In vitro ASSESSMENT OF SELECTED GHANAIAN MEDICINAL PLANTS' EXTRACTS' ACTIVITY AGAINST *Plasmodium falciparum*

BY

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DECLARATION

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

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DEDICATION

I would also like to dedicate this to my sweet sensei, Dr. Mitsuko Ohashi, who has unconditionally supported me in the pursuance of this degree, without whom this dream would not have materialized.

Also to my parents, family and friends who have supported me in one way or the other.

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LIST OF ABBREVIATIONS

ACT	Artemisinin combination therapy
AL	Artemether-lumefantrine
AQ	Amodiaquine
AS	Artesunate
BSC	Bio-safety cabinet
CDC	Centre for Disease Control and Prevention
CI	Confidence Interval
CPD	Citrate phosphate dextrose
CPM	Complete parasite medium
CPMR	Centre for Plant Medicine Research
DMSO	Dimethyl sulfoxide
DNA	Deoxyribose nucleic acid
FACS	Fluorescence activated cell sorting
HRP-2	Histidine-rich protein
IRS	Indoor residual spraying
ITNs	Insecticide-treated nets
KDR	Knock-down resistance gene
LLINs	Long lasting insecticide-treated bed nets
MICS	Multiple indicator clusters survey
NMIMR	Noguchi Memorial Institute for Medical Research
OPD	Out Patient Department
PBS	Phospate buffer saline
PCR	Polymerase chain reaction
pLDH	Parasite lactate dehydrogenase
PMI	The president's Malaria Initiative
pRBCs	Parasitized red blood cells
PWM	Parasite washing medium
RBCs	Red blood cells
RDT	Rapid diagnostic test
RPMI	Roswell Park Memorial Institute 1640
WHO	World Health Organization

ABSTRACT

Previous reports of the existence of chloroquine-resistant *Plasmodium falciparum* strains and in recent times, the increasing reports on the emergence of artemisininresistant strains of the malaria parasites call for continuous search for new antimalarial medicines. The purpose of this study was therefore to screen some selected Ghanaian medicinal plants' extracts for anti-*P. falciparum* activity. Processed extracts of 50 medicinal plants obtained from the Centre for Plant Medicine Research (CPMR), Akuapim-Mampong, and stored at the Department of Parasitology, Noguchi Memorial Institute for Medical Research (NMIMR), were used. *In vitro* screening of the crude extracts for anti-*P. falciparum* activity were performed using *Plasmodium falciparum* (3D7 strains) cultures and Guava FACS analysis. MitoPotential$^{(R)}$ was used to determine the apoptosis induction properties of active plant extracts. Two active extracts were derived from *Mangifera indica* and *Alcornea corifolia*, respectively, with IC_{50} values of 10 µg/mL and 14 µg/mL, were found out of 50 screened extracts. These extracts induced some level of apoptosis in *Plasmodium falciparum*. The active extracts are potential candidates that can be investigated further for their anti-*P. falciparum* activity *in vitro* and *in vivo*, and enhances the prospects of development of a new anti-malarial medicine.

CHAPTER ONE

GENERAL INTRODUCTION

1.0 Introduction

Malaria is an important disease endemic in many tropical and sub-tropical countries, posing a great public health challenge. It is known to be the major cause of morbidity and mortality in endemic areas. An estimated number of 198 million cases were reported worldwide (World Health Organization [WHO] 2013). As high as 90% of malaria deaths is reported in sub-Saharan Africa alone and 77% of these deaths occur in children under 5 years of age (WHO, 2013).

Malaria is caused by apicomplexan parasites belonging to the genus Plasmodium. It infects mostly humans and it is transmitted by the bite of the female *Anopheles* mosquito (WHO, 2014b). Four major species of *Plasmodium* is known to infect humans: *Plasmodium falciparum*, *Plasmodium vivax*, *Plasmodium ovale and Plasmodium malariae*. *Plasmodium falciparum* is the most deadly and is the major cause of malaria deaths. Another Plasmodium species, *Plasmodium knowlesi*, known to infect monkeys has recently been discovered to cause malaria in humans in South East Asia (Kantele et al., 2008). Female *Anopheline* mosquitoes are mainly responsible for transmitting malaria with *Anopheles gambiae*, *Anopheles arabiensis* and *Anopheles funestus* as the main vectors found to transmit the disease in Africa (Sinka et al., 2010). The high dominance of these vector species has resulted in the prominent malaria burden found in the tropics.

Several efforts such as vector control, effective chemotherapy and disease management have been implemented to control the transmission of malaria (Fullman et al., 2013).

The discovery of drugs has been very useful in the treatment of many diseases. Chloroquine- and artemisinin-based drugs, the latter being arguably more active, have helped in reducing morbidity and mortality cases related to malaria (Jones et al., 1996). There have been concerns of parasites developing resistance to these drugs, hence the improvisation in malaria treatment where artemisinin-based drugs have been combined with other anti-malaria drug to effectively clear malaria parasites in hosts (Moonasar et al., 2012).

Insecticide based control of vectors has also made a huge impact in controlling the disease. Massive use of insecticides, through insecticide-impregnated bed nets, and indoor residual spraying (IRS), have altogether helped in effectively reducing human contact with mosquitoes infected with malaria parasites (Kleinschmidt et al., 2009). Furthermore, efforts are being made in the areas of development of a practical effective malaria vaccine, as well as, transformed germ-line mosquitoes which would be resistant to malaria parasites (refractory) to replace wildtype vectors that are susceptible to malaria parasites (Yamamoto et al., 2012). Some of these novel approaches are all being still investigated and being used to help control malaria although success in some of these new methods seems to be far-fetched, for now.

Malaria parasites have developed resistance to many of the available drugs that are used for treating the disease (White, 2004). Anti-malarial drug resistance can be defined, according to Bloland (2001), as the ability of a parasite strain to survive and/ or multiply despite the drug in question gaining access to the parasite or the infected red blood cell for the duration or the time necessary for its normal action. It has to be noted that while drug resistance can cause treatment failure, not all treatment failure is due to drug resistance (Bloland, 2001). Many factors including incorrect dosing,

non-compliance with duration of dosing regimen, poor drug quality and interaction, poor absorption and misdiagnosis may contribute to development and intensifying true drug resistance. Because of these factors, they increase the likelihood of exposure of parasites to suboptimal levels of drugs (White and Pongtavornpinyo, 2003). Resistance appear to occur due to spontaneous mutations that confer decreased sensitivity to a given drug or class of drugs (White, 2004). Biochemical mechanism of resistance has been well described for chloroquine, antifolate combination drugs and atovaquone and this information can be useful in preventing resistance to other anti-malaria drugs available or yet to be developed (Adams, 1959). Natural products tend to have components that *Plasmodium* pathogens have not been exposed to before, thus rendering these parasites susceptible to natural products. Most natural products or organics are found from plants, making plants the best source for natural products and for drug discovery.

Plants used as natural remedies for overcoming health challenges, known as medicinal plants, have existed since prehistoric times (Petrovska, 2012). They have played major roles in the lives of native and indigenous people found in South America, Asia and Africa, by curing them of many ailments and plights (Petrovska, 2012). Medicinal plants have been useful in three ways: 1) as teas or in other extracted forms for their natural chemical constituents; 2) as agents in the synthesis of drugs; and 3) their organic molecules as models for synthetic drugs. Many modern medicines have had their origin from plants including aspirin from willow bark (*Salix* spp.) (Aspirin-foundation.com, 2014) as well as, artemisinin-based antimalarial drugs which were obtained from the sweet wormwood *Artemisia annua* (Tu,

2011; Cui and Su, 2009; Krafts, Hempelmann and Skórska-Stania, 2012). The search for alternative anti-malaria drugs continues and expectedly, many plant extracts have shown considerably good anti-malarial properties (Rukunga et al., 2009).

1.1 Rationale of Study

1.1.1 PROBLEM STATEMENT

Malaria is singly known to affect millions of people in certain parts of the world, particularly Africa, Asia and South America (WHO, 2013). Despite the enormous efforts made in reducing the cases of malaria in endemic areas, by the use of drugs, insecticides and chemical treated mosquito nets, a lot more people are still at risk of getting the disease (WHO, 2014a). The people most at risk are pregnant women and children under 5 years of age (WHO, 2013). This may be due to suppressed immune response in pregnant women or under-developed immune system of children (Rogerson et al., 2008).

The discovery of chloroquine was a breakthrough to the treatment of malaria, at least for a period, after which came the emergence of chloroquine-resistant malaria parasites mainly across Africa (Trape, 2001). The discovery of artemisinin-based medicines as an alternative for effective treatment of malaria overcame that challenge (Duffy and Mutabingwa, 2004). However, there has been increasing reports of artemisinin-resistant *falciparum* strains in Cambodia and this raises concerns for possible worldwide spread of these resistant parasite strains (Kyaw et al., 2013).

Alternative therapies such as artemisinin and its derivatives-based drugs are being combined with chloroquine or other anti-malaria drugs, known as the artemisinin combination therapy (ACT) (WHO, 2010a). Yet, there are concerns that there might be increased spread of artemisinin-resistant strains across the globe and ACT might no more be effective in malaria treatment. This will have a negative toll on current

chemotherapy that are being used in transmission control as there are no alternative drug available for effective treatment. The end result of this unpleasant event, if it should happen, is the increase in malaria cases, high rate of morbidity and mortality especially in children and pregnant women.

1.1.2 JUSTIFICATION OF STUDY

Medicinal plants have had immense impact in the treatment of many diseases, including malaria (Sofowora et al., 2013). Chloroquine- and artemisinin-based drugs which were derived from plants are evidence in the effective treatment of malaria.

Results from screening of extracts from some selected Ghanaian medicinal plants showed a few with high anti-malarial activity (Asase et al., 2005). Local herbalist have used plants for curing more than one disease and this may be due to multiple roles of one particular compound played in the target host or the presence of more than one compound found in a plant extract, hence its ability to be used in treating more than one disease (Sofowora et al., 2013).

This study therefore sought to screen some selected Ghanaian medicinal plants' extracts for anti-*Plasmodium falciparum* activities.

1.2 Aim of study

The main objective of the study is to screen some selected Ghanaian medicinal plants' extracts for anti-*P. falciparum* activities *in vitro.*

1.3 Specific objectives

The specific objectives of this studies are;

1. To undertake an *in vitro* cultivation of *Plasmodium falciparum* using Trager and Jensen principle, with slight modification.
2. To determine the anti-*P. falciparum* activities of some 50 selected plants' extracts *in vitro* using FACS analysis.

3. To determine the apoptosis inducing effect of active plants' extracts on *P. falciparum* using Guava MitoPotential$^{(R)}$ kit.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Malaria

Malaria, a disease found in the tropics and some subtropics, is a very serious and deadly disease that affects a lot of people. TheWorld Health Organization reports that, 3.2 billion people in 97 countries and territories are at risk of getting infected with the disease (WHO, 2014a). Mostly, children under 5 years and pregnant women are at higher risk of disease severity due to immune naivety and immune suppression respectively which directly impacts on the survival of babies to malaria infection. (Monif and Baker, 2004; WHO, 2013). As high as 430,000 deaths are recorded for African children alone, every year (WHO, 2014b). Everyday, more than 1,000 children die from malaria across the sub-Saharan Africa [The President's Malaria Initiative (PMI), 2014]. In the year 2013, approximately 584,000 malaria deaths were reported worldwide. Of these, 90% occurred in Africa and included mostly children who were 5 years or younger (WHO, 2014b). However, the number of mortality cases reported worldwide suggest a decline by 47% from the year 2000 to 2013. In WHO African region, mortality cases have reduced by 54% within the same period (WHO, 2014a). Due to the high cases of morbidity, there is a corresponding increase in healthcare cost; resources that could have been used for national development. Malaria is known to be a disease of poverty [Centre for Disease Control and Prevention (CDC), 2015], however, there are current management protocols established to reduce the high mortality rates. This is basically an integrated management control system where there is improved chemotherapy and vector control approaches. Effective diagnosis at point of care settings ensures prompt effective chemotherapeutic administrations.

2.1.1 Epidemiology of malaria in Ghana

Malaria is still the leading cause of morbidity in Ghana. It was responsible for 13% of the total cases reported (CDC, 2013). In the same year, 11% to 30% of the total deaths recorded in the country were as a result of malaria. Approximately 24 million suspected cases were reported in 2012 (WHO, 2013) which makes Ghana an area of high endemicity. In the year 2013, there were 1,639,451 confirmed cases reported, with a corresponding 2,506 deaths. There were 82 cases of deaths of children under 5 for every 1000 live births, reported by (WHO, 2014a). Furthermore, there was an increase of confirmed malaria cases, admissions and deaths in all age groups observed across 83 hospitals from 2005 to 2013 (WHO, 2014a). *Plasmodium falciparum* is the main parasite observed to be associated with all the cases reported. Thirty-nine percent of children under five used insecticide-treated nets (ITNs) which caused a reduction in mortality rate by 26% from 2006 to 2013 (PMI, 2014). Despite the figures reported by WHO, the generated data is not enough to depict the actual trend of malaria transmission in Ghana.

2.2 Clinical features and pathology of malaria

The asexual erythrocytic stages of the parasites are mainly responsible for causing the pathology of malaria (Ramasamy, 1998). The disease may present itself with or without signs, 10-15 days after the bite of an infected mosquito. Symptoms may vary from mild forms such as chills and headaches to more severe clinical manifestations such as cerebral malaria or anaemia; and ultimately death. The associated symptoms that are observed occur due to the host's immune system in an attempt to respond to the toxin or waste substances that are released into the bloodstream after an invaded red blood cell have been lysed by the parasite (Boström et al., 2012). Most of the

symptoms are observed periodically as the invasion of RBCs and toxin release happen in a cyclic manner during the asexual erythrocytic stage of the parasite's life cycle (Schumacher and Spinelli, 2012). Paroxysms (cycles of fever) are based on the parasite species involved. It takes every 48 hours for paroxyms to occur for *P. falciparum*, *P. vivax* and *P. ovale* (Crutcher and Hoffman, 1996). On the other hand, it takes *P. malariae* every 72 hours to manifest symptoms of infection.

Malaria can be grouped into two forms depending on the parasites and clinical symptoms involved at a particular moment (CDC, 2010). These are uncomplicated and complicated (severe) malaria. The symptoms that come along with uncomplicated malaria are chills and shivers, headaches, vomiting, tiredness and on a rare occasions seizures. On the other hand, symptoms such as acute respiratory distress syndrome, kidney failure, severe anaemia; and cerebral malaria with consciousness impairment, seizures and coma are associated with severe malaria (Kochar et al., 2014; Kochar et al., 2010). Late or wrong diagnosis and failure of prompt treatment of the disease can lead to death.

2.3 Diagnosis of Malaria

Prompt and effective diagnosis of malaria is essential for early treatment and prevention of spread of the disease from infected individuals to uninfected ones (National Malaria Guidelines, 2011). A case can be suspected of malaria infection by considering the travel history of individuals and also observing the symptoms at a particular moment. However, suspected malaria cases cannot be treated for malaria unless it has been confirmed by laboratory tests (WHO, 2010a). Confirming the presence of infection is important for reducing drug resistance, avoiding misdiagnosis and improving the healthcare of patients or individuals infected with malaria.

2.3.1 Microscopy

Malaria parasites can be identified under a microscope by observing their components in the patient's blood. The patient's blood is smeared onto a microscope slide in a thin or thick film and parasites are stained with Giemsa to give them a distinctive appearance (Bailey et al., 2013; Sathpathi et al., 2014). Within an hour or few hours, this simple method of diagnosis is able to provide results or information detailing the parasite species present, the parasitaemia and the percentage of RBCs hosting the parasites (WHO, 2000). However, the proficiency and reliability of the results obtained usually lies on the quality of reagents used, good laboratory set up (Petti et al., 2006) and, the laboratory technician performing the test (Ayalew, Tilahun and Taye, 2014; Moura et al., 2014). There are usually inconsistencies found in one microscopy result produced by different technicians. The expertise of a qualified and proficient technician or haematologist is usually required to provide accurate and reliable results (Abreha et al., 2014). Examination by microscopy remains the standard gold method used for detection of malaria infection despite its low sensitivity in detecting malaria parasites at very low concentrations in individuals' blood (Bejon et al., 2006; Kahama-Maro et al., 2011). This mars the actual parasite population and epidemics of the disease in areas where microscopy is mainly used for screening. The effect of wrong surveillance culminates in influencing policy making and the use of inadequate methods in preventing transmission.

2.3.2 Rapid Diagnostic Tests

A quicker way of determining malaria infection in humans is the use of the rapid diagnostic test (RDT). This method is able to determine malaria infection by detecting specific malaria antigens found in an individual's blood (WHO, 2006). Usually it takes

about 20 mins to obtain results making the use of RDT the preferred choice in a clinical setting of high infection rate, where no expertise of a highly qualified person is required, and urgent medical attention is needed (Wongsrichanalai et al., 2007). The kit is able to show specific bands for *P. falciparum* and one of the other three human malaria parasites (Moody, 2002). There are two commonly used types of RDTs: the HRP-2 type which detects a histidine-rich protein produced only by *P. falciparum* and it is the only RDT kit used for malaria diagnosis in Ghana

(WHO, 2014a), and the pLDH type, detecting the parasite lactate dehydrogenase (pLDH) enzymes produced by all species of *Plasmodium* that cause malaria in humans (WHO, 2010b; Wongsrichanalai, 2007). In as much as RDT is more efficient and quicker in diagnosing malaria, it is flawed in some ways. One is its inability to detect malaria infection in low endemic areas (Baiden et al., 2012; WHO, 2000) and, also not providing accurate results in cases where there are co-infections with other diseases (Meatherall et al., 2014). RDT is also not able to determine parasitaemia or percentage of RBCs infection. As such all negative and positive RDTs have to be followed up with microscopy for confirmation of the presence of parasites (Bailey et al., 2013), but microscopy itself may be less sensitive than RDTs in determining the prevalence of malaria infection in a community.

2.3.3 PCR

A more sensitive and highly efficient method of confirming the presence of malaria parasites is the use of polymerease chain reaction to detect parasites' nucleic acids (Mwingira et al., 2014; Shehzadi et al., 2015). This method is quite expensive to be used for day to day screening of malaria infection in the community as it requires a laboratory and expensive reagents to obtain results (Mens et al., 2006). Results

obtained by this method is not quick enough and therefore delays the necessary treatment needed for patients. As such this tool is only used for confirmatory purposes after suspected cases have been diagnosed either by microscopy or RDT.

2.4 Parasites that cause malaria

Malaria is caused by parasitic microbes that depend on both the mosquito and the human/animal host to complete their complex life cycle. These microbes are apicomplexans of the genus group *Plasmodium*. There are hundreds of *Plasmodium* species that are known to infect vertebrates, however, four species are majorly known to infect humans only and they are the following; *Plasmodium falciparum, Plasmodium vivax, Plasmodium ovale and Plasmodium malariae* (WHO, 2014b). It has been mentioned that there is another *Plasmodium* strain reported to cause zoonotic malaria. This species is known as *P. knowlesi* and is mainly found in South East Asia (Lee et al., 2013; Singh and Daneshvar, 2013; William et al., 2013)

Plasmodium falciparum, the most lethal human malaria parasite, is able to cause critical complications such as huge loss of RBCs causing severe anaemia. This is due to the parasite's ability to multiply rapidly and destroy both immature and mature RBCs (Evans et al., 2005). It causes millions of deaths every year, mainly in Africa (Snow et al., 2005), due to their predominance in the tropics and subtropics.

Plasmodium vivax is the most prevalent malaria parasite found in Asia and Latin America. It is also found in some parts of Africa (Guerra et al., 2010). It has a characteristic dormant liver stage during infection, able to reactivate several months after an infectious bite and invade RBCs causing a relapse. *Plasmodium ovale* is very similar to *P. vivax* but, different by being able to infect negative Duffy blood group individuals (McKenzie and Bossert, 1999). It is prevalent in Africa, particularly West

Africa. *Plasmodium malariae* is able to cause a lifetime lasting chronic infection if untreated. Found worldwide, it is the only species out of the four that has a peculiar quartan cycle (3-day cycle).

2.4.1 *Plasmodium falciparum*

Majority of the cases (85%) and deaths (90%) resulting from malaria is due to *P. falciparum* alone (Snow et al., 2005). It is the deadliest known species of malaria parasites affecting mostly pregnant women and children under five (WHO, 2014a). *Plasmodium falciparum* parasites have the ability of escaping the human host's immune system (Gardner et al., 2002). One way of evasion is by parasitized RBCs acting as adhesins. They do so by expressing certain functional proteins on the cell membrane of RBCs and this leads to an interaction with the surface of the host endothelium. As a result, parasites are able to avoid systemic circulation and clearance by the spleen (Montgomery et al., 2007). This ability of the parasites is a huge disadvantage to the human host, as it causes blood vessel blockages leading to hypertension and heart related complications, as well as other organ complications. The parasites are also able to affect the brain causing cerebral malaria where the central nervous system is affected and complications such as deafness, blindness, paralyses and problems with movements may occur (Miller et al., 2002). These severe complications are seen during the advanced stages of the disease when left untreated.

2.4.2 Life cycle of *Plasmodium falciparum*

Through the bite and saliva of infected female *Anopheles* mosquitoes, malaria parasites are introduced into humans in the form of sporozoites (Figure. 2.1). Injected sporozoites invade liver cells (hepatocytes) and develop into schizonts. Matured schizont-invaded hepatocytes then burst open and release many merozoites into the

bloodstream, which attacks red blood cells (erythrocytes). At this point, there is a cyclic invasion of erythrocytes during which the parasites complete their erythrocytic stage cycle from early trophozoites (rings) to schizonts. This cyclic attack is responsible for the characteristic feature of malaria; chills, sweating, fever, nausea, vomiting, and headache. This cycle occurs approximately every 48 hours. Some of the parasites found in the bloodstream differentiate into sexual forms called gametocytes. The male gametocyte divides its nucleus, producing 8 flagellated microgametes and the female gametocytes differentiate into macrogametes. These gametes are picked by the bite of another female *Anopheles* mosquito and are fused together to form a zygote inside the mosquito midgut. The zygote matures into an elongated structure called ookinete and then moves to invade the midgut wall. In the midgut wall it develops into an oocysts, grows and releases sporozoites after its rapture. These sporozoites then find their way to the salivary glands of the vector, ready to be injected into another human and the transmission continues (Cowman et al., 2012).

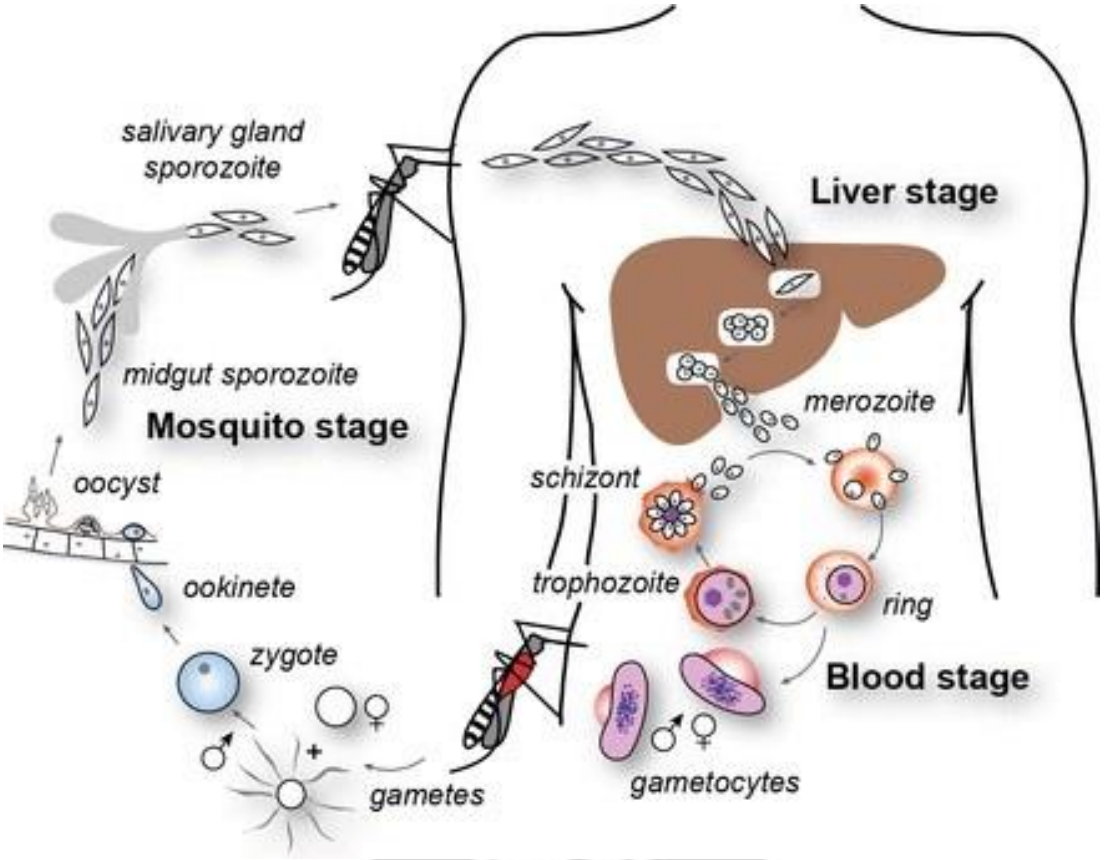

Figure 2.1: Life cycle of *Plasmodium spp*.

Source: Cowman, Berry and Baum, (2012), pp 164.

2.5 Malaria Vectors: Anopheles mosquitoes

Female mosquitoes of the genus Anopheles are the only responsible insects for transmitting malaria from one individual to the other (Mosqutio Malaria Vectors, 2015). About 40 species of Anopheles mosquitoes are capable of acting as vectors of malaria, out of approximately 430 Anopheles species available. After the ingestion of malaria parasites by the mosquito vector, they develop within the vector for a 10 - 18 days before they become infectious to humans (CDC, 2012). This association between humans and the completion of malaria parasites' life cycle within female mosquitoes is due to the blood meal that is required by mosquitoes to develop their eggs for generation continuity. The success rate of parasite survival in vector hosts is dependent

on ambient temperature, humidity and lifespan of vector (Mzilahowa et al., 2012). Most Anopheles mosquitoes found in tropical and subtropical areas have long lifespans due to the hot and humid climatic conditions found there (Mzilahowa et al., 2012), making mosquitoes the perfect reservoir for *Plasmodium* species for continued transmission, rendering these areas endemic for malaria. The predominant species found in these areas are *Anopheles gambiae* (*An. gambiae*), *An. funestus* and *An. ariebiensis* (Lwetoijera et al., 2014; Mzilahowa et al., 2012). *Anopheles gambiae* is the predominant vector responsible for transmitting malaria in Ghana (Dery et al., 2010; Tchouassi et al., 2012). Some species of Anopheles mosquitoes do not allow proper development of malaria parasites within them or at all, rendering them as poor vectors (Marshall and Taylor, 2009). Most Anopheles mosquitoes are endophagic (feed indoors) and crepuscular (active at dawn or dusk) or nocturnal (active at night) (Maxwell et al., 1998), hence increasing factors leading to human contact with malaria parasites since humans are less active at these periods.

2.6 Transmission control

Strategies and innovative methods to best control or end transmission of malaria will depend on better understanding of the biology and behaviour of Anopheles mosquitoes and *Plasmodium* species. The vectors' susceptibility to *Plasmodium* and insecticides, its host choice, its longevity; and the parasites' susceptibility to antimalaria drugs and immune system of human host are all factors that need to be considered when planning transmission control of the disease.

2.6.1 Vector control

The massive usage of indoor residual spraying (IRS) and insecticide-treated bed nets (ITNs) are the main insecticide based control methods used to reduce human

contact with malaria parasites (Lengeler, 2009; Pluess et al., 2010). The free use of ITNs and long lasting insecticide-treated bed nets (LLINs) by children and pregnant women was adopted in Ghana in 2004 and then extended to all age groups 6 years later (WHO, 2014a). The use of ITNs alone reduced mortality of infant children (Howitt et al., 2012) and in Ghana, the mortality of children under five was reduced by 26% from 2006 to 2013 (PMI, 2014). Despite the barrier the nets create between humans and mosquitoes, nets treated with insecticides are more protective than untreated ones (Raghavendra et al., 2011). Nets only treated with pyrethroid insecticides are approved (WHO, 2007) for use on ITNs as they are known to pose very low health risks to humans but deadly to insects at low doses. Nevertheless, repeated washing of LLINs in a short period of time tends to render these nets ineffective of protecting individuals from mosquito borne pathogens (Atieli et al., 2010). As such, a better way of providing long lasting protection has to be improvised, and for now less washing or re-application of insecticides into the nets is recommended. Similarly, the use of IRS was recommended by WHO in 2005. The use of IRS, where insecticides are coated on wall surfaces and remain as residues for several months with the aim of killing mosquitoes when they come into contact with it, is another method for vector control. This may not be as effective as ITNs, it is still capable of reducing malaria vector population (Sharp et al., 2007), since it does not directly prevent humans from being bitten by mosquitoes but rather kills the mosquitoes after a blood meal and if they rest on a sprayed surface.

2.6.2 Parasite control: Drug Treatment of Infected host

Malaria transmission can be reduced hugely through early detection of the disease and subsequent prompt treatment, to help prevent morbidity and deaths. WHO has

recommended that within 24 hours after the first symptoms appear and upon confirmation by rapid diagnostic test (RDT) and/or microscopy, prompt treatment must be given to malaria-inflicted individuals (WHO, 2010a). The use of a particular drug for malaria treatment usually depends on the *Plasmodium* species involved, presence of drug-resistant strains found in an endemic region, the individuals involved and the side effects that may come along with the intake of a drug. Quinine, quinidine, chloroquine, amodiaquine, pyrimethamine, proguanil, chloroproguanil, Mefloquine, halofantrine, artemisinin and its derivatives are some of the available drugs that have been used or are being used for treating malaria (Morrow, 2007; Winstanley, 2000; Winstanley, 2001). These drugs target sporozoites, asexual forms and gametocytes form of malaria parasites. The first-line treatment for unconfirmed malaria in Ghana is artesunate (AS) and amodiaquine (AQ) and this was adopted as far back as 2004 (WHO, 2014a). In situations of confirmed *P. falciparum* cases, the medicine used are artemether-lumefantrine (AL) or AS together with AQ. Qunine was employed for the treatment of severe malaria or treatment failure of *P. falciparum* was encountered as a result of infection with chloroquine-resistant *P. falciparum* strains(WHO, 2014a). Currently, artemisinin and its derivatives have the ability to reduce parasitaemia significantly in the blood of individuals with malaria (White, 1997).

Currently, ACT is the best available drug treatment and the first-line treatment, particularly for *P. falciparum* (Achan et al., 2009; Alecrim et al., 2006). In ACT treatments, another antimalarial drug is added to artemisinin to treat infected people. During this process, artemisinin clears majority of the parasite load in the blood within a short period of treatment time and the partner drug eradicates the remaining parasites (Chotivanich et al., 2000). ACT treatments have also been successful in eliminating artemisinin-resistant strains of malaria (Lefevre et al., 2001), however, it takes a longer

time to cure such individuals as the artemisinin is unable to eliminate the parasites by the third day of treatment. It is of utmost importance in transmission control programmes that parasites are completely cleared from the blood of individuals. Some individuals harbour parasites without showing symptoms and this provides opportunity for re-infection of malaria in a high densely populated community. This is a challenge and new methods have to be employed where nationwide screening is performed to identify people with malaria parasites and then treat them accordingly, as results of intermittent preventive treatment method have shown to be encouraging (Ahorlu et al., 2011). Reduced parasite population in a community, in combination with other control factors, can have a huge effect on eradicating the disease entirely.

2.6.3 Immunity

Despite the wait for an effective vaccine, humans are able to develop some partial immunity to malaria. This developed immunity after repeated exposure to malaria antigens (Brown et al., 1986) is however short lived as *P. falciparum* is able to evade the host's immune system by periodically changing their cellular membrane proteins (Kirkman and Deitsch, 2012). This mechanism is due to the enormous variant genes the parasites possess which gives them the ability to change their antigen markers from lineage to lineage. Individuals who have been previously and repeatedly exposed to malaria parasites, particularly during childhood, tend not to show symptoms of the disease or are able to tolerate its clinical manifestation even though harbouring the parasites in their bloodstream (Doolan et al., 2009). Such individuals are shown to have high immunity to malaria as compared to children or immunonaive individuals who have never been exposed to *Plasmodium* parasites or have been exposed on fewer occasions. Antibodies or proteins that confer such protection on adults or

immunocompetent individuals could be harvested and given to infants for protection and improve their tolerance to the disease at a very delicate age (Raj et al., 2014). However, asymptomatic immunocompetent individuals tend to act as reservoirs of malaria parasites and increase the likelihood of continued transmission via a mosquito bite.

2.7 Resistance

2.7.1 Resistant mosquitoes

Long term exposure of mosquitoes to insecticides over multiple generations produce resistant mosquitoes, where they develop resistance and survive even after contact with an insecticide (Hemingway et al., 2004). Insecticide resistance in mosquitoes arises so quickly due to the ability of mosquitoes to produce a lot of generations within a year. *Anopheles gambiae* which is known to be the dominant vector for malaria transmission in sub-saharan Africa have developed some level of resistance to pyrethroid insecticides (Kerah-Hinzoumbé et al., 2008; Protopopoff et al., 2013; Sharp et al., 2007), which is the main and only recommended chemical used in treating bed nets against mosquito bites. Resistance to pyrethroid arises due to either increased insecticide detoxification (Vulule et al., 1999), which is associated with increased oxidases and esterases activity or target site insensitivity (Ranson et al., 2000), which involves point mutation in the voltage-gated sodium channel gene (knock-down resistance gene). The emergence and increasing spread of pyrethroid resistant strains may have a degrading toll on the ongoing malaria control programmes in place, as some have suggested that mosquitoes' susceptibility to

Plasmodium infection is increased in the presence of the kdr gene (Ndiath et al., 2014). Better understanding of the causes of insecticide resistance will help improvise better and efficient vector control methods. Survey of nationwide, or better still global survey

of vector population and prevalence of resistance among mosquitoes will help manage insecticide resistance more efficiently.

2.7.2 Resistant parasites

The emergence of parasites resistant to anti-malaria drugs and its widespread to other geographical areas is a long term problem that needs resolving. In Ghana, *Plasmodium falciparum* is already known to be resistant to chloroquine and as early as the 1980s, chloroquine-resistant *P. falciparum* became widespread in Africa (WHO, 2005). This resistance is as a result of the parasite's ability to expel chloroquine from within before the amount of chloroquine required to effectively inhibit hemozoin biocrystallization (Krafts et al., 2012). Haemoglobin digestion by parasites leads to the formation of toxic byproducts harmful to the parasites. Hemozoin biocyrstallization is employed by the parasites to render these toxic byproducts harmless to the parasites. The expulsion mechanism is necessary to prevent build-up of the toxic byproducts formed by haemoglobin digestion (Foley and Tilley, 1997). Resistance to amodiaquine, mefloquine, halofantrine and quinine also have similar efflux mechanism. Using antimalarials with similar basic chemical compound background can increase the rate of resistance development, for example cross-resistance to chloroquine and amodiaquine, which are two 4-aminoquinolones; and mefloquine confer resistance to quinine and halofantrine (Bloland et al, 2001). This mechanism reduces the usefulness of newly developed therapies prior to largescale usage.

At the moment, there are no protection or prevention of anti-malarial resistance to chemotherapy under development or to be developed in the seen future. This makes continuous development of resistance to newly developed drugs more credible.

South-East Asian countries such as Cambodia, Vietnam, Thailand, Laos and

Myanmar are areas where malaria parasites resistant to artemisinin, the best antimalaria drug available, have been detected (Bustos et al., 2013; Na-Bangchang and Karbwang, 2013). Due to the devastating nature of an outbreak of artemisinin resistant parasites, as there are no alternative drug treatment that is as efficacious as ACTs, WHO has recommended, in May 2007, that countries endemic to malaria should progressively stop using oral artemisinin-based monotherapies, and is prepared to help with long term routine monitoring of anti-malaria drug resistance. WHO has also recommended that if there is a 10% failure in ACT treatments, that country should switch its malaria treatment policy to an ACT with a different active partner drug. Lastly, areas with no detection of drug resistance must be monitored hugely and sustained, as well as, contain antimalarial drug resistance in reported areas (Satimai et al., 2012).

2.8 Innovative models

Due to the huge impact malaria has on society, several researches, methods and programmes are being employed to reduce its transmission or eradicate the disease entirely as it has been done in most part of the Americas and Europe (CDC, 2012). Innovative models and methods are being welcomed, as some existing methods have failed with the emergence of drug-resistant parasites and insecticide-resistant mosquitoes. Researchers are encouraged to come out with groundbreaking results to overcome this emerging obstacle. Some of the approaches being employed are vaccination (Todryk and Hill, 2007), genetic modifications of malaria vectors and search for an alternative effective and affordable antimalarial drugs (Ridley, 2002).

2.8.1 Vaccination

At the moment, there are no licensed vaccines available to completely provide immunization against malaria. This predicament is a result of the complexity of the

parasite to evade its host's immune system. Nevertheless, malaria vaccine development is ongoing. A lot of vaccine candidates are under investigation and in clinical study phase. Currently, the most advanced malaria vaccine candidate against *Plasmodium falciparum* available is RTS,S/AS01E. This vaccine targets the circumsporozoite protein and blood stage protein, and has been shown to have interesting results (Agnandji et al., 2012; Schwartz et al., 2012). In May 2009, a Phase III trial started where 15,460 children were completely enrolled from seven countries in sub-Saharan Africa, including Ghana. Preliminary results indicate signs of future success of obtaining the first ever effective vaccine against malaria as disease manifestation in infants were reduced by half (Agnandji et al., 2012).

2.8.2 Transgenecity

Lately, a lot of research has been made in the field of genetics and breaking pathways where genetic modification can be used to control transmission by using the vector (Marshall and Taylor, 2009). In the laboratory, it is possible to obtain strains of *An. gambiae* that are refractory to malaria parasites by selection or genetic modification. These strains are able to produce immune response that encapsulates and destroy the parasites after they have invaded the mosquito's gut epithelium (Yamamoto et al, 2012). This approach can help reduce transmission by replacing wildtype mosquitoes with transgenic ones. However, the outcomes or side effects of modifying mosquitoes' genome is unknown and uncontrollable, making its application far-fetched. Hopefully one day when all ethical issues are cleared, they can be used to provide mass vaccination or block transmission of malaria.

2.8.3 Plant medicine & anti-malaria activity

Before the wide use of orthodox drugs for malaria treatment, extracts or concoctions made from medicinal plants were the ideal way of relieving individuals of fever and malaise conditions (Adebayo and Krettli, 2011). Plant medicine have provided enormous avenues from which many purified compounds and drugs have been produced. For instance, the most potent drug known for its fast clearance of malaria parasites, artemisinin was discovered from *Artemisia annua*, a Chinese sweet wormwood plant which has been used for thousands of years (Cui & Su, 2009). Identifications of these compounds have also allowed for structural modifications where similar high activity has been observed against *P. falciparum* (Abiodin et al, 2013). In Ghana, most indigenous individuals prefer the use of medicinal plants to treat fevers and other diseases since they are known to be effective in curing them.

Presently, a lot of medicinal plants have shown some level of anti-malaria activity (Bagavan et al., 2011; Ouattara et al., 2006) and this provides platforms for the development of novel anti-malaria drugs as alternative and effective drug treatment.

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Study Approval & Ethical Clearance

The study was examined thoroughly for standardized methods and correctness by the Scientific Technical Committee of the Noguchi Memorial Institute for Medical Research (NMIMR). Ethical clearance was obtained from the Institutional Review Board of NMIMR. The ethical approval certificate is attached as Appendix 2.

3.2 Blood Collection and RBCs Preparation

RBCs needed to culture malaria parasites were obtained from blood of consented volunteers (Blood group O, Positive Rhesus factor). An amount of 20 ml of venous blood was drawn and collected into vacutainers containing citrate phosphate dextrose. Collected blood were kept at 4 ^{o}C overnight, transferred into 15 ml tubes and centrifuged at 2,000 rpm for 7 mins. Suspended plasma which contains Buffy coat and white blood cells were discarded. Packed RBCs were then washed three times with wash medium which contains 50 µg/mL gentamycin and 2 mM Lglutamine. Each washing step involved adding of wash medium, pipetting up and down thrice, centrifuging at 2,000 rpm for 7 mins and then discarding suspended medium. After washing, wash medium was added to the packed RBCs and stored at 4 ^{o}C until ready for use. Washed RBCs was stored for up to 2 weeks maximum after which new blood was collected.

3.3 *In vitro* cultivation of *Plasmodium falciparum*

Cryopreserved ring stage of 3D7 strain of *Plasmodium falciparum*, were obtained from the Immunology Department of the NMIMR, Legon, and cultivated based on the principle of Trager and Jensen (1976) with slight modifications. Cultures were

maintained in 25cm^2 cell culture flask containing washed RBCs suspended at 4% haematocrit in RPMI 1640 comprising of 5 mg/mL albumax, 2 mM L-glutamine and 50 µg/mL gentamycin. Malaria cultures were provided with a gas mixture (2% O_2, 5% CO_2, and 93% N_2), and incubated at 37°C. Culture media were changed daily and the level of parasitaemia were determined by counting RBCs on a Giemsa-stained thin blood smear under a light microscopy. Every 2 to 3 days or when parasitaemia was at about 5%, infected erythrocytes were subcultured into fresh complete media with uninfected erythrocytes. The stock culture was synchronized with 5% sorbitol to obtain singular life stage of parasites, preferably ring stages (early trophozoites) and then approximately 48 h later, obtained trophozoites, were used in the screening of plant extracts.

3.4 Thin film preparation and parasitaemia determination

To prepare thin smears, a drop of blood was placed on a microscope slide and spread with the aid of another slide set at 45^o to the test slide. Slides were air dried, and the blood was fixed onto the slide by dipping into methanol for 5 secs and allowed to air dry again. 10% Giemsa stain was prepared by adding 10 ml of Giemsa stain to 90 ml of tap water. The top surface of fixed slides were then covered completely with 10% Giemsa stain and left standing for 10 mins. The Giemsa stain was then gently rinsed off with running water. Stained slides were allowed to air dry and then viewed under a light microscope with immersion oil at objective 100x for identification of malaria parasite stages and determination of parasitaemia. Parasitaemia was expressed as a percentage of the number of parasitized RBCs (pRBCs) to the total number of RBCs .

3.5 Plant extracts preparation

Dried plant crude extracts, previously prepared by CPMR, Akuapim-Mampong, whose identity were blinded and stored at the Parasitology Department, NMIMR were randomly selected. The extracts were reconstituted in 50% ethanol to obtain a concentration of 10 mg/mL. The mixture was agitated to obtain a homogenous solution with the help of a vortex. Artesunate (Sigma-Aldrich, USA) which served as a standard drug was also prepared by dissolving in 100% Dimethyl sulfoxide (DMSO) to obtain a concentration of 1 mg/ml. Dissolved extracts and standard drug solutions were sterilized with 0.22 µm sterile bio- filters in a safety cabinet (BSC).

3.6 Screening plants' extracts for anti-*P. falciparum* activities

A total of 50 plants extracts were screened for anti-*P. falciparum* activity by using the SYBR Green I fluorescence assay as described by Smilktein et al. (2004), with some modifications. A two-fold serial dilution from Stock solutions of the standard drug and plants' extracts were prepared in CPM to yield final concentrations ranging from 100 ng/ml to 400 ng/ml and 3.13 µg/mL to 25 µg/mL respectively. An aliquot of 5 µL of each concentration was dispensed into different test wells in triplicates. The synchronized parasite culture were diluted with CPM and washed RBCs to a starting packed volume of 2% haematocrit and 1% parasitaemia. An aliquot of 95 µL of the parasite culture were added to each test well of the 96-well micro-titer plates, containing the drugs or extracts. In addition, wells containing RBCs at 2% haematocrit, pRBCs at 2% haematocrit and CPM alone; served as negative controls, positive controls and blank controls respectively. Furthermore, wells containing pRBCs plus either 0.1% DMSO or 2.5% ethanol served as reference controls. Final volume per well was 100 µL. The plates were then incubated for 48 hr as described above in the

cultivation of malaria parasites. A 100 µL aliquot of 2.5x buffered SYBR Green I (0.25 µL of 10,000X SYBR Green I/mL of 1X phosphate buffer saline) was added to each well after the incubation period and incubated again in the dark for 30 min at 37 ºC. Fluorescence was detected by Guava EasyCyte HT FACS machine (Millipore, USA).

3.7 Apoptosis study

3.7.1 Mitochondrial Potential

Determination of apoptosis-inducing capabilities of crude extracts as indication of high anti-*P. falciparum* activities was done by the use of the Guava MitoPotential$^{(R)}$ kit (Guava Technologies, USA) as described by the manufacturer's instructions. A cationic dye, 5, 5', 6, 6'-tetrachloro-1, 1', 3, 3'-tetrathyl benzimidalyl carbocyanine iodide (JC-1), was used to evaluate mitochondrial membrane potential changes and 7-Aminoactinomycin (7-AAD), a cell-impermeant DNA intercalator, was used to monitor cell membrane permeability changes. Synchronized culture parasites and preparation of 96-well micro-titer plates were taken through the same procedure described above for the screening of the plants' extracts to the point of 24 hr incubation. A staining solution (4 µL of 50x staining solution/100 µL of CPM) of the Guava MitoPotential$^{(R)}$ kit was then added to each well in a ratio of 1:1 (v/v) and incubated for 30 min at 37 ºC in the dark. Stained cells were analyzed on a Guava EasyCyte HT system.

3.8 Data Analysis

The data obtained from the FACS analysis were represented on a trend curve to calculate the 50% inhibitory concentration (IC_{50}) values, the concentration of plants' extracts at which 50% of the parasite population growth are inhibited. For all the culture parasites for which interpretable results were obtained from the SYBR Green I

assays, the following scale was used for interpretation: IC_{50}values > 100 µg/ml was considered as 'no activity', from 50 to 100 µg/ml as 'low activity', from 25 to 50 µg/ml as 'moderate activity' and < 25 µg/ml as 'high activity'. Differences in anti-*P. falciparum* activity and apoptotic effect among active extracts were calculated using GraphPad Prism 6.05 software (GraphPad Software Inc,). Significant differences were set at a confidence interval (CI) of 95%.

CHAPTER FOUR

4.0 RESULTS

4.1 IC_{50} values of screened extracts

A total of 50 crude extracts were screened *in vitro* for their anti-*P. falciparum* activity. This study determined the concentration of the screened extracts at which the parasite population would be reduced to 50%, or inhibit the growth of 50% of the parasite population (IC_{50}). The different levels of activity against *P. falciparum* are represented in Table 4.1 and the percentage of crude extracts showing the different levels of activity is shown in Figure 4.1. As shown on Table 4.1, there were only two extracts shown to have high activity against *P. falciparum* cultures, with IC_{50} values less than 15 µg/ml. Two other extracts were found to have moderate activity in inhibiting the growth of malaria parasites with IC_{50} values between 30 µg/ml and 50 µg/ml. Extracts with relatively low activity with IC_{50} ranging between 50 µg/ml and 100 µg/ml, were also found. The remaining screened extracts, which made up 88% of the total screened extracts (Fig. 4.1), had no activity against *P. falciparum* as their IC_{50} values were found to be more than 100 µg/ml. The inhibition growth among malaria parasites at different concentrations of the active crude extracts were compared with artesunate, the reference drug, and an inactive extract as displayed in Figure 4.2.

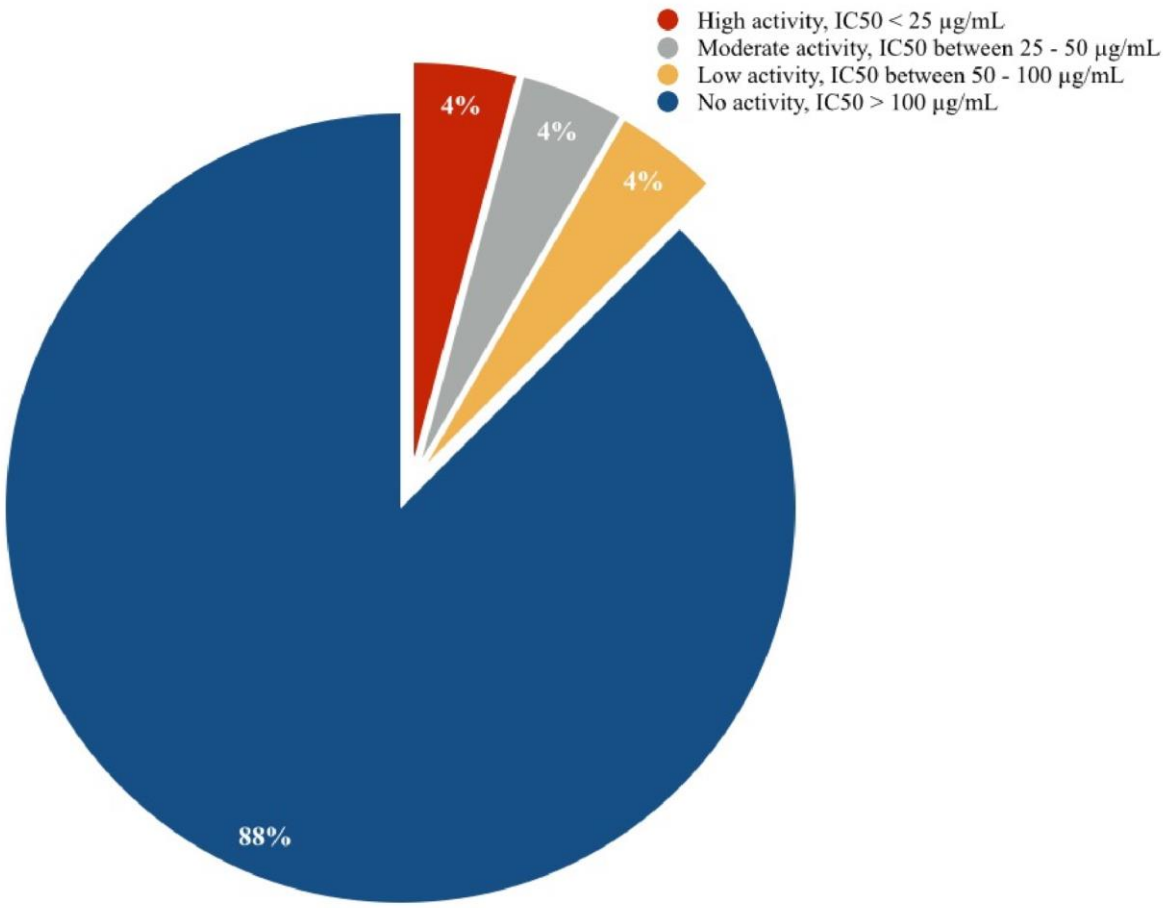

Figure 4.1: Percentage of crude extracts showing the different levels of activity against *Plasmodium falciparum* Table 4.1: Screened crude extracts showing their activity against *Plasmodium*

falciparum growth.

Extract Code	Activity	Extract Code	Activity
CMJA001L	-	CMJA015L	++
CMJA001R	-	CMJA018F	-
CMJA001SB	-	CMJA018L	-
CMJA002L	-	CMJA019L	-
CMJA002R	-	CMJA019SB	-
CMJA003L	-	CMJA020R	-
CMJA003R	-	CMJA020SB	-
CMJA003SB	-	CMJA022L	+++
CMJA004L	-	CMJA023L	-
CMJA004SB	-	CMJA024L	-
CMJA005L	-	CMJA024SB	-

CMJA005SB	-	CMJA025SB	-
CMJA006L	**+++**	CMJA026L	-
CMJA006SB	-	CMJA026SB	-
CMJA007SB	-	CMJA027R	-
CMJA008L	-	CMJA028L	-
CMJA008R	-	CMJA028SB	+
CMJA008SB	-	CMJA029L	+
CMJA009L	-	CMJA029SB	-
CMJA010L	-	CMJA030L	-
CMJA011SB	++	CMJA030R	-
CMJA012L	-	CMJA030SB	-
CMJA013L	-	CMJA031SB	-
CMJA013S	-	CMJA032L	-
CMJA013WP	-	CMJA032SB	-

High activity (+++): IC_{50}< 25 µg/ml, moderate activity (++): 25 µg/ml≤ IC_{50} ≤ 50 µg/ml, low activity (+): 50 µg/ml≤ IC_{50} ≤ 100 µg/ml, no activity (-): IC_{50}> 100 µg/ml

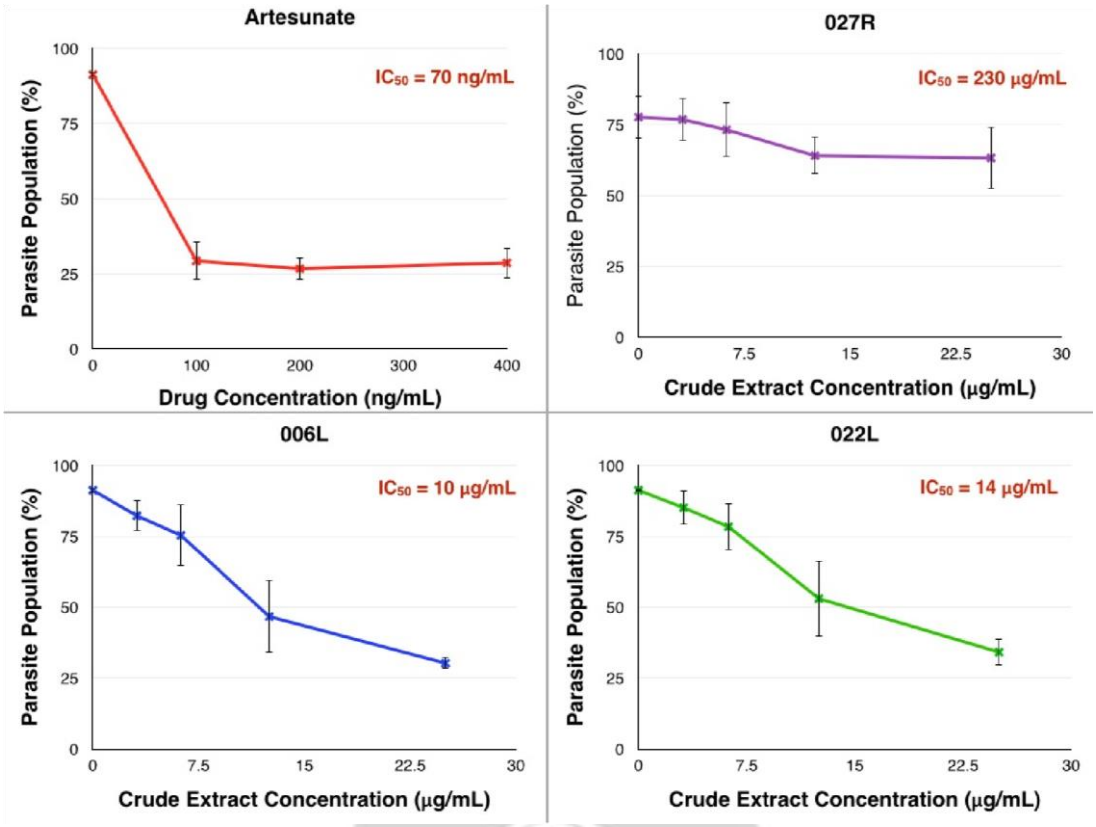

Figure 4.2: Growth inhibition curve of three crude extracts and artesunate on laboratory-reared 3D7 strains of *Plasmodium falciparum*

4.2 Anti-*Plasmodium falciparum* effect of medicinal plants' extracts

Since artesunate is known to clear malaria parasites significantly at very low doses, this study sought to observe the difference in parasite population in the absence and presence of artesunate and crude extracts. Malaria cultures obtained 48 hrs after synchronization to obtain ring stage forms or early trophozoites were exposed to different doses of artesunate and crude extracts of medicinal plants for

48h and then the parasitized cell count was detected and measured. As shown in Figure 4.3, artesunate independently reduced parasitaemia with approximately 70% reduction of pRBCs at a dose of 0.4 µg/ml. Comparable reduction in parasite population was observed for artesunate even at a lower doses of 0.2 µg/ml and 0.1 µg/ml. Figure 4.3 also indicates that, the two extracts found to have high anti-*P. falciparum* acitivity, CMJA006L and CMJA022L, were able to reduce parasitaemia by 18% and 17%,

respectively, at doses of 3.13 µg/ml. Reduction in pRBCs was dose-dependent. For instance, CMJA006L remarkably reduced the parasite

population by 61% at a dose of 12.5 µg/ml (Fig. 4.3).

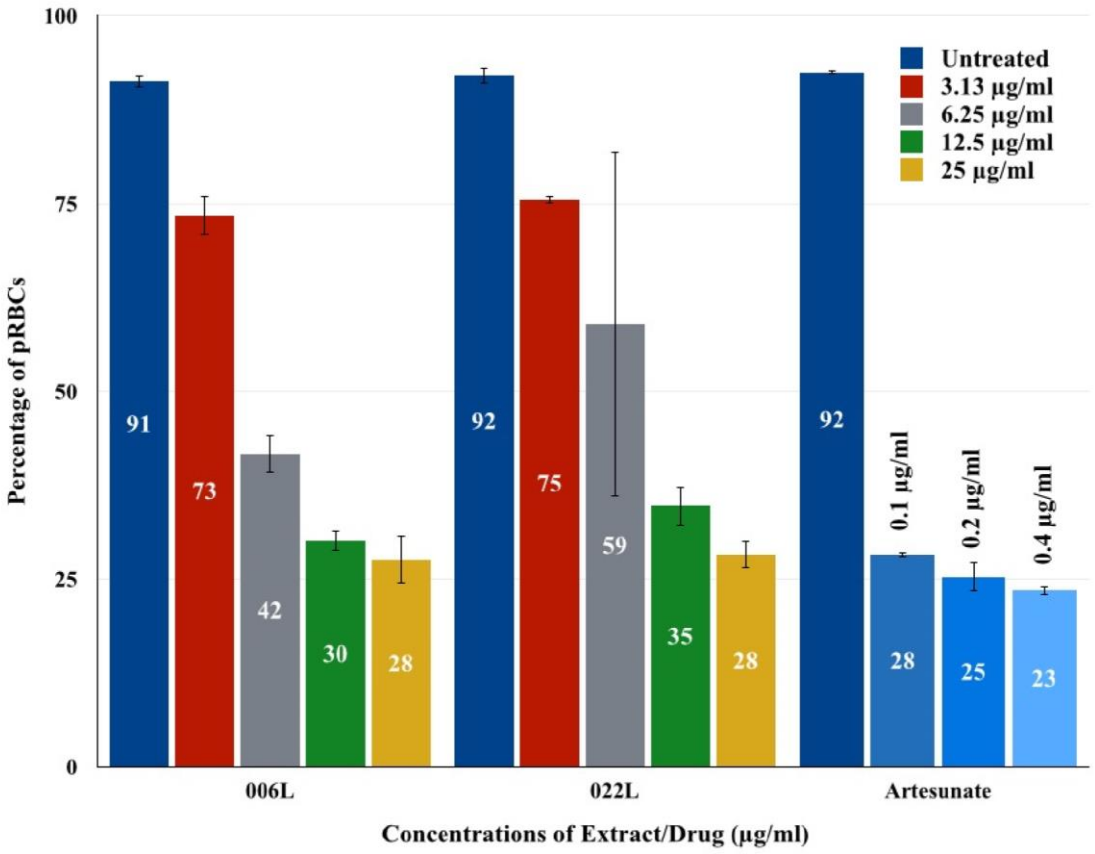

Figure 4.3: Growth inhibiting effect of active crude extracts and artesunate at different doses against *P. falciparum* (Appendix 1b indicates the dot graph of presence of RBCs and iRBCs due to extracts and artesunate)

4.3 Apoptosis inducing effect of active crude extracts

The active crude extracts of medicinal plants observed to have high anti-*P. falciparum* activity were investigated if they induced apoptosis in pRBCs. Signals of apoptosis induction were detected using changes in mitochondrial membrane potential. Parasitized RBCs were exposed to different doses of the active extracts for 24h, using artesunate as the reference drug. Parasitized RBCs with no drug or extract treatment was used as control and had negligible or no apoptotic pRBCs after 24 hours. As seen

in Figure 4.4, CMJA022L induced the most apoptosis in pRBCs, as compared to CMJA006L and artesunate. A lower concentration (6.25 µg/ml) of CMJA022L was required to reach 12.79% (95% CI = 4.09 to 21.49) apoptosis of pRBCs, compared to that required, 12.5 µg/ml, to reach 11.12% (95% CI = 10.26 to 11.98) apoptosis by CMJA006L (Figure 4.4). Even though lesser concentrations of artesunate is required to induce apoptosis in pRBCs compared to CMJA006L and CMJA022L, the effect of apoptosis by artesunate, CMJA006L and CMJA022L is comparable as there were no significant differences between them (artesunate : CMJA006L, p = 0.083, artesunate : CMJA022L; p = 0.076 and CMJA006L :

CMJA022L; p = 0.069).

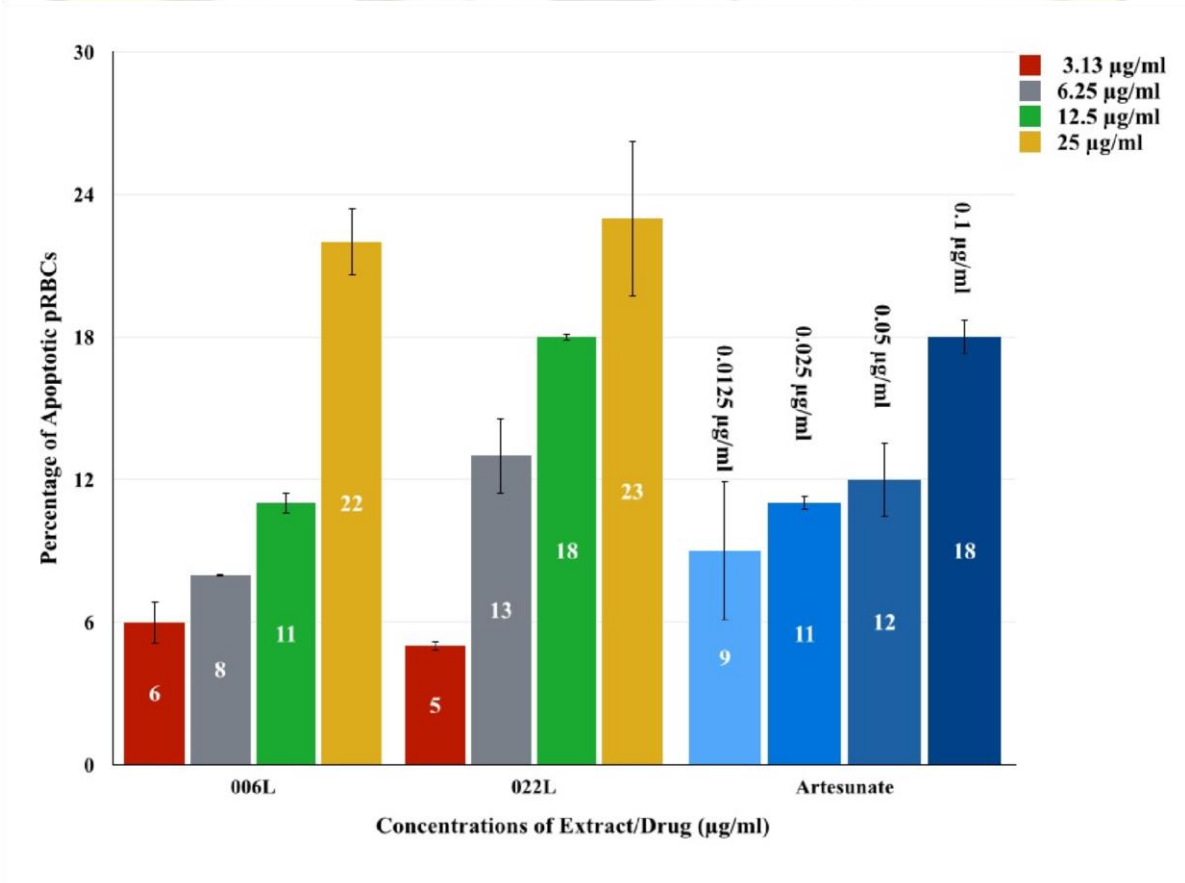

Figure 4.4: Active crude extracts and artesunate inducing apoptosis at different doses

among parasitized erythrocytes (Appendix 1c indicates the dot graph of apoptotic cells among pRBCs due to extracts and artesunate)

CHAPTER FIVE

5.0 DISCUSSION AND CONCLUSION

5.1 Discussion

The existence of chloroquine-resistant strains and the emergence of artemisininresistant strains of *P. falciparum* have stirred up the urgency to find alternative and efficient drug for malaria treatment. This study aimed to find plant extracts with anti*P. falciparum* activity by screening some Ghanaian medicinal plants. Out of the fifty 50% ethanolic plant extracts screened, two were found to be active extracts. These two active extract, coded as CMJA006L and CMJA022L were obtained from the leaves of *Mangifera indica* (Plate 1) and *Alchornea cordifolia* (Plate 2) respectively. *Mangifera indica*, commonly known as mango, comes from the anarcadiaceae family. Mango leaves are known for their anti-parasitic, antioxidant, antibacterial, and other pharmacological and medicinal properties (Shah et al., 2010). Mango leaves contain a chemical constituent, which is mainly responsible for the medicinal properties they possess. This chemical is known as mangiferin, a polyphenolic antioxidant and a glucosylxanthone (Wauthoz et al., 2007). The high anti-*P. falciparum* activity from our mango leaves extract (CMJA006L) is consistent with previous work that have shown that extracts obtained from the leaves, flowers and barks of mango have anti-malaria properties (Bidla et al, 2004; Ibrahim et al., 2012). Crude extract of CMJA006L was able to inhibit parasite growth by 50% at a concentration of 10 µg/ml, which is similar to a previous report by Arrey Tarkang et al., (2014). However, our extract, CMJA006SB, obtained from the stem bark of mango showed no anti-*P. falciparum* activity (Table 4.1), suggesting different parts of a plant

may have different activities towards *plasmodium* species or other microorganisms. On the other hand, *A. cordifolia*, also known as christmas bush, is a shrub commonly found in Africa where it is used for medicinal purposes. It belongs to the plant family, Euphorbiaceae. *Alchornea cordifolia* leaves have been investigated for their antidiarrhoeal (Agbor et al., 2004), antibacterial (Lamikanra et al., 1990) and antioxidant (Olaleye et al., 2007) properties. Although extracts from *A. cordifolia* have shown high activity in this study with an IC_{50} of 14 µg/ml, Adebayo & Krettli, (2011) study using different strains of *Plasmodium falciparum*, excluding 3D7, have reported lower IC_{50} values of 2.30 - 3.50 µg/ml. An active component have been isolated from *A. cordifolia* known as ellagic acid (Banzouzi et al., 2002). Ellagic acid is able to inhibit *P. falciparum* significantly with an IC_{50} range of 0.2-0.5 µM (Banzouzi et al., 2002). The isolation of active compounds from *Mangifera indica* and *Alchornea cordifolia*, respectively, increases the prospects of identifying active anti-*P. falciparum* drugs from other medicinal plants.

Plate 1: Body parts of *Mangifera indica* (Mango)

Plate 2: Body parts of *Alchornea cordifolia* (Christmas Bush) The use of a particular solvent in extracting components from plants can have an effect in the anti-plasmodial properties of extracts that are screened (Adebayo & Krettli, 2011; Banzouzi et al., 2002). Depending on the solvents used in extraction process, active components against parasites may or may not have been extracted from medicinal plants. This study showed that 88% of screened extracts, where 50% ethanol was used as solvent for extraction, were found to have no activity against chloroquine-susceptible strains (3D7) of *P. falciparum*. Perhaps more extracts would have shown some level of antimalarial activity if a different solvent were used. Arrey Tarkang and his colleagues reported in 2014 that, ethanol forms of plants extracts had higher anti-*P. falciparum* activity as compared to their aqueous forms. Accordingly, higher activity may have been observed in extracts that have shown moderate or low activity against malaria parasites. Moreover, crude forms of plant extracts were used in this investigation, which contained different fractions and constituents. These

constituents may have caused some synergistic, additive and protagonistic effects which could have influenced their plant extract's activity against plasmodial species (Dabo et al., 2013). Active components mainly responsible for inhibiting the parasites' growth cannot be known when dealing with crude forms of plant extracts. Hence further isolation and purification steps will help identify active components in CMJA006L and CMJA022L that are mainly responsible for inhibiting growth in *P. falciparum*.

Usually, during life processes of cells, cellular deaths occur which involve apoptosis, necrosis and autophagy (Elmore, 2007). Apoptosis can occur during normal conditions where cellular processes have come to a halt due to maturity or abnormality of an infection or cancer. Necrosis, on the other hand, occur when cells undergo abrupt death. Apoptosis is preferred, because the steps and changes involved in eliminating dead or infected cells are programmed, soothing the immune responses and other cellular modifications. Necrosis tends to shock the immune system of hosts and consequently produce spikes in the levels of immune responses which could be detrimental to the health of individuals (Ameisen, 2002).

In drug development, all these information are necessary to know the particular cellular pathways that are employed by the parasite for survival. However, much information have not been provided for the various extracts that have been screened to be highly active against *P. falciparum*. As such, active anti-*P. falciparum* extracts observed to highly inhibit growth in malaria parasites were investigated for their apoptotic inducing properties. As it can be seen in Figure 4.4, active extracts were able to induce some level of apoptosis by depolarising the mitochondria. The effect of induced apoptosis was dose-dependent of active extracts. It took as low as 3.13 µg/ml

of both CMJA006L and CMJA022L to induce 6% and 5% of apoptosis in pRBCs, respectively. Notably, CMJA022L induced more apoptosis as compared to CMJA006L.

In this study, only changes in the mitochondrion membrane potential was used to determine apoptosis. Loss of mitochondria function plays a vital role in programmed cell death and it is usually associated with early stages of apoptosis (Kroemer et al., 1998). Loss of mitochondria membrane potential triggers a sequence of enzyme related events that leads to programmed cell death (Pradelli et al., 2010). It has to be mentioned that there are other markers and cellular pathways that can be used to indicate apoptosis. Other apoptotic signals that occur in the cascade of events include DNA fragmentation, chromatin condensation, DNA laddering and phosphatidylserine externalisation (Engelbrecht & Coetzer, 2013). If these other markers were employed in addition to this study, perhaps more pRBCs would be observed to be apoptotic in the presence of these active extracts. Also, it could have been determined if the apoptosis-inducing effect of active extracts on *P. falciparum* is progressive and ends with DNA fragmentation which is the last step of apoptosis.

5.2 Recommendation

The use of different solvents in the extraction process for crude forms of medicinal plants tends to influence the anti-plasmodial activity of extracts. Solvents such as water, ethyl ether, methanol, hexane and other solutions should be employed to identify the best solvent that best brings out the active anti-*P. falciparum* constituents of medicinal plants. Moreover, further fractionations, purification and isolation of pure forms should be performed to determine active components or compounds directly responsible for inhibiting malaria parasite growth. More apoptotic markers should be

employed as well to determine if there are any DNA fragmentation and membrane disintegration occurring due to the presence of active anti-*P. falciparum* compounds. Active extracts or compounds should also be investigated against field isolates or resistant-strains of *P. falciparum.* Also determination of which stage of the parasite; whether trophozoites, schizonts or gametocytes, that are mostly affected by active extracts should be made. *In vivo* studies should also be performed to investigate the performance of active extracts in some animal models that are associated with malaria.

5.2 Conclusion

Two active extracts from *Mangifera indica* and *Alchornea cordifolia* were found to have high anti-*P. falciparum* activity with IC_{50} values of 10 µg/ml and 14 µg/ml, respectively. They are also known to induce apoptosis. These two active extracts are potential candidate that can be investigated further in obtaining an alternative and effective drug to treat malaria

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APPENDICES

Appendix 1

Appendix 1a: IC_{50} values of screened crude extracts

Extract Code	IC_{50} (µg/ml)	Extract Code	IC_{50} (µg/ml)
CMJA001L	590	CMJA015L	46.66
CMJA001R	227	CMJA018F	713
CMJA001SB	297	CMJA018L	132
CMJA002L	299	CMJA019L	133
CMJA002R	128	CMJA019SB	188
CMJA003L	229	CMJA020R	135
CMJA003R	221	CMJA020SB	409
CMJA003SB	185	CMJA022L	**17.44**
CMJA004L	183	CMJA023L	535
CMJA004SB	545	CMJA023R	432
CMJA005L	199	CMJA024L	472
CMJA005SB	223	CMJA024SB	120
CMJA006L	**14.25**	CMJA025SB	160
CMJA006SB	248	CMJA026L	210
CMJA007SB	327	CMJA026SB	175
CMJA008L	593	CMJA027R	291
CMJA008R	153	CMJA028L	195
CMJA008SB	126	CMJA028SB	95.19
CMJA009L	454	CMJA029L	50.53
CMJA010L	122	CMJA029SB	186
CMJA011SB	34.83	CMJA030L	318
CMJA012L	129	CMJA030R	509

CMJA013L	233	CMJA030SB	307
CMJA013S	175	CMJA031SB	361
CMJA013WP	233	CMJA032L	730

Appendix 1b: Analysis dot plots of growth trend of *Plasmodium falciparum*

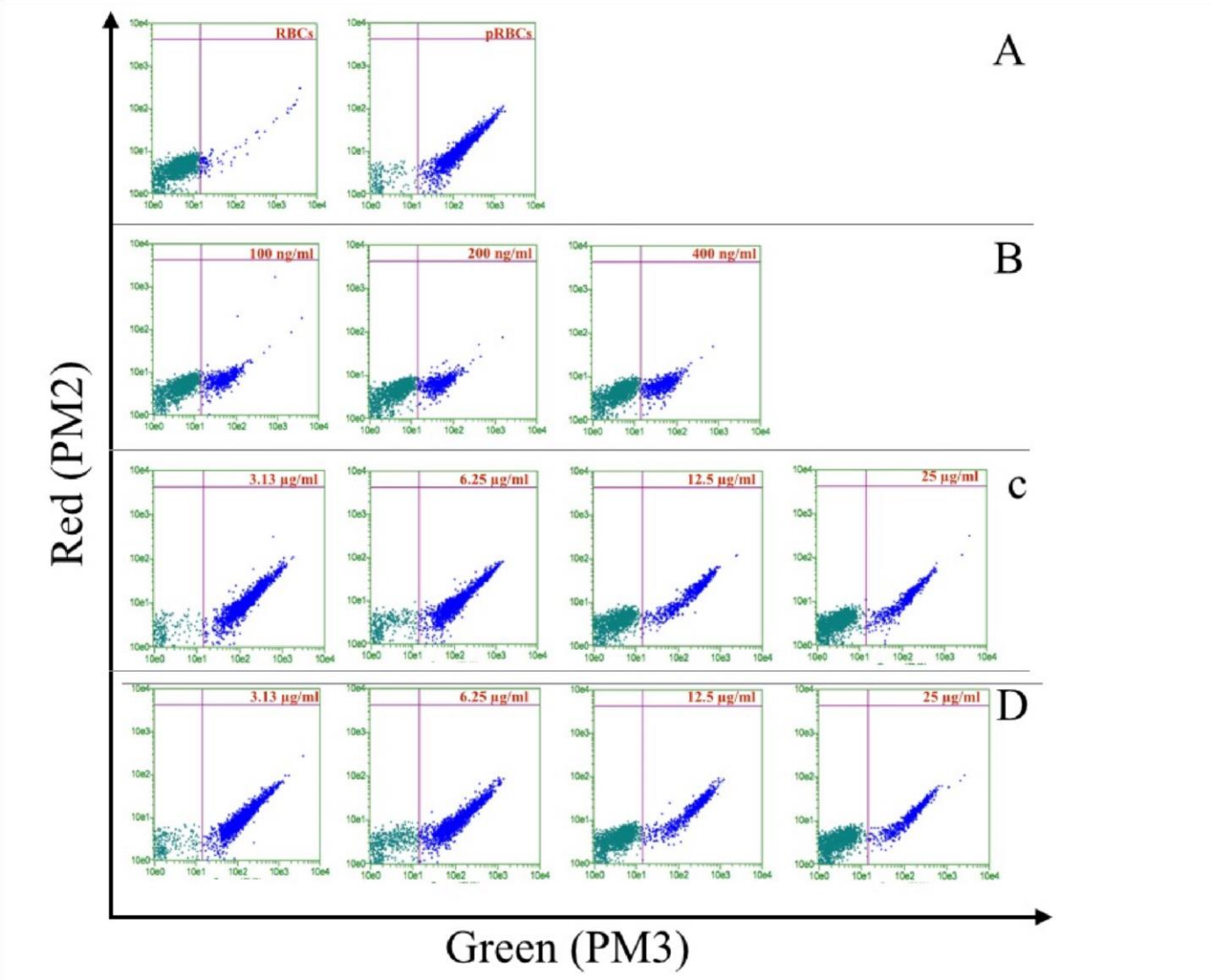

Figure 6: Growth inhibition trend of *Plasmodium falciparum* exposed to different doses of active extracts and artesunate. Controls (A) used for inhibition growth assays. Artesunate (B), 006L (C) and 022L (D) were used to inhibit the growth of malaria parasites.

Appendix 1c: Analysis dot plots of depolarized (apoptotic) cells of *Plasmodium falciparum*

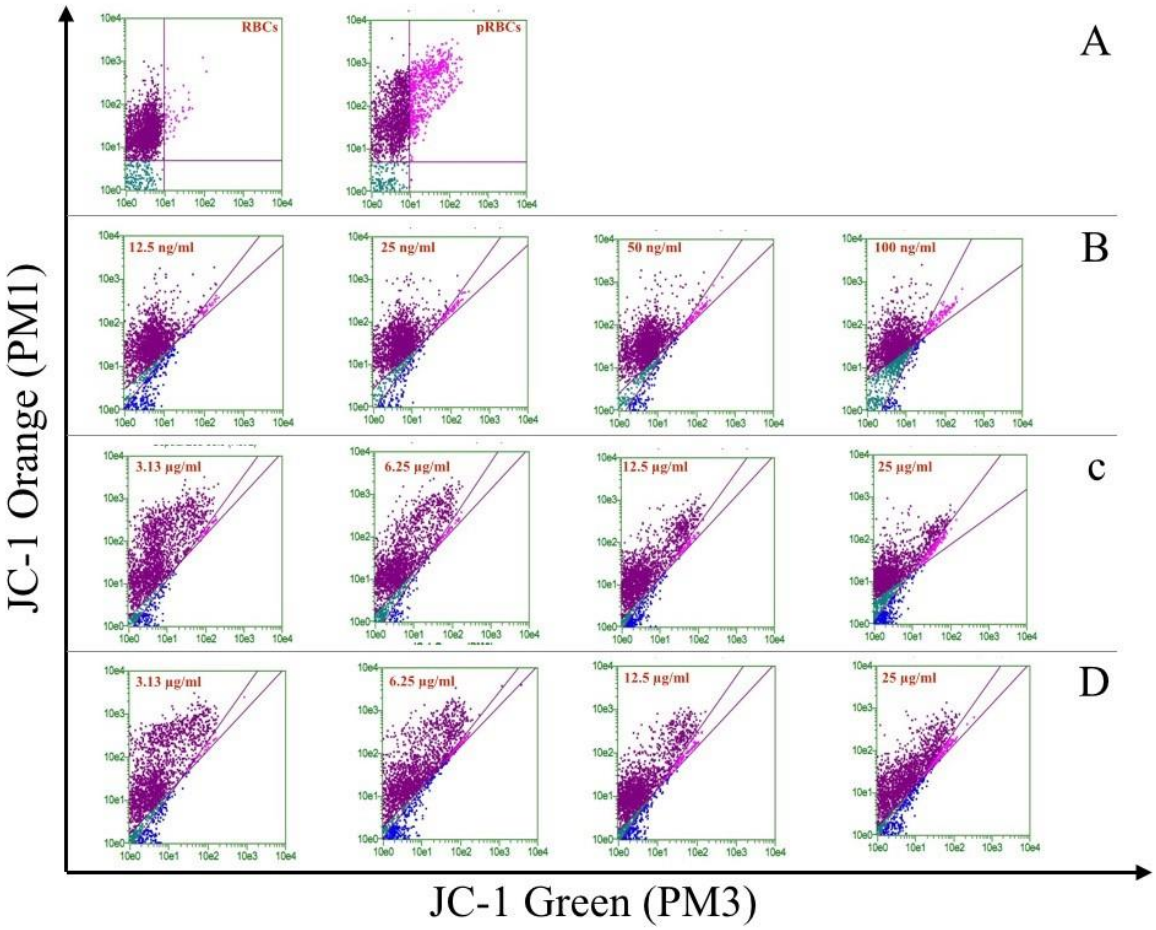

Figure 7: Apoptosis effect of artesunate (B), 006L (C) and 022L (D) on *Plasmodium falciparum* at different doses after 24h. [A]: Controls used for Guava MitoPotential assays, RBCs (left) and pRBCs (right).

Appendix 2: Ethical clearance letter from NMIMR-IRB

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My Ref. No: DF.22
Your Ref. No:

2nd July, 2014

ETHICAL CLEARANCE

FEDERALWIDE ASSURANCE FWA 00001824 **IRB 00001276**

NMIMR-IRB CPN 107/13-14 **IORG 0000908**

On 2nd July 2014, the Noguchi Memorial Institute for Medical Research (NMIMR) Institutional Review Board (IRB) at a full board meeting reviewed and approved your protocol titled:

TITLE OF PROTOCOL : **"*In vitro* Assesment of selected Ghanaian medicinal plants' extracts' Activity against *Plasmodium falciparum*"**

PRINCIPAL INVESTIGATOR : **Jeffrey Agyapong, BSc**

Please note that a final review report must be submitted to the Board at the completion of the study. Your research records may be audited at any time during or after the implementation.

Any modification of this research project must be submitted to the IRB for review and approval prior to implementation.

Please report all serious adverse events related to this study to NMIMR-IRB within seven days verbally and fourteen days in writing.

This certificate is valid till 1st July, 2015. You are to submit annual reports for continuing review.

Signature of Chair: .....
Mrs. Chris Dadzie
(NMIMR – IRB, Chair)

cc: Professor Kwadwo Koram
Director, Noguchi Memorial Institute
for Medical Research, University of Ghana, Legon

Appendix 3: Consent form

NMIMR-IRB CONSENT FORM

Title: "*In vitro* ASSESSMENT OF SELECTED GHANAIAN MEDICINAL PLANTS' EXTRACTS' ACTIVITY AGAINST *Plasmodium falciparum*"

Principal Investigator: Jeffrey Agyapong
Address: Parasitology Department
NMIMR, P. O. Box LG 581
Email: jagyapong@noguchi.ug.edu.gh
Tel: 0242917673

General Information about Research

One of the most commonest and important disease in Ghana is malaria. As a way to control the transmission of the disease is the use of anti-malaria drugs for treatment. However, malaria parasites, particularly *plasmodium falciparum* have grown or continue to grow resistant to the two most used drugs, chloroquine and artemisinin. This calls for continuous search for new and alternative anti-malaria medicines. The present study is purely laboratory based, which will involve the screening of plant extracts for anti-*P. falciparum* activities and therefore will require the growing of the parasite in the laboratory. To be able to undertake this investigation will also require fresh red blood cells (RBCs) from healthy volunteers which the parasites require to be able to grow in the laboratory. **To qualify to be a volunteer, one must have a blood group O and should also be sickling negative.** A 20 ml (4 teaspoons) blood sample will be taken intravenously every 6 months over a two year period. All participants will receive oral and written information regarding the aim of the project and the procedures involved, and that participation will require the signature of the participants on a standard approved informed consent. Only qualified clinical staff will take samples from volunteers.

Possible Risks and Discomforts: There will be no foreseeable harm caused or risk in participating in this study. **Only experienced clinical staff will draw the blood samples using adequate sterile, disposable syringes and needles.**

Possible Benefits: There is no direct benefit to you except that you will be given **GHc 20.00 per visit** for your transportation/snack to and from the NMIMR where the blood will be taken.

Confidentiality: You are assured that your records will be kept in strict confidentiality. **All blood samples taken from you will be used in the laboratories at Noguchi Memorial Institute for Medical Research at Legon for growing malaria parasites only. These samples will be identified using a unique number assigned to you. You will not be identified by name in any publication of the results**

of these tests. You may take away your permission to use the samples taken from you for growing these parasites at any time by writing to the study coordinator. If you do this, no new data that identifies you will be gathered after that date.

Compensation: You will not be compensated for your time except the **GHc 20.00 per visit** that will be given for transportation/snack at all the 4 time points that the samples will be collected.

Voluntary Participation and Right to Leave the Research

Participation in the proposed study will be entirely voluntary and you can withdraw at any time.

Contacts for Additional Information

In case you want more information about the research, you can contact Dr. Irene Ayi on telephone number 0275303819 and in case of research related injury; contact Dr. Michael Ofori at the Immunology Department, NMIMR, Legon.

Your rights as a Participant

This research has been reviewed and approved by the Institutional Review Board of Noguchi Memorial Institute for Medical Research (NMIMR-IRB). If you have any questions about your rights as a research participant you can contact the IRB Office between the hours of 8am-5pm through the landline 0302916438 or email addresses: nirb@noguchi.ug.edu.gh or HBaidoo@noguchi.ug.edu.gh.

VOLUNTEER AGREEMENT

The above document describing the benefits, risks and procedures for the research title:"*In vitro* ASSESSMENT OF SELECTED GHANAIAN MEDICINAL PLANTS' EXTRACTS' ACTIVITY AGAINST *Plasmodium falciparum"* has been read and explained to me. I have been given an opportunity to have any questions about the research answered to my satisfaction. I agree to participate as a volunteer.

Date Name and Signature or mark of volunteer

If volunteers cannot read the form themselves, a witness must sign here:

I was present while the benefits, risks and procedures were read to the volunteer. All questions were answered and the volunteer has agreed to take part in the research.

Date Name and signature of witness

I certify that the nature and purpose, the potential benefits, and possible risks associated with participating in this research have been explained to the above individual.

Date Name Signature of Person Who Obtained Consent