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Bioactivity guided fractionation and antimalarial activity of fractions of ethanol extract of *Clerodendrum myricoides* leaves in mice model

A Thesis Submitted to the School of Graduate Studies of Addis Ababa University in Partial Fulfillment of the Requirements for the Degree of Masters of Science (M. Sc.) in Biomedical Sciences

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LIST OF ABBREVIATIONS

3x	Three times
ACTs	Artemisinin-based Combination Therapies
AL	Artemether-lumefantrine
ANOVA	Analysis of variance
BGF	Bioactivity guided fractionation
Bsf	Buthanol subfraction
CC	Column Chromatography
CQ	Chloroquine
Csf	Chloroform subfraction
DCM	Dichloromethane
DHA	Dehydroartemisinin
DHFR	Dihydrofolate reductase
DHPS	Dihydropteroate synthase
DMSO	Dimethyl sulfoxide
DTB	Drug and Therapeutics Bulletin
EF	Ethyl acetate fraction
EtOAc	Ethyl acetate
FMOH	Federal Ministry of Health
GDP	Gross Domestic Product
HF	Hexane fraction
Hsf	Hexane subfraction

Hsf-1	One to five fractions obtained from hexane subfraction eluted with 1:0 hexane and ethyl acetate respectively.
Hsf-5	Thirteen to eighteen fractions obtained from hexane subfraction eluted with 7:3 hexane and ethyl acetate respectively.
Hsf-7	Twenty three to twenty five fractions obtained from hexane subfraction eluted with 1:1 hexane and ethyl acetate respectively.
Hsf-8	Twenty six to twenty seven fractions obtained from hexane subfraction eluted with 3:7 hexane and ethyl acetate respectively.
Hsf-5	Thirteen to eighteen fractions obtained from hexane subfraction eluted with 7:3 hexane and ethyl acetate respectively.
Hsf-14	Thirty eight to forty one fraction forty two obtained from hexane subfraction fraction eluted with 1:0 ethyl acetate and methanol respectively.
IP	Intraperitoneal
IC50	50% inhibition at a test concentration
ITNs	Insecticide-treated mosquito nets
MF	Methanol fraction
MF-3	Eighteen to twenty fractions obtained from methanol fraction eluted with 0:1:1 cyclohexane:DCM:MeOH respectively
MF-4	Twenty one to thirty fractions obtained from methanol fraction eluted with 0:1:1 cyclohexane:DCM:MeOH respectively
MF-5	Thirty one to forty fractions obtained from methanol fraction eluted with 0:1:3 cyclohexane:DCM:MeOH respectively
MF-6	Forty one to fifty fractions obtained from methanol fraction eluted with 0:1:9 cyclohexane:DCM:MeOH respectively

NC	Negative Control
NMR	Nuclear Magnetic Resonance
PTLC	Preparative Thin Layer Chromatography
RBC	Red blood cells
SEM	Standard error of mean
SP	Sulphadoxine-pyrimethamine
SPSS	Statistical Package for Social Science
SSA	sub-Saharan Africa
TCC	The Carter Center
TLC	Thin Layer Chromatography
TM	Traditional medicine
WHO	World Health Organization
WMR	World Malaria Report

ABSTRACT

Malaria is one of the major health problems in the sub-Saharan African countries including Ethiopia. One of the reasons attributed for its increase is the emergence and spread of antimalarial drug resistant strains. A continuous search for other alternative plant-based antimalarial drugs thus becomes an urgent need. The objective of the present study was, therefore, to evaluate *in vivo* antimalarial activitiy of fractions and subfractions of ethanol extract of *Clerodendrum myricoides* leaves and isolates active ingredients. They were evaluated for their antimalarial activity in 4-day suppressive assay against *Plasmodium berghei* in Swiss albino mice. Methanol fraction (MF) and ethyl acetate fraction (EF) obtained from successive fractionation of ethanol crude extract of *C. myricoides* showed highest activity with suppression of 77.24% and 65.21% at an oral dose of 300 mg/kg/day respectively. Further bioactivity guided fractionation of ethanol extract provided some fractions which exhibited good antiplasmodial activity. Results of 1H- and 13 C-NMR spectra showed the presence of one pure compound which was isolated using preparative TLC in Hsf-5. On the other hand, the hexane fraction did not suppress the parasitaemia significantly. TLC, CC and NMR results of the fractions showed that the activity of the plant leaves was due to synergistic effect of many compounds. The results confirmed that using medicinal plants in the search for new antimalarial compounds from medicinal plant is an important approach.

Key words: Antimalarial activity, BGF, *C. myricoides, Plasmodium berghei*, Medicinal plants, Traditional medicine

1. INTRODUCTION

1.1. Malaria Epidemiology and its Burden

Malaria is a complex disease that varies widely in epidemiology, transmission and clinical manifestations in different parts of the world (Bloland, 2001). Countries at risk of malaria are primarily in tropical and subtropical regions and include: sub-Saharan Africa (SSA), Central and South America, the Indian subcontinent, South-East Asia and Eastern Mediterranean regions (WHO, 2008). Dharani *et al.* (2010) noted that transmission of the disease through mosquito bites depends on factors such as rainfall patterns, how close breeding sites are to people and the types of mosquito species in an area. Some regions have a fairly constant number of cases throughout the year („malaria endemic" areas), while others have seasonal bouts of infection, usually coinciding with rainy season.

Despite the continuous global efforts to fight parasitic infections and the attempts to eliminate the causative organisms, malaria still remains as one of the greatest human killers, causing almost 1 million deaths per year and 300-500 million infections annually (Bero *et al.*, 2009; Tamura *et al.*, 2010). About half of the world"s population is living in malaria risk areas, and there were approximately 863,000 deaths in 2008 from an estimated 243 million cases worldwide. Approximately 90% of malaria deaths occur in Africa (WHO, 2009).

This is due to the majority of infections in Africa being caused by *P. falciparum*, the most severe and life-threatening form of the human malaria parasite, as well as the most efficient and difficult to control malaria vector, *Anopheles gambiae*, which is the most widespread in the continent (Pillay, 2006; Teklehaimanot and Mejia, 2008). Moreover, widespread poverty, lack of infrastructures and resources necessary to mount sustainable interventions against the disease in the continent play a role in the continuing burden of malaria (Teklehaimanot and Mejia, 2008).

Most of the deaths due to malaria occur in African children under the age of 5 years, who have little or no immunity to the disease (Krettli *et al.*, 2009) and in every 40 second a child dies of malaria, resulting in a daily loss of more than 2000 young lives worldwide (Tamura *et al.*, 2010). Pregnant women and their unborn children are also at great risk for malaria because the immune response is suppressed in pregnancy and parasitized red blood cells (RBCs) sequestered in the placenta (Vangapandu *et al.*, 2007). Moreover, non-immune

tourists that move from non-malarious to malarious areas are at risk of severe disease and death (Kaur *et al*., 2009).

However, recent report declared good news about the remarkable reduction in malaria incidence and mortality rates during the past decade in all regions of the world. Statistically, it was estimated that the number of cases of malaria increased from 233 million in 2000 to 244 million in 2005 but interestingly decreased to 225 million in 2009, and then to 216 million in 2010. There was also a reduction of number of deaths from 985,000 in 2000 to 781, 000 in 2009, and then to 655,000 in 2010 (WHO, 2010a). Populations living in SSA have the highest risk of acquiring malaria. Among 216 million episodes of malaria in 2010, approximately 81%, or 174 million cases, were observed from the African region. There were an estimated 655,000 of malaria deaths in 2010, of which 91% were from Africa (WHO, 2011a).

These considerable improvements in malaria burden have been observed throughout the malarious areas of the world, with the largest proportional decreases recorded in Europe and then in America. In Africa an absolute decrease in the number of deaths from the previous time was observed. This achievement is largely the result of a significant scaling-up of malaria prevention and control measures in the last decade, including the widespread use of bed nets, better diagnostics and a wider availability of effective medicines to treat malaria (WHO, 2010a; RBM, 2010; WHO, 2011a).

There are threats to the current progress including the emergence of artemisinin resistance, and unmet needs that will continue to be central to the global research agenda for improving malaria control and eventually achieving eradication (Alonso *et al.*, 2011). Furthermore, while this 5% year-on-year decline represents significant progress, the mortality figures are still high for a disease that is entirely preventable and treatable (RBM, 2010; WHO, 2011a).

In addition to causing significant morbidity and mortality, it is both a disease of poverty and a cause of poverty, which results in significant lost productivity and economic losses, with annual Gross Domestic Product (GDP) estimated to be reduced by as much as 1.3% in countries with high disease rate (RBM, 2010). In Africa, it is estimated that at least USD 12 billion per year is lost directly through illness, treatment and premature death (Byakika-Kibwika *et al.*, 2010; Dharani *et al.*, 2010), with individual African families spending up to 25% of their income on malaria prevention and control (Byakika-Kibwika *et al.*, 2010). In

some countries with a heavy disease burden, malaria accounts for up to 40% of public health expenditure, up to 50% of inpatient hospital admissions, and up to 60% of visits to outpatient health clinics (Dharani *et al.*, 2010). Overall, malaria constitutes 10% of the continent's disease burden (TCC, 2011).

Aggregated losses over time have resulted in substantial differences in GDP between countries with and without malaria, particularly in Africa (Dharani *et al.*, 2010). Furthermore, it also hampers children's schooling and social development through both absenteeism and permanent neurological and other damage associated with severe episodes of the disease, which have important short- and long-term social and economic impacts (Teklehaimanot and Mejia, 2008; TCC, 2011). Malaria disease management is therefore an essential part of global health improvement and economic development (Dharani *et al.*, 2010).

1.2. Human Malaria Causing Parasites and their Life Cycle

Malaria is caused by approximately 200 known *Plasmodium* species that infect particular lineages of primates, rodents, bird and reptiles (Dhangadamajhi *et al.*, 2010). However, *P. falciparum, P. vivax, P. malariae* and *P. ovale* cause malaria in humans (Hobbs and Duffy, 2011; Rahmatullah *et al.*, 2012). The disease is transmitted by more than 30 *Anopheline* mosquito species with diverse breeding and feeding habits, and result in different disease spectra in different population target groups and epidemiological settings (Alonso *et al.*, 2011). *P. falciparum* is the most virulent and lethal malaria parasite and 90% of known human deaths are caused by *P. falciparum* (Hobbs and Duffy, 2011; Rahmatullah *et al.*, 2012). *P. vivax* is less deadly but causes a high morbidity. The ability of *P. vivax* and *P. ovale* to remain dormant for months as hypnozoites in the liver hampers current control and future elimination efforts (Gamo *et al.*, 2010). *P. malariae* does not form hypnozoites, but it can persist for decades as an asymptomatic blood stage infection. *P. knowlesi*, which is newly reported to infect humans has a shorter life cycle of replication than any of the other human malaria causing parasites, can cause high parasite counts, severe complications and death if not treated (Lee *et al.*, 2011; Kantele and Jokiranta, 2011).

All human malaria parasites develop through the same general life cycle which alternates between the human host and the female *Anopheline* mosquito (Figure 1). The cycle begins

when a *Plasmodium* infected female *Anopheline* mosquito injects the sporozoites into the dermis while probing for a blood meal. Sporozoites then migrate to the liver, infect hepatocytes, and remain in a clinically silent stage. Between 8 to 30 days later depending on the *Plasmodium* species, sporozoites undergoes a process of asexual replication releasing 10,000-40,000 liver merozoites per infected hepatocytes into the blood (Rathore *et al.*, 2005; Nogueira and Junqueira, 2010). Some *P. vivax* and *P. ovale* sporozoites are predestined to develop into non dividing hepatocytic forms (hypnozoites) which can remain latent in the liver for months to years until they activate and cause relapse infections (Gamo *et al.,* 2010).

Once inside the bloodstream each merozoite invades an erythrocyte, where it multiply again and differentiate into morphological phases: ring, trophozoite and schizont, and resides in a self created membrane-bound vacuole, and undergoes repetitive rounds of growth, division, and invasion in one-day (*P. knowlesi*), two-day (*P. falciparum, P. vivax*, and *P. ovale*), or three-day (*P. malariae*) periods (Dhangadamajhi *et al.*, 2010). The erythrocyte cycle is the stage responsible for the disease, where all the symptoms and complications take place. It is also the stage when most of the antimalarial drugs (quinolines, antifolates and endoperoxide) take action (Bustamante *et al.*, 2009).

A subset of developing merozoites differentiates into male and female gametocytes, and the Plasmodium life cycle continues when both of these gametocytes are taken up by a female mosquito during blood meals. In the mosquito midgut, gametocytes undergo fertilization and maturation forming an infective ookinete, which migrates through the midgut into the hemocele and develops into the oocyst in which sporozoites are formed. When fully matured, the oocysts burst and release sporozoites, which migrate into the mosquito‟s salivary glands to initiate another life cycle (Rathore *et al.*, 2005; Dhangadamajhi *et al.*, 2010).

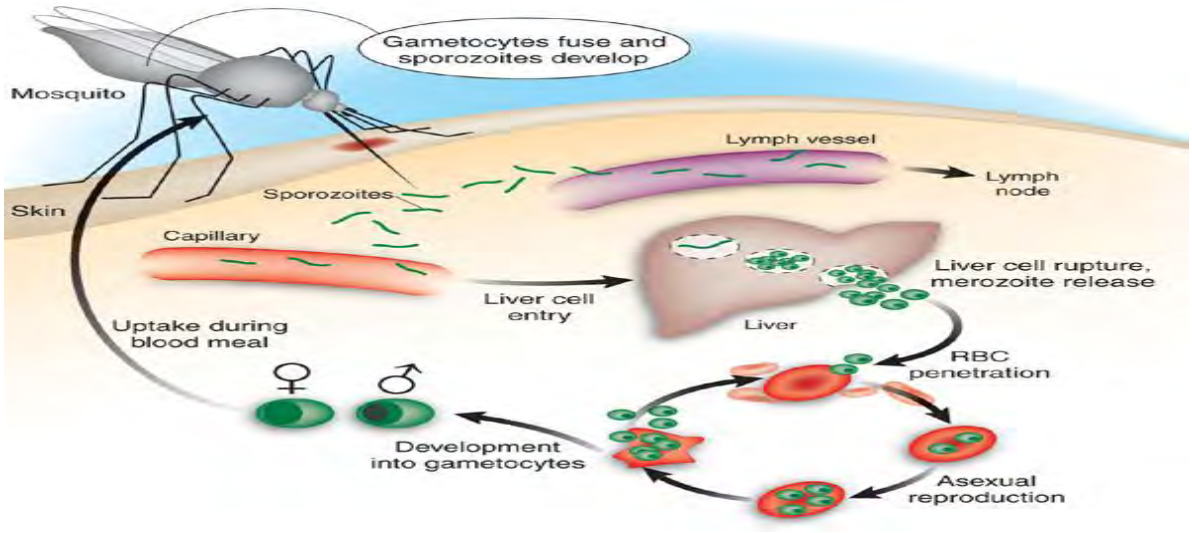

Figure 1: Schematic life cycle of malaria in humans (Batista *et al.*, 2009).

The malaria-associated pathology only occurs during the blood stage of infection (Dhangadamajhi *et al.*, 2010) and involves repeated cycles of Plasmodium replication inside the erythrocytes, which ultimately result in the rupture of the RBCs, releasing merozoites into the blood stream to reinvade other cells (Na-Bangchang and Karbwang, 2009; TCC, 2011). The growing parasite progressively consumes and degrades intracellular proteins, principally haemoglobin, resulting in formation of the „malarial pigment" and haemolysis of the infected RBC. This also alters the transport properties of the RBC membrane, and the RBC becomes more spherical and less deformable, avoid the splenic clearance by sequestering in capillaries and microvenules of the brain and other vital organs (Dhangadamajhi *et al.*, 2010). The rupture of RBCs by merozoites releases certain factors and toxins, which could directly induce the release of cytokines such as tumour necrosis factor and interleukin-1 from macrophages (Vangapandu *et al.*, 2007). This resulting in chills and high-grade fever once in 48/72 hour corresponds to the erythrocytic cycle (Rathore *et al.*, 2005).

The various symptoms of malaria such as chills, muscle ache, fever, nausea and vomiting can develop as soon as 6-8 days after being bitten by an infected *Anopheles* mosquito, or as late as several months after departure from a malaria endemic area (Bangchang and Karbwang, 2009). If it is not cured during its first stage, the infection is complicated by the host"s immune response (Rowe *et al.*, 2009). This leads to clinical features such as impaired consciousness, coma, difficulty in breathing, severe anaemia, and multi organ failure including brain occur during the blood stage of infection (Roepe, 2009), and thought to occur

because of a combination of a high parasite burden (Dhangadamajhi *et al.*, 2010) and the ability of parasitized erythrocytes to adhere to vascular endothelial cells (cytoadherence), uninfected erythrocytes (rosetting), and platelets (clumping or autoagglutination) (Rowe *et al.*, 2009). This increased cytoadherence, rosetting and clumping (characters of *P. falciparum*), resulting in occlusion of blood-flow culminates in damage to vital organs like the brain, kidneys, lungs, liver, and gastrointestinal tract which leads to the various fatally serious complications of *P. falciparum* malaria (Vangapandu *et al.*, 2007).

1.3. Malaria in Ethiopia

In Ethiopia, malaria is one of the leading causes of morbidity and mortality (Petros, 2011). Statistically, Ethiopian malaria death increased from 15268 in 1980 to a peak of 46918 in 2000, decreasing to 22165 in 2010 (Murray *et al.*, 2012), and in 2007-2008, malaria was the first cause of outpatient visits, health facility admissions and in-patient deaths, accounting for 12% of all visits and 10% of admissions (TCC, 2011). Though according to O"Meara *et al.* (2010), country-wide surveillance in Ethiopia revealed a 70% decline in malaria morbidity like other areas of sub-Saharan Africa in the past 3-5 years, malaria has been consistently reported as one of the three leading causes of morbidity and mortality over the past years (Otten *et al.*, 2009).

Almost 75% of the country is malarious, including the fertile low-land areas that are most suitable for agriculture, and an estimated 51 million people (68% of the population) live in areas at risk of malaria (Mekonnen *et al.*, 2010; TCC, 2011). In Ethiopia, the number of annual malaria cases is as high as 10-15 million (FMOH, 2006) and 60-70% of the cases are attributable to *P. falciparum* infection while 30-40% of the cases are attributed to *P. vivax* infections (Jima *et al.*, 2005). However, this relative proportion varies both temporally and geographically, with published ranges of 22.4-73.4% for *P. falciparum* and 22.4-67.4% for *P. vivax* (Ramos *et al.*, 2005).

The epidemiology of malaria in Ethiopia differs from that in most of SSA (Yeshiwondim *et al.*, 2009); transmission in Ethiopia is seasonal and unstable and the transmission patterns and intensity vary greatly throughout the country due to differences in altitude, rainfall, population movement and in part by recent scale up of control measures (TCC, 2011; FMOH,

2011), with peak of malaria incidence follows the main rainfall season (July - September) each year (Jima *et al.*, 2010). This unstable transmission made the country prone to periodic epidemics that resulted in a profound impact on people of all ages (TCC, 2011; Hwang *et al.*, 2011). In the country, malaria is not only a health issue but also a food-security and environmental issue. Since the peak transmission season coincides with the major cultivating and harvesting season of the year, malaria has tremendous impact on the agriculture productivity (Karunamoorthi and Bekele, 2009), which gradually shrinks the household income and eventually lessens the socio-economic development of the nation at large (Karunamoorthi and Tsehaye, 2012).

1.4. Malaria Prevention and Control

Though interruption of malaria transmission is the greatest challenge, by aggressively scaling up control with currently available tools and strategies, much greater gains could be achieved against the disease, including elimination from a number of countries and regions (Alonso *et al.*, 2011). The fight against malaria relies heavily upon, vector control, chemotherapy and chemoprophylaxis (Hyde, 2007; Zofou *et al.*, 2011).

Vector control is used to reduce the transmission capacity of local vector populations below the critical threshold to prevent ongoing or epidemic transmission (Alonso *et al.*, 2011), and involves the use of insecticides to control the vector mosquito and the eggs as well as larvae (Ibezim and Odo, 2008). Other preventive measures are the use of mosquito nets (treated with long-lasting insecticide) that offer overnight protection, through indoor residual spraying of insecticides, closing of doors/windows against mosquitoes and use of mosquito repellents (Dharani *et al.*, 2010). However, pyrethroid resistance in anopheline mosquito vectors pose very serious threats (White *et al.*, 2011; White, 2011).

Chemotherapy is the use of drugs to treat malarial attack and is a very effective way of treating malaria attack, once a person is infected (Ibezim and Odo, 2008). For chemotherapy blood schizonticides that terminate clinical attacks of the disease by acting on the blood stage forms of the parasite are used (Vangapandu *et al.*, 2007). Chemoprophylaxis on the other hand is the use of drugs to prevent the occurrence of malaria (Hobbs and Duffy, 2011). For chemoprophylaxis tissue schizonticides that bring about prophylaxis or prevent relapse by

acting on the exoerythrocytic parasite stages (forming merozoites and hypnozoites) in liver cells are used (Ibezim and Odo, 2008). However, worldwide spread of *P. falciparum* multi-drug resistant parasites in the last decades and, more recently, of *P. vivax* malaria parasites resistant to CQ and primaquine, has made the control of human malaria difficult (Krettli *et al.*, 2009).

According to Alonso *et al.* (2011), a safe and effective vaccine that target different stages of the parasite life cycle, or the mosquito would be an important component of a comprehensive malaria control programme. However, no licensed vaccine is currently available. While one vaccine (RTS,S/AS01) is achieving promising but incomplete levels of protection with clinical efficacy in the 25–60% range in different malaria endemic settings (Schwartz *et al.*, 2012). It is a hybrid construct of the hepatitis B surface antigen fused with a recombinant antigen derived from part of the circumsporozoite protein and its success is associated with the immunogenic polymeric nature of RTS,S particles and the proprietary adjuvant AS01. Though it is a great achievement and an important advance, it is known that this partially protective vaccine is not the sole solution to the control and elimination of malaria (White, 2011). Thus, in the absence of a functional, safe and widely available malaria vaccine, chemotherapy/chemoprophylaxis remains the principal means of combating malaria (Batista *et al.*, 2009; Grimberg and Mehlotra, 2011), and efforts to develop new antimalarial drugs continue being urgently needed now (Oliveira *et al.*, 2009).

The main malaria control strategies in Ethiopia include early diagnosis and treatment of cases, vector control using insecticide treated bed nets (ITNs) and indoor residual spraying, and epidemic prevention and control (FMOH, 2011). The coverage and proper utilization of malaria preventive measure, ITNs, in the country is limited by lack of sustainable distribution and issues related to replacement of free nets, seasonality of malaria, poor knowledge of the community with regard to the link between mosquitoes and malaria (FMOH, 2006). Moreover, more than 85% of the total population of Ethiopia lives in rural areas where a significant proportion cannot easily access basic health facilities because of geographical or economic barriers, even though they recognize their illness as malaria (Kassaye *et al.*, 2006). Although currently artemisinin combination therapy (ACT) serves a pivotal role to treat wide spread *P. falciparum* chloroquine (CQ)-resistant strains that have created severe set-back in malaria control, they are mostly inaccessible and unaffordable in the resource-limited settings (Karunamoorthi and Tsehaye, 2012).

1.5. Malaria Treatment and Drug Resistance

Prompt parasitological confirmation by microscopy or alternatively by rapid diagnostic tests is recommended in all patients with suspected malaria before treatment is started (WHO, 2010b). The choice of malaria treatment depends on the infecting Plasmodium species, drug resistance, the severity of the disease and whether the patient or she is in a special risk group (DTB, 2010). Quick and effective treatment of human malaria using antimalarial drugs played a main role in controlling the spread of malaria by interrupting the blood schizogony (intraerythrocytic asexual multiplication) that causes the pathogenesis and clinical symptoms of the infection (Oliveira *et al.*, 2009), and controlling its transmissibility (Na-Bangchang and Karbwang, 2009).

The currently available antimalarial agents can be classified into groups according to their biological activity and chemical structure (Vangapandu *et al.*, 2007). The first are quinoline based antimalarials, which have been widely used for the treatment of malaria. Drugs belonging to this group include chloroquine (CQ), amodiaquine, primaquine, quinine, halofantrine, lumefantrine and mefloquine, with oral CQ being the preferred therapy (Choi *et al.*, 2008; Grimberg and Mehlotra, 2011). CQ, amodiaquine, quinine, mefloquine, lumefantrine and halofantrine are known to act only on the growing intraerythrocytic stages (Vangapandu *et al.*, 2007), whereas primaquine is effective against the dormant liver form, the hypnozoite (Hobbs and Duffy, 2011). Inhibition of heme dimerization by quinolines leads to the accumulation of free heme in the food vacuole, resulting in the death of the parasite from the toxic by-products of hemoglobin digestion (Vangapandu *et al.*, 2007; Choi *et al.*, 2008; Hobbs and Duffy, 2011).

CQ used for decades as gold standard for the treatment of malaria due to its high efficacy against all species of malaria parasites, its low toxicity, low cost and high tolerance (Fidock *et al.*, 2004). Quinine primarily has an important role for the treatment of severe malaria for children in Africa (Na-Bangchang and Karbwang, 2009). However, according to Hobbs and Duffy (2011), parenteral artesunate has shown superior efficacy over parenteral quinine. Primaquine is still in use today as the only licensed drug effective against hypnozoites (Grimberg and Mehlotra, 2011).

The second group of antimalarial drugs is the antifolates that inhibit the enzymes of the folate pathway to interfere with DNA synthesis by depleting the pool of tetrahydrofolate, a cofactor of DNA synthesis (Winstanley and Ward, 2006). In the treatment of malaria, they are usually used as synergistic combinations of the inhibitors of the folate pathway enzymes dihydrofolate reductase (DHFR) and dihydropteroate synthase (DHPS). The most commonly used antifolate combinations are pyrimethamine and proguanil as a DHFR inhibitor combined with dapsone and sulfadoxine as a DHPS inhibitor (Choi *et al.*, 2008; Grimberg and Mehlotra, 2011). Typical combinations include sulphadoxine-pyrimethamine (SP) or, Fansider which is the most widely used combination (Fidock *et al.*, 2004). Antifolates attack all growing stages of the malaria parasite, and are also found to inhibit the early growing stages in the liver and in the mosquito (Vangapandu *et al.*, 2007). Fansider has been used as second line treatment in most of the second half of the twentieth century to control uncomplicated malaria caused by all four species of *Plasmodium* (Wells *et al.*, 2009).

The third class of antimalarial include the natural endoperoxide artemisinin and its semisynthetic derivatives (dehydroartemisinin (DHA), artemether, arteether, artesunate, artelinic acid) (Pillay, 2006). Combining different classes of drugs is now the best method for malaria treatment. This is because the probability of a single point mutation, resulting in resistance, is considered to be very low (Rathore *et al.*, 2005; Trafford, 2005) and the combination of two antimalarials with different sites of action in the parasite leads to a simultaneous attack on two systems in the parasite (Rathore *et al.*, 2005; Ginsburg and Deharo, 2011). The drug that has this quality, artemisinin-based combination therapy (ACT), has recently been introduced in virtually all countries in which malaria is endemic, represented a shift away from monotherapy for malaria, which was common previously (Hobbs and Duffy, 2011). The rationale for the use of ACTs is based on the facts that artemisinin derivatives are highly potent and fast acting, which causes rapid reduction of the parasite biomass and the partner drug in ACT has a long half-life, which eradicate the small fraction of parasites that escape from the potent action of rapidly metabolized artemisinin (Hyde, 2007; Grimberg and Mehlotra, 2011).

Na-Bangchang and Karbwang (2009) revealed that this group of drug reduce the parasite biomass including gametocytocides very quickly by around 4-logs for each asexual cycle, and this involves the cleavage of endoperoxide bridge within the sesquiterpene lactone

molecule homolytically by heme to give a reactive free radical intermediate that alkylate vital parasite macromolecules (Winstanley and Ward, 2006). Hence, according to Wells (2011), these medicines are extraordinary effective, curing more 98% of patients. However, the cost, production, recrudescence and pharmacological issues associated with artemisinin derivatives and potential partner drugs are hindering the implementation of ACTs (Vangapandu *et al.*, 2007; Na-Bangchang and Karbwang, 2009).

The emergence and spread of antimalarial drug resistance is probably the greatest problem faced by malaria control programs worldwide and is an important public health concern (Achan *et al.*, 2011; WHO, 2010b). WHO (2011b) defined antimalarial drug resistance as the ability of a parasite strain to survive and/or to multiply despite the administration and absorption of a medicine given in doses equal to or higher than those usually recommended but within the tolerance of the subject, provided drug exposure at the site of action is adequate. To date, drug resistance has been documented in three of the four malaria species known to affect humans in nature: *P. falciparum*, *P. vivax* and *P. malariae* (WHO, 2010b).

Drug resistance may arise due to various factors, including the use of antimalarial drugs as monotherapies, over-reliance on a small number of drug classes, use of fake or substandard drugs and self treatment (DTB, 2010). In addition to this, Ibezim and Odo (2008) and Hyde (2007) noted that the use of sub-therapeutic doses of drugs or not completing, mass administration, use of drugs with long half-life, overuse of antimalarial drugs for prophylaxis and high transmission intensity are also responsible for drug resistance.

Over the years, malaria parasites have developed resistance to a number of commonly used antimalarial drugs (Achan *et al.*, 2011). Unfortunately, the malaria parasite has developed resistance to all the above drugs in different parts of the world (Hobbs and Duffy, 2011). CQ resistance was reported in parts of Southeast Asia as early as the 1950s and is now widespread in almost all areas with falciparum malaria (Achan *et al.*, 2011). Mefloquine was introduced in the 1970s, in response to emerging CQ resistance, but mefloquine-resistant strains were reported within six years of its introduction. Resistance also emerged rapidly for members of the other major class of antimalarials, the antifolates (Trafford, 2005) and ACTs (Dondorp *et al.*, 2009).

Resistance appears to occur through spontaneous mutations of drug targets that confer reduced sensitivity to a given drug or class of drugs (Roepe, 2009). Quinoline-based antimalarials resistance is associated with mutations on genes that encode transport proteins localized in the membrane of digestive vacuole (Na-Bangchang and Karbwang, 2009). Pyrimethamine and sulfadoxine both have long-elimination half-lives that equates with a strong selective pressure for resistance, as new infections are ultimately exposed to sub-inhibitory drug concentrations. Resistance to DHFR and DHPS-inhibitors results from specific mutations in the DHFR gene (dhfr) and DHPS gene (dhps) respectively (Winstanley and Ward, 2006) and the use of an alternative pathway to recover folate (Pillay, 2006). Potential mechanisms for resistance in artemisinin derivatives include mutations in genes encoding drug efflux pumps (as were seen for the 4 aminoquinolines) or mechanisms that alter the concentration of free Fe2+ (required for endoperoxide activation) (Wells *et al.*, 2009).

In an effort to delay the development and spread of multiple drug-resistant falciparum malaria and maximize the effectiveness of artemisinin and its derivatives, ACTs are now recommended as the first-line treatment for uncomplicated *P. falciparum* worldwide (WHO, 2010b; Byakika-Kibwika *et al.*, 2010). The current gold standard medicine is the fixed dose ACTs consisting of five combinations: artemether-lumefantrine, artesunate-amodiaquine, artesunate-mefloquine, artesunate-sulfadoxine-pyrimethamine and DHA-piperaquine (Wells, 2011; Rahmatullah *et al.*, 2012). But the choice of drug-combinations varies, and in each endemic area it is influenced by factors like the availability of the drugs, the level and type of resistance and economical restrictions (Krettli *et al.*, 2009), and Lemma *et al.* (2011) recommended that adherence to malaria drug regimens is a key determinant in the success of any malaria control programme.

However, there is genuine concern that the first signs of resistance to artemisinins are emerging, with patients taking longer to clear their fever and parasite in some parts of Cambodia (Dondorp *et al.*, 2009). In addition to this, Noedl *et al.* (2008) reported that artemisinin resistance has already emerged along the border between Cambodia and Thailand and Noedl *et al.* (2009) suggested that the increased failure rate of ACT can probably be attributed to decrease artemisinin susceptibility. This is a warning sign that there is a significant need for the discovery of new, and effective antimalarial drugs with novel

structures that have different modes of action from the currently available drugs to combat the disease (Wells, 2011; Ovenden *et al.*, 2011).

In Ethiopia CQ has been the first line treatment for uncomplicated malaria since 1950. By the late 1990s, 86-88% treatment failure rates with CQ were reported, which prompted change of first-line treatment to SP in 1998 (Kebede *et al.*, 1999). In 2003, a nation-wide study evaluating SP efficacy showed wide-spread resistance to SP (Jima *et al.*, 2005), as a result of this, the country switched its first-line treatment of uncomplicated *P. falciparum* malaria from SP to a fixed ACT, artemether-lumefantrine (AL) in 2004 (Lemma *et al.*, 2011; FMOH, 2011). *In vivo* therapeutic efficacy of AL has shown that AL is a highly efficacious, safe and well-tolerated antimalarial drug for uncomplicated falciparum malaria in Ethiopia (Assefa *et al.*, 2010; Hwang *et al.*, 2011).

1.6. Herbs as Medicines

Traditional medicine (TM) include herbal medicines composed of herbs, herbal materials, herbal preparations, and finished herbal products, that contain as active ingredients parts of plants, or other plant materials (WHO, 2002). Most developing countries, especially those in Asia, Africa, Latin America and the Middle East, 70%–95% of population rely on traditional medicines for their primary health care needs and even in the Western world (WHO, 2011b). The use of herbal medicines is steadily growing with approximately 40% of the population reporting use of herbs to treat medical illness. This growing acceptance for herbal remedies, in developed countries, is partly due to the dissatisfaction with conventional medicines while in developing countries is due to lack of medical doctors, shortage of pharmaceutical products and their unaffordable prices (Bekele, 2007).

Herbal medicines refer to the medicinal products of plant roots, leaves, barks, seeds, berries or flowers that can be used to promote health and treat diseases (Li *et al.*, 2008). The beneficial medicinal effects of plant materials typically result from the combinations of secondary products present in the plant (Ginsburg and Deharo, 2011**)** and when isolated from plants form not only valuable drugs but also valuable lead molecules for having better or similar biological activity as their natural counter parts (Shrivastava and Patel, 2007). They have been used as sources of medicines throughout history and continued to serve as the basis for many pharmaceuticals used today (Ginsburg and Deharo, 2011; Lopez, 2011), and their

potential as the source of drugs is still largely unexplored (Karthishwaran and Mirunalini, 2010).

WHO (2011b) noted that of 119 plant-derived pharmaceutical medicines, about 74 percent are used in modern medicine in ways that correlated directly with their traditional uses as plant medicines by native cultures. Sucher and Carles (2008) have estimated that at least 25% of all modern medicines are derived, either directly or indirectly, from medicinal plants, primarily through the application of modern technology to traditional knowledge. For example, Bekele (2007) reviewed that modern drugs such as bruceatin, coaine, digoxin, morphine, quinine and reserpine are originally discovered through the study of traditional cures and knowledge of indigenous people.

Research in medicinal plants has gained a renewed focus recently. The prime reason is that plant based system of medicine being natural come with fewer side effects (Karthishwaran and Mirunalini, 2010). However, not everything that is natural is safe so TM products must be used with care and as indicated, just like any other medication (Zhang *et al.*, 2008). Secondly, plant products represent an inexpensive virtually inexhaustible reservoir of novel biologically active molecules with enormous structural and chemical diversity, which make them favourable for drug discovery (Na-Bangchang and Karbwang, 2009). Lastly, biologically-derived secondary metabolites and, synthetic compounds derived from them perform better as drugs than do randomly synthesized compounds (Ginsburg and Deharo, 2011).

With this fact, Ginsburg and Deharo (2011) and Kaur *et al*. (2009) have tried to name the limitations facing to the development of drugs from medicinal plants. These are: many compounds cannot be further developed because of their toxicity, low bioavailability and/or poor solubility and large-scale harvesting of medicinal plants from forests may affect the forest ecology or even a total extinction of the prospected species.

Ethiopian people have been using TM since time immemorial (Birhan *et al.*, 2011), with 90% of population dependent on TMs for the management of health and as primary health care to address their health-care needs and concerns (WHO, 2011b). Beside to this, 90% of livestock population depend on TM similar to many developing countries particularly that of SSA countries (Bekele, 2007). Petros (2011) summarized that the wide spread use of TM in both

urban and rural populations of Ethiopia could be generally attributed to acceptability from cultural perspective, efficacy, physical accessibility and economic affordability.

Ethiopia has an enormous resource of plant species that are used as TM (Karunamoorthi and Tsehaye, 2012), with effective medicinal value for some ailments of human and domestic animals, thus medicinal plants and knowledge of their use provide a vital contribution to human and livestock health care needs throughout the country (Bekele, 2007). However, the status of phytomedicine, preparation of crude extracts and isolation of active principles is very minimal (Petros, 2011; Mesfin *et al.*, 2012).

1.7. Plants as Sources of Antimalarial Agents

Plants have been used in the traditional treatment of malaria for thousands of years in various parts of the world (Adebayo and Krettli, 2011; Gathirwa *et al.*, 2011) and have been used as source of antimalarial drugs (Saxena *et al.*, 2003; Bero *et al.*, 2009). In the fight against malaria, an increased drug resistance to conventional antimalarials, unavailability and un affordability of the drugs, increasing resistance of mosquito vectors to insecticides, challenge of having effective vaccines and adverse effects of the existing antimalarial drugs, constitute as the major cause of re-emergence malaria (Na-Bangchang and Karbwang, 2009; Petros, 2011). The emergence of drug resistance is reducing the therapeutic arsenal for the treatment of malaria at a rate that is barely balanced by the development of novel effective drugs (Verma *et al.*, 2011).

The history of antimalarial chemotherapy is highly linked with the history of herbal medicinal products. Previous finding of antimalarial agents such as quinine, the original natural product used in antimalarial chemotherapy, was identified from cinchona tree bark (Pillay, 2006; Wells, 2011). The most recent addition to the global antimalarial arsenal is artemisinin, extracted from *Artemisia annua* which has been a component of Chinese herbal medicine for >2000 years (Trafford, 2005). The effective antimalarial activity of the two plant-based drugs, quinine and artemisinin, has generated much interest to explore other plant resources for their possible antimalarial efficacy (Mishra *et al.*, 2009).

In light of this historic success and the fact that most indigenous people living in malaria endemic areas use TMs to fight this disease, it is possibile that ethnopharmacological approaches canlead for discovery of antimalarial compounds and templates for the synthesis of novel antimalarial agents (Calderon *et al.*, 2012) and it is clearly an approach that must be continued (Nguyen-Pouplin *et al.*, 2007). Thus, there is a strong interest from scientists in disease endemic countries to work on local TM (Wells, 2011). To support this, several initiatives in TM for malaria have been initiated in the last 10 years to develop a strategy for more effective, evidence-based use of TMs (Gathirwa *et al.*, 2011).

The first step in the antimalarial drug discovery process from plants is to evaluate the antimalarial activity of plant extracts or the test compounds (Calderon *et al.*, 2012), which can be done either *in vivo* or *in vitro* (Fidock *et al.*, 2004). *In vitro* the effects of antimalarial activity are evaluated by assessing the parasite growth in drug-exposed cultures, in relation to drug-free control cultures (Krettli *et al.*, 2009), where as in the *in vivo* tests the survival time and clearance of parasitemia is measured in a dose response in lab rodent models (Nogueira and Junqueira, 2010).

Recently, there are several reports on the antimalarial activities of different medicinal plants from different parts of the world. Although many plant species, used traditionally for the treatment of malaria have been evaluated for their antimalarial effect, the studies are often limited to the evaluation of crude plant extracts against malaria parasites *in vitro* (Muregi *et al.*, 2004; Gathirwa *et al.*, 2011; Zofou *et al.*, 2011). In some cases active compounds have been isolated and in relatively few studies compounds or extracts have been assessed for their activities against mice infected with malaria (Wright, 2010).

Reviews by Saxena *et al.* (2003); Kaur *et al*. (2009); Batista *et al.* (2009); Bero *et al.* (2009) and Nogueira and Lopes (2011) indicated that alkaloids, terpenoids, glycosides, flavonoids, quassinoids, phenolics, steroids and limonoids possess antimalarial activities. Alkaloids are one of the major classes of natural products that exhibit antimalarial activity. Indeed, quinine, the first antimalarial drug, belongs to this class (Saxena *et al.*, 2003). They are physiologically active nitrogenous bases derived from biogenetic precursors and have been successfully used for the treatment of parasitic infections (Kaur *et al*., 2009). Terpenoids also form the largest class of natural products and majority of compounds occur in plants

(Gershenzon and Dudareva, 2007). Artemisinin isolated from *Artemisia annua* is a sesquiterpene lactone prescribed in combination therapies to fight CQ-resistant *P. falciparum* (Bero *et al.*, 2009). β-sitosterol and stigmasterol belongs to steroids also have been reported to possess antimalarial activities (Uchoa *et al.*, 2010; Nogueira and Lopes, 2011).

As reviewed by Adebayo and Krettli (2011) the mechanisms of action for antimalarial compounds isolated from plants are: inhibition of hemozoin polymerization in the parasite, inhibition of *P. falciparum* lactate dehydrogenase, interference with the formation of mitotic spindles and the assembly of microtubules into typical axonemes in gametes. Moreover, Mishra *et al.* (2011) also noted inhibition of proteolytic processing of circumsporozoite protein and nucleic acid synthesis as mechanisms of action.

One of the approaches in discovery of new lead antimalarial compounds from plants is bioactivity guided fractionation (BGF) based on either *in vitro* or *in vivo* antiplasmodial assay (Bero *et al.*, 2009). This approach commonly employed in drug discovery research due to its effectiveness to directly link the analyzed extract and targeted compounds using fractionation procedure that followed with certain biological activity (Kaur *et al*., 2009; Batista *et al.*, 2009; Bero *et al.*, 2009). BGF is a procedure of whereby extract is chromatographically fractionated and refractionated until a pure biologically active compound is isolated (Butler, 2004).

This approach always may not lead to the isolation of active compound because total activity may be lost by fractionation from the active extracts (Wells, 2011). This originates from the fact that the efficacy of most natural medicines may lie in the synergy or additivity of diverse components rather than arising from a single compound (Ginsburg and Deharo, 2011), and oxidation or chemical break down of the active molecule lead to inactivation of the extracts (Wells, 2011). Besides, many of them are found in low concentrations in the plant species and usually as part of complex mixtures making their isolation and purification highly expensive (Butler, 2004; Batista *et al.*, 2009). Moreover, according to Wright (2010), the new antimalarial lead compounds should exhibit potent antiplasmodial activities, suppress parasitaemia in mice by close to 100% without showing toxicity, be selectively toxic to malaria parasites compared to their toxicities against human cell lines and orally active. These are often given as a reason for the lack of progress in the identification of new natural product templates for antimalarial agents (Wells, 2011).

With these pros and cons, a number of compounds of various chemical classes have been isolated from plant species that fulfil some of the pre-requisites needed for new lead antimalarial agents (Wright, 2010). For example, two new triterpenoids, salvadione C and perovskone B, which were isolated from *n*-hexane extract of *Salvia hydrangea* evaluated *in vitro* against *P. falciparum* K1 strain and showed good antiplasmodial activity with IC50 values of 1.43 and 0.18μM (Farimani *et al.*, 2011). Andrographolide, the diterpene lactone compound, was purified from the methanolic fraction of *Andrographis paniculata* bark extract. The compound was found to have potent antiplasmodial activity when tested in isolation and in combination with curcumin and artesunate against the erythrocytic stages of *P. falciparum in vitro* (Mishra *et al.*, 2011).

Mbeunkui *et al.* (2012) isolated four known indole alkaloids (geissolosimine, geissospermine, geissoschizoline and vellosiminol) from the methanolic extract of stem bark of *Geissospermum vellosii*. These compounds were tested for *in vitro* antiplasmodial activity against the CQ-sensitive strain of *P. falciparum* and geissolosimine showed the highest antiplasmodial activity (0.96µM). Investigation of the chemical composition of the aerial parts of *Artemisia gorgonum* led to the isolation and identification of a flavone: artemetin. The compound was evaluated for inhibition of *P. falciparum* growth *in vitro* and showed good activity, with IC50 values of 3.5µg/ml (Ortet *et al.*, 2011).

Although recently there are efforts to identify and screen antimalarial plants used in the ethnomedical practice of Ethiopia (Deressa *et al.*, 2010, Mesfin *et al.*, 2012), the studies done are very limited and they are not fully exploratory and most of them focuse on the ethnobotanical uses of plants rather than pharmacological screenings (Bekele, 2007).

In Ethiopia, some of the medicinal plants used traditionally for the treatment of malaria have been screened for their antimalarial activity against *P. berghei*. Mesfin *et al.* (2012) have reported that ethanol and aqueous leaf extracts of *Aloe sp.* and *Azadirachta indica* and fruits of *Tamarindus indica* have significant *in vivo* antimalarial activity against *P. berghei.* According to Dikasso *et al.* (2006) extracts from the roots and aerial parts of A*sparagus africanus* were observed to inhibit *P. berghei* parasitaemia in the Swiss albino mice by 46.1% and 40.7% respectively.

Clerodendrum myricoides is also among the numerous traditionally used medicinal plants in Ethiopia. Ethnomedical value of *C. myricoides* is enormous in Ethiopia and different parts of the plant are used in TM. The bark of the plant is used for abdominal pains, malaria and against snake bites. Its roots and leaves are also used to treat gonorrhea, rabies, measles, glandular TB, colic, eye disease, malaria, swellings, in the body, wound dressings, hemorrhoids and asthma (Person, 2006; Assefa *et al.*, 2007). According to Jeruto *et al.* (2008), roots decoction of *C. myricoides* traditionally used for epilepsy, rheumatism, gonorrhoea, tonsillitis, arthritis, malaria, diabetes, typhoid, cough/cold, eye problems, and proper position of foetus.

Phytochemically, the major groups of chemical constituents present in *Clerodendrum* are phenolics, flavonoides, terpenoides, steroids, alkaloids and oils (Shrivastava and Patel, 2007). Pascaline *et al*. (2011) revealed that *C. myricoides* leaves contain alkaloids, steroids, saponins, glycosides, terpenoides, phenolics and flavonoides.

In pharmacological studies, the methanolic extract of *C. myricoides* leaves showed good antiplasmodial activity and the ethyl acetate extract was within the mild activity range against both sensitive and resistant strains of *P. falciparum* (Muregi *et al.*, 2004). Dichloromethane leaf extracts of *C. myricoides* indicated antimutagenic properties against *Salmonella typhimurium* TA98 and TA100 bacterial strains (Reid *et al*., 2006).

There are several reports that demonstrate the antimalarial properties of the plant *C. myricoides* (Muregi *et al.*, 2004; Assefa *et al.*, 2007; Deressa *et al.*, 2010 and Tadesse, 2011) but there is hardly any report that describes the active compound responsible for the antimalarial property of this plant. In the present study, as a part of continuing efforts directed towards the discovery of the structurally interesting and biologically active compounds from medicinal plants, the *in vivo* antimalarial activity of *C. myricoides* fractions and sub fractions have been identified and evaluated against *P. berghei* infected mice.

2. OBJECTIVES

2.1. General Objective

- To evaluate the antimalarial activities of fractions and subfractions obtained using bioactivity guided fractionation (BGF) of ethanol extract of *C. myricoides* leaves against *P. berghei* in mice.

2.2. Specific Objectives

- To evaluate the effect of fractions of ethanol crude extract of *C. myricoides* against *P. berghei* in Swiss albino mice model.

- Further fractionation the antimalarial active fractions obtained from BGF using column chromatography (CC) and evaluate their antimalarial activity.

- To identify the active subfractions responsible for the antimalarial activity of the active fractions.

- To isolate and characterize active compounds from the active subfractions.

3. MATERIALS AND METHODS

3.1. Plant Material Collection and Authentication

C. myricoides (Lamiaceae) was collected from Lideta Mariam, around Russia Embassy, to the East direction of Addis Ababa in September 2011. Identification and authentication of the plant specimen was done at the National Herbarium of Addis Ababa University by a botanist in October 2011 and voucher specimen was deposited as voucher number (GG04/2011) in the Herbarium.

3.2. Description of the Plant Material

The genus *Clerodendrum* L. (Family: Lamiaceae) is very widely distributed in tropical and subtropical regions of the world and is comprised of small trees, shrubs and herbs. One of them is *Clerodendrum myricoides* which is commonly used in Ethiopian traditional medicine. It is locally called misrch (Amharic), is an open shrub reaching 6 to 10 feet tall by 6 feet wide with 4 inch long dark green glossy leaves. Leaves are arranged opposite or in whorls of 3 or 4, they are sessile or with a petiole and with revolute (rolled under) margins. Flowers are bilaterally symmetrical. The flower has four pedals a light blue color with the bottom petal violet blue and the pistil and stamens arch outward and upward (Person, 2006).

Fig. 2 Pictures of *C. myricoides* aerial part (Lideta Mariam; September, 2011,)

3.3. Plant Extraction and BGF of Ethanol Crude Extract

3.3.1. Preparation and fractionation of ethanol crude extract

The collected leaves of *C. myricoides* were washed with water and air dried at room temperature in open air under shade for two weeks in the Biomedical Science laboratory of Faculty of Life Sciences, College of Natural Sciences of Addis Ababa University. The dried plant material was ground into fine powder with mesh size of less than 0.7mm using an electrical cross bitter mill (IEC, 158VDE 0660, Germany) and the powdered plant material were packed in plastic bag until extraction was done. The crude extract was prepared by maceration of the powder in ethanol (99.5%) at room temperature (Verma *et al.*, 2011); by placing 1 kg of powdered plant material in an Erlenmeyer flask containing six L of absolute ethanol and it was placed on orbital shaker (at 120 rpm) for 24 hours at room temperature. Then filtered out using a Whatman filter paper (No. 1, 15 cm size with retention down to 0.1 ml in liquids) yielded a greenish filtrate, which was concentrated under reduced pressure in a rotary evaporator (Buchi typeTRE121, Switzerland) to give 185 g (18.5%) dark semi-solid ethanol crude extract.

The ethanol extract was subjected to successive fractionation using n-hexane first for non-polar compounds and followed by ethyl acetate to extract moderately polar compounds. This was done by adding the ethanol crude extract into an Erlenmeyer flask containing 300 ml of hexane. Then placed on a shaker for 6 h, filtered and concentrated to afford 6 g (3.49%) hexane fraction (HF). The mark was subsequently fractionated using ethyl acetate (300 ml) by placing it on a shaker for 6 h, filtered and concentrated to obtain the ethyl acetate fraction (EF) 11 g (6.96%). The marc remained (158 g) is methanol soluble (MF). All the fractions were stored at -20ºC until they were subjected to antimalarial test. Following antimalarial evaluation the fractions found to be active (EF and MF) were further fractionated using solvent partitioning and column chromatography (CC) respectively. The schematic diagram of the extraction procedure was depicted as shown below (Fig. 3). The extraction and fractionation were conducted in the laboratory of the Natural Products Chemistry Project, Chemistry Department, AAU.

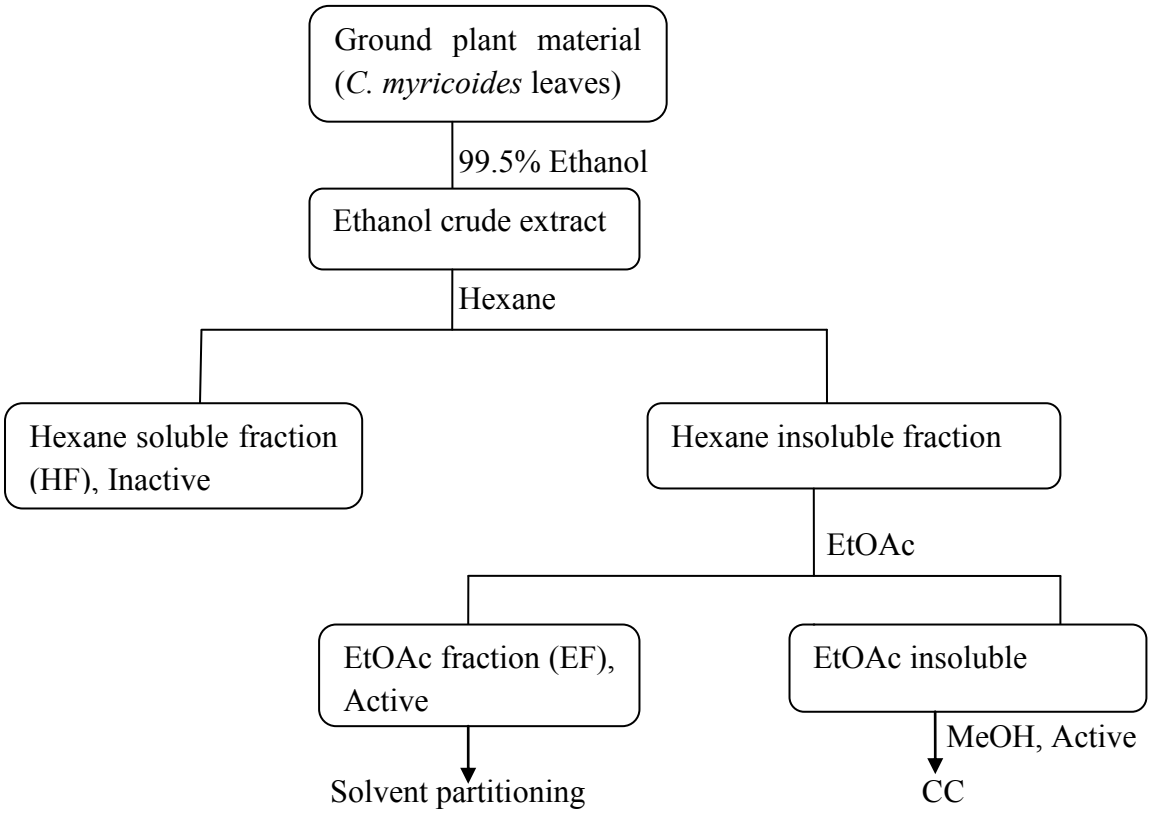

Fig. 3: Flow sheet diagram of fractionation procedure of *C. myricoides* ethanol extract

3.3.2. Preparation of subfractions from EtOAc fraction (EF)

EtOAc fraction (5 g) was allowed to dissolve in MeOH (50 ml) and its volume was reduced to about 30 ml. Then 30 ml water was added to make 50% aqueous solution. It was fractionated three times (3x) each with 20 ml of n-hexane to obtain hexane subfraction (Hsf). The aqueous phase remained after fractionated with n-hexane was further fractionated 3x each with 20 ml of chloroform to obtain chloroform subfraction (Csf) and aqueous phase. Finally the aqueous phase remained after extracted with chloroform was partitioned using n-butanol to obtain n-butanol subfraction (Bsf). Each subfraction was concentrated in rotary evaporator to yield 3.6 g (72%), 1 g (20%) and 0.3 g (6%) of Hsf, Csf and Bsf respectively (Fig. 4).

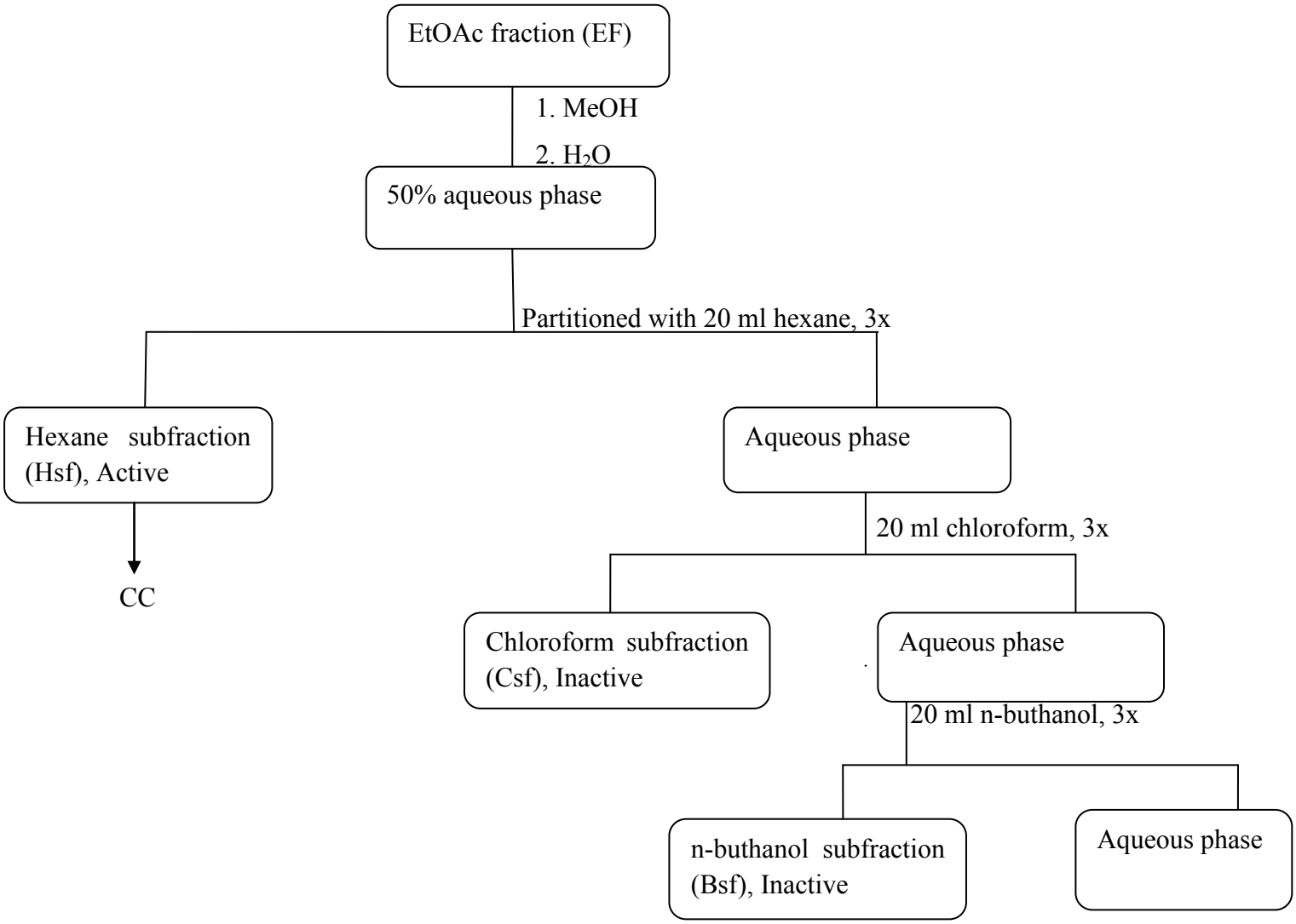

Fig. 4: Flow sheet diagram of solvent partitioning procedure of EF.

3.3.3. Column chromatographic fractionation of MF and Hsf

5 g of the marc (MF) was dissolved in methanol and subjected to column chromatography. Elution was done using cyclohexane: dichloromethane (DCM): MeOH solvents with different ratios (100:0:0, 1:1:0, 0:19:1, 0:1:1, 0:1:3, 0:1:9) by increasing polarity. The eluted fractions were combined based on thin layer chromatography (TLC) profiles to give six (6) methanol subfractions, designated as MF-1, MF-2, MF-3, MF-4, MF-5 and MF-6. Similarly Hsf (3.6 g) of ethyl acetate fraction was adsorbed onto silica gel and applied on top of column chromatography packed with fifty (50 g) grams of silica gel (230-400 mesh). Elusion was

done using hexane: EtOAc and EtOAc: MeOH solvents with different ratios (1:0, 9:1, 4:1, 7:3, 1:1, 3:7, 1:4, 0:1, 1:0, 4:1) by increasing polarity. The eluted fractions were combined based on their TLC profiles to give seventeen (17) subfractions, designated as Hsf-1, Hsf-2, ..., Hsf-17.

Analytical TLC was run on a 0.25 mm thick layer of silica gel GF254 (Merck) on aluminum plate. Spots were detected by observation under UV light and spraying with vanillin in H_2SO_4 and by heating with hot air gun.

3.3.4. Isolation of compounds using Preparative Thin Layer Chromatography (PTLC)

Among the seventeen subfractions collected (Table 2), Hsf-5 was found to be the most active of all. Hence it was applied onto PTLC to get pure compound. In line with this, the PTLC was developed using DCM and ethyl acetate (9:1) to afford two bands. The two bands were separated to give two fractions.

3.4. *In vivo* Antiplasmodial Activity of Fractions and Subfractions

3.4.1. Experimental animals

Male Swiss albino mice weighing 25-35 grams, 6-8 weeks of age obtained from the Animal House of the Faculty of Life Sciences, Addis Ababa University were used in the study. The animals were housed in standard cages and acclimatized for a period of 10 days before using for experiment. They were housed in plastic cages with saw dust as beddings and given a diet and tap water *ad libitum*.

3.4.2. Malaria parasite and inoculation

For *in vivo* antiplasmodial assays of extracts (plant fractions, subfractions and standard drug) the mouse infective CQ sensitive strain of *P. berghei* maintained in the Microbial, Cellular and Molecular Program Unit, Faculity of Life sciences was used. The parasite was maintained by serial passage of blood obtained from infected mice to non-infected ones on weekly basis.

Each mouse used in the experiment was infected intraperitoneally with 0.2ml of infected blood containing about 1 x 10^6-10^7 *P.berghei*-parasitized erythrocytes (Shittu *et al.*, 2011).

For each experiment about 1 ml *P. berghei* infected blood sample was obtained by gentle cardiac puncture of the donor mice with rising parasitaemia of about 25-35% in such a way that 1 ml blood contains 5 x 10^6-10^7 *P. berghei*-parasitized erythrocytes per ml (Mishra *et al.*, 2009; Mesfin *et al.*, 2012). This was prepared by determining the percentage of parasitaemia and diluting 1 ml of blood in 4 ml of physiological saline solution (0.9% NaCl) (Mishra *et al.*, 2009).

3.4.3. Evaluation of suppressive activity of the fractions

The evaluation protocol was based on Peters‟ 4-day suppressive test against *P. berghei* infection in mice (Peters *et al.*, 1975). Twenty infected mice were randomly divided into two test groups and two control groups (each for chloroquine as a standard drug and dimethyl sulfoxide (DMSO) as a negative control) for each of the three: HF, EF and MF. Five mice for each cage of the test groups and the control groups were assigned. The test fractions were prepared in two doses (150mg/kg and 300mg/kg of body weight); CQ at 25mg/kg in a volume of 0.2 ml and negative control group was given the solvent (0.2 ml of 20% DMSO) for the same duration. The standard drug (CQ) and fractions used in the antiplasmodial study were administered as a single dose per day through intragastric route by using standard intragastric gavage to insure safe ingestion of the drug and fractions. Within 3 hours post-inoculation of mice with parasite, on day 0 (D_0) treatments of infected mice were started and continued daily for four days (i.e. from D_0 to day 3) in 24 hour schedule. On the fifth day (D_4) blood samples were collected from tail snip of each mouse and smeared on to microscopic slides to make thin blood smear. The thin blood smear first fixed with methanol for 30 seconds and stained with 10% Geimsa solution, pH 7.2, for 25 min. Then, five uniform fields from tailed region of each stained slide (for each mouse) were examined under the microscope with an oil immersion objective of 100x magnification power to evaluate the percent suppression of each fraction with respect to the control groups (Mesfin *et al.*, 2012). The obtained parasitemia count was varying from one field to another in the same slide, in different slides obtained from a mouse and in different slides obtained from different mice with in one group. The average was taken to determine the percent parasitemia. Then percent parasitaemia and suppression were calculated for each dose by comparing the parasitaemia in treated group with negative control group according to the following formula as indicated by Chen *et al*. (2010).

Percentage parasitemia in each field was calculated as:

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

Where, PRBC= Parasitized Red Blood Cells

RBC= Red Blood Cells.

Percentage suppression was calculated as:

$$\frac{\text{Parasitemia in control (\%) - Parasitemia in treated group (\%)}}{\text{Parasitemia in control (\%)}} \times 100$$

3.4.4. Evaluation of suppressive activity of the MF and EtOAc subfractions

The subfractions; Hsf, Csf and Bsf obtained from EF were evaluated for their antiplasmodial activity in Swiss albino mice in the same way as the fractions but in lower doses. Twenty infected mice were randomly divided into two test groups and two control groups (each for CQ as a standard drug and DMSO as a negative control) for each of the subfractions of MF (MF-4, MF-5, MF-6 and MF-7) and EF (Hsf, Csf and Bsf). Five mice for each cage of the test groups and the control groups were assigned. The test subfractions were prepared in two doses (50 mg/kg and 100 mg/kg of body weight), CQ at 25 mg/kg in a volume of 0.2 ml and vehicles (20% DMSO) at 0.2 ml/mouse. These preparations were administered orally to the infected mice starting 3 hours post infection on D_0 orally. On the fifth day blood samples were taken from the tail snip of the mice to make thin blood smear on microscopic slides and microscopic examination was done following similar procedures as in the fractions. The percent parasitaemia and suppression were calculated using the above formula (Chen *et al*., 2010).

3.4.5 Evaluation of suppressive activity of chromatographic subfractions of Hsf

The chromatographic subfractions of Hsf (Hsf-1, Hsf-5, Hsf-7, Hsf-8 and Hsf-14), which were obtained from the active subfraction of EF were evaluated for their antiplasmodial activity against *P. berghei* infection in mice in the same way as the fractions but in much

lower dose. Twenty infected mice were randomly divided into two test groups and two control groups (each for CQ as a standard drug and 20% DMSO as a negative control) for each of the five tests: Hsf-1, Hsf-5, Hsf-7, Hsf-8 and Hsf-14. Five mice for each cage of the test groups and the control groups were assigned. The test subfractions were prepared in two doses (20 mg/kg and 40 mg/kg of body weight), CQ at 25 mg/kg in a volume of 0.2 ml and vehicles (20% DMSO) at 0.2 ml/mouse. These preparations were administered orally to the infected mice starting 3 hours post infection on D_0 orally. On the fifth day blood samples were taken from the tail snip of the mice to make thin blood smear on microscopic slides and microscopic examination was done following similar procedures as in the fractions. The percent parasitaemia and suppression were calculated using the above formula (Chen *et al*., 2010).

4. STATISTICAL ANALYSIS

Results of the studies were expressed as mean plus or minus standard error of the mean (M±SEM). Comparison of parasitaemia and statistical significance were determined by one way ANOVA followed by Scheffe‟s post-hoc test using SPSS Version 15.0. Level of significance was set as $P<0.05$.

5. RESULTS

5.1. Thin Layer Chromatography (TLC) and Column Chromatography (CC)

5.1.1. TLC analysis of fractions of *C. myricoides*

Ethanol extracts of the leaves of *C. myricoides* was analyzed on analytical TLC. Dried fractions of the plant material were dissolved in solvents and spotted on activated pre-coated aluminium oxide TLC plates. After the TLC of fractions of *C. myricoides* was developed, some spots were visible after spraying with vanillin in sulfuric acid and heating with hot air gun. The result obtained is shown below (Fig. 5).

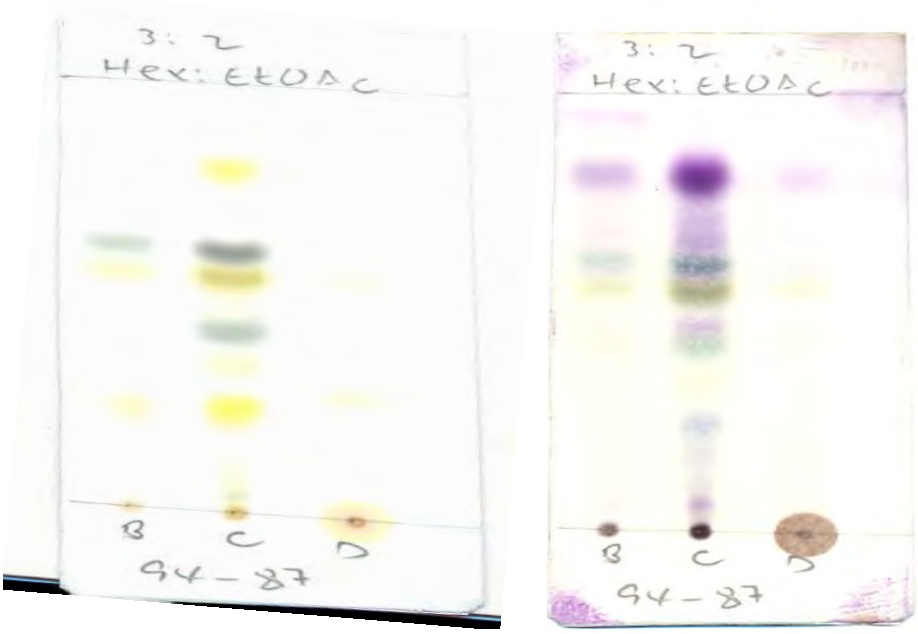

Fig. 5: Analytical thin layer chromatogram for HF (B), EF (C) and MF (D) of *C. myricoides* (Left is before sprayed and right is after sprayed with vanillin in H_2SO_4).

5.1.2. Chromatographic subfractions of the marc, MF

The eluted fractions obtained during chromatographic fractionation of MF were combined based on their TLC results to give six (6) methanol subfractions, designated as MF-1, MF-2, MF-3, MF-4, MF-5 and MF-6 (**Table 1**).

Table 1: CC subfractions of Mfr of *C. myricoides* ethanol extract.

Chromatographic subfractions of MF	Solvent system (Cyclohexane :DCM:MeOH)	Yield in mg	Volume collected in ml	Remark
MF-1(Mf_{1-9})	100% Cyclohexane	20	390	
MF-2(Mf_{10-17})	0:19:1	32	280	
MF-3(Mf_{18-20})	0:1:1	122	120	Inactive
MF-4(Mf_{21-30})	0:1:1	500	400	Inactive
MF-5(Mf_{31-40})	0:1:3	1900	400	Active
MF-6(Mf_{41-50})	0:1:9	2000	400	Active

5.1.3. TLC analysis of EF

The subfractions of the ethyl acetate fraction of *C. myricoides* were analyzed on analytical TLC. They were dissolved in solvents and spotted on activated pre-coated aluminium oxide TLC plates. After the TLC of fractions of *C. myricoides* was developed, some spots were visualized after sprayed with vanillin in sulfuric acid and heating with hot air gun. The result obtained is shown below (Fig. 6).

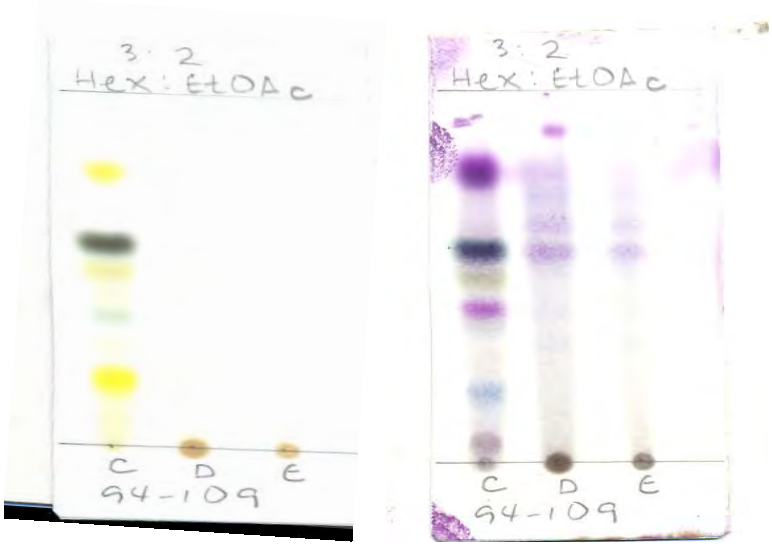

Fig. 6: Analytical TLC of subfractions of EF, hexane (C), chloroform (D) and n-butanol (E) (Left is before spraying and right is after spraying with vanillin in H_2SO_4).

5.1.4. Chromatographic subfractions of Hsf

The eluted fractions obtained during chromatographic fractionation of Hsf were combined based on their TLC results to give seventeen (17) subfractions, designated as Hsfr-1, Hsfr-2, ..., Hsfr-17. Hsf-5 and Hsf-14 were considered to be active in this study against *P. berghei* (Table 2).

Table 2: Column chromatography subfractions of Hsf of *C. myricoides* EF

Chromatographic subfractions of Hsf	Ratio of solvent system Hexane:EtOAc	Yield in mg	Volume calculated in ml	Remark
Hsf-1(F_{1-5})	1:0	290	150	Inactive
Hsf-2(F_{6-7})	1:0	90	60	
Hsf-3(F_{8-10})	9:1	100	90	
Hsf-4(F_{11-12})	4:1	98	60	
Hsf-5(F_{13-18})	7:3	273	150	Active
Hsf -6(F_{19-22})	7:3	113	60	
Hsf-7(F_{23-25})	1:1	301	90	Inactive
Hsf-8(F_{26-27})	3:7	952	60	Inactive
Hsf-9(F_{28})	3:7	42	10	
Hsf-10(F_{29})	3:7	30	10	
Hsf-11(F_{30-33})	1:4	76	90	
Hsf-12(F_{34-35})	0:1	14	40	
Hsf-13(F_{36-37})	0:1	67	40	
	EtOAc:MeOH			
Hsf-14(F_{38-41})	1:0	896	80	Active
Hsf-15(F_{42})	4:1	66	30	
Hsf-16(F_{43})	4:1	41	30	
Hsf-17(F_{44})	4:1	37	30	

5.1.5. TLC Analysis of Hsf-5

The TLC analysis of Hsf-5 was developed using hexane and ethyl acetate (3:2). A mixture of vanillin and sulphuric acid was used as visualizing agent. The appearance of the second spot was observed after heating with hot air gun. The result obtained is presented as below (Fig. 7).

Fig.7: Analytical thin layer chromatogram of Hsf-5

5.1.6. The isolated compound

The second fraction obtained using PTLC was recrystallized from methanol as a white solid crystal. The NMR spectrometer results of Hsf-5 showed the presence of one pure compound. Unfortunately the antimalarial activity of the purified compound cannot be determined because of its low amount obtained. The characterization of the pure compound obtained is in progress in the Chemistry Department of Addis Ababa University using Spectroscopic methods and other techniques.

5.2. *In vivo* Antiplasmodial Suppressive Test of *C. myricoides*

The microscopic examination of Giemsa stained slides of thin smears of blood from mice of each experimental and control groups were done. The fractions and subfractions did not eradicate parasites completely, but the percentage of parasitaemia was lower in all the mice administered with the fractions and subfractions of the *C. myricoides* leaves ethanol extract, indicating that they may have a direct action on the parasites, as compared to the negative control groups that were given the vehicle only. The positive control groups administered with CQ cleared the parasite completely on D_4; while the mice treated with the fractions and subfractions did not show complete parasite clearance. The test fractions and subfractions

which showed greater than 30% suppression were considered to be active (Krettli *et al.*, 2009).

5.1.1. Ethanol extract fractions

After four days treatment of mice with different ethanol extract fractions (MF, EF and HF), significantly reduced mean parasitaemia were found in mice treated with MF and EF as compared to those in the negative control group (treated with 0.2ml of 20% DMSO). The percent parasitaemia was 9.61 ± 0.38 and 8.07 ± 0.39 in those administered with MF at doses of 150 mg/kg and 300mg/kg, respectively, whereas the percent parasitaemia in the control group was 35.47 ± 0.46. Similarly, *P. berghei* parasitaemia was significantly reduced in mice treated with EF as compared to those in the negative control group. The percent parasitaemia was 14.23 ± .34 and 12.34 ± .45 at 150 mg/kg and 300mg/kg, respectively, whereas the corresponding value of the negative control group was found to be 35.47 ± 0.46. The mice treated with CQ were completely free from the parasites on D_4 (**Table 3**).

Statistical analysis using Scheffe"s procedure indicated that groups of mice treated with 150mg/kg and 300mg/kg MF and EF of the leaves of *C. myricoides* ethanol extract showed statistically significant difference in parasitaemia level as compared to the negative control group ($P<0.05$). MF at doses of 150 mg/kg and 300mg/kg showed a suppressive effect of 72.90% and 77.24% respectively, whereas EF at doses of 150 mg/kg and 300mg/kg showed a suppressive effect of 59.88% and 65.21%, respectively, which have statistically significant difference as compared to negative control ($P<0.05$). It was observed that there was a slight increase of percentage suppression with increase in fraction concentration (**Table 3**).

On the other hand, the mice administered with HF of *C. myricoides* leaves ethanol extract did not differ significantly in parasitaemia as compared to the mice in the control group ($P>0.05$). The mean parasitaemia of the study groups were 24.60 ± 0.84% and 23.51 ± 0.64% at 150mg/kg and 300mg/kg oral doses respectively, whereas the corresponding figure in the control group was 28.48 ± 0.41 (**Table3**).

Table 3: *In vivo* effect of methanol, ethyl acetate and hexane fractions of ethanol extract of *C. myricoides* leaves against *P. berghei* in mice.

Fractions of *C. myricoides* ethanol extract	Dose (mg/kg/day)	Antiplasmodial activity	
		% Parasitaemia ± SEM	% Suppression
Methanol fraction (MF)	NC	35.47 ± 0.46^{a}	0.00
	150	9.61 ± 0.38^{b}	72.90
	300	8.07 ± 0.39^{b}	77.24
Ethyl acetate (EF)	NC	35.47 ± 0.46^{a}	0.00
	150	14.23 ± 0.34^{b}	59.88
	300	12.34 ± 0.45^{b}	65.21
Hexane (HF)	NC	28.48 ± 0.41^{a}	0.00
	150	24.60 ± 0.84^{a}	15.77
	300	23.51 ± 0.64^{a}	17.45
Chloroquine	25	0.00^{b}	100.00

Values are Mean ± SEM; n=5, NC: Negative control (0.2 ml vehicle); a, b = Values in the same column followed by the same letter do not differ significantly (P>0.05).

5.2.2. Subfractions of EF

Significant reduction of parasitaemia ($P < 0.05$) was observed in all groups of mice treated with hexane subfraction (Hsf) of EF compared to the negative control. The percent parasitaemia of the mice treated with 50mg/kg of Hsf was 16.37 ± 0.49 and the mice that administered with 100mg/kg was 14.11 ± 0.51. The mean parasitaemia of the mice in the control group was $27.47 \pm 0.48\%$. The subfraction also induced an inhibition of parasitaemia by 40.34% and 46.56% at 50mg/kg and 100mg/kg doses respectively. In the case of treatment of the *P. berghei* infected mice with chloroform and *n*buthanol subfractions of EF, no significant reduction in parasitaemia was observed ($P>0.05$). The percent parasitaemia of Csf and Bsf at 50mg/kg and 100mg/kg were 23.60 ± 1.02 and 23.39 ± 1.28, and 20.32 ± 0.48 and 19.77 ± 0.58, respectively, which did not have significant difference from percent

parasitaemia of 25.40 ± 0.71 and 27.47 ± 0.48 of the control mice treated with 0.2 ml of 20% DMSO (**Table 4**).

Table 4: *In vivo* activity of Hsf, Csf, and Bsf of EF of *C. myricoides* leaves against *P. berghei* in mice.

Subfractions of EF	Dose (mg/kg/day)	Antiplasmodial activity	
		% Parasitaemia ± SEM	% Suppression
Hexane subfraction (Hsf)	NC	27.47 ± 0.48[a]	0.00
	50	16.37 ± 0.49[b]	40.34
	100	14.11 ± 0.51[b]	46.56
Chloroform subfraction (Csf)	NC	25.40 ± 0.71[a]	0.00
	50	23.60 ± 1.02[a]	7.08
	100	23.39 ± 1.28[a]	7.91
Buthanol subfraction (Bsf)	NC	27.47 ± 0.48[a]	0.00
	50	20.32 ± 0.48[a]	26.02
	100	19.77 ± 0.58[a]	28.03
Chloroquine	25	0.00[b]	100.00

5.2.3. Chromatographic subfractions of MF

The chromatographic subfractions (MF-3 - MF-6) obtained from the active fraction (MF) were also evaluated against *P. berghei* in mice. Significant reduction of parasitaemia ($P < 0.05$) was observed in all groups of mice treated with MF-5 and MF-6 compared to the negative control. The percent parasitaemia of the mice treated with MF-5 was found to be 18.63 ± 0.37 and 16.36 ± 0.44 at doses of 50mg/kg and 100mg/kg respectively, where as those treated with MF-6 was found to be 17.33 ± 0.48 and 15.45 ± 0.49 at doses of 50mg/kg and 100mg/kg respectively. The mean parasitaemia of the mice in the control group was 31.55 ± 1.603%. The chromatographic subfractions also induced an inhibition of parasitaemia by 48.14 and 40.95% at 50 mg/kg and 100 mg/kg of MF-5, respectively, and by 51.03 and 45.07% at 50 mg/kg and 100 mg/kg of MF-6, respectively (**Table 5**).

Nevertheless, in the case of treatment of the *P. berghei* infected mice with MF-3 and MF-4, no significant reduction in parasitaemia was observed (P>0.05). The percent parasitaemia of the mice treated with MF-3 was found to be 30.54 ± 0.41 and 30.27 ± 0.52 at doses of 50 mg/kg and 100 mg/kg respectively, where as those treated with MF-4 was found to be 30.17 ± 0.32 and 28.51 ± 0.43 at doses of 50mg/kg and 100mg/kg respectively. This mean parasitaemia of the study group did not have significant difference from 31.55 ± 1.603 parasitaemia of the control mice treated with 0.2 ml of 20% DMSO (**Table 5**).

Table 5. *In vivo* suppressive test of chromatographic subfractions of MF against *P. berghei* in mice.

Chromatographic subfractions of MF	Dose (mg/kg/day)	Antiplasmodial activity	
		% Parasitaemia ± SEM	% Suppression
MF-3	NC	31.55 ± 1.60[a]	0.00
	50	30.54 ± 0.41[a]	3.29
	100	30.27 ± 0.52[a]	4.05
MF-4	NC	31.55 ± 1.603[a]	0.00
	50	30.17 ± 0.32[a]	4.37
	100	28.51 ± 0.43[a]	9.63
MF-5	NC	31.55 ± 1.603[a]	0.00
	50	18.63 ± 0.37[b]	40.95
	100	16.36 ± 0.44[b]	48.14
MF-6	NC	31.55 ± 1.603[a]	0.00
	50	17.33 ± 0.48[b]	45.07
	100	15.45 ± 0.49[b]	51.03
Chloroquine	25	0.00[b]	100.00

5.2.4. Chromatographic subfractions of Hsf

Chromatographic subfractions of Hsf showed varying degrees of antiplasmodial activity. Significant reduction of parasitaemia ($P < 0.05$) was observed in all groups of mice treated with Hsf-5 and Hsf-14 compared to the negative control. The percent parasitaemia of the

mice treated with Hsf-5 was found to be 18.31 ± 0.39 and 16.74 ± 0.36 at doses of 20mg/kg and 40mg/kg respectively, where as those treated with Hsf-14 was found to be 18.50 ± 0.33 and 16.84 ± 0.67 at doses of 20mg/kg and 40mg/kg respectively. The mean parasitaemia of the mice in the control group were 33.84 ± 0.33 and 30.14 ± 0.65% for Hsf-5 and Hsf-14 respectively. These chromatographic subfractions also induced an inhibition of parasitaemia by 45.89 and 50.53% at 20 mg/kg and 40 mg/kg of Hsf-5, respectively, and by 38.61 and 44.12% at 20 mg/kg and 40 mg/kg of Hsf-14, respectively (**Table 6**).

Nevertheless, in the case of treatment of the *P. berghei* infected mice with Hsf-1, Hsf-7 and Hsf-8, no significant reduction in parasitaemia was observed ($P>0.05$). The percent parasitaemia of the mice treated with Hsf-1, Hsf-7 and Hsf-8 was found to be in a range of 25.96 ± 0.61 and 29.80 ± 0.47 at both doses. This mean parasitaemia of the study group did not have significant difference from 33.84 ± 0.33 and 30.14 ± 0.65 parasitaemia of the control mice treated with 0.2 ml of 20% DMSO. These chromatographic subfractions of Hsf also induced inhibition of parasitaemia by about less than 20% (**Table 6**).

Table 6: *In vivo* activity of chromatographic subfractions of Hsf against *P. berghei* in mice.

Chromatographic subfractions of Hsf	Dose (mg/kg/day)	Antiplasmodial activity	
		% Parasitaemia ± SEM	% Suppression
Hsf-1	NC	33.84 ± 0.33[a]	0.00
	20	28.87 ± 0.36[a]	14.68
	40	27.23 ± 0.29[a]	19.53
Hsf-5	NC	33.84 ± 0.33[a]	0.00
	20	18.31 ± 0.39[b]	45.89
	40	16.74 ± 0.36[b]	50.53
Hsf-7	NC	33.84 ± 0.33[a]	0.00
	20	29.80 ± 0.47[a]	11.93
	40	27.76 ± 0.34[a]	17.96
Hsf-8	NC	30.14 ± 0.65[a]	0.00
	20	27.56 ± 0.58[a]	8.56
	40	25.96 ± 0.61[a]	13.86
Hsf-14	NC	30.14 ± 0.65[a]	0.00
	20	18.50 ± 0.33[b]	38.61
	40	16.84 ± 0.67[b]	44.12
Chloroquine	25	0.00[b]	100

6. DISCUSSION

In the present study, methanol fraction obtained from ethanol extract of leaves of *C. myricoides,* did not show much difference in their percent suppression as the dose was doubled. So it is better to treat the mice with lower dose as maximizing the dose might cause toxicity without causing significant inhibition. This result is in agreement with the result of the study of Tadesse (2011) that reported suppressive effect of 69.31% at 150mg/kg oral dose of methanol fraction. Furthermore, the methanol crude extract was found to show high activity, with suppressive effect of 82.25% at 600mg/kg against *P. bergei* in mice as reported by Deressa *et al*. (2010), and with IC_{50}=16.8 + 2.65µg/ml as demonstrated in Muregi *et al.* (2004).

Ethyl acetate fraction obtained from ethanol extract of leaves of *C. myricoides,* did not show much difference in their percent suppression. So it is better to treat the mice with lower dose as maximizing the dose might cause toxicity without causing significant inhibition. The obtained suppression is in agreement with the study of Tadesse (2011) that reported suppressive effect of 61.30 at 150mg/kg oral dose of ethyl acetate fraction. Muregi *et al.* (2004) also reported the mild antiplasmodial activity of ethyl acetate extract *in vitro* with IC_{50} of 48.6 $\pm$ 1.43µg/ml. The TLC result of this fraction showed that there are many compounds that are might responsible for antimalarial activity. Assefa *et al*. (2007) also reviewed that ethanol, petroleum ether, ethyl acetate and aqueous root bark extracts of C. *myricoides* exhibited antimalarial activities *in vitro* with IC_{50} values of 300µg/ml, 47µg/ml, 11µg/ml and 300µg/ml, respectively.

All the above results showed that the antimalarial activity of the plant is mainly contained in the polar and medium polar soluble part of the plant crude extracts and fractions as evident from the suppressions obtained using *in vitro* and *in vivo* activity test (Tadesse, 2011). These observations suggest that the active constituents in the fractions might be cytotoxic for *P. berghei*, thereby inhibiting their development to the erythrocytic stage. Moreover, these studies have confirmed *C. myricoides* is among the plants that displayed interesting antimalarial activity *in vitro* as well as *in vivo* systems.

On the contrary, the HF reduced only in a limited percent the parasitaemia and was considered to be inactive (Krettli *et al.*, 2009). This result is similar with the work of Tadesse

(2011), which reported low suppressive effect of 19.84% at 150mg/kg oral dose of hexane fraction, except slight difference. There was also a report by Muregi *et al*. (2004) which indicated *C. myricoides* hexane extract was found to exhibit limited antiplasmodial activity *in vitro* against *P. falciparum* with IC50 value greater than 100µg/ml. This observation might indicate the antimalarial active components of the crude extract and fractions obtained from it might not have non polar properties (Deressa *et al*., 2010).

Hexane subfraction obtained from EF induced an inhibition of parasitaemia significantly as compared to the negative control and hence considered to be active (Krettli *et al.*, 2009). The parasitemia suppression of Hsf is reduced as compared to EF. This result might indicate that either the active components responsible for the suppression of parasitenia in EF treated mice are partitioned into Hsf, Csf and Bsf or the in active components of EF are important for the enhancement of antiplasmodial activity of EF.

Phytochemical studies of plants used as antimalarial in traditional medicine of different countries revealed the presence of terpinoides, alkaloids, flavonoides, glycosides and quassinoids (Batista *et al.*, 2009; Kaur *et al.*, 2009; Bero *et al.*, 2009) that are responsible for antimalarial activity, but the most important and diverse biopotency has been observed in alkaloids, quassinoids and sesquiterpene lactones (Adebayo and Krettli, 2011). Sesquiterpene lactone found in artemisinin is responsible for the antimalarial activity of the drug (Bero *et al.*, 2009). Alkaloids are also one of the major classes of compounds possessing antimalarial activity. One of the oldest and most important antimalarial drugs, quinine, belongs to this class of compounds (Saxena *et al*., 2003). Pascaline *et al*. (2011) revealed that *C. myricoides* contains alkaloids, saponins, glycosides, terpenoides, steroids, phenolics and flavonoides. So, these compounds might be responsible for the antimalarial activity of the fraction and subfractions of the plant.

In the present study, the MF and the subfractions obtained from it (MF-6 and MF-7) and EF and the subfractions obtained from it (Hsf, Hsf-5 and Hsf-14), which might be enriched in alkaloids, terpenoides, steroids, phenolics and flavonoides are found to show good suppression by acting synergistically against *P. berghei* in infected mice. Furthermore, the TLC results of these fractions showed the presence of many compounds which might inhibit *P. berghei* growth using different mode of action. These compounds might exert their

antiplasmodial activity by causing interference with the parasites ability to eliminate the toxic by product of hemoglobin digestion, inhibition of *P. falciparum* lactate dehydrogenase, directly killing young intraerythrocytic malaria parasites or by inhibiting protein synthesis of the parasite or by other unknown mode of action other than those mentioned above (Adebayo and Krettli, 2011, Mishra *et al.*, 2011; Hobbs and Duffy, 2011).

Hsf-5 and Hsf-14 obtained from the hexane subfraction showed significant reduction of parasitaemia as compared to the negative control. The TLC results of Hsf-5 showed the presence of two spots which may correspond with two different compounds. Hence, the activity of the fraction is due to synergistic effect of two compounds, as the above fractions. The NMR spectrometer analysis of Hsf-5 leads to the isolation of one pure compound, which might be the major component of the subfraction. It also could be the active compound responsible for antimalarial activity of EF in general or Hsf-5 in particular.

Importantly, many cases are known where the crude biological extract is more efficient pharmacologically than the most active purified compound from this extract (Ginsburg and Deharo, 2011). In this study, the antimalarial activity of the plant is generally reducing as we further fractionate the active fractions. The efficient antimalarial activity of the fractions as compared to subfractions could be due to synergism with other compounds present in the fraction, which as such have no pharmacological activity (Ginsburg and Deharo, 2011). This synergism between two or more compounds might result from binding to the same target protein such that a conformational change caused by the binding of compound A enhances the binding of compound B, binding of compound A to a transporter causing increased uptake of compound B into the cell or the sub cellular compartment in which it acts, formation of a complex between compound A and compound B enhance toxicity or stimulation by A of the conversion of B to a more active form (Bell, 2005). Moreover, lost activity of the plant during the fractionation process is could be attributable to the instability of the actives due to oxidation or chemical break down of the active molecule (Wells, 2011). So, the plant should be prepared in the form of crude extract as the highest activity is obtained due the synergistic effect of many compounds in the crude extracts

C. myricoides is commonly grown in many parts of Ethiopia. People prepare it in form of decoction and use it against malaria, diarrhea, relapsing fever, and abdominal colic (Assefa *et al*., 2007). Other traditional uses of *Cleodendrum* plant include treatment for gonorrhea, gout,

swelling, wound dressing and rabies (Shrivastava and Patel, 2007). With regard to interpreting these results in relation to the traditional uses of the plant studied, the claim by traditional practitioners for the use of the plant against malaria medication is in parallel lining with the scientific evidence of this study.

The results of this study in addition to the confirmation of the traditional use of the plant *C. myricoides*, the bioactivity guided fractionation of the ethanol extract yielded one pure compound, which can exhibit good antiplasmodial activity against *P. berghei*. Considering this a large percentage of Ethiopian plants have not been investigated chemically or pharmacologically, they remain a potential source of leads for possible drug development.

7. CONCLUSION

The methanol and the ethyl acetate fractions of the ethanolic extract of *C. myricoides* leaves and some of the sub fractions obtained from them using bioactivity guided fractionation suppress *P. berghei* infection in Swiss albino mice in a dose related manner. Antimalarial activity of the plant is mainly contained in the polar and medium polar soluble part of the plant crude extract. Further fractionation of MF and EF provided MF-6 and MF-7 and Hsf, Hsf-5 and Hsf-14, respectively, which still showed good level of suppressive effect against *P. berghei* but with lower effect. So fractionation resulted in the reduction of antiplasmodial activity of the plant and this leads to the conclusion that the plant should be used in its crude form. The NMR spectrometer analysis of Hsf-5 leads to the isolation of one pure compound. This plant therefore represents a potential source of new compounds, confirming ethnopharmacology as an important approach in the search for new antimalarial compounds. TLC, CC and NMR results of the fractions and subfractions showed that the activity of the plant leaves was due to synergistic effect of many compounds and the results also proved the plant is rich in chemical constituents.

8. RECOMMENDATION

- As toxicity is a very important parameter for a suitable lead candidate in the development of antimalarial drugs, the active fractions and subfractions also have to be further investigated using various cell lines, as well as animal models.

- Further biological studies are needed in order to evaluate and elucidate the mechanism of antimalarial action of the compounds, which were obtained from active fractions, as well as their structure-activity relationships, and then optimize the doses.

- The identification and validation of antimalarial active compounds of the traditional plants of Ethiopia should involve researchers from different disciplines in order to achieve better results.

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Appendixes

Appendix 1: Photographs showing the different laboratory activities


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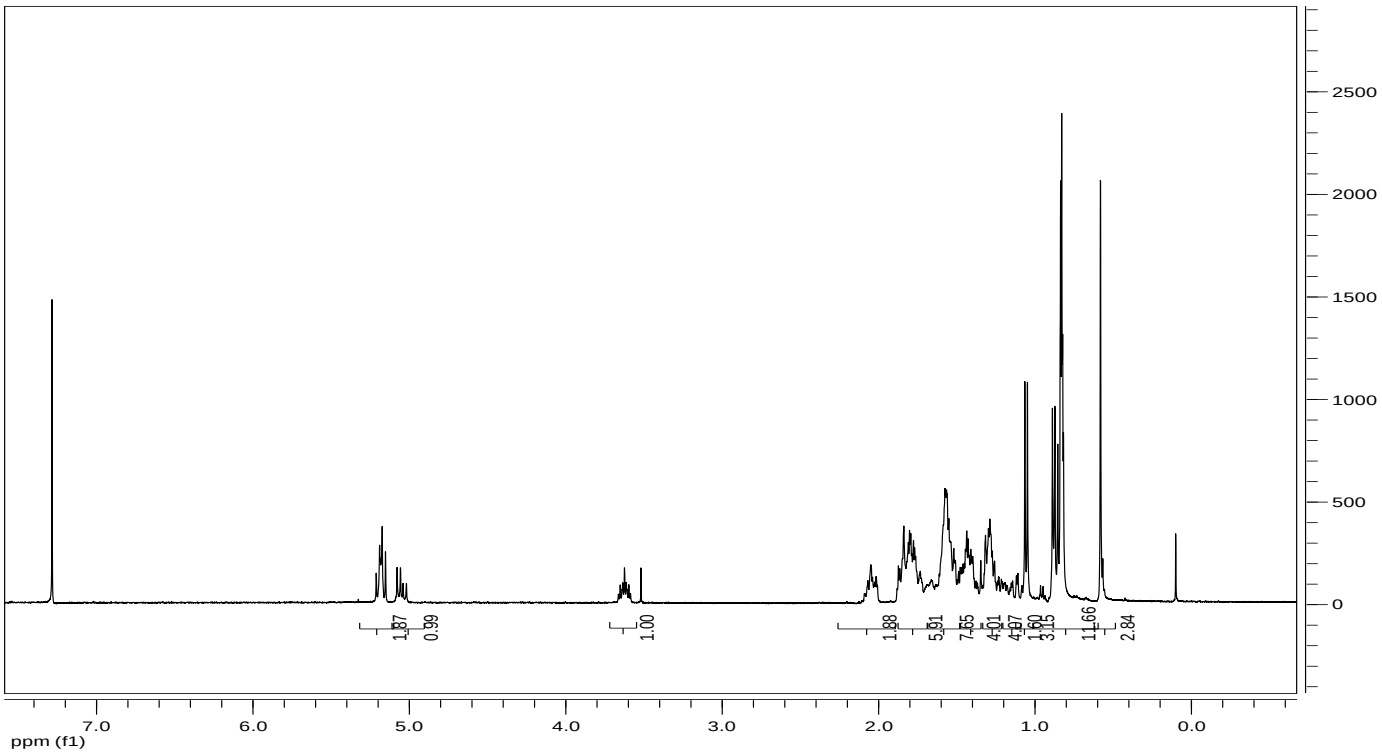
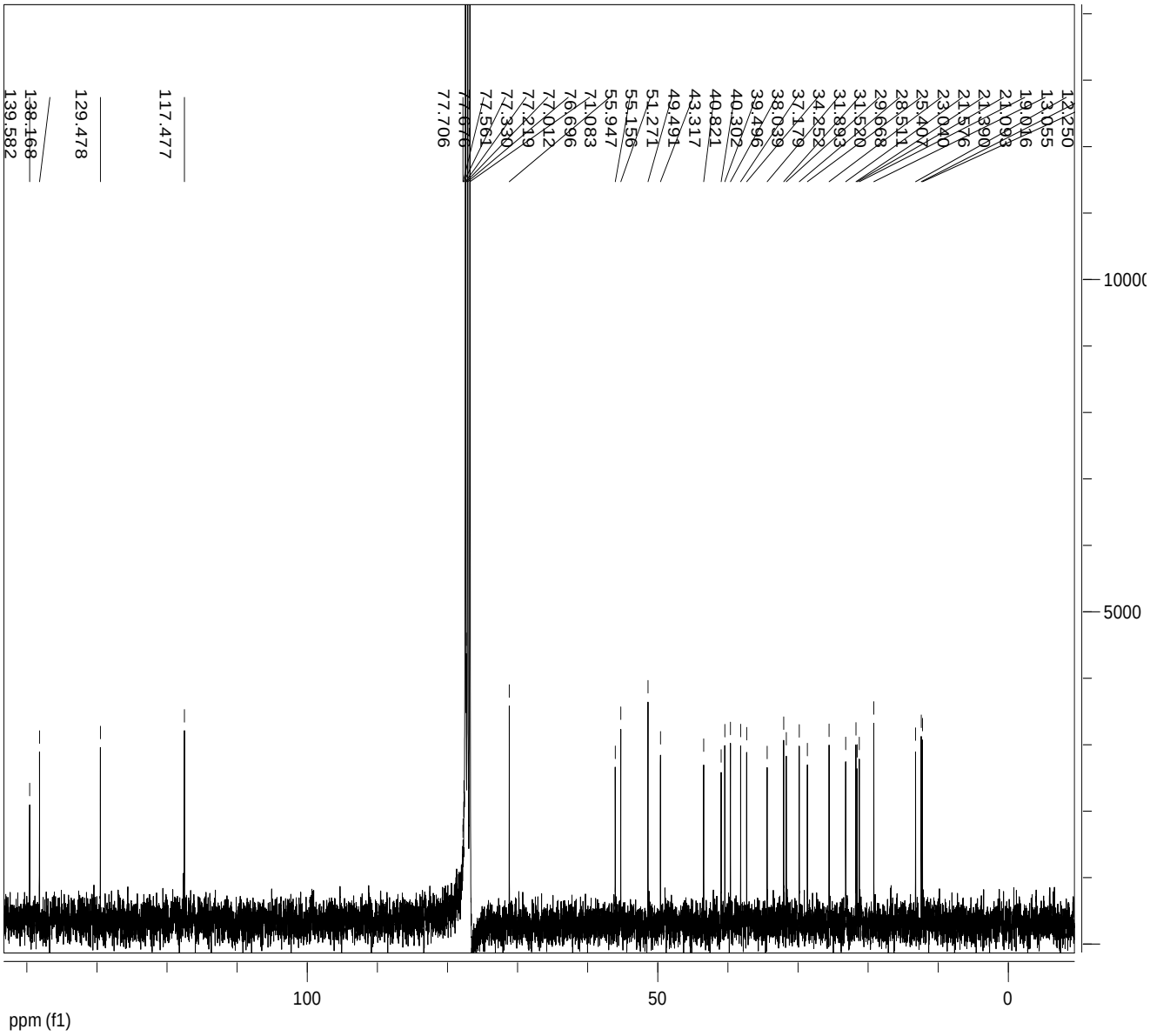
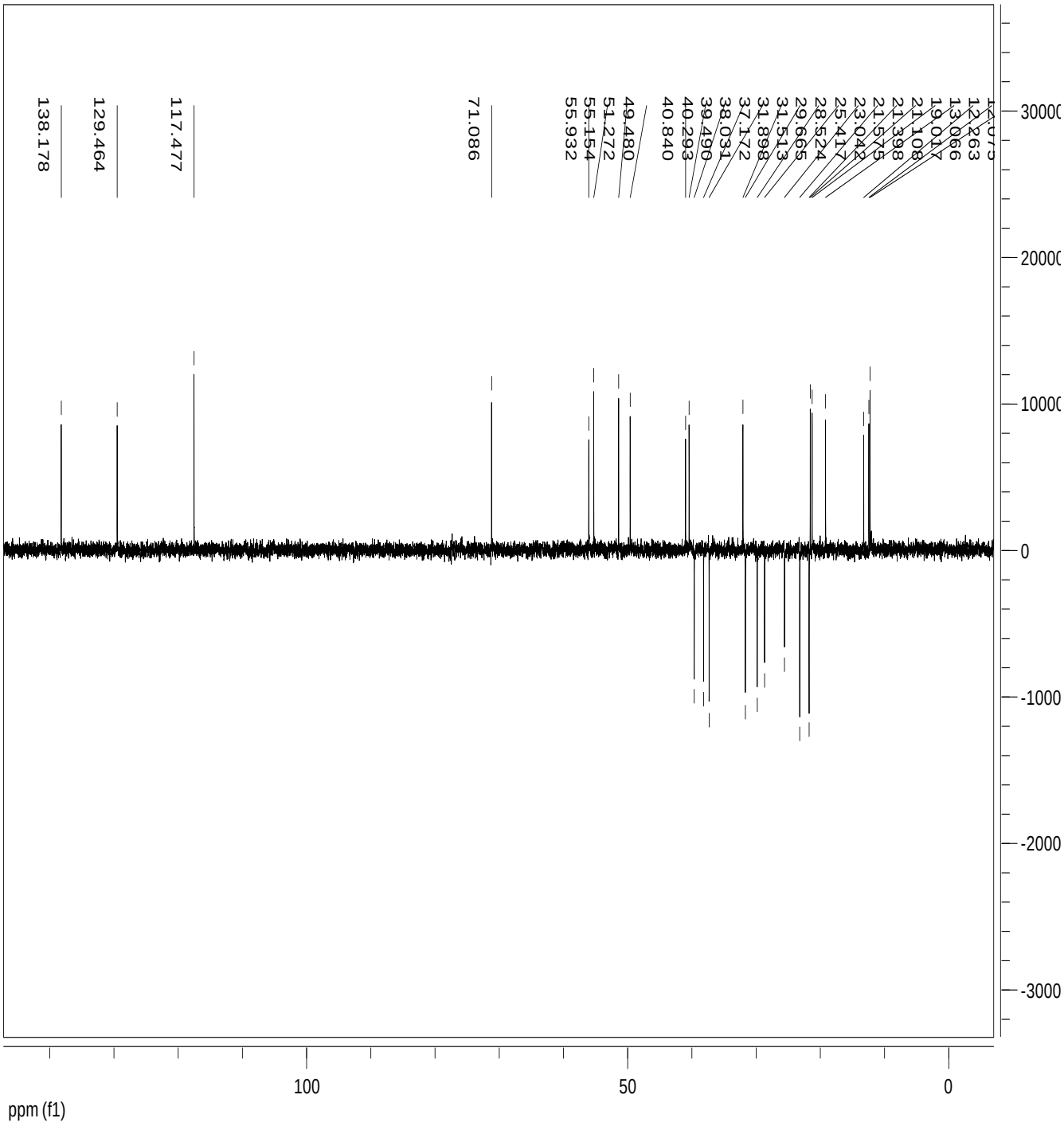
Appendix 2: Proton NMR Spectrum of the pure compound



Appendix 3: 13C NMR Spectrum of the pure compound



Appendix 4: DEPT-135 Spectrum of pure compound



Appendix 5: Average Percent Parasitemia of Ethanol Fractions

NC-1	MF		EF		NC-2	HF	
	Dose (mg/kg/day)		Dose(mg/kg/day)			Dose(mg/kg/day)	
	150	300	150	300		150	300
35.48	9.98	8.07	14.26	12.35	28.48	24.36	23.28
34.24	10.98	9.05	13.13	14.30	26.24	20.16	24.38
36.72	8.02	7.09	15.13	10.40	30.72	28.52	27.31
35.44	11.89	10.09	16.51	12.20	29.72	22.52	25.27
35.52	7.09	6.05	12.01	12.50	27.24	26.16	19.20
36.22	10.09	8.09	13.51	13.40	30.48	27.33	24.18
34.74	8.79	8.05	15.01	11.30	26.48	21.39	22.38
37.06	9.09	7.07	13.22	12.60	28.24	25.16	24.23
33.90	9.89	9.07	15.30	12.10	28.72	26.66	26.31
32.32	10.89	10.13	14.42	15.30	29.28	28.16	21.19
38.64	8.79	6.01	14.10	9.40	27.78	20.51	22.34
35.42	9.88	8.11	14.24	12.34	28.47	24.36	22.12

NC-1= Negative control for Methanol fraction and Ethyl acetate fraction

NC-2= Negative control for Hexane fraction

Appendix 6: Average Percent Parasitemia of Methanol Subfractions

NC	MF-3		MF-4		MF-5		MF-6	
	Dose (mg/kg/day)		Dose (mg/kg/day)		Dose(mg/kg/day)		Dose (mg/kg/day)	
	50	100	50	100	50	100	50	100
25.50	31.00	30.50	30.12	30.27	18.71	16.33	17.24	15.25
40.70	30.21	29.25	29.98	28.25	20.22	17.23	20.24	17.23
39.80	29.91	31.25	29.97	29.75	17.20	15.25	14.24	14.85
36.70	28.41	28.45	28.42	27.25	18.32	18.20	16.44	16.75
35.10	32.85	32.05	31.27	28.45	19.10	14.46	18.02	17.27
28.30	31.45	28.25	30.78	26.45	20.27	15.25	17.20	13.23
30.30	32.43	32.25	29.47	30.25	17.15	17.41	19.23	15.68
25.30	31.21	30.13	28.27	25.89	19.85	18.26	15.25	14.98
26.00	29.11	30.45	32.12	27.78	17.60	14.40	18.65	18.23
26.20	28.22	33.23	31.27	28.17	19.95	14.65	16.21	13.01
32.39	31.18	27.03	30.27	29.45	16.55	18.60	18.03	15.75
32.38	30.59	30.45	30.11	30.25	18.71	16.31	17.28	13.22

Appendix 7: Average Percent Parasitemia of Ethyl acetate Subfractions

NC-1	Hsf		Bsf		NC-2	Csf	
	Dose (mg/kg/day)		Dose(mg/kg/day)			Dose(mg/kg/day)	
	50	100	50	100		50	100
27.47	16.38	14.67	20.33	19.67	30.20	21.24	22.81
25.42	16.66	14.34	20.44	18.24	26.40	25.64	25.51
29.52	16.10	10.00	20.22	20.43	26.30	18.28	20.12
26.84	13.23	17.22	22.13	21.32	24.20	28.60	28.42
28.14	19.53	12.12	18.53	16.34	22.80	26.80	17.20
27.64	17.16	13.14	17.12	23.00	26.00	20.08	29.25
27.30	15.60	15.20	23.52	21.27	22.10	19.22	16.37
24.22	14.27	14.23	19.41	18.07	21.50	27.66	30.15
30.72	18.49	15.11	21.25	17.24	27.20	24.33	22.78
27.27	17.33	14.27	19.21	22.10	27.40	24.55	20.54
27.67	15.43	14.40	21.45	19.23	25.41	20.73	24.80
27.47	16.37	14.67	20.32	20.34	25.40	26.15	22.82

NC-1= Negative control for hexane subfraction and buthanol subfraction

NC-2= Negative control for chloroform subfraction

Appendix 8: Average Percent Parasitemia of Hexane Subfractions

NC-1	Hsf-5		Hsf-1		Hsf-7		NC-2	Hsf-8		Hsf-14	
	Dose (mg/kg/day)		Dose (mg/kg/day)		Dose (mg/kg/day)			Dose (mg/kg/day)		Dose (mg/kg/day)	
	20	40	20	40	20	40		20	40	20	40
34.48	18.24	16.13	29.21	27.98	32.17	26.98	30.63	25.27	27.73	18.48	16.25
33.25	19.38	17.28	28.78	26.21	29.87	28.13	31.27	27.21	28.12	17.92	17.78
35.56	17.45	15.98	26.97	29.31	28.78	27.21	27.28	23.98	25.45	19.27	15.14
34.25	15.26	19.21	30.08	26.78	26.98	28.21	26.29	29.12	27.23	16.79	19.73
33.23	19.34	14.88	31.21	27.32	31.21	30.14	32.41	21.78	30.41	17.98	13.71
32.52	20.28	15.87	27.28	28.13	31.13	28.21	28.21	26.23	25.21	20.34	14.21
31.69	18.13	16.75	28.78	26.78	30.19	29.15	31.43	27.21	28.29	20.09	17.31
34.32	19.21	17.25	29.21	27.21	28.28	25.98	32.19	24.78	26.27	17.49	15.41
35.13	17.98	16.68	28.95	26.98	30.21	27.21	28.47	27.57	29.31	18.12	19.37
33.65	18.21	18.31	27.89	27.11	28.21	26.97	31.21	27.21	24.97	19.21	20.21
34.21	17.98	15.89	29.26	25.73	30.85	27.25	32.18	25.27	30.27	17.81	16.21
33.84	33.84	33.84	33.84	33.84	33.84	33.84	33.84	33.84	33.84	33.84	33.84

NC-1= Negative control for hexane subfraction Hsf-5, Hsf-1and Hsf-7

NC-2= Negative control for hexane subfraction Hsf-8 and Hsf-14