RESISTANCE-MITIGATING EFFECT OF *Artemisia annua* PHYTOCHEMICAL EXTRACTS IN CULTURES OF *Plasmodium falciparum* AND IN *Plasmodium berghei AND Plasmodium yoelii*

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Resistance mitigating effect of *Artemisia annua* phytochemical extracts in cultures of *Plasmodium falciparum* and in -*Plasmodium berghei and Plasmodium yoelii*

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A research thesis submitted in fulfillment for the Degree of Doctor of Philosophy in Biochemistry in the Jomo Kenyatta University of Agriculture and Technology

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DECLARATION

This thesis is my original work and has not been submitted to any other university for the award of a degree.

Signed……..Date……

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This thesis has been submitted for examination with our approval as university supervisors.

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JKUAT, Kenya

DEDICATION

This work is dedicated to my family members especially my husband Joseph, my lovely kids Triza, Mike, Jacinta, Eliza and Ann. I also dedicate the same to my mother Martha Wangeci for her upbringing and sacrifice to be where I am today, and special remembrance to my late dad Samuel Mukinyo who had passion for education.

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ABBREVIATIONS AND ACRONYMS

2% RT	2% Relapse technique
ACT	Artemisinin combination therapy
ART	Artemisinin
CQ	Chloroquine
DNA	Deoxyribonucleic acid
ED_{50}	Effective dose at 50%
ED_{90}	Effective dose at 90%
GFM	Glass Fibre Membrane.
HPLC	High Perfomance Liquid Chromatography
IC_{50}	Inhibitory concentration at 50%
IC_{90}	Inhibitory concentration at 90%
ILRI	International Livestock Research institute
KEMRI	Kenya Medical Research Institute
MDR	Multi drug resistant
PCR	Polymerase chain reaction
PF	*Plasmodium falciparum*
***Pf*ATPASE**	*Plasmodium falciparum* Adenosine triphosphatase
***Pf*CRT**	*Plasmodium falciparum* chloroquine resistant transporter
***Pf*DHFR**	*Plasmodium falciparum* dihydrofolate reductase
***Pf*DHPS**	*Plasmodium falciparum* dihydropteroate synthase
***Pf*MDR1**	*Plasmodium falciparum* Multi drug resistance -1
RSI	Relative sensitivity index
TCTP	Translationally controlled tumour protein

WHO World Health Organization

ABSTRACT

The emergence and spread of multidrug resistant *Plasmodium falciparum* has severely limited therapeutic options for the treatment of malaria. With ever-increasing failure rates associated with chloroquine or sulphadoxine-pyrimethamine treatment, attention has turned to the few alternatives, which include quinine, mefloquine and recently the use of artemisinin. Artemisinin derivatives, particularly in combination with other drugs, are thus increasingly used to treat malaria, reducing the probability that parasites resistant to their components will emerge. Although stable resistance to artemisinin is yet to be reported from laboratory or field isolates, its emergence would be disastrous because of lack of alternative treatments. The project was designed to demonstrate resistance-mitigating effects of phytochemicals in the extract of *Artemisia annua* relative to pure artemisinin against the malaria parasite *Plasmodium falciparum* and on rodent malaria parasite *Plasmodium berghei* ANKA and *P. yoelii.* For the *in vitro* experiments selection was undertaken on two cultures of *P. falciparum,* a CQ-sensitive strain D6 (originally from Sierra Leone) and a CQ-resistant W2 (strain from Indochina), by exposing them to *A. annua* phytochemical extract and the pure artemisinin over 50 cycles at doses initially required to give 50% mortality of the parasites. Dose-response effects of the extract and the pure artemisinin were determined after 10, 20, 30, and 40 exposure cycles and compared to determine if significant difference in percentage mortality of the parasites with increasing exposure cycles. The *in-vivo* experiments were carried out by inoculating mice with the murine *Plasmodiu*m parasites and thereafter given the test drugs using the 4 day test. After 4 days parasitaemia in test mice and control was determined to calculate the effective doses ED_{50} and the ED_{9O}. The ED_{90} was utilized to study resistance under drug pressure and this gave gave rise to "resistant" parasites that were used for molecular characterization. Emergence of resistance of the phenotypes was determined by a quantitative Peter's 4-day test. DNA was extracted from resistant parasites and sensitive parasites using DNA extraction kit by Roche and then amplified using PCR method. The amplicons were then purified before sequencing. The nucleotide sequences of the possible genetic modulators of Artemisinin and *Artemisia annua* extract resistance (*mdr1*, *cg10*, *tctp*, and *atpase 6*) of sensitive and "resistant" parasites were compared. On analyzing the sequences, one mutation was detected on *Pf*MDR 86, in parasites exposed to artemisinin at $IC_{50.}$ No mutations were detected with TCTP, cg10 and MDR 1034.

However the *in-vitro* results showed an increase in (IC_{50}) values where W2 parasites exposed to artemisinin at (IC_{50}) showed a twenty six fold and parasites exposed to artemisinin at (IC_{90}) a ten fold increase was realized. W2 parasites exposed to the blend at (IC_{50}) and at (IC_{90}) showed a twenty nine fold and thirty six fold increases respectively when tested with artemisinin but W2 parasites exposed to *Artemisia annua* remained sensitive to *Artemisia annua* extract. A rather interesting finding was that parasites exposed to the *Artemisia annua* and tested with the extract remained sensitive but parasites exposed to the *Artemisia annua* and tested with artemisinin showed some level of resistance. . A similar trend was observed with D6 parasites in that those exposed the *Artemisia annua* extract and tested with the extract remained sensitive to the blend
The findings indicated that artemisinin resistance will finally occur and the use of *Artemisia annua* whole extract would be one of the ways of mitigating against resistance development.

CHAPTER ONE

INTRODUCTION

1.1 Plasmodium species infecting man

Malaria parasites are micro-organisms that belong to the genus Plasmodium. There are more than 100 species of Plasmodium, which can infect many animal species such as reptiles, birds, and various mammals. Only four species of Plasmodium have been infecting humans; *Plasmodium falciparum, Plasmodium vivax, Plasmodium malariae* and *Plasmodium ovale* (Cowman & Crabb, 2006, Gauthier & Tibayrenc, 2005), but recently *Plasmodium knowlesi* has been found to infect humans. *Plasmodium knowlesi*, a malaria parasite species commonly found in long-tailed and pig-tailed macaques (*Macaca fascicularis* and *Macaca nemestrina*, respectively) and is the only malaria parasite of primates with a 24-hour erythrocytic cycle (Garharm, 1996, Knowles, 1932). Humans were shown to be susceptible to *P. knowlesi* by blood passage soon after the parasite was first isolated in 1932 (Knowles 1932) and until recently, naturally acquired human infections with *P. knowlesi* were thought to be extremely rare. However, following a number of reports of human knowlesi malaria infections detected by molecular methods in various countries in Southeast Asia, *P. knowlesi* is now recognized as the fifth species of *Plasmodium* infecting humans. Smear microscopy remains the gold standard for malaria diagnosis. Microscopy can also be used to determine the species of malaria parasite and quantify the parasitemia—both of which are necessary pieces of information for providing the most appropriate treatment (CDC, 2014). Various test kits are available to detect antigens derived from malaria parasites. Such immunologic tests provide results in 2–15 minutes. These rapid diagnostic tests (RDTs) offer a useful alternative to microscopy in situations where reliable microscopic diagnosis is not immediately available. Although RDTs can detect malaria antigens within minutes, they cannot determine the species or quantify parasitemia. In addition,

positive and negative results must always be confirmed by microscopy. PCR tests are also available for detecting malaria parasites. Although these tests are slightly more sensitive than routine microscopy, results are not usually available as quickly as microscopy, thus limiting the utility of this test for acute diagnosis. PCR testing is most useful for definitively identifying the species of malaria parasite and detecting mixed infections (Arguin & Tani, 2014).

1.2 Morphology, life cycle and diagnosis

Infection in humans begins with the bite of an infected female anopheline mosquito (**Figure 1.1**). 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. 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 hundrends of sporozoites into the mosquito body wall cavity where they eventually migrate to the salivary gland as indicated by **Figure 1.1** (Wallace & Gilles, 1995, Daniel *et al.*, 2004).

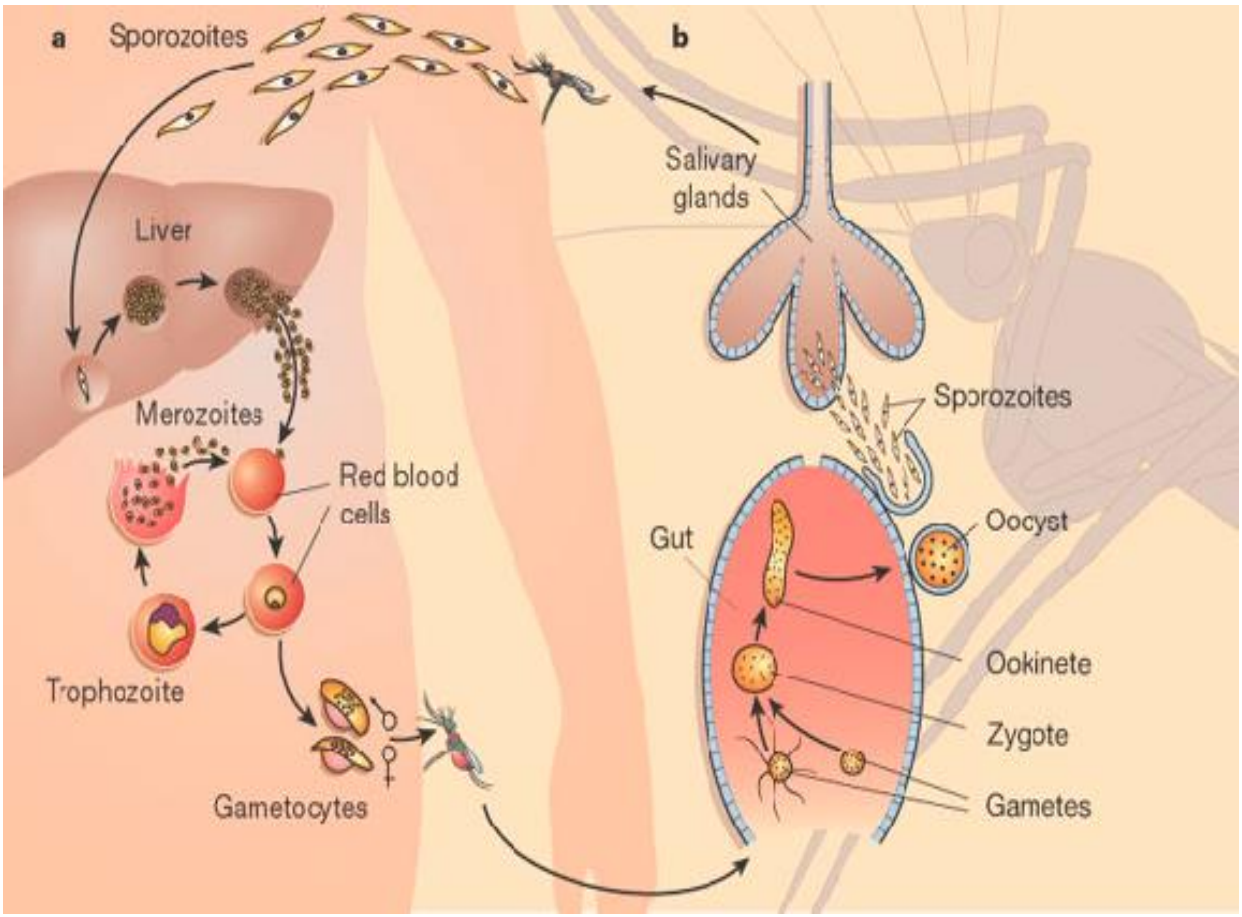

Figure 1.1.Life cycle of *P. falciparum*

Adopted from Singh 2008.

1.2.1 Exo-erythrocytic stages

Exo-erythrocytic stages that include the sporozoites which enter hepatocytes of liver and start multiplying, schizonts which are the dividing forms of liver stages, from where the merozoites arise after the schizont bursts, merozoites are released by hepatocytes. Hypnozoites are the dormant liver stages of the parasite as indicated by **Figure 1.2.**

1.2.2 Blood stages

Merozoites enter erythrocytes and start developing to become trophozoites. Trophozoites are the feeding and growing stages in red cells and they develop to become a schizont. Some of the merozoites develop to become gametocytes **(Figure 1.2)** which are the sexual blood stages while the other merozoites infect new red blood cells.

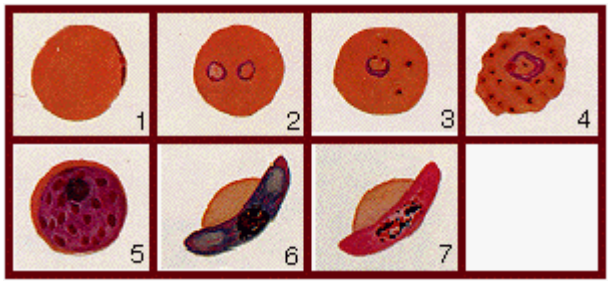

Figure 1. 2. Morphology of malaria parasites *Plasmodium falciparum* inside the infected erythrocyte

1, early trophozoite; 2, early trophozoite (double infection); 3, early trophozoite double chromatin with a few Maurer's dots; 4, late trophozoite with Maurer's dots and crenated red cell; 5, Mature schizont with merozoites and clumped pigment; 6, macrogametocyte with bluish cytoplasm and compact chromatin; 7, microgametocyte with pinkish cytoplasm and dispersed chromatin. Adopted from www.icp.ucl.ac.be/~opperd/parasites/malaria4.htm.

Figure 1.3

Adopted from Singh 2008.Anopheles mosquito after a blood meal

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

1.3 Malaria epidemiology

In 2012, there were an estimated 207 million cases world-wide, causing an estimated 627 000 deaths, mainly among children under 5 years of age in Africa. Ninety percent of all deaths occur in Sub Sahara Africa (WHO 2013). More than a third of the world's population (about 2 billion people) live in malaria endemic areas and 1 billion people are estimated to carry parasites at any one time **(Figure 1.4).** In Africa alone, there are an estimated 200-450 million cases of fever in children infected with malaria each year (Breman *et al.*, 2001).

The number of malaria cases globally fell from an estimated 262 million in 2000 (range: 205–316 million), to 214 million in 2015 (range: 149–303 million), a decline of 18%. Most cases in 2015 are estimated to have occurred in the WHO African Region (88%), followed by the WHO South-East Asia Region (10%) and the WHO Eastern Mediterranean Region (2%). The incidence of malaria, which takes into account population growth, is estimated to have decreased by 37% between 2000 and 2015 (WHO, 2015).

The number of malaria deaths globally fell from an estimated 839 000 in 2000 (range: 653 000–1.1 million), to 438 000 in 2015 (range: 236 000–635 000), a decline of 48%. Most deaths in 2015 were in the WHO African Region (90%), followed by the WHO South-East Asia Region (7%) and the WHO Eastern Mediterranean Region (2%). The malaria mortality rate, which takes into account population growth, is estimated to have decreased by 60% globally between 2000 and 2015 (WHO, 2015).

The number of malaria deaths in children aged under 5 years is estimated to have decreased from 723 000 globally in 2000 (range: 563 000–948 000) to 306 000 in 2015 (range: 219 000–421 000). The bulk of this decrease occurred in the WHO African Region, where the estimated number of deaths fell from 694 000 in 2000 (range: 569 000–901 000) to 292 000 in 2015 (range: 212 000–384 000) (WHO 2015).

Infections in children aged 2–10 years infected with malaria parasites halved in endemic areas of Africa since 2000. Infection prevalence among children aged 2–10 years is estimated to have declined from 33% in 2000 (uncertainty interval [UI]: 31–35%) to 16% in 2015 (UI: 14–19%), with three quarters of this change occurring after 2005.

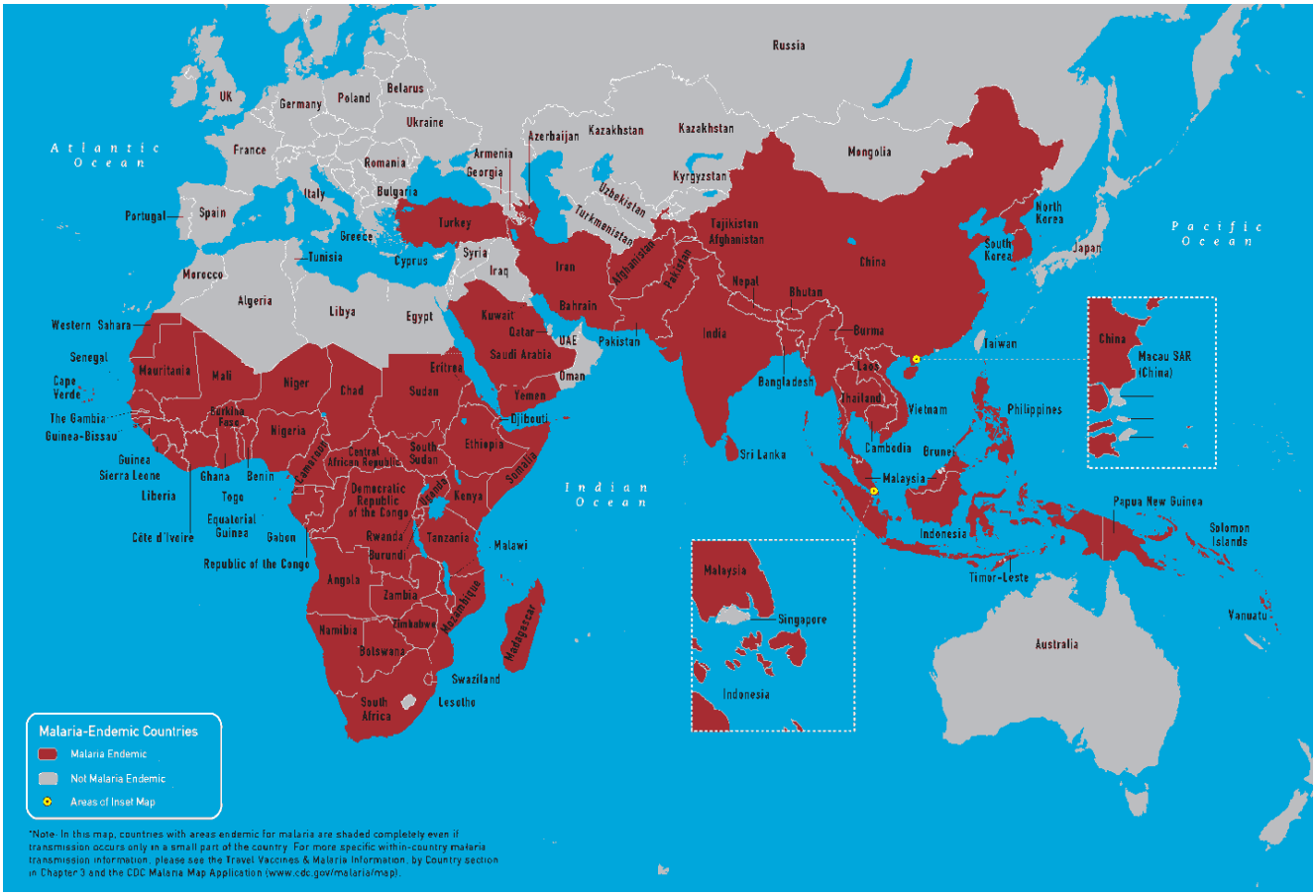

Figure 1.4 Malaria-endemic countries in the Eastern Hemisphere

Adopted from CDC 2014

1.4 Pathogenesis of human malaria parasites

Malaria can begin with flu-like symptoms. In the early stages, infection from *P. falciparum* is similar to infection from *P. vivax*, *P. malariae*, and *P. ovale*.

The time from the initial malaria infection until symptoms appear (incubation period) typically ranges from: 9 to 14 days for *Plasmodium (P.) falciparum*, 12 to 18 days for *P. vivax* and *P. ovale*, 18 to 40 days for *P. malariae* 11 to 12 days for *P. knowlesi* (American Public Health Association (2008). Symptoms can appear in 7 days. The time between exposure and signs of illness may sometimes be as long as 8 to 10 months with *P. vivax* and *P. ovale*. The incubation period may be longer if you are taking medicine to prevent infection (chemoprophylaxis) or because you have some immunity due to previous infections. In the early stages, malaria symptoms are sometimes similar to those of many other infections. Symptoms may include: fever, chills, headache, sweats, fatigue, nausea and vomiting.

Symptoms may appear in cycles. The time between episodes of fever and other symptoms varies with the specific parasite you are infected with. Episodes of symptoms may occur: every 24 hours with *P. knowlesi*, every 48 hours with *P. vivax* or *P. ovale*, every 72 hours *P. malariae*. *P. falciparum* does not usually cause a regular, cyclic

fever.

1.5 Malaria prevention and control options

Control strategies can either target the malaria parasite or the vector **(Figure 1.3)** above. Methods used to prevent the spread of disease, or to protect individuals in areas where malaria is endemic, include prophylactic drugs, mosquito eradication, and the prevention of mosquito bites.

The continued existence of malaria in an area requires a combination of high human population density, high mosquito population density, and high rates of transmission

from humans to mosquitoes and from mosquitoes to humans. If any of these is lowered sufficiently, the parasite will sooner or later disappear from that area, as happened in North America, Europe and much of Middle East.

1.5.1 Vector control

1.5.1.1 Indoor residual spraying

Indoor residual spraying is the practice of spraying insecticides on the interior walls of homes in malaria affected areas. After feeding, many mosquito species rest on a nearby surface while digesting the bloodmeal, so if the walls of dwellings have been coated with insecticides, the resting mosquitos will be killed before they can bite another victim, transferring the malaria parasite.

The World Health Organization (WHO, 2006) currently advises the use of 12 different insecticides in Indoor residual spraying operations. These include DDT and a series of alternative insecticides (such as the pyrethroidspermethrin and deltamethrin) to both, combat malaria in areas where mosquitoes are DDT-resistant, and to slow the evolution of resistance. One problem with all forms of Indoor Residual Spraying is insecticide resistance via evolution of mosquitos.

1.5.1.2 Mosquito nets and bedclothes

Mosquito nets help keep mosquitoes away from people, and thus greatly reduce the infection and transmission of malaria. The nets are not a perfect barrier, so they are often treated with an insecticide designed to kill the mosquito before it has time to search for a way past the net (http://www.eac.int/health/index).

Insecticide-treated nets (ITN) are estimated to be twice as effective as untreated nets, and offer greater than 70% protection compared with no net. For maximum effectiveness, the nets should be re-impregnated with insecticide every six months. This process poses a significant logistical problem in rural areas. New technologies like Olyset or DawaPlus allow for production of long-lasting insecticidal mosquito nets

(LLINs), which release insecticide for approximately 5 years. (http://www.eac.int/health/index).

ITNs have the advantage of protecting people sleeping under the net and simultaneously killing mosquitoes that contact the net. This has the effect of killing the most dangerous mosquitoes. Some protection is also provided to others, including people sleeping in the same room but not under the net (http://www.eac.int/health/index).

1.5.1.3 Prophylactic drugs

Several drugs, most of which are also used for treatment of malaria, can be taken preventively. Generally, these drugs are taken daily or weekly, at a lower dose than would be used for treatment of a person who had actually contracted the disease. Use of prophylactic drugs is seldom practical for full-time residents of malaria-endemic areas, and their use is usually restricted to short-term visitors and travelers to malarial regions. This is due to the cost of purchasing the drugs and negative side effects from long-term use (http://www.eac.int/health/index).

Quinine was used starting in the seventeenth century as a prophylactic against malaria. Today, quinine is still used to treat chloroquine resistant Plasmodium falciparum, as well as severe and cerebral stages of malaria, but is not generally used for prophylaxis. Modern drugs used preventively include mefloquine (Lariam), doxycycline and the combination of atovaquone and proguanil hydrochloride (Malarone). The choice of which drug to use depends on which drugs the parasites in the area are resistant to, as well as side-effects and other considerations. The prophylactic effect does not begin immediately upon starting taking the drugs, so people temporarily visiting malaria-endemic areas usually begin taking the drugs one to two weeks before arriving and must continue taking them for 4 weeks after leaving (with the exception of atovaquone-proguanil that only needs be started 2 days prior and continued for 7 days afterwards (CDC 2011, http://www.eac.int/health/index).

1.5.1.4 Vaccination

Vaccines for malaria are under development, with no completely effective vaccine yet available. Presently, there is a huge variety of vaccine candidates. Pre-erythrocytic vaccines (vaccines that target the parasite before it reaches the blood), in particular vaccines based on circumsporozoite protein (CSP), make up the largest group of research for the malaria vaccine. Other vaccine candidates include: those that seek to induce immunity to the blood stages of the infection; those that seek to avoid more severe pathologies of malaria by preventing adherence of the parasite to blood venules and placenta; and transmission-blocking vaccines that would stop the development of the parasite in the mosquito right after the mosquito has taken a bloodmeal from an infected person. It is hoped that the sequencing of the P. falciparum genome will provide targets for new drugs or vaccines (WHO 2016, http://www.eac.int/health/index 29/10/2016).

CHAPTER TWO
LITERATURE REVIEW

2.1 Antimalarial Drugs

Antimalarial drugs fall into several chemical groups: 8-Aminoquinolines, 4-Aminoquinolines, Quinoline-Methanols, and other Aryl Alcohols. They can also be classified on the basis of the Plasmodium parasite stages against which they are most effective (Tracey *et al.,* 1996). Blood schizontocides are drugs acting on asexual intraerythrocytic stages of malaria parasites. They suppress the proliferation of in the erythrocytic parasites. Tissue schizontocides prevent the development of hepatic schizonts. They are prophylactic because they affect the early developmental plasmodium and prevent the invasion of the erythrocytes. A hypnozoiticide acts on the dorminant and yet persistent intrahepatic stages of *P. vivax* and *P. ovale* in the liver. Gametocides destroy the intraerythrocytic sexual forms (gametes) of the parasite and prevent transmission from human to another mosquito. Antimalarials are however rarely used clinically just for their gametocidal action (Afonso *et al.,* 2006).

2.1.1-aminoquinolines

This is the only class of gametocides that is commonly used in the treatment of malaria. It is a form of aminoquinoline with an amine at the 8-position of quinoline. The group contains three members, primaquine, tafenoquine and pamaquine (Sweeney, 2004). They may be used to eradicate malaria hypnozoites from the liver. The drugs must not be given to patients with glucose 6 phosphate deficiency (G6PD), because they cause potentially fatal haemolysis in these patients. Pamaquine is no longer available anywhere, but primaquine is still used routinely worldwide as part of the treatment of *Plasmodium vivax* and *Plasmodium ovale* malaria.

2.1.1.1 Primaquine

Primaquine 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).

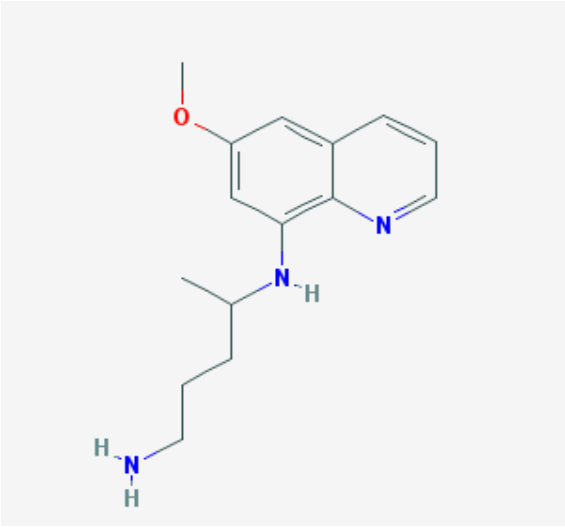

Figure 2.1 Structure of Primaquine adopted from https:// pubchem.ncbi.nlm.nih. gov/compound/primaquine

2.1.1.2 Tafenoquine

Tafenoquine (**Figure 2.2**) (8-[{4-amino-1-methylbutyl} amino]-2, 6-dimethoxy-4-methyl-5-[3-trifluoromethyl-phenoxy] quinoline, succinate is a primaquine analog with a long elimination half life of 14 days. It is useful for *P.falciparum* and for prevention of relapses of vivax malaria (Roberts *et al*, 2001). Tafenoquine can cause acute haemolytic anaemia in G6PD deficient subjects (http://apmen.org/storage/apmen-iii/Dr%20Joerg-Peter%20Kleim.pdf) 5/8/2014. Tafenoquine is well tolerated after oral administration, with only mild and transient gastrointestinal effects (Brueckner, 1998).

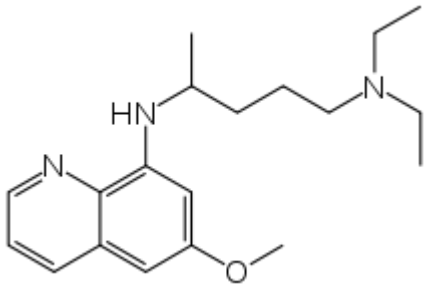

Figure 2.2 Showing structure Pamaquine

adopted from http://quod.lib.umich.edu **/m /medchem1ic/x-289/pamaquine___tif 29/10/2016**

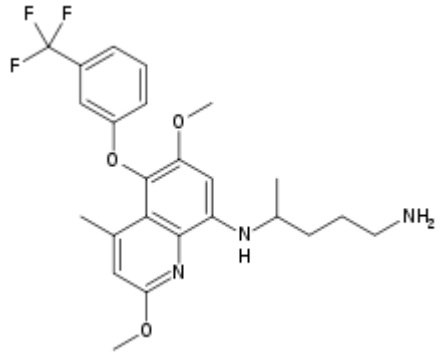

Figure 2.3 Showing structure of Tafenoquine

adopted from https://pubchem.ncbi.nlm.nih.gov/compound/Tafenoquine 29/10/2016

2.1.2 Aminoquinolines

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. They include chloroquine, amodiaquine, and quinine (O'Neill *et al.*, 1998).

2.1.2.1 Chloroquine (CQ)

Chloroquine (**Figure 2.4**) 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 (Roberts *et al*., 2001). Being alkaline, the drug reaches high concentration within the food vacuoles of the parasite and raises its pH. It is found to induce rapid clumping of the heme. Chloroquine inhibits the parasitic enzyme heme polymerase that converts the toxic heme into non-toxic hemazoin, thereby resulting in the accumulation of toxic heme within the parasite. It may also interfere with the biosynthesis of nucleic acids (CDC, 2015).

The mechanism of plasmodicidal action of chloroquine is not completely certain. Like other quinoline derivatives, it is thought to inhibit heme polymerase activity. This results in accumulation of free heme, which is toxic to the parasites. Inside red blood cells, the malarial parasite must degrade hemoglobin to acquire essential amino acids, which the parasite requires to construct its own protein and for energy metabolism. Digestion is carried out in a vacuole of the parasite cell. During this process, the parasite produces the toxic and soluble molecule heme. The heme moiety consists of a porphyrin ring called Fe (II)-protoporphyrin IX (FP). To avoid destruction by this molecule, the parasite biocrystallizes heme to form hemozoin, a non-toxic molecule. Hemozoin collects in the digestive vacuole as insoluble crystals. Chloroquine enters the red blood cell, inhabiting parasite cell, and digestive vacuole by simple diffusion (WHO, 1995). Chloroquine then becomes protonated (to CQ2+), as the digestive vacuole is known to be acidic (pH 4.7); chloroquine then cannot leave by diffusion. Chloroquine caps hemozoin molecules to prevent further biocrystallization of heme, thus leading to heme buildup. Chloroquine binds to heme (or FP) to form what is known as the FP-Chloroquine complex; this complex is highly toxic to the cell and disrupts membrane function. Action of the toxic FP-Chloroquine and FP results in cell lysis and ultimately

parasite cell autodigestion. In essence, the parasite cell drowns in its own metabolic products (WHO, 1995).

CQ 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 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 (Roberts *et al.*, 2001).

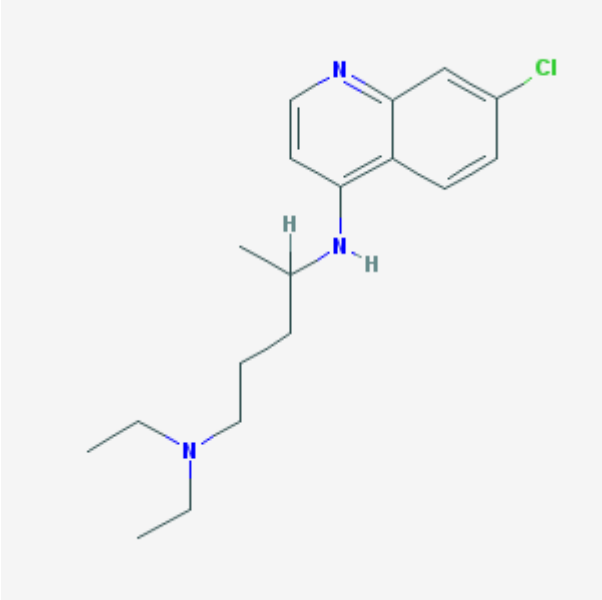

Figure 2.4 Structure of Chloroquine

https://pubchem.ncbi.nlm.nih.gov/compound/chloroquine Accessed on 5/5/2016

2.1.2.2 Amodiaquine

Amodiaquine (**Figure 2.5**) is chemically related to CQ but more effective than CQ 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).

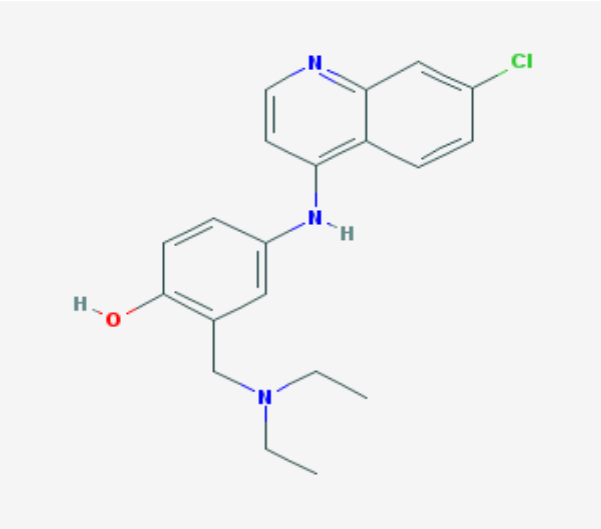

Figure 2.5 Structure of amodiaquine adopted from https://pubchem.ncbi.nlm.nih.gov/compound/amodiaquine#section 5/5/2016

2.1.2.3 Quinine

Quinine (**Figure 2.6**) is one of the four main alkaloids found in the bark of the Cinchona tree, 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). Despite of some observed resistance, it remains an essential antimalarial drug for severe *P. falciparum* where the intravenous infusion is the preferred route of administration (Roberts *et al.*, 2001).

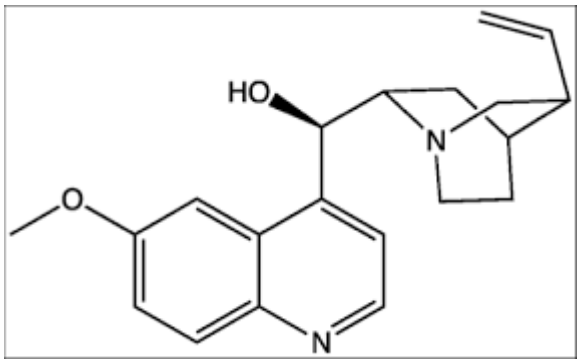

Figure 2.6 Structure of quinine adopted from https://www.google.com/ 5/5 /2016

2.1.3 Quinoline-Methanols

Commonly used antimalarial in this group is mefloquine.

2.1.3.1 Mefloquine (Lariam)

Mefloquine (**Figure 2.7**) was first introduced in 1971, this quinoline methanol derivative is related structurally 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 compound was effective against malaria, resistant to other forms of treatment when first introduced and because of its long half life was a good prophylactic, but widespread resistance has now developed and this together with undesirable side effects have resulted in a decline in its use (Foley *et al.,* 1998).

Because of its relationship to quinine the two drugs must not be used together. There have been reports of various undesirable side effects including several cases of acute brain syndrome, which is estimated to occur in 1 in 10,000 to 1 in 20,000 of the people taking this drug. It usually develops about two weeks after starting mefloquine and generally resolves after a few days (Roberts *et al.*, 2001).

Its mode of action is by binding with high affinity to parasite membranes thus causing morphological changes in the food vacuole, and interacting relatively weakly with free heme (Roberts *et al*., 2001).

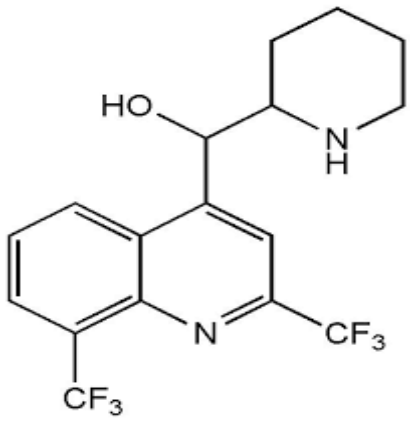

Figure 2.7 Structure of Mefloquine
https://www.google.com/search?q=mefloquine+structure&espv=2&biw 5/5/2016

2.1.4 Other Aryl Alcohols

Antimalarials included in this group are halofantrine, pyronaridine, and benflumetol.

2.1.4.1 Halofantrine

Halofantrine (**Figure 2.8**) is a drug used to treat malaria. It belongs to the phenanthrene-methanols class of compounds that includes quinine and lumefantrine. It appears to inhibit polymerisation of heme molecules (by the parasite enzyme "heme polymerase"), resulting in the parasite being poisoned by its own waste. Halofantrine has been shown to preferentially block open and inactivated HERG channels leading to some degree of cardiotoxicity (http://www.drugbank.ca/drugs/DB01218 25/02/2015). It is effective against CQ 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). The mechanism of action of halofantrine may be

similar to that of Chloroquine, quinine and mefloquine; by forming toxic complexes with ferritoporphyrin IX that damage the membrane of the parasite (WHO 1995). It is an effective antimalarial introduced in the 1980s, but due to its short half life of 1 to 2 days, is therefore not suitable for use as a prophylactic. Halofantrin has been associated with neuropsychiatric disturbances. It is contraindicated during pregnancy and is not advised in women who are breastfeeding. Abdominal pain, diarrhoea, puritus and skin rash have also been reported (http://cmr.asm.org/cgi/content/full/15/4/564).

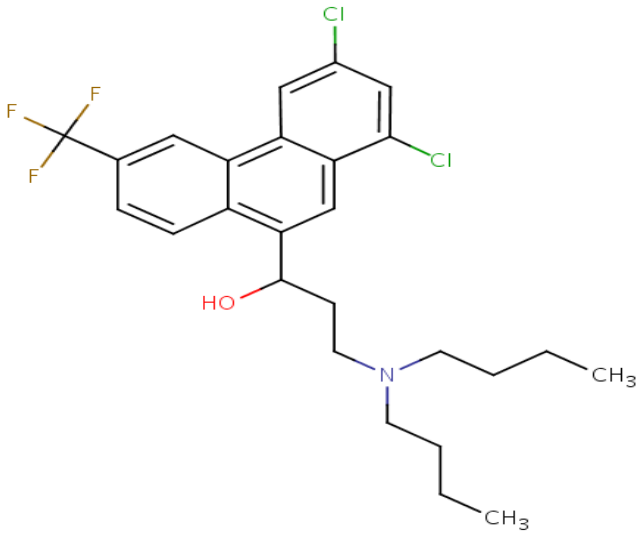

Figure 2.8 Stucture of Halofantrine adopted from http://www.drugbank.ca/drugs/DB01218

2.1.4.2 Pyronaridine

Pyronaridine (**Figure 2.9**) is a potent antimalarial compound which is an acridine derivative developed in China over 40 years ago (Chang *et al.*, 1992) and a synthetic drug widely used in China for multi resistant falciparum malaria (Elueze *et al.* 1996, Ringwald *et al.*, 1999). It inhibits the growth of *P. falciparum* during the asexual stage and early gametocyte stages (I and II) with an IC_{50} value of approximately 10 Nm (Wang *et al.*, 2014, Gupta *et al.*, 2014). It is highly effective against artemisinin-resistant

malaria and can be used in combination therapy, particularly with the artemisinin derivative, artesunate (Henrich *et al.*, 2014, Pascual *et al.*, 2012). 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). Pyronaridine, alone or in combination with artesunate, prevents recrudescence of parasites (Henrich *et al.*, 2014). Resistance to pyronaridine may be linked to genetic variations in the *P. falciparum* multidrug resistance protein 1 (Gupta *et al.*, 2014).

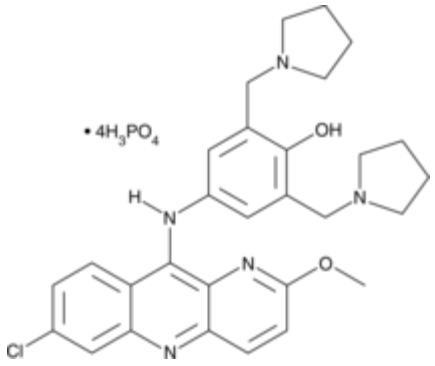

Figure 2.9 Structure of Pyronaridine adopted from

https://www.caymanchem.com/app/template/Product.vm

/catalog/16139 25/02/2015

2.1.4.3. Benflumetol (Lumefantrine)

Lumefantrine (**Figure 2.10**) 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 hemoglobin 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).

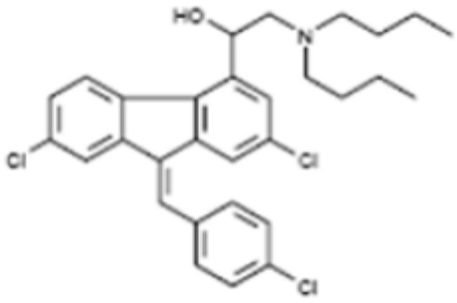

Figure 2.10 Structure of Lumefantrine http://www.rxlist.com/coartem-drug.htm 11/1/2016.

2.1.5 Folate Antagonists

These include pyrimethamine, cycloguanil, proguanil, chlorproguanil, sulfadoxine, and dapsone. These compounds interfere with the synthesis of folic acid and its derivatives, compounds involved in the transfer of C1 groups such as methyl and methylene, essential for the synthesis of thymine base from uracil in the thymidylate synthase reaction, thus inhibit the synthesis of parasitic pyrimidines and of parasitic DNA (**Figure 2.11**). There are two groups of antifolates, Dihydrofolate reductase (DHFR) inhibitors e.g pyrimethamine, proguanil and Dihydropteroate synthase (DHPS) inhibitors that are sulfones and sulphonamides like sulphadoxine and dapsone respectively. Sulfa drugs 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 *Plasmodium vivax* malaria (Roberts *et al*., 2001**).**

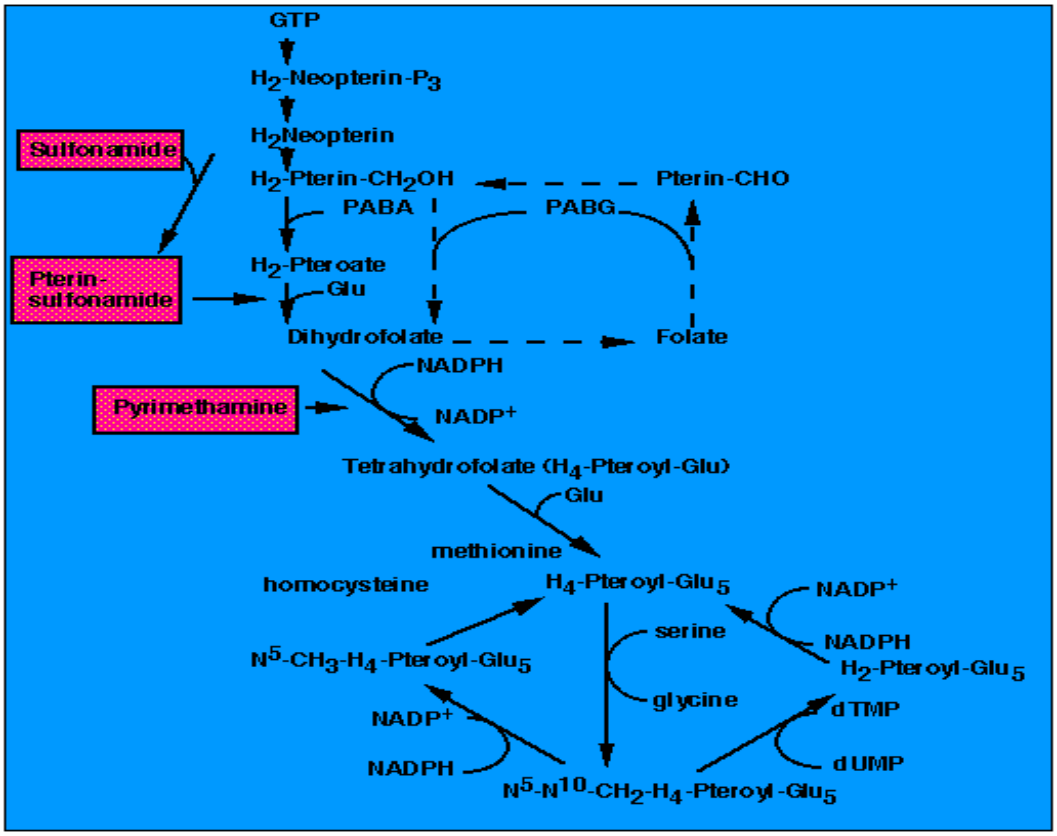

Figure 2.11Folate pathway http://www.icp.ucl.ac.be/~opperd/ parasite /folate_pathw.html 27/7/2014.

2.1.5.1 Fansidar

This is a combination drug, each tablet containing sulphadoxine 500mg. and pyrimethamine 25mg. The anti-malarial activity of Pyrimethamine (**Figure 2.12**) is that it inhibits the dihydrofolate reductase of plasmodia and thereby blocks the biosynthesis of purines and pyrimidines, which are so essential for DNA synthesis and cell multiplication. This leads to failure of nuclear division at the time of schizont formation in erythrocytes and liver. Pyrimethamine is slowly but completely absorbed after oral administration and is eliminated slowly with a plasma half-life of about 80-95 hours. Suppressive drug levels may be found in the plasma for up to 2 weeks. The drug is also

excreted in breast milk. Pyrimethamine can cause occasional skin rashes and depression of hematopoiesis. Excessive doses can produce megaloblastic anemia.

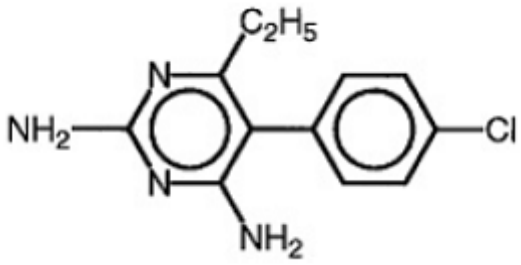

Figure 2.12 Pyrimethamine structure adopted from http://www.rxlist.com/daraprim-drug.htm 5/5 2016

Sulfadoxine (**Figure 2.13**) inhibits the utilization of para-aminobenzoic acid in the synthesis of dihydropteroic acid. The combination of pyrimethamine and sulfa thus offers two step synergistic blockade of plasmodial division. Sulfonamides are rapidly absorbed from the gut and are bound to plasma proteins. They are metabolised in the liver and are excreted in the urine. They pass through the placenta freely. Sulfadoxine is a long acting sulfonamide with a half-life of 7-9 days.

Sulfonamides can cause numerous adverse effects. Agranulocytosis; aplastic anemia; hypersensitivity reactions like rashes, fixed drug eruptions, erythema multiforme of the Steven Johnson type, exfoliative dermatitis, serum sickness; liver dysfunction; anorexia, vomiting and acute hemolytic anemia can also occur. The drug is contraindicated in patients with known hypersensitivity to sulfa, infants below 2 months of age, patients with advanced renal disease and first and last trimesters of pregnancy (Bjorkman & Howard, 1991).

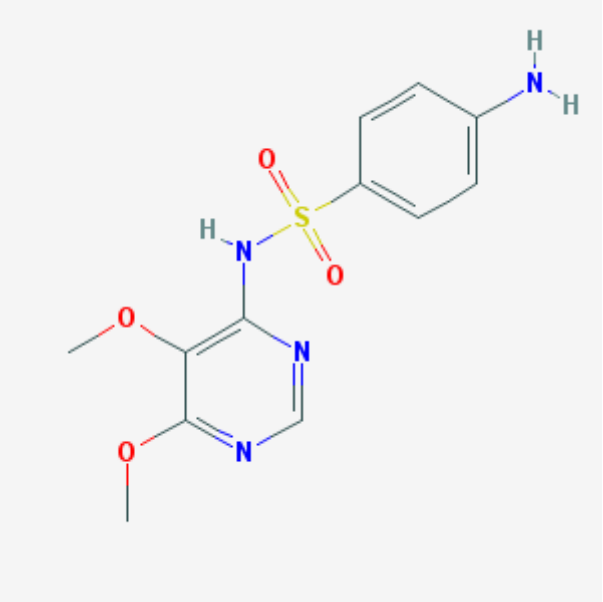

Figure 2.13 Structure of sulfadoxine adopted from https://pubchem.ncbi.nlm.nih.gov/ compound /sulfadoxine 5/5/2016

2.1.5.2 Proguanil

Proguanil (**Figure 2.14**) drug falls into the biguanide class of antimalarials and was first synthesised in 1946. It has a biguanide chain attached at one end to a chlorophenyl ring and it is very close in structure to pyrimethamine. The drug is a folate antagonist and destroys the malaria parasite by binding to the enzyme dihydrofolate reductase in much the same way as pyrimethamine. It is still used as a prophylactic in some countries (CDC 2015). Proguanil is well absorbed after oral dosage.

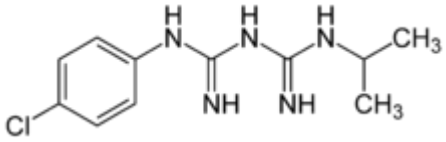

Figure 2.14 Structure of proguanil adopted from https://en.wikipedia.org/wiki/Proguanil 18/5/2016

The half life is about 16.5 hours; about 60 % of a dose of proguanil is excreted unchanged in the urine. It is essentially a pro-drug as it is metabolized to cycloguanil and 4-chlorophenyl-biguanide, the former being a potent antimalarial compound. The dosage is 200 mg adult dose, which may be divided or taken in one dose. Mechanism of resistance is through multiple mutations in the gene coding for the enzyme dihydrofolate reductase (CDC, 2015).

2.1.5.3 Malarone

In 1998 a new drug combination was released in Australia called Malarone (**Figure 2.15**). This is a combination of proguanil and atovaquone. Atovaquone became available in 1992 and was used with success for the treatment of *Pneumocystis carrinii*. When combined with proguanil there is a synergistic effect and the combination is at the present time a very effective antimalarial treatment (CDC 2001). The drug combination has undergone several large clinical trials and has been found to be 95% effective in otherwise drug resistant falciparum malaria. How long it will be before resistant strains of malaria appear remains to be seen. It has been claimed to be largely free from undesirable side effects but it should be noted that proguanil is an antifolate. This is not likely to be a problem with a single treatment course of the drug but some caution should be exercised when using it for prophylaxis. At present it is a very expensive drug (CDC, 2001).

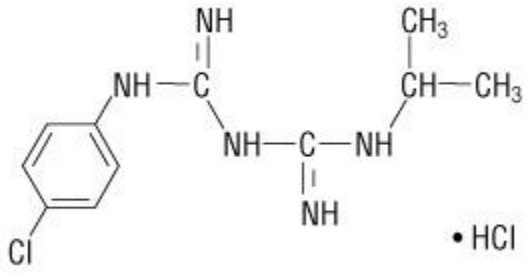

Figure 2.15 Structure of Malarone adopted from

https://dailymed.nlm.nih.gov/dailymed /archives/image 18/5/2016

2.1.6 Artemisinin

This is derived from a Chinese herbal plant called *Artemisia annua* and covers a group of products. The two most widely used artemisinin (**Figure 2.16**) derivatives are artesunate (**Figure 2.19)** and artemether **(Figure 2.18)**, others are artetheer (**Figure 2.20)** and dihydroartemisinin (**Figure 2.17).** While they are widely used in Southeast Asia they are not licensed in much of the so called "Western World", including Australia. It is now being combined with mefloquine for the treatment of falciparum malaria. In Africa artemisinin combination therapy is being recommended (WHO 2001). Prior to 2006, ACT was used to treat significant numbers of cases in just a few African countries, for example Burundi (1.2 million courses in 2005), Ethiopia (3.2 million in 2005) and Kenya (723 000 in 2005). Zambia and Zanzibar (United Republic of Tanzania) started ACT distribution in 2004, but did not report on their distribution or usage before 2006 (WHO 2010). Artemisinins are sesquiterpene trioxane lactone peroxides derived from the plant *Artemisia annua.*

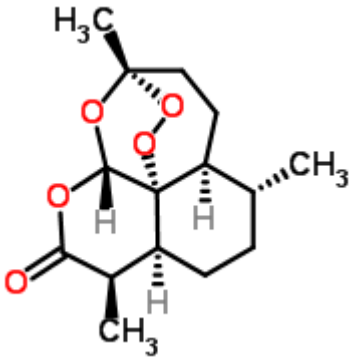

Figure 2.16 Structure of artemisinin adopted from -http://www.chemspider .com/Chemical-Structure.62060.html

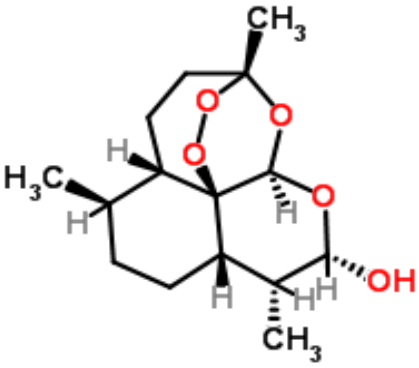

Figure 2. 17 Structure of Dihydroartemisinin from http://www.chemspider.com/Chemical-Structure.2272104.html accessed on 29/10 2016.

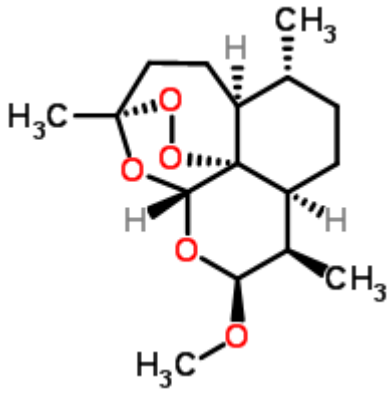

Figure 2. 18 Structure of Artemether adopted from http://www.chemspider.com/Chemical-Structure.62138.html- accessed on 13th Feb 2015.

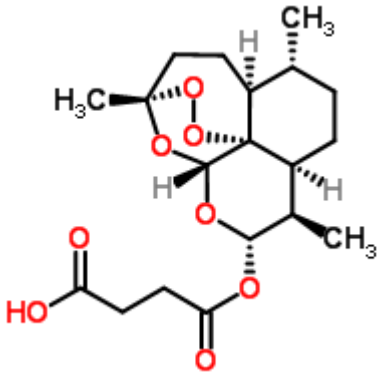

Figure 2.19 Structure of Artesunate http://www.chemspider.com/Chemical-Structure.5293084.html accessed on 13th Feb 2013

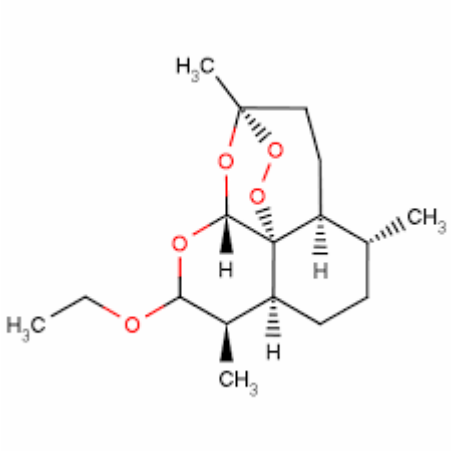

Figure 2.20 Structure of Arteether

https://www.google.com/search?

q=structure+of+arteether accessed 29/10 2016

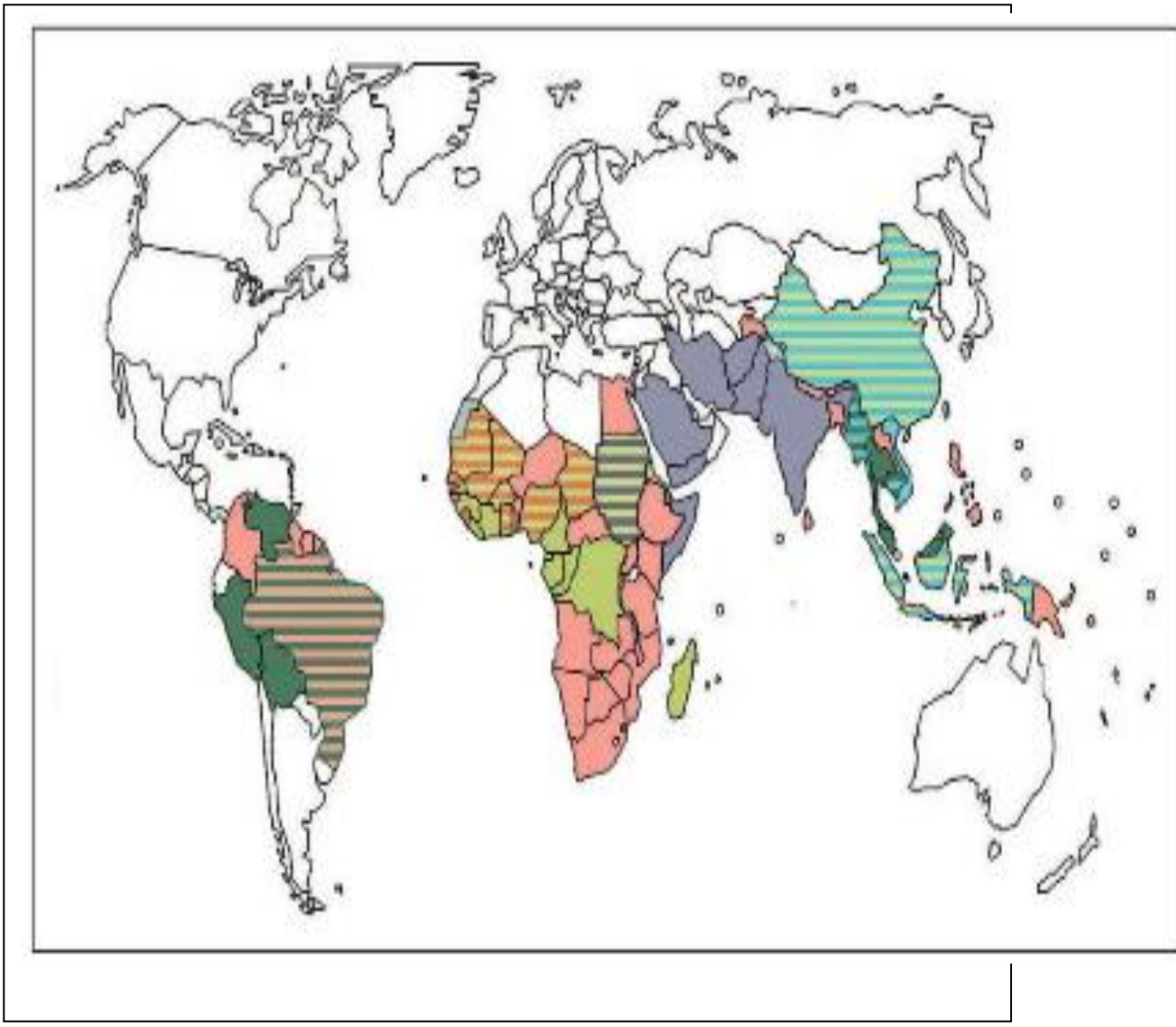

Figure 2.21 Current global distribution of artemisinin-based combination therapies as the first-line treatment of uncomplicated falciparum malaria.

[http://www.who.int/malaria/publications/atoz/9789241500470/en/index.html]. Artemether–lumefantrine; dihydroartemisinin– piperaquine; artesunate–amodiaquine; artesunate-mefloquine; artesunate– sulphadoxine/pyrimethamine.

2.1.6.1 Blend effects

Recent phytochemical research has begun to reveal a variety of blend effects in the bioactivities of plant natural products. Two principal blend effects have been demonstrated: (a) enhanced biological activity resulting from synergistic or other additive effects of moderately active or individually inactive compounds to give mixtures that are more active than a linear summation of individual activities (Romeo *et al.*, 1996; Bekele and Hassanali., 2001); and (b) mitigating effects of structurally related or unrelated compounds against rapid resistance development that characterizes most single-component antibiotics, antiparasitic drugs, and pesticides. Most plants produce a variety of secondary metabolites, which may or may not be structurally related, with multiplicity of defence and non-defence functions against different pathogens and herbivores. This phytochemical and functional diversity has undoubtedly arisen from sustained selective forces in response to succession of attack by pathogens and herbivores and other selective pressures over evolutionary time (Berenbaum, 1985; Isman *et al.*, 1996).

While enhanced biological effects of phytochemical blends have been amply recorded, very few selection experiments to demonstrate resistance-mitigating effects of blends have been demonstrated experimentally. In one selection study, the growth-inhibiting effect of a pure natural product (azadirachtin from the neem plant) and the resulting mortality of an aphid (green peach aphid) were compared with those of crude phytochemical blend (azadirachtin and a series of other limonoids) containing the same amount of the natural product at the same frequency. The pure natural product selected line demonstrated significantly decreased susceptibility to the pure natural product within 10 generations compared to the parental line. No such resistance to the natural product occurred in phytochemical-blend selected line even after 40 generations (Feng and Isman, 1995). The presence of other constituents in the phytochemical blend, many simply structural variants of the active natural product, forestalled any resistance development. A similar contrast was found in another study involving the feeding

behaviour of *Spodoptera litura*, suggesting the generality of mitigating effects of phytochemical blends against different biotic selection processes (Isman *et al.*, 1996). The precise mechanism associated with these effects remains to be elucidated.

2.1.6.2 Artemisinin Combined Therapies (ACTS)

ACTs comprise semi-synthetic artemisinin derivatives paired with distinct chemical classes of longer acting drugs. These artemisinins are exceptionally potent against the pathogenic asexual blood stages of *Plasmodium* parasites and also act on the transmissible sexual stages. These combinations increase the rates of clinical and parasitological cures and decrease the selection pressure for the emergence of antimalarial resistance (Richard and David, 2009). The poor pharmacokinetic properties of ART and its derivatives, including the short half-lives of this chemical class, translate into substantial treatment failure rates when used as monotherapy. (Woodrow and Krishna, 2006); Combining a member of this class with a longer-lasting partner drug assures sustained anti-malarial pressure after the plasma concentrations of the ART derivatives have fallen below therapeutic levels. This increases the antimalarial treatment efficacy and reduces the selective pressure for resistance.

Ideally, antimalarial combination drug partners would have similar pharmacokinetic properties so that no drug is left 'unprotected' by the other. ACTs benefit substantially from the ability of the ART derivative to rapidly reduce the parasite biomass, resulting in few parasites to be cleared by the partner drug and reducing the pool of parasites from which resistance can emerge. Several ACTs have been developed. These include Coartem, the combination of artemether with lumefantrine, and the combination of artesunate with amodiaquine, mefloquine or sulfadoxine-pyrimethamine (Balint, 2001, Bulletin of the World Health Organization 2005; Bunnag *et al*., 1995, Burk *et al.*, 2005; Olliaro *et al*., 2004., Svenson *et al*., 1998, Svensson *et al*., 1999).

Below, represented are the key partner drugs that are currently in use with artemisinin. In addition, SP has also been used in combination with the derivative AS (Bukirwa and Critchley, 2006).

Table 2.1: Plasma half-lives of drugs used in artemisinin based combined therapies adopted from Richard and Fiddock, 2009.

Antimalarial	**T ½ life of artemisinin derivative**	**T 1/2 of partner drug**	**Regions currently using the drug**
Artemether-lumefantrine	3hrs	4-5days	Africa, EM, SE Asia, WP and SA
Artesunate-mefloquine	<1 hrs	14-21 days	Africa, SE Asia, WP and SA
Artesunate-amodiaquine	<1 hr	9-18 days	Africa and EM
DHA-piperaquine	45 min	5 weeks	SE Asia
Artesunate – pyronaridine	<1hr	16 days	NA
Chloroquine	N/A	1-2 months	Africa, EM, SE Asia, WP and SA
Sulfadoxine-pyrimethamine	N/A	4 days (S)',8 days (P)	Africa, EM (IPT in Africa, EM and WP)

Key: The former first-line antimalarials are included as reference drugs. EM, Eastern Mediterranean; IPT, intermittent preventive treatment; NA, not applicable; P, pyrimethamine; S, sulphadoxine; SA, South America; SE Asia, Southeast Asia; $t_{1/2}$-half-life; WP, Western Pacific.

Semisynthetic artemisinins are obtained from dihydroartemisinin (DHA), the main active metabolite of artemisinin (Haynes *et al.*, 2002, Klayman, 1985). The first generation of semisynthetic artemisinins includes artether and artemether, the lipophilic

artemisinins, whereas artesunate is the water soluble derivative (Haynes *et al.*, 2002, Klayman, 1985). Artemisone, a second-generation artemisinin, has shown improved pharmacokinetic properties including longer half-life and lower toxicity (Haynes, 2006). So far, artesunate is the derivative that is commonly used in the antimalarial combination therapy (**Table 2.1**).

Fully synthetic artemisinin derivatives have also been designed by preserving the peroxide moiety which confers potent drug activity. These compounds are easily synthesized from simple starting materials, thus being currently under intense development (Creek *et al.*, 2008, Jefford *et al.*, 2007, Ramirez *et al.*, 2009, Taylor *et al.*, 2004).

2.1.6.3 Site of action of Artemisinin combined therapy drugs

The mechanisms by which ARTs exert their antimalarial activity remain contentious (Golenser, 2006). Nevertheless, most studies concur that the activity of ART and many if not all of its potent derivatives results from reductive scission of the peroxide bridge by reduced haem iron, which is produced inside the highly acidic digestive vacuole (DV) as it digests haemoglobin. In support of this, a recent study with fluorescent ART trioxane derivatives provided evidence for their rapid accumulation in the digestive vacuole and their activation by neutral lipid-associated haem (Hartwig, 2009). Other studies with fully synthetic endoperoxides (including trioxolanes and trioxaquines) as well as ART found that these compounds can alkylate haem, both *in vitro* with *P. falciparum* and *in vivo* in rodent malaria models, and identified a correlation between the degree of alkylation and the potency of trioxolanes against cultured *P. falciparum* asexual blood-stage parasites (Robert, 2005, Bousejra-El, 2008, Creek, 2008, Creek, 2009).

Investigations into potential protein targets of ARTs have included PfATP6, a *P. falciparum* SERCA-type calcium-dependent ATPase in the endoplasmic reticulum. Studies with transgenic *P. falciparum* asexual blood-stage parasites cultured *in vitro*

have found that ART susceptibility can be influenced by genetic changes in the loci encoding *P. falciparum* multidrug resistance protein 1 (*Pf*MDR1; also known as Pgh-1) and *P. falciparum* chloroquine resistance transporter (*Pf*CRT). Point mutations in both genes, as well as *Pfmdr1* gene duplications, are known to affect parasite responses to diverse antimalarials. The potencies of these drugs can be altered by their degree of accumulation inside the DV, which is the site of haem detoxification (Bhisutthibhan and Meshnick, 2001, Olliaro *et al*., 2001, Eckstein-Ludwig *et al.*, 2003).

2.2 *Artemisia annua*

The genus *Artemisia* is a member of the family Asteracae and comprises of more than three hundrend 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 (http://www.public.asu.edu/~ camartin/plants/Plant%20html%20files/artemisiaabsinthium.html). *A. dracunculus* is used both as a flavoring in cooking and for adding taste to vinegar. Mugworts has been featured to be effective to "women ailments" (Lee, 2002).

Artemisia annua is also known as sweet annie or annual wormwood 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 (Klayman, 1989, Klayman, 1993). It is a vigorous weedy annual, short day plant with a critical photoperiod of 13.5 hours (Ferreira *et al.*, 1995). Its total chromosome number (2n) is 36 (Bennet *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. Essential oils of *Artemisia annua* are associated with

secretory cells based on the association of monoterpenes and sesquiterpenes with well-defined secretory structures (Croteau, 1986). The oils are distributed in a way such that 36 % of the total comes from the upper third of foliage, 47% from the middle third and 17 % from the lower third, with only trace amounts in the main stem, side shoots and roots (Charles *et al.,* 1990). Leaves have glandular trichomes that are sites where many terpenoids are biosynthesized (Wallaart *et al.,* 1999).

The extract from Artemisia has marked activity against chloroquine–resistant and chloroquine-sensitive strains of *Plasmodium falciparum* and is thus useful in treatment of cerebral malaria (Yuchun *et al.,* 2002). According to Klayman (1993) the antimalarial coordinating research group (1979), treated 2099 cases of malaria (*P. vivax* and *P. falciparum)* in a ratio of 3; 1 with different dosage of qinghaosu (QHS), leading to clinical cure of all patients. In addition 143 cases of chloroquine–resistant falciparum malaria and 141 cases of cerebral malaria were treated with good results. However clinical trials revealed treatment with arteannuin (one of the compounds in *Artemisia annua*) the disease recurred sooner than following treatment with chloroquine, despite complete disappearance of parasite from patients blood (Zhou, 1986). Dharam *et al.,* (1996) cites that *Artemisia 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 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 (oral, parenteral and suppository formulations) and as three semi-synthetic derivatives artesunate, artemether, and arteether (Ramachandran, 2002). Artemisinin 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 are, however, not soluble in

water or oil and therefore, formulations of artemisinin, other than oral or rectal, are not used. Due to its poor solubility the drug absorption and its bioavailability are also poor (Ramachandran, 2002).

Figure 2.22 *Artemisia annua* plant (Reed magazine 2013)

A. annua has a very rich phytochemistry comprising several classes of compounds, mainly monoterpenes, sesquiterpenes (including artemisinin), and flavonoids (Bhakuni *et al.*, 2002). The composition of each category of these 'secondary' metabolites is variable, both qualitatively and quantitatively. The principal monoterpenoid constituents of *A. annua* from different regions are artemisia ketone (30-80%), artemisia alcohol (7-56%), camphor (3-22%), myrcene (~5%), α-guainene (<5%), germacrane D (0-18%) and 1,8-cineole (~3-13%) (Woerdenbag *et al*., 1993; Hethelyi *et al*., 1995, Ahmad and Mishra, 1994). Of the sesquiterpenes, in addition to artemisinin, a series (up to 30) of compounds related to artemisinin occur in variable amounts in different chemotypes. However, only some of these (including arteannunin B and artemisinic acid) occur in consistently higher relative amounts (Bhakuni *et al.*, 2002). Likewise, a large number of closely related non-glycosidic flavonoids and some glycosidic ones have been isolated from different *A. annua* varieties.

Indirect evidence from studies conducted at the International Centre of Insect Physiology and Ecology (ICIPE) strongly suggests that other constituents of the crude extracts of products derived from *A. annua* contribute to anti-plasmodium activity (Kangethe, 2006). Chloroform extracts from *A. annua*, with no significant amounts of artemisinin, has been found to have moderate antimalarial activity in vitro (IC_{50} ~ 10^{-5} M, compared to 10^{-8}-10^{-7}M for artemisinin) attributable to methoxy flavonoids (Liu *et al*., 1992). At lower concentrations, these flavonoids had a marked and selective potentiating effect on the antiplasmodial activity of artemisinin (Elford *et al.,* 1987; Yang *et al.,* 1989; Liu *et al.,* 1992). Of the sesquiterpenes, so far, one (arteannuin B) has been tested and shown to synergise the activity of artemisinin (Chang and But, 1986). A recent *in vitro* study undertaken at ICIPE by Kangethe 2006 suggests about four centers of anti-plasmodial activity (including one with artemisinin) in the phytochemical profile of *A. annua* (hybrid promoted by Natural Uwemba System for Health) of different polarities, with evidence of synergism between artemisinin-containing fraction and others (Kangethe, 2006).

A major concern on the use of *A. annua* for malaria is that ingestion of sub-lethal doses of artemisinin may accelerate the development of resistance to this drug. Indeed, some degree of resistance to artemisinin may have already occurred in China. The IC_{50} of pure artemisinin varies according to the strain of *P. falciparum* and can be as low as 6nM (Wongsrichanalai *et al.,* 1997). Interestingly, Chinese strains (IC_{50} = 630nM) are much less sensitive than African ones (IC_{50} = 25nM) (Wernsdorfer, 1999). Could this be due to long-standing local use in China of hot aqueous extracts of *A. annua* (with limited phytochemical composition) and/or of pure artemisinin over the last 30 years.

2.3 Parasite resistance

The emergence of resistance to antimalarial medicines is initiated by rare spontaneous mutations that give survival advantages to the parasite when exposed to a specific antimalarial compound.When exposed to the medicine in question ("drug pressure"), the mutant parasite strains that have a survival advantage are selected in favour of sensitive strains. Mutations can originate in populations of parasites from the same geographical area or in parasites from different areas. Migrating populations contribute to the development and spread of resistance by introducing parasites with resistance mutations from other geographical areas, leading to a new pool of mutated and recombined parasites in a certain geographical area. Antimalarial immunity in patients, which increases in proportion to the intensity of malaria transmission, might conceal the effects of drug resistance and delay the detection of drug-resistant infections (White, 2004, WHO/HTM/GMP/2014.3).

The techniques used to demonstrate resistance are: *in vivo*, *in vitro*, animal model testing and molecular techniques.Drug resistant parasites are often used to explain malaria treatment failure. However, they are two potentially very different clinical scenarios : The failure to clear parasitemia and recover from an acute clinical episode when a suitable treatment has been given and anti-malarial resistance in its true form.Drug resistance may lead to treatment failure, but treatment failure is not necessarily caused by drug resistance despite assisting with its development. A multitude of factors can be involved in the processes including problems with non-compliance and adherence, poor drug quality, interactions with other pharmaceuticals, poor absorption, misdiagnosis and incorrect doses being given. The majority of these factors also contribute to the development of drug resistance (http://www.eac.int/health/index.ph 2015).

The generation of resistance can be complicated and varies between plasmodium species. It is generally accepted to be initiated primarily through a spontaneous mutation that provides some evolutionary benefit, thus giving an anti-malarial used a reduced

level of sensitivity. This can be caused by a single point mutation or multiple mutations. In most instances a mutation will be fatal for the parasite or the drug pressure will remove parasites that remain susceptible, however some resistant parasites will survive. Resistance can become firmly established within a parasite population, existing for long periods of time Atovaquone is recommended to be used only in combination with another anti-malarial compound as the selection of resistant parasites occurs very quickly when used in mono-therapy. Resistance is thought to originate from a single-point mutation in the gene coding for cytochrome-b (http://www.eac.int/health/index.ph 2015).

2.3.1 Spread of Resistance

There is no single factor that confers the greatest degree of influence on the spread of drug resistance, but a number of plausible causes associated with an increase have been acknowledged. These include aspects of economics, human behaviour, pharmokinetics, and the biology of vectors and parasites. The biological influences are based on the parasites ability to survive the presence of an anti-malarial thus enabling the persistence of resistance and the potential for further transmission despite treatment. In normal circumstances any parasites that persist after treatment are destroyed by the host's immune system, therefore any factors that act to reduce the elimination of parasites could facilitate the development of resistance. This attempts to explain the poorer response associated with immunocompromised individuals, pregnant women and young children. There has been evidence to suggest that certain parasite-vector combinations can alternatively enhance or inhibit the transmission of resistant parasites, causing 'pocket-like' areas of resistance (http://www.eac.int/health/index.ph 2015).

The use of anti-malarials developed from similar basic chemical compounds can increase the rate of resistance development, for example cross-resistance to chloroquine and amiodiaquine, two 4-aminoquinolones and mefloquine conferring resistance to quinine and halofantrine. This phenomenon may reduce the usefulness of newly developed therapies prior to large-scale usage.

The resistance to anti-malarials may be increased by a process found in some species of plasmodium, where a degree of phenotypic plasticity, allowing the rapid development of resistance to a new drug, even if the drug has not been previously experienced (http://www.eac.int/health/index.ph 2015). .

The pharmokinetics of the chosen anti-malarial are key; the decision of choosing a long-half life over a drug that is metabolised quickly is complex and still remains unclear. Drugs with shorter half-life's require more frequent administration to maintain the correct plasma concentrations, therefore potentially presenting more problems if levels of adherence and compliance are unreliable, but longer-lasting drugs can increase the development of resistance due to prolonged periods of low drug concentration. The pharmokinetics of anti-malarials is important when using combination therapy. Mismatched drug combinations, for example having an 'unprotected' period where one drug dominates can seriously increase the likelihood of selection for resistant parasites. Ecologically there is a linkage between the level of transmission and the development of resistance, however at present this still remains unclear. The treatment regime prescribed can have a substantial influence on the development of resistance. This can involve the drug intake, combination and interactions as well as the drug's pharmokinetic and dynamic properties (http://www.eac.int/health/index.ph 2015).

Four main methods used to monitor antimalarial drug efficacy and drug resistance are therapeutic efficacy studies, in vitro tests, use of molecular markers and measurement of drug concentrations. Therapeutic efficacy studies allow measurement of the clinical and parasitological efficacy of antimalarials and the detection of subtle changes in treatment outcome when monitored consistently over time. They are considered the gold standard for determining antimalarial drug efficacy, and their results are the primary data used by national malaria control programmes to make treatment policy decisions. While therapeutic efficacy studies conducted according to a standard protocol provide an excellent indication of drug efficacy, additional studies are needed to confirm and characterize drug resistance and may be of some use in surveillance (Global report on

antimalarial drug efficacy and drug resistance 2010). These studies include *in vitro* studies of changes in the parasite phenotype, molecular marker studies of genetic mutations of the parasite and pharmacokinetic analyses of drug concentrations in blood.

The identification of genes that control important parasite phenotypes such as drug resistance, growth rate, and strain-specific immunity, is of immense importance in the fight against malaria. Knowledge of the gene(s) controlling resistance to a specific antimalarial drug, for example, enables the monitoring of the spread of resistance, as well as potentially increasing the effectiveness of decisions concerning drug policy. Determination of the genes involved in resistance to a drug can also help us to understand the molecular basis of drug resistance and aid in the design of new versions of drugs that are unaffected by the mutations causing parasite drug resistance (Yuvaniyama *et al,*. 2003). Similarly, the identification of genes involved in the parasite's immunogenicity is crucial in the development of vaccines. Other parasite traits for which the genetic basis is unknown include virulence (i.e., what causes some parasites to be more harmful to a host than others) and transmissibility through particular vectors. Both would be better understood if the genes underlying these traits were known (Richard *et al.*, 2005).

2.3.2 Prevention of Resistance

The prevention of anti-malarial drug resistance is of enormous public health importance. It can be assumed that no therapy currently under development or to be developed in the foreseeable future will be totally protective against malaria. In accordance with this, there is the possibility of resistance developing to any given therapy that is developed. This is a serious concern, as the rate at which new drugs are produced by no means matches the rate of the development of resistance. In addition, the most newly developed therapeutics tend to be the most expensive and are required in the largest quantities by some of the poorest areas of the world. Therefore it is apparent that the degree to which malaria can be controlled depends on the careful use of the current

drugs to limit, insofar as it is possible, any further development of resistance (http://www.eac.int/health/index.ph 2015).

Provisions essential to this process include the delivery of fast primary care where staff are well trained and supported with the necessary supplies for efficient treatment. One method proposed that aims to avoid the fundamental lack in certain countries health care infrastructure is the privatisation of some areas, thus enabling drugs to be purchased on the open market from sources that are not officially related to the health care industry. There are 2 general approaches to preventing the spread of resistance: (a) preventing malaria infections and, (b) preventing the transmission of resistant parasites (http://www.eac.int/health/index.ph 2015).

A hope for future of anti-malarial therapy is the development of an effective *malaria vaccine*. This could have enormous public health benefits, providing a cost-effective and easily applicable approach to preventing not only the onset of malaria but the transmission of gametocytes, thus reducing the risk of resistance developing. Anti-malarial therapy could be also be diversified by combining a potentially effective vaccine with current chemotherapy, thereby reducing the chance of vaccine resistance developing (http://www.eac.int/health/index.ph 2015).

2.4 Chloroquine resistance

The first type of resistance to be acknowledged was to Chloroquine in Thailand in 1957. The biological mechanism behind this resistance was subsequently discovered to be related to the development of an efflux mechanism that expels Chloroquine from the parasite before the level required to effectively inhibit the process of haem polymerization (that is necessary to prevent build-up of the toxic by-products formed by haemoglobin digestion). This theory has been supported by evidence showing that resistance can be effectively reversed on the addition of substances which halt the efflux. The resistance of other quinolone anti-malarials such as amodiaquine, mefloquine, halofantrine and quinine are thought to have occurred by similar mechanisms (http://www.eac.int/health /index.ph 2015).

The ATP-binding cassette (ABC) superfamily is one of the largest evolutionarily-conserved families of protein transporters. ABC proteins play key roles in cellular detoxification of xeno-and endobiotics. Overexpression of certain ABC proteins, among them the multidrug resistance protein (MDR) and the multidrug resistance associated proteins (MRPs), contribute to drug resistance in a variety of organisms ranging from parasitic protozoa to human neoplastic cells (María González-Pons, 2009). Membrane transporters, such as the *Plasmodium falciparum* chloroquine resistant transporter (*PF*CRT), *Plasmodium falciparum mdr1*, have been identified as key contributors in decreasing susceptibility to several anti-malarial drugs (Wilson *et al.,* 1989, Reed *et al.,* 2000, Fiddock *et al.,* 2000, Sidhu *et al.,* 2001, Barker *et al.,* 1986). Considering the genetics of drug resistance, two main genes have been implicated in chloroquine resistance: the *P. falciparum* multi-drug resistance 1 gene (*Pf*MDR1) and the *P. falciparum* chloroquine resistance transporter gene (*Pf*CRT). *PF*MDR1 protein is a membrane protein, belonging to the sub group of ABC-type multidrug transport system. *P. falciparum* gene MDR1 is localized on chromosome 5 while the *PC*MDR1 (gene homologous on *P. chabaudi* of the *PF*MDR1) *P. chabaudi* synteny map gene is localized on chromosome 12. *PF*CRT protein is a digestive vacuole transmembrane protein, associated with chloroquine resistance. *P. falciparum* gene CRT is localized on chromosome 7 and according to the *P. chabaudi* synteny map the *PF*CRT gene referred to as cg10 gene is localized on chromosome 6. It has been shown that some point polymorphisms in the **PF**MDR1 gene can be correlated with chloroquine resistance in some field isolates (Basco *et al.,* 1995, Cox-Singh *et al.,* 1995, Duraisingh *et al.,* 1997, Duraisingh et *al.*, 2000).

2.5 Resistance to Sulphadoxine-pyrimethamine

Plasmodium have developed resistance against antifolate combination drugs, the most commonly used being sulfadoxine and pyrimethamine. Two gene mutations are thought to be responsible (DHFR and DHPS), allowing synergistic blockages of two enzymes involved in folate synthesis. Regional variations of specific mutations give differing

levels of resistance. Sulfadoxine/pyrimethamine (SP) has been used as an affordable alternative treatment of uncomplicated malaria cases in chloroquine-resistant areas of Africa. However, in some East African countries; SP was adopted earlier and has been used extensively as a first-line treatment due to the high prevalence of chloroquine-resistant strains of *P. falciparum*. This antifolate drug was used intensively in these areas, which has led to the selection of resistant strains against the drug (Basco *et al.,* 2000).

Resistance to pyrimethamine is associated with mutations in the gene encoding the parasite enzyme dihydrofolate reductase (DHFR), and resistance to sulfadoxine is correlated with mutations in the parasite gene for dihydropteroate synthetase (DHPS). The level of resistance is associated with the number of mutations in the genes for these two enzymes. Therefore, multiple mutations in the two genes are considered to be responsible for SP treatment failure (Basco *et al*., 2000).

Mutations in DHFR have been reported at codons 16, 51, 59, 108, and 164 in a number of geographic isolates. Mutations S108 to N108 or T108 in DHFR has been proposed as the main mechanism of resistance against pyrimethamine. All multiple mutations emerge from stepwise selection of a single mutant at position 108 of the DHFR gene. It has also been shown that resistance level is significantly increased by additional sequence changes at positions 51 (N51 to I51) and 59 (C59 to R59) in DHFR. In addition, a point mutation at codon 164 has been suggested to be responsible for the development of resistance to chlorproguanil-dapsone. DHFR has been studied at codons 108, 51, and 59, whereas DHPS gene has been typed at positions 436, 437, 540 and 581 (Peterson *et al*, 1988). The major amino acid mutation in the DHPS gene is at residue 437 (A to G), which plays a major role in the development of clinical resistance against sulfadoxine. Mutations in the DHPS gene associated with resistance to sulfadoxine include a change of S436 to F436, A437 to G437, K540 to E540, A581 to G581, and A613 to S613 or

T613. Normally, multiple DHPS mutations result in a synergistic effect on SP resistance (Curtis *et al.,* 1998, Peters., 1999).

2.6 Resistance to artemisinins and its derivatives

On the Cambodia–Thailand border emerging artemisinin resistance has been supported by 3 independently conducted studies. In Pailin, a Province in West Cambodia, clinical monitoring carried out in 2002, indicated declining Artesunate-Mefloquine efficacy. Here the combined treatment with Artesunate (≈12 mg/kg) and Mefloquine (≈20 mg/kg) using co-blister packs (Artesunate for 3 days and Mefloquine for 1 day) showed 85.7% efficacy at day 28 follow-up (Denis *et al*.,2006). A repeat study in Pailin in 2004, which used the same drug combination but more precise dosing and follow-up at 42 days, found efficacy to be 79.3% (Denis *et al.,* 2006). To exclude cases of reinfection from analysis, parasite variants were identified by using nested PCR amplification of 3 polymorphic genes for merozoite surface protein 1 (msp1), merozoite surface protein msp2, and glutamate-rich protein, efficacy of 78.6% (95% confidence interval 66.4%–91.1%) was reported from a 28-day follow-up study of 44 patients in Thailand's Trat Province in 2003 who received the same total dosage of this combination in a 2-day regimen (Vijaykadga 2003).

The clinical observations in Pailin have been supported by molecular evidence. High copy numbers of the *Plasmodium falciparum* multidrug resistance 1 (*PF*MDR1) gene, a marker of multidrug resistance (Dondorp 2009), predicted recrudescence in the 2004 Pailin study. Even after PCR correction and adjustment for age and parasite density, clinical and molecular evidence indicated that artesunate-mefloquine failures are occurring on the Cambodia–Thailand border (WHO, 2014).

In 2008, scientists confirmed the first cases of falciparum resistance to artemisinin derivatives, also in the province of Pailin (Noedl *et al.,* 2008). In this area artemisinins were extensively used as monotherapy during the past decade, which may have

contributed to the development of resistance, with other unidentified factors (White *et al.*, 2004, Dondorp *et al.*, 2009, and Noedl *et al*., 2008). Today, there is great concern that artemisinin resistance may spread beyond the Greater Mekong sub-region or emerge independently on other continents. The WHO Global Malaria Programme published the latest status report on artemisinin resistance (WHO, 2014). Foci of artemisinin resistance have meanwhile been identified in five countries in the Greater Mekong subregion, mainly along international borders: Cambodia, the Lao People's Democratic Republic, Myanmar, Thailand and Viet Nam. Additional foci of artemisinin resistance are suspected in Suriname, Guyana and French Guiana (WHO, 2014).

All artemisinin compounds induce a very rapid reduction of parasitemia, starting almost immediately after administration, killing all stages of the malaria parasite, including young gametocytes. Artemisinin and its derivatives contain a stable endoperoxide bridge, which, it is suggested, is cleaved by intraparasitic heme. The cleaved endoperoxide becomes a carbon-centered free radical which then functions as an alkylating agent, reacting with both heme and parasite proteins (but not DNA) (Akompong *et al.,* 2000). A previous study with *P. falciparum* suggested that a sarcoplasmic and endoplasmic reticulum Calcium ATPase (SERCA)-type protein encoded by a gene denoted *pfATP6* might be the major chemotherapeutic target of these drugs (Eckstein-Ludwig *et al.*, 2003).

Over the past 20 years, clinical trials to assess the efficacy and safety of antimalarial drugs and pharmacokinetic studies in pregnant women have been conducted separately, and the artemisinin derivatives have been the subject of disproportionately more studies than all other drugs used previously (WHO, 2013, Ward *et al.*, 2007, McGready and Nosten., 2010). Although artemisinin derivatives have been implicated in fetal abnormalities in animal studies, specifically in the early stages of fetal development (Ward *et al.,* 2007, McGready and Nosten, 2010), this has not been observed among infants of women with first trimester exposure (Nosten *et al*., 2006). However, the

number of well documented first trimester artemisinin exposures has been relatively few, and at present insufficient data are available to fully assess safety. Therefore, use of artemisinins in the first trimester of pregnancy is contraindicated, and these drugs or their combinations are currently recommended only for treatment in the second and third trimesters. Studies are needed to determine optimal dosing to achieve comparable cure rates to those achieved in non-pregnant adults (O' Brien 2010).

2.7 Murine *Plasmodium*

Malaria research greatly relies on animal models, as studies with human *Plasmodium* species are very limited for ethical reasons. Human *Plasmodium* species are inaccessible for *in vivo* investigations because access to organs such as liver, lungs and spleen is not possible. Laboratory research with human *Plasmodium* species is restricted to culture of blood stages, mosquito infections via membrane feed in infection of primary human hepatocytes; therefore, it is currently not possible to reproduce the whole parasite life cycle. This limits research on host immune responses, parasite adaptations and mechanisms of disease. To overcome these limitations and to complement studies on human malaria, several animal models are available, e.g. avian, primate and rodent malaria models. The murine models are the most widely used, as they are more closely related to the human species than the avian models (Martinsen *et al.,* 2008). They are also cheaper, easier to handle, and arising less ethical concerns than primate models.The rodent *Plasmodium* species *P. berghei, P. yoelii, P. chabaudi and P. vinckei* were initially isolated from thicket rats in Central Africa and have been adapted to grow in laboratory rodents, principally mice and rats (Carlton *et al.,* 2001). The different species and laboratory strains differ in various aspects, such as their preferences to infect reticulocytes or mature erythrocytes. Apart from the great advantage that the whole parasite life cycle can be reproduced and analyzed in the laboratory, rodent malaria parasites offer the possibility of easier and faster genetic modifications than *P. falciparum* (Janse *et al.,* 2006c). Genetic manipulation of mosquitoes (Blandin *et al*., 2009), mice (Kordes *et al*., 2011) and malaria parasites (Mueller *et al.,* 2005a) allows the

investigation of specific gene functions from the host and the parasite perspectives. In addition, the generation of fluorescent parasite lines has opened the possibility to visualize the parasites live in the vertebrate host (Amino *et al*., 2007).

The importance of murine malaria models is highlighted by their wide applications, such as immunopathogenesis (Lamb *et al.,* 2006), vaccine development (Khan *et al*., 2012), drug discovery (Fidock *et al*., 2004), drug resistance (Carlton *et al.,* 2001), host-parasite interactions (Franke-Fayard *et al*., 2010) and placental malaria (Hviid *et al.,* 2010).

2.7.1 Plasmodium berghei Anka

P. berghei is a murine malaria species, originally isolated from thicket rats in Central Africa. The species is one of four common murine models but its relative ease of transfection has made it a particularly popular model for reverse genetics. Although the 'core' genome shares large block of synteny with human malaria parasites, there are several well-characterised breaks in synteny and the gene organisation in the subtelomeres is substantially different. http://www.sanger.ac.uk/resources/downloads/protozoa/plasmodium-berghei.html.

Plasmodium berghei is one of the many species of malaria parasites that infect mammals other than humans. *P.berghei* is one of the four species that have been described in murine rodents of West Africa. The rodent parasites are not of direct practical concern to man or his domestic animals. The interest of rodent malaria parasites is that they are practical models for the experimental study of mammalian malaria. These parasites have proved to be analogous to the malarias of man and other primates in most essential aspects of structure, physiology and life cycle (Carter and Diggs., 1977).

Several species of malaria parasites of rodents other than murines have been described but these have been little studied and, since none of their complete life cycles has been described for any of these parasites, there is insufficient material available for critical comparisons to be made (Killick-Kendrick., 1978).

Figure 2.23 Foci of murine malaria in Africa (from: Landau and Chabaud, 1994).

The species and subspecies of rodent malaria are listed in **Table 2.1**. All parasites have been collected from either murine rodents or from mosquitoes in Central Africa (see **Figure 2.20** for the foci of murine malaria in Central Africa). The original discovery of *P. berghei* was made by Vincke and Lips in 1948. A number of isolates (strains) have since then been collected from the wild (see **Table 2.2** and the overview of *P.berghei* isolates clones from the University of Edinburgh). The four murine malaria species does

not interbreed and can be distinguished from each other by small differences in morphological and developmental characteristics and iso-enzyme patterns (see **Table 2.2** for a number of morphological and developmental differences). Many of the subspecies can interbreed under laboratory conditions. They are separated mainly on the basis of geographical distribution and iso-enzyme patterns (Carter and Diggs, 1977 and Beale *et al.*, 1978).

The genome of both *P. falciparum* and the four rodent parasites are organised into 14 linear chromosomes, ranging in size from 0.5-3.8 Mb. They possess identical telomeric repeats [CCCT (A/G)AA], organised into tandem arrays. The chromosomes are compartmentalised into relatively conserved internal regions (core regions) flanked by highly polymorphic subtelomeric regions. These variable subtelomeric regions are the sites where gene families are located encoding proteins that are transported to the surface of the infected erythrocyte and hence involved in antigenic variation and immune evasion. In addition the subtelomeric regions contain numerous and varied DNA repeats. Both the subtelomeric genes and many of the variant multigene families are species specific and are not conserved between rodent and human parasites. In both *P. falciparum* and *P. berghei* rearrangements in the subtelomeric regions, involving repeat sequences, are the main cause of intra-species size differences between homologous chromosomes. Comparative mapping of genes located in the central regions of the chromosomes has shown that both linkage and gene order appear to be well conserved between human and rodent parasites, resulting in significant level of synteny between these species (Carlton *et al.,* 1998, Lin *et al.,* (2001). Moreover, conservation of specific gene domains, regulatory control elements (Janse *et al.,* 1994)), organisation of complex genomic loci (Thompson *et al.,* (2001) and the presence of conserved multi-gene families in rodent and human parasites (Homewood and Neame 1980) all

emphasize the high similarity of genome organisation, gene content and gene-regulation (Landau and Chabaud 1994).

Table 2.2: The species and subspecies of rodent malaria parasites (adopted from Killick Kendrick 1978 , Landau and Boulard 1978).

(Sub)Species		Host		Vector
P. berghei		*Grammomys surdaster*		*Anopheles dureni*
		Praomys jacksoni		
		Leggada bella		
Isolate:	K173	Isolated from:	*Grammomys surdaster* (1948)	
	SP11		*A.dureni* (1961)	
	ANKA*		*A.dureni* (1966)	
	LUKA		*A.dureni* (1966)	
	NK65*		*A.dureni* (1964)	
P. yoelii yoelii		*Thamnomys rutilans*		?
P. yoelii nigeriensis		*Thamnomys rutilans*		?
P. yoelii killicki		*Thamnomys rutilans*		?
P. chabaudi chabaudi		*Thamnomys rutilans*		?
P. chabaudi adami		*Thamnomys rutilans*		?
P. vinckei vinckei		*?*		*Anopheles dureni*
P. vinckei petteri		*Thamnomys rutilans*		?
P. vinckei lentum		*Thamnomys rutilans*		?
P. vinckei brucechwatti		*Thamnomys rutilans*		?

* isolates collected in the regions Katanga and Kasapa of Democratic Republic of Congo: ANKA - Antwerp/Kasapa; NK - New York-Katanga.

Table 2.3: Different characteristics of the four rodent malaria parasites and human parasites (Carter and Diggs 1977, Kellick Kendrick 1978, Landau and Boulard 1978 and Landau and Chabaud 1994).

	berghei	***yoelii***	***chabaudi***	***vinckei***	**Human parasites**
Merozoites per schizont	12-18	12-18	6-8	6-12	8-16 (32)
Reticulocyte preference	Yes	Yes	No	No	Yes/No
Synchronous blood infection	No	No	Yes	Yes	Yes/No
Optimum temperature mosquito transmission	19-21	23-26	24-26	24-26	>26
Oocyst size (µm)	<45	60-75	50	45-54	50-60
Sporozoites in glands (days after infection)	13-14	9-11	11-13	10-13	dependent on temperature
Mean diameter pre-erythrocytic schizonts (µm)	27	35-50	38-45	35	45-60
Duration of pre-erythrocytic cycle (hours)	48-52	43-48	50-58	60-72	6-15 days
Duration of asexual blood stage cycle (hours)	22-24	18	24	24	48-72
Developmental time gametocytes (hours)	26-30	27	36?	27	48h-12days
Developmental time ookinete (hours)	18-24	18-24	18-24?	18-24?	12-24
Microgamete (size in µm)	15	16	-	-	16-25
Sporozoite (size in µm)	11-12	14-16	10-15	11-21	10-14
Ookinete (size in µm)	10-12	11	-	8-10	11-20

2.7.1.1 Genome size

By comparative and quantitative cytophotometric measurements of DNA of the haploid stages of *P. berghei* the genome size has been estimated at 2.5 x 107 bp (Cornelissen *et al.,* (1984). By comparison of the size of pulsed field gel electrophoretic separated chromosomes the genome size has been estimated at 2.3-2.4 x 107 bp (Ponzi *et al*., 1990). This genome size of *P. berghei* is comparable to the genome size of 22.8 Mb of *P. falciparum* (Gardner *et al.,* 2002).

2.7.1.2 Base composition

Like *P. falciparum*, the nuclear DNA of *P. berghei* has an extremely high overall A+T content of about 82% (McCutchan *et al.,* 1984). This (A+T)-rich bias is unevenly distributed between protein coding and non-coding regions. All open reading frames are relatively (G+C)-rich (25-30%), while the (A+T) composition of the vast majority of the intergenic regions and intragenic introns can rise to more than 90% (Carter and Diggs, 1977, Landau and Chabaud, 1994).

2.8 *Plasmodium yoelii*

Plasmodium yoelii is a parasite of the genus *Plasmodium* subgenus *Vinckeia*. Like all *Plasmodium* species *P. yoelii* has both vertebrate and insect hosts. The vertebrate hosts for this parasite are mammals. Three subspecies are recognised: *P. yoelii killicki*, *P. yoelii nigeriensis* and *P. yoelii yoelli*. It's most notable feature is that it is used in the laboratory to infect mice, as a model of human malaria, particularly with respect to the immune response (Landau and Chabaud 1994).

It is advantageous to have a whole-animal model of malaria because often it is difficult to know which factors to study *in vitro*, particularly in a complex system like the immune system. Moreover, for many experiments it is not ethical or practical to use humans. One of the special things about this particular model is that it has two strains

with vastly different pathogenicity. These are generally referred to as the "lethal" and "non-lethal" strains of the species. Comparison of these two strains can be used to deduce which factors may contribute to more serious malaria infections in humans (Landau and Chabaud, 1994).

2.9 Statement of the problem

Multidrug-resistant strains of *P. falciparum* have compromised the effectiveness of anti-malaria drugs. While no stable artemisinin resistance has been documented in *P. falciparum* high likelihood of emergence of artemisinin resistant parasites. Therefore need to explore avenues to mitigating development of artemisinin resistance by use of whole extract of *Artemisia annua*. Presence of other phytochemical constituents in *A. annua* may therefore have a significant effect on reducing the rate of resistance development in *P. falciparum* and *P. berghei.*

2.10 Justification

Effective treatment is essential for malaria control. However, drug-resistant malaria has become a challenge in malaria control programs in recent years. The emergence of multidrug-resistant strains of *P. falciparum* has compromised the effectiveness of routinely used anti-malaria drugs. This has threatened the use of the cheap and safe drugs in resource-poor countries where the mosquito control has been ineffective. Most of the antimalarial drugs have been shown to have developed varying resistance. This has resulted to the use of a combination of drugs (Artemisinin Combination Therapy) to try and minimize the problem; presently a combination of Arteminisin and Lumefantrine (AL) (Coartem) is the front line drug in Kenya. The present study is designed to determine if resistance can build up in the *Artermisia annua* blend in comparison to pure artemisinin. It would also guide us on whether to isolate the artemisinin first and then combine it with other drugs with a longer half-life or make use of the total crude extracts from this plant. If safe, efficacious and tolerable medication could be produced from

locally-grown medicinal plants; such preparations may offer an additional tool for malaria control, especially in socio-economic circumstances that preclude the availability or accessibility of the more expensive synthetic anti-malarial drugs.

2.11 General objective

To compare resistance development using *Artemisia annua* phytochemical extract and pure artemisinin on *in-vitro* cultures of *P. falciparum* and *in vivo* using *Plasmodium berghei* and *P. yoelii*.

2.11.1 Specific objectives

1. To udertake dose-response activities of the *Artemisia annua* extract on selected strains of *P. falciparum* and pure artemisinin and compute their $IC_{50.}$
2. To determine effective doses (ED_{50} and ED_{90}) of pure artemisinin and *Artemisia annua* extract on *P. berghei*.
3. To determine stability of *P. falciparum* and *P. berghei* 'resistant' strains.
4. To amplify, sequence and detect any mutations in the sequences of resistant parasites of *P. falciparum* and *P. berghei* anka

CHAPTER THREE
MATERIALS AND METHODS

3.1 Study site and study design

The study was conducted in accordance with KEMRI guidelines on animal care and use. Additionally; the study followed the internationally accepted principles for laboratory animal use and care, as found in WHO guidelines). Specifically 21 gauge needles were used in the animal experiments. Mice that died during the experiment as well as those that were sacrificed by exposure to choloroform fumes were at the end of the experiment put in plastic bags and incinerated. Permission to carry out the study was granted by KEMRI'S Scientific Steering Committee and the Ethical Review Committee (Study SSC No. 1340/08).

3.2 Culture work (*in-vitro*)

For the in vitro work serum required for the maintenance of cultures was obtained from KEMRI donors after having signed the consent form indicated in appendix. The donors went through HIV and hepatitis screening before enrolling for the study. Permission to carry out the study was granted by KEMRI'S Scientific Steering Committee and the Ethical Review Committee (Study SSC No. 1340/08).

3.2.1 Source of *A. annua,* artemisinin and optimization of extraction procedure

Artemisia annua plants used in this study were obtained from Tanzania highlands (2000-2200 m altitude) in Arusha. The leaves were harvested just before flowering, dried for approximately 3 weeks under shade, and then crushed, powdered and homogenized. Samples of dried, whole-leaf uniformly powdered *A. annua* were extracted sequentially with solvents of increasing polarity (hexane, Dichloromethane, ethanol, and water), the extracts were analyzed by high performance liquid chromatography (HPLC) and chromatographs were compared to ensure total extraction of the compounds in *Artemisia*

annua. The combined extract was filtered and concentrated under a flow of nitrogen to remove the organic solvents. The crude extract in the final solvent was then freeze-dried. Stock solutions of this and that of pure artemisinin were made by first dissolving in (DMSO) and then diluting with water.

3.2.1 Preparation of *Artemisia annua* extract

Stock solutions were made by dissolving the extract in diemethyl sulfoxide (DMSO) and then diluting with water. The start concentration was 250 µg/ml.

3.2.2 Preparation of pure artemisinin

Stock solutions were made by dissolving the pure artemisinin in diemethyl sulfoxide (DMSO) and then diluting with water. The start concentration was 10 ng/ml

3.3 *In-vitro* study of resistance to *A. annua* extract and pure artemisinin

3.3.1 Cultures of *P. falciparum*

Two different isolates of laboratory–adapted *P. falciparum* cultures were available at Kenya Medical Research Institute (KEMRI) Nairobi, Kenya: D6 (CQ-sensitive strain from Sierra Leone) and W2 (CQ-resistant strain from Indochina) and were used for the study. Blood from human O+ was used to provide erythrocytes (red blood cells) that served as host cells for the parasites. Sera were obtained from blood groups A, B, and O (donors signed appropriate consent forms before donation). These were pooled and utilized for the maintenance of the cultures.

The culture medium used was a variation of that described by Trager and Jensen (1976) and consisted of RPMI 1640 supplemented with 15% human serum in citrate-dextrose anticoagulant (ACD), 25 mM HEPES (N-2-hydroxyethylpiperazine-N’-2-ethylsulfonic

acid) and 25 mM $NaHCO_3$, and were incubated at 37^0C in an atmosphere of 3% CO_2, 5% O_2 and 92% N_2. The human erythrocytes used were less than 28 days old from the day of donation.

3.3.2 Dose-response studies of *Artemisia annua* extract and artemisinin

Standard procedure described by Trager and Jensen (1976) were used. One 96-well micro-titre plate was used to test the *Artemisia annua* extracts as well as the pure artemisinin at different doses in triplicate (3 columns of 8 wells each for 7 drug dilutions and 1 control) against both D6 and W2 cultures (**Figure 3.1**)

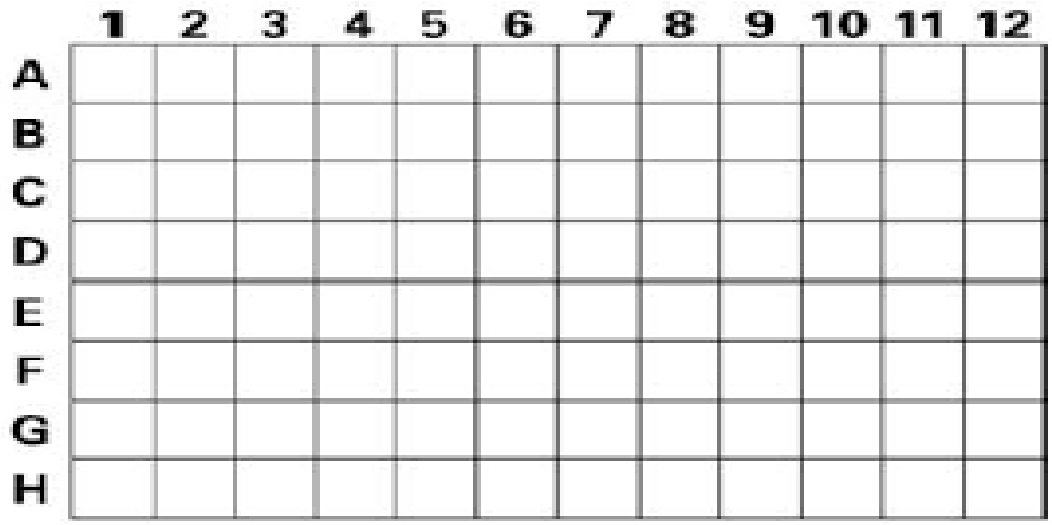

Figure 3. 1 A 96 well plate set up for IC_{50} determination.

Key

Well A1, A2, A3 dosed with *Artemisia annua* extract 250 µg/ml. After doing serial dilution wells B1, B2 B3 will have a concentration of 125µg/ml. A similar trend followed for artemisinin wells A4, A5, and A6 were dosed with 10µg/ml artemisinin and

serial dilution was then done. CQ was used as control. Wells in row H did not have the pure artemisinin or the *Artemisia annua* extract.

Stock solutions of the extract and artemisinin (containing 250μg ml^{-1} of each) were filter-sterilized (using 0.22 μm and 0.45μm filters) before dilutions with the culture medium. Aliquots (200μl) of a 1.5% (vol-vol) suspension of parasitized erythrocytes in the culture medium (0.4% parasitaemia) were added to all test wells and the plate incubated at 37^0 C in a gas mixture of 3% CO_2, 5% 0_2 and 92 % N_2. After 48 hours 0.5 μCi (G-^{3}H) hypoxanthine was added and the test samples incubated for additional 18 hours. The contents of each well were harvested (using a cell harvester machine) on to glass fibre papers and radioactivity measured using a liquid scintillation counter (micro-beta) as indicated by the flow diagram below (**Figure 3.2**).

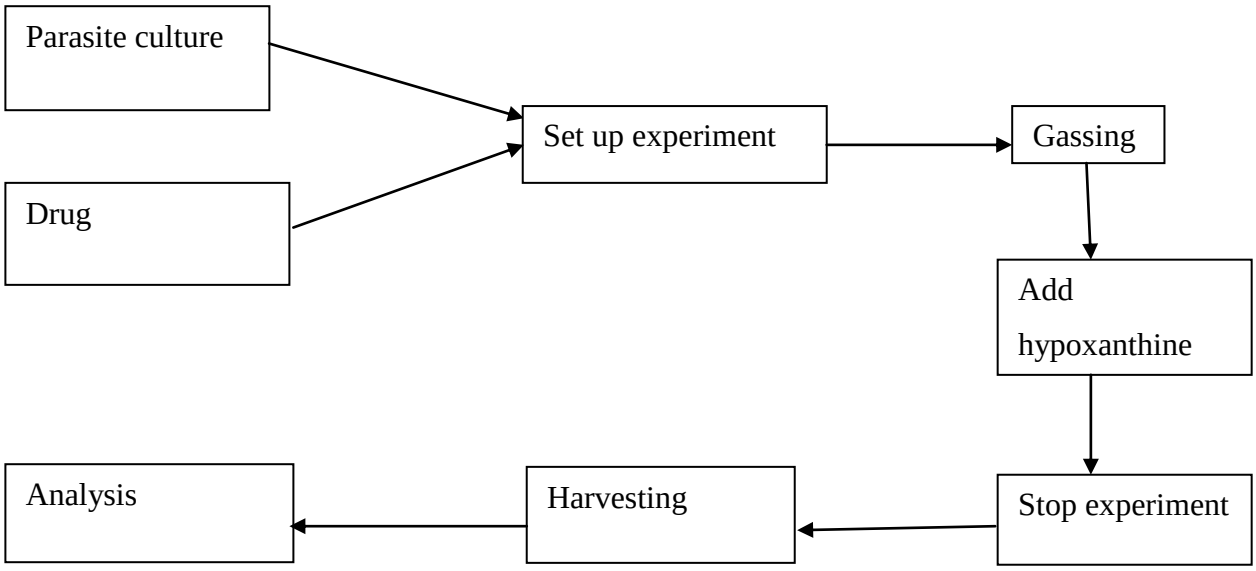

Figure 3.2 Experimental set up of the *in vitro* experiments

Parasitic growth was calculated and expressed as a percentage of the zero-drug control. The concentration of the test materials that inhibited 50% (IC_{50}) was calculated using non-linear regression analysis. This involved computation of the concentration of each

drug causing 50% inhibition of (G-^{3}H) hypoxanthine uptake (IC_{50}).this calculation was carried out using Chemosen Program 2 according to the following formula,

$$IC_{50} = \text{antilog}\ (\log X_I + [\ (\log Y_{50} - \log Y_I) \times (\log X_2 - \log X_I)]/\ \log Y_2 - \log Y_I,$$

where Y_{50} is the cpm (counts min^{-1}) value midway between parasitised and non-parasitised control cultures, and X_I, Y_I, X_2, Y_2 are the concentrations and cpm values, respectively, for the data points above and below the cpm midpoints (Sixsmith *et al*., 1984).

3.3.3 Generation of drug resistant parasite strains

3.3.3.1 Cyclic exposures with *Artemisia annua* and artemisinin

IC_{50} and IC_{90} values of artemisinin and the blend were initially determined with the W2 and D6 strains of *Plasmodium falciparum*. The values obtained were used to calculate appropriate equivalents of artemisinin and the blend, which were separately incorporated in RPMI 1640 media that were enriched with 6% pooled serum. In each set of three replicates of exposures, medium change was done after every cycle of 48 hours. Parasitemia was determined during media change and, if higher than 2.0, it was diluted to 0.4%. At the end of 10 cycles of exposures to artemisinin or *A. annua* blend, sensitivities (i.e. IC_{50} or IC_{90}) to the two materials, as well as chloroquine as a positive control, were measured. The new IC_{50} or IC_{90} equivalents were then used in the next set of 10 cycles of exposures to artemisinin or *A. annua* blend. This was repeated at cycle 20, 30, and 40 cycles (except in exposure experiments with IC_{90} equivalents of artemisinin, when the experiment was terminated at 30 cycles).

3.3.3.2 Resistance development

IC_{50} determination after every drug cyclic exposures of the parasites helped to calculate resistance which was expressed as resistance sensitivity index (RSI), with RSI_{50} defined as the ratio of the IC_{50} of the resistant line to that of the sensitive, parent line. The RSI_{50} values were ranked into four categories, based on previous work (Merkli and Richle, 1980): (1) RSI_{50} = 1.0, sensitive, (2) RSI_{50} = 1.01–10.0, slight resistance, (3) RSI_{50} = 10.01–100.0, moderate resistance and (4) RSI_{50} > 100.0, and high resistance.

3.3.4 Preparation of DNA template

3.3.4.1 Glass Fiber Membranes Samples

Preparation of DNA template was as described by Warhust *et al* (1991). Blood spots on GFM were outlined with a pencil to clearly mark the spot. Each GFM was supported on a fresh 2.5 cm Whatman filter paper disc (Whatman®) and placed in a sintered glass vacuum filter (Millipore®) then washed with 2-3 ml sterile distilled water to lyse blood. Blood proteins were removed by washing with 2-3 ml of sterile normal saline. The prepared GFM was then air dried at room temperature for 1-2 hrs and stored in fresh labelled self seal bags at 4°C with desiccant to maintain dryness.

3.3.4. 2 Filter paper samples

For the samples collected using filter papers (Whatman 903), they were extracted using DNA extraction kit protocol from Roche. The scapel and the forceps were immersed into 5 M HCL followed by neutralization with 5 M NaOH and a brief rinse with distilled autoclaved water. These were then dried using a clean tissue. The filter paper was then

removed from the plastic bag using forceps. The area of filter paper with the blood spot was cut using a scapel on a piece of glass without allowing the scapel blade or the forceps to come into contact with the blood spot. The piece of filter paper was then transferred to a sterile 1.5 ml microfuge tube (eppendorf ®) using the forceps, cleaning scapel blade, glass and forceps between samples.

Table 3.1 Primers for *in vitro* DNA

The **Table 3.3** is a summary of primers used in the *in vitro* studies.

Gene		Primer	Sequence	
*Pf*MDR1-86	Primary	A1	TGT TGA AAG ATG GGT AAA GAG CAG AAA GAG	Forward
		A3	TAC TTT CTT ATT ACA TAT GAC ACC ACA AAC	Reverse
	Inner	A2	GTC AAA CGT GCA TTT TTT ATT AAT GAC CAT TTA	Forward
		A4	AAA GAT GGT AAC CTC AGT ATC AAA GAA GAG	Reverse
*Pf*MDR-1034		B1	TGC ATT TAG TTC AGA TGA TG	Forward
		B2	AAT GTT GCT ACT TCT CTT C	Reverse
		B3	TGG TTT AGA AGA TTA TTT CTG	Forward
		B4	AAA TAA CAT GGG TTC TTG AC	Reverse
TCTP		Tctp1	TTC AAT TGA CCT CCA CTG CTT:	Forward
		Tctp2	CCT GCT TCC ATA TCA AGT GAT TC	Reverse

KEY: *Pf*MDR1-86 *P. falciparum* multidrug resistant gene at codon 86

*Pf*MDR-1034 *P. falciparum* multidrug resistant gene at codon 1034

*P. f*TCTP- *P. falciparum* translationally controlled tumour protein

3.3.5 PCR amplification of PfMDR 1- 86;

3.3.5.1 Primers for PCR amplification of PfMDR 1- 86;

The following primers were used in amplification. Primary primers: MDR A1 and MDR A3 (MDR/A1: TGT TGA AAG ATG GGT AAA GAG CAG AAA GAG ;
MDR /A3: TAC TTT CTT ATT ACA TAT GAC ACC ACA AAC), nested primers MDR /A2 and MDR /A4 (MDR /A2: GTC AAA CGT GCA TTT TTT ATT AAT GAC CAT TTA; MDR /A4: AAA GAT GGT AAC CTC AGT ATC AAA GAA GAG),

3.3.5.2 Primary PCR *Pf*MDR1-86

After the extraction the sample DNA or the clone DNA was put in 0.2 or 0.5 µl eppendorf tubes taking care not to cause cross contamination. DNA for the control parasites was included i.e DNA for negative and positive controls was included for each possible alleles at the locus being amplified. Positive controls were W2, 3D7 7G8, D6, and VIS.

The PCR premix was prepared while always working on ice with the following concentrations 0.1 µM of each nest 1 Primers, 1x standard PCR buffer, (1.5 mM $MgCL_2$, 50 mM KCL, 10mM Tris HCL {PH 8.3}, 0.5% DMSO, 200 µM of each of the dNTPS {100mM dATPs, 100mM dTTPs, 100mM dCTPs, 100mM dGTPs}, 1U of Taq polymerase (Kemri taq), in a final reaction volume of 25 µl.

The samples were placed into the PCR thermocycler (My cycler Bio rad) and the following amplification programme was used. Initial denaturing at 94 $^{\circ}$C for 3 min and then 40 cycles of denaturing at 94 $^{\circ}$C94^{0}C for 1 min, annealing 45 $^{\circ}$C 1min, extension 72 $^{\circ}$C for 1 min, followed with a final extension at 72 $^{\circ}$C for 3 min ,and then held at 4^{o}C. The PCR product was stored at -20^{0} C awaiting further analysis.

3.3.5.3 Nested PCR

The primary PCR product stored at -20° C was thawed and kept on ice when working. Each of the two primary primers was prepared (10 µM solution {50 µl volume} separately using DNase water. PCR tubes were labeled for the nested amplification reactions making sure not to contaminate the tubes when removing them from the packet. Controls were included, two positive from primary PCR, the negative control from the primary PCR and a negative control for the nested reaction mix.
PCR premix was prepared, working on ice at all times. For each sample 25 µl of premix was used.

An aliquot of 23 µl of the premix was added into each labeled tube recapping after use. Appropriate product from primary PCR was added and the tubes placed into the PCR machine (My cycler Bio Rad) and the following programme was run. Primary denaturing at 94 ° C for 3 min, denaturing at 94 ° C for 30 seconds, annealing at 45 ° C for 1min, Extension at 72 ° C for 1 min, cycles were 40 with a final extension at 72 ° C for 3 min, and a hold at 4°C. The PCR product was stored at -20 ° C awaiting further analysis.

3.3.5.4 Analysis of *Pf* MDR 1-86 PCR

Premix (23 µl) was added to each labeled tube and then added the DNA (samples and controls). The tubes were placed into the PCR machine and run using the following programme: primary denaturation at 94 ° C for 3 min, denaturation 94 `° C for 30 sec, 45 ° C for 1 min, extension 72 ° C for 3 min, and an infinite hold at 4 ° C.

An agarose gel was prepared in 1x TBE. Ethidium bromide was included in the gel and also the running buffer at 0.5 ug/ml. Five microlitres of each amplification reaction was

loaded onto the gel with 1 µl gel loading buffer. A molecular weight marker (100bp) was included.

The gel electrophoresis machine was put on and the gel was run at <5V/cm at 0.80 volts and amp. The gel was then visualized on UV transilluminator and a photograph gel taken.

3.3.6 Pf MDR 1 -1034, Pf MDR 1-1042, Pf MDR 1-1246

3.3.6.1 Primers for *Pf* MDR1034

Primary primers: MDR /B1 and MDR /B2 (MDR/B1: TGC ATT TAG TTC AGA TGA TG; MDR /B2: AAT GTT GCT ACT TCT CTT C), nested primers MDR /B3 and MDR /B4 (MDR /B3: TGG TTT AGA AGA TTA TTT CTG; MDR /B4: AAA TAA CAT GGG TTC TTG AC) as indicated in **Table 3.1**

3.3.6.2 Primary PCR

Twenty mM solution of mixed dNTPS was prepared comprising of 20 µl of 100 mM dATP, 20 µl of 100 mM dTTP, 20 µl of 100 mM dCTP, 20 µl of 100 mM dGTP and DNase free water. The solution was kept on ice when working. Ten milimolar solutions (50 µl volumes) of each of the two primary primers were prepared separately using dnase free water. PCR tubes were labeled for primary amplification. Positive and negative controls for each of the possible alleles at the locus being amplified were included (3D7 and 7G8).

DNA sample were thawed, spun briefly in a microfuge (10,000x g) and a PCR premix was prepared. Premix (20 µl) was aliquot into each of the labeled tubes, recapping each tube. Appropriate DNA was added to each tube and keeping tubes capped at all times when not in use. The tubes were placed into the PCR machine and the programme was run with the following conditions: Primary denaturing at 94^0C for 3 min, denaturing at 94 $^\circ$C for 30 sec, annealing at 50 $^\circ$C for 60 sec, extension at 65 $^\circ$C for 2 min, repeating

the above for 30 cycles and a final extension at 65 ° C for 5 min and a hold at 4 ° C. The PCR product was stored at -20°C awaiting further analysis.

3.3.6.3 Nested PCR

The primary PCR product was stored at -20^0 C and 10 um solutions (50 µl volumes) of each of the two nested primers were prepared separately using DNAse free water. PCR tubes were labeled and a PCR premix (25 µl per sample) was prepared. An aliquot (23 µl) of the premix was added to each labeled tube recapping each tube after aliquoting. Three microlitres of the appropriate primary PCR product was added to each and keeping tubes capped at all times when not in use. The tubes were then placed into the PCR machine and he programme was run with the following conditions; Primary denaturing at 94^{0}C for 3 min, denaturing at 94 ^{0}C for 30 sec, annealing at 50^{0}C for 60 sec, extension at 65^{0}C for 2 min, repeating the above for 30 cycles and a final extension at 65^{0}C for 3 min and a hold at 4^{0}C. Agarose gel (1.5%) in 1x TBE buffer was prepared. Ethidium bromide and running buffer at 0.5 ug/ ml was included in the gel. Five µl of each amplification reaction was loaded onto the gel with 1 µl gel loading buffer (containing bromophenol blue). A molecular weight ladder was included and run at <5V /cm for 30 min. the gel was visualized on UV transilluminator and photograph gel. The remaining 20 µl of the PCR product was stored at -20^{0}C.

3.3.6.4 Purification and sequencing *Pf* MDR 1034

The nested PCR products were purified with the thermoscientific purification kit (FE KO701 GeneJET^{TM} PCR purification kit) and the purified products were then sent to International Livestock Research Institute (ILRI) for sequencing. Nested primers (B3, B4) were used for sequencing of the PCR products for *Pf* MDR 1034.

3.3.7 Amplification of Translationally Controlled Tumour Protein (TCTP)

TCTP sequence for *Pf* 3D7 was obtained from the internet using the PlasmoDB and the sequence was used to design primers. The following primers were designed using the primer3plus programme (http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi) TCTP 1: TTC AAT TGA CCT CCA CTG CTT: and TCTP 2: CCT GCT TCC ATA TCA AGT GAT TC and the designed primers were used in the amplification and sequencing of the TCTP gene product. The conditions for the PCR reaction were as follows , primary denaturation at 94^0C or 3 min, 94^0C for 1 min, 53^0C for I min, 65^oC for 2 min, 65^oC for 3 min and the cycle no was 30 with an infinite hold at 4^oC.

3.3.8 Purification and Sequencing of the PCR products

The PCR products were purified with the thermo scientific purification kit (FE KO701 GeneJET^{TM} PCR Purification kit) and they were then sent to International Livestock Research Institute (ILRI) where the sequencing was done using the primers TCTP1 and TCTP2.

3.4 *In-vivo Plasmodium berghei* Anka and *Plasmodium yoelii*

3.4.1 Parasites strains and test

To select artemisinin and the *Artemisia annua* resistance, a strain of *P. berghei* ANKA, resistant to pyrimethamine obtained from the MR4 repository (MRA-865, MR4, ATCC_ Manassas, Virginia) was used. The *Plasmodium yoelii yoelii* (*P. yoelii yoelii* 17x)-isolate used in this study was also obtained from MR4 repository.

3.4.1.1 Infection of experimental mice

Male, random-bred Swiss albino mice (20 ± 2 g), were obtained from KEMRI and were maintained in the animal house. They were each infected intra-peritoneally with donor blood containing approximately 2 X 10^7 parasite red blood cells (PRBC) in 0.2 ml inoculum. Infection was assessed by microscopic estimation of the proportion of infected erythrocytes in Giemsa-stained thin smears made from tail-vein blood. The animals were housed in experimental room in a standard Macrolon type II cages clearly labeled with experimental details at 22 ^{0}C and 60–70% relative humidity and fed on commercial rodent feed and water *ad libitum*. Each cage housed five mice during the test.

3.4.1.2 Drug treatment

Artemisia *annua* used for the in vivo experiments was prepared as described earlier.

Table 3. 2 Experimental layout for *in vivo* tests

Artemisia annua	Pure artemisinin
Conc 1-5 mice	Conc 1-5 mice
Conc 2-5 mice	Conc 2-5 mice
Conc 3-5 mice	Conc 3-5 mice
Conc 4-5 mice	Conc 4-5 mice
Control-5 mice	Control-5 mice

3.4.2. Determination of 50 % and 90 % effective-dose level (ED_{50} and ED_{90})

3.4.2.1 Four day test

Fifty percent and 90% effective doses (ED_{50} and ED_{90}) were measured in a quantitative standard method '4-day test' (4-DT), in which the parasites were exposed to four, daily, drug doses (Peters, 1975). Drugs were administered by oral route starting on the day 0 (4 h post-infection) and continuing for a total of four daily doses, days 0–3 (24, 48 and 72 h

post-infection). Parasite count was estimated by microscopic examination of Giemsa-stained thin smears prepared from tail snips on day 4, 96 h post-infection. Percentage chemosuppression of each dose was then calculated as

Percentage chemosuppression of parasitaemia was calculated using the following formula:

$$\% \text{ chemosuppression} = \frac{\text{Parasitaemia in negative control - Parasitaemia in study group}}{\text{Parasitaemia in negative control}}$$

(Tona *et al*., 2001). ED_{50} and ED_{90} were estimated using a linear regression line.

3.4.2.2 One day test

The drug pressure tests were carried out by treating once with the already determined doses which were measured using the '1-day test' (1-DT), in which the parasites were exposed to a single drug dose (Vennerstrom *et al*., 2004). Drugs were administered by oral route on day 1, (24 h post-infection). Parasite count was estimated by microscopic examination of Giemsa-stained thin smears prepared from tail snips on day 3, 72 h post-infection. Percentage chemosuppression of each dose was then calculated as indicated in the four day test.

3.4.3 Procedures for exerting drug-selection pressure and assessing the level of resistance

After inoculation (2×10^7 parasitized red blood cells contained in 0.2 ml inoculums) of 5 mice on day zero (D0), the mice were then orally treated once with the drug at a concentration equivalent to ED_{90}, on day 3 (D3) 72 h post-infection. Thereafter, parasitaemia was monitored daily until it reached 2–5%, when a mouse was selected for passaging of PRBC to the next naive group of five mice. This was repeated for the next nine passages.

The level of resistance was evaluated after every 10 passages by measurement of ED_{50} and ED_{90} in the standard 4-DT which permits the calculation of an 'index of resistance', RSI_{50} and RSI_{90} (defined as the ratio of the ED_{50} or ED_{90} of the resistant line to that of the sensitive, parent line).

The RSI_{50} and RSI_{90} values were ranked into four categories, based on previous work (Merkli and Richle, 1980): (1) $RSI_{50/90}$ = 1.0, sensitive, (2) $RSI_{50/90}$ = 1.01–10.0, slight resistance, (3) $RSI_{50/90}$ = 10.01–100.0, moderate resistance and (4) $RSI_{50/90}$ > 100.0 high resistance.

3.4.4 Stability study of *P. berghei* and *P. yoelii*

The stability of artemisinin and the blend resistant line was evaluated by measuring drug responses after making 10 drug free passages followed by measurement of ED_{50} and ED_{90}. Stable resistance was defined as the maintenance of the resistance phenotype when drug-selection pressure was removed for at least 10 passages in mice (Gervais *et al*., 1999).

3.4.4.1 Primer design for the *in vivo* work

Using the programme primer 3, primers were designed for sequencing and several pairs were selected for amplification. The designed primers are indicated in the **appendix 1**.

3.4.4.2 *In-vivo* DNA extraction

Plasmodium berghei Anka DNA was extracted from either whole blood or from filter paper using the Roche extraction kit and the procedure used was as described by roche in the manual supplied together with the kit. The DNA was then stored at -20^0C until when the PCR amplification was to be done.

3.4.4.3. *Pb*MDR amplification

Materials required for amplification were similar as those used for amplifying PFMDR except for the primers and amplification conditions. The primers used here were selected from the primers designed for sequencing *PB*MDR. These were 1F and 9R and they had the following sequences.

*PB*MDR 1F: ATCAGGAGCTTCGTTGCCTA

*PB*MDR 9R: GGGCTTGAACAAAAGATCCA

Primary denaturation 95 ^{0} C for 5 minutes, Denaturation 95^{0} C for 1 min , annealing 48^{0} C for 45 sec, extension 68^{0} C for 5 min, final extension 72^{0} C for 10 min and an infinite hold at 4^{0}C.

3.4.4.4 *Pb*CG10 amplification

Similar materials as those used in the amplification of *Pb*MDR were used except for the primers and the amplification conditions. The primers used were *Pb* cg10 1F and *Pb* cg10 6R. Their sequences were as follows.

*PB*CG10:1F: TGATGATCGCTATAAAGAATTGG

*PB*CG10 6R: TTTTCTTACAGCATCGCCcta

Amplification conditions were as follows, Primary denaturation 95^{0} C for 5 minutes, denaturation 95^{0} C for 1 min, annealing 48^{0}C for 45 sec, extension 68^{0}C for 3 min, final extension72^{0}C- for 10 in and an infinite hold at 4^{0}C.

3.4.4.5 *Pb*ATPase amplification

*PB*ATPASE 1F: CGTGGTTTATCCGAAAATGAA

*PB*ATPASE 10R: ACATATTTCGCCAAGGTGGT

Primary denaturation 95 ^{0} C for 5 minutes

Denaturation 95^{0} C for 1 min , annealing 48^{0} C for 45 sec, extension 68^{0} C for 3.5 min,×30 cycles final extension72^{0}C- for 10 in and an infinite hold at 4^{0}C.

3.4.4.6 *P. yoelii* MDR

The following primers and conditions were applied in amplification of *P. yoelii* MDR,

P. yoelii MDR 1F: AGCTTCGTTGCCCATTTTTA

P.yoelii MDR 12R: AGATGCAATTCTGTGAGCAAT

Primary denaturation 95 ^{0} C for 5 minutes

Denaturation 95^{0}C for 1 min, annealing 48^{0}C for 45 sec, extension 68^{0}C for 3.5 min, ×30 cycles final extension 72^{0}C for 10 min and an infinite hold at 4^{0}C.

3.4.4.7 *Py* CG10 gene.

The following primers and conditions were used in the amplification of the Py CG 10

P. yoelii cg10 IF: CCAATGGAAgtaataaccaaacg

P. yoelii cg10 8R: TTGGTTTTCTTACAGCATCACC

Primary denaturation 95 ^{0} C for 5 minutes

Denaturation 95^{0} C for 1 min , annealing 48^{0} C for 45 sec, extension 68^{0} C for 3.5 min,x30 cycles final extension72^{0}C- for 10 minutes and an infinite hold at 4^{0}C.

3.4.4.8 *Py* ATPAse

P. yoelii ATPASE 1F: TGGATGAGAATCGGGGTTTA

P. yoelii ATPASE 10R: ACGGGAAATGACCACAAAAA

Primary denaturation 95^{0} C for 5 minutes

Denaturation 95^{0}C for 1 min, annealing 48^{0}C for 45 sec, extension 68^{0}C for 3.5 min, x 30 cycles final extension 72^{0}C for 10 min and an infinite hold at 4^{0}C.

,

3.4.4.9 Sequencing *P. berghei* and P *.yoelii yoelii*

PCR amplicons were purified using the thermo scientific Purification kit (FE K0701 GeneJETTM. PCR PURIFICATION KIT) and they were then sent to ILRI for sequencing. The sequencing primers for each gene are as indicated in Appendix 1. The primers were designed using the primer 3 programme.

3.5 Statistical Analysis

Relative sensitivity indices, RSI (IC_{50} new /IC_{50} initial, or IC_{90} new/IC_{90} initial) were calculated after each cycle of 10 exposures in each set of treatments. Student–Newman–Keuls (SNK) method was used to compare RSI values obtained from each set of treatments at different 10-fold exposures. Data was entered in excel sheet and analyzed with SPSS.

CHAPTER FOUR

RESULTS

4.1 HPLC chromatographs

After extracting the *Artemisia annua* with the different solvents the extract was analyzed with HPLC and **Figure 4.1** shows the compounds present in *Artemisia annua.*

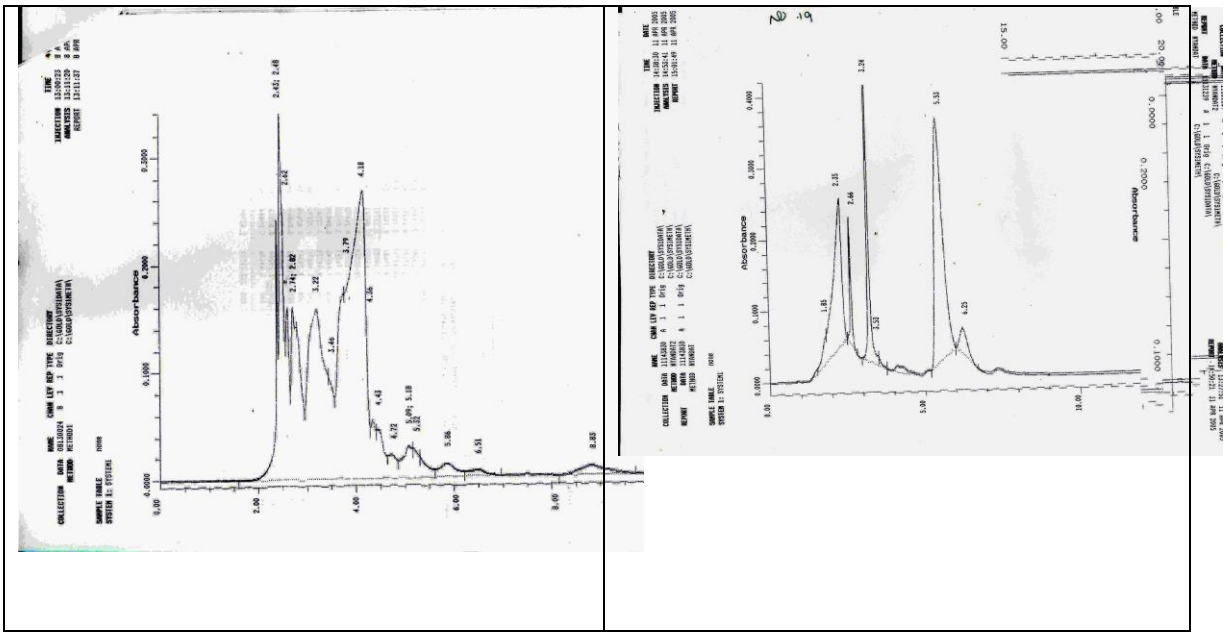

Figure 4.1Chromatographs present in the *Artemisia annua* extract

4.2 *In-vitro* resistance assays using *Plasmodium falciparum*-W2

4.2.1. IC_{50} and Relative sensitive index (RSI) for *P. falciparum*

The following table (**Table 4.1**) indicates values obtained after determining IC_{50} equivalents of artemisinin at different cycles. The RSI was also determined as indicated in the table. When Artemisinin was tested it was 0.2ng/ml at cycle 0, 0 0.362 ng/ml at cycle 10, 0.48 ng/ml at cycle 20, 4.533 ng/ml at cycle 30 and 5.372 ng/ml at cycle 40. Relative sensitivity index (RSI) was used as an indicator of resistance and for parasites tested with artemisinin had an RSI that increased with increase in the number of cycles. RSI was 1.81 for cycle 10, 2.42 for cycle 20, 22.66 for cycle 30 and 26.45 for cycle 40. Chloroquine was included in the study as a control drug.

On the other hand the parasites exposed to artemisinin at IC_{50} and tested with the *Artemisia annua* extract were rather stable and they remained sensitive to the extract. The RSI at cycle 10 was 0.09, at cycle 20 it was 0.09, 1.89 at cycle 30 and 0.07 at cycle 40. It is worth noting that the parasites remained sensitive to the extract even after exposure to artemisinin.

The RSI for parasites cyclically exposed to artemisinin and tested with artemisinin increased with increase in exposure cycles (**Table 4.2**). The parasites cyclically exposed to artemisinin and tested with *Artemisia annua* remained rather stable.

Table 4. 1:IC_{50} and relative sensitivity index (RSI) of *P. falciparum* cyclically exposed to artemisinin at IC_{50} and tested with i) artemisinin and ii) *Artemisia annua* extract and iii) chloroquine

	Cycle 0	**Cycle 10**		**Cycle 20**		**Cycle 30**		**Cycle 40**	
	IC_{50}	IC_{50}	**RS1**	IC_{50}	**RS1**	IC_{50}	**RS1**	IC_{50}	**RS1**
ART	0.2	0.362	**1.81**	0.48	**2.42**	4.533	**22.66**	5.372	**26.45**

Extract	1.6	0.15	**0.09**	0.14	**0.09**	2.995	**1.9**	0.106	**0.07**
CQ	57	57.7	**1.01**	64.5	**1.13**	49.478	**0.87**	81.688	**1.43**

Key:

ART concentrations were in ng/ml, while *Artemisia annua* concentrations were expressed in µg/ml

Table 4. 2: Means and standard errors for Relative sensitivity Indices (RSI) of *P. falciparum* cyclically exposed to artemisinin and tested with (i) artemisinin ii) *A. annua* extract and iii) chloroquine

CYCLES	ART MEAN±SE	Extract MEAN±SE	CQ MEAN±SE
RSI cycle 10	1.81±0.83[a]	0.09±0.01[a]	1.01±0.03[a]
RSI cycle 20	2.42±0.84[a]	0.09±0.01[a]	1.13±0.10[b]
RSI cycle 30	22.66±2.95[b]	1.89±0.14[b]	0.87±0.01[a]
RSI cycle 40	26.45±0.87[b]	0.07±0.00[a]	1.43±0.03[c]
p-value	<0.001	<0.001	<0.001

Key

Mean ± SE followed by the same letter(s) within the same column do not differ significantly from one another (One-way ANOVA, SNK-test, $\alpha = 0.05$).

The results of RSI obtained at successive 10-fold cycles of treatments with artemisinin are depicted in **Figure 4.2** and **Figure 4.3.**

RSI for parasites exposed to artemisinin at IC_{50} equivalents and tested with artemisinin increased with increasing cycles. This showed that the parasites became less sensitive to artemisinin as the exposure period increased. RSI for parasites exposed to IC_{90} equivalents of artemisinin and tested with artemisinin increased with an increase in

cycles. At cycle 10 the parasites were more sensitive to artemisinin but as the cycles increased they became less sensitive.

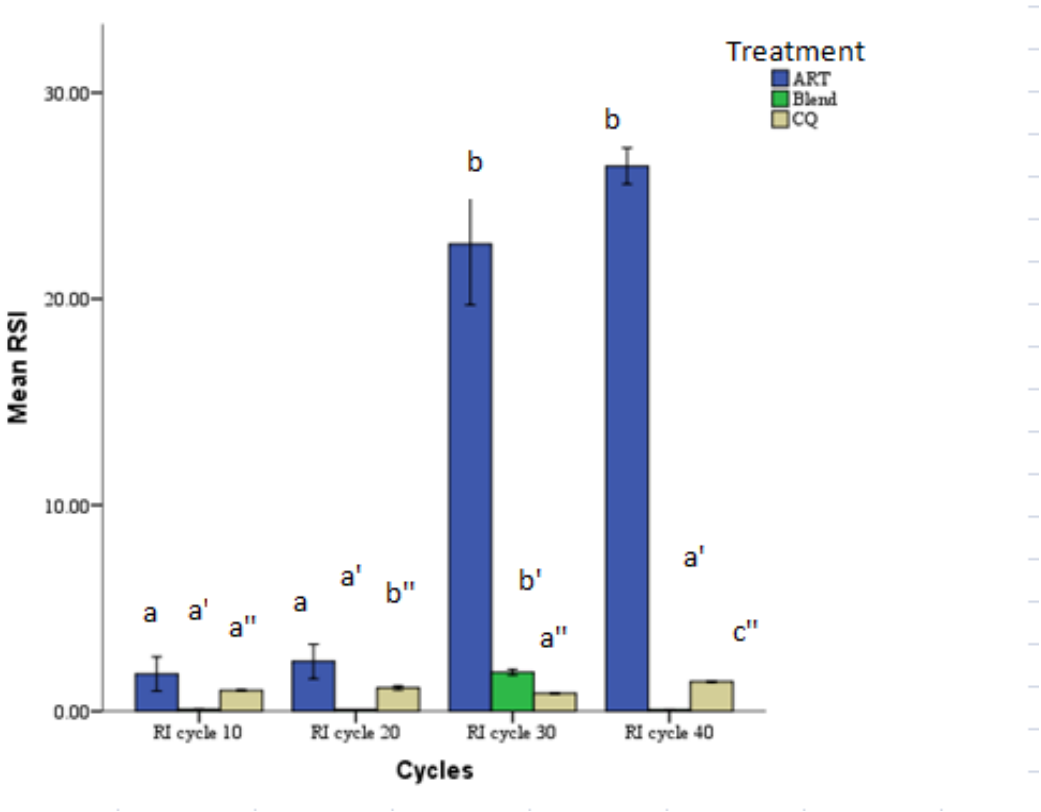

Figure 4.2.Relative sensitivity indices (RSI) of *P. falciparum* cyclically exposed to artemisinin at IC_{50} and tested against (i) artemisinin (ii) *A. annua* extract and (iii) Chloroquine

Key:

Unprimed number (a) represents significance levels in column analyzing exposure with artemisinin.

One prime on the number (a$^{'}$) represents significance levels in analyzing exposure to *A.annua* extract.

Two primes on the number (a$^{''}$) represents significance levels in analyzing exposure to Chloroquine.

Parasites exposed to artemisinin at IC_{90} had the following IC_{50} after determining the activity at different cycles as indicated by **Table 4.3.** At cycle 0 the IC_{50} reading was 0.2 ng/ml, at cycle 10 it was 0.17 ng/ml, at cycle 20 it was 1.47 ng/ml, at cycle 30 it was 3.228 ng/ml. When the same was used with the extract the values were, at cycle 0 it was 1.6 mg/ml, at cycle 10 it gave 0.96 mg/ml , at cycle 20 it gave 2.42 mg/ml, cycle 30 was 0.355 mg/ml. **Table 4.4** indicates the RSI means and standard deviations for parasites exposed to artemisinin at IC_{90} equivalents. Parasites were cyclically exposed to cycle 50 but the micro-beta counter machine broke down hence no *in vitro* results for cycle 40 and cycle 50.

Figure 4.3 indicates the RSI means at cycle 10, cycle 20 and cycle 30. There was an increase of RSI means with increase with the number of cycles for artemisinin exposed parasites that were tested against artemisinin. The RSI of parasites cyclically exposed to artemisinin at IC_{90} and tested with artemisinin had an increase in the RSI with an increase in the number of cycles, 0.82 at cycle 10, 7.36 at cycle 20 and 16.04 at cycle 30. When the parasites were exposed to artemisinin at IC_{90} and tested with the *A. annua* extract the RSI remained very low. It was 0.61 at cycle 10, 1.51 at cycle 20 and 0.22 at cycle 30. The extract tested parasites remained sensitive the *A. annua* extract.

Table 4. 3:IC_{50} and RSI for *P. falciparum* parasites exposed to IC_{90} equivalents of artemisinin and tested with (i) *Artemisia annua* extract (ii) artemisinin and (iii) Chloroquine

	Cycle 0	**Cycle 10**		**Cycle 20**		**Cycle 30**	
	IC_{50}	IC_{50}	**RS1**	IC_{50}	**RS1**	IC_{50}	**RS1**
ART	0.2	0.17	**0.82**	1.47	**7.36**	3.228	**16.04**
Extract	1.6	0.96	**0.61**	2.42	**1.51**	0.355	**0.22**
CQ	57	63.03	**1.11**	87.14	**1.53**	64.149	**1.13**

Table 4. 4:Means and standard errors for Relative Sensitivity Indices (RSI) of *P. falciparum* cyclically exposed to artemisinin at IC_{90} and tested on i) artemisinin ii) *A. annua* extract and iii) Chloroquine

CYCLES	Art MEAN±SE	Extract MEAN±SE	CQ MEAN±SE
RI cycle 10	0.82±0.13[a]	0.61±0.12[b]	1.11±0.01[a]
RI cycle 20	7.36±0.13[b]	1.51±0.08[c]	1.53±0.08[b]
RI cycle 30	16.04±1.24[c]	0.22±0.01[a]	1.13±0.05[a]
p-value	<0.001	<0.001	0.002

Key

Mean ± SE followed by the same letter(s) within the same column do not differ significantly from one another (One-way ANOVA, SNK-test, $\alpha = 0.05$)

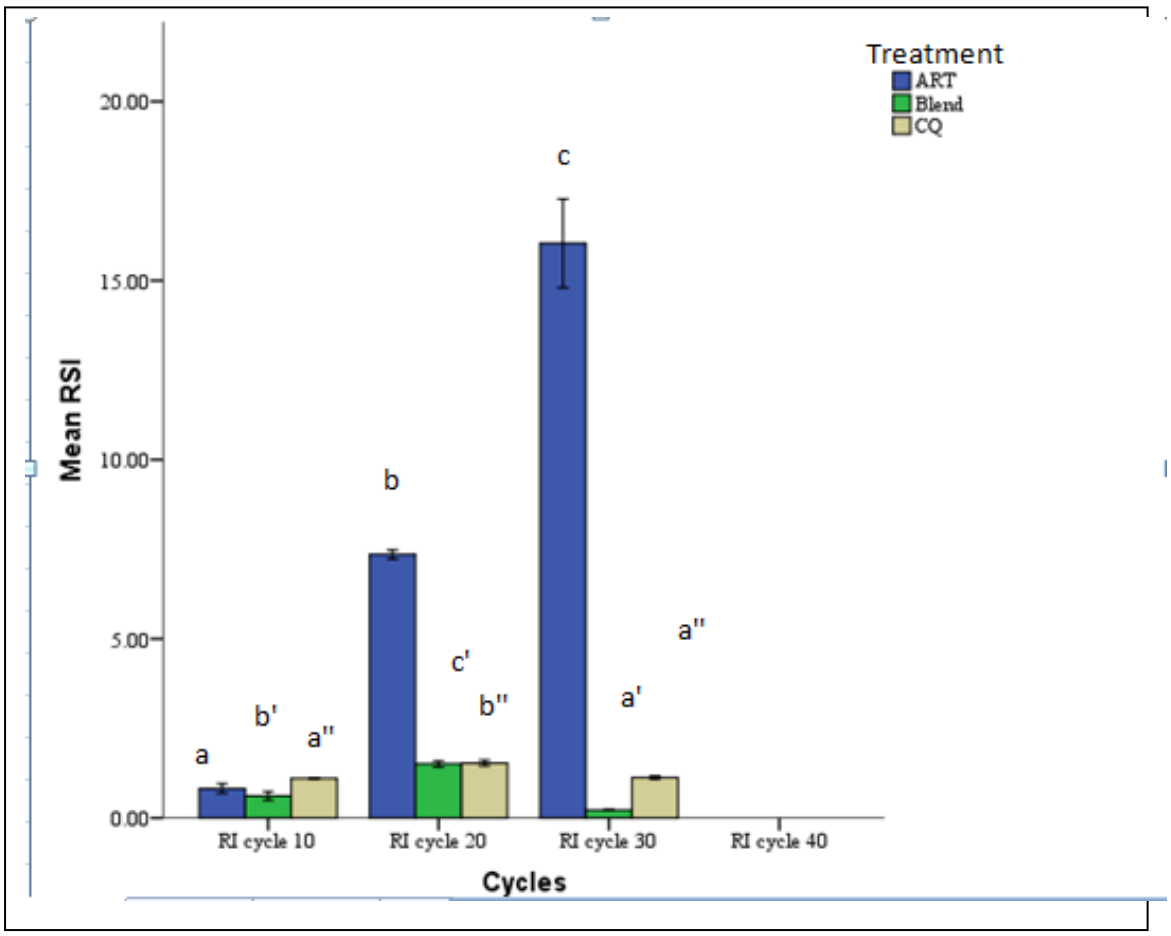

Figure 4. 3 Relative sensitivity indices (RSI) of *P. falciparum* cyclically exposed to artemisinin at IC_{90} and tested against (i) artemisinin, (ii) *A. annua* extract and (iii) Chloroquine

Key:

Unprimed number (a) represents significance levels in column analyzing exposure with artemisinin.

One prime on the number ($a^{'}$) represents significance levels in analyzing exposure to *A.annua* extract.

Two primes on the number ($a^{''}$) represents significance levels in analyzing exposure to Chloroquine

4.2.2. Effects of cyclic treatments of W2 strain of *P. falciparum* to (a) IC_{50}, and (b) IC_{90} values of *A. annua* extract

The results of RSI obtained at successive 10-fold cycles of treatments with *Artemisia annua* are depicted in **Figure 4.3** and **Figure 4.4**. For the parasites that got exposed to the extract equivalent of IC_{50} the following IC_{50} values were got when artemisinin was used, at cycle 0 it was 0.2ng/ml, at cycle 10 it was 0.19ng/ml, at cycle 20 it was 0.61 ng/ml, at cycle 30 it was 5.688 ng/ml, and at cycle 40 it was 5.89 ng/ml. the extract on the other side at cycle 0 was 1.6mg/ml, cycle 10 was 1.33, at cycle 20 was 0.26, at cycle 30 it was 1.344 and at cycle 40 was 3.415 (**Table 4.6**).

The parasites that were cultured in the presence of *Artemisia annua* at IC_{50} equivalents and tested with artemisinin showed an interesting trend in that the RSI increased with increase in cycles, and hence the parasites were less sensitive to artemisinin. With artemisinin the RSI was 0.93 at cycle 10, 3.00 at cycle 20, 27.99 at cycle 30 and 29.00 at cycle 40 (**Table 4.5**)

Table 4. 5:IC_{50} and RSI for parasites exposed to IC_{50} equivalents of *A.annua* extract and tested with (i) artemisinin (ii) *A. annua* extract (iii) Chloroquine

	Cycle 0	Cycle 10		Cycle 20		Cycle 30		Cycle 40	
	IC_{50}	IC_{50}	**RS1**	IC_{50}	**RS1**	IC_{50}	**RS1**	IC_{50}	**RS1**
ART	0.2	0.19	**0.93**	0.61	**3**	5.688	**27.99**	5.89	**29**
Extract	1.6	1.33	**0.83**	0.26	**0.17**	1.344	**0.85**	3.415	**2.14**
CQ	57	54.05	**0.95**	62.58	**1.1**	13.071	**0.23**	16.718	**0.29**

When the parasites exposed to the extract at IC_{50} equivalents were tested with *Artemisia annua* extract the parasites remained sensitive to the extract, and the RSI was 0.83 as at cycle 10, 0.17 at cycle 20, 0.85 at cycle 30 and 2.14 at cycle 40 as indicated in **Table 4.5** and **Figure 4.3.**

RSI for parasites exposed to IC_{50} equivalents of extract and tested with artemisinin at cycle 10 was low as it was 0.93, at cycle 20 the RI was 3.00, at cycle 30 increased to 27.99 while at cycle 40, RSI had increased by 29.0. It is thus worth noting that when parasites were exposed to the extract and then tested with artemisinin, the parasites were less sensitive. When parasites were exposed to the extract and then tested with extract the parasites remained sensitive. This showed that when the parasites exposed to *Artemisia annua* were tested against artemisinin there was an increase in the RSI. However for the parasites exposed to IC_{90} equivalents of the extract they were rather stable and the increase in RSI was quite minimal as indicated by **Table 4.6** and **Figure 4.3**.

RSI for parasites exposed to IC_{50} equivalents of extract and tested with *A. annua* remained sensitive to the extract, at cycle 10 was low as it was 0.83, at cycle 20 the RI was 0.17, at cycle 30 it was 0.85 while at cycle 40, RSI was 2.14.

Table 4. 6: Means and RSI of *P. falciparum* cyclically exposed to *A. annua* extract at IC_{50} and tested on (i) artemisinin (ii) *A. annua* and (iii) chloroquine

Cycles	Art MEAN±SE	Extract MEAN±SE	CQ MEAN±SE
RI cycle 10	0.93 ± 0.06^{a}	0.83 ± 0.02^{b}	0.95 ± 0.02^{c}
RI cycle 20	3.00 ± 0.08^{b}	0.17 ± 0.01^{a}	1.10 ± 0.03^{d}
RI cycle 30	27.99 ± 0.48^{c}	0.85 ± 0.05^{b}	0.23 ± 0.00^{a}
RI cycle 40	29.00 ± 1.15^{c}	2.14 ± 0.08^{c}	0.29 ± 0.01^{b}
p-value	<0.001	<0.001	<0.001

Mean±SE followed by the same letter(s) within the same column do not differ significantly from one another (One-way ANOVA, SNK-test, $\alpha = 0.05$).

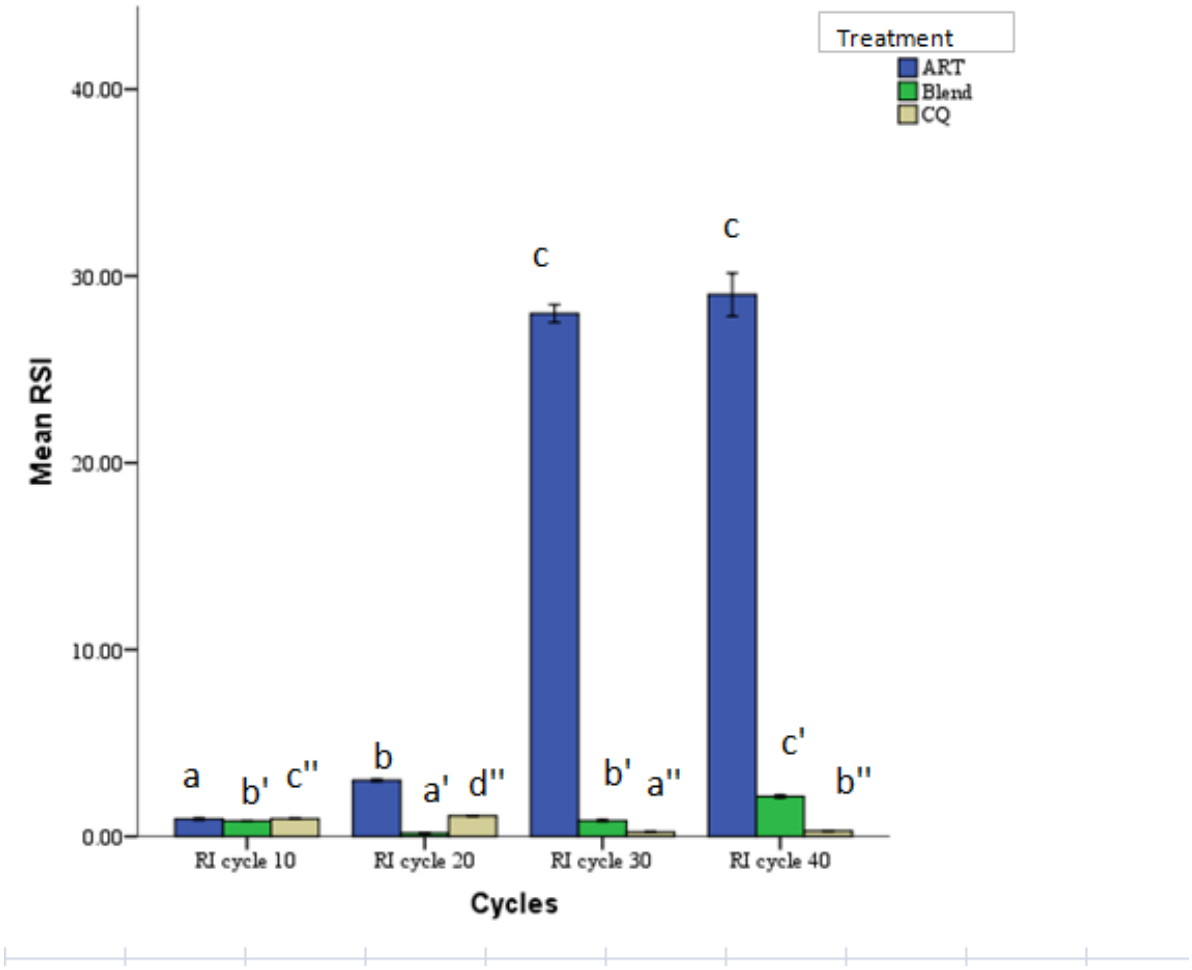

Figure 4.4 Relative sensitivity indices (RSI) of *P. falciparum* cyclically exposed to artemisinin at IC_{50} and tested against (i) artemisinin, (ii) *A. annua* extract and (iii) Chloroquine

Key

Unprimed number (a) represents significance levels in column analyzing exposure with artemisinin.

One prime on the number (a') represents significance levels in analyzing exposure to *A.annua* extract.

Two primes on the number ($a^{''}$) represents significance levels in analyzing exposure to Chloroquine

Table 4.7: IC_{50} and relative sensitivity index for parasites exposed to *Artemisia annua* at IC_{90} and tested with (i) artemisinin (ii) *Artemisia annua* extract and (iii) Chloroquine

	Cycle 0	**Cycle 10**		**Cycle 20**		**Cycle 30**		**Cycle 40**	
	IC_{50}	IC_{50}	**RS1**	IC_{50}	**RS1**	IC_{50}	**RS1**	IC_{50}	**RS1**
ART	0.2	0.26	**1.28**	2.93	**14.4**	3.85	**19.25**	7.396	**36.38**
Extract	1.6	1.69	**1.06**	0.17	**0.11**	1.0845	**0.67**	8.52	**5.37**
CQ	57	57.63	**1.01**	62.04	**1.09**	5.521	**0.1**	8.712	**0.15**

From this observation it was concluded that *Artemisia annua* mitigates against resistance development to the extract only RSI for parasites exposed to IC_{90} equivalents of the extract and tested with artemisinin increased by 1.28 at cycle 10, 14.40 at cycle 20, 19.25 at cycle 30 and 36.38 at cycle 40.

When parasites were exposed to *Artemisia annua* at IC_{90} and tested with the *A.annua* extract, they remained sensitive and the RSIs were as follows, 1.06 at cycle 10, 0.11 at cycle 20, 0.67 at cycle 30 and 5.37 at cycle 40 as indicated by **Table 4.8** and **Figure 4.5**. Interestingly, CQ-resistant parasite strain that was exposed to 40 cycles of artemisinin became less sensitive to the artemisinin. In all the four tests above the RSI for artemisinin increased between 10 fold to 36 fold.

Table 4. 8: Means and standard errors of RSI of *P. falciparum* cyclically exposed to *A. annua* at IC_{90} and tested with (i) artemisinin (ii) *A. annua extract* and (iii) Chloroquine

Cycles	Art MEAN±SE	Extract MEAN±SE	CQ MEAN±SE
RI cycle 10	1.28 ± 0.06^{a}	1.06 ± 0.04^{b}	1.01 ± 0.02^{b}
RI cycle 20	14.40 ± 0.63^{b}	0.11 ± 0.01^{a}	1.09 ± 0.04^{b}
RI cycle 30	19.25 ± 3.25^{b}	0.67 ± 0.10^{b}	0.10 ± 0.02^{a}
RI cycle 40	36.38 ± 0.40^{c}	5.37 ± 0.33^{c}	0.15 ± 0.01^{a}
P-value	<0.001	<0.001	<0.001

Key:

Mean ± SE followed by the same letter(s) within the same column do not differ significantly from one another (One-way ANOVA, SNK-test, $\alpha = 0.05$).

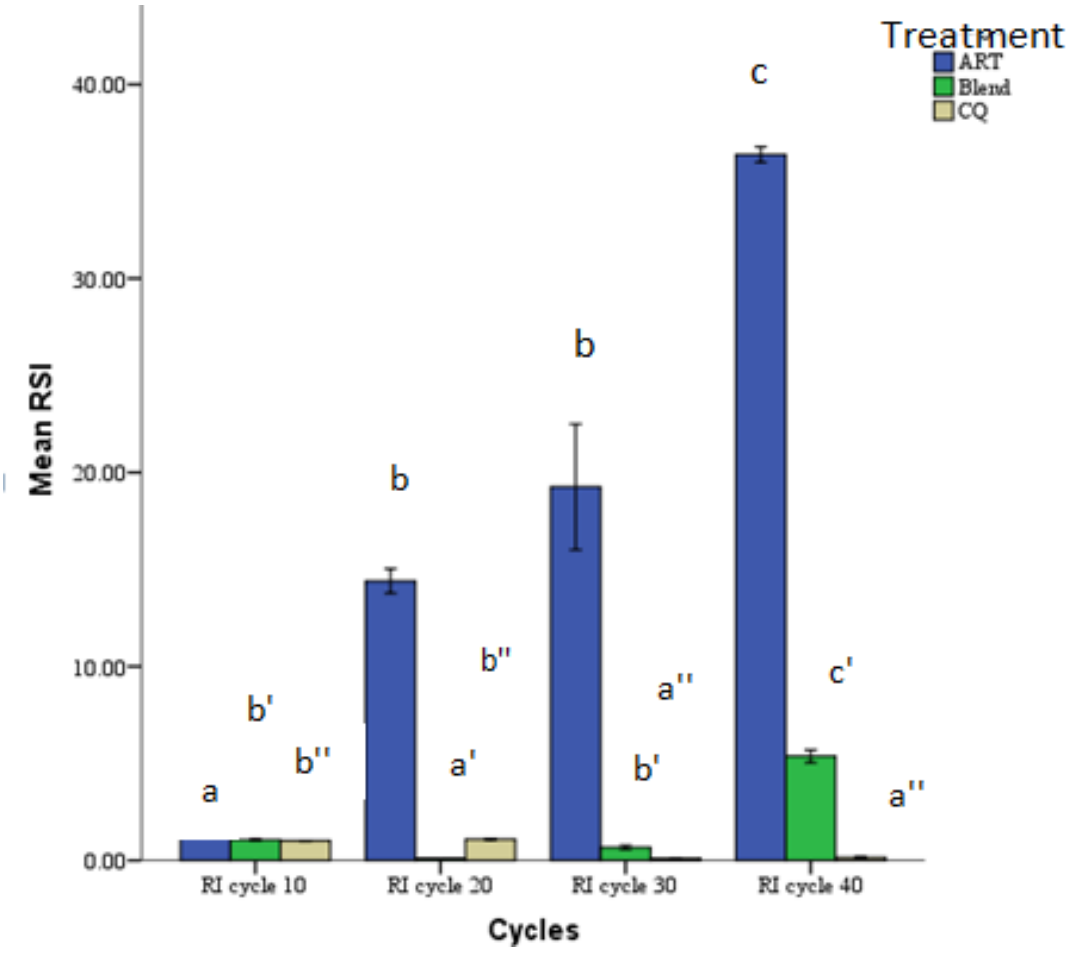

Figure 4.5 Relative sensitivity indices (RSI) of *P. falciparum* cyclically exposed to *A. annua* at IC_{90} and tested against (i) artemisinin, (ii) *A. annua* extract and (iii) Chloroquine

Key: Unprimed number (a) represents significance levels in column analyzing exposure with artemisinin.

One prime on the number ($a^{'}$) represents significance levels in analyzing exposure to *A.annua* extract.

Two primes on the number ($a^{''}$) represents significance levels in analyzing exposure to Chloroquine

4.3. *In vitro* results –with D6 Parasites (Chloroquine sensitive isolate)

4.3.1 D6 parasites exposed to artemisinin at IC_{50} equivalent

After cyclically exposing D6 (Chloroquine sensitive) parasites to IC_{50} equivalents of artemisinin and testing with artemisinin there was an increase in RSI with an increase in the number of cycles, at cycle 10 the RSI was 39. 13, at cycle 30 it was 33.61. On cyclically exposing D6 parasites to artemisinin at IC_{50} equivalents and testing with the extract the RSI increase was minimal as it was 6.06 at cycle 10 and 5.8 at cycle 30 as indicated by **Table 4.9** and **Figure 4.5**. Chloroquine and dihydroartemisinin (DHA) were included as controls. The micro beta counter broke down and therefore was not able to get counts for cycle 40 and cycle 50.

Table 4.9: IC_{50} and RSI values for D6 parasites exposed to artemisinin at IC_{50} equivalents

	Cycle 0	**Cycle 10**		**Cycle 30**	
	IC_{50}	IC_{50}	**RS1**	IC_{50}	**RS1**
ART	0.205	8.022	**39.1317**	6.891	**33.61**
Extract	0.18	1. 091	**6.0611**	1.0505	**5.8**

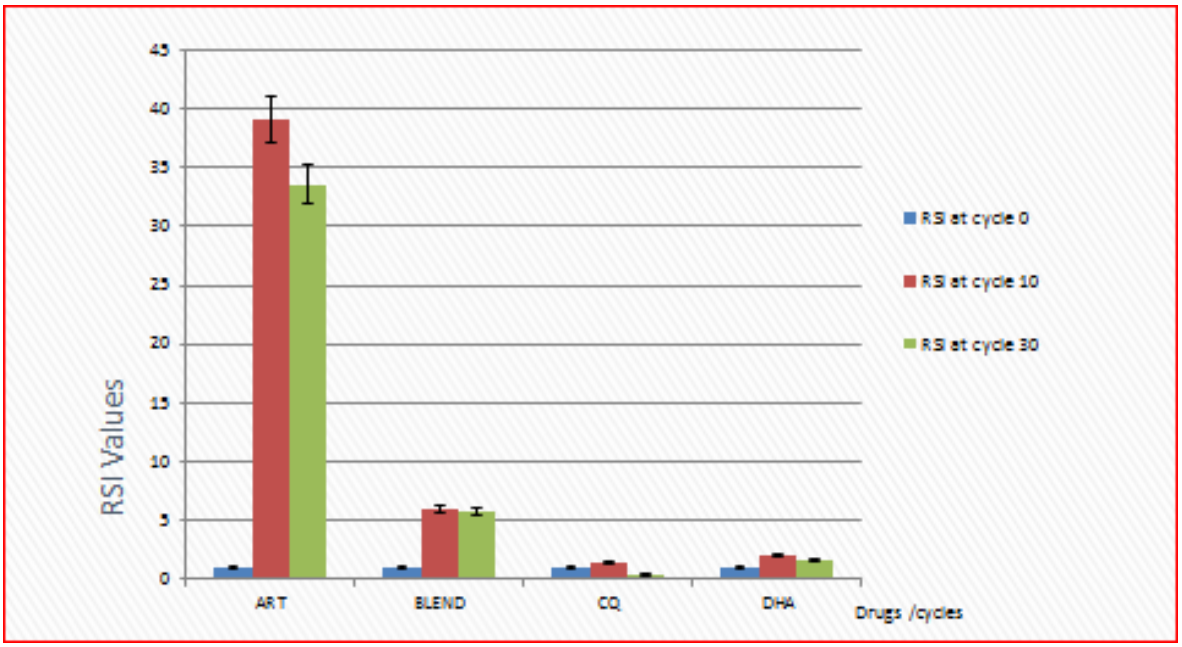

Figure 4.6 Relative sensitivity index of parasites exposed to the artemisinin at $1C_{50}$ equivalents after 10 and 30 cycles.

4.3.2 D6 parasites exposed to artemisinin at IC_{90} equivalents

After cyclically exposing D6 (Chloroquine sensitive) parasites to IC_{90} equivalents of artemisinin and testing with artemisinin there was an increase in RSI with an increase in the number of cycles, at cycle 10 the RSI was 30.87, at cycle 30 it was 43.46. On cyclically exposing D6 parasites to artemisinin at IC_{90} equivalents and testing with the extract the RSI increase was minimal as it was 13.78 at cycle 10 and 9.92 at cycle 30 as indicated by **Table 4.10** and **Figure 4.6**. Chloroquine and dihydroartemisinin (DHA) were included as controls.

Table 4. 10: IC_{50} and RSI for D6 parasites exposed to artemisinin @ IC_{90} and tested with (i) artemisinin (ii) *A. annua* extract (iii) Chloroquine (iv) dihydroartemisinin

	IC_{50} at Cycle 0	IC_{50} at Cycle 10	RSI at cycle 10	IC_{50} at Cycle 30	RSI for cycle 30
ART	0.205	6.3295	30.875	8.910	43.46
Extract	0.18	2.482	13.788	1.787	9.92
CQ	37.825	39.798	1.05216	16.607	0.439
DHA	52.188	150.586	2.8854	80.911	1.550

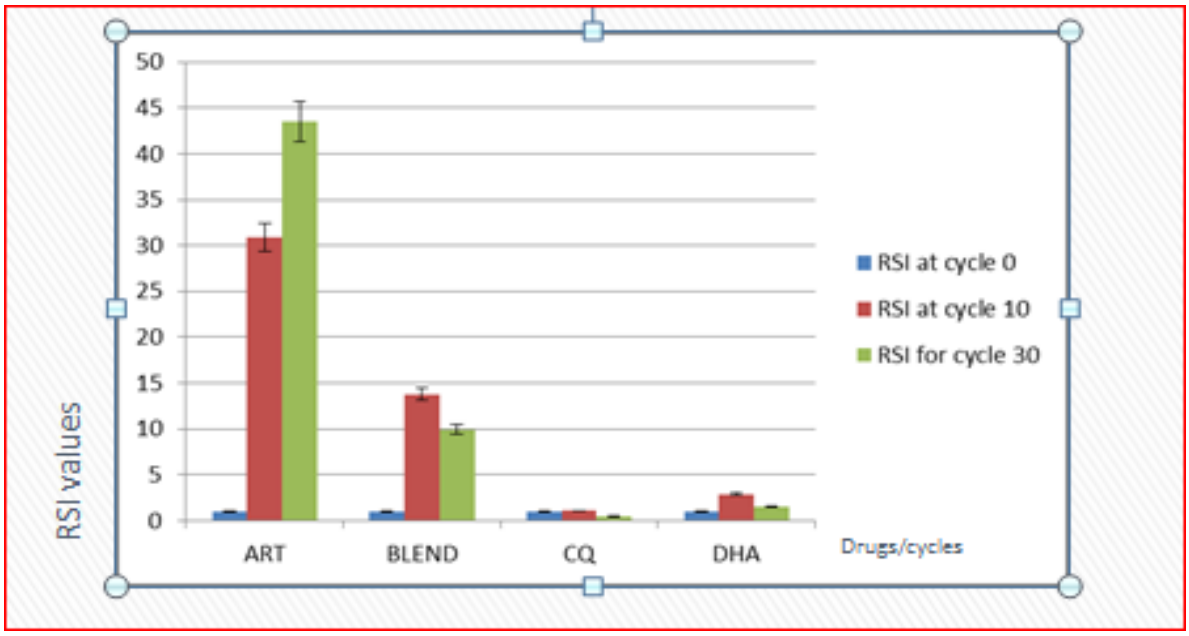

Figure 4.7 Relative index of parasites exposed to the Artemisinin at $1C_{90}$ equivalents after 10 and 30 cycles and tested with (i) artemisinin, (ii) *Artemisia annua* extract, (iii) chloroquine and (iv) dihydroartemisinin

4.3.3 D6 parasites exposed to *Artemisia annua* (extract) at IC_{50} equivalent

Table 4.9 and **Figure 4.7** indicate the IC_{50} and RSI of D6 parasites cyclically exposed to artemisinin and tested with artemisinin, *Artemisia annua*, Chloroquine and dihydroartemisinin after 10 and 30 cycles. The greatest increase in RSI was observed with artemisinin which was 35.2 against the extract that was 4.2 at cycle 10. After 30 cycles the RSI for artemisinin was 40.47 while that for the extract was 4.02. The parasites exposed to *Artemisia annua* and tested with artemisinin became less sensitive to artemisinin. For the artemisinin tested parasites, there was an increase in the RSI with an increase with cycle numbers. On the other hand when the parasites were exposed to *Artemisia annua* and tested with *Artemisia annua* extract they remained sensitive to *Artemisia annua* extract. Chloroquine and dihydroartemisinin were included as controls.

Table 4. 11: Values and Relative Sensitivity Index for parasites exposed to *A. annua* extract at IC_{50} equivalents and tested with (i) artemisinin, (ii) *A. annua* extract (iii) Chloroquine and (iv) dihydroartemisinin**.**

	Cycle 0		Cycle 10		Cycle 30	
	IC_{50}	**RSI**	IC_{50}	**RS1**	IC_{50}	**RS1**
ART	0.205	**1**	7.217	**35.2**	8.29	**40.473**
Extract	0.18	**1**	0.7705	**4.28**	0.72	**4.0255**
CQ	37.825	**1**	50.706	**1.34**	8.79	**0.2325**
DHA	52.188	**1**	42.8805	**0.8216**	155.126	**2.972**

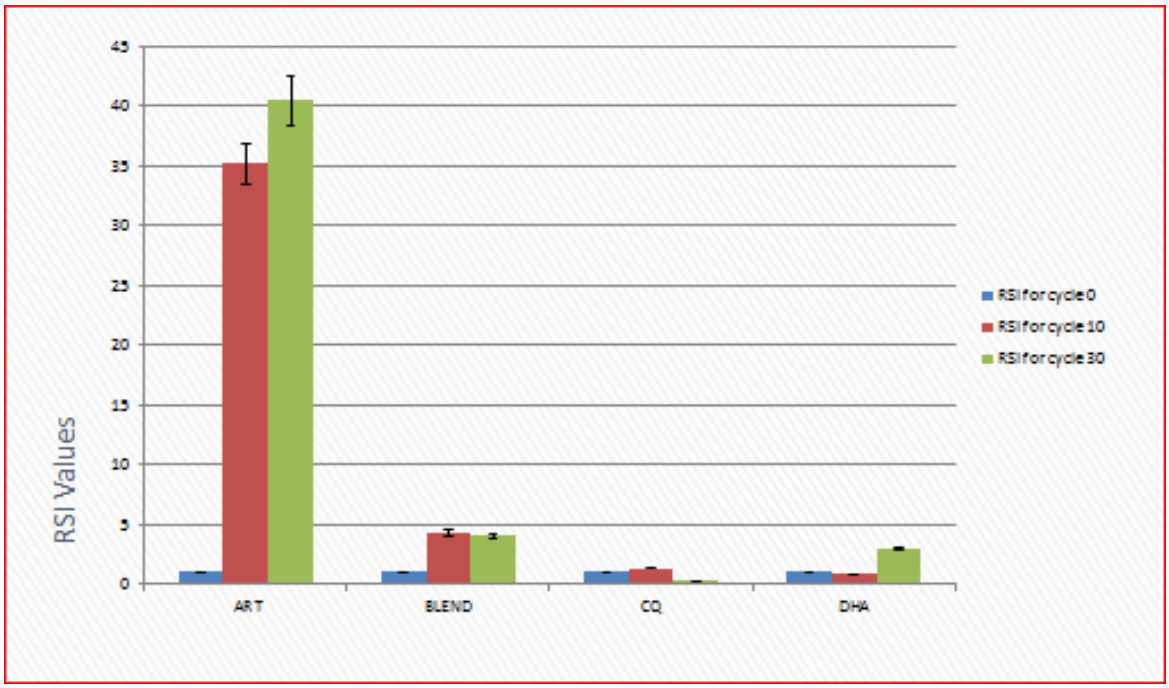

Figure 4.8 Relative sensitivity index of parasites exposed to the extract at $1C_{50}$ equivalents after 10 and 30 cycles

4.4 *In vivo* results with *Plasmodium berghei* Anka

4.4.1 *Plasmodium berghei* ANKA tested with artemisinin

The following data was obtained with *Plasmodium berghei* Anka tested on artemisinin. An ED_{50} of 1.43 mg/ml and an ED_{90} of 7.18 mg/ml was found with *P. berghei* Anka parasites tested with artemisinin at cycle 0.

Table 4. 12: Chemo-suppression of *Plasmodium berghei* Anka with artemisinin at cycle 0

Concentration	Parasitemia	Chemosuppression	Parasite reduction
10 mg/ml	1.0	98.00	2.0
5	5.1	88.00	12.0
2.5	16.0	61.00	39.0
1.25	23	44.00	56.0
Control	41	----------	----------

Values for chemo suppression got in **Table 4.12** were used to draw the graph indicated in **Figure 4.9**.

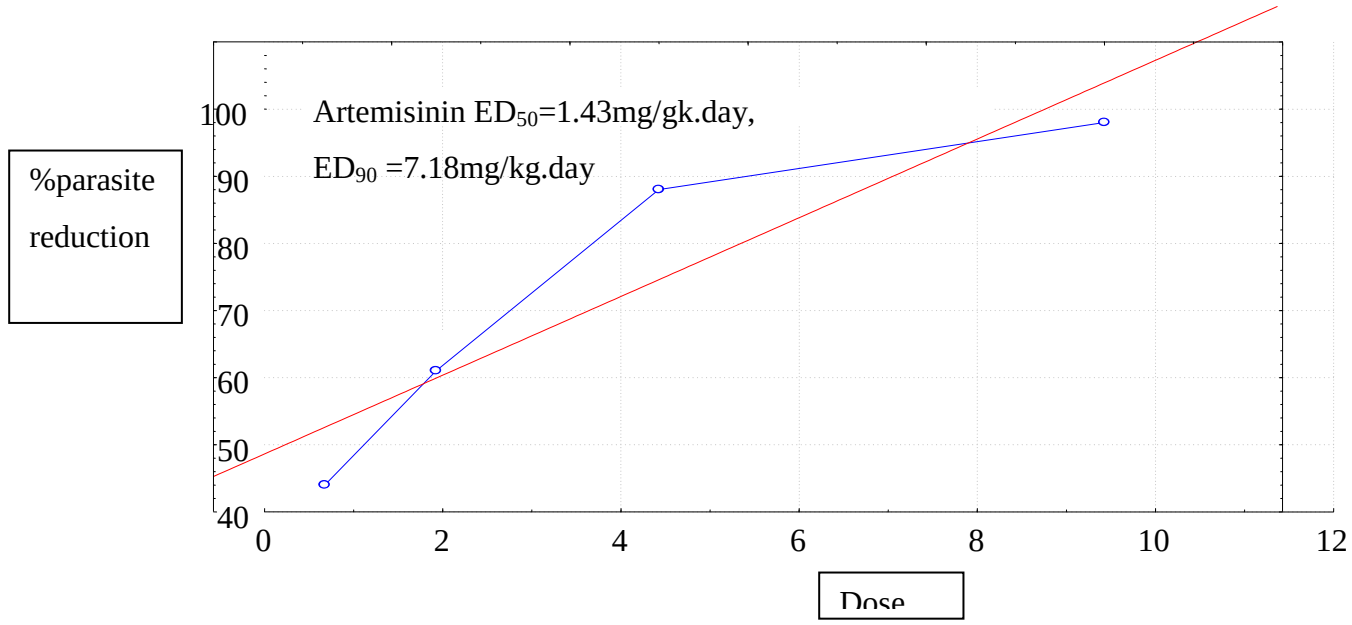

Figure 4.9 ED_{50} and ED_{90} of artemisinin against *P. berghei* at cycle 0

4.4.2 *Plasmodium berghei* ANKA tested with *Artemisia annua* extract

The ED_{50} for *Artemisia annua* extract at cycle 0 was 34.50 mg/kg.day while the ED_{90} was 118.00 mg/kg.day as indicated **Figure 4.9.** This was arrived at after plotting the dose against chemo-suppression of the *Artemisia annua* extract.

Table 4. 13 Concentration and chemo-suppression for the extract with *Plasmodium berghei* Anka at cycle 0

Concentration in mg/kg.day	% Parasitemia	% Chemosuppression	% Parasitemia relative to control
125	3.72	89.37	10.63
62.5	8.61	75.39	24.61
31.25	20.84	40.46	59.54
control	35.00	----------	----------

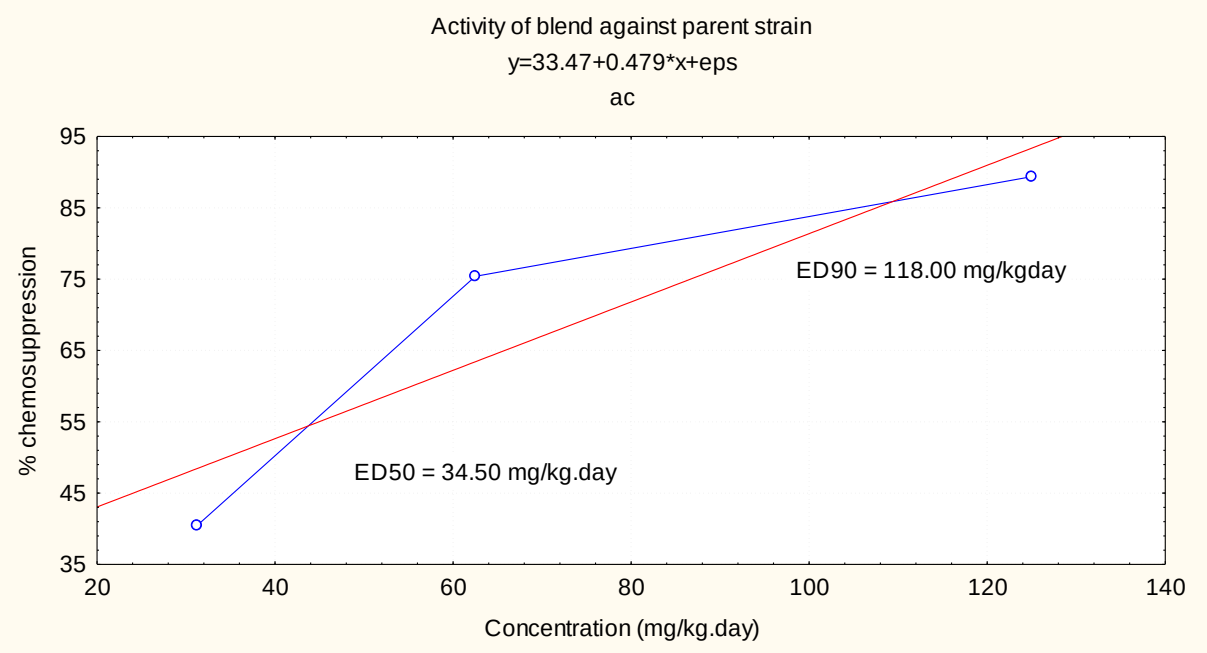

Figure 4.10 Concentration against chemo-suppression for the extract at cycle 0

4.4.3 *Plasmodium berghei* ANKA tested with artemisinin at cycle 10 reversal

P.berghei exposed to the extract and tested with artemisinin at cycle 10 reversal gave an ED_{50} of 15.3mg/ml as indicated below in **Figure 4.11.**

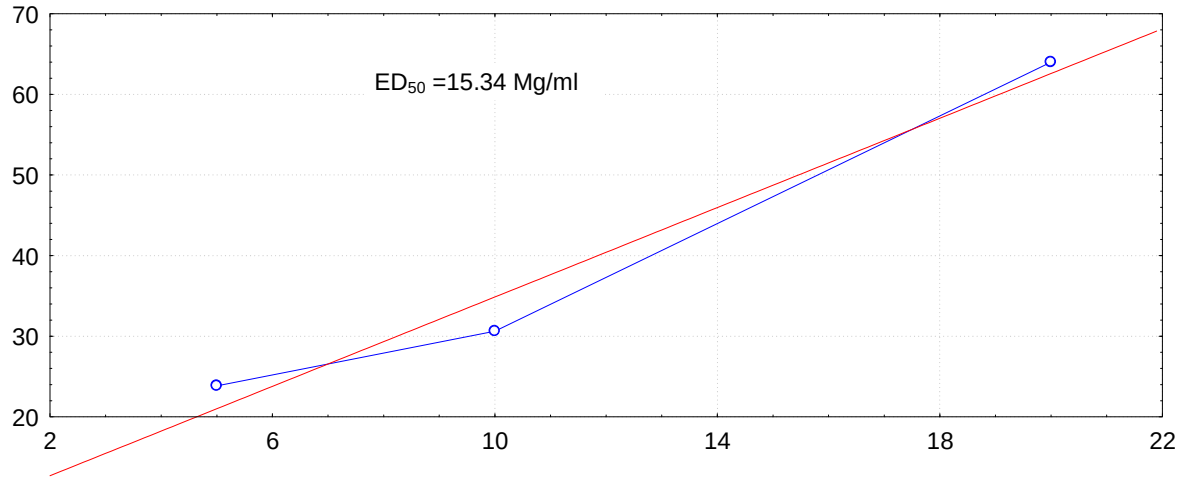

Figure 4. 11 Concentration against chemo-suppression for *P. berghei* and exposed to the extract and tested with artemisinin at cycle 10 reversal

Key;

X axis-concentration in mg/kg.day

Y axis-% parasite reduction

4.4.4 *Plasmodium berghei* ANKA tested with artemisinin at cycle 0 reversal

P. berghei parasites were subjected to drug pressure with (i) artemisinin and ii) *Artemisia annua* for 20 cycles and thereafter the drug pressure was removed and subjected again to another 20 cycles without drug pressure.

When artemisinin exposed parasites were tested with artemisinin at cycle 0 reversal the ED_{50} was 0.86 mg/ml and ED_{90} was 7.52 as indicated by **Figure 4.12**.

After exposing *Plasmodium berghei* Anka to artemisinin for 20 cycles and then removing drug pressure for 20 cycles the ED_{50} got was 0.86 mg/ kg.day and ED_{90} was 7.52 mg/kg.day.

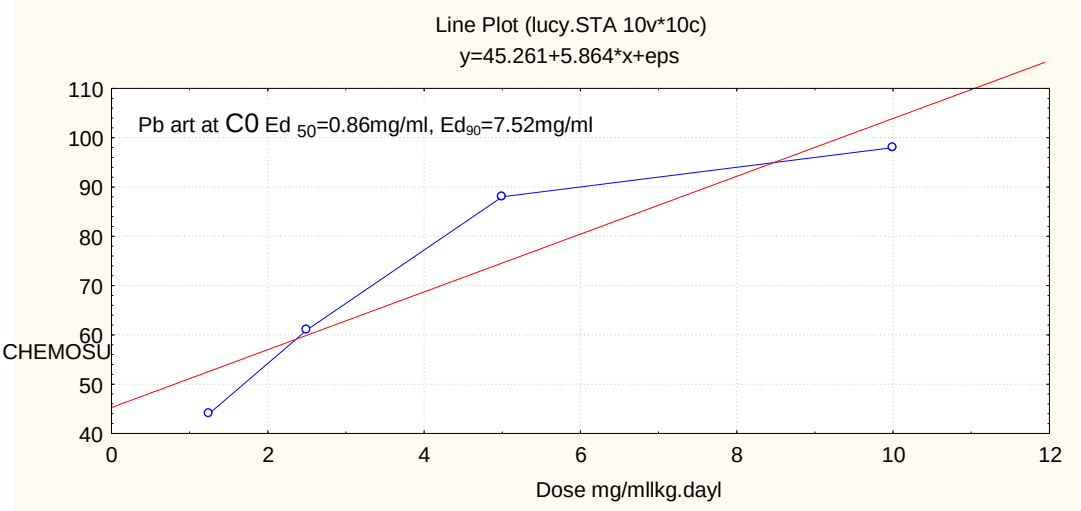

Figure 4.12 Concentration against chemo-suppression for artemisinin at cycle 0 reversal after exposure to artemisinin

4.4.5 *Plasmodium berghei* ANKA tested with dihydroartemisinin at cycle 0

Table 4.14 represents parasitemia and chemo-suppression at different levels of dihydroartemisinin. The ED_{50} was 1.73 mg/kg.day and the ED_{90} was 8.31 mg/kg.day at cycle 0. After 20 cycles with the drug pressure of artemisinin the drug was removed for the next 20 cycles. When these parasites were tested with artemisinin the following values were got. The ED_{50} was 0.86 and ED_{90} was 7.52 mg/ml as shown in **Figure 4.11**.

This indicated that resistance that had been achieved with drug pressure was not maintained after removal of drug pressure.

DHA was used as a control drug

Table 4.14 indicates % parasite reduction with dihydroartemisinin on *Plasmodium berghei* anka. The reduction at 10mg/kg.day was 87%, 5mg/kg.day was 80.33%, 2.5mg/kg.day was 63.58%, while 0.625 mg/kg.day was 38.44%.

Table 4. 14: Concentration and chemo-suppression for dihydroartemisinin with *Plasmodium berghei* anka

Concentration (mg/kg.day)	% Parasitemia	% Chemo-suppression	% Parasitemia relative to control
10	2.61	87.00	13.00
5	4.17	80.33	19.67
2.5	7.72	63.58	36.58
0.625	13.05	38.44	61.56
Control	21.2	----------	----------

4.5 *Plasmodium yoelii* parasites tested with artemisinin

When *Plasmodium yoelii* was tested with artemisinin after removal of drug pressure the ED_{50} was 7.64 mg/ml as indicated in **Figure 4.13**

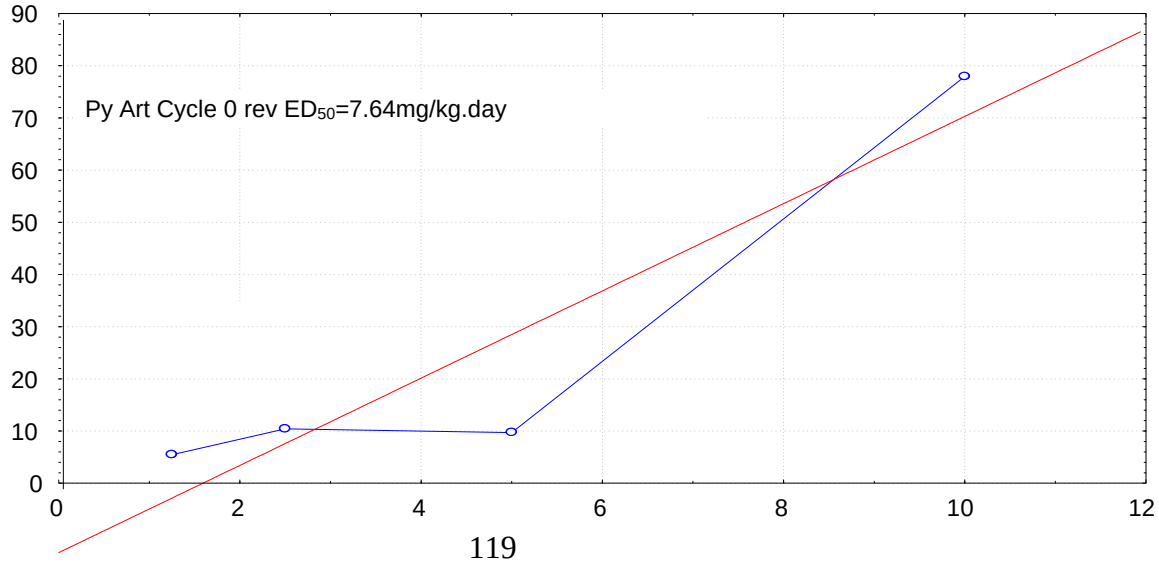

Figure 4.13 *Plasmodium yoelii* cycle 0 reversal with artemisinin

Key :X axis dosage in mg/kg.day

Y axis % parasite reduction

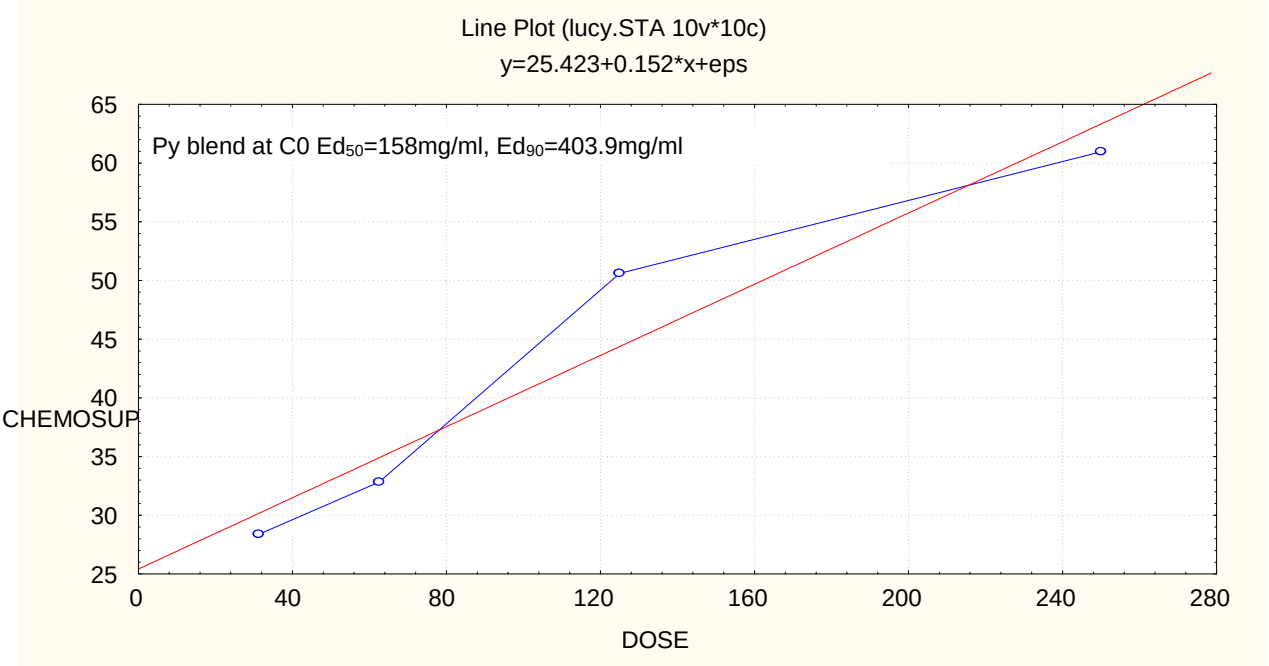

Figure 4. 14 Concentration against chemo-suppression for the extract at cycle 0 with *Plasmodium yoelii*

The sensitive *P. yoelii* was tested with extract and an ED_{50} of 158mg/ml was got, while the ED_{90} was 403.9mg/ml. After 20 cycles of drug pressure with the extract the ED_{50} was 89.4 and after the drug pressure was removed for the next 20 cycles the ED_{50} was determined again and the value of 97.3mg/ml was got. It was evident that the resistance got was not stable in that when the drug pressure was removed the ED_{50} decreased again. This type of resistance has been described as being transient.

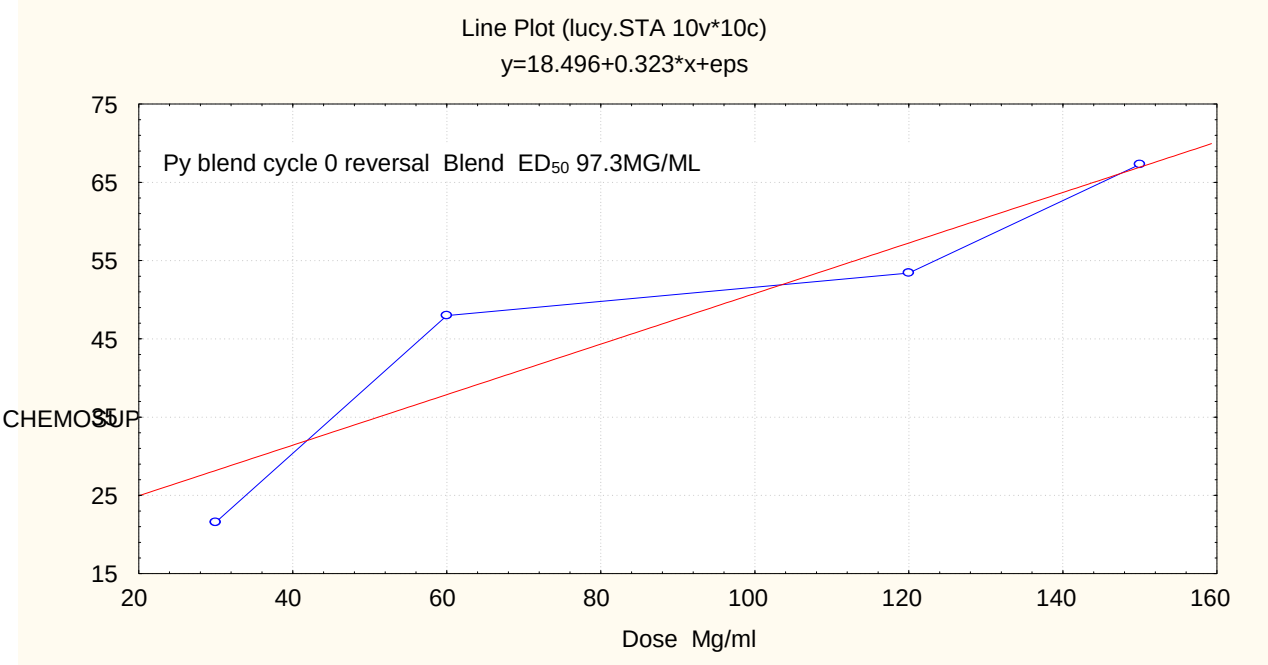

Figure 4.15 Concentration against chemo-suppression for the extract at cycle 0 after removal of drug pressure

After subjecting *P. yoelii* to the *A. annua* for 20 cycles and then removing drug pressure for another 20 cycles the ED_{50} got with the extract was 97.3 mg/ml.

Table 4. 15: Concentration and chemosuppression for artemisinin with *P.yoelii*

Concentration (mg/kg.day	Parasitemia	Chemo-suppression	Parasite reduction
10	2.61	87.00	13.00
5	4.17	80.33	19.67
2.5	7.72	63.58	36.58

0.625	13.05	38.44	61.56
Control	21.2	----------	----------

When *Plasmodium berghei* Anka was exposed to the *Artemisia annua* for twenty cycles and the drug pressure removed for 10 cycles the ED_{50} was determined and it was 17.73mg/kg.day

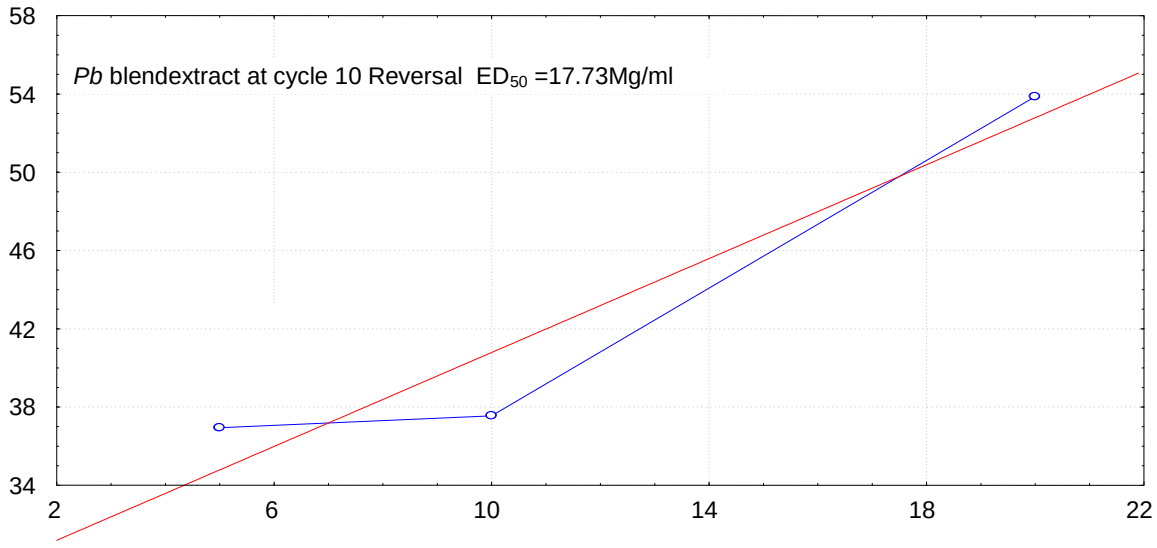

Figure 4.16 Concentration chemosuppression for *P. yoelii* parasites exposed to artemisinin at cycle 10 reversal

Key: x axis concentration mg/kg.day
Y axis % parasite reduction

When *Plasmodium berghei* Anka was exposed to the *Artemisia annua* for twenty cycles and the drug pressure removed for 10 cycles the ED_{50} was determined and it was 17.73mg/kg.day (**Figure 4.16**)

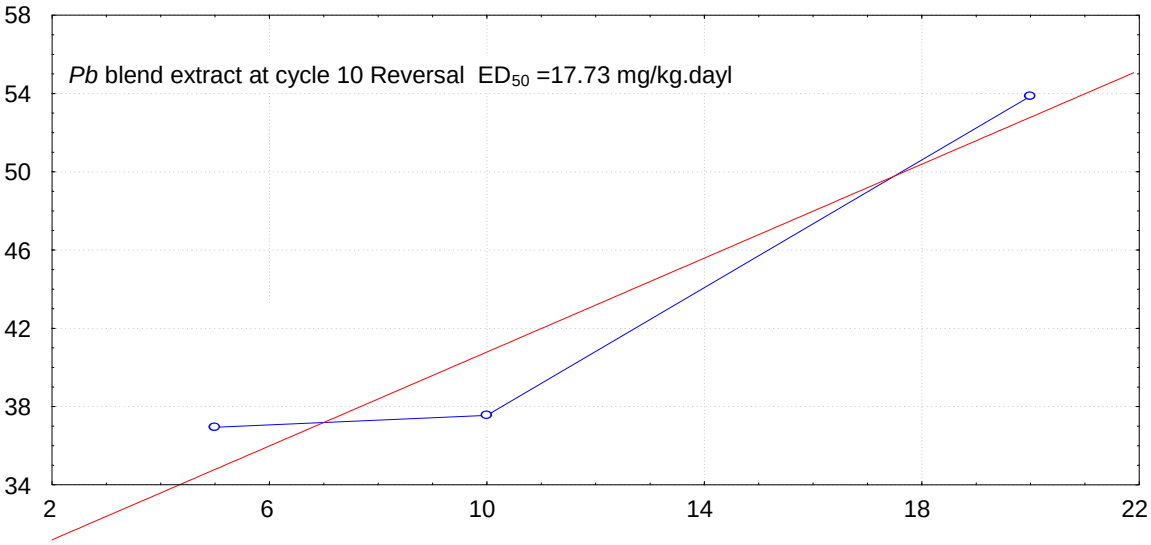

Figure 4.16 Concentration chemosuppression for *P. yoelii* parasites exposed to artemisinin at cycle 10 reversal

Table 4. 16:IC_{50} and Relative Sensitivity Index of *P. berghei* anka parasites exposed to artemisinin and tested with (i) artemisinin and (ii) *A. annua* extract at cycle 0, cycle 10, cycle 20 , cycle 10 reversal and cycle 0 reversal.

	Cyle 0		Cycle 10		Cycle 20		Cycle 10 rev		Cycle 0 rev	
	IC_{50}	**RSI**	IC_{50}	**RSI**	IC_{50}	**RSI**	IC_{50}	**RSI**	IC_{50}	**RSI**
ART	1.43	**1**	20.52	**14.34**	35.64	**24.9**	17.7	**12.39**	5	**3.49**
Extract	34	**1**	70.56	**2.07**	104.6	**3.08**	84.9	**2.29**	48.5	**1.42**

Table 4.16 is a summary of exposure of *P. berghei* Anka to artemisinin and the effect of removal of drug pressure at different cycles. At cycle 10 RSI with artemisinin was 14.34 and at cycle 20 it was 24.9 mg/kg.day. After 10 cycles of drug free with artemisinin the ED_{50} was 17.7 and at cycle 0 reversal it was 3.49. When the artemisinin exposed parasites were tested with *Artemisia annua* at cycle 10 the RSI was 2.07, at cycle 20 it was 3.08, at cycle 10 reversal it was 2.29 and at cycle 0 reversal it was 1.42. The parasites exposed to the *Artemisia annua* remained sensitive to the extract. **Figure 4.17** indicates that some resistance was achieved with artemisinin but on drug pressure removal the parasites reverted to being sensitive. A transient resistance was observed.

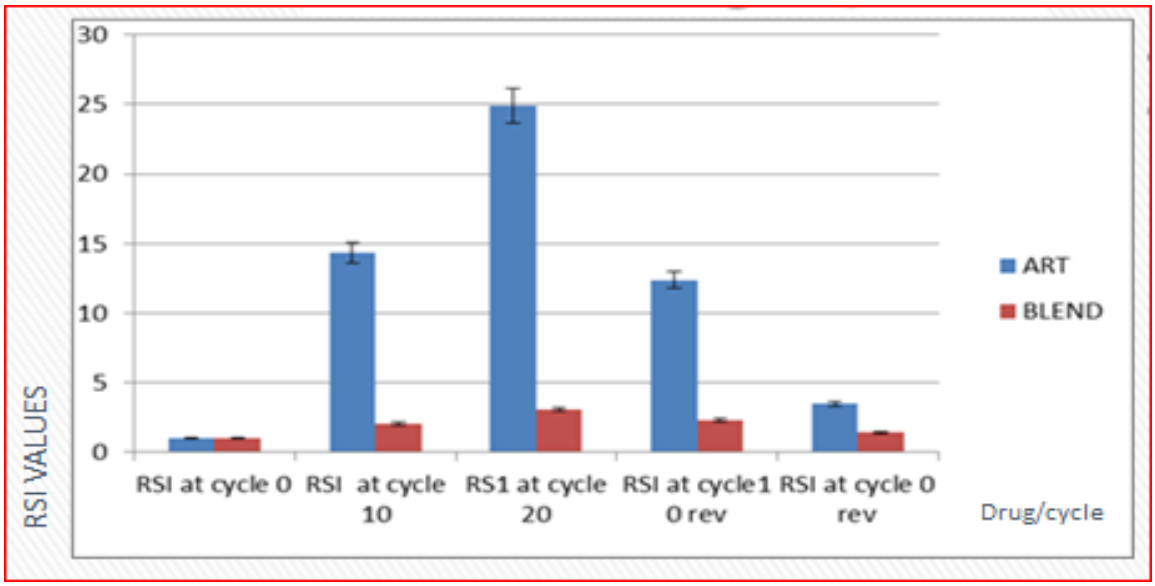

Figure 4.17 RSI for *P. berghei* parasites exposed to artemisinin and tested with (i) artemisinin ii) *Artemisia annua* extract

Table 4. 17:***P.berghei*** **Anka parasites exposed to *A. annua* extract and tested with (i) artemisinin .and (ii) *A. annua* extract at cycles 0, cycle 10, cycle 20 , cycle 10 reversal and cycle 0 reversal**

	Cyle 0		Cycle 10		Cycle 20		Cycle 10 rev		Cycle 0 rev	
	IC_{50}	**RSI**	IC_{50}	**RSI**	IC_{50}	**RSI**	IC_{50}	RSI	IC_{50}	**RSI**
ART	1.43	**1**	3.42	**2.39**	8.56	**5.9**	6.7	4.69	0.86	**0.6**
Extract	34	**1**	56.4	**1.65**	64.8	**1.9**	36.6	1.07	30.5	**0.89**

Table 4.17 and **Figure 4.18** is a summary of the effect of exposing *Plasmodium berghei* parasites to *Artemisia annua* for twenty cycles and then removing drug pressure for twenty cycles. When tested with artemisinin the RSI was 2.39 at cycle 10, 5.9 at cycle

20, 4.69 at cycle 10 reversal and 0.60 at cycle 0 reversal. When the *A. annua* exposed parasites were tested with *Artemisia annua* extract the RSI was 1.69 at cycle 10, RSI at cycle 20 was 1.9 and at cycle 10 reversal was 1.07 and at cycle 0 reversal was 0.89.

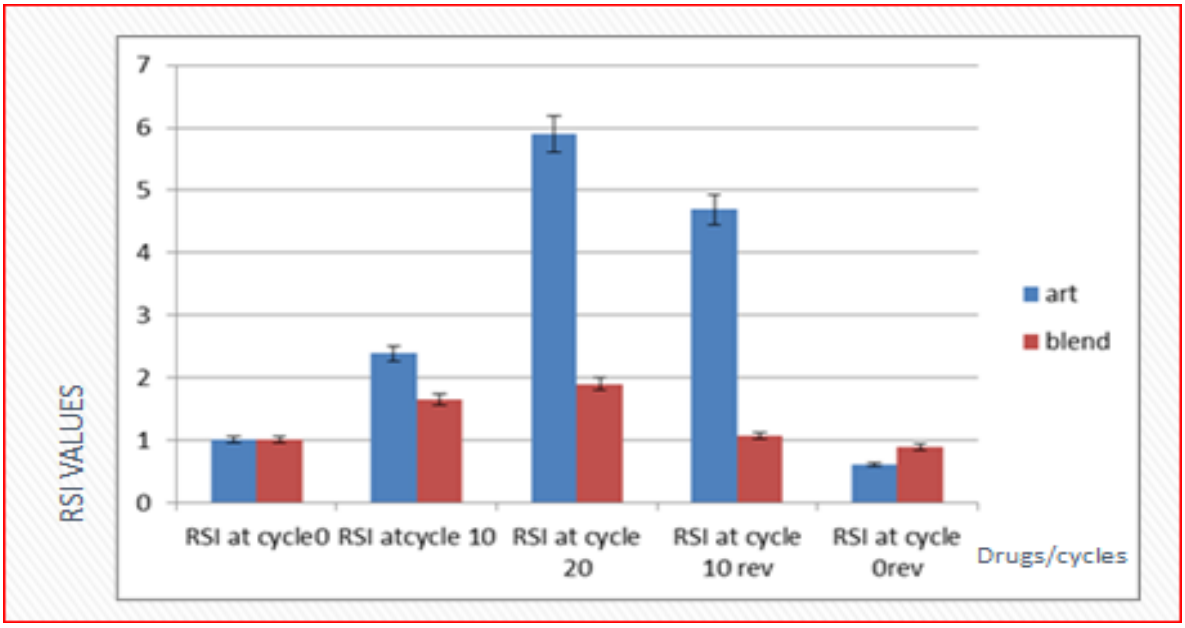

Figure 4.18 RSI for *P. berghei* Anka exposed to *Artemisia annua* and tested with (i) artemisinin (ii) *Artemisia annua*

Table 4. 18: ***P. yoelii*** **parasites exposed to artemisinin and tested with (i) artemisinin and (ii)** ***A. annua*** **extract at cycles 0, cycle 10, cycle 20 , cycle 10 reversal and cycle 0 reversal**

	Cyle 0		Cycle 10		Cycle 20		Cycle 10 rev		Cycle 0 rev	
	IC_{50}	**RSI**	IC_{50}	**RSI**	IC_{50}	**RSI**	IC_{50}	**RSI**	IC_{50}	**RSI**
ART	11.63	**1**	16.4	**1.41**	24	**2.06**	14.6	**1.255**	7.64	**0.65**
Extract	158	**1**	174.6	**1.1**	230.5	**1.45**	199	**1.256**	93.4	**0.59**

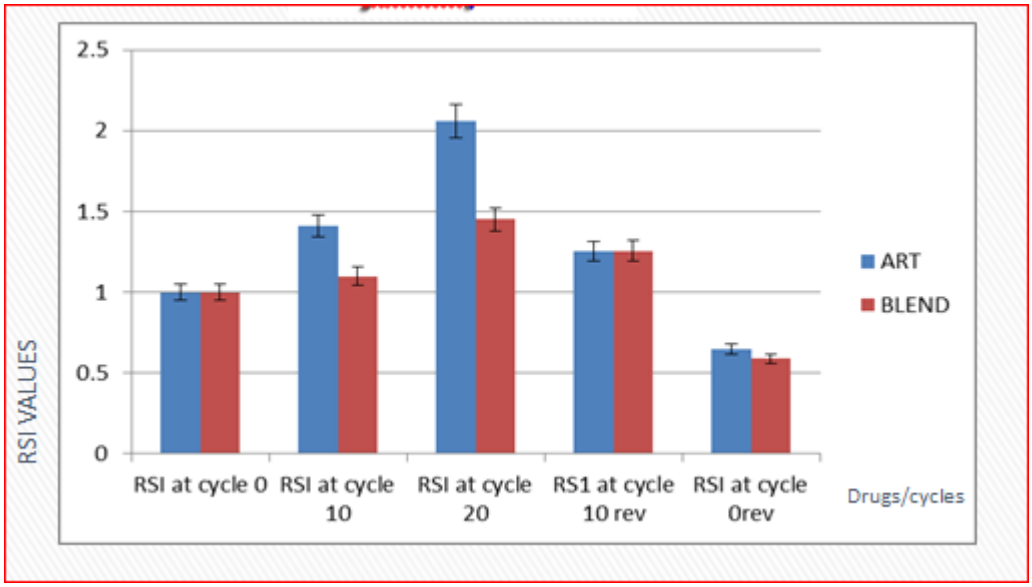

Figure 4.19 RSI for *P. yoelii* exposed to *Artemisinin* and tested with (i) artemisinin (ii) *Artemisia annua*

P. yoelii parasites exposed to *Artemisia annua* and tested with artemisinin showed the following RSI values. RSI at cycle 10 was 1.24, 0.77at cycle 20, 0.73at cycle 10 reversal and 0.63 at cycle 0 reversal as indicated in **Table 4.19** and **Figure 4.20. Figure 4.19** indicates that the RSI increased with increase in number of cyles and upon drug removal the RSI decreased. Some resistance developed in cycles 10 and cycle 20. In all the cases the parasites exposed to artemisinin had a uniform trend of increasing RSI with an increase in the number of cycles, however those exposed to *Artemisia annua* remained sensitive to the extract.

Table 4. 19: *P. yoelii* exposed to *A. annua* and tested with (i) artemisinin and (ii) *A. annua* extract at cycle 0, cycle 10, cycle 20, cycle 10 reversal and cycle 0 reversal.

	Cycle 0		Cycle 10		Cycle 20		Cycle 10 rev		Cycle 0 rev	
	IC_{50}	**RS**	IC_{50}	**RSI**	IC_{50}	**RSI**	IC_{50}	**RSI**	IC_{50}	**RSI**

		I								
ART	11.63	**1**	14.5	**1.24**	9	**0.77**	8.6	**0.73**	7.36	**0.63**
Extract	158	**1**	168	**0.94**	89.4	**0.56**	81.6	**0.51**	97.3	**0.61**

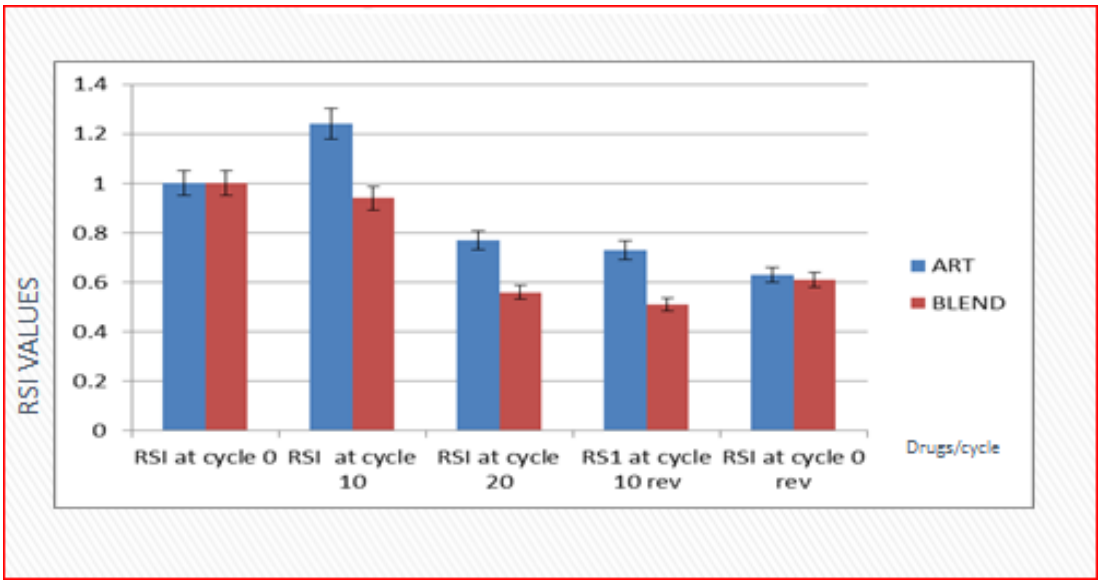

Figure 4.20 RSI for *P. yoelii* parasites exposed to *Artemisia annua* and tested with (i) artemisinin (ii) *Artemisia annua* extract

As a control *P. berghei* Anka was exposed to dihydroartemisinin and tested with artemisinin. The following results were obtained. RSI at Cycle 10 was 9.15, at cycle 20 it was 14.8, at cycle 10 rev 7.22 and at cycle 0 rev it was 0.497.

Table 4. 20: *P. berghei* Anka parasites exposed to dihydroartemisinin and tested with artemisinin at cycles cycle 0, cycle 10, cycle 20, cycle 10 reversal and cycle 0 reversal

	Cycle 0		Cycle 10		Cycle 20		Cycle 10 rev		Cycle 0 rev	
	IC_{50}	**RSI**	IC_{50}	**RSI**	IC_{50}	**RSI**	IC_{50}	**RSI**	IC_{50}	**RSI**

ART	1.73	**1**	15.84	**9.15**	25.67	**14.8**	12.5	**7.22**	0.86	**0.497**

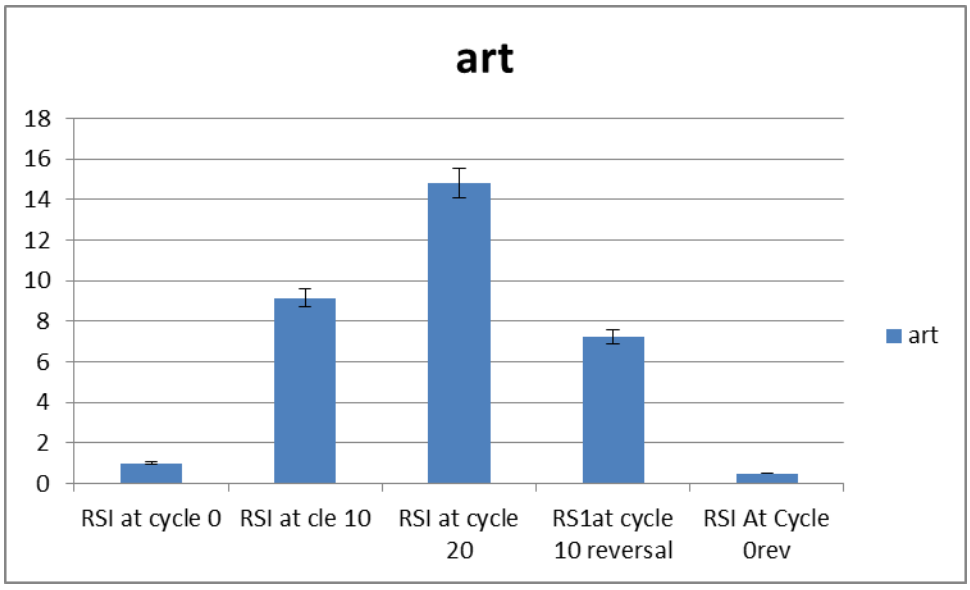

Figure 4.21 RSI for *P. berghei* parasites exposed to dihydroartemisinin and tested with artemisinin

4.4.6 Amplification products with *Plasmodium berghei* Anka and *Plasmodium yoelii*

Amplification for both the *in vitro* and *in vivo* parasites was successfully done and demonstrated by gel electrophoresis tests. The **Figure 4.22** is a PCR amplification products got from *P.berghei* multi drug resistant *(MDR) gene.* The size was 4002 bp. *Pb* cg 10 was also amplified and the size was 2708 bp while the *Pb* Atpase was 3259 bp.

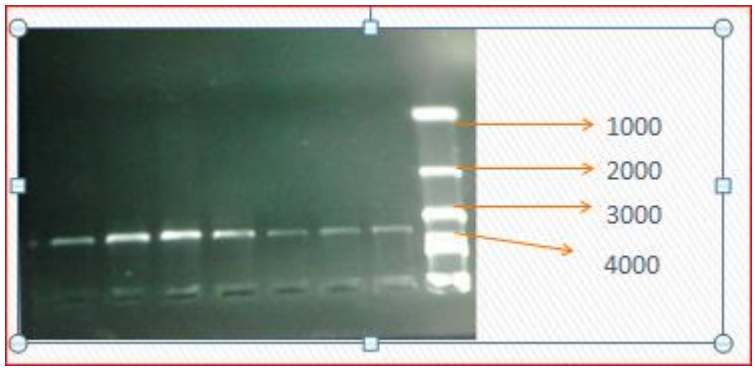

Figure 4.22 *Pb*MDR amplified was 4002 bp

well 1+, well 2+++, well 3+++, well 4++, well 5+, well 6+, well 7+, Well 8- 1 kb ladder,

Py mdr and *Py* atpase and *Py* cg10 were successfully amplified and the *Py* mdr size was =3940 bp, while Py Atpase was 3437 bp.

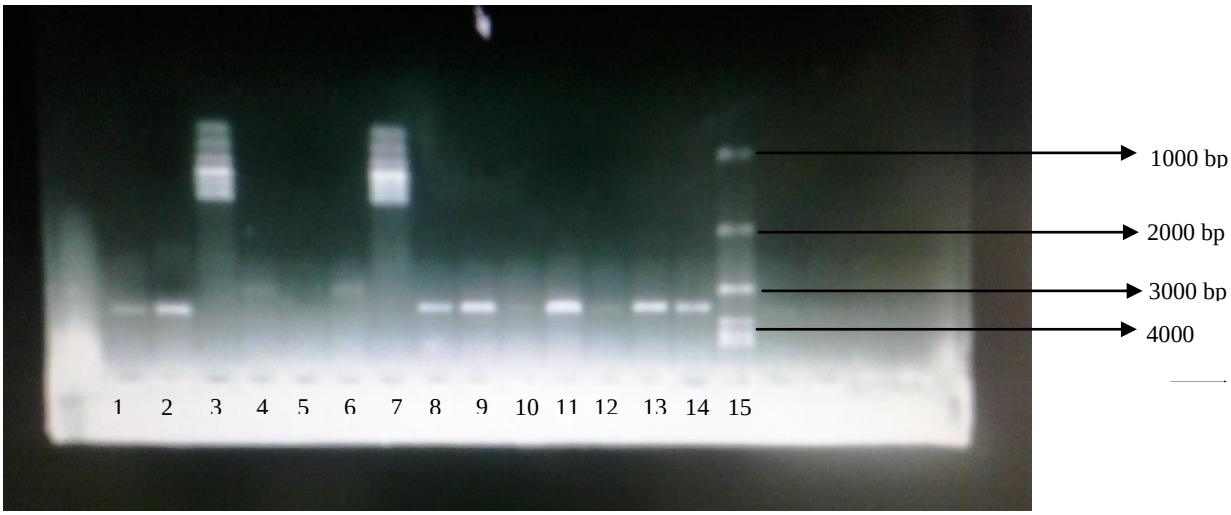

Figure 4.23 *Py* ATPASE 3437 bp

Well 1+, well 2++ , well 3 ladder, well 4-ladder, well 5 negative, well 6 +, well 7 ladder, well 8++, well 9+++, well 10 +, well 11+++,well 12 +, well 13 +,well 14++,well 15 ladder

With *P.falciparum* mdr 1, one mutation was detected at codon 86, this is as shown in **Figure 4.24** and **Figure 4.25.**

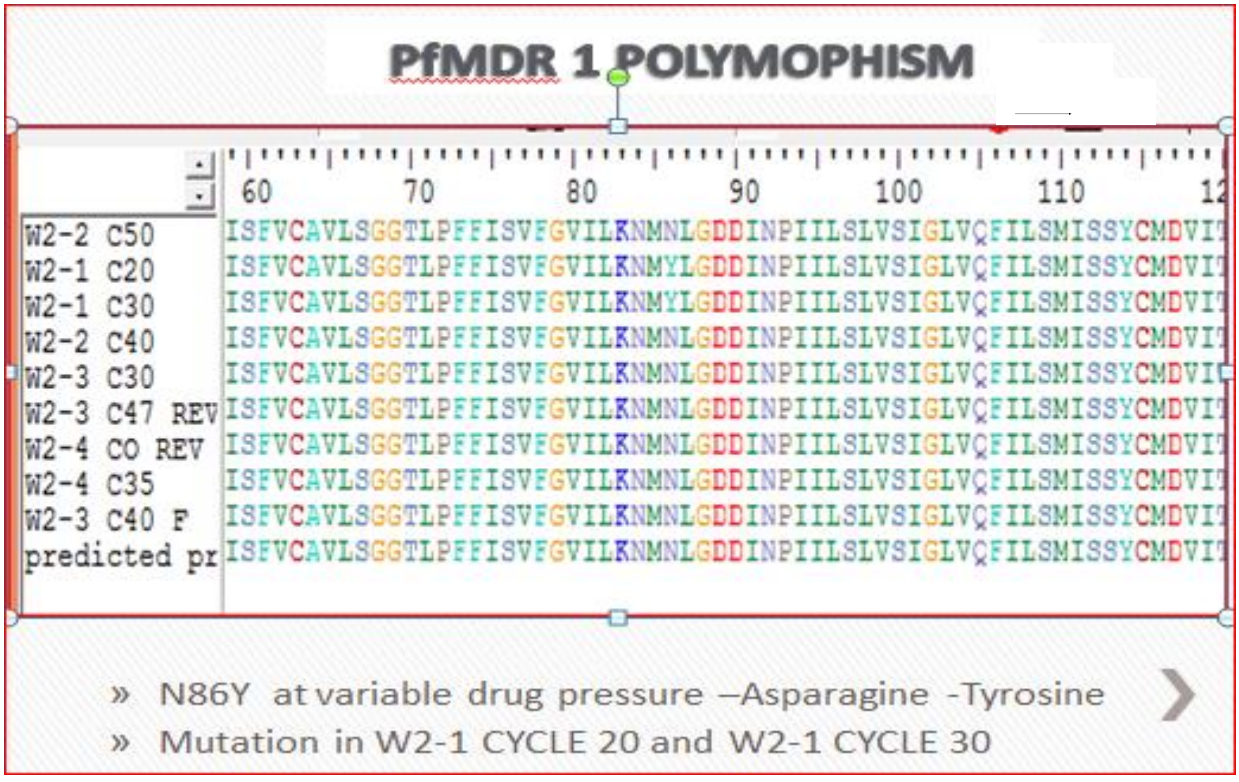

Figure 4.24 *Pf* MDR polymorphisms detected at codon 86

Key:

W2-1 W2 parasites exposed to artemisinin @IC_{50} equivalents

W2-2 W2 parasites exposed to artemisinin @IC_{90} equivalents

W2-3 W2 parasites exposed to *Artemisia annua* @IC_{50} equivalents

W2-4 W2 parasites exposed to *Artemisia annua* @IC_{90} equivalents

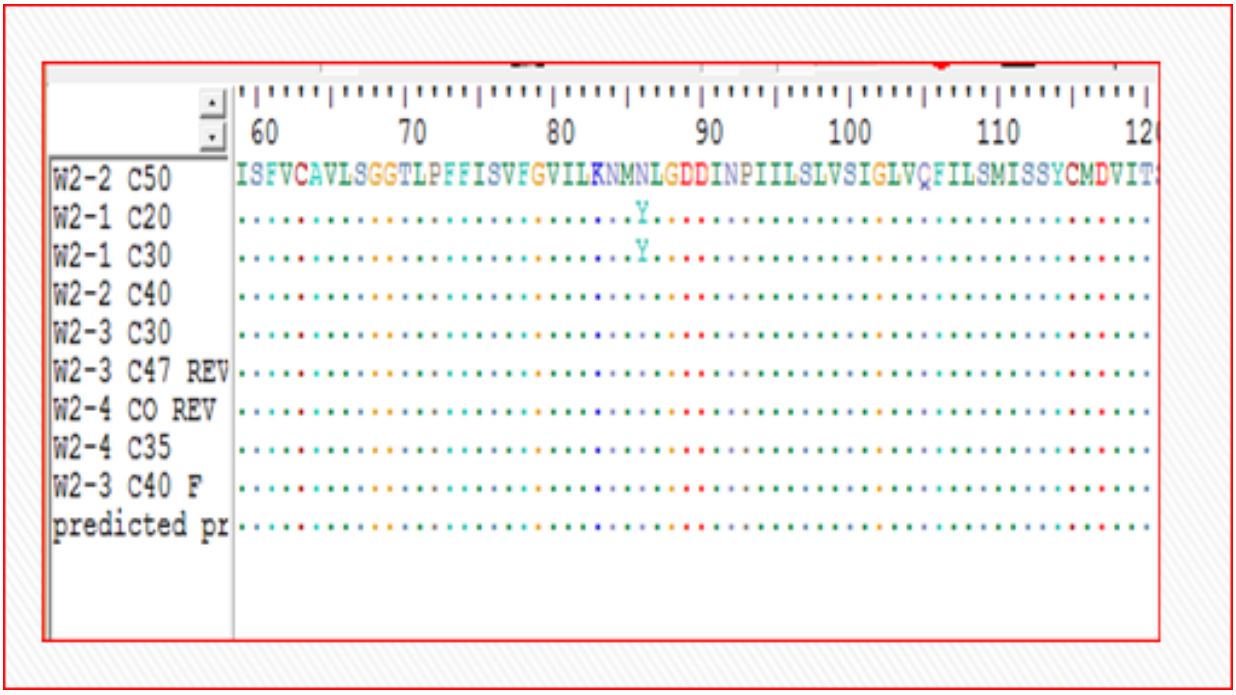

Figure 4.25*Pf* MDR1 amino acid sequence

CHAPTER FIVE

DISCUSSION, CONCLUSION AND RECOMMENDATION

5.1 Discussion

Artemisinin-based combination therapies have been adopted worldwide as official first-line policy for the treatment of *Plasmodium falciparum* malaria. These combine a fast-acting but short-lived artemisinin derivative with one of several longer-lasting partner drugs. Evidence of emerging resistance to artemisinin derivatives has been gathered from clinical studies in western Cambodia, a known source of multidrug-resistant *P. falciparum.* Resistance manifests as prolonged parasite clearance time, as evidenced by microscopically detectable blood stage infections on day 3 after initiation of treatment. Intense efforts are being pursued worldwide to confirm, characterize, and contain resistance to artemisinins. The urgency of these efforts is increased by the paucity of alternative antimalarials should artemisinins fail. Molecular markers have yet to be defined. Nevertheless, evidence is mounting that the ability of early ring-stage intra-erythrocytic parasites to resist artemisinin action, possibly including mechanisms of quiescence or dormancy, might contribute to prolonged clearance times and/or parasite recrudescence after treatment. With the findings in this study indicating that resistance to artemisinin will occur it's the artemisinin combined therapy will save countries where malaria is endemic.

Recent phytochemical research has begun to reveal a variety of extract effects in the bioactivities of plant natural products. Two principal blend effects have been demonstrated: (a) enhanced biological activity resulting from synergistic or other additive effects of moderately active or individually inactive compounds to give mixtures that are more active than a linear summation of individual activities and (b) mitigating effects of structurally related or unrelated compounds against rapid resistance development that characterizes most single-component antibiotics, antiparasitic drugs, and pesticides. Most plants produce a variety of secondary metabolites, which may or may not be structurally related, with multiplicity of defense and non-defense functions against different pathogens and herbivores. This phytochemical and functional diversity has undoubtedly arisen from sustained selective forces in response to succession of attack by pathogens and herbivores and other selective pressures over evolutionary time (Romeo *et al*., 1996; Berenbaum, 1985; Isman *et al*., 1996; Bekele and Hassanali, 2001). Traditional medicine has been utilized for many decades to treat human's ailments; in particular *Artemisia annua* has been used as a tea infusion in China for over 2000 years. Artemisinin was discovered as the active component of A. annua in early 1970s, hundreds of papers have focused on the anti-parasitic effects of artemisinin and its semi-synthetic analogs dihydroartemisinin, artemether, arteether, and artesunate. Artemisinin per se has not been used in mainstream clinical practice due to its poor bioavailability when compared to its analogs. Although artemisinin is a major bioactive component present in the traditional Chinese herbal preparations (tea), leaf flavonoids, also present in the tea, have shown a variety of biological activities and may synergize the effects of artemisinin against malaria and cancer. However, only a few studies have focused on the potential synergistic effects between flavonoids and artemisinin. Artemisia annua has a very rich phytochemistry comprising several classes of compounds mainly monoterpenes, sesquiterpenes and flavonoids (Bhakuni *et al.,* 2002). Other phytochemicals present in *A.annua* has not been used comprehensively in human treatment and there is need to characterize them, determine their potential in treatment,

and do studies on their potential toxicity. Another important potential observed with *A.annua* is its mitigating effect against resistance development.

In our study, *P. falciparum* parasites were exposed to Artemisinin for 40 cycles and inhibitory concentration was determined after every ten cycles. The inhibitory concentration got were compared and expressed as relative sensitivity indices (RSI). Parasites exposed to artemisinin were less sensitive to artemisinin; and the relative sensitivity index was 26.86. When a parallel group of parasites was tested with *A. annua* the parasites were still sensitive as the RSI was 0.06 after 40 cycles of exposure to artemisinin. When the parasites were cultured in the presence of Artemisia annua they remained sensitive. After 40 cycles the RSI was 5.32 and 5.8 at Artemisia annua equivalents of 50 and 90 respectively. These findings demonstrated that *A. annua* was effective in mitigating against resistance development in Plasmodium falciparum. Our findings concurred with Elfawal's finding which indicated higher efficacy of *Artemisia annua* in comparison with the artemisinin.

In a more recent finding, Elfawal and colleagues demonstrated that dried whole-plant *A. annua* effectively kills rodent malaria parasites that are resistant to artemisinin. Mice given a high, single dose of blend showed significantly greater reduction in parasitemia than those given high dose of artemisinin from 16 to 48 h post treatment (Elfawal *et al.*, 2015). Parasitemia of mice treated with a low dose of artemisinin did not differ from control at any point, whereas treatment with *A. annua* was as effective as with high dose of artemisinin, despite the fact the artemisinin high dose contained five times more artemisinin /kg than *A. annua* (Elfawa *et al.*, 2015). In the same study, Elfawal discovered that *A. annua* treatment of 100mg/kg was more resilient than a double dose of pure artemisinin (200mg/kg) (Elfawal *et al.*, 2015).

Since recent research has resolved that artemisinin is nearly insoluble in water, the past success in using *A. annua* for the treatment of various ailments may be attributed either

to the other herbs added to the concoction or to other constituents in *A. annua*, which strengthened the therapeutic effects of artemisinin against such illnesses as malaria (Brown, 2010). Because of artemisinins low solubility in water and oil, semi-synthetic derivatives of artemisin have been synthesized including artemether, arteether, and artesunate and these have unveiled greater therapeutic and pharmacological potential, in addition to enhanced solubility (Brown, 2010).

Artemisinin resistance was achieved in vivo in *Plasmodium berghei* Anka but the resistance phenotype in these parasites was lost after removal of drug pressure, suggesting that resistance was probably due to genetic mutations that are not sustainable by the parasites and therefore was lost in the absence of exposure to treatment, or that continuous exposure of the parasites to artemisinin, might have triggered physiological or epigenetic adaptations that increased the parasite's tolerance to the drug, but were manifested only in a transient manner and were consequently lost after stopping drug treatment.

When *Artemisia annua* extract was used in drug pressure tests and tested using *A. annua* extract, the RSI remained rather low indicating there was no resistance buildup. This correlates with Elfawal's finding with whole plant of *Artemisia annua* with a murine plasmodium (Elfawal *et al.*, 2015).

5.2 Conclusion

The evidence for the emergence of resistance to artemisinins in South-east Asia is compelling and demands aggressive intervention measures. Modeling studies using parasite responses to ACT treatment in western Cambodia argue that as intervention measures decrease the local parasite pool, the relative proportion of resistant parasites will increase (Maude *et al*., 2009). These authors concluded that the spread of resistance can only be halted by eliminating malaria in this region. Proposals to counter resistance include mass drug administration, mass screen and treat campaigns including the use of rapid diagnostic tests, the use of newer ACTs such as DHA–piperaquine or artesunate–pyronaridine that have favorable efficacy and compliance characteristics, and improved case detection and treatment. Vector control measures are also critical and need to include careful monitoring for the emergence and spread of insecticide resistance. Additional efforts include the Affordable Medicines Facility-malaria, an innovative financing mechanism to expand access to affordable ACTs. Defining the molecular basis of resistance will also be critical to effectively monitor for the spread of resistance. With no drugs available to replace ARTs should they fail, the importance of countering resistance cannot be underestimated.

The study has demonstrated that *A. annua* is an effective means of killing malaria parasites in both in vitro cultures and in mouse model (*P. berghei* anka and *P. yoelii yoelii*). A. annua treatment approach could significantly increase the number of patients treated and at significantly lower cost. In fact, the results indicate that treatment with A. annua is a more efficient in slowing resistance development than the purified drug (artemisinin), which is both costly and inefficient. Because *A. annua* has such broad potential therapeutic power against malaria parasites, it could effectively reduce the cost of healthcare in developing and developed nations. Furthermore, use of A. annua could

be implemented locally: a plan for plant cultivation, processing, and drug content validation would require to be formulated. This, in turn, could provide a broad socioeconomic stimulus for developing countries bearing the greatest burden of malaria transmission.

There was resistance development with artemisinin exposed parasites whereas the *A. annua* blend mitigated against resistance development.

5.3. Recommendation

From our finding it is evident that artemisinin resistance will finally take place and this will be quite disastrous as the artemisinins combinations are the main malaria treatment drugs that we have currently. *Artemisia annua* on the other hand have been found to be effective in mitigating resistance debelopment in *P. falciparum*, however more studies need to be carried out to ensure the whole blend does not cause or is not associated with toxicity in humans. Individual phytochemicals present in *Artemisia annua* need to be elucidated and their efficacy and toxicity studied before using the whole blend for human treatment.

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APPENDICES

Appendix 1 Primers designed for sequencing

Primers designed for sequencing *Plasmodium yoelii* and *Plasmodium berghei* Anka genes

set 1	1	py cg10 -1F	CCAATGGAAgtaataaccaaacg
	2	py cg10 -1R	tccttattgtttgccttcgag
set 2	3	py cg10-2F	ttaagctcgaaggcaaacaa
	4	py cg10-2R	tgtttggaaggggttataagaaa
set 3	5	py cg10-3F	GGAGATAATTCAGGACGAAGTTG
	6	py cg10-3R	CCCATTTTATTTAGGGTTCTTTTTG
set 4	7	py cg10-4F	TTTGGATGGCAATTTTTCCT
	8	py cg10-4R	CCCGTTGTTCTTGTGAGACc
set 5	9	py cg10-5F	ccaacagatcccctacaaataca
	10	py cg10-5R	TTGAAGACAACCTCTCTGGTCA
set 6	11	py cg10-6F	TCAAACATGACCAGAGAGGTTG
	12	py cg10-6R	tttggaaaattgccctatcg
set 7	13	py cg10-7F	GCCATTCTCTGAAATGAGCA
	14	py cg10-7R	CATGCCAACACCACAGTTct
set 8	15	py cg10-8F	AGGACCAGCCATAACAATCG
	16	py cg10-8R	TTGGTTTTCTTACAGCATCACC
P. y atpase			
set 1	17	py atpase 1F	TGGATGAGAATCGGGGTTTA
	18	py atpase 1R	AACAACGGGTTCTATGAAATCAC
set 2	19	py atpase 2F	TGGAAATAAAACCCCAGCAG
	20	py atpase 2R	CAGCTGTACATCTACCTGCCACT

set 3	21	py atpase 3F	TGAAGAAACAGATACACCATTGC
	22	py atpase 3R	ATTGCAGCAACTGCTAATGC
set 4	23	py atpase 4F	TGATGATGATGATGATACAGATTATGA
	24	py atpase 4R	GCCAATTCGGTACTATCTCCAA
set5	25	py atpase 5F	TCTGCATGGAGAAACGAATG
	26	py atpase 5R	AATGAATCTGTTAATGGGCGTA
set 6	27	py atpase 6F	CGCCCATTAACAGATTCATT
	28	py atpase 6R	AACCCGAATACCTGCCAAAT
set 7	29	py atpase 7F	TAAATGATGCTCCGGCTCTC
	30	py atpase 7R	AATCGAAGCGACTTCTCCAA
set 8	31	py atpase 8F	CTTGGCATACCCGACAGTTT
	32	py atpase 8R	AGAAGTGGTGGGAAGTGGTG
set 9	33	py atpase 9F	TCTGCTGGAAAGGTCAAGgt
	34	py atpase 9R	CGCCAAGGTGGTAATACGAA
set 10	35	py atpase 10F	TTACCACCTTGGCGAAATATG
	36	py atpase 10R	ACGGGAAATGACCACAAAAA
py mdr			
set 1	37	py mdr 1F	AGCTTCGTTGCCCATTTTTA
	38	py mdr 1R	CATGAAATTCTCCATCTTGTTGA
set 2	38	py mdr 2F	AAATGCAGGAATAGGAACCAAA
	40	py mdr 2R	GCAACAGTTTTAATACCAACTAATGCT
set 3	41	py mdr 3F	TGTTGCAAGTTATTGTGGAGAAA
	42	py mdr 3R	CTGCACTTCCATTGAAACGA
set 4	43	py mdr 4F	TGAACGATTTTATGACCCAACA
	44	py mdr 4R	CCATGCAAGTTGAACCCATT
set 5	45	py mdr 5F	AATGGGTTCAACTTGCATGG
	46	py mdr 5R	TGATGCTACAAAATCGTGGA
set 6	47	py mdr 6F	GACGAAAACAAACAAGGTGCT

	48	py mdr 6R	GCACCACCTGTATCAGAATCTTT
set 7	49	py mdr 7F	GCCAACTAAACCATCATTCTTCA
	50	py mdr 7R	GATATAAACCAGCTGCCACCA
set 8	51	py mdr 8F	CATGCGCCTGGATTTTTATC
	52	py mdr 8R	GATTTTTGCAATCGTGCTCTT
set 9	53	py mdr 9F	AATGCATTTGCTTATTGGTTAGG
	54	py mdr 9R	TTTAGCCTTATCTGCATCTCCTTT
set 10	55	py mdr 10F	AGTTGGAGAAACTGGATGTGG
	56	py mdr 10R	TGCAAGTCGTTCAAATTCTTCA
set 11	57	py mdr 11F	TTGCAATAGTTAACCAAGAACCAA
	58	py mdr 11R	CGTTGTTTTTGACCACCTGA
set 12	59	py mdr 12F	CAGGTGGTCAAAAACAACGA
	60	py mdr 12R	AGATGCAATTCTGTGAGCAAT
pb cg10			
se 1	61	PB CG10-1F	TGATGATCGCTATAAAGAATTGGA
	62	PB CG10-1R	cgcccttattgtttgcattc
set 2	63	PB CG10-2F	TTTTGGATGGCAGTTTTTCC
	64	PB CG10-2R	CCCGTTGTTCTTGTGAGACc
set 3	65	PB CG10-3F	TGAAACACAAGGTGAAAACTCAA
	66	PB CG10-3R	TCCCTGGTCATGTTTGAAAAG
set 4	67	PB CG10-4F	TGACCAGGGAAGTTGTTTTCA
	68	PB CG10-4R	TTGGAACAAAACGACCATAGC
set 5	69	PB CG10-5F	CAAGGACCAGCCATAACAAT
	70	PB CG10-5R	TTTTCTTACAGCATCGCCcta
set 6	71	PB CG10-6F	CAAGGACCAGCCATAACAAT
	72	PB CG10-6R	TTTTCTTACAGCATCGCCcta
pb mdr			
set 1	73	PB MDR 1F	ATCAGGAGCTTCGTTGCCTA

	74	PB MDR 1R	TCCATGCATAAACTTGAAATCG
set 2	75	PB MDR 2F	CAATGTCGATAATTGAAGAAGCA
	76	PB MDR 2R	CCATACCAAAATCCCAAAGC
set 3	77	PB MDR 3F	GCTTTGGGATTTTGGTATGG
	78	PB MDR 3R	TGTCGACAGCTGGTTTTCTG
set 4	79	PB MDR 4F	TCGTCAAGTGGAAATGGTGA
	80	PB MDR 4R	TCTGCAATCTCTTTTTCTTTTCG
set 5	81	PB MDR 5F	GCCCCTGGATTTTTATCGTC
	82	PB MDR 5R	AGCAAATGTTCGCGTTGTAA
set 6	83	PB MDR 6F	TTACAACGCGAACATTTGCT
	84	PB MDR 6R	TTTTCTTCTATCCCCTTTACTGTCA
set 7	85	PB MDR 7F	AGTTGGAGAAACTGGATGTGG
	86	PB MDR 7R	GATGTTGCATCACGCATTTC
set 8	87	PB MDR 8F	TTCGGTAAACAAGATGCAACA
	88	PB MDR 8R	GGCTCTAGCAATAGCAACTCG
set 9	89	PB MDR 9F	GGTGGTCAAAAACAACGAGT
	90	PB MDR 9R	GGGCTTGAACAAAAGATCCA
pb atpase			
set 1	91	PB ATPASE 1F	CGTGGTTTATCCGAAAATGAA
	92	PB ATPASE 1R	CAACGGGCTCTATGAAATCAC
set 2	93	PB ATPASE 2F	CAGTTACAACCAACAAAAGCAAA
	94	PB ATPASE 2R	CCGCTTTAATGCTTGTTGAA
set 3	95	PB ATPASE 3F	GCTGCAATTCCTGAAGGTTT
	96	PB ATPASE 3R	AGCTGTCATTTGGTTTGTTGTT
set 4	97	PB ATPASE 4F	AACAACAAACCAAATGACAGC
	98	PB ATPASE 4R	TGGTTCTTTTTCATAATCTGTATCA
set 5	99	PB ATPASE 5F	CAGAACCAACATTCCCAAGTG
	100	PB ATPASE 5R	TTTCTGGGGCACCTTTACAA

set 6	101	PB ATPASE 6F	CCCTCCAAGAAAATATGTAGGAAA
	102	PB ATPASE 6R	TCGCGTCCATTAAAACAACA
set 7	103	PB ATPASE 7F	ATGGACGCGAATTTGAAGAG
	104	PB ATPASE 7R	TGAGAGCAGGTGCATCATTT
set 8	105	PB ATPASE 8F	CGTCCAGTTGCTTTGGGTTA
	106	PB ATPASE 8R	TGGGCTTGCATTTCATTACA
set 9	107	PB ATPASE 9F	AGGCACATATGTCGGAATAGC
	108	PB ATPASE 9R	TTTTCCAGCAGAAAAATAAGAACA
set10	109	PB ATPASE 10F	TTTTTCTGCTGGAAAAGTTAAGg
	110	PB ATPASE 10R	ACATATTTCGCCAAGGTGGT

PB CG10:1F: TGATGATCGCTATAAAGAATTGG

PB CG10 6R: TTTTCTTACAGCATCGCCcta

PB MDR 1F: ATCAGGAGCTTCGTTGCCTA

PB MDR 9R: GGGCTTGAACAAAAGATCCA

PB ATPASE 1F:CGTGGTTTATCCGAAAATGAA

PBATPASE 10R:ACATATTTCGCCAAGGTGGT

Appendix 2 Informed consent form

Page 1 of 3

Project title: Demonstrating resistance mitigating effect of *Artemisia annua* phytochemical blend with *in vitro* cultures of *Plasmodium falciparum* and *in vivo* with *plasmodium berghei* Anka and *Plasmodium yoelii* in mice.

Consent

I_____ (Name of donor), hereby state that I am over 18 years of age, in good physical health, not pregnant, and wish to participate in a program of research being conducted by Dr Sabah Omar of Malaria Laboratories, KEMRI, the Kenya Medical Research Institute P.O BOX 54840-00200 Nairobi.

Purpose of study

Malaria causes untold suffering and economic losses to the financially challenged sections of the local population.traditionally these diseases are treated with herbal remedies selected through trials over many generation. The herbal remedies consist of crude herbal extracts containing blends of constituents. The identities of such therapies, their sources, and methods of preparation remain difficult as family or community knowledge and sometimes secrets is transferred orally from one generation to the next. In this project we propose to compare the IC_{50} of Artemisinin and that of *Artemisia annua* on in vitro cultures p falciparum (D6 and W2). Dose response studies of *Artemisia annua* extract and artemisinin will be carried out for the next 50 generations as earlier stated. This would help determine if resistance will build in under drug pressure.

age 2 of 3 initials

……..date……

Project title: Demonstrating resistance mitigating effect of *Artemisia annua* phytochemical blend with *in vitro* cultures of *Plasmodium falciparum* and *in vivo* with *Plasmodium berghei* anka and *Plasmodium yoelii* in mice.

Procedure: venous blood will be collected by the standard vein puncture procedure, into an EDTA tube. The area will be cleansed with sterile swab and will be dried with a sterile pad.

Confidentiality: All information collected in this study is confidential to the extent permitted by law. After we collect your blood sample as part of the study on **Demonstrating resistance mitigating effect of *Artemisia annua* phytochemical blend with in *vitro cultures* of *Plasmodium falciparum* and *in vivo* with *Plasmodium berghei* anka and *Plasmodium yoelii* in mice,** we will use your sample (serum/whole blood) for the purpose of this study. Your name will not be linked with your serum or whole blood sample. The original information that you provide with your name and the informed consent form that you sign will be kept in a locked drawer in the office of Dr Sabah Omar, Malaria laboratories KEMRI. The blood that you will provide will be screened for HIV and Hepatities B Viral antigens before blood is used as whole or for obtaining serum you will be counseled by the voluntary counseling centre (VCT) personnel before and after the HIV testing. The blood you provide, if used to obtain serum, will be grouped with other serum from others. At the end of this study all names will be destroyed.

Risks: The risks of participation in this project are minimal. Possible risks of drawing blood include infection, minimal bruising. There may be a small amount of pain accompanying blood collection that may last for a few minutes. Because your sample will be assigned an anonumous numerical identifier and will be kept private by the primary investigator, the chances that anybody could trace information about your sample back will be minimal.

Benefits: You won't benefit directly from giving the sample. This is because we will be making your identity anonymous. Although the experiment is not designed to helpou personally, by participating you may contribute to scientific knowledge about the

potential of natural products for antimalarial drug activity. Participants can ask questions at any time and are free to withdraw at any time to all consent documents.

Page 3 of 3 initials

……..date……

Project title: Demonstrating resistance mitigating effect of *Artemisia annua* phytochemical blend with in vitro cultures of *Plasmodium falciparum* and in vivo with *Plasmodium berghei* Anka and *Plasmodium yoelii* in mice.

I state that I have read the information stated above and that am over 18 years of age in good physical health, and wish to participate in a program of research being conducted by Dr Sabah Omar of Malaria Laboratories, Centre of Biotechnology Research and Development, KEMRI.I understand that I am free to ask questions or to withdraw from participation at any time without penalty.

FOR QUESTIONS ABOUT THE STUDY, CONTACT;

Dr Sabah Omar
Malaria Laboratories,
Centre of Biotechnology Research and Development, KEMRI
P.O Box 54840-00200
Nairobi.
Tel (020) 2722541
Or
Secretary National Ethical Review Board /Director CCR Kemri
P.O Box 54840-00200
Nairobi.
Tel (020) 2722541 FAX (02)715105

Printed name of

subject…….

Signature of subject

……

Date

……

Printed name of researcher

…….

Signature of researcher

……

Date

……

Appendix 3 Reagents for *in vitro* and *in vivo*

The following reagents were prepared for use in the *in vitro* work.

1). 5% $NaHCO_3$

5 gm dissolved in 100 ml of dissolved water. A syringe adaptable filter (mesh size 0.2) was used to filter.

2). **3.5 % NaCL**

3.5 gm dissolved in 100 ml of dissolved water. A syringe adaptable filter (mesh size 0.2) was used to filter.

3). **ROWES Solution**

Glycerol-14 gm, Sorbitol-1.5 gm, Nacl-0.325gm

Top up to 50 ml with distilled water.

4. ACD solution

5. Hypoxanthine

A concentration of 0.05 ml per plate was used

6. Gentamycin- a final concentration of 10ug/ml

7. Ampicilin-a final concentration of 25ug/ml

The following reagents were used in the *in-vivo* work.

1. **Phosphate Saline Glucose Buffer** (PSG) preparation

i. Di- Sodium hydrogen phosphate (Na_2HPO_4)	5.392 gm
ii. Sodium di-hydrogen phosphate ($NaH_2PO_4.2H_20$	0.312 gm
iii. Nacl	1.7 gm
iv. D-Glucose	10 gm

The above were mixed in one litre of distilled water and sterilised by autoclaving at 121^0C for 15 min and stored in a dark place at 4^0 C.

2. **Glycerol PSG**

20 % Glycerol in phosphate saline glucose buffer was used for cryopreserving *P. berghei* and *P. yoelii* parasites.

3. **Heparin** was used as the anticoagulant.

Appendix 4; Sequence data

CLUSTAL W (1.8) *PF* MDR 86 multiple sequence alignment (BioEdit-generated mock-up)

```
W2 plain
TTGTTATAAATTTCGTACCAATTCCTGAACTCACTTGTTCTAAATAAAAA
W2-2 C50
tTGTTATAAATTTCGtACCAATTCCTGAACTCACTTGttCtAAAtaaAAA
W2-1 C20
TTGTTATAAATTTCGTACCAATTCCTGAACTCACTTGTTCTAAATAAAAA
W2-1 C30
TTGTTATAAATTTCGTACCAATTCCTGAACTCACTTGTTCtAAATAAAAA
W2-2 C40
TtGTTATAAATTTCGTACCAATTCCTGAACTCACTTGTTCTAAATAAAAA
W2-3 C30
TTGTTATAAATTTCGTACCAATTCCTGAACTCACTTGTTCTAAATAAAAA
W2-3 C47 REV
TTGTTATAAATTTCGTACCAATTCCTGAACTCACTTGTTCTAAATAAAAA
```

W2-4 CO REV
TTGTTATAAATTTCGTACCAATTCCTGAACTCACTTGTTCTAAATAAAAA
W2-4 C35
TTGTTATAAATTTCGTACCAATTCCTGAACTCACTTGTTCTAAATAAAAA
W2-3 C40 F
tTGTTATAAATTTCGTACCAATTCCTGAACTCACTTGTTCTAAATAAAAA

W2 plain
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W2-2 C50
TCTAAATCAGATCTTAATTTAGATCCAGGATtAttAtcAtGAAATTGTCC
W2-1 C20
TCTAAATCAGATCTTAATTTAGATCCAGGATTATTATCATGAAATTGTCC
W2-1 C30
TCTAAATCAGATCTTAATTTAGATCCAGGATTATTATCATGAAATTGTCC
W2-2 C40
TCTAAATCAGATCTTAATTTAGATCCAGGATTATTATCATGAAATTGTCC
W2-3 C30
TCTAAATCAGATCTTAATTTAGATCCAGGATTATTATCATGAAATTGTCC
W2-3 C47 REV
TCTAAATCAGATCTTAATTTAGATCCAGGATTATTATCATGAAATTGTCC
W2-4 CO REV
TCTAAATCAGATCTTAATTTAGATCCAGGATTATTATCATGAAATTGTCC
W2-4 C35
TCTAAATCAGATCTTAATTTAGATCCAGGATTATTATCATGAAATTGTCC
W2-3 C40 F
TCTAAATCAGATCTTAATTTAGATCCAGGATTATTATCATGAAATTGTCC

W2 plain
ATCTTGATAAAAAACACTTCTTAAATATTCAAGCTTTAAAGTTTTTAATA
W2-2 C50
ATCTTGATAAAAAACACTTCTTAAATATTCAAGCTTTAAAGTTTTTAATA
W2-1 C20
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W2-1 C30
ATCTTGATAAAAAACACTTCTTAAATATTCAAGCTTTAAAGTTTTTAATA
W2-2 C40
ATCTTGATAAAAAACACTTCTTAAATATTCAAGCTTTAAAGTTTTTAATA
W2-3 C30
ATCTTGATAAAAAACACTTCTTAAATATTCAAGCTTTAAAGTTTTTAATA
W2-3 C47 REV
ATCTTGATAAAAAACACTTCTTAAATATTCAAGCTTTAAAGTTTTTAATA
W2-4 CO REV
ATCTTGATAAAAAACACTTCTTAAATATTCAAGCTTTAAAGTTTTTAATA
W2-4 C35
ATCTTGATAAAAAACACTTCTTAAATATTCAAGCTTTAAAGTTTTTAATA
W2-3 C40 F
ATCTTGATAAAAAACACTTCTTAAATATTCAAGCTTTAAAGTTTTTAATA

W2 plain
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W2-2 C50
TTTTTGATGtAATTACaTCCATACAATAACTTGATATCATTGATAATATA
W2-1 C20
TTTTTGATGTAATTACATCCATACAATAACTTGATATCATTGATAATATA
W2-1 C30
TTTTTGATGTAATTACATCCATACAATAACTTGATATCATTGATAATATA
W2-2 C40
TTTTTGATGTAATTACATCCATACAATAACTTGATATCATTGATAATATA
W2-3 C30
TTTTTGATGTAATTACATCCATACAATAACTTGATATCATTGATAATATA
W2-3 C47 REV
TTTTTGATGTAATTACATCCATACAATAACTTGATATCATTGATAATATA
W2-4 CO REV
TTTTTGATGTAATTACATCCATACAATAACTTGATATCATTGATAATATA
W2-4 C35
TTTTTGATGTAATTACATCCATACAATAACTTGATATCATTGATAATATA
W2-3 C40 F
TTTTTGATGTAATTACATCCATACAATAACTTGATATCATTGATAATATA

W2 plain
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W2-2 C50
AATTGTACTAAACCTATAGATACTAATGATAATATTATAGGATTAATATC
W2-1 C20
AATTGTACTAAACCTATAGATACTAATGATAATATTATAGGATTAATATC
W2-1 C30
AATTGTACTAAACCTATAGATACTAATGATAATATTATAGGATTAATATC
W2-2 C40
AATTGTACTAAACCTATAGATACTAATGATAATATTATAGGATTAATATC
W2-3 C30
AATTGTACTAAACCTATAGATACTAATGATAATATTATAGGATTAATATC
W2-3 C47 REV
AATTGTACTAAACCTATAGATACTAATGATAATATTATAGGATTAATATC
W2-4 CO REV
AATTGTACTAAACCTATAGATACTAATGATAATATTATAGGATTAATATC
W2-4 C35
AATTGTACTAAACCTATAGATACTAATGATAATATTATAGGATTAATATC
W2-3 C40 F
AATTGTACTAAACCTATAGATACTAATGATAATATTATAGGATTAATATC

W2 plain
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W2-2 C50
ATCACCTAAATTCATGTTCTTTAATATTACACCAAACACAGATATAAAAA
W2-1 C20
ATCACCTAAATACATGTTCTTTAATATTACACCAAACACAGATATAAAAA
W2-1 C30
ATCACCTAAATACATGTTCTTTAATATTACACCAAACACAGATATAAAAA

W2-2 C40
ATCACCTAAATTCATGTTCTTTAATATTACACCAAACACAGATATAAAAA
W2-3 C30
ATCACCTAAATTCATGTTCTTTAATATTACACCAAACACAGATATAAAAA
W2-3 C47 REV
ATCACCTAAATTCATGTTCTTTAATATTACACCAAACACAGATATAAAAA
W2-4 CO REV
ATCACCTAAATTCATGTTCTTTAATATTACACCAAACACAGATATAAAAA
W2-4 C35
ATCACCTAAATTCATGTTCTTTAATATTACACCAAACACAGATATAAAAA
W2-3 C40 F
ATCACCTAAATTCATGTTCTTTAATATTACACCAAACACAGATATAAAAA

W2 plain
AAGGTAATGTTCCTCCTGATAATACAGCACATACAAATGATATAAATAAT
W2-2 C50
AAGGTAATGTTCCTCCTGATAATACAGCACATACAAATGATATAAATAAT
W2-1 C20
AAGGTAATGTTCCTCCTGATAATACAGCACATACAAATGATATAAATAAT
W2-1 C30
AAGGTAATGTTCCTCCTGATAATACAGCACATACAAATGATATAAATAAT
W2-2 C40
AAGGTAATGTTCCTCCTGATAATACAGCACATACAAATGATATAAATAAT
W2-3 C30
AAGGTAATGTTCCTCCTGATAATACAGCACATACAAATGATATAAATAAT
W2-3 C47 REV
AAGGTAATGTTCCTCCTGATAATACAGCACATACAAATGATATAAATAAT
W2-4 CO REV
AAGGTAATGTTCCTCCTGATAATACAGCACATACAAATGATATAAATAAT
W2-4 C35
AAGGTAATGTTCCTCCTGATAATACAGCACATACAAATGATATAAATAAT
W2-3 C40 F
AAGGTAATGTTCCTCCTGATAATACAGCACATACAAATGATATAAATAAT

W2 plain
AATTTTCTATGTTGTGCAGGTAAACATTTAAACGGTAAAAAAAATGATAT
W2-2 C50
AATTTTCTATGTTGTGCAGGTAAACATTTAAACGGTAAAAAAAATGATAT
W2-1 C20
AATTTTCTATGTTGTGCAGGTAAACATTTAAACGGTAAAAAAAATGATAT
W2-1 C30
AATTTTCTATGTTGTGCAGGTAAACATTTAAACGGTAAAAAAAATGATAT
W2-2 C40
AATTTTCTATGTTGTGCAGGTAAACATTTAAACGGTAAAAAAAATGATAT
W2-3 C30
AATTTTCTATGTTGTGCAGGTAAACATTTAAACGGTAAAAAAAATGATAT
W2-3 C47 REV
AATTTTCTATGTTGTGCAGGTAAACATTTAAACGGTAAAAAAAATGATAT
W2-4 CO REV
AATTTTCTATGTTGTGCAGGTAAACATTTAAACGGTAAAAAAAATGATAT

```
W2-4 C35
AATTTTCTATGTTGTGCAGGTAAACATTTAAACGGTAAAAAAAATGATAT
W2-3 C40 F
AATTTTCTATGTTGTGCAGGTAAACATTTAAACGGTAAAAAAAATGATAT

W2 plain            TTTCTCATTCTTTATTTTT
W2-2 C50            TTTCTCATTCTTTATTTTT
W2-1 C20            TTTCTCATTCTTTATTTTT
W2-1 C30            TTTCTCATTCTTTATTTTT
W2-2 C40            TTTCTCATTCTTTATTTTT
W2-3 C30            TTTCTCATTCTTTATTTTT
W2-3 C47 REV        TTTCTCATTCTTTATTTTT
W2-4 CO REV         TTTCTCATTCTTTATTTTT
W2-4 C35            TTTCTCATTCTTTATTTTT
W2-3 C40 F          TTTCTCATTCTTTATTTTT
```

CLUSTAL W (1.8) D6 MDR 86 multiple sequence alignment (BioEdit-generated mock-up)

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D6A C20         ACTGGCATATGTAAAAATTGTTATAAATTTCGTACCAATTCCTGAACTCA
D6 PLAIN        ACTGGCATATGTAAAAATTGTTATAAATTTCGTACCAATTCCTGAACTCA
D6 D C20        ACTGGCATATGTAAAAATTGTTATAAATTTCGTACCAATTCCTGAACTCA
D6 C C20        -CTGGCATATGTAAAAATTGTTATAAATTTCGTACCAATTCCTGAACTCA

D6A C20         CTTGTTCTAAATAAAAATCTAAATCAGATCTTAATTTAGATCCAGGATTA
D6 PLAIN        CTTGTTCTAAATAAAAATCTAAATCAGATCTTAATTTAGATCCAGGATTA
D6 D C20        CTTGTTCTAAATAAAAATCTAAATCAGATCTTAATTTAGATCCAGGATTA
D6 C C20        CTTGTTCTAAATAAAAATCTAAATCAGATCTTAATTTAGATCCAGGATTA

D6A C20         TTATCATGAAATTGTCCATCTTGATAAAAAACACTTCTTAAATATTCAAG
D6 PLAIN        TTATCATGAAATTGTCCATCTTGATAAAAAACACTTCTTAAATATTCAAG
D6 D C20        TTATCATGAAATTGTCCATCTTGATAAAAAACACTTCTTAAATATTCAAG
D6 C C20        TTATCATGAAATTGTCCATCTTGATAAAAAACACTTCTTAAATATTCAAG

D6A C20         CTTTAAAGTTTTTAATATTTTTGATGTAATTACATCCATACAATAACTTG
D6 PLAIN        CTTTAAAGTTTTTAATATTTTTGATGTAATTACATCCATACAATAACTTG
D6 D C20        CTTTAAAGTTTTTAATATTTTTGATGTAATTACATCCATACAATAACTTG
D6 C C20        CTTTAAAGTTTTTAATATTTTTGATGTAATTACATCCATACAATAACTTG

D6A C20         ATATCATTGATAATATAAATTGTACTAAACCTATAGATACTAATGATAAT
D6 PLAIN        ATATCATTGATAATATAAATTGTACTAAACCTATAGATACTAATGATAAT
D6 D C20        ATATCATTGATAATATAAATTGTACTAAACCTATAGATACTAATGATAAT
D6 C C20        ATATCATTGATAATATAAATTGTACTAAACCTATAGATACTAATGATAAT
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D6A C20        ATTATAGGATTAATATCATCACCTAAATTCATGTTCTTTAATATTACACC
D6 PLAIN       ATTATAGGATTAATATCATCACCTAAATTCATGTTCTTTAATATTACACC
D6 D C20       ATTATAGGATTAATATCATCACCTAAATTCATGTTCTTTAATATTACACC
D6 C C20       ATTATAGGATTAATATCATCACCTAAATTCATGTTCTTTAATATTACACC

D6A C20        AAACACAGATATAAAAAAAGGTAATGTTCCTCCTGATAATACAGCACATA
D6 PLAIN       AAACACAGATATAAAAAAAGGTAATGTTCCTCCTGATAATACAGCACATA
D6 D C20       AAACACAGATATAAAAAAAGGTAATGTTCCTCCTGATAATACAGCACATA
D6 C C20       AAACACAGATATAAAAAAAGGTAATGTTCCTCCTGATAATACAGCACATA

D6A C20        CAAATGATATAAATAATAATTTTCTATGTTGTGCAGGTAAACATTTAAAC
D6 PLAIN       CAAATGATATAAATAATAATTTTCTATGTTGTGCAGGTAAACATTTAAAC
D6 D C20       CAAATGATATAAATAATAATTTTCTATGTTGTGCAGGTAAACATTTAAAC
D6 C C20       CAAATGATATAAATAATAATTTTCTATGTTGTGCAGGTAAACATTTAAAC

D6A C20        GGTAAAAAAAAATGATATTTTCTCATTCTTTATTTTTCTAAATAAT
D6 PLAIN       GGTAAAAAAAAATGATATTTTCTCATTCYTTATTTTTCTAAATAAT
D6 D C20       GGTAAAAAAAAATGATATTTTCTCATTCTTTATTTTTCTAAATAAT
D6 C C20       GGTAAAAAAAAATGATATTTTCTCATTCTTTATTTTTCTAAATAAT
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CLUSTAL W (1.8) PF MDR 1034 multiple sequence alignment (BioEdit-generated mock-up)

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W2-Plain          TAGATCTCTTAGTTATAATCACATATATTAATATCATCTAATAATATTTC
W2-2 C47REV       TAGATCTCTTAGTTATAATCACATATATTAATATCATCTAATAATATTTC
W2-2 C20          TAGATCTCTTAGTTATAATCACATATATTAATATCATCTAATAATATTTC
W2-3 C25REV       TAGATCTCTTAGTTATAATCACATATATTAATATCATCTAATAATATTTC
W2-4 C25REV       TAGATCTCTTAGTTATAATCACATATATTAATATCATCTAATAATATTTC
W2-4 C20          TAGATCTCTTAGTTATAATCACATATATTAATATCATCTAATAATATTTC
W2-4 C0REV        TAGATCTCTTAGTTATAATCACATATATTAATATCATCTAATAATATTTC
W2-2 C40REV       TAGATCTCTTAGTTATAATCACATATATTAATATCATCTAATAATATTTC
W2-3 C10          TAGATCTCTTAGTTATAATCACATATATTAATATCATCTAATAATATTTC
W2-2 C50          TAGATCTCTTAGTTATAATCACATATATTAATATCATCTAATAATATTTC
W2-4 C20          TAGATCTCTTAGTTATAATCACATATATTAATATCATCTAATAATATTTC
W2-2 C40REV       TAGATCTCTTAGTTATAATCACATATATTAATATCATCTAATAATATTTC
W2-32 C42         TAGATCTCTTAGTTATAATCACATATATTAATATCATCTAATAATATTTC
W2-3 C25          TAGATCTCTTAGTTATAATCACATATATTAATATCATCTAATAATATTTC
W2-2 C40          TAGATCTCTTAGTTATAATCACATATATTAATATCATCTAATAATATTTC

W2-Plain          TCCATTATTATTAAATACAGTATAATCTTCTGCAGATCCAGATTGGTTTG
W2-2 C47REV       TCCATTATTATTAAATACAGTATAATCTTCTGCAGATCCAGATTGGTTTG
W2-2 C20          TCCATTATTATTAAATACAGTATAATCTTCTGCAGATCCAGATTGGTTTG
W2-3 C25REV       TCCATTATTATTAAATACAGTATAATCTTCTGCAGATCCAGATTGGTTTG
W2-4 C25REV       TCCATTATTATTAAATACAGTATAATCTTCTGCAGATCCAGATTGGTTTG
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W2-4 C20          TCCATTATTATTAAATACAGTATAATCTTCTGCAGATCCAGATTGGTTTG
W2-4 C0REV        TCCATTATTATTAAATACAGTATAATCTTCTGCAGATCCAGATTGGTTTG
W2-2 C40REV       TCCATTATTATTAAATACAGTATAATCTTCTGCAGATCCAGATTGGTTTG
W2-3 C10          TCCATTATTATTAAAAACAGTATAATCTTCTGCAGATCCAGATTGGTTTG
W2-2 C50          TCCATTATTATTAAATACAGTATAATCTTCTGCAGATCCAGATTGGTTTG
W2-4 C20          TCCATTATTATTAAATACAGTATAATCTTCTGCAGATCCAGATTGGTTTG
W2-2 C40REV       TCCATTATTATTAAATACAGTATAATCTTCTGCAGATCCAGATTGGTTTG
W2-32 C42         TCCATTATTATTAAATACAGTATAATCTTCTGCAGATCCAGATTGGTTTG
W2-3 C25          TCCATTATTATTAAATACAGTATAATCTTCTGCAGATCCAGATTGGTTTG
W2-2 C40          TCCATTATTATTAAATACAGTATAATCTTCTGCAGATCCAGATTGGTTTG

W2-Plain          AAAATTTATTTACATTTTTTAAAACCAATGAATTATTATTATTATTTTGA
W2-2 C47REV       AAAATTCATTTACATTTTTTAAAACCAATGAATTATTATTATTATTTTGA
W2-2 C20          AAAATTCATTTACATTTTTTAAAACCAATGAATTATTATTATTATTTTGA
W2-3 C25REV       AAAATTCATTTACATTTTTTAAAACCAATGAATTATTATTATTATTTTGA
W2-4 C25REV       AAAATTCATTTACATTTTTTAAAACCAATGAATTATTATTATTATTTTGA
W2-4 C20          AAAATTCATTTACATTTTTTAAAACCAATGAATTATTATTATTATTTTGA
W2-4 C0REV        AAAATTCATTTACATTTTTTAAAACCAATGAATTATTATTATTATTTTGA
W2-2 C40REV       AAAATTCATTTACATTTTTTAAAACCAATGAATTATTATTATTATTTTGA
W2-3 C10          AAAATTCATTTACATTTTTTAAAACCAATGAATTATTATTATTATTTTGA
W2-2 C50          AAAATTCATTTACATTTTTTAAAACCAATGAATTATTATTATTATTTTGA
W2-4 C20          AAAATTCATTTACATTTTTTAAAACCAATGAATTATTATTATTATTTTGA
W2-2 C40REV       AAAATTCATTTACATTTTTTAAAACCAATGAATTATTATTATTATTTTGA
W2-32 C42         AAAATTCATTTACATTTTTTAAAACCAATGAATTATTATTATTATTTTGA
W2-3 C25          AAAATTCATTTACATTTTTTAAAACCAATGAATTATTATTATTATTTTGA
W2-2 C40          AAAATTCATTTACATTTTTTAAAACCAATGAATTATTATTATTATTTTGA

W2-Plain          TAATCTTGAAAATTTGTCATATCATTTTTTAATATAATGTGATCATTTTT
W2-2 C47REV       TAATCTTGAAAATTTGTCATATCATTTTTTAATATAATGTGATCATTTTT
W2-2 C20          TAATCTTGAAAATTTGTCATATCATTTTTTAATATAATGTGATCATTTTT
W2-3 C25REV       TAATCTTGAAAATTTGTCATATCATTTTTTAATATAATGTGATCATTTTT
W2-4 C25REV       TAATCTTGAAAATTTGTCATATCATTTTTTAATATAATGTGATCATTTTT
W2-4 C20          TAATCTTGAAAATTTGTCATATCATTTTTTAATATAATGTGATCATTTTT
W2-4 C0REV        TAATCTTGAAAATTTGTCATATCATTTTTTAATATAATGTGATCATTTTT
W2-2 C40REV       TAATCTTGAAAATTTGTCATATCATTTTTTAATATAATGTGATCATTTTT
W2-3 C10          TAATCTTGAAAATTTGTCATATCATTTTTTAATATAATGTGATCATTTTT
W2-2 C50          TAATCTTGAAAATTTGTCATATCATTTTTTAATATAATGTGATCATTTTT
W2-4 C20          TAATCTTGAAAATTTGTCATATCATTTTTTAATATAATGTGATCATTTTT
W2-2 C40REV       TAATCTTGAAAATTTGTCATATCATTTTTTAATATAATGTGATCATTTTT
W2-32 C42         TAATCTTGAAAATTTGTCATATCATTTTTTAATATAATGTGATCATTTTT
W2-3 C25          TAATCTTGAAAATTTGTCATATCATTTTTTAATATAATGTGATCATTTTT
W2-2 C40          TAATCTTGAAAATTTGTCATATCATTTTTTAATATAATGTGATCATTTTT

W2-Plain          CAAGTCATAAAATCTTAATAAGAGATTCATAAAAGTTGATTTTCCACTAC
W2-2 C47REV       CAAGTCATAAAATCTTAATAAGAGATTCATAAAAGTTGATTTTCCACTAC
W2-2 C20          CAAGTCATAAAATCTTAATAAGAGATTCATAAAAGTTGATTTTCCACTAC
W2-3 C25REV       CAAGTCATAAAATCTTAATAAGAGATTCATAAAAGTTGATTTTCCACTAC
W2-4 C25REV       CAAGTCATAAAATCTTAATAAGAGATTCATAAAAGTTGATTTTCCACTAC
W2-4 C20          CAAGTCATAAAATCTTAATAAGAGATTCATAAAAGTTGATTTTCCACTAC
W2-4 C0REV        CAAGTCATAAAATCTTAATAAGAGATTCATAAAAGTTGATTTTCCACTAC
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```
W2-2 C40REV      CAAGTCATAAAATCTTAATAAGAGATTCATAAAAGTTGATTTTCCACTAC
W2-3 C10         CAAGTCATAAAATCTTAATAAGAGATTCATAAAAGTTGATTTTCCACTAC
W2-2 C50         CAAGTCATAAAATCTTAATAAGAGATTCATAAAAGTTGATTTTCCACTAC
W2-4 C20         CAAGTCATAAAATCTTAATAAGAGATTCATAAAAGTTGATTTTCCACTAC
W2-2 C40REV      CAAGTCATAAAATCTTAATAAGAGATTCATAAAAGTTGATTTTCCACTAC
W2-32 C42        CAAGTCATAAAATCTTAATAAGAGATTCATAAAAGTTGATTTTCCACTAC
W2-3 C25         CAAGTCATAAAATCTTAATAAGAGATTCATAAAAGTTGATTTTCCACTAC
W2-2 C40         CAAGTCATAAAATCTTAATAAGAGATTCATAAAAGTTGATTTTCCACTAC

W2-Plain         CTGTTTCTCCAACGATTGCTGTAGTTTTTTTACTATCACATGTAAAAGAT
W2-2 C47REV      CTGTTTCTCCAACGATTGCTGTAGTTTTTTTACTATCACATGTAAAAGAT
W2-2 C20         CTGTTTCTCCAACGATTGCTGTAGTTTTTTTACTATCACATGTAAAAGAT
W2-3 C25REV      CTGTTTCTCCAACGATTGCTGTAGTTTTTTTACTATCACATGTAAAAGAT
W2-4 C25REV      CTGTTTCTCCAACGATTGCTGTAGTTTTTTTACTATCACATGTAAAAGAT
W2-4 C20         CTGTTTCTCCAACGATTGCTGTAGTTTTTTTACTATCACATGTAAAAGAT
W2-4 C0REV       CTGTTTCTCCAACGATTGCTGTAGTTTTTTTACTATCACATGTAAAAGAT
W2-2 C40REV      CTGTTTCTCCAACGATTGCTGTAGTTTTTTTACTATCACATGTAAAAGAT
W2-3 C10         CTGTTTCTCCAACGATTGCTGTAGTTTTTTTACTATCACATGTAAAAGAT
W2-2 C50         CTGTTTCTCCAACGATTGCTGTAGTTTTTTTACTATCACATGTAAAAGAT
W2-4 C20         CTGTTTCTCCAACGATTGCTGTAGTTTTTTTACTATCACATGTAAAAGAT
W2-2 C40REV      CTGTTTCTCCAACGATTGCTGTAGTTTTTTTACTATCACATGTAAAAGAT
W2-32 C42        CTGTTTCTCCAACGATTGCTGTAGTTTTTTTACTATCACATGTAAAAGAT
W2-3 C25         CTGTTTCTCCAACGATTGCTGTAGTTTTTTTACTATCACATGTAAAAGAT
W2-2 C40         CTGTTTCTCCAACGATTGCTGTAGTTTTTTTACTATCACATGTAAAAGAT

W2-Plain         AAATTTTTATAAATAGGTACATTTGGTCTTGAAATATAACGGAAATTTAC
W2-2 C47REV      AAATTTTTATAAATAGGTACATTTGGTCTTGAAATATAACGGAAATTTAC
W2-2 C20         AAATTTTTATAAATAGGTACATTTGGTCTTGAAATATAACGGAAATTTAC
W2-3 C25REV      AAATTTTTATAAATAGGTACATTTGGTCTTGAAATATAACGGAAATTTAC
W2-4 C25REV      AAATTTTTATAAATAGGTACATTTGGTCTTGAAATATAACGGAAATTTAC
W2-4 C20         AAATTTTTATAAATAGGTACATTTGGTCTTGAAATATAACGGAAATTTAC
W2-4 C0REV       AAATTTTTATAAATAGGTACATTTGGTCTTGAAATATAACGGAAATTTAC
W2-2 C40REV      AAATTTTTATAAATAGGTACATTTGGTCTTGAAATATAACGGAAATTTAC
W2-3 C10         AAATTTTTATAAATAGGTACATTTGGTCTTGAAATATAACGGAAATTTAC
W2-2 C50         AAATTTTTATAAATAGGTACATTTGGTCTTGAAATATAACGGAAATTTAC
W2-4 C20         AAATTTTTATAAATAGGTACATTTGGTCTTGAAATATAACGGAAATTTAC
W2-2 C40REV      AAATTTTTATAAATAGGTACATTTGGTCTTGAAATATAACGGAAATTTAC
W2-32 C42        AAATTTTTATAAATAGGTACATTTGGTCTTGAAATATAACGGAAATTTAC
W2-3 C25         AAATTTTTATAAATAGGTACATTTGGTCTTGAAATATAACGGAAATTTAC
W2-2 C40         AAATTTTTATAAATAGGTACATTTGGTCTTGAAATATAACGGAAATTTAC

W2-Plain         ATCTTTAATATCAACTTTACCTTTTATTAAATTTTTATTTATTCTTATTC
W2-2 C47REV      ATCTTTAATATCAACTTTACCTTTTATTAAATTTTTATTTATTCTTATTC
W2-2 C20         ATCTTTAATATCAACTTTACCTTTTATTAAATTTTTATTTATTCTTATTC
W2-3 C25REV      ATCTTTAATATCAACTTTACCTTTTATTAAATTTTTATTTATTCTTATTC
W2-4 C25REV      ATCTTTAATATCAACTTTACCTTTTATTAAATTTTTATTTATTCTTATTC
W2-4 C20         ATCTTTAATATCAACTTTACCTTTTATTAAATTTTTATTTATTCTTATTC
W2-4 C0REV       ATCTTTAATATCAACTTTACCTTTTATTAAATTTTTATTTATTCTTATTC
W2-2 C40REV      ATCTTTAATATCAACTTTACCTTTTATTAAATTTTTATTTATTCTTATTC
W2-3 C10         ATCTTTAATATCAACTTTACCTTTTATTAAATTTTTATTTATTCTTATTC
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W2-2 C50          ATCTTTAATATCAACTTTACCTTTTATTAAATTTTTATTTATTCTTATTC
W2-4 C20          ATCTTTAATATCAACTTTACCTTTTATTAAATTTTTATTTATTCTTATTC
W2-2 C40REV       ATCTTTAATATCAACTTTACCTTTTATTAAATTTTTATTTATTCTTATTC
W2-32 C42         ATCTTTAATATCAACTTTACCTTTTATTAAATTTTTATTTATTCTTATTC
W2-3 C25          ATCTTTAATATCAACTTTACCTTTTATTAAATTTTTATTTATTCTTATTC
W2-2 C40          ATCTTTAATATCAACTTTACCTTTTATTAAATTTTTATTTATTCTTATTC

W2-Plain          CACCATCATCTCTTACATCAATATTTGATTTTCTAATCATTAATGGATAA
W2-2 C47REV       CACCATCATCTCTTACATCAATATTTGATTTTCTAATCATTAATGGATAA
W2-2 C20          CACCATCATCTCTTACATCAATATTTGATTTTCTAATCATTAATGGATAA
W2-3 C25REV       CACCATCATCTCTTACATCAATATTTGATTTTCTAATCATTAATGGATAA
W2-4 C25REV       CACCATCATCTCTTACATCAATATTTGATTTTCTAATCATTAATGGATAA
W2-4 C20          CACCATCATCTCTTACATCAATATTTGATTTTCTAATCATTAATGGATAA
W2-4 C0REV        CACCATCATCTCTTACATCAATATTTGATTTTCTAATCATTAATGGATAA
W2-2 C40REV       CACCATCATCTCTTACATCAATATTTGATTTTCTAATCATTAATGGATAA
W2-3 C10          CACCATCATCTCTTACATCAATATTTGATTTTCTAATCATTAATGGATAA
W2-2 C50          CACCATCATCTCTTACATCAATATTTGATTTTCTAATCATTAATGGATAA
W2-4 C20          CACCATCATCTCTTACATCAATATTTGATTTTCTAATCATTAATGGATAA
W2-2 C40REV       CACCATCATCTCTTACATCAATATTTGATTTTCTAATCATTAATGGATAA
W2-32 C42         CACCATCATCTCTTACATCAATATTTGATTTTCTAATCATTAATGGATAA
W2-3 C25          CACCATCATCTCTTACATCAATATTTGATTTTCTAATCATTAATGGATAA
W2-2 C40          CACCATCATCTCTTACATCAATATTTGATTTTCTAATCATTAATGGATAA

W2-Plain          TATTTCTCAAATGATAATTTTGCATTTTCTGAATCTCCTTTTAAGGACAT
W2-2 C47REV       TATTTCTCAAATGATAATTTTGCATTTTCTGAATCTCCTTTTAAGGACAT
W2-2 C20          TATTTCTCAAATGATAATTTTGCATTTTCTGAATCTCCTTTTAAGGACAT
W2-3 C25REV       TATTTCTCAAATGATAATTTTGCATTTTCTGAATCTCCTTTTAAGGACAT
W2-4 C25REV       TATTTCTCAAATGATAATTTTGCATTTTCTGAATCTCCTTTTAAGGACAT
W2-4 C20          TATTTCTCAAATGATAATTTTGCATTTTCTGAATCTCCTTTTAAGGACAT
W2-4 C0REV        TATTTCTCAAATGATAATTTTGCATTTTCTGAATCTCCTTTTAAGGACAT
W2-2 C40REV       TATTTCTCAAATGATAATTTTGCATTTTCTGAATCTCCTTTTAAGGACAT
W2-3 C10          TATTTCTCAAATGATAATTTTGCATTTTCTGAATCTCCTTTTAAGGACAT
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W2-2 C40REV       TATTTCTCAAATGATAATTTTGCATTTTCTGAATCTCCTTTTAAGGACAT
W2-32 C42         TATTTCTCAAATGATAATTTTGCATTTTCTGAATCTCCTTTTAAGGACAT
W2-3 C25          TATTTCTCAAATGATAATTTTGCATTTTCTGAATCTCCTTTTAAGGACAT
W2-2 C40          TATTTCTCAAATGATAATTTTGCATTTTCTGAATCTCCTTTTAAGGACAT

W2-Plain          TAATTTTCCAGCATAACTACCAGTAAATATAAAAGTAAATAAGGATTTCA
W2-2 C47REV       TAATTTTCCAGCATAACTACCAGTAAATATAAAAGTAAATAAGGATTTCA
W2-2 C20          TAATTTTCCAGCATAACTACCAGTAAATATAAAAGTAAATAAGGATTTCA
W2-3 C25REV       TAATTTTCCAGCATAACTACCAGTAAATATAAAAGTAAATAAGGATTTCA
W2-4 C25REV       TAATTTTCCAGCATAACTACCAGTAAATATAAAAGTAAATAAGGATTTCA
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W2-2 C40REV       TAATTTTCCAGCATAACTACCAGTAAATATAAAAGTAAATAAGGATTTCA
W2-3 C10          TAATTTTCCAGCATAACTACCAGTAAATATAAAAGTAAATAAGGATTTCA
W2-2 C50          TAATTTTCCAGCATAACTACCAGTAAATATAAAAGTAAATAAGGATTTCA
W2-4 C20          TAATTTTCCAGCATAACTACCAGTAAATATAAAAGTAAATAAGGATTTCA
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W2-2 C40REV      TAATTTTCCAGCATAACTACCAGTAAATATAAAAGTAAATAAGGATTTCA
W2-32 C42        TAATTTTCCAGCATAACTACCAGTAAATATAAAAGTAAATAAGGATTTCA
W2-3 C25         TAATTTTCCAGCATAACTACCAGTAAATATAAAAGTAAATAAGGATTTCA
W2-2 C40         TAATTTTCCAGCATAACTACCAGTAAATATAAAAGTAAATAAGGATTTCA

W2-Plain         TAAAGTCATCAACTAATATAGTACCTCTTTTAATTAAGAAGGATCCAAAC
W2-2 C47REV      TAAAGTCATCAACTAATATAGTACCTCTTTTAATTAAGAAGGATCCAAAC
W2-2 C20         TAAAGTCATCAACTAATATAGTACCTCTTTTAATTAAGAAGGATCCAAAC
W2-3 C25REV      TAAAGTCATCAACTAATATAGTACCTCTTTTAATTAAGAAGGATCCAAAC
W2-4 C25REV      TAAAGTCATCAACTAATATAGTACCTCTTTTAATTAAGAAGGATCCAAAC
W2-4 C20         TAAAGTCATCAACTAATATAGTACCTCTTTTAATTAAGAAGGATCCAAAC
W2-4 C0REV       TAAAGTCATCAACTAATATAGTACCTCTTTTAATTAAGAAGGATCCAAAC
W2-2 C40REV      TAAAGTCATCAACTAATATAGTACCTCTTTTAATTAAGAAGGATCCAAAC
W2-3 C10         TAAAGTCATCAACTAATATAGTACCTCTTTTAATTAAGAAGGATCCAAAC
W2-2 C50         TAAAGTCATCAACTAATATAGTACCTCTTTTAATTAAGAAGGATCCAAAC
W2-4 C20         TAAAGTCATCAACTAATATAGTACCTCTTTTAATTAAGAAGGATCCAAAC
W2-2 C40REV      TAAAGTCATCAACTAATATAGTACCTCTTTTAATTAAGAAGGATCCAAAC
W2-32 C42        TAAAGTCATCAACTAATATAGTACCTCTTTTAATTAAGAAGGATCCAAAC
W2-3 C25         TAAAGTCATCAACTAATATAGTACCTCTTTTAATTAAGAAGGATCCAAAC
W2-2 C40         TAAAGTCATCAACTAATATAGTACCTCTTTTAATTAAGAAGGATCCAAAC

W2-Plain         CAATAGGCAAAACTATTAATAAATAATTGAGCGCTTTGACTGAATCCCCA
W2-2 C47REV      CAATAGGCAAAACTATTAATAAATAATTGAGCGCTTTGACTGAATCCCCA
W2-2 C20         CAATAGGCAAAACTATTAATAAATAATTGAGCGCTTTGACTGAATCCCCA
W2-3 C25REV      CAATAGGCAAAACTATTAATAAATAATTGAGCGCTTTGACTGAATCCCCA
W2-4 C25REV      CAATAGGCAAAACTATTAATAAATAATTGAGCGCTTTGACTGAATCCCCA
W2-4 C20         CAATAGGCAAAACTATTAATAAATAATTGAGCGCTTTGACTGAATCCCCA
W2-4 C0REV       CAATAGGCAAAACTATTAATAAATAATTGAGCGCTTTGACTGAATCCCCA
W2-2 C40REV      CAATAGGCAAAACTATTAATAAATAATTGAGCGCTTTGACTGAATCCCCA
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W2-32 C42        CAATAGGCAAAACTATTAATAAATAATTGAGCGCTTTGACTGAATCCCCA
W2-3 C25         CAATAGGCAAAACTATTAATAAATAATTGAGCGCTTTGACTGAATCCCCA
W2-2 C40         CAATAGGCAAAACTATTAATAAATAATTGAGCGCTTTGACTGAATCCCCA

W2-Plain         TAAAGCTGCATTTACAATAATTCTTCTTTTTTGTCCTTTATTTTTATAAT
W2-2 C47REV      TAAAGCTGCATTTACAATAATTCTTCTTTTTTGTCCTTTATTTTTATAAT
W2-2 C20         TAAAGCTGCATTTACAATAATTCTTCTTTTTTGTCCTTTATTTTTATAAT
W2-3 C25REV      TAAAGCTGCATTTACAATAATTCTTCTTTTTTGTCCTTTATTTTTATAAT
W2-4 C25REV      TAAAGCTGCATTTACAATAATTCTTCTTTTTTGTCCTTTATTTTTATAAT
W2-4 C20         TAAAGCTGCATTTACAATAATTCTTCTTTTTTGTCCTTTATTTTTATAAT
W2-4 C0REV       TAAAGCTGCATTTACAATAATTCTTCTTTTTTGTCCTTTATTTTTATAAT
W2-2 C40REV      TAAAGCTGCATTTACAATAATTCTTCTTTTTTGTCCTTTATTTTTATAAT
W2-3 C10         TAAAGCTGCATTTACAATAATTCTTCTTTTTTGTCCTTTATTTTTATAAT
W2-2 C50         TAAAGCTGCATTTACAATAATTCTTCTTTTTTGTCCTTTATTTTTATAAT
W2-4 C20         TAAAGCTGCATTTACAATAATTCTTCTTTTTTGTCCTTTATTTTTATAAT
W2-2 C40REV      TAAAGCTGCATTTACAATAATTCTTCTTTTTTGTCCTTTATTTTTATAAT
W2-32 C42        TAAAGCTGCATTTACAATAATTCTTCTTTTTTGTCCTTTATTTTTATAAT
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W2-3 C25          TAAAGCTGCATTTACAATAATTCTTCTTTTTTGTCCTTTATTTTTATAAT
W2-2 C40          TAAAGCTGCATTTACAATAATTCTTCTTTTTTGTCCTTTATTTTTATAAT

W2-Plain          CAATAGCTTTTTCTATCAAATT
W2-2 C47REV       CAATAGCTTTTTCTATCAAATT
W2-2 C20          CAATAGCTTTTTCTATCAAATT
W2-3 C25REV       CAATAGCTTTTTCTATCAAATT
W2-4 C25REV       CAATAGCTTTTTCTATCAAATT
W2-4 C20          CAATAGCTTTTTCTATCAAATT
W2-4 C0REV        CAATAGCTTTTTCTATCAAATT
W2-2 C40REV       CAATAGCTTTTTCTATCAAATT
W2-3 C10          CAATAGCTTTTTCTATCAAATT
W2-2 C50          CAATAGCTTTTTCTATCAAATT
W2-4 C20          CAATAGCTTTTTCTATCAAATT
W2-2 C40REV       CAATAGCTTTTTCTATCAAATT
W2-32 C42         CAATAGCTTTTTCTATCAAATT
W2-3 C25          CAATAGCTTTTTCTATCAAATT
W2-2 C40          CAATAGCTTTTTCTATCAA-TT
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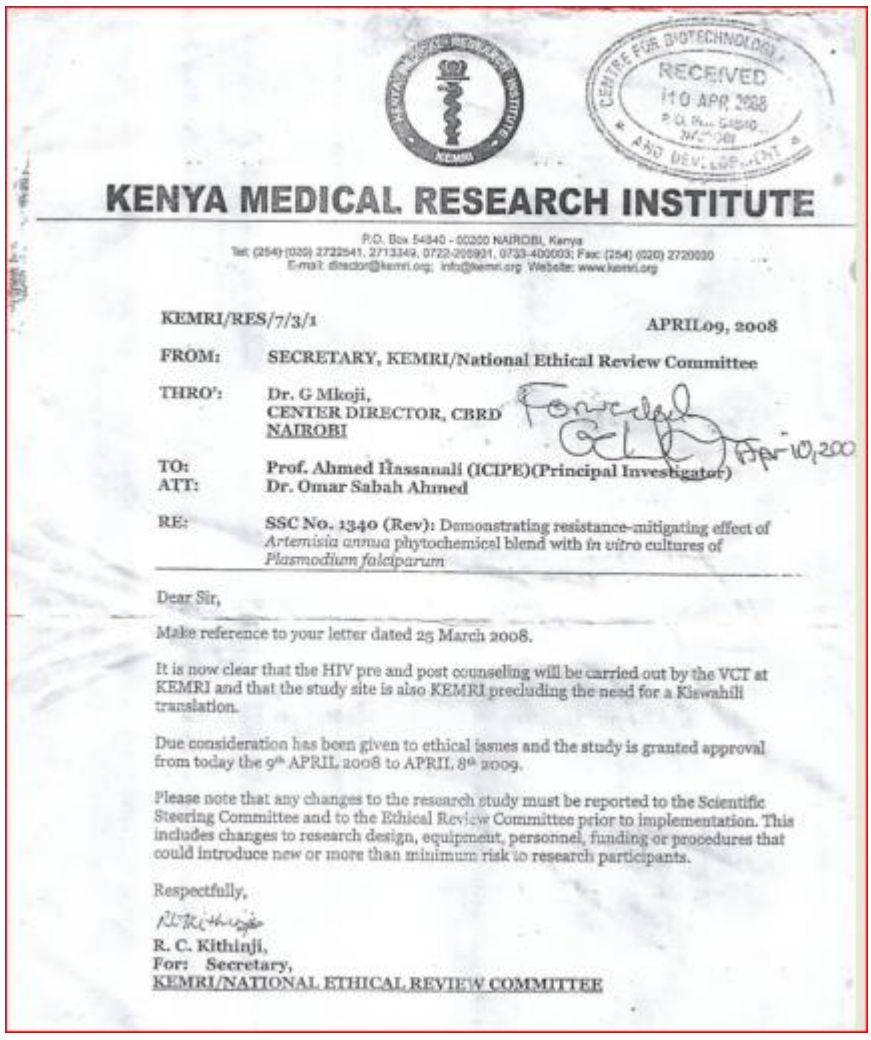
Appendix 5 Ethical clearance



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E-mail: director@kemri.org; info@kemri.org Website: www.kemri.org

KEMRI/RES/7/3/1 APRIL09, 2008

FROM: SECRETARY, KEMRI/National Ethical Review Committee

THRO': Dr. G Mkoji,
CENTER DIRECTOR, CBRD
NAIROBI

TO: Prof. Ahmed Hassanali (ICIPE)(Principal Investigator)
ATT: Dr. Omar Sabah Ahmed

RE: SSC No. 1340 (Rev): Demonstrating resistance-mitigating effect of *Artemisia annua* phytochemical blend with *in vitro* cultures of *Plasmodium falciparum*

Dear Sir,

Make reference to your letter dated 25 March 2008.

It is now clear that the HIV pre and post counseling will be carried out by the VCT at KEMRI and that the study site is also KEMRI precluding the need for a Kiswahili translation.

Due consideration has been given to ethical issues and the study is granted approval from today the 9th APRIL 2008 to APRIL 8th 2009.

Please note that any changes to the research study must be reported to the Scientific Steering Committee and to the Ethical Review Committee prior to implementation. This includes changes to research design, equipment, personnel, funding or procedures that could introduce new or more than minimum risk to research participants.

Respectfully,

R. C. Kithinji,
For: Secretary,
KEMRI/NATIONAL ETHICAL REVIEW COMMITTEE

.