IN-VIVO ANTI-PLASMODIUM ACTIVITY OF CRUDE EXTRACTS AND SOLVENT FRACTIONS OF STRYCHNOS MITIS LEAVES IN PLASMODIUM BERGHEI INFECTED MICE.

By:

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A thesis submitted to the School of Graduate Studies of Addis Ababa University in partial fulfillment of the requirement for the Degree of Master of Science in Pharmacology.

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ADDIS ABABA, ETHIOPIA
This is to certify that the thesis prepared by Selamawit Fantahun, entitled: *In-vivo* anti-malarial activity of crude extracts and solvent fractions of *Strychnos mitis leaves* in *Plasmodium berghei* infected mice and submitted in partial fulfillment of the requirements for the Degree of Master of Science in Pharmacology complies with the regulations of the university and meets the accepted standards with respect to originality and quality.

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Principal advisor __________________Signature_________Date________

CO-advisor ______________Signature_________Date________

CO-advisor __________________Signature_________Date________

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Chair of Department or Graduate program coordinator
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<td>ACT</td>
<td>Artemisinin-based Combination Therapy</td>
</tr>
<tr>
<td>ANOVA</td>
<td>Analysis of variance</td>
</tr>
<tr>
<td>CDC:</td>
<td>Centers for Disease Control and Prevention</td>
</tr>
<tr>
<td>CQ</td>
<td>Chloroquine</td>
</tr>
<tr>
<td>DHEAS</td>
<td>Dehydroepiandrosterone sulfate</td>
</tr>
<tr>
<td>DHFR</td>
<td>dihydrofolate reductase</td>
</tr>
<tr>
<td>DHOD</td>
<td>dihydroorotate dehydrogenase</td>
</tr>
<tr>
<td>GPI</td>
<td>Glycosylphosphatidylinositol</td>
</tr>
<tr>
<td>IRBC</td>
<td>Infected Red Blood Cells</td>
</tr>
<tr>
<td>IRS</td>
<td>Indoor Residual Spraying</td>
</tr>
<tr>
<td>ITNs</td>
<td>Insecticide-treated nets</td>
</tr>
<tr>
<td>LLINs</td>
<td>Long Lasting Insecticide-Treated nets</td>
</tr>
<tr>
<td>OECD</td>
<td>Organization for Economic Cooperation and Development</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase Chain Reaction</td>
</tr>
<tr>
<td>PCV</td>
<td>Packed Cell Volume</td>
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<tr>
<td>RPM</td>
<td>Revolutions per Minute</td>
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<tr>
<td>SPSS</td>
<td>Statistical Packages for Social Science</td>
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<td>WHO</td>
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ABSTRACT

Malaria is a major public health problem in the world which is responsible for death of millions particularly in sub-Saharan Africa. Today, the control of malaria has become gradually more complex due to the spread of drug-resistant parasites. Medicinal plants are the unquestionable source of effective antimalarias. The present study was aimed to evaluate antiplasmodial activity and determine acute toxicity of *Strychnos mitis* in *P.berghi* infected mice. All extracts revealed no obvious acute toxicities on mice up to the highest (2000mg/kg) dose given.

The crude aqueous extract, crude hydromethanolic extract, N--hexane fraction of crude hydromethanolic extract, chloroform fraction of crude hydromethanolic extract and aqueous fraction of crude hydromethanolic extract of the leaf of *Strychnos mitis* significantly (p<0.01) inhibited parasite load. At highest dose (600mg/kg) both aqueous and hydromethanolic extracts showed higher percentage suppression 95.5% and 93.97% respectively, which is comparable to CQ(25mg/kg)(100%). All doses of crude extracts and fractions of *S.mitis* leave prolong the survival time of infected mice dose dependently. The higher two doses of the crude aqueous extract, hydromethanolic extract, chloroform fraction and aqueous fraction prevented the weight loss in a dose dependent manner. Whereas all doses of N- hexane fraction prevented loss of body weight not dose dependently.

The crude aqueous extract at (400mg/kg and 600mg/kg) and hydromethanolic extract at all dose level significantly (p<0.01) prevented the expected reduction in PCV. Crude aqueous extract at (600mg/kg) and hydromethanolic extract at all dose level significantly prevented temperature reduction. The phytochemical screening of the crude aqueous extract showed the presence of Alkaloids, Anthraquinones, Glycosides, Terpenoids, Saponins, Tannins and Phenols. The results of this study provide support for the traditional therapeutic value for treatment of malaria.

**Key words**: *Strychnos mitis*, crude extract, fraction, *Plasmodium berghei*, Antimalarial activity.
1. INTRODUCTION

Malaria remains a critical problem in global public health. It continues to remain among the top three infectious diseases (Malaria, tuberculosis and HIV) affecting billions of people globally (Mboowa, 2014). It is one of the most prevalent, devastating parasitic infectious diseases in the world that are caused by parasites of the genus Plasmodium and kills more than one million individuals in the tropical and subtropical zones annually (Mojarrab et al., 2014). Malaria remains a protracted global disease problem compromising improved health care and life expectancy among the poor especially in South-east Asia and sub-Saharan Africa. Pregnant women and children under 5 years of age are most vulnerable to malaria (Jigam et al., 2011). Even though malaria control programs have been strongly implemented in the last decade, the huge burden of malaria is still persisting especially in sub-Saharan Africa (Korenromp et al., 2013).

1.1. Epidemiology of Malaria

In 2012, an estimated 3.4 billion peoples are at risk of malaria from which 207 million cases and 627,000 deaths of malaria reported globally. The deaths (90%) occurred in the WHO African Region, most deaths (77%) were in children under 5 years of age. In addition, malaria accounts for 40% of public health expenditure, 30-50% of inpatient admissions, and up to 50% of outpatient visits in areas with high malaria transmission (WHO, 2013). Where as in 2013, an estimated 3.3 billion People are at risk of being infected with malaria from which198 million cases and the disease led to 584, 000 deaths of malaria reported globally, representing a decrease in malaria case incidence and mortality rates of 30% and 47% since 2000, respectively. The burden is heaviest in the WHO African Region, where an estimated 90% of all malaria deaths occur, and in children aged less than 5 years, WHO account for 78% of all deaths (WHO, 2014).
A number of factors account for the malaria burden in developing countries, which include climate change, infrastructure, emerging drug and insecticide resistance, massive population and costs of containment and therapy (WHO, 2012).

Similarly, malaria is also the major public health problem in Ethiopia, where it has been the major cause of illness and death for many years. According to records from the Ethiopian Federal Ministry of Health, 75% of the country is malarious with about 68% of the total population living in areas at risk of malaria. That is, more than 50 million people are at risk from malaria, and four to five million people are affected by malaria annually (Ayele et al., 2012). The disease causes 70,000 deaths each year and accounts for 17% of outpatient visits to health institutions (Ferede et al., 2013).

Epidemics of malaria are relatively frequent involving highland or highland fringe areas of Ethiopia, mainly areas 1,000-2,000 m above sea level. Malaria transmission peaks bi-annually from September to December and April to May, coinciding with the major harvesting seasons (Ayele et al., 2013). The majority of Ethiopian regional states (Oromia, Amhara, Tigray, South Nation and Nationalities People’s Region (SNNPR) have experienced malarial epidemic (Mekonen et al., 2014).

Since the year 2000, Malaria mortality rates have decreased by 47% worldwide and by 54% in Africa Region (WHO, 2014). But, this success is fragile it is crucial for to sustain its control activities and decrease malaria admission rates. Recently, malaria control program was jeopardized by lack of accessibly to effective malaria control tools, emergence of resistance to insecticides and first-line drug, artemisinin, and limited effective antimalarial drugs (Blayneh & Mohammed-Awel, 2014). These clearly discovered the need for new anti-malarial compounds with novel mechanisms of action that strengthen the current effort in malaria control.
1.2. Ethiology and transmission of Malaria

The parasites that cause malaria are protozoan organisms that also infect humans and many animal species including primate, lizard and birds. Malaria is caused by five species of the parasite belonging to the genus Plasmodium. Four of these are; *P. falciparum*, *P. vivax*, *P. malariae* and *P. ovale* are human malaria species. In recent years human cases of malaria have also been recorded due to *P. knowlesi* which is a species that causes malaria among monkeys, and occurs in certain forested areas of South-East Asia (WHO, 2014).

In Africa, malaria due to *P. falciparum* is the most predominant and deadly form (WHO, 2013). *P. vivax* has a wider distribution than *P. falciparum* because it is able to develop in the Anopheles mosquito vector at lower temperatures, and to survive at higher altitudes and in cooler climates. It also has a dormant liver stage (known as a hypnozoite) that enables it to survive during periods when Anopheles mosquitoes are not present to continue transmission, such as during winter months (Price et al., 2007). *Plasmodium vivax* and *P. ovale* form resting stages in the liver as hypnozoites and can cause a clinical relapse (Mekonene et al., 2014).

In Ethiopia *P.falciparum* and *P. vivax* are the most dominant malaria parasites, distributed all over the country and accounting for 60% and 40% of malaria cases respectively. However, *P. malariae* accounts for less than 1% and *P. ovale* is rarely reported in the country (Adugna, 2011).

Malaria is transmitted to humans by the bite of an infected female anopheles mosquito. The disease results from the multiplication of Plasmodium parasites within red blood cells, causing symptoms that typically include fever and headache, in severe cases progressing to coma or death. It is widespread in tropical and subtropical regions, including much of Sub-Saharan Africa, Asia, and the Americas (Odeghe Othuke et al., 2012). There are about 400 different
species of *Anopheles* mosquitoes, but only 30 of these are vectors of major importance. *Anopheles gambiae* is the most widespread in Africa and the most difficult to control. In addition, the *Anopheles arabiensis, Anopheles pharoensis, Anopheles funestus, Anopheles nili* are the prominent *Anopheles* species in Ethiopia (WHO, 2013; Adugna, 2011).

1.3 Lifecycle of Malaria Parasite

Malaria is transmitted by the bite of infected female *Anopheine* mosquitoes. Sporozoites from the mosquito salivary glands rapidly enter the circulation after a bite and localize via specific recognition events in hepatocytes, where they transform, multiply, and develop into tissue schizonts which lasts for 5 to 15 days, depending on the *Plasmodium* species (Bannister & Sherman, 2009). Sporozoites infect liver cells and mature into schizonts which rupture and release merozoites and infect red blood cells (Nwazue *et al*., 2013). It is to be noted that *P. vivax* and *P. ovale* infections that release dormant schizonts (hypnozoites) which can persist in the liver and cause relapse by invading the blood stream weeks or even years later. After this initial replication in the liver (exo-erythrocytic schizogony), the parasites undergo asexual multiplication in the erythrocytes (erythrocytic schizogony). The merozoites invade more erythrocytes to continue the cycle, which proceeds until death of the host or modulation by drugs or acquired partial immunity. *P. falciparum* assembles cytoadherence proteins into structures called knobs on the erythrocyte surface. This allows the parasitized erythrocyte to bind to the vascular endothelium, to avoid the spleen, and to grow in a lower oxygen environment. For the patient, the consequences are micro vascular blockage in the brain and organ beds and local release of cytokines and direct vascular mediators such as nitric oxide, leading to cerebral malaria (Rowe *et al*., 2009). Some parasites differentiate into sexual erythrocytic stages (gametocytes). Blood stage parasites are responsible for the clinical manifestations of the disease. The male (microgametocytes) and the female gametocytes (macrogametocytes) are
ingested by anopheles mosquitoes during a blood meal. The parasite multiplication in the mosquito is known as the sporogonic cycle. While in the mosquito stomach, the microgametes penetrate the macrogametes generating zygotes. The zygotes in turn become motile and elongated, and invade the mid-gut wall of the mosquito, where they develop into oocyst. The oocyst grow, rupture and release sporozoites which invade the salivary gland of the mosquito. The insect then can infect a human host by taking a blood meal, perpetuating the malaria or plasmodium lifecycle (Nwazue et al., 2013).

Figure 1. Malaria life cycle (CDC, 2003)

1.4. Pathophysiology of Malaria

Lysis of infected RBCs (iRBCs) resulted in release of newly developed merozoites and other toxic factors glycosylphosphatidylinositol (GPI) in to the blood. These products, particularly the
GPI, activate macrophages and endothelial cells to secrete cytokines and inflammatory mediators such as tumor necrosis factor, interferon-γ, interleukin-1, IL-6, IL-8, macrophage colony-stimulating factor, and lymphotoxin, as well as superoxide and nitric oxide (NO). The systemic manifestations of malaria such as headache, fever and rigors, nausea and vomiting, diarrhea, anorexia, tiredness, aching joints and muscles, thrombocytopenia, immune suppression, coagulopathy, and central nervous system manifestations have been largely attributed to the various cytokines released in response to these parasite and red cell membrane products (Angulo & Fresno, 2002). In addition to these factors, the plasmodial DNA is also highly proinflammatory and can induce cytokinemia and fever. The plasmodial DNA is presented by hemozoin to interact intracellularly with the Toll-like receptor-9, leading to the release of proinflammatory cytokines that in turn induce COX-2-upregulating prostaglandins leading to the induction of fever. Hemozoin has also been linked to the induction of apoptosis in developing erythroid cells in the bone marrow, thereby causing anemia (Miller et al., 2002).

In *P. falciparum* infections, iRBCs become rigid and spherical losing their fluidity unlike the normal RBCs which increases adhesiveness, iRBCs adhere to the capillary and postcapillary venular endothelium in the deep microvasculature (cytoadherence). The infected red cells also adhere to the uninfected red cells, resulting in the formation of red cell rosettes (rosetting)(Adams, 2014). Cytoadherence leads to sequestration of the parasites in various organs which facilitate growing *P. falciparum* parasites in these deeper tissues that is better suited for their maturation and the adhesion to endothelium allows them to escape clearance by the spleen and to hide from the immune system. If the cytoadherence-rosetting-sequestration of infected and uninfected erythrocytes in the vital organs goes on uninhibited, it ultimately blocks blood flow, limits the local oxygen supply, hampers mitochondrial ATP synthesis, and stimulates cytokine production - all these factors contributing to the development of severe disease. Certain
proteins expressed on the surface of the infected red cells mediate the adhesion of parasitized RBCs to the endothelium and to uninfected red cells. The most important of such proteins is the *P. falciparum* erythrocyte membrane protein 1 (PfEMP1), an antigenically diverse protein family that is expressed on the thousands of knob-like excrescences on the surface of red cells infected with *P. falciparum* trophozoites and schizonts (Hughes *et al.*, 2010)

1.5. Clinical manifestations

The signs and symptoms of malaria typically begin 8–25 days following infection (Olasunkanmi *et al.*, 2013) however, symptoms may occur later in those who have taken antimalarial medications as prevention (Nadjm and Behrens, 2012). The first symptoms of malaria are nonspecific and similar to the symptoms of a minor systemic viral illness. They comprise: headache, lassitude, fatigue, abdominal discomfort, muscle and joint aches, usually followed by fever, chills, perspiration, anorexia, vomiting and worsening malaise. The hallmark of malaria is fever. The fever is usually irregular at first and the temperature rises with shivering and mild chills (Olasunkanmi *et al.*, 2013).

Consequences of severe malaria include coma and death if untreated, young children are especially vulnerable in endemic areas. Children with malaria frequently exhibit abnormal posturing, a sign indicating severe brain damage and cognitive impairment (Ekwebene, 2012).

1.6. Diagnosis of malaria

Rapid and accurate diagnosis of malaria is important for effective case management and if implemented well would reduce mortality from this disease. Malaria diagnosis has had several limitations. Microscopy remains the gold standard for diagnosis of malaria in developing endemic countries. Malaria smears permit both species identification and determine the parasite density. However processing and interpretation of malaria smear requires a skilled and well experienced technicians, appropriate equipment and fresh reagent that limit their use in endemic
region (Mekonene et al., 2014). In addition, it is time consuming and is not available in peripheral health facilities such as health posts. Rapid diagnostic tests (RDTs) have been recommended to minimize the problems in areas where microscopy is not available. They are commercially available, in kit form, with all necessary reagents and are rapid when compared to microscopy and can be performed easily by any medical stuff without of the need of laboratory technician (Mohammed et al., 2012).

Malaria RDTs are lateral-flow immuno-chromatographic tests that detect specific antigens produced by malaria parasites (Azikiwe et al., 2012). However, Compared to microscopy, the main disadvantages of currently available RDTs are: inability to quantify parasite density, inability to differentiate between *P. vivax*, *P. ovale* and *P. Malariae*. Therefore, microscopy remains the golden standard of choice (Mekonen et al., 2014).

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

1.7. Management of malaria

Strategies to control and eliminate malaria include vector control, chemoprevention for the most vulnerable populations, particularly pregnant women and infants; and timely treatment with appropriate antimalarial drugs (based on the parasite species and the documented drug resistance). Malaria control relies on effective prevention and case management. Prevention with vector control interventions aims to reduce transmission and thus decrease the incidence and prevalence of parasite infection and clinical malaria (WHO, 2013).
1.7.1. Chemotherapy of malaria

There are several classes of drugs used to treat malaria. All share the feature of targeting the merozoites, while some target gametocytes as well. These drugs include quinolines, antifolates and artemisinin, administered alone or in combination. Quinolines are thought to affect the polymerization of hemozoin, which is toxic to the parasite. Antifolates inhibit the synthesis of folic acid by blocking the dihydrofolate reductase and dihydropteroate synthetase enzymes of the parasite. Although the mechanism of action of artemisinin is not known, the most accepted one is interference with the plasmodial sarcoplasmic endoplasmic calcium ATPase (Santos et al., 2013).

Artemisinin derivatives and 8-aminoquinolines are useful transmission-blocking antimalarials whose optimal actions are on different stages of gametocytes. Quinine remains important for treating complicated *P. falciparum* malaria despite its toxicity when used for extended period of time (WHO, 2013).

Based on high-quality evidence of the efficacy superiority of artesunate over quinine in adults and children with severe malaria, the world health organization guidelines have been revised. Chloroquine is highly effective against erythrocytic forms of *P. vivax, P. ovale, P. malariae* and to chloroquine-sensitive strains of *P. falciparum*. It is the prophylaxis and treatment of choice when these organisms are involved. Artemisinin and semisynthetic derivatives, including artesunate, artemether and dihydroartemisinin are short-acting antimalarial agents that kill parasites more rapidly than conventional antimalarials, and are active against both the sexual and asexual stages of the parasite cycle (Whirl-Carrillo et al., 2012).

1.7.1.1. Treatment of uncomplicated *Plasmodium falciparum* malaria
The causative species, the severity of signs and symptoms as well as patient age, immunity status and other risk determining factors (acute or chronic conditions, pregnancy and/or immune impairment) direct the choice of the most appropriate therapy. In addition, drug therapy should be in conjunction with relevant treatment guidelines and subject to local availability of drugs. Currently, artemisinin-based combination therapy (artemether - lumefantrine) is used as first-line to treat uncomplicated *P. falciparum* malaria (WHO, 2013).

### 1.7.1.2. Treatment of severe malaria

The WHO guidelines for the treatment of malaria recommend that intravenous artesunate should be used for the treatment of severe malaria (falciparum and vivax) for a minimum period of 24 hours. Following initial parenteral treatment, once the patient can tolerate oral therapy, it is essential to continue and complete treatment with an effective oral antimalarial using a full course of an effective ACT (artesunate plus amodiaquine or artemether plus lumefantrine or dihydroartemisinin plus piperaquine) or artesunate (plus clindamycin or doxycycline) or quinine (plus clindamycin or doxycycline) (Singh *et al.*, 2013).

### 1.7.1.3. Treatment of non-falciparum malaria

In areas where *P. vivax* is known to be chloroquine-sensitive, the WHO recommends three days of chloroquine plus two weeks of primaquine (provided the affected individual is not severely G6PD deficient). Where ACT has been adopted for treatment of falciparum malaria and / or in areas where *P. vivax* is known to be resistant to chloroquine, ACT plus primaquine is seen as an “appropriate” alternative, with the exception of artesunate plus sulfadoxine-pyrimethamine which is regarded as ineffective against *P. vivax* in most areas (Douglas *et al.*, 2012).

### 1.7.1.4. Treatment of uncomplicated *P. falcifarum* malaria in special risk groups

Pregnant women are at increased risk of acquiring malaria and are susceptible to more severe disease. At First trimester, quinine plus clindamycin to be given for 7 days (artesunate plus
clindamycin for 7 days is indicated if this treatment fails); an ACT is indicated only if this is the only treatment immediately available, or if treatment with 7-day quinine plus clindamycin fails or uncertainty of compliance with a 7-day treatment and at second and third trimesters, ACTs known to be effective in the country/region or artesunate plus clindamycin to be given for 7 days, or quinine plus clindamycin to be given for 7 days. In case of lactating women, they should receive standard antimalarial treatment (including ACTs) except for dapsone, primaquine and tetracyclines. ACTs used as first-line treatment in infants and young children (WHO, 2010).

1.7.2. Chemoprophylaxis

Primary chemoprophylaxis involves taking a medicine before, during, and after travel to an area with malaria. In addition to primary prophylaxis, presumptive antirelapse therapy (terminal prophylaxis) is used after the exposure period (or immediately thereafter) to prevent relapses or delayed-onset clinical presentations of malaria caused by hypnozoites (dormant liver stages) of *P. vivax* or *P. ovale*. Chloroquine phosphate or Hydroxychloroquine sulfate is used for prevention of malaria only in destinations where chloroquine resistance is not present. Prophylaxis should begin 1–2 weeks before travel to malarious areas and continued by taking the drug once a week during travel in malarious areas and for 4 weeks after a traveler leaves these areas. Atovaquone-proguanil is also a fixed combination of atovaquone and proguanil used for prophylaxis malaria (daily for 1–2 days before travel to malarious areas, at the same time each day, while in the malarious areas, and daily for 7 days after leaving the areas). In addition, Primaquine phosphate is used for malaria prevention as primary and terminal prophylaxis in areas with primarily *P. vivax* prevalent (CDC, 2014; WHO, 2012).

1.8. Antimalarial drug resistance

The main cause of the worsening malaria situation in recent years has been the spread of drug-resistant parasites. This has led to rising malaria-associated mortality (Olasehinde *et al.*, 2014).
Anti-malarial drug resistance has emerged as one of the greatest challenges facing malaria control today and has also been implicated in the spread of malaria to new areas and re-emergence of malaria in areas where the disease had been eradicated. This resistance concerns numerous drugs, but is thought to be most serious with chloroquine (CQ), the cheapest and most widely used drug to treat malaria (Olasehinde et al., 2014).

Thus, there is an urgent need for increased efforts in anti-malarial drug discovery especially in Africa. In recent times, natural products of plant sources have been the center of focus as the main source of new, safer and more effective bioactive compounds with medicinal properties (Ogbuehi et al., 2013).

1.8.1. Mechanisms of antimalarial drug resistance

Generally, resistance depends on the chemical class of the antimalarial and its mode of action. Resistance to 4-aminoquinolines, Quinine and highly hydrophobic arylaminoalcohols, arises from mutations of genes encoding vacuolar trans-membrane proteins which regulate the influx/efflux of the drug at the target and Chloroquine (CQ) resistance in *P. falciparum* is primarily attributable to single nucleotide polymorphisms in *pfcrt* (CQ resistance transporter). Mutations in *P. falciparum* multidrug resistance 1 (PfMDR1), the gene encoding the *P. falciparum* P-glycoprotein homologue-1, seem to be the main cause of resistance to mefloquine but are also implicated in CQ resistance (Warhurst, 2007).

Resistance to antifolates is quite common worldwide and apparently depends on a stepwise accumulation of single point mutations of genes *pfalhps* and *pfalhfr* encoding the drug targets, dihydropteroate synthase and dihydrofolate reductase, respectively. Atovaquone resistance is associated with single point mutations in the cytochrome b gene of *P. falciparum* (WHO, 2010). Although artemisinin derivatives represent the most efficacious class of antimalarial drugs, some cases of resistance have been recently detected. This may be due to mutations or amplifications
of the gene encoding a PfMDR1 or mutations in the gene encoding sarco-endoplasmic reticulum calcium ATPase6 (Marfurt et al., 2010).

Resistance to one chemical class of antimalarial drugs may cross-react with the others and this is the main reason for the poor efficacy of multi-target antimalarial chemotherapy. This capacity of the malaria parasite to counter the multi-target therapy arises from the fact that gene mutations usually do not act in isolation and act synergistically to encode or enhance resistance. Both mutations in different genes and sequential accumulation of mutations in a single gene may determine cross-resistance (Marfurt et al., 2010).

1.9. Traditional medicine

The World Health Organization (WHO) defines traditional medicine as practices, knowledge and belief systems which use minerals, plants and animal based remedies, spiritual therapies and exercises to prevent, treat and maintain wellbeing. According to the WHO, about 80% of the population of the world depends on traditional medicine, mostly herbal remedies, for their primary health care needs. The African continent have a long history with the use of plants and in some African countries, up to 90% of the population relies on medicinal plants as a source of drugs. The recent reports have indicated that, 25% of the modern drugs are derived from the extract of medicinal plants (Wabe et al., 2011).

Medicinal plants have been the focus for the search of new antimalaria drugs in various parts of the world and the present global situation indicates a recent resurgence in the severity of malaria, due to the resistance of malaria parasites to mainstay antimalaria drugs. Hence, there is need to intensify research in the development of new, cheap and effective antimalaria drugs from medicinal plants (Shittu et al., 2011).
The analysis of traditional medicines that are employed for the treatment of malaria represents a potential for discovery of lead molecules for development of antimalarial drugs (Deressa et al., 2010). Historically, traditional medicinal plants have provided the source of the two major families of anti-malarial drugs still in use today, artemisinin and quinine. Artemisinin is a sesquiterpene endoperoxide that has been isolated as the active principle from the Chinese antimalarial herb *Artemisia annua* and quinine from *Cinchona* bark by the French scientists Caventou and Pelletier (Nwaka & Hudson, 2006). The success in isolation of artemisinin has inspired many researchers to look for new antimalarial drugs from plants that are being used to treat malaria in traditional health care systems (Deressa et al., 2010).

Local communities in Africa have practiced traditional medicine in particular herbal remedies as an alternative choice of treatment of malaria for generations. It is therefore, of interest to screen traditional antimalarial medicinal plants evaluation for the *in vitro* antiplasmodial, *in vivo* antimalarial and toxicity tests (Muthaura et al., 2007). Treatment with medicinal plants is a good alternative to modern antimalarial drugs, especially for the majority of those populations at malaria risk, particularly in developing countries that cannot afford to pay for conventional drugs. So; it is encouraging to see such a large number of naturally-occurring antimalarial compounds from the plants with immense structural diversity (Dharani et al., 2008).

In Ethiopia it is estimated that about 80% of the population is still dependent on traditional medicine, which essentially involves the use of plants. Despite their wide use in the traditional health care, the work that has been done to evaluate the safety and efficacy of Ethiopian traditional medicinal plants is not extensive (Deressa et al., 2010).

In Ethiopia, some of the medicinal plants used traditionally for the treatment of malaria have been screened for their antimalarial activity. For example, *Dodonaea Angustifolia* is traditionally claimed to have antimalarial activity in Ethiopia. *In-vivo* antimalarial Activity of *Dodonaea*
*angustifolia* seed extracts against *Plasmodium berghei* in mice model showed that the extracts significantly inhibited parasitemia and prevented packed cell volume reduction as showed by (Mengiste et al., 2012). In the study done by Deressa and his colleagues (2000), crude extracts of *Clerodendrum myricoides* and *Aloe debrana* of the plant showed strong activities against *P. berghei*. In study done on in-vivo antimalarial activity of crude extracts of the leaves of *Adhathoda schimperiana* to ascertain its traditional use in Ethiopia, the hydro-alcoholic extracts of leaves of *A. schimperiana* significantly reduced parasitaemia induced by chloroquine-sensitive *Plasmodium berghei* infection in suppressive, curative and prophylactic models in mice (Petros & Melaku, 2012). In addition, Dikasso et al have reported the hydroalcoholic extracts from *Asparagus africanus* showed appreciable in-vivo antimalarial activity against *Plasmodium berghei* (2006). This study was aimed to evaluate the in vivo antimalarial activity of *Strychnos mitis* since its anti-malarial activity has not been reported yet.

1.10. Investigational antimalarial agents

The goal of developing new antimalarial drugs is to find a molecule that can target multiple stages of the parasite’s life cycle, thus impacting prevention, treatment, and transmission of the disease. The 4(1H)-quinolone-3-diarylethers are selective potent inhibitors of the parasite’s mitochondrial cytochrome bc$_1$ complex. These compounds are highly active against the human malaria parasites *Plasmodium falciparum* and *Plasmodium vivax*. They target both the liver and blood stages of the parasite as well as the forms that are crucial for disease transmission, that is, the gametocytes, the zygote, the ookinete, and the oocyst (Nilsen et al., 2013). Besides, the new antimalarial candidates (TCMDC29, GSK1057714, GNF156, KA609, and MMV390048) developed by the optimization of chemical exhibited a potent activity against blood stages of *Plasmodium falciparum*. In addition, the antimalarial candidates, DSM265 (currently on clinical trial phase II) and P218, were derived from target-based screens against *Plasmodium falciparum*.
dihydroorotate dehydrogenase (DHOD) and *P. falciparum* dihydrofolate reductase (DHFR), respectively (Gamo, 2014).

A trioxolane, OZ439, which is now on clinical trial II was rationally designed to have the parasite killing activities similar to artemisinins, but with a longer half-life. OZ439 is capable of completely curing mice from malaria in a single dose of 20 mg/kg, artemisinin derivatives cannot achieve without the addition of a second drug. Furthermore, OZ439 has significant prophylactic activity, and a single 30 mg/kg oral dose of OZ439 administered 48 hours prior to parasite inoculation is protective activity against malaria (Biamonte et al., 2013; Wang et al., 2013).

Alkaloids such as benzophenanthridines (sanguinarine, chelerythrine), protoberberines (berberine) and protopines (protopine, allocryptopine) from aerial part of *Argemone mexicana* L. (Papaveraceae) were also shown to be potent antimalarial activity. It is now on clinical trial I to investigate the pharmacokinetics, pharmacodynamics and tolerability of the decoction of the aerial parts of *Argemone mexicana*, administered in healthy volunteers (Rubio-Pina & Vazquez-Flota, 2013). The physiological hormone dehydroepiandrosterone sulfate (DHEAS) which is a potent inhibitor of G6PD activity is known to exert antimalarial protection. Combination of CQ with DHEAS or buthionin sulfoximin (BSO, a specific inhibitor of GSH synthesis) significantly increased sensitivity of resistant parasites to CQ and increased the survival period of the infected mice. This reduction of parasitaemia and improvement of the survival of infected mice were associated with intra-parasite depletion of GSH and inhibition of G6PD activity due to DHEAS action (Safeukui, 2004). A number of trials have shown that Eurartesim® (dihydroartemisinin-piperaquine phosphate), artemisinin-based combination therapy (ACT), is highly effective in treating uncomplicated *P. falciparum* malaria. In 2011, the European Commission granted full marketing authorization of Eurartesim® (Ubben & Poll, 2013).
In addition, some antibiotics are also effective against malaria parasites. Fosmidomycin-clindamycin combination is on clinical trial III for treatment of acute uncomplicated *P. falciparum* malaria among Gabonese children. A combination of chlorproguanil HCl and dapsone as chlorproguanil-dapsone has already been shown to be efficacious against *P. falciparum* in adults and children in Sub-Saharan Africa. The addition of artesunate to Chlorproguanil-Dapsone has been also demonstrated to increase the parasite clearance rate as demonstrated in the clinical trial phase II study, and reduce the chance of any parasites escaping treatment over the 3-day course. A clinical trial phase III study is ongoing to compare the efficacy of a fixed ratio combination tablet of Chlorproguanil-Dapsone-Artesunate (CDA) to chlorproguanil-dapsone for uncomplicated malaria. Moreover, KAF156 showed a complete malaria protective activity as a single oral dose of 10 mg/kg in mice model. In addition, KAF156 displays potent Plasmodium transmission blocking activities both *in vitro* and *in vivo*. KAF156 is currently under evaluation in clinical trials for treatment, prevention, and blockage of transmission of malaria (Kuhnen et al., 2014).

Moreover, there are many antigens present throughout the parasite life cycle that potentially could act as targets for the vaccine. More than 30 of these are currently being researched by teams all over the world in the hope of identifying a combination that can elicit immunity in the inoculated individual. Some of the approaches involve surface expression of the antigen, inhibitory effects of specific antibodies on the life cycle and the protective effects through immunization or passive transfer of antibodies between an immune and a non-immune host. The majority of research into malarial vaccines has focused on the *Plasmodium falciparum* strain due to the high mortality caused by the parasite and the ease of a carrying out *in vitro/in vivo* studies. The earliest vaccines attempted to use the parasitic circum sporozoite protein. Circum sporozoite protein is the most dominant surface antigen of the initial pre-erythrocytic phase
Karunamoorthi, 2014). RTS,S/AS01E is the only malaria vaccine available and highly effective in reducing malaria infection in children aged 5-17 months (The RTS,S Clinical Trials Partnership, 2014).

1.11. *Strychnos mitis*

*Strychnos* is a genus of flowering plants, belonging to family Loganiaceae (sometimes Strychnaceae). The genus includes about 100 accepted species of trees and lianas, and more than 200 that are yet unresolved (Babu and Chaudhuri, 2005). The genus is widely distributed around the world's tropics. The member of this genus; *Strychnos nux-vomica*, native to tropical Asia which have a potential antidiabetic and antioxidant activity in alloxan induced diabetic model (Chitr et al., 2010). In addition Katiyar et al., 2010 experimental study revealed that the seed extract of *Strychnos nux-vomica* significantly reduced spontaneous motor activity and inhibited catalepsy. Aqueous and methanolic extract of *Strychnos nux-vomica* also reported to be active against Escherichia coli Enterobacter, Staphylococcus aureus, and Pseudomonas as described by Joy and Appavo, 2014.

The other species of the same genus, *Strychnos potatorum* Linn seeds have antiarthritic activity in Freund's adjuvant induced arthritic rats (Ekambaram et al., 2010). Selected parts of the tree like seeds, ripe fruit and roots are used in traditional system of medicine for the treatment of various ailments like tumors, pain, inflammation, anemia and jaundice, diabetes, diarrhea and gonorrhea; it has also been found to be locally effective for treatment of eye infections (Kirtikar and Basu, 2000, Yadav et al., 2014). And the seeds extract of *Strychnos Potatorum* Linn. definitely possess hypoglycemic potential in a streptozotocin-induced model as evidenced by Biswas et al., 2012 experimental study. *Strychnos potatorum* seed also possesses suppressive
effects on male fertility and could be useful in development of male contraceptive agent (Priya et al., 2012).

In addition, Sanmugapriya and Venkataraman, (2010) revealed that seeds of *Strychnos potatorum* linn. which have antinociceptive and antipyretic effects on experimental rats.

*Strychnos spinosa*is used in traditional medicine for treating snakebite, ulcers, wounds, headache, gastric and intestinal problems, venereal diseases, leprosy, diarrhea, and fever (Neuwinger ,1996). *S. spinosa* also have antiplasmodial activity (Beru et al., 2009). The acetone extract and the chloroform fraction of *Strychnos spinosa* leaves had the highest antibacterial activity that support the use of *S. spinosa* leaves in traditional medicine to treat infectious diseases as evidenced by Isa et al., 2014.

*Strychnos mitis* belonging to a Family:Loganiaceae is commonly known as Yedingamst (Amh), Mulqaa, Satto(Orm), Dankuke (Sidamgna), Suchamecha(Wel) and Hurutia (Anywaa). It is an evergreen tree, 6-35(-40) m tall (rarely shorter);the bark is smooth or slightly fissured and scaly, grey to greyish brown or greenish, inner bark thin, fibrous, pale brown or cream with orange-brown flecks; crown rounded; twigs glabrous, with lenticels. The leaves are opposite, petiole glabrous or sometimes pubescent, 0.2-0.5 cm long, smooth; blade paler beneath, leathery (not thick), narrowly elliptic, oblong, or sometimes ovate or narrowly ovate, 4-11.5 x 1.5-5 cm. Fruits are Yellow/orange, round.1.2 - 2 cm long. Seeds ellipsoid, flattened on one side,1 cm long, smooth and glabrous but with minute pits (Beentje , 1994).  

*Strychnos mitis* is found widely and abundantly in Sudan and Ethiopia southward to Angola, Zimbabwe, Mozambique, eastern South Africa and Swaziland, and in Comoros and Mayotte. The wood of *Strychnos mitis*, known as ‘hard pear’, is often used for building poles, railway sleepers and tool handles. In Ethiopia and Uganda *Strychnos mitis* is used as shade tree in coffee
plantations, in Uganda also in cocoa plantations and as an avenue tree. In Ethiopia the fruits are eaten (Bolza & Keating, 1972).

*Strychnos mitis* has therapeutic application against helmentic infection in ethno veterinary medicine on egg hatching and larval development of *Haemonchus contortus* and having excellent antifungal activities (Adamu *et al*., 2013).

The study by Adamu *et al*., 2014 revealed that *Strychnos mitis* leaf acetone extract have antibacterial and antioxidant activity.

*S. mitis*, is one of the traditionally used antimalarial plants used for treatment of malaria. However, the antimalarial activity and toxicity of *S. mitis* are not investigated. So the traditional claims enforced to evaluate its *in-vivo* antimalarial activity.

![Figure 2: Photograph of *Strychnos mitis* S.Moore](image)

The antiplasmodial activity has been linked to a range of several classes of the secondary plant metabolites including alkaloids, sesquiterpenes, triterpenes, flavanoids, limonoids, quassinoids, xanthones, quinines and phenolic compounds of which alkaloids have been the most important and have shown very interesting antiplasmodial activities (Dharani *et al*., 2008)
1.12. Rationale of the Study

Management of malaria is a big challenge due to four major problems. The most important one is that the parasites which cause malaria are resistant to the most widely available, affordable and safest first line treatments (Yeung et al., 2004). In addition, currently available indoor spraying with insecticides to reduce the transmission of malaria is hampered by insecticide resistance (Blayneh & Mohammed-Awel, 2014), absence of clinically proven vaccine (Mwangoka et al., 2013), lack of the necessary infrastructure and resources to manage and control malaria as well as to ward off fake curing agent (Kazembe et al., 2012).

Indeed, artemisinin resistance to a deadly *Plasmodium falciparum* now poses a threat to the control and elimination of malaria (Ashley et al., 2014). Chloroquine resistant strains can be controlled by artemisinin and artemisinin derivatives. But, currently there is no alternative approved antimalarial drug to replace artemisinin derivatives. Hence, it is extremely important to urgently intensify research in the development of new, cheap and effective antimalarial drugs. Medicinal plants remain the main focus by scientists and researcher as a noble source of new lead compound in the development of new antimalarial agent. Hence, Ethiopia, with a wealth of unexplored natural resources, is an ideal place to search for new drugs from plants.

The finding of this experimental study helps the scientific community to further investigate on the plant, *Strychnos mitis* by initiating advanced studies on formulation and molecular mechanisms of plant source drugs by identifying and isolating a specific antimalarial compound. Therefore, the present study describes the *in-vivo* antimalarial activity of crude extract and solvent fraction of *Strychnos mitis leaves* on mice infected with chloroquine sensitive *Plasmodium berghei*. 

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2. OBJECTIVES OF THE STUDY

2.1 General objective

➢ To evaluate in-vivo antimalarial activities of aqueous and hydromethanolic extract of Strychnos mitis leaves against Plasmodium berghei in mice model.

2.2 Specific objectives

✓ To evaluate the antimalarial activities of the crude aqueous and hydromethanolic extract of Strychnos mitis leaves in P. berghei inoculated mice.

✓ To investigate antimalarial effect of the solvent fractions of the plant extract in P.berghei inoculated mice.

✓ To determine the toxicity of crude extracts of the leaves of S.mitis in Swiss albino mice.

✓ To determine phytochemical constituents of the crude aqueous and hydromethanolic extract of Strychnos mitis leaves.
3. MATERIALS AND METHODS

3.1 Drug and chemicals
Trisodium citrate (BDH chemicals, England), Tween 80 (Lobe chemi, India), absolute methanol (Lobe chemi, India), Geimsa stain (Hightech Health Care, India), chloroquine phosphate tablet and distilled water (Ethiopian pharmaceutical manufacturer, Ethiopian), normal saline (Fresenius kabi AG, Germany), Wagners reagent, Mayer reagent (Fisher Scientific, UK), glacial acetic acid (Lobe chemi, India), ferric chloride (fisher scientific company/USA), dilute ammonia, hydrochloric acid (Riedal de Haen/Germany), chloroform ((Finkem Laboratory Reagent, India), N-hexane (fisher scientific UK limited/Israel), sulphuric acid (Farmitalia carlo erba/Italy).

3.2 Plant material
Fresh leaves of *S. mitis* were collected in February, 2014 from Yergalem of SNNP regional state, 318 km south of Addis Ababa. The fresh leaves were wrapped with plastic sheets during transportation. The specimen of the plant was identified as *Strychnos mitis* by a taxonomist and deposited at the National Herbarium, College of Natural Sciences, Addis Ababa University, with Voucher specimen (No. SF001) for future reference.
3.3 Animal and parasite

Male Swiss albino mice (age 6–8 weeks and weight of 27–32 g) bred and maintained at the Ethiopian public Health Institute were used. They were maintained under standard condition (at room temperature and 12 hr light/12 hr dark cycle), with food and water *ad libitum* in the animal house of Akililu Lemma Institute of Pathobiology, Addis Ababa University. Animals were acclimatized for one week to the experimental environment. All the experiments were conducted in accordance with the internationally accepted laboratory animal use, care and guideline (National Academy of Sciences, 2011).

Chloroquine sensitive strain of *Plasmodium berghei* (ANKA) was obtained from the Ethiopian public health institute. The parasites were maintained by serial passage of blood from infected mice to non-infected once on weekly basis (Fidock *et al.*, 2004). A Blood sample taken from donor mouse with the growing parasitaemia of 20-30% was diluted with normal saline, so that
each 0.2 ml of blood contained $1 \times 10^7$ *P. berghei* infected erythrocytes, the standard inoculums. These parasites were used to infect the experimental animals intraperitoneally (IP).

### 3.4. Preparation of Plant Crude Extracts

The collected plant leaves were, cleaned to eliminate any dead matter or other unwanted particles and air dried under shade at room temperature without exposure to sun light in Akiliilu Lemma Institute of Pathobiology, AAU. The dried specimens were grounded to powder using mortar and pestle. The crude extracts were prepared by cold maceration techniques as outlined by (O’Neill *et al.*, 1985). Considering the fact that organic substance either dissolves in water (polar) or alcohol (non-polar), aqueous and hydro methanolic extracts of the specimen were prepared as follows.

The powdered plant material was weighed by sensitive balance (METTLER TOLEDO, Switzerland), 300g of powdered specimens’ soaked in 2400ml of 80% methanol and 300g of powered specimens’ soaked in 2700ml of distilled water in separate Erlenmeyer flasks and placed on orbital shaker (Thermoforma, USA) at 145 rotations per minute (rpm) for 24 hours of water and 72 hours of 80% methanol at room temperature. The mixtures were first filtered using gauze and then the filtrates were passed through Whatman filter paper number 1 with pore size 150mm diameter (Wagtech international Ltd, England). The residues were re-macerated twice. The methanol from the combined filtrates of the hydro-methanolic extract was removed under reduced pressure by rotary evaporator (Buchi type TRE121, Switzerland) at 45 rpm and 40°C to obtain the crude extract. The extract was further concentrated to dryness with a lyophilizer (Wagtech Jouan Nordic DK-3450 Allerod, Denmark) at -50°C and vacuum pressure (200 mBar) whereas aqueous extract, the filtrate was frozen in deep freezer overnight and then freeze dried with a lyophilizer (Wagtech Jouan Nordic DK-3450 Allerod, Denmark) at -50°C and vacuum pressure (200 mBar). A total of 56.8 grams (yield = 18.8%) of dried hydro methanolic crude
extract and 43.3 grams (yield = 14.4%) of dried aqueous crude extract. All the extracts were stored in screw cap vials in a refrigerator (AKIRA, China) at -4°C until used. The water extract were dissolved in distilled water and 80% methanol extracts in 2% Tween 80 for use in the tests.

3.5 Preparation of fraction of hydromethanolic crude extracts

The Crude hydro methanolic extract was subjected to fractionation using N-hexane and chloroform. Forty gram of the crude hydro methanolic extract was suspended in a separatory funnel in a 240 ml of distilled water and partitioned with 3 ×240 ml N-hexane. The filtrate was concentrated in a rotary evaporator (Buchi type TRE121, Switzerland) at 45 rpm and 40°C to obtain the N-hexane fraction 9.1 grams (yield=22.75%). The aqueous residue was then partitioned with 3 ×240 ml chloroform. The chloroform filtrates was concentrated similarly as N-hexane fraction to have chloroform fraction 15.3 grams (yield=38.25%). The remaining aqueous residue was frozen in deep freezer overnight and then freeze dried with a lyophilizer (Wagtech Jouan Nordic DK-3450 Allerod, Denmark) at -50°C and vacuum pressure (200 mBar) to obtain aqueous fraction 17.3 grams(yield=43.25%). All the fractions were stored in screw cap vials in a refrigerator (AKIRA, China) at -4°C until used. The N-hexane and chloroform fraction were dissolved in 3% Tween 80 and aqueous fraction dissolved in distilled water for use in the tests.

3.6 Phytochemical Screening

The 80% methanol and aqueous extracts of Strychnos mitis leaves were screened for the presence of secondary metabolites to relate the antimalarial activity of the plant with the presence or absence of these constituents. Thus, tests for alkaloids, saponins, cardiac glycosides, flavonoids, terpenoids, steroids, phenols and tannins were performed using standard tests as described below.
**Test for Alkaloids (Wagner’s reagent)**

Crude extract was mixed with 2ml of 1% HCl and heated gently. Mayer’s and/or Wagner’s reagents were then added to the mixture. Turbidity of the resulting precipitate was taken as evidence for the presence of alkaloids (Yadav & Agarwala, 2011).

**Test for Terpenoids (Salkowski’s test)**

Crude extract was dissolved in 2ml of chloroform and evaporated to dryness. To this, 2ml of concentrated H$_2$SO$_4$ was added and heated for about 2 minutes. A grayish colour indicated the presence of terpenoids (Yadav & Agarwala, 2011).

**Test for Steroids (Liebermann-Burchard test)**

Crude extract was mixed with 2ml of chloroform and concentrated H$_2$SO$_4$ was added sidewise. A red color produced in the lower chloroform layer indicated the presence of steroids. Another test was performed by mixing crude extract with 2ml of chloroform. Then 2ml of each of concentrated H$_2$SO$_4$ and acetic acid were poured into the mixture. The development of a greenish coloration indicated the presence of steroids (Yadav & Agarwala, 2011).

**Test for Flavonoids (Alkaline reagent test)**

The extract was treated with few drops of 20% sodium hydroxide solution. Formation of intense yellow colour, which becomes colourless on addition of dilute hydrochloric acid, indicates the presence of flavonoids (Ugochukwu et al., 2013).

**Test for Tannins (Braymer’s test)**

Extract was treated with 10% alcoholic ferric chloride solution and observed for formation of blue orgreenish colour solution (Ugochukwu et al., 2013).
Test for saponins (Foam test)
Crude extract was mixed with 5ml of distilled water in a test tube and it was shaken vigorously. The formation of stable foam was taken as an indication for the presence of saponins (Yadav & Agarwala, 2011).

Test for phenols (Ferric chloride test)
A fraction of the extracts was treated with aqueous 5% ferric chloride and observed for formation of deep blue or black colour (Ugochukwu et al., 2013).

Test for cardiac glycosides (Keller Kelliani’s test)
Extract was treated with 2ml of glacial acetic acid in a test tube and a drop of ferric chloride solution was added to it. This was carefully underlayed with 1ml concentrated sulphuric acid. A brown ring at the interface indicated the presence of deoxysugar characteristic of cardenolides. A violet ring may appear below the ring while in the acetic acid layer, a greenish ring may form (Ugochukwu et al., 2013).

3.8. In vivo antimalarial screening

3.7.1. Acute toxicity test

Acute oral toxicity test was done according to the OECD/OCDE 425:2008 guideline for the crude extracts (OECD, 2008). The crude water and 80% methanol extracts of S. mitis intended for the antimalarial test against P. berghei were evaluated for their toxicity in non-infected female Swiss albino mice aged of 6-8 weeks and weighing 27-32g. The mice were fasted overnight and weighted before test. A single female mouse was given 2000 mg/kg of the extract as a single dose by oral gavage. After administration of the extracts food was withheld for further two hours period. Death was not recorded in the first 24 hrs; another 4 female mice were given the same dose and observed for toxic signs in the next 14 days for both extracts. According to the OECD guideline, the extract of Strychnos mitis leaves did not cause mortality and no body weight
reduction was observed at the administered 2000 mg/kg body weight dose within 24 hours and/or 14 days of observation. Gross physical and behavioral observation also revealed no visible signs of toxicity such as lacrimation, hair erection, and reduction in their motor and feeding activities.

3.7.2. Grouping and dosing of animals

Evaluation of antimalarial activity of two extract (hydromethanolic and aqueous extract) and three fractions (N-hexane, chloroform and aqueous fraction) of Strychnos mitis leaves were carried out by randomly assigning 30 male mice into five groups (three treatment groups and two controls), six mice per group for each extract and fraction.

Three treatment groups- received 200mg/kg, 400mg/kg and 600mg/kg of the crude extracts and 100mg/kg, 200mg/kg and 400mg/kg of the fraction respectively once daily for four days. Two controls (negative and positive) for crude extract and fractions received the vehicle (distilled water) and chloroquine phosphate (25 mg/kg) (standard drug) respectively. The vehicle, the plant and the standard drug were administered orally (by oral gavage). The dose levels of the extracts and fractions were selected for mice based on the result obtained from the oral acute toxicity test.

3.7.3. Inoculation of mice

In-vivo antimalarial activity of crude extract and fraction against early P. berghei ANKA strain infection was carried out according to the method described by Peters et al (1975). Chloroquine sensitive P. berghei was obtained from the Ethiopian public Health Institute. A donor mouse infected by chloroquine sensitive P. berghei (ANKA) strain with a rising parasitemia 20-30% was used for infecting mice in the 4 day suppressive test procedures. Parasites are maintained by continuous re infestation in mice. The parasitemia of the donor mice was first determined. The donor mice were then sacrificed and the collected blood from all donor mice were pooled together to avoid variability in parasitemia in a petri-dish containing 2%
trisodium citrate (BDH chemicals, England) as anticoagulant. The blood was then diluted with 0.9% normal saline. Accordingly each 0.2 ml of the aliquot could contain about $1 \times 10^7 P. \text{berghei}$ infected red blood cells and 1ml of the inoculums consisted of $5 \times 10^7 P. \text{berghei}$ infected erythrocytes. Each mouse used in the experiment was inoculated with 0.2 ml via intraperitoneal route.

On day 0, each mouse was inoculated with 0.2ml of infected blood containing about $1 \times 10^7 P. \text{berghei}$ ANKA strain parasitized erythrocytes intraperitoneally by using a hypodermic needle. Treatment was started three hours after mice had been inoculated with the parasite (Trager and Jensenon, 1976) at day 0 and then continued daily for four days from day 0 to day 3.

On the 5th day (day 4), thin films were made from the tail blood of each mouse and smear on to a microscope slide to make a film (Saidu et al., 2000). The blood films were fixed with methanol, stained with 10% Giemsa at pH 7.2 for 15 min and parasitaemia was examined microscopically to determine parasitemia level and percentage parasite suppression. Moreover, each mouse was observed daily for determination of survival time.

3.7.4. Determination of Body Weight and Temperature

The body weight of each mouse in all the groups was taken before infection (day 0) and on day 4 using a sensitive weighing balance (METTLER TOLEDO, Switzerland). The rectal temperature of the mice was measured with a digital thermometer before infection and then daily up to day 4 to see the effect of the extracts and fractions on body temperature.

3.7.5. Determination of Packed cell volume (PCV)

Packed cell volume (PCV) was measured to predict the effectiveness of the test extract and fractions in preventing hemolysis resulting from increasing parasitemia associated with malaria. Blood was collected from tail of each mouse in heparinized microhaematocrit capillary tubes. The capillary tubes were filled with blood up to $\frac{3}{4}$th of their volume and sealed.
The tubes were sealed by crystal seal and placed in a microhematocrit centrifuge (Hettich haematokrit, Germany) with the sealed ends outwards and centrifuged for 5 min at 11,000 rpm. PCV is a measure of the proportion of RBCs to plasma and measured before inoculating the parasite (day 0) and after treatment (day 4) (Dikasso et al., 2006) using the following relationship (Mengiste et al., 2012)

\[
PCV = \text{volume of erythrocyte in a given volume of blood} \times \frac{\text{Total blood volume examined}}{3.7.6. \text{Determination of Parasitemia}}
\]

On day 4 of the experiment, thin smears were prepared from tail blood on microscopic slides, dried and fixed with methanol. The blood films were stained with Giemsa and examined under the microscope. Five different fields on each slide were examined and the average was taken and percentage parasitemia was determined using the formula described by (Fidock et al. 2004)

\[
\% \text{ Parasitaemia} = \frac{\text{Number of infected RBCs}}{\text{Number of infected RBCs} + \text{Number of non-infected RBCs}} \times 100
\]

The percentage suppression of parasitaemia was calculated for each test concentration by comparing the parasitaemia in infected controls with those received different concentrations of the test extract.

\[
\% \text{ average suppression} = \frac{\text{average parasitaemia in negative control} - \text{average parasitaemia in test group}}{\text{average parasitaemia in negative control}} \times 100
\]

3.7.7. Determination of Mean Survival Time

Mortality was monitored daily and the number of days from the time of inoculation of the parasite up to death was recorded for each mouse in the treatment and control groups throughout the follow up period. The mean survival time (MST) for each group was calculated as follows:

\[
\text{MTS} = \frac{\text{Sum of survival time of all mice in group (days)}}{\text{Total number of mice in that group}}
\]
4. DATA ANALYSIS

Results of the study were expressed as a mean plus or minus standard error of mean (M ± SEM). Data were analyzed using Windows SPSS Version 16.0. One-way analysis of variance (ANOVA) followed by Tukey’s (post-hoc test) was used to determine statistical significance for comparison of parasitemia, % suppression, body weight, PCV, rectal temperature and survival time among groups. The analysis was performed with 95% confidence interval and P-values less than 0.05 was considered to be statistically significant.
5. RESULTS

5.1. Phytochemical screening

The result of phytochemical screening of leaves of *Strychnos mitis* showed the presence of different secondary metabolites as shown in Table 1.

Table 1. Result of phytochemical screening of hydromethanolic and aqueous extracts of leaves of *Strychnos mitis*.

<table>
<thead>
<tr>
<th>Phytochemicals</th>
<th>Hydromethanolic extract</th>
<th>Aqueous extract</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alkaloids</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Tannins</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Saponins</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Flavonoids</td>
<td>_</td>
<td>_</td>
</tr>
<tr>
<td>Terpenoids</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Steroid</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Phenols</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Glycosides</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

Note: (+) indicates the presence and (−) indicates absence of particular metabolites.

5.2. *In-vivo* antiplasmodial Activity of *Strychnos mitis* leaves

5.2.1 Effect of crude extracts of the leaves of *Strychnos mitis* on parasitemia, %suppression and survival time

Four-day suppressive test results indicated that both hydromethanolic and aqueous extract of *Strychnos mitis leaves* have prominent antiplasmodial activity against chloroquine sensitive *P. berghei* infected Swiss albino mice (Table 2). Analysis of the test result indicated that the percentage suppression of hydromethanolic extract was 36.56%, 81.49% and 93.97% at
200mg/kg/day, 400mg/kg/day and 600mg/kg/day of the extract, respectively and percentage suppression of aqueous extract was 29.43%, 74.86% and 95.5% at 200mg/kg/day, 400mg/kg/day and 600mg/kg/day of the extract, respectively after four day suppressive test.

At all dose levels evaluated, both the hydromethanolic and aqueous extract of *Strychnos mitis* showed statistically significant (p<0.001) difference in reducing parasite load as compared to negative control after four day suppressive test. Among themselves, in both extracts at a dose of 400mg/kg and 600mg/kg showed a statistically significant (P<0.001) parasitemia reduction as compared to 200mg/kg dose level. At higher dose (600mg/kg) both aqueous and hydromethanolic extracts showed higher percentage suppression 95.5% and 93.97% respectively, which is comparable to CQ (25mg/kg)(100%).

Mean survival date of *P. berghei* infected mice treated with 400mg/kg and 600mg/kg of hydromethanolic and aqueous extract of *Strychnos mitis* leaves showed statistically significant (p<0.001)difference to the negative control .Whereas at lower dose(200mg/kg) of both extracts stastically significant (p<0.01) differences to the negative control on four day suppressive test.
Table 2. Effect of crude extract of the leaves of *S. mitis* on parasitemia, % suppression and survival time of *P. berghei* infected mice.

<table>
<thead>
<tr>
<th>Test substances</th>
<th>Dose (mg/kg)</th>
<th>% parasitemia</th>
<th>% suppression</th>
<th>Survival time (day)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AE</td>
<td>200</td>
<td>31.65±7.69</td>
<td>29.43 a^3,c^3,d(^{3,})^{,}e(^{3})</td>
<td>9.83±1.32 a(^{2,})c(^{2,})d(^{3,})(^{,})e(^{3})</td>
</tr>
<tr>
<td></td>
<td>400</td>
<td>11.27±4.05</td>
<td>74.86 a(^{3,})b(^{3,})d(^{3,})(^{,})e(^{3})</td>
<td>12.33±1.63 a(^{2,})b(^{2,})d(^{3,})(^{,})e(^{3})</td>
</tr>
<tr>
<td></td>
<td>600</td>
<td>2.01±0.55</td>
<td>95.50 a(^{3,})b(^{3,})c(^{2})</td>
<td>17.50±1.04 a(^{3,})b(^{2,})c(^{3,})(^{,})e(^{3})</td>
</tr>
<tr>
<td>Vehicle</td>
<td>1ml</td>
<td>44.85±5.81</td>
<td>0.00</td>
<td>7.33±0.81</td>
</tr>
<tr>
<td>CQ</td>
<td>25</td>
<td>0.00</td>
<td>100</td>
<td>27.50±0.83</td>
</tr>
<tr>
<td>HE</td>
<td>200</td>
<td>28.45±5.27</td>
<td>36.56a(^{3,})c(^{3,})d(^{3,})(^{,})e(^{3})</td>
<td>10.83±1.72a(^{2,})d(^{3,})(^{,})e(^{3})</td>
</tr>
<tr>
<td></td>
<td>400</td>
<td>8.30±5.03</td>
<td>81.49a(^{3,})b(^{3,})e(^{2})</td>
<td>13.00±2.00a(^{3,})e(^{3,})d(^{2})</td>
</tr>
<tr>
<td></td>
<td>600</td>
<td>2.70±1.10</td>
<td>93.97a(^{4,})b(^{5})</td>
<td>16.50±1.04a(^{4,})b(^{5,})c(^{7})(^{,})e(^{4})</td>
</tr>
<tr>
<td>Vehicle</td>
<td>1ml</td>
<td>44.85±5.81</td>
<td>0.00</td>
<td>7.33±0.81</td>
</tr>
<tr>
<td>CQ</td>
<td>25</td>
<td>0.00</td>
<td>100</td>
<td>27.50±0.83</td>
</tr>
</tbody>
</table>

Data are expressed as mean ± SEM; n = 6; a= compared to distilled water (vehicle); b,= compared to 200 mg/kg; c= compared to 400; d= compared to 600 mg/kg; e= compared to chloroquine 25 mg/kg: 1p=<0.05, 2p=<0.01, 3p=<0.001. AE= aqueous extract, CQ=chloroquine, HE= hydromethanolic extract. Numbers refer to dose in mg/kg.

5.2.2 Effect of crude extracts of *Strychnos mitis* leaves on body weight.

The higher two doses of both hydromethanolic and aqueous extracts protected from parasite induced weight reduction compared to negative control (table 3). At a dose of 400mg/kg both hydromethanolic and aqueous extracts significantly prevented (p<0.05) body weight reduction as compared to negative control. Whereas at highest dose (600mg/kg) hydromethanolic extract
significantly prevented (p<0.001) body weight reduction and aqueous extract significantly prevented (p<0.01) body weight reduction as compared to the negative control.

**Table 3.** Effect of crude extracts of *S. mitis* leaves on body weight of *P. berghei* infected mice.

<table>
<thead>
<tr>
<th>Test substances</th>
<th>Dose (mg/kg)</th>
<th>Body weight</th>
<th>% change</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>D0 (g)</td>
<td>D4 (g)</td>
<td></td>
</tr>
<tr>
<td>AE</td>
<td>200</td>
<td>30.33 ± 1.93</td>
<td>28.28 ± 3.82</td>
</tr>
<tr>
<td></td>
<td>400</td>
<td>29.83 ± 1.40</td>
<td>30.35 ± 1.35</td>
</tr>
<tr>
<td></td>
<td>600</td>
<td>29.40 ± 1.69</td>
<td>30.76 ± 1.73</td>
</tr>
<tr>
<td>Vehicle</td>
<td>1 ml</td>
<td>29.26 ± 1.26</td>
<td>27.30 ± 1.10</td>
</tr>
<tr>
<td>CQ</td>
<td>25</td>
<td>29.11 ± 1.54</td>
<td>30.12 ± 2.03</td>
</tr>
<tr>
<td>HE</td>
<td>200</td>
<td>31.33 ± 0.54</td>
<td>29.88 ± 2.58</td>
</tr>
<tr>
<td></td>
<td>400</td>
<td>29.63 ± 1.45</td>
<td>30.16 ± 1.32</td>
</tr>
<tr>
<td></td>
<td>600</td>
<td>30.41 ± 1.92</td>
<td>32.45 ± 2.23</td>
</tr>
<tr>
<td>Vehicle</td>
<td>1 ml</td>
<td>29.26 ± 1.26</td>
<td>27.30 ± 1.10</td>
</tr>
<tr>
<td>CQ</td>
<td>25</td>
<td>29.11 ± 1.54</td>
<td>30.12 ± 2.03</td>
</tr>
</tbody>
</table>

Data are expressed as mean ± SEM; *n* = 6; a = compared to distilled water (Vehicle); b = compared to 200 mg/kg; c = compared to 400; d = compared to 600 mg/kg; e = compared to CQ 25 mg/kg; 1p = <0.05, 2p = <0.01, 3p = <0.001. D0 = pre-treatment value on day 0, D4 = post-treatment value on day four, AE = aqueous extract, CQ = chloroquine, HE = hydromethanolic extract. Numbers refer to dose in mg/kg.
5.2.3. Effect of crude extracts of the leaves of *Strychnos mitis* on packed cell volume and rectal temperature.

Aqueous extract of *Strychnos mitis* prevented reduction in PCV significantly at higher two doses (p<0.01) and lower dose (p<0.05) as compared to the negative control as indicated on Figure 5. By contrast, all doses of hydromethanolic extract showed significantly (p<0.01) prevented the reduction in PCV as compared to the negative control on Figure 7. In addition all doses of the hydromethanolic extract significantly (P<0.001) at a dose of (600mg/kg) and (p<0.01) at doses of (200mg/kg and 400mg/kg) prevented the reduction in rectal temperature, and at a dose of 600mg/kg, the effect is comparable to that of CQ(25mg/kg) as shown in Figure 6; whereas aqueous extract showed significantly (p<0.05) prevention on the reduction in rectal temperature only at the dose of 600mg/kg when compared with the negative control as indicated in Figure 4.

**Figure 4:** The effect of aqueous extract of *Strychnos mitis* leaves on rectal temperature of *P. berghei* infected mice on four day suppression test. Data are mean ± SEM; n=6; CQ = chloroquine, AE, Aqueous extract of *S. mitis*. Numbers refer to dose in mg/kg/day.
Figure 5: The effect of aqueous extract of *Strychnos mitis* leaves on packed cell volume of *P. berghei* infected mice on four day suppression test. Data are mean ± SEM; n=6; CQ = chloroquine, AE. Aqueous extract of *S. mitis*. Numbers refer to dose in mg/kg/day.

Figure 6: The effect of hydromethanolic extract of *Strychnos mitis* leaves on rectal temperature of *P. berghei* infected mice on four day suppression test. Data are mean ± SEM; n=6; CQ = chloroquine, AE. Aqueous extract of *S. mitis*. Numbers refer to dose in mg/kg/day.
**Figure 7:** The effect of hydromethanolic extract of *Strychnos mitis* leaves on packed cell volume of *P. berghei* infected mice on four day suppression test. Data are mean ± SEM; n=6; CQ = chloroquine, AE, Aqueous extract of *S. mitis*. Numbers refer to dose in mg/kg/day.

### 5.2.4. Effect of fractions of *Strychnos mitis* leaves on parasitemia, % suppression and survival time

At all dose levels evaluated, all the fractions showed statistically significant (p<0.001) difference in reducing parasite load from that of the negative control group after four day suppressive test. Order of percentage suppression of the solvent fraction was N-hexane (42.85%) > Chloroform (39.72%) > aqueous (34.47%). Survival date was significantly prolonged (p<0.001) by all dose level of N-hexane, chloroform and the larger (400mg/kg) and middle (200mg/kg) doses of aqueous fraction whereas lower dose of aqueous fraction (100mg/kg) was significantly prolonged (p<0.01) as compared to negative control as shown on table 4.
Table 4. Effect of fraction of *S.mitis* leave on % parasitemia, % suppression and survival time on *P.berghei* infected mice.

<table>
<thead>
<tr>
<th>Test substances</th>
<th>Dose (mg/kg)</th>
<th>% parasitemia</th>
<th>% suppression</th>
<th>Survival time (day)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NF</td>
<td>100</td>
<td>28.68±6.31</td>
<td>42.11 a&lt;sup&gt;e&lt;/sup&gt;,e&lt;sup&gt;3&lt;/sup&gt;</td>
<td>11.33±1.21 a&lt;sup&gt;e&lt;/sup&gt;,e&lt;sup&gt;3&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>200</td>
<td>28.31±0.89</td>
<td>42.85 a&lt;sup&gt;e&lt;/sup&gt;,e&lt;sup&gt;4&lt;/sup&gt;</td>
<td>11.50±1.76 a&lt;sup&gt;e&lt;/sup&gt;,e&lt;sup&gt;3&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>400</td>
<td>32.81±4.38</td>
<td>33.77 a&lt;sup&gt;e&lt;/sup&gt;,e&lt;sup&gt;3&lt;/sup&gt;</td>
<td>11.00±1.54 a&lt;sup&gt;e&lt;/sup&gt;,e&lt;sup&gt;3&lt;/sup&gt;</td>
</tr>
<tr>
<td>CF</td>
<td>100</td>
<td>36.25±2.14</td>
<td>26.84 a&lt;sup&gt;d&lt;/sup&gt;,e&lt;sup&gt;3&lt;/sup&gt;</td>
<td>10.50±0.54 a&lt;sup&gt;e&lt;/sup&gt;,e&lt;sup&gt;3&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>200</td>
<td>33.83±3.19</td>
<td>31.71a&lt;sup&gt;e&lt;/sup&gt;,d&lt;sup&gt;e&lt;/sup&gt;,e&lt;sup&gt;3&lt;/sup&gt;</td>
<td>10.83±0.75 a&lt;sup&gt;e&lt;/sup&gt;,e&lt;sup&gt;3&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>400</td>
<td>29.86±2.30</td>
<td>39.72 a&lt;sup&gt;b&lt;/sup&gt;,c&lt;sup&gt;e&lt;/sup&gt;,e&lt;sup&gt;3&lt;/sup&gt;</td>
<td>11.33±1.03 a&lt;sup&gt;e&lt;/sup&gt;,e&lt;sup&gt;3&lt;/sup&gt;</td>
</tr>
<tr>
<td>AF</td>
<td>100</td>
<td>37.26±1.84</td>
<td>24.78 a&lt;sup&gt;d&lt;/sup&gt;,e&lt;sup&gt;3&lt;/sup&gt;</td>
<td>10.16±1.47 a&lt;sup&gt;e&lt;/sup&gt;,e&lt;sup&gt;3&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>200</td>
<td>36.10±2.52</td>
<td>27.14 a&lt;sup&gt;d&lt;/sup&gt;,e&lt;sup&gt;3&lt;/sup&gt;</td>
<td>10.66±1.36 a&lt;sup&gt;e&lt;/sup&gt;,e&lt;sup&gt;3&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>400</td>
<td>32.46±2.18</td>
<td>34.47 a&lt;sup&gt;b&lt;/sup&gt;,c&lt;sup&gt;e&lt;/sup&gt;,e&lt;sup&gt;3&lt;/sup&gt;</td>
<td>11.16±0.75 a&lt;sup&gt;e&lt;/sup&gt;,e&lt;sup&gt;3&lt;/sup&gt;</td>
</tr>
<tr>
<td>Vehicle</td>
<td>1ml</td>
<td>49.55±2.53</td>
<td>0.00</td>
<td>7.33±1.03</td>
</tr>
<tr>
<td>CQ</td>
<td>25</td>
<td>0.00</td>
<td>100</td>
<td>27.83±0.75</td>
</tr>
</tbody>
</table>

Data are expressed as mean ± SEM; n = 6; a= compared to distilled water (vehicle); b= compared to 100 mg/kg; c= compared to 200; d= compared to 400 mg/kg; e= compared to chloroquine 25 mg/kg; 1p<0.05, 2p<0.01, 3p<0.001. Numbers refer to dose in mg/kg. CQ = chloroquine, NF=N-hexane fraction of Crude hydromethanolic extract, Chloroquine= CQ, CF= chloroform fraction of Crude hydromethanolic extract, AF=Aqueous fraction of Crude hydromethanolic extract of *Strychnos mitis*.

5.2.5. Effect of fractions of *Strychnos mitis* leaves on body weight

All tested doses of the N-hexane fraction and the higher two doses (200mg/kg and 400mg/kg) of chloroform and aqueous fraction of *Strychnos mitis* leaves protected the mice from body weight loss as compared to negative control after four day suppressive test (table5).
Table 5. Effect of fraction of *S. mitis* leaves on body weight of *P. berghei* infected mice.

<table>
<thead>
<tr>
<th>Test substances</th>
<th>Dose(mg/kg)</th>
<th>Body weight</th>
<th>% Change</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>D0(g)</td>
<td>D4(g)</td>
<td></td>
</tr>
<tr>
<td>NF</td>
<td>100</td>
<td>29.45±1.46</td>
<td>30.48±2.12</td>
</tr>
<tr>
<td></td>
<td>200</td>
<td>29.40±1.65</td>
<td>30.36±2.47</td>
</tr>
<tr>
<td></td>
<td>400</td>
<td>30.25±1.63</td>
<td>30.86±2.34</td>
</tr>
<tr>
<td>CF</td>
<td>100</td>
<td>29.68±1.55</td>
<td>29.28±1.56</td>
</tr>
<tr>
<td></td>
<td>200</td>
<td>29.75±1.42</td>
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<tr>
<td></td>
<td>400</td>
<td>30.46±1.70</td>
<td>31.11±2.35</td>
</tr>
<tr>
<td>AF</td>
<td>100</td>
<td>29.43±2.06</td>
<td>28.93±2.02</td>
</tr>
<tr>
<td></td>
<td>200</td>
<td>29.41±1.79</td>
<td>29.75±2.26</td>
</tr>
<tr>
<td></td>
<td>400</td>
<td>30.76±0.91</td>
<td>31.35±1.28</td>
</tr>
<tr>
<td>Vehicle</td>
<td>1ml</td>
<td>30.51±1.78</td>
<td>28.20±1.59</td>
</tr>
<tr>
<td>CQ</td>
<td>25</td>
<td>29.23±2.00</td>
<td>30.74±2.53</td>
</tr>
</tbody>
</table>

Data are expressed as mean ± SEM; n = 6; a= compared to distilled water (vehicle); b=compared to 100 mg/kg; c= compared to 200; d= compared to 400 mg/kg; e= compared to chloroquine 25 mg/kg; 1p=≤0.05, 2p=≤0.01, 3p=≤0.001. Numbers refer to dose in mg/kg. D0 = pre-treatment value on day 0, D4 = post-treatment value on day four, CQ = chloroquine, NF=N-hexane fraction of Crude hydromethanolic extract, Chloroquine= CQ, CF= chloroform fraction of Crude hydroalcoholic extract crude, AF=Aqueous fraction of Crude hydromethanolic extract of *Strychnos mitis*.

5.2.6. Effect of fractions of *Strychnos mitis* leaves on packed cell volume and rectal temperature

None of the doses of the fraction of *Strychnos mitis* significantly improved body temperature of *P. berghei* infected mice as shown on Figure 8, 10 and 12. All dose levels of chloroform fraction
of *Strychnos mitis* showed significant (p<0.01) prevention on the reduction in PCV as compared to the negative control (Figure 11).

On the other hand, aqueous fraction at the dose levels of 200mg/kg (p<0.01), 400mg/kg (p<0.01) and 100mg/kg(p<0.05) showed significant prevention on the reduction in PCV as compared to the negative control (Figure 13). Whereas N-hexane fraction at the dose levels of 100mg/kg (p<0.01), 200mg/kg (p<0.01) and 400mg/kg (p<0.05) showed significant prevention on the reduction in PCV respectively as compared to the negative control (Figure 9).

![Graph showing rectal temperature changes](image)

**Figure 8:** The effect of N-hexane fraction of *Strychnos mitis* leaves on rectal temperature of *P. berghei* infected mice on four day suppression test. Data are mean ± SEM; n=6; CQ = chloroquine, NF=hexane fraction of *S. mitis*. Numbers refer to dose in mg/kg/day.
**Figure 9:** The effect of N-hexane fraction of *Strychnos mitis* leaves on packed cell volume of *P. berghei* infected mice on four day suppression test. Data are mean ± SEM; n=6; CQ = chloroquine, NF = hexane fraction of *S. mitis*. Numbers refer to dose in mg/kg/day.

**Figure 10:** The effect of Chloroform fraction of *Strychnos mitis* leaves on rectal temperature of *P. berghei* infected mice on four day suppression test. Data are mean ± SEM; n=6; CQ = chloroquine, CF = Chloroform fraction *S. mitis*. Numbers refer to dose in mg/kg/day.
**Figure 11:** The effect of Chloroform fraction of *Strychnos mitis* leaves on packed cell volume of *P. berghei* infected mice on four day suppression test. Data are mean ± SEM; n=6; CQ = chloroquine, CF = Chloroform fraction *S. mitis*. Numbers refer to dose in mg/kg/day.

**Figure 12:** The effect of aqueous fraction of *Strychnos mitis* leaves on rectal temperature of *P. berghei* infected mice on four day suppression test. Data are mean ± SEM; n=6; CQ = chloroquine, AF = aqueous fraction *S. mitis*. Numbers refer to dose in mg/kg/day.
**Figure 13:** The effect of aqueous fraction of *Strychnos mitis* leaves on packed cell volume of *P. berghei* infected mice on four day suppression test. Data are mean ± SEM; n=6; CQ = chloroquine, AF = aqueous fraction *S. mitis*. Numbers refer to dose in mg/kg/day.

### 5.2.7. LD<sub>50</sub> of the crude extracts

The acute toxicity study indicated that both hydromethanolic and aqueous extract of *Strychnos mitis* leaves caused no mortality up to 2000mg/kg oral doses within the first 24 hours as well as for the following 14 days. Physical and behavioral observations of the experimental mice also revealed no visible signs of overt toxicity like lacrimation, loss of appetite, tremors, hair erection, salivation and diarrhea. The acute toxicity result of the present study suggested that the oral medial lethal dose (LD<sub>50</sub>) of the extract could be greater than 2000 mg/kg body weight of the extract as per OECD guideline No 425 (OECD, 2008). The experimental determination of lack of acute toxicity at the dose of extract up to 2000 mg/kg body weight of mice would justify the use of this plant extracts for malaria treatment.
6. DISCUSSION

Malaria is one of the world’s most deadly infectious diseases. Spread and emergence of resistance to the front line antimalarial drugs including artemisinin is the major challenge that jeopardize all recent gains in malaria control and has major implications for public health (Ashley et al., 2014; Marfurt et al., 2010). The scientific community is now underway to combat this problem by searching for new, affordable and effective antimalarial agents from medicinal plants and other sources (Gamo, 2014). The present study was aimed to determine the in vivo antiplasmodial activity of crude extract and fraction of S. mitis in P. berghei infected mice using 4-day suppressive tests.

The 4-day suppressive test is a standard test commonly used for antimalarial screening (Akele, 2013) and the determination of percent inhibition of parasitemia is the most reliable parameter. A mean group parasitemia level of less than or equal to 90% that of mock-treated control animals usually indicates that the test compound is active in standard screening studies (Peter and Anatoli, 1998). Therefore, it is clear from the result indicated in (table 2) that in P. berghei infected mice treated with the extracts of S. mitis, the percentage of parasitemia measured changed significantly from those in the control animals.

Plasmodium berghei (ANKA strain) was used in the prediction of treatment outcome (Bantie et al., 2014) and hence it was an appropriate parasite for the study. Even though the rodent malaria model, P. berghei, is not exactly similar to that of the human Plasmodium parasites, it is the first step to screen most of the in vivo antimalarial activities of test compounds ((Fidock et al., 2004). Moreover, several conventional antimalarial agents, such as chloroquine, halofantrine, mefloquine and more recently artemisinin derivatives have been identified using rodent model of malaria (Madara et al., 2010).
The observation that no death with an oral dose of 2000mg/kg body weight of the extracts could indicate that the hydro-methanolic and aqueous extract of *S. mitis* may imply that this plant could safely be used to treat malaria. This was consistent with the results of the previous study on toxicity of Gardenia lutea by (Akele, 2013) and (Murithi et al., 2014).

*In vivo* antiplasmodial activity can be classified as moderate, good and very good if an extract displayed a percent parasite suppression equal to or greater than 50% at a dose of 500, 250 and 100mg/kg body weight per day, respectively (Deharo et al., 2001 and Muñoz et al., 2000). Based on this classification, crude extracts of *S. mitis* exhibited a good antiplasmodial activity, with a dose dependent inhibition against *P. berghei* infection in mice.

Analysis of the test result indicated that the percentage suppression of hydromethanolic extract of *Strychnos mitis* was produced statistically significant (p<0.001) 36.56%, 81.49% and 93.97% at 200mg/kg/day, 400mg/kg/day and 600mg/kg/day of the extract, respectively when compared to negative control, and statistically significant percentage suppression of aqueous extract of *Strychnos mitis* was also produced (p<0.001) 29.43%, 74.86% and 95.5% at 200mg/kg/day, 400mg/kg/day and 600mg/kg/day of the extract, respectively when compared to negative control after four day suppressive test. This parasite suppression exhibited by the extracts were comparable with the results of the former studies done on methanol leaf extract of *A. debrana* (Deressa et al., 2010), crude extract of *Croton macrostachys* (Bantie et al., 2014) and hexane extracts of *Ficus thonningii* (Falade et al., 2014).

Among the fractions, the N-hexane and chloroform fractions were found to possess higher percentage suppression than aqueous fraction. This was evident from the chemosuppression obtained during the four day suppressive test, suggesting the possible localization of the active ingredients in these two fractions.
All crude extracts and fractions prolonged the mean survival time of the study mice indicating that the plant suppressed *P. berghei* and reduced the overall pathologic effect of the parasite on the study mice. However, neither the extracts nor the standard drug cured the infection. This could be due to recrudescence of *P. berghei* parasites after apparent cure. The result on mean survival time is in agreement with similar studies done on *Croton macrostachys* (Bantie *et al.*, 2014) and *D. Angustifolia* (Mengiste *et al.*, 2012). Particularly the highest dose (600mg/kg) recorded the longest survival time (16.50±1.04) and (17.50±1.04) in hydromethanolic and aqueous extract, respectively compared to negative control could be linked to the presence of active secondary metabolites in sufficient concentration unlike the lower doses. The phytochemical screening of hydro-methanolic and aqueous extract of *S. mitis* indicated the presence of alkaloids, anthraquinones, terpenoids, glycosides, saponins, tannins and phenolic compounds.

As explained by Dharani *et al.* (2010), common antimalarial plants used to treat malaria in traditional medicine contain secondary metabolites, such as alkaloids, terpenoids, coumarins, flavonoids, chalcones, quinones and xanthones. In addition to this, alkaloids and terpenoids have been implicated in anti-plasmodial activity in the previous study (Okokon and Nwafor, 2009). Phenolic compounds detected in this plant could possibly responsible for the antiplasmodial activity as these metabolites have been proved to possess potential antimalarial effect in other studies (Ayoola *et al.*, 2008; Alexandru *et al.*, 2007). In addition to this, phenolic compounds detected in the leaves extract of *Strychnos mitis* showed antioxidant properties (free radical inhibitors or scavengers) (Adamu *et al.*, 2014) that also may contribute to the antiplasmodial activity. Antioxidative activity can inhibit heme polymerization as heme has to be oxidized before polymerization, and the unpolymerised heme is very toxic for the parasite (Taramelli *et al.*, 1999).
Anemia, body weight loss and body temperature reduction are the general features of malaria-infected mice (Langhorne et al., 2002). So, an ideal antimalarial agents obtained from plants are expected to prevent body weight loss in infected mice due to the rise in parasitemia. The crude extracts (aqueous and hydromethanolic) and fractions (chloroform and aqueous) of *Strychnos mitis* leaves significantly prevented weight loss at higher two doses in a dose dependent manner. Whereas N-hexane fraction of *Strychnos mitis* leaves significantly prevented weight loss at all dose in a dose independent manner. This suggests the possibility of the localization of appetite suppressing components even at lower dose in this fraction and nutrients and other immunomodulatory substances in the N-hexane fraction. This result is in agreement with that of the previous studies on hydroalcoholic extract of *Asparagus africanus* (Dikasso et al., 2006) and Bantie *et al.*, 2014).

A decrease in the metabolic rate of infected mice occurred before death and was accompanied by a corresponding decrease in internal body temperature (Mengiste *et al.*, 2012). Active compounds should prevent the rapid dropping of rectal temperature. All doses of hydromethanolic and the highest dose of aqueous crude extract did have protective effect against temperature reduction, which reflects constituents responsible for this effect were likely found in these extracts. The effects on rectal temperature in this study are similar to the one reported by previous study on crude extract and chloroform fraction of *Croton macrostachys* (Bantie *et al.*, 2014). Unlike the crude extracts, all fractions failed to prevent parasite induced rectal temperature reduction compared to negative control unlike the standard drug. This could be attributed to the effect of the extract as it may have hypothermic effect on the treated mice.

After malaria infection, the host (human or mouse) suffer from anaemia (Chinchilla *et al.*, 1998). The underlying cause of anemia includes; loss of infected erythrocytes through parasite maturation, destruction of uninfected red cells in the spleen and liver by macrophages activation
and/or enhanced phagocytosis, reduced erythropoiesis and dyserythropoiesis (Lamikanra et al., 2007). This necessitates hematocrit (packed cell volume) analysis that evaluates the effectiveness of the extract in preventing hemolysis.

The effect of crude extracts and fractions of *S. mitis* leaves extract on the packed cell volume (PCV) was also evaluated. Both the crude extracts and fractions significantly prevented PCV reduction when compared to negative control in a dose dependent manner which is comparable with previous studies on *D. Angustifolia* (Mengiste et al., 2012); *Croton macrostachys* (Bantie et al., 2014)
7. CONCLUSION

The present study indicates that *in vivo* antiplasmodial effect of crude extract and solvent fractions of *Strychnos mitis* have significant antimalarial activity. This plant extracts also exhibited safety profile at the maximum dose of 2000mg/kg. The antimalarial effect of solvent fractions of hydromethanolic extract demonstrated less as compared to the crude extracts. Although the N-hexane fraction had protected body weight loss at all dose level and displayed greater parasite suppression among the fractions. Therefore, the extracts and fractions of hydromethanolic extract could represent a new source for the development of new plant based antimalarial agent. Moreover, the data would provide evidence to uphold the claims made by the Ethiopian traditional medicine practitioners.
8. RECOMMENDATIONS

From the present study, the following works are suggested for further investigation on the plant.

- Mechanism of antimalarial action.

- Further quantitative phytochemical investigation to clearly identify and quantify the antiplasmodial active components from the plant.

- Sub-acute and chronic toxicity study to know the safety of *Strychnos mitis* leaves.
9. REFERENCES


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