UNIVERSITY OF GHANA

COLLEGE OF BASIC AND APPLIED SCIENCE

THE CAROTENOID BIOSYNTHESIS PATHWAY IN THE ASEXUAL INTRAERYTHROCYTIC STAGES OF *PLASMODIUM FALCIPARUM*: *IN VITRO* INHIBITION ASSAYS, HPLC PROFILING OF CAROTENOIDS AND BIOINFORMATICS ANALYSIS

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A THESIS SUBMITTED TO THE DEPARTMENT OF BIOCHEMISTRY, CELL AND MOLECULAR BIOLOGY OF THE UNIVERSITY OF GHANA IN PARTIAL FULFILMENT OF THE REQUIREMENT FOR THE AWARD OF MASTER OF PHILOSOPHY DEGREE IN BIOCHEMISTRY

MARCH, 2016

DECLARATION

This is to certify that this thesis is the result of research undertaken by Richard Nortey-Mensah, towards the award of the Master of Philosophy in the Department of Biochemistry, Cell and Molecular Biology, University of Ghana.

All references have been duly cited.

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ACKNOWLEDGEMENTS

First, I would like to express my sincerest gratitude to my supervisors, Dr. Marian Teye and Dr. Michael Ofori for their time, advice and support throughout the research period and also giving me the opportunity to undertake this project. Their tutoring and mentoring has made me a better person today and I will forever be indebted to them.

I would also like to acknowledge Mr. Emmanuel Kakra Dickson and Mr. Eric Kyei-Baafour for their immense support and contribution towards the successful completion of the lab work. I'm most grateful to Miss Andrea Twumwaa Arku for teaching me everything I know about parasite cultures. I couldn't have done this work without getting training on parasite culturing from her. I'm also grateful to Dr. Regina Appiah-Opong [Head of Dept. for Clinical Pathology, Noguchi Memorial Institute for Medical Research (NMIMR)] for giving me the opportunity to use the HPLC machine in her department and to Mr. Ebenezer Ofori-Atta for the technical support during the HPLC analysis and also his kind gestures towards me.

I would also like to express my appreciation to the Head and entire staff of the department of Biochemistry, Cell and Molecular Biology for nurturing me. I am also grateful to the entire staff and colleague students at the Immunology department (NMIMR) and anyone who has contributed directly or indirectly to the success of this project.

I would like to acknowledge the Cambridge-Africa Partnership for Research Excellence (CAPREx), the University of Ghana Office for Research, Innovation and Development (ORID) and the Noguchi Post-Doc Master's Program for funding this project.

Finally, I would like to thank my family for the love and support they have shown me throughout this journey.

DEDICATION

This work is dedicated to the Almighty God. Thank You for the love and kindness You have shown me throughout this period. I also dedicated it to my family for their care and support.

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LIST OF ABBREVIATIONS

ABA	Abscisic acid
CM	Cerebral Malaria
CPM	Complete Parasite Medium
CRTISO	Carotenoid Isomerase
DMAPP	Dimethylallyl Pyrophosphate or Dimethylallyl diphosphate
DOXP	1-deoxy-D-xylulose-5-phosphate
DXR	1-deoxy-D-xylulose-5-phosphate Reductoisomerase
DXS	1-deoxy-D-xylulose-5-phosphate Synthase
FPP	Farnesyl Pryophosphate
GGPP	Geranylgeranyl Pyrophosphate or Geranylgeranyl Diphosphate
HDR	1-hydroxy-2-methyl-2(E)-butenyl 4-diphosphate Reductase
IPP	Isopentenyl Pyrophosphate or Isopentenyl Diphosphate
MEP	Methylerythritol Phosphate
MTOC	Microtubule Organising Center
OPPS	Octaprenyl Pyrophosphate Synthase
PDS/crtI	Phytoene Desaturase
PSY	Phytoene Synthase
PWM	Parasite Washing Medium
rpm	Revolutions Per Minute
SEM	Standard Error of Mean
SMA	Severe Malaria Anaemia
ZDS	Zeta-carotene Desaturase
ZISO	Zeta-carotene Isomerase
β-CYC	lycopene β-Cyclase
ε-CYC	lycopene ε-Cyclase

ABSTRACT

Plasmodium falciparum, like other Apicomplexans, has retained a relict plastid known as the apicoplast. This organelle represents a new and exciting target for the chemotherapeutic management of malaria because it houses metabolic pathways that are unique to the parasite such as isoprenoid biosynthesis. The phytoene synthase (*PSY*) gene, has been demonstrated to be very important in carotenogenesis, however, little is known about the evolutionary relatedness of this gene in *P. falciparum* and other Apicomplexans. This study therefore aimed at profiling the carotenoids synthesized in the asexual intraerythrocytic stages of *P. falciparum* and to determine the evolutionary history and relatedness of the *PSY* gene in Apicomplexans and other organisms. *In vitro* inhibition assays were performed on the asexual intraerythrocytic stages of *P. falciparum* using fluridone to determine its IC_{50} and effect on parasite population. HPLC was used to profile carotenoids synthesized at the asexual stages and to determine the evolutionary history and relatedness of the *PSY* gene in Apicomplexans and other organisms, an unrooted phylogenetic tree was generated using MEGA 6. A dose-dependent inhibition of parasite population was observed with fluridone treatment on all the asexual stages, with the ring stages being the most susceptible. The carotenoid profiles showed that synthesis of carotenoids in *P. falciparum* is cumulative through the asexual intraerythrocytic stages with carotenoids such lycopene, α-, β-carotene among others being synthesized. An exciting novel finding of this study was the discovery of relatively high amounts of abscisic acid (ABA) in the schizont stages and not in the other stages. This is the first time ABA has been demonstrated to be synthesized by *P. falciparum* and it would be pioneering to further investigate the specific role of ABA in *P. falciparum* schizont stages. The phylogenetic analysis showed that the *P. falciparum PSY* was most related to *P.*

reichenowi, the chimpanzee strain of the malaria causing parasites further lending support to the proposed origin of malaria species in humans.

CHAPTER ONE

1.0 INTRODUCTION

1.1 The Malaria Burden

Malaria remains one of the most important parasitic diseases in humans, causing great morbidity and mortality in tropical regions. The World Malaria Report estimates that 198 million cases of malaria occurred resulting in 580,000 deaths globally (WHO, 2014). Despite on-going and intensive efforts to control the disease, malaria still remains endemic in five continents. Thus, more than half of the world's population is at risk. Human malaria is caused by five species of *Plasmodium* parasites, namely *P. falciparum*, *P. vivax*, *P. ovale*, *P. malariae* and *P. knowlesi* (Jiang *et al.*, 2010; Sermwittayawong *et al.*, 2012) with *P. falciparum* being responsible for the vast majority of malaria-related deaths (Bousema & Drakeley, 2011; Hay *et al.*, 2009). The global expansion of the disease has been attributed mainly to the failure of vector control programs, the absence of a vaccine and the increase in parasite resistance to drugs commonly used for therapy (Sharma & Dutta, 2011; Shiff, 2002). A comprehensive understanding of the metabolic pathways in *Plasmodium* is therefore essential for the development of new chemotherapeutic strategies and agents to combat the disease. These new chemotherapeutic strategies and agents can be achieved in three ways: (*i*) by focusing on previously validated parasite targets to generate new drug candidates, (*ii*) by identifying new potential parasite targets for malaria chemotherapy or (*iii*) by performing high-throughput testing of drug libraries (Choi *et al.*, 2008).

Plasmodium parasites, like other apicomplexans (e.g. *Toxoplasma spp.*) have retained a relict plastid organelle called the apicoplast, which is derived from secondary endosymbiosis millions of years ago of a plastid-bearing red algae (Foth &

McFadden, 2003). The apicoplast is therefore prokaryotic in origin, containing pathways that have no counterpart in the human host. It has a genome related to that of plants and algae and its metabolic functions based on proteome analysis include type II fatty acid and isoprenoid biosynthesis (Sato, 2011). In the case of malaria parasites, especially the most virulent species, *P. falciparum*, a series of new “plant-like” enzymes have been recently discovered with some of these being associated with the apicoplast (Kalanon & McFadden, 2010). These enzymes are known to be involved in many essential biochemical pathways in plants and algae (Foth & McFadden, 2003; Gornicki, 2003; Seeber, 2003). Among these biochemical pathways is the carotenoid biosynthesis pathway. It is an attractive target for investigation, because it is essential in algae, higher plants, bacteria and fungi but absent in mammals and products of this pathway are involved in many important metabolic functions (Paniagua-Michel *et al.*, 2012).

Plasmodium also retains isoprenoid biosynthesis (resident in the apicoplast) which results in the synthesis of the isoprene units, isopentenyl pyrophosphate (IPP) and dimethylallyl pyrophosphate (DMAPP) through the mevalonate-independent, methylerythritol phosphate (MEP) pathway (Jordão, Kimura & Katzin, 2011; Rodríguez-Concepción & Boronat, 2013). These isoprene units are not an end onto themselves but rather serve as building blocks for the synthesis of isoprenoids (for example, carotenoids). Previous work has shown that treatment with fosmidomycin, an inhibitor of non-mevalonate isoprenoid precursor biosynthesis in the apicoplast, inhibits the growth of asexual blood-stage *P. falciparum* (van der Meer & Hirsch, 2012; Wiesner, Borrman & Jomaa, 2003). Yeh & DeRisi (2011) also demonstrated that fosmidomycin inhibition in *P. falciparum* could be chemically rescued by supplementation with IPP, the pathway product. Also, abscisic acid (which is synthesized from the isoprenoid pathway) has been implicated in the control of calcium-dependent parasite egress in *T. gondii*, a related apicomplexan and inhibition of its synthesis resulted in delayed parasite egress from the host cell in a dose dependent manner (Nagamune *et al.*, 2008). These data show that the isoprenoid biosynthesis pathway is essential for the survival of apicomplexan parasites during their infective stages. However, most of the downstream products of this pathway and their functions in the asexual intraerythrocytic stages of *P. falciparum* parasites are currently unknown.

Additionally, many genes have proposed functions in the carotenoid biosynthesis pathway in *Plasmodium*, however, only the phytoene synthase (*PSY*) gene (PfB0130w) has been definitely implicated through functional assays (Rodríguez-Villalón *et al.*, 2009; Toledo-Ortiz *et al.*, 2010). The phytoene synthase gene (*PSY*) encodes the enzyme phytoene synthase (PSY), which is a very important protein because it catalyses the first committed and rate-limiting step in carotenoid biosynthesis pathway (Welsch *et al.*, 2000) and also serves as an important regulatory point for the influx of carbon into the pathway. It catalyses the condensation of two molecules of geranylgeranyl diphosphate (GGPP) to form phytoene – the initial C-40 carotenoid skeleton of all carotenoids (Rodríguez-Villalón *et al.*, 2009; Welsch *et al.*, 2000). The structure (secondary and tertiary), function and the phylogenetic history of the *PSY* protein in apicomplexans, plants, bacteria and algae have been investigated (Agarwal *et al.*, 2015; Tonhosolo *et al.*, 2005). Evolutionary history and relatedness of the *PSY* gene has also been investigated in other species, however, no studies on the evolutionary history and relatedness of the gene in apicomplexans are available.

1.2 Problem Statement

Malaria remains one of the most important causes of morbidity and mortality in the tropical regions of the world since the discovery of the parasite over a hundred years ago. The biology of the parasite is poorly understood (Laishram *et al.*, 2012) and this has made it difficult to control the disease. Combating the malaria epidemic will therefore require the understanding of the biology and metabolic pathways of the parasite in order to control and possibly eradicate the disease which affects productivity in poor, endemic countries.

1.3 Hypothesis

The carotenoid biosynthesis pathway is essential for the survival of the intraerythrocytic stages of *P. falciparum* and is therefore an attractive target for the chemotherapeutic management and treatment of malaria.

1.4 Aim

To profile the carotenoids synthesized in the asexual intraerythrocytic stages of *P. falciparum* and to determine the evolutionary history and relatedness of the *PSY* gene in *P. falciparum*.

1.5 Specific Objectives

1. To investigate the effect of fluridone on the asexual intraerythrocytic stages of *P. falciparum* through *in vitro* inhibition assays
2. To profile the carotenoids synthesized by the asexual intraerythrocytic stages of *P. falciparum* using High Performance Liquid Chromatography (HPLC)
3. To determine evolutionary history and relatedness of the phytoene synthase gene in *P. falciparum* and other organisms through bioinformatics analysis

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Global Statistics and Distribution of Malaria

Malaria still remains a major infectious disease that plagues the world despite extensive efforts spanning more than a century to control this disease (Garcia, 2010). It is a parasitic disease caused by protozoan parasites belonging to the genus *Plasmodium* and there are five species known to infect humans, namely *P. falciparum*, *P. vivax*, *P. ovale*, *P. malariae* and *P. knowlesi* (Garcia, 2010). Malaria that results from *P. falciparum* infection is the most virulent form, predominates in Africa and is associated with the majority of malaria related deaths (Garcia, 2010). *P. vivax* however, is less dangerous in terms of devastation but more widespread and with high morbidity (Arévalo-Herrera & Herrera, 2001). The other three species are found much less frequently (WHO, 2013). Clinical manifestation of *P. falciparum* malaria (which include fever, rigor, vomiting) is attributed to the propagation of the asexual stage of the parasite within human erythrocytes (Cowman *et al.*, 2012).

Globally, it is estimated that 3.3 billion people in 97 countries and territories are at risk of being infected with malaria and developing the disease and 1.2 billion, especially people in sub-Saharan Africa, are at higher risk (WHO, 2014). In 2013, it was estimated that 198 million cases of malaria occurred and the disease led to 584,000 deaths globally. This represented a decrease in malaria case incidence and mortality rates of 30% and 47% respectively since 2000 (WHO, 2014). The WHO Africa region suffers most from the malaria burden, where an estimated 80% of all malaria cases and 90% of deaths occur, with children less than five years of age (accounting for 78% of all death) and pregnant women being the most severely affected (WHO, 2014). Figure 2.1 shows a global distribution of the malaria epidemic while table 2.1 estimates the global malaria morbidity and mortality by region and also indicates that the worst affected region is Africa. Apart from being a major health challenge, the malaria burden also affects the economy and productivity of the endemic areas, especially sub-Saharan Africa (Sachs & Malaney, 2002; WHO, 2013).

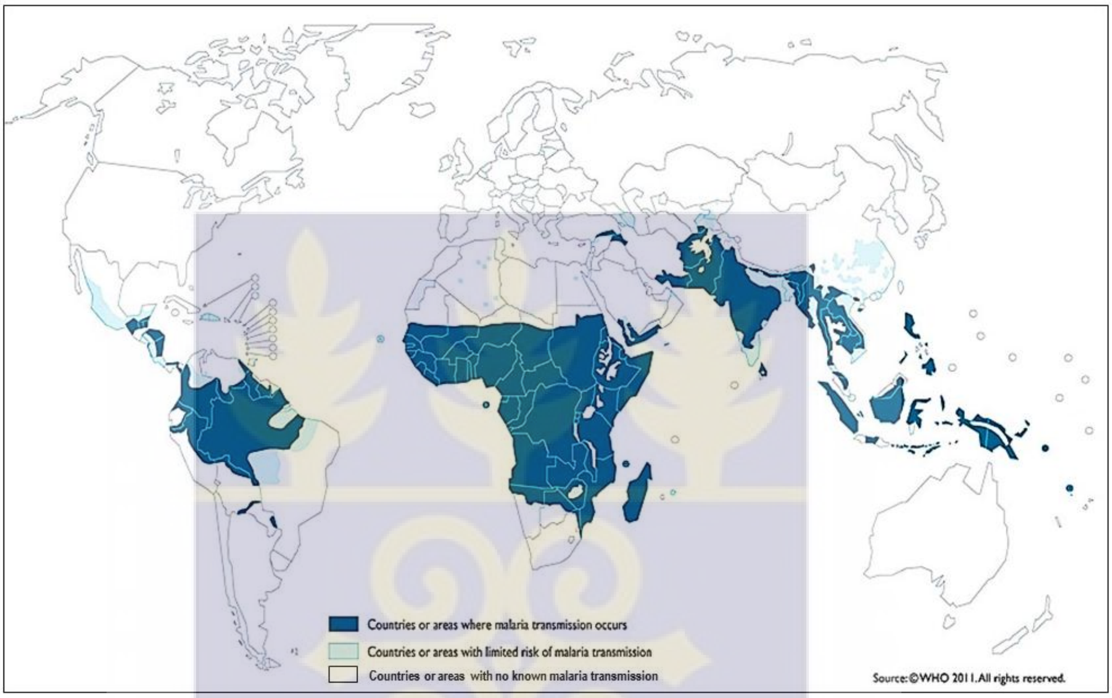


Figure 2.1: Map of global distribution of malaria transmission
(Source: WHO world malaria report, 2013)

Table 2.1: Estimate of malaria cases and deaths by region in 2013
[Source: Henry J. Kaiser Family Foundation fact sheet, March 2015.]

WHO Region	# of Countries with Ongoing Transmission	Cases* (Number in thousands, %)		Deaths* (Number in thousands, %)	
Africa	45	163,000	82%	528	90%
Americas	21	700	<1%	<1	<1%
E. Mediterranean	8	9,000	5%	11	2%
Europe	3	2	<1%	0	0%
South-East Asia	10	24,000	12%	41	7%
Western Pacific	10	1,000	<1%	3	<1%
Global Total	**97**	**198,000**	**100%**	**584**	**100%**

NB: * Represents WHO’s "best estimate" for each indicator.

Malaria is considered an entirely preventable and treatable disease, provided the currently recommended interventions are properly implemented (Campbell & Steketee, 2011; Mendis *et al.*, 2009). These include;

(i) vector control through the use of insecticide-treated nets, indoor residual spraying and in some specific settings, larval control

(ii) chemoprevention for the most vulnerable populations, particularly pregnant women and infants

(iii) confirmation of malaria diagnosis through microscopy or rapid diagnostic tests for every suspected case, and

(iv) timely medication with the appropriate antimalarial medicines (according to the parasite species and any documented drug resistance) (Enayati & Hemingway, 2010; Russell *et al.*, 2013; White *et al.*, 2011; WHO, 2014).

2.1.1 Life Cycle of *Plasmodium falciparum*

Plasmodium species are obligate intracellular parasites that reside within a membrane-bound vacuole for the most part of their life cycle (Cesbron-Delauw *et al.*, 2008; Charpian & Przyborski, 2008; Heussler *et al.*, 2006). The life cycle of *Plasmodium* is a complex one which alternates between the human host and the mosquito vector as shown in figure 2.2. During the life cycle progression, three major stages can be distinguished. These are the liver, blood and the mosquito-specific stages (Aly *et al.*, 2009; Lang-Unnasch & Murphy, 1998). The clinical symptoms of malaria are observed during the erythrocytic stages of the life cycle (Perlmann & Troye-Blomberg, 2000). The erythrocytic stage of the life cycle involves merozoite invasion, growth and multiplication within the infected erythrocyte (schizogony), followed by egress of the daughter merozoites (which results in the destruction of red blood cells) that go on to invade new uninfected erythrocytes (Cowman *et al.*, 2012).

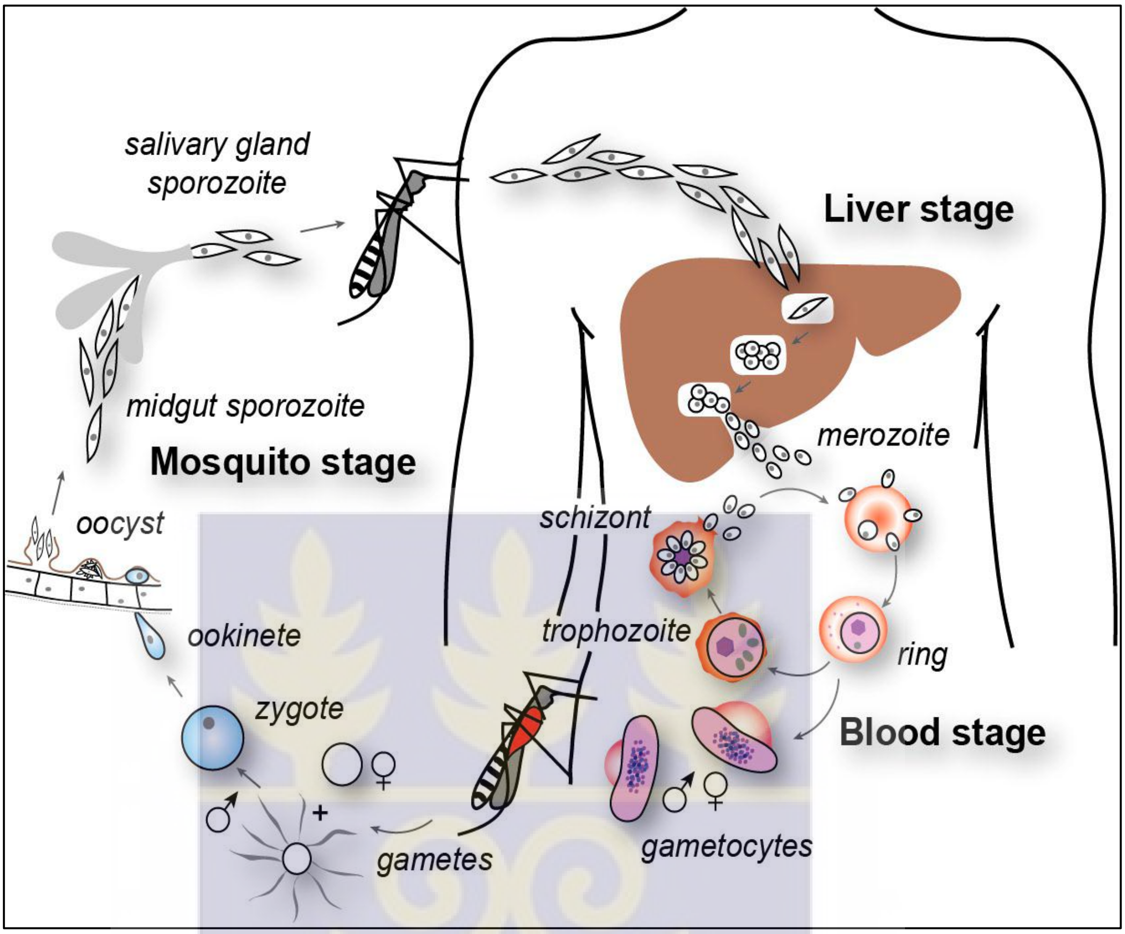


Figure 2.2: Life cycle of *Plasmodium falciparum*
[Source: *J. Cell Biol.*, 2012, *198*(6), 961–971]

Infection in the human host begins when, during a blood meal, an infected female anopheles mosquito releases long, slender, motile, single-celled parasite forms called sporozoites from its salivary glands and introduces them into the dermis (the lower or inner layer of the skin that contains blood vessels, lymph vessels, glands, etc.) where they rapidly find blood vessels and enter the blood circulation (Galinski & Barnwell, 2012). After entering circulation, a sporozoite quickly locates and invades hepatocytes where it rounds up into a trophozoite and undergoes asexual development and maturation to become a multinucleated schizont (Ejigiri & Sinnis, 2009; Stanway *et al.*, 2013). This in turn differentiates into thousands of small (1.5–3µm), invasive, ovoid shaped merozoites that egress from the hepatocyte and invade red blood cells

(RBCs). This process is termed the primary exo-erythrocytic or liver stage cycle (Galinski & Barnwell, 2012; Stanway *et al.*, 2013).

A secondary liver stage cycle is known to be present in two species of the *Plasmodium* parasites causing malaria in humans (*P. ovale* and *P. vivax*) and several other simian *Plasmodium* species (Galinski *et al.*, 2013). In this cycle, some sporozoites invade hepatocytes and develop into a trophozoite but then become dormant and cease further development (Collins & Jeffery, 2005; Markus, 2011). These quiescent, small sized (3–4µm) forms known as hypnozoites may become reactivated at a later time point (weeks, months or even years) depending on the strain and resume development into large schizonts producing thousands of merozoites that are capable of invading erythrocytes and starting a new cycle of blood stage infection and illness (Dembele *et al.*, 2014; Galinski & Barnwell, 2012). This reactivation of hypnozoites resulting in a new blood stage infection is termed a relapse infection. On the other hand, recrudescence infection results from the continuous asexual development of the blood stage parasites leading to increase in numbers and becoming microscopically or clinically evident in the blood (Dembele *et al.*, 2014).

In the red blood cells, the infection propagates itself through cyclic intraerythrocytic growth which progresses from the small vacuolar trophozoite known as the ring stage through to large trophozoites and schizonts which in the process consumes most of the hemoglobin in the RBC to form invasive merozoite (Cowman *et al.*, 2012; Tilley *et al.*, 2011). When released from the membranes of a ruptured host RBC, the merozoites invade new host red blood cells that are in circulation (Tilley *et al.*, 2011). Depending on the species, 8–24 new merozoites may be formed with each cycle of merozoite invasion and multiplication, resulting in an increase in the parasitemia and percentage of parasitized RBCs. This multiplication of parasite numbers may be in the order of 6–20-fold if the host's immunity does not intervene (Galinski & Barnwell, 2012).

A small percentage (less than 1%) of the invading merozoites do not continue to develop asexually into schizonts but may alternatively become gametocytes, which may require 1, 2, 3 or 11 days to mature depending on the species (Bousema & Drakeley, 2011; Galinski & Barnwell, 2012). *P. falciparum* gametocytes develop through five morphologically distinct stages (I to V) and this can take between 10 to 12 days to fully mature into stage V gametocytes (Kariuki *et al.*, 1998; Saliba & Jacobs-Lorena, 2013). Additionally, only mature stage V gametocytes are found in the peripheral blood circulation of an infected patient (Tibúrcio *et al.*, 2013). Once gametocytes are released into the circulatory blood system, they require an additional 2 to 3 days to become infective to a mosquito (Bousema *et al.*, 2010; Talman *et al.*, 2004). When an uninfected mosquito ingests mature gametocytes of both sexes while feeding on the host's blood containing these gametocytes, fertilization and zygote formation can occur in the vector. The male gametocyte undergoes a process known as exflagellation to release eight, sperm-like bodies that fertilize female gametocytes (Kuehn & Pradel, 2010). The zygote that is formed from this union then matures into an ookinete which then goes ahead to penetrate the mosquito gut wall cells to lodge between the gut cells and the exterior gut wall and develops into an oocyst (Angrisano *et al.*, 2012). The oocyst enlarges and develops over an 11–17-day period to produce thousands of maturing sporozoites that emerge into the haemolymph and then migrate to and invade the salivary glands (Angrisano *et al.*, 2012). Once this mosquito bites another human or possibly another primate host to obtain a blood meal, the sporozoites are released and a new malaria infection may begin.

2.1.2 Pathogenesis of Malaria

Malaria most commonly presents as an acute systemic, febrile illness but may manifest more lethargically as chronic anaemia, glomerulonephritis or tropical splenomegaly syndrome (also known as hyper-reactive malarial splenomegaly) as a result of *P. falciparum*, *P. vivax* or *P. malariae* infections. All the clinical and pathological effects of malaria in humans and other non-human primates are attributed to the asexual stage infection in the blood (Amante *et al.*, 2011; Ameri, 2010). General symptoms of malaria include fever, headaches, vomiting, abdominal pain, loss of appetite. However, more severe forms of malaria such as severe malarial anaemia (SMA) and cerebral malaria (CM) are associated with hypoglycaemia, haemoglobinuria, acute respiratory distress syndrome (ARDS), renal failure and coma (Mohan *et al.*, 2008; Trampuz *et al.*, 2003; Van den Steen *et al.*, 2013). Severe manifestations of malaria are caused by a phenomenon known as parasite sequestration and this enables parasites to avoid the host immune system. In this phenomenon, the infected erythrocytes adhere to endothelial cells within the microvascular circulatory system as the *Plasmodium* parasites mature from the late ring stage to young trophozoites (Trampuz *et al.*, 2003).

Typically, when infected RBCs rupture, merozoites and a variety of waste materials and toxins from the metabolism of the parasite in the host cell are released into the bloodstream. These waste materials and toxins include hemozoin, myristolated and palmitolated glycerol phosphatidylinositol (Tilley *et al.*, 2011). This process sets off the classical malaria rigor of high fever (ranging from 38.9°C to over 40°C), shaking chills and fatigued sweats along with other symptoms that can include myalgia and diarrhoea (Heck, 1991; Tilley *et al.*, 2011). The malaria cycle repeats itself approximately every 24, 48 or 72 hours depending on the length of the cycle of development for each particular parasite species or the degree of synchronicity of the parasite population in a given blood infection (Aly *et al.*, 2009; Cowman *et al.*, 2012). This cycle of periodic episodes of fever and chills can occur a number of times during the course of an infection if left untreated, resulting in incapacitation, profound weight loss and possibly the development of severe forms of the disease or a slow return to relative health (Galinski & Barnwell, 2012).

If left untreated, the blood stage infection of malaria can become chronic (if not fatal) and may last 2 or 3 years in the case of *P. falciparum* (Buffet *et al.*, 2011) with many recrudescing waves of parasitemia and clinical patency before the infection apparently resolves or remains undetectable (Tran *et al.*, 2013). In the case of *P. vivax* or *P. ovale*, new blood infections may occur with some frequency even if the blood stage is treated with drugs, due to the periodic reactivation of hypnozoites (Dembele *et al.*, 2014; Orjuela-Sánchez *et al.*, 2009). On the other hand, *P. malariae* does not have a secondary liver stage cycle, but blood infections are incredibly chronic and can last for decades, with one case report of up to 50 years documented (Vinetz *et al.*, 1998).

2.2 Phylum Apicomplexa

The Apicomplexa is an extremely large and diverse group with more than 5000 named species (Templeton *et al.*, 2004) and is a monophyletic group composed almost entirely of parasitic species (Leander & Keeling, 2004; Leander *et al.*, 2003). Along with ciliates and dinoflagellates, they form a higher order group known as Alveolata (Leander & Keeling, 2004). Members of this phylum are some of the most widespread and poorly controlled pathogens in the world (Plattner & Soldati-Favre, 2008; Seeber & Soldati-Favre, 2010). They are primitive, obligate, intracellular eukaryotic parasites that cause an immeasurable impact on both human and animal health worldwide (Mir *et al.*, 2011). Seven genera are known to infect humans and several other apicomplexan species are important pathogens of livestock (Janouškovec *et al.*, 2010). Members of this phylum include *Plasmodium spp*. (the agents of malaria) (Garcia, 2010), *Toxoplasma gondii* (an important opportunistic pathogen in immune-compromised individuals) (Carruthers, 2002), *Eimeria spp*. (pathogens that cause disease in chicken and cattle) (Daugschies & Najdrowski, 2005; Shirley, 1997), *Theileria spp.* (tick-borne parasites of cattle in Africa) and *Cryptosporidium* (a parasite of animal as well as an opportunistic pathogen of humans) (Mir *et al.*, 2011). *Plasmodium*, *Toxoplasma* and *Cryptosporidium* are important human pathogens (Kim & Weiss, 2004).

Apicomplexan parasites are the causal agents for a range of important veterinary and human diseases (Mir *et al.*, 2011). These agents and some of the diseases they cause include, *Eimeria tennella* which causes coccidiosis, a disease that is both persistent and challenging in the poultry industry (Ruff, 1999). Infection with *Babesia* species causes a haemolytic disease resembling malaria (Schnittger *et al.*, 2012). *Theileria annulata* and *T. parva* curtails productivity in sheep, goat and cattle herds (Ica *et al.*, 2007; Silva *et al.*, 2010). *Cryptosporidium parvum* is a water- and food-borne pathogen of livestock that, like *Babesia spp.*, can also infect humans (Fayer *et al.*, 2000). *T. gondii* is a common parasite of cats that causes a mild disease in healthy humans but, like *C. parvum*, *T. gondii* becomes particularly problematic in immuno-compromised individuals such as AIDS/HIV patients (Carruthers, 2002; Hill & Dubey, 2002). The most important members of this family of parasites are the *Plasmodium* species, which are responsible for malaria, a disease that continues to exact a devastating toll on human populations in the tropics (WHO, 2013).

All apicomplexans are obligate intracellular parasites and have a complex life cycle that involves a stage as a parasite in a diverse range of animals such as lizards, birds or mammals (Sibley, 2011; Mir *et al.*, 2011). One characteristic of this group is that they grow and replicate within the parasitophorous vacuole, a nonphagosomal, membrane bound compartment that is isolated from most cellular trafficking pathways (Cesbron-Delauw *et al.*, 2008). Proliferation of these parasites occurs by invasion of a host cell, followed by parasite growth and cell division until the host cell is lysed by the replicating parasites (Dubremetz *et al.*, 1998; Tonkin *et al.*, 2011). Parasites released by host cell lysis do not grow or undergo any cell division extracellularly and must rapidly reinvade other host cells in order to survive (Tonkin *et al.*, 2011). Repeated cycles of host cell invasion, parasite replication, host cell lysis and parasite invasion of new cells account for most of the tissue damage associated with apicomplexan infections (Tonkin *et al.*, 2011).

Despite great variation in the target host cell of each parasite and subsequent disease pathology, these obligate intracellular parasites share many prominent and unique subcellular features that are considered diagnostic for this phylum (figure 2.3), with the most distinguishing one being the apical complex, which gives this phylum its name and it has been conserved throughout evolution (Dubremetz *et al.*, 1998). The apical complex is found at the anterior domain of the invading cell. It consists of cytoskeletal elements and three specialized secretory organelles that mediate movement. Since it is involved in tissue or cell penetration, it is key to infection (Blackman & Bannister, 2001; Dubremetz *et al.*, 1998). The polar ring, conoid and microtubule organising centers (MTOCs) make up the cytoskeletal elements and the secretory organelles are the rhoptries, micronemes and dense granules (Blackman & Bannister, 2001; De Souza, 2006; Dubremetz *et al.*, 1998). The micronemes, rhoptries and dense granules are unique apically positioned secretory structures that contain products secreted into the target host cell in a rapid and coordinated sequence of events and are required for parasite motility, adhesion to host cells, invasion of host cells, and establishment of the parasitophorous vacuole (figure 2.3) (Blackman & Bannister, 2001). The conoid is a small cone-shaped structure composed of a spiral of unidentified filaments. It is thought to play a mechanical role in invasion of host cells and is present in only some apicomplexans (Blackman & Bannister, 2001; Chitnis & Blackman, 2000). The apical polar ring is a hallmark organelle of all members of the Apicomplexa. It serves as one of the three MTOCs in these parasites (the other MTOCs are pole plaques and centrioles/basal bodies) (Morrissette & Sibley, 2002). Another distinguishing and essential feature of this group of organisms is the presence of a chloroplast-like organelle known as the apicoplast (Waller & McFadden, 2005).

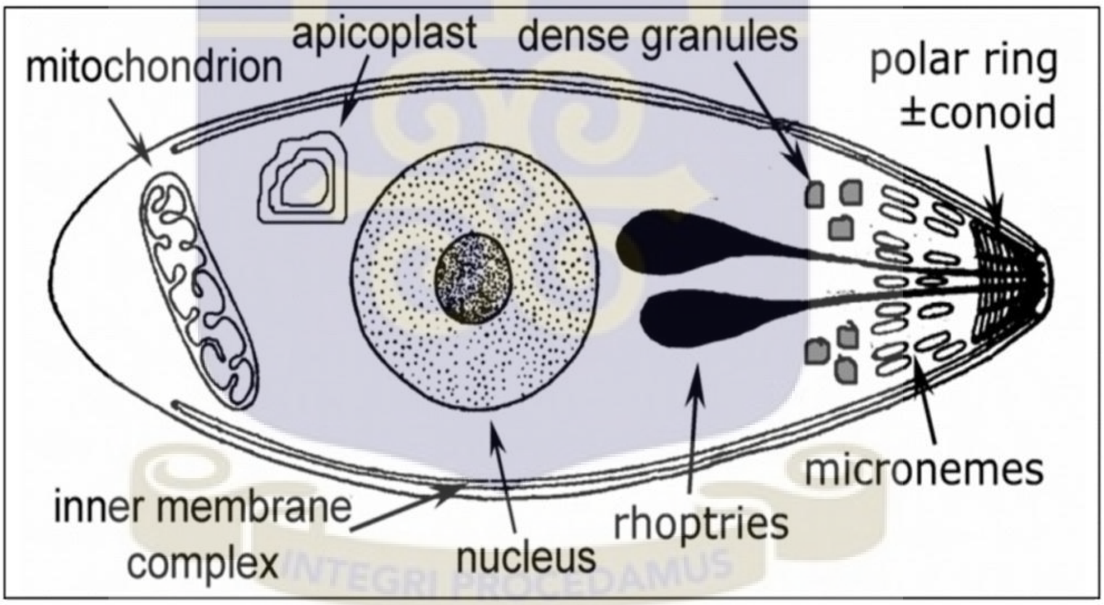


Figure 2.3: Schematic representation of the generalised structure of Apicomplexans. At the most anterior end of the invasive stages are rings of microtubules known as the polar ring. In some species, a hollow cone-shaped structure, called a conoid, is part of the polar ring.
[Source:www.tulane.edu/~wiser/malaria/ch11_draft.pdf]

2.2.1 Apicoplast

Presently, there are no effective vaccines available to prevent diseases caused by apicomplexans in humans and management therefore relies mainly on chemotherapy (Hill, 2011; malERA Consultative Group on Vaccines, 2011; Richie & Saul, 2002). Treatment of malaria, in particular, is threatened by resistance to most anti-malarial drugs by the parasites and documentations of emerging resistance to the current last-line therapy in the deadliest species, *P. falciparum* (O'Brien *et al.*, 2011; Schellenberg *et al.*, 2006). Until an effective vaccine is developed, new drug targets and a constant stream of new drugs with novel modes of action are therefore required to stay ahead of the pathogen (Choi *et al.*, 2008). One of such promising sources for new drug targets is the apicoplast due to its unusual, non-mammalian metabolism (Biot *et al.*, 2012; Yeh & DeRisi, 2011). The discovery of the apicoplast, a plastid organelle in *Plasmodium spp.* and other apicomplexans instantly made it a potential Achilles' heel for the malaria parasite and an important target in the development of new chemotherapeutic agents against these pathogens (Gleeson, 2000; Lim & McFadden, 2010; Sato, 2011; Waller & McFadden, 2005). Several features of this organelle make it both biologically fascinating and an attractive therapeutic target.

The apicoplast is a relict plastid homologous to the chloroplasts of plants (Foth & McFadden, 2003; Maréchal & Cesbron-Delauw, 2001; Waller & McFadden, 2005). It possesses four membranes and is therefore hypothesised to be the result of a secondary endosymbiosis, meaning that at some point in the evolution of these group of pathogens, the parasite engulfed another eukaryote that contained a plastid obtained by primary endosymbiosis of a cyanobacterium-like prokaryote (Foth & McFadden, 2003; Moore *et al.*, 2008). The apicoplast is therefore prokaryotic in origin and its cyanobacterial ancestry is reflected in much of its metabolic machinery (Foth &

McFadden, 2003). It harbours metabolic pathways that are distinct from or have no counterpart to those found in the mammalian host cell and is also dependent on its prokaryotic transcriptional and translational machinery (Seeber & Soldati-Favre, 2010). All of these make the apicoplast an attractive target for the identification and design of potential antimalarial agents. As the structure of the apicoplast has been retained in the parasite, it is therefore thought to have an important function that is required for the parasite's survival (Agrawal *et al.*, 2010; Lim & McFadden, 2010).

The apicoplast has a genome that is related to the plastid genome of plants and algae but has a greatly reduced sequence complexity and lacks the genes that encode proteins involved in photosynthesis (Foth & McFadden, 2003; Waller & McFadden, 2005). This is because during the course of evolution, the apicoplast lost its photosynthetic function and transferred most of its genome to the nucleus (Lim & McFadden, 2010; Sato, 2011) therefore requiring a dedicated protein targeting pathway to localize the majority of its over 500 gene products into the organelle (Waller *et al.*, 1998; Waller *et al.*, 2000). More than 10% of the nuclear genome may therefore encode proteins that function within the organelle. The remaining 35kb [circular, double-stranded DNA with a high A-T content (86%)] genome that is maintained by the apicoplast encodes approximately 50, mostly housekeeping genes, responsible for transcription and translation functions (including ribosome subunits, tRNAs, RNA polymerase) and a chaperone that is thought to be involved in protein import (Maréchal & Cesbron-Delauw, 2001).

The apicoplast represents an ideal drug target for two reasons. Firstly, it has been shown to be essential for the survival of *Plasmodium* and *Toxoplasma* (He *et al.*, 2001; Yeh & DeRisi, 2011). Secondly, since all plastids eventually trace back to a cyanobacteria-like ancestor, the molecular and metabolic processes that occur in plastids are prokaryotic in nature, making the parasites susceptible to the action of certain antibiotics and herbicides (Dahl *et al.*, 2006; Dahl & Rosenthal, 2007; McFadden, 2011). These herbicides and antibiotics include fosmidomycin (which targets DOXP reductoisomerase in the MEP pathway) (Hunter, 2011); fluridone and norflurazon (both inhibit phytoene desaturase activity in the carotenoid biosynthesis pathway) (Tonhosolo *et al.*, 2009); clindamycin, doxycycline and chloramphenicol (which are inhibitors of transcription and translation of prokaryotic genome) (Dahl *et al.*, 2006; Dahl & Rosenthal, 2007). On the other hand, many of these compounds (when used in the appropriate dosages) are harmless to humans and livestock (Pradel & Schlitzer, 2010). This is because humans and animals do not have a plastid in their cells and altogether lack some of the metabolic pathways present in this organelle (Fichera & Roos, 1997). In cases where the parasitized host does share similar pathways, it often happens that the host and plastid pathways or the enzymes involved are sufficiently different to allow for selective inhibition. Such differences offer great potential for chemotherapeutic exploitation (Ralph *et al.* , 2001).

The apicoplast has been shown to be essential during the asexual intraerythrocytic and hepatic stages of *P. falciparum* (Yeh & DeRisi, 2011) and there is strong evidence supporting its essential metabolic functions during the mosquito stages of the parasite (Wiley *et al.*, 2015). The organelle is believed to support three metabolic functions; type II fatty acid biosynthesis, *de novo* heme biosynthesis, and isoprenoid biosynthesis (Gornicki, 2003; Surolia & Padmanaban, 1992; Wiesner & Jomaa, 2007). Type II fatty acid and *de novo* heme biosynthesis are not essential during the asexual and gametocyte intraerythrocytic stages of the parasites since they scavenge lipids and heme from the human host during these stages (Wiley *et al.*, 2015). However, both of these biosynthetic pathways are essential for the development of liver and mosquito stages of the parasite (Nagaraj *et al.*, 2013; van Schaijk *et al.*, 2013; Vaughan *et al.*, 2009). In contrast, biosynthesis of the isoprenoid precursors, isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP) has been shown to be the only essential metabolic function of the apicoplast in the asexual intraerythrocytic stages (Wiley *et al.*, 2015; Yeh & DeRisi, 2011).

2.2.2 Isoprenoid Precursor Biosynthesis Pathway

Isoprenoids, also referred to as terpenoids, are one of the largest and most diverse group of natural products and they include sterols, natural rubber, carotenoids, ubiquinones, menaquinones, dolichols and prenylation moieties (Kirby & Keasling, 2009; Prasain & Barnes, 2009). They are derived from the sequential assembly of two 5-carbon molecules, isopentenyl pyrophosphate (IPP) and its isomer dimethylallyl pyrophosphate (DMAPP), the sole precursors for the synthesis of all isoprenoids (Chang *et al.*, 2013; Kirby & Keasling, 2009). The assembly results in a chain of isoprene units which are subsequently modified through cyclizations, oxidations, reductions and additions of functional groups to generate the array of over 25,000 isoprenoids that are found in nature. Isoprenoids and their derivatives are vital to all classes of organisms and they play key roles in all aspects of life (Holstein & Hohl, 2004), e.g., as gene expression regulators [farnesyl pyrophosphate (FPP) and geranylgeranyl pyrophosphate (GGPP)] (Holstein *et al.*, 2003), constituents of membranes (sterols) (Czub & Baginski, 2006; Dufourc, 2008), vitamins (α- and β-carotene) (Heldt & Piechulla, 2011), antimicrobial agents (geraniol, artemisinin) (Balint, 2001; Nagaki *et al.*,2011), mating pheromones (nepetalactol) (Regnier & Law, 1968; Wyatt, 2003), components of signal transduction pathways (dolichol) (Hamadmad *et al.*, 2006), reproductive hormones (oestrogens) (Stacey, 2003), components of electron transport (ubiquinone) and photosynthetic machinery (chlorophyll) (Heldt & Piechulla, 2011).

Two distinct and independent pathways (figure 2.4) result in the biosynthesis of the isoprenoid precursors IPP and DMAPP; the canonical mevalonate-dependent pathway (first described in yeast and mammals) which is existent in most eukaryotic cells (all mammals), archaea, the cytosol and mitochondrion of plants, fungi and eukaryotic parasites (e.g. *Leishmania* and *Trypanosoma*) and the mevalonate-independent pathway (first described in studies with bacteria and plants) also referred to as MEP/DOXP (2-*C*-methyl-D-erythritol-4-phosphate/1-deoxy-D-xylulose-5-phosphate) pathway (Kuzuyama & Seto, 2012; Lange *et al.*, 2000). In humans and other metazoans, the isoprenoid building blocks are synthesized through the mevalonate-dependent pathway which begins with the production of acetoacetyl-CoA from acetyl-CoA by the enzyme thiolase (Miziorko, 2011). The alternate ‘‘non-mevalonate’’ pathway is present in the plastids of bacteria, algae, cyanobacteria, eubacteria and also the chloroplasts of plants. Apicomplexans (*Toxoplasma* and *Plasmodium spp*.) also rely on this pathway to synthesize the isoprenoid precursors (Hale *et al.*, 2012; Hunter, 2007).

The MEP pathway (figure 2.4) begins with the generation of deoxy-xylulose 5-phosphate (DOXP) from pyruvate and glyceraldehyde 3-phosphate. Methylerythritol-4-phosphate (MEP) (the first dedicated metabolite and also from which the pathway derives its name), is then synthesized from DOXP by the enzyme deoxyxylulose phosphate reductoisomerase (Gräwert *et al.*, 2011; Vranová *et al.*, 2013). While plants contain both the mevalonate and MEP pathways, apicomplexans do not contain the mevalonate pathway and depend exclusively on the MEP pathway to generate their isoprenoid precursors. Both pathways produce IPP and DMAPP as the final products, but the enzymes and chemical intermediates leading to synthesis of these compounds are entirely different (Miziorko, 2011).

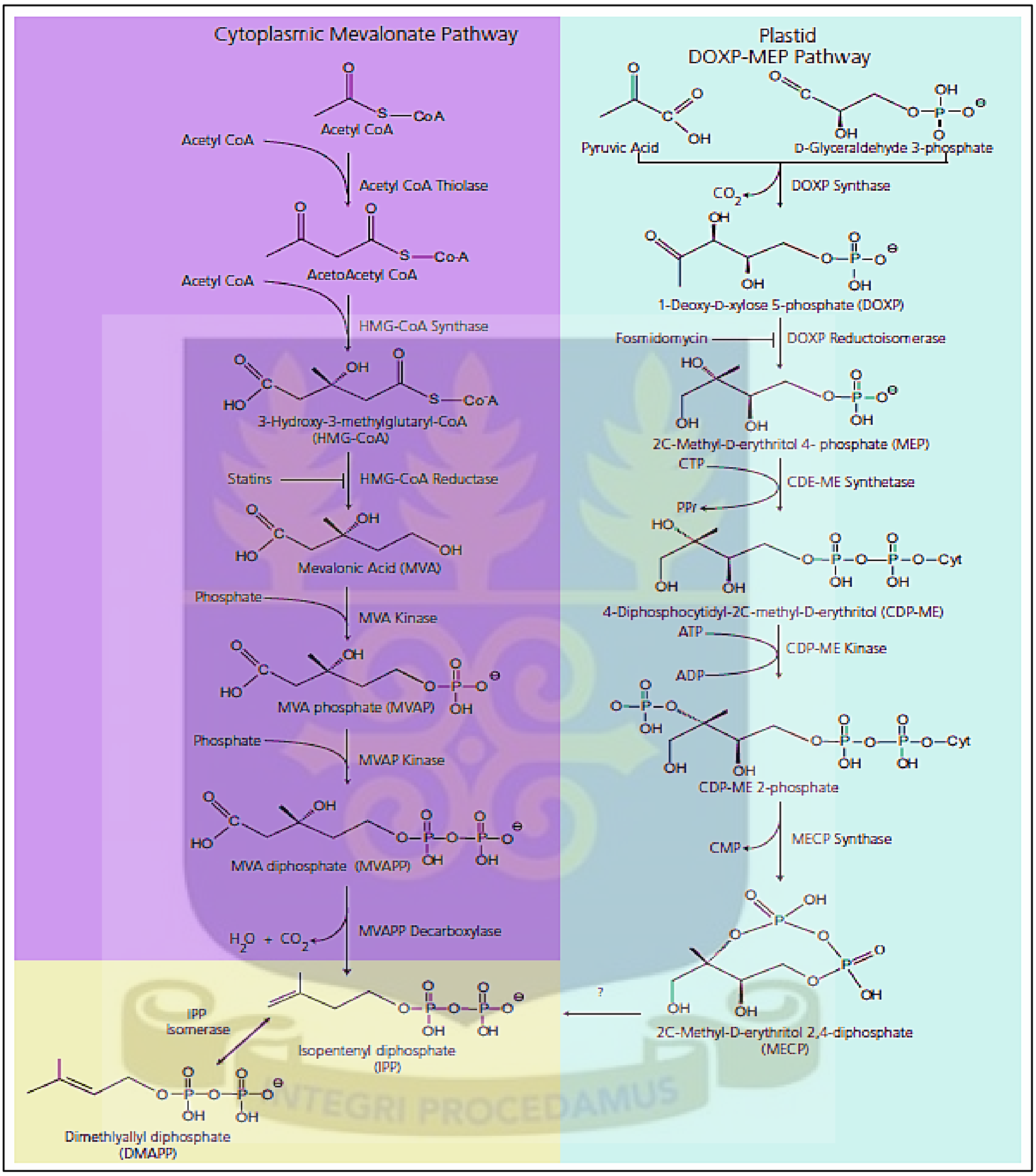


Figure 2.4: Schematic representation of the isoprenoid precursor biosynthetic pathways.

It indicates the location, metabolites and the enzymes involved in each pathway. The formation of IPP and its isomerization to DMAPP occurs independently in both the cytoplasmic and plastid compartments.

[Source:http://www.sigmaaldrich.com/technical-documents/articles/biofiles/dietary-terpenes.html#sthash.6WV8efY4.]

The plastids of plant and algae are the site for many essential biochemical pathways, some of which have already been found in *P. falciparum* (Keeling, 2010; Lopez-Juez & Pyke, 2005). Among the biochemical pathways that are believed to be retained and incorporated in the parasite's metabolism, the carotenoid biosynthesis pathway is an attractive target for investigation. This is because it is essential in algae, higher plants, bacteria and fungi but absent in mammals and its products are involved in many important metabolic functions (Hirschberg, 2001). All carotenoids are derived from the isoprenoid biosynthesis pathway with the isoprenoid precursors as starting materials (Vranová *et al.*, 2012; 2013). They possess a polyisoprenoid structure, a long conjugated chain of double bonds and an almost bilateral symmetry around the central double bond (Meléndez-Martínez *et al.*, 2006; Schmalz & Griffin, 2009). Their biosynthesis starts with the condensation of two molecules of geranylgeranyl pyrophosphate (GGPP) to form phytoene, the initial C40 carotenoid skeleton (Tonhosolo *et al.*, 2009). The different carotenoids in nature are derived essentially by modifications in the base structure such as cyclization of the end groups and by introduction of oxygen functions, resulting in their characteristic colours and antioxidant properties (Namitha & Negi, 2010; Rao & Rao, 2007).

In recent times, the isoprenoid biosynthesis pathway has been explored as a target for the development of chemotherapeutic agents for the treatment of some diseases (malaria and toxoplasmosis) (Coppens, 2013; Goodman & McFadden, 2013; Oldfield, 2010). One of these inhibitors is fluridone (1-methyl-3-phenyl-5-[3-(trifluoromethyl) phenyl]-4-(1*H*)-pyridinone), a fluorine-containing compound that belongs to the class of nitrogen-containing heterocycles (figure 2.5) (McCowen *et al.*, 1979). Fluridone is a known herbicide used in the control of invasive aquatic plants. It targets the enzyme phytoene desaturase which catalyses the conversion of phytoene to lycopene. It is an odourless crystalline solid with a white to pale yellow appearance, with a molecular weight of 329.32g/mol and a melting point between 151°C and 154°C (McCowen *et al.*, 1979). It is soluble in organic solvents such as methanol, diethyl ether, chloroform, ethyl acetate and hexane. It is also partially soluble in water (McCowen *et al.*, 1979).

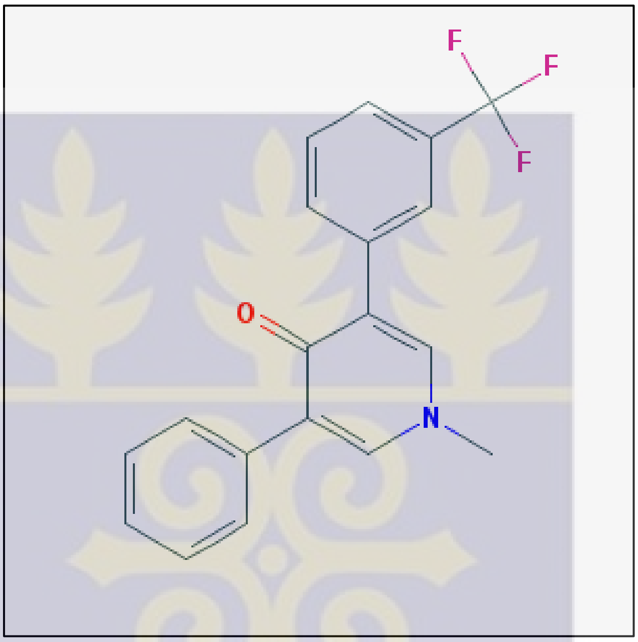


Figure 2.5: Chemical structure of fluridone
[Source: http://www.pubchem.ncbi.nlm.nih.gov/compound/fluridone]

2.3 Carotenoids

Carotenoids belong to a class of compounds known as terpenoids and are the most ubiquitous and widespread natural pigments and are a characteristic of organisms from all major taxa (Mortensen, 2006). Examples of the presence of carotenoids in nature include the pink flesh of salmon (Anderson, 2000), the black carapace of crayfish (Sagi *et al.*, 1995), the variety of bird feather or reptile skin colours (Czeczuga, 1979; Lindgren *et al.*, 2014; Shawkey & Hill, 2005), the yellow stain of mussels (Czeczuga, 1979; Maoka, 2011), the brown and purple coloration of brown algae and purple bacteria respectively (Matsuno, 2001; Takaichi, 2013). They are naturally occurring, lipid-soluble pigments (secondary metabolites) that are widely distributed in nature. They belong to the category of natural products known as tetraterpenoids (molecules containing 40 carbon atoms that are built from four terpene units each containing 10 carbon atoms) comprising a large family of C_{40} polyenes hydrocarbon chain that may be terminated in rings (Namitha & Negi, 2010). In general, carotenoids are compounds made from eight isoprenoid units whose order is inverted at the molecule center (Cazzonelli, 2011; Fraser & Bramley, 2004). All carotenoids can be considered as lycopene ($C_{40}H_{56}$) derivatives as they occur through series of reactions involving: hydrogenation, dehydrogenation, cyclization, oxygen insertion, double bond migration, methyl migration, chain elongation and chain shortening (Delgado-Vargas *et al.*,2000).

They can be found in plants, algae, bacteria, fungi and some animals and form one of the most important classes of plant pigments that play a vital role in defining the quality parameters of fruit and vegetables (Christaki *et al.*, 2013; Eldahshan & Singab, 2013). Carotenoids are predominantly found and synthesized *de novo* in plants, algae and photosynthetic bacteria, where they play a critical role in the photosynthetic process (Cogdell & Frank, 1987; Goodwin, 1980). They also occur in some non-photosynthetic bacteria, yeasts and moulds, where they may perform a protective function against damage by light and oxygen (Vachali *et al.*, 2012). *Plasmodium spp.* and *Toxoplasma spp.* and other members of the phylum Apicomplexa are also known to synthesize carotenoids (Coppens, 2013; Nagamune *et al.*, 2008; Tonhosolo *et al.*, 2009). Mammals are unable to synthesize carotenoids *de novo* and must rely on their diet as a source of these compounds (Fernández-García *et al.*, 2012; Rao & Rao, 2007). In plants, carotenoids are responsible for the distinctive yellow, orange and some reddish colours observed in leaves, fruits, vegetables and flowers as well as several aromas in plants and are also responsible for the colours of some birds (e.g., flamingo), insects (e.g., aphids), fish (e.g., salmon), and crustaceans (e.g., crab, shrimp) (Cazzonelli, 2011).

Carotenoids fulfil a variety of critical functions in the organisms in which they are found (Britton, 2008; Eldahshan & Singab, 2013; Vershinin, 1999). In plants they serve as accessory pigments to harvest light for photosynthesis and constitute the basic structural units of the photosynthesis apparatus (Cogdell *et al.*, 1994; Fraser *et al.*, 2001). They also act as photo-protectors for plants so they could adapt to high light stress and also endow flowers and fruits with distinct colours to attract insects and animals for pollination and seed dispersal (Cazzonelli *et al.*, 2010; Cazzonelli, 2011). In addition, oxidative cleavage of carotenoids results in the production of apocarotenoids, some of which serve as signal molecules in plant development, antifungal agents and also contribute to the flavour and aroma of flowers and fruits (Auldridge *et al.*, 2006). Some of the products of carotenoid catabolism, such as β-ionones, are involved in plant-insect interactions (Auldridge *et al.*, 2006). The phytohormones, abscisic acid and strigolactone (which are derived from carotenoid precursors) regulate a wide range of important biological processes in plants such as responses to environmental stress, embryo development and seed dormancy (Cheng *et al.*, 2013; Chinnusamy *et al.*, 2008; Koornneef *et al.*, 2002). Abscisic acid has been shown to be synthesized by the opportunistic apicomplexan parasite *T. gondii* and it controls calcium signalling in the parasite. Treatment of the *T. gondii* cultures with fluridone (which selectively disrupts abscisic acid synthesis) either delayed or inhibited calcium-dependent egress by the parasites from the infected host cells in a dose-dependent manner (Nagamune *et al.*, 2008). Some carotenoids are precursors of vitamin A and prevent human age-related macular degeneration. Others like lycopene (a potent antioxidant) and astaxanthin are considered to prevent prostate cancer and cardiovascular disease respectively in the human body (Rao & Rao, 2007). Carotenoids are also widely used as colorants in the food and cosmetic industries and some are important supplements in livestock and fish feed formulations (Francis, 2000; Mortensen, 2006).

2.3.1 Classification and Functions of Carotenoids

Carotenoids are classified into two main groups (figure 2.6) based on their chemical structure as:

- oxycarotenoids or xanthophylls (those that have carbon, hydrogen and additionally, oxygen) e.g., lutein and zeaxanthin
- carotenes (those that are composed of carbon and hydrogen only) e.g., β-carotene and lycopene (Fraser & Bramley, 2004)

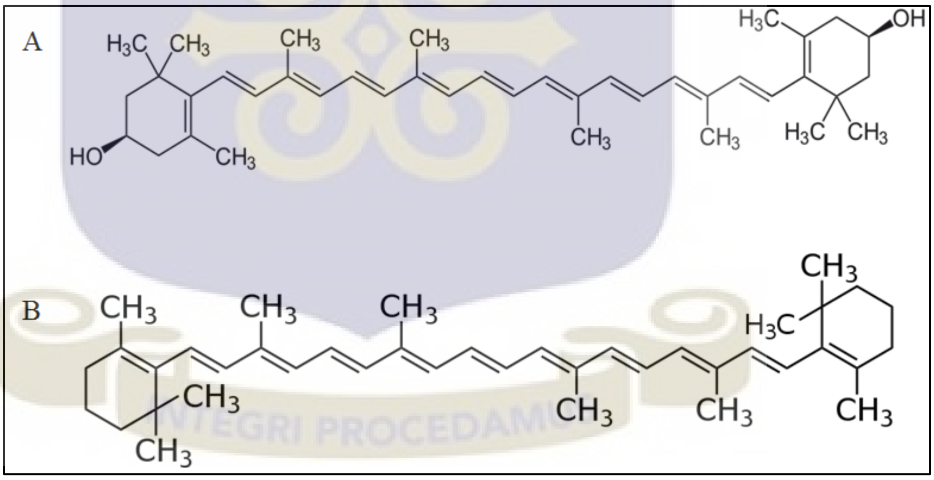


Figure 2.6: The general chemical structure of the two groups of carotenoids (**A**. Xanthophylls, **B**. Carotenes) [Source:https://achemicallife.wordpress.com/2012/08/26/aphids-carotenoids-and-photosynthesis/]

Also, carotenoids have been classified as primary or secondary. The primary carotenoids group are those compounds required by plants in photosynthesis (β-carotene, violaxanthin, and neoxanthin), whereas the secondary carotenoids are localized in fruits and flowers (α-carotene, β-cryptoxanthin, zeaxanthin, antheraxanthin, capsanthin, capsorubin).

Generally, carotenoids absorb blue light and serve two key roles in plants and algae: they absorb light energy for use in photosynthesis and protect chlorophyll from photo-damage (Vershinin, 1999). In animals, carotenoid derivatives promote health, improve sexual behaviour and are essential for reproduction (Cazzonelli, 2011). In most animals, dietary carotenoids are cleaved to provide precursors for vitamin A biosynthesis (of which a deficiency leads to blindness). They are valuable for many physiological functions and thus promote human health (e.g. antioxidant activity, immuno-stimulants, photo-protection, visual tuning as well as limiting age-related macular degeneration of the eye) (Handelman, 2001; Rao & Rao, 2007). In humans, four carotenoids (α-, β- and γ-carotene, and β-cryptoxanthin) have vitamin A activity (i.e. can be converted to retinal) and these and other carotenoids can also act as antioxidants (Bendich & Olson, 1989; Rao & Rao, 2007). In the eye, the carotenoids lutein and zeaxanthin apparently act directly to absorb damaging blue and near-ultraviolet light, in order to protect the macula lutea (Abdel-Aal *et al.*, 2013; Schalch, 2001). People consuming diets rich in carotenoids from natural foods, such as fruits and vegetables, are healthier and have lower mortality from a number of chronic illnesses (Perera & Yen, 2007; Rao & Rao, 2007).

2.3.3 Carotenoid Biosynthesis Pathway

Carotenoids are synthesized from the basic five carbon isoprene building precursor, IPP and its isomer DMAPP (Schwender *et al.*, 1996). As isoprenoids, carotenoid compounds originate from the plastid-localized MEP pathway that starts with the reaction between pyruvate and glyceraldehyde-3-phosphate (Tritsch *et al.*, 2010). The carotenoid biosynthesis pathway from isoprene precursors (via the MEP pathway) is well characterised in higher plants and in microorganisms (Bertrand, 2010; Rodríguez-Concepción, 2010; Tran *et al.*, 2009). The first two steps in the MEP pathway are regulated by 1-deoxy-D-xylulose-5-phostaphate synthase (DXS) and 1-deoxy-D-xylulose 5-phosphate reductoisomerase (DXR) respectively (Lu & Li, 2008) (figure 2.7). A second important regulatory point is the step catalysed by 1-hydroxy-2-methyl-2-(E)-butenyl 4-diphosphate reductase (HDR) which leads to the generation of isopentenyl diphosphate and dimethylallyl diphosphate (Lu & Li, 2008). Geranyl-geranyl diphosphate (GGPP) synthase catalyses the condensation of three molecules of IPP and one molecule of DMAPP to produce GGPP – a 20-carbon molecule (Sandmann, 1994). The first committed step in carotenoid biosynthesis is the condensation of two molecules of GGPP by phytoene synthase (PSY) to form phytoene – the initial C40 carotenoid skeleton (Gas *et al.*, 2009) (figure 2.7). GGPP is also the precursor for several other groups of metabolites, including chlorophylls, ubiquinones and tocopherols (Hirschberg, 2001). Different carotenoids are essentially derived by modifications to the parent skeleton such as cyclization of the end groups and by introduction of oxygen, resulting in their characteristic colours and antioxidant properties (Krinsky, 1998; Namitha & Negi, 2010). After the formation of phytoene, it is then converted to lycopene by phytoene desaturase (crtI) through four sequential reactions (Hirschberg, 2001; Sandmann, 1994). Only one crtI is known to catalyse the conversion of phytoene to lycopene in bacteria; however, in plants, at least four enzymes are required for this step. These enzymes include phytoene desaturase (PDS) and zeta-carotene desaturase (ZDS) which produce respective poly-*cis*-compounds (Schaub *et al.*, 2012). These poly-*cis*-compounds are then isomerized to the *trans* forms by zeta carotene isomerase (ZISO) and carotenoid isomerase (CRTISO) to produce lycopene (Schaub *et al.*, 2012).

In higher plants, the cyclization of lycopene with lycopene ε- and β-cyclases is a critical branch-point in carotenoid biosynthesis (Cazzonelli, 2011). In one branch, a β-ring is introduced at each end of lycopene by the enzyme lycopene β-cyclase (β-CYC) in a sequential two-step reaction to form β-carotene. In the other branch (which leads to the formation of lutein), one β- and one ε-ring are introduced into lycopene in the first dedicated reaction by lycopene β-cyclase (β-CYC) and lycopene ε-cyclase (ε-CYC) respectively to form α-carotene (Cazzonelli *et al.*, 2010). β-ring hydroxylase then acts on α-Carotene to form zeinoxanthin, which is later hydroxylated by an ε-ring hydroxylase to produce lutein (Lu & Li, 2008). Carotenoids with two ε-rings are rare in plants and algae, with an exception in lettuce, wherein a single ε-CYC adds two ε-rings to lycopene to form lactucaxanthin (Cunningham & Gantt, 2001). β-Carotene can be converted to zeaxanthin in a two-step hydroxylation reaction, with β-cryptoxanthin as an intermediate product (Lu & Li, 2008). In green tissues, zeaxanthin can be epoxidized to violaxanthin and a set of reactions known as the xanthophyll cycle rapidly optimizes the concentration of violaxanthin and zeaxanthin in the cell through the action of zeaxanthin epoxidase and violaxanthin de-epoxidase respectively (Chen & Gallie, 2012; Lin *et al.*, 2002; Masojídek *et al.*, 2004) (figure 2.7).

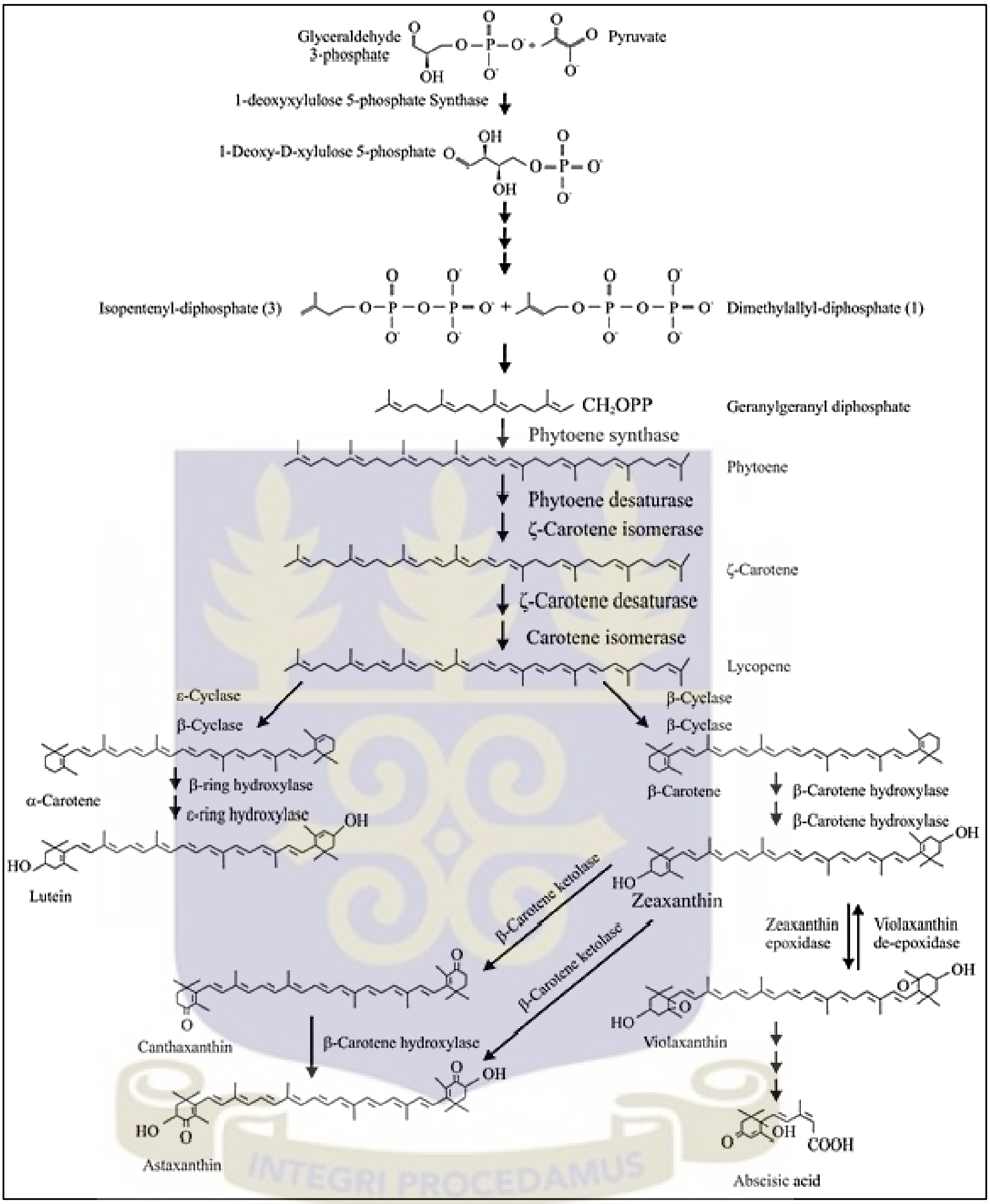


Figure 2.7: Schematic of the carotenoid biosynthesis pathway via the MEP isoprenoid precursor biosynthesis pathway
[Source: http://lipidlibrary.aocs.org/Biochemistry/content.cfm?ItemNumber=40302]

A myriad of other carotenoids with distinctive biosynthetic routes are present in some plants (Cazzonelli *et al.*, 2010). An example is the ketocarotenoid astaxanthin which is accumulated in some higher plants, such as *Adonis aestivalis* and cyanobacteria (Lemoine & Schoefs, 2010; Vachali *et al.*, 2012). Zeaxanthin oxidation results in the formation of astaxanthin, a step catalysed by β-carotene ketolase (Hirschberg, 2001). It can also result from canthaxanthin hydroxylation which is catalysed by β-carotene hydroxylase (Hirschberg, 2001). Canthaxanthin synthesis from β-carotene is catalysed by β-carotene ketolase (Scaife *et al.*, 2012). A similar carotenoid biosynthesis pathway described above has been shown to exist in *Plasmodium* species and some of these carotenoids are synthesised in the intraerythrocytic stages of the parasites (Tonhosolo *et al.*, 2009). However, most of the intermediates or end products and their role and the role of the pathway itself in the parasite are unknown.

2.3.4 Metabolic Profiling of Compounds Using HPLC

Metabolite profiling is an analytical method for the identification and relative quantitation of a number of metabolites from biological samples (Fiehn, 2002). Often, these samples are garnered from a specific tissue or a part of a tissue of interest, however, depending on the biological question, they may also be obtained either from a larger mixture of different organs (such as whole-shoots) or conversely on a micro scale from single cells or purified organelles. The metabolite profiling of a mixture of compounds or compound classes requires the use of chromatography and universal detection technologies such as High Performance Liquid Chromatography/diode array UV detection (HPLC-UV), gas chromatography/mass spectrometry (GC-MS), HPLC-MS/MS, etc. In metabolic profiling, target analysis is constrained to one or a very few target compounds (such as carotenoids). Such targets are usually quantified in an absolute manner using calibration curves and/or stable isotope labelled internal standards (Fiehn, 2002).

HPLC is a technique that is frequently employed for the separation, identification and quantification of compounds in a mixture (Lee, 2011; Snyder *et al.*, 2009). It is basically a highly improved form of column liquid chromatography. Instead of a solvent being allowed to drip through a column under gravity, it is forced through under high pressure which makes it much faster. All chromatographic separations, including HPLC operate under the same basic principle; separation of a sample into its constituent parts because of the difference in the relative affinities of different molecules for the mobile phase and the stationary phase used in the separation (Hanai, 1999). There are variants of HPLC depending on the phase system (stationary) involved in the process. These are;

1. **Normal Phase HPLC**: This method separates analytes on the basis of polarity. NP-HPLC uses polar stationary phase and non-polar mobile phase. Therefore, the stationary phase is usually silica and typical mobile phases are hexane, methylene chloride, chloroform, diethyl ether and mixtures of these. Polar samples are thus retained on the polar surface of the column packing longer than less polar materials (Moldoveanu & David, 2013).
2. **Reverse Phase HPLC:** Reverse phase chromatography has the widest range of applications. The stationary phase comprises non polar (hydrophobic) organic chains bound to inert silica surface and mobile phase comprises of aqueous or aqueous-organic mixtures of polar solvents (such as mixtures of water and methanol or acetonitrile) of varying degrees of polarity. It works on the principle of hydrophobic interactions between the column and the components of the mixture to be separated. Hence, the elution sequence is polar followed by less polar and least polar or nonpolar compounds eluting last through the column (Moldoveanu & David, 2013).
3. **Size-exclusion HPLC:** The column is filled with materials having precisely controlled pore sizes, and the particles are separated according to their

molecular sizes. Larger molecules are rapidly washed through the column; smaller molecules penetrate the pores of the packing particles and elute later (Moldoveanu & David, 2013).

4. **Ion-Exchange HPLC:** The stationary phase has an ionically charged surface of opposite charge to the sample ions. This technique is used almost exclusively with ionic or ionizable samples. The stronger the charge on the sample, the stronger it will be attracted to the ionic surface of the column and thus, the longer it will take to elute. The mobile phase is an aqueous buffer, where both pH and ionic strength are used to control elution time (Moldoveanu & David, 2013).

In this study, the reverse phase HPLC was used to profile carotenoids extracted from *P. falciparum* intraerythrocytic stages.

2.3.5 Significance of Bioinformatics in Evolutionary Studies

Bioinformatics is an important area of study that involves the application of computer science and mathematics to solve biological problems by analysing nucleotide or amino acid sequence to identify relatedness or divergence in a group of organisms. This area of study uses tools such as BLAST, CLUSTAL W, etc. (Larkin *et al.*, 2007), to analyse the nucleotide or amino acid sequences from databases in evolutionary or comparative studies. Bioinformatics studies have helped identify orthologues of genes or proteins in related or unrelated species and also helped to predict the presence and functions of genes and proteins in several organisms (Agarwal *et al.*, 2015).

The first committed and rate-limiting step in carotenogenesis in apicomplexans, plants, algae and bacteria is catalysed by phytoene synthase (figure 2.7) (Rodríguez-Villalón *et al.*, 2009; Tran *et al.*, 2009), a transferase enzyme which catalyses the condensation of two molecules of geranylgeranyl pyrophosphate (GGPP) to form phytoene (the initial C40 skeleton of all carotenoids) (Sieiro *et al.*, 2003). GGPP also serves as a precursor for the synthesis of other terpenoids (e.g., steroids, menaquinones, plastoquinones and chlorophyll) therefore, the expression of the *PSY* gene is highly regulated and this represents an important regulatory point for controlling the flux (of carbon) into the carotenoid biosynthetic pathway. The PSY protein has been well investigated in higher plants, algae and apicomplexans (Agarwal *et al.*, 2015; Salvini *et al.*, 2005; Yan *et al.*, 2005; Zhu *et al.*, 2005). Recent bioinformatics studies in *P. falciparum* have identify PSY as a bifunctional enzyme exhibiting octaprenyl pyrophosphate synthase (OPPS) as well as PSY activity, analogous to some bacterial OPPS enzymes (Tonhosolo *et al.*, 2005, 2009). *T. gondii*, a related apicomplexan is also known to have weak orthologues of *PSY* based on bioinformatics searches using genes from higher plants.

The PSY enzyme is coded for by the *PSY* gene and is well studied in plants, bacteria and algae (Cunningham & Gantt, 1998; Sandmann, 1994, 2001) but is poorly studied in apicomplexans. Amongst all the genes that have been proposed to be involved in the carotenoid biosynthesis pathway, only the *PSY* gene has been definitely implicated through functional assays (Rodríguez-Villalón *et al.*, 2009). Depending on the organism, the *PSY* maybe present as a single gene or a family of related genes that codes for one or more proteins with phytoene synthase activity (Giorio *et al.*, 2008; Li *et al.*, 2009; López-Emparán *et al.*, 2014; Römer *et al.*, 1993). In most organisms, the nucleotide sequence, structure and evolutionary history of the *PSY* gene are known (Cordero *et al.*, 2011; Fraser *et al.*, 2002; Iwata-reuyl *et al.*, 2003; Yan *et al.*, 2005), however, little is known about the structure and evolutionary relationship of the *PSY* gene in apicomplexans in relation to other organisms.

CHAPTER THREE

3.0 MATERIAL AND METHODS

All work relating to parasite culture were performed in a biological safety cabinet (BSC) using aseptic techniques.

3.1 Media Preparation and Parasite Culture

3.1.1 Preparation of Media for Culturing

To make 500ml parasite washing medium (PWM), RPMI 1640 [containing L-glutamine and HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid)] (Gibco, UK) was supplemented with 7.5% $NaHCO_3$ (Sigma-Aldrich, USA) (working concentration, 32mM), 20% glucose (Sigma-Aldrich, USA) solution (working concentration, 20mM) and gentamycin (Gibco, UK) (working concentration, 10µg/ml). Complete parasite medium (CPM) was made by supplementing the PWM with 5mg/ml Albumax II (Invitrogen, USA) and 0.2µg/mL hypoxanthine (Sigma-Aldrich, USA). All culture media were stored at temperatures between 4-8°C.

3.1.2 Thawing of Cryopreserved Parasite

Cryopreserved *P. falciparum* 3D7 strain [obtained from the Immunology Department of the Noguchi Memorial Institute for Medical Research (NMIMR), Ghana] were removed from liquid nitrogen storage and thawed in a water bath set at 37ºC for 1 minute. This was followed the addition of 12% NaCl (Sigma-Aldrich, USA) for every 100µL of the parasite suspension. After incubating for 5 minutes at room temperature, one in ten parasite suspension in 1.6% NaCl was added drop wise, whilst gently swirling the tube. The suspension was then centrifuged at 1500 revolutions per minute (rpm) for 5 minutes at room temperature (RT). The supernatant was removed and the erythrocytes were resuspended in complete parasite medium (RPMI 1640 containing

5mg/mL Albumax II, 10µg/mL gentamycin, 0.2µg/mL hypoxanthine, 2mM L-glutamine, 25mM HEPES, 23.8mM $NaHCO_3$). The suspension was then centrifuged at 1500 rpm for 5 minutes at RT. The supernatant was removed and the parasitized RBCs were added to 200µl of freshly prepared sickling negative, O^+ human erythrocytes placed in a 25ml tissue culture flask (Corning, USA) containing 5ml CPM. The mixture was then gently flushed with a special gas mixture [(5.5% CO_2, 2% O_2 and 92.5% N_2), Air Liquide, UK] for 30 second. The culture flask was capped immediately and kept in an incubator (RS Biotech, USA) set at 37°C.

3.1.3 Parasite Culture and Maintenance

P. falciparum 3D7 strain (NMIMR, Ghana) were maintained in culture as described by Trager & Jensen (1976) with some modifications (Maier & Rug, 2013). Parasite cultures were maintained in CPM with human O^+, sickling negative erythrocytes at 4% haematocrit in either 25ml or 75ml tissue culture flasks (Corning, USA). Five millilitres (5ml) or 25ml of CPM was used to maintain the cultures in 25ml and 75ml tissue culture flasks respectively. The content of the flasks were then gently flushed with the special gas mixture, closed tightly and incubated at 37°C. The RBCs used for maintaining the cultures were initially collected from a donor into citrate phosphate dextrose (CPD) vacutainers (BD biosciences, UK). The blood was stored at 4-8°C for 48 hours before processing. After this storage period, the blood was then transferred into 15ml centrifuge tubes and washed three times with PWM by centrifuging at 1500 rpm for 5 minutes and discarding supernatant. The haematocrit was measured from the packed RBC volume after centrifuging in a swinging bucket rotor at 2,000 rpm for 5 minutes at room temperature. This was then stored at 4-8°C and used within two weeks for culturing. Spent CPM was replaced with fresh one, the population and health of the cultures were also monitored daily unless stated otherwise. Parasite population was determined by making thin smears of the culture on a microscope slide, fixed with methanol and stained with 10% Giemsa (Fluka chemicals, UK) for 15 minutes and visualized by bright-field microscopy. Parasitemia were scored visually with a 100-fold oil-immersion objective by counting at least 1,000 erythrocytes to determine the percentage of infected erythrocytes. This procedure was repeated daily.

3.2 Synchronisation of Parasites

3.2.1 D-Sorbitol Synchronisation for Rings

P. falciparum cultures were synchronized as described by Lambros & Vanderberg (1979) with some modifications. Cultures with high ring stage parasite populations (≥8%) were selected for synchronisation. To establish synchrony, the selected cultures were transferred into 15ml centrifuge tubes and centrifuged at 2000 rpm for five minutes, the supernatant discarded and the pellet resuspended in 5ml of aqueous 5% D-sorbitol solution pre-warmed to 37 °C in a water bath. The cell suspension was then incubated for 10 minutes in an incubator set at 37°C. After the incubation period, the suspension was centrifuged at 2000 rpm for 5 minutes, the D-sorbitol solution removed and the pellet washed once with PWM and then twice with CPM. All washing steps were done at 2000 rpm for 5 minutes. Successful synchronisation was ensured by making a thin smear from the washed cultures on a microscope slide, fixed with methanol, stained with 10% Giemsa for 15 minutes and visualised by bright-field microscopy with the 100X magnification objective lens. Synchronisation was considered successful when majority (about 90% or more) of the parasites observed under the microscope were in the ring stages. A new culture was then set up with the washed pellet by transferring into a new 25ml tissue culture flask containing 5ml CPM and uninfected O^+, sickling negative human RBCs at 4% haematocrit. The content of the flask was then gassed with the special gas mixture for 30 seconds, closed immediately and tightly and then transferred into an incubator set at 37°C.

3.2.2 Percoll® Synchronisation for Schizonts

Ninety percent (90%) Percoll® solution was prepared by gently mixing nine parts of 100% Percoll® (Sigma-Aldrich, USA) with one part of 10X phosphate buffered saline [(PBS), Gibco, USA]. Sixty-five percent (65%) and 35% Percoll® solutions were then prepared from the 90% Percoll® solution by mixing with PWM. This was done by combining 6.5ml of 90% Percoll® with 2.5ml of parasite wash medium (PWM) to make the 65% Percoll® solution and mixing 3.5ml of 90% Percoll® with 5.5ml of PWM to make the 35% Percoll® solution. Subsequently, these were filter sterilised using a 0.22µm filter (Millipore, France). These solutions were to be used to establish a gradient. Meanwhile, the parasite cultures were transferred into 15ml centrifuge tubes (Fisherbrand, USA), centrifuged at 2000 rpm for 5 minutes at room temperature, supernatant discarded and the pellet resuspended in PWM to 10% haematocrit (total of about 2.5ml). The Percoll® gradient (35%/65%) was set up by transferring 3ml of 65% Percoll® into a 15ml centrifuge tube and then with a Pasteur pipette, 3ml of the 35% Percoll® was gently transferred onto it along the wall of the tube. The resuspended parasite culture (2.5ml) was then slowly layered on the freshly prepared Percoll® gradient. This was then centrifuged in a swing-out rotor at 2500 rpm at room temperature for 15 minutes without a brake. After centrifugation, parasites were recovered from the 35-65% interface, transferred to a new 15ml centrifuge tube, washed twice with PWM and then once with CPM and put into culture at 6% haematocrit.

3.3 Preparation and Sensitivity Assays of Test Compounds

3.3.1 Preparation of Test Compounds

Flat-bottom 96-well microtitre plates (Thermo Scientific Nunc™, USA) were uniformly coated with different concentrations of fluridone [test compound (Sigma-Aldrich, Germany)] dissolved in dimethyl sulfoxide [DMSO (Sigma-Aldrich, Germany)] and artemisinin [as control drug (Sigma-Aldrich, USA)]. In the respective wells, two-fold serial dilutions of the test compounds were made in triplicates. The working concentrations used for the sensitivity assays were 500mM, 250mM, 125mM, 62.5mM, 31.25mM and 15.625mM for fluridone and 152nM, 76nM, 38nM, 19nM, 9.5nM and 4.75nM for artemisinin. The drug-coated plates were stored at -20°C until ready for use.

3.3.2 Inhibition Assays of Test Compounds against *P. falciparum* Asexual Stages

In vitro inhibition assays were performed for the asexual (rings, trophozoites and schizonts) stages of *P. falciparum* 3D7 strain at 1% parasitemia and 2% haematocrit. The plates were placed in an incubation chamber, gassed for 6 minutes with special gas mixture and incubated at 37°C for 48 hours. After the incubation period, 100µL of a 1:10,000 SYGR Green I (Sigma-Aldrich, USA) in lysis buffer (20nM Tris base, 5mM EDTA, 0.008% saponin, 0.08%Triton X-100 and pH 7.5) was added to the cultures in the wells and mixed thoroughly. The plates were then incubated in the dark at room temperature for an hour. Using a fluorescence plate reader (Tecan infinite 200pro, Japan) the fluorescence intensity of the cells in each well was measured after incubation at excitation and emission wavelengths of 485nm and 535nm respectively.

3.4 Extraction of Carotenoids from *P. falciparum* Asexual Stages

For each asexual stage, *P. falciparum* 3D7 parasites were cultured in three 75ml tissue culture flasks to initially obtain high ring stage population. These cultures were then treated with 5% D-sorbitol to synchronize for the ring stage parasites. The synchronized cultures were allowed to grow to obtain rings, trophozoites and schizonts at a parasitemia of 8-10%. The cultures for each particular stage were pooled and harvested by centrifuging at a speed of 2000 rpm for 5 minutes to obtain the cell pellet. The pellet was resuspended in 20ml of PBS and using a haemocytometer, the number of RBCs in the suspension was estimated. The number of infected RBCs was also estimated using the parasitemia and the RBC counted. The targeted number of parasites required for the extraction was 500 million parasites. The pooled cultures were centrifuged at 3500 rpm for 10 minutes at RT to obtain the cell pellets. The pellets were then resuspended in 10ml PBS (0.007M Na_2HPO_4, 0.01M Na_2HPO_4, pH 7.4 and 0.15M NaCl) containing 0.1% saponin. The resuspended pellet was washed three times with PBS and centrifuged at 6000 rpm for 10 minutes. When extraction was not carried out immediately, the pellets were stored at -80°C until use. Extraction of the carotenoids was carried out as follows; addition of 1ml of methanol, followed by the addition of 2ml of hexane to the parasite pellet. The suspension was then vortexed for 2 minutes. Subsequently, 1ml of water was added to the mixture and again vortexed for 1 minute. This was then centrifuged at 4,000 rpm for 20 minutes. The supernatant phase was collected and filtered through a 0.2µm nylon filter. The filtrate was then used directly for HPLC analysis.

3.5 HPLC Analysis of Carotenoids Extracted from *P. falciparum* Asexual Stages

The HPLC analysis was performed on a Shimadzu Prominence (Shimadzu Corp.) separation module equipped with a Shimadzu Prominence (CTO-10ASV, Shimadzu Corp.) column oven, SIL-20AC HT auto sampler (Shimadzu Corp.), Shimadzu Prominence DGU-20A3 online degasser (Shimadzu Corp.), Shimadzu Prominence LC-20AB solvent delivery system (Shimadzu Corp.) and a Shimadzu Prominence SPD-M20A UV-VIS and RF-10AXL fluorescence detector (Shimadzu Corp.) controlled by a UCI-1500 universal chromatography interface (Shimadzu Corp.). The analytical scale Acclaim^{TM} C30 reversed-phase column (250mm x 4.6mm internal diameter, 5µm particle size) used was operated at a temperature of 25°C. The mobile phase consisted of a non-linear gradient of acetone/water with 50:50 (*v/v*) as starting composition at a flow rate of 1 ml/min. The total run time was 45 minutes and the injection volume of the filtrate was 20µl. Detection of the eluents was done using a UV-VIS detector at a wavelength of 475nm. The retention times of six carotenoid standards (lycopene, α-carotene, β-carotene, abscisic acid, lutein and apo-carotenal) were determined individually under the above mentioned conditions and compared with those obtained from the filtrate to determine the identity of the peaks observed.

3.6 Bioinformatics Analysis of the *P. falciparum PSY*/OPPS

Nucleotide sequences of phytoene synthase (*PSY*) or octaprenyl pyrophosphate synthase (OPPS) from *P. falciparum* (PF3D7_0202700, PFB0130w) and other organisms were retrieved from the gene databases of National Center for Biotechnology Information (NCBI, www.ncbi.nlm.nih.gov/gene/), PlasmoDB (www.plasmodb.org/) and ToxoDB (www.toxodb.org/). The primary accession numbers or gene ID's of the *PSY* gene obtained for the analysis are listed in table A2 in the appendix. The gene sequence of *P. falciparum* OPPS/PSY was used as query for Basic Local Alignment Search Tool, nucleotide (BLASTn) (blast.ncbi.nlm.nih.gov) analysis for identification of homologues in other organisms. The best blast hits obtained included octaprenyl pyrophosphate synthase of *T. gondii* and *T. thermophilus*. Selection of the nucleotide sequences from the BLAST result was based on the fact that the carotenoid biosynthesis pathway has been extensively studied in these organisms. Multiple sequence alignment of the gene sequences retrieved was carried out using CLUSTAL W2 (http://www.ebi.ac.uk/Tools/msa/clustalw2/) and MEGA6 software used to construct the phylogenetic tree (Larkin *et al.*, 2007). The evolutionary history was inferred using the Neighbor-Joining method (Saitou & Nei, 1987).

CHAPTER FOUR

4.0 RESULTS

4.1 *In vitro* Inhibition Assays

In vitro inhibition assays were carried out on *P. falciparum* 3D7 strain using artemisinin and fluridone as control and test compounds, respectively. The inhibition assays were carried out on the intraerythrocytic asexual stages (rings, trophozoites and schizonts) of the parasite to determine the effect of the compounds on the parasites and also the half maximum inhibitory concentrations (IC_{50}) against each stage. After 48 hours of incubating the parasites with the compounds, they were again incubated with a SYBR Green I in lysis buffer solution for an hour. The absorbance from the respective wells in the microtitre plates were read immediately after this incubation period with a fluorometer. The absorbance obtained from the fluorometer readings and concentrations of the compounds were entered into Excel® spreadsheets and converted to percentage parasitemia and log concentrations respectively. These were then keyed into GraphPad Prism® software (version 5) and a non-linear regression performed to generate the dose-response curves and the IC_{50}'s of the compounds tested.

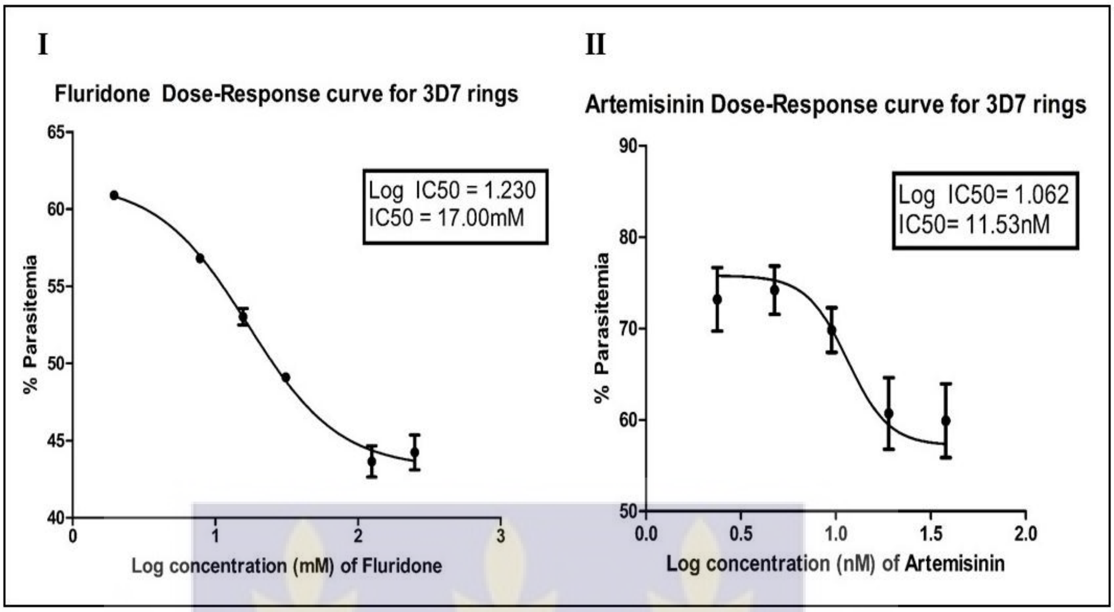


Figure 4.1: Dose-response curves for *P. falciparum* 3D7 ring stage parasites. Growth inhibitions by **(I)** fluridone and **(II)** artemisinin after incubating the parasites with different concentrations of these compounds for 48hrs. Error bars represent the standard error of mean (SEM) from triplicate readings of each concentration used.

A sigmoidal curve and a dose-dependent response are observed in all the results from the inhibition assays performed, as percentage parasitemia is plotted on the *y*-axis against the log of concentration of the test compounds on the *x*-axis. This was expected because high concentrations of the inhibitors, if effective against the parasite, will cause a greater level of inhibition of the targeted cellular process, leading to a decrease in parasite population (lower parasitemia), if that biological process is essential for parasite survival and vice versa. In figure 4.1, a gradual decrease in the ring stage parasite population was observed with the treatment of fluridone, resulting in an IC_{50} of 17.00mM.Treatment of the parasites with artemisinin (figure 4.1) on the other hand, initially showed minimal response of the parasites to the first two lower concentrations of the compound and then a gradual decline in parasite population as the concentration of artemisinin began to increase, resulting in an $IC_{50}$of 11.53nM against the ring stages. Comparing the concentrations at which fluridone and artemisinin achieved their half maximum inhibition, artemisinin (IC_{50} in nanomolar) proved to be a much more effective inhibitor than fluridone was, since its IC_{50} is significantly lower than that of fluridone (IC_{50} in millimolar). This observation was made in all the inhibition assays for the other asexual stages, where half maximum inhibition concentrations of artemisinin were achieved at significantly lower concentrations compared to fluridone against the same stages. The growth inhibition by fluridone ranged between 43%-62% whereas that by artemisinin was between 55%-75% (figure 4.1).

The results from the inhibition assays for both compounds on the trophozoite stages of the parasite (figure4.2) was comparable, where there was virtually no response to the effect of both fluridone and artemisinin at the initial lower concentrations. A fairly flat line was observed at the start of the graph for both compounds (figure 4.2). Then for fluridone (figure 4.2), a sharp increase in the slope of the graph resulting from a decrease in percentage parasitemia was observed as the concentration of fluridone increased. The graph then levelled off after treatment with the fourth highest concentration of fluridone. The percentage parasitemia for artemisinin also begins to level off again after treatment with the fifth highest concentration of the compound.

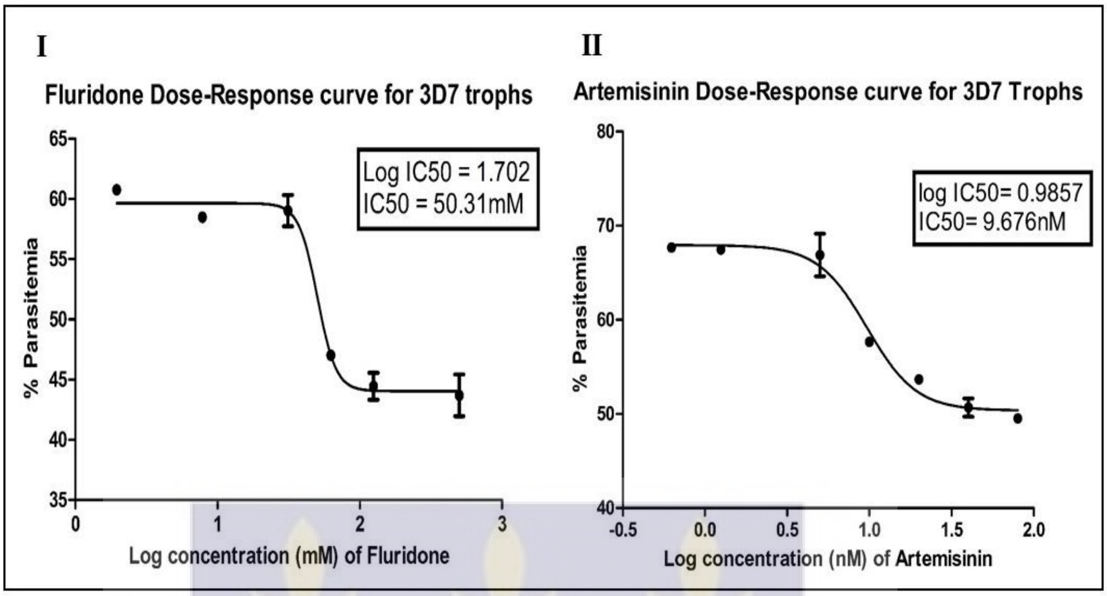


Figure 4.2: Dose-response curves for *P. falciparum* 3D7 trophozoite stage parasites. Growth inhibitions by **(I)** fluridone and **(II)** artemisinin after incubating the parasites with different concentrations of these compounds for 48hrs. Error bars represent the standard error of mean (SEM) from triplicate readings of each concentration used.

Thus, further increase in the concentrations of fluridone and artemisinin had no observable effect on the population of the trophozoite stages of the parasites. The half maximum inhibitory concentration for fluridone treatment was achieved at 50.31mM and that of artemisinin was found to be 9.67nM. The growth inhibition by fluridone ranged between 40%-60% whereas that by artemisinin was between 50%-70%.

The results of the inhibition assays against the schizont stages of the parasite are shown in figure 4.3. Both compounds tested showed a dose-dependent effect on this stage of the parasite life cycle. For fluridone treatment, the graph shows a dose-dependent effect on the population of the schizont stages of the parasite. There was a steep slope observed from the start of the graph. The half maximum inhibitory concentration for fluridone was achieved at 25.10mM and the growth inhibition by fluridone on the schizont stage ranged between 15% and 60%.

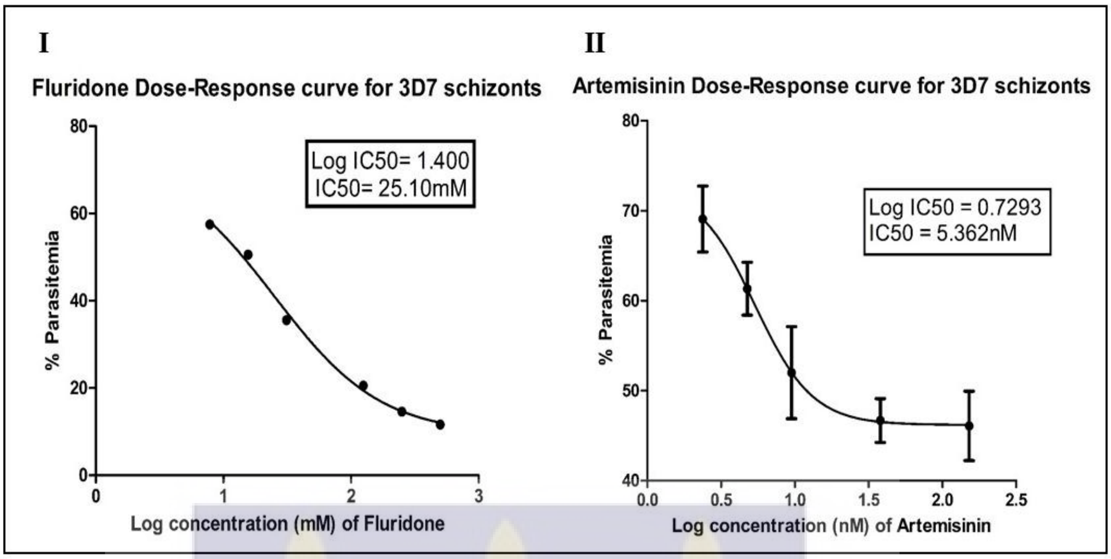


Figure 4.3: Dose-responses curve for *P. falciparum* 3D7 schizont stage parasites. Growth inhibitions by **(I)** fluridone and **(II)** artemisinin after incubating the parasites with different concentrations of these compounds. Error bars represent the standard error of mean (SEM) from triplicate readings of each concentration used.

In the case of artemisinin (figure 4.3), the effect of the compound is observed even with the least concentration, where there is an immediate decline in the parasite population and this continues till the fourth highest concentration when the graph begins to level off. The half maximum inhibitory concentration for artemisinin against the schizont stage was achieved at 5.362nm. This was the least IC_{50} obtained for the asexual stages with respect to both compounds in this study. The growth inhibition ranged between 45%-70%. For fluridone treatment, the ring stages were the most susceptible to this compound, recording an IC_{50} of 17.00mM, almost three-folds and one-and-a-half-folds of the IC_{50}'s recorded for the trophozoite (50.31mM) and schizont (25.10mM) stages respectively.

4.2 Carotenoid Extraction and HPLC Analyses

In the chromatograms (figures 4.4, 4.5, and 4.6) presented below, retention time is plotted on the *x*-axis and milli Absorbance Unit (mAU) is plotted on the *y*-axis. The chromatogram of the extract from the ring stage parasites (figure 4.4) had few detected or observed peaks. The peaks observed at the beginning of the chromatogram with retention times between 2-4 minutes are those of the organic solvents (methanol and hexane) used for the extraction. They elute fairly early at the start of the run. These peaks are also observed in the chromatograms for the trophozoite (figure 4.5) and schizont (figure 4.6) stage extractions. The lone peak that is observed further down the chromatogram from the ring stage extract (figure 4.4) with a retention time of 22.754 minutes is lutein. Identification of the peak was done by comparing its retention time to that of the standards used for the analysis. The quantity of lutein detected in the ring stage was found to be 0.0032mg/ml. This was estimated by using the area under curve for each peak generated by the HPLC machine and the specific equation of line obtained from the standard curves from the individual carotenoid standards (Appendix E). Similar results were obtained for duplicate experiments.

Figure 4.4: Chromatogram of carotenoids extracted from *P. falciparum* ring stage. Carotenoids were extracted from synchronised cultures using a ternary solvent system (methanol/hexane/water) and the organic phase fraction was collected and run on a C-30 column with a mobile phase of acetone/water for 40 minutes. Detection of the eluents was at 475nm.

The chromatogram for the trophozoite stage extraction (figure 4.5) showed a couple more peaks compared to the ring stages. Apart from the initial peaks with retention time between 2-4 minutes, three other peaks were observed in the region of the carotenoid retention times. The identity of the peak with a retention time of 17. 763 minutes, is unknown since this time does not correspond with any of the retention times of the standards used. The next peak was identified as α-carotene with a retention time of 19.573 minutes. The estimated quantity of α-carotene synthesized by the trophozoite stage parasites was 0.0031mg/ml.The last observable peak with a retention time of 30.303 minutes (figure 4.5) was identified to be β-carotene and the estimated quantity of this carotenoid was 0.0019mg/ml. The carotenoids synthesized by trophozoite stage parasites were different from what was synthesized by the ring stage parasites. Again similar results were obtained for duplicate experiments.

Figure 4.5: Chromatogram of carotenoid extracted from *P. falciparum* trophozoite stage.
Carotenoids were extracted from synchronised cultures using a ternary solvent system (methanol/hexane/water) and the organic phase fraction was collected and run on a C-30 column with a mobile phase of acetone/water for 40 minutes. Detection of the eluents was at 475nm.

The chromatogram for the schizont stage extract (figure 4.6) showed several peaks. Some of these peaks had retention times that corresponded to those of the standards and others did not and therefore were not identified. Here again, the peaks observed at the retention time between 3-5 minutes are those of the solvents used for the extraction. The peaks with retention times corresponding to that of a standard used were 19.550 minutes (α-carotene), 23.567 minutes (apo-carotenal), 24.114 minutes (abscisic acid), 30.086 minutes (lycopene) and 30.348 minutes (β-carotene). The rest of the peaks observed did not have retention times corresponding to any of the standards and therefore their identity could not be verified. The estimated quantities of the identified carotenoids in the schizont stage were found to be 0.0824mg/ml (α-carotene), 0.0032mg/ml (apo-carotenal), 0.664mg/ml (abscisic acid), 0.8321mg/ml (lycopene) and 0.0022mg/ml (β-carotene). Similar results were obtained for duplicate experiments. The highest peak observed in figure 4.6 below was identified to be that of abscisic acid. This indicates high levels or quantities of this phytohormone being synthesized and was observed only at the schizont stage of the parasite life cycle. Clearly, the kind and quantity of carotenoid synthesized differed as the parasites progressed from the young stages to the mature stages.

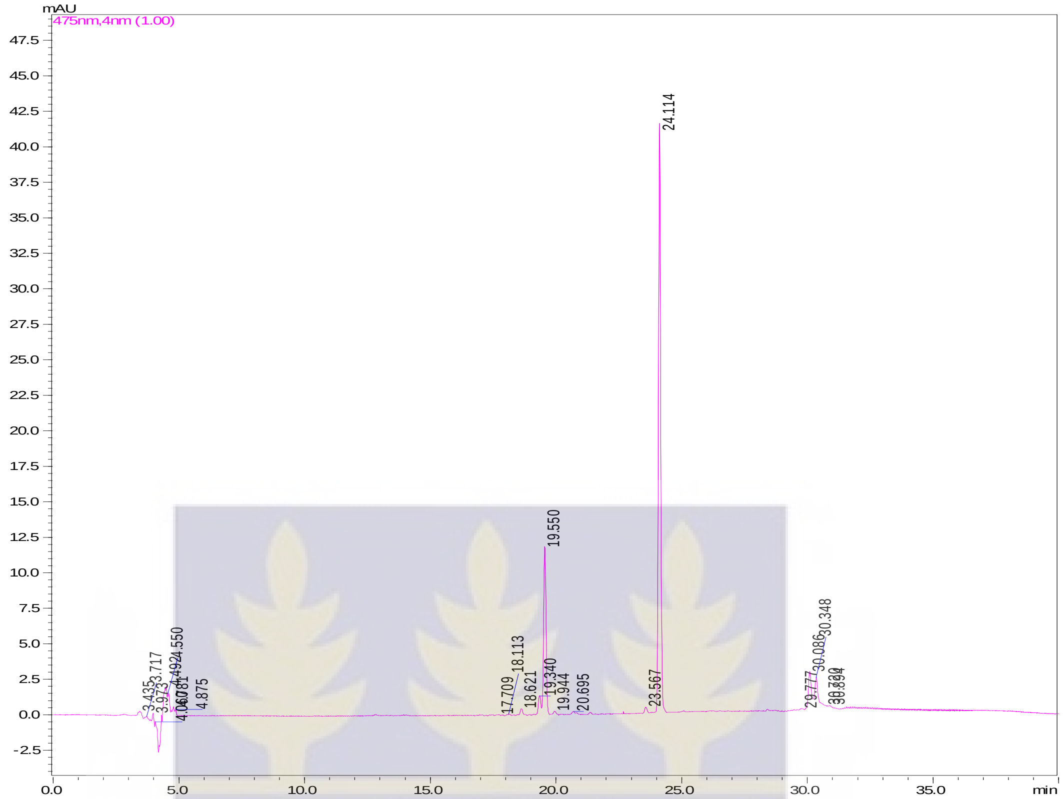
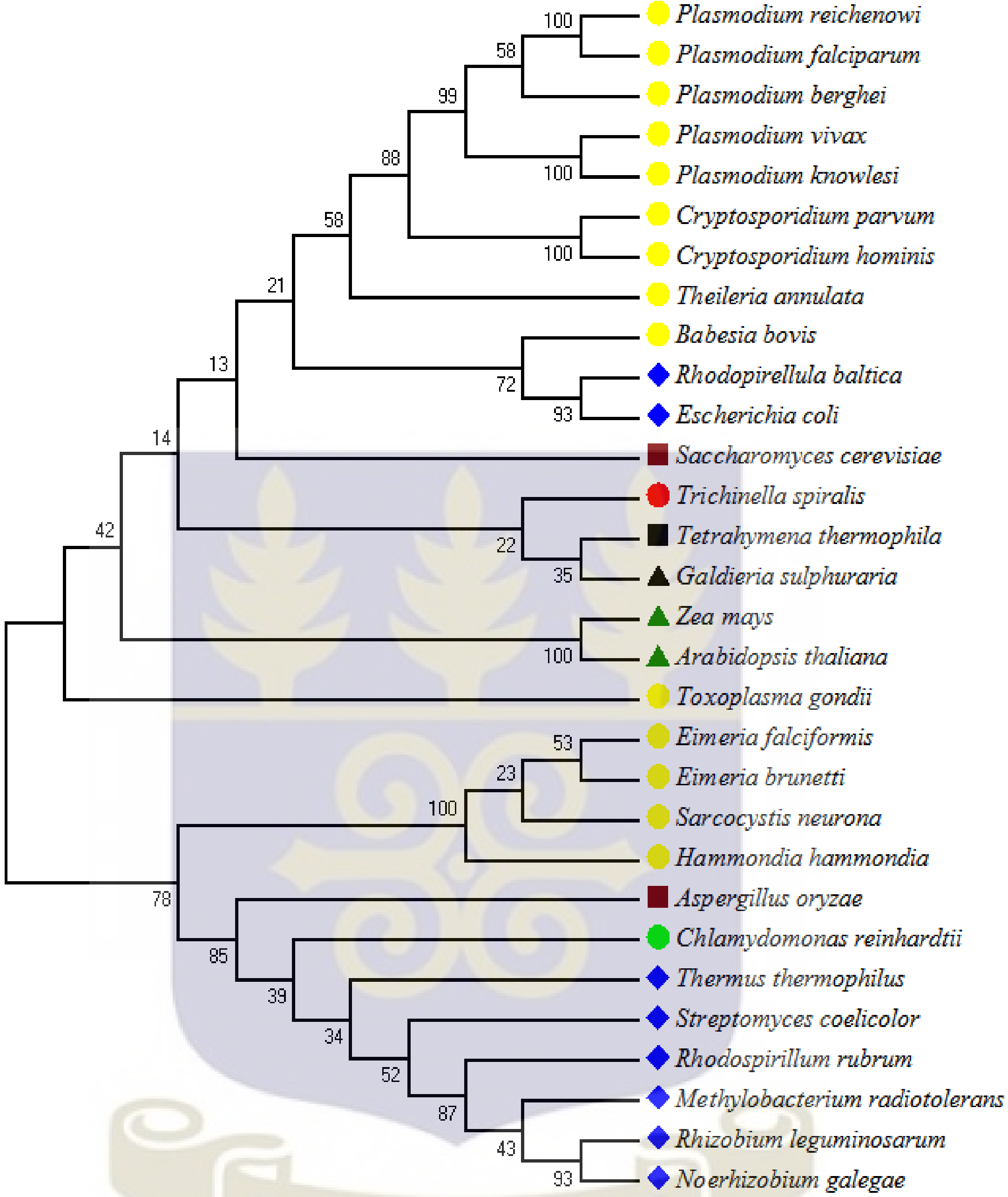


Figure 4.6: Chromatogram of carotenoid extracted from *P. falciparum*schizont stage. Carotenoids were extracted from synchronised cultures using a ternary solvent system (methanol/hexane/water) and the organic phase fraction was collected and run on a C-30 column with a mobile phase of acetone/water for 40 minutes. Detection of the eluents was at 475nm.

4.3 Evolutionary History and Phylogenetic Analysis

Phylogenetic relationship between *P. falciparum PSY* gene and homologues in other species is analysed (figure 4.7). *P. falciparum PSY*/OPPS gene sequence was used as query for BLASTn analysis to find homologues of the gene in other organisms. The choice of selection of the *PSY*/OPPS sequences from BLAST results for the phylogeny analysis was based on the fact that the carotenoid biosynthesis pathway has been extensively studied in these organisms. In all, thirty *PSY*/OPPS gene sequences from different taxonomic groups were chosen, including *P. falciparum PSY*. The organisms selected for the analysis included apicomplexans, fungi, plants and bacteria. A multiple sequence alignment was done by aligning all the selected gene sequences using CLUSTAL W2 and an unrooted phylogenetic tree was generated from the alignment by the Neighbor-Joining Method using MEGA6. The evolutionary distance is indicated by the numbers along the branches and were computed using the Maximum Composite Likelihood method and are in the units of number of base substitutions per site.

Two major clades with several subclades coming off of each major clade are observed on the unrooted tree (figure 4.7). The *Plasmodium*, *Cryptosporidium*, *Theileria*, *Babesia* and *Toxoplasma* species were located on one of the major clades and the *Eimeria* species and the other apicomplexans found on the other major clade. Thus, the *PSY* gene in the apicomplexans (yellow circle) bifurcated earlier during evolution of this group. It is also observed that all the *Plasmodium PSY* gene form a single subclade with a bootstrap value of 99, indicating a higher level of relatedness of the gene among this group and likelihood that this species shared a common ancestor at some point in their evolution. Surprisingly, *P falciparum* formed a subclade with the chimpanzee parasite, *P. reichenowi*, with a bootstrap value of 99, indicating that the *P. falciparum PSY* gene is most closely related to *P. reichenowi* among all the sequences used for this analysis.



● Apicomplexans
▲ Red Algae
▲ Plants
● Green Algae
● Nematode
◆ Bacteria
■ Fungi
■ Alveolate

Figure 4.7: Phylogenetic relationship of *PSY*/OPPS gene in *P. falciparum* and other species.
The evolutionary history was inferred using the Neighbor-Joining method. The optimal tree with the sum of branch length = 15.24929587 is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (100 replicates) are shown next to the branches. The analysis involved 30 nucleotide sequences. All ambiguous positions were removed for each sequence pair. There were a total of 5432 positions in the final dataset. Evolutionary analyses were conducted in MEGA6.

CHAPTER FIVE

5.0 DISCUSSION, CONCLUSION AND RECOMMENDATION

5.1 *In vitro* Inhibition Assay

Efforts have been made over the past decade in several areas including vector control, vaccine and drug development programs in the bid to eradicate malaria from the affected regions of the world (malERA Consultative Group on Vaccines, 2011; Raghavendra *et al.*, 2011; Rieckmann, 2006; Schwartz *et al.*, 2012). The genetic versatility of *P. falciparum* makes it possible for the parasite to evade the host immune system (Jeffares *et al.*, 2007; Wright & Rayner, 2014) and also become resistant to some of the current drugs used in the treatment and management of malaria (Djimdé, *et al.*, 2001; Dondorp *et al.*, 2010; Schellenberg *et al.*, 2006). In order to win the fight against the disease, more effort and resources have to be channelled into vector control, vaccine and drug development. Currently, the search for an effective vaccine candidate to help eradicate the disease is far advanced (Casares *et al.*, 2010; Crompton *et al.*, 2010; Malik *et al.*, 2012). Novel compounds and their effect on new or existing targets in the metabolic pathways of *P. falciparum* are also being investigated (Crowther *et al.*, 2011; Qidwai & Khan, 2012). The apicoplast organelle is an exciting new target discovered in apicomplexans (Kalanon & McFadden, 2010; Maréchal & Cesbron-Delauw, 2001). It houses metabolic pathways such as fatty acid synthesis and carotenoid biosynthesis (Seeber & Soldati-Favre, 2010).

The apicoplast has been the focus of many studies recently, partly because this organelle is unique to the parasite and also opens a new window into understanding the metabolism of the parasite (Goodman & McFadden, 2013; Ralph *et al.*, 2001). An interesting metabolic pathway that occurs in the apicoplast and has received much attention from the scientific community is the carotenoid biosynthesis pathway. This pathway is present in the parasite but absent in the human host (Tonhosolo *et al.*, 2009). A known inhibitor of this pathway in plants is fluridone which targets phytoene desaturase (McCowen *et al.*, 1979). In previous studies, the effect of other inhibitors of carotenoid biosynthesis such as norflurazon and fosmidomycin, on *Plasmodium* and *Toxoplasma* have been determined and found to be effective at inhibiting the growth of parasites in a dose-dependent manner (Tonhosolo *et al.*, 2009; Umeda *et al.*, 2011; Yeh & DeRisi, 2011). The effect of fluridone on *Toxoplasma* has also been studied and results indicated that inhibition caused a delay in egress and induced development of the slow-growing, dormant cyst stage of the parasite (Nagamune *et al.*, 2008), however, the effect of fluridone on *Plasmodium* has not been investigated. This study therefore aimed at studying the effect of fluridone on the carotenoid biosynthesis pathway in *P. falciparum*.

In this study, artemisinin, whose inhibitory capability on *P. falciparum* is known (Klonis *et al.*, 2011), was used as a control drug to serve as a basis for comparison with fluridone, whose inhibitory capability in *P. falciparum* is unknown. Results from the inhibition assays (figures 4.1 to 4.3) performed against the asexual stages revealed that fluridone was not very effective at reducing the population of the parasite as compared to artemisinin, considering the concentrations at which each compound achieved its half maximum inhibitory concentration against each particular stage. This may be due to the inability of fluridone to effectively cross the membrane barriers surrounding the intraerythrocytic parasite, thus, the lipid bilayer of the RBC and parasite and also the parasitophorous vacuole surrounding the parasite, therefore preventing the compound from reaching its intended target. Amongst the three asexual stages the parasite life cycle, the ring stage was the most susceptible to the treatment of fluridone because it was at this stage that the lowest IC_{50} value (17.00mM) was recorded (figure 4.1).

In order for an inhibitor to reach its target, it should be able to traverse any barrier that is between it and its intended target in sufficient amounts to disrupt or inhibit the activity of the target such as enzymes and ligands (Clay & Sharom, 2013). The plasma membrane is selectively permeable as it allows only certain substances to pass through it but not others (Vaara, 1992). The physical and chemical properties of the inhibitor can either aid or prevent it from traversing the phospholipid bilayer. Small, non-polar (hydrophobic) substances such as water, oxygen and carbon dioxide are able to squeeze through the gaps in the membrane by simple diffusion or osmosis (Haines, 1994). Larger, charged molecules including glucose and amino acids on the other hand are transported across the membrane through channels or pores either by active or passive diffusion (Stillwell, 2013). Fluridone is a fluorine-containing compound (figure 2.4) that belongs to the nitrogen-containing heterocyclic class of compounds. This is a relatively large molecule and might require transport across the membrane either by active or passive transport through pores or channels. This could restrict the movement of fluridone across the membranes (parasitophorous vacuole and the membranes of the RBC and parasite) and therefore accounting for the high IC_{50} values observed for fluridone treatment of the three intraerythrocytic stages of *P. falciparum*. Another reason that could account for the high IC_{50} values observed for fluridone treatments is the difference in the targets or mode of actions of artemisinin and fluridone. Artemisinin has been shown to inhibit haemoglobin (the major protein source for the parasite in the infected RBC) uptake in the intraerythrocytic stages of *P. falciparum* (Klonis *et al.*, 2011), while fluridone inhibits the activity of phytoene desaturase, the enzyme that catalysis the conversion of phytoene to lycopene in the carotenoid biosynthesis pathway (Tonhosolo *et al.*, 2009). Lycopene present in the host blood circulation (which is obtained from our diets) could be salvaged by the parasites when phytoene desaturase is inhibited by fluridone, however, the proteins obtained from the digestion of haemoglobin may not be salvaged.

5.2 Carotenoid Profiling and HPLC Analyses

Since the discovery of the apicoplast in 1996 (Mcfadden *et al.*, 1996) and the association of certain pathways including the isoprenoid biosynthesis pathway with this organelle in apicomplexans (Tonkin *et al.*, 2008), it has been the interest of scientists to determine whether this organelle actually serves a function in this group of parasites and could be targeted for chemotherapeutic management of diseases caused by this group or is just a remnant of their evolutionary past. The carotenoid biosynthesis pathway has been linked to the isoprenoid precursor biosynthesis pathway in apicomplexan (Eisenreich *et al.*, 2004). This link is made when phytoene synthase catalyses the condensation of two molecules of GGPP to form phytoene, the first committed step of the carotenoid biosynthesis pathway. The synthesis of carotenoids and inhibition of this pathway subsequently leading to development and growth inhibitions in apicomplexans (*Toxoplasma*) was first demonstrated by Nagamune *et al.*, 2008. Carotenoid biosynthesis in the intraerythrocytic stages of *P. falciparum* has also been demonstrated by Tonhosolo and his colleagues (2009). However, only a few carotenoids includingβ-carotene, phytoene and lutein were reported to be synthesized by *P. falciparum*, painting an incomplete picture of the carotenoid synthesis landscape in *P. falciparum*.

The types and quantities of carotenoids synthesized in the three asexual stages of *P. falciparum* were therefore investigated in this study. The carotenoids were extracted from synchronised *P. falciparum* cultures at ring, trophozoite and schizont stages with a ternary solvent system that included methanol, hexane and water. Since carotenoids are soluble in organic solvents, they partition into the organic (methanol and hexane) phase of the extraction solvent which was subsequently used for a reverse-phase HPLC analyses. The non-linear gradient used, i.e., the gradual increase in the volume of acetone to water in the mobile phase, was to gradually decrease the polarity of the mobile phase. This was to enable the non-polar compounds (e.g. carotenoids and any other compound that might have been extracted) to elute later during the run. The C-30 column is a preferred column for the effective separation of carotenoids, since these compounds are similar in structure and could all elute together. Compared to a C-18 column, the C-30 column provides a greater number of interaction sites for the carotenoids to interact with the column, ensuring complete partitioning of positional isomers, as it occurs in carotenoids. The C-30 column therefore prolongs the retention time by increasing the interaction time of the eluents with the packing elements of the column, thereby greatly enhancing the selectivity of the carotenoid isomers (Breitenbach *et al*., 2001; Emenhiser *et al*., 1995).

The chromatogram for the ring stage extract (figure 4.4) showed only one detectable peak with retention time corresponding to lutein, one of the standards used. This may be due to very low levels of carotenoids synthesized at this stage, such that they may be below detection limits. This suggests that the roles played by carotenoids in the early stages of the parasite life cycle may be discretionary. One of the major roles played by carotenoids in the organisms in which they are found is to protect against oxidative damage by quenching photosensitizers, interacting with singlet oxygen molecules and scavenging peroxy radicals, thereby preventing the accumulation of harmful oxygen species generated during active metabolism of the organism (Shimidzu *et al*., 1996; Stahl & Sies, 2003). Probably at this stage in the parasite life cycle, there are not many of these free radicals present that might lead to activation and robust synthesis of these carotenoids in sufficient quantities, therefore resulting in low levels of the carotenoids at the ring stage.

In the chromatogram for the trophozoite stage extract (figure 4.5), three peaks were observed compared to the single peak observed for the ring stage (figure 4.4). Two of the peaks had retention times corresponding to standards used. These peaks were identified as α-carotene (19.573 minutes) and β-carotene (30.303 minutes). The third peak with a retention time of 17.763 minutes is unknown because its retention time did not correspond with any of the standards used in this study. However, it is suspected to be a carotenoid since its retention time falls within the range for carotenoids in this study. The schizont stage (figure 4.6) synthesized other several carotenoids compared to the ring (figure 4.4) and trophozoite (figure 4.5) stages. It was observed that the schizont stage contained the highest levels of carotenoids when compared with the ring and trophozoite stages. This confirms the study by Tonhosolo *et al.* (2009), which indicated that carotenoid synthesis actually starts from the ring stages and accumulates in the schizont stage. Tonhosolo *et al.* (2009), detected carotenoids such as lutein and β-carotene in their study, which were also observed in this study. This study also provides, for the first time, evidence of other carotenoids such as α-carotene, apo-carotenal, lycopene and abscisic acid being profiled in *P. falciparum.*

A very exciting and interesting observation that was also made was the presence and quantity of abscisic acid (with retention time 24.114 minutes) synthesized in the schizont stage. Abscisic acid (ABA) is a phytohormone that regulates a number of important events in plants, including environmental stress responses, embryo development and seed dormancy (Nakashima & Yamaguchi-

Shinozaki, 2013; Nambara *et al.*, 2010). ABA is known to be synthesized in *T. gondii* (Nagamune *et al.*, 2008), a related apicomplexan, and a rise in abscisic acid levels signalled a calcium-dependent egress of the parasites from the host cells, whereas the inhibition of abscisic acid synthesis resulted in the formation of cysts, a dormant form of the parasite (Nagamune *et al.*, 2008). To further confirm the role of ABA in *Toxoplasma*, it was demonstrated that addition of exogenous abscisic acid induced formation of cyclic adenosine diphosphate ribose (cADPR, a second-messenger) in *T. gondii*, stimulated calcium-dependent protein secretion and induced parasite egress from the infected host cell in a density-dependent manner (Nagamune *et al.*, 2008). Also, selective disruption of ABA synthesis by the inhibitor fluridone delayed egress and induced development of the slow-growing, dormant cyst stage of the *Toxoplasma* parasite. Thus, ABA-mediated calcium signalling controlled the decision between lytic and chronic stage growth, a developmental switch that is central in pathogenesis and transmission in *Toxoplasma*, a closely related apicomplexan to *Plasmodium* (Nagamune *et al.*, 2008).

ABA was not detected in the ring and trophozoite stages probably due to the absence or very low levels of this phytohormone synthesized at these stages, such that they were below detection limits. But interestingly, there was a sudden spike in ABA levels that was detected only in the schizont stage. This may be due to synthesis from the numerous individual merozoites that are still trapped in the parasitophorous vacuole. The rise in abscisic acid levels in only the schizont stage is an exciting finding because this is the first time the phytohormone has been associated with *P. falciparum* and also because it could be serving the same important function of mediating parasite egress in this parasite as it does in *T. gondii*. To date, such a signal for parasite egress from infected RBCs in the schizont stage, during which merozoites are released to infect new RBCs, is unknown. It is not known for sure whether ABA regulates or is associated with merozoite egress in *P. falciparum* or not. However, it is possible to hypothesize that ABA might be playing a similar role in *P. falciparum* as it does in *T. gondii*, where it mediates egress of the parasite from its host cell, considering the fact that *P. falciparum* and *T. gondii* belong to the same Phylum Apicomplexa and share similar features such as the presence of the parasitophorous vacuole and the use of similar machinery for invading their host cells (Kemp *et al.*, 2013).

5.3 Bioinformatics and Phylogenetic Analysis of the Phytoene Synthase Gene

Of the five known *Plasmodium* species that infect humans, *P. falciparum* causes the greatest morbidity and mortality, with millions of clinical cases and more than one million deaths recorded annually (Greenwood *et al.*, 2005; Hay *et al.*, 2004). Although much progress has been made towards the management and control of *P. falciparum* (Kappe *et al.*, 2010), establishing the evolutionary origin and relatedness of this parasite and other related *Plasmodium* pathogens remain controversial and has been of much interest in recent years. Phylogenetic studies of the malaria pathogen can be effective in helping to develop new drugs and vaccines for the disease as well as to understand host pathogen interactions and evolution of drug resistance in the pathogen (Datta & Chauhan, 2010). It has been demonstrated recently that the closest known relative of *P. falciparum* is the chimpanzee parasite, *P. reichenowi*, which was assumed to have diverged from *P. falciparum*, its human counterpart, at the same time as the ancestors of chimpanzees and humans, more than 5 million years ago (Escalante & Ayala, 1994; Jeffares *et al.*, 2007; Rich *et al.*, 1998). Over the past few years, other studies have detected other closely related *Plasmodium* strains in chimpanzees, western gorillas and bonobos, raising the possibility that *P. falciparum* in humans could have arisen as a result of cross-species transmission from one or more of these apes (Krief *et al.*, 2010; Prugnolle *et al.*, 2010; Rich *et al.*, 2009). The presence of a plastid in *Plasmodium* species and other apicomplexan parasites indicates the affinity of this group for cyanobacteria and green algae. Since the apicoplast is derived from secondary endosymbiosis of an algal ancestor, *Plasmodium* species are therefore thought to be of prokaryotic origin (McFadden, 2011). Though the apicoplast lost its photosynthetic function in its new host, it retained some prokaryotic vestiges and is therefore thought that the apicomplexan group of organisms should be related to algae and plants (Arisue & Hashimoto, 2014; Foth & McFadden, 2003).

Several studies have investigated the isoprenoid and carotenoid biosynthesis in plants, algae and their endosymbiont apicomplexans (Botella-Pavía *et al.*, 2004; Cunningham *et al.* , 2007; Janouškovec *et al.*, 2015). The apicomplexans have a different pathway for the synthesis of isoprenoids and carotenoids (Goodman & McFadden, 2013). Though different at many steps, the enzyme phytoene synthase (PSY), catalysing the first committed step of isoprenoid to carotenoid biosynthesis pathway is also present in apicomplexans parasites including *P. falciparum* (Tonhosolo *et al.*, 2009). To date, no genetic evidence has been obtained for the enzymes presumed to be involved in the carotenoid biosynthesis pathway, except for the *PSY* gene coding for the PSY enzyme that has been implicated through functional assay (Rodríguez-Villalón *et al.*, 2009). The structure and evolutionary relationship of the *PSY* gene in plants and other organisms has been investigated (Giorio *et al.*, 2008; Li *et al.*, 2009; López-Emparán *et al.*, 2014). This has also been recently studied in *P. falciparum* (Agarwal *et al.*, 2015). However, not much is known about the evolutionary relationship of the *PSY* gene in apicomplexans and other organisms.

The evolutionary history and relationship of the phytoene synthase (*PSY*) gene in *P. falciparum* and other organisms was investigated in this study. The BLAST search revealed that there were *PSY* orthologues present in all the *Plasmodium* species whose genome sequence were available. Other apicomplexans used in this phylogenetic analysis such as *Toxoplasma*, *Eimeria*, *Cryptosporidium*, *Babesia*, etc. also presented orthologues of this gene. However, all these orthologues in the apicomplexans were not closely related since the apicomplexan species (represented by the yellow circles) bifurcate into two different clades on the tree (figure 4.7). Thus, *Plasmodium*, *Cryptosporidium*, *Theileria*, *Toxoplasma* and *Bebasia* species formed a large monophyletic group with other species including plants (green triangle) and algae (black triangle) and the other remaining apicomplexans (*Sarcocystis*, *Eimeria* and *Hammondia* species) also clustered together to form the other major clade. This finding therefore suggests a major and significant evolutionary event that caused a divergence in this gene but most importantly the *PSY* was still maintained by the apicomplexans clearly suggesting that the *PSY* and carotenoid synthesis is essential to the apicomplexan parasites.

The apicomplexan species (yellow circle) being part of a large monophyletic group that includes plants (green triangle) and algae (black triangle) as shown in figure 4.10 seem to support the theory that the apicoplast was obtained from photosynthetic ancestry (McFadden, 2011). It was also observed that all the *Plasmodium* species used for this analysis formed a subclade with a bootstrap value of 99 on a branch of the tree, indicating a high level of relatedness in the *PSY* sequences in this species. Taxa forming a clade indicate that there is high level of relatedness amongst these taxa or species as compared to members of other clades. Species occupying the same clade on a branch of the tree with a bootstrap value of 70 or greater, indicates that there is a greater level of support for the existence or formation of that node and the organisms that make up the node are likely to have shared the same ancestor at some point in time in their evolution (Soltis & Soltis, 2003). Interestingly, *P. falciparum* and *P. reichenowi* (the chimpanzee strain of *Plasmodium*) occupied the same clade instead of the other human strains used in this study, with a bootstrap value of 100. This data supports the argument that *P. reichenowi* is the closest known relative of *P. falciparum* (Escalante & Ayala, 1994; Jeffares *et al.*, 2007).

5.3 Conclusion

The inhibition assays revealed that fluridone had a dose-dependent effect on all three asexual stages of *P. falciparum*, with the ring stages being the most susceptible to the treatment of fluridone. However, fluridone, a known plant carotenoid biosynthesis inhibitor, was not as effective at inhibiting parasite growth as compared to artemisinin. This study also revealed that carotenoid synthesis in the asexual stages is cumulative, starting from the ring stage and accumulating in the schizont stage, with high levels of abscisic acid being synthesized at this stage. The bioinformatics and phylogenetic analysis showed *PSY* orthologues to be present in all the apicomplexan species used. It also showed a high level of relatedness amongst the *PSY* gene in all the *Plasmodium* species used in this analysis. The *P. falciparum PSY* is most related to that of the chimpanzee malaria parasite, *P. reichenowi*, supporting the argument that *P. falciparum* is of chimpanzee origin. The tree also supports the theory that the apicoplast has a photosynthetic ancestry since the apicomplexans formed a monophyletic group with the plants and algae used in this analysis. The data presented above is very significant because it sheds more light on the carotenoid biosynthesis pathway in *P. falciparum* and apicomplexans as a whole, in terms of the kinds and quantities of carotenoids synthesized, what compounds have effect on the pathway and also strongly supporting the fact that the pathway can be targeted for the control and possibly eradication of the diseases caused by this group of organisms, especially malaria.

5.4 Recommendations

It is recommended that the inhibition assays and the carotenoid profiling be done with more sensitive and specific assays/methods and equipment. For example, ELISA-based inhibition assays could be used to ascertain the IC_{50} of fluridone for the inhibition assays. Metabolic labelling assays and liquid chromatography in tandem with mass spectrometry (LC/MS) or Nuclear Magnetic Resonance (NMR) are very sensitive and advanced techniques that could be employed to accurately quantify and profile the carotenoids synthesized by not only the asexual stages but also the sexual stages of the *P. falciparum*. Carotenoid profiles of fluridone-treated cultures could be done to determine its effect on the levels of carotenoids that are synthesized when the pathway is inhibited. Further studies could be also be carried out using new compounds or modify known compounds that have effect on the carotenoid biosynthesis pathway.

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APPENDIX

A. Reagents

Table A1: List of reagents and companies from which they were obtained

REAGENT	SOURCE
RPMI 1640 (with L-glutamine and HEPES)	Gibco
D-Sorbitol	Sigma-Aldrich
Sodium bicarbonate ($NaHCO_3$)	Sigma-Aldrich
Gentamycin (50mg/ml)	Gibco
Albumax II	Invitrogen
Percoll®	Sigma-Aldrich
Normal Human serum (NHS)	Sigma-Aldrich
Hypoxanthine	Sigma
Fluridone	Sigma
SYBR Green I	Invitrogen
Hexane (HPLC grade)	Sigma
Acetone (HPLC grade)	Sigma-Aldrich
Methanol (HPLC grade)	Sigma-Aldrich
Water (HPLC grade)	Sigma-Aldrich
Phosphate Buffered Saline (0.01M, pH=7.4)	Sigma

B. Materials

Centrifuge tube (15ml and 50ml)

Culture gas

Tissue culture flask (25 ml and 75 ml)

Serological pipette (5ml and 10ml)

Column (HPLC)

96-well microtitre plate

Nitrile gloves

Sterile hypodermic needles

70% ethanol

Filters (0.8μm and 0.22μm)

C. Preparation of Solutions

i. Albumax

1L RPMI 1640

50g Albumax II

0.2g Hypoxanthine

ii. Parasite Wash Medium (PWM)

500ml RPMI 1640 (with L-glutamine and HEPES)

5ml of 20% glucose (in distilled water) solution

500µl Gentamycin (50mg/ml)

13ml of 7.5% $NaHCO_3$

iii. Complete Parasite Medium (CPM)

500ml RPMI 1640 (with L-glutamine and HEPES)

5ml of 20% glucose (in distilled water) solution

500µl of Gentamycin (50mg/ml)

13ml of 7.5% $NaHCO_3$

50ml Albumax

10ml NHS (for culturing clinical isolates)

iv. SYBR Green I/Lysis buffer for Fluorometry

1L distilled water

2.423g Tris base

Adjust pH to 7.5 using conc. HCl

10ml of 0.5M EDTA

80mg saponin

0.8ml Triton X-100

Filter the solution and store at room temperature

v. Lysis buffer for extraction of carotenoids

Phosphate Buffered Saline (0.01M) powder

Saponin (0.1% of PBS volume)

D. List of Organisms used for the Phylogenetic Analysis

Table A2: Table showing the gene ID of PSY/OPPS in the various organisms used for the phylogenetic relationship study

Organism	Gene ID	Database
Plasmodium falciparum 3D7	PF3D7_0202700, PFB0130w	PlasmoDB, NCBI
Plasmodium vivax	PVX_003575	PlasmoDB
Plasmodium berghei ANKA	PBANKA_0300800	PlasmoDB
Plasmodium reichenowi	gi|832044551	PlasmoDB
Plasmodium knowlesi strain H	PKNH_0418400	PlasmoDB
Cryptosporidium hominis TU502	cgd5_4532	ToxoDB
Cryptosporidium parvum Iowa II	cgd7_3730	ToxoDB
Eimeria brunette	EBH_0010260	ToxoDB
Eimeria falciformis	EfaB_MINUS_800.g99_1	ToxoDB
Hammondia hammondia strain H.H.34	HHA_224490	ToxoDB
Theileria annulata	TA03505	ToxoDB
Toxoplasma gondii	TGME49_269430	ToxoDB
Sarcocystis neurona	SN3_00400385	ToxoDB
Arabidopsis thaliana	gi|240256493	NCBI
Aspergillus oryzae	gi|169781061	NCBI
Babesia bovis T2Bo	gi|156087461	NCBI
Chlamydomonas reinhardtii	gi|159486413	NCBI
Escherichia coli	gi|387615344	NCBI
Galdieria sulphuria	gi|545700663	NCBI
Methylobacterium radiotolerans	gi|170746450	NCBI
Neorhizobium galegae	gi|752716557	NCBI
Rhizobium leguminosarum	gi|752843554	NCBI
Rhodopirellula baltica	gi|32470666	NCBI
Rhodospirillum rubrum	gi|83591340	NCBI
Saccharomyces cerevisiae	gi|330443520	NCBI
Streptomyces coelicolor	gi|32141095	NCBI
Tetrahymena thermophila	gi|229594551	NCBI
Thermus thermophilus	gi|593268528	NCBI
Trichinella spiralis	gi|331705224	NCBI
Zea mays	gi|662248888	NCBI

E. Standard Curves of the Carotenoid Standards used for HPLC

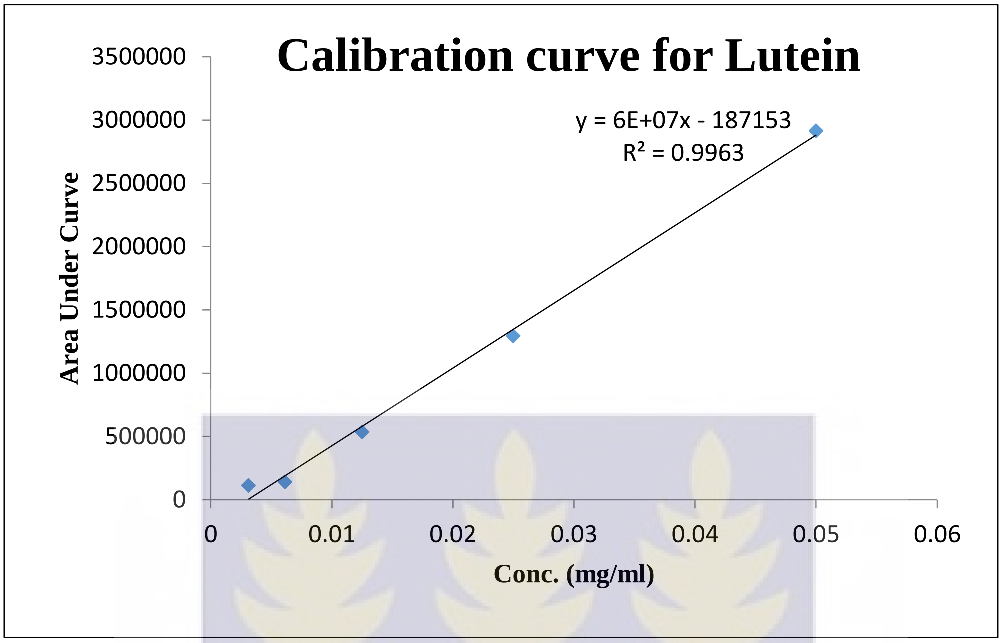

Figure A1: Standard curve for carotenoid standard, lutein.

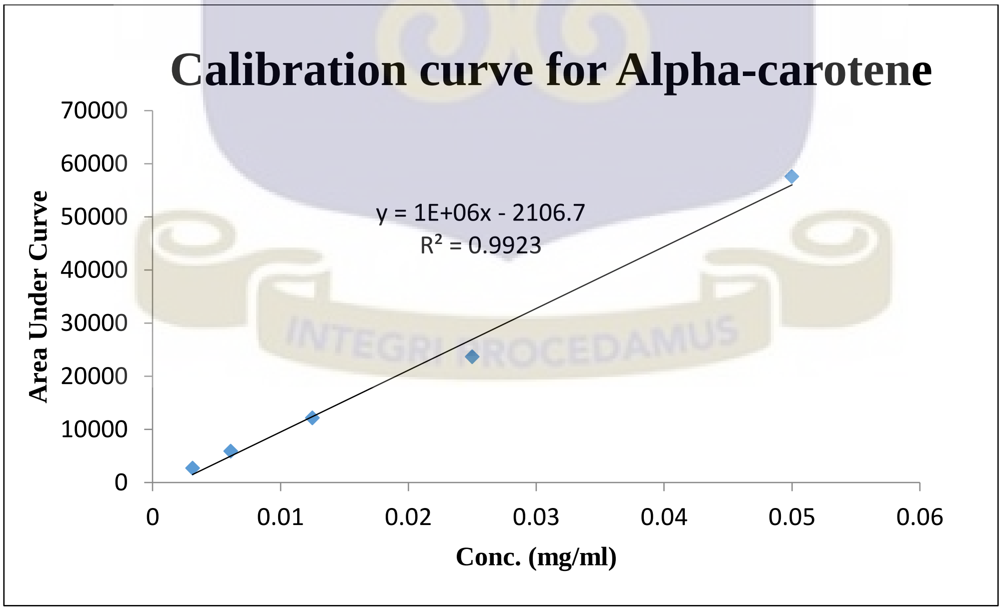

Figure A2: Standard curve for carotenoid standard, α-carotene.

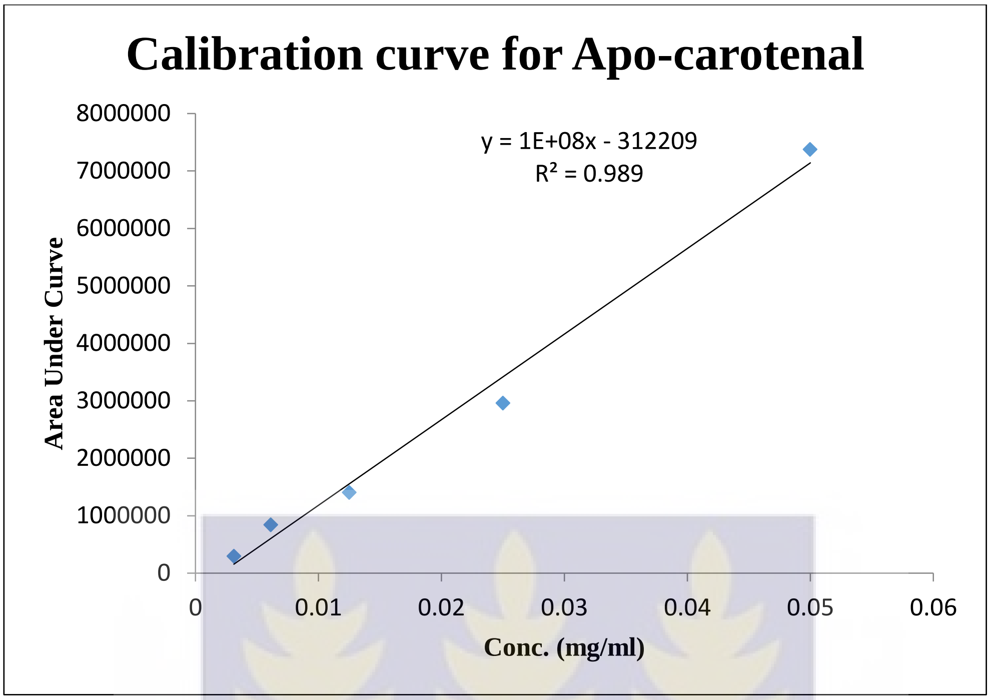

Figure A3: Standard curve for carotenoid standard, apo-carotenal.

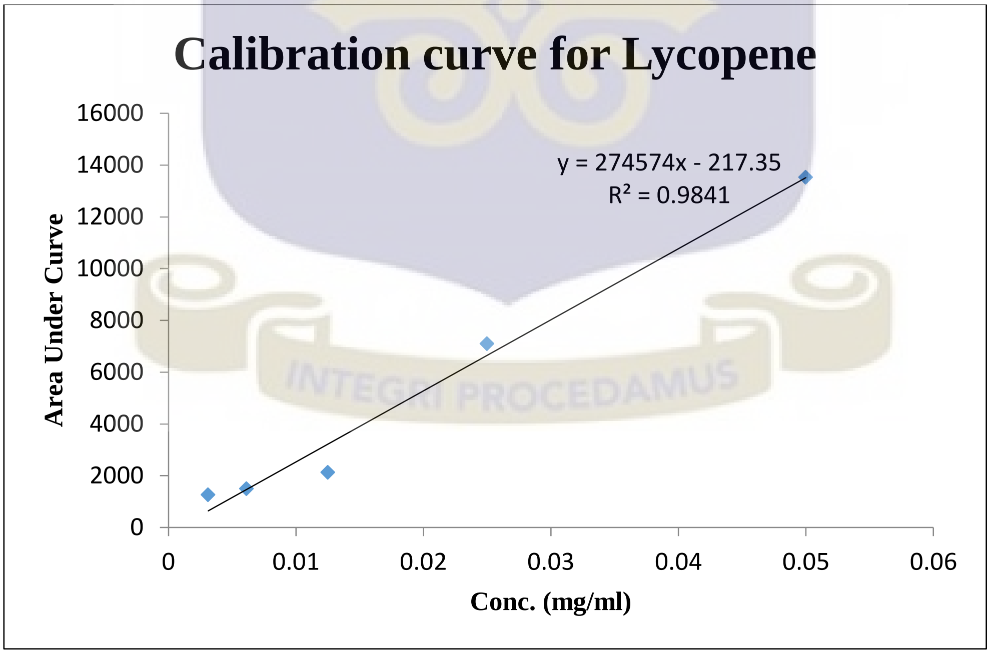

Figure A4: Standard curve for carotenoid standard, lycopene.

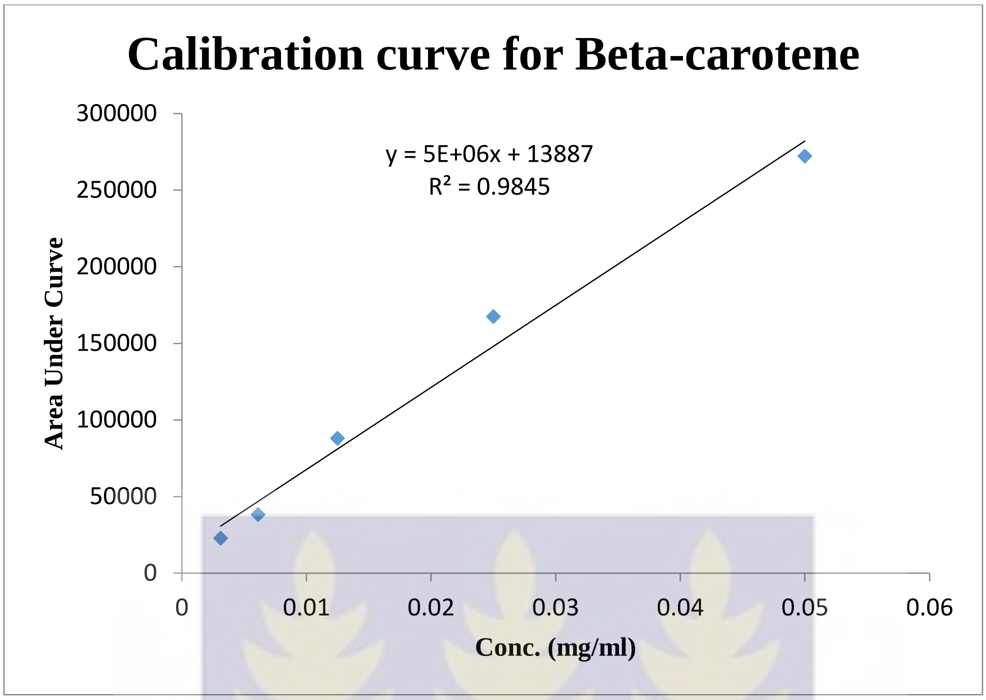

Figure A5: Standard curve for carotenoid, β-carotene.

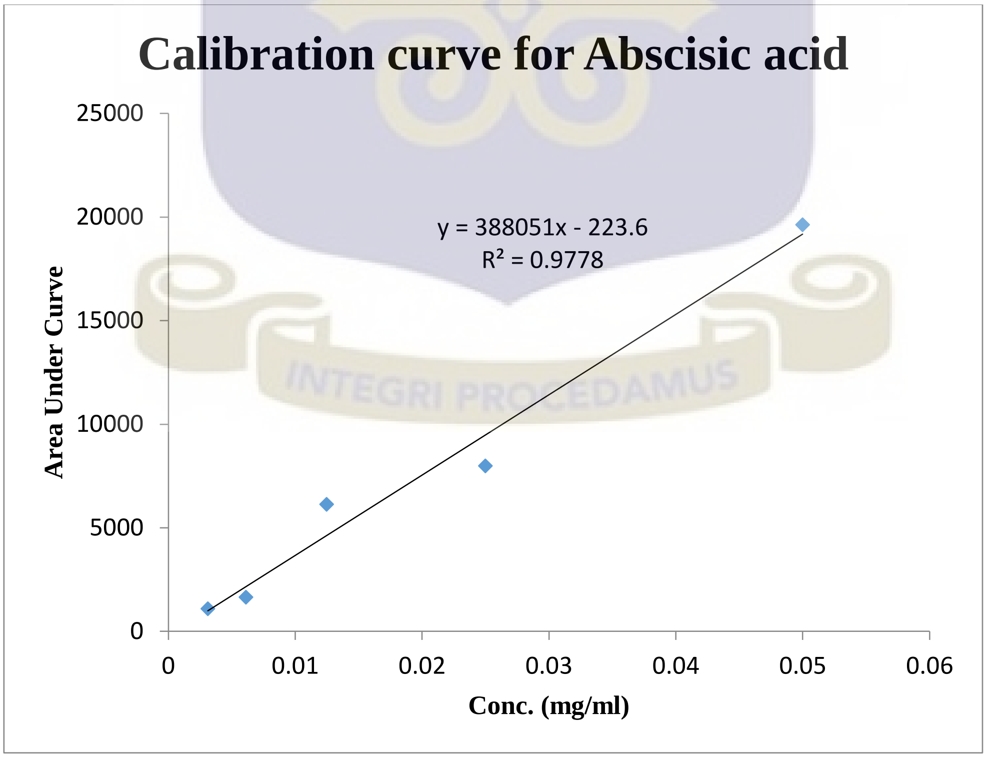

Figure A6: Standard curve for carotenoid standard, abscisic acid.