial activity, defined as the concentrations that inhibited or killed 50% of the parasite, determined (Table 3.3).

Table 3.3 Antiplasmodial activity of the plant extracts on multi-drug resistant *Plasmodium falciparum*

Plant extracts	IC_{50} ± STD (µg/ml)	
	Total extract	**Alkaloidal extract**
A. cissampeloides	34.86 ± 5.48	32.12 ± 0.71
A. nobilis	41.92 ± 4.45	35.44 ± 3.97
C. portoricensis	117.55 ± 3.78	112.30 ± 8.72
E. guineensis	45.80 ± 2.91	27.62 ± 2.08
E. angolense	39.27 ± 2.23	120.38 ± 27.36
M. oppositifolius	36.93 ± 2.14	20.60 ± 2.81
P. macrocarpus	96.45 ± 13.41	118.74 ± 9.25
P. longifolia	22.04 ± 4.23	15.47 ± 1.73
S. latifolius	33.14 ± 1.25	16.63 ± 1.11
T. ivorensis	59.41 ±7.32	Not tested
Chloroquine diphosphate	0.4120 ± 0.015	
Artemether	0.0193 ± 0.005	

3.3.11 Discussion

Many patients from poor resource settings have strong beliefs in the use and efficacy of ethnomedicines (Chinsembu & Hedimbi, 2010) on which they are reliant for their health care needs. Hence scientists, in developing traditional pharmacopeias are concerned among others with the biological activities of medicinal plants (Kone *et al*., 2011; Gupta & Sharma, 2010). Medicinal plant species are a great part of the biodiversity in the sub-Saharan African region. Ten (10) of these tropical plant species which are employed in the ethnomedical treatment of malaria have been screened in this study to ascertain their efficacy.

The results of the study showed various degrees of *in vitro* antiplasmodial activity of these plant species (Table 3.3). The literature indicates that plant extracts with *in vitro* antiplasmodial activity are classified as 'very active' ($IC_{50} \leq 25$ µg/ml), 'active' ($IC_{50} > 25 \leq 50$ µg/ml), 'moderately active' ($IC_{50} > 50 \leq 100$ µg/ml) and 'weakly active' ($IC_{50} > 100 \leq 1000$ µg/ml) (Gessler *et al*., 1994; Prachayasittikul, 2009; Ramazani *et al*., 2010; Omoregie & Sisodia, 2012). Among the 10 plants screened, only three of the total extracts from *C. portoricensis, P. macrocarpus* and *T ivorensis* and three of the alkaloidal extracts from *C. portoricensis, E. angolense* and *P. macrocarpus* exhibited moderate activity with IC_{50} values greater than 50 µg/ml. The rest demonstrated potent antiplasmodial activity. Chloroquine diphosphate and Artemether (all from Sigma-Aldrich, UK) which were used as reference drugs were more active than all the extracts tested.

The stem bark extracts of *P. longifolia* exhibited the highest antiplasmodial activity against the multi-drug resistant *P. falciparum* [IC_{50} 22.04 µg/ml (total extract) and 15.96 µg/ml (alkaloidal extract)]. Although the antiplasmodial activity of other species of *Polyalthia* such as *P. avecta, P. viridis, P. cerasoides, P. oliveri* and *P. debilis* has been reported, this seems to be the first report of the activity for *P. longifolia*. The hexane and dichloromethane extracts of *P. avecta* roots and the ethanol extract of *P. viridis* stem bark have been reported to be active against the multidrug resistant K1 strain of *P. falciparum* with IC_{50} 20 µg/ml (for both extracts of *P. avecta*) and 10 µg/ml (for *P. viridis*) (Kanokmedhakul *et al*., 2006; Kanokmedhakul *et al*., 2003; Ichino *et al*., 2006). The aqueous, methanolic and hexane fractions of *P. oliveri* stem bark extract tested against Cameroonian field isolates of *P. falciparum* exhibited potent activities with IC_{50} values in the range 0.05 – 8.09 µg/ml (Kemgne *et al*., 2012). The hexane extract of *P. debilis* has also been found to exhibit antiplasmodial activity against chloroquine resistant strains of *P. faciparum* (T9.94) (IC_{50} 10 – 100 µg/ml) (Prachayasittikul *et al*., 2009). These previous reports and the

results of this present study seem to support the suggestion that the genus *Polyalthia* might be a rich source of untapped, antiplasmodially active phytocompounds effective against various strains of *P. falciparum* including the drug resistant ones.

The extracts of *S. latifolius* roots and *M. oppositifolius* leaves have also shown potent antiplasmodial activity (IC_{50} 16 - 37 µg/ml). Antiplasmodial activity of *S. latifolius* root bark extracts against the Colombian strain of chloroquine-resistant *P. falciparum* (FcB1) (IC_{50}: 8.9 µg/ml) has been reported (Zirihi *et al.*, 2005). Benoit-Vical *et al*., (1998) have also reported potent activity (IC_{50} 0.6 - 7.5 µg/ml) of the aqueous extracts of *S. latifolius* stem and root against the FcB1 and the Nigerian chloroquine sensitive strains of *P. falciparum*. The results of the alkaloidal extract of *S. latifolius* roots in this current study together with the reported *in vitro* antiplasmodial activity of the stem and roots barks of this plant classifies it as 'very active'. The alkaloidal extract of *S. latifolius* showed higher activity (IC_{50} 16.63 µg/ml) than the alcohol extract (IC_{50} 33.14 µg/ml) and this appears to suggest that plants species containing alkaloids in the Rubiaceae family possess potent antiplasmodial property. The cinchona alkaloids and many other reported alkaloidal plant species with potent antiplasmodial activities (such as *Mitragyna africanus*, *Hallea rubrostipulata*, *Morinda morindoides* and *Cathium setosum*) are of the Rubiaceae family (Mustofa *et al.*, 2000; Karou *et al*., 2011; Addae-Kyereme *et al*., 2001).

Varying levels of antiplasmodial activity have however been reported in other species of *Sarcocephalus.* Examples include *S. diderrichii* stem (IC_{50} of 312.14 µg/ml) (Mustofa *et al*., 2000) and aqueous and ethanol extracts of *S. pobeguinii* stem (IC_{50} 44 and 32 µg/ml respectively) (Mesia *et al*., 2010). Also, lower antiplasmodial activity (IC_{50} between 60 - 260 µg/ml) has been reported for the leaves of *S. latifolius* when tested against the Cameroonian chloroquine resistant (FcM29) and the Nigerian chloroquine sensitive strains of *P. falciparum* (Ménan *et al*., 2006).

This seems to suggest occurrence in higher quantities of the antiplasmodial constituents in the stem and roots of *S. latifolius* than the leaves. It is probable that traditional healer in Ghana have realised higher curative potentials of the stem and roots of this plant since these are the reported commonly used parts (Asase & Oppong-Mensah, 2009; Asase *et al.*, 2010; Personal communication).

The alkaloidal extract of *M. oppositifolius* leaves showed a higher antiplasmodial activity (IC_{50} 20.60 µg/ml) in comparison with the total extract (IC_{50} 36.93 µg/ml). *Mallotus* species are however known to be rich in dimeric phloroglucinols. Two phloroglucinols; mallotojaponin B and C with potent antiplasmodial properties against chloroquine resistant *P. falciparum* (IC_{50} values of 0.75 and 0.14 µM respectively) were recently isolated from the leaves of the Madagascan *M. oppositifolius* (Harinantenaina *et al*., 2013). Phytoconstituents stand the potential of either antagonizing, or synergizing the activity of each other or remaining additive in action when tested in the crude form. They may therefore show a better individual response upon either isolation into individual compounds or separation into chemical groups. Antagonism might have however generally contributed to the differences seen in the activities of the two types of *M. oppositifolius* extracts tested in this study as well as explained the high potencies of the mallotojaponins isolates reported. Bioassay-guided isolated of the individual compounds might therefore throw more light on the above observation.

Elaeis guineensis leaves, *Adenia cissampeloides* stem and *Anthocleista nobilis* stem bark extracts had IC_{50} values ranged between 27 - 46 µg/ml. Higher activity (IC_{50} values < 10 µg/ml) has been reported for *E. guineensis* and *A. cissampeloides* extracts by the WHO micro-test method of evaluation of antiplasmodial activity (Annan *et al*., 2012). This contrasting result reported might

be due to the differences in the strains of the *P. falciparum* used and or the techniques employed in evaluating the antiplasmodial activity.

Among the *Adenia* species, only *A. gummifera* (stem extract) has been reported to exhibit antiplasmodial activity against chloroquine-sensitive strains of *P. falciparum* with an IC_{50} of 50 µg/ml (Kraft *et al*., 2003). In the case of *Anthocleista* species, *in vivo* antiplasmodial activity in *P. berghei* infected mice have been reported for the ethanol extracts of *A. vogelii* leaves and *A. djalonensis* stem bark and leaves (Akpan *et al*., 2012; Alaribe *et al*., 2012; Bassey *et al*., 2009). The results of this current study have therefore shown for the first time that *E. guineensis* leaves, *A. cissampeloides* stem and *A. nobilis* stem bark extracts possess some antiplasmodial activity against the multi-drug resistant K1 strain of *P. falciparum*.

Entandrophragma angolense stem bark total extract showed antiplasmodial activity (IC_{50} 39.27 µg/ml) close to those reported by Bickii *et al*. (2007b) (IC_{50} 18 - 34 µg/ml) for chloroquine resistant W2 strain of *P. falciparum* when different organic and aqueous extracts of the plant were studied. The alkaloidal extract however, showed a weak activity (IC_{50} 120.62 µg/ml).

Terminalia ivorensis stem bark total extract also exhibited a weak antiplasmodial activity. There was no yield in the alkaloidal extraction of this plant and hence not included in the study. It is worthy of note that an earlier report (IC_{50} 6.95 µg/ml) (Annan *et al*., 2012) and the result of this current study (59.41 µg/ml) of *T. ivorensis* extracts are all within the range (IC_{50} 4 – 68 µg/ml) reported for *T. spinosa* stem bark and wood and *T. chebula* seeds which are different species of the genus (Bagavan *et al.*, 2011; Omulokoli *et al.,* 1997).

3.3.12 **Conclusion**

From the study, the alkaloidal extracts of *C. portoricensis, P. macrocarpus* and *E. angolense* exhibited weak antiplasmodial activity (IC_{50} between 112 - 121 µg/ml) while those of *E. guineensis, A. cissampeloides* and *A. nobilis* were active with IC_{50} values between 27 - 36 µg/ml. However, the alkaloidal extracts of *P. longifolia, S. latifolius* and *M. oppositifolius* were very potent (IC_{50} < 21 µg/ml) against the multidrug resistant K1 strain of the *P. faciparum*.

With regard to the total ethanolic extracts, *T. ivorensis, P. macrocarpus* and *C. portoricensis,* exhibited weak antiplasmodial activity (IC_{50} ranged between 60 - 118 µg/ml) while *S. latifolius, A. cissampeloides, M. oppositifolius, E. angolense, A. nobilis* and *E. guineensis* showed average activity (IC_{50} ranged between 33 - 46 µg/ml). The most activity was again shown by *P. longifolia* (IC_{50} < 25 µg/ml). The results of the study appeared to lend support to the traditional use of these plant species as remedies for treating malaria especially *P. longifolia*.

Chapter Four

4 PHYTOCHEMICAL INVESTIGATIONS OF *POLYALTHIA LONGIFOLIA* VAR. PENDULA

4.1 The choice of *Polyalthia longifolia* var. pendula

In the preliminary studies the stem bark extract of *Polyalthia longifolia* var. pendula clearly exhibited amongst others, a significant antiplasmodial activity and as such was a suitable candidate for further antiplasmodial investigations. The plant also has a historical and traditional use in the treatment of malaria in Ghana and other West African countries. It is also employed in other countries including India and Sri Lanka where it originated in the folklore treatment of all kinds of feverish conditions (Mundhe *et al*., 2010).

In view of these reasons, in addition to the fact that very little scientific work of its antimalarial activity has been reported, *P. longifolia* var. pendula was selected for further investigations including the isolation, characterization and the subsequent testing of its active constituents.

4.1.1 Organoleptic characteristics of *Polyalthia longifolia*

In addition to the plant description in Section 2.1.7**,** the powdered stem bark material had the following organoleptic characteristics:

Texture: - coarse powder mixture of fibers and fragments.

Colour: - straw-like to brownish-grey in colour

Odour: - indistinguishably characteristic odour

Taste: - slightly bitter and biting taste with astringent properties when chewed.

4.2 The bulk extraction processes

4.2.1 Total Ethanolic Extraction

A total of one kilogram (1.0 kg) of the powdered *P. longifolia* stem bark was extracted with 70 % ethanol by cold maceration in two portions. Equal quantities (500 g) of the powdered plant material were packed into two 5 litre capacity glass percolators and 2.0 litres of the ethanol was added to each. The percolator lids were put in place and the set-up allowed to stand for 24 hours at room temperature (20 – 25 ^{o}C) after which the extract was drained into 2.5 litre bottles. The marc was repeatedly extracted with more 70% ethanol until the dark colour of the extracts obtained became clearer (which signified exhaustive extraction of the material). The extracts were then pooled together and filtered through Whatman filter paper number 1. The filtrate was concentrated to a syrupy mass under reduced pressure in a rotary evaporator (Buchi, Swaziland) and then dried completely *in vacuo* at room temperature. The dried extract was weighed (yield: 22.8 g; 2.28 % w/w) and then stored in a refrigerator until required for use.

4.2.2 Alkaloid Extraction

Eight hundred grams (800 g) of the powdered *P. longifolia* stem bark was packed into a 5 litre capacity percolator and 2.0 litres of ammoniacal-ethyl alcohol (1 part ammonia solution to 9 parts ethyl alcohol) was added to completely soak the material. The set-up was then treated as in Section 4.2.1 above to obtain the extract in the form of a syrupy mass. This was dissolved in 150 ml of dilute sulphuric acid (1 %) and filtered. The acidic filtrate was alkalinised by adding enough ammonia solution until it changed the colour of a red litmus paper to blue and by means of a separation funnel, the alkaloids were exhaustively extracted (six times; until the initial yellowish colour of the extracted portions became clear) with 100 ml portions of chloroform. The

chloroform portions were pooled and by means of anhydrous sodium sulphate traces of water molecules were removed. It was then concentrated under reduced pressure and dried on a silica gel in a vacuum chamber at room temperature. The dried alkaloid extract obtained was weighed (yield 2.12 g; 0.27 % w/w) and stored in a refrigerator until required for use.

4.3 Chromatography

4.3.1 Flash column chromatography

Silica gel 60 H (Merck, 5-40 µm particle size) was used as the stationary phase for the column. The packing of the column was done by the wet method while gradient elution was employed in developing the column.

4.3.2 Analytical thin layer chromatography (TLC)

A 0.25 mm thick precoated TLC silica gel 60 F_{254} on Aluminium Sheets 20 x 20 cm (Merck, Catalog No. 1.05554.0001) were used to analyse the TLC fingerprints of fractions and chromatographic aliquots. The samples were applied using 5 µl capillary tubes (CAMAG – Merck). The TLC solvent systems compositions that produced the best separation of components of the fractions at the various stages of isolation of the compounds included; ethanol-dichloromethane-hexane (0.5:8:1.5), methanol-chloroform (1:9), methanol-ethyl acetate (0.5:9), ethyl acetate-hexane (4:6) and ethyl acetate-dichloromethane (2:8).

4.3.3 Preparative thin layer chromatography (pTLC)

A 0.75 mm thick of Silica gel 60 $PF_{254+366}$ (Merck, Catalog 1.07748.2500) prepared on glass plates of size 20 x 20 cm and activated at 100 ^{o}C for 24 hours were use. The solvent systems employed were the same as stated in Section 3.5.1.2 above.

4.3.4 Detection of components

The spots on developed TLC plates corresponding to separated compounds were detected under UV light 254 and 365 nm and also by spraying with either mostly Vanillin reagent (2 g vanillin, 100 ml methanol and 2 ml concentrated H_2SO_4), or Dragendorff reagent. The plates sprayed with Vanillin reagent were dried by hot air from a hand held hair-dryer for about 2 minutes.

4.3.5 Chemicals and solvents

The chemicals and solvents used were, unless otherwise stated, obtained from Fisher Scientific and Sigma-Aldrich Chemical Company, UK. Analytical grade chemicals were employed, where as for the solvents, both analytical and laboratory grades were used.

4.4 Fractionation of total ethanol extract

Twenty-one (21 g) grams of the *P. longifolia* stem bark ethanol extract was divided into three equal portion and each was fractionated by column chromatography on 40 g of silica gel 60 G (average particle size 5-40 µm; Merck) (in a glass column of 5 cm diameter) by sequential elution with 600 ml each of n-hexane, dichloromethane, ethyl acetate and 10% methanol-ethyl acetate. The eluates were concentrated under reduced pressure and dried over silica gel *in vacuo* at 40 ^{o}C; resulting in the following fractions:

- Hexane fraction (Hxf) (2.35 g)
- Dichloromethane fraction (Dcf) (5.87 g)
- Ethyl acetate fraction (Eaf) (7.25 g)
- 10% Methanol-ethylacetate fraction (Mef) (4.03 g)

4.5 Antiplasmodial evaluation of the fractions

The antiplasmodial activities of Hxf, Dcf, Eaf, and Mef as well as the alkaloidal extract (Alf) were evaluated by the parasite lactate dehydrogenase assay (Sections 3.3.9) in the 96 well microtiter plates using the multi-drug resistant *P. falciparum* K1 strain as test organism. Complete RPMI 1640 medium (Section 3.3.3) was employed as the growth medium while Chloroquine diphosphate and Artemether were used as reference antimalarial drugs.

4.5.1 Results of antiplasmodial evaluation

The optical densities obtained from the pLDH assay were used to calculate percentage parasite growth inhibitions of the various fractions of the *P. longifolia* stem bark extract. Log dose-response curves were plotted and used to calculate the IC_{50} values of the fractions (Table 4.1).

Table 4.1 Antiplasmodial activities of fractions of *P. longifolia* stem bark extract and reference drugs.

Extract fractions	**IC_{50} ± STD (µg/ml)**
Hexane fraction	47.80 ± 1.33
Dichloromethane fraction	22.91 ± 0.76
Ethyl acetate fraction	**10.07 ± 0.18**
10% Methanol-ethyl acetate fraction	26.06 ± 0.97
Alkaloidal extract	**15.47 ± 1.43**
Chloroquine diphosphate	0.4120 ± 0.015
Artemether	0.0193 ± 0.005

4.5.2 Discussion

The total alcoholic extract of *P. longifolia* stem bark was fractionated and tested against the multidrug resistant K1 strain of *P. falciparum* to determine the potentially active fractions for selection for further investigation. The 10% methanol-ethyl acetate, hexane and dichloromethane fractions exhibited levels of antiplasmodial activity (IC_{50} values of 26.06, 47.80 and 22.06 µg/ml respectively) lower than those obtained for the parent total alcoholic extract (IC_{50} 22.04 µg/ml; Section 3.3.10; Table 3.3) and hence did not merit any further antiplasmodial investigations. The ethyl acetate fraction and the alkaloidal extract however displayed levels of activity (IC_{50} values of 10.07 and 15.47 µg/ml respectively) higher than the parent total ethanolic extract (Table 4.1). These were therefore selected for further investigations.

4.5.3 Conclusion

The ethyl acetate fraction of the ethanolic extract and the alkaloidal extract of *P. longifolia* stem bark were the antiplasmodially most potent against the multi-drug resistant K1 strain of *P. falciparum* with IC_{50} values (10.07 and 15.47 µg/ml) within significantly acceptable thresholds; that merited further scientific investigations.

4.6 Isolation of compounds from *P. longifolia* stem bark

4.6.1 Ethyl acetate fraction of the alcoholic extract

Fifty grams (50 g) TLC grade silica gel 60 H (Merck) was weighed into a 500 ml beaker and 300 ml of n-Hexane was added and shaken to form a slurry. This was transferred into a 5 cm diameter glass chromatographic column. More of the packing solvent (n-Hexane) was added to the column and allowed to drain through the settled silica gel plug until it became homogeneous in

appearance; an indication that the column was well packed. Throughout the packing as well as the elution, it was ensured that the top of the column was never run dried, which would otherwise force air to enter the silica gel plug.

Figure 4.1 Flash column chromatography set-up in a fume cupboard.

Seven grams (7.0 g) of the ethyl acetate fraction of the alcoholic extract of *P. longifolia* stem bark was dissolved in a minimum amount of ethyl acetate, thoroughly mixed with about 14 g of silica gel in a mortar and dried at room temperature. This was gently spread on top of the packed column after triturating into a fine powder. The side of the column was rinsed with about 20 ml of the elution solvent followed by spreading of about 8 g of sand on the surface of the packed silica gel as a protection against disturbing the surface of the column during solvent addition (Fig 4.1). The column was then connected to compressed air to provide positive pressure (Still 2002;

Still *et al*. 1978). The tap of the column was regulated to deliver eluent at a flow rate of about 2.5 ml per minute.

The column was eluted in a gradient manner, started with 1% ethyl acetate in n-hexane and ended with 100 % ethyl acetate. In all, 81 aliquots of 28 ml each were collected and aliquots with similar TLC profiles were combined together resulting into four fractions designated as P_1 (Eaf-1 to 21), P_2 (Eaf-22 to 57), P_3 (Eaf-58 to 74) and P_4 (Eaf-75 to 81). The fractions were concentrated under reduced pressure using the rotary evaporator, dried over nitrogen and weighed: P_1 (1.14 g), P_2 (4.31 g), P_3 (0.65 g) and P_4 (0.25 g).

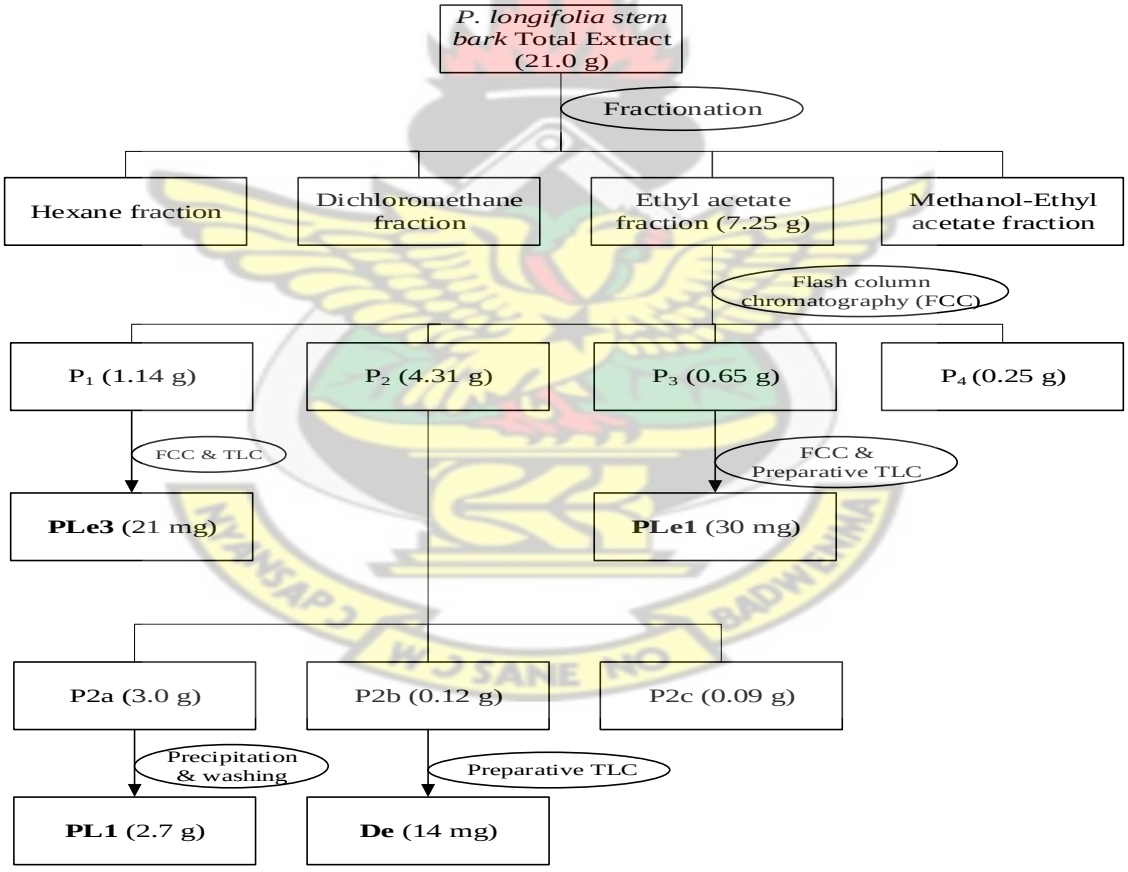


Figure 4.2 Flow chart of isolation of compounds from total extract of *P. longifolia*.

Fractions P_1, P_2 and P_3 which exhibited interesting TLC patterns were subjected to further flash column chromatographic purification followed by preparative TLC. P_1 and P_3 yielded compounds **PLe3** (21 mg, R_f = 0.44 in ethanol-dichloromethane-hexane 0.5: 8: 1.5, whitish flakes in appearance) and **PLe1** (30 mg, R_f = 0.28 in dichloromethane-ethyl acetate 7:3, colourless and semi-solid), respectively. Fraction P_2 however, upon re-fractionation on 15 g of silica gel 60 H in a 3 cm diameter glass column with n-hexane graded with ethyl acetate (in 2 % increments) yeielded forty-six (46) aliquots of about 25 ml each. The aliquots were pooled into three fractions [P_2a (1 - 34), P_2b (35 - 41) and P_2c (42 - 46)] in accordance with their TLC profiles. Precipitation was found to occur in fraction P_2a after it weas left in the fume cup-board overnight. The precipitate, whitish in colour, was washed repeatedly with enough n-hexane and this resulted in compound **PL1** (2.7 g, R_f = 0.71 in ethanol-dichloromethane-hexane 0.5: 8: 1.5). P_2b (150 mg) was purified on preparative TLC to yield compound **De** (14 mg, R_f = 0.41 in ethanol-dichloromethane-hexane 0.5: 8: 1.5, colourless and semi-solid).

4.6.2 Acetylation of PL1

PL1 (1 g) was dissolved in 100 ml of chloroform in a 250 ml beaker and treated with 10 ml of acetic anhydride in the presence of potassium carbonate (as a catalyst). The set up was heated in a hot water bath maintained at 60 ^{o}C. By means of TLC, the end of the reaction (24 hours) was determined; indicated by the absence of original (PL1) spot and the appearance of a new spot for the acetylated product (PL1a).

The reaction mixture was concentrated, mixed with kieselghur (1.4 g) and allowed to dry at room temperature in a porcelain mortar. The mixture was fractionated on silica gel by column chromatography [eluted with hexane-ethyl acetate (6:4)]. **PL1a** was obtained and purified by re-crystallization out of hexane-chloroform (1:1) as a colourless solids (yield 0.67 g).

4.6.3 Alkaloid Extract

The alkaloid extract (2.0 g) was placed on 30 g of TLC grade silica gel 60 H in a column of 4 cm diameter in a similar manner as described in Section 4.6.1. The column was eluted with dichloromethane graded with methanol (in 0.5 % increments up to 10 %). A drop of liquid ammonia (about 0.02 ml) was added and shaken with every 200 ml volume of the elution solvent before introduced into the column.

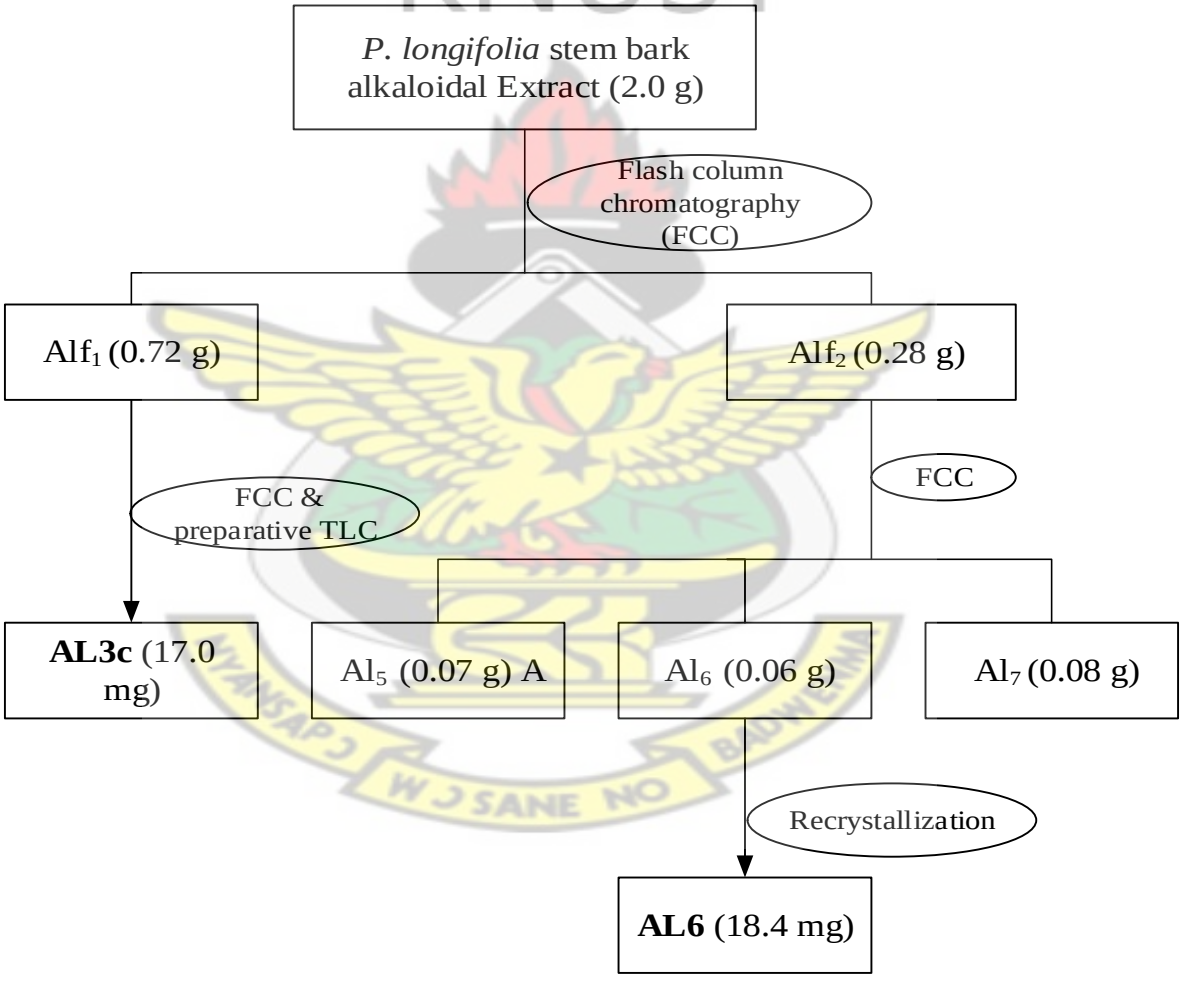


Figure 4.3 Flow chart of isolation of compounds from alkaloidal extract of *P. longifolia*.

The aliquots collected were pooled into two fractions [(Alf_1 0.719 g (from aliquots 1 – 19) and Alf_2 0.208 g (20 – 32)] in accordance with their TLC patterns. Repeated fractionation of Alf_1 through the column chromatographic technique followed by purification on a preparative TLC yielded a yellow powdered compound **AL3c** (17 mg, R_f = 0.57 in methanol-chloroform 1:9). Alf_2 was also further fractionated by the column chromatographic technique using chloroform graded with ethyl acetate up to 100 %. The resultant aliquots were combined into Al_5, Al_6 and Al_7 and left to dry in the fume cup-board overnight. Brownish cuboidal crystals which were insoluble in chloroform formed in fraction Al_6. The crystals were washed thoroughly in chloroform, allowed to dry *in vacuo* and labelled compound **AL6** (18.4 mg, R_f =0.54 in methanol- ethyl acetate 1:9).

4.6.4 Isolated compounds

A total of six compounds were isolated from the ethyl acetate fraction of the ethanolic extract and the alkaloidal extract of *P. longifolia* stem bark: PL1, PLe1, PLe3, De, AL3c and AL6. A reaction of PL1 with acetic anhydride in the presence of K_2CO_3 (as a catalyst) yielded the acetylated product PL1a.

4.7 Materials and methods of spectroscopic analysis of compounds

Chemical compounds are usually identified through a combination of various techniques, including nuclear magnetic resonance (NMR), mass spectrometry (MS), ultraviolet (UV) and infrared (IR) spectroscopy and X-ray crystallography. Other ways of confirming the identity of compounds include comparison of the retardation factor (R_f) values in solvent systems and melting points with reference compounds. In this study, NMR and MS techniques were employed to determine the structure and identity of the isolated compounds.

4.7.1 Nuclear magnetic resonance spectroscopy (NMR)

The NMR (both ^{1}H and ^{13}C) spectra were recorded on either Bruker DPX 400 and/or Joel 600 spectrometers (ppm, *J* in Hz) using TMS as internal standard. DEPT and 2D-NMR experiments were also carried out on the same instruments. The NMR analysis samples were prepared by dissolving 1.0 mg of the compound in a maximum of 1.2 ml of deuterated NMR solvents (Sigma-Aldrich, UK). The solvents of choice were chloroform-d ($CDCl_3$) and methanol-d (CD_3OD) as the compounds of interest were soluble in them. The solution was rendered free of insoluble impurities by filtering through a clean tissue paper plugged Pasteur's pipette directly into a NMR tube and then used for the NMR experiments.

4.7.2 ^{1}H-NMR experiment

Proton NMR was widely used in the analysis. The spectrum appears in the range 0 - 10 ppm downfield from the reference tetramethyl silane (TMS) signal. Proton NMR gives a measure of the absorption of the different proton signals from a compound. The integral of the signal is proportional to the number of protons it represents, and the nature of the hydrogen is established by the chemical shift. The absorption of a signal is usually proportional to the number of protons coming into resonance frequency of the signal, such that the area under the absorption peak is in proportion to the number of protons being detected. A nucleus in a region of high electron density experiences a chemical shift proportionately weaker than those in a region of low electron density, and a higher field has to be applied to bring it into resonance. Such nuclei are said to be shielded by electrons. A high electron density shields a nucleus and causes resonance to occur at relatively high field (with low chemical shift values). Likewise, a low electron density causes resonance to occur at relatively low field (with high chemical shift values) and the nucleus is said to be de-shielded.

4.7.3 ^{13}C-NMR experiment

The ^{13}C-NMR was used to determine the precise frequency at which each carbon comes into resonance and is not only determined by the applied magnetic field but also by minute differences in the magnetic environment experienced by each nucleus. These minute differences are caused by the variation in electrons in the neighbourhood of each nucleus, with the result that each chemically distinct carbon atom in a structure, when it happens to be a ^{13}C, will come into resonance at a slightly different frequency from all the others. Each upward line in a ^{13}C spectrum corresponds to one carbon atom.

4.7.4 DEPT experiments (DEPT-135 and DEPT-90)

Distortionless enhancement through polarization transfer (DEPT) is a technique that allows a separate spectrum to be obtained for the ^{13}C of CH_3, CH_2 and CH. The impulse sequence used in this experiment forces part of the higher sensitivity associated with proton detection on to ^{13}C, a process that enhances the ^{13}C signal intensity by polarization transfer from ^{1}H to ^{13}C. While DEPT-90 spectrum shows signals for CH carbons only, DEPT-135 spectrum shows positive signals for CH and CH_3 carbons and negative signals for CH_2 carbons. Quaternary carbons are however, usually absent in DEPT spectra.

4.7.5 Mass spectrometry

Specific identification of molecules is more certain with the use of mass spectrometer (MS). In electron impact mass spectrometry (EI-MS), the effluent which contains the separated and vaporized compounds, passes into the ion chamber of the mass spectrometer, which is under a high vacuum. A beam of electrons accelerated from a filament bombards the molecules which ionizes and fragments them. Initially, one electron is removed from each molecule to form a

positively charged molecular ion (M^+). Breakage of bonds relative to bond strength occurs rapidly in the molecular ion to generate fragment ions. The various ions are accelerated into the analyser portion of the mass spectrometer where they are sorted according to their mass to charge ratios (m/z values) that are equivalent to the molecular weights of the fragments. The ion signal is amplified by an electron multiplier and the mass spectrum is plotted from low to high mass. The m/z values are plotted against relative abundance of the ions. The most abundant ion (base peak) in the spectrum is assigned as 100%. In the study, Electron-Spray Mass Spectroscopy (ES-MS) spectra were conducted in methanol on Micromass QUATTRO Ultima (with the sample infused through syringe pump). Electron-Impact Mass Spectra (EI-MS) were conducted on a Micromass Autospec M Spectrometer.

4.8 Results of spectral data and identification of isolated compounds

4.8.1 Spectral data of Isolate PL1

A whitish powder; ^{1}H NMR (400 MHz, $CDCl_3$): δ_H 0.80 (s, H_3-20), 0.85 (d, *J*= 6.7 Hz, H_3-17), 1.00 (s, H_3-19), 1.62 (s, H_3-18), 5.19 (br s, H-3), 5.84 (s, H-14), 6.05 (s, H-16).

^{13}C NMR (600 MHz, $CDCl_3$): δ_C (ppm) 16.02 (C-17), 18.03 (C-18), 18.21 (C-1), 18.25 (C-20), 19.90 (C-19), 21.40 (C-12), 26.79 (C-2), 27.38 (C-7), 34.69 (C-11), 36.31 (C-8), 36.69 (C-6), 38.15 (C-5), 38.64 (C-9), 46.43 (C-10), 99.50 (C-16), 116.75 (C-14), 120.47 (C-3), 144.39 (C-4), 171.24 (C-13), 172.40 (C-15).

EIMS m/z (% relative intensity): 355 (5), 353 (10), 318 (M^+, 22), 317 (M^+-1, 100), 273 (10), 176 (75), 126 (40).

4.8.2 Spectra of PL1ac

Amorphous colourless solids; ^{1}H NMR (400 MHz, $CDCl_3$): δ = 0.80 (s, H_3-20), 0.85 (d, *J*= 6.7 Hz, H_3-17), 1.00 (s, H_3-19), 1.62 (s, H_3-18), 5.19 (br s, H-3), 5.84 (s, H-14), 6.77 (s, H-16)

^{13}C NMR (600 MHz, $CDCl_3$): δ_C (ppm) 16.02 (C-17), 18.03 (C-18), 18.21 (C-1), 18.25 (C-20), 19.90 (C-19), 21.40 (C-12), 26.79 (C-2), 27.38 (C-7), 34.69 (C-11), 36.31 (C-8), 36.69 (C-6), 38.15 (C-5), 38.64 (C-9), 46.43 (C-10), 93.82 (C-16), 116.75 (C-14), 120.47 (C-3), 144.39 (C-4), 171.24 (C-13), 172.40 (C-15).

4.8.3 Identification of PL1 and PL1ac (Compounds 1 and 1a)

PL1 exhibited four methyl [two tertiary (δ_H 0.80 and δ_H 1.00), a secondary (δ_H 0.85) and an olefinic (δ_H 1.62)] and three key methine (at δ_H 5.19, 5.84 and 6.05) signals in the ^{1}H NMR spectrum. Twenty carbon resonances attributable to five quaternaries, five methines (-CH), six methylenes ($-CH_2$) and four methyls ($-CH_3$) were observed in the ^{13}C NMR and DEPT spectra (DEPT 135 and 90) of PL1 (Table 4.2). The EI-MS exhibited a maximum peak at 317 (M^+-1, 100%) and a molecular ion (M^+) peak at m/z 318 (22%) corresponding to the molecular formula $C_{20}H_{30}O_3$. The index of hydrogen deficiency (IHD) was calculated from the molecular formula to be six, due to two olefinic functionalities as supported by two down-field pairs of carbon signals at δc 120.47 (C-3)/144.39 (C-4) and δc 171.24 (C-13)/116.75 (C-14) and a carbonyl signal at δc 172.40 (C-15). The remaining IHD is accounted for by a decalin and a five-membered lactone ring moieties. The above assignments are ascribable to a clerodane-type diterpene skeleton (Hara *et al*., 1995; Lee *et al*., 2009).

HSQC correlation studies assigned the key protons to the carbon-positions as recorded (Table 4.3). In the ^{1}H NMR spectrum, a downfield shift of the proton singlet signal at δ_H 6.05 (H-16) to δ_H 6.77 together with an up-field shift of a methine carbon signal at δc 99.50 (C-16) to δc 93.82 upon acetylation (PL1ac; **1a**), are indicative of the presence of a hydroxyl group at C-16 position.

Table 4.2 Comparison of ^{13}C NMR spectral data of PL1 and PL1ac

Position	PL1	DEPT PL1	PL1ac	Compound 1*
1	18.21	CH_2	18.21	18.3
2	26.79	CH_2	26.79	26.8
3	120.47	CH	120.47	120.3/120.4
4	144.39	C	144.42	144.3
5	38.15	C	38.15	38.1
6	36.69	CH_2	36.69	36.7
7	27.38	CH_2	27.38	27.4
8	36.31	CH	36.31	36.3
9	38.64	C	38.64	38.6
10	46.43	CH	46.43	46.5
11	34.69	CH_2	34.69	34.8
12	21.40	CH_2	21.40	21.3/21.4
13	171.24	C	171.24	171.0
14	116.75	CH	116.75	116.8
15	172.40	C	172.40	172
16	99.50	CH (OH)	93.82	99.3

17	16.02	CH_3	16.02	15.9
18	18.03	CH_3	18.03	17.9
19	19.90	CH_3	19.90	19.9
20	18.25	CH_3	18.25	18.1
-CO	-	-	168.16	-
$-CH_3$	-	-	20.65	-

* ^{13}C NMR spectral data reported by Hara *et al.*, (1995)

The above spectral data together with the observations are consistent and compared with those reported (Tables 4.2 and 4.3) and therefore identified PL1 and PL1ac as 16-hydroxycleroda-3,13-dien-16,15-olide (Figure 4.4; compound **1**) and acetyl-16-oxycleroda-3,13-dien-16,15-olide (Figure 4.5; compound **1a**), respectively (Hara *et al*., 1995; Phadnis *et al*., 1988).

Table 4.3 ^{1}H NMR and HSQC spectral data of PL1 and PL1ac

Position	**PL1**	**PL1ac**	**Compound 1***
3	5.19 (br s)	5.19 (br s)	5.18 (br s)
14	5.84 (s)	5.84 (s)	5.83 (s)
16	6.05 (s)	**6.77** (s)	6.03 (s)
17	0.85 (d, *J*=5.8)	0.85 (d, *J*=5.8)	0.82 (d, *J*=6.0)
18	1.62 (s)	1.62 (s)	1.6 (s)
19	1.00 (s)	1.00 (s)	1.01 (s)
20	0.80 (s)	0.80 (s)	0.78 (s)
$COCH_3$	-	1.11 (s)	-

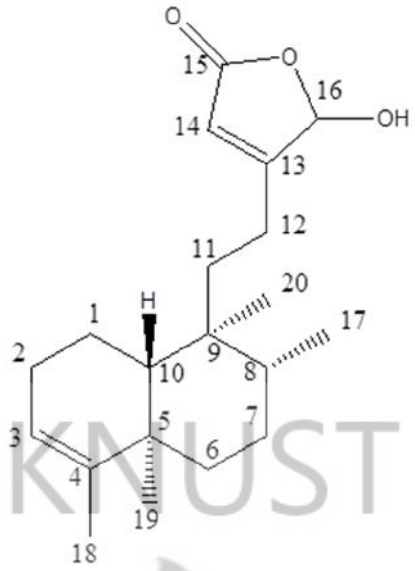


Figure 4.4 16-hydroxycleroda-3,13-dien-16,15-olide (Compound 1; PL1)

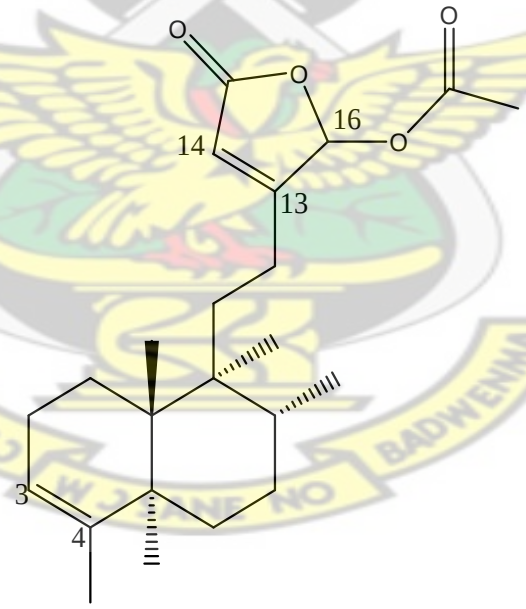


Figure 4.5 Acetyl-16-oxycleroda-3,13-dien-16,15-olide (Compound 1a; PL1ac)

4.8.4 Spectral Data of isolate PLe1

A semi-solid colourless compound: ^{1}H NMR (400 MHz, $CDCl_3$); δ_H = 0.70 (3H, s, C-20), 0.90 (3H, d J=6.6, C-17), 1.05 (3H, s, C-19), 1.60 (3H, s, C-18), 1.80 (2H, m, C-1), 5.23 (1H, br s, C-3), 6.48 (1H, br s, C-14), 9.57 (1H, s, C-16).

^{13}C NMR (400 MHz, $CDCl_3$); δ_C (ppm) 15.89 (C-17), 18.00 (C-20), 18.07 (C-18), 18.08 (C-1), 19.21 (C-12), 19.91 (C-19), 26.79 (C-2), 27.55 (C-7), 36.60 (C-8), 36.81 (C-6), 36.95 (C-11), 38.20 (C-5), 39.33 (C-9), 46.56 (C-10), 120.74 (C-3), 133.74 (C-14), 144.28 (C-4), 157.77 (C-13), 171.65 (C-15), 194.25 (C-16).

EIMS m/z (% relative intensity): m/z 318 (M^+, 20), 317 (M^+-1, 100), 273 (20), 257 (5).

4.8.5 Identification of PLe1 as 16-Oxocleroda-3,13E-dien-15-oic acid (Compound 2)

The EI-MS of PLe1 exhibited similar M^+ peak (m/z 318, 20%) with molecular formula $C_{20}H_{30}O_3$, and a maximum peaks at 317 (M^+-1, 100%) as observed with PL1. In the ^{1}H NMR spectrum, PLe1 showed four methyl (δ_H 0.90, 1.60, 1.05 and 0.70) and an olefinic proton singlet (δ_H 5.23) signals similar to those in the spectrum of PL1. HSQC correlation was used to assign the key protons to the carbon-positions as recorded (Table 4.5). The H-16 singlet at δ_H 6.05 in the spectrum of PL1 (compound 1) was replaced by a singlet at δ_H 9.57 in the ^{1}H NMR spectrum of PLe1. The H-14 singlet (of PL1) at δ_H 5.84 was also found shifted to δ_H 6.48 in the PLe1 spectrum.

Twenty carbon resonances with similar multiplicities and six IHD as in the ^{13}C NMR and DEPT spectra of PL1 were observed for PLe1 except at C-13, C-14 and C-16 where significant variations occurred in the carbon resonance positions: the olefinic carbons of the lactone ring of

PL1 [C-13 (δ_C 171.24)/C-14 (δ_C 116.75)] were shifted [C-13 (δ_C 157.77)/C-14 (δ_C 133.74)] in the spectrum of PLe1, indicating an opened lactone ring. The C-16 at δ_C 99.50 (PL1) also shifted to δ_C 194.25 in the spectrum of PLe1which showed that the hydroxyl group was replaced by an aldehyde. The spectra consistently compared with those reported by Hara et al. (1995) (Tables 4.4 and 4.5). PLe1 is therefore identified as 16-oxocleroda-3,13*E*-dien-15-oic acid (Figure 4.6).

Table 4.4 Comparison of ^{13}C NMR spectral data of PLe1 and PL1

Position	PLe1	DEPT PLe1	PL1	Compound 2*
1	18.08	CH_2	18.21	18.1
2	26.79	CH_2	26.79	26.8
3	120.74	CH	120.47	120.7
4	144.28	C	144.39	144.2
5	38.20	C	38.15	38.2
6	36.81	CH_2	36.69	36.8
7	27.55	CH_2	27.38	27.6
8	36.60	CH	36.31	36.3
9	39.33	C	38.64	39.3
10	46.56	CH	46.43	46.6
11	36.95	CH_2	34.69	37.0
12	19.21	CH_2	21.40	19.2
13	**157.77**	C	171.24	157.7
14	**133.74**	CH	116.75	133.8
15	171.65	C	172.40	170.9
16	**194.25**	CH (O)	99.50	194.2

17	15.89	CH_3	16.02	15.9
18	18.07	CH_3	18.03	18.0
19	19.91	CH_3	19.90	19.9
20	18.00	CH_3	18.25	18.0

* ^{13}C NMR spectral data reported by Hara *et al*. (1995)

Table 4.5 Comparison of ^{1}H NMR spectral data of PLe1 and PL1

Position	PLe1	PL1	Compound 2*
3	5.23 (br s)	5.19 (br s)	5.20 (br s)
14	**6.48** (br s)	5.84 (s)	6.48 (br s)
16	**9.57** (s)	6.05 (s)	9.53 (s)
17	0.90 (d, J=6.5)	0.85 (d, J=5.8)	0.84 (d, J=6.8)
18	1.60 (s)	1.62 (s)	1.59 (s)
19	1.05 (s)	1.00 (s)	0.99 (s)
20	0.70 (s)	0.80 (s)	0.68 (s)

* ^{1}H NMR spectral data reported by Hara *et al*. (1995)

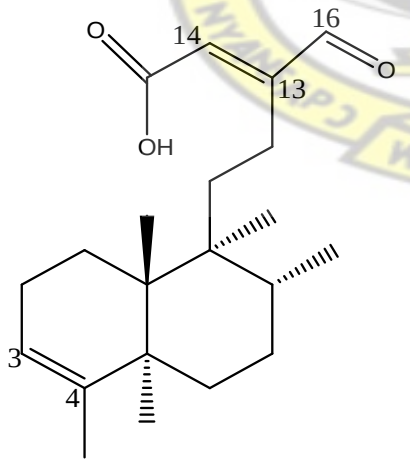


Figure 4.6 16-Oxocleroda-3,13*E*-dien-15-oic acid (Compound 2; PLe1)

4.8.6 Spectral Data of isolate De

A semi-solid colourless compound: ^{1}H NMR (400 MHz, $CDCl_3$); δ_H = 0.72 (3H, s, H-20), 0.75 d/0.77 d (3H, H-17), 0.99 (3H, s, H-19), 2.29 (2H, m, H-2), 4.25 (1H, dd, H-3), 4.67 and 4.85 (2H, s, H-18), 5.75 (1H, s, H-14), 5.91 (1H, d, H-16).

^{13}C NMR (400 MHz, $CDCl_3$); δ_C (ppm) 15.80 (C-17), 17.90 (C-20), 20.40 (C-1), 21.10 (C-11), 21.20 (C-19), 27.10 (C-7), 34.70 (C-12), 36.60 (C-8), 37.20 (C-2), 37.40 (C-6), 39.20 (C-5), 40.20 (C-9), 48.30 (C-10), 69.60 (C-3), 98.8/99.1 (C-16), 99.7 C-18), 117.10/117.10 (C-14), 161.20 (C-4), 170.10/170.20 (C-13), 171.40 (C-15).

EIMS m/z (% relative intensity): m/z 371 ($M^+ + 2H_2O + H^+$; 30), 357 ($M^+ + Na^+$; 80), 352 ($M^+ + H_2O$; 100), 199 (64).

4.8.7 Identification of De (Compound 3)

The EI-MS of De exhibited maximum molecular ion (M^+) peak at m/z 357 ($M^+ + Na^+$) and two other prominent peaks at 371 ($M^+ + 2H_2O + H^+$) and 352 ($M^+ + H_2O$). Unlike PL1 and PLe1, the molecular weight and the formula of De were determined to be 334 and $C_{20}H_{30}O_4$ respectively. Twenty carbon signals attributable to five quaternaries, five methines (-CH), seven methylenes ($–CH_2$) and three methyls ($–CH_3$) were observed in the ^{13}C NMR and DEPT spectra of De. The methyl substituent (C-18) at C-4 which is characteristic of a clerodane skeleton (observed in PL1: δ_H 1.62; δ_C 18.03) was replaced by an exo-methylene group (δ_H 4.67/ 4.85; δ_C 99.7).

Table 4.6 Comparison of ^{13}C NMR and DEPT spectral data of De with compounds 3 (a and b) and PL1

Position	δ_C De	DEPT De	δ_C PL1	Compound 3a*	Compound 3b*
1	20.4	CH_2	18.21	20.4	21.3/21.3
2	37.2	CH_2	26.79	37.0	26.8
3	**69.6**	CH(OH)	**120.47**	69.7	28.5/28.6
4	161.2	C	144.39	161.7	160.2/160.2
5	39.2	C	38.15	39.2	40.0
6	37.4	CH_2	36.69	37.3	37.2
7	27.1	CH_2	27.38	27.1	27.4
8	36.6	CH	36.31	36.6	36.7/36.7
9	40.2	C	38.64	40.2	39.2/39.2
10	48.3	CH	46.43	48.2	48.7
11	21.2	CH_2	34.69	21.2	34.6/34.7
12	34.7	CH_2	21.40	34.7	21.7/21.7
13	170.1/170.2	C	171.24	170.6	170.9/170.9
14	117.0/117.1	CH	116.75	116.9	116.7/116.8
15	171.4	CO	172.40	171.9	172.1
16	99.1/99.8	CH(OH)	99.50	99.3	99.3
17	15.8	CH_3	16.02	15.8	16.0
18	**99.7**	CH_2	**18.03**	99.7	102.7/102.8
19	21.2	CH_3	19.90	21.2	20.8/20.8
20	17.9	CH_3	18.25	17.9	18.0

*^{13}C NMR spectral data reported by Ma *et al*. (1994) (Compound 3a) and Hara *et al*. (1995) (compound 3b).

Table 4.7 Comparison of ^{1}H NMR spectral data of De and compounds 3a, 4 and PL1

Position	δ_H De	δ_H PL1	Compound 3a*	Compound 4**
3	**4.25** (dd)	5.19 (br s)	4.32 (dd)	
14	5.75 (s)	5.84 (s)	5.81 (s)	5.80 (s)
16	5.91 (d)	6.05 (s)	5.98 (s)	6.00 (m)
17	0.75 (d, J 5.6)	0.85 (d, J 5.8)	0.82 (d)	0.81 (d, J 4.0)
	0.77 (d, J 4.0)			0.80 (d, J 6.0)
18	**4.67 (**s); **4.85 (**s)	1.62 (s)	4.74 (s); 4.91 (s)	4.51 (s)
19	0.99 (s)	1.00 (s)	1.05 (s)	1.05 (s)
20	0.72 (s)	0.80 (s)	0.78 (s)	0.78 (s)

* Ma *et al*. (1994); **Hara *et al*. (1995)

From the HSQC spectral study of De (Appendix), the H signals at δ_H 5.91 related to two carbon signals at δ_C 99.1 and 99.8 which suggested a similarity between these two carbons. The NMR spectral data of De are quite close to those published (Table 4.6 and 4.7). However, two differences in the multiplicities of the proton signals were observed: The De spectrum showed a double-doublet for the methyl group at C-17 in contrast to a doublet report by Ma *et al*. (1994). This could be due to a mixture of epimers at C-16 (that is, a mixture of 16α- and 16β-hydroxy-isomers). Usually, the 16α-hydroxy group is closer in space to the C-17 methyl than the 16β-hydroxy group, resulting in a slight change in the δ-values for the C-17 methyl. In support of this theory is the data published by Hara *et al*. (1994): In their data of 16-hydroxycleroda-4(18),13-dien-16,15-olide, these authors noted that the proton spectrum showed two doublets for the C-17 methyl group. The double carbon signal for C-16 also appears to be due to the mixture of epimers. Also, in the spectrum of De, the H-16 showed as a doublet whereas Ma *et al*., 1994 report a

singlet. This could possibly again be due to a mixture of epimers. In light of all the above account, isolate De is identified as a mixture of 3β,16α-dihydroxycleroda-4 (18),13(14)Z-dien-15,16-olide (**3a**) with approximately an equal amount [since the two doublets in the ^{13}C NMR spectrum (in $CDCl_3$) are about equal size] of 3β,16β-dihydroxycleroda-4 (18),13 (14) Z-dien-15,16-olide (**3b**) (Figure 4.7; compound **3**).

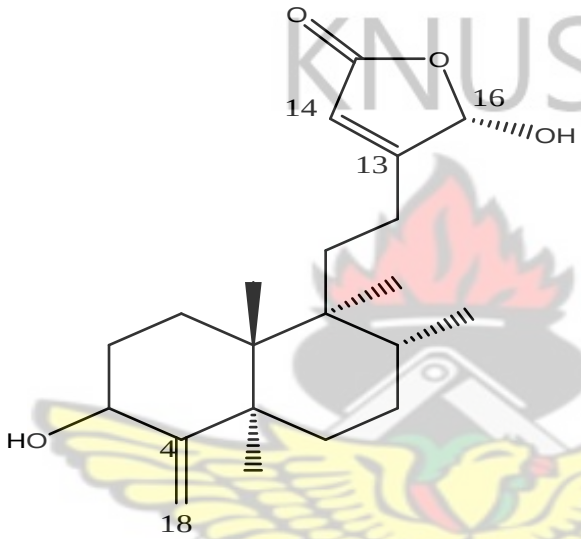


Figure 4.7 3,16-dihydroxycleroda-4(18),13(14) Z-dien-15,16-olide (Compound 3)

4.8.8 Spectral Data of isolate PLe3

Whitish flakes: ^1H NMR (400 MHz, $CDCl_3$); δ_H = 0.67 (3H, H-18), 0.70 (3H, H-27), 0.80 (3H, H-29), 0.82 (3H, H-26), 1.02 (3H, H-19), 1.22 (3H, H-21), 3.45 (H, H-3), 5.12 (H, H-23), 5.26 (H, H-22), 5.30 (H, H-6).

^{13}C NMR (400 MHz, CDCl3); δ_C (ppm) 12.0 (C-18), 12.3 (C-29), 19.0 (C-27), 19.4 (C-19), 21.0 (C-11), 21.1 (C-26), 21.3 (C-21), 24.3 (C-15), 25.4 (C-28), 28.2 (C-16), 31.7 (C-2), 31.9 (C-7), 31.9 (C-8), 32.6 (C-25), 36.5 (C-10), 37.2 (C-1), 39.8 (C-12), 40.5 (C-20), 42.2 (C-13), 42.3 (C-

20), 50.1 (C-9), 51.2 ((C-24), 56.0 (C-17), 56.9 (C-14), 71.8 (C-3), 121.7 (C-6), 129.2 (C-23), 138.4 (C-22), 140.7 (C-5).

EIMS m/z (% relative intensity): m/z 414 (M^+; 10), 397 (70), 383 (15), 255 (10).

4.8.9 Identification of PLe3 (compound 4)

PLe3 was obtained as white amorphous crystals on crystallisation in dichloromethane. Its melting point was determined to be 170-172^{o}C (uncorrected).

The EI-MS spectrum of PLe3 exhibited a molecular ion peak at m/z 414 $[M+2H]^+$ and a maximum peak at 397 $[M-H_3O]^+$. The molecular weight and formula of PLe3 were therefore respectively determined to be 412 and $C_{29}H_{50}O$. The ^{1}H and ^{13}C-NMR data of PLe3 compared with those reported for β-stigmasterol (Woldeyes *et al*., 2012; Table 4.8). The ^{1}H NMR spectrum signals at δ_H 0.67, 0.70, 0.80, 0.82, 1.02, and 1.22 are indicative of the presence of protons of six methyl groups. The signal at δ_H 3.45 indicated the presence of a carbon attached to a hydroxyl (-OH) group whereas those at δ_H 5.12, 5.26 and 5.30 showed the presence of olefinic protons. The ^{13}C-NMR spectrum showed 29 signals while DEPT-135 showed 26 signals which can be assigned to six methyl, nine methylene, eleven methine and three quaternary carbon atoms. In the ^{13}C NMR, the signal at δ_C 71.8 suggested that the β-hydroxyl group is attached to C-3. The signal at δ_C 140.7, 121.7 and 138.4, 129.2 could be assigned to C-5, C-6 and C-22, C-23 double bonds respectively. The observed melting point of PLe3 however compared with the reported values (ie 169-171^{o}C) (Woldeyes *et al*., 2012; Habib *et al*., 2007). The above observations suggested PLe3 to be identical to β-Stigmasterol (**4)** (Figure 4.8).

Table 4.8 Comparison of ^{13}C and ^{1}H NMR data of PLe3 with those reported (compound 4)

Position	δ_C PLe3	DEPT PLe3	δ_C Compound 4*	δ_H PLe3	δ_H Compound 4*
1	37.2	CH_2	37.2		
2	31.7	CH_2	31.6		
3	71.8	CH	71.8	3.45	3.55
4	42.2	CH_2	42.2		
5	140.7	C	140.7		
6	121.7	CH	121.7	5.30	5.38
7	31.9	CH_2	31.9		
8	31.9	CH	31.9		
9	50.1	CH	50.1		
10	36.5	C	36.5		
11	21.0	CH_2	21.0		
12	39.8	CH_2	39.7		
13	42.3	C	42.3		
14	56.9	CH	56.7		
15	24.3	CH_2	24.3		

16	28.2	CH_2	29.7		
17	56.0	CH	56.0		
18	12.0	CH_3	12.2	0.67	0.70
19	19.4	CH_3	19.4	1.02	1.03
20	40.5	CH	40.5		
21	21.3	CH_3	21.1	1.22	1.20
22	138.4	CH	138.3	5.26	5.36
23	129.2	CH	129.2	5.12	5.06
24	51.2	CH	51.2		
25	32.6	CH	39.7		
26	19.0	CH_3	19.0	0.82	0.83
27	21.1	CH_3	21.2	0.70	0.71
28	25.4	CH_2	25.4		
29	12.3	CH_3	12.0	0.80	0.82

*^{13}C and ^{1}H NMR spectral data of β-Stigmasterol reported by Woldeyes *et al*. (2012)

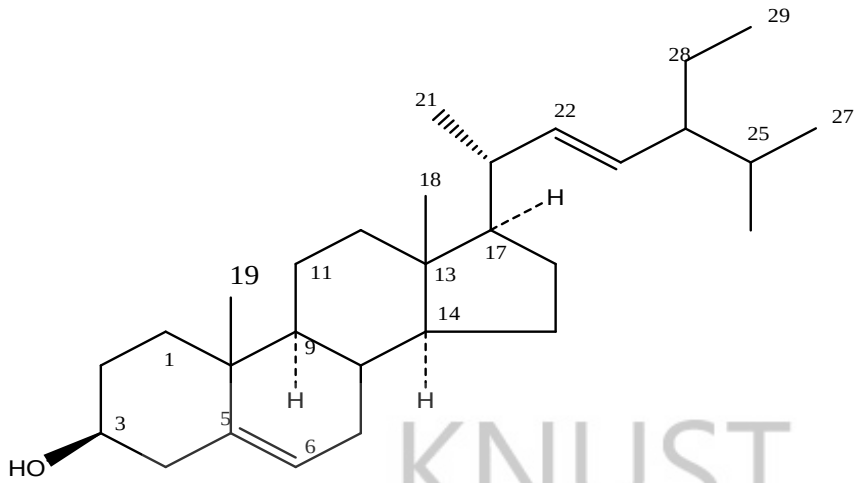


Figure 4.8 β-Stigmasterol (compound 4; PLe3)

4.8.10 Spectral data of isolate AL3c

A yellow powder: ^{1}H NMR (400 MHz, $CDCl_3$); δ_H = 2.58 (3H, *s*, CH_3), 4.06 (3H, *s*, OCH_3), 4.07 (3H, *s*, OCH_3), 6.05 (1H, *s*, OH-7), 6.84 (1H, *d*, H-2), 7.09 (1H, *s*, H-8), 8.39 (1H, *d*, H-3).

^{13}C NMR (400 MHz, $CDCl_3$); δ_C (ppm) 17.40 (CH_3), 61.70 (OCH_3), 61.90 (OCH_3), 107.30 (C-8), 124.50 (C-2), 126.00 (C-4b), 127.50 (C-8a), 131.70 (C-9a), 145.50 (C-6), 147.00 (C-7), 148.70 (C-5), 151.80 (C-1), 152.90 (C-3), 165.10 (C-4a), 192.30 (C-9).

EIMS m/z (% relative intensity): m/z 413 (5), 391 (5), 273 (18), 272 (M^++1, 100), 257 (10), 239 (12), 149 (14), 100 (12).

4.8.11 Identification of AL3c (compound 5)

AL3c reacted positively with Dragendorff's reagent for alkaloids. The EI-MS maximum molecular ion peak appeared at m/z 272 $[M+1]^+$. The molecular weight and formula of AL3c were respectively determined to be 271 and $C_{15}H_{13}NO_4$. The Index of Hydrogen Deficiency (or

double-bond equivalents) was also calculated to be ten. The spectroscopic data of AL3c are in comparison with those reported for compound 5 (Tables 4.9 and 4.10; Figure 4.9), an azafluorenone alkaloid (Arango *et al*., 1987; Mueller *et al*., 2009). The ^{1}H NMR spectrum of AL3c displayed seven [one methyl (δ_H 2.58, -CH_3 protons on C-1), two methoxy (δ_H 4.06, -OCH_3 on C-6; 4.07, -OCH_3 on C-5), three aromatic (δ_H 6.84 H-2; 7.09 H-8; 8.39 H-3) and one hydroxyl proton (δ_H 6.05 7-OH)] signals while the ^{13}C NMR and DEPT spectra showed the presence of 15 signals (nine quaternaries, three methines and three methyl). All direct proton-carbon connectivities were assigned through HSQC data analysis.

The HMBC data of AL3c shows δ_H 2.58 (methyl ^{1}H substituent on C-1) correlating with δ_C 131.7 (C-9a), δ_C 124.5 (C-2) and δ_C 145.5 (C-1) of the pyridine unit which suggested its *para* substitution on the pyridine ring. A confirmation of the above was observed in the HMBC correlation of δ_H 6.84 (H-2) with δ_C 152.9 (C-3; weak), δ_C 131.7 (C-9a; strong) and δ_C 17.4 (methyl ^{1}H substituent on C-1), and the downfield aromatic proton's (δ_H 8.39 H-3) correlation with δ_C 165.1 (C-4a; strong), δ_C 145.5 (C-1; strong) and δ_C 124.5 (C-2; weak).

In the HMBC spectrum also, the aromatic signal at δ_H 7.09 (H-8) correlates with four carbons: δ_C 127.5 (C-4b), δ_C 151.8 (C-6), δ_C 192.3 (C-9; carbonyl) and δ_C 147.0 (C-7). The hydroxyl proton at δ_H 6.05 was found correlating with δ_C 107.3 (C-8) and δ_C 151.8 (C-6) which suggested its substitution on C-7. The above analysis supports the deductions reported (Arango *et al*., 1987) except position C-1 and C-6 as well as C-8a and C-9a which were interchanged. AL3c is therefore identified as Darienine (7-Hydroxy-8,9-dimethoxy-4-methyl-5H-indeno(1,2-b)pyridin-5-one (Figure 4.9; **5**).

Table 4.9 ^{13}C NMR, DEPT and HMBS spectral data of AL3c and compound 5

Position	δ_C AL3c	DEPT 135/90 of AL3c	δ_C Compound 5*
1	145.5	C	151.8
2	124.5	CH	124.3
3	152.9	CH	152.7
4a	165.1	C	164.9
4b	127.5	C	125.9
5	148.7	C	148.6
6	151.8	C	145.4
7	147.0	C	146.8
8	107.3	CH	107.2
8a	131.7	C	127.3
9	192.3	C	192.1
9a	126.0	C	131.6
1-CH_3	17.4	CH_3	17.2
5-OCH_3	61.7	CH_3	61.8
6-OCH_3	61.9	CH_3	61.5

*^{13}C NMR spectral data of compound **5** reported by Arango *et al*. (1987).

Table 4.10 ^{1}H NMR, HSQC and HMBC spectral data of AL3c and compound 5

Position	δ_H AL3c	HMBC	δ_H Compound 5*
2	6.84 (d)	3, 9a, 1-CH_3	6.85 (d 5.4)
3	8.39 (d)	1, 2, 4a	8.40 (d)
8	7.09 (s)	4b, 7, 6, 9	7.11 (s)
7-OH	6.05 (s)	6, 8	-
1-Me	2.58 (s)	1, 2, 9a	2.59 (s)
5-OMe	4.07 (s)	5	4.07 (s)
6-OMe	4.06 (s)	6	4.07 (s)

*^{1}H NMR spectral data reported by Arango *et al*. (1987)

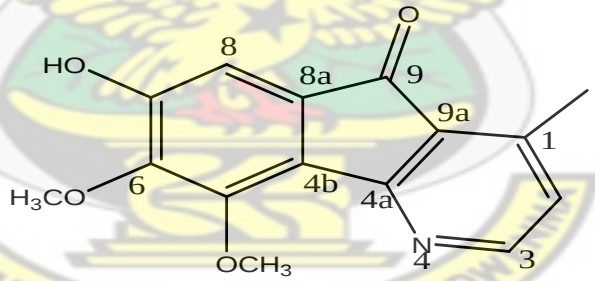


Figure 4.9 Darienine (7-Hydroxy-8,9-dimethoxy-4-methyl-5H-indeno(1,2-b)pyridin-5-one; compound 5)

4.8.12 Spectral Data of (AL6)

Brownish cuboidal crystals: ^{1}H NMR (400 MHz, CD_3OH); δ = 2.59 (1H, ddd, H-6), 2.67 (2H, m, H-5/13), 3.04 (1H, m, H-),3.15 (1H, t, H-6), 3.47 (2H, d, H-8/13a), 3.77 (3H, s, OCH_3), 3.79 (3H, s, OCH_3), 4.15 (1H, d *J*=, H-8), 6.64 (1H, s, H-4), 6.70 (1H, d *J*=, H-11), 6.72 (1H, s, H-1), 6.76 (1H, d *J*=, H-12).

^{13}C NMR (400 MHz, CD_3OH); δ_C (ppm) 28.00 (C-5), 35.20 (C-13), 51.50 (C-6), 53.60 (C-8), 55.00 (OCH_3), 59.10 (OCH_3), 59.50 (C-13a), 111.20 (C-4), 111.80 (C-1), 115.10 (C-11), 124.10 (C-12), 124.90 (C-4a), 125.90 (C-12a), 127.50 (C-8a), 129.50 (C-13b), 143.60 (C-9), 144.70 (C-2), 146.50 (C-3), 147.50 (C-10).

EIMS m/z (% relative intensity): m/z 329 (24), 328 (M^++1, 100), 60 (5).

4.8.13 Identification of AL6 as L-Stepholidine (compound 6)

AL6 reacted positively with Dragendorff's reagent for alkaloids. The EI-MS showed maximum molecular ion peak at m/z 328, (M^+ +1) which corresponded to a molecular weight and formula of 327 and $C_{19}H_{21}O_4N$, respectively. The ^{13}C NMR and DEPT spectra showed a total of 19 carbon signals with hydrogenation patterns (8 non-hydrogenated, four methylene, five methine and two methoxy; Table 4.11) depicting the carbon skeleton of a protoberberine-type of alkaloid.

The two methoxyl and two hydroxyl substituents appear to be on the aromatic rings of the tetrahydroprotoberberine alkaloid structure. Signals for four aromatic protons, two singlets and two doublets in the ^{1}H-NMR spectrum indicates that in one aromatic ring there are two protons *para* to each other while the other has two *ortho* proton substituents. The remaining positions on

the aromatic rings are occupied by the methoxyl and hydroxyl groups. The substitution patterns were determined as follow:

The non-aromatic protons. There are 3 proton spin systems in the molecule: $-CH_2-$ at position 8, $-CH_2-CH_2-$ at positions 5 and 6, and $-CH_2-CH-$ at positions 13 and 13a. From the ^{1}H-NMR spectrum, the signal for the most downfield non-aromatic proton appears at δ_H 4.15 and it is compatible with the equatorial hydrogen at position 8 (H-8eq) based on the data from Hussain *et al*. (1989). The HSQC spectrum shows that H-8eq is attached to C δ_C 53.6 and this carbon also correlates with the ^{1}H-NMR signal at δ_H 3.47 which therefore represents the corresponding axial hydrogen (H-8ax). Further confirmation of this is seen in the ^{1}H-^{1}H COSY spectrum where a strong coupling is seen between H-8ax and H-8eq; in addition, the ^{1}H-NMR shows that the above signals have coupling constants close to 15.5, typical of geminal coupled protons.

The signal at δ_H 3.47 integrates for two protons and the HMQC shows that the second proton is attached to the C δ_C 59.5 and the DEPT spectrum shows that this carbon is a -CH. Therefore C δ_C 59.5 must be the carbon at 13a. H-13a shows a weak COSY correlation to the proton at δ_H 2.68 and the latter shows a strong correlation with the proton at δ_H 3.28 thus suggesting that the above protons are located at C-13. This is confirmed by the HSQC which shows both of the above protons correlating with C δ_C 35.2.

The remaining four non-aromatic protons must therefore be attached to C-5 and C-6 and the HMQC clearly shows that the protons at δ_H 2.6 and 3.17 are at C δ_C 28 while those at δ_H 2.68 and 3.05 are attached to C δ_C 51.5. Carbon δ_C 51.5 must be C-6 (adjacent to N) and therefore C δ_C 28 is C-5.

The aromatic protons. The proton singlet at δ_H 6.64 correlates with carbon δ_C 111.2 in the HSQC spectrum and in the HMBC spectrum shows the following C correlations: δ_C 28 (strong), 124.1

(weak), 129.5 (strong), 144.7 (strong) and 146.5 (less strong). All of these carbons are quaternary except the one at δ_C 124.1 (Table 4.11). The strong correlation at δ_C 28 suggests a ^3J correlation between H-4 and C-5 and therefore the two aromatic protons giving singlets must be at H-4 and H-1.

Table 4.11 ^{13}C, DEPT, HMQC and HMBC spectral data of AL6, compound 6.

Position	δ_C AL6	Compound 6*	DEPT-135/90 of AL6	HMQC of AL6	HMBC of AL6
1	111.8	110.5	CH	6.72	
2	144.7	145.0	C		6.64/6.72
3	146.5	143.8	C		6.64/6.72
4	111.2	111.4	CH	6.64	
4a	124.9	130.5	C		6.72
5	28.0	29.1	CH_2	2.60/3.17	
6	51.5	51.5	CH_2	2.68/3.05	
8	53.6	53.5	CH_2	3.47/4.15	
8a	127.5	128.1	C		6.76
9	143.6	141.4	C		6.70
10	147.5	143.9	C		6.67/6.70
11	115.1	108.9	CH	6.70	

12	124.1	119.3	CH	6.76	
12a	125.9	121.1	C		6.70
13	35.2	36.2	CH_2	2.68/3.28	
13a	59.5	59.1	CH	3.47	
13b	129.5	126.0	C		6.64
3-MeO	55.0	56.1	CH_3	3.79	
9-MeO	59.1	55.0	CH_3	3.77	

*^{13}C NMR spectral data of compound 6 reported by Tavares *et al*. (2005).

In a similar analysis of the proton singlet at δ_H 6.72, C-1 must be carbon δ_C 111.8 with the following HMBC correlations seen: δ_C 59.5 (strong), 124.9 (strong), 144.7 (less strong) and 146.5 (strong). The carbon at δ_C 59.5 has already been identified as C-13a which further confirmed that the proton signal at δ_H 6.72 is H-1.

Carbons at ring junctions have smaller δ_C values than the others (Hussain *et al*., 1989). Hence δ_C 124.9 may be assigned to C-4a and δ_C 129.5 to C-13b. Carbons δ_C 144.7 and 146.5 are thus assigned to C-2 and C-3 respectively but because HMBC correlations are seen for both H-1 and H-4 it is not possible to be sure which way round they are. The observation that the HMBC between H-4 and 146.5 is less strong than the correlation with 144.72 suggests that the one with 146.5 may be a 3-bond correlation while that with 144.7 may be a 2-bond correlation. A similar argument may be made using the correlations seen with H-1.

The methoxyl methyl groups correlate with C δC 146.5 and 143.6. This shows that one methoxyl group is attached to either C-2 or C-3 – if the above argument is valid then it is likely to be attached to C3, so that a hydroxyl group will then be attached to C2. A NOESY correlation between a methoxy methyl protons and H-4 confirms the presence of the methoxy group on C-3 and hence the hydroxyl group must necessarily be positioned on C-2.

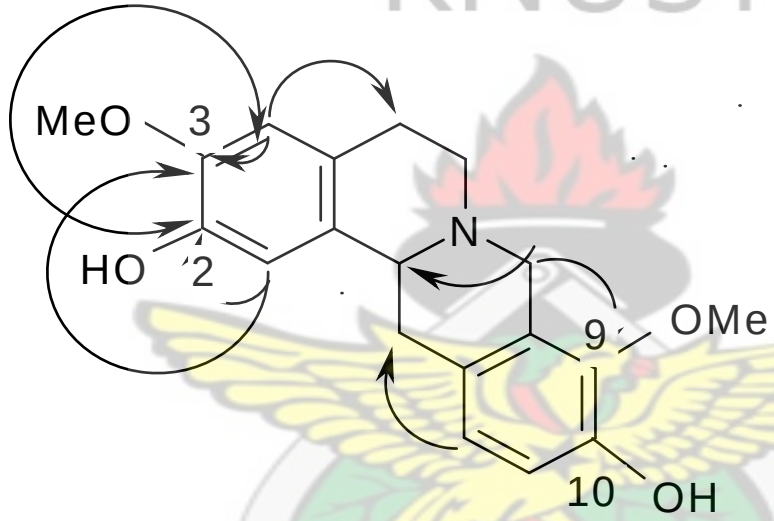


Figure 4.10 L-Stepholidine (AL6; compound 6) showing some important ^{1}H to ^{13}C NOESY correlations.

With regard to the aromatic proton doublet signals, the signal at δ_H 6.76 correlates with δ_C 124.1 in the HMQC and shows strong HMBC correlations to δ_C 35.2, 127.5 and 147.5. The strong correlation with δ_C 35.2 clearly indicates a correlation with C-13 and suggests that this proton (δ_H 6.76) is attached to C-12; thus the other doublet at δ_H 6.7 indicates a proton at C-11. This doublet at δ_H 6.7 (H-11) shows HMBC correlations to δ_C 125.9, 143.6 and 147.5.

The second methoxyl group also shows an HMBC correlation to 143.6 which is C-9. This is confirmed by the HMBC correlations with the proton at δ_H 4.15 (H-8eq); which are δ_C 59.5 (C-13a), 125.9 (C-12a), 127.6 (C-8a) and 143.6 (C-9). The above deductions are in agreement with those reported of L-Stepholidine (Figure 4.10; compound 6) by Tavares *et al*. (2005).

4.9 Conclusion

The bioassay-guided isolation of extracts of *P. longifolia* stem bark yielded six compound which were characterised and identified as 16-hydroxycleroda-3,13-dien-15,16-olide (**1**); 16-oxocleroda-3,13E-dien-15-oic acid (**2**); 3,16-dihydroxycleroda-4(18),13(14)Z-dien-15,16-olide (**3**); β-Stigmasterol (**4**); Darienine (**5**) and L-Stepholidine (**6**). Acetyl-16-oxycleroda-3,13-dien-15,16-olide (**1a**) was also obtained through derivatization of 16-hydroxycleroda-3,13-dien-15,16-olide.

Chapter Five

5 BIOLOGICAL ACTIVITY OF *P. LONGIFOLIA* ISOLATES

The extracts of *Polyalthia longifolia* stem bark displayed significant antiplasmodial activity in the preliminary studies (Section 3.3.10), on which basis the plant was selected for phytochemical studies (section 4.1). The six compounds successfully obtained from the selected active fractions (ethyl acetate and alkaloidal) and the acetylated product were assayed for antiplasmodial activity to ascertain if they were responsible or contributed to the antiplasmodial activity of *P. longifolia* stem back. The isolated compounds were, in addition to the antiplasmodial assay, evaluated for antimicrobial property as some plant-derived antimalarial drugs (for example artemisinins) have as well displayed antibacterial properties (Efferth *et al*., 2008; Srivastava *et al*., 2009; Li *et al.*, 2011).

5.1 Antiplasmodial testing

5.1.1 Materials and methods

The six compounds [16-hydroxycleroda-3,13-dien-16, 15-olide (**1;** PL1) and its acetylated derivative (**1a;** PL1ac); 16-oxocleroda-3,13E-dien-15-oic acid (**2;** PLe1); 3,16-dihydroxycleroda-4(18),13(14) Z-dien-15,16-olide (**3;** De); β-Stigmasterol (**4;** PLe3); Darienine (**5;** AL3c) and L-Stepholidine (**6;** AL6)] were evaluated for antiplasmodial activity by the parasite lactate dehydrogenase (pLDH) method described my Makler and friends (Section 3.3.9) (Markler *et al*., 1993). The *in vitro* cultivation of the malaria parasite (K1 strain of *Plasmodium falciparum*) used in the assay was based on the method described by Jensen and Trager (Section

3.3.5). RPMI 1640 medium supplimented with human serum and red blood cells (Section 3.3.3 and 3.3.4) was employed as the growth medium while chloroquine diphosphate and artemether served as the reference antimalarial drugs.

5.1.2 Drug combination assay

Stock solutions (1 mg/ml) of the compounds and reference drugs were prepared. The Chloroquine H_2PO_4 solution was prepared with water while the isolated compounds were prepared using 1% dimethyl sulfoxide in water, which on dilution had no effect on the parasite growth at final concentrations usually less than 0.05 % v/v. The dilutions of chloroquine in combinations with each of the isolated compounds (or reference chemosensitizers: promethazine and verapamil) were prepared in fixed ratios as described by Fivelman *et al*., (2004) with slight modifications. Six drug combination solutions of each isolated compound were prepared as shown for example in Table 5.1 for Chloroquine and PL1. Combination solutions one to six were prepared at 0:100, 2:80, 4:60, 6:40, 8:20 and 10:0 concentration ratios in micrograms per milliliter, respectively (the first solution being PL1 alone and last solution also being chloroquine diphosphate alone). In the assay, the parasitized red blood cells were added to the various combination solutions which were serially diluted with complete RPMI 1640 medium in flat-bottom 96-well microtiter plates (Section 3.3.9).

The antiplasmodial activity of the combination solutions against the multidrug resistant K1 strain of *P. falciparum* was then evaluated by the pLDH method. The fractional IC_{50}s (FIC) of chloroquine and the agent in the combination were then determined (Table 4.3). Isobolograms (Figure 4.1) were drawn from the recorded FICs to picturise the effect of isolated compounds on the antiplasmodial activity of chloroquine in the various fixed-ratio combinations.

Table 5.1 Fixed ratio combination solutions of chloroquine and isolated compounds

Combination solutions	Ratio of Chloroquine to PL1		Chloroquine (10 µg/ml)	PL1 (100 µg/ml)
	Chloroquine	PL1		
1	0	5	0 ml	10.0 ml
2	1	4	2.0 ml	8.0 ml
3	2	3	4.0 ml	6.0 ml
4	3	2	6.0 ml	4.0 ml
5	4	1	8.0 ml	2.0 ml
6	5	0	10.0 ml	0 ml

5.1.3 Results of antiplasmodial activity of isolated compounds

After growing the malaria parasites in the presence of graded concentrations of the isolated compounds, growth inhibition was evaluated by the pLDH method. Duplicates of three independent experiments were conducted in each case and by means of Microsoft Excel software (Microsoft Inc.) dose-response curves were drown and used to obtain the IC_{50} values (Table 5.2).

5.1.4 Results of antiplasmodial activity of chloroquine-isolate combination assays

In this assay, the IC_{50} values of the second to fifth combination solutions (Table 5.1) were determined as fractions (FIC_{50}) of the two agents (chloroquine and compound/reference chemosensitizer) in the combination (Table 5.3). The first and the last solutions were the agents alone and hence were not different from the IC_{50} values recorded for the respective agents in Table 5.2. The isobolic curves constructed for the various combination solutions depicted the

interactions of chloroquine with the compounds or reference chemosensitizers (Figure 5.1). The IC_{50}s of Chloroquine alone, isolated compounds or reference drugs alone were normalized to one unit. In each combination, IC_{50} was plotted as fraction of agent alone IC_{50} values (FIC). Concave and convex isobolic curves respectively, reveal synergistic and antagonistic effects of chloroquine and isolated compounds or reference drugs. The straight line is the additive effect. Results represent the means of three independent experiments.

Table 5.2 Antiplasmodial activity of isolated compounds against multi-drug resistant K1 strain of *P.falciparum*

Codes	Compound/Drugs	IC_{50} ± std	
		(µg/ml)	µM
PL1	16-hydroxycleroda-3,13-dien-16,15-olide	5.33 ± 0.70	16.76±2.20
PL1ac	Acetyl-16-oxycleroda-3,13-dien-16,15-olide	5.92 ± 0.56	16.44±1.56
PLe1	16-oxocleroda-3,13E-dien-15-oic acid	3.05 ± 0.19	9.59±0.60
De	3,16-dihydroxycleroda-4(18),13(14)Z-dien-15,16-olide	6.15 ± 0.65	18.41±1.95
PLe3	β-Stigmasterol	63.36 ± 9.39	153.79±22.79
Al3c	Darienine	22.05 ± 1.47	81.37±5.42
AL6	L-Stepholidine	104.33 ± 8.95	319.05±27.37
	Chlroroquine diphosphate	0.4365 ± 0.016	0.8462±0.0310
	Artemether	0.0186 ± 0.017	0.0623±0.0570

Table 5.3 Antiplasmodial activity of chloroquine-Isolate combinations

Combination Ratio	Chloroquine H_2PO_4 + PL1(µg/ml)		Chloroquine H_2PO_4 + PL1a (µg/ml)	
	FIC_{50}-CQ	FIC_{50}-PL1	FIC_{50}-CQ	FIC_{50}-PL1a
0:5	0.0	5.327 ± 0.700	0.0	5.918 ± 0.557
1:4	0.2263 ± 0.019	9.023 ± 0.766	0.3295 ± 0.091	4.776 ± 0.488
2:3	0.4079 ± 0.035	6.140 ± 0.562	0.2776 ± 0.045	4.165 ± 0.912
3:2	0.4645 ± 0.031	3.097 ± 0.205	0.2993 ± 0.009	1.995 ± 0.274
4:1	0.4329 ± 0.047	1.082 ± 0.092	0.2704 ± 0.037	0.676 ± 0.087
5:0	0.4365 ± 0.016	0.0	0.4365 ± 0.016	0.0
Combination Ratio	Chloroquine H_2PO_4 + PLe1		Chloroquine H_2PO_4 + De	
	FIC_{50}-CQ	FIC_{50}-PLe1	FIC_{50}-CQ	FIC_{50}-De
0:5	0.0	3.750 ± 0.431	0.0	6.146 ± 0.653
1:4	0.1007 ± 0.037	2.570 ± 0.294	0.1985 ± 0.044	6.010 ± 0.271
2:3	0.2405 ± 0.051	2.881 ± 0.098	0.3498 ± 0.071	5.011 ± 0.422
3:2	0.4000 ± 0.022	2.692 ± 0.425	0.3171 ± 0.034	3.984 ± 0.284
4:1	0.3310 ± 0.024	0.857 ± 0.693	0.2965 ± 0.051	1.341 ± 0.475
5:0	0.4365 ± 0.016	0.0	0.4365 ± 0.016	0.0
Combination Ratio	Chloroquine H_2PO_4 + PLe3		Chloroquine H_2PO_4 + AL3c	
	FIC_{50}-CQ	FIC_{50}-PLe3	FIC_{50}-CQ	FIC_{50}-AL3c
0:5	0.0	63.362 ± 9.386	0.0	22.054 ± 1.474
1:4	0.2512 ± 0.035	25.116 ± 3.771	0.3295 ± 0.024	19.950 ± 2.007
2:3	0.2667 ± 0.071	10.081 ± 0.819	0.2774 ± 0.041	10.957 ± 0.851
3:2	0.3006 ± 0.019	5.009 ± 0.549	0.2993 ± 0.072	5.616 ± 0.497

4:1	0.2848 ± 0.028	1.776 ± 1.097	0.2704 ± 0.009	1.817 ± 0.231
5:0	0.4365 ± 0.016	0.0	0.4365 ± 0.016	0.0
Combination Ratio	Chloroquine H_2PO_4 + AL6		Chloroquine H_2PO_4 + Promethazine	
	FIC_{50}-CQ	FIC_{50}-AL6	FIC_{50}-CQ	FIC_{50}-Pr
0:5	0.0	104.330 ±8.951	0.0	18.174 ± 5.119
1:4	0.2234 ± 0.021	44.267 ± 5.117	0.0661 ± 0.017	2.628 ± 0.383
2:3	0.2371 ± 0.075	17.776 ± 1.706	0.1147 ± 0.022	1.738 ± 0.445
3:2	0.3000 ± 0.046	10.001 ± 0.772	0.1173 ± 0.071	0.794 ± 0.098
4:1	0.2648 ± 0.055	3.305 ± 0.294	0.1906 ± 0.026	0.479 ± 0.154
5:0	0.4365 ± 0.016	0.0	0.4365 ± 0.016	0.0
Combination Ratio	Chloroquine H_2PO_4 + Verapamil			
	FIC_{50}-CQ	FIC_{50}-Ver		
0:5	0.0	30.156 ± 6.052		
1:4	0.0179 ± 0.018	5.754 ± 0.877		
2:3	0.0832 ± 0.021	2.566 ± 0.048		
3:2	0.1245 ± 0.054	1.657 ± 0.102		
4:1	0.1445 ± 0.008	0.724 ± 0.411		
5:0	0.4365 ± 0.016	0.0		

Key: FIC_{50} = Fractional IC_{50}; CQ = Chloroquine H_2PO_4; Pr = Promethazine HCl; Ver = Verapamil

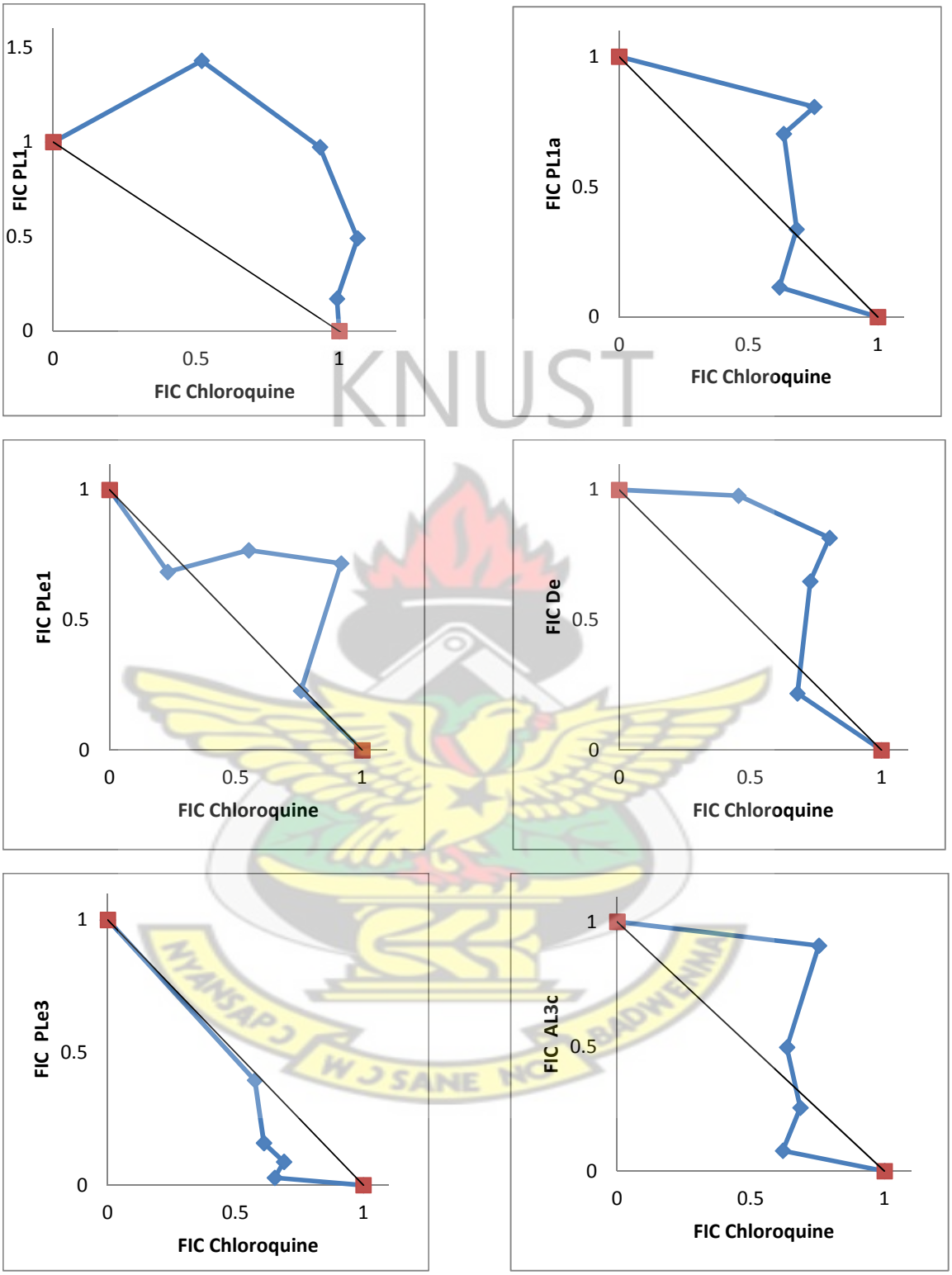
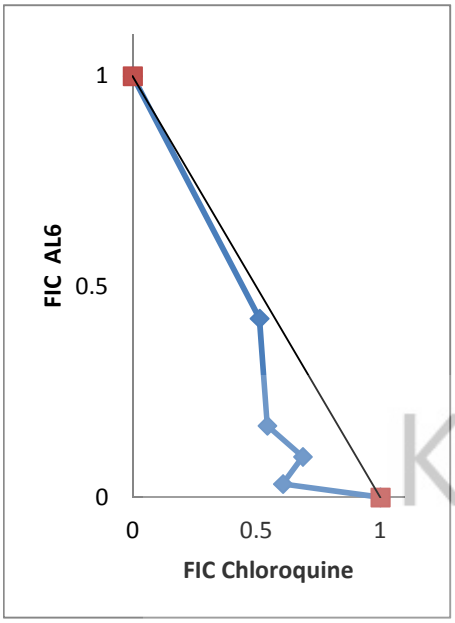
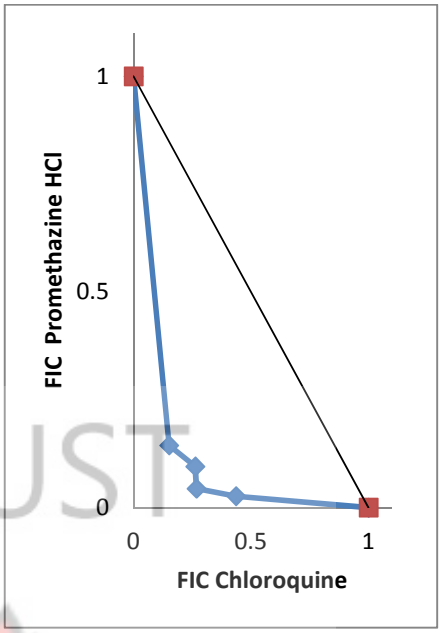


Figure 5.1 Isobolograms of Fractional Inhibitory Concentrations of combination assays.





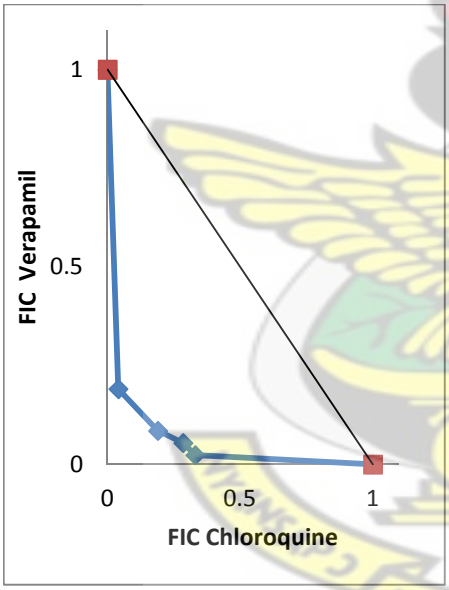


Figure 5.1 continued

5.1.5 Discussion

Plants have played a dominant role in the discovery of antimalarials. Most of the existing antimalarial chemotherapeutic agents are based on phytochemical compounds of plants. The anticipation that new leads for effective antimalarials against the fast spreading drug-resistant *P. falciparum* strains will emerge from plant sources cannot be overemphasized. Within a period of just 23 months (January 2009 to November 2010) a total of 360 phytochemical compounds effective against various strains of the drug-resistant malaria parasite were published (Nogueira & Lopes, 2011). In this present study the phytochemical compounds obtained from *P. longifolia* stem bark [16-hydroxycleroda-3,13(14)-dien-16,15-olide (**1**) and its acetylated derivative (**1a**); 16-oxocleroda-3,13(14)E-dien-15-oic acid (**2**); 3,16-dihydroxycleroda-4(18),13(14) Z-dien-15,16-olide (**3**); β-Stigmasterol (**4**); Darienine (**5**) and L-Stepholidine (**6**)] showed varying degrees of antiplasmodial activity against the blood schizonts of K1 strain of *P. falciparum* in the pLDH assay.

In accordance with the criteria adopted by Basco *et al*., (1994) and Batista *et al*., (2009) for considering the *in vitro* antiplasmodial activity of compounds [IC_{50} 1-10 µg/ml (or 1-20 µM), good activity; IC_{50} of 10-50 µg/ml (or 20-100 µM), moderate activity; IC_{50} of 50-100 µg/ml (100-200 µM), weak/low activity; IC_{50} >100 µg/ml (>200 µM), inactive], L-Stepholidine (**6**), a tetrahydroxyprotoberberine alkaloid, exhibited no appreciable antiplasmodial activity (IC_{50} >100 µg/ml). Contrastingly, potent activity had been reported among the protoberberine alkaloids: Palmatine, jatrorrhizine and berberine (and its derivatives including berberinephenolbetaine dihydroberberine, tetrahydroberberine, oxyberberine and 8-cyanodihydroberberine) have been shown to exhibit antiplasmodial activity comparable to that of quinine (Elford, 1986; Vennerstrom & Klayman, 1988). It has also been reported that the activity of the protoberberine alkaloids is influenced by the type and position of the oxygen substituents and or oxygen functional groups present: methylenedioxy group (-OCH_2O-) at

C-2 and C-3 or C-9 and C-10 and oxygen functional group on C-10 and C-11 of the protoberberine skeletal structure increase their antiplasmodial activity (Iwasa *et al*., 1999). The absence of methylenedioxy and the positioning of $–OCH_3$ and –OH on C-9 and C-10, respectively (instead of C-10 and C-11; Figure 4.10) could therefore possibly explain the inactivity of L-Stepholidine.

Compound **5 (**Darienine, 7-hydroxy-5,6-dimethoxyonyichine), an azaflourene alkaloid however displayed moderate antiplasmodial activity (IC_{50} <50 µg/ml). In a report by Mueller *et al*. (2009), among two related analogs of onychine; 5-hydroxy-6-methoxyonychine and 5,8-dihydroxy-6-methoxyonychine isolated from the dichloromethane-methanol extract of *Mitrephora diversifolia* root, the former exhibited good antiplasmodial activity (IC_{50} 9.9 and 11.4 µM against 3D7 and Dd2 strains of *P. falciparum*, respectively) while the activity of the latter was very weak. These reports together with the results of this current study support the fact that structural differences among the onyichine derivatives affect the level of their antiplasmodial activity. Structural modification studies could therefore, be useful in improving the antimalarial effectiveness of this isolated compound (Darienine, **5**).

Compound **4** (β-Stigmasterol) exhibited a weaker antiplasmodial activity (IC_{50} >50 µg/ml) than darienine (**5**). Generally steroids have not been known to have good antiplasmodial activity. From the reports of Abrantes *et al*. (2008), the dichloromethane extract of *Pycnanthus angolense* (Myristicaceae) stem bark displayed a very potent antiplasmodial activity (IC_{50} 1.60 µg/ml) but when it was fractionated into the various phytoconstituents comprising of steroids (stigmast-4-en-6β-ol-3-one, β-sitosterol and stigmasterol), talaumidin, pycnantolol, hinokinin and the labdane type diterpene ozic acid and evaluated, all the constituents showed weak activity except talaumidin which exhibited moderate activity (IC_{50} 20.70 µg/ml) against the chloroquine-resistant (Dd2) strain of *P. falciparum*. The low IC_{50}

value recorded for the *P. angolense* extract might probably have been a result of potentiation effect of the steroids and or the other weaker constituents on talaumidin.

The clerodane diterpenes (compounds **1, 1a, 2** and **3**) obtained in this study exhibited good antiplasmodial activity (IC_{50} 3.05 - 6.15 µg/ml) (Table 5.2). These diterpenes have earlier been isolated from other species of *Polyalthia* and identified with anticancer, antimicrobial, antileishmanial, anti-inflammatory, antidyslipidemic and antioxidant properties (Ma *et al*., 1994; Hagiwara *et al*., 1995; Rashid *et al*., 1996; Sashidhara *et al.*, 2009; Katkar *et al*., 2010). An antiplasmodial activity (IC_{50} of 3.60 µg/ml) has previously been reported of 16-hydroxycleroda-3,13(14)-dien-16,15-olide which was isolated from the ethanol extract of *P. viridis* leaves (Ichino *et al*., 2006). The results of this present study not only confirmed the antiplasmodial activity of compound **1** but also favourably compared with the reported IC_{50} value of this compound. The antiplasmodial activity of **1a, 2,** and **3** are being reported for the first time and compound **2** (16-oxocleroda-3,13(14)E-dien-15-oic acid; PLe1) is however the most potent (IC_{50} 3.05 µg/ml) of all the compounds isolated.

Many classes of terpenes have been reported exhibiting antiplasmodial and antimalarial activities. The clerodane diterpenes gomphostenin and gomphostenin-A isolated from *Gomphostemma niveum* (Lamiaceae) exhibited levels of activity (IC_{50} 114.90 and 9.80 µM, respectively) against MRC-02 strain of *P. falciparum* (Sathe *et al.,* 2010). The kaurane diterpene ent-kaur-16-en-19-oic acid, isolated from *Schefflera umbellifera* (Araliaceae) was also found to exhibit some antiplasmodial activity (IC_{50} 106.50 µM) against chloroquine-susceptible strain D10 of *P. falciparum* (Mthembu *et al*., 2010). Two diterpenes, (+)-8,11,13-totaratriene-12,13-diol and (+)-8,11,13-abietatrien-12-ol, isolated from *Harpagophytum procumbens* (devil's claw) displayed excellent (IC_{50} < 1 µg/ml) antiplasmodial activity against a K1 and D10 (chloroquine-sensitive) strains of *P. falciparum* (Clarkson *et al*., 2003). However, the development of the artemisinin sesquiterpene lactone with a rare endoperoxide

bridge, as antimalarial drug has revolutionized the treatment of malaria. The potencies of the four clerodane diterpenes in this present study showed that they could be excellent leads for further discovery of useful antimalarial drugs.

Chloroquine resistance in *P. falciparum* is associated with decreased chloroquine accumulation in the vacuole due to reduced uptake of the drug, an increased efflux or a combination of both processes (Wellems & Plowe, 2001; Henry *et al*., 2006). Chloroquine is inexpensive and hence the development of an agent that can increase its accumulation in drug resistant parasites will be of tremendous help in combating the problem of fast spreading drug-resistant malaria parasites in the poor regions of Africa. L-Stepholidine and β-stigmasterol have shown synergistic action in the combination assay with chloroquine against the K1 strain of *P. falciparum* (Figure 5.1). Some structural modifications of these compounds may help improve their synergistic actions. Darienine, 16-hydroxycleroda-3,13(14)-dien-16,15-olide and its acetylated derivative, 3,16-dihydroxycleroda-4(18),13(14) Z-dien-15,16-olide and 16-oxocleroda-3,13(14)E-dien-15-oic acid have however, antagonised the action of chloroquine when combined. This is the first report of chloroquine combination studies of all the isolated compounds. The reference agents (verapamil and promethazine HCl) employed exhibited their classical synergic actions (Pradines *et al*., 2002).

5.1.6 Conclusion

The results of the antiplasmodial activity studies have shown that the clerodane diterpenes are the active constituents of the ethyl acetate fraction of the alcoholic extract of *P. longifolia* stem bark: 16-oxocleroda-3,13(14)E-dien-15-oic acid was the most active (IC_{50} 3.05 µg/ml) followed by 16-hydroxycleroda-3,13(14)-dien-16,15-olide and its acetyl derivative (IC_{50} of 5.33 and 5.92 µg/ml repectively) and then 3,16-dihydroxycleroda-4(18),13(14) Z-dien-15,16-olide (IC_{50} 6.15 µg/ml). β-stigmasterol isolated alongside the clerodane diterpenes, exhibited a weak antiplasmodial activity (IC50 63.36 µg/ml). Darienine and stepholidine from

the alkaloidal fraction had 22.05 and 104.33 µg/ml IC_{50}s respectively. β-stigmasterol and stepholidine were found to synergized the activity of chloroquine against the multi-drug resistant malaria parasite.

5.2 Antimicrobial activity of isolated compounds

The total alcoholic and alkaloidal extracts of *P. longifolia* stem back together with the isolated compounds were screened for antibacterial and antifungal activities by the disk-plate agar diffusion and micro-well broth dilution methods.

5.2.1 Materials and methods

5.2.1.1 Test Microorganisms

The test microorganisms employed were *Staphylococcus aureus* (ATCC 25923), *Bacillus subtilis* (NCTC 10073), *Streptococcus pneumoniae* (clinical isolate), *Pseudomonas aeruginosa* (ATCC 4853), *Escherichia coli* (NCTC 25922), *Salmonella typhi* (NCTC 8385), and *Candida albicans* (NCPF 3179). These test organisms were from the stock kept at the Microbiology laboratory of the Department of Pharmaceutics, KNUST, Kumasi, Ghana. Nutrient Agar and Nutrient Broth (all from Oxoid, Cambridge, UK), were used in cultivation and maintenance of the microorganisms while Mueller-Hinton (MH) Agar and Broth (Sigma-Aldrich, USA) were employed in the susceptibility testing. All the media were prepared in accordance to the manufacturers' directions. 18 to 24 hour cultures of the test microorganisms were prepared and used in the susceptibility testing.

5.2.1.2 Antibiotic disk preparation

Solutions of extracts, isolates and reference compounds in dichloromethane were prepared and 10 µl quantities (containing 1000, 100 and 50, and 20 µg, respectively) were incorporated into

6 mm disks (of Whatmann number 1 filter paper) and dried. The reference drugs solutions were however prepared using water. These disks were used to assess the susceptibility of the test microorganisms by the agar diffusion method.

5.2.2 Antimicrobial susceptibility testing of extracts and isolated compounds

The Kirby-Bauer disk-diffusion method of antimicrobial susceptibility testing (Feglo *et al*., 2010) was employed in assaying the extracts and isolated compounds of *P. longifolia* stem bark. The inocula were prepared by growing the test microorganisms on Nutrient Agar plates for 18 hours and selected colonies were transferred into correspondingly labelled MH Broths (2 ml). The turbidity of the suspensions was adjusted with sterile normal saline to 0.5 McFarland standard (1×10^8 cfu/ml) and then separately and evenly inoculated onto the surfaces of MH agar plates (using sterile cotton swabs) after they were diluted (1000-folds) to yield 1×10^5 cfu/ml. The drug impregnated disks were firmly and equidistantly placed on the surface of the inoculated agar plates using sterile forceps. Amoxicillin and fluconazole were included as reference drugs for bacteria and fungus respectively. The plates were incubated at 37°C for 24 hours and zones of growth-inhibition measured.

5.2.3 Minimum inhibition concentration determination of isolated compounds

Stock solutions of 100 µg/ml (PL1, PL1ac, Ple1 and De) and 1000 µg/ml concentrations (total alcoholic and alkaloidal extracts) were prepared by initially dissolving the compounds or extracts in DMSO and then making up to required volumes with MH broth. The final concentration of DMSO was never more than l% in each well (concentrations of 1% v/v or lower were found to be harmless to microorganisms). Two-fold serial dilutions of the compounds or extracts solutions were made in sterile 96 well flat-bottomed micro-titre plates:

MH broth (100 µl) was dispensed into each well of the 96-microtiter plate except the first column (column A). 100 µl quantities of compound or extract solutions were placed in columns A and B. The two-fold serial dilution was then made from column B to H in each row. This was followed by inoculating each well with 100 ul quantities of 18 h culture of the test microorganism (standardized to 2 x 10^4 cfu/ml) in MH broth (final volume 200 ul). The plates were incubated for 24 h at 37°C. Bacterial growth was determined after addition of 50 µl p-iodonitrotetrazolium violet (0.2 mg/ml, Sigma-Aldrich, USA) (Annan *et al.*, 2009): wells with microbial growth appeared bluish-black while those with no growth were yellow in colour. Duplicates of three independent experiments were conducted in each case.

5.2.4 Results of antimicrobial activity testing of extracts and compounds of *P. longifolia*

The diameters of the clear areas observed around the drug impregnated discs in the agar diffusion method of antimicrobial susceptibility testing were measured as the zones of growth inhibition of the test microorganisms (Table 5.4). With the broth dilution method the MICs were determined as the highest dilution of the compound that just prevented the growth of the microorganisms (Table 5.5).

Table 5.4 Antimicrobial activity of extracts and compounds of *P. longifolia*

Compound/ extract/drug	Mean diameter of zones of growth inhibition ± standard deviation (mm)						
	Sa	***Sp***	***Bs***	***Pa***	***St***	***Ec***	***Ca***
1000 µg/disc							
Total ext.	18.33±1.07	17.70±1.45	24.10±1.04	7.52±1.44	17.50±0.19	21.55±0.63	20.17±1.37
Alkaloid ext.	16.00±0.95	15.19±0.39	18.22±0.76	8.11±1.02	12.91±1.07	20.87±1.24	17.43±1.92
100 µg/dics							
PL1	22.00±1.26	18.23±0.50	17.25±1.77	15.35±1.00	16.34±0.51	16.71±1.01	10.15±1.51
PL1ac	20.15±1.43	16.15±1.10	14.10±0.45	14.50±1.52	15.55±2.01	16.00±0.50	9.50±1.71
PLe1	25.50±0.76	24.60±1.07	27.50±0.15	19.00±1.90	21.40±2.10	18.10±0.53	12.50±0.82
De	16.10±1.22	14.20±1.96	17.50±1.78	11.23±0.49	14.35±0.67	16.15±1.24	10.05±1.82
PLe3	12.03±1.75	7.82±0.11	8.00±1.09	8.42±1.25	9.05±1.00	14.10±0.95	-
AL3c	-	-	-	-	-	-	-
AL6	-	-	-	-	-	-	-
50 µg/disc							
PL1	12.00±1.70	8.00±2.43	8.50±1.00	-	8.00±0.24	-	-
PL1ac	10.16±0.89	7.20±0.13	9.00±0.42	-	7.50±0.50	-	-
PLe1	14.22±0.70	12.17±1.16	12.00±1.36	10.37±0.92	12.15±0.87	10.74±1.03	10.33±0.09
De	11.00±0.13	8.20±0.41	7.22±0.20	-	-	9.10±0.17	-
PLe3	-	-	-	-	-	-	-
AL3c	-	-	-	-	-	-	-
AL6	-	-	-	-	-	-	-
20µg/disc							
Amoxicillin	27.66±1.00	31.50±0.12	28.00±0.45	13.50±0.24	15.33±0.71	13.00±0.16	nd
Fluconazole	nd	nd	nd	nd	nd	nd	21.20±0.51

The results are means of three independent experiments. *Sa = Staphylococcus aureus* (ATCC 25923), *Sp = Streptococcus pneumoniae* (clinical isolate), *Bs = Bacillus subtilis* (NCTC 10073), *Pa = Pseudomonas aeruginosa* (ATCC 4853), *St = Salmonella typhi* (NCTC 8385), *Ec = Escherichia coli* (NCTC 25922), *Ca = Candida albicans* (NCPF 3179), - = no inhibition zone, nd = not determined, ext. = extract.

Table 5.5 Minimum inhibitory concentrations of extracts and isolates of *P. longifolia*

Compound/ extract/drug	Minimum Inhibitory Concentration (µg/ml)						
	Sa	***Sp***	***Bs***	***Pa***	***St***	***Ec***	***Ca***
Total extract	125.00	125.00	31.25	125.00	62.50	31.25	125.00
Alkaloid extract	15.63	15.63	15.63	125.00	31.25	7.81	31.25
PL1	12.50	12.50	12.50	25.00	25.00	12.50	50.00
PL1ac	12.50	12.50	12.50	25.00	25.00	12.50	50.00
PLe1	6.25	6.25	3.13	25.00	12.50	12.50	12.50
De	12.50	12.50	12.50	50.00	50.00	25.00	50.00
PLe3	50.00	>50.00	50.00	>50.00	50.00	50.00	>50.00
AL3c	>50.00	>50.00	>50.00	>50.00	>50.00	>50.00	>50.00
AL6	>50.00	>50.00	>50.00	>50.00	>50.00	>50.00	>50.00
Amoxicillin	0.16	0.08	0.04	>5.00	2.50	2.50	nd
Fluconazole	nd	nd	nd	nd	nd	nd	0.16

The results are means of three independent experiments. *Sa* = *Staphylococcus aureus* (ATCC 25923), *Sp* = *Streptococcus pneumoniae* (clinical isolate), *Bs* = *Bacillus subtilis* (NCTC 10073), *Pa* = *Pseudomonas aeruginosa* (ATCC 4853), *St* = *Salmonella typhi* (NCTC 8385), *Ec* = *Escherichia coli* (NCTC 25922), *Ca* = *Candida albicans* (NCPF 3179), - = no inhibition zone, nd = not determined, ext. = extract.

5.2.5 Discussion

It has been found that patients with severe *P. falciparum* malaria are mostly also predisposed to bacterial infections (Section 1.2). As such, malarial chemotherapeutic agents that can also effectively control bacterial and possibly other microbial infections will be of tremendous benefit in regions of *P. falciparum* infection. Therefore, having established the antiplasmodial property of the extracts and isolated compounds of *Polyalthia longifolia* stem bark, it was necessary to investigate their antibacterial and antifungal activities so that a more comprehensive activity profile can be established as well as possibly justify the plant's folklore use in managing bacterial and fungal diseases.

The extracts of *P. longifolia* stem bark (at 1000 µg/disc) inhibited the growth of all the test microorganisms (three gram positive and three gram negative bacteria, and *Candida albicans*) with zones of growth-inhibition ranged between 7.52 and 24.10 mm by the agar diffusion method. The total alcoholic extract produced higher zones (zone diameter > 20 mm for *B. subtilis*, *S. typhi*, *E. coli* and *C. albicans* zones) than the alkaloidal extract (except zones produced against *E. coli* which was greater than 20 mm) (Table 5.4). In the broth dilution method however, the alkaloidal extract contrastingly exhibited higher activity than the total alcoholic extract; the highest minimum inhibitory concentration of the alkaloidal extract against the test organisms was 31.25 µg/ml (with exception of *P. aeruginosa* which had MIC = 125.00 µg/ml for both extracts). *E. coli* was the most susceptible organisms to the alkaloidal extract (MIC 7.81 µg/ml) follow by *S. pneumoniae* and *B. subtilis* (MIC = 15.63 µg/ml in both cases). With the total alcoholic extract, *E. coli* and *B. subtilis* were the most susceptible organisms (MIC = 31.25 µg/ml in each case).

In a previous study by Faizi *et al*. (2008), the methanol extracts of the leaves, stem, green berries and roots of *P. longifolia* from Pakistan were evaluated and found to possess potent antimicrobial activity against gram-positive and gram-negative bacteria and *C. albicans*. Reports of other earlier studies have also shown that *P. longifolia* var. *pendulla* growing in various geographical regions possessed potent antibacterial and antifungal activity. Rashid *et al*. (1996) showed that the petroleum ether extract (200 µg/disc) of the stem bark of *P. longifolia* growing in Bangladesh exhibited significant antibacterial activity against a wide variety of gram-positive and gram-negative organisms (growth-inhibition zones between 12.66 and 32.00 mm). In India, the petroleum ether extract of the stem bark exhibited significant antibacterial activity against *E. coli*, *S. typhi*, *P. mirabilis*, *P. aeruginosa*, *Klebsiella* species, *B. subtilis* and *Staph. aureus* (growth-inhibition zones and MICs ranged between 15.50 and 37.55 mm and 7.00 and 143 µg/ml respectively) (Ghosh et al., 2011). The antibacterial activity of various solvent extracts (petroleum ether, chloroform, ethyl acetate,

ethanol and aqueous) of the leaves harvested in India was studied on some pathogenic bacteria (Thenmozhi & Sivaraj, 2010). The chloroform extract produced the highest zones of growth-inhibition with *B. subtilis* being the most susceptible bacteria. Another report from India again showed that the methanolic extract of the leaves of *P. longifolia* inhibited the growth of many microorganisms including *Staph aureus, Staph subflava, B subtilis, Proteus mirabilis, Klebsiella pneumonia, C. albicans* and *C. tropicalis* with inhibition zones between 9.00 and 20.00 mm. MICs between 390 and 25000 µg/ml (by the broth dilution method) were also reported in the study (Chanda *et al*., 2011). The results of this present study therefore showed that the Ghana grown *P. longifolia* var. *pendulla* also displayed antimicrobial activity comparable to those reported. The geographical location of this plant therefore has no effect on the antimicrobial property.

In this study, all the isolated compounds exhibited antimicrobial property at the concentrations employed except the two alkaloidal compounds (Tables 5.4 and 5.5). The clerodane diterpenes showed higher activity against the gram-positive bacteria (growth-inhibition zones were in the range of 16.10 and 27.50 mm) than the gram-negative bacteria (inhibition zones ranged between 11.23 and 19.00 mm) and *C. albicans* (zones ranged between 10.15 and 9.50 mm). 16-oxocleroda-3,13E-dien-15-oic acid (**2**) exhibited the highest antimicrobial activity (MICs ranged between 3.13 and 25.00 µg/ml) followed by 16-hydroxycleroda-3,13(14)-dien-16,15-olide (**1**) and its acetyl derivative (**1a**) (MIC: bacteria = 12.50 µg/ml; fungi = 50 µg/ml) while 3,16-dihydroxycleroda-4(18),13(14)Z-dien-15,16-olide (**3**) was the least active of the diterpenes.

Antimicrobial activity of the four clerodane diterpenes with MIC values between 7.8 and 500 µg/ml against a number of microorganisms including *P. aeruginosa, S. typhi, Staph. aureus, Streptococcus pyogenes and C. albicans* have been reported (Faizi *et al*., 2008). In this earliar report, acetyl-16-oxycleroda-3,13-dien-16,15-olide (**1a**) was however less active than the

parent compound (16-hydroxycleroda-3,13-dien-16,15-olide; **1**). In another study by Rashid *et al*. (1996), 16-hydroxycleroda-3,13-dien-16,15-olide and 16-oxocleroda-3,13E-dien-15-oic acid (**2**) (isolated from the petroleum ether extract of the stem bark of *P. longifolia*) showed antimicrobial property against seven strains of gram-positive and twelve strains of gram-negative bacteria as well as seven strains of fungi. In this report, inhibition zones between 14.33 to 25.00 mm were recorded for *Staph. aureus, Strep. β-haemolyticus, B. subtilis, P. aeruginosa* and *Salmonella* species at 100 µg/disc of these two clrodane diterpenes. For *C. albicans* however, a 200 µg/disc concentration was employed and zones obtained were in the range of 7.66 and 10.33 mm. Sashidhara *et al*. (2009) also reported the isolation of seven clerodane diterpenes including compounds **1, 2** and **3** from the leaves of *P. longifolia* and determined their antimicrobial activity. In this present study, while compound **1** exhibited a potent activity against *Staph. aureus* (MIC of 6.25 µg/ml), Compound 2 however was active against both *Staph. aureus* (MIC = 25.00 and *E. coli* (MIC = 50.00 µg/ml). Compound **3** also displayed significant activity ($p > 0.005$) against *P. aeruginosa* and *E. coli* (MIC of 50.00 µg/ml in each case). The present study therefore confirmed the reported antimicrobial property of these clerodane diterpenes as well as showed that *P. longifolia* growing in sub-Sahara African region also contain these bioactive clerodane diterpines.

The isolated phytosteroid (β-Stigmasterol; **4**) demonstrated antibacterial activity against gram-positive and gram-negative bacteria with fairly lower inhibition zones (7.82 and 13.01 mm). A MIC of 50.00 µg/ml was obtained for *Staph. aureus, B. subtilis S. typhi* and *E. coli* while in the case of *S. pneumoniae, P. aeruginosa* and *C. albicans* the MICs of this steroid were far above the concentrations tested. Contrastingly, this compound isolated from *Arctotis arctotoides* was inactive against all the test bacteria employed (Sultana & Afolayan, 2007). However, this phytosteroid isolated from the roots of *Sida rhombifolia* Linn. (Malvaceae) was found to be active against *E. coli, Staph. aureus, P. aeruginosa* and *S. typhi* with inhibition zones ranging between 9.50 and 14.10 mm (Woldeyes *et al*., 2012).

The reference drugs (amoxicillin and fluconazole) used were more potent than the compounds isolated. However, structural modification studies on these lead compounds may afford effective and safe antimicrobial chemotherapeutic agents.

5.2.6 Conclusion

The total ethanolic and alkaloidal extracts of *P. longifolia* stem bark exhibited antibacterial and antifungal properties and the active constituents isolated included 16-hydroxycleroda-3,13(14)-dien-16,15-olide, 16-oxocleroda-3,13E-dien-15-oic acid, 3,16-dihydroxycleroda-4(18),13(14)Z-dien-15,16-olide and β-Stigmasterol. A semi-synthetic acetyl derivative of 16-hydroxycleroda-3,13(14)-dien-16,15-olide also exhibited antimicrobial property comparable to the parent compound. Darienine and stepholidine were however inactive against all the test microorganisms employed.

Chapter Six

6 GENERAL DISCUSSION, CONCLUSION AND RECOMMENDATIONS

6.1 GENERAL DISCUSSION

Malaria is among the top major infectious diseases of the world. About half of the world's population is at risk. It is a major public health challenge as well as a significant economic burden on many developing countries in the African region. People living in extreme poverty are the most vulnerable; they most often suffer from malaria and other infectious diseases simultaneously which affects their ability to make a living and move out of poverty. Malaria research, unlike diseases such as cancer and diabetes, seems to attracts little donor funding due to the fact that it is rare in wealthier developed countries. However, the disease poses a risk to travellers with imported cases increasing in non-endemic areas (Al-Kuwari, 2009; Shiff, 2002). Areas suitable for malaria transmission, particularly higher altitudes and boarders of the malarious regions, are also gradually expanding as a result of the global climate change.

Prevention, diagnosis and treatment of the infection are the key strategies in 'rolling back malaria' on all fronts until the total elimination of the disease. Vaccines are often the most cost-effective preventive tools that have historically contributed to the reduction in the spread and total elimination of some infectious diseases such as smallpox. However, due to the malaria parasite's unique ability to evade destruction by the body's immune system (thus diminishing the immune system from fighting the infection), there is currently no effective malaria vaccine. The malaria parasite's vector control measures are also being hampered by the wide spread resistance developed by the mosquitoes to available classes of insecticides

(Ranson *et al*., 2011). The only terminal option for managing the infection is treatment of clinically infected people and a chemoprophylactic preventive measure.

Over the years, many chemotherapeutic agents have been developed and employed in the treatment of the *P falciparum* infection. However, the appearance and wide spread of drug-resistance *P. falciparum* species has made the treatment of malaria increasingly problematic in virtually all malarious regions of the world ((Na-Bangchang & Karbwang, 2009; Witkowski *et al*., 2010)). The development of effective and safe therapeutic alternatives is therefore essential to ensure the availability of affordable drugs that will help reduce mortality and morbidity due to malaria.

Research into plants has increased in recent years due to the fact that plants have historically served as a valuable source of discovery of drugs for diseases. In Ghana, traditional medicine is the most accessible healthcare option (Gbedema *et al*., 2010); a number of plants are used in the folklore treatment of infectious diseases. This study sought to verify the scientific basis and validate or otherwise the traditional uses of a selection of local medicinal plants for malaria. It also sought to identify and select a suitable candidate plant for further investigation aimed at finding new and effective antiplasmodial compounds or leads for potent and affordable antimalarial drugs and other anti-infective remedies such as antibacterial and anticandida agents.

In the study, extracts of ten local medicinal plant species used in the traditional treatment of malaria were evaluated for antiplasmodial activity using the pLDH assay. The extracts demonstrated a wide range of antiplasmodial activity against the multidrug resistant K1 strain of *P. falciparum*.

The extracts of *C. portoricensis* roots, *P. macrocarpus* stem bark and *T. ivorensis* stem bark displayed weak to moderate antiplasmodial activity with IC_{50} values above 50 µg/ml. The

potencies of these extracts were not significant ($p > 0.005$) for considering these plant species as alternative remedies for malaria.

The extracts of *A. cissampeloides* stem, *A. nobilis* stem bark and *E. guineensis* leaves showed good activity (IC_{50} between 27 and 46 µg/ml). Although the alkaloidal extract of *E angolense* stem bark had a weak antiplasmodial activity, the total alcoholic extract exhibited good activity. These results therefore appeared to lend some support to the traditional use of these plants as remedies for malaria. The potencies were however not significantly ($p < 0.005$) high enough for considering the plants in further scientific anti-infective investigations.

The alkaloidal extracts of *S. latifolius* root, *M. oppositifolius* leaves and *P. longifolia* stem bark displayed the most potent antiplasmodial activities ($IC_{50} < 21$ µg/ml). The total alcoholic extracts of these three plants also exhibited significantly ($p > 0.005$) potent antimplasmodial activity (IC_{50} 37 - 22 µg/ml). These results lend support to the traditional use of these plants by the local communities in the treatment of malaria. Further scientific antiplasmodial investigation of these plants is therefore deemed essential. This investigation may include formulation of phytomedicines for use in the sub-region, isolation of the phytoconstituents, *in vivo* studies of the extracts and the isolated compound and determination of the mechanisms of actions of the phytoconstituents.

The *P. longifolia* stem bark extracts displayed the highest levels of activity (IC_{50} 15.47 µg/ml (alkaloid) and 22.04 µg/ml (alcoholic)] and was therefore ranked the overall most potent plant species in this study. This plant is increasingly being planted in several communities in Ghana, making it easily accessible to many households. Based on the historical evidence of the plant's folklore use in the treatment of malaria coupled with the fact that there is no reported scientific antiplasmodial investigation of it, *P. longifolia* was selected for further investigation which included isolation, characterization and testing of the isolated compounds for antimalarial property.

The bioassay guided fractionation revealed the ethyl acetate fraction of the total alcohol extract and the alkaloidal extract as the most potent of all the fractions. These were therefore selected for further fractionation and separation by chromatographic techniques which led to the isolation of three clerodane diterpenes [16-hydroxycleroda-3,13-dien-16,15-olide (**1**), 16-oxocleroda-3,13*E*-dien-15-oic acid (**2**) and 3,16-dihydroxycleroda-4(18),13(14)*Z*-dien-15,16-olide (**3**), a phytosteroid [β-Stigmasterol (**4**)] and an azafluorene [Darienine (**5**)] and a protobeberine [L-Stepholidine (**6**)] alkaloids. Compound **1** was treated with acetic anhydride and these resulted in its acetylated derivative acetyl-16-oxycleroda-3,13-dien-16,15-olide (**1a**)]. The compounds were characterized and identified through spectroscopic analysis and comparison with reported data. Bioactivity investigations of the compounds revealed that they possessed varying degrees of antiplasmodial, antibacterial and anticandida properties.

The isolated clerodane diterpenes together with the acetylated derivative displayed significatly ($p < 0.005$) potent antiplasmodial activities (with IC_{50} values ranged between 3.0 to 6.2 µg/ml) which could largely have contributed to the antiplasmodial activity observed with the *P. longifolia* stem bark extract. Apart from 16-hydroxycleroda-3,13-dien-16,15-olide (**1**) which was previously reported of possessing antiplasmodial activity, the acetyl-16-oxycleroda-3,13-dien-16,15-olide (**1a**), 16-oxocleroda-3,13*E*-dien-15-oic acid (**2**) and 3,16-dihydroxycleroda-4(18),13(14)*Z*-dien-15,16-olide (**3**) are being reported for the first time of exhibiting antiplasmodial activity against the multi-drug resistant K1 strain of *P. falciparum.*

The antiplasmodial activity of Darienine (**5**) and β-Stigmasterol (**4**) even though weak, could also possibly have contributed to the overall antiplasmodial property observed with the *P. longifolia* stem bark extract. L-Stepholidone (**6**) was inactive against the malaria parasite employed.

Though not comparable to promethazine and verapamil (chemosensitizing agents used), β-Stigmasterol (**4**) and L-Stepholidone (**6**) displayed a potentiation on chloroquine activity

when tested in fixed-ratio combinations against the malaria parasite. It is therefore possible that structural modifications of β-Stigmasterol (**4**) and L-Stepholidone (**6**) could help improve upon their chloroquine chemosensitizing property. The four clerodane diterpenes and the azaflourene alkaloid, darienine (**5**) were however antagonistic to the action of chloroquine. Therefore, the simultaneous administration of *P. longifolia* preparations or other phytomedicines with orthodox medicines in the treatment of malaria should be done with caution since drugs that have modes of action similar to chloroquine may have a high chance of being rendered ineffective.

The antimicrobial investigation of the extracts and the isolated compounds of *P. longifolia* stem bark was to validate and provide a comprehensive bioactivity profile of the plant which could probably lead to a 'holistic phytomedicine' beneficial for malaria patients who simultaneously suffer microbial infections. At 1000 µg/disc the extracts inhibited the growth of *Staphylococcus aureus*, *Bacilus subtilis*, *Streptococcus pneumoniae*, *Pseudomonas aeruginosa*, *Escherichia coli*, *Salmonella typhi*, and *Candida albicans* (zones of inhibition 7.52 – 24.10 mm; MIC 7.81 – 125.00 µg/ml).

The isolated compounds also displayed varying degrees of antibacterial and antifungal properties except Darienine (**5**) and L-Stepholidine (**6**) which were inactive at the concentrations tested. The clerodane diterpenes [100 µg/disc; 16-hydroxycleroda-3,13-dien-16,15-olide (**1**), 16-oxocleroda-3,13*E*-dien-15-oic acid (**2**) and 3,16-dihydroxycleroda-4(18),13(14)*Z*-dien-15,16-olide (**3**) and the acetylated derivative of **1;** acetyl-16-oxycleroda-3,13-dien-16,15-olide (**1a**)] exhibited a broad spectrum of activity (inhibition zones ranged between 9.50 and 27.50 mm). 16-oxocleroda-3,13*E*-dien-15-oic acid (**2)** displayed the most potent activity against all the test microorganisms with minimum inhibitory concentrations between 3.13 and 12.50 µg/ml except *P. aeruginosa* (MIC 25.00 µg/ml). The other diterpenes (**1, 1a** and **3**) were however comparable in terms of their antimicrobial activity (MIC 12.50 –

50.00 µg/ml). The activity of these diterpenes and the phytosteroid (β-Stigmasterol; MIC 50 µg/ml) could be said to have contributed to the antimicrobial property exhibited by the *P. logifolia* stem bark extracts. These results have therefore lent support to the folkloric use of the plant in treating skin and other infectious conditions (Ichino *et al*., 2008). These clerodane diterpenes may serve as useful leads for potent antimicrobial as well as antimalarial drugs.

6.2 CONCLUSION

The study has shown that all the plants evaluated displayed some antiplasmodial property: *C portoricensis* showed a weak activity (IC_{50} 112 µg/ml) while *T. ivorensis* and *P. macrocarpus* displayed 'moderate activity' (IC_{50} 59 – 96 µg/ml). *A. cissampeloides, A. nobilis, E. guineensis* and *E. angolense* also displayed 'good antiplasmodial activity' (IC_{50} 32 – 45 µg/ml). However, the plants that showed the most potent activity were *M. oppositifolius, S. latifolius* and *P. longifolia* (IC_{50} 15 – 20 µg/ml).

Phytochemical compounds: 16-hydroxycleroda-3,13-dien-15,16-olide (**1**); Acetyl-16-oxycleroda-3,13-dien-15,16-olide (**1a**); 16-oxocleroda-3,13E-dien-15-oic acid (**2**); 3,16-dihydroxycleroda-4(18),13(14)Z-dien-15,16-olide (**3**); β-Stigmasterol (**4**); Darienine (**5**) and L-Stepholidine (**6**) were obtained from the stem bark extracts of *P. longifolia*. While compounds **4, 5** and **6** were moderately weak in action (22.05 – 104.33 µg/ml), compounds **1, 1a, 2** and **3** displayed potent antiplasmodial activities (IC_{50} 3.05 – 6.15 µg/ml). Compounds **4** and **6** however synergized the action of chloroquine against the multidrug resistant strain of *P. falciparum* (K1 strain). In addition to the antiplasmodial activity, compounds **1, 1a, 2, 3** and **4** displayed antimicrobial activity against gram positive and gram negative bacteria and yeast (MIC 3.13 – 50 µg/ml).

6.3 RECOMMENDATIONS

The stem bark extract of *P. longifolia* should be scientifically formulated into suitable dosage forms such as capsules (for adults) and syrups (for children) for use in the sub-region. Other parts of the plant such as the leaves and fruits should be studied for antiplasmodial and other anti-infective agents. Cultivation of the plant must also be encouraged to ensure constant supply of the plant material in the sub-region.

Further studies to determine the mode and or mechanisms of action as well as structural modifications to improve upon the potency of the isolated constituents must be carried out.

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