
**ADDIS ABABA UNIVERSITY**

**SCHOOL OF GRADUATE STUDIES**

**FACULITY OF LIFE SCIENCES**

# Bioactivity Guided Study on the Antimalarial Activities of *Clerodendrum myricoides* and *Dodonaea angustifolia*

By

**Yemane Tadesse**

Thesis Submitted to the School of Graduate Studies in Partial Fulfillment of the Requirements for the Degree of Masters of Science in Biology (Biomedical Science)

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Approved by Examining board

| Name | Signature |
|---|---|
| Prof. Yalemtsehay Mekonnen (Advisor) | _____ |
| Dr. Mekuria Lakew | _____ |
| Prof. Beyene Petros | _____ |
| Dr. Fassil Assefa | _____ |

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# List of Abbreviations

| | |
|---|---|
| ACTs | Artemisinin-based Combination Therapies |
| ANOVA | Analysis of variance |
| $CHCl_3$ | Chloroform |
| CQ | Chloroquine |
| CSA | Central Statistical Authority |
| DEPT | Distortionless enhancement polarization transfer |
| DMSO | Dimethyl sulfoxide |
| EtOAc | Ethyl acetate |
| FMOH | Federal Ministry of Health |
| $F_{1\text{-}9c}$ | One to nine fractions obtained from ethyl acetate extract of leaves of *C. myricoides* eluted with 3:2 cyclohexane and ethyl acetate respectively. |
| $F_{1\text{-}3d}$ | One to three fractions obtained from ethyl acetate extract of leaves of *D. angustifolia* eluted with 100% cyclohexane. |
| $F_{4d\text{-}10d}$ | Four to ten fractions obtained from ethyl acetate extract of leaves of *D. andustifolia* eluted with 8:2 cyclohexane and ethyl acetate respectively. |
| $F_{11d}$ | Eleventh fractions obtained from ethyl acetate extract of leaves of *D. anfustifolia* eluted with 7:3 cyclohexane and ethyl acetate respectively. |
| $F_{12d \text{ and } 13d}$ | Twelfth and thirteenth fractions obtained from ethyl acetate extract of leaves of *D. angustifolia* eluted with 1:1 cyclohexane and ethyl acetate respectively. |
| IP | Intraperitonial |
| ITNs | Insecticide-treated mosquito nets |
| NMR | Nuclear Magnetic Resonance |
| % Para | Percentage parasitaemia |
| % Supp | Percentage Suppression |

| | |
|---|---|
| RBC | Red blood cells |
| SEM | Standard error of mean |
| SPSS | Statistical Package for Social Science |
| TLC | Thin Layer Chromatography |
| WHO | World Health Organization |

# Abstract

Malaria constitutes one of the major health problems in Ethiopia. One of the reasons attributed for the increase was the development of resistance of *Plasmodium falciparum* to antimalarial drugs. A continued search for other effective, safe and cheap plant-based antimalarial agents thus becomes urgent in the face of these difficulties. The objective of the present study was therefore to evaluate *in vivo* antiplasmodial activities of successive extracts of *Clerodendrum myricoides* and *Dodonaea angustifolia* leaves and possibly isolate active ingredients. They were evaluated for their antimalarial activity in 4-day suppressive assays against *Plasmodium berghei* strain in Swiss albino mice. The concentrated methanol extract of leaves of *C. myricoides* and *D. angustifolia* presented relatively high activities, which reduced the parasitemia by 76.66% and 80.89% at an oral dose of 300mg/kg/day, 76.99% and 78.27% at an oral dose of 500mg/kg/day respectively. On the other hand, the chloroform/ethyl acetate (1:1) and chloroform extracts of these plants did not inhibit the parasitaemia significantly. Ethyl acetate extract obtained from successive extraction of methanol extract of *D. angustifolia* suppressed parasitaemia significantly (82.24% at 150mg/kg). Similarly the same dose of ethyl acetate and residue (ethyl acetate insoluble) extract of *C. myricoides* methanol extract also showed moderate inhibition activity with 61.30% and 69.31% mean suppression respectively. The TLC, Column chromatography and NMR spectrometer results of the extracts of these plants indicated that they are rich in natural chemical constituents. The results showed encouraging indications for further refined work on the use of these plants as sources of antimalarial plants.



**KEY WORDS**: *Clerodendrum myricoides*, *Dodonaea angustifolia,* malaria, medicinal plants, *Plasmodium berghei*, traditional medicine

# 1. Introduction

## 1.1 Herbal medicine

Herbal medicine is the use of plant's seeds, berries, roots, leaves, bark, or flowers for their therapeutic or medicinal value (Seekers, 2009). According to Barboza *et al.* (2009), herbal medicine is any manufactured medicine obtained exclusively from plants, either in the crude state or as a pharmaceutical formulation. Herbal plants produce and contain a variety of chemical substances that act upon the body. Therefore any plant that contains active materials for treating the body or that can be the origin of pharmacologically active drugs is medicinal plant. Medicinal plants make up the base of heath care systems in many societies as part of traditional medicine (Ahmad *et al*., 2009). Dori *et al.* (2008) defined traditional medicine as "the knowledge, skills and practices of holistic health care, recognized and accepted for its role in the maintenance of health and the treatment of diseases. It is based on indigenous theories, beliefs and experiences that are handed down from generation to generation".

The history of herbal medicine is dated back to ancient mankind who used plants for the treatment of a range of diseases in addition to their food, shelter and aromatic values. A lot of ancient healing traditions gave rise to the familiar herbal medications of the twentieth century (Desalegn, 2000). This can be approved through the written evidences during the great civilizations of the ancient Chinese, Indians, and North Africans that showed man's creativity in utilizing plants for the treatment of a wide variety of diseases. During the civilization of the ancient Greece, scholars classified plants by giving descriptions that help their identification process. This can be mentioned as an example of the focus of ancient society on herbal medicine (Philipson, 2001). Other written documents indicated some of the oldest medicinal systems of the world that were in use thousands of years ago. Ayurveda of Indus Civilization around 2500 BC (Amit *et al*., 2010), Arabian Medicine of Mesopotamia, Chinese and Tibetan Medicine of the Yellow River Civilization of China and Kempo of the Japanese used herbal medicine. During these ancient civilizations it is recorded that there was systematic collection of information and well defined herbal pharmacopoeias (Barboza *et al.* 2009).

As reported by World Health Organization (2010), 80% of the population of the world, particularly in developing countries presently uses herbal medicine for primary health care. Herbal medicine is a major component in all indigenous peoples' traditional medicine. As noted by Maregesi *et al*. (2007), in many countries, traditional medicines are deeply rooted in their cultures. Specially, in the poor communities medicinal plants have remained the most affordable and accessible source of treatment in controlling several human and animal ailments. This means that about 3.5 to 4 billion, which is the majority of the global population rely on plants resources for drugs (Lawal *et al*., 2010).

According to the report of Barboza *et al*. (2009) and Muthee *et al*. (2011), in excess of 50,000 flowering plants serve for medicinal purpose today. These medicinal herbs can be found throughout the world, but they are not as abundant as in the tropical countries. For example Tanzania, a country with 10,000 plant species of which 1,100 are endemic, is located in this zone. Most of the drugs which are currently in clinical use are originated from the tropical area. So it is hopeful to search for bioactive therapeutic substances of plants with special focus on this region (Maregesi *et al*., 2007). According to the information from WHO (2010), from 119 plant derived pharmaceutical medicines, about 74% are used in modern medicine in ways that associated directly with their traditional uses as herbal medicines by cultural practice. Most of these herbs are originated from the tropical forest of the world. This shows that there is high correlation of the tropical traditional herbal knowledge with the modern well designed experimental drug production.

Since recently, science has been isolating the medicinal properties of a large number of botanicals by extracting the healing components and using for production of plant derived modern drugs (Amit *et al*., 2010). Even though a great advancement of modern medicine has been observed in recent decades, plants still make an important contribution to health care. Convincingly, there is a revival of an interest in plant derived modern drugs. As a result, 50% of all drugs in clinical use today are reported to be the natural products (Lawal *et al*., 2010), out of this 25% are the results of plants (Calixto, 2002). Herbal medicine has become a crucial treatment regimens and a subject of interests for the pharmaceutical companies for the following reasons. Primarily, it has little side effects as

opposed to modern drugs (Lawal *et al*., 2010). Secondly, the traditional medicines are cheap and easily available in the markets (raw materials), especially in developing countries as compared to modern drugs which are very expensive (Tariq *et al*., 2008). Thirdly, traditional medicines are the reservoirs of ethnomedical and ethnobotanical information which is the keys for opening many new modern drug leads i.e. they serve as source of important pure chemicals that have become mainstays of modern therapy (Shrivastava and Patel, 2007). Lastly, as reported by Barboza *et al.* (2009) the chemical diversity and versatility of active components of herbal plants as compared to synthetic drug chemicals make them more preferable to conventional synthetic drugs. So the huge body of traditional knowledge of herbal plants is becoming part of the scientific knowledge.

Herbs are available in variety of forms, including fresh, dried, in tablets or capsules, or bottled in liquid form (Bent, 2004). They can be prepared as individually or in mixtures of different remedies. But these different herbal product preparations do not compromise the quality of the raw herb. That is, any form of the preparation whether it is in capsule, tablet, tea, tincture, bath, compress, or ointment form is the same with the quality of the raw herb from which it was prepared (Seekers, 2009). For example, the treatment route of malaria includes oral eating and drinking, bathing and steam inhalation of the aqueous herbal preparations for 4-10 days or until symptoms of malaria disappear (Idowu *et al*., 2010).

As reported by the pupa's health information team (2009), herbal medicines can have an adverse effect like any medicine. Many people believe that because medicines are herbal or traditional they are safe (Bent, 2004). However, traditional medicines and practices can cause harmful, adverse reactions if the product or therapy is of poor quality, or it is taken inappropriately or in combination with other medicines (Anastasi *et al*., 2011). Patients must have good awareness before using herbal medicine (WHO, 2010). According to Anastasi *et al*. (2011), there are some recommendations that must be strictly followed during herbal medicine use. Patients such as pregnant, in surgery operation or with allergens are not advised to take herbal medicines. Moreover, taking herbal

medicine together with modern medicine may have an adverse effect on the body. Optimizing the dosage is also very important since it can have unwanted consequence.

African traditional medicine is mainly based on herbal remedies. In African countries like Ghana, Mali, Nigeria and Zambia, for example, herbal medicine is used for 60 % of children with high fever resulting from malaria as first line of treatment in the home. Traditional herbal medicine is also becoming popular in many other countries of Asia and Latin America. Moreover, it is also showing incredible spreading even in the industrialized countries (Kassaye *et al*., 2006).

As the report of recent studies, the prevalence of herbal medicine has shown steady growth in the developed countries (Anastasi *et al*., 2011). Bent (2004) reported that about one fourth of adults in the United States are known to use herbal medication within the past years to treat different human illnesses. Similarly, in China 30 %- 50% of the overall medicinal consumption nowadays is traditional herbal preparation (Kassaye *et al*., 2006).

Several factors have been related to patients' use of herbal remedies in the developed as well as developing countries. Of these factors doubt about the efficacy of conventional medicine, acceptance of a holistic approaches of herbs, poor health status and chronic health problems have all been found to positively influence the use of alternative therapies (Bennett and Brown, 2002).

In Ethiopia, medicinal plants have been used as traditional medicine to treat number of human and animal ailments by the local people from time immemorial. 80% of the population in Ethiopia use traditional medicine, mainly herbal plants (Kassaye *et al*., 2006). Even though these plants are used extensively, a little has been done to evaluate their toxicity and efficacy in a scientific way (Gidey *et al*, 2010)**.** These medicinal plants are estimated to be over 700 species and most of them are confined to the Southwestern regions of the country. However, since they are not documented scientifically and the skills are easily forgettable as most of the indigenous knowledge transfer in the country is based on oral transmission, this knowledge may disappear after a period of time (Yinger and Yewhalaw, 2007).

## 1.2 The malaria disease

Malaria, the name, is derived from the Italian word *mal'aria,* for ''bad air". This is to describe the swampy areas in Europe in which the disease was prevalent (Amorosa *et al*, 2005). But later, the etiological agent is known to be the protozoan parasites (Ridder *et al.*, 2008). Malaria is one of the world's most serious infectious diseases caused by *plasmodium* parasites (Cropper *et al*., 2004). The four most common *Plasmodium* species that attack humans are *P. vivax*, *P. ovale*, *P. malariae*, and *P. falciparum*, as well as mixed infections. *P. falciparum*, one of the four malaria causing *plasmodium* in humans, is the cause of most of the mortality and morbidity. Malaria has been challenging human health and losing the lives of many people since long period of time (Moss, *et al*., 2008). It is one of the most prevalent, devastating parasitic infectious diseases in the world.

Each year, more than 300–500 million clinical cases and 1.5–2.7 million deaths associated with malaria were reported globally in the past years, most of them were children (Angayarkanni *et al*., 2010; Garcia, 2010). As reported by Garcia (2010), more than 90 countries with an estimated 2400 million people are suggested to be attacked by endemic malaria. Approximately 90% of malaria deaths occur in Africa. According to the World Health Organization (2003), malaria is endemic in many countries, predominantly in Africa, Asia and Latin America. 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 (WHO, 2009).

However, recent world malaria report declared good news about the remarkable reduction in malaria burden. 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. There was also a reduction of number of deaths from 985,000 in 2000 to 781, 000 in 2009. 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 incriminated to rapid diagnostic test prior to treatment, scaling up of insecticide-treated

mosquito nets (ITNs) distribution in the malarious areas and advancing the production of artemisinin-based combination therapies (ACTs) (WHO, 2010).

Malaria is a major public health problem in Africa. It has afflicted man and animals for over a century. Each year an estimated 300–500 million clinical cases of malaria occur (Onwuamah, *et al*., 2010). Nevertheless, there is a spatial and temporal variation in mortality and morbidity of malaria particularly in semiarid and high land regions of Africa. In these regions, unstable and epidemic malaria is the cause of around 12.74 million clinical cases and 155,000–330,000 deaths annually (Yeshiwondim *et al*., 2009).

Africa faces the greatest economic impact of this disease, particularly in Sub Saharan countries with children and pregnant women are the main targets (Idowu *et al*., 2010). According to Chima *et al*. (2003), the economic costs of malaria can be classified as direct and indirect. The direct one is the costs of expenditure on prevention and treatment. The indirect impact is the costs of productive labor time lost due to malaria morbidity and mortality. Therefore, the total economic impact of malaria is the combination of the two components. Its direct and indirect economic costs were reported by Onwuamah, *et al*., (2010), with an estimate of US$73.6 billion for 31 African countries from 1980–1995. Africa is costing more than US$12 million annually for the treatment of malarial acute illnesses and this again slows economic growth in African countries by 1.3% a year (Niringiye and Douglason, 2010). Despite this economic lose, there is a scarcity of chemical treatment in rural areas, hence cultural practices still remain important (Traore-Keita *et al*., 2000).

Malaria is distributed widely, mainly due to the multidrug resistance developed by *P. falciparum*. Of the four species of *plasmodium* parasites that cause malaria in humans, *P. falciparum* is by far the most virulent. Despite over 22 years of efforts, antimalaria vaccine is not yet in use. The fight against this parasite has become more complex over the last few years with prevalence of multidrug resistant strains. The cases of adverse reaction of available antimalarial drugs such as toxicity has been also negatively affect the disease control (Angayarkanni, 2010).

Douglas (2010) revealed that there was a hope for the development of a successful vaccine against malaria for several decades. But at this time it is not as easy as it was expected before to develop effective at the same time sufficient antimalarial vaccine except probably the recently discovered Artemisinin derivatives. The use and misuse of chloroquine has led to widespread appearance of chloroquine resistant parasites in tropical countries. Moreover, the rising costs of non-chloroquine drugs have made the local people to turn to traditional remedies for management of this disease (Bussmann and Njorge, 2006). However, progress has been made. The evidence that humans develop protective immune responses against *P. falciparum* when repeatedly exposed to infection indicates that development of an effective vaccine should be possible. As indicated by Moss *et al*., (2008), the target of vaccine development has been the following stages. These are pre-erythrocytic sporozoite and hepatic forms to prevent infection, asexual erythrocytic forms to reduce morbidity and mortality, and lastly sexual forms within the mosquito to prevent transmission.

Recent literatures reported that the major challenges remained in Sub Saharan countries (Moss *et al*., 2008). It has been also revealed that the key malaria controlling method is availability of new funding sources. This enables to have a well developed infrastructure, treatment policy that ensures rapid diagnosis of patients and effective antimalarial drugs (Douglas *et al*., 2010). Witkowski *et al*. (2009) also indicated the only solution for the current shocking burden of malaria is now considered to be the use of ACTs. It is also hoped to have a promising effect in delaying the emergence of antimalarial resistance.

## 1.3 Malaria in Ethiopia

Malaria stands as the leading cause of morbidity and mortality in Ethiopia. Over the past years, the disease has been consistently reported as the first leading cause of outpatient visits, hospitalization and death in health facilities across the country (Karunamoorthi and Bekele, 2009). Annually, half a million microscopically confirmed cases of malaria are reported to the Federal Ministry of Health (FMOH) from basic health services. According to the 2007/2008 report of the FMOH, malaria was the leading cause of outpatient visit accounting for 12% of cases (ACIPH, 2009; Guthman *et al*., 2007). In Ethiopia, it is

known that the vulnerability of the people to malaria has been aggravated by malnutrition and weak supply of infrastructure (Das, 2003).

In Ethiopia, malaria is at the front among the health problems of the country. As the report of Yeshiwondim *et al*. (2009), about 70% of the population in Ethiopia (approximately 52 million people) is estimated to be at risk for malaria infection each year. Jima *et al*. (2010) also indicated that about 75 % of the land mass in Ethiopia is endemic for malaria. 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 (Ghebreyesus *et al*.; 1996, Deressa *et al*., 2003; Jima *et al.,* 2005). *P. falciparum* is responsible for 13–28% of deaths in children under 5 years of age (Tulu *et al*., 1993).

Due to climatic and geographic factors, the disease occurs in different parts of the country in epidemic form (Jima *et al*., 2010). In consequence, about 40,000 Ethiopians die from malaria every year, more than those dying from HIV and tuberculosis. Rates of morbidity and mortality increase during epidemic years that reappear at irregular intervals of 3–4 years. In 2003, from 6 million cases shot up to 16 million, and over 100,000 people died (Zeleke *et al*., 2010). During this epidemic season transmission usually occurs in about three fourth of the country with altitude below 2,000 m. Most the areas affected by epidemics are highland or highland fringe areas (mainly areas 1,000–2,000 m above sea level), in which the population lacks immunity to malaria. Occasionally, transmission of malaria occurs in areas previously free of malaria, including areas greater than 2,000 m above sea level, in which the microclimate and weather conditions are favorable for malaria (Yeshiwondim *et al*., 2009).

Malaria is also associated with rainfall in Ethiopia. The two peaks of malaria incidence in Ethiopia are from September to December, after the heavy summer rains, and from March to May, after the light rains each year (Jima *et al*., 2010). However, many areas in the south and west of the country have no clearly defined rainfall season (Peterson *et al*., 2009; Jima *et al*., 2010). Depending on these rainfall patterns, transmission tends to be highly heterogeneous geospatially within each year as well as between years (Negash *et al*., 2005).

The main malaria control strategies in Ethiopia include early case detection and immediate treatment, sustainable vector control and prevention and control of epidemics (Adhanom *et al*., 2006). Early diagnosis of cases is accomplished either through laboratory diagnosis at health centers and hospitals or through clinical diagnosis or Rapid Diagnostic Tests at peripheral health facilities where microscopy is not available. Rapid access to early diagnosis and effective antimalarial treatment is one of the major strategies for reducing morbidity and mortality from malaria (WHO, 1993).

However, 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 (CSA, 1996; Kassaye *et al*., 2006). As a result, patients with malaria, particularly children, can die before they reach health services. In rural communities, home management of malaria in the form of self treatment almost is the first choice after self diagnosis based on presumptive symptoms of malaria (WHO, 1998). The inadequacies, uneven distribution, high costs, inaccessibility and inefficiency of health services as well as the high burden of malaria in rural communities of Ethiopia are among the major reasons for current interest in self treatment using herbal remedies (Deressa *et al*., 2003).

## 1.4 Antimalarial drug resistance

Antimalarial drug resistance is one of the greatest threats to the achievement of the malaria control targets (WHO, 2009). According to Okafor and Amzat, 2007, malaria drug resistance has been defined as the ability of a parasite strain to survive and/or multiply despite the administration and absorption of drug which gain access to the parasite or the infected red blood cells for the duration of the time necessary for its normal action, and given in the doses equal to or higher than those usually recommended but within the tolerance of the subject. That is, the ineffectiveness of a certain drug that has been formerly potent in treating a certain disease. *P. falciparum* is outstanding in its virulence and development of resistance from the other *Plasmodia* species. Due to this reason, it becomes potential life threatening infection (Dikasso *et al*., 2006). Furthermore, although drug resistance is a major problem in other parts of the world, it is well known

in Africa mainly because of the enormous scale of the disease, antimalarial drug consumption for fever and the scarcity of the fund located for the control program (Winstanly *et al.*, 2002).

Chemotherapy, and to a lesser extent prophylaxis, is the mainstay of malaria disease so far (Olliaro, 2001). However, since antimalarial drug resistance weakens the impact of chemotherapy now it seems that their use is becoming limited (Olliaro *et al*., 1996). Antimalarial drug resistance is heightened in individuals e.g. children less than 5 years, pregnant women, and nonimmune immigrants to malarious areas, malnourished individuals and in patients with human immunodeficiency virus, who have reduced immunity. Reduced immunity allows the survival of parasites that are able to tolerate treatment. So this plays a role in development, intensification and spread of resistant strains (Enato and Okhamafe, 2005). Witkowski *et al*. (2009) noted the increase in mortality and morbidity of malaria in the last two decades. This is due to the tolerability of the parasite to the currently existing antimalarial drugs, especially to chloroquine.

With this fact, White and Olliara *et al.* (1996) have tried to list the possible factors that can affect the development of resistance in malaria parasites. These are appropriate consumption, sensible prescribing, and patient treatment seeking behavior and compliance with the prescribed regiment. Drugs with long elimination half lives are potentially more likely to develop resistance, because parasites are exposed inevitably to suboptimal drug levels for longer periods. On the contrary, short lived (rapidly eliminated) drugs are less likely to develop resistance. Treatment failure also often results from poor compliance with prescribed regiments. Recent studies indicated that despite the above human factors that influence treatment outcome, indications of the level of resistance to antimalarial drugs can be estimated from the frequency of resistance allele types in known drug target genes (Bonizzoni *et al*., 2009). Another study also shows that lack of chemical diversity among the antimalarial drugs in use, with few exceptions, can aggravate drug resistance as it leads to cross resistance between drugs of the same class of compounds (Olliaro *and* Yuthavong 1999).

The bark of the Peruvian *Cinchona* was recognized for its antimalarial activity by the Pope's physician in Italy around 1650s. At that time he encouraged its widespread

distribution as herbal antimalaria (Amorosa *et al*., 2005). Later, the French scientists Pelletier and Caventou isolated Quinine for the first time from the bark of the *Cinchona* species (Rubiaceae) tree in 1820 (Oliveira *et al*., 2009). Since then it remains as the most effective antimalarial drug for the next three centuries. Quinine, a quinolinemethanol, is now considered too toxic for prophylaxis or routine treatment of malaria. Quinine has also faced resistance in some parts of the world. In Zimbabwe, quinine use is limited because of its relatively side effects and prolonged dosage (Makono and Sibanda, 1999). But it is still used as an intravenous injection to treat severe malaria (Folley and Tilley, 1997). Moreover, Quinine is the most important lead compound against malaria which was used as a template for chloroquine and mefloquine.

In the 1940s as a result of a large scale search for less toxic analogues of quinine, the 4-aminoquinoline, chloroquine, was produced (White, 1994; Foley and Tilley, 1998). However it started its use since 1950s and it has been the mainstay of malaria treatment around the world. Parasites resistant to chloroquine emerged in the early 1960s and by the early 1990s the drug was ineffective in treating *P. falciparum*. Resistance to chloroquine is a major cause of increased morbidity and mortality from malaria in recent decades (Kamya *et al*., 2001; Laufer and Plowe, 2004).

Resistance is also evolved to sulfadoxine-pyrimethamine (SP) which has been globally deployed as first line antimalarial drug together with chloroquine (Hastings, 2004). SP is currently the second line agent for uncomplicated malaria in some African countries, for example Uganda. According to the report of Kamya *et al*., (2001), resistance of the parasite to SP drugs has not been observed in East African countries even though there are some indications of emergence in regions of high drug pressure. SP drug combinations are also in use as second line treatments in most of African countries as well as first line in Malawi, South Africa and Kenya (Brasseur *et al*., 1999). However, as the report of some researchers the lifespan of these drugs is questionable as resistance may develop rapidly under intense drug pressure. In Southeast Asia resistance of the parasite against these drugs has developed which was resulted from its frequent use in malaria prevalent areas (Olliaro *et al*., 1996).

Other drugs, such as mefloquine, halofantrine, atovaquone, proguanil, artemether and lumefantrine are too expensive for widespread use. As reported by Brasseur *et al*. (1999), amodiaquine may be one of the few possible alternatives. Amodiaquine, a 4-aminoquinoline similar to chloroquine, was considered to be potentially toxic in prophylactic use and without any significant advantages over chloroquine in treatment. Nevertheless, a more recent review of effectiveness and tolerability of amodiaquine revealed that it is safe and reliable when given in areas where chloroquine resistance is substantial. As a result amodiaquine was using in many African countries, for instance in Kenya, which are confronted with chloroquine resistance (Van den Broek *et al*., 2003).

With the failure of CQ and SP in Malaria treatment, there is the possibility that other drugs may also fail to treat when used as monotherapies (Okafor and Amzat, 2007). Hence, there is an increasing demand for active compounds with a new mode of action to replace the current ineffective drugs. For thousands of years, plants have formed the basis of traditional medicinal systems for a number of societies in the world. At this time natural products have been a good source of lead compounds, especially against infective diseases like malaria.

The latest antimalarial drugs artimisinin and its derivatives, artemether and artether, isolated from *artemesia annua* are the only effective antimalarial drugs available in the market (Andrade-Neto *et al*., 2007). This is considered as the hope of saving millions of lives especially in Sub Saharan Africa where children are affected worst. However, the artemisinin drugs are much more expensive than chloroquine and other antimalarials and are unaffordable by the poor society in malaria endemic developing countries (Wright, 2005). Furthermore, although artemisinins currently provide effective treatment, resistance to artemisinin may evolve quickly, especially if this class is used intensively as a single ingredient treatment (Laxminarayan *et al*., 2010). There are also evidences of sporadic cases of decrease in sensitivity that have been reported in French, Guyana and Senegal. In addition, resistant genes of parasites to artemisinin-based combination therapies (ACTs) are also found in Cambodia (Witkowski *et al*., 2009).

Douglas *et al*. (2010) advised the treatment policies to focus on combination therapies in response to the rapid spread of drug resistance and the slow rate at which new

antimalarial drugs are developed. The implementation of combination therapy will require modification of the design of the therapeutic efficacy test (WHO, 2002). Development of resistance of antimalarial drugs together with increasing of child mortality in some countries makes the ACTs powerful treatment tools. ACTs are not only efficacious but also protect drugs from emergence of resistance by *P. falciparum*. At this time, the WHO treatment guidelines recommend the artemisinin-combination therapies (ACTs) that contains artemisinin and either of the following drugs with a longer half life, such as lumefantrine, mefloquine, amodiaquine, SP or piperaquine. This type of treatment is suggested to counter the rapid development of *P. falciparum* drug resistance (Sibley *et al*., 2010). The only problem of these drugs is mentioned as the small scale production. This can be also solved by lowering unit costs from manufacturers and maximizing availability of funding from variety of sources.

In general, development of new natural chemical agents with different modes of action to broaden therapeutic options is reported to be crucial in multidrug resistance malaria parasite control. Barnes *et al*. (2008) revealed that implementation of improved dosing regiments is also as important as use of antimalarials in combination as law dosing regiments can induce drug resistance. As written by Laximinarayan *et al*., (2010), combinations are usually more effective than monotherapy and they also help to avoid or at least delay the emergence of resistance. The probability of a malaria parasite emerging that is resistant to two drugs with different modes of action is markedly reduced when they are used in combination. Therefore, as the report of WHO (2009), medicine production needs to include potential partner medicines with different modes of action. Traditionally used antimalarial herbal remedies can fulfill such types of qualities since they provide versatile chemical compounds. So examining plants for new therapeutic agents with different mode of action is the best choice.

## 1.5 Antimalarial herbal medicine

Traditional herbal medicines have been used to manage malaria for thousands of years and are the source of the two main groups (artemisinin and quinine derivatives) of modern antimalarial drugs (Muthaura *et al*., 2007; Adebayo and Krettli, 2011). They could be sustainable source of treatments for malaria which face difficulties of increasing level of resistance and problem of availability and affordability of the costly drugs (Willcox, 2004). Thus, many communities who live in endemic areas have started to look for malaria remedies in plants in their local environments (Miliken, 1997).

Traditional herbal medicines became the most affordable treatments against malaria in endemic regions (Koudouvo *et al*., 2011). Ancolio *et al*. (2002) also indicated that most of the societies in Africa have shifted from modern antimalarials into herbal once due to the reason mentioned earlier. According to several reports Africa is endowed with an important floristic biodiversity and indigenous people have an old tradition of antimalarial plant usage that has been handed from generation to generation (Adebayo and Krettli, 2011). It seems convincing that African traditional plants are effective in treating malaria. That is why our ancestors hundreds of years ago saved Africa from being destroyed from malaria in the absence of modern drugs (Idowu *et al*., 2010).

Ethnobotanical survey is the first step in the identification, selection and development of antimalarial therapeutic agents from medicinal plants (Idowu *et al*., 2010). Thus until recently, over 1,200 plant species from 160 families used in the treatment of malaria or fever throughout the world are already documented from various studies (Willcox and Bodeker, 2004).

As the report of Traore-Keita *et al.* (2000), leaves, roots and stem barks of *Mitragyna inermis* (Rubiaceae), *Nauclea latifolia* (Rubiaceae), *Glinus oppositofolius* (Molluginaceae) and *Trichilia roka* (Meliaceae) were investigated for their antimalarial activity. Aqueous, hydromethanol and chloroform extractions were prepare from their leaves, roots and stem barks and *in vitro* antimalarial activity was evaluated microscopically. This study indicated that the hydromethanol and chloroform extracts of these plants showed significant parasite suppression.

Six plants commonly used in African traditional medicine for treating malaria were evaluated for their antimalarial activity in Mali. Methanol and chloroform extracts were prepared from various parts of *Guiera senegalensis, Feretia apodanthera, Combretum micranthum, Securidaca longepedunculata, Pycnanthus angolensis* and *Morinda citrifolia* were assessed for their *in vitro* activity. The methanol extract of leaves of *Feretia apodanthera* and the chloroform extract of roots of *Guiera senegalensis* exhibited a pronounced antimalarial activity (Ancolio *et al*., 2002). Kaou *et al*. (2010) also confirmed the antiplasmodial activity of ethyl acetate extract of *Flacourtia indica* which have antimalarial traditional usage.

Reports from Ghana also indicated the importance of the following families as possible sources of antimalarial drugs. Anacardiaceae, Meliaceae, Celastraceae, Rutaceae, Asteraceae and Combretaceae are commonly used for the treatment of malaria. The most frequently used species of plants were *Azadirachta indica*, *Senna siamea*, *Citrus aurantifolia* and *Nauclea latifolia*. *A. indica* has been also mostly mentioned as a treatment for malaria in Togo and Kenya (Nguta *et al*., 2010) and found to have good antiplasmodial activity. A study by Asase *et al*., (2010) reported the wide use of *N. latifolia* in central west Cote Divoire *as* treatment of malaria.

*Tiliacora triandra*, from the family Menispermaceae, is reported to have wide spread traditional use as fever treatment. Likewise, antimalarial preparations are also made from this plant and believed to have healing activity traditionally (Saiin and Markmee, 2003).

According to the study of Lukasibanza *et al*. (2010), five plants traditionally used in Congo were evaluated for their *in vivo* antimalarial activity. These are *Anisopappus chinensis, Entandrophragma palustre, Melia azedarach, Physalis angulata* and *Strychnos icaja*. Two of them, *Strychnos icaja* and *Physalis angulata* show potent activity *in vivo*. *Anisopappus chinensis* whole plant also became active with (IC50 < 15µg/ml) *in vitro*. But the rest two plants do not have an appreciable *in vitro* or *in vivo* antimalarial activity.

*Creptolepis sanguinolenta* is a West African climbing shrub. It is traditionally used for the treatment of malaria. The scientific investigation of this plant shows potential lead to new antimalarial drug (Wright, 2005). In an effort to document traditionally used

antimalarials, Nguta *et al*. (2010) conducted a survey in Kenya. Accordingly, twenty seven species of plants were reported from a particular region for the treatment of malaria. From this thirteen plant species which are most commonly used are listed. *Aloe deserti* (Liliaceae)*, Launea cornuta,* (Compositae*), Ocimum bacilicum* (Labiatae), *Teclea simplicifolia* (Rutaceae), *Gerranthus lobatus* (Cucurbitaceae), *Grewia hexaminta* (Tiliaceae), *Canthium glaucum* (Rubiaceae), *Amaranthus hybridus* (Amaranthaceae), *Combretum padoides* (Combretaceae), *Senecio syringitolius* (Compositae), *Ocimum suave* (Labiatae), *Aloe macrosiphon* (Liliaceae*)* and *Laudolphia buchananii* (Apocynaceae) are the common plants.

In Ethiopia, some of the medicinal plants used traditionally for the treatment of malaria have been screened for their antimalarial activity. Dikasso *et al.* (2006) have reported that extracts from plants such as *Hagenia abysinica, Berssama abysinica*, *Artemesia afra*, *Artemesia rehan, Ajuga remota* as well as *Withania somenifer*, and *Vernonia amygdalina* have significant *in vitro* antimalarial activity against *P. falciparum.* The aqueous root extract of *Gnidia stenopylla*, leaf extract of *Vernonia bipontini*, root extract of *Eculea schimperi*, *Cissampelos mucronata*, and *Clerodemdrum myricoides* and methanolic leaf extract of *Vernonia bipontini*, as reported by Assefa *et al*. (2006), also showed appreciable *in vivo* antimalarial activities against *P. berghei* parasite. Among these, three extracts were reported to had very high percent parasitemia inhibition values (>50%).

As indicated in Bekele (2007), *Moringa stenopetala*, a plant which grows widely in Ethiopia, is reported to have medicinal value. Local peoples use boiled leaves as tea or chopped and mixed it with water to treat malaria in Konso area (South Ethiopia). Moreover, according to the report of Dharani *et al*. (2010), *Albizia amara* (mimosaceae) is widely used as traditional treatment of malarial in Ethiopia. Bark stem decoction of *A. amara* is taken three times a day to treat malaria.

Acetone fraction of the stem bark of an Ethiopian medicinal plant, *Combretum molle*, was evaluated for its antimalarial activity *in vivo*. It showed significant activity against trophosoites of *P. falciparum* with $IC_{50}$ value of 8.17µg/ml (Kaur *et al*., 2009). Similarly Bogale and Petros (1996) reported the antimalarial activity of *V. amygdalina* and *W. somnifera in vitro* against *P. falciparum*.

## 1.6 Phytochemical constituents of antimalarial medicinal plants

The challenge in malaria chemotherapy is to find safe and selective agents with potency that will not be compromised by plasmodial resistance. To meet the criteria of efficacy and safety the pharmacological, toxicological and phytochemical report of the plant extracts have to be scientifically evaluated. Hence, the WHO recognizes that the age old use of certain plants as drug resources and their efficacy should be taken into account. In relation to this certain basic procedures are suggested in order to validate plant derived drugs in developing countries (Nguta *et al*., 2010). To isolate the natural active principles *in vitro* or *in vivo* bioassay procedures can be used (Frederick *et al*., 2008).

As revealed by Wright (2005), the recent development of artemisinin derivatives has indicated the potential of plant species to provide effective drugs for the treatment of malaria. Not only this but also other antimicrobial drugs, such as morphine and codeine from the latex of the opium poppy, digoxin from *Digitalis* leaves, atropine from species of the Solanaceae attracted the attention of researchers towards higher plants (Phillipson, 2001). In some cases the constituents responsible for their activity have been isolated but relatively few have been studied further to assess their potential as lead compounds for the development of drugs (Phillipson, 2001; Oliveira *et al*., 2009).

Alkaloids are the most important secondary metabolites of plants. As reviewed by Oliveira *et al*. (2009), they are one of the major classes of natural products that exhibit antimalarial activity. Quinine, the first antimalarial drug, belongs to this class. Saxena *et al*. (2003) indicated that about 100 groups of alkaloids that have antimalarial activity were identified from higher plants with in 10 years duration of time from 1990 to 2000.

Two alkaloids isolated from the active extract of *Guiera senegalensis*, showed antimalarial activity ($IC_{50}$ lower than 4g/mL) (Ancolio *et al*., 2002). Another plant *Stephania rotunda* (Menispermaceae) is used in traditional medicine for the treatment of fever. Four major alkaloids dehydroroemerine, tetrahydropalmatine, xylopinine, cepharanthine were tested against *P. falciparum in vitro*. Dehydroroemerine, cepharanthine were active against *P. falciparum* (Chea, 2007). Similarly, the alkaloid cryptolepine, a constituent of *Cryptolepis sanguinolenta*, used traditionally for malaria

treatment has been also investigated as a potential lead to new antimalarials and inhibited good activity (Wright *et al*., 2001).

Two pure alkaloids, tiliacorinine and tiliacorine, are isolated from *Tiliacora triandra* using chromatography (Saiin and Markmee, 2003). Similarly, *Brunsvigia littoralis*, *B. radulosa* and *Cirinum amabile* have alkaloids and used as antimalarial herbal medicine. Four alkaloids are isolated from *B. littoralis*. From these, lycorine and crinamine have been found to be active against *Plasmodium* parasite (Schwikkard and van Heerden, 2002).

*C. myricoides* and *D. angustifolia* are traditionally used for treatment of several diseases throughout the world. Digestive system disorders, including indigestion, ulcers, diarrhea and constipation are commonly treated with an orally administered decoction of either the leaves or roots of *D. angustifolia*. It has been also confirmed to cause growth inhibition in human pathogens namely *Bacillus* species, *Salmonella* species and *Corynebacterium diphtheria* (Malarvannan *et al.*, 2008).

*D. angustifolia* and *C. myricoides* are also among the numerous traditionally used medicinal plants in Ethiopia. The crude extracts of these plants have been checked for their antiplasmodial effect *in vivo* and showed a desirable activity (Tekalign *et al*., 2010). *D. angustifolia* is proved to contain secondary metabolites such as quinines, saponins, flavonoides, alkaloids, terpenoides, diterpenoides and essential oils (Anilreddy, 2009). Similarly, Pascaline *et al*. (2011) revealed that *C. myricoides* contains alkaloids, saponins, glycosides, terpenoides, phenolics and flavonoides. The reported antiplasmodial use of these plants may be attributed to a single or combined effect of these secondary metabolites. This study investigates the antiplasmodial activity of crude extracts and extracts using different solvent systems of *D. Angustifolia* and *C. myricoides* in an experimental mice model.

# 2. Objectives

## 2.1 General Objective

- To evaluate the antimalarial activities of bioactivity guided successive extracts of two Ethiopian traditional plants against *P. berghei in vivo.*

## 2.2 Specific Objectives

- To evaluate the bioactive extracts of *C. myricoides* and *D. angustifolia* against the *P. berghei* in Swiss albino mice model.

- Fractionation of the bioactive extracts using TLC and Column Chromatography.

- To possibly isolate and characterize the active ingredients which show antiplasomodial effect.

# 3. Materials and methods

## 3.1 Plant materials collection

*C. myricoides* (Lamiaceae) was collected from Entoto forest, around Addis Ababa, and *D. angustifolia* (Sapindaceae) was collected from Lideta Mariam, around Russia Embassy, to the East direction of Addis Ababa in September 2010. After authentication by a botanist, representative plant specimens with voucher numbers 1 for *D. angustifolia* and 2 for *C. myricoides* were kept in the National Herbarium of Addis Ababa University in October 2010.

## 3.2 Description of the plant materials

*C. myricoides*, 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. Stems are angular or terete. 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. Black, fleshy fruit follows with forked style (pollen receptive structure) that overarches the flower (Person, 2006).

Plate 1. Picture of *C. myricoides* aerial part (Entoto forest; September, 2010)

*D. angustifolia* is locally called kitkita (Amharic). It is an evergreen shrub growing up to 3 m long; all parts are glabrous and resinous when young, leaves are simple and petiolate (1-5mm long) with narrowly attenuate lamina. The flowers are dioecious (individual flowers are either male or female, but only one sex is found on any one plant). Inflorrescences terminal and auxillary and sepals are ovate, yellowish green in color and shortly connate. Ovary 2-3 locular, 3mm long; and stigma 2-3mm. Fruits are circular in outline and yellowish with 3-6mm wide. The plant mostly grows at edge of upland forest, upland bush land and grassland (Vollesen, 1989).

Plate 2. Picture of *D. angustifolia* aerial part (Lideta Mariam, in the Eastern part of Addis Ababa, near Russia Embassy; September, 2010)

## 3.3 Preparation of crude extracts and extracts of organic solvents

### 3.3.1 Preparation of crude extracts

Leaves of *C. myricoides* and *D. angustifilia* were air dried at room temperature under shade in the Biomedical Science laboratory of Faculty of Life Sciences, College of Natural Sciences of Addis Ababa University and ground into fine powder using a grinding mill. The crude extracts were prepared by cold maceration technique (Tekalign *et al*., 2010). Methanol (99.5%) extract of each specimen was prepared. The extraction was done by refluxing 50 grams of plant material in 300ml of methanol (99.5%) and the mixtures were placed on orbital shaker (at 160rpm) for 72 hours at room temperature. Then, each sample was filtered out using a Whatman filter paper (No. 1, 15cm size with retention down to 0.1ml in liquids). The methanol (99.5) filtrates were concentrated in a rotary evaporator (Buchi typeTRE121, Switzerland) at a temperature of 45ºC. The residue was taken again and mixed with chloroform/ethyl acetate (1:1) in a volume of 300ml. The same procedure was followed to chloroform. All the extracts were stored at -20ºC until they were applied in the experiment.

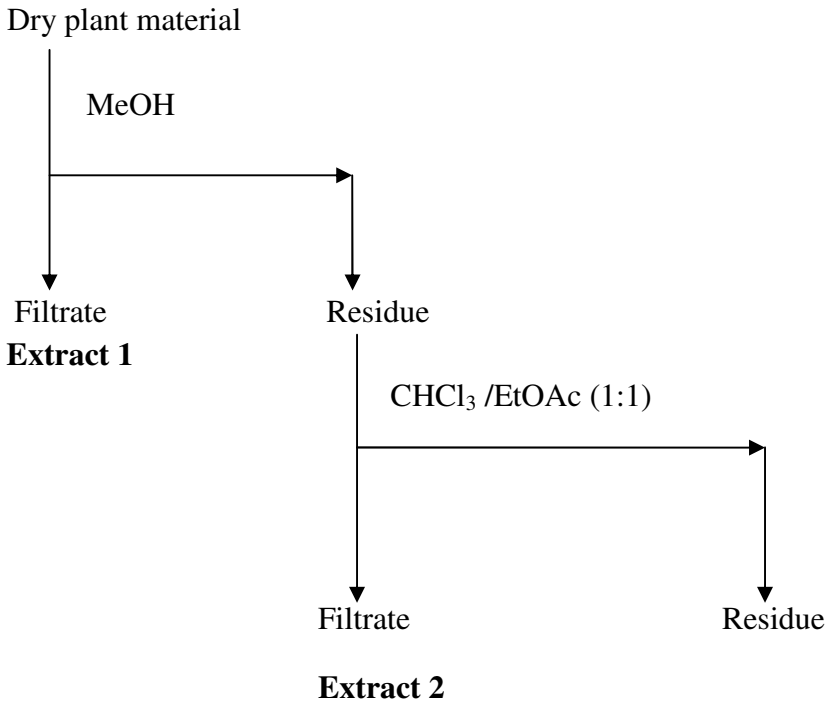


Fig. 1 Flow sheet diagram of extraction procedure of *C. myricoides* and *D. angustifolia.*

### 3.3.2 Preparation of successive extracts of organic solvents

The further extraction process of the methanol extracts of *C. myricoides* and *D. angustifolia* was done in the Organic chemistry Research Laboratory of the Department of Chemistry, College of Natural Sciences, Addis Ababa University.

Five (5) grams of the powdered methanol extract of each plant was weighed using a top loading balance and transferred to a conical flask. Successive extraction was carried out using increasing polarity. Hexane (for non polar compounds) then ethyl acetate (for moderately polar compounds) was added subsequently. First the extracts were soaked in hexane at room temperature by shaking for 3 hours (*D. angustifolia*) and 6 hours (*C. myricoides*). Extracts were filtered through No. 1 Whatman filter paper and the flask containing the filtrate was closed with a stopper. The filtrate was then rotavapoured to concentrate the extracts. Finally 0.402 grams (8%) and 0.3611 grams (7.2%) were produced for *C. myricoides* and *D. angustifolia* respectively.

The marks were subsequently extracted using ethyl acetate by shaking for overnight and the yields were 1.10 grams (22%) for *C. myricoides* and 1.48 grams (29.6%) for *D. angustifolia*. The remaining residues were weighed and found to be 3.2 grams (64%) for *C. myricoides* and 3.1grams (62%) for *D. angustifolia*.

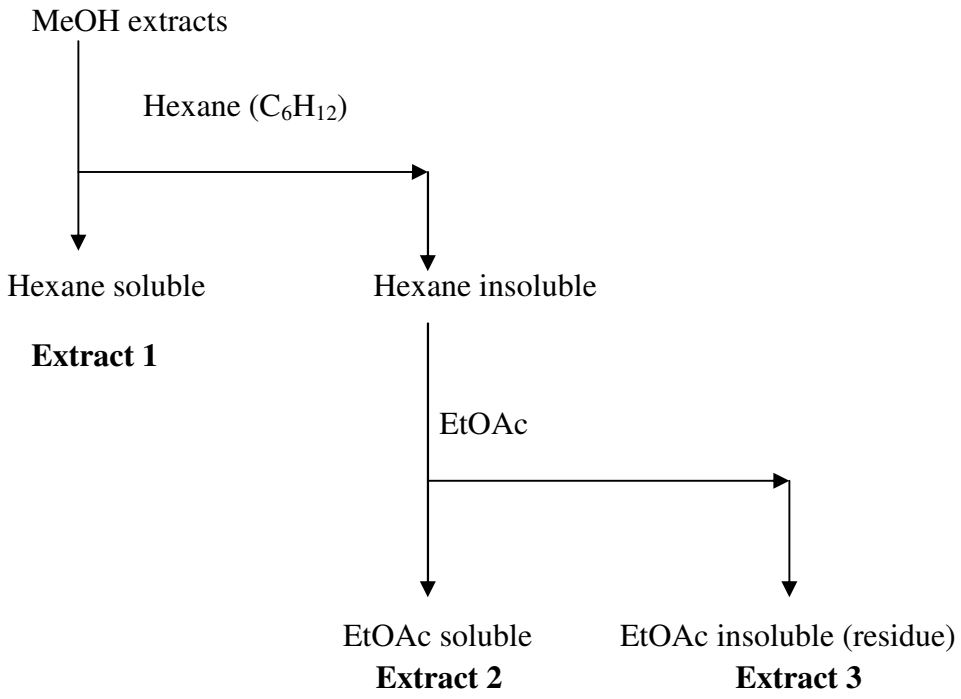


Fig. 2 Flow sheet diagram of further extraction procedure of *C. myricoides* and *D.angustifolia.*

### 3.4 Experimental animals and the *Plasmodium* parasite strain

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. They were given a standard diet and tap water *ad libitum*. For *in vivo* antimalarial assays of plant extracts, the mouse infective chloroquine sensitive strain of *P. berghei* maintained in the Microbial, Cellular and Molecular Program Unit, Addis Ababa University were used. The parasite was maintained by serial passage of blood obtained from the tail snip from infected mice to non-infected ones on weekly basis.

### 3.5 Evaluation of the antiplasmodial activity of crude extracts

In evaluating of the antiplasmodial activity of the plant extracts, the standard four-day suppressive method was used. For each experiment about 1ml blood sample was taken from the tail snip of the donor mice previously infected with chloroquine sensitive *P. berghei* with parasitaemia of about 20%. The blood sample was then diluted with saline so that 0.2ml contained 1 x $10^6$-$10^7$ infected erythrocytes. Each mouse used in the study was infected interaperitonially on day 0.

The infected mice were randomly divided into two test groups and two control groups (each for chloroquine as a standard drug and 3% Tween 80 as a negative control) for each of the three: methanol, ethyl acetate/chloroform and chloroform extracts. The test extracts were prepared in two doses (300mg/kg and 500mg/kg of body weight), chloroquine at 25mg/kg in a volume of 0.2ml and vehicles at 0.5ml/mouse. Each extract was administered as a single dose per day. All the extracts and the drug were given through intragastric route by using standard intragastric tube to insure safe ingestion of the extracts and the drug. Treatments were started after 3 hours of infection on day 0 and continued daily for four days (i.e. from day 0 to day 3). On the fifth day (day 4) blood samples were collected from tail snip of each mouse. Thin smears were prepared and stained with 10% Geimsa solution. Then, each stained slide was examined under the microscope with an oil immersion objective of 100x magnification power to evaluate the percent suppression of each extract with respect to the control groups.

### 3.6 Evaluation of the antiplasmodial activity of extracts of organic solvents

Hexane, ethyl acetate and residue of both plants were collected and evaluated for their antimalarial activity in Swiss albino mice in the same way as the crude extracts. But only one dose (150mg/kg) was used. So the infected mice were randomly divided into three 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 extracts: hexane, ethyl acetate and residue of the two plant species. Chloroquine was prepared at 25 mg/kg in a volume of 0.2ml and vehicles (DMSO) at 0.5ml/mouse. Five mice for each cage of the test groups and the control groups were assigned. These preparations were administered to the mice at an oral dose of 150ml/kg and finally on the fifth day; blood samples were taken from the tail snip of the mice and microscopic examination was done following similar procedures as in the crude extracts. This experiment was done twice to increase the reliability of the result.

Thin Layer Chromatography (TLC): Before giving to the mice, the extracts were checked using TLC by applying various solvent systems and sprayed with suitable reagents. Each of the hexane, ethyl acetate and residue extracts (Fig. 1) of *C. myricoides* and *D. angustifolia* were dissolved in hexane, ethyl acetate and methanol respectively. A solvent system of hexane: EtOAc (3:2) was set for the hexane extracts for TLC. A solvent system of hexane: EtOAc (1:4) was also prepared for the ethyl acetate and residue extracts of both plants. Ethyl acetate extract of *C. myricoides* was subjected to column chromatography over seven (7) grams of silica gel using cyclohexane and ethyl acetate (3:2). Similarly ethyl acetate extract of *D. angustifolia* was applied on top of column chromatography packed with twenty (20) grams of silica gel (230-400 mesh). Elusion was done in cyclohexane/ethyl acetate solvents (with different ratios) by increasing polarity. Then TLC of each sample was analyzed.

Nuclear Magnetic Reasonance (NMR): NMR was also run for ethyl acetate extracts of *C. myricoides* and *D. angustifolia* that had better antimalarial activity, in an effort to isolate and identify the biologically active compounds.

## 4. Data analysis

Values 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 17.0. Level of significance was set as $P<0.05$. Percent parasitaemia and percentage of suppression were calculated using formula indicated in Li *et al*. (2003) and Devi *et al*. (2001) respectively.

$$\%\ \boldsymbol{para.} = \frac{\boldsymbol{Number\ of\ infected\ RBCs}}{\boldsymbol{Number\ of\ infected\ RBCs + Number\ of\ uninfected\ RBCs}}\ \mathbf{X\ 100}$$

$$\%\ \boldsymbol{supp.} = \frac{\boldsymbol{Para.\ in\ negative\ control - Para.\ in\ study\ group}}{\boldsymbol{Para.\ in\ negative\ control}}\ \mathbf{X\ 100}$$

# 5. Results

Microscopic examination of thin smears of blood from mice of each experimental and control groups were done. The percentage of parasitaemia was lower in all the mice administered with the extracts of the two plants, *C. myricoide* and *D. angustifolia*, as compared to the negative control groups that were given the vehicle (Tween 80 or DMSO) only. The positive control groups administered with Chloroquine cleared the parasite completely on day four; while the mice treated with the extracts did not show complete parasite clearance.

## 5.1 *In vivo* antiplasmodial suppressive test of *Clereodendrum myricoides*

### 5.1.1 Crude extracts

After four days treatment of mice with different methanol extract doses of *C. myricoides*, the mean parasitaemia of the test groups were found to be 6.52 ± 0.58% in 300mg/kg and 5.92 ± 0.38% in 500mg/kg while the corresponding value of the negative control group being 25.73 ± 1.57%. The mice treated with CQ were completely free from the parasites on day four. Statistical analysis using Scheffe's procedure indicated that groups of mice treated with 300mg/kg and 500mg/kg of *C. myricoides* leaf methanol extract showed statistically significant difference in parasitaemia level as compared to the negative control group ($P<0.05$). The highest suppression of parasitaemia was observed at the dose of 500mg/kg body weight of mice. It was observed that there was a slight increase of percentage suppression with increase in extract concentration (Table 1).

On the other hand, the mice administered with chloroform/ethyl acetate (1:1) extract of the leaves of *C. myricoides* 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 13.29 ± 0.89 and 12.92 ± 0.79 at 150mg/kg and 300mg/kg oral doses respectively, whereas the corresponding figure in the control group was 15.69 ± 0.61 (Table 1).

In the case of chloroform extract of the leaves of *C. myricoides*, the groups of mice treated with the 300mg/kg and 450mg/kg doses resulted in low parasite clearance in

comparison with the negative control. Treatment with this extract at both doses did not show significant difference in parasite suppression (P>0.05) (Table 1).

**Table 1**. *In vivo* suppressive test of crudes of methanol, ethyl acetate/chloroform (1:1 percentage) and chloroform extracts of leaves of *C. myricoides* against *P. berghei* in mice.

| *C. myricoides* extracts | Dose (mg/kg/day) | Antiplasmodial activity | |
|---|---|---|---|
| | | % Parasitaemia ± SEM | % Suppression |
| Methanol | NC | $25.73 \pm 1.57^{a}$ | $0.00^{a}$ |
| | 300 | $6.52 \pm 0.58^{b}$ | $74.66^{b}$ |
| | 500 | $5.92 \pm 0.38^{b}$ | $76.99^{b}$ |
| Chloroform/ethyl acetate (1:1) | NC | $15.69 \pm 0.61^{a}$ | $0.00^{a}$ |
| | 150 | $13.29 \pm 0.89^{a}$ | $15.31^{a}$ |
| | 300 | $12.92 \pm 0.79^{a}$ | $17.69^{a}$ |
| Chloroform | NC | $23.51 \pm 0.56^{a}$ | $0.00^{a}$ |
| | 300 | $21.22 \pm 0.42^{a}$ | $9.75^{a}$ |
| | 450 | $17.23 \pm 0.68^{a}$ | $26.75^{a}$ |
| Chloroquine | 25 | 0.00 | 100.00 |

Values are Mean ± SEM; n=4, NC: Negative control (0.5 ml vehicle); a,b = Values in the same column followed by the same letter do not differ significantly (P>0.05)

5.1. 2 Successive extracts of organic solvents

Ethyl acetate soluble and insoluble extracts (residue) at a dose of 150 mg/kg of *C. myricoides* leaves showed a suppressive effect of 61.30% and 69.31% respectively which have statistically significant difference as compared to negative control (P<0.05). While the suppressive effect of hexane extract was 16.21% which is not significantly different from the negative control (Table 2).

**Table 2**. *In vivo* suppressive test of successive extracts of the methanol extract of *C. myricoides* leaves against *P. berghei* in mice.

| *C. myricoides* extracts | Dose (mg/kg/day) | Antiplasmodial activity | |
|---|---|---|---|
| | | % Parasitaemia ± SEM | % Suppression |
| Ethyl acetate | NC | $35.48 \pm 0.92^{a}$ | $0.00^{a}$ |
| | 150 | $13.73 \pm 0.41^{b}$ | $61.30^{b}$ |
| Hexane | NC | $35.48 \pm 0.92^{a}$ | $0.00^{a}$ |
| | 150 | $29.73 \pm 0.1.2^{a}$ | $16.21^{a}$ |
| Residue | NC | $35.48 \pm 0.92^{a}$ | $0.00^{a}$ |
| | 150 | $10.89 \pm 0.64^{b}$ | $69.31^{b}$ |
| Chloroquine | 25 | 0.00 | 100.00 |

Values are Mean ± SEM; n=10, NC: Negative control (0.5ml vehicle); a,b= Values in the same column followed by the same letter do not differ significantly (P>0.05).

## 5.2 *In vivo* antiplasmodial suppressive test of *Dondonea angustifolia*

### 5.2.1 Crude extracts

Significant reduction of parasitaemia ($P < 0.05$) was observed in all groups of mice treated with methanol extract of the leaves of *D. angustifolia* compared to the negative control. The percent parasitaemia of the mice treated with 300mg/kg of the extract was 5.99 ± 0.38 and the mice that received 500mg/kg was 6.81 ± 0.49. The mean parasitaemia of the mice in the control group was 31.34 ± 0.85%. The extracts also induced an inhibition of parasitaemia by 80.98 and 78.27% at 300mg/kg and 500mg/kg doses respectively (Table 3).

Nevertheless, in the case of treatment of the *P. berghei* infected mice with ethyl acetate/chloroform (1:1) extract of the leaves of *D. angustifolia,* no significant reduction in parasitaemia was observed (P>0.05). The parasitaemia of the two study groups with 150mg/kg and 300mg/kg were 14.53 ± 0.63 and 13.51 ± 0.58 respectively which did not

have significant difference from 15.69 ± 0.61 parasitaemia of the control mice treated with Tween 80 (Table 3).

As compared to the methanol extract, chloroform extract of the leaves of *D. angustifolia* showed no significant suppressive effect on parasitaemia of the mice administered with the extract. The statistically analyzed result indicated that the effect induced by the chloroform extract of the leaves of the plant was not significant (P>0.05) at all doses administered to the experimental group parasitaemia level as compared to the negative control (Table 3).

**Table 3.** *In vivo* suppressive test of crudes of methanol, ethyl acetate/chloroform (1:1 percentage) and chloroform extracts of leaves of *D. angustifolia* against *P. berghei* in mice.

| *D. angustifolia* extracts | Dose (mg/kg/day) | Antiplasmodial activity | |
|---|---|---|---|
| | | % Parasitaemia ± SEM | % Suppression |
| Methanol | NC | $31.34 \pm 0.85^{a}$ | $0.00^{a}$ |
| | 300 | $5.99 \pm 0.38^{b}$ | $80.89^{b}$ |
| | 500 | $6.81 \pm 0.49^{b}$ | $78.27^{b}$ |
| Chloroform/ethyl acetate (1:1) | NC | $15.69 \pm 0.61^{a}$ | $0.00^{a}$ |
| | 150 | $14.53 \pm 0.63^{a}$ | $7.41^{a}$ |
| | 300 | $13.51 \pm 0.58^{a}$ | $13.91^{a}$ |
| Chloroform | NC | $21.77 \pm 1.20^{a}$ | $0.00^{a}$ |
| | 300 | $20.30 \pm 0.71^{a}$ | $6.80^{a}$ |
| | 450 | $15.27 \pm 0.60^{a}$ | $29.85^{a}$ |
| Chloroquine | 25 | 0.00 | 100.00 |

Values are Mean ± SEM; n=4, NC: Negative control (0.5ml vehicle); a,b= Values in the same column followed by the same letter do not differ significantly (P>0.05).

5.2.2 Successive extracts of organic solvents

The percent suppression effects of mice treated with ethyl acetate extract of the methanol extract of *D. angustifolia* exhibited the highest result with 82.24%. The hexane extract and residue of this plant also exerted parasitaemia suppressive effect of 19.84% and 51.93% respectively in 150mg/kg treatment dose (Table 4).

**Table 4**. *In vivo* suppressive test of successive extracts of methanol extract of *D. angustifolia* leaves against *P. berghei* in mice.

| *D. angustifolia* extracts | Dose (mg/kg/day) | Antiplasmodial activity | |
|---|---|---|---|
| | | % Parasitaemia ± SEM | % Suppression |
| Ethyl acetate | NC | $68.19 \pm 0.86^{a}$ | $0.00^{a}$ |
| | 150 | $12.11 \pm 0.68^{b}$ | $82.24^{b}$ |
| Hexane | NC | $68.19 \pm 0.86^{a}$ | $0.00^{a}$ |
| | 150 | $54.66 \pm 0.99^{a}$ | $19.84^{a}$ |
| Residue | NC | $68.19 \pm 0.86^{a}$ | $0.00^{a}$ |
| | 150 | $32.78 \pm 0.96^{b}$ | $51.93^{b}$ |
| Chloroquine | 25 | 0.00 | 100.00 |

Values are Mean ± SEM; n=10, NC: Negative control (0.5ml vehicle); a,b= Values in the same column followed by the same letter do not differ significantly (P>0.05).

## 5.3 Thin Layer Chromatography (TLC), Column Chromatography and Nuclear Magnetic Resonance (NMR) Spectrometer analysis

After the TLC plates were sprayed using vanillin, MeOH and sulfuric acid mixtures, the results indicated that three and five spots appeared for the hexane extracts of *C. myricoides* and *D. angustifolia* respectively. In the case of ethyl acetate extracts three and six spots were observed for *C. myricoides* and *D. angustifolia* respectively. The residue of *D. angustifolia* showed three spots. However, the residue of *C. myricoides* did not move from the organic spot.

### 5.3.1 *Clerodendrum myricoides*

After evaluation of the antiplasmodial activity of the extracts, residue and ethyl acetate extracts of *C. myricoides* had better activity. Hence, forty (40) milligrams of ethyl acetate extract of *C. myricoides* was subjected to column chromatography over seven (7) grams of silica gel using 3:2 of cyclohexane and ethyl acetate. Nine fractions were collected from this process (Table 5).

**Table 5**. Column chromatography fractions of ethyl acetate extract of *C. myricoides*

| Fractions | Solvent system | Ratio of solvent system | Yield in mg | Percentage yield | TLC results |
|---|---|---|---|---|---|
| $F_{1c}$ | cyclohexane:EtOAc | 3:2 | – | – | No spots |
| $F_{2c\ \&\ 3c}$ | cyclohexane:EtOAc | 3:2 | 11.9 | 30 | 3 spots |
| $F_{4c\ \&\ 5c}$ | cyclohexane:EtOAc | 3:2 | 2.9 | 7.3 | 1 spot |
| $F_{6c,7c,8c\ \&\ 9c}$ | cyclohexane:EtOAc | 3:2 | – | – | No spots |

**Remark**: Fractions $F_{2c\ \&\ 3c}$ and $F_{4c\ \&\ 5c}$ were combined based on TLC results.

Fractions $F_{2c\ \&\ 3c}$ and $F_{4c\ \&\ 5c}$ were subjected to NMR spectrometer. These fractions were selected according to their TLC results. But due to insufficient amounts obtained during fractionation, it was difficult to get good NMR results.

5.3.2 *Dodonaea angustifolia*

About one (1) gram of ethyl acetate extract of *D. angustifolia*, which showed good antiplasmodial suppression effect on infected mice, was applied on top of column chromatography packed with twenty (20) grams of silica gel (230-400 mesh). Elusion was done in hexane/ethyl acetate solvents by increasing polarity to obtain the following fractions (Table 6).

**Table 6**. Column chromatography fractions of ethyl acetate extract of *D. angustifolia*

| Fractions | Solvent system | Ratio f solvent system | Yield in mg | Percentage yield | TLC results |
|---|---|---|---|---|---|
| $F_{1\text{-}3d}$ | cyclohexane:EtOAc | 100 | 201 | 20.1 | many spots |
| $F_{4d\ \&\ 5d}$ | cyclohexane:EtOAc | 8:2 | 51 | 5.1 | 1 spot |
| $F_{6\text{-}10d}$ | cyclohexane:EtOAc | 8:2 | – | – | No spots |
| $F_{11d}$ | cyclohexane:EtOAc | 7:3 | 110 | 11.0 | 1 spot |
| $F_{12d}$ | cyclohexane:EtOAc | 1:1 | – | – | No spots |
| $F_{13d}$ | cyclohexane:EtOAc | 1:1 | 104 | 10.4 | 1 spot |

**Remark**: Fractions $F_{1\text{-}3d}$, $F_{4d\ \&\ 5d}$ and $F_{6\text{-}10d}$ were combined based on TLC results.

Fractions, $F_{4d\ \&\ 5d}$ and $F_{11d}$ were subjected to NMR spectrometer for further characterization of the compound shown as a single spot in TLC result in both. $F_{11d}$ did not show good peaks whereas the compound in the fourth and fifth fractions ($F_{4d\ \&\ 5d}$) gave good NMR peaks. The $^{13}C$ NMR spectrum revealed 38 carbons (Fig.4). The DEPT-135 spectrum contains 18 carbons (Fig.5). From the carbon and DEPT-135 spectra, there are 20 quaternary carbons. The DEPT-135 spectrum also signifies the presence of four $CH_2$ groups and the remaining fourteen are either $CH_3$ or CH. Three carbon bonded together with oxygen were indicated in both $^{13}C$ and DEPT NMR spectra. The $^{13}C$ NMR

also revealed as there are two carbonyl groups which absorb at δ 195 ppm and 179.2 ppm.

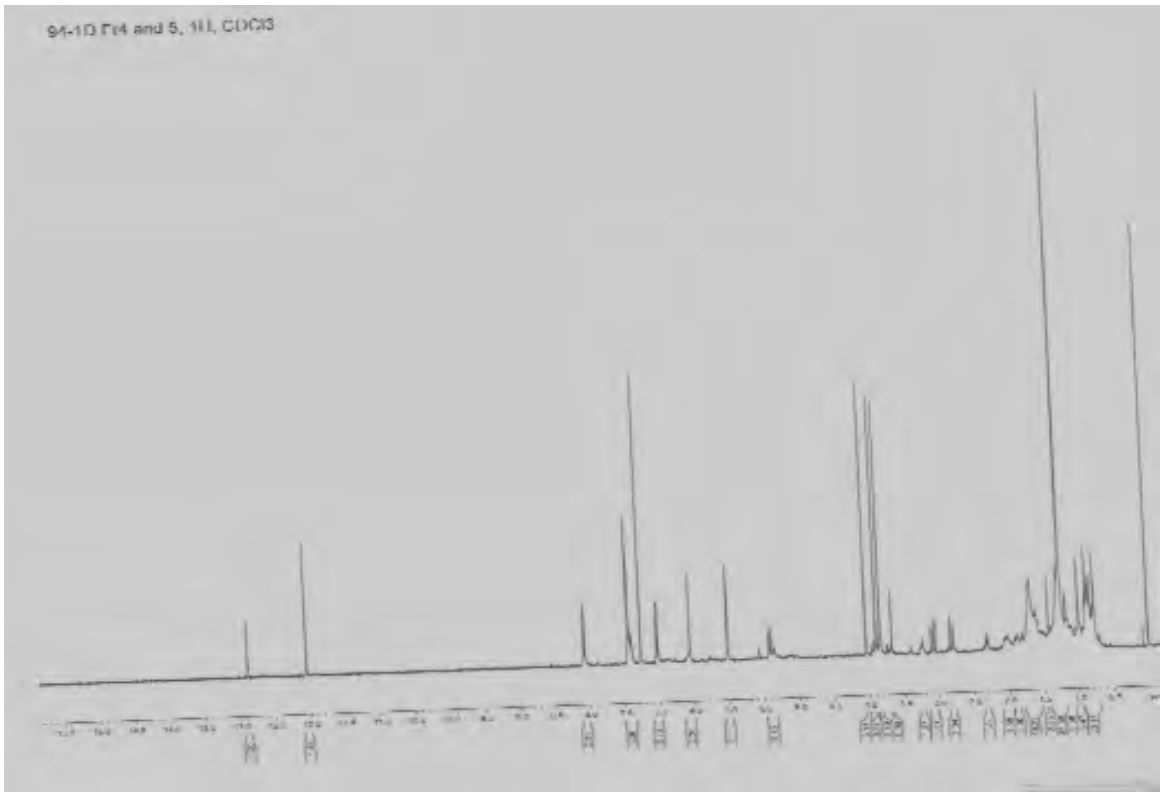

Fig. 3 Proton NMR spectrum of combination of fraction four and five of *D. angustifolia* leaves ethyl acetate extract.

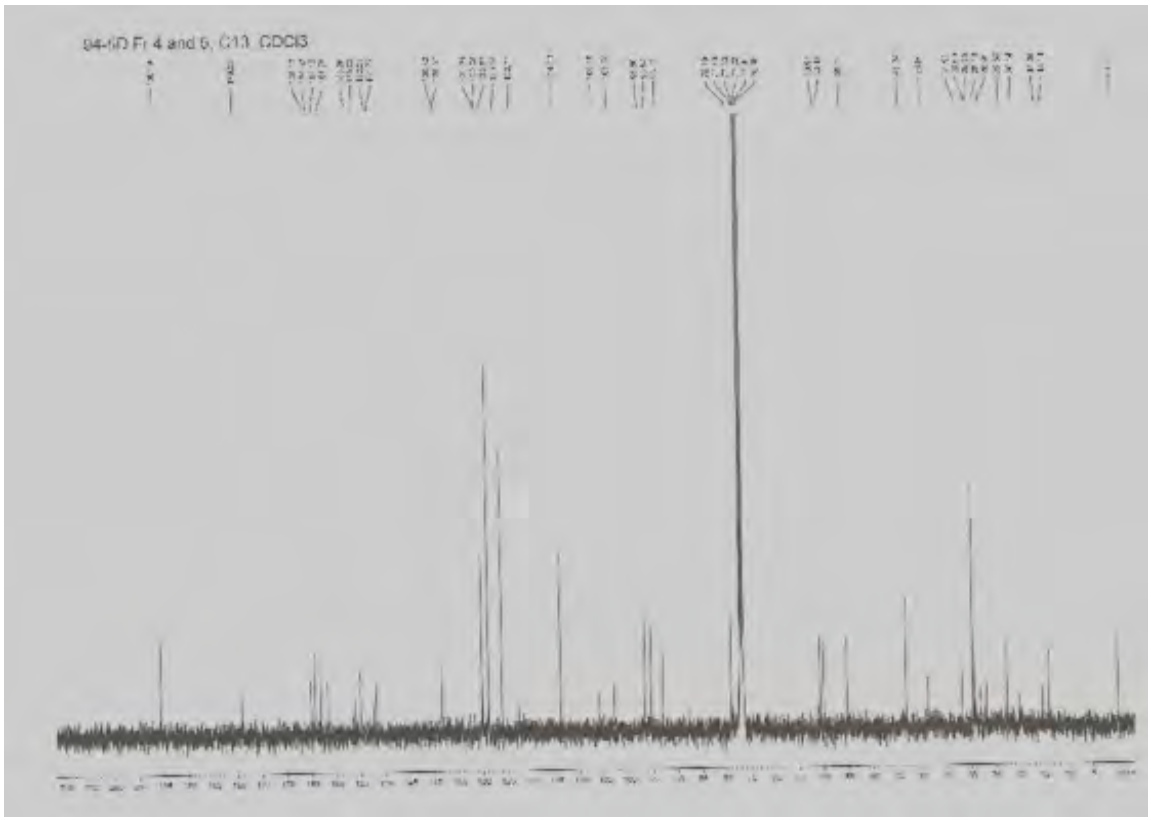

Fig. 4 Carbon-13 NMR spectrum of combination of fraction four and five of *D. angustifolia* leaves ethyl acetate extract.

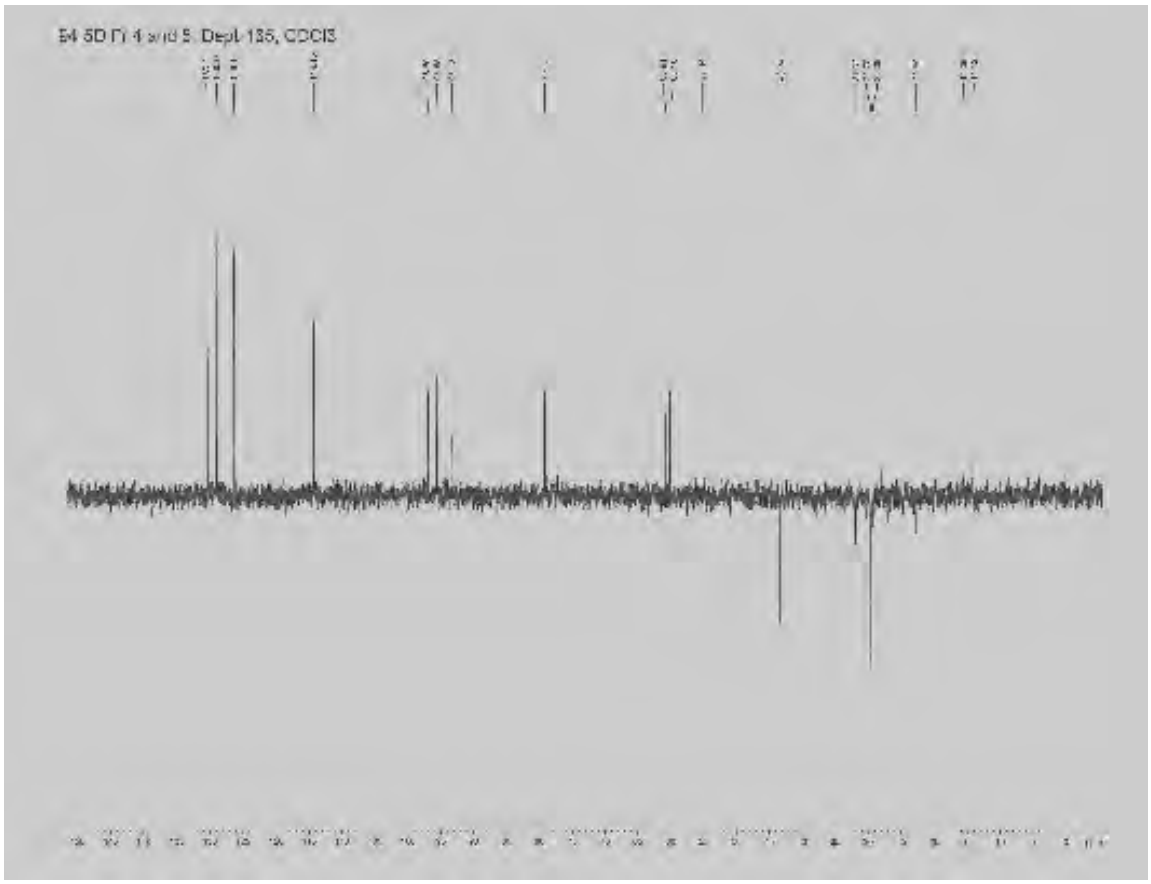

Fig. 5 DEPT-135 NMR spectrum of fraction four and five of *D. angustifolia* leaves ethyl acetate extract.

# 6. Discussion

Research on new antimalarials from natural products is the principal approach for new drugs with novel modes of action. This approach is believed to exploit the active substances for development of drugs from the untouched reservoir in medicinal plants. The relevance of this approach is evidenced from recent works where several traditionally used plants such as *Cinchona* species and *Artemisia annua* became the origin of quinine and artemisinin respectively (Andrade-Neto *et al*., 2007). So the curing capacity of plants possessed by inhabitants of malaria endemic regions is now being assessed. Hence evaluation of extracts of the leaves of *C. myricoides* and *D. angustifolia* against *P. berghei* in Swiss albino mice has relevance in this regard.

### 6.1 *Clerodendrum myricoides*

The non-toxic property of the leaves of *C. myricoides* and *D. angustifolea* extracts in mice has been reported from previous studies (Tekalign *et al.* 2010). Moreover, these plants are widely used as oral administrations for medication purposes and so far there is no know claim of toxicity.

In this study methanol extract of leaves of *C. myricoides* significantly ($P< 0.05$) inhibited parasitaemia of *P. berghei* in mice. The highest suppressive effect of this medicinal plant was 76.99% at 500mg/kg oral dose and this result is in agreement with the result of the study of Tekalign *et al*. (2010) that reported suppressive effect of 82.25% at 600mg/kg oral dose. Assefa *et al*. (2006) also reported that ethanol, petroleum ether, ethyl acetate and aqueous root bark extracts of C. *myrecoids* exhibited antimalarial activities *in vitro* with $IC_{50}$ values of 300µg/ml, 47µg/ml, 11µg/ml and 300µg/ml, respectively. In addition, this methanolic extract also afforded high activity with $IC_{50}=16.8 \pm 2.65$µg/ml as demonstrated in Muregi *et al.* (2004). These studies have confirmed *C. myricoides* is among the plants that displayed interesting antimalarial activity *in vitro* as well as *in vivo* systems.

The chloroform and chloroform/ethyl acetate extracts of leaves of *C. myricoides* did not induce significant *in vivo* suppression on *P. bergei* in mice at different doses. This observation may indicate the antimalarial active components of this medicinal plant

might not have polar properties since the solvents used have less polarity. This result is in parallel with the findings of *in vitro* activity test of Muregi *et al*. (2004), in which *C. myricoides* chloroform extract showed limited antiplasmodial activity against *P. falciparum* with $IC_{50}$ value greater than 100µg/ml.

In the present study upon successive extraction of the methanol extract of leaves of *C. myricoides,* the ethyl acetate and residue (ethyl acetate insoluble) extracts showed percent suppression of 61.30 and 69.31 at a dose of 150mg/kg each. On the contrary, the hexane extract did not show significant antimalarial effect. This finding is consistent with the work of Muregi *et al*. (2004) which indicated that *C. myricoides* hexane extract was found to exhibit limited antiplasmodial activity *in vitro* against *P. falciparum* with $IC_{50}$ value greater than 100µg/ml. However, the ethyl acetate extract was within the activity range of $IC_{50}$=48.6 $\pm$ 1.43µg/ml. The level of suppression induced by these extracts was less than that of the methanol extract of this plant. The antiplasmodial activity of these extracts suggests that the observed suppression of parasitaemia could be due to synergetic effect of the compounds present in the crude extract.

*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. Other traditional uses of *Cleodendrum* plant include treatment for gonorrhea, gout, swelling, wound dressing and rabies (Harbone and Baxter, 1993). Therefore, the present findings indicated that the traditional use of this plant as antimalarial medication is in parallel lining with the scientific evidence of this study. Consequently, since many people of the rural residents of Ethiopia practice traditional use of *C. myricoides* as an alternative choice of treatment of malaria without any available scientific proof on their efficacy, this study contributes a lot to the recognition of this traditional healing. Nevertheless, there is a need of optimization of its dose.

The suppression induced by the extracts might be associated with the presence of chemical ingredients that have antimalarial properties. *C. myricoides* is known to contain alkaloids such as cleromyrin I and II, myricoidine and dihydromyricoidine (Assefa *et al*., 2006). Pascaline (2011) also reported that the methanol extract of leaves of *C. myricoides* contains alkaloids, saponins, glycosides, phenolics, terpenoides and flavonoides. The

activity displayed by this species *in vivo* as well as the presence of these types of alkaloids suggest that the antiplasmodial activity observed might be due to such compounds.

### 6.2 *Dodonaea angustifolia*

In the present study of the antimalarial activity of leaves of *D. angustifolia,* the methanol extract showed a relatively higher suppression of parasitaemia (80.89%) at an oral dose of 300mg/kg. Administration of the mice with 500mg/kg also resulted in parasitaemia suppression of 78.27%. Both test doses showed significant result ($P<0.05$) and this work is in agreement with the report of Tekalign *et al.* (2010) even though a slight decrease in suppressive activity was observed in this study, unlike the concentration dependent increment of antiplasmodial activity as indicated by Tekalign and coworkers (2010).

Nevertheless, chloroform and chloroform/ethyl acetate (1:1) extracts of the leaves of *D. angustifolia* showed no significant suppression of the parasites in the mice ($P>0.05$). Besides, the suppression effect of chloroform/ethyl acetate (1:1) extract of *D. angustifolia* leaves was greater than that of the pure chloroform extract at the same dose (13.91%, 6.80% respectively at 300 mg/kg). This suggests the antiplasmodial active compounds of *D. angustifolia* could probably be more polar compounds as the polarity of chloroform/ethyl acetate combination is greater than pure chloroform.

From the products of further extraction of the methanolic extract of *D. angustifolia* leaves, ethyl acetate extract gave stronger suppression value (82.24 % suppression at 150mg/kg dose) suggesting that the antiplasmodial active components of the plant are probably with moderate polarity that can dissolve in ethyl acetate. Venkatesh *et al.* (2008) have reported that phytochemical analysis of ethyl acetate extract of *D. angustifolia* leaves showed the presence of carbohydrates, steroids and sterols, flavonoids, tannins and saponins. The residue (ethyl acetate insoluble) also exhibited moderate activity (51.93%) at the same dose. However, the third extract, with hexane lacked plasmodial suppression activity.

Rani *et al*. (2009) reported that *D. angustifolia* has worldwide traditional use as source of traditional medicine. It is administered orally or as poultice to treat a great variety of ailments. Stem or leaf infusions are used to treat sore throats; root infusions to treat colds. The stems and leaves are used to treat fever, and seeds (in combination with other plants and coated in honey) to treat malaria.

Different secondary metabolites from *D. angustifolia* have been identified. Out of these, saponins, flavonoides, alkaloids, terpenoids, diterpenoids, p-coumarin acid ester and essential oils are found (Rani *et al*., 2009). Any therapeutic activity in the herb is associated with polyvalent pharmacological effects brought on by synergistic combination of several constituents rather than any single isolated one (Anilreddy, 2009). Many uses of *D. angustifolia* by the indigenous people from various countries show remarkable similarities. These in turn appear to correlate with the active constituents. Although the active compounds are not yet fully identified, the antiplasmodial activity observed in this study could probably have resulted from a single or combined effect of these compounds which can easily dissolve in ethyl acetate.

### 6.3 TLC, Column Chromatography and NMR spectrometer

During the application of extracts of *C. myricoides* in the TLC, the hexane and ethyl acetate extracts gave three spots that may correspond to three compounds each. In the same way, the hexane extract of *D. angustifolia* showed five compounds and the ethyl acetate extract revealed six compounds in the TLC. The residue, which is the ethyl acetate insoluble material, also resulted in three spots.

The ethyl acetate extract of *C. myricoides* was fractionated using column chromatography. From the nine fractions collected, fractions one and two were known to have three compounds, whereas fractions four and five were observed to contain one visible compound. So these compounds could be the possible antiplasmodial active agents.

Many compounds in fractions one, two and three; one compound in each of the fourth, fifth, eleventh and thirteenth fractions of ethyl acetate extract of *D. angustifolia* leaves

became visible. The NMR spectrometer results of the eleventh fraction ($F_{11d}$) showed good proton peaks but the carbon analysis did not show any resolution due to decomposition of the sample. Moreover, the combination of fractions four and five resulted in better NMR spectrometer findings with some possible characterization of the compound such as the presence of 18C-13; presence of aromatic region as well as carbonyl groups. Fragments like –CH, $–CH_2$ and $–CH_3$ are also known to be included in the compound. Even though the above partial interpretation indicated some constituents of the molecule in the fraction, it is not conclusive and it needs further elucidation.

The present study demonstrated the antimalarial effect of *C. myricoides* and *D. angustifolia* as is justified by significant decrease in parasitaemia and the percent suppression of parasitaema in the Swiss albino mice.

# 7. Conclusions

The methanol extracts and the successive extracts of the leaves of *C. myricoides* and *D. angustifolia* possess antiplasmodial activity as seen in their ability to suppress *P. berghei* infection in Swiss albino mice in a dose related manner. These suppressive activities of the extracts suggest their ethnopharmacological usefulness as possible sources of antimalarials. Besides, low antiplasmodial activity of the chloroform and hexane extracts in the present *in vivo* study may indicate that the active ingredients responsible for this activity are not non polar compounds. In particular, the ethyl acetate extract and residue (ethyl acetate insoluble) of methanol extracts of both plant parts, especially the leaves of *D. angustifolia,* displayed higher suppression and may serve as a potential source for isolation of lead antimalarial compounds after further detailed study. TLC, Column chromatography and NMR results of these plants showed that the leaves were rich in chemical constituents even though it was difficult to know the actual compounds.

# 8. Recommendations

- Further phytochemical investigation should be done in order to clearly identify the antiplasmodial active components of *C. myricodes* and *D. angustifolia.*

- To identify the antiplasmodial active compounds of *C. myricoides* and *D. angustifolia,* it is recommended to use polar solvents because the extracts of these plants using non polar solvents did not show appreciable results.

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

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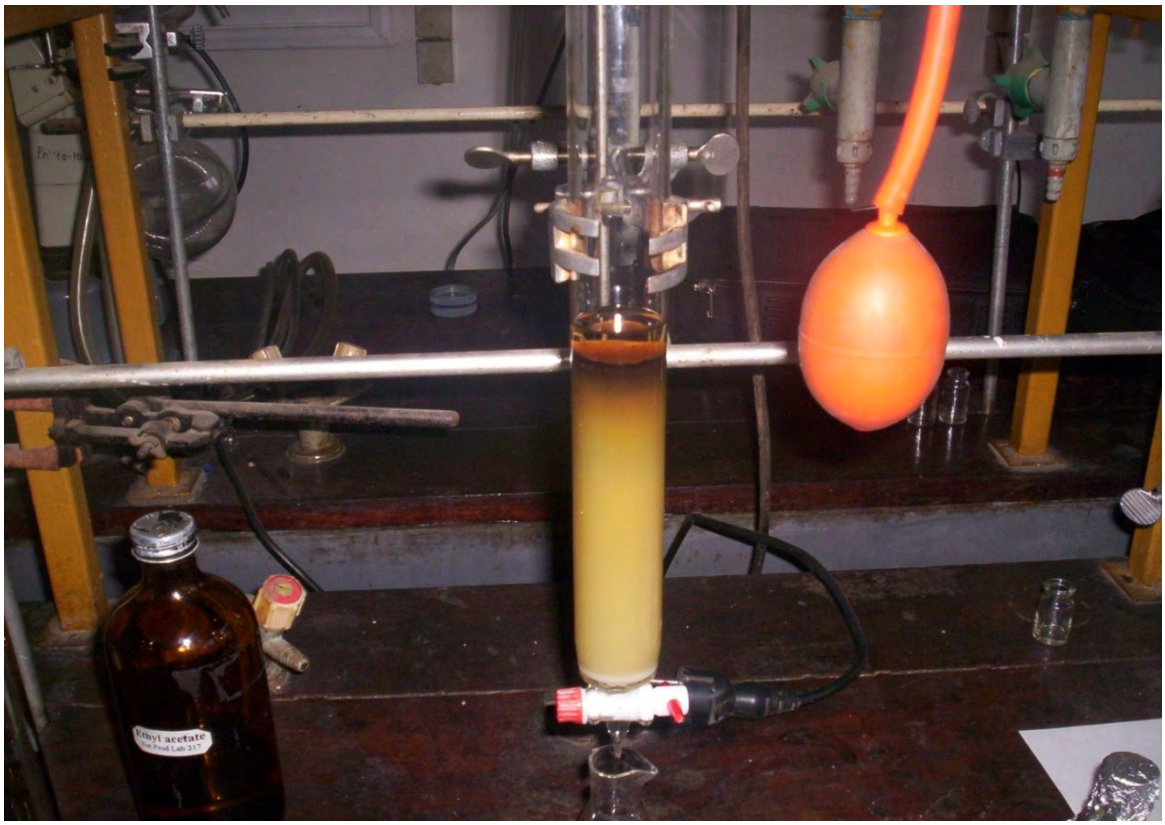
# Appendix

Photographs that show some of the practical works done throughout this study


Ethyl acetate