Antimalarial activity and safety properties of *Clausena anisata* and *Clutia robusta* in a mouse model.

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DECLARATION

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

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

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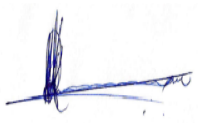
3. Signature…  … …… Date …07.07.2011……

Dr. Romano Mwirichia

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DEDICATION

I dedicate this thesis to my parents Isaiah Murungi and the late Phyllis Mukwanjagi and my siblings for their patience, financial and moral support in the course of this study.

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GOD BLESS YOU ALL

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LIST OF ABBREVIATION AND ACRONYMS

ACT	Artemisinin based combination therapy
ANKA	A strain of *Plasmodium berghei* parasite
4- DT	4- Day Suppressive Test
C*rt*	Chloroquine resistance transporter
CQ	Chloroquine
CTMDR	Centre for Traditional Medicine and Drug Research
DHFR	Dihydro folate reductase
DHPS	Dihydro pteroate synthase
DHA	Dihydroartemisin
DNA	Deoxyribo nucleic acid
I p	Intraperitoneal
KEMRI	Kenya Medical Research Institute
LD_{50}	Lethal dosage that kills 50% of subject under study
Mdr 1	Multi-drug resistance gene 1
Pbmdr	*Plasmodium berghei* multi drug resistance gene 1
PfCRT	*Plasmodium falciparum* chloroquine resistance transporter
PfMdr	*Plasmodium falcipurum* multi-drug resistance 1 gene
PfEMP1	*Plasmodium falciparum* erythrocyte membrane protein 1
PSG	Phosphate saline glucose buffer
SP	Sulfadoxine – Pyrimethamine
WHO	World Health Organization

ABSTRACT

In sub-Saharan Africa, malaria is responsible for approximately a million infant deaths a year, predominantly among the poor who have little or no access to modern medicine. This group represents some 75% of the world's population that relies on herbal remedies. In this project, the antimalarial activities and safety properties of *Clausena anisata* and *Clutia robusta* hexane, chloroform and methanol extracts on *Plasmodium berghei* ANKA, *in vivo* in swiss mouse model of malaria was investigated. The results showed that at a single dose of 5000 mg/kg body weight, *Clutia robusta* extracts had no toxic effects on the mice. *Clausena anisata* chloroform extract doses above 1582 mg/kg were lethal to the mice with animals treated with 5000 and 2811 mg/kg of the extract producing 60% and 40% mortality respectively. LD_{50} of mice treated with chloroform extract was calculated as 3514 mg/kg. Chloroform extract at 500mg/kg/day exhibited high suppressive activities at 72.13%. When established infections were treated with chloroform extracts of *Clausena anisata*, the median survival time of the mice observed at 500 mg/kg/day was longer compared to the untreated control at 9 and 7days respectively. *C. anisata* extracts tested demonstrated a dose dependent chemosuppression of 78.56% at 500 mg/kg/day. PCR was used to detect the presence of *P. berghei* in the dry blood spots from the experimental mice after the drug pressure assay. *C.anisata* chloroform extract showed significant antimalarial activity and enhanced median survival time of mice. This shows that the plant has antimalarial properties that can be explored for the management of malaria**.**

CHAPTER ONE

1.0 Introduction

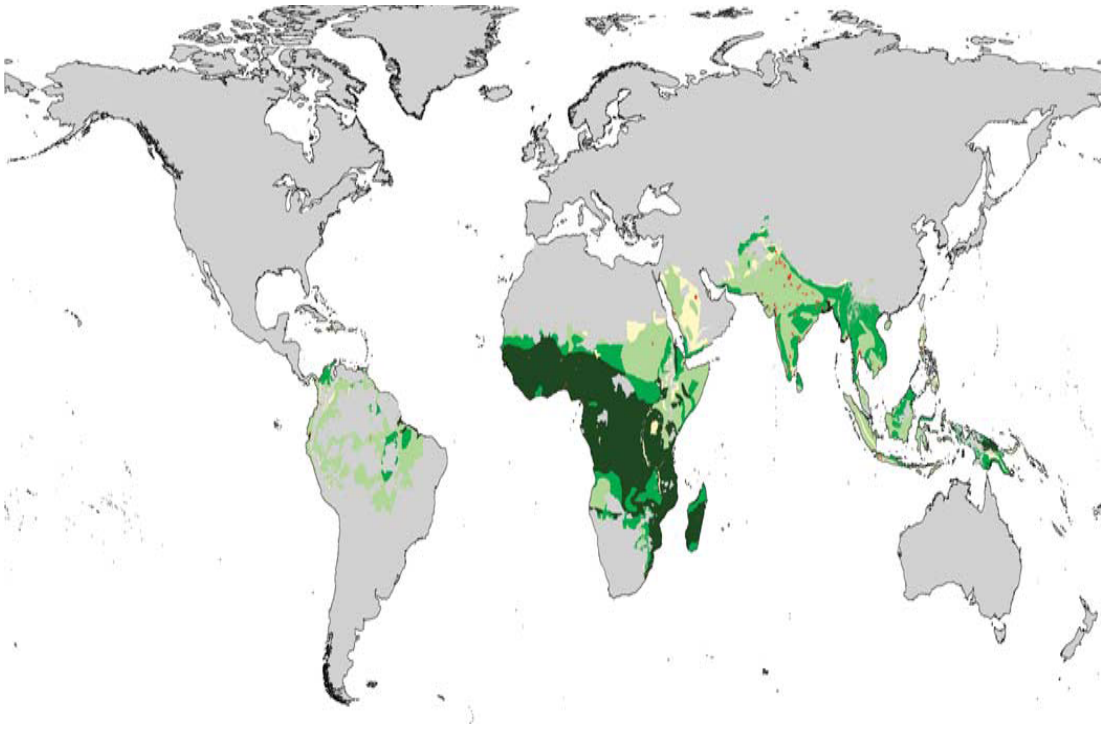
1.1 Background information

Malaria is a vector borne infectious disease caused by protozoan parasites of the genus *Plasmodium* (Phylum Apicomplexa). It is widespread in tropical and subtropical regions, including the Latin America, Asia and Africa. Each year, there are approximately 515 million cases of malaria, killing between one and three million people, the majority of whom are young children and pregnant women in sub- Saharan Africa (Snow *et al*, 2005; WHO 2008). Ninety percent of malaria related deaths occur in sub-Saharan Africa. Despite efforts to reduce transmission and increased treatment, there has been little change in the areas which are at risk of this disease since 1992 (Hay *et al*, 2004). Indeed, if the prevalence of malaria stays on its present upward course, the death rate could double in the next twenty years (Breman *et al*, 2001). Precise statistics are unknown because many cases occur in rural areas where people do not have access to hospitals or the means to afford health care. As a consequence, the majority of cases are undocumented.

Plasmodium falciparum is the most common cause of infection and is responsible for about 80% of all malarial cases, and about 90% of deaths (Escalante *et al.*, 1994; Mendis *et al*., 2001). The malaria situation is aggravated by the appearance of the *Plasmodium falciparum* strain which is resistant to antimalaria drugs as well as by the

resistance of the vector *Anopheles* mosquito to insecticides (Nathan *et al*., 2005). There is no single method of malaria control that is effective in high transmission areas (Lengeler *et al*., 1997; Casmiro *et al.,* 2006*).* In Africa alone the disease is assumed to be responsible for the death of about 1 million children annually (Geoffrey, 1998; Snow *et al*., 2005; WHO, 2008).

Widespread chloroquine resistance forced many countries to use alternative drugs as antimalarials against falciparum malaria, such as the combination of sulphadoxine and pyrimethamine (SP). However, the parasite has since developed resistance to this drug combination associated with point mutations in the genes for the enzymes involved in the obligatory parasite-folate biosynthesis pathway. Such mutations lead to the lowering of the drug binding affinity to the parasite enzymes (Yuvaniyama *et al*., 2003). Resistance to pyrimethamine is attributed to mutations in the gene for the parasite enzyme dihydrofolate reductase (*dhfr*). The increased level of resistance has been found to be associated with increased numbers of mutations in the genes for this enzyme. Studies have shown that multiple mutations in the gene for this enzyme result in exceedingly high pyrimethamine treatment failure (Wang *et al*., 1997). Detection of these mutations in field isolates has been proposed as an alternative strategy for rapid screening of antifolate drug resistance (Kublin *et al*., 2002).



-Infection prevalence of 50% or more

-Infection prevalence between 11% and 50%

- Infection prevalence less than 10%

-Only 6% of the global population at risk

-Areas outside of the transmission limit

Plate 1: Falciparum endemicity distribution within the global limits of risk (WHO world report 2005).

Malaria is not just a disease commonly associated with poverty but also a cause of poverty in itself and a major hindrance to economic development (Sachs *et al.*, 2002).Tropical regions are mostly affected, since the poor do not have the financial

capacity to prevent or treat the disease. (Bremen *et al.*, 2001; Clarkson et *al.*, 2004; Snow *et al.*, 2005).

One of the strategies in the search for new antimalarial compounds is a study of active constituents in medicinal plants. Traditional medicines have been used to treat malaria for thousands of years. Some of the plants used traditionally in malaria treatment have been investigated for their efficacy with positive results. The extracts tested *in vitro* have shown to be active against chloroquine (CQ)-sensitive and resistant strains of *P. falciparum* (Muregi *et al.*, 2003).

Plants have invariably been a rich source for new drugs and some antimalarial drugs in use today were either obtained from plants or developed using their chemical structures as templates (Gessler *et al.*, 1994; Zowai *et al.*, 2003). It is already estimated that 122 drugs from 94 plant species have been discovered through ethnobotanical leads (Fabricant and Farnsworth, 2001).

Plants commonly used in traditional medicine are assumed to be safe due to their long usage in the treatment of diseases according to knowledge accumulated over centuries. However, recent scientific findings have shown that many plants used as food or in traditional medicine are potentially toxic, mutagenic and carcinogenic. The reported toxicity of herbal formulations may be the result of several factors, including contamination with pesticides, microbes, heavy metals, toxins or adulteration with orthodox drugs (El Nahhal, 2004). Therefore, for safety and quality assurance, chemical analytical techniques should be applied at different stages for good practices in quality

assurance of natural or herbal products, including good sourcing and innovative clinical trial practices by researchers (Chan, 2003).

A number of studies have been undertaken to evaluate the inhibitory effects of various plant extracts on *Plasmodium falciparum* (Le Tran *et al*., 2003; Muregi *et al*., 2003). Similarly, the *in vivo* antimalarial properties of several plant extracts have been studied in mice (Agbaje and Onabanjo, 1991; Perez *et al*., 1994; Andrade-Neto *et al*., 2004).

In Africa, it is estimated that, up to 80% of the population use traditional medicine for primary health care (WHO, 2003). However, scientific evidence of their efficacy is limited.

The aim of this project was to identify an active extract from some selected medicinal plants. This is because many of the effective phytomedicines in the market are whole extracts of plants and practitioners believe that synergistic interactions between the compounds of individual or mixtures of medicinal plants are vital for therapeutic efficacy (Williamson, 2001).

1.2 Plants investigated

1.2.1 *Clausena anisata*

Clausena anisata (willd) Benth is a tropical plant that belongs to the family Rutaceae. Its many common / vernacular names include hoarse wood, spirit plant, Mjarikali (Swahili), Mutathi (Kikuyu, Kenya), Munyithia/ Mukibia (Meru, Kenya), Sesadua (Twi, Ghana) and umsanka (Zulu). The plant often grows as a small shrub reaching a height of about 4m. It bears alternately arranged, petiolated, pinnately compound leaflets (7-38 cm long) which may be ovate-lanceolate or obliquely ovate. The leaf apex may be acute, rounded or acuminate and the margin entire, serrated or crenulated.

Clausena anisata has cream or whitish –yellow green flowers, the smell of which resembles oil of aniseed. It bears drupe – like yellow green fruits which turn blue –black on maturity. The bark of the plant is grey or mottled coloured. Medicinally, the plant has been used as an antirheumatic, analgesic (for ear and toothache), anthelminthic (tapeworm) and antiparasitic agent (Hutchings *et al*., 1996).The pounded roots serve as ingredients in a soup preparation taken by women during and after childbirth to cleanse the uterus and for the treatment of headaches, malaria, influenza and indigestion (Kokwaro,1993).The plant is used to treat a wide range of other conditions including dementia, infertility and impotence, gingivitis, respiratory and cardiovascular disorders, constipation, gastroenteritis and leprosy. Decoction of the plant is drunk three times a day to treat syphilis (Beetje, 1994). Carbazole alkaloids active against Gram-positive and Gram–negative bacteria and fungi and with antitumour activity have been isolated from

this plant (Chakraborty *et al.*, 1995; Ito *et al.,* 2000). The antidiabetic, antimicrobial, antifeedant, parasiticidal, central nervous system depressant and spasmolytic properties of *C.ansata* have been investigated *in vivo* and *in vitro* (Ito *et al.,* 2000; Ojwole, 2002). A number of these studies have confirmed the folkloric uses of the plant and speculated that the effects of the plant extract are due to the content of its oil.

The oil of *C.anisata* is yellow liquid with a strong anise odour, over nineteen components have been identified in the leaves with the volatile oil containing phenyl propanoids, methyl havicol, terpenoids and fatty acids (GHP, 1992), coumarins such as xanthotoxins and limonoids such as clausenarin. The plant also contains clausenal and clausemine (Chakraborty *et al*., 1995) and carbozole alkaloids (Ito *et al*., 2000).

1.2.2 *Clutia robusta*

Clutia robusta locally known as *Muthimamburi* by Kikuyus and *Mutemavengi* by the Kamba people of Kenya is a plant of the family Euphorbiaceae. It is a dioecious, erect, lax shrub that grows up to 6 m tall and common in dry forest and wooded grassland on rocky hillsides at 700–3700m altitude. *Clutia robusta* has many medicinal uses against a range of diseases. Roots and leaves are commonly used for medicinal purposes. The Kamba people from Machakos District of Kenya use the root decoction to treat fever and cough and is taken by pregnant women as a tonic, they apply ground roots as an enema to treat gonorrhoea, rub on the head or drink a leaf extract to treat headache, drink sap of leafy twigs to treat chest pain, side pain and shortness of breath, drink an infusion of leafy twigs or leaves and eat the ash to treat skin problems, elephantiasis, diarrhoea and

tachycardia (Schmelzer, *et al*., 2008). The Maasai people drink the root decoction, sometimes in milk, against liver problems and rheumatism, and use pieces of wood as ear plugs as they are thought to be antibacterial.

In most parts of Kenya, the boiled roots are made into a soup which is taken as a remedy for enlarged spleen and kidney problems, and to treat headache, stomach-ache and malaria. A root extract is drunk to cure intestinal worms, influenza, colds and fever, and as a remedy for indigestion. To treat malaria, leaves are boiled to prepare a vapour bath. Leaf extract is drunk to induce the process of childbirth and wood is smoked by women to treat menstrual pains. A maceration of root bark is drunk to treat abdominal problems, as a laxative and to expel intestinal worms (Beentje, 1994; De boer *et al*., 2005).

1.3 Research problem

Malaria a disease caused by *Plasmodium* parasites, though preventable and curable, is still one of the greatest global public health problems especially in sub-Saharan Africa. This can partly be attributed to development of resistant strains to readily affordable medicines such as chloroquine, sulphadoxine/pyrimethamine and amodiaquin. As a result, artemisinin-based combination therapies (ACTs) have been recommended by WHO as a first-line treatment of uncomplicated falciparum malaria in endemic areas. They are seen as the magic 'bullet' that will roll back malaria. Unfortunately, recent reports indicate a decline in efficacy of artemisinin derivatives along Thai-Cambodia border, a site historically known for emerging antimalarial drug resistance. Development and spread of artemisinin resistance to other malaria endemic areas is therefore not a

question of if but when. Hence, new agents active against artemisinin-resistant *P. falciparum* will be needed, possibly soon. Consequently, there is an urgent need for a vibrant drug discovery initiative to produce lead antimalarial agents including phytomedicines. Traditional medicinal plants have been used to treat malaria for thousands of years. However, scientific information about the antimalarial activity of these plants is very limited and only whole extracts of these plants are available in the market.

Most people living in Kenya and other tropical and subtropical regions are unable to afford the pharmaceutical drugs and so they rely on such whole extracts which are sold over the counter or on the streets and are not registered. The current project investigated the antimalarial activities and safety properties of the crude extracts of *C. anisata* and *C robusta* in a mouse model in order to determine their potential as a source of new and cheaper antimalarial agents.

1.4 Justification

Malaria continues to be a major health challenge in sub–Saharan Africa. This is due to increase of resistant strains of malaria parasite and lack of cheaper, safer, and effective antimalarial remedies. Studies indicate that traditional medicinal practices in this region are dynamic. However, most of the effective phytomedicines in the market are whole extracts of the plants and there is limited scientific data available to assess the efficacy and safety of these herbal remedies. In treatment of malaria, development of safe, cheap

and potent antimalarial plant extracts may be the way forward in dealing with the drug resistance problem of malaria.

1.5 Hypothesis

Extracts from *C.anisata and C.robusta* have no suppressive, curative or prophylactic antimalarial properties

1.6 General objective

To investigate *in vivo* antiplasmodial properties of *C.anisata* and *C. robusta* against *Plasmodium berghei* using a mouse model

1.7 Specific objectives

1. To determine suppressive, curative and prophylactic antimalarial properties of the extracts
2. To determine LD_{50} of the extracts
3. To assess the efficacy of the extracts in clearing sensitive and or resistant parasites by targeting the *mdr* 1 resistance gene marker

CHAPTER TWO

2.0 Literature review

2.1 Human plasmodium species

In humans malaria is caused by four distinct species of plasmodium namely *P.falciparum, P. ovale, P.malariae and P.vivax,* although recent research shows that *P. knowlesi* also contributes to the human malaria burden *(Singh et al.,* 2004*; Mueller et al.,* 2007). Parasitic plasmodium species also infect reptiles, birds, monkeys, chipanzees and rodents (Escalante *et al.,* 1994). There have been documented human infections with several simian species of malaria, namely *P. knowlensi, P .inui, P .cynomolgi, P.simiovale, P.brazilianum, P.schwetzi and P .simium;* however , with the exception of *P. knowlensi,* these are mostly of limited public health importance (Permin *et al.,* 2001).

Plasmodium falciparum is the most common cause of infection and is responsible for about 90% of the deaths from malaria. *Plasmodium vivax* remains in the body longer than *P.falciparum* causing more gradual health deterioration. *P. malariae* causes the third most common type of malaria although the progress is slower than the other three species. *P.ovale* causes the less pathogenic and least common malaria in humans (Mendis *et al.*, 2001).

The parasites definitive host and transmission vectors are female mosquitoes of the *Anopheles* genus. Only female mosquitoes feed on blood, thus males do not transmit the

disease. The females of the Anopheles genus of mosquito prefer to feed at night. Malaria parasites can also be transmitted by blood transfusions, although this is rare (Marcucci *et al.,* 2004)

2.2 Rodents plasmodium species

In Africa, there are four rodent parasites used in research as laboratory models in studies aimed at developing strategies to eradicate human malaria (Carlton *et al.,* 2001). They include *P. berghei, P. yoelii, P.chabaudi and P. vinckei* (Smith and pearsons, 1996*).*

Plasmodium berghei was discovered in 1948 by Vincke and Lips and five strains have since been isolated namely ANKA, SP11, NK65, LUKA and K173. *Plasmodium berghei* infects mice, rats and hamsters and all the isolates show a comparable sensitivity to antiplasmodial drugs and no variation in isoenzymes (Janse and Waters, 2006).

2.3 Pathogenesis and the life cycle of malaria parasite

Malaria parasites in human develop via two phases: an exoerythrocytic and an erythrocytic phase. The exoerythrocytic phase involves infection of the hepatic system, whereas erythrocytic phase involves infection of the erythrocytes. When an infected mosquito pierces a person's skin to take blood meal, sporozoites in the mosquito's saliva enter the bloodstream and migrate to the liver. Within 30 minutes of being introduced into human host, the sporozoites infect the hepatocytes, multiplying asexually and asymptomatically for a period of 6 -15 days. Once in the liver, the organisms differentiate to yield thousands of merozoites, which following rapture of their host

cells, escape into blood cells, thus beginning the erythrocytic stage of the life cycle. (Bledsoe *et al.,* 2005). Within the red blood cells, the parasite multiplies further asexually into ring forms, then into trophozoites (a feeding stage), and then schizonts (a reproductive stage) and then back into merozoites. Sexual forms called gametocytes are also produced which, if taken by a mosquito, will infect the insect and continue the life cycle or break out of their hosts to invade fresh red blood cells. Several such amplifications occur. This simultaneous wave of merozoites escaping and infecting the red blood cells causes the classical description of waves of fever.

Plate 2: Mosquito piercing a human skin to take a blood meal

Some *P. vivax* and *P.ovale* sporozoites do not immediately develop into exoerythrocytic-phase merozoites, but instead produce hypnozoites that remain dormant for periods ranging from several months (6-12) to as long as three years. After a period of dormancy, they reactivate and produce merozoites. Hypnozoites are responsible for long incubation and late relapses in these species of malaria (Cogswell, 1992).The parasite is

relatively protected from attack by the body's immune system because for most of its human life cycle it resides within the liver and blood cells and is relatively invisible to immune surveillance. However, circulating infected blood cells are destroyed in the spleen. To avoid this fate, the P. *falciparum* parasite displays adhesive proteins on the surface of the infected blood cells, causing the blood cells to stick to the walls of small blood vessels, thereby sequestering the parasite from passage through the general circulation of the spleen (Chen *et al*., 2000) This "stickiness" is the main factor giving rise to hemorrhagic complications of malaria. High endothelial venules (the smallest branches for the circulatory system) can be blocked by the attachment of masses of these infected red blood cells. The blockage of these vessels causes symptoms such as in placental and cerebral malaria. In cerebral malaria the sequestrated red blood cells can breach the blood brain barrier possibly leading to coma (Adams *et al.*, 2002). Although the red blood cell surface adhesive proteins (called PfEMP1, for *Plasmodium falciparum* erythrocyte membrane protein 1) are exposed to the immune system, they do not serve as good immune targets because of their extreme diversity; there are at least 60 variations of the protein within a single parasite and perhaps limitless versions within parasite populations (Chen *et al.*, 2000). The parasite switches between a broad repertoire of PfEMP1 surface proteins, thus staying one step ahead of the pursuing immune system.

Some merozoites turn into male and female gametocytes. If a mosquito pierces the skin of an infected person, it potentially picks up gametocytes within the blood. Fertilization and sexual recombination of the parasite occurs in the mosquito's gut, thereby defining the mosquito as the definitive host of the disease. New sporozoites develop and travel to

the mosquito's salivary gland, completing the life cycle. Pregnant women are especially attractive to the mosquito (Lindsay *et al.*, 2000) and malaria in pregnant women is an important cause of stillbirths, infant mortality and low birth-weight (Van Geertruyden,,*et al.*, 2004) particularly in *P. falciparum* infection, but also in other species infection, such as *P. vivax* (Rodriguez-Morales *et al.*, 2006).

2.4 Symptoms of malaria

Symptoms of malaria include fever, shivering, arthralgia (joint pain), vomiting, anemia (caused by haemolysis), haemoglobinuria, retinal damage (Beare *et al.*, 2006) and convulsions. The classic symptom of malaria is cyclical occurrence of sudden coldness followed by rigor and then fever and sweating lasting for six hours, occurring every two days in *P.vivax*, and *P.ovale* infections, while every three for *P.malarieae* (http:/www.malasria.am/eng/pathogenesis.php). *P. falciparum* can have recurrent fever every 36-48 hours or less pronounced and almost continuous fever. Children with malaria frequently exhibit abnormal posture, a sign indicating severe brain damage. Malaria has been found to cause cognitive impairments, especially in children. It causes widespread anaemia during a period of rapid brain development and also direct brain damage. This neurological damage results from cerebral malaria to which children and pregnant women are more vulnerable (Boivin *et al.*, 2002).

2.5 Diagnosis of malaria parasites

Severe malaria is commonly misdiagnosed in Africa, leading to a failure to treat other life threatening illnesses. Recent investigation suggests that malarial retinopathy is better (collective sensitivity of 95% and specificity of 90%) than any other clinical or laboratory feature in distinguishing malaria from non-malaria coma (Beare *et al*., 2006).

2.5.1 Microscopic examination of blood films

Two sorts of blood film are used. Thin film and thick film. Thin film allows species identification because the parasite appearance is best preserved in this form of preparation. Thick film allows screening of larger volume of blood and is more sensitive than the thin film, so picking up low levels of infection. The appearance of the parasite is much more distorted and therefore distinguishing between the different species can be much more difficult. (Warhust *et al.*, 1996).

In areas where microscopy is not available, or where laboratory staff is not experienced, immunochromatographic tests (Malaria Rapid Diagnostic Tests, Antigen- capture Assay or "Dipsticks") have been developed. These tests use the *P. falciparum* lactate dehydrogenase, (pLDH), a 33kDa oxidoreductase. It is the last enzyme of the glycolytic pathway, essential for ATP generation and one of the most abundant enzymes expressed by *P. falciparum*. pLDH does not persist in the blood but clears about the same time as the parasite following successful treatment (WHO, 2001). More accurate molecular

methods have been developed that detect parasite nucleic acids using the polymerase chain reaction (PCR) (Mens *et al*., 2006).

2.6 Prevention and disease control

Prevention and spread of malaria requires integrated approaches, including the use of prophylactic drugs, mosquito eradication, vaccine development, use of chemotherapeutic agents and the prevention of mosquito bites (Breman *et al*., 2006).

2.6.1 Prophylactic drugs

Most of the drugs which are used for treatment of malaria can be taken preventively.The 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. Modern drugs used preventively include mefloquine (Lariam), doxycycline and the combination of atovaquone and proguanil hydrochloride (Malarone). The choice of the drug for prophylaxis depends on which drug the parasites in the area are resistant to and the side effects of the drug.

2.6.2 Vector control

2.6.2.1 Sterile insect control (SIT)

SIT is emerging as a potential mosquito control method. Progress towards transgenic or genetically modified insects suggests that wild mosquito populations could be made malaria–resistant (Ito *et al.*, 2002). Successful replacement of existent populations with

genetically modified populations relies upon a drive mechanism, such as transposable elements to allow for non- Mendelian inheritance of genes of interest. Studies published in *PLoS Pathogens* (Plospathogen.3 (12) indicate that the hemolytic C- type lectin CEL-III from *Cucumaria echinata,* a sea cucumber, impaired the development of the malaria parasite when produced by transgenic mosquitoes (Yoshida *et al*., 2007). Although there are scientific and ethical issues to be overcome, such a control tragedy could be potentially used one day to control malaria.

2.6.2.2 Insecticide – Treated Nets (ITN)

Nets are not a perfect barrier, so they are treated with an insecticide designed to kill the mosquito before it has time to search for a way past the net. Insect- treated nets (ITN) are estimated to be twice as effective as untreated nets (Hull, 2006). ITNs have been shown to substantially reduce child mortality in malaria endemic areas of Africa (WHO, 2003). Such nets provide partial protection against biting and reduce the number of infective malaria parasites in mosquitoes but the disease management still relies heavily on chemotherapy (White, 2004).

2.6.3 Vaccination

Vaccines for malaria are under development, with no complete effective vaccine yet available. Presently, there is a huge variety of vaccine candidates on the table, especially pre-erythrocytic vaccines (vaccines that target the parasite before it reaches the blood). In particular vaccines based on the circumsporozoite protein (CSP), which coat the

sporozoite, 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 the placenta; and transmission- blocking vaccines that would stop the development of the parasite in the mosquito right after the mosquito has taken a blood meal from an infected person (Matuschewski, 2006). It is hoped that the sequenced Plasmodium *falciparum* genome will provide targets for new drugs or vaccines (Gardner *et al.*, 2002).

2.6.4 Antima larial drugs

Development of antimalarial drugs was facilitated when *Plasmodium falciparum* was successfully cultured as this allowed *in vitro* testing of new drug candidates (Trager and Jensen, 1976).There are several families of drugs used to treat malaria and are classified according to therapeutic action against the different cycle stages of the parasites (Biagini *et al.*, 2005). These include, the antitrophozoite/ schizonts such as the quinine, halofantrine, mefloquine, chloroquine, amodiaquine, atovaquone, sulfadoxine and artemisinin (Robert *et al.*, 2001); the antigametocyte drugs such as amodiaquine, quinine, artemisinin and chloroquine, and drugs that target the liver schizont, which include primaquine, lumefantrine and the pyrimethamine (Vangapandu *et al*., 2006).

2.6.5 Artemesinin and its derivatives

Artemesinin was originally extracted from Qinghao plant (*Artemisia annua*) in China (Robert and Meunier, 1998). Artemisinin and its derivatives namely, artemether, arteether, artsunate, artelinic and dihydroartemisinin are the most active antimalarial drugs (Meshinick *et al.,* 1996). Artemesinins are well-tolerated, rapidly eliminated and potent drugs (O'Neill and Posner, 2004). Artemisinin and its delivertives are metabolized to the biologically active dihydroartemisin (DHA),which act specifically on blood stage parasites and is effective against multidrug resistant *Plasmodium falciparum* (Mutabingwa, 2005).

Artemisinins have a short half- life of 3-5 hours and are therefore combined with longer half – life drugs to reduce treatment time and increase cure rates in order to lower recrudescence (White, 1998). Despite the high cost of these drugs, their poor oral bioavailability and their action on blood stage parasite only, artemisinin and its derivatives appear to be the best alternative for the treatment of severe malaria (Robert *et al.*, 2001).

2.7 Drug resistance in malaria

Antimalarial drug resistance has been defined as the "ability of a parasite strain to survive and/or multiply despite the administration and absorption of drug given in doses equal to or higher than those usually recommended but within tolerance of the subject" (Bruce-Chwatt *et al.*, 1986). Resistance to antimalarial drugs has been described for two

of the four species of malaria parasite that naturally infect humans, *P. falciparum* and *P. vivax*. *P. falciparum* has developed resistance to nearly all antimalarials in current use, although the geographical distribution of resistance to any single antimalarial drug varies greatly. *P. vivax* infection acquired in some areas has been shown to be resistant to chloroquine and/or primaquine (Murphy *et al*., 1993).

Plasmodium falciparum resistantance against chloroquine was first reported in Thailand in 1961 (Anderson and Roper 2005). To date, resistance *in vivo* has been reported against all antimalaria drugs except artemisinin and its derivatives (Jambou, 2005).

Artemisinin-based combination therapies (ACTs), considered the best current treatment for falciparum malaria (WHO, 2006), have energized worldwide programs to control malaria. Combination therapies, in general, tend to delay the development of microbial resistance. However, several ACT regimens are combinations of artesunate and older antimalarial drugs against which resistance already exists. Preexisting resistance to these older partner drugs could lead to drug failure. This has already happened on the Cambodia–Thailand border (WHO, 2008).

2.7.1 Likely Resistance Factors

Several reasons may explain the emergence of antimalarial drug resistance. First, the concept of rational therapy is poorly reinforced. Improper use of antimalaria drugs based on clinical diagnosis alone or on misdiagnosis as a result of poor microscopy technique or interpretation could have accelerated the onset of resistance. In sub-Saharan Africa,

because of poor transportation and public health infrastructure, antimalarial drugs are made available in the private sector to increase patients' access to the drug. This access, in turn, increases the risks that drug quality will be substandard and drug use will be uncontrolled (Denis *et al*, 2006). Because adherence and indication are not adequately emphasized, drugs are consumed in incomplete dosages or for prophylaxis such as before a jungle trip. Social marketing helps to control drug quality but cannot ensure adherence.

Unreliable services and poor diagnostic capabilities at peripheral health facilities further discourage patients from seeking malaria treatment from the public sector and encourage self-purchase of drugs. The short half-life of artesunate relative to that of partner drug means that tolerance to the partner drug could develop when treated patients are reinfected (Hasting *et al*, 2005)

The malaria parasites in African region could have a unique ability to develop resistance to any antimalaria drugs (Rathod *et al*, 1997). The emergence and spread of drug resistance occurs faster if the number of mutations required to encode resistance is few and their effects on the parasite fitness are minimal (White, 1999). The resistance is due to changes or mutations in the copy number of genes encoding the drug's parasite target or the influx pump that affect intra- parasitic concentration of the drug (Hyde, 2005). Such genetic events that confer antimalarial drug resistance are independent of the drug used and occur spontaneously (White, 2004).

2.7.2 Genetic basis of drug resistance

The researchers' present inability to synthesise a fully protective vaccine means that chemotherapy stands as the only effective measure in the control of the malaria. However, in many parts of the world the parasite *P. falciparum* has become resistant to most drugs presently used (Jambou, 2005), seriously undermining efforts for controlling malaria. Chloroquine (CQ) has long been the drug of choice for the treatment of malaria; however, CQ-resistant parasites are now present in most areas where malaria is endemic (Peters, 1998). The decline in the efficacy of chloroquine led to the use of alternative antimalarials, such as antifolates, mefloquine and artemisinin derivatives, but parasite resistance to these drugs is also becoming a real problem (Jambou, 2005). In this context, understanding the genetic basis of drug resistance is essential for implementing rational measures to overcome the problem. Although significant progress has been made in trying to understand how resistance to CQ may occur, many aspects of it remain unclear, and the genetic mechanisms responsible for mefloquine and quinine resistance are largely unknown. Nevertheless, two main genes have been implicated in quinoline resistance; the *pfmdr1* (*P. falciparum* multi-drug resistance1) and the *Pfcrt* (*P. falciparum* chloroquine resistance transporter). There is evidence from the analysis of a genetic cross which indicates that point polymorphisms in the *pfmdr1* gene may modulate sensitivity to both mefloquine (MF) and artemisinin in *P. falciparum* (Duraisingh, 2000). Furthermore, recent genetic transfection work has suggested that single nucleotide polymorphisms in the *pfmdr1* gene encoding changes in aminoacids 1034, 1042 and 1246 can influence parasite responses to mefloquine, quinine and

halofantrine as well as to the structurally unrelated drug artemisinin, and modulate sensitivity to chloroquine depending on the genetic background of the parasites strains (Reed, 2000). However, chloroquine resistance was shown to segregate independently of the *pfmdr1* gene, following a genetic cross between a CQ-sensitive parasite, *P. falciparum* HB3, and a CQ-resistant one, Dd2 (Wellems, 1990), and the absence of a clear association between *pfmdr1* and chloroquine responses in natural parasite populations (Awad-el-Kariem, 2001), strongly suggests the involvement of other gene(s). Detailed linkage analysis and fine chromosome mapping of progeny clones of the HB3 × Dd2 cross allowed the identification of another gene, *pfcrt*, in which a mutation at amino acid 76 (*pfcrt* K76T) is highly correlated with increased CQ tolerance among field parasite isolates of *P. falciparum* (Labbé , 2001). In addition, a causal relationship between *pfcrt* 76T and chloroquine resistance was confirmed by genetic transfection experiments (Fidock, 2000).

The study of the correlation between drug resistance in natural parasite populations and genetic polymorphisms may allow the development of molecular tools to help predict responses to drugs and, as mentioned above, the *pfcrt* and *pfmdr1* genes have been identified as putative markers of quinoline resistance.

Parasite resistance to CQ is caused by the ability of the resistance parasite to limit the accumulation of CQ in the parasite digestive food vacuole (Verdier *et al.,* 1985). The mechanism leading to low intracellular CQ levels is not yet clear, although, it is proposed that, altered pH (Druilhe *et al.,* 2001), decreased drug uptake into the parasite

food vauole (Sanchez *et al.*, 1997), or increased drug efflux out of the vacuole (Krogstad *e t al.*, 1987) could be contributing factors.

The multi-drug resistance is associated with mutations on the multi- drug resistance 1 gene *(mdr 1)* which encodes a P- glycoprotein homologue -1(Pgh-1) that localizes on the digestive vacuole membrane (Hyde, 2005). Mutations or changes in copy number in *Pfmdr1* may affect accumulation of drug in the digestive vacuole, thus affecting the potency of the drugs (Sidhu *et al.*, 2006). Resistance to pyrimethamine and cycloguanil (antifoliates) results from the sequential acquisition of mutations in DHFR (White, 2004), with each mutation conferring a stepwise reduction in susceptibility. A single point mutation at DHFR Ser108Asn causes pyrimethamine resistance and only moderate loss of susceptibility to chlorocycloguanil (Gregson and Plowe, 2005). Higher resistance to both drugs is caused by addition of fourth lle164Leu mutation (White, 2004).Resistance to the sulfonamides and sulfones often administered in synergistic combination with antifolates results from sequential acquisition of mutations in the *dhps* gene (White, 2004). DHPS mutations associated with resistance seen in sulfonamides and sulfones include ser436ala, ala437gly and ser436phe together with ala613thr/ser (Plowe, 2003).

Atovaquone acts through inhibition of electron transport at the cytochrome *bc1* complex (Cogswell, 1992a). Although resistance to atovaquone develops very rapidly when used alone, when combined with a second drug, such as proguanil (the combination used in

MalaroneTM) or tetracycline, resistance develops more slowly (Looareeesuwan, 1996). Resistance is conferred by single-point mutations in the cytochrome-b gene.

2.7.3 Detection of resistance

Four basic methods have been routinely used to study or measure antimalarial drug resistance: *in vivo* and *in vitro*, animal model studies, and molecular characterization. Additionally, less rigorous methods have been used, such as case reports, case series, or passive surveillance.

2.7.3.1 *In vivo* tests

In vivo test consists of the treatment of a group of symptomatic and parasitaemic individuals with known doses of drug and the subsequent monitoring of the parasitological and/or clinical response over time. One of the key characteristics of *in vivo* tests is the interplay between host and parasite. Diminished therapeutic efficacy of a drug can be masked by immune clearance of parasites among patients with a high degree of acquired immunity (White, 1997). *In vivo* tests most closely reflect actual clinical or epidemiological situations (i.e. the therapeutic response of currently circulating parasites infecting the actual population in which the drug will be used). Because of the influence of external factors (host immunity, variations of drug absorption and metabolism, and potential misclassification of reinfections as recrudescences), the results of *in vivo* tests do not necessarily reflect the true level of pure antimalarial drug resistance. However,

this test offers the best information on the efficacy of antimalarial treatment under close to actual operational conditions (Shakoo *et al.*, 1997).

2.7.3.2 Animal model studies

This is an *in vivo* test conducted in a non-human animal model and, therefore, is influenced by many of the same extrinsic factors as *in vivo* tests. The influence of host immunity is minimized by using lab-reared animals or animal-parasite combinations unlikely to occur in nature, although other host factors would still be present. These tests allow for the testing of parasites which cannot be adapted to *in vitro* environments (Provided a suitable animal host is available) and the testing of experimental drugs not yet approved for use in humans. A significant disadvantage is that only parasites that can grow in, or are adaptable to, non-human primates can be investigated (WHO, 2001)

2.7.3.3 In vitro tests

In vitro tests avoid many of the confounding factors which influence *in vivo* tests by removing parasites from the host and placing them into a controlled experimental environment. In the most frequently used procedure, the micro-technique, parasites obtained from a finger-prick blood sample are exposed in microtitre plates to precisely known quantities of drug and observed for inhibition of maturation into schizonts (Rieckmann *et al.*, 1978). This test more accurately reflects “pure” antimalarial drug resistance. Multiple tests can be performed on isolates, several drugs can be assessed simultaneously, and experimental drugs can be tested. However, the test has certain

significant disadvantages. The correlation of *in vitro* response with clinical response in patients is neither clear nor consistent, and the correlation appears to depend on the level of acquired immunity within the population being tested. Drugs, such as proguanil, which require host conversion into active metabolites cannot be tested. Neither can drugs that require some level of synergism with the host's immune system. Although adaptation of erythrocytic forms of *P. vivax* to continuous culture has been achieved, non-falciparum erythrocytic parasites generally cannot be evaluated *in vitro* (Golend *et al.*, 1997) In addition, because parasites must be cultured, differential die-off of parasites can occur. If, for instance, resistant strains are less likely to adapt, the results would be biased towards sensitive responses. Because venous blood is typically needed, resistance in the more vulnerable younger age groups is often not studied. Finally, these tests are technologically more demanding and relatively expensive, which makes them potentially more difficult to adapt successfully to routine work in the field (WHO, 2001).

2.7.3.4 Molecular techniques

These tests are in the process of being developed and validated, but offer promising advantages to the methods described above. Molecular tests use polymerase chain reaction (PCR) to indicate the presence of mutations encoding biological resistance to antimalarial drugs (Plowe *et al.*, 1995). Theoretically, the frequency of occurrence of specific gene mutations within a sample of parasites obtained from patients from a given area could provide an indication of the frequency of drug resistance in that area analogous to information derived from *in vitro* methods. Advantages include the need

for only small amounts of genetic material as opposed to live parasites, independence from host and environmental factors, and the ability to conduct large number of tests in a relatively short period of time. Disadvantages include the obvious need for sophisticated equipment and training, and the fact that gene mutations that confer antimalarial drug resistance are currently known or debated for only a limited number of drugs (primarily for dihydrofolate reductase inhibitors [pyrimethamine], dihydropteroate synthase inhibitors [sulfadoxine], and chloroquine (Plowe *et al*., 1995; Su *et al., 1997).*

Confirmation of the association between given mutations and actual drug resistance is difficult, especially when resistance involves more than one gene locus and multiple mutations. If these complexities can be resolved, molecular techniques may become an extremely valuable surveillance tool for monitoring the occurrence, spread, or intensification of drug resistance (WHO, 2001).

2.8 *In vivo* screening of antimalarial compounds

Plasmodium species that cause human disease are essentially unable to infect non-primate animal models (with the exception of a complex immunocompromised mouse model that has been developed to sustain *P. falciparum*-parasitized human erythrocytes *in vivo* (Moreno *et al,* 2001). *In vivo* evaluation of antimalarial compounds typically begins with the use of rodent malaria parasites. Of these, *P. berghei*, *P. yoelii*, *P. chabaudi* and *P. vinckei* have been used extensively in drug discovery and early development (Childs *et al*, 1984). Rodent models have been validated through the identification of several antimalarials — for example, mefloquine, halofantrine and more

recently artemisinin derivatives (Sanni, *et al*, 2002). In view of their proven use in the prediction of treatment outcomes for human infections, these models remain a standard part of the drug discovery and development pathway. Individual species and strains have been well characterized, including duration of cycle, time of schizogony, synchronicity, drug sensitivity and course of infection in genetically defined mouse strains (Peters *et al*, 1999; Sanni *et al* 2002). The most widely used initial test, which uses *P. berghei* or less frequently *P. chabaudi*, is a four-day suppressive test (Peters & Robinson,1999) in which the efficacy of four daily doses of compounds is measured by comparison of blood parasitaemia (on day four after infection) and mouse survival time in treated and untreated mice. Rodent infection is typically initiated by needle passage from an infected to a naive rodent via the intraperitoneal or preferably the intravenous route, often using a small inoculum (typically in the range of 10^6–10^7 infected erythrocytes). Compounds can be administered by several routes, including intraperitoneal, intravenous, subcutaneous or oral. CQ is often used as the reference drug and typically has an ED_{50} value against *P. berghei* (ANKA strain) of 1.5–1.8 mg per kg when administered subcutaneously or orally (Peters & Robinson, 1999).

Compounds identified as being active in four-day assays can subsequently be progressed through several secondary tests. In the 'dose ranging, full four-day test', compounds are tested at a minimum of four different doses, by subcutaneous and/or oral routes, to determine ED_{50} and ED_{90} values. This test also provides useful information on relative potency and oral bioavailability. In the 'onset/recrudescence' test, mice are administered a single dose (by the subcutaneous or oral route) on day 3 post-infection and observed

daily to monitor parasitaemia. Results are expressed as the rapidity of onset of activity (disappearance of parasitaemia), time to onset of recrudescence, increase of parasitaemia and survival in number of days. Compounds can also be tested for prophylactic activity by administering the compound prior to infection, followed by daily examination of smears. Additional screens have been developed to assess cross-resistance and the potential for *in vivo* selection of resistant parasites (Peters, 1999).

When using rodent models, several key variables need to be considered during experimental design and interpretation. Foremost is the choice of rodent malaria species and mouse strains. As alluded to before, rodent plasmodia can differ significantly in their degree of infection, lethality and synchronicity, which can dramatically affect the results. These factors also broaden the range of possible assays for compound evaluation. For example, *P. chabaudi* and *P. vinckei* generate a high parasitaemia and produce synchronous infections, enabling studies on parasite stage specificity. Rodent malaria species can also differ significantly in sensitivity to certain classes of compounds. For example, *P. chabaudi* and *P. vinckei* are more sensitive than *P. berghei* to iron chelators and lipid biosynthesis inhibitors (Alanine *et al.*, 2003). The course of infection can also vary enormously depending on the mouse strain, and models exist that are amenable to studies on chronic infection or sequestration (Sanni, 2002). For example, the *P. chabaudi* AS strain in CBA mice produces a chronic infection with a defined immune response that can be used in studies of immunomodulators. The drug sensitivity of a given rodent malaria species does not always mirror that of *P. falciparum* and can limit the types of investigations that can be performed. For example, cysteine proteases in

rodent plasmodia show subtle active site differences to those in *P. falciparum* (Singh *et al.*, 2002).

Primate models have also had an important role in preclinical development, by providing a final confirmation of the choice of a drug candidate. Infection with certain strains of *P. falciparum* has been well-characterized in both *Aotus* and *Saimiri* monkeys (Gysin, 1998). Primate models provide a clearer prediction of human efficacy and pharmacokinetics than rodent models, providing a logical transition to clinical studies (Wengelnik, *et al.*, 2002).

CHAPTER THREE

3.0 Materials and methods

3.1 Study site

The study was carried out in the pharmacology laboratory at Kenya Medical Research Institute (KEMRI), Centre for Traditional Medicine and Drug Research (CTDMR), Nairobi, Kenya.

3.2 Plant materials

Barks of the two plant species were collected in August 2008 from their natural habitats in Kajiado and Machakos districts, southern Kenya and authenticated at University of Nairobi Herbarium, in the School of Biological Sciences. Samples with voucher number BN/2008/1 for *C. anisata* and BN/2008/2 for *C. robusta* were deposited in the herbarium for future reference.

3.3 Preparation of extracts

Plant materials were chopped, air -dried and powdered using a mill. Powder (200g) was extracted successively for 48 hrs at room temperature with 600 ml of hexane, chloroform and methanol. The marc was filtered with a 3mm Whatman filter paper. Extracts were dried under reduced pressure in a rotary evaporator. Water extracts were filtered and the filtrate freeze- dried.

3.4 Laboratory animals

Healthy female Swiss albino mice weighing between 18 -22g supplied by the Laboratory Animal Unit, KEMRI, were used in this study. The rodents were housed in experimental room in animal house in standard Marrolon type 11 cages at room temperature (20-23^{o}C) with free access to commercial food pellets and water. Permission to carry out the study and ethical clearance was sought from KEMRI'S Scientific Steering Committee and the Ethical Review Committee. The experiments were conducted in strict compliance with KEMRI guidelines on animal use and care and, the internationally accepted principles for laboratory animal use and care as contained in WHO guidelines, Technical report series NO 863, Geneva.

The needle size for cardiac puncture and intraperitoneal infection was 26G×5/8". At the end of the experiment, mice were sacrificed by euthanizing in carbon dioxide chamber (CO_2) and placed in biohazard disposable bags and incinerated.

3.5 Parasites

Plasmodium berghei ANKA strain, donated by CTMDR, KEMRI was used in this study. Malaria parasites were maintained by blood passage in Swiss mice when active parasites were required, otherwise it was stored at -80^{0}C in Alserver's solution (2.33% glucose, 0.525% NaCl and 1% sodium citrate in deionised water) and glycerol (9:1 parts by volume).

3.6 Mouse inoculation

Mice were inoculated (0.2 ml, i.p) with blood from a donor mouse with a rising parasitaemia of 5-10% and according to the level of parasitaemia blood diluted with phosphate saline glucose (PSG) buffer to contain 2×10^7 (approximately 1%) *P. berghei* – parasitized red blood cells per 0.2 ml.

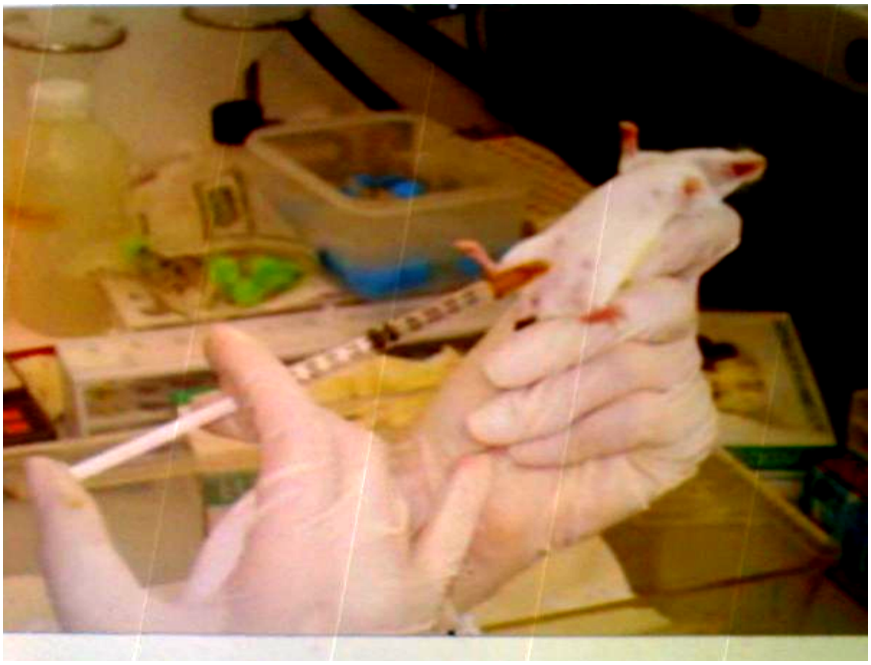


Plate 3: Intraperitoneal treatment with experimental product

3.7 Preparation of extracts solution

On the day of drug administration, each extract was freshly prepared by solubilising in 70% Tween-80 (d=1.08g/ml) and 30% ethanol (d=0.81g/ml) and diluted 10 fold with double-distilled water.

3.8 Suppressive test

The Peters' 4-day suppressive test against *P. berghei* infection in mice was employed (Peters, 1975). Adult Swiss male albino mice weighing 18-20g were inoculated by intra-peritoneal (i.p.) injection with $2x10^7$ infected erythrocytes contained in 0.2ml inoculum. The mice were randomly divided into five groups of five mice per cage and treated for 4 consecutive days (4, 24, 48, 72 hours post infection) with daily doses of the extracts by ip route (500, 250 and 100mg/kg/day). Two control groups were used in each experiment, one treated with chloroquine at total dose of 10 mg/kg/day while the other group was kept untreated. On day 4 (96 hrs post-infection) of the test, thin blood smears were prepared from thin films made from tail snips and blood films were fixed with methanol. The blood films were stained with Giemsa, and then microscopically examined (1000 x magnification). The percentage suppression of parasitaemia was calculated for each dose level by comparing the parasitaemia in infected controls with those of treated mice. The formula **[(A-B)/A] ×100** (Tona *et al.*, 2001) was used. Where

A= is the mean parasitaemia in the negative control group.

B= is the parasitaemia in the test group.

Surviving mice were monitored up to day 28 and checked for the presence of parasite and reported as with or without parasites. Finally, the surviving mice were sacrificed by

euthanizing in carbon IV oxide chamber, terminating the experiment. The dead mice were placed in disposal bags and incinerated.

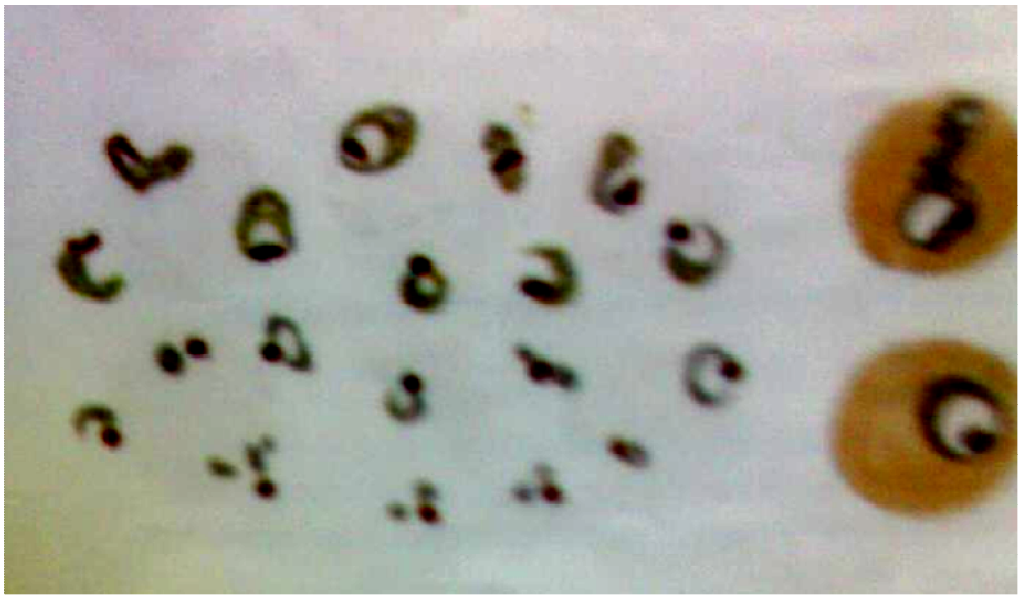


Plate 4: Plasmodium berghei blood –stage forms in Geimsa-stained smears from Swiss mice

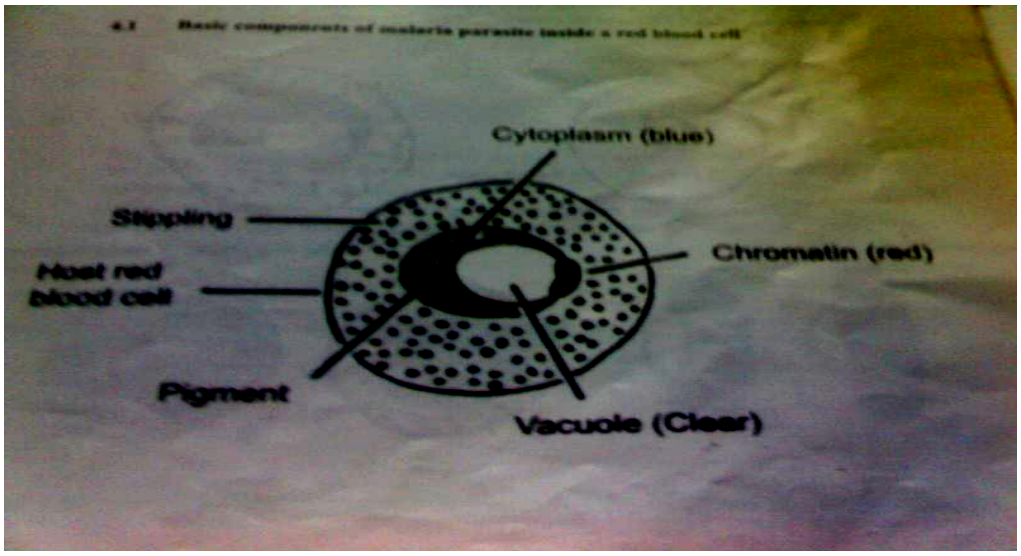


Plate 5: Basic components of malaria parasite inside a red blood cell

3.9 Curative (established infection) treatment test.

Ryley and Peters, (1970) and Isah *et al,* (2003) protocol was followed. The animals were divided into 5 groups of five mice each for each plant extract. Group 1 served as control (infection, no treatment), group 2 received chloroquine (10mg/kg/day), while 3-5 was treated with plant extract. Animals were infected on day 1 (D1) and left for 3 days (72 hours) before commencement of treatment. On day 4 drugs were administered for four consecutive days. Mice were assessed daily for the 4 days. On each day of assessment, percentage parasitaemia and % chemosuppression relative to the control was determined for each of the doses. Parasitaemia was assessed using Giemsa-stained thin films made from tail snip. Percentage chemosuppression was determined. Quantitative expression of drug (extract) efficacy was determined by calculating the survival index (I_s) according to Nathan *et al.,* (1979) and Isah *et al.*, (2003). This was used to express degree of life prolongation in the absence of cure.

Survival Index (I_s) = Nt-Nu / Nt+ Nu

Where Nt = Lifespan of treated mice.

Nu = Lifespan of untreated mice.

3.10 Prophylactic treatment test

This model was performed as described by Peters (1965) and Isah *et al.,* (2003). Five groups of five mice each were used in this experiment for each plant. Group one served

as control, group two received pyrimethamine (1.2 mg/kg/day) while groups 3-5 were treated with the plant extracts in three dose levels (500, 250 and 100 mg/kg/day). The drug was given for three consecutive days. On day four all animals were infected and left for three days after which thin blood smears were taken using Giemsa-stained thin films made from tail blood. Percentage chemosuppression was determined.

3.11 *In Vivo* Safety test (Acute Toxicity).

The acute toxicity test was carried out according to the WHO guidelines (WHO, 1996). Three dose levels of each extract were administered intraperitoneally to the mice. Five mice were used per dose level and 70% Tween- 80 and 30% ethanol diluted 10- fold was administered as the control. The animals were observed over a period of 24-48 hours for signs of acute toxicity such as writhing, gasping, palpitation, closure of the eyes and death. The number of deaths during this period were noted and recorded. LD_{50} was calculated as geometrical mean of maximum dose producing 0% mortality and the minimum dose producing 100% mortality according to Okokon and Nwafor, (2009).

$$LD_{50} = \sqrt{ab}$$

Where a =Dose producing 0% mortality

b =Dose producing 100% mortality

3.12 Drug pressure assay

Selection of the *Clausena anisata* and *C.robusta* extract resistance was a follow up of the *in vivo* work that indicated a dose dependent increase in chemotherapeutic effect at different dose levels employed in the study. The chemosuppression attained for the highest dose was 72.96% at 500 mg/kg/day for the chloroform extract without the drug pressure.

Serial technique (S T) where the extract selection pressure *in vivo* is gradually increased was employed (Peter's *et al.*, 1975). Sensitive parasites were exposed to 500 mg/kg/day as the starting point and increased stepwise by a factor of 0.1 at each subsequent passage until when the extract could not dissolve at 800 mg/kg/day.

Fifteen Swiss albino mice (20±2g) were intraperitoneally infected each with donor blood containing 2×10^7 *P.berghei* ANKA PRBC contained in 0.2 ml inoculum on day zero (D_o). The randomly infected mice were then divided into two test groups and a control group with five mice each and treated for 4 consecutive days (4, 24, 48, 72 hours) with daily doses (500 mg/kg/day) of *C.anisata* chloroform and *C. robusta* methanoic extracts by ip route. The control group was kept untreated. Evaluation of the parasitaemia was done on D4 and according to the level of parasitaemia of the donor mouse, blood diluted with phosphate saline glucose (PSG) buffer to contain 2×10^7 (approximately 1%) *P. berghei* – parasitized red blood cells per 0.2 ml. This was used for passage. This was repeated for seven consecutive weeks after which the blood spot samples were taken.

3.13 Dry Blood Sample Collection

Peripheral blood was collected from the tail snip of each mouse by spotting directly on the 3mm whatman filter paper. Blood spots were allowed to air- dry, then placed individually in plastic bags and stored at 4^0C with a silica gel (sigma) desiccant to prevent dampness until DNA extraction was carried out.

3.14 DNA Extraction by Chelex Method

DNA was extracted by chelex method as per protocol (Plowe *et al.,* 1995). Briefly, a $4mm^2$ piece of filter paper with blood was cut using a sterile scalpel blade. This was incubated in 0.5% saponin in 1x phosphate-buffered saline (PBS) which contained 3.2mM Na_2HPO_4, 0.5mM KH_2PO_4, 1.3mM NaCl, pH 7.4 overnight at 4^0C. The brown solution was then removed and 1x PBS added, then incubated for 15-30 minutes at 4^0C. The solution was removed and 100µl of DNAse free water was added followed by 50 µl of 20% chelex. The tubes were placed into a heated block at 99^0c and voltexed every two minutes, for a total of ten minutes. The solution was centrifuged at 10,000 rpm for 2 minutes and the supernatant containing DNA removed and aliquoted into units of 30 µl to avoid freeze-thawing which could lead to DNA degradation. The aliquots were stored at -20 ^{0}C until PCR amplification was done.

3.15 Amplification of the Pbmdr1 gene by PCR

A nested PCR protocol was adapted

3.15.1 Primary PCR

The amplification of the *Pbmdr1* gene was done on MJ ThermocyclerTM PCR machine (www.mjr.com).In the outer PCR *Pb/1,* CGC GGA AAT AGT GAT TCT TCA and

Pb/2, CCA CCT GAC AAT TTA GAT GAG C were used as forward and reverse primers, respectively. The reaction mix for each tube was constituted in a 30 µl reaction volume containing 5 µl of the DNA, 10 µM of each primer, 2 µM of dNTP mix and 0.24 units of Taq polymerase. For each run, a negative control was included. 5µl of PCR water was used. PCR cycling was done as follows: initial denaturation at 94^0C for 3 minutes, followed by denaturation at 94^0C for 1 minute, annealing temperature of 49^0C for 1minute, and extension at 72^0C for 1minute. These conditions were repeated for 40 cycles followed by a final extension for 3 minutes at 72^oC, then halted at 4^0C.

3.15.2 Inner PCR

Nested PCR was done using forward and reverse primers *Pb/3*, TGG AAA GCG CAC TAG TAT TTT GGA TG and *Pb/4*, TGG TAA GGA TGC TAC AAA TCG.

To the PCR tube (0.2ml size), containing the master mix, 2 µl of the DNA template was added and re-amplified in the nested PCR reaction. The known purified genomic DNA from V1/S laboratory parasite clones were used as positive controls and NT (No template) was included as negative control. PCR cycling was done as done as follows: initial denaturation at 94^0C for 3 minutes, followed by denaturation at 94^0C for 30 seconds, annealing at 48^0C for 1minute, and 72^0C extension for 1minute.

These conditions were repeated for 40 cycles followed by a final extension for 3 minutes at 72^{o}C, then halted at 4^{0}C. Samples with no detectable PCR products were re-examined at least twice starting from the DNA preparation before declared negative.

3.16 Gel electrophoresis.

The PCR amplicons were loaded into the agarose gel for visualization of the amplified DNA. The agarose gel was prepared by taking 1 gram of agarose and dissolving in 1x TBE (Tris Borate EDTA) to make a 1% gel. Samples were mixed with the loading dye and loaded onto the gel together with the molecular weight marker. The gel was then put in an electrophoresis tank for electrophoresis at a constant voltage of 80v for 40 min for outer PCR and 25 mins for inner PCR. The gel was then stained with ethidium bromide and visualized under ultraviolet light, photographed and electronically stored.

3.18 Data analysis

The antiplasmodial activity of the extract and the standard drugs, the percentage parasiteamia at each concentration was determined by expressing the difference of mean parasitaemia of the negative control and the extract / standard drug as the percentage of the negative control (infected without the extract or drug, representing the normal parasite growth). The mean parasitaemia was processed using the analysis of variance and the data separated using Student-Newman-Keuls test. Data was transformed for uniformity using square root transformation before being subjected to analysis of variance. The percentage survival time was calculated for each treatment using the median values.

CHAPTER FOUR

4.0 Results

4.1 Extraction of plant materials

From the extraction experiments it was evident that the extraction using methanol gave the highest yield for both study plants as shown in table 1.

Table1: Yields of extracts from *Clausena anisata* and *Clutia robusta* stem bark extracted using different solvents.

Plant\solvent	Hexane	Chloroform	Methanol
	Weight(gm)	Weight (gm)	Weight (gm)
C.anisata	$3.4 \pm 0.01^{a}{}_{c}$	$4.07 \pm 0.20^{a}{}_{b}$	$4.97 \pm 0.24^{c}{}_{b}$
C.robusta	$1.65 \pm 0.08^{b}{}_{b}$	$2.96 \pm 0.14^{b}{}_{b}$	$4.69 \pm 0.23^{a}{}_{b}$

Numbers represent mean weigh of the extracts in grams
Means separated using Student-Newman-Keuls test by the same letter along the column are not significantly different ($P<0.05$) from each other

4.2 Suppressive test (4-day test)

The Hexane and chloroform bark extracts of *Clausena anisata* produced a dose – dependent chemotherapeutic effect at different dose levels employed in the study. The chemosuppression attained for highest dose levels were 44.07% and 72.13% at 500 mg/kg/day for hexane and chloroform doses respectively (Table 2). Similarly, the chloroform extracts of *Clutia robusta* caused day-4 chemosuppressions of 61.80 % when given intraperitoneally at doses of 500 mg/kg/day (Table 3).

Table 2: Suppressive activity of hexane, chloroform and methanol bark extracts of *Clausena anisata* (4-day test)

Solvent extract	**Chloroform**			**Hexane**			**Methanol**		
Dosage (mg/kg)	Mean Parasitaemia	% Chemo-suppression	Survival time (days)	Mean Parasitaemia	% Chemo-suppression	Survival time (days)	Mean Parasitaemia	% Chemo-suppression	Survival time (days)
10 (CQ)	0.67±0.07[a]	9.85±0.02c	28	0.67±0.07[a]	9.82±0.05[a]	28	0.67±0.07[a]	9.89±0.02[a]	28
100	3.03±0.12[c]	5.83±1.35ab	12	2.68±0.10[b]	6.68±0.96[b]	7	3.87±0.51[b]	8.32±0.05[b]	11
250	3.20±0.24[c]	4.92±1.08a	12	2.73±0.22[b]	6.87±0.23[b]	9	4.09±0.45[b]	7.49±0.30[c]	9
500	1.98±0.18[b]	8.44±0.45b	18	2.51±0.16[b]	6.51±0.65[b]	9	5.26±0.23[b]	1.56±0.00[d]	8
Control	3.90±0.16[d]		7.5	3.50±0.34[c]		7.5	4.29±0.25[b]		7.5

Numbers represent mean parasitaemia, % chemo-suppression and survival time (days) for each treatment.
Means separated using Student-Newman-Keuls test by the same letter along the column are not significantly different (P<0.05) from each other.

Table 3: *P. berghei* suppression by *C. robusta* bark extracts in swiss mice

Solvent extract	**Chloroform**			**Hexane**			**Methanol**		
Dosage (mg/kg)	Mean Parasitaemia	% Chemo-suppression	Survival time (days)	Mean Parasitaemia	% Chemo-suppression	Survival time (days)	Mean Parasitaemia	% Chemo-suppression	Survival time (days)
10(CQ)	0.67±0.06[a]	9.79±0.04[a]	28	0.71±0.09[a]	9.88±0.03[c]	8	0.71±0.09a	9.83±0.07a	28
100	3.45±0.29[c]	5.31±1.59[b]	8	4.68±0.16[c]	2.42±1.24[a]	7	3.87±0.50b	8.32±0.05b	8
250	2.69±0.07[b]	6.00±0.50[b]	9	4.48±0.17b[c]	4.67±0.71[b]	10	4.09±0.45bc	7.49±0.30c	9
500	2.42±0.22[b]	6.18±1.23[b]	10	4.17±0.09[b]	4.81±0.26[b]	19	5.26±0.23c	0.73±0.73d	10
Control	3.36±0.14[c]		7.4	4.76±0.07[c]			4.29±0.25bc		7.5

Numbers represent mean parasitaemia, % chemo-suppression and survival time (days) for each treatment. [*]Means separated using Student-Newman-Keuls test by the same letter along the column are not significantly different (P<0.05) from each other.

The effects produced by the *C.anisata* extracts were significant ($P < 0.05$) compared to the control (untreated) and incomparable to that of the standard drug (chloroquine 10mg/kg/day) with a chemo-suppression of 97.10% (Table.2). However, there was a significant parasite reduction and survival period was high compared to the untreated control for both plants.

4.3 Curative test

In this experiment, malaria infections had become established by the time of treatment with the extracts. The chloroquine treatment caused a significant higher chemosuppression than that of the extracts treated groups. Chloroquine (10mg/kg/day) also produced a daily reduction in parasitaemia. The mean parsitaemia recorded for chloroform and methanol were not significantly different from the control. Table 4.

Table 4: Curative effects of *C. robusta* organic extracts against *P.berghei* in mice.

		Mean parasitaemia			
Solvent extract	**Dosage (mg/kg/day)**	**Day4**	**Day5**	**Day6**	**Day7**
Chloroform	10(CQ)	5.49±0.07[a]	4.44±0.49[a]	2.40±0.27[a]	1.59±0.13a
	100	3.19±0.39[b]	5.30±0.15[a]	7.28±0.24[b]	7.43±0.16bc
	250	3.25±0.50[b]	4.37±0.35[a]	6.90±0.19[b]	8.01±0.18c
	500	2.96±0.17[b]	4.24±0.56[a]	6.28±0.80[b]	6.46±0.99b
	Control	3.84±0.23[b]	5.40±0.21[a]	7.54±0.29[b]	7.44±0.08bc
Methanol	10(CQ)	5.49±0.07[a]	4.38±0.63[a]	2.40±0.27[a]	1.59±0.13[a]
	100	2.67±0.45[b]	4.11±0.41[a]	6.11±0.82[b]	7.03±0.55[b]
	250	2.98±0.07[b]	4.69±0.43[a]	6.03±0.95[b]	6.86±0.40[b]
	500	3.03±0.15[b]	5.46±0.63[a]	6.70±0.33[b]	7.66±0.09[b]
	Control	3.07±0.15[b]	5.26±0.43[a]	6.54±0.72[b]	6.79±0.25[b]
Hexane	10(CQ)	5.49±0.07[a]	4.44±0.49[a]	2.40±0.27[a]	1.59±0.13[a]
	100	3.58±0.12[b]	3.83±0.20[a]	5.87±0.67[b]	6.94±0.15[b]
	250	2.94±0.32[ab]	4.37±0.32[a]	5.05±0.64[b]	5.20±0.94[b]
	500	2.57±0.20[c]	4.08±1.01[a]	5.43±0.83[b]	5.88±0.64[b]
	Control	2.62±0.28[c]	4.49±0.58[a]	4.99±0.54[b]	5.80±0.45[b]

Numbers represent mean parasitaemia for each treatment at time intervals.
Means separated using Student-Newman-Keuls test by the same letter along the column are not significantly different ($P<0.05$) from each other.

Mice given Chloroquine at 10mg/kg/day had an MST (median survival time) of at least 28 days (ie. all were alive on the last day of follow up).This value was longer than the value for mice given the C. *robusta* organic extracts at 500 mg/kg/day of at least 10.

Table 5: Median survival time of mice receiving various doses of *Clutia robusta* chloroform, methanol and hexane extracts during established infection

Drug/ extract	Dose (mg/kg/day)	Median survival time (days)
Chloroform	100	10
	250	9.0
	500	9.5
Methanol	100	8.4
	250	8.6
	500	8.0
Hexane	100	8.0
	250	9.0
	500	7.8
Control (drug vehicle)	0.2ml	7.2
Chloroquine (standard)	10ml	28

There was daily increase in parasitaemia levels in the negative control group as well in the methanol and hexane *C. anisata* bark extract -treated groups. However, there was reduction in parasitaemia levels of the chloroform extract at 500 mg/kg/day (Table 6).On day seven, the mean prasitaemia recorded for the organic extracts was significantly different from that of the standard drug chloroquine.

Table 6: Curative effects of C. anisata organic extracts against P.berghei in mice

		Mean parasitaemia			
Solvent extract	**Dosage (mg/kg/day)**	**Day4**	**Day5**	**Day6**	**Day7**
Chloroform	10(CQ)	5.42±0.09[a]	4.44±0.49[a]	2.40±0.27[a]	1.59±0.13[a]
	100	3.58±0.12[b]	3.50±0.95[a]	5.38±1.71[a]	5.86±1.04[b]
	250	2.94±0.32[ab]	3.75±0.93[a]	4.54±1.08[a]	6.03±0.96[b]
	500	2.57±0.20[c]	4.11±0.50[a]	4.33±0.26[a]	3.90±0.45[b]
	Control	2.62±0.28[c]	4.49±0.58[a]	4.99±0.54[a]	6.23±0.24[b]
Methanol	10(CQ)	5.42±0.09[a]	4.44±0.49[a]	2.40±0.27[a]	1.59±0.13[a]
	100	1.71±0.45[b]	3.07±0.53[a]	4.22±1.03[b]	5.19±0.86[b]
	250	2.35±0.27[b]	4.21±0.40[a]	5.14±0.52[b]	6.20±0.44[b]
	500	2.55±0.31[b]	3.88±0.42[a]	5.42±0.35[b]	5.61±0.95[b]
	Control	2.62±0.28[b]	4.49±0.58[a]	4.99±0.54[b]	6.01±0.38[b]
Hexane	10(CQ)	5.42±0.09[a]	4.44±0.49[a]	2.40±0.27[a]	1.59±0.13[a]
	100	3.58±0.12[b]	3.83±0.20[a]	5.87±0.67[b]	6.92±0.09[b]
	250	2.94±0.32[b]	4.37±0.32[a]	5.05±0.64[b]	5.20±0.94[b]
	500	2.57±0.20[b]	4.08±1.01[a]	5.43±0.83[b]	5.88±0.64[b]
	Control	2.62±0.28[b]	4.49±0.58[a]	4.99±0.54[b]	5.80±0.45[b]

Numbers represent mean parasitaemia for each treatment at time intervals.
Means separated using Student-Newman-Keuls test by the same letter along the column are not significantly different ($P<0.05$) from each other.

4.4 Prophylactic test

Methanol extracts of *C. anisata* exerted a dose dependent prophylactic activity at various doses employed resulting in significant ($p<0.001$) reduction of parasitaemia in extract-treated groups when compared to controls. Chemotherapeutic effects at all dose levels tested were not significantly different from that of the control Pyrimethamine (Table 7).

Table7: Prophylactic activity of orgarnic extracts of *C.anisata*

	Chloroform			Hexane			Methanol		
Dosage	Mean Parasitaemia	% Chemo-suppression	Survival time (days)	Mean Parasitaemia	% Chemo-suppression	Survival time (days)	Mean Parasitaemia	% Chemo-suppression	Survival time (days)
1.2(Pyr)	1.43±0.11[a]	7.88±0.64[b]	15	1.57±0.13[a]	7.41±0.67[a]	16	2.06±0.42[a]	8.44±0.80b	16
100	2.03±0.15[b]	5.88±1.28[ab]	13	2.29±0.13[b]	5.94±0.12[a]	9	2.46±0.32[a]	7.82±0.31b	12
250	2.24±0.07[bc]	3.98±0.90[a]	12	2.36±0.22[b]	7.01±0.55[a]	15	2.05±0.06[a]	7.89±0.87b	12
500	1.40±0.08[a]	8.20±0.34[b]	15	2.22±0.10[b]	6.20±0.83[a]	10	1.62±0.04[a]	8.88±0.11b	14
Control	2.53±0.15[c]		7.4	2.53±0.15[b]		7.2	3.51±0.07[b]		7.5

Numbers represent mean parasitaemia, % chemo-suppression and survival time (days) for each treatment.
Means separated using Student-Newman-Keuls test by the same letter along the column are not significantly different (P<0.05) from each other.

Table 8: Prophylactic activity of orgarnic extracts of *C. robusta*

	Chloroform			Hexane			Methanol		
Dosage	Mean Parasitaemia	% Chemo-suppression	Survival time (days)	Mean Parasitaemia	% Chemo-suppression	Survival time (days)	Mean Parasitaemia	% Chemo-suppression	Survival time (days)
1.2(Pyr)	2.01±0.12a	8.35±0.15a	16	2.01±0.12[a]	7.14±0.47[a]	15	2.01±0.12a	8.85±0.27a	13
100	1.75±0.34a	8.28±0.83a	11	3.23±0.23[c]	6.75±3.25[a]	7.5	3.87±0.50b	8.32±0.05a	11
250	1.89±0.26a	8.23±0.72a	12	3.00±0.25[bc]	1.79±0.00[a]	7.8	4.09±0.01b	7.49±0.30b	11
500	2.13±0.28a	8.05±0.39a	13	2.38±0.28[ab]	5.57±2.26[a]	8.2	5.26±0.23b	1.46±0.00c	13
Control	3.69±0.14b			2.91±0.14[bc]		7.2	4.29±0.25b		7.2

Numbers represent mean parasitaemia, % chemo-suppression and survival time (days) for each treatment.
Means separated using Student-Newman-Keuls test by the same letter along the column are not significantly different (P<0.05) from each other.

C. robusta exhibited repository activity with the doses employed producing suppression comparable to the standard drug (pyrimethamine 1.2mg/kg/day). (Table 8) Chloroform extract at 500mg/kg/day was observed to sustain the mice for over 13 days.

4.5 Acute toxicity

C. anisata: The acute toxicity evaluation of the chloroform extract of *C.anisata* revealed that doses at1582 mg/kg and above was lethal to the mice. Animals treated with 5000 and 2811 mg/kg of the extract produced 60% and 40% mortality respectively (Table 9). The mice produced signs of toxicity ranging from writhing, gasping, palpitation, ptosis, closure of the eyes and death. The LD_{50} of the extract in mice was calculated to be 3514 mg/kg/day. Methanol and hexane extracts were safe at doses below 5000mg/kg/day.

C. robusta: All extracts of *Clutia robusta* were safe in mice at doses below 5000mg/kg/day and devoid of any adverse clinical symptoms.

Table 9: Effects of *C. anisata* chloroform extract on uninfected mice

Group	Dose (mg/kg)	Death	% mortality
1	Vehicle	0/5	0
2	1581	0/5	0
3	2811	2/5	40
4	5000	3/5	60
5	78II	5/5	100

4.6 Drug pressure assay)

Microscopic determination of the parasitaemia indicated that the *Clausena anisata* at 800mg/kg/day drug pressure at 7^{th} passage had a significant ($P < 0.001$) effect on

P.berghei at 94.12 % chemosuppresson while *C.robusta* methanol extract caused 60.68% parasite reduction compared to the control as shown in Table 10.

Table 10: *In vivo* activity of *C.anisata* chloroform and *C.robusta* methanol extracts against *P. berghei* ANKA at 7th passage of drug pressure

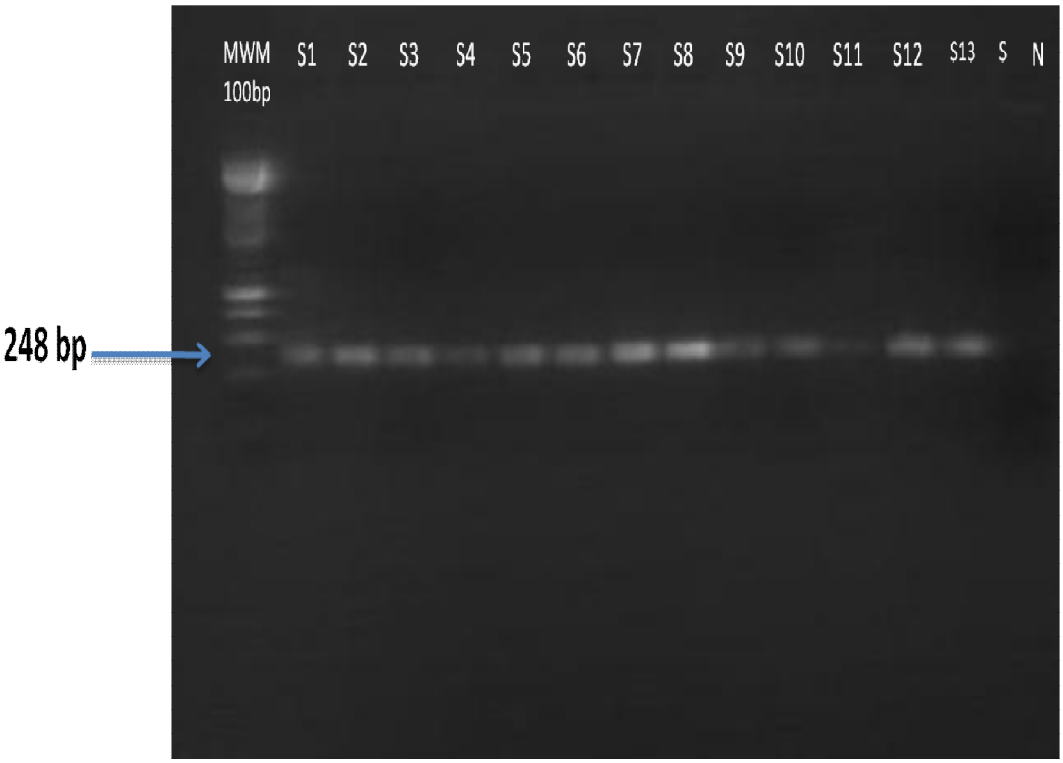
Treatment	**Dose (mg/kg/)**	**%Mean parasitaemia**	**% Decrease in parasitaemia**	**Survival time (Days)**
C.anisata (Chloroform)	800	1.93 ± 1.7^{b}	94.12	22
C.robusta (Methanol)	800	12.92 ± 2.9^{a}	60.68	13
Control		32.86 ± 4.6^{a}		**7**

Numbers represent mean parasitaemia, % chemo-suppression and survival time (days) for each treatment.
Means separated using Student-Newman-Keuls test by the same letter along the column are not significantly different ($P<0.05$) from each other.

4.7 PCR amplification of the *Pbmdr1* gene

PCR amplification of the *Pbmdr1* gene done using DNA extracted from the experimental mice and primers based on *P. berghei* (Gene Bank AFOO2130) (Gervais *et al*., 1999), indicated presence of the parasites in all the samples tested (Fig. 5).This was consistent with the phenotypic results (Table 10)

Plate 6: *Pbmdr* 1 PCR product gel analysis



Expected product 248 bp, 1% Agarose gel electrophoresis, 80V, 40 Mins

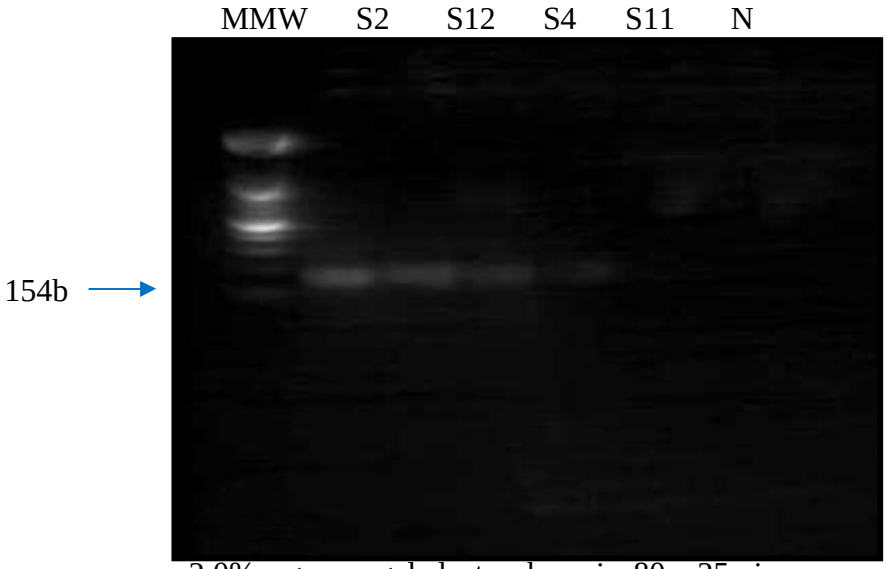
Sample name	Description
MWM 100bp	100bp Molecular weight marker
S1	Control – infected and untreated – 4th passage
S2	Control – infected and untreated -4th passage
S3	Control – infected and untreated- 4th passage
S4	Chloroform extracts treated-C. anisata-4th-passage
S5	Chloroform extract treated-C.anisata-4th passage
S6	Chloroform extract treated- C.anisata-4th passage
S7	Methanol extract treated-C.robusta-4th passage
S8	Control –untreated-7th passage
S9	Chloroform extract treated-C.anisata-7th passage
S10	Chloroform extract treated-C.anisata-7th passage
S11	Chloroform extract treated –C.anisata-7th passage
S12	Methanol extract treated – C. robusta-7th passage
S13	Methanol extract treated –C. robusta- 7th passage
SN	Negative control - not infected and untreated

4.8 Nested PCR

Nested primers used were internal to the first primer pair. The larger fragment produced by the first round of PCR was used as the template for the second PCR. This was meant to increase sensitivity and specificity of the amplification by eliminating any spurious non-specific amplification products. After the first round of PCR the non-specific products were unlikely to be sufficiently complementary to the nested primers to be able to serve as template for further amplification. Thus the desired target sequence was preferentially amplified.

Plate 7: *Pb mdr* 1 inner PCR product gel analysis after treatment

Expected product 154 bp



2.0% agarose gel electrophoresis, 80v, 25mins

Sample name	Description
MWM 100bp	100bp Molecular weight marker
S2	Control-infected and untreated-4th passage
S12	Methanol extract- treated 7th passage
S 4	Chloroform extract treated 4th passage
S11	Chloroform extract treated 7th passage
N	Negative control –Not infected and not treated

CHAPTER FIVE

5.1 Discussion

In sub-Saharan Africa and other parts of the world where malaria is endemic, plants are extensively used for treating periodic malaria. About 75% of the population in Africa does not have direct access to chemical treatment, such as ACTs, but they have access to traditional medicine for treating fevers. Treatment with these remedies has suffered a number of deficiencies. Diagnosis is often a problem, identification of plant extracts may be inccurate and the chemical content of extracts may vary considerably (Azas *et al*., 2002). In this study, four crude organic extracts obtained from medicinal plants used in Kenyan folk medicine for the treatment of malaria were tested *in vivo* against *P. berghei (*ANKA)

Crude extracts of *C.anisata* have been reported to exhibit good *in vitro* antiplasmodial activity against chloroquine resistant strain W2 with IC_{50} of less than 10mc/ml and are not toxic to VERO199 mammalian cell (Irungu *et al.,* unpublished) The present results indicating significant chloroformic crude extract at 500 mg/kg/day antimalarial activity in a mouse model are consistent with the earlier report of significant *in vitro* antimalarial activity (Muthaura *et al*., 2007).

In the present study, the organic extracts from the bark of *C. anisata* were found to posses not only significant suppressive activity (as exhibited by its blood schizonticidal

activity during the 4–day suppressive test. (Table 2), but also a considerable prophylactic activity. (Table 7).The higher doses of the extracts gave results similar to those seen with the standard drugs (CQ and Pyrimethamine). The crude extracts exhibited minimal curative effect during established infection test, incomparable to that of mice in the chloroquine treated groups. The malaria model used in this study lacks the insect vector and it utilizes laboratory parasite inoculation in a manner and in doses that result in rapid infection of erythrocytes without the parasite going through the liver stage. Higher parasite loads are achieved than during natural infection and this would reduce the efficacy of test extracts under investigation. Small mammals metabolise drugs faster and by different metabolic pathways to humans, which might result in less efficacy of the extract under investigation. Indeed, small mammals require 5-10 times per kg body weight the amount of drug taken by humans to produce the same effect. (Freireich *et al*, 1966).

Nineteen components have so far been identified in the leaves of *C anisata***.** with the volatile oil containing phenylpropanoids and an acute toxic principle, methyl havicol (estragole) (Ekundayo *et al*., 1986). Other notable compounds found in the oil include terpenoids and fatty acids, as well as more than 20 coumarins (Ngadjui *et al*., 1989; GHP, 1992). The plant also contains clausenol and clausenine, (Chakraborty *et al*., 1995) and carbazole alkaloids (clausamine D, E, F and G) (Chakraborty *et al* 1995; Ito *et al*., 2000). Many compounds of medicinal interest, including alkaloids, flavonoids, coumarins, and steroids, isolated from other species such as *Phyllanthus niruri* (Calixto *et al.,* 1998); *Psadia arguta voigt* (Govinden-soulange *et al.,* 2002) have been found to

inhibit *Plasmodium falciparum*, in both *in vitro* and *in vivo* tests (Nkunya *et al*., 1991; Marshall *et al* 1994; Bickii *et al*., 2000).

Alkaloids are one of the major classes of compounds possessing antimalarial activity. 0ne of the oldest and most important antimalarial drug, quinine, belongs to this class of compounds and still relevant. Pure alkaloids rank among the most efficient and therapeutically significant plant compounds. Most of the basic medicinal agents eg morphine which is an analgesic,colchine used for treating gout, reserpine which is a tranquilizer, vincristine and vinblastine which have antitumor effects are all pure alkaloids or their derivatives (Haidet, 2003). The phytochemical diversity of a plant is indicative of its high medicinal and therapeutic potentials. This is because these compounds form the basis of the pharmacologic effects of such plants (Jigam *et al*., 2004).

The bark extracts of *C.anisata* had high safety levels (LD_{50}=3514mg/kg), considerable suppressive effects, and significant ($P< 0.001$) prophylaxis against *P.berghei* in mice. This phenomena, together with the other pharmacological effects observed in other parts of the plant, such antidiabetic, antimicrobial, parasiticidal and spasmolytic properties observed in other experiments makes *C.anisata* a better antimalaria than other species with antimalarial potential only (Pascual *et al* ., 2001). This also partly explains the high survival periods of the *P. berghei* challenged mice noted after treatment with *C.anisata* extracts, despite the exhibited minimal curative effect during established infection test. Noedi *et al*, (2003), suggested that crude plant extracts tended to have better

plasmodistatic than plasmodicidal effects because some unpurified bioactive principles may require initial conversions and the time lag allows for parasite proliferation. Moreover the active components might not be present in enough concentrations to effect rapid clearance of target organisms (Fidock *et al.,* 2004). Since the extracts are mixture of various compounds, the antiplasmodial activity could be caused by a different class of molecule. To confirm this, the compounds responsible for the activity should be isolated and characterized.

Various parts of *C. robusta* are used in traditional medicine, for several therapeutic purposes. Several tests with root stem and leaf extracts showed antifungal and antiviral activities, while the antibacterial activities showed divergent results (Kokwaro, 1993; Beentje, 1994).

Phytosterol glycoside and diterpene, have been previously isolated from *C.robusta* (Zerihun, 1987), and from other plants of the same family such as *Clutia abyssinica* (Waigh, *et al.*, 1991) and *Clutia hirsuta* (Kraft, *et al.,* 2003). Ethanolic leaf, stem and root extracts showed moderate antiviral activity *in vitro* against polio virus and Coxsackie virus, moderate antifungal activity against *Aspergillus fumigatus* and *Fusarium culmorum* , but little antibacterial activity . An ethanol extract of the dry leaves showed antifungal activity against *Trichophyton mentagrophytes* (Cos *et al*., 2002; de Boer, *et al.*, 2005).

In the present study, chloroform extract of the bark of *C.robusta* was found to posses significant prophylactic activity (Table 8). The higher doses of the extract gave results

similar to those seen with pyrimethamine and also considerable suppressive activity. This was illustrated by its activity during the 4- day suppressive test although the chemossuppresion obtained was uncomparable with that of Chloroquine used as the standard drug perhaps due to the crude nature of the extract. The mechanism of action of the extract needs to be elucidated although some plants are known to exert antiplasmodial action either by causing elevated red cell oxidation (Elkin, 1997) or by protein synthesis inhibition (Kirby *et al*., 1989). The extract could have elicited its action through any of the above-mentioned mechanisms or by some other means yet to be determined. The organic extracts tested at doses below 5000 mg/kg/day were well-tolerated by the mice for 24 to 48 hours without showing any sings of physical toxicity.

Flavonoids, diterpenes, and diterpenoids isolated from other plants have been implicated in antimalarial activity *in vivo* (Philipson and Wright, 1991; Christensen, 2001). Since the extracts are a mixture of various compounds, the antiplasmodial activity could be caused by a different class of molecule or synergism between the various phytochemical compnents. To confirm this, the compounds responsible for the activity should be isolated and characterized in another project.

The *C.anisata* chloroform extract and *C.robusta* methanol extracts demonstracted a high chemosupression and median survival time during the 4-day and prophylactic tests and so were picked for further investigation. PCR amplification of the *P. berghei* DNA extracted from the dry blood spot of mice challenged with the parasite and treated with *C.anisata* chloroform and *C. robusta* methanoic bark extract showed that the extract was

unable to clear completely the parasite after seven weeks of drug pressure (Plate 6). The results were consistent with chemsuppression at 94.12% for *C.anisata* and 60.68% for *C.robusta* obtained through microscopy. Further molecular analysis in form of mutations–specific PCR and mutations–specific restriction endonuclease digestion should be carried to determine if the extracts inability to clear the parasite was due to induced resistance or due to other factors such as the individual variations in the infected mice, synergism with the mice immune response, and the plants pharmacological action amongst other factors.

5.2 Conclusion

The results of this study showed significant decrease in parasitaemia of *P. berghei* infected mice treated with the crude bark extract of *C. anisata* and *C. robusta*. This significant suppression of parasitaemia observed was dose dependent. The crude extract of *C. anisata* caused 72.13 % suppression in parasitaemia of *P berghei* infected mice while chloroquine a standard antimalaria drug exerted 91.10%suppression. When a standard antimalarial drug is used in mice infected with *P. berghei*, it suppresses parasitaemia to non-detectable levels (Kiseko *et al.,* 2000), just like the effect of chloroquine in this study. The extracts demonstrated a significant prophylactic effect against *P. berghei infected* mice as demonstrated by the significant reduction in the level of parasitaemia of 71.35% using *C. robutsa* hexane extrac. It is evident based on these findings that *C. anisata and C. robusta* possess potential antimalarial activities justifying its folkloric usage in the management of malaria.

5.3 Recommendations

1. *Clausena anisata* and *Clutia robusta,* medicinal plants used in this study which are very popular in Kenya rural and urban centres are potential sources of antimalarial agents and should therefore be the subject of further research to study their active constituents also to test for the cytotoxicity of the extract.
2. Further molecular analysis in form of mutations–specific PCR and mutations–specific restriction endonuclease digestion should be carried to determine if the extracts inability to completely clear the parasite was due to induced resistance or due to other factors such as the individual variations in the infected mice.
3. The new concept in malaria chemotherapy is towards combination therapy, it would be worthwhile to test the effect of the combination of a potent extract such as chloroformic *C. anisata* extract and extracts from other medicinal plants.

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APPENDICES

APPENDIX 1: Preparation of buffers and solutions

Phosphate saline glucose buffer (PSG)

5.392 g	di-sodium hydrogen phosphate (Na_2HPO_4)
0.312 g	sodium di- hydrogen phosphate ($NaH_2PO_4.2H_2O$)
1.7 g	Sodium chloride (NaCl)
10.0 g	D-glucose

Dissolve the mixture in 1 liter of distilled water, sterilize by autoclaving at 121^0c for 15 minutes and store at 4^0c

20% glycerol in Phosphate saline glucose buffer

Two parts of 100% Glycerol solution is mixed with eight parts of PSG to obtain 20% glycerol solution.

Sorensen staining buffer

1.270 g	potassium di- hydrogen phosphate (KH_2PO_4)
4.275 g	di-sodium hydrogen phosphate ($Na_2HPO_4.2H_2O$)

Dissolve the mixture in 2.5 liters of distilled water, pH 7.2. Store at room temperature

Giemsa stock solution

3g	Giemsa powder
300 ml	Methanol
200 ml	100% glycerol

Mix the solutions thoroughly for 45-60 minutes. Store at room temperature

70% Tween-80 and 30% ethanol (stock solution)

7parts of Tween-80 is mixed with 3 parts of analytical ethanol

Primers Design

tctacaattttaaaattacttgaaccattttatgacccaacagacggagatattgttatt
aatgattcacacagtttgaaagacgttgatctaaaatggtggagatctaaaattggagta
gttagtcaagatcctttattatttagcaattctattaaaaataatattaaatatagttta
ataagtccaaatagtttagaagcagtagaaaatgggttcgatttcgcggaaatagtgat
tcttcattaaatagagataatagtaaaaatggaaagtgcactagtattttggatgaaata
tctaagaggaatacaactagcgatttattagaagtaatatcgtctattaattcagttgag
gattcaaaagttgtagatgtatctaagaaagtcttaatcccacgatttgtagcatcctta
ccagataaatatgacactttagtaggttctagctcatctaagttgtcaggtggacaaaaa
aaacgaatatctataggtagagctgttattagaaatcctaaaatttaattcttt
//

1st set of primers

Forward primer

5'CGCGGAAATAGTGATTCTTCA3'

Len: 21 **MW**: 6445.09 **T_m**: 63.82° C **GC**: 42.86% **Sec. Str.**: Very weak **Primer Dimer**: No

Reverse Primer

5'CCACCTGACAACTTAGATGAGC3'

Len: 22 **MW**: 6688.28 **T_m**: 62.56° C **GC**: 50% **Sec. Str.**: None **Primer Dimer**: No

Product size **248bp**

cgcggaaatagtgattcttcattaaatagagataatagtaaaaatggaaagtgcactagtattttggatg
aaatatctaagaggaatacaactagcgatttattagaagtaatatcgtctattaattcagttgag
gattcaaaagttgtagatgtatctaagaaagtcttaatcccacgatttgtagcatcctta
ccagataaatatgacactttagtaggttctagctcatctaagttgtcaggtgg

Forward Primers Set 2

5'TGGAAAGTGCACTAGTATTTTGGATG'

Len: 26 **MW**: 8080.08 **T_m**: 65.66° C **GC**: 38.46% **Sec. Str.**: None **Primer Dimer**: No

Reverse Primers

5'TGGTAAGGATGCTACAAATCG3'

Len: 21 **MW**: 6494.14 **T_m**: 61.42° C **GC**: 42.86% **Sec. Str.**: Very weak **Primer Dimer**: No

Expected product size **154bp**

Preparation of the Agarose gel

Measure 108 g of Tri

55 g of boric acid

8.3 g of EDTA

Add 1000ml of distilled water and stir to mix.

To prepare 1X TBE, measure 900ml of distilled water and 100ml of 10X TBE mix them and you get 1X TBE

Agarose gel electrophoresis

Prior to gel casting, dried agarose was dissolved in buffer by heating and the warm gel solution then was poured into a mold (made by wrapping clear tape around and extending above the edges of an 18 cm X 18 cm glass plate), which was fitted with a well-forming comb. The percentage of agarose in the gel varied. 2% agarose gel was prepared. Ethidium bromide was included in the gel matrix to enable fluorescent visualization of the DNA fragments under UV light. Agarose gel was submerged in

electrophoresis buffer in a horizontal electrophoresis apparatus. The DNA samples were mixed with gel tracking dye and loaded into the sample wells. Electrophoresis usually is at 150 - 200 mA for 0.5-1 hour at room temperature, depending on the desired separation.

When low-melting agarose is used for preparative agarose gels, electrophoresis is at 100-120 mA for 0.5-1 hour, again depending on the desired separation, and a fan is positioned such that the heat generated is rapidly dissipated. Size markers were co-electrophoresed with DNA samples, when appropriate for fragment size determination. Two size markers are used, phi-X 174 cleaved with restriction endonuclease HaeIII to identify fragments between 0.3-2 kb and lambda phage cleaved with restriction endonuclease HindIII to identify fragments between 2-23 kb. After electrophoresis, the gel was placed on a UV light box and a picture of the fluorescent ethidium bromide-stained DNA separation pattern was taken with a Polaroid camera.

Protocol

1. Prepare an agarose gel, according to recipes listed below, by combining the agarose

(low gel temperature agarose may also be used) and water in a 500 ml Ehrlenmeyer flask,

and heating in a microwave for 2-4 minutes until the agarose is dissolved.

Genetic technology grade (800669) or low gel temperature (800259) agarose from Schwarz/Mann Biotech.

2. Add 20X TAE and ethidium bromide (EtBr), swirl to mix, and pour the gel onto a taped plate with casting combs in place. Allow 20-30 minutes for solidification.

3. Carefully remove the tape and the gel casting combs and place the gel in a horizontal electrophoresis apparatus. Add 1X TAE electrophoresis buffer to the reservoirs until the buffer just covers the agarose gel.

4. Add at least one-tenth volume of 10X agarose gel loading dye to each DNA sample, mix, and load into the wells. Electrophorese the gel at 150-200 mA until the required separation has been achieved, usually 0.5-1 hour (100-120 mA for low gel temperature agarose), and cool the gel during electrophoresis with a fan. Visualize the DNA fragments on a long wave UV light box and photograph with a Polaroid camera.

Primers constitution

1µm=1pmol/µl. Therefore 100pmols/ µl=100 µm

To make a volume of 100ul using double distilled water or PCR water (50µL prepared)

C1V1=C2V2

100 µm, V1==10 µl, 100 µm

= 10 x 100/100==10 µl

Therefore 10 µl of stock primer ,top up in double distilled water to 100 µl

(10 µl in 90 µ PCR water)

PCR master mix

Master mix	**Optimization**	
	X1	**X6**
Double distilled water	15.26	91.56
10X PCR buffer	3.0	18.0
25Mm $MgCL_2$	2.5	15.0
10µM (F)	0.5	3.0
10µM (R)	0.5	3.0
Taq	0.24	1.44
Template	5µm	

NB: Constitution of the master mix was done on the ice to minimize the commencement of any reactions. The Taq polymerase was added last as it is sensitive to buffer and also the reaction begins immediately it is added.

Cycling conditions	***Pbmdr***	**outer**	**inner(nested)**
Primary denaturation		94^0c for 3min	94^0c 3min
Denaturation		94^0c for 1min	94^0c for 30 s
Annealing		49^0C for 1min	48^oc for 1min
Extension		72^0c for 1min	72^0c for 1min
Final extension		72^0c for 3min	72^0c for 3min
Halt		4^0C	4^0C
40cycles			