In vivo anti-malarial activity of 80% methanol root bark extract and solvent fractions of *Gardenia ternifolia* Schumach. & Thonn. (Rubiaceae) against *Plasmodium berghei* infected mice

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This is to certify that the thesis prepared by Dejen Nureye, entitled "*In vivo* anti-malarial activity of 80% methanol root bark extract and solvent fractions of *Gardenia ternifolia* Schumach. & Thonn. (Rubiaceae) against *Plasmodium berghei* infected mice" and submitted in partial fulfillment for the requirements of 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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Abstract:

In vivo anti-malarial activity of 80% methanol root bark extract and solvent fractions of *Gardenia ternifolia* Schumach. & Thonn. (Rubiaceae) against *Plasmodium berghei* infected mice

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Irrespective to tremendous reduction in incidence and prevalence during the last decade, malaria continues to remain a major global public health problem. Spread and evolution of resistance to the available antimalarial drugs endanger all the recent gains in malaria control. This issue makes the development of novel drugs a necessity. The key source in search of such drugs is medicinal plants. *Gardenia ternifolia* is used by traditional healers in Africa including Ethiopia for managing malaria. However, no scientific investigations have been carried out to substantiate the claim. Thus, this study was conducted to evaluate *in vivo* antiplasmodial activity of 80% methanolic root bark extract and solvent fractions of the plant in mice model. To this effect, various doses (200, 400 and 600 mg/kg) of the extract and fractions were administered. Parameters, including parasitemia, survival time, body weight, temperature, and packed cell volume were then determined using suppressive, curative and prophylactic tests. Chemo-protective effect exerted by the crude extract and fractions ranged between 30-59% and 14-51%, respectively. The curative and prophylactic effects of the crude extract were in the range of 36-63% and 24-37%, respectively. All dose levels of the crude extract prevented loss of weight, reduction in temperature and anemia on early and established infection. Butanol and chloroform fraction did also reverse reduction in temperature, body weight and packed cell volume at different degree of significance. Phytochemical screening of the extracts showed the

presence of different secondary metabolites. The results indicate that the plant has a promising antiplasmodial activity and it could be considered as a potential source to develop new antimalarial agents.

Key words: *In vivo,* Anti-malarial activity, *Gardenia ternifolia*, *Plasmodium berghei,* Butanol fraction.

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Abbreviations and Acronyms:

AMA	Apical Membrane Antigen
ANOVA	Analysis of Variance
CBP	CX3CL1 Binding Proteins
CD	Cluster of Differentiation
CDC	Centers for Disease Control and Prevention
CX3CL1	C-X3-C Motif Chemokine Ligand 1
FMoH	Federal Ministry of Health
G6PD	Glucose-6-phosphate Dehydrogenase Deficiency
GPI	Glycosyl-Phosphatidyl-Inositol
IRBCs	Infected Red Blood Cells
KAHRP	Knob-associated Histidine Rich Protein
MSP	Merozoite Surface Protein
MST	Mean Survival Time
MVI	Malaria Vaccine Initiative
NIAID	National Institute of Allergy and Infectious Diseases
OECD	Organization of Economic Cooperation and Development
PCR	Polymerase Chain Reaction
PCV	Packed Cell Volume
*Pf*EMP	*Plasmodium falciparum* Erythrocyte Membrane Protein
PM	Plasma Membrane
PMI	President's Malaria Initiative
PRBC	Parasitized Red Blood Cell
PV	Parasitophorous Vacuole
PVM	Parasitophorous Vacuolar Membrane

RBC	Red Blood Cell
RDT	Rapid Diagnostic Test
SEM	Standard Error of the Mean
SPSS	Statistical Package for Social Science
TM	Traditional Medicine
UNICEF	United Nations International Children Emergence Fund
VAR2CSA	Variant Two Chondrotin Sulphate A Antigen
WHO	World Health Organization

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1. INTRODUCTION

1.1 Definition and History of Malaria

The term *malaria* was derived from the Italian "*mala aria*" meaning foul air [Service and Townson, 2002]. It is a protozoal blood infection caused by mosquito-borne apicomplexan parasites of the genus Plasmodium, which are transmitted from one human to another via the bite of infected female Anopheline mosquito species [Carter and Mendis, 2002; Greenwood *et al.*, 2005]. United States National Institute of Allergy and Infectious Diseases (U.S. NIAID) defined malaria as: a disease caused by a parasite that lives part of its life in humans and part in mosquitoes [NIAID, 2007].

Malaria is an ancient disease that could be traced back to the very earliest human history. It was accepted as a disease by Hippocrates in the 4th century BC [Krettli and Miller, 2001]. In the early 17th Century, the Peruvian bark of Cinchona tree was known to treat fever [CDC, 2016]. In 1847, Heinrich Meckel identified black-brown pigment granules in the blood and spleen of insane person [David, 2006]. Othmer Zeidler synthesized Dichloro-Diphenyl-Trichloroethane (DDT) in 1874 for his thesis. Alphonse Laveran noticed parasites, he called *Oscillaria malariae,* in the blood of malaria patient in 1880 [Bruce-Chwatt, 1981]. The genus plasmodium was portrayed by Ettore Marchiafava and Angelo Celli in 1885 [Chavatte *et al.*, 2007]. William MacCallum discovered the sexual stages of malaria parasite in 1897. In 1898, Camillo Golgi and others demonstrated that human malaria was transmitted by anopheline mosquitoes [Cox, 2010].

Chloroquine was discovered in 1934 by Hans Andersag [CDC, 2016]. The liver stage of malaria parasite was elaborated by Henry Shortt and Cyril Garnham in 1948. In early 1950's, malaria was thought to be eliminated from the U.S. Then after, human infection with *P. knowlesi* was recognized in 1965. Artemisinin was isolated from Qinghaosu plant (*Artemisia annua*) in 1971. Next, dormant stages in the liver were demonstrated in 1982 by Wojciech Krotoski [Cox, 2010; David, 2006; CDC, 2016]. Polymerase chain reaction (PCR) based malaria detection was depicted in the early 1990's [Snounou *et al.,* 1993] and mean while malaria rapid diagnostic tests (RDTs) were developed [Dietze *et al.,* 1995].

1.2 Etiology and Vectors of Malaria

The causative agents for malaria infection are among the genus Plasmodium and phyllum Apicomplexa [Morrissette and Sibley, 2002]. The parasite is thought to have originated from Dinoflagellates, photosynthetic protozoa. There are more than 200 different species of Plasmodium. At least 13 species are pathogenic to humans [Chavatte *et al.,* 2007; Liu *et al*., 2010]. Other species infect other animals, including monkeys, rodents, and reptiles [Gueriard *et al.,* 2010]. Five of the human pathogens, *P. falciparum, P. vivax, P. ovale* (two species) *and P. malariae,* are well known etiologic agents for human malaria. Disease with knowlesi, monkey malaria parasite, occur in people when an Anopheles mosquito infected by a monkey bite humans [WHO, 2015].

Malaria is transmitted majorly via bites of the genus Anopheles mosquitoes, which includes 537 recognized species and most (87%) have been formally named [Harbach, 2013]. Nearly, 70 of these species are able to transmit Plasmodium parasite to human hosts and 41 of 70 are considered to be dominant vector species (DVS) [Service and

Townson, 2002]. The two most efficient malaria vectors in the world, *A. gambiae* and *A. funestus*, are primary malaria vectors in Africa [CDC, 2015]. *A. gambiae, A. funestus* and *A. pharoensis* were confirmed as the dominant vectors in Ethiopia [FMoH, 2014].

Due to residence of the parasite in red blood cells (RBCs), malaria can also be transmitted via blood transfusion, organ transplant, or shared use of needles or syringes contaminated with blood. A new born baby may also acquire congenital malaria from her/his mother before or during delivery [Owusu-Ofori, 2010; Manitoba, 2015]. Furthermore, malaria transmission can largely be affected by global weather patterns, including *El Nino* and *La Nina* [Parham *et al.,* 2011; PMI, 2014].

1.3 Epidemiology of Malaria

Around 44% of world population is at risk from malaria [RBMP, 2015]. At the beginning of 2016, malaria was considered to be endemic in 91 countries and territories, down from 108 in 2000. According to the latest estimates, 212 million cases occurred globally in 2015 and the disease led to 429, 000 deaths, most of which were in children aged under 5 years in Africa. In 2015, the number of malaria deaths in children < 5 years is estimated to be 303, 000 worldwide [WHO, 2016]. In the U.S., roughly 1,500–2,000 cases of malaria in recent travelers are reported every year. Pregnant mothers have increased vulnerability to falciparum malaria. In malaria-endemic areas, falciparum contributes to 8-14% of low birth weight, which in turn decreases the chance of a baby's survival [CDC, 2016].

Almost all deaths (99%) due to malaria are resulted from falciparum. About 4% of estimated cases worldwide are caused by vivax, but outside the African continent this

proportion increases to 41%. About 76% of estimated malaria cases in 2015 occurred in just 13 countries. Four countries (Ethiopia, India, Indonesia and Pakistan) accounted for 78% of vivax cases. The malaria case incidence was reduced by 41% and mortality rates by 62% globally between 2000 and 2015. Irrespective to minimization of the world malaria incidence and prevalence, the burden of morbidity and mortality is high in World Health Organization (WHO) African Region, in which most cases (90%) and most deaths (92%) in 2015 were estimated to occur in this region [WHO, 2016]. Not only health related impact but also there is a severe economic burden in terms of lost days of work due to the disease. Of course, malaria is considered to take off 1.3% from the economic growth and 40% of public health expenditure of some African countries. It affects developing countries in many aspects including determent of tourism [Ananya, 2013].

Malaria is one of the top ten diseases in Ethiopia [CDC Global Health-Ethiopia, 2016]. According to the WHO malaria report in 2016, about 27, 034, 284 populations of Ethiopia live at high risk area for malaria infection. In 2015, Ethiopian Federal Ministry of Health (FMoH) reported 1, 867, 059 confirmed malaria cases and 662 deaths [WHO, 2016]. Despite decreased malaria incidence and mortality rate in Ethiopia by > 40% since 2010, high prevalence was observed in contrast to high household coverage of control interventions [Regasa, 2014, WHO, 2016, Ayalew *et. al.,* 2016]. This increment in prevalence is commonly associated with individuals having poor socio-economic status, especially children and female household members [Ayele *et al.,* 2012, 2013].

1.4 Life Cycle of Malaria Parasites

The human malaria parasite has a complex life cycle as shown in Figure-1. The life cycle within the mosquito takes approximately 8 to 35 days, after which the parasite is infective. The motile infectious form is passed to humans when the insect bites the skin, probes for a blood vessel from which to feed, releases various vasodilators to increase its chance of finding a vessel and salivate into the blood to prevent clotting. Within 30-60 min of inoculation, the thread-like sporozoites are carried to the liver by the circulatory system. One single Plasmodium sporozoite in one liver cell multiplies into tens of thousands of exoerythrocytic merozoites [Krettli and Miller, 2001; NIAID, 2007; Manitoba, 2015].

Over a period of 7–12 days, the sporozoites grow in to schizonts and can develop up to 30,000 merozoites, which rupture the hepatocytes [WHO, 2013a; Ricardo *et al.,* 2014]. Alternatively, some vivax and ovale sporozoites turn into hypnozoites, a form that can remain dormant in the liver for months or years and cause relapses in infected people [Greenwood *et al*., 2005; Walker *et al*., 2010]. Interestingly, recurrence of falciparum malaria was reported in patients some years after leaving an endemic area. It tells that at least occasionally falciparum has a dormant stage [Romand *et al.*, 1994; Greenwood *et al*. 2008; Szmitko *et al*., 2008; Theunissen *et al.*, 2009; Poilane *et al.*, 2009]. Then, the asexual erythrocyte cycle begins, with the merozoites invading the RBC to grow by consuming hemoglobin. The parasites can multiply 10 times every 2 days, destroying RBCs and infecting new cells throughout the body. Within the host RBC, the parasite undergoes development from the early ring stage to late trophozoite and then, after mitotic divisions, to the schizont stage, which contains 6–32 merozoites, depending on the parasite species [UNICEF, 2000; Jiraprapa, 2002].

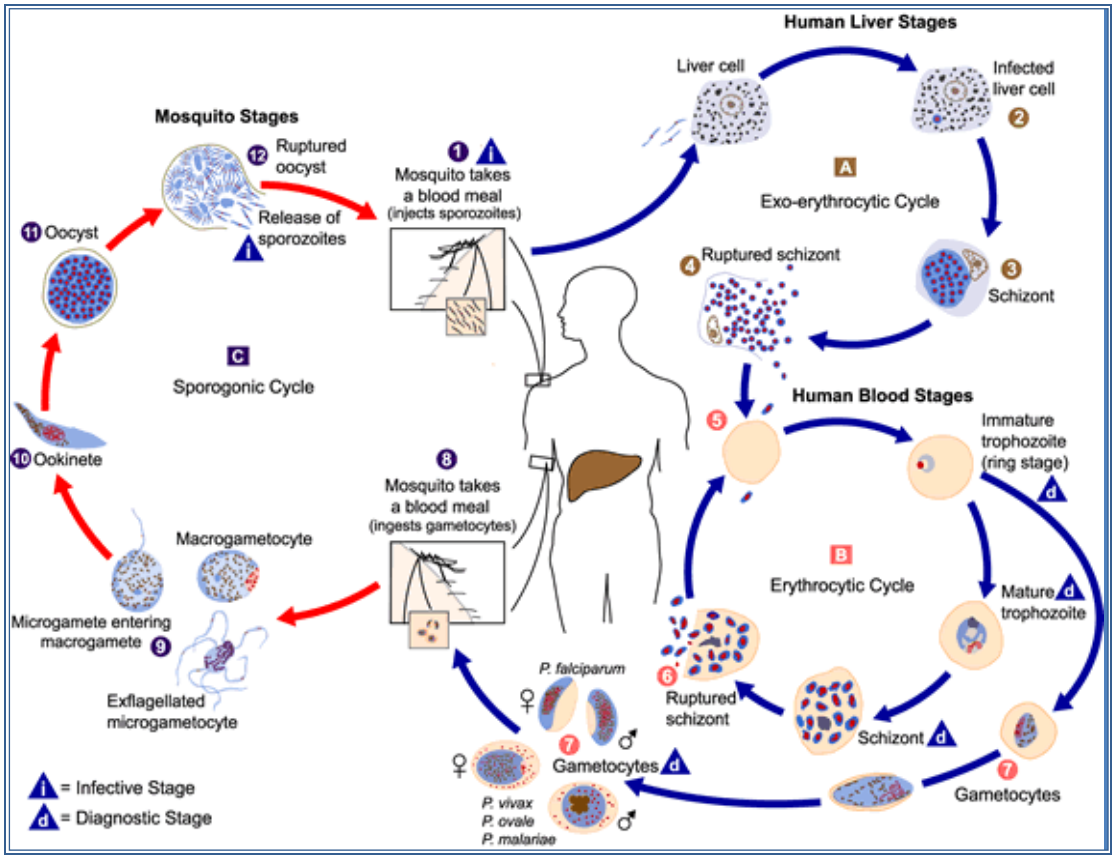

Figure 1: Life cycle of malaria parasites [Bhowmik *et al.*, 2010]. The malaria parasite life cycle involves two hosts. During a blood meal, infected female *Anopheles* mosquito inoculates sporozoites into the human host 1. Sporozoites infect liver cells 2 and mature into schizonts 3, which rupture and release merozoites 4. After this initial replication in the liver (exo-erythrocytic schizogony A), the parasites undergo asexual multiplication in the erythrocytes (erythrocytic schizogony B). Merozoites infect red blood cells 5. The ring stage trophozoites mature into schizonts, which rupture releasing merozoites. Some parasites differentiate into sexual erythrocytic stages (gametocytes) 7. The gametocytes, male (microgametocytes) and female (macrogametocytes), are ingested by an *Anopheles* mosquito during a blood meal 8. The parasites' multiplication in the mosquito is known as the sporogonic cycle C. While in the mosquito's stomach, the microgametes penetrate the macrogametes generating zygotes 9. The zygotes in turn become motile and elongated (ookinetes) 10 which invade the midgut wall of the mosquito where they develop into oocysts 11. The oocysts grow, rupture, and release sporozoites 12, which make their way to the mosquito's salivary glands. Inoculation of the sporozoites 1 into a new human host perpetuates the malaria life cycle [CDC, 2016].

When the erythrocytic schizont ruptures, the released merozoites continue the life cycle by invading other RBCs until it is brought under control. Cyclical fevers are typically happening shortly before or at the time of RBC lysis as schizonts rupture to release new infectious merozoites. This occurs every 48 h in tertian malaria (vivax, ovale and falciparum), and every 72 h in quartan malaria (malariae) infection. During this repeated cycle, some merozoites differentiate into male and female sexual forms known as

erythrocytic gametocytes with one nucleus and then awaiting the arrival of a blood-seeking female Anopheles mosquito [Jiraprapa, 2002; NIAID, 2007]. Intake of gametocytes by the mosquito induces gametogenesis. The flagellated forms of microgametes, formed by a process known as exflagellation, penetrate or fertilize the macrogametes generating zygotes. The zygotes changed into ookinetes and then become a round oocyst. Inside the oocyst, the nucleus divides repeatedly, with the formation of a large number of sporozoites and enlargement of the oocyst. The time necessary for the development of the sporozoites is about 8–15 days [WHO, 2013a].

1.5 Molecular Cell Biology and Pathogenesis of Malaria

Plasmodium species have 14 chromosomes, one mitochondrion, and one apicoplast organelle similar to chloroplast but not photosynthetic [VanDooren and Striepen, 2013]. All Apicomplexa including malaria parasites are characterized by a set of apical organelles called rhoptries, dense granules and micronemes. In Plasmodium, there are three invasive forms involving the apical organelles: sporozoite, merozoite, and ookinete [Bannister *et al.*, 2000]. The co-receptors on sporozoites that mediate invasion [Dame *et al.*, 1984; Robson *et al.*, 1995] bind specifically to the heparan sulfate proteoglycans [Frevert *et al.*, 1993] on hepatocytes and Kupffer cells. CD81 and CD68 are receptors for falciparum invasion in hepatocytes [Cha *et al*., 2015]. After penetrating space of Disse in the liver, sporozoites migrate via several hepatocytes and engage in a final invasion, with the formation of a parasitophorous vacuolar membrane (PVM) [Silvie *et al.*, 2003]. PVM is then ruptured by plasmodial enzymes and merozoite egress from the infected hepatocyte to access blood circulation [Cha *et al*., 2015].

Within the circulation, merozoites are rapidly and specifically enter RBCs, thus implying receptor-ligand interactions. Merozoite surface protein-1 (MSP-1) associated with the parasite membrane via a glycosylphosphatidylinositol (GPI) anchor [Gerold *et al.*, 1996] does binds to the RBC surface proteins [Goel *et al.*, 2003; Baldwin *et al.*, 2015; Boston, 2016]. Eight other merozoite surface-bound GPI-anchored proteins interact with RBC have been reviewed elsewhere [Cowman *et al*., 2012]. After binding to RBC, the merozoite reorients itself using apical membrane antigen 1 (AMA-1) so that the apical end of the parasite will locate adjacent to the RBC membrane with a transient RBC deformation. The contents of apical organelles are going to be expelled as the parasite invades [Mitchell *et al.*, 2004].

Following reorientation, a junction was formed between the parasite and host cell using microneme proteins that recognize and bind to receptors in the host [Maier *et al*., 2003; Mayer *et al.*, 2009; John, 2012]. Proteins in the neck of rhoptry (RON) are inserted into the host membrane and bind to AMA-1 to form the tight junction [Tonkin *et al*., 2011]. Then, the contact area becomes free of RBC membrane proteins. After this, a merozoite enzyme results in a localized disruption of the sub membrane cytoskeleton and lipid architecture of RBC [Zuccala and Baum, 2011]. Formation of the junction triggers the release of rhoptry bulb, providing proteins and lipids required for the parasitophorous vacuole (PV) [Riglar *et al.*, 2011]. So, an incipient PVM will be formed in the junction area [Sam-Yellowe, 1996]. The junction between the parasite and host becomes ring-like and the parasite appears to move via this annulus as it enters the expanding PV [Aikawa *et al.*, 1978]. Following entry, the PVM and host cell membrane will need to be closed [Kappe *et al.*, 2004; Besteiro *et al.*, 2011].

Once inside the RBC, the parasite modifies the host cell to make it a more suitable environment. Formation of knobs, cytoadherence and rosetting (Figure-2) are the major host cell changes happened in the pathogenesis of malaria. Knob-associated histidine rich protein (KAHRP) and erythrocyte membrane protein-2 (*Pf*EMP2) are two of several proteins which reorganize the host RBC submembrane cytoskeleton and induce knob formation [Ryan, 2001]. A polymorphic protein, *Pf*EMP1, has been anchored to the knobs by KAHRP and is exposed on the host RBC surface and function as a ligand. Other cytoadherence ligands [Craig and Scherf, 2001] are shown in the right side of Figure-2.

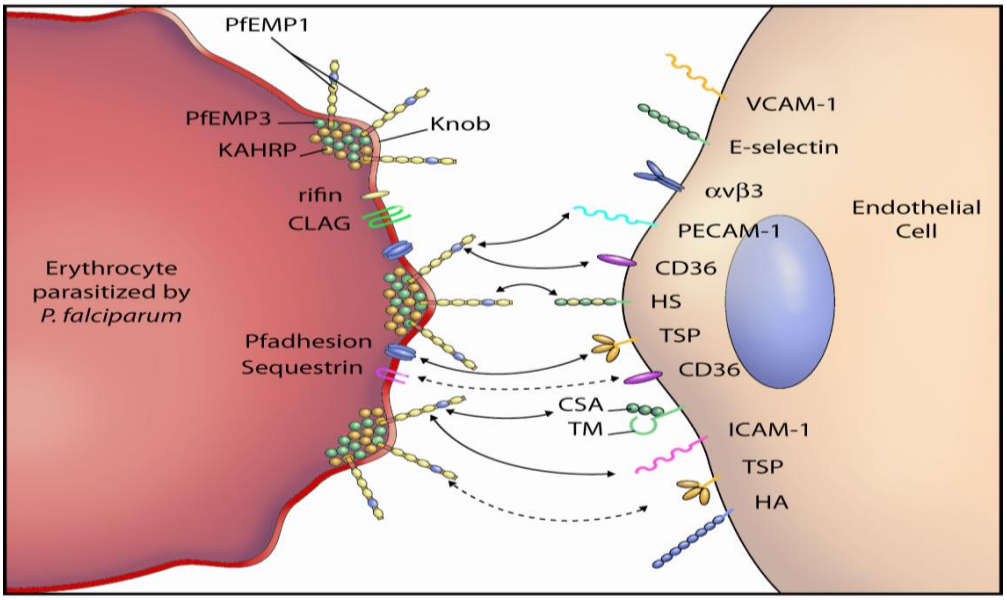

Figure 2: Falciparum cytoadherence (VCAM-1: vascular cell adhesion molecule-1, αvβ3: integrin, PECAM-1: platelet endothelial cell adhesion molecule-1, HS: heparan sulfate, TSP- thrombospondin, CSA: chondroitin sulfate A, ICAM-1: intercellular adhesion molecule-1, HA: hyaluronic acid, CLAG: clag9 ligand) [David, 2006].

Sequestration is also seen during pregnancy when IRBC binds to placental chondroitin sulfate A (CSA), which is mediated by Variant Two chondrotin Sulphate A Antigen (VAR2CSA) [Sander *et al.*, 2011]. So that placental malaria can cause miscarriage, preterm delivery, intrauterine growth retardation, low birth weight and congenital malaria [Bardaji *et al.*, 2011]. Parasites sequestration in tissues provides them the microaerophilic venous environment 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 [Rasti *et al.*, 2004].

The IRBCs also adhere to uninfected RBCs to form red cell rosetting and to other parasitized RBCs to form agglutination [Rasti *et al.*, 2004]. Falciparum, vivax, and ovale are all able to form rosettes [Udomsanpetch *et al*., 1995; Angus *et al*., 1996] but only those caused by falciparum have been associated with severe malaria [Doumbo *et al.*, 2009]. Vivax and ovale show a marked penchant for young RBCs, while malariae prefers old cells. As a result, these parasites have low level of parasitemia in bloodstream. Falciparum, however, can invade RBCs of all ages and produces very high levels of parasitemia [McQueen and McKenzie, 2003; Cowman**,** 2012].

If the above pathophysiologic process goes on uninhibited, it ultimately blocks blood flow, limits the local oxygen supply, impedes mitochondrial ATP synthesis, and stimulates cytokine production – all these factors contributing to development of severe disease [Krishnegowda *et al.*, 2005]. Along with lysis of IRBC [Das *et al*., 2015], toxins (red cell membrane products, hemozoin pigment and GPI) are also released into the blood. These products activate macrophages and endothelial cells to secrete cytokines and inflammatory mediators. The systemic manifestations of malaria including fever have been largely attributed to the released cytokines and toxins [Coban *et al.*, 2005; Ian *et al.*, 2006].

In addition, 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 release of proinflammatory cytokines that in turn induce cyclooxygenouse (COX)-2-upregulating prostaglandins

leading to induction of fever [Ralf, 2007; Peggy *et al.*, 2007]. Hemozoin has also been linked to induction of apoptosis in developing erythroid cells in the bone marrow, thereby causing anemia [Gordon *et al*., 2007; Lamikanra *et al.*, 2009]. The clinical manifestations of severe malaria (coma, severe anemia, metabolic acidosis, hypoglycemia, acute renal failure or acute pulmonary oedema) [WHO, 2015], caused especially by falciparum, are directly correlated with the induction of strong pro-inflammatory immune responses [Schofield and Grau, 2005].

1.6 Diagnosis of Malaria

Malaria must be diagnosed early and accurately to end up with an effective management of patients. Broadly, one can classify diagnosis of malaria in to clinical and parasitological diagnoses. Clinical diagnosis is based on the patient's symptoms and on signs at physical examination [WHO, 2015; CDC, 2016]. WHO recommends malaria should be suspected in any patient presenting with a history of fever or temperature >37.5 ^{0}C in the absence of other obvious causes [WHO, 2015]. Ethiopian FMoH advocates clinical diagnosis of malaria should be made in a patient who has fever or history of fever in the last 48 h and lives in malaria-endemic areas or has a history of travel within the last 30 days to malaria-endemic areas [FMoH, 2012].

All of suspected malaria should be confirmed with a parasitological diagnosis in all settings [WHO, 2015]. Light microscopy and RDTs are routinely employed methods for parasitological diagnosis of malaria. In 2005, single-species RDTs were introduced in Ethiopia. Years after, multi-species RDTs are being supplied by FMoH to health posts [FMoH, 2012]. PCR based method and serology tests are another parasitological

diagnostic means [CDC, 2016]. Recently developed rolling circle enhanced enzyme activity detection (REEAD) - and micromagnetic resonance reflaxometric (MMR) - test are also amenable to deployment in field conditions [Kumar and Renu, 2015].

1.7 Management of Malaria

1.7.1 Conventional Therapeutic Agents

Malaria can lead to fatal outcomes in only few days, thus treatment should be started as soon as possible. The main targets of current antimalarial chemotherapy are the asexual blood stages of the parasite, responsible for the malaria symptoms. Nowadays, the available antimalarial drugs can be grouped into five classes according to their chemical structure and biological activity: (i) Quinoline based antimalarials: 4-aminoquinolines (chloroquine, amodiaquine and piperaquine) and 8-aminoquinolines (premaquine and Tafenoquine) (ii) Arylaminoalcohols– Quinine, Mefloquine, Halofantrine and Lumefantrine (iii) Antifolate compounds (pyrimethamine , proguanil, dapsone, and sulfadoxine), (iv) Artemisinin and derivatives: first generation (dihdyroartemisinin, artesunate, arteether, and artemether) and second generation artemisinin (artemisone) and (v) Hydroxynapthoquinone– Atovaquone [Nicoletta *et al.,* 2015].

WHO recommends artemisinin-based combination therapies (ACT) for the treatment of uncomplicated malaria caused by falciparum parasite or by chloroquine resistant vivax, ovale, malariae and knowlsi. Quinine plus clindamycin is used for uncomplicated malaria treatment in the first trimester of pregnancy [WHO, 2015a]. In Ethiopia, Coartem^{TM} is the

recommended first-line drug for uncomplicated falciparum malaria and chloroquine for other species but oral quinine is used as a second-line drug [FMoH, 2012].

More recently, injectable artesunate becomes the treatment of choice for severe malaria worldwide in infants, children, lactating women and pregnant women in all trimester. After 24 h, the treatment should be completed with oral ACT [WHO, 2015a]. Quinidine plus doxycycline, tetracycline, or clindamycin is the preferred drug in the U.S. [CDC, 2013]. Coming to Ethiopia, injectable artesunate is the preferred drug and intramuscular artemether is an alternative drug. If these two drugs are not available, injectable quinine can be used to manage severe malaria [FMoH, 2012].

1.7.2 Drugs in the Pipeline

The development of strains resistant to commonly used drugs and lack of affordable new drugs are the limiting aspects in the fight against malaria. These factors trigger the continuing need of research for new classes of antimalarial agents, and a re-examination of the existing antimalarial drugs. Hence, dantrolene [Kang *et al*., 2005], antiadhesion adjunctive therapies like levamisole [Rowe *et al*., 2009], acriflavine [Dana *et al*., 2014] and acridiones such as WR249685 and T3.5 [Beteck *et al*., 2014] are under investigation. P218, 21A092 and others are also in preclinical studies [Bvgh, 2015]. The study done by Danny *et al.* (2015) identified for the first time that macrolide antibiotics inhibit Plasmodium species merozoite invasion in to erythrocytes *in vitro*.

A wonderful drug (named ddd107498) for malaria treatment and control has recently been developed in India and about to enter human clinical trial [Das and Dash, 2016]. SJ733

was tested successfully in preclinical studies [Medical Xpress, 2016] and now in human trial [St. Jude Children's Research Hospital, 2016]. MMV39048 has finished phase I trial [Chibale, 2016]. Moreover, CDRI 97/78, ACT451840, Sevuparin and GSK369796 are among drugs under phase I trial [Marco *et al.*, 2013; Janet *et al*., 2016].

The new organometallic drug ferroquine (SR97193) is undergoing Phase II clinical trial as combination therapy with artesunate [Anna *et al*., 2012]. Novartis currently has two new classes of antimalarial drug in Phase II clinical testing: KAE609 (Cipargamin) and KAF156. They have the potential to treat malaria and block transmission [Novartis, 2014]. Ozonides are proved to be useful substitutes for artemisinin. The first-generation OZ277 was developed and subsequently called arterolane. After a limited Phase III programme, the combination of arterolane and piperaquine has got approval under the trade name Synriam in India in 2013. The second-generation OZ439 (artefenomel) is now in Phase IIa studies [Reddy *et al*, 2013] and also being tested in a Phase IIb combination trial with piperaquine, and is about to be tested in another with ferroquine [Janet *et al*., 2016].

A second-generation artemisinin derivative, artemisone, a drug in Phase II trial, provides a single-dose cure when combined with mefloquine [Phillips *et al*., 2015]. Fosmidomycin is under combination therapy trial with piperaquine in phase II [Bvgh, 2015]. AQ-13 (a modified chloroquine) and DSM265 are other compounds in phase II [Martin, 2008; Bvgh, 2015; Janet *et al*., 2016].

Multiple novel combination therapies, including azithromycin-chloroquine [Chandra *et al*. 2013], pediatric pyronaridine-artesunate, pediatric dihydroartemisin in piperaquine [Bvgh, 2015] and trimethoprim-sulfamethoxazole [Janet *et al*., 2016] are in phase III trial. The

primaquine analog tafenoquine is has recently shown great promise in Phase II trial and is currently being tested in pivotal Phase III trial and has proven activity against hypnozoites [Rajapakse *et al*., 2015].

1.7.3 Traditional Medicine

Traditional medicine (TM) use varies among countries depending on a number of factors. In Singapore and the Republic of Korea where the conventional health-care system is quite well established, 76% and 86% of the respective populations still commonly use TMs. About 90% of general hospitals provide TM services for both outpatients and inpatients in China. Over 100 million Europeans are TM users [WHO, 2013b]. In developing countries, 80% of the people almost exclusively use TMs. Virtually, 80% of the population living in Ethiopia is dependent on traditional medicine which essentially involves the use of plants [Agbor *et al*., 2011; Asmare and Kesara, 2015].

More than 1,200 plants that possess antimalarial activities are reported worldwide [Rasoanaivo *et al*., 2011]. It is probable that some of them contain as yet undiscovered active constituents. For example, Ampelozyziphus amazonicus and Strychnopsis thouarsii were commonly used in malaria-endemic areas of Brazil and Madagascar, and their anti-sporozoite activities have been demonstrated [Anna *et al*., 2012].

Ethiopia is rich in a wide range of tropical habitats, remarkable biodiversity and use of traditional remedies for the treatment of various ailments [Deressa *et al*., 2010]. Studies conducted on several traditionally claimed Ethiopian medicinal plants, such as: *Calpurnia aurea* [Mebrahtu *et al*., 2013], *Croton macrostachyus* [Bantie *et al*., 2014], *Asparagus*

africanus [Yared *et al*., 2012], *Withania somnifera* [Dikasso *et al*., 2006], *Dodonaea angustifolia* [Mengiste *et al*., 2012] and *Phytolacca dodecandra* [Mequanint, 2014], confirmed their antimalarial activities.

1.7.4 Control Measures

Vector control is an effective measure of malaria prevention and elimination. Anopheles mosquitoes can be reduced via the use of insecticide-treated bed nets (ITNs) and indoor spraying of residual insecticides (IRSs) [CDC, 2015] even if the effectiveness is threatened by malaria mosquitoes developing resistance. However, the impact of pyrethroid resistance on ITN effectiveness is not yet well established [WHO, 2016]. Individual bite protection methods, insect repellents and protective clothing, have been used to reduce malaria transmission by mosquitoes [MAL WEST, 2016]. Jaleta *et al*. (2016) demonstrated that non-host (Chicken) volatiles can provide protection to mosquito-vectored diseases. Uniquely, some genetic abnormalities in RBCs confer resistance to malaria. This include, among others, deficiency in pyruvate kinase, polymorphic glycophorins, ovalocytosis, spherocytosis, elliptocytosis, sickle cell traits, thalassaemia traits and glucose-6-phosphate dehydrogenase (G6PD) deficiency [Kwiatkowski, 2005; Daily and Pardis, 2008; Hedrick, 2011].

Remarkable ways to interfere transmission are disrupting steroid hormone signaling in mosquitoes [Childs *et al*., 2016], use of transgenic mosquitoes [Taylor and John, 2009; Kokoza *et al*., 2010], using of paratransgenesis for delivering anti-Plasmodium effector molecules by genetically modified symbiotic microorganism of the insect [Hurwitz *et al.*, 2011] and/or transinfection of mosquitoes with symbiotic bacteria and fungi [Walker and

Luciano, 2011]. The use of transgenic procedure can improve the sterile insect technique (SIT) for anopheles induced by radiation [Catteruccia *et al*. 2009].

Intermittent Preventive Therapy (IPT) is another malaria control tool in pregnant women, infants and preschool children where transmission is seasonal [Bardají *et al.*, 2012; Meremikwu *et al.*, 2012]. Repeated Ivermectin Mass Drug Administration (MDA) could also help to control transmission [Alout *et al*., 2014]. Lastly, chemoprophylactic drugs can be used to prevent infection in travelers [Jacquerioz and Croft, 2009].

1.7.5 Malaria Vaccine

The emergence and spread of drug and insecticide resistance has been limiting the current malaria control measures, thus safe and effective vaccine is required to achieve the world malaria eradication programme. So far, three types of vaccine candidate have been intensively investigated: pre-erythrocytic vaccines; blood-stage vaccines; and transmission-blocking vaccines [Janet *et al*., 2016].

Pre-erythrocytic vaccines target the sporozoites and/or hepatic stages of the parasite. Some vaccines of this group are RTS,S/AS01 in post phase III trial [MVI, 2016], *P. falciparum* sporozoite vaccine (PfSPZ) in Phase II trials, cell-traversal protein for ookinetes and sporozoites (CelTOS) under phase Ia clinical trial and vaccine of chimpanzee adenovirus expressing CS (CSVAC) also in Phase I [Arama & Troye-Blomberg, 2014].

Several asexual blood stage vaccines, most target merozoite antigens, are in clinical researches. The commonly known candidates are AMA1 [Sagara *et al*., 2009a], MSP-1

[Ogutu *et al.*, 2009] and MSP-3 [Jepsen *et al*., 2013]. None has resulted in clear clinical protection, probably due to the highly polymorphic nature of the vaccine structures [Takala *et al*., 2009]. But efforts to enhance the efficacy with a novel adjuvants [Ellis *et al*., 2010; Sagara *et al*., 2009b] using viral vector prime-boost strategies [Hill *et al*., 2010] have been increasing. An exception is VAR2CSA, studied in the search of a vaccine for prevention of pregnancy-associated malaria [Rogerson *et al*., 2007]. Multigravidae who have acquired antibodies are indeed protected from pregnancy-associated malaria. Vaccine candidates directed against VAR2CSA are under development [Hviid, 2010]. Conversely, new non-polymorphic falciparum ligands, CX3CL1 binding proteins (CBP 1 and 2), are currently discovered by Hermand and his co-workers, which provides a new opportunity for innovative vaccination approaches [Hermand *et al.,* 2016].

Transmission-blocking vaccines (TBVs) target surface proteins expressed on gametocytes, zygotes and ookinetes to prevent parasite development in the mosquito midgut by specific host antibodies, complement proteins, and cytokines [Sutherland, 2009]. The leading vaccine candidates in this group include falciparum ookinete surface antigens *Pf*s25 [Arakawa *et al*., 2005] and *Pf*s28 and their vivax homologues *Pv*s25 and *Pv*s28 [Hisaeda *et al*., 2000; Arevalo-Herrera *et al*., 2005].

1.8 The Experimental Plant

Gardenia ternifolia Schumach. & Thonn. (Figure-3), commonly known as "Powder-bark or Wild gardenia" in English [Hyde *et al*., 2015], "Gambilo" in Amharic [Gebeyehu *et al*., 2014; Tebkew, 2015], "Kambeelloo" in oromifa [Yineger *et al*., 2008], "Kota" in Gumuz [Gebeyehu, 2016], "Gambela" in Sidama [Kewessa *et al.*, 2015] "Shigidida" in Gedeo

[Tekle, 2014], “Duwong” in Anuak [Lulekal *et al*., 2011], “Brmaiyta” in Konso [Addis *et al*., 2013] and “Bodut” in Menit [Giday *et al*., 2009], is a tree belonging to the Rubiaceae family [Hyde *et al*., 2015].

G. ternifolia is a shrub or small, spreading tree about 5 to 10m high [Burkil, 1997; Omosa *et al*., 2016]. The leaves are in whorls of 3 on short, rigid side branchlets, roughly hairy on both surfaces, but have an ovoid fruits. Whereas the flowers are white, turning yellow when fading, sweetly scented and solitary at the ends of the branchlets. Its flowering time is from September up to December. The worldwide distribution is from Limpopo, South Africa to Kenya and West Africa. The common habitats are wooded grassland, along streams, and termite mounds [Hyde *et al*., 2015].

In Africa, different parts of *G. ternifolia* are thought to be an effective folk medicine. The leaves are used to treat malaria [Alex and Alfred, 2012], arrow poisoning [Ibrahim *et al*., 2013], hypertension [Simplice *et al*., 2011], diabetes [Oulare *et al*., 2015], syphilis [Burkil, 1997] and skin diseases [Erakhrume *et al*., 2010]. The fruit is taken as a remedy for malaria [Kazhila, 2015] and stem is used to halt vomiting [Patricia *et al*., 2014]. Barks have an ethno-medical uses for ascites, leprosy, hepatitis, onchocerciasis, female infertility, wounds, malaria and sexually transmitted disease in elsewhere [Magassouba *et al*., 2007; Baldé *et al*., 2013; Tsobou *et al*., 2015]. The root is believed to have anti-rheumatismal [Baldé *et al*., 2015], anti-malarial and anti-jaundice [Maiga *et al*., 2005], anti-pain [Achigan-Dako *et al*., 2009], anti-epilepsy, anti-hypertensive [Moshi *et al*., 2003] and anti-constipation activity [Von Maydell, 1990]. Traditionally, *G. ternifolia* roots are of purgatives, stomachic, anthelmintic, diuretics, and emetics for ascites, and rickets [Yunana and Dahiru, 2015]. It is also used to manage kwashiorkor [Achigan-Dako *et al*.,

2009], livestock helminthosis [Djoueche *et al.,* 2011], black water fever and cough [Ainslie, 1937].

In Ethiopia, *G. ternifolia* is used medicinally by tribal healers as follows: fruits to treat hemorrhoid lesions [Yineger and Yewhalaw, 2007], dried roots and stem barks against evil eye [Gebeyehu *et al*., 2014], stem barks against malaria [Yineger *et al*., 2008; Giday *et al*., 2009] and to manage leg paralysis and tooth bleeding [Gebeyehu, 2016], barks for weight loss in children & stomachache in livestock [Kewessa *et al.*, 2015]. Leaf is also used in veterinary medicine to treat ulcerative lymphangitis [Tekle, 2014]. Despite non-existence of ethno-botanical study, root barks macerated in cold water are commonly administered orally for treating malaria by traditional medicine practitioners of the local community where the plant material was collected.

Phytochemical screening of the leaves revealed the presence of 15 secondary metabolites [Ibrahim *et al*., 2013; Awede *et. al*., 2015]. *G. ternifolia* fruit contains β-amyrin, sterol, fatty acids, oleanolic acid, and iridoid glucoside [ElGhazali *et al*., 1987]; Tannins [Maiga *et al*., 2005]. Preliminary pharmacognostic analyses carried out on leaves resulted in different values of moisture content, ash and acid-soluble ash. Fruit pulps are composed of crude fat, crude protein, crude fiber, ash, carbohydrate, vitamin C, magnesium, zinc, copper, manganese, iron, potassium, sodium and moisture content [John *et al*., 2016].

Coming to pharmacological investigations, the plant has various biological activities. Extracts from leaves have anti-sickling activities [Ngbolua *et al*., 2015]. The leaves surface exudates showed *in vitro* anti-plasmodial activity against strains of falciparum and larvicidal effects against *Ae. aegypti* larvae [Charles *et. al*., 2010]. The root reliefs sexual

asthenia and has purgative, cholagogue, and diuretic activities [Tsobou *et al*., 2015]. Other pharmacological uses are described under discussion part in this paper. In addition to medical importance, fruits have nutritional value in Africa [Alex and Alfred, 2012; John *et al*., 2016] including Ethiopia [Lulekal *et al*., 2011; Addis *et al*., 2013; Tebkew, 2015]. Moreover, its flowers are used as spices in Ethiopia [Gebeyehu, 2016].

Figure 3: Picture of *Gardenia ternifolia* Schumach & Thonn tree.

1.9 Rationale for the Study

Malaria continues to remain an important cause of illness & death in countries in which it is endemic. For that reason, malaria control requires an integrated approach including prevention and prompt treatment [WHO, 2015]. However, an increasing resistance towards the currently available antimalarial drugs and control measures is a challenge to combat malaria. Despite decades of intense research, no licensed malaria vaccines are available until now and many drugs found in the pipeline are not able to kill both gametocytes and hypnozoites.

If the past is an indicator, resistance to the life-saving antimalarial drugs will spread to Africa. Success and resistance are creating a malaria landscape that requires new tools and approaches [CDC, 2014]. Therefore, the world is in urgent need of new, safe and effective drugs. In discovering new antimalarial drugs, history has taught us how the knowledge of traditional medicines is valuable [Willcox and Bodeker, 2004; Adamsa *et al*., 2011; Liu *et al*., 2013]. Even though they faced widespread development of resistance and difficulties to afford in poor areas, currently used and potent antimalarial drugs such as artemether, chloroquine and quinine are obtained from plant sources.

Hence, it is imperative to focus on traditionally used medicinal plants for the discovery of possible new innovative antimalarial drug for the future. Since the previous study conducted by Charles *et al*., (2010) indicated that extract of *G. ternifolia* leave was endowed with antimalarial activity, the present study was aimed to explore *in vivo* activity of the plant. Thus, antimalarial effects by root bark extracts of the plant were investigated *in vivo* in an effort to contribute to the discovery of lead compound(s).

2. OBJECTIVES

2.1 General Objective

- ⇨ To study the *in vivo* anti-plasmodial activity of 80% methanol extract and solvent fractions of *G. ternifolia* Schumach. & Thonn. root barks against *P. berghei* in mice.

2.2 Specific Objectives

- ⇨ To determine acute toxicity of 80% methanol root bark extract of *G. ternifolia* in mice,
- ⇨ To assess suppressive effect of the root bark crude extract and solvent fractions *in vivo* using Peter's four-day suppressive test,
- ⇨ To investigate curative effect of the root bark crude extract *in vivo* using rane's test,
- ⇨ To evaluate preventive anti-plasmodial activities of the root bark crude extract *in vivo* using peter's repository test,
- ⇨ To perform preliminary phytochemical screening on the hydroalcoholic extract and solvent fractions of *G. ternifolia* root barks.

3. MATERIALS AND METHODS

3.1 Materials

3.1.1 Drug, Reagents and Chemicals

The following chemicals, reagents and drug were used in the present study: distilled water (Ethiopian Pharmaceutical Manufacturing, Ethiopia), absolute methanol (Carlo Erba, France), chloroform (Carlo Erba, France), n-butanol , normal saline (Fresenius Kabi, India), trisodium citrate (BDH Chemicals Ltd, England), oil immersion (Neolab, India), Geimsa stain (BDH Chemicals Ltd, England) and chloroquine phosphate (Ethiopian Pharmaceutical Manufacturing, Ethiopia). All materials used were of analytical grade.

3.1.2 Collection and Authentication of Plant Material

The root barks of *G. ternifolia* were collected in October, 2015 from its natural habitat around Mizan Teferi, South West Ethiopia, which is about 750 km away from Addis Ababa. Identification and authentication of the plant specimens were done by a taxonomist at the National Herbarium, College of Natural and Computational Sciences, Addis Ababa University, where a voucher specimen were kept there for future reference with voucher No. of DN 001/2015.

3.1.3 Experimental Animals

Healthy Swiss albino mice (22-31g), aged 4- 6 weeks, female for toxicity and male for the antimalarial activity study were purchased from Ethiopian Public Health Institute (EPHI), Addis Ababa, Ethiopia and maintained in the animal house at School of Pharmacy, Addis Ababa University. Animals were kept for a week for the purpose of acclimatization, and they were housed in stainless steel cage at room temperature with 12 h light-dark cycle and provided with a commercial food and water *ad libtum*. All procedures and techniques used in this study were in accordance with the Guide for care and use of laboratory animals [National Institute of Health Guidelines for Care and Use of Laboratory Animals, 1996].

3.1.4 Rodent Parasite

For *in vivo* antimalarial assays of plant extract, chloroquine sensitive strain of *Plasmodium berghei* (ANKA) was obtained from EPHI. The parasites were maintained by serial passage of blood from infected mice to non-infected once on weekly basis.

3.2 Methods

3.2.1 Extraction and Fractionation

Fresh root barks of the plant were thoroughly washed with tap water and cleaned with gauze to remove dirt and soil. After that, they were sliced manually into pieces, air-dried under shade and pulverized using a mortar and pestle. The air-dried powdered plant material (200 g) was cold macerated with 600ml of 80% methanol i.e. in the ratio of 1:3 in

an Erlenmeyer flask for three consecutive days at room temperature. The extraction process was facilitated with occasional stirring at 120 rpm by using a mechanical shaker (Bibby Scientific Limited Stone Staffo Reshire, UK). The resulting crude extract was separated from the marc with gauze and further filtered by Whatman filter paper No. 1 (Whatman®, England). This procedure was repeated two times by adding another fresh solvent into the marc. The filtrates were combined and the solvent was removed by evaporation under reduced pressure using rotary evaporator (Buchi Rota vapor R-200, Switzerland) with temperature not exceeding 40^0C. The extract was then freeze dried using lypholizer (Operan, Korea Vacuum Limited, Korea). Finally, the concentrated extract (19 % w/w) was transferred into vials and kept at −20°C until use.

The hydroalcoholic crude extract was subjected to fractionation using solvents with differing polarity, i.e., chloroform, n-butanol and water. For fractionation purpose, 550 g of plant material was macerated in a similar way described above. The crude extract was suspended in distilled water under a separatory funnel and the suspension was shaken with chloroform added. Two layers were formed and chloroform fraction was obtained by separation. The process was continued until the solution became clear. The aqueous layer was then shaken with n-butanol to obtain the butanol fraction. The n-butanol fraction was concentrated in rota vapor and both chloroform fraction and butanol fraction were then dried in dry oven with temperature not exceeding 40^0C. The remaining aqueous residue was also lyophilized to obtain the aqueous fraction. At last, the fractions were kept in an amber glass bottle and stored in a refrigerator (−20°C) for use at the time of treatment. The yield of butanol, chloroform and aqueous fractions was 28.77 gram (28.21 %), 26.80 gram (26.27%), and 46.43 gram (45.52%), respectively.

3.2.2 Acute Toxicity Study

Acute toxicity test was performed according to the Organization of Economic Co-operation and Development (OECD) 425 guideline for crude extract [OECD, 2008]. Three up to four hours fasted female Swiss albino mice of 4-6 weeks were used for the toxicity study. Following the period of fasting, the animals were weighed and 2000 mg/kg of the crude extract was administered by oral gavage. Food was then withheld for further 1 to 2 h. First, one female mouse was dosed and the mouse was observed over a period of 24 h. Since no death was observed, another four female mice were dosed and gross behavioral changes like loss of appetite, hair erection, lacrimation, tremors, convulsions, mortality and other signs of toxic manifestation were observed for 14 days after administration of the dose.

3.2.3 Grouping and Dosing of Animals

To evaluate the crude extract (GT) and solvent fractions (BF=butanol fraction, CF=chloroform fraction and AF=aqueous fraction) for antimalarial activity, infected mice were randomly divided into five groups of 6 mice per group. Three groups (I-III) were treated with the extracts at 200 mg/kg, 400 mg/kg and 600 mg/kg, respectively. The remaining two groups served as negative (CON) and positive controls and received 10 ml/kg of distilled water used for reconstitution and chloroquine (CQ) 25 mg/kg, respectively. Dose selection was made based on the result of acute toxicity and pilot study conducted on the extracts. Each dose was reconstituted by distilled water and administered via the oral route using gavage. Volume administered was calculated based on individual mouse body weight and maximum volume administered was 0.31 ml. Duration of

administration depended on the type of test performed and described along with the respective models.

3.2.4 Parasite Inoculation

Albino mice previously infected with *P. berghei* having different levels of parasitaemia were used as donors. The parasitaemia levels of donor mice were first determined from their blood collected by cutting a 0.5 to 1mm section from tail of the mice with scissor [David *et al.*, 2004; Adumanya *et al.*, 2014; Huang *et al*., 2015]. To infect the mice, the donor mouse with a rising parasitaemia of about 30-37% [Deressa *et al.*, 2010] was euthanized with ethyl ether and infected blood obtained by cardiac puncture was collected in a petri-dish containing 2% trisodium citrate (BDH chemicals, England) as anticoagulant. The blood was then diluted in normal saline so that the final suspension would contain about $1x10^7$ infected RBCs in every 0.2 ml suspension [Basir *et al*., 2012]. The dilution was made based on parasitaemia of the donor mice and RBC count of the normal mice [Waako *et al*., 2005; Huang *et al*., 2015] in such a way that 1 ml blood contains $5x\ 10^7$ infected erythrocytes. Therefore, each mouse used was infected intraperitoneally with 0.2 ml infected blood containing about $1x10^7$ *P. berghei* parasitized RBCs.

3.2.5 Four-Day Suppressive Test

Evaluation of schizonticidal activity of the root barks of *G. ternifolia* extract on early infection was carried out according to the method described by Peters (1975). Thirty male Swiss albino mice were infected and randomly assigned into five groups with six mice for

each as described in section 3.2.3. Treatment was started on the first day 2 h post-infection and continued daily for 4 days.

On the 5th day, blood was collected from the tail of each mouse using clean, non-greasy slides and thin films made accordingly and allowed to air-dry. The films were fixed with few drops of methanol, left for about 15 to 20 min to air-dry and stained with 10% Giemsa at a pH of 7.2 for 15 min. The stain was washed off with distilled water and slides left to air-dry before they were viewed under light microscope (Olympus N-120A, Philippines) using the oil immersion objective and parasitaemia examined microscopically with X100 objective for blood parasite suppression. Weight and rectal temperature were recorded for each mouse just before infection & daily throughout the experiment, while packed cell volume (PCV) was only recorded just before infection & at the end of the experiment.
Mice were followed for 28 days and the mean survival time was determined for each group of mice.

3.2.6 Rane's (Curative) Test

Evaluation of the curative potential of the root barks of *G. ternifolia* was performed according to the method described by Ryley & Peters (1970). Thirty mice were selected & intra-peritoneally inoculated with standard inoculums of 1x10^7 *P. berghei* IRBCs on the first day. Seventy two hours later, the animals were randomly divided into five groups (n=6) and treatment started with the first dose as described in section 3.2.3. Treatment continued once daily until the 7th day. At day 7, blood was then collected from the tail of each mouse and thin films were made as described in section 3.2.5. Weight, temperature & parasitemia level were recorded for each mouse just before the first dose and continued

daily until day 6. However, PCV was recorded only just before the first dose and at day 7 after infection. Thereafter, the mice were followed for 28 days and their survival time was recorded.

3.2.7 Prophylactic Test

The prophylactic activity of the extracts was tested using the residual infection procedure described by Peters (1965). Adult male mice were weighed, randomized into five groups (n=6) and treated for 4 days as described above in section 3.2.3. On the 5th day, all mice were infected with the plasmodium. Seventy two hour post-infection, blood smears were made from each mouse and parasitemia level was determined. Weight and temperature were recorded for each mouse just before inoculation and throughout the experiment, while PCV was recorded only just before inoculation and at the end of the experiment. Then after, the mice were followed for 28 days for their survival to calculate mean survival time.

3.2.8 Packed Cell Volume Measurement

PCV was measured to predict the effectiveness of the crude extract and fractions in preventing hemolysis caused by increment of parasitemia associated with malaria [Dikasso *et al*., 2006]. Heparinized capillary tubes were used for the collection of blood from tail of the mice. The capillary tubes were filled to 3/4th of their height with blood & sealed with sealing clay at their dry end. The tubes were then placed on a micro-hematocrit centrifuge (Centurion Scientific, UK) with the sealed end facing the periphery and centrifuged at 11,000 rpm for 5 min. Finally, the tubes were taken out of the

centrifuge and PCV was determined using the standard hematocrit reader (Hawksley and Sons, England) [Gilmour and Sykes, 1951]. 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]:

$$\text{PCV} = \frac{\text{Volume of erythrocytes in agiven volume of blood}}{\text{Total blood volume examined}} \text{X } 100$$

3.2.9 Parasitemia and Survival Time Measurement

The percentage parasitemia was obtained by counting the number of parasitized red blood cells (PRBC) out of the total erythrocytes in random fields of the microscope. Two stained slides for each mouse were examined [Bantie *et al*., 2014]. Six fields [Mebrahtu *et al*., 2013] approximately 200-500 cells were counted for each slide, average was taken and percentage parasitemia (PP) was determined using the following formula [Adumanya *et al*., 2014].

$$\text{PP} = \frac{\text{Number of PRBC}}{\text{Total number of RBC}} \text{X } 100$$

Percent parasitemia suppression of the extract and fractions was compared with respect to the controls. Percent parasitemia suppression was calculated using the formula [Adumanya *et al*., 2014]:

$$\begin{matrix}\text{Average \% of} \\ \text{Parasitemia} \\ \text{Suppression}\end{matrix} = \frac{\text{Parasitemia in negative control} - \text{Parasitemia in treatment group}}{\text{Parasitemia in negative control}} \text{X } 100$$

Mice were monitored daily and the number of days from the time of inoculation up to death was recorded for each mouse in treatment and control groups throughout the follow up period at all models. The mean survival time (MST) for each group was calculated as follows [Mengiste *et al*., 2012].

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

3.2.10 Body Weight and Rectal Temperature Measurement

Each mouse in a group was weighed using sensitive digital weighing balance (Mettler Toledo, Switzerland) and rectal temperature was measured using digital rectal thermometer (G.S.T Corporation, New Delhi, India). The percentage changes of their mean values that occurred before and after treatment were then calculated.

3.2.11 Preliminary Phytochemical Analysis

Both 80% methanol crude extract and fractions were screened for the presence of different phytochemical constituents following standard procedures.

Test for alkaloids

Five hundred milligrams of the extract was treated in a test tube with 10 ml of 1% HCl for 30 minutes in a water bath and then filtered through cotton in to a test tube. Small portion of the extract was transferred into two test tubes and to one of the test tubes, five drops of Mayer's reagent and to the second five drops of Wagner's reagent were added and the

formation of whitish opalescence (Mayer's reagent) or reddish brown precipitate (Wagner's reagent) was inspected [Debela, 2002].

Test for anthocyanins

Two ml of the extract is added to 2 ml of 2N HCl & NH_3, the appearance of pink red turns blue violet indicates presence of Anthocyanin [Godghate and Rajaram, 2013].

Test for flavonoids

The dried crude extract and fractions were dissolved in a solvent. To 2 ml of the 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].

Test for terpenoids

Five ml of the crude extract and fractions dissolved in the 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].

Test for saponins

Half gram of the sample in 10 ml of distilled water was shaken in a test tube and the formation of honeycomb froth that persists for 30 minutes was considered as positive for saponins [Debela, 2002].

Test for phenols

To 2 ml of filtered solutions of the samples with an appropriate solvent, freshly prepared three drops of a mixture of 1 ml of 1% $FeCl_3$ and 1 ml of 1% $KFe(CN)_6$ were added and the formation of a green blue color was examined [Yadav *et al*., 2010].

Tests for glycosides

Two 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].

Test for steroids

One gram of the each extract was weighed and placed in a test tube. This was dissolved in 2 ml of acetic anhydride, followed by the addition of 4 drops of chloroform. Two drops of concentrated sulphuric acid were then added by means of a pipette at the side of the test tube. The development of a brownish ring at the interface of the two liquids and the

appearance of violet color in the supernatant layer were indicative of the presence of steroids [Yadav *et al*., 2010; Ranjit *et al.*, 2012].

Test for tannins

About 2 ml of each 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].

3.3 Data Analysis

Data were analyzed using windows statistical package for social science (SPSS), version 20. Results of the study are expressed as mean $\pm$ standard error of mean (SEM). One way analysis of variance (ANOVA) followed by Tukey post Hoc test was used to compare the levels of parasitemia, survival time, and changes in body weight, PCV and rectal temperature of the *P. berghei* infected mice between the control and extract-treated groups. In addition, two way repeated measures ANOVA was also used to analyze the development of parasitemia across days of treatment in rane's test. Probability values > 0.05 were considered not statistically significant.

4. RESULTS

4.1 Acute Toxicity Study

The acute toxicity study indicated that the extract caused no mortality in dose of 2000 mg/kg within the first 24 h. Physical and behavioral observations of the experimental mice also revealed no visible signs of overt toxicity; like loss of appetite, tremors, hair erection, salivation and diarrhea. The mice were physically active and fed and drunk as that of the control groups administered with the vehicle within the observation period of 14 days. This suggests that LD_{50} of the extract is greater than 2000 mg/kg.

4.2 Chemosuppressive Effect of the Plant

Results of the four-day suppressive test of the crude extract and solvent fractions at different dose levels on parasitaemia and survival time in mice infected with *P. berghei* are summarized in Table 1. The chemosuppresive study revealed that the extract as well as fractions exhibited a significant inhibition of parasitemia ($p<0.001$) compared to negative controls (CON). However, the effect produced was smaller than that of the standard. The reference drug, CQ, cleared the parasite to undetectable level. Doses among the extract and two fractions (BF, CF) showed a significant difference ($p<0.001$) in chemosuppresive activity when compared to each other. Comparable suppression was also seen when both the lower dose and middle doses of AF were compared, but the higher dose displayed a significantly greater suppression compared to both the lower ($p<0.001$) and middle dose ($p<0.05$). The BF produced the highest parasitemia inhibition than CF but lower than that of GT. Of these, AF showed the lowest chemo-suppression.

Table 1: Antimalarial activity of 80% methanol extract and solvent fractions of *Gardenia ternifolia* root barks against *Plasmodium berghei* in four-day suppressive test.

Group	% Parasitemia	% Suppression	Survival Time
CON	53.95±0. 71	0.00	6.33±0.21
GT200	36.38±0.83	32.58^{a3b3d3e3}	8.50±0.43^{a2b3d3e3}
GT400	28.58±0.65	47.02 ^{a3b3e3}	11.17±0.31^{a3b3e3}
GT600	21.98±0.72	59.25 ^{a3b3}	13.50±0.43^{a3b3}
CQ25	0.00±0.00	100.00^{a3}	28.00±0.00^{a3}
CON	48.64±0.94	0.00	6.50±0.22
BF200	33.61±0.90	30.89^{a3b3d3e3}	8.83±0.60^{a1b3e3}
BF400	27.93±0.51	42.45 ^{a3b3e3}	10.50±0.56^{a3b3e1}
BF600	23.68±0.59	51.33 ^{a3b3}	12.83±0.60^{a3b3}
CQ25	0.00±0.00	100.00^{a3}	28.00±0.00^{a3}
CON	50.49±0.60	0.00	6.33±0.21
CF200	38.12±0.68	24.51^{a3b3d3e3}	7.17±0.31^{b3e3}
CF400	34.77±0.63	31.13^{a3b3e3}	8.00±0.45^{a1b3e2}
CF600	29.93±0.35	40.73^{a3b3}	10.00±0.45^{a3b3}
CQ25	0.00±0.00	100.00^{a3}	28.00±0.00^{a3}
CON	46.86±1.20	0.00	6.67±0.33
AF200	39.99±1.19	14.67^{a3b3e3}	7.00±0.37^{b3}
AF400	37.92±0.57	19.09 ^{a3b3e1}	7.50±0.22^{b3}
AF600	34.80±0.68	25.75 ^{a3b3}	7.83±0.40^{b3}
CQ25	0.00±0.00	100.00^{a3}	28.00±0.00^{a3}

Data are expressed as mean±SEM (n=6); a, compared to CON; b, to CQ 25; c, to 200 mg/kg; d, to 400 mg/kg; e, 600 mg/kg: $^{1}p<0.05$, $^{2}p<0.01$, $^{3}p<0.001$; CON, negative control, received distilled water 10 ml/kg; GT = crude extract of *Gardenia ternifolia*, BF = n-butanol fraction, CF = Chloroform fraction, AF = aqueous fraction and CQ = chloroquine (positive control). Numbers refer to dose in mg/kg.

The mean survival time of mice treated with the crude extract and fractions was increased in a dose dependent manner (Table 1). All doses of the crude extract were capable of significantly increasing survival time compared to negative control. Survival time was also significantly prolonged by the higher ($p<0.001$) and middle doses ($p<0.01$) of CF, but all

doses of BF was significantly ($p<0.001$ for middle and larger dose, $p<0.05$ for lower dose) improved survival time compared to CON mice. CQ-treated groups had a drastically longer ($p<0.001$) MST when compared to the respective control and treatment groups. Significant difference in survival time among doses of the crude extract was noted. With fractions, however, only the higher dose had a significantly greater reduction than the lower and middle doses in both BF and CF. By contrast, suppression was comparable between the different doses of the AF as well as between the doses and the negative control.

The protection provided by crude extract and fraction on parasite-induced body weight loss is shown in Table 2. The crude extract appeared to avert loss of weight associated with infection in a dose-dependent manner compared to controls ($p<0.05$, $p<0.01$, & $p<0.001$ with increasing dose), although no apparent difference was observed between the middle and higher doses of the extract. The effect produced with the fractions was not consistent. The fractions ranged from no effect for the AF through effect with the higher dose for the CF ($p<0.001$) to effect with the higher ($p<0.001$) & middle dose ($p<0.01$) for the BF. Comparison of doses of the different fraction revealed that it was the higher dose of the BF that produced a significant reduction ($p<0.05$) compared to the lower dose. It is of note that the standard provided a significant protection compared to all doses of the crude as well as the fractions.

Table 2: Body weight and rectal temperature of infected mice treated with crude extract and solvent fractions of *Garedenia ternifolia* root barks in four-day suppressive test.

Group	Weight (gram)			Temperature (^{0}c)		
Dose/kg	D_0	D_4	% Change	D_0	D_4	% Change
CON10ml	25.76±1.17	21.65±0.94	-13.55	35.85±0.38	33.15±0.33	-7.51
GT200mg	26.78±1.34	24.73±1.41	-7.80^{a1b3e1}	36.65±0.28	35.10±0.15	-4.21^{a1b3e2}
GT400mg	27.37±1.01	26.37±1.16	-3.67^{a2b3}	36.50±0.25	35.58±0.17	-2.50^{a3b2}
GT600mg	28.51±0.56	28.46±0.81	-0.20^{a3b2}	36.60±0.23	36.30±0.48	-0.81^{a3}
CQ25mg	28.02±1.11	30.95±1.10	10.55^{a3}	36.32±0.34	36.82±0.25	1.40^{a3}
CON10ml	27.78±0.49	24.04±0.53	-11.14	36.75±0.31	34.25±0.26	-6.80
BF200mg	28.91±0.48	26.81±0.42	-7.25^{b3e1}	37.35±0.21	35.53±0.16	-4.85^{b3e1}
BF400mg	29.24±0.31	28.09±0.24	-3.91^{a2b3}	37.45±0.21	36.02±0.30	-3.83^{a2b3}
BF600mg	27.93±0.48	27.77±0.60	-0.61^{a3b3}	36.90±0.24	36.00±0.33	-2.45^{a3b3}
CQ25mg	27.07±0.70	30.23±0.99	11.60^{a3}	36.80±0.43	37.45±0.21	1.80^{a3}
CON10ml	26.33±1.15	22.44±0.97	-12.20	36.30±0.53	33.55±0.33	-7.54
CF200mg	26.85±0.98	24.15±1.24	-10.29^{b3}	36.53±0.40	34.30±0.48	-6.12^{b3}
CF400mg	27.60±0.62	25.43±0.67	-7.91^{b3}	36.70±0.33	35.05±0.43	-4.51^{a1b3}
CF600mg	28.41±0.63	27.20±0.60	-4.27^{a3b3}	36.80±0.41	35.55±0.40	-3.39^{a2b3}
CQ25mg	28.10±0.82	31.39±0.76	11.83^{a3}	36.70±0.40	37.28±0.26	1.65^{a3}
CON10ml	26.93±0.73	23.65±0.90	-10.72	36.45±0.25	33.10±0.50	-9.21
AF200mg	27.74±0.85	25.04±0.91	-9.82^{b3}	36.90±0.31	33.80±0.34	-8.41^{b3}
AF400mg	26.92±0.79	24.60±0.76	-8.61^{b3}	36.35±0.36	33.70±0.34	-7.29^{b3}
AF600mg	28.61±0.61	26.34±0.65	-7.96^{b3}	37.08±0.40	34.52±0.43	-6.92^{b3}
CQ25mg	28.32±0.63	31.21±0.68	10.22^{a3}	37.00±0.35	37.90±0.23	2.45^{a3}

Data are expressed as mean ± SEM (n=6); a, compared to CON; b, compared to CQ 25 mg/kg; c, compared to 200 mg/kg; d, compared to 400 mg/kg; e, compared to 600 mg/kg; $^1p< 0.05$; $^2p<0.01$, $^3p<0.001$; CON, negative control; GT = crude extract, BF = n-butanol fraction, CF = chloroform fraction, AF = aqueous fraction and CQ = chloroquine (positive control); D_0 = pre-Rx value on day zero, D_4 = post-Rx value on day four.

As indicated in Table 2, the extract and the two fractions were able to significantly prevent the decrease in rectal temperature caused by *P. berghei* infection depending on the dose. Analysis of rectal temperature revealed that 80% methanol extract caused a significant ($p<0.05$ for GT200, $p<0.001$ for GT400 and GT600) attenuation of reduction in

temperature compared to vehicle treated group. Both the lower ($p<0.001$) and middle ($p<0.01$) doses of the extract had less effect compared to the standard, while the higher dose had a comparable effect. In addition, the higher dose also produced a significant protection ($p<0.01$) compared to the lower dose. Whilst no apparent difference was observed between the AF and vehicle treated animals, middle and higher doses of both BF ($p<0.01$ & $p<0.001$) and CF ($p<0.05$ & $p<0.01$) protected a significant diminution of temperature compared to controls. On the other hand, the effect attained by chloroquine was higher than of the activity of all fractions ($p<0.001$).

Table 3 shows that dose-related attenuation of anemia was obtained by all doses of the crude extract and fractions, but no detectable changes were noted with any dose of AF as well as lower dose of CF. GT400, GT600, BF400, BF600 and CF600 treated groups showed significantly reduced PCV in a similar fashion ($p<0.001$) compared to their CON mice although the effects were lower than attained by CQ. Significant difference had also been detected when GT200 ($p<0.01$), BF200 ($p<0.05$) and CF400 ($p<0.01$) compared to negative controls. There was a measurable significance in preventing reduction of PCV when the lower doses of GT ($p<0.05$), BF ($p<0.01$) and CF ($p<0.001$) were compared to their own higher doses, respectively. The rank order of protection in anemia was hydroalcoholic extract > butanol fraction > chloroform fraction > aqueous fraction.

Table 3: Effects of crude extract and fractions of *Gardenia ternifolia* root barks on Packed cell volume of infected mice in four-day suppressive test.

Group	Packed Cell Volume		
	D_0	D_4	% change
CON	59.91±2.51	55.11±2.48	-7.99
GT200	64.86±1.79	62.57±1.90	-3.56^{a2b3e1}
GT400	61.98±2.40	61.22±2.63	-1.27^{a3b2}
GT600	56.96±1.43	57.00±1.40	0.09^{a3b1}
CQ25	59.77±1.75	62.19±1.89	4.05^{a3}
CON	63.68±2.72	59.11±2.73	-7.23
BF200	63.10±1.16	60.58±1.07	-3.97^{a1b3e2}
BF400	61.09±1.95	60.11±1.99	-1.58^{a3b3}
BF600	64.02±2.96	63.91±2.94	-0.17^{a3b3}
CQ25	65.21±1.68	68.23±1.72	4.64^{a3}
CON	65.15±1.91	60.26±1.81	-7.51
CF200	62.11±1.44	58.05±1.02	-6.47^{b3e3}
CF400	58.97±1.52	56.62±1.47	-3.97^{a2b3}
CF600	62.13±1.67	60.95±1.77	-1.93^{a3b3}
CQ25	59.82±1.74	62.57±2.15	4.52^{a3}
CON	64.14±3.90	59.55±3.78	-7.22
AF200	62.66±2.50	58.41±2.29	-6.77^{b3}
AF400	64.71±2.30	60.86±2.07	-5.91^{b3}
AF600	58.71±1.29	55.50±1.25	-5.46^{b3}
CQ25	60.48±2.60	63.24±2.44	4.65^{a3}

Data are expressed as mean ± SEM (n=6); a, compared to negative control; b, compared to CQ 25 mg/kg; c, compared to 200 mg/kg; d, compared to 400 mg/kg; e, compared to 600 mg/kg; $^{1}p< 0.05$; $^{2}p<0.01$, $^{3}p<0.001$; CON, negative control (received 10 ml/kg distilled water); CQ, positive control (chloroquine); GT, *G. ternifolia;* D_0 = pre-treatment value on day zero, D_4 = post-treatment value on day four.

4.3 Curative Effect of the Plant

The crude extract evaluated for curative effect reduced ($p<0.001$) parasitemia by 36.29, 52.11 and 63.22% for GT200, GT400 and GT600, respectively, compared to CON mice

(Table 4). In addition, statistically significant ($p<0.001$) differences were observed in suppressing parasitemia when doses of the extract compared to each other. Decline in parasitemia level caused by the standard was also significantly ($p<0.001$) different when compared with control and all treated groups. Survival period was altered significantly by the lower ($p<0.01$) and two higher ($p<0.001$) doses of test sample relative to negative control. Statistically significant ($p<0.001$) prolonged survival time was as well found with regard to comparison between extract doses. However, the increase achieved with the extract did not match with that of chloroquine.

Table 4: Antimalarial activity of the hydroalcoholic extract of *Gardenia ternifolia* root barks against *Plasmodium berghei* in rane's test.

Group	% Parasitemia	% Inhibition	Survival Time
CON	38.12±0.75	0.00	7.17±0.17
GT200	24.29±0.39	36.29 ^{a3b3d3e3}	9.00±0.26^{a2b3d3e3}
GT400	18.26±0.61	52.11 ^{a3b3e3}	11.50±0.43^{a3b3e3}
GT600	14.02±0.81	63.22 ^{a3b3}	14.00±0.58^{a3b3}
CQ25	0.00±0.00	100.00^{a3}	28.00±0.00^{a3}

Data are expressed as mean ± SEM (n=6); a, compared to CON; b, compared to CQ 25 mg/kg; c, compared to 200 mg/kg; d, compared to 400 mg/kg; e, compared to 600 mg/kg; $^{1}p< 0.05$; $^{2}p<0.01$, $^{3}p<0.001$; CON, negative control (received 10 ml/kg distilled water); CQ, chloroquine (positive control); GT = crude extract of *G. ternifolia*. Numbers refer to dose in mg/kg.

Two-way repeated measures ANOVA analysis of parasitemia across days of treatment showed a statistically significant ($p<0.001$) difference in parasitemia level between the three doses of the extract and control groups. Observation of activity across days of treatment revealed that parasitemia level was increased on day 4 (after the first dose) in the presence of placebo and extract but decreased in case of CQ-treated group as presented in Figure 4. After second dose administration, there was a gradual decrement of parasitemia

during the course of treatment in all treated groups in contrast to the negative control. On day 7, CQ completely eradicated the parasite to untraceable level.

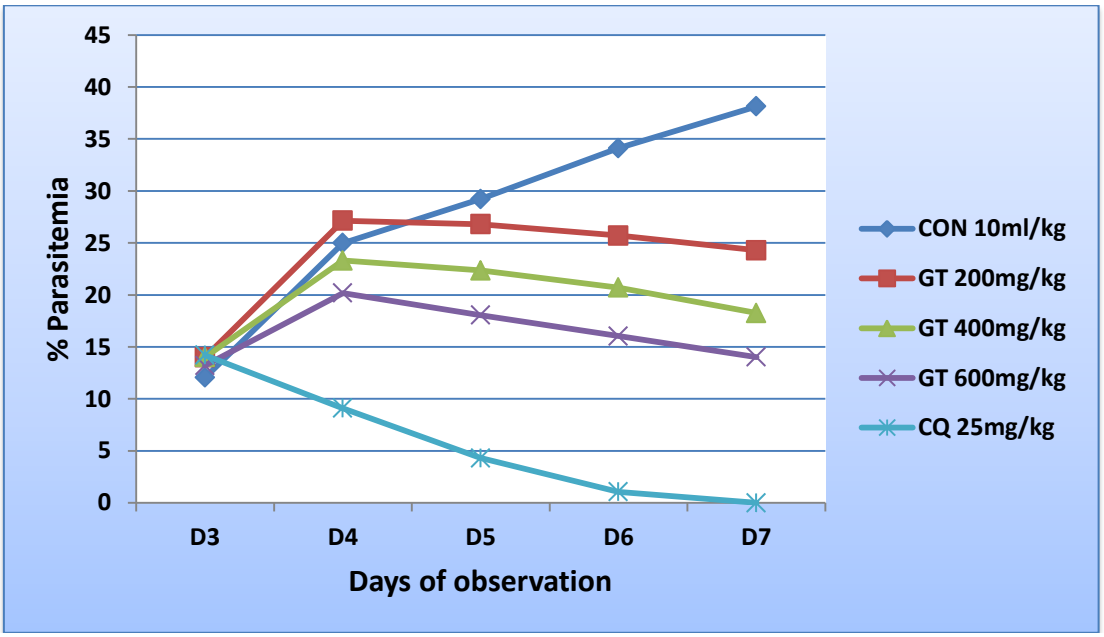

Figure 4: Comparison of parasitaemia chemosuppression among crude extract, chloroquine and vehicle-treated groups from Day 3 until Day 7 after infection in the rane's test. Data are expressed as mean ±SEM; CON, negative control; GT, crude extract; CQ, chloroquine.

The extract exhibited a dose-related preventive effect in body weight and rectal temperature in infected mice as depicted in Table 5. The weight of groups taking the standard drug ($p<0.001$) and the extract ($p<0.05$ for GT200mg and $p<0.001$ for GT400mg and GT600mg) were found to be significantly improved as compared to the negative control although enhanced effect was seen in CQ-treated group. Among extract doses, GT600mg had significant effect ($p<0.01$) in weight improvement relative to GT200mg.

Like in the four-day suppressive test, the reduction in rectal temperature was ablated with increasing doses of the extract (Table 5). CQ and all dose levels of GT significantly ($p<0.001$) prevented the reduction in rectal temperature compared to the CON group even though the effect by CQ was much higher. As comparison within doses of test sample is concerned, GT600mg significantly ($p<0.001$) ameliorated reduction in rectal temperature with respect to GT200mg. Significant difference was also observed by CQ in protecting

the lowering of rectal temperature in relation to all dose levels of the GT-treated groups ($p<0.01$ for GT600mg, $p<0.001$ for GT400mg and GT200mg).

Table 5: Body weight and rectal temperature of malaria infected mice before and after treatement with 80% methanolic extract of root barks of *Gardenia ternifolia* in rane's test.

Group	Weight (gram)			Temperature (^{0}c)		
Dose/kg	D_3	D_7	% Change	D_3	D_7	% Change
CON10ml	25.64±1.20	22.19±1.15	-13.54	36.40±0.25	33.10±0.26	-9.05
GT200mg	24.95±1.14	23.19±0.95	-6.90^{a2b3e2}	35.90±0.22	34.45±0.20	-4.04^{a3b3e3}
GT400mg	25.56±1.07	24.82±1.22	-3.02^{a3b3}	35.87±0.24	35.10±0.35	-2.14^{a3b3}
GT600mg	26.76±0.82	26.72±0.69	-0.09^{a3b3}	36.00±0.23	35.75±0.25	-0.69^{a3b2}
CQ25mg	25.79±1.04	28.85±1.22	11.86^{a3}	36.07±0.25	36.85±0.17	2.18^{a3}

Data are expressed as mean ± SEM (n=6); a, compared to CON; b, compared to CQ 25mg/kg; c, compared to 200mg/kg; d, compared to 400mg/kg; e, compared to 600mg/kg; $^1p< 0.05$; $^2p<0.01$, $^3p<0.001$; CON, negative control; CQ, chloroquine (positive control); GT, Crude extract; D_3 = pre-Rx value on day three, D_7 = post-Rx value on day seven.

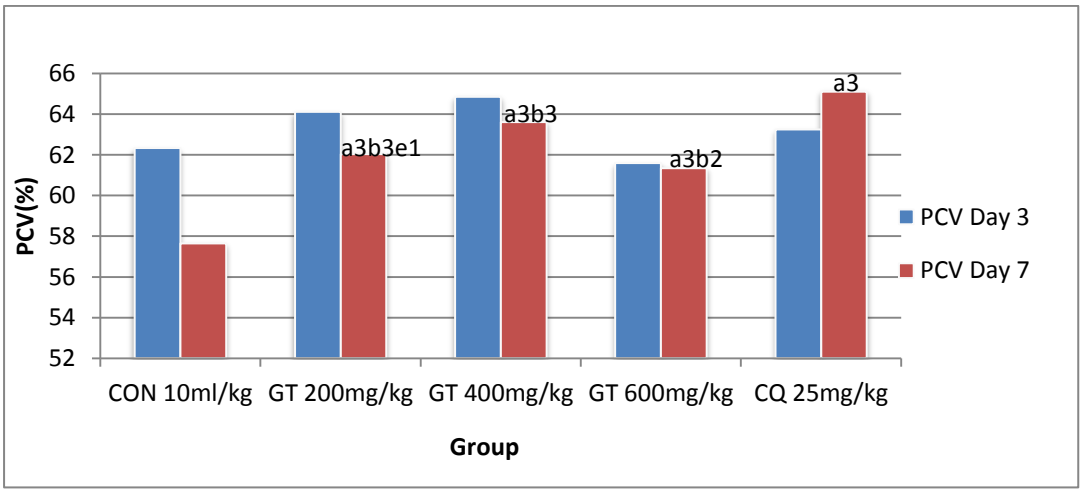

Figure 5: Comparison of packed cell volume in malaria infected mice treated with crude extract, chloroquine and placebo in the rane's test. Data are expressed as mean ± SEM (n=6); a, compared to negative control; b, compared to CQ 25 mg/kg; c, compared to 200 mg/kg; d, compared to 400 mg/kg; e, compared to 600 mg/kg; $^1p< 0.05$; $^2p<0.01$, $^3p<0.001$; CON, negative control; CQ, chloroquine (positive control); GT, crude extract.

Analysis in PCV reduction among the groups taking extract and vehicle revealed that the extract was able to prevent PCV reduction significantly ($p<0.001$) compared to the CON group, with high effect by CQ (Figure 5). Significant difference was also obtained in

protecting PCV reduction by CQ relative to GT-treated groups ($p<0.01$ for 600mg/kg, $p<0.001$ for 400 and 200 mg/kg). A value of $p<0.05$ was attained when the larger dose of GT was compared to its smaller dose counterpart.

4.4 Prophylactic Effect of the Plant

All doses of the crude extract and chloroquine suppressed parasite load significantly ($p<0.001$) when compared to control (Table 6). Maximum suppression 84.83% ($p<0.001$) of parasitemia was noted by the positive control compared to all extract doses, although complete eradication was not achieved. Comparison of suppression among the extract doses themselves indicated that the larger dose was having significant suppression when compared to the lower ($p<0.001$) and middle ($p<0.05$) dose. Survival time of the infected mice pre-treated with the extract on prophylactic study indicated that only GT600 was capable of prolonging survival time compared to control with $p<0.01$ (Table 6). Comparison among doses of the extract showed that the maximum dose significantly ($p<0.05$) prolonged MST when compared to the lowest dose.

Table 6: Parasitemia, percentage suppression and survival time of malaria infected mice treated with hydroalcoholic extract of *Gardenia ternifolia* root barks in repository test.

Group	% Parasitemia	% Suppression	Survival Time
CON	21.49±0.70	0.00	6.67±0.21
GT200	16.24±0.57	24.43^{a3b3e3}	7.33±0.21^{b3e1}
GT400	15.25±0.49	29.04 ^{a3b3e1}	8.50±0.84^{b3}
GT600	13.36±0.33	37.83 ^{a3b3}	9.50±0.56^{a2b3}
CQ25	3.26±0.38	84.83^{a3}	16.00±0.77^{a3}

Data are expressed as mean ± SEM (n=6); a, compared to CON; b, compared to CQ 25 mg/kg; c, compared to 200 mg/kg; d, compared to 400 mg/kg; e, compared to 600 mg/kg; $^{1}p< 0.05$; $^{2}p<0.01$, $^{3}p<0.001$; CON, negative control; CQ, chloroquine (positive control); GT, Crude extract.

Positive control and higher dose of extract showed a significant ($p<0.001$ for CQ and $p<0.01$ for GT) protective effect in body weight reduction compared to CON (Table 7). CQ-treated group considerably prevented weight reduction in comparison to the middle ($p<0.01$) and lower ($p<0.001$) doses of GT.

Both the standard and higher dose of GT appreciably ($p<0.001$) prevented rectal temperature reduction compared to control. However, CQ had significant capacity as compared to the middle ($p<0.01$) and lower ($p<0.001$) doses of GT. Comparison between doses of extract revealed that GT600mg was better ($p<0.05$) in improving rectal temperature than GT200mg as recorded in Table 7.

Table 7: Body weight and rectal temperature of *Plasmodium berghei* infected mice treated with crude extract of *Gardenia ternifolia* root barks in repository test.

Group	Weight (gram)			Temperature (^{0}c)		
Dose/kg	D_0	D_7	% Change	D_0	D_7	% Change
CON10ml	27.82±0.86	26.13±1.02	-6.19	36.90±0.36	34.98±0.45	-5.20
GT200mg	27.68±0.93	26.28±0.86	-5.01^{b3}	36.75±0.29	35.25±0.29	-4.08^{b3e1}
GT400mg	28.58±0.49	27.42±0.40	-4.02^{b2}	37.20±0.22	35.98±0.27	-3.27^{b2}
GT600mg	28.55±0.76	27.88±0.80	-2.36^{a2}	36.80±0.24	36.17±0.38	-1.73^{a3}
CQ25mg	28.54±0.64	28.63±0.76	0.28^{a3}	37.00±0.31	36.85±0.36	-0.41^{a3}

Data are expressed as mean ± SEM (n=6); a, compared to CON; b, compared to CQ 25 mg/kg; c, compared to 200 mg/kg; d, compared to 400 mg/kg; e, compared to 600 mg/kg; $^1p< 0.05$; $^2p<0.01$, $^3p<0.001$; CON, negative control; CQ, chloroquine (positive control); GT, crude extract of *G. ternifolia;* D_0 = pre-inoculated value on day three, D_7 = post-inoculated value on day seven.

Multiple comparison among PCV reductions (Figure 6) indicated that the larger dose ($p<0.001$) as well as the middle dose ($p<0.01$) of GT and the standard ($p <0.001$) were able to significantly attenuate PCV reduction compared to the control. The standard also had significant difference compared to 200 mg/kg ($p<0.001$) and 400 mg/kg ($p<0.05$) of

GT. Comparison between doses of GT showed that 600 mg/kg was better than 200 mg/kg in protecting PCV reduction ($p<0.01$).

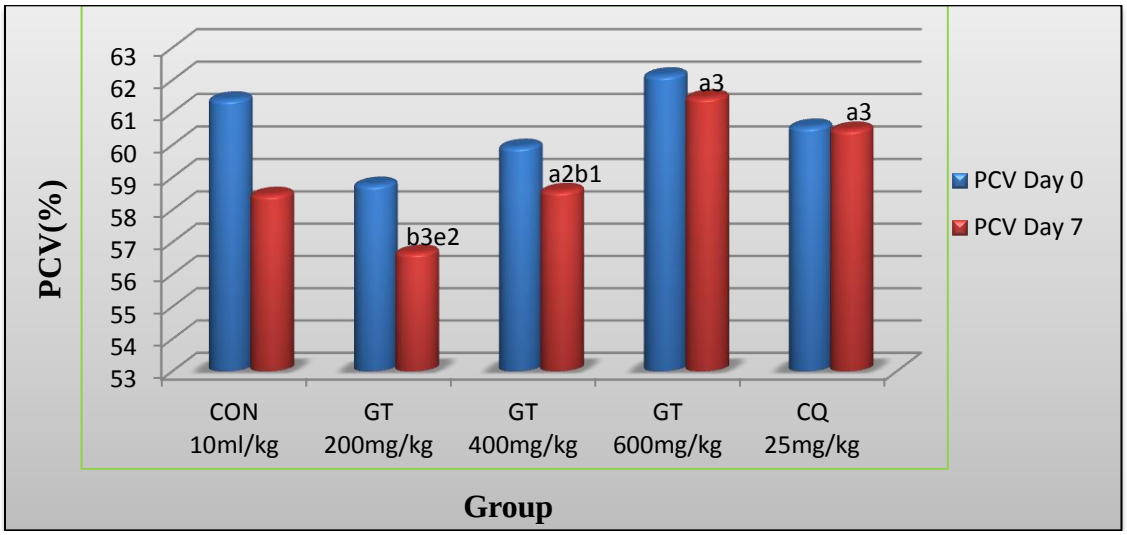

Figure 6: Results on packed cell volume of *Plasmodium berghei* infected mice treated with the 80% methanolic extract of *Gardenia ternifolia* root barks in the prophylactic test. Data are expressed as mean ± SEM (n=6); a, compared to CON; b, compared to CQ 25 mg/kg; c, compared to 200 mg/kg; d, compared to 400 mg/kg; e, compared to 600 mg/kg; $^{1}p< 0.05$; $^{2}p<0.01$, $^{3}p<0.001$, D_0 = pre-inoculated value on day three, D_7 = post-inoculated value on day seven.

4.5 Preliminary Phytochemical Analysis

Phytochemical screening of the hydroalcoholic extract and solvent fractions of *G. ternifolia* root barks revealed the presence of tannins, alkaloids, anthocyanins, terpenoids, saponins, phenols, steroids, and flavonoids in crude extract while only terpenoids were absent from butanol fraction, and chloroform fraction was not represented by anthocyanins and phenols (Table 8). Only saponins and anthocyanins were detected in the aqueous fraction.

Table 8: Preliminary phytochemical screening of the hydroalcoholic extract and solvent fractions of *Gardenia ternifolia* root barks.

Phytoconstituents	Crude Extract	Butanol Fraction	Chloroform Fraction	Aqueous Fraction
Alkaloids	+	+	+	-
Anthocyanins	+	+	-	+
Flavonoids	+	+	+	-
Glycosides	-	-	-	-
Phenols	+	+	-	-
Saponins	+	+	+	+
Steroids	+	+	+	-
Tannins	+	+	+	-
Terpenoids	+	-	+	-

-, absent; +, present.

5. DISCUSSION

Since malaria is a major public health threat in developing countries, it is essential to do extensive researches to be directed towards the search for new drugs [Ukpai and Amaechi, 2012]. Regarding this, plants have proved to be sources of antimalarial agents [Adzu *et al*., 2007]. Therefore, it is reasonable that such studies should be done in screening claimed plants to provide a potential lead for novel antimalarial drug development.

Plasmodium species that cause human disease are essentially unable to infect non-primate animal models with the exception of a complex immunocompromised mouse model that has been developed to sustain falciparum parasitized human RBCs *in vivo* [18, 19]. So, *in vivo* evaluation of antimalarial compound(s) begins with the use of rodent malaria parasite [Kalra *et al.,* 2006]. *In vivo* model was also opted for this study because it takes in to account any prodrug effect and the likelihood of immune system in controlling infection as compared to an *in-vitro* study [Waako *et al*., 2005]. More importantly, it is cost effective to conduct preliminary pharmacological screening studies of crude extract in rodent model [Mohammed *et al*., 2016].

Four African rodent malaria parasites (*P. berghei, P. chabaudi, P. vinckei* and *P. yoelii*) have been adapted for growth in laboratory mice and each of them has similar characteristics to the four human malaria species, making them suitable for parallel study [Nogueira and Virgílio, 2010; Silmon, 2010]. *P. berghei* ANKA has been used in studying the activity of antimalarials in mice since it does not cytoadheres, therefore, all life cycle stages are observed on smears [Huang *et al*., 2015]. Thus, it was used to predict treatment outcomes and is an appropriate parasite for such studies [Madara *et al*., 2012].

The four-day suppressive test is standard and widely utilized rodent model for antimalarial screening [Peter and Anatoli, 1998] even though a new *in vivo* screening paradigm has recently been optimized [Jime´nez-Dı´az, 2013]. Four-day suppressive test using *P. berghei* infected mice provides a preclinical indication of potential bioactivity of the test sample [Peter *et al*., 1975]. In both rane's and four-day suppressive tests, determination of percent inhibition of parasitemia is the most reliable parameter [Bantie *et al*., 2014].

Use of 80% methanol for extraction was justified based on the previous study that alcohol would be a better solvent for extraction of *G. ternifolia* [Ibrahim *et al*., 2013]. Oral dosing of the extract and fractions was used, to replicate the ethno-medical method of administration and the likely route during clinical evaluation [Adzu *et al*., 2007]. None of the test mouse died or showed signs of toxicity within 24 h and the next 14 days of treatment with 2000 mg/kg of the test extract during acute toxicity study. The oral dose of > 2000 mg/kg body weight is 10 times > the minimum effective dose of the extract. Earlier reports have shown that if LD_{50} of a test substance is three times more than the minimum effective dose, the extract is considered as a good candidate for further study [Dikasso *et al*., 2006; Madara *et al*., 2010] and this could explain safe use of the plant to treat malaria by local people in Ethiopia. From previous studies, it was evident that leaves extract of the plant protected induced hepatotoxicity in rats, which can be extrapolated to or further supports the above argument [Yunana and Dahiru, 2015].

From the results, one could see that percentage parasitemia measured in the four-day test was reduced by the crude extract in infected mice. Experimental drugs with suppressive activity against *P. berghei* were known for antimalarial activity [Calvalho *et al.*, 1991]. As revealed in phytochemical analysis, alkaloids, flavinoids, saponins, phenols and terpenoids

present in the crude extract of the study plant could be responsible for its antimalarial activity. The parasite suppression exhibited by the extract was comparable with studies done on *Aloe debrana* [Deressa *et al.,* 2010].

Butanol and chloroform fractions exerted higher chemo-suppressive effects than aqueous fraction in four-day suppressive study, suggesting the possible localization of active ingredients in these two fractions. The highest effect by butanol fraction next to the crude extract might have been resulted from presence of the extract phytoconstituents also in this fraction but not terpenoids as presented above in Table 8. The reason behind a little bit closer effect to the extract by butanol fraction than other fractions might have also been instigated from the alcoholic nature of two solvents and the active ingredient(s) might be concentrated in butanol fraction. This finding is also consistent with other studies in which butanol fraction had high activity than chloroform and aqueous fraction [Kwaghe and Ambali, 2009; Mengiste *et al*., 2012; Asnake *et al*., 2015]. High levels of chemo-suppression were produced at high doses of the extract and fractions, indicating the presence of good concentrations of active compound(s) in higher doses.

Chloroform fraction was found to have the second highest antimalarial activities from fractions. This effect could have been associated with the existence of alkaloids, flavonoids, saponins and terpenoids as in butanol fraction. The fraction lack phenols, which probably explain why it had lower activity than butanol fraction. However, the lowest dose was unable to suppress parasitemia significantly. This might be due to the absence of sufficient concentration of active constituent(s) or might be related to partial loss of active ingredient(s) due to its insufficient uptake to physiologically active level. The result of chloroform fraction is consistent and/or comparable with the effect of

chloroform fraction reported on seed extract of *Dodonaea angustifolia* [Mengiste *et al*., 2012], root extract of *D. angustifolia* [Amelo *et al*., 2014] and leaves of *Vernonia amygdalina* [Bihonegn, 2016].

The parasite suppressive effect of root bark extract and solvent fractions might be via indirect boosting of immune system or by inhibition of other target pathways which are not fully realized [Muthaura *et al*., 2007]. The therapeutic benefits of traditional remedies are often attributed to the presence of non-nutritive bioactive constituents [Ghisalberti, 2008]. The phytosteroids, anthocyanins, flavonoids and saponins noticed in this plant have been proved to possess potential immunomodulatory effects in other plants [Aherne *et al*., 2007; Miguel, 2011; Saxena *et al*., 2013].

On the other hand, aqueous fraction produced the lowest inhibition of parasitemia in the four-day suppressive test. This could probably emanate from the absence of most of the secondary metabolites from this fraction that appeared to be responsible for the observed antimalarial activity. The finding is in line with other studies in which the effect of aqueous fraction is less than chloroform as well as butanol fractions [Bantie *et al*., 2014; Bihonegn, 2016] but comparable to Mengiste *et al*., (2012), Amelo *et al*., (2014) and Asnake *et al*., (2015).

The observed data in aqueous fraction indicates that this extract is gone to be inactive. To be active, the sample should suppress percent parasitemia by ≥ 30% [Krettli *et al*., 2009]. In this study, the chemo-suppressive activity of all three fractions is lower than that of the crude extract. This finding is in agreement with another study although the plants were different [Traoré *et al*., 2006; Bantie *et al*., 2014]. Activity reduction in the fractions could

be explained by the loss of synergistic action among the chemical compounds or differential distribution of secondary metabolites within the fractions.

From views above, we could say that the plant possessed antimalarial activity. This assertion is evidenced by other *in vivo* studies that reported antimalarial activity of other species of the same genus such as *Gardenia Lutea* [Akele, 2013] and *Gardenia sokotensis* [Traoré *et al*., 2006]. Previous study also conveyed that flavonoids and steroids isolated from *G. ternifolia* leaf surface exudates showed an antiplasmodial activity (IC_{50} values 1.06 and 0.94 μg ml^{-1}) against falciparum strains [Charles *et. al*., 2010]. Drugs having antibacterial activity such as doxycycline and clindamycin are employed in the treatment of malaria. The plant *G. ternifolia* also has *in vitro* antimicrobial activities [Ngbolua *et al*., 2015; Tsobou *et al*., 2015]. Those observations further strengthen the notion that the study samples have anti-plasmodial activity. In addition, this study plant possesses *in vitro* anti-parasitic activity against *Theileria lestoquardi* (apicomplexan parasite) that infects erythrocytes [Farah *et al*., 2012]. Hence, the similarity of this ruminant parasite to human parasite Plasmodium both in pathogenesis and phyllum taxonomy fortifies the anti-malarial activity of the investigational plant [Heussler and Stanway, 2008].

The crude extract prolonged survival time in early parasite infection, which is concordant with the studies conducted on ethanolic leaf extract of *Chromolaena odorata* [Ukpai and Amaechi, 2012]; hexane extract of *Pluchea lanceolata* [Mohanty *et al*, 2013] and methanolic extract of *Phytolacca dodecandra* leaves [Mequanint, 2014]. In the four-day test, the crude extract has greater survival time than fractions in line with Bantie *et al*., (2014). From fractions, all dose levels of butanol fraction and two higher doses of chloroform fraction significantly improved the survival time of the study mice on early

infection, which is consistent with Amelo *et al*, (2014) and Bihonegn, (2016). The survival period prolongation effect could be directly associated with the low parasite level and the overall pathologic effect of the parasite on the extract treated groups [Basir *et al*., 2012; Mengiste *et al*., 2012]. However, they did not cure the infection and this could be due to recrudescence of the parasites after apparent cure related to short-lived actions by the extracts [Waako *et al*., 2005; Mengiste *et al*., 2012].

Body weight loss, hypoglycemia and reduction in PCV and body temperature are cardinal signs of malaria-infected mice [Langhorne *et al*., 2002]. Hence, an ideal plant extracts with antimalarial activity are expected to prevent malaria-caused reduction of body weight, PCV and temperature due to the rise in parasitemia. Weight loss protection by the extract on early infection in present study, also experienced in other observations of different plant, is significant compared to untreated control and might have been determined by nutritional components of the plant [John *et al*., 2016] more than other detrimental factors [Tijani *et al*., 2010; Mequanint, 2014; Bantie *et al*., 2014]. The increase in weight was consistent with increase in dose respective to negative group. This finding is discordant with different studies in which the body weight decreased as the dose of extract increased [Dikasso *et al*., 2006; Mengiste *et al*., 2012; Mohammed *et al*, 2016].

The higher doses of butanol fraction and the largest dose of chloroform fraction showed remarkable increase in body weight when compared with the infected untreated group, which is consistent with Asnake *et al*., (2015). This activity might have been resulted from the overall improvement in packed cell volume, rectal temperature and parasite clearance among extract-treated mice as shown in the result section. Vitamins and minerals found in fruit pulps of *G. ternifolia* [John *et al*., 2016] could also exist in its root barks and might

contributed to weight increment in treated mice by enhancing food intake capacity. However, parasite suppression by all doses of both fractions is not translated in to alteration of weight by lower doses as decrease in body weight may be the consequences of disturbed metabolic function associated with malaria infection, and hypoglycemia associated with malaria [Basir *et al*., 2012] and the plant itself [Awede *et. al*., 2015]. Aqueous fraction did not prevent weight reduction here at all dose levels, which might be attributed to accumulation of appetite suppressive chemicals (saponins) in this fraction and too low effects on other parameters [Basir *et al*., 2012]. This finding is in agreement with previous studies on other plants [Amelo *et al*, 2014; Abdela *et al*., 2014] but not concordant with the effect of aqueous fraction on Bihonegn, (2016).

A decrease in the metabolic rate of infected mice occurs before death and is accompanied by a corresponding decrease in internal body temperature [Mengiste *et al*., 2012] contrary to the situation in human subjects [Dikasso *et al*., 2006]. Active compound(s) should prevent the rapid dropping of rectal temperature. In four-day test, all doses of the crude extract as well as middle and larger doses of butanol and chloroform fractions protected the decrease in rectal temperature associated with infection in mice relative to negative control. The observed changes in temperature were correlated to weight changes measured during the experiment rather than parasite suppression since weight loss is indirectly influenced with malaria fever [Kabiru *et al*., 2012]. This could also be attributed to the extract as it may have less amount of hypothermic effect on the treated mice [Mebrahtu *et al*., 2013; Adugna *et al*., 2014]. Overall, this activity might probably indicate that the extracts ameliorate some pathological processes that cause reduction in internal body temperature and metabolic rates [Mengiste *et al*., 2012].

Anemia is caused by: (a) destruction of RBCs, either by parasite multiplication or by spleen reticuloendotelial cell action as the presence of many abnormal RBC stimulates the spleen to produce many phagocytes [Chinchilla *et al*., 1998], (b) erythropoietic suppression and dyserythropoiesis [Lamikanra *et al*., 2007], and (c) oxidative stress, which increases erythrocyte RBC membrane fragility [Iribhogbe *et al*., 2012].

In chemosuppresive study, the extract and fractions of *G. ternifolia* prevented significant PCV reduction in a dose dependent manner like crude extract and fractions of *Dodonaea angustifolia* seeds [Mengiste *et al*., 2012] compared to the respective negative control. The effect is also in line with the outcome produced by crude extract of *Clerodendrum myricoides* leaves [Asnake *et al*., 2015] and chloroform fraction of *Croton macrostachyus* leaves [Bantie *et al*., 2014] as well as fractions of *Ajuga integrifolia* leaves [Asnake *et al*., 2015] when the concern was increase in activity as dose too. This protection effect might have been resulted from significant parasite suppression induced by active constituent(s) in the administered doses of the extract since the increase in blood parameters corresponded to the decreased parasites load [Mequanint, 2014].

Anthocyanins detected in this study and organic acids identified in other study from this plant could also be responsible for the protection of anemia as these metabolites have been proved to possess the ability to interact with proteins and stabilize erythrocytes membrane by preventing the oxidation of membranes phospholipids [Mpiana *et al*., 2010, 2011; Ngbolua *et al*., 2015]. These could counteract hemolysis associated with saponins caused by increasing permeability of RBCs plasma membrane [Yang *et al*., 2005]. Species of this plant are also known for their anti-dehydrating properties which further protect PCV reduction in infected mice [Ngbolua *et al*., 2015]. The effect produced by chloroform

fraction even if less to butanol fraction was obtained probably due to the availability of organic acids and other compounds in this extract, which have protective action in RBC invasion. The better reversal of anemia among crude extract-treated mice than fractions could be due to partition of anthocyanin and other responsible components in to butanol and aqueous fraction. Failure of aqueous fraction to reverse PCV reduction in spite of anthocyanins contained could probably be related to the presence of high concentration of saponins that have strong hemolytic effects [Yang *et al*., 2005].

In established infection, the extract exerted significant suppression of parasitemia. As indicated in the results section, all treated groups brought about reduction of parasitemia after the second dose, however, the standard drug started its activity right after the first dose. This delay of activity may be indicative of the need for a loading dose or the extract might have a slower onset of action compared to chloroquine [Balogun *et al*., 2009]. Since it is desirable to have both suppressive and curative activities in a phytodrug, it may be possible to consider this plant as a potential source of antimalarial agents [Oliveira *et al*., 2009]. This result is comparable with the curative effect of *Piliostigma thonningii* root bark extract [Madara *et al*., 2012], *Languas galanga* rhizomes extract [Al-Adhroey *et al*., 2010] and *Piper betle* leaves extract [Al-Adhroey *et al*., 2011].

The chemosuppresive effect on established infection was higher than the four-day suppressive test probably due to non-selectivity of the extract against the proliferative processes of the parasite. The presence of the parasite alone in the blood does not induce disorder, but the response of the host immune system against foreign pathogenic organism via free radical generation, activation of a phospholipase cascade and generation of prostaglandins and other hemolytic principles such as free fatty acids does [Tijani *et al*.,

2010]. The pronounced anti-malarial activity observed in the established infection test may be due inhibitory effect of the extract on generation of free radicals and hemolytic principles resulting from high parasitaemia level [Calvalho *et al.*, 1991]. These plant species have been scientifically validated for their beneficial effects as anti-aggregating/anti-polymerization and radical scavenging ability [Ngbolua *et al*., 2015]. The suggested mechanism of actions could be supported by the *in vitro* analysis in which flavonoid aglycones from the leaves of *G. ternifolia* have antioxidant activity that can counteract the oxidative damage induced by the malaria parasite [Omosa *et al*, 2016] and it's the root bark was shown, in present study, to contain flavonoids.

Saponins, phenols and tannins of the study plant may also have antioxidant property, like in other medicinal plants [David *et al*., 2004; Alexandru *et al*., 2007; Soh *et al*, 2012], that can inhibit haem polymerization and the unpolymerised haem is very toxic for intraerythrocytic plasmodia [Taramelli *et al*., 1999]. It may also be due to the direct plasmocidal effect of the extract exerted by triterpenes inhibited protein synthesis and flavonoids chelated with nucleic acid base pairing of the parasite [Tijani *et al*., 2010; Okokon *et al.*, 2016]. This finding is consistent with some other findings in which the curing effect is greater than the suppressing effect [Tijani *et al*., 2010; Al-Adhroey *et al*., 2010; Onwusonye and Uwakwe, 2014].

Moreover, anthocyanins, which also exist in the plant, are generally known to have antioxidant, anti-proliferation and anti-inflammatory activity [Miguel, 2011]. Agents with such properties are known to produce additional remedy to malaria patients [Adzu and Salawu, 2009]. Like quinine and proquanil antimalarial drugs, the plant has anti-

hyperglycemic tendency in mice [Awede *et. al*., 2015], which might make it the preferred drug to treat malaria patients with diabetes mellitus.

Parasitemia reduction by the crude extract observed in rane's test which translated in to longer survival time is an additional evidence of antimalarial efficacy of the plant's extract. Protection of weight loss in this study is comparable in significance with findings in other observations compared to untreated control [Tijani *et al*., 2010; Bantie *et al*., 2014]. The extract at all doses protected the decrease in rectal temperature in mice. In a similar fashion, the crude extract in other studies halted the decrement of temperature relative to untreated group [Bantie *et al*., 2014]. It was also noted that extract of *G. ternifolia* prevented reduction in packed cell volume at all dose levels when compared to negative control. This could be as a result of destructive antiplasmodial effect of the extract against the parasitized red blood cell and the parasite, thereby sustaining the availability of new red blood cells produced and released from bone marrow. Impressively, antagonistic effect of the plant on oxidative stress and inflammation would prevent the occurrence of anemia secondary to dys-erythropiosis.

In the prophylactic test, parasite load was decreased by all doses of the extract indicating that the plant has suppressive prophylactic effect. The prophylactic effect of the extract including the standard was; however, lower than effects seen in suppressive and rane's tests. This lower effect may indicate rapid excretion and/or metabolism of the extract and chloroquine [Adzu *et al*., 2007; Tijani *et al*., 2010]. It can also be due to the *in vivo* model used, which lacks the insect vector, and the manner of inoculation and the doses used that result in rapid infection of the erythrocytes without the parasite going through the liver stages [Adzu *et al*., 2007]. Another possibility was that the extract might have acted

through metabolic activation of the immune system and so parasite clearance could not be total [Waako *et al*., 2005]. This finding is in agreement with repository effect reported on root extract of *Aristolochia albida* [Khan *et al*., 2012]. It is also in line with other studies in which repository effect is lower than suppressive and curative effects of extracts [Al-Adhroey *et al*., 2010; Unekwuojo *et al*., 2011; Mebrahtu *et al*., 2013; Onwusonye and Uwakwe, 2014]. The result here was, however, in contrary with other investigations where plants had high residual activity than suppressive and curative activity [Odeghe, 2012]. The higher prophylactic effect seen in other studies were attributed to the pro-drug nature and long duration of action of the active compound(s).

In residual infection, only the higher dose of the extract improved body weight and survival time. The highest dose also showed preventive effect in temperature reduction with similar level of significance to the largest dose effect by methanolic extract of *Syzygium guineense* stem barks [Zeleke, 2015]. Parallel to decrement of those parameters, the preventive effect was not significant in the lower doses of the test sample respective to negative control. Besides, the middle and larger doses turned away the development of anemia in infected mice.

Generally, all the chemo-suppressive, prophylactic and curative tests showed parasitemia suppression in a dose dependent manner, which is similar with Adumanya *et al*., (2014) and Tijani *et al*., (2010). The effects in almost all study parameters are also increased with dose, which is in agreement with Asnake *et al*., (2015) and Adugna *et al*., (2014) as chemo-suppressive test is concerned.

Comparison of crude extract activity with that of its fractions showed that the fractions demonstrated less antimalarial activity. The relatively higher potency of the crude extract may be attributed to the presence of various phytochemical constituents that work singly or synergistically, but might be reduced or lost during fractionation. Some secondary metabolites protect other metabolites as antioxidant for example and breaking of this association could accelerate degradation and consequently reduction of the suppressive effect of the ingredients in fractions [Traoré *et al*., 2006]. The butanol fraction was found to be more active among fractions. This justifies that bioactivity of the plant could be attributed to its moderately polar compounds than non-polar and then polar components or active component(s) responsible for the antiplasmodial activity of this plant might be more concentrated in this fraction. This fraction also prevented PCV fall, body weight loss, temperature reduction significantly.

To summarize, tannins, flavonoids, alkaloids, saponins, terpenoids, steroids and phenols detected in root barks of this study plant have been responsible for its antiplasmodial activity as per suggested in other plants [Oliveira *et al*., 2009; Kaur *et al*., 2009; Soh *et al*, 2012; Arise *et al*., 2012; Saxena *et al*., 2013]. On the other hand, the plant may contain an undiscovered antiprotozoal active compound that could serve as a template for the production of relatively inexpensive or alternative antimalarial drugs. Additionally, presence of more than one class of phytochemicals in a given plant extract determines the nature and extent of its biological activity [Musila, 2013]. If antimalarial activity of a compound displayed a percent growth inhibition of > 50% at a dose of 500, 250 and 100 mg/kg/day, literature grades it as moderate, good and very good, respectively [Munoz *et al*., 2000]. Therefore, the root bark extract had a good antiplasmodial activity.

6. CONCLUSION

In vivo antimalarial test results indicated that crude extract and fractions of the plant materials possess antimalarial activity, with higher effect exhibited by the crude extract. The findings confirmed that the study plant has high curative effect suggesting the direct anti-plasmodial activity of the extract. Butanol fraction was found to be active among the fractions and might contain a potential lead molecule for the development of new drug to treat malaria. The antimalarial action of the extract and solvent fractions has been attributed to the presence of semi-polar to non-polar ingredients in the root barks of the plant. In addition, the data would provide evidence to uphold the earlier *in vitro* investigation on leave surface exudates of *G. ternifolia* as well as the claims made by the Ethiopian traditional medicine healers.

7. RECOMMENDATIONS

- The plant has promising antimalarial activity; therefore, the following works are recommended to be done in future.
  - ☑ *In vitro* activity of the root bark against falciparum malaria,
  - ☑ Bioassay guided isolation to isolate the active principle(s),
  - ☑ Elucidating the structure and mechanism of action of the active principle(s),
  - ☑ Similar studies on the stem, fruit and leave of *G. ternifolia* to compare the antimalarial activity with its root, and
  - ☑ Further toxicological studies to better establish the safety status of the plant.
  - ☑ Since *G. ternifolia* is traditionally used for various ailments, it is an active area for research to justify those claimed activities.

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