SYNERGISTIC EFFECTS OF *ARTEMISIA ANNUA* FRACTIONS AGAINST *IN VITRO* CULTURES OF *PLASMODIUM FALCIPARUM*

BY

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Synergistic effects of
Artemisia Annua Fractions

A THESIS SUBMITTED IN PARTIAL FULFILMENT FOR THE REQUIREMENT OF THE DEGREE OF MASTER OF SCIENCE IN APPLIED MEDICAL PARASITOLOGY

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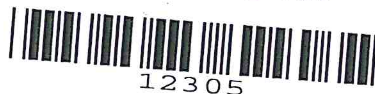

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Declaration

This thesis is my original work and has not been presented before for award of any degree in any university.

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Dedication

Dedicated to

My husband Joseph Nganga

My children

Teresia,

Mike,

Jacinta and

Elizabeth

For their prayers and support.

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Abbreviations

dATP-Deoxyadenosine Triphosphate

dCTP-Deoxycytidine Triphosphate

DDT - dichlorodiphenyltrichloroethane

dGTP-Deoxyguanosine Triphosphate

DMSO- dimethylsulfoxide

dNTP-Deoxyribonucleotide Triphosphate

dTTP-Deoxythymine Triphosphate

HPLC - High Performance Liquid Chromatography

IC_{50}-Inhibitory concentration at 50%

ICIPE- International Centre of Insect Physiology and Ecology

IGRs -Insect Growth Regulators

ITNs - Insecticide Treated Nets

KEMRI -Kenya Medical Research Institute

MSP1-Merozoite surface protein 1

ODS- Octadecyl silane

PBS-Phosphate Buffered Saline

PCR-Polymerase Chain Reaction

RBC - Red blood cell

SIT- Sterile Insect Technique

TAE- Tris Acetate EDTA buffer

TBE-Tris borate EDTA buffer

Abstract

Malaria is an infectious disease that continues to be associated with considerable morbidity and mortality and significant social and economic impact on developing societies. Approximately 300 million people worldwide are affected and between 1 and 1.5 million people die from it every year. The disease is caused by a protozoan parasite of the genus *Plasmodium*. Four species are known to cause malaria in man; these are *Plasmodium falciparum, P. vivax, P. ovale*, and .*P malariae*. The parasite has become resistant to drugs used as antimalarials and this calls for search of new drugs.

The research dealt with *Artemisia annua,* a herb native to China and aimed at understanding the role of different fractions on the *in vitro* cultures of *Plasmodium falciparum*. The fractions were then blend and their activity determined on *in vitro* cultures. Two clones of *P. falciparum* were utilized clone D6 (CQ sensitive) and W2 (CQ resistant). Upper foliage (3.5 kg) was collected, dried, ground to powder form, and sequentially extracted with dichloromethane and methanol. Initially the effect of the crude extract was tested on *in-vitro* cultures of *P. falciparum*. The extract was then fractionated using high performance liquid chromatography (HPLC). The fractions and different blends of these were tested on *P. falciparum* to determine additive or synergistic effects. Nine different fractions of *A. annua* were tested against D6 and W2.

Two fractions (22.2%) had antiplasmodial activity (IC_{50}) activity of above 250 µg/ml, 4 fractions (44.4%) had an IC_{50} of less than 3.9µg /ml whereas the remaining 3 fractions (33.3%) had an IC_{50} of between 4.77-14.76 µg/ml against D6 and W2. After blending the IC_{50} in all the blends

was below 27 μg/ml. It was possible to conclude that there was synergistic effect when the fractions were blended. The fractions were run together with convectional drugs and their IC_{50} compared. When the IC_{50} values were high this indicated low antiplasmodial activity. The purity of the test clones DNA was ascertained by conducting a polymerase chain reaction (PCR) test. The compounds in *Artemisia annua* could be more effective and cheaper in the treatment of *malaria* than the pure artemisinin compound and its derivatives.

Introduction

1.1 Malaria

Malaria is one of the most debilitating tropical diseases, an important cause of fever and morbidity in the tropics, and a significant source of mortality, especially among infants and young children. (Manson-Bahr and Bell, 1987). Malaria is caused by a protozoan parasite of the genus *Plasmodium.* Four species of *Plasmodium* can produce the disease in its various forms. The species responsible for malaria in humans are *Plasmodium .falciparum, Plasmodium vivax, Plasmodium ovale*, and *Plasmodium malariae* (WHO, 2003, Jerome, 1996). *P. falciparum* is the most wide-spread and dangerous of the four associated with the highest morbidity and mortality and causes *falciparum* malaria, a fatal parasitic disease of humans (Jerome, 1996). Infections in Africa are usually transmitted by bites of infected female *Anopheles* mosquito (predorminantly the *Anopheles gambiae* s. s. Giles and *Anopheles funestus)*, which favour temperatures of 20-30° C, and the injection of sporozoites into the bite site (Garnham, 1938, Surtees, 1970).

There are about 300 million malaria cases worldwide and between 1 and 1.5 million people die from it annually (Alibu and Egwang, 2003) the brunt of which are borne mostly in Africa, Asia, and Latin America where malaria is endemic in ninety one countries and 1.5 million people live in endemic areas. Eighty per cent of the cases occur in tropical Africa accounting for 10-30% deaths and responsible for 15-25% of all deaths of children under 5 years. Children and pregnant women are ranked as highly susceptible (WHO, 2003).

Malaria is complex but preventable. Lives can be saved if the disease is detected early and adequately treated. It is necessary to prevent the disease and to avoid or contain epidemics. The technology to prevent, monitor, diagnose and treat malaria exists. It needs to be adapted to local conditions and to be applied through local and national malaria control programmes (WHO, 2003).

1.2 The life cycle of *P. falciparum*

Infection in humans begin with the bite of an infected female anopheline mosquito. Sporozoites released from the salivary glands of the mosquito enter the bloodstream during feeding; quickly invade liver cells (hepatocytes). The sporozoites are cleared from the circulation within 30 minutes. During the next 5-8 days, the liver stage parasites differentiate and undergo asexual multiplication resulting in tens of thousands of merozoites that burst from the hepatocyte.

Individual merozoites invade red blood cells (erythrocytes) and undergo an additional round of multiplication producing 12-16 merozoites within a schizont. The length of this erythrocytic stage of the parasite life cycle depends on the parasite species, 48 hours for *P.falciparum, P.vivax, P. ovale* and 72 hours for *P. malariae*. The clinical manifestation of malaria, fever and chill, are associated with the synchronous rupture of the infected erythrocyte. The released merozoites go on to invade additional erythrocytes. Some of the merozoites differentiate into sexual forms, male and female gametocytes.

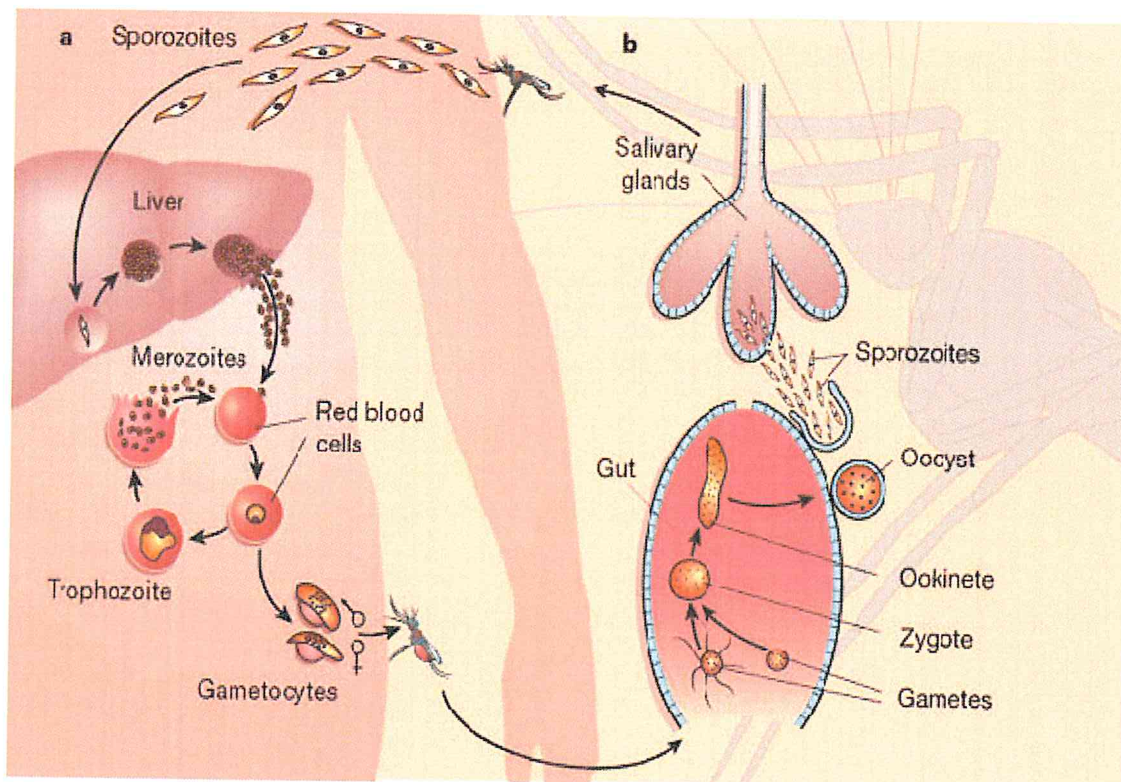


Figure 1. Life cycle of *P.falciparum* 'a' represents the human host stage and 'b' represents the mosquito vector stages.

Figure adopted from (http://encarta.msn.com/media_461541582/html).

These are the ones taken up by a female *anophylean* mosquito during a blood meal. Within the mosquito midgut, the male gametocyte undergoes a rapid nuclear division, producing 8 flagellated microgametes that fertilize the female macrogamete. The resulting ookinete traverses the mosquito gut wall as an oocyst. Soon the oocyst ruptures, releasing hundreds of sporozoites into the mosquito body wall cavity where they eventually migrate to the salivary gland (Figure 1) (Wallace and Gilles, 1995, Daniel *et al,* 2004).

Parasite resistance to existing anti-malarial drugs has already reached alarmingly high levels in Southeast Asia and on the African continent, and therefore there is a dire need for new drugs in the prophylaxis and treatment of malaria (WHO, 1998).

The plant *A. annua* L. (annual wormwood, sweet wormwood, sweet annie) is a highly aromatic annual herb of Asiatic and eastern European origin (Bailey and Bailey, 1985). A compound qinghaosu (artemisinin (**1**) has been isolated from *A. annua* and is reported to have anti-malarial effect (Klayman, 1985). The plant is listed in the Chinese pharmacopoeia as a remedy for various fevers including malaria and contains the well-established anti-malarial compound artemisinin (**1**) (Mueller *et al.* 2000). Chemically artemisinin (**1**) is a sesquiterpene trioxane lactone containing a peroxide bridge, which is essential for its activity (China cooperative research group. 1982). The endoperoxide bridge in artemisinin (**1**) reacts with ferrous iron atom to form free radicals (Zhang *et al.*1992, Posner *et al.* 2000). Artemisinin (**1**) becomes toxic to malaria parasites as they contain a high amount of iron in the form of heme molecules, and generation of free radicals leads to macromolecular damages and cell death (Anderson *et al.*1999).

1.3 Currently available anti-malarials

1.3.1 8-Aminoquinolines

8-Aminoquinolines is the only class of gametocides that is commonly used in the treatment of malaria.

1.3.1.1 Primaquine (5)

Primaquine is the essential co-drug with chloroquine in treating all cases of malaria. It is highly effective against the gametocytes of all plasmodia and thereby prevents spread of the disease to the mosquito from the patient. It is also effective against the dormant tissue forms of *P. vivax* and *P. ovale* malaria, and thereby offers radical cure and prevents relapses. It has insignificant activity against the asexual blood forms of the parasite and therefore it is always used in conjunction with a blood schizonticide and never as a single agent (http://www.malariasite.com/malaria/primaquine.htm). Primaquine(**5**) is widely used for the treatment of hypnozoites (liver reservoirs) common in *P. vivax* and *P.ovale* and have a good oral absorption (Roberts *et al.* 2001).

They have a short half life (4 hours) and needs to be administered daily for effective elimination of the parasites. The drug is however associated with serious toxicity that can be experienced with patients with glucose–6-phosphate dehydrogenase deficiency. The compound acts by interfering with the mitochondrial function of *Plasmodium* (Roberts *et al.* 2001).

1.3.1.2 Tafenoquine (6)

Tafenoquine (2,6-dimethoxy-4-methyl-5-[{3trifluoromethyl}-phenoxy]-8-[{4-amino-1-methyl butyl} amino] quinoline) is a long-acting primaquine analogue that is formulated as the succinate salt, with 250 mg of salt equal to 200 mg base. The long half-life of tafenoquine appears to be 2–3 weeks, thus allowing for infrequent dosing regimens. The drug is well

tolerated except for minor skin rashes and low-level asymptomatic methemoglobinemia, which is anticipated with this primaquine-like compound. This long half-life raises the possibility that short-term travelers to highly endemic malarious areas could be protected by the use of a 3-day regimen taken before travel (Shanks *et al.* 2001). When the drug is taken with food, absorption is increased by ~50% and the severity of gastrointestinal adverse effects is diminished (Brueckner *et al.* 1998; Walsh, 1999).

1.3.2 4-Aminoquinolines

In the last ten years, the widespread increase in *P. falciparum* resistance to chloroquine has prompted research into antimalarial 4-Aminoquinolines, empirically used up to now. The mechanism of action of 4-Aminoquinolines is characterized by the concentration of the drug in the digestive vacuole of the intraerythrocytic parasite. Various hypotheses have been advanced to explain the specificity of action on the parasite; the most recent one is the inhibition of the haem polymerase of the parasite, leading to the accumulation of soluble haem toxin for the parasite. Chloroquine-resistant parasites accumulate the drug to a lesser extent than do sensitive parasites (Pussard and Verdier, 1994).

The 4-Aminoquinolines are easily synthesized, are also cheap and are generally well tolerated by patients. These compounds are active against intra-erythrocytic stages of parasites (Roberts *et al.* 2001). The 4-Aminoquinolines are able to accumulate to high concentration in the acid food vacuole of *Plasmodium* to kill the parasite (O Neill *et al.* 1998).

1.3.2.1 Chloroquine (CQ)

Chloroquine was introduced in 1944-1945 and became the mainstay of therapy and prevention. It is a cheap drug that is non-toxic to malaria patients and has been found to be active against all strains of malaria parasites. CQ (**7**) is still the cheapest and safest of the malaria drugs, but different degrees of resistance, from slight to total, have arisen throughout the tropics since its trumpeted introduction in the late 1940s. CQ (**7**) resistance was first observed in South East Asia and South America at the end of the 1950s and in Africa in the late 1970s (Roberts *et al.* 2001). It still works in China, and in Central America and North Africa. In the 1950s, in combination with DDT against mosquitoes, it was expected to help eliminate malaria from the world — but resistance set in, first in South-East Asia in 1957, and then in South America in 1959. Moving from East to West, it covered tropical Africa between 1978 and 1985. Other drugs, such as mefloquine, halofantrine, pyrimethamine and more recently artemisinin derivatives are effective but so far they are more expensive than chloroquine, and difficult for the poorest countries to afford. Yet malaria is said to kill 700,000 under-fives each year in Africa alone (Roberts *et al.* 2001)

1.3.2.2 Amodiaquine (8)

Amodiaquine is chemically related to CQ (**7**) but more effective than CQ (**7**) for clearing parasitemia in cases of uncomplicated malaria even against some CQ resistant strains (Ringwald *et al.* 1996, O'Neill *et al.* 1998). The parasite resistance to the drug and potential hepatic toxicity limits its use (Roberts *et al.* 2001).

1.3.3 Quinoline-methanols

1.3.3.1 Quinine (9)

Quinine (**9**) is the active ingredient of cinchona bark, which was introduced in Europe from South America in the 17th century, it had the longest period of effective use, but there is now a decrease of the clinical response of *P. falciparum* (Zalis *et al.* 1998). Nevertheless it remains an essential antimalarial drug for severe *P. falciparum* where the intravenous infusion is the preferred route of administration; however resistance has been reported (Roberts et *al.* 2001).

1.3.3.2 Mefloquine (10)

Mefloquine is structurally related to quinine but with a longer half life (14-21days) and probably this has contributed to the rapid development of resistance (Foley *et al.* 1998). The drug has been used in combination with other drugs like chloroquine, quinine and halofantrine (www.drugdigest.org). It binds with the high affinity to membranes, causes morphological changes in the food vacuole of *plasmodium*, and interacts relatively weakly with free heme (Roberts *et al.* 2001).

1.3.4 Other aryl alcohols

1.3.4.1 Halofantrine (11)

Halofantrine (**11**) is effective against CQ (**7**) resistant malaria (ter Kuile *et al.* 1993), but cardiotoxicity limits its use as a therapeutic agent (Nosten *et al.* 1993). It is an expensive drug without parenteral formulation (Roberts *et al.* 2001).

1.3.4.2 Pyronaridine (12)

Pyronaridine is an acridine derivative and a synthetic drug widely used in China for multi resistant falciparum malaria (Elueze *et al.* 1996, Ringwald *et al.* 1999). The current Chinese oral formulation is reported to be effective, well tolerated by malaria patients but its oral bioavailability is low and this leads to a high cost of treatment (Roberts *et al.* 2001).

1.3.4.3 Benflumetol (lumefantrine) (13)

Benflumetol (lumefantrine) is similar to quinine, mefloquine, and halofantrine. Biochemical studies suggest that its antimalarial effect involves lysosomal trapping of the drug in the intra-erythrocytic parasite, followed by binding to toxic haemin that is produced in the course of haemoglobin digestion. This binding prevents the polymerization of haemin to non-toxic malaria pigment (Roberts *et al.* 2001). A combination of artemether lumefantrine can be used for the treatment of uncomplicated infections with *P. falciparum* including strains from multidrug resistant areas. The combination has a long half-life of 88 hours in healthy subjects and twice as long in malaria patients (Roberts *et al.* 2001, WHO, 2001).

1.3.5 Folate antagonists

Folate antagonists include pyrimethamine (**14**), cycloguanil (**16**), proguanil (**17**) chlorproguanil (**18**), sulfadoxine (**15**), dapsone (**19**). These compounds inhibit the synthesis of parasitic pyrimidines and thus of parasitic DNA. There are two groups of antilfolates, Dihydrofolate reductase (DHFR) inhibitors e.g pyrimethamine (**14**), proguanil (**17**) and Dihydropteroate synthase (DHPS) inhibitors that are sulfones and sulphonamides like sulphadoxine(**15**) and dapsone(**19**) respectively. Sulfa drugs (sulfones and sulfonamides) were first used in the 1930s for malaria treatment. Over the next decade, numerous clinical studies indicated that these

drugs were usually effective against *P. falciparum* malaria but much less so against *P. vivax* malaria (Roberts *et al.* 2001).

A drug of the first group is usually used in combination with a drug of the second one, for example pyrimethamine (**14**) is combined with sulfadoxine (**15**) (sp), forming the commonly known drug Fansidar and is a widely used combination. It is effective for the treatment of *P. falciparum* and less effective against *P. vivax*, probably due to the innate refractoriness of parasites to the sulfadoxine component (Roberts *et al.* 2001, Korsinczky *et al.* 2004).

Fansidar is easy to administer since only one dose is needed due to its slow elimination from the body. Currently it is being used in many parts of Africa. However it is poorly active against highly chloroquine –resistant strains (Korsinczky *et al.* 2004). Fansidar (Sp) is also prone to rapid emergence of resistance (Roberts *et al.* 2001).

1.3.6 Artemisinin derivatives

Artemisinin derivatives are the fastest active antimalarials (Meshnick *et al.* 1996) and four compounds have been used i.e.artemisinin from *A. annua* and three derivatives from artemisinin (**1**) itself. Water-soluble hemmisucinate - artesunate and oil soluble ethers artemether and arteether (Roberts *et al.* 2001). Artemisinin(**1**) is converted primarily into inactive metabolites, such as deoxyartemisinin and dihydroxydeoxyartemisinin (Meshnick *et al.* 1996). Artesunate acts as a prodrug, with fast transformation into dihydroartemisinin which probably accounts for its antimalarial effect (Lee and Hufford, 1990). Both artemether and artetheer are converted into a number of different metabolites with retained antimalarial

activity (*Meshnick et al.* 1996). Chemically artemisinin is a sesquiterpene trioxane lactose containing a peroxide bridge which is essential for its activity. The lactone can be easily reduced, resulting in the formation of dihydroartemisinin, which has even more antimalarial activity *in vitro* than artemisinin itself (van Agtmael *et al.1999*).

In vitro testing of drugs on malaria parasites have been used to determine their effect on *Plasmodium* with the note that *P. falciparum* strains resistant to different drugs like chloroquine, mefloquine, are susceptible to artemisinin (**1**) and its derivatives in comparatively low doses (Balint, 2001).

It is worth mentioning that artemisinin (**1**) passes the blood brain and the blood placenta barrier (de Vries and Dien, 1996). For the management of uncomplicated malaria and severe malaria in pregnancy artemisinin (**1**) and its derivatives can be used in second and third trimester, but their use is not recommended in the first trimester (Balint, 2001). Artemisinin(**1**) and its derivatives are safe and well tolerated antimalarial drugs. They should be administered with combination with another effective blood schizontide to reduce recrudescence and to slow the development of resistance. At present the drug of choice for the combined therapy is mefloquine in the dose range of 12-25 mg base/kg (WHO/MAL, 1998), however the combined regimens of mefloquine plus an artemisinin derivative are associated with more side effects than those with an artemisinin derivative alone (Price *et al.* 1999).

Using drugs with short half lives such as artesunate means that if used together with other rapidly eliminated drugs they need to be taken for a longer period resulting in poorer patient

adherence and less likelihood of cure compared with drugs with longer half lives such as mefloquine or fansidar, which can be taken over a three –day period or in a single dose. Artemisinin based combination therapy (ACTS) only require three days of treatment if taken in combination with another effective drug (Shunmay *et al.*2004) although there are neurotoxic and cardiotoxic effects of artemisinin drugs artemether and arteether in experimental animals, the probability of adverse effects in humans is minimal (Balint, 2001). Mutagenicity studies in animals revealed that artemisinin(**1**) is not mutagenic (China Cooperative Research Group, 1982).

1.4 Drug resistance

Drug resistance can be defined as a natural response to the selective pressure of the drug (http://www.who.int/drugresistance/en/). The emergence of antimalarial drug resistance is dependent on the occurrence of a spontaneous genetic change (mutation or gene amplification) in a malaria parasite, which interferes with that parasite's susceptibility to a drug. A single mutation may be sufficient to confer almost complete resistance to some drugs or more usually there is a series of mutations that confer increasing tolerance of the parasite to increasing drug concentrations, as in the case of pyrimethamine and chloroquine (Wellems and Plowe, 2001).

1.5 Hypothesis

The activity of *A. annua* phytochemicals against *in-vitro* cultures of *P. falciparum* is due to a blend of constituents (additive or synergistic).

1.6 Project justification

Plasmodium falciparum resistance to available antimalarial drugs has become widespread allover the world, hence there is need for new drugs that could eliminate the parasite. Artemisinin from *Artemisia annua* and artemisinin derived drugs have been worked on extensively. The other phytochemicals in this medicinal plant have been left out. These certainly warrant investigation of the other phytochemicals in Artemisia annua and determine their antiplasmodial effect. There was need to determine if there was additive effect on the phtytochemicals.

1.7 Objectives

1.7.1 General objective

To determine if the activity of *A. annua* on *in-vitro* cultures of *P. falciparum* is due to a single component or to additive /synergistic effect of the plant constituents.

1.7.2 Specific objectives

1. To test the crude extract of *A. annua* against *in-vitro* cultures of *P.falciparum.*
2. To test the effect of the fractions on *P. falciparum* and also blends of these fractions to determine if there is any additive or synergistic effect.

Literature Review

2.1 Malaria and its control

Malaria has been known since time immemorial, but the true causes were not understood. Previously it was thought mal-aria (bad air or gas from swamps) caused the disease (WHO, 2003). It is the most important insect-transmitted human disease, but progress in its control has been slow especially in Africa. Several factors have contributed to the problem. These include parasites developing resistance to anti-malarial drugs and vectors developing resistance to insecticides (Collins and Pasketwitz, 1995). In view of this some ancient treatments were remarkably effective. An infusion of qinghao (*Artemisia annua*) has been used for at least the last 2000 years in China, its active ingredient artemisinin (1) having only recently been scientifically identified (WHO, 2003).

The disease is diagnosed by clinical symptoms, microscopic examination of the blood and established by finding of parasites in blood (Kamunyi *et al.* 1980). It can normally be cured by antimalarial drugs .The symptoms fever, shivering, pain in the joints and headache, quickly disappear once the parasite is killed (WHO, 2003). In certain regions, however, chemotherapy is failing due to the fact that the parasites have developed resistance to certain antimalarial drugs particularly chloroquine (Greenwood, 1984).

2.2 Control methods

Efforts to control malaria have been largely directed at controlling the vector and at developing effective preventive and therapeutic drugs. These efforts and the hopes of malaria control and

eradication are seriously challenged by the sad reality of 1.5-2.7 million lives that succumb to malaria worldwide each year (Abdulrahman and Husn, 1997)

2.2.1 Chemophylaxis and chemotherapy

Chemophylaxis in malaria refers to use of anti-malarial drugs prior to tissue or blood infection with the an aim of preventing either the infection or its clinical manifestation .It is recommended for high risk groups, who include children under five years and pregnant women (Greenwood, 1984). Countrywide chemophylaxis may enhance selection of resistant parasite strains and prevent development of natural immunity in spite of being expensive and demanding strong organization (Carnevale and Mouchet, 1987).

Chemotherapy on the other hand refers to the treatment of established infections after they have become manifest, whether in the form of clinical symptoms, parasitaemia or both (Greenwood, 1984). Chloroquines, 4-aminoquinolines, sulphadoxine pyrimethamine (SP) are currently in use (Roberts, 1970. Peters, 1970). Chloroquine has been the first line drug since 1940 due to its efficacy, quick onset of action and is cheap while having tolerable adverse effects. However, the emergence of chloroquine resistance and a worldwide scarcity of quinine, have led to the search for newer anti-malarial drugs (Vikas *et al.* 2000).

4-Aminoquinolines, mefloquine, quinine, halofantrine, artemisinin and its derivatives destroy the asexual blood forms (schizonts) of the parasite (WHO, 1995). Tetracyclines are active against tissue forms and blood forms of *P. falciparum* but has limited clinical application

because of their innate toxicity particularly in the foetus and young children as well as their suppressive effect on the normal bowel flora (WHO, 1995).

2.2.2 Malaria vaccine

Malaria vaccine is a new strategy that is being developed. Different types of vaccines being developed include pre-erythrocytic vaccine, asexual blood stage vaccines as well as transmission blocking vaccines (Renshaw and Silver. 2001, Sim *et al.* 1990). An example of a common vaccine is SPf66 that is a synthetic multicomponent peptide vaccine that acts by blocking the sporozoites from entering the liver and inhibit the liver stage development (Pattaroyo *et al.* 1997).

An eagerly awaited study involving 2,022 children in Mozambique, in East Africa, found the vaccine cut by one-third the likelihood of getting malaria and reduced by more than half the risk of developing serious, life-threatening cases of the disease. The vaccine, originally developed by the U.S. military, consists of a genetically engineered molecule that combines a key protein at one stage of the parasite's development with a protein that coats the hepatitis B virus. The combination stimulates a counterattack by the immune system (www.washingtonpost.com/wp-dyn/ articles/A33393-2004Oct14.html -).

Unlike live attenuated or killed whole organism vaccines, a DNA vaccine has the ability to induce immunity to more than one life stage of the parasite (Webster, 2003). Additionally, the DNA vaccine will contain several different genes that code for specific antigens which will induce a more diverse epitope-specific T cell response in the multiple states of parasitic

infection, while also generating a more long-lasting protective immunity (Prieur, 2004). However there have been difficulties in their development in that malaria parasite expresses different antigens at different stages of its life cycle, the parasite is also difficult to cultivate, expensive and time consuming animal model system is required (Renshaw and Silver, 2001).

2.2.3 Vector control methods

These are the methods applied to reduce mosquito population having an ultimate goal of reducing malaria incidences. Mosquitoes (Plate 1) are the vectors of *P. falciparum* There are about 422 species of *Anopheles* that have been described worldwide, of which, 30 species are the main vectors. In Africa, the main vectors are *Anopheles gambiae* complex and *Anopheles funestus* (Kettle, 1994).

2.2.3.1 Insecticides

Chemical insecticides remain fundamental tools in malaria vector control but are limited in their efficacy by various problems due to resistance build-up by insects as well as environment pollution (Majori *et al.* 1987). Larval control with chemical larvicides has been successful in some areas but in others insecticides have been effective against adult mosquitoes (Gratz and Pal, 1988). Some of the insecticides that have been widely used are chlorinated hydrocarbon insecticides like dichlorodiphenyltrichloroethane (DDT), organophosphates like diazinon, fenthion, as well as carbamates like carbaryl and propoxur (Palchick, 1963). Repellants refers to chemical substances that make plants or animals unattractive to their pests and disturb their sensory receptors or are sensed as alarm signals. Repellents may be mildly poisonous to the

target organism (Mafong and Kaplan, 1997) and have also been developed and proven effective (Palchick, 1963).

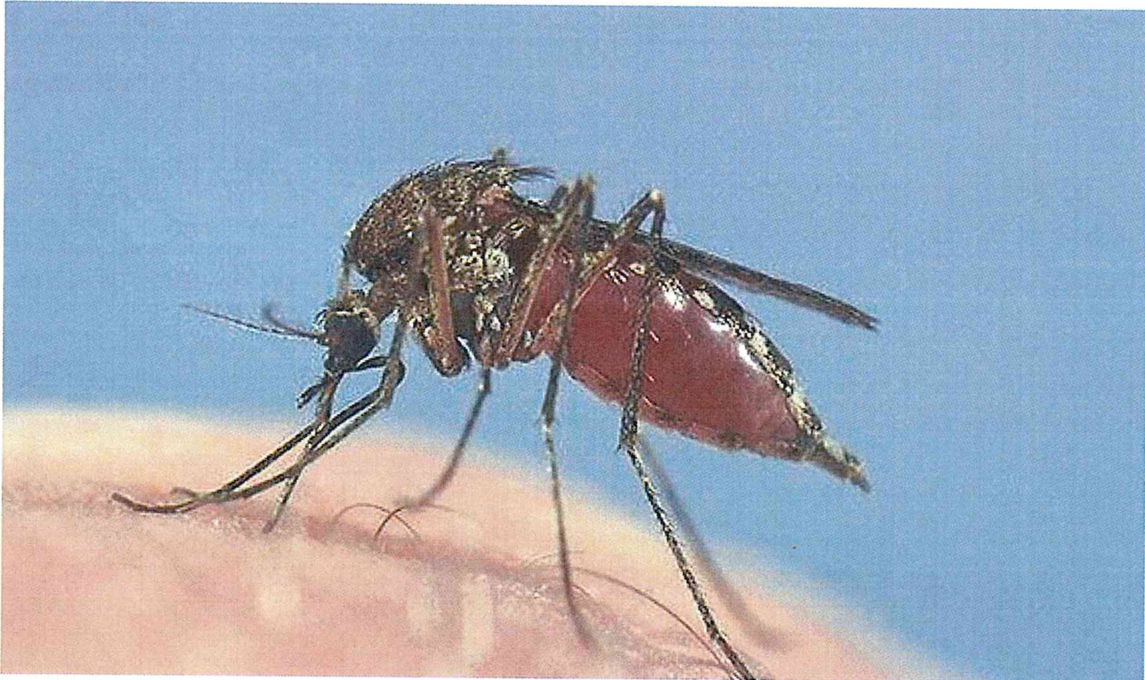


Plate 1: Anopheles mosquito feeding on a blood meal

http://www.mosquito-kill-net.com /more-mosquito-pictures.html

Systemic control of malaria started after the discovery of malaria parasite by Laveran in 1889 and the demonstration that the mosquito was the vector of malaria (WHO, 2003). These discoveries quickly led to control strategies and with the invention of dichlorodiphenyltrichloroethane (DDT) during the Second World War, the hope of global eradication was reached. This was finally abandoned in 1969 when it was recognized that this was unlikely ever to be achieved. According to Raymond *et al.* (1991) in some parts of the world, resistance to insecticides had evolved. Problems associated with insecticide spraying

for vector control include high cost of insecticide, development of insect resistance in vector, and difficulties in maintaining an appropriate infrastructure (Curtis, 1994).

2.2.3.2 Biological control

Biological control involves the use of a living organism to control the mosquito e.g. insect pathogens and predator fish. Certain spiders and other insects like emesine bugs play a role in controlling mosquito numbers in the house (White *et al.* 1972). Use of insect pathogens has been recorded (Federici, 1985). Toxins produced by bacterium *Bacillus sphaericus* and *Bacillus thuringensis israelensis (Bti)* have also shown promising results in larval control (Gratz and Pal, 1988). *Bacillus thuringensis* is more active than B*acillus sphearicus* against malaria vectors (WHO, 1995). Unfortunately the *Bacillus* toxins have no residual activity so require frequent application, or suitable for environment where a one-time control measure produces a valuable outcome (Collins and Paskenitz, 1995).

2.2.3.3 Insect growth regulators

Insect growth regulators (IGRs) or growth inhibitors are products or materials that interrupt or inhibit the life cycle of a pest. They are safe insecticides, active at low concentration and remain effective for 2-20 weeks depending on the compound used, mosquito species and nature of breeding site (Davidson and Becker, 1963). They affect the hormonal control of mosquito growth and development. The main effect of IGRs is the inhibition of adult emergence, but reproduction and ecdysteroid production in surviving females are also affected (Fournet *et al.* 1993, Fournet *et al.* 1995). In general IGRs have high levels of activity and efficacy against various species of mosquito in a variety of habitats (Mulla *et al.*1989).

Examples are Methoprene (trade name ALTOSID) with an analog of juvenile hormone (Jeffrey, 1996), Pyriproxyfen that affects the physiology of morphogenesis, reproduction and embryogenesis of insects (Dong-Kyu, 2001, Kawada *et al.* 1998). Plants have also been used in the control of the mosquito like Ocimum species (Sing et al 2003), neem plant (*Azadirachta indica*) (Van der Nat et al. 1991, Shumutterer 1995) and *Lantana camara(* Seyoum et al. 2002).

2.2.3.4 Genetic control

According to Rai (1963) genetic control can be applied and involves any hereditary manipulation to suppress population. Some of these genetic control mechanisms include sterile insect technique (SIT), hybrid sterility, cytoplasmic incompatibility and competitive displacement. The Sterile Insect Technique (SIT) is the first insect pest control method that uses genetics. The SIT involves mass breeding of huge quantities of target insects in a "factory" and sterilizing the males by exposing them to low doses of radiation. These sterile male flies are then released by air over infested areas, where they mate with wild females. If the sterile males vastly outnumber the fertile wild males, the wild fly population quickly dies out. The proportion of infertile males to fertile wild males must be at least 10:1. Chemical sterilants may also be used in sterilizing insect males (FAO, 1998).

2.2.3.5 Fish

Fish have been used for many years to control mosquitoes particularly malaria vectors and the best known species is *Gambusia affinis* which has been of use in California. Others are *Poecilia reticulata* and grass carp (*Ctenopharyngodon idellus*) (Marshall, 1981). Some of the fish species feed on mosquito larvae and also acts as a food source (Kathleen, 2002).

Predatory fish that eat mosquito larvae have been used for mosquito control for at least a century. In both Africa and Asia, native fish species have been identified that may act as biological control agents. The introduction of exotic larvivorous fish species can also have negative consequences upon local ecosystems (WHO, 2004).

2.2.3.6 Personal protection

People need to be encouraged to protect themselves against biting mosquitoes to stop the spread of malaria. Several approaches applicable include use of diethyltoluamide (DEET), using repellant creams and soaps, burning insecticide coils or local leaves and herbs, as well as use of physical barriers like ITNs, curtains in houses, screening doors and windows and also by wearing long sleeves shirts and long trousers to cover most parts of the body (Renshaw and Silver, 2001, Oketch 2004).

2.2.3.7 Use of bed nets

Initially people were unaware of the origin of malaria but protective measures against the mosquito have been used for many hundreds of years. Inhabitants of swampy regions of Egypt were recorded as sleeping in tower like structures out of reach of mosquitoes whereas others slept under nets as early as 450 B .C (WHO, 2003). Mosquito nets provide a barrier against attacks of indoor night biting mosquitoes especially the malaria vector *Anopheles* (Renshaw and Silver, 2001).

Insecticide treated nets, (ITNS). This involves the treatment of bed nets with a pyrethroid insecticide (Deltamethrin, Permethrin, Cyfluthrin, Bifenthrin), which repels and kills mosquito that come into contact with it. The insecticide prevents the mosquito from biting (Quinones *et al.* 1998. USAID, 1999). ITNs and curtains have proven in large-scale trials to have a high efficiency in reducing mortality and morbidity from malaria in African children and they have been associated with reduction in all-cause child mortality of 33 % in Kenya. The advantages of ITNs include improved personal protection, rational use of insecticides, community and household effect, and high efficacy (USAID, 1999).

2.2.4 Integrated strategy

Other efforts to control human malaria rely on the control of mosquitoes and may involve breeding site destruction and coating marshes with paraffin to block anopheles mosquito larvae spiracles (WHO, 2002). An integrated approach to malaria control is required. No single method is likely to be completely effective. In Africa new methods need to be developed to complement the existing ones (Mathenge, 1999). There is need to come up with a natural extract that is environmentally friendly, cheap, easily available and effective against resistant species of *plasmodium* (Hirt, 2000). It is also necessary to determine the efficacy and safety of locally used preparations as well as to investigate their potential to provide novel compounds as leads to new antimalarial agents. Lacy and Orr (1994) states that environmental methods and biological control are alternatives to chemical control and are key components of the integrated strategy.

Malaria thus has social consequences and is a heavy burden on economic development. The significance of malaria as a health problem is increasing in many parts of the world (WHO, 2003). Epidemics are even occurring around traditionally endemic zones in areas where transmission had been eliminated. These outbreaks are generally associated with social and economic conditions, and main victims are underprivileged rural populations (WHO, 2003). Demographic economic and political pressures compel entire populations to leave malaria free areas and move into endemic zones (WHO, 2003).

In many areas conflict, economic crises, and administrative disorganization can result in the disruption of health services. The absence of adequate health services frequently results in recourse to self-administration of drugs often with incomplete treatment. This is a major factor in the increase in resistance of the parasite to previously effective drugs. Inadequate health structures and poor socio-economic conditions aggravate the problems of controlling malaria. The situation has become even more complex over the last few years with the increase in parasite resistance to the antimalarial drugs. Recent research in the Peoples Republic of China with traditional herbal medicine has brought attention to *A. annua* that shows promise as an antimalarial agent (Klayman, 1985).

2.3 The genus *Artemisia*

The genus *Artemisia* is a member of the family Asteracae and comprises of more than three hundred species of annual, biennial and perennial herbs. Species included here are *Artemisia absinthium* (bitter wormwood), *A. dracunculus* (tarragon), *A. annua* (sweet annie), A. *vulgaris* (the mugwort), *A. aponticum* (the roman wormwood) (Lee, 2002). *A.absinthium* was used to

eliminate intestinal worms and as an insect repellant. *A.dracunculus* is used both as a flavoring in cooking and for adding taste to vinegar. Mugworts has been featured to be effective to all women ailments (Lee, 2002).

A. annua is also known as sweet annie or annual wormwood (Plate 2) in the United States whereas in China it is referred to as qinghao and is an annual herb native to Asia (Klayman, 1989). It has become naturalized in many countries including Argentina, Bulgaria, France, Hungary, Italy, Spain, Romania and former Yugoslavia (Bailey and Bailey, 1976, Klayman, 1989, Klayman, 1993). It is a vigorous weedy annual (Hall and Clements, 1923), short day plant with a critical photoperiod of 13.5 hours (Ferreira *et al.* 1995), and the chromosome number is 2n =36 (Benn *et al.*1982). The plant is usually single stemmed reaching about 2 metres in height with alternate branches and alternate deeply dissected aromatic leaves 2.5 to 5 centimeters in length. It bears tiny yellow flowers (Plate 3) (Benn *et al.* 1982).

Cooperative Group., (1982). However clinical trials have revealed that in cases where patients were treated with artemisinin malaria recurred sooner than when treatment was with chloroquine, despite complete disappearance of parasite from patients blood (Zhou, 1986). Dharam *et al.* (1996) cites that *A. annua* is among the top ten pharmaceutical crops that are receiving intensive world wide scientific attention and generating clinical interest. A*rtemisia* oil has also been found to have strong insecticidal action (Dharam *et al.*1996).

The main active principle, artemisinin (**1**) was isolated and had its structure correctly defined in 1972 in China as a sesquiterpene lactone with an endoperoxide bridge (Chen *et al.* 1991, Duke *et al.* 1987). Currently this class of antimalarials is available as the parent compound artemisinin (**1**) (oral, parenteral and suppository formulations) and as three semi-synthetic derivatives artesunate (**3**), artemether (**2**), and arteether (**4**) (Ramachandran, 2002). Artemisinin (**1**) is now available commercially in China and Vietnam as an antimalarial drug efficacious against drug resistant strains of *Plasmodium* (Duke *et al.* 1987, Chen *et al.* 1991). The white crystals of artemisinin (**1**) are, however, not soluble in water or oil and therefore, formulations of artemisinin (**1**), other than oral or rectal, are not used. Due to its poor solubility the drug absorption and its bioavailability are also poor (Ramachandran, 2002).

Artemisinin (**1**) also has phytotoxic activity and *A. annua* is a candidate as a natural herbicide. (Duke *et al.* 1987, Chen *et al.* 1991). Artemisinin (**1**), artemether (**2**), and artesunate (**3**) have been effective against the erythrocytic stages of two chloroquine resistant Hainan strains of *P. falciparum*, the malarial parasite, at lower minimum effective concentrations than chloroquine (Klayman, 1985). With *P. falciparum* developing resistance to chloroquine and

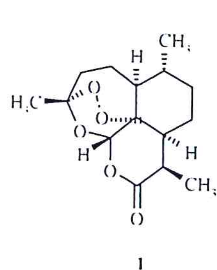
pyrimethamine/ sulfonamide (WHO, 1981), alternative treatments based on new compounds such as artemisinin (**1**) and its derivatives are actively being sought (Zhou, 1986, Xu *et al.*1986).

These antimalarial drugs have a short half-life in the human body and they are eliminated from the body rather quickly (1.6-2.6 hours) and so when used over short periods (less than five days) clearance of parasites from the blood is only temporary in up to 50 per cent of patients (Ramachandran, 2002).

Artemisinin (**1**) production by *A. annua* is usually in the range of 0.01% to 0.4% but some clones produce over 1% (Delabays *et al.* 1993). Essential oils of *Artemisia* can be quantified by various analytical procedures including thin layer chromatography, gas chromatography, high performance liquid chromatography (HPLC) with ultraviolet or electrochemical detection (Ferreira *et al.* 1994), radioimmunoassay, and enzyme-linked immunosorbant assay (Ferreira and Janick, 1996). Artemisinin (**1**) has been detected from aerial parts of the plant, mostly in leaves and inflorescences with low levels in stems and none in pollen or roots.

Analysis of artemisinin (**1**) is difficult as the compound is unstable, concentration of the compound in the plant is low, the intact molecule stains poorly, and other compounds in the crude plant interfere in its detection. A method to analyse artemisinin (**1**) in crude plant extract based upon high pressure liquid chromatography (HPLC) with reductive mode electrochemical detection was first developed by Acton (Acton *et al.* 1985) and later modified by Charles

(Charles *et al.* 1990). This latter method is highly sensitive, rapid, and of value in analyzing large numbers of samples (Charles *et al.* 1990).



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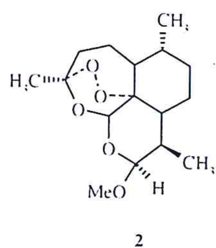

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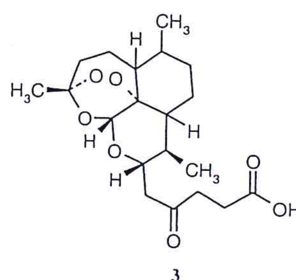

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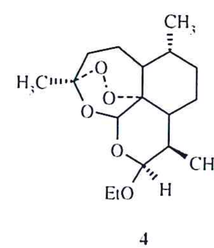

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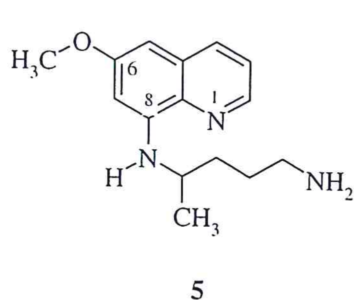

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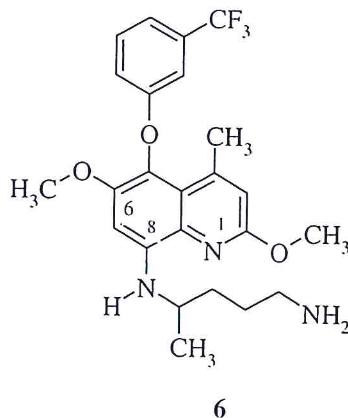

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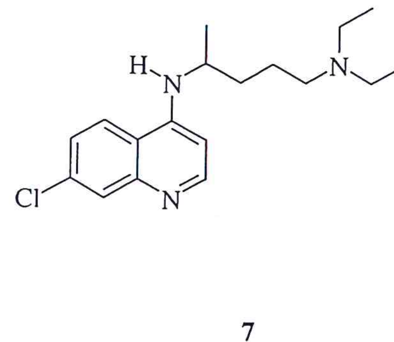

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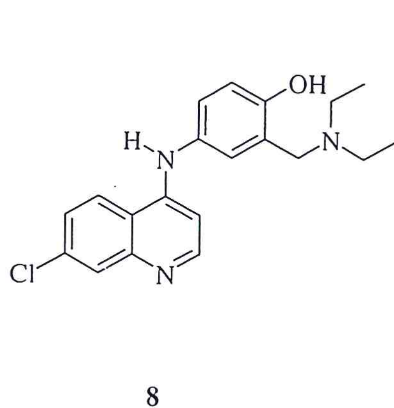

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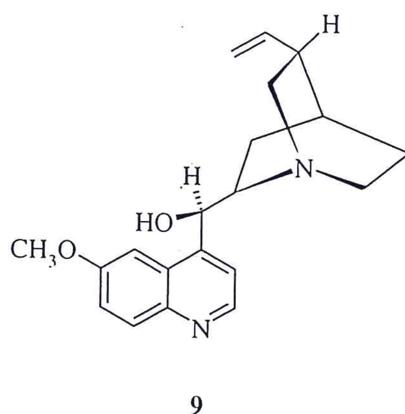

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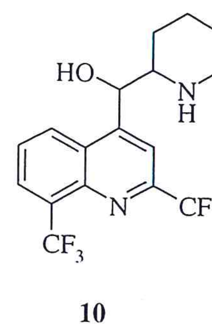

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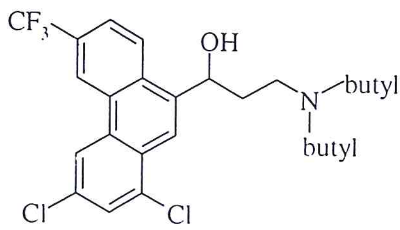

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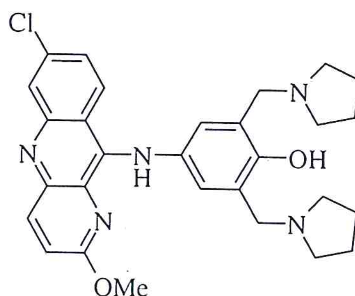

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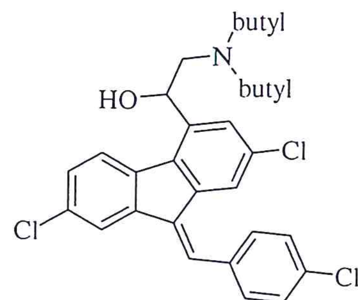

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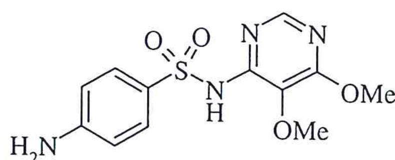

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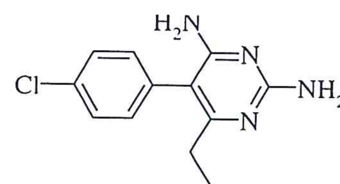

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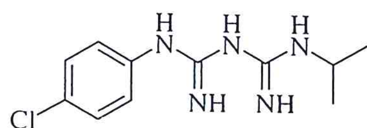

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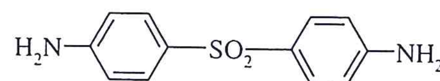

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Artemisinin (**1**) is now available commercially in China and Vietnam as an antimalarial drug efficacious against drug resistant strains of *Plasmodium* (Duke *et al.* 1987, Chen *et al.* 1991). A semisynthetic drug based on artemisinin (**1**), artemether (**2**), has been recently registered in Africa as Paluther. Essential oils of *A. annua* can be extracted via steam distillation and chemically characterize them by gas chromatography (GC). They comprise of α-pinene (0.032%), camphene (0.047%), β-pinene (0.882%), myrcene (3.8%), 1, 8-cineole (5.5 %), artemisia ketone (66.7%), camphor (0.6%), borneol (0.2%), and β-caryophyllene (1.2 %)

(Klayman, 2000). The essential oils contain at least 40 volatile compounds and several non-volatile sesquiterpenes (Charles *et al.* 1991).

2.4 Mode of action

The way in which the plant kills off the malaria parasite is complex and several different mechanisms are involved:

- Disruption of hemoglobin catabolism in the *Plasmodium* parasite.
- Damage to hemoglobin detoxification system of the parasite.
- Generation of free radicals from the sesquiterpene lactone which attack the membranes of the parasite and
- Alkylation of intracellular proteins in the parasite either by free radicals or by the hemoglobin –artemisinin complex (Lee, 2002).

The risk of toxic effects from the use of this plant appears limited, since *A. annua* is included in the pharmacopoeia of the Peoples Republic of China with recommendations of its dose and therapeutic use (Muller, 2000). The isolated ingredient artemisinin (**1**) seems to produce virtually no side effects but careless usage could lead to the development of resistance against *Plasmodium* species (Hans, 2000).

2.5 Polymerase chain reaction

The polymerase chain reaction, now widely used in research laboratories and medical institutions, relies on the ability of DNA-copying enzymes to remain stable at high temperatures. No problem for *Thermus aquaticus*, the sultry bacterium from Yellowstone that

now helps scientists produce millions of copies of a single DNA segment in a matter of hours. In nature, most organisms copy their DNA in the same way. The PCR mimics this process; only it does it in a test tube. When any cell divides, enzymes called polymerases make a copy of the entire DNA in each chromosome.

There are three basic steps in PCR. First, the target genetic material must be denatured-that is, the strands of its helix must be unwound and separated-by heating to 90-96°C. The second step is hybridization or annealing, in which the primers bind to their complementary bases on the now single-stranded DNA. The third is DNA synthesis by a polymerase. Starting from the primer, the polymerase can read a template strand and match it with complementary nucleotides very quickly. The result is two new helixes in place of the first, each composed of one of the original strands plus it's newly assembled complementary strand.

The four nucleotide bases, the building blocks of every piece of DNA, are represented by the letters A, C, G, and T, which stand for their chemical names: adenine, cytosine, guanine, and thymine. The A on one strand always pairs with the T on the other, whereas C always pairs with G. The two strands are said to be complementary to each other. To copy DNA, polymerase requires two other components: a supply of the four-nucleotide bases (dNTPS) and something called a primer. DNA polymerases, whether from humans, bacteria, or viruses, cannot copy a chain of DNA without a short sequence of nucleotides to "prime" the process, or get it started. So the cell has another enzyme called a primase that actually makes the first few nucleotides of the copy. This stretch of DNA is called a primer. Once the primer is made, the polymerase can take over making the rest of the new chain.

A PCR vial contains all the necessary components for DNA duplication: a piece of DNA, large quantities of the four nucleotides, large quantities of the primer sequence, and DNA polymerase. The polymerase is the Taq polymerase, named for *Thermus aquaticus*, from which it was cloned. The three parts of the polymerase chain reaction are carried out in the same vial, but at different temperatures. The first part of the process separates the two DNA chains in the double helix. This is done simply by heating the vial to 90-95 degrees centigrade (about 165 degrees Fahrenheit) for 30 seconds. But the primers cannot bind to the DNA strands at such a high temperature, so the vial is cooled to 55 degrees C. At this temperature, the primers bind or "anneal" to the ends of the DNA strands. This takes about 20 seconds.

The final step of the reaction is to make a complete copy of the templates. Since the Taq polymerase works best at around 75 degrees C (the temperature of the hot springs where the bacterium was discovered), the temperature of the vial is raised. The Taq polymerase begins adding nucleotides to the primer and eventually makes a complementary copy of the template. If the template contains an A nucleotide, the enzyme adds on a T nucleotide to the primer. If the template contains a G, it adds a C to the new chain, and so on to the end of the DNA strand. This completes one PCR cycle.

The three steps in the polymerase chain reaction - the separation of the strands, annealing the primer to the template, and the synthesis of new strands - take less than two minutes. Each is carried out in the same vial. At the end of a cycle, each piece of DNA in the vial has been duplicated. The cycle can be repeated 30 or more times. Each newly synthesized DNA piece

can act as a new template, so after 30 cycles, 1 billion copies of a single piece of DNA can be produced. Taking into account the time it takes to change the temperature of the reaction vial, 1 million copies can be ready in about three hours (Tabitha, 2004, http://users.ugent.be/~avierstr / principles/pcr.html).

To study the *Plasmodium falciparum* genotype there are two types of locithat can be used :those encoding highly polymorphic antigen genes and those encoding microsatellites.the antigenic loci are generally more polymorphic than the microsatellite lociand are more likely to detect differences between the parasites

Amplification of DNA from *P. falciparum* using primers to a highly polymorphic antigen loci merozoite specific protein 1 (MSP1), merozoite specific protein 2 (MSP2), and the glutamate-rich protein GLURP was done. The loci MSP1, MSP2, and GLURP were single copy genes on chromosome 9, 2 and 10 respectively. MSP-1 assists the merozoites in binding to the erythrocytes before invasion (Kakkilaya, 2004). Specifically MSP-119, the C-terminal fragment of this protein shows a promising future as an effective vaccine antigen. MSP-1 as a whole is expressed on the surface of the parasite, and before the parasite enters the erythrocyte most of MSP complex is shed, and what remains is the MSP-119 portion (Morgan, 2004).

The proteins have regions of tandemly repeated amino acids which vary in size in different variants of the protein. At the genetic level this is seen as differently sized PCR products on agarose gel (Ranfold-Cartwright.*et al* 1997).

2.6 Malaria diagnosis

Despite development of serological techniques, conclusive diagnosis of malaria continues to be made through microscopic examination of peripheral blood smears. This is the only method that can differentiate among the four species of *Plasmodium* that cause human malaria. A blood smear is spotted, fixed in thin smear preparation and then stained using Giemsa stain. In thick smear preparation there is no fixing and washing haemolyses the Red blood cells, leukocytes and any malaria parasites present are the detectable elements. The hemolysis and slow drying that occur in thick smear preparation cause distortion of *Plasmodium* morphology, making differentiation of species difficult. Thick smears are used to detect infection, and estimate parasite concentration.

Thin smears are fixed with methanol, preventing haemolysis. Red blood cells are intact, and any Plasmodia present are less likely to be distorted, and remain within erythrocytes. Identification of specific species is usually done using thin smears after detection of parasites on the thick smears.

Materials and Methods

3.1 Plant material-identification and collection

Uppermost foliar portions (top 1/3 of growth maturity) of *Artemisia annua* were collected from Kenyatta University. The foliage was harvested at eight weeks, air dried for three weeks and then ground to powder form using a laboratory milling machine, and the powder gave a weight of 0.5 kg.

3.1.1 Extraction of *Artemisia* components

The ground plant material (0.5 KG) was placed in 1-litre flat-bottomed flask and sequential extraction followed where dichloromethane (DCM) was used first for three days week and then methanol for a similar period of time. The extract was then filtered using a funnel and filter paper. The solvents were removed *in vacuo* to obtain the dry crude extract of the leaves. The dried extract was emptied into a glass vial after dissolving in methanol. This was then air-dried and this gave a dark green gummy extract that had a dry weight of 40 grammes.

3.2 *P. falciparum*

In-vitro cultures of *P. falciparum* were obtained from malaria laboratories of Kenya Medical Research Institute (KEMRI) Nairobi, Kenya where there is a culture bank. Two different strains were used D6 (chloroquine sensitive strain from Sierra Leone) and W2 (chloroquine resistant strain from Indochina). Blood from blood group O+ was used to provide erythrocytes (Red blood cells) that served as host cells to the parasites. Sera was obtained from blood

groups A, B and O, pooled and utilized to maintain the cultures. The adult donors (between 20-40 years) volunteered, and signed a consent sheet before blood donation.

3.3 Maintenance of *in vitro* cultures

In vitro cultures of *P. falciparum* were grown continuously by a modification of the methods of Trager and Jensen (Trager and Jensen 1976) and Haynes (Haynes et *al* 1976). A 6% suspension of human type 0+ erythrocytes was prepared in culture medium that consisted of powdered RPMI 1640, 25mM HEPES (N-hydroxyethylpiperazine-N'-2-ethanolsulfonic acid), 25mM $NaHCO_3$ supplemented with 10% human serum (in acid citrate–dextrose (ACD) anticoagulant). Stock cultures were maintained in 5.0 milliliters of the 6% erythrocyte suspension in 25 ml tissue culture flasks. The flasks were flushed with a gas mixture consisting of 3 % CO_2, 5 % O_2 and 92 % N_2. The cultures were kept in an incubator at 37^0 C and culture media was changed daily and smears prepared after every 48 hours.

3.4 Bioassays

Initially the crude extract 22.5mg was dissolved in 10 ml of sterile distilled water (deionised and autoclaved). The water insoluble extracts were first dissolved in 50μl of Dimethylsulfoxide (DMSO) (solvent concentration <0.02%) (Elueze *et al,* 1996) to enhance solubility. The stock solution was sterilized by passing through a 0.45μm and 0.22 μm micro filters that are syringe adaptable and this was done in a sterile laminar flow hood. The prepared drugs were kept at 4 ^{0}C until use. The stock extract was tested on *in-vitro* cultures of *P. falciparum* and then the individual fractions were prepared the same way and tested on the

cultures. The fractions were then blend and tested on *in-vitro* cultures of *P. falciparum* to determine additive or synergistic effect.

3.5 Sample preparation and culture

Parasitized blood samples (from continous culture) were diluted with uninfected RBCS (blood group 0+) from 6% to 1.5% hematocrit. Parasitemia was diluted from 4% to 0.04) % parasitemia of the *P. falciparum* 80 % ring stage parasites, and with RPMI 1640 medium/ Hepes (with 10% human serum of pooled blood (A, B, & O). Thin and thick films of the cultures were prepared before setting the experiment.

The thin films were vital to verify initial parasitemia. If the parasitemia was 4 % and above the experiment was set and 200 µl of the resulting cell medium mixture was added to each well of the predosed plates. The plates were then incubated for 48 hrs at 37^0 C in a gas chamber with a gas mixture that consisted of 3 % CO_2, 5 % O_2 and 92 % N_2. After the 48 hours 25µl of culture medium containing ($G\text{-}^3H$) hypoxanthine was added to each well and the plate further incubated for 18 hours in a gas mixture of 3 % CO_2, 5 % O_2 and 92 % N_2.The parasites were harvested onto a filter mat using a semi-automated cell harvester. It is advisable to check the gas mixture 24 hours later after setting the experiment and if the gas is low refill the gas chamber with more gas.

The extract was tested on *P. falciparum* by using *in-vitro* semi-automated microdilution technique that measured the ability of the extracts to inhibit the incorporation of [$G^{-3}H$] hypoxanthine into malaria parasite. Using a cell harvester did cell harvesting and cell counts

per minute (cpm) were taken using a beta- cell counter machine. Positive and negative controls were included in the set up and these helped to determine the Y_{50}.

3.6 Pre dosing culture plates with the extract

One 96- well microtitre plate was used to test 4 drugs in triplicate (3 columns of 8 wells each, 1 control and 7 drug dilutions). The stock solution was filtered through 0.45μM and 0.22 μM filter and the stock was diluted to the maximum concentration. Fifty (50μl) of the diluted drug solution was added to well A using the titertek motorized hand diluter (flow laboratories UK) of sterile 96-well flat bottomed micro culture plates. Twenty-five microlitres (25μl) of complete RPMI 1640 medium /double distilled water was added to wells B to H.

Twenty five microlitres of the drug solution was transferred from well A to B, mixed well the drug with the water and transferred 25μl of this solution to well C. This was continued until reaching well G and discarded 25μl from this well (serial 2 fold dilutions) and left 25μl of fluid in each well. As a result well A contained the highest concentration (100μl), well G contained 1.5625 % of the maximum concentration.

Well H (control) contained only drug free medium/water. The plates were then stored at 4 ^{0} C until when the cultures were added. Culture media (25 μl aliquot) was added to all the wells of 96 well flat bottom micro culture plate (costar glass UK). Aliquots 25 μl of the test fractions were added in triplicate or duplicate to the first wells, and titertek motorized hand diluter (Flow laboratories UK) was used to make serial two fold dilutions of each sample. Aliquots (200μl) of 1.5% suspension of parasitized erythrocytes in culture medium 0.4% parasitemia were

added to all test wells. The plates were incubated at 37^0 C in a gas mixture 3% CO_2, 5% O_2, and 92% N_2. After 48 hours each well was added 25 μl of culture media containing 0.5μCi of [G-^{3} H] hypoxanthine and the plates incubated for a further 18 hours. After 60 hours from setting, removing the plates from the gas chamber and covering them with plate sealers stopped the experiment. The plates were frozen at −80 ^{0} C after which they were thawed and then harvested. The contents of each well were then harvested onto glass fiber filters, washed with distilled water, dried and the radioactivity counts per minute (cpm) measured by liquid scintillation.

	1	2	3	4	5	6	7	8	9	10	11	12
	D1	D1	D1	D2	D2	D2	D3	D3	D3	D4	D4	D4
A	250	250	250	250	250	250	250	250	250	250	250	250
B	125	125	125	125	125	125	125	125	125	125	125	125
C	62.5	62.5	62.5	62.5	62.5	62.5	62.5	62.5	62.5	62.5	62.5	62.5
D	31.25	31.25	31.25	31.25	31.25	31.25	31.25	31.25	31.25	31.25	31.25	31.25
E	15.62	15.62	15.62	15.62	15.62	15.62	15.62	15.62	15.62	15.62	15.62	15.62
F	7.81	7.81	7.81	7.81	7.81	7.81	7.81	7.81	7.81	7.81	7.81	7.81
G	3.91	3.91	3.91	3.91	3.91	3.91	3.91	3.91	3.91	3.91	3.91	3.91
H	Nd	Nd	Nd	Nd	Nd	Nd	Nd Np	Nd Np	Nd Np	Nd NP	Nd Np	Nd Np

Figure 1: Layout of drug dosed culture plate.

Nd represents no drug and Np represents no parasites. 96 wells arranged in 8 rows (A-H) and 12 columns (1-12) each extract is present in triplicate columns with the highest concentration in row A and in twofold dilutions to the lowest concentration in row G. Row H serves as a Parasite control (1-6) no drug present and (7-12) serves as erythrocyte control (no drug and no parasite. Concentration varied from highest in row A (250 µg/ml) to lowest in row G (3.91 µg/ml). Columns 1,2 and 3 had one drug designated as D1, D1, D1, columns 4,5,6 had the second drug represented as D2 and likewise for the other columns(Figure 1).

Parasites were grown to attain a parasitemia of between 3-4% and drugs were also prepared. The experiment was set up and gassed and 48 hours later hypoxanthine was added. After another 12 hours the experiment was stopped. Plates were frozen and then harvesting was done, the filters sealed after addition of a scintillant. The filters were then taken to a counting machine (Figure 2).

Computation of the concentration of drug causing 50 % inhibition of $[G^{-3} H]$ hypoxanthine uptake (IC_{50}) was carried out by interpolation after logarithmic transformation of both concentration and cpm values using the formula, IC_{50}=antilog (log X_1+[(log Y_{50}-log Y_1)(log X_2-log X_1)] / (log Y_2 –log Y_1), where Y_{50} is the cpm value midway between parasitized and non parasitized control cultures and X_1, Y_1, X_2 and Y_2 are the concentrations and cpm values for the data points above and below the cpm midpoints.

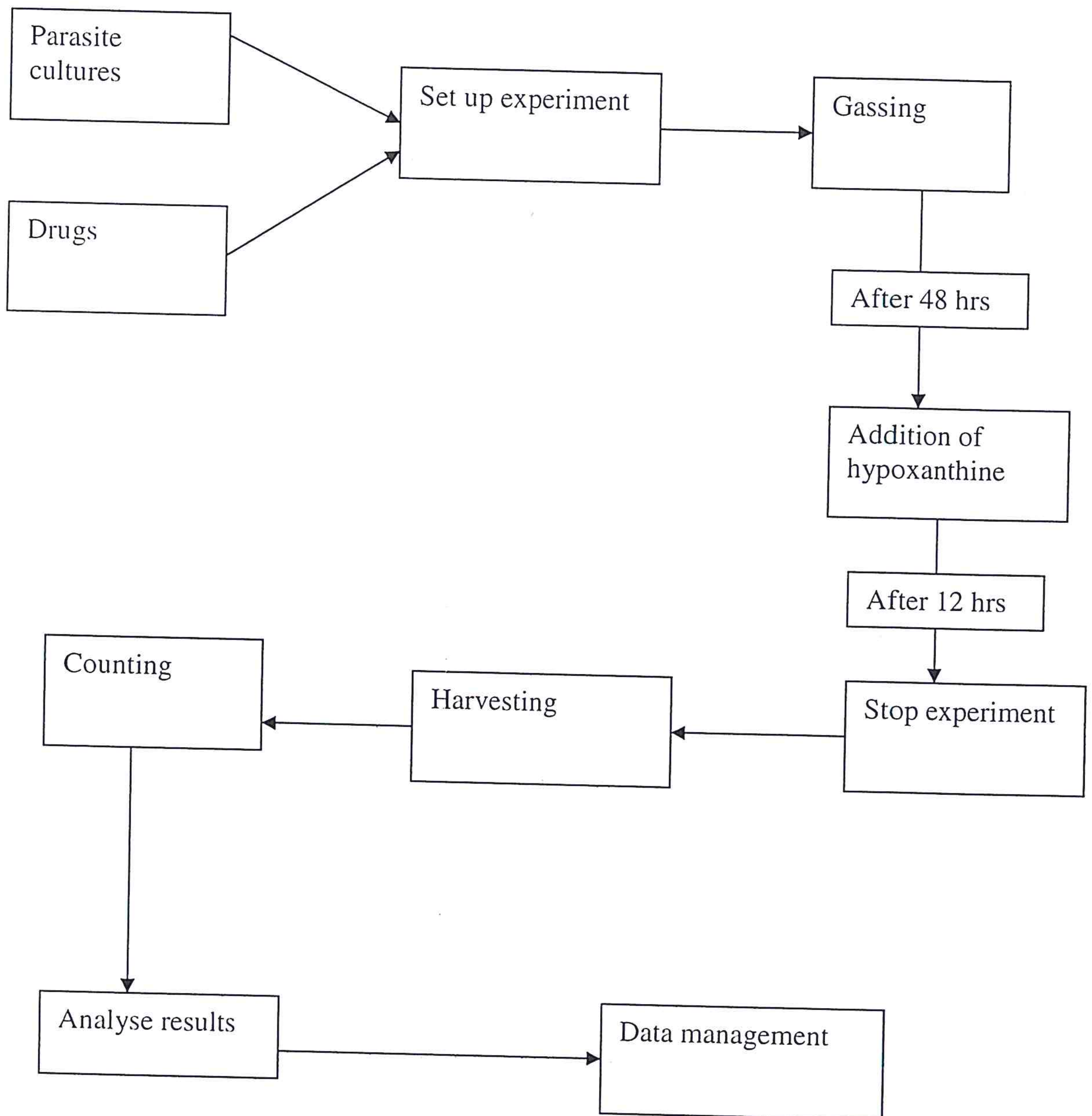

Figure 2: Experimental set up.

3.7 Fractionation

The crude extract was fractionated using high performance liquid chromatography (HPLC) with a 4.6×250mm (5μm) ODS Column 18 (C-18). The eluents were 50% water: 50% acetonitrile within 30 minutes. Flow elution was 3ml min-1 and 100 μl of samples was injected.

3.8 Fractions interactions (blending)

The initial concentrations of the nine fractions were combined in equal ratios, this was followed by combining eight of the fractions minus one compound. The combined fractions were then dispensed into 96 flat bottomed well microtitre plates to give duplicate rows. Incubation and subsequent procedures were followed as described earlier and the IC_{50} determined.

3.9 Parasitemia determination

A clean microscope slide free of any contaminants was taken and a pipette was used to apply a drop of blood to slide(s) for thick smear (step 1). The blood drop was spread with corner of another slide to make an area about 1 cm in diameter. Correct thickness was attained when newsprint was barely legible through the smear (Step 2). A new drop of blood (smaller than the first) was applied with a pipette to a clean slide (Step 3). The edge of the slide was brought with the new drop of blood to the surface of the first slide, placed it at the far end, and waited until the blood spread along the whole edge (Step 4). The slides blood films were prepared as above and allowed to dry before staining was done. For preparation of separate slides for thick

and thin smears, the second slide was used in step 4. Holding the slide at an angle of 45°, the slide was pushed forward with a rapid, gentle movement in step 5 (Figure 3 below).

The smears were air dried, allowing 10 minutes for the thin smear and 30 minutes for the thick smear. Marking of the slide was done using a pencil on the frosted end of the slide. After drying, only thin smears were fixed. Fixing was done using methanol in one of two ways: Thin smear was dipped into methanol for 5 seconds or by dabbing with methanol-soaked cotton ball. The thick smear was not fixed. In the one slide method, exposure of the thick smear to methanol or methanol fumes was prevented by carefully dipping or dabbing the slide, and gently blowing the fumes away from the thick smear area.

For the thick smear freshly prepared Giemsa stain was flooded on the blood film and allowed to stain for 30minutes. After this the slide was washed with tap water, allowed to dry and then mounted on microscope and observed under oil immersion.

The preparation of microscope slides every day was very essential as this enabled the determination of parasitemia of the cultures. The parasitemia in each culture flask had to be determined individually. The preparation also helped to detect any attack by bacteria or fungi. If these were detected in the slides the flask had to be disposed before the infection spread to other flasks.

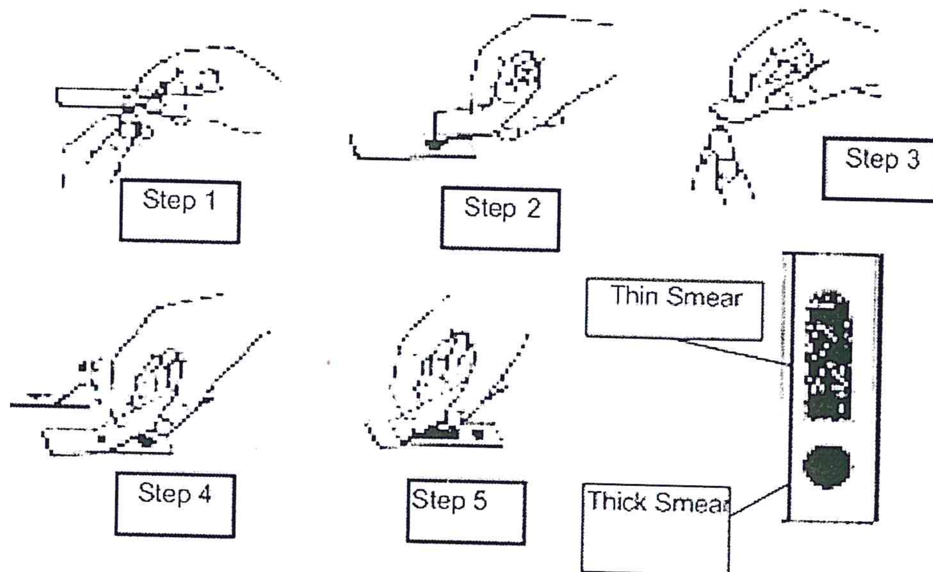

Figure 3: Thick and thin blood smear preparation. Adopted from Davidson, and Henry 1974, Wintrobe, 1974.

For the experiment to be set the parasitemia of 4% had to be achieved. The preferred stages for setting were the ring stages and 80 % of the parasites had to be in the ring stages; this however was not easy to achieve. The parasitemia was calculated by

Parasitemia = (parasitised erythrocytes×100)/ (RBCs per field) × (fields counted)

3.10 Polymerase chain reaction procedure

3.10.1 DNA extraction and amplification

Whatman filter papers 3 mm were blotted with parasitised blood (3%-4% parasitemia) and allowed to dry completely. The blotted section was cut using a razor blade. These were

combined with 1 ml of PBS (PH 7.4) and 50 μl of 10% saponin in 1.5 μl microfuge tubes. These were inverted several times and stored overnight. They were then microfuged at 1400 revolutions per minute (rpm) for five seconds. The reddish PBS saponin was aspirated from the tubes with a clean yellow tip attached to a Pasteur pipette at the end of a vacuum assembly. 1 ml of PBS was added to the tube, inverted several times and incubated at 4 ^{0} C for 2 hrs. Tubes were microfuged and as much fluid as possible was aspirated using the yellow tip and pressing down the filter paper into the lower 3rd of the tube. 100 μl of distilled water (DNase free water) was added to each tube. 50 μl of vortexed chelex (20%) was then added to the samples.

DNA was extracted by incubating the tubes for 10 minutes in 95^{0}C heat block, vigorously vortexing each sample every 2 min. After the incubation the tubes were microfuged for 5 min at 1400 rpm. Meanwhile 2 sets of 0.5 microfuge tubes were labeled for transfer of the second set for final storage of the extracted DNA samples. The solution from the spun tubes was transferred to the first set of microfuge tubes with a plugged 200 μl tip, spun the tubes for ten minutes then transferred the final white to yellowish supernatant avoiding the pelleted chelex to the final set of labeled tubes and stored at –20^{0} C.

3.10.2 Outer MSP1 PCR

Solution of mixed dNTPs were prepared with 100 mM dATP, 20μl, 100 mM dCTP, 20μl, 100 mM dTTP, 20μl, 100 mM dGTP, 20μl, DNase free water 20μl and stored at –20^{0} C and kept on ice when in use. 10 μM (50-100μl volumes) solutions of each of the two primers (01{: CACATGAAAGTTATCAAGAACTTGTC} /02{GTACGTCTAATTCATTTGCACG}) was

prepared separately using DNase free water and stored at -20^0 C. PCR tubes for primary amplification reactions were labeled.

Positive and negative controls were also added; DNA samples were thawed, spun briefly in a microfuge to pellet any chelex matrix and placed immediately in ice. PCR premix was prepared ensuring that there was enough, as each sample required 20 μl. The premix 20 μl was aliquoted into each labeled tube and recapped. 1-2μl of the appropriate DNA was added to each tube keeping tubes capped at all times when not in use. A molecular weight marker was also included and this was a mixture of fragments with known size to compare with the PCR fragments. Tubes were placed into the PCR machine and the programme was run. Primary denaturation was set at 94 ^{0}C for 3 minutes, denaturation was done at 94 ^{o}C for 25 seconds, annealing was done at 50 ^{o}C for 35 seconds initial extension was set at 68 ^{o}C for 2 minutes 30 seconds and finally the final extension at 72 ^{o}C for 3 minutes.

The outer PCR product was run on agarose gel. One and a half (1.5) % agarose gel in 1×TAE buffer was prepared and ethidium bromide was included in the gel. Eight μl of each amplification reaction was loaded onto the gel with 1 μl gel-loading buffer (containing bromophenol blue). This was run until the bromophenol blue dye front had migrated to within 1 cm of the end of the gel. It was visualized on UV transilluminator and photograph gel. The remaining PCR product was stored at –20 C.

3.10.3 Nested MSP1 PCR

Ten μM solutions (50-100 μl volumes) of each of the two primers (N1 {GCAGTATTG ACAGGTTATGG} /N2 {GATTGAAAGGTATTTGAC}) were prepared separately using DNase- free water, stored at –20^0 C and kept on ice when in use. 2 μl of the outer PCR was added as the template. PCR tubes for the primary amplification reactions were labeled, controls were added. One positive and two-negatives. A PCR premix was prepared on ice, and for each sample 20 μl of the premix was needed.

3.10.4 Polymerase chain reaction

PCR premix comprised of 10×PCR buffer, 20 mM mixed dNTPs, 10 μM N1 PRIMER, 10 μM N2 PRIMER, Taq DNA Polymerase, and Dnase free water. The premix 20μl was aliquoted into each of the labeled tubes. 2 μl of the appropriate outer PCR product was added to each tube and keeping the tubes capped at all times when not in use. The tubes were placed into the PCR machine and the programme was run. Primary denaturation was set at 94 ^{0} C for 3 minutes, denaturation at 94 ^{0} C for 25 seconds, annealing was set at 50 ^{0} C for 35 seconds, initial extension was set at 68 ^{0}C for 2 minutes 30 seconds and finally the final extension at 72 ^{0}C for 3 minutes.

One and a half (1.5) % Agarose gel in 1 ×TAE buffer was prepared. Ethidium bromide was included in the gel together with the running buffer. Eight (8) μl of each amplification reaction was loaded onto the gel with 1 μl-loading buffer (containing bromophenol blue). Molecular weight marker was also included and run at <5 v/cm until the bromophenol blue dye front had migrated to within 1 cm of the end of the gel. These were visualized on UV transilluminator and photograph gel. The remaining PCR product was stored at –20^0 C.

3.11 Data analysis

The data collected was analyzed by calculating the IC_{50} i.e. the drug concentration corresponding to 50% uptake of ^{3}H-hypoxanthine by the parasites in drug–free control wells. Computation of the concentration of drug causing 50 % inhibition of [G- ^{3} H] hypoxanthine uptake (IC_{50}) was carried out by interpolation after logarithmic transformation of both concentration and cpm values using the formula, IC_{50}=antilog (log X_1+[(log Y_{50}-log Y_1)(log X_2-log X_1)] / (log Y_2 –log Y_1), where Y_{50} is the cpm value midway between parasitised and non parasitised control cultures and X_1, Y_1, X_2 and Y_2 are the concentrations and cpm values for the data points above and below the cpm midpoints.

Results and Discussion

4.1 Compounds in *Artemisia annua*

After extraction a HPLC chromatograph was run and different compounds were present. Some of these were in very small quantities and exhibited very minute peaks. Others were in higher quantities as indicated by Figure 4. Some of the compounds were very polar and formed the bulk of the extracted compounds.

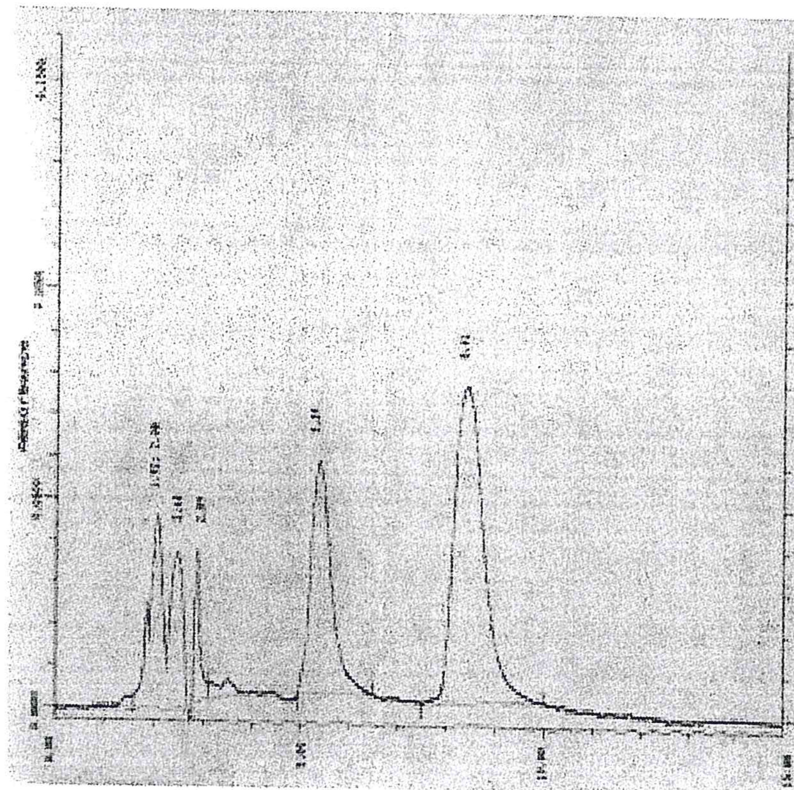

Figure 4: HPLC Chromatograph showing fractions present in *A. annua* extracted with dichloromethane and methanol.

The plant extract had both major and minor compounds that were present in variable amounts. The plant *A. annua* had thirteen compounds as exhibited by the Figure 4 above.

The polar compounds were eluted first followed by those with medium polarity and finally those that were non polar. The first fractions La, Lb, Lc and Ld were the first to be eluted hence more polar as compared to fractions Lh, Li, and Lj that were eluted lastly hence non polar.

4.2 Parasitemia

After culturing the parasites in some instances the parasitemia was very high 12 % (Plate 5), in such a case very many Red Blood Cells had already been infected. If this was happening in a patient the clinical symptoms would have been well exhibited. When the parasitemia was such high dilution was done before setting the experiment. *P. falciparum* have been known to have multiple infections this was when a single red blood cell was infected by more than one parasite (Plate 4). When determining the parasitemia even if a cell had 2 or 3 parasites this was counted as one infected cell. The parasite stage of development was also crucial. Plate 6 indicates the ring stage shown by the arrow (Plate 6).

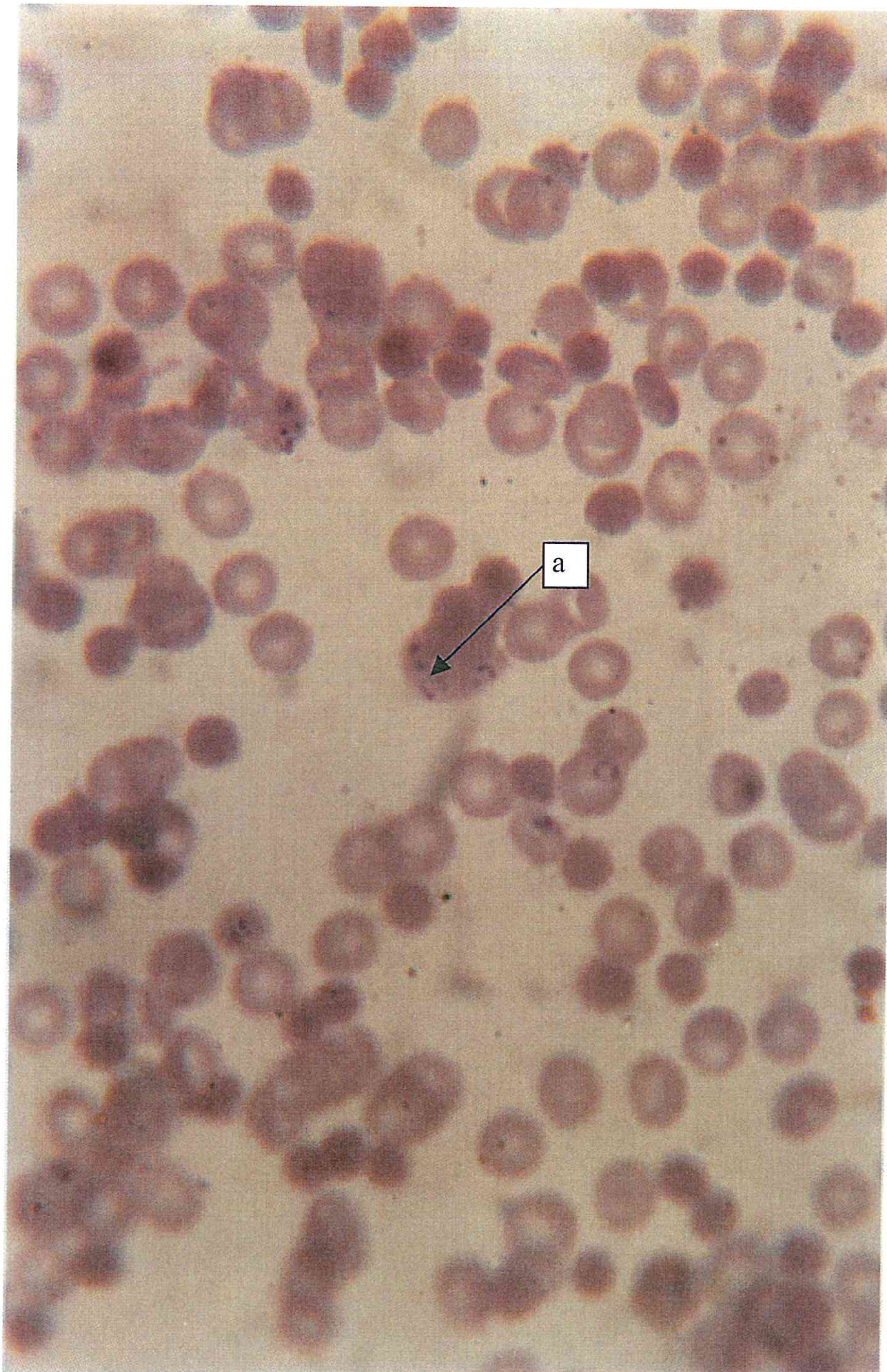

Plate 4: Slide photo showing multiple infections of *P. falciparum*.

'a' represents red blood cell infected by several *P. falciparum* parasites.

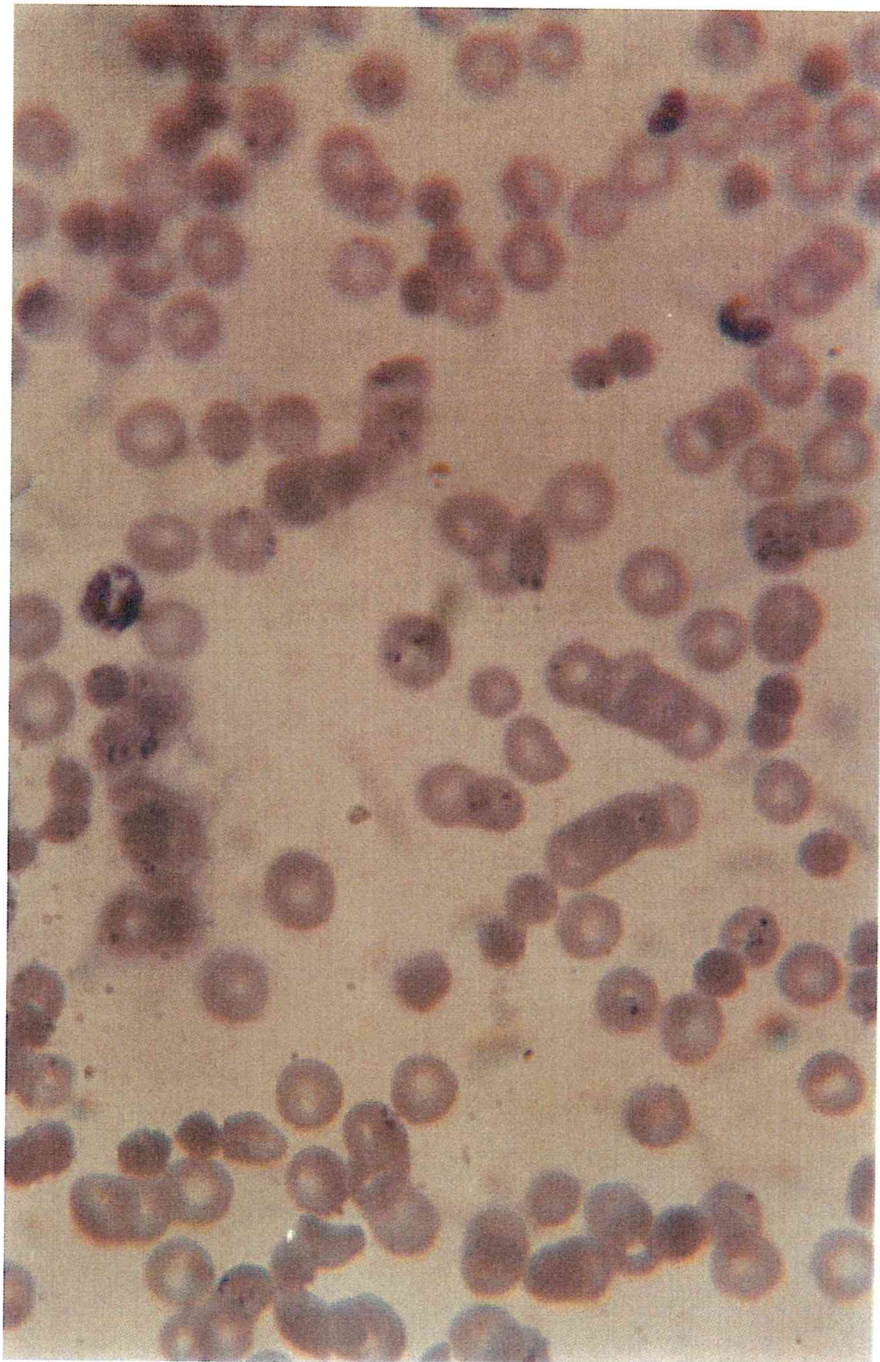

Plate 5: Slide photo showing very high parasitemia of *P. falciparum.*

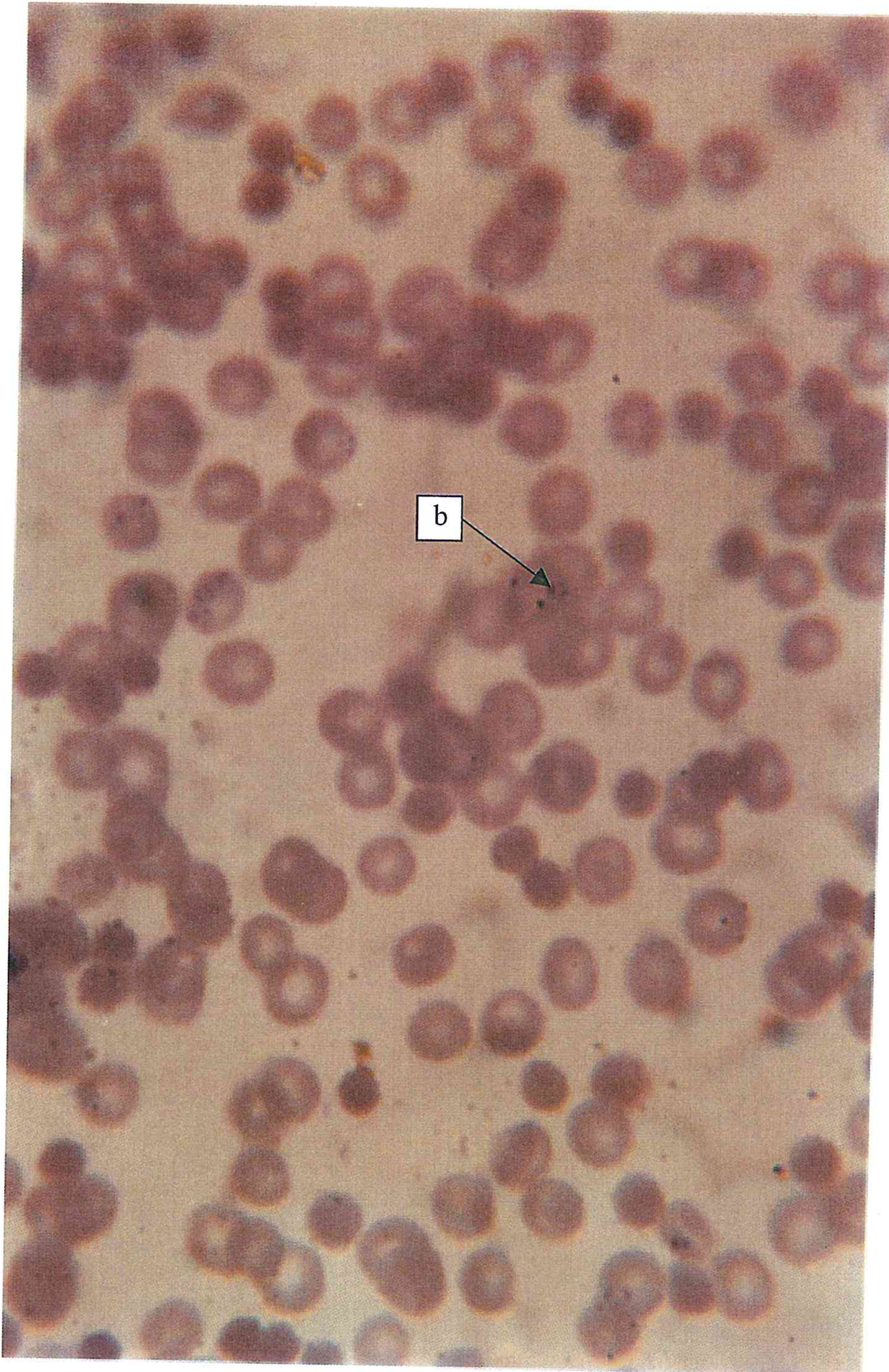

Plate 6: Slide photo showing ring stage of *P.falciparum*.

'b' represents a ring stage of the *P. falciparum*.

4.3 Data analysis

Table 1: IC_{50} for the fractions against D6 clone

Code on plate	IC_{50} D6	comments
La	4.77±1.49μg /ml	polar and active to parasites
Lb	14.763±1.75μg /ml	Polar and also active
Lc	>250μg /ml	No activity to parasites
Ld	>250μg /ml	No activity to parasites
Le	<3.9μg /ml	Active
Lf	6.95±4.34μg /ml	Active
Lh	<3.91μg /ml	Low polarity and active
Li	<3.91μg /ml	low polarity and active
Lj	<3.9μg /ml	active
Lg	10.40±0.50μg /ml	active

In the preliminary studies the fractions were screened against the clone D6 (a chloroquine (**7**) sensitive clone). Nine fractions (La-Lj) were used in the bioassay and the results were summarized in Table 1. Only two fractions (22.2%) showed an IC_{50} of above 250 μg /ml (Lc and Ld) and this showed that these fractions were not active to *in-vitro* cultures of *P. falciparum* against clone D6. 4 fractions (44.4%) (Le, Li, Lh, and Lj) showed an IC of below 3.9μg /ml meaning that these fractions had a very high activity against clone D6. These were assayed further with clone W2 (CQ) (**7**) resistant clone). Three fractions (33.3%) had an IC_{50} of between 4.7±1.49μg /ml and 14.763±1.75μg /ml, reflecting high activity. The crude extract gave a IC_{50} of 10.40±0.50μg /ml against D6, however the crude extract was not very soluble as

the material was very gummy, probably leading to a high IC_{50}. La had an activity of 4.77±1.49μg /ml, Lb had an activity of 14.763±1.75μg /ml, Lc had an activity of >250μg /ml, Ld had an activity of >250μg /ml, Le had an activity of <3.9μg /ml, Lf had an activity of 6.95±4.34μg /ml, Lh had an activity of <3.9μg /ml, Li had an activity of <3.9μg /ml and Lj <3.9μg /ml against clone D6.

Table 2: Comparison of the IC_{50} for D6 and the IC_{50} for W2

Code on plate	IC_{50} D6	IC_{50} W2	Comments
La	4.77±1.49	6.59±0.36	W2 required a higher concentration of the fraction than D6
Lh	<3.91	2.21±0.01	High activity of the fraction
Li	<3.91	13.13±0.20	In W2 a higher concentration was needed than in D6.

A few compounds were randomly picked and tried to determine the IC_{50} with two different clones i.e. D6 and W2 in order to compare if there was any difference between Chloroquine sensitive and Chloroquine resistant clones (Table 2), results showed that the CQ resistant clone (W2) required a higher concentration of the fraction than the CQ sensitive clone (D6). From the assays it was possible to conclude that the Chloroquine resistant clone W2 always required a higher dose of the drug when compared with the Chloroquine sensitive clone D6. Fraction La's activity was tested against the CQ resistant clone W2 which gave an IC_{50} of 6.59±0.36μg /ml this was compared with IC_{50} for D6 that was 4.77±1.49μg /ml; this was confirmatory in that a higher concentration of the drug was required for a CQ resistant clone.

The fractions with activity of less than 3.9 were further diluted and 10μg /ml were the start concentration.

Table 3: IC_{50} for the blends of the nine fractions

Drug	IC_{50} for D6 μg /ml	Comments
LNa	16.19±1.74μg /ml	active
LNb	9.19±0.31μg /ml	active
LNc	10.52±0.35μg /ml	active
LNd	10.12±0.70μg /ml	active
LNe	26.75±8.43μg /ml	active
LNg	10.40±0.50μg /ml	active
LNi	9.72±0.17μg /ml	active

The fractions were further blend and the following codes were used to represent the fractions used in the blending. In each case eight fractions were combined in equal ratio and the concentrations of the individual fractions were similar.

LNa Lb+Lc+Ld+Le+Lf+Lh+Li+Lj

LNb La+Lc+Ld+Le+Lf+Lh+Li+Lj

LNc La+Lb+Ld+Le+Lf+Lh+Li+Lj

LNd La+Lb+Lc+Le+Lf+Lh+Li+Lj

LNe La+Lb+Lc+Ld+Lf+Lh+Li+Lj

LNf La+Lb+Lc+Ld+Le+Lh+Li+Lj

LNg La+Lb+Lc+Ld+Le+Lf+Lh+Li+Lj

LNh La+Lb+Lc+Ld+Le+Lf+Li+Lj

LNi La+Lb+Lc+Ld+Le+Lf+Lh+Lj

LNj La+Lb+Lc+Ld+Le+Lf+Lh+Li

After the fractions were blended all the blends had an IC_{50} of less than 27µg /ml against D6 clone (Table 3). LNk had an activity of 7.95µg /ml against D6 clone. Two blends (LNb ,LNi) had an activity of 9.19±0.31µg /ml and 9.72±0.17µg /ml respectively against D6. Three blends LNc, LNd, LNg had activities of 10.52±0.35µg /ml, 10.12±0.70µg /ml and 10.40±0.50µg /ml respectively against D6 (Table 3). The blend LNa had an activity of 16.19±1.74µg /ml against D6 clone. The lowest activity of 26.75µg /ml against D6 was observed with the blend LNe ((Table 3). This occurred when one of the very active fractions (Le) was absent; this probably indicates the effect of the left out fraction which might be having a very high activity on *in vitro* cultures of *P. falciparum.*

Table 4: Blends of some fractions

Codes	W2	Comments
LNK	7.95±0.08µg /ml	Active
LNM	15.96±0.31µg /ml	Active
LNN	<3.9µg /ml	High activity

The codes above represented the following fractions as used in Table 4 above.

LNK LD+ LH+ LI+ LJ

LNM LD+LH+LI

LNN LH+LI+LJ

The blend LNK had high activity of 7.95±0.08μg /ml against W2 and LNM had antiplasmodial activity of 15.96±0.31μg /ml against W2 (Table4). This indicated additive effect on the activity of the fractions. One blend (LNN) had an activity of >3.9μg /ml against W2 clone; this indicated a very high activity that calls for further research (Table4).

Table 5: The IC_{50} for the blends of the most active fractions against D6 clone

Extract	IC50 for D6 clone	Comments
LKA	6.273±0.90μg /ml	Active
LKB	6.332±0.51μg /ml	Active
LKD	5.063±0.48μg /ml	Active
LKE	5.039±0.32μg /ml	Active

The codes below represented the fractions included in the blending and the antiplasmodial activity as shown in Table 5

LKA Le+Lh+Li+Lj

LKB Lh+Li+Lj

LKC Le+Li+Lj

LKD Le+Lh+Lj

LKE Le+Lh+Li

When the most active fractions were blend they gave a high activity. The IC_{50} of LKE was 5.039±0.32 μg /ml against D6 clone (Table 5). LKB also had a high activity of 6.332±0.51μg

/ml against D6 (Table 5). LKA had a high activity of 6.273±0.90 µg /ml against D6 (Table 5) and, IC_{50} for the same blend screened against D6 had an IC_{50} of 5.063±0.48µg /ml, and this showed that a higher concentration of the drug is required for the resistant strains of the parasite. As indicated by the Tables 4, 5 and 6 all the blends have a very high antiplasmodial activity that indicates that the fractions probably had an additive effect and this calls for further research.

Table 6: The IC_{50} for the blends of the most active fractions against W2

Extract	IC_{50} for W2	Comments
LKA	7.07±0.09µg /ml	Active
LKC	11.87±1.95µg /ml	Active
LKD	7.74±0.09µg /ml	Active

The following fractions were used in the codes below

LKA Le+Lh+Li+Lj

LKC Le+Li+Lj

LKD Le+Lh+Lj

LKA had antiplasmodial activity of 7.07±0.09µg /ml against W2. LKC had antiplasmodial activity of 11.87±1.95µg /ml against W2 and LKD had antiplasmodial activity of 7.74±0.09 µg/ml against W2 (Table 6). All the three blends LKA, LKC and LKD indicated high activity (Table 6). LKA had antiplasmodial activity of 7.07±0.09µg /ml against W2 (Table 6) and 6.273±0.90µg /ml against D6 (Table 5). The blend LKD was also screened against the two

clones, with D6 the activity was 5.063±0.48μg /ml (Table 5) while the activity with W2 was 7.74±0.09μg /ml (Table 6).

Table 7: The IC_{50} for the conventional antimalarials

Drug	IC_{50} for D6 clone	IC50 for W2	Comments
DIHYDROARTEMISININ	2.025±0.002ng/ml	7.7±0.04ng/ml	Correlates with literature
MEFLOQUINE	2.55±0.001ng/ml	6.19±0.19ng/ml	Correlates with literature
CHLOROQUINE	21±0.01ng/ml	28.5±0.00ng/ml	W2 deviates
QUININE	25±0.001ng/ml	67±0.001ng/ml	Correlates with literature

The blends were also compared with the conventional antimalarials, dehydroartemisinin (DHA) gave an antiplasmodial activity of 2.025±0.002 ng/ml against clone D6 and 7.7±0.04 ng/ml against W2 (Table 7). This tallied with documented literature where the activity of DHA is given as the 2.0 ng/ml. Mefloquine had an antiplasmodial activity of 2.55±0.001ng/ml against D6 and 6.19±0.19ng/ml against W2 (Table 7). The values obtained for Mefloquine also correlated with the documented values. Chloroquine had an antiplasmodial activity of 21±0.01ng/ml against D6 and 28.5±0.00ng/ml against W2 (Table 7). The value against W2 was too low as the expected was 100-160 ng/ml, this was probably due to use of the clone D6 and this prompted the use of PCR in order to determine the purity of the clones used in the

bioassays. Quinine had an antiplasmodial activity of 25±0.001ng/ml against D6 and 67±0.001ng/ml against W2, this correlates with the documented literature (Table 7). The screening of the conventional drugs also helped in the conclusion that the in vitro work was correct

4.4 Polymerase chain reaction (PCR)

The PCR image that was taken included the base pair ladder (labeled 1 and 6) that helped to determine the size of the MSP1 DNA results of the test clones (Plate 7). A negative control (labeled 2) was also included. This was not loaded with any DNA hence the blank results. The two D6 and W2 were loaded on wells 3 and 4 respectively (Plate 7). The results indicated that the test DNA was 500 BP in size (Plate 7). This was in line with the expected results as the WHO protocol specifies that 400-600 BP is normal in a nested MSP1 PCR.

The two clones D6 and W2 had the same band size. These were compared with the standard DNA (5) that was obtained from molecular laboratories in KEMRI. The test DNA bands appeared at the same level as the standard DNA as shown by Plate 7 below. This indicated that the test DNA had no multiple infections. The PCR also indicated that the test DNA (both D6 and W2) was clean with no impurities as indicated by the presence of a single band in each case. Presence of different sized bands in one sample amplification is indicative of multiple clone infection.

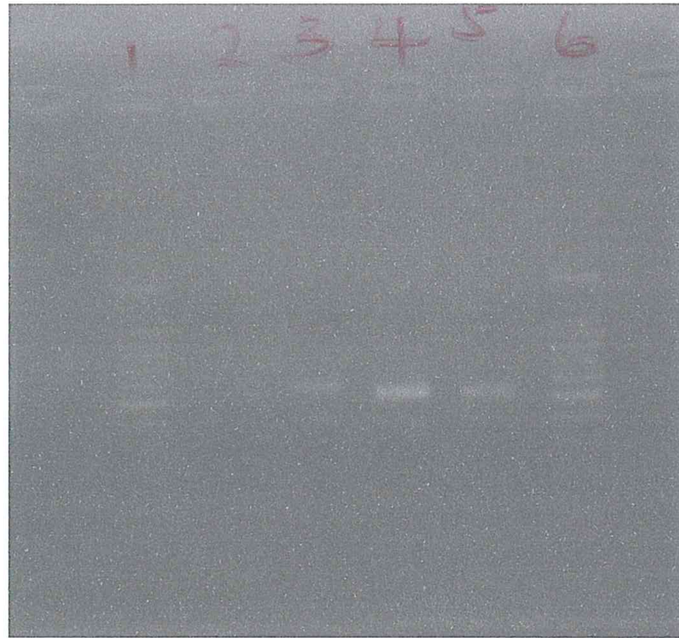

Plate 7: PCR image for clones W2 and D6

1 = 100 BP ladder, 2 = Negative control, 3 = Clone D6, 4 = Clone W2, 5 = Standard clone and 6= 100 BP ladder

4.5 Conclusion and further suggestions

The fractions La, Lb, Le, Lf, Lh, Li and Lj had high antiplasmodial activity. Fractions Lc and Ld were not active. The crude extract was active with antiplasmodial activity of 10.40±0.50μg /ml. The blends of the above fractions also showed very high activity. The fact that fractions and blends of *A. annua* had high *in vitro* antiplasmodial activity would justify their use in the treatment of malaria. The lack of antiplasmodial activity in some fractions may not necessarily imply the same *in vivo* since compounds may either act as prodrugs, which must undergo metabolic changes to achieve the required activity.

Malaria has been considered as a re-emerging disease, due largely to the spread of drug resistant parasite strains, decay of health care infrastructure and difficulties in implementing and maintaining vector control programs in many developing countries. The problem of drug resistance can be attributed primarily to increased selection pressures on *P. falciparum* in particular due to indiscriminate and incomplete drug use for treatment. *P. falciparum* has demonstrated that it is extremely good at adapting to any drugs we may use against it and there is no reason to suspect that this would be different for new compounds. Responsible use of new drugs will be important for the future if artemisinin and artemisinin based combination therapy (ACTs) are not to become as clinically compromised as chloroquine (CQ).

Artemisinin and its derivatives have an essential role to play in the treatment of multidrug-resistant falciparum malaria. The remarkable properties of these drugs are particularly valuable in the treatment of severe and complicated malaria caused by multidrug-resistant *P. falciparum*. (http://www.geocities.com/nutriflip/Naturopathy /Sweet Annie.html). Whole *A.*

annua extracts, with all their synergens, could be better and cheaper than the isolated pure artemisinin compound, or derivatives thereof, at preventing as well as treating malaria, could have fewer side effects, and would less likely lead to multidrug resistance (James, 2002).

In vitro IC_{50} values that are significant markers of antiplasmodial activity have indicated that *P. falciparum* can be eliminated by most of the fractions in *Artemisia annua* and not necessarily the artemisinin alone. The whole extract from *Artemisia annua* could be better and cheaper in the treatment of the CQ resistant srains of *Plasmodium falciparum*. It has also been noted that *P. falciparum* strains resistant to antimalarial drugs like chloroquine, mefloquine, are susceptible to artemisinin and its derivatives in low doses (Balint, 2001).

Biochemical investigations on some of the fractions and blends of *A. annua* compounds may provide useful templates for the development of novel antimalarial drugs that are effective against CQ resistant *P. falciparum*. There is need to isolate the different compounds of *A. annua* characterize them individually and determine their activity on *in vitro* cultures of *P. falciparum*. The isolated compounds can then be blended and tested against *P. falciparum*.

References

Abdulrahman, A. and Husn, H. F. (1997). Chloroquine resistant *Plasmodium falciparum.* The ministry of health *Annual Saudi Medicine* **17**:247-249.

Acton, N. Klayman, D. L. Rollman, J. (1985). Reductive electrochemical HPLC assay for artemisinin (qinghaosu). *Planta Medica* **47**: 445-446.

Alibu, V. P. and Egwang, T. G. (2003). Genomics research and malaria control. Great expectations.**1** (2): 1-3.

Anderson, K.M. Seed, T.O. Harries, J.E (1999). Free radicals and reactive oxygen species in programmed cell death. *Journal of Medical Hypothesis* **52**:451-463.

Arnold, J. Alving, A.S. Hockwald, R.S. (1954). The effect of continuous and intermittent primaquine therapy on the relapse rate of Chesson strain vivax malaria. *Journal of Laboratory Clinical Medicine* **44**:429–438.

Bailey, L.H., and Bailey E.Z. (1976). *Artemisia annua.* Hortus third. MacMillan, NewYork. N.Y., USA. pp 1290.

Balint, G.A. (2001). Artemisinin and its derivatives. An important new class of antimalarial agents. *Journal of Pharmacology and Therapeutics* **90**:261-265.

Benn, M. D. Smith J. B. Helslop-Havison, J. S. (1982). Nuclear DNA amounts in angiosperms. *Proceedings of Royal Society* **216**:179-199.

Bethesda, M.D (2004*)* Polymerase Chain Reaction - Xeroxing DNA.

Brueckner, R. P. Lasseter, K. C. Lin, E. T. Schuster, B. G (1998). First-time-in-humans safety and pharmacokinetics of WR 238605, a new antimalarial. *American Journal of Tropical Medicine Hygiene* **58**:645–649,

Carnevale, P. and Mouchet, J. (1987). Prospects of malaria. *International Journal of Parasitology* **17**:181-187.

Charles, D. J. Cebert, E. and Simon, J. E. (1991).Characterization of the essential oils of *Artemisia annua*. *Journal of Essential Oil Resources* **3**:33-39.

Charles, D. J. Simon, J. E. Wood, K. V. Heinsrtein, P. (1990). Germplasm variations in artemisinin content of *A.annua* using alternative method of artemisinin analysis from crude plant extract. *Journal of Natural Products* **53**:157-160.

Chen, P. K. Leather, G. R. Klayman, D. L. (1987). Allelopathic effect of artemisinin and its related compounds from *Artemisia annua*. *Journal of Plant Physiology* **835**: 406.

Chen, P. K. Leather, G. R. Polatrick, M. (1991). Comparative study on artemisinin, 2,4-d and glycophosphate. *Journal of Agricultural Food Chemistry* **39**:991-994.

China Cooperative Group. (1982). Qinghaosu and its derivatives as anti-malarials. *Journal of Traditional China Medicine* **2**:3–8.

Collins, F.H., and Pasketwitz, S.M. (1995). Malaria –current and future prospects for control. *Annual Review Entomology* **40**:195-219.

Coluzzi, M. Sabatini, A. Petrarca, V. and DiDico, M.A. (1979) Chromosomal differentiation and adaptation to human environment in the *Anopheles gambiae* complex. *Transactions of the Royal Society of Tropical Medicine and Hygiene* **73**: 483-497.

Croteau, R. (1986). Biochemistry of monoterpenes and sesquiterpenes of the essential oils. Herbs, spices and medicinal plants: *Recent Advances in Botany,Horticulture, and Pharmacology* **1**:81-135.

Curtis, C.F. (1994). Approaches to vector. New and trusted appropriate technology for vector control. Impregnated bednets, polystyrene beads,and flytraps. *Transactions of the Royal Society of Tropical Medicine and Hygiene* **88**:144-146.

Daniel, J. C. Malcom, J. G. Herve, T. Leda, C. M. Halmilton, S. O. Mark, A. D. Hoffman, L. and Ventor, J. C. (2004). Life cycle of *P.falciparum* malaria parasite. *Expert Review in Molecular Medicine* http://www-ermm.cbcu.cam.ac.uk.

Davidson, E.W. and Becker N. (1963). Microbial control of vectors: The biology of disease vectors. Chapter 30.

Davidson, I. and Henry, J. (1974). Clinical Diagnosis by Laboratory Methods, I. Davidsohn and J. Henry, eds., W.B. Saunders Co., Philadelphia. pp. 135-137.

De Vries, P. J. and Dien, T. K. (1996). Clinical pharmacology and therapeutic potential of artemisinin and its derivatives in the treatment of malaria. *Journal of Drugs* **52**:818-836.

Delabays, N. Benakis, A. and Collet, G. (1993). Selection and breeding for high artemisinin (qinghaosu) yielding strains of *Artemisia annua. Acta Horticulture* **330**:203-206.

Dharam, C. J. Mathur, A. K. Gupta, M. M. Sing, A. K. Verma, K. R. Gupta, A. P. and Kumar, S. (1996). Isolation of high artemisinin yielding clones of *Artemisia annua. Journal of Phytochemistry* **43**(5) 993-1001.

Dong-Kyu, L. (2001). Field evaluation of insect growth regulators, pyriproxyfen, against *Aedes togoi* larvae in brackish water in South Korea. *Journal of Vector Ecology* **26**(1) 39-42.

Duke, S. O. Vaughn, K. C. Croom, E. M. Elsohly, H. N. (1987). Artemisinin, a constituent of annual wormwood (*Artemisia annua*), is a selective phytotoxin. *Journal of Weed Science* **35**:499-505.

Elueze, E. I. Croft, S. L. Warhust, D. C (1996). Activity of pyronaridine and mepacrine against twelve strains of *P.falciparum* in vitro. *Journal of Antimicrobial Chemotherapy* **37**:511-518.

FAO, (1998). The Sterile Insect Technique http://www.iaea.org/programmes/nafa/

Fedirici, B. A. (1985). Viral pathogens in biological control of mosquitoes, AMCA Bulletin ``No 6, *Mosquito Control Association* 62-74.

Ferreira, J. F. S. and Janick, J., (1996a). Immunoquantitative analysis of artemisinin from *Artemisia annua* using polyclonal antibodies. *Phytochemistry* **41**:97-104.

Ferreira, J.S., Charles, D. J., Wood, K., Simon, J.E., and Janick, J., (1994). A comparison of gas chromatography and high performance liquid chromatography for artemisinin analyses. *Phytochemistry Analyses* **5**:116-120.

Ferreira,J.F.S., Simon ,J.E., and Janick, J., (1995a). Developmental studies of *A.annua* flowering and artemisinin production under green house and field conditions. *Planta Medica* **61**:167-170.

Foley, M. and Tilley, l. (1998). Quinoline antimalarialr,mechanis of activity and application in organic synthesis. *Tetrahedron* 54:4414-4450

Fournet, F. Sannier, C. Montenny, N. (1993). Effects of two insect growth regulators on the reproductive potential of *aedes aegypti. Journal of America Mosquito Control Association* **9**:426-430.

Fournet, F. Sannier, C. Moriniere, M. Porcheron, P. Montenny, N. (1995). Effects of two insect growth regulators on the ecdysteroid production in *Aedes aegypti. Journal Medical Entomology* **32**:588-593.

Garnharm, P.C.C. (1938). Malaria parasites and vectors in Western Kenya *Acta Conventus Tropical Malaria Morbidity* **2**:161-162.

Gratz, N. G. and Pal, R. (1988). Malaria vector control Larviciding. 1213 –1249.

Greenwood, B. M. (1984). The impact of malaria chemoprophylaxis on the immune status of African children. *Bulletin of World Health Organization* **62**:69-75.

Hall, H. M. and Clements, F. F (1923). The phylogenetic method in taxonomy. The north American species of *Artemisia*, *Chrysothamnus* and *Atriplex* Carnegie Inst ,Washington.

Hanriette kress (2005). http://www.henriettesherbal.com/blog/?p=457

Hans, H. M. (2000). www.thetravelforum.com/t161295.html malaria precaution.

Haynes, J. D. Diggs, C. C. Hines, F. A. Desjardins, R. E. (1976). Culture of human malaria parasites *Plasmodium falciparum. Nature* **263**:767-769.

Hirt, H. M., (2000) *Artemisia annua* anamed http://sextocontinente.org/apoyohumano/a-a-anamedA-3.html 1-7.

http://people.ku.edu/~jbrown/pcr.html (1995) What the heck is PCR.

http://users.ugent.be/~avierstr/principles/pcr.html (1997) Principle of the PCR

http://www.geocities.com/nutriflip/Naturopathy/SweetAnnie.html.

http://www.herbalgram.org/herbalgram/articleview.asp?a=2458.

http://www.malariasite.com/malaria/primaquine.htm). (2005) antimalarial drugs-primaquine.

http://www.mosquito-kill-net.com /more-mosquito-pictures.html

http://www.photounit@ars.usda.gov.

http://www.who.int/drugresistance/en/) (2005) WHO drug resistance.

James, A. D. (2002). Artemisia, edited by C.W. Wright. Taylor & Francis. New York, NY.344.

Jeffrey, R .O. N. (1996). The use of insect growth regulators in mosquito larviciding.Sandos Agro. Inc. *North Eastern mosquito control association. http://www.nmca.org/paper15a.htm*

Jembere, B. Hassanali, A. (2001). Blend effects in the toxicity of essential oil constituents of *Ocimum kilimandschscrum* and *Ocimum kenyense* on two-post harvest insect pests *Phytochemistry* **57**:385-391.

Jerome, G. (1996). Physicians guide to arthropods of medical importance 2nd edition 222-258.

Kakkilaya, B. S. (2004). http://www.malariasite.com./malaria.Evolution.htm.

Kamunyi, F. Owaga, M. L Wegesa, P. (1999). Proceedings of 2nd annual medical scientific conference. Nairobi.

Kathleen, W. (2002) A Review of Control Methods for African Malaria Vectors, *Activity Report* **108**:2.2.

Kawada, H. Dohara, K. Shinjo, G. (1988). Laboratory and field evaluation of an insect growth regulator, phenoxyphenol propyl-ether, as a mosquito larvicide. *Journal of Sanitation Zoology* **39**:339-346.

Kettle, D. S. (1994). Medical and Veterinary Entomology. Cambridge University Press, UK, 2nd edn.

Klayman, D. L. (1989). Weeding out malaria. *Natural Histology* **18**:26.

Klayman, D. L. (1993). *Artemisia annua*: From weed to respectable antimalarial plant.242-255.In Kinghorn, A.D., and Balandrin, M.F., Human Medicinal Agents from plants. *American Chemistry Society Symposium Series* ,Washington ,DC.

Klayman, D. L., (1985). Qinghaosu (Artemisinin). An antimalarial drug from China. *Science* **228**:1049-1055.

Korsinczky, M. Fisher, K. Chen, N. Rieckmann and Cheng, Q. (2004) Sulfadoxine resistance in *Plasmodium vivax* is associated with a DHPS sequence polymorphism altering the putative drug-binding site. *Antimicrobial Agents and Chemotherapy* **48**(6):2214-2222.

Lacy, L and Orr, M. (1994). The role of biological control of mosquito in integrated vector control. *American Journal of Tropical Medicine and Hygiene* **50**: 97-115.

Lee, I. S. and Hufford, C. D. (1990). Metabolism of of antimalarial sesquiterpene lactones. *Journal of Pharmacology and Therapeutics* **48**:345-355.

Lee. R. (2002). Plants against malaria. *Artemisia annua* or the sweet wormwood. *Journal of Royal College of Physicians Edinburgh* **32**:300-305.

Mafong, E. A. Kaplan, L. A. (1997) "Insect repellents". What really works? *Postgraduate Medicine* **102**(2):63-74.

Majori, G. Sabatinelli, G. and Colluzzi, M. (1987). Efficacy of permethrin impregnated curtains for malaria control. *Journal of Medical veterinary Entomology* **1**:185-192

Manson-Bahr, P. E .C. and Bell, D. R. (1987). Malaria and babesiosis in Manson's tropical diseases 19th edition: London, Bailliere Tindall, 3-51.

Marshall, L. (1981) .Biocontrol of medical and veterinary pests.

Mathenge, E. M. (1999). The effects of permethrin –impregnated bednets on mosquito behaviour. MSC Thesis.

Meshnick, S. R. Taylor, T. E. Kamchonwongpaisan, S. (1996). Artemisinin and the antimalarial endoperoxides:from herbal remedy to targeted chemotherapy. *Microbial Reviews* **60**:301-315.

Morgan, D. W. Matthew, J. L Thomas, A. F. Murira, G. and Anthony, A. H. (2004). Malaria parasite-inhibitory antibody epitopes on *P.falciparum* merozoite surface protein-119 mapped by TROSY NMR. *Molecular and Biochemical Parasitology* **138**:29-36.

Mueller, M. S. Karhagomba, I. B. Hirt, H. M. and Wemakor, E. (2000). The potential *Artemisia annua* as a locally produced remedy for malaria in the tropics. *Journal of Ethnopharmacology* **73**:487-493.

Mulla, M. S. Darwazeh, H. A. and Schreiber, E. T. (1989). Impact of new insect growth regulators and their formulations on mosquito larval developments in impoundment and floodwater habitats. *Journal of American Mosquito Control Association* **5**:15-20.

Nosten, F. ter Kuile, F. Luxermburger, C. Woodrow, C. Kyle, D. E. Chongsulphajaisiddhi, T. White, T. J. (1993). Cardiac effects of antimalarial treatment with halofantrine, *Lancet* **341**:1054-1056.

O'neill, P. M. Bray, P. G. Hawley, S. A. Ward, S. A. Park, B. K. (1998). Geographic and temporal patterns in toxicopathic liver lesions in english sole (*Pleuronectes vetulus*) from puget sound and relationships with contaminant concentrations in sediments and fish tissues. *Journal of Pharmacology and Therapeutics* **77**:29-58.

Oketch, B. A. (2004). Factors affecting the infectivity and early sporogonic development of *Plasmodium falciparum* in the malaria vector, *Anopheles gambiae ss* .Phd thesis

Palchick, S. (1963). Chemical control of vectors: The Biology of Disease Vectors. Chapter 29: 502-511.

Pattaroyo, M. E. Romero, P. Torres, M. L. Clavijo, P. Moreno, A. Martinez, A. Rodriguez, R. Guzman, F. and Cazebas, E (1987). Induction of protective immunity against experimental infecion with malaria using synthetic peptides. *Nature* **328**:629-632.

Peters, W. (1970). Experimental basis for the use of sulfonamides and sulphones against malaria in African communities-health and disease in Africa.

Posner, G. H. Cumming, J. N. Krasavin, M. (2000). Carbon centered radicals and rational design of new antimalarial peroxide drugs. In Torence P.F, editor, Biomedical chemistry: Applying chemical principles to the understanding and treatment of disease. John Wiley and sons, New York pp 289-309.

Price, R. van Vugt, M. Phaipun, L Luxemburger, C. Simpson , J. McGready, R. ter Kuile, F. Kham, A. Chongsuphajaisiddhi, T. White N. J. Nosten, F. (1999a). Adverse effects in patients with acute falciparum malaria treated with artemisinin derivatives. *American Journal of Tropical Medicine and Hygiene* **60**(4):547-555

Prieur, E. Sarah, C. G. Joerg, S. Anne, C. M. Eric, G. S. Nilu, G. Kathryn, J. Robson, H. and Adrian V.S. H. (2004). A *Plasmodium falciparum* candidate vaccine based on a six-antigen polyprotein encoded by recombinant poxviruses. *Proceeding of the National Academy of Science* **101**:290-295.

Pussard, E. Verdier, F. (1994). Antimalarial 4-aminoquinolines: mode of action and pharmacokinetics. *Journal of Fundamental Clinical Pharmacology* **8**(1):1-17.

Quinones, M. L. Lines, J. D. Thomson, M. C. Jawara, M. Greenwood, B. M. (1998) Permethrin treated bednets do not have a mass killing effect on village population of *Anopheles gambiae* in the Gambia. *Transactions of the Royal Society of Tropical Medicine and Hygiene* **2**:45-51.

Rai, K. S. (1963). Genetic control of vectors; The Biology of Disease Vectors pp 564-574.

Rai, K. S. (1963). A cytogenetic study of the effects of X-irradiation on *Aedes aegypti. Journal of Cryologia* **16**:592-607.

Ramachandran, R (2002). Resistance to anti-malarial drugs. *Indians National Magazine* **19**(13) 1-7.

Ranfold-Cartwright, L. C. Taylor, J. Umasunthar, T. Taylor, L. Babiker, H. A. Lell, B. Schmidt-Ott, J. R. Lehman, L. G. Walliker, D. and Kresmner, P. G (1997). Molecular analyses of recrudescent parasites in a *Plasmodium falciparum* drug efficacy trial in Gabon. *Transactions of the Royal Society of Tropical medicine and Hygiene* **91**:714-724.

Raymond, M. Callaghan, A. Fort, F. and Pasteur, N. (1991). Worldwide migration of amplified insecticide resistance genes in mosquitoes. *Nature* **350**: 151-153.

Renshaw, M. and Silver, J. B. (2001). The encyclopedia of arthropod transmitted infections of man and domesticated animals. New York: CABI Publishing pp 314-327.

Ringwald, P. Eboumbou, E. C. M. Bickii, J. Basco, L. K. (1999). In Vitro Activities of pyronaridine alone and in combination with other antimalarial drugs, against *P.falciparum Journal of Antimicrobial Agents and Chemotherapy* **43**(6):1525 – 1527.

Roberts, A. Francoise, B. Odile, D. C. Benard, M. (2001). From classical antimalarial drugs to new compounds based on the mechanism of action of artemisinin. *Journal of Pure Applied Chemistry* **73**:1173-1188.

Roberts, J. M. (1970). Sulphodoxine alone and sulphodoxine in combination with pyrimethamine as malarial suppressant 161-176.

Schumutterer, H. (1995). The neem tree *Azadirachta indica* A Juss and other Meliaceous plants.VCH Weinheim pp 1-696.

Seyoum, A. Kabiru, E. W. Lwande, W. Killeen, G. F. Hassanali, A. Knols, B. G. (2003). Repellency of live potted plants against *Anopheles gambiae* from human baits in semi-

field experimental huts. *American Journal of Tropical Medicine and Hygiene*: **67**(2):191-195.

Shanks, G. D. Kain, K. C. Keystone, J .S. (2001). Malaria chemoprophylaxis in the age of drug resistance II. Drugs that may be available in the future. *Journal of Clinical Infectious Diseases* **33**:381-385

Shanks, G. D. Oloo, A. Klotz, F. W. (2001). A new primaquine analogue, tafenoquine (WR238605), for prophylaxis against *Plasmodium falciparum* malaria. *Journal of Clinical Infectious Diseases* **33**:1968-1974.

Shunmay, Y. Wirichada, P. Ian, M. H. Anne, J. M. Nicholas, J. W. (2004). Antimalarial drug resistance, artemisinin-based combination therapy, and the contribution of modeling to elucidating policy choices. *American Journal of Tropical Medicine and Hygiene* **71**(2):179-186.

Sim, B. K .L. Orlandi, P. A. Haynes, J. D. Klotz, F. W. Carter, J. M. Cammus, D. Zegans, M. E. and Chulay, J. D. (1990) Primary structure of the 175K *Plasmodium falciparum* binding antigen and the identification of a peptide which elicits antibodies that inhihit malaria merozoites invasion. *Journal of Cell Biology* **111**:1877-1884.

Simon, J. E. Charles, D. Cebert, E. Grant, L. Janick, J. and Whipkey, A. (1990). *Artemisia annuA*. A promising aromatic and medicinal plant pp 522-526. *In*: J. Janick and J.E. Simon (eds.), *Advances in new crops*. Timber Press, Portland,

Sing, N. P. Kumari, V. Chauhan, D. (2003) Mosquito larvicidal properties of the leaf extract of a herbaceous plant, *Ocimum canum* (Family: Labiatae). *Journal of communicable disease*: **35**(1):43-45.

Sixsmith, D. G. Watkins, W. M. Chulay, J. D. Spencer, H. C. (1984). *In vitro* antimalarial activity of tetrahydrofolate dehydrogenase inhibitors. *American Journal of Tropical Medicine and Hygiene* **33**:772-776

Surtees, G., (1970). Mosquito breeding in Kuching area savanna, *Journal of Medical Entomology* **7**:273-276.

Tabitha, M. (2004). http://users.ugent.be/~ avierstr / principles/pcr.html).

ter Kuile, F O. Dolan, G. Nosten, F. Edstein, M. D. Luxemburger C. Phaipun, l. Chongsuphajaisiddhi, T. Webster, H. K. White, N. J. (1993). Halofantrine versus mefloquine in treatment of multidrug-resistant falciparum malaria. *Lancet* **341**:1044-49.

ter Kuile, R. Kham, A. Chongsuphajaisiddhi, T. White, N. J. and Nosten, F. (1999). Adverse effects in patients with acute falciparum malaria treated with artemisinin derivatives. *American Journal of Tropical Medicine and Hygiene* **60**:547-555.

Trager, W. and Jensen, J. B. (1976). Human malaria parasites in continuous culture. *Science* **193**:673-678.

USAID, (1999). Brief on insecticide treated bednets for malaria control. *Malaria Consortium* 1-3.

van Agtmael, M. A. Eggelte, T. A. van Boxtel, C. J. (1999). Artemisinin drugs in the treatment of malaria from medicinal herb to registered medication. *Trends in Pharmacology Science* **20**:199-204

Van der Nat, J. M. Van der Sluis, W. G. de Silva, K. T. D. Labadie, R. P. (1991). Ethnopharmacognostical survey of *Azadirachta indica* A Juss (Meliaceae). *Journal of Ethnopharmacology* **35**:1-24

Vikas, D. K. Vishweshwar, R. Narasu, M. L. (2000). Current status of artemisinin and its derivatives as antimalarial drugs. *Life Sciences* **66** (4): 279-300.

Wallace, P. and Gilles, H. (1995). *Tropical Medicine and Parasitology* 4th edition pp 24-36.

Wallart, T. Uden, W. V. Lubberink, H. G. M. Woerdenbag, H. J. Pras, N and Quax, J. W. (1999). Isolation and identification of Dihydroartemisinic acid from *Artemisia annua* and its possible role in the biosynthesis of artemisinin. *Journal of Natural Products* **62**:430-433.

Walsh, D. S. Looareesuwan, S. Wilairatana, P. (1999). Randomized dose-ranging study of the safety and efficacy of WR 238605 (Tafenoquine) in the prevention of relapse of *Plasmodium vivax* malaria in Thailand. *Journal of Infectious Diseases* **180**:1282–1287

Washington post (2004). Malaria vaccine (www.washingtonpost.com/wp-dyn/ articles/A33393-2004Oct14.html -)

Webster, D. and Adrian, V.S. H. (2003). Progress with new malaria vaccines. Bulletin of the *World Health Organization* **81**:902-908.

Wellems, T. E. and Plowe, C. V. (2001).Chloroquine-resistant malaria. *Journal of Infectious Diseases* **184**:770 -776.

White, G. B. Boreman, P. F. L. Dolling, W. R. (1972). Synathropic emesine bugs (*Ruduvidae, Emesinae*) as a predator of endophilic mosquitoes. *Transactions of the Royal Society of Tropical Medicine and Hygiene* **66**:534-535.

WHO, (1981). Report of the 4th meeting of the scientific working group on the chemotherapy of malaria. Beijing, *Peoples Republic of China.* 6-10.

WHO, (1995). Operational issues in malaria control. *World Health Organization.*

WHO, (1995). Control for malaria and other mosquito- borne diseases *World Health Organization* 22-32.

WHO, (1995). Drugs used in parasitic diseases. 2nd edition *World Health Organization* Geneva.

WHO, (1998). Roll back malaria. A global partnership. *World health organization.* Geneva.

WHO, (1998). The use of artemisinin and its derivatives as anti-malarial drugs. *World Health* Organization Geneva.

WHO, (2002). History of Plasmodium parasites 1-3. *World Health Organization* Geneva.

WHO, (2003). *Plasmodium* Genus protozoa Encyclopedia -family health. *World Health Organization*

WHO, (2004). Standard operating procedures for *in vitro* cultures of *Plasmodium falciparum.*

Wintrobe, M.W. (1974). Clinical Hematology, Lea and Febiger, Philadelphia,pp. 26.

www.drugdigest.org.mefloquine

Xu, X. X. Zhu, J. Huang, D. Z. and Zhou W. S. (1986). Total synthesis of arteannuin and deoxyarteannuin. *Tetrahedron* **42**:819-828.

Zalis, M G., Pang, L., Silveira, M. S., Milhous, W. K. and Wirth, D. F. 1998. Characterization of *Plasmodium falciparum* cloned from the Amazon region of Brazil: evidence for quinine resistance. *American Journal of Tropical Medicine and Hygiene* **58**(5):630-637.

Zhang, F., Gosser D.K., Meshnick S.R., (1992). Hemin catalysed decomposition of artemisinin (qinghaosu). *.Biochemistry Pharmacology* **43**:1805-1809.

Zhou W. S., (1986.) Total synthesis of arteannuin and related compound. *Pure Applied Chemistry*.**58**: 817-824.