In vivo antimalarial activity of solvent fractions of the leaf of *Justicia schimperiana Hochst. Ex Nees* (*Acanthaceae*) against *Plasmodium berghei* in mice

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This is to certify that the thesis prepared by Jemal Abdela, entitled: "*In vivo* antimalarial activity of solvent fractions of the leaf of *Justicia schimperiana Hochst. Ex Nees* (Acanthaceae) against *Plasmodium berghei* in mice" and submitted in partial fulfillment of the requirement for the Master of Science degree in Pharmacology complies with the regulations of the University and meets the accepted standards with respect to originality and quality.

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ABSTRACT

In vivo antimalarial activity of solvent fractions of the leaf of *Justicia schimperiana Hochst. Ex Nees* (*Acanthaceae*) against *Plasmodium berghei* in mice

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In spite of tremendous reduction in malaria-associated morbidity and mortality achieved during the last decade, malaria still remains a major global public health problem accounting for about 219 million infections and 0.66 million deaths in 106 malaria-endemic countries in the year 2010. Increasing resistance of *Plasmodium falciparum* to almost all the available antimalarial drugs urged a search for newer antimalarial drugs. *Justicia schimperiana* is traditionally used for the treatment of malaria and a study conducted previously on the crude extract confirmed that the plant is endowed with antimalarial activity. The present study was therefore aimed to evaluate antimalarial activities of chloroform, methanol and aqueous fractions of the leaves of *Justicia schimperiana* against *Plasmodium berghei* in mice. To this effect, following successive soxhlet extraction and maceration, the resulting fractions were evaluated at doses of 200 mg/kg, 400 mg/kg and 600 mg/kg using the 4-day suppressive, curative and prophylactic tests. All the three fractions had shown significant suppression of parasitemia in the 4-day suppressive test, of which the methanol fraction exerted the highest chemosuppression (65.2%, $p<0.001$) at 600 mg/kg followed by the aqueous fraction (40.93%, $p<0.001$) at

the same dose. The methanol fraction also showed significant suppression of parasitemia in both curative (67.44%, $p< 0.001$) and prophylactic (35.02%, $p<0.01$) tests at 600 mg/kg. Furthermore, all doses of the methanol fraction significantly ($p<0.05$) prevented the reduction in rectal temperature in the 4-day suppressive, curative and repository tests. Therefore, the high antimalarial activity observed in methanol and aqueous fraction indicates the active constituents of the plant are semi-polar and polar in nature.

Key words: *Justicia schimperiana, in vivo*, anti-malarial activity, *Plasmodium berghe*

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LIST OF ABBREVIATIONS

ACT - Artemisinin Based Therapy

CS -Circumsporozoite

CSP - Circumsporozoite Protein

DBP - Duffy Binding Protein

EMA - European Medicines Agency

FCA -Freud's Complete Adjuvants

$GABA_A$ - Gamma Aminobutyric Acid A Receptor

GABA-T - Gamma Aminobutyric Acid –Transaminase

Hbs Ag - Hepatitis B Virus Antigen

HIV - Human Immuno Deficiency Virus

HSD -Honestly Significant Difference

ICAM-1 - Intracellular Adhesion Molecule -1

IL - Interlukin

MDR -Multi-Drug Resisitance

MIP - Malaria in Pregnancy

MST -Mean Survival Time

OECD - Organization for Economic Co-operation and Development

PCV -Packed Cell Volume

P*f*EMP-1 -*Plasmodium falciparum* Erythrocyte Membrane Protein-1

QS21 -Quillaja Saponaria Fraction 21

RBC -Red Blood Cells

RCF - Relative Centrifugal Force

RPM - Revolution Per Minute

SP -Sulphadoxine Pyrimethamine

SPSS -Statistical Package for Social Sciences

TNF-α - Tumor Necrosis Factor -Alpha

VCAM-1 - Vascular Adhesion Molecule -1

WHO -World Health Organization

1. INTRODUCTION

1.1 Etiology of malaria and its vectors

The etiological agents of malaria are protozoans that belong to the genus *Plasmodium,* phyllum *Apicomplexa*, and family *Plasmodidae*. Its vectors are mosquitoes of the genus *Anopheles* and family *Culicidae* (Oliveira et al., 2009). Malaria is usually transmitted by the bite of an infected female *anopheline* mosquito. In addition to this blood-borne transmission such as blood or blood products transfusion, transplantation, needle-sharing among intravenous drug addicts, accidental nosocomial transmission, vertical transmission (mother to the growing fetus or congenital transmission) may occur (Bartoloni and Zammarchi, 2012; Rudrapal, 2011).

In general, there are 512 *Anopheles* species recognized worldwide (Autino et al., 2012), but 70 are believed to be malaria vectors under normal conditions (Usher, 2010). In sub-Saharan Africa, there are 140 *Anopheles* species of which 20 are known to transmit malaria to human beings under natural conditions. Of these, *Anopheles gambiae sensu strict giles*, *Anopheles arabiensis patton* and *Anopheles funestus giles* are the most widely distributed and the most efficient malaria vector species in tropical Africa. *Anopheles pharoensis* is also a major transmitter in arid and semiarid regions with permanent water bodies (Kibret et al., 2009).

Malaria in humans is caused by five *Plasmodium* parasites: *Plasmodium falciparum, Plasmodium vivax, Plasmodium malariae*, *Plasmodium ovale* and *Plasmodium knowlesi*

(Autino et al., 2012; Li et al., 2012; Odeghe et al., 2012). The distribution of human-pathogenic *Plasmodium* species shows preponderance of *P. falciparum* in tropical Africa, while *P. vivax* prevails over *P. falciparum* in South America (Autino et al., 2012). Endemic *P. vivax* is transmitted throughout the tropics in much the same geographical pattern as *P. falciparum*. The major exception is West Africa where *P. vivax* is largely absent. This has been attributed to individuals lacking the Duffy red blood cell (RBC) surface antigen - an important mediator of *P. vivax* red cell invasion (Douglas et al., 2012). Both *P. falciparum* and *P. vivax* are prevalent in South-Eastern Asia and western Pacific. Although *P. malariae* may occur in all malarious areas, its prevalence is generally low. In tropical Africa, *P. falciparum* and *P. malariae* co-infection is sometimes encountered. *P. ovale* is widespread principally in tropical Africa, whereas *P. knowlesi* infection occurs only in certain forested areas of South-East Asia (Autino et al., 2012). The zoonotic infection caused by *P. knowlesi* has emerged as a common and potentially fatal cause of human malaria in Malaysian Borneo, and presents an increasing threat to malaria control. It is transmitted by the forest-dwelling *Anopheles leucosphyrus* group of mosquitoes (Barber et al., 2012).

In addition, there are also other species of *Plasmodium* that cause malaria in a wide range of vertebrate hosts, from snakes and birds to mice and humans. *Plasmodium vinckei, Plasmodium berghei, Plasmodium yoelii, Plasmodium chabaudi and Plasmodium cynomolgi* are commonly used mouse malaria species, each of which has different characteristics in terms of infectivity and used for different types of study (Silmon de Monerri, 2010).

1.2 Epidemiology of malaria

1.2.1 Global scenario

Malaria is not only among the most prevalent tropical diseases worldwide, but it is a major threat to the public health and economic development of many nations (Khan et al., 2013a). In 2010, there were an estimated 219 million cases of malaria and 660 000 malaria deaths globally. Of these, 80% of cases and 90% of deaths are estimated to occur in the African Region (WHO, 2012a). Approximately, 86% of malaria deaths globally were of children under 5 years of age (WHO, 2011; 2012a), and this indicates every 30 seconds a child dies from malaria (Khan et al., 2013b). In addition, malaria in pregnancy (MIP) poses substantial risk to the mother, fetus and neonate (Kazembe et al., 2012; Khan et al., 2013b). World Health Organization (WHO) Malaria Report (2011) also indicated that, 106 countries in the world are at risk of transmission of malaria infection.

More than one billion people live in areas with high malarial risk. Populations living in sub Saharan Africa have the highest risk of acquiring malaria (Khan et al., 2013a). The proportion of people exposed to malaria parasites has decreased during the last century. Nevertheless, the absolute number of people at risk for malaria infection increased from 0.8 billion in 1900 to 3.3 billion in 2010. Moreover, malaria mortality rates showed a global reduction of 26% between 2000 and 2010 (WHO, 2011).

In addition to its health effect, the disease also imposes high and regressive cost burden on households that have a sick family member, with poor households spending a higher proportion of their income on health care than the better-off households (Kioko et al.,

2013). According to WHO 2010 report on economic impact of malaria, the economic losses due to the disease in sub-Saharan Africa estimated to be over 12 billion US dollars annually and it slows economic growth by as much as 1.3 percent per year in many African countries. In some countries with a heavy malaria burden, the disease may account for as much as 40% of public health expenditure (Kazembe et al., 2012; Mushashu, 2012).

1.2.2 Malaria in Ethiopia

Malaria is a leading public health problem in Ethiopia. It is estimated that about 75% of the total area of the country and 65% of the population is at risk of infection **(**Alemu et al., 2011). Malaria was reported as the primary cause of health problems accounting for 17% of out-patient visits, 15% of hospital admissions and 29% of in-patient deaths (Petros, 2011). According to Global malaria mortality report between 1980 and 2010, the death from malaria in Ethiopia in 2010 was 22165 (Murray et al., 2012). It has been a cause of morbidity and mortality as well as low productivity in the country since early days (Woyessa et al., 2002). *P. falciparum* and *P. vivax* are the main two species accounting for 60% and 40% of malaria cases, respectively **(**Alemu et al., 2011)**.** *P. falciparum* has been the major cause of epidemics, and of most malaria deaths (Woyessa et al., 2012). *A. arabiensis* is the major malaria vector followed by *A. pharoensis* and other secondary vectors include *A. funestus* and *Anopheles nili* ***(***Alemu et al., 2011; Woyessa et al., 2012**).**

In Ethiopia, the epidemiological pattern of malaria transmission is generally unstable and seasonal; the level of transmission varies from place to place because of differences in altitude and rainfall patterns (Alemu et al., 2011). Depending on these rainfall patterns, transmission tends to be highly heterogenous geo-spatially within each year as well as between years (Jima et al., 2010). Changes have been observed in the epidemiology of malaria through time. Previously, malaria was known to occur in areas below 2000 m but currently it has been documented to occur indigenously even in areas above 2400 m, such as Addis Ababa (Alemu et al., 2011). Additionally, malaria in Ethiopia is characterized by widespread epidemics occurring every 5-8 years, with the most recent one was between 2003 and 2005 (Jima et al., 2010).

1.3 Life cycle and pathogenesis of malaria

1.3.1 Life cycle of malaria parasites

Plasmodium species, as members of *Apicomplexa*, share many common morphological features. Each of the developmental stages in the life cycle of malaria parasites exhibits a remarkable conservation and distinct patterns of structural organization. Structural, biochemical and molecular biological aspects are different among the complex cycle comprising the erythrocytic schizogony, mosquito stages, and pre-erythrocytic schizogony (Fujioka and Aikawa, 2002).

The *Plasmodium* life cycle is complex, involving both vertebrate and invertebrate hosts (Akinyi, 2010; Fujioka and Aikawa, 2002). A major part of the complexity associated with the malaria parasite life cycle is due to the parasite's ability to (*a*) change its cellular

and molecular makeup, which is controlled by a genome with more than 5000 recognized genes, and (*b*) develop in intracellular and extracellular niches in the mammalian host and the mosquito vector (Aly et al., 2009).

Infection of the human host with a *Plasmodium* parasite begins with the bite of an infected *Anopheles* mosquito, which injects sporozoites from its salivary glands into the skin of a human host when it takes a blood meal (Fig. 1). Then, the sporozoites migrate to the liver via the bloodstream and invade hepatocytes (Akinyi, 2010). Sporozoites infect the hepatocytes and develop into pre-erythrocytic (exoerythrocytic) schizonts during the next 5–15 days depending on the *Plasmodium* species (Fujioka and Aikawa, 2002). The sporozoites reproduce asexually, generating 10,000 to 30,000 descendants, which are contained within a structure called a liver schizont. During this time, the human host is asymptomatic (Betterton-Lewis, 2007). *P.vivax*, *P.ovale* and *P. cynomolgi* have a dormant stage, named hypnozoite that may remain in the liver for weeks to many years before the development of pre-erythrocytic schizogony. This results in relapse of malaria infection (Akinyi, 2010; Fujioka and Aikawa, 2002).

In the erythrocytic stages the merozoites, rupture out of the hepatocytes, migrate through the bloodstream within membrane-bound vesicles known as merosomes that enable them to avoid immune detection (Akinyi, 2010). The parasites invade the RBCs in the bloodstream. There, they consume hemoglobin and transform from delicate rings to larger ameboid forms called trophozoites and then to a blood schizont stage containing 8 to 24 daughter parasites (Betterton-Lewis, 2007).

Mature form of blood schizonts of *P. falciparum* sequester deep within the venous microvasculature where they are difficult to detect on routine blood smears. When these parasites are released from the blood schizonts, recurring attacks of fever, sweats, chills, headache, abdominal and back pain, nausea, diarrhoea, and sometimes vomiting are triggered. The frequency of these attacks is a reflection of the time needed for a single cycle of growth inside RBCs and development, which occurs every 48 h for *P. vivax and P.falciparum* and every 72 h for *P. malariae.* For *P.falciparum,* the rupture of blood schizonts occurs in waves and the cycle of fever, sweats, and chills is less predictable (Betterton-Lewis, 2007).

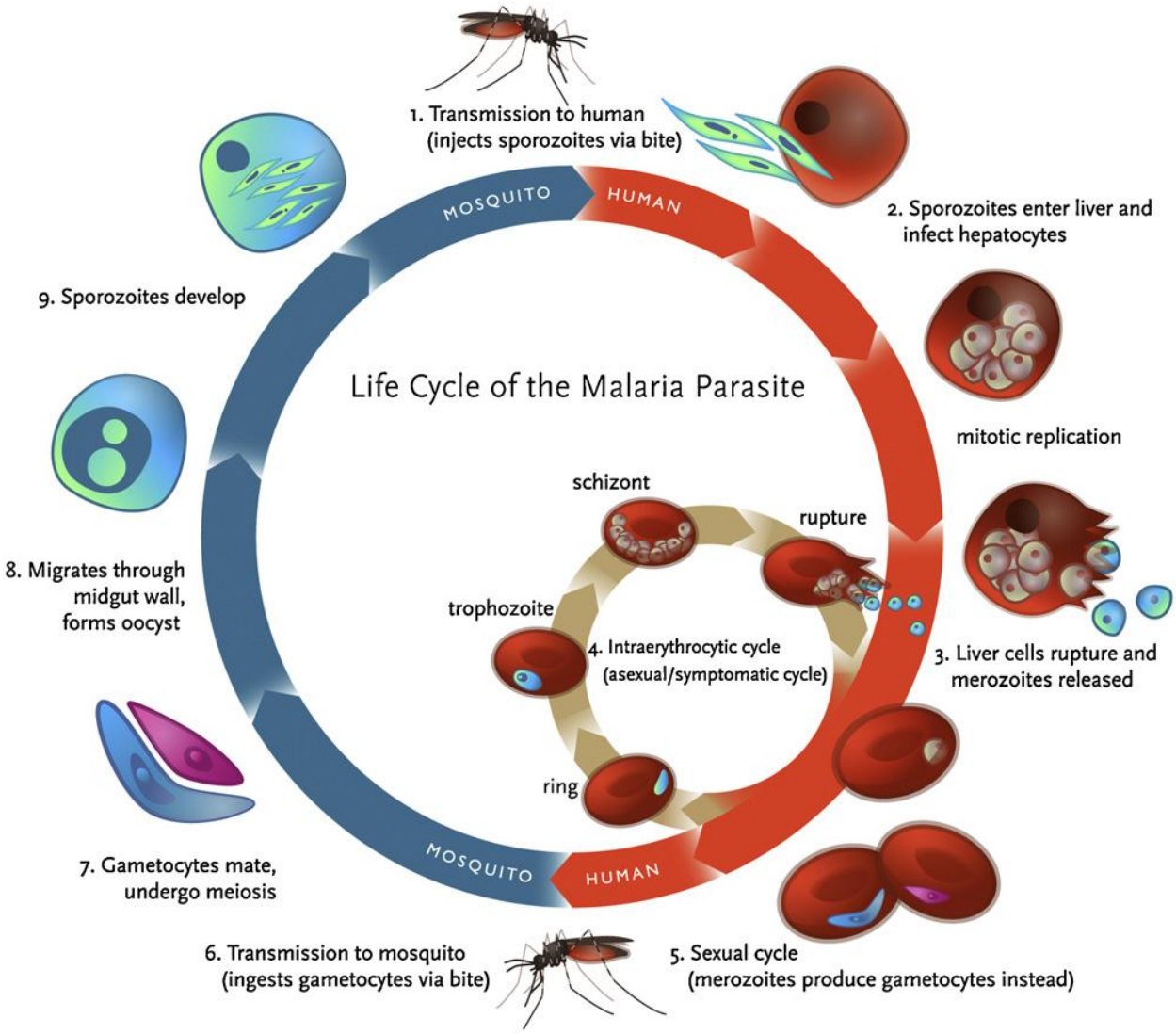

Fig. 1. Life cycle of the malaria parasite (Klein, 2013)**.**

Transmission of malaria occurs through a vector, the mosquito that injected sporozoites into a human during blood meal(s) (1), where they rapidly make their way to the liver and infect hepatocytes and begin asexually (mitotically) replicating (2). After a period of ca. 6–15 days, the liver schizonts rupture, releasing thousands of merozoites into the blood where they invade RBCs (3). Over the next ca. 48 h, the parasite begins replicating mitotically, progressing through a set of stages (ring, trophozoite and schizont), and produces an average of 16 new daughter merozoites per schizont. The schizonts then burst in near synchrony with other parasites, producing the characteristic fever cycle that embodies the clinical manifestations of the disease (4). Some of the merozoites produce gametocytes—the sexual form of the parasite (5), which is ingested by mosquito upon feeding on an infected human (6). Gametocytes, which are

both male and female, mate within the gut of the mosquito, undergo meiosis (7), then migrate through the midgut wall of the mosquito, and form an oocyst (8), within which thousands of sporozoites develop (9), that can then infect susceptible mosquitoes and bringing the transmission cycle full circle.

Within RBCs, merozoites begin cycles of multiplication known as schizogony, which involve the development of rings that then differentiate into trophozoites. The ring and trophozoite stages are marked by increased RNA, DNA and protein synthesis, and the beginning of hemoglobin digestion. *P. falciparum* digests more than 80% of its host's hemoglobin to support growth and replication (Akinyi, 2010). The parasite then matures to first form multinucleated, then segmented, schizonts by asexual reproduction, resulting in the production of daughter merozoites. The merozoites then rupture out of erythrocytes to invade uninfected RBCs and initiate the next round of the erythrocytic stage (Akinyi, 2010).

The mosquito stages: during the erythrocytic stage, some of the merozoites will differentiate into sexual forms known as the male and female gametocytes. When a mosquito takes up these gametocytes during a blood meal, they migrate to the mosquito gut where the male gametocyte (microgamete) fertilizes the female gametocyte (macrogamete) forming a zygote that develops into an ookinete. The ookinete migrates through the midgut epithelium and basal lamina where it differentiates into an oocyst. Within two weeks of differentiation, the oocyst produces daughter sporozoites by asexual multiplication, and the sporozoites migrate to the mosquito's salivary glands where they can then be transmitted to susceptible hosts, beginning a new life cycle for the *Plasmodium* parasite (Akinyi, 2010).

1.3.2 Pathogenesis of malaria

Severe malaria is most commonly caused by infection with *P. falciparum*, although *P. vivax* and *P. knowlesi* can also cause severe disease (Kantele and Jokiranta, 2011). The risk is increased, if treatment of an uncomplicated attack of malaria caused by these parasites is delayed (WHO, 2012b). *P. vivax* infection is much less likely to progress to severe malaria than *P. falciparum* infection. The risk for severe *vivax* malaria is greatest among young children and people with comorbid conditions (WHO, 2012b).

P. falciparum is the only species that induces cytoadherence to vascular endothelium of erythrocytes containing the mature forms of the parasite. As the parasite matures, parasite proteins are transported and inserted into the erythrocyte membrane. The high molecular transmembrane protein of *P. falciparum* erythrocyte membrane protein 1 (*Pf*EMP1) is the most important ligand for cytoadherence. Cytoadherence causes sequestration of parasitized erythrocytes in the microcirculation, mainly capillaries and post-capillary venules (Dondorp, 2005).

The pathogenesis of human *P. falciparum* infection is a complex interplay of parasite-induced RBCs alterations and microcirculatory abnormalities, accompanied by local and systemic immune reactions, resulting in multiple clinical forms of variable severity (Buffet et al., 2011). Infection with *P.falciparum* is more serious than with other malarial species, because of frequency of severe and fatal complications associated with it. This lethal parasite can be the basis to cerebral malaria, acute renal failure, acute malarial hepatitis, hypoglycemia, hyperpyrexia, non-cardiogenic pulmonary edema, adult

respiratory distress syndrome (Ali et al., 2008). The development of severe malaria probably results from a combination of parasite-specific factors, such as adhesion and sequestration in the vasculature and the release of bioactive molecules, together with host inflammatory responses. These include cytokine and chemokine production and cellular infiltrates (Mackintosh et al., 2004).

Therefore, in severe malaria, blood concentrations of proinflammatory cytokines like TNF-α, Interleukin-1(IL-1), IL-6 and IL-18 are raised. Cytokines can upregulate the expression of intracellular adhesion molecule-1 (ICAM-1) and vascular adhesion molecule -1 (VCAM-1) on endothelium cells, and could thus promote sequestration of parasitized erythrocytes in the brain, contributing to coma. High plasma concentrations of TNF-α in patients with *falciparum* malaria correlate with disease severity, including coma, hypoglycemia, hyperparasitemia and death (Dondorp, 2005; Maitland and Marsh, 2004).

When sequestered, mature forms of RBC do not circulate and escape retention and destruction by the spleen. Sequestration of mature forms of *P. falciparum* favors parasite multiplication and explains the initial Log-linear increase of parasite loads in non-immune subjects. Sequestration of parasitized erythrocytes will compromise the microcirculation in vital organs. In addition, the deformability of both parasitized and uninfected erythrocytes is markedly reduced in severe malaria, and this is strongly associated with a fatal outcome of the disease. Acting synergistically with the reduction in lumen caused by sequestration, rigid erythocytes presumably further reduce blood flow

in the microcirculation of vital organs, causing hypoxia with lactic acidosis, organ dysfunction and death (Dondorp, 2005).

1.4 Agents used in the treatment of malaria

1.4.1 Conventional antimalarial drugs

Traditionally, antimalarial agents are classified by the stages of the malaria life cycle that are targeted by the drug: blood schizonticides acting on the asexual intraerythrocytic stages of the parasites. Tissue schizonticides kill hepatic schizonts, and thus prevent the invasion of erythrocytes, acting in a causally prophylactic manner. Hypnozoiticides kill persistent intrahepatic stages of *P. vivax* and *P. ovale*, thus preventing relapses from these dormant stages. Gametocytocides destroy intraerythrocytic sexual forms of the parasites and prevent transmission from human to mosquito (Rudrapal, 2011; Schlitzer, 2007).

Chemotherapy has played an important role in the treatment and control of malaria (Farooq and Mahajan, 2004). Most of the drugs used to treat malaria are quinoline derivatives modeled on the quinine molecule (Krettli et al., 2001). Quinine is still one of the most important drugs for the treatment of uncomplicated malaria, and often the only therapeutic option for the treatment of severe malaria because preparations for intravenous applications are available (Nosten et al., 2006). Generally, a combination of quinine with tetracycline or doxycycline or clindamycin is recommended (Schlitzer, 2008). Chloroquine, the derivative of quinine, was close to the ideal antimalarial drug and used for decades due to its high efficacy against all species of malaria parasites, low toxicity, low cost and high tolerance. It is still widely used to treat malaria in areas where

notable drug resistance has not yet appeared **(**Krettli et al., 2001). This drug can be taken both as a prophylactic and as a treatment (Saifi et al., 2013).

Amodiaquine is also active against most chloroquine-resistant strains; however, hepatitis, myelotoxicity and agranulocytosis restrict its use in treating acute malaria. Mefloquine has been widely used for malaria prevention due to a long half-life (2–4 weeks in human) that necessitates only a once-weekly dosing (Biamonte et al., 2013).

Artemisinin (or qinghaosu) and its derivatives (artesunate, artemether, and arteether) have been used extensively in China and Southeast Asia, where there are high levels of resistance to the majority of the quinoline containing drugs and to all the antifolate drugs. The artemisinin-type compounds currently in use are either the natural extract artemisinin itself or the semi-synthetic derivatives (dihydroartemisinin, artesunate, artemether) (Saifi et al., 2013). Artemisinin-based combination therapies (ACTs) are the current standard of care for uncomplicated malaria. The most popular combination consists of tablets containing artemether (20 mg) and lumefantrine (120 mg) sold as Coartem™ . In 2011, the European Medicines Agency (EMA) approved the combination of dihydroartemisinin and piperaquine, which is taken once a day for 3 days. Parenteral artesunate is the drug of choice for severe malaria (Biamonte et al., 2013).

Currently, confirmed cases of uncomplicated *P. falciparum* malaria should be treated with an ACT. *P. vivax* malaria should be treated with chloroquine where this drug is effective, or an appropriate ACT in areas where *P. vivax* is resistant to chloroquine.

Treatment of *P. vivax* should be combined with a 14-day course of primaquine to prevent relapse (WHO, 2011).

1.4.2 Herbal drugs

Nearly 80 % of the global population still depends upon the herbal drugs for their health care (Bahekar and Kale, 2013). The use of traditional medicine is rapidly spreading in the industrialized countries. In China, traditional herbal preparations account for 30%-50% of the total medicinal consumption. In Ghana, Mali, Nigeria and Zambia, the first line treatment for 60% of children with high fever resulting from malaria is the use of herbal medicines at home (Kassaye et al., 2006). Therefore, traditional medicine is the first-choice for healthcare of at least 80% of Africans who suffer from high fever and other common ailments (Agbor et al., 2011; Elujoba et al., 2005). It is known that over 1,277 plants belonging to 160 families were reported in 2004 to be used traditionally for the treatment of malaria (Rasoanaivo et al., 2011).

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 *F. apodanthera* and the chloroform extract of roots of *G.senegalensis* exhibited a pronounced antimalarial activity (Ancolio et al., 2002). Reports from Ghana also indicated the importance of the following families as possible sources of antimalarial

drugs: *Anacardiaceae*, *Meliaceae*, *Celastraceae*, *Rutaceae*, *Asteraceae* and *Combretaceae*. The most frequently used species of plants were *Azadirachta indica*, *Senna siamea*, *Citrus aurantifolia* and *Nauclea latifolia*. As well *Azadirachta indica* has been mostly mentioned as a treatment for malaria in Togo and Kenya (Nguta et al., 2010) and found to have good antiplasmodial activity**.** On the other hand**,** *Argemone mexicana* had shown the best activity *in vitro*, both for the extracts in polar solvents and the aqueous decoction. The IC_{50} of the methanol extract was 1.0 μg/ml, which is of the same order as the ethanolic extract of *Artemisia annua* (Willcox et al., 2011). A high antimalarial activity was also shown for *Pittosporum viridiflorum* which indicates the interest of *Pittosporum* genus as a potential source of antimalarials (Ramalhete et al., 2008*).*

In Ethiopia, some of the medicinal plants used traditionally for the treatment of malaria have been screened for their antimalarial activity. The 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 *Clerodendrum myricoides* showed appreciable *in vivo* antimalarial activities against *P. berghei* parasite (Tadesse, 2011).

1.5 Antimalarial drugs resistance

For decades, drug resistance has been one of the main obstacles in the fight against malaria. To date, drug resistance has been documented in three of the five malaria species known to affect humans in nature: *P. falciparum*, *P. vivax* and *P. malariae***.** Drug resistance is complicated by cross-resistance, which can occur among drugs that belong to the same chemical family or which have similar modes of action. Multi-drug resistance (MDR) of *P. falciparum* is seen when the parasite is resistant to more than two operational antimalarial compounds of different chemical classes and modes of action. Generally, the two classes first affected are the 4-aminoquinolines and the antifolates (diaminopyrimidine, sulfonamides) (WHO, 2010).

The earliest report of antimalarial resistance was that of *P. falciparum* to quinine reported from Brazil in 1910 (Spencer, 1985). The resistant parasite then has spread out to all known malaria-endemic area except the countries located north of the Panama Canal and Haiti Island.Whereas resistance to chloroquine by *P. falciparum* first appeared virtually simultaneously in Southeast Asia (Thai-Cambodian border) and South America (Colombia) in late 1950s (Farooq and Mahajan, 2004). Increasing chloroquine resistance has driven those countries in which resistance has developed to switch their first line treatment from chloroquine to Sulphadoxine /Pyrimethamine (SP). However, by the late of 1980s, resistance to SP became prevalent on the Thai-Cambodian and Thai-Myanmar borders, which later became an MDR area (Bjorkman and Phillips-Howard, 1990). This facilitated global spread of drug resistance and hence worsening the status of management and control of the disease, escalating morbidity and mortality (WHO, 2006).

Resistance to all known anti-malarial drugs, including the newly introduced ACT has developed to various degrees in several countries (Carrara et al., 2009)**.**

1.6 Development of malaria vaccine

Vaccines hold particular promise for malaria, and recent technological advances and evidence demonstrates that immunizing children against the malaria parasite is feasible (Braich and Malik, 2012). However, development of malaria vaccine poses scientific challenges due to a great complexity of the parasite regarding antigens, limited and incomplete knowledge about acquired immunity developed against malaria, no appropriate animal model for malaria, and high cost of developing a malaria vaccine candidate before it can be marketed (Aide et al., 2007 : Bonn, 2005).

Of the five species of *Plasmodium* that are known to cause disease in humans, two have received attention for vaccine development. Over 90% of malaria-related deaths are caused by *P. falciparum,* and there is a similar dominance for *P. falciparum* projects in the malaria vaccine landscape (Schwartz et al., 2012). P. vivax vaccine**:** two leading P.vivax antigens, circumsporozoites protein (CSP) and RII domain of the Duffy binding protein (DBP), are in clinical testing in human challenge studies and are most promising vaccine candidates for P.vivax **(**Braich and Malik, 2012**).**

Three main strategies were adopted for developing malaria vaccines. The first is pre-erythrocytic stage vaccine that would neutralize sporozoites as they enter the blood stream thereby rendering them incapable of invading the liver. The second is the blood stages vaccine that limits the invasion of erythrocytes and produce mild disease but would not prevent infection. The third one is transmission-blocking vaccines, which stimulate the production of antibodies against the sexual stages thereby rendering them sterile within the mosquito and incapable of infecting a host during a blood meal. Actually, the vaccines that have received the most attention are those directed against the sporozoite stage of the parasite (Uadia, 2007).

It is known that *Plasmodium* circumsporozoite protein (CS) is involved in the adhesion of the sporozoite to the hepatocyte and invasion of the hepatocyte. Anti-CS antibodies have been shown to inhibit parasite invasion and are associated with a reduced risk of clinical malaria (Schwartz et al., 2012). The new malaria vaccine being under development (RTS, S-a recombinant protein vaccine) is a lead candidate in this category (Braich and Malik, 2012).

RTS, S/ASO2A is based on the CS protein and is the vaccine candidate for which clinical development is most advanced. It consists of a hybrid molecule recombinantly expressed in yeast, in which the CS protein and its carboxyl-terminal regions are fused to the N-terminal of the S antigen of hepatitis B virus (Hbs Ag) in a particle that also includes the un-fused S antigen. ASO2A is an adjuvant consisting of an oil-in-water emulsion containing the immunostimulants monophosphoryl lipid A and *Quillaja saponaria* fraction 21 (QS21) (Uadia, 2007). RTS, S, was effective for at least 18 months in reducing clinical malaria episodes by 35% and severe malaria by 49 % (Braich and Malik, 2012; Chatopadhyay and Kumar, 2009). RTS,S is being evaluated in a phase 3 clinical trial at 11 sites in seven African countries (Chatopadhyay and Kumar, 2009; Limbach et al., 2011).

1.7 The experimental plant: Justicia schimperiana

1.7.1 Botanical background

The family and genus of Justicia

The family *Acanthaceae* is a taxon of dicotyledonous flowering plants containing almost 250 genera and 2500 species. Most are tropical herbs, shrubs or twining vines, some are epiphytes. Only a few species are distributed in temperate region. The four main centers of distribution are Indonesia, Malaysia, Africa, Brazil and Central America (Reddy et al., 2013). *Justicia* is the largest genus of *Acanthaceae*, with approximately 600 species that are found in pantropical and tropical regions (Corrêa and Alcântara, 2012; Hedberg et al., 2006). The species of *Justicia* can be easily recognized by their bilabial corolla, with a

posterior lip that is generally two-lobed, an anterior lip that is three lobed, two stamens, a capsule with four seeds, and a basal sterile portion (Corrêa and Alcântara, 2012).

Justicia Schimperiana

Synonyms: *Adhatoda schimperiana/Gendarussa schimperiana*, local name 'dhumuugaa' in Afan Oromo, 'Sensel' or 'simiza'in Ahmaric, 'surpa', 'kasha' or 'keteso' in Sidama. It belongs to the family of *Acanthaceae*. It is a shrub with much branched stems 2-3 m high, with slightly unpleasant smell. It grows in moist mantane forest usually near stream/river, evergreen shrub on hill slopes, waste ground, and village & house hedge from altitude ranging 1300-2700 m (Hedberg et al., 2006).

1.7.2 Ethnopharmacological information for the genus of Justicia

Several species of *Justicia* are widely used in folk medicine for the treatment of respiratory and gastrointestinal diseases as well as inflammation (including applications in rheumatism and arthritis). The plants are also utilized for their effects on the central nervous system as hallucinogens, somniferous agents, sedatives, depressors, and treatments for epilepsy and other mental disorders (Corrêa and Alcântara, 2012).

Crude water extracts of the aerial parts of *Justicia gendarussa* proved to be strongly active against *in vitro* HIV type 1 reverse transcriptase. *Justicia pectoralis* showed positive antimosquito tests, which were observed on the growth and development of IV-stage larvae of *Aedes aegypti* mosquitoes. A brief exposure to concentrations of 0.05 to 0.50 mg/mL of the plant extract is required to produce 100% larvicidal activity. The ethanol extract of the leaves of *J. gendarussa* showed a higher paw edema inhibition than

aspirin-treated rats in the Freund's Complete Adjuvant (FCA)-induced and the collagen-induced arthritic models. The popular use of *J. pectoralis* in the treatment of epilepsy and anxiety was confirmed with the ethanol extract of the leaves. The ethanol extracts of *J. pectoralis, Justicia aurea* Schltdl., and *Justicia albobracteata* Leonard were tested *in vitro* for their ability to inhibit GABA transaminase (GABA-T) or to bind to the $GABA_A$ benzodiazepine receptor, two principal drug targets in epilepsy and anxiety. A significant positive correlation between GABA-T inhibition and the relative frequency of use for epilepsy was observed (Corrêa and Alcântara, 2012*).*

In Ethiopia *J. schimperiana* (Fig.2) is used in the treatment of scabies, fever, asthma and other inflammatory situations, excessive pellagra and constipation (Tariku, 2008), malaria (Mekonnen, 2005; Tariku,2008; Yirga and Zeraburk,2011), gonorrhea, rabies, headache in Gindeberet District western Ethiopia (Yirga and Zeraburk, 2011). It is also used to treat ascaris by people in Zegie Peninsula, Northwestern Ethiopia (Teklehaymanot and Giday, 2007) and for hepatitis B treatment by people in Bale, Southeastern Ethiopia (Yineger et al., 2008). In eastern Ethiopia, the plant is also used as a laxative and in northern Ethiopia the plant alone or in combination with other plants is used for epilepsy, mental illness, eye diseases, jaundice, leprosy, syphilis, measeles, relapsing fever, vitiligo, gout and acute febril illness, venereal diseases; leishmaniasis and cough. Insect repellant, hypotensive, histamine antagonist, cardiac depresant, oxytocic and abortificient effect of the plant also have been recorded. It has also been reported that different parts of the plant are used as expectorant, anthelmintic and antispasmodic (Mekonnen, 2005).

Majority of the Ethiopian population still depends on traditional medicine for their health care practices and more than 95% of traditional medical preparations are of plant origin (Petros, 2011).

A number of plants are traditionally used in Ethiopia for the treatment of malaria. Although recently there are efforts to identify and screen antimalaria herbs used in the country, the studies done are very limited and they are not fully exploratory and most of them focuses on the review of ethnobotanical uses of the plants rather than pharmacological screenings. Scientific screening of potential antimalarial herbs in the country for their *in-vitro* and *in-vivo* antimalarial activities as well as toxicological evaluations (Petros, 2011), along with testing for the particular phytochemicals present in the plants that is responsible for antimalarial effects have an immense role in standardization of the herbal preparations. Therefore, since the previous study conducted by Petros and Melaku (2012) indicated that the hydroalcoholic (methanol 80%) extract of *J. schimperiana* leaf endowed with antimalarial activities, the present study was aimed to further investigate which solvent fraction (s) of the crude hydroalcoholic (methanol 80%) extract would be responsible for the antimalarial activity of the plant.

Fig. 2 Photograph of *Justicia Schimperian* Hochst. Ex Nees (*Acantaceae*)

2. OBJECTIVES

2.1 General objective

- To investigate antimalarial activities of the chloroform, methanol and aqueous fractions of *J. schimperiana* leaves extract in *P. berghei* infected mice

2.2 Specific objectives

- To investigate the effect of the chloroform, methanol and aqueous fractions on percentage parasitemia, packed cell volume (PCV), body weight and rectal temperature using Peter's four day suppressive test
- To evaluate the curative effect of the most active solvent fraction of the plant extract on percentage parasitemia, PCV, body weight and rectal temperature using Rane's test
- To evaluate the effect of the most active solvent fraction of the plant extract on percentage parasitemia, PCV, body weight and rectal temperature using prophylactic test
- To perform preliminary phytochemical screening of the secondary metabolite responsible for antimalarial activities

3. MATERIALS AND METHODS

3.1 Materials

3.1.1 Chemicals, reagents and drug

The following chemicals, reagents and drug were used in the present study: Absolute methanol (Carlo Erba, France), chloroform (Carlo Erba, France), normal saline, Geimsa stain, trisodium citrate (Hunan Dongting, China), Tween 80 (BDH Laboratory Supplies, England), Dragendrof's reagent, Mayer's reagent, 1% $FeCl_3$, 1% $K_3Fe(CN)_6$, 2% lead acetate, concentrated sulfuric acid, glacial acetic acid, sodium chloride and standard chloroquine (Ethiopian Pharmaceutical Manufacturing, Addis Ababa Ethiopia). All the chemicals were of analytical grade.

3.1.2 Collection of plant material

The leaves of *J. schimperiana* were collected in February 2013 from Qanate village in Bidaru Gobata within Gindeberet district, western Ethiopia, which is located 270 km from Addis Ababa. Taxonomic identification was performed by Mr. Assefa Hailu at the National Herbarium, College of Natural Sciences, Addis Ababa University and a voucher specimen was kept there for future reference with voucher No. of 001/HR.

3.1.3 Experimental animals and parasites

A total of 225 male Swiss albino mice (age 6-8 weeks and weight of 25-36g) obtained from the Ethiopian Health and Nutrition Research Institute and School of Pharmacy, Addis Ababa University were used for the pilot and main studies. The animals were housed in the animal house of School of Pharmacy, Addis Ababa University, under

standard environmental conditions (12 h light/dark cycle). All mice were allowed free access to food and water *ad libitum* throughout the experimental period. Good hygiene was maintained by constant cleaning and removal of feces from cages daily. The mice were maintained and cared for according to the international guidelines for the use and maintenance of experimental animals (OECD, 2001).

Chloroquine sensitive *P. berghei* strain ANKA maintained at the Animal House of the Department of Biology, College of Natural Sciences, Addis Ababa University were used. The parasite was subsequently maintained in the laboratory by serial passage of blood from mouse to mouse every 5-7 days.

3.2 Methods

3.2.1 Extraction procedure for crude extract

The collected plant leaves were cleaned with tap water and dried under shade at room temperature for two weeks and coarsely powdered. A total of 600 g of coarsely powdered leaves were macerated with 80% methanol (200 g in 1600 ml) i.e in the ratio of 1:8 in Erlenmeyer flask for 72 h at room temperature. The extraction process was facilitated by using an orbital shaker at 120 rpm with occasional stirring. After 72 h, the extract was separated from the marc (the residue left after the extraction) using gauze and further filtered by Whatman filter paper No. 1 (Whatman®, England). The marc was remacerated twice using the same volume of 80% methanol to exhaustively extract the plant material. After exhaustive extraction, the hydroalcohol (methanol 80%) was removed by evaporation under reduced pressure using rotary evaporator (Buchi Rota

vapor, Switzerland) in distillation flask at 72 rpm and 40 °C to obtain the crude extract of the plant. The extract was further concentrated to dryness by freeze drying using lypholizer (Operon, Korea vacuum limited, Korea). The yield was found to be 134 gm (22.33%). Portion of the yield was used for confirmation of the antimalarial activity of the crude extract and the remaining (126.73 g) was used for fractionation.

3.2.2 Fractionation

The crude extract was subjected to a successive extraction using Soxhlet apparatus with chloroform and absolute methanol in increasing order of their polarity and finally the remaining marc was macerated with distilled water. Accordingly, the dried crude hydroalcoholic (methanol 80%) extract was placed in a cellulose thimble in an extraction chamber, which was placed on top of a collecting flask beneath a reflux condenser. Chloroform was first added to the flask, and the set up was heated under reflux. This process was continued until a drop of solvent from the siphon tube did not leave residue when evaporated. The residue left in the thimble was extracted with methanol following the same procedure of chloroform extraction. After chloroform and methanol was removed from their respective fraction by using rotary evaporator, both fractions were further dried in an oven at < 40^0C. The residue left inside the thimble from the two solvent fractions was macerated in Erlenmeyer flask with distilled water. The major portion of the marc was easily dissolved in distilled water, while a bit of the marc was slightly warmed on the water bath to facilitate dissolution.The aqueous fraction placed in a deep freeze (Aftron, Denmark) overnight and dried using a lypholizer. The dried fractions were then transferred into separate vials and stored in a refrigerator (-20 °C) until use.

3.3 Preliminary phytochemical screening

The fractions of the leaf extract of *J. schimperiana* were screened for the presence of different phytochemical constituents following standard procedures.

Test for alkaloids

The formation of yellowish orange precipitate when one ml of the test solution was treated with a few drops of Dragendorff's reagent or the production of a creamy or white precipitate when the sample solution is mixed with a few drops of Mayer's reagent indicates that the test is positive (Trease and Evans, 1989). Both tests were used for detection of alkaloids.

Saponins test:

For methanol and aqueous fractions: 0.5 g of methanol and aqueous fractions were dissolved in 10 ml of distilled water in a test tube. The test tube was stoppered and shaken vigorously for 30 sec and allowed to stand in a vertical position and observed over 30 min. Formation of "honey comb" froth over the surface of liquid and persistence after 30 min indicates presence of saponins (Jones and Kinghorn, 2006;Trease and Evans, 1989).

For chloroform fraction (CF): The fraction was diluted with an appropriate solvent and made up to 20 ml. The suspension was shaken in a graduated cylinder for 15 min. One cm layer of foam indicates the presence of saponins (Iraqui and Yadav, 2013).

Polyphenols (Phenolic compounds)

A drop wise of a mixture of 1 ml each of 1% $FeCl_3$ and 1% $K_3Fe(CN)_6$ were added to 2 ml of each of aqueous solution of the aqueous fraction (AF) and solution of appropriate solvent for CF and methanol fraction (MF). The presence of polyphenols and phenols was indicated by the apparition of a blue and green precipitate (Kouitcheu et al., 2013).

Test for flavonoids

The dried fraction was dissolved in a mixture of appropriate solvent. To 2 ml of the fraction solution, three to five drops of 2% lead acetate solution were added. Then, it was observed whether it developed yellow or orange color, which indicates the presence of flavonoids (Jones and Kinghorn, 2006;Trease and Evans, 1989).

Test for terpenoids

Five ml of fraction dissolved in appropriate solvent was mixed in 2 ml of chloroform, and 3 ml concentrated H_2SO_4 was carefully added to form a layer. A reddish brown coloration of the interface was formed to show positive result for the presence of terpenoids (Trease and Evans, 1989).

Tests for steroids

i) A red color produced in the lower chloroform layer when 2 ml of organic extract was dissolved in 2 ml of chloroform and 2 ml concentrated sulphuric acid added indicates the presence of steroids (Njoku and Obi, 2009).

ii) The development of a greenish color when 2 ml of the organic extract was dissolved in 2 ml of chloroform and treated with sulphuric and acetic acids indicates the presence of steroids (Njoku and Obi, 2009).

Test for tannins

About 2 ml of the extract was stirred with 2 ml of distilled water and few drops of $FeCl_3$ solution were added. The formation of a green precipitate was an indication for the presence of tannins (Njoku and Obi, 2009).

Tests for glycosides

Keller-Kiliani test: 2 ml of each extract was dissolved in 2 ml of glacial acetic acid containing one drop of $FeCl_3$ solution. The mixture was then poured into a test tube containing 1 ml of concentrated H_2SO_4. A brown ring at the interphase indicates the presence of a deoxy sugar, characteristic of cardenolides (Njoku and Obi, 2009).

3.4 Pharmacological evaluation

3.4.1 Preliminary test for the crude extract

Preliminary test of the crude extract was performed using twenty-five mice. Animals were grouped into five groups and inoculated with 0.2ml of blood infected by P. berghei intraperitoneally. In such a way, group I received 2% Tween 80, group II-IV treated with extract at doses of 200, 400 and 600 mg/kg while group V treated with chloroquine 25mg/kg. Based on the test conducted, the suppressive effect of the crude extract on parasitemia was confirmed using 4-day suppressive test.

3.4.2 Grouping and dosing of animals

The studies of the fractions were performed using forty mice for each solvent fraction. Mice were randomly assigned into three extract treated groups and two controls, eight mice per group for each fraction and inoculated as described below in section 3.4.3. Group I was negative control and treated with vehicle; 2% of Tween 80 v/v in water for CF and MF or distilled water for AF. Group II, III and IV were treated with solvent fractions of the plant extract at doses of 200, 400 and 600 mg/kg, respectively. The doses were selected based on preliminary test. Group V was treated with chloroquine 25 mg/kg. Administration was performed via the oral route using gavage. Volume administered was calculated based on individual mouse body weight and 0.45ml is the maximum volume administered. Duration of administration depends on the type of test performed and described along with the respective models.

3.4.3 Inoculation of parasite

To infect the mice, blood sample was collected from a donor mouse with a rising parasitemia of about 30-37% (Adediji et al., 2012; Deressa et al., 2010). After determination of the percentage parasitemia and erythrocytes count, the donor mouse was sacrificed by decapitation and blood was collected into a petridish containing 0.5% trisodium citrate. The collected blood was then diluted with isotonic saline in proportions indicated by both determinations (Odetola and Basir, 1980; Okokon et al., 2011a). The inoculum consisted of $5x10^7$ *P. berghei* parasitized erythrocytes per ml. Each mouse used in the experiment was infected intraperitoneally with 0.2 ml of infected blood containing about 1×10^7 *P. berghei* parasitized erythrocytes.

3.4.4 Four-Day suppressive test

This test was performed to evaluate schizontocidal activitities of the fractions on early *P. berghei* infection in mice using a 4-day suppressive test described by Knight and Peters (1980). The infected mice were randomly divided into five groups as described in section 3.4.2. Treatment was started three hours after the mice had been inoculated with the parasite on day 0, and then continued for the next four days from day 0 to day 3 with 24 h time interval between the doses. After giving the treatment for four days, thin blood film was made from the tail of mouse on the fifth day (D4) to determine the level of parasitemia and percentage inhibition.

3.4.5 Rane's test (Curative test)

The curative test was undertaken with the MF that shown the highest parasitemia suppression in the 4-day suppressive test. Accordingly, evaluation of the curative potential of the MF was carried out using the method described by Ryley and Peters (1970). The mice were injected intraperitoneally with standard inoculum of 1x 10^7 *P. berghei* infected erythrocytes on the first day (day 0). Seventy-two hours later, the mice were divided into five groups of eight mice per group as described in section 3.4.2 and treated accordingly. The mice were treated once daily for 5 days from day 3 to day 7. Starting from day 3 through day 7 daily thin blood films was prepared from the tail of each mouse to monitor the level of parasitemia.

3.4.6 Prophylactic test

The method of Peters (1967) was adopted in the evaluation of the prophylactic potential of the most active solvent fraction. Mice were randomly divided into five groups with eight mice per group as described in section 3.4.2. Treatments were given for four consecutive days and on the fifth day all the mice were inoculated intraperitoneally with 0.2 ml of infected blood containing $1x10^7$ inoculum of *P. berghei*. Blood smears were then made from the tail snip of each mouse 72 h after the last dose administration to determine the levels of parasitemia.

3.4.7 Determination of body weight and temperature change

The body weight of each mouse in all groups was measured before infection (day 0) and on day 4 in the four-day suppressive test. For prophylactic test, body weight was taken on day 0 before inoculating parasite and on day 3 (i.e 72 h after infection), while in the Rane's test it was measured on day 3 after infection was established and on day seven, the last day of the treatment using a sensitive digital weighing balance (Mettler Toledo, Switzerland). The rectal temperature of each mouse in all groups was measured by a digital thermometer (G.S.T corporation, New Delhi, India) one hour before infection, four hours after infection and then daily to see the effect of fractions on body temperature in both 4-day suppressive and prophylactic tests. On the other hand, in Rane's test, rectal temperature was measured one hour before infection and then daily from day 3- day 7 after infection.

3.4.8 Packed cell volume measurement

Blood was collected from tail of each mouse in heparinized microhematocrit capillary tubes. The capillary tubes were filled to $3/4^{th}$ of their height with blood and sealed at one end with sealing clay. It was then placed in a microhematocrit centrifuge (Hettich hematokrit, Germany) with the sealed ends outwards. The blood was centrifuged at 11,000 rpm (relative centrifugal force (RCF) =11498.63xg) for 5 min. The tubes were then taken out of the centrifuge and PCV was determined using the standard Microhematocrit Reader (Hawksley and Sons, England). It was measured before inoculating the parasite and after treatment in both Peter's 4-day suppressive and prophylactic tests. However, in the case of Rane's test, PCV count was made on the third day after infection was established and on the last day of treatment on the seventh day. PCV is a measure of the proportion of RBCs to plasma in the whole blood and determined using the relation shown below (Dikasso et al., 2006):

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

3.4.9 Determination of parasitemia

Thin blood smears were prepared from tail snip of each mouse on the fifth day (D4) for Peter's 4-day suppressive test, from day 3 after infection was established to day 7 for curative test and 72 h after infection in prophylactic test on microscopic slides. The slides were dried and fixed with absolute methanol. The slides were stained with 10% Giemsa at pH 7.2 for 10 min and then washed gently using distilled water and air dried at room

temperature. Two stained slides were prepared for each mouse in the 4-day suppressive and prophylactic tests, while one slide was prepared in Rane's test daily for five days and examined under microscope with an oil immersion nosepiece of 100x magnification power. Five different fields on each slide were examined to calculate the average parasitemia (Jigam et al., 2011). Percentage parasitemia was calculated by counting infected RBC and total RBC from giemsa stained thin blood films of the blood that was collected from the tail snip of each mouse in all groups using the formula shown below (Oyewole et al., 2008):

$$\% \text{ Parasitemia} = \frac{\text{Number of parasitized RBC}}{\text{Total number of RBC}} X100$$

The mean % parasitemia was recorded for each animal and for each group; it was used to determine variations in parasitemia level with time of infection. The average percentage suppression of parasitemia was calculated for each dose level by comparing the parasitemia in infected controls with those of treated mice with the following formula (Aarthi and Murugan, 2011):

$$\textbf{\%} \text{ suppression} = \frac{(\text{Mean parasitemia of negative control} - \text{Mean parasitemia of treated group})}{\text{Mean parasitemia of negative control}} 100$$

3.4.10 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 of 30 days (D0-D29) for all the models. The mean

survival time (MST) for each group was then calculated using the following formula (Mengiste et al., 2012):

$$MST = \frac{\text{Sum of survival time for all mice in a group (in days)}}{\text{Total numbers of mice in that group}}$$

3.4.11 Statistical analysis

All the results are expressed as mean ± SEM for each group. All the grouped data were statistically evaluated and the significance of various treatments was calculated using one-way ANOVA followed by Tukey's HSD post hoc test. A p-value of < 0.05 was considered significant. All data processing was done using SPSS data analysis soft ware version 16.0.

4. RESULTS

4.1 Percentage yield of extractions

As summarized in Table 1, the MF provided the highest yield followed by the AF, while the lower yield was obtained from the CF.

Table 1: Yield and physical properties of solvent fractions of the leaves of *J. schimperiana*

Types of Extracts	**Nature of Extract**	**Color of Extract**	**Actual Yield (g)**	**Percentage yield(w/w)**
CF	Gummy	Black	3.31*	2.61%
MF	Slightly gummy	Light brown	58.07*	45.82%
AF	Powder (hygroscopic)	Light brown	19.85*	15.66%

* Yield obtained from 126.73g of hydroalcoholic (methanol 80%) extract of the leaves of *J. schimperiana*

4.2 Four-day suppressive test

As presented in Table 2, all the three fractions significantly suppressed parasitemia in the 4-day suppressive test. Of these, the CF showed the lowest chemo-suppression. Despite that the fraction exerted significant ($p<0.05$) inhibition of parasitemia at 400 mg/kg and 600 mg/kg doses while it failed to do so at 200 mg/kg dose as compared to the control group. On the other hand, the MF indicated the highest chemo-suppressive effect in the 4-day suppressive test. All dose levels of the MF significantly ($p<0.001$) reduced the level of parasitemia when compared to the control group. Statistically significant difference ($p<0.01$) was observed only when 600 mg/kg dose compared to 200 mg/kg dose.

The AF produced the second highest inhibition of parasitemia next to the MF in the 4-day suppressive test. Only 400mg/kg and 600mg/kg doses of the AF did significantly ($p<0.001$) suppress the level of parasitemia. Significant difference ($p<0.05$) was seen when 600mg/kg dose compared to 200mg/kg dose in inhibiting parasitemia. The reference drug, chloroquine, cleared parasitemia to undetectable level on the fifth day from a thin blood film. This suppressive effect was statistically significant ($p<0.01$) as compared to the control and entire dose levels of all the fractions (Table 2).

All dose levels of the CF failed to significantly prolong the survival time of the mice as compared to the control group. Only 400mg/kg and 600mg/kg doses of the MF and 600mg/kg dose of the AF were able to significantly ($p<0.05$) improve survival time of the mice as compared to the control group. Statistically significant differences were

observed in prolonging survival time of the mice when 400mg/kg ($p<0.01$) and 600mg/kg ($p<0.001$) doses of the MF compared to 200mg/kg dose. Chloroquine treated mice survived all the 30 days and improvement in survival periods were statistically significant ($p<0.001$) in relation to the control and all the three fractions treated groups (Table 2).

Table 2: Parasitemia, percentage suppression and survival time of mice treated with the fractions of *J.schimperiana* leaves in the 4-day suppressive test

Groups	**Average %Parasitemia**	**% Suppression**	**Survival time (in days)**
2% TW80*	45.55 ±4.83	0	7.63 ±0.5
DW**	23.74±1.7	0	7.75±0.25
CF200mg/kg	38.08±3.55	16.4 ^{b3}	7.75±0.31^{b3}
CF400mg/kg	33.56 ±1.62	26.32 ^{a1b3}	8.0±0.27^{b3}
CF600mg/kg	32.65 ±1.22	28.32 ^{a1b3}	8.57±0.57^{b3}
MF200mg/kg	28.67 ± 1.59	37.1^{a3b3e2}	7.88±0.44^{b3d2e3}
MF400mg/kg	22.59 ± 1.21	50.4 ^{a3b3}	10.5±0.65^{a2b3c2}
MF600mg/kg	15.85 ± 0.44	65.2 ^{a3b2}	12.38±0.5^{a3b3}
AF200mg/kg	19.34±1.39	18.54 ^{b3e1}	8.88±0.88^{b3}
AF400mg/kg	16.12±1.19	32.08 ^{a3b3}	11.00±0.94^{b3}
AF600mg/kg	14.02±0.50	40.93 ^{a3b3c1}	11.25±1.28^{a1b3}
CQ25mg/kg¥	0.00 ±0.00	100^{a3}	30.0±0.00^{a3}

Data are expressed as mean±SEM; n=8, a= compared to negative control, b= to CQ25 mg/kg, c= to 200 mg/kg, d=to 400 mg/kg, e=to 600 mg/kg within the same fraction, 1=P<0.05, 2=P<0.01, 3=P<0.001, AF=aqueous fraction, CF= chloroform fraction, MF= Methanol fraction, 2% TW80= 2% Tween80, DW=distilled water, CQ= chloroquine, * = negative control used for chloroform and methanol fractions, **= negative control used for aqueous fraction, ¥ = suppressive and survival effect of CQ25mg/kg is the same for all the three fractions

As shown in Table 3, all the three fractions did not significantly protect the loss in body weight resulted from infection by the parasite as compared to the control group. In CF and AF, weight loss was not related to the dose, with the highest loss of body weight

observed at 400mg/kg dose. However, the body weight loss increased with increasing the doses of MF in early infection. Chloroquine was also unable to significantly prevent the reduction in body weight as compared to the control group.

All the three fractions did not significantly reverse the reduction in PCV caused by the infection of parasite as compared to the control group. Nevertheless, the reduction in PCV was decreased with increasing the doses of all the three fractions, though it failed to reach statistical significance level. Likewise, the reference drug was also unable to significantly counteract the decrease in PCV in relation to the control group (Table 3).

As indicated in Table 3, all the three fractions were able to significantly prevent the decrease in rectal temperature caused by *P. berghei* infection depending on the dose. Only 400mg/kg and 600mg/kg doses of CF significantly ($p<0.05$) protected the decrease in rectal temperature as compared to the control group. On the other hand, all the three dose levels of the MF significantly ($p<0.05$ for 200mg/kg, $p<0.01$ for 400mg/kg and $p<0.001$ for 600mg/kg) prevented the reduction in rectal temperature in relation to the control group. No apparent diffrence was, however, observed among doses of the MF in protecting the rectal temperature of the mice. In similar manner to CF, only 400mg/kg and 600mg/kg doses of AF significantly ($p<0.01$ and $p<0.001$ respectively) protected the decrease in rectal temperature as compared to the control group. Significant ($p<0.05$) differences were observed among the AF doses when 400mg/kg and 600mg/kg doses compared to 200mg/kg dose in protecting the rectal temperature of the mice.

Furthermore, the reference drug was also significantly ($p<0.01$) prevent the decrease in rectal temperature in relation to the control and 200mg/kg dose of AF treated groups.

Table 3: Body weight, packed cell volume and rectal temperature of mice before and after treatment with the fractions of *J. schimperiana* leaves in the 4- day suppressive test

Groups	**W0 (g)**	**W4 (g)**	**% change**	**T0 (^{0}C)**	**T4 (^{0}C)**	**% Change**	**PCV0**	**PCV4**	**% change**
2%TW80*	25.34±0.69	25.31±0.87	-0.38	37.79±0.2	33.83±0.55	-11.9	62.05±1.92	62.63±4.88	-3.11
DW**	31.35±0.96	30.76±1.19	-2.19	37.86±0.30	33.98±0.26	-11.5	58.75±1.47	56.97±1.34	-3.63
CF200mg/kg	30.19±1.67	29.73±1.55	-1.54	37.89±0.38	36.06±0.3	-5.08	67.9± 1.89	63.49±2.51	-7.53
CF400mg/kg	30.52±0.75	29.62±0.74	-3.11	36.31±0.39	34.51±0.33	-5.22^{a1}	62.67±1.09	60.1± 2.09	-4.96
CF600mg/kg	30.21±1.54	29.97±1.31	-0.63	38.44±0.13	36.41±0.37	-5.62^{a1}	64.05±1.41	63.52±3.87	-2.99
MF200mg/kg	29.42±1.28	29.38±1.26	-0.15	38.4±0.17	36.51±0.29	-5.23^{a1}	60.45±0.92	59.58±1.54	-1.92
MF400mg/kg	30.64±1.81	30.26±1.8	-1.39	37.54±0.41	35.99±0.65	-4.47^{a2}	58.50±3.23	61.16±1.64	-0.19
MF600mg/kg	33.08±1.85	32.38±1.57	-1.9	36.85±0.37	36.85±0.37	-0.06^{a3}	65.56±1.98	65.46±1.77	-0.17
AF200mg/kg	29.65±1.31	29.32±1.37	-1.26	37.95±0.08	34.69±0.28	-9.46^{b2d1e1}	57.79±0.85	57.00±1.09	-1.53
AF400mg/kg	29.34±1.14	28.52±1.34	-3.25	37.49±0.38	35.81±0.29	-4.75^{a2c1}	57.46±1.74	56.88±1.67	-1.11
AF600mg/kg	28.92±0.95	28.13±0.92	-2.89	37.38±0.17	35.99±0.2	-3.88^{a3}	58.88±2.18	58.85±2.83	-0.83
CQ25mg/kg*	25.56±1.53	26.72±1.23	4.64	37.66±0.29	35.93±0.4	-4.91^{a2}	60.34±1.68	65.21± 2.1	7.17
CQ25mg/kg**	30.99±0.51	31.30±0.84	0.75	37.13±0.35	35.95±0.28	-3.27^{a3}	56.70±0.85	57.07±1.76	0.19

Data are expressed as mean±SEM; n=8, a= compared to negative control, b= to CQ25mg/kg , c= to 200mg/kg, d=to 400mg/kg, e=to 600mg/kg within the same fraction, 1=P<0.05, 2=P<0.01, 3=P<0.001, AF=aqueous fraction, CF= chloroform fraction, MF= Methanol fraction, 2% TW80= 2% Tween80, DW=distilled water, CQ= chloroquine, * = negative and positive controls used for the chloroform and methanol fractions, **= negative and positive controls used for the aqueous fraction, 0=pre-treatment value on day 0, 4= post- treatmet value on day 4,W= weight, T= temperature

4.3 Rane's test

In the Rane's test, the solvent fraction with the highest activity in the 4- day suppressive test was further evaluated for its curative effect. Accordingly, the test was undertaken with the MF that revealed the highest chemosuppression in the four-day suppressive test.

As presented in Table 4, despite the first dose administration on the 3rd day, parasitemia level was increased on the 4th day with all dose levels of the MF. Up on the second dose administration on the 5th day, the parasitemia level began to decline with all the three dose levels of the fraction and kept on decreasing across the treatment days. All dose levels of the MF significantly ($p<0.001$) suppressed parasitemia determined on the 7th day from a thin blood film in relation to the control group. In addition, statistically significant ($p<0.001$) differences were observed when 600mg/kg dose was compared to 400mg/kg and 200mg/kg doses as well as the same difference was noted when 400mg/kg dose was compared to 200mg/kg dose in suppressing parasitemia. By contrast, the standard drug caused reduction in parasitemia levels right after the first dose administration on the 3rd day and it completely cleared parasitemia on the 7th day from a thin blood smear. This effect was significantly ($p<0.001$) different when compared with the control and all fraction treated groups.

With respect to the survival time, only 400mg/kg and 600mg/kg doses of the fraction did significantly ($p<0.05$ and $p<0.001$ respectively) improve survival periods of the mice as compared to the control group. Statistically significant ($p<0.001$) difference was observed

in prolonging survival time of the mice when 600mg/kg dose was compared to 200mg/kg dose. Chloroquine was found to prolong survival time significantly ($p<0.001$) with regard to the control and all fraction treated groups (Table 4).

Table 4: Parasitemia, percentage suppression and survival time of mice treated with methanol fraction of *J.schimperiana leaves in* Rane's test

Groups	**Average Parasitemia**					**% suppression**	**Survival time(days)**
	D3	D4	D5	D6	D7		
2% TW80	22.13±2.18	30.57±1.41	36.79±1.36	43.58±1.44	48.77±0.74	0	7.50±0.27
MF200mg/kg	22.70±1.08	26.75±1.84	25.45±1.41	24.71±0.55	24.41±0.74	$49.95^{a3b3d3e3}$	$8.88±0.61^{b3e3}$
MF400mg/kg	21.02±0.67	24.86±1.67	23.18±0.89	21.80±0.90	19.59±0.42	$59.85^{a3b3c3e3}$	$10.25±0.92^{a1b3}$
MF600mg/kg	20.28±0.83	22.09±1.49	19.70±1.22	18.73±1.31	15.88±0.44	67.44^{a3b3}	$12.50±0.73^{a3b3}$
CQ25 mg/kg	18.48±2.02	14.53±1.16	8.25±0.5	1.97±0.36	0.00±0.00	100^{a3}	$30±0.00^{a3}$

Data are expressed as mean± SEM; n=8, a= compared to 2% TW 80, b= to CQ25mg/kg, c= to MF 200mg/kg, d= to MF 400mg/kg, e=to MF 600mg/kg

1=P<0.05, 2=P<0.01, 3=P<0.001, MF= Methanol fraction, 2%TW80= 2% Tween 80, CQ= chloroquine, D=day

As shown in Table 5, the loss in body weight and reduction in PCV resulted from infection by the parasite did not significantly ameliorate with all dose levels of the fraction as well as the standard drug. Like in the four-day suppressive test, the loss in body weight escalated with increasing the doses of the fraction, whilst the reduction in PCV slightly decreased with increasing the fraction doses. However, the decrease in rectal temperature significantly ($p<0.05$ for 200mg/kg dose and $p<0.01$ for 400mg/kg and 600mg/kg doses) protected by all the three dose levels of the fraction with respect to the control group. Likewise chloroquine was able to prevent the reduction in rectal temperature significantly ($p<0.001$) when compared to the control group. Significant ($p<0.05$) difference was also observed in protecting the lowering of the rectal temperature by chloroquine in relation to all dose levels of the MF treated groups.

Table 5: Body weight, packed cell volume and rectal temperature of mice before and after treatment with the methanol fraction of *J. schimperiana leaves* in Rane's test

Groups	**W3 (g)**	**W7 (g)**	**% Change**	**T3 (^{0}C)**	**T7 (^{0}C)**	**% Change**	**PCV3**	**PCV7**	**% change**
2%TW80	29.78±1.7	27.64±1.9	-8.44	37.94±0.3	31.43±0.4	-18.77	61.49±4.2	35.013±2.1	-83.98
MF200mg/kg	28.73±1.44	26.65±1.6	-6.29	37.43±0.2	33.33±0.8	-10.95^{a1b2}	64.99±3.7	38.69±5.5	-79.25
MF400mg/kg	30.63±1.05	28.89±1.1	-6.36	37.19±0.2	33.73±0.6	-10.47^{a2b1}	66.69±2.2	36.33±1.3	-74.91
MF600mg/kg	30.38±1.44	28.44±1.3	-6.92	37.44±0.3	34.03±0.7	-10.30^{a2b1}	63.70±3.0	40.45 ±5.2	-71.12
CQ25mg/kg	29.55±1.07	28.90±1.5	-2.05	37.33±0.2	36.9±0.34	-1.15^{a3}	64.45±3.4	39.64±2.7	-68.46

Data are expressed as mean± SEM; n=8, a= compared to 2% TW 80, b= to CQ25mg/kg, 1=P<0.05, 2=P<0.01, 3=P<0.001, MF= Methanol fraction, 2% TW 80= 2% Tween 80, CQ= chloroquine, 3=pre-treatment value on day 3, 7= post- treatmet value on day 7, W=weight, T=temperature

4.4 Prophylactic (Repository) test

MF had also shown prophylactic activities against *P. berghei* infected mice. Accordingly, all dose levels of the fraction significantly ($p<0.05$ for 200mg/kg dose and $p<0.01$ for 400mg/kg and 600mg/kg doses) suppressed parasitemia relative to the control group. However, there were no significant differences among the doses in suppressing parasitemia. The reference drug resulted in significant ($p<0.001$) chemosuppression with respect to the control group. On the other hand, only 600mg/kg dose of the MF and chloroquine were able to significantly ($p<0.05$) prolong survival time in relation to the control group (Table 6).

Table 6: Parasitemia, percentage suppression and survival time of mice treated with methanol fraction of *J.schimperiana* leaves in the prophylactic test

Groups	**Average Parasitemia**	**% Suppression**	**Survival time(days)**
2% TW80	44.07±2.38	0	6.75±0.31
MF200mg/kg	31.79±1.81	27.86[a1]	8.38±0.63
MF400mg/kg	29.55±2.32	32.93[a2]	8.5±0.38
MF600mg/kg	28.63±2.33	35.02[a2]	8.63±0.53[a1]
CQ25mg/kg	23.56±3.71	46.53[a3]	8.63±0.38[a1]

Data are expressed as mean± SEM; n=8, a= compared to 2% TW 80, b= to CQ25mg/kg, 1=P<0.05, 2=P<0.01, 3=P<0.001, MF= methanol fraction, 2% TW80= 2% Tween 80, CQ= chloroquine

The decrease in body weight and PCV resulted from infection by the parasite not significantly counteracted by all dose levels of the MF and reference drug as compared to the control group. Unlike 4-day suppressive and curative tests, the reduction in PCV escalated with the doses of the fraction in repository test. Moreover, all dose levels of the MF and chloroquine were able to significantly ($p<0.05$) protect the decrease in the rectal temperature relative to the control group(Table 7).

Table 7: Body weight, packed cell volume and rectal temperature of mice before and after treatment with the methanol fraction of *J. schimperiana leaves* in the prophylactic test

Groups	**W0 (g)**	**W3 (g)**	**% change**	**T0 (^{0}C)**	**T3 (^{0}C)**	**% change**	**PCV0**	**PCV3**	**% change**
2%TW80	33.87±1.55	33.08±1.51	-2.38	37.24±0.32	33.85±0.61	-12.42	57.14±1.03	44.11±2.35	-32.50
MF200mg/kg	31.49±1.78	31.26±1.54	-0.51	37.54±0.20	37.74±0.15	-3.24^{a1}	61.16±1.39	50.99±3.86	-23.96
MF400mg/kg	29.64±1.88	29.31±1.87	-1.45	37.28±0.22	35.93±0.71	-4.02^{a1}	52.90±1.22	39.84±2.10	-35.34
MF600mg/kg	35.84±2.04	35.00±2.15	-2.68	36.99±0.13	35.74±0.46	-3.59^{a1}	56.51±0.91	41.44±2.47	-39.59
CQ25mg/kg	30.28±1.38	29.17±1.27	-3.74	37.66±0.11	36.28±0.80	-4.24^{a1}	55.65±1.26	42.60±2.38	-32.46

Data are expressed as mean± SEM; n=8, a= compared to 2% TW 80, b= to CQ25mg/kg, 1=P<0.05, MF= Methanol fraction, 2%TW80= 2% Tween 80, CQ= chloroquine, 0= pre-treatment value on day 0, 3= post-treatment value on day 3, W=weight, T= temperature

4.5 Phytochemical screening

As shown in Table 8, phytochemical analysis indicated the presence of saponin and alkaloid in all the three fractions, while terpenoids detected only in CF and flavonoids in MF and AF.

Table 8: Phytochemical composition of the solvent fractions of the leaves of *J. schimperiana*

Compound screened	**CF**	**MF**	**AF**
Alkaloid	+	+	+
Saponin	+	+	+
Tannin	-	-	-
Glycoside	-	+	-
Phenol	-	+	-
Steroid	-	-	-
Flavonoids	-	+	+
Terpenoids	+	-	-

+ Present, - absent

5. DISCUSSION

Historically, majority of conventional antimalarial drugs have been derived from plants or from structures modeled on plant derived compounds (Nguta et al., 2010) and over 50% of all modern clinical drugs are of natural product origin and natural products play an important role in drug development programs of the pharmaceutical industry (Borokini, 2012). Therefore, it is justified that such studies should be done in screening plant with antimalarial activities to provide a potential lead for novel antimalarial drug development.

This study was undertaken using *in-vivo* model in which the fractions were tested against *P. berghei* infected mice. The *in-vivo* model was employed because it takes into account prodrug effect and possible involvement of immune system in eradication of infection (Waako et al., 2005).

Primate models provide a better prediction of evaluation of the efficacy of anti-malaria in human than the rodent models. However, the rodent models have also been validated through the identification of several conventional antimalarials especially with the success of quinine and more recently artemisinin derivatives (David et al., 2004). *In-vivo* murine *Plasmodium* models such as *P. berghei*, *P. vinckei* and *P.yoelii* are firmly established models in anti-malarial drug discovery (Petros and Melaku, 2012). *P. berghei* has been used in studying the activity of potential antimalarials in mice (Thomas et al., 1998) and in rats (Pedroni et al., 2006). Therefore, it has been used to predict treatment

outcomes and is an appropriate parasite for this study (Gitua et al., 2012; Madara et al., 2012)**.** Since the parasite is sensitive to chloroquine, this drug was used as the standard treatment drug in the present study.

The 4-day suppressive test is a standard test commonly used for antimalarial screening (Peter and Anatoli., 1998). It is the most widely used preliminary test, in which the efficacy of a compound is assessed by comparison of blood parasitemia and mouse survival time in treated and untreated mice (Kalra et al., 2006), and the determination of percent suppression of parasitemia is the most reliable parameter (Ene et al., 2008; Peter and Anatoli.,1998).

The results in this study showed that in the 4-day suppressive test MF and AF exerted higher chemosuppressive effects than the CF. This significantly higher activity of the MF and AF possibly indicate that the most active components of the plant extracts were concentrated in these fractions. The highest chemosuppressive effect of the MF might be resulted from secondary metabolites found in this fraction. Therefore, alkaloids**,** saponin, phenol and flavonoids detected in this fraction could have contributed to the antimalarial activities observed in this fraction. Different reports indicated the antimalarial implication of alkaloids (Oliveira et al., 2009), flavonoids (Aarthi and Murugan, 2011; Okokon et al., 2011a; Oskoueian et al., 2011; Preez, 2012 ; Saxena et al., 2013), phenols (Adesokan and Akanji, 2010; Arise et al., 2012; Oskoueian et al., 2011; Saxena et al., 2013) and saponins (Adewoye et al., 2010; Arise et al., 2012). This finding is also consistent with other study in which the MF had high activity (Jigam et al, 2012a**;** Okokon et al, 2011b; 2012a;

Ukwe et al., 2010) and with the effect of methanolic *Languas galanga* rhizome extracts in early infection (Al-Adhroey et al., 2010). It is also further in agreement with the *in vitro* activities of the root extract of *J. schimperiana* against *P. falciparum* in which the IC_{50} of methanol extract was lower as compared to the IC_{50} of petroleum ether and chloroform extracts (Bogale and Petros, 1996).

The AF found to have the second highest antimalarial activities next to the MF in the 4-day suppressive test. This antimalarial effect could have been associated with secondary metabolites such as alkaloids**,** saponin and flavonoids (Oseni and Akwetey, 2012; Salawu et al., 2010), identified in this fraction. However, the lower dose of AF was unable to suppress parasitemia significantly. This might be due to the absence of sufficient concentration of active constituents with antiplasmodial activities in the lower dose. This result is consistent with the effect of AF reported on the leaf of *Carpolobia lutea* (Okokon et al., 2011a) and with the lower doses (100 mg/kg and 200 mg/kg) of the aqueous leaf extract of *Morinda lucida* (Unekwuojo et al., 2011).

The high antimalarial activity seen in the MF and AF suggests that semi-polar and polar solvents are the preferred media for the extraction of the antiplasmodial principles from the leaves of *J. schimperiana*. This result is in agreement with other study conducted (Jigam et al., 2012a). In addition, a mean group parasitemia level of less than or equal to 90% of the mock-treated control animals usually indicate that the test material is active in standard screening studies (Peter and Anatoli.,1998) or alternatively an antimalarial compound is considered as an active compound when it suppresses percent parasitemia

by ≥ 30% (Krettli et al., 2009). This idea strongly supports the present study in which the AF and MF significantly suppress parasitemia level beyond this cut off point. Therefore, these fractions are active. Further, this signifies that agents with suppressive activity against *P. berghei* were known for antimalarial activity (Akuodor et al., 2010).

On the other hand, CF produced the lowest inhibition of parasitemia level as compared to the MF and AF in the 4-day suppressive test. Probably the low parasitemia suppression by CF as compared to the other two fractions might have emanated from the absence of flavonoid and phenol in this fraction or the absence of enough concentration of the right sub-groups of phytoconstituents responsible for antimalarial activities. This result is in agreement with the activity of CF reported on *Ageratum conyzoides* (Ukwe et al., 2010). Further, it is also in line with the low effect of CF reported on *Enicostema littorale and Smilax krausiana* in early infection (Okokon et al., 2012a; 2012b) as well as *in vitro* activities of chloroform extract of the root of *J. schimperiana* against *P. falciparum* (Bogale and Petros, 1996).

The most active solvent fraction in the 4-day suppressive test was further evaluated for its curative effects. In established infection, MF exerted significant suppression of parasitemia. However, the reduction in parasitemia produced by the MF did not appear right after the first dose administration on the 3rd day, rather parasitemia began to decrease following the second dose administration on the 4th day with all dose levels of the fraction. This might be due to the fact that the extract at the doses administered had not accumulated sufficiently to bring about considerable chemosuppression or it has a

slower onset of action compared to chloroquine (Balogun et al., 2009). This result is in agreement with the curative effect of ethanolic root bark extract of *Piliostigma thonningii* (Madara et al., 2012*),* methanolic *Languas galanga* rhizome extracts (Al-Adhroey et al., 2010) and methanolic leaf extract of *Piper betle* L. (Al-Adhroey et al., 2011). However, the current finding is higher than the curative effect of methanol extract of *Acacia nilotica* reported (Jigam et al., 2010).

After the curative effect of the MF established, it was further evaluated for its prophylactic potential. In the prophylactic test, the MF produced the lowest percentage suppression of parasitemia as compared to its effect in the 4-day suppressive and curative tests. Nevertheless, the fraction demonstrated significant suppressive effect on the level of parasitemia. This might have resulted from some constituents of the extract that increased immunological response in the treated mice against the malaria parasite (Okpani et al., 2013). The lower chemosuppressive effect of the MF in prophylactic test might have arisen from rapid hepatic clearance or metabolism of the active component (Alli et al., 2011; Salawu et al., 2010), of the fraction responsible for antimalarial activities. It may also be explained by the fact that not all anti–malarials are completely active against *Plasmodium berghei* model (Dow *et al*., 1998).This finding is in agreement with repository effect reported on methanol extract of the roots of *Aristolochia albida* (Khan et al., 2012). Furthermore, it is also in line with other studies in which repository effect of aqueous leaf extract of *M. lucida* is lower than its suppressive and curative effects (Unekwuojo et al., 2011).

In general, many anti-malarial herbal remedies may exert their anti-infective effects not only by directly affecting the pathogen but also by stimulating natural and adaptive defense mechanisms of the host. The immune system of the host plays a major role in complete suppression or elimination of the pathogens (Rasoanaivo et al., 2011). As well, some plants are known to exert antiplasmodial action either by causing elevation of RBC oxidation or by inhibiting protein synthesis. Though the mechanism of action of this extract remains to be seen, the plant might exert its action by one or more of the above mechanism depending on their phytochemical constituents. Therefore, the antiplasmodial activity observed in this study could have resulted from a single or combined action of phytochemicals (Bassey et al., 2009; Pradhan et al., 2011), identified in these fractions.

Moreover, chloroquine used as a reference drug in this study since it is highly effective against erythrocytic forms of *plasmodium* parasites (Shapiro and Goldberg, 2006). This drug completely cleared the parasite in both the four-day suppressive and curative tests, which is in line with the activity of chloroquine obtained in other studies (Adewoye et al., 2010; Al-Adhroey et al., 2010; Deressa et al., 2010). However, the reference drug produced the lowest inhibition of parasitemia in prophylactic test in relation to its activities in early and established infection. This low suppressive effect of the reference drug probably resulted from rapid metabolisms that inactivate the drug or this could be attributed to low half-life of chloroquine in rodents compared to human (Cooper & Magwere, 2008).

In addition to significant suppressive effect on parasitemia, the MF and AF also significantly improved the survival time of the study mice at 600mg/kg dose relative to the control. This finding might probably indicated that the extracts suppressed *P. berghei* and reduced the overall pathologic effect of the parasite on the study mice (Mengiste et al., 2012). However, the extracts did not cure the infection and this could be due to recrudescence of *P. berghei* parasites after apparent cure (Mengiste et al., 2012). Moreover, survival time is higher for the MF than AF. This result is concordant with other study in which the MF has longer survival periods (Jigam et al., 2012a). However, this finding is higher than the survival time reported on aqueous extract of *Phyllanthus emblica, Terminalia chebula,* and *Terminalia bellerica* (Pinmai and Hiriote, 2010).

In the 4-day suppressive test, variation in weight loss was observed among the CF, MF and AF evaluated for their activities. Accordingly, unlike the AF and CF weight loss had been increased with doses of the MF. This might indicate the presence of constituents implicated with appetite suppressive effect that escalated with the doses of the MF. Alternatively, it could be attributed to the presence of anti-nutritive factors in the extracts (Jigam et al., 2012b). The presence of saponin might have significantly affected feeding by its astringent and irritating taste (Francis et al., 2002; Saxena et al., 2013**)**.

Measuring rectal temperature of mice is used to predict the effectiveness of the test fractions. It is theoretically accepted that the body temperature of mice decreased in a rapid manner with increasing parasitemia, contrary to the situation in human subjects (Dikasso et al., 2006). In this study the middle and higher doses of CF and AF and all

dose levels of MF significantly protected the decrease in rectal temperature associated with *P. berghei* infection in mice. This might probably indicate that the extracts ameliorate some pathological processes that cause reduction in internal body temperature and modulate the immune system of infected mice as well as counteract the reduction in metabolic rates that occur because of increased parasitemia (Mengiste et al., 2012). Moreover, the presence of saponin could have also attributed to immunostimulant effect of the fractions (Saxena et al., 2013), which augment the immune system of the mice. However, at 200mg/kg dose the CF and AF were unable to significantly protect the reduction in rectal temperature resulted from the infection by the parasite in the 4-day suppressive test. This might be probably arising from the presence of low concentration of active constituents responsible for protecting the reduction in rectal temperature in this dose level of the fractions. The current finding is consistent with the effect of butanol fraction of *Dodonaea angustifolia* Seed (Mengiste et al., 2012), while it was discordant with the report on aerial and roots of *Asparagus africanus* (Dikasso et al., 2006).

The reduction in PCV is decreased with increasing the doses of the fractions in the 4-day suppressive and curative tests. In contrast, the trend of change in PCV in repository test was found to be the reverse of the effect of MF in the 4-day suppressive and curative tests, in which 600mg/kg dose of the MF produced the minimum protective effect on PCV. This could have probably resulted from the ability of the extract to induce metabolic enzymes that accelerate the clearance of constituents of the plant responsible for protective effect on PCV which may be correlated with enzyme inducing activities of

the related species (Singh et al., 2000) or the extract might have inhibited the formation of RBCs (Okpani et al., 2013).

6. CONCLUSION

The solvent fractions exhibited a significant chemosuppressive effect, with the MF and AF producing the highest activity in the 4-day suppressive test. The MF also possessed a significant activity in both curative and repository tests. These results likely imply that the active ingredients responsible for antimalarial activities are semi-polar and polar. The higher antimalarial activity of the MF makes the methanol solvent an appropriate solvent for further investigation of the activity of the plant.

7. RECOMMENDATIONS

- Evaluation of the plant on different *plasmodium* species and animal models are needed to better identify its potential antimalarial activity
- Bioassay guided isolation should be conducted to isolate the active principle responsible for the activity of the plant
- Elucidating the structure and mechanism of action of the active principle is recommended
- Detailed pharmacological and toxicological studies are needed to develop the active principles into lead compound
- Since *J. schimperiana* is traditionally used for various ailments, it is an active area for research to justify those claimed activities

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