



ADDIS ABABA UNIVERISTY
SCHOOL OF GRADUATE STUDIES

***IN VIVO* ANTIMALARIAL ACTIVITY OF THE ROOT EXTRACTS
AND FRACTIONS OF *CLERODENDRUM MYRICOIDES* IN
PLASMODIUM BERGHEI INFECTED MICE**

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List of abbreviations

ACHE-Acetylcholinesterase

ED-Effective dose

EHNRI-Ethiopian Health & Nutrition Research Institute

Pfmdr-Plasmodium falciparum multidrug resistant

RDTs-Rapid antigen detection tests

HDMECs-human dermal microvascular endothelial cells

TNF- α -Tumor necrosis factor alpha

IFN- γ -Interferon gamma

CDKs-Cyclin dependent kinases

NPP- new permeability pathways

KASIII- ketoacyl-ACP synthase

MSP-Merozoite surface protein

GLUR- glutamate rich protein

LDH-Lactate dehydrogenase

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ABSTRACT

Malaria is one of the six infectious diseases that account for half of all premature deaths; and for over 40% of the world's population from more than 90 countries living with the risk of the disease. Plants are essential sources for the battle against it. *C. myricoides* is found in different parts of Ethiopia and its vernacular name is 'misiritch' in Amharic. It is traditionally used in Ethiopia for different purposes such as treatment of gonorrhoea, colic, gout, swelling, measles, gland TB, eye diseases, malaria, rabies, as wound dressing and as aphrodisiac. The present study therefore attempted to evaluate the antimalarial activity of the root extracts of *C. myricoides* in mice infected with *P. berghei* and to determine LD₅₀ and phytochemical screening.

In this study, phytochemical screening has been done using standard methods. 4-days petter's test was used to determine parasite inhibition, PCV was determined by Wintrob's method, effects against loss of body weight, effect on reduction in temperature and improvement on survival time were determined. LD₅₀s of the crude extracts have been also done.

The crude aqueous, crude hydroalcoholic, dichloromethane fraction of crude aqueous, butanol fraction of crude hydroalcoholic extracts of the roots of *C. myricoides* significantly ($P < 0.05$) inhibited parasitemia of malaria. These parasitemia suppression observed was comparable to that observed with *Solanum indicum* leaves. The activity is due to components in the plant which might have retarded the multiplication of the parasites in the blood.

The crude aqueous, crude hydroalcoholic, dichloromethane fraction of aqueous, butanol fraction both crude aqueous and hydroalcoholic extracts of the roots of *C. myricoides* prevented weight loss ($P \geq 0.05$) especially at high doses. This also occurs for plants like *Withania somnifera*. No weight gain was observed which may be due to the presence of compounds with appetite suppressing effect.

The crude aqueous, crude hydroalcoholic, dichloromethane fraction of aqueous, butanol fraction of crude hydroalcoholic and chloroform fraction of crude hydroalcoholic extracts ($P \geq 0.05$) of roots of *C. myricoides* prevented reduction in PCV. The parasites caused destruction of red blood cells leading to reduction in PCV. The prevention in reduction of PCV may be due to the compounds in the extracts. The crude aqueous and crude hydroalcoholic extracts improved ($P \geq 0.05$) survival time.

From this study, it can be observed that the crude aqueous extract, crude hydroalcoholic extract, dichloromethane fraction of crude aqueous extract and butanol fraction of crude hydroalcoholic extract has reduced parasitemia, prevents loss of body weight, protects against PCV reduction and improved mean survival time. Chloroquine (10mg/kg) has shown 100% parasitemia inhibition and all other parameters measured in mice returned to normal. In this study, the crude extracts showed better antimalarial activity than the fractions since different compounds may be found which have synergistic effect and some secondary metabolites protect other metabolites (as antioxidants) and break of this association can accelerate degradation. In this study, alkaloids and saponins have been detected which might be responsible for the antimalarial activity. The hydroalcoholic crude extract likely to contain flavonoids, saponins and phytosterols while polyphenols, saponins, tannins and alkaloids are likely to be present in the crude aqueous extract. Thus the synergistic activity observed in the crude extracts and the activity in the fractions may be due to these compounds.

The oral LD₅₀ of the crude aqueous and hydroalcoholic extracts were 1134.42mg/kg and 1629.99mg/kg, respectively.

From the present study, it can be concluded that crude aqueous extract, crude hydroalcoholic extracts, of roots of *C.myricoides* have antimalarial activity at much lower doses than the LD₅₀s hinting the safe and efficacious nature of the plant. The dichloromethane fraction of crude aqueous extract and butanol fraction of crude hydroalcoholic extract also have antimalarial effect. Further fractionation was also not possible due to low yield. Thus, the active ingredients should be isolated by different extraction methods.

1. Introduction

Infectious diseases are the most important causes of mortality in infants and young children, causing more than 13 million deaths per year, one in every two deaths in resource-poor countries (Julie et.al., 2005). Malaria is one of the six infectious diseases that account for half of all premature deaths; and for over 40% of the world's population from more than 90 countries that live with the risk of the disease (Julie et.al., 2005).

1.1. Epidemiology

Malaria has been responsible for the death of about half of all people who ever lived. Even today, inspite of the several attempts made to intervene worldwide, malaria remains the most significant parasitic disease in the tropics and subtropics, where it causes at least 500 million clinical episodes and claims 1.5 million lives each year, mostly young children and pregnant women (Riscoe et.al., 2005). At the global level, malaria incidence is concentrated in the world's poorest countries, with 90% of malaria deaths occurring in sub-Saharan Africa (Eve et.al., 2005). Its complications may account 30-50% of inpatient admission and up to 50% of outpatient visits (Dunavan, 2005). The majority of deaths result from pediatric cases in developing countries. In Africa alone, one child dies of malaria every 30 seconds (Gundula and Philippe, 2005; Jennifer et.al., 2005).

Malaria is a major public health problem in Ethiopia. It has been consistently reported as one of the three leading causes of morbidity and mortality in the past years. The magnitude of the problem in 2002/03 has even worsened and the disease has been reported as the first cause of morbidity and mortality accounting for 15.5% outpatient consultations, 20.4% admissions and 27.0% inpatient deaths (Gezahegn, 2004). In a non-epidemic year, 5 - 6 million clinical malaria cases and over 600,000 confirmed cases are reported from health facilities in Ethiopia(Gezahegn, 2004). However, as the potential health service coverage is accessible to about 61% of the population and due to the low

service utilization rate (27%); the number of malaria cases reported by health facilities is only a portion of the actual magnitude (Gezahegn, 2004).

1.2. Plasmodium species

Malaria, the disease caused by protozoan parasites of the genus *Plasmodium*, continues to put an enormous burden on global health (Jennifer, 2005). *P. falciparum* is a single-celled eukaryotic parasite responsible for the most lethal form of human malaria, i.e cerebral malaria. The parasite is transmitted to humans by *Anopheles* mosquitoes. Endemic regions encompass approximately 40% of the global human population. *Anopheles gambiae* the most aggressive among the more than 60 mosquito species that transmit malaria to people in sub-Saharan Africa and bears partial responsibility for millions of human deaths per year (Vizioli et.al, 2000; Dunavan, 2005 and Legoff et.al., 2006).

Four species of *Plasmodium* can be transmitted to humans by female *Anopheles* mosquitoes , the most prevalent being *P. falciparum* and *P. vivax* and less frequently *P. malariae* and *P. ovale* (Timothy, 2000; Carvalho et.al., 2002).

P. falciparum and *P. vivax* are the two dominant parasite species with relative frequency of 60% and 40%, respectively in Ethiopia (Gezahegn, 2004). This proportion varies from place to place and from season to season. In malaria epidemic situations, *P. falciparum* is the dominant parasite species that causes severe manifestations, and almost all malaria deaths happen due to infection by this parasite. Moreover, the biological diversity of *P. falciparum* and its ability to develop resistance to a number of anti-malarial drugs has been a major challenge in malaria chemotherapy (Gezahegn, 2004).

In Ethiopia, it was studied that about 13–28% of deaths in children under five years of age are attributed to falciparum malaria. Recently, a considerable increase in malaria morbidity has been noted in the south and southwest of Ethiopia. Possible reasons include climatic changes, drug resistance and migration (e.g. from Sudan). Also, epidemic outbreaks are now being observed in highland areas (Mirjam et.al., 2006).

1.3. Life cycle of malaria parasite

While feeding, an infected female Anopheles mosquito passes sporozoites of the malaria parasite plasmodium into the victim's blood stream. Within 30-60 minutes, the sporozoites enter the liver cells where they reproduce asexually, forming thousands of merozoites. The parasites go through several host cells by breaching their plasma membrane before infecting a final hepatocyte. The schizonts are then released as merozoites to the circulation to invade red blood cells where they get multiplied, causing the cells to rupture releasing yet more merozoites. The cycle repeats, eventually some of the merozoites develop into male and female gametocytes, which can be ingested by a previously uninfected mosquito taking a blood meal. In the mosquito's gut, the gametocytes develop into gametes and fuse to eventually produce an oocyst that releases sporozoites. These travel to the mosquito's salivary glands, ready to be transferred to another victim and the life cycle continues. The rupture of infected blood cells causes fever, chills and progressive anemia. Death may occur from severe anaemia as well as clogging of blood vessels in the brain, lungs and other organs by parasitized blood cells. In pregnancy malaria-laden placentas rob babies of growth before they are even born (Dunavan, 2005). The life cycle is shown in figure 1.

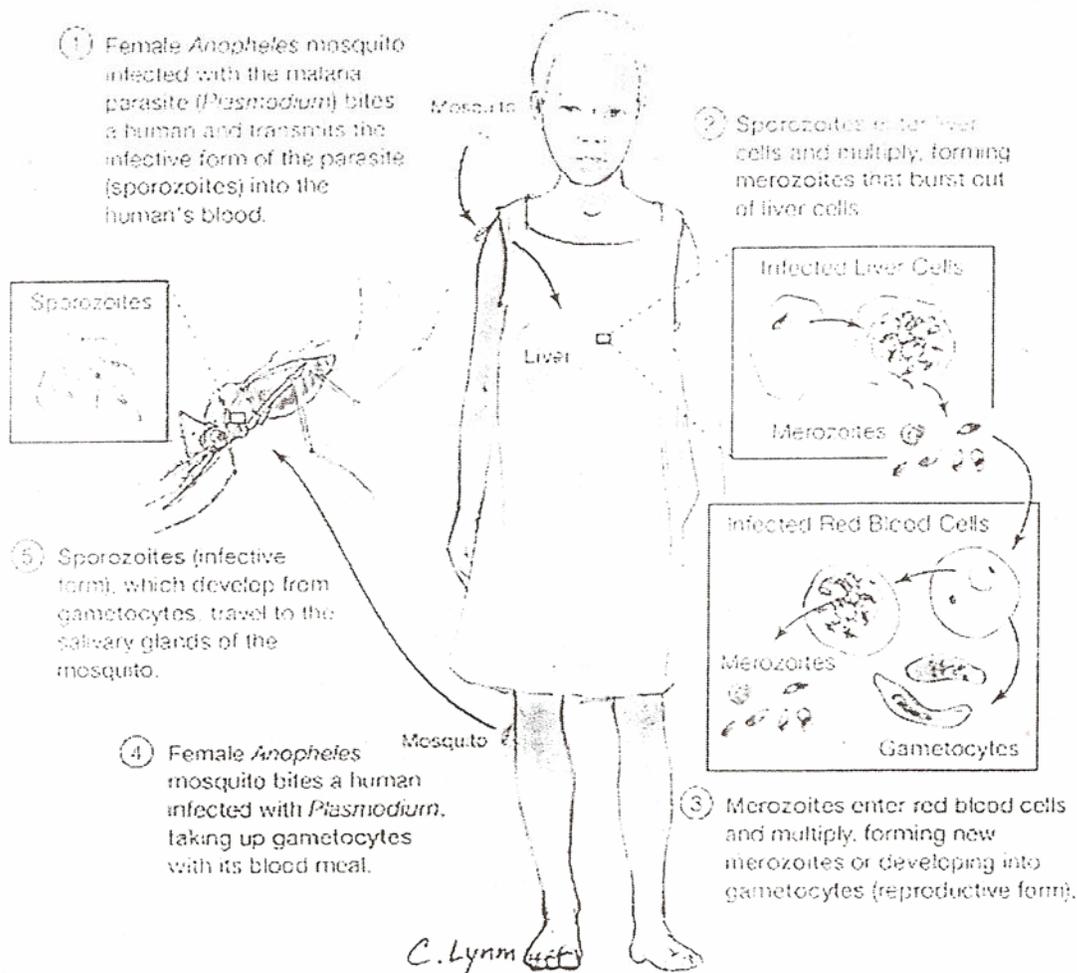


Fig 1. life cycle of malaria parasite (Sharon et.al., 2004)

Fig 1. Life cycle of malaria parasite (Sharon et.al., 2004)

1.4. Pathophysiology

Proliferation of the parasite within the host's erythrocyte takes place by using hemoglobin as predominant source of nutrition. The malaria parasite digests hemoglobin within the digestive vacuole through the sequential metabolic processes involving multiple proteases. Massive degradation of the hemoglobin generates large amount of toxic heme. Malaria parasite, however, evolves distinct mechanism for detoxification of heme through its conversion into an insoluble crystalline pigment known as hemozoin. Hemozoin synthesis is an indispensable process for the parasite and is the target of action of several antimalarials (Tekwani and Walker, 2005).

Malaria pathogenesis could be explained by *Plasmodium falciparum* erythrocyte membrane protein mediated sequestration of parasitized erythrocytes. This protein helps the parasite infected RBC adhere to blood elements including noninfected erythrocytes, leukocytes, and wall of endothelial cells of microcirculation. These binding events enable parasitized erythrocytes to sequester and avoid clearance by the spleen and also contribute to disease by causing microvascular inflammation and obstruction (Rogerson et.al., 2004; Fairhurst and Wellem, 2006). Cytoadherence of IRBCs on human dermal microvascular endothelial cells (HDMECs) is responsible for pathogenesis of malaria. IRBCs were observed to tether, roll, and adhere on resting HDMECs, rosetts will be formed causing clogging of capillaries. IRBCs interact synergistically with multiple adhesion molecules on vascular endothelium. The rolling of IRBCs may be the rate-limiting step in cytoadherence (Bryan, 2000).

During its development in the host red cell, the human malaria parasites cause profound alteration in the permeability of host cell membrane. These transport systems, play a role in the development of intra erythrocytic parasite in its need to take up solutes and nutrients from the extracellular medium and the disposal of metabolic wastes. These can be potential sites for drug design either by inhibiting the transport and hence depriving the parasite of nutrients for development or by designing cytotoxic drugs which selectively entering the parasite through these induced transporter routes and hence can not enter the normal mammalian cells (Ghebreyesus et.al., 2000; Mirjam et.al., 2006).

Different factors participate in the neuropathogenesis of malaria. They seem to include abnormally high production of cell-derived cytokines such as tumor necrosis factor TNF- α and IFN- γ induced by infected erythrocytes. These cytokines may play an important role in causing certain pathological changes, by up-regulating the expression of cell surface markers like ICAM-1 and chondroitin sulfate A, thus leading to the sequestration of infected erythrocytes, leukocytes and monocytes in the cerebral capillaries. This theory was proposed to explain the cytoadherence of parasite-infected red blood cells is the expression of *P. falciparum* erythrocyte membrane protein-1 and up-regulation of neurovascular endothelial adhesion molecules, which may cause the neurovascular lesions which characterize cerebral malaria (Mariama, 1999).

1.5. Clinical manifestations

The clinical symptoms of malaria are varied and nonspecific but commonly include fever, fatigue, malaise, headache, myalgia and sweating (Sean and Sweetman, 2005).

According to World Health Organization, severe malaria refers to a parasitemic person with one or more of the following: prostration, impaired consciousness, respiratory distress or pulmonary edema, seizures, circulatory collapse, abnormal bleeding, jaundice, hemoglobinuria or severe anemia (hemoglobin < 5 g/dL or hematocrit < 15%). Prostration and altered consciousness occur frequently in both children and adults with severe disease; respiratory distress, seizures and severe anemia are more common in children, whereas renal failure and jaundice occur more frequently in adults. Acute respiratory distress syndrome, an immune-mediated complication, often occurs during the second to fourth day of treatment, even when parasitemia is decreasing. Severe malaria usually occurs with parasitemia of 5% or more, and even with optimal management, the mortality rate exceeds 20% (Kathryn et.al., 2004; Njugna and Newton, 2004).

Nonimmune people, children and pregnant women who live in endemic regions are at highest risk of complications from malaria. Complications generally involve the central nervous, pulmonary, renal and hematopoietic systems (Kathryn et.al., 2004).

Hypoglycemia occurs because of parasite consumption of glucose and treatment with quinine (Kathryn et.al., 2004). Acidosis is another common metabolic derangement.

Severe anemia, acute renal failure, respiratory failure, intravascular hemolysis, coagulopathies, and shock may also develop (Kathryn et.al., 2004). Bacterial infection may occur as a complication of malaria itself (e.g., aspiration pneumonia) or may be iatrogenic. One of the most serious complications is cerebral malaria, manifested by altered level of consciousness, focal neurologic findings and seizures (Kathryn et.al., 2004). Mortality is high (15% to 25%), and survivors may have residual neurologic deficits. Although semi-immune people and those living in endemic regions tend not to experience severe malaria, they may still experience complications from recurrent infections. In children, severe anemia is the most common complication of chronic malaria, with hematocrits approaching 15% (Kathryn et.al., 2004). In case of severe malaria, massive splenomegaly causing abdominal pain e associated with both bone marrow and immune dysfunction (hyperactive malarial splenomegaly) may occur. Nephrotic syndrome has also been attributed to falciparum malaria in endemic areas, as has splenic lymphoma (Kathryn et.al., 2004).

The predominant manifestations of severe malaria in African children are cerebral malaria and severe anemia. *P.falciparum* slightly damages blood brain barrier (Gitau and Newton, 2005; Rangué et.al., 2005).

Plasmodium parasitaemia causes cerebral malaria which is characterized by altered consciousness, acute confusional state, seizures, anaemia, hypoglycemia, brainstem involvement and retinal hemorrhage (Carlos,1997; Vokaer et.al., 2004). Malaria is not only a direct cause of death but also contributes indirectly to death due to respiratory infections, diarrhea and malnutrition by impairing immunity (Kager, 2002).

1.6. Laboratory abnormalities

Different abnormalities can be detected in malarial infection i.e. Hematologic abnormalities are common: thrombocytopenia occurs in up to 70% of patients and anemia in 25% with normal or low level of leukocyte count ; leukocytosis is seen in less than 5% of cases and is a poor prognostic factor (Kathryn et.al., 2004). Liver function test results are often abnormal; transaminase levels are elevated in about 25% of cases, bilirubin in one-third and lactose dehydrogenase in up to 80% (Kathryn et.al., 2004). An elevated bilirubin level along with high lactate dehydrogenase level suggests hemolysis and is often a clue for diagnosis. Electrolyte abnormalities, especially hyponatremia, and an elevated creatinine level may also be present. Hypoglycemia is rarely present except in those with very high parasitemias and metabolic acidosis is usually associated with severe disease. Radiologic investigations are often unremarkable, although noncardiogenic pulmonary edema common with severe malaria (Kathryn et.al., 2004).

1.7. Diagnosis

A high degree of suspicion and rapid diagnosis are essential to optimize outcome. Thick and thin peripheral blood smears, stained with Giemsa stain (or, alternatively, Wright's or Field's stains), remain the "gold standard" for routine clinical diagnosis. Malaria smears permit both species identification and quantification (expressed as a percentage of erythrocytes infected or as parasites per microlitre) of parasites but malaria should not be excluded until at least 3 negative blood smears have been obtained within 48 hours. However, processing and interpretation of malaria smears require appropriate equipment as well as considerable training and expertise, factors that limit their use in endemic regions. Furthermore, accurate interpretation of malaria smears remains problematic in many established clinical laboratories, especially those outside major referral centers. Rapid malaria tests, which require minimal skill to perform and interpret, have been developed to overcome the problems of malaria smears. The most practical of these are the rapid antigen detection tests (RDTs), which detect parasite proteins in finger-prick blood samples (Kathryn et.al., 2004). RDTs recently available can identify only *P. falciparum* and *P. vivax*. Important shortcomings of RDTs include their inability to

quantify parasitemia and suboptimal test performance with low-level parasitemia. Furthermore, some RDTs are unreliable as tests of cure because antigenemia may persist for prolonged periods even after treatment. But their simplicity may make them attractive and useful alternatives to blood smears, particularly in laboratories where expertise in reading blood films is lacking or in centers where malaria is infrequently encountered. Based on clinical studies involving both travelers to and residents of endemic areas, the overall sensitivity and specificity of RDTs for the detection of *falciparum* malaria are over 90% (Kathryn et.al., 2004). However, sensitivity falls dramatically with low level parasitemia, and at present RDTs cannot be used alone to exclude malaria (Kathryn et.al., 2004).

Polymerase chain reaction (PCR) is a sensitive and highly specific test. It can detect extremely low numbers of parasites (and thus may be particularly useful in smear-negative cases) and is species specific. However, most PCR assays do not have sufficiently rapid turnaround times to be clinically useful; therefore, PCR remains largely an investigational tool. Serologic tests have no role in the diagnosis of acute malaria (Kathryn et.al., 2004).

1.8. Management of malaria

The current approach to manage the disease includes vector control to disrupt transmission from mosquito to human, prevention of infection and treatment after infection (Tripathi et.al., 2005).

1.8.1. Non pharmacologic therapy

Prevention of the disease includes vector control to disrupt transmission from mosquito to human which can be achieved by using insecticide treated nets and genetically engineered mosquitoes (Grahm et.al., 2005; Pates and Curtis, 2005 and Tripathi et.al., 2005;). But nowadays high insecticide resistance resulting from insensitive acetyl cholinesterase (AChE) has emerged in mosquitoes (Weill et.al , 2004).

The other approach for prevention is vaccination, though it is difficult for the reasons that malaria life cycle encompasses several stages in mosquitoes and humans, malaria's complex genetic makeup and human immune system must work together to achieve ideal response to malaria vaccination (Dunavan , 2005; Pizarro et.al. , 2005).

1.8.2. Pharmacologic therapy

Antimalarial drugs which act at different stages of malaria parasite can be used for prophylaxis or treatment of malaria. They are classified as blood schizonticides, tissue schizonticides and gametocides. Antimalarials have different mechanisms of action such as nucleic acid synthesis inhibition, interference on heme detoxification and protein synthesis inhibition (Michel et.al., 2002).

1.8.2.1. Chemoprophylaxis

Mass chemoprophylaxis can not be done for all people in malaria endemic areas. However, it can be done for those at high risk of malaria like children and pregnant women, particularly primigravidae, though costly (Kathryn et.al., 2004 and Sean and Sweetman, 2005).

Special risk group exposed to malaria such as long term travelers, children, pregnant women, aircrew, migrants to visit malarious areas need prophylaxis (Shanks and Edestein , 2005).

Malaria related morbidity and mortality can be reduced in children less than 5 years of age by either intermittent or continuous chemoprophylaxis. Chemoprophylaxis during pregnancy increases infant birth weight and survival, although this effect is largely limited to primi gravidae. Travelers to malaria endemic areas are also recommended to take drugs to prevent malaria (Kathryn et.al., 2004).

Chemoprophylaxis should be started 2 weeks before departure and continued for four weeks after return from the malarious area. For non-immune travelers visiting malarious areas for a period of 2 – 3 months, weekly mefloquine administered at 5 mg/kg is the recommended drug for chemoprophylaxis (Michel et.al, 2002; Fairhurst and Wellems, 2006).

1.8.2.2. Treatment of malaria

Management of malaria includes general measures to be taken to save life of the person and prevention of recrudescence using drugs and other supportive measures (Kathryn et.al., 2004).

The treatment of malaria depends on the infecting plasmodia species, the geographic area of acquisition (which affects the likelihood of drug resistance) and severity of infection (Kathryn et.al. and 2004 and Sean and Sweetman, 2005).

Falciparum malaria in nonimmune person is a medical emergency and requires rapid initiation of therapy. Even in cases where the species can not be immediately identified, the patient should be assumed to have drug resistant falciparum malaria until proven (Kathryn et.al., 2004).

The first-line treatment of uncomplicated *P. falciparum* malaria is artemether-lumefantrine administered 2 times a day for 3 days. For infants less than five kg of body weight and pregnant women, oral quinine 8mg/kg administered 3 times a day for 7 days is the first line treatment. For the treatment of malaria caused by *P.vivax*, *P.malariae* or *P.ovale*, the drug of choice is chloroquine. In malaria-free areas and where compliance can be insured, in order to eliminate hypnozoite forms (relapsing stages) of *P.vivax* from the liver and to bring about radical cure, primaquine may be administered daily for 14 days starting after chloroquine treatment is completed. However, in malarious areas where there is a high risk of re-infection, and where the main purpose of treatment is to bring about clinical cure rather than radical cure, administration of primaquine is not recommended (Gezahegn , 2004; Olumese, 2006).

Second-line treatment is oral quinine if condition of the patient permits. Otherwise intravenous (IV) or intramuscular (IM) administration of quinine should be done (Gezahegn , 2004; Olumese, 2006).

In treatment of severe malaria, supportive therapy in reducing hyperpyrexia, controlling convulsions, maintaining fluid balance and correcting hypoglycaemia should be done (Sean and Sweetman, 2005).

Along with these measures for management of other complications in severe malaria, loading dose of intramuscular or slow intravenous quinine should be used. Maintenance dose should be followed twelve hours after the start of the loading dose until the patient can take oral medication. Artemether-lumefantrine or oral quinine could be administered if intramuscular or slow intravenous quinine is not available (Gezahegn , 2004; Olumese, 2006).

1.8.2.3. Resistance to antimalarial drugs

Malaria being a public health problem is nowadays worsened by the emergence and worldwide spread of drug resistant parasites (Arav and Shapiro, 2005). Resistance has emerged to all classes of antimalarial drug except to artemisinins, and this is responsible for recent increase in malaria related mortality that occurs particularly in Africa (White, 2004).

The current state of antimalarial chemotherapeutics is particularly important for those living in malaria endemic regions of the world because of the low economic incentive for drug development and the rise of resistant strains. Resistant parasite strains limit the use of affordable drugs in many endemic areas (Klaus et.al., 2004 and Eve et.al., 2005). Cost and the limited number of antimalarial drugs in current use impose considerable constraints on control of the disease, especially in sub-Saharan Africa (Niloofar et.al., 2004; Piero et.al., 2005). There is clear evidence that malaria-related mortality in Africa has risen as a direct consequence of increasing chloroquine resistance since chloroquine has been the mainstay of antimalarial treatment for the past 50 years (Piero et.al., 2005).

In a study conducted in different regions of Ethiopia, resistance of plasmodium to chloroquine has been observed which has led to treatment of patients with sulphadoxine+ pyrimethamine (Alene and Bennett , 1996). But later, studies has shown emergence of parasite's resistance to sulphadoxine+ pyrimethamine (Alene and Bennett , 1996). Consequently Artemether-lumefantrine (AL) was suggested for treatment of malaria in 2004. Yet, availability of AL is limited and 85% of the population are living in rural areas with restricted access to health care (Mirjam et.al., 2006).

Mechanism of resistance emergence to antimalarial drugs

Mechanisms of resistance emergence to antimalarial drugs are described as follows:-

Pyrimethamine :- Resistance for this inhibitor occurs by simple single mutations of the gene encoding for the enzyme dihydrofolate reductase causing a depletion of tetrahydrofolate and inhibition of DNA synthesis (Michel et.al., 2002).

Atovaquone:- Resistance to this drug occurs by mutations in cytochrome B gene, resulting in modifications in the coenzyme Q binding site (Michel et.al., 2002).

Chloroquine- type – 4- aminoquinolones such as chloroquine, amodiaquine and pyronaridine. Resistance occurs by less accumulating capacity of acidic parasitic vacuoles like expulsion by plasmodial glycoprotines (Michel et.al., 2002).

Quinine type aryl amnoquinolones, such as quinine, quinidine, mefloquine, halofanthrine and lumefantrin. Resistance to mefloquine, quinine and haolofanthrine occurs by amplification of the pfmdr-1 gene which is responsible for pgh-1 protein overexpression which decreases accumulation of those drugs (Michel et.al, 2002).

1.8.2.4. Combination chemotherapy

Antimalarial combination is one of the strategies to combat resistance e.g. combining artemisinin derivatives with antifolate drugs, aminoquinolines, mefloquine and lumefantrine. Artemisinin derivatives are the most active of the available drugs nowadays (Geoffrey and Giancarlo, 2006).

Multidrug resistant parasites are the biggest therapeutic challenge to health care in most malaria endemic areas. This resistance is distributed worldwide. Thus new antimalarial agents are required especially aiming at curing patients no longer responding to standard therapies (Peter and Sanjeev, 2004).

Severe malaria affects only small proportion of individuals. Many of the people are affected by uncomplicated *P.falciparum* malaria. Thus, combination therapy will benefit much those with uncomplicated *falciparum* malaria. The purpose of antimalarial combination is to increase efficacy, to shorten duration of treatment in order to improve compliance and decrease the risk of resistant parasite emergence through mutation (Peter and Sanjeev, 2004).

Ideal combination of antimalarials should be safe, well tolerated, efficacious and have pharmacokinetic match to avoid selection pressure for resistance. Pharmacokinetic match should be considered since parasites exposed to declining concentration of one drug in combination are more likely to be transmitted if selection pressure for resistance to that drug occurs. The following combination of drugs has been tried and found to be effective: Artesunate+ mefloquines, Artemether- lumefantrine, Artesunate+ sulphadoxine-Pyrimethamine, Artesunate+ amodiaquine, Sulfadoxine –pyrimethamine, Sulfadoxine +

pyrimethamine + chloroquine, Sulfadoxine-pyrimethamine- mefloquine, Atovaguone-proguanil, Chloroguanil – dapsone, Quinine + tetracycline and Quinine + clindamycin (Peter and Sanjeev, 2004).

1.8.2.5. Medicinal plants used in malaria

Plants have formed the basis of sophisticated traditional medicine systems that have been in existence for thousands of years (Gordon and David, 2001). The use of plants in the traditional medicine systems of many other cultures has been extensively documented. These plant-based systems continue to play an essential role in health care, and it has been estimated by the World Health Organization that approximately 80% of the world's inhabitants rely mainly on traditional medicines for their primary health care (Alan and Dattner, 2003; Tabi et.al., 2006). Plant products also play an important role in the health-care systems of the remaining 20% of the population, who mainly reside in developed countries. (Alan and Dattner, 2003; Tabi et.al., 2006).

The economic value of tropical medicinal resources, both to private pharmaceutical corporations and to society is very high. Each undiscovered plant medicine could be worth in the area of US\$96 million to a pharmaceutical interest, with gross revenue for all drug plant discoveries yielding a total of US\$3.2–4.7 billion (Robert, 2004).

The medicinal use of quinine dates back to 350 years. It is first in 1663 that an Augustinian monk noted the use of powdered *Cinchona* to treat fevers. *Cinchona* was used to treat fevers in Europe. In 1820, Pelletier and Caventou isolated quinine and cinchonine from the bark and in 1994 quinine was synthesized (Michel et.al, 2002).

1.8.2.5.1. Artemisinin and other natural compounds as antimalarials

Artemisinin is extracted from sweet worm wood (*Artemisia annua*) and are the most important antimalarials available, rapidly killing all asexual stages of *Plasmodium falciparum*. They are widely used to treat multidrug resistant malaria. Artemisinin is a sesquiterpene trioxane lactone containing a peroxide bridge (Michel et.al, 2002).

The mechanism of action of Artemisinin based compounds is not fully understood. But the peroxide ring is assumed to play major role. Peroxide bond is cleaved in presence of heme iron. A series of reactions occur finally leading to epoxide, which is highly active alkylating agent. Alkylation of antimalarial proteins lead to death of the parasite in the erythrocytic stage (Michel et.al, 2002).

Many other compounds from different plants are also used for the treatment of malaria, i.e. 2-n-pentyl quinolone from *Galipea longiflora*, roemrefidine isolated from *Sparattanthelium amazonium*, tubulosine and cephaline from *Pogonopus tubulosus* and vismione from *Vismia guinensis* are some of them.

1.8.2.5.3. Clerodendrum myricoides

Members of the family Verbenaceae are varied in their biological activities and the chemical constituents that they possess. *Clerodendrum* species (Verbenaceae) are cosmopolitan in distribution and have been used in Indian and Chinese traditional medicine for ages (Rajasekaran and Poanusamy, 2006).

Clerodendrum is a large genus with 400 species in tropical and warm temperate areas, especially in the eastern hemisphere (Inga et.al, 2006). Several species are grown as ornamentals. *C. acerbianum*, *C. cephalanthatum*, *C. johnstonii*, *C. umbellatum*, *C. alatum*, *C. robecchii*, *C. myricoides* are some of them (Inga et.al, 2006).

The leaves of *Clerodendrum* species are reported to contain several essential oils. These are exploited in traditional medicine and are useful in the chemotaxonomical identification

of the genus. Moreover, the leaf extracts of *Clerodendrum* species are effective in controlling fruit rotting fungi (Rajasekaran and Poanusamy, 2006).

In Indian tribal medicine, leaves of *C. inerme* are used for treating fever, cough, skin rashes and boils, and are used in conjunction with other plant leaves. They are also used to treat umbilical cord infection and for cleaning the uterus in local medicine. *C. inerme* also has antihaemolytic effect. Several constituents have been isolated from *C. inerme*: inerminoside-A1, inerminoside-C, inerminoside-D, 3- Iridoid glycosides, inerminoside-B, 4 α -Methyl-24 β -ethyl-5 α -cholesta-14, 25-dien-3 β -ol and 24 β - Ethylcholesta-5,9, 22E-trien-3 β -ol, sterols and megastigmane glycosides (sammangaosides A and B) (Rajasekaran and Poanusamy, 2006).

A decoction of *C. phlomidis* leaves is used along with other plants for inflammation, and is effective in treating bronchitis, headache, weakness, drowsiness and digestive problems (Rajasekaran and Poanusamy, 2006). *C. phlomidis* has been used in herbal formulations to treat cancer. The methanolic extract of leaves of *C. phlomidis* has been found to significantly reduce the spontaneous activity and behavior of experimental animals and also is shown to have antidiarrhoeal activity. From dried roots and flowers of *C. phlomidis*, α -LRhamnopyranosyl(1-2)-2-D-glucopyranosyl-7-0-naringin-4-0- α & D-glucopyranoside-5-methyl ether 2,4-trihydroxy,6-methoxy chalcone-4,4- α -D Diglucoside were found. Leaf and stem extracts of *C. inerme* and *C. phlomidis* have been reported to be more effective in controlling plant pathogens than human pathogens (Rajasekaran and Poanusamy, 2006).

Recently the anti-inflammatory properties of the leaf extract of *C. trichotomum* were reported. It also seemed to have antipyretic activity. *In vitro* and *in vivo* studies of dried leaf extracts of this plant have demonstrated antioxidant activity (Rajasekaran and Poanusamy, 2006).

Diterpenoid compounds are also found from clerodendrum species. The antibacterial activity of flavonoid isolated from clerodendrum species has been reported by some researchers (Rajasekaran and Poanusamy, 2006).

C.myricoides, is one of the traditionally used antimalarial plants used for treatment of malaria. It is a South African shrub 2 m tall. The leaves are opposite or whorled, lanceolate or obovate, to 9 cm long, cuneate at the base, margins entire or coarsely toothed, particularly above the middle. Flowers have corolla with 4 upper lobes nearly equal, white or pale-blue, lower lobe much larger, usually blue-violet (Fig.2). It is rare in cultivation (Nancy and Wayne, 1994). *C. myricoides* is found in most parts of Africa such as Nigeria, South Africa, and Madagascar (Inga et.al. , 2006). *C. myricoides* is also found in different parts of Ethiopia and its Vernacular name is ‘misiritch’ in Amharic (Desta, 1994).



Fig 2. *Clerodendrum myricoides* (Nancy and Wayne, 1994).

Decoctions of the roots are used in many countries to treat coughs, headaches, abdominal pains, and malaria. The bark has been used against snakebites (Inga et.al., 2006).

Clerodendrum myricoides is used for many purposes in Ethiopia. Among them are gonorrhoea, colic, gout, swelling, measles, gland TB, eye diseases, malaria, rabies, as wound dressing and as aphrodisiac (Abebe et.al, 2003).

Clerodendrum myricoides has been found to contain the following constituents: cleromyrine I,II; myricoidine; and dihydromyricidine (Abebe et.al., 2003). Chromatographic separation of a methanolic extract of *Clerodendrum myricoides* has led to the isolation of myricoidine (Kiprono et.al., 2004).

In one study it has been found that using extracts of *Ekebergia capensis* and *C. myricoides* in combination with chloroquine was more effective than using the drug on its own (Kat , 2004; Ochieng, 2004 and Muregi et.al, 2004).

Methanolic extract of *Clerodendrum myricoides* as well as myricoidine have been subjected to larvicidal tests using the second instar larvae of *Anopheles gambiae* (malaria vector) at concentrations of 100, 75, 50, and 25 ppm. The crude methanolic extract of *Clerodendrum myricoides* and the isolated component (myricoidine) were also found to be active against *Anopheles gambiae* with LD₅₀ values of 14 and 9 ppm, respectively (Kiprono et.al., 2004).

The methanolic extract of roots of *Clerodendrum myricoides* were observed to have parasitaemia suppression effect (Abebe et.al., 2003).

1.9. Drug discovery approaches

In this era of environmental change, resurgent disease and multidrug resistance, the need to explore alternative and novel control strategies is urgent (Whitten et.al.,2006)

To combat malaria, new drugs are desperately needed, but traditional mechanisms for drug development have provided few drugs to treat diseases of the developing world. In this challenging situation, there are some reasons for optimism. First, the determination of the genome sequence of *P. falciparum* offers a multitude of potential drug targets. Second, advances in malaria genetics offer improved means of characterizing potential

targets. Third, the recent increased participation of pharmaceutical companies in the antimalarial drug discovery and development process offers hope for the development of new, affordable drugs. Many malaria discovery and development projects are now underway, involving many organizations including the Medicines for Malaria Venture (David et.al. , 2004).

MMV, established in 1999 in Geneva, was the first public-private partnership of its kind to tackle a major global disease. This expert, not-for-profit organization brings together public, private and philanthropic partners to fund and manage the discovery, development and delivery of affordable new medicines for the treatment and prevention of malaria in disease-endemic countries. MMV aims to develop one new antimalarial drug every five years (David, 2004). It is cultivating new classes of drugs, alone and in combination, as well as existing drugs, such as the artemisinin derivatives (Jennifer, 2003).

1.9.1. Malaria genome and Proteome

Scientists has determined malaria genome and proteome in which the following advantages could be harvested.

Possible harvestings from the genome:-

- The genome will help to understand the *P. falciparum* biology more clearly as well as it will provide a firm foundation for starting new research (Poonam, 2003).
- With the help of the malaria genome data, various biochemical pathways important for the survival of the malaria parasite can be targeted for drug development.
- With the help of genome data, genes encoding transporter proteins as well as proteins themselves can be exploited as drug targets (Poonam, 2003).
- The *P. falciparum* genome will enhance the vaccine development by the detection of the potential antigens, that could be screened for derived characteristics like surface expression or limited antigenic diversity by stage-specific gene expression and proteomic analysis (Poonam, 2003).
- Comparative genomics: Comparative studies are very useful for the annotation of any unannotated genome. The genome sequence of *P. falciparum* will provide a means to the other ongoing sequencing projects of the other species of Plasmodium for their annotation (Poonam, 2003).

●Diagnostics: Nowadays a number of diagnostic approaches are applied of which PCR based approaches have special attention (Poonam, 2003).

Plasmo DB is one of the official databases of the malaria parasite genome project and contains the finished genome for CQ-sensitive *P. falciparum* strain and its official annotation as provided by the members of the genome sequencing consortium (Iwei et.al, 2004)).

Comparative proteomics from sporozoite, merozoite, trophozoite and gametocyte stages showed that they contained 1,049, 839, 1,036 and 1,147 proteins respectively. Only 152 proteins, which constitute 6% of the total proteome, were found to be common to all stages (Poonam, 2003).

On one hand the proteome of the *P. falciparum* will facilitate the discovery of novel subunit vaccines composed of purified proteins and polysaccharide antigens, on the other hand the genomic sequence will facilitate the discovery of new DNA vaccines in which an antigen coding gene is inserted into suitable expression vector (plasmid) and the purified recombinant vector encoding the immunogen is injected into the host (Poonam, 2003).

1.9.2. Rational drug design and new drugs

In addition to drug resistance, the mechanisms of antimalarial activity of several of these drugs are unknown or not very well understood which complicates efforts to design effective derivatives of these drugs. Knowledge-based drug design is needed more than ever in the fight against malaria to introduce new drugs with known mechanisms of actions. Structure-based approaches to malaria drug discovery to include high throughput screening, virtual and computer-aided design, and synthesis of chemical libraries, are currently being applied to develop the next generation of effective antimalarials. In this age of significant drug resistance, these approaches provide an opportunity to introduce novel chemical entities into the malaria drug development pipeline (Norman C, 2005). Rational drug discovery and computer-aided design are emerging as valuable means to develop new medicines. The explosion in the number of possible drug targets arising

from, in part, genomics, gave the move towards rational drug discovery and design (Ivan, 2004).

Many new drugs are emerging as a result of progress in genomic and proteomic analysis. To date, several classes of compounds including quinolines and oxindoles have been identified as selective inhibitors of the plasmodial CDK7 homologue, Pfmrk. From iterative rational drug design process, more potent, selective, and most importantly, chemically unique compound classes have been identified as effective inhibitors of the plasmodial CDKs and the malarial parasite (Susan , 2005).

The malaria parasite, *Plasmodium falciparum*, spends part of its complex life cycle within the red blood cells of a human host. During this time, the parasite alters the permeability of the red blood cell's plasma membrane to allow the uptake of nutrients, the removal of waste and volume and ion regulation of the infected cell. The increased permeability is due to the induction of new permeability pathways (NPP), which are obvious chemotherapeutic antimalarial targets and/or selective routes for drugs, which target the internal parasite (Henry, 2005).

Falcipains (FP) of *P.falciparum* are important virulence factors marked as potential targets for antimalarial drug discovery (Lihuh, and Tiow;2007).Nitric oxide (NO) is a potent regulatory molecule possessing an antiparasitic activity. It inhibit catalytic activity of falcipain, the papain-like cysteine protease involved in *P.falciparum* trophozoite hemoglobin degradation. In particular, NO donors S-nitrosoglutathione , (±)-(E)-4-ethyl-2-[(E)-hydroxyimino]-5-nitro-3-hexenamide , 3-morpholinosydnonimine , and sodium nitroprusside inhibit dose-dependently the falcipain activity present in the *P. falciparum* trophozoite extract, this effect likely attributable to S-nitrosylation of the Cys25 catalytic residue (Giorgio, 2000).

Fatty acid biosynthesis, one of the apicoplast metabolic pathways, has proven to be one of the most attractive for drug discovery. This pathway is the target of several classes of antimicrobial compounds, some of which have antimalarial activity. Recent experiments with these antimicrobial compounds paved the way for drug discovery projects focused on several targets for instance the PfKASI/II enzyme may prove to be an important drug target in malaria. Thiolactomycin and many of its analogues have been shown to inhibit the growth of *P.falciparum* cultured in human erythrocytes (Jeff et.al, 2005).

Members of the methionine aminopeptidase family has been investigated as potential antimalarial targets. The *Plasmodium falciparum* methionine aminopeptidase 1b (PfMetAP1b), one of four MetAP proteins encoded in the *P. falciparum* genome, was cloned, overexpressed, purified, and used to screen a 175,000-compound library for inhibitors. A family of structurally related inhibitors containing a 2-(2-pyridinyl)-pyrimidine core was identified. Structure-activity studies led to the identification of a potent PfMetAP1b inhibitor, XC11, it was highly selective for PfMetAP1b and did not exhibit significant cytotoxicity against primary human fibroblasts. Most importantly, XC11 inhibited the proliferation of *P.falciparum* strains [chloroquine (CQ)-sensitive] and (multidrug-resistant) in vitro and is active in mouse malaria models for both CQ-sensitive and CQ-resistant strains. These results suggest that PfMetAP1b is a promising target and XC11 is an important lead compound for the development of novel antimalarial drugs (Xiaochun et.al., 2006).

In addition to specific targets, metabolic pathways can also be targeted to kill the malaria parasite. Three generations of compounds, (bis-quaternary salts, bis-amidines, and bis-thiazolium salts), were synthesized to target phosphatidylcholine biosynthesis and heme detoxification pathways in the parasite. These compounds have potent activity against malaria parasites in culture and in animal models (Norman, 2005).

A recombinant viral vaccine, NYVAC Pf-7 (*P falciparum*-7), has been developed that encodes seven antigens from various life-cycle stages. Results of a sporozoite challenge study of NYVAC Pf-7 showed encouraging delays in time to parasitaemia, and some antibody and cytotoxic T-lymphocyte immunogenicity, but this candidate has not been further developed. An anti-invasion vaccine based on Merozoite surface protein (MSP-1)

known as falciparum malaria protein (FMP-1) is being clinically assessed and has progressed quickly to an adult phase-I study in western Kenya (Vasee et.al., 2004).

Two blood-stage candidates, glutamate rich protein (GLURP) and Merozoite surface protein-3 (MSP3), have been clinically assessed in Europe (Vasee et.al.,2004).

1.9.3. Model for screening antimalarial compounds

1.9.3.1. *In vitro* method for screening of antimalarial compounds

In vitro screens for activity, constitute a key component for antimalarial drug screening. It is based on the ability to culture *P.falciparum* in human erythrocytes *in vitro*. The development of techniques for continuous cultivation of *P. falciparum* is a reliable source, for continuous stock culture of parasite, apart from drug screening and long term assessment. Culture of *P. falciparum* is now being used to study the mode of entry of parasite into erythrocytes, screening of new drugs, to isolate and characterize strains and clones, to identify immunogenic antigens and genome of parasite. There are many *in vitro* methods useful under different circumstances (Kalra et.al, 2006).

Among the many *in vitro* methods 3sub H Hypoxanthine uptake, Giemsa stained slide method, flow cytometry, LDH activity measurement, Isobologram analysis and Micro-test (David et.al., 2004, Kalra et.al, 2006).

Advantages of *in vitro* methods are they are precise and efficient, rapid, large number of compounds can be evaluated at the same time, synergism or antagonism with drug combinations can be studied, better assessment of intrinsic activity of a drug. Limitations of *in vitro* methods are drugs acting through active metabolite cannot be studied, non reproducibility of pharmacokinetic effects, toxic compounds also get selected, expertise and infrastructure needed, lack of clinical correlation (Kalra et.al, 2006).

1.9.3.2. *In vivo* method for screening of antimalarial compounds

In vivo methods for screening antimalarial compounds:-Compounds effective in *in vitro* screening tests (i.e. those with $IC_{50} < 1 \mu M$) are taken up for *in vivo* evaluation. Plasmodium species that cause human disease are essentially unable to infect non primate animal models (Kalra et.al, 2006).

There are different models for *in vivo* evaluation of antimalarial compounds rodent model, avian models, primate models are some of them (David et.al., 2004, Kalra et.al, 2006).

The choice of malaria model depends upon sensitivity, reproducibility and breadth of response to known antimalarial drug and also on practical consideration such as required rate of testing, technical complexity, quantity of test compound needed and cost per test. Host factors such as natural resistance and immune competence also influence the efficiency of test compounds in different models. Each malaria model system has its individual characteristics, and no single model exists which can be said to be entirely predictive for humans (Kalra et.al, 2006).

1.10. Rationale for the study

The delay to change to new antimalarials as first-line therapy in Africa is constrained by the lack of alternative antimalarials (Fred, 2001).

The developing world will not, in the long term, be able to repay the enormous investment made in developing even a single new drug, and certainly does not itself have the resources to carry out such research (Fred, 2001). Moreover, most of the research on the development of antimalarials is carried out by high-income countries, who have an interest in malaria because of tourists visiting malaria-endemic areas. Since the majority of malaria cases occur in low-income countries, there is very little profit incentive for pharmaceutical companies to invest in antimalarials. Besides, there is no guarantee that even if a new antimalarial drug were developed in the high-income countries, it would be available at an affordable cost for the majority of malaria sufferers in Africa (Fred, 2001).

Thus searching of antimalarial drugs by Africans is very urgent nowadays especially in poor countries like Ethiopia where the problem exists and many traditionally used plants are available. In this study, the pharmacological activity and phytochemical screening of

the root extracts from the traditionally used plant *Clerodendrum myricoides* has been studied.

2. Objectives

2.1. General objective

To evaluate antimalarial activity and determine LD_{50} of the root extracts of *Clerodendrum myricoides* in mice infected with *Plasmodium berghei* .

2.2. Specific objectives

- ❖ Phytochemical screening of *Clerodendrum myricoides*
Using *Plasmodium berghei*:
- ❖ To determine the percentage of parasite inhibition.
- ❖ To evaluate the effect on body weight
- ❖ To evaluate the effect on packed cell volume
- ❖ To evaluate the effect on body temperature
- ❖ To evaluate the effect on mean survival time of *Plasmodium berghei* infected mice.
- ❖ To estimate the LD_{50} s of the plant extracts

3. Materials and Methods

3.1. Materials

3.1.1 Plant materials

Roots of *Clerodendrum myricoides* were collected in January 2007 from Debrebirhan, about 130km North of Addis Ababa, where it is used traditionally for treatment of malaria. Specimens of the plant was identified and deposited at the National Herbarium, Department of Biology, Addis Ababa University, with Voucher specimen Number (002/TIG/PHAR).

3.1.2. Animals

Adult male Swiss albino mice (25- 32g) obtained from EHNRI.

3.1.3. Parasites

Chloroquine- sensitive *Plasmodium berghei* was obtained from EHNRI and was used for the experiment.

3.1.4. Chemicals

Chloroquine phosphate(Addis pharmaceuticals/Ethiopia), Absolute methanol(Fisher Scientific UK limited/UK), n-butanol(E.Merck /Germany),dichloromethane(Sigma Aldrich /Germany), chloroform(Sigma Aldrich /Germany) , hexane(Fisher Scientific UK limited/UK), Antimony chloride(E.Merck /Germany), acetic anhydride(Bio-lab laboratories limited/Israel), picric acid(E.Merck /Germany), lead acetate(Narkarai Chemicals Ltd/China), Antimony chloride(E.Merck /Germany), 3,5 dinitro benzoic acid(Riedal de Haen/Germany), Dragendroffs reagent(bismuth subnitrate-May and Baker Limited/ England; Potassium iodide-Evans medical limited/Liverpool),Mayers reagent(Mercuric chloride-Riedal de haen-Germany), tannin solution(British drug houses/England) ,Ferric chloride(Fisher Scientific Company/USA), sodium carbonate

(The british drug houses/England), potassium hydroxide(Riedal de Haen/Germany), hydrogen peroxide(Labort fine chemie pvt.ltd/India), acetic acid (Pharmitalia carlo/Italy), sulphuric acid(Farmitalia carlo erba/Italy), Ferric sulphate(Trolabo/), sodium hydroxide (Riedal de Haen/Germany), potassium hexacyanoferrate (Fluka AG/ Switherland), ammonia (Labort fine chemie pvt.ltd/India) and hydrochloric acid (Riedal de Haen/Germany).

3.2. Methods

3.2.1. Plant material preparation

Roots of *Clerodendrum* was garbled, dried at an ambient temperature (23 °C -26 °C), and protected from light. The plant material was then ground to powder using a laboratory mill and kept in amber glass bottle till extracted with different solvents.

3.2.1.1. Preparation of aqueous crude extract

Three hundred grams of the powdered roots of *C.myricoides* were soaked in 1500ml distilled water in the first day and shaken using electric shaker for 1 hour. The suspension was then filtered through gauze (0.1mm² mesh) on the second day since the yield has not increased by the third day, and 1000ml water was added to the mark and filtered again. The combined filtrate was then freeze dried or lyophilized with a lyophilizer (Labconco T^o- 47°C, Pressure-346x10⁻³) to give powder (yield =4.66%) that was collected in amber glass bottle and kept in a desiccator until used for experiment.

3.2.1. 2. Preparation of hydroalcoholic crude extract

Two hundred grams of the powdered roots of *C.myricoides* macerated with 1000ml of 80% methanol for 72 hours at room temperature. Then the extract was filtered through Watmann filter paper No.1(18cm) and the marc was remacerated twice using 500ml of methanol each time for exhaustive extraction. The methanol was removed from the extract by evaporation under vacuum in a rotavapour (Buchi at 40°C and RPM 60) (yield=18%) and kept in amber glass bottle in a refrigerator till used.

3.2.1. 3. Fractionation of crude aqueous extract

Aqueous crude extract (13.75 grams) was allowed to dissolve in 750ml of water. Then the suspension was shaken in a separatory funnel by adding 50 ml of dichloromethane repeatedly. This was done 4 times to get the dichloromethane fraction and the aqueous residue. The dichloromethane fraction was then concentrated in rotary vaporizer (Buchi at 40°C and RPM 60) (yield= 4.34%).The residue was then shaken with 50 ml of n-butanol each time 3 times to obtain n-butanol fraction and aqueous residue. The n-butanol fraction was treated similarly as dichloromethane fraction (Yield= 10.9%).The aqueous residue was also then freeze dried or lyophilized in a lyophilizer (Labconco T°- 47°C, Pressure- 346×10^{-3}) to give powder(Yield=49.45%).The fractions were then kept in an amber glass bottle.

3.2.1. 4. Fractionation of crude hydroalcoholic extract

Hydroalcoholic crude extract(23.5grams) was allowed to dissolve in 200ml of water .Then the suspension was shaken in a separatory funnel by adding 50ml of chloroform each time 3 times, and the chloroform fraction was then concentrated in rotary vaporizer(Buchi at 40°C and RPM 60) (Yield=9.23%) and collected in an amber coloured glass vial. The aqueous residue was then shaken with 50 ml of n-butanol 3 times to obtain n-butanol fraction. The n-butanol fraction was treated similarly as chloroform fraction (Yield=17.9%) and aqueous residue. The aqueous residue was also then freeze dried or lyophilized in lyophilizer (Labconco T°- 47°C, Pressure- 346×10^{-3}) to give powder (Yield=24.68%). The fractionations were then kept in an amber glass bottle.

3.2.1. 5. Animal preparation

Adult male Swiss albino mice (25- 32g), Age (2months)were obtained from EHNRI. The animals were kept in cages and housed in a standard animal house under natural 12/12h light dark cycle at room temperature. They were maintained on standard pelleted diet and water *ad libitum*. Before the experiment was started, they were all acclimatized to the test

environment for 10 hours. The animals were then randomly assigned to the control and experimental groups.

3.2.2. Phytochemical screening

Standard tests were employed to detect the major secondary metabolites such as polyphenols, cyanogenic glycosides, saponins, anthranides, phytosteroides & withanides carotenoides, phenolic glycosides, free anthraquinones, flavonoides, cardiac glycosides, tannins, alkaloids and o-anthraquinone glycosides (Asfaw, 2002).

3.2.3. Pharmacologic screening

A method described by Knight and Peters was used for the *in vivo* anti-malarial activity testing on root extracts of *Clerodendrum myricoides* (Okokon et.al., 2005).

Donor *Plasmodium berghei* infected mice (parasitemia of 20%) were killed by head blows. Blood was then collected in slightly heparinized syringe from auxillary vessels. The collected blood was diluted with Trisodium citrate medium in such a way that each 0.2ml contains approximately 1×10^7 infected red cells. Mice were then divided in to ten groups of six each; eight groups of mice received the crude extracts or fractions, while the other two groups were used as positive and negative control.

On day 0 (before starting administering the test substance and standard), each mouse was inoculated intraperitoneally, with 0.2 ml of infected blood containing about 1×10^7 parasitized red blood cells, which was expected to produce steadily rising consistent infection of the required intensity in mice. From pilot study performed, the doses to be given for mice in the group receiving the crude extracts have been determined to be 100mg, 200mg and 400mg while for those groups of mice receiving the fractions 25mg, 50mg and 100mg.

The mice in the test groups received the crude extracts or fractions of the plant extract, once daily for 4 days. One group received distilled water(vehicle) (negative control) and the other group received chloroquine phosphate 10mg/kg(positive control).All administrations were done by the oral route using oral needle, starting from 3 hours of parasite inoculation. The various parameters were then determined to evaluate the antimalarial activity.

3.2.3.1. Determination of parasitemia

On day 4 after infection, thin smears of blood films were taken from the peripheral blood of the tail of each mouse in the test and control groups. The smears on the microscopic slides were fixed with methanol and the blood films were then stained with Giemsa stain at pH 7.2 for 45 minutes and parasitized red blood cells were examined under the microscope. The microscope diaphragm was adjusted in such a way that 100 red blood cells could be counted in each field. The percentage parasitemia was determined by counting the number of parasitized blood cells out of 100 red blood cells in random fields of the microscope. Three different fields were examined on each slide and the number of infected and uninfected red blood cells were counted, and the mean parasitemia was calculated and expressed as follows (Peters ,1993):-

$$\text{Parasitemia} = \frac{\text{Total number of PRBC}}{\text{Total number of RBC}} \times 100\%$$

PRBC= Parasitized red blood cells RBC = Red blood cells

Percentage parasitemia suppression was calculated according to the following formula (Obih et.al, 1985).

$$\% \text{ Parasitemia suppression} = \frac{\text{Av\% Parasitemia in negative control group} - \text{Av\% parasitemia in study group}}{\text{Parasitemia in negative control group}} \times 100$$

Av=Average

3.2.3.2. Determination of body weight

The body weights of the mice were measured to observe whether the test extracts of *C.myricoides* roots part prevent body weight loss. The weights were taken on D-0(after infection is initiated) and D-4 (Dikasso et.al., 2006).

3.2.3.3. Determination of Packed Cell Volume

Packed Cell Volume (PCV) is a measure of the proportion of red blood cells to total blood volume, used in estimating the mean erythrocyte hemoglobin concentration. The PCV was measured to predict the effectiveness of the test extracts using the modified Wintrobe's method. Blood from the tail of the animals was drawn with haematocrit tubes of thick-walled capillary glass. Each was dipped in heparin solution so that the inner wall was wetted with anticoagulant. Blood from the tail of the animals was drawn up to a 100mm mark on the tube. The tube was then closed at both ends with an elastic band and centrifuged. Duplicate determinations were done by measuring the relative volume of the blood occupied by erythrocytes, the PCV was then determined in each case as follow:

$$\text{Packed Cell Volume} = \frac{\text{Volume of erythrocytes in a given volume of blood}}{\text{Total blood volume}}$$

This test was done just before infection (Day 0) and on day 4 after infection (Dikasso et.al., 2006).

3.2.3.4. Determination of mean survival time

Survival time was recorded to observe the effect of the extracts for improvement in survival days. The animals were fed *ad libitum* and observed for about 28 days. Any death that occurred during this period was noted to determine the mean survival time (Eulifoye and Agbedahunsi, 2004).

3.2.3.5. Measurement of body temperature

The daily measurement of rectal temperature was done to predict the effectiveness of the test extracts. It is theoretically accepted that the body temperature of mice decreased in a

rapid manner with increasing parasitemia in contrary to the situation in human beings. The efficacy of the test extracts were determined by observing the protective effect against the rapid fall in temperature. The study as well as the control animals were checked for a change in body temperature, by measuring the rectal temperature 3 times daily for six consecutive days starting from the day prior to parasite inoculation (Dikasso et.al., 2006).

3.2.4. Determination of LD₅₀

Six groups of mice weighing between 20-25gm (10 mice/ group) were acclimatized and fasted overnight. The weight of each mouse was taken and doses were calculated .The test mice received different doses of the extract by oral route, while the control mice received the vehicle. They were all observed for toxicity signs like change in physical appearance, behavioral change and lethality for 24hrs, and LD₅₀ were determined. Those survived were kept under observation up to 14 days after drug administration (Dikasso et.al., 2006). This test was done for the crude aqueous and hydroalcoholic extracts.

3.2.5. Data analysis

To analyze data obtained during the experiment statistical methods SPSS version 15 was used. ANOVA followed by Post Hoc LSD was used to test significance among and within group differences and independent Student's t-test were employed to test significance for the difference between initial and final results within the same group. All the data were analyzed at 95% confidence interval ($\alpha= 0.05$).

4. Results

4.1. Phytochemical screening

The phytochemical screening of the roots of *C.myricoides* showed the presence of secondary metabolites such as saponins, withanides & phytosteroides, polyphenols, flavonoides ,tannins and alkaloids (Table1).This was done to check the presence of different compounds some of them not done in other studies.

Table 1. Phytochemical screening of the roots of *C.myricoides*

Test for secondary metabolite	Observed Results
Chromophers	Yellow coloured filtrate
Polyphenols	Greenish blue coloured solution
Cyanogenic Glycosides	Yellow colour on the filter paper
Saponins	Honey comb froth formation
Anthranides	Cloudy solution
Phytosteroides and withanides	Reddish brown coloured solution
Carotenoides	Cloudy solution
Phenolic glycosides	Cloudy precipitate
Free anthraquinones	Orange coloured solution
Flavonoides	Yellow coloured solution
Cardiac glycosides	Keddle reagent- Yellow coloured precipitate Keller rxn- light yellow solution Salkowski- Brown colour solution

	Liberemann- Cloudy solution
Tannins	Light brown coloured solution
Alkaloides	Dragendroffs reagent -yellow orange precipitate. Mayers reagent –White precipitate Tannin solution(0.5%) - white precipitate
O-anthraquinone glycosides	Orange colour was formed in NH ₃ Phase

4.2. Effect of crude extracts and fractions of the roots of *C.myricoides* on parasitemia

The crude aqueous extract (P=0.000, 100mg; P=0.000, 200mg; P=0.000, 400mg) and the crude hydroalcoholic extract (P=0.000, 100mg; P=0.000, 200mg; P=0.000, 400mg) of the roots of *C.myricoides* significantly (P<0.05) inhibited parasitemia (Table 2) .

The dichloromethane fraction of crude aqueous extract (P=0.012, 25mg; P=0.003, 50mg; P=0.002, 100mg) and the butanol fraction of crude hydroalcoholic extract (P=0.018, 25mg; P=0.020, 50mg; P=0.014, 100mg) of the roots of *C.myricoides* significantly (P<0.05) inhibited parasitemia (Table 2).

The other fractions, i.e. butanol fraction of crude aqueous extract (P=0.796, 25mg; P=0.872, 50mg; P=0.650, 100mg), aqueous residue of both crude aqueous (P=0.664, 25mg; P=0.625, 50mg; P=0.665, 100mg) and hydroalcoholic extracts (P=0.406, 25mg; P=0.574, 50mg; P=0.309, 100mg) and chloroform fraction of crude hydroalcoholic extract (P=0.883, 25mg; P=0.781, 50mg; P=0.756, 100mg) did not inhibit parasitemia (Table 2).

The crude hydroalcoholic extract has comparable parasitemia inhibition to crude aqueous extract (P=0.680, the dichloromethane fraction of crude aqueous extract (P=0.246) and the butanol fraction of crude hydroalcoholic extract (P=0.536) at a dose of 100mg/kg.

4.3. Effect of crude extracts and fractions of the roots of *C.myricoides* on body weight

The crude aqueous (P=0.026, 100mg; P=0.073, 200mg; P=0.744, 400mg) and crude hydroalcoholic extracts (P=0.004, 100mg; P=0.655, 200mg; P=0.820, 400mg) of the roots of *C.myricoides* prevented weight loss especially at high doses, though no weight gain was observed in any of the cases (Table 3).

The dichloromethane fraction of aqueous extract (P=0.018, 25mg; P=0.202, 50mg; P=0.345, 100mg), butanol fraction both crude aqueous (P=0.051, 25mg; P=0.124, 50mg; P=0.207, 100mg), and hydroalcoholic extract (P=0.004, 25mg; P=0.139, 50mg; P=0.087, 100mg) also prevented weight loss.

The other fractions i.e. aqueous residue of both crude aqueous (P=0.917, 25mg; P=0.018, 50mg; P=0.000, 100mg) and hydroalcoholic extract (P=0.000, 25mg; P=0.000, 50mg; P=0.000, 100mg) and chloroform fraction of crude hydroalcoholic extract (P=0.016, 25mg; P=0.001, 50mg; P=0.009, 100mg), however, did not prevent body weight loss (Table 3).

The crude hydroalcoholic has not protected loss of body weight when compared to crude aqueous extract (P=0.000), dichloromethane fraction of crude aqueous extract (P=0.000) and the butanol fraction of crude hydroalcoholic extract (P=0.000) at a dose of 100mg/kg.

4.4. Effect of crude extracts and fractions of the roots of *C.myricoides* on packed cell volume

The crude aqueous (P=0.354, 100mg; P=0.411, 200mg; P=0.951, 400mg) and hydroalcoholic extracts (P=0.125, 100mg; P=0.297, 200mg; P=0.461, 400mg) of roots of *C.myricoides* prevented reduction in PCV (Table 4).

The dichloromethane fraction of aqueous extract (P=0.312, 25mg; P=0.385, 50mg; P=0.296, 100mg), butanol fraction of crude hydroalcoholic (P=0.306, 25mg; P=0.077, 50mg; P=0.147, 100mg) and chloroform fraction of crude hydroalcoholic extracts (P=0.136, 25mg; P=0.104, 50mg; P=0.131, 100mg) of roots of *C.myricoides* also prevented reduction in PCV (Table 4).

The other fractions i.e. butanol fraction of crude aqueous extract (P=0.009, 25mg; P=0.173, 50mg; P=0.027, 100mg), aqueous residue of both crude aqueous (P=0.004, 25mg; P=0.266, 50mg; P=0.006, 100mg) and hydroalcoholic extracts (P=0.171, 25mg; P=0.127, 50mg; P=0.023, 100mg) and did not show protection against reduction in PCV especially at higher doses (Table 4).

The crude hydroalcoholic extract has comparable prevention of reduction in PCV to crude aqueous extract (P=0.580), dichloromethane fraction of crude aqueous extract (P=0.824) and the butanol fraction of crude hydroalcoholic extract (P=0.837) at 100mg/kg.

4.5. Effect of crude extracts and fractions of the roots of *C.myricoides* on mean survival time

The crude aqueous (P=0.218, 100mg; P=0.603, 200mg; P=0.408, 400mg) and crude hydroalcoholic extract (P=0.118, 100mg; P=0.733, 200mg; P=0.610, 400mg) improved survival time (Table 5).

The dichloromethane fraction of crude aqueous extract (P=0.094, 25mg; P=0.047, 50mg; P=0.004, 100mg), improved survival time only at lower doses (Table 5).

The other fractions i.e. butanol fraction of hydroalcoholic extract (P=0.028, 25mg; P=0.007, 50mg; P=0.002, 100mg), aqueous residue of both crude aqueous (P=0.000, 25mg; P=0.000, 50mg; P=0.000, 100mg) and hydroalcoholic extracts (P=0.000, 25mg; P=0.000, 50mg; P=0.000, 100mg), butanol fraction of crude aqueous extract (P=0.000, 25mg; P=0.000, 50mg; P=0.000, 100mg) and chloroform fraction of crude hydroalcoholic extract (P=0.000, 25mg; P=0.000, 50mg; P=0.000, 100mg) of roots of *C.myricoides* did not improve survival time at any of the doses tested (Table 5).

The crude hydroalcoholic has comparable improvement in mean survival time to crude aqueous extract (P=0.425).

4.6. Effect of crude extracts and fractions of the roots of *C.myricoides* on body temperature

The dichloromethane fraction of crude aqueous extract (P=0.062, 25mg; P=0.291, 50mg; P=0.004, 100mg), aqueous residue of crude aqueous extract (P=0.147, 25mg; P=0.000, 50mg; P=0.000, 100mg) and butanol fraction of crude hydroalcoholic extract (P=0.074,

25mg; P=0.000, 50mg; P=0.000, 100mg) shows protective effect against temperature reduction only at lower doses (Table 6).

The crude aqueous (P=0.000, 100mg; P=0.014, 200mg; P=0.000, 400mg) and crude hydroalcoholic extract (P=0.000, 100mg; P=0.001, 200mg; P=0.000, 400mg). , butanol fraction of crude aqueous extract (P=0.004, 25mg; P=0.009, 50mg; P=0.004, 100mg) & aqueous residue of crude hydroalcoholic extract (P=0.000, 25mg; P=0.000, 50mg; P=0.000, 100mg) and chloroform fraction of crude hydroalcoholic extract (P=0.000, 25mg; P=0.000, 50mg; P=0.000, 100mg) did not have protective effects against temperature reduction (Table 6).

The crude hydroalcoholic has not prevented reduction in temperature when compared to crude aqueous extract (P=0.115), the dichloromethane fraction of crude aqueous extract (P=0.201) and the butanol fraction of crude hydroalcoholic extract (P=0.710).

4.7. LD₅₀ of the crude extracts

The oral LD₅₀ of the crude aqueous and hydroalcoholic extracts were 1134.42mg/kg and 1629.99mg/kg, respectively. The toxic manifestations observed for both extracts include muscle weakness and sedation.

Table 2. Effect of the roots of *C.myricoides* crude extracts and fractions on % parasitemia and % parasitemia inhibition

Test substances	Dose(mg/kg)	%Parasitemia	Parasitemia inhibition	P-value
1. Crude aqueous extract	100	28.75±3.59	36.22*	0.000
	200	21.92±5.95	51.37*	0.000
	400	21.70±2.18	51.86*	0.000
2. Crude hydroalcoholic extract	100	30.58±2.87	32.16*	0.000
	200	22.70±4.34	49.65*	0.000
	400	17.25±2.24	61.73*	0.000
3. Dichloromethane fraction of crude aqueous extract	25	38.00±3.88	15.70*	0.012
	50	36.42±2.01	19.21*	0.003
	100	35.75±3.37	20.69*	0.002
4. Butanol fraction of crude aqueous extract	25	44.00±7.84	2.22	0.796
	50	44.40±8.96	1.50	0.872
	100	43.17±4.16	4.23	0.650
5. Aqueous residue of crude aqueous extract	25	43.67±7.00	3.12	0.664
	50	43.50±2.88	3.50	0.625
	100	43.67±4.23	3.12	0.665
6. Butanol fraction of crude hydroalcoholic extract	25	33.83±3.43	24.95*	0.018
	50	34.00±5.92	24.57*	0.020
	100	33.33±11.69	26.06*	0.014
7. Chloroform fraction of crude hydroalcoholic extract	25	44.33±10.3	1.66	0.883
	50	43.67±6.84	3.12	0.781
	100	43.50±10.11	3.50	0.756
8. Aqueous residue of crude hydroalcoholic extract	25	41.50±5.47	7.94	0.406
	50	42.67±6.65	5.34	0.574
	100	40.67±9.60	9.78	0.309
9. Vehicle	—	45.08±6.88	0.00	
10. Chloroquine	10	0.00	100.00	

Day 0=Day infection initiated, Day4=Fifth day of infection, *P<0.05, each result is a mean of 6 mice and expressed as mean ± SD

Table 3. Effect of the roots of *C.myricoides* crude extracts and fractions on body weight

Test substances	Dose(mg/kg)	D-0(g)	D-4(g)%	Loss in Weight	P-value
1. Crude aqueous extract	100	28.50±0.91	25.27±2.50	11.33	0.026
	200	33.76±1.13	31.33±1.33	7.19*	0.073
	400	31.19±2.19	28.18±1.49	9.65*	0.744
2. Crude hydroalcoholic extract	100	36.54±2.16	33.79±2.92	7.50	0.004
	200	31.39±1.58	29.38±2.82	6.40*	0.655
	400	30.78±1.33	28.28±1.47	8.12*	0.820
3. Dichloromethane fraction of crude aqueous extract	25	27.61±2.33	24.43±1.61	11.5	0.018
	50	35.89±1.67	30.85±2.32	14.04*	0.202
	100	30.92±3.63	27.04±3.72	12.55*	0.345
4. Butanol fraction of crude aqueous extract	25	30.56±3.75	24.48±3.61	19.89*	0.051
	50	29.29±2.12	25.42±4.58	13.21*	0.124
	100	31.49±2.63	26.02±2.76	17.37*	0.207
5. Aqueous residue of crude aqueous extract	25	33.05±3.11	28.83±3.41	12.77*	0.917
	50	33.80±5.12	24.49±1.93	27.54	0.018
	100	27.27±2.10	21.76±2.17	20.20	0.000
6. Butanol fraction of crude hydroalcoholic extract	25	28.20±0.95	23.36±2.29	17.16	0.004
	50	32.12±1.93	26.11±2.49	18.71*	0.139
	100	30.88±2.08	25.69±3.11	16.80*	0.087
7. Chloroform fraction of crude hydroalcoholic extract	25	29.10±2.09	24.53±2.70	15.70	0.016
	50	28.43±1.25	22.85±1.61	19.63	0.001
	100	30.59±2.56	24.13±2.85	21.11	0.009
8. Aqueous residue of crude hydroalcoholic extract	25	26.87±1.06	22.08±1.47	17.83	0.000
	50	28.04±0.99	21.81±1.68	22.20	0.000
	100	27.23±1.47	21.19±1.29	22.18	0.000
9. Vehicle	—	34.20±3.94	27.90±3.59	18.4	
10. Chloroquine	10	28.13±2.46	28.65±2.73	+1.85	

Day 0=Day infection initiated, Day4=Fifth day of infection, * $P \geq 0.05$, each result is a mean of 6 mice and expressed as mean \pm SD

Table 4. Effect of the roots of *C.myricoides* crude extracts and fractions on PCV

Test substances	Dose(mg/kg)	D-0	D-4	% reduction	P-value
1. Crude aqueous extract	100	54.95±1.16	45.97±3.86	16.34*	0.354
	200	53.67±2.08	46.30±3.80	13.73*	0.411
	400	52.92±0.79	48.58±5.33	8.20*	0.951
2. Crude hydroalcoholic extract	100	52.92±2.25	44.30±3.53	16.28*	0.125
	200	53.05±1.73	45.77±3.86	17.72*	0.297
	400	54.46±2.28	50.87±4.21	6.59*	0.461
3. Dichloromethane fraction of crude aqueous extract	25	54.56±2.46	45.10±1.84	16.78*	0.312
	50	51.88±1.65	45.63±10.29	12.04*	0.385
	100	52.8±1.26	44.98±2.55	14.80*	0.296
4. Butanol fraction of crude aqueous extract	25	54.30±1.73	40.13±4.84	14.17	0.009
	50	53.42±1.32	44.50±4.58	16.69*	0.173
	100	52.5±3.64	41.60±4.59	20.76	0.027
5. Aqueous residue of crude aqueous extract	25	50.40±1.36	40.02±2.50	20.59	0.004
	50	52.65±0.72	45.65±3.47	13.29*	0.266
	100	48.98±2.04	40.48±4.57	17.35	0.006
6. Butanol fraction of crude hydroalcoholic extract	25	53.84±0.93	45.22±4.23	16.01*	0.306
	50	52.88±1.99	42.48±7.54	19.67*	0.077
	100	53.7±0.96	43.68±5.01	19.21*	0.147
7. Chloroform fraction of crude hydroalcoholic extract	25	49.94±1.41	42.33±9.76	15.23*	0.136
	50	48.01±2.28	41.70±6.38	13.14*	0.104
	100	51.58±2.58	42.23±7.29	18.13*	0.131
8. Aqueous residue of crude hydroalcoholic extract	25	54.14±2.18	44.43±4.26	17.93*	0.171
	50	52.58±2.90	43.92±3.32	16.47*	0.127
	100	51.56±2.72	41.32±6.25	19.56	0.023
9. Vehicle	—	50.96±1.41	41.57±3.97	18.40	
10. Chloroquine	10	53.93±0.97	48.77±7.60	9.56	

Day 0=Day infection initiated, Day4=Fifth day of infection, *P≥0.05, each result is a mean of 6 mice and expressed as mean± SD

Table 5. Effect of the roots of *C.myricoides* crude extracts and fractions on survival time

Test substances	Dose(mg/kg)	Survival time(days)	P-value
1. Crude aqueous extract	100	11.00±2.83*	0.218
	200	12.17±4.36*	0.603
	400	11.67±2.66*	0.408
2. Crude hydroalcoholic extract	100	11.67±1.75*	0.118
	200	12.57±2.64*	0.733
	400	12.50±0.52*	0.610
3 Dichloromethane fraction of crude aqueous extract	25	10.50±4.46*	0.094
	50	10.00±2.61*	0.047
	100	8.50±0.84	0.004
4. Butanol fraction of crude aqueous extract	25	9.50±0.83	0.000
	50	9.17±0.41	0.000
	100	9.17±0.41	0.000
5. Aqueous residue of crude aqueous extract	25	7.67±0.52	0.000
	50	8.83±0.98	0.000
	100	9.50 ±1.38	0.000
6. Butanol fraction of crude hydroalcoholic extract	25	10.5±2.43	0.028
	50	9.83±2.23	0.007
	100	9.33±1.63	0.002
7. Chloroform fraction of crude hydroalcoholic extract	25	7.83±0.41	0.000
	50	8.00±1.10	0.000
	100	7.67±0.52	0.000
8. Aqueous residue of crude aqueous extract	25	8.83±0.98	0.000
	50	8.5±0.55	0.000
	100	8.33 ±0.52	0.000
9. Vehicle		9.00±1.26	
10. Chloroquine	10	13.0±1.41	

Day 0=Day infection initiated, Day4=Fifth day of infection, * $P \geq 0.05$, each result is a mean of 6 mice and expressed as mean± SD

Table 6. Effect of the roots of *C.myricoides* crude extracts and fractions on rectal temperature

Test substances	Dose(mg/kg)	Temperature(^o C)	P-value
1. Crude aqueous extract	100	33.95±1.93	0.000
	200	36.22 ±0.87	0.014
	400	34.72±1.26	0.000
2. Crude hydroalcoholic extract	100	35.17±1.57	0.000
	200	35.93±1.10	0.001
	400	35.22±0.76	0.000
3 Dichloromethane fraction of crude aqueous extract	25	36.87±0.88*	0.062
	50	37.37± 1.14*	0.291
	100	36.15± 1.46	0.004
4. Butanol fraction of crude aqueous extract	25	36.32± 0.88	0.004
	50	36.52± 1.16	0.009
	100	36.33±1.11	0.004
5. Aqueous residue of crude aqueous extract	25	37.08± 0.83*	0.147
	50	35.17± 1.22	0.000
	100	33.47± 1.60	0.000
6. Butanol fraction of crude hydroalcoholic extract	25	36.98±1.03*	0.074
	50	35.78±0.89	0.000
	100	35.45± 1.33	0.000
7. Chloroform fraction of crude hydroalcoholic extract	25	35.22±1.13	0.000
	50	34.62± 1.73	0.000
	100	34.85± 1.13	0.000
8. Aqueous residue of crude aqueous extract	25	34.72±1.46	0.000
	50	35.17±1.39	0.000
	100	33.47± 1.19	0.000
9. Vehicle	—	34.33 ±0.71	
10. Chloroquine	10	37.98 ±0.43	

Day 0=Day infection initiated, Day4=Fifth day of infection, P≥0.05 each result is a mean of 6 mice and expressed as mean± SD

Figure 3. Effect of crude aqueous extract of the roots of *C.myricoides* on rectal temperature ($^{\circ}\text{C}$).

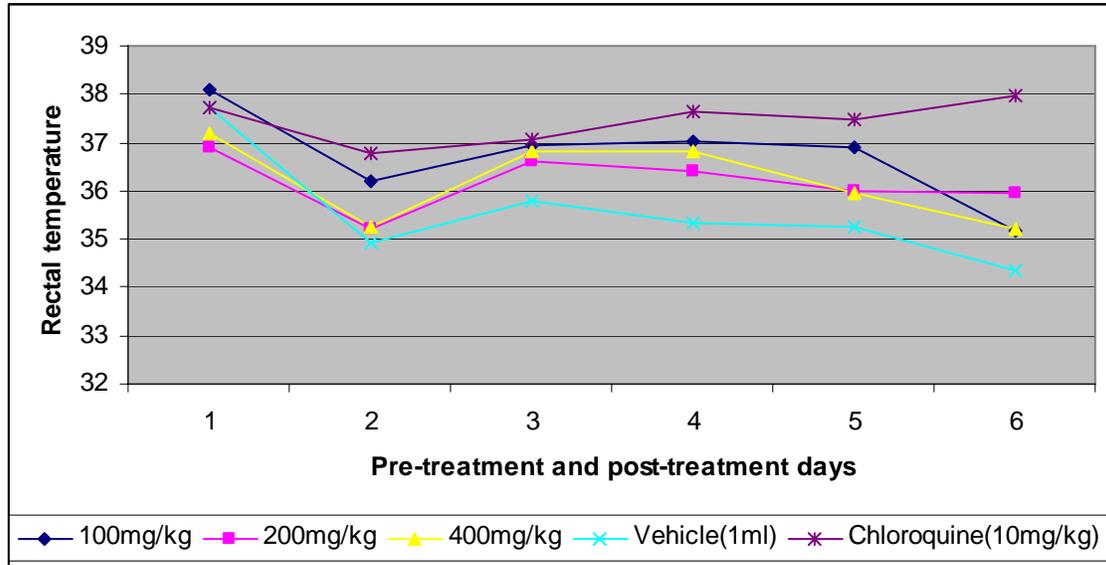


Figure 4. Effect of crude hydroalcoholic extract of the roots of *C.myricoides* on rectal temperature ($^{\circ}\text{C}$).

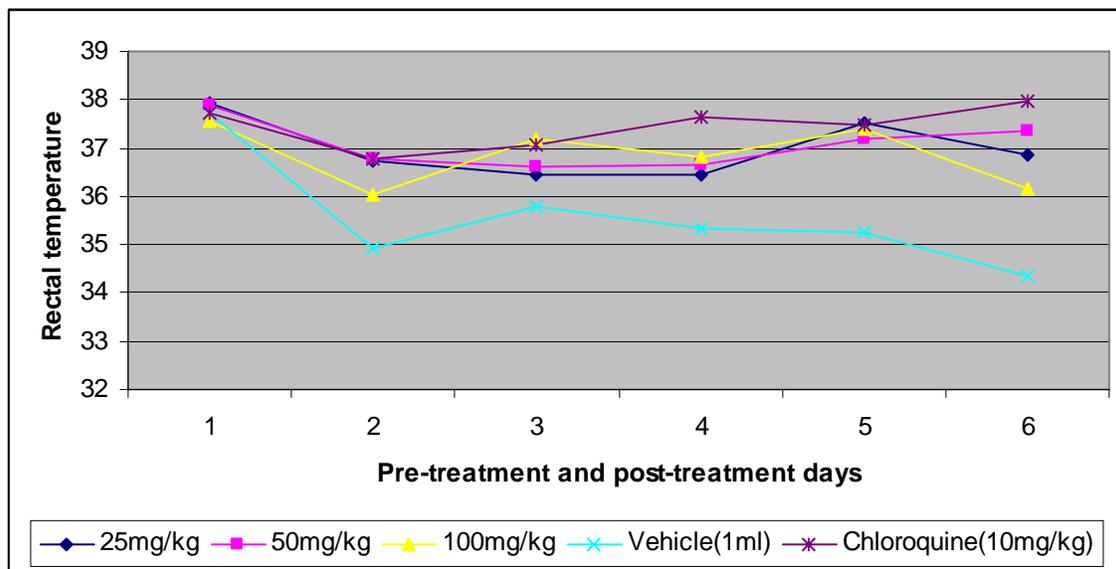


Figure 5. Effect of dichloromethane fraction of aqueous extract of the roots of *C.myricoides* on rectal temperature ($^{\circ}\text{C}$).

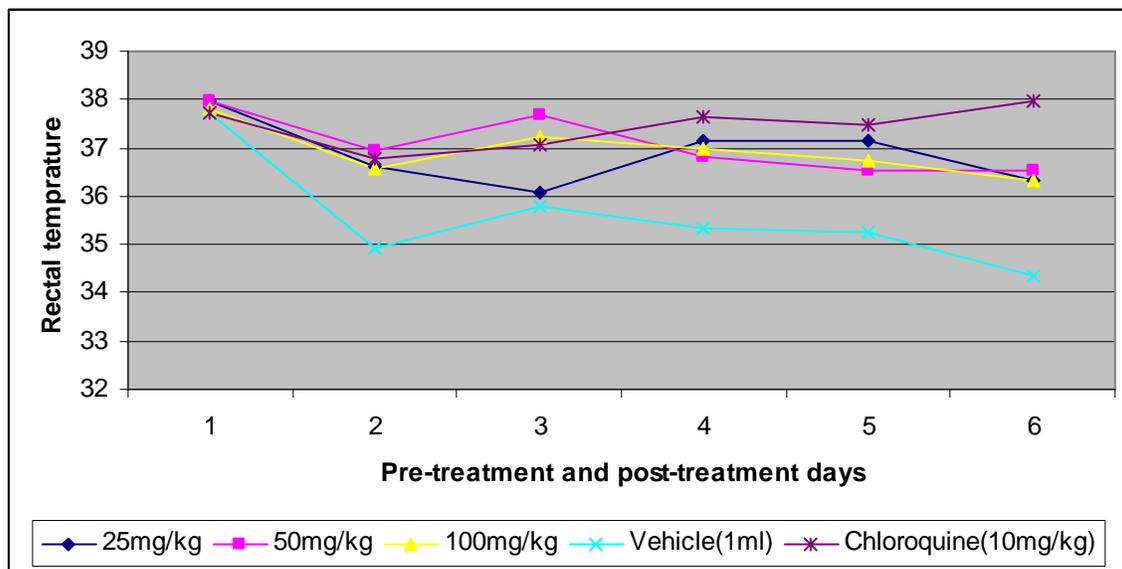


Figure 6. Effect of butanol fraction of aqueous extract of the roots of *C.myricoides* on rectal temperature ($^{\circ}$ C) .

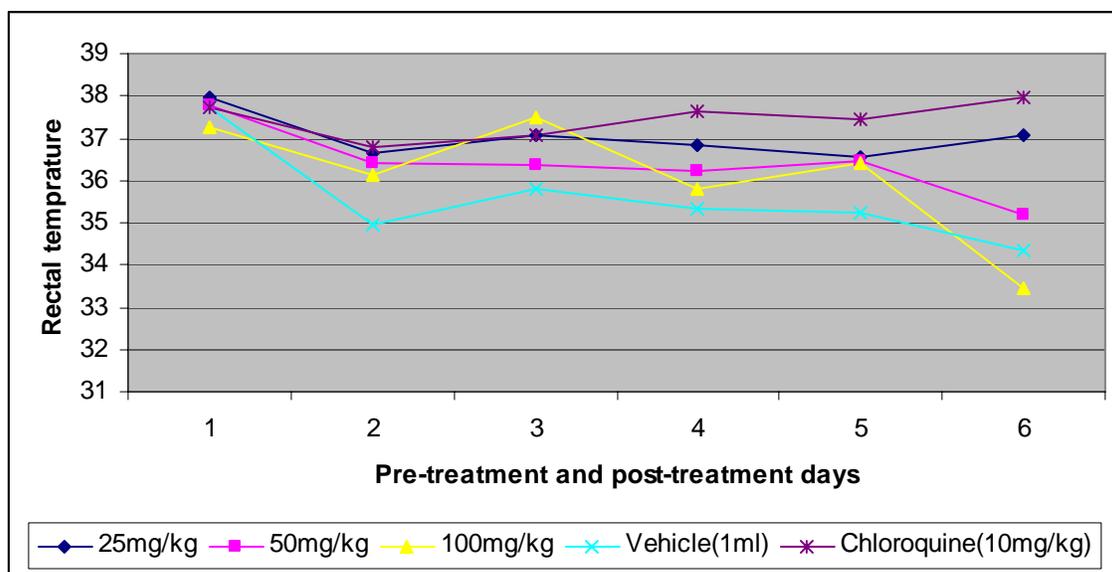


Figure 7. Effect of aqueous residue of aqueous extract of the roots of *C.myricoides* on rectal temperature ($^{\circ}$ C) of *P.berghei* infected mice .

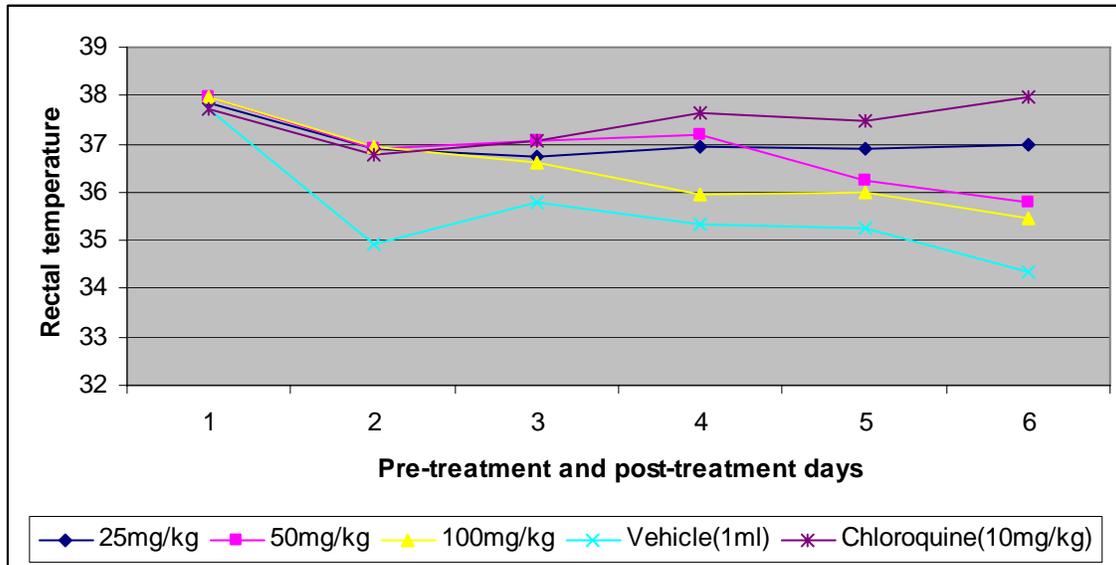


Figure 8. Effect of butanol fraction of hydroalcoholic extract of the roots of *C.myricoides* on rectal temperature ($^{\circ}$ C).

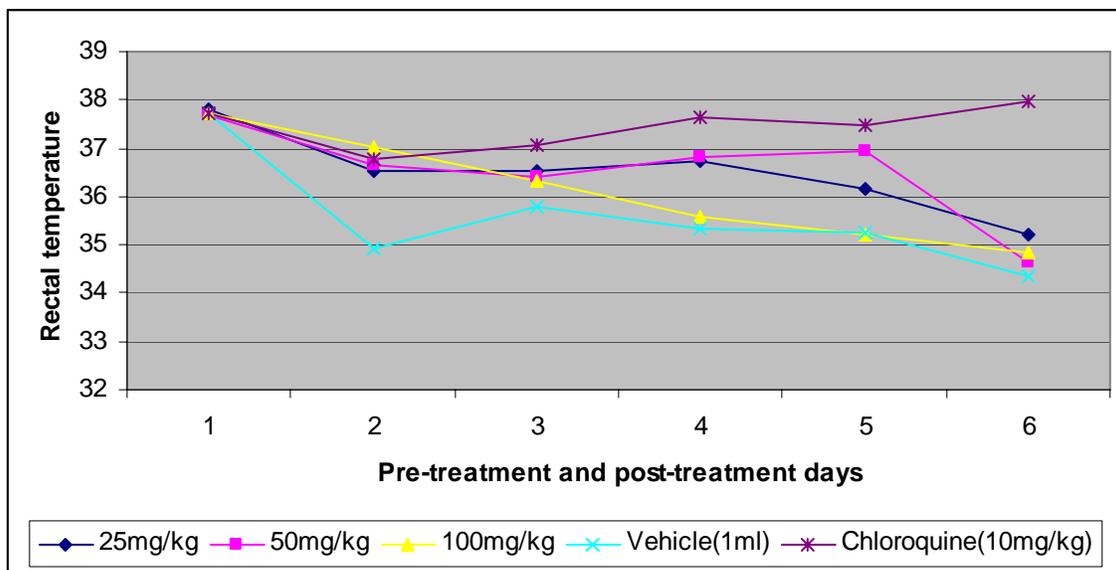


Figure 9. Effect of chloroform fraction of hydroalcoholic extract of the roots of *C.myricoides* on rectal temperature ($^{\circ}$ C).

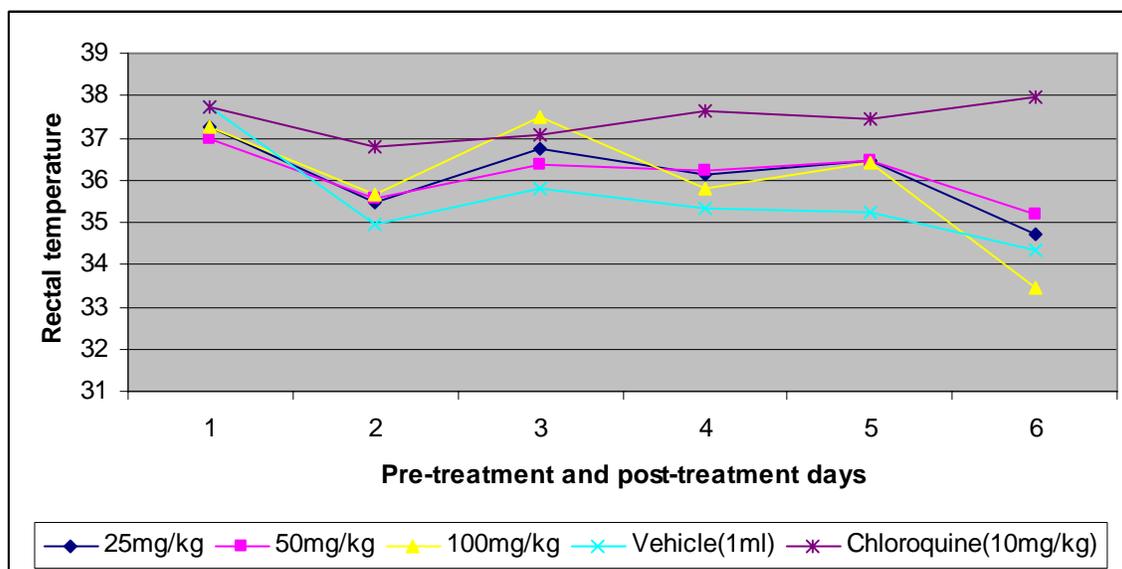


Figure 10. Effect of aqueous residue of hydroalcoholic extract of the roots of *C.myricoides* on rectal temperature ($^{\circ}\text{C}$).

5. Discussion

Sourcing of artemisinin from *Artemisia annua* has encouraged malaria phytotherapists to revisit medicinal plants frequently used in the traditional management of the disease (Elufioye and Agbedahunsi, 2004).

Antimalarials are drugs which can be used for prevention and clinical cure of infection, and interrupt transmission of plasmodia from mosquito to human. In this study chloroquine was used as a positive control since it has rapid acting blood schizontocidal activity (Sean and Sweetman, 2005) and *P.berghei* is sensitive to it (Peters, 1998).

P.berghei infected mice are used for better prediction of antimalarial efficacy of drugs for human use in many studies in search of antimalarial drugs.. The 4-day suppressive test is the standard test commonly used for antimalarial screening (Peters, 1998).

In other study, Cleromyrine I, II; myricoidine; and dihydromyricidine, have been found in *Clerodendrum myricoides* (Abebe et.al., 2003).

The hydroalcoholic crude extract likely to contain flavonoids , whitanides and phytosterols while polypenols , saponins ,tannins and alkaloids are likely to be present in the crude aqueous extract (Asfaw,2002).Thus the synergistic activity observed in the crude extracts and the activity in the fractions may be due to these compounds. The presence of saponins and alkaloids in the plant may also be responsible for the antimalarial activity observed as compounds belonging to this group have been reported to exhibit antimalarial activity (Marjorie, 1999; Mesia et.al., 2005).

The parasitemia suppression with the crude aqueous extract, crude hydroalcoholic extract dichloromethane fraction of crude aqueous extract, butanol fraction of crude hydroalcoholic extracts of *C.myricoides* on infected mice was comparable to that observed with *Solanum indicum* leaves (Dikasso et.al., 2006).

The fractions, i.e. butanol fraction of crude aqueous extract, aqueous residue of both crude aqueous and hydroalcoholic extracts and chloroform fraction of crude hydroalcoholic extract , did not inhibit parasitemia.

The crude aqueous and hydroalcoholic extract , the dichloromethane fraction of aqueous extract, butanol fraction both crude aqueous and hydroalcoholic extract prevented the weight loss. The same was also observed for plants like *Withania somnifera* (Dikasso et.al., 2006). The protective effect of the extracts aganist body weight loss of the infected mice is also in agreement with other studies (Dikasso et.al., 2006). The inconsistency of the results might be due to variation in nutrient content (Dikasso et.al., 2006). No weight gain was observed in any of the cases which may be due to the presence of compounds with appetite suppressing effect.

The parasites caused destruction of red blood cells leading to reduction in PCV. The crude aqueous, crude hydroalcoholic, dichloromethane fraction of aqueous extract , butanol

fraction of crude hydroalcoholic extract and chloroform fraction of crude hydroalcoholic extracts prevented reduction in PCV. The prevention in reduction of packed cell volume may be due to the compounds in the extracts. Our results on PCV is inconsistent with those of the previous studies (Dikasso et.al., 2006).

The fractions, i.e. butanol fraction of crude aqueous extract, aqueous residue of both crude aqueous and hydroalcoholic extracts did not show protection against reduction in PCV especially at higher doses .

As can be seen from the result, the crude aqueous and crude hydroalcoholic extract improved mean survival time and the dichloromethane fraction of crude aqueous extracts improved mean survival time only at lower doses.

The fractions, i.e. butanol fraction of hydroalcoholic extract, aqueous residue of both crude aqueous and hydroalcoholic extracts , butanol fraction of crude aqueous extract and chloroform fraction of crude hydroalcoholic extract of roots of *C.myricoides* did not improve the mean survival time . This may be due to the fact that compounds responsible for the antimalarial activity are faster acting, rapidly metabolized and the toxic components in the extracts may accumulate during repeated dosage.

As shown in the result, the crude aqueous and crude hydroalcoholic extract, butanol fraction of crude aqueous extract & aqueous residue of crude hydroalcoholic extract and chloroform fraction of crude hydroalcoholic extracts of *C.myricoides* did not have protective effects against temperature reduction which is in agreement with other studies (Dikasso et.al., 2006).

The dichloromethane fraction of crude aqueous extract, aqueous residue of crude aqueous extract and butanol fraction of crude hydroalcoholic extract shows protective effect against temperature reduction only at lower doses.

Generally, from this study it can be observed that the crude aqueous extract, crude hydroalcoholic extract, dichloromethane fraction of crude aqueous extract and butanol fraction of crude hydroalcoholic extract has reduced parasitemia , prevents loss of body weight, protects against PCV reduction and improved mean survival time . Chloroquine

(10mg/kg) has shown 100% parasitemia inhibition and all other parameters measured in mice returned to normal (Kiseko et.al , 2000).

In this study, the crude extracts showed higher antimalarial activity than the fractions since different compounds may be found which have synergistic effect and some secondary metabolites protect other metabolites (as antioxidants) and break of this association can accelerate degradation.

This finding is in agreement with another study in which the crude dichloromethane extract of *Cochlospermum tinctorum* reported to show better *in vivo* antimalarial activity than the fractions (Traore et.al., 2006). Three hypotheses were given to explain the loss of activity of the crude extract during fractionation. i.e. The loss of synergistic action among the chemical compounds in an extract, the decline of activity of the fractions may be due to denaturation of the compounds during storage and some secondary metabolites protect other metabolites (as antioxidants) and break of this association can accelerate degradation (Traore et.al., 2006).

Active compounds may be concentrated in crude aqueous extract and crude hydroalcoholic extract, which showed activity, but traces may be found in dichloromethane fraction of crude aqueous extract and butanol fraction of crude hydroalcoholic extract of which would explain the *in vivo* antimalarial activity of roots of *C.myricoides*. In other studies, the plant, *Neurolaena lobata*, has been studied by researchers and compounds were concentrated in the dichloromethane extract, which showed the highest activity, but traces were also found in the aqueous extract, this plant also has *in vivo* antimalarial activity (Sianne, 2002).

In other studies, it was observed that low activity of *Holarrhena.flouribunda* isolated compounds after bioassay guided fractionation as compared to those of crude extracts. This happens due to the fact that those compounds are not solely responsible for the antimalarial activity and there may be synergistic effect with other components of crude extract and effect can be lost when the components are tested individually. Thus it was recommended other fractions should be studied (Jean, 2006). Similarly, the comparable activity of fractions of roots of *C.myricoides* to those of crude extracts indicates that

those compounds in the fractions may not solely be responsible for the antimalarial activity. Thus other fractions should be studied.

When the in vitro antimalarial activity of dichloromethane extract *Onchosiphon Piluliferum* was studied, isolation of additional compounds from active fractions proved unsuccessful due to low yields and marked instability. Some fractions may be unstable thus modifications may be done before doing bioassay (Pamisha, 2007). In this study also, further fractionation of active fractions was difficult due to low yields.

The activity of the crude aqueous extract, crude hydroalcoholic extract, dichloromethane fraction of crude aqueous extract and butanol fraction of crude hydroalcoholic extract might be due to the components in the plant which might have retarded the multiplication of the parasites in the blood (Malagon et.al., 1997).

Once the antiplasmodial effect of the plant is confirmed, the active ingredients could be isolated by different extraction methods.

Generally, if the LD₅₀ value of the test substance is 3 times more than the minimum effective dose the substance is considered a good candidate for further studies (Dikasso et.al., 2006). In our study, the minimum effective dose for both the aqueous extracts and the hydroalcoholic extracts of *C.myricoides* were 11 and 16 times lower than the LD₅₀, respectively.

6. Conclusion

From the present study, it can be concluded that crude aqueous extract, crude hydroalcoholic extracts, of roots of *C.myricoides* have antimalarial activity at much lower doses than the LD₅₀s hinting the safe and efficacious nature of the plant. The dichloromethane fraction of crude aqueous extract and butanol fraction of crude hydroalcoholic extract also have antimalarial effect. Thus, the active ingredients responsible for the activity should be isolated by different extraction methods.

7. Recommendations

-Isolation and identification of active constituents of roots of *C.myricoides* should be done to identify compounds responsible for the antimalarial activity and toxicity.

-*In vivo* antimalarial testing on established malaria infection should be done.

-Subacute and chronic toxicity study should be done to know the safety of this plant.

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