KWAME NKRUMAH UNIVERSITY OF SCIENCE AND TECHNOLOGY, KUMASI

COLLEGE OF SCIENCE

FACULTY OF PHYSICAL SCIENCES

DEPARTMENT OF CHEMISTRY

ANTIPLASMODIAL CONSTITUENTS IN THE LEAVES AND STEM BARKS OF *CARAPA PROCERA* DC (MELIACEAE) AND *ALSTONIA BOONEI* DE WILLD. (APOCYNACEAE)

BY

LINDA MENSAH SARPONG

FEBRUARY, 2011

APPENDIX 3: Concentrations and percentage inhibition of parasitaemia of hot and cold petroleum ether and 70% ethanolic extract of the stem bark and leaves of *A. boonei*

Conc mg/ml	PS_1			H_1			C_1			CONTROL		
100.000000	59.805900	59.805900	59.805900	80.653770	80.653770	80.653770	88.627570	88.627570	87.627570	1.087403	1.087403	1.087403
25.000000	40.238040	40.238040	40.238000	64.174890	64.174890	64.174890	72.026480	72.026480	72.026480	0.9642481	0.9642481	0.9642481
6.250000	32.096420	32.096420	32.096420	53.926030	53.926030	53.926030	69.972360	69.972360	69.972360	0.9642481	0.9642481	0.9642481
1.562500	31.198870	31.198870	31.198870	41.839040	41.839040	41.839040	65.356090	65.356090	65.356090	0.9642481	0.9642481	0.9642481
0.390625	28.105920	28.105920	28.105920	36.871950	36.871950	36.871950	51.927850	51.927850	51.927850	-0.8868874	-0.8868874	-0.8868874
0.0976563	0.230000	0.230000	0.230000	0.134000	0.134000	0.134000	6.236250	6.236250	6.236250	-1.170543	-1.170543	-1.170543

Conc mg/ml	PS_L			H_3			C_3			CONTROL		
100.000000	84.685490	84.685490	84.685490	87.084510	87.084510	87.084510	98.038860	98.038860	98.038860	1.087403	1.087403	1.087403
25.000000	80.428830	80.428830	80.428830	90.913380	90.913380	90.913380	91.849220	91.849220	91.849220	0.9642481	0.9642481	0.9642481
6.250000	42.102990	42.102990	42.102990	84.855640	84.855640	84.855640	73.619510	73.619510	73.619510	0.9642481	0.9642481	0.9642481
1.562500	41.108040	41.108040	41.108040	77.462330	77.462330	77.462330	68.133690	68.133690	68.133690	-0.8868874	-0.8868874	-0.8868874
0.390625	39.952100	39.952100	39.952100	34.450000	34.450000	34.450000	60.410010	60.410010	60.410010	-1.170543	-1.170543	-1.170543
0.0976563	34.837030	34.837030	34.837030	21.560000	21.560000	21.560000	0.105000	0.105000	0.105000	-1.170543	-1.170543	-1.170543

APPENDIX 4: Concentrations and percentage inhibition of parasitaemia of the petroleum ether and hot and cold 70% ethanolic extract of the stem bark of *C. procera*

conc/mg/ml	PC1			H2			C2			CONTROL		
100.000000										1.087403	1.087403	1.087403
25.000000	83.331570	83.331570	83.331570	94.310990	94.310990	94.310990	70.613690	70.613690	70.613690	0.9642481	0.9642481	0.9642481
6.250000	83.331570	83.331570	83.331570	90.006580	90.006580	90.006580	73.138110	73.138110	73.138110	0.9642481	0.9642481	0.9642481
1.562500	81.960400	81.960400	81.960400	79.069230	79.069230	79.069230	60.107550	60.107550	60.107550	0.9642481	0.9642481	0.9642481
0.390625	80.053770	80.053770	80.053770	83.080100	83.080100	83.080100	52.576380	52.576380	53.576380	-0.8868874	-0.8868874	-0.8868874
0.0976563	71.472260	71.472260	71.472260	56.301020	56.301020	56.301020	7.992870	7.992870	7.992870	-1.170543	-1.170543	-1.170543

APPENDIX 5: Concentrations and percentage inhibition of parasitaemia of standard drug artesunate petroleum ether and hot ethanolic (79%) extract of the stem bark of *C. procera*

conc	artesunate		H_{2B}		H_{2C}		H_{2D}		H_{2E}	
31.250000			73.0	73.0	73.	73.	73.	73.	59.0	59.0
7.810000			66.0	66.0	59.	59.	63.	63.	45.0	45.0
1.950000			63.0	63.0	63.	63.	59.	59.	42.0	42.0
0.490000			59.0	59.0	49.	49.	63.	63.	31.7	31.7
0.120000			42.0	42.0	66.	66.	45.	45.	31.7	31.7
0.030000			31.7	31.7	42.	42.	45.	45.	31.7	31.7
12.500000	100.	100.								
3.125000	100.	100.								
0.781000	100.	100.								
0.195000	100.	100.								
0.048800	90.	90.								
0.012200	89.	89.								
0.003000	78.	78.								
0.000760	66.	66.								
0.000190	61.	61.								

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A THESIS SUBMITTED IN PARTIAL

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BY

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KUMASI

FEBRUARY, 2011

DECLARATION

I hereby declare that the experimental work described in this thesis is my own work towards the MSc and to the best of my knowledge; it contains no material previously published by another person or material which has been submitted for any other degree of the university, except where due acknowledgement has been made in the text.

……. …….

Linda Mensah Sarpong Date

Certified by:

……. ……

Mr. G. K. Tuani Date

(Supervisor)

Certified by:

…….. ……..

Dr. J.A.M Awudza Date

(Head of Department)

DEDICATION

This work is dedicated to my family for their prayers, love and support.

ACKNOWLEDGEMENT

To God be the glory great things He has done! I acknowledge the sufficient grace of God that has sustained me through the difficult moments encountered during the course of this work.

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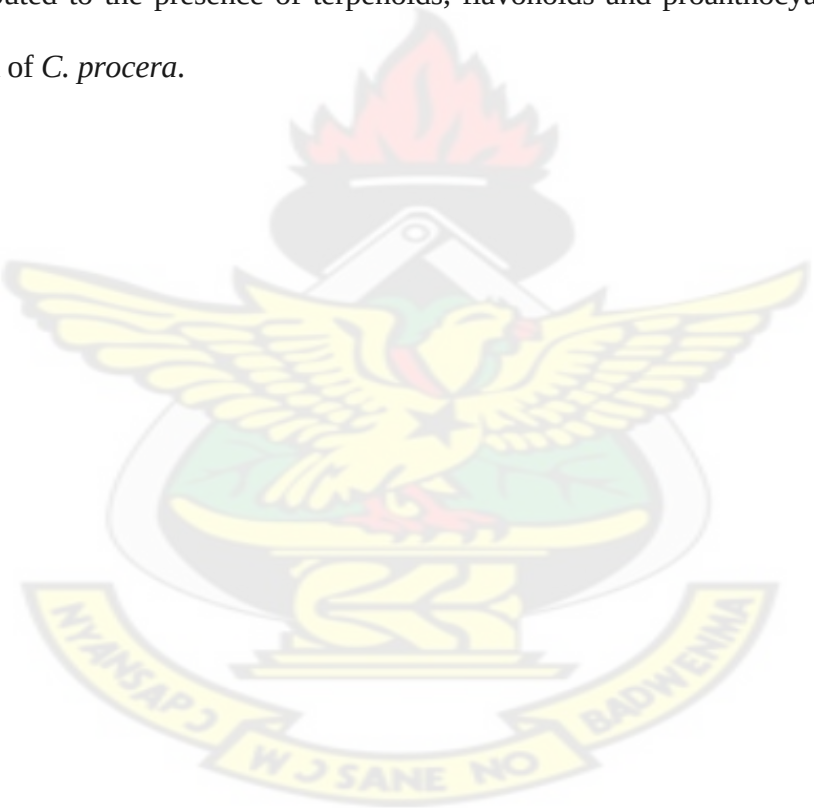
Finally to my husband, Mr. Isaac Kingsley Amponsah, who has always been with me through thick and thin to see to the realization of this dream, I say thank you.

ABSTRACT

Malaria remains the most devastating infectious parasitic disease, inflicting both death and economic losses on at least half the world's population. Presently, the most effective way of dealing with malaria is the administration of chemotherapeutic agents. There is an urgent need for the development of effective anti-malarial drugs due to emergence of resistant strains of *P. falciparum*. The stem and leaf extracts of *Alstonia boonei* are used in various traditional medicines for the treatment of malaria. A decoction of the bark of *Carapa procera* is taken orally for fevers whereas oil obtained from the seeds is applied topically as a mosquito repellent and also for other skin infections. Petroleum ether and hydro-alcoholic extracts of these two plants were tested *in vitro* on choloroquine sensitive (3D7) strains of *Plasmodium falciparum* for their anti-malarial activity. Growth inhibition was determined *in vitro* by counting GIEMSA-stained parasites by light microscopy. The petroleum ether extract of the leaves (PSL) and stem bark (PS_1) of *A. boonei* were both inactive (IC_{50}>100 µg/ml). Also their hot ethanolic extracts were inactive with IC_{50} >100 µg/ml. However, the cold ethanolic extract of the leaves showed weak activity (IC_{50} = 71.24 µg/ml) whereas that of the stem was 88.15 µg/ml. The petroleum ether extract of the stem bark of *C. procera* (PC_1) inhibited the growth of the chloroquine sensitive (3D7) *Plasmodium falciparum* parasite with IC_{50} value of 19.52 µg/ml. The cold ethanolic extract of *C. procera* had moderate activity (IC_{50} = 33.35 µg/ml) whereas the hot ethanolic extract (H_2) showed the highest antimalaria activity (IC_{50} = 11.41 µg/ml). Column chromatography of H_2 on silica gel (70-230 mesh) yielded four bulked fraction designated as H_{2A}, H_{2B}, H_{2C} and H_{2D} which were tested *in vitro* for antiplasmodial activity. Fractions H_{2A}, H_{2C} and H_{2D} showed the highest antimalaria activities (IC_{50}<10µg/ml)

whereas H_{2B} showed moderate antimalaria activity with (IC_{50}<50µg/ml). The activities of fractions H_{2A} and H_{2C} (IC_{50}<1µg/ml) were comparable to the standard antimalaria drug artesunate (IC_{50}<0.01µg/ml). TLC analysis of the fractions described in the present work revealed the presence of steroids and terpenoids (fraction H_{2A}), flavonoids, catechins and proanthocyanidins (H_{2B}, H_{2C} and H_{2D}). Thus cold ethanolic extract of the leaves and stem bark of *A. boonei* have weak antiplasmodial activity whereas the hot ethanolic extract of *C. procera* has very good antiplasmodial activity. Antiplasmodial activity of the stem bark of *C. procera* may be attributed to the presence of terpenoids, flavonoids and proanthocyanidins present in the stem bark of *C. procera*.



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ABBREVIATIONS

AIDS	Acquired Immune Deficiency Syndrome
ANOVA	Analysis of Variance
APAD	3-acetylpyridine Adenine Dinucleotide
ARDS	Acute Respiratory Distress Syndrome
ATS	Artesunate
AUC	Area under Curve
CQS	Chloroquine Sensitive strain
DAPI	4, 6-diamidino-2-phenylindole
DHFR	Dihydrofolate Reductase
DHPS	Dihydrophteroate Synthase
ECG	Electrocardiogram
ELSDS	Evaporative Light-Scattering Detectors
G6PD	Glucose-6- Phosphate Dehydrogenase
GC	Gas Chromatography
HIV	Human Immune Virus

HPLC	High Performance Liquid Chromatography
IC_{50}	Inhibition Concentration
IRBC	Infected Red Blood Cells
LC	Liquid Chromatography
MOH	Ministry of Health
MS	Mass Spectrometer
NCEs	New Chemical Entities
NMR	Nuclear Magnetic Resonance
PDA	Photodiode array
RBCs	Red Blood Cells
RI	Refractive Index
SEM	Standard Error of Mean
SP	Sulfadoxine- Pyrimethamine
TLC	Thin Layer Chromatography
UNDP	United Nations Development Plan
UNICEF	United Nations International Children Educational Fund

URBC Uninfected Red Blood Cells

UV Ultraviolet

WHO World Health Organisation

Chapter 1

INTRODUCTION

1.1 GENERAL INTRODUCTION

Malaria contributes substantially to the poor health situation in Africa. It is on record that, Sub-Saharan Africa accounts for 90% of the world's 300 – 500 million cases and 1.5 –2.7 million deaths annually. About 90% of all these deaths in Africa occur in young children. Between 20 and 40 percent of outpatient visits and between 10 and 15 percent of hospital admissions in Africa are attributed to malaria (WHO, 1999). This burdens the health system. In general, it is estimated that malaria accounts for an average of 3% of the total global disease burden as a single disease in 1990.

Malaria is ranked second after HIV/AIDS accounting for 10.6% of the disease burden. Furthermore, while malaria contributed 2.05% of the total global deaths in 2000, it was responsible for 9.0% of all deaths in Africa (WHO, 2002). The World Health Organisation also estimated that the total cost of malaria to Africa was US$ 1.8 billion in 1995 and US$ 2 billion in 1997 (WHO, 1997). Malaria is therefore a massive problem, which plagues all segments of the society. The effect of malaria on people of all ages is quite immense. It is however very serious among pregnant women and children because they have less immunity. When malaria infection is not properly treated in pregnant women, it can cause anaemia and also lead to miscarriages, stillbirths, underweight babies and maternal deaths. Also, frequent cerebral malaria can lead to disabling neurological problems. Furthermore, malaria in school children is a major cause of absenteeism in endemic countries. It is estimated that about 2% of children who recover from cerebral malaria suffer brain damage including epilepsy (WHO/UNICEF,

2003). Hence, among young children, frequent episodes of severe malaria may negatively impact on their learning abilities and educational attainment. This is a threat to human capital accumulation, which constitutes a key factor in economic development. The debilitating effects of malaria on adult victims are very much disturbing. In addition to time and money spent on preventing and treating malaria, it causes considerable pain and weakness among its victims. This can reduce peoples working abilities. The adverse impact of the disease on household, production and gross domestic product can be substantial. Malaria therefore is not only a public health problem but also a developmental problem. At the national level, apart from the negative effect of lost productivity on the major sectors of the economy, malaria has negative effects on the growth of tourism, investments and trade especially in endemic regions. Malaria presents a major socio-economic challenge to African countries since it is the region most affected. This challenge cannot be allowed to go unnoticed since good health is not only a basic human need but also a fundamental human right and a prerequisite for economic growth (Streeten, 1981).

According to the WHO, drug resistance is probably the most important factor affecting malaria control at the present time. Since its development in the forties, chloroquine has been successfully used by millions of human beings for prevention or treatment of all malaria species (Canfield, 1980). The resurgence and incidence of malaria in many parts of the world have been difficult to control because of resistance of the malaria parasite to chloroquine, sulphadoxine-pyrimethamine (SP) and other conventional anti-malarial drugs. Resistance to these drugs has been reported to be as high as 40-60% in some African and Asian countries. Chloroquine resistance is now universal, and the days of treating malaria with a single cheap drug are generally believed to be numbered. Two relatively cheap drugs could potentially

replace SP as monotherapy; amodiaquine (an older drug with similarities to chloroquine) and chlorproguanil-dapsone. Both are currently effective in many areas where SP resistance already occurs. Resistance to amodiaquine already exists at an appreciable level in some areas (up to 26% in Kenya). New drugs against malaria are thus urgently needed.

1.2 THE PROBLEM

The World Health Organisation reported that up to 500 million people suffer from severe malaria, with most cases occurring in sub-Saharan Africa. In Ghana, statistics show that one in every five childhood deaths results from malaria. On a continent-wide basis, malaria has severe socio-economic effect through increased poverty, impaired learning and decreasing attendance of school and work, as well as direct costs that include a combination of personal and public expenditures on both prevention and treatment of the disease (Makalo, 2009).

Despite efforts to reduce transmission and increase treatment, there has been little change in which areas are at risk of this disease since 1992. Indeed, if the prevalence of malaria stays on its present upwards course, the death rate could double in the next twenty years. Precise statistics are unknown because many cases occur in rural areas where people do not have access to hospitals or the means to afford health care. As a consequence, the majority of cases are undocumented. Malaria is presently endemic in many parts of Asia and much of Africa; however, it is in sub-Saharan Africa where 85– 90% of malaria fatalities occur. It is also a serious public health problem in certain regions of South East Asia and South America. Human malaria transmitted by female Anopheles mosquitoes is caused by four species of Plasmodium, which are, *P. falciparum, P. vivax, P. ovale and P. malariae.* Most cases of

malaria are caused by *P. falciparum*. It is estimated that the world would have to spend more than $5 billion a year to prevent deaths from malaria, nearly five times the current spending to fight the mosquito-borne disease (McInnis, 2008).

The Roll Back Malaria Partnership; comprised of United Nations agencies, the World Bank, leading drug makers and aid experts, estimated that boosting spending on bed nets, medicines and malaria tests could save 4.2 million lives a year by 2015. Its "Global Malaria Action Plan," unveiled in the midst of a global financial crisis that may curb international aid budgets, called for malaria spending to increase to $5.3 billion in 2009, $6.2 billion in 2010 and $5.1 billion annually from 2011-2020. Another $8.9 billion is needed in the next decade for research and development into malaria drugs, vaccines and tests, plus vector-control measures to fight mosquitoes. Spending on malaria, from both national governments and international groups was about $1.1 billion in 2008. The cost of treating malaria, in Ghana, amounted to US $772.4 million in 2009. This amount that equalled to Ghana's entire health budget for 2008, represents 10% of the country's entire Gross Domestic Product for 2006 (Makalo, 2009).

Self prescribed medication is a widespread phenomenon in Ghana. Majority of the malaria victims only seek medical examination and treatment from health facilities when the initial attempts have failed resulting in late presentation (Agyapong, 1992; Asenso-Okyere and Dzator, 1995). Very often malaria treatments in Ghana occur at home with only a few of such home-based treatments being correct and complete. Accessibility to orthodox medical treatment in Ghana is low with per capita outpatient visit of 0.46 in 2000 (MoH, 2002).

One main reason for continued or, in some areas, rising rate of malaria infection are attributable to the spread of drug-resistant strains of *Plasmodium* and the failure to get the existing, effective drugs to be applied in those areas where they can be of most benefit, especially those living in the rural areas. They resort to traditional remedies. There is therefore the need to optimize the use of these traditional antimalarials for the benefit of all.

1.3 JUSTIFICATION OF RESEARCH

The search for new anti-malaria drugs remains indispensable in the face of resistant strains of *P. falciparum*. One of the most promising targets in the search for new anti-malaria drugs is the large repository of medicinal plants used in the treatment of malaria in traditional societies. The first anti-malaria drug provided by ancestral treatment was quinine, derived from the bark of the Peruvian *Cinchona* tree (Camacho *et al*., 2000). Despite the cost and adverse effects, a standard treatment for severe malaria in Africa and in Europe is still the intravenous administration of quinine (WHO, 2001). Still now, resistance against quinine in Africa is absent (Le Bras *et al.,* 2006). One of the new antimalarial molecules discovered by ethnopharmacological plant research was artemisinin. Initially, hot water extracts from *Artemisia annua* were tested in mice infected with a rodent malaria parasite, but no activity was found. Fortunately researchers later tested ether extracts of the plant and these were found to be active. This led to the isolation of the active principle, initially named Qinghaosu (substance from Qing Hao) and later named artemisinin, in 1972. The elucidation of the structure revealed that artemisinin was a sesquiterpene lactone that was highly unusual as it contained an endoperoxide moiety (Wright, 2005). Clinical trials on a large number of patients showed that artemisinin was highly effective in clearing parasitaemia and reducing symptoms

in patients with malaria, including some with chloroquine-resistant malaria and cerebral malaria (Woodrow *et al*., 2005). The success of Artimisinin, isolated from *Artemisia annua*, and its derivatives for the treatment of resistant malaria has focused attention on plants as sources of anti-malaria drugs.

Ethno botanical information about anti-malarial plants, used in traditional herbal medicine, is essential for further evaluation of the efficacy of plant anti-malarial remedies and efforts are now being directed towards discovery and development of new chemically diverse anti-malarial agents. The world's poorest are the worst affected, and many treat themselves with traditional herbal medicines. These traditional herbal remedies are often more available and affordable, and sometimes are perceived as more effective than conventional anti-malaria drugs. Some Ghanaians living in rural areas depend on traditional herbal medicine for treatment of many infectious diseases including malaria. The reputed efficacies of these plants have been experienced and passed on from one generation to the other. Apparently, lack of scientific proof of efficacies claimed by traditional medical practitioners in Ghana called for this study. The present research is aimed at investigating the anti-plasmodial activity (*in vitro*) of two Ghanaian medicinal plants, *Carrapa procera* DC (Meliaceae) and *Alstonia boonei* De Willd (Apocynaceae), used in ethnomedicine for the treatment of malaria.

Carrapa procera belongs to the family meliaceae. Members of the Meliaceae have been used for generations in Africa, India and tropical America to treat malaria (Schwikkard and Van-Heerden, 2002). In America, *Cedrela odorata, Carapa guianensis* and *Swietenia mahagoni* have been used, while in Africa and India the 'Neem'tree or *Azadirachta indica* is used (MacKinnon *et al*., 1997). A decoction of the leaves of *Azadirachta indica* is taken orally for

the treatment of malaria in Ghana. *Carapa procera* commonly called 'monkey kola', is used traditionally for cough, wound dressing, fever, malaria, small pox and worms (Mshana *et al*., 2000). Oil based formulation of *C. Procera* with *Cocos nucifera* had mosquito repellent activity. Limonoids, present in the meliaceae, are reported to be responsible for the antimalarial activity. Work on the leaves of *Azadirachta indica* collected in India resulted in the isolation of four limonoids, of which meldenin was the most active against the chloroquine-resistant K1 strain of *P. falciparum* (Joshi *et al*., 1998). Similarly the stem bark of *Alstonia boonei* is used traditionally in Ghana for the treatment of malaria, boils, hypertension, measles, wounds, cataract and rheumatoid arthritis (Mshana, 2000; Taiwo *et al.*, 1998).

The aim of this research is to give scientific credence to the use of *C. procera* and *A. boonei* for the treatment of malaria and identify the secondary metabolites responsible for the activity

1.4 AIMS AND OBJECTIVES

The main aim of this thesis is to investigate the anti-malaria properties of the leaves and stem barks of *Carapa procera* (meliaceae) and *Alstonia boonei* (Apocynaceae)

OBJECTIVES

1. Extract the phytochemical constituents of the leaves and stem barks of *Carapa procera* and *Alstonia boonei* using different solvents under different conditions.
2. Test the extracts for antiplasmodial activity
3. Column chromatographic fractionation of bioactive extract
4. Antiplasmodial assay of fractions
5. Phytochemical analysis of bioactive fractions

Chapter 2

LITERATURE REVIEW

2.1 MALARIA

Malaria is a life threatening disease caused by parasites that are transmitted to people through the bites of infected mosquitoes. A child dies of malaria every 30 seconds. There were 247 million cases of malaria in 2006, causing nearly one million deaths, mostly among African children. Malaria is preventable and curable. Approximately half of the world's population is at risk of malaria, particularly those living in lower income countries (WHO, 2009).

2.1.1 Prevalence

Each year, 300 to 500 million people develop malaria and 1.5 to 3 million, mostly children, die according to the World Health Organization. In the United States, approximately 1,000 cases are reported annually, which researchers estimate represent only 25 to 50 percent of actual cases. Malaria contributes substantially to the poor health situation in Africa. It is on record that, Sub-Saharan Africa accounts for 90% of the world's 300 – 500 million cases and 1.5–2.7 million deaths annually. About 90% of all these deaths in Africa occur in young children. This has serious demographic consequences for the continent. Between 20 and 40 percent of outpatient visits and between 10 and 15 percent of hospital admissions in Africa are attributed to malaria (WHO, 1999). This burdens the health system. In general, it is estimated that malaria accounts for an average of 3% of the total global disease burden as a single disease in 1990.

Malaria is hyper endemic in Ghana. The parasite rate ranges from 10 – 70% with *P. falciparum* dominating. It is the number one cause of morbidity accounting for over 40 % of outpatient

attendance in public health facilities with annual reported cases of about 2.2 million between 1995 and 2001 (Figure 2.1), with over 10 % ending up on admission. Malaria is a major killer in Ghana and also the leading cause of mortality among children under five years old (UNDP, 2000). The disease accounts for an average of 13.2% of all mortality cases in Ghana and 22% of all mortalities in children younger than 5 years. In the case of pregnant women, out of the total number reporting at the health institutions, 13.8% suffer from malaria (Antwi and Marfo, 1998; Marfo, 2002). It is estimated that malaria prevalence (notified cases) is 15,344 per 100 000 with a malaria death rate for all ages being 70 per 100 000. In the case of the 0 – 4 years, it is 448 per 100 000 reported for the year 2000 (United Nations, 2003)

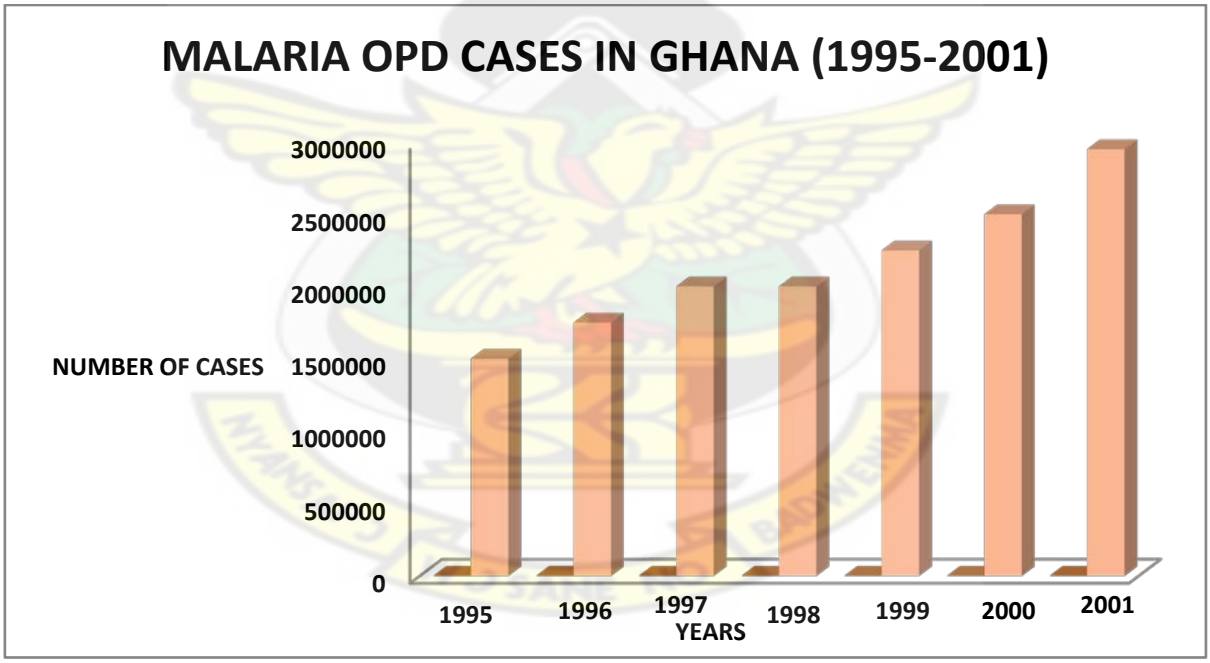


Figure 2.1 Malaria OPD cases from 1995 to 2001.
Source of Data: Centre for Health Information Management, Ghana Health Service, 2003

2.1.2 Distribution of the disease

Malaria has a worldwide distribution, being found in tropical areas, throughout sub-Saharan Africa and to a lesser extent in South Africa, South East Asia, and the Pacific islands, India, Central and South America. *P. falciparum* is the predominant species in most endemic countries, exceptions being India and South America where *P. vivax* is more common. *P. ovale* is mainly found in West Africa. In South–East Asia, particularly along the borders of Thailand with Myanmar and Cambodia, *P. falciparum* is multi-drug resistant with chloroquine, sulphadoxine-pyrimethamine (SP) and mefloquine monotherapies all ineffective and quinine slowly losing its potency. In sub-Saharan and South Africa, chloroquine resistance to *falciparum* malaria is widespread and antifolate resistance is increasing rapidly. A degree of protective immunity develops in individuals infected repeatedly from birth in moderate to high transmission areas. This transient form of immunity is also known as premunition. It is lost rapidly if the host moves out of the endemic area. In all endemic areas, children and pregnant women are at higher risk of malaria and are more susceptible to severe disease (Luxemburger *et al.*, 1997). Pregnant women are also more attractive to some malaria vectors and the non-immune pregnant traveller is at particular risk (McGready *et al.*, 2004).

2.1.3 Signs and symptoms of malaria

Uncomplicated malaria

The early signs and symptoms of malaria tend to be non-specific, characterised by fever, chills, headache, loss of appetite and body aches in adults or fever plus any other symptom in children. *P. falciparum* malaria may progress to severe disease, sometimes very rapidly.

Untreated malaria is associated with co-morbidity in the form of anaemia. *P. malariae* also causes nephropathy and nephrotic syndrome (Ashley *et al.*, 2006).

Severe malaria

Manifestations of severe malaria cover a broad spectrum from prostration to unrousable coma (WHO, 2000). The mortality rate associated with treated cerebral malaria is 20% in non-pregnant adults and 15% in children. Pregnancy puts a woman at much higher risk of developing severe disease (Luxemburger *et al.*, 2001). Cerebral malaria in pregnancy carries a 50% mortality risk even with treatment. Symptomatic pregnant women are also at risk of fever induced contractions leading to abortion or premature delivery. Adults with cerebral malaria are typically comatose. Cranial nerve abnormalities are uncommon. Tone may be increased, decreased or normal and reflexes brisk or depressed. The abdominal reflexes are invariably absent. Fixed jaw closure and teeth grinding (bruxism) may be observed. Seizures are common and may be focal or generalised. A severe metabolic acidosis is often present in severe malaria secondary to tissue hypoxia and is a major cause of death. Acute renal failure is more likely to occur in adults as is pulmonary oedema with Acute Respiratory Distress Syndrome (ARDS) which is also more common in pregnancy.

2.1.4 Pathophysiology

In humans, malaria is caused by four species of the plasmodium protozoa (single celled parasites) – *P. falciparum, P. vivax, P. ovale* and *P. malariae*. Of these species *P. falciparum* accounts for the majority of infections and is the most lethal.

Pre-erythrocyticschizogony: when an individual is bitten by the female anopheles mosquito, sporozoites are injected by the mosquito into the subcutaneous tissue (less frequently directly

into the bloodstream) and travel to the liver either directly or through lymphatic channels. They reach the liver in 30-40 minutes. Approximately 8-15 (up to 100) sporozoites are injected and therefore only a few hepatocytes are infected, therefore this stage of the infection causes no symptoms. Recent evidence indicates that sporozoites pass through several hepatocytes before invasion. Within the hepatocyte, each sporozoite divides into 10000-30000 merozoites. This phase is called pre-erythrocytic schizogony, meaning development of schizoid forms of the parasite before reaching the red blood cells. This phase takes about 10-15 days in *P. vivax* malaria but in *P. falciparum* malaria it takes about 7-10 days.

(http://www.malariasite.com/malaria/Evolution.htm).

Erythrocyticschizogony: At the completion of the pre-erythrocytic schizogony, the mature *schizonts* rupture the liver cells and escape into the blood, wherein they infect the red blood cells. These infective forms are called *merozoites* and they continue their growth and multiplication within the red blood cells. In *P. vivax* malaria, the young red blood cells are predominantly infected, while in *P. falciparum* malaria, red blood cells of all ages are affected. Thus the infective load and severity of infection are more in case of *P. falciparum* malaria. The growth and multiplication cycle within the RBCs (erythrocytic schizogony) takes about 48 hours for one cycle (72 hours in case of *P. malariae*). Each merozoite divides into 8-32 (average 10) fresh merozoites. The merozoites grow in stages into rings (trophozoites) and divide to release more merozoites. At the end of this cycle, the mature schizonts rupture the RBCs and release the new merozoites into the blood, which in turn infect more RBCs.

All the clinical features of malaria are caused by these developments in the blood. The growing parasite progressively consumes and degrades intracellular proteins, principally hemoglobin,

resulting in formation of the 'malarial pigment' and haemolysis of the infected red blood cell. This also alters the transport properties of the red blood cell membrane, and the red blood cell becomes more spherical and less deformable. The rupture of red blood cells by merozoites releases certain factors and toxins (such as lycosylphosphotidylinositol anchor of a parasite membrane protein, phospholipoprotein, RBC membrane products, protease sensitive components with hemozoin, malarial toxins etc.), which could directly induce the release of cytokines such as TNF and interleukin-1 from macrophages, resulting in chills and high grade fever. This occurs once in 48 hours, corresponding to the erythrocytic cycle. In the initial stages of the illness, this classical pattern may not be seen because there could be multiple groups (broods) of the parasite developing at different times, and as the disease progresses, these broods join and the synchronous development cycle results in the classical pattern of alternate day fever. It has been observed that in primary attack of malaria, the symptoms may appear with lesser degree of parasitemia or even with submicroscopic parasitemia. However, in subsequent attacks and relapses, a much higher degree of parasitemia is needed for onset of symptoms (http://www.malariasite.com/malaria/Evolution.htm).

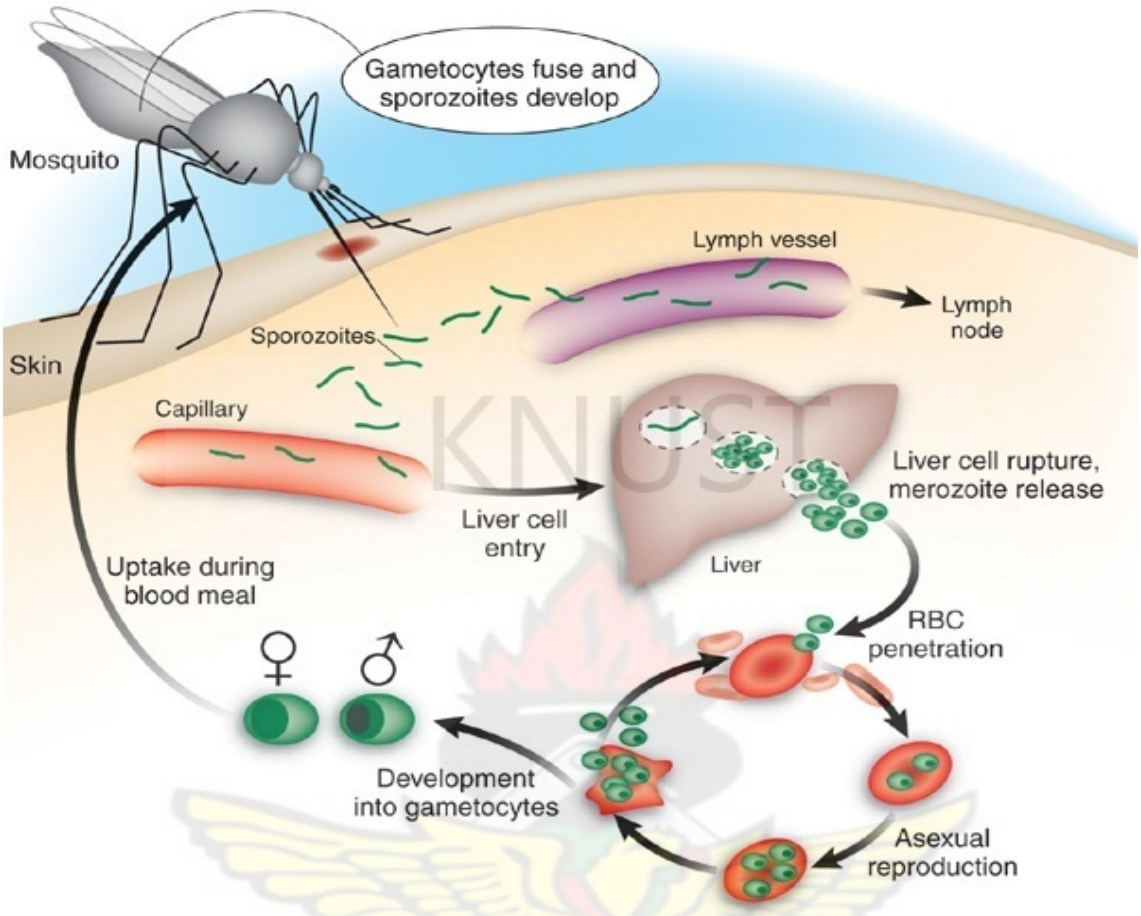


Figure 2.2 The life cycle of the *Plasmodium falciparum* parasite

Exo-erythrocyticschizogony: In *P. vivax* and *P. ovale*, some exo-erythrocytic forms remain as single celled dormant forms called hypnozoites. This helps them to survive in temperate countries. These hypnozoites can get re-activated once in 3-6 months to cause 'relapses'. This phase of the infection is called exo-erythrocyticschizogony. In *P. falciparum* and *P. malariae* infections, relapses from the liver do not occur; however, the blood infection may remain chronic and, if untreated, may remain for years in case of *P. falciparum* and decades in case of *P. malariae*. Some of the merozoites in the blood transform into sexual forms called gametocytes. These appear in the peripheral blood after 7-10 days of the infection in P.

vivax and 10-20 days in *P. falciparum* infection. When anopheles mosquito bites an infected individual, these gametocytes enter the mosquito and continue their sexual phase of development within the gut wall of the mosquito. This completes the asexual-sexual cycle of the malaria parasite.

2.1.5 Treatment

2.1.5.1 Anti-malaria drugs

The antimalarials in common use come from five classes of compounds: the quinolines and arylaminoalcohols, the antifols, the artemisinin derivatives, the hydroxynaphthaquinones and antibacterial agents.

Quinolines and arylaminoalcohols: Examples includes chloroquine, amodiaquine, quinine, quinidine, mefloquine, halofantrine, primaquine, lumefantrine and piperaquine. Chloroquine is a 4-aminoquinoline that acts mainly on the large ring-form and mature trophozoite stages of the parasite. Side-effects include pruritis, rash, headache, gastrointestinal disturbance and rarely bone marrow suppression, hair loss and convulsions. Chloroquine is known to exacerbate psoriasis. It is highly toxic in overdose and may cause fatal cardiac arrhythmias when given intravenously. Cumulative doses of chloroquine (45 years) are associated with retinal damage (Finbloom *et al.,* 1985). Amodiaquine is more active than chloroquine against resistant parasites. The side-effect profile is similar, except that prophylactic use has been associated with an unacceptably high incidence of serious toxicity with approximately 1 in 2000 patients developing agranulocytosis (Hatton *et al.,* 1986) and reports of significant hepatotoxicity (Sturchler *et al.,* 1987).

Quinine is an ancient drug derived from the bark of the cinchona tree. Side-effects are common and include tinnitus, hearing impairment, dizziness and vertigo. Quinine stimulates insulin production and may cause hypoglycaemia (Kremsner *et al.*, 1994), a particular risk in pregnancy (Looareesuwan *et al.*, 1985). Rare adverse events include renal failure, intravascular coagulation and cardiotoxicity. ECG should be monitored in patients with known atrial fibrillation or other conduction defects. For treatment of falciparum malaria infections contracted in regions where sensitivity to quinine is reduced the drug is combined with an antibiotic such as tetracycline/doxycycline or clindamycin (Kremsner *et al.*, 1994). Intravenous or intramuscular quinine is used for the treatment of hyperparasitaemic infections and for severe malaria.

Mefloquine is a quinoline methanol compound with a long terminal elimination half-life. Side effects are frequent and include dizziness, nausea, vomiting, diarrhoea and abdominal pain. Neuropsychiatric side-effects such as seizures, acute psychosis, anxiety neurosis, and major disturbances of sleep–wake rhythm are estimated to occur in 1 in 159 to 1 in 2089 patients following treatment doses (Nosten and Vugt, 1999; Weinke *et al.*, 1991). The use of mefloquine is therefore contraindicated in patients with epilepsy, a history of neuropsychiatric disease or in patients recovering from cerebral malaria. It should not be given to treat patients who have already received the drug in the preceding 4 weeks or whose ability to work safely may be impeded by drug side-effects e.g. machine operators. As a treatment it should be combined with 3 days of artesunate (Nosten *et al.*, 1994). A fixed co-formulation of the two medicines is under development. In Thailand, a retrospective study of 3587 pregnancies of which 208 were exposed to mefloquine treatment found that the drug was associated with an increased risk of stillbirth but not with abortion, low birth weight, or malformations of the fetus

(Nosten and Vugt, 1999). However a study in Malawi did not conclude there was an increased risk of stillbirth with mefloquine (Steketee *et al*., 1996).

Primaquine is an 8-aminoquinoline which eradicates hypnozoites of *P. vivax* and *P. ovale* in the liver. It has potent gametocytocidal properties. It is contraindicated in pregnancy or in individuals with glucose-6-phosphate dehydrogenase (G6PD) deficiency, as it may cause massive haemolysis (Clyde, 1981). Side-effects are usually gastrointestinal and dose-related. Tafenoquine is a promising newer 8-aminoquinoline which has a much longer half-life than primaquine meaning shorter courses are effective to eradicate hypnozoites. It is also contraindicated in patients with G6PD deficiency. This drug is still in Phase III trials. Lumefantrine is a racemic 2, 4, 7, 9-substituted fluorine derivative synthesised in China. It is manufactured as a fixed combination with artemether, one of the artemisinin derivatives. It is important to note that lumefantrine absorption varies widely between individuals and is dependent on co-administration with fat. Each dose should be taken at the same time as a fatty meal or a 200ml carton of milk (Ezzet *et al.*, 2000).

Piperaquine is a bisquinoline compound related to chloroquine developed in the 1960s which was given as mass prophylaxis and treatment for falciparum malaria in China in the 1970s and1980s when resistance began to develop. In recent years it has been co-formulated with dihydroartemisinin as a fixed dose combination that has been registered in a number of Asian countries. Experience with the drug so far suggests it is a highly effective and safe treatment for uncomplicated falciparum malaria (Ashley *et al.*, 2004).

Antifols (folate biosynthesis inhibitors) used in malaria management includes pyrimethamine, proguanil, chlorproguanil, trimethoprim.

Pyrimethamine acts by inhibiting plasmodialdihydrofolatereductase (DHFR) while the sulpha drugs, with which they are combined e.g. SP, inhibit dihydropteroate synthase (DHPS). There is marked synergy between the two compounds used in combination. Prolonged administration may cause dyserythropoiesis by interfering with folic acid metabolism; skin rashes and hypersensitivity have been described. Other side-effects include atrophic glossitis, abdominal pain and vomiting. Proguanil is a biguanide and, like chlorproguanil, acts as a prodrug for an active metabolite which also inhibits DHFR. It is a weak antimalarial and when used alone resistance developed quickly. Proguanil is well tolerated but can cause mild gastric intolerance, diarrhoea and aphthous ulceration. Doses must be reduced in patients with renal impairment. Chlorproguanil-dapsone is the latest addition to this class. The fixed combination has been registered. It is thought to be more effective than SP against *P. falciparum* in Africa (Winstanley, 2001). Haemolysis and methoglobinaemia are the most frequent adverse effects reported with dapsone. Antifolate resistance results from the sequential accumulation of point mutations in the DHFR gene. Acquisition of four DHFR mutations renders available antifols completely ineffective.

Artemisinin derivatives include artemisinin, dihydroartemisinin, artemether, artesunate. These drugs, known as quinghaosu in China, are derived from *Artemisia annua*, the sweet wormwood plant. Use of this plant in traditional Chinese medicine as a treatment for fever dates back to at least 300 AD (Li *et al.*, 1990). These drugs have a very short terminal elimination half-life of a matter of hours. They form the mainstay of the modern treatment of malaria (Price *et al.*, 1999). They act rapidly, have a broad stage specificity of action and are extremely well tolerated. Evidence of their safety and efficacy comes from large randomised trials in tens of thousands of patients. Mild gastrointestinal disturbance, dizziness, tinnitus and neutropenia

have been reported but in general the artemisinin derivatives are very well tolerated. The only potentially serious adverse effect is a type 1 hypersensitivity reactions in approximately 1:3000 patients (Leonardi *et al.,* 2001). Artesunate is a water-soluble hemisuccinate derivative of dihydroartemisinin available as oral, suppository and intravenous preparations. Artemether is the methyl ether of dihydroartemisinin and exists in oral and intramuscular forms. Its use has been associated with an usual pattern of neuronal damage to certain brain stem nuclei in animals (Brewer *et al.,* 1994). However this toxicity has not been found in humans (Hien *et al.,* 2004). The short half-life of these drugs means they are best suited to treating uncomplicated malaria combined with another drug rather than as monotherapy. If used alone a 7 day course must be prescribed. While artemisinin derivatives are not recommended for use in the first trimester of pregnancy since they cause fetal resorption in animal studies there has been no evidence of reproductive toxicity or teratogenicity from published data on their use for malaria treatment in hundreds of pregnant women (McGready *et al*., 2009).

Hydroxynaphthaquinones e.g. atovaquone.

Atovaquone is used in a fixed combination with proguanil (Malarone TM GlaxoSmithKline) for malaria prophylaxis and treatment. Used alone atovaquone resistance develops at an alarmingly rapid rate (Looareesuwan *et al.,* 1996). This combination is active on the ubiquinone metabolic pathway. Pharmacokinetic studies have shown absorption is also improved if taken with fat. Mild gastrointestinal side-effects have been described.

Examples of antibacterial drugs with antimalarial activity are clindamycin and tetracyclines. These drugs are weak antimalarials and slow acting and should not be used as monotherapy to treat malaria. Doxycycline is used for chemoprophylaxis in travellers but photosensitivity may

be a troublesome side-effect. Gastrointestinal effects such as nausea, vomiting and diarrhoea are common.

2.2 *IN VITRO* ANTIMALARIA ASSAYS

In vitro screening of compounds for antimalaria activity is based on the ability to culture *P. falciparum in vitro* in human erythrocytes. Typically, parasites are propagated in leukocyte-free erythrocytes at 2–5% haematocrit at 37°C under reduced oxygen (typically 3–5% O_2,5% CO_2,90–92% N_2) in tissue culture (RPMI 1640) media containing either human serum or Albumax (a lipid-rich bovine serum albumin). Multiple drug-resistant and drug-sensitive isolates from around the world have now been culture-adapted and can be obtained from the Malaria Research and Reference Reagent Resource Center (Noedl *et al.*, 2003). In 1976, the continuous *in vitro* cultivation of the human malaria parasite, *Plasmodium falciparum* was reported (Trager and Jensen, 1976), and this paved the way for the development of micro-titre plate assays for the determination of antiplasmodial activity in which parasite growth was assessed by measuring the incorporation of ^{3}H-hypoxanthine, which is taken up by the parasite for purine salvage and DNA synthesis (Desjardins *et al.*, 1979).

In most applications, parasites are cultured in the presence of different concentrations of test compound in media containing reduced concentrations of hypoxanthine, after which ^{3}H-hypoxanthine is added for an additional incubation period before cell harvesting and measurement of radioactive counts. IC_{50} values can be determined by linear regression analyses on the linear segments of the dose–response curves (Fidock *et al.*, 2004). Although ^{3}H-hypoxanthine incorporation is the most commonly used method to assay antimalarial activity *in vitro*, it is costly, radioactive and quite complex, and therefore problematic for

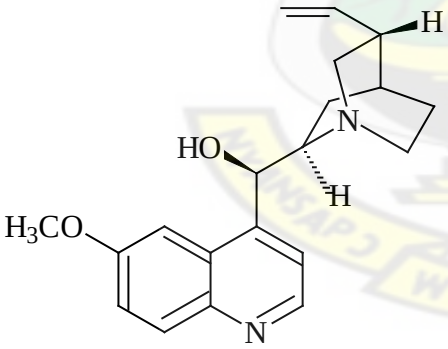
resource-poor locations or for high-throughput screening (Noedl *et al.*, 2003). A low-cost alternative for testing small numbers of compounds is to incubate parasites with test compounds (typically for 48 or 72 hours), and then to compare parasitaemias of treated and control parasites by counting GIEMSA-stained parasites by light microscopy (Fidock *et al.*, 2004).

More recently, a colorimetric method that utilises 3-acetylpyridine adenine dinucleotide (APAD) as a substrate for malaria parasite lactate dehydrogenase has been developed (Makler and Hinrichs, 1993) with the advantage that radiolabelled substrates are not required, and since parasite growth is shown clearly by the production of a dark blue colour, tests can even be carried out without the need for a spectrophotometer. The above assays have been very successfully applied to the screening of plant extracts as well as phytochemicals. Flow cytometry has also been used to test candidate antimalarial compounds, and takes advantage of the fact that human erythrocytes lack DNA. In the simplest use of this technology, parasites are fixed after the appropriate period of incubation with test compounds, then either the parasitized cells are stained with hydroethidine (which is metabolized to ethidium) or the parasite nuclei are stained with DAPI (4′,6-diamidino-2- phenylindole) (Heyde *et al.*, 1995). Counts of treated and control cultures are then obtained by flow cytometry. Recent advances in molecular biology including the sequencing of the genome of *Plasmodium falciparum* are also impacting on the development of antiplasmodial tests. Transgenic malaria parasites have been produced that express green fluorescent protein and this is expected to lead to the development of new high-throughput assays in the near future (Anon, 2005). In addition to assays that detect activity against erythrocytic parasites, methods have been developed to assess activity against liver stage parasites as well as gametocytes and we now have the

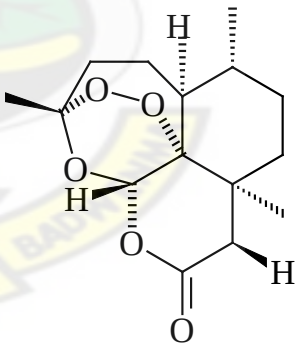
potential to examine plant extracts and compounds for their abilities to interfere with the pathological processes involved in cerebral malaria such as the adhesion of infected erythrocytes to cerebral micro vessels and the action of cytokines (Mazier *et al*., 2004).

2.3 NATURAL ANTIMALARIA AGENTS

Nature remains an ever evolving source for compounds of medicinal importance. The use of medicinal plants for the treatment of parasitic diseases is well known and documented since ancient times. For example, use of *Cinchona succiruba* (Rubiaceae) for the treatment of malaria infection is known for centuries. Several compounds isolated from nature also form a rich source of diverse structures for optimization to obtain improved therapeutic agents (Kaur *et al.,* 2009). The most important lead compound against malaria is quinine, isolated from *Cinchona* bark, which was used as a template for chloroquine and mefloquine. More recently, artemisinin, isolated from the Chinese plant *Artemisia annua*, has been used successfully against malaria that has become resistant to chloroquine (Schwikkard and Heerden, 2002).



Quinine



Artemisinin

2.4 THE FAMILY MELIACEAE

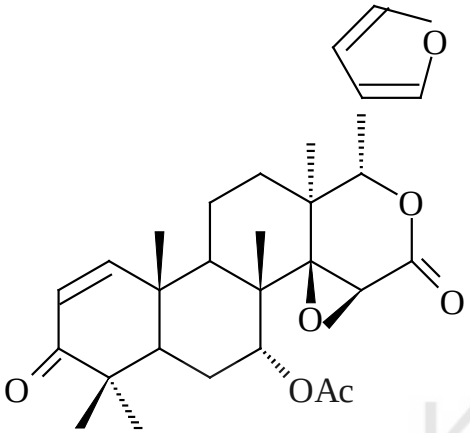
The Meliaceae, or the Mahogany family, is a flowering plant family of mostly trees and shrubs (and a few herbaceous plants). They are characterised by alternate, usually pinnate leaves without stipules and bisexual flowers borne in panicles, cymes, spikes, or clusters. Most species are evergreen, but some are deciduous, either in the dry season or in winter. The family includes about 50 genera and 550 species, with a pantropical distribution; one genus (*Toona*) extends north into temperate China and south into southeast Australia, and another (*Melia*) nearly as far north.

Various species are used for vegetable oil, soap-making, insecticides, and highly prized wood (mahogany). Some economically important species belong to this family. Examples includes sapele (*Entandrophragma cylindricum)* found in tropical Africa and mahogany species.

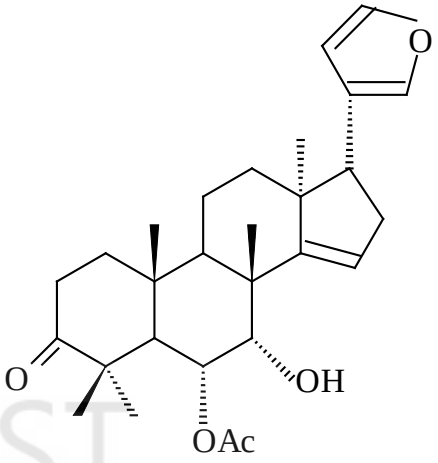
2.5 ANTIMALARIAL COMPOUNDS FROM THE FAMILY MELIACEAE

Members of the Meliaceae have been used for generations in Africa, India and tropical America to treat malaria. In tropical America *Cedrela odorata, Carapa guianensis* and *Swietenia mahagoni* have been used while in Africa and India the 'Neem' tree or *Azadirachta indica* is used. Sixty extracts of twenty-two plants of the Meliaceae were tested for activity against *P. falciparum* (MacKinnon *et al.,* 1997), using both chloroquine-sensitive and chloroquine-resistant strains. The extracts showing the highest activity against the chloroquine-sensitive strain were the leaves of *Azadirachta indica, Cedrela salvadorensis* and *Chukrasia tabularis*, the bark of *Trichilia glabra* and the wood of both *Cedrela odorata* and *Dysoxylum fraseranum*. The leaves of *A. indica, C. tabularis* and *C. salvadorensis* and the wood of *C.*

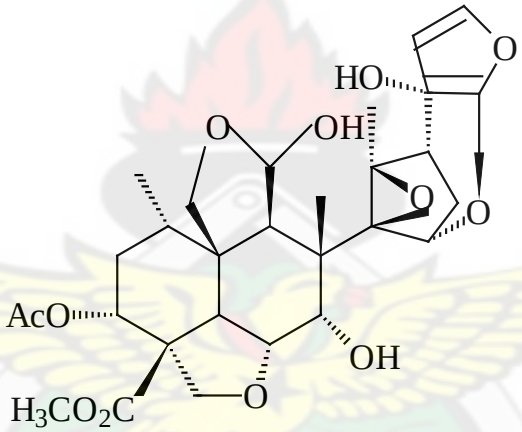
odorata and *Guarea pyriformis* showed the most activity against the chloroquine-resistant strain. The common denominator in the Meliaceae was the presence of limonoids, in particular the limonoid gedunin. In a study of *A. Indica* wood extracts from different locations, the activity increased as the percentage of gedunin increased. MacKinnon *et al.* (1997) prepared a series of nine derivatives of gedunin in an attempt to establish some sort of structure–activity relationship. None of the derivatives was as active as gedunin but a number of important characteristics were identified. It was found that the presence of an α,β-unsaturated ketone in ring A was vital for activity and that the presence of a 7α-acetate group as well as the furan ring also contributed to the activity. However, despite the promising *in vitro* activity of gedunin, it did not inhibit *Plasmodium berghei* in mice (Bray *et al.,* 1990). Work on the leaves of *Azadirachta indica* collected in India resulted in the isolation of four limonoids, of which meldenin was the most active against the chloroquine-resistant K1 strain of *P. falciparum* (Joshi *et al.,* 1998). Further investigation on *A. indica* has been carried out by Jones *et al.,* (1994) and Dhar *et al.,* (1998). They looked at azadirachtin and a series of seventeen semisynthetic derivatives and their effect *in vitro* on male gamete production from malarial microgametocytes. Azadirachtin and three of the semisynthetic derivatives were found to inhibit the formation of mobile male gametes *in vitro*. This study indicated that the presence of a hemiacetal group at C-11 was vital to the activity. Also the seeds of *A. indica,* were found to be active against all the erythrocytic stages of *P. falciparum*. In addition to inhibiting the asexual stages of the parasite, the neem extracts also revealed a gametocytocidal effect. All stages of maturation of the gametocytes were affected, unlike artemisinin and primaquine that just affect the immature stages (Dhar et al., 1998; Jones *et al*., 1994).



Gedunin



Meldenin



Azadirachtin

2.5.1 The genus *Carapa*

This is a poorly known genus of small to medium-sized trees of rain forest in western tropical Africa, central and northern South America, and the West Indies. The species present in Africa includes *C. augustifolia, C. batesii, C. macrantha, C. parviflora and C. procera (Forget et al., 2009).* Trees in the genus *Carapa* offer many ecological and economical

services to two categories of users: animals and human beings. Fruit and seeds are a staple food sources for frugivores and granivores, among them the main seed dispersers, i.e. rodents in the neotropics, and rodents and elephants in the paleotropics, which allow seedling recruitment and survival of both plants and animals on the long term. On the other hand, the trees produce timber and non-timber forest products for usage of human beings. Seeds are collected and oil extracted and used as repellent, in ethnomedecine or cosmetics (Degen *et al.*, 2001).

2.5.2 Phytochemistry and biological activities of the genus *Carapa*

One of the most widely investigated members of this genus is *Carapa guianensis.* Oil obtained from the seeds (andiroba oil) exhibited anti-inflammatory and pain-relieving properties using zymosan-induced arthritis in mice model (Penido, 2006). It has also been found to have antiparasitic and/or insecticidal actions (Mackinnon, 1997; Mesquita, 2005). It's being reported that the seed oil could prevent and even reverse cervical dysplasia (a precancerous condition that can oftentimes develop into cervical cancer) (Moura, 2002). In addition, the leaf, bark, seeds, and flowers have shown some activity against sarcoma cancer cells *in vitro*, and the crude oil passed a preliminary screening test to predict antitumor activity (Cohen, 1996). The bark has also demonstrated in vitro antibacterial activity in another clinical study (Nakanishi, 1965). Caventou isolated bitter component of *Carapa* bark called touloucounin from *Carapa touloucouna* bark. The bark contained bitter triterpenes which were non-toxic natural repellent (Caventou, 1859). Investigations confirmed that the bark have anti-bacterial and anti-tumor properties (Hammer and Johns, 1993). Main chemicals found in andiroba oil include andirobin, arachidic acid, acetoxy-gedunins, epoxyazadiradiones, deacetoxygedunins, hydroxylgedunins,

gedunins, hexadecenoic acid, linoleic acid, linolenic acid, oleic acid, palmitic acid, palmitoleic acid, and stearic acid.

2.6 CARAPA PROCERA DC

This is a tree which is widely distributed throughout the tropics. It is known synonymously as *C. touloucouna, C. gummiflua, C. velutina* and *C. microcarpa*. It is commonly called Crabwood or monkey kola. It is known locally by the names irrere, agogo and ninku (Nigeria), andiroba (Guinea), Gobi, Kowi (Sierra Leone), Toon-kor-dah (Liberia), Alla, Dona (Ivory Coast), Mujogo, Mutongana (Uganda) (Chalk *et al.,* 1933). In Ghana it is known by names such as kwakue bese (Twi), kraa bese (Fante) and asokoro (Nzema) (Busia, 2007).

2.6.1 Ecological and geographical distribution

C. procera is a lowland rainforest plant. It occurs in Ghana, Ivory Coast, Nigeria, Cameroun, Zaire and Tanzania. Its habitat range includes lake-shores, mid-altitude forest, especially where drainage is impeded, and typically at 1100-1800 m altitude. It has also frequently been recorded growing on sandy soils (Iwu, 1993).

2.6.2 Botanical description

Carapa procera varies from being a sprawling tree in swamp forest, to a tall tree in lowland rain forest. It is a tall tree often attaining a height of up to 17m with wide spreading branches and large leaves that are clustered at the ends of the twigs. The bark is reddish and smooth. The leaves are large up to 2m long with 8-20 pairs of opposite or alternate leaflets. Leaflets vary enormously in shape and size, usually from 12 to 24cm long and 1.8 to 9cm broad. They are elliptic to elongate-oblong in shape. The leaf surface displays about 10 pairs of lateral

veins, widely spaced out. The flowers appear from August to October and January to February. They are creamy white with reddish or pinkish centres, sweet scented, about 6mm long and borne on long stalks. The fruits are almost globular in shape with long or short beaks. The seeds are 15-20 per fruit, somehow resembling cola-nuts. Each seed is about 2.5cm in diameter obliquely ovoid with flattened surface, reddish –brown and have a thick woody oily kernel (Iwu, 1993).

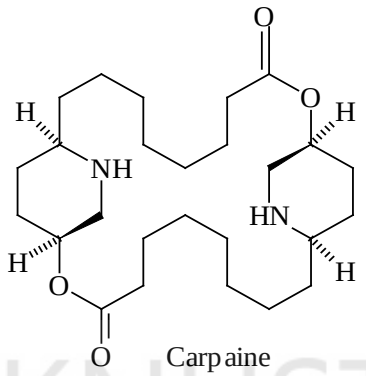
2.6.3 Medicinal and Non-medicinal uses

The good quality reddish-brown timber, known as 'Uganda crab wood', resembles true mahogany and is used for furniture and interior fittings. The fruit is edible and oil can be extracted from the seeds. Although little is known about how the species behaves in agro forestry systems, its potential as a multipurpose (oil wood) crop seems great.

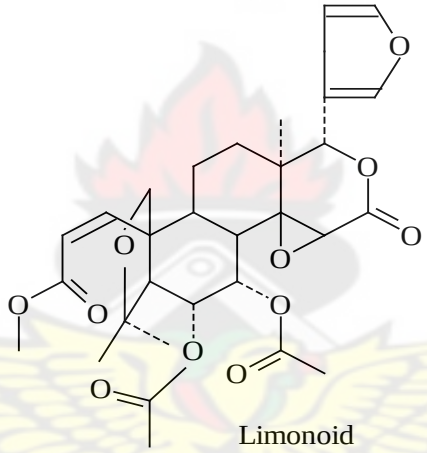
The seeds yield fat that is used for skin and hair, for treating sores, burns, rheumatic pains, insect bites, eruptions, ring worms and yaws. It is also used as a vermifuge for both tapeworms and roundworms (Iwu, 1993).

2.6.4 Phytochemistry and Biological activities

Oil-based formulations of *Cocos nucifera*, *Elaeis guineensis* and *C. procera* were effective as mosquitoe repellent (Konan *et al*., 2003). *Carapa procera* is reported to contain tannins, glycoside, triterpnoids (limonoids), fatty acids and a bitter principle tulukinin (Busia, 2007).



Carpaine



Limonoid

2.7 THE FAMILY APOCYNACEAE

The family Apocynaceae is very large, made up of about 250 genera and 2000 species (Dutta, 1981; Trease and Evans, 1989). The most important genera in terms of numbers are Tabernaemontana (110 spp.), Rauwolfia (100 spp.), Ervatamia (80spp.), Alstonia (50 spp.) and Landolphia (50 spp.) (Dutta, 1981).

The members of the family have diverse habits. Some members exist as trees e.g., *Alstonia*, *Holarhena*, *Writghtia* and *Carissa.* Other members of the family also exist as shrubs e.g.

Rauwolfia or as herbs e.g. *Catharanthus* (MetCalfe and Chalk 1950). Members of the family are found throughout the tropical and subtropical world.

2.7.1 The genus *Alstonia*

Alstonia is a widespread genus of evergreen trees and shrubs of the dogbane family (Apocynaceae). *Alstonia* consist of about 40-60 species, native to tropical and subtropical Africa, Central America, South East Asia and Australia. These trees can grow very large, for instance *A. pneumatophora* is recorded with a height of 60m and a diameter of more than 2m. Alstonia trees are used in traditional medicine. The bark of *Alstonia constricta* and *Alstonia scholaris* is a source of remedy for malaria, toothache, rheumatism and snake bites. The latex is used in treating coughs, throat sores and fever. Many Alstonia species are commercial timbers (Sidiyasa, 1998).

2.7.2 Phytochemistry

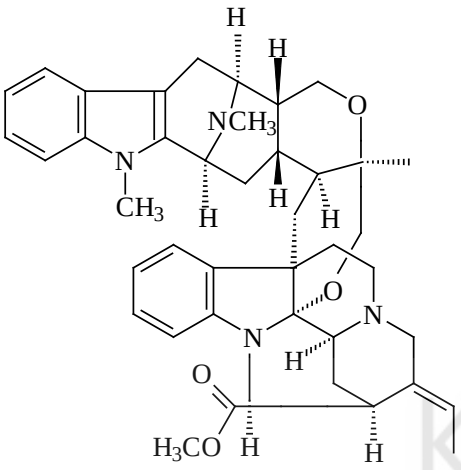
The plant has elaborate and wide range of compounds, from simple but rare monosaccharide to complex secondary metabolites, including alkaloids, terpenes and steroids. Most of the alkaloids reported are of the indole type but other alkaloids groups, e.g. furoquinolines have also been reported in the family. The distribution of alkaloids within the family is not even. Of the 250 genera in the family, alkaloids have been reported in only 25 genera (Trease and Evans 1989). Many other non-alkaloidal compounds have been reported in the family. The family Apocynaceae has a variety of plants with elaborate identical secondary metabolites, some of which are useful medicinal compounds, e.g. Reserpine which is antihypertensive (Cordell, 1981) has been isolated from several species of *Alstonia* (Glasby 1975). A variety of physico-

chemical characteristics are shown by alkaloids of the family Apocynaceae. Most compounds have medium to high polarizabilities, high melting point and highly capable of isomerisation.

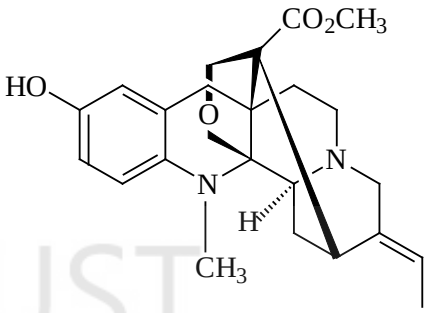
Echitamine isolated from *Alstonia boonei* De wild has three molecules of water of crystallization but it dehydrates slowly at 100°C to give a more stable monohydrate (Glasby, 1975).

2.8 ANTIMALARIA COMPOUNDS FROM THE FAMILY APOCYNACEAE

There are 43 species of the genus *Alstonia* distributed throughout Africa, Central America, China, South East Asia, and the Pacific. A number of species have been reported to be used by traditional healers in the treatment of malaria (Wright *et al.*, 1993). More than 130 alkaloids have been isolated from *Alstonia* species, only a few of which have been assessed for antimalarial activity. Wright *et al.* (1993) investigated *Alstonia angustifolia*, a plant that is used in South-East Asia to treat malaria and dysentery. Nine alkaloids were isolated and tested and the most active one against *P. falciparum* was villalstonine. François *et al.,* (1996) and Kapadia *et al.,* (1993) investigated the antiplasmodial activity of *Picralima nitida*. They investigated the organic and aqueous extracts of the roots, stem bark, fruit rind, seeds and leaves. A wide range of activities were noted with the highest activities being found in the root dichloromethane extract, the stem bark dichloromethane extract and the fruit rind aqueous extract (IC_{50} values of 0.188, 0.545 and 1.581 μg/ml respectively). An active alkaloid, akuammine, was found in the seeds of *P. Nitida* (François *et al.,* 1996 ; Kapadia *et al.,* 1993)



Villalstonine



Akuammine

2.9 ALSTONIA BOONEI

Alstonia boonei De Willd. is commonly known as pattern wood or stool wood. It is synonymously known as *Alstonia congensis* Engl.

Local names

In Ghana it is known by the local names ‘onyamedua’ (Twi), ‘adawura’ (Ga-Adangbe), ‘Nyamenlebaka’ (Nzema) and Siaketekre (Ewe).

2.9.1 Ecological and geographical distribution

It is a deciduous tree that grows to about 35m; found in the forest zones of Ghana and throughout tropical Africa.

2.9.2 Botanical description

Alstonia boonei is a tree about 25–40m high with white latex. The trunk has a diameter of about 1.4 m, with or without buttresses. It has a grey, white or yellowish bark which can be smooth or scaly. Leaves occur in whorls of 4–9.

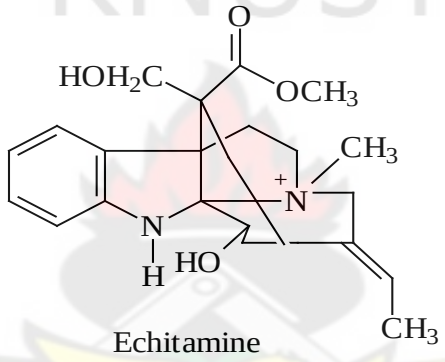
2.9.3 Ethnomedicinal uses

The stem bark is commonly used in malaria treatment, and is listed in the African pharmacopoeia as an anti-malarial drug. An infusion of the bark is used as anti-venom for snake bites, and in the treatment of arrow poisoning. The stem bark is also an astringent, alternative tonic and a febrifuge for relapsing fevers. The leaves and latex of *A. boonei* are used topically to reduce swellings for the treatment of rheumatic pains, muscular pains and hypertension (Dalziel, 1937; Irvine 1961; Oliver-Bever, 1986; Iwu, 1993). Ojewole (1984) and Asuzu and Anaga (1991) reported that the plant stem bark is used in traditional medicine for treating ailments such as malaria, painful micturition and rheumatic conditions. A closely related species, *Alstonia congensis* was reported by Awe and Opeke (1990), to be effective against *Plasmodium berghei* in mice.

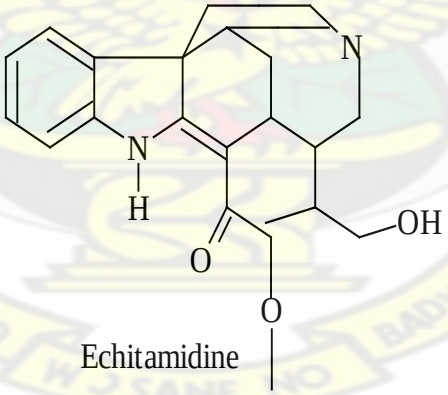
2.9.4 Phytochemistry and biological activities

Alstonia boonei exhibits a wide range of pharmacological activities such as antipyretic, anti-inflammatory, analgesic, anti-rheumatic and antimicrobial activities (Olajide *et al*., 2000). The analgesic and anti-inflammatory activity have been shown in many laboratory studies. Aqueous extracts of the stem bark had a contractile effect on both guinea pig ileum and rat stomach strip *in vivo*; the effect was more pronounced on rat stomach strip than on guinea pig ileum (Taiwo and Makinde, 1996). The extracts inhibited carragenan-induced paw oedema, cotton pellet

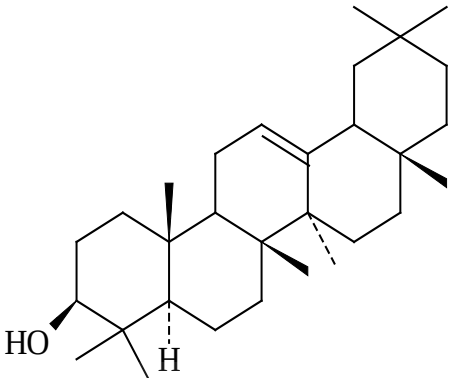
granuloma and acetic acid induced vascular permeability (Olajide *et al*., 2000). The alcoholic extract demonstrated protection against egg white–induced rat hind paw oedema (Osadebe, 2002). Extracts of *A. boonei* have potential anti-helminthic effects by the ability to inhibit gluthatione S- transferase from parasitic nematodes. A number of chemical constituents have been isolated from *A. boonei*, some of which includes the alkaloids echitamine, echitamidine and related alkaloids and triterpenoids (lupeol, ursolic acid, β-amyrin); iridoids, loganin.



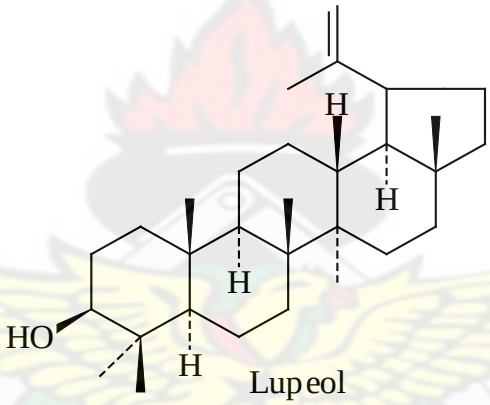
Echitamine



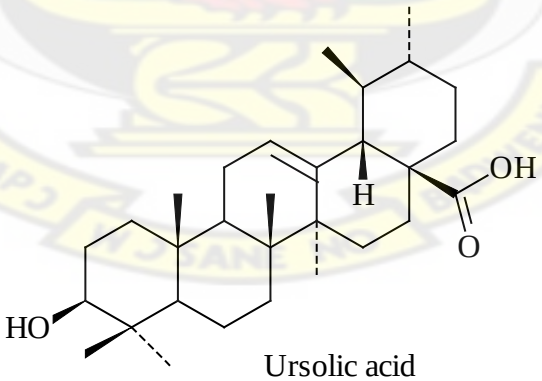
Echitamidine



β-amyrin



Lupeol



Ursolic acid

2.10 NATURAL PRODUCTS AND DRUG DISCOVERY

Plants are indispensable to man as far as life is concerned. They have been used as food, as sources of shelter, medicines and even as poisons since the beginning of mankind (Cordell, 1981). The Industrial Revolution and the development of organic chemistry resulted in a preference for synthetic products for pharmacological treatment. The reasons for this were that pure compounds were easily obtained, structural modifications to produce potentially more active and safer drugs could be easily performed and the economic power of the pharmaceutical companies was increasing (Rates, 2001). Furthermore, throughout the development of human culture, the use of natural products has had magical-religious significance and different points of view regarding the concepts of health and disease within each culture. Western societies considered drugs from natural sources as either an option for poorly educated or low income people or simply as religious superstition of no pharmacological value. However, even if we only consider the impact of the discovery of the penicillin, obtained from micro-organisms, on the development of anti-infection therapy, the importance of natural products is clearly enormous. About 25% of the drugs prescribed worldwide come from plants, 121 such active compounds being in current use. Of the 252 drugs considered as basic and essential by the World Health Organisation (WHO), 11% are exclusively of plant origin and a significant number are synthetic drugs obtained from natural precursors (Rates, 2001). Examples of important drugs obtained from plants are digoxin from *Digitalis* spp., quinine and quinidine from *Cinchona* spp., vincristrine and vinblastine from *Catharanthus roseus*, atropine from *Atropa belladonna* and morphine and codeine from *Papaver somniferum*. It is estimated that 60% of anti-tumour and anti-infectious drugs already

on the market or under clinical trial are of natural origin (Yue-ZhongShu, 1998). The vast majority of these cannot yet be synthesised economically and are still obtained from wild or cultivated plants. Natural compounds can be lead compounds, allowing the design and rational planning of new drugs, synthesis and the discovery of new therapeutic properties not yet attributed to known compounds (Hamburger and Hostettmann, 1991). In addition, compounds such as muscarine, physostigmine, cannabinoids, yohimbine, forskolin, colchicines and phorbol esters, all obtained from plants, are important tools used in pharmacological, physiological and biochemical studies (Williamson *et al.*, 1996). In recent years, there has been growing interest in alternative therapies and the therapeutic use of natural products, especially those derived from plants. This interest in drugs of plant origin is due to several reasons, namely, conventional medicine can be inefficient (e.g. side effects and ineffective therapy), a large percentage of the world's population does not have access to conventional pharmacological treatment, and folk medicine and ecological awareness suggest that "natural" products are harmless.

It is estimated that, in 1997, the world market for over the- counter phytomedicinal products was US$ 10 billion, with an annual growth of 6.5% (Soldati, 1997; Vulto and Smet, 1988). The WHO considers phytotherapy in its health programs and suggests basic procedures for the validation of drugs from plant origin in developing countries (Vulto and Smet, 1988). Eastern countries, such as China and India, have a well-established herbal medicines industry and Latin American countries have been investing in research programs in medicinal plants and the standardisation and regulation of phytomedicinal products, following the example of European countries, such as France and Germany. In Germany, 50% of phytomedicinal products are sold on medical prescription, the cost being refunded by health insurance (Gruenwald, 1997). Thus

research on medicinal plants used in folk medicine represents a suitable approach for the development of new drugs (Calixto, 2000; Elisabetsky and Posey, 1986).

If the lead compound (or active principle) is present in a mixture of other compounds from a natural source, it has to be isolated and purified. Therefore, in more recent history, the use of plants as medicines has involved the isolation of active compounds, beginning with the isolation of morphine from opium in the early 19th century (Kinghorn, 2001). Drug discovery from medicinal plants led to the isolation of early drugs such as cocaine, codeine, digitoxin, and quinine, in addition to morphine, of which some are still in use (Butler, 2004; Newman *et al.*, 2000; Samuelsson, 2004). Isolation and characterization of pharmacologically active compounds from medicinal plants continue today. More recently, drug discovery techniques have been applied to the standardization of herbal medicines, to elucidate analytical marker compounds. The methods utilized to acquire compounds for drug discovery includes isolation from plants and other natural sources, synthetic chemistry, combinatorial chemistry, and molecular modeling (Geysen *et al.*, 2003; Ley and Baxendale, 2002; Lombardino, 2004).

Natural products have played an important role as new chemical entities (NCEs) approximately 28% of NCEs between 1981 and 2002 were natural products or natural product-derived (Newman *et al.*, 2003). Another 20% of NCEs during this time period were considered natural product mimics, meaning that the synthetic compound was derived from the study of natural products (Newman *et al.*, 2003). Combining these categories, research on natural products accounts for approximately 48% of the NCEs reported from 1981–2002. Natural products provide a starting point for new synthetic compounds, with diverse structures and often with multiple stereocenters that can be challenging synthetically (Clardy and Walsh, 2004; Koehn and Carter, 2005; Nicolaou and Snyder, 2004; Peterson and Overman, 2004).

Thus not all natural products can be fully synthesized. Some have very complex structures that are too difficult and expensive to synthesize on an industrial scale. These include drugs such as vincristine, morphine, and paclitaxel (Taxol). Such compounds can only be harvested from their natural source; a process which can be tedious, time consuming, and expensive, as well as being wasteful on the natural resource.

The best example of drug development based on traditional medicine is Artemesinin, a sesquiterpene lactone with potent antimalaria activity against multiple-resistant plasmodium falciparum. It is the first line drug for the treatment of malaria in most countries. It was rescued from a millenary traditional use of the plant *Artemisia annua* L. (Gen and Lin, 1986; Philpson, 1986). This has resulted in investigation of most folk medicines used for the treatment of malaria.

2.11 ANALYTICAL METHODS IN NATURAL PRODUCT SEPARATION

2.11.1 Extraction methods

Extraction methods employed in natural medicinal product processing takes into account knowledge of several physicochemical properties of the compound(s) of interest. These include partition coefficients in water or organic solvents, relative polarity of the molecule, stability of the molecule in light or dark, as well as the temperature employed during the extraction process. So, if the compounds of interest are highly soluble in water, hot or cold water is employed to obtain an aqueous extract. If, on the other hand, the compound is highly soluble in a particular organic solvent, that solvent is employed to obtain an organic-solvent extract (Cseke *et al.*, 2006).

2.11.2 Aqueous extraction of compounds

Traditional Methods of Aqueous Extraction*:* The preparation of herbal remedies based on traditional methods of water extraction utilizes two different approaches: if extracting herbaceous tissues of leaves, roots, and flowers, or soft-textured fruits with a relatively high water content (in the range of 60 to 95% water) with hot water or cold water, relatively mild physical conditions are used to obtain what is called an infusion. However, for woody highly lignified tissues with relatively low water content (in the range of 5 to 50% water), such as roots, barks, twigs, and some dry fruits, more vigorous physical extraction procedures are employed, using longer extraction times and boiling water, to obtain what is called a decoction (Cseke *et al*., 2006).

Laboratory Methods of Aqueous Extraction: In contrast with the traditional methods of aqueous extraction described above, laboratory methods for aqueous extraction rely on the use of more stringent quantitative procedures and more sophisticated equipment.

2.11.3 Laboratory methods of organic solvent extraction of compounds

If the compounds of interest are not soluble in water because of their non-polar nature, an organic solvent (e.g., acetone, methanol, ethanol, chloroform, diethyl ether, methylene chloride, or a combination of more than one organic solvent) is selected to carry out the extraction. The temperature of this extraction depends on the boiling point of the solvent chosen and must be carefully watched due to the special equipment that is used. One can use a Soxhlet extractor, which is basically a specialized glass refluxing unit that is used for such organic-solvent extractions. When higher temperatures are used, there is always the risk of

degrading some of the active compounds; thus, low-boiling-point solvents, such as dichloromethane or diethyl ether, are usually the best choice (Dai *et al*., 1999).

2.11.4 Ultrasonic Extractions

Ultrasonic extraction (often called sonication) uses high-frequency sound to liberate phytochemicals from the plant materials. This extraction process is fast compared with traditional laboratory methods, such as maceration or Soxhlet extraction, because of particle disruption of the plant material. This type of extraction was recently used for the isolation of essential oils (Salisova *et al*., 1997), polysaccharides (Hromadkova *et al.*, 1999; 2002), and bioactive phytochemical (Vinatoru *et al.*, 1997), including menthol (Shotipruk *et al*., 2001), cardiac glycosides (Ikeda *et al.*, 1995) and pyrethrins (Romdhane and Gourdon, 2002). However, the procedure presents problems when attempting to isolate very large molecules, such as DNA or large proteins, due to the shearing forces that occur during sonication. The process of sonication can also generate a lot of heat, so various heat labile compounds, such as proteins; require steps to keep them cold during sonication.

2.11.5 Chromatographic separation methods

Chromatography is one of the most useful means of separating mixtures of compounds, as a technique to both purify the components and identify them. In chromatography, the mixture is separated by distribution of the components between a stationary phase and a mobile phase. Primary methods of chromatography in isolation and analysis of natural products include the following: thin layer chromatography (TLC), liquid column chromatography (LC), gas chromatography (GC), high-performance liquid chromatography (HPLC) and immobilized metal-ion affinity chromatography (Heftmann, 1992a; 1992b). There are probably five major

separation mechanisms of chromatography. They include adsorption chromatography, gas chromatography, liquid–liquid partition chromatography, ion-exchange chromatography, and size-exclusion chromatography.

2.11.5.1 Gas chromatography

Gas chromatography is a chromatographic technique that can be used to separate volatile organic compounds. A gas chromatograph consists of a gaseous mobile phase, an injection port, a separation column containing the stationary phase, and a detector. The organic compounds are separated due to differences in their partitioning behavior between the mobile gas phase and the stationary liquid phase in the column. The volatilities of the compounds, which strongly correlate to their boiling points, are mostly responsible for the partitioning between the liquid phase and the gas phase (Marongiu *et al*., 2004).

In natural products, GC instruments are typically used for the evaluation of highly volatile essential oils. Because, in many cases, retention time is the only parameter to distinguish individual compounds.

2.11.5.2 Ion-exchange chromatography

In this method, separation is based on ionic interactions of the individual components of a mixture with a stationary phase that is an ionically charged surface of opposite charge to the sample ions. The mobile phase is an aqueous buffer, where both pH and ionic strength are used to control elution time. The stronger the charge on the sample, the stronger it will be attracted to the ionic surface and thus, the longer it will take to elute it from the column. Typical applications in natural products chemistry are in the separation of fruit acids (Cseke *et al.*, 2006).

2.11.5.3 Affinity Chromatography

This is a powerful means of purifying proteins. The principle of this technique is based on the fact that some proteins have a very high affinity for specific chemical groups (ligands) covalently attached to a chromatographic bed material (the matrix). After loading and running the protein mixture through the column, only proteins with a high affinity to the ligand can bind to the column matrix. Other proteins, in contrast, run through the column, because they are unable to bind to the ligand (Porath, 1988). The bound proteins can then be eluted from the column by a solution containing a high concentration of the soluble form of the ligand or, in some cases, by changes in the buffer conditions that cause the bound proteins to change conformation. A number of affinity columns are commercially available, depending on the particular protein purification desired (Bruno, 1991).

2.11.5.4 Size-exclusion chromatography

Size-exclusion chromatography is also referred to as gel filtration or permeation chromatography (Bruno, 1991). The separation of mixtures is based strictly upon size. It involves the use of porous gel molecules of agarose, cross-linked dextran, or polymers of acrylamide that allow the separation of compounds based on their molecular sizes. The column is filled with material that has precisely controlled pore sizes, and the sample is simply screened or filtered according to its solvated molecular size. Molecules that are too big to fit in the pores of the solid support will travel straight through the gel, and hence, elute from the column first. Smaller molecules, however, penetrate inside the porous packing particles, which leads to a longer path through the column, and as a result, makes them elute later. Typical applications for size-exclusion chromatography are separations of biomacromolecules, such as proteins (Cseke *et al.*, 2006).

2.11.5.5 Partition Chromatography

Partition chromatography, often called liquid–liquid partition chromatography, involves two liquid phases. The stationary phase is an adsorbed solvent held on the surface or within the grains or fibers of an inert solid supporting matrix. Examples of inert supports include sheets of paper (cellulose) as used in paper chromatography. The separation of mixtures is based on the partitioning of the individual components between two immiscible liquid phases i.e the solubility differences of each component in the mobile phase and the stationary phase. In paper chromatography, a thin film of water on the paper constitutes the stationary phase. Separation is achieved when the mobile phase travels up the paper via capillary action. After the mobile-phase trails off the paper sheet, the paper is hung to dry in a fume hood, where it can then be sprayed with reagents (e.g., ninhydrin reagent for amino acids) that give colour to the separated compounds of interest in white or UV light. Some compounds of interest have their own distinctive colours (e.g., chlorophylls), and hence, can be purified using this technique.

2.11.6 Adsorption chromatography

In adsorption chromatography, finely divided inert adsorbent materials (e.g., silica gel or alumina) serve as the stationary phase, and organic solvents serve as the mobile phase. Separation of the mixture, then, is achieved by differences in polarity of the individual components. Adsorption chromatography can be further subdivided into normal-phase chromatography and reversed-phase chromatography. In normal-phase chromatography, the stationary phase is of a polar nature (hydrophilic), typically silica. In order to achieve separation, the polarity of the solvent is adjusted to the polarity of the mixture. It ranges from non-polar solvents such as hexane to very polar methanol or even water. Polar samples are

retained on the polar surface of the column packing longer than less-polar materials. On the other hand, reversed-phase chromatography consists of modified silica surfaces, such that the nature of the stationary phase becomes non- polar. The mobile phase is a polar solvent, such as water–methanol mixtures or water–acetonitrile mixtures. Examples of adsorption chromatography include thin layer chromatography and liquid column chromatography.

2.11.6.1 Thin-Layer Chromatography (TLC)

In thin-layer chromatography (TLC), the adsorbent is coated on one side of a plate of glass or a strip of plastic or aluminum. Common adsorbents are silica gel and alumina. A few microliters of a solution of the sample to be analyzed are spotted onto the plate as a single small dot near one end of the plate using a microcapillary tube. The plate is developed by placing it in a jar or developing chamber that contains a small amount of solvent. The solvent rises up to the plate by capillary action, carrying the components of the sample with it. The different compounds are separated based upon their interaction with the adsorbent coating. The detection of spots is generally achieved using an ultraviolet (UV) lamp (if the TLC plates have the fluorescent indicator) or iodine vapour. The detection of components of TLC plates may also be accomplished by spraying the plates with a suitable reagent (e.g. Anisaldehyde reagent).

2.11.6.2 High-Performance Liquid Chromatography (HPLC)

HPLC typically uses small particle sizes for the stationary phase, which results in a fairly large backpressure when the mobile phase is passed through this bed. As a consequence, the only way to achieve flow of the mobile phase is to use pump systems. The pressure of HPLC typically reaches 150 to 200 bars. In certain cases, larger pressures are unavoidable. Modern instrumentation is able to handle up to 400 bars. Typically, an HPLC system consists of the

following components: solvent reservoir, injection system, column, HPLC pump, detector, sample collector (optional), and a computer serving as a data station for the detector information as well as a way to control and automate the HPLC pump.

More sophisticated instruments consist of two or three pumps as well as a number of detectors to characterize the sample. The advantage of having more than one pump is that solvent gradients can be programmed, meaning that the solvent composition can be changed continuously throughout the chromatography. This allows for the separation of a much larger range of compounds because the mobile phase can be adjusted to the changing polarity of the mixture. As an additional benefit, chromatographic peaks get sharper, they elute in a smaller volume from the column and the separation can be done in a much shorter time period.

Almost every HPLC system is equipped with a UV detector in order to detect compounds of interest. Traditionally, these UV detectors were single-wavelength detectors; however, with cheaper hardware, photodiode-array (PDA or DAD) detectors that permit the scanning of the full UV-visible range to 650 nm) are becoming more popular. For the separation of natural products, this is of great advantage because depending on the compounds involved, there can be very large differences in their UV maxima. Natural products, such as sugars, that absorb in the range of the solvent systems are detected using refractive-index (RI) detectors or evaporative light-scattering detectors (ELSDs). In addition, MS and NMR can be used to detect compounds. The latter techniques are always used in combination with UV detection. In most cases, HPLC is implemented as an analytical technique with either reversed-phase or normal phase materials. The typical load for an analytical column is well below 1 mg for the overall mixture. However, up to 5 mg of sample are possible in favorable cases. Common flow rates for analytical separations are 1ml min^{-1}. Silica columns are commonly used. However, the use

of alumina packing or modified silica columns, such as diol, cyano, or amino phases, is possible. The advantage of the modified silica phases is their ability to equilibrate quickly, contrary to silica or alumina phases.

Chapter 3

MATERIALS AND METHODS

3.1 PLANT COLLECTION AND IDENTIFICATION

The stem bark of *Carapa procera* DC(Meliaceae) and the leaves and stem bark of *Alstonia boonei* De Willd(Apocycnaceae) were harvested from a farm in Effiduase, a town in the Sekyere-East district of the Ashanti Region. The plants were identified by Mr. George Henry Sam of the herbarium section of the department of herbal medicine, Faculty of Pharmacy and Pharmaceutical sciences, Kwame Nkrumah University of Science and Technology.

3.2 PLANT MATERIAL PROCESSING

The leaves and stem barks were initially screened for foreign matter like 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 on the leaves. The barks were then chopped into smaller bits about 2cm x 2cm with a cutlass and air dried in the organic laboratory of chemistry department for seven days. The materials, thus dried, were coarsely milled and packed into a brown paper bag and kept in the laboratory until required for use.

3.3 EXTRACTION

3.3.1 Solvents and chemicals

Petroleum ether and ethanol (70%) were the main solvents used for the extraction of the plant materials. Solvents used for the extraction of the powdered plant materials were

supplied from B.D.H. Ltd (England) and they were of the British pharmacopoeia (BP) grade.

3.3.2 Preliminary extraction of powdered plant material

65.5g of coarsely powdered stem bark of *C. procera* and 72.5g of *A. boonei* leaves and stem barks were packed into a calico gray baft and tied. These were packed separately into a soxhlet apparatus and extracted successively with petroleum ether and 70% Ethanol over 48hours until the material was completely exhausted. The extraction was considered complete when the colour of the percolate had turned to colourless. This was further confirmed by thin layer chromatographic examination of the percolate. Each extract was concentrated under reduced pressure to a small volume by means of rotavapor (R-114, Buchi, Switzerland) at a temperature of 40ºC. Each concentrate was then evaporated to dryness on a water bath. They were then kept in a desiccator in the laboratory until needed. Similar weights of powdered plant materials were extracted again by cold maceration.

From the results of the antiplasmodial bioassay (section 4.2.2), the hot ethanolic extract of *C. procera* showed the highest antimalarial activity. Hence about 350g of the powdered stem bark was subsequently soxhlet extracted sequentially with petroleum ether and 70% ethanol. About 40g of the 70% ethanolic extract was obtained which was kept in a dessicator for subsequent bioassays and column chromatographic fractionation.

3.4 ANTI-MALARIA ASSAY

3.4.1 *In vitro* cultivation of malaria parasite

Frozen laboratory strains of *P. falciparum* (chloroquine sensitive 3D7 strain) were cultured according to the method described by (Hout *et al.,* 2006) with slight modifications.

Parasite vials were taken from a liquid nitrogen tank, thawed quickly in a water bath set at 37 ºC. The vials were spun 10 minutes at 2000rpm and the supernatant discarded. An equal volume of thawing mix (3.5% NaCl in distilled water) was added to each of the pellet, thoroughly mixed and spun at 2000rpm for 10 minutes. The pellets were gently disengaged and 1ml of complete parasite medium (5ml of L-glutamine, 2.5ml of 10mg/ml and 50ml Albumax in 500ml of RPMI 1640) was added and span again at 2000rpm for 10 minutes. This washing step was repeated and the supernatant discarded. The parasites were suspended in 25ml culture flask (BD Falcon) containing 200µl freshly prepared pack RBC (sickling negative; O rhesus positive) and 5ml of complete parasite medium to have a haematocrit of 4%. The culture was then gassed with a special gas mixture (2% Oxygen, 5.5% Carbon dioxide and 92.5% Nitrogen) for 30 seconds for 25ml culture flask or 90 seconds for 75ml culture flask. The flasks were quickly closed and placed in an incubator (RS Biotech) set at 37^{o}C. The spent culture medium was changed daily using fresh complete parasite medium. Spent complete parasite medium was aspirated out from the culture flask using pipette and each time a thin smear was prepared on microscope slides under sterile conditions in the laminar flow safety cabinet (Hitachi Clean Bench, Japan). The slides were fixed in absolute methanol, stained with 10% giemsa in phosphate buffer for 10 minutes and after adding a drop of immersion oil (Fluka, BDH England) examined using 100X magnification light microscope to check the level of parasitaemia, growth stages and viability of the parasites. The culture flasks were then sent back into the incubator after adding appropriate amount of complete parasite medium and gassing (Hout *et al.*, 2006).

The parasitaemia was determined as below:

$$\% \text{ Parasitaemia} = \frac{\text{Number of infected RBCs X 100\%}}{\text{Total RBC count (IRBC} \pm \text{URBCs)}}$$

Where: IRBCs = RBCs infected with *P. falciparum*

URBC = RBCs not infected with *P. falciparum*

At a parasitaemia of about 5%, subcultures (of the 3D7) were made by adding known amount of fresh and pack sickling negative O rhesus positive (O^+) RBCs to the culture to reduce the parasitaemia and always maintaining 200ul or 1ml of the pack red cells in 25ml or 75ml culture falsk respectively The parasites were maintained in continuous culture to obtain a stable parasitaemia before they were used for the efficacy assay.

3.4.2 Incubation of Parasites with Plant Extracts

Thin smears were prepared and the level of parasitaemia determined. The parasites were then diluted with non-infected O^+ RBC to obtain 1% parasitaemia. Complete parasite medium was then added to the culture in the culture flask to have a haematocrit of 4.44%. Nine hundred microlitres (900ul) aliquots of the culture at 1% parasitaemia were separately dispensed into each of twenty-four micro titre plates at haematocrit. One hundred microlitres (100µl) of the prepared plant extract at the various dilutions (100-0.09mg/ml) were then added to the labelled wells except the first 2 wells which served as positive controls and the last 4 in order to observe the effect of the drug on normal RBC whiles the

remaining 2 wells served as drug negative controls while the remaining two wells served as non-drug negative controls. Standard antimalaria drug artesunate was tested at dilutions between 0.000048-12.5µg/ml. Assays were performed in duplicates and under aseptic conditions. In all the cases, the addition of 100ul/medium to the culture in the microtitre plate broght the haematocrit to 4%. The micro titre plates were covered and placed in a clean modular incubator chamber (Califonia, USA) tightly closed and gassed for 6 minutes. The chamber containing the plates were then placed in the incubator previously set at 37^{o}C for 48 hrs. After 48 hours, cultures were removed from incubator and 2 thin smears prepared from each of the wells on double frosted microscope slides (Fisher, USA). Slides were air-dried, fixed with methanol and stained with 10% giemsa in phosphate buffer (Fluka, BDH England). The slides were then examined using the light microscope at 100X magnification after a drop of immersion oil was added.

The same procedure was used for the fractions $H_{2B,}$ $H_{2C,}$ H_{2D} and H_{2E} except that they were tested at dilutions between 0.03 and 31.25 µg/ml.

3.5 STATISTICAL ANALYSIS OF DATA

Growth inhibition due to each extract defined as the difference between the % parasitaemia of each treatment group and the corresponding positive control was calculated as follows (Ene *et al.*, 2009).

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

Where CIRBC = % parasitaemia of infected RBC without extracts i.e. control

DIRBC = % parasitaemia of infected RBCs incubated with extract or standard drug

Total parasitaemia over the 48 hour period was calculated in arbitrary unit as the area under the curve (AUC). Differences in AUCs were analyzed by one way analysis of variance (ANOVA) followed by Student-Newman-Keuls' *post hoc t* test.

Doses and concentrations responsible for 50% of the maximal effect (IC_{50}) for each drug/extract were determined using an iterative computer least squares method, with the following nonlinear regression (three-parameter logistic) equation.

$$Y = \frac{a + (b - a)}{(1 + 10^{(LogIC50 - X)})}$$

Where, *X* is the logarithm of dose and *Y* is the response. *Y* starts at *a* (the bottom) and goes to *b* (the top) with a sigmoid shape (Miller, 2003; Motulsky *et al.*, 2003). Using regression equations of best fit of plotted growth inhibition versus concentration curves, the IC_{50} of each plant extract against each of the parasite strains were obtained.Graph Pad Prism for Windows version 5.0 (Graph Pad Software, San Diego, CA, USA) was used for all statistical analyses. $P < 0.05$ was considered statistically significant (Rowe, 2007).

3.6 PHYTOCHEMICAL SCREENING OF EXTRACT

Preliminary phytochemical screening of the extracts was performed to identify the main phytochemical groups or secondary metabolites present in the stem bark of *Carapa procera*. This included the test for alkaloids, phenolic compounds, reducing sugars, flavonoids, saponins, triterpenes and phytosterols (Harbone, 1976).

3.6.1 Tannins

About 0.1 g of the powdered plant material was boiled with 5mls of water for 5 minutes. It was then cooled, filtered and the volume adjusted to 10mls. To 1ml aliquot of the aqueous extract was added 10mls of water and 5 drops of 1% lead acetate. The colour and amount of precipitate, if any, was noted and recorded. The procedure was repeated using 5 drops of 1% ferric chloride (Odebiyi and Sofowora, 1978).

3.6.2 Alkaloids

A small amount of the extract of *C. procera* was extracted with 30 ml of ammoniacal alcohol (ammonia/alcohol 1:9) and filtered. The filtrate was then evaporated to dryness and the residue extracted with 1% H_2SO_4. This was then filtered and the filtrate rendered alkaline with dilute ammonia solution. The alkaline solution of the extract was then put in a separating funnel and partitioned with chloroform. The chloroformic layer was then separated and evaporated to dryness. The residue was again dissolved in 1% H_2SO_4 and few drops of Dragendorff's reagent added. An orange precipitate indicates the presence of alkaloid (Harbone, 1976).

3.6.3 Test for phytosterols (Lieberman's test)

The ethanolic extract of *C. procera* was extracted with chloroform. 2 ml of acetic anhydride was then added to the chloroformic extract and few drops of concentrated H_2SO_4 was added along the sides of the test tube. A violet, blue or green colouration indicates the presence of steroids (Harbone, 1976)

3.6.4 Terpenoids (Salkowski test)

The ethanolic extract of *C. procera* was mixed with 5 ml chloroform. It was then warmed for 30 minutes. The chloroform solution was then treated with a small volume of

concentrated sulphuric acid and mixed properly. A reddish brown colouration of the inter face shows a positive results for the presence of terpenoids (Harbone, 1976)

3.6.5 Test for flavonoids

The extract was heated with 10 ml of ethyl acetate over a steam bath for 3 min. The mixture was filtered and 4 ml of the filtrate was shaken with 1 ml of dilute ammonia solution. A yellow colouration was observed indicating a positive test for flavonoids (Odebiyi and Sofowora, 1978)

3.6.6 General test for glycosides

About 200 mg of the extract was warmed with 5 ml dilute H_2SO_4 on a water bath for 2 minutes. It was then filtered and the filtrate rendered distinctly alkaline with 2 to 5 drops of 20% NaOH. 1 ml each of Fehling's solution A and B was then added to the filtrate and heated on the water bath for 2 minutes. A brick red precipitate indicates the presence of glycosides (Harbone, 1976).

3.7 CHROMATOGRAPHY

3.7.1 Column chromatography

Silica gel 60 (70-230 mesh ATSM) was used as the stationary phase for the column. The packing of the column was done by the dry method while gradient elution was employed in developing the column.

3.7.2 Detection for analytical thin layer chromatography

The zones on TLC plates corresponding to separated compounds were detected under U.V light 254nm and 365nm and also by spraying with anisaldehyde 0.5%w/v in HOAC-H_2SO_4-MEOH (10:5:85) followed by heating at 105°C for 5-10 minutes (Ergon Stahl).

3.7.3 Solvents and chemicals

The solvents and chemicals used were obtained from Fissons Company Ltd., May & Baker Limited and Philip Harris Ltd., all in the United Kingdom, Merck in Germany and were of analytical grade.

3.7.4 Solvent systems

The column was developed by gradient elution using varying proportions of Petroleum ether, chloroform and methanol.

3.7.5 Column chromatographic fractionation of extract

90g of silica gel 60 (70-230 mesh ASTM) was packed dry into a glass column (90cm × 5cm). 15g of H_2 was dissolved in about 50 ml of ethanol (90%) and mixed with 30g of silica gel 60 (70-230 mesh ASTM), allowed to dry to attain the same consistency as the silica in the column, and spread gently on top of the packed column. A wad of cotton wool was placed on top of the packed column in order not to disturb the surface of the packing.

The column was eluted with a gradient of Pet-ether, chloroform and methanol. Aliquots of 50ml each were collected based on the polarity of the eluent. By means of thin layer chromatography (TLC), the profiles of the aliquots were determined employing anisaldehyde reagent as detecting agent. Similar fractions were bulked together based on the TLC profiles resulting in four bulked fractions (H_{2A}, H_{2B}, H_{2C} and H_{2D}). The fractions

were weighed and subjected to thin layer chromatographic studies to ascertain their chromatographic fingerprint. Their proportions and order are presented in Table 3.1

Table 3.1Bulked fractions and aliquotes numbers for the fractionation of H_2

Bulked Fractions	**Aliquot Numbers**	**Weight/ g**
H_{2A}	1-25	2.269
H_{2B}	26-40	3.741
H_{2C}	41-55	3.6
H_{2D}	56-75	3.668
Total weight of fractions		**12.278**

The figure below shows the column chromatographic procedure for the fractionation of the hot ethanolic extract (70%) of the stem of *C. procera* using silica gel (70-230 mesh).

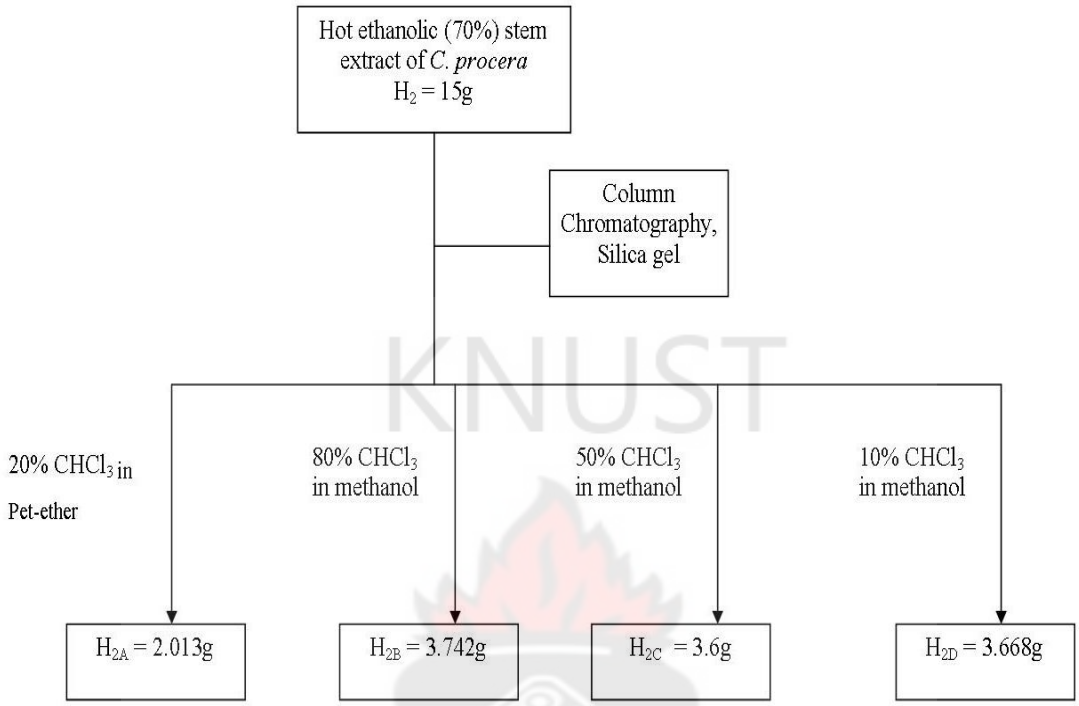


Figure 3.1 Schematic representation of the fractionation of the extract H_2 from the stem of *C. procera.*

3.7.6 Thin layer chromatography (TLC) analysis of fractions

Very small amounts of each fraction (about 200mg) were reconstituted in various solvents: fraction H_{2A} in chloroform, H_{2B} in ethanol, H_{2C} and H_{2D} in methanol. The identification of major chemical groups was carried out by TLC on silica gel 60 F254 Merck (layer thickness 0.25 mm) as follows; for polyphenols (flavonoids), *n*-butanol/acetic acid/water 4:1:5 (top layer) was used as solvent system, 5% vanillin (BDH laboratory) in concentrated HCl as detecting reagent (Markham, 1982). The developed chromatogram was exposed to fumes of ammonia and observed under UV at 365 nm. Flavanols give a

blue-green fluorescence whereas catechins and proanthocyanidins form a pinkish red colour after spraying with vanillin in Conc. HCl (Markham, 1982). Alkaloids were detected with Dragendorff's reagent and chloroform/ acetone/diethylammine: 5:4:1 was used as mobile phase. Anthraquinones detected with NaOH 10% using ethylacetate/methanol/water: 8:1:1 as mobile phase. Coumarins were detected under UV (366 nm) by their blue fluorescence which becomes intense after spraying with KOH 10%. Terpenes or steroids were identified with Liebermann–Burchard test (Harborne, 1976).

Chapter 4

RESULTS

4.1 YIELD OF EXTRACTS

The stem barks of *A. boonei* and *C. procera* as well as the leaves of *A. boonei* were extracted with different solvents under different conditions. The percentage yields of the extracts are presented in table 4.1.

Table 4.1 Percentage yields of extracts of *A. boonei* and *Carapa procera*

Solvents	*% Yield of extracts*		
	Alstonia stem	*Alstonia leaf*	*Carapa stem*
Petroleum ether	PS_1=2.51	PSL=4.1	PS_2=3.6
Hot ethanol (70%)	H_1=7.2	H_3=11.1	H_2=8.81
Cold ethanol (70%)	C_1= 6.8	C_3= 8.7	C_2=6.51

4.2 ANTIPLASMODIAL ACTIVITY OF EXTRACTS

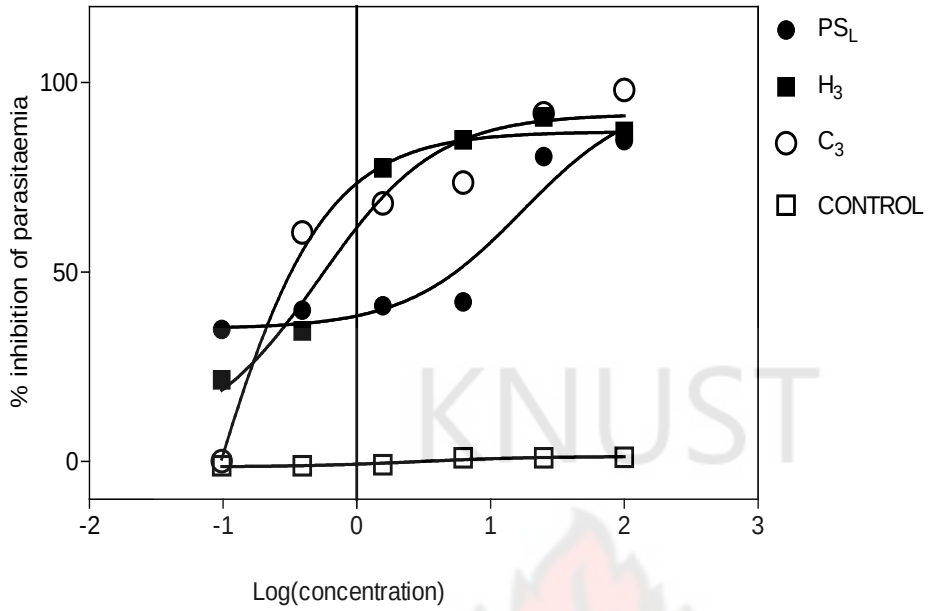
The effects of the leaf and stem extracts (Table 4.1) on the growth and development of the in-vitro *Plasmodium falciparum* cultures were determined by adding various concentrations of the extracts to erythrocyte suspensions with ringstage synchronised parasites distributed in triplicate into 24-well plates at 1% parasitaemia and 4% haematocrit. Aliquots were taken from each well for preparation of Giemsa-stained thin

films and degree of parasitaemia determined by microscopic examination of the film preparations (Kassim *et al.*, 2009).

4.2.1 Antiplasmodial activity of *Alstonia boonei* extracts

Petroleum ether extracts of the leaves and stem (PSL and PS_1) as well as hot (H_3 and H_1) and cold ethanolic extracts (C_3 and C_1) of the leaves and stem respectively were tested for their antiplasmodial activity as described (section 3.4.2) at concentrations of 0.09-100 mg/ml. The effect of the extracts, at various concentrations, on the growth of the malaria parasite was observed. Figure 4.1 shows the dose dependent chemosuppressive effect of the leaf extracts at various doses employed in this study. The extracts showed various degrees of growth inhibition against the *Plasmodium falciparum* parasite as shown by the area under the curve (AUC) in figure 4.1. C_3 showed a significant anti-malaria activity ($p<0.001$) against the parasites compared to the untreated group (control). This was followed by H_3 and PSL respectively. Thus cold ethanolic extract of the leaves of *Alstonia boonei* showed the highest antimalaria potential with $IC_{50}<100$ (Table 4.2).

The stem extracts of *Alstonia boonei* were also tested as described above. The petroleum ether and hot ethanolic extracts were practically inactive ($IC_{50}>1000$) whereas the cold ethanolic extract showed very weak antiplasmodial activity (Table 4.2). The hot ethanolic extracts of both stems and leaves of *A. boonei* showed weak antiplasmodial activity whereas the cold ethanolic extracts showed some degree of antimalarial activity. The leaf extracts of *A. boonei* was more active than the stem extract.



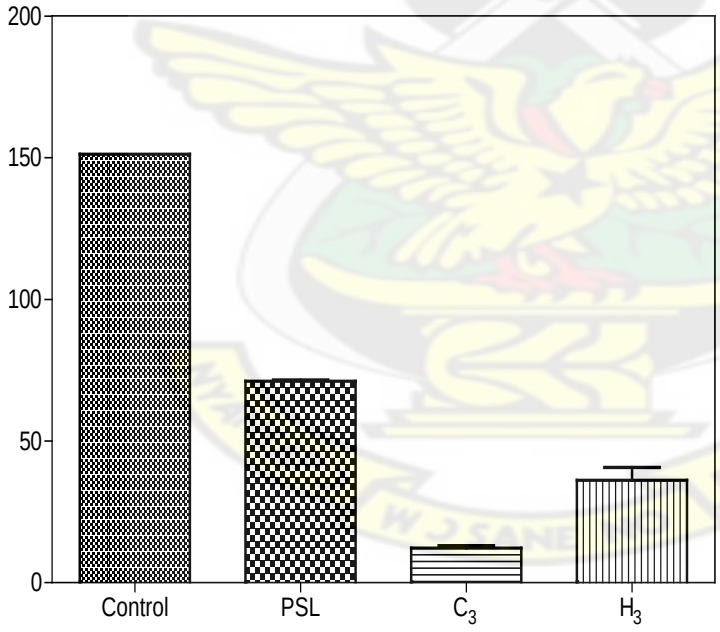
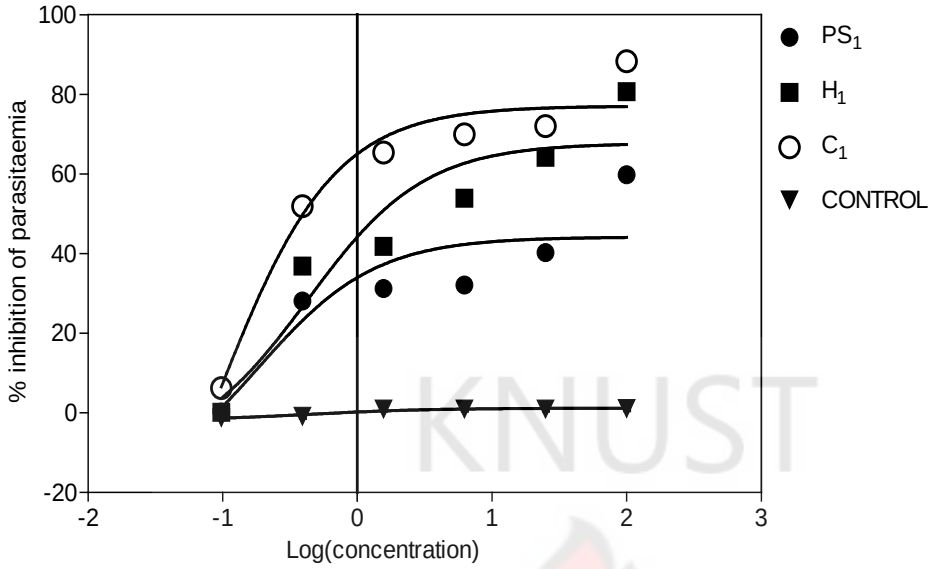


Figure 4.1 Effect of *Alstonia* leaf extracts: C_3, H_3 and PSL (0.09-100 mg/ml) on the total parasitaemia for 48 hours. Values are means ± S.E.M (n=3) *** $p < 0.001$, ** $p < 0.01$. *$P<0.05$ compared to control group (One-way ANOVA followed by Newman-Keul's post hoc test)



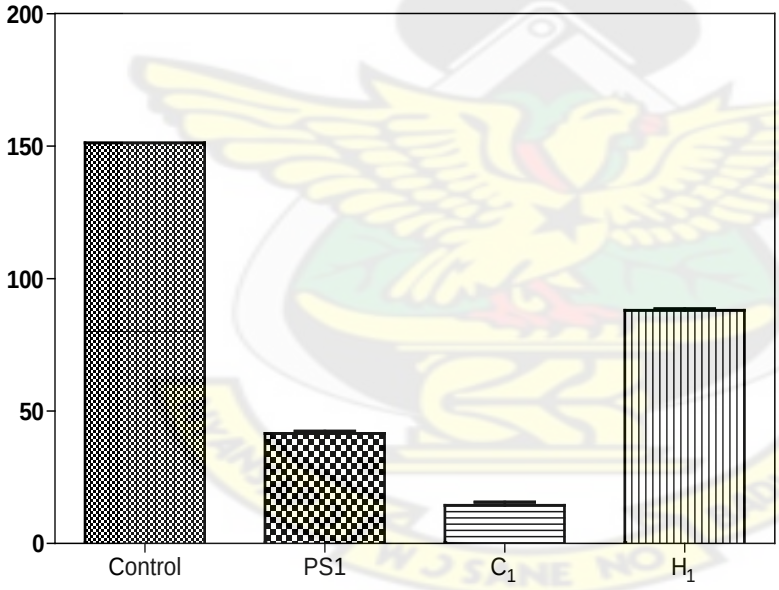


Figure 4.2 Effect of *Alstonia* stem extracts: C_1, H_1 and PS1 (0.09-100 mg/ml) on the total parasitaemia for 48 hours. Values are means ± S.E.M (n=3) *** $p < 0.001$, ** $p < 0.01$. *P<0.05 compared to control group (One-way ANOVA followed by Newman-Keul's post hoc test)

Table 4.2 *In Vitro* antiplasmodial activity of extracts of *A. boonei*

Extracts	$IC_{50}/\mu gml^{-1}$
PSL	>100
H_3	>100
C_3	71.24
PS_1	>100
H_1	>100
C_1	88.15

H_3 and C_3= Hot and cold ethanolic extracts of Alstonia leaves, H_1 and C_1=Hot and cold ethanolic extracts of Alstonia stem, PSL and PS1=Petroleum ether extract of Alstonia leaves and stem.

4.2.2 Antiplasmodial activity of *Carapa procera* extracts

The effects of the extracts on the growth and development of the *in vitro Plasmodium falciparum* cultures were determined with ring stage synchronised parasites distributed in triplicate into 24-well plates at 1% parasitaemia and 4% haematocrit. Petroleum ether (PS_1), 70% hot (H_2) and cold (C_2) ethanolic stem extracts of *Carapa procera* were used at concentrations of 0.09-100 mg/ml. The differential effect of the extracts, at various concentrations, on the growth of the malaria parasite was observed. Figure 4.3 shows the dose dependent chemosuppressive effect of the extracts at various doses employed in this study. There was total clearance of the parasites at concentrations greater than 25mg/ml for

petroleum ether extract and the hot ethanolic extract. The total parasitemia is represented by the area under curve (AUC) shown in Figure 4.3. The extracts showed various degrees of growth inhibition against the *Plasmodium falciparum* parasite. H_2 showed a significant anti-malaria activity ($p<0.001$) against the parasites compared to control groups. This was followed by PC_1 and C_2 respectively.

Table 4.3 *In vitro* antiplasmodial activity of *C. procera* extracts

Extracts	$IC_{50}/\mu gml^{-1}$
PC1	19.52
H2	11.41
C2	33.35

PC_1=Petroleum ether extract, H_2= hot ethanolic extract and C_2= cold ethanolic extract

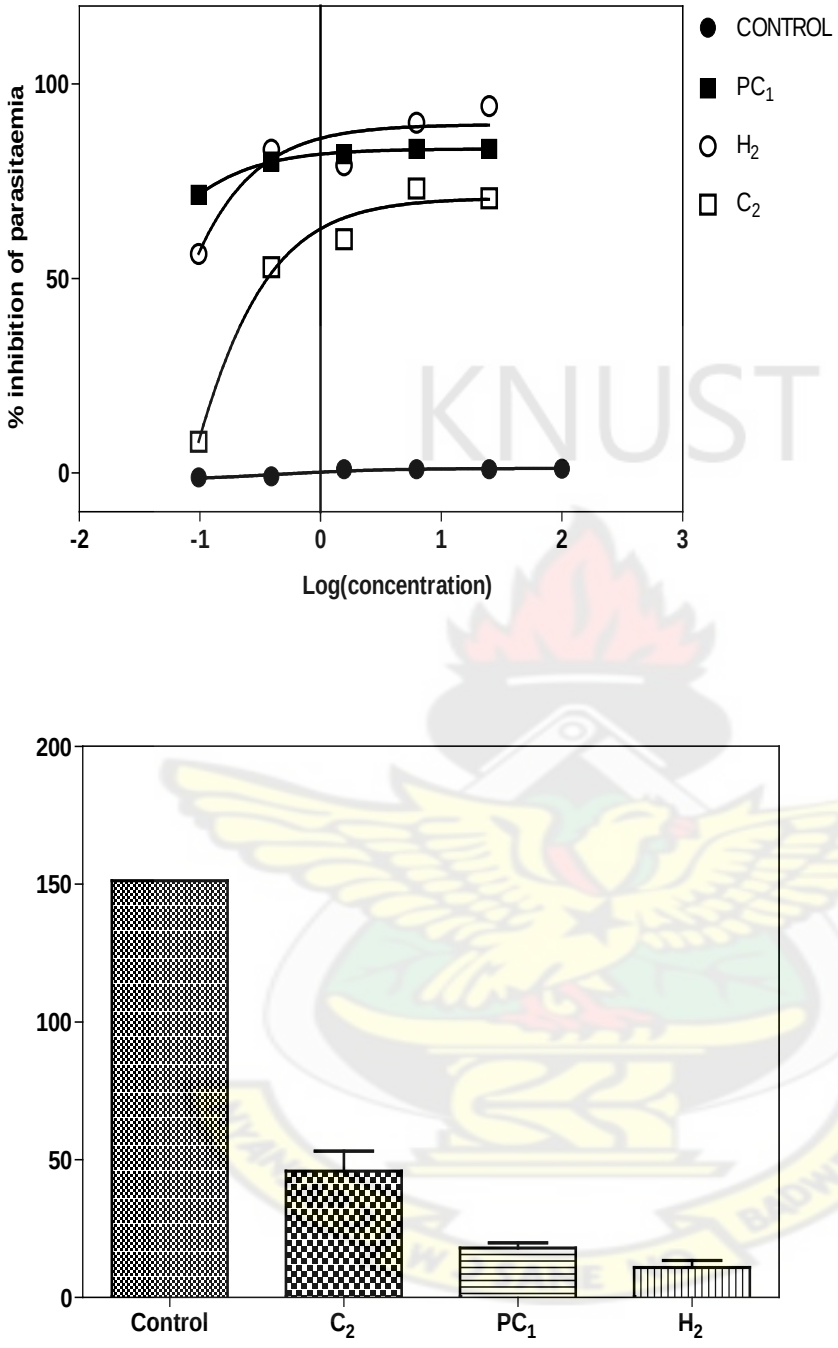


Figure 4.3 Effect of H_2, C_2 and PC1 (0.09-100 mg/ml) on the total parasitaemia for 48 hours. Values are means ± S.E.M (n=3) *** $p < 0.001$, ** $p < 0.01$. *$P<0.05$ compared to control group (One-way ANOVA followed by Newman-Keul's post hoc test).

The concentration of extracts which inhibit 50% of the parasites (IC_{50}) using regression equations of best fit of plotted growth inhibition versus concentration curves (Figure 4.3) is shown in table 4.3.The order of inhibitory potency is as follows: H_2>PC_1>C_2

4.3 PHYTOCHEMICAL ANALYSIS OF H_2

Due to the relatively high antiplasmodial activity shown by H_2, it was analysed for its phytochemical constituents. The results of the phytochemical screening of the hot ethanolic extract of *Carapa procera* (H_2) as shown in table 4.4 indicates the presence of terpenoids, flavonoids, phytosterols, tannins and glycosides in the stem extract. Alkaloids were absent.

Table 4.4 Phytochemical analysis of hot ethanolic extract of *C. procera* (H_2)

Secondary metabolite	*Inference*
Tannins	Present (hydrolysable tannins)
Terpenoids	Present
Flavonoids	Present
Glycosides	Present
Alkaloids	Absent
Phytosterols	Present

4.3.1 Antiplasmodial activity of fractions of hot ethanolic extract of *C. procera*

The hot ethanolic extract of the stem bark of *C. procera* (H_2) showed the highest anti-malaria activity *in vitro* as shown in table 4.3 (IC_{50}= 11.41µg/ml) . Upon fractionation of

H_2, four bulked fractions were obtained designated as H_{2A}, H_{2B}, H_{2C} and H_{2D}. Fraction H_{2B} contributed the highest yield (30.47%) of the total fractions eluted, while the total fraction eluted was 81.85% of the 15g of the extract fractionated (Table 3.1).

Dose-dependent *in vitro* antiplasmodial assay of the fractions revealed that combined fraction H_{2A} exhibited the highest antiplasmodial activity (IC_{50}= 0.1916µg/ml). However it was 108 fold less potent than the standard antimalarial drug artesunate (IC_{50}= 0.00177µg/ml) as shown in table 4.5

Table 4.5 *In Vitro* antiplasmodial activity of fractions of *C. procera* stem extract (H_2)

Fraction	IC_{50}/µgml^{-1}
H_{2A}	0.1916
H_{2B}	23.14
H_{2C}	0.3084
H_{2D}	8.999
ATS	0.00419

ATS= Standard drug artesunate

The rank order of inhibitory potencies of the fractions and standard drug is: Artesunate> H_{2A}> H_{2B}>H_{2C}> H_{2D}. A statistically significant difference was observed between the parasitemia of the infected cells treated with the fractions and the cells not treated (control group).

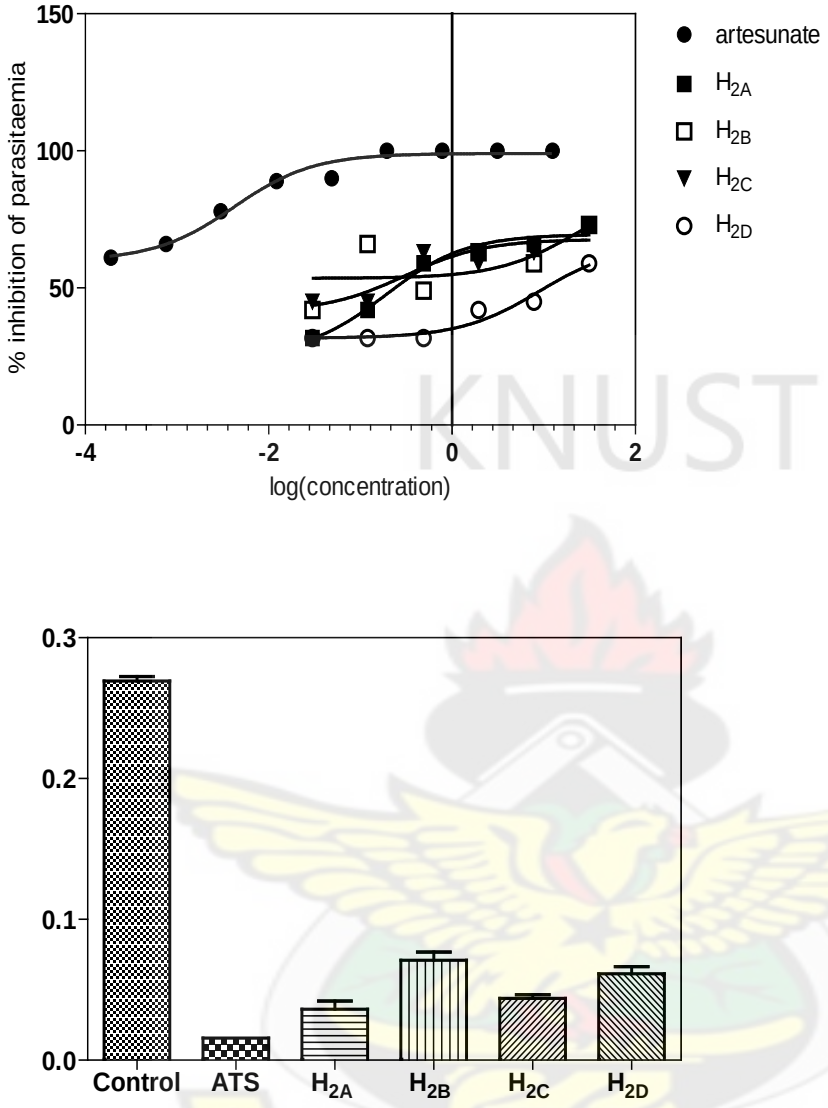


Figure 4.4 Effect of H_{2A-D} and (0.03-31.25 μg/ml) and standard drug artesunate (0.00019-12.5μg/ml) on the total parasitaemia for 48 hours. Values are means ± S.E.M (n=3) *** $p < 0.001$, ** $p < 0.01$. *$P<0.05$ compared to control group (One-way ANOVA followed by Newman-Keul's post hoc test).

4.3.2 Phytochemical analysis of fractions of H_2

The phytochemical constituents of the fractions were analysed by thin layer chromatography (TLC). The presence or otherwise of the secondary metabolites were established by spraying the TLC plates with various derivatising reagents. The results are presented in table 4.6 below.

Table 4.6 Phytochemical constituents of fractions of ethanolic extract of *C. procera*

Fraction	H_{2A}	H_{2B}	H_{2C}	H_{2D}
Secondary metabolites	Sterols terpenoids	Flavanoids Catechin proanthcyanidins	Flavanoids Catechin proanthcyanidins	Flavanoids Catechin proanthcyanidins

Fraction H_{2A} was found to contain stigmasterol. This was done by co-chromatography of H_{2A} with authentic stigmasterol (Merck, Lot number 5298307) Plate 1a and 1b.

Chapter 5

DISCUSSION

5.1 EXTRACTS OF *A. BOONEI* AND *CARAPA PROCERA*

Extracts of *A. boonei* and a few other plants in combination are marketed commercially on the Ghanaian market as antimalaria. Traditionally a decoction of the stem bark is taken orally for the treatment of malaria (Asase and Oppong-Mensah, 2009). In Togo the leaves of *Azadirachta indica, Picralima nitida* and *Alstonia boonei* are reported as being used to treat malaria (Gbeassor *et al.*, 1996). However, there is lack of scientific credence for most of the medicinal plants used in traditional medicine for the treatment of malaria. Therefore this study was designed to investigate the antimalaria potential of the leaves and stem bark of *A. boonei* as well as the stem bark of *Carapa procera* using clinical isolates of chloroquine sensitive (CQS) strains of *P. Falciparum* (3D7). Antimalaria activity was determined *in vitro* by counting GIEMSA-stained parasites by light microscopy (Fidock *et al.*, 2004). The plant materials were extracted by cold maceration and also by soxhlet extraction using petroleum ether and 70% ethanol. This was to investigate the effect of heat on the secondary metabolites of the plant extracts which may be responsible for the antiplasmodial activity (if any) as well as the appropriate solvent for the extraction of the bioactive constituents towards further development of appropriate dosage form.

Earlier studies on the *in vitro* antiplasmodial activity of furoquinoline and acridone alkaloids reported the results on IC_{50} values expressed in μg/ml, the following criteria being adopted: IC_{50}<10 μg/ml, good activity; IC_{50}>100 μg/ml, inactive; IC_{50} of 10–50

µg/ml, moderate activity; and IC_{50} of 50–100 µg/ml, low activity (Basco *et al*., 1994). In this research, the criteria considered by Basco *et al.* (1994) are being adopted.

Alstonia boonei (stem and leaves) is widely used in traditional medicine in some African countries to treat malaria. In the present investigation, the petroleum ether extract of the leaves (PSL) and stem bark (PS_1) were both inactive (IC_{50}>100 µg/ml). Also their hot ethanolic extracts were inactive with IC_{50} >100 µg/ml. However, the cold ethanolic extract of the leaves showed weak activity (IC_{50} = 84.52 µg/ml) whereas that of the stem was 71.24 µg/ml as shown in table 4.3. This result indicates that extracts of the leaves and stem bark of *A. boonei* have very weak antiplasmodial activity and therefore does not justify it use as an antimalaria agent. In many cases plants that were reported as being used for the treatment of malaria were found to have antipyretic properties and had no real antiplasmodial properties (Addae-Kyereme *et al.*, 2001). Antipyretic agents relieve fevers associated with malaria. Thus a patient may be asymptomatic (for a short period) but present with high parasitaemia levels. Also herbal preparations containing extracts of *A. boonei*, for the treatment of malaria, are found in combination with other plants such as *Picralima nitida* and *Azacdrichta indica (*Zirihi *et al*., 2005). The root, stem bark and fruits of *P. nitida* were found to exhibit potent antiplasmodial activities with respective IC_{50} values of 0.188, 0.545 and 1.581. This activity is attributed to the active alkaloid akuammine (Schwikkard and Heerden, 2002). Therefore antimalaria preparations containing extracts of *A. boonei*, may owe their activity to the other plant extracts.

Carapa procera (meliaceae) stem bark, is used in many traditional societies in Ghana and other African countires for the treatment of malaria. Members of the meliaceae have been used for generations in Africa to treat malaria (Schwikkard and Heerden, 2002). In this

present study extracts of the stem bark of *C. procera* exhibited good antimalaria activity. The petroleum ether extract of the stem bark (PC_1) inhibited the growth of the chloroquine sensitive (3D7) *Plasmodium falciparum* parasite with IC_{50} value of 19.52. The cold ethanolic extract had moderate activity (IC_{50} = 33.35) whereas the hot ethanolic extract (H_2) showed the highest antimalaria activity (IC_{50} = 11.41). The difference in activity observed, between the hot and cold ethanolic extract may be attributed to the more exhaustive extraction of the active metabolites in H_2 (yield= 8.81%) than in C_2 (yield= 6.51%) as shown in table 4.1.

The bitter taste and antimalaria activity of *Carapa* species have been attributed to a group of terpene chemicals called meliacins, which are very similar to the bitter antimalarial chemicals found in tropical plants. One of these meliacins, named gedunin, has recently been documented to have antiparasitic properties and antimalarial effect equal to that of quinine (Nayak *et al*., 2009). Chemical analysis of the oil obtained from the seeds of closely related specie (*C. guianensis***)** has also identified the presence of another group of chemicals called limonoids. The anti-inflammatory and insect repellent properties of *C. guianensis* seed oil, are attributed to the presence of these limonoids (Roy and Saraf, 2006), including a novel one which has been named *andirobin*. Another limonoid called *epoxyazadiradione* is found in *C.guianensis* oil. The three chemicals present in *C. guianensis* have been found to have antiparasitic and insecticidal actions (Silva *et al*., 2004). Thus the antimalaria activity exhibited by the pet-ether extract of *C. procera* may be due to the presence of one or more of such compounds. The results shown by the hot ethanolic stem bark extract (H_2) of *C. procera*, underlined the interest to fractionate and investigate it further for antiplasmodial activity and also know it phytochemistry.

5.2 FRACTIONS OF *CARAPA PROCERA*

The ethanolic extract, H_2, was subjected to column chromatography yielding four bulked fraction designated as H_{2A}, H_{2B}, H_{2C} and H_{2D}. The IC_{50} values obtained with these fractions on the chloroquine sensitive *plasmodium falciparum* (3D7) are summarized in Table 4.5. Fractions H_{2A}, H_{2C} and H_{2D} showed the highest antimalaria activities (IC_{50}<10µg/ml) whereas H_{2B} showed moderate antimalaria activity with (IC_{50}<50µg/ml). The activities of fractions H_{2A} and H_{2C} (IC_{50}<1µg/ml) were comparable to the standard antimalaria drug artesunate (IC_{50}<0.01µg/ml). TLC analysis of the fractions described in the present work revealed the presence of steroids and terpenoids (fraction H_{2A}), flavonoids, catechins and proanthocyanidins (H_{2B}, H_{2C} and H_{2D}) section 4.3.2. Representatives of these phytochemical classes have already been reported to inhibit *Plasmodium falciparum* growth *in vitro* and/or *in vivo* (Wright and Phillipson, 1990). Terpenoids and steroids (Krafi *et al*., 2003; Phillipson and Wright, 1991) saponins (Oketch-Raban and Dossaji, 1997; Traore *et al*., 2000), tannins, anthocyanins and flavonoids especially methoxylated flavonoids (Oliver-Bever, 1986) have been reported to exhibit antiplasmodial activity. Stigmasterol was found in fraction H_{2B} by co-chromatography with standard stigmasterol. Thus the high antiplasmodial activity shown by fraction H_{2A} may be due it steroidal and more importantly terpenoidal contents. The antiplasmodial activity of fraction H_{2B}, H_{2C} and H_{2D} may be attributed to the polymeric flavonoids catechin and proanthocyanidins. The exact mechanism of antimalarial action of flavonoids is unclear but some flavonoids are shown to inhibit the influx of L-glutamine and myoinositol into infected erythrocytes (Kaur *et al.*, 2009). It is reported that catechins are potent inhibitors of mammalian facilitative glucose transporter 1

(GLUTT1)-mediated d-glucose transport in human erythrocytes (Naftalin *et al.*, 2003). It is hypothesized that catechins exhibit antimalaria activity by a similar inhibition of d-glucose uptake via P. falciparum hexose transporter (PfHT), a parasite plasma membrane-localised protein that is a major route for parasite d-glucose and d-fructose uptake (Woodrow *et al*., 2000; Woodrow *et al*., 1999). Thus these secondary metabolites may be responsible for the antimalaria activity of *Carapa procera.*

CONCLUSION

It is concluded that some constituents of the stem bark of *Carapa procera* exhibit *in vitro* antiplasmodial activity and this results justifies and confirms the traditional usage of this plant as a malarial remedy. The antiplasmodial activity resides in the hot ethanolic extract. This activity may be attributed to secondary metabolites such as catechins, proanthocyanidins, terpenoids and flavonoids which were found in the phytochemical analysis of the extract and fractions. This is the first time the antiplasmodial activity of the stem bark of *C. procera* is being reported. The stem bark and leaves of *Alstonia boonei* exhibit very weak antiplasmodial activity. The solvent and conditions employed in the extraction of plant secondary metabolites affects their bioactivity. This was shown by the disparity in the antiplasmodial activity of the extracts as shown in this study.

RECOMMENDATION

Based on the results of this research, the following recommendations are made;

- *In vivo* antimalaria studies on the stem bark of *C. procera* must be conducted and should seek to determine the toxicity of the active constituents, serum attainable levels and pharmacokinetic properties.
- Isolation and structural elucidation of the active constituents responsible for the antimalaria activity. Knowledge of the phytoconstituents will be useful in the standardization of this plant as an antimalaria phytomedicine.
- *In vivo* and *in vitro* antiplasmodial studies of the isolated compounds
- Cytotoxity studies as well as selectivity index of active compounds
- Moreover cultivation and preservation of this promising plant (*C. procera*) is needed.

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APPENDICES

APPENDIX 1: Picture of whole plant and stem bark of *C. procera* from a farmland in Effiduase, Kumasi

APPENDIX 2: Picture of whole plant, stem bark and leaves of *A. boonei* from a farmland in Effiduase, Kumasi

APPENDIX 3: CONCENTRATIONS AND PERCENTAGE INHIBITION OF PARASITAEMIA OF HOT AND COLD PETROLEUM ETHER AND 70% ETHANOLIC EXTRACT OF THE STEM BARK AND LEAVES OF *A. BOONEI*

APPENDIX 4: CONCENTRATIONS AND PERCENTAGE INHIBITION OF PARASITAEMIA OF THE PETROLEUM ETHER AND HOT AND COLD 70% ETHANOLIC EXTRACT OF THE STEM BARK OF *C. PROCERA*

APPENDIX 5: CONCENTRATIONS AND PERCENTAGE INHIBITION OF PARASITAEMIA OF STANDARD DRUG ARTESUNATE PETROLEUM ETHER AND HOT ETHANOLIC (79%) EXTRACT OF THE STEM BARK OF *C. PROCERA*

APPENDIX 6: CHROMATOGRAPHIC FINGERPRINT OF FRACTIONS H2A-D AND STIGMASTEROL (ST) DEVELOPED WITH PET-ETHER AND CHLOROFORM (80:20)