

***IN VITRO* EFFECTS OF NATURAL COCOA EXTRACT ON  
ERYTHROCYTE MEMBRANE AND ASEXUAL ERYTHROCYTIC STAGE  
OF *PLASMODIUM FALCIPARUM***

**BY**

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## DECLARATION

I hereby declare that this research undertaken at Noguchi Memorial Institute for Medical Research in partnership with the Department of Animal Biology and Conservation Sciences, University of Ghana, is my own work. It was done under the supervision of Dr Neils Ben Quashie, Dr Fred Aboagye-Antwi and Dr Regina Appiah-Opong.

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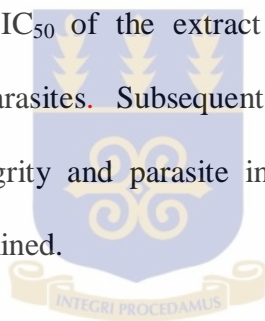
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## ABSTRACT

Malaria remains a life-threatening disease in the tropical and sub-tropical world, affecting mostly children and pregnant women. One of the biggest threats to malaria control is the ability of the causative parasite to develop resistance to anti-malarials and how quickly the resistance spreads. This makes it necessary for further research to discover new anti-malarial drugs. Plant products offer a great promise in this direction. This study tested the anti-plasmodial activities of crude methanol extract of natural cocoa powder.

Cold maceration was used to produce the crude cocoa extract and the flavonoids and phenolic content determined. SYNERGY BRAND (SYBR<sup>®</sup>) Green I assay was used to determine the *in vitro* IC<sub>50</sub> of the extract against 3D7 and DD2 strains of *Plasmodium falciparum* parasites. Subsequently, the effects of the extract on erythrocyte membrane integrity and parasite invasion as well as sorbitol-induced haemolysis were also determined.



The extract tested positive for flavonoids and showed a high total phenolic content (358.94 mg GAE/g of extract). A stronger inhibitory activity (about 12 fold) against the 3D7 than DD2 strains (IC<sub>50</sub> values of 51.4 µg/L and 622.6 µg/mL respectively) was demonstrated. However, pre-treatment of uninfected RBCs with the IC<sub>50</sub> concentration of the extract resulted in 47.3 and 36.0% inhibition of invasion in DD2 and 3D7 strains, respectively. Pre-treating schizont- and late trophozoite-infected RBCs with the extracts resulted 26.7 % and 17.7 % inhibition of sorbitol-induced haemolysis in DD2 and 3D7 strains respectively.

The anti-plasmodial activity of the crude methanol natural cocoa extract could be mainly due to its ability to enhance erythrocyte membrane integrity and protect from

parasite invasion. Erythrocyte membrane integrity enhancement inhibited sorbitol-induced haemolysis of infected erythrocytes. The direct effects of the bioactive ingredients of extract on the intracellular parasite could have contributed the inhibition of sorbitol-induced haemolysis of infected erythrocytes.

It can thus be concluded that crude methanol extract of natural cocoa has anti-plasmodial activity.



## DEDICATION

This work is a further proof that difficulties can be overcome when we persist. I dedicate it to my parents who continue to empty their lives so that ours can be filled. Also to the next generation: Edudzi, Kekeli, Dzidzornu, Awoenam and Dzidefo. The future is yours!



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Marian, you virtually had to drag me over the finish line. You are amazing. Thanks and God bless you.

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## LIST OF ABBREVIATIONS

ACD	Adenine citrate dextrose
ARS	Artesunate
BSC	Bio-safety cabinet
CDC	Centre for Disease Control (and Prevention)
CPM	Complete Parasite Media
CQ	Chloroquine
CME	Crude Methanol Extract
DDT	Dichlorodiphenyltrichloroethane
DMSO	Dimethylsulfoxide
HCl	Hydrochloric acid
HEPES	N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid
IC <sub>50</sub>	Concentration of drug inhibiting schizogony by 50%
iRBC	Infected red blood cells
iRPMI	Incomplete RPMI
MOH	Ministry of Health, Ghana.
MSF	Malaria SYBR Green 1 based fluorescence
NaHCO <sub>3</sub>	Sodium Hydrogen Carbonate

<i>RBC</i>	<i>Red blood cells</i>
RDT	Rapid Diagnostic Test
RFU	Relative fluorescence unit
RPMI 1640	Roswell Park Memorial Institute 1640
<i>SOP</i>	<i>Standard operating procedure</i>
PT	Parasitaemia
UNICEF	United Nations' Children Fund
WHO	World Health Organization

## CHAPTER ONE

### INTRODUCTION

#### 1.1 MALARIA

Malaria remains a major public health problem especially in tropical and sub-tropical countries of the world (Mustofa *et al.*, 2007). There are 109 malarious countries worldwide and according to the WHO's World Malaria Report, 219 clinical cases of malaria reported (Doolan *et al.*, 2009; WHO, 2010). It is a major cause of mortality and morbidity in endemic regions and results in 660,000 deaths annually (WHO, 2010). Globally, malaria in combination with other parasitic diseases accounts for approximately one million deaths in children below age 5 (Greenwood *et al.*, 2005; WHO, 2010).

The greatest impact of the disease is felt in sub-Saharan Africa where about 90% of all cases occur. In sub-Saharan Africa and many other parts of the world where the disease is endemic, the problem is further compounded by the challenge of limited availability and ready access to modern health facilities and quality drugs (WHO, 2010). The disease affects mostly pregnant women and children below age 10 and accounts for about 20% of all infant deaths in the region (WHO, 2010).

In Ghana, malaria is the leading cause of morbidity and accounts for 35.7% of all outpatient consultations at the country's health units (MOH, 2008). Approximately 3.5 million people contract the disease in the country each year, killing about 20,000 children aged less than 5 years (UNICEF, 2007). This figure represents 25% percent of all infant deaths that occur each year.

Malaria is caused by a protozoan parasite of the genus *Plasmodium* and is transmitted through the bite of female mosquitoes of the genus *Anopheles*. Five species of *Plasmodium* are known to cause human malaria; *P. falciparum*, *P. vivax*, *P. ovale*, *P. malariae* and *P. knowlesi*. The most virulent species, *P. falciparum*, is the most common cause of malaria in sub-Saharan Africa (Perlmann and Troye-Blomberg, 2000). The high mortality and morbidity associated with malaria caused by *P. falciparum* is due to the high number of merozoites formed in each schizont, how rapidly the parasite multiplies once it infects an erythrocyte, its ability to sequester to blood vessels to avoid host defense mechanisms and a lack of preference for host erythrocytes of a particular age, unlike the other species (Wiser, 2000; Olliaro, 2008)

The clinical symptoms of the disease are as a result of the asexual stage of the parasite life cycle which occurs in the erythrocytes. The symptoms of the disease start as a mild infection with flu-like symptoms like chills, fever, diarrhoea, headache and vomiting, especially in children. However, if untreated within 24 hours, it can degenerate into complicated malaria, characterized by severe anaemia, hepatomegaly, splenomegaly, renal failure, respiratory distress, cerebral malaria or even death (Dondorp *et al.*, 2004).

Despite efforts made over the years to control the disease, malaria is still a life-threatening disease in most endemic parts of the world. This is mainly due to the ability of the most important etiological agent of the disease, *P. falciparum* to develop resistance to conventional anti-malarial drugs quickly and the fast rate at which resistance spreads. Thus there is the need for novel anti-malarial drugs and drug combinations with modes of action that are different from those of the anti-malarial agents in current use (Ramalhete *et al.*, 2008). There is also the need for compounds that will potentiate the anti-malarial



drugs by delaying the development of resistance by the parasite. Plant products offer a great promise in this direction and this is imperative considering the fact that two of the most common anti-malarial agents, quinine and artemisinin and their derivatives were obtained from the plants, *Cinchona pubescens* and *Artemisia annua*, respectively (Challand and Wilcox, 2009).

Interestingly, most malaria-endemic countries are found in the developing world and their populations still rely heavily on traditional medicines made from plants for their primary health-care delivery. A WHO report (2002), indicates that over the years there has been about 80% dependence on traditional plant medicines worldwide and a lot of research has gone into validating the efficacy and scientific basis of these medicines, sometimes with very promising outcomes (Madureira *et al.*, 2002; Ramahelte *et al.*, 2008; Ramazani *et al.*, 2010; Appiah-Opong *et al.*, 2011).

Although anecdotal reports tout the anti-malarial activity of natural cocoa (Addai, 2009), there is little scientific data to support its efficacy and mechanisms of anti-plasmodial action. Addai (2010) proposes that active ingredients found in cocoa, including flavonoids, boost the immune system, act directly on the parasite and could enhance erythrocyte membrane integrity.

Research shows that infected erythrocytes are markedly different from uninfected erythrocytes (Lopez *et al.*, 2009). This is as a result of the intracellular parasite modifying the membrane to aid it in obtaining the nutrients and solutes needed for survival and growth from the blood or growth medium. Kirk (2001) suggested that there are compounds that enhance membrane integrity, reducing membrane susceptibility to

parasite invasion and further inhibiting or reversing parasite modifications. Treatment of uninfected erythrocytes with steroids isolated from *Solanum nudum* has been reported to reduce susceptibility to invasion by 3D7 strain of *P. falciparum* and protect infected erythrocytes from solute-induced haemolysis (Lopez *et al.*, 2009). Go *et al.* (2004) also identified bioflavonoids as compounds that have effects on erythrocyte membrane in a manner similar to that reported for steroids. Other works have revealed high amounts of bioflavonoids in natural cocoa (Keen *et al.*, 2004; Engler and Engler, 2006).

## **1.2 AIMS**

The main aim of this study was to assess the crude methanol extract of natural cocoa for its anti-malarial activity *in vitro*.

### **1.2.1 SPECIFIC OBJECTIVES**

The specific objectives were to:

- a) To detect the presence of flavonoids in crude methanol extract of natural cocoa and to estimate the total phenolic content.
- b) Evaluate the activity of the extract against chloroquine-sensitive 3D7 and chloroquine-resistant DD2 laboratory strains of *P. falciparum in vitro*.
- c) Assess the effects of crude methanol natural cocoa extract on invasion of RBCs by the 3D7 and DD2 parasite strains.

- d) Determine the effect of the extract on susceptibility of RBCs to induced haemolysis.

### 1.3 JUSTIFICATION

Malaria is a threat to human life. This is due to the increasing incidence of drug resistance by the parasite to established anti-malarials such as chloroquine and antifolates (Tan and Sebisubi, 2010). Recently, resistance or delayed parasite clearance has been reported for ACTs (Noedl *et al.*, 2007; Dondorp *et al.*, 2009; Anderson *et al.*, 2010) which are the first line drugs for the treatment for *P. falciparum* malaria in many endemic countries including Ghana. This makes it necessary to investigate further for leads to the development of new drugs which are inexpensive, have relatively shorter course of treatment and higher cure rate and drug targets with limited side effects.

The limited availability of health-care facilities and quality drugs in many malaria endemic regions make their populations reliant on crude plant products as alternatives to the conventional drugs (Mustofa *et al.*, 2000). Besides the fact that they serve as sources of primary health-care for the treatment of malaria (Joy *et al.*, 2001), the basis of the anti-malarial properties of some of them have been established and further research is on-going to isolate the active ingredients (Tan and Sebisubi, 2010).

To test susceptibility to plant extracts, according to guidelines established by the WHO (WHO, 1973) the preliminary test involves evaluating anti-plasmodial activity of crude extract *in vitro* by monitoring parasite viability when cultured with the extracts. Parasite viability is monitored using different assays. The SYNERGY BRAND (SYBR<sup>®</sup>) Green

assay has gained currency in malaria drug sensitivity and susceptibility tests because of its relative technical simplicity and high sensitivity (Johnson *et al.*, 2007; Vossen *et al.*, 2010).

Results of epidemiological and laboratory studies show evidence that the anti-malarial activity of some plants products is attributable to their polyphenolic content (Aziza *et al.*, 1999; Go *et al.*, 2005).

Cocoa is a rich source of polyphenols, especially flavonoids (Lamuella – Raventos *et al.*, 2001) and anti-malarial properties have been proposed for natural cocoa (Addai, 2009). *In vitro* anti-plasmodial activity of crude methanol extract of natural cocoa against chloroquine-sensitive 3D7 strain has been reported and so has its immune-regulatory effect on malaria (Amponsah *et al.*, 2012). However, there is no information available on the anti-plasmodial activity of natural cocoa extracts against chloroquine-resistant strains of *P. falciparum*. How the extract affects erythrocyte membrane susceptibility to parasite invasion and the haemolysis of erythrocytes induced by the parasite has also not been investigated.

According to Jain *et al.* (2006), many biological activities have generally been reported for polyphenols and flavonoids in particular. These are a result of their ability to interact with cell surface proteins as well as their anti-oxidant, iron-chelating, and free radical scavenging abilities. Although these interactions on the erythrocyte membrane and how they affect the pathology of many diseases have been studied, their effect on the erythrocytic stage of malaria has not been elucidated. Studying the ability of the extract to protect erythrocytes against invasion by the merozoites and solute-induced haemolysis

*in vitro* is useful in determining the role of the extract in fighting malaria and how it may apply *in vivo*.

Traditionally, plant products have shown a high success rate against *Plasmodium* and results of initial research into the anti-plasmodial activity of natural cocoa shows promising results (Amponsah *et al*, 2012). This makes it necessary for more extensive studies to be conducted into its anti-plasmodial activity.

The extract was tested on *P. falciparum* because it is the species that causes the most severe form of the disease and is the most prevalent species in sub-Saharan Africa.

#### **1.4 RESEARCH HYPOTHESIS**

Crude methanol extract of natural cocoa is a rich source of flavonoids for which anti-plasmodial activity has been reported. Natural cocoa extract enhance erythrocyte integrity and its anti-plasmodial activity can be partly attributed to the effects of the extracts on the erythrocyte membrane.

## **CHAPTER TWO**

### **LITERATURE REVIEW**

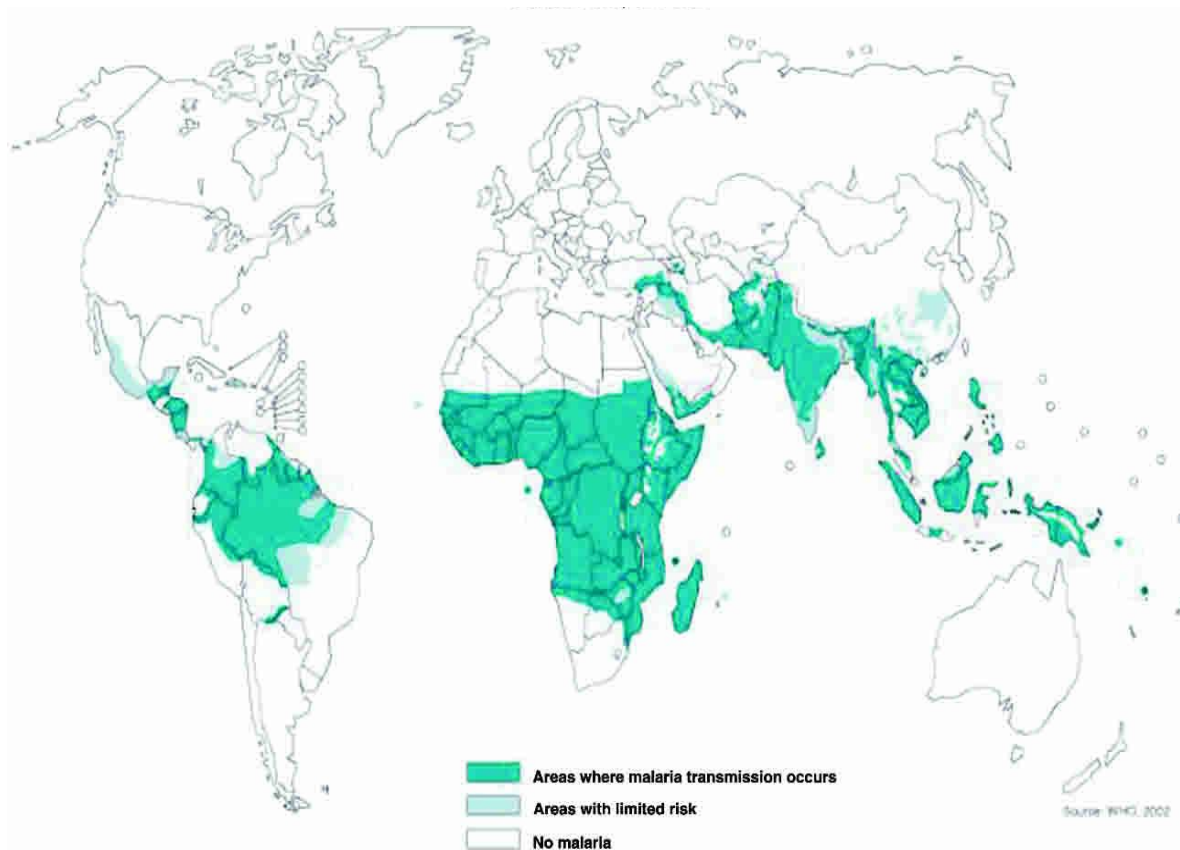
#### **2.1 MALARIA**

Malaria is a life-threatening parasitic infection of great global importance (Kain and Keystone, 1998; Snow *et al.*, 2004). Although it is mainly transmitted by female Anopheline mosquitoes, cases of infection through blood transfusion and vertical transmission have been extensively reported (Shinger *et al.*, 1999; Bruneel *et al.*, 2004; Garrou, 2006; Vareil, 2011). It is a killer disease, especially in sub-Saharan Africa, and is the most important infectious tropical disease, according to Tangpudkee (2009). The threat the disease poses and the attention it attracts is demonstrated by the fact that four Nobel prizes have been awarded for malaria-related research (CDC, 2004).

Malaria has been with man for millennia and has affected human life politically, economically, socially and geographically and in many other ways. According to World Health Organization (WHO, 2009), the disease is endemic and perennial problem in 109 countries, mostly in Africa, South and Central America and Asia. The disease killed nearly 655,000 people in 2010 accounting for 2.3% of all deaths worldwide (WHO, 2011).

The greatest impact of the malaria is felt in sub-Saharan Africa where 90% of malaria-related mortality and morbidity occur, especially in children under 5 years (Kuhn and McCarthy, 2006; WHO, 2006; Schantz-Dunn and Nour, 2009). In sub-Saharan Africa, it

is estimated that a child dies of malaria every 30 seconds (WHO, 2010) and, Schantz-Dunn and Nour (2009) suggest that it accounts for almost 25% of all maternal deaths.



**Figure 1:** A world map showing the global distribution of malaria in 2002 (Amponsah *et al.*, 2012).

As illustrated in Figure 1, the disease is endemic in tropical and sub-tropical countries of the world. Although only about 40% of the world's population live in these areas, malaria is considered a global health problem because of the phenomenon of globalization and the increasing incidence of traveler's with malaria (Pasvol, 2006).

Malaria is still a health challenge in Ghana. It is the number one cause of morbidity and the leading cause of workdays lost due to illness, accounting for 40-60% of outpatients' consultations in the country's health units, according to Asante and Asenso-Okyere (2003). Despite the control programmes adopted over the years, between 1985 and 2003, there has been an increase in malaria cases from 37.5% to 48.7% (Adams *et al.*, 2004).

## **2.2 SOCIO-CULTURAL AND ECONOMIC COST OF MALARIA**

Malaria is not only a disease of health in terms of mortality and morbidity, but also a disease of great social, cultural and economic importance. The disease affects both the infected individual and the society at large. People who are down with malaria may not be able to go to work and may have to be attended to by other members of the family at home. The social, cultural and economic effects have been studied and well-documented (Sachs and Malaney, 2001; Chima *et al.*, 2003; Jones and Williams, 2003; Chuma *et al.*, 2006, Jukes *et al.*, 2006; Drake *et al.*, 2011).

At the national level, malaria affects direct foreign investment, tourism, productivity and trade and expenditure. Countries in which the disease is endemic are generally shunned by foreign investors and tourists. The disease reduces productivity and a large amount of endemic countries' Gross Domestic Product (GDP) is spent on malaria interventions to control the disease. These factors affect national development and growth. According to Chuma *et al.* (2006), they account for why the per capita GDP is one-fifth lower in endemic regions than in non-endemic regions.



At the household level, malaria is known to cause and aggravate poverty as result of the expensive cost of treatment and the man-hours lost by both the infected individual and the care-taker. In a study conducted in Ghana, Chima *et al.* (2003), found that the cost of treatment of uncomplicated malaria in Ghana, a malaria-endemic country, was as high as \$7.38. This represented up to 7.1% of household income (Onwujekwe, 2000; Chuma *et al.*, 2006);

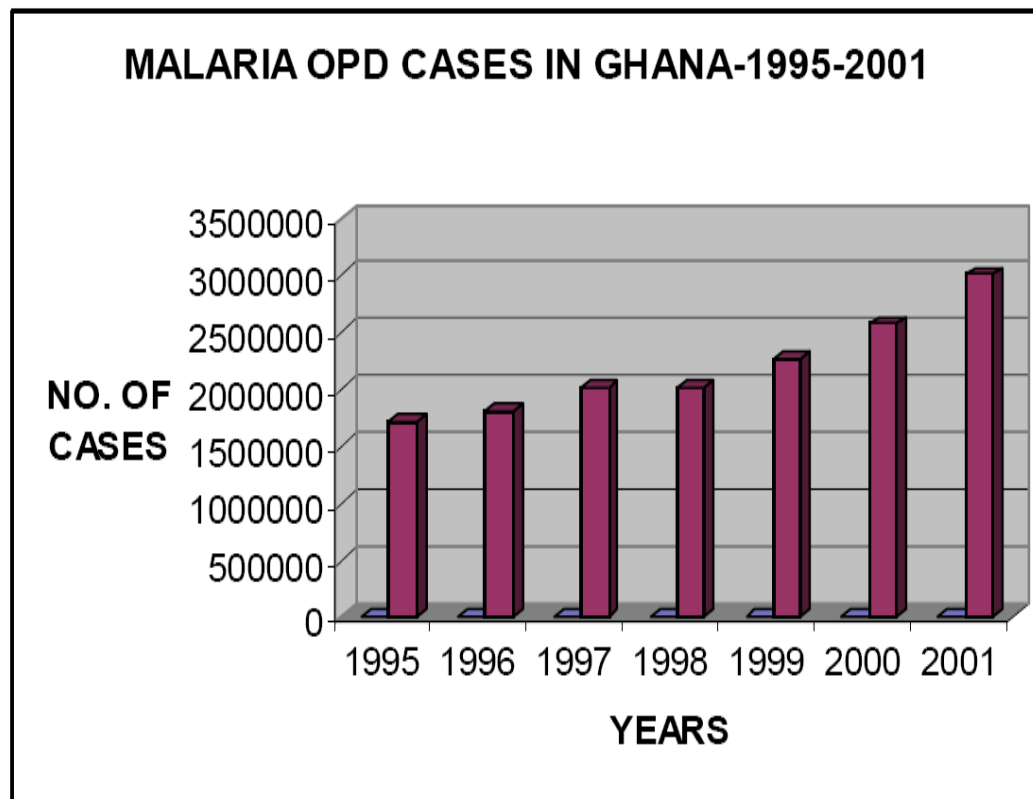
According to the United Nations Children's Fund (UNICEF) Report, 2007:

1. Malaria is a disease of the poor and is a cause of poverty. Countries in which the disease is endemic constitute some of the world's poorest countries.
2. The cost of preventing and treating malaria results in about 1.3% reduction in economic growth in Africa.
3. On the average, when malaria strikes a family, a quarter of the family's income is spent on treatment and preventing further spread. Research shows that a malaria-inflicted family is able to harvest only 40% of crops a healthy family is able to harvest.
4. Repeated bouts of malaria cause and increase absenteeism and drop-out rate in school children.

In children, who are the most vulnerable group to malaria, the effects of the disease can be a life-time burden. Cerebral malaria can cause life-long posture deformities and other physical deformities (Idro *et al.*, 2005). Jukes *et al.*, (2006) and Fernando *et al.*, (2010), showed that malaria affects the cognitive abilities, attention and ultimately, the intellectual development of children.

Pain, emotional distress and changes in household behavior, social action, social structure, and reproduction are aspects of malaria burden that cannot be measured (Jones and Williams, 2006).

In a study conducted by Asante and Asenso-Okyere (2003) on reported malaria OPD cases in some selected district hospitals in Ghana, from 1995 to 2001 it was reported that the disease increased annually, as illustrated in Figure 2.



**Figure 2:** A graphical illustration of malaria OPD cases in Ghana (Asante & Asenso-Okyere, 2003).

Malaria imposes a great burden on health institutions in Ghana, especially those at the primary health care level. Though the treatment of malaria is not free in Ghana, health sector resources are stretched during preventive and treatment services. The cost imposed on health institutions by malaria is assumed to contribute substantially to their annual recurrent expenditures. On the average, malaria cost to public health facilities in three districts (Sekyere East, Bole and Awutu-Efutu-Senya) amounted to GH¢ 96,767 in 2002, financed mainly from their internally generated funds (Asante and Asenso-Okyere, 2003). This amount represented 48.5% of the total recurrent expenditure from internally generated funds, with the average cost per case being estimated at GH¢1.61.

### **2.3 SYMPTOMS OF MALARIA**

Malaria is a disease of often potentially fatal consequences (Amexo, 2004). Clinical symptoms of malaria usually start 2 weeks after infection, although it can delay for up to 2 months, a year or even more (Kain and Keystone, 1998). Generally, malaria starts with symptoms that mimic flu and other common viral infections (Murphy and Oldfield, 1996). It is characterized by headache, fever, joint and muscle pains, dizziness, fatigue, nausea, vomiting, bitterness in the mouth and loss of appetite. These may be accompanied by diarrhoea, cough and sore throat (Kuhn and McCarthy, 2006). These symptoms may occur in episodes of sudden feeling of coldness followed by fever and sweating at 2-3 day intervals depending on the parasite species (Maitland and Marsh, 2004). *Plasmodium vivax* and *P. ovale* have a 2-day cyclical pattern, while that of *P. malariae* follows a 3 day cycle (Ntoumi, 2009). Cyclical patterns for *P. falciparum* are

more frequent but irregular. In uncomplicated malaria, these symptoms may progress to jaundice and haemoglobinuria (Maitland and Marsh, 2004).

Although malaria affects all age groups, clinical symptoms are more severe in children below five years, pregnant women and people from non-endemic areas, with accompanying high mortality. According to Trampuz *et al.*, (2003), in children, *P. falciparum* malaria may progress to severe or complicated malaria within 6-14 days of infection if not effectively and promptly treated. It results in extensive destruction of erythrocytes and the release of anti-malarial cytokines. This may result in neurologic, circulatory, pulmonary, cardiac, metabolic and renal complications such as hepatosplenomegaly, metabolic acidosis, severe anaemia, cerebral malaria, hypoglycaemia, ischemia, kidney failure, coma and brain damage (Maitland and Marsh, 2004) and brain damage may affect posture in children (Idro *et al.*, 2005). Approximately 20% mortality in children worldwide is associated with severe malaria, even under intensive care (Kain *et al.*, 1998; Trampuz *et al.*, 2009).

However, chronic non-symptomatic infections occur commonly in endemic areas (Malaney *et al.*, 2004). It has the advantage of preventing non-target drug effects, the high cost of unnecessary treatment and lowering the risk of drug resistance (Rafael *et al.*, 2006).

## 2.4 THE MALARIA PARASITE

The protozoan parasite of genus *Plasmodium*, was discovered by Louis Alphonse Laveran in 1880 as the cause of malaria. It is a single-celled eukaryotic organism of the parasitic phylum Apicomplexa. More than 200 species have been isolated in reptiles, birds and mammals (Tuteja, 2007). Five species are known to cause human malaria namely: *P. falciparum*, *P. ovale*, *P. malariae*, *P. vivax* and *P. knowlesi* (Jongwutiwes *et al.*, 2004; Betterton-Lewis, 2007).

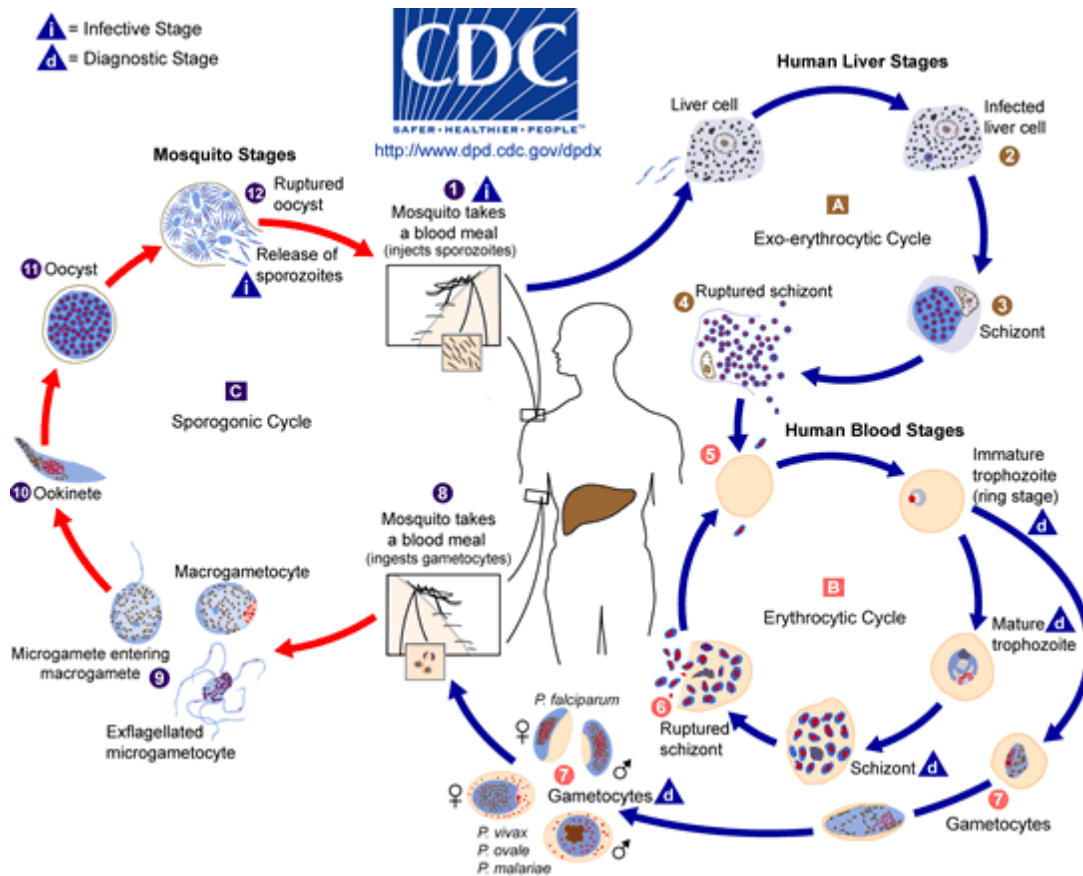
According to Tuteja (2007), the species vary morphologically, immunologically and in terms of geographical distribution and drug response. However, mixed infections are common in some malaria cases. Although *P. vivax* is rarely fatal, it is the most geographically widespread and accounts for most malaria cases worldwide while *P. falciparum* is the most virulent species and is responsible for 90% fatalities due to the disease (Tuteja, 2007; WHO, 2010). According to Carter and Mendis (2002) and Mendis *et al.* (2001), *P. vivax* is found in Asia, Central and South America and the Middle East. *P. falciparum* is restricted almost exclusively to sub-Saharan Africa (Snow *et al.*, 2004) whereas *P. malariae* is found in Africa, India and South America, and *P. ovale* is restricted to sub-Saharan Africa and South-eastern Asia (Tuteja, 2007). *P. knowlesi* was only known to cause simian malaria until recently when it was reported in human malaria cases in Malaysia, Thailand, Vietnam, Philipines and Burma (Jongwutiwes *et al.*, 2004; Betterton-Lewis, 2007; Muller *et al.*, 2007; Cox-Singh and Singh, 2008; Daneshvar *et al.*, 2009; Putaporntip *et al.*, 2009; Figtree *et al.*, 2010; Lee *et al.*, 2011). But because there have not been any reported cases of human-human transmissions, *P. knowlesi* malaria is considered a zoonotic disease with wild macaque monkeys as the reservoirs (Cox-Singh

*et al.*, 2007). Rare cases of human malaria caused by other *Plasmodium* species have been reported, but they are not considered to be of public health importance (Oguike *et al.*, 2011).

#### **2.4.1 LIFE CYCLE OF THE MALARIA PARASITE**

*Plasmodium* has a complex multiple stage lifecycle, which it completes in two different organisms: the vector mosquito and the vertebrate host (Floren *et al.*, 2002). The different stages are morphologically, structurally and functionally different and survive in different intracellular and extracellular environments. Survival is enhanced by a set of genes and their proteins, which enable the parasite to evade host defense mechanisms (Floren *et al.*, 2002; Greenwood *et al.*, 2005).

The complex life cycle is divided into three distinct stages: the sporogonic, the exo-erythrocytic and the erythrocytic stages. The sporogonic stage occurs in the digestive tract of the mosquito, the exo-erythrocytic stage occurs in the liver and erythrocytic stage occurs in the erythrocytes as illustrated by the C, A and B parts of Figure 3 respectively.



**Figure 3:** An illustration of the malaria parasite life cycle (CDC, 2010)

Man is the intermediate host of the human malaria parasite. The erythrocytic cycle begins when an infested mosquito takes a blood meal. Tens to a few hundred infective sporozoites from the salivary glands are inoculated into the skin. Experiments by Yamauchi *et al.* (2007) showed that the sporozoites remain at the site of infection. Some are destroyed by macrophages and some find their way into the lymphatic system (Vaughan *et al.*, 2008). Majority, however, find their way into the blood and are transported to the liver where they penetrate a few hepatocytes within a few hours after infection. Exo-erythrocytic schizogony occurs within the hepatocytes with each sporozoite multiplying into 10000 – 30000 merozoites, depending on the parasite

species (Jones and Good, 2006; Amino *et al.*, 2008). The liver stage takes 5–16 days to complete (Tuteja, 2007). The liver stage is referred to as the pre-patent phase and is characterized by little histopathology of the liver and no clinical symptoms, according to Vaughan *et al.* (2008).

The merozoites remain enveloped in host-cell derived membrane called merosome that exits the liver cells intact and may only rupture on entering the bloodstream (Silvie *et al.*, 2008). In *P. vivax* and *P. ovale* infections, the sporozoites in the hepatocytes may go into dormancy and only reactivate after a few months, or even years after primary infection. This explains the phenomenon of relapse in malaria caused by these parasites (Collins and Jeffrey, 1999). Merozoites do not re-infect hepatocytes.

Erythrocytes are the primary targets of the merozoites and it is the erythrocytic stage of the parasite life cycle that is responsible for much of the clinical symptoms of the disease. The merozoites recognize, attach and invade the erythrocytes by complex receptor-ligand interactions between parasite membrane proteins and erythrocyte membrane proteins. Greenwood *et al* (2008), suggest that the time taken to complete these interactions is so rapid that within 60 seconds of their release into the bloodstream, the merozoites disappear completely. Following merozoite invasion, the parasite undergoes erythrocytic schizogony

The erythrocytic stage of the parasite has two easily distinguishable phases: the trophic phase and the replicative phase. In the trophic phase, the parasite is enclosed in an intracellular ‘ring’ and develops into a trophozoite. This stage is marked by high metabolic activity and increase in size of the ‘ring’ (Cowman and Crabb, 2006; Haldar



and Mohandas, 2007). The parasite breaks down haemoglobin into constituent amino acids and haem. The amino acids are the substrates for parasite protein synthesis. Haem which is toxic to the parasite is polymerized into haemozoin (malaria pigment) which is stored in food vacuoles (Miller *et al.*, 2002). The final part of the trophic phase is marked by the parasite undergoing multiplication without undergoing cytokinesis.

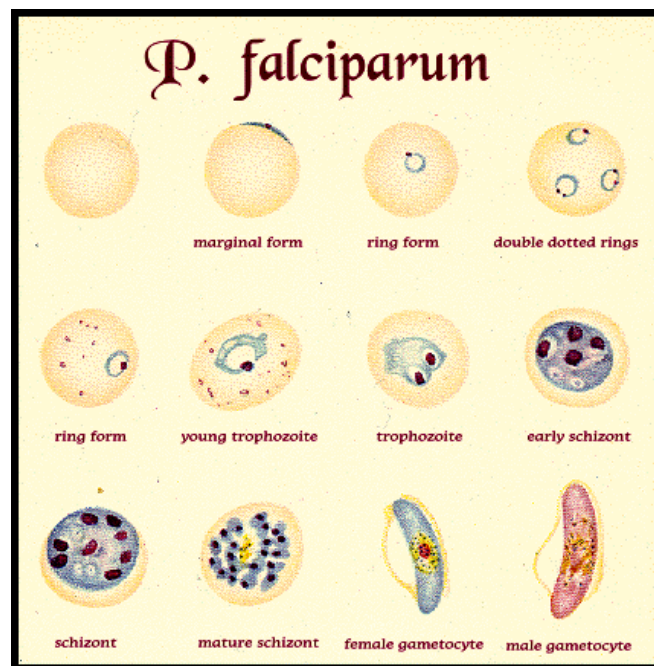
This is followed by the replicative stage. During this stage, the trophozoites develop into schizonts accompanied by cytokinesis. Each schizont contains 8 – 32 merozoites (Greenwood *et al.*, 2005; Tuteja, 2007). The end of the replicative phase is marked by infected cell lysis and the release of merozoites that in turn infect new cells and the cycle is repeated.

For *P. knowlesi*, the cycle takes 24 hours to complete, 48 hours for *P. falciparum*, 72 hours for *P. vivax* and *P. malariae* (Silvie *et al.*, 2008). In *P. falciparum* infections, multiple parasites are commonly seen in a single erythrocyte. Erythrocytic schizogony increases parasite burden.

A few of the merozoites upon invading erythrocytes differentiate into sexual forms of the parasite called gametocytes. They contain the male microgametes and female macrogametocytes. The gametocytes are non-pathogenic. Gametogenesis is stimulated especially by stressful conditions like reduced temperature, high carbon dioxide concentration and parasite waste products (Pukrittayakamee, 2008).

The gametes are ingested by the female *Anopheles* mosquito when it takes a blood meal from an infected person (Jones and Good, 2006; Tuteja, 2007; Amino *et al.*, 2008). In the mid-gut of the mosquito, they fuse to form a zygote which develops into a motile

ookinete after sometime. The ookinete penetrates the walls of the midgut and develops into an oocyst. Asexual sporogony occurs in the oocyst to produce several sporozoites, which are released into the haemocoel from where they migrate to the salivary glands. Sporozoites can be seen in the salivary glands of the mosquito 8 – 18 days after the ingestion of sporozoites. When the infected mosquito bites a person, the sporozoites are inoculated and the cycle is repeated.



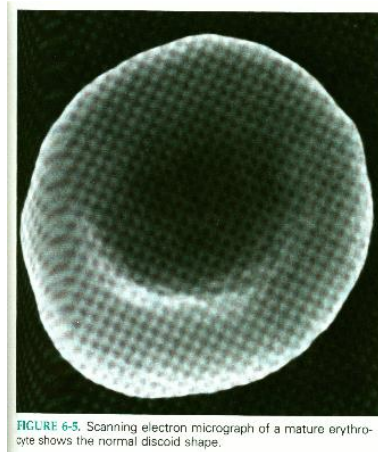
**Figure 4:** An illustration of the different erythrocytic stages of the parasite ([www.mahaarogya.gov.in](http://www.mahaarogya.gov.in))

## 2.5 ERYTHROCYTES

Erythrocytes are the primary targets of the merozoites. They contain nutrients for the survival of the parasite and they keep them protected against the host's defense mechanisms (Cowman and Crabb, 2006). A better understanding of the characteristics of

the erythrocyte and what roles they play in parasite entry would present opportunities to be exploited in the development of new anti-malarials.

Typically, a microlitre of blood contains between 4–6 million erythrocytes, although this may be slightly higher for those living at high altitudes due to low oxygen tension.



**FIGURE 6-5.** Scanning electron micrograph of a mature erythrocyte shows the normal discoid shape.

**Figure 5:** An illustration of a simplified structure of the erythrocyte membrane (Ogedegbe, 2003).

Erythrocytes have a flexible membrane and morphologically, they are biconcave disc-shaped, (as illustrated in Figure 5) and appear dumb-bell shaped in cross-section (Ogedegbe, 2003). They vary in size greatly from 2–8 microns in diameter and about 2 microns in thickness (Ogedegbe, 2003).

In the mammalian body, there are more erythrocytes than any other cell type, but unlike most other mammalian cells, erythrocytes are anucleate, lack mitochondria and many other cell organelles (Ogedegbe, 2003).

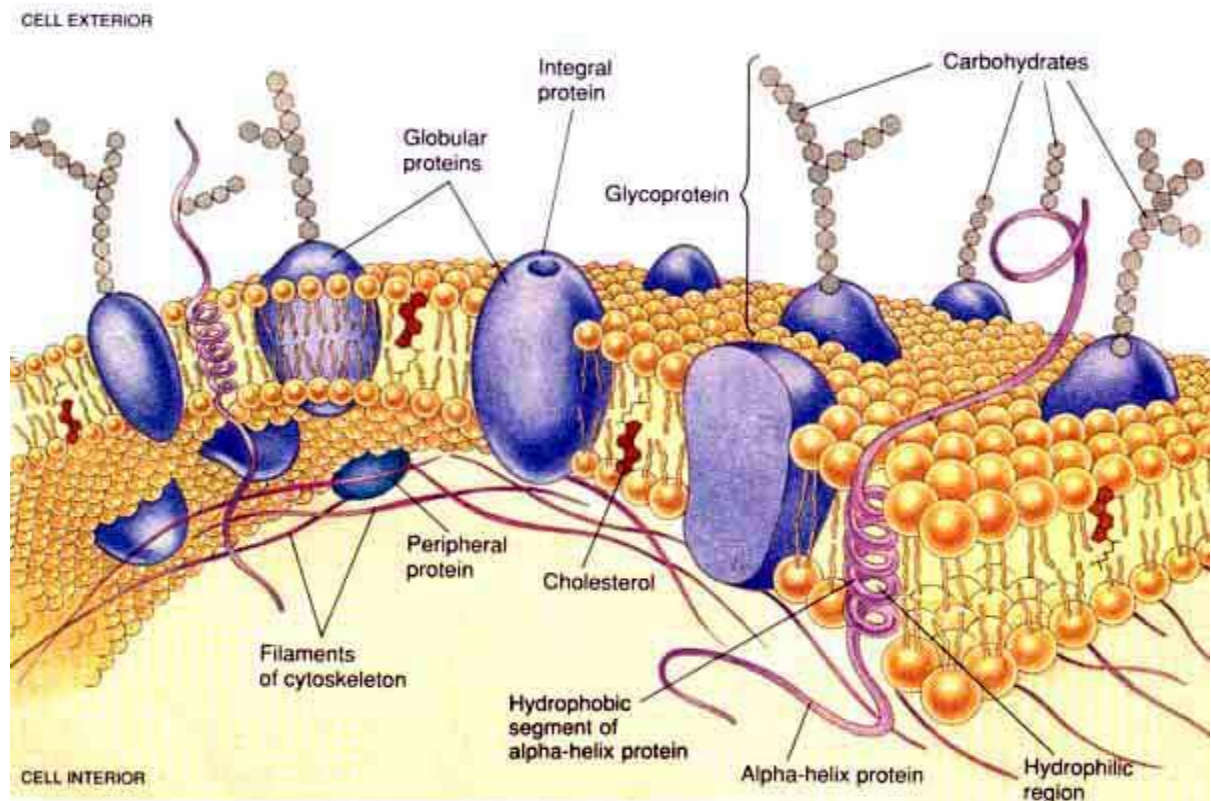
They are continuously synthesized through a process of erythropoiesis which takes place in the marrow of the long bones at a normal rate of about 2 million cells per second (Morera and MacKenzie, 2011). Newly synthesized erythrocytes are called reticulocytes and are nucleate. They constitute only about 1% of total erythrocytes in circulation.

Erythrocytes have a lifespan of up to 120 days. It has been suggested that *P. malariae* has a higher affinity for more mature erythrocytes and *P. ovale* and *P. vivax* have higher affinity for reticulocytes, *P. falciparum* has equal affinity for erythrocytes of all ages (Billig *et al.*, 2012). This phenomenon is one of the reasons why *P. falciparum* is the most deadly of all the species.

At the end of its lifespan, erythrocytes are broken down into biliverdin and bilirubin pigments and iron in the spleen (Ogedegbe, 2003). The broken down components are transported to the liver where the iron is recycled and the pigments are excreted in bile. Excessive destruction of erythrocytes by the parasite is what results in anaemia and hepato-splenomegaly associated with severe malaria (Schantz-Dunn and Nour, 2009).

Some of the structural and morphological features that adapt the erythrocyte for its main function of oxygen transport are what make them primary targets for merozoites (Cohn *et al.*, 2003). The membrane is flexible because it is made up of lipids interspersed with proteins (Asgary *et al.*, 2005). This enables it to undergo deformation to squeeze between cells to deliver oxygen in tissues. The structural proteins play a role in recognition by the

parasite and the transport proteins help in the movement of parasite nutrients and wastes into and out of the erythrocyte respectively. Also, haemoglobin, the oxygen-binding molecule is broken down by the parasite to release the constituent amino acids for use by the parasite (Tan and Sebisubi , 2010).



**Figure 6:** A schematic diagram of the ultra-structure of the erythrocyte membrane ([www.winona.edu](http://www.winona.edu)).

The erythrocyte membrane is organized into three layers:

1. The glycocalyx: This is the exterior carbohydrate-rich layer. It has covalently bound lipid that are protein substrates of the blood group antigens.

2. The lipid bi-layer: This consists of lipids with polar heads facing both extracellular and intracellular environment. It is also characterized by transport proteins
3. The membrane skeleton: This comprises of a network of proteins found in the inner lipid bi-layer.

The membrane lipids are either phospholipids or neutral lipids predominantly consisting of unesterified cholesterol. The phospholipids are found on the outer and inner surfaces of the bilayer leaflets with the cholesterol interspersed in the phospholipid molecules. The relative amounts of the phospholipids and the cholesterol determine the flexibility and the fluidity of the erythrocyte membrane.

Many proteins have been mapped to the erythrocyte membrane and are distributed over the entire lipid bilayer as illustrated in Figure 6. They are known to perform structural, enzymatic, transport and receptor functions. Others, such as glycophorin A and B and receptor X, function as ligands in erythrocyte-merozoite interactions during invasion (Sim *et al.*, 1990; Dolan *et al.*, 1994). Actin, spectrin and Band 4 are the most important structural proteins. They form a complex network of interconnected strands within the membrane forming a mesh. This is known as the membrane skeleton. This determines the shape, the deformability and the integrity of the cell membrane.

Band 3 is an example of transport proteins mapped to the erythrocyte membrane. It is the channel that transports ions and water molecules into and out of the cell. Glycophorin-A is a glycoprotein that plays receptor roles. Disruptions in these proteins and lipids have

been demonstrated to inhibit erythrocyte invasion by malaria parasite (Cowman *et al.*, 2003; Cohn *et al.*, 2003).

*Plasmodium* is an obligate intracellular parasite (Tuteja, 2007). The erythrocyte-invasive stage of the parasite is the motile merozoite which is produced by exo-erythrocytic schizogony in the liver cells and erythrocytic schizogony. Although the merozoites lack flagella, the parasite translocates protein molecules through microtubules posteriorly resulting in a forward gliding movement of the parasite to locate erythrocytes to initiate the process of invasion (Pinder *et al.*, 1998).

Goel *et al.*, (2003) and Cowman and Crabb (2006), suggest there are surface proteins on the merozoite such as Merozoite Surface Proteins (MSPs), Erythrocyte Binding Proteins (EBPs) and Reticulocyte Binding Proteins (RBPs) which function as ligands in parasite invasion.

Wiser (2010), categorizes the process of parasite invasion into four distinguishable steps:

1. Initial merozoite binding step
2. Re-orientation and erythrocyte deformation step
3. Junction formation step
4. Parasite entry step

To avoid the body's immune response to the parasite, the process is so rapid that it is completed within about 60 seconds (Cowman and Crabb, 2006). Once the parasite invasion of an erythrocyte is complete, it modifies the erythrocyte membrane structure inducing the formation of new permeability pathways (Kirk and Horner, 1995; Kirk,

2001). These serve as channels through which nutrients needed for the survival and growth of the parasite i.e. nucleosides, amino acids, sugars and organic and inorganic ions are taken up and waste products are also removed (Kirk, 2001). This makes the inhibition of parasite-induced channels a target for potential anti-malarial agents (Gero and Weiss, 2001; Cohn *et al.*, 2003).

## **2.6 MALARIA CONTROL**

For as long as malaria has been with man, there have been attempts to control the incidence and prevalence of the disease and the morbidity and mortality it causes. Over the years, two strategies have been adopted, mostly in combination to control the disease:

1. Transmission Prevention Strategies
2. Chemotherapy

### **2.6.1 TRANSMISSION PREVENTION STRATEGIES**

This involves prompt and accurate diagnosis of infection and the use of prophylaxis to suppress infection and prevent disease, preventing infection and transmission.

It is an important component of the malaria control programmes adopted over the years. In parts of the world where the disease has been eradicated, vector control programmes played an important role (William, 1963) and the countries in which the disease is still endemic, it is because vector control programmes have not been adopted or have not been



successful (Kouyaté *et al.*, 2007). The strategy involves the use of insecticides to control mosquitoes in human habitations through the technique of Indoor Residual Spraying (IRS). Killing mosquitoes breaks transmission.

Another transmission control strategy aims to prevent vector-host contact. This is achieved by the use of protective clothing in the night and bed-nets which protects people against mosquito bites (Korenromp *et al.*, 2003). This is effective because most mosquito species that transmit malaria only bite at dawn and at dusk. Recently, insecticide-treated nets (ITNs) have been introduced and are estimated to be doubly more effective than ordinary bed-nets. ITNs combine vector control and personal protection.

Advancements in genetics and molecular biology suggest it is possible to produce *Plasmodium*-resistant Anopheles species (Ito *et al.*, 2002). Because research shows that *Plasmodium* compromises the fitness of infected mosquitoes, the transgenic species will be fitter than infective species which will result in their removal from the environment sometime after the introduction of the transgenic species. However, the cost of this technique is high and the other techniques used have not produced the needed results in reducing malaria disease burden. This makes dependency on chemotherapy very heavy.

### **2.6.2 CHEMOTHERAPY**

Although, malaria is preventable, it is also curable if promptly and accurately diagnosed and effectively treated. *Plasmodium falciparum*-malaria can be fatal; however, complete recovery from the disease is possible. Chemotherapeutic agents target the parasites in an infected person. There are two modes of malaria chemotherapy:

1. Prophylactic chemotherapy
2. Treatment chemotherapy

### **2.6.3 PROPHYLACTIC CHEMOTHERAPY**

It is evident that people who live in malaria endemic areas have some level of immunity to the disease. However, children below 5 years, pregnant women and people from non-endemic areas are very vulnerable to the disease when exposed to the parasite (Schantz-Dunn and Nour, 2009). This makes preventive chemotherapy or prophylaxis necessary for these vulnerable groups to offer protection against the disease. Intermittent Preventive Therapy (IPT) is particularly recommended for pregnant women and people from non-endemic areas travelling to endemic areas are encouraged to take malaria prophylaxis (Schantz-Dunn and Nour, 2009).

Over the years, atovaquone-proguanil (malarone), mefloquine, doxycycline, chloroquine and hydrochloroquine have been used in malarial prophylaxis (Dow *et al*, 2006). These suppress infection and prevent the development into full disease and are available as over-the-counter or prescription medications and may be administered before, during or after exposure to mosquito bites, depending on the type of medication. However, the choice of prophylactic agent and regimen depends upon the region and personal characteristics of the patient.

However, there are no known prophylactic drugs that confer 100% protection against malaria.

#### **2.6.4 TREATMENT THERAPY**

Many anti-malarial agents for treatment are available and choice is based on:

1. Severity of disease
2. Geographical location of where the infection was acquired
3. Host factors such as age, weight and pregnancy status

In most malaria-endemic regions, ACTs are used as the first line drugs for the treatment of malaria (WHO, 2010).

#### **2.7 SUCCESS AND CHALLENGES OF MALARIA CONTROL PROGRAMMES**

The malaria burden in many countries especially in tropical Africa where the disease exacts its heaviest toll has reduced (Mendis, 2009). The success of vector control programmes in Burundi (Beier, 2008; Protopopoff *et al.*, 2008), Eritrea, Brazil, India and Vietnam (Barat, 2006) has led to a reduction in mortality. For example, infant mortality due to malaria has reduced by 20% globally (Sketekee and Campbell, 2011). According to Sambo *et al.* (2011), there has been a 50% reduction in malaria cases, admissions and mortality from the disease in 11 African countries. The success of these programmes has led to a 20% reduction of *P. falciparum* malaria prevalence in sub-Saharan Africa (Eiselle and Sketekee, 2011).

Over the years, a lot of research has gone into developing malaria vaccines. Trials have been going on, with some reaching very advanced stages and with promising results (Graves and Gelband, 2006; Thera and Plowe, 2011).

These interventions have led to the eradication of the disease in North America and Europe and major reductions in mortality and morbidity in South Asia, but the disease is still a major killer in sub-Saharan Africa (Cowman and Crabb, 2006). This is because of the challenges these strategies are faced with in the region.

Although according to the WHO (WHO, 2010) vector control is the most important strategy in reducing mortality and morbidity due to malaria, it has not been very successful in many countries, especially in sub-Saharan Africa. It is often interrupted by wars and disasters, poor sanitation, breakdown of healthcare delivery services and lack or failure of health education programmes.

The first insecticide used for IRS was DDT (Kelly-Hope *et al.*, 2008). However, its use was discouraged because of health risks associated with it and other chemicals including pyrethroid and permethrin have been introduced in its place. However, this strategy has not been sustainable because of the reduced susceptibility of the vector mosquitoes to the insecticides (Kelly-Hope *et al.*, 2008).

Cases of side effects of anti-malarials have also been reported, but the most formidable challenge to the fight against the disease is the emergence and increasing spread of drug-resistant parasite strains. This places a heavy burden on malaria control programmes and health delivery systems in sub-Saharan-Africa, especially, and has rendered the more common and affordable anti-malarial drugs like sulfadoxine-pyrimethamine, chloroquine

and mefloquine less effective (WHO, 2005). There have been recent reports of delayed parasite clearance for the ACTs (Noedl *et al.*, 2008; Dondorp *et al.*, 2009; Anderson *et al.*, 2010), a comparatively more expensive drug which is the drug of choice for the treatment of uncomplicated malaria.

Malaria is a disease of the poor (WHO, 2010). Thus, coupled with treatment failures as a result of poor compliance, non-availability or accessibility of primary healthcare units leaves many people in sub-Saharan Africa and other parts of the world where the disease is endemic solely reliant on traditional plant products to treat malaria. Use of traditional plant products is an important source of health delivery to these populations.

## **2.8 TRADITIONAL PLANT MEDICINES AS ANTI-MALARIAL AGENTS**

According to Tiburt and Kaptchuk (2008), traditional plant medicines are natural products obtained from plants with little or no industrial processing that are used to treat or prevent diseases or to enhance health. They come in different forms including concoctions, decoctions dietary supplements, pills, teas, powders, creams, syrups and steam therapies.

In many parts of the world, traditional medicines have been used long before the advent of allopathic medicine and were the only means of treating or preventing diseases (Tiburt and Kaptchuk, 2008). Even in the era of allopathic medicine, due to lack of access, poverty, inconveniences of side effects and increasing cases of drug resistance, many populations still rely on traditional herbal medicines. In Africa WHO, (2002) and Joy *et*

*al.* (2001) puts the percentage of people who still rely on at least some form of traditional herbal medicine at 80%. The situation is not very different in other parts of the world. In countries like China and India, it has become integrated and is part of the mainstream health-care delivery system (Ssegawa and Kesenene, 2007).

For centuries, traditional herbal medicines have been used as anti-malarials and even in modern times the active ingredients in them are used as starting point for the production of allopathic drugs. The best-known and best-studied traditional anti-malarials are *Cinchoma pubescens* and *Artemesia annua* (Challand and Wilcox, 2009). In China, herbal teas prepared from *A. annua* are still used to treat malaria in areas where there is limited or no access to allopathic anti-malarials. Quinine and its derivatives and ACTs which constitute the drugs of choice for the treatment of malaria in most parts of the world are obtained from *C. pubescens* and *A. annua* respectively (Challand and Wilcox, 2009).

A study by Wilcox and Bodeker (2004), revealed the following:

1. More than 1200 plant species in over 600 plant families worldwide are used as anti-malarials
2. As much as 75% of people living in many malaria-endemic areas rely on traditional anti-malarials.

Traditional herbal medicines are administered either as monotherapy or in combination for synergistic effect and have been shown in some cases to achieve 100% parasite clearance (Oguntola, 2011). Research into their modes of action suggests they function in

different ways in preventing or treating malaria. According to Challand and Wilcox, (2009):

1. They disrupt the life cycle of the parasite by interfering with nutrient uptake and metabolism.
2. They stimulate the immune system to produce substances like nitric oxide and other substances that directly kill the parasite or inhibit its activity.

Many plant species have been identified but due to the high interest in traditional anti-malarials, the list is ever increasing (Philippe, 2011). Some of the well-researched traditional anti-malarials include: *Psidium guajava*, *Anacardium occidentale*, *Alchornea caudifolia*, *Citrus sinensis*, *Khaya senegalensis*, *Morinda lucida*, *Nauclea latifolia*, *Magnifera indica*, *Crptolepis sanguinolenta*, *Acasia nilotica*, *Carissa edulis*, *Fagaropsis angolensis*, *Lantana camara*, *Harrissonia abyssinica*, *Zanthoxylum usambarensis*, *Bideus pilosa*, *Nuxia verticulata*, *Plumbago indica*, *Garcinia mangostana*, *Dracaena loureiri*, *Dioscorea membranacea*, *Piper chaba*, *Myristica fragrans*, *Kaempferia galanga* and *Psiadia arguta* (Abbiw, 1990; Andrade-Neto *et al.*, 2004; Wilcox and Bodeker, 2004; Kirira *et al.*, 2004; Jonville *et al.*, 2008; Oguntola, 2011; Adebayo *et al.*, 2011; Thiengsusuk *et al.*, 2013).

Different parts of these plants are used as anti-malarials including the seeds, leaves, roots, bark and pods.

Different bioactive compounds in these plants are responsible for their medicinal properties. Sack and Froehlich (1982) grouped them into different broad groups depending on their chemical structures namely: alkaloids, anthocyanins, anthraquinones,

glycosides, coumarins, flavonoids, glucosilates, minerals, mucilages, volatile oils, saponins, tannins and vitamins. Cocoa has been shown to be an important source of flavonoids (Steinberg *et al.*, 2003; Lee *et al.*, 2003).

*Theobroma cacao* or cocoa tree is a semi-deciduous plant thought to be native to the Amazon in South America (Hirst, 2011). It is of plant family Sterculiaceae and is widely grown in humid tropical countries in West Africa, South America and Asia. In Cote d'Ivoire, Ghana, Malaysia, Nigeria and Brazil, it is grown as a major cash crop and important source of foreign exchange to these countries.

There is hardly any database available on the number of species of the plant there are. However, three varieties of the plant are recognized namely: *Theobroma cacao ssp. cacao*, *Theobroma cacao ssp. sphaerocarpum* and a hybrid of the two species commonly called *Trinitario* (Hirst, 2011).

The cocoa plant grows up to between 4 and 8 metres with oblong leaves 10 – 40 cm long and 5–20 cm broad (Amponsah *et al.*, 2012). The leaves appear red when young. The plant has a short trunk with branches occurring in whorls. At maturity, the plant bears flowers with pink, rosy, white, yellow or bright red calyx (depending on the sub-species) in clusters directly on the stem. The plant starts flowering after 4 – 5 years after planting.

A ripe cocoa fruit called a pod is bright yellow to orange in colour and ovoid in shape. Each pod is divided into ten sections by prominent ridges along the length of the fruit. The pod contains between 20–60 dicotyledonous seeds called cocoa beans. They are embedded in a white, sweet and sticky pulp.



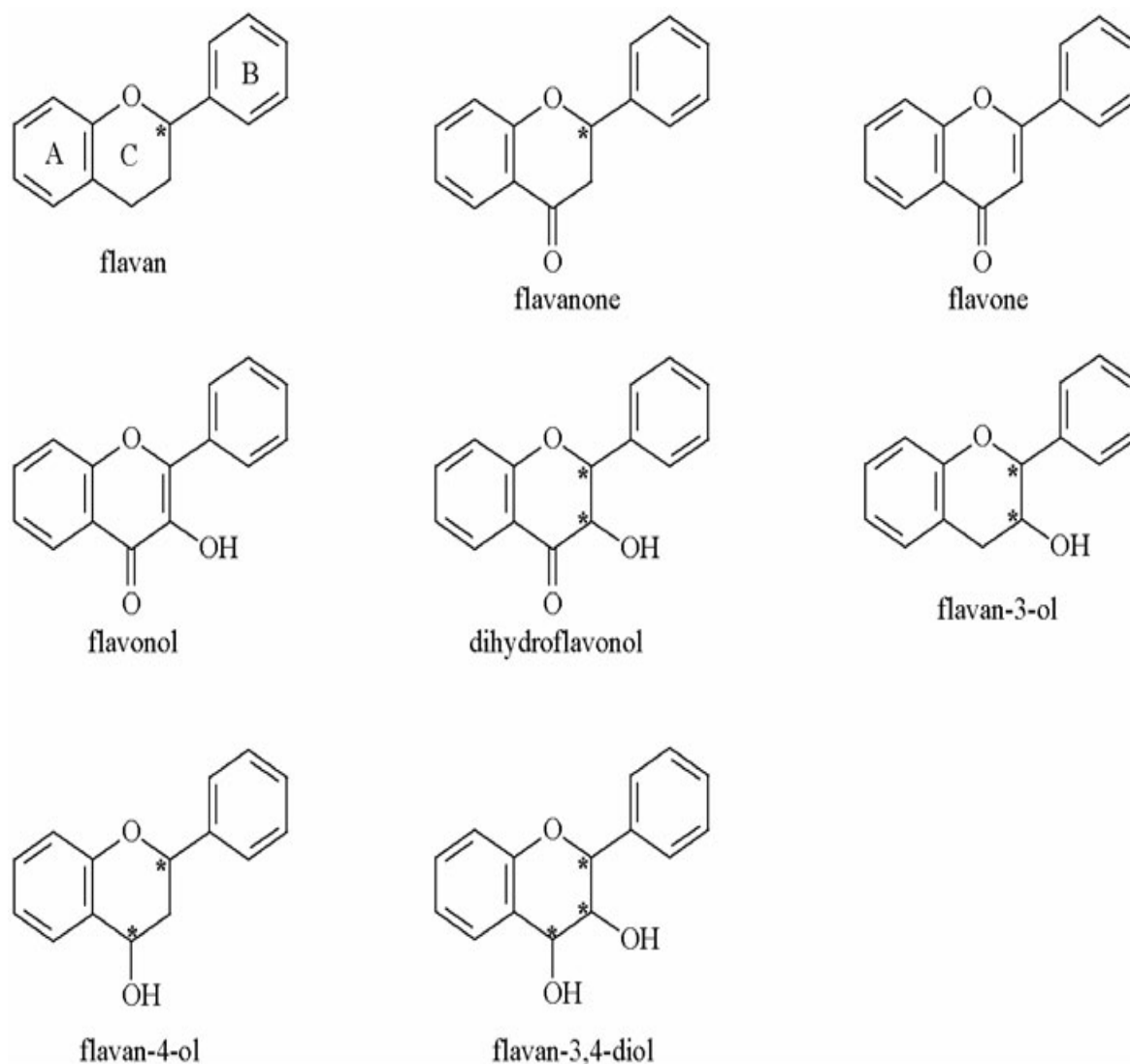


**Figure 7:** An illustration of cocoa pod cut open to expose beans and pulp (www.fs.fed.us).

When the pods are harvested, they are cut open and the beans removed and fermented. Fermentation is done over 3 to 6 days, according to Hui *et al.*, (2007). The beans are then dried, roasted and winnowed before grinding is done using a micropulverizer to produce chocolate paste (Afoakwa *et al.*, 2008). The paste is subjected to hydraulic pressing to separate it into cocoa butter and the residue. Natural cocoa powder is obtained from the dry residue (Hui *et al.*, 2007).

Polyphenols or phenols are secondary metabolites and are widely distributed in leaves, seeds, stems, flowers, bark, grains and fruits (Middleton, 1998; Lamuela-Raventos *et al.*, 2005; Biesaga, 2011). Asgary *et al.* (2005) suggest that polyphenols are found in every product of plant origin. Many different polyphenols have been identified with diverse and complex chemical structures (de Groot and Rauen, 1998) and they are known to constitute 12% to 18% of the total weight of dried cocoa nibs (Porter *et al.*, 1991).

Flavonoids are the most abundant polyphenolic micronutrients in diets and account for 60% of dietary polyphenols (Hodek *et al.*, 2002; Chahar *et al.*, 2011; Scalbert *et al.*, 2005). As illustrated in Figure 9, flavonoids have a skeleton that consists of a linear three-carbon chain that forms an oxygenated heterocycle (Chahar *et al.*, 2011) with two phenyl groups attached (Kuhnau, 1976; Hodek *et al.*, 2002; Chahar *et al.*, 2011; Biesaga *et al.*, 2011). It is the numbers and positions of methoxyl, hydroxyl or other substituents attached to the backbone that determine their diversity and different chemical and physical characteristics.



**Figure 8:** An illustration of the structures of the major sub-classes of flavonoids (Grotewold, 2006).

Most of the flavonoids found in cocoa are of the flavanol sub-class namely the monomers catechin, epicatechin and procyanidin and their oligomers (Vinson *et al.*, 1999; Adamson *et al.*, 1999; Osakabe *et al.*, 2001; Steinberg *et al.*, 2003; Ranganath *et al.*, 2008).

Thus, cocoa has been used as medicinal food for a long time and has been known as treatment therapeutic agent for many chronic diseases (Dillinger *et al.*, 2000; Weisburger,

2001). Dark chocolate and natural cocoa powder have been proven to have greater flavonoid content than most other cocoa products, according to DeNoon (2004). This is because processing destroys up to half the flavonoid content of cocoa and these products undergo very little processing.

**Table 1:** A table showing general composition of cocoa beans (Baba *et al*, 2007)

<b>Component</b>	<b>Amount (Units/ 100 g)</b>
Protein	23.0 g
Fat	11.5 g
Carbohydrate	23.1 g
Fibre	26.9 g
Minerals	7.7 g
Epicatechin	377 mg
Cathechin	135 mg
Procyanidins B2	158 mg
Procyanidin C1	96.1 mg
Theobromine	2192 mg

The traditional and the most frequently used method of flavonoid extraction is the solvent extraction technique by cold maceration (Sultana, 2009; Biesaga, 2011). However, recent years have seen the introduction of novel techniques including microwave-assisted techniques (Biesaga, 2011). The type of solvent and its extraction capability depends upon its polarity. Both keto-group bearing and alcohol-group flavonoids are polar. This

makes polar solvents including methanol, ethanol and their mixtures (Bonoli *et al.*, 2008) and their combinations with water and ethyl acetate, good solvents for the extraction of flavonoids (Sultana *et al.*, 2009). Results of work by Ghasemzadeh (2010) showed that methanol has the highest flavonoid extraction capability. Generally, aqueous methanol gives higher extract yield than its absolute counterpart (Sultana *et al.*, 2009).

Flavonoids have been extracted from other plant sources and their anti-malarial and other medicinal properties investigated *in vitro* (Bero *et al.*, 2009). However, there is not much information available on the anti-malarial properties of natural cocoa extract.

## **2.9 IN VITRO ANTI-PLASMODIAL ASSAYS**

Since Trager and Jensen (1976) described the technique for culturing the asexual erythrocytic stages of the malaria parasite *in vitro*, it has been extensively used to provide information supplementary to that obtained by epidemiological studies (WHO, 2007). This has led to a better understanding of the malaria parasite in terms of immunology, biochemistry, molecular biology and pharmacology (Ringwald *et al.*, 1999).

The *in vitro* malaria culture technique has found great use in drug sensitivity assays. This involves culturing parasite isolates in different concentrations of the drug or the potential drug and monitoring parasite growth and development at each concentration. The gold standard method of microscopic examination of smears is tedious and time-consuming. The alternative method of measuring drug effect by assessing radioactive hypoxanthine incorporation is also limited because it is not stage-specific (Noedl *et al.*, 2011). The other

method of measuring parasite lactate dehydrogenase activity uses relatively expensive reagents and is not suitable for large-scale drug screening efforts (Vossen, 2010). Many assays have been developed to quantitatively assess the effects of drugs on parasite growth and development *in vitro* (Noedl *et al*, 2011). The SYBR<sup>®</sup> Green assay is an inexpensive, fast and easy-to-use assay developed to monitor parasite viability *in vitro* drug sensitivity tests (Johnson *et al.*, 2007; Vossen, 2010).

### **2.9.1 SYBR<sup>®</sup> GREEN ASSAY**

SYBR<sup>®</sup> Green assay is a fluorescence assay that detects malaria parasite DNA in infected erythrocytes. The amount of parasite DNA detected is a measure of parasite growth and degree of inhibition by the anti-malarial agent the culture is exposed to (Vossen *et al.*, 2010).

According to Bennett *et al.* (2004), SYBR<sup>®</sup> Green is a cyanide dye that binds to double-stranded DNA preferring G and C base pairs. When it binds to DNA, it becomes fluorescent and absorbs light at wavelengths between 390 and 505 nm, with the highest point at 497nm and has a secondary peak near 254 nm. It gives off light at 505 to 615 nm, with a peak at 520 nm (Sigma-Aldrich, 2008).

Although SYBR<sup>®</sup> Green is not selective for malaria parasite DNA and therefore binds to all double-stranded DNA including that of contaminant microorganisms and host white blood cells leading to high background reading, it is fast, easy to use and inexpensive, compared to other assays (Vossen *et al*, 2010).

## CHAPTER THREE

### MATERIALS AND METHODS

#### 3.1 MATERIALS

The following were obtained from the sources indicated:

25cm<sup>3</sup> culture flasks (Corning Inc)

75cm<sup>3</sup> culture flasks (Corning Inc)

96 well microtitre plate (Corning Inc)

24 well microtitre plate (Corning Inc)

1ml plastic pipettes (Corning Inc)

5ml serological pipettes (Corning Inc)

10ml serological pipettes (Corning Inc)

25ml serological pipettes (Corning Inc)

Filter unit 500ml (Nalge Nunc Int)

15ml centrifuge tubes (Corning Inc)

50ml centrifuge tubes (Corning Inc)

25 cm<sup>3</sup> tissue culture flask (Corning Inc)

75 cm<sup>3</sup> tissue culture flask (Corning Inc)

Frosted end microscope slides (Thermo Scientific)

Vacutainer bld collection set G23 (BD)

Vacutainer tubes, 5ml (BD)

RPMI1640 powder (GibCo)

Glucose (Sigma-Aldrich)

AlbuMAX II (Invitrogen)

Hypoxanthine (GibCo)

HEPES (Sigma)

SYBR<sup>®</sup> Green 1 (Sigma-Aldrich)

Ethanol (Sigma-Aldrich)

Methanol (Sigma-Aldrich)

Immersion oil (Sigma-Aldrich)

Giemsa stain (VWR Int.)

Adenine (Sigma-Aldrich)

Saponin (Sigma-Aldrich)

L-Glutamine (Sigma-Aldrich)

Na<sub>2</sub>HPO<sub>4</sub> (Sigma-Aldrich)

NaCl (Sigma-Aldrich)

KH<sub>2</sub>PO<sub>4</sub> (Sigma-Aldrich)

Sorbitol (Sigma-Aldrich)

Gas mix (92.5% N<sub>2</sub>, 5.5 % CO<sub>2</sub>, 2% O<sub>2</sub>,)

Good Food Natural cocoa powder (Kakawa Entreprise Ltd, Accra)

Artesunate powder (Sigma-Aldrich)

Chloroquine powder (Sigma-Aldrich)



Natural cocoa powder was obtained from Kakawa Entreprise Limited, Accra and extracted using aqueous methanol and analysed for the presence of flavonoids and total phenolic content. It was then assayed to determine its activity against chloroquine-sensitive 3D7 and chloroquine-resistant DD2 strains of *Plasmodium falciparum*.

The proposal was approved by the Scientific and Technical Committee and the Institutional Review Board of the Noguchi Memorial Institute for Medical Research, University of Ghana.

### **3.2 EXTRACTION**

Four hundred grammes of the natural cocoa powder was weighed out into two separate reagent bottles with a volume of 2L each. One litre of aqueous methanol (98% v/v) was added to each bottle and extraction was done by percolation over three weeks. At the end of each week, the supernatant was decanted and the extract solution filtered using a Buchner funnel with filter paper. The filtered extract solution was evaporated to dryness using rotary vacuum evaporator (Büchi, Germany) set at 60 °C, 20 rpm and reduced pressure of 26 mm Hg. The extract was transferred into a storage bottle and the recovered solvent was added back to the reagent bottles containing the cocoa powder to maximize the extraction process.

The crude methanol extract was put in a glass flask and covered with cellophane. It was wrapped in aluminium foil and stored at 4 °C to preserve it for use in assays.

### 3.3 QUALITATIVE ANALYSIS FOR FLAVONOIDS

Shinoda test as described by Kumar *et al* (2007) was carried out to determine the presence of flavonoids in the crude methanol natural cocoa extract. Two hundred milligrams of the crude methanol extract was dissolved in 20 ml of 80% (v/v) ethanol. The solution was filtered using a Whatman filter paper and 3 ml portion of the filtrate aliquoted into a test tube. Five pieces of magnesium turnings and 0.5 ml of concentrated hydrochloric acid (HCl) were added to the tube and shaken. The appearance of a pink or red color in the solution indicates the presence of flavonoids.

### 3.4 QUANTITATIVE ANALYSIS FOR TOTAL PHENOLIC CONTENT

The total phenolic content (TPC) assay was carried out to estimate the quantity of flavonoids in the extract. It does not provide an exact measure of the quantity of flavonoids in the extract, but it gives a fair estimate.

The Folin-Cicalteau assay described by Chum *et al.*, (2003) was used with a slight modification. Ten  $\mu\text{l}$  of stock extract solution was aliquoted into a microtube and 0.79 ml of distilled water was added, followed by the addition of 50  $\mu\text{l}$  of Folin-Ciocalteau reagent and mixed thoroughly by triturating with a pipette. The set-up was incubated at room temperature for about 8 minutes.

After incubation, 150  $\mu\text{l}$  of sodium carbonate ( $\text{Na}_2\text{CO}_3$ ) solution was added, mixed and incubated again at room temperature for 2 hours. The solution was aliquoted into wells of a 96-well microtitre plate and absorbance measured at 765nm using a spectrophotometer

(Tecan Plate Reader, Infinite Pro.). Gallic acid was used as the standard for the calibration curve and TPC was expressed as milligram gallic acid equivalent (GAE) per gram of extract. The equation of the standard calibration curve was  $y = 0.2794x - 0.0374$  ( $r^2 = 0.9997$ ) with  $y$  as the absorbance and  $x$  as the concentration of the GA expressed as mg/ml.

### **3.5 CRUDE EXTRACT SOLUTION PREPARATION**

The crude methanol natural cocoa extract was first dissolved in absolute methanol at a concentration of 100 mg/ml and shaken for 30 minutes to ensure a complete solubilization. This was considered the stock extract solution. It was stored at 4<sup>o</sup> C. Each prepared stock solution was used for one month.

To prepare working concentrations, this was further diluted with complete parasite media (CPM) to obtain a concentration of 1000 µg/ml. The solution was serially diluted two-fold 9 times to give 10 different concentrations of the extract solution. Standard drugs, chloroquine dissolved in double-distilled water and artesunate dissolved in DMSO were used as positive controls in all experiments. Aliquots of each concentration (12.5 µl) were transferred into 96-well microtiter plates. Working solutions were stored at -20<sup>o</sup>C until use. The highest concentration of the solvents to which the parasites were exposed was less than 1%, which according to Ramazani *et al.* (2010), has no significant effect on parasite growth. Experiments were performed in triplicates and each was repeated at least once.

### 3.6 PREPARATION OF BLOOD FOR USE IN PARASITE CULTURES

Venous blood was taken from individuals who belonged to blood group O<sup>+</sup> and were tested as sickling negative. They also had no recent history of inflammatory disease and were not on any anti-malarial medications as at the time the blood was taken. The blood was collected in vacutainer tubes pre-dosed with anticoagulant, adenine citrate dextrose (ACD) by venu-puncture. The blood was stored at 4 °C overnight to kill any external malaria parasite. It was transferred into 15 mL centrifuge tubes (Corning Inc.) and centrifuged at 2500 revolutions per minute (rpm) for 5 minutes. The plasma and buffy coat were aspirated and discarded (the buffy coat contains white blood cells which are detrimental to *in vitro* parasite growth). The same volume of incomplete RPMI was added; centrifugation repeated and supernatant aspirated and discarded again. The washing process was repeated three more times. The washed RBCs were re-suspended in CPM at 50% haematocrit and kept at 4 °C to be used to culture malaria parasites. Blood washing was done in the bio-safety cabinet to ensure sterility. Each blood sample was used within two weeks after collection.

### 3.7 MALARIA PARASITE CULTURES

The asexual erythrocytic stages of two laboratory strains of *P. falcipraum*: 3D7 (chloroquine-sensitive) and DD2 (chloroquine-resistant) were obtained from the Immunology Department, Noguchi Memorial Institute for Medical Research, University of Ghana, Legon.

The frozen parasites were thawed in a water-bath set at 37 °C for 15 minutes and transferred into sterile 15 ml falcon tube. For 0.5 ml of thawed sample, 0.1 ml of thawing solution A was added drop-wise, while stirring. It was allowed to stand for 3 minutes and 5 ml of thawing solution B was added drop-wise. It was mixed well by triturating with the pipette and then centrifuged at 2500 rpm for 5 minutes.

The supernatant was aspirated and discarded. Five ml of thawing solution C was then added drop-wise and the pellet suspended in it. Centrifugation was repeated at 2500 rpm for 5 minutes and supernatant removed and discarded. The pellet was re-suspended in 5 ml of CPM in a 25 ml culture flask, 0.5 ml of freshly washed RBCs was added, flushed with mixed gas and incubated at 37°. The cultures were checked after 48 hours to monitor parasite viability.

### **3.8 CULTURING OF MALARIA PARASITES**

The asexual intra-erythrocytic stage of laboratory strains were kept in continuous cultures based on a slightly modified form of the standard technique described by Trager and Jensen (1976). The parasites were hosted within erythrocytes (from O<sup>+</sup> donors) in RPMI 1640 solution supplemented with 0.5% AlbuMAX II and hypoxanthine and buffered with 0.4% sodium bicarbonate (NaHCO<sub>3</sub>) and 0.72% N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid (HEPES). The broad-spectrum antibiotic, gentamycin was also added to a final concentration of 0.005mg/ml. The parasites were grown in culture flasks and incubated at 37 °C. Optimum growth environment was created in the flask by flushing with mixed gas of 92% N<sub>2</sub>, 5% CO<sub>2</sub> and 3% O<sub>2</sub> before incubation.

Synchronization to ring stage was done using 5% D-sorbitol solution (Lambros and Vanderberg, 1979).

Malaria parasite cultures were maintained daily by changing the media and monitoring parasite viability and growth by light microscopy. Picked RBCs were transferred onto a clean glass slide and thin smear was made. After drying, it was fixed with absolute methanol for 15 seconds and stained with freshly-prepared 10% Giemsa. After 15 minutes of staining, the slides were washed by dipping in clean water 5 -8 times and dried. It was then examined under the light microscope using x100 (oil immersion) objective lens.

The number of infected RBCs and total number of RBCs in a field were counted and recorded. This was done for three fields. Enough fields were counted to make up to at least a total of 300 RBCs. The percentage parasitaemia in each field was calculated using the formula:

$$\% \text{ Parasitemia} = \frac{\text{Number of infected RBCs} \times 100}{\text{Total RBCs counted}}$$

The percentage parasitaemia of each flask was estimated as the average of the three fields. When the parasitaemia level rose beyond 4%, the parasites were harvested and used for assays or it was reduced to 1% by dilution with fresh RBCs or by sub-culturing.

### 3.9 *IN VITRO* ANTI-PLASMODIAL ASSAYS

The anti-plasmodial activity of the crude methanol extract of natural cocoa was assessed against *P. falciparum*: 3D7 (chloroquine-sensitive) and DD2 (chloroquine-resistant) strains *in vitro* using SYBR<sup>®</sup> Green assay. The activity of the extract was measured over a wide range of final concentrations: 1000 µg/ml, 500 µg/ml, 250 µg/ml, 125 µg/ml, 62.5 µg/ml, 31.25 µg/ml, 15.62 µg/ml, 7.81µg/ml, 3.90 µg/ml, 1.95 µg/ml, obtained by two-fold serial dilution with CPM. Chloroquine and artesunate were used as positive controls. All experiments were performed in triplicates. At least two independent experiments were performed.

For SYBR<sup>®</sup> Green I assays, the haematocrit of cultures were adjusted to 2% by dilution with CPM and parasitaemia was stepped down to 1.5% with washed uninfected RBCs.

Hundred microlitres of the cultures were aliquoted into the wells of 96-well microtitre plates into which 12.5 µl of extract or drug solution had already been dispensed. The plates were covered and shaken slightly to ensure a thorough mixing. The plates were arranged in a clean modular incubation chamber (Billups-Rothenberg Inc, USA) and flushed with mixed gas for 1 minute. The set-up was then incubated at 37 °C for 72 hours.

After incubation, the plates were wrapped in aluminium foil and stored at -20 overnight to lyse the cells. They were taken out and thawed. The SYBR<sup>®</sup> Green assay was performed using a modified form of the method described previously (Roepe *et al.*, 2004). Hundred µl of SYBR<sup>®</sup> Green solution (diluted with lysis buffer made of saponin solution) was added to each well and incubated in the dark at room temperature for two

hours. The SYBR<sup>®</sup> Green fluorescence was measured with a fluorescence multi-well plate reader (Qiagen Lake Constance, Stockach, ESRE02-MB-0200) with excitation and emission wavelength at 485 and 530 nm, respectively. The fluorescence readings were plotted against the extract and drug concentrations using GraphPad Prism (GraphPad 3 Software, San Diego). The 50% inhibitory concentration (IC<sub>50</sub>) was determined by analysis of dose–response curves.

### **3.10 EFFECTS OF NATURAL COCOA EXTRACT ON MEMBRANE OF UNINFECTED RBCs**

A modified form of the technique described by Lopez *et al.* (2009) was used to assess the effect of natural cocoa extract on the susceptibility of erythrocytes to *P. falciparum* invasion.

Hundred microlitres each of uninfected erythrocytes at 50% haematocrit were pre-treated with 100 µl of natural cocoa extract solution to final concentrations of 51.4 and 662.6 µg/mL which were the IC<sub>50</sub> concentrations obtained for the 3D7 and DD2 strains respectively. The sets up were incubated at 37 °C for 2 hours. This was to allow the components of the extract to interact with the erythrocyte membrane (Lopez *et al.*, 2009).

After incubation, the treated erythrocytes were harvested and washed twice with incomplete RPMI to remove excess extract solution. This was to avoid any direct effects of the extract on the parasite when the treated erythrocytes were introduced into culture.



Cultures were then set up by introducing schizont-infected RBCs into the treated erythrocytes at 1.5% parasitaemia in 16-well microtitre plates. The CPM was added to set final haematocrit at 2%. The plates were arranged in an incubation chamber, flushed with mixed gas and incubated at 37 °C for 48 hours. After the incubation an aliquot was taken from each well and thin smears made. Microscopy was done to determine the parasitaemia and to observe the morphology of the parasites. Percentage inhibition of *P. falciparum* invasion was determined using the formula:

$$\frac{[\text{PT of cultures with untreated RBCs} - \text{PT of cultures with treated RBCs}]}{\text{PT of cultures with untreated RBCs}} \times 100\%$$

PT stands for parasitaemia.

### **3.11 EFFECTS OF NATURAL COCOA EXTRACT OF *P. FALCIPARUM*-INFECTED RBCs**

The technique described previously (Go *et al.*, 2004; Lopez *et al.*, 2009) was used with a slight modification. In this experiment, 100 µl of synchronized late stage trophozoite-infected erythrocytes was pre-treated with the extract and drug solutions at the IC<sub>50</sub> concentrations determined for 3D7 and DD2 strains and incubated for 2 hours at 37 °C. After incubation, the pre-treated cultures were washed twice with RPMI incomplete medium to remove excess extract and to minimize direct effect on the intracellular parasites.

The treated cultures were then re-suspended in 5% D-sorbitol solution and allowed to stand for 10 minutes. The D-sorbitol solution was washed off with incomplete RPMI. Aliquots were taken and thin smears prepared. Microscopy was done to determine

parasitaemia and to observe changes in the morphology of the parasites. Percentage inhibition of sorbitol-induced haemolysis was determined using the formula:

$$\frac{[(PT_i - PT_{f(\text{untreated})}) - (PT_i - PT_{f(\text{treated})})]}{(PT_i - PT_{f(\text{untreated})})} \times 100\%$$

$PT_i$ ; Initial parasitaemia of culture before treatment and sorbitol-induced haemolysis

$PT_{f(\text{untreated})}$ ; Final parasitaemia of untreated culture after subjection to sorbitol-induced haemolysis without treatment.

$PT_{f(\text{treated})}$ ; Final parasitaemia of treated culture after subjection to sorbitol-induced haemolysis.

### 3.12 STATISTICS AND DATA ANALYSIS

Experiments were performed in triplicates and were repeated at least once. Curves with coefficient of correlation greater than or equal to 0.8 were used. Data are presented as mean  $\pm$  SEM. Statistical analysis was done using student t-test at confidence interval of 95% using SPSS (SPSS Corp, IL). The P-values less than 0.05 were considered significant. Microsoft Excel 2007 software was used in plotting graphs and  $IC_{50}$  were obtained from the log-linear regression analysis of log-dose response curves using GraphPad Prism 3 (GraphPad Software, San Diego, California, USA).

## **CHAPTER FOUR**

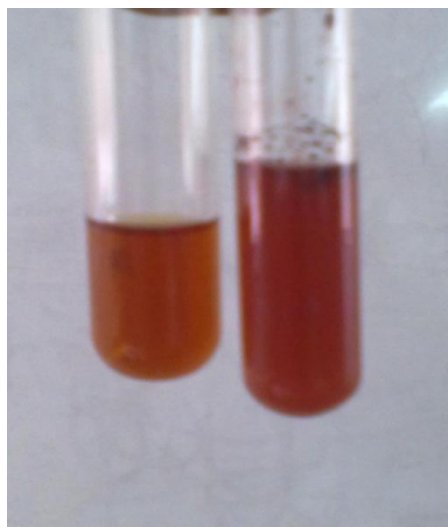
### **RESULTS**

#### **4.1 FLAVONOID ANALYSIS OF CRUDE METHANOL EXTRACT**

Two chemical tests were performed on the crude methanol extract of natural cocoa to determine qualitatively the presence of flavonoids using the Shinoda test and then to estimate the quantity of total phenolics using the Folin-Cicalteau assay.

##### **4.1.1 SHINODA TEST**

The Shinoda test showed the presence flavonoids in the crude methanol extract of natural cocoa. The initial colour of the extract solution was yellowish. However, on the addition of magnesium turnings, there was effervescence and heat production accompanied by a change in the colour of the solution to reddish indicating the presence of flavonoids. This is illustrated in Figure 9. The test tube on the left shows the colour of the solution before the addition of the magnesium turnings. The test one on the right shows the colour of the solution after the addition of magnesium turnings.

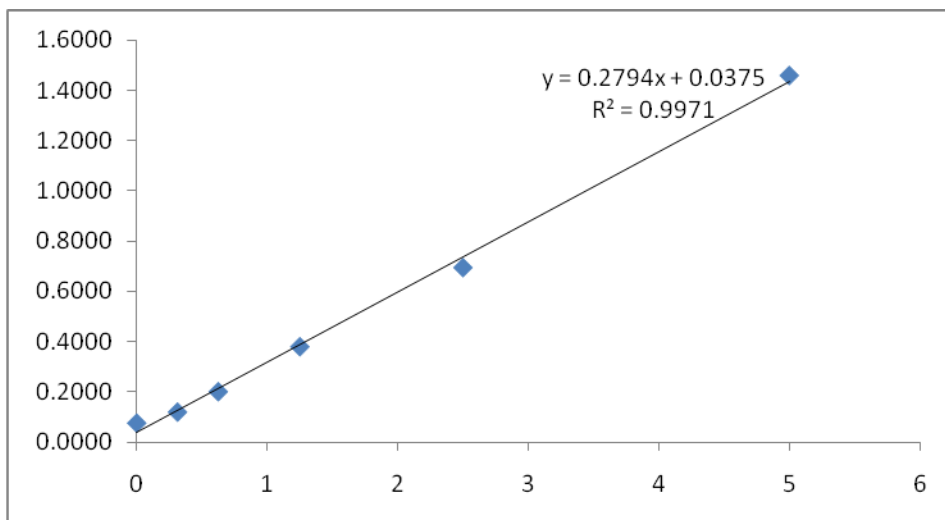


**Figure 9:** An illustration of the results of Shinoda test.

The test tube on the left shows the yellowish colour of the solution formed when extract was dissolved in absolute ethanol. However after the addition of hydrochloric acid and magnesium turnings, the colour of the solution changed to reddish as shown in the test tube on the right.

#### **4.1.2 TOTAL PHENOLIC CONTENT**

The total phenolic content of the crude methanol extract was assessed using the Folin-Ciocalteu assay with gallic acid as the standard phenolic compound. As illustrated in Figure 10, a linear calibration curve was plotted for the gallic acid.

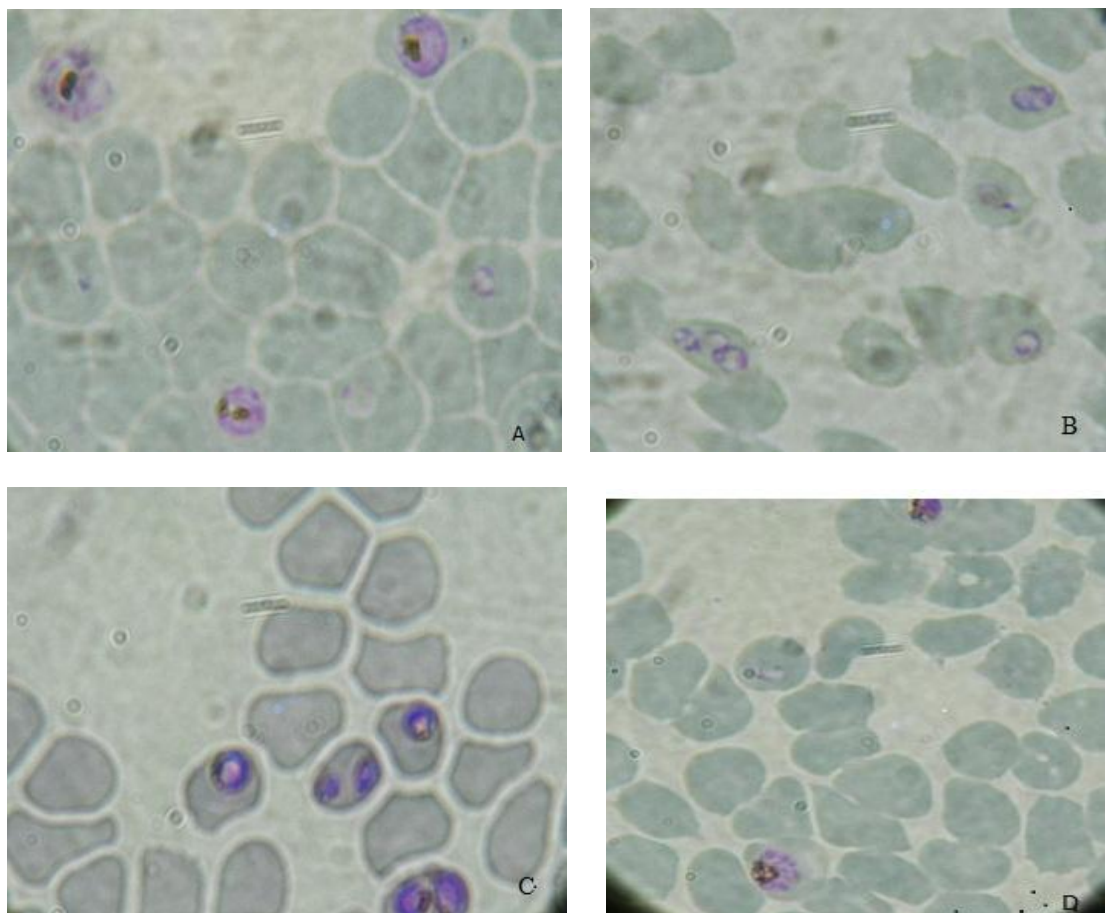


**Figure 10:** Calibration curve of gallic acid used as the standard for the determination of the total phenolic content of crude methanol extract.

Methanol (98%) showed an excellent phenol-extracting ability for the natural cocoa powder and yielded total phenolic content of 358.94 mg GAE/g of extract.

#### **4.2 PLASMODIUM FALCIPARUM PARASITE CULTURES**

The DD2 and 3D7 strains of *P. falciparum* were cultured and monitored at 24-hour intervals. Growth and viability was monitored by preparing thin smears and carrying out light microscopy. Figure 11 shows the different asexual erythrocytic stages of the cultured parasites.



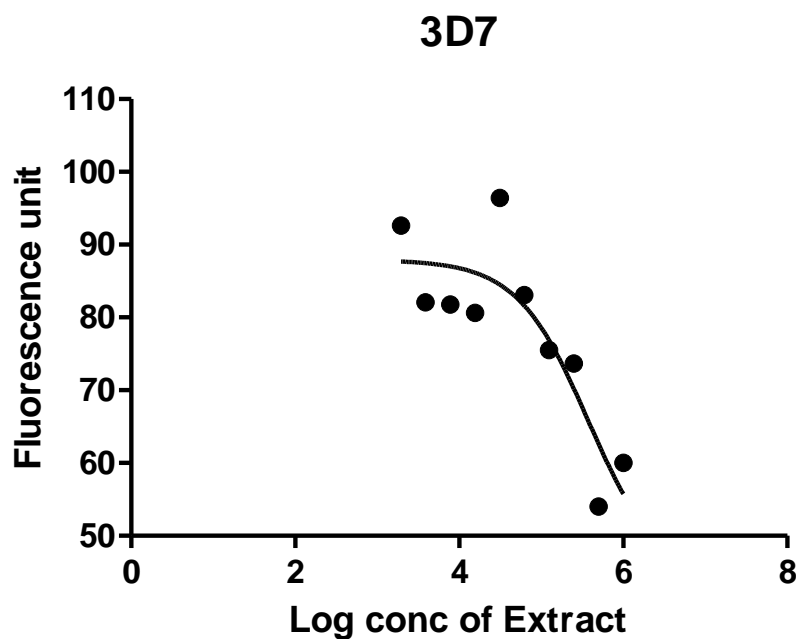
**Figure 11:** Micrographs illustrating the different asexual erythrocytic stages of *P. falciparum*.  $\times 100$ .

The DD2 and 3D7 strains of *P. falciparum* were not morphologically distinguishable from each other by light microscopy. Micrograph A shows a slide of unsynchronized cultures with rings, trophozoites and a schizont. Micrographs B, C, and D show slides of synchronized cultures. Slide B was prepared 48 hours after synchronization and has only ring stages of the parasite. Slide C was prepared 70 hours later after synchronization and shows only trophozoites. Two hours later, the trophozoites matured into schizonts as shown on slide D.

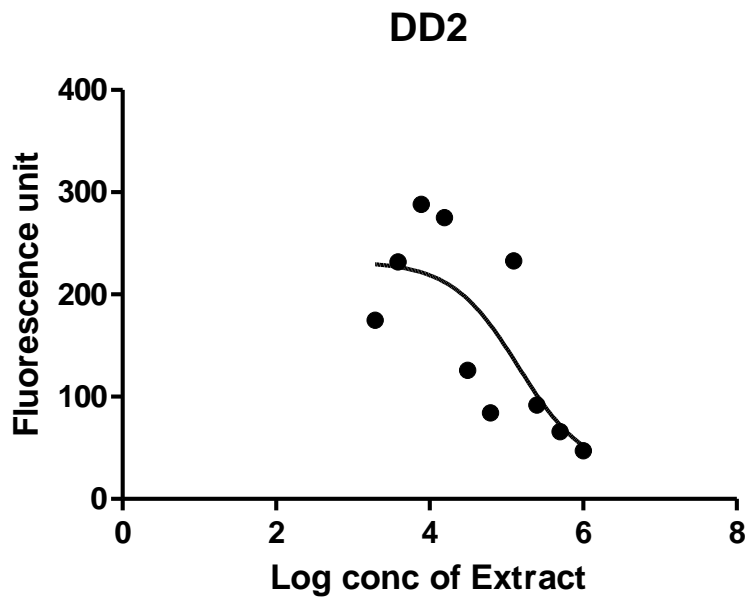
### 4.3 ANTI-PLASMODIAL ACTIVITY OF NATURAL COCOA POWDER

To determine the effect of natural cocoa on malaria parasites, the crude methanol extract was assayed for its anti-plasmodial activity against chloroquine-sensitive 3D7 and chloroquine-resistant DD2 strains of *P. falciparum* *in vitro*. The IC<sub>50</sub> values of the crude methanol natural cocoa extract were determined. Two established anti-malarial agents, chloroquine and artesunate were used as positive controls.

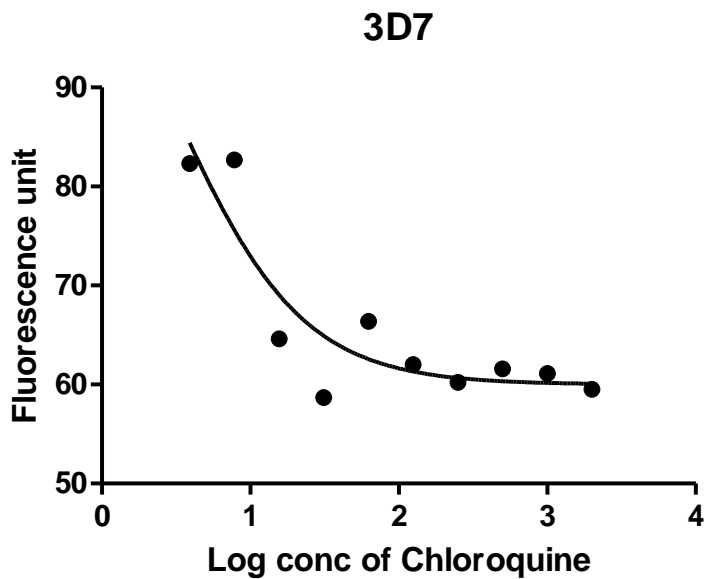
Figures 12-17 show the pattern of inhibition exhibited by the extract and the control drugs against the 3D7 and the DD2 strains of *P. falciparum*.



**Figure 12:** Dose-response curve of effect of crude extract of natural cocoa on 3D7 strain of *P. falciparum*.

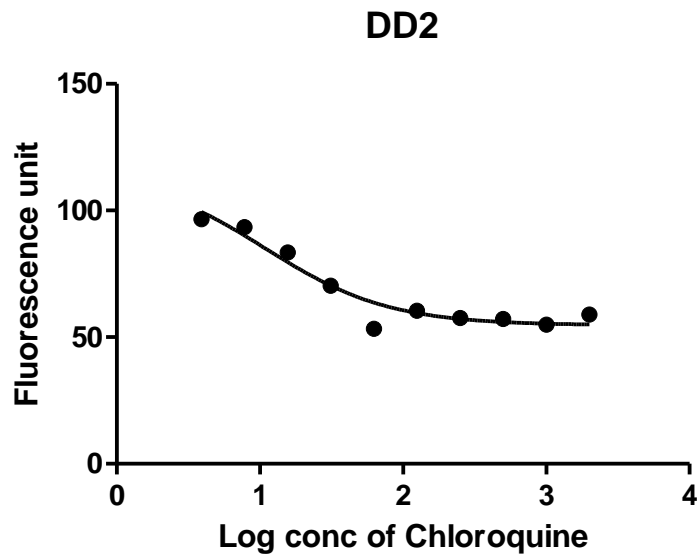


**Figure 13:** Dose-response curve of effect of crude extract of natural cocoa on DD2 strain of *P. falciparum*.

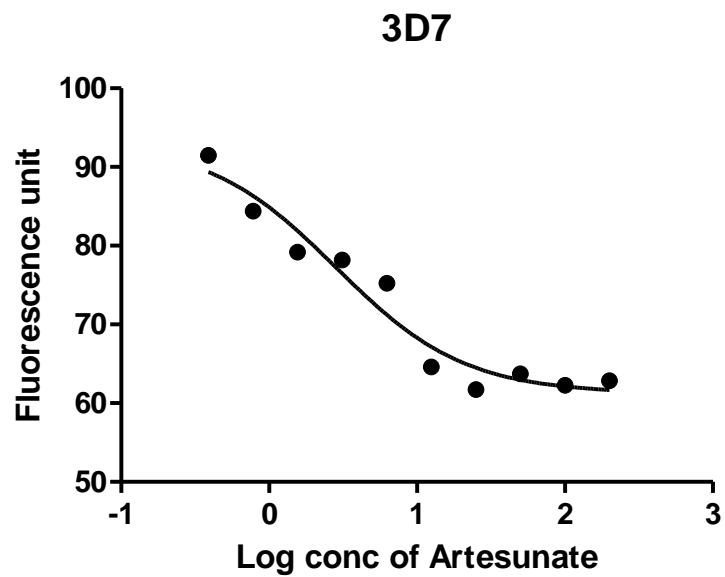


**Figure 14:** Dose-response curve of effect of chloroquine on 3D7 strain of *P. falciparum*.

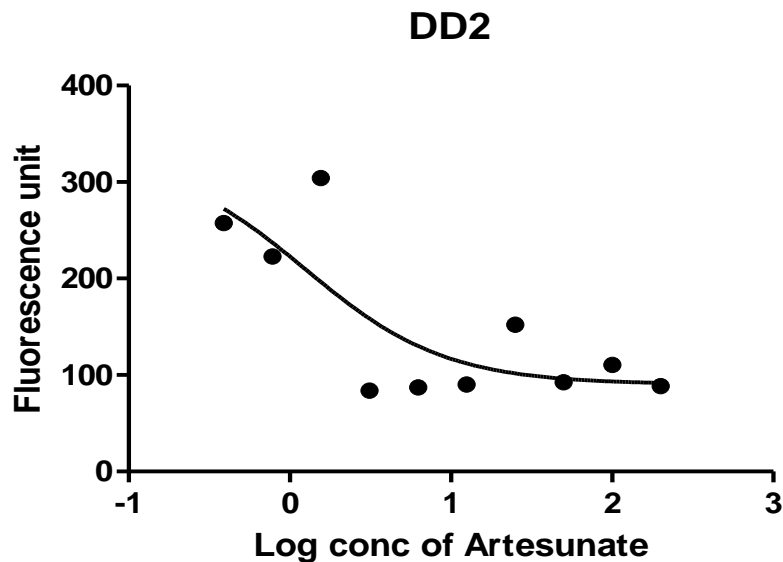




**Figure 15:** Dose-response curve of effect of chloroquine on DD2 strain of *P. falciparum*.



**Figure 16:** Dose-response curve of effect of artesunate on 3D7 strain of *P. falciparum*.



**Figure 17:** Dose-response curve of effect of artesunate on DD2 strain of *P. falciparum*.

Tables 2 and 3 summarize the  $IC_{50}$  values of the crude methanol extract, chloroquine and artesunate against the 3D7 and DD2 strains of *P. falciparum*, respectively.

**Table 2:** Summary of the  $IC_{50}$  values on activities of Crude Methanol Extract, Artesunate and Chloroquine against 3D7 strain of *P. falciparum*.

Treatment	$IC_{50}$ ( $\mu\text{g/ml}$ )	SEM
Crude Methanol Extract	51.4	$\pm 18.38$
Artesunate	$5.0 \times 10^{-3}$	$\pm 1.5 \times 10^{-3}$
Chloroquine	$3.2 \times 10^{-3}$	$\pm 1.3 \times 10^{-3}$

**Table 3:** Summary of the IC<sub>50</sub> values on activities of Crude Methanol Extract, Artesunate and Chloroquine against DD2 strain of *P. falciparum*.

Treatment	IC <sub>50</sub> (µg/ml)	SEM
Crude Methanol Extract	622.6	±152.02
Artesunate	6.6 x 10 <sup>-3</sup>	±3.5x10 <sup>-4</sup>
Chloroquine	1.3 x 10 <sup>-2</sup>	±2.4x10 <sup>-3</sup>

As shown on Tables 2 and 3, there was marked difference between the activity of the extract against 3D7 and DD2 parasite strains. The IC<sub>50</sub> value of the crude methanol extract on DD2 strain was 622.6 ±152.02 µg/mL and that for 3D7 was 51.40 ± 18.38 µg/mL.

#### 4.4 EFFECT OF NATURAL COCOA EXTRACT ON ERYTHROCYTE SUSCEPTIBILITY TO *P. FALCIPARUM* INVASION

The appearance of the erythrocyte membrane and parasites was observed for changes in their morphology. There were no changes in the morphology of the erythrocytes after treatment with the extract and incubation when they were observed under the microscope.

The parasitaemia determined for the negative controls after 48 hours were 8.5± 1.8 and 5.6± 0.7 (n=6) for DD2 and 3D7 (n=5) respectively. The parasitaemia recorded for the positive controls, artesunate and chloroquine were 8.3±1.9 for DD2 (n=5), 5.3± 1.2 for

3D7 (n=5); and  $7.5 \pm 2.6$  for DD2 (n=6) and  $5.4 \pm 0.6$  for 3D7 (n=5) percent, respectively. The parasitaemia recorded for cultures with RBCs treated with the natural cocoa extract solution were  $4.5 \pm 0.43$  for DD2 (n=6) and  $3.6 \pm 0.45$  for 3D7 (n=6).

#### **4.4.1 PERCENTAGE INHIBITION OF *P. FALCIPARUM* INVASION**

The determined  $IC_{50}$  concentration of the crude methanol extract against the 3D7 strain was  $51.4 \mu\text{g/ml}$ . However, when RBCs were pre-treated with extract solution at final concentration of  $51.4 \mu\text{g/ml}$ , it only resulted in 1.8% percentage inhibition of RBC invasion by 3D7 strain.

The ability of the extract and the control drugs to inhibit parasite invasion of erythrocytes was tested at the following final concentrations:  $662.6 \mu\text{g/ml}$  (for the extract),  $6.6 \times 10^{-3}$  and  $1.3 \times 10^{-2} \mu\text{g/ml}$  for artesunate and chloroquine, respectively. The percentage inhibition of invasion values for DD2 and 3D7 strains of *P. falciparum* are summarized in Tables 4 and 5.

**Table 4:** Inhibition of *P. falciparum*, DD2 strain invasion by Crude Methanol Extract of Natural Cocoa, Artesunate and Chloroquine

<b>Treatment</b>	<b>Percentage Inhibition (%)</b>
Crude Methanol Extract	47.26
Artesunate	1.88
Chloroquine	1.77

**Table 5:** Inhibition of *P. falciparum*, 3D7 strain invasion by Crude Methanol Extract, Artesunate and Chloroquine.

<b>Treatment</b>	<b>Percentage Inhibition (%)</b>
Crude Methanol Extract	36.02
Artesunate	4.64
Chloroquine	3.51

#### 4.5 EFFECT OF CRUDE METHANOL NATURAL COCOA EXTRACT ON SORBITOL-INDUCED HAEMOLYSIS OF LATE TROPHOZOITES- AND SCHIZONT-INFECTED ERYTHROCYTES

Cultures containing late stage trophozoite-infected RBCs were pre-treated with the extract and control drugs at final concentrations values which are the same as the IC<sub>50</sub> values. Table 6 illustrates the percent inhibition of sorbitol-induced haemolysis of infected erythrocytes by the extract and control drugs.

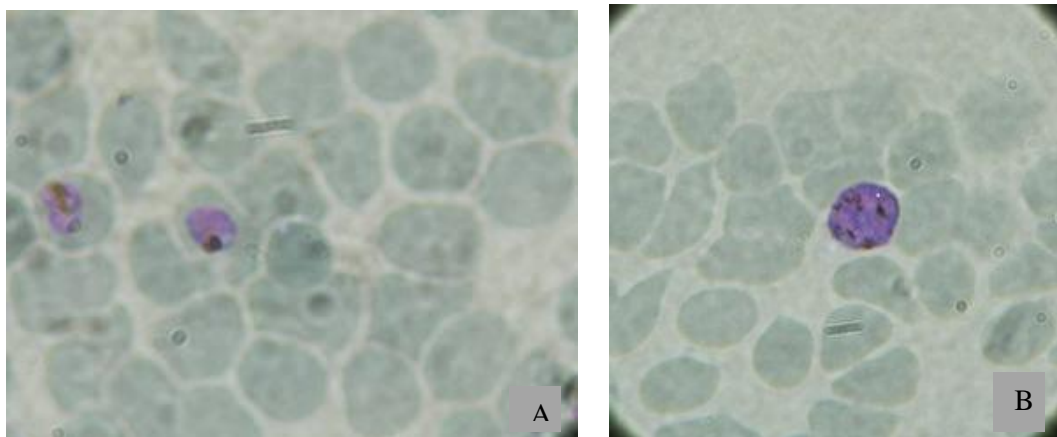
**Table 6:** Percentage inhibition of sorbitol-induced haemolysis of late trophozoite- and schizont-infected erythrocytes.

Treatment	Percentage Inhibition of Sorbitol-Induced Haemolysis of Infected Erythrocytes (%)	
	DD2	3D7
Crude Methanol Extract	26.7	17.7
Artesunate	0.5	4.7
Chloroquine	1.4	3.5

Similar to the earlier observation there were no observable changes in the morphology of red cell membranes of either the infected or uninfected erythrocytes in the test and control assays. However, the trophozoite- and schizont-infected RBCs that were not

haemolyzed by sorbitol solution treatment appeared turgid with the parasite appearing to have taken up the whole of the erythrocyte space. Although uninfected cells looked deformed and out of shape, the infected cells appeared bigger and more rounded in shape.

With regards to infected cells which were not treated with sorbitol solution, the merozoites were distinguishable at all stages of the parasite. However, in sorbitol-treated infected cells, there were no clearly distinguishable merozoites.



**Figure 18:** Micrographs of late stage trophozoite-infected cells before and after treatment with natural cocoa extract and 5% sorbitol solution,  $\times 100$ .

Micrograph A shows trophozoite-infected cells before treatment with natural cocoa extract and haemolysis induction and micrograph B shows a trophozoite-infected cell after treatment..

## CHAPTER FIVE

### DISCUSSIONS

Plant products remain a vital source of primary healthcare for many populations, especially in sub-Saharan Africa and are used to treat viral, bacterial, fungal, protozoal and helminthic infections, often with excellent results (Joy *et al.*, 2001). Furthermore, in medicinal chemistry, plant-derived compounds with specific activity offer an approach to chemotherapy by serving as starting points for useful chemotherapeutic agents (Tan and Sebisubi, 2010). As a result, there has been an extensive investigation into the medicinal properties of plant products in recent years to provide information on the components of these products and scientific basis for their therapeutic properties.

The study of plant extracts for their anti-plasmodial activities forms an important part of these on-going investigations. Natural cocoa powder has been shown to contain plant phenolic compounds and flavonoids, for which anti-plasmodial activity has been reported (Sanchez-Rabaneda *et al.*, 2003; Amponsah *et al.*, 2012). Several anecdotal reports suggest that persons who take natural cocoa beverages regularly are less susceptible to malaria (Addai, 2009).

This study was done to determine the presence of flavonoids in crude methanol extract of natural cocoa powder and its activity against the asexual erythrocytic stage of the chloroquine-sensitive and chloroquine-resistant strains of *P. falciparum*.

Screening and quantifying the bioactive components of plant extracts is an important part of medicinal plant product research. The bioactive components have been isolated and



studied for their chemical and biological properties individually and in synergy (Sanchez-Rabaneda *et al.*, 2003).

As illustrated by the positive Shinoda test result in Figure 9, the crude methanol extract of natural cocoa contained flavonoids.

The presence of flavonoids in crude methanol extract of natural cocoa has been previously studied by Amponsah *et al.* (2012) and the results of this study are consistent with what was reported. Sanchez-Rabaneda *et al.* (2003) actually isolated flavonoids such as catechin and epicatechin, quercetin, apigenin and luteolin from cocoa nibs as well as the fermented and dried cocoa seeds from which natural cocoa powder is made. Crude methanol extract of *Ficus thonningii* showed similar results (Usman *et al.*, 2009).

However, the amount of flavonoid present in a cocoa product depends largely on the post-harvest processing (Amponsah *et al.*, 2012). This makes it necessary to quantify the flavonoid content of the crude methanol extract of natural cocoa powder.

The total phenolic content (TPC) analysis of the crude methanol extract of natural cocoa was determined using the Folin-Ciocalteu assay. Although it only gives a crude estimate of the phenolic content of extracts, it is relatively simple, fast and inexpensive and is used extensively in determining the crude phenolic content of plant extracts. The assay yielded a high total phenolic content of 358.94 mg GAE/g.

Although there is no universally accepted procedure and solvent for the extraction of phenols, high phenol extracting properties of methanol and the use of the maceration method have been advocated (Marinova and Yanishlieva, 1997; Dai and Mumper, 2010;

Stankovic *et al.*, 2012). In a study to compare the phenol extracting properties of water, acetone, methanol, ethanol and petroleum ether of *Teucrium polium*, it was found that the aqueous methanol extract produced the highest yield of 157.84 mg of GAE/g of extract (Stankovic *et al.*, 2012). Solubility of phenols in organic solvents is determined by polarity. The high phenol-extracting ability of aqueous methanol is explained by the fact that the chemical nature of especially low molecular weight phenols makes them highly soluble in methanol because of the polarity (Stankovic *et al.*, 2012).

No data has been found so far in literature on the total phenolic content of crude methanol extract of natural cocoa, but plant extracts obtained using methanol generally have high TPC. In an experiment, methanol extracts of *Dyera costulata*, *Alstonia angustiloba* and *Calotropis gigantea* yielded TPC values of 319, 354 and 279 mg GAE/g of extract, respectively (Wong *et al.*, 2011). Stankovic *et al.* (2012) reported the total phenolic content of dry crude methanol extract for green tea to be 233.68 mg of GAE/g. According to Lee *et al.* (2003), cocoa generally contains more phenolic compounds than teas.

Most phenols found in natural cocoa are flavonoids and there is a direct correlation between the flavonoid content and the total phenolic content of plant extract. A high total phenolic content suggest high flavonoid content. According to Forsyth (1955), approximately 35% of the total content of polyphenols in cocoa nibs is the flavonoid epicatechin.

However, extraction with aqueous methanol is not specific for only polyphenols (Tawaha *et al.*, 2007). The other active ingredients extracted may interfere with the Folin-Cicalteau assay leading to apparently high total phenolic content values. The medicinal

properties of many plant products have been attributed to their phenolic content (Singhai and Dixit, 2006; Rohman *et al.*, 2010).

The *in vitro* anti-plasmodial activity of the crude methanol extract of natural cocoa against chloroquine-sensitive 3D7 and chloroquine-resistant DD2 strains of *P. falciparum* was studied using the SYBR<sup>®</sup> Green I assay. SYBR<sup>®</sup> Green I is a cyanide-based dye (Zipper *et al.*, 2004) that specifically binds to double-stranded DNA in biological systems. The complex thus formed results in fluorescence -absorbance of blue light at an excitation wavelength of 497 nm and emission of green light at 520 nm. This was used to quantify the DNA present, thus the parasitaemia, at each concentration of the extract or drug after incubation. Dose-response inhibition curves were plotted to determine the IC<sub>50</sub> values of the extract and drugs.

Many anti-plasmodial studies on plant extracts have been reported in terms of the IC<sub>50</sub> values (Mbatchi *et al.*, 2006; Ngemenya *et al.*, 2010; Sahal *et al.*, 2012 and Kovendan *et al.*, 2012). Rasoanaivo *et al.* (1992), categorizes plant extracts into very active if IC<sub>50</sub> is less than 5 µg/mL, active if IC<sub>50</sub> is between 5 µg/mL and 50 µg/mL, weakly active if between 50 µg/mL and 100 µg/mL, and inactive if greater than 100 µg/mL. According to Gessler *et al.* (1994), an extract shows very good anti-plasmodial activity if it has an IC<sub>50</sub> less than 10 µg/mL; 10 to 50 µg/ml, moderate; and over 50 µg/ml the extract was considered to have low activity. The standard classification of anti-plasmodial activity of plant extracts by Kamaraj *et al.* (2012) is illustrated in Table 7.

**Table 7:** An illustration of the categories of the activity of plant extracts against malaria parasites (Kamaraj *et al.*, 2012)

IC <sub>50</sub> Value (µg/mL)	Category of Activity
<10	Promising
10–20	Moderate
20–40	Good
40–70	Marginally Potent
> 70	Poor

Although the criteriae used in classifying the potency of plant extracts based on the IC<sub>50</sub> vary from one scientist to another, the IC<sub>50</sub> value is still considered a good measure of the anti-plasmodial activity of plant extracts.

The results of these studies showed that crude methanol extract of natural cocoa powder showed some activity against *P. falciparum*. Although according to the criteria used by Kamaraj *et al.* (2012), the activity was poor (IC<sub>50</sub> of 622.6 µg/mL) against the chloroquine resistant DD2 strain, it was moderately potent (IC<sub>50</sub> of 51.40 µg/mL) against the chloroquine-sensitive 3D7 strain. To the best of my knowledge, there is no information in literature on the activity of crude methanol extract of natural cocoa on DD2, but an earlier research by Amponsah *et al.* (2012) using [3H]-hypoxanthine incorporation assay suggested poor anti-plasmodial activity or inactivity against the 3D7

strain. They reported an IC<sub>50</sub> value was  $1.6 \times 10^3 \mu\text{g/mL}$ . However, it is difficult to compare the results of the two studies because of the differences in the *in vitro* culture assays used as well as other factors such as differences in duration for which parasite clones have been in culture and the number of passages the parasites have been through in the different studies.

On the other hand, the anti-plasmodial activities of phenols and flavonoids of plant extracts have been extensively demonstrated. Bero *et al.* (2009) attribute the anti-plasmodial activity of many plant extracts against chloroquine-sensitive, chloroquine-resistant and multiple drug-resistant strains of *P. falciparum* to their high polyphenolic and flavonoids content. These include extract of the leaves of *Piptadenia pervillei* Vatke from which phenolic compounds: catechin 5-gallate was isolated; extract of root of *Bauhinia purpurea* L from which a flavanone; stem bark and root bark of *Friesodielsia obovata* which contained demethoxymatteucinol and root bark of *Artocarpus rigidus* from which two known flavonoids, artonin and cycloartobiloxanthone were isolated.

Flavonoids have been isolated in *A. annua* and have been shown to augment the antiplasmodial activities of artemisinin by 3–5 folds (Elford *et al.*, 1987). Work by Lehane and Saliba (2008) tested the anti-plasmodial properties of eleven common dietary flavonoids on chloroquine-sensitive 3D7 strain of *P. falciparum* and the chloroquine-resistant 7G8 strain *in vitro*. All showed activity against the 7G8 strain and eight showed activity against the 3D7 strain. This confirmed similar results by Tasdemir *et al.* (2005). Desjadins (1979), Kraft *et al.* (2000) and Harsteen (2002) have also demonstrated the anti-plasmodial activities of flavonoids.

The activity of the extract against *P. falciparum* as the results showed is supported by the results of the flavonoid analysis and earlier research which shows that flavonoids are active components of natural cocoa powder. Sanchez-Rabenada *et al.* (2003) found the flavonoids luteolin, quercetin, hyperoxide and apigenin in methanol extracts of natural cocoa.

According to Amponsah *et al.* (2012), there is a strong correlation between total flavonoid concentration of plant extracts and their anti-plasmodial activity ( $r^2 = 0.9145$ ). Growth inhibition of the malaria parasite was directly linked to the total flavonoid concentration of the extracts. According to them, as the total flavonoid content of the extract increased, the inhibitory effect on the malaria parasite increased accordingly and *vice versa*. This is consistent with results by Sadhu *et al.* (2003) who isolated 4 flavonoids from extracts of *Leucas aspera*, which showed anti-plasmodial activity. Juan-Badaturuge *et al.* (2011) also isolated flavonoids kaempferol-3-O-rutinoside, kaempferol, quercetin and luteolin from leaf extracts of *Cassia auriculata*, a plant used for the traditional treatment of malaria -for which anti-plasmodial activity has been reported by Kamaraj *et al.* (2011). Earlier work by Lehane and Saliba (2008) also demonstrated high anti-plasmodial activity of the luteolin against both chloroquine-sensitive and chloroquine-resistant strains of the malaria parasite.

Anti-plasmodial activity has been reported for many plant extracts, both *in vitro* and *in vivo*, and has been attributed to immune-regulatory effects on the immune system and antioxidant effect on the membrane of erythrocytes, but the mechanism of their activities on the malaria parasite itself is still under investigation and is still a subject of speculation. Li *et al.* (1996) suggested that flavonoids inhibit parasite cysteine protease.

Results of other studies suggest the anti-plasmodial activity of flavonoids is attributable to their ability to inhibit mitochondrial enzymes such as cytochrome C reductase, thus affecting the energy metabolism pathways of the parasite (Uyemura *et al.*, 2000).

Also, inhibition of parasite-induced pathways in the erythrocyte membrane suggests inhibition of permeability. This will interfere with the intracellular parasite's ability to take up nutrient and dispose off waste out of the erythrocyte (Lopez *et al.*, 2009). The deficiency in nutrients supply and the build-up of wastes will negatively affect parasite development. According to Fraga *et al.*, (2004), cocoa flavonoid, procyanidin, interferes with the proper functioning of parasite-induced pathways.

Results of earlier *in vitro* investigations into anti-plasmodial activities of some other plant extracts showed varying comparisons between the IC<sub>50</sub> values for chloroquine-sensitive and chloroquine-resistant strains of *P. falciparum*. There was a significant difference between the IC<sub>50</sub> values of methanol extracts of *Lantana camara* against chloroquine-sensitive 3D7 (5.7 µg/mL) and chloroquine-resistant W2 (14.1 µg/mL) strains of the parasite (Jonville *et al.*, 2008). In another study by Mustofa *et al.* (2007), crude methanol extracts of *Phyllanthus niruri* showed comparable *in vitro* anti-plasmodial activity against chloroquine-resistant FCR-3 (IC<sub>50</sub> of 2.3–3.9) and chloroquine-sensitive D-10 (3.2–2.5 µg/mL) strains of *P. falciparum*.

This experiment, however, shows a rather significant difference between the activity of crude methanol extract of natural cocoa powder against the chloroquine-sensitive 3D7 (IC<sub>50</sub> = 51.40 µg/mL) and chloroquine-resistant DD2 (IC<sub>50</sub> = 622.6 µg/mL) strains. Studies into the invasion mechanism of *P. falciparum* show that the different strains of

the parasite have different invasion pathways. The DD2 parasite strain, for example shows a sialic acid-dependent pathway, while 3D7 strain shows a sialic acid-independent pathway (Cowman and Crabb, 2006). According to Cowman *et al.* (2003), some compounds inhibit the receptor-ligand interactions associated with some of the pathways. It is possible that this is what accounts for the difference in the IC<sub>50</sub> values for the DD2 and 3D7 strains. Although the mechanism of action of flavonoids and many other anti-plasmodial plant products are not known and are still under investigation, information on differences in activity against the difference strains in terms of drug resistance can serve as leads to elucidating them.

However, it has been postulated that the anti-plasmodial activity of plant extracts is in some cases as a result of the effects they have on the erythrocyte membrane integrity (Kirk *et al.*, 2001; Go *et al.*, 2004; Lopez *et al.*, 2009).

Erythrocytes are the most common blood cells and their membrane comprises of proteins and lipids that determine the shape, deformability and permeability of the erythrocytes (Kirk, 2001). The membrane gives the cell integrity and controls what enters or exits it.

The main function of erythrocytes is oxygen transport and its features adapt it for that function (Morera and Mackenzie, 2011). However, these features and adaptations make them the primary targets of the merozoite stage of the malaria parasite, *P. falciparum*.

Receptor-ligand interactions between the erythrocytes membrane proteins and parasite extracellular surface proteins such as Merozoite Surface Proteins (MSPs), Erythrocyte Binding Proteins (EBPs) and Reticulocyte Binding Proteins (EBPs) are what mediate parasite invasion of erythrocytes. The mechanisms of parasite invasion and remodeling of



erythrocyte membrane after invasion provide information on what points can serve as target for new anti-malarial agents (Kirk *et al.*, 1994; Kelly *et al.*, 2007; Baum, 2008).

As illustrated by Tables 4 and 5, the invasion of erythrocyte by DD2 and 3D7 strains of *P. falciparum* was significantly inhibited by treatment with the extract. The percentage inhibition values for DD2 and 3D7 strains of the parasite were 47.3% and 36.0%, respectively. The effects of plant extracts on erythrocyte membrane in relation to some diseases have been extensively studied, but there is limited data on how it affects the malaria parasite and the progress of the disease. However, research by Lopez *et al.* (2009) has shown that the treatment of uninfected erythrocytes with extracts of *Solanum nudum* significantly reduced their susceptibility to invasion by *P. falciparum*.

According to Ziegler *et al.* (2004) and Lopez *et al.* (2009), what accounts for the inhibition of invasion of extract-treated erythrocytes by *P. falciparum* was the incorporation of the flavonoids into the erythrocyte membrane ultrastructure. This, they suggested, led to the modification of membrane curvature. This limits contact between the merozoite and the erythrocyte membrane thereby inhibiting the initial merozoite binding, re-orientation and erythrocyte deformation and junction formation steps during parasite invasion.

Addai (2010) proposed that flavonoids found in natural cocoa enhance membrane protection against oxidation. This significantly protects the erythrocytes against invasion by the malaria parasite when exposed to it, as the by-products of oxidation compromise the integrity of the erythrocyte membrane and increases their susceptibility to parasite invasion.

Reactive oxygen species (ROS) are by-products of energy metabolism formed in a series of reactions catalyzed by enzymes such as dismutase, catalase and superoxidase and co-factors such as iron ions in the mitochondria of cells (McCord and Fridovich, 1988, Selivanov *et al.*, 2011). Higher levels of ROS have been reported in individuals with uncomplicated malaria (Sack and Froehlich, 1982; Sharma *et al.*, 1998; Garba *et al.*, 2005). Nijveldt *et al.* (2001) demonstrated that the elevated levels of ROS disrupt membrane structure of uninfected erythrocytes and leads to an increased susceptibility to invasion by merozoites. However flavonoids found in cocoa such as quercetin and luteolin inhibit these enzymes thereby interfering with the ROS formation process (Pabon, 2003). Quercetin has been identified as an iron chelating agent.

There was a significant difference between the percentage inhibitions of invasion in the two strains even when treated with the same concentration of the extract. This can be explained by the differences in the invasion mechanisms of the two strains and the effects of the extract on the membrane surface receptors as well as the parasite surface ligands that play vital roles during invasion.

The second asexual reproductive stage of the malaria parasite occurs in the erythrocyte, the first being the exo-erythrocytic schizogony which occurs in the liver cells. It is this stage of the parasite life cycle that is responsible for much of the symptoms of malaria.

Soon after, 12–15 hours post-invasion, changes in the erythrocyte membrane caused by the intracellular parasite becomes apparent (Kirk, 2001). It establishes New Permeability Pathways (NPPs) for the transport of different metabolites the parasite needs to survive and thrive (Kirk, 2001) including ions (Staines *et al.*, 2000; Cohn *et al.*, 2005), sugars

(Kanaani and Ginsburg, 1992) and nucleosides (Upston and Gero, 1995). In later blood stages, the intracellular parasite and its biochemical reactions disrupt the erythrocyte ultra-structure (Glushakova *et al.*, 2005). These modifications render the erythrocyte membrane more permeable and more susceptible to haemolysis which allows daughter merozoites to be released after schizogony. Proteases released by the parasite also play a role (Salmon *et al.*, 2001)

Although uninfected erythrocyte membrane is impermeable to D-sorbitol, trophozoite- and schizont-infected membranes erythrocytes are permeable as a result of parasite modification (Go *et al.*, 2004). This information provides a strong basis for obtaining data on the effect extracts, for example, have on parasite-infected erythrocyte membranes (Staines *et al.*, 2005). In this study, it was determined by comparing schizont parasitaemia in cultures before and after extract treatment and haemolysis induction.

As illustrated on Table 6, it was found that the extract treatment resulted in 26.7% and 17.7% inhibition of D-sorbitol-induced haemolysis of DD2 and 3D7-infected erythrocytes, respectively. This is consistent with results by Fraga *et al.* (2004), who reported that procyanidins, a cocoa flavonoid, protect erythrocytes against experimentally induced membrane disruptions.

Furthermore, effects of natural plant products on erythrocyte membrane and their correlation to anti-plasmodial activity has been demonstrated by results of earlier research by (Go *et al.* 2004, Kang *et al.* 2005, Lisk *et al.* 2006, Staines *et al.* 2004; Lopez *et al.*). Lopez *et al.* (2009) reported that the anti-plasmodial activity of extracts of *Solanum nudum* was attributable to its inhibitory effects on the parasite-induced channels

on the erythrocyte membrane. In another research, it was revealed that isolated bioflavonoids reduced D-sorbitol-induced haemolysis to up to 40% the value of control experiments and there was a good correlation between haemolysis inhibition and anti-plasmodial activity (Go *et al.*, 2004).

The bioflavonoids had negligible effects on haemoglobin metabolism by the parasite and did not appear to inhibit parasite cysteine protease (Liu, 2003) cited by Go *et al.* (2004). This view is supported by the Lopez *et al.* (2009) who suggested that because the IC<sub>50</sub> value for parasite growth inhibition is lower than the value required to inhibit parasite-induced pathways, the inhibition of the parasite-induced pathways accounts more for the anti-plasmodial activity.

A number of factors are accountable for the haemolysis inhibitory properties of plant products. One of them is lipophilicity. Cohn *et al.* (2003) reported that compounds with higher lipophilicity generally exhibited greater membrane inhibitory activity. This is explainable by the fact that extracellular part of the erythrocyte membrane has a high distribution of lipids. This ensures a stronger interaction between the functional group of the compound and membrane receptors and parasite-induced channels (Cohn *et al.*, 2003).

Another factor influencing the anti-plasmodial activity of the plant extract is the chemical structure of the active compounds. There is a good correlation between the degree of hydroxylation of a compound and its anti-plasmodial activity (Liu *et al.*, 2001) as this affects the compounds lipophilicity, charge distribution, volume and surface area. This in

turn affects their interactions with erythrocyte membrane surface proteins, hence, membrane-inhibitory properties.

These interactions affect the parasite life cycle in a number of ways. They cause a blockage in membrane parasite-induced channels. This inhibits the intracellular parasite's ability to take up nutrients needed for growth and development from the extracellular environment and to transport wastes into the extracellular environment (Lopez *et al*, 2009). In their study with compounds isolated from extracts of *Solanum nudum*, Lopez *et al* (2009) reported a mechanism similar to that of 5-Nitro-2-(3-phenylpropylamino) benzoic Acid (NPPB), a known ion channel-blocking compound which also inhibited haemolysis.

Addai (2010) proposed that flavonoids enhance membrane integrity and protect it against haemolysis. As has been shown in this study, the schizont-infected erythrocytes appeared rounded and turgid on examination under the light microscope after treatment with the extract. This lends credence to the idea that although the flavonoids did not completely inhibit parasite activity on cell membranes, it was able to protect the erythrocyte against lysis.

Although there were differences between the percentage inhibition in the DD2 and 3D7 strains, the non-haemolyzed infected erythrocytes looked fairly indistinguishable from each other. This is illustrated in Figure 18. The membrane of the infected erythrocyte appeared intact, apparently as a result of enhancement due to the effects of the extract, although the shape suggested that it had taken up the sorbitol solution. The absence of a clearly distinguishable merozoite, unlike that seen in untreated infected cells suggests

that the merosome ruptured and released its content of the parasite into the general erythrocyte space.

To the best of my knowledge, this study is the first of its kind to show a significant difference between the response of the DD2 and 3D7 strains to treatment with crude methanol extract of natural cocoa. The difference observed in the percentage RBC protection against the DD2 and 3D7 strains is traceable to the difference seen in the anti-plasmodial activity of the extract on each of the two strains. The DD2 strain showed an  $IC_{50}$  value of 622.6  $\mu\text{g/ml}$  and that of 3D7 is 51.4  $\mu\text{g/ml}$ . The ability of the extract to inhibit the activity of intracellular parasites is not completely ruled out in the outcome of sorbitol-induced haemolysis. It is possible that lesser effect of the extract on the parasite (as shown by the low  $IC_{50}$  for DD2) accounted for the greater protection against D-sorbitol-induced haemolysis observed for the DD2 strain.

## CHAPTER SIX

### CONCLUSIONS AND RECOMMENDATIONS

The results of this study confirmed what has been reported in literature that natural cocoa is a source of flavonoids. It also showed that crude methanol extract of natural cocoa has a moderate anti-plasmodial activity against chloroquine-sensitive 3D7 strain of *P. falciparum* but poor activity against chloroquine-resistant DD2 strain of the parasite. The difference in activity against the two strains may be indicative of the mode of action of the extract.

It also showed a significant ability to protect erythrocytes against invasion by the malaria parasite and to reduce susceptibility of schizont-infected erythrocytes to D-sorbitol-induced haemolysis. The extract also showed a greater protection of the erythrocyte membrane against infection by DD2 strain.

This study provides a scientific basis for the belief that natural cocoa consumption can protect one against malaria infection.

However, it is recommended that the extract be tested against field isolates of the parasite. Also because only one solvent was used, it is recommended that other solvents are used to extract the active ingredients from the cocoa powder and to test their anti-plasmodial activity. Additionally, it is recommended that the individual constituent active ingredients of natural cocoa powder be isolated and their anti-plasmodial activity investigated.

The compound 5-Nitro-2-(3-phenylpropylamino)benzoic Acid (NPPB) which is known to block parasite-induced pathways in the erythrocyte membrane could also be used as controls in determining the effects of extracts on the erythrocyte membrane.

Finally, it is recommended that other *in vitro* assays such 3H-hypoxanthine uptake and the newly developed tetrazolium-based colorimetric assays be used to assess anti-plasmodial activity of plant extracts and their results compared to identify the most ideal assay suitable for determining bioactivity of plant extracts.



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## **APPENDIX**

### **7.0 REAGENT PREPARATION**

#### **7.1 Preparation of Albumax**

One hundred grams of Albumax powder was dissolved in 500 ml of incomplete RPMI medium using a magnetic stirrer.

The solution was filter-sterilized and 5ml aliquots were made and stored at -20°C until use.

#### **7.2 Preparation of Sodium Hydrogen Carbonate**

Approximately 37.5g of NaHCO<sub>3</sub> powder in 500 ml of distilled water.

The solution was filter-sterilized and stored at 4°C until use.

#### **7.3 Preparation of Sorbitol**

Twenty-five grammes of sorbitol-D powder was dissolved in 500 ml of distilled water.

The solution was filter-sterilized and stored at room temperature for use.



#### **7.4 Preparation of ACD**

Approximately 2.4 g citric acid was dissolved in 400 ml of distilled water.

About 6.6 g sodium citrate and 7.35 g of D-glucose were added and the flask shaken carefully to dissolve completely.

The volume was made up to 500 ml and the solution filter-sterilized with 0.22  $\mu\text{m}$  filter unit.

It was stored at 4°C until use.

#### **7.5 Preparation of Incomplete RPMI 1640**

Approximately 10.4g (1 pack) of RPMI 1640 powder was dissolved in about 850 ml of distilled water in a large conical flask using a magnetic stirrer.

Two grammes Dextrose, 7.15g HEPES, 500 $\mu\text{l}$  gentamycin solution and 2ml of hypoxanthine solution (25mg/ml in NaOH) were added.

The volume was made up with distilled water to 1L and filter-sterilized with 0.2 $\mu\text{m}$  filter in a BSC and stored at 4°C until use.

The solution has a shelf life of up to 2 months.

## **7.6 Preparation of Complete RPMI 1640 (CPM)**

To approximately 188.6 ml of incomplete RPMI 5 ml of 20% Albumax solution and 6.4 ml of 7.5%  $\text{NaHCO}_3$  were added.

The solution was filter-sterilized and stored at  $4^\circ\text{C}$  until use.

## **7.7 Preparation of Giemsa buffer**

Three grammes of  $\text{Na}_2\text{HPO}_4$  powder and 0.6g of  $\text{KH}_2\text{PO}_4$  powder were dissolved in 1 L of distilled water.

The solution was filter-sterilized and stored at room temperature until use.

## **7.8 Preparation of thawing solutions**

### **7.8.1 Thawing Solution A:**

Six grammes of  $\text{NaCl}$  powder was dissolved in 50 ml of distilled water.

It was filter-sterilized stored at  $4^\circ\text{C}$  until use.

### **7.8.2 Thawing Solution B:**

Approximately 6.66 ml of Thawing Solution A was added to 43.33 ml of distilled water.

The resulting solution was filter-sterilized and stored at  $4^\circ\text{C}$  until use.

### **7.8.3 Thawing Solution C:**

Approximately 0.1g of dextrose and 0.45g of NaCl were dissolved in 50 ml of distilled water.

It was filter-sterilized and stored at 4°C until use.

## **7.9 Preparation of SYBR Green 1 Solution (1L)**

### **7.9.1 Preparation of SYBR Green Lysis Buffer**

Approximately 2.423g Tris base was added to 1L cell culture water (20mM<sub>final conc.</sub>) and dissolved using magnetic stirrer.

The pH was adjusted to 7.5 using conc. HCl.

Approximately 10ml 0.5M EDTA (5mM final conc.) and 80mg saponin (0.008% w/v final) and 0.8ml Triton X-100 (0.08% w/v final) were added.

The solution was thoroughly mixed, filter-sterilized and stored at room temperature until use. Solution is good indefinitely.

### **7.9.2 SYBR Green 1 Stock Solution**

Stock SYBR Green 1 solution was added aliquoted into 10µl portions into amber microtubes or into clear tubes and cover with aluminum foil.

They were stored at -70°C until use.

### **7.9.3 Preparation of MSF Lysis Buffer containing SYBR Green 1 (10 ml)**

SYBR Green1 stock aliquots in vials were thawed.

Two microlitres of SYBR Green stock was added to 10 ml MSF lysis buffer (0.2  $\mu$ l SYBR Green 1/ml of lysis buffer) and mixed well.

10 ml MSF lysis buffer is enough for only one 96 well plate.