

**EVALUATION OF THE *IN-VIVO* ANTIMALARIAL ACTIVITY OF
L-ISOLEUCINE HYDROXAMATE**

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NOVEMBER, 2015

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L-ISOLEUCINE HYDROXAMATE**

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MSc/Sci/30655/2012-2013**

**A DISSERTATION SUBMITTED TO THE SCHOOL OF POSTGRADUATE
STUDIES,
AHMADU BELLO UNIVERSITY, ZARIA**

**IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE AWARD OF
MASTER DEGREE IN
BIOCHEMISTRY**

**DEPARTMENT OF BIOCHEMISTRY,
FACULTY OF SCIENCE
AHMADU BELLO UNIVERSITY, ZARIA
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NOVEMBER, 2015

DECLARATION

I declare that this dissertation titled “**Evaluation of the *In-vivo* Anti-malarial Activity of L-Isoleucine Hydroxamate**” was performed by me in the Department of Biochemistry, Faculty of Science Ahmadu Bello University Zaria, under the supervision of Prof. K.M. Anigo and Prof. I.A. Umar. The information derived in the text has been duly acknowledged in a list of reference provided. No part of this work has been previously presented for another degree or diploma at any institution.

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CERTIFICATION

This dissertation titled “**Evaluation of the *In-vivo* Anti-malarial Activity of L-Isoleucine Hydroxamate**” meets the regulations governing the award of Masters of Science of the Ahmadu Bello University, and is approved for its contribution to knowledge and literary presentation.

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DEDICATION

I dedicate this research work to Yahweh who is the pillar of my strength and also my parents, Mr. and Mrs. Atuluku for their love and support.

ACKNOWLEDGMENTS

I sincerely express my heartfelt gratitude to my distinguished supervisors, Prof. K.M. Anigo and Prof. I.A. Umar for their scholarly ideas, constructive criticism and open dialogue towards the improvement of the research.

My sincere thanks to the teaching and non-teaching staff of the department of Biochemistry, notably; Prof. S.E. Atawodi, Prof. D.A. Ameh, Prof. H.C. Nzelibe, Dr. I. Idowu, Dr. A.B. Sallau, Mr. Y. Apeh, and staff of the Faculty of Veterinary Medicine Ahmadu Bello University Zaria. I acknowledge the immense contribution of Miss Yemisi of the Protozoology Laboratory.

I appreciate my wonderful parents, Mr. and Mrs. Atuluku, my siblings and friends for their encouragement, prayers, patience and goodwill throughout the period of the study.

ABSTRACT

Malaria control still remains a challenge in Africa where 45 countries, including Nigeria, are endemic for malaria, and about 588 million people are at risk with an attendant mortality rate of roughly 125 deaths per minute. Using mice model, the study was undertaken to evaluate the antimalarial activity of L-isoleucine Hydroxamate in early and established malaria infection by carrying out Peter 4-day suppressive test and Rane's test respectively. The oral acute toxicity for Isoleucine Hydroxamate using the fixed dose procedure was found to be greater than 2000mg/kg body weight. For the anti-malarial evaluation, all experimental groups except the healthy control group (HC) were intraperitoneally inoculated with approximately 1.5×10^7 million parasitized erythrocytes and grouped as; negative control group (NCPD) which received 0.2 ml/day distilled water, Standard group (SGPC) which received 10mg/kg/day Chloroquine, treatment group that received 50 mg/kg body weight L-Isoleucine hydroxamate (TGPIH 50), treatment group that received 100 mg/kg body weight L-Isoleucine hydroxamate (TGPIH 100) and treatment group that received 200 mg/kg body weight L-Isoleucine hydroxamate (TGPIH 200). The antimalarial activity of L-Isoleucine hydroxamate in early malaria infection showed that the TGPIH200 group recorded an average parasitemia of $20.45 \pm 6.05\%$ which was not significantly different ($P > 0.05$) from the SGPC group which recorded $5.8 \pm 2.21\%$ nor the NCPD group which recorded $34.96 \pm 9.10\%$. Evaluation of blood glucose, plasma protein, packed cell volume, rectal temperature and Body weight during early malaria infection showed that L-Isoleucine hydroxamate significantly affected only the packed cell volume and rectal temperature. Packed cell volume for the NCPD group ($19.00 \pm 6.60\%$) was significantly lower than the TGPIH 50 group ($30.52 \pm 5.94\%$) and the TGPIH 200 group ($26.00 \pm 3.81\%$). Similarly, rectal temperature for the NCPD group ($34.56 \pm 0.50\text{ }^\circ\text{C}$) was significantly lower than the L-Isoleucine hydroxamate treatment groups; TGPIH 50 ($35.90 \pm 1.58\text{ }^\circ\text{C}$), TGPIH 100 ($35.70 \pm 0.93\text{ }^\circ\text{C}$) and TGPIH 200 ($35.98 \pm 0.18\text{ }^\circ\text{C}$) groups. The HC group ($52.00 \pm 3.06\%$, $37.9 \pm 0.34\text{ }^\circ\text{C}$) and the SGPC group ($45.80 \pm 1.90\%$, $38.24 \pm 0.46\text{ }^\circ\text{C}$) recorded significantly higher ($p < 0.05$) packed cell volume and rectal temperature values respectively, when compared to the other groups. The antimalarial activity of L-Isoleucine hydroxamate in established malaria infection was lost on day-4 of the Rane's test. The average parasitemia for the TGPIH 200 group was $18.09 \pm 9.02\%$ and was significantly ($P < 0.05$) higher than that of the NCPD group ($6.19 \pm 1.30\%$) and the SGPC group ($0.00 \pm 0.00\%$). Thus there was no significant difference ($P > 0.05$) in blood glucose, plasma protein, Packed cell volume, rectal temperature and Body weight, when especially the TGPIH 200 group was compared with the NCPD group. The SGPC group recorded significantly ($P < 0.05$) longer survival time of 28.00 ± 0.00 days compared to the other groups. There was no significant difference ($P > 0.05$) in survival time between the NCPD group (19.60 ± 2.39 days) and the TGPIH group (19.20 ± 3.78 days). These findings show that L-Isoleucine Hydroxamate has antimalarial activity in early malaria infection. However, in established malaria, its antimalarial activity was not sufficient to provide a lasting suppressive action to bring about a curative response.

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ABBREVIATIONS

μL	Microliter
kg	Kilogram
ml	Milliliter
mg	Milligram
dl	Deciliter
°C	Degree Celsius
ACT	Arthemisinin combined therapy
ALT	Alanine aminotransferase
AST	Aspartate aminotransferase
CHR-2863{(S)-[(R)-2-((S)-hydroxycarbamoyl-methoxy-methyl)-4-methyl-Pentanoylamino]-phenyl-acetic acid cyclopentyl ester}	
CSP	Circumsporozoite protein
DHFR	Dihydrofolatereductase
DHPS	Dihydropteroatesynthetase
DNA	Deoxyribonucleic acid
DOXP	Deoxy-D xylulose-5-phosphate
DPAP	dipeptidyl aminopeptidase
DXR	1-deoxy-D-xylulose-5-phosphate reductoisomerease
ELISA	Enzyme linked immunosorbent assay
GDP	Gross domestic product
HC	Healthy control group
HRP	Histidine rich protein
ICAM-1	Intracellular adhesion molecule-1
IHDX	Isoleucine Hydroxamate

IFA	Immuno fluorescence antibody
IRS	IsoleucyltRNAsynthetase
NADH	Nicotinamideadeninucleotide
NCPD	Negative control parasitized group and given 0.2 ml distilled water
OECD	Organization for economic cooperation and development
tRNA	Transfer ribonucleic acid
PCR	Polymerase chain reaction
PCV	Packed cell volume
PfEMP1	<i>Plasmodium falciparum</i> Erythrocyte membrane protein 1
PfCRT	<i>Plasmodium falciparum</i> Chloroquine resistant
PfMDR-1	<i>Plasmodium falciparum</i> multidrug resistance 1
PfM1AAP	<i>Plasmodium falciparum</i> M1 alanine aminopeptidase
PfM17LAP	<i>Plasmodium falciparum</i> M17 leucine aminopeptidase
PPr	Plasma Protein determined by refractometer
RBC	Red Blood Cell
RDTs	Rapid diagnostic techniques
SGPC	paratize Standard group administered 10 mg/kg/body weight
Chloroquine	
TCA	Tricarboxylic acid cycle
TGPIH	Parasitized treatment group administered L-Isoleucine hydroxamate
UV	Ultra violet

CHAPTER ONE

1.0 INTRODUCTION

1.1 Background of the Study

Malaria is a protozoal disease caused by infection with parasites of genus *Plasmodium* and transmitted to man by certain species of infected female Anopheles mosquito (Naser, 2007). The disease was named (*mala aria*) by the Romans who thought that it was due to bad air, which refers to the association of the disease with the foul-smelling air of the Tiberian marshes (Luzzatto and Notaro, 2001), its prevalence, virulence, and drug resistance, make it one of the most serious and widespread parasitic disease encountered by mankind (Vangapanduet *al.*, 2007).

In humans, five *Plasmodium* species are pathogenic namely; *Plasmodium falciparum*, *Plasmodium vivax*, *Plasmodium malariae*, *Plasmodium ovale* and *Plasmodium knowlesi* (World Health Organization, 2014). The current distribution of human-pathogenic *Plasmodium* species shows preponderance of *P. falciparum* in tropical Africa (Culleton *et al.*, 2008). While *P. vivax* prevails over *P. falciparum* in South America (Arevalo-Herrera *et al.*, 2011), both *P. falciparum* and *P. vivax* are prevalent in south-eastern Asia and western Pacific (Beatrice *et al.*, 2012). Although *P. malariae* may occur in all malarious areas, its prevalence is generally low (Beatrice *et al.*, 2012). It is a disease associated with warm, humid climates, where pools of water constitute perfect breeding grounds (Jamieson, 2006).

1.2 Antimalarial Efforts and Attendant Challenges

Malaria control still remains a challenge in Africa where 45 countries, including Nigeria, are endemic for malaria, and about 588 million people are at risk (WHO, 2011).

The protection of children and particularly pregnant women living in malaria endemic countries has been of particular interest to many National Malaria Control Programs because of the reduction in immunity associated with pregnancy as well as weak immune response of children which compromise resistance to other infectious diseases (Meeusen *et al.*, 2001).

Although, substantial success has been achieved in subtropical regions, however, the control of malaria in the tropics proved far more challenging leading to the abandonment of such efforts (Philippe *et al.*, 2002). Malaria-prevention efforts have since gravitated towards appropriate local protection methods. Provision and deployment of impregnated bed nets in China and Africa has been successful in reducing malaria morbidity and mortality (Aikins *et al.*, 1998; Lengelar, 2000).

Widespread resistance in parasites to most anti-malarial drugs, constitute a potential source of frustration, the limited armamentarium of drugs used thus far to treat malaria, development of strains resistant to commonly used drugs, the lack of affordable new drugs, policies and practices constrained by limited resources are limiting factors in the fight against malaria (Olliaro, 2005). All drugs in use are affected except, thus far, artemisinin derivatives (Vangapanduet *al.*, 2007).

To exacerbate the imminent dread, artemisinin, the drug of last resort currently shows decreased sensitivity in few places (World Health Organization, 2011; Elizabeth *et al.*, 2014). Current efforts focuses on optimization of therapy with available drugs, including the use of combination therapy, the development of analogs of existing agents, the discovery of natural products, the use of compounds that were originally developed against other diseases, the evaluation of drug resistance reversers, and vaccine development (Philip, 2003; Shankar *et al.*, 2012).

1.3 Isoleucine Hydroxamate

Analogous to L-Isoleucine, L-Isoleucine hydroxamic acid only differ in having a CONHOH Group instead of a COOH group (Masahiko *et al.*, 1971; David *et al.*, 2014). Generally amino acid hydroxamates have low toxicities (Ahmed *et al.*, 2001). Although in the past, amino acid hydroxamates were not recognized as antagonists, an earlier study by Masahiko *et al.*, (1971) show that Isoleucine hydroxamate exhibited potent bacteriostatic action, by antagonizing Isoleucine metabolism. Hydroxamate based compounds are widely reported to mediate broad activities in biological systems principally by chelating metal ions in the active site of enzymes, thus offering promising leads for medicinal chemistry (Yeng and De lombart, 1999; Munster *et al.*, 2001; Lanzkron *et al.*, 2008; Yajima *et al.*, 2004; Skinner-Adams *et al.*, 2012).

AB

Figure 1.1

Molecular structures of (A) L-Isoleucine and (B) L-Isoleucine Hydroxamate (Masahiko *et al.*, 1971).

1.4 Statement of Research Problem

The World Health Organization reports there were 198 million cases of malaria worldwide in 2013 which resulted in an estimated 584,000 to 855,000 deaths, the majority (90%) of which occurred in Africa. (World Health Organization, 2014).

The vulnerability of pregnant women exacerbates this scourge as 125 million pregnant women are at risk of infection each year with 200,000 estimated infant deaths yearly (Hartman *et al.*, 2010; Bartoloni *et al.*, 2012; Murray *et al.*, 2012).

Malaria is commonly associated with poverty and has a negative effect on economic development (Vanderberg, 2009). In endemic regions, average per capita GDP has risen in a pitiable trend in relation to non endemic areas. In areas of stable transmission, the average per capital Gross Domestic Product has risen pitifully by only 0.4% per year while a 2.4% increase was accounted for in non-endemic countries. In its entirety, the economic impact of malaria has been estimated to cost Africa 12 billion U.S Dollars every year (Worrall, Basu and Hanson, 2005; Humphreys, 2010). This cost include; cost of health care, working days lost due to sickness, days lost in education, decreased productivity due to cerebral damage associated with *Plasmodium falciparum* infection and loss of investment and tourism (Sachs and Malany, 2002; Greenwood *et al.*, 2005).

In addition to the cost of antimalaria drugs, The huge health problem of malaria is exacerbated by the alarming ability of this protozoan to develop resistance to chemotherapeutic agents, hence synthetic interventions that provides cheap and effective chemotherapeutic agents is needful (White, 2004; Marvin, 2010).

1.5 Justification for the Research

From independent studies by McCormic (1970) as well as Rowenna and Kiaran (2007) Isoleucine, which is not incorporated in adult Hemoglobin accumulated significantly (up to 20 fold) in infected cells than in normal cells.

During active and continuous proliferation, blood stage parasites are sensitive to perturbation in the protein translational machinery (Phamet *et al.*, 2014). Hence, *In-vivo* antagonization of Isoleucine using Isoleucine Hydroxamate holds the potential for effectively depressing erythrocytic schizogony-critical to clearing the parasite and abating the disease.

The diverse biological activity of Hydroxamates and hydroxamate based compounds have been exploited in treatment of cancer (Munster *et al.*, 2001), sickle cell anemia (Lanzkron *et al.*, 2008) and management of cardiovascular diseases (Yeng and De Lombaert, 1997). Hydroxamates such as fosmidomycin and desferrioxamine B are potent antimalarial agents (Cabantchik *et al.*, 1999; Yajima *et al.*, 2004).

Ampicoplanin parasites are also dependent on their apicoplast which retains much of the cyanobacterial protein translation apparatus of plastid ancestry, this evolutionary difference in translation process in the parasite and that of the human host may offer an exploitable target. (Istvan *et al.*, 2010; Jackson *et al.*, 2011).

The outcome of this evaluation could provide valuable data for development of a potent anti-malarial intervention with enhanced effectiveness as monotherapy or in combination with other anti-malarials, (Bartoloni *et al.*, 2012; Murray *et al.*, 2012).

1.6 Null Hypothesis

Isoleucine Hydroxamate has no anti-malarial activity in Mice.

1.7 General Aim

The aim of this work is to evaluate anti-malarial activity of L-Isoleucine Hydroxamate in Mice.

1.8 Specific Objectives

- 1) To determine the acute oral toxicity of L-Isoleucine Hydroxamate in mice model and then classify based on the Global Harmonised System.
- 2) To evaluate the effect of oral administration of Isoleucine Hydroxamate on serum ALT, serum AST, weight change and packed cell volume.
- 3) To determine the effect of graded doses of L-Isoleucine Hydroxamate on the blood glucose, plasma protein, packed cell volume, rectal temperature and body weight of *P. berghei* infected Mice after Peter's 4-Day suppressive test and Rane's test.
- 4) To assess the antiplasmodial activity of the oral administration of L-Isoleucine Hydroxamate via Peter's 4-Day suppressive test and Rane's test.
- 5) To assess the curative potential of Isoleucine Hydroxamate in established malaria infection.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1. *Plasmodium*

Commonly known as the malaria parasite, *Plasmodium* is a large genus of parasitic protozoa belongs to the family; *Plasmodiidae*, order; *Haemosporidia* and phylum; *Apicomplexa*, Infection with these protozoans is known as malaria (Yotoko and Elisei, 2002). Believed to have originated from Dinoflagellates-a photosynthetic protozoa, the genus *Plasmodium* was first described by Marchifava and celli and currently contains about 200 species divided into several subgenera with at least ten species pathogenic to humans (Chavatte *et al.*, 2007; Weiminet *al.*, 2010).

Currently all species examined have 14 chromosomes which vary in length (500 kilobases-3.5 mega bases), one mitochondrion and one plastid (apicoplast) which is an organelle similar to a chloroplast but is not photosynthetic. Although the function of the plastid is not fully known, it has been demonstrated to contain some essential metabolic pathways responsible for isoprenoid, Fe-S clusters, fatty acid and phospholipid biosynthesis (VanDooren and Striepen, 2013).

2.2 Cycle of Host Infection of Malaria

This apicomplexan protozoan, is also famous for its heteroxenous cycle of development, this complex process begins with a bite from an infected female *Anopheles* mosquito-notably *Anopheles gambiae*. Via its saliva, motile infective form sporozoite pass into either, the blood or the lymphatic system of a vertebrate host such as a human (Center for Disease Control and Prevention, 2013).

The sporozoite(s) travels through the blood vessels to liver, where it reproduces asexually (tissue schizogony), producing thousands of merozoites (Collins and Barnwell, 2009). Rupturing the liver cells, they return to the bloodstream where infect red blood cells and initiate a series of asexual multiplication cycles (blood schizogony) that produce 8 to 24 new infective merozoites which developed from trophozoites and schizonts, at this point the cells burst and the infective cycle begins anew (Collins and Barnwell, 2009). Other merozoites develop into gametocytes capable of sexual reproduction, Erythrocytes with such forms do not rupture but instead are taken up by the next mosquito that bites, infecting the mosquito and possibly more people (Wiser, 2013).

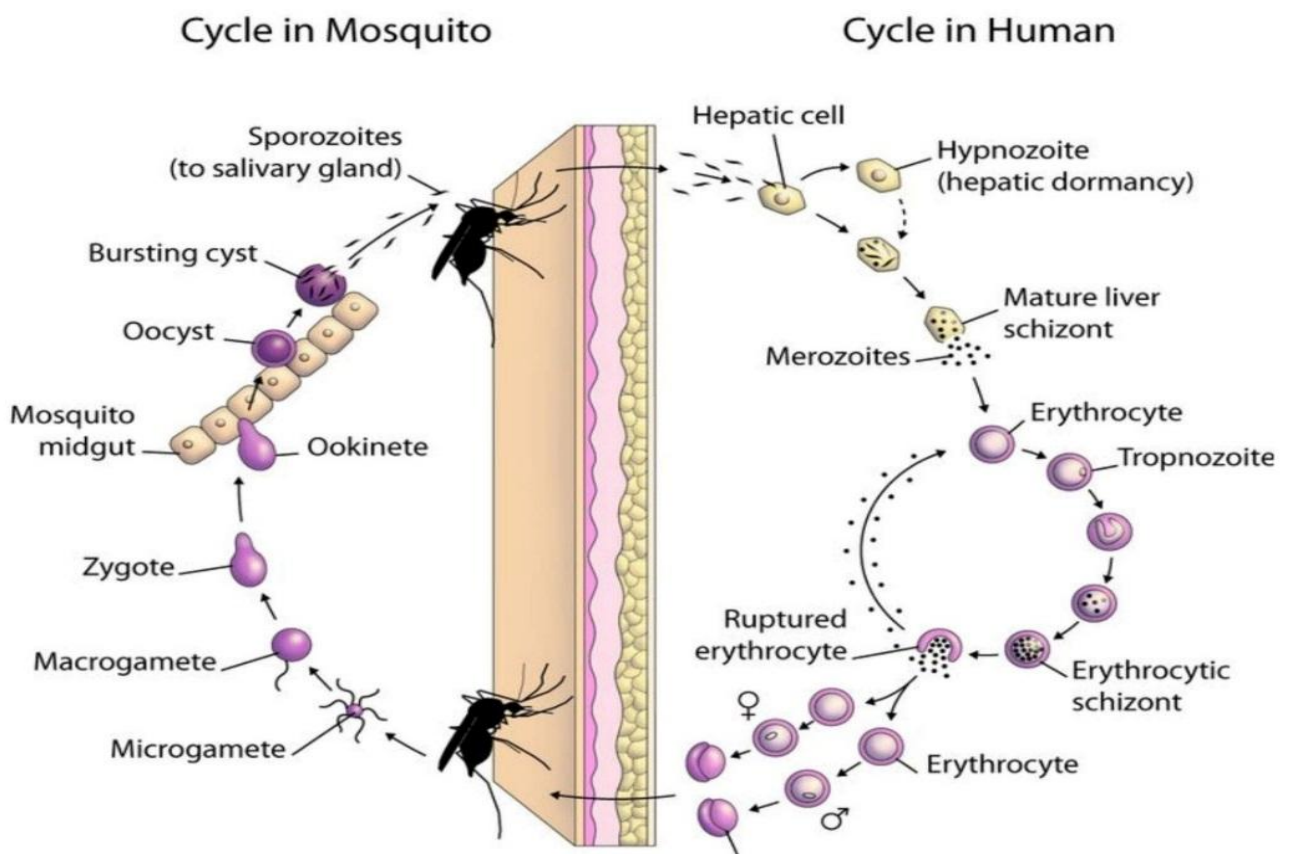


Figure 2.1: Heteroxenous Life Cycle of *Plasmodium falciparum* (Open course ware).

2.3 *Plasmodium* Nutrition and Energy Metabolism

The malaria parasite exhibits a rapid growth and multiplication rate especially during intraerythrocytic developmental stage, necessitating the parasite to devise mechanisms requisite for uptake of host cell nutrients to perpetuate survival and replication and obviously the parasite's metabolism will be intertwined with that of the host's because of the intimate relationship between the host and parasite (Kellen *et al.*, 2009). Repertoire from early studies reveals that glucose uptake in parasitized erythrocytes increases up to 79-fold compared with uninfected erythrocytes (Mc kee, 1951), through alteration in membrane permeability of the host cell (Neame and Homewood, 1975).

In recent times, Onyesome *et al.*, observed significant depression in both plasma and brain glucose in *P. berghei* infected mice with respect to the healthy control (Onyesome *et al.*, 2013). Elased and Playfair (1994) had suggested from an earlier study that hypoglycemia observed in murine malaria, may be largely secondary to increased insulin secretion. Alvaro (1986) admitted glycolysis as a major pathway for carbohydrate metabolism in all plasmodium species, but that the TCA cycle is partly functional (unlike in avian plasmodia) in rodent and mammalian plasmodia.

In contrast to previous findings, Bozdech *et al.* (2003) reported that the *P. falciparum* genome encodes all the necessary enzymes to run a complete TCA cycle. In same vein, James *et al.* (2013) by a metabolomic approach discovered that carbon skeletons derived from both glucose and glutamine are catabolized in a canonical oxidative TCA cycle in both the asexual and sexual blood stages, hence chemical inhibition of the gametocyte TCA cycle led to arrested development and death.

In spite of the significant dependence on glycolysis, oxidative phosphorylation is reported to be little or absent (Lang-Unnasch and Murphy, 1998). According to Ke *et al.* (2011) despite their reliance on glycolysis, the asexual stages of *P. falciparum* retain a single mitochondrion and via its transport chain, transport of proteins and metabolites are mediated as well as the re-oxidation of inner-membrane dehydrogenases, such as the dihydroorotate dehydrogenase involved in *de novo* pyrimidine biosynthesis. As a result, asexual stages and gametocytes are sensitive to electron transport chain inhibitors, including the antimalarial atovaquone (Fleck *et al.*, 1996; Painter *et al.*, 2007).

Digesting up to 75% of the total host hemoglobin into amino acids and incorporating 16% of these amino acids into proteins of blood stage parasites, the degradation of ingested hemoglobin is the major source of amino acid while uptake of free amino acids from the host plasma and *de novo* synthesis are minor sources. (Krugliak *et al.*, 2002). Independent studies by McCormick (1970) and Collins and Barnwell (2009) reported that isoleucine, which is not incorporated in adult Hemoglobin accumulated significantly (up to 20 times) in parasitized cells than in normal cells.

The proposed pathway of hemoglobin digestion involves an initial cleavage by plasmepsin-1 (and possibly falcipain-2) then the combined actions of several plasmepsins and falcipains. The peptide fragments produced by these digestions are then digested into smaller peptides by falcilysin (Benerjee *et al.*, 2002; Goldberg *et al.*, 2005). Dalal reported two food vacuole amino peptidases which converts these peptides into amino acids Dalal *et al.*, 2007). Furthermore, Kemba and Klemba (2004) identified dipeptidyl aminopeptidase (DPAP) activity within the food vacuole, which was postulated to possibly remove dipeptides from the N-termini of the peptides

generated through the actions of the various endopeptidases in the food vacuole and then the amino peptidases can convert these to free amino acids.

Earlier biochemical studies on blood-stage *Plasmodium* malarial parasites first demonstrated its proficiency at scavenging and modifying lipids obtained from the host (Vial *et al.*, 1982). Free fatty acids can be obtained directly from the serum or from sources such as high-density lipoprotein (Grelier *et al.*, 1991; Krishnegowda and Gowda, 2003).

Fatty acids are suitably modified by elongating or desaturating the lipids and incorporating them into phospholipids, di-acylglycerols and tri-acylglycerols (Palacpac *et al.*, 2004; Mi-Ichi *et al.*, 2006), further more scavenged phospholipids can be incorporated into membranes without modification (Krishnegowda and Gowda, 2003).

According to Ross *et al.* (2003) the long held notion that *Plasmodium* species are incapable of *de-novo* fatty acid synthesis has recently been debunked by the emergence of data, revealing the presence of a type II pathway in the parasite's apicoplast. The use of derivatives of thiolactomycin, resulted in heightened sensitivity in the ring stage (Mc Fadden *et al.*, 1996; Kohler *et al.*, 1997; Ross *et al.*, 2003). In contrast, Tarun *et al.* (2008, 2009) opined that *de novo* fatty acid synthesis is only required for parasite late liver-stage development given the relatively high expression of FAS-II genes during this stage. Conclusive evidence supporting the role of *de novo* fatty acid synthesis of plasmodium parasites in the liver was established as targeted deletion of fatty acid biosynthetic enzymes in experimental parasites exhibited deficiencies in late liver-stage development characterized by slow rate of growth, abnormal nuclear division, lack of cytomere formation, and lack of expression of late-stage proteins such as merozoite surface protein-1, however blood-stage growth rates were not impaired by such deletion (Yu *et al.*, 2008; Pei *et al.*, 2010).

Vial *et al.* (2003) reported the incapacitation of *Plasmodium* species for cholesterol synthesis.

Also, no biological pathway involved in sterol production can be demonstrated in

Plasmodium species genome databases (Catron *et al.*, 2004).

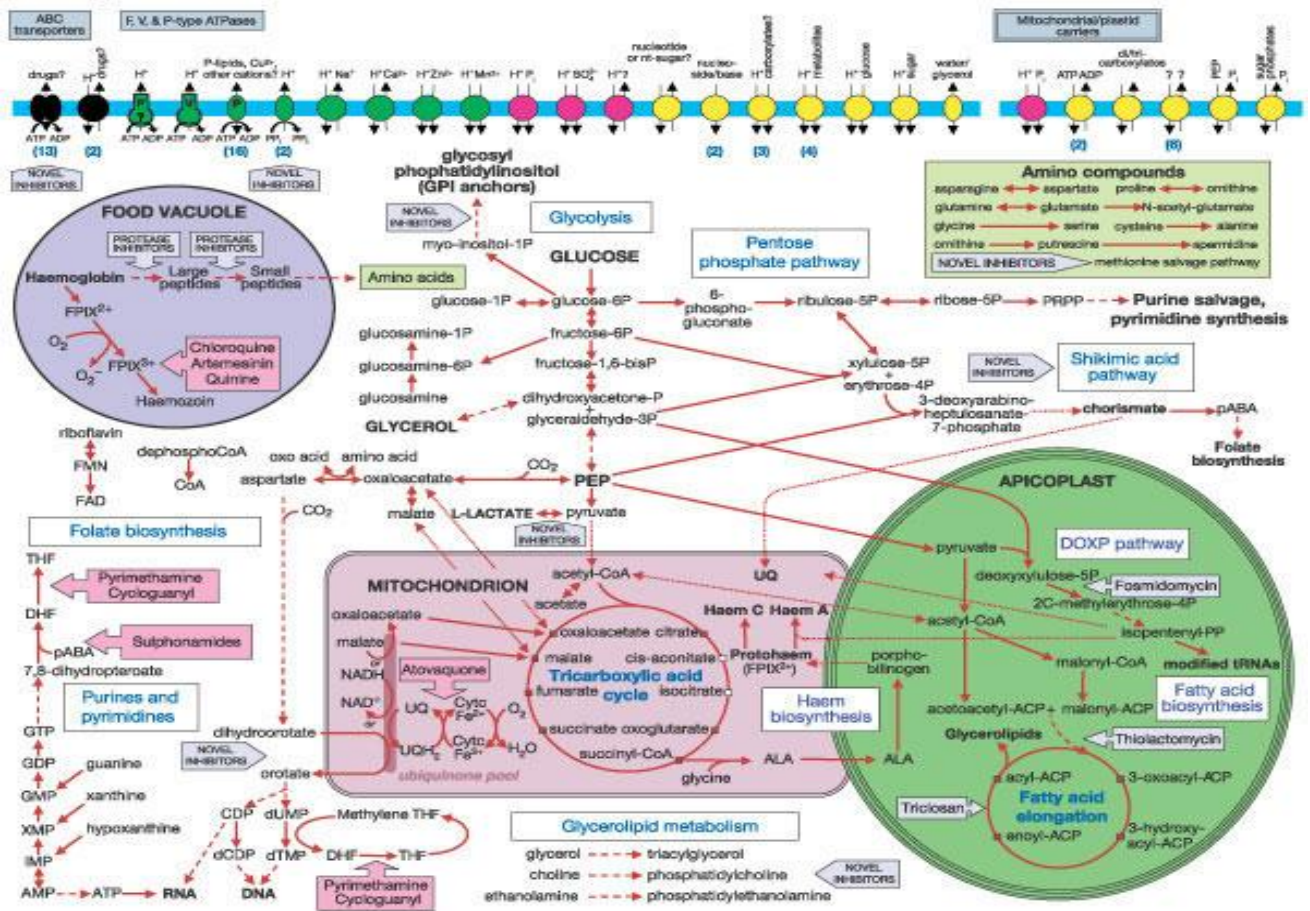


Figure 2.2: Metabolism of *Plasmodium falciparum* (Gardner *et al.*, 2002).

The Pink block arrows indicate the steps inhibited by antimalarial drugs within the mitochondrion, cytosol, and food vacuole while the grey block arrows highlight potential drug targets.

2.4 Pathogenesis and Virulence

The infection of the red cells by malaria parasites, particularly *P. falciparum*, results in progressive and dramatic structural, biochemical, and mechanical modifications of the red cells with a potential for life-threatening complications (Tjitra *et al.*, 1999; Janet *et al.*, 2010).

Several pathophysiological factors such as the parasite biomass, malaria toxin(s) and inflammatory response; cytoadherence, rosetting and sequestration; altered deformability and fragility of parasitized erythrocytes; endothelial activation, dysfunction and injury; and altered thrombostasis have been found to be involved in the development of severe malaria as seen in *P. falciparum* infection (Qijun *et al.*, 2000; Louis *et al.*, 2002).

The pathogenesis of severe malaria therefore involves a cascading interaction between parasite and red cell membrane products, cytokines and endothelial receptors, leading to inflammation, activation of platelets, hemostasis, a procoagulant state, microcirculatory dysfunction and tissue hypoxia, resulting in various organ dysfunctions manifesting in severe malaria (Henri *et al.*, 2006).

Hommelet *al.* (2013) buttressed on *Plasmodium falciparum's* clever avoidance of the spleen and immune system by sequestration in vital organs especially the brain (leading cerebral malaria) by promoting red blood cell membrane surface expression of multiple versions of erythrocyte membrane protein 1 (PfEMP1)-an adhesive ligand protein (virulent knobs) encoded by the multigene segment-Var, thus creating a parasite almost impossible to eradicate from the host without anti-malaria medication.

Bultrini (2013) considered RIFIN- repetitive interspersed family one of the most abundant multigene family also expressed on parasitised erythrocyte surface as an adherence factor,

and with PfEMP1 are considered crucial cornerstones for virulence mainly due to their ability to avoid immune response through antigenic variability.

According to Owain *et al.* (2006) Malaria parasites not only escape the host's immune response but also modulate the immune response and cause significant immune suppression, as they were found (in parasitized erythrocytes) to inhibit the maturation of antigen presenting dendritic cells, thus diminishing their association with T cells, resulting in immunosuppression and increased risk for secondary infections. (Owain *et al.*, 2006).

A study led by Shalon *et al.* (2012) demonstrated the remarkable resilience (a hibernatory-like state) of *P. falciparum* to starvation for the single amino acid isoleucine. A dramatic decrease in metabolic rate to conserve energy and resources, ensured increased survival once growth-permissive conditions were restored.

2.5 Immune Response

Malaria infection gives rise to host responses which are regulated by both the innate and adaptive immune system as well as by environmental factors (Perlmann and Troye-Blomberg, 2002). Infection induces strongly elevated blood concentrations both polyclonal and specific immunoglobulins with IgG been most predominant, mediating parasite neutralization (Stevenson and Riley, 2004). Immunoglobulins protect against malaria

by a variety of mechanisms which include; inhibition of merozoite invasion of erythrocytes (Guevara *et al.*, 1997), enhance clearance of infected erythrocytes from the circulation by binding to their surface, thereby preventing sequestration in small vessels,

promoting elimination by the spleen (Molineaux, 1996), and In particular, opsonization of infected erythrocytes which significantly increases their susceptibility to phagocytosis, cytotoxicity and parasite inhibition by various effector cells such as neutrophils and monocytes/macrophages (Oeuvray *et al.*, 1994).

Neutrophils, mononuclear Phagocytes and natural killer (NK) cells appear to play a role in innate immunity seen early in malaria infections. In particular, NK cells have been shown to be able to lyse *Plasmodiumfalciparum*-infected erythrocytes in-vitro, produceing cytokines such as interferon-(IFN) which mediates parasitocidal macrophage activation, a role considered crucial for innate immunityto malaria (Srinivas, 2015). CD4 + T cells are essential for immune protection against asexual blood stages in both murine and human malaria, responding to malaria antigen by proliferation and/or secretion of cytokines, e.g. IFN or IL4 (Srinivas, 2015). Contributing to protection against severe malaria, it has been proposed that CD8+ T cells may regulate immunosuppression in acute malaria, downmodulate inflammatory responses and perform important effector functions in pre-erythrocytic immunity and which contribute to protection against severe malaria (Perlmann and Troye-Blomberg, 2002).

Other Innate defence mechanism against malaria such as; the alteration in hemoglobin structure particularly certain thalasseмииs, Duffy negativity on red cells and enzyme alteration such as Glucose 6 phosphate dehydrogenase deficiency have been found to offer 50-90 % protection against sever malaria and associated mortality (Mary and Eleanor, 2004; Carolina *et al.*, 2010).

However natural infection with malaria parasites leads to only a slow, partial and short lived immunity that is unable to protect the individual against a new infection which is caused by an expression of a variety of proteins at different stages that keeps changing often (Langhorne *et al.*, 2008). With a couple of more infections, the host acquires several adaptive immune responses mediated by antibodies and immunity cells, graded as anti-disease immunity; that protects against clinical disease, anti-parasite immunity; protecting against high parasitemia, and sterilizing immunity; which protects against new infections by maintaining a low-grade, asymptomatic parasitemia (Denise *et al.*,2009).

2.6 Clinical Signs of Malaria

According to Idro *et al.*, (2005)Symptoms of malaria usually start to appear 10-15 days after the bite of an infected mosquito and all four species can exhibit non-specific prodromal symptoms a few days before the first febril attack. These prodromal symptoms are generally described as 'flu-like' and include: headache, slight fever, muscle pain, anorexia, nausea and lassitude which tend to correlate with parasite density (Idro *et al.*,2005).In severe malaria (caused by *Plasmodium falciparum*), clinical findings include; confusion, coma, neurologic focal signs, severe anemia and respiratory difficulties (center for disease control and prevention, 2015).

2.7 Malaria and Climate

Malaria is climate sensitive and the pivotal balance of climatic elements creates ideal conditions, requisite for mosquitoes to breed and to transmit malaria parasites,

thus is suggestive of the malaria thrive in tropical climates as against deserts or arctic areas (Elizabeth, 2014).

Pascual *et al.* (2006) had opined that Infection rates tend to increase with increase in temperature, stemming from increase in reproduction rate inside vector mosquitoes when it's warmer. Warming temperatures expand the risk area for malaria, pushing the disease burden farther uphill in regions of higher altitude, thus having the potential for devastating outcomes especially for people who dwell in high land cities, (ClimateWire, 2012; Elizabeth, 2014).The aforementioned phenomenon is exacerbated by rapid population growth and significant land use changes and the proliferation of greenhouse gas emissions by industrialized nations (Jonathan and Sarah, 2006).

2.8 Laboratory Diagnosis of Malaria

2.8.1 Microscopic examination

Direct microscopic examination of intracellular parasites on both thick and thin Giemsa stained blood films is the current standard and the most widely practiced and useful method for definitive malaria diagnosis (Bloland, 2001). Advantageously providing; specie differentiation, quantification of the parasite density, and ability to distinguish clinically important asexual parasite stages from gametocytes it is critical for proper case-management and evaluating parasitological response to treatment, however slide collection, time-consuming staining/reading protocol; training and supervision of microscopist as well as skepticism associated with obtained results are limiting factors (Warhurst and William, 1996: Bloland, 2001).

Anthony (2002) reported the development of certain fluorochromes which cause nuclear fluorescence in association with plasmodial nucleic acid by UV light at an appropriate excitation wavelength of 470 nm to 490 nm. The quantitative buffy coat (QBC) method as a modification of the light microscope has been adapted for malaria diagnosis (Hakim *et al.*, 1993). By concentrating parasites at a predictable location after microhaematocrit tubes precoated with fluorescent Acridine Orange (AO) are centrifuged, the parasites can be viewed through the capillary tube using a special long-focal-length objective (paralens) with a fluorescence microscope (Craig and Sharp, 1997).

Although the QBC is more sensitive, requires less operational training and is less time consuming (Tharavani, 1990). Electricity is always required, special equipment and supplies are needed, the per-test cost is higher than simple light microscopy, and species diagnosis is non-specific (Bloland, 2001).

2.8.2 Rapid diagnostic tests (Rdts)

In view of the World Health Organization (WHO) recognition for the urgent need for new, simple, quick, accurate, and cost-effective diagnostic tests for determining the presence of malaria parasites and to overcome the deficiencies of light microscopy, numerous new malaria-diagnostic techniques were developed (Lowe *et al.*, 1996). According to Anthony (2002), these tests target plasmodial proteins expressed on the red blood cell or soluble in blood, such as; *P. falciparum* Histidine Rich Protein (HRP) 1 and 2, a pan-malarial Aldolase and lactate dehydrogenase.

RDTs have been developed in different test formats with the dipstick and test strip being more satisfactory a device for safety and manipulation (Gaye *et al.*, 1996).

RDTs detect malaria antigen in blood flowing along a membrane containing specific anti-malaria antibodies bound to a detectable marker; they do not require laboratory equipment (Tangpukdee *et al.*, 2009). RDTs are convenient for utility and speed, however they fail to satisfy crucial diagnostic indices such as; detection threshold, quantification, species differentiation and reliability of test result (Clinton *et al.*, 2009).

2.8.3 Serology

Serology detects anti-malarial antibodies against malaria parasites in serum, via either indirect immunofluorescence antibody (IFA) test or enzyme-linked immunosorbent assay (ELISA) (Center for Disease Control, 2015). Although serological markers are available for the four predominant species of human malaria, a positive test is of no clinical relevance to diagnosis of acute infection as it only measures past experience, hence serology bridges the divide between diagnosis and treatment and is useful in blood screening prior transfusion (Maria *et al.*, 2009).

2.8.4 Molecular tests

Detection of plasmodium genomic DNA using polymerase chain reaction techniques are becoming a more frequently used tool in the diagnosis of malaria, as well as the diagnosis of malaria and surveillance of drug resistance to chemotherapy (Bloland, 2001).

According to Beck (1999), one important use of this technology is in detecting mixed infections or differentiating between infecting species when microscopic examination is inconclusive.

With specific primers developed for each of the four species of human malaria, mixed infection detection and speciation is achievable when microscopic diagnosis is inconclusive (Beck, 1999).

Although PCR cannot be considered a rapid technique for the initial diagnosis of malaria, its value lies in its heightened sensitivity, specificity, and speciation (Snounou *et al.*, 1993; Dakic *et al.*, 2014). Primary disadvantages to these methods are overall high cost, high degree of training required, need for special equipment, absolute requirement for electricity, and potential for cross-contamination between samples (Bloland, 2001).

2.8.5 Mass spectrometry

A novel approach for the *in-vitro* detection of the malaria parasite at a sensitivity of 10 parasites/ μ L of blood has been reported using direct ultraviolet laser desorption time-of-flight mass spectrometry (Mann, 2002). Quantification is rapidly achieved by intense ion signal associated with ferriprotoporphyrin IX (sequestered by malaria parasites during their growth in human red blood cells) which correlate with the sample parasitemia, hopefully, future improvements in the equipment and technique can make this method deployable and useful (Demirev *et al.*, 2002; Mann, 2002).

2.9 Prevention and Control

The prevention and control against malaria employs three non-mutually exclusive approaches;

The effective use of impregnated bed nets, repellants, protective clothing and house spraying which minimizes human-mosquito contact.

Environmental modification by the use of larvicides/ insecticides as well as biological control which reduces Vector density and parasite reservoir reduction by early case detection and treatment, as well as the use of chemoprophylaxis (Kakillaya, 2014: Center for Disease Control and Prevention, 2015).

2.10 Current Drugs for Malaria Treatment

The first effective treatment of malaria came from the bark of the Quinine containing cinchona tree, along with its dextroisomer Quinidine, (Kaufman *et al.*, 2005) since then, Treatment of malaria has relied on a limited collection of ethno based as well as synthetic drugs (Olliaro, 2001: Brian, 2011).

Three classes of drugs are currently in use for the treatment of malaria, the first class are the quinolones, which include; type-1 quinolines, chiefly the 4-aminoquinolines; chloroquine, amodiaquine and pyronaridine and type 2 quinolines, which include; mefloquine, halofantrine, and lumefantrine(white, 2004). Quinolones are active against all forms of the schizonts except resistant *P. falciparum* and *P. vivax* strains and the gametocytes of *P. vivax*, *P. malariae*, *P. ovale* as well as the immature gametocytes of *P. falciparum*(white, 2004).

The antifolates are the second class; they comprise various combinations of dihydrofolate-reductase inhibitors such as; proguanil, chlorproguanil, pyrimethamine, and trimethoprim and dihydropteroate synthase inhibitors such as sulfa drugs which include; dapsone, sulfalene, sulfamethoxazole, sulfadoxine, and so forth(Bloland, 2001).

Acting primarily on the schizonts during the erythrocytic phase as well on the tissue stages of *P. falciparum*, *P. vivax* and *P. ovale*, when used in combination, they produce a synergistic effect, minimizing chemotherapeutic resistance (Bloland, 2001). Typical combinations include sulfadoxine/pyrimethamine, sulfalenepyrimethamine (metakelfin), and sulfamethoxazole-trimethoprim (co-trimoxazole), pyrimethamine-sulfadoxine (Fansidar), proguanil-atovaquone (Malarone) and chlorproguanil-dapsone (LapDap) whose use in Africa was withdrawn in 2008 due to haemolytic anaemia in glucose-6-phosphate dehydrogenase (G6PD) deficient patients (Premji *et al.*, 2009).

Artemisinin and its derivatives such as; Artesunate, Arteether and Artemether belong to the third class of antimalarial drugs (Olliaro,2005).Acting more rapidly than any other class of antimalarial drug, artemisinins and their various combination regimensare recommended as the first-line treatment for malariacaused by *P. falciparum* (World Health Organization, 2010; Eyasu, 2015).Their relatively broad stage-specificity of action extends to an ability to impede the development of gametocytes (Price *et al.*, 1996). At present, the level of interest in artemisinin drugs peaks, due to their well-recognized pharmacological advantages (Schlitzer, 2008).

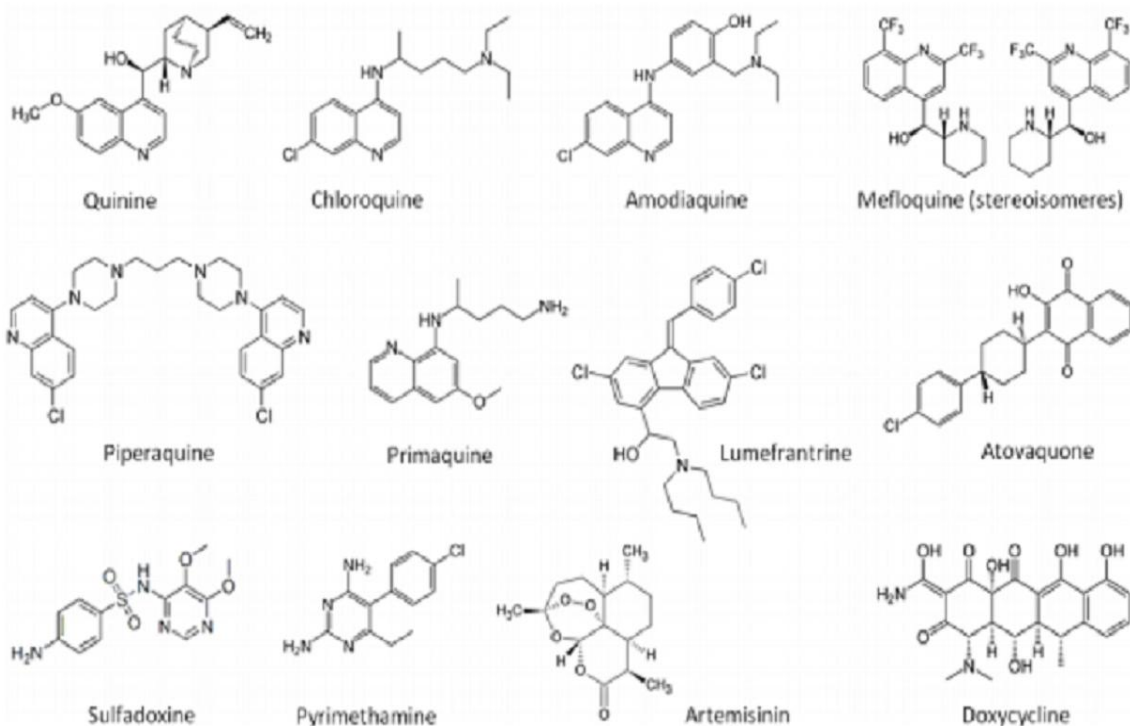


Figure 2.3: Antimalarial Drugs and their Molecular Structures (Aminake and Pradel, 2013).

Miscellaneous compounds not in the class above include; Halofantrine-a phenanthrene-methanol compound recommended in areas with multiple drug-resistant cases. Having activity against the erythrocytic stages of the malaria parasite, fatal cardiac side-effects limit its usefulness (Nosten, *et al.*, 1993). Atovaquone which is a hydroxynaphthoquinone is used most widely for the treatment of opportunistic infections in immunosuppressed patients and is effective against chloroquine-resistant *P. falciparum*, but because, when used alone, resistance develops rapidly, atovaquone is usually given in combination with proguanil (Rodloff *et al.*, 1996). Antibiotics such as; Clindamycin, tetracycline and its derivatives, such as doxycycline, which are consistently active against all species of malaria, and in combination with quinine, are particularly useful for the treatment of severe falciparum malaria (Schlitzer, 2008).

2.11 Mechanism of Anti-Malarial Drug Action

Quinolones mediate their antimalarial activities by primarily accumulating in the parasite's vacuoles resulting in a raise in the internal pH which impairs the conversion of heme to hemozoin thus inhibiting the biocrystallization of toxichemozoin which poisons the parasite (White, 2004). Other potential mechanisms through which they may act include interfering with the biosynthesis of parasitic nucleic acids and the formation of a quinine-haem or quinine-DNA complex. (Olliaro, 2005).

Type 1 antifolates; Sulfadoxine and sulfamethoxypyridazine are specific inhibitors of the enzyme dihydropteroate synthetase in the tetrahydrofolate synthesis pathway of malaria parasites, structurally analogous to *para*-aminobenzoic acid (PABA), they compete with PABA to block its conversion to dihydrofolic acid (Olliaro, 2005). Type 2 antifolates; pyrimethamine and biguanides prevent the NADPH-dependent reduction of dihydrofolate to tetrahydrofolate by DHFR thus preventing the biosynthesis of purines and pyrimidines, thereby halting the processes of DNA replication, cell division and reproduction (Olliaro, 2005).

It is generally believed that the various reactive intermediates of Artemisinin are the actual parasitocidal agent rather than the intact molecule. Acting via mechanisms that are distinct from other anti-malarial classes (Sanjeev *et al.*, 2004). Avery *et al.*, (1993) had reported that the peroxide within the 1,2,4-trioxane system of artemisinins is essential for anti-malarial activity (Avery *et al.*, 1998). Sanjeev *et al.* (2004) reviewed 2 popular hypotheses, the first of which is complexation of the intact molecule into an active site, one crucial evidence for this is the *in vitro* anti-malarial activity that is sensitive to steric effects,

as observed by the replacement of the methyl group Carbon 3 of artemisinin and 10 deoxodihydro artemisinin by phenyl ethyl or other radical stabilizing groups (Avery *et al.*, 1996b).

The second hypothesized mode of its anti-plasmodial action stems from activation by heme ferrous iron or free ferrous iron which decomposes the peroxide bridge to yield 2 C-centered alkoxy radicals which believed to possess anti-malarial activity by reacting with sensitive biomolecules in the parasite. (Butler *et al.*, 1998; Wu *et al.*, 1998). From experimental findings, Eckstien *et al.*, (2003) suggested that due to structural similarities in sesquiterpene moieties, artemisinins may act in a similar manner as thapsigargin against Sacroplasmic reticulum Ca^{2+} transporting ATPases (SERCAs) of plasmdial PfATP6 but spares mammalian pumps.

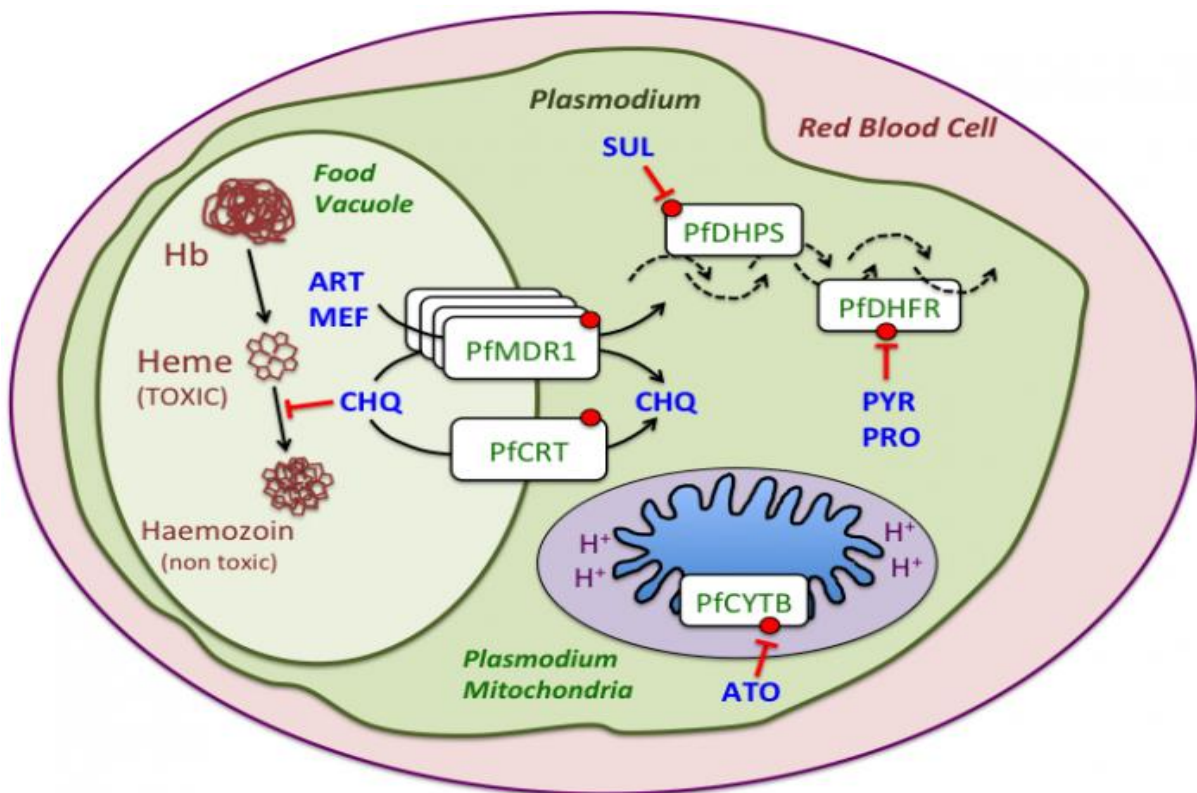


Figure 2.4: Mechanisms of Antimalarial Drug Action and Plasmodial Drug Resistance (tmedweb.tulane.edu).

Red circles= resistance to antimalarial drugs. (CHQ)= Chloroquine PfCRT= chloroquine resistance transporter, Hb= haemoglobin, T=drug target, PfMDR1= multidrug resistance protein-1, mefloquine MEF= mefloquine, ART= artemisinin derivatives, PYR= Pyrimethamine, PRO =Proguanil, PfDHFR= dihydrofolate reductase, SUL= Sulfadoxine, PfDHPS= dihydropteroate synthase, ATO =atovaquone, PfCYTB= cytochrome BC1.

2.12 Malaria and Chemotherapeutic Resistance

The limited anti-malarial armory is now severely compromised because of the parasite's remarkable ability to develop resistance to these compounds, increasingly limiting the control of this serious disease (Wernsdorfer and Pyne, 1991; Hyde, 2005). Resistance enables parasite strain to survive and/or multiply despite the administration and absorption of a drug given in recommended (or higher) doses. Resistance can thus be described as a shift in the concentration-effect (dose-response) relationship toward higher concentrations to inhibit parasite growth. (White, 1997).

According to Olliaro (2005) the emergence of resistance is a two-step process consisting of de novo selection of a genetic mutation or gene amplification in the presence of levels of drug that are inadequate to suppress their growth. In some cases, there are mutations in the gene encoding the drug target and especially multiple mutations of the same gene may accumulate, conferring increasing degrees of susceptibility to resistance.

De novo selection of genetic mutation is followed by the spread of resistance from one parasite to another, which requires that the sexual forms (gametocytes) occur and infect *Anopheline* mosquitoes, and that the resistance genotype is conserved during the sexual cycle (Olliaro, 2005). Lately, Diara and collaborators, by introducing extra copies of the Novel gene PF10 0355 from a resistant *P. falciparum* parasite into a drug-sensitive one rendered it resistant to 3 standard anti-malarial agents (Diara *et al.*, 2012). Until 2009, no noticeable clinical resistance to artemisinin drugs was reported. However, a number recent study has raised concerns about the efficacy of artemisinin combined therapies (ACTs), particularly in Southeast Asia (Jessica, 2009; WHO 2011).

2.13 Predisposing Factors

It is believed that the selection of parasites harboring polymorphisms, particularly point mutations, associated with reduced drug sensitivity, is the primary basis for drug resistance in malaria parasites (Hayton and Su, 2004; Hyde, 2007). Drug-resistant parasites are more likely to be selected if parasite populations are exposed to sub-therapeutic drug concentrations (Bloland, 2001).

White *et al.* (2009) emphasized a resistance selection opportunity in patients with low dosing and high parasite burdens. Similarly Boland (2001) reported that when blood drug concentration levels drop below the concentration that fully inhibits parasite growth, but remain above the concentration of drug that produces 5% inhibition of parasite growth, selection of resistant parasites occurs. Furthermore fraudulently manufactured drugs and/or drugs with spurious packaging are characterized by poor quality or low concentration of active pharmacological ingredient and create sub therapeutic levels of drug *in vivo* and therefore may lead to develop drug resistance (Harshul, 2013).

Olliaro (2005) emphasized on some pharmacological factors such as the drug's pharmacokinetic and pharmacodynamic characteristics, as well as its intrinsic propensity to generate resistance. Reliance on presumptive treatment can facilitate the development of antimalarial drug resistance by greatly increasing the number of people who are treated unnecessarily but will still be exerting selective pressure on the circulating parasite population (Oliver, 1991).

Earlier studies conducted by Ettling *et al.*, (1995) suggest that resistance rates are higher in urban and periurban areas than rural communities, where access to and use of drug is greater. Many anti-malarial drugs in current usage are closely related chemically therefore development of resistance to one can lead to cross resistance (Boland, 2001). Chloroquine and amodiaquine are both 4-aminoquinolines and cross-resistance between these two drugs is well known (Hall *et al.*, 1975; Basco, 1991). Feikins (2001) reiterated that this increased risk of resistance due to Sulphadoxine/Pyrimethamine use for treatment of malaria may even affect non-malarial pathogens as evident in the increased resistance to trimethoprim/sufamethoxazole among respiratory pathogen.

There is an interesting theory that development of resistance to a number of anti-malarial drugs among some falciparum parasites produces a level of genetic plasticity that allows the parasite to rapidly adapt to a new drug, even when the new drug is not chemically related to drugs previously experienced (Rathod *et al.*, 1997). The underlying mechanism of this plasticity is currently unknown, but this capacity may help explain the rapidity with which South-East Asian strains of falciparum develop resistance to new antimalarial drugs (Boland, 2001).

2.14 Mechanisms of *Plasmodial* Resistance

In recent years, significant progress has been made to unravel the genetic/molecular mechanisms underlying drug resistance in malaria parasites, and resistance has been well described for chloroquine, the antifolate combination drugs, and atovaquone (Boland, 2001). With signs of *Plasmodial* resistance to chloroquine first emerging in the 1950s, and widespread resistance reported late in the 20th century, the drug has now lost its potency in South-East Asia, Sub-Saharan Africa and South America (Ward and Boulton, 2013). *Plasmodial* resistance to chloroquine is related to an increased capacity for the parasite to expel chloroquine at a rate (40-50 times faster than in sensitive parasites), hence chloroquine cannot reach levels required for inhibition of haem polymerization (Foley and Tilley 1997).

Further evidence supporting this mechanism is provided by an earlier study conducted by Martin *et al.* (1987) who used verapamil to interfere with this efflux system and hence reversed chloroquine resistance. According to Fidock *et al.* (2000) there is now evidence from

isolates around the world that chloroquine resistance correlates with a single amino acid mutation (Lys→Tyr) at codon 76 (K76T) of the parasite *pfcr* gene.

Other satellite *pfcr* mutations occur, but their patterns tend to vary in different geographic areas (Wellem and Plowe,2001). Resistance to dihydrofolate reductase (DHFR) and dihydropteroate synthetase (DHPS) inhibitors is conferred by point mutations of the gene encoding for the respective enzymes, resulting in substitutions in the amino acid chain (Sibley *et al.*, 2001).Plowe *et al.* (1998) in their study identified DHFR and DHPS mutations found in isolates that fail to respond to pyrimethamine-sulfadoxine treatment. They reported mutations at codons 108 (Ser→Asn), 51 (Asn→Ile), 59 (Cys→Arg), and 164 (Ile→Leu) of the DHFR gene and mutations at codons 436 (Ser→Ala), 437 (Ala→Gly), 540 (Lys→Glu), 581 (Ala→Gly), and 613 (Ala→Thr/Ser) of the DHPS gene.

Nzila *et al.* (2000) reports that isolates carrying the triple mutation in DHFR (S108N, C59R, and N51I), with or without the double mutation in DHPS (A437G and A581G or K540E), are bound to cause treatment failure. Resistance to atovaquone develops at a redlatively high frequencyaffecting about 30% of individuals treated with atovaquone monotherapy (Korsinczky *et al.*, 2000; Winstanley,2001), however when combined with a second drug, such as proguanil (the combination used in Malarone) or tetracycline, resistance develops more slowly (Looareesuwan *et al.*, 1996).Given the sensitivity decline reports on the Thai–Cambodian border, a historic a site for emerging antimalarial-drug resistance, there are recent concerns about the efficacy of artemisinin based combination therapies (Alker *et al.*, 2007: Wongsrichanalai *et al.*, 2008:Arjen *et al.*, 2009).

A Cambodian study, led by Alker showed that increased numbers of copies of the *PfMDR1* gene(a well-established cause of mefloquine resistance), was associated with high failure

rates in artesunate–mefloquine therapy (Alker *et al.*, 2007). However, Arjen *et al.*,(2009) in their study, reports that despite artesunate resistance, the efficacy of artesunate–mefloquine use was excellent in Pailin-Western Cambodia. Inconclusively, they suggested that the forgoing outcome was attributed a large number (95%) of parasite isolates with a single *PfMDR1* copy.

2.15 The Way Forward

In spite of the associated complexities of plasmodial resistance, Olliero in his review accounts for some elements of a sensible strategy, they include;

The use of effective drugs uninfluenced by both economic reasons and inadequate information, encouragement and sustenance of Novel development of anti-malarial drugs, Protection of drugs against resistance in malaria by Combination therapy, Access to prompt, effective treatment and an update/change in the way national malaria treatment policies are made so as to provide alternative solutions to improve efficacy and cost (Olliero, 2005).

2.16 Current Trends in Anti-Malarial Therapy

2.16.1 Combination therapy

According to Naser (2007) the current practice in anti-malarial chemotherapy is multifaceted. Offering several advantages, the first line of action in combating malaria, is the optimization of therapy by combining two or more existing anti-malarial agent (Philip, 2003). Combination therapy in common use, include; artemisinin based combination therapy,

artovaquone/proguanil, amodaquine/sulfadoxine/pyrimethamine and chlorproguanil/dapsone (Naser, 2007).

Muheki *et al.* (2004) reported that the use of artemether-lumefantrine (Coartem) in an area of resistance to pyrimethamine-sulfadoxine resulted in very high cure rates (99%) and reduced gametocyte carriage 2 years after, malaria cases fell by 94% and costs by 88% due to the combined effects of the introduction of effective treatment as well as control measures. The forgoing result was in consonance with earlier studies on artesunate-mefloquine combination therapy by Nosten *et al.* (2000).

According to Kublin *et al.* (2000) another intriguing possibility is the reuse of chloroquine, ideally in combination regimens, in areas where it has not been used for an extended period. In view of the forgoing, Pfizer developed a fixed dose combination of Azithromycin (250mg) and Chloroquine (150mg) for prophylactic use during pregnancy. Data from phase III clinical trial indicates synergy and high efficacy in areas where chloroquine resistance is high (Olliro *et al.*, 2009). Phillip (2003) reiterated that the key advantage of chlorproguanil/dapsone is a relatively short half-life, long enough to provide effective therapy with 3-day daily dosing but short enough to readily select for resistance. He also suggested that its optimal use may be with the rapid potency of artesunate but definitive studies of this combination are still needed (Philip, 2003).

2.16.2 Chemical modification of existing anti-malarial agents

Improvement upon existing anti-malarials by chemical modifications of these compounds is another approach to improving chemotherapeutic outcome.

This strategy birthed novel anti-malarials. For example, chloroquine, primaquine and mefloquine were discovered through chemical strategies to improve upon quinine (Stocks *et al.*, 2001).

4-aminoquinolines that are closely related to chloroquine appear to offer the antimalarial potency of the parent drug, even against chloroquine-resistant parasites (Kaschula *et al.*, 2002). An 8-aminoquinoline, tafenoquine, offers improved activity against hepatic-stage parasites over that of the parent compound primaquine (Walsh *et al.*, 1999), and is effective for anti-malarial chemoprophylaxis (Naser, 2007).

The analog lumefantrine was developed from halofantrine due to its toxicity and is now a component of the new combination co-artemether (vanVugt *et al.*, 2000). Better tolerated in G6PD deficiency and a propensity for anti-relapse activity is Bulaquine a congener of primaquine (Naser, 2007). New folate antagonists (Tarnchompoo *et al.*, 2002) and new endoperoxides related to artemisinin (Vennerstrom *et al.*, 2000; Posner *et al.*, 2003) are also under study.

2.16.3 Exploration of ethno-based products

Currently, Plant-derived natural products are the sources of the two most important drugs currently available to treat severe falciparum malaria and folkloric knowledge/use of medicinal plants among natives from malarious regions continues to provide ethnomedical data (Shanker *et al.*, 2012).

From *in-vitro* and *in-vivo* studies, Uchoa *et al.* (2010) observed a high therapeutic index and confirmed the anti-malarial activity of fractions; chiefly beta-sitosterol and tormentic acid

from *Cecropia pachystachya* (Cecropiaceae) a plant species largely used to treat fever (including that caused by malaria parasites) and as food by native indigenous populations in the Amazon Region of Latin America.

An *in-vitro* and *in-vivo* assessment of the essential oils obtained from *Vanillosmopsis arborea*, *Lippiasidoides* and *Croton zehntneri*, revealed robust representation of Monoterpenes and Sesquiterpenes in the fractions. Individual essential oil constituents includes α -bisabolol, estragole, and thymol, which exhibited good activity against *P. falciparum* as well as low toxicity (Mota *et al.*, 2012). Botte' *et al.* (2012) reported that Molecules such as polyketide cycloperoxides isolated from the marine sponge, *Plakortis simplex* and xestoquinone from the sponge, *Xestospongia* sp., have antiplasmodial activity.

2.16.4 Anti-malarial evaluation of compounds active against other diseases

Although Folate antagonists, tetracyclines and other antibiotics were developed for their antibacterial properties, they were later found to be active against malaria parasites (Clough and Wilson, 2001). Atovaquone development was expedited by the discovery of its activity against *Pneumocystis*, its potential as an anti-malarial in combination with proguanil was re-explored, and found to have marked anti-malarial synergy (Canfield *et al.*, 1995).

It is thus expedient to screen new antimicrobial agents and other available compounds for anti-malarial activity, as they may provide a relatively inexpensive means of identifying new anti-malarials. (Gelb *et al.*, 2003).

2.16.5 Anti-sequestration compounds

Inhibition of cell adhesion processes is becoming increasingly interesting in the discovery of novel therapeutics (Simmons,2005). An interesting possibility lies in compounds which prevents the parasite from causing severe forms of the disease rather than killing, by way of hampering the parasite's ability to perform cytoadhesion as mediated by adhesins (Naser, 2007; Krinratunet *al.*, 2007). Sequestration in brain and placenta play an important role in the pathogenesis of cerebral and placental malaria, respectively (Rowe *et al.*, 2009; Rogerson *et al.*, 2010).

Efforts at inhibiting cytoadherence mediated by plasmodia receptors have been rewarded. CD36-mediated cytoadherence can be inhibited *in vitro* by antiretroviral such as; ritonavir and saquinavir (Nathoo *et al.*, 2003), and polysaccharides (carrageenans) derived from seaweed (Andrew *et al.*, 2005; Adams *et al.*, 2007). Using the crystal structure of intracellular adhesion molecule 1 (ICAM1), (+)-epigalloylcatechin gallate, a naturally occurring polyphenol compound in green tea, inhibited cytoadherence to ICAM1 in a dose-dependent manner (Dormeyer *et al.*,2006).

Although detailed molecular mechanisms of parasite adhesion is still unclear, the foregoing findings open up the possibility of developing therapeutic interventions aimed at blocking or reversing parasite adhesion (Brain and Rajeev, 2011).

2.16.6 Exploring drug resistance reversers

Although chloroquine- a first-line anti-malarial in most of the world appears to already have failed, this inexpensive, rapid acting, well-tolerated anti-malarial may be resurrected

by its combination with effective resistance reversers (Martin *et al.*, 1987; Bitonti *et al.*, 1988; Sowunmi *et al.*, 1997). Although unacceptably high concentrations of the resistance reversers are needed for their effects in many cases, however Van and colleagues demonstrated that combinations of two or more of these agents (verapamil, desipramine and trifluoperazine) at non-toxic concentrations may provide clinically relevant resistance reversal, hence restore chloroquine efficacy (Van Schalkwyk *et al.*, 2001).

Tri-cyclic Acridones possessing a short alkyl amine chain attached to the central nitrogen atom, in addition to its anti-malarial propensity, could make chloroquine resistant parasites susceptible to the drug again by means of blocking the PfCRT Pump protein (Kelly *et al.*, 2009).

2.16.7 Discovery of compounds active against new drug targets

In contrast to the cytosol, the Apicoplast of plasmodium species has a prokaryote-like genome and includes a number of biochemical pathways that are present in bacteria, plants and apicomplexan parasites but are absent in the human host and thus provide obvious opportunities for chemotherapy (Ralph *et al.*, 2001; Roos *et al.*, 2002; Istvan *et al.*, 2010).

Globin hydrolysis mediated by a number of classes of proteases appears to offer potential targets for chemotherapy (Haque *et al.*, 1999; Jiang *et al.*, 2001; Nezami *et al.*, 2002; Noteberg *et al.*, 2003). Falcipain inhibitors have been shown to prevent hemoglobin hydrolysis with the accumulation of intact hemoglobin in the food vacuole, inhibition of parasite development and curative activity (Rosenthal, 2001b; Rosenthal *et al.*, 2002; Batra *et al.*, 2003; Shenai *et al.*, 2003). Razakantoanina *et al.* (2000) demonstrated the *in-vitro* antimalarial activity of some *plasmodial* lactate dehydrogenase (pLDH).

In view of developmental demands, host dependent Synthesis of phosphatidylcholine (the most abundant lipid in plasmodial membranes) is one obvious target with great potential. Blockage of choline transport has been identified as a promising therapeutic strategy (Vial and Calas, 2001). From independent studies carried out by Calas *et al.* (2000) and Wengel *et al.* (2002), the lead compound-G25, inhibited the development of cultured *P. falciparum* parasites displaying an outstanding *in-vivo* therapeutic index.

Transport pathways unique to malaria parasites also constitute membrane targets. Although parasite transport mechanisms is not well understood (Haldar and Akompong, 2001; Kirk, 2001). Exploiting the selective transport of cytotoxic compounds into *P. falciparum*-infected erythrocytes was reported by Gero *et al.* (2003) using dinucleoside phosphate dimers conjugated to anti-malarial compounds to improve selective access to parasite targets. Using thiolactomycin, Waller *et al.* (1998) in an earlier study exploited the type II fatty acid biosynthesis pathway (β -ketoacyl-acyl-carrier protein-synthase-FabH) and showed that this antibiotic was active against cultured malaria parasites.

2.16.8 Malaria vaccine development

With the growing trend of both insecticide and anti-malarial drug resistance, the development of a malaria vaccine carries herculean expectations, about 6000—8000 malaria proteins have so far been identified, however few have been the subject of clinical trials (Philippe *et al.*, 2002). Vaccines can target different stages of the parasite's cycle offering protective immunity (Philippe *et al.*, 2002).

Though reports from experimental means of achievement have so far failed (Brown *et al.*, 2002; Hoffman *et al.*, 2002), moreover immunization with sporozoites was reported to confer

protective immunity (Riley *et al.*, 2013). In view of the forgoing, pivotal challenges include; substantial polymorphism in immunologically important regions of the proteins and low immunogenicity. Thus emphasis is being placed on molecules derived from the latter stages (Druilhe *et al.*, 1998). While asexual blood-stage vaccines aim at reproducing antibody-mediated protection acquired through repeated exposure to infection (Philippe *et al.*, 2002).

Gamete-stage vaccine aims to prevent mosquitoes that are feeding on an infected individual from acquiring and transmitting the parasite. This altruistic approach however does not confer protection on the vaccinated individual but contributes to protection in the community (Carter *et al.*, 2000). A completely effective vaccine is not yet available for malaria; the SPf66 developed by Pattarroyo did not show any efficacy (Nosten *et al.*, 1996; Bojang *et al.*, 1998). Other vaccine candidates, targeting the blood-stage of the parasite's life cycle such as; MSP1-3, AMA1, EBA 175 and Pfs25 have also been insufficient on their own (Graves and Gelband 2006).

The engineering of RTSS (Mosquirix) by Glaxo Smith Kline induced high antibody titers that block the parasite from infecting the live it is currently the most developed vaccine (Malaria vaccine initiative PATH, 2013). It provided protective immunity to 7 out of 8 volunteers challenged with *P. falciparum* as well as against both clinical and severe malaria in young infants (Agnandji *et al.*, 2012; Malaria vaccine initiative PATH, 2013). In view of the failures with initial available candidates, recent trends gravitate towards multistage vaccines that use combinations of components that are individually not sufficiently effective, hence the use of mixtures of five or more antigens (Hoffman *et al.*, 1998; Okenhouse *et al.*, 1998).

However this combo strategy must surmount; immunogenic, financial, safety, technical and analytical challenges (Philippe *et al.*, 2002). In view of the foregoing, the malaria vaccine advisory committee to the world health organization outlined a "Malaria Vaccine Technology Roadmap" that has as one of its landmark objectives: "to develop and license a first-generation malaria vaccine that has a protective efficacy of more than 50% and lasts longer than one year" by 2015 (Hoffman *et al.*; 2002).

2.16.9 Plasmodial Biochemistry

Elucidation of novel plasmodial biochemical pathways continues to open up drug targets crucial to plasmodial metabolism (Calas *et al.*, 2000; Roos *et al.*, 2002; Gero *et al.*, 2003; Shenai *et al.*, 2003). In view of the crucial role Aminoacyl-tRNA synthetases play in protein translation, Pham *et al.* (2014) acknowledged the considerable shift in research interest from bacteria and fungi aminoacyl-tRNA synthetases to that found in eukaryotic parasites.

Crystal structures have now been solved for many parasite tRNA synthetases, and opportunities for selective inhibition are becoming apparent. Istvan *et al.* (2011) showed that the *Plasmodium* cytosolic IleRS was inhibited by the isoleucine analogue-Thiaisoleucine whose rapid *ex-vivo* antimalarial activity was largely attributed to competition with isoleucine for binding to the synthetase prior to its incorporation into newly translated proteins.

According to Shibata *et al.* (2011) few differences exist in active site residues between parasite and host. An *in-vitro* study by Istvan *et al.* (2011) revealed that *P.falciparum*'s cytoplasmic IleRS (PF13_0179) is 47.5% identical to human IRS, whereas its apicoplast IRS (PFL1210w) is more similar to bacterial IRS. Although Thiaisoleucine was not considered a potential drug lead due to its limited potency against parasites and possibly low therapeutic

index, it was regarded a valuable tool for studying Isoleucine acquisition and show that simple isoleucine analogs can be incorporated by cytosolic IRS resulting in parasite toxicity. A potential source of concern is the development of bacterial resistance and treatment failure reported for this drug target (Patel *et al.*, 2009; Ranade *et al.*, 2013).

According to Holm *et al.* (1996), approximately one-third of proteins are metalloproteins, which serve to execute a wide array of functions in vivo, including regulating blood pH, facilitating matrix degradation, modulating DNA transcription, and many others.

Given the importance of these functions, metalloenzyme misregulation plays a significant role in human disease, and its inhibition offers an appealing approach to disease treatment (Day and Cohen, 2013). Typically, metalloenzyme inhibitors especially hydroxamic acids, are drug-like small molecules that incorporate a metal binding group (that is, the hydroxamate moiety) in order to coordinate the active site metal ion, leading to enzyme inactivation (Day and Cohen, 2013).

The zinc metalloenzymes; M1 alanine aminopeptidase (PfM1AAP) and M17 leucine aminopeptidase (PfM17LAP) are believed to play a role in the terminal stages of digestion of host hemoglobin, generating a pool of free amino acids that are essential for parasite growth and development (Skinner-adams *et al.*, 2012). Furthermore, Skinner-adams *et al.* (2012) reported that the experimental hydroxamate based compound-CHR-2863 is a potent inhibitor ($K_i = 2.4 \mu\text{M}$) of malaria aminopeptidases, binding strongly and chelating the active site's metal ion via the terminal hydroxamic acid region.

Similarly Umeda *et al.* (2011) reported that Fosmidomycin a hydroxamate based compound proven to be efficient in the treatment of *P. falciparum* malaria by inhibiting 1-deoxy-D-

xylulose 5-phosphate reductoisomerase (DXR), an enzyme of the non-mevalonate pathway, absent in humans. Crystal structures of fosmidomycin-bound complete quaternary complexes of *plasmodium falciparum* DXR revealed that *cis* arrangement of the oxygen atoms of the hydroxamate group of the bound inhibitor is essential for tight binding of the inhibitor to the active site metal. Indiscriminate metalloenzyme inhibition or metal ion dysregulation by metal ion removal from non-target metalloproteins, especially by structurally simple hydroxamate based compounds creates the perception of a greater risk in obtaining clinically successful therapeutics (Day and Cohen, 2012).

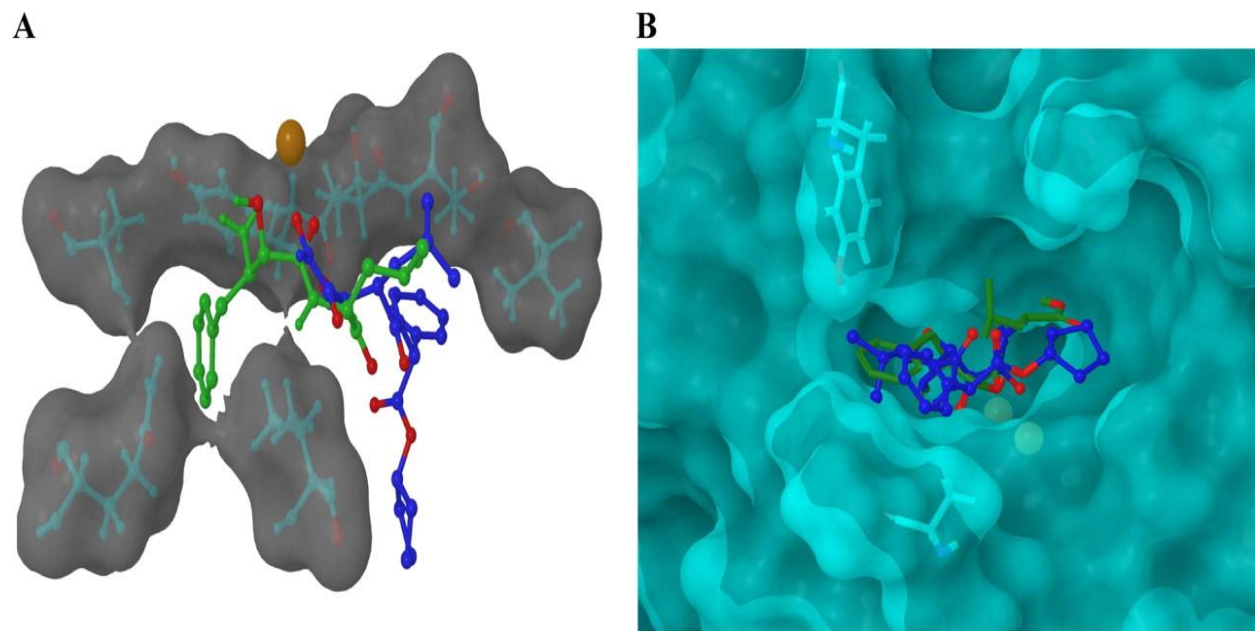


FIGURE 2.5: CHR-2863 and Bestatin within the binding site of *Plasmodium falciparum* M1 Alanine Aminopeptidase (Skinner-Adams *et al.* 2012)

These findings, provide the theoretical frame work for this research, that is, the evaluation of the potential of L-Isoleucine Hydroxamate to possibly interfere with the activity (inactivation) of enzymes critical to plasmodial biochemistry such as Isoleucyl tRNA

synthetase, Aminopeptidases, 1-deoxy-D-xylulose-5-phosphate reductoisomerase, ribonucleotide reductase, and so forth (Istvan *et al.*, 2011; Umeda *et al.*, 2011; Day and Cohen 2012; Skinner-adams *et al.*, 2012), there by arresting plasmodial intraerythrocytic growth to possibly cure malaria (Wiser 2013).

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Chemicals and Reagents

Chemicals used were of analytical grade and were sourced from the Protozoology Laboratory, Department of Veterinary Medicine, Ahmadu Bello University Zaria. They include; Normal saline, Formalin, Methanol, Xylene, Paraffin, Hematoxylin, Turk's solution, bouin's solution, Giemsa, and Glycerol. L-isoleucine hydroxamic acid acetate of analytical grade was purchased from BOC Sciences 14-16 Ramsey Road Shirley, New York 11967, U.S.A.

3.2 Equipment

Equipment include; Hematocrit centrifuge (Saitexiangyi TG 12MX), Hawkshy and sons Hematocrit reader, Micro haematocrit tubes (sodium heparinized Ref161313), Sky tech microscopic slides (25.4 x76.2mm), New improved Neubauer counting chamber, Microscope (Olympus CH XSZ-107BN), Digital thermometer (Kris Aloy 12-507G), Digital weighing balance (Mettler AE240), Microtome machine (Sorvall porter-blue LB-4), Digital weighing

scale (Labtech bl 20001), Adam clay laboratory counter, Hand held refractometer (RHC 200), Colorimeter (Optima AC114), On call glucometer (G133-211) and a Digital camera (Scopetech DCM 500).

3.3 *Plasmodiumberghei*

Chloroquine-sensitive strain of *Plasmodium bergheibergeri* (NK 65 strain) was obtained from the National Institute for Medical Research (NIMR), Lagos.

The parasite was suspended in normal saline and promptly intraperitoneally administered to 4 female mice whose whole blood were be used for the *in-vivo* anti-malaria evaluation.

3.4 Animal Procurement and Grouping

A total of 60 mice of about 4-5 weeks of age were obtained from the Faculty of Pharmaceutical Sciences Ahmadu Bello University, Zaria, Nigeria and subsequently kept in the faculty's animal house, having a temperature of 24 °C and humidity of 19 %. Mice were kept in groups of fifteen in separate cages and acclimatized for 1 week while they are allowed access to food and water *ad-libitum*. They were subsequently grouped in accordance with the phase requirement for the research. The grouping are as presented in the table below;

Table 3.1: Experimental Groups, Codes and Description

Experimental group	Code	Description
Test	ATGIH2000	Mice orally administered 2000mg/kg L- isoleucine hydroxamate in toxicity study.
Control	ACGD	Mice orally administered distilled water alone (0.2ml) in toxicity study.
Healthy control	HC	Un-parasitized Mice orally administered distilled alone (0.2ml).
Negative control	NCPD	Parasitized mice orally administered distilled alone (0.2ml).
Standard group	SGPC	Parasitized Mice treated with 10mg/kg chloroquine.
Treatment 1	TGPIH50	Parasitized Mice treated with 50mg/kg L-Isoleucine hydroxamate.
Treatment 2	TGPIH100	Parasitized Mice treated with 100mg/kg L-Isoleucine Hydroxamate.
Treatment 3	TGPIH200	Parasitized Mice treated with 200mg/kg L-Isoleucine hydroxamate.

3.5 Acute Toxicity Test

The fixed dose procedure (OECD 420) was used to determine the acute oral toxicity of L-Isoleucine hydroxamate. It does not use death as an end point, but instead uses observed definitive signs of toxicity developed at one of a series of fixed doses. Such dose is then classified according to the Globally Harmonized Classification System for Chemical Substances and Mixtures which cause acute toxicity (Organization for Economic Cooperation and Development, 2001). Reported Signs of toxicity for the main dose were; anesthesia, arching and rolling, salivation, tremors, diarrhea, convulsion, tremor and fatigue (Deora *et al.*, 2010).

3.5.1. Selection of starting and main dose

A sighting study was initially carried out, to allow selection of the appropriate starting dose to be used for the main study. In the sighting study, one nulliparous female mouse after being fasted for 5-hours was administered 300mg/kg Isoleucine Hydroxamate and observation for toxicity signs was carried out (Organization for Economic Cooperation and Development, 2001). Based on results from the sighting study, a healthy nulliparous mouse after being fasted for 5-hours was administered 2000mg/kg Isoleucine Hydroxamate via an intubation canula and observation for toxicity signs was carried out. During and after this period there

were no obvious signs of neither toxicity nor mortality respectively, 4 more mice in addition to the first were again fasted for 5 hours and thereafter orally dosed with 2000mg/kg Isoleucine Hydroxamate via an intubation canula (Organization for Economic Cooperation and Development, 2001).

At the end of the study, all the mice (including those that died during the test, or are removed from the study for animal welfare reasons) were subjected to gross necropsy. Blood collected from sacrificed animals was used for packed cell volume determination as well liver function assay, while the Liver was removed for histopathological analysis (Organization for Economic Cooperation and Development, 2001).

3.5.2 Weight changes

Body weights of the mice were recorded on day 0, 7, and 14. Weight changes at the end of the first week was calculated using the formulae;

Weight change= weight on day 7 - weight on day 0.

Similarly at the end of week two, which marked the end of the experimental phase, weight change was calculated using the formulae;

Weight change= weight on day 14 - weight on day 7. (Organization for Economic Cooperation and Development, 2001).

3.5.3 Packed cell volume measurement

Blood samples obtained from the caudal vein of both test and control groups were placed in heparinized capillary tubes and sealed at the dry end with sealing clay. The tubes were then placed in a micro-hematocrit centrifuge with the sealed end outwards and centrifuged for 5 min at 11,000 rpm. The tubes were then taken out of the centrifuge and packed cell volume (PCV) was determined using a standard Micro-Hematocrit Reader manufactured by Hawksley and Sons, England (Laychiluh *et al.*, 2014).

PCV=Volume of erythrocytes in a given volume of blood /Total blood volume.

3.5.4 Liver function test

The hepatotoxic effect of L-Isoleucine Hydroxamate was evaluated by measurement of serum alanine amino transferase (ALT) and Aspartate amino transferase (AST) which was determined using Accucare Assay kit (Teitz, 1976). For AST measurement, 1000µl of the accucare serum AST working reagent was mixed with 100 µl of sample and incubated for 1 minute at 37⁰C. Decrease in absorbance is measured per minute during 3minutes. The activity of the enzymes was calculated as shown below;

$$\text{AST (U/I)} = \text{Change in Absorbance per minute} \times 1746$$

For Serum ALT measurement, 1000µl of the accucare serum ALT working reagent and 100 µl of the sample were well mixed and incubated for 1minute at 37⁰C. At 340nm absorbance was read, and decrease in absorbance value was measured every 30seconds for 2minutes. The concentration of ALT in the sample was calculated as shown below;

ALT (U/I) = Change in Absorbance per minute \times 1746

3.5.5 Histopathological examination of the liver

Histological sections prepared from the liver samples suspended in 10% formalin solution and fixed in bouin's solution (mixture of 75 mL of saturated picric acid, 25 mL of 40% formaldehyde and 5 mL of glacial acetic acid) for 12 hours and then embedded in paraffin and cut into 5 micrometer thick sections using a microtome machine, the cut sections were stained using hematoxylin-eosin dye and finally mounted in diphenyl xylene. The sections were then observed under the microscope for histopathological changes in the liver architecture (Greaves, 2007).

3.6 *In-vivo* Antimalarial Evaluation

3.6.1 Grouping and dosing of animals

For evaluating the Anti-plasmodial activity of isoleucine hydroxamate, 25 mice were randomly divided into five groups having 5 mice each. The study on the curative activity of L-Isoleucine Hydroxamate was conducted using 15 Mice. The Mice were randomly assigned into three groups, 5 mice per group while the Negative control group (NCPD) was orally administered 0.2ml distilled water, while the TGIH200 group was orally administered 200 mg/kg Isoleucine Hydroxamate, and the SGPC group was treated with 10mg/kg chloroquine (Ishih *et al.*, 2006).

3.6.2 Determination of total red blood count

Total erythrocyte count in donor mice was performed using a Neubauer hemacytometer. Heparinized whole blood was collected in a 20 μ L pipette, diluted with normal saline and gently inverted repeatedly to mix.

The ruled area of the counting chamber was covered with a cover slip and the tip of the pipette was placed between the ruled area and the cover slip at 45 degrees to spread the diluted blood. The chamber was placed on the stage of the microscope and allowed 2 minutes for the cell to settle. Counting was done using the x 40 objective lens (Laychiluh *et al.*, 2014).

The total number of red cells was estimated as shown below;

The total number of red cells = $N \times 10,000$.

Where N is the number of red cells found in the 5 squares.

3.6.3 Determination of average parasitemia of donor mice

Clean, well labelled glass slides were used to make thin blood smears from a drop of blood from the caudal vein of each donor mouse. The smears were air dried and subsequently fixed in methanol for 5 minutes, and then stained in freshly Prepared Giemsa stain for 25 minutes. These stained slides were then washed thrice with water and air dried. The slides were oiled at the body region of the smear and then examined for ring forms as well as trophozoites under an Olympus microscope using an oil immersion nose piece of 100 x magnification. The average % parasitemia for each mouse was determined by taking the ratio of the number of parasitized red blood cells to non-parasitized red blood cells generated from 7 microscopic fields (Hilou *et al.*, 2006), as shown below;

Average % Parasitemia= $A/B \times 100$

Where; A=Number of parasitized red blood cell in microscopic field.

B= Number of non-parasitized red blood cell in microscopic field.

3.6.4 Standard inoculum preparation and inoculation

After obtaining the average % parasitemia as well as the total blood count for the donor mice, the number of Parasitized red blood cell/ μl (thin blood film) was calculated by multiplying the average % parasitemia by the Total blood count. The resulting value was then converted to ml. The total blood obtained was diluted with 13.8ml of normal saline to yield approximately 1.5×10^7 million parasitized Erythrocytes per 0.2ml. This volume served as the standard inoculum and was intraperitoneally administered (Summiya, 2009).

3.6.5 Peter 4-day suppressive test

This test was used to evaluate the antiplasmodial activity of L-Isoleucine hydroxamate against *Plasmodium berghei* infected mice (Peter *et al.*, 1975). Infected mice were randomly divided into their respective groups as described under grouping and dosing. Treatment commenced three hours after mice had been inoculated with the parasite on day 0 and then continued daily for four days from day 0 to day 3. After treatment was completed, thin blood film was prepared from the tail of each animal on day 4 to determine parasitemia and also percentage suppression (Kalra *et al.*, 2006). Some selected parameters such as; packed cell volume, blood glucose and plasma protein associated with malaria infection were assayed.

Where; Average % suppression= $\{(A-B)/A\} \times 100$

A= Average % parasitemia in control group.

B= Average % parasitemia in treated group.

3.6.6 Rane's test

Evaluation of the curative potential of the most active dose in Peter's test was carried out as described by Ryley and Peters (1995). On Day 0, the standard inoculum was intraperitoneally administered in all the mice, except the healthy control. Seventy-two hours later, the respective treatment protocols for the treatment groups commenced and lasted for four days. Geimsa stained thin blood film was prepared from the tail of each mouse intermittently for 3 days to monitor parasitemia level. Some selected parameters such as packed cell volume, blood glucose and plasma protein associated with malaria infection were assayed (Ryley and Peters, 1995).

Mean survival time for each group was determined arithmetically by calculating the average survival time (days) of mice starting from date of infection over a period of 30 days (Christian *et al.*, 2014).

3.6.7 Blood glucose measurement

Blood glucose was measured using a glucometer which uses transient electric current formed in the reaction cell to calculate the blood glucose concentration. After calibration with

Control solution 1, whole blood obtained from the caudal vein was applied to the strip's sample well. And reading was taken when displayed.

3.6.8 Plasma protein measurement

Plasma protein of all experimental subjects was measured using a hand held refractometer (George, 2001). It simply measures refractive index, or the degree to which light is slowed in a solution converting such index to protein concentration in g/100ml. Blood plasma protein is the dominant solute; thus the contribution of other components such as electrolytes, glucose, urea, and lipids have a minimal effect on the refractive index.

Calibration was performed using distilled water. A drop of distilled water was put on the glass plate and after 30 seconds, thermal harmonization was achieved and the scale was zeroed. Spun capillary tubes containing whole blood were carefully broken above the buffy coat area and the plasma smeared on the glass plate, while holding the plastic cover in place, the refractometer was pointed towards sunlight. The sharp horizontal line between the bright and dark area was used to read and record the plasma protein concentration.

3.6.9 Monitoring of body weight and temperature changes

For Peter's test and Rane's test, body weight of each mouse was measured before infection (day 0) and on day 4 after the tests, using a digital weighing balance. Rectal temperature was also measured using a digital thermometer before infection and at the end of the tests.

3.7 Statistical Analyses

Data obtained were expressed as mean \pm standard deviation (SD). Data were analyzed using Windows IBM SPSS Statistics 20. One-way analysis of variance (ANOVA) followed by Duncan *post-hoc* test was used to determine statistical significance

for comparison of %parasitemia, 0%suppression, blood glucose concentration, plasma protein, weight, Packed cell volume, rectal temperature and survival time among groups. P-value <0.05 was considered statistically significant.

CHAPTER FOUR

4.0 RESULTS

4.1: Oral Acute Toxicity of Isoleucine Hydroxamate and Classification

Table 4.1 show that the limit dose of L-Isoleucine Hydroxamate used in the main study resulted in neither observable toxic signs nor death, within 24hours of monitoring, hence the acute oral toxicity of L-Isoleucine Hydroxamate is greater than 2000mg/kg body weight. And its acute oral toxicity hazard was categorized as 5/unclassified based on the global harmonized system for the classification for Chemical Substances and Mixtures (Organization for Economic Cooperation and Development, 2001).

Death was however recorded for treatment subjects 1 and 3 on days 10 and 12 respectively during the 14-day period of sub-acute toxicity evaluation.

Table 4.1: Acute Oral Toxicity of L-Isoleucine Hydroxamate

GSH classification Of main dose	Main study dose (mg/kg)	Subjects	OTS (minute)	DTS (minute)	A/R	CONV	F	D	S	COM	DD (Day)
5/unclassified	2000	S ₁	-	-	-	-	-	-	-	-	10
		S ₂	-	-	-	-	-	-	-	-	-
		S ₃	-	-	-	-	-	-	-	-	12
		S ₄	-	-	-	-	-	-	-	-	-
		S ₅	-	-	-	-	-	-	-	-	-

GHS=global harmonized system, S= experimental subject, OTS= onset of toxic sign, DTS= duration of toxic sign,- = no observable effect, A/R= Arching and or rolling, CONV= convulsion, F=fatigue, D= diarrhoea, S= salivation, COM= comma, DD= delayed death.

L-Isoleucine Hydroxamate at the limit dose permitted (2000mg/kg) is classified as category 5/unclassified since it resulted in neither observable toxic signs nor death within 24hours of monitoring.

4.2:Effect of Oral Administration of L-Isoleucine Hydroxamate on Serum ALT,Serum AST, Weight Change and Packed Cell Volume in Mice

Table 4.3 shows that there is no significant difference ($P>0.05$) in serum ALT activity between the test group (8.57 ± 2.91 U/I) and control group (7.44 ± 3.68 U/I). For AST activity, there was also no significant difference between the test group (16.23 ± 6.02 U/I) and the control group (15.26 ± 5.11 U/I).

Mean Weight changes after the sub-acute toxicity evaluation was not significantly different ($P>0.05$) when the test group (3.53 ± 1.27 g) and control group (3.98 ± 1.83 g) were compared. Similarly there was no significant difference ($P>0.05$) in packed cell volume when the test group ($57.33\pm 2.52\%$) and control group ($55.80\pm 4.71\%$) were compared.

Plate I, II and III shows hepatocyte architecture for a control and 2 test subjects respectively. A non-significant change in hepatocyte architecture when the control group was compared with the test group was observed. Furthermore Plate IV show clumping of erythrocytes which was observed for test subjects whereas that for the control showed a normal distribution of erythrocytes as shown on plate V.

Table 4.2: Effect of Oral Administration of L-Isoleucine Hydroxamate on Serum ALT, Serum AST, Weight Change and Packed Cell Volume.

	Experimental groups	Mean
Serum ALT (U/I)	Control	7.44±3.68 ^a
	Test	8.57±2.91 ^a
Serum AST (U/I)	Control	15.26±5.11 ^a
	Test	16.23±6.02 ^a
Weight change (g)	Control	3.98±1.83 ^a
	Test	3.53±1.27 ^a
Packed Cell Volume (%)	Control	55.80±4.71 ^a
	Test	57.33±2.52 ^a

Values are Mean ± Standard Deviation, values for each parameter with different superscript are significantly different (P<0.05) down the column, n= 5, ALT=Alanine aminotransferase, AST=aspartate aminotransferase

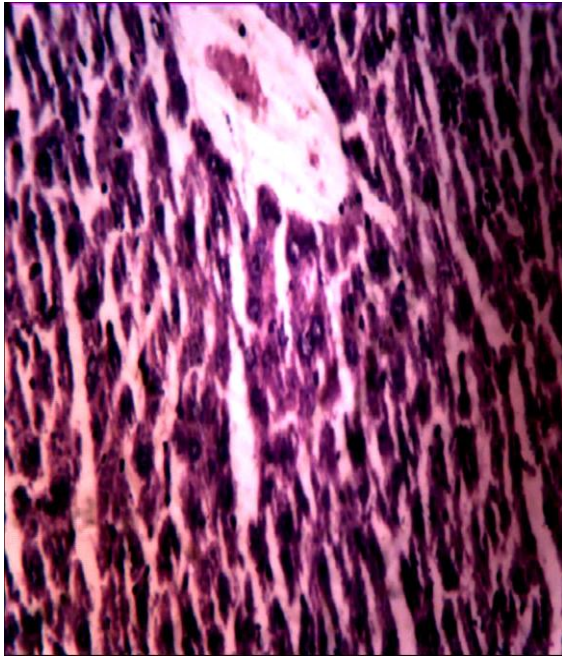


Plate I: Hematoxylin and Eosin Stained Liver Section Prepared from a Control Subject

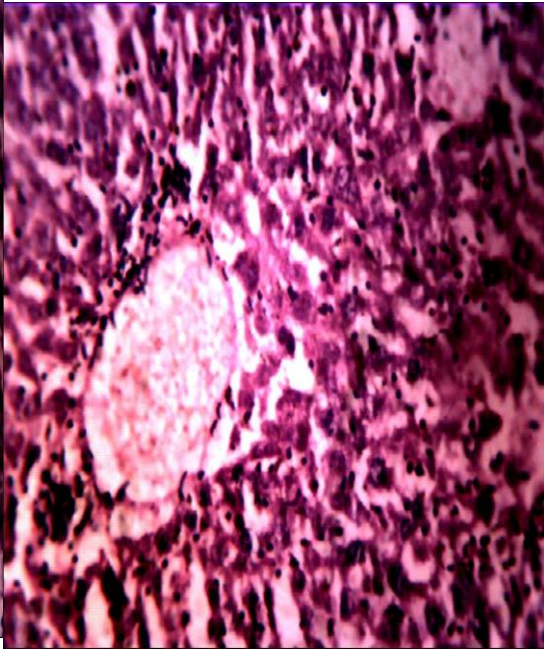


Plate II: Hematoxylin and Eosin Stained Liver Section Prepared from Test subject 2

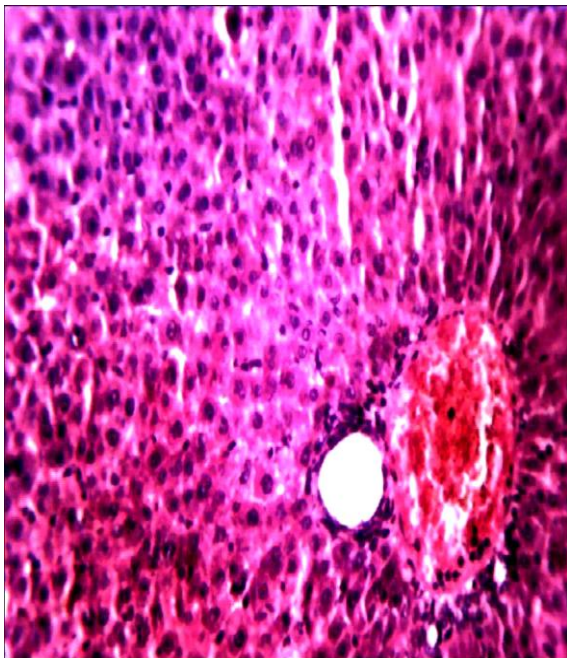
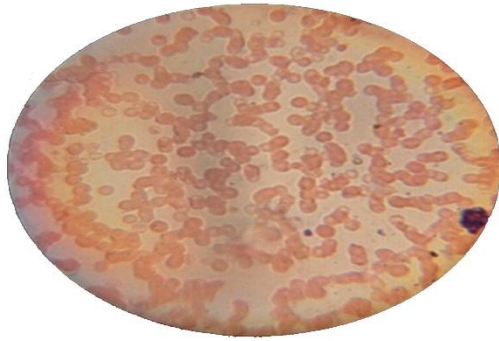


Plate III: Hematoxylin and Eosin Stained Liver Section of a Test Subject 1

Magnification= x100 oil immersion objective lens



Thin Blood Film Prepared from Test Subject-2

Plate IV: Giemsa Stained

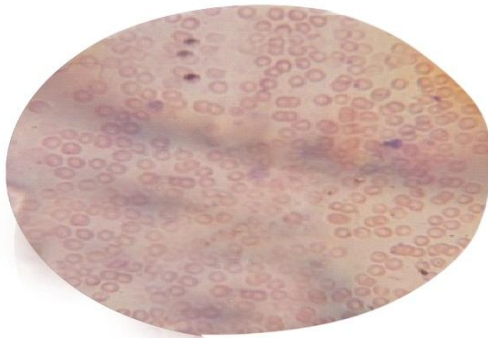


Plate V: Giemsa Stained Thin Blood Film prepared from a Healthy Control Subject.

Magnification= x100 oil immersion objective lens.

4.3(a): Effect of Graded Doses of Isoleucine Hydroxamate on some selected Parameters in Parasitized Mice after Peters 4-Day Suppressive Test.

The effect of chloroquine as well as the graded doses of Isoleucine Hydroxamate on some selected parameters which are usually altered in malaria infection is shown in Table 4.3(a). After the Peter 4-day suppressive test, there was no significant difference ($P>0.05$) when the blood glucose concentration for the standard group (152.4mg/dl), L-Isoleucine Hydroxamate treatment groups; TGPIH200 (198mg/dl), TGPIH100 (182mg/dl), TGPIH50 (169mg/dl) and Negative control group (165mg/dl) were compared. Fasting blood glucose concentration for the healthy control group (263.29mg/dl) was significantly ($P<0.05$) higher when compared to the rest of the groups.

The plasma protein concentration for the standard group (6.6g/100ml), and L-Isoleucine Hydroxamate treatment groups; TGPIH200 (6.04g/100ml), TGPIH100 (5.5mg/100ml), and TGPIH50 (6.0mg/100ml) were not significantly different ($P>0.05$) from both the healthy control group (7.10g/100ml) and the negative control group (4.8g/100ml).

Packed cell volume for the negative control group (19.00%) was significantly lower ($P<0.05$) than the TGPIH200 group (26.00%) and TGPIH50 group (30.52%) but not the TGPIH100 group (20.72%). No significant difference ($P>0.05$) was observed between the TGPIH100 group and TGPIH200 group, as well as between the TGPIH200 group and TGPIH50 group. The Standard group (45.80%) recorded significantly lower ($P<0.05$) PCV value than the Healthy control group (52.00%), however both the standard group and healthy control group had significantly higher ($P<0.05$) PCV values when compared with the rest of the groups.

Rectal temperature for the negative control group (34.56⁰C) was significantly ($P < 0.05$) lower than the rest of the experimental groups. There was however no significant ($P > 0.05$) difference when the various L-Isoleucine Hydroxamate treatment groups were compared; TGPIH200 (35.98⁰C), TGPIH100 (35.7⁰C) and TGPIH50 (35.9⁰C). L-Isoleucine Hydroxamate treatment groups recorded significantly ($P < 0.05$) lower rectal temperature when compared to the Standard group (37.9⁰C) and the Healthy control group (38.24⁰C), which did not differ significantly ($P > 0.05$) in their rectal temperature values. There was no significant difference ($P > 0.05$) in body weight, when all the experimental groups were compared.

Table 4.3(a): Effect of Oral Administration of L-Isoleucine Hydroxamate and Chloroquine on some Selected Parameters in Parasitized mice after Peter 4-day Suppressive Test

Groups	Parameters				
	BG (Mg/dl)	PPr (g/100ml)	PCV (%)	RT (⁰ C)	BW (g)
HC	263.29±90.27 ^b	7.10±1.1 ^b	52.00±3.06 ^c	37.90±0.34 ^c	18.34±0.60 ^a
NCPD	165.40±31.24 ^a	4.80±0.5 ^a	19.00±6.60 ^a	34.56±0.50 ^a	15.20±2.28 ^a
SGPC	152.40±17.16 ^a	6.60±0.8 ^{ab}	45.80±1.9 ^d	38.24±0.46 ^c	17.96±1.90 ^a
TGPIH 200	198.00±18.90 ^a	6.04±0.9 ^{ab}	26.00±3.81 ^{bc}	35.98±0.18 ^b	17.56±3.34 ^a
TGPIH 100	182.40±13.69 ^a	5.52±1.1 ^{ab}	20.72±6.30 ^{ab}	35.70±0.93 ^b	16.20±3.34 ^a
TGPIH 50	169.20±53.03 ^a	6.26±1.6 ^{ab}	30.52±5.94 ^c	35.90±1.58 ^b	15.20±2.77 ^a

Values are Mean ± Standard Deviation ,Values with different superscript are significantly different(P<0.05) down the column, n= 5, HC=Healthy Control group, NCPD= Negative Control group given distilled water, SGPC=standard group treated with10mg/kg chloroquine phosphate, TGPIH; 200, 100, 50= treatment groups dosed with 200mg/kg/day, 100mg/kg/day and 50mg/kg/day L-Isoleucine Hydroxamate respectively, BG= Blood Glucose, PPr=Plasma Protein, PCV=Packed Cell Volume, RT= Rectal Temperature, BW=Body Weight.

4.3 (b): Effect of Graded Doses of Isoleucine Hydroxamate on some selected Parameters in Parasitized Mice after Rane's Test.

The effect of the graded doses of L-Isoleucine Hydroxamate and Chloroquine on some selected parameters in parasitized mice after the Rane's test is shown in table 4.4. The blood glucose concentration for the negative control group (215.20mg/dl) and the TGPIH200 group (206.25mg/dl) was not significantly ($P>0.05$) different from both the standard group (148.80mg/dl) and the healthy control group (263.29mg/dl). Although the healthy control group had a significantly higher ($P<0.05$) blood glucose concentration when compared with the standard group.

For Plasma protein concentration, there was no significant difference ($P>0.05$) between the negative control group (5.40g/100ml), TGPIH200 group (5.5g/100ml) and the standard group (6.4g/100ml). Also Plasma protein concentration for the standard group was not significantly different from the healthy group (7.05g/100ml).

The negative control group had the lowest Packed Cell Volume (16.20%), which was however not significantly different ($P>0.05$) from the TGPIH200 group (20.50%). Packed cell volume for the Standard group (40.80%) as well as the healthy control group (52.00 %) were significantly higher ($P<0.05$) than the rest of the groups. There was however no significant difference ($P>0.05$) when the packed cell volume was compared between the standard group and healthy control group.

Both the negative control and the TGPIH200 treatment group recorded rectal temperature values which did not differ significantly ($P>0.05$) from each other (35.22⁰C and 35.67⁰C respectively). These values were significantly lower when compared to the Standard group (37.90⁰C) and Healthy control group(38.02⁰C).

There was no significant difference ($P>0.05$) in body weight throughout the groups as the negative control group, TGPIH200 group, the standard group and the healthy control group recorded; 16g 16.25g, 16.6g and 17g respectively.

Table 4.3(b): Effect of Oral Administration of L-Isoleucine Hydroxamate and Chloroquine on some selected Parameters in Parasitized Mice after Rane's Test

Parameters					
Experimental Groups	BG (mg/dl)	PPr (g/100ml)	PCV (%)	RT (⁰ C)	BW (g)
HC	263.29±90.2 ^b	7.06±1.1 ^b	52.00±3.05 ^b	37.90±0.3 ^b	17.01±0.45 ^a
NCPD	215.20±40.8 ^{ab}	5.40±0.42 ^a	16.20±3.90 ^a	35.22±1.7 ^a	16.00±1.41 ^a
SGPC	148.80±28.2 ^a	6.40±0.5 ^{ab}	40.80±7.46 ^b	38.02±0.1 ^b	16.60±1.34 ^a
TGPIH200	206.25±67.7 ^{ab}	5.50±0.4 ^a	20.50±2.08 ^a	35.68±0.7 ^a	16.25±2.06 ^a

values are Mean ± Standard Deviation, values with different superscript are significantly different (P<0.05) down the column, n= 5, HC=Healthy Control group, NCPD= Negative Control group that received distilled water only, STD=standard group treated with 10mg/kg chloroquine phosphate, TGPIH200= treatment group dosed with 200mg/kg Isoleucine Hydroxamate, BG= Blood Glucose, PPr=Plasma Protein as determined by Refractometer, PCV=Packed Cell Volume, RT= Rectal Temperature, BW=Body Weight.

4.4: Antiplasmodial Activity of the Oral Administration of the Graded Doses of L-Isoleucine Hydroxamate after Peter's 4-Day Suppressive Test.

Result from the peter's 4-day suppressive test is shown in Table 4.5 which showed that the Standard group that received 10mg/kg chloroquine had the lowest parasitemia value (5.80%), which was not significantly different ($P>0.05$) from the TGPIH200 group (20.45%). Parasitemia value for the TGPIH200 group was lower but not significantly different ($P>0.05$) from the rest experimental groups; TGPIH100 group (23.63%), TGPIH50 group (29.76%) and Negative control group (34.96%).

The Standard group thus recorded the highest plasmodial suppression (81%) while a dose dependant relationship for plasmodial suppression was observed for the various L-Isoleucine Hydroxamate treatment groups; TGPIH 200 (45%), TGPIH100 (42%) and TGPIH 50 (22%).

Table 4.4: Antimalarial activity of Chloroquine and L-Isoleucine Hydroxamate in Early Malaria Infection in Mice (%)

Experimental Group	Parasitemia	Suppression
NCPCD	34.96±9.10 ^b	Nil
SGPC	5.83±2.21 ^a	81
TGPIH 50	29.76±18.93 ^b	22
TGPIH100	23.63±9.55 ^b	42
TGPIH200	20.45±6.05 ^{ab}	45

Values are Mean ± Standard Deviation, Values with different superscript are significantly different (P<0.05) down the column, n= 5, NCPC= negative control group given distilled water only, SGPC=standard group treated with 10mg/kg Chloroquine phosphate, TGPIH;50, 100, 200= treatment groups dosed with 50mg/kg/day, 100mg/kg/day and 200mg/kg/day L-Isoleucine Hydroxamate respectively.

4.5: Curative Effect of L-Isoleucine Hydroxamate in Established Malaria.

Table 4.6 shows Parasitemia evaluated at an interval of one day, beginning from day-0, which marked the pre-patent period. Initial parasitemia values taken for the experimental groups reveals that the standard group (39.64%), had a significantly higher ($P < 0.05$) parasitemia value compared to the rest groups. There was no significant difference ($P > 0.05$) between the negative control group (14.20%) and the TGPIH200 group (21.12%).

On Day-2 of treatment, parasitemia value for the TGPIH200 group (49.94%) was not significantly different from the standard group (42.87%), while the negative control recorded a significantly higher ($P < 0.05$) parasitemia value (87.97%).

On Day-4 of treatment, the TGPIH200 treatment group (18.09%) lost plasmodial suppression and thus had a percentage parasitemia value which was significantly higher ($P < 0.05$) than the negative control group (6.19%) and the standard group (0.00%) which showed complete suppression.

After treatment, observation for mortality within a 28 day period from the point of infection was conducted. Table 4.7 shows that there is no significant difference ($P > 0.05$) in survival time in days between the TGPIH 200 group that survived 19.20 days and the negative control group that survived for 19.60 days. The standard group had a significantly ($P < 0.05$) longer survival time which exceeded the duration of observation.

Table 4.5: Curative Activity of L-Isoleucine Hydroxamate and Chloroquinein Established Malaria Infection in Mice. (%)

Experimental group	Day-0		Day-2		Day-4	
	Average Parasitemia	Chemo Suppression	Average Parasitemia	Chemo-Suppression	Average Parasitemia	Chemo-Suppression
NCPD	14.20±7.87 ^a	X	87.97±40.35 ^a	x	6.19±1.30 ^a	x
SGPC	39.64±22.40 ^b	X	42.87±40.75 ^a	51	0.00±0.0 ^a	100
TGPIH200	21.12±11.00 ^a	X	49.94±6.78 ^a	43	18.09±9.0 ^b	-192

Values are Mean ± Standard Deviation, values with different superscript are significantly different (P<0.05) down the column, n= 5 NCPD= Negative Control group that received distilled water only, SGPC=standard group treated with 10mg/kg chloroquine, TGPIH200= treatment group dosed with 200mg/kg L-Isoleucine Hydroxamate, x = not determined

Table 4.6: Mean Survival Time of Experimental Groups after Infection

Experimental Groups	Survival time (days)
NC	19.60±4.39 ^a
STD	28.00±0.00 ^b
TGPIH 200	19.20±3.78 ^a

Values are Mean ± Standard Deviation, Values with different superscript are significantly different (P<0.05) down the column, n= 5, NC= Negative Control group given distilled water only, STD=standard group dosed with 10mg/kg chloroquine, TGPIH 200= treatment group dosed with 200mg/kg L-Isoleucine Hydroxamate

CHAPTER FIVE

5.0DISCUSSION

The study was conducted to ascertain the antimalarial activity of L-Isoleucine Hydroxamic acid acetate in mice infected with *Plasmodiumberghei*. Peter's 4-day suppressive test as well as Rane's test were pivotal tools in assessing the schizonticidal and curative activity of the test compound (Ryley and Peter, 1995). The rodent model of malaria was employed in this study for prediction of efficacy of anti-malarial effect of L-Isoleucine hydroxamate, this is substantiated by the fact that several mainstream anti-malarial agents been identified using rodent malaria model (David *et al.*, 2014). Chloroquine sensitive *P.berghei berghei* strain was used in the prediction of treatment outcomes; hence chloroquine was used as the standard drug in this study. The *in-vivo* model was employed for this study because it takes into account most importantly possible pro-drug effect (Waako *et al.*, 2005).

Oral Acute Toxicity

At the limit dose (2000mg/kg) used in the study, there was no significant difference ($P>0.05$) in ALT and AST activities, weight change and packed cell volume when the test and control groups were compared. This points to the relatively low oral toxicity hazard of L-Isoleucine hydroxamate and explains the non-appearance of toxic signs beyond the stipulated 24-hour period. Researchers had reported on the negligible toxicity associated with the amino acid hydroxamate-L-Glutamic acid (γ)monohydroxamate (Itzak *etal*; 1999; Ahmed *et al*; 2001).

Delayed toxicity recorded on day 10 and 12 might in part, be explained by observation made from Hematoxylin and Eosin stained liver sections (plates 1-III) and giemsa stained thin blood films from experimental subjects (plate IV-V). Kupffer cell hypoplasia was observed in liver sections while clumping of erythrocytes in thin blood film was observed.

Kupffer cells are closely associated with hepatic responses to infection, ischemia, toxins, acute and chronic response to toxic compounds, repair and regeneration of the liver (Bilzer *et al.*, 2006). This suggests that Isoleucine Hydroxamate probably elicit a toxic response in the liver, but such, is not sufficient to induce significant hepatic damage. The probable drug induced clumping of erythrocytes might have been due to Rouleux formation causing weak binding between cells as RBC surface now have dissimilar ionic charges (Thrall, 2004).

This clumping may have contributed not only to vascular congestion seen in the liver but also in critical blood vessels, as seen in sickle cell anemia which can lead to deep vein thrombosis /pulmonary embolism which is lethal (Center for Disease Control and Prevention, 2015). The onset and progression of malaria infection is characterized by notable alterations in hematological and biochemical parameters, and such alterations have been widely reported in malaria infection (Bidaki and Dalimi, 2003: Mishra and Mohanty, 2003: Udosen, 2003).

Blood Glucose

In early malaria infection, a lower but non-significant decrease ($p > 0.05$) in blood glucose concentration for the negative control when compared, especially to L-Isoleucine hydroxamate treatment groups was observed. Such decrease is most likely associated with extra energy demands for intra-erythrocytic development and replication as well as largely secondary to increased insulin secretion (Elased and Playfair, 1994).

Onyesome *et al.*, (2013) observed significant depression in both plasma and brain glucose in untreated group in *p. berghei* infected mice with respect to the control. In harmony with previous research work by Adeosun *et al.*, (2007) reduced blood glucose concentration for the standard group dosed with chloroquine was observed to be lower than the rest experimental group, such hypoglycemic effect have recently been found to stem from chloroquine's ability to stimulate glucose uptake and glycogen synthase through activation of protein kinase B (Halaby *et al.*, 2013).

Total Protein

This study also showed lower but non-significant ($p > 0.05$) decrease in the concentration of total protein of the negative control group. Earlier studies by Adekunle *et al.* (2007), Adebisi *et al.* (1998) and Adeosun *et al.* (2007) reported significant decrease of plasma total proteins in malaria infections. Adebisi *et al.* (2002) opined that such depletion of protein stores stems from anorexia which induces gluconeogenesis.

Packed Cell Volume

Packed cell volume (PCV) was measured to evaluate the effectiveness of the analogue in preventing haemolysis due to the escalating parasitaemia level. Packed cell volume was significantly reduced ($p < 0.05$) in the negative control group compared to the rest of the treatment groups. This could be as a result of increasing breakdown of red blood cells by the parasites, thus causing anaemia. Goselle *et al.* (2009) and Ogbodo *et al.* (2010) reported reduced PCV levels during malaria infection. L-Isoleucine Hydroxamate displayed significant anti-haemolytic activity in treated Mice. This finding is in agreement with observations from the administration of other hydroxamate based antimalarials such as; desferrioxamin B (Khalifa *et al.*, 1989), Fosmidomycin and the related Fosfomycin in G6PD patients. Borrmann *et al.*, (2004) thus opined that any observed case of haemolysis was disease related rather than drug induced.

Rectal Temperature

Rectal temperature was measured to evaluate the ability of the analogue to prevent a decrease in metabolic rate which manifests in a rapid drop in internal body temperature due to escalation in parasitemia (Mengiste *et al.*, 2012). A significant decrease ($P < 0.05$) in rectal temperature was observed when the negative control was compared with the rest treatment groups. This finding is in consonance with that of Basir *et al.* (2012) and Laychiluh *et al.* (2014), who buttressed that active compounds should prevent the rapid dropping of rectal temperature.

Body Weight

A non-significant ($P>0.05$) but lower body weight was observed in the negative control group compared to other treatment groups, this observation is consonance with findings of Langhorne *et al.*, (2002), they described Anaemia, body weight loss and body temperature reduction as the general features of malaria-infected mice. Moreover, malaria is often associated with extreme nausea and high fever, which can induce loss of appetite leading to weight loss. Also disturbed metabolic function and hypoglycemia are implicated factors (Basir *et al.*, 2012).

Antiplasmodium Activity of L-Isoleucine Hydroxamate in Early Malaria Infection

The Peter 4-day suppressive test is a standard test commonly used for antimalarial screening and the determination of percent suppression of parasitaemia is the most reliable parameter. A mean group parasitemia level of less than or equal to 90% of the negative control animals usually indicate that the test material is active in standard screening studies (Ryley and Peter, 1995). Antiplasmodium activity (plasmodial suppression) was observed at all doses orally administered. Increase in administered dose was associated with increase in plasmodial suppression, such dose dependent related effect has been widely reported. (Madara *et al.*, 2010; Builders *et al.*, 2012; Dawat and Yakubu, 2014; Laychiluh *et al.*, 2014).

According to Deharo *et al.*, (2001), *in-vivo* anti-plasmodial activity can be classified as moderate, good, and very good if an extract displayed percentage parasitemia suppression equal to or greater than 50% at a dose of 500mg/kg, 250mg/kg and 100 mg/kg body weight per day, respectively. L-Isoleucine Hydroxamate at 200mg/kg showed a good anti-plasmodial activity which possibly stems from inactivation, by bidentate ligand formation with particularly, aminopeptidases and other metalloenzymes such as 1-deoxy-D-xylulose-5-

phosphate reductoisomerase (Yajema *et al.*, 2004; Skinner-Adams *et al.*, 2012; Day and Cohen, 2013). Furthermore L-Isoleucine Hydroxamate might have possibly antagonized isoleucine utilization by Plasmodial IRS to either poison protein translation which is toxic to the parasite (Istvan *et al.*, 2010).

Antiplasmodial Activity of L-Isoleucine Hydroxamate in Established Malaria Infection

The most active dose observed in Peter's test was evaluated for curative antimalarial activity in established malaria (Ryley and Peters, 1995). Significant ($P < 0.05$) increase in percentage parasitemia and complete loss of plasmodial suppression on day 4 for the L-Isoleucine Hydroxamate treatment group compared to the negative control and standard groups may suggest that in established malaria, the possible mechanisms of antimalarial activity of L-Isoleucine hydroxamate, may not have been sufficient to result in a lasting suppressive action in order to bring about a curative response.

There is also the possibility that 200mg/kg L-Isoleucine hydroxamate was sub-therapeutic, hence a higher dose might be required for effectively suppress parasitemia, to bring about a curative response or that bioavailability of the amino hydroxamate was poor (Istvan *et al.*, 2011). Day and Cohen (2013), opined that structurally simple hydroxamate based inhibitors are often weak and exhibit off-target inhibition. Moreover, findings from Shalon *et al.*, (2012) may offer a possible explanation for this phenomenon. They demonstrated resilience of *in vitro P. falciparum* to starvation of Isoleucine.

A dramatic decrease in metabolic rate to conserve energy and resources was observed, hence there was increased survival once growth-permissive conditions were restored.

CHAPTER SIX

6.0 SUMMARY CONCLUSION AND RECOMMENDATIONS

6.1 Summary

This study was conducted to ascertain the antimalarial activity of L-Isoleucine Hydroxamic acid acetate in mice infected with *Plasmodiumberghei*. Peter's 4-day suppressive test as well as Rane's test provided requisite data used in the assessment of the schizonticidal and curative activity of the test compound. Findings from this study are summarized as follows;

- i) The global harmonized system of classification for L-Isoleucine hydroxamate is category 5/unclassified.
- ii) L-Isoleucine hydroxamate has a relatively low oral toxicity hazard, hence there was no significant ($P>0.05$) difference in the activities of the liver enzymes-ALT and AST, packed cell volume and weight change, when the control and test groups were compared.
- iii) In early malaria infection L-Isoleucine hydroxamate administration provided significant ($P<0.05$) increase in packed cell volume and rectal temperature when compared to the negative control. Fasting blood glucose concentration, plasma protein and body weight were although higher but not significantly ($P>0.05$) different from the negative control.
- iv) In established malaria infection L-Isoleucine hydroxamate administration did not provide significant increase in fasting blood glucose concentration, plasma protein, packed cell volume, rectal temperature and body weight compared to the negative control.
- v) In early malaria infection, L-Isoleucine hydroxamate administration showed antimalarial activity.

- vi) In established malaria infection L-Isoleucine hydroxamate administration lost its antimalarial activity, hence could not provide a lasting plasmodial suppression, which would have resulted in a curative response.

6.2 Conclusion

This study shows that L-Isoleucine Hydroxamate has antimalarial activity in early malaria infection in mice. However this antimalarial activity was lost in established infection.

6.3 Recommendation

- i) In view of the antimalarial activity observed for L-isoleucine hydroxamate, elucidating the pharmacological properties of the drug, especially its metabolism and molecular mechanism of antimalarial action is needful
- ii) The possibility for enhanced anti-malarial activity by combination with other existing anti-malarial drugs should be explored, especially those whose activity has been compromised by resistant strains.
- iii) The antimalarial activity for other routes of administration should be considered.
- iv) From a pilot study co-conducted, Leucine appears to serve as an adjuvant for the anti-malarial activity of Isoleucine Hydroxamate. Thus a structured experimental design to validate the forgoing claim is imperative.
- v) The anti-parasitic activity of Isoleucine Hydroxamate should also be explored for other *in-vivo* intracellular and extracellular parasites.

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APPENDICES

Appendix I: Total Erythrocyte count of donor mice

DMEC 1	DMEC 2	DMEC 3	DMEC4
20	22	83	90
22	12	60	84
21	27	68	64
29	30	67	65
32	24	52	61
$\Sigma= 124$	$\Sigma=215$	$\Sigma=330$	$\Sigma=364$

DMEC=Donor mice erythrocyte count

Total blood count/ μl =erythrocyte count x10,000

Appendix II: Average parasitemia of Donor Mice

MF	DM 1		DM2		DM3		DM4	
	NPC	NUPC	NPC	NUPC	NPC	NUPC	NPC	NUPC
1	40	119	19	86	52	102	109	300
2	42	106	21	108	45	121	87	267
3	39	72	26	82	69	109	86	277
4	44	98	40	94	75	137	63	210
5	55	97	40	105	64	135	90	335
6	47	135	30	102	69	125	60	276
7	43	122	23	586	73	141	31	130
Σ	310	749	199	663	447	870	526	1797
% parasitemia	41		30		50		30	

Where; DM= donor mouse, MF=Microscopic Field, NPC=Number of Parasitized Red blood Cell,

NUPC=Number of un-Parasitized Red blood Cell, % parasitemia= $\text{NPC}/\text{NUPC} \times 100$

Appendix III: Calculation of Standard Inoculum

Number of Parasitized red blood cell/ μl (thin blood film)

$$= \frac{\text{Total Number of counted asexual parasites in RBC} \times \text{total RBC count}/\mu\text{L of Mice}}{\text{Total number of counted healthy RBC in 7 fields}}$$

Total number of counted healthy RBC in 7 fields

$$199+310+526+447/663+749+1797+870 \times 1240000+2150000+3640000+3300000$$

$$= 1482/4079 \times 100330000$$

$$= 15309060000/4079$$

$$3753140 = \text{parasitized red blood cells} / \mu\text{l}$$

Thus in 2.2ml (total volume of blood obtained from 4 Mice), the number of parasitized

$$\text{Erythrocytes} = 3753140 \times 2.2\text{ml}/0.001\text{ml}$$

$$= 8256908000 \text{ parasitized red blood cells}/2.2\text{ml}$$

Total volume of blood required/40 mice (based on 0.2ml inoculums volume) = 8ml

Volume of dilution used = 13.8ml

Final volume=16ml

But how many parasitized red blood cells are in 13ml?

$$C_1V_1=C_2V_2$$

$$C_2= 8256908000 \times 2.2/16$$

$$= 1135324850 \text{ parasitized red blood cells}/16\text{ml}$$

Thus, In 0.2ml

1135324850 x 0.2/16 = 15137664 million parasitized red blood cells

~1.5x10⁷ million parasitized red blood cells.

Appendix IV: Microscopic Estimation of Parasitemia for NCPD Group
after the Peter 4-day Suppressive Test

NCPD Group								
MF	S1		S2		S3		S4	
	NPC	NUPC	NPC	NUPC	NPC	NUPC	NPC	NUPC
1	82	248	177	350	210	319	63	235
2	45	228	174	371	36	387	70	271
3	80	239	188	343	45	372	52	196
4	45	233	222	258	78	89	80	256
5	64	192	264	381	69	135	41	380
6	52	185	124	320	48	85	66	168
7	74	94	78	297	69	268	81	233
8	72	218	118	278	84	251	57	251
9	70	163	143	275	77	215	69	143
10	71	187	42	315	57	300	50	209
Σ=	655	1981	1530	3188	773	2421	629	2342

Where; S= Subject, MF=Microscopic Field, NPC=Number of Parasitized Red blood Cell, NUPC= Number of un-Parasitized Red blood Cell, % parasitemia=NPC/NUPC×100

Appendix V: Microscopic Estimation of Parasitemia for SGPC Group
after the Peter 4-day suppressive test

SGPC Group								
MF	S1		S2		S3		S4	
	NPC	NUPC	NPC	NUPCC	NPC	NUPCC	NPC	NUPC
1	40	352	12	376	26	314	33	328
2	49	371	10	382	13	331	16	259
3	18	335	21	368	12	242	3	367
4	36	387	9	385	17	231	12	305
5	25	410	8	351	12	352	20	320
6	40	311	20	338	25	347	49	338
7	41	359	23	377	16	368	16	257
8	17	297	9	381	16	366	0	360
9	21	335	7	358	12	357	5	226
10	26	365	15	378	23	297	9	216
Σ=	313	3522	134	3694	172	3305	163	2916

Where; S= Subject, MF=Microscopic Field, NPC=Number of Parasitized Red blood Cell, NUPC= Number of un-Parasitized Red blood Cell, % parasitemia= $\text{NPC}/\text{NUPC} \times 100$

Appendix VI: Microscopic Estimation of Parasitemia for TGPIH 200 group
after the Peter 4-day Suppressive Test.

TGPIH 200								
MF	S1		S2		S3		S4	
	NPC	NUPC	NPC	NUPC	NPC	NUPC	NPC	NUPC
1	38	276	81	216	20	120	25	261
2	17	178	44	165	18	110	57	110
3	21	201	55	206	45	115	28	263
4	13	202	66	304	22	141	30	197
5	19	216	51	278	35	156	38	243
6	39	273	78	242	32	162	34	227
7	31	241	49	325	42	152	71	133
8	22	248	54	300	28	115	99	248
9	36	237	55	279	36	102	83	175
10	31	255	56	327	35	109	25	219
Σ=	267	2327	589	2642	313	1282	490	2076

Where; MF=Microscopic Field, NPC=Number of Parasitized Red blood Cell, NUPC= Number of un-Parasitized Red blood Cell, % parasitemia= $\text{NPC}/\text{NUPC} \times 100$

Appendix VII: Microscopic Estimation of Parasitemia for TGPIH 100 group after Peter the 4-day Suppressive Test.

TGPIH 100								
MF	S1		S2		S3		S4	
	NPC	NUPC	NPC	NUPC	NPC	NUPC	NPC	NUPC
1	50	380	29	165	65	147	40	300
2	40	266	24	129	38	123	29	220
3	24	336	27	122	59	140	61	216
4	12	155	28	223	43	157	35	276
5	29	286	33	164	25	95	88	252
6	35	317	44	127	51	156	48	146
7	68	338	33	120	60	131	53	225
8	56	297	30	165	48	135	33	253
9	67	355	50	141	38	111	66	288
10	37	349	53	137	46	100	62	293
Σ=	420	3079	351	1493	473	1295	515	2469

Where; S= Subject, MF=Microscopic Field, NPC=Number of Parasitized Red blood Cell, NUPC= Number of un-Parasitized Red blood Cell, % parasitemia= $\text{NPC}/\text{NUPC} \times 100$

Appendix VIII: Microscopic Estimation of Parasitemia for TGPIH 50 group after the 4-day Suppressive Test.

TGPIH 50								
MF	S1		S2		S3		S4	
	NPC	NUPC	NPC	NUPC	NPC	NUPC	NPC	NUPC
1	69	192	38	115	22	430	168	291
2	54	247	26	131	28	350	132	300
3	73	182	29	103	25	375	176	312
4	69	209	66	198	20	400	164	299
5	75	179	64	191	24	358	191	323
6	72	214	52	178	21	425	108	223
7	61	186	48	203	19	381	170	400
8	55	210	50	155	28	332	180	394
9	61	164	14	62	27	358	153	256
10	50	194	28	160	26	333	166	262
Σ=	639	1977	415	1496	240	3742	1608	3060

Where; S= Subject, MF=Microscopic Field, NPC=Number of Parasitized Red blood Cell, NUPC= Number of un-Parasitized Red blood Cell, % parasitemia= $\text{NPC}/\text{NUPC} \times 100$

Appendix IX: Microscopic Estimation of Parasitemia for the NCPD Group after Rane's test on Day4.

MF	NCPD									
	S1	S2		S3		S4		S5		
	NPC	NUPC	NPC	NUPC	NPC	NUPC	NPC	NUPC	NPC	NUPC
1	1	45	11	200	8	55	7	180	6	209
2	1	70	9	170	5	52	14	220	21	231
3	1	75	17	235	10	62	28	250	17	225
4	4	70	24	218	1	53	10	200	11	196
5	4	75	10	173	1	70	13	180	20	240
6	7	70	8	62	3	60	9	150	15	150
7	3	40	6	74	2	100	21	150	13	235
8	5	55	15	205	6	180	10	200	10	246
9	5	55	17	217	4	220	16	250	9	219
10	5	75	4	83	3	180	8	250	21	215
Σ=	36	630	121	1637	43	1032	136	2050	153	2166

Where; S= Subject, MF=Microscopic Field, NPC=Number of Parasitized Red blood Cell, NUPC= Number of un-Parasitized Red blood Cell, % parasitemia= $\text{NPC/NUPC} \times 100$

Appendix X: Microscopic Estimation of Parasitemia for the SGPC Group
after Rane's test on Day 4.

MF	SGPC									
	S1	S2		S3		S4		S5		
	NPC	NUPC	NPC	NUPC	NPC	NUPC	NPC	NUPC	NPC	NUPC
1			0	250	0	320	0	300	0	274
2			0	400	0	350	0	250	0	348
3			0	450	0	250	0	200	0	410
4			0	300	0	200	0	380	0	367
5			0	400	0	300	0	320	0	300
6			0	350	0	450	0	250	0	400
7			0	600	0	400	0	300	0	287
8			0	520	0	300	0	350	0	350
9			0	300	0	350	0	300	0	430
10			0	350	0	280	0	400	0	328
Σ =			0	3920	0	3200	0	2630	0	3494

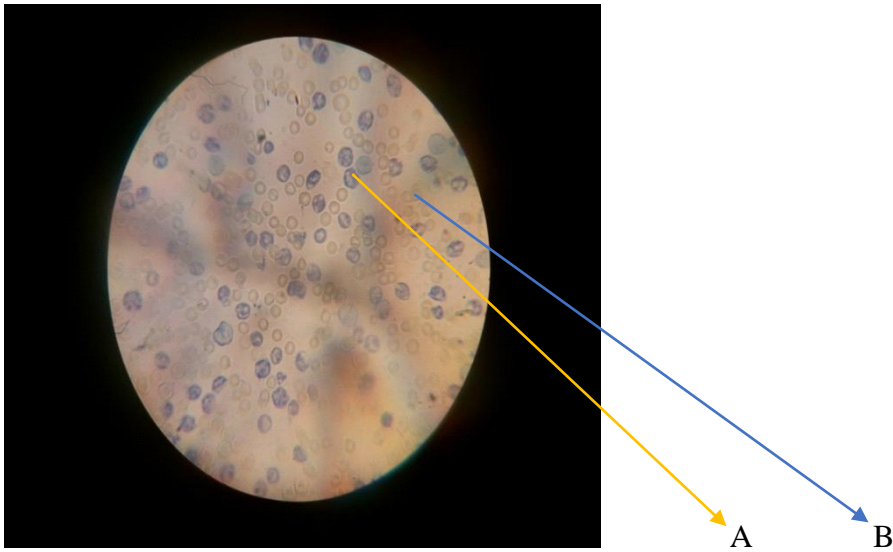
Where; S= Subject, MF=Microscopic Field, NPC=Number of Parasitized Red blood Cell, NUPC= Number of un-Parasitized Red blood Cell, % parasitemia= $\text{NPC}/\text{NUPC} \times 100$

Appendix XI: Microscopic Estimation of Parasitemia for TGPIH 200 group
after Rane's test on Day-4.

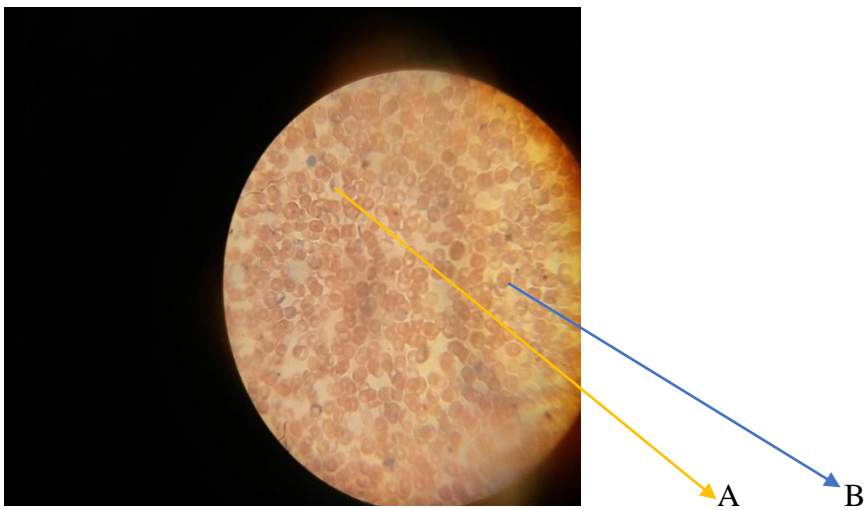
TGPIH 200							
S1	S2		S3		S4		
NPC	NUPC	NPC	NUPC	NPC	NUPC	NPC	NUPC
42	300	57	200	50	350	4	20
21	280	96	250	62	380	12	30
23	250	110	300	70	400	6	20
24	200	168	400	56	350	3	15
16	180	110	350	54	380	8	25
31	200	98	400	73	400	8	15
13	150	146	350	62	300	4	25
23	100	94	700	53	350	12	25
36	300	98	600	25	250	5	30
41	380	82	400	54	280	1	25
270	2340	1059	3950	559	3440	63	225

Where; S= Subject, MF=Microscopic Field, NPC=Number of Parasitized Red blood Cell, NUPC= Number of un-Parasitized Red blood Cell, % parasitemia= $\text{NPC/NUPC} \times 100$

PLATES

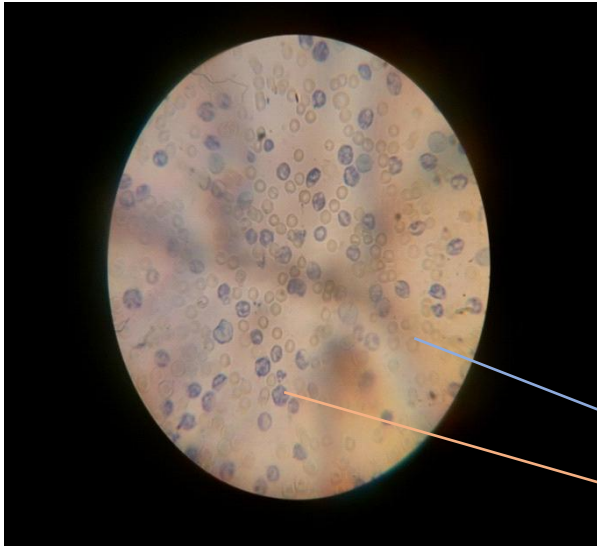


PlateVI: Giemsa Stained Thin Blood Film prepared after Peter's 4-Day Suppressive Test from an NCPD subject

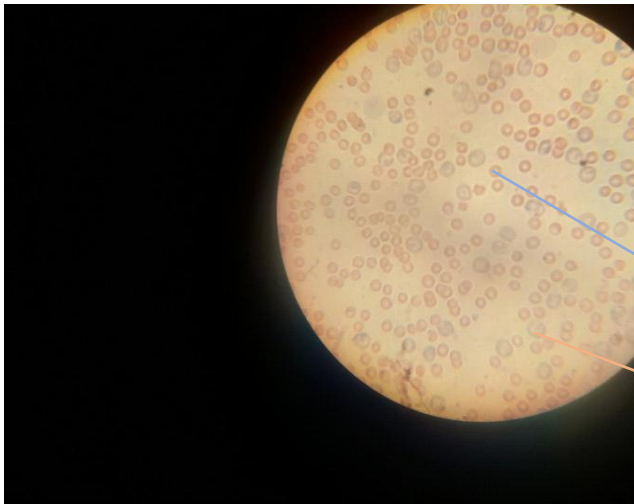


PlateVII: Giemsa Stained Thin Blood Film from an SGPC subject after Peter's 4-Day Suppressive Test.

A= parasitized red blood cell, B=Non-parasitized red blood cell,
x100 magnification oil immersion objective lens

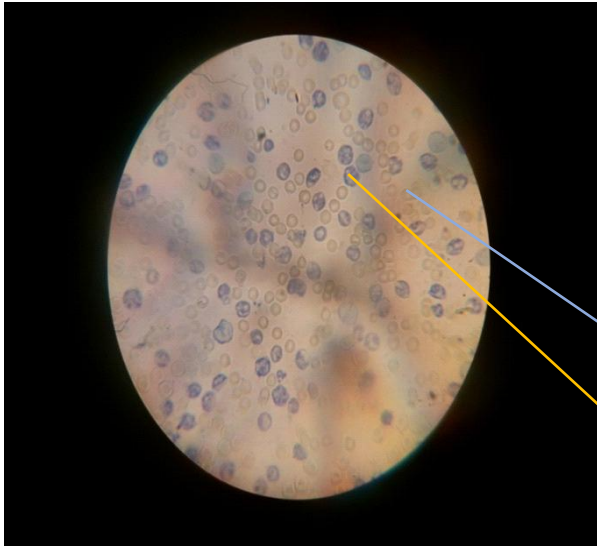


PlateVI: Giemsa Stained Thin Blood Film made from an NCPD subject after Peter's 4-Day Suppressive Test

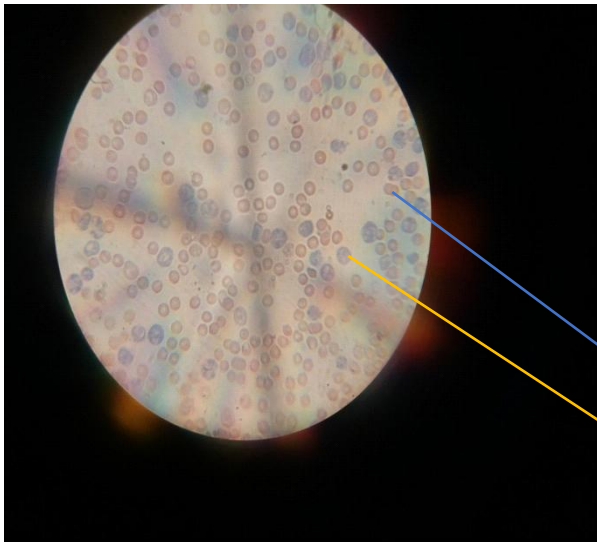


PlateVIII: Giemsa Stained Thin Blood Film from a TGPIH 200 Subject after Peter's 4-Day Suppressive Test.

A= parasitized red blood cell, B=Non-parasitized red blood cell,
x100 magnification oil immersion objective lense

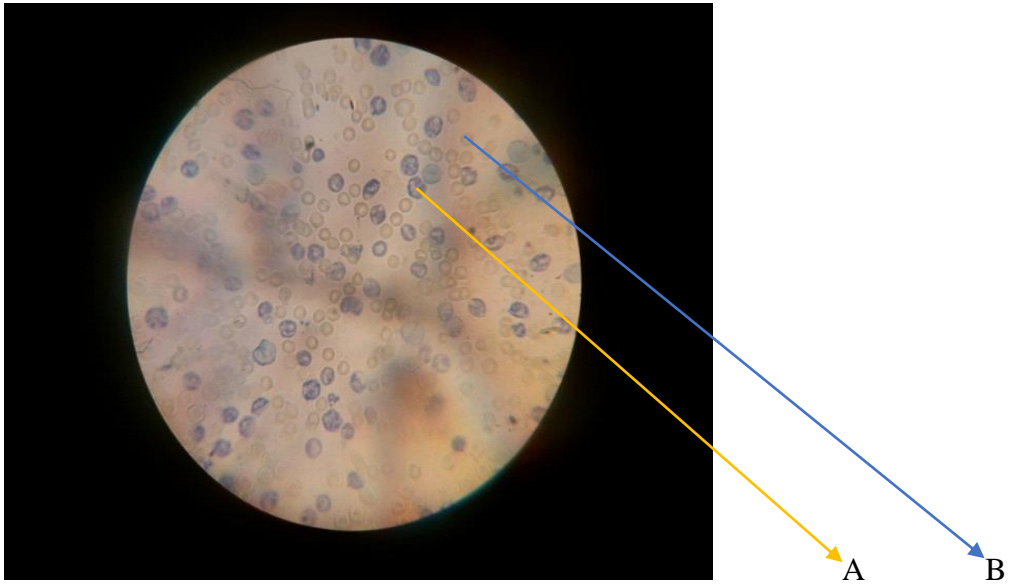


PlateVI: Giemsa Stained Thin Blood Film prepared from an NCPD Subject after Peter's 4-Day Suppressive Test

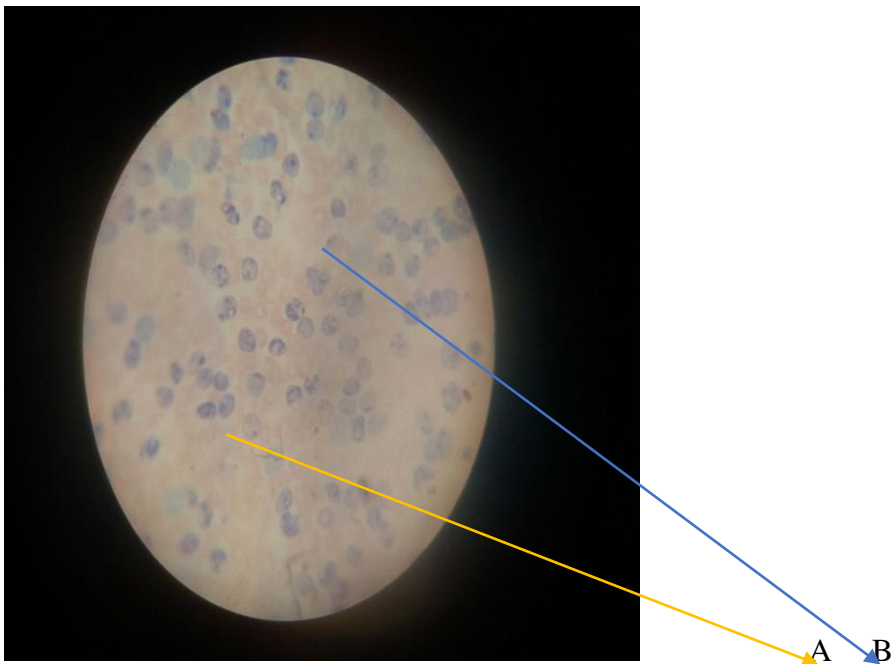


PlateIX: Giemsa Stained Thin Blood Film prepared from a treatment group II subject after Peter's 4-Day Suppressive Test.

A= parasitized red blood cell, B=Non-parasitized red blood cell,
x100 magnification oil immersion objective lense



PlateVI: Giemsa Stained Thin Blood Film prepared from an NCPD Subject after Peter's 4-Day Suppressive Test



PlateX: Giemsa Stained Thin Blood Film prepared from a TGPIH 50 Subject after Peter's 4-Day Suppressive Test.

A= parasitized red blood cell, B=Non-parasitized red blood cell, x100 magnification oil immersion objective lens.

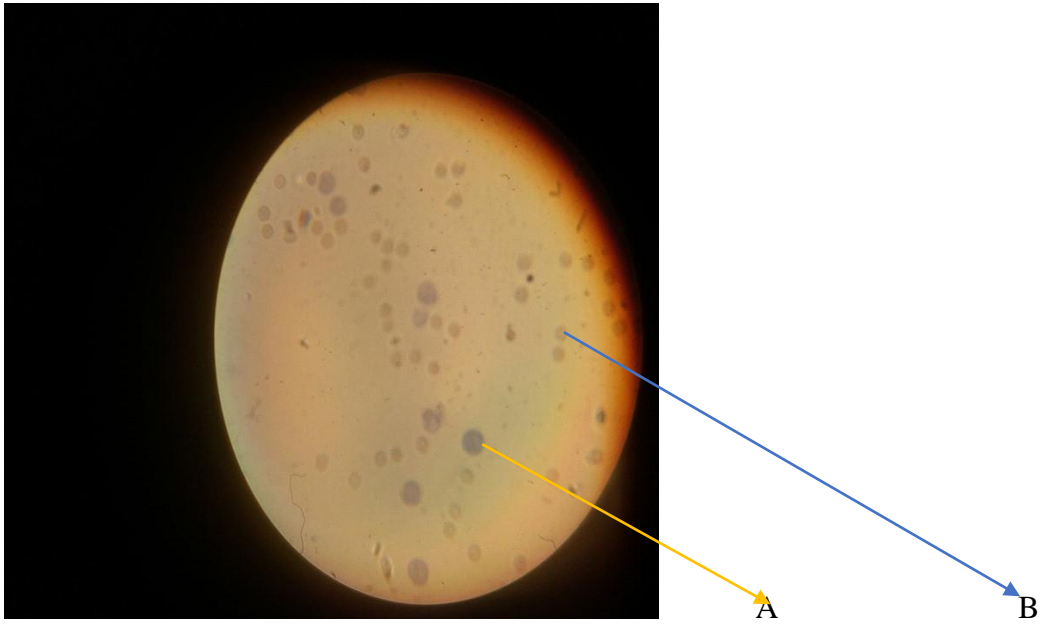


Plate XI: Giemsa Stained Thin Blood Film prepared from an NCPD Subject after Rane's Test.

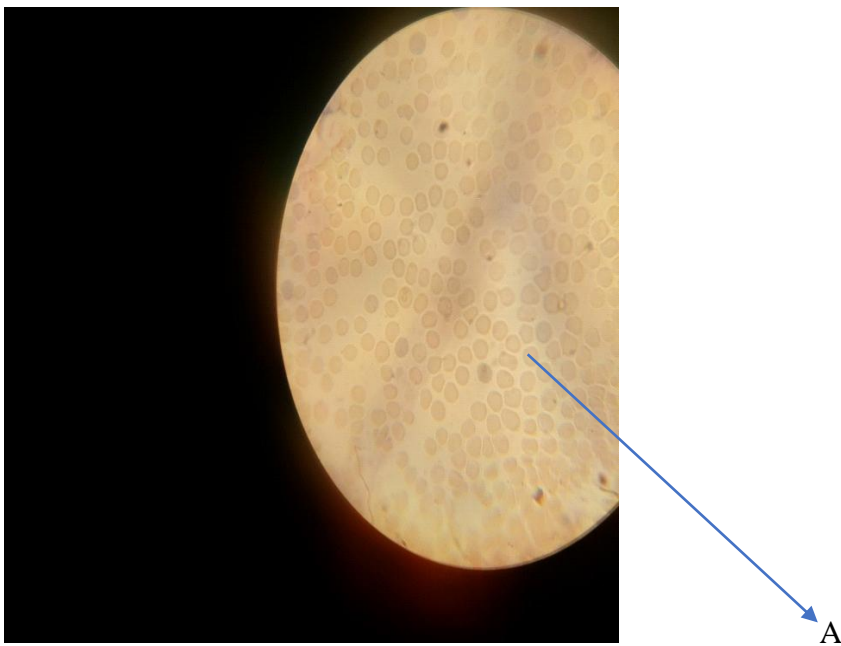


Plate XII: Giemsa Stained Thin Blood Film from a standard group Subject after Rane's Test

A= parasitized red blood cell, B=Non-parasitized red blood cell,
x100 magnification oil immersion objective lens.

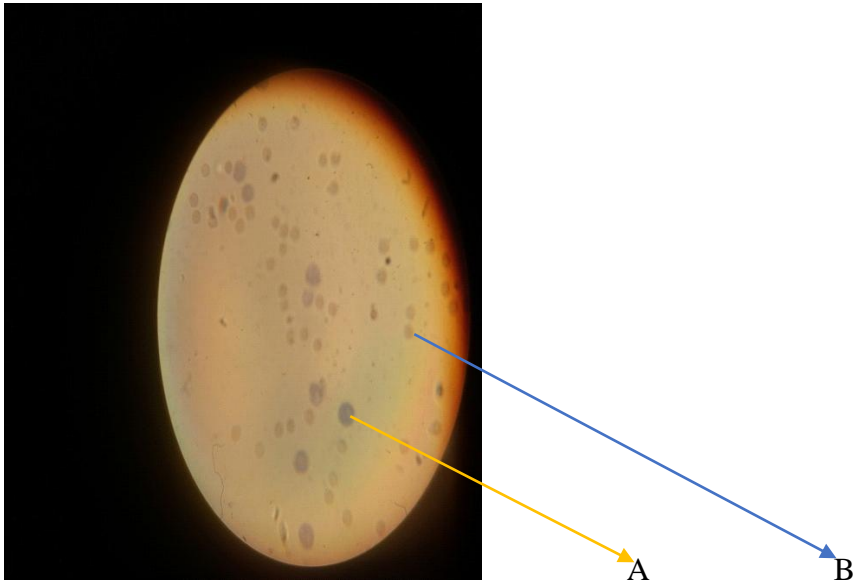
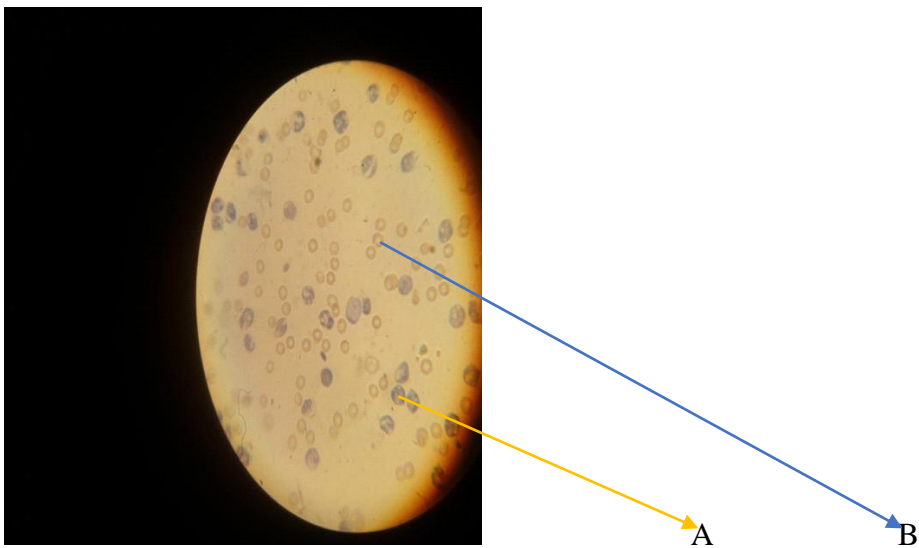


Plate XI: Giemsa Stained Thin Blood Film prepared from an NCPD Subject after Rane's Test.



PlateXIII: Giemsa Stained Thin Blood Film from a TGPIH 200 Subject after Rane's Test

A= parasitized red blood cell, B=Non-parasitized red blood cell, x100 magnification oil immersion objective lens.