THE EFFECTS OF CRYPTOLEPIS SANGUINOLENTA ROOT EXTRACTS ON
PLASMODIUM FALCIPARUM GAMETOCYTE DEVELOPMENT

A THESIS SUBMITTED
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DECLARATION

THIS EXPERIMENTAL WORK DESCRIBED IN THIS THESIS WAS DONE BY ME AT THE IMMUNOLOGY DEPARTMENT, NOGUCHI MEMORIAL INSTITUTE FOR MEDICAL RESEARCH, UNIVERSITY OF GHANA AND UNDER THE SUPERVISION OF DR MICHAEL F. OFORI AND PROF. GORDON A. AWANDARE.

REFERENCES CITED IN THIS WORK HAVE FULLY BEEN ACKNOWLEDGED.

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DEDICATION

TO JEHOVAH GOD ALMIGHTY AND MY FAMILY
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LIST OF ABBREVIATIONS

WHO – World Health Organization
CDC – Centre for Disease Control
NADH – Nicotinamide adenine dinucleotide (reduced)
NADP⁺ - Nicotinamide adenine dinucleotide phosphate (oxidized)
FADH₂ – Flavin adenine dinucleotide (reduced)
ATP – Adenosine triphosphate
CoA – Coenzyme A
CO₂ – Carbon dioxide
FAD – Flavine adenine dinucleotide (oxidized)
α – Alpha
β - Beta
PF10_0218 – Citrate synthase, mitochondrial precursor, putative
(Plasmodium falciparum 3D7, location: chromosome 10, tag value: 0218)
PF13_0229 – Aconitase
(Plasmodium falciparum, 3D7, location: chromosome 13, tag value: 0229)
PF13_0242 – Isocitrate dehydrogenase (NADP), mitochondrial precursor
(Plasmodium falciparum 3D7, location: chromosome 13, tag value: 0242)
PF11_0097 – Succinyl-CoA synthetase alpha subunit, putative
(Plasmodium falciparum 3D7, location: chromosome 11, tag value: 0097)
PF14_0295 – ATP-specific succinyl-CoA synthetase beta subunit, putative
(Plasmodium falciparum 3D7, location: 14, tag value: 0295)
PF10_0334 – Flavoprotein subunit of succinate dehydrogenase
(Plasmodium falciparum 3D7, location: chromosome 10, tag value: 0334)
E1 (PF08_0045) – 2-oxoglutarate dehydrogenase E1 component
(Plasmodium falciparum 3D7, location: chromosome 8, tag value: 0045)
E2 (PF13_0121) – Dihydrolipamide succinyltransferase component of 2-oxoglutarate dehydrogenase complex (Plasmodium falciparum 3D7, location: chromosome, tag value: 0212)
E3 (PFL1550w) – Lipoamide dehydrogenase, component of 2-oxoglutarate dehydrogenase complex (Plasmodium falciparum 3D7)
PFL0630w – Iron-sulphur subunit of succinate dehydrogenase (Plasmodium falciparum 3D7)
ABSTRACT

Current efforts to eliminate malaria worldwide are soaring and one of the approaches to this is the mass screening of medicinal plants for their possible potential anti-malarial property. The current study conducted was to determine the effect of one such plant, *Cryptolepis sanguinolenta* on gametocyte development. *In vitro* growth inhibition assays on early and late gametocytes stage using ethanolic and aqueous extract of the *C. sanguinolenta* roots was performed. Enzyme activity assays were conducted to determine the effect of *C. sanguinolenta* ethanolic extract on aconitase, citrate synthase and α-ketoglutarate dehydrogenase activity in late gametocyte stages. Rhodamine 123 and bodipy-tr ceramide dye labelling of *C. sanguinolenta* treated gametocytes was also performed to determine the effect of *C. sanguinolenta* ethanolic root extract on the gametocytes mitochondrial function and the intracellular membranes integrity respectively. *C. sanguinolenta* inhibited gametocytes with IC\(_{50}\) obtained for *C. sanguinolenta* ethanolic and aqueous extracts on early gametocyte stages, 307 ± 47.4μg/ml and 305 ± 42.8μg/ml respectively. The IC\(_{50}\) obtained for *C. sanguinolenta* ethanolic and aqueous extracts on late gametocyte stages were 291.2 ± 24.668μg/ml and 307.3 ± 20.42μg/ml respectively. In the treated late gametocytes stage, *C. sanguinolenta* ethanolic extract at 90% inhibitory concentration significantly inhibited citrate synthase activity (P = 0.04) and aconitase activity (P = 0.001) but not α-ketoglutarate dehydrogenase activity (P = 0.1). Loss of uptake of Rhodamine 123 dye in the matrix of the ethanolic extract of the *C. sanguinolenta* treated late gametocytes stage suggested total collapse of the gametocyte’s mitochondrial membrane potential. Consequently, this led to the loss of membrane integrity of other intracellular membranes within the treated late gametocytes stage as observed from the reduced uptake of bodipy-tr ceramide dye. This study suggests that the ethanolic roots extract of the *C. sanguinolenta* reduced the energy production capability of late gametocyte stages, which consequently impaired their growth and therefore possible loss of their infectivity.
CHAPTER ONE

INTRODUCTION AND LITERATURE REVIEW
1.1 INTRODUCTION

There has been a growing interest in the use of plant medicines in the Ghanaian population for the treatment of many diseases (Ankrah, 2010). With its proven effectiveness and safety, the World Health Organization (WHO) has shown acknowledgement for the essential role that plant medicine plays in the formal health system and as such is promoting research in the area (WHO, 2009). For example, preparations of Radix Echinaceae which consist of fresh and dried roots of *Echinacea angustifolia* or *Echinacea pallida*, also known as coneflower and pale coneflower root respectively, are clinically reported to be effective for cold, respiratory and urinary tract infections in the WHO monographs on selected medicinal plants (WHO, 1999). Data from studies indicates that its effect in the treatment of these is primarily due to its stimulation of the immune system (Bauer & Wagner, 1991; Schöneberger, 1992; Melchart et al., 1994). Another popular plant medicine under research is *Cryptolepis sanguinolenta*, which is used for the treatment of malaria (Wright et al., 1996).

Due to the emergence of resistant strains of *P. falciparum* to chloroquine and currently to artemisinin (Dondorp et al., 2009; Noedl et al., 2008; WHO, 2005), there is increasing interest in research on *C. sanguinolenta* plant for future use as an antimalarial agent. Its formulation into tea-bags for consumption has proven to be effective for the treatment of acute uncomplicated malaria and showed antimalarial cure rate of 93.5% in a patient population (Bugyei et al., 2010). It has been found that the active anti-plasmodial component in the root is the indoquinoline alkaloid cryptolepine, with reports indicating its effect against *P. falciparum* asexual stages both in vitro and in vivo (Cimanga et al., 1997; Wright et al., 1996). In many Ghanaian communities, the use of *C. sanguinolenta* plant has become very common for the treatment of malaria due to its affordability and accessibility in contrast to the known orthodox drugs (Ankrah, 2010).
result, most herbal preparations for the treatment of malaria sold on the market consists of dried roots of *C. sanguinolenta* plant (Osei-Djarbeng *et al.*, 2015).

A study conducted by Paetey *et al.*, (2009), demonstrated that the use of some orthodox drugs for the treatment of malaria has rather resulted in an increase in gametocyte population *in vitro* and therefore possibly enhances transmission of the malaria parasite from the host into the mosquito. As a result, it may turn out that the therapeutic use of the plant medicine as an alternative to the orthodox drugs would become a popular choice, although its effect on gametocytes which is the sexual stage of *Plasmodium spp.* is not yet clearly understood.

The gametocytes have been demonstrated to utilize the conventional Tricarboxylic Acid (TCA) cycle in the mitochondrion for energy production (MacRae *et al.*, 2013). Demonstration of the utilization of the radioactively labelled carbon incorporated in glucose which was identified in the mitochondrial TCA cycle intermediates within gametocytes gives sufficient proof of this assertion (MacRae *et al.*, 2013). Another possible ground for the existence of this metabolic pathway is the identification of the mitochondria-located branched chain α-keto acid dehydrogenase (BCKDH) complex in gametocytes. This enzyme is reported to function in the glycolytic pathway to convert pyruvate to acetyl coA. Since it is shown that gametocytes lack the mitochondrial isoform of the enzyme pyruvate dehydrogenase, BCKDH is thought to function in its place (Oppenheim *et al.*, 2014). In addition, it has been revealed that a transcriptional up-regulation of key subunits in the BCKDH complex within the gametocytes occurs during starvation conditions (Daily *et al.*, 2007; Oppenheim *et al.*, 2014). It has therefore been speculated that gametocytes utilize glucose via the glycolytic and TCA pathway for the production of NADH and FADH₂. These reducing equivalents are essential for the respiratory chain function, energy production and the synthesis of succinyl-CoA for haem biosynthesis (Mogi & Kita 2010).
It has also been shown from a study by Lovegrove et al., (2008), that gametocytes transcriptionally up-regulate the TCA cycle enzymes in response to glucose limitation or other metabolic stresses in the human host. It is speculated that the switch from glycolysis to a more efficient energy metabolism is this TCA metabolic pathway (Lovegrove et al., 2008). It is therefore suggested that an inhibition of the TCA enzymes may lead to strong metabolic phenotype such as reduced yield of NADH which will in turn affect the respiratory chain function and mitochondrial membrane potential within the parasite. Also it may lead to the accumulation of intermediates such as citrate to toxic levels which could result in adverse consequences for the gametocytes (del Pilar Crespo et al., 2008).

It is a suggestion that C. sanguinolenta extract, being used as an antimalarial, may have an effect on gametocytes via this metabolic pathway. Furthermore, based on the report that a drug like primaquine kills gametocytes by acting on an enzyme in the gametocytes mitochondria (Oduola et al., 1988), it is believed that C. sanguinolenta extract may also act via inhibition of key enzymes in the TCA pathway which would essentially impair the gametocyte’s development.

With no current information on the effect of C. sanguinolenta on the TCA cycle enzymes, however, it would be necessary to determine whether this plant extract would have effect on key regulatory enzymes involved in the mitochondrial TCA metabolic cycle of gametocytes. Also, considering the current malaria situation, the fact that there is reported resistance to known antimalarial drugs and also no transmission blocking vaccine on the market, it is highly necessary to search for drugs that may be effective against gametocyte development in order to block transmission. It is against this background that the study was designed to investigate the effect of C. sanguinolenta extracts on gametocyte development in vitro. It was therefore hypothesized that C. sanguinolenta root extract inhibits gametocyte development in P. falciparum via the inhibition of the metabolic pathway of the parasite.
The aim of this work therefore was to determine the effect of *C. sanguinolenta* on gametocyte development and identify its potential property as a transmission blocking agent by experimentally determining its mechanism of action on the parasite. With regard to this, the objectives outlined included the following:

- Determination of the general effect of *C. sanguinolenta* extract on the asexual and sexual stage (stages I-V) of *P. falciparum*

- Determination of the effect of *C. sanguinolenta* extract on the metabolic activity of gametocytes by assessing their effect on some enzymes (aconitase, citrate synthase, alpha ketoglutarate dehydrogenase) involved in the parasite’s metabolic TCA pathway.
1.2 LITERATURE REVIEW

1.2.1 THE DISEASE MALARIA

The latest estimate reported for malaria in the 2014 WHO malaria report for 2013 is an approximately 198 million cases of morbidity in people affected and 584 thousand deaths worldwide (WHO, 2014). The disease is caused by the protozoan parasite *Plasmodium* and five species of this parasite are known to infect humans with a sixth one just recently discovered (Kantele & Jokiranta 2011; Luchavez et al. 2008; Ta et al. 2014). The six species are *Plasmodium falciparum*, *P. vivax*, *P. malariae*, *P. ovale*, *P. knowlesi* and *P. cynomolgi*. The virulent form of all the species is *P. falciparum* which is known in some cases to cause cerebral malaria, a severe form of malaria and causes majority of the deaths (James & Miller, 2000). *Plasmodium falciparum* which is causing the major health issue is reported to result in 1-2 million deaths annually worldwide. Regions most affected by this species is Africa (95%), Southeast Asia (3%) and Eastern Mediterranean region (1%) (WHO, 2010).

The *Plasmodium* species have a complex life cycle that occurs in the mosquito vector and the human host where the emergence of clinical symptoms is observed as a result of the cyclical intraerythrocytic development (Bannister et al., 2000). The life cycle begins in the mosquito after a male (microgametocytes) and female (macrogametocytes) gametocytes are ingested during a blood meal by a female Anopheles mosquito (Fig. 1.1). The male and female gametes then fuse within the stomach of the mosquito to form a zygote which in turn develops into an ookinete (motile and elongated). The ookinete invades the midgut of the mosquito where it develops into an oocyst which grows, eventually ruptures and release sporozoites which cross the epithelial layer of the mosquito midgut and move to the salivary gland (Fig. 1.1).
During another blood meal by this same mosquito, the sporozoites are inoculated into the human host which finds its way to infect the liver cells and mature into schizonts (exo-erythrocytic schizogony). The schizonts rupture to release merozoites which proceed to infect red blood cells where they form rings or early trophozoites (Fig. 1.1). These trophozoites mature into schizonts (erythrocytic schizogony) and rupture to release daughter merozoites. Some of the merozoites repeat this cycle by invading new erythrocytes and most clinical manifestation of the disease is as a result of these blood stage parasites. Other merozoites upon invading red blood cells differentiate into sexual erythrocytic stages called gametocytes which is later picked up by a mosquito during the next blood meal (Fig. 1.1, CDC, 2013a).
1.2.1.1 High risk malaria groups

High risk groups to the disease include those whose immunity has not yet developed such as young children in endemic areas, travellers from non-endemic regions of the world and those whose immunity is suppressed such as pregnant women and people from endemic areas who have ceased to be routinely exposed to infection (WHO, 2014). In fact, the disease burden in the endemic regions is known to impede economic and social development (James & Miller, 2000). Due to the re-emergence of the disease as a result of the spread of the drug-resistant parasite strains, health-care infrastructure deterioration and some impediments in the implementation and maintenance of vector control programs in most developing countries (James & Miller, 2000), there is a growing concern for the introduction of new potent drugs into the market to help
control the disease and most importantly used as possible targets against resistant strains in order to protect these risk groups.

### 1.2.2 MALARIA CONTROL STRATEGIES

Malaria as a public health concern has attracted a lot of attention worldwide and effort to control or manage the disease, especially in areas where it is most endemic began since 1946 (CDC, 2012a). Newer technologies are being practiced today with the same aim of eradicating the disease. Some of the control measures currently employed by many of the countries affected include the reduction of parasite-vector contact, reduction of vector population and most importantly reduction of parasite reservoir in the human host (Ministry of Health, 2006).

#### 1.2.2.1 Parasite–vector contact control

**Insecticide treated bed nets** - The use of insecticide treated nets worldwide has been shown to reduce the prevalence of malaria in many communities (CDC, 2014). The use of insecticide treated nets was adopted in Ghana as one of the methods for malaria control (Teye & Awetoriyaro, 2013). It is considered an effective method of preventing malaria. These nets reduces the incidence of death of children below the age of five by 20% by acting as a protective barrier from mosquitoes for those sleeping under it (CDC, 2014). In the Accra urban area where insecticide treated nets are used, reports shows that malaria prevalence has decreased by 4.1% (Roll Back Malaria, 2015). Widespread usage of the nets could not be achieved in the country due to limited financial resources, the discomfort and the belief that it does not work (Teye & Awetoriyaro 2013; Adeyeri 2011).

**Indoor spray with residual insecticides**- The World Health Organization in 2006 recommended indoor residual spray as one of the control measures for the malaria vector. It was shown that DDT and pyrethroids were successful for indoor residual spray (WHO, 2012). The impact of indoor residual spraying was shown from the report in the Obuasi area of Ghana. In 2
years of indoor residual spray implementation, malaria prevalence reduced by 74% with reported cases declining from 6711 in 2005 to 973 in 2009 and 238 in 2013 (George, 2015). The effort in eradicating malaria via this method however was not achieved in Africa due to the fact that logistics, human and financial resources needed to sustain the program was woefully inadequate in many of these African countries (WHO, 2012).

**Mosquito repellent and protective clothing** - The mosquito repellents help reduce the risk of being bitten by a mosquito and therefore reduce the risk of the disease malaria (Scott *et al.*, 2014). There are many products on the market which are used as mosquito repellents today. Some of these products contain N,N-diethyl-meta-toluamide (DEET), picaridin, IR3535, lemon oil eucalyptus and para-methane-diol which provides long lasting protection (CDC, 2015). There are also permethrin-treated clothings and shoes that provide protection against mosquito bites (CDC, 2015). Evidence from study also shows that there are some plant-based repellents which are effective against mosquitoes and similarly provide long lasting protection (Bissinger *et al.*, 2014).

**1.2.2.2 Vector control**

Due to the popular occupation of most people in some areas of Ghana to sleep outdoors and perform regular night activities, the implementation of the control strategy by means of indoor residual spraying and use of insecticide treated net has become inadequate to curb the transmission of malaria (Monroe *et al.*, 2015). As a result, control intervention methods such as environmental and biological modification of the mosquito vector have been employed to reduce the population thereby reducing the malaria parasite transmission level (Caputo *et al.*, 2015; Whyard *et al.*, 2015).
Environmental modification- This approach is based on the use of insecticides to target the larvae or adult stage of the mosquito (WHO, 1995). The larval source control has been shown to be successful in US, Canada, Brazil, Singapore and throughout Europe and is believed would be successful in Africa (Fillinger & Lindsay, 2011). It involves the use of larvicides to kill the mosquito larva at their source (Djènontin et al., 2014). The operation when carried out in some parts of Africa was shown to be successful, completely eliminating the larva of mosquitoes within 2 to 3 days although it did not exhibit longer residual effect (Djènontin et al., 2014). Also, adult mosquitoes targeted for control in many lands by the use of adulticides and other interventions showed a high efficacy (Abramides et al., 2011). The use of the mosquito adulticides effectively reduced the adult population by 77% (Fulcher et al., 2015; Doyle et al., 2009).

Biological modification- The sterile insect technique has been employed as a biological control tool to reduce mosquito population by releasing a large number of adult male mosquitoes sterilised by radiation to compete with the wild males during mating periods. The females mosquitoes were found sterilized (Knipling, 1959). The use of chemicals such as pyriproxyfen to similarly induce sterility in adult female mosquitoes has been explored with relative success (Mbare et al., 2014). Other avenues of research presently being explored includes miRNAs targeting mRNAs to control mosquito development and reproductive processes in order to reduce mosquito borne infectious disease (Jain et al., 2015).

1.2.2.3 Reducing parasite reservoir

Reduction of the malaria parasite in the human host is primarily necessary in order to prevent the disease and or its complications (Cedillos et al., 1978). This require prompt diagnosis to identify the presence of the parasite in the host after which appropriate treatment is
given (CDC, 2012b). There are many diagnostic methods available as well as treatment procedures in order to eliminate the parasites. Most health facilities in Africa follow the diagnosis and treatment policies provided by WHO in the WHO report series of 2013 (World Health Organization 2013).

**Diagnosis**- The PCR method is shown to be more sensitive than Rapid diagnostic test (RDT) or microscopy method for malaria parasite detection (Ganguly *et al.*, 2013). However, due to lack of resources and financial constraint in the underdeveloped countries, WHO recommends standard microscopy or RDT as a method for diagnosis (CDC, 2012b). Prompt diagnosis for early treatment is very important to prevent disease progression and parasite transmission to other human hosts (CDC, 2012b).

**Treatment**- The treatment of human malaria parasite infection is directed towards the elimination of the blood stages of the parasite that causes the clinical symptoms mostly observed in clinical cases (Guerin *et al.*, 2002). Prompt treatment is mostly effective and reduces the severity of the disease (Guerin *et al.*, 2002). Treatment is especially important for asymptomatic malaria infected people in order to reduce parasite reservoir and transmission (Ganguly *et al.*, 2013).

Treatment based methods include both prevention of the disease using chemoprophylaxis and chemotherapy. Artemisinin based combination therapy (ACT) is recommended as first-line treatment for malaria caused by *P. falciparum* whiles chloroquine is used for treatment of *Plasmodium vivax*, except for areas where vivax is resistant to chloroquine (WHO, 2013). Artemether-Lumifantrine and artesunate-amodiaquine is registered for use in Africa as ACT and kills malaria parasite more efficiently than the other drugs such as quinine, doxycycline (used in combination with quinine), tetracycline, clindamycin (used in combination with quinine),
mefloquine and chloroquine which are single based therapies (CDC 2013b). Primaquine is considered effective against the sexual stage of the malaria parasites, gametocytes, although it is reported to result in complication for G6PD deficient individuals since it induces haemolytic toxicity (Shekalaghe et al., 2010). Primaquine use at the optimal dosage is considered for *P. vivax* and *P. ovale* (Shimizu et al., 2014). Chemoprophylaxis is also required especially for travellers and malaria naive individuals entering malaria endemic regions in order to prevent infection and disease manifestation (Chen & Keystone, 2005). Atovaquone-proguanil is used as a short prophylactic course in most cases and is shown to effectively clear the malaria parasites although cases of resistance has been reported in some areas (Boggild et al., 2014; Leshem et al., 2014).

1.2.3 MALARIA CHEMOTHERAPY

1.2.3.1 Drugs used as anti-malarials

Chloroquine is one of the oldest antimalarial drug and it is still in use in some parts of the world today (Saxena et al., 2003). As a result of the emergence of chloroquine resistant strains of *Plasmodium falciparum* in 1979, new compounds were identified for their effect on the resistant strains (WHO, 2001). The goal was to develop a drug with high potency against malaria. This goal led to the development of artemisinin and its subsequent derivative which is widely used today for the treatment of malaria (WHO, 2008). Following this, other compounds have been developed which are effective against different stages of the malaria parasite in the human host (Ministry of Health, 2006; Guerin et al., 2002).

Some of the drugs like halofantrine, lumefantrine, piperaquine, amodiaquine and mefloquine act by diffusing into and remaining in the erythrocyte and subsequently inhibiting the later asexual growth phase of parasites (Wilson et al., 2013). The drugs artesunate, artemisinin, cyclohexamide and trichostatin A inhibit parasite growth significantly at ring stage (Wilson et
al., 2013). Despite these positive effects, the emergence of drug resistance has put a strain on the treatment therapies administered and compelling WHO to advocate for the introduction of drug combination therapies which started full implementation from 1994 (WHO, 2001). The use of Artesunate Combination Therapy (ACT), most especially artesunate-amodiaquine and artesunate-lumefantrine was endorsed worldwide (WHO, 2001).

1.2.3.2 Mechanism of action of some selected anti-malarials

The mode of action of most antimalarials on the market today is not clearly understood, however, due to the methods employed by the parasite to develop resistance to these drugs, enough information has been gathered (Gregson & Plowe, 2005). The sesquiterpene lactones typified by artemisinin, folate inhibitors and atovaquone are believed to act as blood schizonticides. The class of 8-aminoquinolines and some folate inhibitors also act as liver schizonticides (Guerin et al., 2002). Primaquine is thought to destroy hypnozoites, the latent form of parasite observed in infections by *P. vivax* (Carson, 1984). Quinine, chloroquine and amodiaquine are thought to act on the food vacuole of the asexual parasite stages (Gabay et al., 1994). The malaria parasites have been shown to contain an acidic food vacuole where erythrocyte haemoglobin is hydrolyzed (Rathore et al., 2006). Several antimalarials are shown to act in this vacuole by interacting with haem which is the product of the hydrolysed haemoglobin (Rathore et al., 2006). Thus, this vacuole serves as site of action for some of the antimalarials. Within this oxygen-rich lysozome-like food vacuole are several parasite proteases (plamepsins, falcipain 2, metallopeptidases) and they are involved in the hydrolysis of haemoglobin to produce the Fe(II) haem and is also oxidized to Fe(III) haematin prior to being sequestered in the form of an inert pigment called hemozoin (another name being β-haematin) (Deharo et al., 2002).
The haem produced from haemoglobin hydrolysis is very toxic to the parasite due to its effect on the cellular metabolism by inhibiting enzymes, peroxidizing membranes and producing free radicals (Rathore et al., 2006). To counteract the toxic effect, haem is detoxified to allow for uninterrupted growth and parasite proliferation. Detoxification of haem is by polymerization to obtain the insoluble crystalline hemozoin (Rathore et al., 2006; Sullivan 2002; Deharo et al., 2002). Perturbation at the point of haem detoxification is a unique drug target and for which several antimalarials such as chloroquine, quinine and amodiaquine is demonstrated to act by the route of binding noncovalently to the iron protoporphyrin IX (FPIX) and preventing the conversion of haem to hemozoin (Leed et al., 2002; Rathore et al., 2006; Warhurst 1981). There are other alternative targets that can be selected from biosynthetic pathways or metabolic pathways that may be absent in humans or in the case of enzymes be the isoforms or homologues.

The bioactive indole alkaloid cryptolepine which is a major component in Cryptolepis sanguinolenta root is thought to act like chloroquine on asexual malaria parasite (Dassonneville et al., 2000). The effect on the sexual stages (gametocytes) is, however, not clearly understood.

The sexual stages (gametocytes) of the malaria parasite accounts for less than 1% of the parasite population during infection (Alano 2007; Dixon et al., 2008). To ensure the preservation of generations of the parasite, the development of these gametocytes is highly necessary for the malaria parasite since that is the reproductive stage of its life cycle. Reports from clinical trials and in vitro studies have provided information on the effect of existing antimalarials on the gametocyte development in humans (Butcher, 1997). Some of the drugs such as chloroquine and quinine have been demonstrated to exhibit activity against immature gametocytes by the same route of target of the metabolic pathways in asexual blood stages of
the parasite (Butcher, 1997). Atovaquone is reported to target the mitochondrial electron transport chain (Bousema & Drakeley, 2011). Artemisinin is also observed to inhibit immature sexual stages (Nosten et al., 2000; Targett et al., 2001). MacRae et al., (2013), reported on the dependence of gametocytes on glucose uptake, glycolysis and the TCA cycle for ATP synthesis and survival. Information on this gametocytes metabolic pathways has made easy the identification of potential targets for the control of their development (MacRae et al., 2013).

1.2.3.3 Effect of anti-malarial drugs on gametocytes

The *P. falciparum* gametocytes is reported to exhibit insensitivity to most of the antimalarials except for artemisinin and primaquine (Butcher, 1997). Even though observed to be less profound, chloroquine and quinine as mentioned earlier have been demonstrated to exhibit activity against immature gametocytes by targeting their food vacuole as in the asexual blood stages of the parasite (Butcher, 1997). Artemisinin is also observed to inhibit immature sexual stages (Nosten et al., 2000; Targett et al., 2001) whereas primaquine eliminates the gametocytes in the peripheral blood stream by inhibiting the mitochondrial function via dihydroorotate dehydrogenase complex involved in pyrimidine biosynthesis (Oduola et al., 1988). Primaquine, however, causes the lysis of red blood cells and thus patients on this medication may experience massive hemolysis which is not restricted to only G6PD-deficient patients (Shekalaghe et al., 2010). In other studies conducted, mefloquine, chloroquine, pyronaridine and primaquine is reported to increase gametocyte numbers at IC$_{10}$ concentration by having effect on their formation. The drugs quinine, artemisinin, and piperaquine also showed a similar significant induction of gametocyte numbers but effective treatment doses of these drugs is reported to annul the situation (Peatey et al., 2009). The drug atovaquone is reported to target the mitochondrial electron transport chain (Bousema & Drakeley, 2011).
1.2.4 ALTERNATIVE DRUG FOR MALARIA TREATMENT

1.2.4.1 Traditional plant medicine and malaria

The first discovery of an antimalarial drug which is named quinine was from the bark of the Cinchona spp. (Rubiaceae) used by the Peruvian Indians (Duran-Reynals, 1947). The structure was established in 1908 by Rabe and the synthesis made by Woodward and Doering in 1944. Currently, it is extracted from the Cinchona spp., which is found in the wild in South America and are cultivated in Java (Boulos et al., 1997). Chloroquine was derived from quinine by synthetic preparation in 1940 (Fidock et al., 2004).

The emergence of chloroquine resistant strains of the malaria parasite led to the search for new antimalaria compounds. This led to the discovery of the compound artemisinin in 1972. Artemisinin was isolated from the Artemisia annua plant species which was used in China for many traditional purposes for several millions of years. From artemisinin, semi-synthetic derivatives such as arteether, artemether and sodium artesunate were obtained (Wright, 2005). The persistent development of resistant strains of the parasite to the artemisinin derivatives such as artesunate made it necessary for the introduction of the antimalarial drug combination therapy (WHO, 2005). Artemether-lumifantrine and artesunate-amodiaquine popularly known as artemisinin combination therapy (ACT) is widely used in Africa for treatment of malaria (CDC, 2013b). Another antimalarial combination therapy is the antimalarial atovaquone which is used in combination with proguanil for treatment of malaria (Fidock et al., 2004). Atovaquone is synthetic 2-alkyl-3-hydroxynaphthoquinone compound which is an analog of lapachol (a prenynaphthoquinone from Tabebuia species; Bignoniaceae) (Looasreesuwon et al., 1999). The search for new compounds from medicinal plants for the treatment of malaria is still ongoing. One such medicinal plant currently under study is Cryptolepis sanguinolenta (Bugyei et al., 2010).
1.2.4.2 *Cryptolepis sanguinolenta* (Lindl.) Schlechter

*Cryptolepis sanguinolenta* is of the family Apocynaceae (subfamily: Periplocoideae) (McGuffin *et al.*, 2000) and appears as a scrambling shrub, 8 metres tall, with a thin twining stem. It contains a yellow-colored juice in the cut stem which is used for dyeing purposes (Paulo & Houghton, 2003).

The plant is distributed from the east to the west coast of Africa. Its existence has been reported from Ghana, Cameroon, Central African Republic, Congo, DR Congo, Uganda, Tanzania and Angola, mostly growing in the tropical rain forest regions (Fig. 1.2, Jansen & Schmelzer, 2010).

![Figure 1.2. Geographical distributions (shaded area) of *Cryptolepis* in Africa (Jansen & Schmelzer, 2010).](image-url)
In some countries like Nigeria and Cote d’Ivoire, the roots which contain the yellow dye are used to tan goat skins (Jansen & Cardon, 2005). Also, the roots and leave extracts are used for the treatment of various forms of diseases such as malaria in Ghana (Bugyei et al., 2010), hepatitis in Guinea Bissau (Silva et al., 1996), hypertension, urinary and upper respiratory tract infections in Ghana and Nigeria (Mills-robertson et al., 2009), colic, stomach complaints, amoebic dysentery and diarrhoea in Senegal, Ghana, Nigeria, Uganda and DR Congo (Tona et al., 1998; Paula et al. 1994) and diabetes (Ajayi et al., 2012). The root extract used as tonic is taken on a daily routine for years and has shown no evidence of toxicity (Appiah, 2009). The plant is known locally in Ghana as Nurubima (Guans), Kadze (Ewe) and Nibima (Twi) (Ameyaw, 2012).

The plant is found commonly on the mountainous territories in Ghana, areas being especially the Akwapim and Kwahu mountains (Addy, 2003). It is used by some indigenous inhabitants in the Akwapim and Kwahu mountains for the management of fever, malaria and some bacterial infections (Boye and Oku-Ampofo, 1990). The antimalarial medicine ‘Phytolaria’ was developed using this plant in Ghana for the treatment of malaria (Bugyei et al., 2010). The leaves and roots of C. sanguinolenta contain the indole bioactive alkaloid, cryptolepine (Fig. 1.3), a major component of the root bark and the first of the indole alkaloid to be isolated from the plant. Also isolated from the roots are other structurally related alkaloids such as hydroxycryptolepine, isocryptolepine, cryptospirolepine, neocryptolepine, neocryptine and quindoline. In the aqueous root extract which is used for the treatment of various ailments, only cryptolepine is detected (Jansen & Schmelzer, 2010). In the ethanolic root extract, apart from cryptolepine, the cryptolepine isomer called neocryptolepine and two others called biscryptolepine and cryptoquindoline which are dimeric alkaloids are isolated (Cimanga et al., 1998). In the case of its use for the treatment of malaria, the bioactive indole alkaloid cryptolepine which is the major constituent of the root bark is reported to possess an
antiplasmodial property among other antiparasitic (Jansen & Schmelzer, 2010), anti-thrombotic (Oyekan & Okafor 1989), noradrenergic (Noamesi & Bamgbose, 1980), vasodilator (Oyekan & Okafor 1989) hypoglycaemic (Herzenberg et al., 2006; Fort et al., 1998) properties. Although its mode of action is not clear, previous studies showed that cryptolepine acts in a similar way to chloroquine on asexual malaria parasites (Feiz Haddad et al., 2004; Wright et al., 2001). Other studies have indicated the role of cryptolepine as cytotoxic, genotoxic, DNA intercalating and topoisomerase II inhibition agent and therefore can be useful as an anticancer agent (Dassonneville et al., 2000). During a treatment survey of malaria using the aqueous extract of C. sanguinolenta, results revealed that the time taken for parasite clearance was only a day longer than using chloroquine (3.3 days for Cryptolepis and 2.2 days for chloroquine). However, the clearance of fever occurred in a much shorter time than with chloroquine (36 hours for Cryptolepis and 48 hours for chloroquine) (Willcox & Bodeker, 2004).

![Structure of Cryptolepine](http://ugspace.ug.edu.gh)

Figure 1.3. Structure of Cryptolepine. The indole bioactive alkaloid from the aqueous root extract of Cryptolepis sanguinolenta (Ablordeppey et al., 1990)
1.2.5 GAMETOCYTE MORPHOLOGY

1.2.5.1 P. falciparum gametocyte development

Gametocytes begin development from the erythrocytic asexual stages (Garnham, 1966). It has been suggested that merozoites emerging from one schizont develop into asexual stages or into gametocytes (Silvestrini et al., 2000; Smith et al., 2000). There are five different developmental stages of gametocytes, the stage I to the matured stage V (Field, J.W. & Shute, P.G., 1956). The early gametocytes (stage I and II) cannot be distinguished morphologically from early asexual stage parasites. The late stage, however, are characterized by their unique crescent or falciform shape (Baton and Ranford-Cartwright, 2005; Dixon et al., 2008). The Stage I gametocytes are indistinguishable from the small round trophozoites (Fig. 1.4) due to their larger round shape but with granular distribution of pigment in their food vacuoles (Day et al., 1998; Sinden, 1982). This stage can more specifically be distinguished by the formation of a subpellicular membrane flattened vesicle and a microtubule array as well as the sexual dimorphism in nuclear size (Sinden, 1982). With the stage II gametocytes (Fig. 1.4), they are noted for their elongated and D-shaped structure within the erythrocyte (Day et al., 1998; Sinden, 1982). Additionally, the subpellicular membrane and microtubule complex are expanded to give an asymmetrical structure. The nucleus is located at the terminal site or elongated across the long axis of the cell with some spindle observed within it (Sinden, 1982). Stage III gametocytes (Fig. 1.4) are also elongated within slightly distorted erythrocytes or are sometimes D-shaped with Pink/blue distinction of the male/female by Giemsa-stain (Day et al., 1998; Sinden, 1982). At this stage, there is further development of the subpellicular membrane complex. The male nucleus is larger (lobbed shaped) then that of the female (Sinden, 1982). However, the female contains slightly more ribosomes, ER and mitochondria then the male gametocyte (Sinden, 1982). The stage IV gametocytes (Fig. 1.4) are also elongated and thin with most red cell distorted. Males pigment tends to be scattered whereas female pigment are mostly
denser (Day et al., 1998; Sinden, 1982). Again male’s nucleus is larger than female nucleus (Sinden, 1982). The membrane and microtubule complex surrounds the gametocyte completely enabling the restoration of symmetry with appearance of membrane bound osmophillic bodies (Sinden, 1982). In the female gametocytes, there is sexual dimorphism and more mitochondria, ribosomes and osmophillic bodies and the existence of a transcription factory (Sinden, 1982). Stages V gametocytes tend to be sausage shaped with more rounded ends (Fig. 1.4). The male stage V’s also called the microgametocytes are pinkish with pigment scattered whereas the females which are known as macrogametocytes are light violet with a dense pigment (Day et al., 1998; Sinden, 1982). This stage Vs gametocytes are characterised by loss of subpellicular microtubules by depolymerisation (Sinden, 1982) and the microgametocytes tend to have reduced ribosomal density with very few mitochondria and a large nucleus with a kinetochore complex attached to the nuclear envelope (Sinden, 1982). The macrogametocytes in contrast have numerous mitochondria, ribosomes and osmophillic bodies with smaller nucleus and a transcription factory (Field & Shute, 1956; Hawking et al., 1971; Langreth et al., 1978; Sinden, 1982).
Figure 1.4. Stages of development of *P. falciparum* gametocytes *in vitro*. These pictures originate from Giemsa-stained thin smear of gametocyte culture (Josling & Llinás, 2015).
1.2.6 GAMETO CYTE METABOLISM

1.2.6.1 Gametocyte energy consumption capacity

Gametocytes depend heavily on glucose consumption for glycolysis and pyruvate for tricarboxylic acid (TCA) cycle metabolism. A study by Oppenheim et al., (2014), provides evidence of the utilization of a conventional TCA cycle for the catabolism of pyruvate by gametocytes. This energy efficient metabolism reportedly sustains gametocyte development during hypoglycaemic state in severe malaria patients (Daily et al., 2007). This suggests that gametocytes survival is dependent on the TCA metabolic pathway and as a result demand for energy requires the switch to such more efficient energy metabolism via upregulation of transcription of the TCA cycle enzymes (Oppenheim et al., 2014). Also, demand for energy in female gametes for the purpose of preparing for post-fertilization stages in the mosquito hemolymph where glucose availability to them is limited may account for the increase in energy production during the gametocytogenesis stage (Talman et al., 2004). Hino et al., (2012), providing evidence of the requirement of a functional TCA cycle by the developing ookinete, all support the view that gametocytes highly depend on the TCA cycle for their high energy demanding physiological processes.
1.2.6.2 Gametocyte mitochondrial metabolism

*P. falciparum* gametocyte’s mitochondrion undergoes certain morphological development during gametocytogenesis. This mitochondrial development is associated with the activation of its metabolism in the gametocytes (Okamoto *et al*., 2009). Further development of the tubular mitochondrial cristae becomes a requirement for the functioning of the mitochondria in the gametocyte (Krungkrai *et al*., 2000). In 2014, a study performed by Lamour *et al*., provided evidence of acetate production as the end product of glucose metabolism by gametocytes. The utilization of this acetate in the tricarboxylic acid (TCA) cycle within the mitochondria of the gametocytes was demonstrated by MacRae *et al*., 2013, and it was shown that the TCA cycle functions to produce NADH and FADH$_2$ which eventually enters the electron transport chain for energy (ATP) production (Fry & Beesley 1991; Uyemura *et al*., 2004; Uyemura *et al*., 2000). In the gametocyte mitochondria, the generation of the membrane potential in the electron transport chain like in other organisms function to translocate proteins across membranes and also import solutes apart from its known role as a contributing factor to the generation of ATP (Laloi, 1999; Pfanner & Geissler, 2001). It is also suggested to be involved in cellular calcium homeostasis (Uyemura *et al*., 2000). Thus, the malfunctioning of the mitochondrion is shown to have pleiotropic effect on other cellular processes within the gametocyte. Owing to this, the mitochondrion is reported to play an important role for the development and survival of the gametocyte (MacRae *et al*., 2013).
1.2.6.3 Tricarboxylic acid (TCA) Cycle in the gametocyte mitochondria

Homologues of enzymes required for the proper functioning of the TCA cycle are all encoded by the *P. falciparum* parasite (Foth *et al*., 2005). An interruption in their activity leads to the impairment of the gametocyte’s TCA metabolic cycle which consequently leads to a loss in the energy production. The strong metabolic phenotype such as reduced yield of NADH observed affects the respiratory chain function and the generation of the mitochondrial membrane potential within the parasite (del Pilar Crespo *et al*., 2008). Study conducted by MacRae *et al*., (2013), provided proof of the existence of the conventional TCA cycle in the mitochondria of gametocytes. In fact, in this parasite stage, work in transcriptomics reveals that 15 of the 16 mitochondrial TCA cycle enzymes are up-regulated (Young *et al*., 2005). The enzymes which are notable in the conventional TCA cycle exist as possible drug targets in gametocytes for the inhibition of their development. The key enzymes known to play a regulatory role in a conventional TCA cycle include pyruvate dehydrogenase, isocitrate dehydrogenase, citrate synthase and alpha ketoglutarate dehydrogenase (Campbell and Reece, 2005).

Although pyruvate dehydrogenase is reported to be missing in the *Plasmodium* parasite species (Foth *et al*., 2005), the mitochondrion-located branched chain α-keto acid dehydrogenase (BCKDH) complex is a possible candidate proposed to function in the place of pyruvate dehydrogenase (Seeber *et al*., 2008). This enzyme is one regulatory point that could targeted for drug action. Another regulatory point to inhibit gametocyte development is at the point of conversion of citrate to isocitrate. Inhibition of the aconitase enzyme which converts citrate to isocitrate results in the subsequent accumulation of citrate which is toxic to the gametocyte and also may reduce the yield of NADH which is very vital for the mitochondrial respiration and energy production in the gametocyte (MacRae *et al*., 2013). Below is a diagram (Fig. 1.5) depicting the conventional TCA cycle of *P. falciparum*. Homologues of TCA cycle enzymes in
*P. falciparum* are depicted in the grey spheres (Fig. 1.5) whereas those that are absent in the *P. falciparum* are in blue spheres and the substrates are shown in blue writing (van Dooren et al., 2006).

Figure 1.5. Schematic depiction of the possible TCA cycle of gametocytes (van Doore et al., 2006).
The initial step of the TCA cycle is also the first committed step and it involves the production of citrate using oxaloacetate and acetyl-CoA as the substrate by the enzyme citrate synthase (Fig. 1.6a). The reaction is an irreversible one which is inhibited by ATP and NADH.

Figure 1.6. The function of some key enzymes in the tricarboxylic acid cycle (Alberts et al., 2004)
Tonkin *et al.*, (2004), reported the existence of a human citrate synthase homologue, (PF10_0218) in *P. falciparum* that contains an N-terminal presequence enabling their transport into the mitochondria. An inhibition of citrate synthase results in a dysfunctional TCA cycle in the gametocytes and the eventual decline in the parasite viability (MacRae *et al.*, 2013). The next step is the isomerisation of citrate to isocitrate which is catalysed by the enzyme aconitase (Fig. 1.6b). The *P. falciparum* aconitase-like-protein (PF13_0229) is believed to have dual localization both in the mitochondrion and cytosol and therefore may possess dual function in iron regulation and aconitase activity (Hodges *et al.*, 2005; Loyevsky *et al.*, 2003; Loyevsky *et al.*, 2001). This is due to the findings that the aconitase belong to a group of protein family that included the iron-responsive element binding proteins (IRPs) and therefore suggests that the *P. falciparum* aconitase function also like IRPs in binding to mRNA species to regulate cellular iron levels (Hodges *et al.*, 2005; Loyevsky *et al.*, 2003; Loyevsky *et al.*, 2001). An inhibition of the aconitase enzyme results in accumulation of citrate which is toxic to gametocytes and may reduce the yield of NADH vital for the mitochondrial respiration and energy production in the sexual stage parasite (MacRae *et al.*, 2013).

The conversion of isocitrate to α-ketoglutarate leads to the production of CO₂ and the reducing equivalent NADH (Fig. 1.6c). The enzyme involved in this step is the isocitrate dehydrogenase. The enzyme in the *P. falciparum* has been identified to require NADP⁺ as cofactor (Chan & Sim, 2003; Wrenger & Müller, 2003) and therefore considered as NADP⁺-dependent isocitrate dehydrogenase (PF13_0242) (Wrenger & Müller, 2003). This enzyme contains an N-terminal extension that enables its transport across the mitochondrion (Wrenger & Müller, 2003). Studies by Wrenger and Muller, (2003), further reported the up-regulation of the *P. falciparum* isocitrate dehydrogenase in the event of oxidative stress. The production of NADPH in the isocitrate dehydrogenase reaction is essential as it serves as reducing agent for
antioxidant enzymes involved in the mitochondrial reductive reactions (Wrenger & Müller, 2003). The *P. falciparum* isocitrate dehydrogenase may hence function for the generation of α-ketoglutarate and not NADH which is required by the *Plasmodium* electron transport chain (van Dooren et al., 2006). In other words, it is involved in the mitochondrial reductive process and not generation of energy (Wrenger & Müller, 2003). The allosteric inhibitor ATP inhibit the function of the enzyme whereas activator ADP is thought to activate the activity (Campbell & Reece, 2005).

The conversion of α-ketoglutarate to succinyl-CoA by the enzyme complex α-ketoglutarate dehydrogenase proceeds after isocitrate dehydrogenase’ activity. Alpha-ketoglutarate dehydrogenase is a multi-enzyme complex belonging to the keto-acid dehydrogenase family. It is composed of three subunits namely E1 (PF08_0045), E2 (PF13_0121) and E3 (PFL1550w) subunits (McMillan et al., 2005). The E3 subunit is shared between all mitochondrial keto-acid dehydrogenases such as the pyruvate and branch-chain amino acid dehydrogenases earlier mentioned and it is believed to target the *P. falciparum* mitochondrion (McMillan et al., 2005). The E2 subunit is said to contain a lipoate cofactor which may be inserted by Lipoate ligase A (LpIA) protein of the mitochondria (Wrenger & Müller, 2004). Inhibition of α-ketoglutarate dehydrogenase results in the abrogation of the process for the synthesis of haem (Figure 1.6d) due to the absence of succinyl-CoA product. The remaining steps of the tricarboxylic acid cycle (steps 5-8) serve to regenerate oxaloacetate for step 1. The enzyme succinyl-CoA synthetase in the 5th step which consist of two subunits, α (PF11_0097) and β (PF14_0295) subunits, converts succinyl-CoA to succinate with the generation of GTP. Succinate is converted to fumarate by succinate dehydrogenase with a reduction of FAD in the process (van Dooren et al., 2006). Succinate dehydrogenase also has two major subunits, flavoprotein (PF10_0334) and iron-
sulphur (PFL0630w) of which their homologues are present in *P. falciparum* (Takeo *et al.*, 2000). Fumarate is converted to malate by fumarate hydratase (PF11340w) which is followed by malate being converted to oxaloacetate to complete the tricarboxylic acid cycle by the malate-quinone oxidoreductase homologue (PFF_0815w) in *P. falciparum*. This process results in the reduction of FAD to FADH$_2$ (Uyemura *et al.*, 2004). Malate dehydrogenase although present in *P. falciparum* just as the malate-quinone oxidoreductase is however not localized in the mitochondrion (Lang-Unnasch, 1992) and therefore is suggested not to be involved in the tricarboxylic acid cycle. The malate-quinone oxidoreductase is found in the *P. falciparum* genome and in some bacteria and is shown to reduce FAD during the generation of oxaloacetate which is fed into the electron transport chain (van Dooren *et al.*, 2006).
CHAPTER TWO

MATERIALS AND METHODS
2.1 PROCESSING OF CRUDE EXTRACT OF CRYPTOLEPIS SANGUINOLENTA (C. sanguinolenta)

Dried roots of *C. sanguinolenta* obtained from Centre for Scientific Research into Plant Medicine (CSIR), Mampong, was pulverized using a grinder and the powdered product taken separately through ethanol and aqueous fractionation.

2.1.1 Ethanolic extraction of crude powdered *C. sanguinolenta* roots

The root powder taken through ethanolic fractionation was initially weighed at 50 g and suspended in 500 ml of 70% ethanol in a flat bottom conical flask. It was left overnight on a shaker after which centrifuged at 450 g for 15 minutes in 50 ml falcon tubes using FORMA 3L GP 4500R centrifuge from Thermo Corporation and the supernatant filtered into flat bottom conical flask and stored at 4°C. The ethanol in the stored supernatant was evaporated using a rotary evaporator (BUCHI Rotavapor R-205) and the residue from the evaporation which is a semi-fluid extract was freeze-dried to obtain powdered ethanolic extract of *C. sanguinolenta*. The freeze drying process was performed by first preserving the sample by freezing it and then subjecting it to high vacuum which removed the ice by sublimation to obtain the dried powdered product.

2.1.2 Aqueous extraction of crude powdered *C. sanguinolenta* roots

Powdered roots of *C. sanguinolenta* was weighed at 50 g and suspended in 500 ml double distilled water (ddH₂O). The aqueous suspension was placed in a water bath and heated at 80°C for 1 hour. After heating, it was cooled, dispensed into 50 ml falcon tubes and centrifuged at 450 g for 15 min. The supernatant was filtered, stored at -80°C and later freeze-dried to obtain a powdered aqueous extract of *C. sanguinolenta*. 

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2.1.3 Preparation of stock concentrations

A weighed mass of 0.2 g of both ethanol and aqueous powdered extract were separately dissolved in 1 ml of 70% ethanol and double distilled water respectively to obtain a 0.2 g/ml stock solution. The filtered sterilised stock concentrations were each used to prepare a working dilution of 10 µg/ml and 100 µg/ml. These were then serially diluted to obtain two sets of five concentrations, from 1 µg/ml to 8 µg/ml and 3.125 µg/ml to 50 µg/ml in complete parasite medium. Using artesunate and primaquine as positive controls, stock concentrations were prepared at 0.5 g/ml and 1mg/ml respectively in DMSO. Using the stocks, concentrations 20 µg/ml and 100 µg/ml were prepared in complete parasite medium. These concentrations were then serially diluted to obtain two sets of five concentrations, from 0.625 µg/ml to 10 µg/ml and 3.125 µg/ml to 50 µg/ml in complete parasite medium. All preparations were stored at -30°C wrapped in aluminium foil until required for drug inhibition assay.

2.2 LONGTERM IN VITRO CULTIVATION OF P. falciparum

2.2.1 Cultivation of the asexual stage malaria parasites

Culturing P. falciparum in vitro, a modified version of Trager and Jensen method (Trager and Jensen, 1976) was employed. Briefly, 3D7 P. falciparum strain retrieved from liquid nitrogen storage was thawed in 37°C water bath and centrifuged at 450 g for 5 minutes. The supernatant was discarded and the pellet resuspended in a thawing solution (3.5% NaCl). The mixture was centrifuged again at 450 g for 5 minutes and the supernatant discarded. A volume of 500 µl complete parasite medium (CPM) containing 10 mg/ml gentamicin, 25 mM HEPES, 2 mM L-glutamine, 7.5% sodium bicarbonate solution (final = 0.2% NaHCO3), 20 mM Glucose and 10% albumax II was added to the pellet and washed by centrifugation at 450 g for 5
minutes. The wash was repeated again and the pellet resuspended in 1ml culture medium. The parasites were then transferred into a T25 cm$^2$ culture flask already containing 4 ml complete culture medium and 200 µl of O$^+$ red blood cells to obtain a total volume of 5 ml parasite culture. To enable the parasites to survive, the culture was gassed with a special gas mixture (90% Nitrogen, 5% CO$_2$ and 5% O$_2$) and then placed in an incubator at 37°C for optimal parasite growth. Culture was maintained daily by changing of the spent medium and addition of fresh CPM and thin smear of culture prepared on slide was fixed with absolute methanol and stained with 10% giemsa for parasitemia estimation under a light microscope. The culture was maintained for 2 weeks to allow for a stable growth of the parasite.

### 2.2.2 Cultivation of the sexual stage (gametocytes) malaria parasite

Culture of asexual stage parasites at a parasitemia of 1%, an initial hematocrit level of 6% and 12.5 ml complete parasite medium was set up in a T75 cm$^2$ culture flask, gassed with a special gas mixture (90% Nitrogen, 5% CO$_2$ and 5% O$_2$) and then placed in an incubator at 37°C for optimal parasite growth. The spent culture supernatant was replenished with fresh 12.5 ml medium on day 3 and day 6 and then replenished everyday with 25 ml medium to day 9. On day 9, ring culture (10-15% parasitemia) obtained was synchronised using 5% sorbitol and thereafter treated with 60% percoll to eliminate any gametocytes. The synchronised rings were put back in culture and monitored daily for the development of gametocytes. Upon development of early gametocytes (stage I & II) on day 4 from the start of asexual synchronisation, 50 mM NAG (N-acetyl Glucosamine) was added to the medium to eliminate asexual parasites in the culture. The gametocytes obtained at the desired stage were enriched by 60% percoll (see appendix IV) synchronisation. Each gametocyte stage took approximately 2 days to develop. On day 16 from start of culture, gametocyte infected RBCs (mainly stage IIIIs) were enriched by 60% percoll
density gradient centrifugation (refer to appendix IV). The enriched gametocytes were washed twice in incomplete parasite medium (without albumax) and put back in culture overnight. The gametocytemia was determined the next day by thin smear preparation, methanol fixation, 10% giemsa staining and microscopy.

2.3 IN VITRO DRUG INHIBITION ASSAY ON 3D7 MALARIA PARASITES

2.3.1 Determination of the effects of *C. sanguinolenta* extracts on asexual stages of *P. falciparum*

**Ring stage inhibition assay:** *Plasmodium falciparum* parasites cultured for 2 weeks and at a parasitemia of 5% rings were sorbitol synchronised twice at an 8 hour interval to obtain early rings. The culture of rings was adjusted to 1% parasitemia, 4% hematocrit and 900 µl was added to each well of 48-well-flat-bottom plate containing 100 µl of either *C. sanguinolenta* ethanolic or aqueous extract at concentrations ranging from 1 µg/ml to 8 µg/ml. Artesunate which was originally dissolved in DMSO and then prepared concentrations made in CPM was used as drug control. DMSO and ethanol (70%) were included as solvent controls. Positive control which is only the infected culture and the negative control which is uninfected RBCs were included. All experiment were setup in triplicates. The prepared plates were then placed in an incubating chamber, gassed using a special gas mixture (90% Nitrogen, 5% CO₂ and 5% O₂) for 6 minutes after which it was placed in an incubator set at 37°C. The assay was incubated for 48 hours and after the plates were removed and placed on the bench at room temperature for 30 minutes. The cells were harvested by removing the supernatant and thin smears were prepared from each well. The smears were allowed to air dry and then fixated in absolute methanol. The slides were stained with 10% giemsa (freshly prepared) for 15 minutes, air dried and observed under a light microscope (Olympus CH3ORF200, Japan) to determine the parasitemia of the culture. The
latter involves estimating the population of infected red blood cells against the total population of red blood cells ($\geq 200$) and then finding the percentage. Positive and negative controls included in the plates were also harvested.

**Trophozoite stage inhibition assay:** The 3D7 parasites cultured for 2 weeks and at a parasitemia of 5% rings were sorbitol synchronised and put back in culture. It was allowed to incubate for another 12hrs in order to obtain late trophozoites. The trophozoites were adjusted to 1% parasitemia, 4% hematocrit and 900 µl was added to each well of 48-well-flat-bottom plate containing 100 µl of either *C. sanguinolenta* ethanolic or aqueous at concentrations ranging from 1 µg/ml to 8 µg/ml. As described earlier, artesunate which was originally dissolved in DMSO and then prepared concentrations made in CPM was used as drug control. DMSO and ethanol (70%) were included as solvent controls. Positive control which is only the infected culture and the negative control which is uninfected RBCs were included. All experiment were setup in triplicates. The prepared plates were placed in an incubating chamber, gassed (90% Nitrogen, 5% CO$_2$ and 5% O$_2$) for 6 minutes after which placed in an incubator at 37°C. Assay was incubated for 48 hours and then harvested following the procedure described for the rings stages above.

**Schizont stage inhibition:** The 3D7 parasites cultured for 2 weeks and at a parasitemia of 5% schizonts were 65% percoll synchronised (see appendix III) and then put back in culture for another 48 hours. The schizonts stage was adjusted to 1% parasitemia, at 4% hematocrit and 900 µl was added to each well of 48-well-flat-bottom plate containing 100 µl of either *C. sanguinolenta* ethanolic or aqueous at concentrations ranging from 1 µg/ml to 8 µg/ml. Artesunate which was originally dissolved in DMSO and then prepared concentrations made in CPM was used as drug control. DMSO and ethanol (70%) were included as solvent controls. Positive control which is only the infected culture and the negative control which is uninfected
RBCs were included. All experiments were conducted in triplicates. The prepared plates were placed in an incubating chamber, and gassed with a special gas mixture (90% Nitrogen, 5% CO₂ and 5% O₂) for 6 minutes after which they were incubated at 37°C for 48 hours and then harvested following the procedure described for the rings stages above.

2.3.2 Determination of the effects of *C. sanguinolenta* extracts on sexual stages of *P. falciparum* parasite by colorimetric assay

The culture of enriched gametocytes obtained by percoll density gradient centrifugation as described in section 2.2.2. was adjusted to 2-5 x 10⁵ gametocytes (5-10% parasitemia) in 8 x 10⁶ – 10 x 10⁶ RBC/90 µl CPM and 90 µl of the culture was added to each well of 96-well-flat-bottom plate containing 10 µl of the either *C. sanguinolenta* ethanolic or aqueous extract at concentrations ranging from 3.125 µg/ml to 50 µg/ml. Primaquine at concentrations ranging from 3.125 µg/ml to 50 µg/ml was used as drug control. The prepared plates were placed in an incubating chamber, gassed for 6 minutes with the special gas mixture to allow for optimal growth and then placed in an incubator set at 37°C for 48 hours. After 48 hrs, 10 µl alamar blue was added to each well as a fluorometric/colorimetric indicator to detect the level of metabolic activity of the gametocytes in the culture and was incubated for another 24 hrs. The plates were then read at a fluorescence emission and excitation wavelength of 550/700 nm using a fluorescence microplate reader (Infinite M200 PRO, TECAN, Austria). The optical density (OD) values obtained from the fluorescence reading were used for the calculation of the % inhibition of the extracts. Negative and positive controls were included in the test plate.
2.4 ENZYME ACTIVITY ASSAYS

2.4.1 Development of gametocytes for enzyme activity assays

The laboratory strains of *P. falciparum* (NF54) were cultured for 14 days to generate gametocyte. On day 14, gametocyte infected RBCs were enriched by 60% percoll density gradient centrifugation (see appendix IV for 60% percoll synchronisation). The enriched gametocytes were washed twice in incomplete parasite medium (without albumax) and put back in culture overnight. The gametocytemia (stage I-V) was determined the next day by thin smear preparation, methanol fixation, 10% giemsa staining and microscopy reading. At gametocyte stage III, the culture was split into 5 different flasks and each treated separately with different concentrations (IC₁₀, IC₅₀ and IC₉₀ generated from the gametocytes inhibition assay) of the ethanolic *C. sanguinolenta* extract, using sodium acetate (NaAc) which is known to be an inhibitor of aconitase as the reference control at a concentration of 10 µg/ml. A control setup was prepared with untreated gametocytes in the fifth flask. All five flasks were gassed with the special gas mixture (90% Nitrogen, 5% CO₂ and 5% O₂) and incubated for 48 hours at 37°C in an incubator.

2.4.2 Gametocytes mitochondrial isolation

The mitochondria of the gametocytes in each of the five flasks were isolated separately using a commercial mitochondrial isolation kit obtained from Biovision (USA). Briefly, gametocytes at a concentration of 2 x 10⁵ cells were centrifuged at 600 g for 10 minutes. The pellet was resuspended in 1ml of mitochondrial isolation buffer and vortexed for 5 seconds followed with incubation on ice for 2 minutes to solubilise the gametocytes to release the mitochondria. A volume of 10 µl of reagent A provided in the kit was added and vortexed again for 5 seconds.
And then incubated on ice for 5 minutes while vortexing every minute for 5 seconds. The sample was then centrifuged at 600 g for 10 minutes at 4°C. Collected supernatant was centrifuged at 7000 g for 10 minutes at 4°C. The pellet which is mitochondria obtained after centrifugation was washed with mitochondrial isolation buffer and the supernatant discarded. The pellet of mitochondria was used in the next experiment for the enzyme activity assay.

2.4.3 Determination of the effect of C. sanguinolenta ethanolic extract on gametocyte aconitase activity

The gametocyte’s aconitase activity was determined using commercially available aconitase assay kit obtained from Abcam (USA). Briefly, mitochondria isolated as described above in section 2.4.2 were resuspended in 0.1ml cold assay buffer on ice and then centrifuged at 800g for 10 minutes at 4°C to solubilise the mitochondria in order to release the aconitase into suspension. To 100 µl of the supernatant was added 10 µl of activation solution and incubated on ice for 1 hour which led to the activation of the aconitase. After incubation, 10 µl of the sample was aliquoted into a 96-well plate in triplicates and the volumes adjusted to 50 µl with the aconitase assay buffer. Into separate wells were also added in triplicate different concentrations (0 nmol, 4 nmol, 8 nmol, 12 nmol, 16 nmol and 20 nmol) of isocitrate standard as positive standard. A control background well was also prepared in triplicate using the test sample. To each test well (sample, control and standards) was added 50 µl of reaction mixture composing of 46 µl assay buffer, 2 µl enzyme mix and 2 µl substrate, bringing the whole assay volume to 100 µl in each well. To each of the background control wells was added 50 µl of background control mixture composing of 48 µl assay buffer and 2 µl enzyme mix. All contents were thoroughly mixed and incubated for 30 minutes at 25°C. A volume of 10 µl developer was
added to each well after incubation and mixed well. Again, it was incubated for 10 minutes at 25°C room temperature and the optical density (OD) was measured at 450 nm using a colorimetric reader (EL808, BioTek microplate reader, USA).

2.4.4 Determination of the effect of *C. sanguinolenta* ethanolic extract on the gametocyte citrate synthase activity

The gametocyte citrate synthase activity assay was determined using commercially available citrate synthase activity assay kit obtained from Abcam (USA). Mitochondria isolated as described in section 2.4.2 were centrifuged at 500 g for 10 minutes at 4°C. Cells were rinsed twice with phosphate buffered saline (PBS) and the cell pellet solubilised in 9 volumes citrate synthase extraction buffer in order to release the citrate synthase into suspension. The sample mixture was incubated on ice for 20 minutes and then centrifuged at 16000 g for 20 minutes at 4°C. The supernatant was collected and pellet discarded. The supernatant was aliquoted at 100 µl per well in triplicate into microplate strips. Background control consisting of only the supernatant or sample and a zero standard consisting of only incubation buffer was also added to wells in triplicates. The wells of the microplate strip were already coated with anti-citrate synthase antibody which captures any citrate synthase enzyme present in the sample mixture prepared. This makes easier the separation of the citrate synthase present in the sample mixture for the reaction process while the rest of the sample mixture were washed off. The microplate strips were covered and incubated for 3 hours at room temperature. After incubation, samples were aspirated off in each well and wells washed twice with 300 µl wash buffer. Complete removal of liquid in each well was performed at the last wash by inverting the plate and blotting it against clean paper towel to remove excess liquid. Gently, 100 µl fresh mixed activity solution was added into each well, minimizing the production of bubbles. Absorbance was immediately
read at 412 nm using a colorimetric microplate reader at two time points, 5 minutes and 15 minutes at an ambient temperature of 27°C and with shaking at 20 seconds intervals.

2.4.5 Determination of the effect of *C. sanguinolenta* ethanolic extract on gametocyte α-Ketoglutarate dehydrogenase activity

The alpha-ketoglutarate dehydrogenase activity was determined using commercially available alpha-ketoglutarate dehydrogenase activity assay kit (Abcam, USA). In brief, the mitochondria isolated as described above in section 2.4.2 were resuspended in 100 µl ice cold α-ketoglutarate dehydrogenase (KGDH) assay buffer on ice and centrifuged at 10,000 g for 5 minutes. After centrifugation, the supernatant was collected and pellet discarded. The supernatant was aliquoted at 20 µl per well into a 96-well microtitre plate in triplicate and the volumes adjusted to 50 µl with KGDH assay buffer. KGDH positive control solution provided in the kit was added to the well also in triplicate at 10 µl per well and the volumes also adjusted to 50 µl with KGDH assay buffer. Background control consisting of only the test sample was also added to wells in triplicates. Again, NADH standard was added to wells at different concentrations (0, 2.5 nmol, 5.0 nmol, 7.5 nmol, 10 nmol and 12.5 nmol) in triplicates. To each test well (sample, positive control and standards) was added 50 µl of reaction mixture composing of 46 µl KGDH assay buffer, 2 µl KGDH developer and 2 µl KGDH substrate, bringing the whole assay volume to 100 µl in each well. To each of the background control wells was added 50 µl of background control mixture composing of 48 µl KGDH assay buffer and 2 µl KGDH developer and all wells were thoroughly mixed. The absorbance was immediately read at an optical density (OD) of 450 nm in a kinetic mode and choosing two time points 10 minutes (T₁) and 30 minutes (T₂) at a temperature of 37°C to calculate the α-Ketoglutarate dehydrogenase activity. The NADH standard was read at the end of the incubation time point of 30 minutes.
2.5 EXAMINATION OF THE EFFECT OF C. SANGUINOLENTA ETHANOLIC EXTRACT ON LATE GAMETOCYTE STAGE MITOCHONDRIAL MEMBRANE POTENTIAL

Enriched stage III P. falciparum (NF54) gametocytes were used to start gametocyte culture at 1% parasitemia, 1% hematocrit in complete parasite medium and then divided into four treatment groups. The treatment groups included gametocytes treated with the ethanolic C. sanguinolenta extract at different concentrations (IC$_{10}$, IC$_{50}$ and IC$_{90}$) and that treated with 1 mM sodium acetate (NaAc) as the positive control. All were gassed and incubated for 96 hours in T25 cm$^2$ culture flasks. Untreated gametocytes in a separate flask were used as control. This set up was performed for two different experiments involving the effect of C. sanguinolenta ethanolic extract on the gametocytes mitochondrial membrane potential and on the gametocytes intracellular membranes. For the study of the effect of C. sanguinolenta ethanolic extract on the mitochondrial membrane potential, treated and untreated cultures were incubated for 30 minutes with Rhodamine 123 dye at a final concentration of 0.1 µg/ml. After the incubation, cultures were centrifuged at 450 g for 5 minutes and resuspended in complete parasite medium and incubated for an additional 30 minutes at 37°C. Cells were then washed at 450 g for 5 minutes with phosphate buffered-saline (PBS). Pellets obtained were resuspended in PBS and a drop placed on a glass slide with a cover slip placed on it. A nail polish was applied at the end of the coverslip on the slide to prevent flow of the suspension. The fluorescent images of the labelled gametocytes were obtained using Nikon standard fluorescence microscope with accompanying cellsense imaging software program. The images were later analysed with FIJI image software program.
2.6 EXAMINATION OF THE EFFECT OF *C. SANGUINOLENTA* ETHANOLIC EXTRACT ON LATE GAMETOCYTE STAGE INTRACELLULAR MEMBRANES

Enriched stage III *P. falciparum* (NF54) gametocytes were used to start gametocyte culture at 1% parasitemia, 1% hematocrit in complete parasite medium and then divided into four treatment groups. The treatment groups included gametocytes treated with the ethanolic *C. sanguinolenta* extract at different concentrations (IC$_{10}$, IC$_{50}$ and IC$_{90}$) and those treated with 1 mM sodium acetate (NaAc) as the positive control. All were gassed and incubated for 96 hours in T25 cm$^2$ culture flasks. Untreated gametocytes were used as control. With regard to the study of the effect of *C. sanguinolenta* ethanolic extract on the intracellular membranes, the treated and untreated cultures were incubated overnight in the presence of Bodipy-TR ceramide dye at a final concentration of 0.7 µM. After incubation, cells were washed at 450 g for 5 minutes with phosphate buffered saline (PBS). Pellets obtained were resuspended in PBS and a drop placed on a glass slide with a cover slip placed on it. A nail polish was applied at the ends of the coverslip on the slide to prevent flow of the suspension. The fluorescent images of the labelled gametocytes were obtained using Nikon standard fluorescence microscope with accompanying cellsense imaging software program. The images were analysed with FIJI image software program.
2.7 DATA ANALYSIS

The results obtained from the various experiments were imported to excel and analysed. Percentage parasitemia estimated from each of the drug assays performed were used to calculate the % parasite growth inhibition as shown from the formulae below. The % growth inhibition values obtained were input in Graphpad prism version 5 statistical software. Using the nonlinear regression, log (dose) vrs response analysis on the graphpad prism version 5 statistical software, the 10%, 50% and 90% inhibition concentration (IC) values were obtained. The IC\textsubscript{50} of both the ethanolic and aqueous \textit{C. sanguinolenta} extracts obtained for all the treated stages of the malaria parasite showed the clearance of half the population of the parasites. For the enzyme activity assays, comparison of results between groups and across groups was determined by using one-way ANOVA and Dunnett’s multiple comparison test analysis method. The difference was considered significant if the P value was less than 0.05 (P < 0.05). The Cellsense image acquisition software from Nikon was used to acquire the fluorescent labelled gametocyte images and then analysed with FIJI image software program.

\[
\% \text{Parasitemia} = \frac{\text{Test Parasitemia}}{\text{Control Parasitemia}} \times 100
\]

\[
\% \text{Parasite Growth Inhibition} = \frac{\% \text{Control Parasitemia} - \% \text{Test Parasitemia} \times 100}{\% \text{Control Parasitemia}}
\]
CHAPTER THREE

RESULTS
3.1 CRYPTOLEpis Sanguinolenta (C. sanguinolenta) EXTRACTION

Dried roots of the C. sanguinolenta were processed via two extraction methods, ethanolic and aqueous extraction, to obtain powdered products as shown in Fig. 3.1. The percentage yield from the extraction process for both products was 6.7% and 8.2% respectively. The aqueous fractionated product was a powder with a yellow-orange colour appearance whilst the ethanol fractionated product was also a powder but with a dark-brown coloured appearance (Fig. 3.1).

Figure 3.1. Powdered products of C. sanguinolenta dried roots processed via ethanolic (left) and aqueous (right) extraction methods.
3.1.1 Effects of the ethanolic and aqueous extracts of *C. sanguinolenta* on the asexual stages of *P. falciparum*

Ethanolic and aqueous extracts of *C. sanguinolenta* roots were used for the asexual stage inhibition assays. The values of the IC$_{50}$’s generated from the graphs are shown in Fig. 3.2 (A-C). Higher activity was exhibited by the ethanolic extract on the rings stage, giving rise to an IC$_{50}$ of 9.208 ± 1.36 µg/ml compared to the aqueous extract with IC$_{50}$ 99.98 ± 10.8 µg/ml (Fig. 3.2 A). Synchronous culture of trophozoites was also treated with the ethanolic and aqueous extracts of *C. sanguinolenta* independently. The value of the IC$_{50}$’s generated from the graphs in Fig. 3.2 (B) for both the ethanolic and aqueous extracts were 3.3 ± 0.79 µg/ml and 14.16 ± 3.98 µg/ml respectively. Again a higher activity was exhibited by the ethanolic extract on the schizonts stages of the parasite with IC$_{50}$ 0.28 ± 0.05 µg/ml compared to the aqueous extract with IC$_{50}$ 1.5 ± 0.7 µg/ml (Fig. 3.2 C). Across all *C. sanguinolenta* treated asexual parasite groups, a weak trend of dose dependent effect was observed for the activity of both the ethanolic and aqueous extracts. As a reference drug, artesunate yielded an IC$_{50}$ of 5.36 ± 3.6 ng/ml (Fig. 3.2 D) on the schizonts stage which was much lower than the IC$_{50}$’s obtained for both the ethanolic and aqueous *C. sanguinolenta* extracts. A dose-dependent inhibition curve was observed for the graph of the effect of artesunate on the schizonts stage in Fig. 3.2 (D). Activity of artesunate on the rings and trophozoites stage to determine the IC$_{50}$’s was not conducted (Table 3.1). The artesunate was tested on the schizont stages because report indicates that the artesunate which is a derivative of artemisinin exert their effect as blood schizonticides (Guerin *et al.*, 2002).
Figure 3.2. Effects of *C. sanguinolenta* ethanolic and aqueous roots extracts and artemesunate on asexual stages of *P. falciparum*. (A) Growth-inhibitory activity of *C. sanguinolenta* ethanolic and aqueous roots extracts when incubated with rings for a total of 48 hrs. (B) Growth-inhibitory activity of *C. sanguinolenta* ethanolic and aqueous roots extracts when incubated with trophozoites with total incubation time of 48 hrs (12-15 postinvasion). (C) Growth-inhibitory activity of *C. sanguinolenta* ethanolic and aqueous roots extracts when incubated with schizonts with total incubation time of 48 hrs (24-28 hrs postinvasion). The growth-inhibitory activity of *C. sanguinolenta* on schizonts was compared to the growth-inhibitory activity of artemesunate. The LogIC_{50} and IC_{50} are shown on the graphs. Data represent the means of three experiments; error bars represent standard errors of the means.
3.1.2 Effects of the ethanolic and aqueous root extracts of *C. sanguinolenta* on the sexual stages of *P. falciparum*

Unsynchronised gametocytes (stage I-IV) culture was treated with both ethanolic and aqueous extracts of *C. sanguinolenta*. As shown in Fig. 3.3 (A), a greater inhibition was exhibited by the ethanolic extract on the unsynchronised gametocytes with the IC\(_{50}\) of 207.6 ± 27.58 µg/ml than the aqueous extract with IC\(_{50}\) of 374 ± 35.4 µg/ml. It was observed that the effect of the extracts followed a weak trend of dose-dependent activity (Fig. 3.3 A). A weak inhibitory response was observed against synchronous early stage gametocytes (stage I & II) when treated with both the ethanolic and aqueous root extract with IC\(_{50}\)’s of 307 ± 47.4 µg/ml and 305 ± 42.8 µg/ml respectively (Fig. 3.3 B). Synchronous late gametocytes stage (III) treated with the ethanolic and aqueous extracts of *C. sanguinolenta* also exhibited weak susceptibility to the extracts. As shown in Fig. 3.3 (C), the value for the IC\(_{50}\)’s generated were 291.2 ± 24.66 µg/ml for the ethanolic extract, showing greater activity compared to 307.9 ± 20.42 µg/ml for the aqueous *C. sanguinolenta* extracts. As a reference drug, primaquine yielded an IC\(_{50}\) of 314.8 ± 9.8 ng/ml (Fig. 3.3 D) on the synchronised late gametocytes stage which was much lower than the IC\(_{50}\)’s obtained for both the ethanolic and aqueous *C. sanguinolenta* extracts. The inhibition in this case also followed a weak trend of dose dependent activity. Again activity of primaquine was tested only on the late gametocytes stage (Table 3.1) since report indicates that primaquine only kills mature stage gametocytes (White, 2008).
Figure 3.3. Effects of C. sanguinolenta ethanolic and aqueous roots extracts and primaquine on sexual stages of P. falciparum. (A) Growth-inhibitory activity of C. sanguinolenta ethanolic and aqueous roots extracts when incubated with unsynchronised gametocyte stages for a total of 48 hrs. (B) Growth-inhibitory activity of C. sanguinolenta ethanolic and aqueous roots extracts when incubated with early gametocytes stage with total incubation time of 48 hrs. (C) Growth-inhibitory activity of C. sanguinolenta ethanolic and aqueous roots extracts when incubated with late gametocytes stage with total incubation time of 48 hrs. The growth-inhibitory activity of C. sanguinolenta on late gametocytes stage was compared to the growth-inhibitory activity of primaquine. The Log IC\textsubscript{50} and IC\textsubscript{50} are shown on the graphs. Data represent the means of three experiments; error bars represent standard errors of the means.
### 3.1.3 Summary of the inhibitory concentrations of *C. sanguinolenta* ethanolic and aqueous roots extracts against 3D7 *P. falciparum* asexual and gametocytes stage.

<table>
<thead>
<tr>
<th></th>
<th>IC$_{50}$ (Crypt_ETOH)</th>
<th>IC$_{50}$ (Crypt_AQ)</th>
<th>IC$_{50}$ (Reference drug)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rings Inhibition</td>
<td>9.208± 1.36µg/ml</td>
<td>99.98± 10.8µg/ml</td>
<td>ND</td>
</tr>
<tr>
<td>Trophozoite Inhibition</td>
<td>3.3± 0.79µg/ml</td>
<td>14.16± 3.98µg/ml</td>
<td>ND</td>
</tr>
<tr>
<td>Schizont Inhibition</td>
<td>0.28± 0.05µg/ml</td>
<td>1.5± 0.07µg/ml</td>
<td>5.36± 3.6ng/ml*</td>
</tr>
<tr>
<td>Gametocyte Inhibition-All stages</td>
<td>207.6± 27.58µg/ml</td>
<td>374 ± 35.4µg/ml</td>
<td>ND</td>
</tr>
<tr>
<td>Gametocyte Inhibition-Early stage</td>
<td>307± 47.4µg/ml</td>
<td>305± 42.8µg/ml</td>
<td>ND</td>
</tr>
<tr>
<td>Gametocyte Inhibition-Late stage</td>
<td>291.2± 24.66µg/ml</td>
<td>307.3±20.42µg/ml</td>
<td>314.8 ± 9.8ng/ml**</td>
</tr>
</tbody>
</table>

Table 3.1. Inhibitory concentrations of *C. sanguinolenta* ethanolic and aqueous roots extracts. Effects on the asexual and the sexual stages were determined using graphpad prism version 5 software. * = Artesunate, ** = Primaquine, ND = Not done.
3.2 EFFECT OF THE ETHANOLIC ROOTS EXTRACT OF *C. SANGUINOLENTA* ON LATE GAMETOCYTES STAGE MORPHOLOGY

The effect of the *C. sanguinolenta* ethanolic roots extract on the morphology of the late gametocyte stage was determined. The *C. sanguinolenta* ethanolic extract at IC\(_{10}\) (58.24 µg/ml), IC\(_{50}\) (291.2 µg/ml) and IC\(_{90}\) (524.16 µg/ml) as well as sodium acetate (NaAc) at 1 mM concentration were tested against stage III gametocytes. Untreated gametocytes were used as control during the experiment. The setup was incubated for 48hrs followed by smear preparations. Observation under light microscope showed significant changes in the physical appearance of the treated gametocytes compared to the control (Fig. 3.4). The elongated or spindle shaped appearance of a stage IV observed for the healthy gametocyte control seemed to have been deformed in the treated gametocytes. The pigment within the deformed gametocytes was randomly dispersed throughout whereas it was localised in the centre within the control gametocytes. Although starting with the same parasite material the results after the experiment showed a complete disorganization of the cell outer membrane unit leading to the abnormal shape of the IC\(_{10}\), IC\(_{50}\) and IC\(_{90}\) treated gametocytes (Fig. 3.4). NaAc treated gametocytes appeared to have maintained their outer membrane rigidity allowing them to retain the elongated or spindle shaped characteristic of a stage IV gametocyte just as the control. The results therefore indicate a possible defect in the overall developmental process of the ethanolic *C. sanguinolenta* treated late gametocytes stages.
Figure 3.4. Effect of ethanolic *C. sanguinolenta* root extract on late gametocyte stage morphology. Treated groups include gametocytes treated with IC$_{10}$ (58.24 µg/ml), IC$_{50}$ (291.2 µg/ml), IC$_{90}$ (524.16 µg/ml) concentration of *C. sanguinolenta* ethanolic roots extract, sodium acetate (NaAc) at 1 mM (10 µg/ml) concentration and an untreated gametocyte setup as control. The same parasite material was used for all setups before 48 h incubation. Gametocytes images were obtained before and after 48hrs incubation under an Olympus light microscopy.
3.2.1 Effect of the ethanolic *C. sanguinolenta* roots extract on citrate synthase activity in the tricarboxylic acid (TCA) pathway of late gametocytes stage

The setup of the experiment described in the methods (section 2.4) was used for the enzyme activity assay performed. Citrate synthase activity was measured in stage III gametocytes grouped into five experimental setups. The histogram plot for the mean of triplicate values for the citrate synthase activity against the treated groups showed a dose-dependent activity (Fig. 3.5). Citrate synthase activity was relatively lower for the *C. sanguinolenta* treated gametocyte groups compared to the untreated gametocytes as shown in Fig. 3.5 (P = 0.04). It was observed that the lowest activity obtained was for the NaAc (1mM) treated gametocyte (III), followed by the IC$_{90}$ (524.16 µg/ml), IC$_{50}$ (291.2 µg/ml) and then IC$_{10}$ (58.24 µg/ml) treated gametocytes (Fig. 3.5). The NaAc was used as a positive control in the experiment and in using this compound, there was an expected feedback inhibition on citrate synthase as a result of the accumulation of the end product, citrate, from citrate synthase catalysis. This accumulation is due to the inhibition of aconitase which converts citrate to isocitrate by the NaAc. From the histogram plot in Fig. 3.5, inhibition of the citrate synthase was found to be significant for the IC$_{90}$ treated and the NaAc treated gametocytes compared to the untreated or control gametocytes (P = 0.04). Using the One-way ANOVA statistical analysis, results showed significant difference for the across group comparison test (P = 0.04). A statistical significance was also obtained for the pair-wise group comparison test using the Dunnet’s multiple comparison tests (P = 0.04).
### Table 3.5

<table>
<thead>
<tr>
<th>Treatment Group</th>
<th>Citrate Synthase Activity (Δm OD/min @ 412nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IC10</td>
<td>0.00</td>
</tr>
<tr>
<td>IC50</td>
<td>0.01</td>
</tr>
<tr>
<td>IC90</td>
<td>0.02</td>
</tr>
<tr>
<td>NaAc</td>
<td>0.03</td>
</tr>
<tr>
<td>Control</td>
<td>0.04</td>
</tr>
</tbody>
</table>

**Figure 3.5.** Effect of the ethanolic roots extract of *C. sanguinolenta* on the citrate synthase activity in the tricarboxylic acid (TCA) pathway of late gametocytes stage. Treated groups included gametocytes treated with IC10 (58.24 μg/ml), IC50 (291.2 μg/ml), IC90 (524.16 μg/ml) concentration of *C. sanguinolenta* ethanolic roots extract, sodium acetate (NaAc) at 1mM (10 μg/ml) concentration and an untreated gametocyte setup as control. Measurement of citrate synthase activity was obtained from the OD of the enzyme product formed per minute per μl of sample used. Values are means ± S.D. Across group comparison using ANOVA: *P = 0.04. Pair-wise group comparison using Dunnet’s multiple comparison test: *P = 0.04.
3.2.2 Effect of the ethanolic roots extract of *C. sanguinolenta* on aconitase activity in the TCA pathway of late gametocytes stage

The histogram plot for the mean of triplicate values for the aconitase activity against the treated groups showed a dose-dependent activity (Fig. 3.6). Results from the enzyme activity assay revealed inhibition of aconitase by the *C. sanguinolenta* ethanolic roots extracts at IC$_{10}$, IC$_{50}$ and IC$_{90}$ (Fig. 3.6). Inhibition of aconitase activity was significant for both the IC$_{50}$ and IC$_{90}$ concentrations of the extract compared to the control (P = 0.001 and P = 0.001 respectively). NaAc (1 mM or 10 µg/ml) which is reported to directly inhibit aconitase activity was used to treat gametocytes as a positive control and a significant inhibition was observed for its effect on aconitase as expected (P = 0.001; Fig. 3.6). Compared to the untreated or control gametocytes, all treated gametocytes showed significant level of inhibition of the aconitase activity (P = 0.001). Across group comparison of all treated gametocytes together with the control using ANOVA was also statistically significant (P = 0.0001).
Figure 3.6. Effect of the ethanolic roots extract of *C. sanguinolenta* on the aconitase activity in the tricarboxylic acid (TCA) pathway of late gametocytes stage. Treated groups include gametocytes treated with IC$_{10}$ (58.24 µg/ml), IC$_{50}$ (291.2 µg/ml), IC$_{90}$ (524.16 µg/ml) concentration of *C. sanguinolenta*, sodium acetate (NaAc) at 1 mM (10 µg/ml) concentration and an untreated gametocyte setup as control. Measurement of aconitase activity was obtained from the amount of the enzyme product formed taken over two time points per ml of sample used. Values are means ± S.D; Pair-wise comparison using Dunnet’s multiple comparison test: ***P = 0.001.
3.2.3 Effect of the ethanolic roots extract of *C. sanguinolenta* on α-Ketoglutarate dehydrogenase activity in the TCA pathway of late gametocytes stage

Stage III gametocytes obtained by synchronisation and treated with the ethanolic roots extract of *C. sanguinolenta* at the IC\textsubscript{10} (58.24 µg/ml), IC\textsubscript{50} (291.2 µg/ml), IC\textsubscript{90} (524.16 µg/ml) concentration and also NaAc (1 mM or 10 µg/ml) were analysed for α-Ketoglutarate dehydrogenase activity over a kinetic mode at two time points. Results from the enzyme activity assay revealed weak inhibition of the enzyme α-Ketoglutarate dehydrogenase by the ethanolic *C. sanguinolenta* extracts (Fig. 3.7). Inhibition of α-Ketoglutarate dehydrogenase activity was not significant for both the IC\textsubscript{50} and IC\textsubscript{90} concentrations of the extract (P > 0.05). The NaAc (1 mM or 10 µg/ml) was used to treat gametocytes as a negative control and was expected to show no reduction in the α-Ketoglutarate dehydrogenase activity. From the histogram plot in Fig. 3.7, the level of the effect of the NaAc seemed to parallel with that of the untreated or control group, giving an indication of no effect at all as expected. Comparison of all the treated gametocytes together with the control using ANOVA was statistically not significant (P = 0.1).
Figure 3.7. Effect of the ethanolic roots extract of *C. sanguinolenta* on the α-Ketoglutarate dehydrogenase activity in the tricarboxylic acid (TCA) pathway of late gametocytes stage. Treated groups include gametocytes treated with IC<sub>10</sub> (58.24 µg/ml), IC<sub>50</sub> (291.2 µg/ml), IC<sub>90</sub> (524.16 µg/ml) concentration of *C. sanguinolenta*, sodium acetate (NaAc) at 1mM concentration and an untreated gametocyte setup as control. Measurement of α-Ketoglutarate dehydrogenase activity was obtained from the amount of the enzyme product formed taken over two time points per ml of sample used. Values are means ± SD; Across group comparison using ANOVA: P = 0.1.
3.3 EFFECT OF *C. SANGUINOLENTA* ETHANOL ROOTS EXTRACT ON THE MITOCHONDRIAL MEMBRANE POTENTIAL OF LATE GAMETOCYTES STAGE BY RHODAMINE 123 LABELLING

Examination of the functioning of the mitochondrial membrane potential of the gametocytes treated with the ethanolic *C. sanguinolenta* roots extract at the IC\textsubscript{10} (58.24 µg/ml), IC\textsubscript{50} (291.2 µg/ml) and IC\textsubscript{90} (524.16 µg/ml) was conducted by fluorescence staining with rhodamine 123 dye. The relatively high negative electric potential generated across the inner mitochondrial membrane creates an attractive force that sequesters the positively charged rhodamine molecule at physiological pH in the mitochondrial matrix (Johnson *et al*., 1980). As shown in Fig. 3.8, rhodamine 123 uptake was observed within the matrix of the mitochondria of healthy gametocytes because of an actively functioning membrane potential. Rhodamine 123 staining was reduced in the mitochondrial matrix of the ethanolic *C. sanguinolenta* extract treated gametocytes groups (Fig. 3.8). Treatment of gametocytes with sodium acetate (NaAc) was associated with the loss of mitochondrial membrane potential and this was shown by the absence of rhodamine 123 uptake (Fig. 3.8). From critical observation of the images obtained, it could be said that gametocytes treated with the ethanolic *C. sanguinolenta* roots extract at IC\textsubscript{10} (58.24 µg/ml) concentration appeared to show small amounts of rhodamine 123 labelling within the mitochondrial matrix which perhaps reflects partial collapse of the mitochondrial membrane potential (Fig. 3.8). There was, however, complete collapse of the membrane potential within the IC\textsubscript{50} and IC\textsubscript{90} *C. sanguinolenta* ethanolic extract treated gametocyte groups. The phase-contrast view produced from the same images highlights the distinct alterations in gametocyte shape (Fig. 3.8). In contrast to the gametocytes treated with the *C. sanguinolenta* ethanolic extract and the NaAc, the morphology of the untreated gametocyte was very intact.
Figure 3.8. Effects of *C. sanguinolenta* ethanolic roots extract on the mitochondrial membrane potential of late gametocytes stage. Phase-contrast visualisation and rhodamine 123 stained images of NF54 gametocytes. Images examined for reticular rhodamine123 staining include both the untreated and *C. sanguinolenta ethanolic* extract treated gametocytes groups using the $IC_{10}$ (58.24 µg/ml), $IC_{50}$ (291.2 µg/ml) and $IC_{90}$ (524.16 µg/ml) concentration. The NaAc (1mM) treated gametocytes represent the positive control images from the setup. Images were obtained using Nikon standard fluorescence microscope, exposure time of 5s. Scale bar = 10 µm.
3.4 EFFECT OF *C. SANGUINOLENTA* ETHANOL ROOTS EXTRACT ON THE INTRACELLULAR MEMBRANES OF LATE GAMETOCYTES STAGE BY BODIPY-TR CERAMIDE LABELLING

The integrity of the intracellular membranes within treated gametocytes groups was indicated by an uptake of the bodipy-tr ceramide dye by the membranes. Loss of the mitochondrial membrane potential which is associated with the absence of the reticular rhodamine 123 staining (Del Pilar Crespo *et al.*, 2008) is also associated with the reduced labelling of intracellular membrane by bodipy-tr ceramide dye within gametocytes (MacRae *et al.*, 2013). Results obtained from the bodipy-tr ceramide labelling of the gametocytes in this study revealed that the effect of the ethanolic *C. sanguinolenta* extract on the mitochondrial membrane potential subsequently had an effect on other membrane organelles within the treated gametocytes (Fig. 3.9). This was shown by the observed absence of bodipy-tr ceramide dye uptake by the membrane of the intracellular organelles in the treated gametocyte whereas the untreated gametocyte exhibited an uptake of the dye by the appearance of distinct blobs within it. Treatment of gametocytes with the ethanolic *C. sanguinolenta* extract at the IC$_{10}$ (58.24 µg/ml), IC$_{50}$ (291.2 µg/ml) and IC$_{90}$ (524.16 µg/ml) as well as with the positive control, 1mM NaAc, was associated with a loss of the membrane integrity of the intracellular organelles as shown by the absence of the bodipy-tr ceramide labelling. The dye redistributed to the gametocyte’s plasma membrane (Fig. 3.9). Phase contrast images showed alterations in the gametocytes shape in the treated groups but not the untreated gametocyte.
Figure 3.9. Effects of *C. sanguinolenta* ethanolic roots extract on the intracellular membranes of late gametocytes stage. Phase contrast view and bodipy-tr ceramide stained images of NF54 gametocytes. Images examined for bodipy-tr ceramide fluorescence include both the untreated and ethanolic *C. sanguinolenta* root extract treated gametocytes groups using the IC$_{10}$, IC$_{50}$ and IC$_{90}$ concentration. The NaAc treated gametocytes represent the positive control images from the setup. Images were obtained using Nikon standard fluorescence microscope, exposure time of 5s. Scale bar = 10 µm.
CHAPTER FOUR

DISCUSSION AND CONCLUSION
4.1 Discussion

The efforts toward malaria elimination are partly dependent on blocking the disease transmission. Targets for this goal include disrupting the sexual stage that occurs within the host or the vector or eliminating the mosquito itself. Most antimalarial compounds used clinically are known to inhibit the development of the asexual stages by inhibiting schizont maturation and rupture, merozoite invasion or the ring-stage development post-invasion (Wilson et al., 2013). In some studies, however, these compounds have been shown to increase gametocyte production in vivo (Drakeley et al., 2006) or have no significant effect on gametocyte population in vitro (Paetey et al., 2009). In this study, the effect of the ethanolic and aqueous C. sanguinolenta root extracts on the asexual and sexual stage (gametocyte) development Plasmodium falciparum was determined. The stage-specific effects of the extracts on both the asexual and gametocyte stages were determined as performed in a previous study (Reader et al., 2015).

For the asexual stages, inhibition of the schizonts stage of P. falciparum by the ethanolic roots extract of C. sanguinolenta was five times more effective than the aqueous roots extract of C. sanguinolenta based on their respective IC₅₀ value (Table 3.1; Fig. 3.2 C). The effect of the ethanolic extract on the trophozoites stage was four times more effective than the aqueous extract (Table 3.1; Fig. 3.2 B) and was eleven times more effective on the ring stages than the aqueous extract (Table 3.1; Fig. 3.2 A).

As shown in a study by Edeoga and colleagues, (2005), the alkaloids which occur in high concentrations in the C. sanguinolenta roots extracts was considered to be responsible for the observed activity of the extracts against the malaria parasites. Again, it has been reported (Cimanga et al., 1998; Jansen & Schmelzer, 2010) that the main alkaloid in the aqueous C. sanguinolenta roots extract is cryptolpine whiles the ethanolic extract contains neocryptolpine, biscryptolepine and cryptoquindoline in addition to cryptolepine. Although cryptolepine is
reported to be the most potent compound against the malaria parasites, the other three alkaloids in the ethanolic extract have also been shown to have some level of antiplasmodial activity (Jansen & Schmelzer, 2010; Cimanga et al., 1998). It is therefore believed that the combined effect of all these alkaloids in the ethanolic extract accounted for its profound activity on the malaria parasites as shown in this study compared to the aqueous extract. The activity of the ethanolic extract was 32 folds greater on the schizonts stages compared to the ring stages (Table 3.1), but the activity was less pronounced (11x) when compared to the trophozoites stages based on their IC$_{50}$ values (Fig. 3.2 A, B & C). Reasons for the observed greater effect on the schizonts may be that *C. sanguinolenta* acts profoundly on the metabolism of this stage rather than the trophozoites or the ring stages (Rosenthal & Meshnick, 1996).

*C. sanguinolenta* is thought to behave like chloroquine, interrupting the heme degradation process within the asexual stage malaria parasites by inhibiting heme polymerization to hemozoin (Feiz Haddad et al., 2004), a process which is believed to lead to the generation of amino acids, iron and other nutrients required by the parasite for their growth and multiplication (Rosenthal, 1995; Rosenthal et al., 1998). Since the schizonts have high demand for these supplies as a result of their higher metabolic activity compared to the trophozoites and rings, an interruption of this process by *C. sanguinolenta* is believed to have profound effect on their development (Yayon et al., 1983).

With the sexual stages, the activity of both the ethanolic and aqueous *C. sanguinolenta* roots extracts was determined on the late and early gametocytes stage. The ethanolic extract was observed to exhibit greater activity on the late gametocytes stage than the aqueous extract (Table 3.1, Fig. 3.3C). The understanding that the ethanolic extract is composed of compounds apart from cryptolepine which may confer their synergistic effect to inhibit the gametocytes development could possibly be the reason for the greater activity (Jansen & Schmelzer, 2010;
Cimanga et al., 1998). The activity on the early gametocyte stage showed little difference in the IC\textsubscript{50} values obtained for both the ethanolic and aqueous extracts (Table 3.1; Fig. 3.3 B). From the study, it was observed that the ethanolic extract was relatively more effective on the late gametocyte stages than the early stages based on the IC\textsubscript{50} values (Table 3.1, Fig. 3.3 B & C). Unsynchronised gametocytes stages tested against the extracts exhibited some level of susceptibility for the ethanolic extract which was almost the same as that on the late gametocytes stage based on their IC\textsubscript{50} values (Table 3.1, Fig. 3.3 A &C). The aqueous extract, however, exhibited just as much activity on the unsynchronised gametocyte stages as on the late gametocytes stage (Fig. 3.3 A & C). From the results, it could be speculated that the ethanolic extract of \textit{C. sanguinolenta} exhibited greater activity on all the gametocytes stages than the aqueous extract (Table 3.1). Reasons for this may be as explained above that the ethanolic extract is composed of greater proportions of the alkaloids than the aqueous extract (Jansen & Schmelzer, 2010; Cimanga et al., 1998) and that the combined effect of these alkaloids may have resulted in the profound activity observed.

As demonstrated in a study, an extract is very active if IC\textsubscript{50} < 5 \textmu g/ml, active if 5 \textmu g/ml < IC\textsubscript{50} < 50 \textmu g/ml, weakly active if 50 \textmu g/ml < IC\textsubscript{50} < 100 \textmu g/ml and inactive if IC\textsubscript{50} > 100 \textmu g/ml (Rasoanaivo et al., 1992). Thus it can be reported from this study that the IC\textsubscript{50}’s obtain for the effect of the ethanolic \textit{C. sanguinolenta} roots extract on the schizonts and trophozoites stage was very active and active for the rings while inactive for late gametocytes, the early gametocytes and unsynchronised gametocytes stages. Furthermore, IC\textsubscript{50}’s for the effect of the aqueous extract on the schizonts stage was very active, trophozoites stage active, rings stage weakly active whiles late gametocytes stage, early gametocytes and unsynchronised gametocytes stage were inactive. This implies that the IC\textsubscript{50}’s which were obtained from this study for the \textit{C. sanguinolenta} extracts were considered too high to be used on the parasites,
especially the gametocytes for the elimination of their population. A possible explanation for 
this is that the effect of the *C. sanguinolenta* extracts on the gametocytes was not to immediately 
kill them within the 48 hours incubation period and therefore reduce their population but rather 
it produces changes in their metabolic pathway that eventually leads to a deformed growth and 
development and the eventual loss of their infectivity rate into the mosquito.

Thus, the effect of the ethanolic *C. sanguinolenta* extract on the functionality of the 
gametocyte mitochondria was determined. It is believed that the inhibition of the gametocyte 
development by the ethanolic *C. sanguinolenta* extract may probably be through the inhibition 
of the functioning of the gametocyte’s respiratory and energy producing organ, the mitochondria 
(MacRae *et al.*, 2013). As demonstrated by MacRae and his colleagues, (2013), the late 
gametocyte stage highly depends on energy (ATP) supply via their mitochondrial metabolic 
pathways for survival. This energy generated from their tricarboxylic acid cycle (TCA) and 
electron transport chain caters for majority of their cellular activities (MacRae *et al.*, 2013; 
Srivastava *et al.*, 1997).

One major finding from this study was the demonstration of the inhibitory effect of the 
ethanolic roots extract *C. sanguinolenta* on some of the TCA cycle enzymes (Fig. 3.5-3.7). 
Results obtained from this study suggest that inhibition of the enzymes, specifically citrate 
synthase and aconitase, could have led to a decline in the energy production capability of the 
mitochondria within the gametocytes (Fig. 3.8). This finding is in harmony with the study 
conducted by MacRae and his colleagues (2013), where it was observed that the inhibition of the 
enzyme aconitase in the TCA cycle of late gametocyte stage resulted in the collapse of the 
gametocyte’s mitochondrial membrane potential (Fig 3.8). The effect as explained by MacRae 
and his colleagues, (2013), may be as a result of the reduced yield of NADH and/or the 
accumulation of toxic levels of citrate, the substrate of aconitase, which adversely affected the
gametocytes by reducing the energy supply and subsequently causing their death (MacRae et al., 2013). The overall effect as observed from this study was the impaired development of the gametocytes treated with the ethanolic *C. sanguinolenta* roots extract (Fig. 3.4).

In a study conducted to determine the effect of primaquine on gametocytes, it was reported that primaquine was most effective on late gametocytes stage, however, this effect was not statistically significant when determined *in vitro* (Paetey et al., 2009). It was therefore suggested that, one or more of the metabolites of primaquine breakdown were responsible for the effect on late gametocytes stage *in vivo* (Bates et al., 1990). In this study, the effect of primaquine on the late gametocyte stage *in vitro* was also not significant (Fig. 3.3). In comparison with the *C. sanguinolenta* extracts however, the IC₅₀ for primaquine was so many folds (970x) less than the ethanolic extract which indicates greater activity of primaquine on the late gametocytes stage (Table. 3.1; Fig. 3.3 C).

Findings from this study revealed that the ethanolic *C. sanguinolenta* extract inhibited the activity of some of the enzymes involved in the TCA cycle of the late gametocytes stage. With regard to the enzyme aconitase, there was an observed reduction of its level of activity when exposed to the ethanolic *C. sanguinolenta* extract (Fig. 3.6). This enzyme when deactivated is thought to result in the accumulation of the substrate, citrate, to levels that are toxic to the gametocyte and may lead to its death (Savarie 1984; MacRae et al., 2013). From this study, the aconitase activity within the gametocytes reduced significantly (P = 0.0001) after treatment with the ethanolic extract in comparison with the control or untreated gametocytes (Fig. 3.6). The inhibition of aconitase by sodium acetate (NaAc), a positive control, was significant compared with the control (P = 0.0001, Fig. 3.6). The inhibition of aconitase by NaAc was, however, not significantly different from the inhibition of aconitase by the two different concentrations of the ethanolic fractionated *Cryptolepis* extract, IC₅₀ = 291.2 µg/ml and IC₉₀ = 524.16 µg/ml (P > 0.05,
The result obtained for the inhibition of aconitase by NaAc is consistent with result obtained in a study by MacRae and his colleagues (2013) as explained above. With this information, it can be speculated that the ethanolic roots extract of *C. sanguinolenta* works as effectively as NaAc in inhibiting gametocyte’s aconitase activity which could eventually lead to the gametocyte’s death as a result of accumulation of citrate (MacRae *et al.*, 2013).

Again, citrate synthase, which is an enzyme found in the TCA cycle is responsible for conversion of the two carbon molecules from acetyl coA and the four carbon molecules of oxaloacetate to form the six carbon citrate (Wiegand & Remington, 1986). This enzyme was found to be inhibited by the ethanolic *C. sanguinolenta* roots extract. Being one of the four key regulatory enzymes in the TCA pathway, its function is very crucial to the survival of the parasite (MacRae *et al.*, 2013). In comparison with the control, there was a strong inhibition ($P = 0.04$) of this enzyme by the ethanolic *C. sanguinolenta* extract (Fig. 3.5) and this was observed to be concentration dependent. Even though NaAc is reported to inhibit aconitase, it was shown in other studies conducted that the inhibition of aconitase results in the accumulation of citrate, the substrate and this negative feedback inhibits citrate synthase within the TCA pathway (MacRae *et al.*, 2013; Wiegand & Remington, 1986). In this study, a strong inhibition of citrate synthase by NaAc (1 mM) within the treated gametocytes was observed ($P = 0.04$, Fig. 3.5). It could therefore be speculated that the ethanolic extract might as well have inhibited citrate synthase activity through the same negative feedback mechanism or perhaps inhibited directly.

The α-ketoglutarate dehydrogenase is another key regulatory enzyme in the TCA cycle within the mitochondrion of *P. falciparum* (Hansford, 1980). It was observed from this study that inhibition of this enzyme by the ethanolic *C. sanguinolenta* roots extract was not statistically significant ($P = 0.1$, Fig. 3.7). Although the effect of the extract on the enzyme was concentration dependent, it was very weak when compared with the control ($P = 0.1$).
Despite this observation, the collapse of the mitochondrial membrane potential within the treated gametocytes was observed (Fig. 3.8). Thus it could be speculated that the *C. sanguinolenta* ethanolic extract might have had additional inhibitory effects on other enzymes downstream of the α-ketoglutarate dehydrogenase’s activity such as succinyl coA synthetase, succinate dehydrogenase, fumarate hydratase and malatequinone oxidoreductase. From Fig. 3.7 it was observed that the treatment of gametocytes with NaAc had no effect on the α-ketoglutarate dehydrogenase activity as expected. NaAc is known to only inhibit aconitase activity (MacRae *et al.*, 2013).

It was reported in some studies that inhibition of the enzymes in the TCA cycle of the gametocytes leads to the loss of production of reducing equivalents such as NADH, NADPH and FADH$_2$ which is utilized via their electron transport chain (van Dooren, 2006; Uyemura *et al.*, 2000, Uyemura *et al.*, 2004). Also known is that electrons from the NADH and FADH$_2$ are utilized by the electron transport chain for the generation of the mitochondrial membrane potential across the inner mitochondrial membrane leading to the production of ATP and also for oxygen consumption (Evans and Guy, 2004; van Dooren, 2006; Uyemura *et al.*, 2000, Uyemura *et al.*, 2004, ). This therefore means that loss of these reducing equivalents could lead to the collapse of the mitochondrial membrane potential (Fry and Beesley, 1991; Uyemura *et al.*, 2000).

Results obtained from this study showed that using concentrations of the ethanolic *C. sanguinolenta* extract that result in 50% and 90% reduction of the gametocytes population, rhodamine 123 dye uptake was reduced within the mitochondrial matrix of the treated gametocytes (Fig. 3.8). It has been explained that the existence of the mitochondrial membrane potential creates a highly negative charge in the matrix of the mitochondria which allows for the
equilibration with rhodamine 123 dye, a cationic molecule. The dye therefore sequesters in the matrix of a mitochondrion with an actively generating membrane potential (Johnson et al., 1980). Thus, the loss of uptake of the rhodamine 123 dye observed in this study may be attributed to a collapse of the mitochondrial membrane potential across the inner mitochondrial membrane. Possibly, the inhibition of the TCA enzymes by the *C. sanguinolenta* ethanolic extract could have resulted in the loss of production of NADH, NADPH and FADH$_2$ in the TCA pathway of the gametocytes, which may have affected the electron transport chain function (MacRae et al., 2013). The suspected absence of these reducing equivalents could have caused the collapse of the mitochondrial membrane potential since they are reported to stimulate electron transport and O$_2$ consumption (Fry and Beesley, 1991; Uyemura et al., 2000, 2004).

Additionally, reports indicate that the ATP generated as a result of the combined action of the mitochondrial membrane potential and the proton gradient to force protons through the ATP synthetase is utilized for many biochemical processes such as protein translocation, solute import, cellular differentiation and membrane organization (Evans and Guy, 2004; Laloï, 1999; Pfanner and Geissler, 2001). The membrane organization ensures the establishment of intact intracellular membranes and the plasma membrane of the parasite (van Dooren, 2006). Some processes such as the protein translocation, uptake of molecules and solute import are able to then occur across membranes within the parasite (van Dooren, 2006). In this study, uptake of bodipy-tr ceramide dye by intracellular membranes within healthy control gametocytes was demonstrated to occur as a result of an intact membrane (Fig. 3.9). The intracellular membranes of the ethanolic extract treated gametocytes, however, lost their ability to take up the bodipy-tr ceramide dye due to the compromise of their membrane integrity (Fig. 3.9). As shown in a study, the collapse of the mitochondrial membrane potential, which may have led to the absence of the production of ATP, could have caused the subsequent effect on the intracellular
membranes within the gametocytes resulting in the loss of their membrane integrity (MacRae et al., 2013).

Lastly, it is reported that the unique crescent shape of the gametocytes, especially the stage V, enables their survival and circulation in the host long enough before transmission into the mosquito vector (Day et al., 1998; Hayward et al., 1999). The reason is that the crescent shape of this stage of the gametocytes enables their evasion of the filtering process within the spleen and thus prevents their clearance from circulation (Glenister et al., 2002; Safeukui et al., 2008). Another consequence of the elongated shape of the late gametocytes (III-IV) is that it enables adhesion to lower affinity receptors on the surface of the host capillary walls as they are able to flatten their body against the wall (Dearnley et al., 2012). Any alteration in the shape therefore may confer some disadvantage on the parasite.

Starting with the same gametocyte population of stage IIIIs, the control untreated gametocytes was observed to maintain their elongated stage IV shape with pointed ends and with pigment clustered in the middle as expected after the 48 hours incubation (Fig. 3.4). The ethanolic extract of C. sanguinolenta treated gametocytes using the IC$_{50}$ and IC$_{90}$ concentrations appeared distorted in shape with scattered pigment (Fig. 3.4). About 85% of the gametocytes population for the IC$_{90}$, 75% for the IC$_{50}$ and 59% for the IC$_{10}$ concentration were distorted in shape. The distortion of shape of these treated gametocytes may render them defective in evading the host’s clearance mechanism of the spleen. This may therefore affect the population of gametocytes available in circulation, their infectivity in the mosquito and subsequently their transmission rate into the other potential host from the mosquito (Day et al., 1998; Hayward et al., 1999).

Clearly it can be shown from the results obtained from this study that the ethanolic roots extract of C. sanguinolenta prevented the proper function of the canonical TCA cycle of the gametocytes, leading to a systematic breakdown of the gametocytes developmental process.
4.2 Conclusion

*C. sanguinolenta* extracts were effective on the late gametocyte stages with the ethanolic extract being the most potent. This plant extract inhibited the general development of the gametocytes partly through the disruption of the parasite energy producing metabolic process in the mitochondria. It affected the activity of the tricarboxylic acid cycle enzymes citrate synthase and aconitase but not α-Ketoglutarate dehydrogenase. This resulted in a loss of the gametocyte’s mitochondrial membrane potential and subsequently led to the compromise of the membrane integrity of other intracellular membranes within the gametocytes. The active compounds in this extract therefore may be explored further for their potential as malaria transmission blocking agents.

4.3 Recommendation

Studies on the effect of *Cryptolepis sanguinolenta* on field isolates of *P. falciparum* sexual stage needs to be considered for a clearer understanding of what happens within these field isolates as compared to the laboratory strains. Also, since *C. sanguinolenta* ethanolic extract did not affect alpha-ketoglutarate dehydrogenase activity but had effect on the collapse of the mitochondrial membrane potential within the treated gametocytes, it could be speculated that it had effect on other enzymes (such as succinyl coA synthetase, succinate dehydrogenase, fumarate hydratase and malate oxidoreductase) downstream of the alpha-ketoglutarate dehydrogenase. Further studies on assessing the effect of the *C. sanguinolenta* ethanolic roots extract on enzymes downstream of alpha-ketoglutarate dehydrogenase needs to be conducted. Additionally, a high resolution fluorescence microscope such as a confocal microscope will be very useful for the biological imaging of the labelled parasites. It is believed that highly resolved and better quality images can be obtained for a more comprehensive image analysis.
REFERENCES


Mbare, O., Lindsay, S.W. & Fillinger, U., 2014. Pyriproxyfen for mosquito control: female sterilization or horizontal transfer to oviposition substrates by Anopheles gambiae sensu stricto and *Culex quinquefasciatus*. *Parasites & Vectors*, 7(1), 280.


Savarie, P.J., 1984. Toxic characteristics of fluorocitrate, the toxic metabolite of compound 1080.


WHO, 2014. WORLD MALARIA REPORT.


APPENDICES

Appendix I

Buffers & Solutions

(a) Washing Medium

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>RPMI-1640</td>
<td>500ml</td>
</tr>
<tr>
<td>Gentamycin (10mg/ml)</td>
<td>2.5ml/500ml RPMI</td>
</tr>
<tr>
<td>L-glutamine</td>
<td>4.0ml/500ml RPMI</td>
</tr>
</tbody>
</table>

(Conc. Of L-glutamine: 2.92g/100ml in saline)

(b) Complete Parasite Medium

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>RPMI-1640</td>
<td>500ml</td>
</tr>
<tr>
<td>Gentamycin (10mg/ml)</td>
<td>2.5ml/500ml RPMI</td>
</tr>
<tr>
<td>L-glutamine</td>
<td>4.0ml/500ml RPMI</td>
</tr>
<tr>
<td>NHS (A or O positive)</td>
<td>5%, filtered through</td>
</tr>
<tr>
<td>0.8μm pore filter</td>
<td></td>
</tr>
<tr>
<td>Albumax</td>
<td>50ml/500ml RPMI</td>
</tr>
</tbody>
</table>

Filter sterilize medium and store at 4°C.

(c) CPD Buffer, pH 5-6

<table>
<thead>
<tr>
<th>Concentration</th>
<th>Component</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>17mM</td>
<td>Citric acid</td>
<td>5.0g</td>
</tr>
<tr>
<td>90mM</td>
<td>Sodium citrate</td>
<td>26.47g</td>
</tr>
<tr>
<td>175mM</td>
<td>Glucose</td>
<td>31.54g</td>
</tr>
<tr>
<td>16mM</td>
<td>NaH$_2$PO$_4$</td>
<td>2.21g</td>
</tr>
<tr>
<td>2mM</td>
<td>Adenine (96-Aminopurine)</td>
<td>270.0mg</td>
</tr>
</tbody>
</table>

Distilled water up to 1.0 litre

Filter sterilize medium and store at 4°C
Use solution: 3ml per 20ml whole blood
(d) Giemsa Buffer, pH 7.2

\[
\begin{align*}
\text{Na}_2\text{HPO}_4 & \quad 1.0\text{g} \\
\text{KH}_2\text{PO}_4 & \quad 0.7\text{g} \\
\text{Distilled water up to} & \quad 1.0\text{ liter}
\end{align*}
\]

Store at 4°C

(e) Freezing mix

4.2% sorbitol in 0.9% sodium chloride (sorbitol solution)
Add 72% sorbitol solution to 28ml glycerol
Filter sterilize and store at 4°C

(f) Parasite thawing mix

3.5% sodium chloride in distilled water
Filter sterilize and store at 4°C
Appendix II

5% Sorbitol Synchronisation for rings

Select culture with a high ring population. Warm 5% sorbitol to 37°C in incubator.

Transfer culture into a 15ml tube, centrifuge for 5 mins at 2000rpm. Remove all medium above pellet.

Resuspend to original volume in 5% sorbitol (in distilled water). Mix the contents of the tube and allow to stand in an incubator at 37 °C for 10 mins.

Centrifuge for 5 mins at 2000rpm, remove sorbitol and wash once in incomplete medium and twice with complete parasite medium (CPM) at 2000rpm for 5 mins.

Set up a new culture with the pellet. Prepare a slide of the culture.
Appendix III

65% Percoll Synchronisation for schizonts

Prepare 90% percoll by mixing 10X PBS with 100% percoll (eg. 20ml of 90% percoll – mix 2ml of 10X PBS with 18ml of 100% percoll).

Prepare 65% percoll by combining 6.5ml of 90% percoll with 2.5ml of incomplete medium (ICM).

Prepare 35% percoll by mixing 3.5ml of 90% percoll with 5.5ml of ICM. Sterilise the mediums by filtering through a 0.22µm filter.

Transfer parasite culture to a 15ml falcon tube, centrifuge at 2000rpm for 5 mins at room temperature. Discard supernatant. Resuspend cells with incomplete medium to 10% hematocrit (total about 2.5ml).

Set up a percoll gradient:
Pipe 3ml of 65% percoll into a 15ml falcon tube.
Gently pipette 3ml of the 35% percoll onto it with a pasteur pipette.
Slowly layer cell suspension (2.5ml) on top of the freshly prepared percoll gradient.

Centrifuge in a swing-out rotor at 1500 x g (2500rpm) at room temperature for 15 mins without a brake.

Recover lower interface and transfer to a new 15ml tube. Wash pellet and put into culture.
Appendix IV

60% Percoll Synchronisation for Gametocytes

Prepare 90% percoll by adding 3.6ml percoll (100) to 0.4ml parasite washing medium (PWM) in 15ml falcon tube.

Dilute further to 60% by adding 2ml PWM

Transfer culture into 15ml tube and spin at 1500rpm for 10min. Discard supernatant and leave for a while. Resuspend pellet to about 25% haematocrit (1 part pellet to 3 parts medium)

Using a sterile Pasteur pipette and tilting the falcon tube with the 60% percoll at 60° angle, carefully lay one volume of the resuspended culture (3) over 2 volume of the 60% percoll ie. (1.5ml of resuspended culture to 3ml of 60% percoll) ensuring the resuspended culture runs along the side of the tube into the top of 60% without mixing.

Centrifuge at 1450 rcf at room temperature for 10min. 60% percoll beneath and used medium (top)

Collect parasites at interphase and transfer into a fresh 15ml tube

Centrifuge at 1500rpm for 10min and discard supernatant

Resuspend in 3ml PWM and repeat centrifuging

Repeat (8), prepare slides

Resuspend in 5ml CPM-NAG (adding 100µl of RBC). NAG~50mM NAG.