In vitro anti-plasmodial and *in vivo* anti-malarial activity of some medicinal plants used by the Meru community in Kenya for treatment of malaria

By

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DECLARATION

I, Gathirwa Waweru Jeremiah, dully declare that this thesis is my original work and has not been presented for a degree in any other university or for any other award.

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We confirm that the work reported in this thesis was carried out by the candidate under our supervision.

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DEDICATION

Dedicated to my wife Cecilia Wanjiku and daughter Cassidy Mbaire

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ABSTRACT

Malaria is a serious disease affecting approximately 500 million people worldwide resulting in 3 million deaths every year. Increasing resistance to the commonly used anti-malarial drugs has impacted negatively to its treatment. Concequently, there is an urgent need to develop new drugs. Plants have been considered to be a possible alternative and a rich source of new drugs. Most traditional healers in Africa use medicinal plants in combination and not singly. Research to justify use of medicinal plants in treatment of malaria alone and in combination is a priority. The objective of this study was to investigate the efficacy and safety of traditional anti-malarial plant extracts individually and incombination. Aqueous and methanol extracts of 15 plants traditionally used for treatment of malaria in Meru District, Kenya were tested *in vitro* and *in vivo* against *Plasmodium falciparum* (D6 and W2 clones) and *P. berghei*. Toxicity of the active extracts was evaluated *in vitro* and *in vivo*, while their interactions in combination were tested by the sum-FIC method. Of the plants tested *in vitro,* 25.0% were highly active (IC_{50} <10 µg/ml), 45.59% moderately active (IC_{50} 10-50 µg/ml), 16.18% had weak activity of 50-100 µg/ml while 13.24% were not active IC_{50} >100 µg/ml. Both the water and methanol extracts of *Boscia salicifolia* Oliv. and *Artemisia afra* Jacq. ex Willd. were the most active against both the chloroquine (CQ) sensitive (D6) and the CQ resistant (W2) *P. falciparum* clones. When tested *in vivo* in a mouse model, *A. afra* and *Rhus natalensis* Bernh. ex Krauss. depicted the highest percent parasite clearance and a chemossupresion greater than 70%. Evaluating effect of combining some of these extracts with one another or with CQ against the multi-drug resistant *P. falciparum* clone W2 revealed some synergistic effect. Marked synergy/additive effect was among blends of plant extracts as opposed to blends with CQ that in many cases was antagonistic. The highest synergy was between *Sclerocarya birrea* (A.Rich.) Hochst + *Lannea schweinfurthii* (Engl.) Engl., *A. afra* + *Clausena anisata* (Willd.) Hook. f. ex. Benth., *A. afra* + *L. sweinfurthii, and A. afra* + *Clutia robusta* Pax, E, FZ. The interaction between *Tabernaemontana holstii* K. Schum + chloroquine was largely additive. Impressive cytotoxicity results were obtained with most of the plants tested revealing high selectivity indices an indication of enabling achievement of therapeutic doses at safe concentrations. *In vivo* acute toxicity on these medicicinal plants revealed that most were not toxic at a high dose of 5000 mg/kg body weight. The plants with low IC_{50} values, high percent chemossupresion and low toxicity profiles are potential sources for novel antiplasmodial agents. These findings also justify use of combined medicinal plants in traditional medicine practices.

GLOSSARY OF ABBREVIATIONS

ACTs	Artemisinin-based combination therapies
ART	Artemisinin
CMS	Complete media with serum
CPM	Counts per minute
CQ	Chloroquine
CTMDR	Centre for Traditional Medicine and Drug Research
CVR	Center for Virology Research
DAW	Distilled-autoclaved water
DDW	Double Distilled water
DMSO	Dimethyl sulphoxide
ELISA	Enzyme linked immunosorbent assay
FIC	Fractional inhibitory concentration
Hct	Hematocrit
HEPES	*N*-2-hydroxyethylpiperazine-*N*-2-ethanesulfonic acid
HIV	Human immune virus
Hrs	Hours
Hyp	Hypoxanthine
IC	Inhibitory concentration
ITN	Insecticide treated nets
KEMRI	Kenya Medical Research Institute
KU	Kenyatta University
LA	Lactic acid

MEM	Minimum essential media
MQ	Mefloquine
PABA	*p*-aminobenzoic acid
PCV	Parked cell volume
PRBC	Parasitized red blood cells
QU	Quinine
RBC	Red blood cells
RPM	Revolutions per minute
RPMI	Rose Park Memorial Institute
SP	Sulfadoxine-pyrimethamine
UPRBC	Un-parasitized red blood cells
UK	United Kingdom
USA	United States of America
V/V	Volume by volume
WM	Wash media
WHO	World Health Organization
W/V	Weight by volume

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CHAPTER ONE

INTRODUCTION AND LITERATURE REVIEW

1.1 The disease

Malaria is one of the most important infectious diseases in the tropics (Breman, 2001). It is estimated to cause 2.3 and 9% of disease burden in the world and Africa, respectively (WHO, 2000) thus ranking 3rd after pneumococcal acute respiratory infections (3.5%) and tuberculosis (TB) (2.8%). The incidence of malaria is increasing, with an estimated 300 million to 500 million cases and 3 million deaths annually (Trape, 2001). Malaria mortality and morbidity are highest in children under five in African (Snow *et al.,* 2001). Increased resistance of the parasites to the available drugs is a major contributor to mortality (Olliaro and Bloland, 2001).

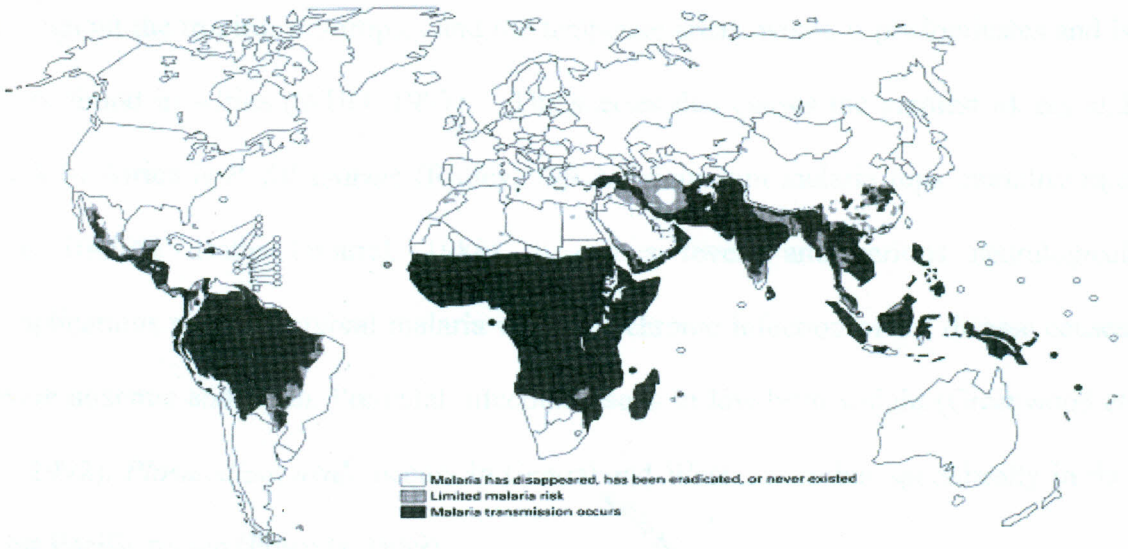

Figure 1: Epidemiological assessment of the global status of malaria (WHO, 1997)

Drug resistance by *P. falciparum,* the species that causes the greatest malaria cases and death in Africa (Ridley, 1997), has become an issue of utmost concern (Harald *et al.,*

2003; Majori, 2004), and was implicated in enhanced mortality in hyper- and holoendemic areas and the development of new and expanding foci of *falciparum* malaria in the last 5 decades (Trape, 2002). It has been identified as a major factor in the economic constraints in malaria control (Bloland, 2001). Consequently, there is an urgent need to discover, develop and avail new anti-malarial drugs.

1.1.1 Causative agents

Malaria is caused by protozoa of genus *Plasmodium*. Of the 120 species identified, only four *(P. ovale*, *P. vivax*, *P. malariae* and *P. falciparum)* are capable of infecting humans. *Plasmodium knowesli* and *P. semiovale* are rarely known to cause malaria in humans (Singh *et al.,* 2004). *Plasmodium vivax* is the most wide-spread species extending throughout the tropics, sub-tropics and the temperate areas, where it predominates and is rarely found in Africa (WHO, 1997). The species that causes the greatest illness and death in Africa is *P. falciparum* (Ridley, 1997). Falciparum malaria tops mortality rate with 10-50% deaths (Warrel, 1988). It causes fevers and serious neurological complications such as cerebral malaria while the chronic infection of the disease causes severe anaemia and death. Pre-natal infection results in low birth weight (Greenwood *et al.,* 1992). *Plasmodium ovale* occurs in Central and West Africa but sporadically in the West Pacific region (Powells, 1989).

1.1.2 *Plasmodium* life cycle

Malaria is transmitted by the bites of infected anopheline mosquitoes. Sixty anopheline species are responsible for transmission of human malaria (Kakkilaya, 2002). *Anopheles gambiae* complex is the most efficient and responsible for the transmission of malaria in Africa. The life cycle of *P. falciparum* parasite consists of two phases; the schizogonic (asexual) in the vertebrate and sporogonic (sexual) phase in the mosquito (Jensen, 1983). Infected female *Anopheles* mosquitoes carry *Plasmodium* sporozoites in their salivary glands. During blood feeding on human hosts, the sporozoites are injected into the blood circulation system and migrate to the liver where they multiply within the hepatic liver cells. They turn into merozoites and infect red blood cells, multiply further, periodically rapture the erythrocytes. The parasite is protected from attack by the host immune system because it stays inside liver and red blood cells. However, circulating infected blood cells are destroyed in the spleen. To avoid this fate, the parasite produces certain surface proteins (SP) which infected blood cells express on their cell surface, causing them to stick to the walls of blood vessels. The surface proteins are highly variable and cannot serve as a reliable target for the immune system. The stickiness of the red blood cells is particularly pronounced in *P. falciparum* and is responsible for hemorrhagic complications of malaria. Some merozoites turn into male and female gametocytes and are ingested during blood feeding.

The gametocytes fuse in the mosquito gut to form a zygote that penetrates the stomach to form an oocyst (Garnham, 1966). Within the oocyst, large numbers of sporozoites

develop and travel to the salivary gland, completing the cycle. The life cycle depend on environmental temperature, and below 16 and 18 °C *P. vivax* and *P. falciparum,* respectively, cannot complete their developmental cycle (Wernsdorfer and MacGregor, 1988).

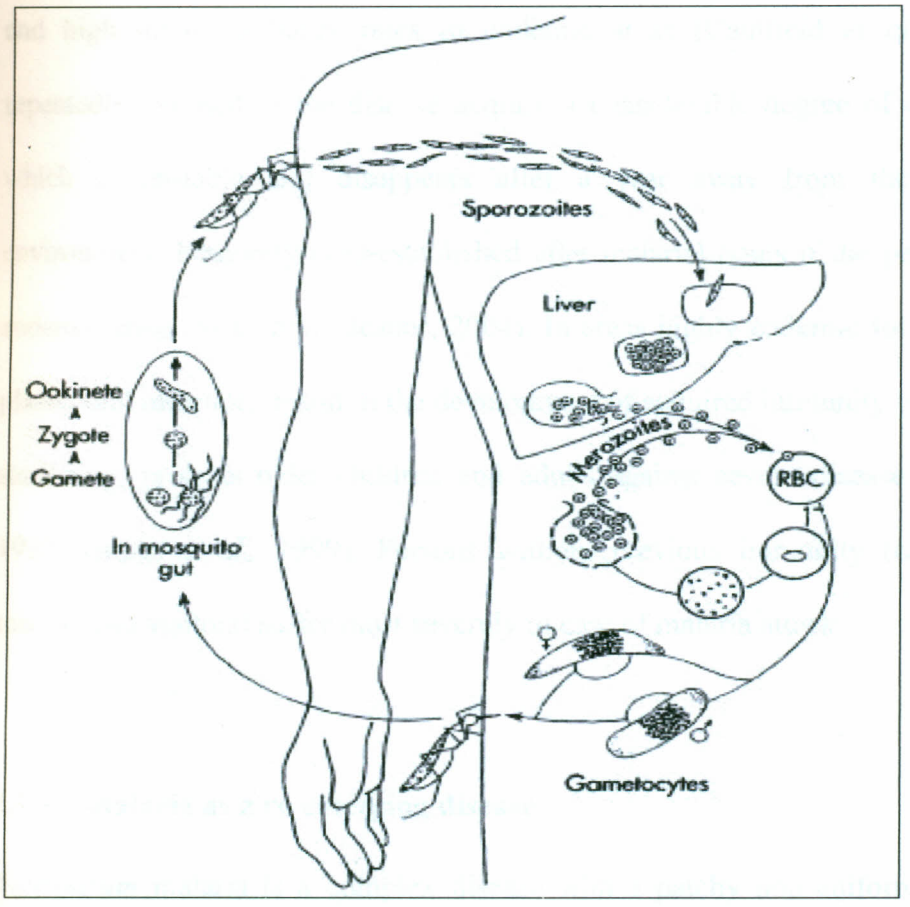

Figure 2: Life cycle of *Plasmodium falciparum* (Wirth, 2002).

1.1.3 Disease manifestation

The main symptom of malaria is fever every 3-4 days. The classical description of waves of fever coming every three or four days correspond to simultaneous bursting of red blood cells to release merozoites. Other symptoms include arthralgia (joint pain),

vomiting, and convulsions. There may be the feelings of tingling in the skin, particularly for *P. falciparum* inflicted malaria. The most severe manifestations include cerebral malaria (children and naive persons), anemia (children and pregnant women), kidney and other organ dysfunction (respiratory distress syndrome). The disease is fatal in young children if left untreated. Malaria in pregnant women is an important cause of still births and high infant mortality rates in endemic areas (Caulfield *et al.,* 2004). Persons repeatedly exposed to the disease acquire a considerable degree of clinical immunity, which is unstable and disappears after a year away from the disease-endemic environment. Immunity is re-established after malarial bouts if the person returns to an endemic zone (Siske and Eleanor, 2004). In areas highly endemic for malaria, repeated plasmodial infections result in the development of acquired immunity which although not sterilizing, protects older children and adults against severe disease (Barragan *et al.,* 1998; Gupta *et al.,* 1999). Persons without previous immunity (children, travelers, tourists and visitors) suffer most severely in case of malaria attack.

1.1.4 Malaria as a re-emerging disease

Falciparum malaria is a complex disease with a patchy non-uniform distribution and clinical manifestations that vary from one area to another within a disease-endemic zone, often showing space-time clustering of severe malaria in the community (Snow *et al.,* 1993). The relationship between fevers, clinical disease, anaemia, and cerebral malaria remains the subject of current research. The determinants of severe life-threatening malaria need further elucidation. Current research, focusing on the disease rather than the infection and the dynamics of its transmission, is bringing in new vision about the

disease, particularly the immunologic aspects. Persons with asymptomatic parasitemia constitute an important reservoir.

The epidemiology of malaria (particularly the relationship between the clinical patterns of the disease in different locations, the pattern of severe disease, and causes of deaths due to malaria) needs further research (Carlson *et al.,* 1995). In the last decade, the prevalence of malaria has been escalating at an alarming rate, especially in Africa. Between 1994 and 1996, malaria epidemics in 14 countries of sub-Saharan Africa caused an unacceptably high number of deaths, many in areas previously free of the disease (Thomas, 1998). Adolescents and young adults are now dying of severe forms of the disease. Air travel has brought the threat of the disease to the doorsteps of industrialized countries, with increasing incidences of imported cases and deaths from malaria by visitors to disease-endemic regions (Parija *et al.,* 2003).

1.1.5 Malaria diagnosis

Malaria caused by *P. falciparum* symptomatology is extremely diverse and inherently ambiguous, making a differential diagnosis based on the clinical presentation difficult (Warrell, 1993). Frequent signs and symptoms are fever, headache, joint pain, dizziness, nausea, vomiting and diarrhoea. Illness sets in abruptly and quite severely. Fever can appear in a 48-hour rhythm though these symptoms greatly vary. Its varied clinical picture often does not remind one of malaria and misdiagnosis or late recognition is frequent. The sudden and rapid progress from uncomplicated to complicated malaria with

potential irreversible and fatal outcomes makes prompt treatment absolutely essential in an early phase of clinical manifestations (Wiesman, 1986; Warrell, 1993).

Since differential diagnosis primarily of *falciparum* malaria is unreliable, etiological confirmation by demonstration of malaria parasites in the blood is essential. The most widely used diagnostic technique is the microscopic screening of blood slides for parasites. Usually a thick drop and thin film of finger-prick blood are prepared on a glass slide, chemically fixed, stained with Giemsa solution (or other formulations) and screened under oil immersion microscopy (magnification of 400 to 1'000 times). While the thick drop provides results with an increased sensitivity due to the relatively large volume of blood, the thin film permits one to quantify the blood infection rate and to determine the malaria species. This kind of diagnosis requires skilled technicians and high power microscopes otherwise misdiagnosis is likely (Trape, *et al.*, 1985; McGregor, 1986; Oaks *et al.*, 1991). A serious problem for diagnosis is that the intake of anti-malarial drugs prior to the blood test, which in many areas occurs as a consequence of the widely practised self-administration of drugs (Foster, 1991), may lead to temporary or partial parasite clearance, so the infection is microscopically undetectable (Benenson, 1985). Rapid diagnostic tests (RDTs) already in use and other antigen-based diagnostic assays that are being developed offer another window of diagnosis (Olliro and Taylor 2003).

1.2 Malaria control

The current strategy for malaria control, adopted by the Ministerial Conference on Malaria in Amsterdam in 1992, is to prevent death, reduce illness, and decrease social and economic loss due to the disease (WHO, 1998). Its practical implementation requires two main tools: first, drugs for early treatment of the disease, management of severe and complicated cases, and prophylactic use of drugs on the most vulnerable group (children ≤5 and pregnant women); second, insecticide-treated nets for protection of the most vulnerable group against mosquito bites.

1.2.1 Vaccine development

Various characteristics of the *P. falciparum* parasite make it difficult to make an effective vaccine. Each infection is known to present several thousand antigens to the human immune system. Secondly, the parasite changes through several life stages even while in the human host. Thirdly, it is possible to have multiple malaria infections of different species and strains at the same time (Hoffman *et al.,* 1996). However, there have been several initiatives to develop malaria vaccines. Important candidate antigens for vaccine development include merozoite surface protein (MSP) (Cowman *et al.,* 2002) of asexual (Kumar *et al.,* 2002) and pre-erythrocytic stages (Ballou *et al.,* 2002).

Candidate vaccines include nucleic acids (Doolan and Hoffman, 2002). But peptide-based vaccines have also been used successfully though they face the challenge of toxic adjuvant, which are critical for immunogenicity of synthetic peptides (Ben Mohamed *et*

al., 2002). In Tanzania, a double-blind phase III trial of the Colombian SPf66 peptide vaccine demonstrated that it reduced the numbers of first malarial fevers in children by one third (WHO, 1996). A randomized trial of the efficacy of RTS,S/AS02; a pre-erythrocytic vaccine candidate based on *P. falciparum* circumsporozoite surface protein (CSP) (antigen) against natural *P. falciparum* infection in semi-immune adult men in Gambia revealed efficacy of 71% during the first 9 weeks of follow-up (Bojang *et al.*, 2001). Recent trials in Mozambique with RTS,S/AS02A showed 57.7% reduction of severe malaria prevalence (Alonso *et al.*, 2004). The vaccine was found to be safe, well tolerated and immunogenic (Alonso *et al.*, 2004).

1.2.2 Vector control

Controlling mosquito population at larval or adult stages of their life cycle has been effective for malaria control. Insecticides have been used in adult vector control. Organochlorines like 1,1,1-trichloro-2,2-*bis*-(*p*-chlorophenyl)ethane (DDT) have been used to control mosquitoes (Gunasekaran *et al.*, 2005). By 1964, malaria was eradicated from parts of India by use of DDT house spraying (Sharma and Mehrotra, 1986). DDT is non-biodegradable hence a major environmental problem. It is also toxic to non-target organisms including man (Charles *et al.*, 1995). Other synthetic insecticides such as organophosphate, organocarbamates and synthetic pyrethroids such as allethrin and cyclethrin have been used as effective insecticides (Shono *et al.*, 1991). However, they suffer from resistance developed by mosquitoes against them and toxicity to non-target organisms.

The use of insecticide-treated nets (ITNs) can reduce malaria incidences (Ter Kuile *et al.*, 2003). The efficacy of ITNs for reducing infant deaths was demonstrated in Dakar (Alonso *et al.*, 1991). Subsequent large-scale multi-center studies in six countries across Africa confirmed this finding (Binka *et al.*, 1996; Lengeler *et al.*, 1996). Currently, several synthetic pyrethroids are recommended with permethrin topping the list (Ter Kuile *et al.*, 2003). Fear of increased resistance to permethrin have been expressed and demonstrated (Vulule *et al.*, 1994). Improper use of ITNs, non-compliance and the unaffordable re-treatment regime has reduced their impact on malaria transmission (Lines and Addington, 2001). The costs of the nets and treatment still inhibit wide-scale use (Worrall *et al.*, 2005).

Synthetic repellents such as dimethyphthalate and 2-ethyl-1, 3- hexanediol have not provided a great impact in controlling the rate of mosquito bites and transmission of malaria parasites since most of them are highly volatile and thus provides only short-lived protection against the vector (Frances and Cooper, 2002). The use of some mosquito repellents such as *N, N*-diethyltoluamide (DEET), IR and KBR (also known as picaridin, or by trade name Bayrepel) can reduce malaria as well as the discomfort of insect bites (Costantini *et al.*, 2004). DEET is currently the most used synthetic repellent (Frances and Cooper, 2002). However, concerns have been raised about its safety (McGready *et al.*, 2001).

The discovery of *p*-menthane-3, 8-diol as an effective mosquito repellent from *Eucalyptus citriodora* (Trigg, 1996) has increased the choice of topical repellents. This compound is rapidly replacing DEET from the market (Govere *et al.,* 2000). Larval population management has also been employed for control of malaria. In the U.S.A oil sprays on water has been used to control mosquito larvae (Wigglesworth, 1976). However, concerns about fate of other non-target organisms have discouraged this practice (Wigglesworth, 1976).

Inorganic larvicides like Paris green have also been used but the high heavy copper content and its toxicity to other aquatic organisms have limited the practice (Birn, 1998). The synthetic insecticides (organochlorines, organophosphates, organocarbamates and pyrethroids) have all been used in larval control (Walker, 2000). However, their persistence in environment, toxicity to non-target organism and the increase in resistance developed by mosquitoes have discouraged their widespread use (Ali *et al.,* 1999). One of the earliest reports of the use of plants extracts against mosquito larvae found that plant alkaloids like nicotine, anabasine, methylanabasine and lupinine extracted from Russian weed, *Anabasis apylla*, killed larvae of *Culex pipens* Linn, *C. territans* Walker and *C. quinquefasciatus* Say (Campbell *et al.,* 1933). Other bioorganic larvicides include rotenone, azadaractin and unsaturated amides (Jacobson, 1989). Evaluation of 3 insect growth regulators (IGRs) (diflubenzuron, methoprene, and pyriproxyfen) against *Culex quinquefasciatus* larvae showed efficacy in the order of pyriproxyfen, diflubenzuron and methoprene (Ali *et al.,* 1999).

The discovery of the selective mosquito-pathogenic bacterium *Bacillus thuringiensis* var. *israelensis* (Bti) and *B. sphaericus* (Bs) (Das *et al.,* 1997; Scholte *et al.,* 2004; Lee *et al.,* 2005) curtailed widespread interest in the search for other suitable biological control agents. Fungal pathogens Lagenidium, Coelomomyces and Culicinomyces have been shown to affect mosquito populations (Scholte *et al.,* 2004). Unlike insecticides, these bio control agents are host specific and safer to the environment (Scholte *et al.,* 2003).

1.2.3 Treatment and prevention

If diagnosed early, malaria can be treated, but prevention is always much better and substances that inhibit the parasite are widely used by visitors to the tropics. Since the 17th century quinine (QU) has been the prophylactic drug of choice for malaria (Mengesha and Makonnen, 1999). The development of quinacrine, chloroquine, mefloquine, amodiaquine and primaquine in the 20th century reduced the reliance on quinine. These anti-malarial drugs can be taken for prevention of the disease, and is recommended for travelers to affected regions (Goodyer and Gibbs 2004). Recently, certain strains of *P. falciparum* have developed resistance to chloroquine and its analogs that have been used on the first line of treatment in many countries, thus complicating treatment of malaria. In West Africa, where the local *P. falciparum* strains are particularly virulent, Lariam is now the recommended prophylactic, despite being the cause of psychological problems in some vulnerable people (Juckett, 1999).

1.2.3.1 Chemoprophylaxis

8-Aminoquinolines is the only class of gametocytocides used in chemoprophylaxis. Very few of these have been developed into effective anti-malarial drugs but they are useful in chemoprophylaxis. Primaquine was recently reconsidered for malaria chemoprophylaxis to eliminate *P. falciparum* at the early stage of infection, when parasites develop in the liver, thus preventing the clinical symptoms (Basco *et al.,* 1999). Despite its good oral absorption, the drug has a short half-life and needs to be administered daily. Serious toxicity can be a major problem in patients with glucose-6-phosphate dehydrogenase deficiency (Robert *et al.,* 2001). Primaquine interferes with the mitochondrial function of *Plasmodium* parasites (Robert *et al.,* 2001). Tafenoquine is a primaquine analog with a longer elimination half-life (14 days compared to 4 hours for primaquine) (Peters *et al.,* 1993). It has a larger therapeutic index than primaquine. This drug may be useful for chemoprophylaxis of *P. falciparum* and for prevention of relapses of *P. vivax* inflicted malaria (Lell *et al.,* 2000). Primaquine has been widely used for the treatment of the hypnozoites (liver reservoirs) responsible for the relapsing forms of *P. vivax* and *P. ovale* (Robert *et al.,* 2001).

1.2.3.2 Chemotherapy

1.2.3.2.1 Anti-malarial drugs and their mechanism of action

Few compounds are active against gametocytes and the intra-hepatic stages of the parasite. This involves use of chemical agents to clear the parasites once they are in the body. In most cases, anti-malarial drugs target the asexual erythrocytic stage of the

parasite (Francis *et al.,* 1997). The available commercial anti-malarial drugs can be classified into 5 groups based on their chemical structural relationships. The main chemical classes of active schizontocides are 4-aminoquinolines, aryl alcohols including quinoline alcohols and anti-foliate compounds which inhibit the synthesis of parasitic pyrimidines (Robert *et al.,* 2001).

The latest class of anti-malarial drugs is based on the natural endoperoxide, artemisinin and its hemi-synthetic derivatives and synthetic analogs (Benoit, *et al.,* 2000). Some antibiotics are also used, generally in combination with quinoline alcohols (Pukrittayakamee *et al.,* 2000). Quinoline-based anti-malarial drugs act by inhibiting the polymerisation of haeme, thus killing the parasite with its own metabolic waste (Egan *et al.,* 2002). The most common pathway for detoxification of haeme moieties is polymerization as malaria pigment (Slater *et al.,* 1991; Pagola *et al.,* 2000). The parasites degrade haemoglobin in its acidic vacuole, producing free haeme that reacts with molecular oxygen to generate reactive oxygen species as toxic by products (Francis *et al.,* 1997).

1.2.3.2.1.1 Quinolines

4-Aminoquinolines are the most successful class of compounds for the treatment and prophylaxis of malaria (Robert *et al.,* 2001). They are easily synthesized, cheap and generally well tolerated. Together with quinoline alcohols, they are active against the erythrocytic stages of the parasite (Anne *et al.,* 2001). They accumulate to high concentrations within the acid food vacuole and kill the parasite (O'Neill *et al.,* 1998).

Several of them are available in the market. 4-Aminoquinolines generally have two highly electronegative nitrogen atoms while the related aryl alcohols have one. These drugs act on the growing intra-erythrocytic stages, actively digesting haemoglobin. The drugs have a rapid effect on the haemoglobin containing digestive vacuole of intra-erythrocytic parasites causing fusion of adjacent vesicles followed by sequestration of the fused vesicles into a large autophagic vacuole (Warhurst, 1986).

Quinine (QU), the active ingredient of cinchona bark, was introduced in the 17th century (White, 1992). It has the longest period of effective use, but there is a decrease of the clinical response of *P. falciparum* in some areas (Pukrittayakamee *et al.,* 1994; Zalis *et al.,* 1998). Quinine interacts weakly with haeme, but has been shown to inhibit its polymerization *in vitro* (Robert *et al.,* 2001). The mechanism of resistance to quinine is unknown, but a similar one to mefloquine has been suggested (Foley and Tilley, 1998).

Chloroquine (CQ) was introduced in 1944 and soon became the mainstay of therapy and prevention, since the drug is cheap and non-toxic (Nuwaha, 2001). In 1994, CQ was the third most widely consumed drug in the world after aspirin and paracetamol (Foster, 1994). Chloroquine resistance was observed in South East Asia and South America at the end of the 1950s and in Africa in the late 1970s (Legarde *et al.,* 1998). Chloroquine and related quinoline drugs exert their anti-malarial activity by interfering with detoxification of ferriprotoporphyrine ix (FP IX), a hemoglobin digestion product (WHO, 1984). This is by complexing with FP IX in the acid food vacuoles and the toxic FP IX-drug poison the food vacuole thus killing the parasite (WHO, 1987).

Mefloquine (*Lariam*®) another quinoline methanol is structurally related to quinine. It has long half-life (14–21 days) that has contributed to the rapid development of resistance (Wongsrichanalai *et al.,* 2002). For this reason, mefloquine should be used in combination with other anti-malarial agents (Robert *et al.,* 2001).

Amodiaquine (AQ) is chemically related, but more effective than CQ in clearing parasitaemia in cases of uncomplicated malaria. It is effective against some chloroquine-resistant strains (Ringwald *et al.,* 1996; O'Neill *et al.,* 1998). AQ has been shown to bind to haeme and inhibit its polymerization *in vitro*, with better efficiency than CQ (Foley and Tilley, 1998).

1.2.3.2.1.2 Acridines

Include pyronaridine, a synthetic drug widely used in China and may be useful for multi-resistant *falciparum* malaria (Elueze *et al.,* 1996; Ringwald *et al.,* 1999). The current oral formulation is reported to be effective and well tolerated (Guerin *et al.,* 2002). However, its oral bioavailability is low, contributing to an unacceptably high cost of the treatment (Guerin *et al.,* 2002). It is likely that drug resistance would emerge rapidly if pyronaridine is used as monotherapy (Robert *et al.,* 2001).

1.2.3.2.1.3 Aryl methanol's

Include halofantrine (Halfan®) which is effective against chloroquine-resistant malaria (Ter Kuile *et al.,* 1993). However, cardiotoxicity and high cost have limited its use as a therapeutic agent (Nosten *et al.,* 1993). Mefloquine usage appears to lead to selection of parasites that are cross-resistant to halofantrine (Wongsrichanalai *et al.,* 1992).

1.2.3.2.1.4 Foliate antagonists

These compounds inhibit the synthesis of folic acid and DNA in the parasite (Sirotnak, 1984). There are two groups of anti-foliates: (i) the dihydrofoliate reductase (DHFR) inhibitors, like pyrimethamine and proguanil and (ii) the dihydropteroate synthase (DHPS) inhibitors including sulfones and sulphonamides like sulfadoxine and dapsone, respectively (Stanley *et al.,* 1991). Due to the marked synergistic effect between the two groups, a drug in the first group is usually used in combination with another in the second one (Cowman, 2001). Typical examples of the combinations include sulfadoxine-pyrimethamine (Fansidar®), sulfalene-pyrimethamine (Metakelfin®), sulfamethaxazole-trimethoprim (Co-trimoxazole®), and chlorproguanil-dapsone (Lapdap®). Fully reduced folate cofactors are essential for the key one-carbon transfer reactions needed for nucleotide biosynthesis and amino acid metabolism (Sherman, 1998). Unfortunately, resistance to this group of anti-malarial drugs is widespread in Asia, India, and Africa (Ogutu *et al.,* 2000; Cowman 2001).

Examples of dihydrofolate reductase (DHFR) inhibitors include proquanil (N'[*p*-chrolophenyl-*n*-isopropyldiguanidine), cycloquanil, embonate, pyrimethamine (2,4-

diamino-5-*p*-chlorophenyl-6-ethylpyrimidine) and trimethoprin (2, 4-diamino-5-[3', 4', 5'-trimethoxybenzene]. The use of dihydrofolate reductase inhibitors as chemo prophylactic agents is now obsolete due to widespread resistance of *P. falciparum.* Dihydrofolate reductase is a key enzyme in folate metabolism since it converts the pteridine ring to the tetrahydro reduction state required for reactions in which the folate cofactors are synthesized. DHFR inhibitors competitively inhibit the enzyme by competing with dihydrofolic acid. Their affinity for plasmodial enzyme is 100-1000 times more than the host enzyme, hence the selective toxicity (Wernsdorfer and Trigg, 1988).

Dihydropteroate synthase (DHPS) inhibitors (PABA blockers) are drugs which compete with *para*-aminobenzoic acid (PABA) and include both sulfonamides and sulfones. Some sulfonamides include sulfadoxine (*N*'-[5, 6-dimethoxy-4-pyrimidinyl] sulfanilamide and sulfaline (*N*-3-methoxy-2-pyracinyl] sulfanilamide). Sulfones include dapsone (4, 4-diamino diphenylsulfone), which is a blood schizontocide with no activity against sporozoites and trophozoites and works like sulfonamides (Peters and Richards, 1984). Activity against human malaria parasites by PABA blockers appears to be restricted to an effect on the asexual blood cycle. No effect is observed on the gametocyte of any species. These have selective toxicity as they compete with *para*-amino benzoic acid (PABA) for the binding site of the enzyme DHPS which catalyses the condensation of PABA with phosphorylated pteridine to form dihydropteroate. This in turn is converted to dihydrofolate, which is used as a cofactor in the formation of precursors of purines

required for nucleic acid synthesis. Mammalian cells can produce their dihydrofolate directly from dietary folic acid (Wernsdorfer and Trigg, 1988).

1.2.3.2.1.5 Other anti-malarial drugs

Atovaquone, a hydroxynapthoquinone is successful against uncomplicated *P. falciparum* malaria and should be taken with food, to improve absorption (Delmont *et al.,* 1992). When used alone resistance to atovaquone develops rapidly, thus it is used in combination with proguanil (Canfield *et al.*, 1995; Looarsecuwan *et al.*, 1996).

Lumefantrine (beflumetol) was first registered in China as an anti-malarial drug (Frederich *et al.,* 2002). Little is known about its cross resistance with other drugs (WHO, 1990).

Promethazine is affordable anti-histamine that acts through competition for H-1 receptor sites, on effector cells, with histamine. The H-1 antagonist is also used as adjunct therapy in the treatment of malaria in English-speaking West African countries (Oduola *et al.,* 1998).

Artemisinin (sesquiterpene lactones) derivatives are the latest anti-malarial drugs (Meshnick *et al.,* 1996). Four compounds have been used, the parent one, artemisinin extracted from *Artemisia annua* (Ranasinghe *et al.,* 1993) and three other derivatives that are more active than the parent compound (Meshnick *et al.,* 1996; Cumming *et al.,* 1998). They include the water-soluble artesunate, the oil-soluble artemether and arteether (Anne

et al., 2001). All of them are readily metabolized to the biologically active metabolite, dihydroartemisinin (Newton and White, 1999). Artemisinin is active at nanomolar concentrations *in vitro* on both CQ-sensitive or resistant *P. falciparum* strains (Price *et al.,* 1999). During the treatment of several million patients with artemisinin derivatives for uncomplicated malaria no significant toxicity was reported (Newton and White, 1999; Price *et al.,* 1999; Van Vugt *et al.,* 2000), even in pregnant women (McGready and Nosten, 1999). Artemisinin and its derivatives appear to be the best alternative for the treatment of uncomplicated malaria (Dhingra *et al.,* 2000), and artemether has been included in the WHO List of Essential Drugs (Robert *et al.,* 2001) for the treatment of uncomplicated malaria.

Artemisinin semi-synthetic derivatives; artesunate, artemether and arte-ether have shown very rapid parasite clearance times and faster fever resolution as opposed to quinine and thus are anticipated to reduce chances of development of drug resistance to partner drugs (White, 1999). These drugs kill growing parasites including young rings by interacting with haeme to produce carbon centered free radicals that alkylate proteins and damage parasite micro-organelles and membranes (Meshnick *et al.*, 1996). Artemisinins concentrate in the food vacuole and are thought to exert their activity through the interaction with haeme. They undergo oxido-reductive cleavage of their peroxide bond in the food vacuole, most probably through interaction with Fe (II) haeme (Meshnick *et al* 1996; Meshnick, 2001). This generates fatal free-radical-induced damage to the parasite. However, the exact mechanisms by which free radicals are generated and the mechanism of parasite death are still matters of debate (Olliaro *et al.,* 2001). Different researchers

have suggested that SERCA-pfATPase is the target for artemesinins (Jambou *et al.*, 2005; Uhlemann *et al.*, 2005). It has been demonstrated that artemesinins inhibit the sarcoendoplasmic reticulum Ca++ ATPase ortholog of *P. falciparum* with marked specificity. A single amino acid in trans-membrane segment 3 of SERCA can determine susceptibility to artemesinins. An L263E replacement of a malarial by a mammalian residue abolishes inhibition by artemesinins. Introducing residues found in other *Plasmodium* species also modulates artemesinin sensitivity, suggesting that the artemisinins interact with the thapsigargin-binding cleft of the susceptible SERCAs (Eckstein-Ludwig *et al.*, 2003; Uhlemann *et al.*, 2005). The short half-life of both the parent semi-synthetic derivatives and the dihydroartemisinin metabolite necessitate treatment over a long period (5-7 days) when these compounds are used alone. They are therefore being used together with long half-life drugs to reduce treatment time and increase individual compliance (WHO, 2001).

1.2.3.2.1.6 Antibiotics

Anti-biotics such as tetracyclines, doxycycline and minocycline are used in combination with other anti-malarial drugs to augment their activity (Kremsner *et al.*, 1994). It has been postulated that the anti-malarial effects of anti-biotics are as a result of inhibition of mitochondrial protein synthesis by direct action in the ribosomes. This explains the relatively slow clinical effects of anti-biotics as anti-malarial drugs (Wernsdorfer and Trigg, 1988). They are also thought to inhibit parasite growth through the inhibition of "prokaryote like" protein synthesis in the apicoplast an organelle unique to the apicomplexa parasites (Waller, 2000).

1.2.4 Drug resistance

Over the past five decades, drug resistance of *P. falciparum* has become an issue of utmost concern (Harald *et al.,* 2003). Drug sensitivity has become an issue of utmost importance for the development of therapeutic guidelines and policies. Drug resistance in malaria has been defined as the 'ability of a parasite strain to survive and/or multiply despite the administration and absorption of a drug given in doses equal to or higher than those usually recommended, but within the limits of tolerance of the subject (WHO 1973). One reason for the failure of a drug to clear the parasite may be that not enough of the drug, or its active metabolite, reaches the parasite (Harald *et al.,* 2003). Unlike drug resistance, drug failure is a process not directly related to the parasite and is almost solely dependent on the host organism and the properties of the drug. As long as chemotherapy remains a key factor in the fight against malaria, the need for a sound knowledge of the efficacy of anti-malarial drugs cannot be overemphasized.

The limited armoury of safe, effective and affordable anti-malarial drugs calls for utmost prudence in use of available drugs (Winstanley *et al.,* 2000). In Kenya, chloroquine (CQ) has been discontinued as the first line treatment for malaria due to overwhelming presence of resistant *P. falciparum* strains (Dianne *at al.*, 2003). Drug resistance has been implicated in enhanced mortality from malaria in hyper- and holoendemic areas (Trape *et al.,* 2002) and in the development of new and expanding foci of *falciparum* malaria, but above all it has been identified as a factor in the economic constraints of malaria control

(Bloland *et al.*, 2001). Due to overwhelming resistance to monotherapy combination of drugs has been adopted to combat malaria (Staedke *et al.*, 2001).

1.2.4.1 Mechanism of drug resistance

One of the most important factors limiting success in the treatment of malaria, whether for preventive or for curative purposes, is the varying response of individual parasites to the drugs used. Parasite populations may adapt to the introduced chemical environment and thereby enter the state of drug resistance. They are capable of passing on their genetic information to future generations of drug-resistant malaria parasites. Another reason for failing to clear the parasite may be that not enough of the drug, or its active metabolite, reaches the target, an event that is referred to as drug failure. Emerging and spreading resistance to an increasing number of antimalarial drugs has been a major concern especially in Asia, Africa and South America (Olliaro and Taylor, 2004).

Although mechanism of resistance of the parasite to 4-aminoquinolines such as CQ is not well understood, there is strong evidence that resistant strains of *P. falciparum* accumulate less CQ than the sensitive ones (Rasoanaivo *et al.*, 1998). However, the mechanism by which it occurs, either rapid efflux of pre-accumulated CQ mediated by p-glycoprotein or decreased CQ uptake is still being debated (Rasoanaivo *et al.*, 1998).

1.2.4.2 Assessment of drug resistance

There are several different approaches to the assessment of *P. falciparum* susceptibility to anti-malarial drugs (White, 2002). The most traditional approach is the assessment of

therapeutic (*in vivo*) response, which was originally defined by the WHO in terms of parasite clearance [sensitive (S) and three degrees of resistance (RI, RII, RIII)] (WHO, 1973). As re-infection is difficult to exclude in areas with intense malaria transmission, the WHO introduced a modified protocol based on clinical outcome (adequate clinical response, early treatment failure and late treatment failure) for areas with intense transmission, where parasitemia in the absence of clinical signs or symptoms is common (WHO 1996). The genetic diversity of parasites has been exploited to develop molecular protocols whereby true recrudescence could be distinguished from re-infections (Nevill *et al.,* 1996), but these methods add to the cost of *in vivo* drug-sensitivity studies. All *in vivo* tests have to be carried out with set standard therapeutic doses of drugs within the limits of general tolerability.

1.2.5 Combinations of anti-malarial drugs

Chemotherapy and chemoprophylaxis now depend on combinations that achieve drug potentiation to circumvent or delay resistance (Zucker *et al.,* 1993; WHO, 2000). Combination therapy involves the simultaneous use of two or more blood schizontocidal anti-malarial drugs with independent modes of action and different biochemical targets in the parasite (Majori, 2004). Combination therapy is based on the synergistic or additive potential of two or more drugs, to improve toxicity, sensitivity, therapeutic efficacy and delay the development of resistance to the individual components (Kremsner and Krishna, 2004). A study done in a large field trial showed that the combination of an artemisinin derivative and mefloquine remarkably delayed the emergence and spread of

drug resistance (Nosten and Brasseur, 2002). This has opened the way for a new approach to the deployment of anti-malarial drugs (Nosten *et al.,* 2000).

Synergism in drug combination regiments permits the quantity of each drug to be reduced, enhance efficacy and delay resistance. Drug combination is one of the effective means to counter parasite resistance in anti-malarial chemotherapy (Anne *et al.,* 2001; Olliaro and Taylor 2003). For instance when artemisinin is used in combination, its derivatives are rapidly eliminated and reduce the parasitaemia considerably within a single life cycle of the parasites, and residual parasites may be eliminated by a second drug with a minimal risk of selecting mutant, resistant parasite populations (White and Olliaro, 1996). It is difficult to predict *in vivo* drug interactions in humans on the basis of the *in vitro* results, although the findings from studies with animal models may be more predictive (Chawira *et al.,* 1987). Although certain drug combinations may show antagonism *in vitro*, the effects are often not apparent *in vivo*.

1.2.5.1 Double therapy

The combination of sulfadoxine and pyrimethamine (Fansidar®) represents one of the most important chemotherapeutic agents currently used in treatment of chloroquine-resistant malaria (Sowumni, 2002). However, more recent studies have indicated there is evidence of increasing resistance to Fansidar® by *P. falciparum* in East Africa (Ogutu *et al.,* 2000). Maloprim®, a combination of dapsone and pyrimethamine has also been developed though resistance to the drug is now widespread and its use in malaria chemotherapy is no longer recommended (Canfield *et al.,* 1995). Atovaquone, an agent

first marketed for *Pneumocystis* has been combined with proguanil, an old dihydrofoliate reductase (DHFR) inhibitor, to provide synergistic anti-malarial activity against parasites resistant to individual agents in the combination therapy (Canfield *et al.,* 1995; Vaidya and Mather, 2000). Malarone®, a new drug combination that was released in Australia in 1998, is a combination of proguanil and atovaquone (Fivelman *et al.,* 1999). It is effective for treatment of acute uncomplicated malaria caused by *P. falciparum* resistant to first line anti-malarial drugs. However, it is very expensive (Kremsner *et al.,* 1999). A related combination of atovaquone and tetracycline has also shown good synergism (Canfield *et al.,* 1995).

In 2004, the Kenya government changed the first-line anti-malarial therapy to Coartem® (artemether-lumefantrine) due to the high levels of resistance to Fansidar®, chloroquine mefloquine and amodiaquine (Falade *et al.,* 2005). Artesunate has been studied in combination with amodiaquine in Africa and found to be efficacious (Adjuik *et al.,* 2002). In Thailand, where drug resistance is particularly severe, the combination of artesunate and mefloquine has been shown to be effective, even in areas where mefloquine resistance was previously common (Price *et al.,* 1997). A combination of artemether and lumefantrine, a new agent related to halofantrine, provides an effective therapy against uncomplicated malaria (Lefevre *et al.,* 2001). Combination of artemether or artesunate and mefloquine has been used and is currently the standard treatment in areas of multi-drug resistance in South East Asia (Robert *et al.,* 2001). When used in association with lumefantrine (benflumetol), artemether clears most of the infection, and the lumefantrine concentration that remains at the end of the 3-5 day treatment course is responsible for

elimination of the residual parasites (Looareesuwan *et al.,* 1996; Robert *et al.,* 2001). The major benefit of artemisinin combinations is the 90% reduction in gametocyte levels in treated patients although they are expensive and are toxic (especially where mefloquine is involved) (Price *et al.,* 1996). Artemisinin combinations decrease malaria parasite density more rapidly than any other anti-malarial drug (White, 1997).

1.2.5.2 Triple therapy

The most recent addition to malaria treatment in Africa is Lapdap®, a combination of chloroquine-proguanil and dapsone (Mutating *et al.,* 2001). It has been specifically devised for the treatment of malaria in Africa, where resistance to chloroquine is very common and resistance to sulfadoxine/pyrimethamine is increasing. Although the new regimen shares the targets of sulfadoxine-pyrimethamine, it is generally effective against sulfadoxine/pyrimethamine-resistant parasites, as the common DHFR and DHPS mutations that mediate this resistance do not lead to clinical resistance to Lapdap®, and additional mutations that lead to higher level anti-foliate resistance are rare in Africa (Nzila *et al.,* 2000; Mutabingwa *et al.,* 2001; Kublin *et al.,* 2002). It is cheap to produce and has a short half-life, which may be associated with better safety, although further assessment of the drug is required (Kayak, 2003).

Triple anti-malarial therapy like use of chloroquine plus sulfadoxine-pyrimethamine (SP), amodiaquine in combination with SP and Lapdap® (a combination of chlorproguanil and dapsone) have been used successfully and are also affordable (Winstanley *et al.,* 2002). Artesunate has been studied in combination with sulfadoxine-pyrimethamine (Von

Seidlein *et al.,* 2000) with good efficacy, although underlying resistance to the two artesunate partners may lead to unacceptable rates of late recrudescence in many areas, as seen with artesunate-sulfadoxine-pyrimethamine in Uganda (Dorsey *et al.,* 2002). The combination of amodiaquine and sulfadoxine-pyrimethamine has also shown excellent anti-malarial efficacy in some regions of East Africa, with high levels of resistance to individual agents (Staedke *et al.,* 2001; Dorsey *et al.,* 2002; Schellenberg *et al.,* 2002).

1.2.5.3 Quadruple therapy

A few cases of combination of 4 anti-malarial agents have been reported. This includes a combination of artesunate sulfadoxine-pyrimethamine and primaquine (Chokejindachai *et al.,* 1999).

1.2.6 Drug resistance reversers

Combinations of previously effective agents with compounds that reverse parasite resistance to these agents offer another approach to chemotherapy. Many drugs have been shown to reverse the resistance of *P. falciparum* to chloroquine *in vitro*, most notably the anti-hypertensive, verapamil (Martin *et al.,* 1987), and the anti-depressant, desipramine (Bitonti *et al.,* 1988). In many cases, unacceptably high concentrations of the resistance reversers are needed for their effects, but combinations of two or more of these agents at pharmacological concentrations may provide clinically relevant resistance reversal, as demonstrated by studies with verapamil, desipramine and trifluoperazine (Van Schalkwyk *et al.,* 2001).

The commonly used and inexpensive anti-histamine chlorpheniramine reversed resistance at safe dosing levels, although the common side-effect of drowsiness might limit its acceptance (Sowunmi *et al.,* 1997). Efforts to design new reversers of chloroquine resistance are underway (Batra *et al.,* 2000; Alibert *et al.,* 2002). Although chloroquine appears to have already failed as a first-line anti-malarial in most parts of the world, this inexpensive, rapid acting, well-tolerated drug may be resurrected by combination with effective resistance reversers.

1.2.7 Natural products

Plant-derived compounds offer an alternative approach to chemotherapy (Basco *et al.,* 1994; Mueller *et al.,* 2000; Tagboto and Townson, 2001). It is evident that most of the anti-malarial drugs in use today such as quinine (from *Cinchona* spp.) and artemisinin (from *Artemisia annua*) were either obtained directly from plants or developed using chemical structures of plant-derived compounds as templates (Basco *et al.,* 1994). Relatively simple chemical modifications of artemisinin have led to a series of potent anti-malarial drugs (artemether, arteether and artesunate) that are playing an increasingly important role in the treatment of malaria (Meshnick, 2001). In tropical countries, modern medicines are not available to most of the rural populations (or the price is prohibitive). It is estimated that 80% of the world's population use botanical medicines for their primary health care needs (Farnsworth *et al.,* 1985; Arvigo and Balick, 1993).

In Kenya, plant extracts are still widely used in the treatment of malaria and other ailments (Njoroge and Bussmann, 2006). A large number of local plants have been

studied to establish their potential in malaria treatment (Omolo *et al.,* 1997; Omulokoli *et al.,* 1997; Wanyonyi *et al.,* 2002; Muregi *et al.,* 2003; 2004; Wanyoike, 2004; Kirira *et al.,* 2006). It is estimated that some 20,000 species of higher plants are used medicinally throughout the world (Tagboto and Townson, 2001). However, only about 20% of the plants with acclaimed medicinal values have been subjected to rigorous scientific screening and evaluation (Houghton, 2001). *Azadirachta indica* (neem), a widely used medicinal plant has been shown to have aspirin-like activity, which may be involved in anti-pyretic effect of the plant (Subapriya and Nagini, 2005). Other examples include *Harrisonia abyssinica* with anti-plasmodial (El-Tahir *et al.,* 1999) and anti-bacterial (Fabry *et al.,* 1998) activity. *Ajuga remota* has anti-plasmodial (Kuria *et al.,* 2002; Muregi *et al.,* 2004) and anti-fungal (Kariba, 2001) activity. *Plumbago zeylanica* has both anti-plasmodial (Simonsen *et al.,* 2001) and anti-mycobacterial activity (Mossa *et al.,* 2004). *Quassia amara* is effective as anti-malarial (Ajaiyeoba *et al.,* 1999), anti-bacterial and anti-fungal agent (Ajaiyeoba and Krebs, 2003).

Compounds of immense pharmacological importance have been isolated from nature. Phenolic compounds extracted from several plants have been shown to inhibit *P. falciparum* growth *in vitro* (Decosterd *et al.,* 1991; Oketch-Rabah *et al.,* 1997; Oketch-Rabah *et al.,* 2000). Chalcone glycosides such as phlorizidin from *Micromelum tephrocarpum* (Rutaceae) have been reported to exhibit anti-parasitic activity (Kayser *et al.,* 1998). Flavonoids are found in a large variety of plants including *Artemisia annua* (Asteraceae) a strong anti-malarial herb. Exiguaflavanone extracted from this plant is

1.3 Plants under investigation

1.3.1 *Clutia robusta* (Euphorbiaceae)

This plant is locally known as *mutemagengi* (Meru). Roots are boiled in water, the decoction mixed with milk and taken as a remedy for colds and rheumatism (Kokwaro, 1976). In a previous study, extract of *C. abyssinica* was found to have anti-fungal and anti-bacterial activity (Boer *et al.,* 2005). Literature on compounds isolated from *C. robusta* could not be found.

1.3.2 *Turraea robusta* (Meliaceae)

The plant is commonly known as *muringa* (Meru). A decoction of boiled roots is drunk 2-3 times a day to cure stomach pain, diarrhoea, and other stomach troubles. Leaves are used as an antidote for general poisoning (Kokwaro, 1976). The plant has been reported to contain triterpenoid compounds (Bentley *et al.,* 1992; Rajab *et al.,* 1988).

1.3.3 *Tabernaemontana holstii* (Apocynaceae)

The plant is locally known as *muerere* (Meru). A decoction of the roots is taken as a remedy for stomachache and headache (Kokwaro, 1976). A number of alkaloidal compounds including; conodurine, voacamine, tabernamine, 19*S*-heyneanine, coronaridine and voacangine have previously been isolated from *T. laeta* (Walter *et al.,* 2001).

1.3.4 *Artemisia afra* (Asteraceae)

The plant is commonly known as African wormwood and as *muhato* (Meru). A fermentation of the heated herb is given to children with sore throat to cure fever (Kokwaro, 1976). Dried aerial parts are used for stomachache (Kloos *et al.,* 1978; Nkunya *et al.,* 1992), diabetes (Watt and Breyer-Brandwijk 1962), malaria (Nkunya *et al.,* 1992; Kuria *et al.,* 2001; Kraft *et al.,* 2003) and fevers (Nkunya *et al.,* 1992). Several compounds including flavonoids (Wollenweber and Mann 1989; Nkunya *et al.,* 1992; Kraft *et al.,* 2003), terpenoids (Libbey and Sturtz 1989; Moody *et al.,* 1994; Chagonda *et al.,* 1999; Jenett-siems *et al.,* 2002), benzenoids (Nkunya *et al.,* 1992), and fatty acids (Mwangi *et al.,* 1995) have been isolated from this plant.

1.3.5 *Rhus natalensis* (Anacardiaceae)

The plant is commonly known as *mirimathua* (Meru). Leaves and root decoctions are used for treatment of coughs and colds (Kokwaro, 1976); headache and neck pain (Chhabra *et al.,* 1987). Methanolic extracts of the roots have strong anti-giardiac activity at 100 ppm (Johns *et al.,* 1995). Flavanoids are among major compounds isolated from this plant (Furth and Young, 1988).

1.3.6 *Cyathula schimperiana* (Amaranthaceae)

The plant is commonly known as *rusirusiru* (Meru). Decoction of root bark is used to treat malaria, as an emetic and a purgative (Kokwaro, 1976). Among the compounds recently isolated include 4-[(1-ethoxy-2-hydroxy)ethyl]phenol, 2, 3-

isopropylidenecyasterone, 24-hydroxycyasterone and 2, 3-isopropylideneisocyasterone (Zhou *et al.*, 2005).

1.3.7 *Clausena anisata* (Rutaceae)

The plant is commonly known as *matathi* (Kikuyu). Roots boiled in soup are highly recommended for headache, malaria, influenza, indigestion (Kokwaro, 1976; Chhabra *et al.*, 1991), anti-fungal, anti-nematicidal and anti-bacterial therapy (Mc-Gaw *et al.*, 2000). It is reported to contain phenylpropanoid (Okunade and Olaifa 1987), benzenoids, coumarins (Okorie, 1975; Lakshmi *et al.*, 1984; Ekundayo *et al.*, 1986; Ngadjui *et al.*, 1989; Ngadjui *et al.*, 1991; Reisch and Wickramasinghe 1998), terpenoids (Reisch *et al.*, 1985) and alkaloidal compounds (Ito *et al.*, 1998; Ito *et al.*, 2000).

1.3.8 *Lonchocarpus eriocalyx* (Fabaceae)

The plant is commonly known as *muthingiri* (Meru). Powdered roots of this plant are used in water for pimples (Kokwaro, 1976). *Lonchocarpus* flavanones are cytotoxic (Fang and Casida, 1999). Previous anti-plasmodial studies on the plant were not found in literature.

1.3.9 *Ximenia americana* (Oleacaceae)

The plant is called *mutura* (Meru). It is used for schistosomiasis (Sparg *et al.*, 2000), gastro-intestinal problems and as anti-malarial (Johns *et al.*, 1990; Mwangi *et al.*, 1994;

Johns *et al.*, 1995). It contains triterpenes (Fatope *et al.*, 2000; D'Agostino *et al.*, 1994), flavonoids and benzenoids (Mwangi *et al.*, 1994).

1.3.10 *Lannea schweinfurthii* (Anacardiaceae)

This plant is referred to as *mwethi* (Meru). It is used to treat gastro-intestinal problems (Johns *et al.*, 1995). Methanolic extracts of bark, leaves or root have strong anti-giardiac activity at 100 ppm (Johns *et al.*, 1995).

1.3.11 *Sclerocarya birrea* (Anacardiaceae)

The plant is known by the local community as *mura* (Meru). It is used as anti-malarial, anti-bacterial (Adoum *et al.*, 1997; Sparg *et al.*, 2000) and anti-schistosomiosis drug. Compounds isolated from it include flavonoids (Peralta *et al.*, 1992; Galvez *et al.*, 1992) and alkaloids (Hussain and Deeni, 1991).

1.3.12 *Catharanthus roseus* (Apocynaceae)

The plant is used to cure fever, stomach pains (Gurib-Fakim *et al.*, 1996), leishmaniasis, malaria (Marshall *et al.*, 2000) and as anti-bacterial (Srinivasan *et al.*, 2001). The plant contains sesquiterpenes, alkaloids (Hirose and Ashihara 1984), proteins, indole alkaloids (Stockigt *et al.*, 1980; Kurz *et al.*, 1981; Gueritte *et al.*, 1983; Kohl *et al.*, 1983), steroids (Fujimoto *et al.*, 1998), flavonoids, monoterpenes (Vimala and Jain 2001), iridoid monoterpenes (Guarnaccia *et al.*, 1974), secoiridoid monoterpenes and triterpenes (Ali *et al.*, 1979).

1.3.13 *Boscia salicifolia* (Capparidaceae)

The plant is locally known as *mulule* (Meru). Its traditional use includes treatment of domestic livestock. A hot water extraction is given orally to relieve fever in cattle (Hussain *et al.,* 1990). It is also used externally in healing of wounds (Pauli and Sequin, 1996). Weak anti-bacterial activity (100 mg/ml) has been demonstrated (Omer *et al.,* 1998). A sesquiterpene (boscialin-4'-O-β-D-glucoside) and flavonols (rhametin-3-O-β-neohesperidoside and rhamnocitrin-3-O-β-D-glucoside) have been isolated (Pauli and Sequin, 1996).

1.3.14 *Terminalia brownii* (Combretaceae)

The plant is known by the local community as *muuku* (Meru). The organic extract of the stem bark has weak activity against a wide range of bacteria (Omer *et al.,* 1998). Previous anti-plasmodial study on the plant was not found in literature.

1.3.15 *Trimeria grandifolia* (Flacourtiaceae)

The plant is known locally as *muhindihindi* (Meru). Dried leaf decoction is used traditionally for abdominal troubles (Hedberg *et al.,* 1983; Chhabra *et al.,* 1984) while gum from bark is used for healing of old wounds and mouth sores (Hedberg *et al.,* 1983). Compounds such as coumarins, sterols, tannins and terpenoids have been isolated (Chhabra *et al.,* 1984).

1.4 Problem statement

The increasing resistance of malaria parasites to available anti-malarial drugs is a major contributor to the re-emergence of the disease as a major public health problem. Traditional herbal anti-malarial drug combinations have not been assessed for treatment of multiple drugs resistant malaria.

1.5 Hypothesis

Plants used by the Meru community for malarial treatment have antimalarial activity singly or in combination with others against multiple drug resistant malaria parasites.

1.6 Justification

Over the last 70 years, extensive efforts, including the screening of hundreds of thousands of compounds, have led to the development of a number of effective natural and synthetic anti-malarial drugs. CQ has been the mainstay of anti-malarial chemotherapy for the last 60 years. However, resistance to CQ has been steadily increasing in South America, South East Asia and Africa. CQ resistance is now widespread in most disease endemic areas. The use of CQ for presumptive treatment of *falciparum* malaria or for chemoprophylaxis is no longer appropriate. Analogs like MQ and AQ have equally become less effective. Resistance to anti-folate drugs is reaching alarming levels in most parts of the world. Artemisinin-based drugs are the only ones still effective against *P. falciparum* inflicted malaria. New effective anti-malarial drugs are urgently required.

Some of the most important anti-malarial drugs known today were originally obtained from plants. Herbal medicines are still considered as a potential source of new drugs or templates for developing new drugs. Plant based drugs continue to play an essential role in cultural health care. It is estimated that approximately 80% of the world population rely on traditional medicines for primary health care. Most of the traditional healers in Africa use medicinal plants in combination possibly to enhance efficacy and delay resistance development by the target pathogen. This study aims to establish presence of synergism among anti-malarial plants used by traditional health practitioners and the local community in Meru. Furthermore, the study seeks to investigate possible chloroquine potentiation by local traditional anti-malarial plants.

1.7 Study objectives

1.7.1 General objective

To investigate the efficacy and safety of traditional anti-malarial plant extracts individually and incombination.

1.7.2 Specific Objectives

i. To investigate anti-plasmodial and anti-malarial activity of medicinal plant extracts *in vitro* and *in vivo.*

ii. To evaluate the toxicity of active anti-malarial plants *in vitro* and *in vivo.*

iii. To investigate anti-plasmodial and anti-malarial activity of combination of plant extracts on *P. falciparum* and *P. berghei,* respectively.

iv. To investigate *in vitro* anti-plasmodial activity of combinations of plants extracts and chloroquine.

CHAPTER TWO

MATERIALS AND METHODS

2.1 Assay requirements

2.1.1 Reagents

Sodium hydrogen carbonate, sodium chloride and RPMI 1640 powdered medium were purchased from Gibco Laboratories, California, U.S.A. Giemsa stain, glycerol, DMSO and HEPES were purchased from Sigma Chemical Company, St. Louis, U.S.A. [^{3}H]-Hypoxanthine was obtained from Armasham Bioscience, UK. Culture gas mixture (92% N_2, 5% CO_2 and 3% O_2) was purchased from British Oxygen Company (BOC), Nairobi, Kenya. Industrial methylated spirit was used in sterilization of the working surfaces while methanol was used in fixing of dried thin blood smears. Methanol (Analar grade) for use in extraction of plants material was purchased from Sigma Chemical Company, St. Louis, U.S.A.

2.1.2 Equipment for the *in vitro* culture

The following pieces of capital equipment were used in the *in vitro* procedures: laminar flow hood, liquid scintillation counter, microscopes, refrigerators (4, -20 and -80 °C), incubator, gas chambers, cell harvester, analytical balance (with sensitivity of 0.1 mg), vacuum pump, thermostated centrifuge, adjustable Eppendorf micro-pipette, automatic pipet pump, vibro mixer and electrically heated water bath.

2.1.3 Disposable plastic and glassware

The following disposables were used in running the assay: 15 and 50 ml sterile centrifuge tubes (Brinkmann Instruments Company, Westbury, U.S.A), 0.22 µm filter units (Naglene®, Naglene Company, U.S.A), 50 and 250 ml culture flasks (Corning® U.S.A), froasted-end microscope slides (Sigma Chemicals Company, U.S.A), serological Pasteur pipettes (Fischer Scientific, Pittsburgh, U.S.A), 1-200 µl pipette tip, anti-coagulant-free blood collecting bags and sterile gloves (Triflex®).

2.1.4 Sterilizing materials

Industrial methylated spirit (70%) was used to sterilize laminar flow hood and other sterile benches. Only new sterile disposable plastics and glassware were used in culturing. Culture reagents were sterilized by filtration through 0.22 microfilters or by autoclaving the temperature stable. Where possible all experimental procedures were carried out in laminar flow hood. Sterilised Pasteur pipettes, lids and disposable pipettes were passed over a Bunsen burner flame at least thrice before use. Disposable apparatus were disinfected with 20% sodium hypochlorite before being incernalated.

2.1.5 Plant materials

2.1.5.1 Plants sampling

Plants were selected following a survey conducted on ethno-medical information on materials used by traditional health practioners (THPs) in treatment of malaria or fever in Meru District, Kenya. Further information was obtained from Kokwaro (1993). The

selected plants include: *Artemisia afra*, *Tabernaemontana holstii*, *Catharanthus roseus*, *Sclerocarya birrea*, *Lannea schweinfurthii*, *Clutia robusta*, *Lonchocarpus eriocalyx*, *Clausena anisata*, *Trimeria grandifolia*, *Rhus natalensis*, *Terminalia brownii*, *Ximenia americana*, *Cyathula schimperiana*, *Turraea robusta* and *Boscia salicifolia*. They were identified and botanically authenticated at the East African Herbarium based at National Museum of Kenya (NMK). Voucher specimens were deposited at the Herbarium for future reference. Part of plants collected included leaves, roots, stem or stem-barks depending on the part used by the THPs. Collection process was carried out between the months of February and August 2004.

2.1.5.2 Processing and extraction of plant extracts

Samples of roots, stem or leaves for the respective plant were chopped into small pieces and dried under shade for 14 days. Roots, stems (twigs), stem-bark or root-bark were ground using a laboratory mill (Christy and Norris Ltd, Chelmsford. England) while a kitchen blender was used to grind the leaves. The ground material was catalogued and stored in plastic bags at room temperature in dry condition. Ground plant material (100 g) was extracted in 300 ml of double distilled water in a water bath at 60 °C for 1 hr, cooled and filtered using Whatman filter paper. An equal amount sample was extracted in 300 ml of methanol by soaking overnight at room temperature followed by filtration through Whatman filter paper. Water and methanol extracts were dried by lyophilization and rotatory evaporation respectively.

2.2 *In vitro* anti-plasmodial assay

2.2.1 Parasites cultures

Two strains of *Plasmodium falciparum,* originally obtained from the Division of Experimental Therapeutics, Walter Reed Army Institute of Research (WRAIR), Nairobi were used. These strains were the Sierra Leonean chloroquine sensitive D6 and Indochinese chloroquine resistant W2.

2.2.2 Parasite cultivation

Parasite cultivation was carried out aseptically in a laminar flow hood based upon *in vitro* technique described by Trager and Jensen (1976). The culture medium consisted of RPMI 1640 (10.4 g/l) dissolved in 960 ml of distilled-autoclaved water (DAW). This was supplemented with 10% human serum, 25 mM (5.94 g/l) HEPES and 25 mM $NaHCO_3$ (Schlichtherle *et al.,* 2000). The medium was filter-sterilized through 0.22 μm filters using a vacuum pump and stored at 4 °C for use within 4 weeks.

2.2.3 Preparation of culture reagents

2.2.3.1 Acid dextrose (ACD)

ACD was used as an anti-coagulant for collecting RBCs. This was prepared by dissolving 22.0 g tri-sodium citrate, 8.0 g citric acid and 24.4 g dextrose in 1 litre of sterile water. The ACD was re-sterilized by filtration through 0.22 μm and kept at 4 °C until required.

2.2.3.2 Wash medium

Wash medium (WM) was prepared by mixing 95.8% (v/v) RPMI 1640, 4.2% (v/v) HEPES and 5% (w/v) sodium bicarbonate (Rowe *et al.,* 1968). The WM was flushed with a gas mixture (92% N_2, 5% CO_2 and 3% O_2), well corked and kept at 4 °C for use within 2 weeks.

2.2.3.3 Host cells

Uninfected human blood group O Rhesus positive erythrocytes (<28 days old) served as host cells. Briefly, blood from recruited volunteers who had not contracted malaria or visited malaria endemic area in the past two months was drawn into 15% (v/v) ACD. By use of a questionnaire and consent form, it was ascertained that the donor had not taken any anti-malarial or antibiotic drugs for the last one month prior to recruitment. Enzyme linked immunosorbent assay (ELISA) was used to screen blood for HIV and hepatitis B infections at the Centre for Virology Research (CVR), KEMRI. Blood donation that tested positive for either was promptly incinerated.

The blood was washed to remove plasma and white blood cells before use in the culture by centrifugation at 3600 rpm for 10 min at 4 °C. The plasma and buffy coat at the top of the cell pellet was aspirated and discarded. The red cell pellet was washed twice with 2 volumes of wash medium (WM) and the resulting suspension centrifuged at 3600 rpm for 10 min at 4 °C. After the last wash, packed cells were re-suspended in an equal volume

of WM to obtain a haematocrit of 50%. The cells were exposed to a gas mixture (92% N_2, 5% CO_2 and 3% O_2) and stored at 4 °C for use within 2 weeks.

2.2.3.4 Human serum

Pooled human serum groups (A,O,B) was used to supplement the media. Briefly, consent for one unit of blood from at least three adults volunteers with blood groups A+, B+ and O+ and who had not contacted malaria or visited malaria endemic area in the past two months was obtained. One pint (500 ml) of blood from the volunteers was drawn aseptically into blood bags (Fenwell 4R0001) without anti-coagulants. About 2 ml of the blood from each bag was sampled into a 7 ml Bijou bottle and tested using ELISA for HIV and hepatitis B infections. Blood donation that tested positive for either was promptly discarded. The blood was allowed to clot at room temperature for 90 minutes followed by an overnight storage at 4 °C. The serum was carefully dispensed into sterile 50 ml centrifuge tubes 24 hrs later and centrifuged at 3000 rpm for 10 min at 4 °C to separate contaminating cells. The serum was transferred into 75 cm^2 (250 ml) sterile corning flasks heat inactivated in a water bath at 56 °C for 40 min. Using each batch of serum separately, a small amount (50 ml) of CMS was prepared and used to maintain a culture for one week to test whether it supported growth. Batches that supported growth were pooled into a 250 ml corning flask and aseptically aliquoted into sterile 10 ml snap-top tubes. The tubes were kept in upright position at -20 °C overnight and then at -80 °C until they were required for use.

2.2.4.2 Parasite culture growth

The method described by Trager and Jensen (1976) was used for parasite culture. Briefly, after washing the parasites, the packed cell volume (pcv) of the parasitized erythrocytes was estimated and the volume of the RBC adjusted to 6% (v/v) (6% haematocrit) by the addition of the CMS. Cultures were exposed to a gas mixture (92% N_2, 5% CO_2 and 3% O_2) and incubated at 37 °C for 24 hr. Medium was changed daily to remove toxic bio-products and smears prepared after every 48 hr to determine percentage parasitaemia (% P), growth rate and monitor contamination.

2.2.4.3 Estimation of the percentage parasitaemia

This was done according to Trager and Jensen (1976). Briefly, thin blood smears were prepared using sterile plugged Pasteur pipettes after carefully aspirating and discarding the spent medium. A small drop of cell suspension was placed on a clean frosted glass microscope slide and a thin film made by touching the drop with the edge of another slide held at angle of 45 degrees to the first. This spread the cells across the width of the slide and a smear was made along the length of the slide with quick smooth movement. The blood films were air-dried, fixed with absolute methanol and stained in 10% Giemsa for 10 min. The slides were rinsed gently under flowing tap water, air-dried and observed in oil immersion under microscope at a magnification of 1000 times.

Parasitaemia is defined as the number of parasitized red blood cells in 10,000 red blood cells. It is expressed as a percentage. Parasitized cell (PRBC) is defined as red blood cells

containing parasites (multiple or single infection). Un-parasitized cell (UPRBC) is defined as red blood cell that does not contain infection.

Appropriate number of fields equal to 10,000/RBC in 1 field, were scanned and the total number of erythrocytes infected counted, ensuring that the same field was not observed twice. This was done by counting from one end of the selected area, then moving the slide to another field as illustrated in figure 3.

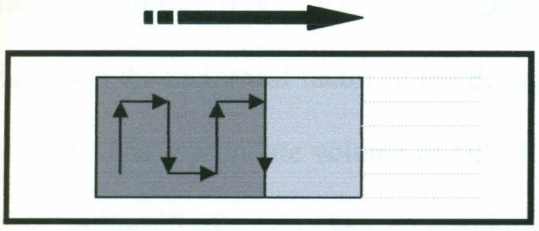

Figure 3. Illustration of a thin blood smear analysis

Percentage parasitaemia (%P) was calculated using the formula:

$$\%P = \frac{\text{No. of parasitized erythrocytes}}{\text{RBC per field x Fields counted}} \times 100$$

Growth rate (GR) was calculated at every 48 hr cycle using the formula:

$GR = [(P_f/P_i)xD]^{2/d}$ (Schlichtherle *et al.*, 2000).

Where P_f is the final parasitaemia, P_i is the initial parasitaemia before dilution, D is dilution factor, and d is number of days since culture was diluted.

2.2.4.4 Dilution of cultures

Dilution or sub-culturing was usually done when the percentage parasitaemia was high (>4%), and no contamination found on examining the slide under the microscope. The necessary volumes of culture 50% fresh erythrocytes and medium needed for 5 ml, 6% hematocrit culture were calculated from the formulae:

Culture Volume (CV) = 5/D

50% Erythrocyte Volume (EV) = 6/ (50-CV)

Medium Volume = (CV+EV) (Schlichtherle *et al.,* 2000)

where D is the reciprocal factor of the desired dilution factor (for instance D=10 for 1:10 dilution). The appropriate volume of 50% RBC and medium were mixed together in new 25 cm^3 culture flasks using sterile technique, gassed (92% N_2, 5% CO_2 and 3% O_2) and incubated for 20 min at 37 °C. The desired volume of old culture was then added, gassed and incubated.

2.2.4.5 Cryopreservation of parasites

For cryopreservation of parasites to ensure enough supply of laboratory-adapted isolates as well as having manageable number of culture flasks, the method of Rowe *et al.* (1968) was adapted. A thick smear was made to ascertain the cultures to be frozen are not contaminated. No parasites were cryopreserved at merozoite stage. Briefly, the culture to be cryopreserved was transferred into 15 ml centrifuge tube and centrifuged at 1500 r.p.m (400 g) for 5 min at 20 °C. After aspirating the supernatant, PCV was estimated and one equivalent of Rowe's cryosolution added. Aliquots of 0.25 ml were put into 2 ml

cryovials (Nunc®, U.S.A), placed in aluminium canes and placed into liquid nitrogen. Proper catalogue of the kept vials was made for easier retrieval.

2.2.5 Procedure for drug sensitivity assays

2.2.5.1 Preparation of plant extracts and standard reference drugs for *in vitro* bioassays

Stock solutions for *in vitro* assay of plant extracts were made in DAW and re-sterilized by passing through 0.22 µm micro-filters in a laminar flow hood. The water insoluble extracts were dissolved in 100% DMSO (Sigma Chemical Co, St Louis, MO, USA) heated or sonicated where necessary followed by a subsequent dilution to lower concentration of DMSO to $\leq$ 1% to avoid solvent carry over effect (Dominique *et al.*, 2001). Briefly, the dry plant extract samples were retrieved from storage and dissolved so that the final highest concentration in the micro-titre plates was 125 µg/ml. For these experiments, 0.0225 g of the plant extract was dissolved in 20 ml (stock solution of 1,125 µg/ml).

Since the final volume in each well was 225 µl, this stock solution was meant to give the first row concentration of 125 µg/ml using the formula:

$C_1V_1=C_2V_2$

Where; C_1 = initial concentration, V_1 = initial volume, C_2 = final concentration, V_2 = final volume. Taking into account that the volume of each drug in each well was 25 µl, the highest concentration (125 µg/ml) was calculated so that:

1,125 µg/ml x V_1 = 125 µg/ml x 225 µl

$V_1 = 125 \times 225/1,125 = 25\ \mu l$

This meant that 25 µl of stock solution (1,125 µg/ml) was used in the first row (Elueze *et al.*, 1996). Each drug was filter-sterilized with syringe adaptable 0.22 µm filters into sterile Bijoux bottles and stored at -20 °C.

Stock solutions (1 µg/ml) of chloroquine diphosphate, artemisinin and mefloquine were similarly prepared for use as reference drugs. All the drug solutions were stored at -20 °C until when required for bioassay.

Similarly reference standards were prepared so that the plate's first concentration was 1 µg/ml (9 µg/ml stock solution, C_1), sterilized and stored at –20 °C.

$9\ \mu g/ml \times V_1 = 1\ \mu g/ml \times 225\ \mu l$

$V_1 = 1\ \mu g/ml \times 225\ \mu l / 9\ \mu g/ml$

$= 25\ \mu l$

$C_1 = 25\ \mu l$ in the first row of the micro-titre plate

2.2.5.2 *In vitro* drug sensitivity protocol

The semi-automated micro-dilution technique of Desjardins *et al.*, (1979) for assessing *in vitro* anti-plasmodial activity as modified by Le Bras and Deloron (1983) was adapted in the drug sensitivity studies for extracts and standard drugs against *P. falciparum* isolates. The test relies on the ability of viable parasites to incorporate [^{3}H] label from

hypoxanthine, whereas non-viable parasites will not take up label. Briefly, 96 well flat-bottom micro-titre plates (8 rows x 12 columns) were set such that all wells except control contain 25 µl of doubling concentrations of drug solutions. PRBCs and CMS (200 µl) were added so that the total volume per well was 225 µl. Under sterile conditions in laminar flow hood (Bellco Glass Inc., U.S.A), 25 µl of CMS were added with a Titertek motorized hand diluter (Flow Laboratories, Uxbridge, UK) from row B to H, exempting row A.

Test samples (50 µl) were added in duplicate into wells of row A (each drug held two columns and one plate therefore accommodated 6 drugs in duplicate). At least one reference standard drug was included in every plate. Two-fold dilutions were done by transferring 25 µl of the drug with a 12-multi-channel pipette from row A down to row G discarding the last 25 µl from G wells. Row H wells were exempted since they served as controls. Thus, row A wells had a concentration of 125 µg/ml, B wells 62.5 µg/ml as concentrations halved down to G, which had the lowest concentration of 1.95 µg/ml. Consequently, wells of row A contained the highest concentration (100%) while those of G contained only 1.5625% of the test sample. All wells of row H (control) contained only drug-free medium/water. The plates were covered with plate sealers and kept at 4 °C.

2.2.5.3 Addition of parasites to the pre-dosed plates

This was done according to Desjardins *et al* (1979). Briefly, the test culture at ≥80% ring stage, having a percentage parasitaemia (% P) ≥ 4% and growth rate (GR) ≥ 3 % was

used for sensitivity tests. After examining the parasites under a microscope, the % P of the test culture to be added to the wells of pre-dosed plates was adjusted to 0.4 % and hct adjusted to 1.5 % with 50 % RBCs. The mixture (200 µl) was added into each well except for H_9 to H_{12}. If for instance the % P of the test culture (V_i) was 4 % and the number of plates to be set was 1 (n =1), the following calculations were done for the cultures maintained at 5 ml and 6 % haematocrit.

$C_iV_i = C_fV_f$

Where C_i and C_f = initial and final concentrations, respectively.

V_i and V_f = initial and final volumes, respectively

$C_i = 4\ \%$, $C_f = 0.4\ \%$

The volume of the plate (V_f) was calculated as follows approximating 96 wells to 100 wells.

V_f = 1 plate x 100 wells x 200 µl (volume of culture per well) = 20000 µl

= 20 ml

The volume of the test culture (5 ml, 6 % hct) that was used (V_i) was calculated as follows:

$C_iV_i = C_fV_f$

$4\ \% \times V_i = 4\ \% \times 20$ ml

$V_i = 0.4\ \% \times 20$ ml$/4\ \%$

= 2 ml

Since 5 ml has 6 % hct, or 6/100 x 5 ml = 0.3 ml (100 % RBC)

2 ml culture has 0.12 ml (100 % RBC)

To adjust hct to 1.5 % of V_f

1.5/100 x 20 ml = 0.3 ml (100 % RBC)

But the V_i (2 ml) has 0.12 ml (100 % RBC) and (0.3-0.12) ml = 0.18 ml (100 % RBC) are required. This requires the addition of 50 % RBC. Since the remaining 0.18 ml hct is 100 % RBC, 0.18 ml x 2 = 0.36 ml of 50 % RBC is needed.

The final volume of 20 ml, needed is achieved by addition of CMS to 2 ml test culture and 0.36 ml of 50 % RBC.

CMS needed = 20 ml- (2 +0.36) ml

= (20 – 2.36) ml = 17.64 ml

This means that to set 1 plate using a culture whose % P = 4, you require 17.64 ml CMS, 0.36 ml of 50 % RBC and 2 ml test culture, to achieve 0.4 % P and 1.5 % hct.

The pre-warmed CMS (37 °C) was put into 25 cm^2 flask, the appropriate volume of 50 % RBC added, flushed with the gas mixture (92 % N_2, 5 % O_2 and 3 % CO_2) (BOC, Kenya) and kept at 37 °C incubator for 5 min.

Using sterile technique in a laminar flow hood, the appropriate volume of test culture was added into the flask containing CMS and 50 % RBC, and gently swirled in a circular motion to mix. The pre-dosed plates were warmed at 37 °C for about 20 min, retrieved, placed in the laminar flow hood and the test culture put into sterile tissue culture dishes (Lux®, U.S.A). Using 1-200 µl pipette tips (Fisherbrand®, U.S.A) and a multi-channel pipette, aliquots of 200 µl were dispensed into the wells except for H_9 to H_{12} (4 wells). To these, UPRBC were added (negative control) so that H_1-H_8 served as PRBC positive

control since they had no drug and the former served as UPRBC control. For 1 plate (4 wells):

Volume of CMS/RBC mixture = 4 wells x 200 µl = 800 µl

= 0.8 ml

1.5 % hct = 1.5/100 x 0.8 = 0.012 ml (100 % RBC)

= 0.024 (50 % RBC)

CMS = (1.2 – 0.024) ml

= 1.176 ml

0.024 ml of 50 % RBC was mixed with 1.176 ml CMS and 200 µl of mixture dispenced into wells H_9 to H_{12} using a multi-channel pipette. The same procedure and calculations were done for all plates.

2.2.5.4 Incubation of the plates

Culture plate lids were replaced, plates agitated gently and placed into gas-tight box, which had a damp tissue to maintain a humid atmosphere in the chamber. The gas box lid was replaced and the airtight box flushed with the gas mixture (92 % N_2, 5 % CO_2, 3 % O_2) and incubated at 37 °C. The chamber was flushed with the gas mixture twice a day (08:00 and 17:00) for the entire period that the experiment was on.

2.2.5.5 Addition of [^{3}H]-hypoxanthine to test cultures

After 48 hr. incubation, [G-^{3}H]hypoxanthine was added to paratisized and non-paratisized erythrocytes in the 96 well plates. Each micro-culture (225 µl) was treated

with 0.5 µCi of [^{3}H]-hypoxanthine (1 mCi/ml stock, Amersham Biosciences) after incubation of parasitized RBCs with drug. CMS+ [^{3}H]-hypoxanthine mixture to be added in each plate was calculated as follows:

1 well = 25 µl (CMS+Hyp)

96 wells per plate approximated to 100 wells

Hence total volume = 100 wells x 25 µl

= 2500 µl

One 96-well micro-culture plate is labelled with 56 µl of [^{3}H]-hypoxanthine

Therefore, volume of CMS = 2500 µl – 56 µl

= 2444 µl

Therefore, 56 µl of [^{3}H]-hypoxanthine (1 mCi/ml) was added to 2444 µl pre-warmed CMS and mixed gently. Using an Eppendorf multipipette plus 4981, 25 µl of the mixture was dispensed into each well in order of URBC control, PRBC control, then drug containing wells, from the lowest concentrations to the highest. Upon addition of [^{3}H]-hypoxanthine, the micro-culture plates were incubated for further 18 hr. in the same environment as described previously.

2.2.5.6 Termination of experiment

The experiment was terminated after 66 hr. incubation by removing the plates from the gas box, covering them with plate sealers and then freezing at –20 °C until harvested.

2.2.5.7 Harvesting of cells

This was done using a 96-well harvester (Wallac). Briefly, plates were thawed for 30 minutes at 37 °C to lyse the cells. A five-wash-cycle protocol was adopted for harvesting. Contents of the plates were washed onto printed-glass fibre filters (Wallac, Turku, Finland). The filters were dried in an oven for 1 h at 40 °C, placed in sample bags (Wallac) and sealed. One edge of the sample bag was cut and 5 ml of scintillation fluid (Ecoscint A; National Diagnostics, Atlanta, GA) added. The fluid was spread to wet the mats, excess fluid squinted out and the mats re-sealed.

2.2.5.8 Scintillation counting

Radioactive emissions were counted in a 1205 Beta plate reader (Wallac Micro-Beta TriLux). Briefly, filter mats loaded into reading cassettes were inserted into the beta plate reader and ran based on tritium protocol. Counts per minute (CPM) for each well was given in an excel format and used for further analysis. CPMs for each well represented the incorporated [^{3}H] hypoxanthine into the parasite nucleic acids.

2.2.5.9 Inhibitory concentration 50 (IC_{50})

Percentage reduction in [^{3}H] hypoxanthine uptake in presence of test samples was analyzed as a measure of sample efficacy. This was calculated according to Sixsmith *et al.*, (1984). The 50% inhibitory concentration (IC_{50}) refers to the drug concentration

inhibiting 50% of the parasite from incorporating [^{3}H] hypoxanthine in nucleotide synthesis. The UPRBC's CPM values were taken as the background count and corrected CPM values of each well by subtracting UPRBC's CPM values from CPM values of each wells. To calculate IC_{50}, the mid-point (Y_{50}) was calculated by the formula:

Y_{50} = (PRBC CPM values – UPRBC CPM value)/2

The drug concentration capable of inhibiting 50% of the *P. falciparum* (IC_{50}) was determined by computatation after logarithmic transformation of both concentration and cpm values using the formula:

IC_{50} = Antilog ($LogX_1$ + [(Log Y_{50} – Log Y_1)($LogX_2$ – $LogX_1$)/ ($LogY_2$ – $LogY_1$)],

IC_{50} = inhibitory concentration 50, X_1 and X_2 = lower and higher concentrations respectively, Y_1 = CPM values which correspond with X_1, Y_2 = CPM values which correspond with X_2.

CQ resistance is declared when the IC_{50} > 100 nM ≡ 52 ng/ml (Basco and Le Bras, 1992; Basco *et al.*, 1995). MQ resistance on the other hand is declared when the IC_{50} > 10 ng/ml. In this study, activity for extracts was considered high when $IC_{50} \leq 10$, moderate when between 10 and 50 and low when between 50 and 100 µg/ml. Samples with IC_{50} exceeding 100 µg/ml were considered as inactive.

2.2.5.10 Drug combination bioassay

Template plates were used in preparation of drugs combinations. Briefly, test drugs were aliquoted into an extra plate in various ratios of blends. The combined test samples were well mixed and transferred to multiple daughter plates or test plates, such that 3 daughter

plates were made per sample. One set of plates was used in the assay while the other daughter plates were kept at –20 °C for running repeats experiments on different days.

2.3 Studies of interaction of plant extracts and chloroquine

The method described by Canfield *et al.* (1995) was adopted. Briefly solutions of initial concentrations 20-50 times the estimated IC_{50} values was combined in various ratios of various drugs. Thus fixed ratios of pre-determined concentrations needed to inhibit parasite growth by 50% (IC_{50}) was used to determine the interaction of 2 drugs. Plants that had been cited by the traditional health practioners as being used in combination were assayed against chloroquine-resistant clone W2. At least 3 were assayed in combination with chloroquine phosphate (MW = 515.9). Single and combined drug solutions were dispensed into the 96 well microtitre plates to give 9 combinations ratios of 90:10, 80:20, 70:30, 60:40, 50:50, 40:60, 30:70, 20:80, and 10:90 (CQ:extract or extract A:extract B) (Fivelman *et al.,* 1999). Incubation and subsequent procedures were followed as previously described. Corresponding IC_{50} values were determined for each drug alone and in combination using the method of Sixsmith *et al.,* (1984). The degree of synergy was evaluated according to the method of Berenbaum (1978). Briefly, fraction inhibition concentration (sum FIC) was calculated using the formula;

$$A_c/A_e + B_c/B_e = K,$$

where A_c and B_c are the equally effective concentrations (IC_{50}) when used in combination, and A_e and B_e are the equally effective concentrations when used alone.

Sum FICs <1 denote synergism, sum FICs ≥1 and <2 denote additive interaction, Sum FICs ≥2 and <4 denote slight antagonism, and Sum FICs ≥4 denote marked antagonism. Sum FICs <0.5 indicate substantial synergism (Gupta *et al.*, 2002).

2.4 *In vivo* anti-malarial assay

2.4.1 Drug and sample preparations

Chloroquine was used as a standard anti-malarial drug. It was dissolved in distilled water. Water-soluble plant extracts were dissolved in water while water insoluble extracts were dissolved in 10% (w/v) tween 80.

2.4.2 Animal model

Male Balb C mice (6-8 weeks old, weighing 20 ± 2 g) were used as the test model. The mice were bred at KEMRI animal facility in standard macrolon type II cages in air conditioned rooms at 22 °C and 50-70% relative humidity and fed with the standard diet and water *ad libitum*.

2.4.3 Maintenance of *Plasmodium berghei*

Plasmodium berghei strain ANKA was used to assay for anti-malarial activity. The parasite was maintained by serial passage of infected blood by interperitonial injection. Blood from *Plasmodium berghei*-infected mice was obtained by heart puncture on Day 5 and mixed with 1% (w/v) sodium citrate or heparin in phosphate buffer saline (1:1). Each mouse was injected intra-peritoneally (ip) with 0.2 ml solution of infected red blood cells.

2.4.4 *In vivo* anti-malarial efficacy using *Plasmodium berghei*

The test protocol was based on the 4-day suppressive test described by Peters *et al.*, (1975). Briefly, mice were randomly selected into groups of five each for one test sample. Heparinized blood was taken from a donor mouse with approximately 30% parasitaemia on day 0 and diluted in physiological saline to $\approx 10^8$ parasitized erythrocytes per ml. An aliquot of 0.2 ml ($2x10^7$ parasitized erythrocytes) of this suspension was injected ip into experimental groups of mice (Gessler *et al.*, 1995; Ajaiyeoba *et al.*, 1999).

The experimental groups were treated with a single dose of test sample at 100 mg/kg at a volume of 0.2 ml by ip injection (Gessler *et al.*, 1995) 2-4 hr post infection. On days 1 to 3 (24, 48 and 72 h post-infection), the experimental groups of mice were treated again with the same dose by ip injection. Two goups (5 mice each) served as negative and positive controls, respectively. The negative group received a placebo (saline) while the positive one was treated with 5 mg/kg/day of the reference drug (CQ). CQ at 5 mg/kg was used as the standard reference drug.

Parasitaemia was determined daily (24 hr interval) by a thin blood film sampled from the tail of the mouse and stained in 10% giemsa solution. A thin blood smear was similarly made on day 4 (24 hr after the last treatment or 96 hr post-infection). Parasitaemia was determined microscopically by counting 4 fields of approximately 100 erythrocytes per field. The difference between the mean value of the control group (100%) and those of

the experimental groups was calculated and expressed as percent parasitaemia suppression (PS) (activity) according to the following formula:

$$PS = [(A-B)/A] \times 100$$

where *A* is the mean parasitaemia in the negative control group on day 4, and *B* the corresponding parasitaemia in the test group (Tona *et al.*, 2001). The standard deviations for the mean values were calculated as described by Armitage and Berry (1991). Untreated control mice typically died approximately one week after infection. For treated mice the survival time (days) was recorded and the means calculated. The mean survival time for untreated and standard drug treated groups were also calculated.

Plant extracts were categorized as being highly active when parasitaemia suppression was above 60%, while those between 30 and 60% were considered as moderately active and below 30% as weak in anti-malarial activity.

2.5 Toxicity evaluation

2.5.1 Cytotoxicity evaluation

Actively dividing sub-confluent Vero cell growth-inhibition assay was done (Kurokawa *et al.*, 1995). Briefly, Vero cells were grown in Eagle's minimum essential medium (MEM; GIBCO, Grand Island, N.Y.) supplemented with 5% foetal bovine serum (FBS) in 25 ml cell culture flasks incubated at 37 °C in 5% CO_2 incubator. Upon attainment of confluence, the cells were seeded at a concentration of 5×10^4 cells/well in 24-well plates and incubated at 37 °C for 2 days. The culture medium was replaced by fresh MEM

containing test extracts at different concentrations, and the cells incubated further for 2 days. Cells in triplicate wells for each sample were detached by trypsinization, and the number of viable cells determined by a trypan blue exclusion test. A haemocytometer was used to aid in counting viable cells. Inhibition data was plotted as dose–response curves, from which CC_{50} (concentration required to cause visible alterations in 50% of intact cells) was estimated. Selectivity index (SI) calculated as CC_{50} (Vero)/IC_{50} was used as parameter of clinical significance of the test samples by comparing general toxins and selective inhibitory effect on *P. falciparum* (Wright and Phillipson, 1990).

2.5.2 *In vivo* acute toxicity

Male Balb C mice 6-8 weeks old weighing 20 ± 2 g. were used in determining the lethal dosage of the test samples (Lorke, 1983; Tona *et al.,* 2000). Briefly mice were acclimatized to laboratory conditions and randomly divided into groups of 5 (one group for each extract concentration plus one control group given a placebo). The mice were starved for 24 hr prior to dosing. The first group was treated with normal saline and taken as the control. The other six groups were treated with the test extract dissolved in tween 80 at an increasing dosage of 250, 500, 750, 1000, 1500 and 5000 mg/kg body weight. Treatment was via oral route at a volume of 0.2 ml. The number of deaths within 24-48 hr was noted. Probit-log analysis (Finney, 1964) was used to analyse data and calculate the LD_{50} values.

2.6 Data management and statistical analysis

The *in vitro* anti-plasmodial activity was determined by measuring IC_{50} values of the test extracts preparations for *P. falciparum* and compared with reference synthetic drugs (chloroquine, quinine, mefloquine and artemisinin).

The *in vivo* anti-malarial activity was determined by the percentage suppression of *P. berghei* parasitaemia and survival rate of treated and control mice.

Toxicity study was determined by measuring LD_{50} (for mice), CC_{50} (for vero cells) and by selectivity index.

Computer program for data analysis - As first proposed by Desjardins, concentration response data for each compound were fit to a sigmoidal function by the Marquadt algorithm. GraFit® (Erithacus Software Limited) computer software was used to give dose–response curves of the effect of the test drug on the growth of the parasites. Four parameters for the hyperbolic tangent function: IC_{50}, slope, upper asymptote and lower asymptote were similarly obtained. Thus percentage reductions are used to plot percentage inhibition of growth as a function of drug concentrations. IC_{50} values were determined by linear regression analyses on the linear segments of the curves.

In analyzing the data, Statistica® and Statview® programmes were used to express results as mean ± standard deviation. Significant differences between control and experimental groups were assessed by Student's *t*-test (Benoit *et al.*, 2000). The comparisons were

done by aid of computer software (PC-pcsm, version 6.0, 1992; Delta-soft, Meylan, France). Two-tailed Welch's unpaired *t*-test was used to compare treatment between D6 and W2 as well as water and methanol extracts (Benoit *et al.,* 2000). Tukey-Kramer Multiple Comparisons Test and the Dunnett Multiple Comparisons Test were used to compare significance of mean differences (Dunnett, 1964). $P < 0.05$ was considered statistically significant while $P > 0.05$ was considered not statistically significant.

CHAPTER THREE

RESULTS

3.1 Collection and processing of plant material

The plants and part used, collected between February and August 2004, are listed in table 1.

Table 1: Plants and parts collected

Botanical name	Family	Voucher specimen no.	Part used	Extracts yield	
				Water extracts (%)	Methanol extracts (%)
C. roseus	Apocynaceae	(K4) CM030	Leaves	4.00 (8.0)	1.40 (2.8)
X. americana	Olacaceae	(K4) CM033	Root bark	7.00 (14)	3.20 (6.4)
L. eriocalyx	Fabaceae	(K4) CM032	Stem bark	6.95 (13.9)	2.00 (4.0)
C. robusta	Euphorbiaceae	(K4) CM031	Leaves	5.00 (10.0)	0.95 (1.9)
T. robusta	Meliaceae	(K4) CM036	Root bark	4.10 (8.2)	0.85 (1.7)
T. holstii	Apocynaceae	(K4) CM034	Leaves	5.15 (10.3)	1.05 (2.1)
			Fruit	6.30 (12.6)	1.60 (3.2)
A. afra	Asteraceae	(K4) CM035	Leaves	2.25 (4.5)	0.60 (1.2)
			Stem bark	2.10 (4.2)	0.05 (1.0)
R. natalensis	Anacardiaceae	(K4) CM037	Stem bark	2.45 (4.9)	1.10 (2.2)
C. anisata	Rutaceae	(K4) CM041	Root bark	2.90 (5.8)	0.95 (1.9)
L. schweinfurthii	Anacardiaceae	(K4) CM038	Stem bark	6.65 (13.3)	1.15 (2.3)
S. birrea	Anacardiaceae	(K4) CM042	Stem bark	6.30 (12.6)	1.30 (2.6)
B. salicifolia	Capparidaceae	(K4) CM039	Stem bark	5.60 (7.2)	1.60 (3.2)
T. brownii	Combretaceae.	(K4) CM040	Fruit	4.30 (8.6)	1.25 (2.5)
T. grandifolia	Gentianaceae	(K4) CM044	Leaves	4.30 (8.6)	1.25 (2.5)
C. schimperiana	Amaranthaceae	(K4) CM043	Root	4.30 (8.6)	1.00 (2.0)

Differences in yields between dried water and methanol extracts were not significant ($P > 0.05$) in all cases. The 15 plants collected for the study were processed and extracted in water and in methanol and their extracts yields recorded. The aqueous extract of *X. americana* had the highest yield (7g equivalent to 14%). Stem bark of *A. afra* had the least extracted material (0.05g equivalent to 1%). A higher percentage of material was extracted by water as opposed to methanol in all the cases.

3.2 *In vitro* bioassay

Plasmodium falciparum strains D6 and W2 were propagated for bio-screening. Figure 4 shows the growth pattern of the cultured parasites under normal conditions.

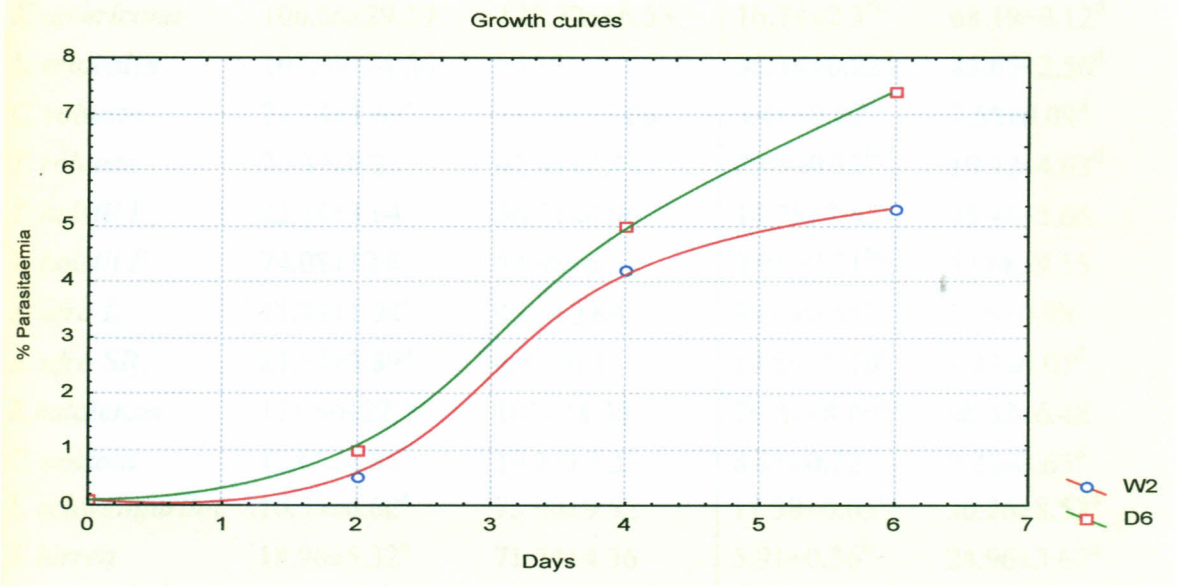

Figure 4: Growth curves for *Plasmodium falciparum* cultures. Parasitaemia for the two parasite strains were determined daily for 6 days and the percentage parasitaemia plotted against numbers of days. *Plasmodium falciparum* D6 strain was the most robust followed by W2.

Methanolic and aqueous extracts of the 15 selected medicinal plants were subjected *to in vitro* anti-plasmodial assay using D6 and W2 *P. falciparum* strains. The results are summarized in table 2.

Table 2: *In vitro* anti-plasmodial activity (IC_{50}±SD) for methanolic and water extracts of selected medicinal plants

	IC_{50} ± SD (µg/ml)			
	Aqueous		Methanol	
Plant	D6	W2	D6	W2
C. roseus	32.36±4.31	36.168±7.79	4.65±0.88^{c}	5.34±0.65^{d}
X. americana	106.66±29.39	122.225±6.55	16.74±2.3^{bc}	68.19±0.12^{d}
L. eriocalyx	103.08±24.36	>125	35.34±0.22^{c}	85.65±2.56^{d}
C. robusta	71.24±1.69^{a}	117.81±5.66	3.41±0.02^{bc}	7.58±0.09^{d}
T. robusta	25.32±0.25^{a}	42.41±2.41	2.09±0.13^{bc}	10.32±4.63^{d}
T. holstii L	25.36±3.64	36.21±4.96	14.75±2.45^{c}	25.44±5.65
T. holstii F	74.08±12.81	54.98±6.36	3.91±0.21^{bc}	53.68±9.15
A. afra L	11.23±1.98^{a}	4.65±0.64	9.04±0.54^{b}	3.98±0.98
A. afra SB	21.63±3.89^{a}	4.11±0.11	17.80±5.70^{b}	1.23±0.07^{d}
R.natalensis	111.60±22.76	105±18.36	76.84±8.09^{b}	98.32±6.48
C. anisata	12.65±4.85	19.21±2.25	8.45±0.22	9.25±3.65^{d}
L. schweinfurthii	10.55±0.62^{a}	75.90±9.52	11.38±0.65^{b}	36.26±8.52^{d}
S. birrea	18.96±5.32^{a}	71.74±4.36	5.91±0.36^{b}	24.96±3.62^{d}
B. salicifolia	3.65±0.78^{a}	10.09±1.56	1.03±0.03^{b}	8.86±0.24
T. brownii	41.52±0.21^{a}	55.82±5.47	22.17±0.99^{c}	30.25±7.41^{d}
T. grandifolia	54.12±7.98^{a}	108.52±19.21	33.75±2.09	17.91±6.82^{d}
C. schimperiana	115.92±14.45	>125	10.40±1.25^{c}	17.61±2.61^{d}

Three reference drugs included as positive control their IC_{50} values given ng/ml: artemisinin 1.64±0.11 and 4.38±0.49; chloroquine 3.11±1.21 and 57.34±2.95 and mefloquine 16.08±2.65 and 2.55±0.03 for D6 and W2, respectively. Values significantly different (p<0.05) when comparing; a - water D6 vs W2, b - methanol D6 vs W2, c - water vs methanol D6, d - water vs methanol W_2.

D6, a CQ-sensitive *P. falciparum* clone, gave IC_{50} of 3.11±1.21 and 16.08±2.65 ng/ml for chloroquine and mefloquine respectively. It was confirmed that D6 is susceptible to CQ and resistant to MQ. Conversely, *P. falciparum* clone W2 that had IC_{50} of 57.34±2.95 and 2.55±0.03 ng/ml for CQ and MQ respectively is CQ resistant and MQ sensitive.

Methanolic extract of *B. salicifolia* was the most active against D6 IC_{50} (1.03±0.03 µg/ml). Others with high anti-plasmodial activity against *P. falciparum* D6 clone include: *T. robusta, C. anisata, C. robusta, A. afra, S. birrea, T. holstii* and *C. roseus* methanol extracts IC_{50} (<10 µg/ml). The water extracts of the same plants had moderate activity. *Artemisia afra, C. roseus, C. robusta, C. anisata* and *B. salicifolia* methanolic extracts exhibited high anti-plasmodial activity against W2 clone. A trend similar to one observed for D6 between the water and methanol extracts was observed with methanol extract exhibiting higher activity than aqueous ones. *Lonchocarpus eriocalyx* and *C. schimperiana* were the least active water extracts (IC_{50} > 125 µg/ml). Interestingly, the methanol extract of the later was moderately active (< 17.61 µg/ml). In summary 25% of the extracts were found to be highly active (IC_{50} < 10 µg/ml), 45.59% moderately active, 16.18% slightly active and 13.24% inactive.

The dose response curves for *P. falciparum* clones treated with CQ, *B. salicifolia* and *A. afra* extracts were constructed (Fig 5-7)

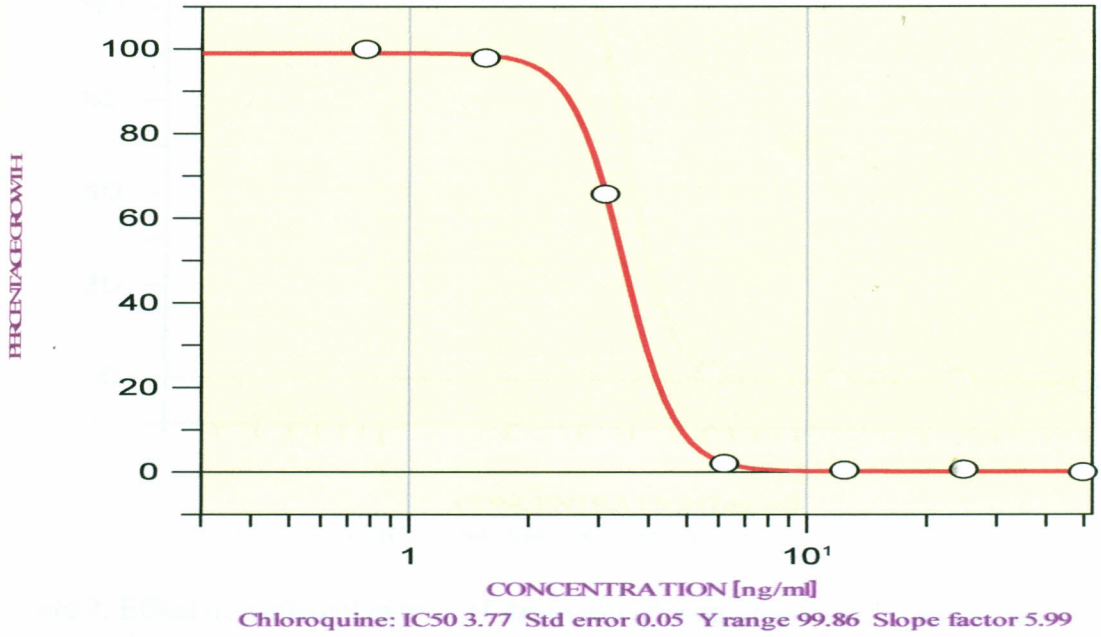

Figure 5: Effect of CQ on growth of *Plasmodium falciparum* D6 clone

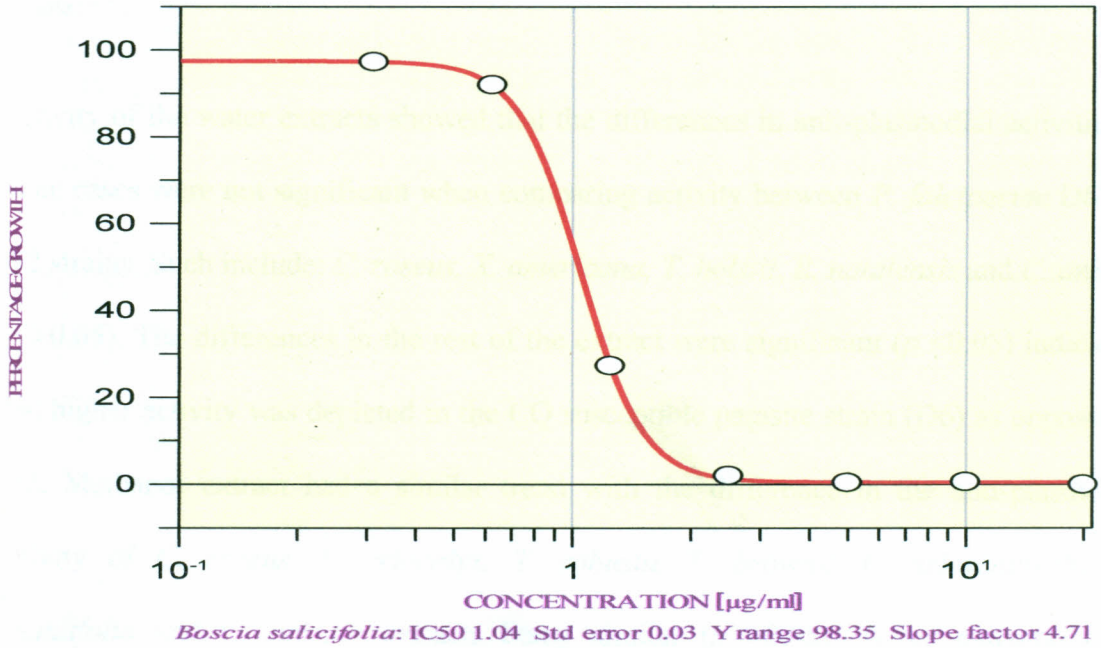

Figure 6: Effect of methanol extract of *Boscia salicifolia* on growth of *Plasmodium falciparum* D6 clone

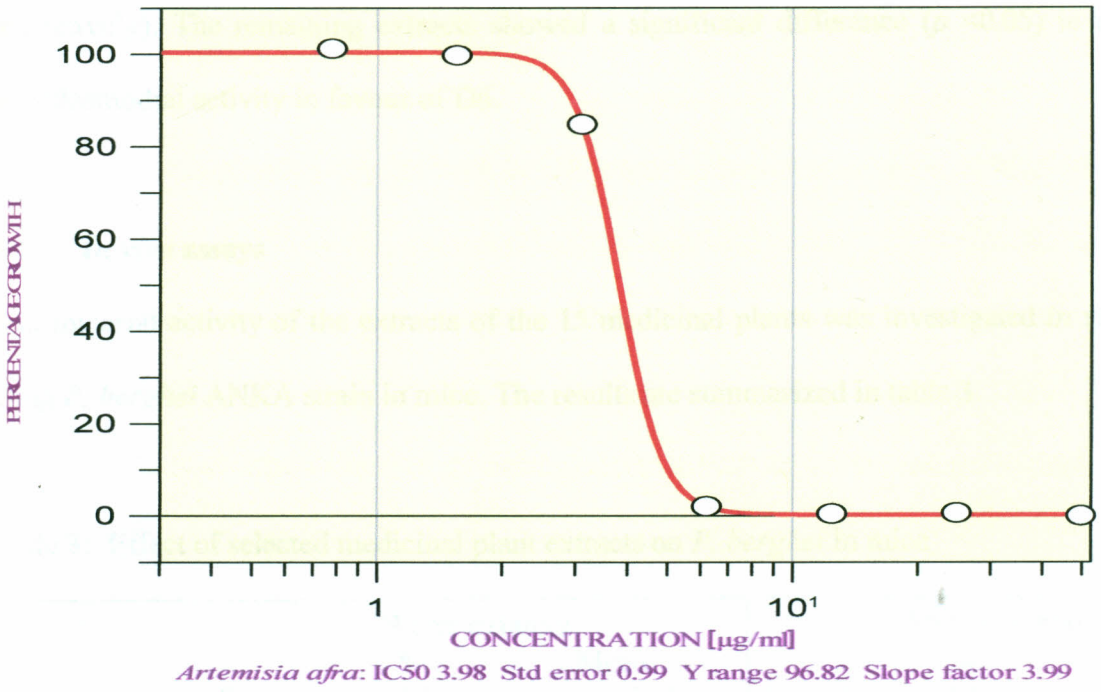

Figure 7: Effect of methanol extract of *Artemisia afra* on growth of *P. falciparum* W2 clone.

The above graphs describe changes in parasitaemia density as the concentration of the test sample increases. The y-axis represents the percentage parasitaemia while the x-axis is the concentration of the test sample.

Activity of the water extracts showed that the differences in anti-plasmodial activities in some cases were not significant when comparing activity between *P. falciparum* D6 and W2 strains. Such include: *C. roseus, X. americana, T. holstii, R. natalensis* and *C. anisata* ($p > 0.05$). The differences in the rest of the extract were significant ($p < 0.05$) indicating that higher activity was depicted in the CQ susceptible parasite strain (D6) as opposed to W2. Methanol extract had a similar trend with the difference in the anti-plasmodial activity of *C. roseus, L. eriocalyx, T. robusta, T. brownii, C. schweinfurthii, T. grandifolia* and *C. anisata* extracts being similar ($p > 0.05$). Water extracts of *C. schimperiana, R. natalensis, X. americana and L. eriocalyx* exhibited the least activity against D6 (IC_{50} 115.92±14.45, 111.60±22.76, 106.66±29.39 and 103.08±24.36 µg/ml

respectively). The remaining extracts showed a significant difference (p <0.05) in the anti-plasmodial activity in favour of D6.

3.3 *In vivo* assays

Anti-malarial activity of the extracts of the 15 medicinal plants was investigated *in vivo* using *P. berghei* ANKA strain in mice. The results are summarized in table 3.

Table 3: Effect of selected medicinal plant extracts on *P. berghei* in mice

	Water extarcts			Methanol extracts		
Plant	Parasite density	% Chemos-supression	Mean survival time (days)	Parasite density	% Chemos-supression	Mean survival time (days)
C. roseus	21.60±2.51	42.35±4.87	12.39±2.95	24.51±4.32	34.60±8.77	10.85±2.50
X. americana	29.40±3.89	21.55±6.27	6.72±1.52^{c}	23.80±7.30	36.49±2.82	7.85±1.59^{c}
L. eriocalyx	29.92±3.71	20.15±0.23	7.52±2.89^{c}	31.85±6.15	15.00±2.54	6.11±2.42^{c}
C. robusta	21.60±2.39	42.35±2.15	12.82±4.70	28.81±1.40	23.10±6.43	10.45±3.25
T. robusta	13.56±1.82^{a}	63.81±5.27	13.62±1.33	8.00±2.35^{ab}	78.20±7.59	11.81±5.27
T. holstii	25.69±4.61	31.45±4.82	7.38±0.54^{c}	24.08±2.65	35.65±9.41	7.61±1.65^{c}
A. afra	11.15±3.01^{a}	70.25±5.59	17.85±2.81	8.45±0.27^{ab}	77.45±3.06	19.21±3.57
R. natalensis	6.31±0.97^{ab}	83.15±3.61	16.52±1.87	16.40±1.82^{a}	56.24±4.85	13.43±5.24
C. anisata	33.63±5.09	10.24±2.46	7.20±1.91^{c}	35.23±2.51	5.97±0.82	6.85±0.08^{c}
L. schweinfurthii	6.34±0.83^{ab}	83.08±9.53	13.27±5.21	3.17±1.42^{ab}	91.37±9.33	12.39±4.21
S. birrea	12.55±2.42^{a}	66.51±7.21	15.22±2.43	13.50±4.29^{a}	63.49±8.86	13.28±3.54
B. salicifolia	21.08±4.52	43.75±6.90	15.71±5.41	5.06±0.84^{ab}	86.50±4.37	19.08±4.22
T. grandifolia	31.21±2.51	16.70±2.36	6.37±2.43^{c}	31.58±0.49	15.86±1.17	6.11±2.32^{c}
C. schimperiana	30.33±2.45	19.06±5.33	7.65±1.89^{c}	20.58±1.69	45.08±6.93	15.38±4.18
Control	37.47±6.62	0.00±0.00	7.14±2.14			
CQ	0.38±0.03^{a}	99.02±1.26	18.25±5.28			

a - significant difference (p<0.05) in parasite density (PD) of treatment goups vs untreated group, b - difference in PD of treatment groups not significant (p>0.05) to CQ group, c - mean survival time of treatment groups not significantly difference (p>0.05) to the untreated group

Suppression of parasitaemia (chemosuppression) and mean survival time were used as measures of efficacy. Methanol extracts of *L. schweinfurthii, B. salicifolia* and the water extracts of *R. natalensis* were the most active showing chemosuppression of 91.37±9.33, 86.50±4.37 and 83.15±3.61%, respectively. The methanol extract of *T. robusta*, water and methanol extracts of *A. afra* and *S. birrea* followed in activity with chemosuppression of 78.20±7.59, 70.25±5.59, 77.45±3.06, 66.51±7.21 and 63.49±8.86% respectively. The water extract of *R. natalensis*, *B. salicifolia, C. roseus* and *C. robusta* also showed moderate activity with chemosuppression of 56.24, 43.75, 42.30 and 42.35%, respectively. Similarly, the methanol extracts of *C. schimperiana*, *C. roseus, T. holstii* and *X. americana* showed moderate activity with 45.08, 34.60, 35.65 and 36.49% chemosuppression, respectively. The remaining extracts (*C. robusta* (methanol), *X. americana* (water), *C. schimperiana* (water), *T. grandifolia* (water and methanol), *L. eriocalyx* (methanol) and *C. anisata* (methanol)) had weak anti-malarial activity (23.10±6.43; 21.55±6.27; 19.06±5.33, 16.70±2.36/15.86±1.17, 15.00±2.54 and 5.97±0.82%).

Mean parasitaemia in the reference drug control (CQ) group was 0.38±0.03%, while that in the untreated control was 37.47±6.62%. A similar reduction in parasitaemia was evident in the treatment groups with the highest being exhibited by *L. schweinfurthii* and *R. natalensis* (3.17±1.42 and 6.31±0.97%). There was a significant parasite density reduction in most of the test samples compared to the un-treated control ($P<0.05$).

Mean survival time of the experimental groups of test animals correlated to treatment. The survival period of mice that received extracts shown to have high percent

chemosuppression lived longer compared to those with low anti-malarial activity. Mice treated with the methanol extract of *A. afra* had a mean survival time of 19.21±3.57 days while that of the untreated group had 7.14±2.14 days. The difference in mean survival time for many of the treatment groups was significant when compared to the untreated control group of mice ($p<0.05$) except for water and methanol of *X. americana, L. eriocalyx, T. holstii, C. anisata, T. grandifolia* and the water extract of *C. schimperiana.*

3.4 Toxicity study

3.4.1 *In vitro* cytotoxicity

In vitro cytotoxicity of selected medicinal plant extracts was done using VERO cells and the results summarized in table 4.

Table 4: Cytotoxicity (CC_{50}) of selected medicinal plants extracts to VERO cells

Plant	Extract	CC_{50} (µg/ml)	SI* (D6)	SI* (W2)
C. roseus	MeOH	167.52	36.03	31.37
	H_2O	1285.74	39.73	35.55
X. americana	MeOH	198.11	11.83	2.91
	H_2O	1720.38	16.13	13.31
C. robusta	MeOH	460.29	134.98	60.72
	H_2O	4352.76	61.10	27.50
T. robusta	MeOH	24.38	11.67	2.36
	H_2O	45.72	1.81	1.08
A. afra L	MeOH	594.85	65.80	149.46
	H_2O	2825.21	251.58	607.57
A. afra SB	MeOH	628.27	35.30	157.86
	H_2O	2964.02	137.03	721.17
R. natalensis	MeOH	211.78	2.76	2.15
	H_2O	3958.16	35.47	37.70
B. salicifolia	MeOH	304.92	293.19	34.42
	H_2O	1683.95	461.36	166.89
L. schweinfurthii	MeOH	225.25	19.79	6.21
	H_2O	3256.52	308.67	42.91
S. birrea	MeOH	361.24	61.12	14.47
	H_2O	3375.22	178.02	47.05
C. schimperiana	MeOH	241.34	23.21	13.70
	H_2O	2907.12	16.52	nd
T. brownii	MeOH	235.20	10.61	7.78
	H_2O	3283.61	79.09	58.82
T. holstii	MeOH	120.25	30.75	2.19
	H_2O	1235.45	16.68	23.05

*SI-selectivity index calculated as CC_{50}/IC_{50}, nd-not done since IC_{50} >125 µg/ml

The highest selective index (ratio of 50% cytotoxic concentration to 50% effective anti-plasmodial concentration) of *P. falciparum* was observed with the water and methanol extracts of *A. afra* and water extract of *B. salicifolia* with selectivity index (SI) ratios of >100. Methanol extract of *C. robusta* also depicted a high selective index. Methanol extracts of *T. holstii* and *R. natalensis* were the most cytotoxic (CC_{50} 120.25 µg/ml, SI 2.19 and CC_{50} 211.78 µg/ml, SI 2.15-2.76, respectively) followed by *X. americana* and *C. schimperiana* (SI 2.9-23.21). *C. roseus* was moderately cytotoxic. *T. robusta* exhibited cytotoxicity (CC_{50}=24.38, 45.72 µg/ml for methanol and aqueous extracts, respectively) with a low selectivity index for aqueous extract against D6 (1.81). Its selectivity indices for W2 were also low (2.36 and 1.08 for methanol and aqueous extract respectively). The methanol extract of *L. schweinfurthii* had a slightly low selectivity index against W2 (6.21) indicative of mild toxicity.

3.4.2 *In vivo* toxicity

In vivo toxicity of the plant extracts was tested in mice. The results on *in vivo* toxicity are summarized in Table 5. The LD_{50} values were obtained when groups of 5 mice each were given an increasing dose of the test extracts to a maximum of 5000 mg/kg body weight.

Table 5: Toxicity of extracts of selected medicinal plants to mice

Species	LD^{50}
C. roseus	>5000
X. americana	3896
L. eriocalyx	>5000
C. robusta	>5000
T. robusta	1237
T. holstii	>5000
*A. afra L** and *SB*	>5000
R. natalensis	>5000
C. anisata	>5000
L. schweinfurthii	>5000
S. birrea	>5000
T. brownii	>5000
B. salicifolia	>5000
T. grandifolia	>5000
C. schimperiana	>5000

**L leaves, SB stem bark*

Where no deaths were recorded at 5000 mg/kg body weight, the extracts were considered safe. Majority of the plant extracts were found to be safe according to this classification.

The LD_{50} values for 13 plants were greater than 5000 mg/kg body weight. *T. robusta* and *X. americana* were the only ones which were toxic to mice (LD_{50} 1237 and 3896 mg/kg).

3.5 Drug interaction studies

3.5.1 *In vitro* drug interaction

In vitro drug interaction studies of selected methanol and water extracts with one another or chloroquine against *P. falciparum* W2 (CQ resistant clone) were undertaken as previously described elsewhere (Muregi *et al.*, 2004). The Sum FIC values were calculated as previously described (Berenbaum, 1978). The results are summarized in Tables 6-11.

Table 6: Interaction of methanolic extracts of selected medicinal plants with chloroquine

Combination ratios	90:10	80:20	70:30	60:40	50:50	40:60	30:70	20:80	10:90
S. birrea	2.01^a	2.03^a	2.05^a	1.97^b	1.94^b	2.25^a	2.32^a	2.99^a	2.87^a
T. holstii	1.58^b	1.47^b	1.38^b	1.42^b	1.19^b	1.70^b	1.41^b	1.28^b	1.12^b
L. sweinfurthii	1.2^b	1.57^b	1.62^b	1.90^b	2.15^a	2.37^a	2.11^a	1.71^b	1.24^b
B. salicifolia	1.69^b	1.32^b	1.76^b	1.96^b	1.19^b	2.47^a	2.75^a	2.55^a	2.06^a
A. afra	1.86^b	1.64^b	1.61^b	1.58^b	2.66^a	2.70^a	2.70^a	2.81^a	3.75^a

a – antagonistic, b – additive, the table above shows the combination ratios and the SumFic values obtained when chloroquine was combined with the selected medicinal plants

The interaction between methanol extract of *S. birrea* and chloroquine is to a large extent antagonistic. However, weak additive effect is realized towards equal concentration

(50:50). The interaction between extract of *T. holstii* with chloroquine is additive at all concentration ratios. At combination ratios of 50:50-70:30, the interaction between *L. sweinfurthii* and chloroquine is antagonistic flanked by a synergistic interaction at lowered concentration of both. At high chloroquine concentration the interaction between *B. salicifolia* and chloroquine is antagonistic while as the concentration of the extract increases at 50:50 combination ratios onwards, the interaction changes to addition. Thus the extract tends to add chloroquine potency. Interaction between *A. afra* and Chloroquine was additive with high amount of the extract in combination. However the interaction changes to antagonism as the amount of chloroquine increase in the blend.

Table 7: Interaction of methanol extracts of *Sclerocarya birrea* with selected medicinal plants

Combination ratios	90:10	80:20	70:30	60:40	50:50	40:60	30:70	20:80	10:90
C. robusta	0.87^{b}	1.36^{b}	1.26^{b}	1.17^{b}	1.00^{b}	1.00^{b}	1.00^{b}	1.05^{b}	1.16^{b}
L. sweinfurthii	0.96^{c}	0.64^{c}	0.59^{c}	0.65^{c}	0.65^{c}	0.33^{c}	0.52^{c}	0.58^{c}	0.92^{c}

b – additive, c – synergistic, the table above shows the combination ratios and the SumFic values obtained when *Sclerocarya birrea* was combined with the selected medicinal plants

Apart from the 90:10 combination of *S. birrea* with *C. robusta* that has a weak synergist effect all the other combinations are additive. Moderate synergistic interaction exists between *S. birrea* and *L. sweinfurthii* at all combinations except 90:10 and 10:90.

Table 8: Interaction of methanolic extracts of *Artemisia afra* with selected medicinal plants

Combination ratios	90:10	80:20	70:30	60:40	50:50	40:60	30:70	20:80	10:90
C. robusta	0.86^{c}	0.75^{c}	0.96^{c}	1.00^{b}	0.96^{c}	0.87^{c}	0.86^{c}	0.77^{c}	0.61^{c}
L. schweinfurthii	1.00^{b}	1.00^{b}	1.00^{b}	1.00^{b}	1.00^{b}	1.00^{b}	1.00^{b}	1.00^{b}	1.00^{b}
X. americana	1.67^{b}	2.47^{a}	1.86^{b}	1.95^{b}	1.78^{b}	1.69^{b}	1.45^{b}	1.25^{b}	1.32^{b}
C. anisata	0.54^{c}	0.54^{c}	0.63^{c}	0.42^{c}	0.68^{c}	0.66^{c}	0.78^{c}	0.84^{c}	0.92^{c}
T. robusta	1.54^{b}	1.42^{b}	1.31^{b}	1.39^{b}	1.73^{b}	1.78^{b}	2.11^{a}	2.02^{a}	1.69^{b}
L. eriocalyx	0.99^{c}	1.02^{b}	1.06^{b}	1.14^{b}	1.18^{b}	1.23^{b}	1.24^{b}	1.27^{b}	1.11^{b}
M. undata	0.65^{c}	0.64^{c}	0.78^{c}	0.74^{c}	0.66^{c}	0.63^{c}	0.54^{c}	0.69^{c}	0.77^{c}

a – antagonistic, b – additive, c – synergistic, the table above shows the combination ratios and the SumFic values obtained when *Artemisia afra* was combined with the selected medicinal plants

Weak synergistic interaction was observed between *A. afra* and *C. robusta* methanol extracts. However, at 60:40 combinations the interaction is strongly additive. Strong additive interaction is observed at all combinations of *A. afra* and *L. schweinfurthii.* The interaction between *A. afra* and *X. americana* was weak addition with most combinations except 80:20 with a moderate antagonistic effect. Moderate synegy was observed with most combinations between *A. afra* and *C. anisata.* However, the synergy was very weak at high concentrations of *C. anisata.* Weak additive interaction was observed with combinations that had more *A. afra* upto 40:60 combinations with ≥70% of *T. robusta* exhibiting antagonism. Apart from the combination with 90% *A. afra* which exhibited weak synergy, all the other combinations between *A. afra* and *L. eriocalyx* resulted in

strong additive interactions. Moderate synergy was observed at all combinations of *A. afra* and *M. undata*

Table 9: Interaction of aqueous extracts of *Turraea robusta* with selected medicinal plants

Combination ratios	90:10	80:20	70:30	60:40	50:50	40:60	30:70	20:80	10:90
L. schweinfurthii	0.90^{c}	0.84^{c}	0.76^{c}	0.83^{c}	1.04^{b}	1.65^{b}	0.68^{c}	0.68^{c}	0.56^{c}
S. birrea	1.21^{b}	1.02^{b}	1.02^{b}	1.01^{b}	1.17^{b}	0.78^{c}	1.00^{b}	1.01^{b}	1.16^{b}
B. salicifolia	1.03^{b}	1.95^{b}	1.25^{b}	1.23^{b}	1.50^{b}	1.09^{b}	1.03^{b}	0.92^{c}	0.92^{c}

b – additive, c – synergistic, the table above shows the combination ratios and the SumFic values obtained when *Turraea robusta* was combined with the selected medicinal plants

Moderate synergy was observed in most combination between *T. robusta* and L. *schweinfurthii* except for two combinations towards equal proportion of each extract where the interaction was additive. Only the 40:60 combination between *T. robusta* and *S. birrea* where synergistic interaction was exhibited. The rest of the combinations were additive. A similar scenario was observed in combination involving *T. robusta* and *B. salicifolia* with only the last two combinations exhibiting synergy.

Table 10: Interaction of aqueous extracts of *Rhus natalensis* with *Sclerocarya birrea*

Combination ratios	90:10	80:20	70:30	60:40	50:50	40:60	30:70	20:80	10:90
	1.19^{b}	1.70^{b}	1.79^{b}	2.06^{a}	2.16^{a}	2.06^{a}	1.15^{b}	1.08^{b}	0.54^{c}

a – antagonistic, b – additive, c – synergistic, the table above shows the combination ratios and the SumFic values obtained for combination of *Rhus natalensis* and *Sclerocarya birrea*

Interaction of *R. natalensis* and *S. birrea* ranged from addition at high concentration of *R. natalensis*, antagonism towards equal proportions of the two extracts to synergy at low concentration of *R. natalensis.*

Table 11: Interaction of aqueous extracts of *Lannea schweinfurthii* with *Boscia salicifolia*

Combination ratios	90:10	80:20	70:30	60:40	50:50	40:60	30:70	20:80	10:90
	1.12^{b}	1.11^{b}	0.92^{c}	1.01^{b}	1.03^{b}	1.06^{b}	1.16^{b}	1.24^{b}	1.28^{b}

b – additive, c – synergistic, the table above shows the combination ratios and the SumFic values obtained for combination of *Lannea schweinfurthii* and *Boscia salicifolia*

Only the 70:30 combination of *L. schweinfurthii* and *B. salicifolia* was synergistic the other combinations were additive.

3.5.2 *In vivo* interactions

The drug combinations were selected from the *in vitro* interaction studies that exhibited strong additive or synergistic interactions.

Table 12: *In vivo* anti-malarial results for combined methanol plant extracts

Sample combination	Combi -nation ratios	*In vitro* combina -tion Sum Fic	*In vivo combination* Parasite density	Chemos-supression	Survival time (days)
A. afra/C. anisata	50:50	0.68	22.50±5.21	43.89±2.20	11±1.00
A. afra/C. robusta	60:40	1	6.50±2.25	83.79±5.63	14±1.27
A. afra/C. robusta	10:90	0.61	13.47±9.55	75.47±3.20	12.67±2.12
S. birrea/C. robusta	90:10	0.87	1.33±1.19	97.58±2.17[a]	19.11±3.40
A. afra/T. robusta	30:70	2.11	25.00±3.11	37.66±3.79	14.13±2.55
S. birrea/L. schweifurthii	40:60	0.33	20.00±1.49	50.12±4.52	10.71±3.52
A. afra/L. schweifurthii	50:50	1	10.11±1.22	75.06±3.14	9.62±1.69
CQ/*L. schweifurthii*	40:60	1.9	4.97±1.00	80.33±6.51	16.84±2.71
CQ/L. schweinfurthii	90:10	1.2	6.43±4.05	88.34±7.35	18.72±2.66
negative control	-	-	40.10±3.80	0.00±00	6.94±1.27
positive control (CQ)	-	-	0.45±0.00	98.29±2.52	17.83±3.24

a – values not significantly different (p>0.05) to positive control

Combination of *S. birrea* and *C. robusta* exhibited the highest chemossupression (97.58%). The other combinations with high chemossupression involved CQ/*L. schweifurthii* (10:90 and 40:60), *A. afra/C. robusta* (60:40 and 10:90), and *A. afra/L. schweifurthii* were among the most active with of 88.34, 80.33, 83.79, 75.47 and 75.06% respectively. Next was the combination involving *S. birrea/L. schweifurthii* whose chemossupression was 50.12%. Combinations of *A. afra/C. anisata* and *A. afra/T.*

robusta were moderately active (43.89 and 37.66%, respectively). Low activity (28.04%) was exhibited by a combination involving *S. birrea/C. robusta.*

Drug combinations of aqueous extracts of medicinal plants giving the optimum anti-plasmodial activity (synergistic or additive effects) were selected from the *in vitro* interaction studies (Table 9-11) and subjected to further investigation for anti-malarial potential in mice. Results of the *in vivo* interaction of the combinations are summarized in table 13.

Table 13: *In vivo* anti-malarial assay of combinations of aqueous extracts of selected plants extracts

Combination	Ratio	SFIC	Parasite density	Chemo-suppression	Survival time (days)
T. robusta and *S. birrea*	40:60	0.78	3.52 ± 2.77^{b}	93.61 ± 3.74^{g}	16.43 ± 2.28^{l}
T. robusta and *B. salicifolia*	20:80	0.92	55.23 ± 6.12^{f}	0.00^{j}	8.33 ± 1.16^{n}
T. robusta and *L. schweinfurthii*	10:90	0.56	23.43 ± 3.81^{e}	57.47 ± 9.59^{i}	10.25 ± 1.71^{m}
R. natalensis and *L. schweinfurthii*	60:40	1.00	15.3 ± 4.21^{d}	71.87 ± 5.74^{h}	15.21±3.25l
R. natalensis and *S. birrea*	10:90	0.54	7.05 ± 3.46^{c}	87.21 ± 6.29^{h}	12.82 ± 1.25^{m}
R. natalensis and *T. robusta*	10:90	0.98	3.1 ± 1.78^{b}	94.38 ± 3.44^{g}	8.04 ± 1.53^{n}
R. natalensis and *B. salicifolia*	70:30	0.88	3.02 ± 0.99^{b}	94.51 ± 5.27^{g}	14.80 ± 2.74^{l}
L.schweinfurthii and *B. salicifolia*	70:30	0.92	5.17 ± 2.67^{c}	90.62 ± 8.48^{g}	18.96 ± 1.88^{k}
L. schweinfurthii and *S. birrea*	80:20	1.16	33.6 ± 3.58^{e}	39.02 ± 2.96^{i}	8.75 ± 1.52^{n}
B. salicifolia and *S. birrea*	60:40	1.08	2.29 ± 1.01^{b}	95.84 ± 1.99^{g}	18.45 ± 2.44^{k}
CQ diphosphate	-	-	0.20 ± 0.00^{a}	99.64 ± 0.48^{g}	19.43 ± 3.52^{k}
PBS	-	-	55.10 ± 5.54^{f}	0.00^{j}	8.11 ± 2.53^{n}

Values with same letters are not significantly different ($p>0.05$), values with different letters are significantly different ($p<0.05$)

No activity was exhibited by the combination of *T. robusta* and *B. salicifolia* (20:80). The following combinations: *B. salicifolia* and *S. birrea, T. robusta* and *S. birrea, R. natalensis* and *B. Salicifolia, R. natalensis* and *T. Robusta* and *L. schweinfurthii* and *B.*

salicifolia exhibited high chemo-supression (95.84, 93.61, 94.51, 94.38 and 90.62, respectively). Testing these combinations *in vivo* revealed enhancement of anti-malarial activity with some giving chemo-suppression close to that of CQ. The mean survival times of mice treated with blends of *L. schweinfurthii* and *B. salicifolia* as well as that of *B. salicifolia* and *S. birrea* were not significantly different ($p<0.05$) from the control group treated with chloroquine. The test samples were able to suppress parasite significantly thereby alleviating deaths associated with parasitaemia effects. It was however noted that despite high parasite suppression in mice treated with a combination of *R. natalensis* and *T. robusta,* the mean survival time was greatly reduced.

Daily changes of the parasite density in the test animals during the dosing period for a few samples are shown in Figure 8.

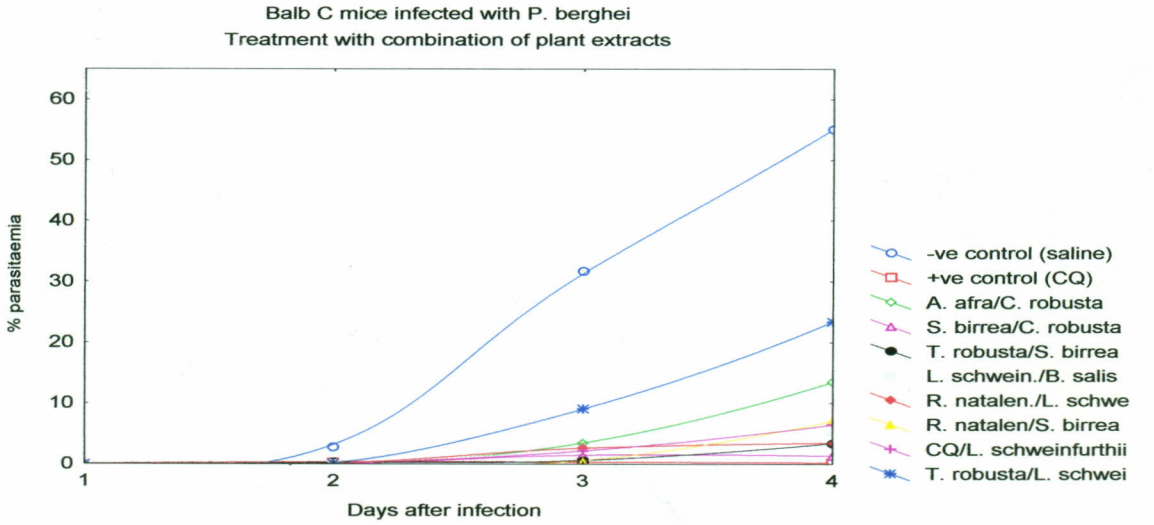

Figure 8: Effect of selected medicinal plants on *Plasmodium berghei* density in mice. Percentage parasitaemia in mice were plotted against days from day 1 to day 4. Parasitaemia for the negative control (PBS) increased steadily while that of the positive control (CQ) was along the base line.

Combination of *T. robusta/S. birrea, R. natalensis/L. schweinfurthii, L. schweinfurthii/B. salicifolia* and *R. natalensis/S. birrea* exhibited high chemossupression (93.83, 93.71, 90.62 and 87.21%, respectively). Combination of *T. robusta/L. schweinfurthii* exhibited moderate *in vivo* anti-malarial activity (57.47). No activity was exhibited by *T. robusta/B. salicifolia* combination. Considering that the two had exhibited moderate activity *in vivo* when tested singly, it can be assumed combination was antagonistic.

CHAPTER FOUR

DISCUSSION, CONCLUSIONS AND RECOMMENDATIONS

4.1 Discussion

Natural products isolated from plants used in traditional medicine, which have potent anti-plasmodial action *in vitro*, represents potential sources of new anti-malarial drugs (Gasquet *et al.*, 1993; Wright and Phillipson, 1990). It is estimated that 122 drugs from 94 plant species have been discovered through ethno-botanical leads (Fabricant and Farnsworth, 2001).

In this study 7 of the 15 plants used by traditional healers to treat malaria showed good anti-plasmodial properties with the IC_{50} values <10 µg/ml for both *P. falciparum* clones tested. Only two water extracts did not exhibit activity *in vitro*.

Methanol and water extracts had different activity profile with methanol being more active in a number of cases. A general trend seen in most of the tested samples indicated that the methanolic extracts are more active than the water ones. Screening with D6 revealed that the anti-plasmodial activity of *C. roseus, A. afra, R. natalensis, C. anisata, L. schweinfurthii, S. birrea, B. salicifolia,* and *T. grandifolia* water and methanol extracts were similar ($p > 0.05$). Active principles in these plants appear to have been extracted in methanol as well as water. While for the other plants, methanol extracts seems to have contained more of the bioactive principles.

A similar comparison against the resistant parasite strain (W2) indicated that activity of methanol and water extracts of *C. roseus, T. holstii, A. afra, R. natalensis* and *B. salicifolia* were similar, while for the other plants methanol extracts were more active. One may be tempted to conclude that the active principles in such plants are selectively extracted in methanol. However, in some cases activity for water and methanol was the same. Such include *B. salicifolia* and *A. afra* extracts. In addition, the two depicted the highest activity compared to all the other plant extracts tested.

Another notable observation is that the two parts of *A. afra* tested (leaves and stem) gave similar IC_{50} values. When tested against the W2 clone, IC_{50} values were 4.65±0.64 and 4.11±0.11 for leaves and stem bark aqueous extracts. This justifies the use of the whole plant by the Meru community, which claims to use plant shoot of the plant for management of malaria and its symptoms. Unlike in other instances where *in vitro* activity was lost *in vivo*, the high *in vitro* activity of *A. afra* was also reflected *in vivo*. This may suggest that the active molecules from this plant were not biotransformed in mice to inactive forms and are available. Gastrointestinal uptake, half-life in plasma among other factors seems to favor active forms of compounds.

Previously, aerial parts of *A. afra* were shown to have good *in vitro* activity against *P. falciparum* PoW (chloroquine-sensitive) and Dd2 (chloroquine-resistant) strains (Kraft *et al.*, 2003). Seven flavanoids have been isolated from this plant through bioassay-guided fractionation from which acacetin, genkwanin and 7-methoxyacacetin showed *in vitro*

anti-plasmodial activity (IC_{50} 4.3 - 12.6 µg/ml) (Kraft *et al.,* 2003). This study confirmed the high activity of *A. afra.*

In vitro anti-plasmodial activity of *B. salicifolia* ranged from IC_{50} 1.04-10.08 µg/ml against *P. falciparum* D6 and W2 clones. However, the activity was moderate *in vivo.* This is the first report of the anti-malarial activity of *B. salicifolia.* However, weak anti-bacterial activity has been demonstrated previously (Omer *et al.,* 1998). *Cantharanthus roseus* exhibited moderate *in vitro* anti-plasmodial activity. Studies on this plant have isolated alkaloids (Battersby and Gibson, 1971), terpenoids (Collu *et al.,* 2001), flavonoids (Vimala and Jain, 2001) and sesquiterpenes (Hirose and Ashihara, 1984).

Previous studies showed that methanol extract of dried bark of *X. americana* is mildly active against *P. falciparum* (IC_{50} 50-65 µg/ml) (Benoit *et al.,* 1996; Verotta *et al.,* 2001; Mainen *et al.,* 2004). Similarly, the stem bark extract evaluated in this study exhibited low anti-plasmodial activity both *in vitro* and *in vivo.* The low activity observed confirms the previous reports. Other researchers reported that the plant exhibits activity against other parasitic micro-organisms such as Gram-negative (*Escherichia coli* and *Pseudomonas aeruginosa*) and Gram-positive (*Staphylococcus aureus, Enterococcus faecalis, Streptococcus pyogenes* and *Bacillus subtilis*) bacteria and viruses (Asres *et al.,* 2001).

Potential of anti-malarial efficacy is evident from the *in vivo* results obtained. Extract of *Lannea schweinfurthii* exhibited intrinsic anti-malarial activity. The chemosuppression

was similar ($p<0.05$) to that of chloroquine. *Rhus natalensis* also had similar results. No previous reports on the activity of these plants have been reported, and the data presented here is thus novel and would be interesting to undertake further studies on these two plants.

Treatment with most of the samples prolonged the mean survival time of mice. Where survival time was not significantly different from the untreated control group, high parasitaemia or toxicological effects of the extract may have caused deaths. Parasite density for the treated group compared to the untreated indicated that treatment significantly ($p<0.05$) reduced parasite level in mice. Such included water and methanol extracts of *T. robusta, A. afra, R. natalensis, L. schweinfurthii, S. birrea* as well as the methanol extract of *B. salicifolia.*

It was noted that *in vitro* activity for a number of plants was lost *in vivo* while some plants whose *in vitro* activity was low demonstrated high *in vivo* activity. Previous studies have shown that owing to many factors, an extract lacking *in vitro* activity may still possess *in vivo* activity (Gessler *et al.,* 1995). This phenomenon of non-correlated findings has been described for other extracts or compounds such as cryptolepine from *Cryptolepis sanguinolenta,* berberine from *Enantia chlorantha* (Phillipson *et al.,* 1993), and nimbolide from *Azadiracta indica* (Rochanakij, 1985). The *in vitro* test reflects the intrinsic properties of the parasite and its response to the drug while the *in vivo* test is influenced by the immune system (Koella, 1993). For extracts not active *in vitro* yet displaying *in vivo* activity, the bioactive compounds may be in pro-drug forms that are

metabolized in the body to release bioactive molecules. The active principles from such plant extracts may also act by more than one mechanism, such as a direct effect on the immune system (biological response modifier) or other pathways that are not yet understood (Rasoanaivo *et al.,* 1992). Bio-transformation or poor bio-availability of plant compounds in rodents may result in loss of *in vivo* activity of an extract that had exhibited *in vitro* activity.

Several reasons could possibly be attributed to the extracts that showed no activity yet the traditional health practitioners had cited them as anti-malarial drugs. In traditional medicine practices, some of these plants are probably taken as relief of symptoms including fever, convulsions, and headache or may possibly have immunostimulatory effects (Rasoanaivo *et al.,* 1992). The plant extracts may not have been prepared using the exact methods of preparation used by the traditional health practitioners. The plants are in some instances used in combination with others where efficacy is observed as a result of synergy and additive interactions (Gessler *et al.,* 1994). In this study, drugs were administered via intraperitoneal injection an oral administration may give differing results as a results of gastro- intestinal modification.

This study showed that most of the *in vitro* effects of plant extracts on parasite development were clearly synergistic or additive when tested in combination. Some of the unpurified plant extracts investigated seemed to have an additive interaction with CQ. Whether the extracts increased the retention of CQ in the food vacuole (reduced CQ efflux) or increased CQ uptake due to better transport of the combination into the food

vesicles is an issue for further investigation. The mechanism of action of each extract need to be evaluated and then studied to establish exactly what happens when they are in combination. Additive effect could be as a result of the two separate entities binding on the same receptor in the parasite while in synergy different sites on the parasite may be the target points. Antagonism which was observed in some combinations could have been as a result of competitive inhibition.

Testing these combinations *in vivo* revealed an enhancement in activity in some cases. Combinations of *A. afra* and *C. robusta* methanolic extracts exhibited 83.79% chemossupression which was higher than either plant tested singly. A 60:40 combination ratio of these extracts tested *in vitro* had depicted a strong additive interaction ($\sum$fic=1) that seems to have been retained *in vivo*. Combinations involving aqueous extracts of *T. robusta* and *S. birrea* showed the highest parasite suppression (93.83%) closely followed by that of *R. natalensis* and *L. Schweinfurthii* (93.71%). The two together with combined methanol extracts of *S. birrea* and *C. robusta* (97.58%) were not significantly different (p>0.05) from CQ parasite suppression (99.64%). Aqueous extracts of *L. schweinfurthii/B. salicifolia* and *R. natalensis/S. birrea* also exhibited high chemossupression (90.62 and 87.21%, respectively). The antagonistic effect exhibited *in vitro* between the combination of *A. afra* and *T. robusta* (>2), was also observed *in vivo* where chemossupression (37.66%) was lower than that exhibited by the plants when tested singly (77.45 and 78.20% for *A. afra* and *T. robusta,* respectively). The exact mechanism by which synergism, addition or antagonism was realized *in vivo* is open to further studies.

It appears that these combinations might help in dealing with the chloroquine-resistance problem. These findings demonstrate that it is useful to combine drugs in a traditional preparation and justify their use in traditional medicine. These results encourage the testing of other plant combinations. Traditional healers seem to have already taken advantage of enhanced efficacy of combined decoctions. For example, in Mali, *Nauclea latifolia* was combined with *Mitragyna inermis* or *Guiera senegalensis* and *Feretia apodanthera* with *M. inermis* (Azas *et al.*, 2004).

Aqueous extracts of combined *T. robusta* and *B. salicifolia* was antagostic. A strong antagonism, by definition, refers to the loss of schizontocidal effect when the drugs are used in combination, requiring higher concentrations of the drugs to produce the same effect as the drugs alone (Berenbaum 1978). The single plants exhibited chemosupression of 63.81 and 43.75%, respectively while the combination did not exhibit parasite suppression (0% chemossupression). Mice in this cage died the same time as the negative control. If drugs in combination are antagonistic, the efficacies of such regimens might be compromised and the chances of resistance development and spread increased, as less effective drugs may be allowing weakly resistant clones to survive and be transmitted.

Combination involving aqueous extracts of *T. robusta* and *L. schweinfurthii* (10:90) also exhibited antagonistic interaction with chemossupression of the combination (57.47%) that is lower than that of the single plants (63.81 and 83.08%, respectively). The

highlighted cases raise a concern of resistance development to herbal preparations. However, since *T. robusta* extract was shown to be weakly cytotoxic, the explanation to loss of activity would be the potentiated toxicity resulting into immune-compromised mice thus exposing them to enhanced parasite attack. It was also noted that despite high parasite suppression in mice treated with a combination of *R. natalensis* and *T. robusta,* the mean survival time was greatly reduced. Combining the two plants may have resulted to enhanced toxicity.

Although, poisonous plants are ubiquitous (Kingsbury, 1964), herbal medicine is used by up to 80% of the population in the developing countries despite recent scientific findings showing many plants used as food or in traditional medicine to be potentially toxic, mutagenic and carcinogenic (Schimmer *et al.,* 1994). Only few scientific studies have been undertaken to ascertain the safety of most traditional remedies. For *in vivo* acute toxicity study, of most of the plant extracts administered in this study were safe at concentration >5000 mg/kg body weight. Only *X. americana* and *T. robusta* had LD_{50} values less <5000 mg/kg body weight with the later being more toxic. Most researchers consider a sample with LD_{50} > 5000 mg/kg body weight as safe (Abere and Agoreyo, 2006). Other symptoms of the acute toxicity including depression, shallow respiration and convulsion were also monitored. No obvious toxic symptoms were observed in most of the extracts by this classification and in particular those with LD_{50} > 5000 mg/ kg body weight. The other pointer to the test samples safety is that all the animal groups were alive for the entire period of the 4-day suppressive test. Normally if the test mice die

before day 5, the cause of death is usually attributed to the effect of the test drug rather than the parasites (Jutamaad *et al.,* 1998) suggesting that the therapeutic index is low.

The present investigation shows that extracts of *T. robusta*, and *X. americana* have toxicological manifestation both *in vivo* and *in vitro.* However, the plants showing indication of toxicity have been used in the Eastern Province of Kenya for treating malaria without serious toxic effects being reported. It may be assumed that since the treatments are often given as decoctions consisting of mixtures of medicinal plants, there is possibility that the other added plants mop up the toxic compounds.

4.2 Conclusions

- Anti-plasmodial and anti-malarial effects of the 15 selected medicinal plants commonly used by the *Ameru* for the treatment of malaria have been demonstrated.
- The large number of the samples that depicted high anti-plasmodial activity demonstrates that the ethno-pharmacological approach used in this study is useful in search for new anti-malarial drugs from natural sources.
- The aqueous extracts are generally less toxic than methanol ones and may explain the use of the former in traditional systems of health care delivery.
- Combination of medicinal plants as is the practice with THPs results in enhanced activity though the choice of combination 'partners' is vital as evidenced by some which were antagonistic in this study

- Cytotoxicity evaluation of a few of the selected plants revealed low selectivity indices an indication that they may contain toxic principles.
- Accept alternate hypothesis that plants used by the Meru community for malaria treatment have anti-malarial activity singly or in combination with others against multiple drug resistant malaria parasites.

4.3 Recommendations for future work

- Thorough toxicological study on these plants is recommended before they are prescribed for human use. Such studies should look at both acute and chronic toxicity by oral and intraperitonial drug administration and pathological examination.
- Bioassay-guided fractionation of the anti-plasmodial extracts is recommended in order to identify, isolate and characterize the active compounds that may be used as leads in anti-malarial drug development or as markers of quality in phytomedicines.
- Undertake combination studies of isolated plant compounds with each other or with standard anti-malarial drugs to investigate potentiation, additive or the antagonistic effects. This could find application in either delaying or reversal of chloroquine resistance.
- The apparent synergistic/additive anti-malarial combinations should be tested to evaluate whether they are accompanied by any apparent synergistic/additive toxicity. It is hoped that the curative mixtures chemical interactions may modulate the toxicity of possibly present toxic compounds.

- Further studies are needed to evaluate the mechanism of action of the plant extracts. This should also contribute to understanding how plant extracts express synergistic or additive effects when used in combination.
- Development of plant extracts or powders as capsules or tablets (phytomedicines) can be undertaken after evaluating dosage and toxicity requirements as is practised in S.E Asian countries to avail crude drugs that are cheaper and affordable to the local population.
- Bio-conservation of the active plants should be exercised by establishment of botanical gardens and through educating the local communities on the value of specific medicinal plants.

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APPENDIX I

Table 14: Example of counts per minute (CPM) obtained in a duplicate plate setting

Drug 15		Drug 16		Drug 18		Drug 19		Drug 20		Art	
140	183	398	378	482	424	128	182	188	262	235	346
120	170	1533	1684	28667	30802	147	130	235	219	250	261
144	133	11309	9099	30059	31413	117	158	220	381	493	513
120	367	23630	24772	31411	31552	140	136	308	387	21912	25252
22490	25977	24135	32584	34394	34686	770	772	1118	1120	32106	32248
30155	43201	27048	34139	37078	39352	4196	4288	9310	8122	32525	33140
47035	45654	35857	42739	38626	42903	23222	23623	36851	37500	42637	41846
43825	47726	37770	35779	40258	34236	47325	48749	251	198	265	185

IC_{50} calculation for artemisinin in the above table

Artemisinin concentration capable of inhibiting 50% of the *P. falciparum* (IC_{50}) was determined by computatation after logarithmic transformation of both concentration and cpm values using the formula;

$$IC_{50} = \text{Antilog}\,(\text{Log}X_1 + [(\text{Log}\,Y_{50} - \text{Log}\,Y_1)\,(\text{Log}X_2 - \text{Log}X_1)/\,(\text{Log}Y_2 - \text{Log}Y_1)],$$

Where: IC_{50} = inhibitory concentration 50, X_1 and X_2 = lower and higher concentrations respectively, Y_1 = CPM values which correspond with X_1, Y_2 = CPM values which correspond with X_2 (Sixsmith *et al.,* 1984).

Mid-point (Y_{50}) was calculated by the formula:

$$Y_{50} = \frac{\text{(PRBC CPM values} - \text{UPRBC CPM value)}}{2}$$

$$= \frac{(43825 + 47726 + 37770 + 35779 + 40258 + 34236 + 47325 + 48749) - (251 + 198 + 265 + 185)}{2}$$

$$= 20866.88$$

Table 15: Counts per minute for artemisinin from previous table

row	Drug conc. ng/ml	Art	
A	25.00	235	346
B	12.50	250	261
C	6.25	493	513
D	3.13	21912	25252
E	1.56	32106	32248
F	0.78	32525	33140
G	0.39	44637	45846
H	CONTROL		

Substituting values from table into the formula above;

$$IC_{50} = \text{Antilog}\left(\log 3.13 + \frac{(\log 20866.88 - \log 21912)(\log 6.25 - \log 1.56)}{(\log 493 - \log 21912)}\right)$$

$$= \text{Antilog}\left(0.49 + \frac{(4.32 - 4.34)(0.80 - 0.19)}{(2.69 - 4.34)}\right)$$

$$= \text{Antilog}\left(0.49 + \frac{(-0.02)(0.61)}{(-1.65)}\right)$$

= Antilog of 0.4974

= 3.143

APPENDIX II

Example of exl. data-sheet used in calculation of IC_{50} for duplicate plate setup

NOTE. X(SD), MIDPOINT, AND IC50 ARE CALCULATED AUTOMATICALLY SO NO ENTRY REQUIRED																
ONLY THE FIRST WELL DIL NEEDS TO BE ENTERED THE REST DILUTIONS ARE CALCULATED AUTOMATICALLY																
The drug units (ug/ml, ng/ml) need only to be entered on the table they will appear at the bottom automatically																
P.FALCIPRUM STRAIN		D6						GROWTH RATE		>3 FOLD		CARRIED OUT BY			JWG	
DRUGS TESTED			Plant extracts					DATE OF TEST		28/03/2006						
TEST CULTURE MEDIUM			physiological folate					FILE	3							
PRBC CONTROLS		28298	28001	26719	29020	30473	30800	31981	34606		X (SD)	29987.25				
URBC CONTROLS		72	116	56	100						X (SD)	86.00		MIDPOINT	15036.63	
DRUG	A	B	DRUG	C	D	DRUG	E	F	DRUG	G	H	DRUG	I	J	DRUG	K
MTX	ng/ml		MTX	ng/ml		MTX	ng/ml		MTX	ng/ml		MTX	ng/ml		DDS	ug/ml
58.89	384	444	88.75	256	306	55.470	298	336	33.82	412	408	22.18	488	494	1.67	9568
29.45	9712	14027	44.38	236	258	27.735	236	260	16.91	1201	1132	11.09	3128	2856	0.84	10407
14.72	28536	28113	22.19	410	520	13.868	2976	2934	8.46	4063	5138	5.55	5511	6034	0.42	11755
7.36	29112	29711	11.09	6344	6012	6.934	5509	5803	4.23	6014	6592	2.77	6737	6580	0.21	12773
3.68	29578	24594	5.55	9985	9658	3.467	6815	7203	2.11	7178	7969	1.39	8714	7436	0.10	13446
1.84	26017	24977	2.77	10589	12076	1.733	8855	8330	1.06	8207	7951	0.69	7656	6809	0.05	16805
0.92	26510	28185	1.39	11507	12429	0.867	9146	8486	0.53	7909	7785	0.35	7428	7384	0.03	17806
IC50	24.387	ng/ml	IC50	0.076	ng/ml	IC50	0.000	ng/ml	IC50	######	ng/ml	IC50	0.000	ng/ml	IC50	0.082