

**THE EFFECTS OF LOCAL ANTIMALARIAL DRUGS ON
PLASMODIUM FALCIPARUM GAMETOCYTES**

BY

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DECLARATION

This thesis is the result of research work undertaken by Courage Kakaney under the supervision of the following names listed below and all references stated have dully been acknowledged.

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DEDICATION

I dedicate this thesis to the 2013/2014 academic year form 3 biology students of Adidome Senior High School, who had faith in me to continue as their elective biology teacher despite my postgraduate engagements. Their patience, joy and trust during the inconvenient meetings I had to arrange to prepare them for their final exams, gave me a lot of encouragement.

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LIST OF ABBREVIATIONS

WHO	World Health Organisation
G6PD	Glucose-6-phosphate dehydrogenase
CSIR	Center for Scientific and Industrial Research
ACT	Artemisinin based combination therapy
OPD	Outpatients' department
GMP	Global Malaria Programme
IC ₅₀	Inhibitory concentration of 50%
IC ₁₀	Inhibitory concentration at 10%
HIV/AIDS	Human Immuno-Deficiency Virus / Acquired Immune Deficiency Syndrome
GDP	Gross Domestic Product
RBM	Roll Back Malaria
MMV	Medicines for Malaria Venture
MVI	Malaria Vaccine Initiative
RBC	Red Blood Cell
RDT	Rapid Diagnostic Test
pLDH	Parasite Lactate Dehydrogenase
HRP-II	Histidine Rich Protein II
Ht	Hematocrit
AL	Arthemether-lumefantrine
AS	Artesunate
PQ	Primaquine
AQ	Amodiaquine
MQ	Mefloquine
SP	Sulphadoxine-pyrimethamine
DHA	Dihydroartemisinin

PPQ	piperaquine
CQ	Chloroquine
Pgh1	P- glycoprotein homologue 1
Pfmdr1	<i>Plasmodium falciparum</i> multidrug resistance protein 1
Pfcr1	<i>Plasmodium falciparum</i> chloroquine Resistance transporter
pABA	p-aminobenzoic acid
DHPS	dihydropteroate synthase
DHFR	dihydrofolate reductase
SERCA	sarcoplasmic-endoplasmic reticulum calcium ATPase
RNA	Ribonucleic Acid
TB	Taabea Herbal Mixture
MS	Masada Mixture
HB	Herbaquin
TF	Top Fever Syrup
KG	Kingdome Mixture
YF	Yafo Fever Mixture
CM	Class Malakare
AD	Adutwumwa Malamix
AN	New Angel Herbal Mixture
RT	Rooter Mixture
EIR	Entomological inoculation rate
ELISA	Enzyme linked immunosorbent assay
DDT	Dichlorodiphenyltrichloroethane
TB	Tuberculosis
VIMT	Vaccines that interrupt malara transmission
FDA	Foods and Drugs Authority
BSC	Biosafety cabinet

CPM	Complete parasite media
PWM	Parasite wash media
NAG	N-Acetyl Glucosamine
NHS	Normal Human Serum
Rpm	Revolutions per minute
Rcf	Relative centrifugal force
DMSO	Dimethyl sulfoxide
IRB	Institutional review board
NMIMR	Noguchi Memorial Institute for Medical Research

ABSTRACT

The gametocytocidal effect of antimalarial drugs has become important in malaria research owing to the global quest to eradicate the disease. In Ghana and most parts of Africa, people still depend on herbal medications despite advances in western medicine. However, these drugs lack the necessary scientific assessment. The IC_{50} s of 10 known herbal antimalarial drugs on the Ghanaian market were determined. The development of sexual stages of the *Plasmodium falciparum* was monitored and the growth inhibitory effect of the herbal drugs on gametocytes was also assessed *in vitro*. All tested herbal drugs showed either high activity ($IC_{50} < 10 \mu\text{g/ml}$) or moderate activity ($10 \mu\text{g/ml} < IC_{50} < 50 \mu\text{g/ml}$) on asexual stages. Top fever showed the lowest IC_{50} value of $0.06 \pm 0.028 \mu\text{g/ml}$, Kingdom herbal: $11.07 \pm 1.010 \mu\text{g/ml}$, Rooter mixture: $2.80 \pm 0.363 \mu\text{g/ml}$, Class malakare: $0.35 \pm 0.135 \mu\text{g/ml}$, Masada: $0.25 \pm 0.035 \mu\text{g/ml}$, Angel herbal: $1.31 \pm 0.160 \mu\text{g/ml}$, Yafo mixture: $0.53 \pm 0.065 \mu\text{g/ml}$, Herbaquin: $5.86 \pm 0.540 \mu\text{g/ml}$, Adutwumwa malamix: $7.49 \pm 0.248 \mu\text{g/ml}$ and Taabea showed the highest IC_{50} value of $11.58 \pm 2.484 \mu\text{g/ml}$. On day 14 parasites treated with IC_{10} of Angel herbal mixture had the highest late stage gametocytemia of 8.99% and Rooter mixture had the lowest late stage gametocytemia of 0.58%. Five herbal drugs showed $> 50\%$ growth inhibition of early stage gametocytes at $7 \mu\text{g/ml}$. Only Masada herbal gave $> 50\%$ growth inhibition of late stage gametocytes at $7 \mu\text{g/ml}$. Though all the herbal drugs were potent antimalarials, only Masada and Yafo fever showed strong gametocytocidal effects and can therefore be recommended as an alternative to orthodox antimalarial treatment.

CHAPTER ONE

GENERAL INTRODUCTION

1.1 Background to the study

Malaria, a disease caused by *Plasmodium* spp. remains a major health problem in sub-Saharan Africa (World Health Organization, 2012a). It is responsible for an estimated 207 million cases worldwide and 627,000 deaths in 2012 with 80% of the cases and 90% of the deaths occurring in Africa (World Health Organization, 2014). The life cycle of the *Plasmodium* sp. shows the gametocyte stage as the infectious stage to the *Anopheles* sp., which as the vector, it harbours and transforms the parasite into sporozoites, the infective stage to humans. Since the pathophysiology of malaria is mostly due to the asexual stages of the *Plasmodium* parasite within the human host (Miller *et al.*, 2002), most antimalarial drugs target the asexual stages of the parasite, which is responsible for the clinical symptoms of malaria. All currently available antimalarial compounds were discovered on the basis of their activity against the asexually reproducing red blood cell stages of the *Plasmodium* spp. (Buchholz *et al.*, 2011). However, for the transmission of parasites to be possible, the gametocyte stage of the parasite must be transmitted from the human host to the *Anopheles* mosquito. Compared to the asexual parasites, gametocytes are less sensitive to all the commonly used antimalarial drugs except for primaquine (Benoit-Vical *et al.*, 2007). Primaquine is currently the only licensed antimalarial drug that is effective against late stage *Plasmodium falciparum* gametocytes but has certain drawbacks such as its tendency to cause acute haemolysis in individuals with glucose-6-phosphate dehydrogenase (G6PD) deficiency (Peatey *et al.*, 2012).

Due to the emergence of resistance against orthodox drugs coupled with unsuccessful attempts to develop vaccines based on asexual blood stage antigens makes it important to focus on gametocyte stages of the *Plasmodium* parasite to prevent transmission instead. The Bill and Melinda Gates Foundation in 2007 spearheaded the campaign for a global eradication of malaria, which will be possible through the elimination of malaria transmission (Buchholz *et al.*, 2011). The elimination and a possible complete eradication of malaria can be achieved if the transmission cycle of the *Plasmodium* parasite is interrupted.

The earlier mentioned problems with orthodox medication coupled with the high cost of western medical care as well as socio-cultural practices make more than 80% of populations in Africa prefer the use of local medicine (World Health Organization *et al.*, 2009). A major problem about traditional medicine in Ghana is the lack of test on their efficacy and toxicity. That notwithstanding, the Council for Scientific and Industrial Research (CSIR) and Ghana Standards Authority conduct tests into herbal drugs that are licensed and sold on the Ghanaian market. Antimalarial herbal drugs used in Ghana are usually concoctions with one or more active plant extracts. Even though some individual active ingredients like *Azadirachta indica* have shown reduction in asexual parasitemia and gametocytes of *Plasmodium falciparum* (Udeinya *et al.*, 2008), it is not known if a combination of these active ingredients could be synergetic. There is also little or no information about the concentration of the active ingredients and their mode of action. For now the efficacy of these local drugs are based on the adverts and the testimony of users. With the new worldwide agenda on the total eradication of malaria, local antimalarial drugs must be tested to see if they actually block the transmission of parasites.

A major challenge in testing the gametocytocidal activity of compounds is the difficulty in developing an effective assay (Lelièvre *et al.*, 2012). The gold standard which involves counting

gametocytes under the microscope is labour intensive and time consuming (Peatey *et al.*, 2012). To add further, *Plasmodium falciparum* mature gametocytes are hard to get in large quantities in *in vitro* cultures. This has resulted in sparse information on *in vitro* studies since most of the available information on gametocytocidal activity of existing antimalarials was obtained from field-based clinical trials (Butcher, 1997). The awakened interest in parasite transmission blocking has made it important to develop new reliable and effective methods in determining gametocytotoxicity.

It therefore, informs on which of these local antimalarial drugs should be endorsed as a herbal alternative in championing the course for malaria eradication in Ghana. It also provides insight into prospects of developing new antimalarial drugs from herbal sources, citing the problems of isolated drug resistant of Artemisinin and its derivatives, the linchpin of current drug of choice (Phyo *et al.*, 2012).

1.2 Rationale of the study

In Ghana, malaria still accounts for 32.5% of outpatients' department (OPD) cases despite efforts to curb its incidence (Sesay and Esena, 2013). In accordance with the World Health Organization (WHO) regulations, the first drug of choice for malaria is artemisinin-based combination therapy (ACT) (World Health Organization, 2012a). Artemisinin-based combination therapy has been known to induce some gametocytocidal effects (Broek *et al.*, 2005). The use of ACTs coupled with vector control measures has dramatically reduced the morbidity and mortality of malaria worldwide (Chong *et al.*, 2013). However resistance to ACT has been discovered in Cambodia and Thailand (Dondorp *et al.*, 2010), which is feared to hamper the progress of malaria eradication by 2015. The resistance to ACTs will be a dangerous consequence to the global

malaria control, because there might be no suitable drugs for replacements (World Health Organization, 2014). Artemisinin being a drug developed from the plant *Artemisia annua* (Miller and Su, 2011), like its predecessor quinine, gives the hope that other herbal compounds could also be developed into very potent antimalarials in the future. Studies on local antimalarial drugs have proven to be effective against the symptoms of malaria (Asase *et al.*, 2010). However since gametocytes do not elicit clinical symptoms, the gametocytocidal effects of these local antimalarial drugs cannot be assessed by way of consumption by patients. It may be possible that some of these drugs promote gametocytogenesis, hence inhibiting the symptoms and enhancing malaria transmission. Based on the high patronage of local antimalarial drugs by majority of Ghanaians (Tabi *et al.*, 2006), identifying the gametocytotoxicity of these drugs would help inform on the drug of choice for those who seek herbal treatment. This would in a long way help eradicate malaria in Ghana.

1.2.1 Aim of study

The main aim of the study was to determine the gametocytocidal effects of local antimalarial drugs. The specific objectives are:

1. To calculate IC_{50} for selected local antimalarial drugs
2. To identify local drugs that promote gametocyte production
3. To identify local drugs that inhibited the growth of gametocytes

CHAPTER TWO

LITERATURE REVIEW

2.1. Malaria

Malaria is the most important parasitic disease of human beings. In 2012, there were an estimated 207 million cases with 627 000 deaths (World Health Organization, 2014). More than 85% of malaria cases and 90% of malaria deaths occur in sub-Saharan Africa, mainly in young children (White *et al.*, 2014). In Africa, a child dies from malaria every 30 seconds, which translates to 2880 children per day (Finkel, 2007). Malaria, together with Human Immunodeficiency Virus / Acquired Immuned Deficiency Syndrome (HIV/AIDS) and tuberculosis, is one of the world's most vital public health challenge compromising developing countries and it accounts for up to an overwhelming 2.7 million deaths per year (Bremam, 2001). Malaria is endemic in 108 countries inhabited by about 3 billion (approximately 40%) people. As such, the disease is largely responsible for the poor economic growth of these areas, which further contributes to more cases of malaria (Gardner *et al.*, 2002).

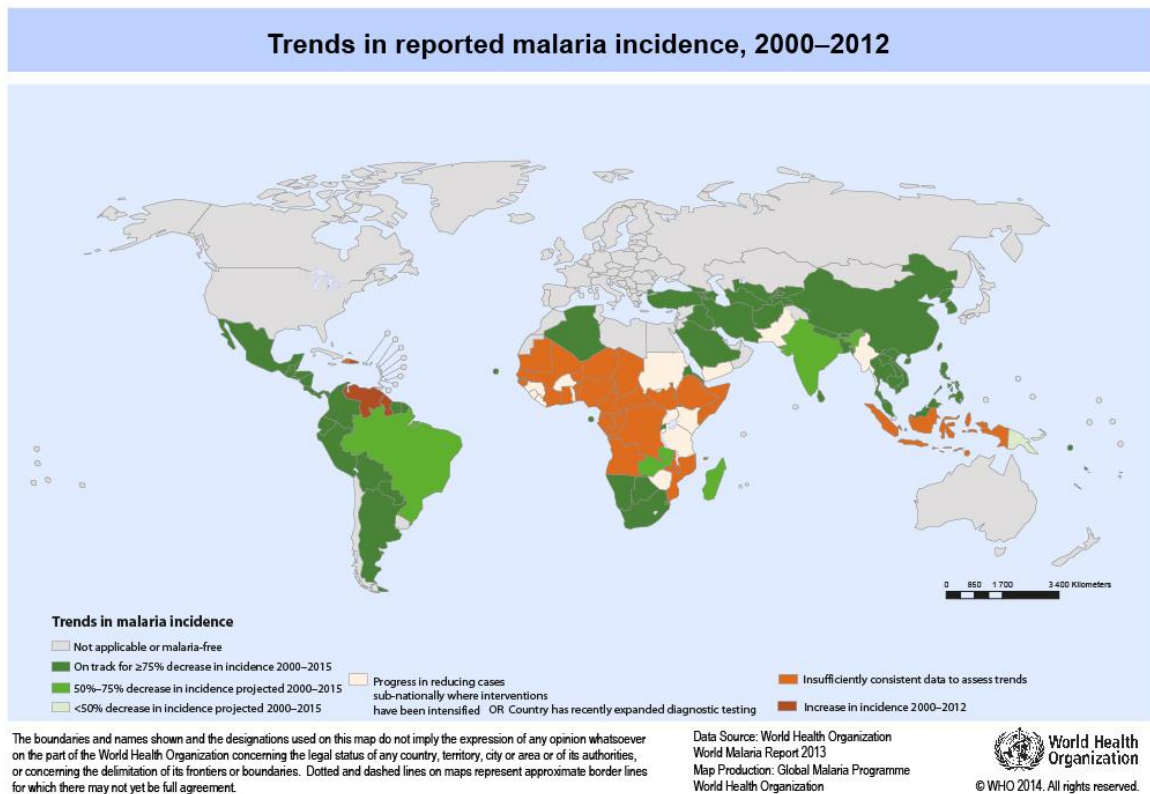


Plate 1: Map showing trends in malaria incidence (World Health Organization, 2014)

Malaria is commonly associated with poverty and may also be a major hindrance to economic development (Teklehaimanot and Mejia, 2008). In countries with endemic malaria, the annual economic growth rates over a 25-year period were 1.5% lower than in other countries. This implies that the cumulative effect of the lower annual economic output in a malaria-endemic country was a 50% reduction in the per capita GDP compared to a non-malaria endemic country (Gallup and Sachs, 2001).

Malaria is a complicated disease and its spread may be attributed to a variety of factors such as ecological and socio-economic conditions, displacement of large populations, agricultural malpractices that cause increase in vector breeding sites, parasite resistance to antimalarial drugs and vector resistance to insecticides. In 1998, The World Health Organization (WHO)

established a global partnership called Roll Back Malaria (RBM) in an attempt to half the world's malaria frequency by 2010 (Nabarro, 1999). Apart from RBM, a number of promising antimalarial drug and vaccine discovery projects have also been launched. This includes the Medicines for Malaria Venture (MMV) funded by a number of organizations including The Bill and Melinda Gates Foundation, for the development of novel antimalarial drugs. The later also contributed more than 300 million US dollars to the Malaria Vaccine Initiative (MVI) (Gross, 2003).

2.2. Causative agent and vectors

Five species of *Plasmodium* are known to infect and cause clinical cases in human beings. They are *Plasmodium falciparum*, *Plasmodium malariae*, *Plasmodium vivax*, *Plasmodium ovale* and *Plasmodium knowlesi*. The most important of these protozoans are *Plasmodium falciparum* and *P. vivax* because they cause majority of deaths. *Plasmodium falciparum* which is the most lethal of the plasmodium species happens to be the most common human malaria parasite in sub-Saharan Africa (Greenwood, 2009). *Plasmodium ovale* and *P. malariae* cause milder forms of malaria and are less fatal (Mueller *et al.*, 2007). *Plasmodium knowlesi* is a zoonotic species prevalent in Southeast Asia, causing malaria in macaques but can also cause severe malaria in humans (Collins, 2012). Effective transmission of these parasites depends on the density, longevity, biting habits, and efficiency of the anopheline vector. Out of the more than 400 anopheline species, only 25 are good transmitters of malaria (Sinka *et al.*, 2010). The most effective vector is the *Anopheles gambiae* complex found in Africa (Bass *et al.*, 2007). Having the most lethal plasmodium species and most effective anopheline vector, puts sub-Saharan Africa in constant risk of high malaria mortality and morbidity.

2.3. Biology and life cycle of the parasite

The *Plasmodium* is an intracellular Apicomplexan parasite. Parasites of the Apicomplexan phylum are characterized by the presence of a special apical complex that is involved in host-cell invasion; the apical complex is made up of the microneme, dense granules and rhoptries (Cowman and Crabb, 2006).

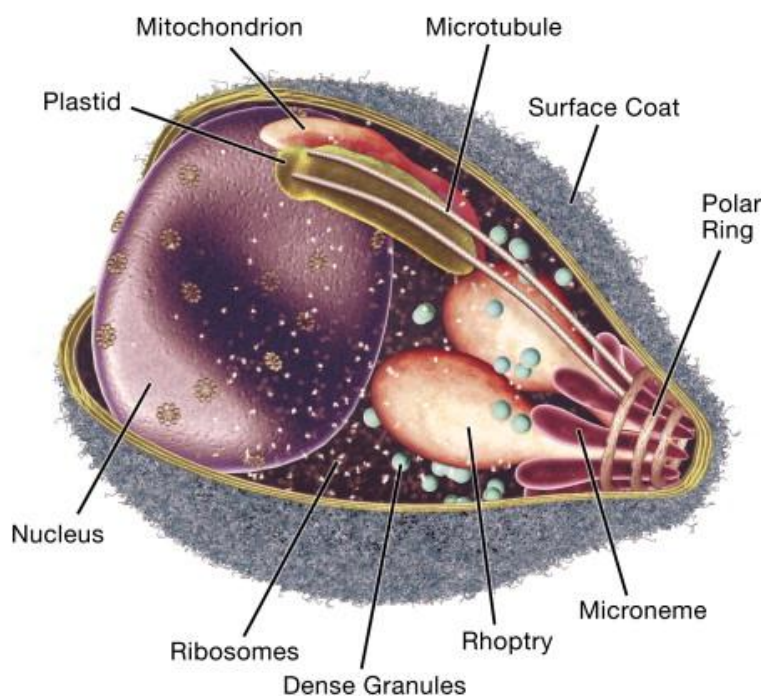


Plate 2: *Plasmodium falciparum* merozoite showing apical complex and other cellular organelles (Cowman and Crabb, 2006).

2.3.1. Life cycle of the vector phase (the definitive host)

The life cycle begins with a blood meal by the female anopheline mosquito. The female mosquito takes regular blood meals to help in the development of its eggs (Briegel, 1985). The peripheral blood taken by the mosquitoes contains gametocytes of plasmodium which end up in the mid-gut of the mosquito. The male and the female gametocytes move from the red blood

cells and differentiate to microgametes (male) and macrogametes (female). Fertilization takes place when a microgamete penetrates a macrogamete to form a zygote (the only diploid stage during the life cycle of the *Plasmodium* parasite). The zygote transforms into an ookinete, an elongated mobile cell inside the mosquito mid-gut, about 18 hours after blood meal. The ookinete penetrates the gut wall and is attached to the outer aspect of the mosquito gut, then the ookinete differentiate to a spherical oocyst. In the oocyst, multiplication takes place (i.e. meiosis) and the parasite becomes haploid again. This stage of multiplication is called sporogony, where thousands of sporozoites are produced. When the oocyst is mature, it ruptures to release sporozoites which migrate and accumulate in the salivary glands and complete maturation. The average duration of the sporogony is about 2 weeks with a range of 1-5 weeks, depending on species and temperature. Inoculation of the sporozoites into a new human host continues the life cycle.

2.3.2. Life cycle of the human phase (intermediate host)

Sporozoites enter blood with mosquito saliva; which serves as an anticoagulant during blood meal (Stark and James, 1996). Usually an infected mosquito injects on average 10-100 sporozoites per blood meal. The sporozoites take few minutes to an hour to reach the liver and invade liver cells (hepatocytes). In the hepatocytes, the sporozoites transform to trophozoites which grow and divide by schizogony, to produce thousands of infective merozoites, about 10,000 for *P. vivax* and 30,000 for *P. falciparum*. The duration of hepatic schizogony is about 5 days for *P. falciparum*, but 13 days for *P. malariae* (Vaughan *et al.*, 2008). Clinical symptoms of malaria are not apparent during liver stage maturation. In *P. vivax* and *P. ovale*, sporozoites may become dormant in the hepatocytes, as hypnozoites, which reactivate at variable intervals (most

often 3-35 weeks) to cause relapses of malaria (Krotoski, 1989). The hepatic schizonts rupture and merozoites are released into the blood stream and rapidly invade RBCs.

In the red blood cell, the merozoites transform into trophozoites, feeding on the content of the red blood cell and form food vacuoles where haemoglobin is digested. After a period of growth, a mature trophozoite starts dividing (erythrocytic schizogony) and produces a new generation of merozoites. A mature schizont of *P. falciparum* contains approximately 16 merozoites. The erythrocytic cycle lasts for about 48 hours for *P. falciparum*, *P. vivax* and *P. ovale*, but 72 hours for *P. malaria* and only 24 hours for *P. knowlesi*. When this stage is reached, the schizonts rupture releasing merozoites and other parasite byproducts (pyrogenic molecules) into the bloodstream. The pyrogenic molecules are responsible for sequence of events leading to fever and other clinical symptoms and signs of malaria. Immediately after release, the merozoites invade uninfected RBCs and repeat the cycle of erythrocytic schizogony. Some merozoites differentiate into sexual stages known as gametocytes (gametogony)- as early as 4 days for *P. vivax* and 10 days for *P. falciparum*. Adherence of infected RBCs to endothelial cells in various organs is called sequestration, which is characteristic of *P. falciparum* infections. Sequestration plays an important role in pathophysiology of severe malaria particularly cerebral malaria and malaria during pregnancy (Miller *et al.*, 1994).

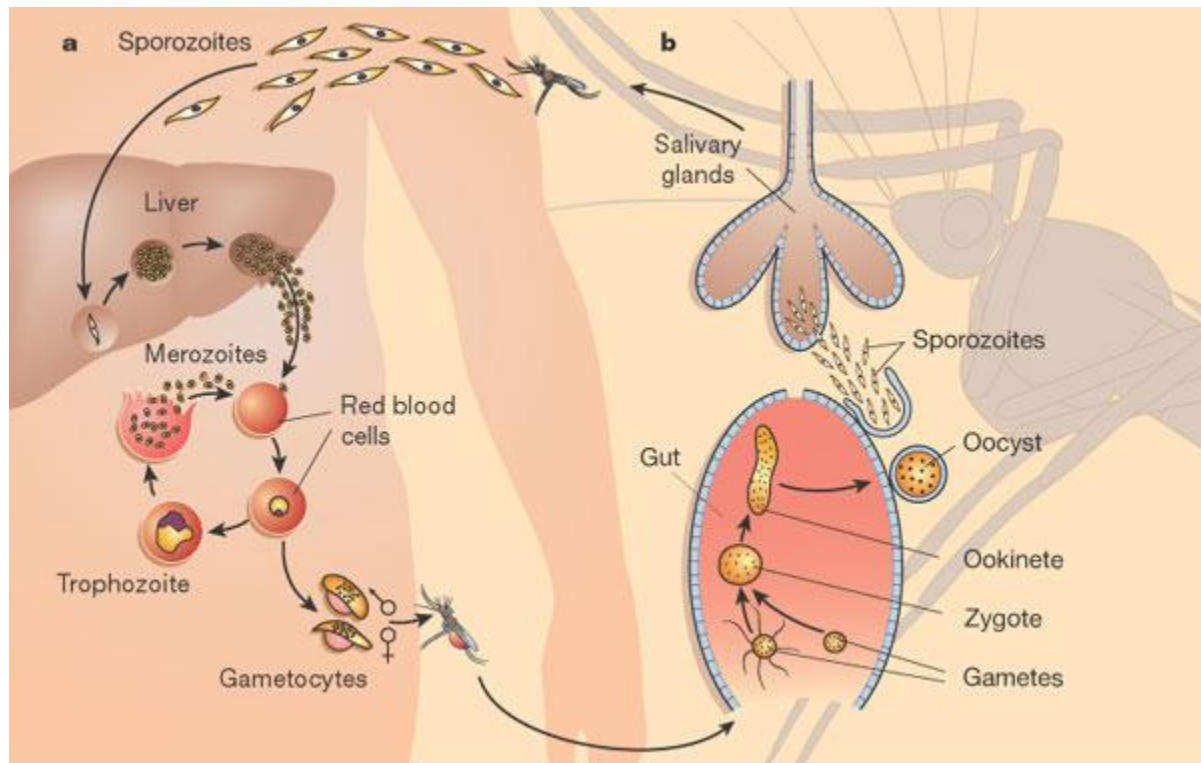


Plate 3: The *Plasmodium falciparum* life cycle; a. asexual stage in human and b. sexual stage in mosquito (Wirth, 2002).

2.4 Clinical diagnosis

Prompt and accurate diagnosis of malaria is an essential part of malaria case management and a key to reducing morbidity and mortality. Accurate diagnosis is also important for differential diagnosis of other febrile illnesses including sepsis, meningitis and pneumonia in which delayed diagnosis and treatment may be a cause of mortality (Berkley *et al.*, 1999). The diagnosis of malaria is based on recognizing clinical symptoms and confirmation with detection of parasites in the blood. In sub-Saharan Africa, many primary health care facilities lack diagnostic equipment, such as microscopes, and well-trained staff for high-quality laboratory diagnosis (Ngasala *et al.*, 2008). Malaria diagnosis is therefore still based on clinical features such as fever and presence of anaemia (World Health Organization *et al.*, 2009). However, symptoms and signs of malaria are nonspecific. It is common to have symptoms of malaria overlap with many

other acute febrile illnesses and co-infections (Berkley *et al.*, 1999; Källander *et al.*, 2004; Luxemburger *et al.*, 1998; O'Dempsey *et al.*, 1993; Tahita *et al.*, 2013), as a result malaria misdiagnosis and over-diagnosis are very common in sub-Saharan Africa (Amexo *et al.*, 2004; Gwer *et al.*, 2007; Reyburn, 2004).

The need to confirm parasites in the blood before therapy has become very important because of resistance of *P. falciparum* to the available monotherapies such as chloroquine, which is now replaced with artemisinin-based combination therapy (World Health Organization *et al.*, 2009). The laboratory methods of malaria diagnosis include light microscopy and parasite antigen detection also known as the rapid diagnostic tests (RDTs).

2.4.1 Light microscopy

Laboratory diagnosis by microscopy examination of stained blood smears remains the gold standard for case management, epidemiological studies, clinical trials of antimalarial drugs and vaccines, and a quality assurance of other malaria diagnostics tests. The microscopy of Giemsa stained blood films has high sensitivity and specificity when used by well-trained staff, which can detect as low as 50 parasites/ μ l blood under field conditions (Moody, 2002; Payne, 1988). Other advantages of microscopy include low cost, determination of parasite densities, distinction between parasite species, differentiation between parasite stages and the possible diagnosing of other diseases. However the accuracy and usefulness of microscopy depends on factors such as the quality of the microscope, reagents and experience of the technician (Makler *et al.*, 1998).

2.4.2 Rapid diagnostic tests

Rapid diagnostic tests use immune-chromatographic technology to detect antigens derived from malaria parasites (Wongsrichanalai *et al.*, 2007). Malaria antigens currently used as diagnostic targets include histidine-rich protein II (HRP-II) specific for *P. falciparum* (Murray and Bennett, 2009). Another antigen is parasite lactate dehydrogenase (pLDH) for detection of *Plasmodium* species. Monoclonal antibodies against pLDH can differentiate between *P. falciparum* and *P. vivax* (Murray and Bennett, 2009). There is limited data on the use of RDTs to identify *P. knowlesi*, however findings from a study show that the pLDH antibodies that detect *P. falciparum* and *P. vivax* can also be used to detect and distinguish *P. knowlesi* (McCutchan *et al.*, 2008). *Plasmodium* aldose enzyme can also be used as a universal antigen target for all malaria parasites (Murray *et al.*, 2008). Unlike HRP-II, pLDH is cleared from the blood about the same time as the parasites following successful treatment (Moody *et al.*, 2000; Wongsrichanalai *et al.*, 2007). However both PLDH and aldolase are expressed in gametocytes, limiting their use in monitoring response to therapy (Mueller *et al.*, 2007). Rapid diagnostic tests offer a good alternative to microscopy, they are simple to perform and do not require skilled personnel (Bell *et al.*, 2001). However limitations to RDTs include high cost, lack of density determination, persistent positivity and low sensitivity (Murray *et al.*, 2003).

2.5 Clinical presentation of uncomplicated *P. falciparum* malaria

Malaria is an acute illness. The first common symptoms are nonspecific and include; fever, headache, joint aches, malaise, vomiting, diarrhoea, body weakness, chest pain and poor appetite. These clinical symptoms usually appear 10-15 days after a person is infected with sporozoites. The symptoms of malaria vary according to the state of immunity, species of infecting parasites, and the epidemiology of malaria (Bruce *et al.*, 2000; Doolan *et al.*, 2009).

In *P. falciparum* malaria, fever starts after few days of prodromal symptoms which starts during the last days of the incubation period which ranges from 9 to 14 days. At the onset, the fever is irregular, but usually occurs daily. It may be intermittent or continuous, and shows no sign of periodicity until the illness has continued for a week or more. The symptoms present in the prodromal phase continue and increase, so configuring a flu-like syndrome. Anorexia, dyspepsia, epigastric discomfort, nausea, vomiting and watery diarrhoea are frequent and may be misdiagnosed as a gastrointestinal infection. A dry cough and an increase in the respiration rate may becoming noticeable, arising the suspicion of an acute respiratory infection. Other non-specific physical findings are tender hepatosplenomegaly, orthostatic hypotension, and some degree of jaundice. The pulse rate of the infected person may become rapid measuring 100 to 120 beats/min and the blood pressure may be low (90–100 mmHg, systolic) (Strickland, 2001). When periodic febrile paroxysms occur, they may be daily (quotidian), every third day (tertian) or at about 36-hour intervals (subtertian).

Laboratory exams may show little or no anaemia in mild cases and in otherwise healthy individuals. When present, it is usually normochromic and normocytic. The reticulocyte count is normal or depressed despite haemolysis, and increases only after the parasitemia is cleared. Serum haptoglobin may be undetectable indicating haemolysis. However, the degree of anaemia may greatly vary and is not always an indication of the severity of the attack.

The white cell count is usually normal, but leucopenia with a count of 3,000–6000 cells/ μ l may be observed. Thrombocytopenia is common (usually around 100,000/ μ l) and does not correlate with severity. Serum transaminases and lactic dehydrogenase usually are moderately elevated as well as bilirubin concentrations, both conjugated (hepatocyte dysfunction and/or cholestasis) and unconjugated (haemolysis). Prothrombin and partial thromboplastin time may be prolonged while

fibrinogen levels are usually elevated. C-reactive protein and procalcitonin value are usually increased in both uncomplicated and severe malaria with levels correlated with parasitemia and prognosis according with some studies (Ahiboh et al., 2008; Chiwakata et al., 2001; Uzzan et al., 2006). Hyponatremia may arise in some cases, a transient increase in serum creatinine and blood urea nitrogen may be observed. Urine analysis reveals albuminuria and urobilinogen.

Examination of peripheral blood film mostly reveals the presence of early trophozoites (typical small ring forms), often numerous, with multiple infected red blood cells. Late trophozoites, as well as schizonts, are seldom observed, being their presence an indicator of very heavy infections and poor prognosis. Gametocytaemia usually occurs in the second week after the onset of parasitemia and may persist for some weeks after cure (Wernsdorfer and McGregor, 1988).

Although the subject may not appear very ill, serious complications may develop at any stage. In non-immune people *P. falciparum* malaria may progress very rapidly to severe malaria unless appropriate treatment is started. If the acute attack is rapidly diagnosed and adequately treated, the prognosis of falciparum malaria is good, even if complications may still occur. The response to treatment is usually rapid with resolution of fever and most symptoms within 3 days (White, 2011). Recrudescence with renewal of clinical manifestation and/or parasitemia, due to persistent erythrocytic forms, may occur.

2.6 Clinical presentation of complicated *P. falciparum* malaria

Clinical features of uncomplicated malaria may progress to severe malaria if a patient is untreated or treated with ineffective drugs. The WHO has developed specific diagnostic criteria

for severe malaria (World Health Organization, 2010). A patient is classified to be suffering from severe malaria if one or more of the following features are present: severe anaemia (haemoglobin < 5 g/dL or Ht $< 15\%$), a common feature in infants and young children in malaria endemic areas; cerebral malaria (unarousable coma), with a Blantyre coma scale < 3 (in children) and Glasgow coma scale < 10 (in adults), a common feature in adult and children in areas of low transmission; Prostration; Respiratory distress induced by metabolic acidosis. Other criteria include Convulsions (> 1 episode/ 24 hours); Hypoglycaemia (< 2.2 mmol/L); Extreme weakness; Acidosis (plasma bicarbonate < 15 mmol/L); Renal failure (serum creatine above normal range for age); Shock; Macroscopic haemoglobinuria; Spontaneous bleeding; Pulmonary edema; Clinical jaundice; Hyperlactataemia (venous lactic acid > 5 mmol/L); and Hyperparasitaemia ($> 25000/\mu\text{L}$ or $> 5\%$ of RBCs) (Pasvol, 2005). For patients with severe malaria receiving treatment, case fatality rate can reach 36.5% and if untreated severe malaria is fatal in majority of cases (Olliaro, 2008).

2.7 Treatment of malaria

Due to development and spread of *P. falciparum* resistance to conventional monotherapies such as chloroquine and sulphadoxine-pyrimethamine (Myint *et al.*, 2004), the WHO has recommended treatment of malaria with antimalarials that have different modes of action and different biochemical targets in the parasite, with the preference falling on ACTs (Wells *et al.*, 2009). Artemisinin derivatives are extremely potent antimalarials that can also decrease malaria transmission because of its considerable effect on gametocytes (Barnes *et al.*, 2005; Price *et al.*, 1996; Sutherland *et al.*, 2005). The WHO currently recommends the following ACTs: arthemether-lumefantrine (AL), artesunate-amodiaquine (AS+AQ), artesunate-mefloquine (AS+MQ), artesunate-sulphadoxine-pyrimethamine (AS+SP) and dihydroartemisinin-

piperaquine (DHA+PPQ) (World Health Organization, 2010). Many countries in Africa, including Ghana have recently adopted ACTs, which are being deployed for treatment of uncomplicated malaria. The treatment of severe malaria includes clinical assessment of the patient, specific antimalarial treatment, adjunctive therapy and supportive care (World Health Organization, 2012b). The specific antimalarial treatments for severe malaria include the cinchona alkaloids (quinine and quinidine) and artemisinin derivatives (artesunate, artemether and artemotil). Quinine is also a drug of choice for malaria in the first trimester of pregnancy, and malaria in children weighing <5 kg. Artesunate suppositories, which can be administered rectally, are recommended for pre-referral management of severe malaria in areas where parenteral antimalarial treatment would not be feasible (World Health Organization, 2010).

2.8 Antimalarial drug resistance

The spread of resistance to antimalarial drugs is a major obstacle to effective case management; Chloroquine resistance in Africa resulted in increased morbidity and mortality (Björkman and Bhattarai, 2005; Korenromp *et al.*, 2003; Marsh, 1998; Trape, 2001; Zucker *et al.*, 2003), hence a major setback to malaria eradication. The switch to ACTs to treat uncomplicated malaria looks to be facing a hindrance with reported cases of resistance on the Thai-Cambodia border (Dondorp *et al.*, 2010). Preserving the efficacy of the antimalarial drugs in use is of critical importance to public health in malaria endemic areas. Widespread use of antimalarial drugs for all types of fever and exposure to sub-therapeutic doses of drugs due to non-compliance of recommended dosages contribute markedly to drug resistance and necessitate continual change in malaria treatment guidelines (Rajakaruna *et al.*, 2008).

In 1973, the WHO defined antimalarial drug resistance as “the ability of a parasite strain to survive and/or multiply despite the proper administration and absorption of an antimalarial drug in the doses equal to a higher than those normally recommended but within the tolerance of the subject”. This definition was modified in 1986 to include the phrase “drug must gain access to the parasite or the infected red blood cell for the duration of the time necessary for its normal action” (Bruce-Chwatt, 1979). Recent development of techniques for *in vitro* drug-sensitivity testing, high-performance liquid chromatography and molecular biology have improved our understanding of mechanisms of drug resistance. In this definition it is important to consider pharmacokinetic properties of antimalarial drugs, which vary in different individuals. For instance lumefantrine has inter-individual variation in bioavailability up to 20 fold (Ezzet *et al.*, 2000). Currently, to confirm true drug resistance the blood concentration of the drug and its metabolite must be considered, also to be considered are recrudescence of the parasites, results of *in vitro* drug sensitivity and genetic markers of resistance (Ezzet *et al.*, 2000; Vestergaard and Ringwald, 2007).

2.9 Current orthodox antimalarial drugs

Malaria has been fought against with several drugs that have been developed to curb its menace on mankind. These drugs have varied mechanisms, which interfere with essential life processes of the parasite for its survival.

2.9.1 Quinolines

The bark of the *Cinchona officinalis* tree has been used for centuries to treat fever associated with malaria infection from which the active ingredient is quinine. It remained the antimalarial drug of choice until the 1940s, when other antimalarial drugs such as its derivative; chloroquine

replaced quinine. Quinine, however, is still used to treat clinical malaria and not as a prophylaxis due to its side effects and poor tolerability. Chloroquine is a 4-aminoquinoline derivative of quinine and for many years it was the main antimalarial drug used in the treatment of malaria until parasite resistance developed in the 1950s (Payne, 1987). It remains the most popular antimalarial developed to date due to its safety, low cost and efficacy (Björkman and Bhattarai, 2005). Today, the widespread of resistance to the drug has rendered it useless as a therapeutic agent, even though it still shows some efficacy in the treatment of the other human malaria parasites (Trape *et al.*, 1998). Despite years of research, the actual molecular mechanism of chloroquine action remains an elusive topic. It is believed that chloroquine accumulates in the food vacuole of the parasite and prevents haem detoxification (Bray *et al.*, 2005). Chloroquine resistance in malaria parasites has been attributed to the reduced concentrations of the drug in the food vacuole possibly due to drug efflux, pH modification in the vacuole, the role of a Na^+/H^+ exchanger and transporters (Bray *et al.*, 2005; Foley and Tilley, 1998). In *P. falciparum*, two genes have been implicated in resistance, namely *Pfmdr1* and *Pfcrt*, which encodes Pgh1 and PfCRT proteins, respectively. Both of these proteins are localized to the food vacuole membrane. Mutations in these genes could lead to small increases in the food vacuole pH thus reducing the amount of chloroquine that can accumulate, rendering the drug ineffective (Spiller *et al.*, 2002). Alternatively, PfCRT may increase the efflux of chloroquine by directly interacting with the drug (Cooper *et al.*, 2007). Resistance is associated with several mutations in the PfCRT protein, while the loss of a Lys residue at position 76 has been shown as the critical mutation rendering the *P. falciparum* parasites resistant to the drug. This residue is located within the first transmembrane segment of PfCRT and may therefore play an important role in the properties of the channel or transporter (Cooper *et al.*, 2005). Mutations in the *Pfmdr1* gene is associated with resistance to mefloquine, quinine and halofantrine (Price *et al.*, 2004; Reed *et al.*, 2000).

A number of related aminoquinolines have since been developed and applied. These include the following;

- Amodiaquine – effective against chloroquine resistant strains, possible cross-resistance with chloroquine, has safety limitation (Bathurst and Hentschel, 2006).
- Atovaquone – usually used in combination with proguanil (Malarone[®]), resistance reported in 1996, it has cost limitation (Bathurst and Hentschel, 2006).
- Lumefantrine – usually co-formulated with arthemeter (Co-ArtemTM) and is highly effective against multi-drug resistant *P. falciparum* (Falade *et al.*, 2005).
- Halofantrine (Halfan): resistance reported in 1992, it has cost and safety limitations (Bathurst and Hentschel, 2006).
- Mefloquine (Lariam[®]) – resistance reported in 1982, it has cost and safety limitations (Bathurst and Hentschel, 2006).
- Primaquine – used for its gametocytocidal effect (*P. falciparum*) and its efficacy against intra hepatic forms of all types of malaria, no resistance, safety limitations (Bathurst and Hentschel, 2006; Chauhan and Srivastava, 2001).

2.9.2 Antifolates

The antifolates are some of the most widely used antimalarial drugs. However, their role in malaria prevention is hampered by the rapid emergence of resistance once the parasites are placed under drug pressure. The direct effect of folate biosynthesis inhibition is a reduction in the synthesis of the amino acids serine and methionine as well as in pyrimidines, which leads to decreased synthesis of DNA. The antifolate drugs target the intra-erythrocytic stages as well as gametocytes of *P. falciparum* (Olliaro, 2001). The antifolates can generally be divided into two

classes; the type-1 antifolates mimic the p-aminobenzoic acid (pABA) substrate of dihydropteroate synthase (DHPS) and include the sulfonamides (sulfadoxine) and sulfone (dapsone). While the type-2 antifolates (pyrimethamine and proguanil) inhibit dihydrofolate reductase (DHFR) (Olliaro, 2001).

Interestingly both of these classes of target proteins are arranged on separate bifunctional enzymes (hydroxymethyldihydropterin pyrophosphokinase/dihydropteroate synthase or PPPK/DHPS and dihydrofolate reductase/thymidylate synthase or DHFR/TS) (Ivanetich and Santi, 1990). In addition, malaria parasites are capable of *in vivo* folate salvage from the extracellular environment as well as synthesizing folate derivatives from simple precursors. The mechanism of exogenous folate uptake by a carrier-mediated process has important implications in increasing the sensitivity of the antifolate inhibitors and is being investigated as a novel drug target (Wang *et al.*, 2007). Pyrimethamine is a diaminopyrimidine and is mostly used in combination with sulfadoxine (Fansidar™) or depose leading to the simultaneous inhibition of DHFR and DHPS. Pyrimethamine crosses the blood-brain barrier as well as the placenta. Resistance to sulfadoxine-pyrimethamine combination therapy, however, emerged rapidly due to the appearance of point mutations in the active sites of the target enzymes resulting in reduced drug binding capacity (Cowman and Lew, 1990; Plowe *et al.*, 1998).

2.9.3 Artemisinin

Artemisinin is a sesquiterpene lactone extracted from the leaves of *Artemisia annua*. It is a potent, fast acting blood schizontocide that allows efficacy against all Plasmodium stages (Klayman, 1985). Its efficacy is especially broad and shows activity against all the asexual stages of the parasites including gametocytes (Mutabingwa, 2005; Skinner *et al.*, 1996). The effects of

artemisinin on gametocytes make it a good candidate for the prevention of malaria transmission. Originally, the mechanism of action of artemisinin was thought to be mediated by the peroxide ring of the drug, which is cleaved and activated by ferrous iron in the heme stores into toxic free radicals that can subsequently damage intracellular targets via alkylation (Meshnick *et al.*, 2003). Recently, however, this theory was challenged by evidence that artemisin exerts its inhibitory effects on the malarial sarcoplasmic-endoplasmic reticulum calcium ATPase (SERCA) resulting in an alteration of intracellular calcium levels (Eckstein-Ludwig *et al.*, 2003). The actual mechanism of action, however, remains elusive and there have been different results from various studies (Krishna *et al.*, 2004).

Several derivatives of artemisinin have been developed since artemisinin itself is poorly absorbed and include dihydroartemisinin, artesunate (sodium salt of the hemisuccinate ester of artemisinin), artemether (methyl ether of dihydroartemisinin) and arthemether (ethyl ether of artemisinin) (Frédérich *et al.*, 2002). Currently, the WHO recommends artemisinin-based combination therapy (ACT) as the first-line treatment against malaria infections where resistance to other antimalarial drugs is prevalent. One of the obvious disadvantages of using ACT for malaria case-management in Africa is the increased cost involved in combining therapies. Even so, several reasons exist for combining antimalarial drugs with an artemisinin derivative, namely an increase in the efficacy of antimalarial drugs, a decrease in the treatment period and a reduced risk of resistant parasites arising through mutation (Kremsner and Krishna, 2004). Several ACTs that have been developed and may have entered clinical trials are listed below (Gelb, 2007):

- Pyramax: artesunate and the 4-aminoquinoline pyronaridine
- Co-ArtemTM: artemether and lumefantrine
- ArtekinTM: dihydroartemisinin and the quinolone-based drug piperazine

- Lapdap™: artesunate, chlorproguanil and dapsone
- ASAQ: artesunate and amodiaquine

There are several reasons why the appearance of parasite resistance to artemisinin was originally thought to be unlikely or at least delayed. These were spelt out by Meshnick (2002) as follows;

1. Parasites are not exposed to the drug for prolonged periods due to the short half-life of the drug.
2. Artemisinin is gametocytocidal, which reduces the transmission potential and spread of the parasite and
3. The frequent use of artemisinin combined with other antimalarial drugs (ACT) specifically introduces delay in the onset of resistance

Evidence for *in vitro* resistance to an artemisinin derivative, however, appeared in field isolates from French Guiana in 2005. The increased arthemether IC₅₀s were ascribed to the presence of a mutation in the SERCA PfATPase6 gene and was attributed to inappropriate drug use that exerted selection pressures, favouring the emergence of parasites with an arthemether-resistant *in vitro* profile. There have been reports of resistance to artemisinin in Western Cambodia (Noedl *et al.*, 2008). Even though reduced *in vitro* drug susceptibility is not tantamount to diminished therapeutic effectiveness, it could lead to complete resistance and thus called for the rapid deployment of drug combinations (Jambou *et al.*, 2005). Lapdap™, a combination of chlorproguanil (targets DHFR), dapsone (targets DHPS) and the artemisinin derivative artesunate, was introduced in 2003 as malaria therapeutics to replace sulfadoxine-pyrimethamine treatment in Africa (Edwards and Biagini, 2006).

2.9.4 Antibiotics

Evidence from *in vivo* experiments and clinical studies has shown that several antibiotics such as tetracycline, doxycycline and minocycline are active against the *P. falciparum* parasite (Geary and Jensen, 1983). The tetracyclins are antibiotics that were originally derived from *Streptomyces* species, but are usually synthetically prepared. They interfere with aminoacyl-tRNA binding and therefore inhibit protein synthesis in the parasite's apicoplast or mitochondrion (Dahl and Rosenthal, 2008). This is due to the presence of genes in the mitochondrion and apicoplast that encode prokaryote-like ribosomal RNAs, tRNAs and various proteins (Dahl *et al.*, 2006). Doxycycline is a synthetic tetracycline derivative with a longer half-life than tetracycline. The disadvantage of these antibiotics as antimalarial drugs is the development of abdominal cramps, diarrhoea, vaginitis and photosensitivity during treatment (Andersen *et al.*, 1998). *In vitro* studies have shown that the effectiveness of antibiotics used as antimalarial drugs is inhibited by the presence of iron (Pradines *et al.*, 2001).

2.10 Local antimalarial drugs

There are numerous local antimalarial drugs on the Ghanaian market. They are mostly made up of one or more herbal extracts. The Foods and Drugs Authority has licensed a number of them to be sold even in pharmacies. However one can still find some unlicensed local antimalarial drugs on the open market. Below are 10 local antimalarial drugs licensed by the FDA for malaria treatment.

Table 1: Some examples of local antimalarial drugs on Ghanaian market

Trade name	Dosage		Indications	Ingredients
	Adults	Children		
Taabea herbal mixture	2 tablespoonful three times daily	1 tablespoonful three times daily	Jaundice, typhoid, malaria, menstrual pains, menstrual disorder, candidiasis, general body pains, loss of appetite	<i>Ocinum viride, Azadirachtha indica,</i> <i>Paullinia pinnate, Tetrapleura</i> <i>tetraptera, Theobroma cacao,</i> <i>Cymbopogon citratus, Moringa</i> <i>oleifera</i>
Masada mixture	2 tablespoonful (30 ml) three times daily	1 tablespoonful (15 ml) three times daily	Malaria	<i>Cryptolepis sanguinolenta</i>
Herbaquin	5 tablespoonful (75 ml) twice a day Prophylaxis dosage: 1-2	2 tablespoonful (30 ml) twice a day	Malaria	<i>Cryptolepsis sanguinolenta, Alstonia</i> <i>boonei, Azadirachta indica, Monodora</i> <i>myristica, Xylopi aethiopica</i>

	tablespoonful three times daily			
Top fever syrup	2 tablespoonful (30 ml) three times daily	1 tablespoonful (15 ml) three times daily	Malaria fever	<i>Azadirachta indica, Alstonia boonei</i>
Kingdom mixture	3 tablespoonful (45 ml) three times daily	1 tablespoonful (15 ml) three times daily	Malaria	<i>Nauclea latifolia, Phyllanthus fratemus, Cryptolepsis sanguinolenta</i>
Yafo fever mixture	4 tablespoonful (60 ml) two times daily		Malaria	<i>Cryptolepsis sanguinolenta, Azadirachta indica</i>
Class malakare	2 tablespoonful (30 ml) three	1 tablespoonful (15 ml) three	Malaria, typhoid, body pains, headache, tiredness, stress	<i>Carapa procera, Cryptolepsis sanguinolenta</i>

	times daily	times daily		
Adutwumwa malamix	2 tablespoonful (30 ml) three times daily	2 tablespoonful (30 ml) two times daily	Malaria, typhoid	<i>Anthocleista nobilis</i> , <i>Vitex grandifolia</i> , <i>Phyllanthus fraternus</i>
New angel herbal mixture	2 tablespoonful (30 ml) three times daily	1 tablespoonful (15 ml) three times daily	Jaundice, menstrual pain, malaria, fever, loss of appetite, body pains	<i>Cola gigantean</i> , <i>Solanum torvum</i> , <i>Spathodea campanulata</i> , <i>Bombax buonopozense</i> , <i>Vernonia amygdalina</i>
Rooter mixture	4 dessert spoonful, three times daily	3 desert spoonful, three times daily	Malaria, typhoid, jaundice	<i>Aloe schweinfurthil</i> , <i>Khaya senegalensis</i> , <i>Pileostigma thornnigil</i> , <i>Cassia siamea</i>

2.11 Herbal plants used in local antimalarial drugs

The local antimalarial drugs are made of the extracts of herbal plants that have traditionally been used in the treatment of malaria. With more interest in the interest of herbal medicine, some of these herbal plants have been studied scientifically to ascertain their efficacy and curative properties.

2.11.1 *Azadirachta indica*

Azadirachta indica, also known as neem or nimtree is a tree within the family Meliaceae. It is one of the two species of the genus *Azadirachta*. It is native to India, Pakistan and Bangladesh; it grows in tropical and semi-tropical regions. Neem-based products from *Azadirachta indica* have been used extensively in traditional medicine in India for over 2000 years (Thakurta *et al.*, 2007). It has been widely used for the treatment of helminthic, fungal, bacterial, viral and protozoan infections. It is a major component in the treatment of diabetic, skin and hepatic disorders (Thakurta *et al.*, 2007). In Ghana *Azadirachta indica* is mainly used in the treatment of malaria. The leaves are boiled and concoction drunk or used for steam bath (Asase *et al.*, 2010).

There is substantial scientific research evidence to support the therapeutic effects of *Azadirachta indica*. For example extracts from neem have been shown to inhibit the growth of bacteria (Biswas *et al.*, 2002). There have also been reports of the antiviral activities of neem tree extracts on viruses in dengue fever (Parida *et al.*, 2002) and HIV (Mbah *et al.*, 2007; Udeinya *et al.*, 2004). Susceptible to neem tree extracts are important protozoa; *Trypanosome* (Yanes *et al.*, 2004) *Leishmania* (Tahir *et al.*, 1998) and *Plasmodium*; both asexual stages and gametocytes (Udeinya *et al.*, 2008). The gametocytocidal activity of neem and transmission blocking properties (Lucantoni *et al.*, 2010), makes it a potential candidate for the eradication of malaria.

Of equal importance is the study which showed that neem extracts protects neurons from apoptosis during cerebral malaria (Bedri *et al.*, 2013).

2.11.2 *Vernonia amygdalina*

Vernonia amygdalina is a perennial shrub of the Asteraceae family that grows in tropical Africa. It is commonly known as “bitter leaf” due to the bitter taste of its leaves. *Vernonia amygdalina* typically grows to a height of 2-5 m. The leaves are elliptical and up to 20 cm long (Ijeh and Ejike, 2011). In West Africa the plant has been domesticated and is used for both nutritional and medical purposes (Igile *et al.*, 1994). The leaves which are edible are used in preparing the popular bitter leaf soup and its leaf extracts are also used as a tonic. It contains 79.93 % moisture, 20.08% dry matter, 35.81g protein, 68.35g carbohydrates, 25.47g dietary fibre and 4.70g lipids per 100g of dry weight, it also contains important vitamins and minerals such as ascorbic acid, carotenoid, calcium, iron, phosphorus (Ejoh *et al.*, 2007). Other nutritional elements found are potassium, sulphur, sodium, manganese, copper, zinc, magnesium and selenium (Atangwho *et al.*, 2009; Bonsi *et al.*, 1995; Eleyinmi *et al.*, 2008).

Vernonia amygdalina has widely been used in traditional medicine as an antihelminthic, antimalarial and laxative herb. It was observed that a sick chimpanzee in the wild chewed the leaves of the *Vernonia amygdalina* plant and after some time returned its normal state (Jisaka *et al.*, 1993) . This led to further research into the therapeutic effects of the plant. It was shown to affect the weight, liver, urine and faecal output, plasma and liver cholesterol concentrations in mice (Igile *et al.*, 1994). The antidiabetic effects of aqueous extract of leaves of *Vernonia amygdalina* was reported (Akah and Okafor, 1992). Reports show that low concentrations of

water-soluble leaf extracts of *V. amygdalina*, potently retards the proliferative activities of human cancerous cells (Gresham *et al.*, 2008). In Ghana the leaves are used in treating cough (Akinpelu, 1999). The antiplasmodium activity of *Vernonia amygdalina* has also been reported against *Plasmodium falciparum* *in vitro* and *in vivo* (Omoriegie *et al.*, 2011), showing adequate clinical response in the treatment of uncomplicated malaria cases (Challand and Willcox, 2009).

2.11.3 *Ocimum viride*

Ocimum viride also known as basil is a tender-growing aromatic annual herb native to West Africa (Danso-Boateng, 2013). *Ocimum viride* leaves are used for the treatment of malaria in Ghana (Shankar *et al.*, 2012). *Ocimum* sp. contains thymol (Keita *et al.*, 2000), which gives the plant its known anti-fungal and antibiotic properties (Mbata and Saikia, 2005).

2.11.4 *Paullinia pinnata*

Paullinia pinnata is an African tropical plant whose roots and leaves are used in traditional medicine for many purposes; notable among them is the treatment of erectile dysfunction (Zamble *et al.*, 2006). *Paullinia pinnata* has also been investigated to possess antibacterial properties (Anani *et al.*, 2000). The plant has also been traditionally used for dressing wounds; research has proved its wound healing properties and cyto-protective activities (Annan and Houghton, 2010). *Paullinia pinnata* also showed some anti-plasmodial effects against *Plasmodium berghei* (Maje *et al.*, 2007).

2.11.5 *Moringa oleifera*

Moringa oleifera which belongs to the family Moringaceae is a highly valued medicinal plant, distributed in many countries of the tropics and subtropics. It has so many nutritional and

medicinal benefits (Anwar *et al.*, 2007). *Moringa* has been used to combat malnutrition in developing countries especially among infants and nursing mothers (Fahey, 2005). *Moringa* is known to have anti-bacterial properties (Fahey *et al.*, 2002) and anti-fungal properties (Chuang *et al.*, 2007). The susceptibility of *Helicobacter pylori* to compounds derived from *Moringa* (Haristoy *et al.*, 2005) makes it efficacious in the treatment of gastritis and ulcers. *Moringa* has also shown good prospects in the prevention of cancer (Guevara *et al.*, 1999). *Moringa* is also a widely used natural antimalarial product (Gbeassor *et al.*, 1990).

2.11.6 *Solanum torvum*

Solanum torvum belongs to the family Solanaceae, commonly known as Turkey berry is native to and cultivated in tropical Africa and West Indies. The fruits are used in most parts of Africa as vegetable and for medicinal purposes (Jaiswal, 2012). *Solanum spp.* seeds have antioxidant properties (Waghulde *et al.*, 2011). *Solanum torvum* contains a number of important active phyto-chemicals like isoflavonoid sulfate and steroidal glycosides (Arthan *et al.*, 2002), chlorogenone and neochlorogenone (Carabot Cuervo *et al.*, 1991). Flavonoid intake has been known to be inversely related to mortality from coronary heart disease in epidemiological studies (Hertog *et al.*, 1993). *Solanum sp.* is used as an antimalarial by the Ga-Adangbe people of Ghana (Asase *et al.*, 2010)

2.12 Malaria transmission and epidemiology

Vectors are the key determinants of malaria transmission despite other factors such as parasite strain and health of individual are also involved. The distribution and abundance of an effective

vector depends on environmental conditions like optimum temperature, altitude, humidity, seasonal pattern of rainfall and suitable water for breeding of larval stages (Robert *et al.*, 2003). The intensity of malaria transmission is assessed by how often people are bitten by anopheline mosquitoes infected with sporozoites. The entomological inoculation rate (EIR) is the number of infective mosquito bites received per person per unit time (Hay *et al.*, 2000). Measurements of sporozoites index from mosquito samples can be determined either microscopically by dissecting the mosquitoes for their salivary glands or using immunological assays such as Enzyme-linked immunosorbent assay (ELISA) to detect Plasmodium-specific circumsporozoite antigens (Gilles and Warrell, 2002).

The vectorial capacity is a transmission probability index, i.e. the expected number of humans infected per infected humans, per day, assuming perfect transmission efficiency (Smith and McKenzie, 2004) or number of new infections the population of a given vector would distribute per case per day at a given place and time, assuming conditions of non-immunity (World Health Organization, 2010). The vectorial capacity is an indicator of stability of transmission, and depends on relative density of female anophelines, probability of mosquito to take blood meal, proportion of mosquitoes surviving incubation period and probability of daily survival. The key factor in determining intensity of transmission is the probability of daily survival of mosquitoes (Gilles and Warrell, 2002)

2.12.1 Levels of endemicity

Geographical areas can be classified by intensity of transmission based upon the percentage of children, aged 2-9 years with splenomegaly or malaria parasitemia (Ngasala, 2010):

Holo-endemic: (>75%) transmission occurs all year long

Hyper-endemic: (50-75%) intense, but with periods of no transmission during dry season

Meso-endemic: (11-50%) regular seasonal transmission

Hypo-endemic: (<10%) very intermittent malaria transmission

Malaria transmission can also be classified as stable, if the populations are continuously exposed to a fairly constant rate of malaria inoculations ($EIR > 10/\text{year}$) or unstable if ($EIR < 5/\text{year}$) and when the level of acquired immunity determines patterns of disease (World Health Organization, 2010). Stable and high malaria transmission occurs in holoendemic and hyperendemic areas, such as in tropical Africa. Unstable and low to moderate transmission occurs in meso and hypoendemic areas, such as much of tropical Asia, Central Asia and Latin America (Hay *et al.*, 2004).

2.14 Malaria eradication

In the 1950s, the World Health Organization and other international organizations made a pledge to eradicate malaria (Pampana and Russell, 1956) based on two assumptions that would later be seen as false. That is firstly, human malarial infection could be eliminated by treatment with antimalarial drugs such as chloroquine and secondly, transmission could be eliminated with residual insecticides such as dichlorodiphenyltrichloroethane (DDT). Firstly, the prevalence of chloroquine resistance, which was boosted by massive chloroquine use during the malaria eradication campaign made it impossible to clear *Plasmodium falciparum* parasitemia in many regions of Southeast Asia and South America with chloroquine. Secondly, both insecticide resistance and exophilic transmission undermined the efficacy of residual insecticides. In addition, massive problems with logistics, planning, resource allocation, and a lack of operational research contributed greatly to the failure of malaria eradication (Bruce-Chwatt, 1979).

Due to the major logistical problems involved, the malaria eradication campaign formulated by the World Health Organization in the 1950s focused primarily on Southeast Asia and South America (Pampana and Russell, 1956) rather than sub-Saharan Africa, where the intensity of transmission and the morbidity and mortality of malaria were greatest. Even though, biologically and technically, malaria is not an ideal candidate for eradication (Aylward *et al.*, 2000; Henderson, 1987; Stuart-Harris, 1984), there have been renewed efforts in malaria eradication. Malaria eradication is the permanent reduction to 0 of the worldwide incidence of malaria infection caused by a specific agent; i.e. applies to a particular malaria parasite species (World Health Organization, 2010). During the past 5 years, there has been a substantial increase in international funding for malaria control through major international financing mechanisms such as the Global Fund to fight HIV, TB and Malaria, the US President's Malaria Initiative and the World Bank's Booster Programme (Mendis *et al.*, 2009). This, together with a high level of political commitment in endemic countries, has resulted in increased coverage of malaria interventions in endemic areas, and a reduction in malarial disease and death in several countries, including several in sub-Saharan Africa where the burden of malaria is greatest. Inspired by these achievements and by the momentum created by global advocacy, the possibility of malaria eradication has been placed again on the agenda of global health (Feachem and Sabot, 2008; Okie, 2008; Tanner and de Savigny, 2008). Therefore after a lapse of almost 40 years, the malaria eradication has been re-introduced on the global health agenda inspired, by the Gates Malaria Forum in October 2007 (Greenwood, 2009; Roberts and Enserink, 2007). Although there is lack of sufficient knowledge, systems and tools to eradicate malaria today, there is hope in the window of political will and financial resources to achieve the goal of eliminating the disease globally (Tanner and de Savigny, 2008).

Vaccines can be very important in the efforts to eradicate malaria. Current strategies in developing malaria vaccines are primarily focused on *Plasmodium falciparum* and are directed towards reducing morbidity and mortality (Bojang *et al.*, 2001). However if malaria vaccines are to eliminate malaria, they will need to have an impact on malaria transmission (Kaslow *et al.*, 1988). This has led to the introduction of “vaccines that interrupt malaria transmission” (VIMT), which includes not only “classical” transmission-blocking vaccines that target the sexual and mosquito stages but also pre-erythrocytic and asexual stage vaccines that have an effect on transmission (The malERA Consultative Group on Vaccines, 2011). Vaccines that interrupt malaria transmission also include vaccines that target the vector to disrupt parasite development in the mosquito. Importantly, if eradication is to be achieved, malaria vaccine development efforts will need to target other malaria parasite species, especially *Plasmodium vivax*, where novel therapeutic vaccines against hypnozoites or preventive vaccines with effect against multiple stages could have enormous impact (Alonso *et al.*, 2011).

CHAPTER THREE

MATERIALS AND METHODS

3.1 Chemicals, reagents and equipment

The sources and manufacturers of reagents, buffers, solutions and equipment used in the study are shown in the Appendix.

3.2 Study design

The study is a part of an ongoing project to identify local antimalarial drugs that will prevent malaria transmission and recommend them as alternative to the first line drug of choice in malaria treatment in Ghana. The study looked at the susceptibility of chloroquine resistant 3D7A *Plasmodium falciparum* asexual stage to ten known herbal antimalarial drugs on the Ghanaian market. The herbal drugs were selected based on their approval by the Foods and Drugs Authority and their availability on the Market. The study also looked at the gametocytogenic ability of the herbal drugs at very low concentrations. Finally the growth inhibition of gametocytes by the ten herbal drugs was studied. All experiments were done *in vitro*.

3.3 Herbal drug preparations

Ten known local antimalarial drugs [Yafo (YF), Masada (MS), Adutwumwa malamix (AD), Kingdom herbal (KG), Herbaquin (HB), Class malakare (CM), Angel Herbal (AN), Top Fever (TF), Rooter Mixture (RT) and Taabea (TB)] (all in liquid form) that have been approved by the Foods and Drugs Authority (FDA) were obtained from various herbal shops. About 10 ml of each drug was transferred into a 50 ml falcon tube under sterile conditions. The drugs were kept

in -80 freezer for 48 hours. The drugs were then freeze-dried (lyophilized) using the LABCONCO Freezone⁶. About 10 mg of each freeze-dried drug was dissolved in 10 ml of distilled water [giving a 10x initial concentration (1000 µg/ml)]. The drugs were sterile filtered with 0.2 µm filters and stored in a -20 freezer.

3.4 Cultivation of *Plasmodium falciparum* cultures

Continuous *P. falciparum* asexual cultures of the chloroquine-sensitive 3D7 strain were maintained *in vitro* using a modified method of Trager and Jensen (1976).

3.4.1 Culturing for asexual assay

Complete parasite media consisted of RPMI 1640 supplemented with Herpes, L-glutamine, NaHCO₃, Glucose, Gentamycin and Albumax II. Parasites were cultured in O⁺ RBCs and placed in a 37°C incubator with 92.5% Nitrogen, 2% Oxygen and 5.5% Carbon dioxide. The culture was then maintained with daily media change and after 48 hours the parasitemia was reduced to 1%.

3.4.1.1 Sorbitol synchronisation for ring forms

Culture was maintained to get $\geq 5\%$ synchronous ring forms in parasitemia. This was done by treating the parasite culture with 5% sorbitol, which resulted in approximately 95% of the parasites in the ring stage of development after 48 hours (Lambros and Vanderberg, 1979). In brief, a culture that had $\geq 5\%$ rings in parasitemia was selected to be synchronized. About 5% sorbitol was warmed to 37°C in a water bath (Thomastat Shaker T-225). In the Biosafety cabinet [BSC (SterilGARD Hood, the BARKER COMPANY Inc.)], the culture was transferred into a 15 ml tube and centrifuged at 2000 rpm for 5 minutes using the Sanyo HARRIER 18/80 centrifuge.

All the media above the pellet was removed, and then 5% sorbitol was added to the pellet. The pellet in sorbitol was mixed thoroughly and made to stand in an incubator (RS Biotech Galaxy S) at 37°C for 10 minutes. After 10 minutes the culture was centrifuged at 2000 rpm for 5 minutes and transferred into the BSC where the sorbitol was removed using a serological pipette. Parasite wash media [PWM (CPM without Albumax)] was added to the culture and mixed well. The culture was centrifuged at 2000 rpm at 5 minutes. The supernatant was aspirated and replaced with CPM. The contents were mixed thoroughly and the culture was centrifuged at 2000 rpm for 5 minutes. The culture was washed again with CPM. The pellet from the culture was transferred into a new T-75 cm³ flask with 25 ml CPM. About 20 µl of the culture was placed in an Eppendorf tube and was centrifuged at 3000 rpm for 30 seconds using a Labnet, Spectrafuge 24D . About 80% of supernatant was taken off and 5 µl of the pellet was placed on a labeled slide and a thin smear prepared to assess the synchronization. The culture was gassed for 60 seconds and placed in the 37°C incubator.

3.4.1.2 Plating of drugs for asexual assay

About 100 µl of 5 different concentrations of each drug was plated in a 4-fold serial dilution starting at 100 µg/ml. The required concentration of each drug was added in triplicates. About 100 µl of 1% parasitemia culture was added to each treated well. The plates were placed into an incubating chamber and gassed for 6 minutes. The incubating chamber was then placed into the 37°C incubator for 72 hours. After 72 hours a thin smear was prepared from each well. The smears were fixed in 100% methanol and stained with 10% Giemsa for 15 minutes. The slides were dried and observed under a light microscope using a 100x oil immersion objective lens. The parasitemia for each drug concentration in triplicates was estimated. The parasitemia estimates were used to calculate percentage growth inhibition and IC₅₀s.

3.4.2 Culturing of gametocytes for drug assay

Gametocytes were generated by a modified method of Tanaka and Williamson (2011). Culture was started at 0.2% parasitemia at 6% hematocrit in a T-75 cm³ flask (750 µl of RBCs in 12.5 ml CPM) on day 1. The culture was maintained under standard culture conditions (37°C, 92.5% nitrogen, 2% oxygen and 5.5% carbon dioxide) with the preparation of thin smears to monitor parasite growth. Media was replaced with 12.5 ml CPM on day 3. On days 4-11 media was replaced daily with 25 ml of CPM to reduce hematocrit to 3%. On days 9 –11, the spent media was taken off and replaced with N-Acetyl Glucosamine (NAG) treated CPM (CPM + 50 mM NAG) to get rid of asexual parasites. On day 12, the culture was treated with 60% percoll to obtain synchronous gametocyte. The gametocytes were put back into culture and after 3-4 hours, the gametocytes were transferred unto drug coated 96-well tissue culture plates for early stage gametocyte (I-II) assay. Left over gametocytes was maintained with daily change of NAG treated CPM till day 14. On day 15 a thin smear was prepared to examine the stage of the gametocytes. Late stage gametocytes (III-V) were transferred unto drug coated 96-well plates for late stage gametocyte assay.

3.4.2.1 Purification of gametocytes

This was performed to obtain a $\geq 80\%$ purified gametocytes in culture (Knight and Sinden, 1982). 90% percoll was prepared by adding 3.6 ml of 100% percoll to 0.4 ml parasite wash medium (PWM) in 15 ml falcon tube. The 90% percoll was further diluted to 60% by adding 2 ml PWM. The culture was transferred into a 15 ml tube and centrifuged at 15000 rpm for 10 minutes. The supernatant was discarded and pellets were resuspended in CPM to a 25% haematocrit. The falcon tube was tilted at 60° angle and a sterile Pasteur pipette was used to transfer the 60%

percoll unto the culture slowly ensuring that there was no mixing. The volume of 60% percoll used was twice the volume of the culture. The tube was centrifuged at 1450 rcf for 10 minutes at room temperature without brake using the Sanyo HARRIER 18/80 centrifuge. Parasites were collected at interphase and transferred into a new 15 ml tube. The parasites collected were centrifuged at 1500 rpm for 10 minutes. The supernatant was discarded and resuspended in 3 ml PWM. The tube was centrifuged at 1500 rpm for 10 minutes and supernatant was discarded. Pellets were transferred into a T-25 cm³ flask with 5 ml of CPM with 50 mM N-Acetyl Glucosamine (NAG). About 20 µl of the culture was aliquot into an Eppendorf tube and centrifuged at 3000 rpm for 30 seconds using the Labnet, Spectrafuge 24D centrifuge. The culture was flushed with gas mixture and placed back into the 37°C incubator. Thin smears were made from the aliquot in the Eppendorf tube.

3.4.2.2 Plating of drugs for gametocyte assay

A 96-well tissue culture plate was filled with 10 µl of three different concentrations (100 µg/ml, 7 µg/ml and 1 µg/ml) of each drug to be tested with artesunate (200 µg/ml), primaquine (200 µg/ml), DMSO (1%) serving as controls. The required concentration of each drug was added in triplicate. Gametocytemia was adjusted to 5% by adding appropriate volume of RBCs to the culture. About 90 µl of culture (CPM + 2 µl of RBCs) was aliquot into each treated well. The plates were put into an incubating chamber and gassed for 6 minutes. The incubating chamber was placed into the 37°C incubator. After 72 hours, thin smears were prepared from each well and stained with 10% Giemsa. The slides were dried and observed unto the 100x immersion oil objective lens to estimate gametocytemia.

3.5 Kinetics of herbal antimalarial drugs

Eleven different CPMs were prepared by treatment with the IC₁₀s of the herbal drugs and artesunate. Twelve separate cultures (11 with drug treatments and 1 with no drug treatment) were set up at 1% parasitemia at normal culture conditions (37°C, 92.5% Nitrogen, 5.5 % Carbon dioxide, 2% Oxygen) on day 1. The appropriate modified media was used to replace spent media daily from days 2-8. Thin smears were prepared daily for each culture treatment and observed under the light microscope. The parasitemia and stages of parasites were examined and recorded for each culture treatment. On days 9-14 media was changed every 48 hours and thin smears were prepared with each media change. The parasitemia and stages of the parasites were examined and recorded for each culture treatment.

3.6 Statistical analysis

Data was analysed using Microsoft Excel 2010 and R-Excel 2.15.3.

3.7 Ethical clearance

The study was approved by the Institutional Review Board (IRB) of Noguchi Memorial Institute for Medical Research (NMIMR).

CHAPTER FOUR

RESULTS

4.1. IC₅₀s of herbal drugs

All tested herbal drugs showed IC₅₀s < 12 µg/ml on asexual stages. Top fever showed the lowest IC₅₀ value being 0.06 µg/ml and Taabea showed the highest IC₅₀ value of 11.58 µg/ml. The table below gives the IC₅₀s of the tested drugs in ascending order, using artesunate as a control drug.

Table 2: The herbal drugs showed IC₅₀s significantly higher than artesunate with p<0.05.

DRUG	IC ₅₀ (µg/ml) ± S.D
Artesunate	2.9e-2± 0.002
Top Fever	6.0e-2± 0.028
Masada	2.5e-1± 0.035
Class malakare	3.5e-1± 0.135
Yafo fever	5.3e-1± 0.065
Angel Herbal	1.31 ± 0.160
Rooter Mixture	2.80 ± 0.363
Herbaquin	5.86 ± 0.540
Adutwumwa malamix	7.49 ± 0.248
Kingdom herbal	1.107e+1± 1.010
Taabea	1.158e+1± 2.484

4.2. Kinetics of the herbal drugs

After 14 days of monitoring the cultures to see the progress of the kinetics and dynamics in growth into the production of gametocytes. The cultures were treated with very low concentration i.e. IC_{10} s of the herbal drugs to monitor the rate at which they produce gametocytes

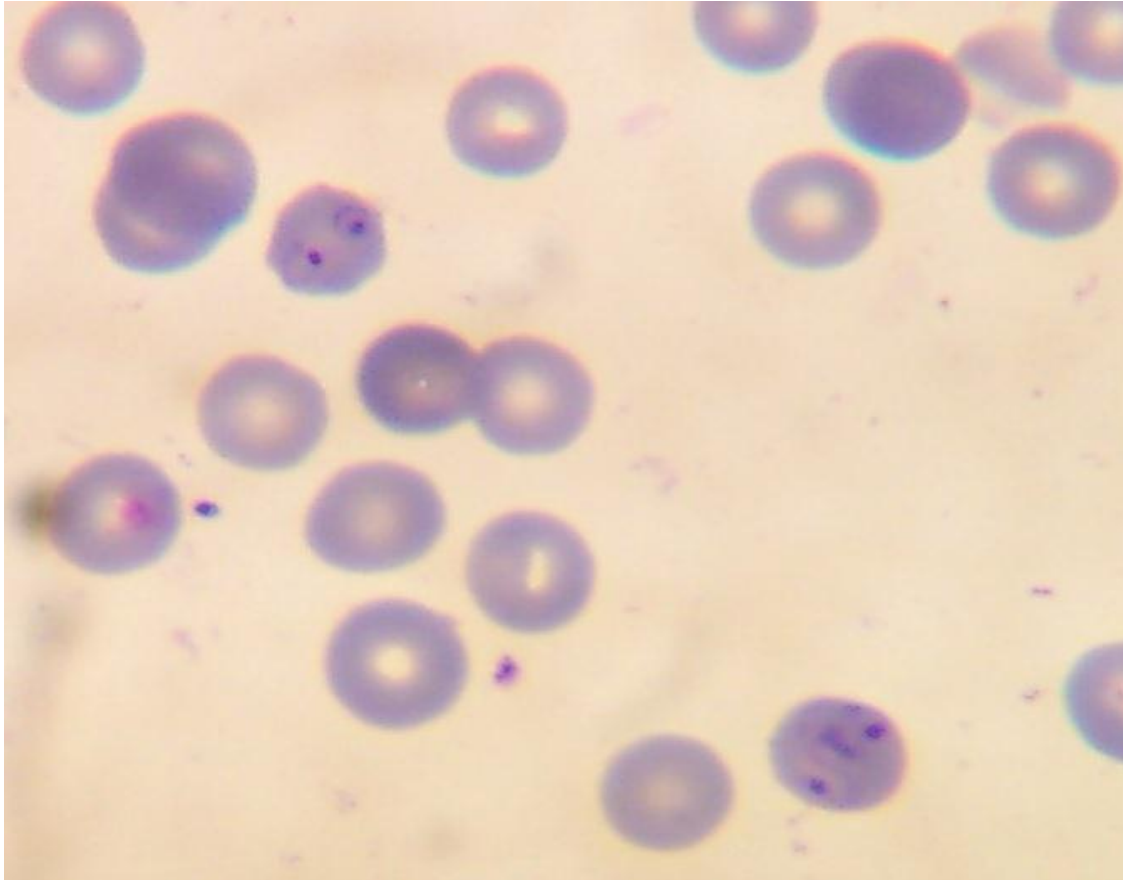


Plate 4: Picture of ring stage *Plasmodium falciparum*

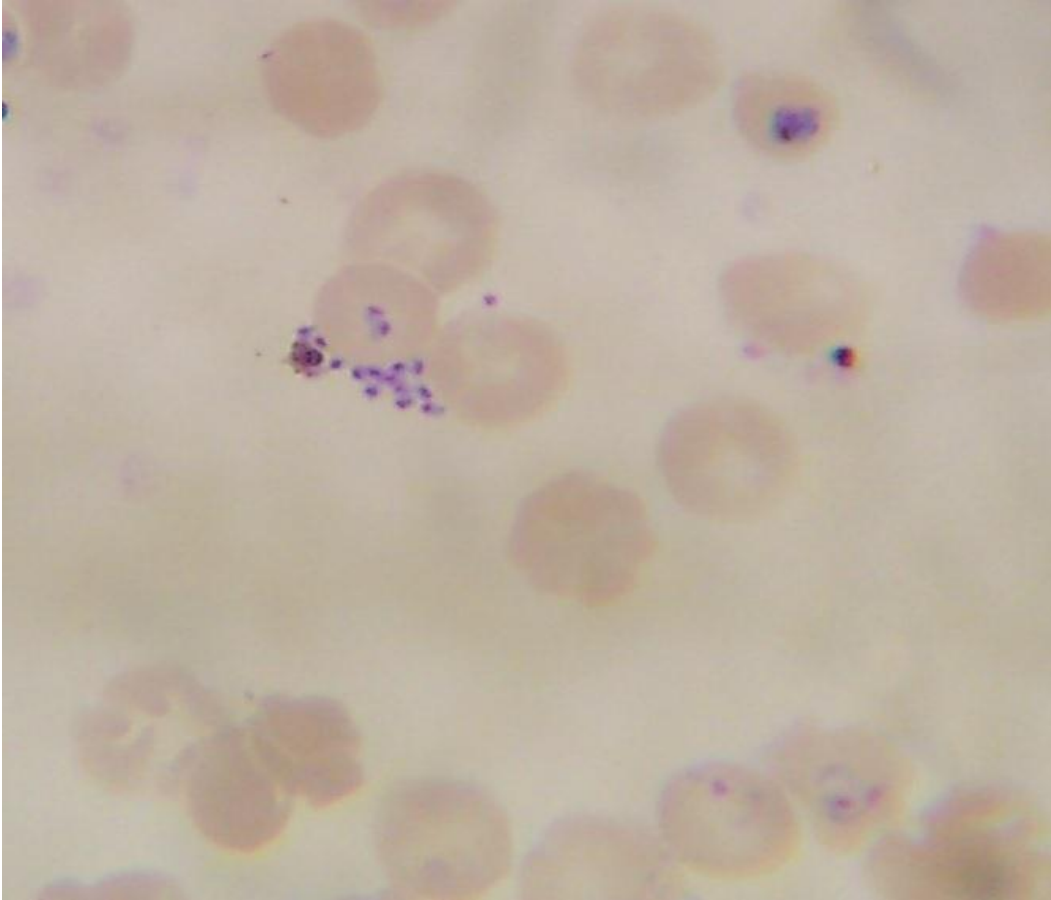


Plate 5: Picture of a ruptured schizont of *Plasmodium falciparum*

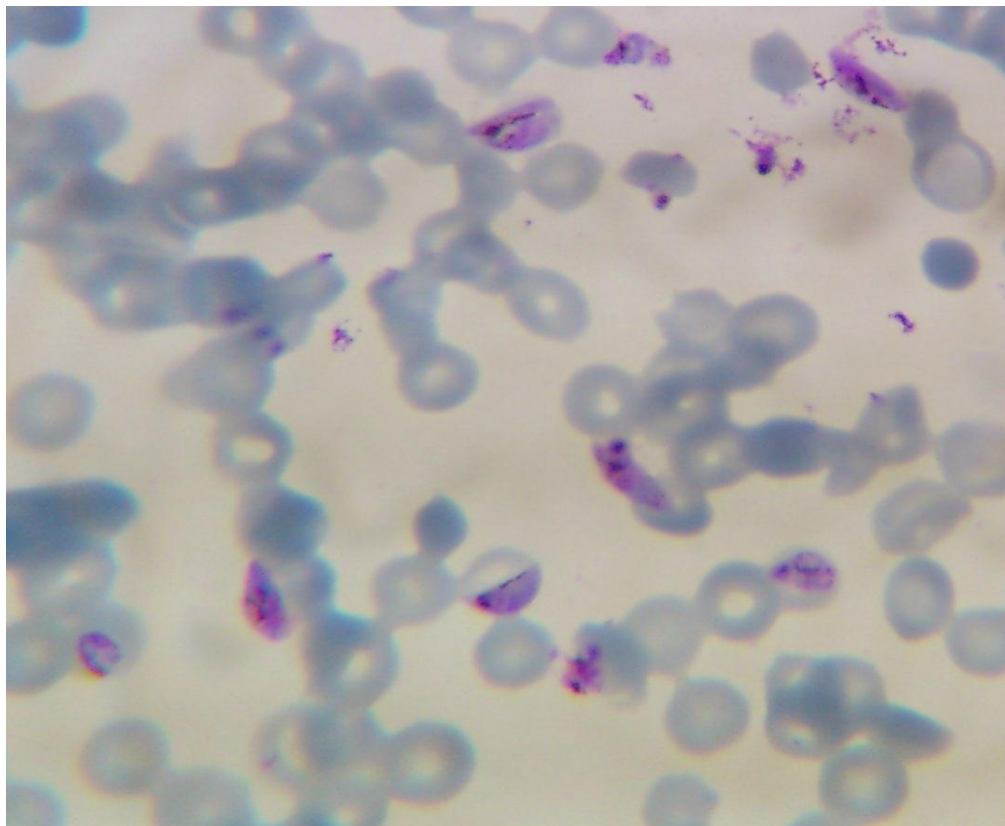


Plate 6: Picture of early stage *Plasmodium falciparum* gametocytes

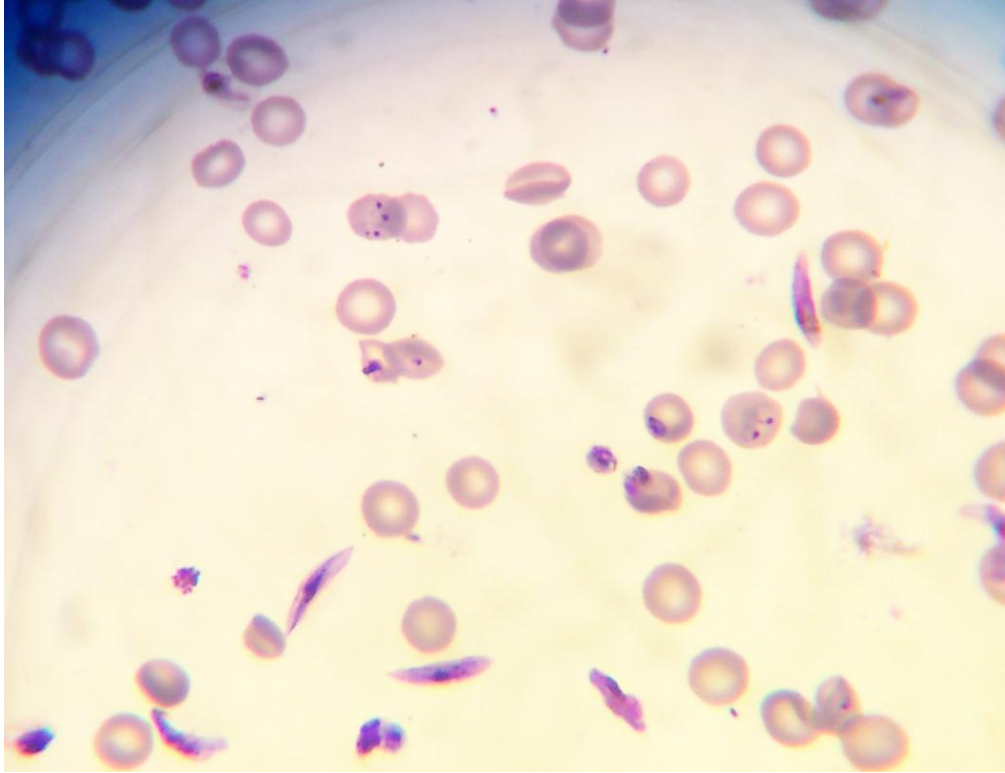


Figure 7: Picture of late stage *Plasmodium falciparum* gametocytes

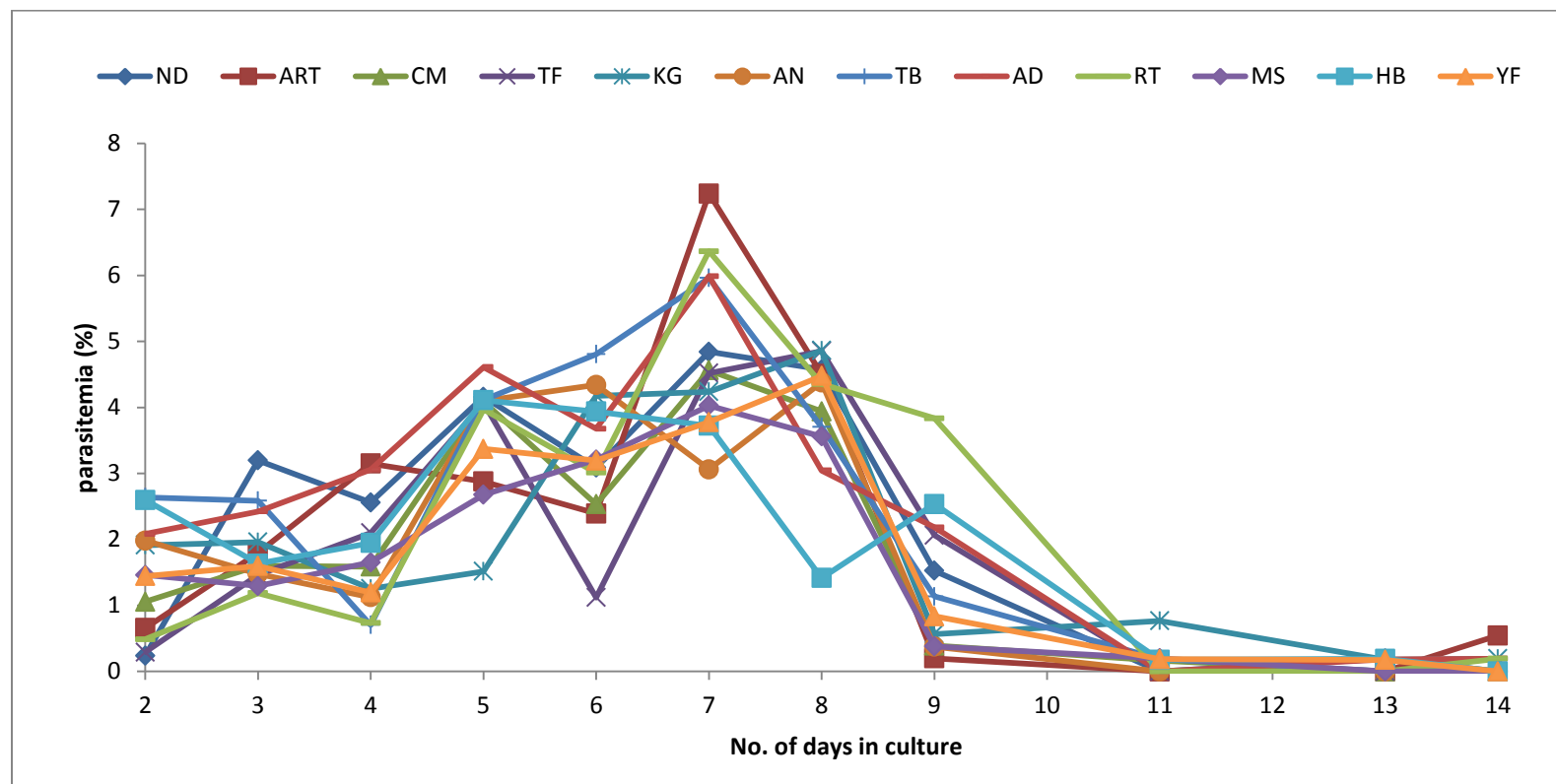


Figure 1: Effect of drug IC₁₀s on the ring stage development

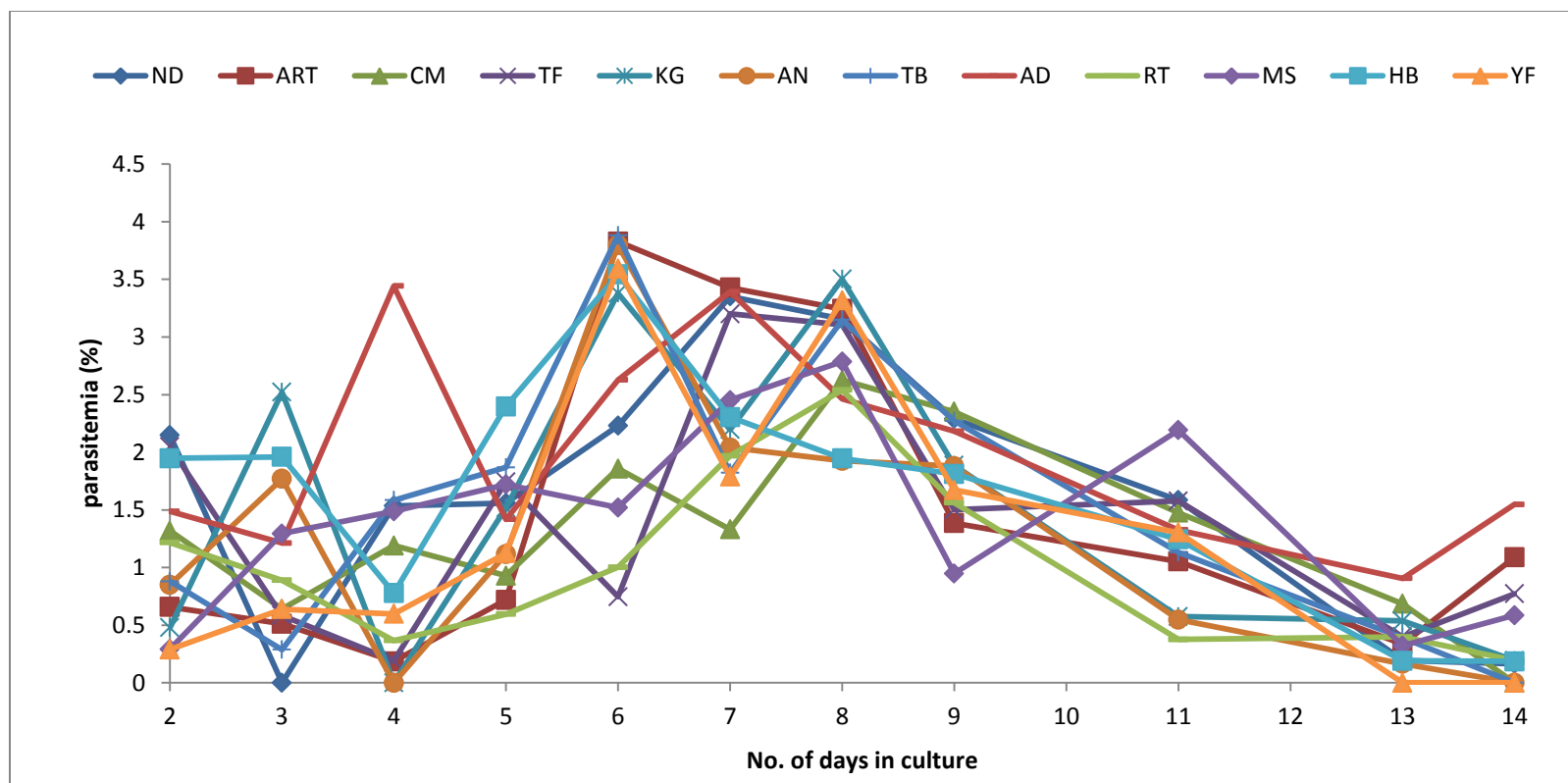


Figure 2: Effects of drug IC₁₀s on trophozoite development

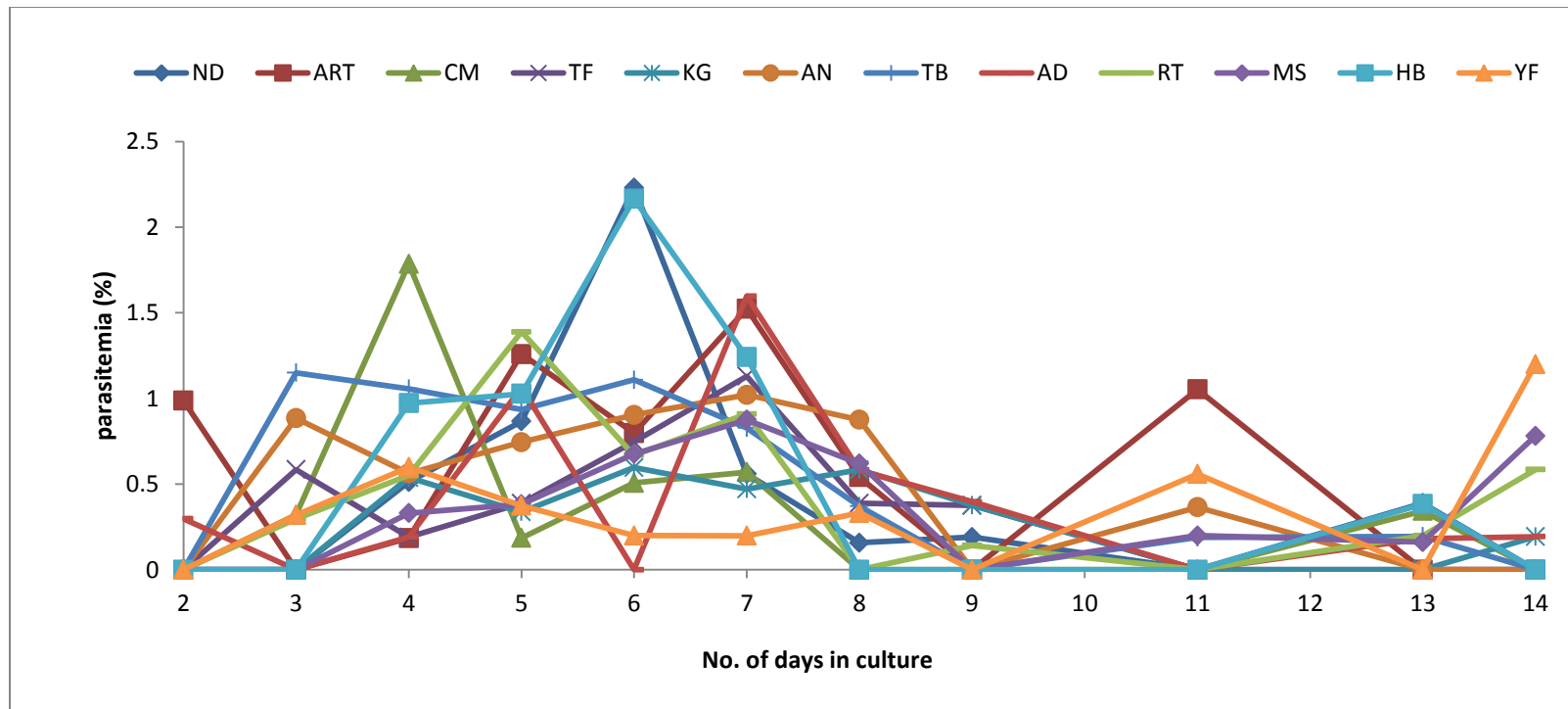


Figure 3: Effects of drug IC₁₀s on schizont development

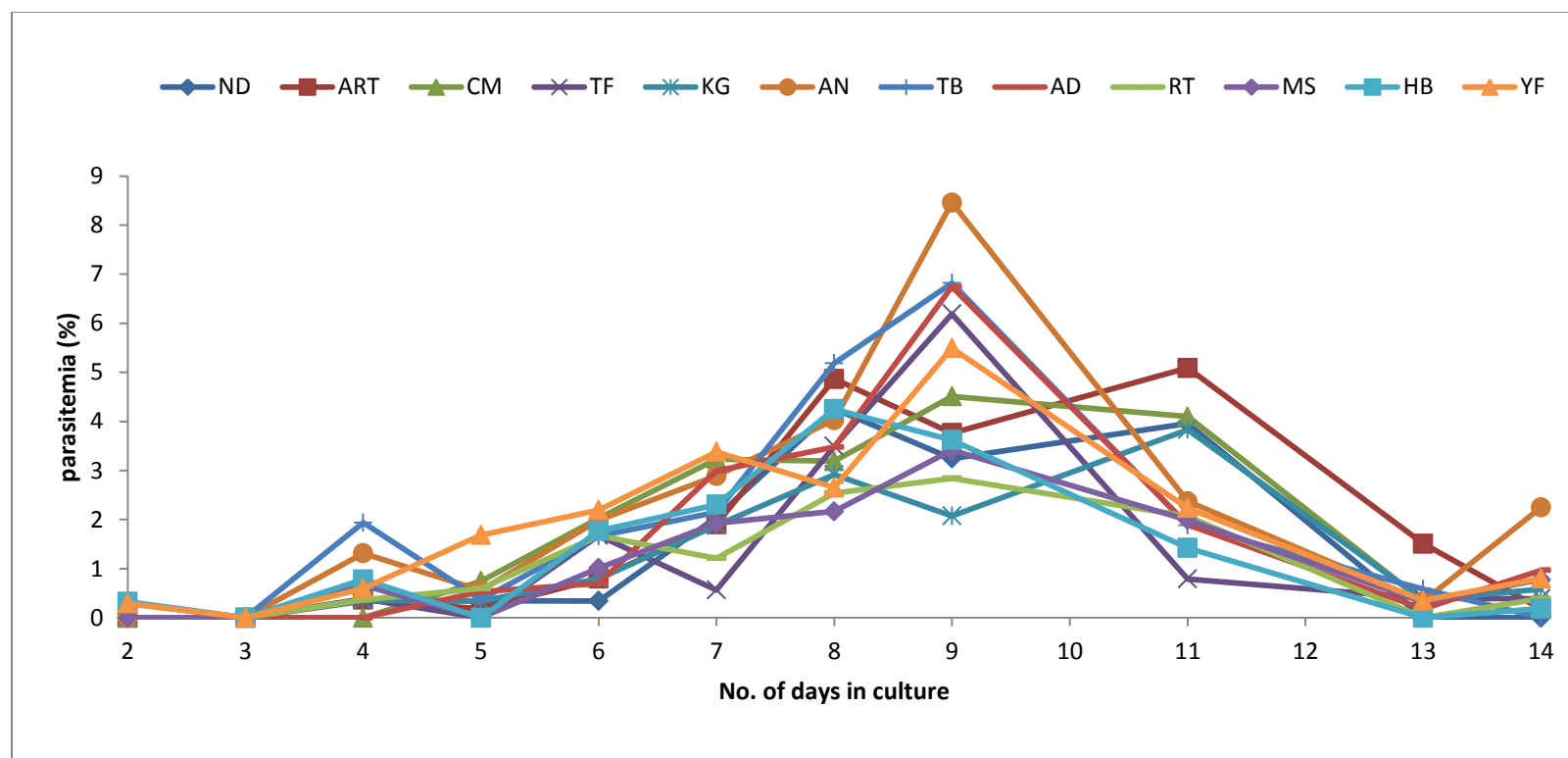


Figure 4: Effects of drug IC₁₀s on early gametocyte development

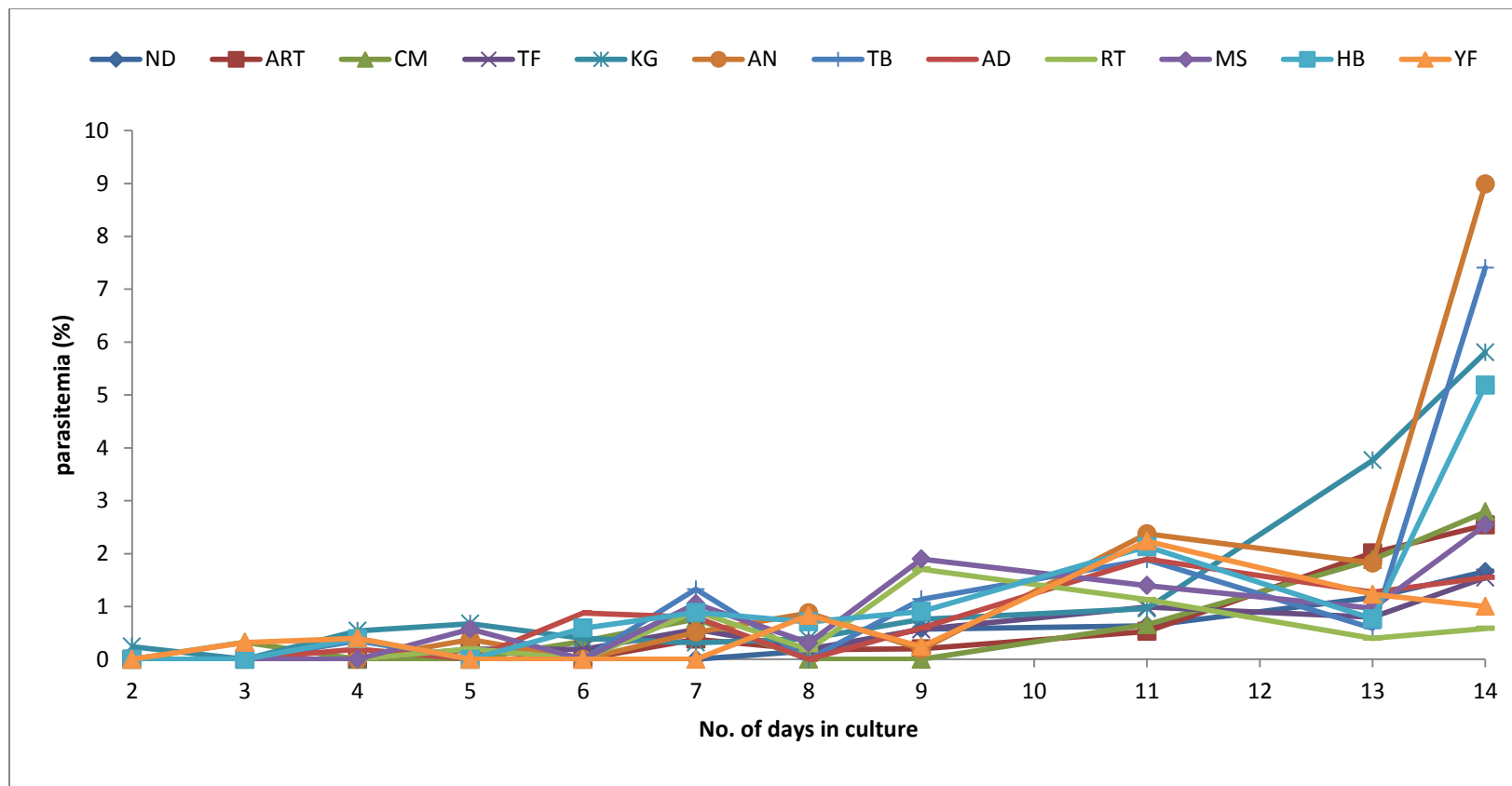


Figure 5: Effects of drug IC₁₀s on late stage gametocyte development

4.3. Early stage growth inhibition by herbal drugs

All the drugs tested, except for Taabea, at 100µg/ml showed more than 50% inhibition of early stage gametocytes. Four drugs; Kingdom herbal, Masada, Yafo fever and Herbaquin at 7 µg/ml showed a more 50% inhibition for early stage gametocytes. At 1µg/ml only Masada and Yafo gave a more than 50% early stage gametocyte inhibition.

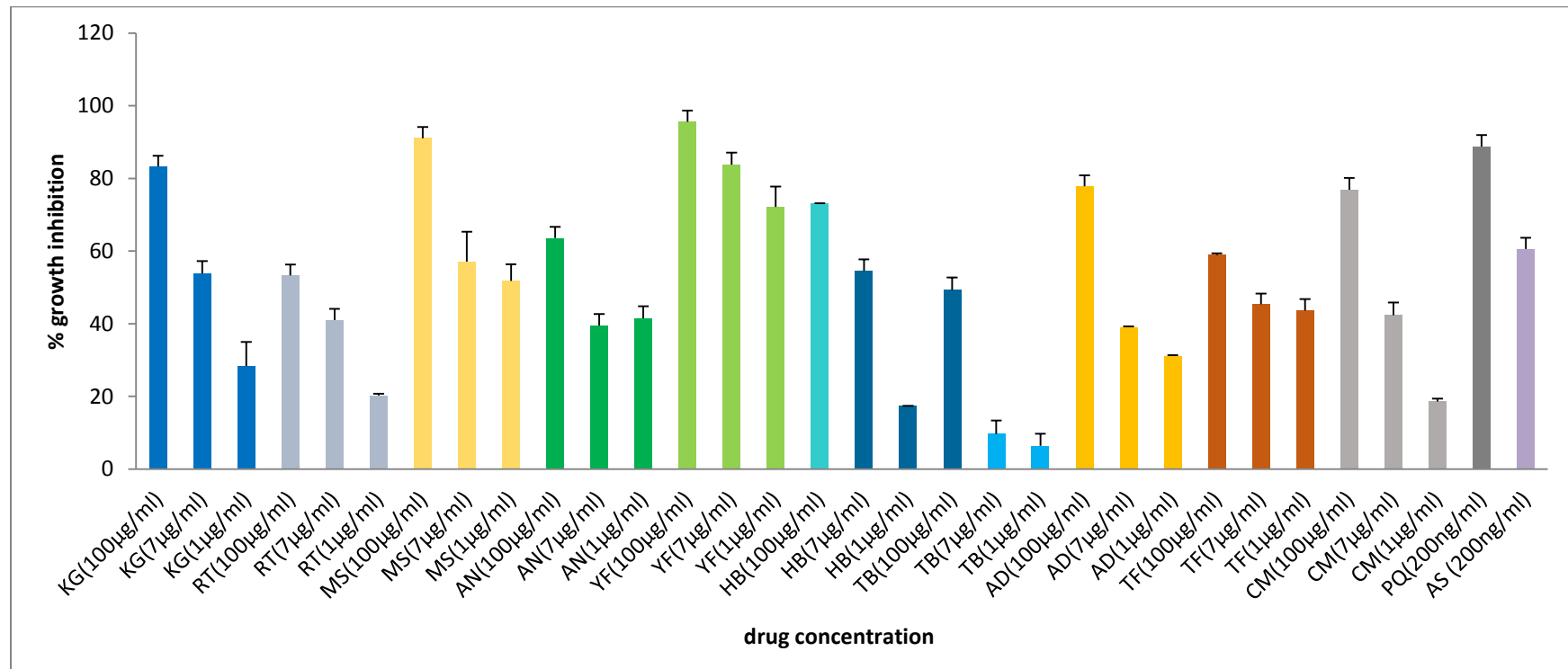


Figure 6: Inhibition of early stage gametocytes by drugs

4.4. Late stage growth inhibition by herbal drugs

Primaquine at 10µg/ml gave a 100% late stage gametocyte growth inhibition. At 100µg/ml, Yafo, Herbaquin, Masada, Top fever, Class malakare and Rooter mixture showed a more than 50% late gametocyte growth inhibition. Only Masada showed $\geq 50\%$ inhibition of late stage gametocytes (III-V) at 7 µg/ml. with none of the herbal drugs show a more than 50% growth inhibition at 1µg/ml

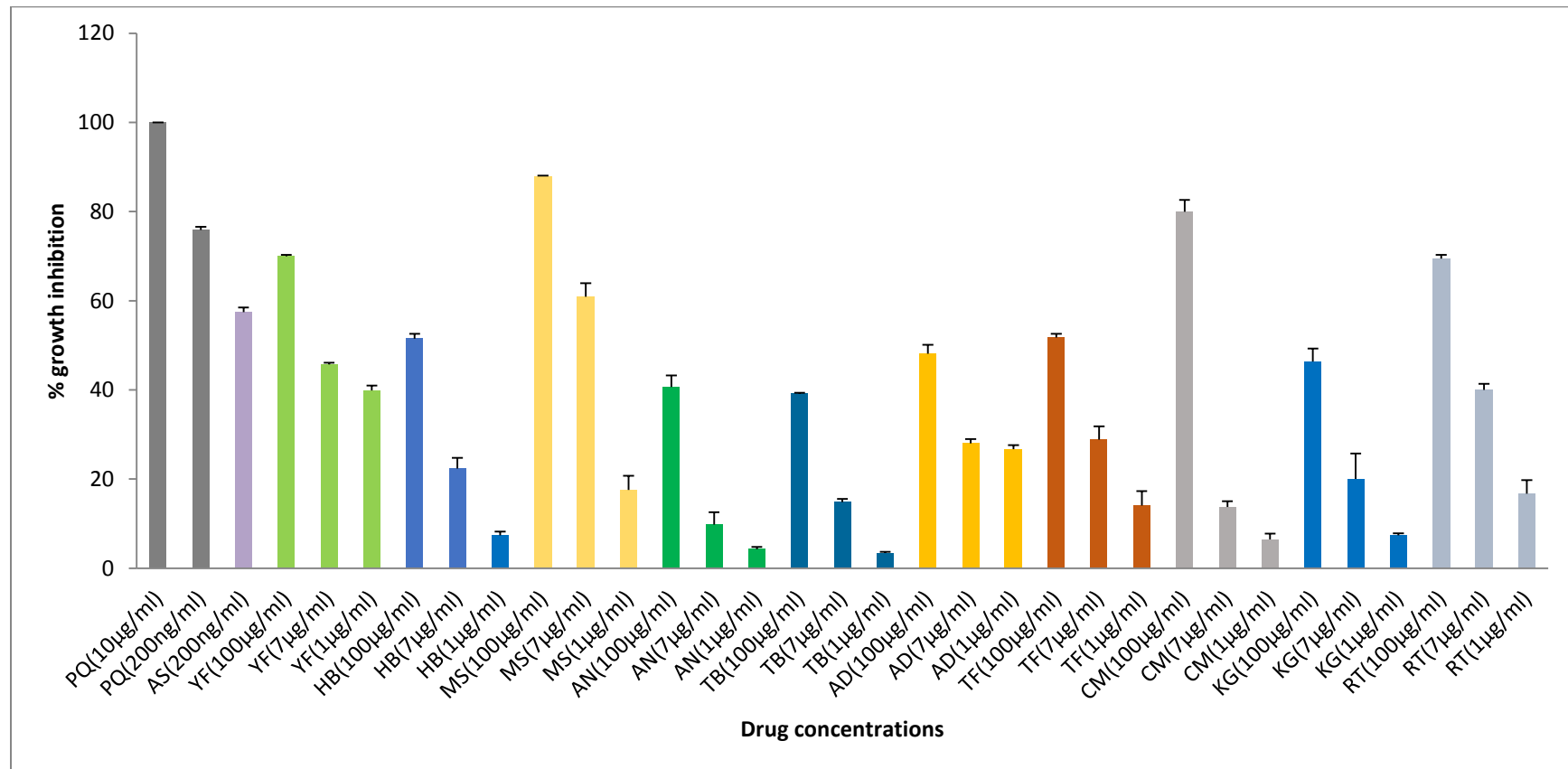


Figure 7: Inhibition of late stage gametocytes by drugs

CHAPTER FIVE

DISCUSSION AND CONCLUSION

5.1 DISCUSSION

Although artemisinin and its analogues have given much help in the treatment of chloroquine-resistant malaria, these drugs are often unavailable and/or unaffordable to many people who live in malaria prone areas. An alternative to manufactured drugs is the use of traditional medicines which are readily available for the treatment of malaria. The discovery of artemisinin and its derivative from the herbal plant *Artemisia annua* and in the last decade has increased interest in the potential of locally grown plants to provide new and more potent drugs in the treatment of malaria and its eradication. The anti-malarial activity of the herbal drugs tested was based on their IC_{50} s on the asexual stage of the *Plasmodium falciparum* parasite. IC_{50} is a measure of how effective a drug is. It indicates how much of a particular drug is needed to inhibit a given biological process or microorganism growth by half. In other words, it is the minimal concentration of a drug that can reduce the growth of parasites by half. Eight of the herbal drugs showed high anti-malarial activity ($IC_{50} \leq 10 \mu\text{g/ml}$) with 2 having moderate anti-malarial activity (between $IC_{50} \geq 10 \mu\text{g/ml}$ and $IC_{50} \leq 50 \mu\text{g/ml}$) (Ramalhete *et al.*, 2008; Sanon *et al.*, 2013). It can therefore be said that the tested drugs were effective in killing malaria parasites.

The IC_{10} of Top fever syrup gave the lowest parasitemia (0.29%) of rings on day 2 which corresponds to it having the lowest IC_{50} value. Taabea also being the least anti-malarial with the highest IC_{50} value gave the highest ring parasitemia (2.64%) on day two. Top fever syrup also had a delayed peak of 4.85% parasitemia on day 8 along with Kingdom herbal, New angel herbal and Yafo fever that also had their peaks on day 8 with parasitemias of 4.86%, 4.39% and 4.48%

respectively. The IC10 of Artesunate gave the highest ring parasitemia of 7.24% but gave a comparatively lower schizont parasitemia of 1.52%, and could be attributed to the schizonticidal effects of artesunate (Li and Weina, 2010; Lin *et al.*, 1987; Mohanty *et al.*, 2004).

All the herbal drugs had a schizont parasitemia < 0.5% on day 2, with Class malakare, Top fever syrup, Kingdom herbal, New angel herbal, Taabea, Rooter mixture, Masada, Herbaquin and Yafo fever giving 0% parasitemia. Herbaquin gave the highest schizont parasitemia of 2.17% on day 6. Comparing the two asexual graphs, the parasitemia for rings were higher than schizont parasitemia suggesting that the anti-malarial effects of the herbal drugs are likely to be schizonticidal. This is confirmed with reported schizonticidal effects of *Azadirachta indica*, a component of the herbal preparation (Dhar *et al.*, 1998; Udeinya *et al.*, 2008). Parasites treated with New angel herbal developed the highest early stage gametocytemia of 8.46% and 2.25% on days 9 and 14 respectively, this may be attributed to the fact that the herbal plants used in the preparation of New angel herbal do not have reported gametocytocidal activities despite *Vernonia amygdalina* being reported to have an anti-malarial effect (Clarkson *et al.*, 2004; Kraft *et al.*, 2003). There were no gametocytes in the presence of Taabea on day 14 however it gave the second highest late stage parasitemia of 7.41%. This could mean there is a rapid transformation from early to late stage gametocytes in the presence of Taabea. Even though Taabea is composed of *Azadirachta indica* which has reported gametocytocidal activities (Dhar *et al.*, 1998; Lucantoni *et al.*, 2010; MacKinnon *et al.*, 1997; Udeinya *et al.*, 2006), it is likely that the added herbal plants lowered the gametocytocidal effects of the neem plant.

Parasite cultures treated with New angel herbal gave the highest late stage gametocytemia of 8.99% on day 14, this coupled with it being the herbal drug with the highest early stage

gametocytemia would imply that New angel herbal is gametocytogenic. Parasite cultures treated with Rooter mixture gave the lowest late gametocytemia of 0.58% on day 14. Yafo fever and Top fever treated parasite cultures gave the second and third lowest late stage gametocyte levels of 1.00% and 1.54% respectively. These results suggest that these drugs when taken in low concentrations will reduce gametocyte production as compared to Herbaquin, Kingdom herbal, Taabea and New angel herbal. These herbal drugs at low concentrations in parasite cultures have higher gametocyte production than artesunate which is known to be gametocytocidal (Bhatt *et al.*, 2006).

At a concentration of 100 µg/ml, all herbal drugs gave a more than 50% inhibition of early stage gametocytes. Yafo fever at 100 µg/ml concentration gave the highest early stage gametocyte inhibition of 95.45%. Yafo fever also recorded the highest inhibition of early stage gametocytes at 7 µg/ml and 1 µg/ml with 83.78% and 72.13% respectively. Recording an IC_{50} at 0.53 µg/ml would imply that Yafo fever has good effect on asexual stages of the parasite and early stage gametocytocidal effects too. Masada inhibited early stage gametocytes by 91.04%, 57.11% and 51.81% at 100 µg/ml, 7 µg/ml and 1 µg/ml respectively. With Masada exhibiting an IC_{50} of 0.25 µg/ml, it would imply that it has gametocytocidal effects on early stage gametocytes. Taabea showed the lowest inhibition for early stage gametocytes, having inhibitions of 49.26%, 9.62% and 6.39% at 100 µg/ml, 7 µg/ml and 1 µg/ml respectively.

Yafo, Herbaquin, Masada, top fever, Masada, Class malakare and Rooter mixture gave a higher than 50% inhibition of late stage gametocytes at 100 µg/ml with Masada giving the highest inhibition of 87.91%. Masada also gave the highest late stage inhibition among the ten drugs at 60.86% at 7 µg/ml. Masada's gametocytocidal activities can be linked to the only herbal plant

that it is composed of *Cryptolepis sanguinolenta*. Even though there are no reported gametocytocidal effects on this plant, it has high antimalarial activity and cryptolepine, a compound derived from the plant is a very potent antimalarial compound which is currently under study (Kumar *et al.*, 2003; Wright, 2005; Yarnell and Abascal, 2004). At 1 µg/ml Yafo fever gave the highest late stage inhibition of 39.87%. Taabea gave the lowest late stage gametocyte inhibition (39.13%) at 100 µg/ml and 3.42% for 1 µg/ml. New angel herbal mixture gave a below 50% inhibition at 100 µg/ml, also giving the lowest value of 9.77% at 7 µg/ml and 4.25% at 1 µg/ml. Class malakare showed a high late stage gametocyte inhibition (79.93%) at 100 µg/ml but low inhibitions of 13.74% and 6.44% at 7 µg/ml and 1 µg/ml respectively. The trend in Class malakare could not be accounted for.

Primaquine and artesunate both at 200 µg/ml (i.e. 0.77 µM for primaquine and 0.52µM for artesunate) showed a more than 50% growth inhibition of early and late stage parasitemia. This is in line with literature about the gametocytocidal activities of both orthodox drugs (Price *et al.*, 1996). Artesunate has reported a much lower IC₅₀ value of 108nM for *Plasmodium falciparum* gametocytes (Benoit-Vical *et al.*, 2007). Increasing the concentration of Primaquine to 10 µg/ml (38.56µM) showed a 100% growth inhibition of late stage gametocytes. This is supportive of reported IC₅₀ values of primaquine and artemisinin (from which artesunate is derived) of 17.6 µM and 1.0 µM, respectively (Chevalley *et al.*, 2010).

From table 1, it can be deduced that all the herbal drugs tested had an active to moderate antimalarial activity (IC₅₀ < 20µg/ml) and would be effective in the eliminating of asexual stage parasites. However, Figures 4-7 show New angel herbal mixture, kingdom herbal and Taabea to be gametocytogenic whiles Yafo fever and Masada have produced gametocytocidal effects due to the strong gametocytocidal effects of *Cryptolepis sanguinolenta* and *Azadirachta indica*.

Since all the drugs were highly or moderately active against asexual stages of *P. falciparum*, those that were not gametocytocidal will potentially be aiding the transmission of malaria parasites even after patients have been cleared of clinical symptoms.

Top fever had the lowest IC_{50} value of 0.06 ± 0.028 $\mu\text{g/ml}$ making it the most effective herbal drug on the asexual stages of *Plasmodium falciparum*. This drug is composed of two plants; *Azadirachta indica* and *Alstonia boonei*, which have been widely used in the treatment of malaria in Ghana (Asase and Oppong-Mensah, 2009; Asase *et al.*, 2010). *Alstonia boonei* tested on the chloroquine-resistant FcB1/Colombia strain showed inactivity ($IC_{50} > 50$ $\mu\text{g/ml}$) (Zirihi *et al.*, 2005). However another test using the same chloroquine-resistant FcB1/Columbia strain showed a moderate anti-malarial activity with an IC_{50} value of 12.3 ± 0.2 $\mu\text{g/ml}$ (Guédé *et al.*, 2010). Different plant varieties may have accounted for the different values in IC_{50} since both experiments used ethanol extracts of *Alstonia boonei* with similar drying techniques. *Azadirachta indica* extracts tested on chloroquine resistant DD2 and chloroquine sensitive 3D7 strains of *Plasmodium falciparum* showed high anti-malarial activity with IC_{50} values of 1.7 $\mu\text{g/ml}$ and 5.8 $\mu\text{g/ml}$ respectively (El Tahir *et al.*, 1999). Against the chloroquine-resistant W2 Vietnam strain of *Plasmodium falciparum*, *Azadirachta indica* showed a high anti-malarial activity of 4.7 $\mu\text{g/ml}$ (Hout *et al.*, 2006). The high anti-malarial activity of Top fever could mainly be due to the *Azadirachta indica* constituent or a possible unreported anti-malarial synergy between *Alstonia boonei* and *Azadirachta indica*.

Yafo fever, Masada mixture and Class malakare exhibited IC_{50} values ≤ 1 $\mu\text{g/ml}$ showing very high anti-malarial activities. All three drugs have the herbal plant *Cryptolepsis sanguinolenta* in them. Apart from Masada that is made up of only *Cryptolepsis sanguinolenta*, Yafo fever and

Class malakare combined *Cryptolepis sanguinolenta* with *Azadirachta indica* and *Carapa procera* respectively. *Cryptolepis sanguinolenta* is used in the traditional treatment of malaria in West Africa (Tempesta, 2010). *Carapa procera* is not a known antimalarial drug but there has been reports of its anti-filarial properties (Titanji *et al.*, 1990). *In vitro* studies of *C. sanguinolenta* alkaloid isolates Cryptolepine and Isocryptolepine gave IC₅₀ ranging between 0.2 - 0.8 µM, making the plant a very potent anti-malarial drug (Cimanga *et al.*, 1997; Grellier *et al.*, 1996). The high antimalarial activity of these three herbal preparations can be attributed to the strong anti-malarial activity of *Cryptolepis sanguinolenta* for Yafo fever and Class malakare. The potency of Masada mixture may be attributed to the combined anti-plasmodial effect of both *Cryptolepis sanguinolenta* and *Azadirachta indica*.

New Angel Herbal and Rooter Mixture showed a high anti-plasmodial activity with IC₅₀ ≥ 1 µg/ml but ≤ 5 µg/ml. Both drugs have completely different herbal compositions. New Angel Herbal is made up of *Cola gigantean*, *Solanum torvum*, *Spathodea campanulata*, *Bombax buonopozense* and *Vernonia amygdalina* whiles Rooter Mixture is made up of *Aloe schweinfurthii*, *Khaya senegalensis*, *Pileostigma thornnigii* and *Cassia siamea*. Even though *Cola gigantean* and *Solanum torvum* have anti-microbial and anti-inflammatory properties (Agyare *et al.*, 2012; Chah *et al.*, 2000), there is no *in vitro* anti-plasmodial report on these plants. Experiments with ethanol extracts of *Solanum torvum* have shown high anti-plasmodial activity with IC₅₀ of 7.5±1.25 µg/ml (Dhanabalan, 2008). *Bombax buonopozense* has shown anti-plasmodial activity in mice against *Plasmodium berghei* (Akuodor *et al.*, 2012; Iwuanyanwu *et al.*, 2013). *Vernonia amygdalina* when tested *in vitro* showed (5 < IC₅₀ < 10 µg/ml) which is lower compared to the IC₅₀ of New angel herbal (Tona *et al.*, 2004). The anti-plasmodial activity for New Angel Herbal can be attributed to the high anti-malarial activity of *Vernonia*

amygdalina. Despite a vast array of medicinal uses for related species of *Aloe schweinfurthii* (Eshun and He, 2004), there has been no report of the plant's anti-plasmodial activity *in vitro*. *In vitro* studies with *Khaya senegalensis*, *Pileostigma thonningii* and *Cassia siamea* have shown these plants to have high anti-malarial properties with their extracts giving $IC_{50} < 5 \mu\text{g/ml}$ (El Tahir *et al.*, 1999), IC_{50} within 6.20 - 15.06 $\mu\text{g/ml}$ range (Kwaji *et al.*, 2010), and $IC_{50} = 5 \mu\text{g/ml}$ (Ajaiyeoba *et al.*, 2008) respectively. These IC_{50} values will be the basis for the high anti-malarial activity of Rooter mixture.

Adutwumwa malamix and Herbaquin had $5 \mu\text{g/ml} < IC_{50} < 10 \mu\text{g/ml}$. Herbal plants used in Adutwumwa malamix are *Anthocleista nobilis*, *Vitex grandifolia* and *Phyllanthus fratermus*. Apart from *Vitex grandifolia* that had no records of *in vitro* anti-malarial activity, extracts of related species of *Phyllanthus fratermus* when tested on chloroquine sensitive 3D7 strain of *Plasmodium falciparum* *in vitro* showed IC_{50} s of 7.25 $\mu\text{g/mL}$ (Bagavan *et al.*, 2011) which corresponds to IC_{50} value attained in this study. When *Anthocleista nobilis* ethanoic extracts were tested on chloroquine resistant K1 *Plasmodium falciparum* strain *in vitro*, IC_{50} value of 10.8 $\mu\text{g/ml}$ was obtained (Sanon *et al.*, 2013). Therefore the active anti-malarial effect of Adutwumwa malamix can be attributed to *Anthocleista nobilis* and *Phyllanthus fratermus*. Herbaquin is made of *Cryptolepis sanguinolenta*, *Alstonia boonei*, *Azadirachta indica*, *Monodora myristica* and *Xylopia aethiopica*. The anti-malarial activities of *Azadirachta indica*, *Alstonia boonei* and *Cryptolepis sanguinolenta* contribute to the anti-malarial effect of Herbaquin (Cimanga *et al.*, 1997; Grellier *et al.*, 1996; Hout *et al.*, 2006; Zirihi *et al.*, 2005), since records show that *Monodora mysritica* and *Xylopia aethiopica* demonstrate only anti-bacterial and anti-fungal properties with no anti-malarial effect (Tatsadjieu *et al.*, 2003).

Kingdom herbal and Taabea were the two drugs that showed moderate anti-malarial activity of 11.07 $\mu\text{g/ml}$ and 11.58 $\mu\text{g/ml}$ respectively. In addition to *Phyllanthus fratemus* and *Cryptolepsis sanguinolenta*, Kingdom herbal includes *Nauclea latifolia* which in previous experiments by Ajaiyeoba *et al.* (2005) was far less active towards malaria parasites as it showed IC_{50} of 478.9 $\mu\text{g/ml}$. This is higher than the recorded IC_{50} for Kingdom herbal of which *Nauclea latifolia* is a constituent. Taabea is composed of *Ocimum viride*, *Azadirachta indica*, *Paullinia pinnate*, *Tetrapleura tetraptera*, *Theobroma cacao*, *Cymbopogon citratus* and *Moringa oleifera*. *Ocimum viride* is known for its anti-fungal and anti-bacterial properties (Hammer and Carson, 2011). *Paullinia pinnate*, *Tetrapleura tetraptera* and *Theobroma cacao* though have been used for traditional treatment of malaria, do not have IC_{50} records of their extracts (Batista *et al.*, 2009; Gessler *et al.*, 1994; Idowu *et al.*, 2010). The moderate anti-malarial property of Taabea can be attributed to additional effects of *Cymbopogon citratus* which have a reported IC_{50} of 12.1 $\mu\text{g/ml}$ (Melariri *et al.*, 2011). *Moringa oleifera* could not have contributed to the moderate antimalarial activity of Taabea due to the reports of its relatively low anti-malarial activity as it an IC_{50} value of more than 50 was recorded by Köhler *et al.* (2002).

Not all the herbal plants used in the herbal drugs have reported anti-malarial activity. This may be due to the fact that some of these drugs have indications for other ailments. Only five (Masada mixture, Herbaquin, Top fever syrup, Kingdom mixture and Yafo fever mixture) out of the ten drugs had indications for malaria alone. Only Masada out of the five had only one herbal plant for only malaria treatment. Masada mixture being made of only *Cryptolepsis sanguinolenta* whose extracts have high anti-malarial activity accounts for the drug's potency (Kirby *et al.*, 1995). Herbaquin, despite being indicative for only malaria, is composed of five plants out of which only three are known anti-malarial drugs. Taabea showed the lowest anti-malarial activity

among the ten drugs also had the highest number of herbal plant composition and highest number of disease indications.

5.2 CONCLUSION

All drugs tested showed potent antimalarial activity, however only Yafo fever and Masada showed very strong gametocytocidal activities. Such drug effects will reduce the transmission of the plasmodium parasite among its hosts. Even though Taabea and New angel fever syrup had anti-malarial effects on the asexual stages of the parasite, they were found to promote gametocyte growth and as such must not be used alone.

RECOMMENDATIONS

Based on the data gathered from this experiment, advocacy for the use of Yafo fever and Masada to achieve malarial eradication should be encouraged and incorporated into malaria control programmes. However, there is the need to test the herbal drugs on field isolates to make a much stronger claim for the drugs gametocytocidal potency. The gametocytocidal effects of cryptolepine, an isolated compound from *Cryptolepis sanguinolenta* should be screened since Masada showed strong gametocytocidal effects and was made up of only *Cryptolepis sanguinolenta*.

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APPENDIX 1

MATERIALS AND EQUIPMENT

Equipment

Bio safety cabinet (SterilGARD Hood, the BARKER COMPANY Inc.)

Water bath (Thomastat Shaker T-225) set at 37°C

Centrifuge (Sanyo HARRIER 18/80 and Labnet, Spectrafuge 24D)

Modular incubator chamber

Incubator (RS Biotech Galaxy S) set at 37°C

Electric vacuum pump

Light Microscope

Refrigerator

Freezer

Weighing balance (AND EK-3000i)

Vortex (Fisher Scientific)

Oven (Bakura, Clini oven TF-31) set at 70°C

Double water distillation unit (Fistreem International Ltd)

Cell counters

Freeze-drier (LABCONCO Freezone⁶)

Materials

Weigh boat

96 well Tissue Culture plate (CytoOne)

5ml serological pipettes

10ml serological pipettes

25ml serological pipettes

20µl pipette tips

200µl Pipette tips

1000µl pipette tips

Pasteur pipettes

Plastic aspirators

Cryo vials

Nalgene Filter unit 500ml

15ml centrifuge tubes

50ml centrifuge tubes

25 cm³ tissue culture flask

75 cm³ tissue culture flask

10ml Syringes

Microscope slides

Tissue

Non-powdered Gloves

0.2µm Filters

Slide staining trough

Aluminium foil

Biohazard disposable bags

Blood Lancets

ACD vacutainers

APPENDIX 2

PREPARATION OF MEDIA AND REAGENTS

Thawing mix

3.5 grams of Sodium Chloride (NaCl) was weighed and transferred into a conical flask with 100ml of distilled water. The solution was made to dissolve and transferred into the biosafety cabinet (BSC). The solution was filtered into two 50ml falcon tubes using a needle, syringe and 0.22µm filter. The thawing mix was stored at 4°C.

Freezing mix

4.2grams of sorbitol and 0.9 grams of NaCl were dissolved in 100ml of distilled. 72ml of the solution was added to 28ml of glycerol. The solution was sent into the BSC and sterile filtered into falcon tubes using 0.22µm filters. The freezing mix was stored at 4°C.

7.5% sodium hydrogen carbonate (NaHCO₃)

7.5g of Sodium Hydrogen Carbonate (NaHCO₃) was weighed and dissolved into 100ml of distilled water in a conical flask. The solution was sent into the biosafety cabinet and filtered using a 0.2µm filter. The solution was stored at 4°C.

20% glucose

20g of glucose was weighed and dissolved in 100ml distilled water in a conical flask. The solution was transferred into the biosafety cabinet and filtered using a 0.2µm filter. The solution was stored at 4°C

Albumax

0.2g of Hypoxanthine was dissolved in 1L of RPMI 1640 in a conical flask. 50g of Albumax powder was added to the solution and placed on a magnetic stirrer till everything dissolved. The

albumax solution was transferred into the BSC and filtered using a 0.8µm filtering unit and an electric vacuum pump. The solution was filtered again using a 0.2µm filtering unit and an electric vacuum pump. The albumax was then aliquoted into 50ml falcon tubes. The aliquots were stored below -20°C.

Incomplete media (ICPM) or parasite wash media (PWM)

In the BSC, 13.5ml of 7.5% Sodium hydrogen carbonate (NaHCO₃), 5ml of 20%Glucose, 0.5ml of 50mg/ml gentamycin were added to 500ml of RPMI-1640(with L-glutamine). The solution is mixed thoroughly and kept at 4°C.

Complete medium

20ml of Albumax was added to 180ml of incomplete media.

Complete media + 2%NHS

1ml of Normal Human Serum (NHS) was added to 49ml of CPM in a 50ml falcon tube. It was stored at 4°C.

Complete media + NAG

0.56g of N-Acetyl Glucosamine powder was weighed and dissolved in 50ml of CPM. The solution was sterile filtered using a 0.2µm filter. It was stored at 4°C.

Preparation of 5% sorbitol

5g of sorbitol was weighed and dissolved in 100ml of distilled water in a conical flask. The solution was transferred into the biosafety cabinet and filtered using a 0.2µm filter. It was stored at 4°C

APPENDIX 3

PLATE DESIGNS

Plate design for asexual assay

	1	2	3	4	5	6	7	8	9	10	11	12
A	CPM	CPM	CPM	CPM	CPM	CPM	CPM	CPM	CPM	CPM	CPM	CPM
B	CPM	YF (100µg/ml)	YF (100µg/ml)	YF (100µg/ml)	HB (25µg/ml)	HB (25µg/ml)	HB (25µg/ml)	AS (0.11µg/ml)	AS (0.11 µg/ml)	AS (0.11 µg/ml)	RBC	CPM
C	CPM	YF (25µg/ml)	YF (25µg/ml)	YF (25µg/ml)	HB (6.25µg/ml)	HB (6.25µg/ml)	HB (6.25µg/ml)	AS (0.03µg/ml)	AS (0.03 µg/ml)	AS (0.03 µg/ml)	RBC	CPM
D	CPM	YF3 (6.25µg/ml)	YF3 (6.25µg/ml)	YF3 (6.25µg/ml)	HB (1.56µg/ml)	HB (1.56µg/ml)	HB (1.56µg/ml)				RBC	CPM
E	CPM	YF4 (1.56µg/ml)	YF4 (1.56µg/ml)	YF4 (1.56µg/ml)	HB (0.39µg/ml)	HB (0.39µg/ml)	HB (0.39µg/ml)				IRBC	CPM
F	CPM	YF5 (0.39µg/ml)	YF5 (0.39µg/ml)	YF5 (0.39µg/ml)	AS (1.8µg/ml)	AS (1.8µg/ml)	AS (1.80µg/ml)				IRBC	CPM
G	CPM	HB (100µg/ml)	HB (100µg/ml)	HB (100µg/ml)	AS (0.45µg/ml)	AS (0.45µg/ml)	AS (0.45µg/ml)				IRBC	CPM
H	CPM	CPM	CPM	CPM	CPM	CPM	CPM	CPM	CPM	CPM	CPM	CPM

Table 3: Plate 1 for asexual assay

	1	2	3	4	5	6	7	8	9	10	11	12
A	CPM	CPM	CPM	CPM	CPM	CPM	CPM	CPM	CPM	CPM	CPM	CPM
B	CPM	MS (100µg/ml)	MS (100µg/ml)	MS (100µg/ml)	AN (25µg/ml)	AN (25µg/ml)	AN (25µg/ml)	AS (0.11µg/ml)	AS (0.11 µg/ml)	AS (0.11 µg/ml)	RBC	CPM
C	CPM	MS (25µg/ml)	MS (25µg/ml)	MS (25µg/ml)	AN (6.25µg/ml)	AN (6.25µg/ml)	AN (6.25µg/ml)	AS (0.03µg/ml)	AS (0.03 µg/ml)	AS (0.03 µg/ml)	RBC	CPM
D	CPM	MS (6.25µg/ml)	MS (6.25µg/ml)	MS (6.25µg/ml)	AN (1.56µg/ml)	AN (1.56µg/ml)	AN (1.56µg/ml)				RBC	CPM
E	CPM	MS (1.56µg/ml)	MS (1.56µg/ml)	MS (1.56µg/ml)	AN (0.39µg/ml)	AN (0.39µg/ml)	AN (0.39µg/ml)				IRBC	CPM
F	CPM	MS (0.39µg/ml)	MS (0.39µg/ml)	MS (0.39µg/ml)	AS (1.8µg/ml)	AS (1.8µg/ml)	AS (1.80µg/ml)				IRBC	CPM
G	CPM	AN (100µg/ml)	AN (100µg/ml)	AN (100µg/ml)	AS (0.45µg/ml)	AS (0.45µg/ml)	AS (0.45µg/ml)				IRBC	CPM
H	CPM	CPM	CPM	CPM	CPM	CPM	CPM	CPM	CPM	CPM	CPM	CPM

Table 4: Plate 2 for asexual assay

	1	2	3	4	5	6	7	8	9	10	11	12
A	CPM	CPM	CPM	CPM	CPM	CPM	CPM	CPM	CPM	CPM	CPM	CPM
B	CPM	TB (100µg/ml)	TB (100µg/ml)	TB (100µg/ml)	AD (25µg/ml)	AD (25µg/ml)	AD (25µg/ml)	AS (0.11µg/ml)	AS (0.11 µg/ml)	AS (0.11 µg/ml)	RBC	CPM
C	CPM	TB (25µg/ml)	TB (25µg/ml)	TB (25µg/ml)	AD (6.25µg/ml)	AD (6.25µg/ml)	AD (6.25µg/ml)	AS (0.03µg/ml)	AS (0.03 µg/ml)	AS (0.03 µg/ml)	RBC	CPM
D	CPM	TB (6.25µg/ml)	TB (6.25µg/ml)	TB (6.25µg/ml)	AD (1.56µg/ml)	AD (1.56µg/ml)	AD (1.56µg/ml)				RBC	CPM
E	CPM	TB (1.56µg/ml)	TB (1.56µg/ml)	TB (1.56µg/ml)	AD (0.39µg/ml)	AD (0.39µg/ml)	AD (0.39µg/ml)				IRBC	CPM
F	CPM	TB (0.39µg/ml)	TB (0.39µg/ml)	TB (0.39µg/ml)	AS (1.8µg/ml)	AS (1.8µg/ml)	AS (1.80µg/ml)				IRBC	CPM
G	CPM	AD (100µg/ml)	AD (100µg/ml)	AD (100µg/ml)	AS (0.45µg/ml)	AS (0.45µg/ml)	AS (0.45µg/ml)				IRBC	CPM
H	CPM	CPM	CPM	CPM	CPM	CPM	CPM	CPM	CPM	CPM	CPM	CPM

Table 5: Plate 3 for asexual assay

	1	2	3	4	5	6	7	8	9	10	11	12
A	CPM	CPM	CPM	CPM	CPM	CPM	CPM	CPM	CPM	CPM	CPM	CPM
B	CPM	TF (100µg/ml)	TF (100µg/ml)	TF (100µg/ml)	CM (25µg/ml)	CM (25µg/ml)	CM (25µg/ml)	AS (0.11µg/ml)	AS (0.11 µg/ml)	AS (0.11 µg/ml)	RBC	CPM
C	CPM	TF (25µg/ml)	TF (25µg/ml)	TF (25µg/ml)	CM (6.25µg/ml)	CM (6.25µg/ml)	CM (6.25µg/ml)	AS (0.03µg/ml)	AS (0.03 µg/ml)	AS (0.03 µg/ml)	RBC	CPM
D	CPM	TF (6.25µg/ml)	TF (6.25µg/ml)	TF (6.25µg/ml)	CM (1.56µg/ml)	CM (1.56µg/ml)	CM (1.56µg/ml)				RBC	CPM
E	CPM	TF (1.56µg/ml)	TF (1.56µg/ml)	TF (1.56µg/ml)	CM (0.39µg/ml)	CM (0.39µg/ml)	CM (0.39µg/ml)				IRBC	CPM
F	CPM	TF (0.39µg/ml)	TF (0.39µg/ml)	TF (0.39µg/ml)	AS (1.8µg/ml)	AS (1.8µg/ml)	AS (1.80µg/ml)				IRBC	CPM
G	CPM	CM (100µg/ml)	CM (100µg/ml)	CM (100µg/ml)	AS (0.45µg/ml)	AS (0.45µg/ml)	AS (0.45µg/ml)				IRBC	CPM
H	CPM	CPM	CPM	CPM	CPM	CPM	CPM	CPM	CPM	CPM	CPM	CPM

Table 6: Plate 4 for asexual assay

	1	2	3	4	5	6	7	8	9	10	11	12
A	CPM	CPM	CPM	CPM	CPM	CPM	CPM	CPM	CPM	CPM	CPM	CPM
B	CPM	KG (100µg/ml)	KG (100µg/ml)	KG (100µg/ml)	RT (25µg/ml)	RT (25µg/ml)	RT (25µg/ml)	AS (0.11µg/ml)	AS (0.11 µg/ml)	AS (0.11 µg/ml)	RBC	CPM
C	CPM	KG (25µg/ml)	KG (25µg/ml)	KG (25µg/ml)	RT (6.25µg/ml)	RT (6.25µg/ml)	RT (6.25µg/ml)	AS (0.03µg/ml)	AS (0.03 µg/ml)	AS (0.03 µg/ml)	RBC	CPM
D	CPM	KG (6.25µg/ml)	KG (6.25µg/ml)	KG (6.25µg/ml)	RT (1.56µg/ml)	RT (1.56µg/ml)	RT (1.56µg/ml)				RBC	CPM
E	CPM	KG (1.56µg/ml)	KG (1.56µg/ml)	KG (1.56µg/ml)	RT (0.39µg/ml)	RT (0.39µg/ml)	RT (0.39µg/ml)				IRBC	CPM
F	CPM	KG (0.39µg/ml)	KG (0.39µg/ml)	KG (0.39µg/ml)	AS (1.8µg/ml)	AS (1.8µg/ml)	AS (1.80µg/ml)				IRBC	CPM
G	CPM	RT (100µg/ml)	RT (100µg/ml)	RT (100µg/ml)	AS (0.45µg/ml)	AS (0.45µg/ml)	AS (0.45µg/ml)				IRBC	CPM
H	CPM	CPM	CPM	CPM	CPM	CPM	CPM	CPM	CPM	CPM	CPM	CPM

Table 7: Plate 5 for asexual assay

Plate design for gametocyte assay

	1	2	3	4	5	6	7	8	9	10	11	12
A	CPM	CPM	CPM	CPM	CPM	CPM	CPM	CPM	CPM	CPM	CPM	CPM
B	CPM	YF (100µg/ml)	YF (100µg/ml)	YF (100µg/ml)	MS (100µg/ml)	MS (100µg/ml)	MS (100µg/ml)	PQ (200ng/ml)	PQ (200ng/ml)	PQ (200ng/ml)		CPM
C	CPM	YF (7µg/ml)	YF (7µg/ml)	YF (7µg/ml)	MS (7µg/ml)	MS (7µg/ml)	MS (7µg/ml)	AS (200ng/ml)	AS (200ng/ml)	AS (200ng/ml)		
D	CPM	YF (1µg/ml)	YF (1µg/ml)	YF (1µg/ml)	MS (1µg/ml)	MS (1µg/ml)	MS (1µg/ml)	DMSO	DMSO	DMSO		CPM
E	CPM	HB (100µg/ml)	HB (100µg/ml)	HB (100µg/ml)	AN (100µg/ml)	AN (100µg/ml)	AN (100µg/ml)	PQ (10µg/ml)	PQ (10µg/ml)	PQ (10µg/ml)	IRBC	CPM
F	CPM	HB (7µg/ml)	HB (7µg/ml)	HB (7µg/ml)	AN (7µg/ml)	AN (7µg/ml)	AN (7µg/ml)	RBC	RBC	RBC	IRBC	CPM
G	CPM	HB (1µg/ml)	HB (1µg/ml)	HB (1µg/ml)	AN (1µg/ml)	AN (1µg/ml)	AN (1µg/ml)				IRBC	CPM
H	CPM	CPM	CPM	CPM	CPM	CPM	CPM	CPM	CPM	CPM	CPM	CPM

Table 8: Plate 1 for gametocyte assay

	1	2	3	4	5	6	7	8	9	10	11	12
A	CPM	CPM	CPM	CPM	CPM	CPM	CPM	CPM	CPM	CPM	CPM	CPM
B	CPM	TB (100µg/ml)	TB (100µg/ml)	TB (100µg/ml)	TF (100µg/ml)	TF (100µg/ml)	TF (100µg/ml)	PQ (200ng/ml)	PQ (200ng/ml)	PQ (200ng/ml)		CPM
C	CPM	TB (7µg/ml)	TB (7µg/ml)	TB (7µg/ml)	TF (7µg/ml)	TF (7µg/ml)	TF (7µg/ml)	AS (200ng/ml)	AS (200ng/ml)	AS (200ng/ml)		
D	CPM	TG (1µg/ml)	TG (1µg/ml)	TG (1µg/ml)	TF (1µg/ml)	TF (1µg/ml)	TF (1µg/ml)	DMSO	DMSO	DMSO		CPM
E	CPM	AD (100µg/ml)	AD (100µg/ml)	AD (100µg/ml)	CM (100µg/ml)	CM (100µg/ml)	CM (100µg/ml)	PQ (10µg/ml)	PQ (10µg/ml)	PQ (10µg/ml)	IRBC	CPM
F	CPM	AD (7µg/ml)	AD (7µg/ml)	AD (7µg/ml)	CM (7µg/ml)	CM (7µg/ml)	CM (7µg/ml)	RBC	RBC	RBC	IRBC	CPM
G	CPM	AD (1µg/ml)	AD (1µg/ml)	AD (1µg/ml)	CM (1µg/ml)	CM (1µg/ml)	CM (1µg/ml)				IRBC	CPM
H	CPM	CPM	CPM	CPM	CPM	CPM	CPM	CPM	CPM	CPM	CPM	CPM

Table 9: Plate 2 for gametocyte assay

	1	2	3	4	5	6	7	8	9	10	11	12
A	CPM	CPM	CPM	CPM	CPM	CPM	CPM	CPM	CPM	CPM	CPM	CPM
B	CPM	KG (100µg/ml)	KG (100µg/ml)	KG (100µg/ml)				PQ (200ng/ml)	PQ (200ng/ml)	PQ (200ng/ml)		CPM
C	CPM	KG (7µg/ml)	KG (7µg/ml)	KG (7µg/ml)				AS (200ng/ml)	AS (200ng/ml)	AS (200ng/ml)		
D	CPM	KG (1µg/ml)	KG (1µg/ml)	KG (1µg/ml)				DMSO	DMSO	DMSO		CPM
E	CPM	RT (100µg/ml)	RT (100µg/ml)	RT (100µg/ml)				PQ (10µg/ml)	PQ (10µg/ml)	PQ (10µg/ml)	IRBC	CPM
F	CPM	RT (7µg/ml)	RT (7µg/ml)	RT (7µg/ml)				RBC	RBC	RBC	IRBC	CPM
G	CPM	RT (1µg/ml)	RT (1µg/ml)	RT (1µg/ml)							IRBC	CPM
H	CPM	CPM	CPM	CPM	CPM	CPM	CPM	CPM	CPM	CPM	CPM	CPM

Table 10: Plate 3 for gametocyte assay

APPENDIX 4

ASEXUAL GROWTH INHIBITION CURVES

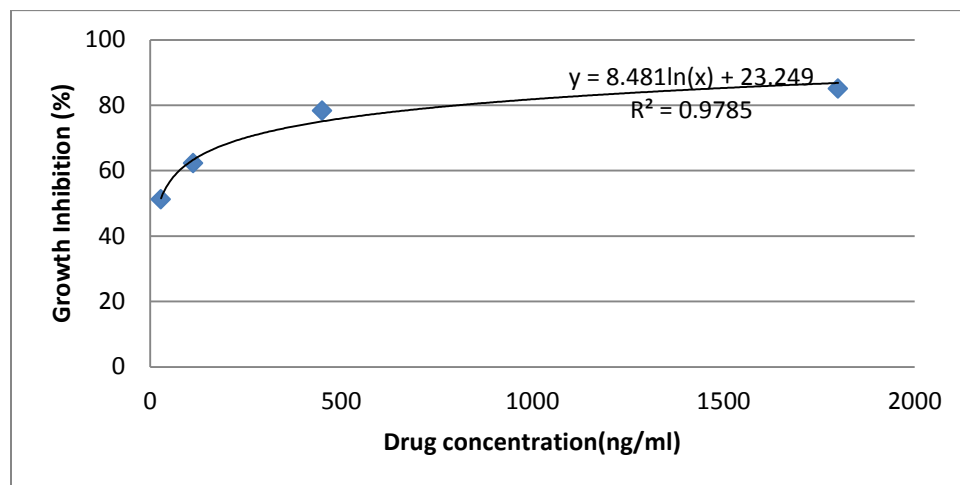


Figure 8: Growth inhibition curve for AS

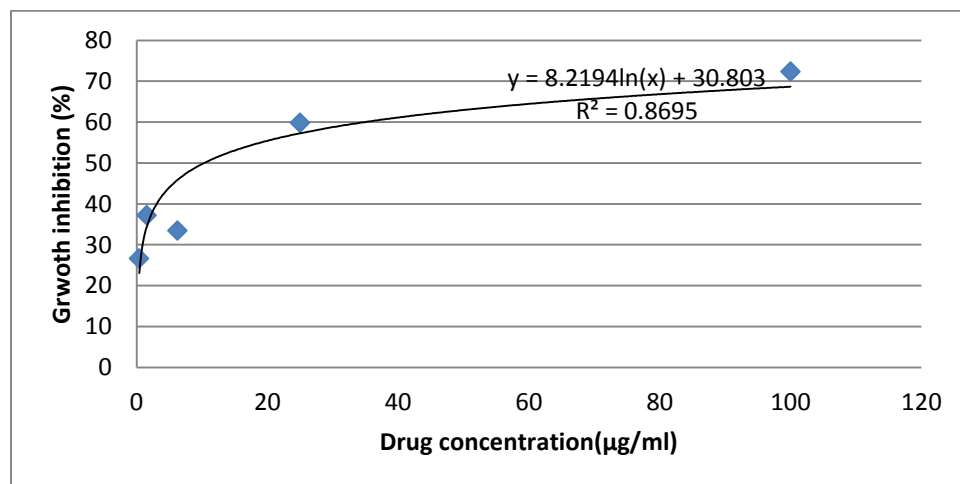


Figure 9: Growth inhibition curve for KG

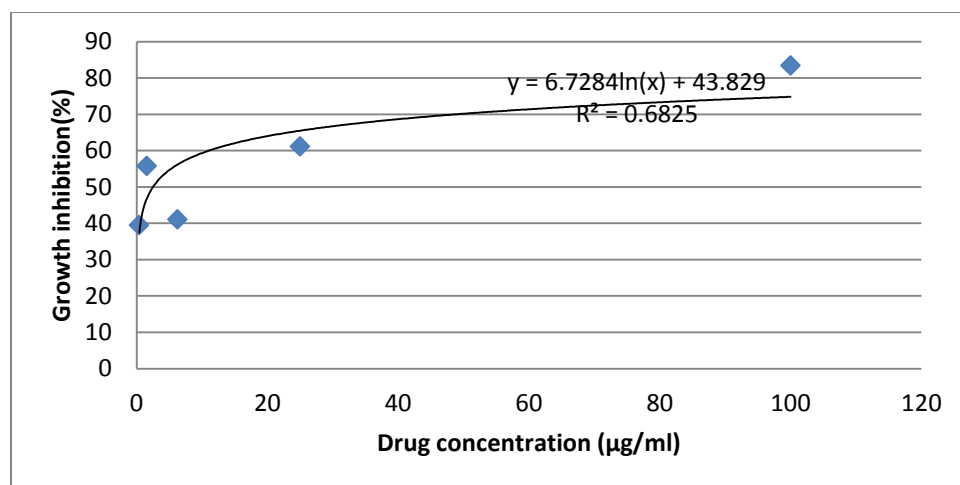


Figure 10: Growth inhibition of RT

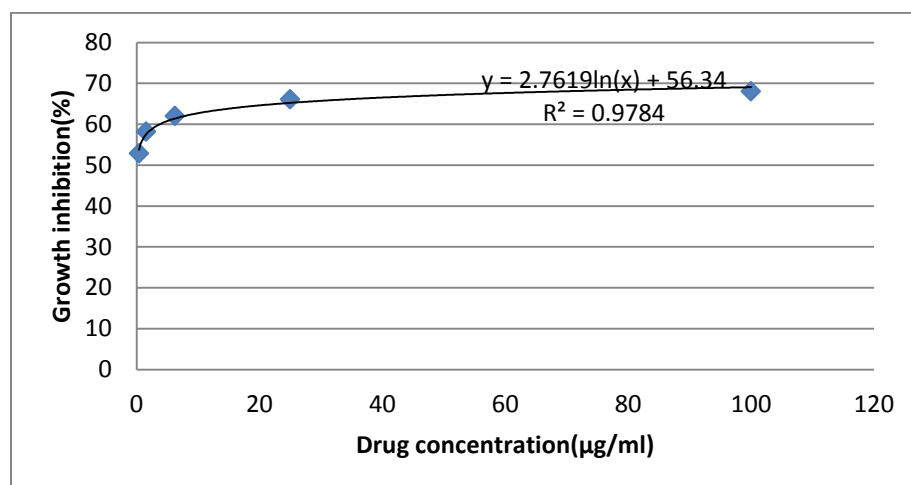


Figure 8: Growth inhibition curve of TF

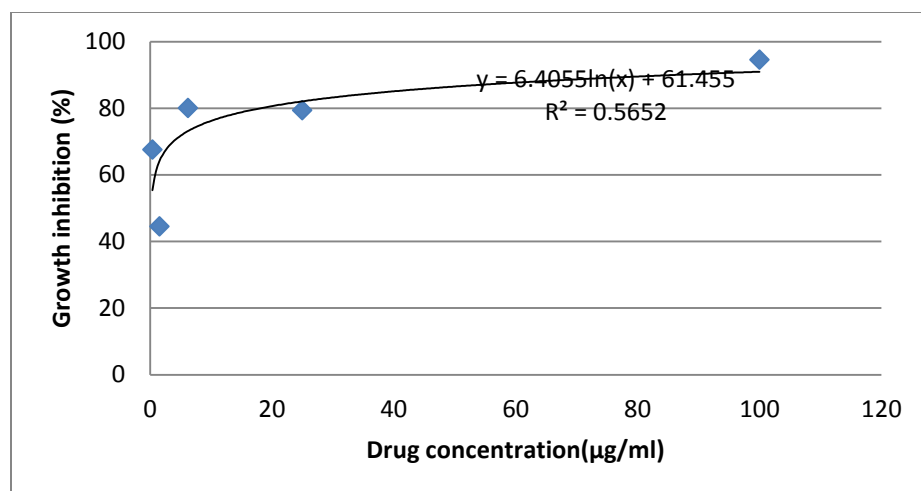


Figure 9: Growth inhibition of CM

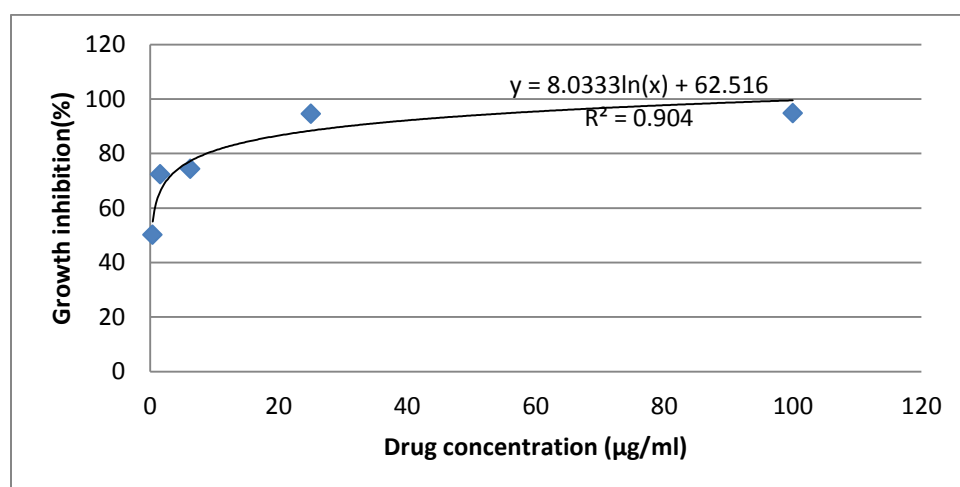


Figure 13: Growth inhibition curve of MS

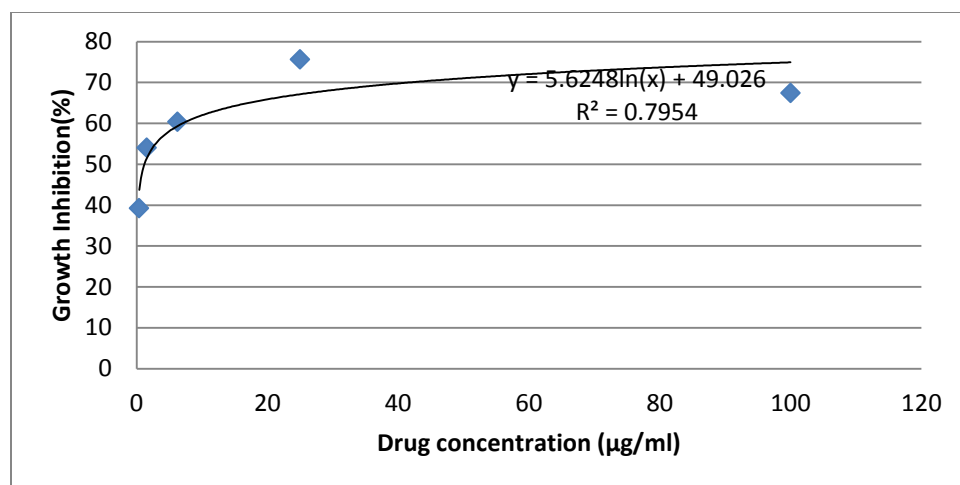


Figure 14: Growth inhibition curve of AN

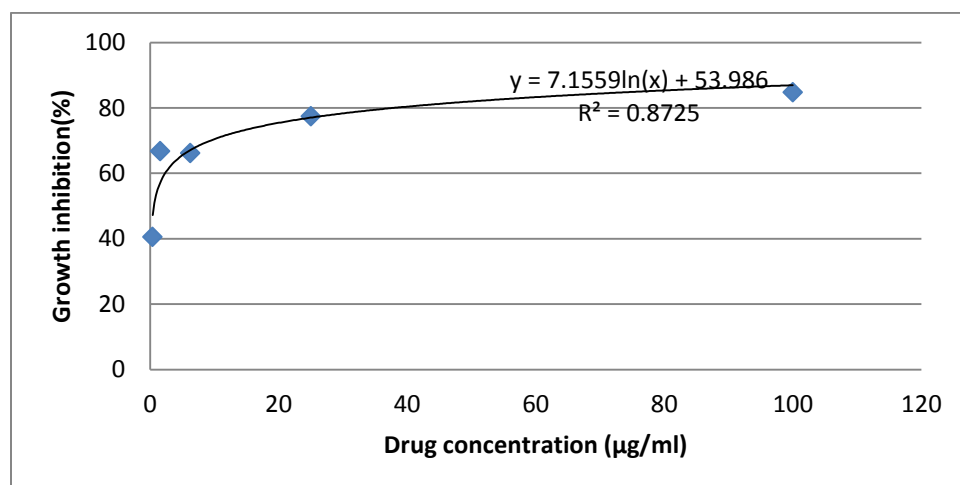


Figure 15: Growth inhibition curve of YF

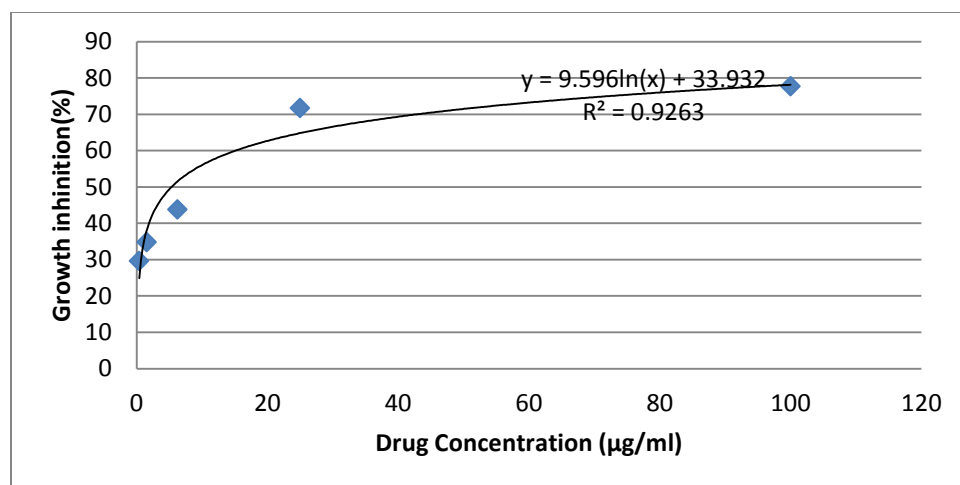


Figure 16: Growth inhibition curve of HB

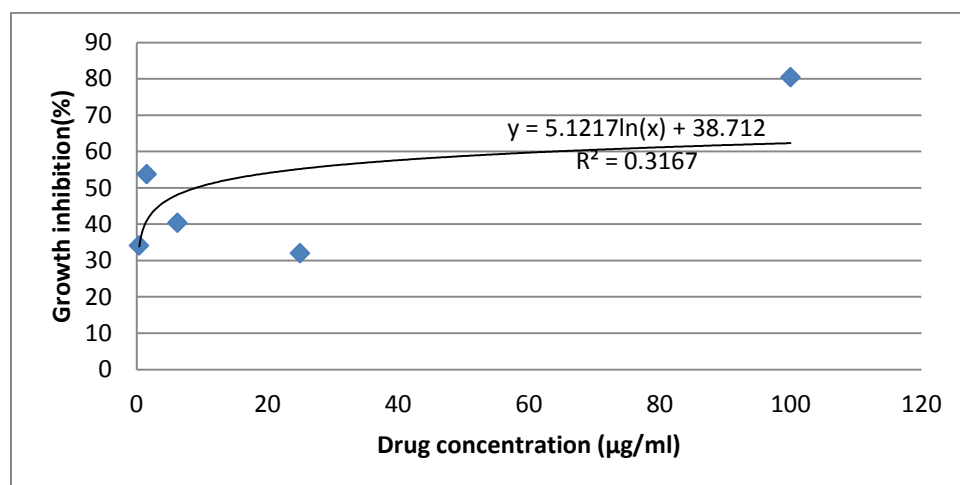


Figure 17: Growth inhibition curve of TB

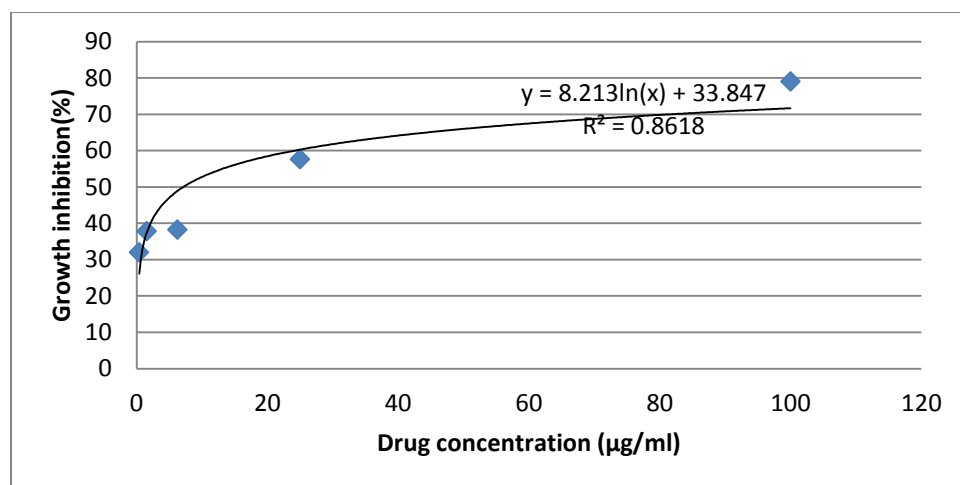


Figure 18: Growth inhibition curve of AD

8. APPENDIX 5

MISCELLANEOUS PICTURES

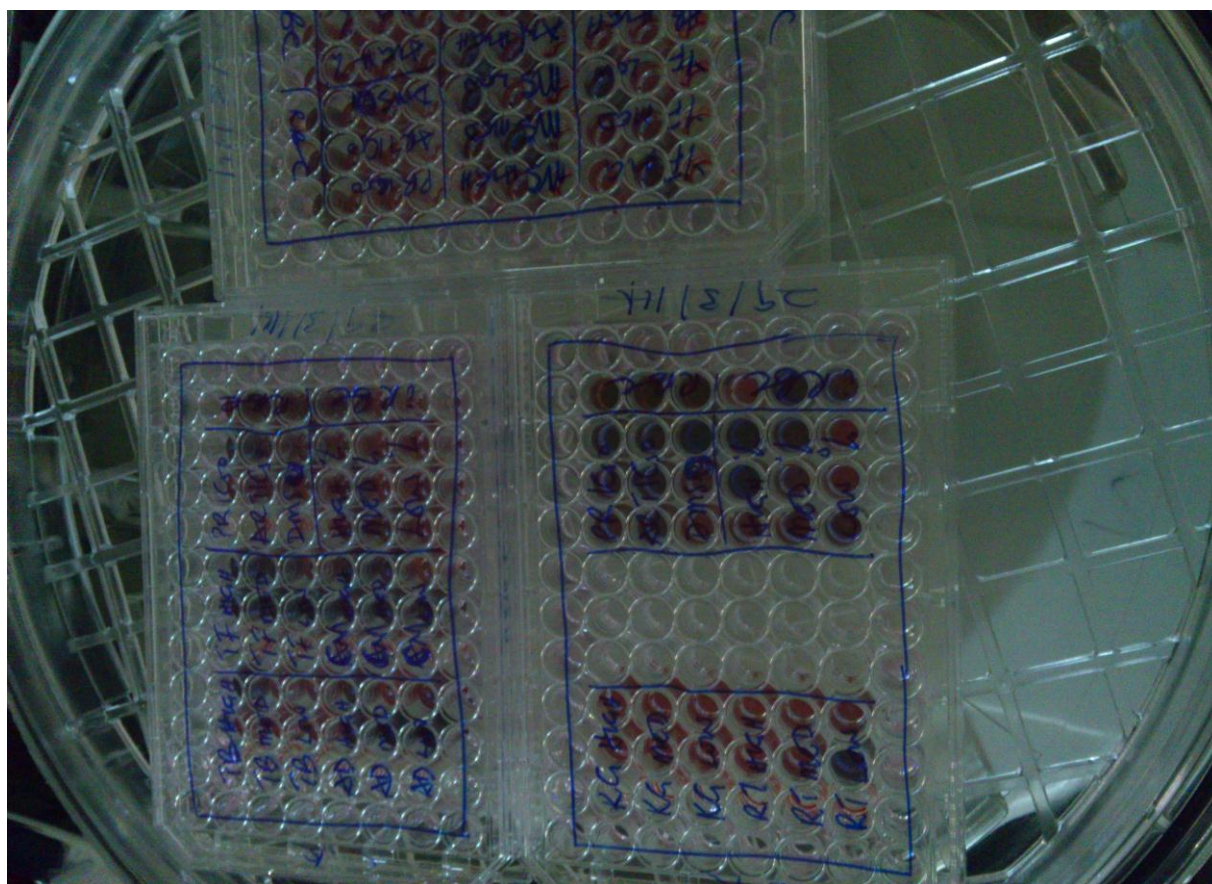


Plate 8: Plated drugs in an incubating chamber

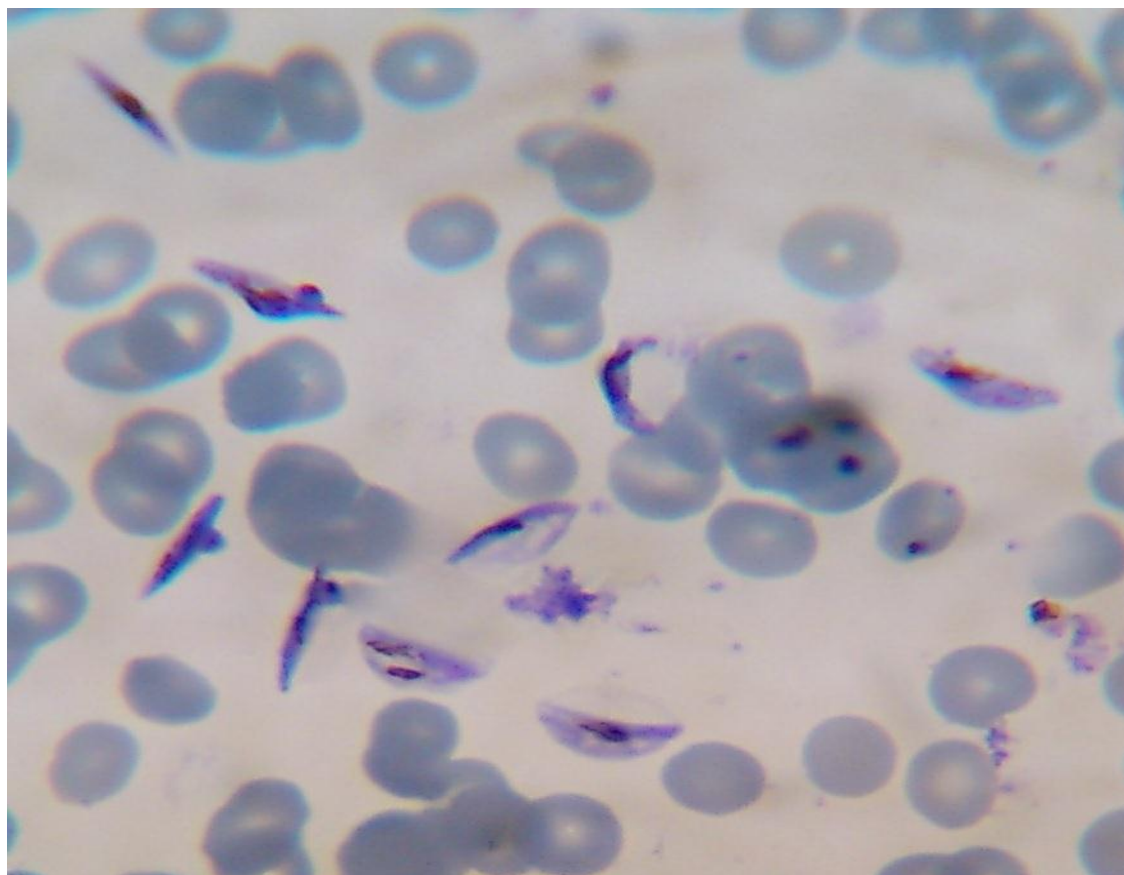


Plate 9: Gametocytes after percoll purification