ANTIMALARIAL ACTIVITY OF SOME KENYAN PLANTS USED IN TRADITIONAL MEDICINE

BY

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DECLARATION:

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

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This work has been dedicated to my dear mum Teresiah, who sacrificed so much for me, and to my loving wife, Anne, who has always been a faithful friend.

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ABSTRACT

Plasmodium falciparum, the commonest etiological agent for human malaria is becoming increasingly resistant to standard antimalarial drugs in many malaria endemic areas. This necessitates a continuous effort to search for new and novel antimalarial drugs. Plants have always been a rich source for new drugs and many antimalarial drugs such as quinine and artemisinine were either obtained from plants, or developed using their chemical structures as templates.

One hundred and fifteen aqueous and organic extracts of 22 plant species from 15-taxonomical families from Kisii District were prepared and screened for their *in vitro* antimalarial activity against four laboratory-adapted *P. falciparum* isolates. In the preliminary screening, the extracts were screened against K39, a chloroquine (CQ) sensitive strain and the extracts which had high activity (IC_{50}<20 μg/ml) were subsequently screened against ENT 30, V1/S and NF 54. Data were expressed in terms of mean 50% inhibitory concentration. The effect of combining CQ with selected plant extracts against the multi-drug resistant V1/S was also investigated.

Out of the 22 plants screened against *P. falciparum* isolate K39, 15 plant species showed an activity of IC_{50}<100 μg/ml. Of these, 5 plant species were highly active with IC_{50} values ranging from 1.01-19.15 μg/ml against all strains of *P. falciparum* tested (K39, NF 54, ENT 30 and VI/S). The best activities were from extracts of *Vernonia lasiopus* whose IC_{50} values ranged from 1.01–4.13 μg/ml for both CQ-sensitive and CQ-resistant isolates. In addition, several

extracts in combination with CQ exhibited good synergistic effects against V1/S.

The lower IC_{50} values exhibited by many extracts against both chloroquine-sensitive and -resistant isolates suggested that there was no cross-resistance to chloroquine. The active principles could therefore be isolated and used to standardize a popular drug based on traditional use of these medicinal plants as antimalarials. The potentiation of CQ by some extracts suggested that the active principles in medicinal plants could be used in combination with standard antimalarials to enhance its activity. These results could account for the ethnopharmacological use of the plants investigated as antimalarials.

CHAPTER ONE

GENERAL INTRODUCTION AND LITERATURE REVIEW

1.1 General Introduction

A World Health Organization (WHO) study has shown that 80% of the world population relies solely on medicinal plants for their primary health care needs (Geoffrey, 1996). It is estimated that the numbers of medicinal plants in the world vary between 30,000 and 75,000 (Norman *et al.*, 1985).

There is a new trend in the world to turn back to natural substances due to various side effects associated with synthetic drugs (Huang *et al.,* 1992). Production of unrefined drugs for export is also becoming very common in some countries. In China and India, pharmaceutical companies are already marketing preparations of tablets and capsules made directly from appropriate plant extracts for treatment of specific diseases (Norman *et al.,* 1985). Examples include morphine from opium poppy (*Papaver sominferum*), one of the best-known painkillers. Coca leaves are also known to contain a strong stimulant (Cocaine) while some steroid products with medicinal value have been isolated from plants (Kee Chang Huang, 1993). For example gossypol from cotton seeds is used in China as a male contraceptive, diosgenin from *Dioscorea* species and sterols from the Soya beans are used as sex hormones (Kee Chang Huang, 1993). Plants also provide useful active compounds used for making insecticides, fungicides (pyrethrum plant) and industrial raw materials (WHO, 1987).

The majority of drugs active against infectious agents are in fact developed from natural products. However, plants remain grossly under-studied and under-used as a source of novel drugs, especially in the developed world (Norman *et al.*, 1985). This could be due to over-reliance with chemically synthesized drugs as well as lack of well-documented literature or catalogue of medicinal plants. The clinical utility of quinine and quinidine isolated from the *Cinchona* tree bark and the Chinese discovery of artemisinin from the herb *Artemisia annua* have stimulated much interest in plants as potential source of new anti-malarial drugs (Basco *et al.*, 1994). Furo-[2,3,β]-quinoline and acridone alkaloids have been isolated from plants belonging to the *Rutaceae* family (Basco *et al.*,1994). So far the furoquinoline alkaloids isolated from these plants have not been studied for their anti-malarial activities (Basco *et al.*, 1994). Except for antifolate antimalarial drugs, all the other commonly used antimalarial synthetic molecules are modelled upon plant-derived compounds (Geoffrey, 1996). Many of the synthetic antimalarial drugs in use at present were either obtained from quinine and artemisinin or have been modelled on the chemical structure of quinine or more recently the peroxide moiety of artemisinin as a template (Gessler *et al.*, 1994). With the emergence of chloroquine resistant *P.falciparum* malaria and reports of parasites resistant to alternative drugs such as the 4-aminoquinolines (e.g. chloroquine), antifolics and even amino alcohols, (quinine, mefloquine, halofantrin) there has been renewed interest in antimalarial activity of the acridine (Figgit *et al.*, 1992; Basco *et al.*, 1995). Also of great interest are dihydro-acridinediones and acridones (Berman *et al.*, 1994).

Malaria is one of the major parasitic diseases in many tropical and sub-tropical regions of the world and is characterized by chills, fever and profuse sweating. Human pathogenic plasmodia need to infect arthropod hosts to complete their life cycle. The *Anopheles* mosquitoes serve as the arthropod host (WHO, 1987). There are about 400 species of anopheline mosquitoes throughout the world, but only some sixty of these are important vectors of malaria under natural conditions (A.I.D., 1985; WHO, 1987). In Kenya, the vector species are members of the *Anopheles gambiae* complex and *A. funestus.* The important members of the *A. gambiae* complex are *A. arabiensis* and *A. gambiae sensu stricto* (s.s). Where as *A. gambiae* s.s. have been distinguished from *A. arabiensis,* the former appear to be the more efficient vector (WHO, 1987). Natural susceptibility or resistance (refractory) of *Anopheles* to infection is largely unexplained, although it is related to the metabolism of the mosquito. The refractory species have been shown to possess substances that are toxic to the parasite and prevent parasite development in these mosquito species (Dimopoulos *et al.*, 1999; Shahabuddin *et al.*, 1998). In addition resistant species have been shown to have trypsin-like proteinases in their midgut which might inhibit the development of the parasites (WHO, 1987). The environment also influences the development of the parasite in susceptible species and there is no single species of the infective *Anopheles* which is a universal vector (A.I.D., 1985).

Attempts to control the spread of malaria in sub-saharan Africa through insecticide application or synthetic anti-malarial drug administration have so

far failed (Gessler *et al.,* 1995). This failure is attributed to two major factors. First, there is a problem of resistance of malaria parasite to the current drugs and secondly the ethno-medical perceptions and practices relating to the illness 'malaria' are very often different from the biomedical ones on which malaria control measures are based (Basco *et al.,* 1994).

It is estimated that 40% of the people living in the tropics are exposed to the risk of being infected with malaria and about 10% of these people are actually infected with malarial parasites (Bryce *et al.,* 1994). Malaria inflicts between 300-500 million people each year causing between 1.5 to 2.7 million deaths of which more than 90% are children under 5 years of age in Africa (WHO, 2000). Malaria ranks third (2.3%) among major infectious disease threats in Africa after pneumococcal acute respiratory infection (3.5%) and tuberculosis (TB) (2.8%) (Nchinda, 1998). Malaria cases in Africa account for approximately 90% of all the cases in the world. The estimated annual direct and indirect costs of malaria were US$ 800 million in 1987 and were expected to exceed US$ 1.8billion by 1995 (Nchinda, 1998; Geoffrey, 1996; Radloff *et al.,* 1996). *Plasmodium falciparum* is the commonest malaria parasite which causes human malaria in Kenya and it accounts for 98% of the cases. It is associated with significant morbidity and mortality. Other species, which include *P. malariae, P. ovale*, form upto 2% of the cases and *P. vivax* is very rare clinically (MoH, 1992).

During the last 20 years there has been an emergence of strains of *P.falciparum* which are resistant to all the widely used antimalarials, especially chloroquine. Resistance to chloroquine by *P.falciparum* isolates was initially reported in East Africa in 1978. Resistance apparently spread via central African regions around 1983, to South West Africa by 1984 and had reached Cameroon in 1985. By 1987 several isolates resistant i*n vitro* to chloroquine and mefloquine in Nigeria were reported (Serpa *et al.,* 1988).

Scientific evaluation of medicinal plants used in the preparation of traditional medicine has in the past provided modern medicine with very effective drugs for the treatment of parasitic diseases (Iwu *et al.*, 1994). Many of the antimalarial drugs in use today were obtained from plants such as quinine from *Cinchona* bark and artemisinin from *Artemisia annua* (Bryce *et al.,* 1994). Some antimalarial drugs have been developed using quinine or more recently the peroxide moiety of artemisinin as template (Phillipson and Wright, 1991). Compounds like pyronaridine and mepacrine have shown high anti-malarial activity against *P. falciparum* (Elueze *et al.,* 1996). These compounds have been modelled using the chemical structure of quinine.

Many plants have already been screened for anti-malarial activity and have been shown to have activities against *P. faciparum* both i*n vitro* and i*n vivo.* Compounds such as furoquinolines and acridone alkaloids have been isolated from several plants such as *Geijera balansai, Sarcomelicope glauca,*

Sarcomelicope dogniensis and have shown some antimalarial activities both *in-vitro* and *in-vivo* (Basco *et al.,* 1994; Mitaku *et al.*, 1985; 1986; 1987).

Table 1 shows some of the plants from several taxonomical families whose extracts demonstrated an *in vitro* IC_{50} of less than 10 μg/ml against *P. falciprum* strains K1 and NF 54 in Africa by Gessler *et al.,* (1994).

Table 1: Tanzanian plants with *in vitro* antimalarial activity ($IC_{50} \leq 10$ μg/ml) against *P. falciparum* isolates K1 and NF 54 (Gessler *et al.,* 1994).

Plant Species		Plant Family
1	*Keetia zanzibarica*	Rubiaceae
2	*Zanthoxylum chalybeum*	Rutaceae
3	*Cissampelos mucronata*	Menispermaceae
4	*Harungana madagascariensis*	Guttiferae
5	*Achyranthes aspera*	Amaranthaceae
6	*Maytenus senegalensis*	Celastraceae
7	*Cussonia zimmermannii*	Araliaceae
8	*Erythrina sacleuxii*	Fabaceae

Although chloroquine has been the first line drug for the treatment of uncomplicated malaria, reports from clinicians and research scientists show that it is no longer effective in certain areas of Kenya due to the development of resitance. *P. falciparum* resistance was first reported in Kenya by a tourist in

1979 (Fogh *et al.,* 1979) and thereafter chloroquine sensitivity pattern studies in Kenya were initiated and an indigenous case of resistance to chloroquine reported in 1983 in Kisumu (Spencer, 1983). Other subsequent chloroquine sensitivity studies in various parts of the country have shown *P. falciparum* resistance levels of 0% in Turkana, 56% in W. Kenya and 72% in Coast (Watkins, 1996).

In the Kenyan highlands, which are situated at altitudes ranging from 1700 m to 2500 m above sea level, malaria epidemics have consistently been reported from 1988. High mortality was reported in 1926-1945 epidemics initially (Ministry of Health, 1992) and according to Ministry of Health (MoH), the out break in 1994 affected more than 12 districts of varying climatic conditions ranging from the highlands (Kisii, Nyamira, Kericho) to the semi-arid lands (Narok and Turkana).

Hospital records (1990-1997) of a Tea company Brooke Bond Central Hospital, Kericho district in Rift Valley showed malaria epidemic almost annually from May to July, with an annual attack rate of 50% and 32% of deaths in hospitalised patients (Malakooti, *et al.,* 1998). Between May and July 1999, the medical report from MoH showed that malaria epidemic in Kisii and Nyamira claimed more than 700 lives (only documented cases).

Claims regarding the use of indigenous plants in folk remedies for the treatment of malaria and control of mosquitoes have been made in China,

India, Africa, South East Asia and Latin America (Rosoanaivo *et al.*, 1992). These reports need to be investigated through scientific evaluation and validation with the aim of discovering new non-toxic and non-cross-resistant drugs. The research was therefore geared towards the evaluation and validation of some medicinal plants. The basis for the selection of the plants came from ethnomedical information on treatment and management of malaria as used by the herbal medicine practitioners and the local communities in the region studied.

1.2 Literature Review

1.2.1 Epidemiology of malaria in Africa

Malaria is one of the major health problems in sub-Saharan Africa, affecting 46 endemic countries. In these contries, 92% of the total population is exposed to malaria risk and malaria accounts for about 10% of of the total burden of disease in the continent (Teklehaimanot and Bosman, 1999). Malaria accounts for about one million deaths per year in children under 5 years in Africa and it is estimated that both direct and indirect global costs of malaria exceeded US$ 2 billion in 1997 (Teklehaimanot and Bosman, 1999).

Malaria in Africa accounts for more than 90% of malaria morbidity and mortality world-wide. The major contributory factor for this is the environment which is highly conducive to malaria transmission (Teklehaimanot and Bosman, 1999). The dominant parasite in Africa is *Plasmodium falciparum*, which is the most pathogenic of the four human plasmodia and responsible for

almost all malaria mortality (Teklehaimanot and Bosman, 1999). The optimal temperature, rainfall and humidity allow a perfect synchronisation between the vector`s (almost exclusively the complexes of *A. gambiae* s.l and *A.funestus* in Africa) multiplication and longevity and the parasite`s replication capacity in the vector and the man (Teklehaimanot and Bosman, 1999). As a result, *falciparum* malaria has an exceptionally high reproductive rate, which is unprecedented in other parts of the world.

In Kenya, malaria transmission patterns are influenced by several factors such as rainfall, relative humidity, vector species, intensity of biting and altitude. Patterns of endemicity are described in terms of stable, unstable, epidemic and malaria-free zones (MoH, 1992).

a). Stable malaria: This occurs in zones which have continuously high transmission rates throughout the year (perennial transmission). Areas affected include most parts of Coast, Nyanza and Western provinces. However, stable malaria areas do have seasonal fluctuations in morbidity and mortality patterns (MoH, 1992).

b). Unstable malaria: Occurs where there is seasonal endemicity, with one or two annual transmission peaks. The areas under this category include parts of Eastern (Machakos, Embu, Kitui) and Rift Valley provinces (Marigat). Highly unstable malaria is found in most of the arid and semi-arid zones and in Rift Valley province (MoH, 1992).

c). Epidemic malaria: Occurs in highland areas bordering endemic zones. Since 1988 after three decades of quiescence, there has been a series of highland epidemics. According to the Ministry of Health, considerable child mortality was reported during 1988, 1989, 1990, 1997, 1999 epidemics in Uasin Gishu, Nandi, Kericho, Kisii and Nyamira.

d). Malaria-free Zones: These include all land that lies at altitudes above 1600m above sea level, such as Nairobi, Mount Kenya and its surrounding area (MoH, 1992).

Changes in malaria endemicity and the appearance of epidemics in Kenya can be attributed to the development of drug resistance, and to many social-cultural and environmental factors. Examples include development projects, population trends, urbanization, political and economic pressures. Global warming is thought to be a contributing factor in the emergence of malaria at higher altitudes (MoH, 1992).

1.2.2 Human malaria parasites

Four species of Plasmodia cause malaria in human namely – *P. vivax*, *P. falciparum*, *P. malariae* and *P. ovale* (Nchinda, 1998). Paniker (1989) reported that malaria periodicity had been recognized from early times and the colloquial terms tertian, quartan and quotidian have been applied to the different types of malaria as follows:

P. vivax: This causes Benign tertian malaria (tertian because the fever recurs after intervals of 48 hours or every third day and Benign because it is relatively less pathogenic than falciparum malaria).

P. falciparum: Malignant tertian malaria because the cycles are often poorly synchronized and fever recurs at intervals of less than the expected 48 hours. It is also called *pernicious* malaria because of it is lethal nature.

P. malariae: Quartain malaria – occurring every fourth day, as it has a cycle of 72 hours.

P. ovale: Ovale tertian because of its tertian periodicity which means that the fever occurs at intervals of 48 hours.

1.2.3 The life cycle of malaria parasites

The malaria parasite life cycle comprises two phases – the *asexual phase* and *sexual phase.* The former occurs in human and the latter in the mosquito. There is therefore an alternation of generations in the life cycle of malaria parasites – asexual and sexual generations alternatively. There also occurs an *alternation of hosts*, as the asexual phase takes place in human followed by the sexual one in the mosquito. The sexual cycle occurs in the mosquito which is considered as the *definitive host* of malaria parasites while human is the *intermediate host.*

In the asexual phase, the parasite multiplies by division or splitting, a process designated *schizogony* (*schizo* – to split, *gene* – generation). Since the asexual cycle occurs in the vertebrate host, it is also called the *vertebrate, intrinsic* or endogenous phase. Schizogony occurs in two locations, in the red blood cells (*erythrocytic schizogony*) and in the liver cells (exoerythrocytic schizogony) or the tissue phase. Because the latter is an essential step before the parasites can invade erythrocytes, it is called *pre-erythrocytic* schizogony. The products of schizogony, whether erythrocytic or exoerythrocytic, are called *merozoites.*

The sexual phase takes place in the female *Anopheles* mosquito, even though the sexual forms of the parasite (gametocytes) originate in human red blood cells. Maturation and fertilization take place in the mosquito giving rise to a large number of sporozoites (*sporos-seed*). Hence this phase of sexual multiplication is called *sporogony.* It is also called the *extrinsic, exogenous* or invertebrate phase (Paniker, 1989; WHO, 1987).

1.2.3.1. The human phase

The human gets infected through the bite of the female *Anopheles* mosquito. The sporozoites, which are the infective forms of the parasite, are present in the salivary gland of the mosquito. They are transferred into the blood capillaries when the mosquito feeds on blood after piercing the skin. The sporozoites pass into the blood stream and some reach the liver and enter the parenchymal cells (hepatocytes).

a). Exo-erythrocytic (tissue) stage: Within an hour of being injected into the blood stream, the sporozoites reach the liver and enter the hepatocytes where they start the *pre-erythrocytic* schizogony. In this stage they undergo repeated nuclear division and each daughter nucleus is surrounded by cytoplasm. This stage of the parasite is called *pre-erythrocytic* or *primary exo-erythrocytic schizont*. In 6 to 16 days, the schizonts mature and burst, releasing thousands of merozoites (Paniker, 1989; WHO, 1987).

b). Erythrocytic stage: The merozoites, released by the *pre-erythrocytic* schizonts invade the red blood cells. They enter by endocytosis and the red cell membrane seals itself. The entry process takes about 30 seconds. In the red blood cells, these develop into forms having annular or 'signet ring' appearance and therefore are called *ring forms*. The parasite feed on the haemoglobin of the red blood cells. It does not metabolise haemoglobin completely and so leaves behind as residue a haematin-globin pigment (haemozoin), called the malaria pigment. This iron-containing pigment accumulates in the body of the parasite as dark granules, which become more prominent as the parasite grows. The ring form develops, enlarging in size and become irregular in shape and show amoeboid motility. This is called the *amoeboid form*. When the amoeboid form reaches a certain stage of development, its nucleus starts dividing. The parasite within the erythrocyte before its nucleus starts dividing is called the *trophozoite form* (*trophos* – growth). The ring form is called the *early trophozoite* and the amoeboid form the *late trophozoite*. From the time the latter's nucleus start dividing the

parasite within the erythrocyte is called the *schizont.* During the *early schizont* stage, only the nucleus divides but not the cytoplasm. This is followed by the *late schizont* stage where each daughter nucleus is surrounded by cytoplasm. The *mature schizont* is the fully-grown form, in which a number of small merozoites are seen, each having a nucleus with surrounding cytoplasm. The mature schizont bursts releasing the merozoites into the circulation. The merozoites invade fresh erythrocytes in which they undergo the same process of development. This process of erythrocytic schizogony is repeated sequentially, leading to progressive increase in intensity of parasitaemia.

c). Gametogony: After many cycles of erythrocytic schizogony, some merozoites that infect erythrocytes do not proceed to become schizonts but instead develop into sexually differentiated forms, the gametocytes. Gametocyte development generally takes place within the internal organs, such as the spleen and bone marrow and only the mature forms appear in peripheral circulation. The mature gametocytes are round in shape, except in *P. falciparum*, where they are crescent shaped. The *macrogametocyte* (female) and *microgametocyte* (male) do not cause any clinical illness in the host but are essential for transmission of the infection. They do not develop further in the vertebrate host and unless taken up by the vector mosquito, they die in a few days (Paniker, 1989; WHO, 1987).

1.2.3.2 The mosquito phase

The microgametocytes taken with a blood meal by an *Anopheles* mosquito divide into 5 to 8 nuclei in the gut. From each nucleus, there protrudes a long,

actively motile, whip-like filament. These filaments, which are the male gametes (microgametes) lash about for sometime and then break free. This process of formation of male gametes from the gametocyte is called *exflagellation* and can be demonstrated *in vitro*. The macrogametocyte does not divide, but matures, into a female gamete or *macrogamete*. It is fertilized by one of the microgametes to produce a *zygote*. Fertilization occurs in half to two hours after the blood meal. The zygote develops into an *Ookinete*, which in turn develops into an *Oocyst*. The later matures, increasing in size, with the nucleus undergoing multiple divisions.

This *sporogony* leads to the development within the oocyst of about 1000 sporozoites. The rupturing of a mature oocyst releases sporozoites into the haemocoel. The sporozoites reach the salivary glands situated in the thorax of the mosquito, penetrate the acinar cells and enter the salivary ducts. The mosquito is now infective and when it feeds on a susceptible human host, the sporozoites are injected into the skin capillaries to initiate human infection, and the cycle is repeated (Paniker, 1989; WHO, 1987).

1.2.4 The distribution and pathology of *Plasmodiun falciparum* malaria

Each year, there are 300 – 500 million cases of malaria and about 1.5 to 2.7 million deaths from *P. falciparum* infection (Radloff *et al.,* 1996; Nchinda, 1998). This is the commonest malaria parasite that is associated with significant mortality and morbidity in tropical and subtropical countries (WHO, 2000). In Kenya, *P. falciparum* inflicted malaria accounts for over 90% of all

the malaria cases (Republic of Kenya, 1992). The name falciparum comes from the characteristic sickle shape of the gametocytes of this species (from *falx,* meaning sickle). It is the most pathogenic of all the plasmodia, and is responsible for over 80% of malaria cases worldwide (Paniker, 1989).

P. falciparum malaria has several pathophysiological effects such as depression of the immune system. It has been suggested that immune depression caused by endemic malaria is responsible for the Burkitt lymphoma seen in African children (Paniker, 1989). Some of the most important complications of *P. falciparum* malaria include:

- Cerebral malaria which results from cerebral anoxaemia due to reduced blood flow rate, because of sequestation of mature parasite forms in the brain vasculature (WHO, 2000).

- Blackwater fever as a result of *P. falciparum* parasites causing red blood cell to become auto-antigenic and thus produce haemolysis through an antigen-antibody reaction (Charters, 1983).

- Anaemia due to loss of iron as the parasite breaks down erythrocyte haemoglobin and multi-organ dysfunction (e.g respiratory distress syndrome) (Nchinda, 1998).

1.2.5 Nucleic acid metabolism by plasmodium

The purines and pyrimidine necessary for the synthesis of parasite nucleic acids are derived from two distinct sources. Purines cannot be synthesized *de*

novo but are obtained preformed by the parasite. It appears that the parasite-preferred purine is hypoxanthine (WHO, 1987). During parasite metabolism, adenosine triphosphate (ATP) is converted to adenosine diphosphate (ADP) and adenosine monophosphate (AMP) (WHO, 1987). The later (AMP) is further catabolized, yielding hypoxanthine (6-hydroxypurine). The purine salvage pathways have been identified in only a few plasmodium species yet it is thought that they exist in all plasmodia. The parasitisised erythrocytes primarily use hypoxanthine for the synthesis of adenylates and guanylates (WHO, 1987). The purine salvage enzymes which have been isolated from *P. falciparum* include adenosine deaminase, purine-nucleoside phosphorylase, hypoxanthine phophoribosyl transferase and adenosine kinase (WHO, 1987).

Unlike purines, *P. falciparum* synthesizes pyrimidines *de novo* and this forms the basis of using exogenous radiolabelled hypoxanthine in drug sensitivity studies.

1.2.6 Antimalarial drugs and their mechanisms of action

WHO (1984) categorized the existing antimalarial drugs using two major criteria namely:-

- How they exert their action on target organisms (Biological activity)
- The chemical class to which they belong (Chemical stucture)

Over the past few years, scientific progress in malaria chemotherapy has concentrated on the improvements of already existing drugs than the development of new ones. Development of residual insecticides such as

dichlorodiphenyl trichloroethane (DDT), and malathion in the early 1960s somewhat overshandowed development of new drugs. However, the search for new antimalarial drugs has regained importance due to resurgence of malaria in many countries.

1.2.6.1 Classification based on where they exert their biological action

Conventional antimalarials can broadly be grouped into blood schizontocides or tissue schizontocides depending on how they exert their action on the target organisms.

1.2.6.1.1 Blood Schizontocides

These are drugs that exert their action primarily on the asexual intraerythrocytic stages of human plasmodia. Table 2 shows some of the drugs in this group.

Table 2: Classification of Blood Schizontocides (WHO, 1984)

Main site of Action	Compound	Chemical Class
Para- aminobenzoic acid (PABA) incorporation (PABA Blockers)	Dapsone	Sulfone
	Sulfadoxine	Sulfonamide
	Sulfalene	Sulfonamide
Folate metabolism (dihydrofolate reductase inhibitors)	Proguanil	Biguanide
	Pyrimethamine	Pyrimidine
Haemoglobin digestion products	Chloroquine	4-aminoquinoline
	Amodiaquine	4-aminoquinoline
	Quinine	Aminoalcohol
	Mefloquine	Aminoalcohol
	Halofantine	Aminoalcohol
Protein Metabolism	Artemisinin	Sesquiterpene lactone
	Artesunate	Sesquiterpene ester
	Tetracyclines	Napthacene derivative

1.2.6.1.2 Tissue Schizontocides

These are of two types namely **primary tissue schizontocide** (causal prophylactic drugs) that affect the pre-erythrocytic (PE) schizonts, and **hypnoitocides** that affect the latent forms, that is, hypnoizoites (dormant forms in the liver which cause relapses in *P. vivax* and also possibly in *P. ovale* infection) (WHO, 1984). Some of these are summarized on table 3.

Table 3: Classification of Tissue Schizontocides (WHO, 1984)

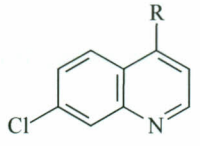
Stage affected	Site of Action	Compound	Chemical Class
PE Schizont	Mitochondria	Primaquine	8-aminoquinoline
PE Schizont	Folate metabolism	Proguanil	Biguanide
		Pyrimethamine	Pyrimidine
	PABA incorporation	Sulfadoxine	Sulfonamide
Hypnozoite	unknown	Primaquine	8-aminoquinoline

PE = Pre-erythrocytic

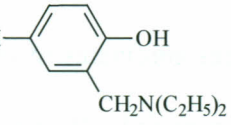
1.2.6.2 Chemical classification

1.2.6.2.1 4-Aminoquinolines

Examples are chloroquine (**1**) (7-chloro-4 [4`-diethyl-amino-1`- methylbutyl-amino]-quinoline and amodiaquine (**2**) (7-chloro-4-[3`-diethylamino-methyl-



1 R = $NHCH(CH_3)(CH_2)_3N(C_2H_5)_2$

2 R = 

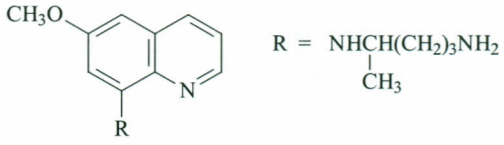
4`-hydroxyanilino]-quinoline).

Chloroquine is the most effective blood schizontocide against *P. vivax, P. ovale, P. malariae* and susceptible forms of *P. falciparum.* It also has the gametocidal activity against *P. vivax, P. ovale, P. malariae* and immature *P. falciparum.* Amodiaquine is a blood schizontocide and more active than chloroquine against resistant strains of *P. falciparum.* However, amodiaquine is not recommended for chemoprophylaxis because of agranulocytosis and toxic hepatitis reactions (Powells, 1989).

The therapeutic value of this group of drugs is seriously reduced in the *P. falciparum* malarious areas due to the development of resistance (Alder, 1992).

1.2.6.2.2 8-Aminoquinolines

The most commonly used is primaquine(**3**) (6-methoxy-8-[4`-amino-1`-



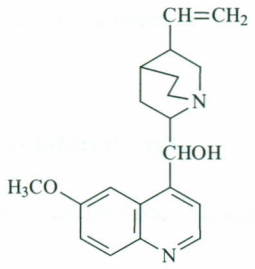
3

methylbutylamino] quinoline).

Tissue schizontocides such as primaquine are thought to act by inhibiting mitochondrial respiration in the parasite. Primaquine is used to treat liver stages of *P.vivax* and *P. ovale* infections (anti-relapse drug) and is also a gametocytocidal drug against *P. falciparum* especially in areas with chloroquine or multidrug-resistance (Wernsdorfer and Trigg, 1988).

1.2.6.2.3 Quinolinemethanols

These include quinine(**4**) (6-methoxy-α[5-vinyl-2-quinuclidinyl]-4-quinolinemethanol) and mefloquine(**5**) (α-[2-piperidyl)-2,8-bis[tri-fluoromethyl]-4-quinolinemethanol).



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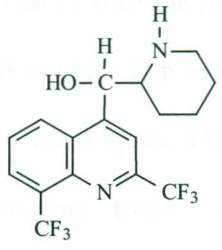
Quinine, isolated from cinchona tree is a blood schizontocide which was neglected after discovery of chloroquine and has regained use due to widespread resistance to chloroquine.

Structure activity studies show that OH group on the bridge between the two moieties (quinoline and quinuclidine) is essential for activity (Hofheinz and Merkli, 1984). It is effective against the asexual blood forms of all plasmodia. In addition, it has activity on sporozoites and primary exo-erythrocytic stages. Several synthetic quinolines including chloroquine, mefloquine and primaquine were developed using quinine as a template.

Mefloquine is a blood schizontocide. It has been used to treat *P. falciparum* malaria resistant to chloroquine and Fansidar®. Its combination with sulfadoxine and pyrimethamine (MSP) was used to treat malaria in Thailand



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but resistance to MSP has been reported (Nosten *et al.*, 1991).

Mode of action of quinolines and related drugs.

Chloroquine, Quinine and related quinoline drugs are effective blood schizontocides. These drugs accumulate in the intracellular acid vesicles because of their weak base properties (Schlesinger *et al.*, 1988; Warhust, 1986). The main function of these acid food vacuoles where these drugs accumulate in the parasite is proteolysis of ingested erythrocyte haemoglobin to provide the parasite with essential amino acids. Digestion of haemoglobin releases haematin (ferriprotoporphyrin 1X or FP 1X) within the parasite (WHO, 1984). This compound, which is soluble, disrupts plasmodial and host membranes as well as inhibits a variety of enzymes. It is suggested that the parasite synthesises a "segregating protein' which aggregates with haematin to form the insoluble crystalline compound, haemazoin, or malaria pigment. It is now generally accepted that chloroquine and related quinoline drugs exert their antimalarial activity by interfering with the detoxification of FP 1X (Foley and Tilley, 1998). They do so by complexing with FP 1X in the acid food vacuoles and the toxic FP 1X-drug complexes poison the food vacuoles, thus killing the parasite (Fitch *et al.*, 1982; Fitch , 1986; WHO. 1987; Warhust 1987; Blauer *et al.,* 1993). It has been suggested that the final toxic event mediated by the build up of haematin (FP 1X) is the peroxidation of the parasite lipids. Interestingly, some evidence have been provided to show that artemisinin exerts its activity by a mechanism similar to that of chloroquine (Macreadie *et al.*; 2000). In addition a haem polymerase enzyme from extracts of *P. falciparum*

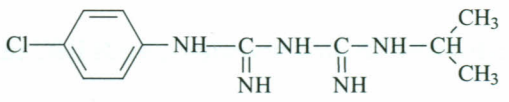
trophozoites has been identified and characterised and it has been suggested that quinoline-containing drugs inhibit this enzyme thereby disrupting the ordered conversion of haem into haemazoin (Wellens, 1992).

1.2.6.2.4 Antifolate drugs

Antifolate drugs are drugs that inhibit the synthesis of folic acid (Figure 1.2) (Jawetz, 1989). These include dihydrofolate reductase inhibitors and Para-aminobenzoic acid incorporation inhibitors (PABA blockers)

1.2.6.2.4.1 Dihydrofolate reductase inhibitors

Examples include proguanil(**6**) (N`[p-chlorophenyl]-$N^5$-isopropyldiguanide), chlorproguanil(**7**)(N`-[3,4-dichlorophenyl]-N^5-isopropyldiguanide), cycloguanil embonate, pyrimethamine(**8**) (2,4-diamino-5-p-chlorophenyl-6-ethylpyrimidine) and trimethoprim (2,4-diamino-5-[3`,4`,5`- trimethoxybenzyl] pyrimidine).



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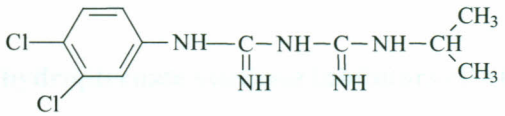

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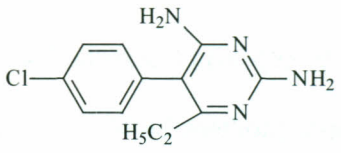

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The use of dihydrofolate reductase inhibitors as chemoprophylactic agents is now obsolete due to widespread resistance of *P. falciparum*.

Mode of action of dihydrofolate reductase inhibitors

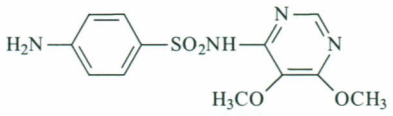
Although the selective toxicity of sulfonamides and sulfones depend on the possession by the malaria parasite of a metabolic pathway distinct from that of the host, both the parasite and the host convert dihydrofolate to tetrahydrofolate using dihydrofolate reductase (DHFR). The selective action of dihydrofolate reductase inhibitors such as pyrimethamine and cycloguanil resides in the greater affinity of the drug to the parasite enzyme than to the host enzyme. 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 synthesised. Dihydrofolate reductase inhibitors competitively inhibit DHFR by competing with dihydrofolic acid. Their affinity for plasmodial enzyme is 100-1000 times more than the host enzyme hence their selective toxicity (Wernsdorfer and Trigg, 1988).

1.2.6.2.4.2 Dihydropteroate synthase inhibitors (PABA Blockers)

These are drugs which compete with para-aminobenzoic acid (PABA) and include both sulfonamides and sulfones.

Some sulfonamides include sulfadoxine(**9**) (N`-[5,6-dimethoxy-4-pyrimidinyl]-sulfanilamide) and sulfalene(**10**) (N`-[3-methoxy-2-pyrazinyl]-sulfanilamide). The pyrimethamine and sulfadoxine are used in combination (Fansidar ^{R}) since they exhibit potentiating effect (synergism) (Alder, 1992).

The combination is effective against chloroquine resistant strains of *P. falciparum.*



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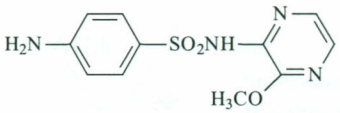

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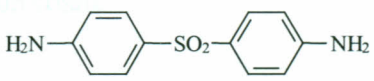
Sulfones include dapsone(**11**) (4,4`-diaminodiphenylsulfone), which is a blood schizontocide with no acivity against sporozoites or hypnozoites and works like sulfonamides (Peters and Richards, 1984).



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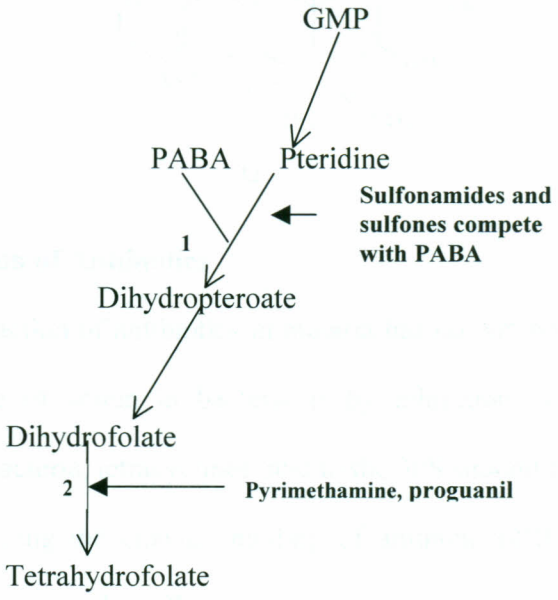
Mode of Action of Sulfonamides and Sulfones

Activity against human parasites by these PABA blockers appears to be restricted to an effect on the asexual blood cycle. No effect is observed on the

gametocyte stage of any species. The selective toxicity of the sulfonamides and sulfones resides in the fact that they compete with para-aminobenzoic acid (PABA) for the binding site of the enzyme dihydropteroate synthase 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 (Fig. 1.2). Mammalian cells can produce their dihydrofolate directly from dietary folic acid (Wernsdorfer and Trigg, 1988).



1- Dihydropteroate synthase

2- Dihydrofolate synthase

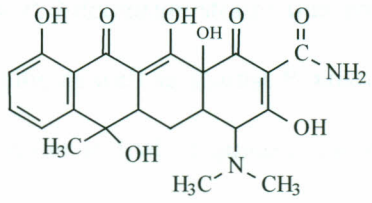
GMP = guanosine monophosphate

Figure 1.2 The sites of action of sulfonamides and sulfones

1.2.6.2.5 Antibiotics

These include tetracycline(**12**), doxycycline and minocycline.

Tetracycline has a potent but slow action against asexual erythrocytic stages of all plasmodia and is active against the primary intrahepatic stages of P. *falciparum*. Doxycycline and minocycline have similar activities.



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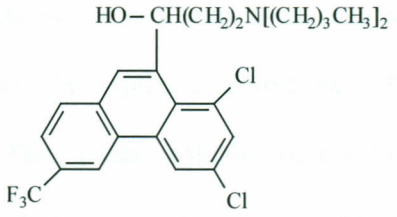
Mode of Action of Antibiotics

The mode of action of antibiotics in malaria has not yet been established. The primary mode of action in bacteria is by inhibition of ribosomal protein synthesis. In bacteria, tetracyclines bind to the 30S subunit of the 70S ribosome and so preventing the enzyme binding of aminoacyl-tRNA to the adjacent ribosomal acceptor site. The available evidence suggests that molecular mechanisms of plasmodial protein synthesis are typically eukaryotic. Therefore, it has been postulated that the antimalarial effects of antibiotics are as a result of inhibition of mitochondrial protein synthesis by a direct action in the mitochondrial ribosomes. Such hypothesis could explain the relatively slow clinical effects of antibiotics as antimalarials (Wernsdorfer and Trigg, 1988).

1.2.6.2.6 Phenanthrenemethanols

These include halofantrine(**13**) (1,3-dichloro-α[2- (dibutylamino)-ethyl]-6-(trifluoromethyl)-9-phenanthrene methanol.



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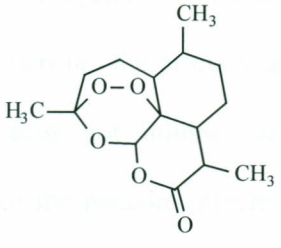
This is effective against *P. falciparum* strains that are resistant to chloroquine, pyrimethamine and quinine as well as against *P. vivax.*

Its mode of action is similar to that of quinine and chloroquine (Peters 1987, Blauer, 1988).

1.2.6.2.7 Sesquiterpene lactones

These include qinghaosu (artemisinin)(**14**), artemether(**15**) (methyl ether



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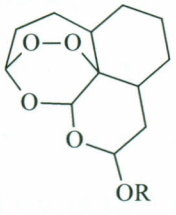


15 R = CH_3

16 R = CH_2CH_3

17 R = $CO(CH_2)_2CO_2Na$

derivative of artemisinin, artesunate(**17**) – sodium salt (sodium succinyl salt of artemisinin) and Arteether(**16**).

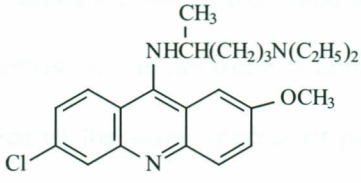
Artemisinin, the most recently developed antimalarial was originally isolated from the Chinese herb, *Artemisia annua* L., which has been used as a febrifuge for many centuries.The endoperoxide bridge present in one of the rings is thought to be the one which confers activity to this molecule. Artemisinin is effective against chloroquine-sensitive and chloroquine- resistant strains of *P.falciparum*. The water soluble derivative of artemisinin, artemether, arteether, and sodium artesunate are more effective antimalarial agents than the parent compound (Peters, 1987).

Mode of Action of Artemisinin and Related Compounds

It has been suggested that the mode of action of artemisinin and its derivatives may be on parasite protein synthesis (Gu *et al.,* 1983) since inhibition of incorporation of ^{3}H-isoleucine into proteins of *P. falciparum in vitro* was observed within one hour of drug administration. The relatively slow onset of inhibition of ^{3}H-hypoxanthine incorporation into parasite nucleic acids when compared with rapidity of action of these drugs *in vivo* suggests that nucleic acid synthesis is not the primary target of the drug (Li, *et al.*, 1983). Qinghaosu apparently does not inhibit carbohydrate metabolism. Gu *et al.,* (1984) indicated that the parasite infected cells concentrate drugs of this species in a similar but not identical manner to that observed with 4-aminoquinolines. However, artesunate does not inhibit the digestion of haemoglobin. More evidence has been provided to show that artemisinin exerts its antimalarial activity by a mechanism similar to that of chloroquine (Pandey, 1999).

1.2.6.2.8 Acridines and other miscellaneous compounds

Acridines include mepacrine(**18**), (2-methoxy-6-chloro-9-[4`-diethylamino-1`-methylbutylamino]-acridine) and miscellaneous compounds include dabequine(**19**) (WHO, 1984; A.I.D, 1985).



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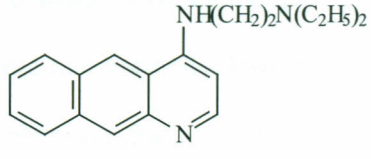

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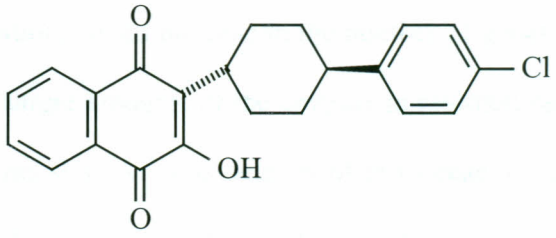
1.2.6.2.9 Newer antimalarial drug – Atovaquone

Atovaquone(20) (hydroxynapthoquinone) is a novel drug developed by Welcome Research Laboratories. It is a new antimalarial agent currently in advanced clinical trials stage (Canfield *et al.*, 1995). Its combination with proguanil had been shown to have a synergistic effect *in vitro* (Canfield *et al.*, 1995).



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1.2.7 Mechanisms of drug resistance

Drugs resistance has been defined as "the ability of a parasite strain to multiply or to survive in the presence of concentrations of a drug that normally destroys parasites of the same species or prevent their multiplication. Such resistance may be relative (yielding to increased doses of the drug tolerated by the host) or complete (withstanding maximum doses tolerated by the host)" (Wernsdorfer and Trigg, 1988)

Drug resistance in microorganisms may be due to a variety of mechanisms including non-genetical physiological adaptations of the organisms to the drug, genetical changes such as mutational events followed by drug selection, non-adaptive changes or a combination of all these mechanisms (Wernsdorfer and Trigg, 1988).

1.2.7.1 Resistance to dihydrofolate reductase inhibitors

Schimke (1980) has shown that resistance to dihydrofolate reductase inhibitors such as pyrimethamine can be attributable to mutants showing alterations in the enzyme, alterations in the transport of the drug across the cell membrane and

gene amplification, i.e, an increase in the number of genes which results in the production of larger amounts of the enzyme in resistant cells. Pyrimethamine-resistant mutants show various degrees of cross-reaction to sulfonamides and sulfones. Mechanisms through which parasites may survive the action of antifolates include use of alternative pathways, modifications of drug transport mechanisms, increase in drug-inactivating enzymes, production of mutant enzymes which have low drug affinity and gene amplification leading to increased synthesis of the blocked enzymes (WHO, 1984).

1.2.7.2 Resistance to sulfonamides and sulfones

The parasite may survive the action of these drugs by by-passing the step at which PABA is incorporated into dihydropteroate and it is possible that they may be able to achieve this by utilizing host cell folates (Wernsdorfer and Trigg, 1988). It has been shown that some *P. falciparum* lines can make efficient use of exogenous folates either as folic acid or folinic acid. This salvage of folates can bypass the blockage of the endogenous folate biosynthetic pathway by the sulfur-based drugs, such as sulfadoxine and dapsone. Analysis of the progeny of a genetic-cross revealed that the folate utilization allele was mapped to a region of chromosome 4 close to the *dhfr* gene but was distinguishable from the gene itself (Macreadie *et al.,* 2000).

Pyrimethamine/sulfadoxine combination such as Fansidar^{R} is increasingly being used as a firstline treatment for falciparum malaria in almost all regions where malaria is a problem. This has led to the selection of populations of *P.falciparum* resistant to this drug combination and the resistance phenotype is

strongly correlated with mutations in dihydrofolate reductase (DHFR) and dihydropteroate synthase (DHPS), the respective targets of these drugs (Macreadie *et al.*, 2000).

1.2.7.3 Resistance to 4-aminoquinolines

It has been suggested that chloroquine resistant parasites produce no haematin. This is thought to be a result of more efficient proteolytic activity of such parasites compared to the chloroquine-sensitive ones (Aissi *et al.,* 1983). Mahoney and Eaton (1981) reported that the protease activity in resistant parasites was 700-800 times than in sensitive ones. Red cells infected with chloroquine-resistant *P.falciparum* accumulate less chloroquine than those infected with comparable chloroquine-sensitive strains. Fitch *et al., (*1982) suggested that ferriprotoporphyrin 1X is more efficiently sequestered in resistant parasites so that it is not available for chloroquine binding.

Another hypothesis suggest that resistance to these class of compounds is a multigenic phenomenon which involves mutations of the genes which code for proteins involved in the uptake of these drugs (Macreadie *et al.*, 2000).

1.2.7.4 Resistance to compounds that inhibit protein synthesis

Resistance to compounds which act by inhibition of protein synthesis could be due to several factors such as changes in drug transport mechanism into the parasites, increase in drug-inactivating enzymes and increased production of

"segregating protein" thus reducing free haematin that would otherwise disrupt parasite and host membranes (WHO, 1984)

1.2.7.5 Resistance to Tissue Schizontocides

For the tissue schizontocides, resistance is carried through all stages of the life cycle. Blood schizontocidal action of primaquine studies have shown that the initial disruptive effect of the compound on parasite mitochondria appears to be rapidly compensated for by an increase in the synthesis of these organelles (WHO, 1984).

1.2.8 Drug resistance in *P. falciparum*

Resistance to 4-aminoquinolines: Chloroquine resistance of *P. falciparum* was first suspected in South America in the late 1950s and confirmed in 1959 in Thailand. More countries in Eastern Asia and South America became affected in the following years. In 1978, East Africa became affected, beginning in Kenya and Tanzania (WHO, 1984).

Resistance to sulfadoxine/pyrimethamine: The combination of sulfadoxine and pyrimethamine is being extensively used against *P. falciparum* malaria. In Kenya, the MoH has declared a ban on the use of chloroquine as the first line drug against malaria, replacing it with sulfadoxine/pyrimethamine drugs (e.g. Fansidar®). Fansidar^{R} is also widely used for prophylaxis (suppression) and this might have accelerated the occurrence of resistance. Resistance has been reported in Thailand, Vietnam, Burma and Malaysia. Failures of prophylaxis

and treatment with sulfadoxine/pyrimethamine have also occurred in persons who had contracted *P. falciparum* malaria in E. Africa, especially in Kenya and Tanzania (Markwalder and Meyer, 1982). Resistance to other drugs such as mefloquine has also been reported and cross-resistance of *P. falciparum* to drugs is not a rare occurrence (Wernsdorfer and Trigg, 1988).

1.2.9 Combinations of Drugs

The use of drugs in combination where scientifically justified, provides a means of reducing the doses of individual drugs and also a possible way of circumventing or delaying the induction of drug resistance (Peters, 1987). Malarial chemotherapy and prophylaxis is now targeting combinations of drugs due to increased resistance to individual conventional antimalarials such as chloroquine. The antimalarial activity of the combined drugs may either be potentiated (synergism), additive or antagonistic. Development of resistance to the compounds may be delayed, unchanged or enhanced and toxicity may also be reduced, additive or potentiated (WHO, 1984). Thus an ideal combination is one that is potentiating and well matched (Pharmacokinetic considerations – absorption, distribution, metabolism, excretion), has reduced toxicity and delays the emergence of resistance to the individual components. Such a combination has been found for sulfadoxine/pyrimethamine (e.g. Fansidar^{R}) that has replaced chloroquine as the first line drug in Kenya for the treatment of uncomplicated *P. falciparum* malaria. Oduola *et al.*, (1998) found that chloroquine-promethazine combination reversed chloroquine resistance in standard *P. falciparum* clones and patient parasite isolates in Nigeria. The

combination reduced the inhibitory concentration fifties (IC_{50S}) for chloroquine against the resistant parasite by between 32-92 %.

Another combination atovaquone (250mg)/proguanil hydrochloride (100mg) (Malarone^{R}) (Kremsner *et al.,* 1999) is undergoing clinical trials in seven countries of Africa including Kenya. This has been found to be well tolerated and to have about 98.5% cure rate and over 98% effective in preventing malaria. Canfield *et al.*, (1995) had shown that atovaquone/proguanil combination have the best synergy *in vitro*. Atovaquone/tetracycline, combination also showed good synergy (Canfield *et al.*, 1995). However, combinations such as sulfadoxine/pyrimethamine do not provide a 100% cure rate. Resistance of the parasite to sulfadoxine/pyrimethamine has been reported in Asia, Malaysia, some parts of E. Africa especially Kenya (Markwalder *et al.,* 1982) and Tanzania (Stahel, 1982).

Several researchers are turning their interest to increasing the lifespan of antimalarial drugs by using herbal preparations and/or their derivatives. In S.E. Asia, use of existing first line drugs with artemisinin derivative (such as artesunate) have been shown to increase efficacy, protect drugs against resistance, reduce transmission of malaria and increase the lifespan of antimalarial compounds (TDR news, 2000). Addition of artesunate to standard pyrimethamine/sulfadoxine treatment for malaria showed increased cure rate and parasite clearance (TDR news, 2000). Studies of artemisinin derivative, artesunate have shown that its combination with mefloquine gives synergy *in*

vitro (Fivelman *et al.*, 1999) and *in vivo* (Chawira *et al.*, 1987). Report from Madagascar has shown that bisbenzyl-isoquinoline (BBIQ) frangchinoline and its enantiomer limacine isolated from *Strychnopsis thouarsii* Baillon and *Spirospermum penduliflorum* Thouars, reversed *in vitro* chloroquine resistance in malaria.

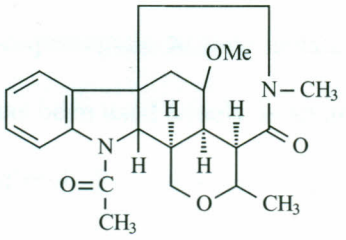
Until recently, chloroquine has been the most effective and widely used drug in malaria therapy because of its onset of action, good tolerability and low cost. The sulfadoxine–pyrimethamine drugs that have replaced chloroquine as the first line drug in Kenya are very expensive (about 10 times more expensive than chloroquine for a full dose). Very few antimalarial drugs are in development today and the whole process is time-consuming and costly. One reasonable approach of solving these problems is potentiation of chloroquine action against resistant parasites using herbal preparations.

1.3 Plants as sources of antimalarial Drugs

Natural products have always been used as a source of medicinal chemicals. Quinine is the best known antimalarial isolated from *Cinchona* tree. *Qinghaosu*, the active component of the Chinese medicinal plant *Artemisia annua* L. (Qinghao), is also an antimalarial compound of novel structure and mode of action and is active against strains of *P.falciparum* known to be multi-drug resistant (WHO, 1984).

The water extracts of *Dichroa febrifuga* Lour. are quite effective against *P. falciparum* malaria and the plant`s total alkaloids are 26 to 50 times more potent than quinine in antimalarial activity (Kee Chang Huang, 1993). Among them, γ-dichroine is the most effective, and is about 100 times more potent than quinine. However, it is 150 times more toxic than quinine (Kee Chang Huang, 1993). Extracts from plants of several families including Amaryllidaceae, Saxifragaceae and Simorubaceae also exhibit antimalarial activity but the active principles are in such low concentrations that the crude extracts may not always show activity in drug screens (WHO, 1984). Certain quassinoids from Simarubaceae have shown good antimalarial activity *in vitro* (Trager and Polonsky, 1981). Modifications of natural products can give derivatives which are more potent than the 'mother' compound. Artesunate, a water-soluble succinyl formulation is a modification of artemisinin which seem to be a promising drug for the treatment of cerebral and other complicated forms of falciparum malaria (WHO, 2000).

Crude alkaloids from *Strychnos myrtoides* Gilg and Buss (Loganiaceae) significantly enhanced *in vitro* and *in vivo* chloroquine action (Rosoanaivo *et al.*, 1998). Malagashanine(**21**) and myrtoidine(**22**), compounds isolated from *Strychnos myrtoides* significantly reversed chloroquine resistance (Rosoanaivo *et al.*,1998).



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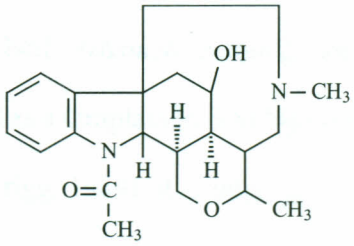

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1.4 Plants of this study

Out of the 22 plant species screened, several plant species that include *Vernonia lasiopus, Ekebergia capensis*, *Rhamnus prinoides*, *Ficus sur* and *Clerondedrum myricoides* gave very positive results (chapter 3) and are discussed below.

1.4.1 The Genus *Vernonia*

The genus *Vernonia* Schreb is classified under the family compositae and more that 1000 members in the new and old worlds have been described. In Kenya, over 50 different species distributed all over the country have been described and the genus consists of diverse plant types ranging from herbs through shrubs to trees (Beenjte, 1994).

Many *Vernonia* species have been investigated chemically and found to contain several metabolites including triterpenes and oxygenated sesquiterpenes, flavones and vernolic acid (Oketch-Rabah, 1996). The most frequently occurring secondary metabolites of the genus *Vernonia* are oxygenated sesquiterpene lactones that have been shown to possess biological activity (Oketch-Rabah, 1996). Since artemisinin is an oxygenated

sesquiterpene lactone isolated from the Chinese herb *Artemisia annua* L. and has been used widely as an antimalarial agent and as a template in development of multidrug resistant strains of *P. falciparum* (Trigg, 1989), it is important to investigate *Vernonia* species further.

Among the oxygenated sesquiterpene lactones isolated from *Vernonia* species include vernolepin isolated from *Vernonia hymenolepsis* A. Rich that was found to have antitumor properties (Oketch-Rabah, 1996). Several steroid glycosides such as vernonioside A1 from *V. amygdalina* that have anti-parasitic activity have been reported. *V.brasiliana* extracts had 40-50% inhibition in rodent malaria *in vivo* against *P. berghei* in mice and *in vitro* activity against *P. falciparum* of IC_{50} value of 40 μg/ml (Oketch-Rabah, 1996)

Vernonia auricurifera Hiern is a woody herb or shrub 1.8-7.5m, well distributed across many regions of Kenya and grows at altitude ranging from 1600-2650m. It is known as musabakwa (Kisii), muchatha or muthakwa (Kikuyu), olusia (Luo), ol-masakwa (Maasai), or tunguet (Tugen) (Beentjee, 1994). *Vernonia lasiopus* is also a woody herb or shrub which grows between 0.9-3m. It is known as muchatha (Kikuyu), muvatha (Kamba), kwamtebenguet (Kipsigis), olusia (Luo), ol-euguru (Maasai). It is used against malaria by Kikuyu, against scabies by Kamba, against venereal diseases by Luo and pounded leaves are applied to sores by Maasai. *V.lasiopus* is well distributed across the country more than *V. auricurifera* and grows in regions of altitude between 1050-2550m (Beentjee, 1994).

1.4.2 *Ekebergia* Species

Ekebergia is a small genus of trees belonging to the tribe Trichiliae of the Meliaceae family (Mulholland *et al*; 1998). In Kenya, 2 species are known namely *Ekebergia capensis* Sparrm or *E. rueppeliana* (Frescen) A. Rich, and *E. benguelensis* C.DC (Beentjee, 1994).

E. capensis is a tree which grows upto 30m and is widely distributed into many regions of Kenya. It is common in dry forests. It is known as mununga (Kikuyu), araruet or aradwi (Kipsigs), omonyamari (Kisii), tido (Luo), ol-subukiai (Maasai), Kerbut (Marakwet), muchogomo (Meru), teldet (Nandi), eng`amwa (Turkana), temwana arariet or kondilwa (Tugen). It grows in altitudes between 1-50m (Coast) and 1300-2600m(Beentjee, 1994). *E. capensis* bark is used as an emetic for heart burn and for respiratory complaints, to treat abscesses and boils and in hot water infusions for pimples in South Africa. It is also used to treat heart ailments and infertility (Muholland *et al.*, 1998). The hexane extracts of *E. capensis* bark and wood yielded atraric acid, β-sitosterol, palmitate and oleate esters of β-sitosterol, lupeol and oleanonic acid (Muholland *et al.*, 1998). The chloroform extracts yielded lupeol and the methanol extracts yielded 3-epioleoleanolic acid. Oleanonic acid has been proposed to have anti-arthritic and anti-inflammatory activity and lupeol has been found to be anti-arthritic agent (Muholland *et al.*, 1998).

E. benguelensis grows to 10 m; it is a rare tree which is used in Tanzania to treat heart and mental illnesses (Nkunya and Jonker, 1998). Several compounds

which include oxysterols and 2 polysqualenes that showed no antimalarial activity were isolated from the crude root bark extracts and the 2 polyoxysqualenes were recently isolated from Kenyan *E. capensis* (Nkunya and Jonker, 1998).

1.4.3 *Rhamnus prinoides*

Rhamnus prinoides L` Herit is a shrub or a tree that grows to 1.2-9 (12) m and has a grey bark. Leaves are shiny above, ovate or elliptic with rounded or cuneate base. The flowers are yellowish (green) and fruits are usually red, turning (purple-) black. It is well distributed in the Rift Valley and the western regions of Kenya and grows in altitudes of between 1500-3150m above sea level. It is found in forest (edges) and less often in secondary bushland or bamboo/heath zone (Beenjte, 1994).

This plant is known as kosisityet (Kipsigis), ol-kokola or ol-konyel (Maasai), zambizi (Kamba), mukarakinga (Kikuyu), omungura (Kisii), kosisit (Marakwet) and mugorona (Meru). The Meru use the root decoction against indigestion and gonorrhea, the Maasai use it against rheumatism and the Kipsigis use it against malaria (Beenjte, 1994; Kokwaro, 1976)

1.4.4 Clerondedrum myricoides

Clerondedrum myricoides (Hochst) Vatke is a shrub that grows 1-3.5 m. The leaves are opposite or in threes/fours and flowers are blue or purple, or occasionally greenish with one lobe blue, or blue with two lobes white. The

fruits are black (Beenjte, 1994). It is found in dry or semi-evergreen bushland, bushed grassland, or wooden grassland and often on rocky sites. It grows in altitudes between 150-2400m and is found in all regions of Kenya but more commonly distributed in the central, Rift Valley and western regions of Kenya (Beenjte, 1994).

The plant is known as chemogong` (Kipsigis), munguya or muvweia (Kamba), munjugu (Kikuyu), shikuma (Luo) and ol-magotogot (Maasai) (Kokwaro, 1976; Beenjte, 1994). The roots are cooked in soup or some broth and drunk for treatment of chest pains. The root extract is drunk for the treatment of colds and for the stoppage of gum-bleeding. In cattle, the root extract is used for the treatment of East Coast fever. In humans, the root extract is drunk to relieve indigestion for the treatment of sore throat, tonsilitis, malaria and rheumatism. A decoction of the roots is also taken as a purgative, as an emetic and for treating gonorrhea (Kokwaro, 1976; Beenjte, 1994).

1.4.5 *Ficus sur*

Ficus sur Forssk. is also known as *Ficus capensis* Thunb. and is a tree that grows to 4.5-25m (Beenjte, 1994). The bark is grey or whitish and figs grow on leafless branches that are orange or red. It is well distributed in the western, central and Rift Valley regions of Kenya but sparsely distributed in the coast (Beenjte, 1994). It is found in the riverine forests and bush, ground water forests, and occasionally in forests away from the water. It grows in altitudes between 1-2000m (Beenjte, 1994).

It is known as odaa (Boran), mukuyu (Kikuyu, Digo), mogoyuet (Kipsigis), omoraa (Kisii), musingu (Luhya), ng`owo matundo (Luo), ol-oboni or ol-ngaboli (Maasai) and mukuu (Meru) (Kokwaro, 1976; Beenjte, 1994). The fruits are edible. Digo use root decoction as cough remedy and Maasai use a bark infusion against stomach-ache and babies diarrhoea. The milky sap that exudes from a cut shoot is put on an aching tooth to relieve pain (Kokwaro, 1976; Beenjte, 1994).

1.5 Justification for this study

Malaria is one of the major parasitic diseases in many tropical and sub-tropical regions (WHO, 2000). *Plasmodium falciparum* is becoming increasingly resistant to 4-aminoquinolines, antifolics and even aminoalcohols, but only a few drugs are currently under development necessitating urgent efforts to identify novel classes of anti-malaria drugs (Basco *et al.*, 1994). It has also been reported that modern synthetic medicines often produce considerable side effects in comparison to those attributed to natural product based drugs (Bjorkman and Phillips-Horward, 1990). Furthermore, the benefits of this investigation are quite evident, as earlier work along the same line had enabled the discovery of useful potent drugs from plants such as quinine and artemisinin (Norman *et al.;* 1985).

1.6 Objectives of this study

1.6.1 General Objective

The study was designed to examine potential antimalarial activity of extracts from different plants used for the treatment of malaria in Kisii district, a malaria epidemic region of Kenyan Highland.

1.6.2 Specific Objectives

1. To catalogue medicinal plants from Kisii District used by traditional healers to treat malaria.
2. To prepare organic and aqueous extracts of selected medicinal plants.
3. To determine the *in vitro* antimalarial activity of the plant extracts against chloroquine-sensitive and -resistant strains of *P. falciparum.*
4. To investigate the effects of combining chloroquine with selected plant extracts on chloroquine-resistant *Plasmodium falciparum* isolate *in vitro.*

CHAPTER TWO

MATERIALS AND METHODS

2.1 Plant Samples

Twenty-two medicinal plants species used in Kisii by traditional medical practitioners to treat malaria were collected based on ethno-medical data. The plant samples were collected either as whole plants, stems, root barks or even leaves depending on which part of the plant had been reported by herbal medical practitioners to be of medicinal value. Taxonomist Simon Mathenge from University of Nairobi, Botany Department, identified these plants and Voucher samples were deposited in the University of Nairobi herbarium.

2.2 Extraction

The plant samples were air-dried and shred using laboratory Warring Blender (for leaves) and electrical mill (for roots and barks). Each sample (25g) was weighed and put in 250ml conical flasks. Cold sequential extraction was done with distilled organic solvents of increasing polarity, which included n-hexane ($[CH_3 (CH_2)_4 CH_3]$, b.p. 69°C), chloroform ($[CHCl_3]$, b.p. 61°C), ethylacetate ($[CH_3COOC_2H_5]$, b.p 77°C), and methanol ($[CH_3OH]$, b.p 64.7°C). n-Hexane (150 ml)was added and flasks placed on a shaker and soaked for 48 hours. The sample was then filtered using a Buchner funnel under vacuum until the sample was dry. The plant sample was further soaked with 150ml of n-hexane for 24 hours until the filtrate remained clear. The filtrate was concentrated under vacuum by rotary evaporation (Buchi model, Switzerland) at 30 - 35°C (Harborne, 1994). The concentrate was then transferred to a sample bottle and

dried under vacuum and the weight of the dry extract taken. The extracts were stored at -20°C. The whole procedure was repeated sequentially for chloroform, ethylacetate and methanol for the same plant sample.

For aqueous extraction, 25g of dried plant sample in 150ml of water was boiled for 5 minutes, loaded in a shaker and soaked for 12 hours, filtered and the process repeated until the sample was clear. The filtrate was then freeze dried and the dry sample weighed and stored at -20°C until used for biossay.

2.3. Cultures of *P. falciparum* and bioassays

2.3.1 Preparation of medium (RPMI 1640/HEPES)

The methods used were adopted from Trager & Jensen (1976). The medium contained 10.4g of Rosewell Park Memorial Institute (RPMI) 1640 with L-glutamine, without PABA and lactic acid (GIBCO^R, U.S.A), 25mM N-[2-Hydroxyethyl] piperazine-N`-[2-ethane-sulfonic acid] ($C_8H_{18}N_2O_4S$)] HEPES (5.94g) (Sigma^R, U.S.A) in 960ml of distilled autoclaved water. It was filter sterilised using 0.22μm filter units (CORNING®, U.S.A), stored at 4^OC and used within 2 weeks.

2.3.2 Preparation of 5% (w/v) Sodium bicarbonate ($NaHCO_3$)

A solution of 5% (w/v) of $NaHCO_3$ (Analar, England) was prepared by dissolving in distilled autoclaved water and filter sterilised using syringe-adaptable 0.2μm filters, stored at 4^0C and used within 2 weeks.

2.3.3 Collection of serum

Human serum was prepared by draining blood into blood bags without anticoagulant from ABO blood system donors. The blood was allowed to clot for 90 minutes at room temperature and overnight storage at 4°C. This was followed by centrifuging at 3600 r.p.m (960g) for 10 minutes at 4°C (Hitachi®, Japan - refrigerated centrifuge). The obtained serum was heat inactivated at 56°C in a water bath for 40 minutes, stored at -20°C overnight and then stored at -70°C.

2.3.4 Preparation of complete culture medium with 10% serum (CMS)

The CMS was prepared by thoroughly mixing 86.22 % (v/v) of RPMI 1640/HEPES, 3.78% (v/v) of 5% $NaHCO_3$ and 10% (v/v) human serum until a uniform colour was attained. This was then flushed with 3% CO_2, 5% O_2, 92% N_2 gas mixture (BOC gases, Kenya), stored at 4°C and used within 1 week of preparation.

2.3.5 Collection of fresh erythrocytes

Blood was obtained from 0^+ donors, who were HIV and Hepatitis-B Virus negative, had not contracted malaria nor visited a malaria endemic region in the past two months and had not taken any antimalarial drugs or antibiotics within the same period. The blood was drawn into 15% (v/v) acid-citrate-dextrose (ACD). Before their use in culture, the cells were washed free of plasma and white cells by centrifuging at 3600 r.p.m (960g) for 10 minutes at 4°C. The red cell pellets were washed twice with 2 volumes of wash medium

which consisted of 95.8% (v/v) RPMI 1640/HEPES and 4.2% of 5% (w/v) $NaHCO_3$. The cells were flushed with 3% CO_2, 5% O_2 and 92% N_2 gas mixture (BOC gases, Kenya), stored at 4°C and used within 2 weeks of preparation.

2.3.6 Thawing of cryopreserved parasites

The method of Rowe *et al.*, (1968) was adapted for this work. Both chloroquine susceptible and resistant laboratory adapted *P. falciparum* strains were used for the tests. Local isolate, K39 originally isolated from a patient in Kisumu District, which was chloroquine-sensitive and non-local isolate, NF 54, (an Amsterdam airport isolate) were used for chloroquine-sensitive isolates studies. Local isolate, ENT 30 from Entosopia, Narok District, Kenya and non-local isolate V1/S, from Vietnam which are chloroquine resistant were also used for this study.

Warm water at 37°C was added into a basin for thawing the frozen parasites. The specimens of laboratory adapted K39, NF 54, ENT 30 and V1/S were then identified from the logbook. The vials were removed, thawed in water bath and sterilely transferred to 15ml-centrifuge tube, in laminar flow hood. They were then centrifuged at 1500 r.p.m (400g) for 5 minutes at 20°C. The supernatant was aspirated (using a sterile unplugged pasteur pipette) and 0.3 ml of 3.5% NaCl (w/v) added, mixed well and centrifuged (1500 r.p.m, 5 min and 20°C). The supernatant was aspirated, 1ml CMS added, mixed and centrifuged as above. The supernatant was aspirated, and 50% RBC and CMS added as required (see Appendix 11).

2.3.7 Maintaining revived cultures

The method described by Trager and Jensen (1976) was used in this study. Cultures were maintained in volumes of 5ml, 6% haematocrit and incubated at 37°C with daily change of the medium and gassing (5% O_2, 3% CO_2 and 92% N_2).

Slides were usually made after 48 hours to assess the status of the culture by applying a small drop of the cell suspension after aspirating the medium onto the glass slide. Thin smears were fixed with absolute methanol, allowed to dry, and stained for 10 minutes with 10% Giemsa stain. The slides were gently washed with water, dried and examined under microscope (100x oil immersion).

Dilution or sub-culturing refers to preparing new cultures from old cultures. This was usually done while the percentage parasitaemia is high, and no contamination found on examining the slide under the microscope. The necessary volumes of culture 50% fresh erythrocytes and medium needed for 5ml, 6% hematocrit culture were calculated from the formulae:

Culture volume (CV) =5/D

50% erythrocytes (RV) = 6/(50-CV)

Medium Volume = 5 – (CV+RV)

D is the reciprocal of the desired dilution factor (e.g. D=10 for 1:10 dilution).

The appropriate volume of 50% RBC and medium were mixed together in new $25cm^2$ (50 ml) flasks using sterile technique, gassed (3% CO_2, 5% O_2 and 92% N_2) and incubated (37°C) for 20 minutes. The desired volume of old culture was then added, gassed and incubated.

2.3.8 Freezing of parasites (Cryopreservation)

The method of Rowe *et al.*, (1968) was adapted for cryopreservation of parasites to ensure enough supply of laboratory-adapted isolates as well as having manageable culture flasks. Thick smear was usually made to ascertain the cultures to be frozen are not contaminated. The culture to be cryopreserved was transferred into 15ml centrifuge tube and centrifuged at 1500 r.p.m (400g) for 5 minute at 20°C. After aspirating the supernatant, packed cell volume (PCV) was estimated and one PCV of Rowe's cryosolution added. Aliquots of 0.25ml were then put into 2ml cryovials (NUNC®, U.S.A) placed in aluminum canes, which were placed into liquid nitrogen freezer.

2.4 *In vitro* drug sensitivity test

The semi-automated microdilution technique of Desjardins *et al.*, (1979) for assessing *in vitro* antimalarial activity and later modified by Le Bras and Deloron (1983) was adapted in the drug sensitivity studies for chloroquine, plant extracts and chloroquine/extracts combination against *P.falciparum* isolates. In brief, the 96 flat-bottomed well plates (a matrix of 8 rows and 12 columns) are set such that all wells except for control wells contained 25µl of

doubling concentrations of drug solutions. Parasitisized red blood cells (200µl) were added so that the total volume per well is 225µl and set up as described in sections 2.4.1 through to 2.4.5.

2.4.1 Preparation of plant extracts and chloroquine for *in vitro* bioassays:

The dry plant extract samples were retrieved from 4°C and dissolved in distilled water so that the final highest concentration in the microtitre plates was 250 µg/ml. For these experiments 0.045g of the plant sample was dissolved to a final volume of 20ml (stock solution of 2,250 µg/ml)

Since the final volume in each well was 225µl this stock solution was meant to give the first row concentration of 250 µg/ml using the formula,

$$C_1V_1 = C_2 V_2$$

Where C_1 = Initial concentration

V_1 = Initial Volume

C_2 = Final concentration

Final Volume = V_2

Taking into account that the volume of each drug in each well was 25µl the highest concentration (250 µg/ml) was calculated so that:

2,250 μg/ml x V_1 = 250 μg/ml x 225 μl

V_1 = 250 x 225/2250 = 25 μl

This meant that 25 μl of stock solution (2250 μg/ml) was used in the first row.

Each of the drugs was filter sterilized with syringe adaptable 0.22 μm filters into sterile bijoux bottles and stored at -20°C until used.

Similarly chloroquine diphosphate (MW 515.9 g) was prepared so that the plate's first row concentration was 1μg/ml (9μg/ml stock solution, C_1), sterilised and stored at -20°C.

9μg/ml x V_1 = 1 μg/ml x 225 μl

V_1 = 1 μg/ml x 225 μl / 9 μg/ml

= 25 μl

C_1 = 25 μl in the first row of the microlitre plate.

The drugs were sterilised using 0.22 μm syringe-adaptable filters in the laminar flow hood (Bellco Glasses Inc., U.S.A). For drugs that were not readily soluble in water, especially non-polar extracts of hexane and chloroform, they were dissolved in 50 μl of dimethyl sulfoxide (DMSO) (solvent concentration in tests did not exceed 0.02%), and the volume adjusted to 20ml with distilled water (Elueze *et al.,* 1996).

2.4.2 Preparation of microtitre plates

The 96 well flat-bottomed micro-titre plates (NUNC^{R}, U.S.A) with covers were used for drug sensitivity tests. Under sterile conditions in the laminar flow hood (Bellco Glas Inc., U.S.A), the plates were laid along the columns (1-12). Sterile deionized water (25 µl) was added with a multi channel pipetter from row B to H, exempting row A wells. The drugs (50 µl) were added in duplicate into wells of row A (This meant that each drug held two columns and one plate therefore accommodated 6 drugs in duplicate). Two-fold dilutions were done by transferring 25 µl of the drug with a multi-channel micropipette from row A down to row G (last 25µl from G wells discarded). Row H wells were exempted since they served as controls (wells without drugs). Thus, A wells had a concentration of 250 µg/ml, B wells 125 µg/ml as concentrations halved down to G, which had the lowest concentration. The final volume per well was 25µl. The plates were covered and kept at 4°C.

2.4.3 Addition of parasites to the pre-dosed plates.

The test culture which was at ring stage, had percentage parasitaemia (%P) of 4% or more and growth rate (G.R) of 3% or more was used for sensitivity tests. (See appendix 11 for calculation of % P and G.R). After examining the parasites under the microscope the % P of the test culture to be added to the wells of predosed plates was adjusted to 0.4% and haematocrit (hct) adjusted to 1.5% with 50% RBC. This mixture (200 µl) was then added into each well

except for H_7 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 [Recall that the cultures are maintained at 5ml and 6% haematocrit]

$C_i V_i = C_f V_f$

Where C_i & 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: (96 wells can be approximated to 100 wells).

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

= 20ml

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

$C_i V_i = C_f V_f$

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

$V_i = 0.4\% \times 20ml/4\% = 2mls$

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

2ml culture has 0.12ml (100% RBC)

To adjust haematocrit to 1.5% of V_f

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

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

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

CMS needed =20ml – (2ml + 0.36ml)

= 20ml – 2.36ml = 17.64ml

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

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

Using sterile technique in 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. Meanwhile the predosed plates were warmed at 37°C for about 20 minutes. The plates were then retrieved and placed in the laminar flow hood and the test culture put into sterile tissue culture dishes (Lux®, U.S.A). Using 1-200 µl tips (Fisherbrand®, U.S.A) and a multichannel pipette, aliquots of 200 µl were dispensed into the wells except for H_7 to H_{12} (6 wells). To these, unparasitisized red blood cells (UPRBC) were added (negative control) so that $H_1 - H_6$ served as parasitisized red blood cells (PRBC) control (positive control) since they had no drug and the former served as UPRBC control. For 1 plate (6 wells);

Volume = 6 well x 200µl = 1200µl

= 1.2ml

1.5% hct = 1.5 /100 x1.2 = 0.018ml (100% RBC)

= 0.036 (50% RBC)

CMS =1.2 –0.036 = 1.164ml

0.36ml of 50% RBC were mixed with 1.164 ml CMS and 200µl were aliquoted into wells H_7 to H_{12} using a multichannel pipette. The same procedure and calculations were done for **n** number of plates (**n** = 2,3… etc).

2.4.4 Incubation of the plates

After replacing the lids of microtitre plates and agitating the plates gently, they were placed into the 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 3% CO_2, 5% O_2 and 92% N_2 (BOC^R, Kenya) and incubated at 37°C. After 48 hours, [G-^{3}H] hypoxanthine (1 μCi/well) was pulsed in aliquots of 25μl into each well and the plates incubated for further 18 hours.

2.4.5 Harvesting of cells and scintillation counting

The cells were harvested using a multiple semi-automatic cell harvester ($Skatron^R$, Norway) onto glass fibre filters ($Skatron^R$, Norway) for each row, from A to H. The filters were then dried at 37°C overnight, then the discs were introduced into scintillation vials, and 1ml of scintillation fluid (ecolume) added and the vials loaded into a liquid scintillation β-counter (1211 MINIBETA, England). Disintegrations per minute were calculated for each sample. The count per minute (CPM) for each sample represented the incorporation of [G-^{3}H] hypoxanthine into the parasite nucleic acids.

2.4.6 Calculations of inhibitory concentration 50 (IC_{50s}).

The method of Sixsmith *et al.*, (1984) was adapted. In brief, the 50% inhibitory concentration (IC_{50}) refers to the drug concentration inhibiting 50% of the parasite incorporation of [$G^{-3}H$] hypoxanthine found in the drug-free PRBC wells.

The UPRBC CPM values were taken as the background count and corrected CPM values of each well were obtained by subtracting UPRBC CPM values from each well`s CPM values.

To calculate IC_{50}, the mid-point (Y_{50}) was calculated by the formula:

Y_{50} = (PRBC CPM values-UPRBC CPM values)/2 and IC_{50} from the formula:

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

(See Appendix 11 for an example).

2.5 Drug interaction experiments

Availability of an effective drug combination for chemotherapeutic use against malaria can not only serve to reverse resistance but also attempt to delay it (White, 1998; Fivelman *et al.,* 1999).

2.5.1 Methodology

A technique adapted from Canfield *et al.,* (1995) using fixed ratios of predetermined concentrations needed to inhibit parasite growth by 50% (IC_{50s}) was used to determine the interaction of 2 drugs. The 8 plant extracts which exhibited good activity against the chloroquine-resistant isolate V1/S (Chloroquine IC_{50} = 0.073 μg/ml) were combined with chloroquine phosphate (m.w = 515.9g). Drugs were prepared by dissolving in distilled water so that the highest initial concentrations of each drug in the first wells ranged between 20–50 times the predetermined IC_{50s}. Plants extracts that were not soluble in water were initially dissolved in 50 μl of DMSO before dissolving in water such that the final concentration of DMSO in the solution did not exceed 0.02% (Elueze *et al.*, 1996). When studying drug combinations, solutions of these initial concentrations were combined in ratios of 90:10, 80:20, 70:30, 60:40, 50:50, 40:60, 30:70, 20:80 and 10:90 of chloroquine and the second drug solution, in this case the plant extract.

Portions (25 μl) of single and combination drug solutions were introduced into 96 flat-bottomed well micro-titration plates to give duplicate rows of

chloroquine alone, the test drug (extract) and the 9 combinations. Two-fold serial dilutions of the drugs with millipore (filter-sterilised) water were made to fill the plate using a 12-channel pipetter, leaving the wells of row H for both positive and negative controls. Parasitisized erythrocytes (PRBCs) (200 µl) were then added into all wells except wells H_7 to H_{12}, to which unparasitisized erythrocytes (UPRBC) were added. Incubations were carried out for 48 hours at 37^0 C, after which radiolabelled hypoxanthine was pulsed, and further incubation carried out for 18 hours at 37°C. The cells were harvested onto glass fibre mats, dried and scintillation counting done. The corresponding IC_{50S} were determined for each drug alone and in combination using the method of Sixsmiths *et al.*, 1984 (See section 2.4.6). Results were expressed as the sum of the fractional inhibitory concentration (sum FIC) as described by Berenbaum (1978).

Sum FIC

= (IC_{50} of A in Mixture/ IC_{50} of A Alone) + (IC_{50} of B in Mixture/ IC_{50} of B Alone).

Sum FIC value < 1 indicate synergism, those > 1 indicate antagonism and those equal to 1 indicate addition.

In this study, drug A was taken as chloroquine and drug B as plant extracts. (See Appendix 11 for an example on calculation of sum FIC).

CHAPTER THREE

RESULTS

3.1 A catalogue of plant species screened and yields of plant samples

Table 4 shows a catalogue of the 22 plant species from 15 taxonomical families that were screened for antimalarial activities. These plant samples were collected between March and September 1999.

Table 5 shows the yield in grams of each plant sample per 25g of plant material.

3.2 Screening plant extracts against K39 *in vitro*

Preliminary screening of the 115 plant extracts was done against a Kenyan laboratory-adapted chloroquine-sensitive *P. falciparum* isolate K39. Chloroquine diphosphate (m.w 515.9g) was used as the control drug. Table 6 show the 50% inhibitory concentrations (IC_{50}s) of the extracts against K39. The extracts are grouped according to their taxonomical families. The values are the mean IC_{50} (x) ± standard deviations (S.D) for 2 independent experiments done in duplicate (n=2).

Table 4: Families and plant parts collected between March and September 1999 based on ethnomedical data:

Botanical name	Family	Vernacular (Gusii)	Part collected
Leucas calostachys	Labiatae	Ekemwa	Leaves
Ajuga remota	Labiatae	Omonyantira	Leaves
Leonotis mollisina	Labiatae	Risibi	Leaves
Senna didymobotria	Caesalpinaceae	Omobeno	Leaves
Clerodendrum myricodes	Verbanaceae	Omonyasese	Leaves/Root bark
Utrica masaica	Utricaceae	Rise	Aeria/parts
Euphorbia inaequilatera	Euphorbiaceace	Ogota Kwembeba	Whole plants
Cucumis figarei	Cucurbitacease	Egwagwa	Whole plants
Rhamnus staddo	Rhamnaceae	Omontontono	Root bark
Rhamnus prinoides	Rhamnaceae	Omonguroro	Root bark
Ficus sur	Moraceae	Omoraa*	Stem bark
Acacia hoockii	Leguminosae	Omokonge	Root and Root bark
Spermacoce princeae	Rubiaceae	Omonhabiebo or Omontakiebo	Whole plant
Stephania abyssinica	Menispermaceae	Omotabararia	Roots
Vernonia lasiopus	Compositae	Omosoricho	Leaves
Vernonia auriculifera	Compositae	Omosabakwa	Leaves
Microglossa pyrifolia	Compositae	Mote O' Kebaki	Leaves
Aspilia pluriseta	Compositae	Rirangera	Leaves
Justicia betonica	Acanthaceae	Mokera	Aerial parts
Melia azadarach	Meliaceae	Omwarubaine	Leaves
Ekebergia capensis	Meliaceae	Omonyamari	Stem bark
Parinari curaterifolia	Rosaceae	Omoraa*	Aerial parts

* The Abagusii use the same name for the two plants species

Table 5: Yield in grams for each plant extract per 25 g of plant material

Plant	Hexane	Chloroform	Ethylacetate	Methanol	Water
S. didymobotria	1.6798	1.4922	1.9023	1.8659	3.4714
C. myricodes					
Leaves	0.6005	0.4295	0.2024	1.8772	2.9421
Root bark	0.3112	0.2751	0.1482	0.8972	1.7894
Leonotis mollisina	1.2945	1.4641	1.2070	1.5217	2.1142
Ajuga remota	1.7025	1.2533	1.9028	1.1280	2.6819
Leucas calostachys	1.4543	1.2774	1.1242	1.6181	3.7871
Utrica masaica	1.2145	1.3678	1.1879	1.4321	2.6743
Cucumis figarei	1.2851	1.3142	1.3214	1.5191	1.7432
Rhamnus staddo	1.1578	1.2143	1.2890	1.3101	1.0121
Rhamnus prinoides	1.1981	1.1141	1.2819	1.2701	0.9781
Ficus sur	1.1214	1.2409	1.3114	1.1361	1.1472
Acacia hoockii	1.1321	1.1467	1.1328	1.1409	2.1439
Melia azadarach	1.6563	1.5663	1.5793	1.7508	3.9461
Ekebergia capensis	1.2132	1.1767	1.1614	1.1327	2.6434
S. princeae	1.3891	1.4612	1.3289	1.3116	3.4341
S. abyssinca	1.0121	1.1432	1.1328	1.0579	1.4101
M. pyrifolia	1.3201	1.3987	1.3810	1.4162	1.3427
P. curaterifolia	1.3981	1.4307	1.3781	1.2714	2.5473
Vernonia lasiopus	1.3814	1.3964	1.3610	1.2989	4.0021
V. auriculifera	1.3957	1.3614	1.3732	1.3202	2.9407
Justicia betonica	1.4111	1.4201	1.3941	1.3521	2.4334
Aspilia pluriseta	1.3881	1.4014	1.3981	1.2412	3.8961
E. inaequilatera	1.4108	1.3949	1.3994	1.2794	2.6972

Table 6: The mean IC_{50} values (x ± S.D) for plants screened against K39

Plant species	The IC_{50}± S.D (μg/ml)				
	Hexane	Chloroform	Ethylacetate	Methanol	Water
Melia azadarach	>100	>100	>100	>100	>100
Ekerbegia capensis	>100	3.92±0.11	4.66±0.14	4.61±0.28	3.87±0.07
Justicia betonica	>100	>100	>100	69.55±1.89	>100
Stephania abyssinca	>100	67.07±2.94	>100	63.87±3.44	22.90±1.03
Rhamnus staddo	>100	>100	35.15±3.67	25.64±2.41	>100
Rhamnus prinoides	>100	96.80±1.04	50.74±2.97	15.05±2.26	82.30±2.44
Ficus sur	19.15±1.31	8.99±3.24	>100	>100	>100
Microglossa pyrifolia	72.54±2.46	58.35±1.93	79.52±2.71	68.35±3.48	>100
Aspilia pluriseta	62.70±8.91	>100	>100	40.81±5.67	>100
Vernonia lasiopus	>100	1.21±0.13	1.01±0.07	3.15±0.28	>100
V. auriculifera	>100	37.73±1.29	40.30±1.98	55.17±2.31	>100
E. inaequilatera	>100	>100	>100	>100	>100
P. curatelifolia	>100	>100	>100	>100	>100
S.princeae	>100	>100	>100	>100	>100
Acacia hookii	>100	>100	>100	>100	91.48±4.78
Ajuga remota	>100	>100	>100	21.60±1.36	>100
Leonotis mollisina	50.77±5.66	35.66±2.34	>100	80.74±4.07	>100
Leucas calostachys	66.47±2.10	36.22±3.98	>100	87.69±1.77	>100
Cucumis figarei	>100	>100	>100	>100	>100
C. myricoides					
Leaves	>100	>100	48.63±1.43	16.78±2.65	>100
Rootbark	>100	>100	>100	8.55±3.51	>100
Senna didymobotria	>100	>100	>100	>100	>100
Utrica masaica	>100	>100	>100	>100	>100

Chloroquine IC_{50} value = 0.021 μg/ml. It was included as positive control.

Table 6 shows that for *E.capensis* the hexane extract was not active. However, the chloroform, ethyl acetate, methanol, and water extracts gave very low IC_{50} values (<5μg/ml) suggesting that the plant extracts have a high antiplasmodial activity. *Melia azadarach* showed no activity for all the extracts (IC_{50}>100μg/ml). However, lack of *in vitro* antiplasmodial activity does not rule out *in vivo* activity and therefore this plant warrants further investigation. It is interesting to note that although the 2 plants belong to the same family (Meliaceae), one shows activity (*E. capensis*) but the other does not (*Melia azadarach*).

The two plants from Rhamnaceae family, namely *R. prinoides* and *R. staddo* showed mild activity that is comparable with each other. The active compounds seem to be in the polar portions, especially in the methanol extracts whose values are 15.05 μg/ml and 25.64 μg/ml for *R. prinoides* and *R. staddo* respectively. The fact that four of the five extracts of *R. prinoides* were active (IC_{50}<100μg/ml) suggests that the plant could be investigated further.

For all the 22 plant species investigated the compositae family exhibited the best antimalarial activity with *Vernonia lasiopus* giving the lowest IC_{50} values. Both *V. auricurifera* and *Microglossa pyrifolia* had their extracts within the mild activity range (20-100 μg/ml) whereas the hexane and methanol extracts of *A.pluriseta* showed mild activity of IC_{50} values of 62.70 μg/ml and 40.81 μg/ml respectively.

The *in vitro* activities of the Labiatae plant species *L. mollisina* and *L. calostachys* were within the mild activity range whereas *Ajuga remota* had only its methanol extracts with good activity (IC_{50}=21.60 μg/ml). Of all the five extracts of *J. betonica,* only the methanol extract had mild activity (IC_{50}=69.55 μg/ml). It seems that the most active compounds are polar in nature.

Apart from the hexane and ethylacetate extracts of *S. abyssinca* that had no activity, its other extracts were within the mild activity range. It is worth noting that the aqueous extracts exhibited good activity (IC_{50}=22.90 μg/ml) which is the second best to *E. capensis* (IC_{50} = 3.87 μg/ml) in Table 6.

Ficus sur is the only plant whose extract showed high antiplasmodial activity in the hexane and chloroform extracts while its more polar portions were inactive. All the extracts of *E. inaequilatera, P. curatelifolia* and *S. princeae* (Table 6) were inactive (IC_{50}>100 μg/ml).

Only the aqueous extracts of *A. hoockii* showed a mild activity of IC_{50}=91.48 μg/ml whereas the rest were inactive and all *C. figarei* extracts were also inactive.

The *C. myricodes* leaves showed good antiplasmodial activity in its methanolic extracts. The ethylacetate extract was within the mild activity range. Although

Table 7: The mean IC_{50} values (x ± S.D) for selected plant extracts against NF 54, ENT 30 and V1/S (K39 values included for comparison).

Extracts	IC50 ±S.D (µg/ml)			
	Chloroquine –sensitive isolates		Chloroquine –resistant isolates	
	K39	NF 54	ENT 30	V1/S
E. capensis				
Chloroform	3.92±0.11	8.68±1.32	8.31±1.13	13.45±2.09
Ethylacetate	4.66±0.14	15.36±2.71	4.87±1.04	23.14±1.87
Methanol	4.61±0.28	8.89±2.66	6.07±1.38	8.27±2.14
Aqueous	3.87±0.07	22.94±1.94	17.58±2.34	26.04±2.83
R. prinoides				
Methanol	15.05+0.26	>62.5	23.15+1.81	29.87+1.76
Ficus sur				
Hexane	19.15±1.31	>62.5	27.43±6.94	>100
Chloroform	8.99±0.24	>62.5	11.32±3.81	34.56±6.63
V. lasiopus				
Chloroform	1.21±0.13	1.67±0.31	3.61±1.02	3.38±0.32
Ethylacetate	1.01±0.07	1.62±0.43	1.37±0.11	1.59±0.87
Methanol	3.15±0.28	3.52±0.15	3.81±1.32	4.13±1.11
C. myricoides				
Leaves				
Methanol	16.78±1.06	12.81±1.25	13.46±4.39	15.96±2.06
Root bark				
Methanol	8.55±0.15	5.81±2.03	6.71±3.61	3.96±2.51
* Chloroquine	0.021 µg/ml	0.016 µg/ml	0.066 µg/ml	0.073 µg/ml

*Chloroquine is included for comparison and to assess resistance or susceptibility.

Although both K39 and NF 54 are chloroquine sensitive isolates, it seems that the latter is less susceptible to *E. capensis* extracts. K39 seem to be twice as susceptible to the chloroform extracts of *E. capensis* (IC_{50}=3.92 μg/ml) than NF 54 (IC_{50}=8.68 μg/ml). K39 is about three times susceptible to *E. capensis* ethylacetate extract (IC_{50}=4.66 μg/ml) than NF 54 (IC_{50}=15.36 μg/ml), twice to methanolic extracts (IC_{50}=4.61 μg/ml) than NF 54 (IC_{50}=8.89 μg/ml) and about seven times to the aqueous extracts of *E. capensis* (IC_{50}=3.87 μg/ml) than NF 54 (IC_{50}=22.94 μg/ml).

For both K39 and NF 54 *V. lasiopus* seem to give comparable values for chloroform, ethylacetate and methanolic extracts respectively. These were IC_{50} values of 1.21 μg/ml, 1.01 μg/ml, 3.15 μg/ml for K39 and 1.67 μg/ml, 1.62 μg/ml and 3.52 μg/ml for NF 54.

It is however noted in Table 7 that the NF 54 is more susceptible to *C. myricoides* methanolic extracts of leaves (IC_{50}=12.81 μg/ml) than K39 (IC_{50}=16.78 μg/ml). A similar observation is made for the *C. myricoides* rootbark methanol extracts which seem to be more active for NF 54 (IC_{50}=5.81 μg/ml) than for K39 (IC_{50}=8.55 μg/ml).

ENT 30, a local Kenyan isolate and V1/S, a Vietnam isolate are both chloroquine resistant with comparable chloroquine IC_{50} values of 0.066 μg/ml

and 0.073 μg/ml respectively. From Table 7, three observations can be made from the antiplasmodial activity of the plant extracts against ENT 30 and V1/S:

a) There are cases where the two isolates were less susceptible to plant extracts when compared with the chloroquine susceptible isolates. For example, *E. capensis* aqueous extracts exhibited an IC_{50} value of 3.87 μg/ml and 26.04 μg/ml against K39 and V1/S respectively.

b) Cases in which both the chloroquine sensitive and resistant isolate seem to be more or less equally susceptible to the same extracts. For example, *V. lasiopus* ethylacetate extracts had IC_{50} values comparable for all isolates. These were 1.01, 1.62, 1.37, 1.59 μg/ml against K39, NF 54 (both chloroquine sensitive), ENT 30 and V1/S (both chloroquine resistant) respectively.

c) Cases in which the chloroquine resistant isolates seemed to be more susceptible to the plant extracts than the chloroquine susceptible ones. For example, V1/S is more than two times susceptible to *C. myricoides* rootbark methanolic extracts (IC_{50}=3.96 μg/ml) than K39 (IC_{50}=8.55 μg/ml).

From Table 7, it is clear that methanolic root-bark extract of *C. myricoides* is more active (about two times) than the methanolic extracts of the leaves.

3.4 Drug Interaction studies

The results of the drug interaction studies between chloroquine and various plant extracts against the chloroquine resistant V1/S isolate are presented in tables 8-15.

Table 8: Interactions of chloroquine with *E. capensis* Chloroform extracts against V1/S[a].

	Combination Ratios								
	90:10	80:20	70:30	60:40	50:50	40:60	30:70	20:80	10:90
Sum FIC[b]	0.32	0.25	0.23	0.19	0.21	0.23	0.33	0.31	0.34
DESCRIPTION	S[c]	S	S	S	S	S	S	S	S

a - V1/S is a chloroquine resistant strain.

b - FIC values <1 = synergism, >1 = antagonism and values of 1 = addition

c - S denotes synergy.

Since all the sum of fractional inhibitory concentration (sumFIC) values are <1, this implies that at all drug combinations, exhibited synergy. Synergism is most marked at 60:40 combination, which corresponds with a sum FIC of 0.19.

Table 9: Interaction of chloroquine with *E. capensis*, ethylacetate extracts against V1/S.

	Combination Ratios								
	90:10	80:20	70:30	60:40	50:50	40:60	30:70	20:80	10:90
Sum FIC[b]	1.76	1.69	1.44	1.27	0.87	0.63	0.54	0.45	0.35
DESCRIPTION	A[d]	A	A	A	S^w	S	S	S	S

d – A denotes antagonism

S^w – weak synergy

Ethylacetate extract of *E. capensis* seems to antagonise chloroquine as indicated by the sum FIC values > 1. On the other hand, chloroquine seems to potentiate the extract as depicted by values of <1 as the amount of the extract increases in the combination.

Table 10: Interaction of chloroquine with *E. capensis* methanol extracts against V1/S

	Combination Ratios								
	90:10	80:20	70:30	60:40	50:50	40:60	30:70	20:80	10:90
Sum FIC	2.97	3.50	1.69	0.74	0.78	0.78	0.94	0.96	0.82
DESCRIPTION	A	A	A	A	S^w	S^w	S^w	S^w	S^w

S^w = weak synergy (sum FIC value approach 1).

The extract highly antagonises chloroquine and even the synergy on the left side is generally a weak one (approaching additive effect) since the values are approaching 1.

Table 11: Interaction of chloroquine with *E. capensis* water extracts against V1/S

	Combination Ratios								
	90:10	80:20	70:30	60:40	50:50	40:60	30:70	20:80	10:90
Sum FIC	0.56	0.54	0.32	0.18	0.28	0.45	0.51	0.78	0.66
DESCRIPTION	S	S	S	S	S	S	S	S	S

The interaction of chloroquine with *E. capensis* aqueous extract is a synergistic one, which is highly marked at 60:40 combination.

Table 12: Interaction of Chloroquine with *C. myricodes* (Rootbark) methanol extract against V1/S

	Combination Ratios								
	90:10	80:20	70:30	60:40	50:50	40:60	30:70	20:80	10:90
Sum FIC	0.18	0.20	0.15	0.18	0.18	0.08	0.09	0.07	0.13
DESCRIPTION	S	S	S	S	S	S	S	S	S

Chloroquine and *C. myricoides* rootbark methanolic extract seem to interact very well confering good synergy to each other.

Table 13: Interaction of chloroquine with *V. lasiopus* chloroform extracts against V1/S.

	Combination Ratios								
	90:10	80:20	70:30	60:40	50:50	40:60	30:70	20:80	10:90
Sum FIC	0.16	0.20	0.14	0.15	0.32	0.16	0.15	0.13	0.97
DESCRIPTION	S	S	S	S	S	S	S	S	S^w

S^w = weak synergy where the value approach the additive line.

Chloroquine and *V. lasiopus* chloroform extract exhibited good synergistic effect.

Table 14: Interaction of chloroquine with *V. lasiopus* ethylacetate extracts against V1/S.

	Combination Ratios								
	90:10	80:20	70:30	60:40	50:50	40:60	30:70	20:80	10:90
Sum FIC	0.55	0.32	0.31	0.26	0.29	0.29	0.31	1.00	1.24
DESCRIPTION	S	S	S	S	S	S	S	Ad	A

Ad = Additive effect (sum FIC = 1).

The combination of chloroquine with *V. lasiopus* ethylacetate extract exhibited good synergy, which goes through addition to antagonism.

Table 15: Interaction of chloroquine with *V. lasiopus* methanol extracts against V1/S.

	Combination Ratios								
	90:10	80:20	70:30	60:40	50:50	40:60	30:70	20:80	10:90
Sum FIC	0.24	0.31	0.17	0.17	0.11	0.14	0.73	1.17	1.84
DESCRIPTION	S	S	S	S	S	S	S	A	A

Chloroquine in combination with *V. lasiopus* methanolic extract showed good synergy that was highly marked at the middle combination of 50:50.

As expected, the results obtained indicate that extract interactions with chloroquine ranged from synergism, through addition to antagonism.

CHAPTER FOUR

DISCUSSION

4.1 The *in vitro* antimalarial activity of the investigated plant extracts

Chloroquine resistance is defined as an IC_{50} >100 nM (about 0.052 μg/ml) (Basco *et al.*, 1995; Basco and Le Bras, 1992). K 39 (local isolate) and NF 54 (Amsterdam Airport isolate) fell far below this cut-off concentration (IC_{50} values were 0.021 μg/ml and 0.016 μg/ml respectively) and therefore are chroloquine sensitive. Conversely isolates ENT 30 (local) and V1/S (non-local) which had IC_{50} values of 0.066 μg/ml and 0.073 μg/ml respectively are chloroquine resistant.

For crude extracts, most researchers consider IC_{50} values above 100 μg/ml to be inactive (Basco *et al.*, 1994). Values ranging between 20-100 μg/ml are considered to be within the mild or moderate activity range. The values of <20 μg/ml are considered to be in the high activity range for crude plant extracts (Mackinnon *et al.*, 1997). Gessler *et al.,* (1994) have grouped plant species by their *in vitro* antimalarial activity into 3 groups: Group A consists of plant extracts whose IC_{50s} are <1 μg/ml, B are in the range of 1-5 μg/ml and C lies between 6-10 μg/ml.

Weenen *et al* (1990) have investigated several Tanzanian medicinal plants to determine plants with the highest *in vitro* antimalarial activity. Among the first category (<1 μg/ml) is the methanolic crude extract of *Cinchona* species (from

which quinine is isolated) which had IC_{50} of 0.5 μg/ml. In general, this study will use the criteria of Basco *et al.*, (1994) and Mackinnon *et al.*, (1997) to classify activity.

4.1.1 Preliminary Screening against K39

Out of the 115 extracts screened against K39, thirty nine (33.9%) were active (IC_{50}<100 μg/ml) of which 12 (30.8 %) fell within the high activity range (IC_{50}<20 μg/ml). These include 4 extracts of *Ekebergia capensis,* 2 extracts of *Ficus sur*, 3 extracts of *Vernonia lasiopus*, the *Clerodendrum myricoides* methanolic extracts (root bark and leaves) as well as the methanolic extracts of *Rhamnus prinoides*. Out of the 23 hexane extracts screened against K39 (table 6), only 5 extracts (21.7%) were within the active range of IC_{50}<100 μg/ml. Only the hexane extract of *Ficus sur* fell below 20 μg/ml range of activity. The other 4 extracts were within the mild activity range. Chloroform ($CHCl_3$) which is more polar than hexane had 9 plant extracts (39.1%) within the active range (IC_{50}<100 μg/ml). Three of these extracts (33.3%), *E. capensis*, *F. sur* and *V. lasiopus* were below 20 μg/ml. This was unlike the hexane extracts where only *F. sur* extract was below 20 μg/ml, suggesting increasing activity with increasing solvent polarity. Despite ethylacetate (EtOAc) extracts having only 7 extracts active against K39 (30.43%), it had the plant extracts with the lowest IC_{50} value of 1.01 μg/ml. Fourteen (60.9%) of all the 23 methanolic extracts were active against K39 isolate with IC_{50} values which ranged from 3.15 μg/ml –87.69 μg/ml. Five of these extracts (35.7%) had activity of less

than 20 μg/ml. The general trend was higher activity with increasing polarity of organic solvent extracts, suggesting that the active compounds in these extracts could be polar in nature.

The best extracts against K39 were those of *V. lasiopus* (table 6) which gave IC_{50} values of 1.21, 1.01 and 3.15 μg/ml from chloroform, ethylacetate and methanol extracts respectively. These values are slightly double the IC_{50} values of the crude extracts of *cinchona* species (IC_{50}=0.5 μg/ml) against *P. falciparum* (Weenen *et al.*, 1990). The chemistry and biological activity of other *Vernonia* species such as *Vernonia brachycalyx* and *V. amygdalina* have been reported. Oketch-Rabah (1996) had reported good *in vitro* antimalarial activity of *V. brachycalyx* $CHCl_3$: EtOAc (1:1) crude extract (leaves) against K39 (IC_{50} = 6.62 μg/ml) and V1/S (IC_{50} = 8.43 μg/ml). The methanol extract had IC_{50} values of 29.62 μg/ml and 16.64 μg/ml against K39 and V1/S respectively. The aqueous extract was the highest with IC_{50} values of 31.22 μg/ml and 30.22 μg/ml against K39 and V1/S respectively. These findings are consistent with the findings of this study in which the best activity were those of ethylacetate and chloroform against all the 4 isolates used. The activity of $CHCl_3$, EtOAc and MeOH extracts ranged from 1.01 μg/ml to 4.13 μg/ml for both the chloroquine-sensitive and resistant-isolates. Although several compounds were isolated from *V. brachycalyx*, 16, 17-dihydrobrachycalyxolid was shown to be the major antiplasmodial principle (Oketch-Rabah, 1997). This showed IC_{50} values of 4.2 and 13.7 μg/ml against K39 and 3d 7 (both CQ-

sensitive *P.falciparum* isolates) respectively, 3.0 and 16.1 μg/ml against V1/S and Dd 2 (both CQ-resistant *P.falciparum* isolates) respectively. Since the crude extracts of *V. lasiopus* exhibited good activity in our study than even the active principle of *V. brachycalyx*, it is therefore important to investigate the plant further for this might reveal novel antiplasmodial compounds.

E. capensis showed good activity (table 6) within category B (1 – 5 μg/ml) of 3.92, 4.66 and 4.71 μg/ml for $CHCl_3$, EtOAc and MeOH extracts respectively. Some chemistry on *E.capensis* (see section 1.3.2) had been reported but not antiplasmodial activity (Mulholland *et al.,* 1998). Nkunya *et al.*, (1997) has reported that no antimalarial activity on compounds isolated from *E. benguelensis,* a plant of the same family with *E. capensis*. In fact, of all the aqueous extracts (table 6), only *E. capensis* extract had an activity of below 20 μg/ml (IC_{50}=3.87 μg/ml), which compare with its organic extracts IC_{50s}. There are cases where the isolated compounds show no activity but crude extracts do, just as it has been found that some extracts may show *in vitro* activity and no *in vivo* activity and vice versa (Gessler *et al*., 1995).

Methanolic extracts of leaves and root bark of *C. myricodes* showed very good activity (table 6) of 16.78 and 8.55μg/ml respectively against K39. The hexane, chloroform and ethylacetate extracts were inactive suggesting that the bioactive compound in this plant is highly polar. *Rhamnus prinoides* showed good activity ranging from 15.05-96.80 μg/ml. This plant warrants detailed study

especially on methanolic extracts, which had IC_{50} value of 15.05 μg/ml against K39.

Ficus sur (table 6) was the only plant that had an active hexane extract (IC_{50} 19.15μg/ml). Its chloroform extract (IC_{50} = 8.99μg/ml) was also amongst the highest active against K39 and the plant warrants further detailed biological and chemical studies.

For the water extracts, only 4 extracts were below 100 μg/ml (table 6) and only *E. capensis* water extract (IC_{50} 3.87μg/ml) was comparable with its organic solvent extracts. Lack of *in vitro* activity may not have *in vivo* extrapolations since compounds may act as pro-drugs which must undergo metabolic changes for them to be active. In addition, the inactive plants may not have direct effect to the parasite but may deal with malaria symptoms such as fever. Therefore, they may either be febrifuges or immuno-modulators. Most of the rest of the plant extracts fell into the moderate activity range when they were screened against K39. Amongst the plants which were around the upper limit margin of 20 μg/ml were *Stephania abyssinca* (table 6) water extract (22.90 μg/ml) and *Rhamnus staddo* methanolic extract (25.64 μg/ml). *Ajuga remota* methanolic extract (IC_{50} 21.60 μg/ml) also fell close to the margin (table 6). Its leaves had been shown (Kuria and Muriuki, 1984) to have an aglycone of cardenolide type, which had cardiotonic activity. For these 3 plants detailed evaluation on their biological activity is necessary. The *Leucas calostachys* and *Leonotis*

mollisina (both Labiatae) showed mild activities (table 6), especially their chloroform extracts which had IC_{50} values of 36.22 and 35.66 μg/ml respectively against K39.

Of the 22 plants species screened against K39, only 7 plant species (31.82%) showed no activity (IC_{50} >100 μg/ml) for all the 5 extracts. These included *Parinani curatelifolia, Spermacoce princeae, Euphorbia inaequilatera, Senna didymobotria, Utrica masaica, Cucumis figarei* and *Melia azadarach*. The latter's bark had been reported to contain gedunin (Mackinnon *et al.*, 1997). *M. azadarach* and *Azadirachta indica* are both of meliaceae family, and gedunin has been shown to be their bioactive antimalarial compound. However, activity depends on many factors such as season in which the plant is collected, the age of the plants, intraspecies variation, part collected (in my case the leaves were collected) the environment etc. Therefore, lack of *in vitro* activity in this case does not disqualify the plants use as antimalarials outrightly. In addition, plant extracts may not display *in vitro* activity but display *in vivo* activity, as Gessler *et al.*, (1995) had observed.

Out of the 22 plant species studied, 15 of them (68.18%) showed antiplasmodial activity (IC_{50} <100 μg/ml) and none of the plant species showed activity for all of the 5 extracts. However, some plant species had as many as 4 out of 5 extracts (80%) active such as *E. capensis*, *R. prinoides*, others 3 out of 5 extracts (60%) such as *V. lasiopus* active, some 2 out of 5 extracts (40%) and

others 1 (20%), against K39 (table 6). This implies that there are bioactive compounds present in the various extracts that require investigation.

4.1.2 Screening against NF 54

The 12 extracts which showed IC_{50} values of less than 20 μg/ml against K39 also exhibited good activity against the laboratory adapted CQ-sensitive NF 54 isolate (table 7). The parasite susceptibility to these extracts followed the order of K39 except for a few cases where the IC_{50} values were generally higher for NF 54 than for K39. The best extracts against NF 54 were those of *V. lasiopus*, which were 1.67, 1.62 and 3.52 μg/ml for $CHCl_3$, EtOAC and CH_3OH respectively which compared well with 1.21, 1.01 and 3.15 μg/ml values of the same plant against K39. Also, *E. capensis* exhibited good activity against NF 54 but the values were between 2 to 6 times higher than that of K39. The $CHCl_3$ and MeOH (8.68 and 8.87 μg/ml respectively) extracts were about 2 times that of K39 values (3.92 and 4.61 μg/ml), EtOAC (15.36 μg/ml) extract was about 3 times (K39: 4.66 μg/ml) and H_2O extract was about 6 times. *R. prinoides* (MeOH), *Ficus sur* (hexane and chloroform) extracts showed mild activities against NF 54 (IC_{50}>62.5 μg/ml). Both MeOH extracts of *C. myricoides* root bark and leaves were lower for NF 54 (IC_{50}=5.81 and 12.81 μg/ml respectively) than for K39 (IC_{50}=8.55 and 16.78 μg/ml respectively). Out of the 12 extracts screened which showed IC_{50} values of <20 μg/ml against K39, 8 extracts (66.67%) fell below 20 μg/ml against NF 54. The other 4 extracts (33.33%) moved into the mild activity range (20-100 μg/ml). As a

general trend, K39 seems to be more susceptible to the plant extracts than NF 54 which may suggest that the plant extracts have different target sites for the two isolates.

4.1.3 Screening against ENT 30

ENT 30 and V1/S are CQ-resistant isolates (table 7) and it is worth noting that out of the 12 extracts screened against ENT 30, only 2 extracts (16.67%) fell above 20 μg/ml into the moderate activity range. These were the *R. prinoides* methanol (23.15 μg/ml) and *Ficus sur* hexane (27.43 μg/ml) extracts. The other extracts had IC_{50} of between 1.37 – 17.58 μg/ml that compare well with those of K39 (1.01-19.15 μg/ml) and NF 54 (1.62-15.36 μg/ml). *V. lasiopus* (table 7) gave the best activity against K39, NF 54 and ENT 30 and in all the 3 cases, ethylacetate extract gave the highest activity with values which were comparable. The IC_{50} values for *E. capensis* extracts against ENT 30 compared well with those of NF 54. For example, the chloroform extract gave IC_{50} values of 8.31 μg/ml and 8.68 μg/ml for ENT 30 and NF 54 respectively. There are cases where extracts had better activity against ENT 30 than for NF 54. For example, *E. capensis* ethylacetate extract had IC_{50} values of 4.87 and 15.36 μg/ml for ENT 30 and NF 54 respectively. It is clear that this extract was 3 times better in activity against ENT 30 than for NF 54. This could possibly imply that the extracts and CQ have different modes of action or different target sites.

4.1.4 Screening against V1/S

Out of the 12 extracts screened against V1/S (table 7), only 4 of them fell into the moderate activity range (20-100 μg/ml) but the rest of the extracts showed values comparable to those of the other isolates. For instance, *V. lasiopus* chloroform, ethylacetate and methanol extracts had IC_{50} values 3.38, 1.59 and 4.13 μg/ml for V1/S which compare with ENT 30 values of 3.61, 1.37 and 3.81 μg/ml respectively.

Within the high activity range of less than 20 μg/ml, the V1/S showed comparable values for *E. capensis* methanol extract (IC_{50}=8.27 μg/ml) and *C. myricoides* root bark methanol extract (IC_{50}=3.96 μg/ml) with that of NF 54 (IC_{50}=8.87 μg/ml and 5.81 μg/ml respectively). *C. myricoides* root bark methanol extracts, just like with the other isolates had better activity against V1/S than the leaves methanol extracts (IC_{50}=3.96 μg/ml and 15.96 μg/ml respectively). This seems to suggest that the accumulation of secondary metabolites or the bioactive compounds in the plant is dependent, among other factors, the part of the plant.

Out of the 12 extracts from 5 species screened (which showed activity of <20 μg/ml against K39), only *R. prinoides* methanolic extract and *Ficus sur* hexane extract showed mild activity (>20 μg/ml) against NF 54, ENT 30 and V1/S. All the 12 extracts (100%) were active against NF 54 within the range of IC_{50} values of 1.62 to <100 μg/ml and ENT 30 within the range of 1.37–27.43

μg/ml. For V1/S, 11 extracts (91.67%) within the range of 1.59 – 29.87 μg/ml were active, and only *Ficus sur* (hexane) was inactive (>100 μg/ml). The fact that most of the extracts screened against all the 4 isolates were active against all of them serves as evidence that these medicinal plants contained antimalarial compounds worthy further investigation. For example, *V. lasiopus* extracts were active within a range of 1.01-4.13 μg/ml against all the isolates with its ethylacetate extracts being quite comparable (K39=1.01 μg/ml, NF 54=1.62 μg/ml, ENT 30=1.37 μg/ml and V1/S=1.59 μg/ml).

4.1.5 Summary of screening experiments

Out of all the 22 plant species screened, 15 plant species (68.18%) had at least one of the extracts falling within the active range (<100 μg/ml). Only 7 plant species (31.82%) had no activity, (>100 μg/ml). Out of the 23 water extracts, only 4 extracts (17.39%) had activity (table 6) and out of these, only *E. capensis* extract was significant (3.87 μg/ml) against K39. Although many extracts did not show any *in vitro* activity, it should be noted that lack of *in vitro* activities does not always indicate lack of *in vivo* activities. In some instances extracts with no *in vitro* activities have shown good *in vivo* activities in animal models (Gessler *et al*, 1995). This could be due to low absorption or the parent molecule may need to undergo structural changes during metabolism for it to be active (i.e pro-drugs). Drugs or herbal preparations may also act by more than one mechanism, such as having an indirect effect on the immune system or other pathways that are not yet understood. Therefore, it is important to investigate even the extracts >20 μg/ml for *in vivo* studies since they might

show better activities against the plasmodia. Care must be taken when interpreting the results of antimalarial screening of extracts since seasonal and regional variations have not been considered, but can be significant. However, although lack of antimalarial activity may not be ruled out for the 7 plants that showed no *in vitro* antimalarial activity, they could also work as antipyretic compounds or febrifuges (fever-suppressors) since fever is one of the symptoms associated with the uncomplicated severe *P. falciparum* malaria.

Some of the extracts (table 7) had similar or even better activity against CQ resistant than CQ-sensitive isolates which suggests no cross-resistance with chloroquine probably due to differences in the mode of action of compounds present in the extracts. This suggests that the extracts from this plants have potential use in solving the problem of multidrug resistance.

4.2 Drug Interaction Experiments

In the case of *E. capensis*, the ethylacetate and methanol extracts (tables 9 & 10) seem to be more of antagonistic interactions with chloroquine, and where synergism is recorded, it is a weak one. However, *E.capensis* chloroform (tables 8 & 11) and aqueous extracts gave very good synergistic effects of as low as 0.19 and 0.18 respectively.

Clerodendrum myricodes (rootbark) methanol extract gave the best synergistic effects when compared to the rest with values as low as 0.07 (table 12) and *V. lasiopus* extracts seemed to potentiate chloroquine at all combinations (tables

13, 14, 15) except for a few combinations which either showed antagonistic or additive effects. *V. lasiopus* showed strong synergy with values as low as 0.12, 0.26, and 0.11 for chloroform, ethylacetate and methanol extracts respectively.

Although several molecules have been shown to restore chloroquine sensitivity to resistant *P. falciparum* strains (Oduola *et al.*, 1998), very little is known about reversal of resistance from herbal remedies. Rasoanaivo *et al.,* (1998) have investigated several medicinal plants used by local populations in Madagascar in association with chloroquine, called adjuvants to chloroquine. They have showed that not only do the crude alkaloids significantly enhance *in vitro* and *in vivo* choloroquine action but also isolated compounds markedly reversed chloroquine resistance (see section 1.3).

Although mechanism of resistance of 4-aminoquinolines such as chloroquine by the parasite is not well understood there is strong evidence that resistant strains *of P. falciparum* accumulate less chloroquine than the sensitive ones (Rasoanaivo *et al.,* 1998). However, the mechanism by which it occurs, either rapid efflux of pre-accumulated chloroquine mediated by p-glycoproteins or decreased chloroquine uptake is still being debated (Rasoanaivo *et al.,* 1998). Most of the crude extracts investigated seemed to synergise chloroquine. Whether the extracts increased the retention of chloroquine in the food vesicles (rapid chloroquine efflux) or increased chloroquine uptake due to better transport of the combination into the food vacuole is an issue for further investigation. Further studies on these extracts are important since they

probably can serve as biochemical tools for the understanding of the chloroquine resistance and its reversal.

4.3 CONCLUSIONS

a. From the results, 15 out of the 22 plant species (68%) investigated had *in vitro* antimalarial activities. This would account for their ethnopharmacological uses as antimalarials.

b. The *V. lasiopus* chloroform, ethylacetate and methanol extracts showed activity in the range of 1.01-4.13 μg/ml against all the 4 isolates (both CQ-sensitive and CQ-resistant). Comparable results were obtained for *C. myricoides* (both leaves and root bark) and *E. capensis* methanol extracts. The bioactive principles could be isolated from these plants and used to standardize a popular crude drug based on traditional use of these plants as antimalarials.

c. Some extracts showed IC_{50} values against the CQ-resistant isolates that were comparable with those of CQ-sensitive isolates. This could imply that there is no cross-resistance between the extracts active principles and chloroquine. This could further be investigated to tackle the problem of multi-drug resistance.

d. Combination of several extracts with chloroquine seemed to have a potentiation effect *in vitro*. If this can be demonstrated *in vivo*, then the

crude preparations and/or isolated compounds from these plants could be used in combination with chloroquine against resistant *P. falciparum* strains of malaria.

4.4 RECOMMENDATIONS FOR FUTURE WORK

a. The extracts that exhibited good *in vitro* antimalarial activity could be subjected to *in vivo* studies in animal models so as to extrapolate *in vitro*/*in vivo* activities of these extracts.

b. The isolation of compounds present in the active extracts should be undertaken, and subjected to bioassays, to investigate whether the individual isolated compounds are more active than their mother extracts since there are cases where this is not so. The bioactive compounds could be characterised using spectroscopic methods.

c. Except for a few cases where more than one part of the plant was used, the studies were done using only one part of the plant (e.g. leaves). It would be necessary to study different parts of the same plant such as root bark, stem bark, leaves etc. and compare their activities.

d. In addition to *in vivo* studies, it is important to carry out cytotoxicity and clinical studies with the plant extracts and isolated compounds. Concentrations and dosage of the active plant principles can be

formulated to improve their effectiveness and this knowledge availed to the traditional medical practitioners.

e. Development of plant extracts as capsules or tablets can be undertaken as has been done in S.E Asian countries. This could avail crude drugs that are cheaper and affordable to the local population.

f. Plant extracts/chloroquine (or any other standard antimalarial drug) combination could be investigated *in vivo* to ascertain positive *in vitro* synergy. Correct doses of such a combination could be formulated and the knowledge availed to both traditional and conventional medical practitioners.

g. Since much has been done on drug-drug interactions and very little on plant extracts-drug interactions, it could be interesting to undertake isolated compounds combination with standard antimalarials to investigate potentiating (synergy), additive, or antagonistic effects. This could find application in either delaying or reversal of chloroquine resistance.

h. Tissue culture techniques could be employed (e.g. callus-cultures) to generate enough bioactive antimalarial compounds/principles for detailed studies and use in future, since most of these plants are rare or may avail active principles in very low concentrations.

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APPENDIX I

Table 16: The IC_{50} values for the Meliace Plant extracts against K39

Plant Extract	IC_{50}s ±S.D (µg/ml)	
	Ekebergia capensis	*Melie azadarach*
Hexane	>100	>100
Chloroform	3.92±0.11	>100
Ethylacetate	4.66±0.14	>100
Methanol	4.61±0.28	>100
Aqueous	3.87±0.07	>100
Chloroquine	0.021 µg/ml	0.021 µg/ml

*Chloroquine included as a positive control.

Table 17: The IC_{50} values for the Rhamnaceae Plant extracts against K39

Plant extract	IC_{50}±S.D.(µg/ml)	
	Rhamnus prinoides	*Rhamnus staddo*
Hexane	>100	>100
Chloroform	96.80±1.04	>100
Ethylacetate	50.74±2.97	35.15±3.67
Methanol	15.05±2.26	25.64±2.41
Aqueous	82.30±2.44	>100

Table 18: The IC_{50} values for Compositae Plant extracts against K39

	IC_{50}±S.D. (µg/ml)			
Plant Extract	*V. lasiopus*	*A. pluriseta*	*V. auriculifera*	*M. pyrifolia*
Hexane	>100	62.70±8.91	>100	72.54±2.46
Chloroform	1.21±0.13	>100	37.73±1.29	58.35±1.93
Ethylacetate	1.01±0.07	>100	40.30±1.98	79.52±2.71
Methanol	3.15±0.28	40.81±5.67	55.17±2.31	68.35±3.48
Aqueous	>100	>100	>100	>100

Table 19: The IC_{50} values for Labiatae Plant extracts against K39

Plant Extract	IC_{50}±S.D. (µg/ml)		
	Ajuga remota	*Leonotis mollisina*	*Leucas calostachys*
Hexane	>100	50.77±5.66	66.47±2.10
Chloroform	>100	35.66±2.34	36.22±3.98
Ethylacetate	>100	>100	>100
Methanol	21.60±1.36	80.74±4.07	87.69±1.77
Aqueous	>100	>100	>100

Table 20: The IC_{50} values for Acanthaceae plant extracts against K39

Plant Extract	IC_{50}±S.D. (µg/ml)
	Justicia betonica
Hexane	>100
Chloroform	>100
Ethylacetate	>100
Methanol	69.55±1.89
Aqueous	>100

Table 21: The IC_{50} values for Menispermaceace Plant extracts against K39

Plant Extract	IC_{50}±S.D. (µg/ml)
	Stephania abyssinca
Hexane	>100
Chloroform	67.07±2.94
Ethylacetate	>100
Methanol	63.87±3.44
Aqueous	22.90 ±1.03

Table 22: The IC_{50} values for Moraceae Plant extracts against K39

Plant Extract	IC_{50}±S.D. (µg/ml)
	Ficus sur
Hexane	19.15±1.31
Chloroform	8.99±3.24
Ethylacetate	>100
Methanol	>100
Aqueous	>100

Table 23: The IC_{50} values of Euphorbiaceae Plant extracts against K39

Plant Extract	IC_{50}±S.D. (µg/ml)
	Euphorbia inaequilatera
Hexane	>100
Chloroform	>100
Ethylacetate	>100
Methanol	>100
Aqueous	>100

Table 24: The IC_{50} values of Rosaceace Plant extracts against K39

Plant Extracts	IC_{50}±S.D. (µg/ml)
	Parinari curatelifolia
Hexane	>100
Chloroform	>100
Ethylacetate	>100
Methanol	>100
Aqueous	>100

Table 25: The IC_{50} values of Rubiaceae Plant extracts against K39

Plant Extract	IC_{50}±S.D. (µg/ml)
	Spermacoce princeae
Hexane	>100
Chloroform	>100
Ethylacetate	>100
Methanol	>100
Aqueous	>100

Table 26: The IC_{50} values of Leguminosae Plant extracts against K39

Plant Extract	IC_{50}±S.D. (µg/ml)
	Acacia hoockii
Hexane	>100
Chloroform	>100
Ethylacetate	>100
Methanol	>100
Aqueous	91.48±4.78

Table 27: the IC_{50} values of Curcubitaceae Plant extracts against K39

Plant Extract	IC_{50}±S.D. (µg/ml)
	Cucumis figarei
Hexane	>100
Chloroform	>100
Ethylacetate	>100
Methanol	>100
Aqueous	>100

Table 28: the IC_{50} values of Verbanaceae Plant extracts against K39

Plant extract	IC_{50}±S.D. (µg/ml)	
	Clerondedrum myricodes	
	Leaves	Rootbark
Hexane	>100	>100
Chloroform	>100	>100
Ethylacetate	48.63±1.43	>100
Methanol	16.78±2.65	8.55±3.51
Aqueous	>100	>100

Table 29: The IC_{50} values of Caesalpinaceae Plant extracts against K39

Plant Extract	IC_{50}±S.D. (µg/ml)
	Senna didymobotria
Hexane	>100
Chloroform	>100
Ethylacetate	>100
Methanol	>100
Aqueous	>100

Table 30: The IC_{50} values for the Utricaceae Plant extracts against K39

Plant Extract	IC_{50}±S.D. (µg/ml)
	Utrica masaica
Hexane	>100
Chloroform	>100
Ethylacetate	>100
Methanol	>100
Aqueous	>100

APPENDIX II

1. Thawing of cultures

Cultures are often kept at 5ml volume, 6% haematocrit (Hct) in 25 cm^2 (50 ml) flasks. 6% x 5ml = 0.3ml (100% RBC in 5ml culture)

In thawing, if the PCV=0.1 ml,

then, (0.3 – 0.1 ml) = 0.2 ml (100% RBC) is required

to make 0.3 ml or 6% hct in 5ml culture.

But since the washed blood is 50% RBC and 50% WM instead of 100% RBC,

then (0.2 ml x 2) =0.4 mls (of 50% RBC) is added.

The new volume = 0.1 ml + 0.4 ml = 0.5 ml.

To make to 5ml culture, 5ml-0.5ml = 4.5 of CMS is required,

2. Calculation of percentage parasitaemia

A thin smear is scanned under low power to locate the area of slide where erythrocytes are evenly distributed. Observe under 100 x oil immersion, count the number of erythrocytes in one field using a tally counter and record. Divide 10,000 by number of erythrocytes per field (RBC) to calculate number of fields to be counted. Scan appropriate number of fields, counting parasitisized erythrocytes. Record number of fields and number of parasites counted. Calculate % parasitaemia (%P) from the formula:

$$\%P = \frac{\text{no. of parasitisized erythrocytes}}{(\text{RBC per field}) \times (\text{fields counted})} \times 100$$

3. Calculation of Growth Rate (G.R) per 48 hour cycle from the formula:

$G.R. = (pf \times D)^{2/d}/pi$

Where

Pf = final parasitaemia

Pi = initial parasitaemia before dilution

D = dilution factor

d = number of days since culture was diluted.

4. Calculation of dilution factor (D) for subculture from the formula;

$D = (GR)^{d/2} \times Pd/Po$

Where GR = growth rate during previous culture period

d = number of days until next subculture

Pd = desired parasitaemia after d days and Po = Parasitaemia on the day of subculture(Pf in 3 above).

5. Calculation of IC_{50}s

As an example, the *E. capensis* chloroform extract corrected CPM values, were as follows:

Down the column	Concentration (μg/ml)	Chloroform extract CPM values in Duplicate	
A	250	126	117
B	125	155	157
C	62.5	385	506
D	31.25	1160	893
E	15.625	1689	1554
F	7.8125	1906	1606
G	3.90625	3201	3194

Midpoint, $Y_{50} = (6423 - 49)/2 = 3187$

This value lies between (1906 + 1606)/2

and (3201 + 3194)/2 which correspond

with concentrations $X_1 = 3.90625$ and

$X_2 = 7.8125$ respectively

$Y_1 = 3198$

$Y_2 = 1756$

Antilog (log3.90625 + [(log3187–log3198) (log7.8125-log3.90625)]/(log1756-log3198) = **3.92 µg/ml**

6. Calculation of sum FICs

	Drug concentration in the 50:50 (CQ:Extract) combination in µg/ml	
CPM values	**Chloroquine**	**Extract**
561	0.50	125
938	0.25	62.5
1023	0.13	31.25
1659	0.06	15.625
2870	0.03	7.8125
3486	0.02	3.90625
5588	0.01	1.953215

IC_{50} of CQ in the mixture = 0.024 µg/ml

IC_{50} of plant extract in the mixture = 0.680 µg/ml

FIC of CQ = 0.024 / 0.073 µg/ml = 0.328

FIC of plant extract =0.680 / 7.91 µg/ml = 0.086

The sum FIC = 0.328 +0.086 = **0.413**

Since 0.413 is < 1, this indicates synergism.