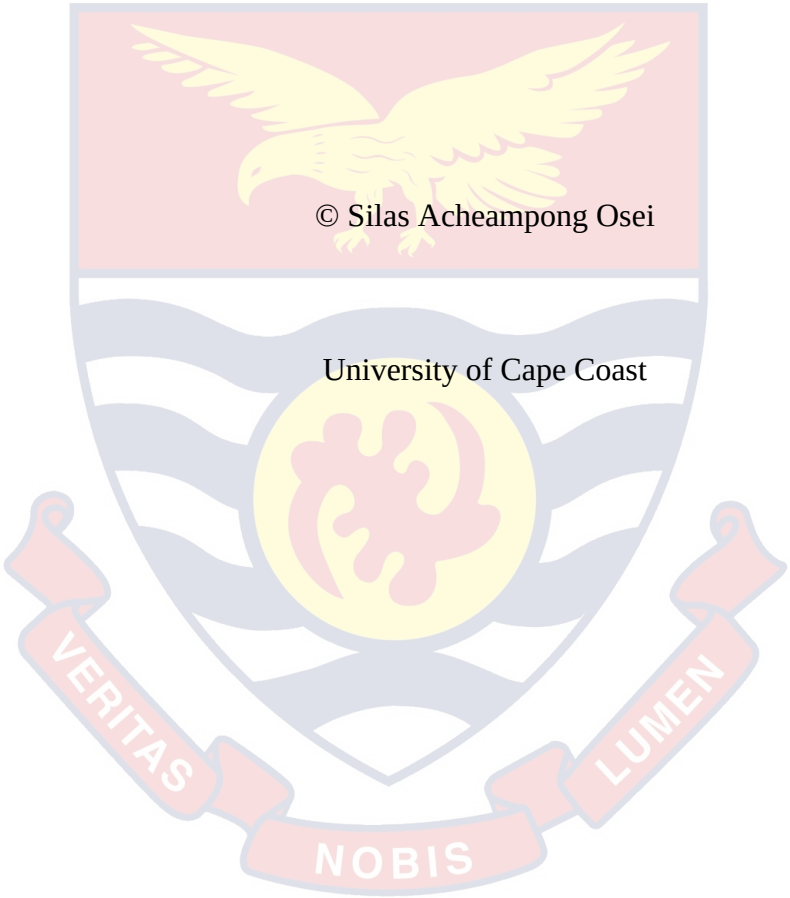
UNIVERSITY OF CAPE COAST

EVALUATION OF THE EFFICACY OF XYLOPIC ACID — ARTESUNATE, AND XYLOPIC ACID —AMODIAQUINE CO-ADMINISTRATION IN *PLASMODIUM BERGHEI* MALARIA MOUSE MODEL.

SILAS ACHEAMPONG OSEI

2020



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BY

SILAS ACHEAMPONG OSEI

Thesis submitted to the Department of Biomedical Sciences of the School of Allied Health Sciences, College of Health and Allied Sciences, University of Cape Coast, in partial fulfilment of the requirements for the award of Master of Philosophy degree in Drug Discovery and Development.

OCTOBER 2020

DECLARATION

Candidate's Declaration

I hereby declare that this thesis is the result of my own original research and that no part of it has been presented for another degree in this university or elsewhere.

Candidate's Signature…… Date……

Name…….

Supervisors' Declaration

We hereby declare that the preparation and presentation of the thesis were supervised in accordance with the guidelines on supervision of thesis laid down by the University of Cape Coast.

Principal Supervisor's Signature…… Date……

Name…….

Co-Supervisor's Signature…… Date……

Name…….

ABSTRACT

Evidence of Plasmodium resistance to some of the current antimalarial agents makes it imperative to search for newer, and effective drugs to combat this challenge, and combination therapy remains key in the quest to address this problem. Therefore, this study evaluated the interaction that exist when xylopic acid-artesunate and xylopic acid-amodiaquine are co-administered in managing malaria in mice. Antiplasmodial effect of xylopic acid (XA: 3, 10, 30, 100, 150 mg kg-1), artesunate (ART: 1, 2, 4, 8, 16 mg kg-1), and amodiaquine (AQ: 1.25, 2.5, 5, 10, 20 mg kg-1) were evaluated in mice infected with *Plasmodium berghei* ANKA and treated for 5 days with the respective drugs p.o. Using iterative curve-fitting of the log-dose responses, the respective ED_{50}s for the three compounds were determined. XA and ART, and XA and AQ were subsequently administered in a fixed-dose combination of their ED_{50}s (1:1) along with the combination fractions of (1/2, 1/4, 1/8, 1/16, and 1/32) to get the experimental ED_{50}S (Zexp). An isobologram was constructed to determine the nature of interaction between xylopic acid —artesunate, and xylopic acid —amodiaquine combinations by comparing Zexp with the theoretical ED50 (Zadd). Also, the treatments ability to relieve other signs of malaria such as weight loss and pyrexia were assessed. All animals were continuously observed further after treatment in a 30-day survival test. ED_{50}S for xylopic acid, artesunate, and amodiaquine were 9.0±3.2, 1.61±0.6, and 3.1±0.8 mg/kg. The Zadd and Zexp for xylopic acid and artesunate co-administration (XA—ART) was 5.3±2.61 and 1.98±0.25, respectively, with an interaction index of 0.37. The xylopic acid and amodiaquine combination therapy (XA—AQ) gave Zadd and Zexp of 6.05±2.0 and 1.69±0.42, respectively, with an interaction index of 0.28. The Zexp for both combination therapies lied significantly ($p<0.001$) below the additive isoboles showing that xylopic acid acts synergistically with both artesunate and amodiaquine in clearing the parasites. XA/AQ and the high dose XA/ART combination significantly ($p<0.05$) increased the survival days of infected mice and also reduced their weight loss.

KEY WORDS

Anti-malaria

Artemisinin-based combination therapy

Parasitaemia

Amodiaquine

Artesunate

Xylopic acid

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DEDICATION

I dedicate this work to my parents: Mr. and Mrs. Osei Acheampong

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LIST OF ABBREVIATIONS

AMA1	Apical membrane antigen-1
ACTs	Artemisinin derivatives
ART-	Artesunate
AQ-	Amodiaquine
CDK6-	Calcium independent protein kinase-6
CELTOS-	Cell traversal for proteins and sporozoites
CM-	Cerebral malaria
CPE-	Cryptolepine
CSP-	Circumsporozoite protein
CyRPA-	Cysteine-rich protective antigen
DCTs-	Drug combination therapies
EBLs-	Erythrocyte binding-like proteins
ED_{50}-	Median effective dose
GEST-	Gamete egress and sporozoite traversal protein
GWAS-	Genome wide association study
HSD-	Honest significant difference
HSPGs-	Heparin sulfate proteoglycans
ICR-	Institute for cancer research
LPS-	Lipopolysaccharide
MAC/PF-	Membrane attack complex/ Perforin-like domain
MSP-	Merozoite surface protein
PBA-	*Plasmodium berghei* ANKA
pRBC-	Parasitized red blood cell
SEM-	Standard error of mean
SP-	Sulfadoxine-pyrimethamine
SPECT-	Sporozoite microneme protein essential for traversal

TLP-	Trap-like protein
TRAP-	Thrombospodin related anonymous protein
TSR-	Thrombospodin repeat
TSR-	Thrombospodin repeat
UWTO-	United nations world tourism organization
XA-	Xylopic acid
Z_{add}-	Theoretical ED_{50}
Z_{exp}-	Experimental ED_{50}

CHAPTER ONE

INTRODUCTION

Regardless of the efforts put in place in the 21st century to eradicate the staggering toll of malaria on human health, the global burden of the disease remains. World Health Organization (WHO) estimates that 40% of the world's population is susceptible to malaria infections (WHO, 2016). A recent report indicates that 228 million cases of malaria occurred in 2018, which resulted in 405,000 deaths, mostly in sub-Sahara Africa (W.H.O, 2019). About 93% (213 million) of the cases in 2018 were recorded in the WHO African Region.

There is an unrelenting need for the discovery and development of novel antimalarial agents. The aim to search for novel antimalarials is to develop affordable and safer new drugs which can prevent the spread of malaria parasites and the resistance to existing agents. Drug combination therapies (DCTs) are pertinent to the optimum control of malaria in developing countries (Guerin et al., 2002) because they provide improved efficacy and might also give synergistic activity. Drug combinations enhance the probability that one agent can be at least clinically active in the case of parasite resistance to the drug. Example, in East Africa, malaria parasites are resistant to both amodiaquine and sulfadoxine-pyrimethamine (SP), but the combination of these two agents still gives an excellent antimalarial efficacy (Dorsey et al., 2002; Staedke et al., 2001; Zuber & Takala-Harrison, 2018).

Natural products are essential in the drug discovery process, and there is no exception in antimalaria agents. Medicinal plants extracts have been a source for

antimalaria drug discovery for long, and its treatment for malaria has been successful (Kaur, Jain, Kaur, & Jain, 2009). Example of established antimalarial drugs such as quinine and artemisinin were isolated from *Cinchona* and *Qinghaosu*, respectively. Xylopic acid, extracted from the ripe fruits of *Xylopia aethiopica*, has also been reported to have antimalaria properties (Boampong et al., 2013). Current reports of the malaria parasite's development of resistance to established drugs (Blasco, Leroy, & Fidock, 2017; Venkatesan et al., 2014), makes it prudent to search for newer and more effective drugs to combat the global burden of the disease.

1.1 Background to the Study

Malaria is a relapsing infection in humans, which is characterized by periodic attacks of chills and fever, anaemia, splenomegaly, and often fatal complications. Malaria is caused by the genus *Plasmodium* (*P)*, a one-celled parasite, and transmitted to humans by a female *Anopheles* mosquito. Five related *Plasmodium* species cause malaria in humans; *P. knowlesi*, *P. falciparum*, *P. vivax*, *P. ovale*, and *P. malaria*. The most harmful is *P. falciparum* while *P. vivax* is the most common (Kumar, Bhushan, & Bhattacharya, 2017).

Usually, it takes about 7 to 15 days for a victim who is bitten by a malaria-carrying mosquito to experience the symptoms (Abiodun, Witbooi, & Okosun, 2018). Patients usually experience headache, muscle ache, abdominal cramps, vomiting, fever, and chills at the beginning. Mostly, the chills and fever occur periodically; lasting for 4-10 hours together with a stage of shaking and chills, followed by severe headache and fever, and a final stage of profuse sweating

dropping the temperature to normal or below, which occurs at 48 (tertian malaria) or 72 (quartan malaria) hours intervals (Anochie, 2013).

P. falciparum infection is the most fatal of the *Plasmodium* species. There is a possibility that a victim of *P. falciparum* infection with "malignant tertian" type of the disease may deteriorate immediately after symptoms of coma if not adequately treated on time. *P. falciparum* is capable of affecting a large "percentage" of erythrocytes; ten times the number of parasites per cubic millimetre of blood higher than other human infecting *Plasmodium* species. Also, the erythrocytes which are infected have the capability of adhering to the walls of micro blood vessels and capillaries resulting in blood flow obstruction in several organs, but the results are graving when micro-vessels in the brain are affected. This results in what is known as cerebral malaria characterized by late-stage complications involving ataxia, respiratory distress, coma and death (Martin, 2017).

Malaria is still a health menace globally with a risk of infection in about 3.3 billion people which leads to about 200 million cases (Cowman, Healer, Marapana, & Marsh, 2016). Throughout history, *P. falciparum* has undoubtedly caused higher selective pressure on human evolution compared to any other pathogen. Although its presence is predominantly in the tropics, the impact on health is mostly uneven; a majority of *P. falciparum* infected individuals are found in South Asia and Asia, but Africa records about 90% of death which are mostly children (Cowman *et al*., 2016; WHO, 2016).

Xylopic acid (XA) is an isolate from the fresh ripe fruits of *Xylopia aethiopica*, a common ingredient in several Ghanaian folklore medicines and foods,

and has been investigated to establish its attributable prophylactic, curative antimalarial, and antipyretic properties (Boampong et al., 2013). The antimalarial properties of xylopic acid were indicated by employing the compound in ICR mice infected with *Plasmodium berghei*. Xylopic acid exerted significant effects on *P. berghei* infection similar to artemether/lumefantrine, the standard drug. Furthermore, it significantly reduced the lipopolysaccharide- (LPS) induced fever in Sprague-Dawley rats similar to prednisolone. Thus, xylopic acid possesses prophylactic and curative antimalarial along with antipyretic properties, making it an ideal antimalarial agent (Boampong et al., 2013).

1.2 Problem Statement

Malaria remains a major health burden in several tropical countries. WHO estimates that 40% of the world's population is susceptible to malaria infections. This results in about 200-300 million cases each year and causing mortality in approximately 2 million children, mostly in sub-Sahara Africa. WHO reported that 216 million patients were diagnosed with malaria in 2015, resulting in 238,000 deaths (WHO, 2016). Majority of cases presented as severe or complicated malaria is caused by *P. falciparum.* Children under five years and pregnant women succumb to the devastating effects of the disease, making the disease a major global infectious disease.

Cinchona alkaloids (quinine and quinidine) and artemisinin derivatives (artesunate, artemether and artemotil) are the two classes of medicines available for the treatment of severe and uncomplicated malaria, respectively. *Plasmodium falciparum* has developed resistance to antimalarial agents in the past (an example

is chloroquine) and a recent report in South-east Asia indicates that there is growing resistance of *P. falciparum* to artemisinin derivatives (Woodrow & White, 2017). Although malaria vaccines are being developed, it has not yet been accepted (Kaslow et al., 2018). This makes it crucial to search for newer, more effective antimalarial agents.

1.3 Justification

Plant-derived compounds have played a significant role in antimalarial drug discovery and development. About 160 plant families have been established to have antimalarial properties. From these families, more than 1200 species have been documented to have antimalarial properties (WHO, 2016). *Xylopia aethiopica* and *Cryptolepis sanguinolenta* from Annonaceae and Apocynaceae families, respectively, are two medicinal plants used to treat malaria by Ghanaian herbal practitioners (Boampong *et al*., 2013; Woode et al., 2012). Xylopic acid, a kaurene diterpene, is the major constituent of *Xylopia aethiopica*, and has been reported to possess antimalarial properties. The antimalarial properties of xylopic acid were indicated by using the compound in ICR mice infected with *Plasmodium berghei*. Xylopic acid exerted significant effects on *P. berghei* infection similar to artemether/lumefantrine, the standard drug. Furthermore, it significantly reduced the lipopolysaccharide- (LPS) induced fever in Sprague-Dawley rats similar to prednisolone (Boampong et al., 2013). Thus, xylopic acid possesses prophylactic and curative antimalarial along with antipyretic properties, making it an ideal antimalarial agent. Also, xylopic acid have shown to be effective in combination therapies for managing malaria A recent study by Ameyaw et al., reported the

synergistic effect of xylopic acid in combination with cryptolepine in clearing malaria parasites in a malaria experimental model (Ameyaw et al., 2018).

Hence the present study seeks to investigate the effectiveness of co-administration of xylopic acid and artesunate in managing malaria using experimentally induced malaria mice.

1.4 Aim

To investigate the effectiveness of the co-administration of xylopic acid/artesunate, as well as xylopic acid/amodiaquine in managing malaria in an experimental model malaria in ICR mice.

1.5 Specific objectives

3. To confirm the efficacy (determine ED_{50S}) of artesunate, amodiaquine, and xylopic acid monotherapies in *P. berghei* ANKA-infected mice.
4. To test for synergism, potentiation or inhibition by performing an isobolographic analysis in *P. berghei ANKA*-infected mice after co-administration of
   i. Artesunate and xylopic acid
   ii. Amodiaquine and xylopic acid

1.6 Hypothesis

The co-administration of xylopic acid/amodiaquine and xylopic acid/artesunate produce synergistic anti-malarial effect *in vivo*.

1.7 Significance of the Study

If the effectiveness of co-administration of xylopic acid and artesunate or xylopic acid and amodiaquine in managing malaria is ascertained through this research, it can be developed into a therapeutic drug and used in clinical settings. A single drug containing both xylopic acid and artesunate or xylopic acid and amodiaquine can be formulated which will help treat malaria than individual drug and also reduce the toxicity of administering the individual drugs. Since malarial causative agent *Plasmodium falciparum*, has proven resistance to many antimalarial agents over the past years, constant research into developing new antimalarial agents is needful to prevent the devastating effect caused by this parasite when left untreated.

1.8 Delimitations

This study examines the antiplasmodial properties of combining xylopic acid and artemisinin derivatives (amodiaquine and artesunate) in experimental malaria model which involve the use of female ICR mice. It also examines the ability of the combined compounds to reduce symptoms of malaria such as hypothermia and loss in body weight. However, the study does not include Safety profile of co-administering xylopic acid-amodiaquine and xylopic acid-artesunate in the treatment of malaria.

1.9 Limitations

The study required the use of a lot of animals. Thus, study was done in batches which may affect the outcome of the study. The results of the study cannot

directly be extrapolated to humans due to biological differences between rodents and primates.

1.10 Organization of Study

This study is organized into five chapters with each chapter being a continuation of the preceding one:

Chapter One gives a brief background, purpose, the objectives and the significance of the study.

Chapter Two talks about the conceptual base of the work exploring theories around which the work is built.

Chapter Three describes the method or study design employed to achieve the goal of this research including data collection, processing and Analysis.

Chapter Four contains results and discussion in which the results are presented by the research questions. The implications of the findings are evaluated with respect to current theoretical positions on the problem along with practical applications.

Finally, Chapter Five gives a summary of the study which includes an overview of the importance of this study, hypothesis and the research methods employed to achieve the hypothesis and the results. Also, this chapter concludes the theses, pointing out anomalies, deviations, with logical explanations for them and the overall opinion regarding the study.

1.11. Chapter summary

Malaria is an infection caused by the bite of a female *Anopheles* mosquito. The symptoms of malaria ranges from common fever to death if not treated properly and immediately. World health Organization estimates 40% of the world population are at risk of malaria infection. Malaria causes the death of about 500, 000 people annually with pregnant women and children being the most victims.

P. falciparum, the most lethal species of the malaria parasites, over the years has been developing resistance to Antimalarials. Recently, there have been reports in Southeast Asia regarding the development of resistance to current established antimalarial drugs (Artemisinin derivatives). This makes it imperative to continue the search for newer and safe antimalarial agents against the fight to combat the menace caused by malaria.

Xylopic acid which is isolated from unripe fruits of *Xylopia aethiopica* has been established to have antipyretic and antimalarial properties. Combination therapies have been the ideal drug regimen for treating malaria because of the ability of the parasite to develop resistance to drugs. The idea is that the combination of the two drugs will provide synergistic activity and potentiate the drugs potency along with reducing the ability of the parasite to develop resistance to the drugs. Xylopic acid, amodiaquine, and Artesunate have all shown to be effective in combination therapies in managing malaria, thus, it is ideal to check their efficacy when they are co-administered.

This study is organized into five chapters to achieve the aim of the study which is to assess the effectiveness of the co-administration of xylopic acid and

artesunate and xylopic acid and amodiaquine in experimental malaria model in mice.

CHAPTER TWO

LITERATURE REVIEW

Introduction

This study seeks to evaluate the effects of the co-administration of xylopic acid and artesunate, and xylopic acid and amodiaquine in experimental model malaria in ICR mice. This chapter discusses the conceptual base of the study, and it also explores the fundamental theories and the interpretation in which the study was built.

The chapter is organized into epidemiology and disease biology, followed by a discussion on antimalarial drug discovery and a conclusion or summary.

1.1. Epidemiology of Malaria

To monitor and quantify the effect of the efforts put in place to control the reduction of malaria morbidity, transmission, and eventually eliminate permanently, it is prudent to understand the epidemiological features and metrics of malaria in populations endemic to this infection.

World Health Organization (WHO) recently released a World Malaria Report, which shows the massive progress towards elimination and control of malaria (WHO, 2016). The report indicated a decline in malaria incidence by 41% since the year 2000 and 21% since 2010. Also, death caused by malaria infection has decreased by 61% since 2000 and 29% since 2015. Seventeen countries have successfully eradicated malaria between 2000 to 2015, while 13 countries are "approaching elimination". These data signify substantial achievements; yet, significant challenges remain including an increase in resistance to insecticides by mosquitoes, resistance to artemisinins and artemisinin combination therapies, and funding shortfalls.

A recent report shows that the different endemicity class and local transmission of malaria depends on the geographical distribution (WHO, 2016). In Africa, 395,000 deaths were estimated in 2016. Pregnant women mostly habor chronic low-density infections with poor outcome for both the child and mother (Steketee, Nahlen, Parise, & Menendez, 2001) and children below the ages of five accounted for 70% mortality rate. Worldwide mortality was estimated to be 445,000 (WHO, 2017) indicating that Africa alone has about 89% of worldwide mortality of malaria. A study conducted from 2010-2016 involving 16 countries in sub-Sahara Africa countries in 8,116 locations provided a detailed report on the

epidemiology of malaria in these endemic regions; prevalence of malaria in children under age 5 ranged from 2.2% to 76.1% in Rwanda and Burkina Faso, respectively. All West African countries had higher prevalence of malaria than East African countries except Senegal. The highest mortality rate of children under age five was Burkina Faso, Guinea, and Cameroon in descending order. Ghana ranked the second-lowest under age five mortality rate, although they had high parasitemia (Papaioannou, Utzinger, & Vounatsou, 2019).

The increase in the number of travellers to malaria-endemic regions along with continued immigration and refugee migrations from endemic areas contribute to a substantial increase in the burden of malaria cases in industrialised countries. The world migration Unit reported in 2018 the staggering increase in tourist arrivals to Sub-Saharan Africa, most endemic region, from 2000 to 2011 with a percentage increase of 22% over the last 5 years with about 40 million arrivals in 2016 (McAuliffe & Ruhs, 2017).

Malaria cases in Europe have remained stable since 2011, with an approximate infection of 6500-7000 annually following a significant decline from 2000 to 2008 (Sinka et al., 2010). France and United Kingdom represent half of the total reported cases; this may be due to a large number of travellers and an immigrant population who regularly visit their birth country. In contrast, reported cases in the United States have risen from 2000 to 2016 with more than 1700 cases reported in 2014 (Costa, 2015) . An annual mean of 433 cases was reported in Australia in 5 years to 2014 (Murray et al., 2014)

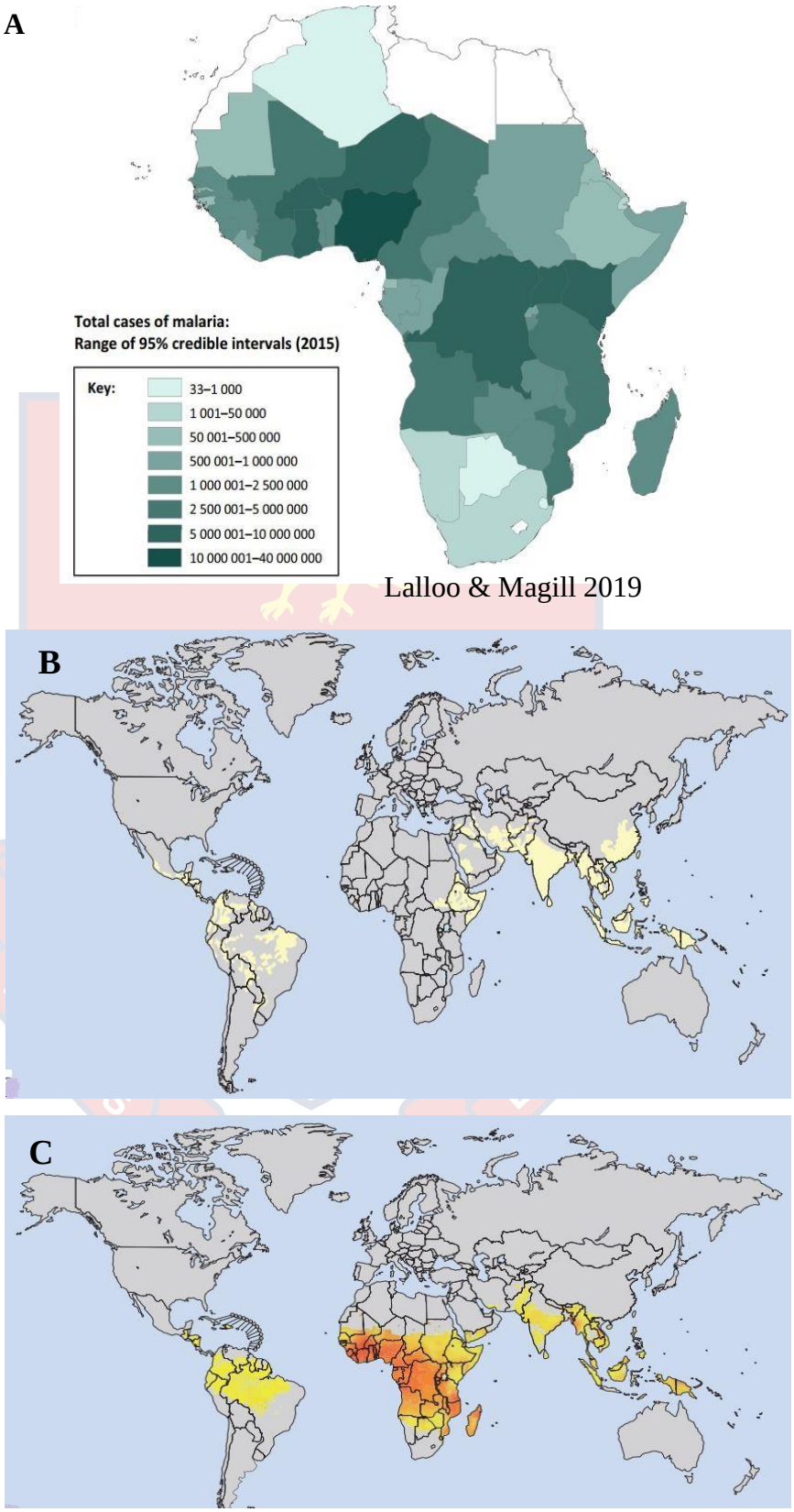


FIG 2.1. Maps are showing (**A**) the prevalence of malaria in Africa in 2015. Current estimate of the intensity of transmission of (**B**) *P. falciparum* and (**C**) *P. vivax* worldwide.

2.1.1 Relationship between transmission and disease burden

The intensity of malaria transmission ranges over five log orders from less than one infectious bite annually to several hundred a day. It is usually expressed in terms of the proportion of a population of defined age with parasitemia at a particular time. In stable endemic countries, adults (older subjects) develop a level of protective immunity, but young children usually suffer from severe diseases and death (figure 4). Immunity acquisition is proportional to the rate of transmission which affects the clinical disease spectrum, making severe anemia dominate areas in which the transmission is very high, while cerebral malaria becomes more dominant under low-to-moderate transmission settings. The relationship amongst transmission and severe malaria are non-linear (Snow et al., 1997), which means control measures in areas with high-transmission can bring about a significant reduction in transmission with less impact on the disease till transmission fails to a crucial point, after which disease reduction may be rapid. The current observations in most areas in Africa support this picture. Even more relevant is the relationship between all-cause mortality and transmission. It is suggested that malaria exposure causes an unreasonably high number of deaths because of other causes which have been backed strongly with epidemiologic studies and direct intervention results (Scott *et al*., 2011).

2.1.2 Complications of malaria

P. falciparum infection results in potentially fatal complications, including cerebral malaria, acute renal failure, pulmonary involvement, black water fiver, and severe anemia. These complications developed in 1.1% of 3,300 Vietnam cases,

resulting in a total fatality of about 0.3%. Intense parasitaemia and delay in treatment or improper diagnosis can lead to the development of crucial complications (Ahmad et al., 2017).

Pregnant women and children under five years succumb mostly to the complications of malaria. The prevalence of malaria in women attending antenatal care was 29.5% in southern and East Africa, and 35% in West Africa. The complications of malaria in pregnant women vary according to the intensity of transmission, geographical area, and the immunity level of the patient. In areas of high transmission, acquired immunity levels are high, and *P. falciparum* infection usually is asymptomatic. Parasites can adhere to the placenta and cause anemia in pregnant women without a documented peripheral parasitaemia. The combination of placental involvement and maternal aneamia results in low birth weight, which is a critical factor for infant mortality. In low endemicity areas where women have low immunity, malaria can result in severe anemia, which can cause spontaneous abortion, prematurity, stillbirth, and foetal death (Conroy, McDonald, & Kain, 2012; McGready et al., 2012; Takem & D'Alessandro, 2013).

There is an increasing concern about the incidence of pulmonary manifestations in recent years. Pulmonary complications in malaria can be asymptomatic or oligosymptomatic. A dry cough is manifested in 20-50% of malaria patients (Nayak et al., 2011). Occasionally, the lung disease, fever or aneamia can cause tachypnoea (abnormally rapid breathing) in patients with *P. falciparum* infection. Pneumonitis occurs in about 1.5% in some series although very rare. Some authors suggest that the pneumonitis is caused by intercurrent

pneumonia, pulmonary oedema or metabolic acidosis (Maguire et al., 2005; Mansanguan & Phumratanaprapin, 2018). The most lethal is respiratory distress, caused by insufficient respiration, due to an increase in alveolar permeability (Taylor, Hanson, Turner, White, & Dondorp, 2012).

Cerebral malaria (CM) is a major fatal complication of *Plasmodium falciparum* infection, characterized by sequestration of parasitized red blood cells (pRBC), specifically in the deep cerebral vascular beds and raised levels of protein inflammation (Hora, Kapoor, Thind, & Mishra, 2016). The etiology of CM malaria is very difficult to assess, but hospital admissions report that 1% of all malaria cases progress to CM, which results in 10-20% fatal cases, accounting for approximately 300000-500000 deaths each year (WHO, 2016).

2.2 Biology of Malaria

2.2.1 Life cycle of *Plasmodium*

Plasmodium spp. complex life cycle alternates between a female *Anopheles* mosquito and a vertebrate host that needs the formation of exclusive zoite forms which can be able to invade different types of cells at a particular stage (see figure 2.2). When sporozoites enter hosts, they invade the hepatocytes, beginning the asexual cycle in blood. (Kumar et al., 2017). An infected female *Anopheles* mosquito spread the parasites by feeding on the human blood to nourish their eggs. They inject sporozoites (an immature form of the parasite) into the host's bloodstream. Usually, it takes about 48 hours for the sporozoites of *P. falciparum, vivax, P. ovale,* and 72 hours for *P. malariae* to develop into the second generation

(merozoites) respectively (Burns et al., 2019). The merozoites reproduce asexually, with the absence of parent genetic material; yet, a few develop into a sexual form of the parasite, also known as the gametocyte. The gametes mate when a female Anopheles mosquito feeds on the blood of an infected person and the gametes get into the gut of the mosquito. These ookinetes which embed themselves in the gut of the mosquito until they mature into oocytes in about 9-14 days, which in turn burst and release numerous sporozoites that migrate to salivary gland of the insect, available to infect the next person in the cycle (Burns et al., 2019).

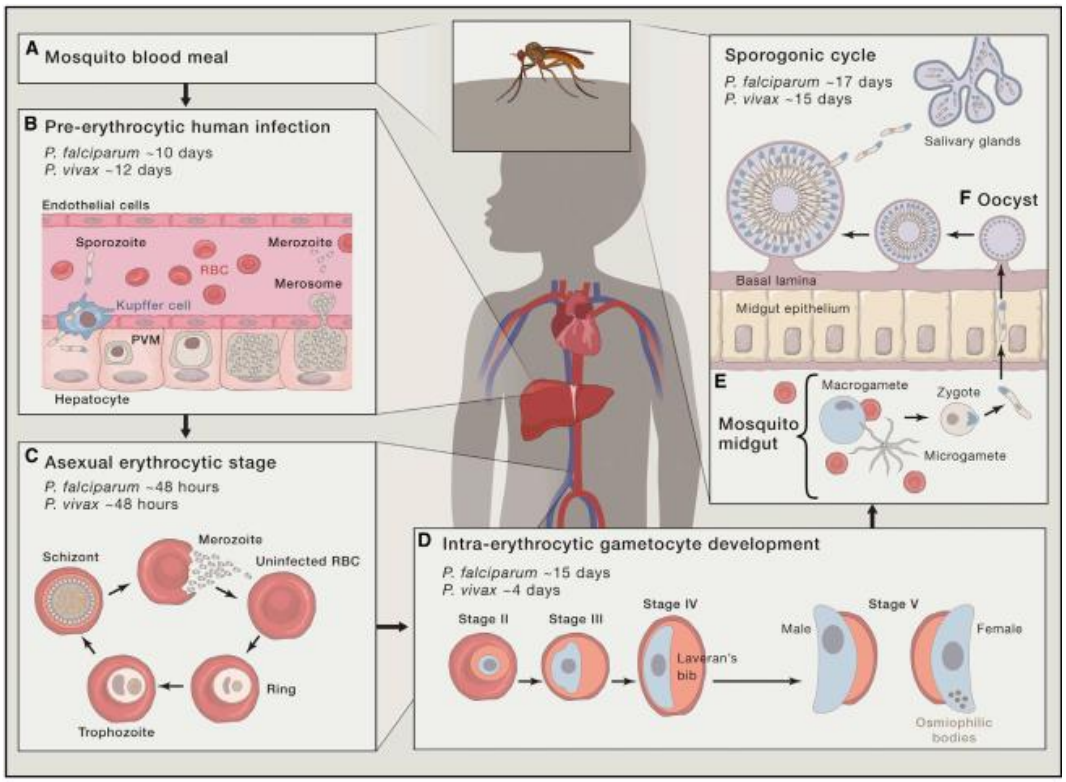


Figure 2.2 *P. falciparum* life cycle. **A**. Malaria infection starts when a feeding female Anopheles mosquito injects sporozoites (spzs) into the dermis of the host. **B.** The sporozoites enter the blood vessels and migrate to the liver. Upon entering the liver, they exit the sinusoids via endothelial cells and enter the hepatocytes. When a suitable hepatocyte is found via cellular traversal, the sporozoites undergo schizogony until several thousands of merozoites are released into the bloodstream in the form of merosome packets. **C**. The merozoites begin a continuous cycle of asexual schizogony in the bloodstream after encountering erythrocytes. **D.** A percentage of the merozoites develop into gametocytes and undergo gametocytogenesis. **E.** In about 15 days, the gametocytes sequester and develop within the bone marrow, and when mature, they enter peripheral circulation for ingestion by a mosquito. In the midgut of the mosquito, they develop into male and female gametes. **F**. A micro and macrogamete fuse (mate) to form a zygote which transforms into an ookinete over 24 hours. Ookinetes migrate via the midgut epithelium of the mosquito and encyst to become oocysts which is capable of undergoing sporogenic replication. Oocysts rupture and release motile sporozoites into hemocoel and migrate into salivary glands of the mosquito and injected into the next host upon feeding. (Cowman *et al.*, 2016).

2.2.1.1 Hepatic Stage of Infection

Female *Anopheles* mosquitoes inject sporozoites into the dermis of the host during blood feed (figure 2.2). The fate of the sporozoites have not been fully elucidated, but they can reach and infect erythrocytes either by gliding motility or random processes. Sporozoites enter the bloodstream by penetrating through the blood vessel. Host immune system is generated to clear the remaining sporozoites in the debris via the lymphatics. Trap-like protein (TLP) helps in the exit of mutant sporozoites which may have gliding motility but unable to enter circulation from the dermis. Those who enter circulation use traversal process to enter the liver. Thus, they cross the sinusoidal barrier, which comprises of macrophage-like Kupffer cells and fenestrated endothelial cells (Tavares et al., 2013). The proteins needed for this traversal process are SPECT (sporozoite microneme protein essential for traversal), SPECT2 (also called PLP1, perforin-like protein 1), GEST (gamete egress and sporozoite traversal protein), and CelTOS (cell traversal protein for ookinetes and sporozoites). These protein functions are fully understood, but SPECT2 has been reported to have MAC/PF (membrane attack complex/perforin-like domain) signifying that it can punch holes of membranes. By creating a temporal vacuole, sporozoites can traverse via cells, with the involvement of pH sensing and SPECT2 in egress from this structure (Risco-Castillo et al., 2015). Sporozoites can only develop in hepatocytes. Thus, sporozoites traverse via the sinusoidal barrier, while priming them for hepatocyte invasion. When sporozoites are initially injected into the dermis, they are in the migratory mode until they interact with hepatocytes to convert to the "invasive mode." This switch is achieved

when sporozoites recognize hepatocytes via binding higher sulfated forms of heparin sulfate proteoglycans (HSPGs) which activates calcium-independent protein kinase 6 (CDK6) (Coppi *et al*., 2007).

Human hepatocyte surface proteins such as B1(SR-1), and CD81, a tetraspanin and a scavenger receptor, respectively, are needed by *P. falciparum* to form parasitophorous vacuoles (Rodrigues et al., 2008). Circumsporozoite protein (CSP), which consist of type 1 thrombospondin repeat (TSR) and a highly repetitive region is a major protein which is part of the dense coat that covers the sporozoite. Sporozoites can invade hepatocytes by binding CSP to HSPGs, which activates the processing of CSP and removes the N-terminus exposing the TSR domain (Herrera *et al.,* 2015). Following steps involve proteins such as TRAP (thrombospondin-related anonymous protein), and AMA1 (apical membrane antigen-1) if the sporozoite successfully infects a hepatocyte, it transforms over the following 2-10 days to the exo-erythrocytic form (EEF). The fully developed EEF in a hepatocyte burst and release about 40,000 merozoites into the bloodstream by budding merosomes; parasite filled vesicles (Sturm et al., 2006) (figure 2.2). Human liver chimeric mice have provided insight into the transformation of *P. falciparum* EEF *in vivo* (Vaughan *et al*., 2012). The study reported that parasite DNA replication begins on day-3 post-invasion, and they remain in a parasitophorous vacuole membrane until the LS development.

2.2.1.2 Erythrocytic stage of infection

Merozoites invade erythrocytes immediately after their release into hepatic circulation via three processes; pre-invasion, active invasion, and echinocytosis

(Weiss et al., 2015) all completed in 2 minutes (Figure 2.2). Merozoites initially interact with erythrocytes, but little is understood regarding the molecular basis of this step. Holder reported that the major glycophosphatidylinositol (GPI)-associated protein found on the surface of merozoites is merozoite surface protein 1 (MSP-1) (Holder, 1994). This MSP1 has recently been reported to act as a platform on the merozoite surface that hosts three major complexes with distinct extrinsic proteins that bind red blood cells (Lin et al., 2016). Das and colleagues previously showed that MSP1 may not be a requirement for erythrocyte invasion, since merozoites without MSP1 can also invade erythrocytes (Das *et al*., 2015).

The first step for merozoite invasion in erythrocytes is the pre-invasion process. This step results in parasite actomyosin motor-driven deformation of the host cell as merozoites interact robustly with erythrocytes (Weiss et al., 2015) (figure 2.2). Erythrocyte binding-like proteins (EBLs*)*, and Type 1 membrane proteins in *P. falciparum* are two ligand families involved in this process, and they bind to specific receptors such as glycophorin A, B, C and complement factor 1 (CR1). PfRh and EBL proteins also have relevant signalling activation role of subsequent steps in invasion. When *P. falciparum* is exposed to low-potassium ion concentrations in blood plasma, following host cell egression, it leads to elevation of systolic calcium levels via a phospholipase C-mediated pathway, which triggers the release of EBL family member, EBA-175 (Singh, Alam, Pal-Bhowmick, Brzostowski, & Chitnis, 2010). When EBA-175 binds to glycophorin A, its receptor, it activates release of proteins from rhoptries. This gives evidence of the relevance of PfRh and EBL protein families in sensing and binding of merozoites

to erythrocytes and the signalling downstream invasion events. A report by Paul et al. showed the role of calcineurin involvement in pre-invasion; responsible for the attachment of merozoites to erythrocytes, maybe via EBL and PfRh proteins stabilization and dimerization, as this is crucial for host-receptor binding and signal transduction for subsequent events in invasion (Paul *et al*., 2015).

Merozoites reorient after distortion of erythrocytes so that the end of the apex abuts the membrane of the erythrocytes. A review by Tham and colleagues reported that a member of the PfRh family, PfRh5, is involved in this process (Tham *et al*., 2015). Another report by Teddy et al. indicates PfRh5 is not a type 1 membrane protein, but forms a complex with Rh5-interacting protein, PfRipr, and a cysteine-rich protective antigen, CyRPA (Reddy *et al*., 2015). PfRh5 binds to basigin, a host receptor, forming a complex in which its interaction is linked with the influx of Ca^{2+} into the host cell (Volz et al., 2016; Weiss *et al*., 2015) crucial for merozoite invasion (Crosnier et al., 2011)

Merozoites attach themselves irreversibly to erythrocytes by forming a tight junction between AMA1 and RON complex. RON complex and AMA1 are parasite-derived proteins. RON complex is embedded in erythrocytes, but RON2 is responsible for spanning the host membrane and AMA1 binding on merozoite surface (Besteiro, Dubremetz, & Lebrun, 2011). Parasites propel into erythrocytes using energy generated by parasite actinomyosin motor (Riglar *et al*., 2011). Following the active invasion phase, membranes at the posterior end of merozoites fuse to seal the parasite in the erythrocyte and the parasitophorous vacuole. This process is followed by echinocytosis, which causes shrinkage of erythrocytes and

formation of spiky protrusions. Weiss and colleagues reported that the influx of Ca^{2+} into erythrocytes during interaction of basigin with PfRh5 complex might happen because of echinocytosis (Weiss *et al*., 2015).

After the infection is established in erythrocytes, schizogony (asexual reproduction by multiple fission) occurs within the next 48 hours resulting in the egress of 16-32 merozoites which destroys the erythrocyte membrane and release of parasites to access and invade new host cells. This coordinated process is strongly regulated and includes several protein kinases, such as cGMP-dependent protein kinase (Collins *et al*., 2013), and plant-like calcium-dependent protein kinase PfCDPK5 (Dvorin *et al*., 2010).

2.2.2 Transition to transition

Some merozoites switch their development during several rounds schizogony; developing into male and female gametocytes in the bloodstream of the host. Transmission of malaria from the host (humans) to the vector (mosquitoes) depends on this developmental switch and has been known as a probable point of intervention, either via transmission-blocking drugs or vaccines.

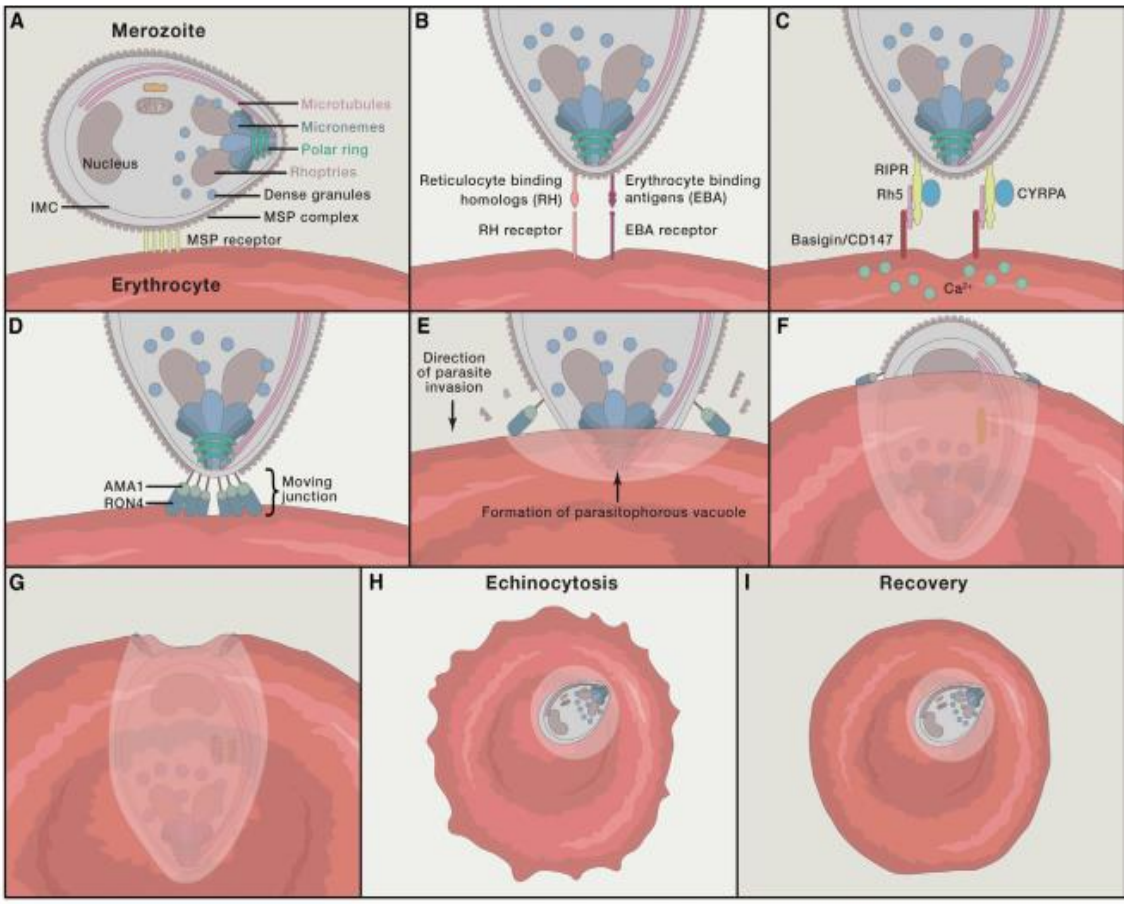


Figure 2.3 Erythrocytes invasion by merozoites. The attachment of merozoite to erythrocytes is caused by low-affinity interactions between the merozoites and the erythrocytes surface coat proteins. **A.** Erythrocytes membrane is wrapped around the merozoite for reorientation to occur, which brings the end of the apex directly in contact with the membrane of the erythrocyte. This is followed by specific ligand-receptor interaction, **B**, facilitated by PfRh and EBA protein family members. This process induces invasion events downstream involving PfRh5 complex binding to basigin, a host cell receptor. The downstream events are linked with the influx of calcium into the red blood cells and microneme secretion which allows deposits of RON complex into the membrane of the red blood cells and then allowing the binding of AMA-1 directly to form a moving junction. **D**. Merozoites force into erythrocyte membrane by reversely mobilizing merozoite-internal actomyosin complex concurrently with the discharge of rhoptries contents which helps the formation of a parasitophorous vacuole membrane (PVM) that encloses the merozoite (E-G). **H**. When the PVM and the erythrocyte membrane is sealed, echinocytosis occurs as a result of water loss from the erythrocyte cytosol. I. Homeostasis follows to recover erythrocytes. (Mantel *et al*., 2013).

Although the molecular events around this shift have not been fully elucidated, the timing of transition happens at some point in the previous schizogony cycle, and daughter from a single-infected cell is committed to either develop asexual schizonts or gametocytes.

Exposure to resistant drugs and high parasitaemia are some of the environmental factors which increase the conversion of gametocyte production. This also shows that parasites can sense their environment (Mantel *et al*., 2013; Regev-Rudzki *et al*., 2013). AP2-G is a major transcription factor that regulates gametogenesis, while the regulation of epigenetics is crucial for controlling sexual differentiation (Kafsack *et al*., 2014). The maturation process of *P. falciparum* gametocytes is extended compared to other species. It takes about 11 days for infectious gametocytes to develop once a commitment is initiated, and they remain sequestered in the bone marrow during this process to avoid clearance by the spleen until appearing in peripheral circulation until an uptake when a mosquito feeds on an infected individual.

2.3 Antimalaria Drug Discovery

Quinine, a natural product, remained an antimalarial agent for several years but was largely replaced by a series of synthetic drugs such as folic acid synthesis (e.g. proguanil, pyrimethamine), 8-aminoquionolines (e.g. primaquine), and 4-aminoquinolines (e.g. chloroquine, amodiaquine). Malaria was expected to be entirely eradicated by the mid50's but was hugely undermined due to resistance problems (Phillipson & O'Neill, 1987). The malaria vector became resistant to DDT while some *P. falciparum* strains became resistant to chloroquine.

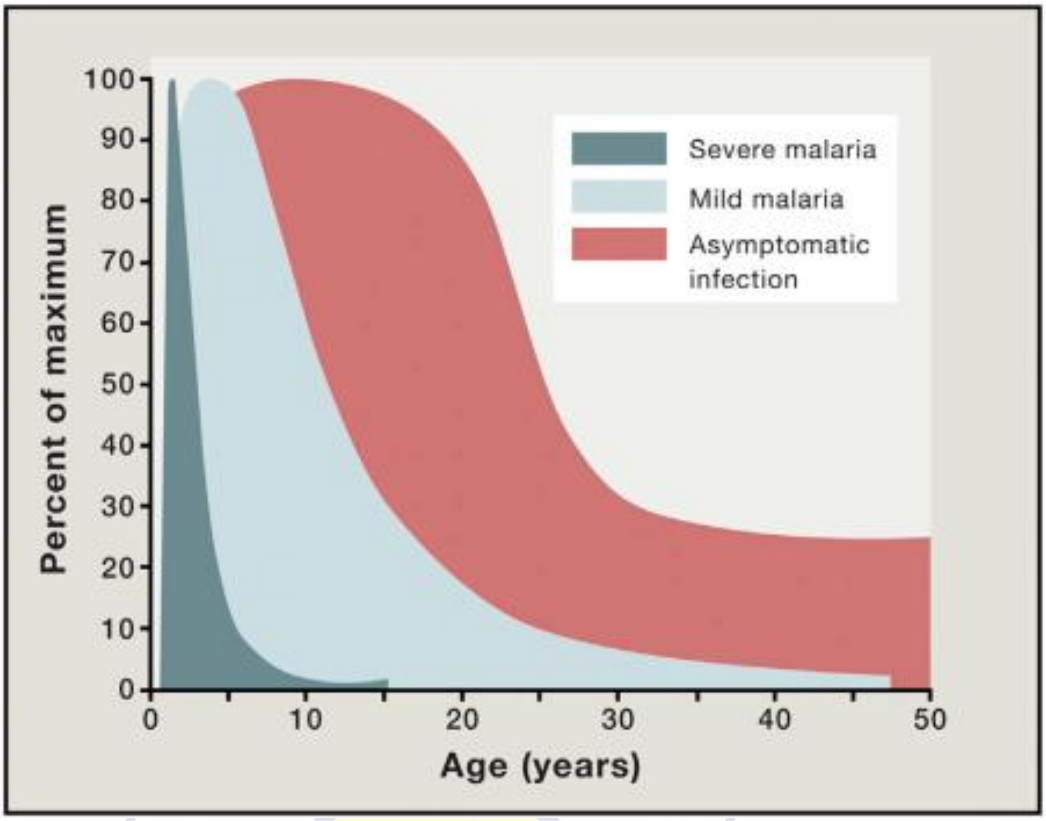


Figure 2.4 The scheme is showing the Immunity Development to diverse Manifestations of Malaria infection in an Endemic population (Langhorne, Ndungu, Sponaas, & Marsh, 2008).

Later several other strains of *P. falciparum* became a multi-drug resistant, and currently, resistance to chloroquine is widespread in endemic areas of *P. falciparum* infection such as East Africa, Subsahara Africa, S. America and South-East Asia. The rise in international travellers in recent years indicates the risks of non-endemic areas resistance to chloroquine.

The issue of resistance of malaria parasites means that there will be a continuous demand for chemotherapeutic agents to completely manage the disease and that the urgent need to discover new antimalarial agents is optimum. Traditional medicinal plants have proven in history to play a significant role in the quest to

discover antimalaria agents and thus, there is the need to establish their efficacy and safety.

2.3.1 Natural products

One of the well known and most valuable plants used in malaria treatment is the *Cinchona species*; its alkaloid quinine is still used for managing malaria. Maybe, quinidine, the stereoisomer (Figure 2.5) is less widely known, but it is as potent or, perhaps more potent than quinine (White, 1985). *Artemisia annua*, a compositae, is used by the Chinese in their traditional malaria treatment, and its active compound, artemisinin, is recently under considerable interest (figure 2.5). The chemotherapeutic index of artemisinin is higher than chloroquine, and it is effective in strains of the human malaria parasite, which are chloroquine-resistant (Warhurst, 1985). Plants used for the management of malaria extends to other parts of the world such as Africa (*Crytolepis sanguinoleta, Piper guianiense*, *Mormodica foetida*) (Sofowora, 1980), Americas (*Momordica charantia, Rhaphidophora decisive, Culcasia lancifolia)(*Ahmad, Hasan, Ahmad, Zishan, & Zohrameena, 2016; Frausin, Lima, Hidalgo, Ming, & Pohlit, 2015), and Asia (*Trichilia megaliths* , *Vernonia amygdalina, Stephania abyssinica*) (R. Kaur & Kaur, 2017). The natural product database, NAPRALERT, lists about species of plants from 152 genera which have folklore reputations for antimalarial properties. It is prudent that modern biological technologies are being employed in evaluating the acclaimed properties of these plants and establish their efficacy and safety.

2.3.1.1 Research on antimalarial plants

Artemisinin (figure 2.5) discovery and development is arguably the most significant development regarding antimalaria drug discovery in recent years. Artemisinin is the active component of *Artemisia annua*, used traditionally as antimalaria agent (Phillipson & O'Neill, 1987). It has been reported to have low toxicity and principally active against chloroquine-resistant *P. falciparum in vivo*. Nevertheless, the average recurrence rate is relatively high (10%) when a patient takes the usual dose of 0.6 mg/day for three days (Phillipson & O'Neill, 1987). The lipophilic nature of Artemisinin causes inherent problems when administered. Thus, several derivatives such as sodium artesunate and artemether (mehyldihydroartemisinin) have been developed.

Some protagonists of herbal medicine argue that the total plant extract contains different substance mixtures which synergistically act for a better efficacy hence the need for a patient to take whole plant or extract than the single isolate or active ingredient. This is because, like most naturally-occurring therapeutic agents, little quantity of the active ingredient exists in the whole plant, and sometimes it is difficult to get the quantity of the active compound in the plant. Example, some Chinese workers did not find artemisinin in 30 other *Artemisia* species. Likewise, a group at Walter Reed Army Institute of Research studied other 70 *Artemisia* species and did not find artemisinin in any of them (Kinghorn & Balandrin, 1993).

Another plant similar to *Artemisia,* which contains endoperoxides in its active compound, yingzhausu, is the Annonaceae, *Artabotrys hexapetalus (Xaio, 1983). Artemisia diffusa* also contains endoperoxides in the principle agent,

tehranolide (Rustaiyan, Sigari, Jakupovic, & Grenz, 1989). The antiplasmodial activity of *A. diffusa* crude extracts against *P. berghei* has been established *in vivo* in mice. The study indicated that *A. diffusa* active constituents are cytotoxic against *P. berghei*, hence, inhibiting the erythrocytic development stages of the parasite. Expressly, the results showed the inhibitory effects of *A. diffusa* crude extracts and the fraction which contains the tehranolide on the *P. berghei* developmental stages by decreasing parasitemia (Rustaiyan *et al*., 1989).

Simaroubaceae species are pantropically used for treating malaria *in vivo*. The antimalarial activity has been established in several quassinoids, bitter upon tasting, biosynthetically degraded triterpenes are characteristics of the family. (Fandeur *et al*.,1985, Pavanand *et al*., 1986, O'Neill *et al*., 1986).

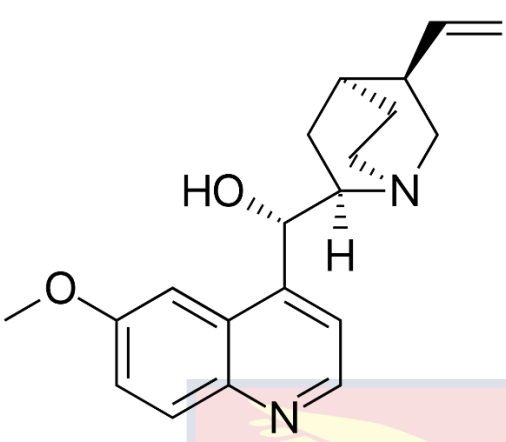
Cryptolepine (CPE), isolated from *Cryptolepis sanguinolenta*, is an indoquinoline whose several pharmacological activities have been investigated. It has been established to have potent antimalarial activities against chloroquine-resistant and Chloroquine sensitive *P. falciparum* strains (Mensah, Benneh, Forkuo, & Ansah, 2019). Forkuo and colleagues recently demonstrated the gametocytocidal activities of cryptolepine against the asexual stage of *P. falciparum* (NF54)(Forkuo *et al*., 2017). Also, cryptolepine showed synergistic interaction with arteminins and xylopic acid when they were co-administered in managing malaria in mice infected with *Plasmodium berghei* (Ameyaw *et al*., 2018; Arnold D Forkuo *et al*., 2016).

Several countries have numerous experiences in the use of plants with medicinal properties and the knowledge extends across several states. A review by

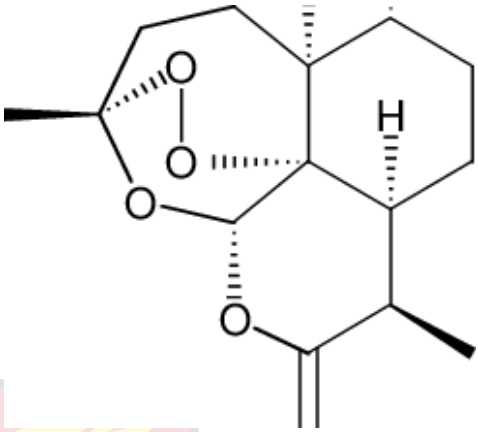
Rajandeep and Harpereet (2017) lists several plants from around the globe with antimalaria properties.

2.3.1.1.2 Xylopia aethiopica

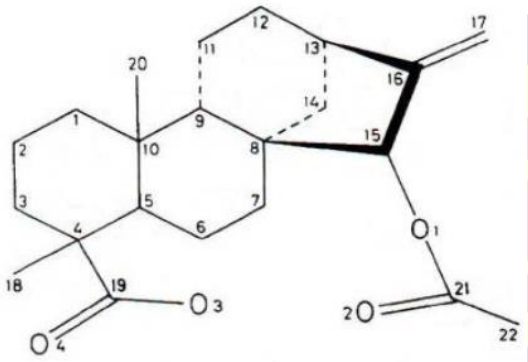
Xylopia aethiopica, commonly known as the Ethiopian pepper, is an evergreen tree which belongs to the Annonaceae family and found in moist fringe forest in several parts of Africa (Johnson & Murray, 2018; Senthilkumar & Murugesan, 2012). Dried fruits of *Xylopia aethiopica* are used as spice and herbal medicine. Its pure compound isolated from the unripe fruits, xylopic acid (figure 2.5), has been reported to have antiplasmodial properties (Boampong et al., 2013). Boampong and colleagues also reported its prophylactic and antipyretic properties. Xylopic acid has shown anti-inflammatory properties and the ability to reduce fever in *Sprague-Dawley* rats following lipopolysaccharide(LPS)-induced fever similar to prednisolone (Boampong et al., 2013). A recent report by Ameyaw and colleagues indicated the synergistic property of xylopic acid in combination with cryptolepine resulting in almost total clearance of malaria parasites in comparison with artemether-lumefantrine in an experimental malaria model (Ameyaw *et al*., 2018).



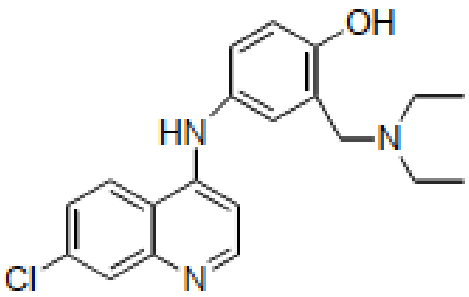
Quinine, Quinidine



artemisinine



Xylopic acid



Amodiaquine

Figure 2.5: Examples of antimalaria natural product

2.3.2 The need for drug combinations

There is a developing general agreement about the relevance of drug combinations to the best management of malaria in endemic regions (Guerin *et al*., 2002). Combination possibly gives several important advantages to monotherapy. First, they should improve their efficacy relatively, compared to individual drugs. Suitably chosen combinations should be able to add at least some potency and give synergistic activity.

Nevertheless, drug combinations which solely rely on synergy will probably not be able to offer higher protection against reluctance selection as expected. The reason being that resistance to any of the component of combination will to lead to a significant decrease in efficacy. Indeed, the well known synergistic activity of SP behaves as a single agent in this regard, with quick selection of resistance (Dorsey, Vlahos, Kamya, Staedke, & Rosenthal, 2003; Rabinovich *et al*., 2017), and similar concerns are reported in the new atovaquone/proguanil combination (Travassos & Laufer, 2017). Second, the likelihood that one drug can become active when there is a resistance of the other is high in combination therapies (Blasco *et al*., 2017). Third, resistance should be reduced in combination therapies; this is arguably the most essential factor for combination therapies in antimalaria drug development. Areas of low endemicity have usually been indicated to develop resistance which probably might be due to high parasitemia and symptoms in non-immune individuals. Example, a current report (Nakoja, 2017) shows a selected resistance for SP for resistances-conferring mutations and subsequent failure in treatment, but a combination of artesunate and SP prevented the SP-resistant parasites selection in subsequent infections (Madanitsa *et al*., 2016).

Combination regimens are supposed to incorporate two individual agents into one, give potent efficacy and if possible have similar pharmacokinetic profiles. Unfortunately, the requirements are challenging, and currently, there is no combination available which has met the requirements. Artemisinins are widely advocated for combination therapies in antimalarials. The reason is that, although their shorter half-lives expose it to late recrudensces, there are no significant reported resistance problems (Hemingway *et al*., 2016). Hopefully artemisinins potent action will block substantial parasite resistance selection to the component which is longer-acting (example, lumefantrine/ artemether chlorproguanil/ dapsone/artesunate (Lang & Greenwood, 2003), mefloquine/artesunate (Phyo *et al*., 2016), or amodiaquine/artesunate (Adjuik *et al*., 2002; Feng, Li, Yan, Feng, & Xia, 2015)). Although there are widespread artemisinin combinations currently, there is an urgent need for novel antimalarial agents, because artemisinins are natural products and their synthesis are expensive especially in endemic areas like Africa, where the need is greatest, and resources are limiting.

2.3.2.1 Drugs resistance in Plasmodium

Before the introduction of artemisinin-based combination therapies (ACTs), chloroquine and later sulfadoxine-pyrimethamine were efficacious, relatively cheaper, safe, and used to treat infection with *P. falciparum* worldwide (Jensen & Mehlhorn, 2009). Spread of resistance and clinical failure to sulfadoxine-pyrimethamine, and chloroquine (Ariey et al., 2014; Mixson-Hayden et al., 2010; Shah *et al*., 2011), in Africa led to the use of ACTs. Artemisinins give quick and potent effectiveness in ACTs, while the second, longer-lived antimalarial offset

their rate of plasma clearance as a partner drug. The reason being that artemisinins quickly clear most of the parasites within days with a different mechanism from the partner, which clear the remaining parasites over weeks, giving the idea that parasite that develops resistance to the artemisinin drug would still be cleared by the partner drug.

Genome-wide association studies (GWAS) have helped to identify the resistant molecular rmarkers of parasites in the study of drug-resistant parasites (Ariey *et al*., 2014; Cheeseman *et al*., 2012). Apart from the detection of molecular resistant markers identification, understanding the mechanism of resistance is critical to the development of strategies to contain and treat to eradicate malaria. Several studies have reported efflux pumps, and single nucleotide polymorphisms (SNPs) amplification in the target catalytic enzymes to cause many antimalarials resistances (Blasco *et al*., 2017; Fidock *et al*., 2000; Sidhu, Verdier-Pinard, & Fidock, 2002). Artemisinins kill parasites by alkylating their main target proteins via their inhibitory mechanisms results in cleavage of parasite's endoperoxide bridge, which yields free radicals. Nevertheless, a recent proteomic study has revealed the probability of several targets, which means the killing of the parasite may be general destruction of the protein milieu (proteopathy) (Ismail *et al*., 2016). The major cause of artemisinin-resistance marker is the gene that codes for *P. falciparum* Kelch 13 (PfKelch13) found on chromosome 13 (Straimer *et al*., 2015). PfKelch13 belongs to a eukaryotic evolutionary gene family of 60 members and its assumed to regulate the quality control of proteins (Gupta & Beggs, 2014). PfKelch13 mammalian orthologues cause drug-resistance in cancer drugs that kill

by the induction of proteopathy, confirming the postulation that, there is a role played by PfKelch13 in the restoration of protein complex system functions in the parasite which promotes its survival from artemisinin-induced proteopathy (Nikesitch & Ling, 2016). In mechanical terms, this so-called proteostasis is different from pump mutation or amplification of a single target enzyme which causes resistance. Apart from Kelch 13, mutations in other genes can also contribute to the resistance of *P. falciparum* against antimalarial agents. Example, the mammalian orthologue Kelch-like ECH-associated protein 1, which is responsible for the binding and ubiquitylating nuclear transcription factor, to control the protein levels of the parasite (Taguchi & Yamamoto, 2017). When this protein is mutated, it causes elevated NFE2L2 protein levels and its translocation to the nucleus. Hence it is emphasiszed that PfKelch13 might also regulate an analogues transcription factor n the parasite.

Two main distinct mechanisms have been proposed for artemisinin resistance; the activation of unfolded protein response (UPR) and prostatic dysregulation of *P. falciparum* phosphatidylinositol 3-kinase (PfP13K), leading to elevated levels of phosphatidylinositol-3-phosphate (PtdIns3P), a lipid product (Mbengue *et al*., 2015). Concurrent resistance to partner drugs have fast-tracked clinical failure of ACTs (Amato *et al*., 2017; Witkowski *et al*., 2017), which emphasizes that there is a need to manage present ACTs and other antimalarials better in addition to development of novel antimalarial drug combination agents via a discovery pipeline informed mechanisms of resistance to ACTs. A recent suggestion that immune system of the host contributes to resistance to ACTs

(Ataide *et al*., 2017), which looks like a distinct characteristic of artemisinin resistance, may affect malaria elimination if new drugs are not discovered.

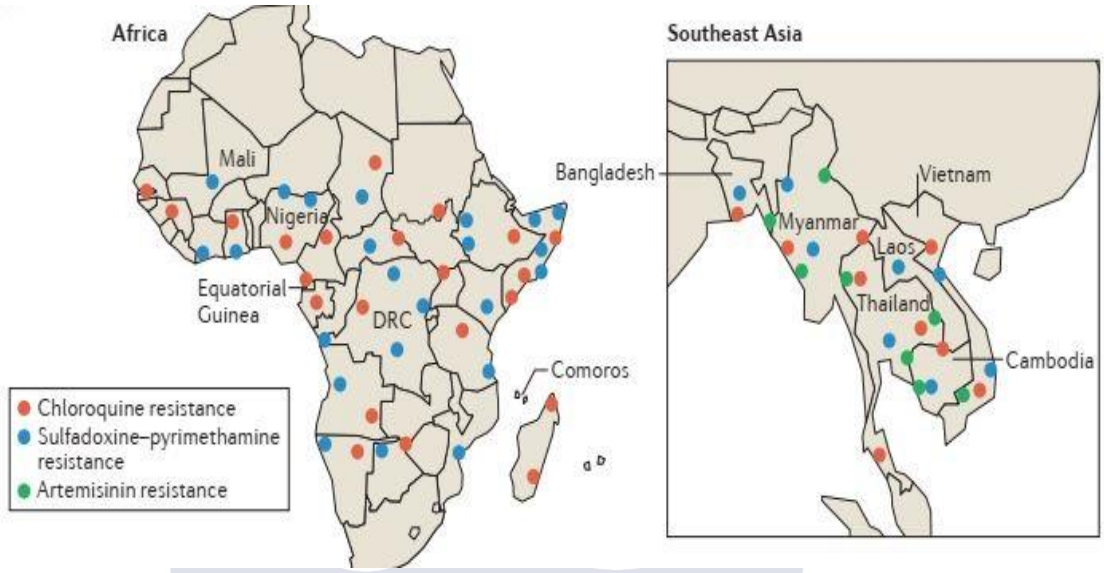


Figure 2.6. Antimalarial drug resistance epidemiology. Maps are showing in detail, the dispersal of *P. falciparum* resistance to known antimalaria drugs; sulfadoxine-pyrimethamine, chloroquine, and artemisinin in Southeast Asia and Africa (Ariey *et al*., 2014; Ménard *et al*., 2016). Each dot represents a region of emergence of drug resistance. DRC means the Democratic Republic of Congo.

2.4 Experimental malaria models

In vivo testing of antimalarial agents is time-consuming, and expensive, but a requirement for initial screening for isolated compounds from plants. Until recently that *in vitro* tests for antimalarial drugs has been established, but it has not eliminated the relevance of *in vivo* tests for antimalarial discovery. A breakthrough *in vitro* antimalarial testing following the development of a method for the continuous *in vitro* cultured *P. falciparum*, the human malaria parasite (Trager & Jensen, 1976). Desjardins *et al*. in 1979 designed a qualitative assessment technique for anti-*P. Falciparum in vitro* activity. Though the method has since been modified

by Fairlamb and colleagues, it still depends on the ability to block ^{3}H-hypoxanthine incorporation into plasmodia Fairlamb (Fairlamb, Warhurst, & Peters, 1985).

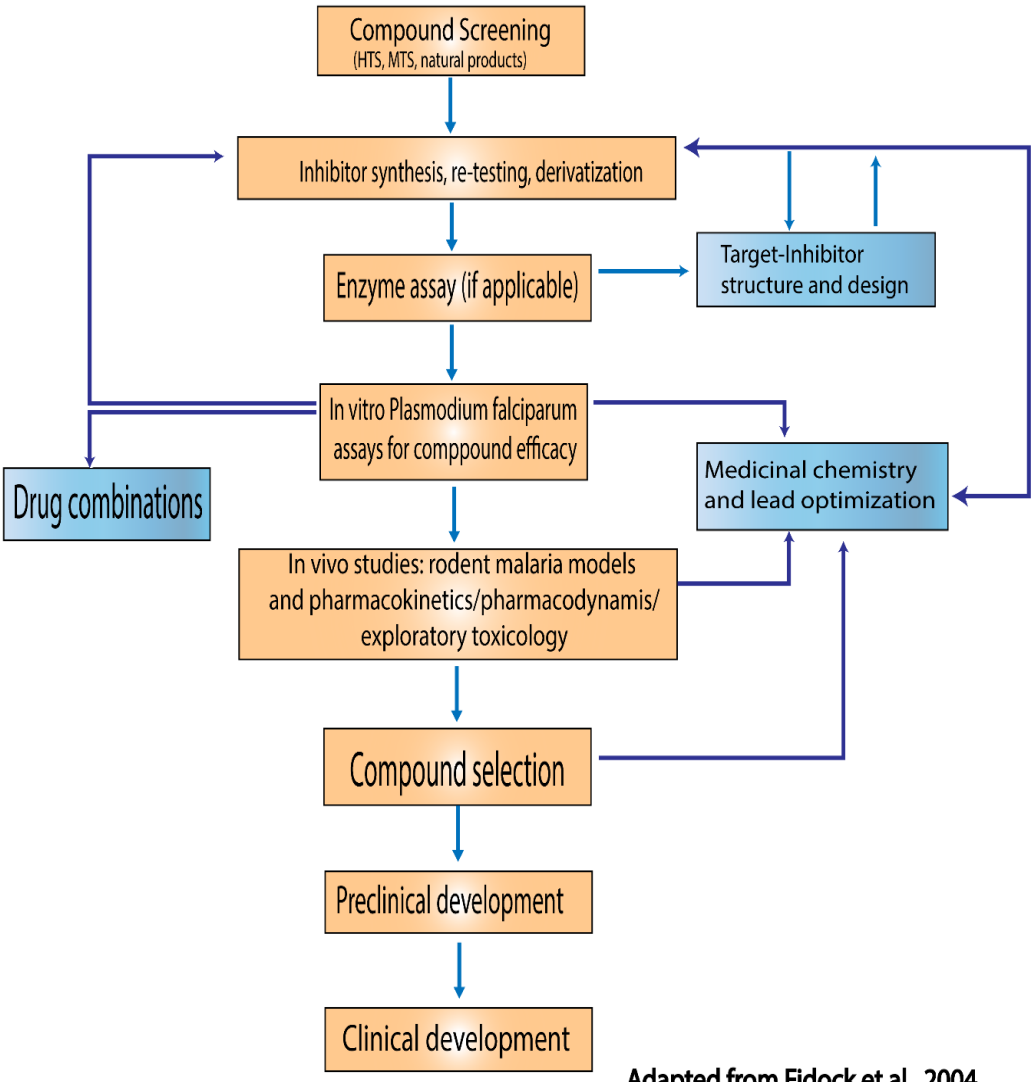
World Health Organization (WHO), prepared in 2015, guidelines for antimalarial screening. The four stages described in the guidelines; 1. Primary screening establishes the effectiveness of compounds against the malaria parasite. 2. Secondary screening should be able to quantify and qualify the antiparasitic activity of compound and determine safety compared to activities on analogues. 3. The tertiary screening is done in order to study human and non-human primates other than man preceding the fourth stage of the clinical testing. Techniques outlined by Peters in 1987 can be used to assess isolated compounds from plants for the presence of their antimalarial activity. Both *in vivo* and *in vitro* are used for initial stages (primary and secondary screening). *In vitro* tests discriminates between species of the same genus. For example, an ethanolic extract of *A. bulgaris* and *Artemisia annua* was prepared, but only *Artemisia annua* was revealed to contain artemisinin. Upon tenfold dilution and twofold dilutions within narrow ranges of concentration, the IC_{50} values proved to be 3.9 µg/ml and 250 µg/ml respectively. Thus, there was discrimination in the in vitro test, because, between species of the same genus, artemisinin, the antimalarial compound was found in only one of the two species.

The plasmodia used for *in vivo* tests cannot parasitise humans. Nonetheless, several important pieces of information can be obtained from in vivo tests for antimalarial activity in rodent infections. *Plasmodium berghei* ANKA model is widely accepted model for experiment malaria. The reason being that it replicates

most of the events seen in human malaria infection. When a suitable strain of malaria mice including ICR is infected *P. berghei* ANKA, it reveals all the clinical signs of a fatal malaria pathology, such as; anaemia, fitting, respiratory distress and coma (De Souza & Riley, 2002).

2.5 Antimalarial drug discovery critical paths.

An antimalarial discovery programme will usually include a screening of compounds *in vivo* against rodent plasmodia and *in vitro* against *P. falciparum. In vivo* studies cut-off values vary based on the programmatic decisions and family of compounds and could be in the range of <5-25 mg/kg. Natural products screens are more focused on chemical families who are already known for their antimalarial activity. *In vivo* assays involve initial four-day suppressive tests in rodent parasitemia, but *in vitro* assays involves the determination of IC_{50} against drug-sensitive and drug-resistant *P. falciparum*. Both studies (*in vitro and in vivo*) are directed towards the selection of compounds. Lead optimisation and medicinal chemistry play a crucial role in this critical path. A secondary *in vivo* tests is not mandatory in this critical path; however, it gives details in evaluation of the compounds, such as dose-ranging, recrudescence, prophylaxis, onset of action and drug resistance. Transgenic rodent malaria models assay can offer additional screening, by assaying the *P. falciparum* target in an *in vivo* setting, using *P. falciparum* transgenic lines that express the mammalian orthologue or overexpress the target, and screens to determine the biochemical impact and frequency of acquiring resistance. (Fidock, Rosenthal, Croft, Brun, & Nwaka, 2004).



Adapted from Fidock et al., 2004. :

Figure 2.7. An Antimalarial drug discovery critical path.

2.6 Chapter Summary

Attempts to discover and develop novel antimalarials have risen dramatically in recent years, because of the increasing resistance of plasmodium parasites to current and past antimalarials and the recognition of the relevance of fighting malaria globally.

Biological researches into natural products employed traditionally for primary health care is one basic method for searching novel leading compounds. Several countries have numerous experiences of the use of medicinal plants, and the needed knowledge spans several centuries. In antimalarial drug discovery, medicinal plants have contributed substantially in providing lead and active compounds for the development of potent antimalarial agents including the currently available antimalarial drugs (artemisinins, and cinchona alkaloids). Xylopic acid, a kuarene diterpene, extracted from the unripe fruits of *Xylopia aethiopica*, has been investigated to establish antimalarial properties, as well as provide a synergistic activity when combined with cryptolepine, a compound extracted from *Cryptolepis nana*. Artemisinins also show good activity in combination therapies against *P. falciparum*, the human malaria parasite. It is only prudent that such a compound (xylopic acid) with established antimalarial activity be combined with Artemisinins in order to develop a drug that will combat the growing resistance of *P. falciparum* and *Plasmodium vivax*.

The growing ineffectiveness of the first two drugs for treating malaria infection, chloroquine and sulfadoxine-pyrimethamine and the increasing resistance to current artemisinin combination therapies call for the search of newer effective antimalarials. Current zeal for combining scientific innovation with expertise in drug discovery and development process provide hope that strenuous efforts can give us an advantage in curbing this disease. Time is a cruel judge, and this window of opportunity to discover and develop new, effective and affordable antimalarial agents cannot be missed.

CHAPTER THREE

METHODS

Introduction

This study seeks to evaluate the effects of the co-administration of xylopic acid and artesunate, and xylopic acid and amodiaquine in an experimental model malaria in ICR mice. This chapter is organized to achieve the specific objectives of the study i.e. to determine ED_{50S} of artesunate, amodiaquine, and xylopic acid monotherapies in *P. berghei* ANKA-infected mice and subsequently test for synergism, potentiation and inhibition by performing an isobolographic analysis in *P. berghei* ANKA-infected mice after co-administration of artesunate and xylopic acid, and xylopic acid and amodiaquine.

This chapter is organised into the extraction of xylopic acid, reagents used, and parasite acquisition and inoculation, the experimental design and software employed to achieve the goal of this research.

3.1. Methods

3.1.1.Xylopic acid extraction

Xylopic acid was extracted from *Xylopia aethiopica* as previously described (Ameyaw et al., 2014; Woode, Ameyaw, Boakye-Gyasi, & Abotsi, 2012; Woode et al., 2016). Fresh unripe fruits of *Xylopia aethiopica* were purchased from Ho Central Market. They were shade dried and pulverized. For every 100 g of plant material, 300 ml of petroleum ether was used as a solvent for maceration. The mixture of *Xylopia aethiopica* and petroleum ether was left to stand for three days with continues shaking every 24 hours. Whatman filter paper (11 µm pore size) was used to filter the mixture and left to stand overnight under dark condition. The filtrate was then concentrated with Rotary evaporator (Heidolph Labo Rota, 4002) at 120 revolutions per minute and 40-55ºC. The concentrate was left was to stand for 72 h and 3 drops of ethyl acetate added to facilitate crystallization of crude xylopic acid crystals. Crude xylopic acid was washed several times with petroleum ether, and dissolved in absolute ethanol for purification by recrystallization (Ameyaw *et al*., 2014; Woode, Ameyaw, Boakye-Gyasi, & Abotsi, 2012; Woode *et al*., 2016).

The purity of the xylopic acid was checked by thin layer chromatography using petroleum ether and ethyl acetate (9:1) as solvent system and anisaldehyde as the indicator as established by Biney *et al*. (2014). The isolated purified compound was dissolved in chloroform and directly spotted onto a pre-coated *silica gel* 60 plate. Pure xylopic acid was used as a reference. Both the isolated purified compound and the reference compound gave Rf value of 0.53.

3.1.2.Experimental animals

Six to ten weeks old female ICR mice purchased from Centre for Medicinal Plant Research, Akuapim Mampong, Ghana, were used for the study. A total of one-hundred and fifty-five animals were used for the study. They were housed in stainless steel cages (16.5 × 11.0 × 13.5 cm^3) with beddings made from softwood shaving under appropriate laboratory conditions and fed with normal commercial pellet diet purchased from Agricare, (Kumasi, Ghana) and water *ad libitum*. The cages were kept in the Department of Biomedical Sciences animal holding facility, University of Cape Coast and the wood shavings were replaced every 3 days and disinfected with 70% alcohol. The facility had a 12/12 h light/dark cycles and a mean temperature of 21°C.

3.1.3. Drugs, Chemicals and Reagents

Artemether/lumefantrine combined tablets (20/120 mg), artesunate, and amodiaquine were acquired from Novartis Pharma AG Basel, Switzerland. Hydrochloric acid, Giemsa stain, absolute methanol, chloroform, petroleum ether, ammonium hydroxide, 96% ethanol, liquid paraffin, and ammonium chloride were also purchased from Sigma Aldrich. St Louis, MO, USA.

3.1.4. Parasite acquisition and inoculation

Chloroquine-sensitive strain rodent malaria parasite, *Plasmodium berghei* ANKA was acquired from Noguchi Memorial Institute for Medical Research (NMIMR), University of Ghana. The parasites were maintained by a continuous passage in mice intraperitoneally every six days (Ishih *et al*., 2004). After three

days of passaging, blood was collected from the tail for thin smears; to check the parasitaemia level before re-infecting another mouse.

Once high parasitaemia (30-40%) is established in a donor mouse, it is sedated under chloroform, following Hoff's technique (Nagamori, 2016). Blood was collected by cardiac puncture and transferred into EDTA tubes, capped, and topped up with Phosphate Buffered Saline (PBS). The mixture containing blood, EDTA and PBS are washed by centrifuging three times with haematocrit centrifuge, at 15000 rpm for 6-7 minutes to obtain pellets, with PBS being added following each centrifugation. Total inoculum concentration of 17.4×10^7 *P. berghei* ANKA parasitized erythrocytes were prepared and each mouse was inoculated with 0.20 ml of PBS containing 1.2×10^6 parasitized red blood cells.

3.1.5. Body weight measurement

Mice body weights were measured on days 0 and day 7 post-infection following a procedure described by Dikasso and colleagues (Dikasso et al., 2006) using a TOLEDO® Metler Balance (Japan). The weight was taken to observe the reported weight loss that usually occurs with increasing parasitaemia in infected mice.

3.1.6. Rectal temperature measurement

The body temperature of mice was observed on days 0, before parasite infection and day 7 post-infection. The rectal temperature was measured by inserting the pre-lubricated round tip of a digital thermometer into the anus of mice

until a beep sound was heard to confirm the reading (Belay, Endale Gurmu, Wubneh, & Medicine, 2018)

3.1.7. Antimalarial activity

3.1.7.1. In vivo anti-malarial assay of xylopic acid, artesunate, and amodiaquine monotherapies

To confirm the reported anti-malaria properties of xylopic acid (XA), artesunate (ART), and amodiaquine (AQ), and also determine their ED_{50} values for the isobolographic analysis, the anti-plasmodial activity of each compound was assessed. After infection, mice were assigned to 18 groups (n=5). Mice in all groups were inoculated with *P. berghei* except Group 18 mice which served as naïve control. Seventy-two hours post-inoculation (day 3), all groups of animals were treated once daily by oral administration with a gastric gavage with either xylopic acid (3, 10, 30, 100, 150 mg kg^{-1}), artesunate (1, 2, 4, 8, 16 mg kg^{-1}), amodiaquine (1.25, 2.5, 5, 10, 20 mg kg^{-1}), artemether/lumefantrine (1.14/6.9 mg kg^{-1}), or vehicle, 10 ml kg^{-1} (naïve and sham control). The ED_{50} values obtained as fitted midpoints of XA, ART, and AQ were determined by iterative curve fitting of log-dose responses of XA, ART, and AQ. Mice were observed at 12 h intervals for death and the median survival and hazard ratio over a 30-day period computed.

3.1.7.2. In vivo isobolographic assessment of xylopic acid-artesunate co-administration on **PbA-***induced malaria*.

To assess the antiplasmodial property of xylopic acid-artesunate (XA/ART co-administration) on established *Plasmodium berghei* infection, 60 female mice were each inoculated with 1.2×10^6 in 0.2 ml PBS and assigned to 8 groups (n=5). On day 3 (seventy-two hours later) each group received fixed ratio (1:1) or

combinations of fractions of the respective ED_{50} values of (9+1.6 mg kg^{-1}), (4.5+ 0.8 mg kg^{-1}), (2.25+0.4 mg kg^{-1}), (1.13+ 0.2 mg $^{kg-1}$), (0.6 + 0.1 mg $^{kg-1}$), being ED_{50} (XA/ART), ED_{50} (XA/ART)/2, ED_{50} (XA/ART)/4, ED_{50} (XA/ART)/8, and ED_{50} (XA/ART)/16, respectively. Positive control (AL) and negative control (sham) mice received 1.14/6.9 mg kg^{-1} AL and 10 ml kg^{-1} vehicle, respectively.

3.1.7.3. In vivo isobolographic assessment of xylopic acid-amodiaquine co-administration on PbA-induced malaria.

To assess the anti-plasmodial property of xylopic acid-artesunate (XA/AQ co-administration, on established *Plasmodium berghei* infection, 60 female mice were each inoculated with 1.2 $\times 10^6$ in 0.2 ml PBS and assigned to 8 groups (n=5). Seventy-two hours later, each group received fixed ratio (1:1) or combinations of fractions of the respective ED_{50} values of (9 +3.1 mg kg^{-1}), (4.5+ 1.6 mg kg^{-1}), (2.25+0.8 mg kg^{-1}), (1.125+ 0.4 mg $^{kg-1}$), (0.6+ 0.2 mg $^{kg-1}$), ED_{50} (XA+AQ), ED_{50} (XA+AQ)/2, ED_{50} (XA+ AQ)/4, ED_{50} (XA+ AQ)/8, and ED_{50} (XA+ AQ)/16, respectively Positive control (AL) and negative control (sham) mice received 1.14/6.9 mg kg^{-1} AL and 10 ml kg^{-1} vehicle, respectively.

3.1.7.4. Percentage chemo-suppression and parasitaemia evaluation.

Thin blood smears were made daily for 5 days by collecting three drops of blood from the tail of each mouse, fixed in absolute methanol, and stained in 10% Giemsa for 10 minutes to determine parasitaemia. The slides were microscopically examined at ×100 magnification. Parasitaemia was checked by counting infected red blood cells in a hundred fields, divided by the total red blood cells in the

hundred fields and then multiplied by hundred. The following formula were used to calculate the parasitaemia.

$$\% \text{ Parasitemia} = \frac{Number\ of\ Plasmodium\ berghei{-}infected\ erythrocytes}{Total\ number\ of\ erythrocytes} \times 100$$

Chemosupression or percentage inhibition of parasitaemia was computed by employing the following formula;

$$\% \text{ inhibition} = \frac{(Mean\ parasitaemia\ of\ negative\ control) - (Mean\ parasitaemia\ of\ test\ drug)}{Mean\ parasitaemia\ of\ negative\ control} \times 100$$

3.2. Data analysis

All statistical analyses were computed with the windows version of GraphPad Prism 7.0 (GraphPad Software, San Diego, CA, USA). Data is presented as mean ± SEM and analysed using One-way analysis of variance (ANOVA). Tukey's honest significant difference (HSD) test was used as a *post hoc* test was used for multiple comparison when ANOVA was significant.

Two isobolograms which consisted of the ED_{50} of XA on the ordinate and ED_{50} of ART or AQ on the abscissa connected with a line of additivity were constructed. The ED_{50} of each drug was determined by linear regression analysis of log dose-response curve (and a T-test was used for the comparison to a theoretical additive ED_{50} i.e. Z_{add}). Z_{add} was computed with the following formulae

$$Z_{add} = (f)\ ED50\ of\ ART + (1\text{-}f)\ ED50\ of\ XA$$

and

$$Z_{add} = (f)\ ED50\ of\ AQ + (1\text{-}f)\ ED50\ of\ XA$$

where f = fraction of each component in the mixture/ combination while the Var (variance) of Z_{add} was computed as follows:

$$\text{Variance of } Z_{add} = f2\ (VarED50\ of\ CYP) + (1\text{-}f)\ 2VarED50\ of\ XA$$

SEMs were calculated from these variances and fixed according to drug's ratio in the combination. If the effect of a drug combination was statistically different (ED_{50} significantly lower) and higher than the theoretically calculated equieffect of a drug combination in the same proportion, it has a supra-additive or synergistic effect.

3.3. Chapter Summary

Xylopic acid was extracted from unripe pulverized dried fruits of *Xylopia aethiopica* following a method described by Ameyaw and colleagues (Ameyaw et al.,2012). Thin-layer chromatography of xylopic acid, as described by Biney et al. ((2014), was used to confirm the purity of the xylopic acid. Six to 10 weeks old male mice used for the study.

PbA was acquired from Noguchi Institute for Medicinal Research, Legon. Each mouse was inoculated with 1.2×10^6 parasitized red blood cells for each drug group for the monotherapies as well as the combination therapies including controls (Positive, Negative and Naïve). Seventy-two hours post-inoculation (Day 3), parasitaemia was checked and their ED_{50}s examined after day 5 of treatment.

In another experiment, Mice were inoculated with 1.2×10^6 parasitized erythrocytes. Groups for combination therapies were as follows ED_{50}, $ED_{50}/2$, $ED_{50}/4$, $ED_{50}/8$, and ED_{50} /16.

All statistical analysis was computed using windows version of GraphPad Prism 7.0 (GraphPad Software, San Diego, CA, USA). Data was considered significant at $p<0.05$ and were presented as the mean ± SEM. Turkey's honest significant difference (HSD) test.

CHAPTER FOUR

RESULTS AND DISCUSSIONS

4.1. Results

The study aims to determine whether the co-administration of xylopic acid and artesunate, and xylopic acid and amodiaquine will be effective in managing malaria in experimental malaria mice. GraphPad prism for windows version 7.0 (GraphPad Software, San Diego, CA, USA) was used to analyze the results quantitatively. Data are presented as mean ± SEM (standard error of the mean) and considered statistically significant at $p<0.05$ using Tukey's honest significant difference (HSD) test.

4.1.0 Extraction and purification of xylopic acid.

Xylopic acid isolated from the extract revealed a single spot on the thin layer chromatograph which showed the presence of a single compound similar to pure xylopic acid. The spot for both for both the isolated and extracted xylopic compound occurred at 5.3 after the solvent front had been allowed to travel a total distance of 10 cm.

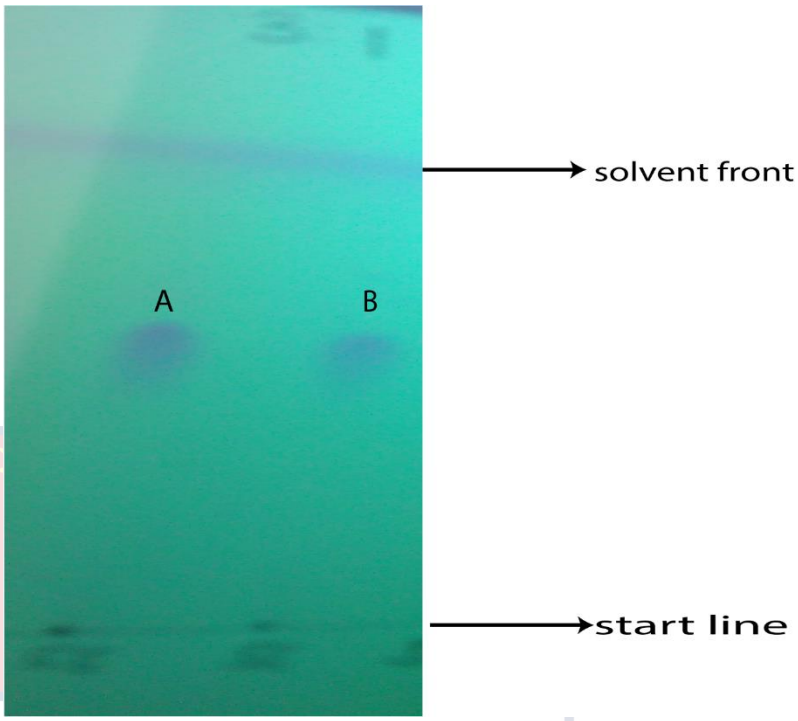


Fig 4.1. TLC results of A) xylopic acid isolated form from fresh unripe extract, and B) pure xylopic acid showing the presence of a single spot suggesting the presence of a single compound.

4.1.1. *In vivo* anti-malarial assay of xylopic acid, artesunate, and amodiaquine monotherapies

4.1.1.1. Effects of monotherapy on body weight

Amodiaquine and artesunate treatment groups significantly prevented the reduction of animal body weights (p=0.001). Although XA-treated groups were able to avoid to some extent, loss in body weight, this was not statistically significant. The highest doses of artesunate (8 mg/kg) and amodiaquine (10 mg/kg) showed an increase in animals' body weight similar to the naïve and AL group animals (Fig 4.2).

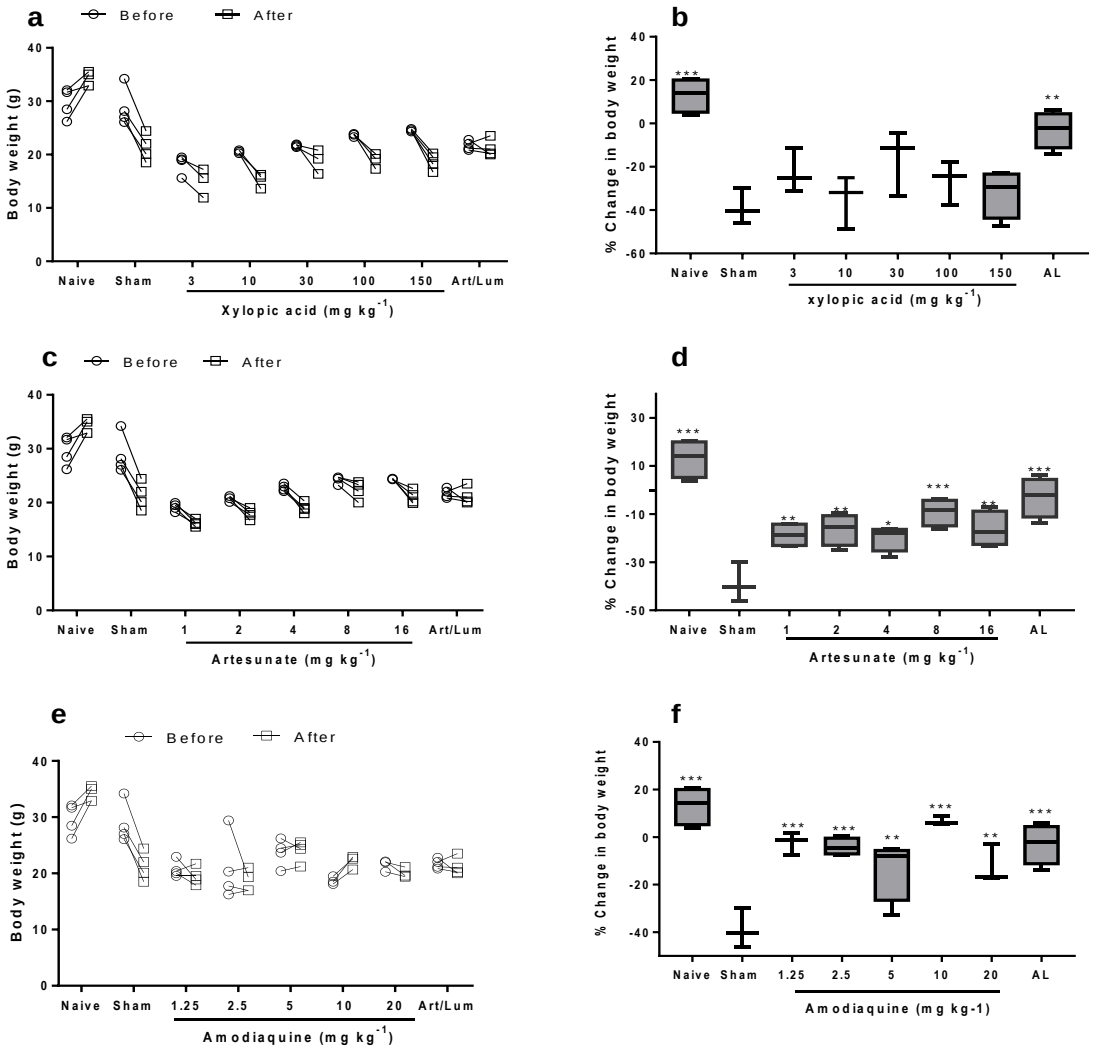


Fig 4.2 Body weight before infection and after treatment (left panel) and percentage change in body weight (right panel) for (a, b) xylopic acid, (c, d) artesunate, and (e, f) amodiaquine treated groups. Data is presented as mean ± SEM and analysed using One-way ANOVA followed by Tukey's honest significant difference (HSD) test used as a *post hoc* test for multiple comparison, (n=5), $p<0.05$

4.1.1.2. Effects of monotherapy on rectal temperature

Rectal temperature of the sham group was highly decreased (13.6%) on day 6. AL and naïve control groups showed a significant prevention in the rectal temperature decline in all the experiments ($p<0.05$). Also, the 16 mg/kg ART and 20 mg/kg AQ treated groups significantly stabilized rectal temperature ($p=0.001$),

whereas the 150 mg/kg XA-treated animals showed a slight reduction in rectal temperature of 8.7%. Only the AQ 20 mg/kg treatment group showed an increase in rectal temperature (2.3%) while 4 mg/kg ART-treated groups showed the highest reduction in rectal temperature (12%) among all the treated groups. Almost all AQ-treated groups stabilized rectal temperature except for the 2.5 mg/kg group.

Table 4.1. Rectal temperature of *Plasmodium berghei*-infected mice before (T_0) and after (T_6) treatment with xylopic acid.

Treatment (mg//kg)	T_0 (°C)	T_6 (°C)	% change in Rectal temperature
Naïve	38.28±0.22	37.58±0.30	-1.9*
Sham	38.10±0.19	33.33±0.42	-13.6
XA 3	37.8±0.21	35.17±0.57	-8.0
XA 10	37.95±0.27	35.17±0.31	-7.2
XA 30	37.68±0.25	36.30±0.57	-2.6*
XA 100	37.38±0.28	35.47±1.04	-5.0
XA 150	38.13±0.17	35.15±0.43	-8.7
AL 1.14	37.2±0.47	36.7±0.26	-1.5*

Data are presented as mean ± SEM, $p< 0.05$, n=5. T_0: rectal temperature measured on day 0 (before parasite infection), T_6: means rectal temperature measured on day 6 (after treatment). * = $p>0.05$. All temperatures are compared to the rectal temperature of sham group.

Table 4.2. Rectal temperature of *Plasmodium berghei*-infected mice before (T_0) and after (T_6) treatment with artesunate.

Treatment (mg/kg)	T_0 (°C)	T_6 (°C)	% change in rectal temperature
Naïve	38.28±0.22	37.58±0.30	-1.9*
Sham	38.10±0.19	33.33±0.42	-13.6
ART 1	38.53±0.12	36.33±0.56	-6.4
ART 2	38.48±0.11	36.75±0.33	-4.8
ART 4	38.93±0.11	33.78±0.6	-15.6
ART 8	38.78±0.19	36.13±0.34	-7.4
ART 16	37.18±0.22	38.05±0.09	2.3
AL 1.14	37.2±0.47	36.7±0.26	-1.5*

Data are presented as mean ± SEM, $p< 0.05$, n=5. T_0: rectal temperature measured on day 0 (before parasite infection), T_6: rectal temperature measured on day 6 (after treatment). * = $p<0.05$. All temperatures are compared to rectal temperature of sham group

Table 4.3. Rectal temperature of *Plasmodium berghei*-infected mice before (T_0) and after (T_6) treatment with Amodiaquine.

Treatment mg/kg	T_0 (°C)	T_6 (°C)	% change in rectal temperature
Naïve	38.28±0.22	37.58±0.30	-1.9*
Sham	38.10±0.19	33.33±0.42	-13.6
AQ 1.13	37.3±0.05	35.8±0.43	-4.2*
AQ 2.5	37.3±0.14	33.3±0.18	-12.0
AQ 5.0	37.5±0.08	36.0±0.11	-4.0*
AQ 10	36.4±0.46	37.3±0.17	0.0***
AQ 20	35.9±0.61	36,8±0.34	-0.6**
AL 1.14	37.2±0.47	36.7±0.26	-1.5*

Data are presented as mean ± SEM, $p< 0.05$, n=5. T_0: rectal temperature measured on day 0 (before parasite infection), T_6: rectal temperature measured on day 6 (after treatment). * = $p<0.05$, **=$p<0.01$, *** $p<0.001$. All temperatures are compared to rectal temperature of sham group

4.1.1.3. Monotherapy survival analysis

Artemether/lumefantrine-treated animals had 26 median survival days with a hazard ratio of 0.20 in the 30 days survival test (fig 4.3), representing the highest survival days and lowest hazard ratio in the treatment groups. Middle doses of XA (30 mg/kg), AQ (10 mg/kg) and high doses of ART treated groups also significantly increased survival days in the 30 days survival test ($p < 0.05$)

Table 4.4. 30-day survival analysis of *Plasmodium berghei* ANKA-infected mice after treatment with either xylopic acid, artesunate, amodiaquine or artemether/lumefantrine

Treatment (mg/kg)	Median Survival (days)	Hazard Ratio (Log-rank)	*p*-value
Naïve	30	-	-
Sham	11	-	-
XA			
3	14	0.49	0.2017
10	15	0.49	0.2017
30	21	0.15	0.0269*
100	17	0.46	0.1401
150	17	0.32	0.0272*
ART			
1	12	0.84	0.7501
2	15	0.51	0.2088
4	17	0.39	0.0689
8	18	0.25	0.0064**
16	24	0.20	0.0064**
AQ			
1.25	15	0.47	0.1843
2.5	15	0.48	0.1842
5	16	0.53	0.2097
10	23	0.25	0.0064**
20	16	0.40	0.0812
AL	26	0.20	0.0018**

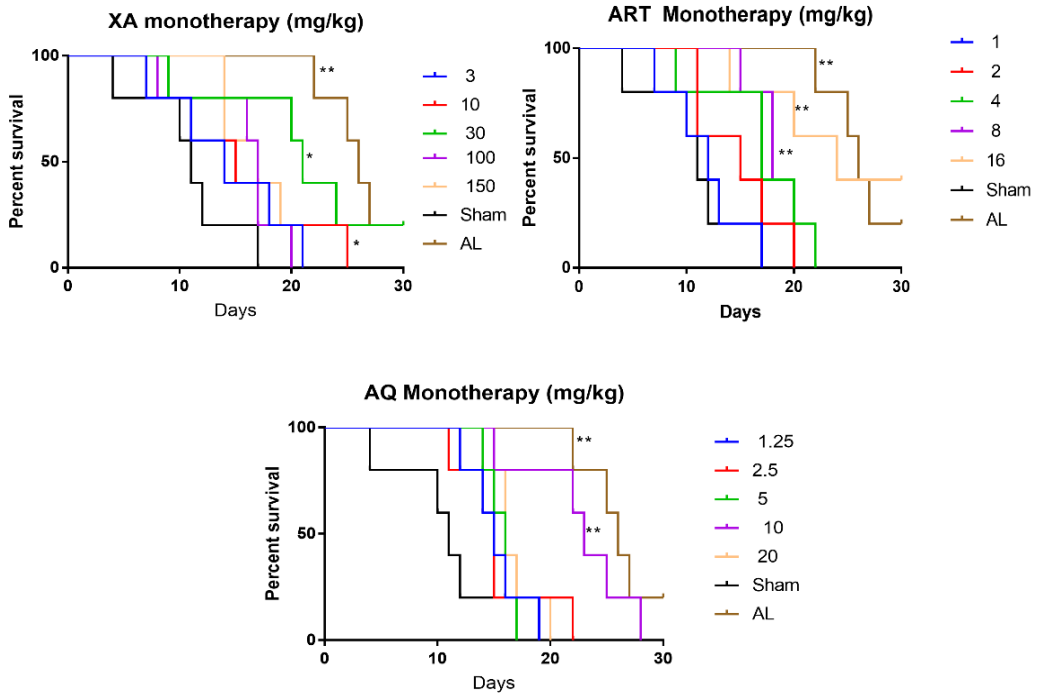


Figure 4.3. Kaplan Meier survival curves comparing the survival of malaria mice between sham and various treatment groups for 30 days after infection with *P. berghei*.

4.1.1.4. Effects of xylopic acid, artesunate, and amodiaquine monotherapies in vivo anti-malarial assay

ED_{50}s for xylopic acid, artesunate, and amodiaquine were 9.0±3.2, 1.61±0.6, and 3.1±0.8 mg/kg. By these results, artesunate was 1.9 times more potent than amodiaquine, and amodiaquine was 2. 9 more potent than xylopic acid (see fig 4.4).

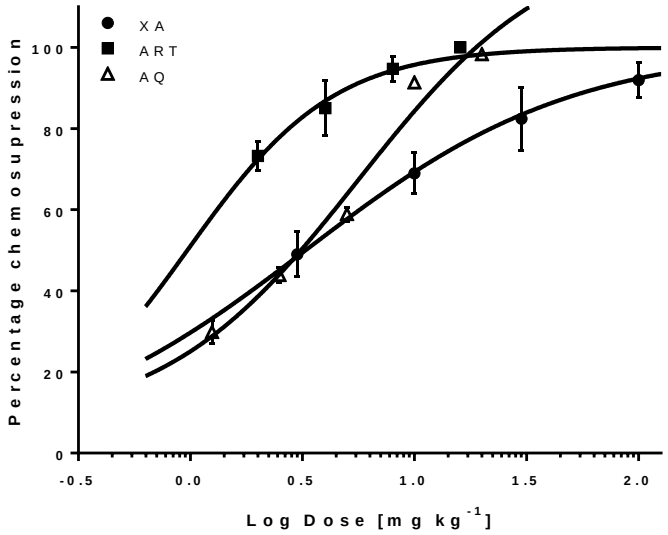


Figure 4.4. Log dose-response curves of percentage chemosupression in *P. berghei*-infected mice administered daily with xylopic acid, artesunate, or amodiaquine over 5 days. Data are presented as mean ± SEM (n=5)

4.1.2. *In vivo* assessment of xylopic acid-artesunate and xylopic acid amodiaquine co-administration on PbA-induced malaria

4.1.2.1. Effects on combination therapy on body weights

AL, 5.3 mg/kg XA/ART, 10.6 mg/kg XA/ART, 6.1 mg/kg XA/AQ, and 12.1 XA/AQ treated groups significantly prevented loss in body weight compared to the sham-treated group ($p < 0.05$)

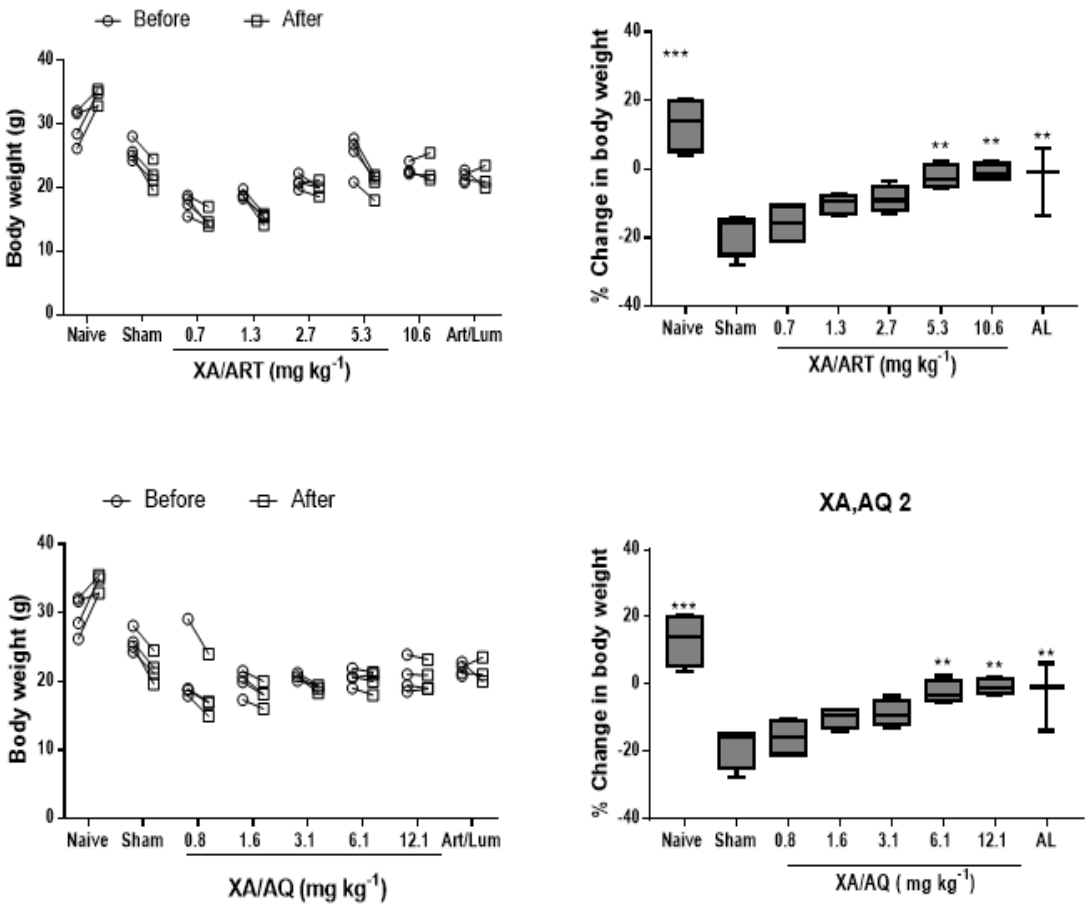


Figure 4.5 Bodyweight before infection and after treatment and percentage change in body weight for (a, b) xylopic acid-artesunate, and (c, d) xylopic acid- amodiaquine combination-treated groups. Data is presented as mean ± SEM and analysed using One-way ANOVA followed by Tukey's honest significant difference (HSD) test used as a *post hoc* test for multiple comparison(n=5), $p<0.05$.

4.1.2.2. Effects of combination therapy on rectal temperature

The lowest dose of xylopic acid-artesunate combination showed a significant decrease in rectal temperature compared to the naïve control. Highest dose combination of XA—AQ and AL control-treated groups stabilize the rectal temperature with a percentage change of 1.5% and 3.4%, respectively, which is similar to the naïve control group mice.

Table 4.5 Mean rectal temperature of *Plasmodium berghei*-infected mice before and after co-administration with xylopic acid and artesunate.

Treatment (ED50 mg/kg)	T_0 (°C)	T_6 (°C)	% change in rectal temperature
Naïve	37.23±0.48	36.70±0.26	-2.5
Sham	38.28±0.22	37.58±0.22	-7.2
(XA+ART)/16	38.18±0.17	33.40±0.26	-14.4
(XA+ART)/8	37.85±0.14	33.19±0.13	-14.5
(XA+ART)/4	38.00±0.26	34.98±0.31	-8.7
(XA+ART)/2	37.98±0.06	34.73±0.42	-9.5
XA+ART	37.98±0.18	35.35±0.55	-7.6
AL 1.14	37.24±0.47	36.70±0.26	-1.7

Data are presented as mean ± SEM, $p< 0.05$, n=5. T_0: rectal temperature measured on day 0 (before parasite infection), T_6: rectal temperature measured on day 6 (after treatment). All temperatures are compared to the rectal temperature of sham group.

Table 4.6. Mean rectal temperature of *Plasmodium berghei*-infected mice before and after co-administration with xylopic acid and amodiaquine

Treatment (ED_{50} mg/kg)	T_0 (°C)	T_6 (°C)	% change in rectal temperature
Naïve	38.28±0.22	37.58±0.22	-2.5
Sham	38.28±0.22	37.58±0.22	-7.2
(XA+AQ)16	38.18±0.17	33.4±0.26	-7.4
(XA+AQ)/8	37.85±0.26	33.1±0.13	-7.8
(XA+AQ)/4	38.00±0.26	34.98±0.31	-10.0
(XA+AQ)/2	38.00±0.06	34.73±0.42	-9.4
(XA+AQ)	38.00±0.18	35.35±0.48	-3.4
AL 1.14	37.23±0.48	36.70±0.26	-1.7

Data are presented as mean ± SEM, $p< 0.05$, n=5. T_0: rectal temperature measured on day 0 (before parasite infection), T_6: rectal temperature measured on day 6 (after treatment). All temperatures are compared to the rectal temperature of sham grou

4.1.2.3. In vivo isobolographic assessment of xylopic acid-artesunate and xylopic acid-amodiaquine co-administration on PbA-induced malaria.

The parasitaemia on day 1 of treatment, final day chemosupression, and the survival days for various treatment groups for xylopic acid-artesunate, and xylopic acid-amodiaquine are presented in tables 4.6-4.8. Percentage chemosupression for highest combination doses of both XA/ART and XA/AQ suppressed parasite replication similar to artemether-lumefantrine (Fig 4.6). ED_{50}s for xylopic acid-artesunate combination, and xylopic acid-amodiaquine was 1. 98 ±0.33 and 1. 69 1.69 ± 0.83, respectively.

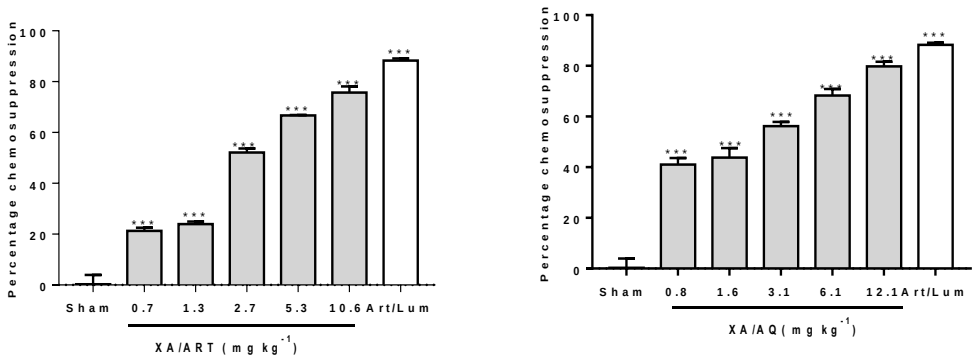


Figure 4.6. Percentage chemosupression for (a) xylopic acid-artesunate and (b) xylopic acid-amodiaquine treated groups in the antiplasmodial combination therapy assay. Data is presented as mean ± SEM and analysed using One-way ANOVA followed by Tukey's honest significant difference (HSD) test used as a *post hoc* test for multiple comparison(n=5), $p<0.05$.

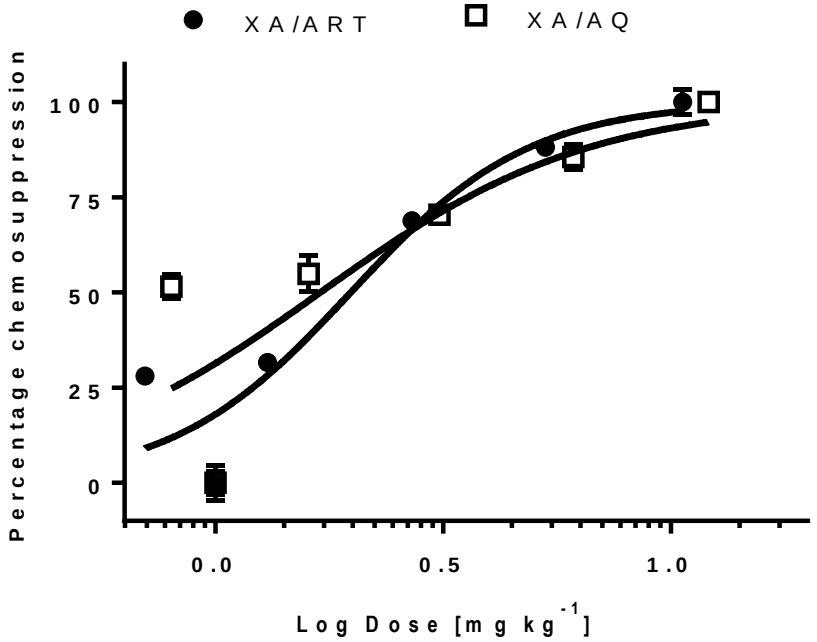


Figure 4.7. Log dose-response curves for *P. berghei*-infected mice treated daily with xylopic acid/ artesunate (XA/ART) combination, and xylopic acid-amodiaquine (XA/AQ) combination over 5 days. Data are presented as mean ± SEM (n=5).

Table 4.7. Parasitaemia, and chemosupression, of mice treated with different combinations of xylopic acid + artesunate for five days.

Treatment (ED_{50} mg/kg)	Dose (mg kg^{-1})	% parasitaemia Day 1	% Chem supp Day 6
Sham	0.50 ml	9.5±0.1	-
(XA+AQ)16	0.6+0.1	9.8±0.2	21.5±0.6
(XA+AQ)/8	1.125+0.2	11.5±0.1	24.1±0.5
(XA+AQ)/4	2.25+0.4	10.6±0.2	52.2±0.8
(XA+AQ)/2	4.5+0.8	10.2±0.2	66.8±0.1
(XA+AQ)	9.0+1.61	10.0±0.1	75.7±1.2
AL 1.14	1.12+16.9	9.8±0.3	84.6±1.6

Data are presented as mean ± SEM

Table 4.8. Parasitaemia, and chemosupression, of mice treated with different combinations of xylopic acid + amodiaquine for five days.

Treatment (ED_{50}mg/kg)	Dose (mgkg-1)	% parasitaemia Day 1	% Chem supp Day 6
Sham	0.50 ml	11.0±0.3	-
(XA+AQ)16	0.6+0.2	11.8±0.1	41.2±1.3
(XA+AQ)/8	1.125+0.4	11.3±0.6	43.9±1.9
(XA+AQ)/4	2.25+0.8	12.8±0.2	56.4±0.8
(XA+AQ)/2	4.5+1.6	11.7±0.3	68.4±1.3
XA+AQ 12.1	9.0+3.1	11.3±0.3	79.8±0.9
AL 1.14	1.12+16.9	11.3±0.2	88.3±0.4

Data are presented as mean ± SEM.

4.1.2.4. Survival analysis for combination therapy

The XA/AQ combination doses significantly delayed death in PbA infected mice in a 30-days survival test, similar to the AL treated group. Likewise, the high doses of XA/ART combination increased the survival days of the PbA infected mice (table 4, fig 8)

Table 4.9 30-day survival analysis *Plasmodium berghei* ANKA-infected mice after treatment with xylopic acid and amodiaquine, and xylopic acid and artesunate

Treatment (mg/kg)	Median Survival (days)	Hazard Ratio (Log-rank)	*p*-value
Sham	10.5		
(XA/ART)			
0.7	10	0.52	0.1766
1.3	12	0.59	0.2941
2.7	16	0.35	0.0374*
5.3	16	0.35	0.0374*
10.6	19	0.23	0.0021**
(XA/AQ)			
0.8	13	0.34	0.0297*
1.6	15	0.14	0.0297*
3.1	15	0.33	0.0224*
6.1	20	0.29	0.0112*
12.1	24	0.23	0.0021**
AL	27	0.21	0.0021**

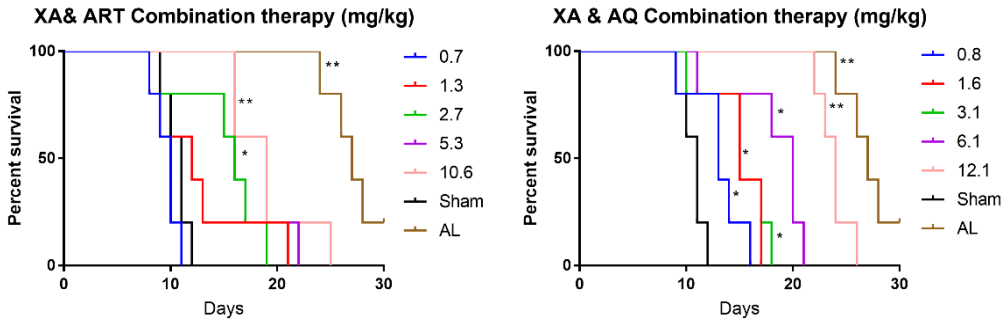


Figure 4.8. Kaplan Meier survival curves comparing the survival of malaria mice among sham and treatment groups of combination therapy for 30 days after infection with *P. berghei*.

4.1.3. Isobolographic analysis of antiplasmodial effects of XA and ART, and XA and AQ co-administration.

Xylopic acid-artesunate co-administration had a theoretical ED_{50} (Z_{add}) of 5.3± 2.61, whereas the experimental ED_{50} (Z_{exp}) was obtained as 1.98±0.25. Also, the co-administration of xylopic acid and amodiaquine had theoretical ED_{50} value of 6.05±2.0; however, the experimental ED_{50} was 1.69 ± 0.42. Thus, the Z_{exp} for both combinations lies significantly below the line of additivity since the interaction index was calculated to be 0.37 and 0.28 for xylopic acid-artesunate and xylopic acid-amodiaquine co-administration, respectively. (Table 4.9 and 4.10).

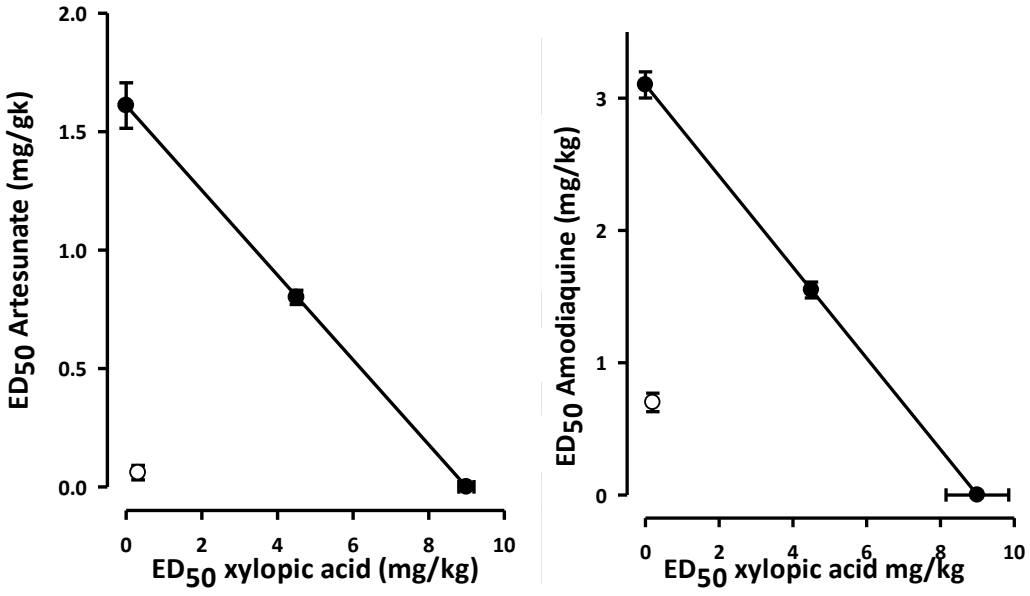


Figure 4.9. Isobologram of the co-administration of xylopic acid and artesunate, and xylopic acid and amodiaquine. Filled circles show theoretical ED_{50}±SEM, while open circles show experimental ED_{50} ± SEM. The line of additivity connects the ED_{50} of xylopic acid on the abscissa to that of artesunate and amodiaquine on the ordinate.

Table 4.10 Theoretical (Zadd), and experimental (Zexp) ED50 of xylopic acid and artesunate, and xylopic acid and amodiaquine co-administration in the anti-malarial assay

$ED_{50}s$ (XA+ART 1:1)	Anti-malarial activity	$ED_{50}s$ (XA+AQ 1:1)	Anti-malarial activity
Z_{add} (mg/kg)	5.3± 2.61	Z_{add} (mg kg^{-1})	6.05±2.0
Z_{exp} (mg/kg)	1.98±0.25	Z_{exp} (mg kg^{-1})	1.69 ± 0.42
Interaction index	0.37	Interaction index	0.28

Data are presented as values ± SEM.

4.2. Discussion

Combination therapies (XA/ART, and XA/AQ) showed a remarkable suppression in parasite growth similar to the artemether/lumefantrine group. Similarly, the monotherapies of XA, ART and AQ also suppressed parasite growth compared to artemether/lumefantrine but at higher dose.

An isobolographic analysis was employed to determine the enhanced or improved potency and efficacy of xylopic acid-artesunate, and xylopic acid-amodiaquine combination therapies. An isobolographic analysis gives a central basis for evaluating if a biological response induced by a mixture of agents is smaller, equal or greater on the concept of dose additivity and the basis of the components or agents' individual activities (Boakye-Gyasi et al., 2018; Gessner, 1995). The co-administration of xylopic acid and artesunate showed significant antiplasmodial activity in comparison to the sham-treated mice. The isobologram showed that when xylopic acid and artesunate are administered together, the Z_{exp} was significantly below the line of additivity ("additive" isobole) and the Z_{add}, which means the two drugs have a synergistic anti-plasmodial effect. The

interaction index of 0.37, which is significantly less than 1, confirms a synergistic relationship (Miranda, Prieto, Puig, & Pinardi, 2008) and supra-additive effect between artesunate and xylopic acid. Compared to a recent study by Ameyaw et al. (2018), combining xylopic acid and artesunate gives a higher supra-additivity and synergistic interaction than xylopic acid and cryptolepine combination, probably, due to the high synergistic property of artesunate (Chou et al., 2011; Mishra, Dash, & Dey, 2011; Okoye et al., 2014). Nevertheless, xylopic acid-cryptolepine co-administration showed a higher parasite clearance rate of 78% for the higher dose combination compared to the 75% for the higher dose combination of xylopic acid and artesunate. Another study which examined the chemotherapeutic interactions between antimalarial drugs and antiretroviral drugs observed the increase in antimalaria activity when ART was combined with lopinavir/ritonavir (LR) on day 5 post-infection in mice infected with *P. berghei* (Abiodun et al., 2016) confirming the synergistic interaction of artesunate with other potent drugs.

The observed increased antiplasmodial activity of XA/ART combination could also be attributed to the two drugs interacting with several targets in the parasite. XA inhibits plasmodium dehydrogenase (Santos et al., 2016), an enzyme which catalyses the reduction of pyruvate to lactate, crucial for energy production, whilst artemisinin derivatives are believed to undergo reductive activation of the peroxide group in the presence of ferrous ion which is released upon haemoglobin digestion within the food vacuole of the parasite (Ameyaw et al., 2018; Tilley, Charman, & Vennerstrom, 2011). This forms a carbon-centred radical which alkylates vital parasite proteins such as haem and membrane-associated parasite

proteins (Asawamahasakda, Ittarat, Pu, Ziffer, & Meshnick, 1994; Sharma, 2005). Thus, the inhibition of metabolic steps in plasmodium haemoglobin digestion combined with the inhibition of parasite glycolysis might contribute to the enhanced antiplasmodial activity of ART and XA.

Also, the anti-inflammatory properties of xylopic acid may have contributed to the limiting survival of the parasite. Inflammation plays a key role in the pathogenesis of malaria. Following *PBA* infection, splenic dendritic cells, $CD8\alpha^+$ and $Clec9A^+$, phagocytose and cross-present parasite antigens which lead to priming of parasite-specific $CD4^+$ and $CD8^+$ T cells. Furthermore, circulating parasitized red blood cells (pRBC) adhere to the endothelium of blood vessels releasing inflammatory ligands such as hemozoin crystals which contain parasite DNA. These stimuli are responded by the release of cytokines and chemokines leading to the upregulation of adhesion molecules (ICAM, VCAM) and receptors (CXCR3) capable of presenting antigens (Howland, Claser, Poh, Gun, & Rénia, 2015). When adhesion molecules are upregulated, they aid in the primary rolling and tethering interactions between lymphocytes, granulocytes, and monocytes to endothelial cells at sites of tissue injury. If perturbed endothelial cells interact with monocytes along with synergistic action of proinflammatory molecules, they potentially exacerbate tissue factor expression and subsequently activate endothelial cells sustaining coagulation-inflammation cycle (Charo & Taubman, 2004; Hezi-Yamit et al., 2005; Liu, Pelekanakis, & Woolkalis, 2004; Shimizu et al., 2004), hence, promoting the “vicious” cycle of coagulation-inflammation of sepsis, which is found to be crucial in malaria pathogenesis. Also, the adherence of

parasite to the endothelium with the help of upregulated adhesion molecules following inflammation helps in the survival of parasites.

Plasmodium parasites have over the years evolved several biomolecular strategies for escaping immune response to secure parasite survival in the host. One-way parasites achieve immune escape is via the exploitation of host components such as inflammation and platelets that can cause infected red blood cells (iRBCs) and uninfected RBCs to agglutinate promoting the appropriate microenvironment for sequestration (Helmby, Cavelier, Pettersson, & Wahlgren 1993). The release of a collection of mediators of inflammation may either result in an exacerbated immune response leading to pathology (Perkins et al., 2011). CD4+ T-helper cells have been reported to be involved in malaria conferring protection. However, they have also been implicated in immune evasion and malaria pathogenesis (Wykes *et al*., 2014). The function of regulatory T cells is controversial in immunity. For instance, T-regs absence in human cerebral malaria correlates with higher parasitaemia; meanwhile, in mice models of experimental cerebral malaria, it renders them more susceptible to the disease. In spite of all this, XA has demonstrated significant anti-inflammatory properties (Osafo & Obiri, 2016). Thus, its inhibition of inflammation may contribute to the observed antimalaria effects since inflammation is significantly involved in malaria pathogenesis which lead to the poor outcome of the disease.

Combination of xylopic acid and amodiaquine showed enhanced activity due to their synergistic interaction. Like the XA/ART combination, XA/AQ interaction also showed additivity with an interaction index of 0.13, which is

significantly different from 1. XA/AQ isobologram lies below the line of additivity, confirming the synergistic interaction between the two compounds. As discussed above, the precise molecular mechanisms by which these two agents act is not known, but several proteins in the parasite might be a target. AQ is assumed to accumulate in parasites food vacuole preventing the conversion of toxic haem produced due to intraerythrocytic parasite digestion of haemoglobin into crystalline hemozoin which is non-toxic to the host but irreversibly toxic to the parasite as a result of the build-up of haem levels (Sharma, 2005). Previous works on antimalarial combination therapies have shown that, when aspartyl PI is combined with other haemoglobin digestion inhibitors, it acts synergistically (Semenov, Olson, & Rosenthal, 1998; Sharma, 2005) but acts antagonistically with vacuole plasmepsin inhibitors (Mungthin, Bray, Ridley, & Ward, 1998). These mechanisms employed by individual drugs of the combination to inhibit metabolic steps in the digestion of haemoglobin may result in the enhanced antimalarial activity of XA in the presence of AQ and ART shown in this study.

In malaria treatment, like any other infectious diseases, it is crucial not only to pay attention to the pathogen, but also the reduction of the symptoms of the infection which independently increase the pathogen burden (Vale *et al*., 2016).

Among the several general features of malaria infection is loss of body weight. Bodyweight decrease can be attributed to metabolic function disturbance and hypoglycaemia caused by malaria parasite infection (Li *et al*., 2003; Miller, Good, & Milon, 1994; Segura, Matte, Thawani, Su, & Stevenson, 2009). Hypoglycaemia in malaria patients can also be attributed to the increase in glucose

uptake by the febrile host and the parasite. Alternatively, the host's glucose production may be impaired (Thien, Kager, & Sauerwein, 2006). Thus, an ideal antimalarial drug is anticipated to prevent the decrease in body weight of mice due to rising parasitaemia, which is crucial for mice survival. AQ and ART prevented the loss of weight of infected mice significantly ($p=0.001$) when compared to the sham animals in this study. Although the XA monotherapy experiment did not significantly prevent weight loss, the combination therapy with ART and AQ showed significant prevention in loss of body weight in the 10.6 mg/kg and 12.1 mg/kg combination doses, respectively ($p< 0.05$). This observation correlates with another study where a combination of xylopic acid and cryptolepine prevented a loss in body weight in mice infected with *P. berghei* ANKA (Ameyaw *et al.*, 2018; Woode, Ameyaw, Abotsi, & Boakye-Gyasi, 2015).

Another characteristic of a potent antimalarial agent is to stabilize the temperature in mice infected with the parasite (Böttger & Melzig, 2013). Infection of rodents with parasite causes a decrease in metabolic rates and severe hypothermia that could result in death (Dascombe & Sidara, 1994; Hintsa, Sibhat, & Karim, 2019; Okokon, Bassey, Udobang, & Bankehde, 2019). A recent study by Cumnock and colleagues (2018) revealed a range of symptoms such as hypothermia, anorexia and lethargy experienced by mice infected with malaria. Only the highest dose combination of XA-AQ was able to stabilize the temperature of the mice, although there was parasite clearance in all the XA-AQ and XA-ART combination graded doses. This observation can be as a result of the combined agents controlling the mice immune system in addition to adjusting some

pathological processes and offsetting the decrease in the rate of metabolism which caused the drop-in mice internal temperature (Lucius & Poulin, 2016).

A good antimalaria agent should be able to prevent death caused by parasites. Increase in parasite growth causes various symptoms of malaria which eventually leads to death of the hosts (Coban, Lee, & Ishii, 2018). The XA and AQ monotherapy showed significant increase in the survival days for the middle doses while the high doses showed increased parasite clearance but reduced median survival days and increased hazard ratios. Notwithstanding, the high doses of ART treated group showed significant increased median survival days and reduced hazard ratio similar to AL. Surprisingly, in the combination therapy, the XA/ART treatment groups showed higher parasite clearance compared to XA/AQ, but their median survival day was only significant in the high doses with a mean hazard ratio 0.40, meanwhile, XA and AQ which showed significant increased survival days and reduced hazard ratio in only the middle doses during the monotherapy, had a significant increase in survival days for all the combination doses with a mean hazard ratio of 0.27 similar to AL. It is possible the early death of the animals in the XA/ART could have been due to toxicity of the combination since there was high parasite clearance (Tang, Wu, Beland, Chen, & Fang, 2019). Thus, hypothetically, the combination of XA/AQ reduced the toxicity of AQ. Median survival for both AL and XA/AQ was statistically significant, confirming the antimalarial properties of both agents.

4.3. Chapter Summary

The results confirmed the antiplasmodial effects on xylopic acid, artesunate and amodiaquine with ED_{50}s of 9.0±3.2, 1.61±0.6, and 3.1±0.8 mg/kg with corresponding E_{max} of 98.72 ±5.13, 107.1±9.13, and 114.6±7.37, respectively. Rectal temperature was significantly stabilized in AL, 30 mg/kg, XA1.25 mg/kg, AQ 5 mg/kg ,10 mg/kg and 20 mg/kg treated groups. Also, all AQ and ART groups significantly prevented loss in body weight when compared to the sham group ($p< 0.05$). Although the XA appeared to prevent loss in body weight, it was not statistically significant.

About the combination therapies, both XA/ART and XA/AQ treated groups significantly suppressed parasite replication ($p< 0.05$) with ED_{50}s of 1. 98±0.33 and 1.69±0.83, and E_{max} of 98.95±12.64, and 97.14±18.47, respectively. The 12.1 mg/kg XA/AQ and 10.6 XA/ART-treated groups suppressed parasite growth similar to artemether/lumefantrine. The AL 5.3 mg/kg, XA/ART 10.6 mg/kg, XA/AQ 6.1 mg/kg and XA/AQ 12.1 mg/kg treated groups significantly prevented loss in body weight compared to the sham group. All the treated groups stabilized rectal temperature, but it was not significant.

CHAPTER FIVE

SUMMARY

4.3.1.Overview of the purpose of the study

This study aimed at investigating the effectiveness of combining two individual artemisinins, amodiaquine and artesunate, with a compound reported to have an antiplasmodial effect, xylopic acid.

The results of the study showed that combining either of two derivatives of artemisinin, amodiaquine or artesunate, with xylopic acid gives a synergistic interaction. This implies that combining xylopic acid with amodiaquine or artesunate can be further developed as a combination therapy and employed in clinical settings. The history of *Plasmodium* resistance to established drugs and the recent report of growing resistance to current artemisinin (Khera & Mukherjee, 2019) makes the findings of this research essential. The combination of xylopic acid and either amodiaquine or artesunate is assumed to have a reduced toxicity since lower doses of each of the agents were required to produce a significant therapeutic effect. Development of antimalarials is still relevant since newly produced vaccines have low efficacy.

4.3.2. Summary of Results

The study first investigated the antimalarial activities of artesunate, amodiaquine and xylopic acid monotherapy in *P. berghei* ANKA-infected mice to determine their ED_{50s} for the combination study. The results of the study are summarized as follows:

- Artemisinin derivatives, artesunate and amodiaquine, showed good antimalarial activity with ED_{50s} of 1.61±0.6, and 3.1±0.8 mg/kg. Xylopic acid, which was extracted from *Xylopia aethiopica*, also showed good parasite clearance rate with ED_{50} and E_{max} of 9.0±3.2 and 98.72 ±5.13, respectively.
- 16 mg/kg ART and 20 mg/kg AQ treated groups along with AL treated groups significantly stabilized rectal temperature compared to the sham (p=0.001). Also, Amodiaquine and artesunate treatment groups significantly prevented the reduction of animal body weights (p=0.001).
- XA/ART and XA/AQ combination suppressed parasites replication similar to artemether-lumefantrine. ED_{50s} for fixed-dose combinations of XA/ART and XA/AQ were 1. 98 ±0.33 and 1. 69 1.69 ± 0.83, with.
- Isobolographic analysis of antiplasmodial effects of XA and ART, and XA and AQ co-administration was done to reveal the type of interaction that existed between the combined drugs. XA/ART and XA/AQ had theoretical ED_{50} (Z_{add}) of 5.3±2.6 and 12±.20 with experimental ED_{50} of 1.98±0.33 and 1.69±0.83, respectively. This gives the interaction index of XA/ART to be

0.12 while that of XA/AQ is 0.13, both of which are significantly different from 1 thus indicating synergism.

4.4. Conclusion

The antiplasmodial activity of xylopic acid, amodiaquine, and artesunate have been confirmed from the results of this study. Further demonstration indicated that co-administration of xylopic acid with artesunate, and xylopic acid with amodiaquine exhibit a synergistic interaction along with preventing the loss of body weight of infected of malaria parasite-infected mice.

4.5. Recommendations

This study has given the scientific evidence to the use of combined xylopic acid and amodiaquine, and xylopic acid and artesunate in the treatment of uncomplicated malaria in rodents. These findings, although, cannot be directly applied in humans, it provides the basis for novel antimalarial development, and hence, care should be taken when used in human subjects. Also, Herbal medicine industries can incorporate these findings into their drug formulations in the quest to fight against malaria and its symptoms.

4.6. Further Research

Subsequent experiments can be done to expand the knowledge and understanding of these drug combinations by

1. testing the safety profile of combining xylopic acid and artesunate, and xylopic acid and amodiaquine,

2. performing *in vitro* tests for the effectiveness of combining xylopic acid and artemisinin derivatives on the human parasite: *P. falciparum*, and
3. evaluating the pharmacokinetic profile on artesunate/xylopic acid and amodiaquine xylopic acid combinations.

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