ANALYSIS OF MEDICINAL ACTIVITIES OF *WARBURGIA UGANDENSIS* AND *ZANTHOXYLUM USAMBARENSE* AGAINST *PLASMODIUM BERGHEI* AND *PLASMODIUM KNOWLESI*

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Analysis of Medicinal Activities of *Warburgia ugandensis* and *Zanthoxylum usambarense* against *Plasmodium berghei* and *Plasmodium knowlesi*

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A Thesis Submitted in Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy in Zoology (Parasitology) of the Jomo Kenyatta University of Agriculture and Technology

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DECLARATION

This thesis is my original work and has not been presented for a degree in any other University

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DEDICATION

To Martha, my loving soulmate, for being there for me and with me throughout the study period. And to my son Trevin; and daughters Tonya, Tara and Tatiana-Verna. The five endured long hours of my absence.

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ACRONYMS AND ABBREVIATIONS

ACTs	Artemisinin-Combined Therapies
ANOVA	Analysis of Variance
AR/ART	Amodiaquine and Artesunate
AQ+AS	Amodiaquine and Artesunate
BALB/c mouse	Mutant albino mice
CAM	Complimentary or Alternative Medicine
CC	Column Chromatoghraphy
CCM	Complete Culture Medium
CN	Quinine
CQ	Chloroquine
CSP	Circumsporozoite Protein
DHFR	Dihydrofolate Reductase
DHPS	Dihydropteroate Synthase
DMSO	Dimethylsulfoxide
DNA	Deoxyribose Nucleic Acid
ED_{50}	Effective Dose that kills 50% of test organisms *in vivo*
EE	Exo-Erythrocytic
ELISA	Enzyme Linked Immunosorbent Assay

EIR	Entomological Inoculation Rates
EtOAc	Ethyl acetate
EtOH	Ethanol
FCS	Foetal Calf Serum
FT-IR	Fourier Transform-Infra Red
FP	Ferriprotoporphyrin IX
GC-MS	Gas Chromatography-Mass Spectroscopy
GFP	Green Fluorescent Protein
GMEC	Global Malaria Eradication Campaign
G6PD	Glucose-6-Phosphate Dehydrogenase
HI	Heat Inactivated
HPLC	High Performance Liquid Chromatography
HTS	High Throughput Screening
i.p	Intraperitoneal
i.v	Intravenous
IC_{50}	Drug Concentration killing 50% of test organisms *in vitro*
ICM	Incomplete Culture Medium
IFA	Indirect Immunofluorescence Assay
IPR	Institute of Primate Research

IRS	Indoor Residual Spraying
IRC	Institutional Review Committee
ITNs	Insecticide Treated Nets
KBr	Potassium bromide
KEMRI	Kenya Medical Research Institute
LBW	Low Birth Weight
LC-MS	Liquid Chromatography-Mass Spectroscopy
LD_{50}	Lethal Dose that kills 50% of experimental organisms
LDL	Low Density Lipoprotein
LF	Lumefantrine
MeOH	Methanol
MST	Mean Survival Time
NAD	No abnormalities detected
NMR	Nuclear Magnetic Resonance
NMK	National Museums of Kenya
PABA	*P*-Aminoparabenzoic Acid
PAN	*Papio anubis* (Olive baboon)
PBA	*Plasmodium berghei* Anka
PBS	Phosphate Buffered Saline

PCR	Polymerase Chain Reaction
PCV	Packed Cell Volume
PE	Pre-Erythrocytic
Pf	*Plasmodium falciparum*
PfHRP	*Plasmodium falciparum* Histidine Rich Protein
PfLDH	*Plasmodium falciparum* Lactate Dehydrogenase
PfMDR	*Plasmodium falciparum* Multi-Drug Resistance
$_{P}$H	Power of Hydrogen ion concentration
PQ	Primaquine
PUFAs	Polyunsaturated Fatty Acids
RBCs	Red Blood Cells
RDTs	Rapid Diagnostic Tests
RES	Reticulo Endothelial System
RNA	Ribose Nucleic Acid
RPM	Revolutions per Minute
RPMI	Rowal Park Memorial Institute
rRNA	Ribosomal Ribose Nucleic Acid
SEM	Standard Error of Mean
SP	Sulphadoxine-Pyrimethamine

SPSS	Statistical package for social scientists
TLC	Thin Layer Chromatography
TLR	Tool-like Receptors
TI	Therapeutic Index
TM	Traditional Medicine
TNFs	Tumour Necrosis Factors
WHO	World Health Organization

DEFINITION OF TERMS

Term	Definition
Alkaloid	A bioactive plant molecule containing nitrogen atoms
Alternative Therapy	Treatment of disease by means other than conventional medical, pharmacological and surgical techniques.
Antimalarial	Substance that is therapeutically effective against malaria
Antiplasmodial	An agent that kills or inhibits growth of *Plasmodia*
Complementary and Alternative Medicine	Forms of treatments used in addition to (complementary) or instead of (alternative), standard treatments
Cryoprotectant	A freeze medium used in cryopreservation of parasites
BALB/c mouse	Mutant strain of albino mouse
Bioactive principle	A phytochemical compound sufficiently efficacious for herbal medicament in which it is comprised
Effective dose (ED_{50})	Amount of drug that kills 50% of the test population
Ethnomedicine	Traditional medicines used by different communities
Herbal Medicine	A medicament whose active principles are not chemically synthesized and are constituent(s) of a plant
Lethal dose (LD_{50})	Amount of drug that kills 50% of the test model
Lyophilization	Removal of a solvent from a solution to obtain solid plant extracts
Maceration	Steeping in a liquid (or) to separate into constituents by soaking

Miscella	Crude extracts of a plant material in extraction solvent
Parasitaemia	Level of parasite infection in an organism's blood
Pharmacognosy	Screening the plants for bioactive moieties
Phytochemical	Chemical constituents present in plant fruits and vegetables
Phytomedicine	Medicine of plant origin
Placebo	A pharmacologically inactive substance used as a control in scientific experiments
Prophylactic	Any agent that prevents an infection or a disease
Pulverize	Grind into fine powder
Recruscedence	Re-emergence of an infection without new infections
Schizonticide	A substance that destroys the schizonts, mature blood stage in the development of *Plasmodia*
Therapeutic Index	Correlation in biological activity between effective dose and lethal dose

ABSTRACT

Medicinal plants play a key role in the worlds' health care, with about 80% of Africans depending on herbal medicines for treatment of bacterial, parasitic, viral and fungal infections. In Kenya, two plants; *Warburgia ugandensis* and *Zanthoxylum usambarense* are credited for treatment of malaria and other ailments by the communities where they grow naturally. To be accepted as viable alternatives, herbal medicines must be subjected to the modern rigorous testing and validation procedures as used in convention medicines. This study was designed to determine the antimalarial activity and safety of *W. ugandensis* and *Z. usambarense*, and characterize bioactive constituents present in their extracts. A total of 288 male adult BALB/c mice and six (6) male adult olive baboons were used for *in vivo* assays. Experimental animals were challenged with chloroquine-sensitive *Plasmodium berghei* and *P. knowlesi* parasites respectively and used to test for efficacy and safety of the herbal extracts in parasite suppressive tests. To isolate and characterize the bioactive moieties, highly efficacious extracts were subjected to phytochemical analyses through Fourier Transform-Infrared (FT-IR) spectrophotometry as well as Gas-Chromatography-Mass Spectroscopy (GCMS) and Liquid Chromatography-Mass Spectroscopy (LCMS) respectively on a 7890A gas Chromatograph linked to a 5975C mass selective detector. Parasitaemia, heamatology, biochemistry, gross pathology and histopathological changes were measured as indicators of efficacy and safety of the extracts. Data were managed and analyzed using GraphPad Prism Version 5.00. Tukey's Multiple Comparison Test ($P < 0.05$) and Bartletts' Test for equal variances confirmed significant differences. In the present study, both polar and non-polar extractions from *W. ugandensis* and *Z. usambarense* displayed low IC_{50} value of 3.2μg/ml and suppressed parasites growth up to 64% while increasing survivorship of extract-treated animals. No abnormalities were observed in extract treated animals. Further, pharmacologically active compounds including alkaloids, terpenoids, flavonoids and terpenes were isolated. These findings justify the use of *W. ugandensis* and *Z. usambarense* as herbal medicines and present them as suitable candidates for development of antimalarial phytomedicines.

CHAPTER ONE

INTRODUCTION

1.1 Background information

Malaria is caused by infection of red blood cells with protozoan parasites of the genus *Plasmodium.* The parasites are inoculated into the human host by a feeding female *Anopheline* mosquito. It can be caused by any of the five species of *Plasmodia;* namely *P. falciparum*, *P. vivax, P. malariae, P. ovale* and *P. knowlesi* (Luchavez *et al*., 2008; Cox-Singh and Singh, 2008; Cox-Singh *et al*., 2008; WHO, 2016). Malaria caused by *P. falciparum*, the deadliest species, predominantly occurs in Africa and is responsible for about 92% of all world malaria deaths (WHO, 2019). In spite of considerable control efforts in many countries malaria remains a major cause of global morbidity and mortality with substantial global public health costs and with most of the burden in Sub-Saharan Africa (Greenwood *et al*., 2005; Muller, 2011). Global investment for malaria has steadily increased between 2000 and 2010, but has since levelled, totalling US$ 2.7 billion in 2018, being put to malaria control, research and elimination programs (WHO, 2019). Despite these massive investments, malaria remains a major public health problem especially in sub-Saharan Africa, with clinical episodes in about 213 million people annually, resulting in about 405,000 deaths (WHO, 2019). In endemic African countries, malaria accounts for 25-35% of all outpatient visits, 20-45% of hospital admissions and 15-35% of hospital deaths, imposing a great burden on healthcare systems (WHO, 2005). In Kenya, malaria continues to be a national concern as it plays a major role in child and infant mortality, with an estimated 3.5million cases per year. It is responsible for about 30% of outpatient treatments, 19% admissions, 8-10 million treatments per year and 13,000 deaths, accounting for 2% of all global malaria cases (WHO, 2019, Njoroge and Bussmann, 2006).

Malaria control requires an integrated approach comprising prevention through vector control and treatment with effective antimalarials. Although continued attempts to develop a vaccine for malaria are ongoing, distribution of mosquito nets, household spraying, and prophylaxis remain the primary prevention methods and

antimalarial drugs remain the only treatment option (Sirma and Gansane, 1997). The affordable and widely available antimalarial drug chloroquine that was in the past a mainstay of malaria control is now ineffective in most *falciparum* malaria endemic areas and resistance to sulfadoxine–pyrimethamine is increasing rapidly. Resistance to the majority of available antimalarial drugs has been reported in a growing number of countries worldwide and such resistance threatens future progress in malaria control (WHO, 2012). In order to decrease the risk of chemo-resistance to most of the antimalarial drugs, the World Health Organization (WHO) recommended artemisinin-based combination therapies (ACTs) for the management of uncomplicated *P. falciparum* malaria cases (WHO, 2012).

Artemisinin-based combination therapies (ACTs) are now generally considered as the best current treatment for uncomplicated *falciparum* malaria (Noedl *et al.*, 2008). However, resistance to artemisinin derivatives has been recently described in Southeast-Asia (Carrara *et al.*, 2009; Dondorp *et al.*, 2010). In addition, these drugs are expensive, limiting their use in a population with average annual income of about $100 (Pyae Phyo *et al.*, 2012). Other limitations to the use of conventional antimalarials include prohibitive cost and inaccessibility to antimalarial drugs. For instance, the current standard antimalarial drug, artemisinin combination therapy (ACTs), consists of chemical derivatives of the Chinese natural product artemisinin (WHO, 2008). Though, these medicines are effective, safe and fairly cheap, there is need to develop new therapies since parasite resistance to artemisinin has now been detected in five countries of the Greater Mekong subregion: Cambodia, Myanmar, Thailand, Vietnam and Lao PDR (WHO, 2016; Dondorp *et al.*, 2010). In fact, in Cambodia's Pailin province, resistance has been found to both components of multiple ACTs (WHO, 2016). If artemisinin resistance were to spread to India or sub-Saharan Africa, the global consequences could be dire, because no alternative antimalarial medicine is available at present with the same level of efficacy and tolerability as ACTs (WHO, 2013). This therefore presents a critical need to develop alternative antimalarial drugs.

Medicinal plants have played a key role in the world health care with about 80% of Africans depending on herbal medicine (Addae-Mensah *et al.*, 2011). These herbal

medicines have more beneficial effect than their conventional counterparts through being accessible, safer, acceptable, affordable and culturally compatible (WHO, 2013). Unfortunately, the average time and cost of discovering, developing and launching a new drug is extremely prohibitive, estimated to 10-15 years and at a cost of between 800 million and 1.5 billion US dollars (WHO, 2013).

Though, thousands of natural extracts have been screened and many of active compounds isolated, they stand very limited chance of moving to clinical studies owing to poor absorption and adverse effect profiles in animal studies (Simoes-pires *et al.,* 2014). As a faster alternative therefore, development of phytomedicine through reverse pharmacology is currently considered. This is a transdisciplinary approach integrating traditional knowledge, experimental observations and clinical experiences, with the aim of reversing from the classical laboratory to clinic process; to a clinic to laboratory approach (Willcox *et al*., 2011; Wells, 2011; Simoes-Pires *et al*., 2014; Patwardhan and Vaidya, 2010).

Phytomedicine, herbal preparations produced by subjecting plant materials to extraction, fractionation, purification and concentration, have been an important source of drugs over a long period of time (WHO, 2013). Moreover, the WHO Regional Committee for Africa strategy for promoting Traditional Medicine (TM) includes the development of local production and conservation of medicinal plants, legislation on TM practice and its integration into conventional health services, since effective health care cannot be achieved in Africa by using conventional medicine alone (WHO, 2013). Unfortunately, TM still remains a largely untapped health care resource despite their being crucial sources of new leads for drug discoveries. Today, only about 50% of useful drugs are obtained from natural sources (WHO, 2013).

Many medicinal plants have been used in ethno medicine for development of phytomedicines. Of these medicinal plants, some have been documented but others are still yet to be documented and their active medicinal compounds characterized (Kokwaro, 2009). One such priority plant is *Warburgia ugandensis*. *Warburgia ugandensis,* a canaleaceae, also known as the East African Greenheart, is a species of evergreen tree native to Africa and a highly valued species within the traditional

health systems of the communities where it naturally grows (Beentje, 1994; Kokwaro, 1976). The species of the genus *Warburgia* are known to be rich in sesquiterpenes, which have been shown to possess insect antifeedant, antimicrobial, anticancer, molluscidal and antifungal properties (Olila *et al*., 2001; Kubo and Taniguchi, 1988). Medicinally, dried bark of *W. ugandensis* is commonly chewed and the juice swallowed as a remedy for stomachache, constipation, toothache, cough, fever, muscle pains, weak joints and general body pains (Olila *et al*., 2001). Fresh roots are boiled and mixed with soup for the prevention of diarrhoea whereas leaf decoction baths are used as a cure for several skin diseases (Kubo and Taniguchi, 1988).

Another medicinal plant under investigation is *Zanthoxylum usambarense*, in the family Rutaceceae. It is a commonly used plant species by some Kenyan communities to treat common ailments (Olila *et al*., 2001). A mixture made from both its bark and its roots is taken for relief from coughs (Ombito, 2021). A decoction made from its stem bark is drunk for relief from rheumatism and for relief from malarial fevers (Asante *et al*., 2019). Its edible fruits are eaten to relieve one from coughs. Both its roots and its leaves are taken for relief from severe colds and for pneumonia treatment. An infusion made from its fruits is mixed together with milk and drunk for relief from fevers, sore throats, tonsilitis and chest pains (Olila *et al*., 2001; Kubo and Taniguchi, 1988).

1.2 Statement of the problem

Falciparum malaria is responsible for high infant mortality and pregnant mothers. However, the high cost of conventional medicines used for treatment of malaria, the spread of resistance to arteminisinin-based antimalarial drugs and other conventional drugs, as well as the emergence of *P. knowlesi* as the fifth human malaria present the need for urgent search of new antimalarial candidates. In developing countries, most people resort to herbal medicines for relief from clinical manifestations of malaria. These herbal medicines require validation for safety and efficacy.

1.3 Justification of the study

Herbal medicines (HM) are used as a source of primary health care for treatment of malaria by a significant percentage of rural population in Kenya. However, the current regulatory and operational framework is not sufficient to support dispensation of HM in the mainstream health care. Most herbal products on the market today have not been subjected to drug approval process to demonstrate their safety and effectiveness. To be accepted as viable alternative to modern medicine, the same methods of scientific and clinical validation must be applied to prove their safety and effectiveness. *Warburgia ugandensis* and *Z. usambarense*, though popularly used by many communities in Kenya for treatment of malaria and other ailments, they have not been subjected to drug approval process to demonstrate their efficacy and safety on vertebrate tissue. This study was therefore designed to determine the efficacy and safety of the two medicinal plants and to isolate and characterize phytoactive constituents in their extracts.

1.4 Research questions

i. What are the *in vitro* and semi-*in vitro* antiplasmodial activities of extracts from *W. ugandensis* and *Z. usambarense* against *P. knowlesi* and *P. berghei?*
ii. What are the *in vivo* activities of extracts from *W. ugandensis* and *Z. usambarense* against *P. knowlesi?*
iii. What are the bioactive constituents of extracts from *W. ugandensis* and *Z. usambarense* effective against *Plasmodia?*
iv. To what extent are extracts from *W. ugandensis* and *Z. usambarense* safe in vetebrate tissues?

1.5 Null hypotheses

i. There are no *in vitro* and semi-*in vitro* antiplasmodial activities of extracts from *W. ugandensis* and *Z. usambarense* against *P. knowlesi* and *P. berghei*
ii. There are no *in vivo* activities of extracts from *W. ugandensis* and *Z. usambarense* against *P. knowlesi*

iii. There are no bioactive constituents of extracts from *W. ugandensis* and *Z. usambarense* effective against *Plasmodia*

iv. Extracts from *W. ugandensis* and *Z. usambarense* are not safe in vetebrate animals.

1.6 Overall study objective

This study was designed to determine antiplamodial activities and Safety of extracts from *W. ugandensis* and *Z. usambarense* and isolate and characterize phytoactive constituents in their extracts.

1.6.1 Specific objectives of study

i. To determine the *in vitro* and semi-*in vitro* antiplasmodial activities of extracts from *W. ugandensis* and *Z. usambarense* against *P. knowlesi* and *P. berghei*

ii. To determine the *in vivo* activities of extracts from *W. ugandensis* and *Z. usambarense* against *P. knowlesi*

iii. To determine the bioactive constituents of extracts from *W. ugandensis* and *Z. usambarense* effective against *Plasmodia*

iv. To determine the safety of extracts from *W. ugandensis* and *Z. usambarense* in *Papio anubis.*

CHAPTER TWO

LITERATURE REVIEW

2.1 Malaria: The global situation and transmission

Nearly 3.4 billion people, that is, almost half of the world's population is at risk of malaria. In 2018, there were about 228 million malaria cases and an estimated 405 000 malaria deaths (WHO, 2019). According to World Health Organization, 90% of all malaria deaths occur in the African Region (Table 2.1) mostly among children under 5 years of age (WHO, 2019). Malaria is transmitted exclusively through the bites of female *Anopheles* mosquitoes (Gething *et al*., 2012). The intensity of transmission depends on factors related to the parasite, the vector, the human host, and the environment (Gething *et al*., 2012).

About 20 different *Anopheles* species are locally important around the world (Ayala *et al.,* 2009). *Anopheles* mosquitoes breed in water and each species has its own breeding preference; for instance, some prefer shallow collections of fresh water, such as puddles, rice fields, and hoof prints. Transmission is more intense in places where the mosquito lifespan is long enough to allow the parasite time to complete its developmental cycle inside the mosquito and where it prefers to bite humans rather than other animals (Ayala *et al.,* 2009). For example, the long lifespan and strong human-biting habit of the African vector species is the main reason why about 90% of the world's malaria deaths are in Africa. Transmission also depends on climatic conditions that may affect the number and survival of mosquitoes, such as rainfall patterns, temperature and humidity. In many places, transmission is seasonal, with the peak during and just after the rainy season.

Malaria epidemics can occur when climate and other conditions suddenly favour transmission in areas where people have little or no immunity to malaria. They can also occur when people with low immunity move into areas with intense malaria transmission, for instance to find work, or as refugees. Human immunity is another important factor, especially among adults in areas of moderate or intense transmission conditions (Crompton *et al.,* 2014). Partial immunity is developed over

years of exposure, and while it never provides complete protection, it does reduce the risk that malaria infection will cause severe disease. For this reason, most malaria deaths in Africa occur in young children, whereas in areas with less transmission and low immunity, all age groups are at risk (Mueller *et al*., 2013).

Africa is the most affected due to a combination of factors that include: a very efficient mosquito vector, *Anopheles gambiae* complex that is responsible for high transmission (Crompton, 2014), and the predominant parasite species of *P. falciparum*, which is the species that is most likely to cause severe malaria and deaths; local weather conditions that allow transmission to occur year round; and scarce resources and socio-economic instability that hinder efficient malaria control activities (Sinka, 2012. In other areas of the world malaria is a less prominent cause of deaths, but can cause substantial disease and incapacitation, especially in rural areas of some countries in South America and South Asia (WHO, 2012).

Table 2.1: Estimated malaria burden by WHO in 2018

WHO region	Malaria cases	Malaria deaths
African	**213 million**	**380,000**
Americas	976,000	630
East Mediterranean	4.4 million	8,300
South-East Asia	11.3 million	19,700
Western Pacific	1.9 million	3,620
World	**228 million**	**405,000**

Source: *World malaria report, 2019*

2.2 Social and economic impact of malaria

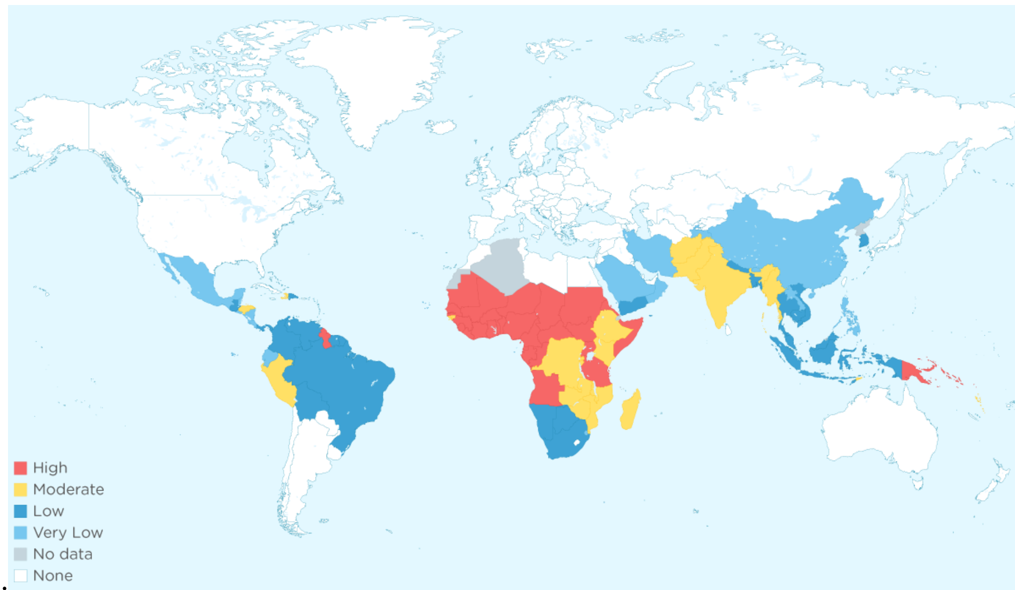
Malaria imposes substantial costs to both individuals and governments. Costs to governments include maintenance, supply and staffing of health facilities; purchase

of drugs and supplies, public health interventions against malaria; such as insecticide spraying and distribution of insecticide-treated bed nets (WHO, 2017; Kipyab *et al.,* 2013), lost days of work with resulting loss of income and lost opportunities for joint economic ventures and tourism. Costs to individuals and their families include purchase of drugs for treating malaria at home, expenses for travel to, and treatment at dispensaries and clinics, lost days of work, absence from school, expenses for preventive measures and expenses for burial in case of deaths (WHO, 2017).

2.3 Groups at risk of malaria

Approximately half of the world's population is at risk of malaria (WHO, 2019). Most malaria cases and deaths occur in sub-Saharan Africa (Figure 2.1), and to a lesser extent; Asia, Latin America, Middle East and parts of Europe (WHO, 2019). According to WHO, specific population risk groups include: young children in stable transmission areas who have not yet developed protective immunity against the most severe forms of the disease, non-immune pregnant women and semi-immune pregnant women in areas of high transmission (Crompton *et al*., 2014).

Malaria can result in miscarriage and low birth weight, especially during first and second pregnancies and semi-immune HIV-infected pregnant women in stable transmission areas, during all pregnancies (Luxemburger *et al.,* 1997). Pregnant women are particularly susceptible to malaria because the parasites have a high affinity for the mother’s placenta (Harrington *et al.,* 2021). This high concentration of malaria parasites in the placenta compromises the passage of nutrients and oxygen, often resulting in premature delivery and low birth weight of babies, people living with HIV/AIDS, international travelers from non-endemic areas because they lack immunity and immigrants from endemic areas and their children living in non-endemic areas and returning to their home countries to visit friends and relatives are similarly at risk because of waning or absent immunity (McGready *et al*., 2012).



(***WHO, 2019***)

Figure 2.1: World's population at risk of malaria

2.4 Human malaria parasites

Malaria is caused by infection of red blood cells with protozoan parasites of the genus *Plasmodium*. The parasites are inoculated into the human host by a feeding female *Anopheline* mosquito. The five *Plasmodium* species that infect humans are *Plasmodium falciparum, P. vivax, P. ovale, P. malariae* and *P. knowlesi. Plasmodium knowlesi* (White, 2008; Tek *et al*., 2008) is zoonotic (Assefa *et al*., 2015), affecting both humans and non-human primates (Cox-Singh and Singh, 2008; WHO, 2016).

2.4.1 General life cycle of *Plasmodia*

Plasmodium species have a complex life cycle, which is shared between a vertebrate host and an insect vector (Crompton *et al.,* 2014). The parasite enters the bloodstream through the bite of an infected female *Anopheles* mosquito. The cyclic development of malaria parasites involves three distinct cycles (Figure 2.2); sporogonic, which occurs in the mosquito vector, pre-erythrocytic phase that takes place in the liver cells of the vertebrate host, and erythrocytic cycle that takes place in the red blood cells (RBCs). Sporozoites, injected by female *Anopheles* mosquitoes as they bite into the skin of mammalian hosts, rapidly enter the blood circulation to

reach liver hepatocytes, where they mature in an entirely asymptomatic phase that lasts for approximately two weeks. Sporozoites of *P. vivax* and *P. ovale* remain dormant (hypnozoites) in the human hepatocyte, where they mature months to years later. These forms cause late malaria relapses under conditions that are not well understood and are related to host stress and low Primaquine (PQ) doses (Townell *et al.,* 2012); such relapses require new drug treatment. The ideal antimalarial drug should destroy sporozoites soon after they are inoculated into the vertebrate host by the mosquitoes (Townell *et al.,* 2012). However, no effective prophylactic anti-sporozoite drug is currently in use. Merozoites are liberated as merosomes from liver cells and then bud off from the hepatocytes to invade and develop in RBCs (Sturm *et al.,* 2006). In the RBCs, the parasites undergo asexual multiplication by schizogony and release merozoites, which invade other RBCs, thereby reinitiating the blood-stage cycle (Greenwood *et al*., 2008).

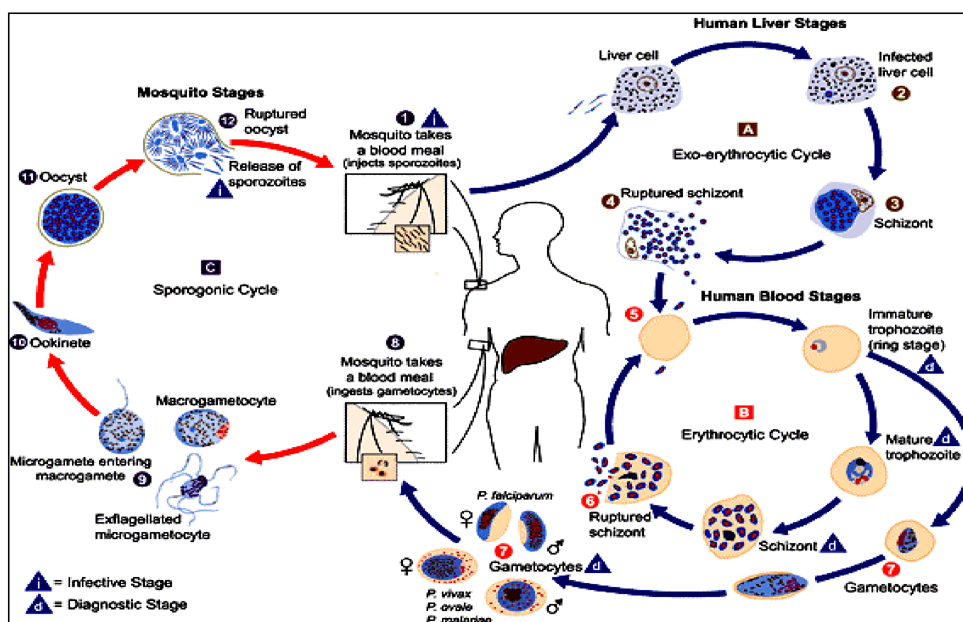


Figure 2.2: The *Plasmodium* life cycle

2.4.1.1 Vertebrate phase of *Plasmodium* parasites

The *Plasmodium* parasites have two forms in which they appear in the vertebrate host; pre-erythrocytic and erythrocytic stages. The pre-erythrocytic stage, also referred to as exo-erythrocytic (EE) cycle, begins when an infected female *Anopheles*

mosquito injects sporozoites into a vertebrate host as it takes a blood meal. The infection sporozoites cross the endothelium of the capillaries in the skin, enter the blood and travel to the liver. In the liver the sporozoites traverse Kupffer cells and hepatocytes then invade a small number of hepatocytes (Crompton, 2014). Within hepatocytes the sporozoites replicate within a week, increasing in number and and differentiating, giving rise to a large number of asexual blood stage parasites, the merozoites (Gueirard *et al*., 2010). These merozoites penetrate erythrocytes in the blood and transform into trophozoites by ingesting the hosts' haemoglobin and cytoplasm. The parasite grows filling more than half of the blood cell and develops asexually into mature schizonts, each consisting of a mean of ten erythrocytic merozoites (Ozwara *et al*., 2003). When development of merozoites is complete, the hosts' erythrocytes lyse, releasing merozoites, the parasites' metabolic wastes and haemozoin. Many merozoites are destroyed by the hosts' reticulo-endothelial system (RES) and leukocytes while others immediately re-invade uninfected red cells (Mota *et al.,* 2001).

Erythrocytic schizogony lasts one to four days depending on the species. It takes 24 hours for *P. knowlesi*, 25 hours for *P. berghei* and 48 hours for *P. falciparum* (Ozwara *et al*., 2003; Garnham, 1966). The life cycle of *Plasmodia* in the human host is completed when, the asexual blood-stage parasites differentiate into male and female gametocytes that are taken up in a mosquito's blood meal. In the mosquito midgut, male and female gametes fuse to form ookinetes that cross the midgut epithelium and ultimately differentiate into sporozoites that invade the mosquito's salivary glands, completing the parasite's life cycle in the invertebrate host (Crompton *et al.,* 2014).

2.4.1.2 Invertebrate phase of malaria parasites

The sexual cycle of the *Plasmodium* parasite takes place in the gut of a suitable mosquito. Mosquitoes become infected when they ingest gametocytes that transform into mature gametes in the midgut lumen (Jaramillo-Gutierrez *et al*., 2010). Fertilization takes place, giving rise to a zygote that matures into an ookinete, a motile stage that invades the mosquito midgut (Mota *et al.,* 2001). Ookinetes

transform into oocysts when they reach the midgut basal lamina and begin to divide continuously, generating thousands of sporozoites that are released into the mosquito circulatory system, invade the salivary glands and are injected into a new host when the mosquito takes a second blood meal (Crompton *et al.,* 2014).

2.4.2 Malairia parasites of interest

The murine malaria parasite; *P. berghei* and the primate parasite; *P. knowlesi*, were used in this study.

2.4.2.1 *Plasmodium berghei* (ANKA)

Plasmodium berghei is one of the four species that have been described in murine rodents (Junaid *et al*., 2017). Other murine parasites are *P. yoelii, P. chabaudi* and *P. vinckei* (Goodman *et al*., 2013). *Plasmodium berghei* originates from the forests of Central Africa where its natural cyclic hosts are the thicket rat *Grammomys surdaster* and the mosquito *Anopheles dureni* (Garnham, 1966; Junaid *et al*., 2017). The vector is restricted to the Katanga Gallery in the Congo Forest at an attitude of between 1000 -1700 meters. *Plasmodium berghei* is therefore localised in Central Africa due to the restricted freedom of range of its arthropod vector (Garnham, 1966). In the laboratory the natural hosts have been replaced by a number of commercially available laboratory mouse strains, and the mosquito *A. stephensi*, which is easily reared and maintained under laboratory conditions (Garnham, 1966). Rodent parasites are recognized as valuable model organisms for the investigation of human malaria because they are similar in aspects such as morphology, physiology and life cycle (Goodman *et al.,* 2013). Moreover, manipulation of the complete life cycle of these parasites, including mosquito infections, is simple and safe. *Plasmodium berghei* is used in research programs for development and screening of antimalarial drugs and for the development of vaccines against malaria (Goodman *et al.,* 2013).

Like all malaria parasites of mammals, *P. berghei* parasites enter the liver after being injected into the bloodstream by a bite of an infected female *Anopheles* mosquito. After a short period of development and multiplication, these parasites leave the liver and invade erythrocytes (Junaid *et al*., 2017). The multiplication of the parasites in

the blood causes anaemia and damage of essential hosts' organs (Okokon *et al.,* 2005). *Plasmodium berghei* has a strong preference for reticulocytes, with parasites growing in mature RBCs producing 6-12 merozoites per schizont while those growing in reticulocytes produce 12-18 merozoites per schizont (Okokon *et al.,* 2005). In BALB/c mice, *P. berghei* (ANKA) causes rapidly fulminating infections leading to death within one week (Okokon *et al.,* 2005).

2.4.2.2 *Plasmodium knowlesi* (H-strain)

Plasmodium knowlesi (H) is prevalent among crab-eating macaques, *Macaca fascicularis*, in the Malaysian Peninsula and the Philippines (Rajahram *et al.,* 2012). Other known natural hosts include pig-tailed macaques, *M. nemestrina*, and leaf monkeys, *Presbytis melalophos* (Singh and Daneshvar, 2013). The great home of *P. knowlesi* is the Malayan jungle and swampy forests where monkeys are naturally infected (Chin *et al.,* 1965; (Assefa *et al*., 2015). The natural mosquito vectors are *A. hackeri* and *A. pujutensis* (Garnham, 1966). The life cycle of *P. knowlesi* is similar to that of *P. vivax* with sporogony taking 10 to 12 days at a temperature of 26 ^{o}C to 28°C while the exo-erythrocytic phase lasts 5.5 days (Ozwara *et al*., 2003).

2.5 Pathology associated with *Plasmodia*

Malaria is an acute febrile illness (Crompton *et al.,* 2014). In non-immune individuals, symptoms appear within seven to fifteen days following an infective mosquito bite (Crompton *et al.,* 2014). The first symptoms of malaria are nonspecific and similar to the symptoms of a minor systemic viral illness. They comprise: headache, lethargy, fatigue, abdominal discomfort and muscle and joint aches, followed by fever, chills, perspiration, anorexia, vomiting and worsening malaise. This is the typical picture of uncomplicated malaria (Miller *et al.,* 2002). The infected RBCs (iRBCs) are responsible for the disease symptoms, particularly high and periodic fever (paroxysms), headaches (common to all human malaria species) and anaemia (Franklin *et al*., 2011). If not treated within 24 hours, *P. falciparum* malaria can progress to severe illness often leading to death (Franklin *et al*., 2011). Children with severe malaria frequently develop one or more of the following symptoms: severe anaemia, respiratory distress in relation to metabolic acidosis, or

cerebral malaria. In adults, multiple organ involvement is common (Miller *et al.,* 2002). Major symptoms of *P. falciparum* include cerebral malaria and respiratory distress. These are life-threatening manifestations that are related to iRBC cytoadherence on microvascular endothelial cells and blockage of deep capillaries (Miller *et al.,* 2002).

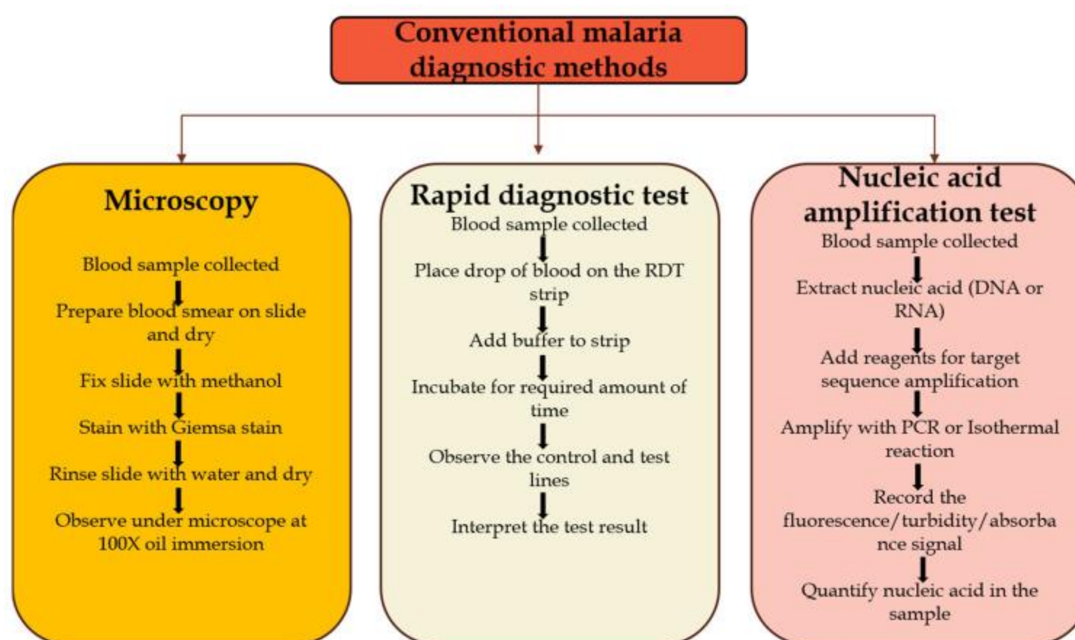
The pathogenesis of severe malaria is not completely understood, although proinflammatory cytokines contribute to the suppression of erythropoiesis, particularly in infected children (Perkins *et al.,* 2011). Evidence supports the role of type 1 pro-inflammatory cytokines that increase the expression of adhesion molecules on vascular endothelium and iRBCs sequestration (Schofield and Grau, 2005). Experimental data demonstrate that E6446, a synthetic antagonist of nucleic acid-sensing tool-like receptors (TLRs), diminishes the activation of TLR9 and prevents the increased production of cytokines in response to *Plasmodium* infections, consequently preventing severe malaria symptoms (Franklin *et al*., 2011).

In malaria endemic areas, persons may develop partial immunity, allowing asymptomatic infections to occur (Gueirard *et al.,* 2010). For both *P. vivax* and *P. ovale*, clinical relapses may occur weeks to months after the first infection, even if the patient has left the malarious area (Schofield and Grau, 2005). These new episodes arise from dormant liver forms known as hypnozoites (absent in *P. falciparum* and *P. malariae*); require special treatment targeted at these liver stages for a complete cure and primaquine has proved effective (Harrington *et al*., 2021).

2.6 Malaria diagnostic methods

Malaria must be diagnosed promptly in order to treat the patient in time to prevent further progression and lower the severity of the disease and to prevent further spread of infection in the community (White *et al*., 2014). Malaria is a febrile illness and clinical symptoms of uncomplicated malaria include fatigue, headaches, muscle aches, malaise, abdominal discomfort, fever, nausea and vomiting (Looareesuwan *et al.,* 1999; Tangpukdee *et al*., 2009). Specific diagnostic methods are needed to differentiate between malaria and other febrile illnesses (White *et al.,* 2014; McMorrow *et al.,* 2008). Currently used diagnostic methods (Figure 2.3) include

microscopy, rapid diagnostic tests (RDT), polymerase chain reaction (PCR), Loop-mediated isothermal amplification (LAMP), nucleic acid sequence-based amplification (NASBA), isothermal thermophilic helicase-dependent amplification (tHDA), saliva-based test for nucleic-acid amplification, saliva-based test for *Plasmodium* protein detection, urine malaria test (UMT) and transdermal hemozoin detection (Mbanefo and Kumar, 2020).



(*Mbanefo and Kumar, 2020*)

Figure 2.3: Malaria diagnostic methods

2.6.1 Clinical diagnosis

Clinical diagnosis is based on the patient's symptoms and on physical findings at examination (Looareesuwan *et al.,* 1999). The first symptoms of malaria include nonspecific and variable, and include fever, headache, weakness, myalgia, chills, dizziness, abdominal pain, diarrhea, nausea, vomiting, anorexia, and pruritus (Looareesuwan *et al.,* 1999). In severe malaria; clinical findings such as confusion, coma, neurologic focal signs, severe anaemia and respiratory difficulties are more striking and may increase the suspicion index for malaria (Larry and Gerald, 1996). Symptomatic diagnosis is however complicated in highly endemic areas due of overlap of malaria symptoms with other tropical diseases and other potentially life-

threatening diseases, such as common viral, bacterial and other febrile illnesses, which impairs diagnostic specificity and can therefore promote the indiscriminate use of antimalarials and compromise the quality of care for patients with non-malarial fevers (McMorrow *et al., 2008;* Tangpukdee *et al*., 2009). Thus, in most cases the early clinical findings in malaria are not typical and need to be confirmed by a laboratory test (Larry and Gerald, 1996).

2.6.2 Microscopy in malaria diagnosis

Malaria parasites can be identified by examining under the microscope a drop of the patient's blood, spread out as a blood smear on a microscope slide. A drop of blood is collected from a patient via a finger stick or venipuncture (Mathison and Pritt, 2017). When a venipuncture is used for blood collection, it is suggested that the blood is spread onto a slide immediately after collection to prevent prolonged exposure to anticoagulants in the collection tube that may alter parasite morphology (Mathison and Pritt, 2017). Thick smears are more sensitive and involve placing one to two drops of blood on a slide in a circle. The red blood cells are lysed and the various malaria parasite blood-stages, trophozoites, gametocytes and schizonts are released. Thin smears are used to detect the morphology of the parasite species and are prepared by spreading a drop of blood across a slide to create a feathered edge that contains a single layer of cells Cordray *et al.,* 2012). The slide is fixed in alcohol and stained with Giemsa stain and examined under x100 in oil immersion using using an Olympus bright-field microscope (BH-2, Tokyo, Japan).

Microscopy gives sensitivity and specificity of up to 95% (Cordray *et al.,* 2012; Kolluri *et al*., 2018). The limit of detection for this method is approximately 50-200 parasites per µL of blood (Pham, *et al.,* 2018). Microscopy remains the gold standard for laboratory confirmation of malaria (Mathison and Pritt, 2017). It is advantageous since a blood film examination under a microscope allows for the identification of parasitemia percentage, parasitic morphology and speciation (Mbanefo and Kumar, 2020). However, microscopy requires trained personnel and sensitivity and specificity may vary based on the quality of the reagents and skill of personnel (Larry and Gerald, 1996; Pham, *et al.,* 2018). Moreover, the time it takes for

parasitemia detection and quantification is long and may lead to delay in treatment (Mbanefo and Kumar, 2020). The limit of detection is also not ideal, because sub-microscopic asymptomatic individuals with low parasitemia remain undiagnosed and untreated, and also enable the transmission cycle to continue in the community (Mbanefo and Kumar, 2020).

2.6.3 Polymerase chain reaction

The parasite nucleic acids are detected using polymerase chain reaction (PCR). Polymerase chain reaction (PCR) based methods identify the presence of malaria target genes in a blood sample (Cordray and Kortum, 2012). Using molecular amplification techniques, nested conventional PCR, multiplex real-time PCR and reverse transcriptase PCR have been developed for human malarial species using target 18S single stranded ribosomal-ribose nucleic acid (rRNA) and circumsporozoite stage deoxyribose nucleic acid (DNA) sequences (Vasoo and Pritt, 2013). The sensitivity and specificity for the various PCR types ranges from 98% to 100%, thus are useful in identifying asymptomatic and submicroscopic patients or individuals with low parasite burden (Roth *et al.,* 2015; Cordray and Kortum, 2012). PCR-based methods however require the acquisition of a thermocycler which may be a financial hindrance for resource-limited settings to adopt the method. It also needs highly skilled personnel to perform the test and is not feasible for use in field settings (Mbanefo and Kumar, 2020).

2.6.4 Serological tests

Serology detects antibodies against malaria parasites antibodies against asexual blood stage malaria parasites using either indirect immunofluorescence (IFA) or through enzyme-linked immunosorbent assay (Tangpukdee *et al*., 2009). Immunofluorescence antibody testing (IFA) is useful in epidemiological surveys, for screening potential blood donors, and occasionally for providing evidence of recent infection in non-immunes (Tangpukdee *et al*., 2009). The principle of IFA is that, following infection with any *Plasmodium* species, specific antibodies are produced within 2 wk of initial infection, and persist for 3-6 months after parasite clearance (She *et al*., 2007). Immunofluorescence antibody testing (IFA) uses specific antigen

or crude antigen prepared on a slide, coated and kept at -30℃ until used, and quantifies both IgG and IgM antibodies in patient serum samples. Titers > 1: 20 are usually deemed positive, and < 1: 20 unconfirmed. Titers > 1: 200 can be classified as recent infections (Chotivanich *et al.,* 2006). Although IFA is simple and sensitive, it is time-consuming and cannot be automated, which limits the number of sera that can be studied daily (Tangpukdee *et al*., 2009). It also requires fluorescence microscopy and trained technicians; readings can be influenced by the level of training of the technician, particularly for serum samples with low antibody titers. Moreover, the lack of IFA reagent standardization makes it impractical for routine use in blood-transfusion centers, and for harmonizing inter-laboratory results (Tangpukdee *et al*., 2009).

2.6.5 Rapid diagnostic tests

Development of rapid diagnostic tests (RDTs), for malaria offers a useful alternative to microscopy in situations where reliable microscopy is not available (Mbanefo and Kumar, 2020). Rapid diagnostic tests kits are designed to detect antigens using an immunochromatographic strip where finger-prick blood is dropped into one end and the results are depicted by lines on the strip surface (Wilson, 2012). Three antigens; *Plasmodium* histidine-rich protein (pHRP-2), *Plasmodium* lactate dehydrogenase (pLDH) and *Plasmodium* aldolase are employed (Amir *et al*., 2018). *P*HRP-2 is specific to *P. falciparum*, while pLDH and *Plasmodium* aldolase are found in all species More than 90% of commercially available RDTs target pHRP-2 (Mouatcho and Goldring, 2013). Antibodies immobilized on the surface of the test strip detect these parasitic antigens when the blood and buffer mixture migrate across it. Each RDT contains a positive control to indicate the validity of the test (Mbanefo and Kumar, 2020). Currently available species-specific RDTs are only able to identify *P. falciparum* and *P. vivax* species (Mukkala *et al.,* 2018). For other species, the RDT is only able to indicate the presence of the parasite alone without speciation (Mbanefo and Kumar, 2020). RDTs represent a fast and affordable method for malaria diagnosis (White, 2014). RDTs are easy to deploy in resource-limited and hard-to-reach settings. The personnel training required is much less intensive as compared to microscopy and PCR (Mbanefo and Kumar, 2020). The

sensitivity of RDTs ranges from 85% to 94.8% and the specificity ranges from 95.2% to 99% (Abba *et al.,* 2011). The limit of the detection is comparable to that of microscopy, 50-100 parasites per μL of blood, and trained personnel are able to produce results in 15 to 20 min from the time of blood collection (Kolluri *et al*., 2018).

2.6.6 Isothermal thermophilic helicase-dependent amplification

In Isothermal Thermophilic Helicase-Dependent Amplification (tHDA) technique, the double-stranded DNA is separated by helicase and single-stranded DNA-binding proteins are attached to the separated strands (Li *et al.,* 2013). Specific primers bind to the strands and DNA polymerase synthesizes new strands, and the test is performed at 65°C in about two hours (Oriero *et al*., 2015). In the application of tHDA for malaria diagnosis, the 18S rRNA gene is amplified from whole blood directly without heat denaturation or nucleic acid amplification (Mbanefo and Kumar, 2020. Probes labeled with either fluorescein (FAM) or digoxigenin (DIG) hybridize to the amplicon and the amplification product is detected with a lateral-flow strip that contains anti-FAM or anti-DIG antibodies (Mbanefo and Kumar, 2020). The sensitivity and specificity of this method are 96.6% and 100%, respectively when microscopy is used as the gold standard (Li *et al.,* 2013). The limit of detection is 200 parasites per μL of blood and the results can be obtained in one to two hours (Li *et al.,* 2013; Oriero *et al*., 2015).

2.6.7 Loop-mediated isothermal amplification

Loop-mediated Isothermal Amplification (LAMP) is a method for nucleic-acid amplification that allows for ease of visualization of amplified product using a fluorescent or colorimetric dye such as calcein and hydroxy naphthol blue (HNB) respectively (Notomi *et al.,* 2000; Abdul-Ghani *et al*., 2012). The LAMP procedure can be carried out in a 65°C bath or in a heat block for 30 to 60 min (Oriero *et al*., 2015), with sensitivity ranging from 98.3% to 100% and specificity from 94.3% to 100% when compared to microscopy (Abdul-Ghani *et al*., 2012). The limit of detection by LAMP is comparable to that of PCR because they are both in the range of 0.5-5 parasites per μL of blood. It is faster than PCR and the results can be

assessed visually without the need for any expensive thermocycler (Mbanefo and Kumar, 2020). However, the method requires moderately skilled personnel and has a complex primer design (Mbanefo and Kumar, 2020).

2.6.8 Transdermal hemozoin detection

This method involves the detection of hemozoin-generated vapor nanobubbles using an ultrasound sensor (Mbanefo and Kumar, 2020). Hemozoin is the by-product of hemoglobin digestion by blood-stage malaria parasites (Mbanefo and Kumar, 2020). A short laser pulse administered to blood vessels through the skin localizes heat and evaporates the liquid around the hemozoin crystals. This liquid evaporation creates expanding and collapsing small sized vapor nanobubbles inside the malaria parasite (Lukianova-Hleb *et al*., 2014). After laser is activated, the probe is able to detect acoustic pulse and generates an electrical signal as an acoustic trace. Transdermal haemozoin detection method is noninvasive, requires no reagents and the results can be obtained within seconds. The method has a very low limit of detection and may potentially detect subclinical carriers which is helpful for disease surveillance (Mbanefo and Kumar, 2020).

2.6.9 Nucleic acid sequence-based amplification

Nucleic Acid Sequence-Based Amplification (NASBA) is a diagnostic method that involves the use of three enzymes; reverse transcriptase, T7 RNA polymerase and RNase H, to amplify RNA targets in a double-stranded DNA background (Cordray and Kortum, 2012). The RNA target, such as 18S RNA, is copied into complementary DNA (cDNA) using reverse transcriptase and then the cDNA is amplified using T7 RNA polymerase Cordray and Kortum, 2012). NASBA, like LAMP, does not require the use of a thermocycler because the reaction can be carried out at 41◦C resulting in more than 10^8-fold amplification of the target RNA sequence. The sensitivity of the method when compared to microscopy ranges from 97.40% to 100% while the specificity ranges from 80.90% to 94%, with a limit of detection is 0.01–0.1 parasites per µL of blood (Oriero *et al*., 2015).

2.6.10 Urine-based malaria test

Urine malaria tests involve the detection of *Plasmodium* protein pHRP-2 using a commercially available test (Mbanefo and Kumar, 2020). The test, known as urine malaria test (UMT) involves dipping the test strip into a urine sample for two minutes, followed by incubation for twenty minutes (Oguonu *et al*., 2014). A positive result is indicated by dark-colored lines on the test strip (Oguonu *et al*., 2014). The urine-based malaria test is affordable, non-invasive, therefore suitable for febrile children under five years old and does not require highly trained personal (Oyibo *et al.,* 2016). The limitation for this test is that it only detects pHRP-2 from *P. falciparum* parasites (Mbanefo and Kumar, 2020).

2.7 Antimalarial drugs currently in use

The discovery of the first antimalarial treatment almost 400 years ago resulted from observations that acutely ill patients were cured of malaria after treatment with infusions of bark obtained from plants growing in the Peruvian Amazon (Garnham, 1966). Such activity in *Cinchona calisaya* and *C. succirubra* plants was later attributed to the alkaloid quinine (QN), which was characterized by French chemists in 1820 (Collins Discovery Encyclopedia, 2005). Quinine remains important for treating complicated *P. falciparum* malaria despite its toxicity when used for extended periods of time (WHO, 2010a, b). Based on the QN ring, several 4-aminoquinolines were later synthesized; among them, Chloroquine (CQ), which is the safest and least expensive drug and most frequently used to treat malaria worldwide as an essential component of the Global Malaria Eradication Campaign (GMEC). This campaign, launched in 1955, was based on the treatment of malaria patients using CQ in association with mosquito control measures. In the late 1960s, *P. falciparum* CQ-resistant strains appeared in Latin America and South East Asia and gradually spread to most endemic regions. Chloroquine (CQ) is now used only in drug combinations against *P. falciparum* or as the schizonticide of choice to treat *P. vivax* and other human *Plasmodia* species (WHO, 2010a, and b).

Artemisinin (ART), a sesquiterpene endoperoxide, a natural product that is isolated from *Artemisia annua* (Asteraceae), is a medicinal plant recommended by the WHO

for use in drug combinations (ACTs) to treat uncomplicated *P. falciparum* malaria and *P. vivax* in areas of CQ resistance (WHO, 2010a, b). Drugs that complement the use of ACTs include lumefantrine (LF), amodiaquine (AQ), mefloquine (MQ), sulfadoxine-pyrimethamine (SP) and antibiotics. However, reduced susceptibility to ACT derivatives has been described in *P. falciparum* (Dondorp *et al.* 2010). The radical cure of *P. vivax* and *P. ovale* requires Primaquine (PQ), an 8-aminoquinoline that prevents relapses (Wells, 2011). Primaquine also targets the primary liver exo-erythrocytic forms, iRBCs and gametocytes. However, PQ metabolites cause severe haemolytic anaemia in patients who are genetically deficient in glucose-6-phosphate dehydrogenase (G6PD) (Carmona-Fonseca *et al*., 2013). This side effect imposes a pre-screening requirement for G6PD deficiency in *P. vivax* malaria patients and limits PQ use. The control of malaria has become gradually more complex due to the spread of drug-resistant *P. falciparum* strains and drugs that block the transmission of malaria are recommended by the WHO, especially in areas of high *P. falciparum* transmission. The use of PQ to control the transmission of malaria in endemic areas requires medical supervision due to the haemolytic toxicity of the drug (WHO, 2010a). Interventions to reduce mosquito density, human-vector contact and the vectorial capacity of *Anopheles* species are not simple (Karunamoorthi, 2011). Some methods that are available to reduce the transmission of malaria include indoor spraying with insecticides, ultra-low volume space spraying, the chemical or biological control of larvae and personal protection using repellents or insecticide-treated nets. These methods are hampered by insecticide resistance and the high maintenance costs required to sustain the measures (Karunamoorthi, 2011).

Resistance to antimalarial medicines is a recurring problem (Plowe, 2003). Resistance of *P. falciparum* to previous generations of medicines, such as Chloroquine and sulfadoxine-pyrimethamine (SP), became widespread in the 1970s and 1980s, undermining malaria control efforts and reversing gains in child survival. In recent years, parasite resistance to artemisinins has been detected in 5 countries of the Greater Mekong subregion: Cambodia, Laos, Myanmar, Thailand and VietNam. While there are likely many factors that contribute to the emergence and spread of resistance, the use of oral artemisinins alone, as monotherapy, is thought to be an important driver. When treated with an oral artemisinin-based monotherapy, patients

may discontinue treatment prematurely following the rapid disappearance of malaria symptoms. These results in incomplete treatment and such patients still have persistent parasites in their blood. Without a second drug given as part of a combination, these resistant parasites survive and can be passed on to a mosquito and then another person. If resistance to artemisinins develops and spreads to other large geographical areas, the public health consequences could be dire (Simoes-Pires *et al.,* 2014).

2.7.1 Need for new antimalarial drugs

The resilience and ingenuity of the malaria parasite in preserving itself, whatever the threat, requires the constant evaluation of control programs and searching for new antimalarials. An ideal antimalarial drug combination to replace ACTs and halt the spread of resistant parasites is urgently needed. If we are to avoid an ever-increasing toll of malaria on tropical areas, it is imperative to rapidly put into action strategic plans for the discovery and development of novel antimalarial compounds that are not encumbered by pre-existing mechanisms of drug resistance. Ideally, new drugs for uncomplicated *P. falciparum* malaria should be efficacious against drug-resistant strains, provide cure within a reasonable time to ensure good compliance, be safe, be suitable for small children and pregnant women, have appropriate formulations for oral use and, above all, be affordable. Drug development necessarily requires trade-offs among desired drug features, but for the treatment of malaria in the developing world the provision of affordable, orally active treatments that are safe for children is, for practical purposes, mandatory.

2.7.2 Drug development models and screening

Most programs aiming to discover new antimalarials are based on microtests against *P. falciparum* in blood cultures and animal models (Krettli, 2009). New methods have replaced traditional assays and various stages of the malaria parasites are currently used. Large scale drug-screening and techniques based on the computer analysis of drug banks are available and being used. One of the promising approaches currently being used in the discovery of new antimalarials aimed at drug-resistant parasites and the interruption of the transmission of malaria is the testing of

commercially available drugs that are currently prescribed for other indications (Penna-Coutinho *et al*., 2011). If successful, this approach will rapidly accelerate the production of new antimalarials at a lower cost. High-throughput screening (HTS) and molecular drug modeling have been successfully used in collaborative projects to select potential antimalarial candidates (Penna-Coutinho *et al*., 2011).

2.7.2.1 Tests for blood schizonticides

The evaluation of *P. falciparum* drug susceptibility *in vitro*, formerly based on microscopy, has been replaced by the incorporation of radioactive [^{3}H] hypoxanthine into live parasite DNA (Desjardins *et al.,* 1979); however, the need to store radioactive solid wastes for decades, expensive equipment required and the need for well-trained technicians restrict the use of this method, making microscopy to remain the gold standard method.

2.7.2.2 Enzyme-linked immunosorbent assays

These assays use monoclonal antibodies that are specific for the *P. falciparum* lactate dehydrogenase (*Pf*LDH) (Druilhe *et al.,* 2001) or a parasite histidine-rich protein (HRP II) (Noedl *et al.,* 2002). The assessment of parasite growth is effective in both of these tests. However, the existence of genetic diversity amongst *Plasmodium* strains interferes with the performance of *Pf*HRP2 tests (Baker *et al.,* 2005).

2.7.2.3 Use of transfected parasites

Transfected parasites expressing nucleic acid dyes may be detected by flow cytometry or fluorescence microscopy. For instance, green fluorescent protein (GFP)-recombinant *P. berghei* and *P. falciparum* parasites have been successfully used (Sanchez *et al*., 2007, Sanchez *et al*., 2004, Wilson *et al*., 2010).

2.7.2.4 Inhibition of haemozoin test

Inhibition of β-haematin (heamozoin) formation *in vitro* is an indirect test that does not require *P. falciparum* blood cultures and has been reported to be useful for antimalarial screening because the majority of 4-aminoquinoline antimalarials test

positive, i.e., they inhibit the formation of heamozoin crystals. The results, which are measured using enzyme-independent reactions, are rapidly obtained (Parapini *et al*., 2000, Ncokazi and Egan, 2005). In the 4-day suppressive test, drug activity is measured based on the inhibition of *P. berghei* parasitaemia in comparison with untreated control mice, as described by Peters (1965). Drugs that act on liver stages and block the transmission of malaria - the primary asexual development of *P. yoelii* or *P. berghei* sporozoites in mice or *P. knowlesi* in monkeys have also been reproduced in cultured hepatoma cells. However, few laboratories produce these sporozoite stages in *Anopheles* mosquitoes. When available, the tests are useful tools to investigate drug activity against pre-erythrocytic stages of the parasites (Mazier *et al*., 2009).

2.7.3 Reverse pharmacology

Reverse pharmacology is a transdisciplinary approach integrating traditional knowledge, experimental observations and clinical experiences (Figure 2.4) with the aim of reversing from the classical laboratory to clinic process (Figure 2.5) to a clinic to laboratory approach. As a faster alternative to drug development, development of phytomedicine through reverse pharmacology is currently considered (Willcox *et al*., 2011; Willcox, 2011; Willcox and Bodeker, 2000; Wells, 2011; Simoes-Pires *et al*., 2014; Patwardhan and Vaidya, 2010).

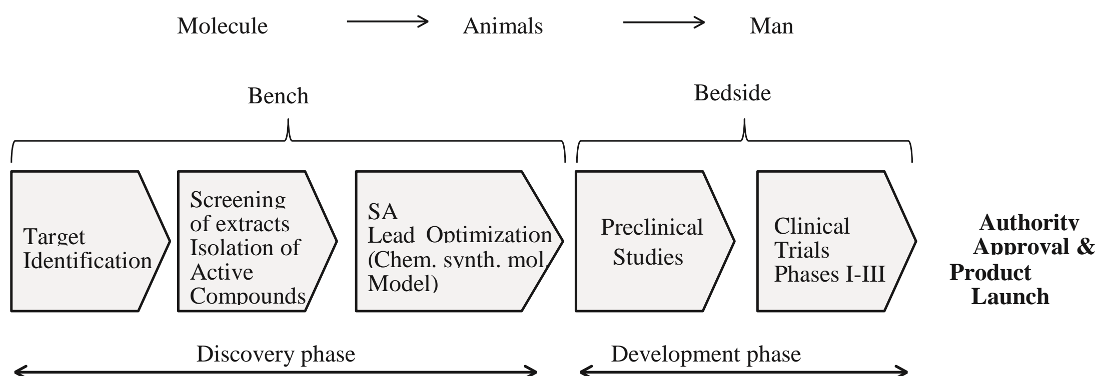


Figure 2.4: Laboratory to clinic drug development

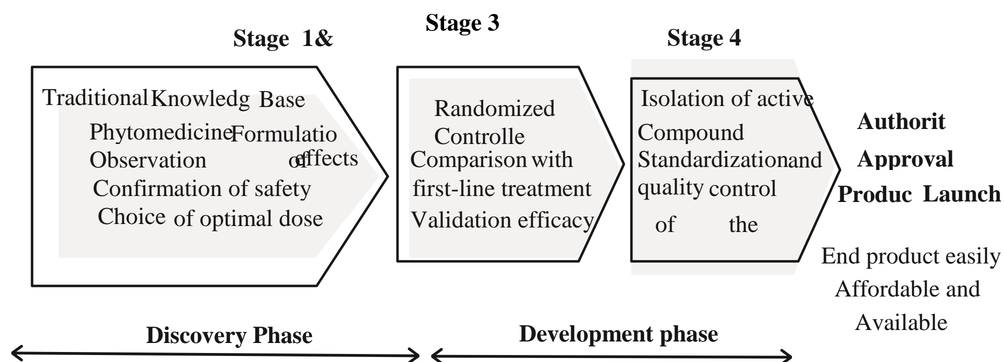


Figure 2.5: Reverse pharmacology approach

2.8 Pharmacology of antimalarial drugs and drug targets

Antimalarial drugs are chemically classified as aminoquinolines, sesquiterpene lactones, quinoline-methanols, sulfonamides, alkaloids, tetracyclines and biguanides. These antimalarials have different mechanisms and targets as summarized in Table 2.2 below.

Table 2.2: Targets for antimalarial chemotherapy

Target Location	Pathway	Target Molecule	Examples of	Therapies
			Existing therapies	**New compounds**
Cytosol	Folate metabolism	Dihydrofolate reductase	Pyrimethamin e proguanil	Chlorproguanil
	Glycolysis	Thymidylate synthase		5-fluoroorotate
		Lactate dehydrogenase		Gossypol derivatives
	Glutathione metabolism	Glutathione reductase		Enzyme inhibitors
	Unknown	Ca^{2+}-ATPase	Artemisinins	
Membrane	Membrane transport	Unique channels	Quinolines	Dinucleoside dimers
		Hexose transporter		Hexosederivatives
Food Vacuole	Haem polymerization	Haemozoin	Chloroquine	New quinolines
	Haemoglobin hydrolysis	Plasmepsins		Protease inhibitors
	Free-radical generation	Unknown	Artemisinins	New peroxides
Mitochondrion	Electron transport	Cytochrome *c* oxidoreductase	Atovaquone	
Apicoplast	Protein synthesis	Apicoplast ribosome	Tetracyclinesc lindamycin	
	DNA synthesis	DNA gyrase	Quinolones	
	Transcription	RNA polymerase	Rifampin	
Extracellular	Erythrocyte invasion	Subtilisin serine proteases		Protease inhibitors

2.8.1 Aminoquinolines used in malaria treatment

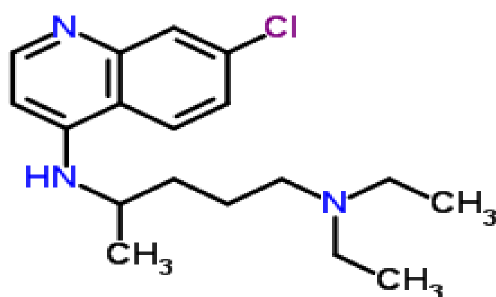
This is a class of antimalarial drugs with an amine group substituting for a carbon atom in their molecular structures (Simooya *et al.,* 1998). They include chloroquine, amodiaquine and primaquine

2.8.1.1 Chloroquine as antimalarial

Chloroquine (CQ) is a 4-aminoquinoline, which has been the drug of choice in most parts of malaria endemic areas for a long time until resistance was developed by the *falciparum* species (Mwai *et al*., 2009). It is a very potent schizonticidal drug against erythrocytic stage of all human *Plasmodia* species (Ginsburg, 1999). It however has no effect on sporozoites, hypnozoites and gametocytes (Ginsburg, 1999). Chloroquine (Figure 2.6), still maintains considerable efficacy for the treatment of *P.*

vivax, P. ovale and *P. malariae* infections (Aguiar *et al.,* 2012). Mechanistically, it interferes with parasite haem detoxification (Aguiar *et al.,* 2012). Resistance to CQ is related to genetic changes in transporters (PfCRT, PfMDR), which reduce its concentration at its site of action; the parasite food vacuole (Table 2.2). It is a weak base, uncharged at neutral pH while it carries a positive charge at acidic pH. Owing to this property, CQ, with plasma half-life of 10 days, selectively accumulates in the lysosomes. The uncharged compound rapidly diffuses through the plasma and lysosomal membranes, where once charged, it becomes trapped inside the acidic lysosomal compartment of the parasite.

The intracellular trophozoite feeds on the haemoglobin of the RBC that serves as a source of amino acids (Winstanley *et al.,* 1987). Digestion of the globin protein takes place inside the parasites' lysosome resulting in the generation of ferriprotoporphyrin IX (FP), which is a free heam (Ginsburg, 1999). The FP is insoluble and therefore precipitates as a black malaria pigment inside the lysosomes. (Collins *et al*., 2018). Normally, plasmodial haem polymerase converts haem to harmless haemozoin. Chloroquine thus inhibits this enzyme and so a built up of haem kills the parasite by membranolytic action. (Collins *et al*., 2018). Chloroquine is administered orally, unless where not feasible or in severe attack, then it is given by continuous intravenous infusion, frequent intramuscular or subcutaneous injection (Braga *et al.,* 2015). It is completely absorbed and extensively distributed throughout the tissues though poorly metabolized in the liver, with 70% coming out as intact drug. Acute overdosage is extremely dangerous and death can occur within a few hours (Simooya *et al.,* 1998). The patient may progress from feeling dizzy and drowsy with headache and gastrointestinal upset, to developing sudden visual disturbance, convulsions, hypokalaemia, hypotension and cardiac arrhythmias (Braga *et al.,* 2015). Side effects include nausea, vomiting, diarrhoea, psychoses, rashes, pruritis, dizziness, blurring vision, headache, and urticarial symptoms (Simooya *et al.,* 1998). Some 60% of CQ is bound to plasma proteins, and the drug is eliminated slowly from the body via the kidneys, with an estimated terminal elimination half-life of 1–2 months. Chloroquine is metabolized in the liver, mainly to monodesethylchloroquine, which has similar activity against *P. falciparum* (Simooya *et al.,* 1998).



(*ChemSpider ID 2618)*

Figure 2.6: Structure of Chloroquine

2.8.1.2 Amodiaquine as an antimalarial

Amodiaquine is a mannich base 4-aminoquinoline with with the amino group at the 4-position (Figure 2.7) of the quinolone (Espinoza *et al.,* 2020). Like other quinoline derivatives, it is thought to inhibit heme polymerase activity. This results in accumulation of free heme, which is toxic to the parasites (Espinoza *et al.,* 2020). The drug binds the free heme preventing the parasite from converting it to a form less toxic. It is effective against some chloroquine-resistant strains of *P. falciparum*, although there is cross-resistance. (Winstanley *et al.,* 1987*).* After oral administration amodiaquine hydrochloride is readily absorbed from the gastrointestinal tract (half-life 5.2 hours), and undergoes rapid and extensive metabolism to desethylamodiaquine that concentrates in blood cells. It is desethylamodiaquine, and not amodiaquine, which contributes nearly all of the antimalarial effect (Winstanley *et al.,* 1987*).* The adverse effects of AQ are similar to those of CQ (Winstanley *et al.,* 1987). Amodiaquine is associated with less pruritus and is more palatable than CQ, but is associated with severe adverse reactions such as neutropenia, agranulocytosis, and liver damage (Caims *et al*., 2010).

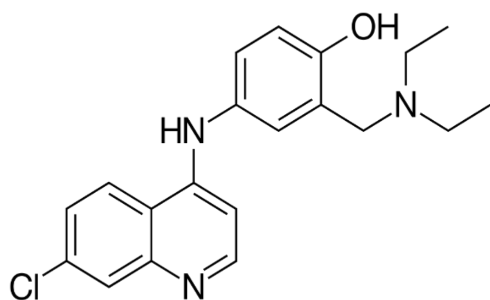


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Figure 2.7: Structure of Amodiaquine

2.8.1.3 Primaquine as an antimalarial

Primaquine (Figure 2.8) is an 8-aminoquinoline, which is a drug of choice for *vivax* malaria and is used against relapsing species of *Plasmodia*, and the only potent gametocytocide in *falciparum* malaria (Ashley *et al*., 2014). Primaquine is given as its base, 15 mg/day for 14 days, and has half-life of 3-6 hours. It is rapidly absorbed and metabolized *in vivo* via cytochrome P450 to reactive intermediates (mainly through CYP2D6) thought to mediate both anti- genetic polymorphisms conferring reduced enzyme activity may have reduced primaquine efficacy (Ashley *et al*., 2014). The major metabolite is carboxyprimaquine, which may accumulate in the plasma with repeated administration (Pybus *et al*., 2012). The most important adverse effects are haemolytic anaemia in patients who are glucose-6-phosphate dehydrogenase (G6PD) deficient and haemoglobinopathy (Chauhan and Sanjay, 2001). In patients with the African variant of G6PD deficiency, the standard course of primaquine generally produces a benign self-limiting anaemia (Ashley *et al*., 2014). Larger doses can cause nausea and vomiting. Other uncommon effects include mild anaemia and leukocytosis White *et al.,* 2012). Overdosage may result in leukopenia, agranulocytosis, gastrointestinal symptoms, haemolytic anaemia and methaemoglobinaemia with cyanosis (Pybus *et al*., 2012).

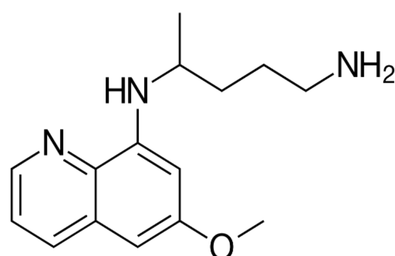

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Figure 2.8: Structure of Primaquine

2.8.2 Quinoline-methanols

These include antimalarial drugs such as quinine and mefloquine.

2.8.2.1 Quinine as an antimalarial

Quinine (Figure 2.9) is an alkaloid derived from the bark of the *Cinchona* tree (Achan *et al.*, 2011). Four anti- malarial alkaloids can be derived from the bark: quinine, quinidine, cinchonine and cinchonidine. Quinine acts principally on the mature trophozoite stage of parasite development and does not prevent sequestration or further development of circulating ring stages of *P. falciparum*. Like other structurally similar antimalarials, Quinine also kills the sexual stages of *P. vivax, P. malariae* and *P. ovale*, but not mature gametocytes of *P. falciparum* (Achan *et al.*, 2011). It does not kill the pre-erythrocytic stages of malaria parasites. Its mechanism of action is similar to CQ, causing cytotoxicity of the parasite by inhibiting plasmodial haem polymerase with the subsequent built up of toxic haem (Achan *et al.*, 2011). It is used in severe *P. falciparum* infection, where slow intravenous (i.v.) infusion is administered or in patients who are unable to retain oral intake, it is administered parenterally (Dondorp *et al.*, 2005). It has analgesic, but not antipyretic properties (Achan *et al.*, 2011). It is well absorbed in the gut where 80% is bound to plasma protein. Quinine has half-life of 10 hours and is metabolized in the liver and excreted in urine within 24 hours (Esamai *et al.*, 2000). Side effects include depression in heart action, irritation of the gastric mucosa, nausea, vomiting, blindness, headaches and hypersensitivity reactions. Other side effects include cardiac arrythmias, central nervous system (CNS) disturbances, hypoglycaemia as

well as blackwater fever, a fatal case of acute haemolytic anaemia, which is associated with renal failure (Dondorp *et al*., 2005). Rimchala *et al*., 1996). The pharmacokinetic properties of quinine are altered significantly by malaria infection, with reductions in apparent volume of distribution and clearance in proportion to disease severity. Extensive metabolism via the cytochrome P450 enzyme CYP3A4 occurs in the liver and elimination of more polar metabolites is mainly renal. The initial metabolite 3- hydroxyquinine contributes approximately 10% of the antimalarial activity of the parent compound, but may accumulate in renal failure. Administration of quinine or its salts regularly causes a complex of symptoms known as *cinchonism*, which is characterized in its mild form by tinnitus, impaired high tone hearing, headache, nausea, dizziness and dysphoria, and sometimes disturbed vision (Karlsson *et al.,* 1990). More severe manifestations include vomiting, abdominal pain, diarrhoea and severe vertigo. Hypersensitivity reactions to quinine range from urticaria, bronchiospasm, flushing of the skin and fever, through antibody-mediated thrombocytopenia and haemolytic anaemia, to life- threatening haemolytic-uraemic syndrome (Karlsson *et al.,* 1990). Intramuscular injections of quinine dihydrochloride are acidic (pH 2) and cause pain, focal necrosis and in some cases abscess formation, and in endemic areas are a common cause of sciatic nerve palsy. Hypotension and cardiac arrest may result from rapid intravenous injection, thus intravenous quinine should be given only by infusion, not injection. Overdosage of quinine may cause oculotoxicity, including blindness from direct retinal toxicity, and cardiotoxicity, and can be fatal (Rimchala *et al*., 1996).

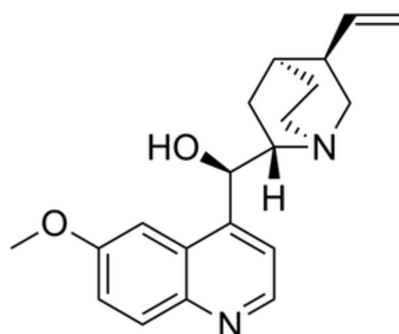

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Figure 2.9: Structure of quinine

2.8.2.2 Mefloquine as an antimalarial

Mefloquine (MQ) is a methanol quinoline and is related to quinine (Foley and Tilley, 1997). Mefloquine (Figure 2.10) is an effective schizonticidal, active against the blood stages of all malaria species that infect humans, including *P. knowlesi* as well as hypnozoites if given as a combination treatment with primaquine (Bronner *et al.,* 2009). Taken orally, it is rapidly absorbed though it has a slow onset of action, though long acting, with a plasma half-life of 30 days. It used for uncomplicated CQ-resistant *falciparum* malaria and as a short-term chemoprophylaxis when entering CQ-resistant zones (Schlagenhauf *et al.,* 2009). Minor adverse effects are common following mefloquine treatment, most frequently nausea, vomiting, abdominal pain, anorexia, diarrhoea, headache, dizziness, loss of balance, dysphoria, somnolence and sleep disorders, notably insomnia and abnormal dreams (Hellgren *et al.,* 1997). Other side effects reported rarely include skin rashes, pruritus and urticaria, hair loss, muscle weakness, liver function disturbances and very rarely thrombocytopenia and leukopenia (Hellgren *et al.,* 1997). Cardiovascular effects have included postural hypotension, bradycardia, and hypertension, tachycardia. Mefloquine works by interfering with transportation of haemoglobin products and other substances from the host cell to the parasite's food vacuole (Foley and Tilley, 1997).

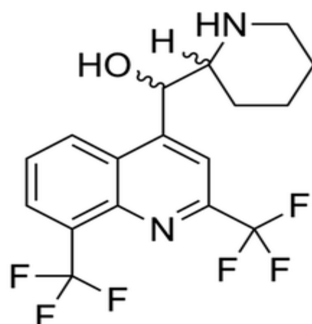

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Figure 2.10: Structure of Mefloquine

2.8.2.3 Halofantrine as an antimalarial

Halofantrine (Figure 2.11) is an amino-alcohol that is schizonticidal against the erythrocytic form of *P. falciparum* resistant to CQ (Bouchaud *et al*., 2009). It is also active against *P. vivax* erythrocytic forms but not the hypnozoites. It is effective against multi drug resistant (including mefloquine resistant) *P. falciparum* malaria. (Tse *et al.,* 2019). If taken orally, halofantrine it is slowly and irregularly absorbed with a peak plasma concentration at 4-6 hours later (Nosten *et al.,* 2000). It has a half-life of 1-2 days and elimination is through faeces. Toxicity includes abdominal pain, gastrointestinal disturbances headache, a transient rise in hepatic enzymes, pruritus, haemolytic anaemia and convulsions (Nosten *et al.,* 2000). The mechanism of action of Halofantrine may be similar to that of chloroquine, quinine, and mefloquine; by forming toxic complexes with ferritoporphyrin IX that damage the membrane of the parasite (Bouchaud *et al*., 2009). It appears to inhibit polymerisation of heme molecules (by the parasite enzyme "heme polymerase"), resulting in the parasite being poisoned by its own waste (Bouchaud *et al.,* 2009). Halofantrine, under the brand name Halfan™, is only used in cases where patients are known to be free of heart disease and where infection is due to severe and resistant forms of malaria (Croft, 2007; Tse *et al.,* 2019). It is only used as a curative drug and not for prophylaxis due to the high toxicity risks and its unreliable pharmacological properties (Croft, 2007).

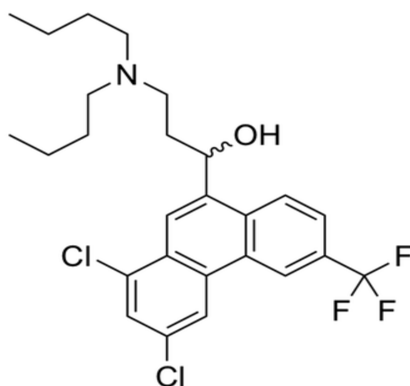

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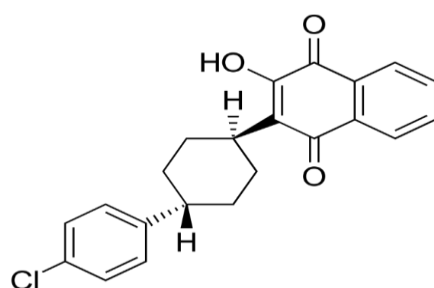
Figure 2.11: Structure of halofantrine

2.8.2.4 Atovaquone as an antimalarial

Atovaquone (Figure 2.12) is a naphthoquinone with broad-spectrum antiprotozoal activity (Baggish and Hill, 2002). Atovaquone is a hydroxynaphthoquinone with broad-spectrum antiprotozoal activity. It is effective for the treatment and prevention of *Pneumocystis carinii* pneumonia (PCP), it is effective in combination with proguanil for the treatment and prevention of malaria, and it is effective in combination with azithromycin for the treatment of babesiosis (Baggish and Hill, 2002). It active against all *Plasmodia* species. It inhibits pre-erythrocytic development in the liver, and oocyst development in the mosquito (Looareesuwan *et al.,* 1996). Atovaquone interferes with cytochrome electron transport and is available for the treatment of malaria in a co-formulation with proguanil (Pudney *et al.,* 1999). It is poorly absorbed from the gastrointestinal tract but bioavailability following oral administration can be improved by taking the drug with fatty foods (Rolan *et al.*, 1994; Beerahee, 1999). It is excreted almost exclusively in the faeces as unchanged drug (Rolan *et al.,* 1997). Atovaquone is generally well tolerated though skin rashes, headache, fever, insomnia, nausea, diarrhoea, vomiting, raised liver enzymes, hyponatraemia and, haematological disturbances, such as anaemia and neutropenia are the commonest side effects (Baggish and Hill, 2002).

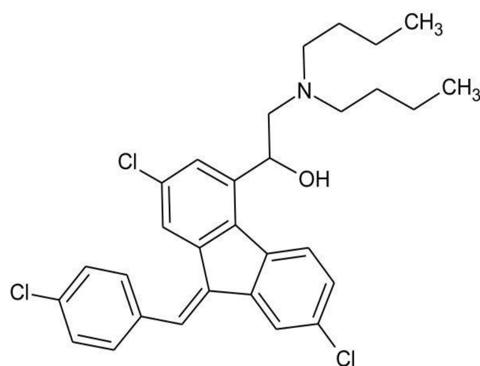


(PubChem CID74989)

Figure 2.12: Structure of Atovaquone

2.8.2.5 Lumefantrine as an antimalarial

Lumefantrine (Figure 2.13) belongs to the aryl amino alcohol group of antimalarials, which also includes quinine, mefloquine and halofantrine. It is only available in an oral preparation coformulated with artemether (Tarning *et al.,* 2009). This combination therapy exerts its effects against the erythrocytic stages of *Plasmodia* and may be used to treat infections caused by *P. falciparum* and unidentified *Plasmodium* species, including infections acquired in chloroquine-resistant areas (Tun *et al.,* 2018). Oral bioavailability is variable and is highly dependent on administration with fatty foods. Ashely *et al.,* 2007). Absorption increases by 108% after a meal and is lower in patients with acute malaria than in convalescing patients. Peak plasma levels occur approximately 10h after administration. The terminal elimination half- life is 3 days. Lumefantrine is well tolerated, with mild side effects such as nausea, abdominal discomfort, headache and dizziness (Mwesigwa *et al*., 2010).



(PubChemCID 6437380)

Figure 2.13: Structure of Lumefantrine

2.8.3 Artemisinin and its derivatives in malaria treatment

Artemisinin (ART), also known as *qinghaosu*, is a sesquiterpene lactone extracted from the leaves of *Artemisia annua* (Martino *et al*., 2019; Balint, 2001). It has been used in China for the treatment of fever for over a thousand years (van Agtmael *et al.,* 1999; WHO, 1998). It is a potent and rapidly acting blood schizontocide and is active against all *Plasmodia* species (Nosten *et al*., 1998, WHO, 1998). It has an unusually broad activity against asexual parasites, killing all stages from young rings to schizonts. In *P. falciparum* malaria, artemisinin also kills the gametocytes, including the stage 4 gametocytes (Nosten *et al*., 1998). The antimalarial properties of ART derivatives is related to the formation of free radicals that alter biochemical pathways within the parasites (WE *et al.,* 2014). In particular, the trophozoite parasite takes up and digests the hemoglobin of the host in its food vacuole to obtain amino acids. Hemoglobin digestion releases hem groups that are toxic for the parasite and are hence polymerized into hemozoin (Phillips *et al.,* 2017).

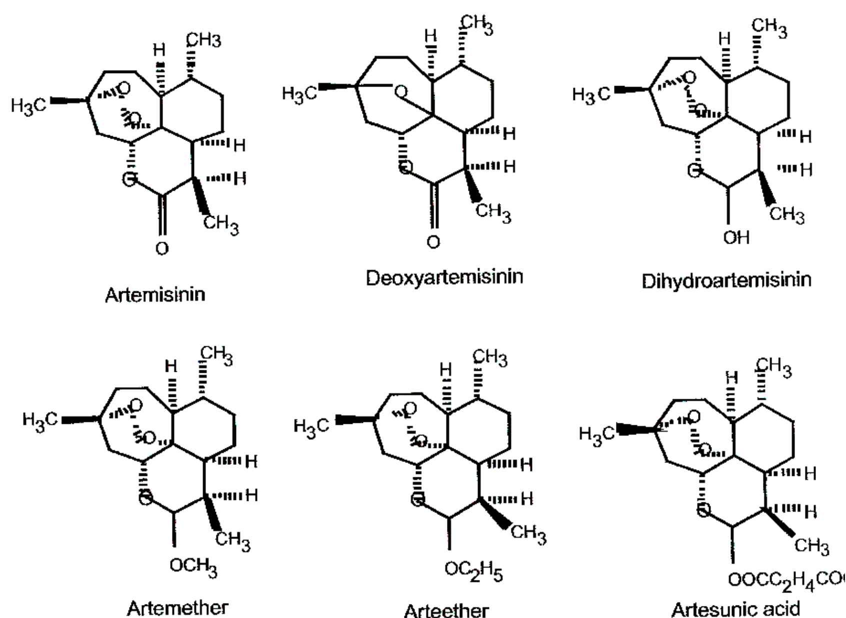
The ART derivatives are activated by the iron in hemozoin: the cleavage of the endoperoxide bridge happens through a Fe(II) Fenton reaction that produces reactive oxygen species (ROS) and carbon-centered radicals. Fröhlich *et al*., 2016). Free radicals are responsible for: alkylation of hem molecules and interference with the hem detoxification pathway, inactivation of the ATPase 6 (PfATPase6) calcium pump (SERCA) in the sarcoplasmic endoplasmic reticulum of *P. falciparum*,

alkylation of PfTCTP (tumor protein likely related to parasite replication) and other cytosolic proteins and disruption of mitochondrial function (Ho *et al.,* 2014).

Active derivatives have been synthesized - artemether, arteether and artesunate - which are used for oral, intramuscular, rectal and intravenous administration (van Agtmael *et al.,* 1999). The fat-soluble derivatives artemether and arteether are approximately twice as active (de Vries and Dien, 1996). The water-soluble dihydro-artemisinin and artesunate are 4 to 5 times more active *in vitro*. Artemisinin is available only for oral and rectal administration. Absorption is incomplete and elimination is fast, with and elimination half-life of 2 to 5 hours. Biotransformation into the active metabolite dihydro-artemisinin occurs rapidly--almost immediately for artesunate (de Vries and Dien, 1996). The three latter derivatives are converted back *in vivo* to dihydroartemisinin. These drugs should be given as combination therapy to protect them from resistance. However, since the peroxide bridge of the compound is stable under certain chemical reactions, several oil and water-soluble derivatives of AR have been synthesized (Figure 2.14). Artesunate, a semi-synthetic derivative of AR, is used for the treatment of both uncomplicated and severe malaria. It is formulated for oral, parenteral and rectal administration (Navaratnam *et al*., 2000). Artemisinin is hydrophobic and passes biological membranes easily (Agustijns *et al.,* 1996). *In vitro* studies have suggested an uptake of AR by both healthy and malaria infected red blood cells (Asawamahasakda *et al.,* 1994). It is known that AR binds to haem, either in haemoglobin or in haemozoin. Through an iron-mediated cleavage of the peroxide bridge, AR free radicals are formed. These free radicals are destructive to different parasite membranes; including mitochondria, rough endoplasmic reticulum, and plasma membranes, thereby killing them (Asawamahasakda *et al.,* 1994).

It is believed that the haem-rich internal environment of the parasites is one of the reasons for the selective toxicity of AR toward the malarial parasites as haem has been shown to interact with the compound (Hong *et al.,* 1994). This heam-rich internal environment of schizonts also account for schizonticidal activity of AR. Artemisinin has an absorption lag-time of 0.5-2 hours after oral intake, with peak plasma concentrations at 1-3h post-administration (Tituler *et al.,* 1990). It is

eliminated by enzymatic metabolism to inactive metabolites, lacking the peroxide bridge (Lee *et al.,* 1988). Only trace amounts are detectable in urine in both healthy volunteers and malaria patients after oral administration (Navaratnam *et al.,* 2000). The ether and ester derivatives are metabolized to dihydroartemisinin, which accounts for most of the clinical effect of these derivatives after intake (Price, 1996; Hong *et al.,* 1994). Artemisinin, artesunate and artemether are well-tolerated in both adults and children, with no evidence to date of serious clinical toxicity (Price, 2000).



(*en.wikipedia.org, 2009)*

Figure 2.14: Artemisinin and its derivatives

2.8.4 Folate antagonists in malaria treatment

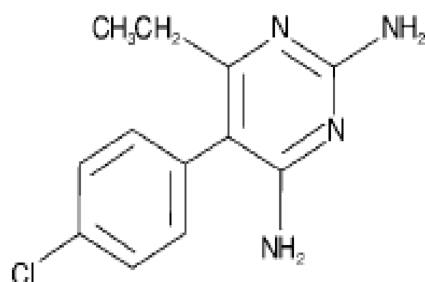
These drugs inhibit the dihydrofolate reductase activity of the dihydrofolate reductase-thymidylate synthetase (DHFR-TS) bifunctional enzyme (Hyde, 1990; Fernández-Villa *et al.,* 2019). Folate is an important co-factor in one-carbon substitutions, especially synthesis of thymidylate (Fernández-Villa *et al.,* 2019). Antifolates have similar structures to trimethoprim (of the pteridine ring of dihydrofolate reductase) and so able to block its activity (Koehn *et al*., 2012).

Blockage of folate synthesis results in a decrease in synthesis of pyrimidines, leading to the arrest of DNA replication, decreased methionine synthesis and reduced conversion of glycine to serine which leads to all cycle arrest and finally death of the parasite (Plowe *et al.,* 1998). Dihydrofolate reductase (DHFR) and dihydropteroate synthase (DHPS) both important in this pathway, are targeted by these drugs (Plowe *et al.,* 1998). DHFR inhibitors are administered for treating different types of cancer, autoimmune diseases and protozoal infections such as malaria or toxoplasmosis (Raimondi *et al.,* 2019). In treatment of malaria, the use of antifolates is less effective since many strains of *P. falciparum* can internalize exogenous folate and metabolize it (Fernández-Villa *et al.,* 2019). To overcome this challenge, combination therapies that act synergistically in different steps of the route are preferred (Metz, 2007). These combinations include chlorproguanil with dapsone (LapDap), pyrimethamine with sulfadoxine (Fansidar) and pyrimethamine with dapsone (Maloprim) (Metz, 2007).

2.8.4.1 Pyrimethamine as an antimalarial

Pyrimethamine (Figure 2.15) is a diaminopyrimidine used in combination with a sulfonamide, usually sulfadoxine or dapsone (Nzila, 2006). It exerts its antimalarial activity by inhibiting plasmodial dihydrofolate reductase thus indirectly blocking the synthesis of nucleic acids in the malaria parasite (Nzila 2006). Pyrimethamine is almost completely absorbed from the gastrointestinal tract and peak plasma concentrations occur 2-6h after an oral dose. Nyunt *et al.,* 2010). It is mainly concentrated in the kidneys, lungs, liver and spleen, and about 90% is bound to plasma proteins Agomo *et al.,* 2011). It is metabolized in the liver and slowly excreted via the kidneys Agomo *et al.,* 2011). Its plasma half-life is 4.16 days Nyunt *et al.,* 2010). Pyrimethamine crosses the blood-brain barrier and the placenta and is detectable in breast milk. Though it is well tolerated, prolonged administration may cause depression of haematopoiesis due to interference with folic acid metabolism Gutman *et al* 2012). Larger doses may cause gastrointestinal symptoms such as atrophic glositis, abdominal pain, vomiting and haematological effects including megaloblastic anaemia, leukopenia, thrombocytopenia and pancytopenia, and central nervous system effects such as headache and dizziness Gutman *et al* 2012). Acute

overdosage of pyrimethamine can cause gastrointestinal effects and stimulation of the CNS with vomiting, excitability and convulsions Gutman *et al* 2012).

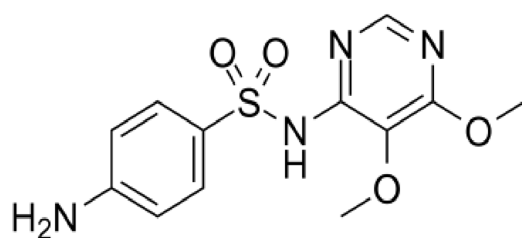


(PubChem 4993)

Figure 2.15: Structure of Pyrimethamine

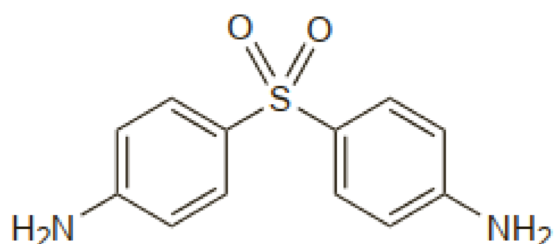
2.8.4.2 Sulfadoxine and Dapsone

Sulphonamides such as sulphadiazine and sulphadoxine (Figure 2.16), with half-life of 180 hours, and dapsone (Figure 2.17); inhibit dihydropteroate synthase (DHPS) by acting as analogues of *P*-aminoparabenzoic acid (PABA), a folate precursor (Foote and Cowman, 1994). They are effective against asexual blood forms of *P. falciparum*, but not sporozoites or hypnozoites (Chauhan and Sanjay, 2001). Sulfonamides are structural analogues and competitive antagonists of *p*-aminobenzoic acid (*p*ABA). They are competitive inhibitors of dihydropteroate synthase, the bacterial enzyme responsible for the incorporation of *p*ABA in the synthesis of folic acid. Sulfadoxine may be administered orally or by the intramuscular route (Chauhan and Sanjay, 2001. The terminal elimination half-life is 8.33 days Nyunt *et al.,* 2010). It is widely distributed to body tissues and fluids, passes into the fetal circulation and is detectable in breast milk. The drug is excreted in urine with a small fraction in stool Agomo *et al.,* 2011; Nyunt *et al.,* 2010). Sulfadoxine shares the adverse effect profile of other sulfonamides, although allergic reactions can be severe because of its slow elimination. Nausea, vomiting, anorexia and diarrhoea may occur (Kuile *et al.*, 2007; Gutman *et al* 2012). Hypersensitivity reactions may affect different organ systems. Cutaneous manifestations can be severe and include pruritus, photosensitivity reactions, exfoliative dermatitis, erythema nodosum and toxic epidermal necrosis Gutman *et al* 2012).



(PubChem ID, 65404)

Figure 2.16: Structure of Sulfadoxine

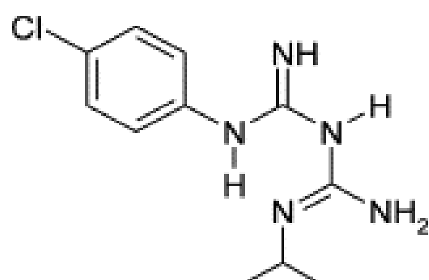


(PubChem 2955)

Figure 2.17: Structure of Dapsone

2.8.4.3 Proguanil as antimalarial drug

Proguanil (Figure 2.18) is a biguanide compound that is metabolized to the active antifolate metabolite, cycloguanil (Nzila, 2006). Cycloguanil inhibits plasmodial dihydrofolate reductase (Nzila, 2006). It is active against pre-erythrocytic forms of the parasite and is a slow blood schizontocide. Proguanil also has sporontocidal activity, rendering the gametocytes non-infective to the mosquito vector. Proguanil is administered as the hydrochloride salt in combination with atovaquone (Kain, 2003). Proguanil is readily absorbed from the gastrointestinal tract following oral administration. Peak plasma levels occur at about 4h, and are reduced in the third trimester of pregnancy (Kain, 2003).



(PubChem CID 9570076)

Figure 2.18: Structure of Proguanil

2.8.5 Combination drug therapy in malaria treatment

Combination therapy with antimalarial drugs is the simultaneous use of two or more blood schizontocidal drugs with independent modes of action and different biochemical targets in the parasite (Alven and Aderibigbe, 2019). Combination therapy is currently preferred to treat malaria, as it is likely to decrease the rate of development of resistance and expand the useful lifetime of the drugs (WHO, 2001 Alven and Aderibigbe, 2019). Combination of drugs is based on various criteria: Drugs targeting different metabolic pathways (SP+AQ); drugs targeting different stages of the parasite [AQ (blood schizonts) and Primaquine (gametocyte)]; and, drugs with different half life; short half life with rapid parasitaemia clearance, such as AS and mefloquine (WHO, 2001). The risk of having a patient being infected with a parasite strain resistant to two antimalarials simultaneously is very small (Nosten *et al.,* 2000). Moreover, by reducing the number of parasites rapidly by AR or its derivatives, the risk of selecting for resistance against the second drug of the combination therapy will be reduced (White, 2002). Trends of such effects have been reported for combinations of AS and mefloquine in which halted progression of mefloquine resistance had been observed (Nosten *et al.,* 2000). Although different combinations of an artemisinin derivative with mefloquine have been the most used ones, other compounds have also been used in combination with an artemisinin compound (Ezzet *et al.,* 2000). One such compound is lumefantrine, a new Chinese drug previously known as benflumetol (Ezzet *et al.,* 2000). A fixed dose of Coartem® has been studied and is now available for treatment of uncomplicated *falciparum* malaria (Vugt *et al.,* 1999). More clinical studies with different

combinations of artemisinin compounds and other antimalarials are being undertaken. Indeed, a recent study to compare monotherapy and combination therapy in Mozambique showed that, in monotherapy the therapeutic efficacy of AQ (91.6%) was better than that of SP (82.7%) and CQ (47.1%); after 14 days (Abacassamo *et al.,* 2004). The same study showed that combination therapy of AQ+SP, AR+SP and AQ+AR was safe and had 100% clinical efficacy at day 14 of follow up (Abacassamo *et al.,* 2004).

2.9 Prevention of malaria

Integrated prevention, which include training, use of insecticide-treated nets (ITNs), symptom recognition and environmental management are extremely effective in combating malaria. Vector control is the main way to reduce malaria transmission at the community level (Musoke *et al.,* 2013). For individuals, personal protection against mosquito bites represents the first line of defense for malaria prevention. Two forms of vector control are effective in a wide range of circumstances (Tangena *et al.,* 2020).

2.9.1 Indoor residual spraying

Indoor residual spraying (IRS) with insecticides is a powerful way to rapidly reduce malaria transmission (Tangena *et al.,* 2020). Its full potential is realized when at least 80% of houses in targeted areas are sprayed. Indoor spraying is effective for 3-6 months, depending on the insecticide used and the type of surface on which it is sprayed (Tangena *et al.,* 2020). Longer-lasting forms of existing IRS insecticides, as well as new classes of insecticides for use in IRS programs, are under development. Indoor Residual Spraying involves use of long-acting insecticides including DDT on the walls and roofs of houses, public buildings and domestic animal shelters in order to kill malaria-carrying mosquitoes that land on these surfaces (Dengela *et al*., 2018).

2.9.2 Use of insecticide-treated nets

Long-lasting insecticidal nets (LLINs) are the preferred form of Insecticide-treated mosquito nets (ITNs) for public health distribution programs (Curtis, 1996). World

Health Organization recommends coverage for all persons at risk of malaria (WHO, 2013). Long-lasting insecticide-treated nets simultaneously provide a protective covering for the body while releasing chemicals to repel and kill the infection-carrying mosquitoes (Sousa *et al.,* 2019). They are designed to retain their efficacy against mosquito vectors for a minimum of 3 years or 20 standard washes under laboratory conditions (WHO, 2013). Long-lasting insecticide-treated nets mark a new era in fighting malaria Shiff, 2002). Major recent efforts to scale-up the availability of ITNs in Africa are yielding impressive results (WHO, 2012). By 2011, 78 countries worldwide had adopted the policy to provide nets to all persons at risk of malaria - "universal coverage", of which 89 have policy of distributing them free of charge to the end user (WHO, 2012).

2.9.3 Chemoprophylaxis in malaria control

Antimalarial medicines can also be used to prevent malaria. For travellers, malaria can be prevented through chemoprophylaxis, which suppresses the blood stage of malaria infections, thereby preventing malaria disease (WHO, 2012). Chemoprophylaxis is an effective tool for individuals to minimize their risk of contracting malaria and serves an important public health role in preventing imported malaria (Rodrigo *et al.,* 2020).

For non-immune travelers, chemoprophylaxis may be given as primary prophylaxis (a schizonticide agent given before, during, and after travel in an endemic area) or as terminal prophylaxis (hypnozoiticide agent against *P. vivax*) given after leaving the endemic area (Hill *et al*., 2006). For primary prophylaxis, the Centers for Disease Control and Prevention (CDC) recommend mefloquine (weekly dosing), Atovaquone/proguanil combination (daily dosing), doxycycline (daily dosing), chloroquine (weekly dosing), primaquine (daily dosing), and tafenoquine (weekly dosing) (CDC, 2020). In addition, WHO recommends intermittent preventive treatment (IPT) with sulfadoxine-pyrimethamine for pregnant women living in high transmission areas, at each scheduled antenatal visit after the first trimester (2012).

2.9.4 Malaria vaccines

Currently, no effective vaccine is available to fight human malaria (Rogers and Hoffman, 1999). However, various antigen formulations are undergoing field trials (Shiff, 2002). In particular, a vaccine based on the *P. falciparum* circumsporozoite protein (CSP) and blood stage parasite proteins, has demonstrated promising results (Cohen *et al*., 2010). Two other vaccines under trial are based on the anti-merozoite surface protein of blood stages (McCarthy *et al*., 2011) and a transmission-blocking (Arevalo-Herrera *et al*., 2010).

2.10 Phytochemicals and malaria control

The phytochemical research based on ethno-pharmacological information is generally considered an effective approach in the discovery of new anti-infective agents from higher plants (Njoroge and Bussmann, 2006). Knowledge of the chemical constituents of plants is desirable, not only for the discovery of therapeutic agents, but also because such information may be of value in disclosing new sources of such economic materials as tannins, oils, gums and even precursors for the synthesis of complex chemical substances (Njoku and Obi, 2009). Moreover, the knowledge of the chemical constituents of plants would further be valuable in discovering the actual value of folkloric remedies (WHO, 2012). Chemical constituents may be therapeutically active or inactive. The ones which are active are called active constituents and the inactive ones are called inert chemical constituents (WHO, 2012). According to WHO more than 80% of the world's population relies on traditional medicines for their primary health care needs. The medicinal value of plants lies in some chemical substances that produce a definite physiologic action on the human body (Edeoga *et al*., 2005). The most important of these bioactive compounds of plants are alkaloids, flavonoids, tannins and phenolic compounds (Basirat *et al*., 2019).

2.10.1 Phytochemical screening of medicinal plants

A phytochemical is a naturally occurring bioactive compound present in plants. Common phytochemicals include alkaloids, tannins, terpenoids, flavonoids, saponins

and phenols (Sharma *et al.,* 2018). Phenolic compounds are attracting interest in the field of food, chemistry and medicine due to their effective antioxidant activity (Sharma *et al.,* 2018). Several different methods are used to screen phytochemicals in order to know the composition of the plant material (Trease and Evans, 1989). The presence or absence of a phytochemical can be tested by the addition of appropriate chemical reagent to the plant material (Gul *et al*., 2017). For example, alkaloids give a precipitate with heavy metal iodides and give a coloured precipitate in the presence of Mayer's and Dragendroff's reagent whilst saponins present in plant materials may be tested by frothing (Trease and Evans, 1983).

2.10.2 Extraction solvents

The selection of the solvent is crucial for solvent extraction. Selectivity, solubility, cost and safety should be considered in selection of solvents Zhang *et al.,* 2018). The properties of the extraction solvent, the particle size of the raw materials, the solvent-to-solid ration, the extraction temperature and the extraction duration will affect the extraction efficiency (Napolitano *et al*., 2017). Based on the law of similarity and intermiscibility, solvents with a polarity value near to the polarity of the solute are likely to perform better and vice versa. Alcohols are universal solvents in solvent extraction for phytochemical investigation Zhang *et al.,* 2018). Temperature also plays an important role by increasing solubility and diffusibility, it affects the quality and effectiveness of some heat-labile components of the material (Li *et al.,* 2008).

2.10.3 Extraction and standardization of herbal extracts

Extraction is the first step to separate the desired natural products from the raw materials. Extraction methods include solvent extraction, distillation method, pressing and sublimation (Zhang *et al.,* 2018). On the other hand, standardization of herbal medicines involves prescribing a set of standards or inherent characteristics, constant parameters, definitive qualitative and quantitative values that carry an assurance of quality, efficacy, safety and reproducibility (Kunle *et al.,* 2012). The separation depends on the physical or chemical difference of the individual natural product efficiency (Zhao *et al.,* 2011). Chromatography, especially column chromatography, is the main method used to obtain pure natural products from a

complex mixture (Popova *et al.,* 2009). Modern or greener extraction methods such as super critical fluid extraction (SFC), pressurized liquid extraction (PLE) and microwave assisted extraction (MAE), have also been applied in natural products extraction, and they offer some advantages such as lower organic solvent consumption, shorter extraction time and higher selectivity efficiency (Zhao *et al.,* 2011).

2.10.4 Metabolites structure elucidation

Structure elucidation of compounds can be achieved by the GC-MS, UV, IR and NMR (Popova *et al.,* 2009). All these methods can be used co-operatively to provide different information about the structure of the compound obtained, thus, helping to identify the compound. NMR provides information on the number and type of hydrogen and carbon atoms present (Napolitano *et al*., 2017). The molecular mass, molecular formula and fragmentation pattern is determined by the use of MS (Zhao *et al.,* 2011). Ultra-Violet radiation gives information about the chromophores present in the compound whereas IR helps to identify functional groups present in the compound being identified (Altemimi *et al*., 2017).

2.11 WHO policy on herbal medicines

WHO has recognized the important contribution of traditional medicine to provide essential care (WHO, http://www.who.int/topics/traditional_medicine/en/). Before proceeding to clinical studies, WHO guidelines state that if a product has been traditionally used without demonstrated harm, no specific restrictive regulatory action should be undertaken unless new evidence demands a revised risk-benefit assessment (WHO, 2004). Pre-clinical toxicity testing is only required for new medicinal herbal products which contain herbs with no established traditional history of use (WHO, 2004). Moreover, WHO recommends national policies that define the role of traditional medicines in national health care programs, ensuring that the necessary regulatory and legal mechanisms are established for promoting and maintaining good practice, assuring the authenticity, safety, and efficacy of traditional medicines and therapies (Wachtel-Galor and Benzie, 2011).

2.12 Herbal medicines of interest

Two plants, *Warburgia ugandensis* Sprague and *Zanthoxylum usambarense,* popularly used in African traditional medicine, were investigated in this study.

2.12.1 *Warburgia ugandensis*

Warburgia ugandensis Sprague, a canellaceae, is commonly referred to as East African greenheart; Kenya greenheart; pepperbark tree (English); Muthiga (Kikuyu); Soget (Kipsigis); Apacha (Luhya); sogo-maitha (Luo); Ol-sogunoi (Maasai); Sokoriori (Samburu district); Soket (Tugen), (Kokwaro, 2009; Dharani *et al.,* 2010; Wamalwa *et al*., 2006). It is a spreading evergreen tree (Plate 2.1), native to Africa, 4.5-30 m tall, 70 cm in diameter, with scaly pale brown bark (Plate 2.2). Early Indian immigrants to Kenya, working on the construction of the railway, used the leaves to flavour their curries before the chilli plant was commonly introduced Nanyingi *et al*., 2008. The flavour is fiercely hot and subtly different to chillies. Widely used medicinally, including as a treatment for Venereal Diseases by Maasai men, who tie a cylinder of bark around the requisite part of the body that troubled them Nanyingi *et al*., 2008. Medicinally, dried bark is commonly chewed and the juice swallowed as a remedy for stomachache, constipation, toothache, cough, fever, muscle pains, weak joints and general body pains (Nanyingi *et al*., 2008). Fresh roots are boiled and mixed with soup for the prevention of diarrhoea (Ngure *et al*., 2009). The inner bark is reddish, bitter and peppery and has a variety of applications (Wube *et al.* 2005). It provides treatment for the common cold; dried and ground to a snuff it is used to clear sinuses; and it is chewed, or smoke from the burning bark inhaled, as a remedy for chest complaints (Ngure *et al*., 2009). The bark, roots, leaves or fruits (Plate 2.3) can be boiled in water and the decoction drunk to treat malaria (Maroyi, 2014). Chemically, it contains sesquiterpenoids such as bemadienolide, cinnamide, drimenol, muzigadial, polygodial, ugandensolide, warburganal, warburgiadione, warburgin, ugandensidial and (Maroyi, 2014; Dharani *et al.,* 2010). These compounds exhibit anti-feedant activity against armyworms (Maroyi, 2014). In addition, they exhibit very potent antifungal, antiyeast and plant-growth regulating

activity (Kokwaro, 2009; Nanyingi *et al*., 2008; Ngure *et al*., 2009; Wamalwa *et al*., 2006; Maroyi, 2014).

Plate 2.1: ***Warburgia ugandensis*** **twig**

Plate 2.2: ***Warburgia ugandensis*** **harvested stem bark**

Plate 2.3: *Warburgia ugandensis* fruit

2.12.1.1 Phytochemical investigations of the genus *Warburgia*

The genus *Warburgia* Engl., a canellaceae consisting of 16 species (Maroyi, 2014); with *Warburgia ugandensis*, *W. elongata, W. salutaris* and *W. stuhlmannii* as the most extensively investigated (Maroyi, 2014). Phytochemical investigations have revealed high pharmaceutical value of *Warburgia* species, due to the abundance of drimane and colorotane sesquiterpenoides (Frum *et al*., 2005). Phytochemical investigations of *W. stuhlmannii* leaves showed the presence of mukaadial 6-*O*-β-D-glucopyranoside, mukaadial 6-*O*-α-L-rhamnopyranoside, a novel flavonol glycosides 3′,5′-*O*-dimethylmyricetin 3-*O*-β-D-2″,3″-diacetylglucopyranoside and 3′-*O*-methylquercetin 3-*O*-β-D-2″,3″,4″-triacetylglucopyranoside (Manguro *et al.,* 2003). Further, a number of drimane sesquiterpenes have been isolated from *W. salutaris* include: 11α-hydroxycinnamosmolide, isopolygodial, warburganal, Salutarisolide, (Mashimbye *et al.,* 1999). Phytochemical investigations of *W. ugandensis* showed the presence of ugandensolide, ugandensidial, warburgin and warburgiadione, mukaadial muzigadiolide, ugandensolide, muzigadial and waburganal (Wube *et al.* 2005).

2.12.2 *Zanthoxylum usambarense*

Known locally as Mugucuwa (Kikuyu), Sagawaita (Kipsisgis), *Zanthoxylum usambarense* belongs to family rutaceceae (Dharani *et al.,* 2010). A mixture made from its bark and roots is taken for relief from coughs; whereas a decoction made from its stem bark (Plate 2.4) is taken for the treatment of malaria and for relief from malarial fevers (Njoroge and Bussman, 2006). Both its roots and leaves (Plate 2.5) are taken for relief from severe colds and for pneumonia treatment (Ombito, 2021). More over, a decoction made from its bark is drunk for relief from rheumatism (Kokwaro, 2009). An infusion made from its fruits is mixed with milk and drunk for relief from fevers, sore throats, tonsilitis and chest pains. Both its bark and its root extracts exhibit fungicidal and insecticidal properties (Kokwaro, 2009).

Plate 2.4: ***Zanthoxylum usambarense*** **stem**

Plate 2.5: ***Zanthoxylum usambarense*** **leaves**

2.12.2.1 Phytochemical investigations of the genus *Zanthoxylum*

The genus *Zanthoxylum* (Rutaceae) is a rich source of structurally diverse secondary metabolites with promising pharmacological activities (Ombito, 2021). Approximately 126 new secondary metabolites, including alkaloids, amides, lignans and neolignans, coumarins, peptides, terpenoids, and flavonoids have been isolated and identified from different *Zanthoxylum* species (Phuyala, 2019). The numerous studies have shown that compounds isolated from the genus *Zanthoxylum* exhibit pharmacological activities, including anti-inflammatory, antimicrobial, cytotoxic, larvicidal, antioxidant, anticancer/ antiproliferative, analgesic and antimycobacterial activities (Ombito, 2021; Asante *et al*., 2019). Three new alkaloids zanthocadinanine, 7-methoxy-8-demethoxynitidine and zanthonitiside are obtained from genus *Zanthoxylum* which is used for the treatment of numerous ailments such as fever, gingivitis, and toothache (Alam *et al.,* 2020).

A Novel amide N-(4'-methoxyphenyl ethyl)-3,4- methylenedioxy cinnamoyl amide was isolated and characterized from *Z. armatum* and norchelerythrine, magnoflorine and (-) (S)-Omethylbalfourodinium cation from *Z. scandens* bark (Nguyen *et al.,* 2002). Also isolated from the genus *Zanthoxylum* are 6-(2',3'-dihydroxy-3'-

methylbutyl)-7-hydroxy-5-methoxy-2H-1-benzopyran-2-one,6- (2',3'-dihydroxy-3'-methyl-butyl)-7-methoxy-8-(3'-methylbut-2'-enyl)-2H-1-benzopyran-2-one, 6-(2',3'-dihydroxy-3'- methyl-butyl)-7-hydroxy-2H-1-benzopyran-2-one, 6-(2',3'- oxiranyl-3'-methyl-butyl)-7-methoxy-8-(3-methyl-but-2- enyl)-2H-1-benzopyran-2-one, 7-methoxy-8-(3'-methylbut-2'-enyl)-2H-1-benzopyran-2-one (Tao *et al.,* 2005).

In another study, Coumarins and alkaloids such as bergarpten, umbelliferone, skimmianine and schinifoline were isolated from *Z. nitidum (*Roxb.) DC (Qin *et al.,* 2019). These data show that the genus *Zanthoxylum* has great pharmacological potential and therefore presents the need for further exploration of the genus.

2.13 Scope of the study

This study covered six (6) broad areas that include: preparation of plant extracts; *in vitro* antiplasmodial activity of extracts from *W. ugandensis* and *Z. usambarense*; i*n vivo* antiplasmodial activity of the extracts; phytochemical groups present in active plants extracts; compounds in extracts with potent antiplasmodial activity and the safety of antiplasmodial phytomedicines in non-human primates.

CHAPTER THREE

MATERIALS AND METHODS

3.1 Chemicals for analysis

All solvents and chemicals used were of analytical grade (Sigma Aldrich). All experiments were performed under sterile conditions in a laminar flow. Re-usable glassware was sterilized by auto-claving, with 70% ethanol being used for general sterilization.

3.2 Selection of plant materials and study site

This study was carried out in the Malaria Laboratory, Institute of Primate Research (IPR), Karen, Kenya. The Institute of Primate Research is located within Oloolua Forest. The forest, at the foot of Ngon Hills, Kajiado County, is approximately 22km west of Nairobi. It is a tropical dry forest that is host to a wide range of fauna and flora. *Warburgia ugandensis* and *Z. usambarense* used in this study, were collected from this forest with a written consent from the IPR and authenticated at the herbarium, National Museums of Kenya, where voucher specimens were deposited.

3.3 Collection of plant materials

Various plant materials; *Warburgia ugandensis* stem bark (WUSB), *W. ugandensis* root bark (WURB), *W. ugandensis* leaves (WUL), *Zanthoxylum usambarense* stem bark (ZUSB) and *Z. usambarense* root bark ZURB) were harvested at their secondary stage of growth and used to prepare extracts. The two medicinal plants were selected on the basis of their popularity as herbal medicine against malaria and other ailments in communities where they grow naturally.

3.4 Study population and experimental design

The experimental design involved two hierarchical levels. An *in vitro* assay for all extracts against *P. knowlesi* and for those that gave IC_{50} values of lower than 5.5μg/ml, an *in vivo* assay against *P. berghei* and for safety against *P. knowlesi* in a

hierarchical randomized design. A total of two hundred eighty eighty (288) male adult BALB/c mice and six (6) adult male olive baboons were used resectively.

3.5 Preparation and extraction process

Four solvent systems with decreasing polarity; hot water, methanol, ethyl acetate and chloroform were used to prepare extracts from stem barks, root barks and leaves of *W. ugandensis* as well as stem and root barks of *Z. usambarense*. Prior to extraction, plant materials at their secondary stage of growth were harvested and washed to remove physical impurities; cut into small pieces, air-dried and pulverized. Two hundred grams of each of the ground powder were each exhaustively extracted in 1000 ml of each of the solvents for for 72 hours and filtered using whatmann filter paper no.1, giving a total of twenty (20) extracts. Methanol, ethyl acetate and chloroform were evaporated in vacuo using a rotor evaporator at reduced temperature of $40^{o}C$ (Appendix v), whereas aqueous extracts were lyophilized through freeze drying using Freeze Dryer (Modulyo Edwards Model), (Appendix vi). Extracts recovered were weighed, put in sterile vials, and stored at $4^{o}C$ until required for reconstitution. For testing, 0.2mg of each extract was reconstituted in Tween 80 under mild sonication and topped up with phosphate buffered saline (PBS).

3.6 Parasites models used in the study

The rodent malarial parasites, Chloroquin-sensitive *P. berghei*, Anka (PbA) were used to test the extracts for their antimalarial activities in BALB/c mice. *Plasmodium berghei* is a useful in vivo rodent model of malaria. Its lifecycle can be reproduced in the laboratory. Moreover, its successful adaptation to a range of rodent and mosquito species establishes it as a malaria model parasite for research (Joachim *et al.,* 2017). Preliminary *in vitro* bioassays were carried out using *P. knowlesi (H strain). Plasmodium knowlesi* infects human and primates and therefore presents a viable drug testing model due to close phylogenetic and pathophysiology between humans and non-human primates (Carlton *et al*., 2008). Non-human primates are highly valued in biomedical research because of the genetic similarity to humans, which means they can be especially useful for testing the safety of new drugs and studying infectious diseases (SCHER, 2009). Moreover, the primate parasite *P. knowlesi* is an

excellent model for *in vivo* studies of human malaria since, in many aspects, the genomes of *P. falciparum* and *P. knowlesi* are uniform, ranging from 23 to 27 Mb across 14 chromosomes, and comprising ~5,500 genes (Carlton *et al*., 2008).

3.7 Retrieval and culture of *P. knowlesi*

Plasmodium knowlesi malaria parasites were cultured and used to evaluate the *in vitro* activities of the plant extracts. Cryopreserved parasites were retrieved from liquid nitrogen using the Behring-Werk method (Kocken *et al.,* 2002). Briefly, an ampoule containing parasitized erythrocytes was collected from liquid nitrogen (Appendix viii) and thawed at 37°C after which the parasites were aseptically transferred into a labelled 50ml sterile centrifuge tube. An equal amount (300µl) of sterile solution of 3.5% NaCl was added dropwise while shaking the tube for proper mixing. The mixture was then spun down at 1200 RPM for 10 minutes and the supernatant was discarded. This step was repeated with half the original volume (150µl) of 3.5% NaCl solution after which the parasites were transferred to a starting erythrocyte packed cell volume (PCV) of 2.5%, 20% of PAN serum and 77.5% of RPMI 1640 medium in a sterile 25cm^2 tissue culture flask. The total volume of the culture in the tissue flask was 5ml. In order to avoid microbial contamination of the culture, 15µg/ml of gentamycin was added to the culture. The culture was then aerated with a gas mixture of 5% O_2, 5% CO_2 and 90% N_2, for 30 seconds before it was incubated at 37°C (Trager and Jansen, 1976; Butcher, 1979). Finally, the initial parasitaemia and subsequent development of parasites was monitored daily through microscopy with cultures being refreshed every 48 hours for two weeks (Kocken *et al*., 2002). Refreshment of the culture, which was preceded with checking of the culture conditions and level of parasitaemia, involved washing off the used culture medium by centrifugation at 1500 RPM (Sorvall RT 6000D) for 10 minutes and re-suspending the parasites in fresh culture medium. A normal PCV of 5% was maintained with fresh erythrocytes added within five days of culturing (Rowe *et al*., 1968).

3.8 Storage and retrieval of *P. berghei*

Approximately 0.8ml of infected blood was collected by cardiac puncture from a mouse with parasitemia of 1-10%. The blood was mixed with 1ml of a glycerol/PBS solution (30% glycerol v/v), containing 0.05ml of stock heparin solution and transferred to nunc cryovials, 0.5ml per vial. The cryovials were left for 5-15 minutes at 4 ^{o}C and gently transferred into liquid nitrogen tank until required for infection. To retrieve the parasites, an ampoule of parasite culture from liquid nitrogen was collected on dry ice quickly thawed at 37°C, and used to start an infection. The blood suspension of 0.5ml (500ul) was used to infect 2-10 mice interperitoneally.

3.9 Experimental animals and ethical appproval

Male adult BALB/c mice and male adult baboons were obtained from the Animal Resources Facility at the Institute of Primate Research (IPR), Nairobi, Kenya. Only male baboons and male BALB/c mice were selected to ensure no mating takes place during the experimental period, since multiple mice were housed in the same cage. The Institutional Review Committee (IRC) comprising animal care and use committee (ACUC), scientific review and the research ethics and integrity approved all protocols and use of animals in this study.

3.9.1 BALB/c mice

Two hundred eighty eighty (288) male adult BALB/c mice Sixty (60) adult male BALB/c mice (average weight = 20 ± 3g) bred in the animal house of the Animal Science Facility at the Institute of Primate Research (IPR) were used to determine semi-*in vivo* activities of the extracts. The PbA infected mice were kept in Standard Macrolon type II cages at room temperature and supplied with mice feed diet with *p*-aminobenzoic acid content of 45mg/kg, and drinking water *ad libitum.*

3.9.2 Olive baboons (*Papio anubis*)

A minimum number of six (6) male adult baboons (*Papio anubis*) weighing between 12 Kg and 23 Kg were acquired from the animal facility of Institute of Primate Research (IPR), Kenya, and housed in individual squeeze-back cages (dimensions

0.6×0.6×0.68 m) in the biocontainment facility according to institutional standards. The animals were fed daily on a standard non-human primate diet, water provided *ad libitum* and their general health monitored throughout the experimental period. Prior to use, the animals were determined to be free from simian immunodeficiency virus (SIV), haemoprotozoan and gastrointestinal parasites. In order to assess the progression of the disease, baboons were monitored for parasitaemia and clinical symptoms, such as fever, appetite, lethargy, and weight changes. Peripheral parasitaemia was determined daily by finger-prick method from day 4 post infection for a period of twenty-one (21) days. The olive baboons have genetic similarity to humans, which means they can be especially useful for testing the safety of new drugs and studying infectious diseases (SCHER, 2009).

3.10 *In vitro* activities of plant extracts against *P. knowlesi*

The antiplasmodial activities of twelve (8) extracts from *W. ugandensis* and eight (4) *Z. usambarense* were evaluated *in vitro* against *P. knowlesi* as described by Desjardins *et al.* (1979), with slight modifications. Briefly, 50µl of Dimethylsulfoxide (DMSO) was added to 5mg of each extract, which was then dissolved in RPMI 1640 culture medium under mild sonication and sterilized through a 0.45µm pore size membrane filter. The final concentration of DMSO was less than 0.1%, which is not inhibitory to the test parasites (Tona *et al.*, 2004). Using a micropipette, 100µl of Complete Culture Media (CCM) were dispensed into each well on a microtitre plate followed by 100µl of extract at a concentration of 100µg/ml. Four-fold serial dilutions (25µg/ml - 0.4µg/ml) were made down the plate using 100µl drawn from the first well. Finally, 10µl of parasitized erythrocytes at 1.6% parasitaemia were dispensed to each well, bringing the total volume per well to 110µl. Tests were performed in duplicate in 96-well microtitre plates. Extract-free controls were included to provide reference IC_{50} values. The concentration of each plant extract that inhibited 50% (IC_{50}) of parasite growth was calculated as a fraction of the initial parasitaemia relative to the growth in the control wells. Only extracts that displayed IC_{50} values of less than 6.0µg/ml were selected for *in vivo* antiplasmodial assays. Microscopic examination of Giemsa-stained thin smears from

each well was done after 30 hours of incubation to assess the extent of parasites growth inhibition.

3.10.1 Inoculums and infection of donor mice

The antimalarial activity tests were performed using the Four-day suppressive test (Peters, 1965; Okokon *et al*., 2005). The compounds were prepared at an appropriate concentration, as a suspension containing 70% Tween 80/ 3% ethanol. This solution was further diluted 10-fold with sterile PBS to result in a stock solution containing 7% Tween 80 and 3% ethanol then sterilized through a 0.45µm pore size membrane filter. This solution was stored at 4°C and used over a period of 3 weeks. An aliquot of 0.1 ml diluted blood containing 1x 10^7 *PBA* parasitized erythrocytes was injected intraperitoneally (i.p.) into donor mice and used to start infection.

3.10.2 Determination of parasitaemia levels in mice

Parasitaemia profiles in *P. berghei*-infected mice were monitored by microscopy. A drop of tail blood was collected on the frosted end of an appropriately labelled slide, which was then evenly spread towards the other end using another slide, held at an angle of 45°. The smears were air-dried and fixed using absolute methanol before staining with 10% Giemsa for 10 minutes (Jansen *et al.,* 2006). Excess stain was washed off by running a gently stream of tap water over the slide tilted at 45°. Air-dried slides were then observed under x100 of microscope (*Olyimpus,* Germany) (Appendix ix). A minimum of 2000 RBCs were counted using at least six fields and the number of infected erythrocytes was recorded and used to calculate the mean percentage parasitaemia as:

$$\%\,Parasitaemia = \left(\frac{Number\ of\ parasitized\ RBCs}{Total\ Number\ of\ RBCs}\right) \times 100$$

3.10.3 Propagation of *Plasmodium berghei* parasites

Experimental mice were infected through mechanical passage using blood stages from donor mouse previously infected with *PBA*. A drop of tail blood (5µl) from the

donor mouse at parasitaemia of 5-15% was collected in 10ml PBS and 0.2ml of the suspension injected *i.p.* into naïve mouse to begin an infection.

3.11 Antiplasmodial activity of selected extracts in BALB/c mice

Tests were performed using the four-day suppressive test (Okokon *et al.*, 2005) using *P. berghei* (ANKA) infected mice. Prior to inoculation, the extracts were dissolved in 0.2% tween 80 and diluted with PBS (pH = 7.3) to a final concentration of 25mg/ml, which was sterilized through a 0.45µm pore size membrane filter. A donor mouse infected with Chloroquin-sensitive *PbA* parasites with ascending parasitaemia was bled into sterile heparinized tubes, diluted with PBS and used to infect experimental mice. To determine the antiplasmodial effects of the three plant extracts on patent *PbA* infection, sixty (60) adult mice were each injected with 0.1ml of diluted blood containing $1x10^5$ *PbA* parasitized erythrocytes and treatment withheld for 72 hours to allow parasites to establish. On the third day of infection, tail blood from each mouse was used to make Giemsa-stained thin films that were examined under the microscope to confirm infection and parasitaemia. The mice were then randomized into five groups of 12 mice per cage. Each mouse in the first group received 0.2ml of the stem bark extract of *W. ugandensis* administered interperitoneally at a standard dose rate of 250mg/kg/day (Okokon *et al.*, 2005) for four consecutive days, with the negative and positive control groups receiving 0.2 ml sterile PBS and Chloroquine over a similar period of time. This procedure was repeated with the methanol extracts of root bark and leaf from the same plant with the remaining groups.

3.12 *In vivo* effects of selected extracts on *Papio anubis*

Cryopreserved *P. knowlesi* were retrieved as described and kept in an overnight culture before they were used to intravenously infect the baboons. Approximately $1.0x10^6$ parasites were used to infect each animal. As soon as infection was confirmed through daily smears, two crude extracts packaged as capsules, both aqueous and organic, were tested for activity and safety in olive baboons (*P. anubis).*

3.12.1 Antiplasmodial activity of selected extracts in olive baboons

A single standard oral dose of 5000mgkg/body weight of each extract was administered to three groups of as: infected and treated; infected and not treated and treated and not infected. Bleeding for heparinized blood samples and serum was done before infection, one-week post infection and on the 21st day post infection and treatment to determine parasitaemia, the Clinical Chemistry and heamatological changes over the experimental period of 21 days. Various tissues were harvested for inestigation of histopathological changes resulting from extacts treatment once the animals were euthanized.

3.12.2 Haematology of extract treated *P. knowlesi*-infected baboons

Venous blood was collected in heparin tubes from each of the experimental baboons at three different time points; before infection, one week after confirmed infection. Blood was analyzed in a Coulter counter (Beckman-Coulter, Mijdrecht, The Netherlands) to determine full counts of haemoglobin (Hb), red blood cells (RBC) and white blood cells (WBC) and other haematological changes over the experimental period.

3.12.3 Biochemistry of extract treated *P. knowlesi*-infected baboons

Serum was collected at three time points; before infection, one week after confirmed infection and two weeks post infection and used to analyze blood creatinine, Gamma-glutamyl transpeptidase; (GGT), Alanine Aminotransferase (ALT) and urea. Commercial kits for Creatinine (Biotech Laboratories, Ipswich, United Kingdom), and urea (Randox Laboratories, Antrim, United Kingdom) were used according to manufacturer's instructions.

3.12.4 Pathology of extracts treated *P. knowlesi*-infected baboons

Gross pathology and histopathological examination of tissue was done on postmortem by a resident veterinary pathologist. For histopathological assessment, several tissue organs that included liver, heart, kidneys and pancreas were harvested on necropsy and fixed in 10% neutral buffered formalin. The tissues were later

sectioned, stained with haematoxylin-eosin (H&E) and examined under the microscope (x10) for any morphological alterations

3.13 Analyses of active extracts from *W. ugandensis* and *Z. usambarense*

The methanolic and ethyl acetate extracts of *W. ugandenssis* as well as aqueous extracts of *Z. usambarense* exhibited high antiplasmodial activity and were therefore subjected to phytochemical analyses to isolate, identify and quantify the phytoactive metabolites responsible for observed activities. The functional groups, chemical families and molecular weights these phytochemicals were determined through Thin Layer Chromatography (TLC), Fourier Transform-Infrared (FT-IR), Gas Chromatography-Mass Sectrophotometry (GCMS) and Liquid Chromatography-Mass Spectrophotometry (LCMS).

3.13.1 TLC for extracts of *W. ugandensis* and *Z. usambarense*

The methanolic and ethyl acetate extracts of *W. ugandenssis* as well as aqueous extracts of *Z. usambarense* were subjected to thin layer column chromatography separate the various bioactive compounds based on difference in polarities. Toluene-diethyl amine in the ratio of 9:1 was used as the solvent system. Glass column wet packing method was used in which 120 grams of silica gel were mixed with methanol solvent to slurry. A padding of cotton was placed at the bottom of the glass column and slurry mixture allowed to settle at the bottom of column while gently tapping the column to allow proper packing and to get rid of air bubbles. Two (2) grams of each extract was placed on the column and a padding of cotton placed above it. The column was first eluted by a non-polar solvent so as to allow the compounds to adsorb to the stationary phase on TLC plate. The polarity of the solvent was increased slowly and progressively to desorb the compounds and allow elution with the mobile phase.

3.13.2 FT-IR analysis of MeOH and EtoAc extracts of *W. ugandensis*

Fourier transform infrared spectrometry (FT-IR) measures the vibrations of bonds within chemical functional groups and generates a spectrum that can be regarded as a

biochemical or metabolic fingerprint of the sample. Air-dried samples of methanolic and EtoAc extracts of *W. ugandensis* were separately analyzed for identification of characteristic functional groups using FT-IR spectrophotometer (Shimadzu 8400) at the Department of Chemistry, Jomo Kenyatta University of Agriculture and Technology. A small quantity (0.01mg) of each extract was dispersed in dry potassium bromide (KBr). The mixture was properly mixed in a mortar and pressed to form a KBr thin disc, placed in a sample cup of a diffuse reflectance accessory and scanned 5 times to increase the signal to noise ratio in a band width ranging from 4000 to 400cm^{1}. The percentage transmittance in the region of infra red was measured.

3.13.3 GCMS/LCMS for extracts of *W. ugandensis* and *Z. usambarense*

Gas chromatography-mass spectroscopy (GCMS) and Liguid Chromatography-mass spectroscopy (LCMS) in full mode were used to detect and identify phytochemical compounds in aqueous, methanolic and ethyl acetate extracts of *W. ugandense* and *Z. usambarense*. Extracts samples were analyzed by GCMS/LCMS on a 7890A gas Chromatograph (Agilent Technologies, Inc.,Santa Clara, CA,USA) linked to a 5975C mass selective detector (Agilent Technologies, Inc.,Santa Clara, CA, USA) by using the following conditions: inlet temperature 270°C, transfer line temperature of 280°C, and column oven temperature programmed from 35 to 285°C with the initial temperature maintained for 5 min then 10°C/min to 280°C held at the temperature for 10.5 min and finally 50°C/min to 285°C and held at this temperature for 29.9 min. The GC was fitted with a HP-5 MS low bleed capillary column (30m x 0.25 mm i.d., 0.25um) (J&W, Folsom, CA, USA). Helium at a flow rate of 1.25ml/min served as the carrier gas. The mass selective detector was maintained at an ion source temperature of 230°C and a quadruple temperature of 180°C. Electron Impact (EI) mass spectra were obtained at the acceleration energy of 70eV. A 1.0µl aliquot of each extract was injected in the split/splitless mode using an autosampler 7683 (Agilent Technologies, Inc., Beijing, China). Fragment ions were analyzed over 40-550 *m/z* mass range in full scan mode. The filament delay time was set at 3.3 min for underivatized samples and 8.6 min for derivatized samples. Various phytochemical compounds were identified on the basis of molecular mass and by comparing gas

chromatographic retention times and fragmentation pattern with that of the authentic standards. When there was lack of a corresponding reference standard, the structures were proposed on the basis of their general fragmentation and reference spectra published by library MS databases.

3.14 Determination of percentage parasite suppression

Parasitaemia suppression was determined microscopically by counting 4 fields of approximately 100 erythrocytes per field. For low parasitemias (<1%), up to 4000 erythrocytes had to be counted. Percentage parasitaemia suppression was established by counting the number of parasitized erythrocytes out of 1000 erythrocytes on random fields under the microscope. Infected cells were then expressed as percentage suppression relative to parasitemia level of the control group. The difference between the mean value of the negative control group (taken as 100%) and those of the experimental groups was calculated and expressed as percent reduction (activity) according to Tona *et al*., 2001 as follows:

$$Suppression = \left(\frac{Parasitaemia\ in\ control - Parasitaemia\ in\ test}{Parasitaemia\ in\ control}\right) \times 100\%$$

3.15 Statistical analyses

Data were managed and analyzed using GraphPad Prism Version 5.00 and reported as means ± SEM. Tukey's Multiple Comparison Test ($P < 0.05$) and Bartletts' Test for equal variances confirmed significant differences. A One-way ANOVA was used to compare antiplasmodial activity within plant parts from *W. ugandensis.* These were reported as means ± SEM, giving a *P-value* of 0.0021 and *F-value* of 5.595 at confidence level of 95%. Separation of means was achieved by Post-ANOVA Turkey test.

CHAPTER FOUR

RESULTS

The antiplasmodial activities, safety on animal tissues as well as phytoconstituents of extracts of *W. ugandensis* and *Z. usambarense* were analyzed and presented in this section.

4.1 Antiplasmodial activities of *W. ugandensis* and *Z. usambarense* extracts

The *in vitro* activities of hot water, methanol, chloroform and ethyl acetate stem bark and root bark extracts of *W. ugandensis* and *Z. usambarense* are presented in Table 4.1. The methanolic and ethyl acetate stem bark extractions of *W. ugandensis* (WUSB) were most effective, with IC_{50} values of 3.2µg/ml and 5.0µg/ml respectively, while only the aqueous extractions from *Z. usambarense* stem bark (ZUSB) and root bark (ZURB) were active with IC_{50} value of 5.1µg/ml and 5.8 µg/ml respectively.

Table 4.1: Activities of *W. ugandensis* and *Z. usambarense* extracts on *P. knowlesi*

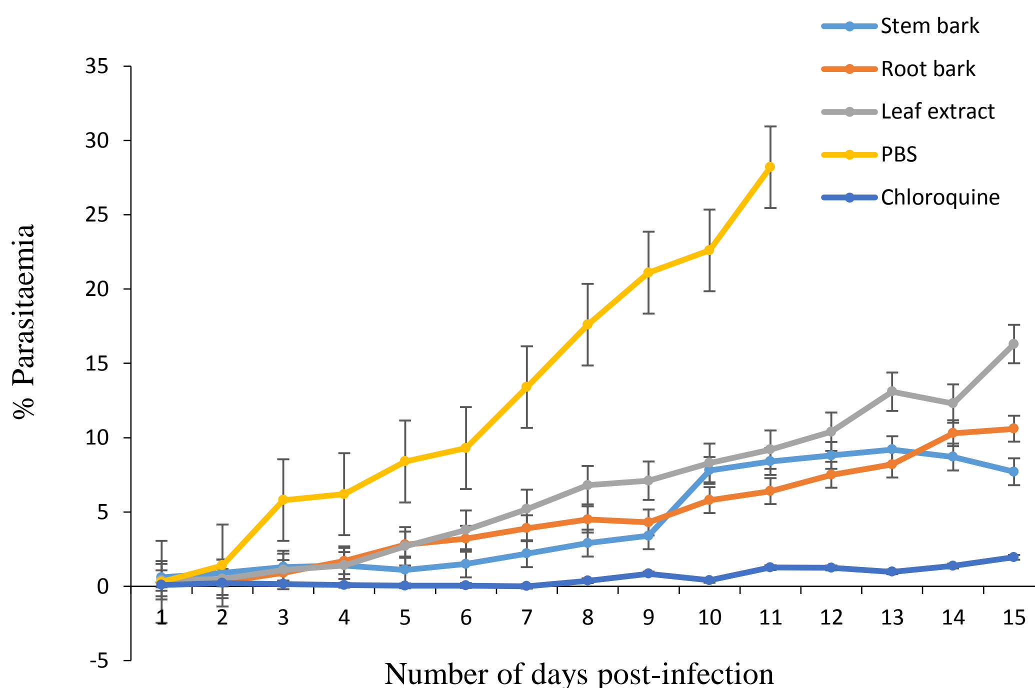
Plant extract	IC_{50} values (µg/ml)			
	Hot water	**Methanol**	**Chloroform**	**Ethyl acetate**
WUSB	**29.8**	**3.2**	**13.2**	**5.0**
WURB	**31.6**	**11.2**	**5.8**	**8.4**
ZUSB	**5.1**	**9.5**	**14.8**	**28.5**
ZURB	5.8	18.7	21.3	28.2

WUSB – *W. ugandensis* stem bark; WURB – *W. ugandensis* root bark; ZUSB – *Z. usambarense* stem bark; ZURB – *Z. usambarense* root bark. Data are expressed as means for five groups with replication (n = 6). F = 5.95, $P < 0.0021$, 99% CI

4.2 *In vitro* activities of extracts from *W. ugandensis* against *P. berghei*

The inhibitory activities of methanolic extracts from leaves, stem bark and root bark extracts of *W. ugandensis* against latent *P. berghei* infection in BALB/c mice are demonstrated in Figure 4.1. The stem bark extract displayed the highest inhibition of

64%, with root bark and leaf extracts at 61.4% and 46.7% respectively. Compared to the negative control (PBS), all extracts displayed higher survivorship and were significantly more efficacious.

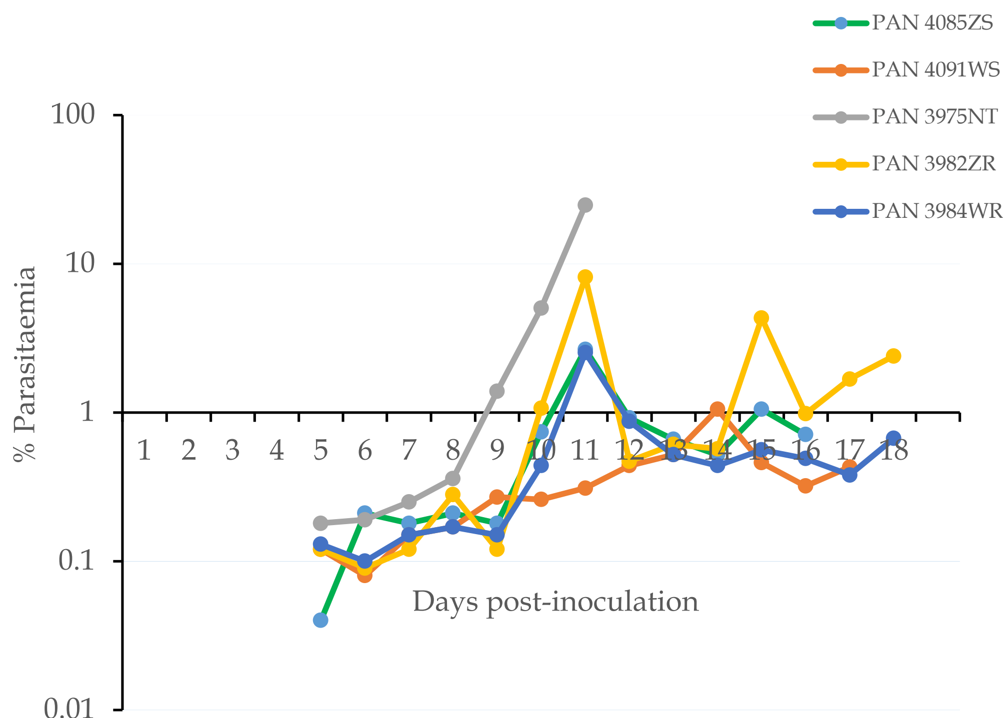


Extracts from stem bark, root back, and leaves inhibited 64%, 61.4% and 46.7% respectively. Data are expressed as mean ±SEM for five groups with replication (n = 6). Significance differences at $P < 0.05$, when compared with control (PBS).

Figure 4.1: Semi-*in vitro* activities of *W. ugandensis* against *P. berghei* infection.

4.3 Activity of MeOH extracts on *P. knowlesi*-infected *Papio anubis*

Parasiteamia profiles of *P. knowlesi* infection in olive baboons *(PAN)* treated with stem bark extracts of *W. ugandensis* (WUSB) and *Z. usambarense* (ZUSB) were determined (Figure 4.2). PAN 3975 (untreated control) died by 12th day post infection from high parasitaemia of 24%. Parasitaemia levels in treated animals remained significantly low until the 21st day post infection when they were euthanized.



PAN 4085 and PAN 4091- Infected and treated with extract from *W. ugandensis* stem bark; PAN 3984 - Infected and treated with extract from *W.* ugandensis root bark; PAN 3982-Infected and treated with extract with stem bark extract from *Z. usambarense*; PAN 3975 - infected, untreated control; PAN 3763* (no parasitaemia) - not infected, treated with extract from *W. ugandensis* stem bark.

Figure 4.2: Activity of MeOH extracts on *P. knowlesi*-infected baboons.

4.4 Safety of extracts in olive baboons (*Papio anubis)*

Heamatology, biochemistry, gross pathology and histopathological assessments were performed post treatment on experimental animals to establish the safety of the extracts on vertebrate tissues. The results are presented in this section.

4.4.1 Haematological profiles of plant extracts-treated baboons

Most haematological values fell within normal ranges except in a few, though not significantly varied. In PAN 3763 (treated and not infected control): A slight elevation of WBC then normalized in week 2 was observed (Table 4.2). Raised LYM and depressed GRA; PAN 3975: Depressed GRA, raised LYM, depressed RBCs count; PAN 3984: LYM raised, GRA depressed. Increased WBC count; PAN

3982: GRA depressed, LYM raised, HGB highly depressed; PAN 4085: GRA depressed, LYM raised. Further, WBC raised, HGB depressed.

Table 4.2: Hematological profiles for plant extracts-treated baboons

PAN	3763			3982			4091			4085			3975		3984			STD
Parameter	**NI-WST**			**I-ZST**			**I-WST**			**I-ZRT**			**I-NT**		**I-WRT**			**Range**
Time point	A	B	C	A	B	C	A	B	C	A	B	C	A	B	A	B	C	
WBC	6	13	6	9	7	9	8	8	7	11	7	14	10	7	8	10	15	4-10
GRA	57	3	5	68	5	4	63	39	6	50	5	12	57	5	64	2	5	40-70
LYM	31	93	90	19	88	91	26	50	88	40	88	80	34	89	26	94	88	20-45
HGB	14	12	11	13	12	4	15	16	7	15	12	4	12	11	17	15	6	12-18
RBC	5	8	5	5	5	1	5	6	3	6	5	1	4	5	6	6	2	4-6
MCHC	32	30	29	32	30	24	29	32	26	32	29	25	34	29	32	32	26	31-46
HCV	42	39	39	39	40	15	40	49	26	47	40	14	35	38	51	46	22	36-54

PAN 4085 and PAN 4091- Infected and treated with extract from *W. ugandensis* stem bark; PAN 3984- Infected and treated with extract from *W. ugandensis* root bark; PAN 3982-Infected and treated with extract with stem bark extract from *Z. usambarense*; PAN **3975** - infected, untreated control; PAN 3763- not infected, treated with extract from *W. ugandensis* stem bark. LYM - lymphocyte count, WBC - white blood cell count, GRA - granulocyte count, HGB - haemoglobin level, MCH- average weight of hemoglobin per red cell; MCV - mean corpuscular volume MCHC-average concentration of hemoglobin per erythrocyte. A, B and C are time points. Before treatment (A); 1 week after treatment (B) and two weeks after treatment (C).

4.4.2 Clinical Chemistry for plant extracts-treated baboons

Biochemical analysis involved measurement of creatinine, urea, Gamma-glutamyl transpeptidase (GGT) and Alanine Aminotransferase (ALT). No significant variation (Table 4.1) was noted when compared to standard references, confirming that, at concentration used, the extracts were safe on vertebrate tissues.

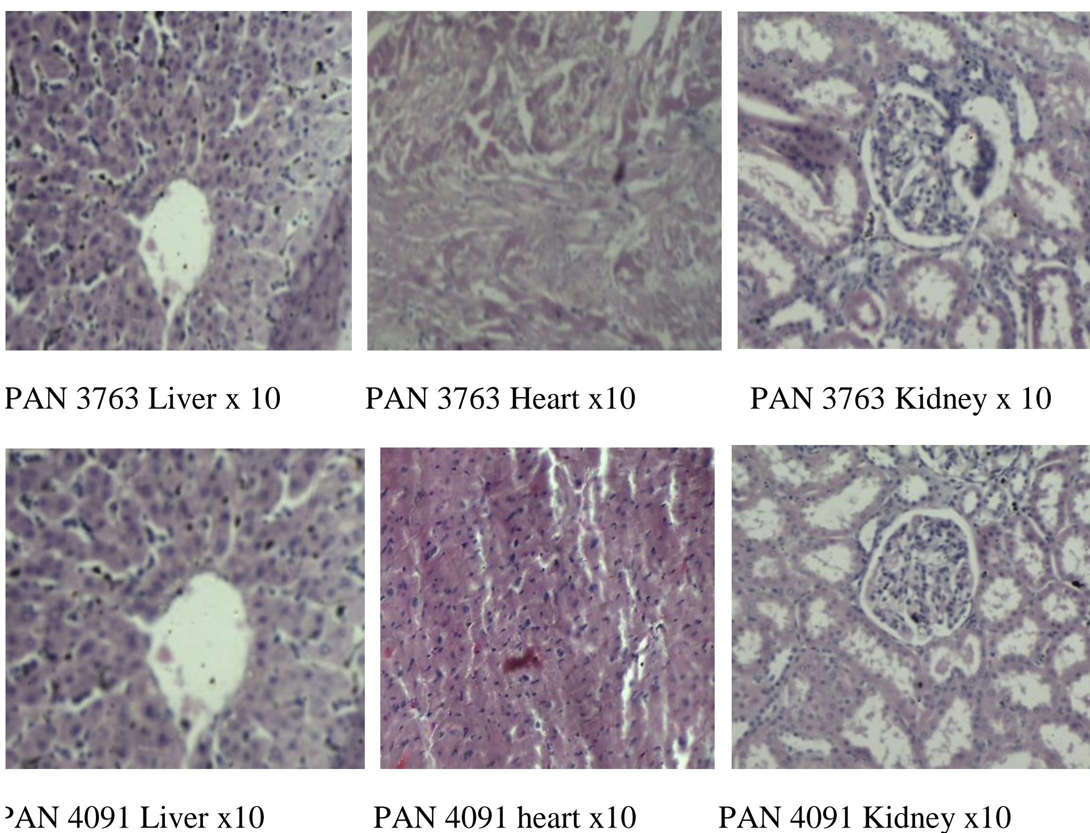
Table 4.3: Biochemical profiles for plant extracts-treated baboons

Factor	Range	PAN 3975		PAN 3963		PAN 3984		PAN 3982		PAN 4085		PAN 4091	
		A	B	A	B	A	B	A	B	A	B	A	B
Urea	**2.5-7.8 mmol/L**	6	0	6	6	5	7	5	5	9	4	7	4
Crtn	**53-124 mmol/L**	81	0	100	109	108	92	106	78	**46**	79	122	79
ALT	**7-56U/L**	**72**	0	**43**	12	34	63	50	**27**	46	**61**	18	55
GGT	**9-45U/L**	22	0	21	22	18	18	18	25	19	26	32	29

Crtn-Creatinine; GGT- Gamma-glutamyl transpeptidase; ALT - Alanine Aminotransferase, PAN- *P. anubis.* Urea, Creatinine, ALT and GGT appear to fall within normal range. A and B are time points before and after treatment.

4.4.3 Pathology and histopathology of extracts-treated *Papio anubis*

Examination of tissues from treated animals revealed normal tissue architecture (Figure 4.3). The liver tissues were of normal size though appeared darkened owing to raised haem metabolism. The heart and kidneys were normal, and no obvious lesions were seen in lungs. PAN 4091 and PAN 3763, both treated with stem back extracts of *W. ugandensis*, presented with normal organs: i.e no signs of cardiac atrophy, no signs of nephritis, no indications of haem metabolism, normal liver, pancreas and spleen sizes, non-engorged gall bladder and clear lungs. The heart musculature showed normal myofibrils with liver tissue presenting with normal hepatocellular architecture. Overally, no abnormalities were detected (NAD), implying that the extracts, at concentration used, had no adverse effect on the mammalian tissue.



PAN 4091 and PAN 3763 H&E staining. All the tissues, treated with W. ugandensis stem bark extracts present with normal tissue archetecture

Figure 4.3: Morphology of tissues from *W. ugandensis*-treated baboons.

4.5 Phytoconstituents in fractions of *W. ugandensis* and *Z. usambarense*

Fractions from *W. ugandensis* and *Z. usambarense* that exhibited high antiplasmodial values were subjected to various phytochemical analyses to identify and quantify the major phytochemical compounds responsible for observed activities. The functional groups, chemical families and molecular weights of major phytoactive metabolites present in extracts of *W. ugandensis* and *Z. usambarense* were determined through TLC, FT-IR, GC-MS and LC-MS. The results from the phytochemical analyses are presented in this section.

4.5.1 TLC for extracts of *W. ugandensis* and *Z. usambarense*

Chromatographic films revealed the presence the presence of a number of major phytochemical groups. All fractions contained phenolic compounds and terpenes. None of the fractions however contained tannins. Alkaloids too were present in all fractions apart from fraction F. Least abundant were the glycosides, being seen only in two fractions; D and F (Table 4.4).

Table 4.4: TLC for fractions of *W. ugandensis* and *Z. usambarense* extracts

Phytochemical	Fractions									
	A	B	C	D	E	F	G	H	I	J
Phenols	+	+	+	+	+	+	+	+	+	+
Terpenoids	+	+	+	+	+	+	+	+	−	−
Flavonoids	+	+	+	+	+	+	+	−	−	−
Steroids	+	+	+	+	+	+	+	+	−	−
Alkaloids	+	+	+	+	+	−	+	+	+	+
Tannins	−	−	−	−	−	−	−	−	−	−
Glycosides	−	−	−	+	−	+	−	−	−	−
Saponins	+	+	+	+	+	−	−	−	−	−
Anthraquinones	+	+	+	+	+	−	−	−	−	−
Terpenes	+	+	+	+	+	+	+	+	+	+

Phytoconstituents present in ethylacetate fractions from *W. ugandensis.* + present − absent

4.5.2 FT-IR analysis of MeOH and EtoAc extracts of *W. ugandensis*

The functional groups present in methanol stem bark extract from *W. ugandensis* as determined through Fourier Transform Infra-Red Spectroscopy (FT-IR) are presented in Table 4.5. From the wavelengths, alkaloids were shown to be the most abundant (50%) followed by terpenes (25%). Other groups present in small quantities were phenols and saponins. Unidentified bands; 3859.3, 3745.5, and 2366.5 were noticed.

Table 4.5: FT-IR peak values for MeOH extracts of *W. ugandensis*

λ (cm^{-1})	Bond type	Functional group	Phyto-constituents
3859.3*	-	-	-
3813.0	$-NH_2$	amines	Alkaloids
3745.5*	-	-	-
3342.4	O-H	Alcohols	Phenols
2931.6	C-H (s)		Terpenes
2366.5*	--	-	-
1724.2	C=O (s)	Unsat. esters	Saponins
1614.3	N-H (b)	Prim. amines	Alkaloids
1523.7	N-O as (s)	Nitro cpds	Alkaloids
1450.4	C-H (b)	alkanes	Terpenes
1336.6	C-N (s)	Arom. amines	Alkaloids
1205.4	C-H wag	Alkyl halides	
1091.6	C-N	Aliph. amines	Alkaloids
1031.8	C-N	Aliph. amines	Alkaloids
887.2	C-H 'loop'	aromatics	-
632.6	C≡C-:C-(b)	alkynes	Terpenes
518.8	C-Br (s)	Alkyl halides	Halides

λ – Wavelength; (s) - stretch; (b) - bend; * unknown

4.5.3 Detection of phenols and nitriles

Strong presence of phenols and nitriles was detected each in six fractions at 3458.1 to 3624.0 (A to G) and D to J (with exception of E) respectively, suggesting abundance of alkaloids (Table 4.6).

Table 4.6: FT-IR Peaks for *W. ugandensis* extracts at 4000-2250 cm-1

FRACTIONS											
A	**B**	**C**	**D**	**E**	**F**	**G**	**H**	**I**	**J**	**Bond type**	**Compound type**
3870.9	3822.7		3988.5	3872.8	3874.7	3861.2	3859.3	3857.4	3892.1	-	-
3801.4	3735.9		3845.8	3811.1	3824.6	3805.3	3813.0	3813.0	3805.9		
3737.8			3801.4 3743.4	3743.6	3739.7	3743.6	3747.4	3747.4	3749.7		
3458.1	3427.3	3404.1	3438.8	3423.4		3624.0				O-H s	Alcohols, **phenols**
	3107.1	3101.3				3560.3				O-H stretch	Carboxylic acids
3084.0					3427.3	3456.2	3434.1	3436.9	3407.1	C-H stretch	Aromatics
2923.9	2929.7	2925.8	2927.7	2925.8 2680.9 2549.7		3388.7				O-H stretch	Carboxylic acids
2864.1	2866.0	2860.2	2862.2	2858.3		3273.0				C-H stretch	Alkanes
						3226.7				-	-
2725	2734.9		2736.6			3151.5				H-C=O	Aldehydes
										C-H	
	2408.9 2283.6		2497.6	2441.7	2929.7	3111.0 2925.0	2927.7	2927.4	2929.7	-	-
			2252.7		2862.2	2858.3	2862.2	2862.2	2864.1	C=-N stretch	**Nitriles**

FT-IR peak values and functional groups for frequency range 4000-2250 cm^{-1} - (uknown)

4.5.4 Detection of aromatic compounds

Aromatic amines compounds were detected at 1334.6 to 1651.0 in six fractions (Table 4.7). Amines were present in three fractions; E, F, G at wavelengths 1614.9, 2260.2, 2206.4 N-H bend. Apart from Fraction E, all fractions displayed the presence of un identified groups at 1371.3 (A), 1371.3 (B), 1371.2 (C), 1371.2 (D), 2088.8 (F), 1730, 1610.5 (G), 1965.7, 1724.2 (H), 1924.8,1728.1,1596.9 (I), 2098.4, 1645.2 (J).

Table 4.7: FT-IR Peaks for *W. ugandensis* extracts at 2800 - 1150 cm-1

FRACTIONS											
A	B	C	D	E	F	G	H	I	J	Bond type	Compound
	2135.1		2206.4							-C=C- stretch	alkynes
			2050.2		2738.7	2675.1	2362.6	2376.1	2721.4	-	-
			2002.0		2543.9	2592.2					
1724.2	1731.6	1735.8	1735.8	1728.1	2497.6	2399.3		2327.9	2329.3	C-O s	sat.aliphatic Aldehydes
1676.0		1647.1	1651.0	1652.9	2447.5					-C=C- s	alkenes
				1614.9	2260.4	2206.4				N-H bend	1 amines
156	1560.3			1564.2						-	-
151	1515.9	1514.0	1515.9	1514.0		2125.4			2183.3	N-O asym	Nitro cpds
145	1452.3	1458.1	1458.1	1458.1						C-H bend	alkanes
1371.3	1371.3	1375.2	1373.2		2088.8	1730.0	1965.7	1924.8	2098.4	-	-
							1724.2	1728.1			
				1369.4		1689.5				C-H rock	alkanes
1334.6				1325.0	1651.0	1651.0	1651.0	1651.0		C-N s	**Aromatic amines**
						1610.5		1596.9	1645.2	-	-
1244.0	1242.1	1244.4	1242.1	1242.1						C-O	Esters ethers

FT-IR peak values and functional groups for frequency range of 2800 - 1150 cm^{-1}

4.5.5 Detection of amines

Both primary and secondary amines were detected in all fractions apart from (J) at wavelengths 896.8 to 1278.7. Aliphatic amines were also seen in all fractions apart from J at between 1022.2 and 1566.1 spectral frequencies (Table 4.8).

Table 4.8: FT-IR Peaks for *W. ugandensis* extracts at 1600 - 400 cm-1

FRACTIONS											
A	**B**	**C**	**D**	**E**	**F**	**G**	**H**	**I**	**J**	**Bond type**	**Compound**
1022.2	1164.1 1024.1	1170.7 1124.4 1022.2	1168.8 1120.6 1024.1	1116.7	1560.3 1515.9 1458.1	1558.4 1514.0 1458.1	1521.0 1454.2	1566.1 1462.5		C-N s	aliphatic amines
					1371.3			1380.9		-	-
			974.0						1355.9	=C-H bend	alkenes
896.8	906.5	900.2	900.7	894.9	1247.9	1265.2	1272.9	1278.7		N-H wag	1,2 amines
					1022.2	1078.1	1124.4 1076.2	1126.4 1078.1	1137.9	-	-
844.8 **802.3** **738.7**	839.0 694.3	837.0 767.6	796.5 756.0		840.9		898.8	894.9	997.1	C-Cl	Alkyl halides
							746.4	748.3	817.8	-	-
		698.2			642.3		617.2	648.0	624.9	-C=C C-H bend	alkynes
603.7	638.4	638.4	651.9	644.2	594.0 538.1	542.0			543.9	C-Br	Alkyl halides
545.8	592.1	596.0	594.0	596.0	416.6	416.6	422.2		410.8	-	**-**

FT-IR peak values and functional groups for frequency range of 1600- 400 cm^{-1} **:** -(unknown)

4.5.6 GCMS for bioactive metabolites from *W. ugandensis*

Gas Chromatography-Mass Spectrophotometry for retention times from 8.26 minutes to 11. 47 minutes' reveal predominantly the presence of terpenes (Table 4.9). The most abundant terpene recorded are benzene, 1, 3-dimethyl- (8.826) at a concentration of 1.7µg/mg and p-Xylene (9.32 min) at 0.97µg/mg respectively. P-Xylene also exhibited the highest quality at 97%. Other important terpenes detected in this range are alpha Pinene at 10.19 min and Eicosane at 11.47 minutes. 1-Butanol, 3-methyl-, acetate, a flavanoid with quality of 95% and concentration of 0.2µg/mg was detected at 9.03 minutes.

Table 4.9: Metabolites in *W. ugandensis* at retention time 8.3 to 11.5 minutes

RT	Library ID	Quality	Conc	Phytochemical
(min)			(µg/mg)	family
8.266	Butanoic acid, 3-methyl-	72	0.2	alkanol
8.624	Ethylbenzene	91	0.5	terpene
8.826	**Benzene, 1,3-dimethyl-**	**95**	**1.7**	terpene
9.028	1-Butanol, 3-methyl-, acetate	90	0.2	flavanoid
9.319	**p-Xylene**	**97**	**0.9**	**terpene**
10.013	Benzene, (1-methylethyl)-	93	0.2	terpene
10.192	**Pinene<alpha->**	**94**	**0.2**	terpene
10.483	3,3-Dimethyl-6-methylenecyclohexene	93	0.2	Terpene
10.775	1,3-Cyclopentadiene,1,2,5,5-tetramethyl-	87	0.3	Terpene
10.887	Benzene, 1,2,3-trimethyl-	87	0.2	Terpene
11.312	Cyclobutane, 1-butyl-2-ethyl-	89	0.6	Terpene
11.469	**Eicosane**	**64**	**0.3**	Terpene

Gas Chromatography-Mass Spectrophotometry for retention times from 8.26 minutes to 11. 47 minutes showing predominantly the presence of terpenes in *W. ugandensis* non-polar extracts

Table 4.10 shows GCMS for retention times of 11.72 to 16.24 minutes. Naphthalene, 1,2,3,4-tetrahydro-1,5,7-trimethyl, a terpene, is the most abundant, at a concentration of 4.0µg/mg and Limonene (12.03 min) at 0.4µg/mg, which exhibited the highest quality at 98%. Benzothiazole, the only non-terpenoid phytoconstituent in this time range, is an important alkaloid that was detected at 15.28 min and has a concentration of 0.32µg/mg, with quality of 93%.

Table 4.10: Metabolites in *W. ugandensis* at retention time of 11.7 to 16.2 minutes

RT (min)	Library ID	Quality	Concn (µg/mg)	Phytochemical Family
11.715	Benzene, 1,3-dichloro-	97	0.2	Terpene
11.805	1,3-Cyclohexadiene,1-methyl-4-(1-methylethyl)-	94	0.2	Terpene
11.939	Cymene<ortho->	94	0.2	Terpene
12.029	Limonene	98	0.4	Cyclic terpene
12.342	1,3,6-Octatriene, 3,7-dimethyl-,(E)	64	0.2	Terpene
12.880	Benzene, 1-ethyl-3,5-dimethyl-	87	0.3	Terpene
13.529	Benzene, 1,2,3,5-tetramethyl-	91	0.3	Terpene
14.672	Dodecene<1->	96	0.6	Terpene
15.008	Octane, 3,5-dimethyl-	80	0.3	Terpene
15.276	Benzothiazole	93	0.3	alkaloidal
15.971	Heptadecane, 2-methyl-	78	0.3	Terpene
16.240	Naphthalene,1,2,3,4-tetrahydro-1,5,7-trimethyl-	91	4.0	Terpene

GC-MS identification and quantification of phytochemicals in *W. ugandensis* for retention time 11.7-16.24 min

Table 4.11 gives the phytochemicals eluted within the retention time of 16.53 to 19.31 minutes. Delta amorphene and Caryophyllene were detected with 99% quality as well as higher quantities of 1.2 and 1.5µg/mg respectively. The highest quantity observed was that of Pentadecane, at 1.8µg/mg. Vanillin, at 91%, is a phenolic aldehyde detected at 17.72 minutes.

Table 4.11: Metabolites in *W. ugandensis* at retention time 16.5-19.3 minutes

RT (min)	Library ID	Quality	Concn (µg/mg)	Phytochemical family
16.531	2-Methoxy-4-vinylphenol	87	0.5	Phenol
17.001	Benzene, 1,3,5-trimethyl-2-(1-methylethenyl)-	94	0.6	-
17.315	1,3-Cyclohexadiene, 2,6,6-	78	0.4	-
17.718	Vanillin	91	0.5	Phenolic aldehyde
18.054	Caryophyllene	99	1.5	terpenoids
18.345	Phenol-2-methoxy-4-(1-propenyl)	95	1.2	-
18.502	a-Humulene (a-Caryophyllene)	93	1.4	terpenoids
19.308	Amorphene<delta->	99	1.2	Sesquiterpene

GC-MS identification and quantification of phytochemicals in *W. ugandensis* for retention time 16.5-19.3mins

Table 4.12 shows GC-MS ranges in the retention time between 19.71 min and 26.74 minutes. Flavanoids are predominantly present and in abundance. Ugandensidial, an alkaloid, Drimenin, muzigadial and nerolidol are in abundance. Also detected in abundance at 26.74 min is a cuomarine, 4, 4, 5, 8-tetramethyl-chroman-2-one. The most abundant terpene is gamma Patchoulene, with a concentration of 36.5µg/mg, at quality of 90%, eluted after 24.82 minutes. Cyclocolorenone, with relative abundance of 13.9 µg/mg and the highest quality of 98%; and heptanal, 2-(phenylmethylene quality of 60% and relative abundance of 38.8 µg/mg, both flavonoids, were recorded at 22.13 min and 24.01 min respectively.

Table 4.12: Metabolites in *W. ugandensis* at retention time of 19.7 to 26.7 minutes

RT (min)	Library ID	Quality	Concn (µg/mg)	Phytochemical family
19.711	Nerolidol<Z->	94	6.1	alkanol
20.629	1,2,3,3a,4a,5,6,7,8,9,9a,9b-Dodecahydrocyclopentaphenanthrene	68	4.8	terpene
20.853	1(2H)Phenanthrenone, 3,4,4a,9,10,10a-hexahydro-4a-methyl-	90	5.1	flavonoid
22.130	Cyclocolorenone<epi->	98	13.9	flavonoid
23.272	1,1,4a-Trimethyl-5,6-dimethylenedecahydronaphthalene	72	7.1	terpene
24.011	Heptanal, 2-(phenylmethylene)-	60	38.8	flavonoid
24.258	1,4-Naphthalenedione, 2-hydroxy-3-(1-propenyl)-	87	17.9	flavonoid
24.818	Patchoulene<gamma->	90	36.5	terpene
25.131	Canellal (Muzigadial)	91	30.5	flavonoid
25.714	Drimenin	93	35.3	flavonoid
26.094	1,4-Naphthoquinone, 6-acetyl-2,5,7-trihydroxy-	89	14.0	flavonoid
26.497	Ugandensidial	91	20.7	alkaloid
26.744	4,4,5,8-Tetramethyl-chroman-2-one	64	21.8	coumarins

GC-MS identification and quantification of phytochemicals in *W. ugandensis* for retention time 19.7-26.7 mins

The GC-MS retention times between 26.86 min and 42.02min revealed predominantly the presence of terpenes, alkaloids and flavonoids (Table 4.13). The most abundant phytoconstituent being 2-ethyl-phenol at 89.1µg/mg, with quality of 64% and a retention time of 28.87 minutes. Notable quality was observed at retention times 29.25 min (Bumetrizole, 3.3 µg/mg); 35.05min (1-docosene, 5.2 µg/mg); and 36.89 min (Triacontylacetate, 1.6µg/mg), all at 99%.

Table 4.13: Metabolites in *W. ugandensis* at retention time 26.9 - 42.0 minutes

RT (min)	Library ID	Quality	Concn (µg/mg)	Phytochemical family
26.856	4,4,5',5'-Tetramethyl-bicyclohexyl-6-ene-2,3'-dione	64	9.8	flavonoid
28.872	Phenol, 2-ethyl-	64	89.1	Flavonoid
29.252	Bumetrizole	99	3.3	Alkaloid
30.238	1-Bromobenzene,4-(4-bromobenzylideneamino)-	78	5.0	alkaloid
30.484	Octacosane	92	6.5	terpene
31.447	Squalene	91	6.1	terpene
35.053	1-Docosene	99	5.2	Terpene
35.747	dl-.alpha.-Tocopherol	95	3.3	Ketone
36.89	Triacontyl acetate	99	1.6	flavonoid
38.01	Stigmasterol	97	5.5	Phenol
39.443	Stigmast-7-en-3-ol, (3.beta.,5.alpha.,24S)-	86	2.4	alkaloid
40.988	9, 19-Cyclolanost-24-en-3-ol, (3.beta.)-	97	2.0	alkaloid
42.019	Ethanone, 2-(1H-imidazo[4,5-b]pyridin-2-yl)-1-(4-morpholyl)-	94	5.1	alkaloid

GC-MS identification and quantification of phytochemicals in *W. ugandensis* for retention time 26.8-42.0 mins

4.5.7 LCMS for polar extracts of *W. ugandensis* and *Z. usambarense*

The LCMS was used to detect and identify, on the basis of molecular weights and standard retention times, polar moieties present in aqueous extracts of *Z. usambarense.* revealed predominantly the presence of glycosides and flavonoids. Robinetinidol-(4alpha->8)-catechin-(6->4alpha)-robinetinidol, a polyphenol, and mesembrinol, an alkaloid, were also present. The LCMS for hot water and methanol extracts of *W. ugandensis* revealed predominantly the presence of glycosides and alkaloids. Glycosides included; (+)-Catechin 3-O-glucose, Cyanidin 3-(6''-p-coumaryl-2'''-sinapylsambubioside)-5-(6-malonylglucoside) and Urdamycin F. Two novel alkaloids; cancentrine and buxaminol were detected. Syringaresinol, a furanoid lignin, was also present.

Table 4.14: LCMS for polar extracts of *W. ugandensis*

RT (min)	Molecular mass	Library ID	Class
0.248	453.787	(+)-Catechin 3-O-glucose	Glycoside
3.336	419.085	Syringaresinol	Furanoid
5.248	1203.259	Cyanidin3-(6''-p-coumaryl-2'''-sinapylsambubioside)-5-(6-malonylglucoside)	Glycoside
5.444	607.238	Cancentrine	alkaloid
5.848	863.350	Urdamycin F	glycoside
5.980	445.185	Buxaminol	alkaloid

RT-Retention Time; Two glycosides, a furanoid lignans and an alkaloid were identified on the basis of molecular weight and standard retention times.

Table 4.15 gives the LCMS for hot water extracts of *Z. usambarense*. The water soluble flavonoids and glycosides are predominantly present. Robinetinidol-(4alpha->8)-catechin-(6->4alpha)-, also detected were mesembrinol, an alkaloid and occidentoside, a glycoside.

Table 4.15: LCMS for polar extracts of *Z. usambarense*

RT	Molecular mass	ID	Class
0.227	453.7863	(+)-Catechin 3-O-glucose	glycoside
3.181	705.1854	6-Hydroxyluteolin 5,6,3',4'-tetramethyl eter 7-cellobioside	Flavonoid
4.928	867.2128	Robinetinidol-(4alpha->8)-catechin-(6->4alpha)-	polyphenol
5.216	272.1285	2-Acetyl-4,6-dimethoxyfuro[2,3-b]quinoline-2-Acetylpteleine	glycoside
5.319	727.1509	Occidentoside	glycoside
5.484	344.1863	3-Hydroxyestra-1,3,5(10)-trien-17-one-O-(carboxymethyl)oxime	
5.659	314.1764	Mesembrinol	Alkaloid
5.979	455.1835	7,8,3',4',5'-Pentamethoxy-6'',6''-dimethylpyrano[2'',3'':5,6]flavones	flavonoid
8.067	481.183	Myricetin 3-O-glucoside	Flavonoid

RT-Retention Time. Glcosides, alkaloid, flavonoids and a polyphenol were detected and identified on the basis of molecular weights and standard retention times.

4.5.8 Selected moieties from *W. ugandensis* and *Z. usambarense*

This section presents some selected phytoconstituents with confirmed antimicrobial and antiplasmodial activities detected in extracts of *W. ugandensis* and *Z. usambarense.* These phytochemical compounds were identified by comparing the gas chromatographic retention times and fragmentation pattern with that of the authentic standards. When there was lack of a corresponding reference compounds, the structures were proposed on the basis of their general fragmentation and reference spectra published by library MS databases.

4.5.8.1 Benzothiazole from *W. ugandensis*

Benzothiazole. an alkaloid with confirmed antiplasmodial activity (Sadhavisan *et al., 2016*) was present in MeOH extraction of *W. ugandensis*. Benzothiazole, a fused heterocyclic moiety, is a valuable scaffold that possesses diverse biological activities, such as anticancer, anti-inflammatory, antimicrobial, antiviral, antimalarial, and anticonvulsant effects **https://doi.org/10.1002/ardp.201800170.**

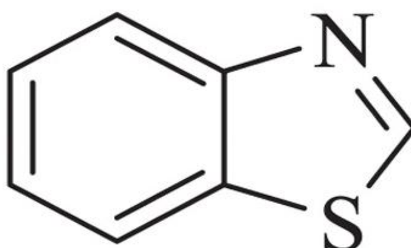


Figure 4.4: Structure of Benzothiazole

4.5.8.2 Bumetrizole from *W. ugandensis*

Bumetrizole is an alkaloid with confirmed antiplasmodial activity (Newness, 2013) was present in MeOH extraction of *W. ugandensis*.

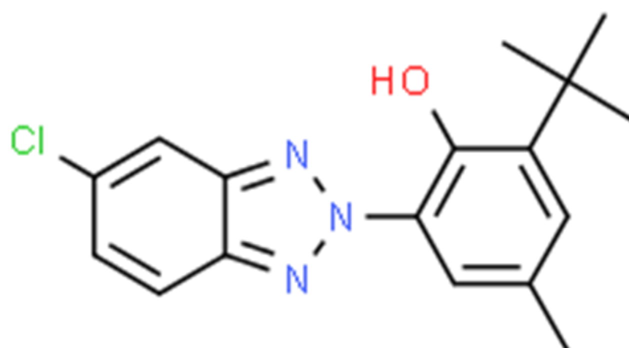


Figure 4.5: Structure of Bumetrizole

4.5.8.3 Drimenin from *W. ugandensis*

Drimenin is a flavanoid with confirmed antiparasitic, antimicrobial and antifungal activities (Newness, 2013). It is present in MeOH extraction of *W. ugandensis.*

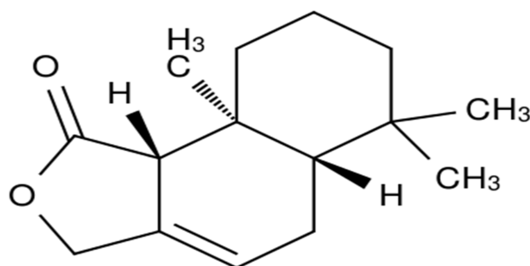


Figure 4.6: Structure of Drimenin

4.5.8.4 Caryophyllene from *W. ugandensis*

Caryophyllene is a terpene present in MeOH of *W. ugandensis* with confirmed antiplasmodial activity (Sadhavisan *et al*., 2016).

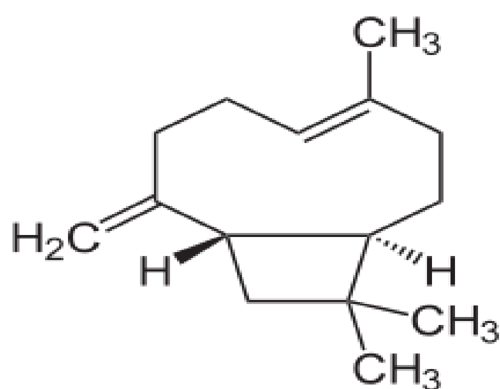


Figure 4.7: Structure of Caryophyllene

4.5.8.5 *(-)*-syringaresinol from *W. ugandensis and Z. usambarense*

(-)-syringaresinol is a lignin furan that isolated from both MeOH and aqueous of extracts from *W. ugandensis* and *Z. usambarense.* It has confirmed antiplasmodial activity and has the ability to inhibit *Helicobacter pylori* motility (Sadhavisan *et al.,* 2016; Newness, 2013).

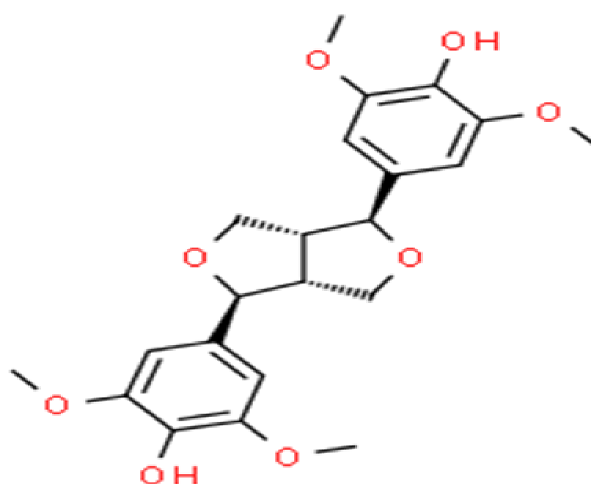


Figure 4.8: Structure of (-)-syringaresinol

4.5.8.6 Buxaminol from *W. ugandensis* and *Z. usambarense*

Buxaminol is an alkaloid isolated in an aqueous extraction of both W. *ugandensis* and *Z. usambarense.* It has antiplasmodial activity (Sadhavisan *et al.,* 2016).

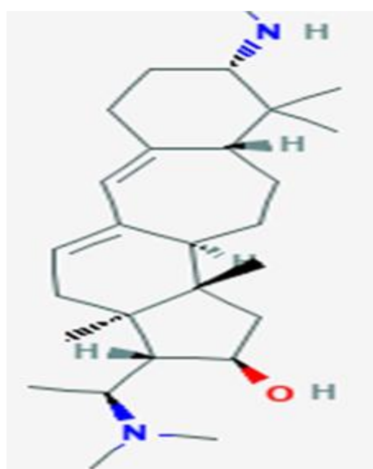


Figure 4.9: Structure of Buxaminol

4.5.8.7 Canellal from *W. ugandensis*

A flavanoid extract in MeOH of *W. ugandensis* with confirmed antiplasmodial activity *(Newness, 2013).*

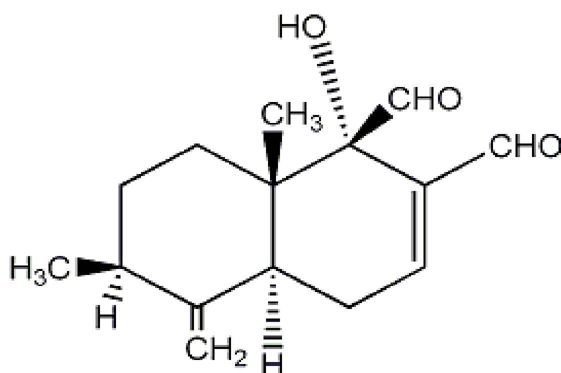


Figure 4.10: Structure of Muzigadial/Canellal

4.5.8.8 Stigmasterol from *W. ugandensis and Z. usambarense*

Stigmasterol is a phytosterol present in MeOH of both *W. ugandensis* and *Z. usambarense*. It has confirmed antimicrobial activity (Sadhavisan *et al.,* 2016).

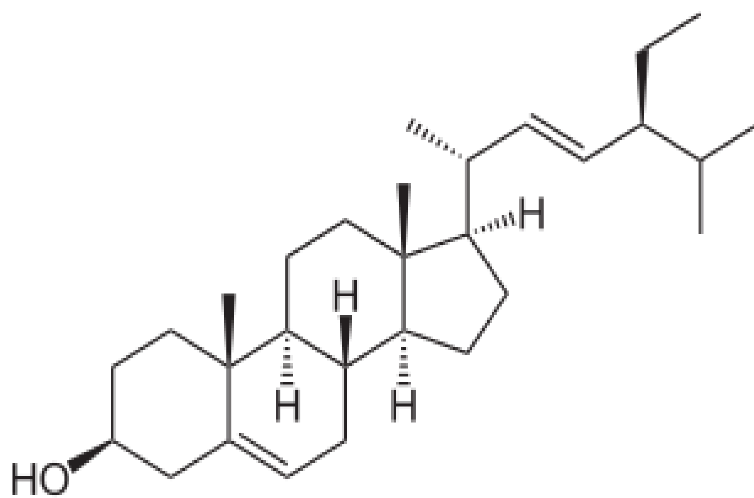


Figure 4.11: Structure of Stigmasterol

CHAPTER FIVE

DISCUSSION

5.1 Antiplasmodial activities of *W. ugandensis* and *Z. usambarense*

Table 4.1 shows IC_{50} values that reveal a high potential for the development of phytomedicines. The methanol and ethyl acetate extracts from the stem bark of *W. ugandensis* (WUSB) were most effective, with IC_{50} values of 3.2µg/ml and 5.0µg/ml respectively. Compared among the plant parts, the stem bark from *W. ugandensis* was more effective compared to root bark (WURB). These results compare well with the findings of Githinji *et al.,* 2009, where a concentration of 1.1444 mg/ml of *W. ugandensis* crude extract killed 50 % of *Leishmania* promastigotes *in vitro.* *Leishmania,* like *Plasmodia,* is a haematoprotozoan. Indeed, *W. ugandensis* is a medicinal plant whose bark has been used for ages as treatment of respiratory tract infections, malaria and catarrhal fevers (Nanyingi *et al.,* 2008). In a related study, *W. salutaris*, a plant in the same genus, displayed trypanocidal activity with IC_{50} value of 6. 59µg/ml (Kuglerova, 2011).

Analysis of *Z. usambarense* showed that the aqueous extractions from stem and roots were more effective at 5.1µg/ml and 5.8µg/ml respectively. This observation agrees with screening of root bark extract from the same genus, *Z. chalybeum* on CQ-resistant *P. falciparum* K1 strain, which gave an IC_{50} value of 4.2 µg/ml (Gessler *et al.,* 1994; Gessler *et al.,* 1995). Moreover, Rukunga *et al.,* (2009), established, in a related study that *Z. chalybeum,* a rutaceae, gave an IC_{50} for aqueous extracts as 6.0 µg/ml, while the methanolic extract produced 4.2µg/ml and 1.7µg/ml g/ml respectively against the 3D7 (CQ-resistant) and W2 (CQ-susceptible) strains of *P. falciparum*. In both cases, the present study findings are consistent with results established in an earlier study (Were *et al*., 2010), and another study**,** Kirira *et al.,* (2006) evaluated the activity of the aqueous, chloroform and methanol extracts from *Z. usambarense* on *P. falciparum* and demonstrated the IC_{50} values of 6.04, 3.14 and 6.12 µg/ml respectively and the IC_{50} value for the aqueous extract from the same plant fell between 6 and 15 µg/mL against both CQ-sensitive and resistant *P. falciparum* strains, while that of methanolic extract was found to be lower than

6μg/mL (Lima *et al.*, 2015; Satish and Sunita, 2017). On the basis of these observations, three extracts: methanol and ethyl acetate extracts from stem bark of *W. ugandensis*, as well as hot water stem bark extract (ZUSB) from *Z. usambarense* were selected for further analyses.

In vivo assays with extracts from *W. ugandensis* on patent *P. berghei* (ANKA) infection in BALB/c mice gave a significant reduction in parasitaemia. The most efficacious plant part was the stem bark which displayed almost four-fold parasitaemia suppression corresponding to 64% activity. The lowest activity, observed in extracts from the leaf was equally significant, with 47% activity, while the root bark yielded 62% suppression (Were *et al.*, 2015). The stem bark extract from this plant had earlier been shown to exhibit a significant *in vitro* and *in vivo* antiplasmodial activities against *P. knowlesi* (IC_{50} = 3.14μg/ml and 6.12μg/ml) and PBA parasite suppression of 41% to 69% at dose rate of 200mg/kg/day (Were *et al.*, 2010a). The present study is consistent with earlier observation and further indicates that other parts of the plant (leaves and root bark) are equally efficacious and could be used in place of the stem bark (Figure 4.1). The study findings further confirm the ethno medicinal knowledge about the effectiveness of *W. ugandensis* against malaria. No significant difference in antiplasmodial activity among the various plant parts was displayed (Appendices xi and xii). The stem bark and root bark extracts (q = 0.2498) depicted nearly same level of activity. Similarly, the difference in activity when compared to leaf extracts was statistically insignificant (q = 1.488). It was however significantly different from the negative control, with q = 5.583. Compared between root bark and leaf extracts, no significant difference was observed, with q = 1.738. The root bark and leaf extracts however displayed significant difference from the negative control, with q = 5.813 and q = 4.214 respectively. Compared to CQ, whose activity over the same period was recorded at 95% suppression, the activity exhibited by the extracts from leaves was significantly low, with a mean difference of 5.96. Interestingly, there was no significant difference observed with regards to stem and root barks extracts. Overally, WUSB and ZUSB maintained parasitaemia levels below 10% over the entire experimental period and increased mice survivorship until they were euthanized (Figure 4.2).

5.2 Antiplasmodial metabolites from *W. ugandensis* and *Z. usambarense*

Fractions that exhibited high antiplasmodial values during bioassays were subjected to various phytochemical analyses to identify and quantify the major phytochemical compounds responsible for activities. The functional groups, chemical families and molecular weights were determined through Thin Layer Chromatography (TLC), Fourier Transform Infra-Red (FT-IR), integrated Gas-Chromatography-Mass spectrophotometer (GC-MS) and Liquid Gas-Mass Spectrophotometer (LC-MS). The results are discussed in this section.

5.2.1 Phytochemical groups from EtoAc extract of *W. ugandensis*

The Chromatographic films from ethyl acetate extract from *W. ugandensis* revealed the presence of a number of major phytochemical groups. All fractions contained phenolic compounds and terpenes (Table 4.4). None of the fractions however contained tannins. Alkaloids too were present in all fractions apart from fraction F. Least abundant were the glycosides, being seen only in two fractions; D and F. The observed antiplasmodial activity observed in the extract could therefore be attributed to mainly the phenolic and terpenoids groups present in the extract. Fraction D contains most of the phytochemical groups; at 89%, while I and J have the least, at 33% each. The most abundant photochemical compounds are the phenols and terpenes, being present in all the 10 fractions. Alkaloids too are abundantly present at 90%, missing in only one fraction; F. The glycosides are least abundant, being present only in fractions D and F, at 20%. This observation implies that antiplasmodial activity observed in the extract, including the arrest of *Plasmodium knowlesi*-infected olive baboons could therefore be attributed to mainly the phenolic, terpenoids and alkaloidal groups present in the extract.

5.2.2 Phytochemical groups from MeOH extract of *W. ugandensis*

The Fourier transform infrared (FT-IR) spectroscopy of methanolic extract of *W. ugandensis* revealed the presence of a number of phytochemicals (Tables 4.5- 4.8). The peaks showed the presence of unsaturated esters, alkanes, aromatic amines, asymmetric nitro compounds, aliphatic amines, alkynes and alkyl halides.

Specifically, the FT-IR revealed C=O stretching for unsaturated esters (1724.2cm^{-1}), N-H stretch for primary and secondary amines (1614.3 cm^{-1} and 3342.2 cm^{-1}), C-H stretching for alkanes (2931.6, 1450.433 cm^{-1}), N-O asymmetric stretching for nitro compounds (1523.7), C-N stretch for aromatic amines (1336.6cm^{-1}), C-N stretch for aliphatic amines (1091.6 and 1031.8 cm^{-1}), C-H ''loop'' at 887.2 cm^{-1} for aromatics, -C≡C-H: C-C-H bending for alkynes, C-H wagging for alkyl halides (1205.4 cm^{-1}) and C-Br stretching for alkyl halides. Clearly, *W. ugandensis* predominantly contains alkaloids, as indicated by strong peaks at 1614.3 cm^{-1}, 1336.6 cm^{-1}, 1091.6 cm^{-1}, and 1031.8 cm^{-1}. The presence of terpenes was indicated at 2931.6 cm^{-1} and 1450.1 cm^{-1}, while C=O pointed at the presence of saponins. Phenols were also present in high concentration as revealed by the frequency 3342.4 cm^{-1}. In terms of phytocomponents, the extract is a rich source of phytochemical compounds with the presence of phenols, alkaloids, saponins and terpenes as depicted by FT-IR spectral peaks.

Strong and broad absorption band of hydroxyl occurred at 3431.1, 3458.1, 3427.3, 3404.1, 3438.8, 3423.4, 3624.0, 3427.3, 3456.2, 3436.9 and 3407.1cm^{-1}, indicating the presence of O-H stretching in all fractions, depicting the presence of alcohols and phenols. The FT-IR Peaks obtained at 3107.1, 3101.3, 2931.6, 2923.9, 2929.7, 2925.8, 2925.8, 2680.9, 2549.7, 3273.0, 3226.7, 3151.5, 3111.0, 2543.9, 2675.1, 2362.6, 2376.1 and 2592.2cm^{-1} showed C=O stretching for Carboxylic acids. While Peaks obtained at 3388.7, 1645.2, 1614.9, 1610.5, 1596.9, 1645.2 cm^{-1} are N-H bending for primary amines with 898.8, 896.8, 906.5, 900.2, 900.7, 894.9cm^{-1} N-H wagging that indicated the presence of secondary amines. Moreover, the medium peaks generated at 2738.7, 2721.4, 2725.2, 2725.2, 2734.9 and 2736.61cm^{-1} represent H-C=O: C-H stretching for aldehydes, with strong absorption peaks at 1689.5, 1724.2, 1731.6, 1735.8, 1735.8 and 1728.1cm^{-1} are assigned to C=O stretching vibration in carbonyl compounds; which may be characterized by the presence of high content for unsaturated aldehydes, esters and ethers. The medium peaks at 1244.0, 1244.4, 1164.9, 1164.1, 1170.7, 1168.8, 1128.3, 1124.4, 1120.6, 1116.7, 1026.1, 1022.2, 1024.1 and 1024.1cm^{-1}; represent C-N stretching for aliphatic amines.

The observed sharp peaks at 1515.9, 1514.0, 1521.0cm^{-1} for N-O asymmetric stretching and N-O symmetric medium stretching at 1355.9cm^{-1}, revealed the presence of nitro compounds. Other strong peaks at 1334.6, 1325.0 cm^{-1} C-N stretching indicates the presence of Aromatic amines. Also present were the medium band for Alkanes; C-H stretching at 2929.7, 2925.0, 2858.3, 2927.7, 2927.4, 2929.7, 2864.1, 2866.0, 2860.2, 2862.2 and 2858.3 cm^{-1}; medium C-H bending for alkanes at peaks 1458.1, 1454.2, 1462.5, 1452.3 cm^{-1} and C-H medium rocking at 1369.4cm^{-1}. The double bond -C=C- medium stretching at 1678.0, 1676.0, 1647.1, 1651.0 and 1652.9 cm^{-1} as well as the double bond -C=C sharp bending at 974.0, 997.1, 894.9 and 898.8 cm^{-1} depicted the availability of alkenes. Alkynes were represented by -*C≡C*- weak stretching at 2206.4, 2183.3, 2135.1, and 2125.4 cm^{-1}; and -C≡C: C-H strong and broad bending at 698.2cm^{-1}. A strong haoalkene, C-Cl appeared at 840.9 cm^{-1}.

Based on the functional groups identified, The FT-IR spectroscopic analysis of ethyl acetate fractions from *W. ugandensis* (Tables 4.8a-c) revealed the presence of alkaloids due to N-H stretch at 3388.7 (G), 1645.2 cm^{-1} (J), 1614.9 (H, E), 1610.5 (G), 1596.9 (I); N-H bending 898.8 (H), 896.8 (A), 906.5 (B), 900.7 cm^{-1} (D, C), 894.9 cm^{-1} (I, E); C-N stretch at 1334.6 cm^{-1} (A) and 1325.0 cm^{-1} (E) cm^{-1} which are fingerprint peaks found in primary, secondary as well as tertiary amines. Indeed, these alkaloids were contained in all fractions apart from F. Also predominantly present were the terpenoids and flavanoids as indicated by peaks for C=O at 2738.7, 2721.4, 2725.2, 2725.2, 2734.9 and 2736.61cm^{-1}, represented as H-C=O: C-H stretching for aldehydes, as well as C=O stretching at 1724.2, 1731.6, 1735.8 and 1735.8cm^{-1} for saturated aliphatic compounds. The C=O stretching at 1689.5 cm^{-1} pointed at α and β- unsaturated aldehydes or ketones. The unsaturated aromatic lactones with C=O at the 1735.8, 1730.0, 1728.1 and 1689.5 cm^{-1} indicated the presence of coumarin glycosides, with the nitrile peak at 2252.7 cm^{-1} at C≡N stretch, showing the presence of cyanogenic glycosides. The presence of phenolic compounds was depicted by O-H stretch at peak values 3431.1, 3458.1, 3427.3, 3404.1, 3438.8, 3423.4, 3624.0, 3427.3, 3456.2, 3436.9 and 3407.1cm^{-1}. Anthraquinones were present as aromatic ethers with C-O stretch at 1244.4, 1244.0 and 1242.1cm^{-1}. The medium band for C-H stretch at 2929.7, 2925.0, 2858.3,

2927.7, 2927.4, 2929.7, 2864.1, 2866.0, 2860.2, 2862.2 and 2858.3 cm^{-1}; the -C=C- medium stretch at 1678.0, 1676.0, 1647.1, 1651.0, 1652.9; and -C=C- sharp bending at 974.0, 997.1, 894.9, 898.81 cm^{-1} revealed large quantities of terpenes. The results of FT-IR are consistent with results from TLC that revealed abundance of alkaloids and terpenes in the extract. The spectral peaks revealing the major functional groups present in the crude stem bark methanolic extract from *W. ugandensis.* Alkaloidal presence was indicated by strong bands at 3813.0, 1614.3, 1523.7, 1336.6, 1091.6 and 1031.8. Terpenes were indicated by three strong bands; 2913.6, 1450.4 and 632.6. Saponins and phenols are indicated at 1724.2 and 3342.4 respectively (Were *et al*., 2015b).

5.2.3 Non-polar metabolites from *W. ugandensis*

A number of previous phytochemical studies on *W. ugandensis*, have reported the presence a large number of drimane-type sesquiterpenoids such as ugandensolide, ugandesidial, warburgin and warburgiadione, muzigadiolide, deacetylugandensolide, cinnamolide, mukaadial, ugandensidial, muzigadial (Canellal) and waburganal (Olila, 2002; Opiyo *et al.,* 2015; Xu *et al*., 2009). Also characterized are flavonol glycosides and monoterpenes (Xu *et al*., 2009). Also isolated from the ethyl acetate extract of the bark of *W. ugandensis* is ugandenial A, a new drimane-type sesquiterpenoid (Xu *et al*., 2009). Indeed, the bark of *W. ugandensis* has been used as a medicinal source for a long history in many African countries. Its biological activities including antifungal activity and trypanocidal activity being attributed to the sesquiterpenes (Olila, 2002). The presence of such diverse terpenoids and abundant polyunsaturated fatty acids (PUFAs), give credence to these pharmacological properties.

In this study, the GC-MS analysis identified over 45 phytoconstituents within the retention time of 8.26 to 42.02 minutes (Tables 4.9 - 4.13). Of these compounds, the most important phytoconstituents include benzothiozole (Figure 4.4), bumetrizole (Figure 4.5), drimenin (Figure 4.6), caryophyllene (Figure 4.7), muzigadial (Figure 4.10), pentadecane, vanillin, 4, 4, 5, 8-tetramethyl-chroman-2-one, phenol, 2-ethyl-, ugandensidial, amorphene, heptanal, 2-(phenylmethylene)-, 1-butanol, 3-methyl-,

xylene, pinene, eicosane, humulene, limonene, acetate and naphthalene, 1,2,3,4-tetrahydro-1,5,7-trimethyl. Of these compounds, terpenes, and especially the sesquiterpenoids, are responsible for the plants' medicinal effects (Wang *et al.*, 2015).

Table 4.9 showing GC-MS for retention times from 8.26 minutes to 11. 46 minutes reveals predominantly the presence of terpenes. The most abundant terpenes recorded are benzene, 1, 3-dimethyl- (8.826) at a concentration of 1.7µg/mg and p-Xylene (9.32 min) at 0.97µg/mg respectively. P-xylene also exhibited the highest quality at 97%. Other important terpenes detected in this range are alpha pinene at 10.19 min and eicosane at 11.47 minutes. 1-butanol, 3-methyl-, acetate, a flavanoid with quality of 95% and concentration of 0.2µg/mg was detected at 8.83 minutes. Further, GC-MS for retention times from 11.72 to 16.24minutes reveal predominantly the presence of terpenes. The most abundant terpenes recorded is naphthalene, 1,2,3,4-tetrahydro-1,5,7-trimethyl (16.24 min) at a concentration of 4.0µg/mg and limonene (12.03 min) at 0.4µg/mg exhibited the highest quality at 98%. Benzothiazole, the only non-terpenoid phytoconstituent in this time range, is an important alkaloid that was detected at 15.28 min and has a concentration of 0.32µg/mg and with quality of 93%. Within the retention time of 16.42 to 19.31 min, delta amorphene and Caryophyllene were detected at 99% quality as well as higher quantities of 1.2 and 1.5µg/mg respectively. The highest quantity observed was that of pentadecane, at 1.8µg/mg. Vanillin, at 91%, is a phenolic aldehyde detected at 17.72 minutes. Phenol, 2-ethyl-, a flavanoid (28.87 min) with a relative abundance of 89.1µg/mg, was the most abundant compound detected. Table showing GC-MS ranges in the retention time between 19.71 min and 26.74 minutes. flavanoids are predominantly present and in abundance. Canellal, also known as muzigadial, a flavanoid with well-known antiplasmodial activity was identified at 25.13 minutes, with abundance value of 30.5µg/mg. the quality of 91%. In an earlier study, muzigadial was isolated from *W. ugandensis* and found to have potent *in vitro* trypanocidal activity (Olila *et al.*, 2001) whereas ugandensidial, also a well described sesquiterpene dialdehyde with potent antiplasmodial properties was also revealed at 26.49 minutes, with relative abundance of 20.7µg/mg and at 91% quality. The sesquiterpene had earlier been isolated from *W. ugandensis*, demonstrated to have

strong anti-trypanosomal activity against *Trypanosoma brucei rhodesience*, with an IC_{50} of between 0.56 to 6.4mM (Schmidt *et al.*, 2014).

Table 4.13 shows the GC-MS ranges in the retention time between 26.86 minutes and 42.02 minutes. Alkaloids are predominantly present. For instance, bumetrizole, an alkaloid with described antiplasmodial activity, was identified at 29.25 minutes, with abundance value of 3.3µg/mg, with relative quality of 99%. Benzothiazole derivatives have diverse and broad spectrum of biological activity such as antitumour, antimicrobial, analgesics, anti-inflammatory and anti-HIV. In one study, compounds containing a core benzothiazole scaffold inhibited *P. falciparum* at IC_{50} of 50mM (Sadhasivan *et al.*, 2016). It was observed that benzothiazoles derivatives with furoyl group substitution were most effective, with IC_{50} value of 12.3mM in 48hours (Sadhasivan *et al.*, 2016). In a different study, a nitro containing benzothiazole was reported to exhibit potent antiplasmodial activity against *P. falciparum* (Hout *et al.*, 2004). Since benzothiazoles have however not been widely investigated (Sadhasivan *et al.*, 2016), the detection of a benzothiazole in *W. ugandensis* extracts, partly account for high antiplasmodial activity seen in our study and; points at an important bioactive resource that calls for further investigation. One phytochemical a study on composition and antiplasmodial activities of essential oils from some Cameroonian medicinal plants, established that delta cardinene (d-amorphene) and caryophyllene oxide were active against *P. faciparum* in culture (Boyom *et al*., 2003; Gleason and Chollet, 2011). In a similar study, caryophyllene, a sesquiterpenoid extensively researched, was found to be a major component of *Tetradenia riparia* extract that exhibited moderate antiplasmodial activity against *P. falciparum* (Campbell, 1997). Moreover, Okokon *et al*., (2005), extracted it from *Croton zambesicus* and demonstrated its activity against *P. berghei* in mice. Delta-amorphene and caryophyllene were invariably detected in our extract in substantially appreciable quantities. Two other important phytoconstituents extensively researched are limonene ana beta-pinene. Limonene, a monoterpene is found mainly in essential oils of citrus fruits and other plants, and has been identified as a non-toxic agent with potential for cancer chemotherapy (Crowell *et al.*, 1991). It has been found to prevent formation of chemically induced tumuors and displays significant antitumour effects (Vigushin *et al.*, 1998). Due to its activity on fast-growing cells such as

tumour cells, it was indeed assessed for activity on *Plasmodium* cultures (Moura *et al.*, 2001). When *P. falciparum* (S20) culture was treated with 0.5nM of limonene, it was observed that isoprenylation of proteins was significantly reduced, arresting progression of parasite growth from ring stage to trophozoite. At 1.22nM, it was observed that 50% of the parasites died after the first cycle (Moura *et al*., 2001). In another study, limonene, together with beta pinene, were extracted from *Cymbopogon citratus* and *Ocimum gratissimum* and found to significantly reduce parasite growth in a 4-day suppressive tested against *P. berghei* (Tchoumbougnang *et al.,* 2005). On the basis of these studies, the observed low parasitemia and arrested parasite growth and increased survivorship in mice and baboons treated with extract from *W. ugandensis* and *Z. usambarense* could therefore be attributed to the presence of limonene and pinene in these extracts. In another study, bioassay-guided fractionation of the dichloromethane extracts of the roots and the bark of *Z. usambarense* led to the isolation of two physiologically active compounds; canthin-6-one 1 (fungicide) and pellitorine 4 (insecticide). Other phytochemicals; isolated in the same study include oxychelerythrine 2, norchelerythrine 3, (+)-sesamin 5 and (+)-piperitol-3,3-dimethylallyl ether 6 (He *et al.,* 2002)

5.2.4 Polar metabolites from *W. ugandensis* and *Z. usambarense*

Tables 4.14 present the LC-MS values for aquous and methanolic extractions respectively from *W. ugandensis*. Two glycosides, (+)-catechin 3-O-glucose and cyanidin 3-(6''-p-coumaryl-2'''-sinapylsambubioside)-5-(6-malonylglucoside) with molecular masses 453.8 and 1203.3 were present in both extractions. Other compounds present are Syringaresinol (Figure 4.8), (Furanoid lignans), Buxaminol (Figure 4.9), and cancentrine (alkaloidal), Urdamycin (glycoside) and Picrasin F (quassinoid). Table 4.15 identifies the phytochemical components in an aqueous extract of *Z. usambarense.* They include two flavanoids; Occidentoside and 6-Hydroxyluteolin 5,6,3',4'-tetramethyl eter 7-cellobioside; Robinetinidol-(4alpha->8)-catechin-(6->4alpha)-robinetinidol (a polyphenol) and mesembrinol (alkaloidal). Other compounds that have displayed antiplasmodial properties that can be harnessed include 3-Hydroxyestra-1,3,5(10)-trien-17-one O-(carboxymethyl) oxime

and7,8,3',4',5'-Pentamethoxy-6'',6''-dimethylpyrano [2'',3'':5,6] flavones, and 2-Acetyl-4,6-dimethoxyfuro[2,3-b] quinoline-2-Acetylpteleine.

In previous investigations on *Z. usambarense*, Kokwaro *et al*. (1983) isolated the coumarin derivative O-methylcedrelopsin. Moreover, a number of alkaloids which were identified as (+)-tembetarine, (+)-magnoflorine, (-)- edulinine, (+)-N-methylplatydesmine, (-)-blongine, (-)-usambarine, usambanoline, (-)-*cis*-N-methylcanadine, nitidine and chelerythrine were isolated from the stems and the roots of *Z. usambarense* (Kato *et al*., 1996; Lawal and Olagoke, 2016). Our study thus identifies a number of new compounds whose structural elucidation and individual bioactivity remains unexplored.

5.3 Preclinical efficacy and safety of plant extracts on *P. anubis*

The antiplasmodial activity of stem bark extracts from *W. ugandensis* (WUSB) and *Z. usambarense* (ZUSB) was investigated in olive baboons *(P. anubis)* by measuring parasitemia profiles in infected animals, clinical observations, heamatological profiles, biochemistry and necropsy. PAN 3975 (untreated control) died by 12th day post infection from high parasitemia of 24% (Figure 4.2).

5.3.1 Parasiteamia profiles of extract-treated baboons

Parasitaemia levels in treated animals remained significantly low until the 21st day post infection when they were euthanized. PAN 3975 (untreated control) died by 12th day post infection from high parasitaemia of 24% while parasitaemia levels in treated animals remained significantly low (under 2%). This clearly indicates a remarkably high level of antiplasmodial activity in extract-treated animals. Apart from maintaining low parasitaemia levels, the extract also increased survivorship of treated animals for over 10 days. This observation agrees with our earlier observation (Were *et al*., 2015; Were *et al*., 2010) in which extracts from the same plant cleared parasites and increased survivorship in *P. berghei*-infected mice and, yielded low *in vitro* IC_{50} values against *P. knowlesi.*

When parasitaemia profiles were subjected to statistic test, significant variation was observed in means between treated and untreated baboon. Calculated mean values are 4.60, 1.36, 0.54, 0.67 and 0.34 for PAN 3975, PAN 3982, PAN 3984, PAN 4085 and PAN 4091 respectively. The variances P value < 0.0001; are significantly different ($P < 0.05$). The Tukeys' Multiple Comparison Test revealed significant difference between PAN 3975 and PAN 4091 ($q = 4.19$) and PAN 3975 and PAN 3984 ($q = 3.99$). This finding indicates some level of antiplasmodial activities by the two extracts used as treatment. Apart from maintaining low parasitemia levels, they also increased survivorship of treated animals.

5.3.2 Pathology and histopathology of extract-treated baboons

Examination of tissues from treated animals showed normal size of spleen, haem metabolism and follicular proliferation. The liver tissues were of normal size though appeared darkened owing to raised haem metabolism. The heart and kidneys were normal, and no obvious lesions were seen in lungs. PAN 3763, the negative control, presented with normal organs: i.e no signs of cardiac atrophy, no signs of nephritis, no indications of haem metabolism, normal liver, pancreas and spleen sizes, non-engorged gall bladder and clear lungs. Overally, no abnormalities were detected(NAD), implying that the extract had no adverse effect on the mammalian tissue. In fact, necropsy examinations of PAN 3975 (infected and not treated) revealed severe hepatosplenomegaly, engorged gall bladder and bile imbibition, proliferation of white follicles, haem metabolism and jaundice. No pathological changes were however observed in the lungs, clearly demonstrating that the animal died due to anaemia, resulting from heavy parasitaemia.

5.3.3 Heamatological profiles of extract-treated baboons

The Complete Blood Count (CBC) tests for the amount of RBCs, haemoglobin, hematocrit, reticulocytes, mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC) and platelets were carried out to evaluate the effects of extracts on heamatological profiles (Table 4.2). All experimental animals that were infected with *P. knowlesi* presented with varying degrees of anaemia and other heamatological alterations.

Severe anaemia, defined as Hb < 5 g/dL (Akinosoglou *et al.*, 2012) was observed in PAN 3982 and PAN 4085, while PAN 4091 and 3984 were mildly anaemic, Hb < 11g/dL, (Akinosoglou *et al.*, 2012). PAN 3763 (non-infected but treated) remained non-anaemic, presenting with a mean Hb value of 12.3g/dL. Clearly, the observed anaemia and low heamatocrit values as well as corresponding low erythrocytes count resulted from the rapture of parasitized RBCs. The sharp rise in parasitaemia observed in PAN

3975 (positive control) as opposed to treated animals strongly displayed the effect of the extract in limiting parasitaemia and increasing animal survivorship. PAN 3975 died early as it suffered hypovolemic shock due to massive destruction of RBCs resulting from exponential parasiteamia levels observed. In terms of differential counts, granulocytes and lymphocyte presented the most important leukocytic changes observed in this study. The levels of granulocytes in all infected animals were significantly low, which is in line with the report of Philipose and Umashankar, 2016.

This observation however, contrasted previous studies that recorded increase in neutrophils attributable to activated neutrophil production and suppressed peripheral removal (Aitken *et al.,* 2018; Maina *et al.,* 2010). It is also not clear why the amount of granulocytes in non-infected but treated animal declined. Moreover, significant lymphocytosis resulted following infection and treatment in all experimental animals, just as was the case in a study by Ourives *et al.,* 2018, but in sharp contrast to decrease in peripheral blood lymphocytes usually observed in patients with acute malaria due to lymph nodes sequestration and apoptosis (Antwi-Baffour *et al.,* 2018; Kassa *et al*., 2006). Interestingly, an increase in lymphocytes was also observed in non-infected treated animal, suggesting that the extract was highly immunostimulatory in its antiplasmodial activity.

5.3.4 Biochemical profiles of extract-treated baboons

Creatinine is critically important in assessing renal function (Francis *et al.,* 2014). The normal range for creatinine in the blood may be 74.3-107 mmol/dL. In this study, it was observed that all creatinine values fell within normal range for all

experimental animals (Table 4.3). The reduction in creatinine levels in PAN 4085 that was treated with stem bark extract of *W. ugandensis* points at sudden decrease in activity, arising from massive destruction of RBCs by *P. knowlesi* (H) parasites. On the other hand, serum concentration of urea reflects the balance between production by the liver and elimination by the kidneys. It is therefore one of all important tools of assessing the renal function status (Francis *et al.,* 2014). From table 4.20, all values were within normal range of 2.5-7.8 mmol/L, confirming normal liver and kidney function. Further, the normal functioning of the liver in treated animals in indicated by liver enzymes; Gamma-glutamyl transpeptidase (GGT) and Alanine Aminotransferase (ALT). Gamma-glutamyl transpeptidase is concentrated in the liver, but it's also present in the gallbladder, spleen, pancreas, and kidneys whereas ALT is produced in liver. A low level of ALT in the blood is expected and is normal in liver between 7 to 56 IU/L (Shrivastar *et al*., 2014). Mild elevations are generally considered to be 2-3 times higher than the normal range. Very high levels suggest drug-induced hepatitis [(Shrivastar *et al*., 2014; Singh and Daneshvar, 2013). In this study, all experimental animals, including PAN 3763 had ALT values within normal range, an indication of normal liver function, confirming safety of extracts.

CHAPTER SIX

CONCLUSIONS AND RECOMMENDATIONS

6.1 Conclusions

i) Extracts from stem barks and root barks of *W. ugandensis* and *Z. usambarense* are equally efficacious against *Plasmodia* parasites and could therefore be used in the development of phytomedicines for treatment of malaria.

ii) Both polar and non-polar extractions from *W. ugandensis* are highly antiplasmodial. Only polar extractions from *Z. usambarense* however displayed antiplasmodial activity. Treated animals presented with low parasiteamia and longer survival times, clearly indicating that the treatment was effective in limiting parasite growth and development of anaemia.

iii) *Warburgia ugandensis* and *Z. usambarense* contain pharmacologically active compounds as demonstrated by phytoanlyses, making them suitable candidates for development of antimalarial pytomedicines.

iv) Animals treated with methanoic extracts from *W. ugandensis* and *Z. usambarense* did not show any abnormalities. Most heamatological and biochemical parameters were observed to be within normal ranges, with a few exceptions, though not significantly varied. These extracts did not display any toxicities on the mammalian tissue as demonstrated by histopathological tissue sections, implying that its safe for use as phytomedicines.

6.2 Recommendations

i. Since no significant difference in efficacy was observed with regard to plant part of *W. ugandensis* used, it is therefore suggested here that any part of this plant could be used as a phytomedicine so as to avoid over reliance on stem barks as is currently the case. Further analyses are however required to establish the optimal dosage for eah part.

ii. Both aqueous and organic extractions should be used since phytochemical analyses of the extracts revealed the presence of both polar and non-polar bioactive constituents.

iii. The two medicinal plants; *W. ugandensis* and *Z. usambarense* have been used by many communities in Kenya as herbal medicines. Preliminary preclinical tests carried out in this study confirm that these plants are safe and efficacious at the dosage used and their use in traditional medicine is therefore recommended.

iv. The preclinical assessments were limited in sample size, dosage forms and routes of administration. Further assessments need to be carried out to determine the optimal concentration of the extracts that completely kill the *Plasmodium* parasites. Such experiments could be designed based on dose escalation experiments and other treatment regimens as well as different routes of drug administration.

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APPENDICES

Appendix I: Air-drying of plant materials in the GK Lab, JKUAT

Appendix II: Air-dried stem bark of *W. ugandensis*

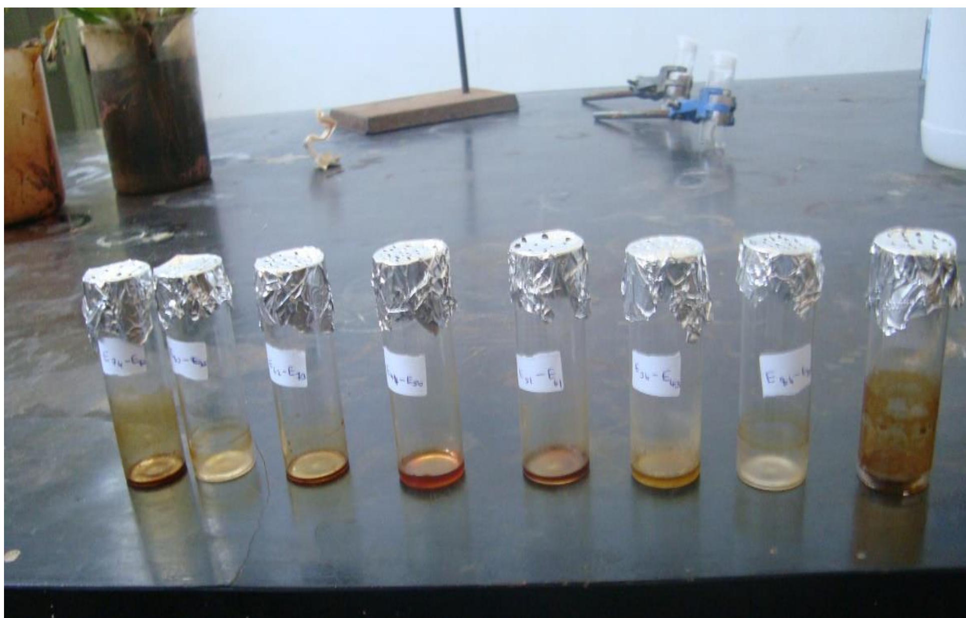
Appendix III: Pulverization of plant material for extraction

Appendix IV: Aqueous extracts of *W. ugandensis* and *Z. usambarense*

Appendix V: *In vacuo* evaporation of organic extracts

Appendix VI: Lyophilisation of plant extracts on a Freeze Dryer

Appendix VII: TLC Fractions from *W. ugandensis* and *Z. usambarense*



Appendix VIII: Retrieval of cryopreserved *Plasmodium* parasites

Appendix IX: Determination of parasitaema through microscopy

Appendix X: Mean parasitaemia for *W. ugandensis* extracts on *P. berghei*

Treatment		Mean % Parasitaemia[a]	Mean % Inhibition
Root bark		4.71±0.86	61.8
Stem bark		4.39±0.91	64.4
Leaf		6.57±1.29	46.7
PBS		12.32±2.82	-
Chloroquin		0.62±0.16	94.9
	F value		9.670
	$P < 0.05$		0.0001

[a]Data are expressed as mean ±SEM for five groups with replication *(n = 6)*

Appendix XI: ANOVA for *in vivo* antiplasmodial activity of plant extracts

Plant part			Difference in means	q value	Significance level ($p < 0.05$)
WUSB	Vs	**WURB**	0.313	0.249	NS
WUSB	Vs	**WUL**	(1.867)	1.488	NS
WUSB	Vs	**PBS**	(7.612)	**5.583**	**S**
WUSB	Vs	**CQ**	4.090	3.261	NS
WURB	Vs	**WUL**	(2.180)	1.738	NS
WURB	Vs	**PBS**	(7.925)	**5.813**	**S**
WURB	Vs	**CQ**	3.777	3.011	NS
WUL	Vs	**PBS**	(5.745)	4.214	S
WUL	Vs	**CQ**	5.957	4.750	S
PBS	Vs	**CQ**	11.700	8.583	S

F = 5.95, $P < 0.0001$, 99% CI

Appendix XII: Tukey's comparison of means for activity of plant parts

	PAN 3975	PAN 3982	PAN 3984	PAN 4085	PAN 4091
Means	4.600	1.363	0.543	0.667	0.338
St Dev	9.080	1.820	0.616	0.677	0.248
Std Error	3.430	0.489	0.165	0.188	0.062
Variances		Diff sig	$P < 0.05$		

WUSB - *W. ugandensis* stem bark, WURB - *W. ugandensis* root bark, WUL - *W. ugandensis* leaf extract, CQ -Choroquine, PBS - Phosphate buffered saline.

Appendix XIII: Tukey's comparison of means for *in vivo* activity of extracts

PAN			Difference in means	q value	Significance level ($p < 0.05$)
4085	Vs	**4091**	0.329	0.389	NS
4085	Vs	**3975**	(3.933)	3.820	NS
4085	Vs	**3982**	(0.695)	0.823	NS
4085	Vs	**3984**	0.124	0.147	NS
4091	Vs	**3975**	(4.262)	4.194	S
4091	Vs	**3982**	(1.021)	1.247	NS
4091	Vs	**3984**	(0.205)	0.247	NS
3975	Vs	**3982**	3.237	3.185	NS
3975	Vs	**3984**	4.057	3.990	S
3982	Vs	3984	0.820	0.988	NS

Treatment for PAN 4091 (WUSB) and PAN 3984 (WURB) are significantly different from the control/untreated (PAN 3975)

Appendix XIV: GCMS peaks for ethyl acetate extracts of *W. ugandensis*