STUDY OF ANTIPLASMODIAL ACTIVITY, CYTOTOXICITY AND ACUTE TOXICITY OF *Zanthoxylum chalybeum* ENGL, and *Vernonia lasiopus* O. HOFFMAN

A Thesis submitted in partial fulfillment of requirements for Master of Science Degree of University of Nairobi, (Pharmacology and Toxicology)

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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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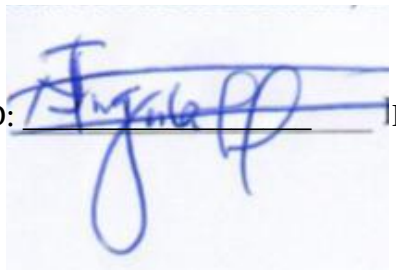
SIGNED:  DATE: _____

DEDICATION

This work is dedicated to my family for support and determination in my education.

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ABBREVIATIONS

CC_{50}	Cytotoxic concentration 50%
CO_2	Carbon dioxide
CQ	Chloroquine
DMSO	Dimethyl Sulphoxide
GDP	Gross Domestic Product
G6PDH	Glucose 6 Phosphate Dehydrogenase
IC_{50}	Inhibition concentration 50%
KEMRI	Kenya Medical Research Institute
LD_{50}	Lethal dose 50% (Median Lethal Dose)
MTT	3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide
Nacl	Sodium Chloride
OECD	Organization for Economic Co-operation and Development
PCR	Polymerase Chain Reaction
PET	Petroleum Ether
RPMI 1640	25mM HEPES (N-Hydroxyethylpiperazine-N'-2-ethanol sulfonic acid)
WHO	World Health Organization

ABSTRACT

Malaria continues to cause heavy morbidity and mortality and it is the fifth leading cause of death globally. The disease causes over one million deaths annually and affects many more, particularly due to increasing multi-drug resistant strains of *Plasmodium falciparum*. Sustained investigations in both curative and prophylactic interventions have supported the ethno-pharmacological approach to identify novel compounds as a major channel towards achieving a solution. The medicinal plants *Zanthoxylum chalybeum* and *Vernonia lasiopus* are traditionally used for the treatment/prophylaxis of malaria by some of the local communities in Kenya. The aim of this study was to evaluate antiplasmodial activity and safety of the two selected plants. Their activity on combination was also investigated. *Zanthoxylum chalybeum* leaves, stem bark, root bark and *Vernonia lasiopus* aerial parts and roots were collected and extracted with methanol: dichloromethane (1:1) solvent mixture. The resulting crude extracts were each fractionated into six fractions by vacuum liquid chromatography using solvents of different polarities, and investigated for antiplasmodial activity (IC_{50}) using the chloroquine (CQ) sensitive D6 and chloroquine (CQ) resistant W2 laboratory adapted *Plasmodium falciparum* strains. The safety profile was done by determining the *in vitro* cytotoxicity (CC_{50}) on Vero 199 cells using the MTT based colorimetric assay and *in vivo* median lethal dose (LD_{50}) in mice. The selectivity index (CC_{50}/IC_{50}) was used as an indicator of their antiplasmodial viability.

The results showed that *Z. chalybeum* roots fraction 6 and *Z. chalybeum* stem bark fraction 6 are highly active and active respectively with IC_{50} values of 0.78 0µg/ml and 6.0µg/ml against the *P. falciparum* D6 strain. They had selectivity indices of >128.21and >16.67 respectively, and LD_{50} values of >5000mg/kg indicating safety. *V. lasiopus* crude root extract showed the most promise at IC_{50} of 13.1 µg/ml against D6 strain, with selectivity index >

7.63 and LD_{50} >5000mg/kg.

Extracts of both *Z. chalybeum* and *V. lasiopus* were found to have antiplasmodial activity and favourable safety profile with the highly active *Z. chalybeum* roots fraction 6 being the most viable sample. Further chemical analysis is recommended for this fraction and the active samples, and they will hopefully be used as candidates for development as anti-malarial medicine. The crude roots extracts of the plants showed activity (IC_{50}) of 1.11 µg/ml and 13.1 µg/ml respectively and this supports their use traditionally. Some samples classified as active also had good safety margin and may also be explored further. Detailed combination studies of the various samples may be carried out to as they may bear different pharmacologic properties whose interactions could result in beneficial outcomes such as potentiated activity and curtailing resistance.

Key words: *Plasmodium falciparum,* Z. chalybeum, *V. lasiopus*, fractions, selectivity index, LD_{50}.

CHAPTER ONE

1.0 INTRODUCTION

1.1 BACKGROUND

Malaria is a mosquito-borne infectious disease of humans and other animals caused by eukaryotic protists of the genus *Plasmodium*. In humans the disease is transmitted by the female mosquito of the genus *Anopheles.* The *Plasmodium* species that cause malaria in human include *P. falciparum, P. vivax, P. malariae, P. ovale and* the zoonotic is *P. knowlesi (*mainly monkey parasite, similar to *P. malariae* and confirmed by PCR) (WHO, 2010). Of the five *Plasmodium* species infecting humans, *P. falciparum* is responsible for a high proportion of the morbidity and nearly all the mortality (White, 2010). Investigation of the *Plasmodium* species present in an infection is necessary because, firstly *P. falciparum* and *P. knowlesi* infections cause rapidly deteriorating severe illness or death and prompt commencement of treatment is crucial. The other species are less likely to cause severe malaria. Secondly, *P. vivax* and *P. ovale* infections also require treatment for the dormant hypnozoite liver forms that can cause infection relapse. *P. vivax* causes more gradual and predictable progressing disease. Finally, the resistance patterns for *P. falciparum* and *P. vivax* vary in various regions (CDC, 2011).

The disease mainly results from the multiplication of Plasmodium parasites within erythrocytes, causing symptoms that typically include fever and headache. In severe cases there is progression to coma or death. Symptoms coincide with rupturing of erythrocytes to release plasmodium which infect more cells. This cyclic event occurs at different span of times among the plasmodium species hence the naming of tertian malaria for *P. falciparum* and quartan malaria for *P. malariae.*

A preventable and curable disease, malaria causes over 1 million annual deaths globally, mostly affecting children below the age of 5 years (86% of total deaths) and pregnant mothers. It is responsible for 8% of children's global deaths, 16% being in Africa. It is widespread in tropical and subtropical regions, including much of sub-Saharan Africa, Asia, and the Americas. There are approximately 9000-10000 cases reported annually in Europe, and there is estimated to be 1.1% fatality rate among *Plasmodium falciparum* cases. These may also be among travellers exposed in other areas (White, 2010). Malaria exerts heavy socio-economic impact on the society; especially due to *Plasmodium falciparum* strains hence the very important need for solutions.

Conventional antimalarial drugs have been the mainstay of clinical management, both for prophylaxis and treatment. Artemisinin based combination drugs are the frontline for treatment currently with artemisinin-lumefantrine being the first line and artemisinin-piperaquine being second line oral treatment. Parenteral artesunate is initially instituted with intravenous quinine being the last line of defence for severe malaria. Previously used drugs such as sulphadoxine-pyrimethamine (SP), mefloquine, chloroquine, primaquine, amodiaquine have been limited due to development of resistance.

Emergence of multidrug resistant strains which has accompanied each new class of antimalarial drugs may be viewed as one of the most significant threats to the health of tropical populations. While it is widely agreed that a new approach to prevention and treatment is needed, solutions have targeted more of development of new drug classes. With renewed interest and funding, there are over 15 new antimalarials in various development stages. The main concern is that they act at known targets and therefore may be subject to common resistance mechanisms. New drugs for new plasmodia targets are needed.

The use of combination therapies of existing drugs having different target sites has also been promoted in order to prevent resistance. Combination therapies existed in traditional medicines before successful extraction could be done. Various remedies were used concurrently for higher effectiveness. Flavonoids in *Artemisia annua*, which are structurally unrelated to artemisinin, enhance the *in vitro* antiplasmodial activity of artemisinin (Bodekar, 2004). Also synergism has been observed between the alkaloids of *Ancistrocladus peltatum.*

The total alkaloid extract of this plant has much greater antiparasitic activity than any of the six alkaloids isolated individually (Bodekar, 2004). Studies on some antimalarial plants have found alkaloids that potentiate chloroquine *in vitro* and in some cases *in vivo*, and these plant preparations are under tests as adjuvants to chloroquine therapy in Madagascar. These alkaloids are bisbenzylisoquinoline, novel pavine and benzyl tetrahydroisoquinolines (Bodekar, 2004). Malaria patients sometimes combine convectional and traditional medicine simultaneously or as first and second line treatments as they perceive better efficacy. This however comes with risk of adverse interactions (Ankrah, 2003).

The ethno-pharmacological approach to identify novel antimalarial compounds is of major potential towards achieving a solution, supported by that main drugs used historically were obtained directly from plants and their variations. These include quinine and artemisinin, the two most important drugs in clinical use currently (Sudhanshu, et. al). As a result, the need for the integration of traditional medicine with modern medicine has been recognized. Integration of traditional medicine as viable treatment options provides an opportunity to introduce novel antimalarials, as well as providing treatment alternatives for communities that do not readily accept Western medicine. The WHO Beijing declaration of 2008 acknowledged the need to integrate traditional medicine into national health systems (WHO,

2008a). This project studied the antimalarial efficacy and safety of *V. lasiopus* and *Z. chalybeam*, which are medicinal plants traditionally used in Kenya for treatment of malaria.

1.2 Justification of the study

Malaria is one of the most important parasitic diseases in the world. It remains a major public health problem in Africa responsible for the annual death of over one million children below the age of five years (White, 2010). *Plasmodium falciparum* is becoming increasingly resistant to standard antimalarial drugs which necessitate a continuous effort to search for new drugs, particularly with novel modes of action. Plants have invariably been a rich source for new drugs and some antimalarial drugs in use today (quinine and artemisinin) were either obtained from plants or developed using their chemical structures as templates. *V. lasiopus* and *Z. chalybeam* have been used traditionally in Kenya for many years to treat malaria symptoms and other conditions (Kokwaro, 2009), but scientifically generated knowledge on their efficacy and toxicity is not well documented. Combination of these plants was important because it was practiced in traditional medicine to treat resistant malaria. This study researched on these promising plants and furthered the work previously done on their potency by Irungu (Irungu, 2007), by determination of the efficacy and safety of the fractions directly and by blending.

1.3 Objectives of the study

1.3.1. General Objective

To determine the anti-plasmodial properties of crude extracts and fractions of *V. lasiopus* and *Z. chalybeum* and their toxicological profile

1.3.2 Specific objectives of the study

1) To determine *in vitro* anti-plasmodial activity (IC_{50}) of the crude extracts, fractions and blends of *V. lasiopus* and *Z. chalybeum*.
2) To test the effect of the combining the most active fractions from each of the two plants at varying concentrations.
3) To determine the *in vitro* cytotoxicity (CC_{50}) on Vero 199 cells, and *in vivo* median lethal dose (LD_{50}) in female swiss mice.

1.4. Hypothesis

V. lasiopus and *Z. chalybeum* have potent antiplasmodial activity and have good safety profile.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Malaria Disease

2.1.1. Aetiology of Malaria

Malaria is caused by plasmodium parasites which are protozoa and it is transmitted by the female mosquito of the genus *Anopheles* in humans. The *Plasmodium* species in human include *P. falciparum, P. vivax, P. malariae, P. ovale and* the zoonotic species is *P. knowlesi*. *P. knowlesi,* mainly a monkey parasite, is similar to *P. malariae* and is confirmed by PCR (WHO, 2010). Of the five *Plasmodium* species infecting humans, *P. falciparum* is responsible for a high proportion of the morbidity and nearly all the mortality. Investigation of the *Plasmodium* species present in an infection is necessary because, firstly *P. falciparum* and *P. knowlesi* infections cause rapidly deteriorating severe illness or death and prompt commencement of treatment is crucial. The other species are less likely to cause severe malaria. Secondly, *P. vivax* and *P. ovale* infections also require treatment for the dormant hypnozoite liver forms that can cause infection relapse. *P. vivax* causes more gradual and predictable progressing disease. Finally, the resistance patterns for *P. falciparum* and *P. vivax* vary in various regions (CDC, 2011).

P. falciparum is widespread with highest density being in tropical regions of Africa, Asia and South America. *P. vivax* is widespread but chloroquine resistant strains are mostly occur in Papua New Guinea and Indonesia. *P. vivax* infections occurring in Papua New Guinea or Indonesia should initially be treated with a regimen recommended for chloroquine-resistant P. vivax infections which includes quinine sulphate plus doxycycline or tetracycline, or, Atovaquone-proguanil, or mefloquine. These three treatment options are equally recommended. Chloroquine is effective for *P. ovale* infections.

2.1.2. Life Cycle and Transmission of Plasmodium

Stages in Man. Sporozoites are inoculated in humans for the asexual phase by female anopheles mosquitoes upon feeding on human blood meal, which they require to nourish eggs. They are injected during probing before aspirating blood, and enter circulation directly or through lymph channels (approx. 20%). They invade human liver cells, being cleared from the blood stream within 45 minutes, differentiate and multiply to release tens of thousands of merozoites. Only few cells are infected and this stage is asymptomatic. This lasts 5.5 and 15 days for *P. falciparum* and *P. malariae* respectively. In the case of *P. ovale and P. vivax* some intra hepatic ones do not develop and rest as inert hypnozoites to awaken and cause relapse weeks to months later.

The parasites migrate to the blood and infect erythrocytes with individual merozoites undergoing further multiplication to produce 12-16 merozoites within schizonts. The length of this erythrocytic stage depends on the species and is usually 48hours for *P. falciparum, P. vivax, and P. ovale,* 24hr for *P. knowlesi* and 72 hours for *P. malariae*. These then burst out infecting more red cells, at usually 10-fold per cycle, leading to fever and damage of vital organs. Only a subpopulation of cells is infected mainly as a result of their age. *P.* (*vivax* and *falciparum*) prefer younger cells, with 13% and 40% uncomplicated malaria cell invasion respectively, while *P. malariae* prefer older erythrocytes. However *P. falciparum* infections in South East Asia have shown unrestricted cell invasion. The erythrocytes also release gametocytes which get ingested by feeding mosquitoes thus completing the lifecycle, while ingested sporozoites do not survive and get digested in the mosquito (White, 2010).

Stages in the Vector. The sexual (sporogony) intra-vector phase starts with ingestion of gametocytes by the feeding mosquito. The male and female gametocyte ration is about 1:4,

with each male having 8 microgamete and female as a macrogamete. One of each sex is needed for successful infection to occur. In the mosquito's midgut, the male gametocytes undergo rapid nuclear division, to produce 8 flagellated micro gametes for fusion and meiosis with the female macrogametes and form zygotes, which become diploid and then develop to the rapidly moving ookinete. These transverse the mid gut wall and form oocysts, each of which differentiates into thousands of Sporozoites which migrate to the mosquito's salivary glands. Sporogony takes 8-35 days depending on temperature and mosquito species (White, 2010). The lifecycle is as shown in figure 2.1.

Malaria can also be spread by inoculation of blood from an infected person. In this form, asexual forms are directly inoculated into the host's blood, by-passing the pre-erythrocytic hepatic development of the parasite (Jain, 2002). Hence, this malaria type has a shorter incubation period.

The most efficient malaria vector is the African *Anopheles gambiae*. This can be attributed, partly, to its relatively long life, strong anthropophily and endophily (the tendency to target humans for blood meal and the tendency to enter and rest inside of houses, respectively). Other species are *Anopheles gambiae, Anopheles arabiensis* and *Anopheles funestus*, all found in Africa and are some of the sixty anopheline mosquitoes able to transmit in humans.

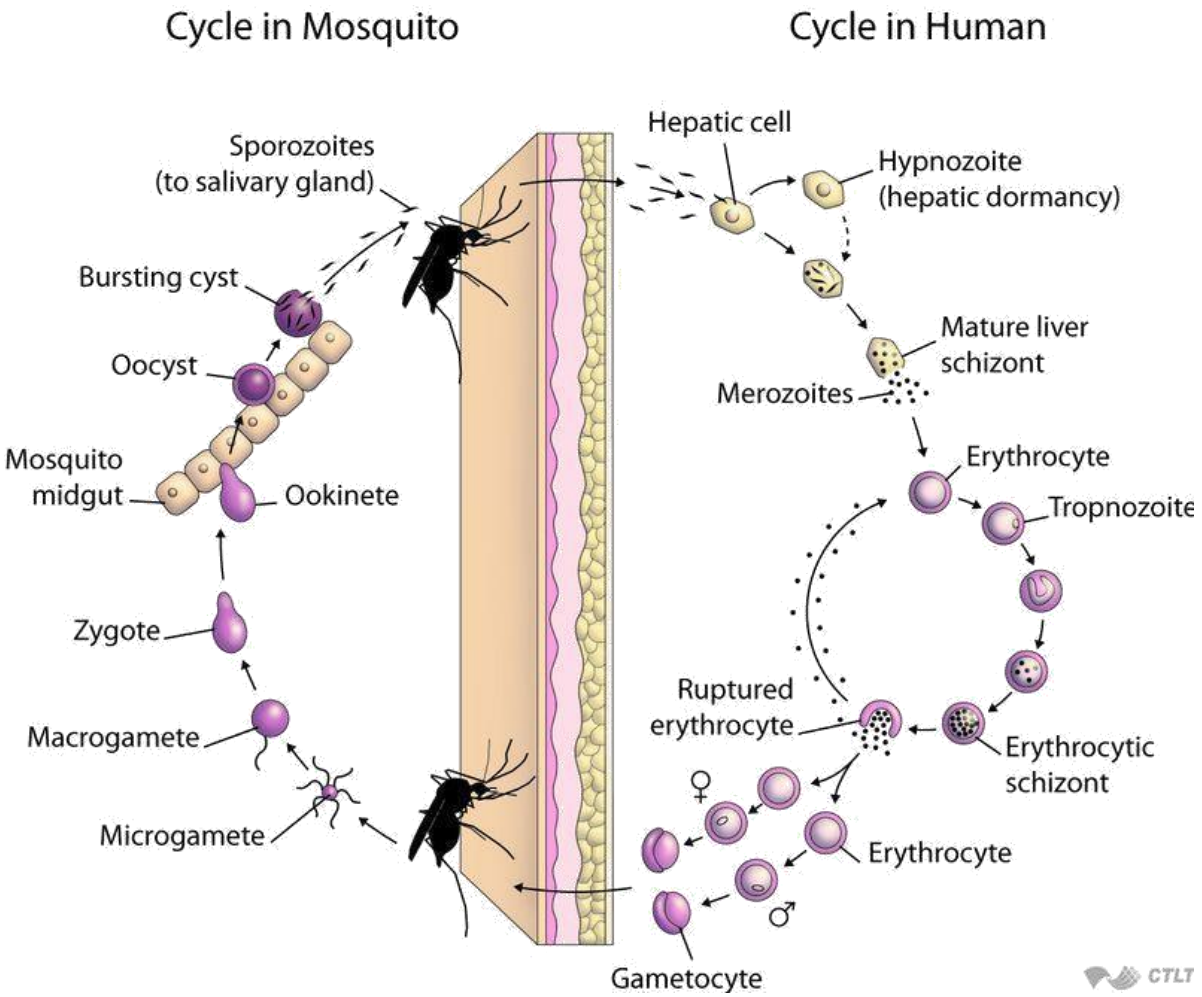


Figure 2.1: Lifecycle of the *Plasmodium* indicating the development stages in man and vector (from malariasite.com)

2.2 Epidemiology of Malaria

Malaria is the world's most widespread infection and there were 216 million episodes in 2010. Compared to a century earlier, the area of malaria risk has reduced from 53% to 27% of the Earth's land surface and the number of countries exposed to some level of malaria risk has fallen from 140 to 106. Africa bore more than 81% of the infected cases and 91% of the deaths in 2010, with sub -Sahara Africa affected the most, (Hay, 2009; WHO, 2011). It is the fifth leading cause of death worldwide with almost half the world's population at risk. Malaria is a classic disease with high morbidity, affecting productivity and has been estimated to cost 1.3% of GDP in endemic countries, like in Asia, Latin America, and Africa (WHO 2010). In Africa alone, an economic burden exceeding US$ 12 billion of lost GDP is encountered annually. However, there has been approximately 17% global fall in malaria incidences between 2000 and 2010 however (WHO, 2011).In Kenya the disease is endemic in the lowlands, particularly the coastal strip and Lake Victoria basin where transmission is sufficiently intense. Both incidence and prevalence of infection reach more than 90% of the population within 10 - 12 weeks after the beginning of the rainy season in Kenya (MOH, 2006).

2.2.1. Travellers and malaria

The infection risk depends on various vector and host factors, with the selection of prophylaxis depending on risk of infection, chances of side effects and adherence of the individual. A way to asses risk is by the entomological inoculation rate (EIR), which is the number of infective *P. falciparum* mosquito bites per person annually. The EIR in East Thailand is about 0.91, around one infective bite per year, 667 in rural Tanzania, around 2 infective bites per night. In Kenya it ranges at 17-299.3, around 1 infective bite in 3 weeks to 1 per night).

P. falciparum malaria is the predominant form imported into Europe and North America (58-63%) with 77683 reported cases in Europe over a 10 year period. Kenya is a popular destination for travellers and malaria rates vary from country to country for visitors, at a range of 50-135 cases per 10,000 visitors. Case mortality also varied with 3.5% for Germany and 0.65% for United Kingdom. Rates for other regions are also at similar risk order (White, 2010).

Malaria transmission does not occur at temperatures outside 16-33 ^{o}C, because mosquito stage development cannot take place. High humidity and 20-30 ^{o}C provide optimal conditions. The vectorial capacity, ability to transmit malaria, varies among anopheline mosquitos with over 400 species known. Of these species, 80 can transmit malaria with 66 considered natural vectors and 45 considered important vectors. Each vector has its own behavioural pattern (White, 2010)

Traditional epidemiologic classification lists areas as hypoendemic, mesoendemic, hyperendemic or holoendemic. Asymptomatic human hosts act as reservoirs in areas with long dry seasons or low transmission areas, until the rainy/transmission periods. In high transmission areas infants and young children show higher gametocyte densities for transmission. However the contribution to transmission is not clear between the young who have higher *plasmodium* densities and receiving more treatment versus the older less symptomatic individuals with more immunity, less density and less likely to receive treatment (White, 2010).

2.2.2. Clinical epidemiology

Severe malaria is rare in the first six months of life but with high mortality if it does. This is due to passive transfer of maternal immunity and their higher haemoglobin F which retards parasite development. In young children progression can be rapid with seizures suggesting that sequestration causes significant damage even in conscious patients. A history of less than a day's illness is common in these cases of cerebral malaria. In holoendemic areas, although infants are repeatedly inoculated, severe malaria is rare for them with the clinical impact being anaemia. Indigenous adults do not develop severe malaria unless they leave the transmission area. Immunity is boosted continually and malaria awareness leads to early interventions, preventing high parasitaemia. Most infections are asymptomatic. In less intense or unstable transmission areas young children, besides infants, are also affected by severe malaria with cerebral malaria also dominating. In low transmission areas seasonal infections are common, symptomatic infections occur at any age with cerebral malaria mainly manifesting for severe cases. Epidemics are associated with migration of new host, introduction of new vectors or change in their habitats. (White, 2010)

2.2.2.1. Mixed Infections

Infection with *P. falciparum* mostly suppresses simultaneous *P. vivax* infection although the reverse is sometimes observed. In Thailand about 30% of *P. falciparum* infected patients show symptomatic *P. vivax* infection in 2 months without exposure to new malaria infections, and the reverse in 8% of patients. In low transmission areas co-infection with the two species lowers severe malaria risk four-fold, but in high transmission areas higher morbidity is seen (White,2010)

2.2.2.2. Diagnosis and clinical symptoms

The severity of the disease varies from mild asymptotic infection to the critical disease which causes death. Common symptoms include periodic flu-like symptoms such as fever, chills, sweating, muscle aches and headaches. Other symptoms include nausea, vomiting, coughing, diarrhoea, abdominal pain, myalgia (limbs and back), loss of appetite, orthostatic hypotension, jaundice, anaemia, liver or kidney failure, enlarged liver and spleen and convulsions. Severe malaria is regarded as a multisystem disorder and includes cerebral malaria and severe malarial anaemia. Severe malaria delirium, metabolic acidosis and multi-organ dysfunction which may occur, if untreated could result in coma and eventually death. Cyclical symptoms such as fever, seizures, chills and anaemia are said to correspond with the erythrocytic stage of the Plasmodium life cycle during which merozoites are released into the bloodstream. Together with this release, there is also a deposit of parasitic waste products and debris, which is believed to give rise to the malarial paroxysm, i.e. the sequenced events of shaking chills, fever and sweating.

Symptoms for complicated or severe malaria includes seizures, altered state of consciousness (or coma), excessive sleepiness, prostration (feelings of helplessness), respiratory distress, inability to ingest any fluids, bleeding problems, jaundice, the absence of urine and the dark coloration of urine if there is any. Splenomegaly and anaemia are major/principal clinical symptoms of malaria. The latter is caused by the destruction of RBCs and the simultaneous loss of haemoglobin or by the removal of the infected erythrocytes as an immune response. Anaemia may also be caused by the ceased production of RBCs due to the bone marrow suppression. Complications in malaria infections are mainly a cause of blocked capillaries. The obstruction of these capillaries is as a result of infected RBCs and or the parasitic RBC debris. The serious complications that can result from this obstruction includes

glomerulonephritis, nephrotic syndrome and renal failure, which are all renal complications that may be life-threatening. Cerebral malaria infections involve severe headaches, cortical blindness, stroke and death (White, 2010).

2.3 Pathophysiology

Malaria pathophysiology is from erythrocyte destruction, release of plasmodium and erythrocyte material into circulation and the host's response to these. *P. falciparum* infected cells sequester in microvasculature of vital organs, interfering with perfusion and tissue metabolism. Erythrocytes with mature forms are sequestered by attaching to vascular endothelium, mainly venules, and thus disappear from circulation. As haematocrit rises blood viscosity rises and also the ease of cell adherence to endothelium. Viscosity may also affect secondary events like cardiac workload hence blood pressure. Cells with ring stages are also sequestered, although to a lesser extent, mainly in the spleen and placenta. Infected cells also undergo resetting by adhering to uninfected ones, with up to five times force as compared to normal adherence, hence clumping and reducing blood flow with subsequent events such as raised cardiac workload. They also aggregate around platelets and obstruct vessels.

Infected cells are also deformed and in *P. falciparum* become spherical and rigid from the biconcave shape. These are removed at the spleen as they are not easily filtered. Sequestration may be a mechanism to avoid removal at the spleen as they cluster and hold back from getting to the spleen. It is opposite for *P. vivax* where cells become larger and more deformable. In both cases the cells are compromised in their ability to transport oxygen.

Mild generalized permeability of systemic vessels also occurs, and may marginally contribute to oedema especially in cerebral malaria cases. Other effects are coma, renal failure,

pulmonary oedema, fluid and electrolyte changes, anaemia, hypoglycaemia, blackwater fever, coagulopathy, gastrointestinal dysfunction, end-organ dysfunction(liver, spleen, placenta, brain, heart, lungs, kidneys and bone marrow), and secondary bacterial infections.

Only the first one third of stages is diagnosed well by microscopy as plasmodia are already sequestered in the second two thirds of the asexual lifecycle. This can misguide clinicians and contribute to the outcome of the patient. The presence of more mature parasites on a blood film, even with a lower parasitaemia, might suggests worse prognosis for a patient than early stage ring forms. The mature forms may be the remnants of much greater sequestration. In this case two patients with the same peripheral parasitaemia may have a hundred fold differences in number of parasites (White, 2010).

2.4 Treatment of Malaria

Fevers have always haunted mankind and several ingenious remedies were tried to combat the fevers. In the ancient times, limb blood-letting, emesis, amputation and skull operations were tried in the treatment of malarial fever. In England, opium from locally grown poppies and opium-laced beer were tried. Even the help of astrology was sought as the periodicity of malarial fevers suggested a connection with astronomical phenomena. Malaria is a focal disease which differs in its characteristics among regions (WHO, 2010), mainly depending on climatic conditions, hence varying malarial control strategies are applicable. Prevention is based on awareness by risk recognition, avoiding mosquito bites, compliance with appropriate chemoprophylaxis, and diagnosing quickly with prompt treatment.

2.4.1. Chemotherapy of Malaria

2.4.1.1 Antimalarial drugs

Antimalarial drugs can be classified as blood schizonticidal, tissue schizonticidal, gametocidal, hypnozointicidal, or sporozointicidal. Drugs can also be classifies in 3 main groups: aryly-aminoalcohols (quinoline related) compounds (quinine, quinidine, chloroquine, amodiaquine, mefloquine, halofantrine, lumefantrine, piperaquine, pyronaridine, primaquine, tafenoquine); antifolates (pyrimethamine, proguanil, chlorproguanil, trimethoprim); and artemisinin compounds (artemisinin, dihydroartemisinin, artemether, artemotil, artesunate) and antibiotics (sulphonamides, sulphones, tetracyclines, clindamycin, macrolides, chloramphenical, fosmidomycin (under investigation). After rapid clinical assessment and confirmation of the diagnosis, full doses of parenteral antimalarial treatment should be started without delay with whichever effective antimalarial that is first available (WHO, 2006). Two classes of drugs are currently available for the parenteral treatment of *P. falciparum* severe malaria: the cinchona alkaloids (quinine and quinidine) and the artemisinin derivatives (artesunate, artemether and artemotil). Although there are a few areas where chloroquine is still effective, parenteral chloroquine is no longer recommended for the treatment of severe malaria because of widespread resistance (WHO, 2010)

2.4.1.2 Resistance to Antimalarial Treatment

The main concern remains that *P. falciparum*, especially in Africa, has developed resistance to commonly prescribed drugs such as quinine, chloroquine, mefloquine, amodiaquine, primaquine , halofantrine (Halfan®) , atovaquone, proguanil , sulphadoxine and dapsone (Madrid *et al.*, 2005; Gareth, 2004; Rang *et al*., 2003; Polrat *et al*., 2002). In Kenya, chloroquine (CQ) and sulphadoxine-pyrimethamine (SP) had been the drugs of choice, due to effectiveness and affordability. Quinine resistance in *P. falciparum* was first reported in1910,

but has neither been high grade nor compromised its use.). Mefloquine replaced quinine in Thailand in1984, as combination with SP in order to delay resistance, but resistance rapidly developed. Its cure rates however were raised on usage with artemisinin based therapy. Resistance to antifolates, proguanil and pyrimethamine, occurred within years of introduction and compromised their use, in both *P. falciparum* and *P. vivax*, but was not treated seriously until CQ resistance was first reported in 1957. The selection to CQ and pyrimethamine may have been due to impregnated salt as mass prophylaxis (White, 2010). CQ resistance is by efflux mechanism, before the level required to effectively inhibit the process of haem polymerization is attained. Polymerization is necessary to prevent build-up of the toxic by-products formed by haemoglobin digestion (Arav, 2005). Resistance to SP, due to sequential mutation at Dihydrofolate reductase (DHFR) and dihydropteroate synthatase (DHPS) enzymes (Hyde, 2002), led to the use of artemisinin based drugs (WHO, 2006). The first line drug for uncomplicated malaria in Kenya is artemether-lumefantrine and for severe complicated cases is quinine (WHO, 2006). Artemisinin acts rapidly on asexual blood stages particularly early forms, hence lowering the number of parasites that will mature and sequester in micro capillaries of vital organs. Also by acting on gametocytes, they limit transmission to new hosts and slow down the spread of resistant forms. This is different from CQ where drug resistant parasites have been found to have higher gametocyte density, favouring their transmission over sensitive strains (Hyde, 2002).The value of drug combinations, notably those with artemisinin derivatives is widely adopted in order to improve efficacy, delay development of the disease and select the drug-resistance parasites thus prolonging their therapeutic life (Polrat *et al*., 2002). However, artemisinin resistance has also been reported in murine models of malaria (Arjen *et al.*, 2009). Artemisinin resistance to P. falciparum, which is now prevalent across mainland Southeast Asia, is associated with mutations in kelch13. Prolonged courses of artemisinin-based combination

therapies are currently efficacious in areas where standard 3-day treatments are failing.

The use of diagnostic tests prior to medication prevents resistance by avoiding unnecessary drug usage, leads to large savings and improves malaria surveillance (WHO, 2010). There is need to eliminate exposure of the parasites to sub-therapeutic drug doses in order to avoid reducing therapeutic life of the drugs. If treatments lose their effectiveness, human cost from malaria will inevitably continue to rise. Tetracyclines are active against tissue and blood forms although clinical application is limited due to their toxicity, particularly foetal and infant, and also normal bowel flora suppression (WHO 2010).

Simple and fast tests that detect when parasites get resistant to front line anti- malarial drugs have been developed and are an important step in monitoring. These tests are taken together and enable monitoring and countering plasmodium's ability to adapt to every new drug. They make it possible to quickly determine if a person has a form of malaria that's resistant to artemisinin, the most potent anti-malaria drug currently available. Also map the spread of resistant malaria parasites in entire communities and regions so that control of infected mosquitoes can be focused. Additionally they pinpoint artemisinin-resistant malaria parasites so scientists can identify the responsible genes and develop new drugs to get ahead of resistance. These are better than the method of administering drugs and monitoring patient's blood at six hour intervals which is cumbersome for both patients and clinicians. One way to test how a patient's parasites respond to artemisinin, the blood is taken and treated with artemisinin in a test tube for six hours, then after washing out the drug and incubating parasites for 66 hours parasitaemia is determined. A more elaborate test involves testing resistance on ring-stage parasites. Once fresh erythrocytes are infected and ring stages form they are exposed to the drug and those that get resistant can be studied to determine the

mechanism involved and avail information on genetic markers which will facilitate tracking the spread of resistant parasites.

2.4.1.3 Future Chemotherapy

Heat shock protein 101 (HSP101) inhibition. The ability of parasites to tap into resources from a surrounding a cell and cause its death may be targeted to entomb them. This would be by targeting a single pore that a parasite uses to secret proteins which enable it to adapt as it invades a cell, including the ability to take part of the host cell membrane in order to shield itself. The HSP101 proteins are involved in normal functioning of the pores. By blocking this single step for secretion of the hundreds of proteins, parasites stopped growing and died in experimental cultures (Beck, 2014). Other researchers at the Burnet Institute neutralized the parasite in a similar fashion by disabling another protein thought to be involved in the passage of proteins through this pore. That suggests there are multiple components of the process that we may be able to target with drugs. In addition, many of the proteins involved in secretion are unlike any human proteins, which means we may be able to disable them without adversely affecting important human proteins hence selective toxicity (Beck, 2014).

2.4.2. Prophylaxis

2.4.2.1 Chemoprophylaxis

Chemoprophylaxis is the use of drugs before blood or tissue infection so as to prevent the infection or clinical manifestation. It has been found that antimalarial medicines significantly reduce Plasmodium reservoirs in populations, including ACT treatments (either artesunate-sulphadoxine, artesunate chloroquine or artemether lumefantrine) (White, 2008). It targets risk populations such as children below five years, pregnant mothers and travellers. Intermittent preventive therapy in pregnancy (IPTp) is given to pregnant women in high

transmission areas has been successful. However the policy recommended in 2009, to give infants 3 doses of SP (IPTi) during immunization visits has not been adopted by countries.(WHO 2011). IPTc, IPT to older children during malaria transmission season, was effective in Mali and Senegal. IPTa, for adults has also been evaluated for rainy seasons (Nicholas White, 2010).

Various drugs are available for travellers to areas with chloroquine resistant malaria. Mefloquine has a protective efficacy of over 90% in sub-Saharan Africa. It is used weekly and usable in the young, elderly and also during most of the pregnancy. It has some level of neuropsychiatric side effects such as vivid and strange dreams and also gastrointestinal effects. Doxycycline is an effective option for areas with mefloquine or chloroquine resistance, though contraindicated in pregnant or breast feeding mothers and children under 12-years of age. Atovaquone and proguanil, are manufactures as a fixed dose combination (Malarone), and has fewer side effects. Its cost may be a limitation given alternatives. Primaquine provides protection of 95% and 90% against *P. falciparum* and *P. vivax* respectively. It is used as 30 mg daily from 1 day before exposure to 1 week upon exiting the area. Its serious side effect is causing acute intravascular haemolysis in people with G6P deficiency. Chloroquine and Proguanil combination has been used for over two decades and is safe for all age groups, in pregnancy and in long term use (over 5 years). Its effectiveness has fallen though. Travellers to very low transmission areas may have stand-by medication for treatment instead of prophylactic ones, and preferably use them under supervision to avoid self-medication.

2.4.2.2 Vector Control

Insecticides are basic in eradicating mosquitoes which spread plasmodia during feeding on the human blood meal. See figure 2.2.

Repellents: Diethyltoluamide (DEET) is safe including on pregnancy and can be topically applied or included in soap. Permethrin and deltamethrin can be applied to clothes.. Pyrethroids are the only insecticide class currently approved for use on ITNs.

Some natural substances act as repellents including coconut oil birch tree, lemon eucalyptus (*Corymbia citriodora), citronella* oil, neem oil *(Azadirachta indica)* and permethrin (a contact insecticide).Indoor residual spraying with Organochlorines (e.g dichlorodiphenyltrichloroethane (DDT) and organophosphates (Malathion and temephos) controls mosquitoes (Mark Frandin, 1998) is also used. There have been reports of insecticide resistance, especially to pyrethroids which are dominantly used; in 27 sub-saharan countries. However, use of these chemicals has raised environmental risk concerns over their residual limits and toxicity (Malcolm, 1988).

Recent strategies of evolution-proof insecticides (that include fungal biopesticides, Wolbachia, and Denso virus) that manipulate the life cycle of the mosquitoes are undergoing further research (Read, 2009).

Insect growth regulators (IGRs): Growth inhibitors (IGRs) interrupt or inhibit the larvae of mosquitoes, and are safe and effective at low concentrations and remain effective for 2-20 weeks. They have good safety margin to non-target biota. Genetic manipulation and sterile insect techniques (SIT) are being developed with the hope of successful widespread use

(Arjen, 2009). SIT involves massive breeding of target insects in a 'factory' sterilizing the males by exposure to low radiation, which are then released over infested areas where they mate with wild females. Larval Control Stagnant water places can be abolished or targeted for treatment by chemical spraying with petroleum oils and their derivatives or application of organophosphates such as methoprene, larvicidal oils and insect growth regulators like diflubenzuron (WHO, 1995). Natural constituents of *Azadirachta indica* (limonoinds) and pyrethrum (pyrethrins) also form a group of effective larvicides. Biological methods entail the use of living organisms to control mosquitoes at various life stages of development. These include parasites, pathogens and predators. Some spiders, like the black and yellow Argiope, and insects such as emesine bugs contribute to lowering mosquito numbers. Adult and naiad stage dragon flies consume adult and larvae mosquitoes respectively, and so do some species of lizard and gecko. Birds, bats, frogs and other predators have varying impact on control. Mosquito pathogens including viruses, fungi, bacteria, nematodes, protozoa and microsproidia could be exploited (Jain, 2002). Dead spores of natural soil bacteria Bacillus thuringiensis (israelensis) interfere with larvae digestion. They can be dispersed from the air but have no residual activity so require repeated application and the larva however may turn to papae as they stop feeding. Two fungi species, Metarhizium anisopliae fungus, and the Beauveria bassiana fungus, are being used against adult mosquito (Ernst *et al*, 2005). Larvivorous Fish. Predatory fish feeding on mosquito larvae have been used to control malaria. *Gambusia affini* (mosquito fish) is widely known, some cyprinids (carps and minnows), killifish and Tilapia will also consume mosquito larvae (Chandra, 2008). Indigenous fish have been found to be the most appropriate although different types can be used. Direct introduction of tilapia and mosquito fish into ecosystems around the world have had disastrous consequences (Carles and Emili, 2007).

Figure 2.2: Anopheles mosquito feeding

(Accessed from http://naturecalendar.files.wordpress.com/2008/05/mosquito-cdc-sm.jpg)

2.4.2.3 Antimalarial Vaccine Development

The development of clinical immunity after continuous exposure to parasites in individuals living in endemic areas, gives hope for vaccine(s) against malaria. There have also been observed higher sickle cell anaemia in these regions as an indication of selection pressure of malaria on humans with patients who have one allele for sickle blood cells (sickle cell trait) having survival advantage against malaria. Vaccines are generally categorized as pre-erythrocytic, blood stage, transmission blocking, multistage multi-antigen, and whole organism vaccines (Thera, 2012). Pre-erythrocytic stage vaccines are intended to prevent infection and, if highly effective, would also prevent disease and block transmission. Most blood stage vaccines are based on antigens that coat the surface of the invasive merozoites and/or are involved in the process of erythrocyte invasion. Transmission blocking is specifically intended to block transmission target molecules that are unique to gametocytes or to subsequent mosquito stages. Whole organism vaccines have been developed targeting sporozoites The most advanced vaccine in development (RTS,S/AS01) in over 20 projects, is in Phase III clinical trials (WHO,2011).

Integrated Approach: An integrated way to control malaria is required as adoption of a single method may not be effective enough or practical. There is need to come with a natural extract that is environmentally friendly, affordable, easily available and effective against mosquitoes and plasmodium falciparum. It is necessary to determine the efficacy of locally used preparations and whether they can be exploited further.

2.5 Ethnomedicine in Treatment and Prophylaxis of Malaria

Local communities in tropical regions have used local flora as a means of preventing and treating malaria. It can be argued that these medicines, based on the use of whole plants with

multiple ingredients or of complex mixtures of plant materials, constitute combination therapies that may as well combat the development of resistance to therapy. Natural products account for 30 percent of chemotherapeutic agents.

Plants are known to synthesize compounds such as secondary metabolites that they use as protection against herbivores (i.e. these compounds act as a deterrent in plants). Secondary metabolites also protect plants from disease-causing agents (pathogens). These compounds have also been shown to produce certain therapeutic effects on the human body.

The first antimalarial drug, quinine, was isolated from the bark of *Cinchona ledgeriana*

(Rubiaceae) in 1820, and is one of the most important and oldest therapeutic options today (Sudhanshu *et al*. 2003). Other semi synthetic and synthetic aminoquinoline based analogues such as chloroquine, amodiaquine, primaquine and mefloquine have been derived from its structure, (Hyde, 2002). Artemisinin, an endoperoxysesquiterpene lactone first isolated in 1970, is the parent compound from a Chinese anti-malarial plant *Artemisia annua*

(Asteraceae). Several structural analogues having varying pharmacokinetic properties such as, sodium artesunate, dihydroartemisinin, arteether and artemether have been derived from it and constitute the most potent and safe plant-derived anti-malarial drugs in clinical use today. Several higher vascular plants are known to constitute potential anti-protozoa and other therapeutic activities (Sudhanshu *et al*, 2003).

2.5.1. Secondary metabolites

Plants in general constitute chemicals known as primary and secondary metabolites. Primary metabolites includes compounds that are necessary for cellular processes such as amino acids, nucleic acids, lipids and simple sugars whereas secondary metabolites include compounds which are produced in response to stress that is induced by abiotic (e.g. heat, drought) and biotic (e.g. herbivores, pathogens, humans) factors on the plant (Keeling & Bohlmann, 2006). Often, secondary metabolites are referred to as natural products, as these compounds exhibit effects on other organisms.

Some of these compounds have been reported as exhibiting curative effects on the human body Secondary metabolites produced by higher eukaryotes such as plants are highly toxic. These toxins are said to be stored in specific vesicles or in the vacuole of the plant; this kind of storage functions have been found as a detoxification of the plant itself and generates a reservoir of, for example, nitrogen-rich molecules. Even though some secondary plant products are very common, not every plant can produce every product. Some secondary metabolites are restricted to single species, others to related groups, but they are nearly always found only in certain specific plant organs.

Also, they are often generated only during a specific developmental period of the plant. Plants are important sources of secondary metabolites that have played an outstanding role in the development of chemotherapies. For example, morphine or mescaline have been developed directly from plants products.

2.5.2. Economic relevance of medicinal plants

The use of medicinal plants plays an essential role in the primary healthcare in Latin

America, Asian and African countries. In Africa up to 80 % of the population uses traditional medicine for primary healthcare (WHO, 2003). Plant-based 33 traditional medicines have been used to treat malaria for thousands of years in various parts of the world In Kenya, as well as in other African countries, a variety of plant species are used as herbal remedies by traditional healers in local communities as curative means for malarial symptoms (Njoroge and Bussmann, 2006)

In areas with limited access or resistance to hospital visits folk medicine is available and accessible for prompt interventions to prevent the development of severe malaria. Thus, presumptive treatment given to patients with ailments similar to the symptoms of malaria, together with plant-based research that could give rise to affordable, accessible and safe phytotherapy, may contribute to a healthy/good socio-economic status of a country.

Due to popular use of medicinal plants in the traditional setting, these plants have become lucrative in the global market. According to WHO (2003), there was a significant rise in the demand for herbal medicines both in the developing and developed countries resulting in global sales of about US$60 billion dollars.

The increased interest in natural products has resulted in the expanding of this sector in Kenya, which in turn elucidates benefits to the livelihoods in rural setting especially to women who are the ones mainly involved in the harvesting, as well as the whole community hence increasing the supplementary household

2.5.3. Conservation of medicinal plants

The use of non-destructive plant parts such as leaves or related species by traditional

herbalists to improve plant conservation should be encouraged (ICIPE). The medicinal application of the roots, bulbs and bark of many medicinal plants is considered to be the main factor contributing to the unsustainable use of these vascular plant species. The roots and other underground plant parts, according to many herbalists, are the most potent principles. Substitution of plant parts and use of similar plant species have been recommended as plant species from one genus appear to have the same bioactive compounds present in similar plant parts and the different parts for one may possess similar compounds, thus exhibiting similar pharmacological activity. This would significantly reduce the pressure on the natural-occurring medicinal plant populations (ICIPE, 2011).

Laws focusing on species conservation and biodiversity protection should be enforced and population awareness raised (Institute of Economic Affairs, 2011). Furthermore, traditional healers or communities should also be encouraged to cultivate the medicinal plants on a communal scale (ICIPE, 2011).

2.6 Phytochemical Compounds

Phytochemical investigations of several trees, shrubs and lianas have yielded compounds with anti-malarial activity and other efficacies. These compounds include:

2.6.1. Phenols

Simple phenols that are widely distributed in nature have shown characteristic inhibition of malaria parasite growth. From *Hypericum calycinum* (Hypericaceae), a prenylated phloroglucinol derivative, inhibited *P. falciparum* growth *in vitro* with an EC_{50} value of 0.88µg/ml. Anti-plasmodial activity of 2'-epicycloisobrachycoumarinone epoxide and its

sterioisomer isolated from *Vernonia brachycalyx* (Asteraceae) have been reported to show similar *in vitro* activity against chloroquine-sensitive and chloroquine-resistant strains of *P. falciparum* with EC_{50} values of 0.11 and 0.15 µg/ml respectively (Najma *et al.*, 2010).

2.6.2. Chalcones

Phlorizidin, from *Micromelum tephrocarpum* (Rutaceae), was one of the first chalcone glycoside reported to exhibit anti-parasitic activity. The most promising compound in this class of natural products is licochalcone A. It was first isolated from *Glycyrrhiza glabra* (Fabaceae) and became the subject of intensive preclinical studies starting with licochalcone A as a lead structure, a large number of chalcones have been synthesized and structure-activity relationships documented (Najma *et al.*, 2010).

2.6.3. Flavonoids

Flavonoids are widespread in the plant kingdom. Following the detection of anti-plasmodial flavonoids from *Artemisia annua* (Asteraceae) this class of compound has attracted renewed interest. Methoxylated flavonones artemetin and casticin act synergistically with artemisinin against *P. falciparum in vitro*. In studies carried out previously on *Artemisia* species, exigua flavanone A and B were isolated from *Artemisia indica* (Asteraceae) and exhibited *in vitro* activity against *P. falciparum* with EC_{50} values of 4.6 and 7.1 µg/ml, respectively (Najma *et al.*, 2010).

2.6.4. Sesquiterpenes

The anti-protozoal potential of sesquiterpenes is well established since artemisinin and its derivatives were identified as new drugs with high clinical preference. In addition to sesquiterpene endoperoxides, other sesquiterpenes with anti-plasmodial activity have been reported. The sesquiterpene lactone parthein has an EC_{50} value of 1.29 µg/ml against *P.*

falciparum in vitro. From *Neuroleaena lobata* (Asteraceae), a medicinal plant used in Guatemala for the treatment of malaria infection, activity was documented for germacranolide sesquiterpene lactones, neurolenin A and B. Two sesquiterpenes, 5-isopropyl-3, 3, 9-tri- methylbicyclo-nona-5-en-4-ol and 9, 10-*tris*-epoxy-pentadec-12-1, 2-diene with significant anti-plasmodial activities (EC_{50} < 4 µg/ml) were isolated from red marine algae *Laurencia implicate* (Rhodomelaceae) and brown algae *Potriera hornemannii* respectively (Rhizophylladaceae) (Najma *et al.*, 2010).

2.6.5. Diterpenes

Diterpenes from many species are well known for their biological activity and are among the most widely distributed terpenes in the plant kingdom. However, most of them combine high anti-parasitic activity with high cytotoxicity to mammalian cells. The macrocyclic germacrane dilactone, 16, 17-dihydrobrachy-calyxolide, from *Vernonia brachycalyx*

(Asteraceae) has good anti-plasmodial activity (EC_{50} 17 µg/ml on *P. falciparum*) but also inhibits the proliferation of human lymphocytes at the same concentration indicating general toxicity. Other anti-plasmodial diterpenes are phytol and 6-*E*-geranylgeraniol-19-oic acid isolated from *Microglossa pyrifolia* (Asteraceae). They have been found to have high anti-plasmodial activity: IC_{50} 8.5 µg/ml and 11.5 µg/ml (Dd2); IC_{50} 12.9 µg/ml and 15.6 µg/ml (Dd2), respectively (Najma *et al.*, 2010).

2.6.6. Triterpenes

Quassinoids are biosynthetically related to triterpenes and share the same metabolic precursors. The most active compound in this group is simalikalactone D from *Simaba guianensis* (Simaroubaceae) (EC_{50} < 0.02 µg/ml) but was found to be too toxic *in vivo*.

Quassinoids inhibits protein synthesis. Limonoids are also known as bitter terpenoids. One well-known plant family rich in these is Meliaceae. *Azadirachta indica*, the neem tree, widely used as an anti-plasmodial plant in Asia belongs to this family. Nimbolide (EC_{50} = 0.95 ng/ml, *P. falciparum* K1 was the first to be identified as the active anti-plasmodial principle of the neem tree. Subsequently, gedunin was also found to be active *in vitro* against *P. falciparum* parasites with EC_{50} values in the range of 0.72-1.74 µg/ml (Najma *et al.*, 2010).

2.6.7. Alkaloids

Alkaloids constitute one of the most important classes of natural products providing drugs for humans since ancient times. Most alkaloids are well known because of their toxicity or use as psychotropic drugs, for instance cocaine and morphine, but many alkaloids have a deep impact on the treatment of parasitic infection, for example quinine. A number of different bisbenzylisoquinolines with anti-protozoal activity have been identified. *In vitro* anti-plasmodial activity of most bisbenzylisoquinolines is < 1.0 µg, close to the IC_{50} of chloroquine (IC_{50} ~ 0.2 µg/ml). For instance, pycnamine from *Trichilia sp.* was found to have IC_{50} value of 0.15µg/ml. However, monomeric benzylisoquinolines do not have potential anti-plasmodial activity. Some aporhinoids, like isoguattouredigine from *Guatteria foliosa* have been tested for anti-plasmodial activity. Indoles comprise a group of alkaloids with varied biological activity. The indole sub-structure is widely distributed in the plant kingdom. Some indoles are reported to possess anti-protozoal activity. For instance, cryptolepine and related indole-quinolines isolated from *Cryptolepis sanguinolenta* were active *in vitro* (EC_{50} = 27-41 ng/ml) against *P. falciparum* (W2, D6 and K1) though they were found to be mildly active *in vivo* (10.8-19.4% through suppression of *P. yoelii* at 100 mg/kg/day. Phenanthridine and benzophenanthridine alkaloids are mostly found within three plant families only; Papaveraceae, Fumariaceae and Rutaceae. Some examples of benzophenanthridine alkaloids

obtained from plant sources are fagaronine (14), nitidine (15), sanguinarine and chelirubine. Anti-malarial activity of nitidine has been reported (IC_{50} = 9 - 108 ng/ml) against *P. falciparum* (Najma *et al.*, 2010).

2.7 Documentation and validation of traditional knowledge

Traditional knowledge (TK) is defined as "a body of knowledge built by a group of people through generations living in close contact with nature" (KIPI). It may be useful in the search for new medicine and the development of ethnomedicine from plants that are affordable and accessible to local people. However, TK is being lost at an increasing rate because of rapid population growth, changes in educational systems, environmental degradation, and development processes all leading to lifestyle changes, modernization and cultural homogenization (KIPI).Documentation of TK is, therefore important in conserving this information.

Validation is an important process which must be carried out to allow the acceptance of traditional medicine as a mainstream alternative to conventional medicine. Traditional medicines from plants are usually not characterized hence their composition in terms of beneficial compounds and harmful compounds is unknown. Furthermore, questions are raised on whether their use is beneficial or just anecdotal; this may arise from the fact that two people with similar ailments may have different clinical outcomes after using traditional medicines.

This study was undertaken to evaluate the efficacy of antimalarial plants used in the traditional setting to prevent, cure and/or alleviate symptoms of malaria. As symptoms are associated with the release of parasites from ruptured red blood cells into the blood stream,

the target for this study was to treat the erythrocytic (blood) stage of the parasites. In vitro work was carried out to assess the effects of the plant extracts against the asexual forms of the malaria parasites.

2.7.1. Vernonia lasiopus

Vernonia lasiopus is commonly known as vernonia. It is known by different names by the local communities: Muvatha (Kamba), Mucatha (Kikuyu), Olusia (Luo), Ol-euguru (Maasai) and Nkaputi (Samburu). This plant is a shrub that grows up to 3 meters high mainly in the African tropics. The bark is greyish brown and smooth. It has oval-shaped and densely hairy leaves. Flowers are pale mauve or white, in heads, flat or slightly rounded, 5 to 10 mm across. See figure 2.4. The plant is found in disturbed areas, bush land, grassland and riverine woodland or forest, growing at an altitude between 1,000 and 2,500 m.

It is reputed to have several health benefits traditionally where an infusion of powdered leaves is used to cure indigestion, severe stomach-ache, malaria and also as a purgative. A root decoction is said to be one of the most effective treatments for stomach ache (Najma *et. al*, 2010). Its use in treating other diseases varies among communities in Kenya, for example malaria (Kikuyu), scabies (Kamba), venereal diseases (luo) and sores (Maasai) (Erasto and Grierson, 2001). The organic fraction extracts of the plant was shown to possess sedative, analgesic (leave and seeds), anti ulcerogenic (leaves and seeds), and membrane stabilizing activity (leaves and roots) shown by reduced RBC lies (Erasto and Grierson, 2001).

The organic extracts of leaves of *V. lasiopus* show significant antimalarial activity (Erasto and Grierson, 2001). The chemical compounds isolated from alcoholic extracts of dried aerial parts of *V. lasiopus* are the elemanolides, epivernodalol and lasiopulide. These are carbon-10

epimers of the sesquiterpene lactones vernodalol and demethyl acroylated vernodalol, which have been found present in other species of *Vernonia*, such as *vernonia amygdalina* (Najma *et. al*, 2010).

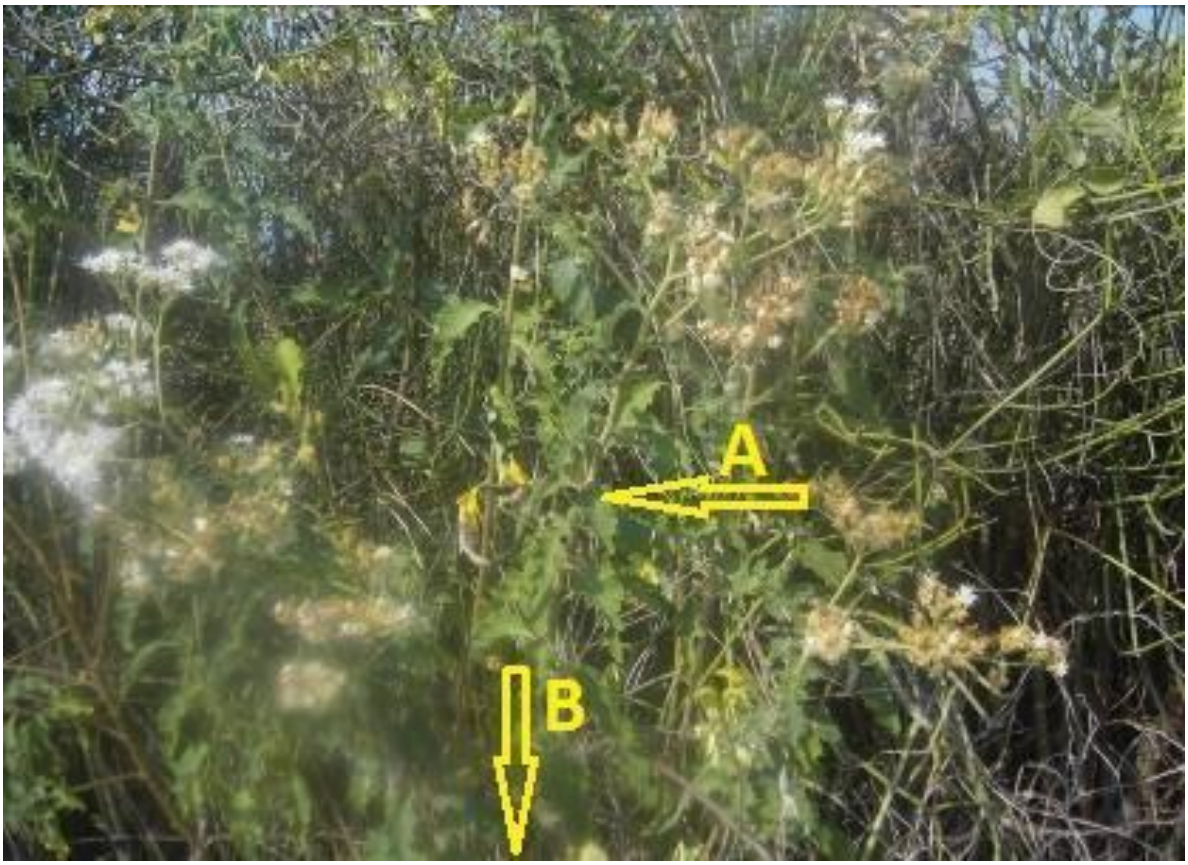


Figure 2.3: A flowering *Vernonia lasiopus* plant, showing aerial parts (A) and an arrow pointing towards the roots (B)

2.7.2. Zanthoxylum chalybeum

Zanthoxylum chalybeum is commonly known as knob wood, and bears different names among local communities: Mukenea/Mukanu (Kamba), Roko (Luo), Oloisuki (Maasai), Loisugi/Loisuki (Samburu), Mjafari (Swahili),Entare/Yeirungo (Haya), Mulungu (Ran), Eusuk (Ate), Ntaleyedungu (Lug) , Roki (Luo). Zanthoxylum means 'yellow wood', from the Greek 'xanthos' (yellow) and'xylon' (wood). The specific epithet chalybeum means steel grey.

The plant is a spiny deciduous tree growing to 8 meters in height, with a rounded open crown. The bole has characteristic large, conical woody knobs with sharp prickles and the branches also bear scattered thorns with conspicuous dark scales. The bark is pale grey and fissured. It has compound leaves, with a strong lemon smell if crushed, with 6 to 9 pairs of shiny leaflets. Flowers are sweet scented, yellow-green, in short sprays 5-10cm long below leaves on new branchlets with male and female flowers on different trees. It bears red-brown-purple, berry-like fruits 5mm in diameter, splitting to allow the shiny black seed to partly protrude. See figure 2.5. It is found in dry woodland, bush land or grassland, often on termite mounds and in rocky areas, on the coast and also in dry forest and closed thicket at an altitudinal range from sea level to 1,800 m (Najma *et.al*, 2010). In Kenya *Zanthoxylum chalybeum* fruits in March or July–August depending on the ecological region.

Extensive use of this plant as a food source and medicinally has endangered it in some areas (Maundu *et. al,* 1999). The plant is claimed to have therapeutic benefits. The Maasai tribe in Kenya, add the root decoction to the milk of their babies to boost appetite. It has found use in sickle cell anaemia where the decoction is administered for life. The root benzoic acid derivative is attributed to the anti-sickling effects due to its membrane stabilizing properties. In a repeat-dose effect study, the extracts did not negatively affect haemoglobin formation or

increase erythrocyte breakdown even at high doses that would lead to anaemia for most drugs (Ogwang *et al*., 2008).

In malaria a *Zanthoxylum* extract is reported to inhibit liver cycle of *P. falciparum* at IC50 of 4.9 ug/ml (Ogwang *et al*., 2008). The lack of growth retardation at high dose in experimental animals may suggest its fitness for use in malaria prevention in children with sickle cell, instead of chloroquine which retards growth. (Ogwang *et al*., 2008). The Stem bark from *Z. chalybeum* shows strong antimalarial activity.

The decoction is used to treat measles in Uganda. Olila in his study showed some *in vitro* antiviral property of the seed extracts, which was attributed to the alkaloid skimmianine, while the stem bark had no activity. This may be environmentally advantageous in an effort to conserve the plants. This may be responsible for curing measles by the plant. (Olila *et al*., 2002). The plant causes a significant rise in lymphocytes hence its potential as an immune system stimulant (Ogwang *et al*., 2008).

It is also used to manage sexually transmitted diseases, flu and cold, enhance digestion, throat infection (Warui, 2006). Bark or root decoction is also used as a cure for chest pains and respiratory diseases such as asthma and tuberculosis. The plant has other uses such as drink (dried leaves used to infuse tea), flavouring (stem pieces for soup), fragrance (crushed seed), toothbrushes. *Zanthoxylum usambarense* and *Z. chalybeum* contain similar alkaloids, but coloured protoberberines have been found in *Z. chalybeum* only. Phytochemical investigations of *Z. chalybeum* seed have yielded the alkaloid skimmianine. However, the antiplasmodial principles have not yet been identified. However, important biological activity of *Z. chalybeum* has been attributed to the rich alkaloidal content which include; fagaronine, fagaramide, chelythrine and berberine (Trease and Evans, 2002)

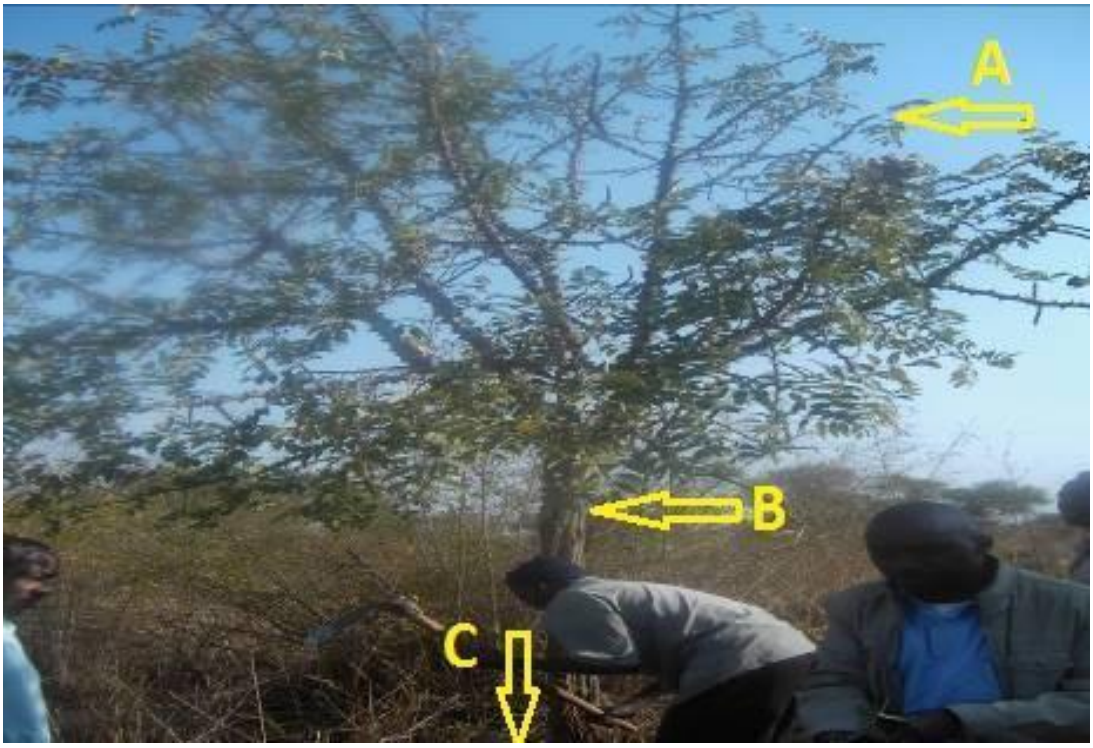


Figure 2.4: *Zanthoxylum chalybeum*; collection of (A) Leave, (B) Stem bark and (C) Root bark

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 MATERIALS

3.1.1. Reagents

The reagents were used mainly for extraction of raw materials from *Z. chalybeum* and *V. lasiopus*. These were the organic solvents: methanol, dichloromethane, *n*-hexane, acetone, ethyl acetate. All the solvents were distilled prior to use.

3.1.2. Apparatus

All reusable apparatus was soaked in hot water with liquid detergent, washed thoroughly and rinsed with distilled water, then dried in an electric oven at 105 ^{0}C for 1 hour and let to cool slowly at room temperature. Just before use, all apparatus were rinsed with a mixture of distilled solvents to remove any organic impurities.

3.1.3. Plant Materials

The plants, *V. lasiopus* (aerial parts and roots) and *Z. chalybeum* (leaves, stem bark and root bark), were collected from their natural habitats in Kionyweni sub location, Muthetheni location, Machakos County. Authentication was done at the University of Nairobi Herbarium School of Biological Sciences University of Nairobi and voucher specimen deposited

3.1.4. Animals

Eight weeks old, female Swiss mice obtained from KEMRI breeding unit of the animal house were used for *in vivo* testing. They were weighed and randomly housed in cages, each containing five mice, with bedding changed twice a week. Mice weighing 20±2 grams were selected. The cages were placed in a well aerated room with access to natural light during the

day. Feed [mice pellets (Unga feeds)] and water were given *ad libitum* and animals allowed 7 days to acclimatize

3.1.5. Parasites

Chloroquine (CQ) sensitive D6 and CQ resistant W2 plasmodium falciparum strains were obtained from cryopreserved banks of KEMRI, revived (as shown in section 3.2.4.3.) and used for *in vitro* assay.

3.2 METHODS

3.2.1. Preparation of plant material

The plant parts from *Z. chalybeum* and *V. lasiopus* were air-dried for two weeks at room temperature then ground separately into fine powder using a Willy mill grinder. The powders for *V. lasiopus* aerial parts and roots were 213gm and 594gm while *Z. chalybeam* leaves, stem bark and roots were 388gm, 518gm and 500gm respectively. They were kept in paper bags till extracted with different solvents.

3.2.1.1. Preparation of crude extracts

The powder plant material was extracted by repeatedly soaking in a mixture of methanol and dichloromethane (1:1) to obtain extracts which were filtered then evaporated to dryness using a rotary evaporator.

The various extracts were coded as follows; *Z. chalybeum* roots (ZCR), *Z. chalybeum* stem bark (ZCS), *Z. chalybeum* leaves (ZCL). *V. lasiopus aerial* parts (VLA), *V. lasiopus* roots (VLR).

Samples weighing 0.1g were separated for thin layer chromatography and the remainder stored in airtight vial at 4° C, in readiness for fractionation and biological evaluation; *in vitro* antiplasmodial activity and cytotoxic, and *in vivo* lethal dose 50 testing.

3.2.1.2 Thin Layer Chromatography: methanol (1:1) mixture.

Samples weighing 0.1 grams were dissolved in 0.5ml dichloromethane: methanol (1:1) mixture. The extracts were spotted on thin layer chromatography plates, which were developed in 10% ethyl acetate/Petroleum ether mixture. They were then sprayed with vaneline and concentrated sulphuric acid and heated in an oven. The plates were visualized under ultraviolet light.

3.2.1.3 Fractionation of crude extracts

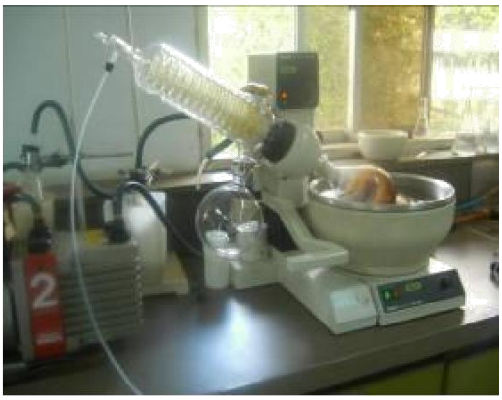
Each crude extract was fractionated. Vacuum liquid chromatography (VLC) using solvents of increasing polarity was carried out to yield six fractions of each crude extract; F1 (5% ethylacetate/Petroleum ether), F2 (20% ethylacetate/Pet ether), F3 (40% ethylacetate/Pet ether), F4 (70% ethylacetate/Pet ether), F5 (20% methanol/Pet ether) and F6 (100% methanol). See figure3.1.

For the *Z. chalybeum*, 17gm of the crude root bark extract was used, and adsorbed on silica gel in a 1:1 ratio. For the stationery phase 68g of silica gel was used. Stem bark: 13g of crude extract was used and adsorbed on 1:1 silica. 52 g silica gel was used as the stationery phase. Leaves: 14g crude extract was used, adsorbed on 1:1 silica gel and 56 g silica used as the stationery phase.

For the *V. lasiopus* 14gm of the aerial part extract was adsorbed on 1:1 silica. 56gm of silica was used as stationery phase. Roots: 11 gm. of extract was adsorbed on 1:1 silica and 44gm silica gel used as the stationery phase.

The fraction were coded as follows; *Z. chalybeum* roots fractions (ZCR1, ZCR2, ZCR3, ZCS4, ZCS5, and ZCS6), *Z. chalybeum* stem bark fractions (ZCS1, ZCS2, ZCS3, ZCS4, ZCS5, and ZCS6), and *Z. chalybeum* leaves fractions (ZCL1, ZCS2, ZCS3, ZCS4, ZCS5, and ZCS6). *V. lasiopus aerial* parts fractions (VLA1, VLA2, VLA3, VLA4, VLA5, VLA6), and *V. lasiopus* roots (VLR1, VLR2, VLR3, VLR4, VLR5, VLR6).

Crude Extracts in Solvents



Drying of Crude Extracts

Dried crude extract adsorbed in equal weight of silica gel

Crude Extract on silica column

VLC Fractionation

Six fractions from a crude extract

Figure 3.1: Pictures of the samples fractionation process

3.2.2. Preparation of Blends

Percentage yield of each fraction from the crude extract determined the amount to be included in blends formation. Subtractive bioassay was done by omitting one fraction at a time. Thus, Blend 1 (B1) was prepared by omitting fraction 1 (F1) Likewise for B2, B3, B4, B5 and B6 (Nelson *et al*., 1999). The antiplasmodial activity of the blend was compared with that of the total extract with the difference indicating the contribution of the omitted fraction.

Blending was done for each plant part as below:

1. Made stocks of each fraction (10,000µl/ml) and mixed respective volumes to make blend stocks of 1.0 ml (10,000µl/ml).

2. Example using *Z. chalybeum* blend 1

ZCR Blend 1 = F2 + F3 + F4 + F5 + F6

That is,

$$\text{Amount of F2 to add} = \frac{\text{\% yield F2}}{\text{(total \%yield f2 – f6)}} \times 1 \text{ ml Total}$$

$$\text{\%yield (F2 + F3 + F4 + F5 + F6)}$$

$$= 8.27 + 2.82 + 1.44 + 3.89 + 52.9 = 69.32$$

$$F2 = \frac{8.27}{69.32} \times 1 = 0.1193 \text{ ml}$$

The volume was added using a pipette aid as 119.3 l µl

$$F3 = \frac{2.82}{69.32} \times 1 = 40.68 \text{ µl}$$

$$F4 = \frac{1.44}{69.32} \times 1 = 20.77 \text{ µl}$$

$$F5 = \frac{3.89}{69.32} \times 1 = 56.12 \text{ µl}$$

$$F6 = \frac{52.9}{69.32} \times 1 = 763.1 \text{ µl}$$

Total volume = 1.0ml

3.2.3. Combinations to Test for Interaction

To study interaction the isobole method (Ohrt *et al*., 2002) for evaluating synergy or antagonism, was applied. The most potent sample from each plant was used alone combined with the other as shown in table 3.1.

Table 3.1: Layout of combinations

	Z. chalybeum : *V. lasiopus*				
Sample	1:0	1:3	1:1	3:1	0:1
IC_{50}W2					
IC_{50}D6					

3.2.4. Preparation for Antiplasmodial screening

3.2.4.1. *In vitro* testing for antiplasmodial activity

Erythrocytic stages of *Plasmodium falciparum* strains were obtained from a cryo preserved culture bank at malaria laboratories of Kenya Medical Research Institute (KEMRI) Nairobi, Kenya. Two laboratory reference strains were used namely the chloroquine sensitive, D6 strain, from Sierra Leone and the chloroquine resistant, W2 strain, from Indochina. Erythrocytes from blood group O+ donors were used as host cells for the parasites. Serum used was pooled from blood groups A, B and O. The donors (adults between 20-40 years) volunteered and signed a consent sheet before donation.

3.2.4.2 Drug Constitution

Stock test sample solutions were prepared at a concentration of 10,000mcg/ml in 100% DMSO and kept at 4^{o}C until use. The working solutions, 100mcg/ml, were made by diluting the stock in RPMI just before performing the assays. When added into the components in the 96-well plates, the eventual DMSO concentration was below the non-toxic concentration of 1%.

3.2.4.3 Reviving cultures

Cryopreserved plasmodia were removed from -80^{o}C or from the liquid nitrogen and thawed. The contents of the cryo -tube were then transferred to a 15ml centrifuge tube and then centrifuged at 1500rpm, 20^{o}C for 5 minutes. The supernatant was aspirated and the packed cell volume (PCV) washed by suspending in 3.5 % NaCl solution. This released them from red blood cells in which they were Cryopreserved, and centrifuged as above. The PCV was suspended in RPMI twice and centrifuged each time. The PCV was transferred to a 25 ml culture flask containing 5 ml of CMS (complete media and serum) and 0.2 ml of RBC added. This makes the culture at 6 % haematocrit. The parasites were gassed using a plugged pasteur pipette and then placed in the incubator at 37^{o}C.

3.2.4.4 Maintenance of *in vitro* cultures

In vitro P. falciparum cultures were grown continuously following a modified procedure described by Trager and Jensen (1976) and Haynes (Haynes et al 1976). Parasite levels were be maintained between 1-10 % parasitaemia at a haematocrit of 6 %, in a culture containing RPMI 1640, 25mM HEPES (N-Hydroxyethylpiperazine-N'-2-ethanolsulfonic acid), 25mM NaHCO3, 10% human serum and human type O+ erythrocytes (in acid citrate dextrose, ACD, anticoagulant). They were incubated in T-25 culture flasks at 37^{o}C and flushed with 92% N_2, 5% CO_2, and 3% O_2 gas atmosphere.

3.2.4.5 Preparation of thin smears

Culture containing erythrocytes was picked using a plugged sterile pasteur pipette and placed on a frosted microscope slide. A second slide was placed on the first slide at 45 to 60^{o} angle and moved back into the drop and then moved forward, smearing a film of blood across the first slide. The speed was varied according to the size and haematocrit of the drop. The smear was heat fixed using a hair dryer after it was fixed with methanol. The slide was then covered with 10 % giemsa solution for 20 minutes to stain. The staining solution was then rinsed with water and allowed to dry, where after it was studied microscopically (1000x). Parasitaemia was determined by counting both uninfected and infected erythrocytes, expressing the parasite count as a percentage of infected erythrocytes per total erythrocyte count. See appendix 1showing mixed stage parasite infected cells.

3.2.4.6 Culture dilution for testing

Thick and thin smears were made to determine the parasitaemia and stages of growth Parasitized continuous cultures were diluted from 6% to 1.5 % haematocrit with uninfected erythrocytes. Cultures at parasitaemia levels of 4% and ring stage parasites (appendix 2) of

80% or above were used to set experimental plates. See appendix 2. This was diluted to 0.4% parasitaemia for addition to the plate using the culture medium with serum used to grow them. Once in the plates the eventual parasitaemia after dilution was lowered from 0.4% to 0.04% due to mixture with the other contents in the wells. Some erythrocytes had multiple ring stage parasites within and this was counted as one.

3.2.5. Determination of Inhibitory Concentration 50 (IC50)

In vitro serial micro-dilution assay technique that measures the ability of the compounds to inhibit the incorporation of radio labelled [G-^{3}H] hypoxanthine by *P. falciparum* was used (Desjardins *et al.,* 1979). Experiments were conducted on 96 well plates (12 columns by 8 rows) and arranged as shown in table 3.1.

Row A was untreated (no test sample was added) and contained 200µl of plasmodium culture to serve as control for normal growth and blank red blood cells to serve as no growth control. 25 µl of media was added to the microtitre plate except to row B. 50 µl (100 µg/ml) of the test agent was added in duplicate wells in row B (six tests per row) and two-fold serial dilution performed down to row H (1.56 µg/m) followed by 200µl of parasite culture mixture (except last 4 wells of row A). Chloroquine and artemisinin were included in one plate as the reference drugs for standardizing the experiment. Chloroquine was reconstituted with water as it is not readily soluble in DMSO. Each well contained 25 µl of test sample and 200 µl culture mixture. The plates were incubated at 37°C (3% CO_2, 5% O_2, 92% N_2) for 48 hr, then Radio labeled-Hypoxanthine added to each well (0.5 µCi in 25 µl of culture medium), and further incubated for 18 hr. to allow its uptake by surviving parasites. Plates were then frozen overnight at -20°C. A complete experimental setup was as shown in figure 3.2.

Table 3.2: Layout of experiment in a 96 well micro titre plate

	1	2	3	4	5	6	7	8	9	10	11	12
A	prbc		prbc		prbc		prbc		rbc		rbc	
B	1	1	2	2	3	3	4	4	5	5	6	6
C	1	1	2	2	3	3	4	4	5	5	6	6
D	1	1	2	2	3	3	4	4	5	5	6	6
E	1	1	2	2	3	3	4	4	5	5	6	6
F	1	1	2	2	3	3	4	4	5	5	6	6
G	1	1	2	2	3	3	4	4	5	5	6	6
H	1	1	2	2	3	3	4	4	5	5	6	6

Key:

Row A: prcb- parasitized red blood cells (Positive control)

rbc- red blood cells with no plasmodium (Negative control/Blank)

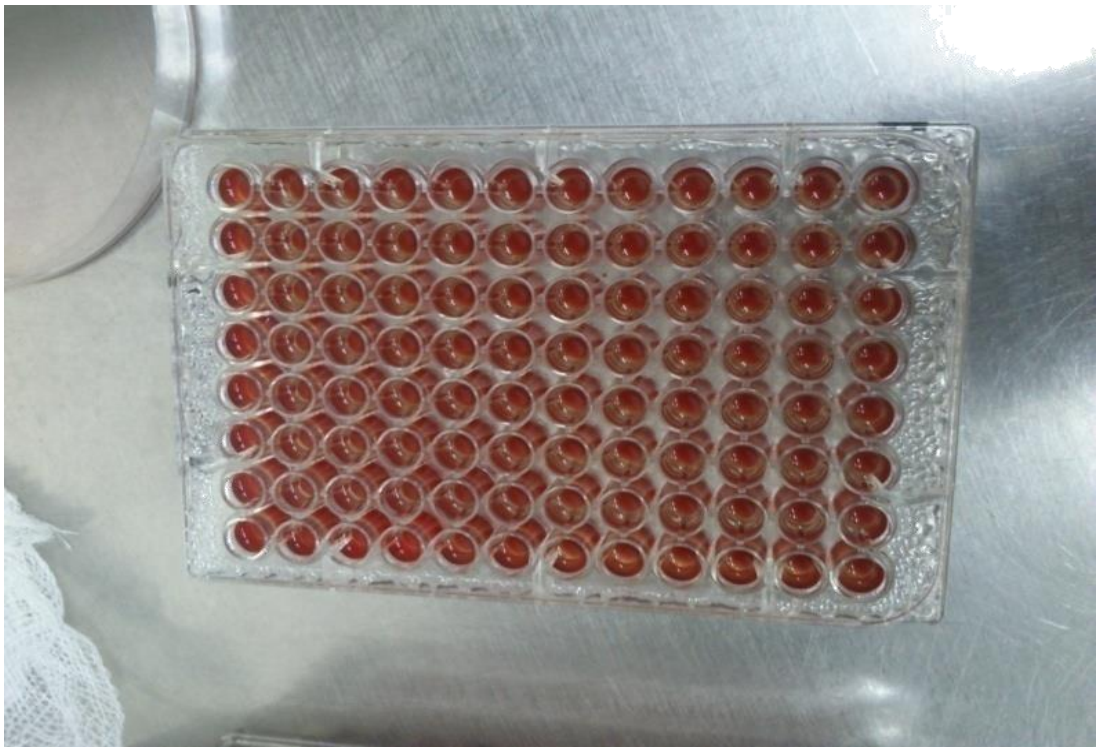


Figure 3.2: A 96 well micro-titre plate showing an experimental setup for a Median Inhibitory Concentration (IC_{50}) Test

3.2.6 Harvesting of the experimental plates

The 96-well plates were thawed at room temperature for 1.5 hours before harvesting. The plates were then harvested with a BetaplateTM cell harvester (Wallac, Zurich, Switzerland), which transfers the red blood cells onto a glass fiber filter and washes with distilled water. The dried filters was inserted into a plastic foil with 10 ml of scintillation fluid and counted in a BetaplateTM liquid scintillation counter (Wallac, MicroBeta TriLux). The results was recorded as counts per minute (cpm) per well at each drug concentration.

Data was be transferred to EXCEL and expressed as percentage of the untreated controls.

The drug concentration capable of inhibiting 50% of the *P. falciparum* (IC_{50}) was determined by logarithmic transformation of drug concentration and radioactive counts per minutes (cpm) using the formula (Sixsmith *et al.*, 1984):

$$IC_{50} = \text{antilog}\left(\log X_1 + \left[\frac{(\log Y_{50} - \log Y_1)(\log X_2 - \log X_1)}{(\log Y_2 - \log Y_1)}\right]\right)$$

Where Y_{50} is the cpm value midway between parasitisized and non-parasitisized control cultures and X_1, Y_1, X_2, and Y_2 are the concentrations and cpm values for the data points above and below the cpm midpoints.

3.3 Cytotoxicity Assay

The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) based assay is a colorimetric assay for measuring the activity of cellular enzymes that reduce the tetrazolium dye, MTT, to insoluble formazine, giving a purple colour was used. *In vitro* cytotoxicity assay was done using vero cells obtained from mammalian kidneys following a modified rapid

calorimetric assay of Mosmann (1983). The cells were revived and grown in Eagle's Minimum Essential Medium (MEM) containing 10 % foetal bovine serum (FBS), and contained in T-25 flasks. Once they were confluent, they were detached from the flask surface by adding trypsin and sub-cultured in the larger T-75 flasks using 10% media. Media change was done every 48 hours. The cells were then maintained in 2.5% FBS media once confluent and then harvested by detaching using trypsin. Trypsinization was done by draining the used up media and rinsing the cells with PBS buffer. Trypsin was pippeted and spread evenly on the cells and let to settle for 2-3 minutes, and by gently tapping the sides of the flask the cells were made to detach. Some media was introduced immediately in order for the serum contained in it to deactivate the trypsin, as trypsin would damage the cells if left exposed for long. The cell density was read using a haemocytometer on an inverted microscope and appropriate quantity of media added to dilute them to a cell density of 2×10^5cells/ml.

The cells were continuously cryo-preserved in order to replenish and build up the stocks in the bank. Storage was done at -85°C. 100µl (at a cell density of 2×10^5cells/ml) was seeded on 96-well plates, with every third column skipped, and left over night to attach. All media was then drained from row H using a micropipette and 150 µl of the test samples introduced in triplicate. Row H carried the highest drug concentration and serial dilution was carried out up to row B by pippeting 50 µl to the subsequent row and mixing with the 100 µl media already there, therefore achieving three fold dilution. Row A served as the untreated control. The plates were incubated for 48 hours at 37^oC / 5 % CO_2, after which 10 µL of MTT reagent was added and incubated for another 4 hours. The MTT gets up taken by viable cells and reduced by enzymes to purple coloured formazin crystals. The experimental setup with formazine is as shown in figure 3.3. Then all media was removed from the plates and 100 µl of DMSO added to lyse the vero cells in order to release and solubilize formazine for homogeneity. The plates were read

on a scanning multiwell spectrophotometer (Multiskan Ex labssystems) at 562 nm and 620 nm as reference. Chloroquine (diphosphate salt. Anhydrous. Mwt 515.9 Sigma chemicals) and podophylotoxin were used as controls.

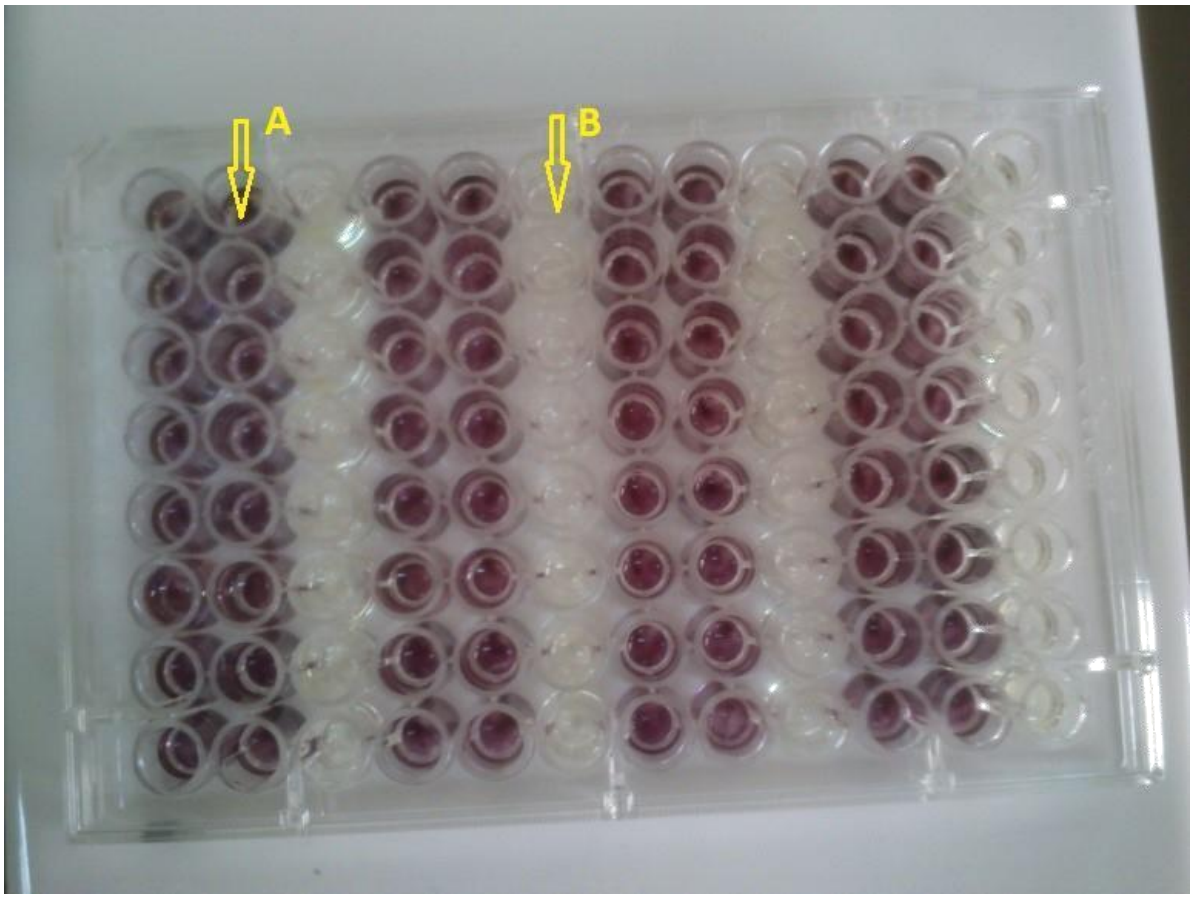


Figure 3.3: A Vero 199 cell cytotoxicity assay showing wells with purple coloured formazine (A) with cells present and controls (B) with no cells present

3.4 Acute Oral Toxicity determination

The assay was conducted according to the internationally accepted OECD guidelines and observed the KEMRI guidelines on laboratory animal use and care.

3.4.1. Drug constitution and mice inoculation

Test samples were dissolved in 0.1% Tween 80 aqueous solution, to make 500mg/ml concentration. Each mouse received 0.2ml (100mg) orally (5000mg/kg) for the acute toxicity test. The mice were deprived of feed 12 hours before and 3 hours after administration of the 35 test samples.

3.4.2. Determination of median lethal dose (LD_{50})

The LD_{50} of *Z. chalybeum* and *V. lasiopus* was determined *in vivo*. This was carried out as described by Lorke, (1983). Five dose levels ranging 500-5000mg/kg of each compound were administered orally to the mice. i.e. Dose 1= 5000, Dose 2= 2812.14, Dose 3= 1581.6, Dose4= 889.56 and Dose 5= 500mg/kg. The number of deaths within this period were noted and recorded. The LD_{50} was calculated by probit. See appendix 3.

They were obtained by the formula

$$\text{Dose 2} = \text{Dose 1} \div r$$

Where

$$r = \frac{(n-1)^{(\ )}}{\sqrt{L \div l}}$$

L=Highest dose (5000), l=least dose (500).

Five mice were used as negative control (receiving the vehicle) and groups of five mice used per test sample dosage. In total 215 mice were used. Animals were observed individually after dosing at least once within the first 30 minutes, then periodically during up to 48 hours. Those

receiving 5000mg/kg were observed twice daily for a total of 14 days. At the end of each experiment the live animals were immediately euthanized in chloroform chamber and incinerated. The parameters of interest were; changes in skin and fur, eyes and mucous membranes, respiratory, circulation, autonomic and central nervous system for signs of toxicity that may include tremors, convulsions, salivation, diarrhoea, lethargy, somnolence or coma and mortality.

3.5 Statistical Analysis

In vitro antiplasmodial activity was evaluated by determining IC_{50} while acute toxicity and cytotoxicity was evaluated by determining CC_{50} values. They were both calculated by logarithmic transformation. LD_{50} were determined *in vivo* and analysed by probit. The significance for statistical analysis was at ρ-values of 0.05 or less.

CHAPTER FOUR

4.0 RESULTS

4.0 To determine *in vitro* anti-plasmodial activity (IC50) of the crude extracts of crude extract, fractions and blends of V. lasiopus and Z. chalybeum.

4.1.1. Preparation of Plant material

The raw materials were Z. *chalybeum* root bark, stem bark and leaves, and *Vernonia lasiopus* aerial parts and roots which were dried and crushed into fine powder. The weight of the powders was 500,518, 388, 594 and 213 grams respectively.

4.1.2. Preparation of Crude Extract

The raw material parts for Z. *chalybeum* and *V. lasiopus* were extracted by serially soaking in dichloromethane: methanol (1:1) and dried by rota-vaporization. The percentage yield of the crude extracts obtained was then determined. The raw materials were Z. *chalybeum* root bark, stem bark and leaves, and *Vernonia lasiopus* aerial parts and roots .The crude extract yield was 6.93, 10.93, 5.93, 4.81 and 11.86 grams percent respectively.

See table 4.1.

Table 4.1: Percentage (%) Yield of crude extracts obtained from powders of *V. lasiopus* and *Z. Chalybeum*

Plant Part	Starting Powder (gm.)	Crude Extract (gm.)	Yield (%)	Description
	Vernonia lasiopus			
Roots	212.9g	25.26g	11.86	Yellowish crystalline powder
Aerial parts	593.9g	28.58	4.81	Dark yellowish gum
	Z. chalybeum			
Stem bark	517.9g	56.62	10.93	Dark yellow gum
Root bark	499.9g	34.67	6.93	Dark yellowish gum
Leaves	387.9g	23	5.93	Dark green gum

4.1.3. Fractionation of Crude Extract

The percentage yield of fractions, obtained by vacuum liquid chromatography of their parent crude samples, was determined. The methanol extract had the highest yield in all cases as shown in table 4.2.

For *Z. chalybeam* roots, 17g of the crude extract was used and yielded fractions 1 to 6 namely ZCR1, ZCR2, ZCR3, ZCR4, ZCR5 and ZCR6. The weight were 0.386, 1.4059, 0.4789, 0.2455, 0.6615 and 9.0g respectively. The 13g stem bark crude extract yield was ZCS1, ZCS2, ZCS3, ZCS4, ZCS5 and ZCS6 weighing 0.8847, 0.7693, 0.4011, 0.2349, 0.6057 and 5.9554g respectively. The 14g leaves crude extract yielded ZCL1, ZCL2, ZCL3, ZCL4, ZCL5, ZCL6 weighing 1.6786, 0.3062, 0.8669, 0.179, 1.0312, and 7.9337g respectively. The 14g *V. lasiopus* aerial parts crude extract yielded six fractions namely VLA1, VLA2, VLA3, VLA4, VLA5, VLA6 and weighing 0.4297, 0.6397, 0.1965, 0.2315, 1.2038 and 8.31g respectively. The 11g *V. lasiopus* roots crude extract yielded fractions VLR1, VLR2, VLR3, VLR4, VLR5 and VLR6 weighing 0.0387, 0.038, 0.2213, 0.6723, 0.4478 and 7.0g respectively. See table 4.2.

Table 4.2: Percentage (%) Yield of the fractions obtained from crude extracts of *V. lasiopus* and *Z. chalybeum* by VLC

Fraction	Weight of fraction	Weight of Crude Extract (gm.)	Yield (%)
ZCR1	0.386	17.0	2.27
ZCR2	1.4059		8.27
ZCR3	0.4789		2.82
ZCR4	0.2455		1.44
ZCR5	0.6615		3.89
ZCR6	9.000		52.9
ZCS1	0.8847	13.0	6.81
ZCS2	0.7693		5.92
ZCS3	0.4011		3.09
ZCS4	0.2349		1.81
ZCS5	0.6057		4.66
ZCS6	5.9554		45.81
ZCL1	1.6786	14.0	11.99
ZCL2	0.3062		2.19
ZCL3	0.8669		6.19
ZCL4	0.179		1.28
ZCL5	1.0312		7.37
ZCL6	7.9337		56.67
VLA1	0.4297	14.0	3.07
VLA2	0.6375		4.55
VLA3	0.1965		1.4
VLA4	0.2315		1.65
VLA5	1.2038		8.6
VLA6	8.31		59.35
VLR1	0.0387	11.0	0.35
VLR2	0.038		0.35
VLR3	0.2213		2.01
VLR4	0.6723		6.11
VLR5	0.4478		4.07
VLR6	7.000		63.64

Key: ZCR (*Zanthoxylum chalybeum* root bark); ZCS (*Z. chalybeam* stem bark); ZCL (*Z. Chalybeum* leaves); VLA (*Vernonia lasiopus* aerial parts); VLR (*V. lasiopus* roots). Example: ZCR1= ZCR fraction 1

4.1.4. Thin Layer Chromatography

The fractions were developed in 10% Ethyl acetate/petroleum ether mixture on commercial plates, sprayed with vaneline/ conc. sulphuric acid and then heated in an oven. The fractions separated into distinctive spots showing various ingredients, which could be the basis for further chemical analysis of potent ones.

4.1.5. Antiplasmodial Activity (IC_{50})

The median Inhibitory concentration (IC_{50}) values were obtained upon reading the radio label Hypoxanthine signal on the plates' setup and the counts per minute per well at each drug concentration expressed as percentage of untreated controls on EXCEL worksheet. The IC_{50} values were categorized depending on level of activity as per the legend below for un-purified plant extracts (a) and not for the purified (b) as shown below (Charity *et. al,* 2012).

Legend:

(a) Activity criteria for un-purified plant extract

1. If the IC_{50} is > 50 µg/ml, the extract is classified as inactive
2. If the IC_{50} is 11- 50 µg/ml, the extract is classified as moderately active
3. If the IC_{50} is 5-10 µg/ml, the extract is classified as active
4. If the IC_{50} is < 5 µg/ml, the extract is classified as highly active. A new range of concentrations is chosen depending on the IC_{50} determined (e.g. 2000 ng/ml).

(b) Activity criteria for purified plant extract

1. If the IC_{50} is > 5 µg/ml, the extract is classified as inactive
2. If the IC_{50} is 1-5 µg/ml, the extract is classified as moderately active
3. If the IC_{50} is <1 µg/ml, the extract is classified as active and is further evaluated using lowered range of concentrations depending on the IC_{50} determined (e.g. 100 ng/ml).

Below are results of the test samples with graphs of the highly active and active test samples.

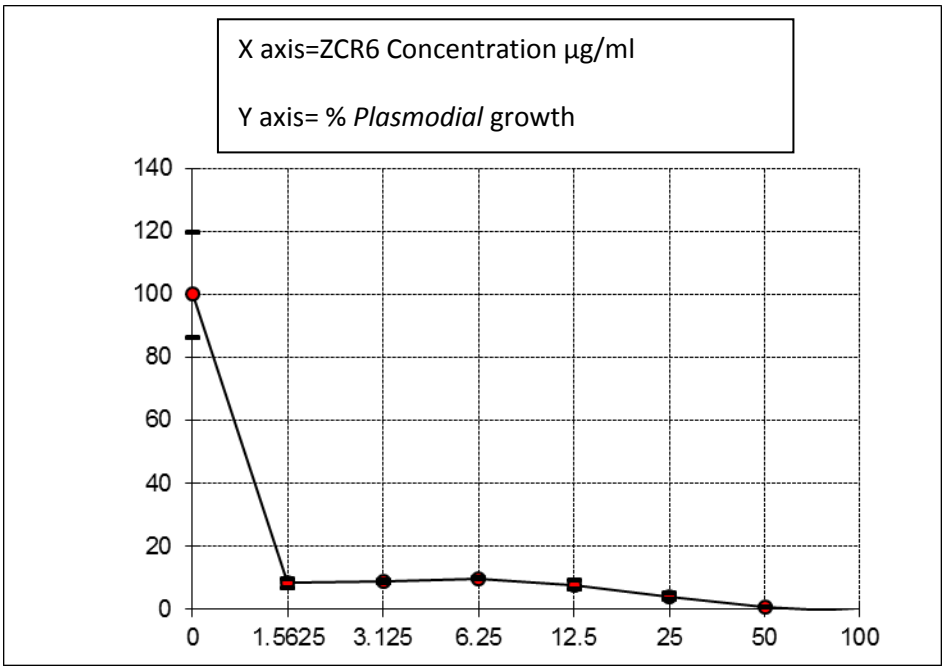


Figure 0.1: *Z. chalybeum* Root Fraction 6 (**IC_{50}: 0.78µg/ml**). Highly activity on *P. falciparum* D6 strain

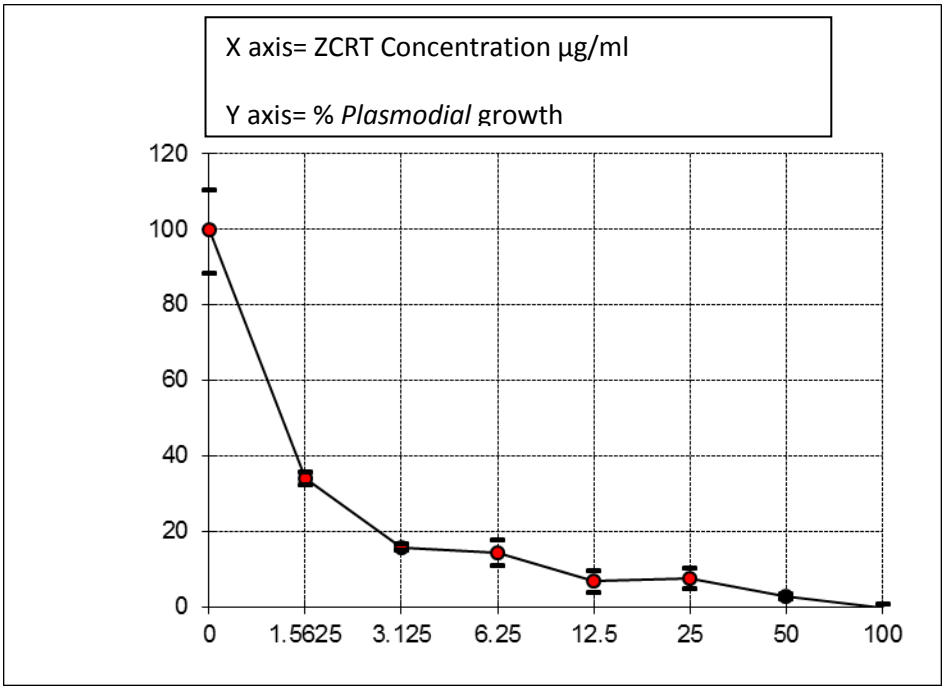


Figure 0.2: *Z. chalybeum* Root crude extract (**IC_{50}:1.11µg/ml**). Highly activity on *P. falciparum* D6 strain

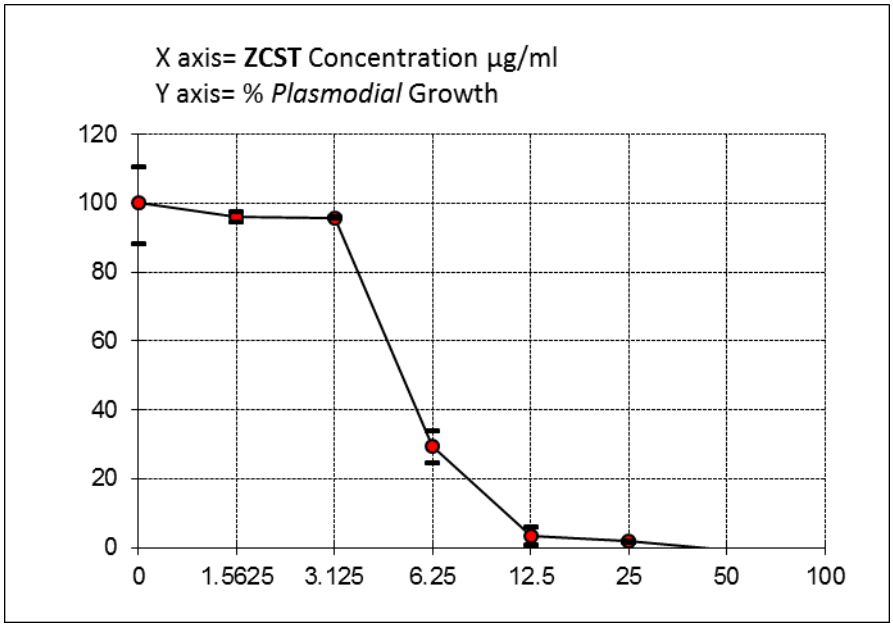


Figure 0.3: *Z. chalybeum* Stem Bark Crude extract (**IC_{50}:5.2 µg/ml**). Active on *P. falciparum* D6 strain

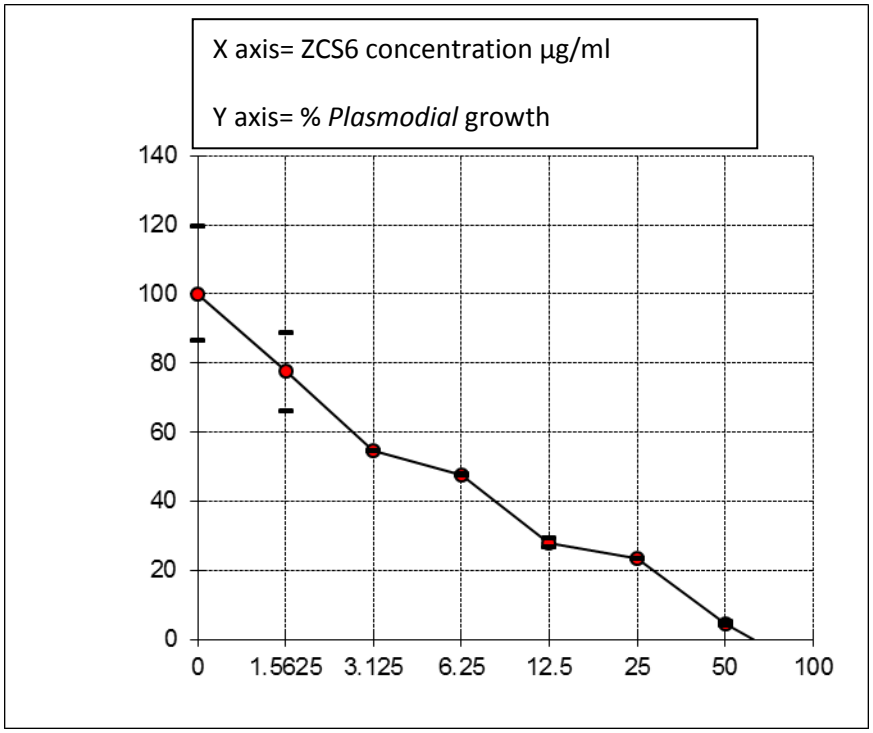


Figure 0.4: *Z. chalybeum* Stem Bark Fraction 6 (**IC_{50}: 6.0 µg/ml**). Active on *P. falciparum* D6 strain

Moderate activity on *P. falciparum* D6 strain (IC_{50}:11-50µg/ml): ZCLT,ZCL2, ZCL3, ZCL4, ZCL5, ZCR2, ZCR3, ZCR4, ZCR5, VLRT, VLR1,VLR2, VLR3, VLR4, VLR5, VLR6, VLAT, VLA2, VLA3, VLA4, VLA5, VLA6, ZCS3, ZCS4, ZCS5,

Inactive on *P. falciparum* D6 strain (IC_{50}: >50µg/ml): ZCL1, ZCL6, ZCR1, VLA1, ZCS1, ZCS2

The IC_{50} and CC_{50} for the extracts were done on the two strains of *P. falciparum*, D6 and W2, and their results presented as shown in Table 4.3 below. A total of 7 different samples were obtained per plant part, being one total extract and the six fractions. Overall, ZCR fraction 6 gave the lowest IC_{50} in D6 (0.78±0.048) and W2 (2.3±0.365).

In D6, there was a significant difference in IC_{50} ($\rho<0.05$) among the ZCR samples. The mean IC_{50} was highest (lowest activity) in fraction 1 (57.7±1.889) and lowest in fraction 6 (0.78±0.048). There was also significant difference in IC_{50} for ZCS samples ($\rho<0.05$). The mean IC_{50} was highest in fraction 1 (>100) and lowest in whole extract sample (5.2±0.424).

In W2 there was a significant difference in IC_{50} among the ZCR samples ($\rho<0.05$). The mean IC_{50} was highest in fraction 1 (57.6±0.963) and lowest in fraction 6 (2.3±0.365). There was also significant difference in IC_{50} on comparing ZCS samples ($\rho<0.05$). The mean IC_{50} was highest in fraction 1 (56.2±0.683) and lowest in fraction 6 (10.5±0.476).

Table 4.3: Viable Crude Extracts and Fractions in Descending Order (SI)

	SAMPLE	ACTIVITY (IC_{50}) on D6 strain.	CYTOTOXICITY (CC_{50})	SI	LD_{50}
	CQ	**0.06**	**>100**	**>1667**	
HIGHLY ACTIVE	ZCR6	0.78±0.048	>100	>128.21	> 5000
	ZCRT	1.11±0.356	>100	90.09	3020
(IC_{50} : < 5 µg/ml)					
ACTIVE	ZCST	5.2±0.424	>100	>19.2	> 5000
	ZCS6	6.0±0.560	>100	>16.67	> 5000
(IC_{50} : < 5-10 µg/ml)					
MODERATELY ACTIVE	ZCL2	11.3±1.627	>100	>8.85	ND
	VLRT	13.1±0.959	>100	>7.63	> 5000
	ZCL4	13.4±0.876	>100	>7.46	ND
	ZCR5	15.1±1.278	>100	>6.62	855.256
(IC_{50} : 11- 50 µg/ml)	VLR1	16.8±0.392	>100	>5.95	ND
	VLA2	18.2±0.497	>100	>5.49	> 5000
	VLA3	18.4±0.408	>100	>5.43	> 5000
	ZCL3	15.6±0.770	84.53±1.184	5.42	>5000
	ZCR4	19.4±1.042	>100	>5.14	1837.94
	VLR5	22.2±0.698	>100	>4.5	> 5000
	VLAT	25.0±1.472	>100	>4.00	> 5000
	ZCS4	27.1±1.042	>100	>3.69	> 5000
	VLR6	29.5±0.753	>100	>3.39	> 5000
	VLA6	30.6±0.606	>100	>3.27	> 5000
	ZCLT	31.0±2.160	>100	>3.23	> 5000
	VLA5	21.4±0.595	65.22±1.843	3.05	>5000
	ZCS3	33.3±1.074	>100	>3.00	3180.39
	VLR3	33.5±0.906	>100	>2.99	ND
	ZCL5	33.7±1.927	>100	>2.97	> 5000
	ZCS5	36.9±1.296	>100	>2.71	> 5000
	ZCR2	43.2±1.438	95.25±1.438	>2.21	> 5000
	ZCR3	19.3±1.120	42.01±0.771	2.18	>5000
	ZCS2	54.6±1.017	>100	>1.83	> 5000
	VLR2	13.7	41.05	1.73	ND
	VLA4	11.2±0.523	19.2±0.778	1.71	>5000
INACTIVE	VLR4	37.5±0.775	63.835±8.998	>1.70	ND
	VLA1	68.8±1.186	>100	>1.45	> 5000
	ZCL1	81.3±2.368	>100	>1.23	> 5000
	ZCL6	91.6±2.895	>100	>1.09	> 5000
(IC_{50} : > 50 µg/ml)	ZCS1	>100	>100	Approx.1	> 5000
	ZCR1	57.7±1.889	50.41±3.231	0.874	> 5000

Key: ND (not determined)

The IC_{50} and CC_{50} for the different blends of fractions was done, on the two strains of *P. falciparum* (D6 and W2), and their results presented as shown in Table 4.4 below.
In total, there were six blends per plant part and there was a significant difference ($\rho<0.05$) in overall IC_{50} when D6 was compared to W2 strain.

In D6, there was a significant difference ($\rho<0.05$) on comparing the IC_{50} for the six different blends for ZCR. The mean IC_{50} was highest in blend 6 (37.4±1.273) and lowest in blend 5 (9.6±0.698). There was also significant difference ($\rho<0.05$) among the six blends of ZCS. The mean IC_{50} was highest in blend 6 (54.7±0.935) and lowest in blend 5 (25.1±1.117).

In W2, there was a significant difference ($\rho<0.05$) among the six blends of ZCR. The mean IC_{50} was highest in blend 6 (39.9±1.042) and lowest in blend 5 (13.5±0.258). There was also significant difference ($\rho<0.05$) among the six blends of ZCS with the highest being in blend 6 (72.1±1.143) and lowest in blend 1 (2.0±0.337).

These findings were consistent with those of individual fractions, as removal of fraction 6 led to loss of activity for the respective blends in both ZCR and ZCS, as evidenced by the high IC_{50} (low activity).

Table 4.4: Antiplasmodial Activity, Cytotoxicity and Lethal Dose 50 of Blends

SAMPLE	CC_{50}	IC_{50}D6 ±SD	SI value D6	IC_{50}W2±SD
VLRB1	>100	31.2±1.134	>3.21	44.5±0.983
B2	>100	34.7±1.726	>2.88	42.7±1.451
B3	>100	16.3±2.020	>6.13	44.5±1.086
B4	>100	22.6±0.963	>4.42	76.9±1.158
B5	>100	34.4±1.687	>2.91	21.3±1.086
B6	>100	28.3±0.956	>3.53	12.4±0.622
VLAB1	59.5±5.549	69.1±1.778	0.86	74.4±1.117
B2	>100	72.3±1.598	>1.38	74.1±0.997
B3	>100	67.5±1.344	>1.48	68.7±1.117
B4	>100	47.3±0.876	>2.11	48.9±0.627
B5	>100	72.2±1.485	>1.39	74.1±0.490
B6	76.2±12.302	43.8±1.299	1.740	45.6±0.876
ZCRB1	>100	25.3±1.180	>3.95	22.8±0.698
B2	>100	13.2±1.055	>7.58	27.2±1.160
B3	64.43±1.213	12.9±0.606	4.995	22.8±0.775
B4	59.83±2.688	13.8±0.956	4.336	17.6±1.160
B5	>100	9.6±0.698	>10.4	13.5±0.258
B6	>100	37.4±1.273	>2.67	39.9±1.042
ZCSB1	>100	34.9±1.117	>20.4	2.0±0.337
B2	>100	33.4±0.560	>29.4	47.8±0.762
B3	>100	35.1±1.146	>19.6	57.9±0.762
B4	>100	41.9±0.920	>20.4	48.0±2.074
B5	>100	25.1±1.117	>19.61	33.4±1.061
B6	>100	54.7±0.935	>1.83	72.1±1.143
ZCLB1	>100	69.9±2.137	>1.43	61.7±0.698
B2	>100	60.3±0.770	>1.66	62.0±1.802
B3	>100	64.0±0.906	>1.56	65.4±1.152
B4	>100	62.1±1.988	>1.61	67.1±1.296
B5	>100	70.2±1.503	>1.42	72.4±1.042
B6	>100	51.9±1.122	>1.93	63.9±1.534

4.2 To Test The Effect Of The Combining The Most Active Samples Of The Two Plants At Varying Concentrations.

4.2.1. Combination studies

The most active samples from each plant, *Z. chalybeam* roots fraction 6 (ZCR6) and *V. lasiopus* aerial parts fraction4 (VLA4) were combined at various ratios and antiplasmodial activity determined as IC_{50} values. The results on W2 strain are shown in table 4.5 below.

Table 4.5: IC_{50} of Combinations of *Z. chalybeum* and *V. lasiopus* at Various Ratios

Sample	1:0	1:3	1:1	3:1	0:1
IC_{50}W2	2.3	2.9	17.1	2.6	6.4
IC_{50}D6	ND	ND	ND	ND	ND

4.3 To Determine The In Vitro Cytotoxicity (CC50) On Vero 199 Cells, And Acute Toxicity By Determination Of Median Lethal Dose (LD50) In Female Swiss Mice.

4.3.1. Cytotoxicity (CC_{50})

CC_{50} values of ≥90 µg/ml were considered safe on vero 199 cells and fit for further exploration. The test samples passed except for ZCL3, ZCR1, ZCR2, ZCR3, VLR2, VLR4, and VLA4 which were cytotoxic at CC_{50} values of 84.53, 50.41, 95.25, 42.01, 41.05, and 63.84 and 19.2 respectively, see graphs in appendix 4. The complete list of CC_{50} values are as per table 4.3 above.

4.3.2. Acute Oral Toxicity

The number of test mice in a test group that died per dose, within 48 hours of administration of test sample was recorded. The mice mostly displayed loss of appetite, marked pilo-erection and crowding at a corner. They elevated the front legs and the whole body violently shook before sudden death. A large majority of deaths (63%) occurred within 15 minutes, 25% occurred beyond 30 minutes and 12% after 3 hours. In total 43 deaths out of 145 were recorded for acute oral toxicity testing at 5000mg/kg. To determine the LD_{50} the doses used were 500, 889.56, 1581.60, 2812.14 and 5000mg/kg body weight, and deaths occurring within 48hours recorded as per table 4.6 below. The LD_{50} value was calculated using probit analysis.

SAMPLE	Number of mice Dead per Dose(mg/kg)					LD_{50}
	5000	2812.14	1581.6	889.558	500	
ZCLT	0					> 5000
ZCL1	0					> 5000
ZCL2	ND					-
ZCL3	0					> 5000
ZCL4	ND					-
ZCL5	0					> 5000
ZCL6	1					> 5000
ZCRT	5	2				3020.68
ZCR1	1					> 5000
ZCR2	1					> 5000
ZCR3	5	1				3180.39
ZCR4	5	4	1			1837.94
ZCR5	4	3	4	2	2	855.256
ZCR6	0					> 5000
ZCST	0					> 5000
ZCS1	0					> 5000
ZCS2	2	2				> 5000
ZCS3	5	1				3180.39
ZCS4	4	4				2268.67
ZCS5	1	1				> 5000
ZCS6	0					> 5000
VLRT	1			1 (day 4)		> 5000
VLR1	ND					-
VLR2	ND					-
VLR3	ND					-
VLR4	ND					-
VLR5	1					> 5000
VLR6	1					> 5000
VLAT	0					> 5000
VLA1	2					> 5000
VLA2	1					> 5000
VLA3	0					> 5000
VLA4	1					> 5000
VLA5	1					> 5000
VLA6	1		1			> 5000

Key: ND (not determined)

ZCRT, ZCR3, ZCR4, ZCR5, ZCS2, ZCS3, ZCS4 killed ≥3 mice at the limit dose (5000mg/kg) and had varied results at lower doses. The other samples showed LD_{50} values ≥ 5000mg/kg and were considered to be safe.

4.4 Findings on viability

The samples were listed according to their activity and safety profile. Upon obtaining IC_{50} (Inhibitory Concentration) and CC_{50} (Cytotoxic Concentration) values, the selectivity index (CC_{50}/ IC_{50}) was used as a guide for viability besides the activity only, as it considers the possibility of parasites that may have died secondary to cell death due to cytotoxic properties of the test sample. The LD_{50} was also considered. The test samples were listed in descending order according their viability of exploration into clinically used antimalarial drugs, as per the scope of this project. This is in table 4.3 above.

5.0 DISCUSSION, CONCLUSION AND RECOMMENDATIONS

5.1 Extraction and Phytochemical Screening of Plant Materials

The dried and ground plant parts from *Z. chalybeum* and *V. lasiopus* were soaked and extracted with dichloromethane: chloroform (1:1). This organic solvent mixture was to ensure extraction of components from the whole spectrum of polarity, both polar and non-polar. Organic solvents were preferred to aqueous extraction as compounds have been shown to be are more soluble in organic solvents (Bodekar, 2004). Bodekar (2004) further states that organic solvents are a good alternative in evaluating antimalarial plant properties as they have the ability to extract wide spectrum of chemical constituents. Each crude extract was fractionated by Vacuum liquid chromatography using solvents of serially varying polarities starting with the non-polar to polar. These were F1 (5% ethylacetate/Petroleum ether (PET)), F2 (20% ethylacetate/PET), F3 (40% ethylacetate/ PET), F4 (70% ethylacetate/PET), F5 (20% methanol/PET) and F6 (100% methanol. These separated constituents which were screened by thin layer chromatography. The most polar fractions (fraction 6) had the highest yield in each case.

The phytochemical tests used in this study were thin layer chromatography (TLC), which works by isolating and separating the different compounds present on an aluminium plate coated with silica gel. The preliminary phytochemical test worked on the basis of colour change to determine presence or absence of certain plant constituents. Both tests had distinct observations and results as colour presentations were observed in the different fractions, and samples were distinctly eluted on the TLC plates. The phytochemical analysis results avail preliminary data on some potential antiplasmodial compounds that are found in the plant parts being investigated. Previous studies demonstrate that the composition of phytochemicals in the plant is directly related to the pharmacological activity (Aremu, 2009).

5.2 Antiplasmodial Screening and Toxicity of Plant Extracts

For the *Z. chalybeum* Leaves fraction 2 had the best activity at IC_{50} values of 11.3 µg/ml followed by fractions 4 and 3 at 13.4 and 15.6µg/ml respectively as shown in table 4.5. Fractions 1 and 6 (ZCL1 and ZCL6) were inactive at IC_{50} values of 81.3 and 91.6µg/ml and upon their removal from the crude extract, the blend's activity does not change radically (blends 1 and 6, suggesting that they do not interact much with the other. See table 4.6.

In Z. chalybeum Stem bark the crude extract (IC_{50} 5.2 µg/ml) showed higher activity than any fraction by itself, although Fraction 6(ZCS6) with IC50 6.0 also had high activity. This is consistent with synergism among the fractions producing higher net activity.

For Z. chalybeum Roots, ZCR3, ZCR4, ZCR5 and ZCRT were toxic to the test mice. ZCR3 was also cytotoxic and this suggests one of the ways of which the mice may have died. ZCR4 and ZCR5 were not cytotoxic but had higher *In vivo* toxicity than ZCR3 hence may have killed the mice via a different process such as respiratory centre depression. These fractions may be responsible for toxicity observed in the total/crude extract, ZCRT.

The total extract, fractions 5 and 6 (ZCR5 and ZCR6) showed high activity and may be candidates for further exploration. The activity of the total extract may be largely but not exclusively due to the presence of the fractions 5 and 6.

One of the documented toxicities of *Z. chalybeum* is by Ogwang (2008) who in his study found high creatinine levels at the dose of 4000 mg/kg, predicting the potential of the extract to compromise renal output if used in very large doses. Also reported was the risk of intestinal neoplasms with long-term use. This concurs with findings of this work as there was

high toxicity leading to100% mortality of test mice at the highest dose (5000mg/ml). However, the work reported long term administration of low dose root bark extract to be safe in experimental animals (Ogwang, 2008). At low dose there were no In vivo fatalities. Negi et.al (2011) found antiplasmodial and cytotoxic activity for *Z. chalybeam*, which is similar to these findings.

The crude extract of *Z. chalybeum* (ZCRT) is the form of the plant mainly used in traditional malaria therapy for the roots. It showed LD_{50} of 3020.68 mg/kg. Fractions 5 and 4 showed higher toxicity given their lower LD_{50} values of 855.26 and 1837.94 mg/kg respectively. These are likely to be the most responsible for toxicity of the crude extract where they occur in lesser concentration.

At the highest dose of 500mg/kg, ZCRT killed all 5 mice but 2 died at 2812.14mg/kg, suggesting high toxicity if overdosed. However, in usual traditional medicine low doses of plant extracts are used and such high doses are only likely to occur in cases of acute poisoning or over dose. This study used a mice model to study the safety of the extracts, rodents are however known to metabolize drugs more rapidly than higher mammals hence the need to interpret results with care. The doses of the extracts used in traditional use are not well documented.

For *V. lasiopus* aerial parts, Blend 2 and Blend 5 show marked lowered activity, theorizing that removal of corresponding fractions leads to less activity as they may be involved in potentiating. The fractions 2 and 5 (VLA2 and VLA5) had activities of 18.2 and 21.4 respectively, in the same range as some others. Their removal however affected the overall activity more hence suggesting more they are involved in interactions to a greater extent.

VLA4 showed antiplasmodial activity of IC_{50}11.2 µg/ml, but due to the high cytotoxicity (CC_{50} 19.2 µg/ml) the selectivity index was low at 1.71, hence not very viable as an antiplasmodial agent. However it was not toxic *in vivo* hence may need to be tested if activity is maintained *in vivo*. An assumption would be that it may be deemed nontoxic by other components before it reaches the cells, such as low pH in the stomach and protective mucous lining, and so it would need to be checked if activity is also affected or is left intact.

In *V. lasiopus* roots, the crude extract (VLRT) is more active than any fraction by itself clearly showing synergy among the various fractions. For example the removal of VLR3, which has an IC_{50} of 33.5 µg/ml, leads to a drop of activity of the crude extract from 13.1 µg/ml to 16.3 µg/ml as represented by blend 3. This may mean that besides simply adding its own activity this fraction may act in synergy with others. VLRT had a kill at day 4, and it shows evidence of delayed toxicity even though this was past than the 48hr observation period.

Presence of antiplasmodial/antimalarial activity in *V. lasiopus* concurs with findings by Francis (Francis *et.al*, 2005) and Najma (Najma *et.al*, 2010), who reported favourable activity.

5.3 CONCLUSION

1. Both *Z. chalybeum* and *V. lasiopus* had viable antiplasmodial activity, through investigation of the crude extracts, fractions isolated from the crude extract and blends (as subtractive bio assay). The highly active fraction ZCR6 (IC_{50} 0.78 µg/ml) and active ZCS6 IC_{50} (6.0 µg/ml) were the most viable for exploration. Their safety profile was also good as indicated by the selectivity index (128.21 and 90.09) and LD_{50} values (>5000 and 3020 mg/kg) respectively. The fractions of *V. lasiopus* roots showed higher net activity combined than individually, as seen in VLRT (IC50 13.1 and S.I >7.63). VLR1 was the most viable fraction at IC_{50} of 16.8 and S.I of >5.95. Both had LD50 >5000mg/kg.

   The crude extracts (ZCRT and ZCST) from which ZCR6 and ZCS6 were obtained were also categorized as highly active (IC_{50} 1.11 µg/ml) and active (IC_{50} 5.2 µg/ml) respectively, corresponding to their fractions. This supports their use traditionally for treatment of malaria.

2. *Z. chalybeam* and *V. lasiopus* were found to have potent antiplasmodial activity when combined.

3. *Z. chalybeam* and *V. lasiopus* were found to be safe for medicinal use based on the high selectivity index and LD_{50} values. Crude extracts, which are commonly used traditionally, and their fractions were tested.

5.4 RECOMMENDATION

The following recommendations were made.

1. Further chemical analysis and purification is recommended for *Z. chalybeum* root bark fraction 6, and stem bark fraction 6 should be done. The moderately active samples (IC_{50}:11-50 µg/ml) may also be investigated further as some had good safety margins and may produce potential compounds if purified. *Z. chalybeum* leaves and *V. lasiopus* aerial parts may be promoted for use in traditional practice as this ensures conservations of these medicinal plants. ZCL2 (IC_{50} 11.3 µg/ml) and VLA2 (IC_{50} 18.2 µg/ml) showed good activity and safety profiles. Traditional practitioners would require to be trained on how to better extract and concentrate their treatment to boost effectiveness from these aerial parts.

2. Detailed combination studies of the various samples may be done as their interactions could result in high activity and other beneficial outcomes such as curtailing resistance. A sample without high activity may also interact favourably with others, such as by being protective against deactivation or may facilitate entry of another compound into a body compartment making it accessible hence better treatment.

3. VLA4 may require to be tested for *in vivo* antiplasmodial activity to determine if activity is maintained as it did not show *in vivo toxicity* (LD_{50} >5000mg/kg), unlike *in vitro* (CC_{50} of 19.2 µg/ml). It had the highest *in vitro* IC_{50} of all *V. lasiopus* samples at 11.4 µg/ml, which was inconclusive due to the cytotoxicity.

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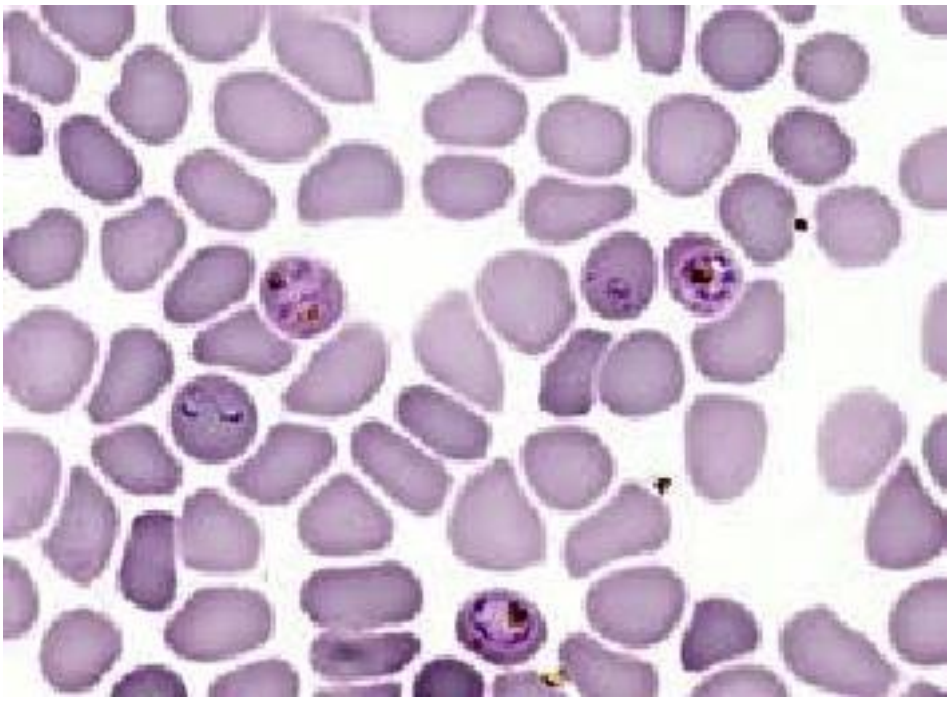
APPENDICES

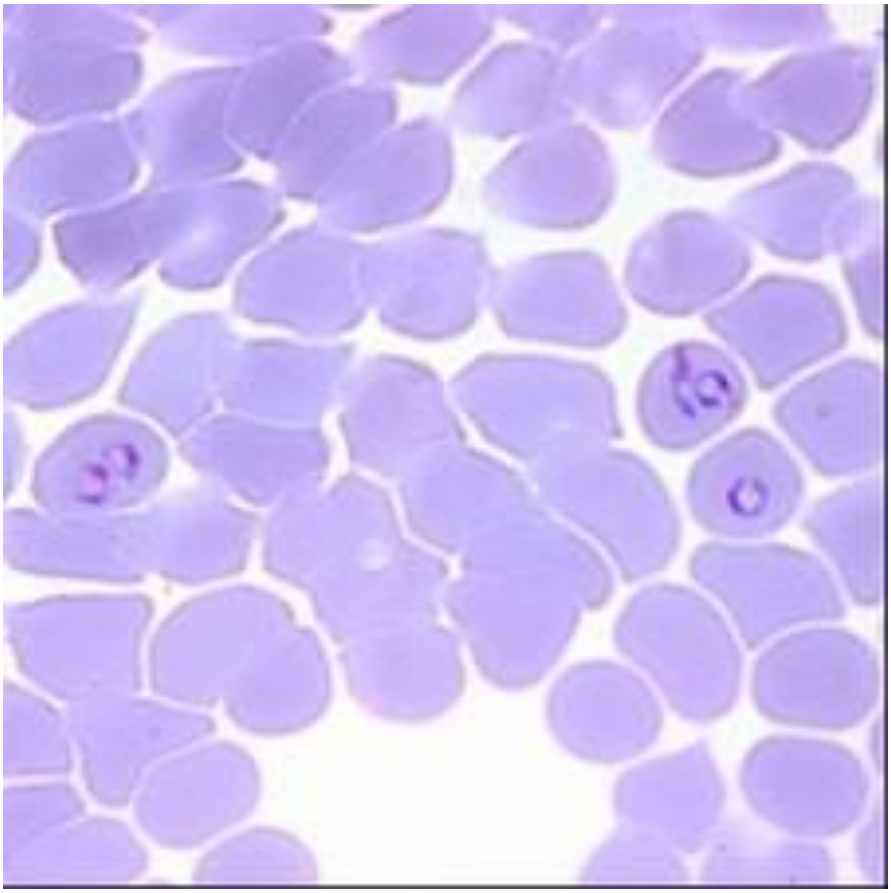
Appendix 1

Photomicrograph at ×1000 magnification, showing mixed stages of *P. falciparum* infected erythrocytes.



Appendix 2

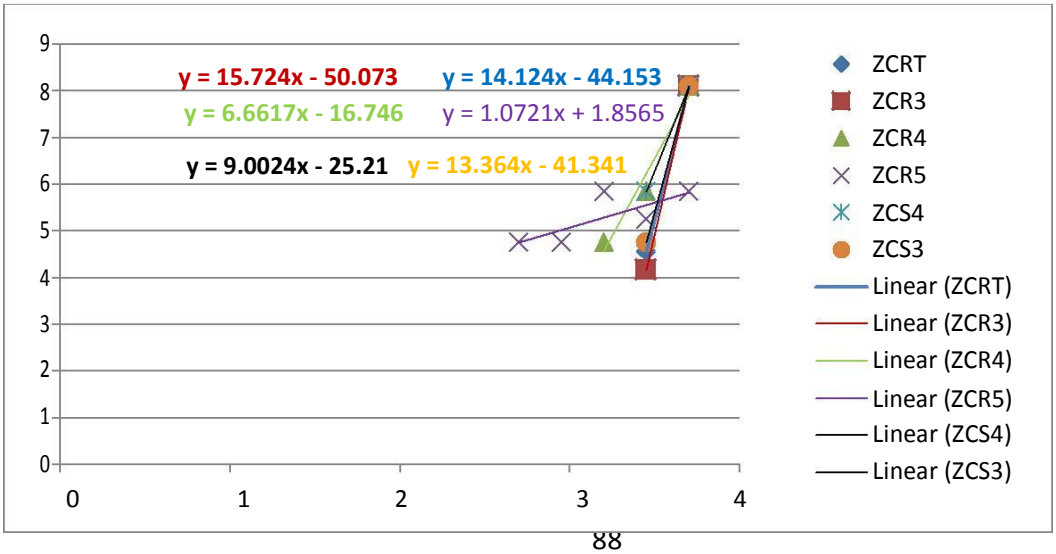
Photomicrograph at ×100**0** magnification showing ring stage *P. falciparum* infected erythrocytes



Appendix 3

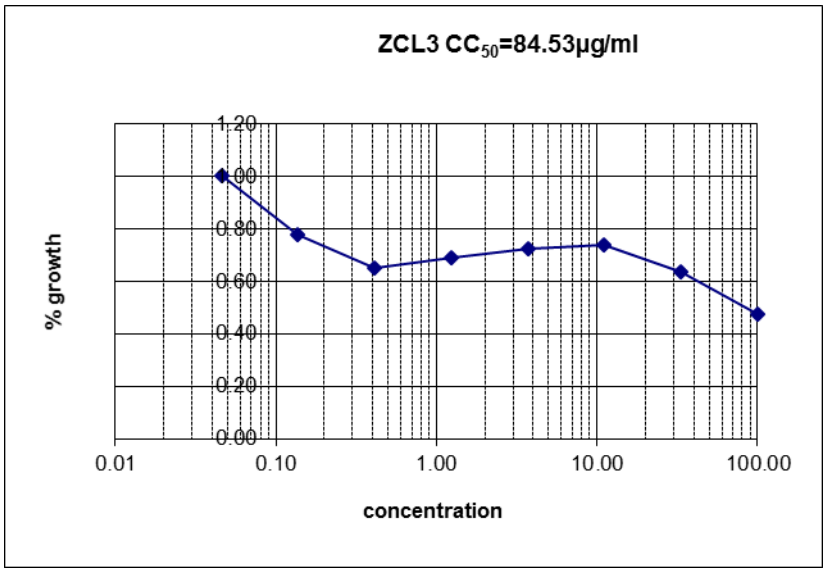
Lethal dose 50 (LD_{50}) calculation, by probit, of the various samples on *in vivo* testing on female swiss mice

Sample	Doses	Total	Dead	% mortality	Probit	Log Dose	LD50
ZCR4	5000	5	5	100	8.09	3.69897	1837.94
	2812.14	5	4	80	5.84	3.449037	
	1581.6	5	1	20	4.76	3.199097	
	889.558	5	0	0		2.949174	
	500	5	0	0		2.69897	
	Doses	Total	Dead	% mortality	Probit	Log Dose	LD50
ZCR5	5000	5	4	80	5.84	3.69897	855.256
	2812.14	5	3	60	5.25	3.449037	
	1581.6	5	4	80	5.84	3.199097	
	889.558	5	2	40	4.75	2.949174	
	500	5	2	40	4.75	2.69897	
	Doses	Total	Dead	% mortality	Probit	Log Dose	LD50
ZCS3	5000	5	5	100	8.09	3.69897	3180.39
	2812.14	5	1	20	4.75	3.449037	
	1581.6	5	0	0		3.199097	
	889.558	5	0	0		2.949174	
	500	5	0	0		2.69897	
	Doses	Total	Dead	% mortality	Probit	Log Dose	LD50
ZCS4	5000	5	5	100	8.09	3.69897	**2268.67**
	2812.14	5	4	80	5.84	3.449037	
	1581.6	5	0	0		3.199097	
	889.558	5	0	0		2.949174	
	500	5	0	0		2.69897	

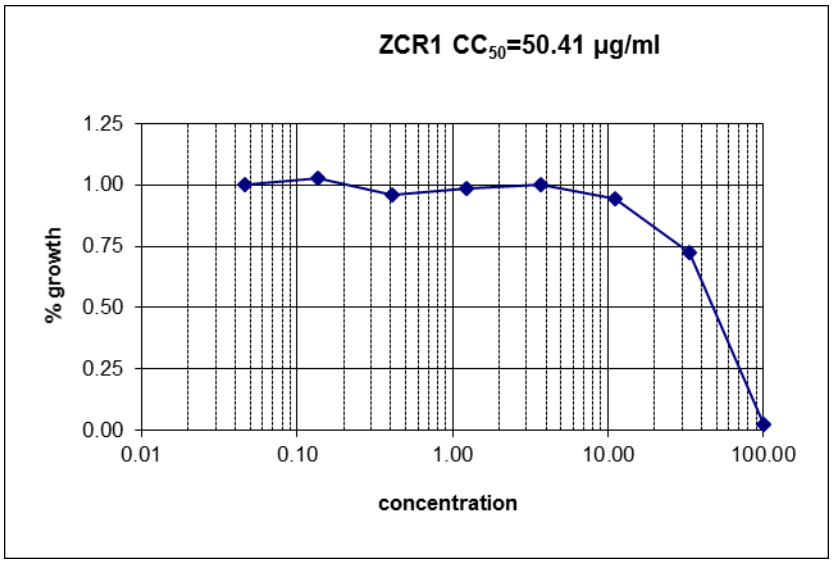


Appendix 4

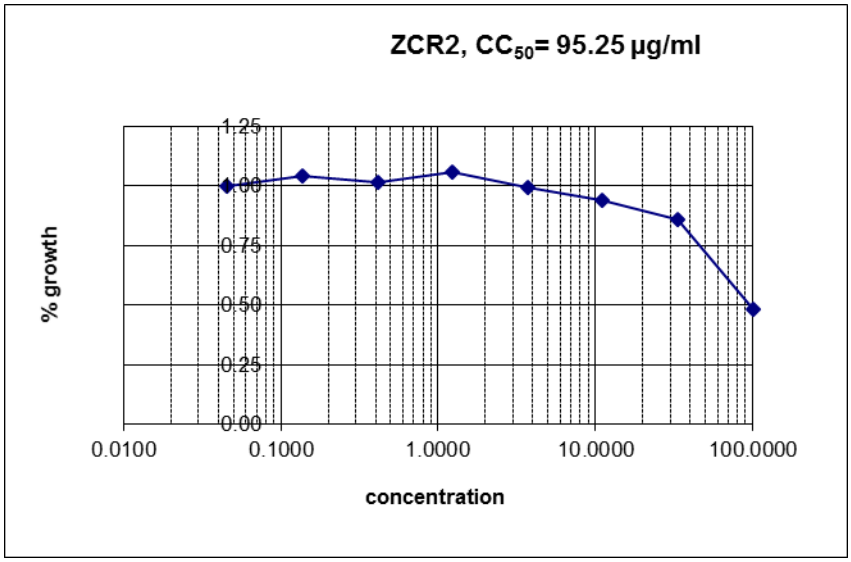
Graphs of Cytotoxic Samples (with CC_{50} < 90 µg/ ml)



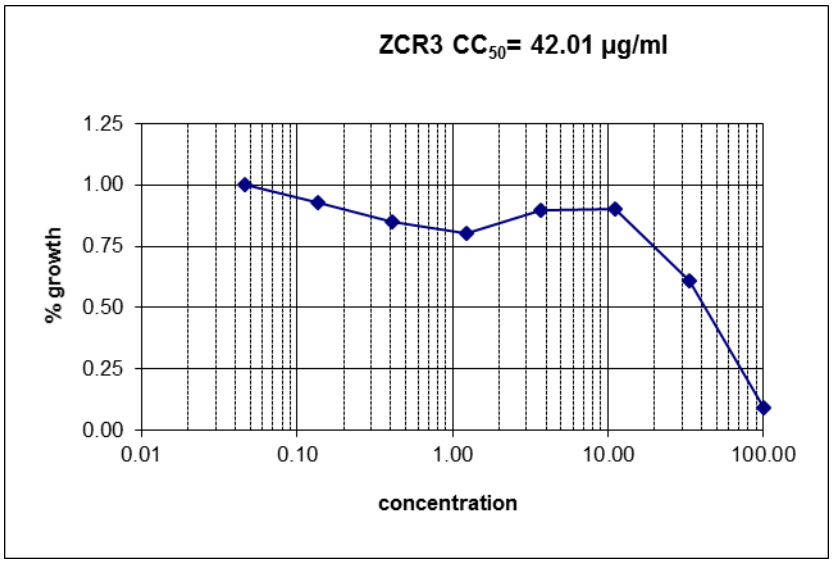
Z. chalybeum leaves fraction 3 (ZCL3)



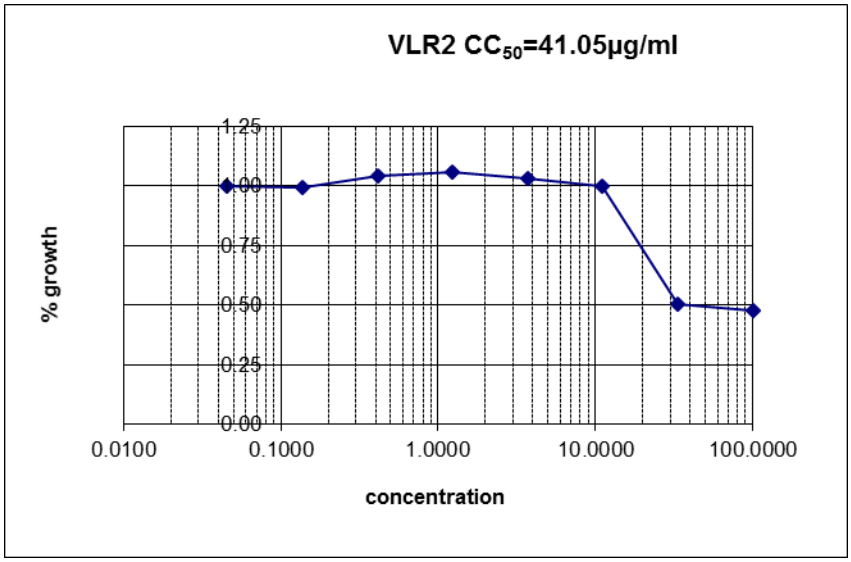
Z. chalybeum roots fraction 1 (ZCR1)



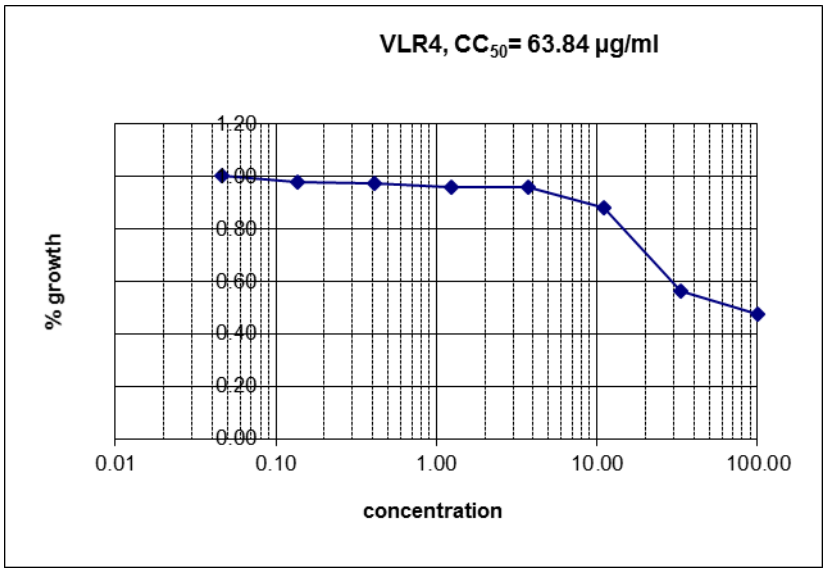
Z. chalybeum roots fraction 2



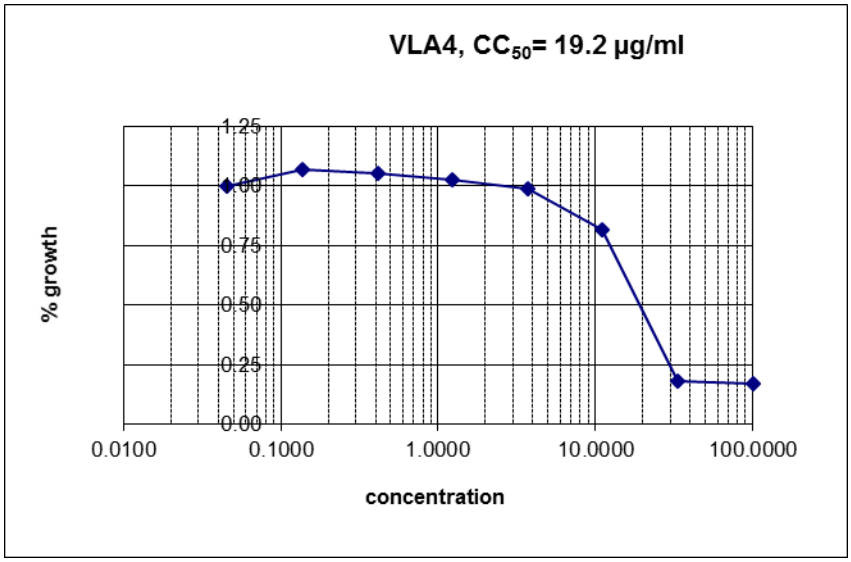
Z. chalybeum roots fraction 3 (ZCR3)



V. lasiopus roots fraction 2 (VLR2)



V. lasiopus roots fraction 4



V. lasiopus aerial parts fraction 4